INTERACTION BEWTEEN IMMUNOSUPPRESSIVE AND CHOLINERGIC MARKERS IN COLORECTAL CANCER

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This thesis is submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Colorectal cancer (CRC) is amongst the leading diagnosed cancers worldwide. Despite the increasing interest to understand, the roles that the nervous and immune systems play in influencing the tumour microenvironment to promote cancer development and progression, more studies are required to understand the mechanism. Cancer cells can influence their microenvironment and bi-directionally communicate with other systems such as the immune and nervous systems. The immune system plays a key role in the eradication of cancer cells. Studies have shown that multiple mechanisms are responsible for the suppression of the immune system in cancer, one of which being the expression of immune checkpoints inhibitors such as programmed death 1 (PD-1), PD-L1, programmed death ligand 1 and 2 (PD-L1, PD-L2), sialic acid-binding lectins 9 (siglec-9) and IDO (indoleamine-2,3-dioxygenase). These molecules function by inhibiting anti-tumour effects of T cell-mediated immune responses. In addition to these molecules, studies have shown that several cancers can release acetylcholine (ACh) and express cholinergic receptors (muscarinic receptor 3 (M3R) and alpha 7 nicotinic receptor (α7nAChR)), overexpress choline acetyltransferase (ChAT), a precursor enzyme required for ACh synthesis and VAChT, essential for transporting of ACh, and excitatory receptor. Currently, there are no data available in determining the interaction between the expression of immunosuppressive and cholinergic markers in cancer, thus, this thesis aims to determine the interaction between the expression of immunosuppressive and cholinergic markers in CRC

The results of this study in *ex vivo* human specimens from patients diagnosed with stages I-IV of CRC demonstrated high expression of immunosuppressive (PD-L1, PD-L2, siglec-9 and IDO) and cholinergic (M3R and ChAT) markers, which were correlated with, advanced stages of CRC. In addition, elevated levels of immunosuppressive markers were correlated with a high risk of CRC and poor patients' survival outcomes, while low levels of cholinergic markers were associated with a high risk of CRC and poor patients' survival outcomes. Furthermore, low levels of M3R and high levels of ChAT were associated with metastasis. On the other hand, α7nAChR expression was not associated with any clinicopathological parameters.

The effect of blocking muscarinic receptors on the expression of immunosuppressive (PD-L1 and PD-L2), cholinergic and angiogenic markers as well as tumour-infiltrating immune cells was evaluated. *In vitro* findings showed that 4-DAMP and atropine treatment significantly decreased PD-L1 and PD-L2 expression in human colon cancer cells and murine colon cancer cell line via the inhibition of EGFR/ERK/AKT/STAT3 pathway. Similarly, 4-DAMP and atropine treatment significantly attenuated M3R expression in human colon cancer cells and murine colon cancer cell line via EGFR/ERK/AKT/STAT3 signalling pathway. Atropine and 4-DAMP had no effect on human colon cancer cells ability to express ChAT; however, both atropine and 4-DAMP decreased ChAT expression in murine colon cancer cell line. *In vivo* findings demonstrated that 4-DAMP treatment significantly decreased the expression of PD-L1, M3R, ChAT and angiogenic markers through AKT/ERK signalling pathways, leading to an enhanced immune response against cancer noted with the increased CD4 and CD8 T cells. These findings suggested that cholinergic signalling might stimulates the expression of immunosuppressive markers and could explain the inconsistency in prognostic value.

The effect of blocking siglec-9/siglec-E on the expression of IDO, cholinergic and angiogenic markers as well as tumour-infiltrating immune cells was evaluated. Blocking siglec-9 with human anti-siglec-9 antibody significantly reduced IDO expression in HT-29 and siglec-9 in LIM-2405 and HT-29. In addition, blocking siglec-9 decreased M3R expression in T4056 but not human colon cancer cells. Anti-siglec-9 antibody unveil its effects in a normal epithelial cell via suppressing EGFR/ERK/STAT3 signalling pathway, while in human colon cancer, LIM-2405 it acts by inhibiting ERK/STAT3 signalling pathway. Similar to human colon cancer cells, mouse anti-siglec-E antibody significantly inhibited the expression of siglec-9 and cholinergic markers in CT-26 cells via inhibiting EGFR/AKT/ERK signalling pathway. Mouse anti-siglec-E antibody significantly reduced tumour weight, volume and size, and inhibited the expression of siglec-9, cholinergic and angiogenic markers *in vivo* via inhibition of ACh production by reducing the amount of ChAT, an enzyme crucial for ACh synthesis, VAChT, essential for transporting of ACh, and excitatory receptor, α7AChR through inhibition of EGFR/AKT/ERK signalling pathway. Moreover, the anti-siglec-E treatment augmented M3R expression, suggesting that anti-siglec-E might exerts its effects through ACh production and α7AChR but not M3R.

Taken together, results presented in this thesis suggested that blocking of M3R and siglec-9/siglec-E have the potential to be used in conjunction with current immune checkpoint inhibitors or traditional cancer therapeutic. In conclusion, it is important to evaluate the expression status of some or all these immunosuppressive molecules and cholinergic markers in order to develop appropriate therapeutic strategies in cancer patients. The findings of this work have important clinical relevance and created a new therapeutic avenue, which could target both immunosuppressive and cholinergic markers that might be beneficial as a treatment regimen for CRC patients.

DECLARATION

I, Nyanbol Kuol, declare that the PhD thesis entitled "Expression of Immunosuppressive and Cholinergic Markers in Colorectal Cancer" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Nyanbol Kuol

February 2020

DECLARATION OF CONTRIBUTION TO WORK

The following people have made the stated contributions to this work:

Chapter 1

Assistance in preparing the manuscript for this chapter was provided by L Stojanovska, V Apostolopoulos and K Nurgali.

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Technical assistance and training for immunohistochemistry was provided by V Jovanovska. Technical assistance with statistical analysis was provided by S Vogrin. Human tissues from patients diagnosed with stages, I-IV of CRC were provided by J Godlewski. Assistance in preparing the manuscript for this chapter was provided by V Apostolopoulos and K Nurgali.

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PRESENTATIONS

Invited Presentations

1. **Nyanbol Kuol**, Janusz Godlewski[c] Majid Hassanzadeganroudsari, Rhiannon Filippone, Margaret Veale, Sarah Fraser[a], Vasso Apostolopoulos[a,d,*], Kulmira Nurgali[a,d,*]. (2019) Expression of immunosuppressive and cholinergic markers *in vitro* and *in vivo* animal studies and *ex-vivo* human samples studies. **Invited speaker** at the 78th Annual Meeting of the Japanese Cancer Association.

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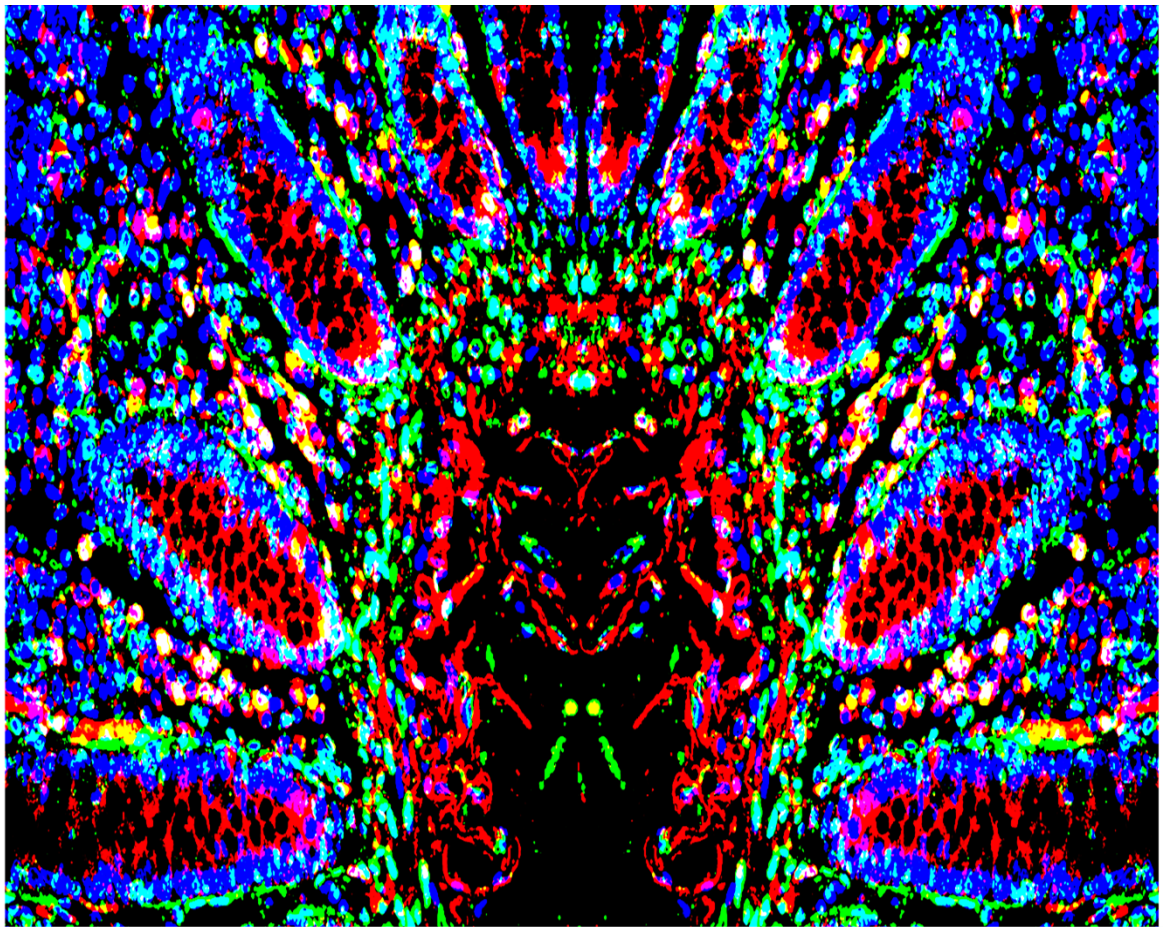
CHAPTER ONE

Literature Review

Some of the material presented in this Chapter is based on published reviews and has been reproduced here with the permission of the publishers.



1.1. Introduction

Cancer remains as one of the leading causes of death worldwide, and colorectal cancer (CRC) are among the leading diagnosed and cause of cancer-related death worldwide due to the aging population and unhealthy lifestyle [1, 2]. Although they are highly treatable when localised, metastatic or recurrent cancer has a poor prognosis. The development of cancer involves an intricate process, wherein many identified and unidentified factors play a role. Although most studies have focused on the genetic abnormalities, which initiate and promote cancer, there is overwhelming evidence that tumours interact within their environment by direct cell-to-cell contact and with signalling molecules, suggesting that cancer cells can influence their microenvironment and bi-directionally communicate with other systems including the immune system.

Studies have shown that tumour microenvironment is more likely to influence the behaviour of cancer cells [3-5]. The tumour microenvironment is composed of numerous stromal cell types, including endothelial cells, immune cells, fibroblasts, and nerve fibers [4, 6]. The tumour microenvironment constituents play a significant role in tumour progression and metastasis, and, as a result, is of concern in cancer treatment [5, 7-10]. In addition to the tumour microenvironment, tumour angiogenesis is one of the major prerequisites for tumour growth and metastatic spread as tumour cells rely on adequate oxygen and nutrient supply as well as the removal of waste products [11]. The impact of the tumour microenvironment in tumour cell invasion, angiogenesis and metastasis has attracted much interest in recent years [4, 7, 12, 13]. The role of the nervous system has surfaced as one of the major contributors to cancer progression and metastasis. The nervous system governs functional activities of many organs, and, as tumours are not independent organs within an organism, this system is integrally involved in tumour growth and progression [14, 15]. However, the contributing role of the nervous system in tumour progression and metastasis has been largely overlooked. As the interaction of these factors makes early detection of cancer important, it is necessary to identify the combination of biomarkers that may predict metastasis before it manifests in the patient. Although understanding of CRC development and progression has improved over the

years the way in which complex interactions between biological, immunological and neurological factors interact to influence the course of CRC is still unclear.

1.2. Molecular pathways involved in CRC initiation

Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide [16]. CRC presents vague or no symptoms in the early stages, hence, making early detection crucial as it is more often diagnosed at advanced stages. There several risk factors associated with CRC development, which include, but not limited to, the family history of inflammatory bowel disease or colon cancer, age, race, diet, smoking, alcohol intake, obesity and physical inactivity [17]. These risk factors cause alterations in colon epithelial cells that, together with inherited genetic traits, may result in the development of a tumour. About 70% of CRC occurs sporadically and arise due to the presence of an accumulation of gene mutations (tumour suppressor genes) that induce cancer [17], such as, *p53*, *K-ras* and *adenomatous polyposis coli* [18, 19].

Three distinct pathways of genomic instability are believed to play an essential role in CRC initiation. These include a mutation in mismatch repair genes, leading to microsatellite instability (MSI), mutations in *APC* and other genes that activate Wnt pathway, categorised by chromosomal instability (CIN), and global genome hypermethylation, resulting in tumour suppressor genes switch off, shown as CpG island methylator phenotype (CIMP) [20, 21]. Microsatellite instability (MSI) is a hypermutable phenotype caused by the loss of DNA mismatch repair activity. MSI constitutes 15 to 20% of early-stage and 3% of metastatic CRC [22]. MSI tumours exhibit innate hostile environment towards tumorigenic cells, but, yet are characterised by enhanced expression of several immune checkpoint markers including cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), lymphocyte activation genes-3 (LAG-3), programmed death-1 (PD-1), programmed death ligands (PD-L1) and indoleamine-2,3-dioxygenase (IDO) [23, 24]. This suggests that MSI CRC subsets are distinguished by expression of immune effector cells, but also possess an immune exhaustion phenotype within the

microenvironment. On the other hand, microsatellite stable (MSS) CRC is mainly negative for PD-1 expression [25].

Specific pathological features, mechanisms of carcinogenesis and process of tumour development might be influenced by each of these pathways. The molecular aspects of these pathways have been used clinically in the diagnosis, screening and management of patients with colorectal cancer. However, their influence on tumour microenvironment remains to be defined.

1.3. Tumour Microenvironment

1.3.1. Mechanisms of tumour cell escape from immune detection

The “survival of the fittest” theory generally describes how tumour cells are able to adapt to host immune surveillance and invade the host. Tumorigenic cells can adapt host immunity via upregulating the expression of molecules such as programmed death-ligand 1 (PD-L1), indoleamine-2,3-dioxygenase (IDO), siglec-9, and downregulating other molecules, including the major histocompatibility complex (MHC) class I (**Figure 1.1**). In addition to these immunosuppressive molecules, tumour cells can recruit and educate immune cells to promote immune evasion [26]. PD-L1, a transmembrane protein, plays a crucial role in suppressing the immune system. T cells express the receptor PD-1, and upon interaction with PD-L1, inhibitory signals are triggered, resulting in apoptosis of cytotoxic T lymphocytes (CTLs, $CD8^+$ T cells) [27]. Interestingly, PD-L1 serves as an anti-apoptotic factor on tumour cells, leading to their resistance to cytolysis by CTLs as well as drug-induced apoptosis [28]. Moreover, in a functional immune system, T cells are activated by interacting with MHC expressed on antigen-presenting cells [29, 30]. In addition to the interaction between co-inhibitory and co-stimulatory receptors, these interactions prevent the host against auto-immune reactivity. The balanced interaction between co-inhibitory and co-stimulatory receptors determines whether T cells are stimulated or whether they become anergic to a specific antigen displayed on the MHC. The balance of co-stimulation and co-inhibition appears to be skewed by cancer cells

towards co-inhibition due to dysregulation of several cell surface markers, such as MHC class I, B7 and CD28 [31]. Furthermore, tumour-associated immune (TAI) cells play an intriguing role in immune evasion. In fact, the presence of TAI cells within the tumour microenvironment correlates with poor prognosis as noted in several cancers [32, 33]. TAI cells are capable of expressing immunosuppressive factors, such as IL-10, tumour growth factor-beta (TGF-β), and prostaglandin E_2 (PGE_2) to exhibit their effects on T cell inhibition [34-36]. These factors play a crucial role in supporting tumour immune evasion by regulating TAI cells or suppressing systemic immune cell function, particularly T cells, which are responsible for immunosurveillance. Herein, we focus on the role of immunosuppressive molecules PD-L1, IDO, siglec-9, downregulation of MHC class I, infiltration of TAI cell and their secreted factors that promote immune evasion, leading to metastasis and/or disease recurrence in patients with cancer.

1.3.1.1. Upregulation of programmed death ligand 1

The expression of PD-L1 by tumour cells plays an essential role in the establishment of an immunosuppressive force that facilitates tumour cells escape from immune. Indeed, the expression of PD-L1 in head and neck squamous cell carcinoma, carcinomas of the lung, ovary, breast, endometrium, and melanoma, contribute significantly to evading the immune system [30, 31, 37-61]. Higher PD-L1 expression is associated with tumour node metastasis, poor prognosis and shorter survival in patients with colorectal cancer [62, 63]. The expression of PD-L1 by tumour cells is dependent on interferon-gamma (IFN-γ) production by tumour-infiltrating immune cells [64]. In addition, downstream signalling molecules such as, nuclear factor-kappaB (NF-κB), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), mammalian/mechanistic target of rapamycin (mTOR) and Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) which act via toll-like receptors and IFN-γ receptor, regulate the nuclear translocation of transcription factors to the PD-L1 promoter leading to PD-L1 induction [65]. PD-L1 upregulation enhances regulatory T cells (Tregs) via AKT and mTOR phosphorylation leading to immunosuppression [66]. Interestingly, the expression of PD-L1 by tumour-associated macrophages (TAMs) also mediates immunosuppression and is important for tumour escape from the immune response. Furthermore, the expression of PD-1 on

tumour infiltrating lymphocytes (TILs) correlates with aggressive features of breast cancer cells, including lack of oestrogen receptor expression, higher TIL counts, high tumour grade, and, the triple-negative breast cancer subtype [50, 54, 61, 67]. In addition, PD-1 on TILs is associated with poor survival of luminal B and basal-like carcinomas [55] and is highly noted in non-small cell lung cancer (TILs and Treg cells) [68]. Similarly, PD-L1 expression in stromal or tumour cells inversely correlates with $Foxp3^+$ cell density (Treg cells) in colorectal cancer, further reinforcing the fundamental role of Treg cells in the tumour microenvironment [69]. In contrast, there are studies suggesting that the expression of PD-L1 is associated with better prognosis in breast cancer patients [68]. PD-L1 expression correlated with elevated TIL infiltration and longer recurrence-free survival in breast cancer and in pulmonary adenocarcinoma patients [57, 70, 71]. The inconsistencies in findings warrant further research into the mechanisms of action of PD-L1 on cancer cells. It is possible that the expression levels of PD-L1 on cancer cells and the co-infiltration of TILs, TAMs and Tregs within the tumour microenvironment vary according to the different stages of the disease, prognosis and ability of tumour cells to evade the host immune system.

1.3.1.2. Downregulation of MHC class I

The MHC class I also known as human leukocyte antigen (HLA)-A, -B, -C in humans is present on all nucleated cells and presents small processed antigenic peptides on its surface to $CD8^+$ T cells to activate the adaptive immune response [72]. MHC class I downregulation on tumour cells is a well-documented mechanism used by tumours to escape host immune detection [73]. In laryngeal squamous cell carcinoma, downregulation of MHC class I correlates with decreased $CD8^+$ T cell infiltration, which associates with poor survival [74]. Similarly, in breast cancer, MHC class I downregulation associates with lymphatic invasion, lymph node metastasis and venous invasion [75]. It has been shown that MAPK signalling adversely regulates the expression of MHC class I in MDA-MB-231 breast cancer cell lines providing mechanistic insights [76]. High intratumoral T cell infiltration and MHC class I expression associates with better survival via nuclear STAT1 stimulation in colorectal cancer patients; downregulation of these markers signified tumour escaping immunosurveillance [77]. Likewise, tumours

expressing high levels of MHC class I associate with good prognosis in non-small cell lung carcinoma patients; however, the effects of $CD8^+$ T cells are abolished in tumours expressing non-classical HLA-E [78]. Consistent with these findings, expression of non-classical HLA-E and HLA-G (key modulators of immune responses interfering with $CD8^+$ T cells and natural killer (NK) cell action) correlates with poor prognosis in serous ovarian carcinoma patients, suggesting that therapies targeting HLA-E and HLA-G hold potential benefit [79]. In papillary thyroid cancer, MHC class I expression associates with lower levels of $Foxp3^+$ Treg cells and $CD16^+$, $CD3^+$ and $CD8^+$ tumour-associated immune effector cells [80]. Administration of IFN-γ or selumetinib MEK1/2 inhibitor increases HLA-A, -B, -C expression in papillary thyroid cancer cell lines (BCPAP, TPC-1 and K-1) *in vitro* [80]. Likewise, we demonstrated that murine mammary adenocarcinoma cell line with low levels of MHC class I and ability to grow in mice without being rejected, was reversed by IFN-γ stimulation which upregulated MHC class I and resulted in tumour inhibition in mice [81]. Hence, tumour cells have evolved into ways to escape from the immune system by downregulating the expression of MHC class I molecules. Thus, it is important to check the expression of MHC class I on cancer tissues in order to develop appropriate treatment modalities for cancer patients.

1.3.1.3. Overexpression of the enzyme indoleamine-2,3-dioxygenase

Indoleamine-2,3-dioxygenase is an enzyme in which its overexpression leads to increased degradation of the essential amino acid L-tryptophan along the kynurenine pathway resulting in T cell inhibition, hence, promoting a mechanism of tumour escape from host immune detection [82]. IDO provokes L-tryptophan deficiency, which impairs T cell proliferation in the tumour microenvironment by inducing apoptosis [83]. In addition, L-tryptophan deficiency impairs $CD8^+$ T cell function via downregulation of the T cell receptor ζ-chain [84]. IDO not only exhibits its effect on T cells but also on other immune cells including NK cells and supports the activity and generation of TAI cells such as Treg cells and myeloid-derived suppressor cells (MDSC) [85-87]. IDO can inhibit NK cells and $CD4^+$ and $CD8^+$ T cell proliferation, however, has no effect on B cells [88]. Overexpression of IDO by a number of cancer cells holds poor prognostic value as noted in colorectal cancer [89], breast cancer [82], glioma [90] and non-small cell lung carcinoma [91]. In

colorectal cancer patients, the expression of IDO by tumour cells is associated with liver metastases and inversely correlates with infiltrating T cells as well as a clinical outcome [89]. Similarly, to the expression of PD-L1, the expression of IDO is dependent on IFN-γ. Moreover, cancer-associated fibroblasts (CAFs) expressing IDO are linked to stage III and poor prognosis in breast cancer patients as well as enhanced invasiveness of breast cancer cells *in vivo* in mice [82]. IDO expression is associated with estrogen receptor but not progesterone receptor or epithelial receptor 2 status. For example, low IDO expression correlates with estrogen receptor negative breast cancers and higher neoangiogenesis [92]. IDO expression correlates with increased Foxp3 Treg cells and is associated with a lower five year survival rate in non-small cell lung carcinoma patients [91]. Likewise, higher IDO expression in glioma patients associates with poor prognosis and high grade; and in orthotopic GL261 bearing mice models, IDO expression increases the recruitment of Treg whilst simultaneously decreasing $CD8^+$ T cells [90]. In contrary, high expression of IDO has an independent good prognostic value in basal-like breast carcinomas [93]; high IDO expression associates with estrogen receptor positive breast cancers and better overall survival [92]. Whether these findings are based on tumour specificity warrants further studies into mechanisms and pathways contributing to immunosuppression.

1.3.1.4. Sialic acid-binding lectin-9 and tumour growth

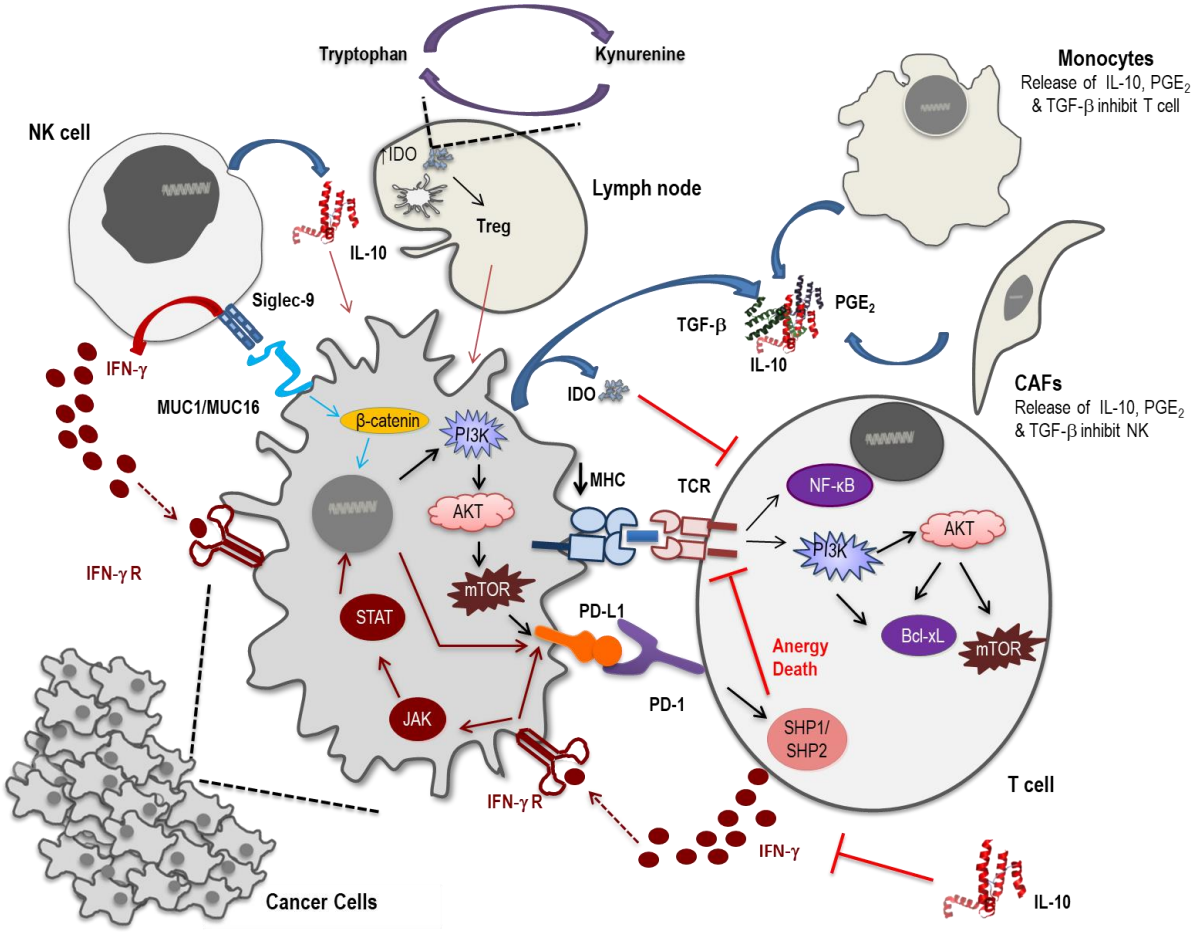
Sialic acid-binding lectins, or siglecs, play an important role in modulating the immune response. Siglecs are expressed by some immune cells such as macrophages, monocytes, neutrophils, B cells, dendritic cells and NK cells [94]. In particular, siglec-9, which is expressed on the surface of immune cells such as, NK cells, B cells and monocytes, has been shown to interact with transmembrane epithelial mucins (MUC), MUC1 and MUC16 [95]. MUC1 (CD227) is overexpressed on adenocarcinomas, and haematological cancers [96] whilst MUC16 (CA125) is primarily overexpressed on ovarian cancer cells although studies show that it is expressed on a number of cancers [97, 98]. Siglec-9 enhances the chemotactic potential and mature phenotype of NK cells and cytokine secretion (tumour necrosis factor-alpha (TNF-α), IFN-γ and macrophage inflammatory protein-1b (MIP-1b)) in neuraminidase-treated K562 cell line [99].

Interestingly, enhanced expression of siglec-9 is noted in melanomas, chronic lymphocytic leukemias and acute myeloid leukemias; however, in the peripheral blood of these patients, siglec-9 positive NK cell population is decreased [99]. Expression of siglec-7 and siglec-9 protects tumour cells from NK cell lysis *in vitro* (K562, A375, LAU2106, and HCT116 cell lines) and in huNSG mouse model, suggesting an immunosuppressive mechanism by tumour cells [99]. The interaction of siglec-9 with MUC16 has been shown to inhibit immune cell (NK and T cells) priming as noted in OVCAR-3 cell line leading to tumour cell evasion [100]. In human breast and colon cancer tissues, siglec-9 positive cells associate with the MUC1 positive cells suggesting siglec-9 to be a counterreceptor for MUC1 [101]. In addition, *in vitro* binding of siglec-9 to MUC1 expressed on HCT116 human colon cancer cell line, results in β-catenin recruitment in tumour cells where it is transported to the nucleus, leading to cell growth [101]. Inhibition of TAMs via siglec-9 leads to M1 polarisation and reduced growth promoting inflammation within the tumour microenvironment [94]. In addition, blocking of siglec-9 enhances neutrophil activity against tumour cells; likewise, siglec-E, equivalent to siglec-9, deficient mice show increased immunosurveillance against tumour cells [94]. However, this outcome is dependent on the stage of tumour and the microenvironment. These findings suggest that the expression of siglec-9 on immune cells and its interaction with MUC1 or MUC16 on tumour cells may be involved in tumour growth, however, the nature of this interaction as well as the cellular framework *in vivo* remains to be defined.

Figure 1.1. Immunosuppressive factors involved in tumour escape mechanisms from host immunity

There are several mechanisms that are involved in the tumour evasion; however, this schematic diagram demonstrates mechanisms discussed in this paper. Tumorigenic cells exhibit various immunosuppressive mechanisms to evade host immune responses, either to circumvent immune recognition or to immobilise effector T cells. These comprise modification of components of the antigen presentation machinery (such as downregulation of MHC class I) and secretion of immunosuppressive factors, including PD-L1, IDO, siglec-9, IL-10, PGE_2 and TGF-β. These mechanisms assist cancer to suppress the ability of the host immune system to restrain from tumour evasion. Understanding the regulation of these mechanisms might contribute to overcoming the tumour immunosuppressive microenvironment.

Abbreviations: Bcl-xL, B-cell lymphoma-extra large; CAFs, cancer-associated fibroblasts; IDO, Indoleamine-2,3-dioxygenase; IFN-γ, interferon gamma; IFN-γR, interferon gamma receptor; IL-10, Interleukin; JAK, Janus kinase; MHC, major histocompatibility complex; mTOR, mammalian/mechanistic target of rapamycin; MUC, Mucin; NK, natural killer; NF-κB, nuclear factor-kappa B; PI3K, phosphoinositide 3-kinase; PD-1, programmed death-1; PD-L1, programmed death-ligand1; PGE_2, prostaglandins; Treg, regulatory T cell; AKT, serine/threonine kinase or protein kinase B; Siglec-9, Sialic acid-binding lectins 9; STAT, Signal transducer and activator of transcription; SHP, Src homology protein-tyrosine phosphatase; TCR, T cell receptor; TGF-β, tumour growth factor-beta.


Tryptophan
Kynurenine
Monocytes
Release of IL-10, PGE2
& TGF-β inhibit T cell
NK cell
IDO
Lymph node
Treg
IL-10
Siglec-9
IFN-γ
MUC1/MUC16
TGF-β
PGE2
IL-10
CAFs
Release of IL-10, PGE2
& TGF-β inhibit NK
IDO
β-catenin
PI3K
AKT
MHC
TCR
NF-κB
PI3K
AKT
IFN-γ R
mTOR
STAT
PD-L1
Anergy
Death
Bcl-xL
mTOR
PD-1
JAK
SHP1/
SHP2
T cell
IFN-γ R
IFN-γ
IL-10
Cancer Cells

1.3.2. Immunosuppressive factors secreted by tumour cells

Cancer cells induce immunosuppression resulting in escape mechanism from the host immune system by secreting factors such as interleukin (IL)-10, prostaglandins, cyclooxygenase and TGF-β (**Figure 1.2**). These factors are secreted within tumour microenvironment and are associated with poor prognosis and overall survival of cancer patients [102-105].

1.3.2.1. Interleukin-10

The cytokine IL-10 (also known as cytokine synthesis inhibitory factor) is an anti-inflammatory cytokine primarily secreted by monocytes, T helper (Th)-2 cells and Treg cells. IL-10 downregulates Th1 cytokines and blocks NF-kB activity. IL-10 plays a vital role in regulating host immune response to pathogens, thus averting damage to the host and maintaining normal tissue homeostasis. Dysregulation of IL-10 increased risk for development of many autoimmune diseases [106]. Interestingly, tumour cells utilise IL-10 to suppress T cell function [107]. In fact, high expression of IL-10 at the tumour site associates with poor prognosis [102, 108, 109]. Cancer cells and TAI cells such as, TAMs, secrete IL-10 into the tumour microenvironment resulting in tumour growth [102, 105]. It is likely that IL-10 induces immunosuppression by downregulating MHC class I expression on cancer cells, resulting in tumour escape from the host [110]. In addition, elevated levels of IL-10 in the serum of cancer patients is associated with increased peripheral monocytes correlating with poor prognosis in lymphoma patients [111]. Enhanced expression of IL-10 receptor on tumour cells and its interaction with PD-1, regulates $CD8^+$ T cells of advanced melanoma patients [102]. Consistent with this finding, high expression of IL-10 positively correlates with B7-H3 (CD276) resulting in lymph node metastasis, advanced disease stage II-IV and large tumours [112]. Furthermore, IL-10 mediates immunosuppressive effects via suppressing T cell expansion through inhibition of IL-2 and IFN-γ secretion [106]. Upregulation of IL-10 is associated with HER-2/neu positive breast cancers; however, there is no correlation with age, estrogen receptor or progesterone receptor status in ductal and lobular breast cancer tissues [112]. It is clear

that IL-10 aids in tumour escape from the host immune system leading to metastasis or recurrence.

1.3.2.2. Prostaglandin E_2 and Cyclooxygenase-2

Cyclooxygenase 2 (COX-2) is an enzyme responsible for the production of prostanoids, including prostaglandins (PGE_2). COX-2 is expressed by several malignancies including breast cancer which associates with an aggressive tumour phenotype, contributing to the high metastatic capacity of cancer cells [103]. COX-2 suppresses NK cells, dendritic cells (DCs) and T cells leading to tumour escape from host immune detection. In fact, enhanced expression of PGE_2 and COX-2 inhibits T cells and DC function in breast cancer patients [113]. PGE_2 mediates cancer growth via stimulation of a family of G-protein coupled receptors. Tumour-bearing mammary adenocarcinoma cells escape immune detection as PGE_2 inhibits the function of NK cells to migrate, secrete IFN-γ and exert cytotoxic effects [114]. In fact, inhibition of PGE_2 reduces breast cancer metastasis in mice [114]. Furthermore, it was noted that prostaglandins in 4T1 breast cancer tumour-bearing mice, results in tumour escape mechanism via inducing myeloid-derived suppressor cells (MDSCs) which leads to $CD4^+$ T cells suppression and to some extent, $CD8^+$ T cells [115]. These findings concur with studies using prostaglandin E_2 receptor 2 (EP2) knockout mice, which show a decrease in MDSC accumulation and impede tumour growth, suggesting immunosuppression [115]. In addition, PGE_2 mediates immunosuppression by enhancing IDO expression by CAFs via STAT3 and EP4/signal transducer signalling pathways as noted in MCF-7 and MDA-MB-231 tumour-bearing mouse model [82].

1.3.2.3. Tumour growth factor-beta

Tumour growth factor-beta is produced by a number of immune cells including macrophages. Its increased expression often correlates with malignancy of cancer cells. TGF-β is an immunosuppressive cytokine leading to tumour growth and progression [116]. It is known that TGF-β supports $CD4^+$ T cell polarisation to Th2 rather than Th1 cells reducing anti-tumour immune responses [117]. In addition, TGF-β regulates the differentiation and expansion of NK cells, macrophages (M2 pro-tumour phenotype

instead of M1 anti-tumour phenotype), DCs and $CD4^+$ T cells. Several studies have determined the mechanisms of how TGF-β impedes with anti-tumour immunity. For example, enhanced levels of TGF-β correlates with an aggressive tumour phenotype and is a good indicator of poor prognosis in several cancers [104, 118, 119]. Furthermore, TGF-β suppresses NK cell cytolytic activity via NKG2D receptor activation, further enhancing poor anti-tumour response [120]. In addition, inhibiting several cytolytic gene expression molecules including FAS ligand, IFN-γ, and, granzyme A and B, TGF-β is able to suppress tumour cell lysis by $CD8^+$ T cells [121]. The presence of TGF-β at the tumour site signifies immunosuppression via stimulation of signalling pathways including IL-6/STAT3, PI-3/AKT pathways. In fact, in C57BL/6 mice, TGF-β requires Foxp3 to inhibit $CD8^+$ T cell responses via stimulating the translocation of downstream molecules Smad 2 and Smad 3 [122]. Overexpression of TGF-β is also associated with enhanced Treg cells and tumour associated neutrophils (TAN) in mice [104]. Interestingly, tumour cells stimulate DCs to release TGF-β which promotes the expansion of Treg cells and indirectly inhibits T cell effectors [123]. Similarly, in mice-bearing melanoma or breast cancer cells, reduced expression of type III TGF-β receptor (TGFBR3) enhanced TGF-β signalling which correlate with elevated Foxp3 Treg cells and reduced $CD8^+$ T cells within the tumour microenvironment [124]. In addition, TGF-β regulates IDO expression [124] and blocking of TGF-β *in vitro* using DNTβRII plasmid, improves the anti-tumour effects of NK cells to MDA-MB-231 and T47D breast cancer cell lines [125]. It is clear that, TGF-β mediates immunosuppression via regulating Treg cells, TAN and reduces $CD8^+$ T cells, resulting in a pro-tumour phenotype for enhanced metastasis and/or recurrent disease. Hence, anti-TGF-β therapy may be a viable treatment strategy for cancer patients.

1.3.3. Immunosuppressive effects of tumour-associated immune cells

Tumour-associated immune cells such as TAMs, CAFs, TILs (particular Tregs), MDSCs and TANs, are key immunosuppressive cells that promote tumour progression via their ability to suppress host anti-tumour responses and stimulate tumour angiogenesis [126-128].

1.3.3.1. Tumour-associated macrophages

Tumour-associated macrophages particularly of the M2 phenotype are associated with poor prognosis in several cancers including breast cancer [129]. However, what triggers TAMs to differentiate into M2 pro-tumour phenotype and not M1 anti-tumour phenotype? TAMs exposed to tumour microenvironment stimuli such as, TGF-β, IL-10, monocyte colony stimulating factor (M-CSF) and other immunosuppressive factors, induce M2 differentiation [34, 130, 131]. Furthermore, the presence of TAMs in hypoxic (avascular) environment modifies their gene expression promoting M2 pro-tumour phenotype [132]. In addition, TAMs inhibit $CD8^+$ T cell proliferation as well as TAM-derived IL-10 suppresses IL-12 secretion by intratumoral DCs as noted in an animal model of breast cancer [133]. Likewise, overexpression of IL-10 by TAMs correlates with advanced stages of disease and poor prognosis in non-small cell lung carcinoma patients [134]. TAMs isolated from renal cell carcinoma cells induce Foxp3 Treg cells and IL-10 derived from T cells leading to immune evasion via 15-lipoxygenase-2 pathway activation [135]. Upregulation of TAMs expressing B7-H1 mediates immunosuppression of glioma cells via autocrine/paracrine IL-10 signalling modulation [136]. These studies clearly demonstrate the fundamental role TAMs play in the tumour microenvironment leading to tumour escape mechanisms.

1.3.3.2. Cancer-associated fibroblasts

Cancer-associated fibroblasts are the main stromal components, which play an essential role within the tumour microenvironment resulting in modulation of tumour growth. CAFs mediate immunosuppression via promoting several other factors including immune infiltrating cells, factors secreted by tumour cells (cytokines/chemokines) and immunosuppressive molecules including IDO [82, 137-140]. CAFs are overexpressed in esophageal carcinomas and correlate with poor prognosis [138]. High expression of CAFs and M2 correlate with clinical outcome of colorectal cancer patients [141]. CAFs inhibit NK cells function creating nourished environment for tumour growth, however, these effects are reduced following administration of IDO and PGE_2 inhibitors in a murine model of hepatocellular carcinoma, suggesting that CAFs possess immunosuppressive abilities

[35]. CAFs suppress T cell proliferation by promoting the expression of PD-L1 and PD-L2 by cancer cells [140].

1.3.3.3. Tumour-associated neutrophils

Tumour-associated neutrophils play an essential role in tumour evasion and are often present within the tumour microenvironment; however, their role in immunosuppression has recently surfaced [142, 143]. Neutrophils orchestrate innate and adaptive immunity during inflammation. In fact, TANs at the tumour site signify tumour evasion and in most malignant tumours including colorectal cancer, enhanced expressions of TANs are associated with poor prognosis [144-147]. TGF-β regulates the expression of N2 pro-tumour phenotype and reduces $CD8^+$ T cell stimulation [148], whilst other studies demonstrate that N1 anti-tumour phenotype is regulated by IFN-β [149]. Elevated levels of intratumoral neutrophils correlate with advanced stage, lymph node metastasis and poor patient survival in esophageal squamous cell carcinoma [144]. In 4T1 tumour-bearing mice, neutrophils (N2 pro-tumour phenotype) are noted to enhance tumour progression and metastasis [150]. However, contrastingly, high TAN density is associated with better prognosis in advanced colorectal cancer patients [151]. Similarly, findings in early stages of lung cancer patients demonstrate that infiltration of TANs enhances $CD4^+$ and $CD8^+$ T cell proliferation rather than inducing immunosuppression [152]. TANs at early stage of disease secrete high levels of nitric oxide, TNF-α and H_2O_2 and exhibit cytotoxicity towards tumour cells, as noted in Lewis lung carcinoma and mesothelioma models [153]. Thus, what triggers TANs to become pro-tumorigenic in advanced stages of lung cancer but not in colorectal cancer? This could be due to cancer specific signalling pathways activated or combination of other immunosuppressive molecules secreted within the tumour microenvironment. These inconsistencies in findings warrant further studies to better understand the role of TAN in tumour growth, metastasis and recurrent disease.

1.3.3.4. Regulatory T cells

Regulatory T cells are distinct $CD4^+$ Th cell subset defined by the $CD25^+CD4^+$ phenotype, which suppress effector T cells, believed to be dependent on IL-10 and/or TGF-β [154].

Tregs are regulated by Foxp3 and hold prognostic value in several cancers [154-158]. Tregs inhibit anti-tumour responses mediating tumour escape mechanisms through the expression of well-known immunosuppressive factors, PD-1, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), lymphocyte activation genes-3 (LAG-3), IL-10 and TGF-β [36]. In fact, enhanced $Foxp3^+$ Treg infiltrates with elevated PD-L1 expression correlate with high grade, basal-like subtype, and negative estrogen receptor and progesterone receptor status [159]. This suggests that $Foxp3^+$ Tregs work synergistically with PD-L1 to endorse immune evasion in breast cancer. In peripheral blood of non-small cell lung carcinoma patients, enhanced expression of $CD4^+CD25^+Foxp3^+$ Tregs was co-expressed with immunosuppressive molecules CTLA-4, PD-1 and LAG-3 [36]. Similarly, in colorectal cancer, enhanced expression of intratumoral $CD4^+Foxp3^+$ Tregs associates with suppressive markers, CTLA-4 and ectonucleotidase CD39; whilst $CD4^+Foxp3^-$ Tregs associate with regulatory markers including LAG-3, latency-associated peptide (LAP) and CD25 [158]. Enhanced intratumoral expression of $Foxp3^+$ Tregs expressing LAP and CD39 is noted in head and neck carcinomas [160]. Enhanced expression of $CD4^+CD25^{high}Foxp3^+$ T cells expressing elevated IL-10 and decreased TGF-β and IFN-γ is reported in gastric cancer patients, challenging the theory that $CD4^+CD25^{high}$ T cells are the main makers of TGF-β [161]. These findings suggest that CD39 and CTLA-4 are commonly co-expressed on several $CD4^+Foxp3^+$ Tregs, suggesting that these markers may play an essential role in regulatory functions of Tregs *in situ*.

1.3.3.5. Myeloid-derived suppressor cells

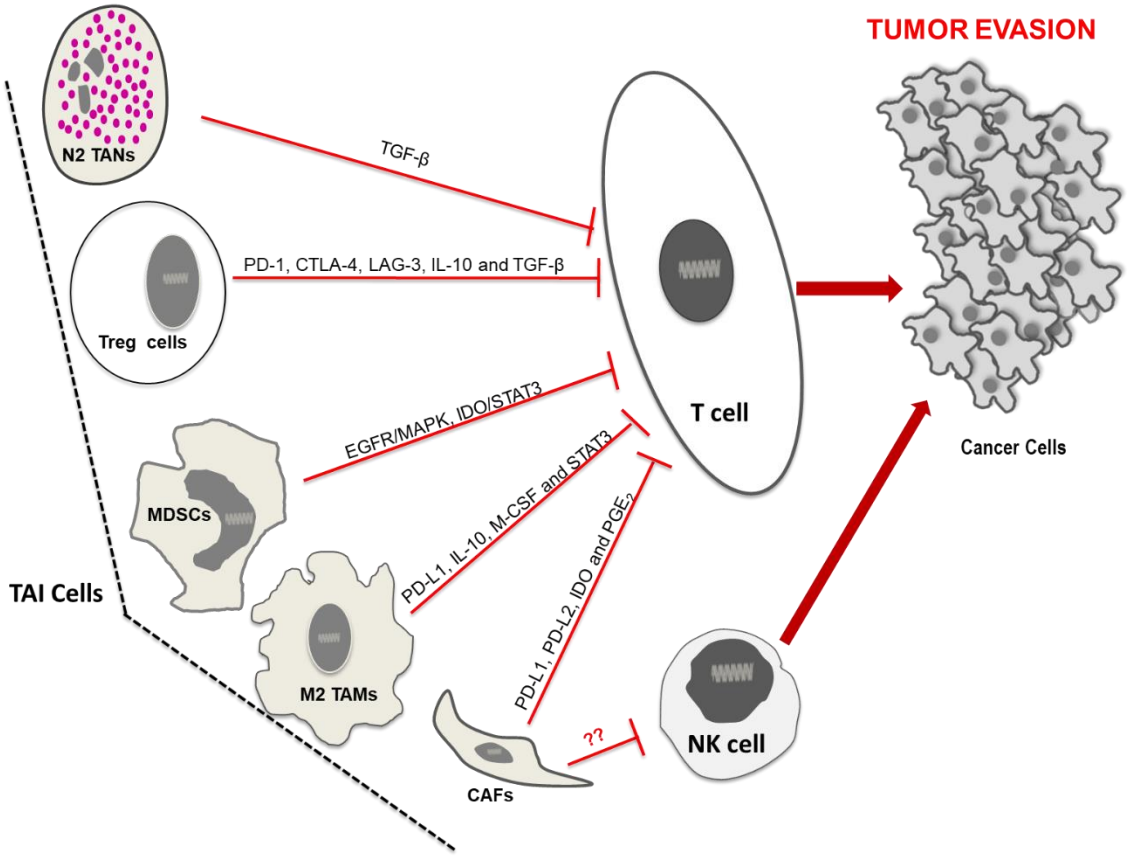
Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid progenitor cells that contribute to the development of tumour and chronic inflammation [162]. The presence of MDSCs within the tumour microenvironment induces immunosuppression as noted in several cancers including breast, colon, pancreatic and non-small cell lung cancer [32, 163-166]. MDSCs utilise several mechanisms to influence innate and adaptive immune responses, such as, inducing PD-L1 expression on tumours leading to $CD8^+$ T cell inhibition. MDSCs crosstalk with other immunosuppressive factors such as IDO and Treg cells. For instance, IDO inhibition or Treg exhaustion results in decreased MDSCs, thus reversing immunosuppression in B16 melanoma cell bearing

mice [167]. In breast cancer patients, MDSCs mediates immunosuppression via upregulating IDO expression dependent on STAT3 phosphorylation [32]. Hence, MDSCs play a significant immunosuppressive role within the tumour microenvironment leading to tumour escape from host immunity.

Figure 1.2. The role of tumour associated immune cells inducing immunosuppression

This schematic diagram illustrates tumour-associated immune cells that enhance tumour evasion discussed in this paper. Tumorigenic cells can utilise host immune cells to promote tumour progression via the expression of immunosuppressive factors.

Abbreviations: CAFs, cancer-associated fibroblasts; CTLA-4, cytoxic T lymphocyte-associated antigen-4; EGFR, epidermal growth factor receptor; IDO, indoleamine-2,3-dioxygenase; IL-10, interleukin; LAG-3, lymphocytes activation genes-3; MAPK, mitogen-activated protein kinase; MDSCs, myeloid-derived suppressor cells; NK, natural killer; PD-1, programmed death-1; PD-L1, programmed death-ligand1; PD-L2, programmed death-ligand 2; PGE_2, prostaglandins; Treg, regulatory T cell; STAT3, signal transducer and activator of transcription 3; TAI, tumour-associated immune cells; TANs, tumour-associated neutrophils; TAMs, tumour-associated macrophages; TGF-β, tumour growth factor-beta.


TUMOR EVASION
N2 TANs
TGF-β
PD-1, CTLA-4, LAG-3, IL-10 and TGF-β
Treg cells
T cell
Cancer Cells
EGFR/MAPK, IDO/STAT3
MDSCs
PD-L1, IL-10, M-CSF and STAT3
PD-L1, PD-L2, IDO and PGE2
TAI Cells
M2 TAMs
??
NK cell
CAFs

1.4. Role of the Nervous System in Tumour Angiogenesis

New growth in the vascular network (angiogenesis) is a normal physiological phenomenon that tumours utilise to aid in their growth, proliferation and metastatic spread. Angiogenesis involves migration and division of endothelial cells, generation of new basement membrane, arrangement into tubular structures and coverage by pericytes. Angiogenesis is regulated by a plethora of pro- and anti-angiogenic molecules such as, interleukin (IL)-8, tumour necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-α, TGF-β, angiogenin, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [168, 169]. The level of angiogenic factors in tissues reflects the aggressiveness of tumour cells which play a significant role in prognostic outcomes [170, 171]. In cancer, the balance between pro- and anti-angiogenic factors is lost, resulting in uncontrolled angiogenesis with irregular blood vessels lacking a clear hierarchal arrangement [168, 172]. Consequently, anti-angiogenic therapies (in particular anti-VEGF) have been approved for cancer treatment [171, 173-175]. The interaction between VEGF with its receptor, VEGFR2, is responsible for the majority of the angiogenic stimulatory signals *in vivo*, however, their therapeutic value for long-term patient survival is relatively modest [170].

In addition to these factors, the impact of the tumour microenvironment in tumour angiogenesis has attracted much interest in recent years as another regulator of angiogenesis [4, 176-178]. Furthermore, the role of the nervous system has also surfaced as one of the major contributors to cancer progression through the regulation of tumour angiogenesis via release of neurotransmitters. The nervous system governs functional activities of many organs, and, as tumours are not independent organs within an organism, this system is integrally involved in tumour growth and progression [179, 180]. Here we present an overview of the nervous system role in tumour angiogenesis.

1.4.1. Neurotransmitters influencing tumour angiogenesis

Neurotransmitters are group of neurological chemical messengers synthesised by neurons and secreted at nerve terminals where they transmit signals to target cells

through binding to their receptors. Studies have demonstrated that various cancers express receptors for different neurotransmitters, which have been identified to play essential role in the control of tumour angiogenesis (**Table 1.1**, **Figure 1.3**).

1.4.1.1. Catecholamines

Catecholamines are a group of neurotransmitters that are synthesised from amino acid tyrosine. These neurotransmitters are intricately involved in the normal physiological response of fight or flight response during stress [181, 182]. Epinephrine and norepinephrine released during chronic stress play an important role in tumorigenesis via regulation of angiogenesis through β-adrenergic signalling. The β-adrenergic signalling pathway is involved in regulation of cancer initiating factors such as apoptosis, DNA damage repair, inflammation, cellular immune response, angiogenesis and epithelial-mesenchymal transition. Numerous *in vitro* and animal studies have demonstrated that epinephrine and norepinephrine acting on their receptors expressed on tumour cells, stimulate angiogenesis via increased VEGF synthesis [181-185] through the cAMP-PKA signalling pathway [183]. In fact, activation of the β-adrenergic signalling pathway in primary mammary tumours has been shown to elevate tumour-associated macrophages (TAMs) expressing *vegf* gene which enhances angiogenesis [186]. Moreover, in some breast cancer cell lines, direct activation of β-adrenergic signalling can amplify expression of VEGF and cytokines, IL-6, and IL-8 that stimulate tumour angiogenesis [187]. Jagged 1 is essential factor mediating Notch signalling which regulates tumour angiogenesis through β2-AR-PKA-mTOR pathway. Upregulation of Jagged 1 in breast cancer patients correlates with poor prognosis [188, 189]. Knockdown of Jagged 1 by siRNA in MDA-231 breast cancer cells inhibits Notch signalling in endothelial cells and impairs tumour angiogenesis induced by norepinephrine [190].

In contrary, dopamine inhibits angiogenesis by downregulation of VEGFR-2-mediated signalling pathway in both tumour endothelial and endothelial progenitor cells through D_2 dopamine receptors (DR2) [181, 182, 191, 192]. Furthermore, in mouse models of breast cancer induced by MCF-7 cell line and colon cancer induced by HT29 cell line, dopamine administration in combination with anti-cancer drugs (e.g. doxorubicin and 5-fluorouracil) impairs tumour growth and improves survival outcome [193]. However,

dopamine effect was found to have no direct impact on tumour growth and survival but by inhibiting tumour endothelial cell proliferation and migration via the suppression of VEGFR-2 and mitogen-activated protein kinase as demonstrated *in vitro* [193]. In tissues from gastric cancer patients and in rats with chemically-induced as well as mice with Hs746T cell-induced gastric cancer, administration of dopamine decelerates tumour growth by suppressing angiogenesis via inhibition of VEGFR-2 phosphorylation in endothelial cells [194]. This concurs with results obtained in ovarian cancer mouse models induced by systemic injection of SKOV3ip1 and HeyA8 cells in which exogenous administration of dopamine inhibits angiogenesis by a stimulation of DR2, however stimulation of DR1 stabilises tumour blood vessels via cAMP-PKA signalling pathway [195].

1.4.1.2. Acetylcholine and nicotine

Nicotinic acetylcholine receptors (nAChRs) can have either stimulatory or inhibitory effect on the production and release of angiogenic factors [196]. Indeed, the expression of VEGF, TGF-β, FGF and PDGF in endothelial cells is increased by nicotine [197-200]. Nicotine-mediated angiogenesis via activation of α7 and α9-nAChRs is cell-type specific, e.g. in lung cancer cells angiogenesis is promoted via activation of α7-nAChRs [200, 201], whereas in breast tumours overexpression of α9-nAChRs [202] stimulates release of pro-angiogenic factors [203]. In colon tumour tissues from HT-29 cell-bearing BALB/c mice, VEGF expression is elevated by nicotine which correlates with enhanced microvessel density [204]. The molecular pathways of nicotine-induced angiogenesis have been extensively reviewed [205]. The role of muscarinic acetylcholine receptors (mAChRs) in tumour angiogenesis is not well understood, however administration of autoantibodies against mAChRs in mouse models of breast cancer (**Table 1.1**) mediates tumour angiogenesis via activation of mAChRs through release of VEGF-A [206]. In addition, in BALB/c mice bearing LMM3 mammary adenocarcinoma cells, administration of muscarinic agonist, carbachol, in the presence or absence of various muscarinic antagonists shows an increase in VEGF expression [207, 208]. Furthermore, tumour macrophages stimulate angiogenesis via activation of M1 and M2 mAChRs which trigger arginine metabolic pathway [207].

1.4.1.3. Gamma-Aminobutyric acid, neuropeptide Y, nitric oxide and serotonin

Gamma-Aminobutyric acid (GABA), neuropeptide Y (NPY), nitric oxide (NO) and serotonin have varying effects on angiogenesis and tumour progression. In a mouse model of cholangiocarcinoma, GABA inhibits VEGF-A/C, decreases cell proliferation and tumour mass [209]. NPY enhances the expression of VEGF and its secretion, promoting angiogenesis and breast cancer progression [210]. The suggested mechanism by which NPY induces angiogenesis is by its influence on endothelial cells dependent on endothelial nitric oxide synthase (eNOS) activation and partly on VEGF signalling pathway The release of NO results in endothelial activation inducing tumour cells lysis [211], although NO can also promote tumour growth and metastasis by enhancing angiogenesis [211-218]. For instance, NO increases VEGF-C and nitrite/nitrate production in MDA-MB-231 breast cancer cells and high levels of nitrotyrosine correlate with increased VEGF-C, lymph node metastasis, reduced disease-free and overall survival in invasive breast carcinoma [219]. The expression of iNOS and VEGF in colorectal cancer correlates with enhanced intratumour micro-vessel density suggesting that NO can promote tumour angiogenesis [212]. In gastric cancer, overexpression of NOS III via abnormal activation of sequence-specific DNA-binding protein (Sp1) correlates with enhanced micro-vessel density and poor survival [220]. Serotonin has also been implicated in tumour angiogenesis. In C57BL/6 mice bearing MC-38-induced tumours, serotonin regulates angiogenesis by plummeting matrix metalloproteinase 12 (MMP-12) expression (e.g. [221]) in macrophages infiltrating the tumour, as well as reducing angiostatin (an endogenous inhibitor of angiogenesis) levels [222].

1.4.1.4. Glutamate

Glutamate is an excitatory neurotransmitter that regulates synaptic and cellular activity via binding to its receptors including metabotropic glutamate receptors (mGluRs). The expression of mGluRs has been implicated in tumour angiogenesis as noted in mouse models of melanoma and breast cancer [223-225]. As such, decreased activity of mGluR1 inhibits angiogenesis in an orthotopic breast cancer (4T1) model suggesting that mGluR1 acts is a pro-angiogenic and pro-tumorigenic factor [223]. Likewise, in an experimental

non-small cell lung cancer in A549-bearing nude mice, inhibition of mGlu1 receptor with BAY36-7620 led to suppression of angiogenesis via inhibiting AKT/HIF-1α/VEGF signalling pathway [226]. Similarly, high expression of glutamate receptor GRM1 in several human melanoma cell lines (**Table 1.1**) leads to increased expression of IL-8 and VEGF via activation of the AKT/mTOR/HIF1 signalling pathway [225].

Hence, these studies clearly demonstrate involvement of neurotransmitters in tumour angiogenesis; however, most of the studies have been performed mainly in animal models and cell lines. Understanding their relevance to human pathology may aid in the development of better anti-angiogenic therapies.

Table 1.1. Neurotransmitters influencing tumour angiogenesis

Neurotransmitters	Receptor	Type of cancer	Model	Mechanism/pathway	Ref.
NE	β2-AR	Breast cancer	MCF-7, MDA-453, and MDA-231 cell lines, subcutaneous injection of 4T1 cells in BALB/c mice	β2-AR expression is elevated in MDA-453, decreased in MCF-7 and intermediate in MDA-231 cells. Administration of β-AR agonist, isoproterenol upregulates Jagged 1 expression and enhances tumour microvessel density via NE-induced β2-AR/PKA/mTOR pathway *in vivo*.	[190]
		Colorectal cancer	HT-29 and CT26 cells *in vitro* and subcutaneous injection of HT-29 cells in nude mice and CT26 cells in BALB/c mice	Activation of β2-AR by NE enhances expression of VEGF, IL-8 and IL-6 *in vitro* and *in vivo* → stimulation of tumour angiogenesis via β-AR -cAMP-PKA signalling pathway.	[184]
		Melanoma	B16-F1 cells *in vitro* and subcutaneous injection in the flanks of C57BL/6 mice		[227]
		Lung adenocarcinoma	A549 cells *in vitro*		

DA		Ovarian cancer	SKOV3p 1, HeyA8 cells *in vitro* and intraperitoneal injection of these cells in a chronic stress C57BL/6 mouse model	Activation of DR2 mediates inhibitory effect of DA on tumour angiogenesis cAMP-PKA signalling pathway.	[195, 228]
	DR1 & DR2	Gastric cancer	Human gastric cancer tissues, subcutaneous injection of Hs746T cells in nude mice, MNNG-induced gastric cancer in rats	DA suppresses gastric cancer growth by inhibition of VEGF-stimulated angiogenesis. In both human gastric cancer and MNNG-induced animal model, DA is depleted. Suppression of VEGFR-2 phosphorylation in endothelial cell → inhibition of angiogenesis.	[194]
		Lung cancer	Orthotopic injection of LLC1 cells in C57BL/6 mice and A549 cells in SCID mice	Administration of DR2 agonists inhibits *in vivo* lung tumour progression via suppressing angiogenesis and reducing myeloid-derived suppressor cells infiltration.	[229]
GABA	GABA	Cholangiocarcinoma	H-69, Mz-ChA-1, HuH28, and TFK-1 cells, subcutaneous	$GABA_A$, $GABA_B$, and $GABA_C$ receptors were expressed by cells *in vitro,* which inhibit cell growth	[209]

			injection of Mz-ChA-1 cells in BALB/c mice	and proliferation via IP3 /cAMP, PKA phosphorylation, and ERK1/2 dephosphorylation. GABA ↓ tumour size and VEGF-A/C expression *in vivo*.	
	$GABA_A$	Ovarian cancer	OVCAR-3 cells *in vitro*	↑ Level of GABARBP inhibited VEGF expression and ↓ HIF-1α protein via PI3K-mTOR-4E-BP1 signalling pathway *in vitro*.	[230]
5-HT	5-HT receptor	Colon cancer	Subcutaneous injection of MC-38 cells in *Tph1*$^{-/-}$ mice	5-HT regulates angiogenesis by reducing MMP-12 expression in TAMs, thus affecting the production of circulating angiostatin.	[222]
Glu	mGluR1 on endothelial cells	Breast cancer	4T1 cells injected into the mammary fat pads of BALB/c mice	↓mGluR1 expression results in ↓ angiogenesis *in vivo*.	[223]
	GRM1	Melanoma	UACC903-G2, UACC903-G4, C8161-G21, C81-61-G6, and C81-61-G7 cells, subcutaneous injection of these cells into each flank of nude mice	*In vitro* ↑ expression of GRM1 → ↑ expression of IL-8 and VEGF via the AKT-mTOR-HIF1 signalling pathway activation. *In vivo* ↑ expression of GRM1 → larger melanoma tumours.	[225]

ACh	α7-nAChRs	Lung cancer	Human NSCLC A549 and H157 cell lines	Nicotine increases HIF-1 & VEGF expression. Nicotine mediates tumour angiogenesis through PI3K/Akt and ERK1/2 signalling pathway.	[231]
		Colon cancer	Subcutaneous injection of HT-29 cells in BALB/c mice	Administration of nicotine ↑ VEGF expression → ↑ microvessel densities and angiogenesis via stimulation of β2-AR.	[204]
	mAChR	Breast cancer	Intradermal injection of MCF-7 and MCF-10A cells in nude mice, intradermal injection of LMM3 cells in BALB/c mice	mAChR activation promotes VEGF-A production and neovascularisation in breast cancer models.	[206]
		Mammary adenocarcinoma	Subcutaneous injection of LMM3 cells in BALB/c mice	↑ Expression of VEGF by activation of M1 and M2 mAChRs via arginine metabolic pathway.	[207]
NPY	Y5R	Breast cancer	4T1 cell line	Activation of NPY ↑ the expression and secretion of VEGF → angiogenesis.	[210, 232]
	Y2R	Melanoma	Subcutaneous injection of B16F10 cells into C57BL/6 mice	Blockade of the Y2R inhibited tumour growth by ↓ tumour angiogenesis.	[233]

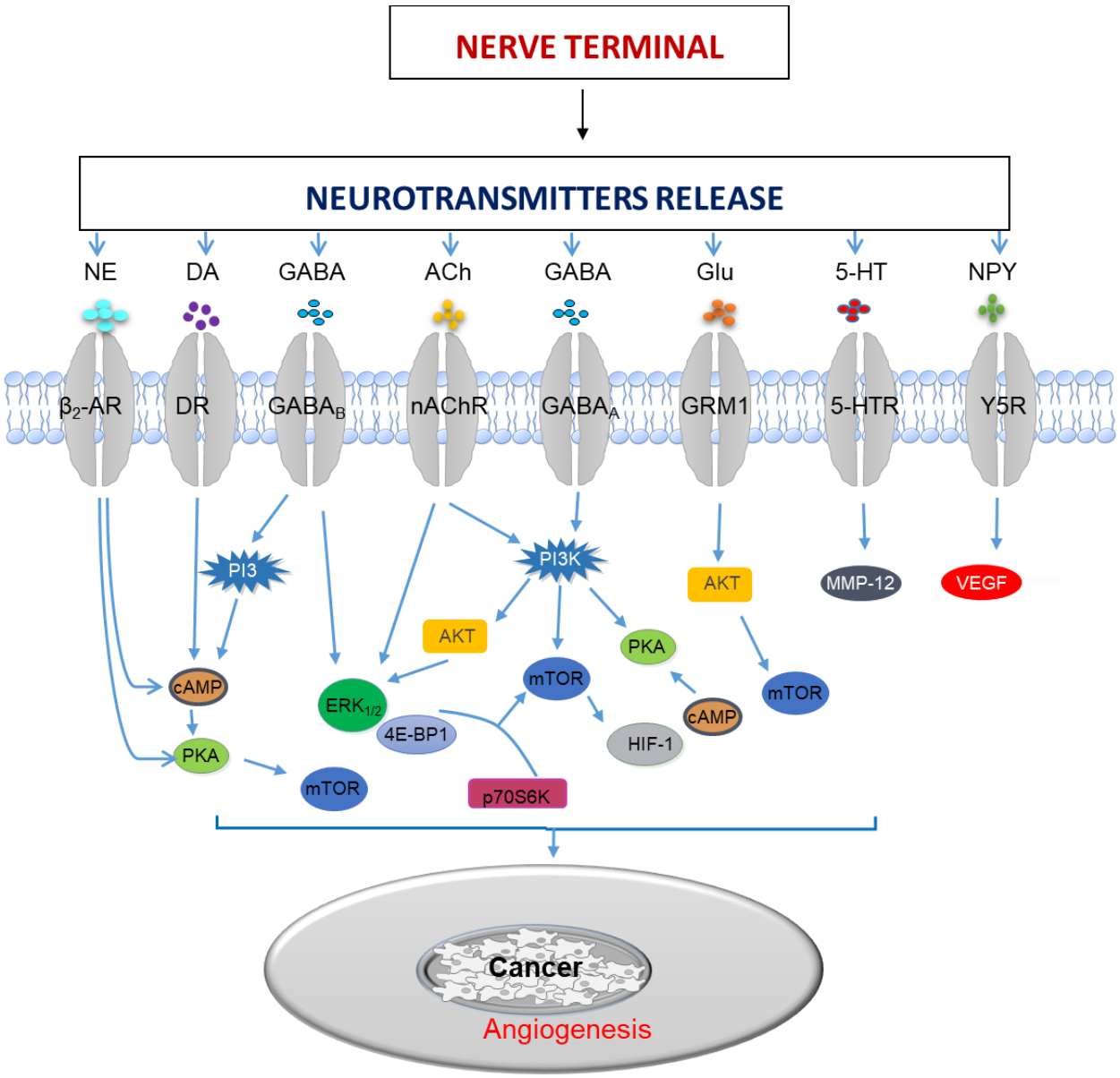
	Neuroblastomas	Human tissue	Y2R expression is observed in both tumour and endothelial cells.	[234]
NO	Breast cancer	MDAMB-231cell and human invasive breast cancer tissues	NO induces the expression of VEGF-C in both breast cancer cell line and human tissues.	[219]
	Ovarian cancer	Cystic fluid samples and human tissues	The expression of iNOS correlates with the degree of tumour differentiation; level of intracystic NO metabolite correlates with tumour stage.	[217]
	Gastric cancer	Human tissues (all stages)	NOS III protein is ↑ in both primary gastric tumours and lymph node metastases.	[220]

Abbreviations: α7nAChR, α7 nicotinic acetylcholine receptor; ACh, acetylcholine; β2-AR, β_2-adrenergic receptor; cAMP, cyclic adenosine monophosphate; DA, dopamine; DR1 & DR2, dopamine receptor 1 & 2; $ERK_{1/2}$, extracellular signal-regulated kinase; GABA, gamma-aminobutyric acid; Glu, glutamate; GABARBP, $GABA_A$ receptor-binding protein; mGluR1, metabotropic glutamate receptor 1; GABAA,B&C, gamma-aminobutyric acid receptor A,B&C; GRM1, glutamate receptor metabotropic 1; HIF-1α, hypoxia inducible factor-1alpha; 5-HT, 5-hydroxytryptamine (serotonin); iNOS, inducible nitric oxide synthase; IL-6, interleukin 6; IL-8, interleukin 8; mTOR, mammalian/mechanistic target of rapamycin; MMP12, matrix metallopeptidase 12; mAChRs, muscarinic acetylcholine receptors; M1 & M2, muscarinic 1 & 2 receptors; NPY, neuropeptide Y; Y2R & Y5R, neuropeptide Y receptor 2 & 5; MNNG, N-methyl N'-nitro-N-nitrosoguanidine; NO, nitric oxide; NOS, nitric oxide synthase; NSCLC, non-small cell lung carcinoma; NE, norepinephrine; PI3K, phosphoinositide 3-kinase; 4E-BP1, phosphorylated 4E binding protein 1; PKA, protein kinase A; AKT, serine/threonine kinase or protein kinase B; TAMs, tumour-infiltrating macrophages; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Figure 1.3. Neurotransmitter signalling pathways in cancer angiogenesis

Neuro-cancer communication is through the release of neurotransmitters activating different signalling kinases which promote cancer progression via angiogenesis.

Abbreviations: Ach, acetylcholine; β2-AR, β_2-adrenergic receptor; cAMP, cyclic adenosine monophosphate; DA, dopamine; DR, dopamine receptor; $ERK_{1/2}$, extracellular signal-regulated kinase; GABA, gamma-aminobutyric acid; GABAA&B, gamma-aminobutyric acid receptor A&B; Glu, glutamate; GRM1, glutamate receptor metabotropic 1; HIF-1, hypoxia inducible factor 1; 5-HT, 5-hydroxytryptamine (serotonin); 5-HTR, 5-hydroxytryptamine receptor (serotonin); mTOR, mammalian/mechanistic target of rapamycin; MMP12, matrix metallopeptidase 12; NPY, neuropeptide Y; Y5R, neuropeptide receptor; nAChR, nicotinic acetylcholine receptor; NE, norepinephrine; PI3, phosphoinositide 3; PI3K, phosphoinositide 3-kinase; 4E-BP1, phosphorylated 4E binding protein 1; PKA, protein kinase A; p70S6K, serine/threonine kinase; AKT, serine/threonine kinase or protein kinase B; VEGF, vascular endothelial growth factor.


NERVE TERMINAL
NEUROTRANSMITTERS RELEASE
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GABAA
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Y5R
PI3
PI3K
AKT
MMP-12
VEGF
AKT
PKA
cAMP
ERK1/2
mTOR
mTOR
cAMP
4E-BP1
HIF-1
PKA
mTOR
p70S6K
Cancer
Angiogenesis

1.4.2. Other factors influencing tumour angiogenesis

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF)**,** angiogenin (ANG), FGF, TNF-α, TGF-β, hepatocyte growth factor (HGF) and epidermal growth factor receptor (EGF) are important signalling molecules promoting angiogenesis (**Table 1.2**, **Figure 1.4**). NGF is a neurotrophic factor that is upregulated in tumour microenvironment of various cancers including breast cancer [235]. NGF, secreted by MDA-MB-231 breast cancer cells, stimulates angiogenesis *in vivo* after injection of these cells subcutaneously to immunodeficient mice and enhances endothelial cell proliferation, invasion, migration and tubule formation *in vitro* [235]. Furthermore, NGF enhances secretion of VEGF by breast cancer cells; *in vivo* administration of anti-VEGF antibody inhibits its angiogenic capacity [235]. In human glioma microvascular endothelial cells, NGF mediates tumour angiogenesis by interaction with α9β1 integrin [236-239]. Another neurotrophic factor, BDNF has been shown to play a role in tumour angiogenesis. For instance, in chondrosarcoma patients, BDNF and VEGF protein expression is significantly higher which is correlated with tumour stage [240]. Furthermore, BDNF knockdown decreases the expression of VEGF and abolishes angiogenesis in *in vitro* studies and animal models of chondrosarcoma [240].

In addition to neurotrophic factors, angiogenic factor ANG is upregulated in number of cancers [241-243] and is associated with worse clinical prognosis in urothelial carcinoma patients [244]. ANG regulates tumour angiogenesis via activation of endothelial and smooth muscle cells triggering various molecular pathways involved in the initiation of angiogenesis (Fig. 2) [245-248]. Elevated expression of ANG associates with high grade and muscle-invasive human bladder tumours involving increase p-ERK1/2 and MMP2 expression [247]. Similarly, downregulation of ANG inhibits tumour angiogenesis via AKT/GSK3β/ mTOR pathways [248]. FGF is involved in angiogenesis by suppressing VEGF-C expression and stimulating expression of pro-lymphangiogenic factors including integrin α9, VEGFR-3, prox1 and netrin-1 [249]. In fact, blocking of FGF2 with anti-FGF2 monoclonal antibody results in impaired angiogenesis of B16-F10 cell induced melanoma in mice [250]. In addition, TNF-α binding to TNFR1/p55 and TNFR2/p57 receptors has been implicated in the secretion of cytokines and pro-angiogenic factors [251]. For

example, blocking p75 by short-hairpin RNA in cultured Lewis lung carcinoma cells results in decreased TNF-mediated expression of VEGF, placental growth factor and HGF, suggesting that p75 is essential factor for tumour angiogenesis [251]. Similarly, blocking TNF-α inhibits angiogenesis in metastatic oral squamous cell carcinoma cells (sh-IFIT2 meta cell) in NOD/SCID mice [252]. TGF-β negatively regulates VEGF-A expression via a PKA- and Smad2-independent and Smad3-dependent pathways as demonstrated in FETα/DNRII colon cancer cell lines [253]. HGF is an angiogenic factor secreted predominantly by fibroblasts; it stimulates invasiveness of cancer cells via c-Met receptor tyrosine kinase activation [254-256]. In fact, high HGF serum levels is correlated with VEGF and IL-8 expression, advanced tumour stage and poor survival of esophageal squamous cell carcinoma (ESCC) patients [257]. High expression of another pro-angiogenic factor, EGFR correlates with increased microvessel density resulting in enhanced tumour angiogenesis via the HIF-1α and Notch1 pathways in tissues from head and neck squamous cell carcinoma patients [258]. Neuropilin is a transmembrane glycoprotein, which serves as a receptors or co-receptor for multiple ligands including VEGF, HGF, EGF and FGF, which are involved in tumour angiogenesis [259, 260]. In gastric cancer, high expression of neuropilin correlates with advanced clinical stages (III and IV) [261]. Depletion of neuropilin-1 inhibits the activation of EGF/EGFR, VEGF/VEGFR2 and HGF/c-Met angiogenic pathways activated by recombinant human VEGF-165, HGF and EGF proteins [255, 261]. Thus, the role of neurotrophic factors such as NGF, BDNF and their molecular pathways should be considered in the development of anti-angiogenic therapies.

Table 1.2. Other factors influencing tumour angiogenesis

Factors	Type of cancer	Model	Mechanism/pathway	Ref.
ANG	Breast cancer	Human tissues	The level of ANG correlates with clinical progression. ANG derived from tumours activates angiogenesis via suppression of miR-543-2p.	[246]
	Bladder cancer	Human tissues, T24, UROtsa and HeLa cells subcutaneously injected in athymic BALB/c (nu/nu) mice	↑ ANG expression correlates with high grade, and muscle-invasive tumours via ERK 1/2 and MMP2. Downregulation of ANG inhibits tumour angiogenesis via AKT/GSK3β/ mTOR pathways.	[247, 248]
TNF-α	Lung cancer	LLC1 cells subcutaneously injected in wild type, p75 knockout (KO) and double p55KO/p75KO mouse xenograft models	Tumour growth ↓ in both LLC and B16 p75KO mice. Decreased tumour growth correlates with ↓ VEGF expression and capillary density via TNFR2/p75.	[251]
	Melanoma	B16 cell subcutaneously injected in C57BL/6 mice. Wild type, p75 knockout (KO) and double p55KO/p75KO mouse tumour xenograft models		

TGF-β	Colon cancer	Human tissues, FETα/DNRII cell	TGF-β signalling is inversely correlates with the expression of VEGF-A in tissues. TGF-β ↓ VEGF-A expression via ubiquitination and deterioration in a PKA- and Smad3-dependent and Smad2-independent pathways *in vitro*.	[253]
BDNF	Chondrosarcoma	JJ012 cell line, JJ012 cells subcutaneously injected in CB17-SCID mice	The expression of BDNF and VEGF correlates with tumour grade. BDNF knockdown ↓ angiogenesis and tumour growth in mouse model. BDNF ↑ expression of VEGF and stimulates angiogenesis via the TrkB receptor, PKCα, PLCγ and HIF-1α signalling pathways.	[240]
FGF	Mammary cancer	Mouse 66c14 mammary carcinoma and inguinal mammary fat pad injection in BALB/c mice	In tumour cells suppression of FGFR signalling inhibits expression of VEGF-C and induces VEGFR-3, netrin-1, prox1 and integrin α9 expression.	[249]
	Glioma	Rat C6 glioma cancer cells injected subcutaneously into rats		

EGFR	HNSCC	Human tissues, CAL27 cells subcutaneously injected in nude mice	In human tissues, ↑ EGFR correlates with ↑ HIF-1α and microvessel density. EGFR inhibitors ↓ the regulation of HIF-1α & Notch1 → ↓ angiogenesis and tumour size.	[258]
NGF	Breast cancer	MDA-MB-231 cells subcutaneously injected into SCID mice	NGF ↑ the release of VEGF in breast cancer cells and mediates angiogenic effect via the activation of PI3K-Akt, ERK, MMP2 and NO synthase pathways.	[235]
HGF	ESCC	Serum samples, human tissues, HKESC-1, HKESC-2 and SLMT cells	In tissues, ↑ level of HGF correlates with tumour metastasis and poorer survival. In serum samples, ↑ HGF level correlated with expression of VEGF and IL-8. HGF stimulates cells to express VEGF and IL-8 *in vitro* via extracellular signal-regulated kinase signalling pathways.	[257]
	Prostate cancer	Castration-resistant prostate cancer blood samples and PC3 cell line	HGF levels ↑ in both blood samples and cell line.	[254]

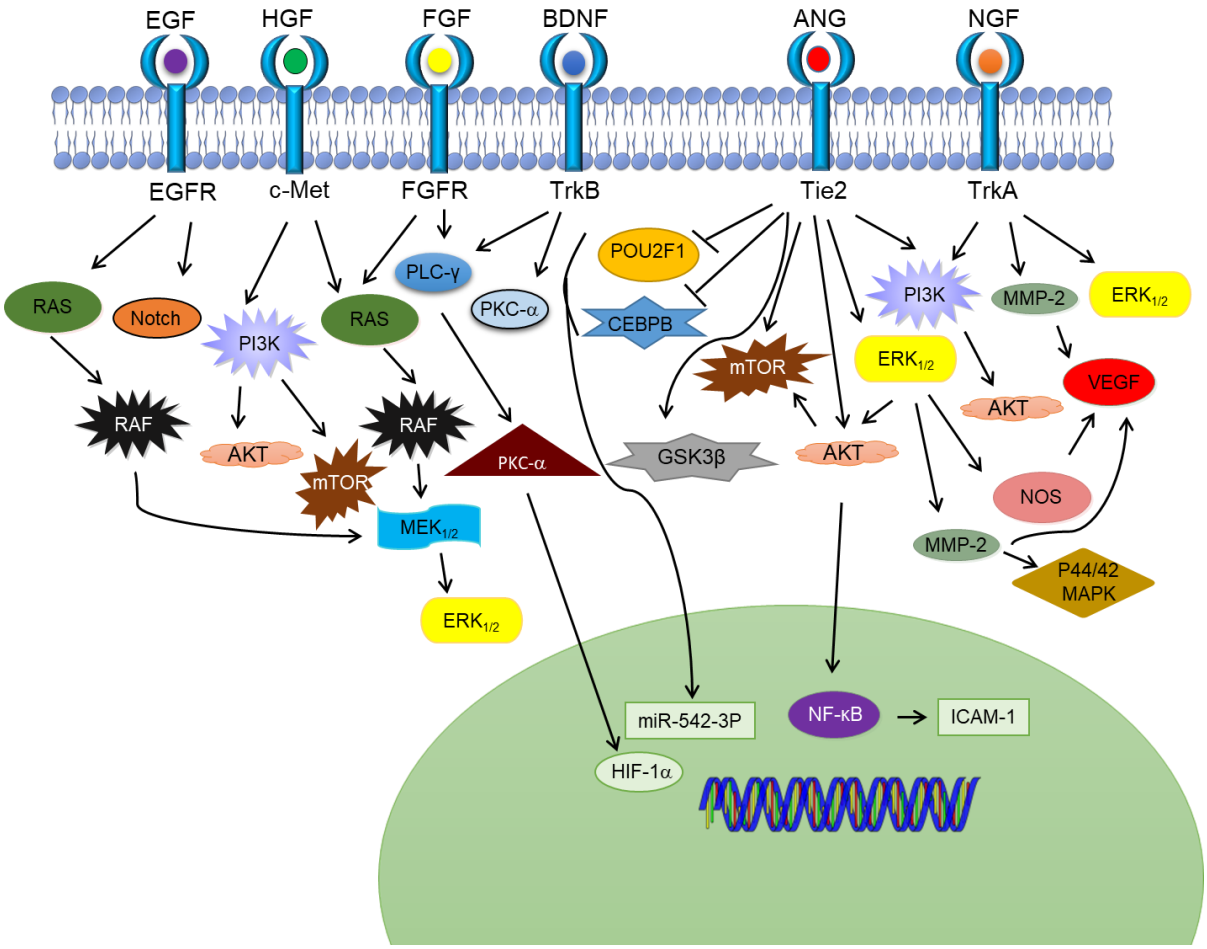
Abbreviations: ANG, angiogenin; BDNF, brain-derived neurotrophic factor; EGFR, epidermal growth factor receptor; ESCC, esophageal squamous cell carcinoma; $ERK_{1/2}$, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GSK3β,

glycogen synthase kinase 3β; HNSCC, head and neck squamous cell carcinoma; HGF, hepatocyte growth factor; HIF-1α, hypoxia inducible factor 1α; IL-8, interleukin-8; mTOR, mammalian/mechanistic target of rapamycin; MMP2, matrix metalloprotease 2; NGF, nerve growth factor; NO, nitric oxide; PI3K, phosphoinositide 3-kinase; PLCγ, phospholipase Cγ; PKA, protein kinase A; PKCα, protein kinase C alpha; AKT, serine/threonine kinase or protein kinase B; TNF-α, tumour necrosis factor alpha; TNFR2/p75, tumour necrosis factor receptor 2/neurotrophin receptor; TGF-β, transforming growth factor beta; TrkB, tropomyosin related kinase B; VEGF, vascular endothelial growth factor.

Figure 1.4. Growth factors intracellular signalling pathways in cancer angiogenesis

The binding of growth factors to their respective receptors (eg, EGF to EGFR) activates multiple kinase pathways which are involved in cancer angiogenesis.

Abbreviations: ANG, angiogenin; Tie2, angiopoietin receptor 2; BDNF, brain-derived neurotrophic factor; CEBPB, CCAAT/enhancer-binding protein beta; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; $ERK_{1/2}$, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GSK3β, glycogen synthase kinase 3 beta; HGF, hepatocyte growth factor; c-Met, hepatocyte growth factor receptor; HIF-1 α, hypoxia inducible factor 1 alpha; ICAM-1, intercellular adhesion molecule-1; mTOR, mammalian/mechanistic target of rapamycin; MMP2, matrix metallopeptidase 2; $MEK_{1/2}$, MAPK/ERK kinase; MAPK, mitogen activated protein kinase; RAS, mitogen activated protein kinase; RAF, mitogen activated protein kinase; NGF, nerve growth factor; NOS, nitric oxide synthase; NF-kB, nuclear factor-kappa B; PLC-γ, phospholipase C-gamma; PI3K, phosphoinositide 3-kinase; POU2F1, POU domain class 2 transcription factor 1; PKC-α, protein kinase C alpha; AKT, serine/threonine kinase or protein kinase B; TrkA, tropomyosin related kinase A; TrkB, tropomyosin related kinase B; VEGF, vascular endothelial growth factor.


EGF
HGF
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BDNF
ANG
NGF
EGFR
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FGFR
TrkB
Tie2
TrkA
RAS
Notch
PI3K
RAS
PLC-γ
PKC-α
POU2F1
CEBPB
mTOR
PI3K
MMP-2
ERK1/2
ERK1/2
VEGF
AKT
RAF
RAF
AKT
mTOR
PKC-α
GSK3β
AKT
NOS
MMP-2
P44/42 MAPK
MEK1/2
ERK1/2
miR-542-3P
NF-κB
ICAM-1
HIF-1α

1.5. Role of the Nervous System in Cancer Metastasis

1.5.1. The role of the nervous system in metastatic cascade

Studies have demonstrated that the nervous system facilitates development of tumour metastasis by modulating metastatic cascades through the release of neural-related factors from nerve endings such as neurotrophins, neurotransmitters and neuropeptides [262-264]. The process of metastasis formation involves tumour cells breaking away from the primary tumour and overcoming the obstacles of primary tissue inhibition (initiation and clonal expansion), anoikis inhibition (evasion from apoptosis), breakdown of base membranes [epithelial-mesenchymal transition (EMT) and invasion], extravasation and colonisation, angiogenesis, evasion of immune response and establishment of tumour microenvironment.

1.5.1.1 Initiation and clonal expansion

Tumour metastasis initiation and clonal expansion is a complex process where contributing factors are not well understood. It is believed that metastasis process is initiated when genetically unstable tumour cells adjust to a secondary site microenvironment [177]. This process involves selecting traits that are beneficial to tumour cells and affiliated recruitment of traits in the tumour stroma that accommodate invasion by metastatic cells. Metastasis-initiating cells possess these traits and can hijack some of the normal stem cell pathways to increase cellular plasticity and stemness [265]. Proteolytic enzymes such as matrix metalloproteinases (MMPs) facilitate this process by degrading the surrounding normal tissues. MMPs are regulated by neural-related factors and neurotransmitters and are overexpressed in tumours [266-270]. Hence, nervous system stimulates the initiation and clonal expansion via the expression of MMPs and the stimulation of metastasis-initiating cells.

1.5.1.2 Evasion from apoptosis

Anoikis is a programmed cell death induced upon cell detachment from extracellular matrix, acting as a critical mechanism in preventing adherent-independent cell growth and

attachment to unsuitable matrix, thus avoiding colonising of distant organs [271, 272]. For tumour metastasis to progress, tumour cells must be resistant to anoikis. Tumour cell resistance to anoikis is attributed to alteration in integrins' repertoire, overexpression of growth factor receptor, activation of oncogene, activation of pro-survival signals, or upregulation/mutation of key enzymes involved in integrin or growth factor receptor signalling [272]. Neurotransmitters and neurotrophins play a role in tumour evasion from anoikis. Increased expression of brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) induces anoikis inhibition in rat intestinal epithelial cells [262]. Similarly, TrkB overexpression induces anoikis inhibition protecting colorectal cancer cells [273]. Application of recombinant human BDNF to gastric cancer cells inhibited anoikis and stimulated cellular proliferation, invasion and migration [274]. Nicotine exposure promotes anchorage-independent growth of A549, MDA-MB-468 and MCF-7 cell lines by downregulation of anoikis [275]. Furthermore, tumour microenvironment contributes to anoikis resistance of cancer cells by producing pro-survival soluble factors, triggering EMT, enhancing oxidative stress, regulating matrix stiffness, as well as leading to metabolic deregulations of cancer cells [272]. These events assist tumour cells to prevent the apoptosis mechanism and sustain pro-survival signals after detachment, counteracting anoikis.

1.5.1.3 EMT and invasion

EMT is a fundamental process for tumour progression by increasing invasiveness and resistance to anoikis and significantly elevating the production of extracellular matrix constituents leading to metastasis [276-278]. EMT development results in the degradation of basement membrane and formation of mesenchymal-like cells [277]. Studies have demonstrated that nervous system regulates EMT development via the release of neurotransmitters and neurotrophins [275, 279]. The overexpression of TrkB or activation by BDNF in human endometrial cancer cell lines results in altered expression of EMT molecular mediators [279]. Nicotine treatment induces changes in gene expression associated with EMT in lung and breast cancer cells [275].

1.5.1.4 Extravasation and colonisation

Nervous system stimulates the function of vascular system, which is essential for tumour cell extravasation and colonisation. It has been found that neuropeptides such as substance P (SP) and bradykinin enhance vascular permeability promoting tumour cell extravasation and colonisation [263, 264]. In a mouse model bearing sarcoma 180 cells, bradykinin enhances tumour-associated vascular permeability [263]. SP regulates physiological functions of vascular system including smooth muscle contractility, and vascular permeability [264]. Cell extravasation and colonisation are prerequisites for angiogenesis, which is a crucial step in the development of cancer metastasis.

1.5.1.5 Angiogenesis

Development of tumour angiogenesis is essential for tumour growth and progression. Vascular endothelial growth factor (VEGF) plays significant role in tumour angiogenesis, leading to metastasis [280-282]. Studies have demonstrated the important role of neurotransmitters and neuropeptides in regulating angiogenesis. In the xenograft models of ovarian cancer, chronic stress mediates the vascularisation of intraperitoneal metastasis and enhances tumour angiogenesis via increasing VEGF expression [183, 283]. In breast cancer cell lines, direct activation of β-adrenergic signalling can amplify expression of VEGF and cytokines, interleukin (IL)-6, and IL-8 that stimulate tumour angiogenesis [187]. In colon tumour tissues from HT-29 cell-bearing BALB/c mice, VEGF expression is elevated by nicotine which correlates with enhanced microvessel density [204]. Neuropeptide Y (NPY) enhances the expression of VEGF and its secretion promoting angiogenesis and breast cancer progression [210].

1.5.1.6 Evasion of immune response

The nervous system plays a fundamental role in regulating immune responses [284]. Inflammatory mediators can activate sensory nerves that send signals regarding inflammation to the central nervous system, which in turn leads to the release of neuromediators modulating local inflammation and influencing immune cells [285]. Since inflammatory signals are important for tumour progression in both the early and late

stages, the anti-inflammatory role of the vagus nerve may play an important role in cancer metastasis [286]. β-adrenergic receptor agonist suppressed natural killer (NK) cell activity resulting in increased lung metastasis in murine metastatic mammary adenocarcinoma [287]. In addition, pharmacological or stress-associated β-adrenergic stimulation results in increased macrophage infiltration and cancer metastasis in breast cancer model [186].

1.5.1.7 Tumour microenvironment

Tumour microenvironment (mainly contain stromal cells and signal molecules) plays essential role in the formation of cancer metastasis. Stromal cells produce neural-related factors and express β-adrenergic receptor that facilitated tumour cell proliferation and survival in the primary site and secondary organ [176, 288]. Tumour-associated macrophages play a role in β-adrenergic signalling pathways, by accelerating angiogenesis, chemokine secretion to attract tumour cells, secretion of pro-inflammatory cytokines (IL-1, IL-6, IL-8, and tumour necrosis factor (TNF)-α) and escape of anti-tumour responses [289-291]. Hence, tumour microenvironment creates a feedback loop with the nervous system enabling the growth of primary and secondary tumours. Overall, these studies have demonstrated that the nervous system stimulates each step of cancer metastasis through the release of neural-related factors.

1.5.2. Role of perineural invasion in cancer metastasis

Perineural invasion (PNI) also known as neurotropic carcinomatous spread is a process mainly categorised by neoplastic invasion of the nerves. PNI is defined as the presence of cancer cells in the perineurium; it is believed to be a common route for cancer metastasis can cause cancer-related pain [292-299]. The presence of PNI is mostly associated with poor prognosis and high recurrence in colorectal [300], gastric [295], oral tongue squamous cell carcinoma (OTSCC) [293], and pancreatic [292] cancers. In stage II and III colorectal cancer patients, the presence of PNI is associated with tumour grade, metastasis to lymph nodes and poor patient survival [294]. However, in invasive breast carcinoma the presence of PNI has been demonstrated to have no prognostic value [298, 301].

PNI is influenced by the interaction between the nerve microenvironment and neurotrophic molecules expressed by cancer cells such as nerve growth factor (NGF), BDNF, glial cell line-derived neurotrophic factor (GDNF) and their receptors [292, 299, 302]. A number of studies demonstrated correlation between the presence of PNI with high expression of NGF and its receptor tropomyosin related kinase A (TrkA) [292, 303, 304]. It is speculated that neurotrophins released by neural tissue act as chemotactic factors, and in cancer cells where TrkA are overexpressed, they provide mechanism to invade the perineural space. High expression of NGF or TrkA and $P75^{NTR}$ receptors is associated with lymph node metastasis in a mouse model of breast cancer [305]. In OTSCC patients [306], the presence of PNI and NGF is associated with larger tumour size and lymph node metastasis, suggesting that its presence can be a valuable marker to predict the disease progression and prognosis [296]. Overexpression of TrkA associates with enhanced growth, invasion and migration of breast cancer cells *in vitro* as well as enhanced metastasis in xenografted immunodeficient mice via the PI3K-AKT and ERK/P38 MAP kinases [307]. Conversely, immuno-histochemical evaluation of tissues from patients with extrahepatic cholangiocarcinoma shows that intra-tumoral NGF expression does not correlate with PNI, absence of disease recurrence and overall patient survival [308]. GDNF has been demonstrated to induce cancer cells migration. In human pancreatic adenocarcinoma tissues and MiaPaCa-2 cell lines, binding of GDNF to its receptor GFRα1 stimulates PNI via GDNF-(Ret proto-oncogene) RET signalling pathway [302]. Activation of GDNF-GFRα1-RET signalling triggers the MAPK signalling pathway leading to pancreatic cancer cell migration toward nerves in both *in vitro* and animal models of PNI [309]. Cancer-nerve interaction studied in *in vitro* co-cultures of DRG and MiaPaCa-2 pancreatic cancer cells demonstrated that GFRα1 facilitates migration of cancer cells along neurites toward the centre of the DRG [302]. Furthermore, decreased release of soluble GFRα1 from DRG inhibits migration of cancer cells towards nerves *in vivo* providing further evidence that GFRα1 expression is important in facilitating PNI [302]. In a metastatic breast cancer model, *in vivo* inhibition of Ret suppresses tumour outgrowth and metastatic potential [310].

BDNF facilitates cancer metastasis via binding to its receptors, TrkB/ TrkC and/or p75NTR as demonstrated in breast [311], colorectal [312, 313], clear cell renal cell carcinoma [314] and non-small cell lung cancer (NSCLC) [315]. The expression of TrkB associates with nodal metastasis and peritoneal metastasis; whereas, TrkC expression associates with liver metastasis in colorectal cancer patients [313]. BDNF-TrkB signalling pathway mediates metastatic effect through modulation of cancer-associated fibroblasts (CAFs) as demonstrated in mouse model co-injected with OSC19-Luc transfected cell line and CAFs [316]. In melanoma, neurotrophin (NT)-3, NT-4, and NGF induce cell migration, with a stronger effect on metastatic cell lines via binding to p75NTR coreceptor sortilin [317]. In breast cancer, NT-3 enhances breast cancer metastasis in the brain via promoting the mesenchymal–epithelial transition of breast cancer cells to a more epithelial-like phenotype and via increasing the ability of these cells to proliferate in the brain [318].

Collectively, these studies demonstrate that neurotrophins and their receptors play crucial role in PNI. These studies also suggest that the presence of PNI could be an effective predictor of metastatic potential and patient survival.

1.5.3. Tumour innervation influencing cancer metastasis

1.5.3.1 Tumour innervation

Cancer-related neurogenesis (tumour innervation) is attributed to the ability of cancer cells to attract normal nerve fibers via the secretion of signalling molecules and neurotrophic factor. However, recent study has demonstrated that cancer stem cells are capable of directly initiating tumour neurogenesis [319]. Cancer stem cells derived from human gastric and colorectal cancer patients generate neurons including sympathetic and parasympathetic neurons which promote tumour progression [319]. Knocking down their neural cell generating abilities inhibit tumour growth in human xenograft mouse model. Neurogenesis and its putative regulatory mechanisms have been reported in prostate [320], gastric [321], colorectal [322] and breast [323] cancers. There is a correlation between the expression of a pan-neuronal marker protein gene product 9.5 with clinicopathological characteristics of breast cancer [323]. In fact, neurogenesis is associated with aggressive features including tumour grade, poor survival as well as

angiogenesis, especially in estrogen receptor-negative and node-negative breast cancer subtypes [323, 324]. In prostate cancer, infiltration of the tumour microenvironment by nerve fibers associates with poor clinical outcomes [325] and is driven by the expression of granulocyte colony-stimulating factor (G-CSF) [326] and proNGF [327]. Similarly, in orthotopic PC3-luc xenografts model of prostate cancer, neurogenesis and axonogenesis correlate with aggressive features including metastatic spread, which is attributed to the neo-cholinergic parasympathetic nerve fiber [326]. These findings indicate that neurogenesis, like angiogenesis, is also a trait of cancer invasion and can alter tumour behaviour.

1.5.3.2 Tumour denervation

On the other hand, disruption of tissue innervation might cause accelerated tumour growth and metastasis [287, 328-333]. For instance, in humans, decreased vagal nerve activity correlates with advanced stages of cancer [328-330]. Similarly, modulation of vagal nerve activity enhances metastasis of breast cancer in mice [331, 332]. In addition, capsaicin-induced inactivation of sensory neurons enhances metastasis of breast cancer cells [287, 333]. On contrary, pharmacological or surgical denervation supresses the tumour progression as noted in three independent mice models of gastric cancer [321]. Thus, these findings suggest that there might be differences in the effects of local tumour innervation and extrinsic innervation on cancer progression.

1.5.4. Neurotransmitters influencing cancer metastasis

Tumour innervation influences metastasis as the ingrown nerve endings release neurotransmitters (such as norepinephrine, dopamine and substance P), which enhance metastatic spread [334]. To date, several neurotransmitters and neuropeptides involved in tumour metastasis have been identified (**Table 1.3** and **Figure 1.5**). In fact, several cancer cells express receptors for a number of neuropeptides and neurotransmitters, like norepinephrine, epinephrine, dopamine, GABA, acetylcholine, SP and NPY which have stimulatory effects on migration of cancer cells [306, 335-343].

1.5.4.1. Catecholamines

The increased expression of β-adrenergic receptor for catecholamines is associated with poor prognosis in breast cancer [344]. Stress stimulation leads to macrophage infiltration to the tumour site which activates β-adrenergic signalling pathways leading to increased metastasis in an orthotopic breast cancer model in BALB/c mice [186]. In this model, administration of β-adrenergic antagonist, propranolol, decreases breast cancer metastasis [186]. Similarly, the use of β-blockers in breast cancer patients inhibits metastasis and disease recurrence as well as improving survival of patients [344, 345]. In ovarian cancer patients, the grade and stage of tumours correlate with higher tumour norepinephrine levels associated with stress [346]. In an orthotopic mouse model of ovarian cancer, chronic stress elevates tumour noradrenaline levels and increases the aggressiveness of tumour growth [183]. In prostate cancer C42 xenografts in nude mice and Hi-Myc mice with prostate cancer, plasma adrenaline promotes carcinogenesis via β_2 adrenergic receptor/protein kinase A/BCL2-associated death protein anti-apoptotic signalling pathway [347]. Hence, stimulation of catecholamines plays a major role in activation of signals for breast cancer metastasis. Therefore, inhibition of the sympathetic nervous system signalling pathways with β-blockers holds great promise in preventing metastasis of various tumours including breast cancer. On the other hand, involvement of α-adrenergic receptors in cancer metastasis is not well understood. In the murine model of metastatic mammary adenocarcinoma induced by 4T1 cells in BALB/c mice, activation of α_2-adrenergic receptors increases tumour growth rate and the number of metastasis [348]. In contrast, blockade of α-adrenergic receptors in the absence of stress increases distant metastasis in the orthotopic model of mammary adenocarcinoma induced by MDA-MB-231HM cell line in nude mice [349].

The role of dopamine in cancer metastasis is not clear. Low levels of dopamine have been reported in stressed mice with ovarian carcinoma [228]. In contrary, in hepatocellular carcinoma (HCC) patients, dopamine levels are elevated in the blood samples compared to healthy individuals [350]. Moreover, enzymes such as monoamine oxidase A (MAOA) degrading catecholamines and serotonin [351] may also play an important role in influencing cancer metastasis [352-354]. Studies have demonstrated

that MAOA expression is decreased in HCC patients; it suppresses HCC cell metastasis by inhibiting adrenergic and epidermal growth factor receptor (EGFR) signalling pathways [355]. Inhibition of MAOA stimulates malignant behaviour in MDA-MB-231 breast cancer cells [356]. On the other hand, high expression of MAOA in human tissues correlates with poor prognostic in prostate cancer patients and increased tumour metastasis in xenograft mouse model of prostate cancer via HIF1-α/VEGF-A/FOXO1/TWIST1 signalling pathway [354]. These limited studies on the role of MAOA in cancer metastasis are controversial.

1.5.4.2. γ-Aminobutyric acid

γ-Aminobutyric acid (GABA) plays a role in cancer metastasis via activation of ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors [357]. It has been demonstrated that GABA mediates its inhibitory effect through $GABA_A$ receptor. For example, HCC cell lines and human adjacent non-tumour liver tissues, express $GABA_A$ receptor. GABA inhibits HCC cell migration through the activation of $GABA_A$ receptor [358]. However, there are studies demonstrating that $GABA_A$ receptor enhances metastasis. The activation of $GABA_A$ receptors upregulates brain metastasis of breast cancer patients [359]. Expression of the $GABA_A$ receptor subunit, Gabra3, which is normally not present in breast epithelial cells, is increased in human metastatic breast cancer which correlated with poorer patients survival [339]. Gabra3 overexpression promotes migration and metastasis of breast cancer cells via activating serine/threonine kinase or protein kinase B (AKT) signalling pathway demonstrated in a mouse orthotopic model induced by MCF7 and MDA-MB-436 breast cancer cell lines [339]. Mechanistically, the activation of AKT signalling pathway enhances metastasis via downstream molecules such as focal adhesion kinase and MMPs [360, 361]. Therefore, it could be speculated that the effect of $GABA_A$ receptor depends on the activated downstream molecules and signalling pathways. Murine (4T1) and human (MCF7) breast cancer cell lines and human breast cancer tissues express $GABA_B$ receptor [338]. In mice, $GABA_B$ receptor mediates 4T1 cell invasion and pulmonary metastasis via $ERK_{1/2}$ signalling [338]. $GABA_B$ activation inhibits migration of PLC/PRF/5 and Huh 7 malignant hepatocyte cell lines *in vitro* [362].

1.5.4.3. Acetylcholine

Acetylcholine (ACh) plays a functional role in cellular proliferation, differentiation and apoptosis. In HCC, the release of ACh acting on androgen receptor promotes SNU-449 cell invasion and migration via activation of AKT and signal transducer and activator of transcription 3 (STAT3) signalling pathways [363]. Nicotine stimulation of nicotinic acetylcholine receptor (nAChRs) enhances SW620 and LOVO colorectal cancer cell invasion and metastasis *in vitro* via the activation of p38 mitogen-activated protein kinases (MAPK) signalling pathway [343]. Similarly, nicotine pre-treatment stimulates the activation of α9-nAChR which mediates MCF-7 and MDA-MB-231 breast cancer cell migration via the expression of epithelial mesenchymal transition markers [364]. Furthermore, implantation of CD18/HPAF pancreatic cancer cells into immuno-deficient mice, demonstrates that nicotine treatment activates α7-nAChR and mediates tumour metastasis via Janus kinase 2 (JAK2)/STAT3 signalling in synergy with mitogen activated protein kinase (Ras/Raf/MEK/ERK$_{1/2}$) signalling pathway [365]. ACh promoted cancer metastasis and associate with poor clinical outcomes in prostate adenocarcinoma via M1R; and pharmacological blockade or genetic disruption of the M1R inhibit tumour invasion and metastasis leading to improved survival of the mice-bearing PC-3 prostate tumour xenografts [325]. In addition, ACh acting on M3 muscarinic receptor (M3R) associates with metastasis and low survival rate of NSCLC patients [366]. M3R activation increased invasion and migration of NSCLC cells and increased release of interleukin (IL)-8 via the activation of EGFR/PI3K/AKT pathway [367]. In human SNU-C4 and H508 colon cancer cell lines, administration of muscarinic receptor inhibitor, atropine, abolished SNU-C4 cell migration; however, H508 cell migration requires the activation of MMP7 [368, 369].

Conversely, studies have shown that administration of Ach or inhibition of acetylcholinesterase (AChE) with physostigmine and pyridostigmine attenuated cancer cell invasion and viability *in vitro* and *in vivo* through the inhibition of ERK phosphorylation in pancreatic cancer [370]. In LSL-*Kras* $^{+/G12D}$;*Pdx1*-Cre (KC) mice, subdiaphragmatic vagotomy augmented pancreatic cancer development, while administration of muscarinic agonist bethanechol reinstated the normal KC phenotype [371]. Similarly, in syngeneic

and orthotopic mouse models of pancreatic cancer, subdiaphragmatic vagotomy promoted tumour growth and poor overall survival through enhance expression mediated through TNF-α and TAMs [372]. In addition, in mice-bearing 4THM breast carcinoma cells who underwent surgical or chemical vagotomy showed enhanced metastasis [373]. In pancreatic cancer patients, high tumour grade and advanced stages correlate with decreased expression of choline acetyltransferase (ChAT) and AChE [370]. Patients who underwent vagotomy for gastric ulcer disease in the past showed a higher incidence of pancreatic cancer [374]. Furthermore, a higher vagal nerve activity correlated with a lower risk of death in metastatic pancreatic cancer patients [330].

The mechanisms behind these inconsistencies in findings is unclear, thus warrant further studies.

1.5.4.4. Neuropeptides

Expression of SP is shown to exert functional effects on small cell lung cancer [375], pancreatic [376], colon [377], prostate [378, 379] and breast cancer [380] cells. SP acting on neurokinin-1 (NK-1) receptors enhances pancreatic cancer cell migration and perineural invasion to the dorsal root ganglia (DRG) mediated by MMP-2 demonstrating its essential role in metastasis [381]. Enhanced expression of SP correlated with lymph node metastasis and poor prognosis in colorectal cancer patients [377]. NPY stimulates cell proliferation, differentiation and survival via acting on its G protein-coupled receptors designated Y1R–Y5R leading to the development of metastasis [232, 382]. High levels of systemic NPY associates with metastatic tumours as noted in Ewing sarcoma patients [383]. Similarly, in the SK-ES1 xenograft model, elevated levels of NPY associates with bone invasion and metastases [384]. NPY mediates 4T1 cell proliferation and migration via the activation of NPY Y5 receptor [232]. Neurotensin mediates metastasis by binding to neurotensin receptors 1 (NTSR1). In breast cancer, the expression of NTSR1 correlates with lymph node metastasis [385]. These studies demonstrate the important role of neuropeptide signalling in cancer metastasis.

Table 1.3. Neurotransmitters influencing tumour metastasis

Neurotransmitters	Receptor	Type of cancer	Model	Mechanism/pathway	Ref.
NE	β2-AR	Pancreatic cancer	CFPAC1, MiaPaCa2 Panc1, and IMIM-PC2 cells	NE treatment reduces migratory activity of pancreatic cancer cells. NE mediates inhibitory effect via imbalanced activation of PKC/PLC signalling pathway → to activation of anti-migratory cAMP/PKA signalling.	[386]
		Prostate cancer	Subcutaneous injection of PC-3 cells in BALB/c nude mice	↑ NE leads to lumbar lymph node metastasis in an animal model.	[387, 388]
DA	DR1 & DR5	HCC	Tumour and non-tumour adjacent tissues from patients; LM3, Huh7 and SNU449 cells; subcutaneous injection of LM3 cells in BALB/c nude mice	DR5 is upregulated in tumour tissue and DR1 is upregulated in non-tumour human tissues. Dopamine ↑ cell proliferation in SNU449 cells. Administration of DR antagonist (thioridazine) inhibits cell proliferation *in vitro* and *in* and cell migration through EMT → ↓ tumour metastasis	[350]
GABA	$GABA_A$		Human primary and adjacent	$GABA_A$ receptor subunit ε1 expression is lower in	[358]

		HCC	non-tumour tissues, and Orthotopic inoculation of SMMC-7721 cells into the liver of BALB/c nude mice	human HCC tissues than in non-tumour liver tissues. GABA inhibits invasion and migration of human liver cancer cells *in vitro.* In mice, inoculation of SMMC-7721 cells pre-treated with GABA ↓ tumour metastasis.	
	$GABA_B$		PLC/PRF/5 and Huh cells	Administration of $GABA_B$ agonist (baclofen) ↓ cell migration associated with ↓ in intracellular cAMP levels.	[362]
		Breast cancer	Human tissues, 4T1 and MCF-7 cells	Administration of $GABA_B$ agonist (baclofen) promotes invasion and migration of breast cancer cells *in vitro* and metastasis *in vivo* via $ERK_{1/2}$ and MMP-2signaling pathway.	[338]
		Prostate cancer	Human prostate and lymph node tissues, C4-2 cells	↑ Expression of GABA → cell invasion *in vitro* and lymph node metastasis in patients mediated by activation of MMPs signalling.	[389]
		HCC	Human primary and adjacent non-tumour tissues	The mRNA levels of $GABA_B$ R1.2 and $GABA_B$ R1.4 are higher in HCC tissues than in non-tumour liver tissues	[358]
ACh	AR	HCC	SNU-449 cells	ACh activates AR receptors → ↑ invasion and migration	[363]

			of SNU-449 cells via activation of AKT and STAT3 signalling pathways.	
α7-nAChR	Pancreatic cancer	CD18/HPAF, Capan1, FG/Colo357 cells *in vitro* and orthotopically implanted CD18/HPAF cells in immunodeficient mice	Nicotine treatment stimulates the expression of α7-nAChR and MUC4 *in vitro.* In the *in vivo* model, exposure to low and high cigarette smoking increases the tumour metastasis and MUC4 expression compared to sham controls. Nicotine induces tumour metastasis by upregulating MUC4 via α7-nAChR-mediated JAK2/STAT3 signalling in collaboration with Ras/Raf/MEK/$ERK_{1/2}$ signalling pathway.	[365]
	Lung cancer	Line 1 cells *in vitro*, and subcutaneous injection of Line 1 cells in BALB/c mice	Intraperitoneal injection of nicotine ↑ tumour growth and metastasis through change in gene expression via nAChR signalling pathway.	[201]
nAChR β2	Lung cancer	B16 cells intravenous injection in C57BL/6 mice	↑ Nicotine exposure → activation of nAChR β2 on NK cells mediates metastasis	[390]

	α9-nAChR	Breast cancer	MDA-MB-231 and MCF-7 cells	Nicotine treatment enhances the migratory abilities of both cells by activating α9-nAChR through elevated expression of EMT markers	[364]
	mAChR	Colon cancer	Hh508 and SNU-C4 cells	Administration of muscarinic inhibitor (atropine) → ↓ cell invasion and migration. ACh binding to M3R mediates cell migration via the activation of post-ERBB1, ERK and PI3K-dependent RhoA pathway.	[368, 369]
		NSCLC	Human tissues, micA549, PC9, SPC-A1, GLC82, L78 and HLF cells	M3R expression correlates with clinical stage and poor survival in patients. M3R stimulation by ACh enhances *in vitro* cell invasion and migration via PI3K/AKt pathway.	[366, 367]
		Prostate cancer	Human tissues, Hi-Myc transgenic mice-bearing PC-3	Presences of cholinergic nerve fibers associate with poor clinical outcome in human patients. Pharmacological blockade or genetic disruption of the M1R inhibit metastasis leading to improved survival of the mice	[325]
SP	NK-1R	Pancreatic cancer	MiaPaCa-2, BxPC-3, CFPAC-1,	Binding of SP to NK-1R promotes cell invasion and migratory potential, which is	[381, 391]

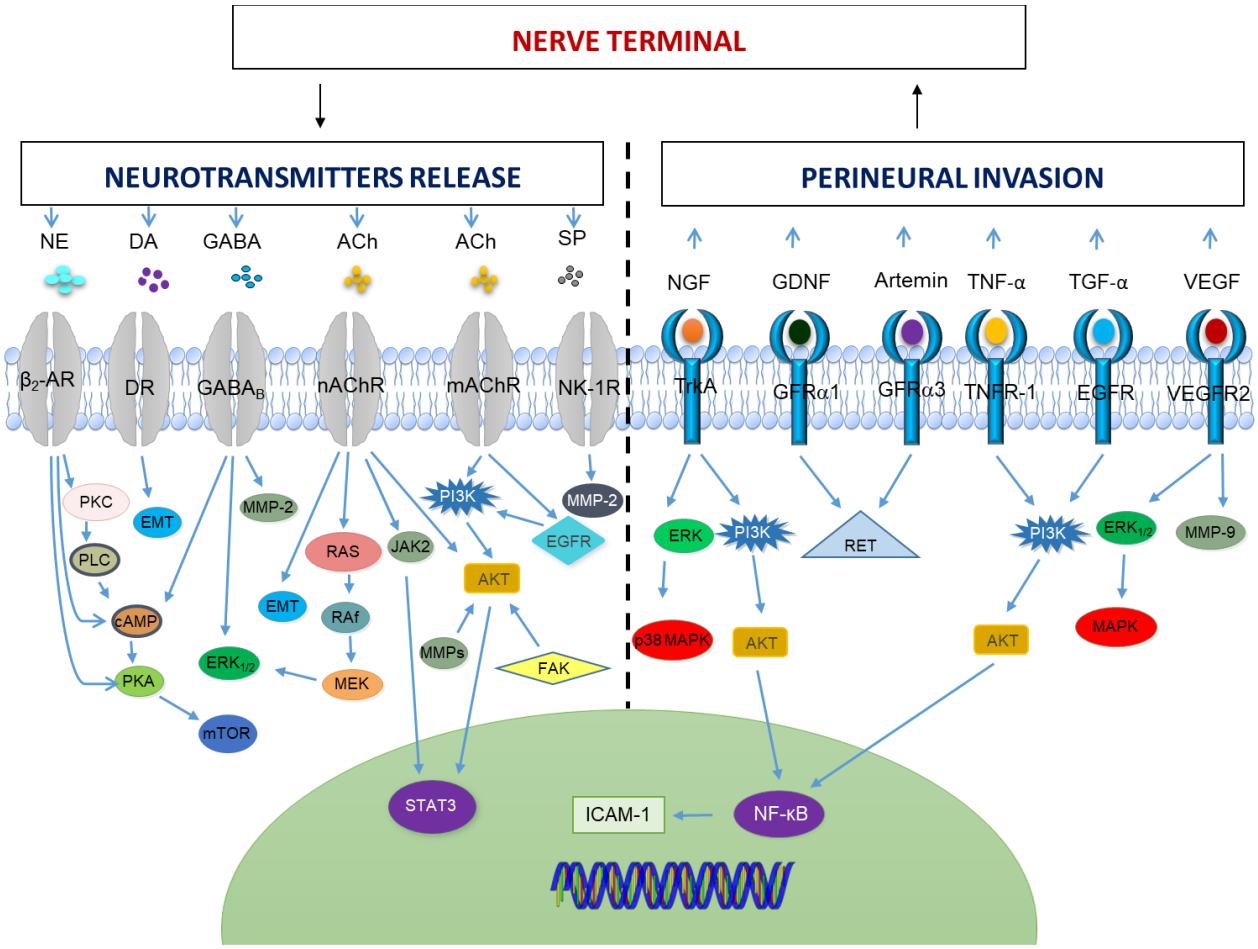
			HAPC, Panc-1, and SW1990 cells	mediated by expression of MMP-2. SP also increases cell migration and neurite outgrowth toward DRG demonstrating important role in metastasis and PNI.	
NPY		Ewing sarcoma	Human serum, SCID/beige mice bearing SK-ES1 cells	Enhanced level of systemic NPY associate with metastatic tumours. In the xenograft model, NPY expression associate with bone metastases.	[383, 384]
	NPY Y5	Breast cancer	4T1 cell line	NPY mediates metastatic effect via the activation of NPY Y 5.	[232]
Neurotensin	NTSR1	Breast cancer	Human tissues	The expression of NTSR1 associates with lymph node metastasis.	[385]

Abbreviations: Ach, acetylcholine; AR, androgen receptor; β2-AR, β_2-adrenergic receptor; cAMP, cyclic adenosine monophosphate; DA, dopamine; DR, dopamine receptor; DRG, dorsal root ganglia; ERBB1, epidermal growth factor receptor 1; EMT, epithelial–mesenchymal transition; $ERK_{1/2}$, extracellular signal-regulated kinase; GABA, gamma-aminobutyric acid; GABAA&B, gamma-aminobutyric acid receptor A&B; HCC, hepatocellular carcinoma; JAK2, janus kinase 2; MEK, MAPK/ERK kinase; MMP, matrix metallopeptidase; RAF, mitogen activated protein kinase; RAS, mitogen activated protein kinase; MUC4, mucin 4; mAChRs, muscarinic acetylcholine receptors; M3R; muscarinic receptors 3; NK, natural killer cells; NK-1R, neurokinin-1 receptor; nAChR, nicotinic acetylcholine receptor; NSCLC, non-small cell lung cancer; NE, norepinephrine; PNI, perineural invasion; PLC, phospholipase C; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A;PKC, protein kinase C; RhoA, Ras homolog gene family member A; AKT, serine/threonine kinase or protein kinase B; STAT3, signal transducer and activator of transcription 3; SP, substance P.

Figure 1.5. Neurotransmitters signalling pathways in cancer

Cancer neuro-immune communication is through the release of neurotransmitters using different signalling kinases which promote cancer progression via metastasis. Perineural invasion mediates cancer metastasis through the release of the NGF and GDNF via the activation of different signaling pathway.

Abbreviations: Ach, acetylcholine; β2-AR, β_2-adrenergic receptor;cAMP, cyclic adenosine monophosphate; DA, dopamine; DR, dopamine receptor; EGFR, epidermal growth factor receptor; EMT,epithelial–mesenchymal transition; $ERK_{1/2}$, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GABA, gamma-aminobutyric acid; GABAB, gamma-aminobutyric acid receptorB; GDNF, glial cell line-derived neurotrophic factor; GFRα, glial cell line-derived neurotrophic factor receptor 1; ICAM-1, intercellular adhesion molecule-1; JAK2, janus kinase 2; MEK, MAPK/ERK kinase; mTOR, mammalian/mechanistic target of rapamycin; MMP, matrix metallopeptidase; MAPK,mitogen-activated protein kinases; RAF, mitogen activated protein kinase; RAS, mitogen activated protein kinase; mAChRs, muscarinic acetylcholine receptors; NK-1R, neurokinin-1 receptor; NGF, nerve growth factor; nAChR, nicotinic acetylcholine receptor; NE, norepinephrine; NF-kB, nuclear factor-kappa B; PLC, phospholipase C; PI3K, phosphoinositide 3-kinase;PKA, protein kinase A; PKC, protein kinase C;RET, proto-oncogene; AKT, serine/threonine kinase or protein kinase B; STAT3,signal transducer and activator of transcription 3; SP, substance P; TrkA, tropomyosin related kinase A.


NERVE TERMINAL
NEUROTRANSMITTERS RELEASE
PERINEURAL INVASION
NE
DA
GABA
ACh
ACh
SP
NGF
GDNF
Artemin
TNF-α
TGF-α
VEGF
β2-AR
DR
GABAB
nAChR
mAChR
NK-1R
TrkA
GFRα1
GFRα3
TNFR-1
EGFR
VEGFR2
PKC
EMT
MMP-2
PI3K
MMP-2
PLC
RAS
JAK2
EGFR
ERK
PI3K
RET
PI3K
ERK1/2
MMP-9
AKT
cAMP
EMT
RAf
p38 MAPK
AKT
AKT
MAPK
ERK1/2
MMPs
FAK
PKA
MEK
mTOR
STAT3
ICAM-1
NF-κB

1.6. Crosstalk between Cancer and the Neuro-Immune System

Cancer is the major health related cause of death worldwide due to unhealthy lifestyle and other factors [2]. Although the mechanisms of cancer progression have been extensively studied in the last decades, these have been predominantly focused on cellular pathways of proto-oncogene, tumour suppressor gene mutations and mechanisms by which immune cells can eliminate cancer cells [4, 5, 392, 393]. More recently, the impact of the tumour microenvironment in tumour cell invasion has attracted much interest [4, 176]. Multiple cellular and extracellular components within the tumour microenvironment, such as, immune cells, endothelial cells, mesenchymal stromal cells (fibroblasts and myofibroblasts), and their secretory products, exert active functions to stimulate gene expression patterns of tumour cells which have an impact on their biological behaviour [394-396]. Invariable crosstalk among these components within the tumour microenvironment triggers pro-survival, invasion and metastatic spread of tumour cells [397-400]. In addition, tumour cells interact with other cells to form organ-like structures that drive and promote cancer growth [4, 393]. The interaction between the tumour microenvironment and the complex immune system plays a major role in tumour progression and as a result, is of concern in cancer treatment [393]. However, it is only in recent years that the role of the neuro-immune network has surfaced as a major contributor to cancer progression. The mechanisms by which neuro-immune signalling in cancer influences its progression are not clear.

The nervous system plays a fundamental role in regulating immune responses to a range of disease states [401]. Its dysfunction influences the progression of disease outcomes including cancer cell growth. The role of the nervous system in tumour progression is of relevance to the immune system since they can bi-directionally communicate via neurotransmitters and neuropeptides, common receptors and cytokines [378, 402]. However, the crosstalk between these cells is highly complex in nature, and numerous variations are possible according to the type of cancer involved [331]. The interaction of the nervous system in modulating immune responses, innervation of lymphoid organs, affects various neurotransmitters influencing cancer. This review presents an overview of

the neuro-immune interaction in cancer progression: lymphoid organs innervation; neurotransmitters and immune cells in cancer, tumour associated immune cells and the nervous system.

1.6.1. Innervation of lymphoid organs

The link between the nervous and immune systems is via direct innervation of lymphoid organs. In particular, sympathetic noradrenergic fibers innervate primary (thymus and bone marrow) and secondary (lymph nodes and spleen) lymphoid organs [403]. In lymphoid organs, the immune responses against pathogens or tissue damage are altered by the release of neuropeptides and neurotransmitters such as, neuropeptide Y, substance P (SP), norepinephrine and dopamine from nerve endings [403, 404]. Dysregulation of this interaction promotes pathogenesis and progression of many diseases including cancer [403]. The spleen plays an important role in response to pathogens or tissue damage; however, its response to cancer has been less empathized. In systemic inflammation, the vagal afferents activate the central nervous system (CNS) which triggers the efferent via the celiac ganglion and, as a result, activates immune cells in the spleen (as reviewed by Matteoli et al, 2013 [405]). Consequently, the activation of adrenergic fibers innervating the spleen results in the release of norepinephrine leading to the activation of T cells secreting acetylcholine.

The spleen accumulates monocytic and granulocytic precursors that directly replenish tumour-associated macrophages (TAMs) and neutrophils, as noted in lung adenocarcinoma [406, 407]. Moreover, the cords of the splenic subcapsule red pulp contain a reservoir of monocyte subsets (e.g. Ly-6C^{high} and Ly-6C^{low}) that are promptly released in the bloodstream following acute injury [408]. Therefore, it can be speculated that the spleen would detect cancer as a pathogen and respond to it in a similar manner. However, cancer invades tissues without the spleen influencing it, in the same way as viruses invade target tissues by inactivating immune responses. In fact, stress or central inflammatory stimulation of the sympathetic nervous system (SNS) inhibits splenic macrophage function, thus, beta-adrenergic mechanisms influence splenic macrophages [409]. This supports the speculation that the spleen’s response to pathogens is via

catecholamine release which acts on beta-adrenergic receptors to inhibit splenic macrophage activity. However, specific mechanisms of this action in cancer are not clear.

Detailed neuro-anatomical description of lymph node (LN) innervation is scarce [403], however, sympathetic fibers in LNs have been reported [409]. In LNs, immune responses to antigens are initiated [409, 410]. During antigen detection, immune cells (dendritic cells (DCs), T cells, etc.) are recruited into regional LNs, which activate immune responses against the antigen. The decision process within LNs to either induce an active immune response or be tolerant is not clear, although in most instances an active immune response is initiated [411-413]. Just like other foreign antigens, cancer cells can escape LN surveillance. It is suggested that the lack of LNs innervation may be a contributing factor to cancer escaping immune surveillance. Thus, information of LN innervation could aid in the understanding of the decision process within LNs to induce protective responses and its lack of response in cancer initiation. Furthermore, understanding the interaction between LN and cancer may aid therapeutic modalities at the early stages of disease.

The SNS regulates bone marrow function [409]. Innervation within the bone marrow is also scarce and likely due to the fact that there is close contact with surrounding mineralized bone which receives sympathetic and sensory innervation [414]. However, sensory fibers containing SP and calcitonin gene-related peptide together with noradrenergic sympathetic fibers and veins are distributed throughout the bone marrow and surrounding bone. Distinguishing between innervated bone and bone marrow is not clear [403], even though, in rodents bone marrow innervation occurs late in fetal life, just prior to hemopoietic activity. Understanding innervation of the bone marrow will enhance our knowledge of bone marrow cancers including but not limited to, lymphoma, leukaemia, and myeloma.

1.6.2. Neurotransmitters and immune cells in cancer

Neurotransmitters play an essential role in the modulation of immunity. A number of immune cells such as, T cells, DCs, natural killer (NK) cells, microglia and myeloid-derived suppressor cells (MDSCs) express cell surface neurotransmitter receptors including

substance P (SP), glutamate, gamma-aminobutyric acid (GABA), serotonin, dopamine, epinephrine, norepinephrine and acetylcholine (**Table 1.4** and **Figure 1.6**) [415-419]. Furthermore, studies have shown that various cancers express receptors for different neurotransmitters, which play an essential role in the control of tumour progression [416, 417, 420-422].

1.6.2.1. Substance P

Substance P (SP) is expressed in both the central and peripheral nervous systems (PNS) and plays an essential role in the neuroimmune system crosstalk. Of the sensory neuropeptides, SP is distributed widely and regulates immune functions, including that of B and T cells [391] and cytokine secretion by monocytes [331] and macrophages. Binding of SP to its receptor NK1 triggers activation of intracellular pathways including cAMP, MEK, ERK1/2, mTOR and NF-kB resulting in proinflammatory cytokine production [423]. In addition, SP enhances lymphocyte proliferation and lymphokine-activated killer cell cytotoxicity, NK cell cytotoxicity, augments tumour necrosis factor alpha (TNF-alpha), interleukin (IL)-10 and IL-12 secretion by macrophages, and, decreases the number of tumour-infiltrating MDSCs [331]. The effects of chronic administration of low dose SP to the brain in a murine model of metastatic breast cancer co-treated with radiation treatment, increased the antigenicity of cancer cells [424]. Hence, SP through neuro-immune modulation can avert an immunosuppressive tumour microenvironment and consequently inhibiting metastatic growth.

1.6.2.2. Glutamatergic, GABAergic and serotonergic signalling

Glutamate is the principal excitatory neurotransmitter that regulates synaptic and cellular activity in the CNS via binding to its receptors including metabotropic glutamate receptors (mGluRs) or ionotropic glutamate receptors (iGluRs). In addition, glutamate also plays a fundamental role in the neuroimmune system crosstalk and it stimulates immune cell functions via the expression of its functional receptors on immune cells [425]. Furthermore, immune cells such as T cells, DC, monocytes and macrophages release glutamate where they act in both an autocrine and paracrine fashion [426]. Although the

role of glutamate and its receptors is well-established in neurological disorders and neuroprotection, it has become evident that glutamate plays a functional role in cancer via regulating immune cells as noted in head and neck, glioma, melanoma, gastric, prostate, squamous cell carcinoma, colorectal and breast cancers [417, 421, 422]. For instance, in head and neck cancer patients, elevated levels of glutamate increase spontaneous migration of peripheral T cells [417].

GABA is the main inhibitory neurotransmitter in the CNS. Nevertheless, GABA exerts physiologic effects in non-neuronal peripheral tissues and organs via the activation of ionotropic ($GABA_A$ or $GABA_C$) and metabotropic ($GABA_B$) receptors [427]. GABA plays a functional role in the proliferation, migration and differentiation of cells including tumorigenic cells [428]. It has been noted that GABA mediates its inhibitory effect through $GABA_A$ receptor. For instance, GABA inhibits hepatocellular carcinoma cell migration through the activation of $GABA_A$ receptor [358]. In addition, administration of GABA agonist Nembutal suppresses tumour metastasis in colon cancer [429]. However, there are studies demonstrating that $GABA_A$ receptor enhances metastasis. The activation of $GABA_A$ receptors upregulates brain metastasis of breast cancer patients [359]. It is speculated that since GABA mediates it functional effect on T lymphocytes and DC through the activation of $GABA_A$ [430]. Further studies are warranted to explain the inconsistency in findings.

5-hydroxytryptamine (5-HT), also known as serotonin, is a monoamine neurotransmitter synthesized in the serotonergic neurons in the brain and it plays an essential role in the modulation of immune response. Ninety percent of the body's 5-HT is secreted by enterochromaffin cells of the gut mucosa. 5-HT regulates a wide range of behavioural, cognitive and physiological functions in pathological disease including cancer [431]. In mouse models of melanoma, administration of selective serotonin reuptake inhibitors decreases tumour growth via enhancing mitogen-induced T cell proliferation, IL-1 beta production, and by inhibiting IFN-gamma and IL-10 production [432]. Furthermore, in a mouse model of colon cancer allografts, serotonin regulates macrophages-mediated tumour angiogenesis [222]. These findings demonstrate the essential role of glutamate,

GABA and serotonin in regulating tumour growth; however, further studies mechanistic studies are required.

1.6.2.3. Dopaminergic signalling

Dopamine is an important monoamine neurotransmitter in the CNS; however, it also plays a role in immune modulation. Elevated levels of dopamine increase spontaneous migration of peripheral T cells in head and neck cancer patients [417]. Dopamine inhibits cytotoxicity and proliferation of T cells via the activation of dopamine receptor 1 (DR1) mediated by intracellular cAMP in lung cancer [433]. Dopamine treatment induces M2 (pro-tumour phenotype) shift to M1 (anti-tumour phenotype) of RAW264.7 cells and mouse peritoneal macrophage in rat C6 glioma [434]. Similarly, in human blood samples from lung cancer patients (stage I-IV) and mouse models using Lewis lung carcinoma and B16 melanoma cell lines, application of dopamine inhibits the effects of MDSC on T cell proliferation via the activation of DR1 [418], suggesting a possible mechanism of inhibition by dopamine. Moreover, inhibition of DR3 signalling in DCs enhances antigen cross-presentation to CD8+ T cells favouring anti-tumour immunity [435]. Dopamine acting on DR4 causes impairment in the endolysosomal system, a block in autophagic flux, and eventual cell death in glioblastoma [420]. It has been shown that CD8+ T cells express functional dopamine receptors DR1-DR5 in both humans and mice, and dopamine plays a significant role in migration and homing of naive CD8+ T cells via DR3 [436, 437]. Moreover, dopamine activates resting effector T cells (Teffs) and suppresses regulatory T cells (Tregs) [437]. Hence, it can be speculated that dopamine inhibits tumour growth via regulating DC antigen presentation to CD8+ T cells. Furthermore, screening cancer patients that present with elevated levels of dopamine for DCs and CD8+ T cells could aid in delivering an effective targeted therapy.

1.6.2.4. β-Adrenergic signalling

SNS activation regulates an array of cancer-related molecular pathways by beta-adrenergic signalling and via beta-adrenergic receptors expressed by tumour cells, immune and vascular cells [288, 438]. beta-adrenergic receptors mediate a range of catecholamine effects on target cells and immune cells, as well as cancer cells, i.e. breast

cancer cells [202, 439, 440];. Several cellular and molecular processes (such as inflammation, angiogenesis, epithelial mesenchymal transition and apoptosis) mediate beta-adrenergic influences on tumour progression [288] and recruitment of macrophages into primary tumours [186, 441]. Moreover, beta-adrenergic signalling influences the secretion of pro-inflammatory cytokines (IL-1, IL-6 and IL-8) by immune cells [441-444], upregulation of vascular endothelial growth factor (VEGF) resulting in increased angiogenesis [445], matrix metalloproteinase (MMP) related increase of tissue invasion [445, 446], tumour cell assembly and motility [447, 448]. Furthermore, beta-adrenergic signalling suppresses CD8 T cell and NK cell responses [449] and inhibits the expression of type I interferons [186, 450]. In fact, in murine metastatic mammary adenocarcinoma, beta-adrenergic receptor agonist suppressed NK cell activity resulting in increased lung metastasis [287]. In addition, either stress or pharmacological beta-adrenergic stimulation results in increased macrophage infiltration and cancer metastasis which can be prevented by injection of a beta-adrenergic antagonist, propranolol [186]. Furthermore, the use of beta2-adrenergic agonist in experimental animals' reverse muscle wasting (cachexia) associated with cancer [451]. Catecholamines can induce apoptosis of lymphocytes, alter the distribution of NK cells and suppress NK cell activity, which are all required for anti-tumour immunity [452], leading to tumour cell escape mechanisms. Thus, persistent release of neurotransmitters from nerve terminals may promote tumour growth and metastasis via modulation of the immune system.

1.6.2.5. Cholinergic signalling

Modulation of the immune system by the sympathetic nervous system (SNS) has been extensively studied [405, 453, 454]. However, the role of the parasympathetic nervous system has gained attention only recently [455]. Inflammatory mediators can activate sensory nerves that send signals regarding inflammation to the CNS, which in turn leads to the release of neuromediators modulating local inflammation and influencing immune cells [285]. Consequently, the nervous system can regulate immune responses in peripheral tissues and restore local immune homeostasis [456]. Since inflammatory signals are important for tumour progression in both the early and late stages, the anti-inflammatory role of the vagus nerve may play an important role in tumorigenesis [286].

It has been established that acetylcholine (ACh) acting on α7 nicotinic receptors (nAChRs) stimulates splenic macrophages and inhibits TNF-alpha production in the spleen [457, 458]. In addition, vagus nerve activation stimulates ACh synthesis by splenic T lymphocytes leading to inhibition of cytokine production [458]. In lipopolysaccharide-induced inflammation in C56BL/6J mice, activation of α7 and α9 nAChRs expressed by bone marrow cells stimulates secretion of anti-inflammatory cytokines (IL-10 and transforming growth factor beta (TGF)-beta) and inhibits production of pro-inflammatory cytokines (TNF-alpha, IL-1beta and IL-12) [415]. Similarly, secretion of TNF-alpha, IL-1beta, IL-6 and IL-18 induced by endotoxin was significantly inhibited by ACh and nicotine in human macrophage cultures [459]. ACh receptors including both muscarinic (mAChRs) and nAChRs are functionally expressed by cancer cells [460-462]. Moreover, cancer cells synthesize and secrete ACh [462]. In a mouse bearing B16 melanoma cells, administration of nicotine inhibits the release of cytokines and cell killing by NK cells via nAChR β2 [416]. Overexpression of α7nAChRs by cancer cells (i.e. human colon cancer cell line HT-29) promotes cancer angiogenesis [463, 464], cell proliferation and metastasis [201, 465-468]. α9nAChRs are reported to play a crucial role in breast cancer development; the correlation between expression levels of α9nAChR mRNA and disease outcome was found in breast cancer patients [202]. Similarly, it has been demonstrated that mAChRs antagonists inhibit small cell lung carcinoma growth both *in vitro* and *in vivo* via inhibiting MAPK pathway [462]. In BALB/c mice bearing LMM3 mammary adenocarcinoma cells, tumour macrophages express M1 and M2 mAChRs which trigger arginine metabolic pathway leading to tumour angiogenesis [469]. Understanding the principal mechanisms of cholinergic signalling in regulating the immune system may highlight the significance of ACh inhibitors in cancer therapy.

Table 1.4. Modulation of immune cells by neurotransmitters

Neurotransmitter	Cancer type	Model	Function on immune cells	Ref.
ACh	Melanoma	B16 melanoma cells *in vitro* and intravenous injection of B16 cells in C57BL/6 mice	Nicotine inhibits NK cells capability to release cytokines and kill target cells via nAChR β2	[416]
Dopamine	Lung cancer	Human patients, *in vitro* dopamine concentration	Plasma levels of dopamine is elevated in lung carcinoma patients Dopamine inhibits the cytotoxicity and proliferation of T cells via the activation of dopamine receptor 1 mediated by intracellular cAMP	[433]
		Human blood from lung cancer patients (stage I-IV); Lewis lung carcinoma and B16 melanoma cells *in vitro* and their subcutaneous injection in C57BL/6 mice	Dopamine administration inhibits the suppressive function of Gr-1$^+$ CD115$^+$ MDSC on T cell proliferation via the activation of DR1 both in human blood *in vitro* and *in vivo*	[418]

	HNC	Human patients	Dopamine increases spontaneous migration of peripheral T cells in HNC patients	[417]
Epinephrine	Leukemia	CRNK-16 leukemia cells *in vitro* and intravenous injection of CRNK-16 cells in F344 rats	Administration of epinephrine reduces NK activity.	[449]
Glutamate	HNC	Human patients	Glutamate increases spontaneous migration of peripheral T cells in HNC patients	[417]
NE	Breast cancer	66c14 mammary adenocarcinoma cell injected into mammary fat pad of BALB/c mice	NE acts on β2-AR enhancing CD11b+F4/80+ macrophage and CD11b+ Gr^{lo}Ly6Chi myeloid-derived suppressor cell infiltration	[186]
SP	Breast cancer	4TBM cells *in vitro* and orthotopic injection of 4TBM cells in BALB/c mice	SP increases $CD4^{+}CD25$ cells in draining LNs Prevents tumour-induced degeneration of sensory nerve endings Alters CAFs releasing of angiogenic factors Enhances lymphokine-activated killer cell	[331]

	cytotoxicity, NK cell cytotoxicity, TNF-alpha, IL-10 and IL-12 secretion by macrophages Decreases tumour-infiltrating myeloid-derived suppressor T cells

Abbreviations: ACh, acetylcholine; DR1, dopamine receptor 1; NE, norepinephrine; SP, substance P; cAMP, cyclic adenosine monophosphate; nAChR, nicotinic acetylcholine receptor; IL-10, interleukin 10; IL-12, interleukin 12; CAFs, cancer associated fibroblasts; TNF-alpha, tumour necrosis factor-α; NK, natural killer cells; LN, lymph node; beta2-AR, beta2-adrenergic receptor; HNC, head and neck cancer.

1.6.3. Tumour-associated immune cells and the nervous system

The role of nervous system in modulating tumor-associated immune (TAI) cells is not well understood. However, various TAI cells within tumor microenvironment play essential role in promoting tumor growth. It could be speculated that nervous system stimulates TAI cells in its original form as normal immune cells.

1.6.3.1. Tumour-associated macrophages

Tumour-associated macrophages (TAMs) play a role in β-adrenergic signalling pathways, by accelerating angiogenesis, chemokine secretion to attract immune and tumour cells, secretion of pro-inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α) and escape of anti-tumour responses [289-291]. Hence, TAMs are sensitive to sympathetic signalling and raise the likelihood that stress-response pathways influence macrophage infiltration within the tumour microenvironment and, as a result, enhance metastasis. In the early or regression stages of tumours, TAMs, in particular, M1 macrophages (pro-inflammatory; releasing IL-1β, IL-6, IL-12, TNF-α, monocyte chemoattractant protein-1 (MCP-1)) inhibit angiogenesis and activate an anti-tumour immune response. In contrast, TAMs shift to a M2 phenotype (anti-inflammatory, releasing IL-1 receptor antagonist, TGF-β, IL-4, IL-10, IL-13) which enhance tumour angiogenesis in advanced tumours [470-474], tumour growth [472], invasion, migration [475], metastatic spread [476] and possess immuno-suppressive activities which are regulated by neuromediators [477]. In breast cancer, infiltrating TAMs correlate with higher tumour and vascular grade [440] and increased necrosis [478] leading to poor prognosis [289, 440, 479]. In fact, eliminating macrophages from the tumour site, either genetically or therapeutically, results in reduced tumour progression in breast cancer [440]. However, the detailed understanding of neuro-immune interaction influencing TAMs in human breast cancer needs further elucidation.

1.6.3.2. Cancer-associated fibroblasts

The role of nervous system in modulating cancer-associated fibroblasts (CAFs) remains scare. To understand how nervous system might stimulate CAFs, studies need to understand the origin of CAFs. It is believe that CAFs originated from bone marrow-

derived mesenchymal stem cells, fibroblasts or cancer cells that undergo endothelial - or epithelial -mesenchymal transition [480]. Therefore, it is possible that nervous system may regulate CAFs via modulating bone marrow-derived mesenchymal stem cells or fibroblasts. CAFs are the key constituent cells within the tumour microenvironment which interact with cancer cells promoting tumour growth and metastasis [481]. For example, in the tumour microenvironment of 4T1 metastatic breast cancer model, *in vivo* abolition of CAFs causes Th2 shift to Th1 polarization which is characterized by increased expression of IL-2 and IL-17, suppressed TAMs, T regulatory cells, MDSCs and decreased angiogenesis [482]. In addition, CAFs enhanced the aggressive phenotype of T47D, MCF-7 and MDA-MB-231 breast cancer cells via epithelial mesenchymal transition induced through paracrine TGF-β signalling [483]. Similarly, in human sample of squamous cell carcinoma, CAFs mediate angiogenesis and inflammation via employing macrophages and stimulating angiogenesis, consequently enhancing tumour growth [484]. These findings demonstrate significant importance of CAFs in mediating tumour progression. Understanding the origin of CAFs could lead to better understanding of how nervous system stimulates it, resulting in better therapies design.

1.6.3.3. Tumour-infiltrating lymphocytes

Nervous system plays essential in modulation of T cell. T cell expressed adrenergic and cholinergic receptor it communicates with nervous system. Tumour-infiltrating lymphocytes (TILs) particularly CD8+ T cells are associated with positive prognostic relevance in various tumours. For example, in a prospective-retrospective study of a primary triple-negative breast cancer demonstrate elevated levels of TILs present at diagnosis were considerably associated with reduced distant recurrence rates [485, 486]. Similar findings are reported in patients with oro- and hypopharyngeal carcinoma showing increased expression of intraepithelial CD8+ TIL in metastatic tumours to be associated with favourable outcome [487]. In prostate cancer, infiltration of CD4+ T cells enhances LNCaP, CWR22RV1 and C4-2 cell invasion and metastasis via fibroblast growth factor 11→miRNA-541→androgen receptor→ matrix metalloproteinase 9 signalling [488]. In addition to the presence of T lymphocytes at the tumour site, B lymphocyte infiltration also plays a role within the tumour microenvironment. Infiltration of B cell subset called

tumour evoked Bregs (B regulatory) plays a crucial role in lung metastasis by converting CD4+ T cell to Foxp3+ Treg cells through induction of TGF-β-dependent which promote immune escape in the 4T1 tumour-bearing mouse model of breast cancer [489]. Similarly, B cell infiltration facilitates the switch of M1 macrophages to a pro-tumoral M2 phenotype via IL-10 secretion [490]. On the contrary, elevated expression of peritumoural B-cells in lymph node metastases in patients with oro- and hypopharyngeal carcinoma is associated with favourable outcome [487]. Correspondingly, tumour-infiltrating B-cells correlate with improved survival outcome in the immunoreactive ovarian cancer subtype and HER2-enriched and basal-like breast cancer subtypes [491]. Although B cells normally do play active roles in anti-tumour immunity, these studies have demonstrated the capacity of the tumour microenvironment to modify immune function to promote tumour progression.

1.6.3.4. Eosinophils

Eosinophils release an array of cytokines, including IL-1β, TNF-α, and interferon-gamma (IFN-γ) and eosinophil derived neurotoxin (EDN) that are potentially toxic to nerve cells. Eosinophils localize to nerves (eosinophil-nerve interaction) and are associated with enhanced nerve activity [492]. In addition, eosinophils infiltrate cancer cells leading to either favourable or unfavourable prognosis [493]. For instance, in Hodgkin's lymphoma, eosinophils infiltration correlate with an unfavourable prognosis [494] whereas in colon cancer the presence of eosinophils leads to a favourable prognosis [495, 496]. However, the role of eosinophils and nerve interactions in cancer aetiology is not clear. The presence of eosinophils in necrotic regions of the tumour suggests that they may have anti-tumour effects associated with a favourable prognosis [497, 498]. Conversely, it has been noted that eosinophils may contribute to tumour invasion via activation of gelatinase [496, 497, 499, 500]. Furthermore, eosinophils at the tumour site can influence angiogenesis via VEGF secretion [501]. Moreover, TNF-α-stimulated eosinophils release pro-angiogenic factors such as, basic fibroblast growth factor, IL-6, IL-8, platelet-derived growth factor and MMP-9 [499]. However, pro-angiogenic factors such as IL-15 and TNF-α-stimulated eosinophils have only been noted, and theirs role in tumours is not clear [400]. Secretion of eosinophilic granular proteins has been noted in breast cancer [502]

and is associated with increased survival. However, some studies report lack of eosinophils [503] which warrants further investigation into eosinophil infiltration in breast cancer.

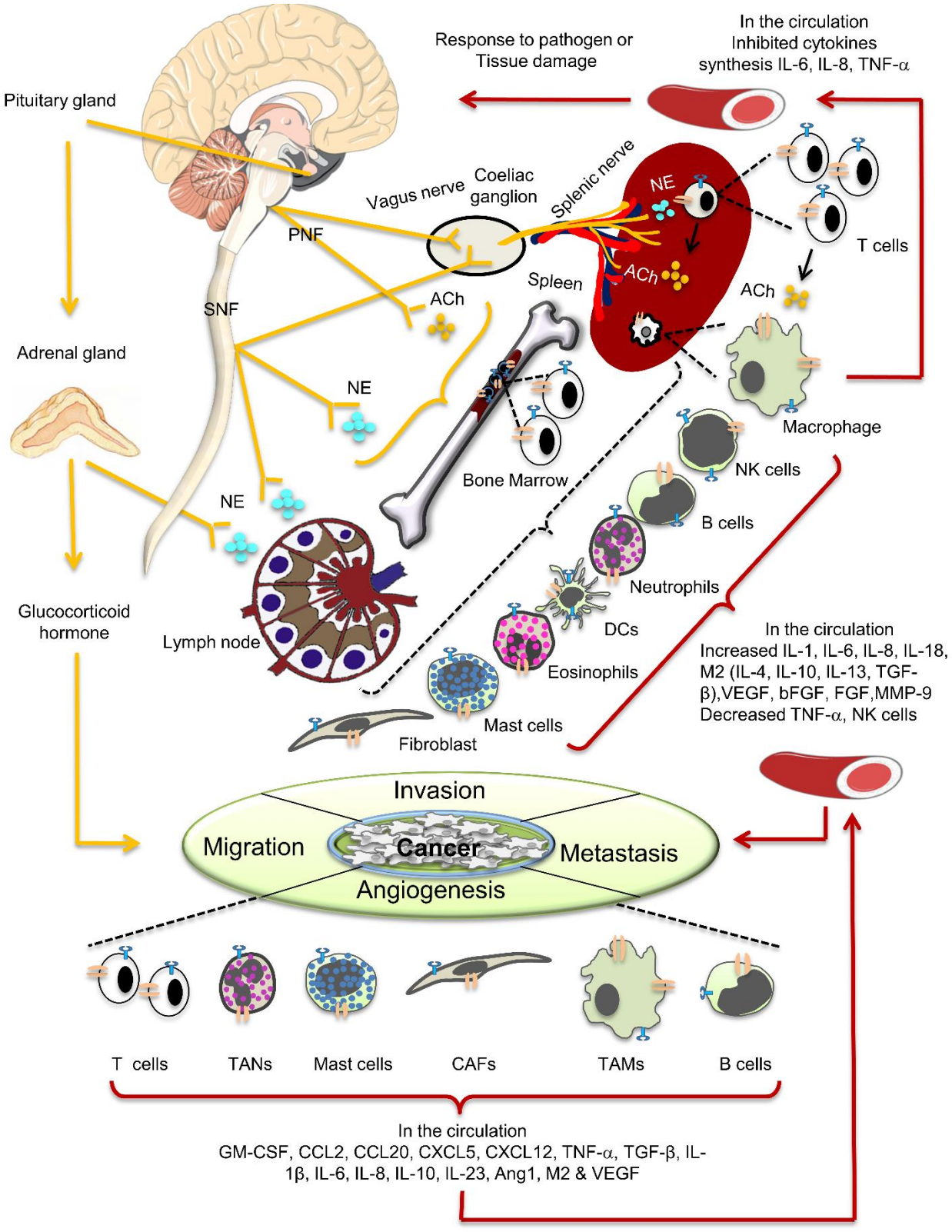
1.6.3.5. Mucosa-associated invariant T cells

The role of nervous system in regulating mucosa-associated invariant T (MAIT) cells is not clear. However, since MAIT cells are subset of T cell, it could be in the same manner T cells get innervated is how MAIT cells get innervate. MAIT cells have anti-microbial specificity [504-506] and are present in a number of cancers [507]. Their presence correlates with the level of pro-inflammatory cytokines within the tumour microenvironment [507], suggesting they have anti-cancer functions. However, enhanced expression of tumour-associated MAIT cells associates with poor prognosis in colorectal cancer contradicting norm that MAIT cell may have anti-tumour effect [508]. In fact, tumour-associated MALT cells are increased while circulating CD8+ MAIT cells decreased in advanced colorectal cancer patients [161]. Co-culture of HCT116 cells with MAIT cells stimulated with phorbol 12-myristate 13-acetate results in enhanced TNF-α, IFN-γ and IL-17 expression and reduced HCT116 cells feasibility, suggesting MAIT cells may contribute to colorectal cancer immunosurveillance [161]. Whether this effect of MAIT cells is cancer type specific, warrant further research. Thus, considering the important role of MAIT cells in response to infections, understanding their potential in cancer would aid in a better understanding of the cancer environment. Furthermore, further studies are warranted to establish the interaction between nervous system and MAIT cells.

Figure 1.6. Schematic diagram highlighting the critical function of the nervous system in modulating immune responses to cancer

ACh released from vagus nerve in macrophages binds to α7 nicotinic receptors on tissue macrophages and inhibits the release of pro-inflammatory cytokines. In the functional immune response to pathogen invasion or tissue damage, these are recognized by macrophages within the spleen, which triggers secretion of pro-inflammatory cytokines. Stress initiates a cascade of responsive neural pathways in the central nervous system, leading to the activation of sympathetic nervous systems and HPA axis. The stress response results in release of catecholamines (principally norepinephrine and epinephrine) and glucocorticoids from sympathetic nerve fibers located within organs and the adrenal medulla. Prolonged exposure to catecholamines under chronic stress importantly affects the process of tumour development. Glucocorticoids are associated with a decreased immune response, which further enhances tumour progression. Most immune cells and cancer cells express adrenergic and cholinergic receptors. Through these receptors, the nervous system is able to communicate with cancer cells via the release of neurotransmitters, cytokines and chemokines from both ends, which eventually influences tumour growth.

Abbreviations: Ach, acetylcholine; HPA, hypothalamic-pituitary-adrenal; IL, interleukin; NK, natural killer cells; NE, norepinephrine; PNF, peripheral nerve fibers; SNF, sympathetic nerve fibers; TNF-alpha, tumour necrosis factor-alpha; Ang1, angiopoietin 1; bFGF, basic fibroblast growth factor.


Pituitary gland
Response to pathogen or
Tissue damage
In the circulation
Inhibited cytokines
synthesis IL-6, IL-8, TNF-α
Vagus nerve
Coeliac
ganglion
Splenic nerve
NE
PNF
ACh
Spleen
T cells
SNF
ACh
Adrenal gland
NE
Macrophage
Bone Marrow
NK cells
NE
B cells
Neutrophils
Glucocorticoid
hormone
Lymph node
DCs
In the circulation
Increased IL-1, IL-6, IL-8, IL-18,
M2 (IL-4, IL-10, IL-13, TGF-
β),VEGF, bFGF, FGF,MMP-9
Decreased TNF-α, NK cells
Eosinophils
Mast cells
Fibroblast
Invasion
Migration
Cancer
Metastasis
Angiogenesis
T cells
TANs
Mast cells
CAFs
TAMs
B cells
In the circulation
GM-CSF, CCL2, CCL20, CXCL5, CXCL12, TNF-α, TGF-β, IL-
1β, IL-6, IL-8, IL-10, IL-23, Ang1, M2 & VEGF

1.7. Contribution to Knowledge and Significance

Despite the increasing interest to understand, the roles that the nervous and immune systems play in influencing the tumour microenvironment to promote cancer development and progression, more studies are required to understand the mechanism. Studies have demonstrated that modulation of the immune system by relentless release of neurotransmitters from the nerve terminals can promote tumour growth and metastasis. Revealing the interaction between the immune and nervous systems in cancer may open new avenues for understanding mechanisms of tumour development and progression. Thus, it is necessary to identify a combination of markers that may predict colorectal cancer (CRC) metastasis before it manifests in patients.

There is no existing comprehensive study available that correlates a range of immunosuppressive and cholinergic markers at different stages of CRC with clinical outcome. It is reasonable to hypothesise that different stages of CRC may be controlled by the changes in cholinergic signalling influencing the immunosuppressive markers, immune response, tumour microenvironment and vice versa. This thesis aims to evaluate the interaction between immunosuppressive and cholinergic markers in CRC. The findings of this work have important clinical relevance and might create a new therapeutic avenue, which could target both immunosuppressive and cholinergic markers that might be beneficial for CRC treatments.

1.8. Hypothesis and Aims

It was hypothesised that:

I) Different stages of CRC are controlled by the changes cholinergic signalling influencing the immune response, immunosuppressive and tumour microenvironment and vice versa.

II) At different stages of CRC, an interaction between immunosuppressive and cholinergic markers correlates with patients' clinical outcomes.

III) Cholinergic signalling can influence the expression of immunosuppressive markers *in vitro* and *in vivo*.

IV) Immunosuppressive molecule siglec-9/siglec-E can influence the expression of cholinergic markers *in vitro* and *in vivo*.

The overall aim of this thesis is to conduct a comprehensive evaluation of the complex interaction between immunosuppressive markers and cholinergic signalling in *ex vivo* human specimens from patients diagnosed with stages I-IV of CRC, *in vitro* and *in vivo* models of CRC.

Specific Aims:

Aim 1. To determine the expression of immunosuppressive molecules in *ex vivo* of human specimens from patients diagnosed with stages I-IV of CRC, *in vitro* and *in vivo*.

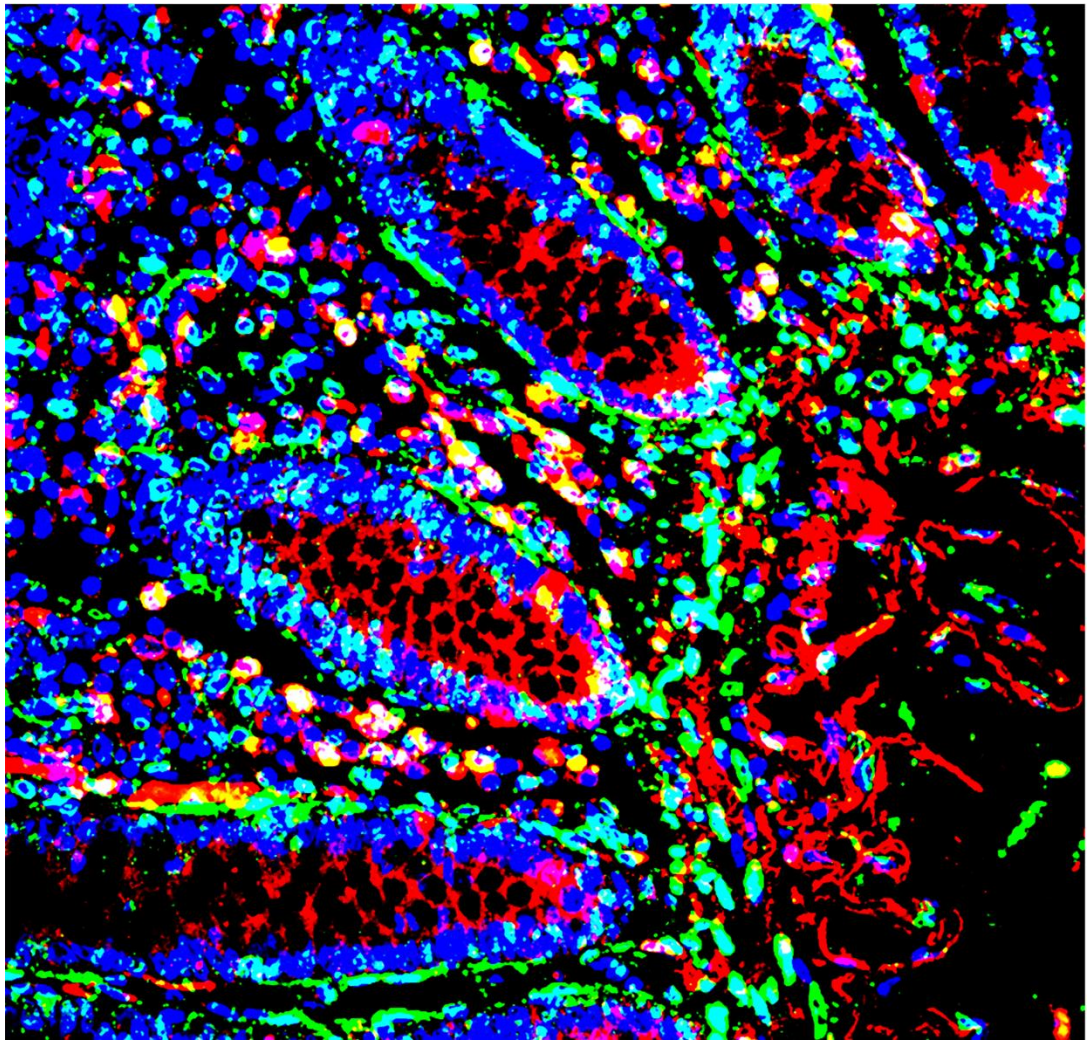
Aim 2. To determine cholinergic markers *ex vivo* of human specimens from patients diagnosed with stages I-IV of CRC, *in vitro* and *in vivo*.

Aim 3. To correlate the interaction between immunosuppressive molecules and cholinergic markers with stage of disease and clinical outcomes.

Aim 4. To determine angiogenic markers in tumour tissues collected from orthotopic model of colon cancer.

CHAPTER TWO

Expression of Immunosuppressive and Cholinergic Markers in Patients Diagnosed with Stages I-IV of CRC



2.0. Abstract

Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide. Tumour cells have evolved to express immunosuppressive molecules enabling their evasion from the host's immunity. These molecules include programmed death ligands PD-L1 and PD-L2. Cancer cells can also produce a neurotransmitter, acetylcholine, which have been shown to play a role in tumour progression. Moreover, tumours can stimulate vascularisation by producing angiogenic and neural factors, leading to tumour growth and metastasis. We correlated the expression of immunosuppressive (PD-L1 and PD-L2) and cholinergic muscarinic receptor 3 (M3R), alpha 7 nicotinic receptor (α7nAChR) and ChAT markers with CRC stages (I-IV), gender, age, metastasis and survival outcomes. Immunofluorescence was used to determine the expression of these molecules in paraffin-embedded tissues from patients with CRC. There was significantly high expression of PD-L1 at stages III and IV compared to stages I and II of CRC. In addition, PD-L2 was highly expressed at stages II, III and IV compared to stage I. Similarly, M3R and ChAT were elevated at stages III and IV compared to early stages. However, there was no significant difference in the expression of α7nAChR at all stages of CRC. Increased expression of PD-L1, PD-L2, M3R and ChAT were associated with a high risk of CRC and poor survival outcome. However, there was no significant correlation between the expression of these markers with patients' gender and age. In conclusion, the expression of immunosuppressive and cholinergic markers may increase the risk of recurrence or second cancer in patients. Therefore, these markers might be used in determining prognosis and treatment regimens for CRC patients.

2.1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide as a result of a predominantly unhealthy lifestyle and genetic factors [2]. CRC presents vague or no symptoms at the early stages; hence, it is more often diagnosed at the advanced stages of a disease. About 70% of CRC occurs sporadically due to the accumulation of mutations in the tumour suppressor genes that induce cancer [17], such as, *p53*, *K-ras* and *adenomatous polyposis coli* [18, 19]. However, studies have demonstrated that immunosuppression and cholinergic signalling play an important role in the development and progression of CRC [509-514].

The immune system plays a pivotal role in the development of tumour, not only it can suppress the growth, but it can also advance tumour growth by creating the immunosuppressive environment. The ability of cancer cells to evade T cell responses and avoid immune recognition by disabling effector T cells is dependent on the multiple immunosuppressive mechanisms controlled by immune checkpoints of inhibitory pathways, including, but not limited to, PD-L1 and PD-L2. These immune checkpoints are initiated by ligand-receptor interactions to enhance anti-tumour immunity [515]. Cancer cells overexpress immunosuppressive factors, such as PD-L1 and PD-L2, leading to suppressed T cell activation and apoptosis [514]. PD-L1 is a transmembrane protein that plays a major role in suppressing the immune system. Several tumour cells and antigen-presenting cells express PD-L1 [516]. T cells express the receptor PD-1 and, upon interaction with PD-L1, inhibitory signals are triggered, resulting in reduced activation or exhaustion of CD8+ T cells [27]. PD-L2 has a similar function to PD-L1, whereby it interacts with PD-1 on activated CD8+ T cells; however, its role in maintaining tumour cell immunity is not clear [517]. These findings have suggested the importance of the interaction between tumour and host's immune system that allows cancer cells to evade the immune responses leading to tumour growth.

Furthermore, the nervous system also plays a functional role in cancer growth and progression. Studies have demonstrated that the nervous system promotes the development of tumour by facilitating angiogenesis and metastasis through the release

of neural-related factors from nerve endings such as neurotrophins, neuropeptides and neurotransmitters [510, 511]. One of the major neurotransmitters in the central and peripheral nervous system is acetylcholine (ACh), which can cause diverse effects depending on the type of receptor it interacts with. Recently, studies have shown that several cancers can release ACh and express cholinergic receptors, suggesting that ACh could play a major role in cancer cell growth, vascularization, invasion and metastasis [518, 519]. In fact, ACh has been shown to promote proliferation and migration of cancer cells, tumour angiogenesis and metastasis through the activation of muscarinic receptor 3 (M3R) and alpha 7 nicotinic receptor (α7nAChR) [520-523]. In addition, cancer cells can also overexpress choline acetyltransferase (ChAT), a precursor enzyme required for ACh synthesis [521].

Currently, there are no studies correlating the expression of immunosuppressive and cholinergic markers with CRC stages and clinical parameters. Therefore, in this study, the expression of immunosuppressive (PD-L1 and PD-L2) and cholinergic (M3R, α7nAChR and ChAT) markers was correlated with CRC stages (I-IV), patients' age, gender, survival status and survival outcomes. The expression of these molecules may be important for the early detection of cancer; hence, it is necessary to identify the combination of factors expressed by cancer cells that may predict cancer progression in patients.

2.2. Materials and Methods

2.2.1. Human tumour samples

Tumour samples were collected at the Ministry of Internal Affairs and Administration Hospital in Olsztyn, Poland from 2010 to 2013. The collection of human samples used in this study was approved by the Bioethics Committee of the University of Warmia and Mazury in Olsztyn, Poland. The study comprised of 139 patients with CRC (out of 139, 91 patients had clinical follow up). All patients signed a written informed consent for the use of their tissues for research purposes. Patients had no evidence of bowel obstruction or other colonic diseases. None of the CRC patients had a second neoplastic disease or had previously undergone chemo- or radiotherapy. Patients' demographical, clinical and overall survival data were collected. Type of cancer and grading were described by a pathologist according to the World Health Organization criteria and staging according to the 7th edition of Cancer Staging Manual of the American Joint Committee on Cancer (AJCC). However, due to a small patient number with grade III (n=7) compared to grade II (n=84) CRC, the grade was excluded from the multivariate analysis and Chi-Square test. Similarly, metastasis status was excluded due to small sample size; nine patients had metastatic cancer compared with 82 patients without. Samples from the neoplasm lesion were collected into 10% neutral buffered formalin, dehydrated in ethanol/xylene and embedded in the paraffin wax. Paraffin-embedded blocks of tissue were cut into 4μm thickness sections and mounted onto the microscope slides.

2.2.2. Immunohistochemical analysis of the paraffin-embedded samples

Samples were deparaffinised and hydrated through the series of washes with xylene and a graded alcohol. Antigen retrieval was performed using citrate buffer pH 6.0, 10x (Sigma-Aldrich, Melbourne, Australia). Citrate buffer was heated until bubbles start to form. Samples were emerged into the buffer and placed on a hot plate pre-set at 100°C for 15 minutes (mins) and left to cool at room temperature for another 20 mins. Using a liquid blocker super pap pen, samples were outlined to reduce the volume of antibody used. Endogenous activity was blocked using 10% donkey serum for 1 hour (hr) at room

temperature. Samples were then incubated overnight at room temperature with primary antibodies (**Table 2.1**). After washing the tissues in phosphate buffered (PBS) saline plus Triton X-100 (PBST), samples were incubated at room temperature for 2hr with secondary antibodies (**Table 2.1**) diluted in PBS containing 2% donkey serum and 0.01% Triton X-100. Samples were incubated for 1 min with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (D1306, Life Technologies, Australia) and mounted with DAKO mounting medium (Agilent Technologies, Australia). Then coverslips were placed on and left to dry overnight before imaging.

2.2.3. Data analysis

Images were captured on a Nikon Eclipse Ti multichannel confocal laser scanning system (Nikon, Japan). Z-series images were acquired at a nominal thickness of 1µm (1024 x 1024 pixels). Image J software (National Institute of Health, Bethesda, MD, USA) was employed to convert images from RGB to 8-bit binary; particles were then analysed to obtain the percentage area of immunoreactivity [524]. For localisation data analysis, the number of cells within the tumour specimen expressing markers were counted within eight randomly captured images at x40 magnification.

All slides were coded, and immunohistochemistry images were quantified blindly. Statistical analysis was performed by one-way *ANOVA* followed by Turkey′s pos-hoc test. For correlation of markers expression with the clinicopathological parameters, Cox regression test for survival analysis, Chi-Square test and multivariate for correlation analyses was used. Low and high expression of the markers was defined by determining the median. A cumulative risk refers to the likelihood that patients expressing low or high markers at different stages of CRC would die from CRC. Pearson Correlation was performed to analyse the relationship between the overall expression of immunosuppressive with cholinergic markers. Microsoft Excel, SPSS and Prism (Graph Pad Software, La Jolla, CA, USA) were utilised to aid in the statistical analysis and $p<0.05$ was considered significant.

Table 2.1. Primary and secondary antibodies used in this study

Primary antibodies				
Markers	**Host Species & Clonality**	**Dilution**	**Source**	**Catalogue no.**
PD-L1	Mouse, monoclonal	1:500	Abcam, Australia	ab210931
PD-L2	Rabbit, polyclonal	1:500	Abcam, Australia	ab200377
M3R	Rabbit, polyclonal	1:500	Abcam, Australia	ab126168
α7nAChR	Mouse, monoclonal	1:500	Novus, Australia	7F10G1
ChAT	Goat, polyclonal	1:500	Abcam, Australia	ab134021
Secondary antibodies				
Alexa Fluor 488	Anti-mouse Anti-goat	1:250	Jackson ImmunoResearch Laboratories, United States	
Alexa Fluor 594	Anti-rabbit	1:250	Jackson ImmunoResearch Laboratories, United States	
Alexa Fluor 647	Anti-mouse	1:250	Jackson ImmunoResearch Laboratories, United States	

2.3. Results

We examined the expression levels of immunosuppressive markers, PD-L1 and PD-L2, and cholinergic markers, M3R, α7nAChR and ChAT, in colon samples resected from 139 patients with colorectal carcinoma. All patients had surgery before commencing chemo- or radiotherapy. To evaluate the expression of these markers, we used tissues obtained from patients diagnosed with stages I-IV of CRC. In this study, immunofluorescence was used to determine the expression of these molecules in paraffin-embedded tumour tissues.

2.3.1. Expression of immunosuppressive markers in CRC tissues

2.3.1.1. Expression of PD-L1 and PD-L2 in patients diagnosed with stages I-IV of CRC

Tumour tissues were immunolabelled with immunosuppressive marker antibodies as described in the Materials and Methods. PD-L1 expression was predominantly localised in the cell membrane and is abundantly expressed at stages III and IV compared to stages I and II. Representative images of PD-L1 expression from each clinical stage are depicted in **Figure 2.1A'-D'**. Quantitative analysis of PD-L1 expression demonstrated a linear relationship between cancer progression and PD-L1 expression (**Figure 2.1E**, *p<0.0001***)**. PD-L2 expression was enhanced at stages II, III and IV, compared to stage I. Moreover, PD-L2 expression was overexpressed at stage IV compared to stages I, II and III (**Figure 2.1A''-D''** and **Figure 2.1F**, *p<0.0001*). Overall, PD-L1 and PD-L2 expression was overexpressed at stage IV.

2.3.1.2. Number of cells within tumour specimen expressing PD-L1 and PD-L2

We evaluated the number of cells within tumour specimens expressing PD-L1 and PD-L2. The results demonstrated that immunosuppressive markers, PD-L1 and PD-L2 are predominantly localised in close proximity to each other. Elevated number of cells within tumour specimens overexpressing PD-L1 and PD-L2 was observed at stages II, III and IV compared to stage I (**Figure 2.2A**-**F**). Moreover, the number of cells overexpressing

PD-L1 and PD-L2 was higher at stage IV compared to stages I, II and III. Furthermore, there was a high number of cells expressing PD-L1 in mucosa and muscularis mucosa at advanced stages III and IV compared to early stages I and II (**Figure 2.2A**-**D**). Cells expressing PD-L2 were confined to the mucosal layer at stages I, II and III, while at stage IV, cells expressing PD-L2 were observed in both mucosa and muscularis mucosa. Overall, PD-L1 is expressed in mucosa and muscularis mucosa layers, whereas PD-L2 is predominantly expressed in mucosa with exception to stage IV where it was expressed in both layers.

Figure 2.1. Intensity of immunosuppressive markers expression

PD-L1 and PD-L2 in human specimens from CRC patients diagnosed with stage I (**A-A'''**), stage II (**B-B'''**), stage III (**C-C'''**) and stage IV (**D-D'''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-D**), PD-L1 (green; **A'-D'**), PD-L2 (red; **A''-D''**) and all markers merged (**A'''-D'''**). Scale bar represents 50μm. Bar graphs displaying the mean fluorescence of PD-L1 (**E**) and PD-L2 (**F**) in tumours from patients with stages I-IV CRC. Data presented as mean ± standard error of the mean (SEM), stage I n=14, stage II n=59, stage III n=57 and stage IV n=9. One-way *ANOVA, **p<0.01, ***p<0.001, ****p<0.0001*.

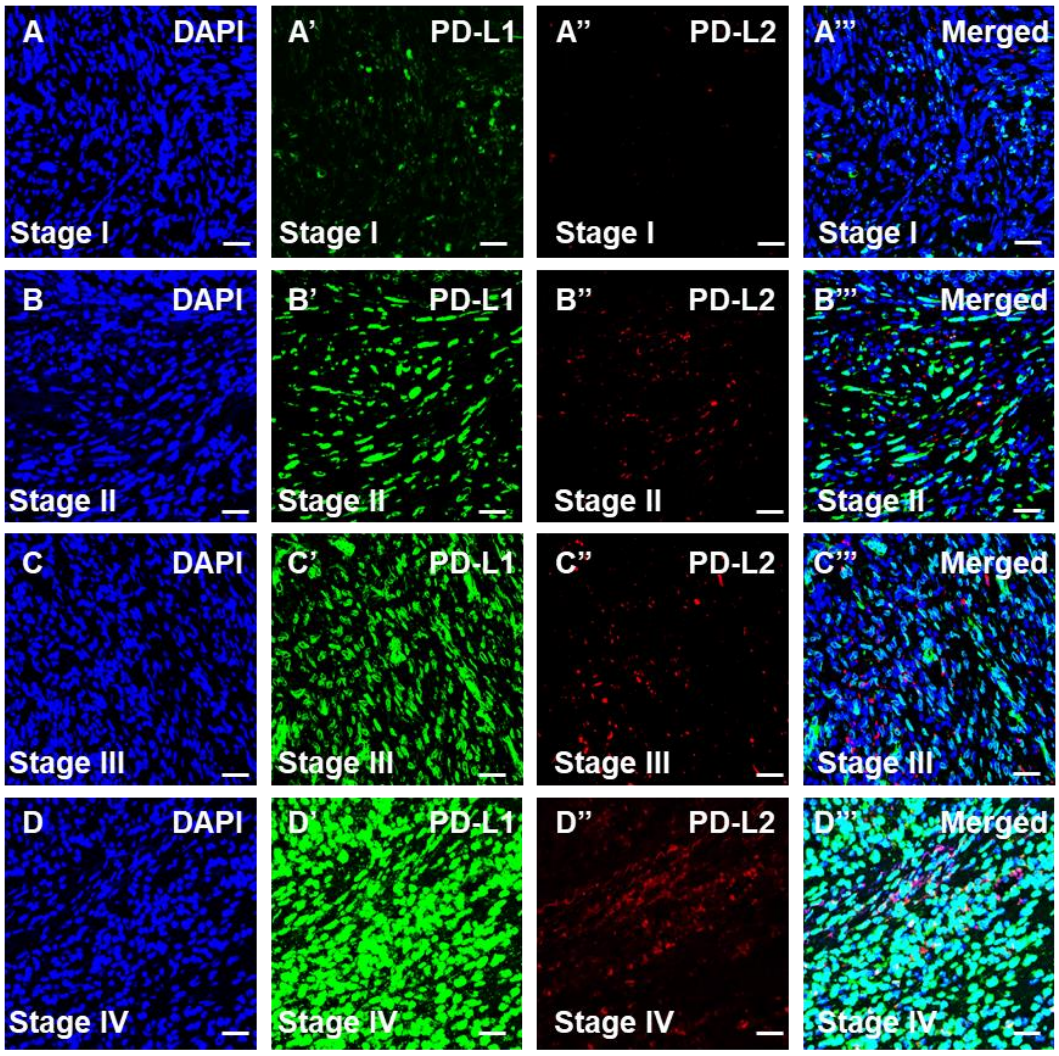
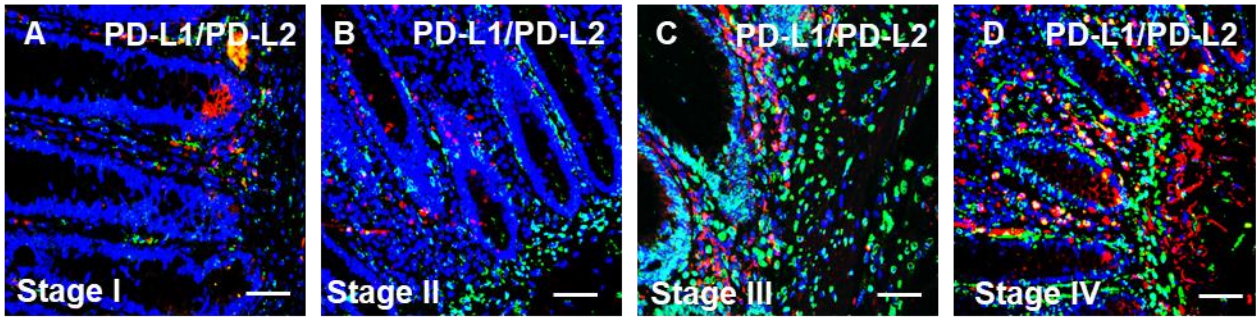
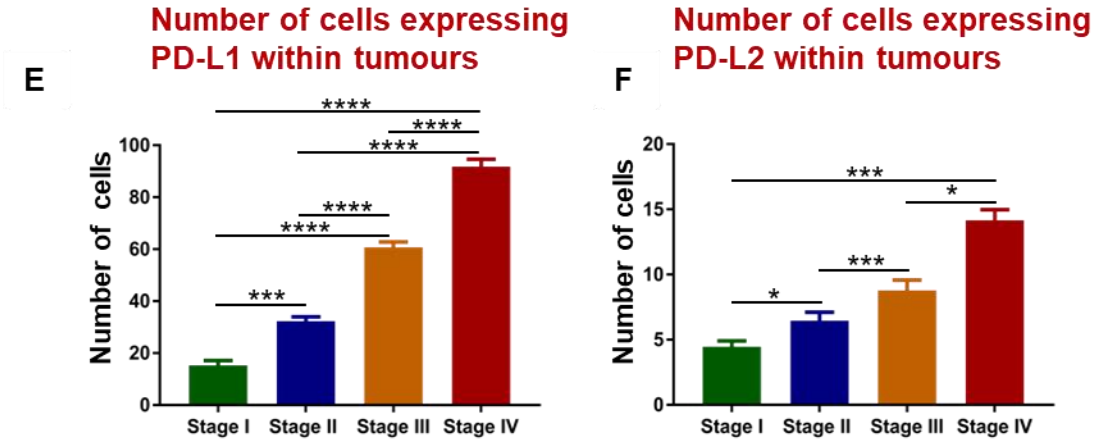

A
DAPI
Stage I
A'
PD-L1
Stage I
A''
PD-L2
Stage I
A'''
Merged
Stage I
B
DAPI
Stage II
B'
PD-L1
Stage II
B''
PD-L2
Stage II
B'''
Merged
Stage II
C
DAPI
Stage III
C'
PD-L1
Stage III
C''
PD-L2
Stage III
C'''
Merged
Stage III
D
DAPI
Stage IV
D'
PD-L1
Stage IV
D''
PD-L2
Stage IV
D'''
Merged
Stage IV
E
PD-L1
Fluorescence (Arb. Units)
0
10
20
30
****
****
****
****
****
Stage I
Stage II
Stage III
Stage IV
F
PD-L2
Fluorescence (Arb. Units)
0
2
4
6
8
****
**
***
****
****
Stage I
Stage II
Stage III
Stage IV

Figure 2.2. Number of cells expressing PD-L1 and PD-L2 within tumour specimens from patients at stages I-IV CRC

PD-L1 and PD-L2 expression within colonic mucosa from CRC patients diagnosed with stage I (**A**), stage II (**B**), stage III (**C**) and stage IV (**D**). All cells were labelled with the nuclei marker DAPI (blue), PD-L1 (green) and PD-L2 (red). Scale bar represents 50μm. Bar graphs displaying the mean number of cells within tumour specimen expressing PD-L1 (**E**) and PD-L2 (**F**) in tumours from patients with stage I-IV CRC. Data presented as mean ± SEM, stage I n=14, stage II n=59, stage III n=57 and stage IV n=9. One-way *ANOVA, *p<0.05, ***p<0.0001, ****p<0.0001*.


A
PD-L1/PD-L2
Stage I
B
PD-L1/PD-L2
Stage II
C
PD-L1/PD-L2
Stage III
D
PD-L1/PD-L2
Stage IV


E
Number of cells expressing PD-L1 within tumours
Number of cells
100
80
60
40
20
0
****
****
****
****
****
***
Stage I
Stage II
Stage III
Stage IV
F
Number of cells expressing PD-L2 within tumours
Number of cells
20
15
10
5
0
***
*
***
*
Stage I
Stage II
Stage III
Stage IV

2.3.1.3. Clinicopathological and demographic parameters of CRC patients and their relevance to immunosuppressive markers expression

The average patients' age was 65 years ranging from 33 to 91 and there were 51 male patients and 40 female patients in the cohort. Among these patients, 14 patients were diagnosed with clinical stage I, 35 with stage II, 33 with stage III and 9 with stage IV of CRC. The clinicopathological and demographic parameters of 91 patients with clinical follow up are presented in **Table 2.2**.

To determine the correlation between the expression of immunosuppressive markers and clinicopathological parameters, the Chi-Square test was used. PD-L1 expression was correlated with gender, age, stage, and survival status (**Table 2.3**). Out of 91 patients, 38 (41.8%) expressed low levels of PD-L1 and 53 (58.2%) expressed high levels. There was a significant difference observed between the expression of PD-L1 and stages of CRC. Lower stages were noted to mainly expressed low levels of PD-L1, 13 (14.3%) at stage I and 26 (28.6%) at stage II compared to 11 (12.1%) at stage III and 0 (0%) at stage IV. High level of PD-L1 expression was associated with stages III and IV.

There was no statistical difference observed in PD-L1 expression and patients' gender as 21 (23.1%) males and 17 (18.7%) females expressed low levels of PD-L1, whereas 30 (32.9%) males and 23 (25.3%) females expressed high levels. In addition, patients were divided into two age groups, under 65 (<65) and over 65 (>65) years old. Seventeen (18.7%) <65 and 21 (23.1%) >65 patients expressed low levels of PD-L1, and 24 (26.4%) <65 and 29 (31.9%) >65 patients expressed high levels. Nevertheless, there was no significant correlation between the level of PD-L1 expression and patients' age. Furthermore, there was correlation between PD-L1 expression and patients' survival status.

On the other hand, 75.8% of patients expressed low levels of PD-L2, and 24.2% expressed high levels. In regards to patients' age, gender and survival status, there were no significant differences observed between the level of PD-L2 expression and these parameters (**Table 2.4**). However, there was a weak association between PD-L2 expression and stages of CRC. Stages, I and II predominantly expressed low levels of

PD-L2 while advanced stages, 24 patients out 33 at stage III and 7 patients out 9 at stage IV expressed high levels of PD-L2. This suggests that PD-L2 expression might hold predictive value for advanced stages of CRC.

Moreover, PD-L1 and PD-L2 expression was correlated with the risk of CRC and patients' survival outcomes. The correlation between PD-L1 and PD-L2 with survival outcome was analysed by hazard ratio (HR) and corresponding 95% confidence interval (CI) using Cox regression survival analysis. The results of this analysis demonstrated a significant correlation between high expression of PD-L1 and an increased risk of CRC and poor survival outcomes (**Figure 2.3A & B**, HR=2.942, 95% CI=1.841-4.699). Furthermore, high levels of PD-L2 expression was also associated with a higher risk of CRC and poor patients' survival outcomes (**Figure 2.3C & D**, HR=2.691, 95% CI=1.725-4.20).

Table 2.2. Clinicopathological and demographic parameters of CRC patients

Parameters	No. of cases	Percentage (%)
Total	91	100
Gender		
Male	51	56
Female	40	44
Age		
<65	41	45.1
>65	50	54.9
Stage		
I	14	15.4
II	35	38.5
III	33	36.3
IV	9	9.9
Survival status		
Event	52	57.1
Censor	39	42.9

Table 2.3. Correlation of clinicopathological and demographic parameters of CRC patients with PD-L1 expression

Parameters	No. of cases	Percentages (%)	PD-L1 expression		*P* values
			Low	High	
Total	91	100	38 (41.8%)	53 (58.2%)	
Gender					
Male	51	56	21 (23.1%)	30 (32.9%)	0.534
Female	40	44	17 (18.7%)	23 (25.3%)	
Age					
<65	41	45.1	17 (18.7%)	24 (26.4%)	0.565
>65	50	54.9	21 (23.1%)	29 (31.9%)	
Stage					
I	14	15.4	13 (14.3%)	0 (0%)	
II	35	38.5	26 (28.6%)	9 (9.9%)	0.0001
III	33	36.3	11 (12.1%)	23 (25.3%)	
IV	9	9.9	0 (0%)	9 (9.9%)	
Survival status					
Event	39	42.9	7 (7.7%)	32 (35.2%)	0.018
Censor	52	57.1	18 (19.8%)	34 (37.4%)	

P values are based on the frequency of PD-L1 expression within each parameter.

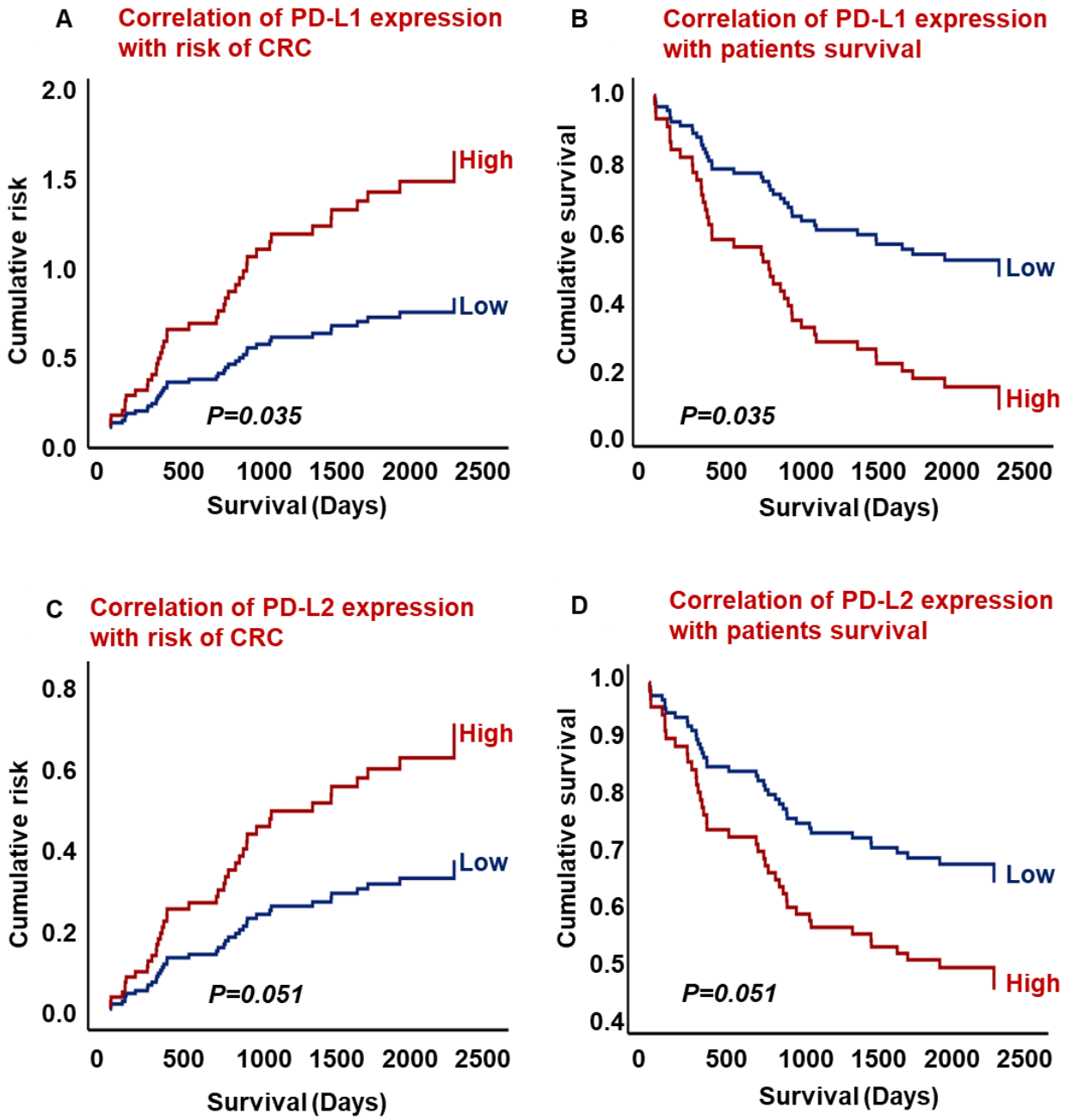
Table 2.4. Correlation of clinicopathological and demographic parameters of CRC patients with PD-L2 expression

Parameters	No. of cases	Percentages (%)	PD-L2 expression		*P* values
			Low	High	
Total	91	100	69 (75.8%)	22 (24.2%)	
Gender					
Male	51	56	39 (42.9%)	12 (13.2%)	0.531
Female	40	44	30 (33.0%)	10 (11.0%)	
Age					
<65	41	45.1	31 (34.1%)	10 (11%)	0.579
>65	50	54.9	38 (41.8%)	12 (13.2%)	
Stage					
I	14	15.4	10 (11%)	3 (3.3%)	
II	35	38.5	27 (29.1%)	8 (8.8%)	0.012
III	33	36.3	9 (9.9%)	24 (26.4%)	
IV	9	9.9	2 (2.2%)	7 (7.7%)	
Survival status					
Event	39	42.9	12 (13.2%)	27 (29.7%)	0.699
Censor	52	57.1	18 (19.8%)	34 (37.4%)	

P values are based on the frequency of PD-L2 expression within each parameter. The median defined low and high expression of the markers.

Figure 2.3. Correlation of PD-L1 and PD-L2 expression with the risk of CRC and patient's survival outcomes

Correlation of PD-L1 expression with the risk of CRC (**A**). PD-L1 expression association with survival outcomes (**B**). PD-L1 expression (HR=2.942, 95% CI=1.841-4.699, *p<0.0001* for both). Correlation of PD-L2 expression with the risk of CRC (**C**). PD-L2 expression association with survival outcomes (**D**). PD-L2 expression (HR=2.691, 95% CI=1.725-4.20, *p<0.0001* for both). Stage I n=14, stage II n=35, stage III n=33 and stage IV n=9. Low and high expression of the markers was defined by the median.


A
Correlation of PD-L1 expression with risk of CRC
Cumulative risk
2.0
1.5
1.0
0.5
0.0
High
Low
P=0.035
0
500
1000
1500
2000
2500
Survival (Days)
B
Correlation of PD-L1 expression with patients survival
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
Low
High
P=0.035
0
500
1000
1500
2000
2500
Survival (Days)
C
Correlation of PD-L2 expression with risk of CRC
Cumulative risk
0.8
0.6
0.4
0.2
0.0
High
Low
P=0.051
0
500
1000
1500
2000
2500
Survival (Days)
D
Correlation of PD-L2 expression with patients survival
Cumulative survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Low
High
P=0.051
0
500
1000
1500
2000
2500
Survival (Days)

2.3.2. Expression of cholinergic markers in patients diagnosed with stages I-IV of CRC

2.3.2.1. Intensity of expression of cholinergic markers in CRC tissues

The expression of cholinergic markers was evaluated in specimens obtained from patients diagnosed with stages I-IV CRC. Representative images of α7nAChR expression from each clinical stage are demonstrated in **Figure 2.4A'-D'**. Although there was a tendency to increase α7nAChR expression at stages, III and IV, the difference was not significant (**Figure 2.4E**, *p-0.0675*). Conversely, M3R expression showed a linear increase with significantly high levels at stages, III and IV (**Figure 2.4A''-D''** and **F**, *p<0001*). Similarly, results also showed overexpression of ChAT at advanced stages, III and IV, compared to the lower stages, I and II (**Figure 2.4 A'''-D'''** and **G**, *p<0001*).

2.3.2.2. Correlation between cholinergic markers and clinicopathological and demographic parameters of CRC patients

To determine the association between the expression of cholinergic markers and demographic and clinicopathological parameters of the patients diagnosed with CRC, the Chi-Square test was utilised. The expression of α7nAChR was associated with patients' age, gender, stage of CRC and survival status. The results show that 49 (53.8%) patients expressed low levels of α7nAChR, while 42 (46.2%) expressed high levels. However, there was no association noted between α7nAChR and patients' age, gender, stage and survival status (**Table 2.5**).

Similarly, M3R expression was not associated with patients' age and gender; however, was correlated with survival status (**Table 2.6**). Stages, I and II mostly expressed low levels, while advanced stages, 23 patients out 33 at stage III and 8 patients out 9 at stage IV patients expressed high levels of M3R, suggesting that high levels of M3R expression might hold a prognostic value for advanced stages of CRC.

Furthermore, out of 91 patients, 32 (35.2%) expressed low levels of ChAT and 59 (64.8%) expressed high levels. High level of ChAT expression was not associated with patients' age, gender and survival status but was associated with CRC stage (**Table 2.7**). Early

stages, I and II, expressed low levels, whereas advanced stages, III and IV expressed high levels of ChAT.

Moreover, the correlation of α7nAChR, M3R and ChAT expression with the risk of CRC and patients' survival outcomes were analysed. There was no correlation between the expression of α7nAChR, risk of CRC and patients' survival outcomes (**Figure 2.5 A & B**, HR=0.066, 95% CI=0.508-1.790). There was a significant correlation observed between the high expression of M3R and a high risk of CRC and poor survival outcomes (**Figure 2.5 C & D**, HR=2.647, 95% CI=1.690-4.147). Furthermore, high level of ChAT expression was also associated with a higher risk of CRC and poor patients' survival outcomes (**Figure 2.5 E & F**, HR=2.692, 95% CI=1.721-4.264).

Figure 2.4. Intensity of expression of cholinergic markers α7nAChR, M3R and ChAT in tumour tissues from CRC patients

Tissues from patients diagnosed with stage I (**A-A’’’’**), stage II (**B-B’’’’**), stage III (**C-C’’’’**) and stage IV (**D-D’’’’**) of CRC. Tissues were labelled with the nuclei marker DAPI (blue; **A-D**), α7nAChR (magenta; **A’-D’**), M3R (red; **A’’-D’’**), ChAT (green; **A’’’-D’’’**) and all markers merged (**A’’’’-D’’’’**). Scale bar represents 50μm. Bar graphs displaying the mean fluorescence (arb. units) expression of α7nAChR (**E**), M3R (**F**) and ChAT (**G**) in patients with stages I-IV CRC. Data presented as mean ± SEM, stage I n=14, stage II n=59, stage III n=57 and stage IV n=9. One-way *ANOVA, ***p<0.001, ****p<0.0001*.

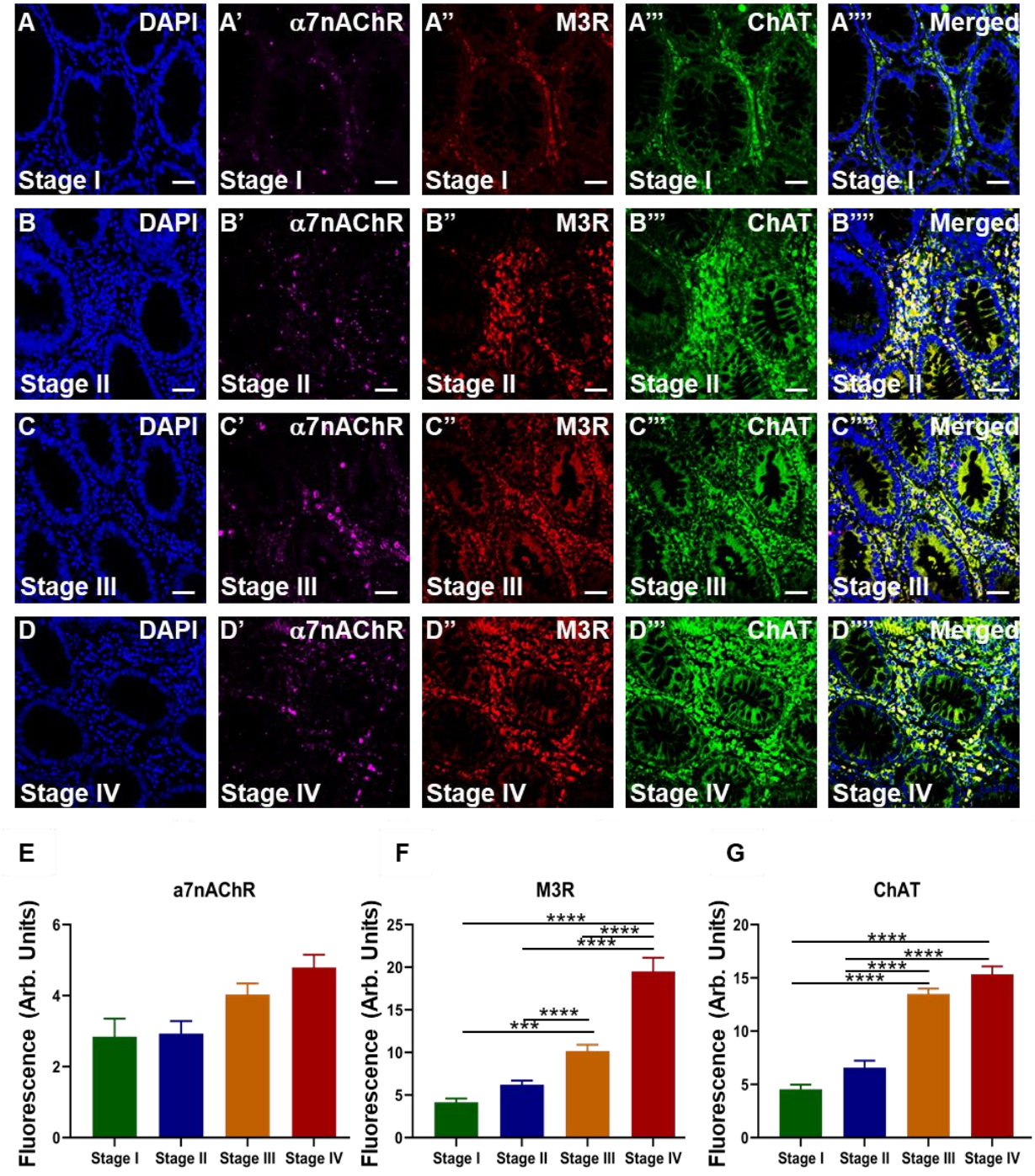

A DAPI
A' α7nAChR
A'' M3R
A''' ChAT
A'''' Merged
Stage I
B DAPI
B' α7nAChR
B'' M3R
B''' ChAT
B'''' Merged
Stage II
C DAPI
C' α7nAChR
C'' M3R
C''' ChAT
C'''' Merged
Stage III
D DAPI
D' α7nAChR
D'' M3R
D''' ChAT
D'''' Merged
Stage IV
E
a7nAChR
Fluorescence (Arb. Units)
0
2
4
6
Stage I
Stage II
Stage III
Stage IV
F
M3R
Fluorescence (Arb. Units)
0
5
10
15
20
25
****
****
****
****
***
Stage I
Stage II
Stage III
Stage IV
G
ChAT
Fluorescence (Arb. Units)
0
5
10
15
20
****
****
****
****
Stage I
Stage II
Stage III
Stage IV

Table 2.5. Correlation of clinicopathological and demographic parameters of CRC patients with α7nAChR expression

Parameters	No. of cases	Percentages (%)	α7nAChR expression		*P* values
			Low	High	
Total	91	100	49 (53.8%)	42 (46.2%)	
Gender					
Male	51	56	30 (32.9%)	21 (23.1%)	0.194
Female	40	44	19 (20.9%)	21 (23.1%)	
Age					
<65	41	45.1	24 (26.4%)	17 (18.7%)	0.274
>65	50	54.9	25 (27.5%)	25 (27.5%)	
Stage					
I	14	15.4	9 (9.9%)	4 (4.4%)	
II	35	38.5	24 (26.4%)	12 (13.2%)	0.130
III	33	36.3	12 (13.2%)	21 (23.1%)	
IV	9	9.9	4 (4.4%)	5 (5.5%)	
Survival status					
Event	39	42.9	14 (15.4%)	25 (27.5%)	0.531
Censor	52	57.1	22 (24.2%)	30 (33%)	

P values are based on the frequency of α7nAChR expression within each parameter. The median defined low and high expression of the markers.

Table 2.6. Correlation of clinicopathological and demographic parameters of CRC patients with M3R expression

Parameters	No. of cases	Percentages (%)	M3R expression		*P* values
			Low	High	
Total	91	100	58 (63.7%)	33 (36.3%)	
Gender					
Male	51	56	31 (34.1%)	20 (22%)	0.508
Female	40	44	27 (29.7%)	13 (14.3%)	
Age					
<65	41	45.1	22 (24.2%)	19 (20.9%)	0.070
>65	50	54.9	36 (39.6%)	14 (15.4%)	
Stage					
I	14	15.4	9 (9.9%)	5 (5.5%)	
II	35	38.5	30 (33.0%)	5 (5.5%)	0.005
III	33	36.3	10 (11%)	23 (25.3%)	
IV	9	9.9	1 (1.1%)	8 (8.8%)	
Survival status					
Event	39	42.9	4 (4.4%)	35 (38.5%)	0.007
Censor	52	57.1	18 (19.8%)	34 (37.4%)	

P values are based on the frequency of M3R expression within each parameter. The median defined low and high expression of the markers.

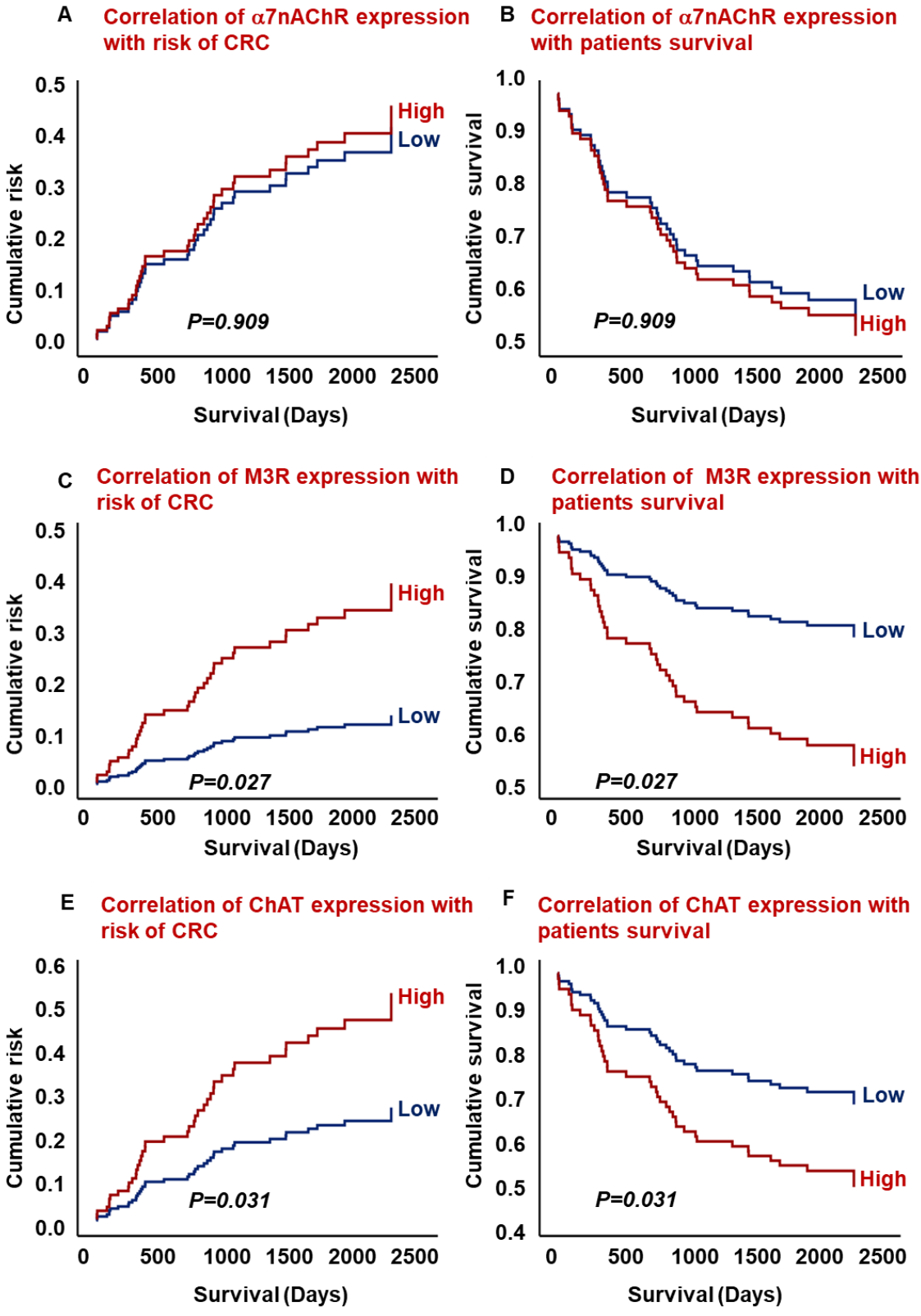
Table 2.7. Correlation of clinicopathological and demographic parameters of CRC patients with ChAT expression

Parameters	No. of cases	Percentages (%)	ChAT expression		*P* values
			Low	High	
Total	91	100	32 (35.2%)	59 (64.8%)	
Gender					
Male	51	56	30 (32.9%)	21 (23.1%)	0.194
Female	40	44	19 (20.9%)	21 (23.1%)	
Age					
<65	41	45.1	24 (26.4%)	17 (18.7	0.274
>65	50	54.9	25 (27.7 %)	%)	
				25 (27.5	
				%)	
Stage					
I	14	15.4	9 (9.9%)	5 (5.5%)	
II	35	38.5	19 (20.9%)	16 (17.6%)	0.0001
III	33	36.3	5 (5.5%)	29 (31.9%)	
IV	9	9.9	0 (0%)	9 (9.9%)	
Survival status					
Event	39	42.9	6 (6.6%)	33 36.3%)	0.807
Censor	52	57.1	9 (9.9%)	43 (47.3%)	

P values are based on the frequency of ChAT expression within each parameter. The median defined low and high expression of the markers.

Figure 2.5. Correlation of cholinergic markers with survival outcomes

Correlation of α7nAChR expression with the risk of CRC (**A**) and survival outcomes (**B**). Correlation of M3R expression with the risk of CRC (**C**) and survival outcomes (**D**). Association of ChAT expression with the risk of CRC (**E**) and survival outcomes (**F**). Stage I n=14, stage II n=35, stage III n=33 and stage IV n=9. Low and high expression of the markers was defined by the median.


A
Correlation of α7nAChR expression with risk of CRC
Cumulative risk
0.5
0.4
0.3
0.2
0.1
0.0
High
Low
P=0.909
0
500
1000
1500
2000
2500
Survival (Days)
B
Correlation of α7nAChR expression with patients survival
Cumulative survival
1.0
0.9
0.8
0.7
0.6
0.5
Low
High
P=0.909
0
500
1000
1500
2000
2500
Survival (Days)
C
Correlation of M3R expression with risk of CRC
Cumulative risk
0.5
0.4
0.3
0.2
0.1
0.0
High
Low
P=0.027
0
500
1000
1500
2000
2500
Survival (Days)
D
Correlation of M3R expression with patients survival
Cumulative survival
1.0
0.9
0.8
0.7
0.6
0.5
Low
High
P=0.027
0
500
1000
1500
2000
2500
Survival (Days)
E
Correlation of ChAT expression with risk of CRC
Cumulative risk
0.6
0.5
0.4
0.3
0.2
0.1
0.0
High
Low
P=0.031
0
500
1000
1500
2000
2500
Survival (Days)
F
Correlation of ChAT expression with patients survival
Cumulative survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Low
High
P=0.031
0
500
1000
1500
2000
2500
Survival (Days)

2.3.3. Correlation between immunosuppressive and cholinergic markers

We further evaluated the overall correlation between the expression of immunosuppressive markers with cholinergic markers (**Table 2.8**). Overall, the expression of PD-L1 was strongly correlated with M3R expression, moderately associated with PD-L2 and ChAT, while lowly correlated with α7nChR expression. Whereas, PD-L2 overall expression was strongly associated with α7nChR, moderately correlated with PD-L1 and ChAT, but not with M3R expression. Moreover, α7nChR expression was strongly associated with PD-L2 and ChAT, while lowly correlated with PD-L1 and M3R. Furthermore, M3R was strongly correlated with PD-L1 and ChAT, whereas lowly associated with α7nChR. Overall, the expression of ChAT was strongly correlated with cholinergic receptors and moderately associated with immunosuppressive markers. These findings suggest that there might be crosstalk between immunosuppressive markers with cholinergic markers.

Table 2.8. Correlation of immunosuppressive with cholinergic markers in CRC patients

		PD-L1	PD-L2	M3R	α7nChR	ChAT
PD-L1	Pearson Correlation	**1**	**0.351****	**0.562****	**0.235***	**0.323****
	Sig. (2-tailed)		0.001	0.000	0.025	0.002
	N	91	91	91	91	91
PD-L2	Pearson Correlation	**0.351****	**1**	0.153	0.**561****	**0.370****
	Sig. (2-tailed)	0.001		0.147	0.000	0.000
	N	91	91	91	91	91
M3R	Pearson Correlation	**0.562****	0.153	1	**0.298****	**0.571****
	Sig. (2-tailed)	0.000	0.147		0.004	0.000
	N	91	91	91	91	91
α7nChR	Pearson Correlation	**0.235***	**0.561****	**0.298****	1	**0.679****
	Sig. (2-tailed)	0.025	0.000	0.004		0.000
	N	91	91	91	91	91
ChAT	Pearson Correlation	**0.323****	**0.370****	**0.571****	**0.679****	1
	Sig. (2-tailed)	0.002	0.000	0.000	0.000	
	N	91	91	91	91	91

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

Strong correlation, ± 0.50 and ± 1; Moderate correlation, ± 0.30 and ± 0.49; Low correlation, ± 0.29.

2.4. Discussion

The results of this study show that level of PD-L1 was significantly elevated at stages, III and IV compared to stages, I and II of CRC, while PD-L2 expression was enhanced at stages, II, III and IV compared to stage I. In addition, there were more cells within tumour overexpressing PD-L1 and PD-L2 at stages, II, III and IV compared to stage I. PD-L1 was expressed in mucosa and muscularis mucosa layers, whereas PD-L2 was predominantly expressed in mucosa with exception to stage IV where it was expressed in both layers. M3R and ChAT were also elevated at stages, III and IV compared to stages, I and II. No significant difference was observed in the expression of α7nAChR at all stages of CRC. High levels of immune checkpoint inhibitors (PD-L1 and PD-L2) and cholinergic marker (M3R and ChAT) expression were associated with a high risk of CRC and poor patient survival outcome. However, there was no significant correlation between the expression of these markers and patients' gender, age and survival status, with exception to PD-L1 and M3R expression, which correlate with survival status In addition, α7nAChR expression was not associated with patients' age, gender, survival status and survival outcome.

Tumours use multiple mechanisms to avoid being recognised and to downregulate the hosts' immune system by expressing PD-L1 and PD-L2, which interact with PD-1 receptor on tumour-infiltrating lymphocytes [525]. The presence of PD-L1 and PD-L2 on the surface of tumours functions as an immune resistance mechanism allowing tumours to go undetected, leading to cancer cell proliferation and progression of tumour growth.

In contrast, some studies have demonstrated that the expression of PD-L1 on immune cells has a favourable prognostic factor in some cancers [526]. Increased expression of PD-L1 on tumour cells has been used in clinical trials to identify patients that will benefit from immunotherapy. As CRC progresses from stage I to stage IV, the expression of immunosuppressive factors such as PD-L1 and PD-L2 would be expected to be significantly upregulated. Generally, it is conceivable to believe that patients with stage IV CRC have an overall increase in the expression of immunosuppressive factors in order to facilitate tumour evasion of the host's immune system. In this study, PD-L1 expression

was significantly enhanced at advanced stages, III and IV compared to early stages of CRC. These findings are in line with studies demonstrating PD-L1 association with cancer stages. For instance, high PD-L1 expression was associated with tumour node metastasis, poor prognosis and shorter survival in CRC patients [62, 527]. Although, our results found no correlation of PD-L1 expression with tumour metastasis, however, there was a strong association with poor prognosis and shorter survival in CRC patients, concurring with previous studies. Furthermore, the expression of PD-L1 was associated with cholinergic markers, suggesting there is crosstalk between PD-L1 and cholinergic markers. Elevated expression of PD-L1 on tumour cells might be influenced by the immune microenvironment, thus allowing immune evasion. The number of cells within tumour overexpressing PD-L1 were enhanced at stages, II, III and IV compared to stage I, and these cells were localised in mucosa and muscularis mucosa layers. On the contrary, some studies demonstrated that PD-L1 expression was associated with early stages, lower tumour grade, absence of vascular invasion and lymph node metastasis, significantly leading to improved patient survival in mismatch repair-proficient microsatellite stable CRCs via enhancing CD8+ T cell infiltration [528]. These studies suggest that the prognostic value of PD-L1 expression could be dependent on the subset of CRCs as well as the presence of infiltrating immune cells. Furthermore, these contradictions in findings could be attributed to the expression of cholinergic markers as we found the link between the expression of immunosuppressive and cholinergic markers in this study.

Studies identifying the role of PD-L2 in cancer progression are scarce. PD-L2 is induced by the IFN-γ as observed in human colon cancer cell lines LOVO and RKO [529]. Studies demonstrated that PD-L2 exerts its function in immune tolerance by modulating and dampening T helper types 2 (Th2) response; however, Th1 response is crucial for anti-tumour immunity [530, 531]. PD-L2 expression in esophageal squamous cell carcinoma is negatively associated with PD-1 positive tumour-infiltrating lymphocytes, suggesting a role in tumour escape mechanism from the host's immunity [532]. In the present study, PD-L2 expression was significantly elevated at stages, II, III and IV compared to stage I. The number of cells within tumour overexpressing PD-L2 were enhanced at stages, II, III

and IV compared to stage I and these cells were predominantly expressed in mucosa with exception to stage IV where these cells were expressed in both mucosa and muscularis mucosa layers. Furthermore, PD-L2 expression was correlated with a high risk of CRC and poor patients' survival outcome. PD-L2 was strongly associated with α7nAChR expression and moderately correlated with PD-L1 and ChAT but not with M3R. This suggests that PD-L2 might be influenced by ACh binding to α7nAChR but not M3R activation. Similarly, PD-L2 expression associated with worse survival in patients with oesophageal cancer [533]. In renal cell carcinoma, PD-L2 expression associates with shorter progression-free survival [534]. More recently, it was noted that PD-L2 expression was independently associated with poor survival of CRC patients [529]. Contradictory to other findings, several studies found no correlation between PD-L2 expression and survival outcomes as noted in hepatocellular carcinoma, pancreatic and ovarian cancer patients [535-537]. In oesophageal adenocarcinomas, PD-L2 expression associates with smaller tumour size, early-stage and well-differentiated grade; however, PD-L2 is not associates with lymph node infiltration or metastasis or patient survival [538]. This could be due to a small number of patients in these studies reporting PD-L2 expression. Furthermore, studies have shown that depending on the molecules present in the microenvironment, the expression of PD-L2 can be increased on immune as well as non-immune cells [539]. The inconsistency in these results warrants further studies to validate the mechanisms involved.

Studies have demonstrated that ACh binding to α7nAChR plays a functional role in the oncogenic processes [540-543]. However, current findings mostly focused on the differences in expression between non-smokers and smokers, as there is evidence that smoking increases the expression of nicotinic receptors [544, 545]. In colon cancer, limited number of studies that specifically focus on the expression of α7nAChR in cancer without the influence of smoking have been studied. In lung cancer, there are several lines of evidence implicating the role of α7nAChR in cancer growth and metastasis [201, 546]; however, in colon cancer, less is known. Human HT-29 colon cancer cells overexpress α7nAChR, which facilitates cell proliferation, tumour angiogenesis [463, 547] and metastasis [201, 522, 548-550]. The results of the present study demonstrated no

significant difference in the expression of α7nAChR at all stages of CRC. Similarly, no association between α7nAChR expression and patients' risk of CRC and survival outcome was observed. However, α7nChR expression was strongly associated with PD-L2 and ChAT, while lowly correlated with PD-L1 and M3R. Further studies to elucidate the mechanisms underlying the role of α7nAChR in cancer cell proliferation, tumour angiogenesis and metastasis are required.

M3R signalling is reported in non-neuronal tissues including, but not limited to, colon, lung, skin and pancreas; and ACh is shown to act as a growth factor in lung and gastric cancers [518, 520, 521, 551-553]. Muscarinic receptor 3 is reported in many tissue types and plays an important role in the progression of many cancers, including breast, prostate, lung and CRC [554-556]. Most of the studies in regards to the role of M3R in CRC were performed mainly in the cell lines or animal models. Only few studies have reported that M3R is overexpressed in human colon cancer tissues compared to normal samples. There are limited studies associating the expression of M3R with different stages of CRC and clinical parameters. For instance, studies have shown that M3R is expressed in 60% of colon cancer cell lines [551, 553]. In addition, studies have reported 8-fold increased expression of M3R in 62% of colon cancers compared to normal adjacent and normal colon epithelium [552]. In non-small cell lung cancer, M3R expression associated with tumour metastasis and poor patients' prognosis [523]. The present study has illustrated that M3R is expressed at all stages but predominantly elevated at advanced stages, III and IV, compared to early stages, I and II. High levels of M3R expression was correlated with a high risk of CRC, survival status and poor patients' survival outcome. In addition, M3R expression was strongly associated with PD-L1 and ChAT, whereas lowly correlated with α7nChR but not with PD-L2, suggesting that M3R has no role in PD-L2 regulation. These findings are supported by a study demonstrating no association between M3R expression and metastasis [520]. Studies have suggested that the intracellular distribution of M3R can have detrimental effects on cancer progression [520, 557]. M3R in a normal colon epithelium is disseminated in the basolateral membrane, which forms a protective barrier between the cells and the blood and/or other cells. However, in cancer, M3R can translocate to a more dynamic

environment that enhances cancer growth, such as cytoplasmic [520]. We speculate that at early stages of CRC, M3R might be expressed on the surface membrane and then translocates into the cytoplasm at the advance stages of CRC. The activities and localisation of M3R within tumour cells at various stages of CRC should be further investigated.

Although studies examining the role of M3R and α7nAChR in cancer have been relatively studied, there are limited studies identifying the role of ChAT in colon cancer progression. High ChAT is noted in cytoplasmic localisation of H508 and Caco-5 colon cancer cells [521]. Furthermore, ChAT was found to be overexpressed in colon cancer specimens compared to normal colon samples [521]. Similarly, ChAT was significantly upregulated in squamous cell lung carcinoma compared to adjacent healthy specimen [557]. In the present study, the expression of ChAT was correlated with advanced stages of CRC, III and IV, compared to the early stages, I and II. In addition, low levels of ChAT were associated with a high risk of CRC and poor patients' survival outcome; however, high levels of ChAT were associated with metastasis. Overall, the levels of ChAT expression strongly correlated with expression of cholinergic receptors and moderately associated with expression of immunosuppressive markers. Increased levels of ChAT leads to higher ACh production; however, it might not be binding to the receptors to stimulate tumour growth at the early stages of CRC.

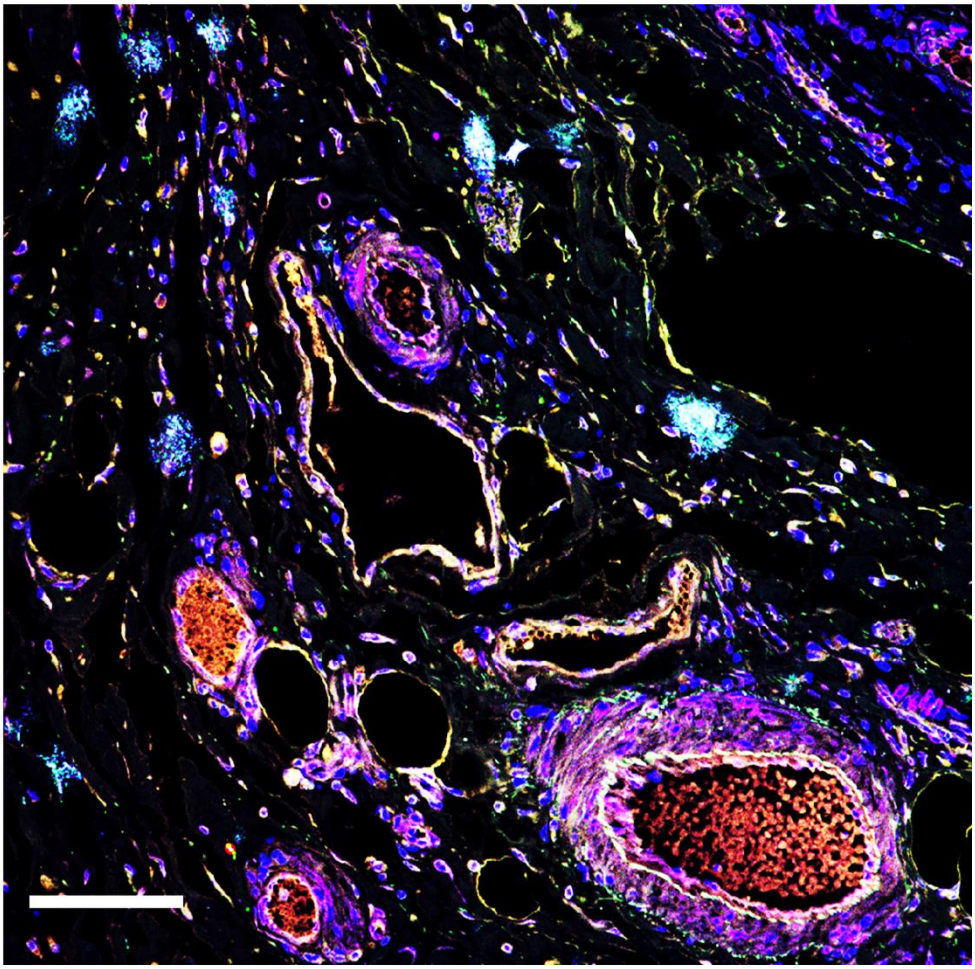
These findings suggest that there is crosstalk between immunosuppressive and cholinergic markers. In fact, our findings are supported by a recent study by Kamiya et al (2019) in breast cancer patients demonstrating that decreased parasympathetic nerve density, determined by VAChT expression, was associated with poor clinical outcomes and elevated levels of immune checkpoint molecules [558]. Similarly, in chemically-induced and xenograft models of breast cancer, sympathetic nerve denervation, and parasympathetic neurostimulation suppressed immune checkpoints molecules, such as PD-1 and PD-L1, leading to attenuated tumour growth [558].

2.5. Conclusion

Nervous and immune systems play an essential role in influencing the tumour microenvironment to promote cancer development and progression. Studies have demonstrated that modulation of the immune system by the relentless release of neurotransmitters from the nerve terminals and cancer cells can promote tumour growth and metastasis; however, underlying mechanisms are not understood. Therefore, revealing the interaction between the immunosuppressive and cholinergic factors in cancer is imperative for the understanding mechanisms of CRC tumour development and progression. Hence, the expression of these markers may increase the risk of recurrence or second cancer in patients. Immunohistochemical staining for immunosuppressive and cholinergic markers might, therefore, be used in determining prognosis and treatment regimens for CRC patients.

CHAPTER THREE

Effect of Blocking Cholinergic Signalling on the Expression of PD-L1 and PD-L2 in Human Colon Cancer Cells



3.0. Abstract

Colorectal cancer (CRC) remains one of the most commonly diagnosed cancers and a leading cause of cancer-related deaths worldwide. Cancer cells have evolved a number of mechanisms to suppress the immune system by increasing cell surface markers, including programmed death ligands 1 and 2 (PD-L1 and PD-L2). In addition, cancer cells can synthesise and secrete acetylcholine (ACh) acting as an autocrine or paracrine hormone to promote their proliferation, differentiation and migration. In the present study, the effects of a general muscarinic receptor blocker, atropine, and a selective muscarinic receptor 3 (M3R) blocker, 1,1-dimethyl-4 diphenylacetoxypiperidinium iodide (4-DAMP), on the expression of immunosuppressive (PD-L1 and PD-L2) and cholinergic (M3R and choline acetyltransferase ChAT) markers were evaluated in human colon cancer cell lines,LIM-2405 and HT-29, and normal epithelial cell line, T4056. The results demonstrated that atropine and 4-DAMP suppressed proliferation and migration of human colon cancer cells and normal epithelial cells as well as induced apoptosis of human colon cancer cells. Furthermore, atropine and 4-DAMP decreased the expression of PD-L1 and PD-L2 in human colon cancer cell lines, however, in normal epithelial cell line atropine had no effect but 4-DAMP significantly attenuated these markers. Similarly, both atropine and 4-DAMP attenuated M3R expression in LIM-2405 and HT-29 cells, but only atropine showed similar effects in T4056 cells. One of the mechanisms attributed to the observed effects involves the modulation of epidermal growth factor receptor (EGFR), phospho extracellular signal-regulated kinase (pERK) and phospho signal transducer and activator of transcription 3 (pSTAT3). Thus, blocking muscarinic receptors inhibits proliferation, migration and induces apoptosis of human colon cancer cells via decreasing the expression of PD-L1, PD-L2 and M3R through the activation of EGFR and phosphorylation of ERK and STAT3 signalling pathways. These effects allow the immune system to recognize and eliminate cancer cells.

3.1. Introduction

Colorectal cancer (CRC) remains one of the most commonly diagnosed cancers and a leading cause of cancer-related death worldwide [2]. CRC is often diagnosed at late stages as it presents indistinct or no symptoms at the early stages. Cancer cells exploit a number of mechanisms, including the expression of immunosuppressive markers, programmed death-ligand 1 and 2 (PD-L1 and PD-L2) [392]. For instance, high expression of PD-L1 is associated with tumour node metastasis, poor prognosis and shorter survival in CRC patients [62, 527]. PD-L1 expression in stromal or tumour cells inversely correlates with FOXP3+ regulatory T (Treg) cell density in CRC patients [69]. It is known that tumour cells can stimulate PD-L1 expression via multiple oncogenic signalling pathways mediated by interferon (IFN)-γ produced by infiltrating immune cells [37, 559-563].

Acetylcholine (ACh) synthesis and secretion by non-neuronal tissues, acting as an autocrine or paracrine hormone, is well established and plays an essential role in cellular proliferation, differentiation and apoptosis [363, 368, 521, 564]. Nonetheless, a direct association between ACh and immunosuppressive markers has not been demonstrated. ACh plays an essential role in several cancers, including CRC [363-366, 368, 518]. Choline acetyltransferase (ChAT) is the enzyme responsible for ACh synthesis. ChAT catalyses the transfer of an acetyl group from the coenzyme acetyl-CoA to choline, resulting in ACh synthesis. Human colon cancer cells (H508, WiDr, Caco-2) and colon cancer specimens overexpress ChAT [521]. In CRC, ACh mediates it effect by binding to alpha7 nicotinic receptor (α7nAChR) and muscarinic receptor 3 (M3R) [565]. Stimulation of M3R enhances colon cancer cell proliferation [521]. In addition, administration of muscarinic receptor inhibitor, atropine, abolishes SNU-C4 colon cancer cell migration; however, H508 cell migration requires the activation of matrix metalloproteinase 7 (MMP7) [368, 369]. M3R activation increases non-small cell lung carcinoma cell invasion and migration via the activation of epidermal growth factor receptor (EGFR)/ Phosphoinositide 3-kinases (PI3K)/ serine/threonine kinase or protein kinase B (AKT) pathway [367]. Nicotine stimulation of nAChRs enhances invasion and

metastasis of SW620 and LOVO colon cancer cells via the activation of p38 mitogen-activated protein kinase (MAPK) signalling pathway [343].

It was hypothesized that ACh could influence the ability of human colon cancer cells to express immunosuppressive and cholinergic markers. To the best of our knowledge, there are no data available, which determine the effect of ACh receptor blockers on the expression of immunosuppressive markers in human colon cancer cells. Hence, in the present study the effects of a general muscarinic receptor blocker, atropine, and a selective M3R blocker, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), were evaluated on the expression of immunosuppressive and cholinergic markers *in vitro* in human colon cancer cell lines, LIM-2405 and HT-29, and normal epithelial cell line, T4056. The influence of cholinergic signalling on the expression of immune checkpoint molecules may play an essential role in cancer development; therefore, it is necessary to understand molecular pathways involved *in vitro*.

3.2. Materials and methods

3.2.1. Cell culture

Primary human colon epithelial cell line, T4056 was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Castle Hill, Australia) and human colon cancer cell lines, LIM-2405 and HT-29, were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich, Castle Hill, Australia). Culture media were supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% glutamine. Cells were cultured at 37°C, 5% CO_2 and 95% air atmosphere. When cells grew into confluent or semiconfluent monolayers in 75 cm^2 medium flasks, they were either passaged or used. The passage of cells was conducted with 0.25% trypsin every 3-4 days.

3.2.2. Cell proliferation

The water-soluble tetrazolium-1 (WST-1) assay kit (Roche Diagnostics GmbH, Germany) was used to determine the viability of T4056, LIM-2405 and HT-29 cells. WST-1 is cleaved to form formazan dye via a complex cellular interaction at the cell surface. This interaction is contingent on the glycolytic nicotinamide adenine dinucleotide phosphate (NADPH) production of the viable cells. Hence, the amount of formed formazan dye correlates to the number of viable cells in the culture. T4056, LIM-2405 and HT-29 cells were seeded and cultured at 1×10^4 cells per well in 96 well plates for 24hrs. Cells were then treated with various concentrations of the general muscarinic receptor blocker, atropine (Sigma-Aldrich, Australia) for 1-48hrs, selective M3R blocker, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) (Abcam, Australia), cholinergic agonist, carbachol (Abcam, Australia) and acetylcholinesterase inhibitor, donepezil (Abcam, Australia) for 8hrs. All treatments were performed in triplicates, and three independent experiments were conducted. WST-1 reagent (10µL) was added to each well and incubated at 37°C for 1hr. Cellular proliferation at the absorbance of 450nm was measured using a microplate reader (Varioskan Flash, Thermo Scientific, Australia).

3.2.3. Migration assay

T4056, LIM-2405 and HT-29 cell lines were used in migration analysis using Boyden chambers with 8µm pore size membrane filter inserts (Corning Costar Corp., Kennebunk, ME, USA) in 24 well tissue culture plates. The cells were trypsinised and resuspended in serum-free RPMI-1640 and DMEM media at the density of 2×10^5 cell per mL. A total of 200µL of cells suspension was seeded in the upper chamber of the Transwells, and 600µL of media into the lower chamber. Cells were then treated with 0µm and 100µm atropine (Sigma-Aldrich, Melbourne Australia) for 8hrs, 24hrs and 48hrs. The chambers were incubated at 37°C, in 5% CO_2 incubator. After 8-48hrs, the non-migrating cells on the upper surface of the insert were removed, and cells that migrated to the underside of the membrane were counted using a light microscope. In all experiments, two independent experiments were conducted in duplicates.

3.2.4. Annexin V apoptosis assay

LIM-2405 and HT-29 cell lines were cultured overnight in six wells at the density of $5x10^5$ cells per well. Cells were treated with 100µM atropine and 4-DAMP for 8hrs. Following treatments, flow cytometry was utilised to determine the apoptotic and necrotic cells. Cells were collected and resuspended in fluorescence-activated cell sorting (FACS) buffer and labelled with 100µL per well with Annexin V at 1:1,000 dilution and 0.5µg/mL of propidium iodide (PI). In all experiments, two independent experiments were conducted in duplicates.

3.2.5. Choline/acetylcholine assay

The choline/acetylcholine assay kit (Abcam, Australia) was used to measure the concentration of choline in CT-26 cell lysates. The assay was carried out in accordance with the instructions provided by the manufacturer. Briefly, CT-26 ($1x10^6$) cells were cultured overnight after which cells were treated with 100µM of cholinergic antagonists, atropine and 4-DAMP and 300µM of cholinergic agonist, carbachol and acetylcholinesterase inhibitor, donepezil. Cells were lysed in 500µL choline assay buffer before commencing choline measurements using a microplate reader (Varioskan Flash, Thermo Scientific, Australia) at an absorbance of 570nm. All treatments were performed in duplicates and in two independent experiments were conducted.

3.2.6. Immunocytochemistry staining

LIM-2405 and HT-29 cell lines were grown on chamber slides (Ibidi, Australia) overnight at 37°C, in 5% CO_2 incubator. Cells at a density of 1×10^5 cells per well were treated with atropine (0µM and 100µM) for 8hr and fixed in 4% paraformaldehyde for 10 minutes (mins). Cells were permeabilised for 15 mins in 0.1% phosphate buffered saline (PBS). The endogenous activity was blocked using 10% donkey serum for 1hr. Cells were incubated overnight at 4°C with primary antibodies (**Table 3.1**). After washing the cells in PBS, cells were incubated at room temperature for 2hr with secondary antibodies (Table 3.2). All antibodies were diluted in PBS containing 2% donkey serum and 0.01% Triton X-100. Cells were incubated for 1 min with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (D1306, Life Technologies, Australia) and DAKO mounted with mounting medium (Agilent Technologies, Australia). Then coverslips were placed on and left to dry overnight before imaging.

3.2.7. Western blot

Expression of immunosuppressive and cholinergic markers (**Table 3.1**), as well as cell signalling pathways, pSTAT3, pERK½ and EGFR (**Table 3.2**) in T4056, LIM-2405 and HT-29 cells, were examined by western blot. Cells were incubated with 100µM atropine and 4-DAMP for 8hrs. After treatments, cells were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer (pH 7.4, 150mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate, 1% NP-40 in PBS, Sigma) containing a protease and phosphatase inhibitors cocktail (Roche Applied Science).Cellular proteins (20µg) from each sample were separated by 8% to 12% SDS/polyacrylamide gel electrophoresis. The separated fragments were transferred to 0.22µm polyvinylidene fluoride membranes, which were blocked with 5% skim milk in PBS containing 0.1% Tween 20 overnight at 4ºC at 40 revolutions per minute (RPM) speed shaker. The membranes were incubated with primary antibodies overnight at 4ºC followed by the incubation with HRP-conjugated secondary antibodies for 2hrs at room temperature. The membranes were washed three times in PBS plus 0.1% Tween 20, and protein detection was performed using enhancing chemiluminescence reagents. Glyceraldehydes-3-phosphate dehydrogenase (GADPH) was used as a loading control.

Table 3. 1. Primary antibodies used in this study

Primary antibodies				
Markers	**Host Species & Clonality**	**Dilution**	**Source**	
PD-L1	Mouse, monoclonal	1:500	Abcam, Australia	ab210931
PD-L2	Rabbit, polyclonal	1:500	Abcam, Australia	ab200377
M3R	Rabbit, polyclonal	1:500	Abcam, Australia	ab126168
ChAT	Goat, polyclonal	1:500	Abcam, Australia	ab134021
EGFR	Rabbit, monoclonal	1:1,000	Cell signalling, Australia	#4267
pSTAT3	Mouse, monoclonal	1:1,000	Cell signalling, Australia	#9145
pERK$_{1/2}$	Rabbit, monoclonal	1:1,000	Cell signalling, Australia	#3192

Table 3. 2. Secondary antibodies used in this study

Secondary antibodies			
Alexa Fluor 488	Anti-goat	1:250	Jackson ImmunoResearch Laboratories, United States
Alexa Fluor 594	Anti-rabbit	1:250	Jackson ImmunoResearch Laboratories, United States
Alexa Fluor 647	Anti-mouse	1:250	Jackson ImmunoResearch Laboratories, United States
Anti-mouse	IgG HL HRP	1:10,000	Abcam, Australia
Anti-rabbit	IgG HL HRP	1:10,000	Abcam, Australia
Anti-goat	IgG HL HRP	1:10,000	Abcam, Australia

3.2.8. Data analysis

Images were captured on a Nikon Eclipse Ti multichannel confocal laser scanning system (Nikon, Japan). Z-series images were acquired at a nominal thickness of 1µm (1024 x 1024 pixels). Image J software (National Institute of Health, Bethesda, MD, USA) was employed to convert images from RGB to 8-bit binary; particles were then analysed to obtain the percentage area of immunoreactivity For western, chemiluminescent signal was captured using the FluorChem FC2 System (Alpha Innotech, USA). The expression level of each protein was quantified using ImageJ software (National Institute of Health, Bethesda, MD, USA). For apoptosis assay, BD FACs Canto II and FACS Diva software (BD Biosciences, Australia) were used to aid in analysis. *Two-way ANOVA* was used for multiple group comparison. Excel and Prism (Graph Pad Software, La Jolla, CA, USA) was used to aid in the statistical analysis, and $p<0.05$ was considered significant.

3.3. Results

3.3.1. Effects of blocking muscarinic receptors on the proliferation of normal epithelial cells and human colon cancer cells

3.3.1.1. Effect of atropine on the proliferation of colon cancer cell lines

Cellular proliferation is an essential step in the development and progression of cancer. Non-neuronal ACh is reported to play a crucial role in colon cancer cells proliferation [363]. To determine the effect of blocking muscarinic receptors, cells were treated with various concentrations of atropine and at different time points. The effect of atropine on cellular proliferation of two human colon cancer cell lines (LIM-2405 and HT-29) and normal epithelial cell line (T4056) was assessed using WST-1 assay. Three independent experiments were performed in triplicate wells. Atropine significantly decreased cell proliferation of all cells at 1-4hrs compared to 8-48hrs (**Figure 3.1A-C**). However, high doses of atropine (400-1,000µm) significantly inhibited cell proliferation of T4056 at 1-2hrs and LIM-2405 and HT-29 at 1-4hrs. Though there was a trend of lower proliferation at 8-48hrs, atropine decreased proliferation in a dose dependent manner. In all subsequent experiments, cells were treated with 100µm of atropine for 8hrs as changes were noticeable at this concentration and time point with high cell viability, hence reducing the auto-fluorescence staining from dead cells.

3.3.1.2. Effect of cholinergic antagonists and agonists on cell proliferation

T4056, LIM-2405 and HT-29 cells were incubated with various concentrations of atropine and 4-DAMP for 8hrs. In addition, cells were also incubated with various concentrations of carbachol, which acts as ACh receptor agonist and donepezil that inhibits ACh breakdown. **Figure 3.2A-D** shows proliferation of normal epithelial cells and human colon cancer cells treated with atropine, 4-DAMP, carbachol and donepezil. Atropine inhibited proliferation of all cells; however, LIM-2405 cells were more sensitive to atropine compared to HT-29 and T4056 cells (**Figure 3.2A**). Atropine decreased cell proliferation in a dose dependent manner. Similarly, 4-DAMP suppressed proliferation of all cells, but LIM-2405 and HT-29 cells were more sensitive to 4-DAMP effect compared to T4056 cells

(**Figure 3.2B**). To determine if the effect of atropine and 4-DAMP can be reversed by ACh agonists, cells were incubated with carbachol and donepezil. The results show that both carbachol and donepezil increased cell proliferation in a dose dependent fashion (**Figure 3.2C-D**).

Figure 3.1. Effect of atropine on cell proliferation of normal epithelial cell and human colon cancer cells

Cell proliferation dose-response curve for T4056 (**A**), LIM-2405 (**B**) and HT-29 (**C**) cells treated with atropine for 1-48hrs. Representative figures of three independent experiments in triplicate wells.

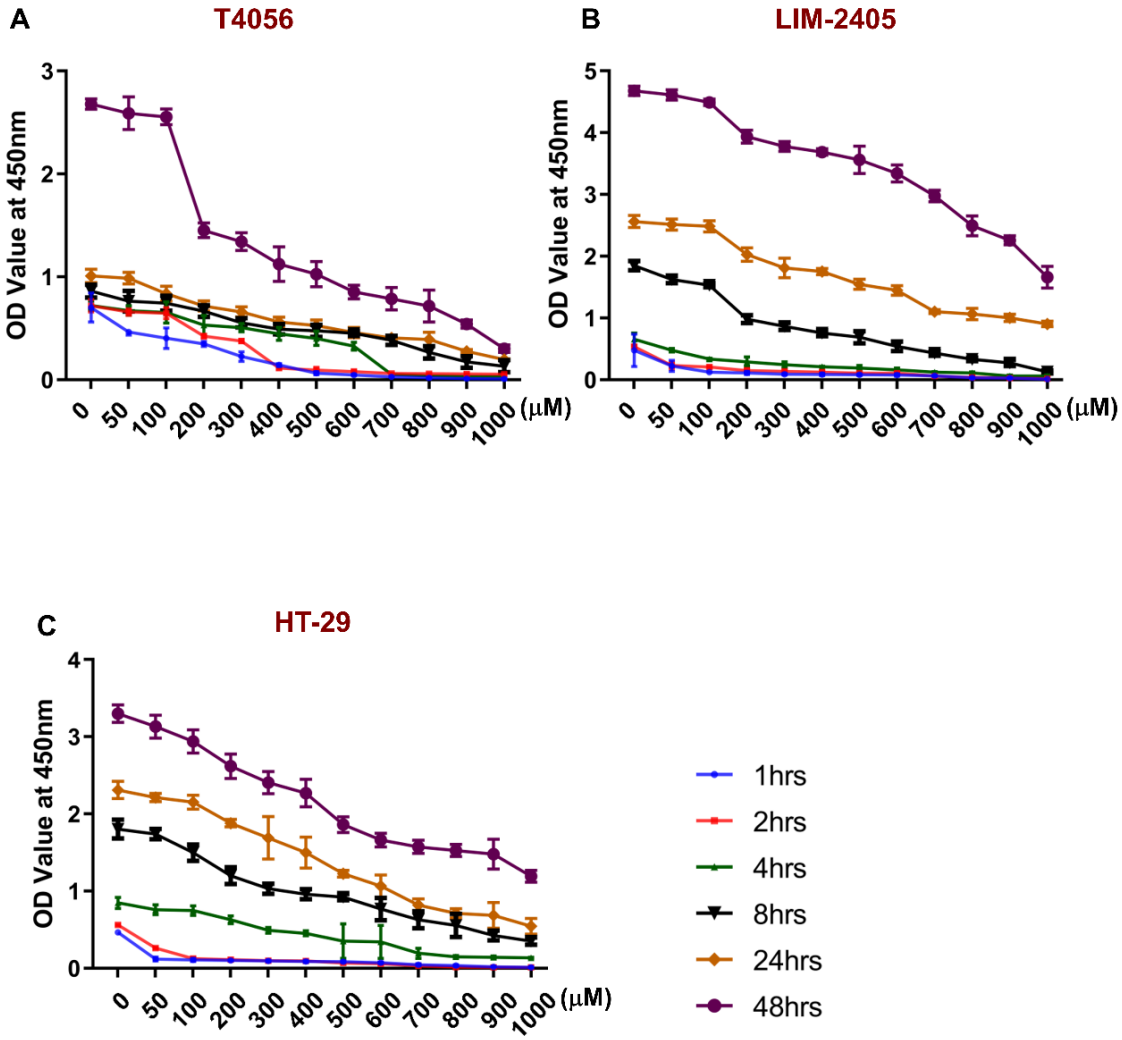
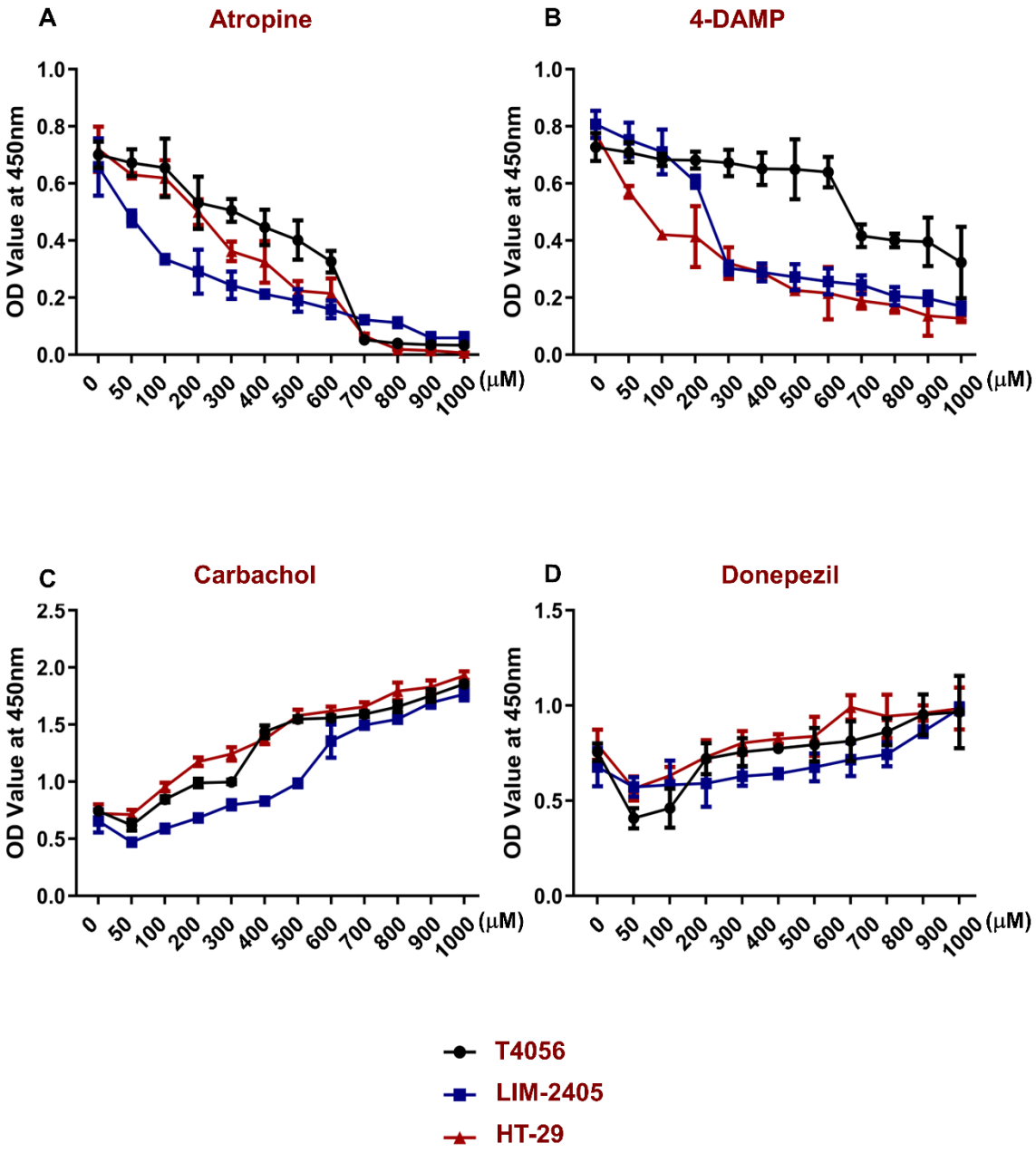

A
T4056
OD Value at 450nm
0
1
2
3
0 50 100 200 300 400 500 600 700 800 900 1000 (μM)
B
LIM-2405
OD Value at 450nm
0
1
2
3
4
5
0 50 100 200 300 400 500 600 700 800 900 1000 (μM)
C
HT-29
OD Value at 450nm
0
1
2
3
4
0 50 100 200 300 400 500 600 700 800 900 1000 (μM)
1hrs
2hrs
4hrs
8hrs
24hrs
48hrs

Figure 3.2. Effect of cholinergic antagonists and agonists on cell proliferation

Proliferation of T4056, LIM-2405 and HT-29 cell lines treated with atropine (**A),** 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) (**B**), carbachol (**C**) and donepezil (**D**) for 8hrs. Values in **A**, **B, C** and **D** are mean ± standard error of the mean (SEM) from at least 3 independent experiments.


A
Atropine
B
4-DAMP
C
Carbachol
D
Donepezil
OD Value at 450nm
0 50 100 200 300 400 500 600 700 800 900 1000 (μM)
T4056
LIM-2405
HT-29

3.3.2. Effect of atropine and 4-DAMP on cell migration

Cell migration is an essential step in cancer progression as it is the main hallmark of cancer metastasis. In the present study, Boyden chamber was used to determine the effects of atropine and 4-DAMP on T4056, LIM-2405 and HT-29 cell migration. Cells were incubated with 100µm of atropine and 4-DAMP for 8hrs, 24hrs and 48hrs (**Figure 3.3A-C**). At 8hrs, both atropine and 4-DAMP decreased migration of T4056, LIM-2405 and HT-29 cells compared to controls. At 24hrs and 48hrs, lower migration rate was noted compared to 8hrs. At 48hrs, there was no significant differences noted between control and atropine-treated T4056 cells (**Figure 3.3A**), but significant differences were observed in both cancer cell lines treated with 4-DAMP when compared to controls.

3.3.3. Effect of atropine and 4-DAMP on cell apoptosis

Acetylcholine receptors, especially muscarinic receptors, play a significant role in the regulation of cell apoptosis [363]. To determine whether atropine and 4-DAMP induces apoptosis or necrosis in LIM-2405 and HT-29 cancer cell lines, cells were incubated with Annexin V and PI. Non-apoptotic cells are both Annexin V and PI negative, while apoptotic cells are Annexin V positive and PI negative. Necrotic or dead cells are PI positive and Annexin V negative. It is clear that blockade of all muscarinic receptors with atropine and the selective block of muscarinic receptor 3 with 4-DAMP induced apoptosis in both cancer cell lines (**Figure 3.4**).

Figure 3.3. Effect of atropine and 4-DAMP on cell migration *in vitro*

Boyden chamber was used to determine the effect of atropine and 4-DAMP on the migration of T4056 (**A**), LIM-2405 (**B**) and HT-29 (**C**) cells. Cells were incubated with 100µm of atropine and 4-DAMP for 8hrs, 24hrs and 48hrs (**A**-**C**). Values in **A**, **B,** and **C** presented as mean ± SEM from at least two independent experiments in duplicates. *Two-way ANOVA* followed by Tukey's multiple comparisons test was used, and the significance value is marked with asterisks, ****p<0.001, ****p<0.0001*.

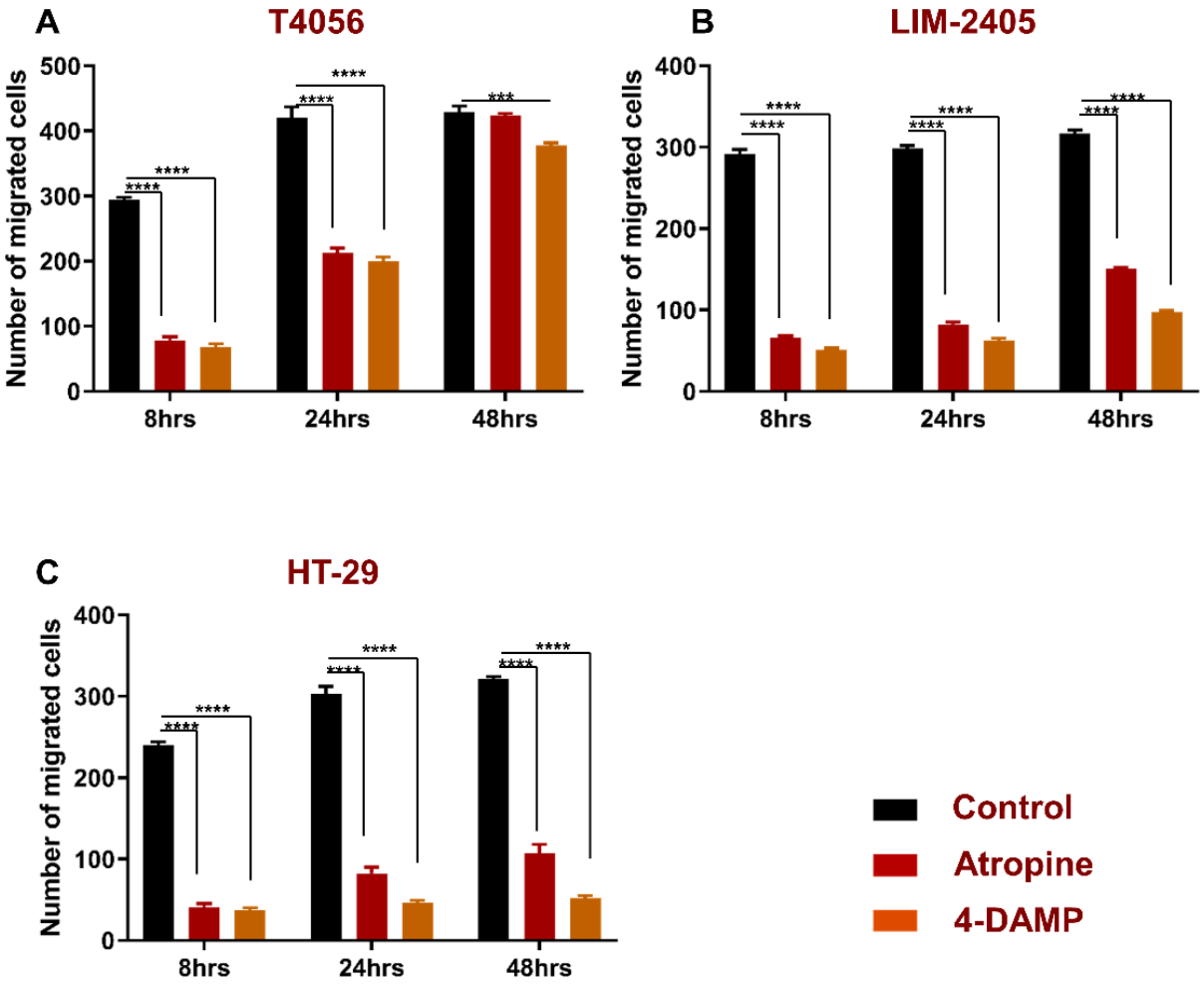
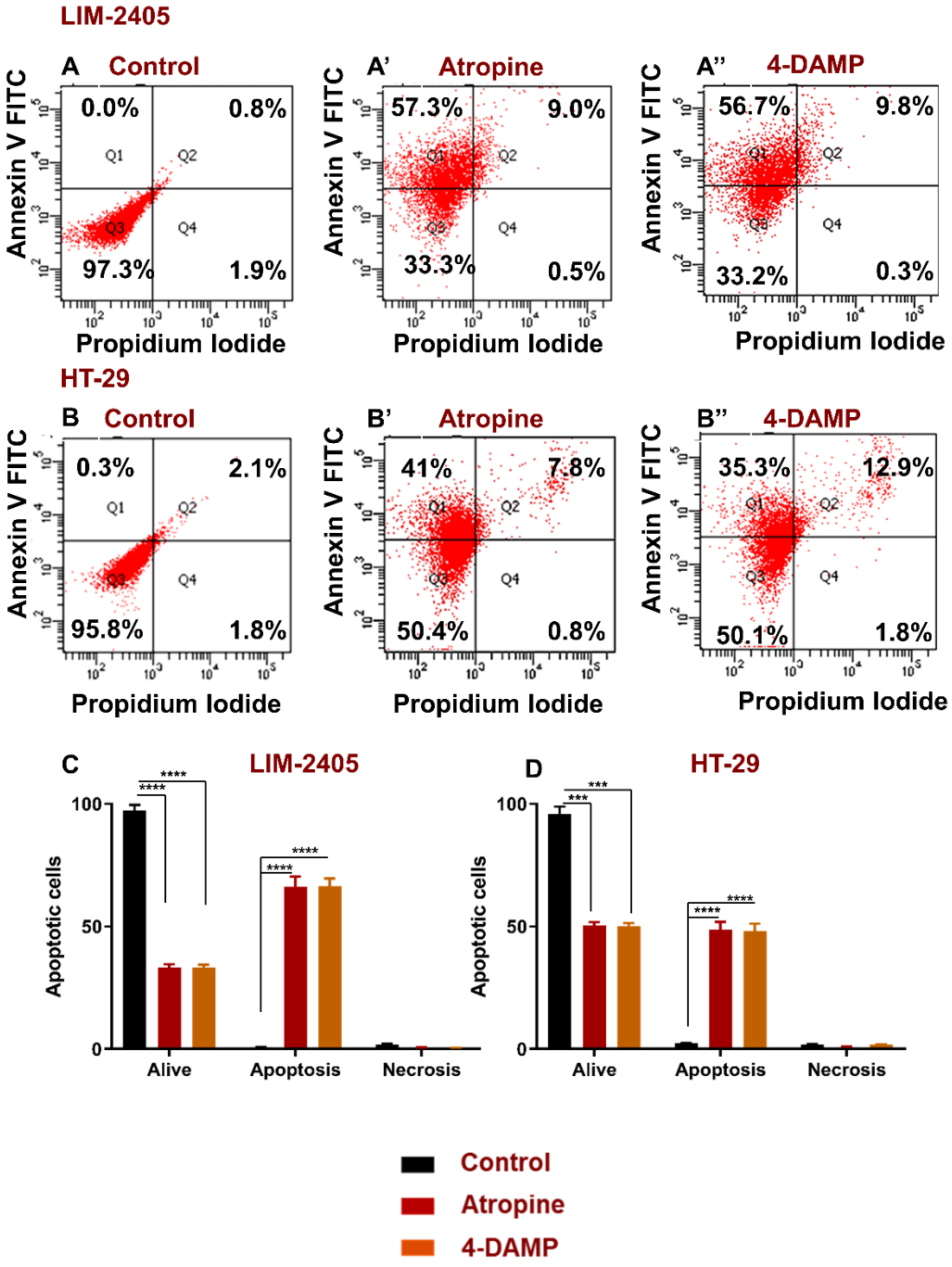

A
T4056
Number of migrated cells
500
400
300
200
100
0
8hrs
24hrs
48hrs
B
LIM-2405
Number of migrated cells
400
300
200
100
0
8hrs
24hrs
48hrs
C
HT-29
Number of migrated cells
400
300
200
100
0
8hrs
24hrs
48hrs
Control
Atropine
4-DAMP

Figure 3.4. Effect of atropine and 4-DAMP on apoptosis *in vitro*

Annexin V-FITC/ PI staining of human colon cancer cells incubated with atropine (**A**-**A''**) and 4-DAMP (**B**-**B''**). (**A**) LIM-2405 controls, (**A'**) LIM-2405 treated with atropine, (**A''**) LIM-2405 treated with 4-DAMP, (**B**) HT-29 controls, (**B'**) HT-29 treated with atropine, (**B''**) HT-29 treated with 4-DAMP. Three independent experiments were performed in triplicate wells. *Two-way ANOVA* followed by Tukey's multiple comparisons test was used, and significance value is marked with asterisks, ****p<0.001, ****p<0.0001*.

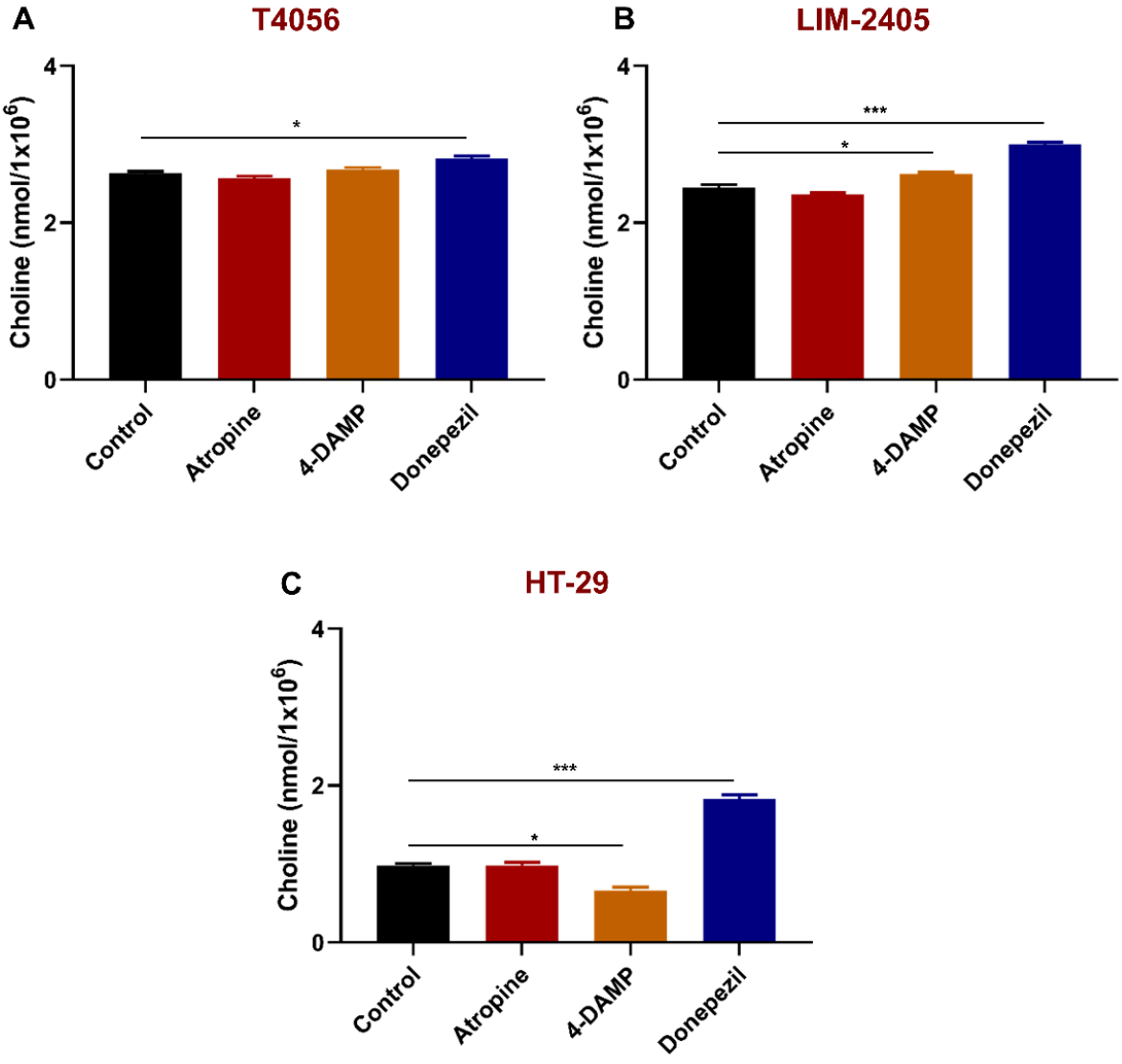

LIM-2405
A Control
A' Atropine
A'' 4-DAMP
Annexin V FITC
Propidium Iodide
0.0%
0.8%
97.3%
1.9%
57.3%
9.0%
33.3%
0.5%
56.7%
9.8%
33.2%
0.3%
HT-29
B Control
B' Atropine
B'' 4-DAMP
0.3%
2.1%
95.8%
1.8%
41%
7.8%
50.4%
0.8%
35.3%
12.9%
50.1%
1.8%
C
LIM-2405
D
HT-29
Apoptotic cells
Alive
Apoptosis
Necrosis
Control
Atropine
4-DAMP

3.3.4. Effect of cholinergic antagonists and agonists on cells ability to produce choline

Cancer cells synthesise their own ACh. To determine whether LIM-2405 and HT-29 cells could synthesize ACh, the amount of choline, a precursor for ACh synthesis was measured in cell lysate ($1x10^6$ cells). Choline/acetylcholine assay kit, which is rapid, sensitive and accurate, was used to measure choline in the cell lysate. There were no significant differences between cells treated with atropine compared to controls (**Figure 3.5A-C**). Similarly, there was no significant difference in T4056 epithelial cells treated with 4-DAMP when compared to control (**Figure 3.5A**). However, 4-DAMP significantly increased choline production in LIM-2405 cells (**Figure 3.5B**), whilst HT-29 cells showed decreased choline when compared to control (**Figure 3.5C**). In addition, cells were treated with acetylcholinesterase inhibitor, donepezil, to determine its effects on T4056, LIM-2405 and HT-29 cells which showed that donepezil significantly augmented choline production in all cell lines.

Figure 3.5. Effect of cholinergic antagonists and donepezil on choline production

The amount of choline was measured in T4056 (**A**), LIM-2056 (**B**), HT-29 (**C**) cells. Values in **A**, **B** and **C** are mean ± SEM, from at least 2 independent experiments. *Two-way ANOVA* followed by Tukey's multiple comparisons test was used, and the significance value is marked with asterisks*, *p<0.05 ***p<0.001*.


A
T4056
Choline (nmol/1x10^6)
4
2
0
*
Control
Atropine
4-DAMP
Donepezil
B
LIM-2405
Choline (nmol/1x10^6)
4
2
0
***
*
Control
Atropine
4-DAMP
Donepezil
C
HT-29
Choline (nmol/1x10^6)
4
2
0
***
*
Control
Atropine
4-DAMP
Donepezil

3.3.5. Effect of atropine and 4-DAMP immunosuppressive and cholinergic markers

3.3.5.1. Effect of atropine and 4-DAMP on PD-L1 and PD-L2 expression

The muscarinic receptors antagonist, atropine has been reported to inhibit cancer cell growth both *in vitro* and *in vivo* [566]. However, no studies have shown the effects of muscarinic acetylcholine receptor (mAChR) blocking on the expression of immunosuppressive markers, PD-L1 and PD-L2. T4056, LIM-2405 and HT-29 were used to determine the effect of atropine on the expression of these markers. The level of PD-L1 and PD-L2 expression was evaluated by immunofluorescence and western blot analyses. Cells were pre-treated with 0µM and 100µM atropine and 4-DAMP for 8hr followed by incubation with the antibody's diluent with or without primary antibodies, followed by incubation with secondary antibodies. No labelling of cells was observed without primary antibodies (**Figure 3.6A-C**) indicating that the positive labelling is specific for the primary antibodies of interested. Atropine had no effect on the expression of PD-L1 in T4056 cells (**Figure 3.6A'', D-F**); however, in LIM-2405 (**Figure 3.6B'', D-F**) and HT-29 (**Figure 3.6C'', D-F**) cells, significantly decreased expression of PD-L1. 4-DAMP decreased PD-L1 in T4056 (**Figure 3.6A''', D-F**), LIM-2405 (**Figure 3.6B''', D-F**) and HT-29 (**Figure 3.6C''', D-F**) cells. T4056 cells expressed low levels of PD-L1, whilst human colon cancer cell lines expressed high levels of PD-L1. Likewise, the effect of atropine and 4-DAMP on the expression of PD-L2 was evaluated (**Figure 3.7A-C**). Atropine significantly decreased the expression of PD-L2 in all cell lines when compared to control (**Figure 3.7A''-C'', D-F**). Similarly, 4-DAMP had no effect on the expression of PD-L2 on T4056 cells (**Figure 3.7A''', D-F**) but decreased PD-L2 in LIM-2405 (**Figure 3.7B''', D-F**) and HT-29 (**Figure 3.7C''', D-F**) cells.

3.3.5.2. Effect of atropine and 4-DAMP on M3R and ChAT expression

Muscarinic receptors, in particularly M3R, play a significant role in the progression of CRC. The effect of atropine and 4-DAMP on M3R and ChAT expression was evaluated in human colon epithelial cell line T4056 and human colon cancer cell lines, HT-29 and LIM-2405 by immunofluorescence and western blot analyses (**Figure 3.8A-C**). Atropine treatment significantly reduced M3R expression in all cell lines (**Figure 3.8A''-C''**).

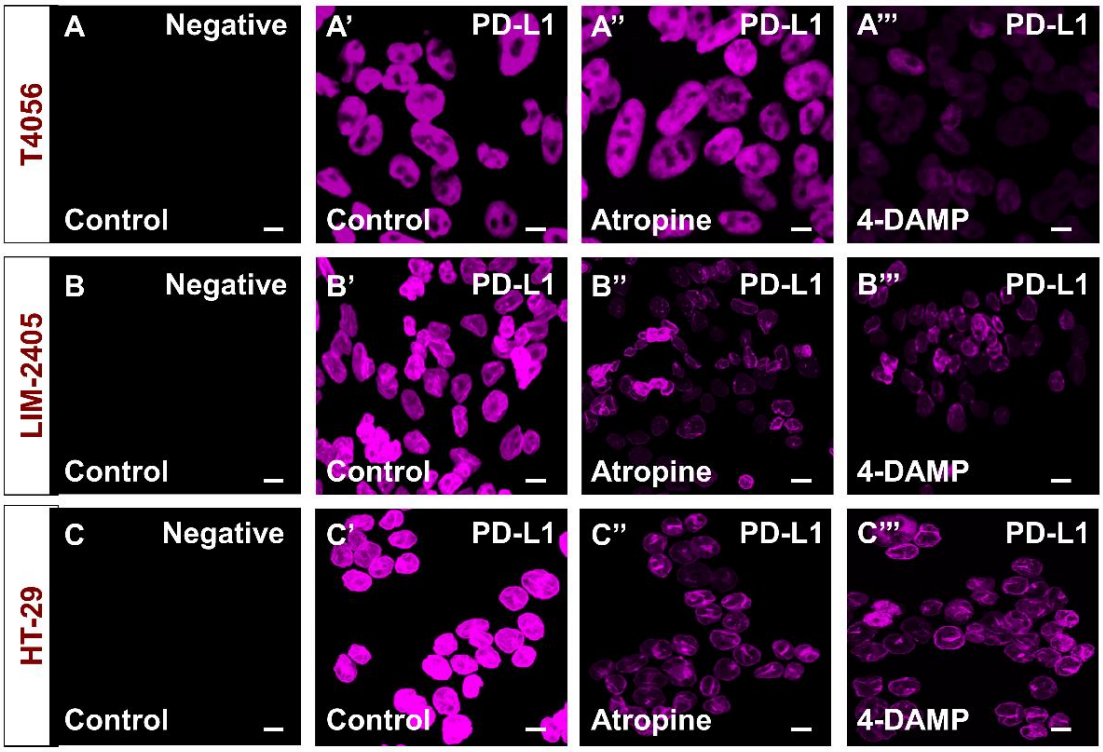
However, specific blocking of M3R with 4-DAMP had no effect on the expression of M3R in T4056 (**Figure 3.8A’’’, D-F**), but significantly decreased M3R in LIM-2405 (**Figure 3.8B’’’, D-F**) and HT-29 (**Figure 3.8C’’’, D-F**) cells when compared to the control.

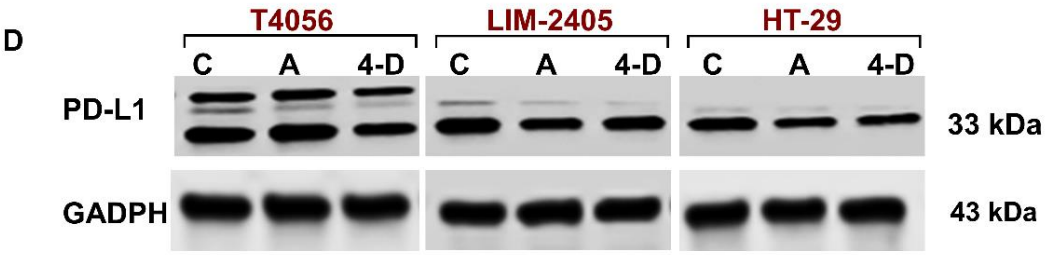
Blocking all muscarinic receptors with atropine had no effect on the expression of ChAT in colon epithelial cell line (**Figure 3.9A’’, D-F**) when compared to control (**Figure 3.9A’, D-F**). Similarly, there were no significant differences between human colon cancer cell lines treated with atropine and control (**Figure 3.9B’’, C’’, D-F**). Moreover, specific blocking of M3R with 4-DAMP significantly augmented ChAT expression on colon epithelial cell line (**Figure 3.9A’’’, D-F**), but had no effect on the expression of ChAT in colon cancer cell lines (**Figure 9B’’’, C’’’, D-F**). Overall, M3R and ChAT are expressed in human colon epithelial cells and overexpressed in colon cancer cell lines.

Overall, these findings correlate with the results presented in Chapter 2, where PD-L1 expression correlates with M3R, reinforcing that M3R may play a role in the induction of PD-L1 by tumour cells.

Figure 3.6. Effect of atropine and 4-DAMP on the expression of PD-L1 *in vitro*

Negative control labelling of T4056 cells is shown in (**A)**, control (**A')** and atropine and 4-DAMP in (**A" & A"')**, respectively. Negative control labelling of LIM-2405 cells is presented in (**B**), control (**B'**), atropine (**B"**) and 4-DAMP (**B"'**). HT-29 negative control is displayed in (**C**), control (**C'**), atropine (**C"**) and 4-DAMP (**C"'**). Scale bar represents 50µm. Western blot bands for T4056, LIM-2405 and HT-29 are shown in (**D**). Bar graphs displaying the mean fluorescence of PD-L1 (**E**) and western blot expression intensity (**F**). Data presented as mean ± SEM. Two-*way ANOVA, *p<0.05, **p<0.01, ****p<0.0001*.


T4056
A
Negative
Control
A'
PD-L1
Control
A''
PD-L1
Atropine
A'''
PD-L1
4-DAMP
LIM-2405
B
Negative
Control
B'
PD-L1
Control
B''
PD-L1
Atropine
B'''
PD-L1
4-DAMP
HT-29
C
Negative
Control
C'
PD-L1
Control
C''
PD-L1
Atropine
C'''
PD-L1
4-DAMP


D
T4056
LIM-2405
HT-29
C
A
4-D
PD-L1
33 kDa
GADPH
43 kDa

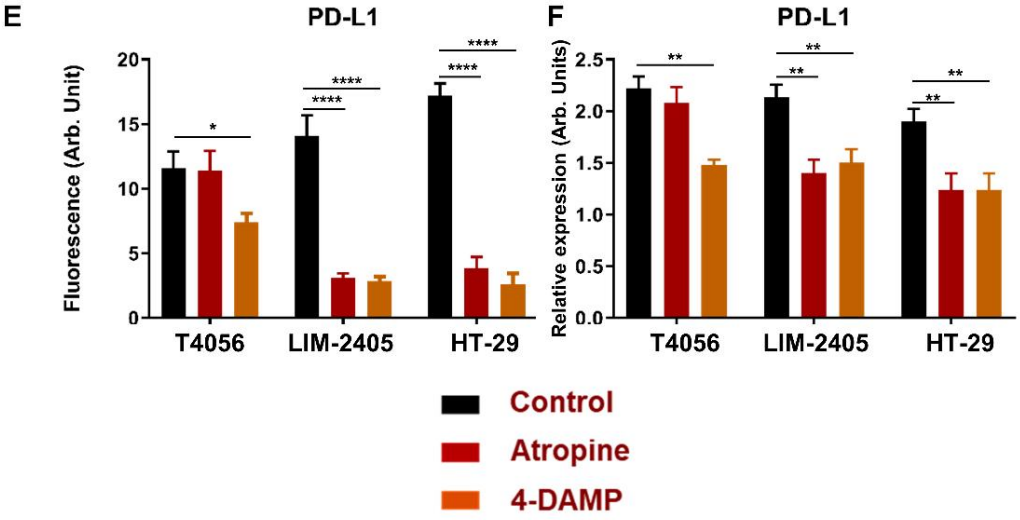
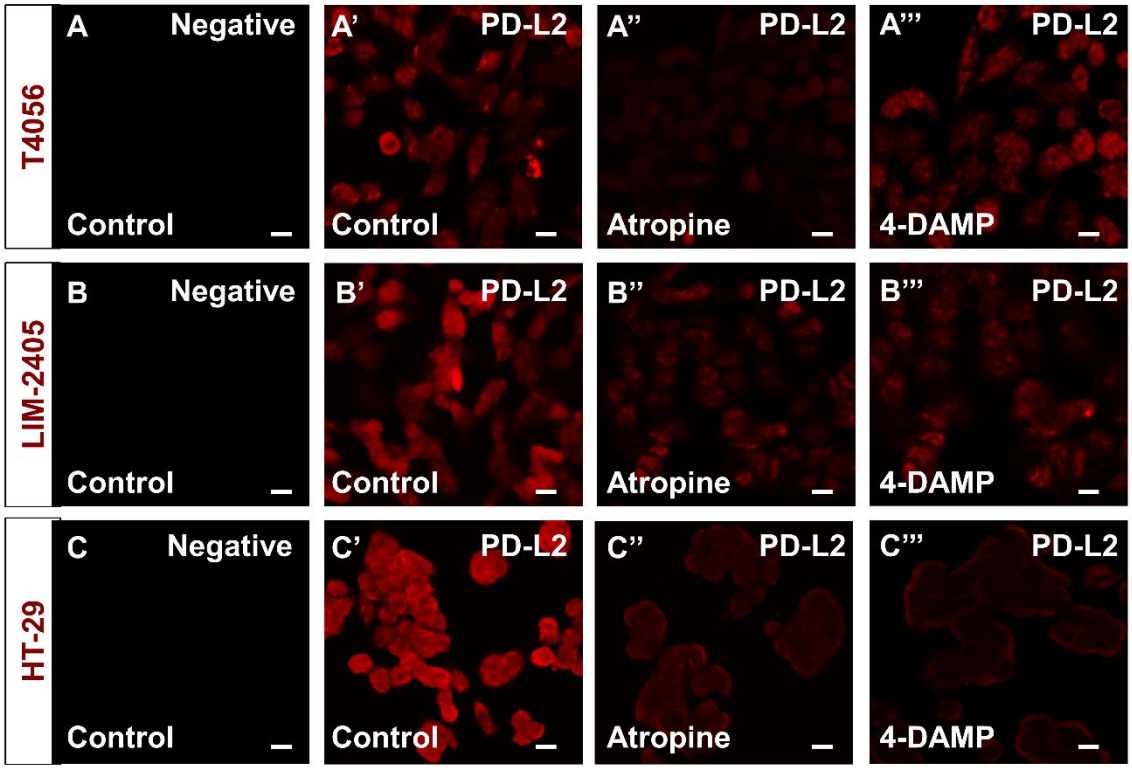
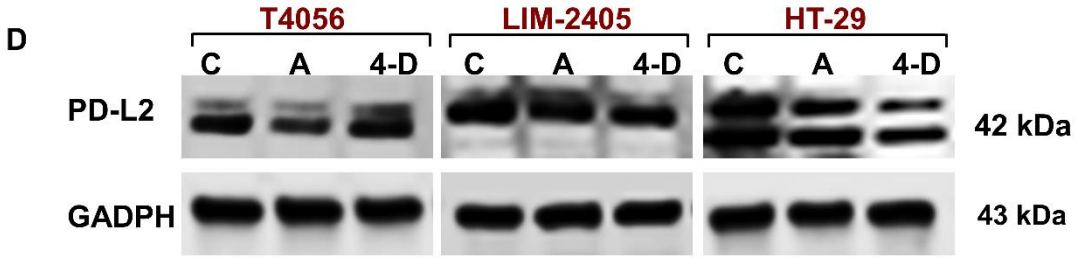

E
PD-L1
Fluorescence (Arb. Unit)
T4056
LIM-2405
HT-29
F
PD-L1
Relative expression (Arb. Units)
T4056
LIM-2405
HT-29
Control
Atropine
4-DAMP

Figure 3.7. Effect of atropine and 4-DAMP on the expression of PD-L2 *in vitro*

Negative control labelling of T4056 cells is shown in (**A)**, control (**A')** and atropine and 4-DAMP in (**A" & A"')**, respectively. Negative control labelling of LIM-2405 cells is presented in (**B**), control (**B'**), atropine (**B"**) and 4-DAMP (**B"'**). HT-29 negative control is displayed in (**C**), control (**C'**), atropine (**C"**) and 4-DAMP (**C"'**). Scale bar represents 50µm. Western blot bands for T4056, LIM-2405 and HT-29 cells are shown in (**D**). Bar graphs displaying the mean fluorescence of PD-L2 (**E**) and western blot expression intensity (**F**). Data presented as mean ± SEM. Two-*way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*.


T4056
A
Negative
Control
A'
PD-L2
Control
A''
PD-L2
Atropine
A'''
PD-L2
4-DAMP
LIM-2405
B
Negative
Control
B'
PD-L2
Control
B''
PD-L2
Atropine
B'''
PD-L2
4-DAMP
HT-29
C
Negative
Control
C'
PD-L2
Control
C''
PD-L2
Atropine
C'''
PD-L2
4-DAMP


D
T4056
LIM-2405
HT-29
C
A
4-D
PD-L2
42 kDa
GADPH
43 kDa

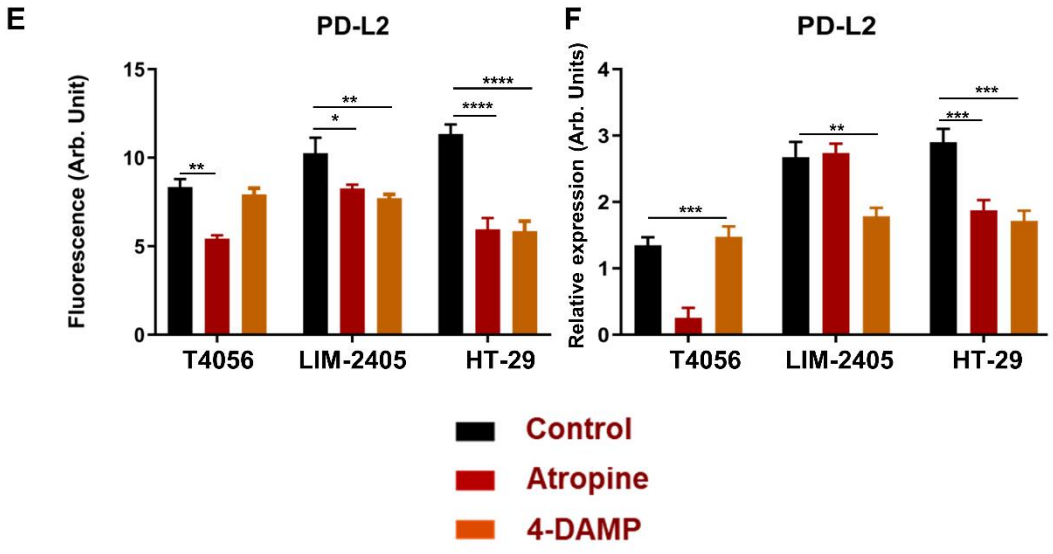
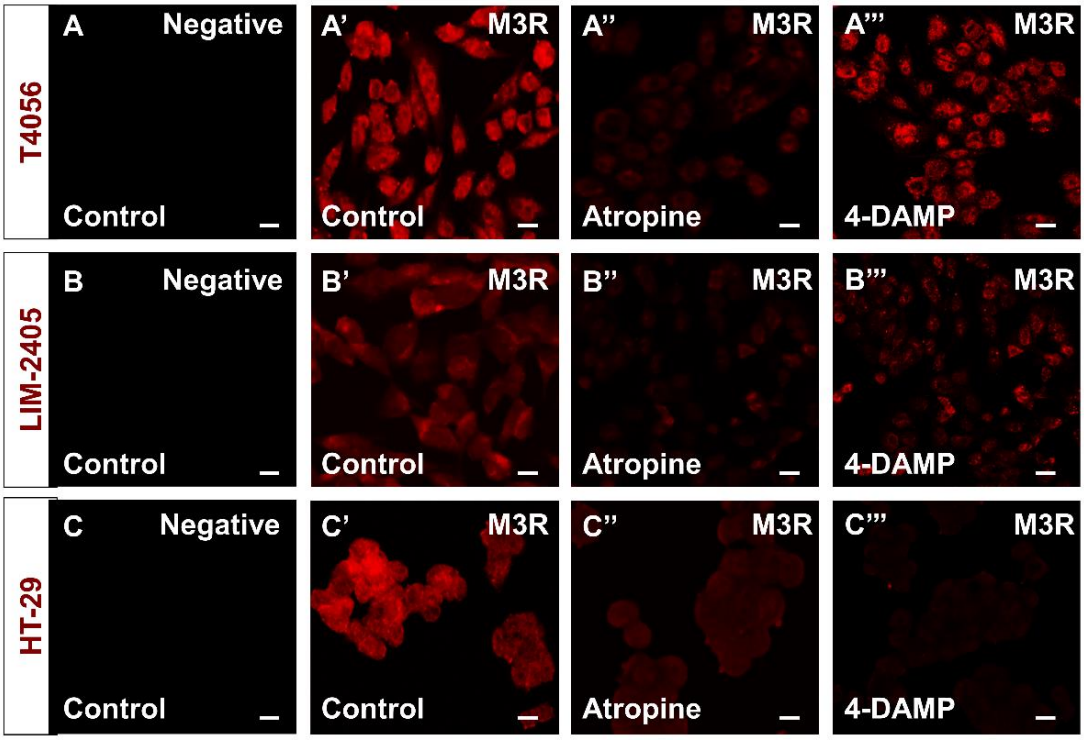
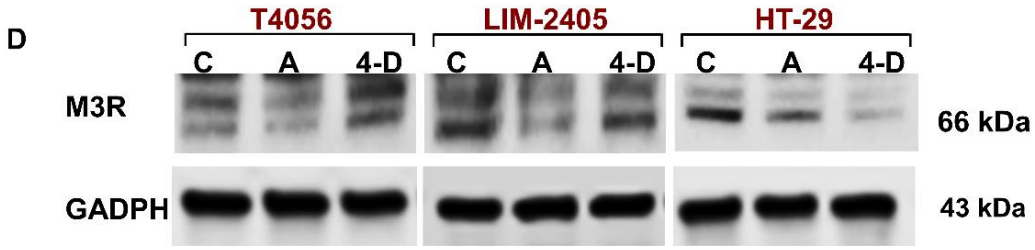

E
PD-L2
Fluorescence (Arb. Unit)
T4056
LIM-2405
HT-29
F
PD-L2
Relative expression (Arb. Units)
T4056
LIM-2405
HT-29
Control
Atropine
4-DAMP

Figure 3. 8. Effect of atropine and 4-DAMP on the expression of M3R *in vitro*

Negative control labelling of T4056 cells is shown in (**A)**, control (**A')** and atropine and 4-DAMP in (**A" & A"')**, respectively. Negative control labelling of LIM-2405 cells is presented in (**B**), control (**B'**), atropine (**B"**) and 4-DAMP (**B"'**). HT-29 negative control is displayed in (**C**), control (**C'**), atropine (**C"**) and 4-DAMP (**C"'**). Scale bar represents 50µm. Western blot bands for T4056, LIM-2405 and HT-29 cells are shown in (**D**). Bar graphs displaying the mean fluorescence of M3R (**E**) and western blot expression intensity (**F**). Data presented as mean ± SEM. Two-*way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*.


T4056
A
Negative
Control
A'
M3R
Control
A''
M3R
Atropine
A'''
M3R
4-DAMP
LIM-2405
B
Negative
Control
B'
M3R
Control
B''
M3R
Atropine
B'''
M3R
4-DAMP
HT-29
C
Negative
Control
C'
M3R
Control
C''
M3R
Atropine
C'''
M3R
4-DAMP


D
T4056
LIM-2405
HT-29
C
A
4-D
M3R
66 kDa
GADPH
43 kDa

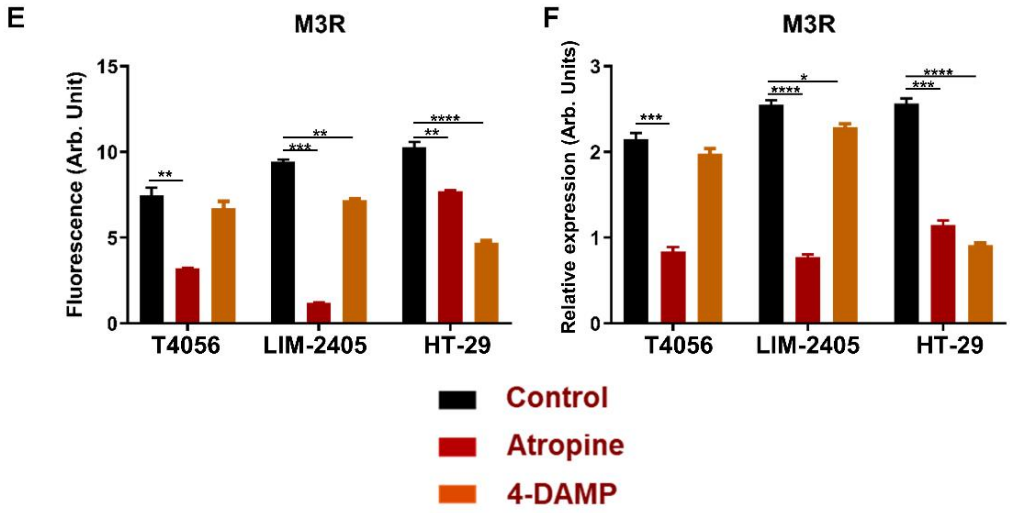
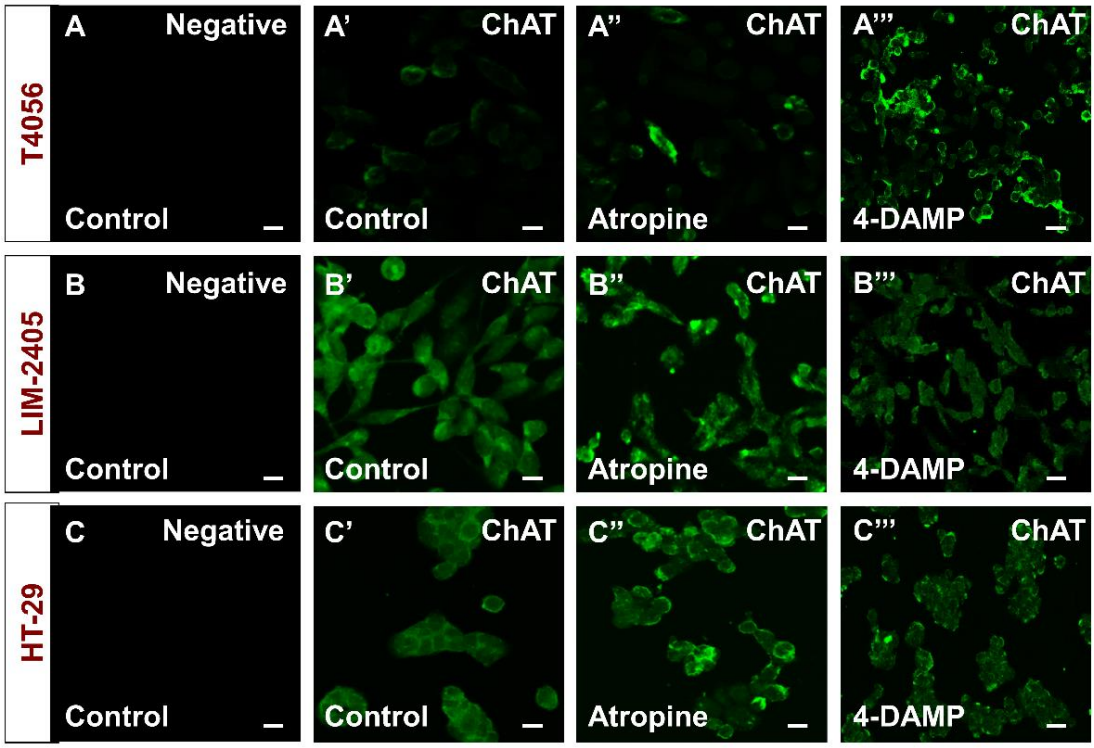
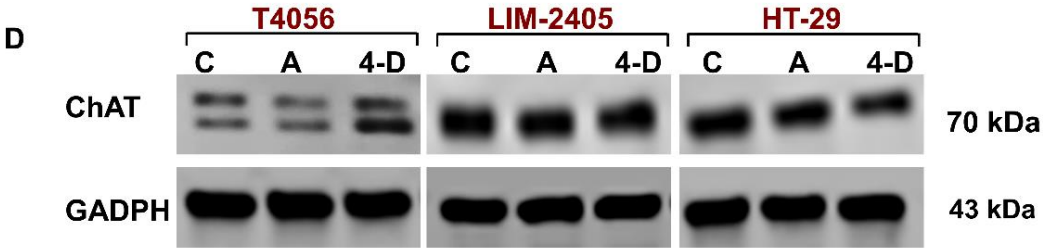
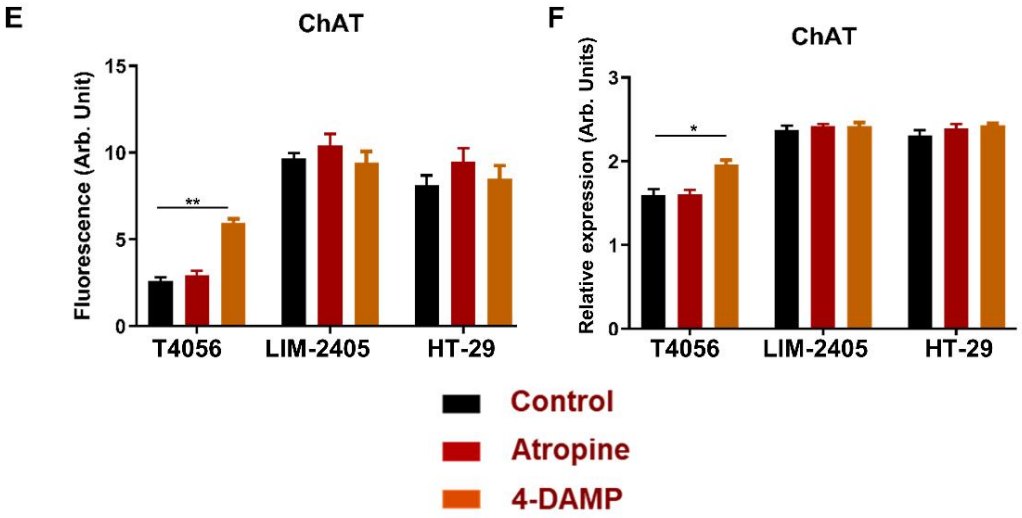

E
M3R
Fluorescence (Arb. Unit)
T4056
LIM-2405
HT-29
F
M3R
Relative expression (Arb. Units)
T4056
LIM-2405
HT-29
Control
Atropine
4-DAMP

Figure 3.9. Effect of atropine and 4-DAMP on the expression of ChAT *in vitro*

Negative control labelling of T4056 cells is shown in (**A)**, control (**A')** and atropine and 4-DAMP in (**A" & A"')**, respectively. Negative control labelling of LIM-2405 cells is presented in (**B**), control (**B'**), atropine (**B"**) and 4-DAMP (**B"'**). HT-29 negative control is displayed in (**C**), control (**C'**), atropine (**C"**) and 4-DAMP (**C"'**). Scale bar represents 50µm. Western blot bands for T4056, LIM-2405 and HT-29 cells are shown in (**D**). Bar graphs displaying the mean fluorescence of ChAT (**E**) and western blot expression intensity (**F**). Data presented as mean ± SEM. Two-*way ANOVA, *p<0.05, **p<0.01*.


T4056
LIM-2405
HT-29
A Negative
A' ChAT
A'' ChAT
A''' ChAT
B Negative
B' ChAT
B'' ChAT
B''' ChAT
C Negative
C' ChAT
C'' ChAT
C''' ChAT
Control
Atropine
4-DAMP


D
T4056
LIM-2405
HT-29
C
A
4-D
ChAT
GADPH
70 kDa
43 kDa


E
ChAT
Fluorescence (Arb. Unit)
15
10
5
0
**
T4056
LIM-2405
HT-29
F
ChAT
Relative expression (Arb. Units)
3
2
1
0
*
T4056
LIM-2405
HT-29
Control
Atropine
4-DAMP

3.3.6. Effect of atropine and 4-DAMP on EGFR and phosphorylation of STAT3 and ERK kinases

It is known that muscarinic receptors suppress cell apoptosis through the activation of phosphatidylinositol-3-OH (PI3) kinase and its downstream targets, protein kinase B (PKB)/AKT and MAPK/ERK [343, 367]. Furthermore, the activation of signalling pathways is essential in the development of CRC.

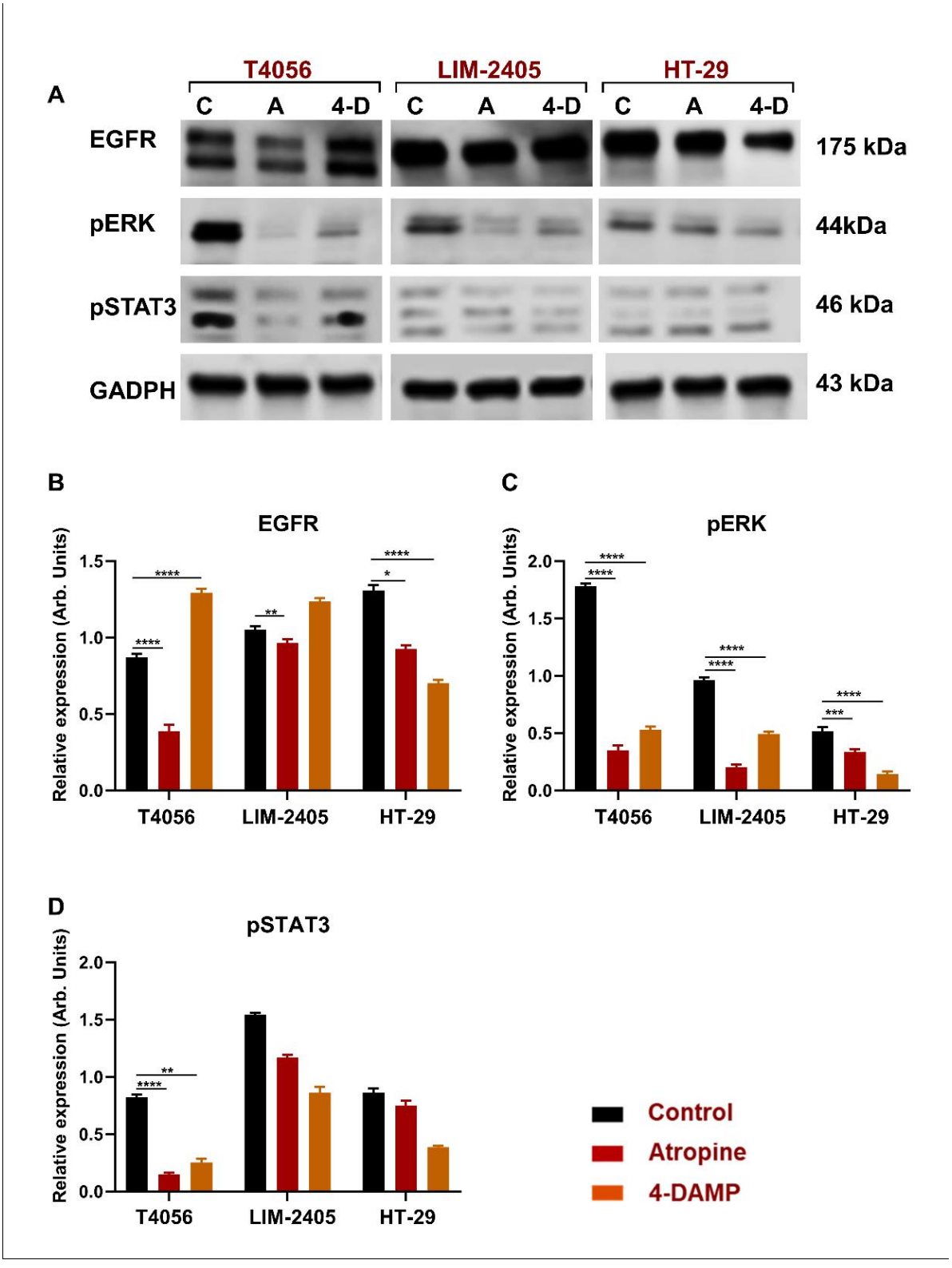
To gain insights into the mechanism of actions of atropine and 4-DAMP, immunoblotting of pSTAT3, pERK and EGFR were performed. It is clear that atropine treatment significantly reduced EGFR expression in T4056, LIM-2405 and HT-29 cell lines (**Figure 3.10A** and **Figure 3.10B**). 4-DAMP treatment significantly augmented EGFR expression in T4056 cells and showed a trend towards increased EGFR expression in LIM-2405 cells, albeit not significant. However, in the HT-29 cell line, 4-DAMP significantly attenuated EGFR expression compared to control. In addition, both atropine and 4-DAMP significantly decreased pERK expression in all cell lines (**Figure 3.10A** and **Figure 3.10C**). Interestingly, both atropine and 4-DAMP showed no effect on the phosphorylation of STAT3 in human colon cancer cell lines; however, both treatments significantly reduced pSTAT3 in normal epithelial cells (**Figure 3.10A** and **Figure 3.10D**).

Overall, the data suggest that atropine exhibits its effect through inhibition of EGFR, pERK and pSTAT3 in colon epithelial cell line, whilst in human colon cancer cell lines through EGFR and pERK. Similarly, blocking of M3R with 4-DAMP exerts its effects via increasing EGFR in T4056 cells and decreasing in HT-29 cells as well as inhibiting phosphorylation of ERK in all cells and STAT3 in T4056 cells. Hence, these findings suggest that atropine and 4-DAMP suppress the expression of immunosuppressive and cholinergic markers, cellular proliferation, migration and induce apoptosis via EGFR/ ERK/ STAT3 signalling pathways.

.

Figure 3.10. Signalling pathways involved in the *in vitro* effect of atropine and 4-DAMP

Western blot bands for T4056, LIM-2405 and HT-29 cells are shown in (**A**). The mean relative expression of EGRF (**B)**, pERK (**C)** and pSTAT3 (**D**). Bar graphs are presented as mean ± SEM. Two-*way ANOVA, *p<0.05, ***p<0.001, ****p<0.001*.


A
T4056
LIM-2405
HT-29
C
A
4-D
EGFR
pERK
pSTAT3
GADPH
175 kDa
44kDa
46 kDa
43 kDa
B
EGFR
C
pERK
D
pSTAT3
Relative expression (Arb. Units)
T4056
LIM-2405
HT-29
Control
Atropine
4-DAMP

3.4. Discussion

To the best of our knowledge, this study is the first to determine the interaction between immunosuppressive markers expressed by cancer cells and the release of acetylcholine acting on muscarinic receptors in CRC. The results of this study show that immunosuppressive molecules, PD-L1 and PD-L2, were significantly elevated in human colon cancer cells compared to colon epithelial cells. Treatment with muscarinic blockers, 4-DAMP and atropine, decreased cellular proliferation and migration similarly in both normal epithelial cell line and human colon cancer cells; as well as induced apoptosis. However, the expression levels of the markers evaluated in this study vary between normal epithelial cells and human colon cancer cells. These effects involve suppression of PD-L1 and PD-L2 expression on cancer cells via inhibition of EGFR activation and phosphorylation of ERK and STAT3 protein kinases.

Overexpression of PD-L1 has been observed in a number of cancers, including CRC [567]. The increase in the expression of PD-L1 on tumour cells could be influenced by the immune microenvironment, thus allowing immune evasion. In contrary, other studies demonstrated that PD-L1 expression is associated with early-stage, lower tumour grade, absence of vascular invasion and lymph node metastasis, significantly leading to improved patient survival from CRCs via enhancing CD8+ T cell infiltration [528]. These studies suggest that the prognostic value of PD-L1 expression could be dependent on the subset of CRCs as well as the presence of infiltrating immune cells.

The expression of PD-L2 in cancer is not well understood, as there are scarce studies identifying the role of PD-L2 in cancer progression. However, studies have reported that about 40% of cancer tissues from patients with CRC overexpressed PD-L2 [568]. Similar to PD-L1, PD-L2 is induced by the IFN-γ as noted in LOVO and RKO human colon cancer cell lines [529]. Furthermore, studies have shown that treating HCT116 human colon cancer cells with PD-L2 Fc fusion protein increased their invasion ability [569]. In esophageal adenocarcinomas, PD-L2 expression associates with smaller tumour size, early-stage and well-differentiated grade; however, PD-L2 is not associated with lymph node infiltration or metastasis or patient survival [538]. In contrary, PD-L2 expression is

associated with worse survival in patients with esophageal cancer [533]. In renal cell carcinoma, PD-L2 expression associated with shorter progression-free survival via c-MET and vascular endothelial growth factor [534]. More recently, it was noted that PD-L2 expression was independently associated with poor overall survival in CRC [529]. Furthermore, studies have shown that depending on the molecules present in the microenvironment, this can increase the expression of PD-L2 on immune as well as non-immune cells [539]. The role of ACh interaction with the immune system in cancer development and progression has attracted attention due to its influence on tumour microenvironment; however, the mechanisms underlying this interaction are not well understood.

The role of ACh in cancer immunomodulation is not clear; however, treatment of spleen cultures with ACh enhances T cell proliferation, suggesting the possible role of ACh in the activation of anti-cancer immune response [570, 571]. Many studies have reported that ACh and other constituents of cholinergic signalling, including ChAT and cholinergic receptors, are present in a variety of non-neuronal tissues and many cancers [368, 554, 564, 566]. ACh plays an important role in cellular proliferation, migration and apoptosis, which are essential for cancer development and progression. For instance, ChAT is upregulated in non-small cell lung carcinoma (NSCLC) while cholinesterase enzymes are downregulated, leading to increased ACh in cancer tissues [572, 573]. In the present study, LIM-2405 and HT-29 human colon cancer cells expressed enhanced levels of ChAT, the main enzyme precursor required for ACh synthesis. This concurs with previous studies demonstrating the role of ACh in cellular proliferation and migration. The data herein shows that blocking muscarinic receptors resulted in decreased cellular proliferation and migration. Likewise, administration of a non-selective muscarinic receptors inhibitor, atropine, suppressed SNU-C4 colon cancer cell migration; however, H508 colon cancer cell migration requires the activation of MMP7 [368, 369]. M3R activation increases invasion and migration of NSCLC cells and enhances the release of interleukin (IL)-8 [367]. Furthermore, muscarinic receptors mediate proliferation of HT-29 cell line [574], supporting our findings. The release of ACh acting on androgen receptors promotes SNU-449 liver cancer cell invasion and migration [363]. The activation of nicotinic receptors (nAChRs) by nicotine enhances LOVO and SW620 colon cancer cell

invasion and metastasis [343]. Similarly, nicotine pre-treatment stimulates the activation of α9-nAChR, which mediates MCF-7 and MDA-MB-231 breast cancer cell migration via the expression of epithelial-mesenchymal transition markers [364]. The present study suggests that ACh might play a role in the induction of immunosuppressive molecules. The results of this study suggest that ACh not only stimulates the expression of PD-L1 and PD-L2 but could explain the inconsistency in prognostic value. The findings presented in this chapter demonstrated that atropine and 4-DAMP significantly decreased the expression of PD-L1 and PD-L2 in colon cancer cells when compared to control.

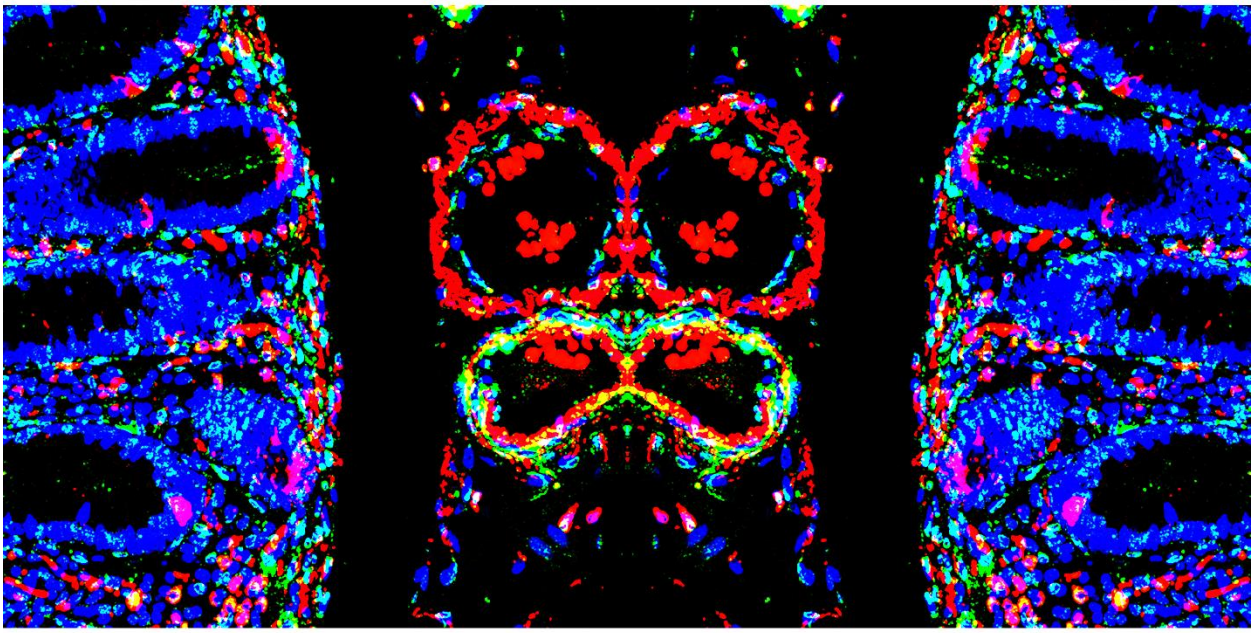
PD-L1 can be induced by the presence of signalling molecules such as nuclear factor-kappa B, mitogen-activated protein kinase, phosphoinositide 3-kinase, mammalian target of rapamycin and Janus kinase/signal transducer and activator of transcription, providing a pathway for tumour evasion [575]. The present study demonstrates that atropine exhibits its effect through suppression of pSTAT3 and pERK in LIM-2405, whereas in T4056 and HT-29 through pSTAT3, pERK and EGFR. Similarly, blocking of M3R with 4-DAMP induced apoptosis, reduced cellular proliferation, migration and the expression of immunosuppressive and cholinergic markers via inhibiting phosphorylation of STAT3 and ERK as well as suppressing activation of EGFR. Most of the immunosuppressive markers of interest are partially induced by INF-γ and oncogenic signalling pathways including EGFR, ERK and STAT3. Furthermore, activation of these signalling pathways is essential in the development of CRC. STAT3 and ERK play a key role in cancer cell proliferation and migration. Therefore, it is crucial to determine the effects of atropine and 4-DAMP on their expression. In order to determine the mechanisms by which atropine and 4-DAMP decrease the expression of immunosuppressive markers, immunoblotting of pERK and pSTAT3 were undertaken. These results indicate that atropine and 4-DAMP inhibit the activation of STAT3 and ERK in LIM-2405 and HT-29 cells. Hence, atropine and 4-DAMP decreased the expression of immunosuppressive markers via inhibiting the activation of EGFR and phosphorylation of pERK and pSTAT3 proteins.

3.5. Conclusion

Cancer cells have evolved to suppress the immune system by increasing cell surface markers. Their ability to synthesise and secrete ACh promotes their proliferation, differentiation and migration via acting as an autocrine or paracrine hormone. Findings presented in this chapter demonstrate that normal epithelial cell line and human colon cancer cells express PD-L1 and PD-L2. Atropine and 4-DAMP attenuate the expression of PD-L1, PD-L2 and M3R, cellular proliferation, migration and induced apoptosis via inhibiting the activation of EGFR and phosphorylation of ERK and STAT3 signalling pathways. Thus, allowing the immune system to recognise and eliminate cancer cells.

CHAPTER FOUR

Effect of Blocking Muscarinic Receptor 3 on the Expression of Immunosuppressive and Cholinergic Markers in Orthotopic Mouse Model of Colorectal Cancer



4.0. Abstract

Colorectal cancer is the third leading cause of cancer-related death worldwide. Tumour cells have evolved to express immunosuppressive molecules allowing their evasion from the host's immune system. These molecules include programmed death-ligands 1 and 2 (PD-L1 and PD-L2). Cancer cells can also produce acetylcholine (ACh), which plays a role in tumour development. Moreover, tumour innervation can stimulate vascularisation leading to tumour growth and metastasis. The effects of atropine and muscarinic receptor 3 (M3R) blocker, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), on cancer growth and spread were evaluated *in vitro* using murine colon cancer cell line, CT-26, and *in vivo* in an orthotopic mouse model of colorectal cancer. Molecular mechanisms and signalling pathways involved in these effects were studied. In the *in vitro* model, atropine and 4-DAMP significantly inhibited CT-26 cells proliferation in a dose dependent manner and induced apoptosis. Atropine attenuated the expression of immunosuppressive and cholinergic markers via inhibition of EGFR/AKT/ERK signalling pathways. However, 4-DAMP showed no effect on the expression of immunosuppressive markers (PD-L1 and PD-L2) on CT-26 cells but attenuated cholinergic markers by suppressing phosphorylation of AKT and ERK. Blocking of M3R *in vivo* decreased tumour growth, expression of immunosuppressive, cholinergic and angiogenic markers through inhibition of AKT and ERK phosphorylation, leading to an improved immune response against cancer. These findings suggest that there is crosstalk between the cholinergic system and the immune system during cancer development and the evasion of cancer from the host's immunity is influenced by the cholinergic system. Taken together, these findings suggest that tumour microenvironment constituents might influence the effects of M3R blocker on the expression of immunosuppressive markers. Thus, targeting both immunosuppressive and cholinergic markers may be beneficial to colorectal cancer treatment.

4.1. Introduction

Colorectal cancer (CRC) is ranked third amongst the most commonly diagnosed cancers and is responsible for 862 000 deaths worldwide [2, 576, 577]. Due to the complex nature of CRC and the lack of early clinical symptoms, it is often detected in the advanced stages. Despite the incessant advancement in treatment technology, the 5-year survival rate of patients with metastatic disease remains at less than 10% [578, 579], which could be partly due to the lack of specific markers for early diagnosis, cancer progression and patients' prognosis. Thus, it is indispensable to develop effective diagnostic and therapeutic approaches.

Resistance against cancer cells and their annihilation is reliant on the induction of cytotoxic CD8+ T cells and their differentiation into cytolytic and T helper-1 (Th1) cells. Cancer cells can avoid host's immune scrutiny by using a number of defensive mechanisms, including upregulation of immunosuppressive factors, such as programmed death-ligand 1 and/or 2 (PD-L1 and PD-L2), downregulation of major histocompatibility complex (MHC)-I and co-stimulatory molecules, secretion of angiogenic factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor receptor α (PDGFRα), the AXL receptor tyrosine kinase (AXL), anti-inflammatory cytokines, i.e. interleukin (IL)-10 and transforming growth factor-β (TGF-β), thus preventing activation of T cells, resulting in cancer invasion [509, 510, 512, 514, 580]. Cancer cells overexpress PD-L1 and/or PD-L2 on their surface, which upon binding to programmed death protein 1 (PD-1) expressed by activated CD8+ T cells leads to their inhibition and/or apoptosis [581]. Interestingly, PD-L1 serves as an anti-apoptotic factor on cancer cells, leading to resistance of lysis by CD8+ T cells as well as apoptosis [28].

The role of PD-L1 overexpression remains contradictory, with some papers reporting that overexpression associate with poor prognostic outcomes while other papers report better survival outcomes. For instance, high PD-L1 expression associates with tumour metastasis, poor prognosis and shorter survival in CRC patients [62, 527]. Similarly, PD-L1 expression in stromal or tumour cells is inversely correlated with FOXP3+ cell density in CRC patients, further reinforcing the fundamental role in modulating regulatory T cells

(Treg) in the tumour microenvironment [69]. More recently, in cohort studies of 181 CRC patients, PD-L1 expression was associated with high CD8+ tumour-infiltrating lymphocytes (TILs), *BRAF* mutation, microsatellite instability (MSI), lower frequency of *K-ras* and poor prognosis [582]. In contrast, other studies have suggested that the expression of PD-L1 is associated with good patients' survival outcomes. For example, PD-L1 expression correlates with elevated TIL infiltration and longer recurrence-free survival in breast cancer and pulmonary adenocarcinoma patients [57, 583, 584].

The role of PD-L2 in human cancers is not as well studied as PD-L1. PD-L2 is expressed by a number of immune and non-immune cells such as T cells, dendritic cells and macrophages, depending on the microenvironmental stimuli [517]. In breast cancer patients, expression of PD-L2 correlates with overexpression of human epidermal growth factor receptor 2 (HER-2) and estrogen receptor (ER)-negative tumours, recurrence at distant sites and younger patients' age [584]. In CRC, PD-L2 expression is independently associated with worse overall survival [529].

In addition to forming immunosuppressive microenvironment, there is compelling data suggesting that there is bi-directional signalling between the nervous system and the tumour microenvironment via the release of neurotransmitters, neuropeptides and other factors, implicating their influence on tumour development [585]. Neurotransmitters play an essential role in the activation of signalling pathways such as phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) which are related to cell proliferation and survival [586]. For example, neurotransmitter acetylcholine (ACh) can stimulate CRC cell proliferation, invasion, vascularisation and migration by binding to muscarinic receptor 3 (M3R) through activation of the epidermal growth factor receptor (EGFR), PI3K, extracellular signal-regulated kinase $(ERK)_{1/2}$ and AKT pathways as well as alpha 7 nicotinic receptor (α7nAChR) through activation of Janus kinase 2 (JAK2)/Signal transducer and activator of transcription 3 (STAT3) pathway [520-523, 587-589]. In addition, cancer cells can also overexpress choline acetyltransferase (ChAT), a precursor enzyme required for ACh synthesis, and vesicular acetylcholine transporter (VAChT), essential for transfer of ACh from the cytoplasm into synaptic vesicles [521, 590].

Furthermore, the cholinergic nervous system plays an important role in tumour angiogenesis and metastasis [510, 580]. Nicotine stimulation enhances VEGF expression and micro-vessel density in human colon cancer xenografts in nu/nu (nude) mice [204]. Administration of autoantibodies against muscarinic ACh receptors (mAChRs) in mouse models of breast cancer mediated tumour angiogenesis via activation of mAChRs through the release of VEGF-A [206]. In addition, administration of muscarinic agonist, carbachol, in the presence or absence of various muscarinic antagonists shows an increase in VEGF expression as noted in LMM3 murine mammary adenocarcinoma-bearing BALB/c mice [207].

Overall, the synergism of these neuro-immune markers has yet to be explored as potential targets in CRC development. In this study, using an orthotopic mouse model of CRC, we determined (i) the effect of blocking M3R on tumour growth, (ii) expression of immunosuppressive, cholinergic and angiogenic markers and (iii) presence of tumour-infiltrating immune cells. The influence of cholinergic signalling on the expression of immune checkpoint molecules may play an essential role in cancer development; therefore, it is necessary to understand the synergism of these neuro-immune markers *in vivo.*

4.2. Materials and Methods

4.2.1. Mice

Male BALB/c mice aged 5-8 weeks (n=16) were purchased from the Animal Resources Centre and housed in groups of 4 per cage. All animals were kept in a temperature-controlled environment with 12-hour (hr) light/dark cycle at approximately 22°C with access to food and water. The mice were allowed to acclimatise for at least one week before undergoing surgery. All animal experiments in this study complied with the guidelines of the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes under the approval of the Victoria University Animal Experimentation Ethics Committee (ethics number AEETH 15-011). All efforts were made to lessen animal suffering.

4.2.2. Cell culture

As previously described in Chapter 3, murine colorectal cancer cell line (CT-26) was cultured in Roswell park memorial institute (RPMI) 1640 culture media supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% Glutamine, at 37°C, in 5% CO_2 and 95% air atmosphere. Passage of cells was conducted with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) every 3-4 days. When cells grew into confluent or semiconfluent monolayers in the 75cm^2 medium flasks, they were either passaged or used.

4.2.3. Cell viability

The water-soluble tetrazolium-1 (WST-1) assay kit (Roche Diagnostics GmbH, Germany) was used to determine the viability of CT-26 cells. WST-1 is cleaved to form formazan dye via a complex cellular interaction at the cell surface. This interaction is contingent on the glycolytic nicotinamide adenine dinucleotide phosphate (NADPH) production of the viable cells. Hence, the amount of formed formazan dye correlates to the number of viable cells in the culture. CT-26 cells were seeded and cultured at 1×10^4 cells per well in 96 well plates for 24hrs. Cells were then treated with various concentrations of the general muscarinic receptor blocker, atropine (Sigma-Aldrich, Australia), selective M3R blocker,

1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) (Abcam, Australia), cholinergic agonist, carbachol (Abcam, Australia) and acetylcholinesterase inhibitor, donepezil (Abcam, Australia) for 8hrs. All treatments were performed in triplicates, and three independent experiments were conducted. WST-1 reagent (10µL) was added to each well and incubated at 37^{o}C for 1hr. Cellular proliferation at the absorbance of 450nm was measured using a microplate reader (Varioskan Flash, Thermo Scientific, Australia).

4.2.4. Annexin V apoptosis assay

CT-26 cells were cultured overnight in six well plates at 5x10^{5} cells per well. Cells were treated with 100µM of atropine and 4-DAMP for 8hr. Cells were collected and resuspended in fluorescence-activated cell sorting (FACS) buffer and labelled with 100µL per well with Annexin V at 1:1,000 dilution and 0.5µg/mL of propidium iodide (PI). Flow cytometry was utilised to determine the percentage (%) of apoptotic and necrotic cells. All treatments were performed in duplicates, and two independent experiments were conducted.

4.2.5. Choline/acetylcholine assay

The choline/acetylcholine assay kit (Abcam, Australia) was used to measure the concentration of choline in CT-26 cell lysates. The assay was carried out in accordance with the instructions provided by the manufacturer. Briefly, CT-26 (1x10^{6}) cells were cultured overnight, after which cells were treated with 100µM of cholinergic antagonists, atropine and 4-DAMP, and 500µM of acetylcholinesterase inhibitor, donepezil. Cells were lysed in 500µL choline assay buffer before commencing choline measurements using a microplate reader (Varioskan Flash, Thermo Scientific, Australia) at an absorbance of 570nm. All treatments were performed in duplicates, and two independent experiments were conducted.

4.2.6. Orthotopic implantation of CT-26 tumour cells

Mice were anaesthetised using xylazine (10mg/kg), and ketamine (80mg/kg) injected intraperitoneally. The level of anaesthesia during the surgery was monitored using the paw pinch reflex test. The eyes of the animals were treated with ViscoTears to protect

them from drying out during the surgery. Mice were placed on an operating table on a heat mat (30-36°C), and all procedures were performed under aseptic conditions. All instruments were autoclaved and only opened when ready to operate. The abdomen was shaved and swabbed with 70% ethanol and covered with sterile film. A small midline abdominal incision was made, and the caecum was exteriorised on sterile gauze. Matrigel (25µL) containing CT-26 cell suspension (1 x 10^6 cells) was injected into the caecum wall of BALB/c mice using an insulin needle. After injection, the abdominal muscle wall was sutured using polygalactone and skin using surgical silk or dissolvable skin sutures. The incision area was sterilised by saline followed with iodine. Mice were given an analgesic Temgesic/Buprenorphine (0.05mg/kg) subcutaneously. Mice were then monitored visually during recovery time (about 1-1.5hrs) and, when fully conscious, they were returned to an animal holding room in the animal facility.

4.2.7. Intraperitoneal injections and tissue collection

After five days post-surgery, vehicle BALB/c mice received intraperitoneal injection of 0.1% dimethyl sulfoxide (DMSO) treatment and study group received 10mg/kg of 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) per a day [587]. The volume of the administered solution was calculated per body weight with the maximum volume of 200µL per injection. Mice were culled at 28 days post-surgery via lethal injection of phenobarbital and tumours were removed, weighed and used for western blot, flow cytometry, proteome profiler array and immunohistochemistry. Tumour tissues were used to assess angiogenesis, tumour-infiltrating immune cells, and expression of immunosuppressive and cholinergic markers. Tumour tissues used for flow cytometry analysis were collected into RPMI media, for western and proteome profiler arrays were snap-frozen in liquid nitrogen and samples used for immunohistochemistry were placed in Zamboni's fixative (2% formaldehyde 0.2% picric acid).

4.2.8. Immunohistochemistry in cross-sections

Tumour tissues collected from vehicle-treated and 4-DAMP-treated groups were fixed with Zamboni's fixative overnight at 4°C. Next day, fixative was cleared off by washing samples for 10 mins three times with DMSO (Sigma-Aldrich, Australia) followed by three

times 10 mins washes with phosphate buffered saline (PBS). Tissues were then embedded in optimum cutting temperature medium (OCT) and frozen using 2-methyl butane (isopentane) and liquid nitrogen. Samples were stored in -80°C freezer until cryo-sectioned. Tissues were cut at 10µm section thickness using a Leica CM1950 cryostat (Leica Biosystems, Germany), adhered to slides and allowed to dry at room temperature for 1hr before commencing staining process. OCT was washed off with PBS containing 0.01% Triton X-100 (PBST) for 5 mins. Using a liquid Blocker Super Pap Pen, samples were outlined to reduce the volume of antibody used. The endogenous activity was blocked using 10% normal donkey serum for 1h at room temperature, followed by PBST washes. Samples were then incubated with primary antibodies (**Table 4.1**) against immunosuppressive, cholinergic and angiogenic markers overnight at 4°C. Sections were then washed in PBST before incubation with secondary antibodies labelled against primary antibodies (**Table 4.2**) for 2hrs at room temperature in the dark, followed by PBST washes. The sections were incubated with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (D1306, Life Technologies, Australia) for 1min. Sections were given final washes in PBST and then mounted with DAKO mounting media (Agilent Technologies, Australia). Coverslips were placed over each section and left to dry overnight before imaging. Sections were viewed under a Nikon Eclipse Ti laser scanning confocal microscope (Nikon, Japan), whereby eight randomly chosen images from each sample were captured with a 40× objective and analysed using image analysis software (Nikon, Japan).

4.2.9. Immunoperoxidase staining

Tumour samples were collected and placed in Zamboni's fixative overnight and processed as described in Section 4.2.8. Using a liquid blocker super pap pen, samples were outlined to reduce the volume of antibody used. Endogenous activity was blocked using 3% hydrogen peroxide for 30 mins. Samples were then incubated overnight at room temperature with primary antibody against CD31 (1:100). After PBST washes, samples were incubated at room temperature for 2hr with secondary antibodies at 1:250 dilution. CD31 antibody was diluted in 0.5% bovine serum albumin (BSA)-RPMI. Samples were incubated with developer reagent 3,3′-diaminobenzidine tetrahydrochloride (DAB) liquid substrate in peroxidase buffer for 30 mins. Samples were counterstained with Mayers

Haematoxylin in a beaker, rinsed in tap water, Scott's tap water, 100% ethanol and xylene. Samples were then mounted in an aqueous mounting medium and imaged using slide scanning imaging systems.

4.2.10. Western blot

Proteins extracted from CT-26-induced tumour tissues and CT-26 cells were evaluated for the expression of immunosuppressive, cholinergic and angiogenic markers as well as cell signalling pathways, phospho signal transducer and activator of transcription 3 (pSTAT3), phospho extracellular signal-regulated kinase (pERK½), phospho serine/threonine kinase or protein kinase B (pAKT) and epidermal growth factor receptor (EGFR) by western blot. CT-26 cells were incubated with 100µM atropine and 4-DAMP for 8hr. After treatments, cells were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer (pH 7.4, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 in PBS, Sigma) containing protease and phosphatase inhibitors cocktail (Roche Applied Science, Switzerland). For tumour samples, 100mg of tumour tissues per mouse were weighed, and tumour samples from 3 mice per band for the first two bands and tumour samples from 2 mice for the third band were pooled together. Samples were then homogenised in 500µL of RIPA buffer containing protease and phosphatase inhibitors cocktail. Cellular proteins (20µg) from CT-26 cell line and 25µg protein from tumour samples were separated by 8% to 12% sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis. The separated fragments were transferred to 0.22µm polyvinylidene fluoride membranes, which were blocked with 5% skim milk in PBS containing 0.1%Tween 20 and incubated overnight at 4^{o}C in platform shaker at 40rpm speed. The membranes were incubated with primary antibodies (**Table 4.1**) overnight at 4^{o}C. The membranes were then incubated with HRP-conjugated secondary antibodies (**Table 4.2**) for 2hrs at room temperature followed by three times PBS-0.1% Tween 20 washes. Glyceraldehydes-3-phosphate dehydrogenase (GADPH) was used as a loading control. Protein detection was performed using enhanced chemiluminescence reagents. Chemiluminescent signal was captured using the FluorChem FC2 system. The expression level of each protein was quantified using ImageJ software.

4.2.11. Proteome profiler mouse phospho-RTK array kit

The Proteome Profiler Mouse Phospho-RTK Array Kit is a membrane-based immunoassay, which captures antibodies spotted in duplicate on nitrocellulose membranes binding to specific target proteins present in the sample. The assay was carried out in accordance with the instructions provided by the manufacturer. Briefly, tumour samples from each group were pooled and lysed in Lysis Buffer 17 prepared with protease inhibitors. Samples were mixed by pipetting up and down to resuspend, and lysates were gently rocked at 4°C for 30 mins on a rocking platform shaker. Tumour lysates were centrifuged at 1500rpm for 5 mins at 4°C and supernatants were transferred into the clean test tubes. Array membranes were placed onto 4-well multi-dish and incubated with Array Buffer 1 for 1hr at room temperature on a rocking platform shaker. After 1hr incubation, Array Buffer 1 was aspirated out, and membranes were incubated with tumour lysates overnight at 4°C on a rocking platform shaker. Membranes were then washed with 1x Wash Buffer for 3x10 mins. Membranes were incubated with the anti-phospho-tyrosine-HRP antibody at room temperature for 2hr on a rocking platform shaker. Membranes were then washed with 1x Wash Buffer for 3x10 mins. Membranes were then incubated with chemiluminescence reagent mix, and chemiluminescent signal was captured using the FluorChem FC2 system.

4.2.12. Flow cytometric cell staining

On the day of culls, tumours were collected into RPMI media, and tumour tissues were processed into single-cell suspensions for FACS analysis. Single-cell suspensions were performed by mechanically dissecting tumours into small pieces and incubating with 2mL of collagenase (0.1%w/v in 1mL of α-MEM) at 37°C for 2hr with 30 mins intervals of mechanical dissociation. Tumour suspensions were filtered with 40µm cell strainers Falcon® into 50mL Falcon® tubes and were then centrifuged at 1500rpm for 5 mins at 4°C. Cell pellets were incubated with 1x red blood cell lysing buffer for 3mins at 37°C. Cell pellets were then resuspended in 1mL of FACS buffer to create a single cell suspension and accounted using a hemocytometer.

Viable cell pellets were incubated with two different antibody cocktails (**Table 4.3**). Cocktail 1 contained leukocyte infiltration markers (CD45, CD11b, CD4, CD8a, CD193(CCR3), Siglec-F and Fc Block), while cocktail 2 was comprised of (CD45, CD11b, CD19, CD206, CD115, F480, Ly-6C, Ly-6G and Fc Block). Tumour cells ($10x10^6$) cells (400µL) were aliquoted in BD Falcon® FACS tubes. Cells were centrifuged at 1300rpm for 3mins at 4°C. Cells were then incubated with 200µL of antibody cocktails for 1hr at 4°C. After incubation, cells were centrifuged at 1300rpm for 3 mins at 4°C and supernatants aspirated. Cells were then resuspended in 200µL FACS buffer and filtered through 35µm filters in a 5mL BD Falcon® tube. Prior to FACS analysis, cells were incubated with viability solution, 7-amino-actinomycin D (7-AAD, 1:20) to gate on the viable cell populations.

Table 4.1. Primary antibodies used in this study

Primary antibodies				
Markers	**Host Species & Clonality**	**Dilution**	**Source**	**Catalogue no.**
PD-L1	Mouse, monoclonal	1:500	Abcam, Australia	ab210931
PD-L2	Rabbit, polyclonal	1:500	Abcam, Australia	ab200377
M3R	Rabbit, polyclonal	1:500	Abcam, Australia	ab126168
α7nAChR	Mouse, monoclonal	1:500	Novus, Australia	7F10G1
ChAT	Goat, polyclonal	1:500	Abcam, Australia	ab134021
VAChT	Sheep, polyclonal	1:500	Abcam, Australia	ab31544
FOXP3	Mouse, monoclonal	1:500	Abcam, Australia	ab20034
VEGF	Rabbit, polyclonal	1:500	Abcam, Australia	ab46154
CD31	Rat, monoclonal	1:500	Abcam, Australia	ab7388
TGF-β	Rabbit, polyclonal	1:500	Abcam, Australia	ab155264
EGFR	Rabbit, monoclonal	1:1,000	Cell signalling, Australia	#4267
pAKT	Rabbit, monoclonal	1:1,000	Cell signalling, Australia	#4060
pERK	Rabbit, monoclonal	1:1,000	Cell signalling, Australia	#3192

Table 4.2. Secondary antibodies used in this study

Secondary antibodies			
Alexa Fluor 488	Anti-goat Anti-sheep Anti-rat	1:250	Jackson ImmunoResearch Laboratories, United States
Alexa Fluor 594	Anti-rabbit	1:250	Jackson ImmunoResearch Laboratories, United States
Alexa Fluor 647	Anti-mouse	1:250	Jackson ImmunoResearch Laboratories, United States
Anti-mouse	IgG HL HRP	1:10,000	Abcam, Australia
Anti-rabbit	IgG HL HRP	1:10,000	Abcam, Australia
Anti-rat	IgG HL HRP	1:10,000	Abcam, Australia
Anti-goat	IgG HL HRP	1:10,000	Abcam, Australia

Table 4.3. Antibodies used for flow cytometry experiments in this study

Cocktail 1				
Cells	**Primary antibody**	**Conjugate**	**Host species**	**Dilution**
Pan-leukocyte marker	CD45	FITC	Mouse	1:400
Granulocytes	CD11b	PE-Cy7	Mouse	1:400
Cytotoxic T cells	CD8a	APC-Cy7	Mouse	1:200
Helper T cells	CD4	BV480	Mouse	1:400
Gamma delta (γδ) T cells	γδ-TCR	PE	Mouse	1:400
Eosinophils	Siglec-F, CD193	BV421, AF647	Mouse	1:50
Cocktail 2				
Pan-leukocyte marker	CD45	FITC	Mouse	1:400
Granulocytes	CD11b	PE-Cy7	Mouse	1:400
Macrophages	F4/80, CD206	PE, AF647	Mouse	1:100, 1:50
Monocytes	CD11b, Ly6C, Ly6G, CD115	BV480	Mouse	1:400
B cells	CD19	APC-Cy7	Mouse	1:800
Neutrophils	Ly6C, Ly6G	PE-CF594 BV421	Mouse	1:50

4.2.13. Data analysis

Images were captured on a Nikon Eclipse Ti multichannel confocal laser scanning system (Nikon, Japan). Z-series images were acquired at a nominal thickness of 1µm (1024 x 1024 pixels). Image J software (National Institute of Health, Bethesda, MD, USA) was employed to convert images from RGB to greyscale 8 bit binary; particles were then analysed to obtain the percentage area of immunoreactivity [591]. All immunohistochemistry images and western blot bands were quantified and statistical analysis was performed by Student's *t-test*.

For flow cytometry analysis, the characterisation of tumour-infiltrating immune cells was performed and quantified by flow cytometry (BD FACS Aria I, BD Bioscience, CA, USA) with the aid of BD FACs DIVA software (BD Bioscience, CA, USA.). Two hundred thousand (200,000) events were collected per samples and analysed by BD FACS DIVA Software. As each antibody conjugate produces a distinctive emission spectrum, each experiment employed single colour compensation controls to optimise photo-multiplier tube (PMT) voltages and calculate spectral overlap (where applicable). Excel, SPSS and Prism (GraphPad software, La Jolla, CA, USA) were utilised to aid in the statistical analysis and $p<0.05$ was considered significant.

4.3. Results

4.3.1. Effect of blocking muscarinic receptors on CT-26 cells proliferation

The effect of blocking all muscarinic receptors on the proliferation of CT-26 cells was assessed using WST-1 assay following treatment with various concentrations of atropine at different time points. Three independent experiments were performed in triplicates. Atropine significantly decreased CT-26 cell proliferation at 1-8hrs compared to 24-48hrs (**Figure 4.1A**) with 300-1,000µM being most significant. It appears that atropine decreases proliferation in a dose-dependent rather than a time-dependent manner. A trend of lower proliferation in a dose-dependent manner was noted at 24-48hrs. In order to compare the effects of atropine and a selective M3R blocker, 4-DAMP, cells were incubated with 4-DAMP and atropine for 8hrs and no significant differences were noted between atropine and 4-DAMP with the exception at 600µM and 700µM doses (**Figure 4.1B**). However, there was a trend of 4-DAMP being less effective compared to atropine. In addition, cells were incubated with carbachol, which activates cholinergic receptors and donepezil, which prevents the breakdown of ACh, for 8hrs with various concentrations and dose curve was generated (**Figure 4.1C**). Incubation with a cholinergic agonist, carbachol, and acetylcholinesterase inhibitor, donepezil, reversed the effect of 4-DAMP and atropine. There was no significant difference between carbachol and donepezil at 50-500µM; however, at high doses (600-1,000µM), differences were noted. These findings further enforced the vital role of ACh in cellular proliferation.

In all subsequent experiments, cells were treated with 100µM of atropine and 4-DAMP for 8hrs as this concentration and time point induced prominent inhibition of cell proliferation and viability. Since no difference between the effects of carbachol and donepezil was observed, only donepezil at 500µM dose was applied for 8hhrs in all subsequent experiments.

4.3.2. CT-26 cells can produce choline required for ACh synthesis

Colon cancer cells are capable of synthesising and releasing ACh [587, 592-594]. To determine whether mouse colon cancer CT-26 cells were able to synthesise ACh, the amount of choline, a precursor for ACh, was measured in cell lysates ($1x10^6$ cells). Choline/acetylcholine assay kit was used to measure the amount of choline in cells lysates as described in the Materials and Methods section. It was noted that CT-26 cells can produce a higher amount of choline when treated with donepezil compared to control, 4-DAMP and atropine treatments (**Figure 4.2**). There were no significant differences observed between choline released in the cells treated with atropine and 4-DAMP when compared to the control. Overall, atropine and 4-DAMP had no effect on the CT-26 cells' ability to produce choline; however, donepezil increased choline production.

4.3.3. Effect of atropine and 4-DAMP on apoptosis in CT-26 cells

Apoptosis is a natural cellular process that safeguards all the body systems. As reviewed in Chapter 1, tumour cells must be resistant to anoikis (evasion from apoptosis) which is one of the essential steps in cancer metastasis [580]. Studies have demonstrated that several neurotransmitters, including ACh play a significant role in the regulation of cell apoptosis [510]. To determine whether atropine and 4-DAMP induce apoptosis or necrosis in CT-26 cells, Annexin V and PI were used and analysed by flow cytometry. Blocking all muscarinic receptors with atropine and muscarinic receptors 3 with 4-DAMP induced apoptosis in CT-26 cells compared to control (**Figure 4.3A-C**). In fact, 93.6% of atropine-treated and 76.6% of 4-DAMP-treated cells underwent apoptosis compared to 1.9% of control cells.

Figure 4.1. Effect of atropine on CT-26 cell proliferation

CT-26 cells treated with different concentrations of atropine with at different time points (**A**). Number of viable cells after 8hrs incubation with various concentrations of atropine and 4-DAMP (**B**). CT-26 cells treated with various concentrations of carbachol and donepezil for 8hrs (**C**). Values in **A**, **B** and **C** are mean ± standard error of the mean (SEM) from at least three independent experiments performed in triplicate wells

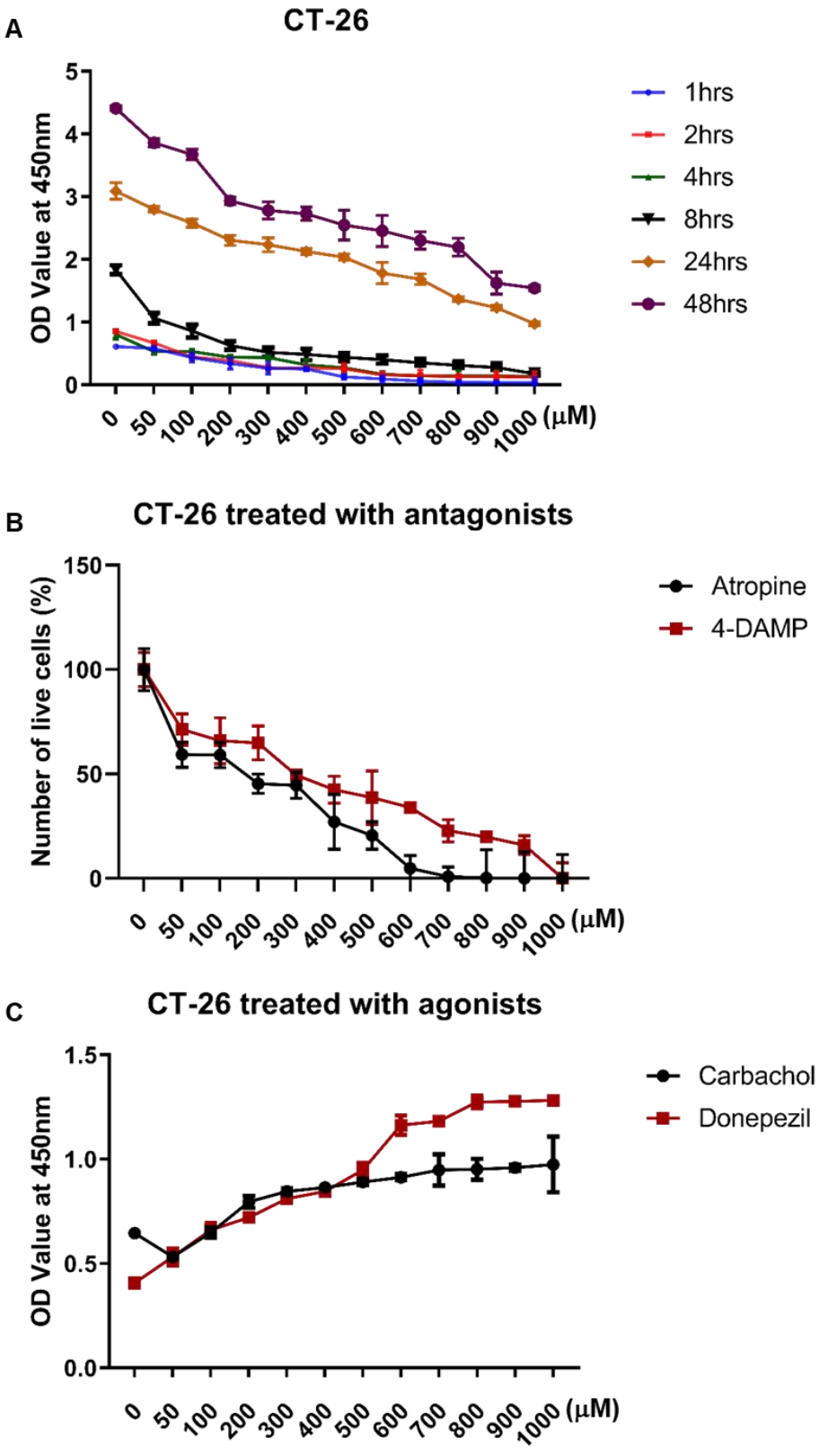

A
CT-26
OD Value at 450nm
0
1
2
3
4
5
0
50
100
200
300
400
500
600
700
800
900
1000 (μM)
1hrs
2hrs
4hrs
8hrs
24hrs
48hrs
B
CT-26 treated with antagonists
Number of live cells (%)
0
50
100
150
0
50
100
200
300
400
500
600
700
800
900
1000 (μM)
Atropine
4-DAMP
C
CT-26 treated with agonists
OD Value at 450nm
0.0
0.5
1.0
1.5
0
50
100
200
300
400
500
600
700
800
900
1000 (μM)
Carbachol
Donepezil

Figure 4.2. Effect of cholinergic antagonists and donepezil on choline production in CT-26 cells

The amount of choline was measured in CT-26 cells treated with atropine, 4-DAMP and donepezil. Values presented as mean ± SEM from at least two independent experiments. *Two-way ANOVA,* followed by Tukey's multiple comparison test, was used and considered significant when *$*p<0.05$*.

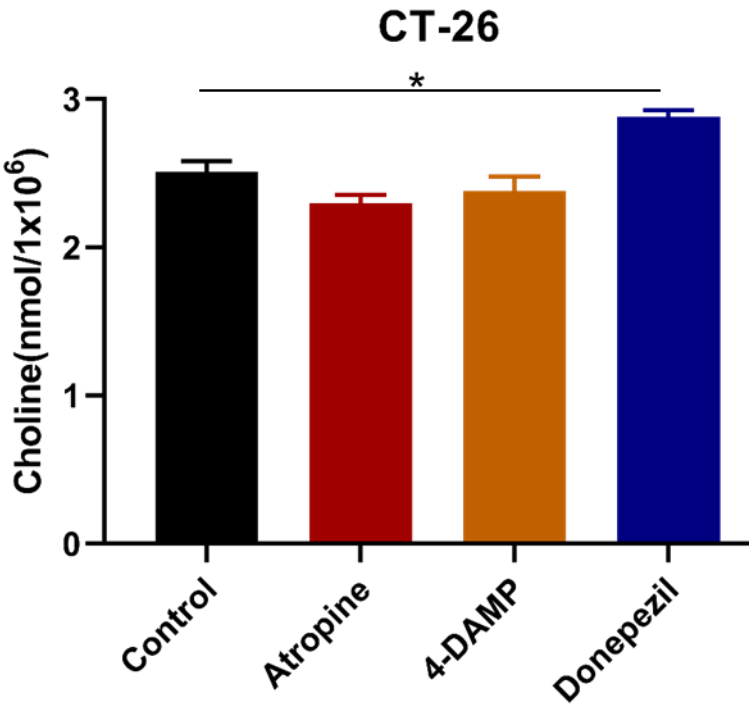
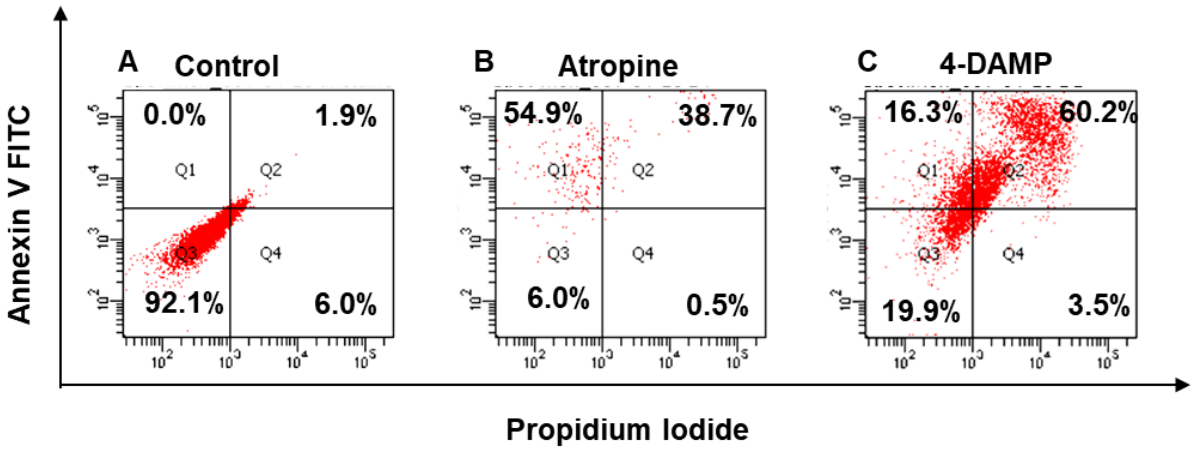

CT-26
*
Choline(nmol/1x10^6)
3
2
1
0
Control
Atropine
4-DAMP
Donepezil

Figure 4.3. Effect of atropine and 4-DAMP on apoptosis of CT-26 cells

Annexin V-FITC/PI staining of CT-26 murine colon cancer cell line treated with control (**A**), atropine (**B**) and 4-DAMP (**C**). Two independent experiments were performed in triplicates.


A Control
0.0%
1.9%
Q1
Q2
Q3
Q4
92.1%
6.0%
B Atropine
54.9%
38.7%
Q1
Q2
Q3
Q4
6.0%
0.5%
C 4-DAMP
16.3%
60.2%
Q1
Q2
Q3
Q4
19.9%
3.5%
Annexin V FITC
Propidium Iodide

4.3.3. Effect of atropine and 4-DAMP on the expression of immunosuppressive and cholinergic markers in an *in vitro* model

4.3.3.1. Effect of atropine and 4-DAMP on PD-L1 and PD-L2 expression in CT-26 cells

In Chapter 3, it was noted that human colon cancer cells overexpressed PD-L1 and PD-L2 when compared to normal epithelial cells, and this was dampened by atropine and 4-DAMP treatment. In this chapter, the effects of atropine and 4-DAMP were determined in murine colon cancer cell line, CT-26. To determine whether atropine and 4-DAMP can influence the expression of PD-L1 and PD-L2, CT-26 cells were pre-treated with 100µM atropine and 4-DAMP for 8hr prior to protein expression via western blot staining. Atropine significantly decreased the expression of PD-L1 (**Figure 4.4A** and **B**). Similarly, atropine attenuated PD-L2 expression compared to control (**Figure 4.4A** and **C**). However, specific blocking of M3R with 4-DAMP had no effect on CT-26 expression of PD-L1 and PD-L2 (**Figure 4.4A-C**).

4.3.3.2. Effect of atropine and 4-DAMP on M3R and ChAT expression in CT-26 cells

Muscarinic receptors expressed by colon cancer cells play in tumour growth and progression. CT-26 cells produce choline, but the expression of ChAT, an enzyme required for ACh synthesis, and M3R were not determined. Here, we assessed the expression of ChAT and M3R in CT-26 cells and the effects of muscarinic receptor blockade on their expression. Western blot was employed to evaluate the effects of atropine and 4-DAMP on CT-26 ability to express M3R and ChAT. The results show that CT-26 cells have prominent expression of M3R and ChAT. Atropine and 4-DAMP treatments significantly reduced M3R expression (**Figure 4.5A** and **B**). Similarly, atropine and 4-DAMP treatment significantly attenuated ChAT expression (**Figure 4.5A** and **C**).

4.3.4. Effect of atropine and 4-DAMP on the phosphorylation of kinases and EGFR activation in CT-26 cells

There is a close link between M3R expression and phosphorylation of AKT and ERK as well as activation of EGFR [587]. Thus, in the present study, the effect of atropine and 4-DAMP on the phosphorylation of AKT and ERK and the activation of EGFR was evaluated in CT-26 cells *in vitro*. The results showed that atropine inhibits the activation of EGFR and suppresses phosphorylation of AKT and ERK in CT-26 cells (**Figure 4.6A-D**). Similarly, 4-DAMP suppresses phosphorylation of AKT and ERK (**Figure 4.6A, C** and **D**), whereas, no significant effect on EGFR was observed (**Figure 4.6A** and **B**). These findings suggest that atropine exhibits its effect through inhibition of EGFR/AKT/ERK pathway, while 4-DAMP exerts its effect via suppression of AKT/ERK signalling pathway.

Figure 4.4. Effect of atropine and 4-DAMP on the expression of immunosuppressive markers in CT-26 cells

Western blot bands for PD-L1 and PD-L2 expression in CT-26 cells treated with atropine and 4-DAMP (**A**). Bar graphs displaying the mean intensity of PD-L1 (**B**) and PD-L2 (**C**) expression in CT-26 cells treated with 4-DAMP and atropine. Data presented as mean ± SEM. Two-*way ANOVA, *p<0.05*.

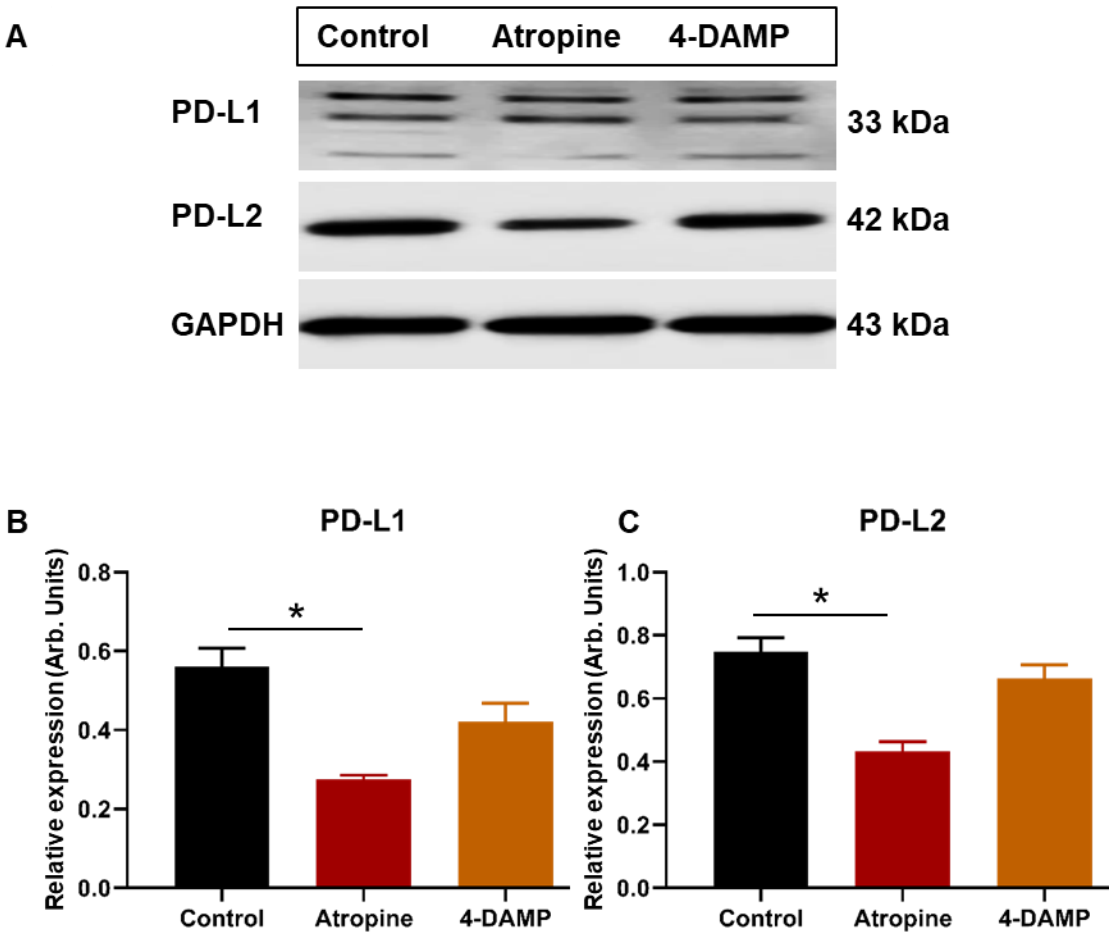

A
Control
Atropine
4-DAMP
PD-L1
33 kDa
PD-L2
42 kDa
GAPDH
43 kDa
B
PD-L1
Relative expression (Arb. Units)
0.0
0.2
0.4
0.6
0.8
*
Control
Atropine
4-DAMP
C
PD-L2
Relative expression (Arb. Units)
0.0
0.2
0.4
0.6
0.8
1.0
*
Control
Atropine
4-DAMP

Figure 4.5. Effect of atropine and 4-DAMP on the expression of cholinergic markers in CT-26 cells

Western blot bands for M3R and ChAT expression in CT-26 cells treated with atropine and 4-DAMP (**A**). Bar graphs displaying the mean intensity of M3R (**B**) and ChAT (**C**) expression in CT-26 cells treated with 4-DAMP and atropine. Data presented as mean ± SEM. Two-*way ANOVA, *p<0.05, **p<0.01*.

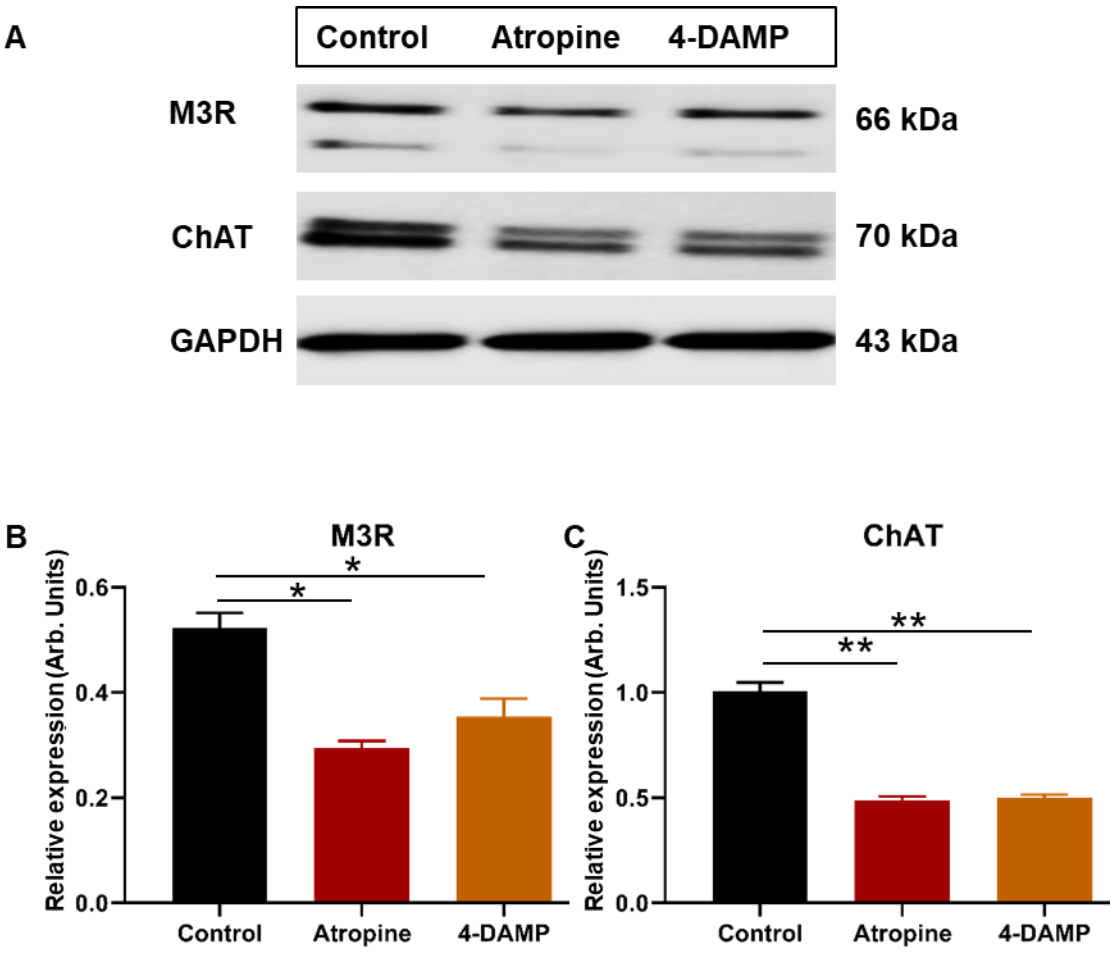
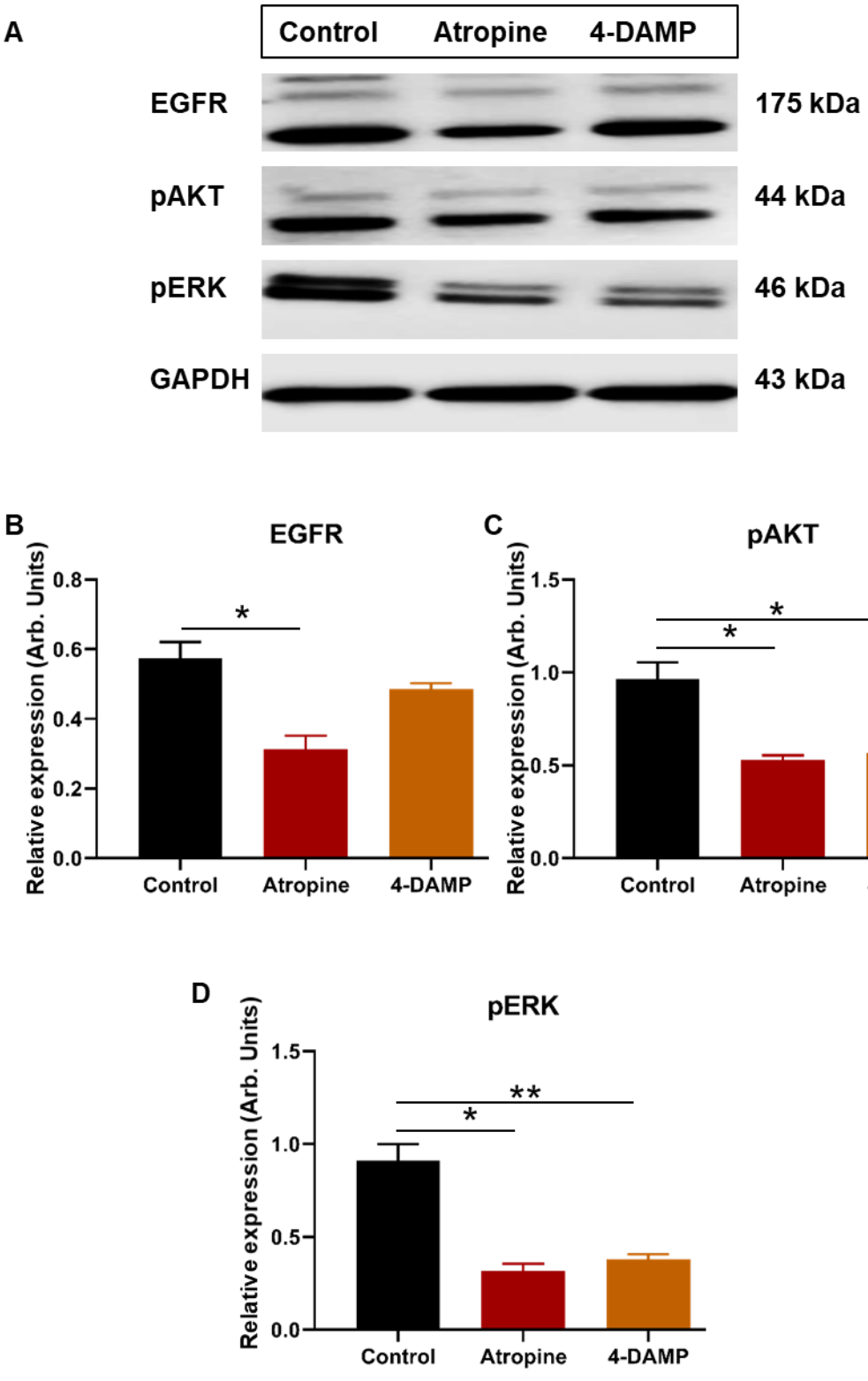

A
Control
Atropine
4-DAMP
M3R
66 kDa
ChAT
70 kDa
GAPDH
43 kDa
B
M3R
Relative expression (Arb. Units)
0.6
0.4
0.2
0.0
*
*
Control
Atropine
4-DAMP
C
ChAT
Relative expression (Arb. Units)
1.5
1.0
0.5
0.0
**
**
Control
Atropine
4-DAMP

Figure 4.6. Effect of atropine and 4-DAMP on the expression of protein kinases in CT-26 cells

Western blot bands for EGFR, pAKT and pSTAT3 expression in CT-26 cells treated with atropine and 4-DAMP (**A**). Bar graphs displaying the mean intensity of EGFR (**B**), pAKT (**C**) and pERK (**D**) expression in CT-26 cells treated with 4-DAMP and atropine. Data presented as mean ± SEM. Two-*way ANOVA, *p<0.05, **p<0.01*.


A
Control
Atropine
4-DAMP
EGFR
175 kDa
pAKT
44 kDa
pERK
46 kDa
GAPDH
43 kDa
B
EGFR
Relative expression (Arb. Units)
0.8
0.6
0.4
0.2
0.0
*
Control
Atropine
4-DAMP
C
pAKT
Relative expression (Arb. Units)
1.5
1.0
0.5
0.0
*
*
Control
Atropine
4-DAMP
D
pERK
Relative expression (Arb. Units)
1.5
1.0
0.5
0.0
**
*
Control
Atropine
4-DAMP

4.3.5. Effect of 4-DAMP on tumour growth *in vivo* in an orthotopic mouse model CRC

Mouse CT-26 colon cancer cells can form fast-growing and extremely vascularised tumours, which make it useful for assessing the effects of therapeutic agents and their mechanisms of action [595]. To assess whether blocking M3R influences CRC tumour growth, CT-26 cells were implanted into the mouse caecum wall. Five days post-surgery, tumour-bearing mice were injected with either DMSO (vehicle solution), or 4-DAMP intraperitoneally daily for 28 days. Mice were culled, tumours removed, and tumour weight, size and volume were measured. In addition, tumours around the caecum were counted and collected. 4-DAMP significantly attenuated tumour size compared to DMSO treatment (**Figure 4.7A** and **B**). There was a significant reduction of tumour weight in the 4-DAMP-treated compared to DMSO-treated mice (**Figure 4.7C**). In addition, treatment with 4-DAMP significantly decreased tumour volume compared to DMSO (**Figure 4.7D**). Furthermore, tumour-bearing mice treated with DMSO had more polyps and invasive tumours around the caecum compared with 4-DAMP-treated mice (**Figure 4.8A-C**, difference between values (Δ): -22.00±4.40, $p<0.001$).

Figure 4.7. Effect of 4-DAMP on tumour growth *in vivo*

Images of tumour size from DMSO-treated group (**A**) and 4-DAMP-treated group (**B**). Bar graphs displaying the mean weight (**C**) and volume (**D**) of tumours collected from DMSO and 4-DAMP-treated groups. Data presented as mean ± SEM, n=8 mice per group. Student's *t-test, ****p<0.0001*.

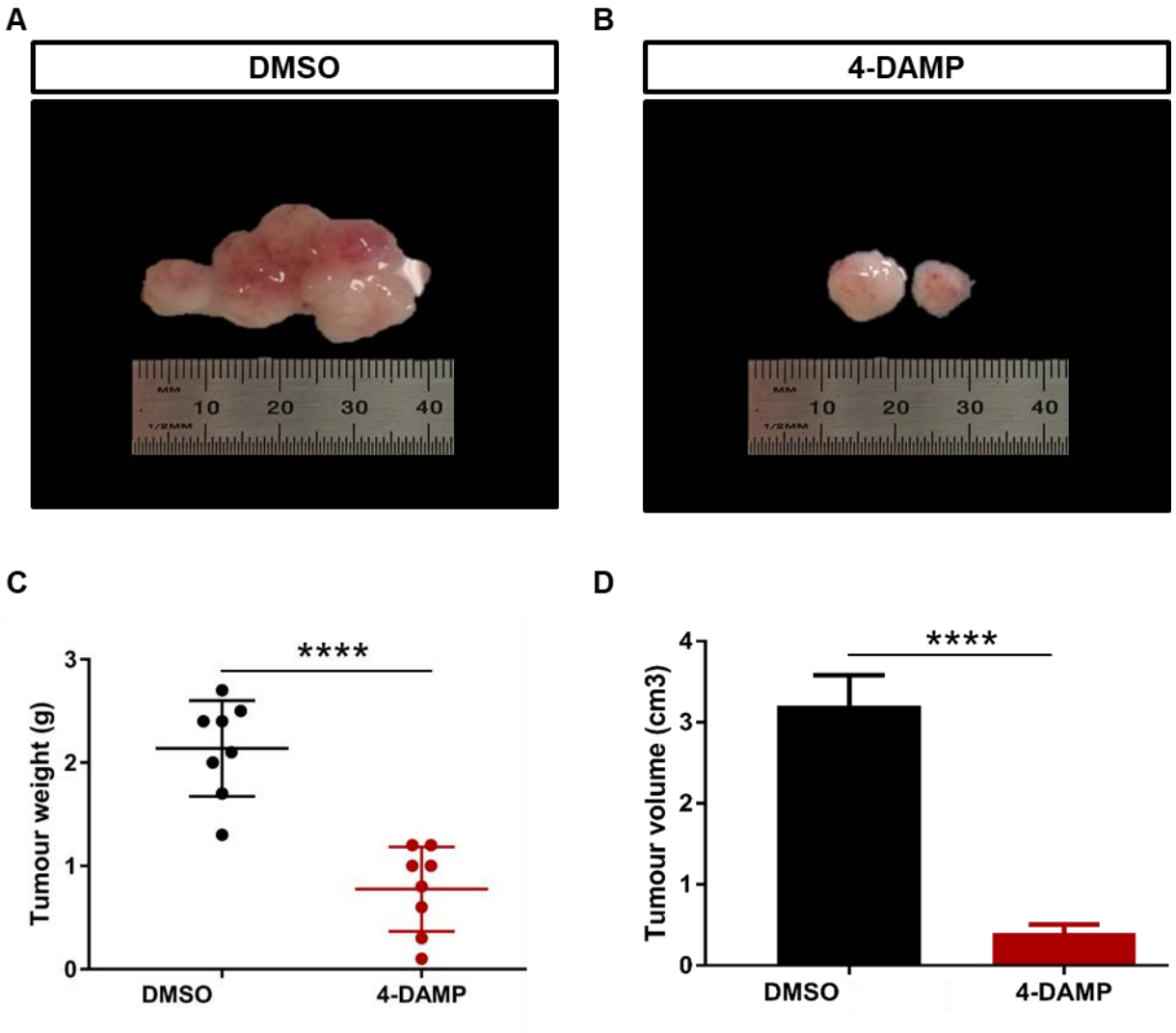
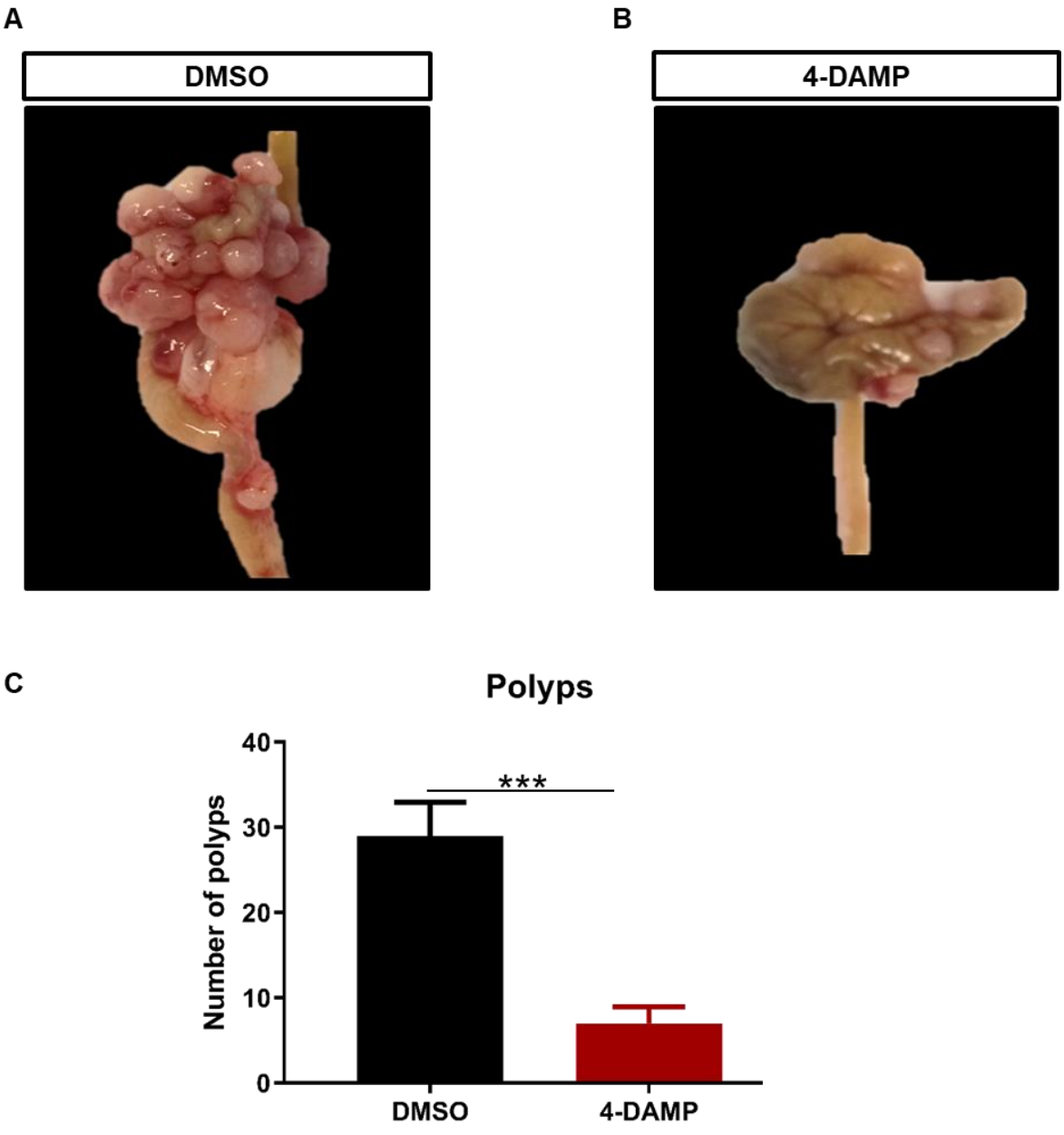

A
DMSO
B
4-DAMP
C
Tumour weight (g)
****
DMSO
4-DAMP
D
Tumour volume (cm3)
****
DMSO
4-DAMP

Figure 4.8. Effect of 4-DAMP on tumour polyps' formation

The caecum samples removed from tumour-bearing mice treated with DMSO (**A**) and 4-DAMP (**B**). Bar graph displaying the mean number of tumour polyps from DMSO and 4-DAMP-treated groups (**C**). Data presented as mean ± SEM, n=8 mice per group. Student's *t-test, ***p<0.001*.


A
DMSO
B
4-DAMP
C
Polyps
Number of polyps
40
30
20
10
0
***
DMSO
4-DAMP

4.3.6. Expression of immunosuppressive and cholinergic markers in tumours from an *in vivo* model of CRC

4.3.6.1. Expression of immunosuppressive markers *in vivo*

Studies identifying the role of PD-L1 expression in CRC have been controversial. Some studies associate PD-L1 expression with poor prognosis, whereas others with good prognosis [48, 392, 516]. Infiltration of immune cells within the tumour microenvironment has been highly implicated in the disease progression and prognosis. In addition, the expression of PD-L1 inversely correlates with FOXP3 in tumour samples from CRC patients [596]. Hence, PD-L1 and FOXP3, a marker used to label the regulatory T cells, were both evaluated in tumour samples from mice with CT-26 cell-induced CRC. The results demonstrated that *in vivo* treatment of tumour-bearing mice with M3R blocker, 4-DAMP, significantly reduced the expression of PD-L1 compared to DMSO-treated controls (**Figure 4.9A'**, **B' C** and **E**, Δ**:** -22.88±0.80, $p<0.0001$). These results concur with findings that FOXP3 inversely correlated with PD-L1 expression (**Figure 4.9A'', B'' D** and **E**, Δ**:** 15.00±1.16, $p<0.0001$).

In addition, the expression of PD-L2 was also evaluated. Although PD-L2 has a similar function to that of PD-L1, there was no significant difference noted between the expression of PD-L2 in tumours from 4-DAMP-treated and DMSO-treated mice (**Figure 4.10**, Δ**:** 0.38.88±0.82, $p=0.6553$). These findings were further confirmed by western blot. The results obtained from *in vitro* model demonstrated that 4-DAMP attenuated PD-L2; however, this was not confirmed *in vivo*. These findings reinforced the influence of tumour microenvironment on the expression of immunosuppressive markers.

4.3.6.2. Correlation of PD-L1 expression with cholinergic markers

It was hypothesised that the neurotransmitters, in particularly ACh, might play a significant role in the induction of immunosuppressive markers such as PD-L1. *In vitro* results of studies in human colon cancer cells described in Chapter 3 and in murine CT-26 cell lines, described in this Chapter, confirmed this hypothesis. To further evaluate this hypothesis *in vivo*, tumour-bearing mice implanted with CT-26 cells were treated with either DMSO

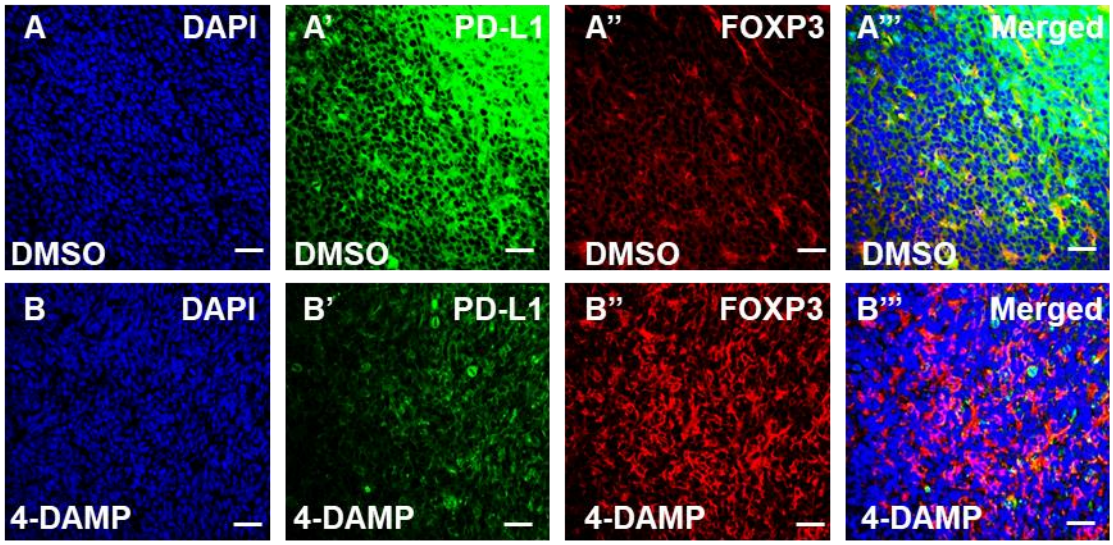
or 4-DAMP administered intraperitoneally daily for 28 days. PD-L1 was co-labelled with ChAT, a cholinergic enzyme crucial for ACh synthesis, and VAChT, a vesicular ACh transporter essential for packaging of ACh into vesicles. Results demonstrated that 4-DAMP treatment significantly reduced the expression of PD-L1 (**Figure 4.11A'**, **B'** and **C**, **Δ:** -24.56±3.15, *p<0.0001*) as well as the expression of ChAT compared to DMSO treatment (**Figure 4.11A''**, **B''** and **D**, **Δ:** -10.23±2.27, *p<0.001*). In addition, VAChT expression was attenuated in tumours from 4-DAMP-treated mice compared to DMSO-treated mice (**Figure 4.11A'''**, **B'''** and **E**, **Δ:** -5.82±0.91, *p<0.05*). Furthermore, PD-L1 was co-localised with ChAT and VAChT in tumours from DMSO-treated mice; however, after 4-DAMP treatment, this co-localisation was abolished. This reinforces the interaction between PD-L1 and cholinergic markers in CRC.

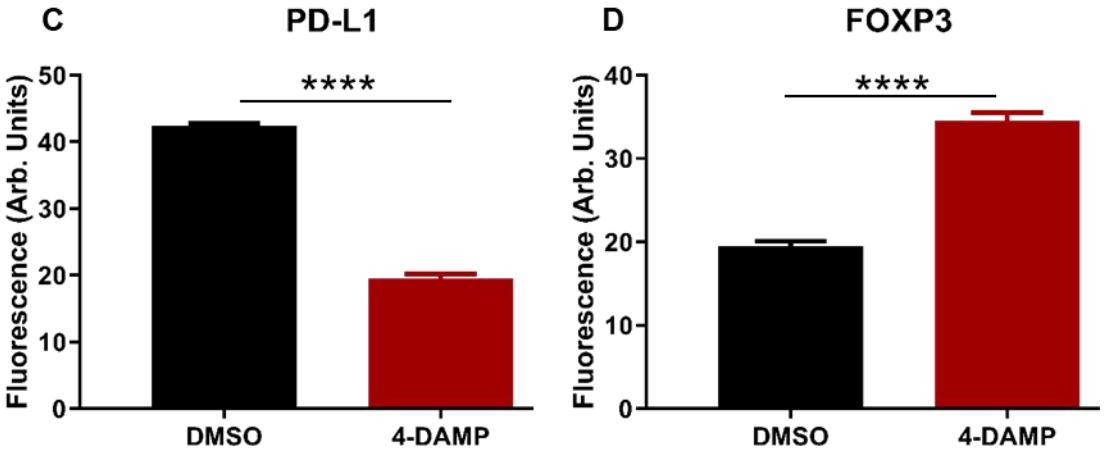
4.3.6.3. Expression of cholinergic markers *in vivo*

Although both ChAT and VAChT have been implicated in CRC, several studies have reported the important role of ACh receptors, especially M3R, in CRC progression. ACh binds to muscarinic M3R and nicotinic α7nAChR stimulating CRC cell proliferation, angiogenesis, tumour growth and metastasis [463, 587, 593, 594]. In this study, ACh receptors were co-labelled with ChAT in tumours from DMSO and 4-DAMP-treated mice. 4-DAMP treatment induced a significant increase in α7nAChR expression (**Figure 4.12A'**, **B', C** and **F, Δ:** 6.38±0.34, *p<0.0001*), however, the underlining mechanisms for this augmentation are unclear. On the other hand, 4-DAMP treatment induced a significant reduction in M3R expression (**Figure 4.12A''**, **B''** and **D, Δ:** -7.38±0.31, *p<0.0001*) as well as ChAT expression compared to DMSO treatment (**Figure 4.12A'''**, **B'''** and **E, Δ:** -12.50±0.62, *p<0.0001*). These findings were further confirmed by western blot (**Figure 4.12F**).

Figure 4.9. Effect of 4-DAMP on the expression of PD-L1 and FOXP3 *in vivo*

Intensity of PD-L1 and FOXP3 in tumour samples from mice bearing-CT-26 cell-induced CRC treated with DMSO (**A-A'''**) and 4-DAMP (**B-B'''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), PD-L1 (green; **A'-B'**), FOXP3 (red; **A''-B''**) and all markers merged (yellow; **A'''-B'''**). Scale bar represents 50µm. Bar graphs displaying the mean fluorescence of PD-L1 (**C**), FOXP3 (**D**) and images of western blot bands (**E**) in tumour samples from DMSO-treated and 4-DAMP-treated mice. Data presented as mean ± SEM, n=8 mice per group. Student's *t-test, ****p<0.0001*.


A
DAPI
DMSO
A'
PD-L1
DMSO
A''
FOXP3
DMSO
A'''
Merged
DMSO
B
DAPI
4-DAMP
B'
PD-L1
4-DAMP
B''
FOXP3
4-DAMP
B'''
Merged
4-DAMP


C
PD-L1
Fluorescence (Arb. Units)
50
40
30
20
10
0
****
DMSO
4-DAMP
D
FOXP3
Fluorescence (Arb. Units)
40
30
20
10
0
****
DMSO
4-DAMP

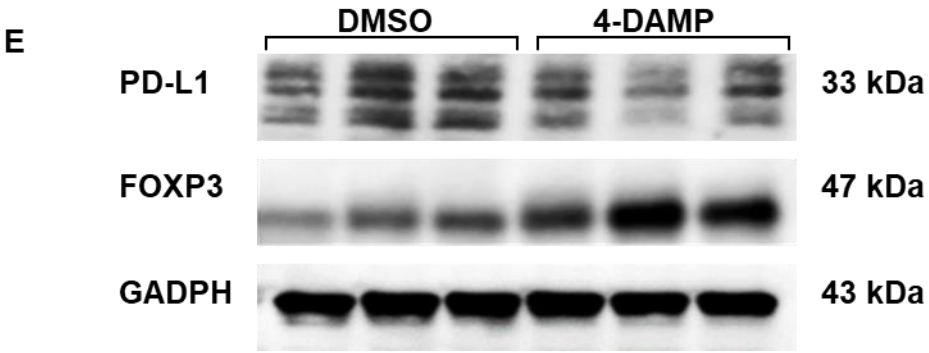
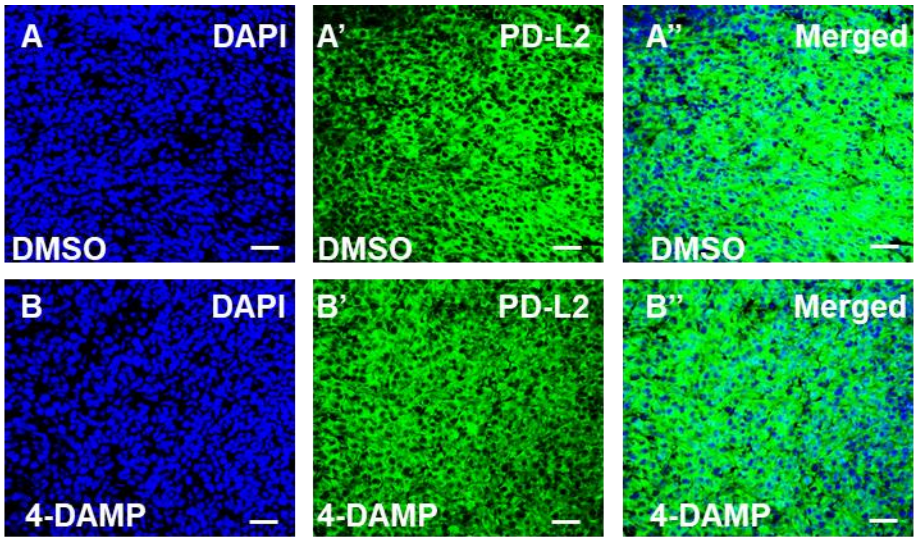
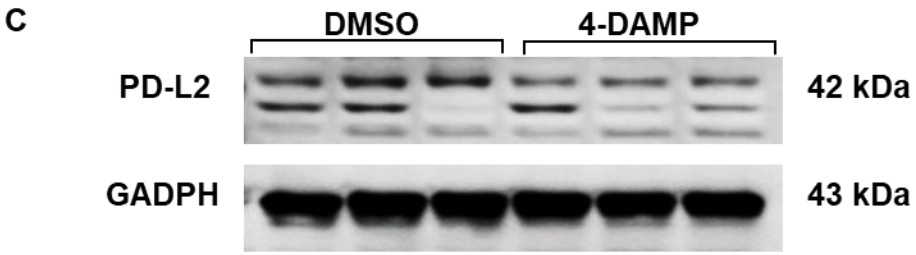

E
DMSO
4-DAMP
PD-L1
33 kDa
FOXP3
47 kDa
GADPH
43 kDa

Figure 4.10. Effect of 4-DAMP on the expression of PD-L2 in mice bearing CT-26 cell-induced tumours

PD-L2 expression in tumour samples from mice bearing-CT-26 cell-induced CRC treated with DMSO (**A-A''**) and 4-DAMP (**B-B''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), PD-L2 (green; **A'-B'**) and all markers merged (**A''-B''**). Scale bar represents 50µm. Western blot bands for PD-L2 expression (**C**) in tumour samples from DMSO-treated and 4-DAMP-treated mice. Bar graphs displaying the PD-L2 mean fluorescence intensity (**D**) and western blot expression level (**E**) in tumour samples from DMSO-treated and 4-DAMP-treated groups. Data presented as mean ± SEM, n=8 mice per group. Student's *t-test*.


A
DAPI
DMSO
A'
PD-L2
DMSO
A''
Merged
DMSO
B
DAPI
4-DAMP
B'
PD-L2
4-DAMP
B''
Merged
4-DAMP


C
DMSO
4-DAMP
PD-L2
42 kDa
GADPH
43 kDa

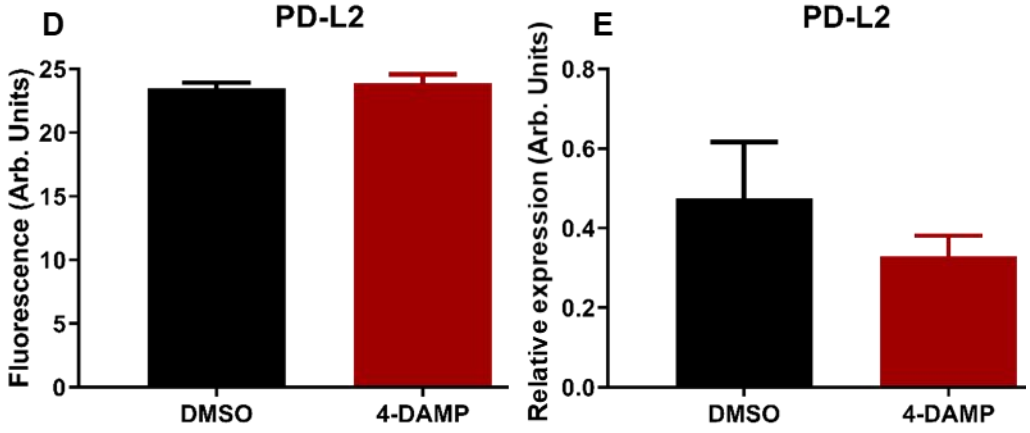
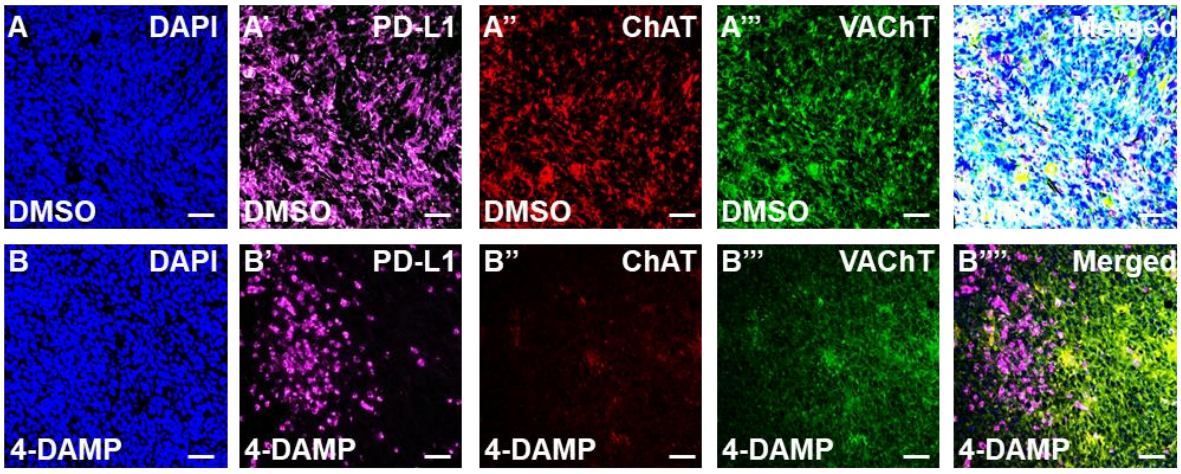

D
PD-L2
Fluorescence (Arb. Units)
25
20
15
10
5
0
DMSO
4-DAMP
E
PD-L2
Relative expression (Arb. Units)
0.8
0.6
0.4
0.2
0.0
DMSO
4-DAMP

Figure 4.11. Correlation of PD-L1 expression with cholinergic markers in tumour samples from mice bearing CT-26 cell-induced tumours

Expression of PD-L1 and cholinergic markers (ChAT and VAChT) in tumour samples from mice bearing CT-26 cell-induced CRC treated with DMSO (**A-A’’’’**) and 4-DAMP (**B-B’’’’**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), PD-L1 (magenta; **A’-B’**), ChAT (red; **A’’-B’’**), VAChT (green; **A’’’-B’’’**) and all markers merged (**A’’’’-B’’’’**). Scale bar represents 50µm. Bar graphs displaying the mean fluorescence of PD-L1 (**C**), ChAT (**D**) and VAChT (**E**) in tumour samples from DMSO-treated and 4-DAMP-treated mice. Data presented as mean ± SEM, n=8 mice per group. Student’s *t-test, *p<0.05, ***p<0.001, ****p<0.0001*.


A
DAPI
DMSO
A'
PD-L1
DMSO
A''
ChAT
DMSO
A'''
VAChT
DMSO
Merged
B
DAPI
4-DAMP
B'
PD-L1
4-DAMP
B''
ChAT
4-DAMP
B'''
VAChT
4-DAMP
B''''
Merged
4-DAMP

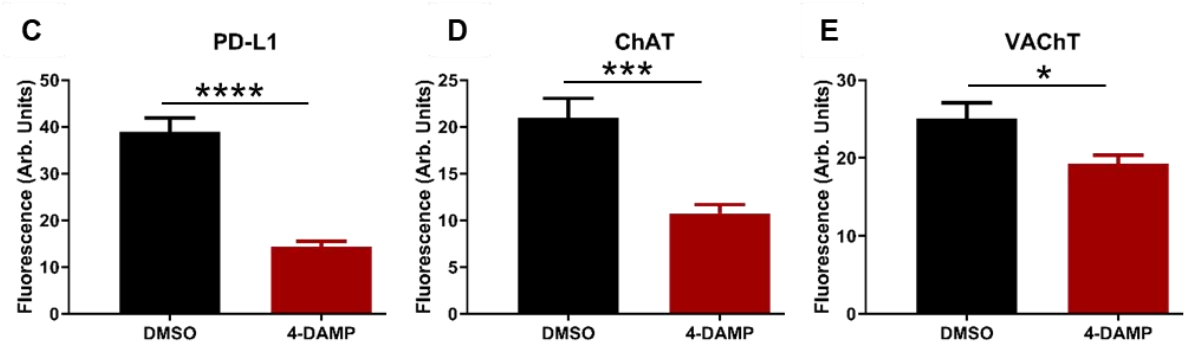
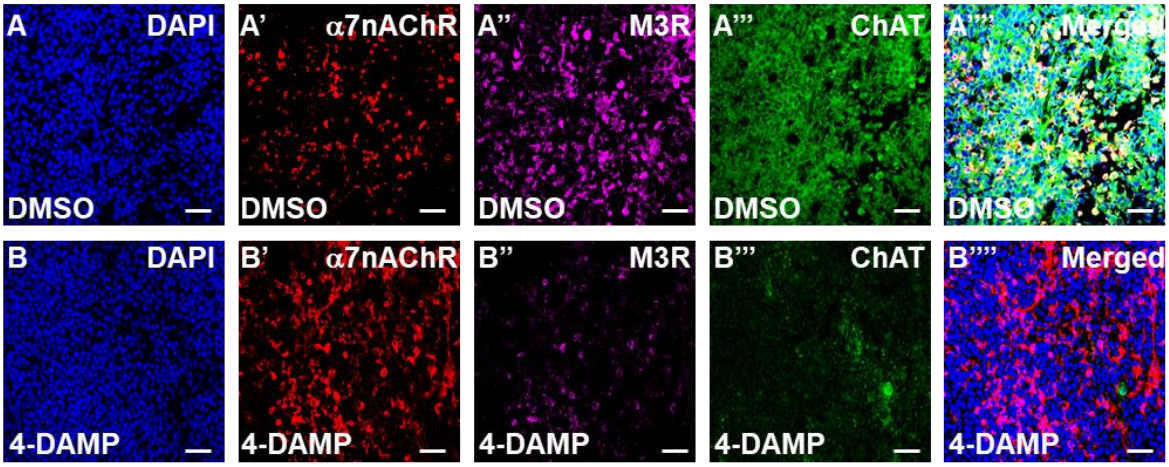
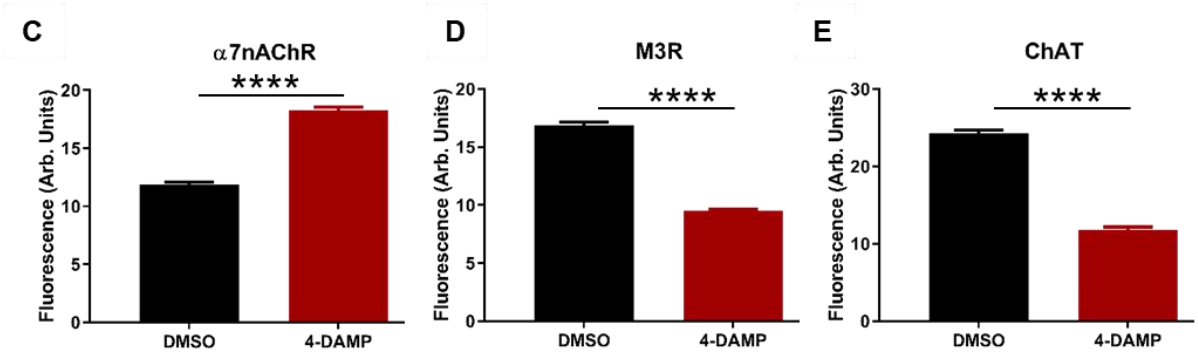
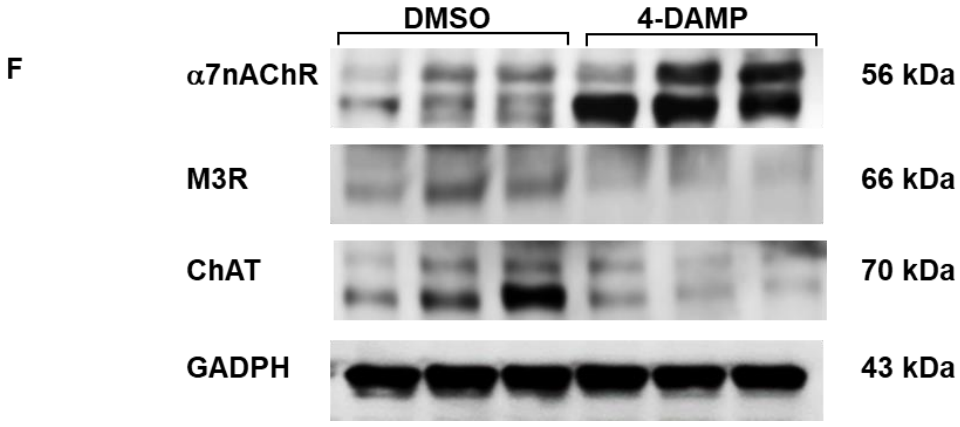

C
PD-L1
****
Fluorescence (Arb. Units)
50
40
30
20
10
0
DMSO
4-DAMP
D
ChAT
***
Fluorescence (Arb. Units)
25
20
15
10
5
0
DMSO
4-DAMP
E
VAChT
*
Fluorescence (Arb. Units)
30
20
10
0
DMSO
4-DAMP

Figure 4.12. Expression of cholinergic markers in tumour samples from mice bearing CT-26 cell-induced tumours

Expression of cholinergic markers in tumour samples from mice bearing CT-26 cell-induced CRC treated with DMSO (**A-A’’’’**) and 4-DAMP (**B-B’’’’**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), α7nAChR (red; **A’-B’**), M3R (magenta; **A’’-B’’**), ChAT (green; **A’’’-B’’’**) and all markers merged (**A’’’’-B’’’’**). Scale bar represents 50µm. Bar graphs displaying the mean fluorescence of α7nAChR (**C**), M3R (**D**), ChAT (**E**) and western blot bands (**F**) in tumour samples from DMSO-treated and 4-DAMP-treated mice. Data presented as mean ± SEM, n=8 mice per group. Student’s *t-test, ****p<0.0001*.


A
DAPI
DMSO
A'
α7nAChR
DMSO
A''
M3R
DMSO
A'''
ChAT
DMSO
A''''
Merged
DMSO
B
DAPI
4-DAMP
B'
α7nAChR
4-DAMP
B''
M3R
4-DAMP
B'''
ChAT
4-DAMP
B''''
Merged
4-DAMP


C
α7nAChR
****
Fluorescence (Arb. Units)
20
15
10
5
0
DMSO
4-DAMP
D
M3R
****
Fluorescence (Arb. Units)
20
15
10
5
0
DMSO
4-DAMP
E
ChAT
****
Fluorescence (Arb. Units)
30
20
10
0
DMSO
4-DAMP


F
DMSO
4-DAMP
α7nAChR
56 kDa
M3R
66 kDa
ChAT
70 kDa
GADPH
43 kDa

4.3.7. Effect of 4-DAMP on tumour-infiltrating immune cells

The prognostic value of PD-L1 or other immune checkpoint inhibitors is influenced by the profile of infiltrating immune cells within the tumour microenvironment. Therefore, to determine the profile of tumour-infiltrating immune cells, fresh tumours from mice with CRC were collected into RPMI media. Tumours were then mechanically dissected into small pieces and incubated at 37˚C for 1hr with collagenase before commencing labelling with antibodies of interest as depicted in the Material and Methods. To characterise leukocyte populations in tumour samples from DMSO and 4-DAMP treated mice, flow cytometry was used. From scatter plots, only viable cells were analysed, and compensation was performed (when applicable) to prevent false-positive/false-negative results (**Figure 4.13A-B**). The gating strategy for CD45+ cells was defined from single-cell doublets (**Figure 4.13C-D**). Lymphocyte populations were gated from CD45+ cells (**Figure 4.13E-F**; **G-H**). No significant differences were observed in CD45+ cells in tumours from both DMSO and 4-DAMP-treated mice (**Figure 4.14A**, **Δ:** 2.17±6.47, *p=0.7428*). However, the results showed that 4-DAMP treatment significantly increased CD4+ T cell infiltration compared to DMSO treatment (**Figure 4.14B**, **Δ:** 4.58±0.88, *p<0.001*). Similarly, CD8+ T cell infiltration was significantly increased in tumours from 4-DAMP-treated compared to DMSO-treated mice (**Figure 4.14C**, **Δ:** 8.55±2.13, *p<0.01*). Moreover, there was no significant differences noted in CD4+/CD8+ T cells ratio in tumours from 4-DAMP-treated compared to DMSO-treated mice (**Figure 4.14D**, **Δ:** 0.79±2.16, *p=0.2315*). Conversely, 4-DAMP treatment significantly attenuated infiltration of γδ T cells compared to DMSO (**Figure 4.14E**, **Δ:** -17.02±1.067, *p<0.0001*).

Furthermore, infiltration of B cells and eosinophils was gated from CD45+ cells. Results showed that the proportions of infiltrating B cells and eosinophils were significantly reduced in 4-DAMP-treated compared to DMSO-treated mice (**Figure 4.15A-B**). The proportions of all macrophages (defined as M0) and M2 phenotype macrophages were statistically increased in 4-DAMP-treated compared to DMSO-treated mice; however, M2 macrophages presented only a small fraction of all infiltrating M0 macrophages (**Figure 4.15 C**). This suggests that most of the M0 macrophages could be exhibiting M1 phenotypes, which have an anti-tumour effect. In regards to neutrophil infiltration, as there

are no specific markers to distinguish between N1 and N2, overall neutrophil infiltration was evaluated. Neutrophil infiltration was enhanced in tumours from 4-DAMP-treated compared to DMSO-treated mice (**Figure 4.15D**). We speculate that these infiltrated neutrophils are N1 phenotype as they exert an anti-tumour effect.

Figure 4.13. Gating strategy for tumour-infiltrating leukocytes from DMSO and 4-DAMP treated mice

Gating strategy for debris exclusion (**A**), viable cells (**B**), single cell doublets discrimination (**C**), single cells (**D**), CD45-positive cells from DMSO-treated group (**E**) and T lymphocytes from DMSO-treated group (**F**), CD45-positive cells from 4-DAMP-treated group (**G**) and T lymphocytes from 4-DAMP-treated group (**H**).

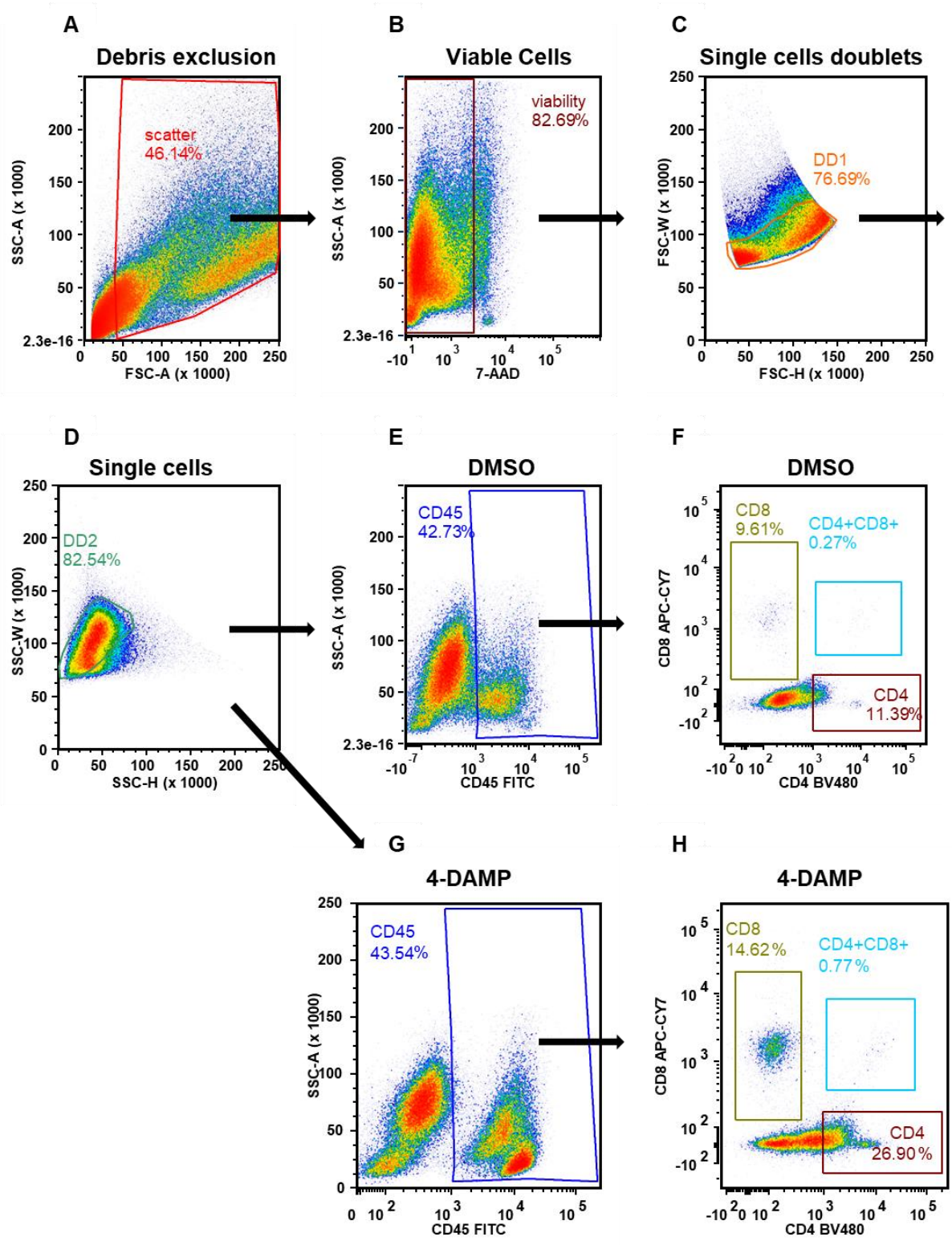

A
Debris exclusion
scatter
46.14%
SSC-A (x 1000)
FSC-A (x 1000)
B
Viable Cells
viability
82.69%
SSC-A (x 1000)
7-AAD
C
Single cells doublets
DD1
76.69%
FSC-W (x 1000)
FSC-H (x 1000)
D
Single cells
DD2
82.54%
SSC-W (x 1000)
SSC-H (x 1000)
E
DMSO
CD45
42.73%
SSC-A (x 1000)
CD45 FITC
F
DMSO
CD8
9.61%
CD4+CD8+
0.27%
CD4
11.39%
CD8 APC-CY7
CD4 BV480
G
4-DAMP
CD45
43.54%
SSC-A (x 1000)
CD45 FITC
H
4-DAMP
CD8
14.62%
CD4+CD8+
0.77%
CD4
26.90%
CD8 APC-CY7
CD4 BV480

Figure 4.14. Flow cytometric analysis of CD45+ cells and T lymphocytes

Proportion of the CD45+ cells (**A**), CD4+ T cells (**B**), CD8+ T cells (**C**), CD4+/CD8+ T cells ratio (**D**) and $\gamma\delta$ T cells (**E**). Data presented as mean ± SEM, DMSO-treated (n=4), 4-DAMP-treated (n=7) mice per group. Student's *t-test*, ***p<0.01, ***p<0.001, ****p<0.0001*.

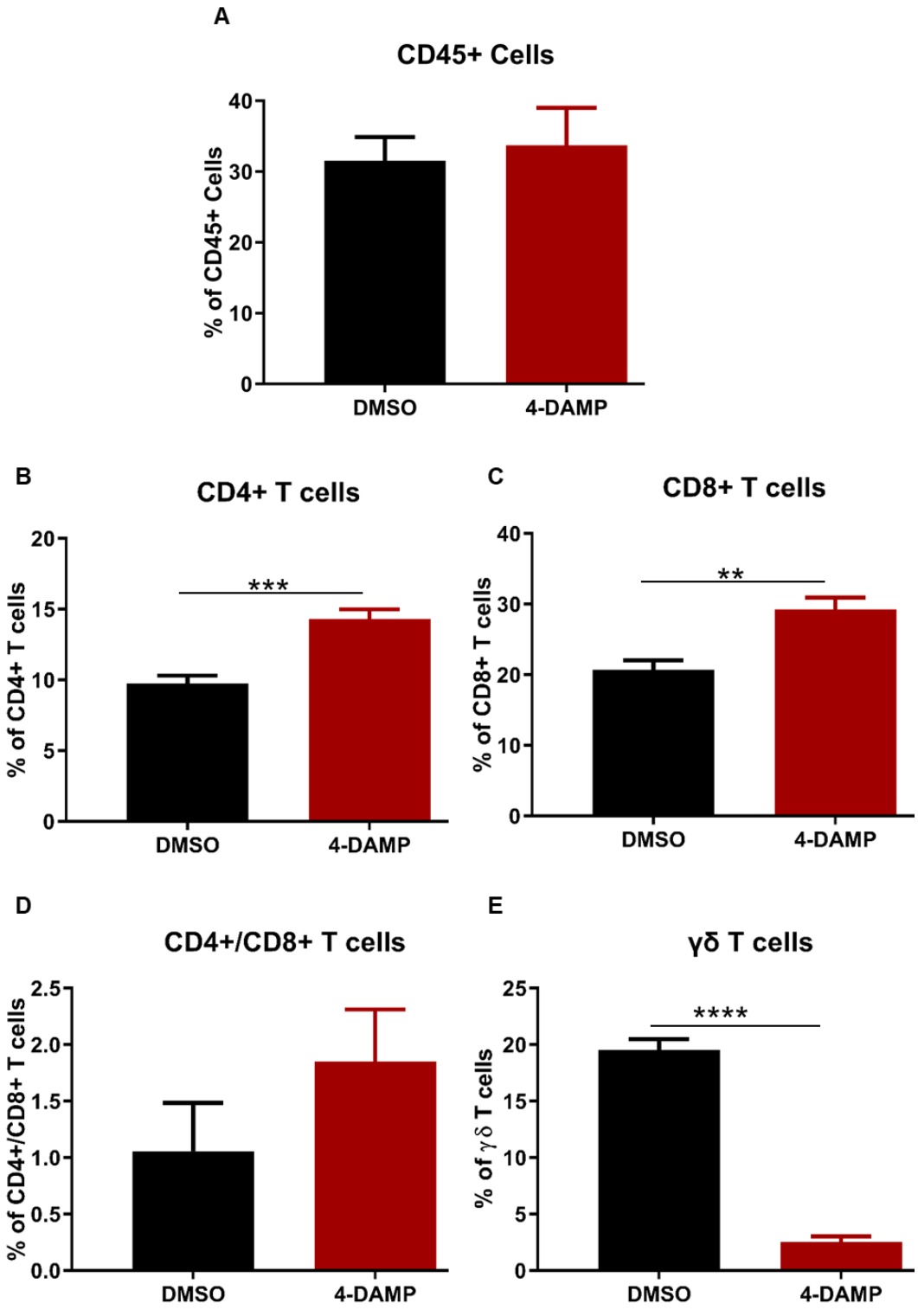
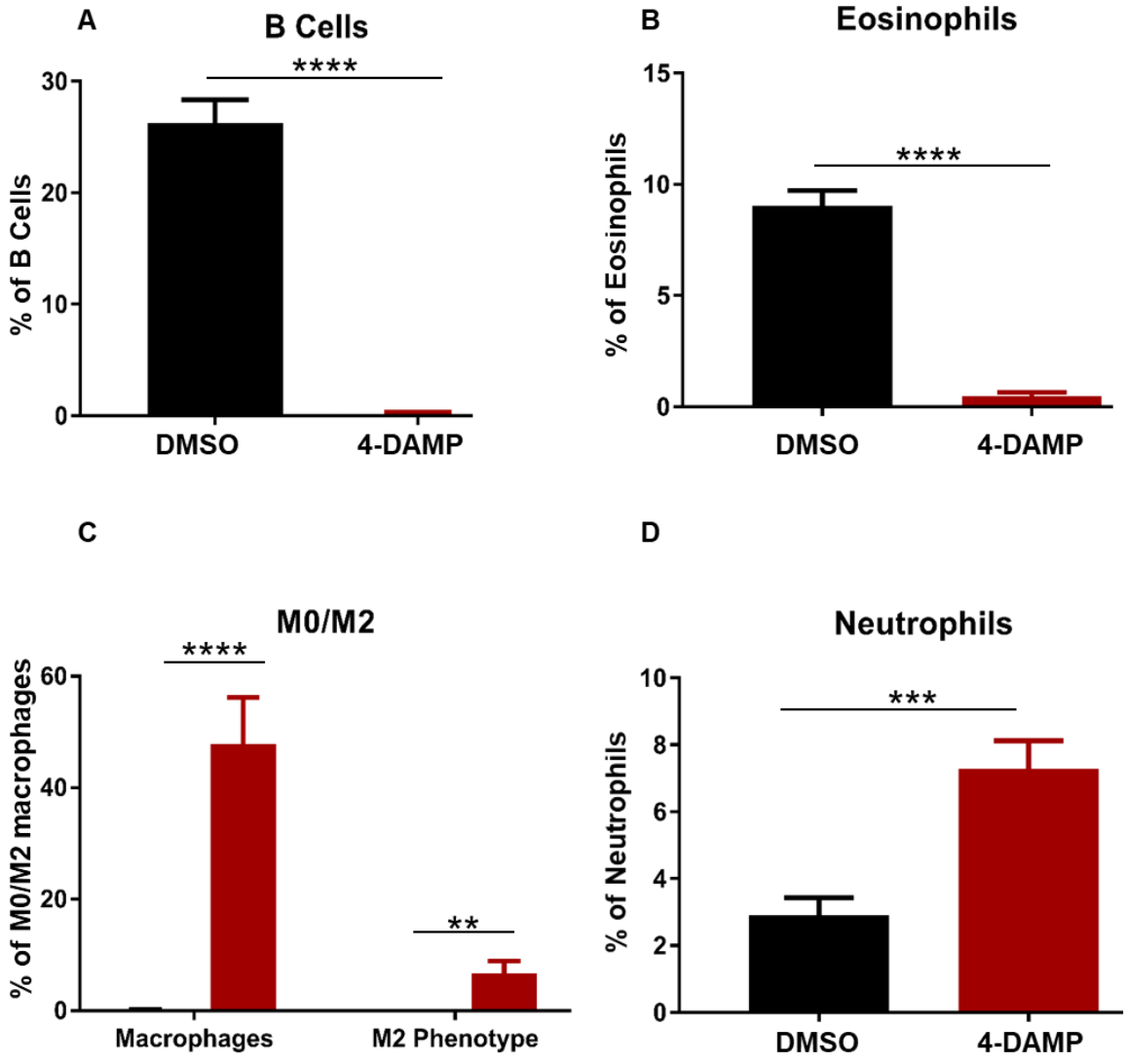

A
CD45+ Cells
% of CD45+ Cells
40
30
20
10
0
DMSO
4-DAMP
B
CD4+ T cells
% of CD4+ T cells
20
15
10
5
0
***
DMSO
4-DAMP
C
CD8+ T cells
% of CD8+ T cells
40
30
20
10
0
**
DMSO
4-DAMP
D
CD4+/CD8+ T cells
% of CD4+/CD8+ T cells
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
4-DAMP
E
γδ T cells
% of γδ T cells
25
20
15
10
5
0
****
DMSO
4-DAMP

Figure 4.15. Flow cytometric analysis of tumour-associated immune cells

Proportion of B cells (**A**), eosinophils (**B**), macrophages (**C**) and neutrophils (**D**) in tumours from DMSO-treated and 4-DAMP-treated groups. Data presented as mean ± SEM, DMSO-treated (n=4), 4-DAMP-treated (n=7) mice per group. Student's *t-test*, ****p<0.001, ****p<0.0001*.


A
B Cells
****
% of B Cells
30
20
10
0
DMSO
4-DAMP
B
Eosinophils
****
% of Eosinophils
15
10
5
0
DMSO
4-DAMP
C
M0/M2
****
**
% of M0/M2 macrophages
60
40
20
0
Macrophages
M2 Phenotype
D
Neutrophils
***
% of Neutrophils
10
8
6
4
2
0
DMSO
4-DAMP

4.3.8. Effect of blocking M3R on tumour angiogenesis

Tumour angiogenesis is one of the critical steps in cancer growth and metastasis [398, 510, 580, 597]. To evaluate the effect of blocking M3R on tumour angiogenesis, the expression of angiogenic markers, VEGF and CD31, was evaluated. The results demonstrated that administration of 4-DAMP suppressed the expression of VEGF (**Figure 4.16A' B', C** and **E**; Δ: -16.75±1.60, *p<0.0001*). CD31 is a transmembrane glycoprotein expressed by endothelial cells and is used as a specific endothelial marker in paraffin sections. Thus, tumour neovascularisation was evaluated in mice bearing-CT-26 tumours by immunohistochemical labelling tumours with anti-CD31 antibody in both freshly-fixed tumours. Treatment with 4-DAMP attenuated the expression of CD31 (**Figure 4.16A'' B'', D**; Δ: -13.85±1.10, *p<0.0001*). There was more intense CD31 staining observed in freshly-fixed tumours from DMSO compared with 4-DAMP-treated mice, supporting the immunofluorescence results in freshly-fixed samples (**Figure 4.17**).

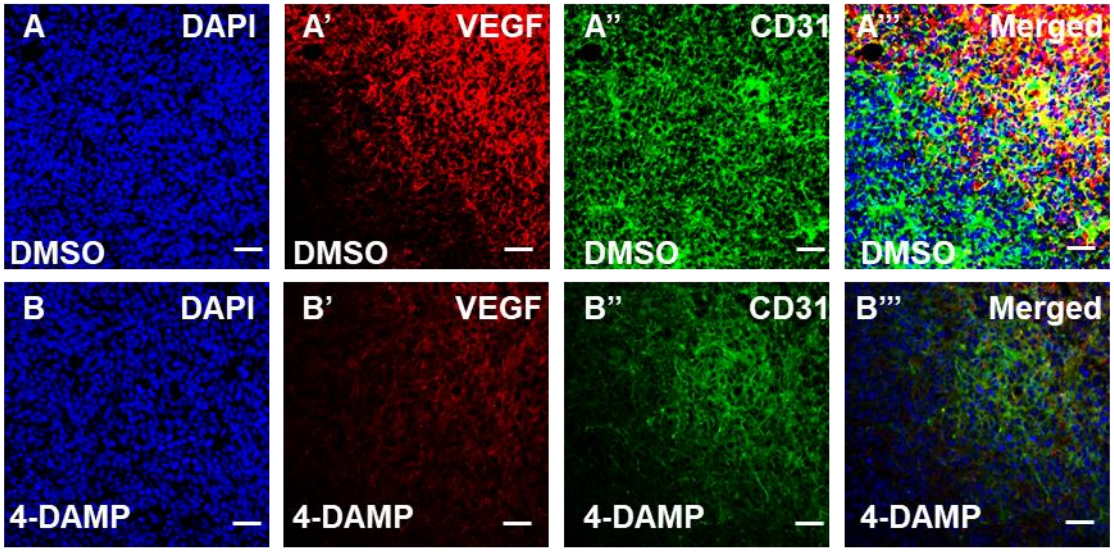
In addition, tyrosine kinases in tumour tissue lysates were evaluated using mouse Phospho-RTK Array. Tumour samples from each group were pooled, and the expression level of kinases was measured as described in the Materials and Methods. The results showed that DMSO group overexpressed platelet-derived growth factor receptor α (PDGFRα) and Axl tyrosine kinases (**Figure 4.18A**), both of which are involved in cancer cell proliferation, invasion, angiogenesis and migration. Treatment with 4-DAMP significantly reduced expression levels of PDGFRα and abolished Axl (**Figure 4.18B**). Although TGF-β is not a marker for tumour angiogenesis per se, studies have demonstrated a strong association between tumour expression of TGF-β and tumour angiogenesis [598, 599]. Administration of 4-DAMP significantly reduced TGF-β expression compared to DMSO treatment (**Figure 4.19**, Δ: -13.85±1.03, *p<0.0001*). Taken together, these findings reinforced the important role of ACh in tumour angiogenesis, and M3 receptors could be a potential therapeutic target for inhibition of angiogenesis.

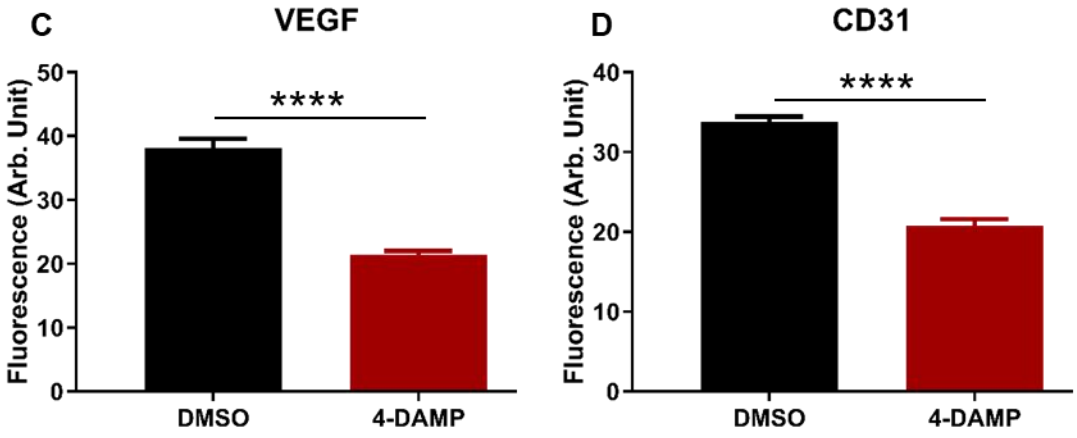
4.3.9. Effect of 4-DAMP treatment on phosphorylation of AKT and ERK *in vivo*

ACh acting on M3R has been shown to trigger the activation of EGFR signalling to persuade the phosphorylation of AKT and $ERK_{1/2}$ [587]. *In vitro* study of normal epithelial and human colon cancer cells presented in Chapter 3, 4-DAMP exerts it effects through suppression of EGFR, pAKT, pSTAT3 and pERK signalling pathways. To determine the effect of blocking muscarinic receptor 3 with 4-DAMP on the phosphorylation of EGFR, STAT3, AKT and ERK *in vivo*, western blot was used. The results demonstrated that 4-DAMP treatment inhibited the phosphorylation of ERK compared to DMSO (**Figure 4.20A** and **C**; Δ: -0.25±0.07, p<*0.05*). Similarly, 4-DAMP treatment induced a significant reduction in phosphorylation of AKT when compared to DMSO (**Figure 4.20B** and **C**; Δ: -0.26±0.09, *p<0.05*). However, both EGFR and pSTAT3 were not detected in tumour tissues; this could be due to fast degradability of these proteins.

Figure 4.16. Effect of 4-DAMP treatment on the expression of VEGF and CD31 *in vivo*

VEGF and CD31 expression in tumour samples from mice bearing CT-26 cell-induced CRC treated with DMSO (**A-A'''**) and 4-DAMP (**B-B'''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), VEGF (red; **A'-B'**), CD31 (green; **A''-B''**) and all markers merged (yellow; **A'''-B'''**). Scale bar represents 50µm. Bar graphs displaying the mean fluorescence level of VEGF (**C**), CD31 (**D**) and image of VEGF western blot bands (**E**) in tumour samples from DMSO-treated and 4-DAMP-treated mice. Data presented as mean ± SEM, n=8 per group. Student's *t-test, ****p<0.0001*.


A
DAPI
DMSO
A'
VEGF
DMSO
A''
CD31
DMSO
A'''
Merged
DMSO
B
DAPI
4-DAMP
B'
VEGF
4-DAMP
B''
CD31
4-DAMP
B'''
Merged
4-DAMP


C
VEGF
Fluorescence (Arb. Unit)
50
40
30
20
10
0
****
DMSO
4-DAMP
D
CD31
Fluorescence (Arb. Unit)
40
30
20
10
0
****
DMSO
4-DAMP

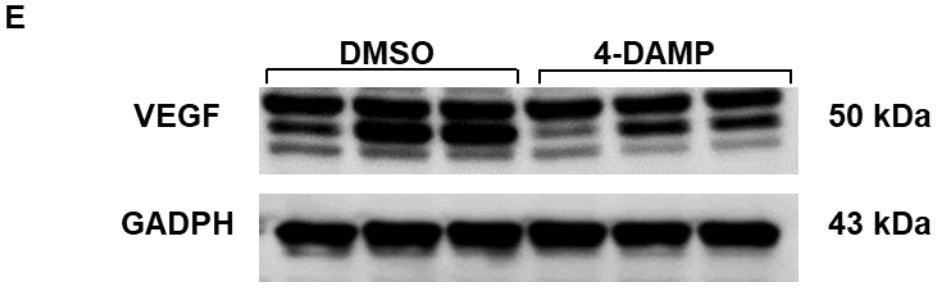

E
DMSO
4-DAMP
VEGF
50 kDa
GADPH
43 kDa

Figure 4.17. Effect of 4-DAMP treatment on the expression of CD31 *in vivo*

Image showing blood vessels and intensity of CD31 in tumour samples from mice bearing CT-26 cell-induced tumours treated with DMSO (**A-A'**) and 4-DAMP (**B-B'**). Scale bar represents 100µm, n=8 mice per group.

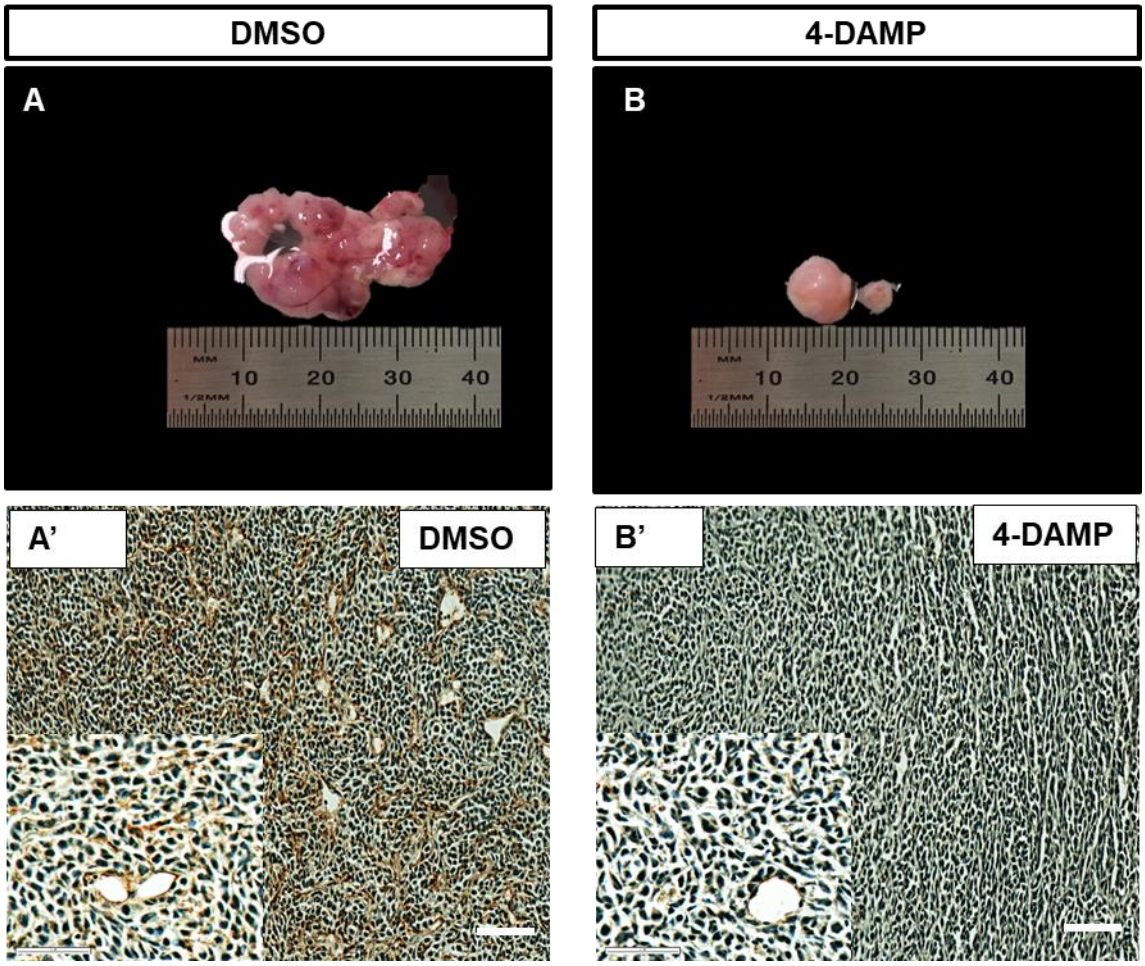
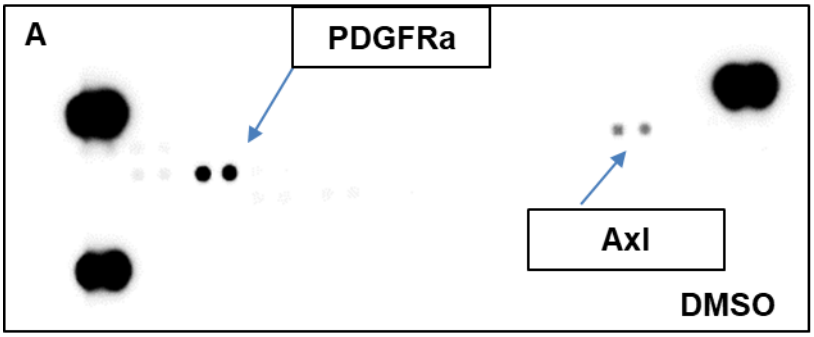

DMSO
4-DAMP
A
B
10
20
30
40
A'
DMSO
B'
4-DAMP

Figure 4.18. Effect of 4-DAMP treatment on the expression of phospho kinases *in vivo*

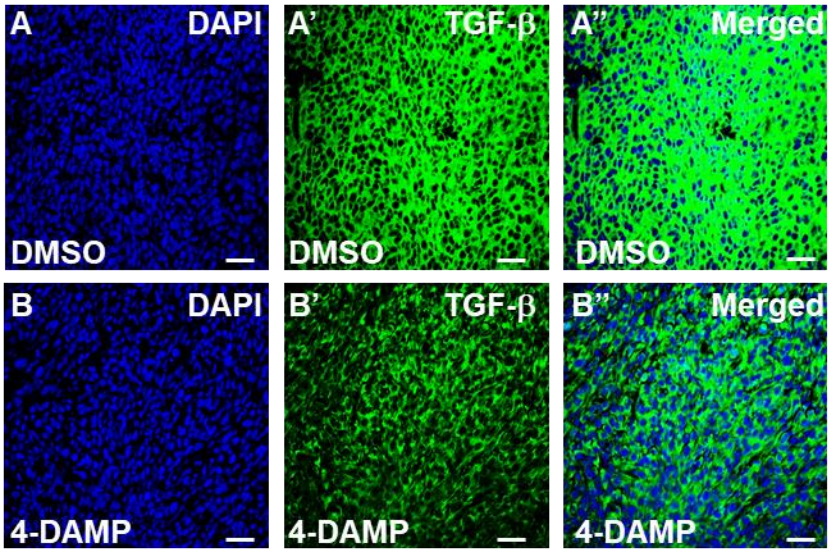
Mouse Phospho-RTK Array measuring phospho-RTK activity in tumours from mice with CT-26-induced CRC treated with DMSO (**A**) and 4-DAMP (**B**); n=8 mice per group.


A
PDGFRa
Axl
DMSO


B
PDGFRa
4-DAMP

Figure 4.19. Effect of 4-DAMP treatment on the expression of TGF-β *in vivo*

TGF-β in tumour samples from mice bearing CT-26 cell-induced tumours treated with DMSO (**A-A''**) and 4-DAMP (**B-B''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), TGF-β (green; **A'-B'**) and all markers merged (**A''-B''**). Scale bar represents 50 µm. Bar graphs displaying the mean fluorescence level of TGF-β (**C**) in tumour samples from DMSO-treated and 4-DAMP-treated mice. Data presented as mean ± SEM, n=8 per group. Student's *t-test, ****p<0.0001*.


A
DAPI
DMSO
A'
TGF-β
DMSO
A''
Merged
DMSO
B
DAPI
4-DAMP
B'
TGF-β
4-DAMP
B''
Merged
4-DAMP

C

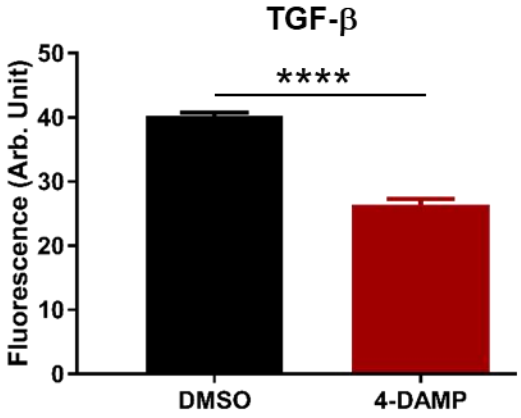
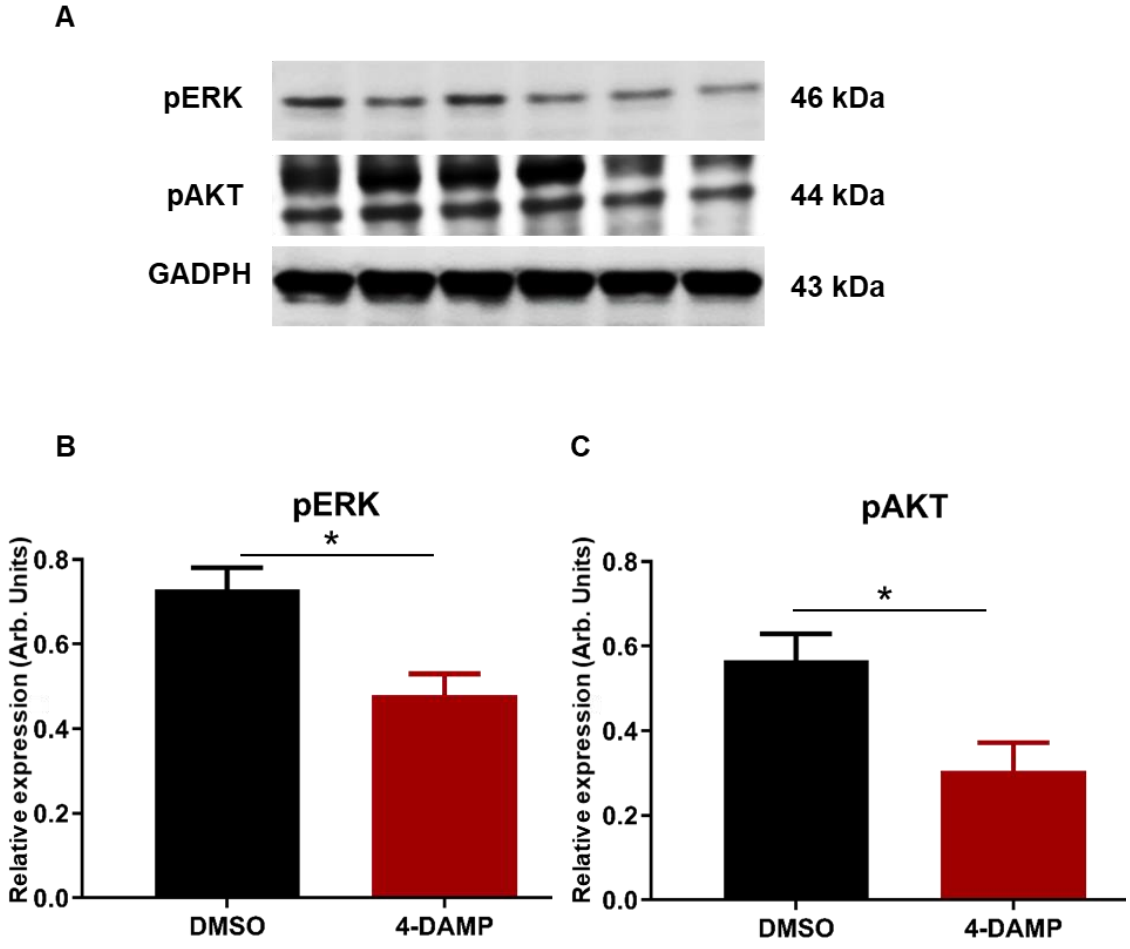

TGF-β
****
Fluorescence (Arb. Unit)
50
40
30
20
10
0
DMSO
4-DAMP

Figure 4.20. Effect of 4-DAMP treatment on the expression of protein kinases *in vivo*

Western blot bands for pERK and pAKT expression in tumour samples from mice bearing CT-26 cell-induced CRC treated with DMSO and 4-DAMP (**A**). Bar graphs displaying the mean intensity of pERK (**B**) and pAKT (**C**) expression in tumour samples from mice treated with DMSO and 4-DAMP. Data presented as mean ± SEM, Student's *t-test, *p<0.05*.


A
pERK
pAKT
GADPH
46 kDa
44 kDa
43 kDa
B
pERK
*
Relative expression (Arb. Units)
0.8
0.6
0.4
0.2
0.0
DMSO
4-DAMP
C
pAKT
*
Relative expression (Arb. Units)
0.8
0.6
0.4
0.2
0.0
DMSO
4-DAMP

4.4. Discussion

The immune system plays a key role in the eradication of cancer cells. However, multiple mechanisms are responsible for the suppression of the immune system in cancer, one of which being the expression of immune checkpoint inhibitors, including PD-1, PD-L1 and PD-L2 [509, 514].

These molecules function by inhibiting the anti-tumour effects of T cell-mediated immune response. Although there are current therapies targeting these molecules, they have shortcomings such as causing adverse events. Therefore, it is crucial to understand the possible mechanisms influencing the expression of these molecules. It was hypothesised that the cholinergic system might play a significant role in the induction of immunosuppressive markers such as PD-L1 and PD-L2. Indeed, the results presented in this Chapter demonstrated that CT-26 cells expressed PD-L1 and PD-L2 *in vitro*, which was attenuated by cholinergic blockers, atropine and 4-DAMP. To further evaluate whether the effects of 4-DAMP in the *in vitro* model could be achieved in the *in vivo* model of CRC, mice bearing CT-26 cell-induced tumours were injected daily with a vehicle, DMSO, or 4-DAMP for 3 weeks. It was clear that treatment with 4-DAMP decreased tumour weight, volume and size when compared to the vehicle-treated group. Furthermore, 4-DAMP treatment significantly decreased PD-L1 but not PD-L2 expression in CRC mouse model. Furthermore, 4-DAMP administration significantly decreased cholinergic and angiogenic markers when compared to DMSO treatment. In addition, 4-DAMP treatment augmented anti-tumour immune response through increased infiltration of CD4+ and CD8+ T cells.

Acetylcholine is one of the main neurotransmitters found abundantly in the body. For a long time, it was believed that only neurons can synthesise and secrete ACh; however, studies have proved that many other cells, including tumour cells, can also produce ACh. In CRC, the expression of M3R plays an important role in the cellular processes such as proliferation, differentiation, angiogenesis, invasion, metastasis and the establishment of cell-cell contact [510, 580] via the activation of various signalling pathways such as AKT, ERK and EGFR [600]. In the present study, we evaluated whether blocking of all

muscarinic receptors with atropine and a selective M3R blocker, 4-DAMP, had a detrimental effect on murine CT-26 colon cancer cells. Thus, blocking of all muscarinic receptor and M3R significantly suppressed CT-26 cell proliferation in a dose-dependent manner and induced apoptosis through the phosphorylation of AKT and ERK signalling pathways. These findings concur with current literature implicating that ACh acting on muscarinic receptors promotes cancer cell proliferation, invasion and metastasis. For example, in human colon cancer cell lines SNU-C4, HT-29 and H508, administration of atropine, muscarinic receptors inhibitor, eradicated SNU-C4 cell migration and HT-29 invasion; however, H508 cell migration entails the activation of MMP7 via EGFR and ERK signalling pathways [592, 593]. Although blocking of M3R had no effect on the activation of EGFR as shown in this *in vitro* study, other studies have demonstrated that there is a link between EGFR and M3R [587, 601].

In addition, activation of nicotinic acetylcholine receptors (nAChRs) with nicotine, facilitates cellular invasion and metastasis of human colon cancer cells, LOVO and SW620, via the activation of p38 mitogen-activated protein kinase (MAPK) signalling pathway [343]. Likewise, nicotine stimulates the activation of α9-nAChR, which facilitates cellular migration of MDA-MB-231 and MCF-7 breast cancer cells via the expression of epithelial-mesenchymal transition markers [364]. Furthermore, in hepatocellular carcinoma, ACh acting on androgen receptor endorses SNU-449 cell invasion and migration via activation of signal transducer and activator of transcription 3 (STAT3) and AKT signalling pathways [363].

The *in vivo* data presented in this Chapter showed that blocking M3R with 4-DAMP in an orthotopic mouse model of CRC significantly reduced tumour weight, volume and size compared to DMSO-treated controls. These findings are supported by previous studies demonstrating that administration of M3R antagonists, darifenacin and 4-DAMP, significantly decreased tumour growth in a mouse model of gastric cancer [587]. Furthermore, 4-DAMP treatment significantly decreased PD-L1 expression in CRC mouse model; however, there was no significant difference observed in the expression of PD-L2 between DMSO and 4-DAMP treated groups. In addition, administration of 4-DAMP significantly attenuated the expression of M3R and ChAT; nevertheless, there was

an increase in a7nAChR expression. We suggest that the possible mechanism involved in attenuation of tumour growth by the 4-DAMP treatment could be through the inhibition of immunosuppressive (PD-L1) and cholinergic (M3R and ChAT) markers. Findings by Kamiya et al (2019) in chemically-induced and xenograft models of breast cancer, demonstrated that presynaptic cholinergic neurostimulation resulted in decreased immune checkpoints molecules (PD-1 and PD-L1) expression and attenuated tumour growth [558]. Our findings provide evidence that blocking cholinergic receptors on a postsynaptic membrane reduced immunosuppressive markers and decreased tumour growth. Thus, in both cases, inhibition of immunosuppressive molecules had pronounced anti-tumour effects.

The tumour microenvironment is complex as it involves many factors including resident and infiltrating immune cells, various stromal cells and blood and lymphatic vessels all of which are concealed in an extracellular matrix [602, 603]. In fact, the nervous system and tumour microenvironment communicated through a feedback loop mechanism that facilitates tumour growth and progression [580, 604]. There is also a complex interaction between immunosuppressive markers and the tumour microenvironment [509]. In the present study, the profile of tumour-associated immune cells was evaluated using flow cytometry cell sorting. Blocking of M3R significantly improved immune response against cancer as noted by the increased expression of CD4+ and CD8+ T cells leading to reduced tumour size, weight and volume. Furthermore, other infiltrating immune cells such as $\gamma\delta$ T cell, B cell and eosinophils, which have a deleterious effect on immune response, were abundantly decreased.

Tumour angiogenesis essential for oxygen and nutrients supply is one of the tumour traits promoting its growth. Studies have reported that tumours not only can form their own blood vessels, but they also produce neurotransmitters and immunosuppressive molecules such as PD-L1 and PD-L2 to promote tumour angiogenesis [510]. The present study showed that 4-DAMP treatment significantly decreased tumour angiogenesis as demonstrated by the decreased expression of VEGF, CD31 and TGF-β. VEGF promotes proliferation and expansion of endothelial cells via interaction with its receptors [605]. Studies have demonstrated elevated levels of VEGF and its receptors in human colon

carcinomas and tumour-infiltrating endothelial cells [605-607]. Aside from its role in facilitating tumour angiogenesis, VEGF also exerts deleterious effects on the immune system by dampening a number of immune cells [608]. Studies have suggested that cholinergic signalling and the expression of immunosuppressive markers such as PD-L1 and PD-L2 play a functional role in tumour angiogenesis. For instance, expression of PD-L1 correlates with VEGF as noted in primary human glioma samples [609]. Similarly, the expression of PD-L1 and PD-L2 was positively associated with VEGF expression in renal carcinoma [534]. In CRC, high expression of VEGF and CD31 was correlated with poor patients' survival [610]. Expression of immune checkpoint molecule, B7-H3, correlates with CD31 expression in tissue samples from patients with CRC and induced VEGFA through the activation of NF-κB pathway as observed *in vitro* and *in vivo* [611].

TGF-β acts as an anti-tumourigenic factor at the early stages and as a pro-tumourigenic factor at late stages of tumour progression [612]. High levels of TGF-β in the tumour microenvironment have been associated with angiogenesis, contributing to tumour development and metastasis [598, 599]. In human gastric cancer cell lines, MKN45 and KATOIII, TGF-β1 induced VEGF-C expression leading to lymphangiogenesis through the activation of Smad2/3 and Smad pathway [613]. In addition, 4-DAMP significantly attenuated PDGFRα and abolished Axl, both of which play an essential role in tumour angiogenesis. In tumour specimens from CRC patients, PDGFRα/β expression correlates with lymphatic dissemination and metastasis [614, 615]. Similarly, in invasive ductal carcinoma, overexpression of PDGFRα correlates with metastasis [616]. Axl promotes survival of endothelial cells and remodelling of endothelial barriers in wound healing and vessel impairment [617]. Axl is essential for angiogenesis mediated by VEGF-A activation of VEGFR-2 via PI3K/AKT pathway [618]. In *in vitro* and *in vivo* models of breast and prostate cancers, inhibition of Axl suppressed pro-angiogenic factors and impaired functional properties of endothelial cells [619].

4.5. Conclusion

The results of this study demonstrated that enhanced expression of PD-L1, PD-L2, M3R and ChAT and angiogenic markers was attenuated by treatments with cholinergic receptor blockers *in vitro*. *In vivo* results demonstrated that blocking M3R has pronounced anti-tumour effects via several mechanisms including inhibition of immunosuppressive molecules, enhancement of anti-tumour immune response and suppression of tumour angiogenesis via suppression of AKT/ERK signalling pathway. Although immunotherapies have shown great efficacy in many solid tumours, there is still a need to develop improved therapies with less side-effects. The results presented in this Chapter suggest that blocking M3R has the potential to be used in conjunction with immune checkpoint inhibitors. Further studies are required to evaluate the anti-tumour efficacy and side-effects of combination therapies with M3R blockers and immune checkpoint inhibitors.

CHAPTER FIVE

Expression of IDO and Siglec-9 in Patients Diagnosed with Stages I-IV of CRC and Effect of Blocking Siglec-9 in Human Colon Cancer Cell Lines

5.0. Abstract

Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide. Tumour cells have evolved to express immunosuppressive molecules enabling their evasion from the host's immune response. These molecules include indoleamine-2,3-dioxygenase (IDO) and sialic acid-binding lectin 9 (siglec-9). Furthermore, cancer cells can also secrete a neurotransmitter, acetylcholine, which has been shown to play a role in tumour progression by stimulating tumour vascularisation leading to tumour growth and metastasis. The expression of immunosuppressive markers, IDO and siglec-9, was correlated with CRC stages (I-IV), patients' gender, age, metastasis and survival outcomes. Immunofluorescence was used to determine the expression of these molecules in paraffin-embedded tumour tissues from patients with CRC. Furthermore, the expression of these markers in human colon cancer cell lines (LIM-2405 and HT-29) and normal human primary colon epithelial cell line (T4056) treated with human anti-siglec-9 antibody was also evaluated. There was significantly higher expression of IDO and siglec-9 at stages, III and IV compared to early stages, I and II of CRC. IDO and siglec-9 expression were associated with a high risk of CRC and poor patients' survival outcomes. However, there was no significant correlation between the expression of these markers and patient's gender, age and metastasis. *In vitro* results showed that LIM-2405, HT-29 and T4056 all expressed immunosuppressive markers, IDO and siglec-9, at varying degrees; however, colon cancer cells overexpressed these molecules compared to normal epithelial cells. Blocking siglec-9 with human anti-siglec-9 antibody significantly attenuated cell proliferation and choline production in all cells. Blocking siglec-9 significantly decreased IDO expression in HT-29 cell but had no effects on T4056 and LIM-2405, while it significantly reduced siglec-9 expression in human colon cancer cells (LIM-2405 and HT-29) but not T4056 cell. Interestingly, blocking siglec-9 decreased M3R expression in T4056 but not human colon cancer cells and had no effects on ChAT expression in all cells. Furthermore, blocking siglec-9 significantly inhibited phosphorylation of ERK and STAT3 in T4056 and LIM-2405 but not HT-29. Similarly, blocking siglec-9 significantly suppressed the activation of EGFR in T4056 but not LIM-2405 and HT-29 cells. These findings suggest that in T4056, human anti-siglec-9 antibody

displayed it effects via suppressing EGFR/ERK/STAT3 signalling pathway, while in LIM-2405, it acts by inhibiting ERK/STAT3 signalling pathway, however, no pathway was observed in HT-29.

5.1. Introduction

Tumour progression involves complex interactions between a number of different factors, one of which being the evasion of the host's immunity via the establishment of the immunosuppressive environment [514]. The immune system plays an essential role in tumour development, not only it can impede the growth, but it can also progress its growth by providing immunosuppressive milieu. Tumourigenic cells utilise a number of mechanisms including the expression of immunosuppressive markers including programmed death-ligand 1 & 2 (PD-L1) and (PD-L2) as demonstrated in previous chapters, but it also overexpresses indoleamine-2,3-dioxygenase (IDO) and siglec-9. IDO is an enzyme that degrades the amino acid tryptophan within the kynurenine pathway, and its overexpression by cancer cells results in immunosuppression [620]. IDO exacerbates L-tryptophan deficit which impairs T cell proliferation within the tumour microenvironment via inducing T cells apoptosis providing an immune escape mechanism [514]. IDO exhibits its effect not only on T cells but also on other immune cells including natural killer (NK) cells and supports the recruitment of tumour-associated immune cells such as myeloid-derived suppressor cells and regulatory T cells [85].

Studies have demonstrated that tumours can alter glycosylation as a consequence of downregulation of innate immune response through immunoregulatory siglecs. Siglecs or sialic acid immune-globulin like binding lectins are transmembrane surface proteins that play a role in hindering T cell activation/signalling promoting tumour progression [100]. Although T cells rarely express siglecs, studies have reported that CD8+ T cells can express siglec-9 leading to inhibition of cytotoxic CD8+ T cells via phosphorylation of inhibitory protein tyrosine phosphatase (SHP)-1, as noted in melanoma [621]. In addition, siglec-9 is expressed on the surface of immune cells such as NK cells, B cells and monocytes, and it interacts with transmembrane mucins (MUC)1 and MUC16 [100]. The interaction of siglec-9 with MUC1 and/or MUC16 provides escape of tumour cells from the immune response via the inhibition of NK cells and T cells [101].

In addition to these immunosuppressive molecules, tumour cells can also express neurotransmitters such as acetylcholine (ACh) to encourage immune circumvention. ACh plays an essential role in the establishment of cell-cell communication and other cellular processes such as proliferation, differentiation, angiogenesis, invasion, and migration, the main hallmarks of cancer [622]. The present study evaluated the expression of immunosuppressive markers, IDO and siglec-9, and their correlation with CRC stages (I-IV), patients' age, gender, metastatic status and survival outcomes. Furthermore, the effect of human anti-siglec-9 antibody on cellular proliferation, choline production, and expression of immunosuppressive and cholinergic markers as well as protein kinases was evaluated *in vitro*. The interaction between siglec-9, IDO and cholinergic signalling may be necessary for the early detection, prognosis of cancer; hence, it is crucial to identify this interaction that may predict cancer progression in patients and *in vitro*.

5.2. Materials and Methods

5.2.1. Human tumour samples

As previously stated in Chapter 2, tumour samples were collected at the Ministry of Internal Affairs and Administration Hospital in Olsztyn, Poland, from 2010 to 2013. The collection of human samples used in this study was approved by the Bioethics Committee of the University of Warmia and Mazury in Olsztyn, Poland. The study comprised of 139 patients with CRC (out of 139, 91 patients had clinical follow up). All patients signed a written informed consent for the use of their tissues for research purposes. Patients had no evidence of bowel obstruction or other colonic diseases. None of the CRC patients had a second neoplastic disease or had previously undergone chemo- or radiotherapy. Patients' demographical, clinical and overall survival data were collected. Type of cancer and grading were described by a pathologist according to the World Health Organization criteria and staging according to the 7th edition of Cancer Staging Manual of the American Joint Committee on Cancer (AJCC). However, due to a small patient number with grade III (n=7) compared to grade II (n=84) of CRC, the grade was excluded from the multivariate analysis and Chi-Square test. Similarly, metastasis status was excluded due to small sample size; nine patients had metastatic cancer compared with 82 patients without. Samples from the neoplasm lesion were collected into 10% neutral buffered formalin, dehydrated in ethanol/xylene and embedded in the paraffin wax. Paraffin-embedded blocks of tissue were cut into 4μm thickness sections and mounted onto the microscope slides.

5.2.2. Immunohistochemical analysis of the paraffin-embedded samples

Samples were deparaffinised and hydrated through the series of washes with xylene and graded alcohol. Antigen retrieval was performed using citrate buffer pH 6.0, 10x (Sigma-Aldrich, Melbourne Australia). Citrate buffer was heated until bubbles start to form. Samples were emerged into the buffer and placed on a hot plate pre-set at 100°C for 15 minutes (mins) and left to cool at room temperature for another 20 mins. Using a liquid blocker super pap pen, samples were outlined to reduce the volume of antibody used.

Endogenous activity was blocked using 10% donkey serum for 1 hour (hr) at room temperature. Samples were then incubated overnight at room temperature with primary antibodies (**Table 5.1**). After washing, the tissues were incubated in phosphate buffered saline (PBS) plus Triton X-100 (PBST) solution at room temperature for 2hrs with secondary antibodies (**Table 5.2**) diluted in PBS containing 2% donkey serum and 0.01% Triton X-100. Samples were incubated for 1 min with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (D1306, Life Technologies, Australia) and mounted with DAKO mounting medium (Agilent Technologies, Australia). Then coverslips were placed on and left to dry overnight before imaging.

5.2.3. Cell culture

As previously described in Chapter 3, human colon cancer cell lines (LIM-2405 and HT-29) were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Sigma-Aldrich, Castle Hill, Australia) and normal human primary colon epithelial cells (T4056) was Dulbecco's Modified Eagle Medium (DMEM) culture media (Sigma-Aldrich, Castle Hill, Australia). Culture media were supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% glutamine. Cells were cultured at 37°C, in 5% CO_2 and 95% air atmosphere. When cells grew into confluent or semiconfluent monolayers in 75cm^2 medium flasks, they were either passaged or used. The passage of cells was conducted with 0.25% trypsin every 3-4 days.

5.2.4. Cell proliferation

A water-soluble tetrazolium-1 (WST-1) assay kit (Roche Diagnostics GmbH, Germany) was used to determine the viability of cancer cells. WST-1 is cleaved to form formazan dye via a complex cellular interaction at the cell surface. This interaction is contingent on the glycolytic nicotinamide adenine dinucleotide phosphate (NADPH) production of the viable cells. Hence, the amount of formazan dye formed correlates to the number of viable cells in the culture. T4056, LIM-2405 and HT-29 cells were seeded and cultured at 1×10^4 cells per well in 96 well plates for 24hrs and then treated with varies concentration of human anti-siglec-9 antibody (MAB1139; R&D Systems, Australia) for 8hrs. All treatments

were performed in triplicates. WST-1 reagent (10µL) was added to each well for 1hr incubation period at 37^{o}C. Cell proliferation was measured using a microplate reader (Varioskan Flash, Thermo Scientific) at the absorbance of 450nm.

5.2.5. Choline/acetylcholine assay

A Choline/Acetylcholine Assay Kit (Abcam, Australia) was used to measure the concentration of choline in cell lysates. The assay was carried out in accordance with the instructions provided by the manufacturer, as previously described in Chapter 3. Briefly, T4056, LIM-2405 and HT-29 (1x10^{6} cells) were cultured overnight, then treated with 50ng of human anti-siglec-9 antibody for 8hrs. Cells were then lysed in choline assay buffer before commencing choline measurement. The level of total choline was measured in each cell line. All experiments were conducted in duplicates.

5.2.6. Western blot

Expression of immunosuppressive and cholinergic markers (**Table 5.1**) as well as cell signalling pathway, phospho signal transducer and activator of transcription 3 (pSTAT3), phospho extracellular signal-regulated kinase (pERK½), phospho serine/threonine kinase or protein kinase B (pAKT) and epidermal growth factor receptor (EGFR) (**Table 5.2**) in T4056, LIM-2405 and HT-29 cells was examined by western blot. Cells were incubated with 50ng of human anti-siglec-9 antibody forhrs. After treatments, cells were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer (pH 7.4, 150mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate, 1% NP-40 in PBS, Sigma) containing a protease and phosphatase inhibitors cocktail (Roche Applied Science). Cellular proteins (20µg) from each sample were separated by 8% to 12% SDS/polyacrylamide gel electrophoresis. The separated fragments were transferred to 0.22µm polyvinylidene fluoride membranes, which were blocked with 5% skim milk in PBS containing 0.1% Tween 20 overnight at 4^{o}C in a platform shaker at 40RPM speed. The membranes were incubated with primary antibodies overnight at 4^{o}C. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2hrs at room temperature. The membranes were washed three

times in PBS plus 0.1% Tween 20 and protein detection was performed using enhancing chemiluminescence reagents. Glyceraldehydes-3-phosphate dehydrogenase (GADPH) was used as a loading control.

Table 5.1. Primary antibodies used in this study

Primary antibodies				
Markers	**Host Species & Clonality**	**Dilution**	**Source**	**Catalogue no.**
IDO	Mouse, monoclonal	1:200	Abcam, Australia	ab55305
Siglec-9	Rabbit, polyclonal	1:200	Abcam, Australia	ab197981
M3R	Rabbit, polyclonal	1:500	Abcam, Australia	ab126168
ChAT	Goat, polyclonal	1:500	Abcam, Australia	ab134021
EGFR	Rabbit, polyclonal	1:1,000	Cell signalling, Australia	#4267
pERK$_{1/2}$	Rabbit, polyclonal	1:1,000	Cell signalling, Australia	#3192
pAKT	Rabbit, monoclonal	1:1,000	Cell signalling, Australia	#4060
pSTAT3	Mouse, monoclonal	1:1,000	Cell signalling, Australia	#9145

Table 5.2. Secondary antibodies used in this study

Secondary antibodies			
Alexa Fluor 488	Anti-mouse	1:250	Jackson ImmunoResearch Laboratories, United States
Alexa Fluor 594	Anti-rabbit	1:250	Jackson ImmunoResearch Laboratories, United States
IgG HL HRP	Anti-goat	1:10,000	Abcam, Australia
IgG HL HRP	Anti-rabbit	1:10,000	Abcam, Australia
IgG HL HRP	Anti-mouse	1:10,000	Abcam, Australia

5.2.7. Data analysis

Images were captured on a Nikon Eclipse Ti multichannel confocal laser scanning system (Nikon, Japan). Z-series images were acquired at a nominal thickness of 1µm (1024 x 1024 pixels). Image J software (National Institute of Health, Bethesda, MD, USA) was employed to convert images from RGB to 8-bit binary; particles were then analysed to obtain the percentage area of immunoreactivity [591]. For localization data analysis, the number of cells within the tumour specimen expressing markers were counted within eight randomly captured images at x40 magnification.

All slides were coded, and immunohistochemistry images were quantified blindly. Statistical analysis was performed by one-way *ANOVA* followed by Turkey's pos-hoc test. For correlation analysis of marker expression with clinicopathological parameters, Cox regression test for survival analysis, Chi-Square test and multivariate test were used. Pearson correlation was performed to analyse the relationship between the overall expression of immunosuppressive markers and cholinergic markers.

For *in vitro* data analysis, chemiluminescent signal was captured using the FluorChem FC2 System (Alpha Innotech, USA). The expression level of each protein was quantified using Alpha View and ImageJ software with GADPH used as a loading control. Two-way *ANOVA* followed with Dunnett's pos-hoc test was used. Microsoft Excel, SPSS and Prism (Graph Pad Software, La Jolla, CA, USA) were utilised to aid in the statistical analysis and $p<0.05$ was considered significant.

5.3. Results

5.3.1. Expression of IDO and siglec-9 in patients diagnosed with stages I-IV of CRC

To determine the expression of IDO and siglec-9, tissues obtained from patients diagnosed with stage I-IV of CRC (n=139) were used. In this study, immunofluorescence was used to determine the expression of these molecules in formalin paraffin-embedded tumour tissues. IDO was overexpressed at stage IV when compared to stages, I, II and III (**Figure 5.1A'-D'**, *p<0.0001*). Moreover, there was a statistical difference observed at stage III when compared to stage I. However, no statistical difference was noted between stages, I and II as well as stages, II and III. Quantitative analysis of IDO expression is shown in **Figure 5.1E**. Similarly, siglec-9 expression was associated with the advanced stages, III-IV, compared to stages, I-II (**Figure 5.1A''-D''**, *p<0.0001*). There was a significant difference observed at stage III when compared to stages, I and II. No statistical difference was noted between stage II, and I. Quantitative analysis of siglec-9 expression is shown in **Figure 5.1F**. Overall, overexpression of both IDO and siglec-9 was associated with advanced stages, III and IV, when compared to early stages of CRC.

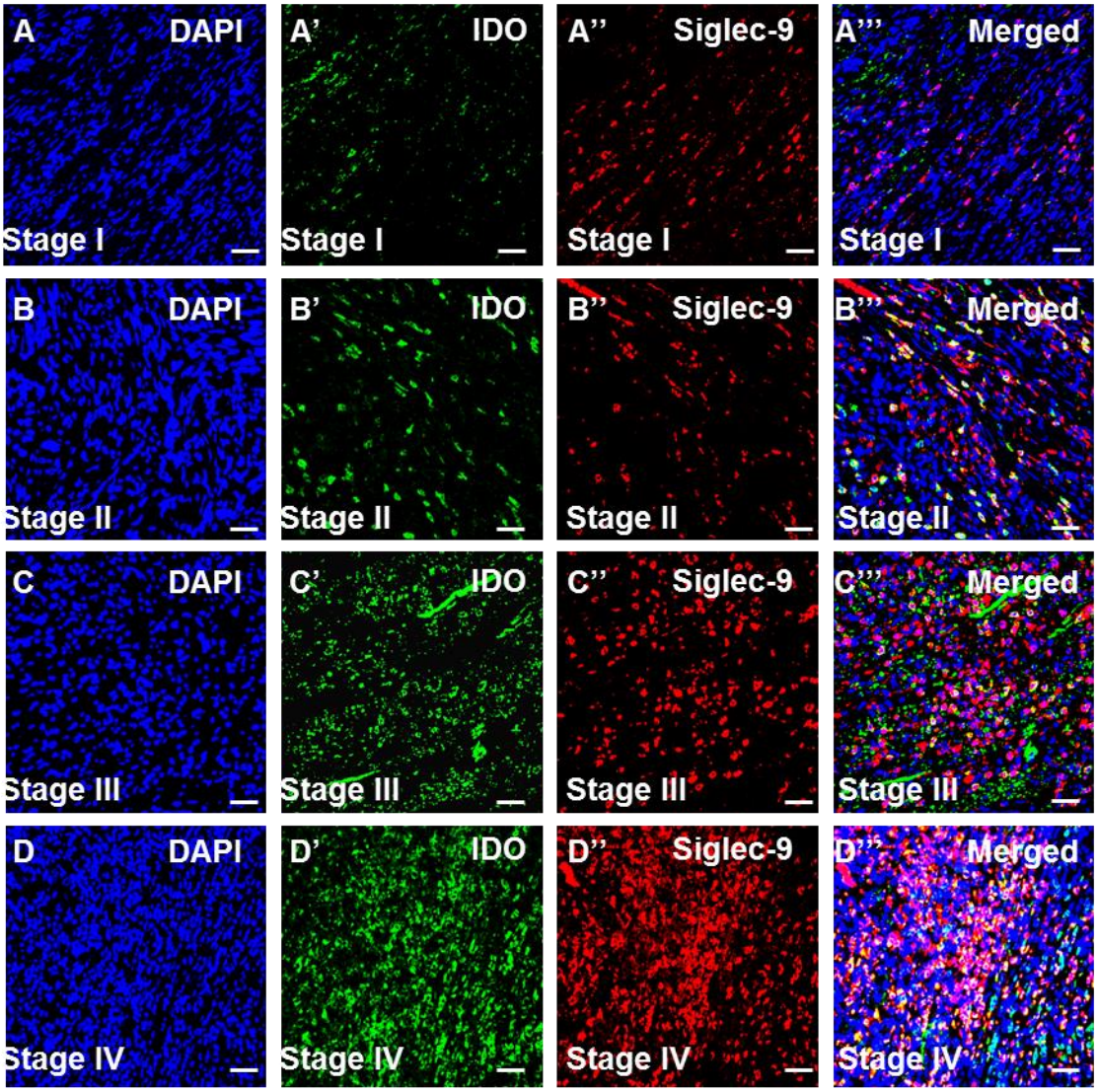
We evaluated the number of cells within tumour expressing IDO and siglec-9. The results demonstrated that the number of cells expressing IDO was augmented at stage IV compared to stages, I, II and III (**Figure 5.2A**-**E**, *p<0.0001*). Moreover, there was an increased number of cells expressing IDO at stage III compared to stages, I and II. No statistical difference was observed in the number of cells expressing IDO at stages, I and II. A number of cells overexpressing siglec-9 were elevated at stage IV compared to stages, I-III (**Figure 5.2A**-**F**, *p<0.0001*). In addition, there were more cells expressing siglec-9 at stage III compared to stage I. No statistical difference was observed in the number of cells within tumour expressing siglec-9 at stage III compared to II, as well as stage II compared to stage I.

In addition, the results demonstrated that IDO and siglec-9 are co-localised, suggesting some interaction. The number of cells co-expressing IDO and siglec-9 within the tumour were augmented at stage III compared to stages, I, II and IV (**Figure 5.2A**-**E**, *p<0.0001*).

Interestingly, the number of cells co-expressing IDO and siglec-9 within the tumour was attenuated at stage IV compared to stage III.

Figure 5.1. Expression of immunosuppressive markers in tumour tissues from CRC patients

IDO and siglec-9 expression in tumour specimens from patients diagnosed with stage I (**A-A'''**), stage II (**B-B'''**), stage III (**C-C'''**) and stage IV (**D-D'''**) of CRC. Tumours were labelled with the nuclei marker DAPI (blue; **A-D**), IDO (green; **A'-D'**), siglec-9 (red; **A''-D''**) and all markers merged (yellow; **A'''-D'''**). Scale bar represents 50μm. Bar graphs displaying the mean fluorescence of IDO (**E**) and siglec-9 (**F**) in tumours from patients with stages I-IV of CRC. Data presented as mean ± standard error of the mean (SEM), stage I n=14, stage II n=59, stage III n=57 and stage IV n=9. One-way *ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*.


A
DAPI
Stage I
A'
IDO
Stage I
A''
Siglec-9
Stage I
A'''
Merged
Stage I
B
DAPI
Stage II
B'
IDO
Stage II
B''
Siglec-9
Stage II
B'''
Merged
Stage II
C
DAPI
Stage III
C'
IDO
Stage III
C''
Siglec-9
Stage III
C'''
Merged
Stage III
D
DAPI
Stage IV
D'
IDO
Stage IV
D''
Siglec-9
Stage IV
D'''
Merged
Stage IV

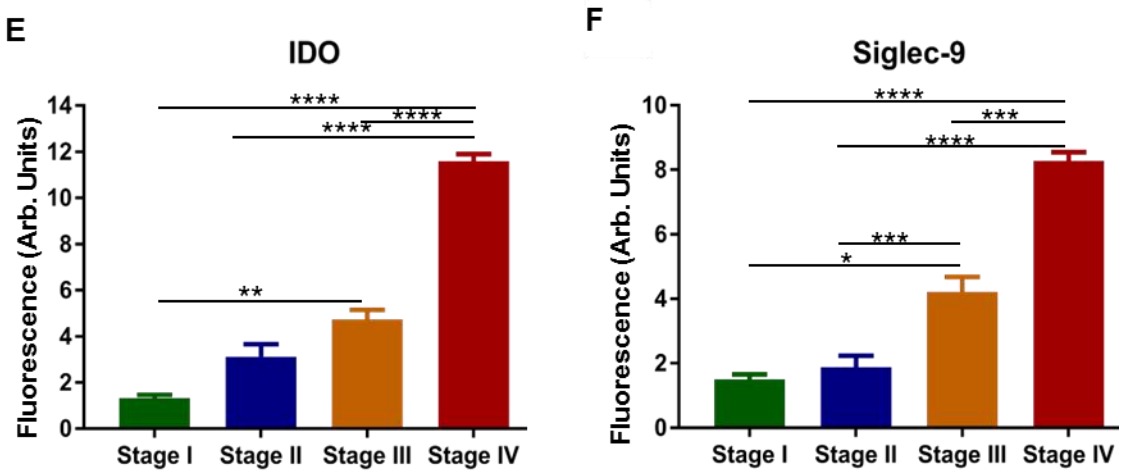
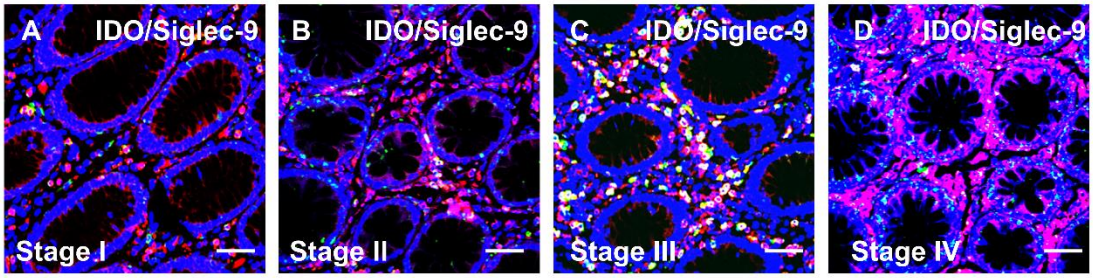
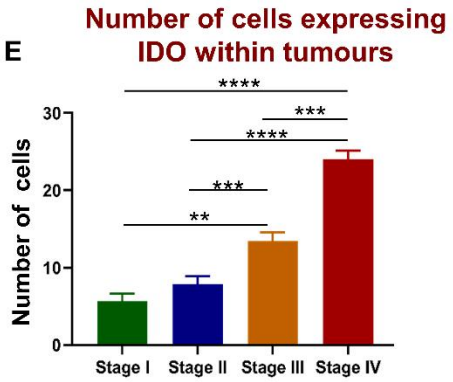
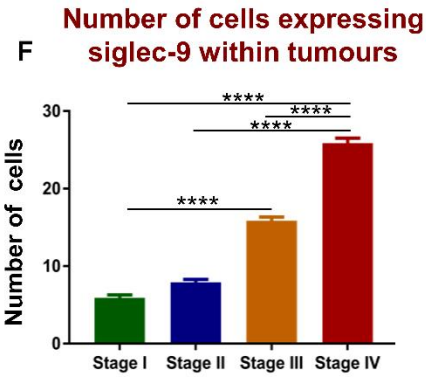

E
IDO
Fluorescence (Arb. Units)
14
12
10
8
6
4
2
0
****
****
****
**
Stage I
Stage II
Stage III
Stage IV
F
Siglec-9
Fluorescence (Arb. Units)
10
8
6
4
2
0
****
***
****
***
*
Stage I
Stage II
Stage III
Stage IV

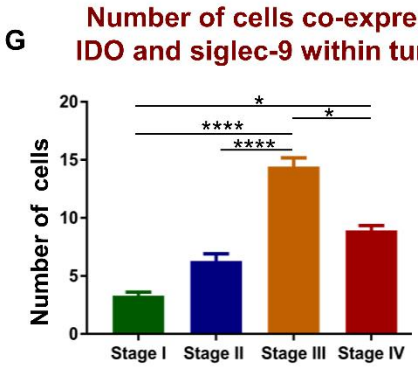
Figure 5.2. Number of cells expressing IDO and siglec-9 within tumour specimens from patients at stages I-IV of CRC

IDO and siglec-9 expression within tumour tissues from patients diagnosed with stage I (**A**), stage II (**B**), stage III (**C**) and stage IV (**D**) of CRC. Tumours were labelled with the nuclei marker DAPI (blue), IDO (green), siglec-9 (red) and all markers merged (yellow). Scale bar represents 50 μm. Bar graphs displaying the mean number of cells expressing IDO (**E**), siglec-9 (**F**) and co-expressing IDO and siglec-9 (**G**) in tumours from patients with stages I-IV of CRC. Data presented as mean ± SEM, stage I n=14, stage II n=59, stage III n=57 and stage IV n=9. One-way *ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*.


A IDO/Siglec-9
Stage I
B IDO/Siglec-9
Stage II
C IDO/Siglec-9
Stage III
D IDO/Siglec-9
Stage IV


E
Number of cells expressing IDO within tumours
Number of cells
0
10
20
30
****
***
****
***
**
Stage I
Stage II
Stage III
Stage IV


F
Number of cells expressing siglec-9 within tumours
Number of cells
0
10
20
30
****
****
****
****
Stage I
Stage II
Stage III
Stage IV


G
Number of cells co-expressing IDO and siglec-9 within tumours
Number of cells
0
5
10
15
20
*
*
****
****
Stage I
Stage II
Stage III
Stage IV

5.3.2. Clinicopathological and demographic parameters of CRC patients and their relevance to IDO and siglec-9 expression

As previously mentioned in Chapter 2, the average patients' age was 65 years ranging from 33 to 91. In the present cohort, 56% (51) of the patients were males, and 44% (40) were females. Among these patients, 15.4% (14) of patients were diagnosed with clinical stage I, 38.5% (35) with stage II, 36.3% (33) with stage III and 9.9% (9) with stage IV of CRC. To determine the correlation between the expression of immunosuppressive markers and clinicopathological parameters, specimens were labelled with IDO and siglec-9. The Chi-Square test was used to determine whether there is a significant difference between the expression of IDO and siglec-9 and the clinicopathological parameters listed in **Table 5.3**.

Among 91 patients with clinical follow-up, 59.3% (54) expressed low levels of IDO and 40.7% (37) expressed high levels (**Table 5.4**). A significant difference was observed between IDO expression and stages of CRC. Lower stages were noted to mainly express low levels of IDO (stage I, 12.1% (11) and stage II, 33% (30)), while elevated levels of IDO expression was associated with advanced stages, III and IV (stage III, 14.3% (13) and stage IV, 0% (0)). Moreover, IDO expression was associated with survival status but not with patients' gender and age.

Similarly, we evaluated the correlation between siglec-9 expression and patients' gender, age, stage and survival status. Out of 91 patients with clinical follow-up, 71.4% (65) expressed low levels of siglec-9, and 28.6% (26) expressed high levels of siglec-9 (**Table 5.5**). There was a significant difference observed between siglec-9 expression and stages of CRC. Stages, I, II and III predominantly expressed low levels of siglec-9 while 8 patients out 9 at stage IV expressed high levels of siglec-9. This suggests that siglec-9 expression might hold predictive value for stage IV.

There was no significant correlation between siglec-9 expression and gender as 36.3% (33) males, and 35.2% (32) females expressed low levels of siglec-9 while 19.8% (18) males and 8.8% (8) females expressed high levels. In addition, the correlation between siglec-9 expression and age was evaluated. Among 41 patients under age 65, 30.8% (28)

expressed low and 14.3% (13) expressed high levels. Low levels of siglec-9 expression was found in 40.7% (37) patients over age 65, whereas 14.3% (13) of patients in this age group expressed high levels of siglec-9, however, there was no significant correlation between siglec-9 expression and patients' age. There was a significant correlation found between siglec-9 expression and survival status.

Moreover, IDO and siglec-9 expression were correlated with the risk of CRC and patients' survival outcomes. The correlation between IDO and siglec-9 with survival outcome was determined by the analysis of hazard ratio (HR) and corresponding 95% confidence interval (CI) using Cox regression survival analysis. The results of this study demonstrated a significant correlation between high expression of IDO and a high risk of CRC and poor survival outcomes (**Figure 5.3A & B**, HR=2.741, 95% CI=1.749-4.295). Similarly, siglec-9 expression was also associated with a higher risk of CRC and poor patients' survival outcomes (**Figure 5.3C & D**, HR=2.792, 95% CI=1.752-4.450).

5.3.3. Correlation between immunosuppressive and cholinergic markers

Furthermore, we evaluated the overall correlation between the expressions of immunosuppressive with cholinergic markers (**Table 5.6**). Expression of IDO correlated with siglec-9, but no association was observed between the overall expression of IDO and cholinergic markers. Overall expression of siglec-9 was strongly correlated with M3R expression, suggesting that siglec-9 might crosstalk with M3R. There was no correlation between siglec-9 and expression of α7nChR and ChAT.

Table 5.3. Clinicopathological and demographic parameters of CRC patients

Parameters	No. of cases	Percentage (%)
Total	91	100
Gender		
Male	51	56
Female	40	44
Age		
<65	41	45.1
>65	50	54.9
Stage		
I	14	15.4
II	35	38.5
III	33	36.3
IV	9	9.9
Survival status		
Event	52	57.1
Censor	39	42.9

Table 5.4. Correlation of clinicopathological and demographic parameters of CRC patients with IDO expression

Parameters	No. of cases	Percentages (%)	IDO expression		*P* values
			Low	High	
Total	91	100	54 (59.3%)	37 (40.7%)	
Gender					
Male	51	56	28 (30.8%)	23 (25.3%)	0.330
Female	40	44	26 (28.6%)	14 (15.4%)	
Age					
<65	41	45.1	22 (24.2%)	19 (20.9%)	0.318
>65	50	54.9	32 (35.2%)	18 (19.8%)	
Stage					
I	14	15.4	11 (12.1%)	2 (2.2%)	
II	35	38.5	30 (33%)	5 (5.5%)	0.0001
III	33	36.3	13 (14.3%)	21 (23.1%)	
IV	9	9.9	0 (0%)	9 (9.9%)	
Survival status					
Event	52	57.1	5 (5.5%)	34 (37.4%)	0.004
Censor	39	42.9	21 (23.1%)	31 (34.1%)	

P values are based on the frequency of IDO expression within each parameter. The median defined low and high expression of the markers.

Table 5.5. Correlation of clinicopathological and demographic parameters of CRC patients with siglec-9 expression

Parameters	No. of cases	Percentages (%)	Siglec-9 expression		*P* values
			Low	High	
Total	91	100	65 (71.4%)	26 (28.6%)	
Gender					
Male	51	56	33 (36.3%)	18 (19.8%)	0.109
Female	40	44	32 (35.2%)	8 (8.8%)	
Age					
<65	41	45.1	28 (30.8%)	13 (14.3%)	0.549
>65	50	54.9	37 (40.7%)	13 (14.3%)	
Stage					
I	14	15.4	13 (14.3%)	0 (0%)	
II	35	38.5	32 (35.2%)	3 (3.3%)	0.0001
III	33	36.3	19 (20.9%)	15 (16.5%)	
IV	9	9.9	1 (1.1%)	8 (8.8%)	
Survival status					
Event	52	57.1	12 (13.2%)	27 (29.7%)	0.001
Censor	39	42.9	34 (37.4%)	18 (19.8%)	

P values are based on the frequency of siglec-9 expression within each parameter. The median defined low and high expression of the markers.

Figure 5.3. Correlation of IDO and siglec-9 expression with the risk of CRC and patients' survival outcomes

Correlation of IDO expression with the risk of CRC (**A**). IDO expression association with survival outcomes (**B**). Correlation of siglec-9 expression with the risk of CRC (**C**). Siglec-9 expression association with survival outcomes (**D**). Low and high expression of the markers was defined by the median. Stage I n=14, stage II n=35, stage III n=33 and stage IV n=9.

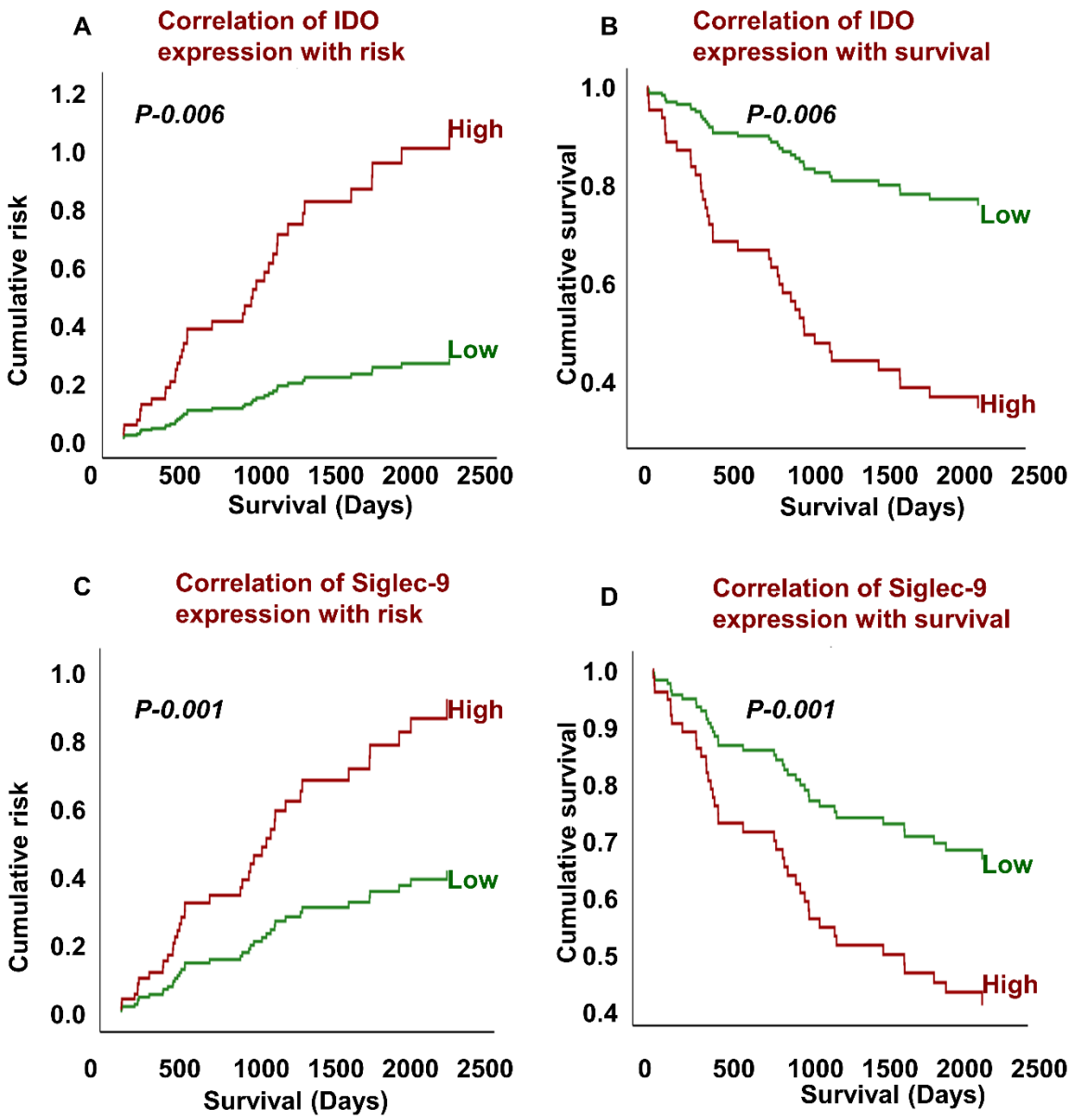

A
Correlation of IDO expression with risk
P-0.006
High
Low
Cumulative risk
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
2500
Survival (Days)
B
Correlation of IDO expression with survival
P-0.006
Low
High
Cumulative survival
1.0
0.8
0.6
0.4
0
500
1000
1500
2000
2500
Survival (Days)
C
Correlation of Siglec-9 expression with risk
P-0.001
High
Low
Cumulative risk
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
2500
Survival (Days)
D
Correlation of Siglec-9 expression with survival
P-0.001
Low
High
Cumulative survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0
500
1000
1500
2000
2500
Survival (Days)

Table 5.6. Correlation of immunosuppressive with cholinergic markers in CRC patients

		Siglec-9	IDO	M3R
Siglec-9	Pearson Correlation	1	0.401**	0.522**
	Sig. (2-tailed)		0<.0001	<0.0001
	N	91	91	91
IDO	Pearson Correlation	0.401**	1	
	Sig. (2-tailed)	<0.0001		
	N	91	91	

** Correlation is significant at the 0.01 level (2-tailed).

5.3.4. Effect of blocking siglec-9 ligand on cellular proliferation

In Chapter 3, we evaluated the effect of blocking cholinergic muscarinic receptors on the proliferation of human colon cancer cells and normal epithelial cells. In this chapter, we aimed to assess the effect of blocking siglec-9 ligand with human anti-siglec-9 antibody on the proliferation of human cancer cell lines (LIM-2405 and HT-29) and normal colon epithelial cells (T4056) using WST-1 assay. Three independent experiments were performed in triplicates. The result demonstrated that human anti-siglec-9 antibody significantly attenuated the proliferation of human colon cancer cells at 50-400ng compared to normal epithelial cells (**Figure 5.4A**). Interestingly, at 500ng, there was a significant difference between T4056 and LIM-2405, but not HT-29 cells. However, at high doses (700-800ng), there was a weak inhibition of human colon cancer cell proliferation when compared to T4056 cells. Overall, human anti-siglec-9 antibody attenuated cell proliferation in a dose dependent manner. Similarly, blocking siglec-9 with human anti-siglec-9 antibody decreased normal epithelial and human colon cancer cells viability in a dose dependent manner (**Figure 5.4B**). Interestingly, human anti-siglec-9 antibody at 50ng concentration significantly reduced human colon cancer cells viability by 50% and normal epithelial by 10%. Hence, in the subsequent experiments, cells were treated with 50ng of human anti-siglec-9 antibody for 8hrs, as changes were noticeable at this low concentration.

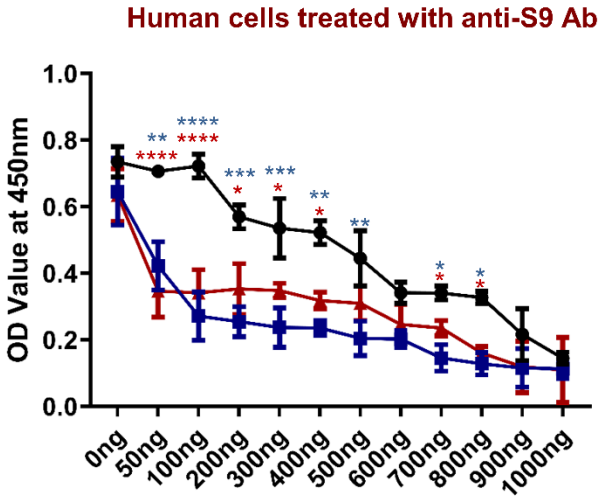
5.3.5. Effect of blocking siglec-9 on the cells ability to produce choline

To determine the effect of human anti-siglec-9 antibody on the cells' ability to produce choline, a precursor for ACh synthesis, cells were treated with 50ng of human anti-siglec-9 antibody. The results demonstrated that human anti-siglec-9 antibody significantly reduced the amount of choline released by a normal epithelial cell, T4056 (**Figure 5.5A**). Similarly, human anti-siglec-9 antibody significantly reduced the amount of choline produced by human colon cancer cells, LIM-2405 (**Figure 5.5B**) and HT-29 (**Figure 5.5C**).

Figure 5.4. Effect of blocking siglec-9 on the cell proliferation of normal epithelial and human colon cancer cells

Dose-response curve for cell proliferation for normal epithelial cell (T4056) and human colon cancer cells (LIM-2405 and HT-29) treated with a human anti-siglec-9 antibody for 8 hrs (**A)**. Percentage of viable cells (T4056, LIM-2405 and HT-29) after treatment (**B)**. Three independent experiments were performed in triplicate wells. Two-way *ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*. Anti-S9 Ab, anti-siglec-E antibody.

A


Human cells treated with anti-S9 Ab
OD Value at 450nm
1.0
0.8
0.6
0.4
0.2
0.0
0ng
50ng
100ng
200ng
300ng
400ng
500ng
600ng
700ng
800ng
900ng
1000ng

B

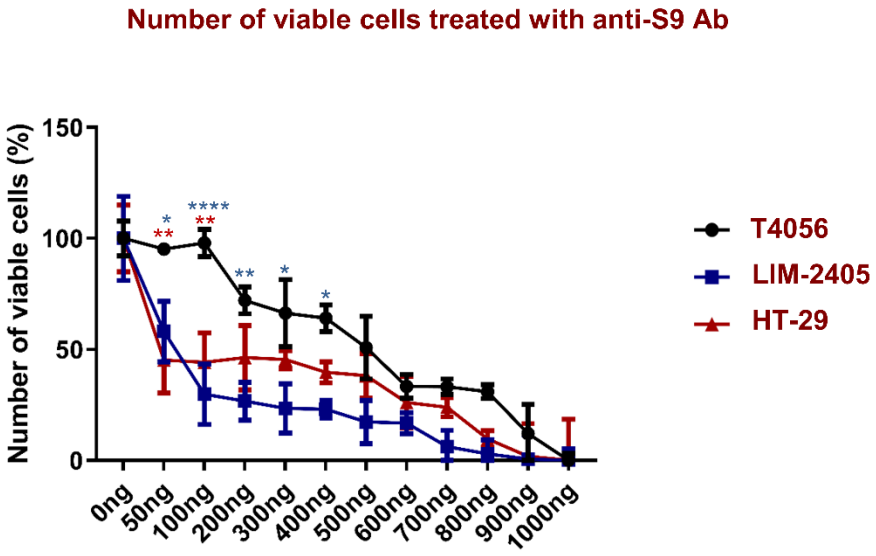
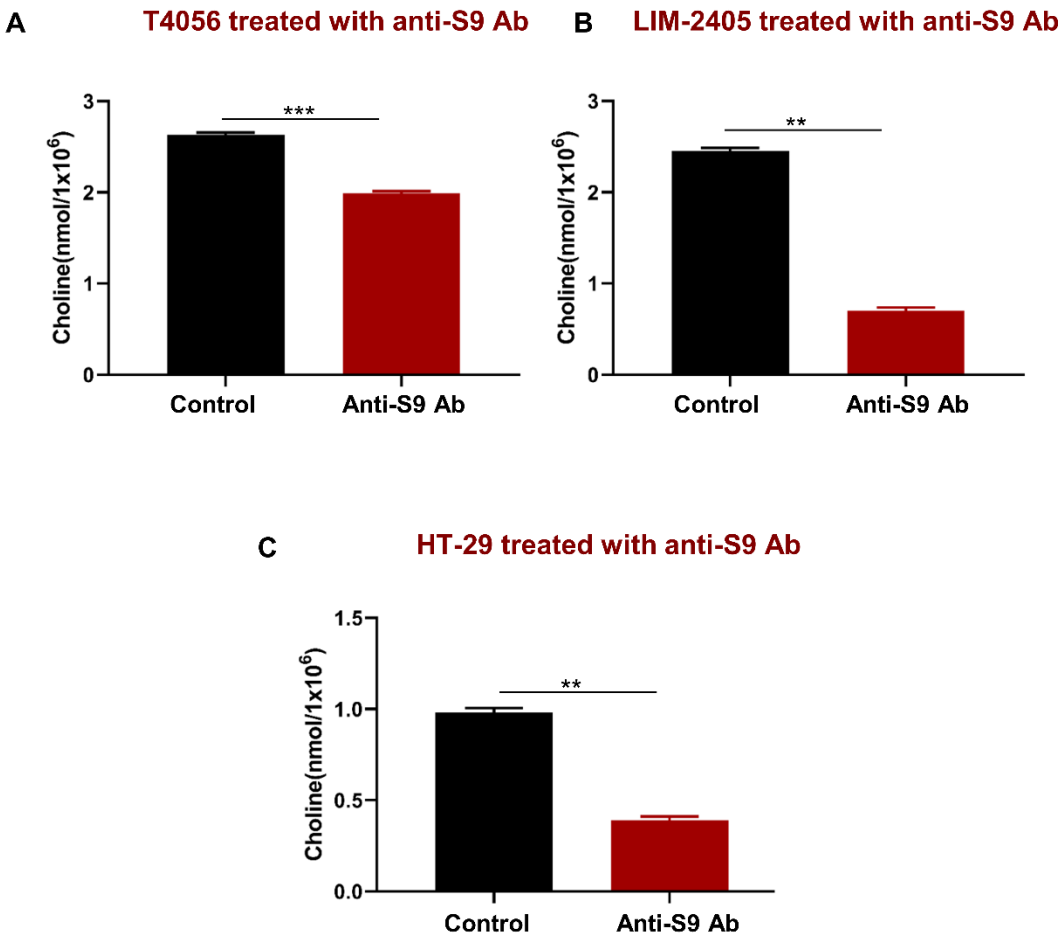

Number of viable cells treated with anti-S9 Ab
Number of viable cells (%)
150
100
50
0
0ng
50ng
100ng
200ng
300ng
400ng
500ng
600ng
700ng
800ng
900ng
1000ng
T4056
LIM-2405
HT-29

Figure 5.5. Effect of blocking siglec-9 on the amount of choline produced by normal epithelial and human colon cancer cells

The amount of choline was measured in normal epithelial (**A**) and human colon cancer cells LIM-2056 (**B**) and HT-29 (**C**) before (control) and after treatment with anti-siglec-9 (anti-S9) antibody. Values in **A**, **B** and **C** are mean ± SEM from at least two independent experiments. Two-way *ANOVA, **p<0.01, ***p<0.001*.


A
T4056 treated with anti-S9 Ab
B
LIM-2405 treated with anti-S9 Ab
Choline(nmol/1x10^6)
3
2
1
0
***
**
Control
Anti-S9 Ab
C
HT-29 treated with anti-S9 Ab
1.5
1.0
0.5
0.0

5.3.6. Effect of blocking siglec-9 on the expression of immunosuppressive markers *in vitro*

The effects of blocking siglec-9 ligand with human anti-siglec-9 antibody on the expression of immunosuppressive markers, IDO and siglec-9, in a normal human epithelial cell line and human colon cancer cells were evaluated. The results demonstrated that human anti-siglec-9 antibody has no effect on the expression of IDO in T4056 and LIM-2405; however, it decreased IDO expression in HT-29 (**Figure 5.6A** and **Figure 5.6B**). No significant difference in expression of siglec-9 was observed in T4056 cells (**Figure 5.6A** and **Figure 5.6C**). However, human anti-siglec-9 antibody significantly decreased siglec-9 expression in LIM-2405 and HT-29 cells when compared to untreated cells. These findings suggest that there is no crosstalk between IDO expression and siglec-9 in T4056 and LIM-2405 cells, but there might be an interaction between IDO and siglec-9 in HT-29 cells. Furthermore, normal epithelial cells expressed lower levels of siglec-9 compared to human colon cancer cells. Moreover, there seem to be molecular differences in siglec-9 expressed by normal epithelial cell and human colon cancer cells. In a normal epithelial cell, two siglec-9 protein bands were noted after treatment with the anti-siglec-9 antibody, while in human colon cancer cells three bands were observed in the controls and these bands were reduced to two bands after treatment with human anti-siglec-9 antibody (**Figure 5.6A**). The addition of a band in cancer cells might imply the functional role of siglec-9 expression in cancer cells that might promote their functional growth, proliferation and migration.

5.3.7. Effect of blocking siglec-9 on the expression of cholinergic markers *in vitro*

The effects of human anti-siglec-9 antibody on the expression of M3R and ChAT was determined. The results demonstrated that human anti-siglec-9 antibody significantly attenuated M3R expression in T4056 cells compared to control untreated cells (**Figure 5.7A** and **Figure 5.7B**). However, human anti-siglec-9 antibody had no effect on the expression of M3R in human colon cancer cells, LIM-2405 and HT-29. Similarly, human anti-siglec-9 antibody had no effect on the expression of ChAT in all cells (**Figure 5.7A** and **Figure 5.7C**). Furthermore, there was a molecular difference observed in the

expression of ChAT in normal epithelial cells, T4056, presented with two bands and human colon cancer cells with a single band (**Figure 5.7A**). Overall, human anti-siglec-9 antibody had no effect on ChAT expression, although the amount of choline was reduced. It might be that human anti-siglec-9 antibody reduced choline before it is converted to ACh or has no functional role on the cholinergic enzymes.

5.3.8. Effect of blocking siglec-9 on the expression of signalling pathways *in vitro*

To determine the mechanism involved in the effects of the human anti-siglec-9 antibody on T4056, LIM-2405 and HT-29 cells, the expression of EGFR, pERK and pSTAT3 *in vitro* was evaluated. The results showed that human anti-siglec-9 antibody attenuated EGFR expression in normal epithelial cells but not in human colon cancer cells (**Figure 5.8A** and **Figure 5.8B**). These findings correspond to results demonstrating lack of blocking effect on the expression of M3R in human colon cancer cells, reinforcing the interaction between M3R expression and EGFR. Phosphorylation of ERK was significantly inhibited in T4056 and LIM-2405, but not in HT-29 cells (**Figure 5.8A** and **Figure 5.8C**). Similar results were noted for pSTAT3, human anti-siglec-9 antibody suppressed phosphorylation of STAT3 in T4056 and LIM-2405 cells, but not in HT-29 cells (**Figure 5.8A** and **Figure 5.8D**). These findings suggest that in normal epithelial cells, human anti-siglec-9 antibody exhibited its effects via suppressing the activation of EGFR and inhibiting phosphorylation of ERK and STAT3. In LIM-2405, human anti-siglec-9 antibody acts by suppressing phosphorylation of ERK and STAT3; however, activation of these pathways was not observed in HT-29 cells. Further studies are warranted to evaluate the possible mechanisms involved in siglec-9 effects on HT-29 cells.

Figure 5.6. Effect of blocking siglec-9 on the expression of immunosuppressive markers *in vitro*

Western blot bands for T4056, LIM-2405 and HT-29 cells are shown in (**A**). Bar graphs displaying the mean intensity of expression of IDO (**B**) and siglec-9 (**C**) in normal epithelial and human colon cancer cell lines treated with human anti-siglec-9 antibody. Data presented as mean ± SEM. Two-*way ANOVA, **p<0.01, ***p<0.001, ****p<0.0001*.

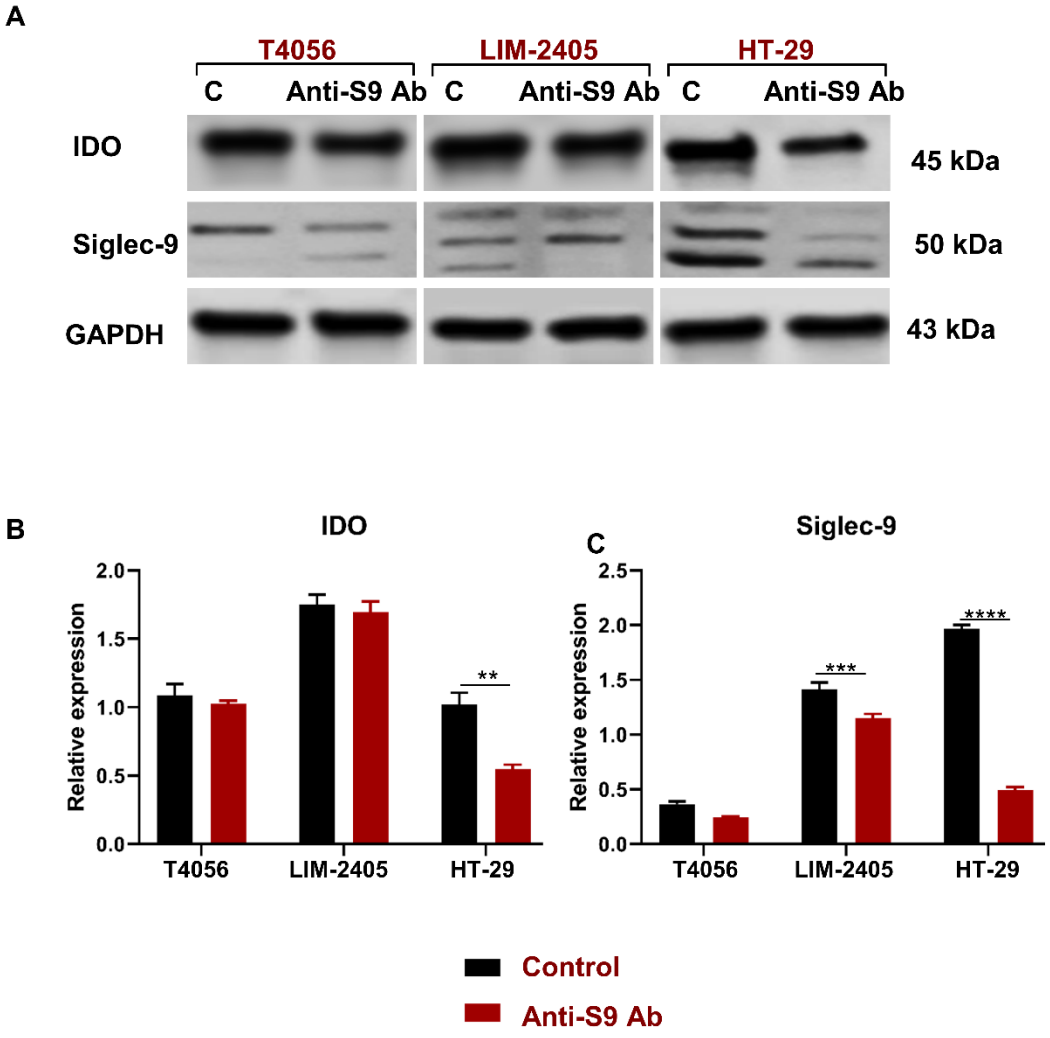

A
T4056
LIM-2405
HT-29
C
Anti-S9 Ab
C
Anti-S9 Ab
C
Anti-S9 Ab
IDO
45 kDa
Siglec-9
50 kDa
GAPDH
43 kDa
B
IDO
Relative expression
2.0
1.5
1.0
0.5
0.0
**
T4056
LIM-2405
HT-29
C
Siglec-9
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
***
****
T4056
LIM-2405
HT-29
Control
Anti-S9 Ab

Figure 5.7. Effect of blocking siglec-9 on the expression of cholinergic markers *in vitro*

Western blot bands for T4056, LIM-2405 and HT-29 cells (**A**). Bar graphs displaying the mean intensity of expression of M3R (**B**) and ChAT (**C**) in normal epithelial and human colon cancer cell lines treated with human anti-siglec-9 antibody. Data presented as mean ± SEM. Two-*way ANOVA, ****p<0.0001*. Anti-S9 Ab, anti-siglec-E antibody.

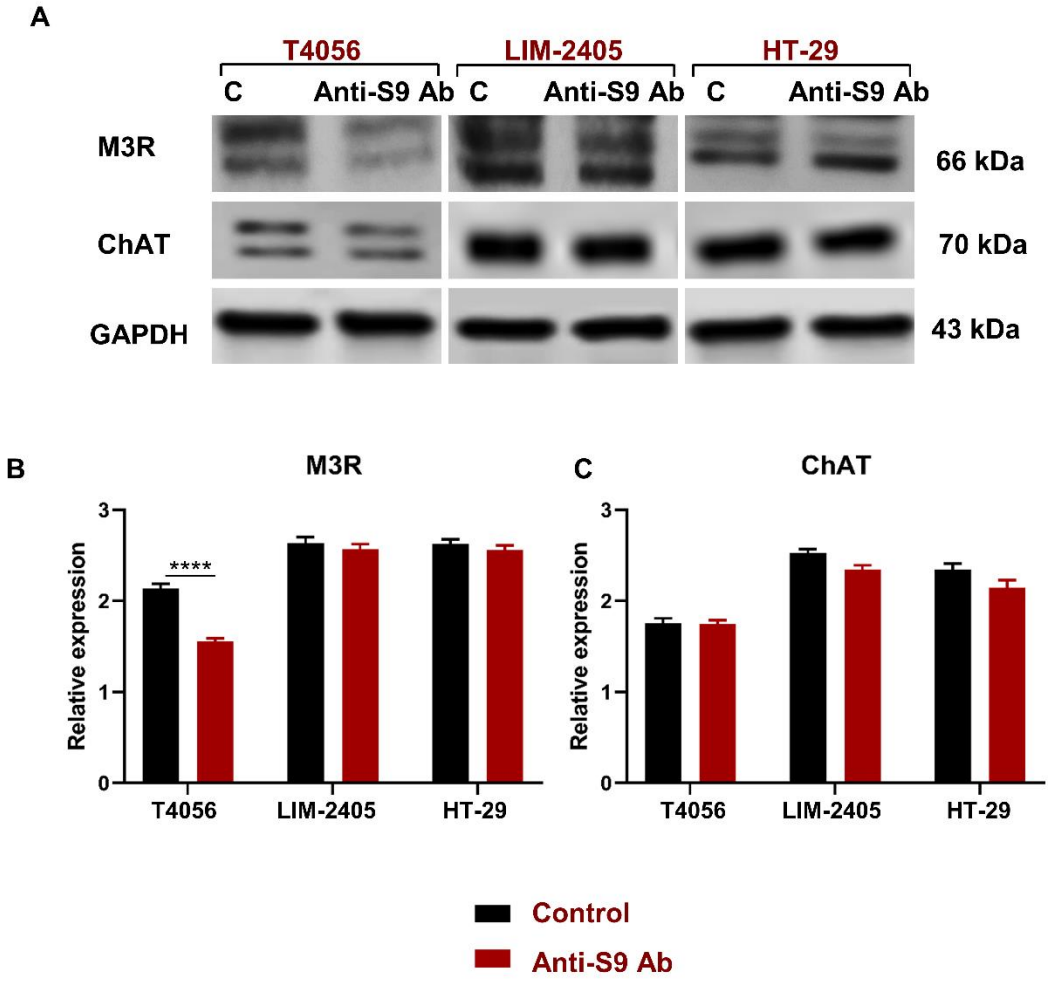
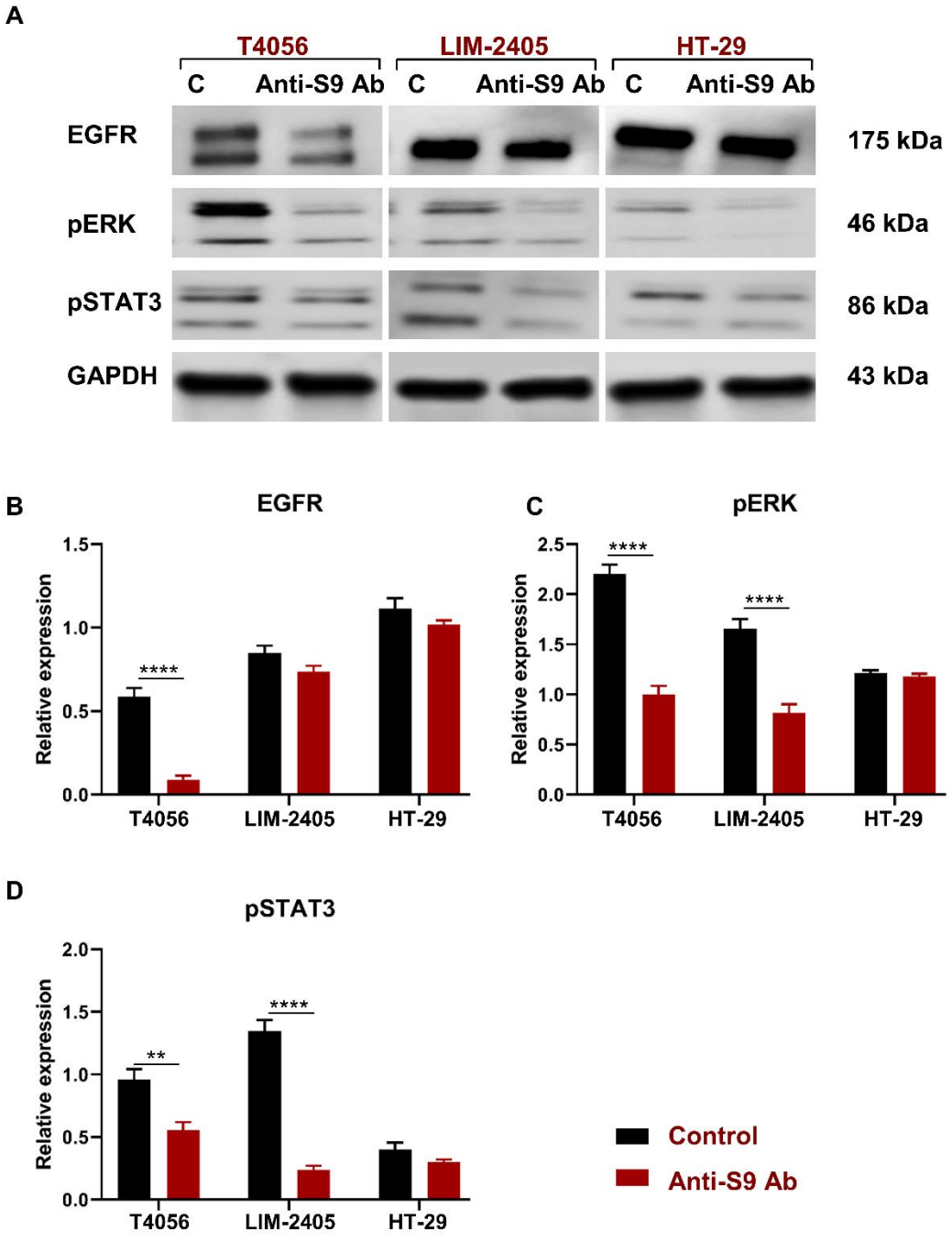

A
T4056
LIM-2405
HT-29
C
Anti-S9 Ab
C
Anti-S9 Ab
C
Anti-S9 Ab
M3R
66 kDa
ChAT
70 kDa
GAPDH
43 kDa
B
M3R
Relative expression
3
2
1
0
****
T4056
LIM-2405
HT-29
C
ChAT
Relative expression
3
2
1
0
T4056
LIM-2405
HT-29
Control
Anti-S9 Ab

Figure 5.8. Effect of blocking siglec-9 on the expression of signalling pathways *in vitro*

Western blot bands for T4056, LIM-2405 and HT-29 cells are shown in (**A**). Bar graphs displaying the mean intensity of EGFR (**B**), pERK (**C**) and pSTAT3 (**D**) expression in normal epithelial and human colon cancer cell lines treated with human anti-siglec-9 antibody. Data presented as mean ± SEM. Two-*way ANOVA, **p<0.01, ****p<0.0001*. Anti-S9 Ab, anti-siglec-E antibody.


A
T4056
LIM-2405
HT-29
C
Anti-S9 Ab
C
Anti-S9 Ab
C
Anti-S9 Ab
EGFR
pERK
pSTAT3
GAPDH
175 kDa
46 kDa
86 kDa
43 kDa
B
EGFR
Relative expression
1.5
1.0
0.5
0.0
****
T4056
LIM-2405
HT-29
C
pERK
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
****
****
T4056
LIM-2405
HT-29
D
pSTAT3
Relative expression
2.0
1.5
1.0
0.5
0.0
**
****
T4056
LIM-2405
HT-29
Control
Anti-S9 Ab

5.4. Discussion

Several lines of evidence have implicated that interaction between the nervous and immune systems plays essential roles in cancer progression. Dysfunction of the nervous system may influence cancer progression by inhibiting functions of the immune system and vice versa. The sympathetic and cholinergic nerves are involved in the control of inflammation, which is believed to be a driving force for cancer metastasis [622]. It has been reported that catecholamines can inhibit the generation of anti-tumour CD8+ T cells, induce apoptosis of lymphocytes, alter the distribution of NK cells and suppress NK cell activity, which is important in the defence against cancer [622, 623]. Thus, the persistent release of neurotransmitters from the nerve terminals may promote tumour growth and metastasis via modulation of the immune system. In fact, we have demonstrated in the previous chapters that dysfunction in cholinergic signalling influenced the expression of immunosuppressive molecules, PD-L1 and PD-L2. Here we evaluated expression of IDO and siglec-9 in human specimens and the effects of blocking siglec-9 on the expression of IDO, siglec-9, cholinergic markers as well as studied signalling pathways involved in these effects. This study is one of the first to report the expression of siglec-9 on normal epithelial cell and human colon cancer cells.

Immune escape mechanisms have emerged to play an essential role in the development and metastasis of tumours. Multiple immunosuppressive mechanisms in the tumour microenvironment can impair the functions of CD8+ T cells. One of these immunosuppressive mechanisms is mediated by the IDO. In the present study, IDO was significantly elevated at the advanced stage of CRC, stage IV, compared to early stages, I, II and III. Furthermore, there was a statistical difference observed at stage III when compared to I. Similarly, the number of cells expressing IDO were augmented at stage IV compared to I, II and III. Moreover, there was a high number of cells expressing IDO at stage III compared to stages I and II. IDO expression is associated with T cell inhibition and/or apoptosis via depleting tryptophan in the tumour microenvironment. It has been noted that the expression of IDO is associated with advanced stages of the disease leading to poor prognosis [624]. Similar to these findings, our results demonstrated

correlation between IDO expression and a high risk of CRC and poor survival outcome. However, no association was observed between IDO expression and patients' age, gender and metastatic status. In CRC patients, the expression of IDO is associated with liver metastases and inversely correlates with infiltrating T cells as well as clinical outcome [625]. However, other studies suggest that the prognostic value of IDO expression may depend on the tissue type as IDO is associated with good outcomes in breast cancer [626].

Siglec-9 expression was associated with the advanced stages of CRC, III-IV, compared to stages I-II. Similarly, there was a significant difference observed at stage III when compared to stages I and II. In addition, number of cells overexpressing siglec-9 were elevated at stage IV compared to stages I-III. Moreover, there were more cells expressing siglec-9 at stage III compared to stage I. These findings may be due to the upregulation of the tumour-associated antigen, mucin 1 (MUC1) on CRC cells [96, 627], as siglec-9 binds to MUC1 [101, 628]. The increase in the expression of siglec-9 provides a protective mechanism for tumour cells via inhibiting NK cell and T cell lysis [101, 629]. In addition, the interaction between siglec-9 and MUC1 expressed on HCT116 human colon cancer cells results in β-catenin recruitment in tumour cells where it is transported to the nucleus, leading to cell growth [101]. In human breast and colon tumour tissues, siglec-9-positive cells associate with the MUC1-positive cells suggesting that siglec-9 might be a counter receptor for MUC1 [101]. In addition, *in vitro* binding of siglec-9 to MUC1 expressed on HCT116 human colon cancer cell line, resulted in β-catenin recruitment in tumour cells where it is transported to the nucleus, leading to cell growth [101]. These findings suggest that the interaction of siglec-9 expressed on immune cells with MUC1 expressed on tumour cells may perhaps be involved in tumour growth, however, the nature of this interaction as well as the cellular framework *in vivo* remains to be defined. Although studies have reported siglec-9 expression in tumours, this study is one of the first to correlate siglec-9 expression with different stages of CRC and clinical parameters. Here we report that high expression of siglec-9 associates with high risk of CRC and poor patients' survival.

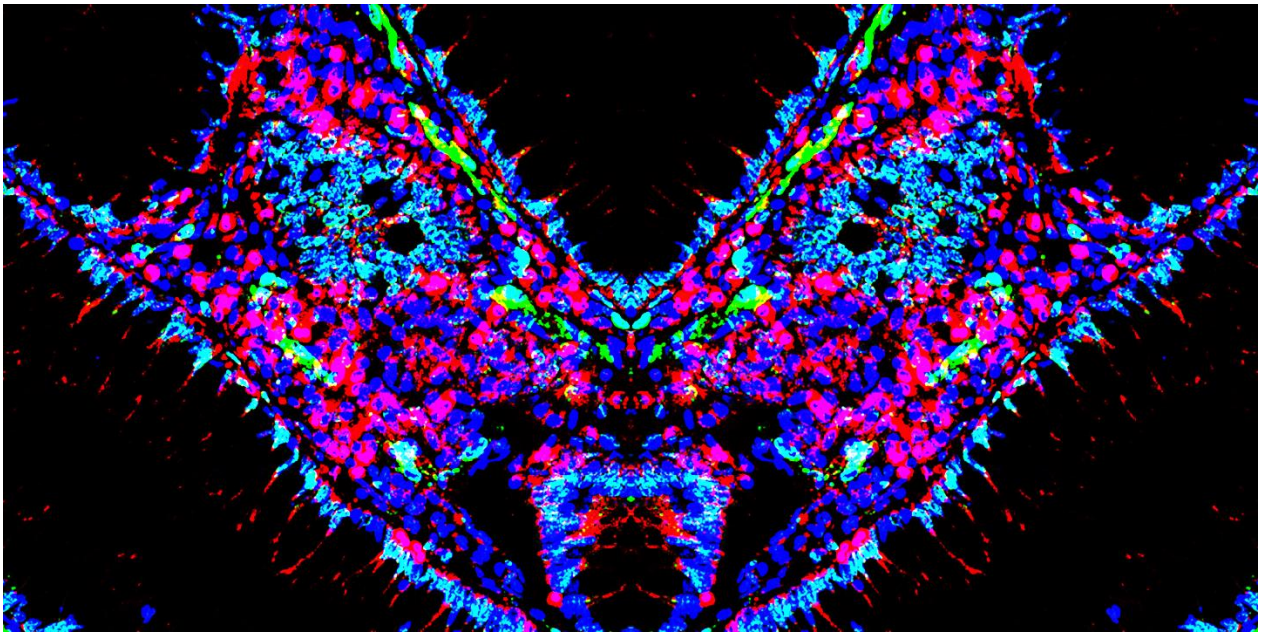
Cholinergic signalling has been showed to promote cancer cells proliferation, invasion and migration by activating M3R [363, 521]. For instance, ACh acting on M3R associated with metastasis and low survival rate of non-small cell lung cancer (NSCLC) patients [366]. In addition, administration of muscarinic receptor inhibitor, atropine, abolished SNU-C4 human colorectal cancer cell migration, however, H508 human colorectal cancer cell migration requires the activation of matrix metalloproteinase-7. In the present study, blocking of siglec-9 significantly reduced normal epithelial cell and human colon cancer cells' ability to produce choline, a precursor for ACh synthesis leading to decreased cell proliferation. Interestingly, blocking siglec-9 exerted no difference in M3R expression on human colon cancer cells, however, M3R expression on normal epithelial cells was significantly attenuated by blocking siglec-9. Furthermore, there was no significant differences observed between controls and treated cells and their expression of ChAT. These findings suggest that siglec-9 might not be exhibiting its affect through M3R or ChAT, but possibly through inhibition of ACh synthesis via reduction of choline production. Therefore, further studies are warranted to elucidate these mechanisms.

5.5. Conclusion

Cancer cells are able to evade the hosts' immune system by upregulating immunosuppressive (IDO, siglec-9) and cholinergic markers (M3R, ChAT) which aid in their invasion, metastasis and/or recurrent disease. The data presented in this chapter associated high levels of siglec-9 and IDO with advanced stages, higher risk of CRC and poor patients' survival outcomes. Overall expression of siglec-9 was associated with M3R, suggesting there might be bi-directional communication. *In vitro* findings demonstrated that human anti-siglec-9 antibody significantly attenuated cell proliferation and choline production in all cells. Blocking siglec-9 had no effects on T4056 and LIM-2405 but significantly decreased IDO expression in HT-29. Similarly, human anti-siglec-9 antibody had no effects on T4056 but significantly reduced siglec-9 expression in human colon cancer cells. Interestingly, blocking siglec-9 decreased M3R expression in T4056 but not human colon cancer cells and had no effects on ChAT expression. This suggests that siglec-9 effects on cholinergic markers might be influenced by the tumour microenvironment. Moreover, blocking siglec-9 significantly inhibited ERK and STAT3 phosphorylation in T4056 and LIM-2405 but not HT-29 cells. Similarly, blocking siglec-9 significantly suppressed the activation of EGFR in T4056 and but not human colon cancer cells. Taken together, human siglec-9 antibody unveiled its effects in a normal epithelial cell via suppressing EGFR/ERK/STAT3 signalling pathway, while in LIM-2405 human colon cancer cells it acts by inhibiting ERK/STAT3 signalling pathway. Therefore, it is imperative to determine the expression status of some or all of these immunosuppressive molecules in order to develop appropriate therapeutic strategies in cancer patients.

CHAPTER SIX

Effect of Inhibiting Siglec-E on the Expression of Immunosuppressive and Cholinergic Markers in an Orthotopic Mouse Model of CRC



6.0. Abstract

Cancer cells have evolved to create favourable microenvironment by expressing immunosuppressive, such as indoleamine-2,3-dioxygenase (IDO) and sialic acid-binding lectins 9 (siglec-9) and cholinergic markers, enabling them to avoid the host's immune detection. Molecular mechanisms and signalling pathways involved in the effects of blocking siglec-E, a murine orthologous protein of human siglec-9, were examined in mouse colon tumour and CT-26, cell line *in vitro*. The effects of *in vivo* treatment with mouse anti-siglec-E antibody on cancer growth, expression of immunosuppressive, cholinergic and angiogenic markers, and tumour spread were evaluated in a mouse orthotopic model of colorectal cancer (CRC) induced by CT-26 cells. *In vitro*, mouse anti-siglec-E antibody significantly inhibited CT-26 cells proliferation in a dose dependent manner. Mouse anti-siglec-E antibody significantly suppressed the expression of siglec-9, cholinergic and angiogenic markers via inhibition of EGFR/AKT/ERK signalling pathway. Interestingly, IDO was not detected in both control and mouse anti-siglec-E antibody treated CT-26 cells, suggesting that tumour microenvironment might influence the expression of IDO. *In vivo* administration of mouse anti-siglec-E antibody reduced tumour growth, expression of immunosuppressive, cholinergic and angiogenic markers via improved hosts' immune response against cancer as demonstrated by increased infiltration of CD4+ and CD8+ T cells through inhibition of ERK phosphorylation. These findings suggest that siglec-9 might influence the expression of IDO, cholinergic and angiogenic markers as well as tumour-associated immune cells. Taken together, these findings suggest that the development of therapies targeting IDO, siglec-9 and cholinergic markers might be beneficial for CRC treatment.

6.1. Introduction

The expression of siglecs in tumours have been reported; however, only recently the role of siglec-9 in cancer has been investigated. Siglecs, sialic acid immunoglobulin-like binding lectins, are involved in discrimination between body cells ('self') and foreign materials ('non-self') [630, 631]. Siglec-9 and its counterpart siglec-E, expressed on murine immune cells, are members of the CD33-related siglecs. Expression of siglec-9 a mouse orthologous protein, siglec-E, have been shown to play a key role in the inhibition of the T cell receptors (TCR)-mediated signalling [631]. Most siglecs contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), and the consensus is that these ITIMs allow the inhibition of certain immune cells that carry activating receptors with tyrosine-based activation motifs (ITAMs) [632, 633]. ITAMs are found on most immune cells and are associated with the activation of B/T cell and natural killer (NK) cell receptors [634]. Some immune cells such as macrophages, B cells, dendritic cells and NK cells can express siglecs [94]. Overexpression of siglec-9 on tumour-infiltrating immune cells creates immunosuppressive microenvironment favourable for tumours to grow [514]. The expression of siglec-9 downregulates innate and acquired immunity [635]. Siglec-9 inhibits the secretion of TNF-α and enhances Il-10 production in macrophages as demonstrated in lipopolysaccharide (LPS) model [636], both cytokines play an important role in the progression of cancer.

In addition, siglec-9 is shown to interact with transmembrane mucins, including mucin (MUC)1 and MUC16 [95]. In human breast and colon tumour tissues, siglec-9-positive cells were associated with the MUC1-positive cells, suggesting that siglec-9 might be a counterreceptor for MUC1 [101]. Furthermore, binding of siglec-9 to MUC1 expressed on HCT116 human colon cancer cell line resulted in β-catenin recruitment in tumour cells where it is transported to the nucleus, leading to cell growth [101]. Inhibition of tumour-associated macrophages via siglec-9 leads to M1 polarization and reduced tumour growth-promoting factors within the tumour microenvironment [94]. Similarly, expression of siglec-9 alters polarisation of macrophages from M1 phenotype (anti-tumour) to M2 (pro-tumour) [637]. In addition, *in vitro* blocking of siglec-9 enhances neutrophil activity

against tumour cells, while siglec-E deficient mice show improved *in vivo* immunosurveillance against tumour cells [94]. However, this outcome is dependent on the stage of tumour development and the microenvironment. These findings suggest that siglec-9 or siglec-E expressed on immune cells can interact with MUC1 expressed on tumour cells, which might be involved in tumour growth, however, the nature of this interaction as well as the cellular framework *in vivo* remains to be defined.

In addition to siglec-9 expression, tumours can express indoleamine-2,3-dioxygenase (IDO); overexpression of this enzyme leads to increased degradation of the essential amino acid L-tryptophan along the kynurenine pathway. A decrease in L-tryptophan leads to T cell inhibition, promoting a favourable environment and a mechanism for tumours to avoid the host's immune detection [514, 620]. Expression of IDO is dependent on the presence of cytokines such as interferon-gamma (INF-γ) [624, 638]. IDO is mainly expressed by dendritic cells in tumour stroma and tumour draining lymph nodes; however, other tumour microenvironment constituents such as macrophages and fibroblasts are capable of expressing IDO [100, 639, 640]. In CRC patients, IDO expression associates with liver metastases and inversely correlates with infiltrating T cells and patients' clinical outcome [624]. In basal-like breast carcinoma, high expression of IDO has an independent good prognostic value [93]. These findings indicated the important role of IDO in cancer progression; however, the mechanisms of action need further elucidation.

Moreover, cholinergic signalling has been reported to play an essential role in cancer progression, as reviewed in Chapter 1 [371, 641]. For example, studies have demonstrated that acetylcholine (ACh) acts as a paracrine growth factor in lung and gastric cancers [518, 521]. Cholinergic signalling plays an important role in the regulation of immunosuppressive markers, as demonstrated in Chapters 3-5. In this chapter, the effects of blocking siglec-E with a mouse anti-siglec-E antibody were evaluated (i) on murine CT-26 cell line, (ii) tumour growth in mice bearing CT-26 cell-induced CRC (iii) expression of immunosuppressive, cholinergic and angiogenic markers in both CT-26 cells and tumour tissues and (iv) presence of tumour-infiltrating immune cells in tumours. The expression of siglec-9, IDO and cholinergic signalling play a significant role in cancer

development. Understanding crosstalk between siglec-E and cholinergic signalling in vivo is crucial as it this interaction plays a vital role in cancer progression.

6.2. Materials and Methods

6.2.1 Mice

As previously stated in Chapter 4, male BALB/c mice aged 5-8 weeks (n=16) were purchased from the Animal Resources Centre and housed in groups of 4 per cage. All animals were kept in a temperature-controlled environment with 12-hour (hr) light/dark cycle at approximately 22°C with access to food and water. The mice were allowed to acclimatise for at least one week before undergoing surgery. All animal experiments in this study complied with the guidelines of the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes under approval of the Victoria University Animal Experimentation Ethics Committee (ethics number AEETH 15-011). All efforts were made to lessen animal suffering.

6.2.2. Cell culture

As previously described in Chapter 4, murine colorectal cancer cell line (CT-26) was cultured in Roswell park memorial institute (RPMI) 1640 culture media supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% Glutamine, at 37°C, 5% CO_2 and 95% air atmosphere. Passage of cells was conducted with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) every 3-4 days. When cells grew into confluent or semiconfluent monolayers in the 75cm^2 medium flasks, they were either passaged or used.

6.2.3. Cell viability

The water-soluble tetrazolium-1 (WST-1) assay kit (Roche Diagnostics GmbH, Germany) was used to determine the viability of CT-26 cells. WST-1 is cleaved to form formazan dye via a complex cellular interaction at the cell surface. This interaction is contingent on the glycolytic nicotinamide adenine dinucleotide phosphate (NADPH) production of the viable cells. Hence, the amount of formed formazan dye correlates to the number of viable cells in the culture. CT-26 cells were seeded and cultured at 1×10^4 cells per well in 96 well plates for 24hrs. Cells were then treated with various concentration of a mouse anti-

siglec-E antibody (AF5806; R&D Systems, Australia) for 8hrs. All treatments were performed in triplicates, and three independent experiments were conducted. WST-1 reagent (10µL) was added to each well and incubated at 37^{o}C for 1hr. Cellular proliferation at the absorbance of 450nm was measured using a microplate reader (Varioskan Flash, Thermo Scientific).

6.2.4. Choline/acetylcholine assay

The choline/acetylcholine assay kit (Abcam, Australia) was used to measure the concentration of choline in CT-26 cell lysates. The assay was carried out in accordance with the instructions provided by the manufacturer. Briefly, CT-26 (1x10^{6} cells) were cultured overnight, after which cells were treated with 50ng of a mouse anti-siglec-E antibody for 8hrs. Cells were lysed in 500µL choline assay buffer before commencing choline measurements using a microplate reader (Varioskan Flash, Thermo Scientific) at absorbance of 570nm. All treatments were performed in duplicates, and two independent experiments were conducted

6.2.5. Orthotopic implantation of CT-26 tumour cells

Mice were anaesthetised using xylazine (10mg/kg), and ketamine (80mg/kg) injected intraperitoneally. The level of anaesthesia during the surgery was monitored using the paw pinch reflex test. The eyes of the animals were treated with ViscoTears to protect them from drying out during the surgery. Mice were placed on an operating table on a heat mat (30-36°C), and all procedures were performed under aseptic conditions. All instruments were autoclaved and only opened when ready to operate. The abdomen was shaved and swabbed with 70% ethanol and covered with sterile film. A small midline abdominal incision was made, and the caecum was exteriorised on sterile gauze. Matrigel (25µL) containing CT-26 cell suspension (1 x 10^{6} cells) was injected into the caecum wall of BALB/c using an insulin needle. After injection, the abdominal muscle wall was sutured using polygalactone and skin using surgical silk or dissolvable skin sutures. The incision area was sterilised by saline followed with iodine. Mice were given an analgesic Temgesic/Buprenorphine (0.05mg/kg) subcutaneously. Mice were then monitored

visually during recovery time (about 1-1.5hrs) and, when fully conscious, they were returned to an animal holding room in the animal facility.

6.2.6. Intraperitoneal injections and tissue collection

Five days post-surgery, mice were randomly divided into two groups: sham/vehicle-treated and anti-siglec-E antibody-treated. Vehicle-treated BALB/c mice received intraperitoneal injections of sterile water, and the study group received 50ng/g of a mouse anti-siglec-E antibody dissolved in sterile water every third day [642] The volume of the administered solution was calculated per body weight with the maximum volume of 200µL per injection. Mice were culled at 28 days post-surgery via lethal injection and tumours were removed, weighed and used for western blot, flow cytometry, proteome profiler array and immunohistochemistry. Tumour tissues were used to assess angiogenesis, tumour-infiltrating immune cells, and expression of immunosuppressive and cholinergic markers. Tumour tissues used for flow cytometry analysis were collected into RPMI media, for western and proteome profiler arrays were snap-frozen in liquid nitrogen and samples used for immunohistochemistry were placed in Zamboni's fixative (2% formaldehyde 0.2% picric acid).

6.2.7. Histopathology

For histological examination, liver samples from mice treated with vehicle and a mouse anti-siglec-E antibody were collected into Zamboni's fixative overnight at 4˚C. The next day, tissues were washed in DMSO (3 times x 10 min) and in PBS (3 times x 10 min) and placed into a solution consisting of 100mL of PBS + 30g of sucrose + 0.1g of sodium azide overnight. Samples were then embedded into OCT, and 5µm transverse sections were cut and placed onto 1% gelatin-coated glass slides. The sections were allowed to dry at room temperature for at least one hour then processed for standard haematoxylin and eosin staining. Following staining, they were dehydrated through graded ethanols, transferred into histolene and mounted using fluorescent mounting medium. Images were taken on a slide scanner microscope (Zeiss, Germany).

6.2.8. Immunohistochemistry in cross sections

Tumour tissues collected from vehicle-treated and a mouse anti-siglec-E antibody-treated group were fixed with Zamboni's fixative overnight at 4°C. Next day, fixative was cleared off by washing samples for 10 mins three times with DMSO (Sigma-Aldrich, Australia) followed by three times 10 mins washes with phosphate buffered saline (PBS). Tissues were then embedded in optimum cutting temperature medium (OCT) and frozen using 2-methyl butane (isopentane) and liquid nitrogen. Samples were stored in -80°C freezer until cryo-sectioned. Tissues were cut at 10µm section thickness using a Leica CM1950 cryostat (Leica Biosystems, Germany), adhered to slides and allowed to dry at room temperature for 1hr before commencing staining process. OCT was washed off with PBS containing 0.01 % Triton X-100 (PBST) for 5 mins. Using a liquid Blocker Super Pap Pen, samples were outlined to reduce the volume of antibody used. The endogenous activity was blocked using 10% normal donkey serum for 1h at room temperature, followed by PBST washes. Samples were then incubated with primary antibodies (**Table 6.1**) against immunosuppressive, cholinergic and angiogenic markers overnight at 4°C. Sections were then washed in PBST before incubation with secondary antibodies labelled against primary antibodies (**Table 6.2**) for 2hrs at room temperature in the dark, followed by PBST washes. The sections were incubated with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (D1306, Life Technologies, Australia) for 1 min. Sections were given final washes in PBST and then mounted with DAKO mounting media (Agilent Technologies, Australia). Coverslips were placed over each section and left to dry overnight before imaging. Sections were viewed under a Nikon Eclipse Ti laser scanning confocal microscope (Nikon, Japan), whereby eight randomly chosen images from each sample were captured with a 40× objective and analysed using image analysis software (Nikon, Japan).

6.2.9. Western blot

Proteins extracted from CT-26-induced tumour tissues and CT-26 cells were evaluated for the expression of immunosuppressive, cholinergic and angiogenic markers as well as cell signalling pathways, phospho signal transducer and activator of transcription 3 (pSTAT3), phospho extracellular signal-regulated kinase (pERK½), phospho serine/threonine kinase or protein kinase B (pAKT) and epidermal growth factor receptor

(EGFR) by western blot. CT-26 cells were incubated with 50ng of mouse anti-siglec-E antibody for 8hrs. After treatments, cells were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer (pH 7.4, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 in PBS, Sigma) containing protease and phosphatase inhibitors cocktail (Roche Applied Science, Switzerland). For tumour samples, 100mg of tumour tissues per mouse were weighed, and tumour samples from 3 mice per band for the first two bands and tumour samples from 2 mice for the third band were pooled together. Samples were then homogenised in 500µL of RIPA buffer containing protease and phosphatase inhibitors cocktail. Cellular proteins (20µg) from CT-26 cell line and 25µg protein from tumour samples were separated by 8% to 12% sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis. The separated fragments were transferred to 0.22µm polyvinylidene fluoride membranes, which were blocked with 5% skim milk in PBS containing 0.1% Tween 20 and incubated overnight at 4^{o}C in platform shaker at 40rpm speed. The membranes were incubated with primary antibodies (**Table 6.1**) overnight at 4^{o}C. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (**Table 6.2**) for 2hr at room temperature followed by three times PBS-0.1% Tween 20 washes. Glyceraldehydes-3-phosphate dehydrogenase (GADPH) was used as a loading control. Protein detection was performed using enhanced chemiluminescence reagents. Chemiluminescent signal was captured using the FluorChem FC2 system. The expression level of each protein was quantified using ImageJ software.

6.2.10. Proteome profiler mouse phospho-RTK array kit

As previously described in Chapter 4, the assay was carried out in accordance with the instructions provided by the manufacturer. Briefly, tumour samples from each group were pooled and lysed in Lysis Buffer 17 prepared with protease inhibitors. Samples were mixed by pipetting up and down to resuspend, and lysates were gently rocked at 4°C for 30 mins on a rocking platform shaker. Tumour lysates were centrifuged at 1500rpm for 5 mins at 4°C and supernatants were transferred into clean test tubes. Array membranes were placed onto 4-well multi-dish and incubated with Array Buffer 1 for 1hr at room temperature on a rocking platform shaker. After 1hr incubation, Array Buffer 1 was

aspirated out, and membranes were incubated with tumour lysates overnight at 4°C on a rocking platform shaker. Membranes were then washed with 1x Wash Buffer for 3x10 mins. Membranes were incubated with anti-phospho-tyrosine-HRP antibody at room temperature for 2hr on a rocking platform shaker. Membranes were then washed with 1x Wash Buffer for 3x10 mins. Membranes were then incubated with chemiluminescence reagent mix, and chemiluminescent signal was captured using the FluorChem FC2 system.

6.2.11. Flow cytometric cell staining

On the day of culls, tumours were collected into RPMI media and tumour tissues were processed into single-cell suspensions for fluorescence-activated cell sorting (FACS) analysis. Single-cell suspensions were performed by mechanically dissecting tumours into small pieces and incubating with 2mL of collagenase (0.1%w/v in 1mL of α-MEM) at 37°C for 2hrs with 30 mins intervals of mechanical dissociation. Tumour suspensions were filtered with 40µm cell strainers Falcon® into 50mL Falcon® tubes and were then centrifuged at 1500rpm for 5 mins at 4°C. Cell pellets were incubated with 1x red blood cell lysing buffer for 3 mins at 37°C. Cell pellets were then resuspended in 1mL of FACS buffer to create a single cell suspension and accounted using a hemocytometer.

Viable cell pellets were incubated with two different antibody cocktails (**Table 6.3**). Cocktail 1 contained leukocyte infiltration markers (CD45, CD11b, CD4, CD8a, CD193 (CCR3), Siglec-F and Fc Block), while cocktail two was comprised of (CD45, CD11b, CD19, CD206, CD115, F480, Ly-6C, Ly-6G and Fc Block). Tumour cells ($10x10^6$) cells (400µL) were aliquoted in BD Falcon® FACS tubes. Cells were centrifuged at 1300rpm for 3 mins at 4°C. Cells were then incubated with 200µL of antibody cocktails for 1hr at 4°C. After incubation, cells were centrifuged at 1300rpm for 3 mins at 4°C and supernatants aspirated. Cells were then resuspended in 200µL FACS buffer and filtered through 35µm filters in a 5mL BD Falcon® tube. Prior to FACS analysis, cells were incubated with viability solution, 7-amino-actinomycin D (7-AAD, 1:20) to gate on the viable cell populations.

Table 6. 1. Details of primary antibodies used in this study

Primary antibodies				
Markers	**Host Species & Clonality**	**Dilution**	**Source**	**Catalogue no.**
Sigec-9	Rabbit, polyclonal	1:500	Abcam, Australia	ab197981
IDO	Mouse, monoclonal	1:200	Abcam, Australia	ab55305
M3R	Rabbit, polyclonal	1:500	Abcam, Australia	ab126168
α7nAChR	Mouse, monoclonal	1:500	Novus, Australia	7F10G1
ChAT	Goat, polyclonal	1:500	Abcam, Australia	ab134021
VAChT	Sheep, polyclonal	1:500	Abcam, Australia	ab31544
FOXP3	Mouse, monoclonal	1:500	Abcam, Australia	ab20034
VEGF	Rabbit, polyclonal	1:500	Abcam, Australia	ab46154
CD31	Rat, monoclonal	1:500	Abcam, Australia	ab7388
TGF-β	Rabbit, polyclonal	1:500	Abcam, Australia	ab155264
EGFR	Rabbit, monoclonal	1:1,000	Cell signalling,	#4267
pAKT	Rabbit, monoclonal	1:1,000	Cell signalling,	#4060
pERK	Rabbit, monoclonal	1:1,000	Cell signalling,	#3192

Table 6. 2. Details of secondary antibodies used in this study

Secondary antibodies			
Alexa Fluor 488	Anti-goat Anti-sheep Anti-rat	1:250	Jackson ImmunoResearch Laboratories, United States
Alexa Fluor 594	Anti-rabbit	1:250	Jackson ImmunoResearch Laboratories, United States
Alexa Fluor 647	Anti-mouse	1:250	Jackson ImmunoResearch Laboratories, United States
Anti-mouse	IgG HL HRP	1:10,000	Abcam, Australia
Anti-rabbit	IgG HL HRP	1:10,000	Abcam, Australia
Anti-rat	IgG HL HRP	1:10,000	Abcam, Australia
Anti-goat	IgG HL HRP	1:10,000	Abcam, Australia

6.2.12. Data analysis

Images were captured on a Nikon Eclipse Ti multichannel confocal laser scanning system (Nikon, Japan). Z-series images were acquired at a nominal thickness of 1µm (1024 x 1024 pixels). Image J software (National Institute of Health, Bethesda, MD, USA) was employed to convert images from RGB to greyscale 8-bit binary; particles were then analysed to obtain the percentage area of immunoreactivity [591]. All immunohistochemistry images and western blot bands were quantified, and statistical analysis was performed by Student's *t-test*.

The characterisation of tumour-infiltrating immune cells was performed and quantified by flow cytometry (BD FACs Aria I, BD Bioscience, CD, USA) with the aid of BD FACS DIVA software (BD Bioscience, CA, USA.). Two hundred thousand (200,000) events were collected per sample and analysed by BD FACS DIVA software. As each antibody conjugate produces a distinctive emission spectrum, each experiment employed single colour compensation controls to optimise photo-multiplier tube (PMT) voltages and calculate spectral overlap (where applicable). Excel, SPSS and Prism (GraphPad software, La Jolla, CA, USA) were utilised to aid in the statistical analysis and $p<0.05$ was considered significant.

6.3. Results

6.3.2. Effect of blocking siglec-E on CT-26 ability to produce choline *in vitro*

In Chapter 4, it was demonstrated that CT-26 mouse colon cancer cells can synthesise ACh. Here, the effect of a mouse anti-siglec-E antibody on the ability of CT-26 cells to synthesise ACh was evaluated. The amount of choline was measured in CT-26 cell lysates ($1x10^6$ cells). The results showed that a mouse anti-siglec-E antibody significantly reduced choline production when compared to control (**Figure 6.2**).

Figure 6.1. Effect of a mouse anti-siglec-E antibody on CT-26 cells proliferation *in vitro*

CT-26 cells treated with different concentrations of a mouse anti-siglec-E antibody (**A**). Number of viable cells after 8hrs incubation with various concentrations of a mouse anti-siglec-E antibody (**B**). Values in **A** and **B** are mean ± standard error of the mean (SEM) from at least three independent experiments performed in triplicates.

CT-26 cells treated with anti-siglec-E antibody

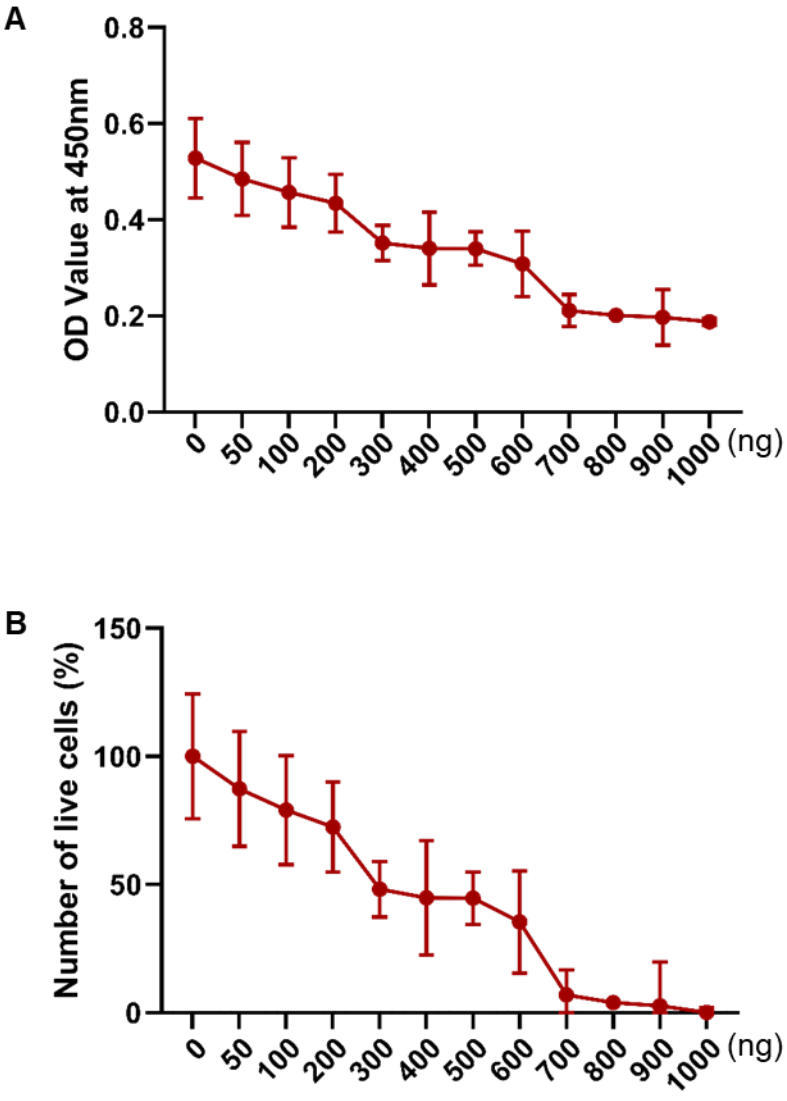
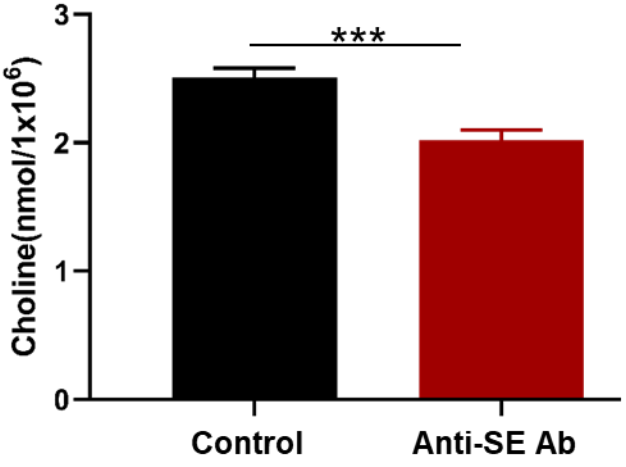


Figure 6.2. Effect of a mouse anti-siglec-E antibody on choline production *in vitro*

The amount of choline was measured in CT-26 cells treated with a mouse anti-siglec-E antibody. Values presented as mean ± SEM from at least two independent experiments. Student's *t-test, ***p<0.001*. Anti-SE Ab, anti-siglec-E antibody.

CT-26 cells treated with anti-siglec-E antibody



6.3.3. Expression of immunosuppressive and cholinergic markers *in vitro*

6.3.3.1. Effect of blocking siglec-E on the expression of siglec-E and IDO in CT-26 cells

In Chapter 5, it was shown that human colon cancer cells overexpressed siglec-9 and IDO when compared to the normal epithelial cell, and this was dampened by recombinant human anti-siglec-9 antibody treatment. In this chapter, the effect of a mouse anti-siglec-E antibody on CT-26 cells ability to express immunosuppressive and cholinergic markers was determined. CT-26 cells were pre-treated with 100ng of a mouse anti-siglec-E antibody for 8hrs prior protein analyses via western blot staining. Treatment with anti-siglec-E antibody significantly decreased the expression of siglec-E (**Figure 6.3A** and **B**); however, IDO was not detected in both control and treated cells.

6.3.3.2. Effect of blocking siglec-E on the expression of M3R and ChAT in CT-26 cells

Cholinergic receptors expressed by colon cancer cells involved in tumour growth and progression. *In vitro* data demonstrated that CT-26 cells can produce choline, but, whether CT-26 cells can express ChAT, an enzyme required for ACh synthesis, and M3R was evaluated by western blot. Mouse anti-siglec-E antibody treatment significantly reduced M3R expression compared to control (**Figure 6.4A** and **B**). Similarly, application of a mouse anti-siglec-E antibody significantly attenuated ChAT expression compared to control (**Figure 6.4A** and **C**).

6.3.4. Effect of blocking siglec-E on the phosphorylation of kinases and EGFR activation in CT-26 cells

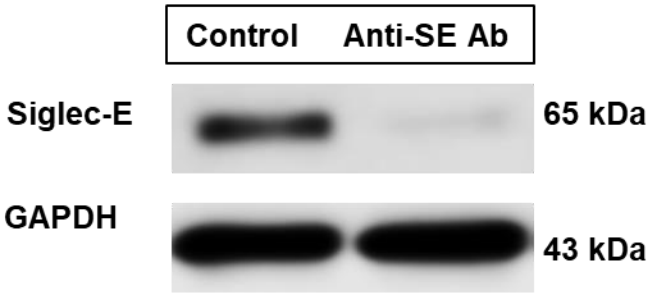
The effects of blocking siglec-E on the phosphorylation of AKT, ERK and the activation of EGFR was assessed. The results showed that a mouse anti-siglec-E antibody inhibits the activation of EGFR (**Figure 6.5A** and **B**, difference between values (Δ): -0.70±0.11, *p<0.01*) and suppresses phosphorylation of AKT (**Figure 6.5A** and **C**, **Δ:** -0.52±0.04, *p<0.001*) and ERK (**Figure 6.5A** and **D**, **Δ:** -1.22±0.07, *p<0.0001*) in CT-26 cells. These

findings suggest that anti-siglec-E antibody exhibits its effects *in vitro* via inhibition of EGFR/AKT/ERK signalling pathway.

Figure 6.3. Effect of a mouse anti-siglec-E antibody on the expression siglec-E in CT-26 cells

Western blot bands for siglec-E expression in CT-26 cells treated with a mouse anti-siglec-E antibody (**A**). Bar graphs displaying the mean intensity of siglec-E expression in CT-26 cells treated with a mouse anti-siglec-E antibody (**B**). Data presented as mean ± SEM, Student's *t-test, ***p<0.001*. Anti-SE Ab, anti-siglec-E antibody.

A


Control
Anti-SE Ab
Siglec-E
65 kDa
GAPDH
43 kDa

B

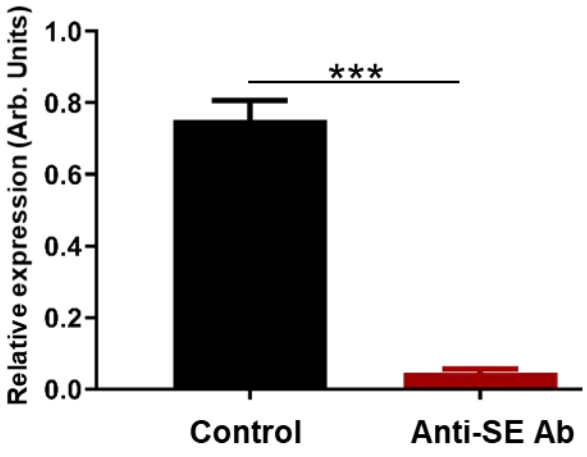

Relative expression (Arb. Units)
1.0
0.8
0.6
0.4
0.2
0.0
***
Control
Anti-SE Ab

Figure 6.4. Effect of a mouse anti-siglec-E antibody on the expression of cholinergic markers in CT-26 cells

Western blot bands for M3R and ChAT expression in CT-26 cells treated with a mouse anti-siglec-E antibody (**A**). Bar graphs displaying the mean intensity of M3R (**B**) and ChAT (**C**) expression in CT-26 cells treated with a mouse anti-siglec-E antibody. Data presented as mean ± SEM, Student's *t-test, **p<0.01, ***p<0.001*. Anti-SE Ab, anti-siglec-E antibody.

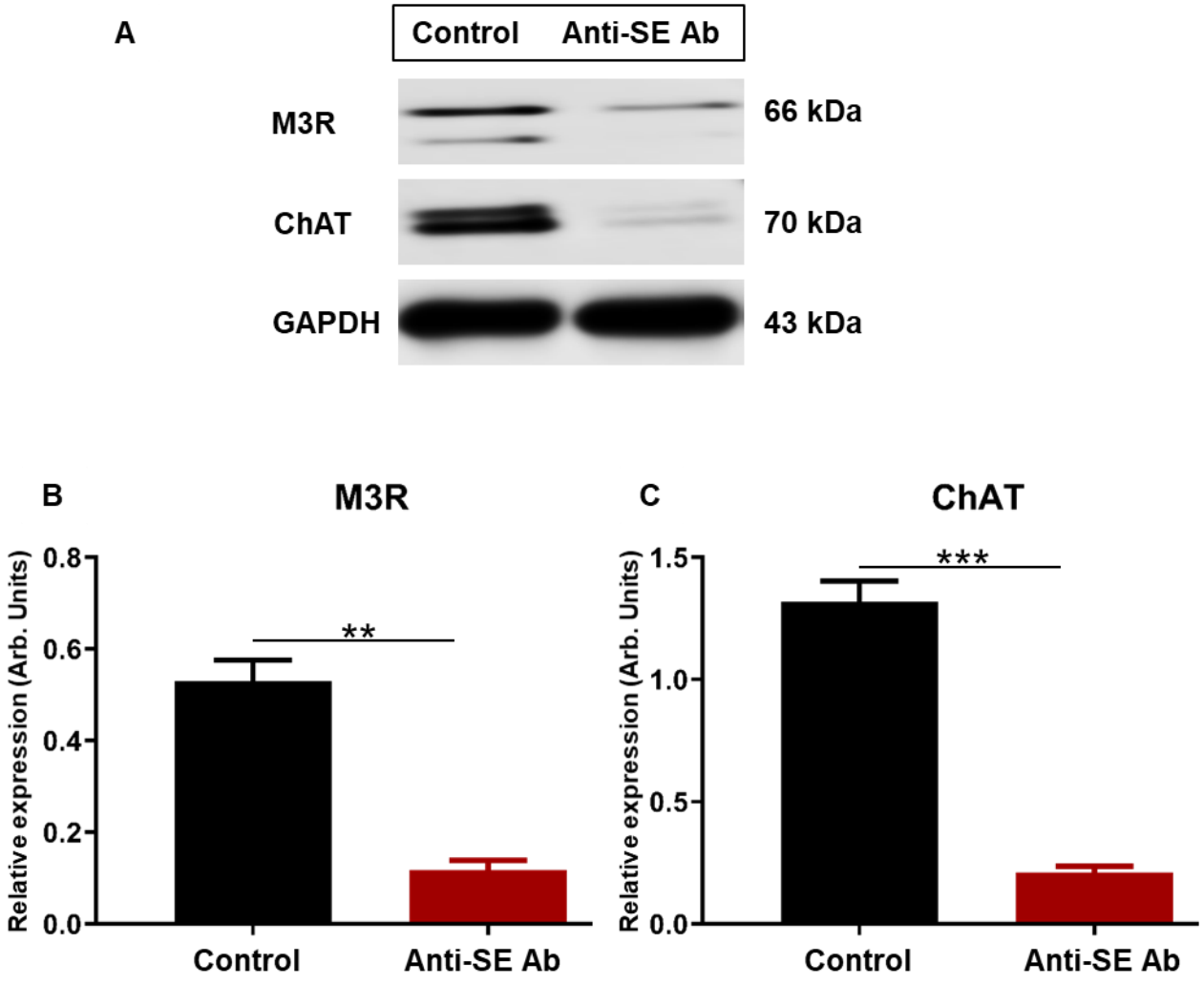
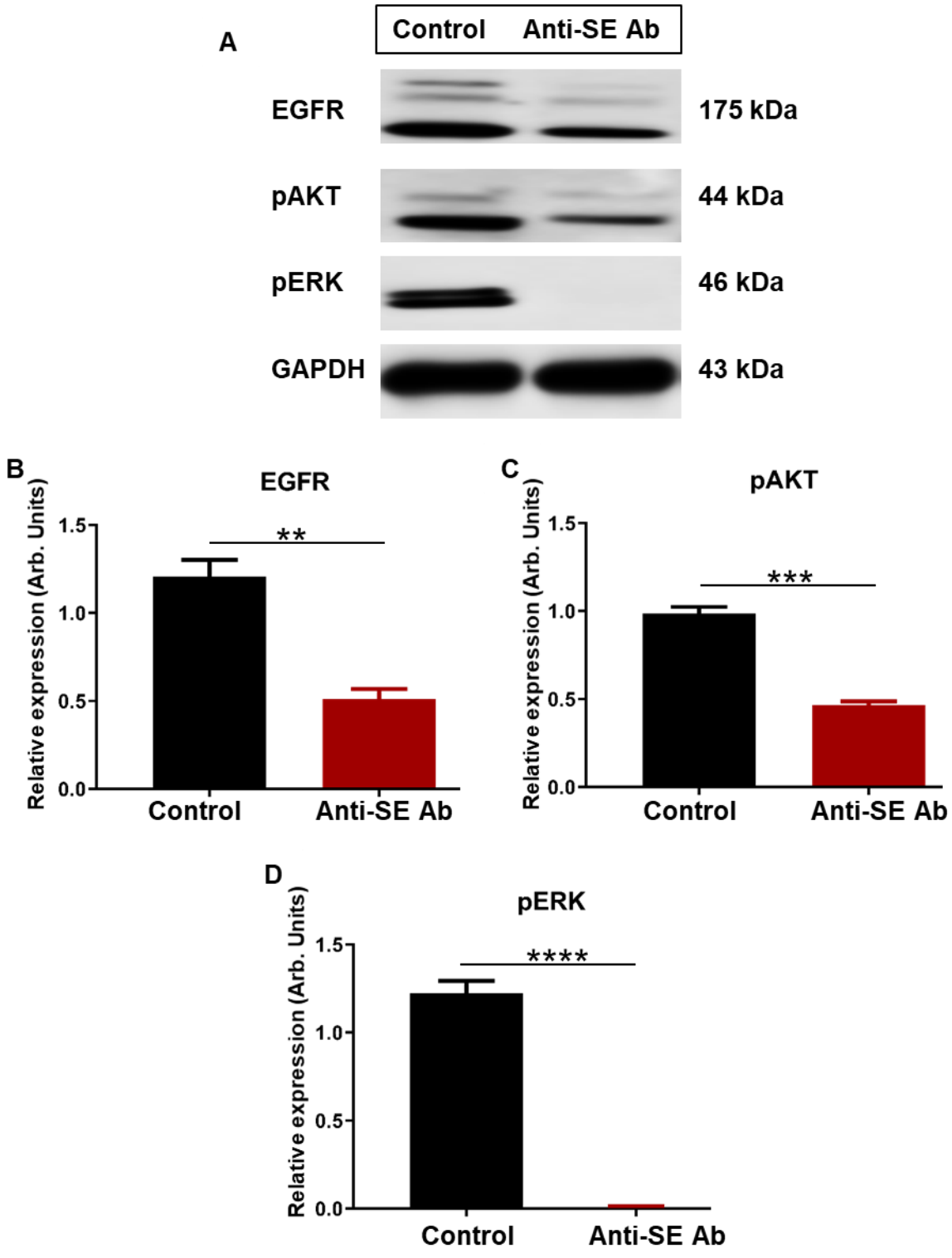

A
Control
Anti-SE Ab
M3R
66 kDa
ChAT
70 kDa
GAPDH
43 kDa
B
M3R
Relative expression (Arb. Units)
0.8
0.6
0.4
0.2
0.0
**
Control
Anti-SE Ab
C
ChAT
Relative expression (Arb. Units)
1.5
1.0
0.5
0.0
***
Control
Anti-SE Ab

Figure 6.5. Effect of blocking siglec-E on the expression of protein kinases in CT-26 cells

Western blot bands for EGFR, pAKT and pERK expression in CT-26 cells treated with a mouse anti-siglec-E antibody (**A**). Bar graphs displaying the mean intensity of EGFR (**B**), pAKT (**C**) and pERK (**D**) expression in CT-26 cells treated with a mouse anti-siglec-E antibody. Data presented as mean ± SEM, Student's *t-test, **p<0.01, ***p<0.001, ****p<0.0001.* Anti-SE Ab, anti-siglec-E antibody.


A
Control
Anti-SE Ab
EGFR
175 kDa
pAKT
44 kDa
pERK
46 kDa
GAPDH
43 kDa
B
EGFR
Relative expression (Arb. Units)
1.5
1.0
0.5
0.0
**
Control
Anti-SE Ab
C
pAKT
Relative expression (Arb. Units)
1.5
1.0
0.5
0.0
***
Control
Anti-SE Ab
D
pERK
Relative expression (Arb. Units)
1.5
1.0
0.5
0.0
****
Control
Anti-SE Ab

6.3.5. Effect of blocking siglec-E on tumour growth *in vivo*

The orthotopic mouse models of CRC has been shown to replicate features (such as regional or distant metastasis) shown in the human CRC condition with high fidelity, which feature cancer cells growing in their natural location [643]. To investigate whether blocking siglec-E influences tumour growth, an orthotopic model of colorectal cancer was established by implanting CT-26 murine colon cancer cells into the mouse caecum wall. Five days post-surgery, tumour-bearing mice were intraperitoneally injected with either vehicle solution (sterile water) or a mouse anti-siglec-E antibody every third day for 28 days. Mice were culled, tumours removed, and the weight, size and volume were measured. In addition, tumours around the caecum were counted and collected. The results showed a significant reduction of tumour size in mice treated with anti-siglec-E antibody compared to vehicle-treated group (**Figure 6.6A** and **B**). Blocking siglec-E significantly decreased tumour weight (**Figure 6.6C**) and tumour volume compared to sterile water treatment **(Figure 6.6D**). Furthermore, tumour-bearing mice treated with sterile water had more polyps and invasive tumours around the caecum compared to anti-siglec-E antibody-treated group (**Figure 6.7**, Δ: -30.00±5.120, $p<0.0001$).

6.3.6. Effect of blocking siglec-E on tumour metastasis *in vivo*

Cancer metastasis is one of the leading causes of cancer-related death in CRC. The liver is the main location of haematogenous metastases in about 10-30% of patients at the time of diagnosis. To study the effect of blocking siglec-E on liver metastases in mice with orthotopic CRC, histological assessment of livers taken from vehicle-treated and anti-siglec-E antibody-treated groups was performed. Tissues were processed for histopathology staining, and results demonstrated that livers from mice treated with sterile water had visible tumours and showed some structural abnormalities of the liver (**Figure 6.8A-A'**). However, livers from tumour-bearing mice treated with a mouse anti-siglec-E antibody demonstrated normal liver structure (**Figure 6.8B-B'**).

Figure 6.6. Effect of blocking siglec-E on tumour growth *in vivo*

Images of tumour size from sterile water-treated group (**A**) and anti-siglec-E antibody-treated group (**B**). Bar graphs displaying the mean weight (**C**) and volume (**D**) of tumours collected from sterile water and anti-siglec-E antibody-treated groups. Data presented as mean ± SEM, n=8 mice per group. Student's *t-test, ****p<0.0001*. Anti-SE Ab, anti-siglec-E antibody.

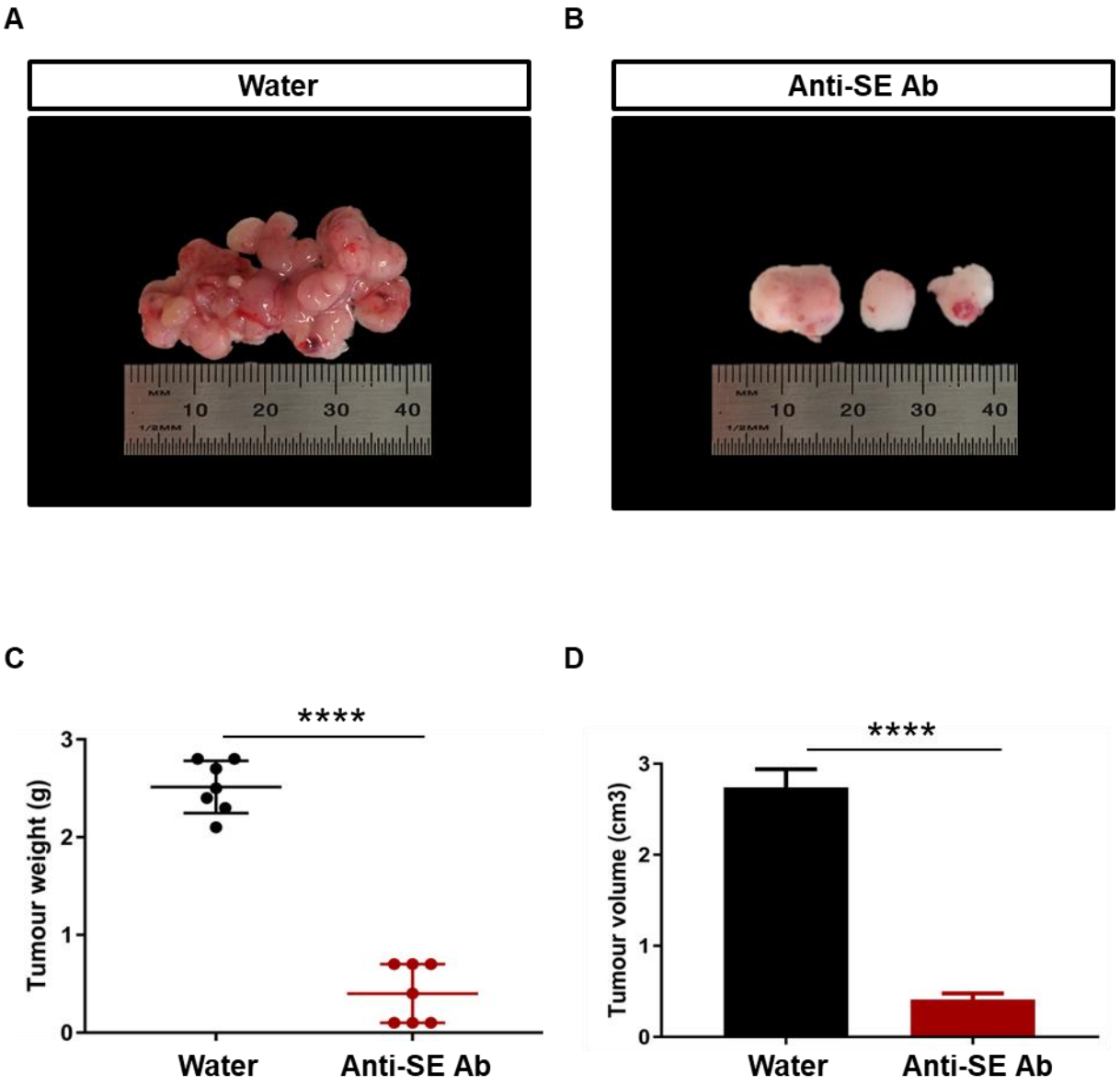
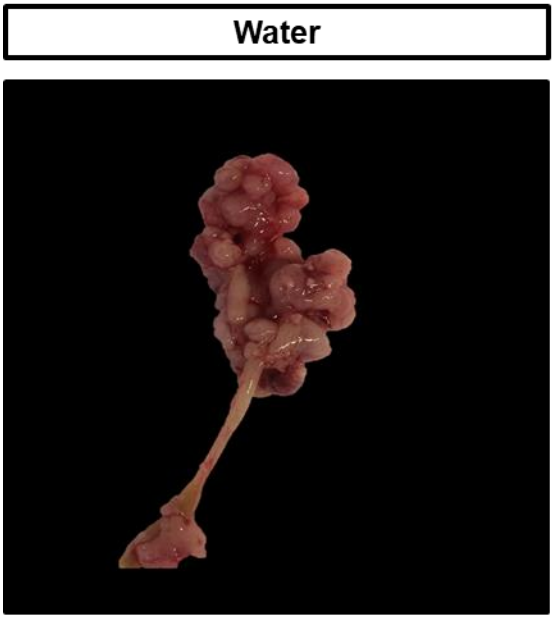

A
B
Water
Anti-SE Ab
10
20
30
40
C
D
****
Tumour weight (g)
Tumour volume (cm3)
0
1
2
3
Water
Anti-SE Ab

Figure 6.7. Effect of a mouse anti-siglec-E antibody on the number of polyps around the caecum

The caecum samples removed from tumour-bearing mice treated with sterile water (**A**) and anti-siglec-E antibody (**B**). Bar graph displaying the mean number of tumour polyps from sterile water and anti-siglec-E antibody-treated groups (**C**). Data presented as mean ± SEM, n=8 mice per group. Student's *t-test, ****p<0.0001*. Anti-SE Ab, anti-siglec-E antibody.

A


Water

B


Anti-SE Ab

C

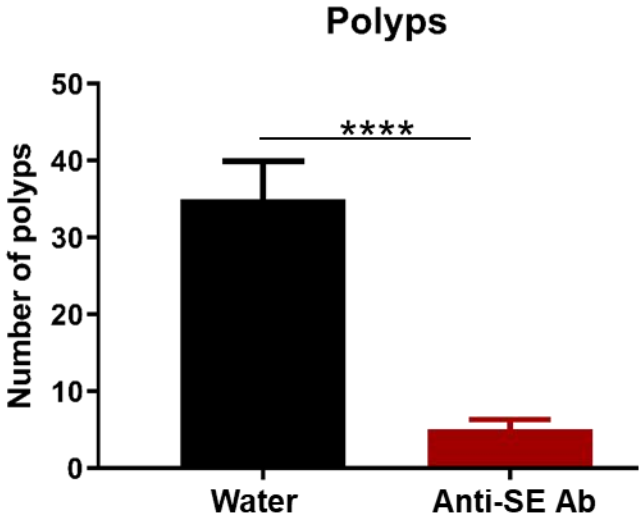
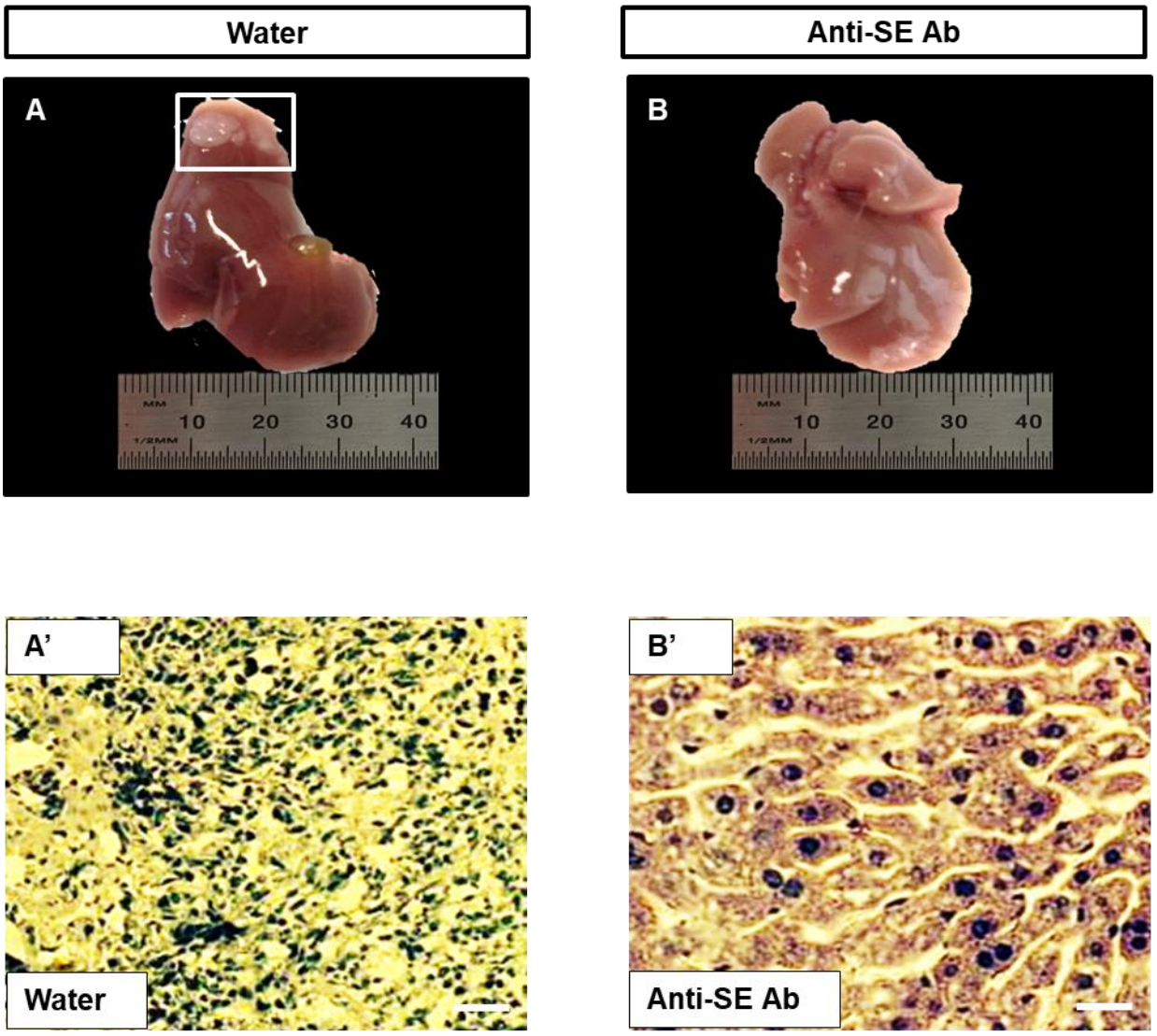

Polyps
Number of polyps
50
40
30
20
10
0
****
Water
Anti-SE Ab

Figure 6.8. Effect of a mouse anti-siglec-E antibody on tumour metastasis

Livers from tumour-bearing mice treated with sterile water (**A**) and with anti-siglec-E antibody (**B**). Haematoxylin and eosin staining of the liver sections from sterile water-treated (**A'**) and anti-siglec-E antibody-treated (**B'**) mice. Images were taken at 20x magnification. Scale bar represents 100µm, n=8 mice per group. Anti-SE Ab, anti-siglec-E antibody.


Water
Anti-SE Ab
A
B
10
20
30
40
A'
B'
Water
Anti-SE Ab

6.3.7. Expression of immunosuppressive and cholinergic markers in tumours from an *in vivo* model of CRC

6.3.7.1. Expression of immunosuppressive markers in the *in vivo* model

Studies identifying the role of siglec-9 or its mouse counterpart, siglec-E, in CRC are scarce. Siglec-9 has been shown to be expressed by a number of immune cells within human colorectal tumour microenvironment, as demonstrated in Chapter 1. In this chapter, the effects of *in vivo* treatment of CRC tumour-bearing mice with a recombinant anti-siglec-E antibody on the expression of siglec-E and IDO were studied. Siglec-E was co-labelled with FOXP3, a marker labelling the regulatory T cells. Blocking of siglec-E with a mouse anti-siglec-E antibody significantly reduced the expression of siglec-E compared to sterile water treatment (**Figure 6. 9A', B' C** and **E**; ∆: -9.33±1.52, *p<0.0001*). However, there was no significant difference observed in the expression of FOXP3 between anti-siglec-E antibody-treated and sterile water-treated groups (**Figure 6.9A'', B'' D** and **E**; ∆: -1.82±1.21, *p=0.1580*).

Furthermore, the effect of a mouse anti-siglec-E antibody on the expression of IDO was evaluated. In this study, IDO was not detected in CT-26 cells *in vitro* (Section **6.3.3.1**) but was detected *in vivo*, suggesting that tumour microenvironment might influence the expression of IDO. Although there was a trend toward a decrease, no significant difference in the expression of IDO was observed between anti-siglec-E antibody-treated and sterile water-treated groups (**Figure 6.10A-D**, ∆: -0.17±0.40, *p=0.6785*). These findings were further confirmed by western blot.

6.3.1. Effect of blocking siglec-E on CT-26 cells proliferation *in vitro*

To determine the effect of blocking siglec-E on CT-26, cells were treated with various concentrations of a mouse anti-siglec-E antibody for 8hrs. The effect of a mouse anti-siglec-E antibody on CT-26 cell proliferation was assessed using WST-1 assay. Three independent experiments were performed in triplicates. Blocking siglec-E significantly attenuated CT-26 cells proliferation in a concentration dependent manner (**Figure 6.1A-B**). Though there was a trend for lower cell proliferation at 50-600ng, there was no

significant difference observed when compared to control (0ng). However, high doses (700-1000ng) of a mouse anti-siglec-E antibody significantly inhibited CT-26 cells proliferation (**Figure 6.1A-B**).

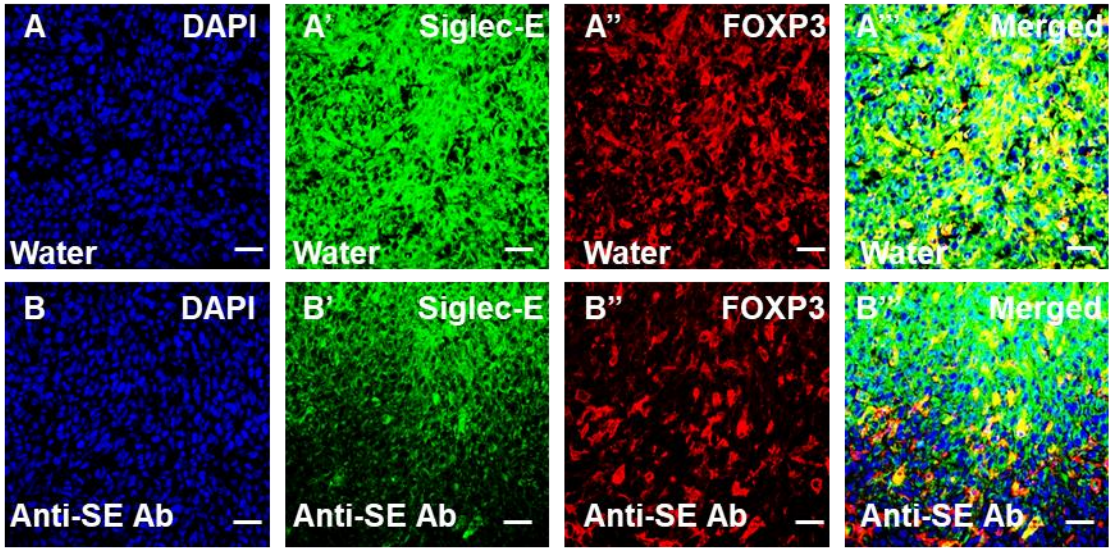
6.3.7.2. Correlation of siglec-E expression with cholinergic markers

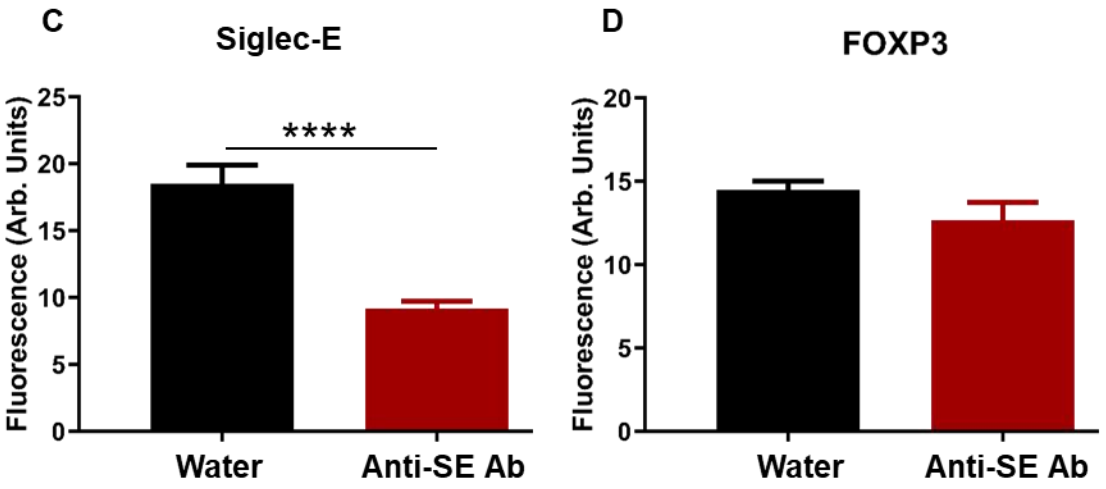
It was hypothesised that siglec-9/siglec-E expression might crosstalk with cholinergic signalling and, in fact, *in vitro* study on human colon cancer cells as demonstrated in Chapter 5 and murine CT-26 cells confirmed this hypothesis. To further evaluate this *in vivo*, tumour-bearing mice implanted with CT-26 cells were injected with sterile water or mouse anti-siglec-E antibody. Siglec-E was co-labelled with a cholinergic enzyme crucial for ACh synthesis, ChAT, and a vesicular ACh transporter, VAChT, essential for packaging of ACh into vesicles. Results demonstrated that mouse anti-siglec-E antibody treatment significantly decreased siglec-E expression compared to sterile water treatment (**Figure 6.11A' B'** and **C**; Δ: -12.92±2.68, $p<0.001$). Cholinergic markers, ChAT (**Figure 6.11A'' B''** and **D**; Δ: -7.96±1.39, $p<0.0001$) and VAChT (**Figure 6.11A''' B'''** and **E**; Δ: -8.20±2.08, $p<0.01$) were significantly attenuated in anti-siglec-E antibody-treated animals compared to sterile water-treated group. Interestingly, in sterile water-treated group, siglec-E was mostly co-localised with ChAT and VAChT, however, in anti-siglec-E antibody-treated group, this co-localisation was abolished, reinforcing that there is an interaction between siglec-E and cholinergic markers.

To determine the overall expression of cholinergic signalling, ACh receptors were co-labelled with ChAT. Anti-siglec-E treatment induced a significant reduction in α7nAChR expression compared to sterile water-treated group (**Figure 6.12A'**, **B'**, **C** and **F**, Δ: -5.72±1.36, $p<0.01$). On the other hand, anti-siglec-E treatment induced a significant increase in M3R expression compared to sterile water treatment (**Figure 6.12A''**, **B''**, **D** and **F**, Δ: 8.94±1.72, $p<0.001$), the underlining mechanisms for this augmentation is not clear. ChAT expression was significantly decreased in anti-siglec-E antibody-treated group compared to sterile water-treated group (**Figure 6.12A''', B''', E** and **F**, Δ: -9.25±4.04, $p<0.05$).

Figure 6.9. Effect of a mouse anti-siglec-E antibody treatment on the expression siglec-E and FOXP3 *in vivo*

Intensity of siglec-E and FOXP3 in tumour samples from mice bearing-CT-26 cell-induced CRC treated with sterile water (**A-A'''**) and mouse anti-siglec-E antibody (**B-B'''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), siglec-E (green; **A'-B'**), FOXP3 (red; **A''-B''**) and all markers merged (yellow; **A'''-B'''**). Scale bar represents 50µm. Bar graphs displaying the mean fluorescence siglec-E (**C**), FOXP3 (**D**) and images of western blot bands (**E**) in tumour samples from sterile water-treated and anti-siglec-E antibody-treated mice. Data presented as mean ± SEM, n=8 mice per group. Student's *t-test, ****p<0.0001*. Anti-SE Ab, anti-siglec-E antibody.


A
DAPI
Water
A'
Siglec-E
Water
A''
FOXP3
Water
A'''
Merged
Water
B
DAPI
Anti-SE Ab
B'
Siglec-E
Anti-SE Ab
B''
FOXP3
Anti-SE Ab
B'''
Merged
Anti-SE Ab


C
Siglec-E
Fluorescence (Arb. Units)
25
20
15
10
5
0
****
Water
Anti-SE Ab
D
FOXP3
Fluorescence (Arb. Units)
20
15
10
5
0
Water
Anti-SE Ab

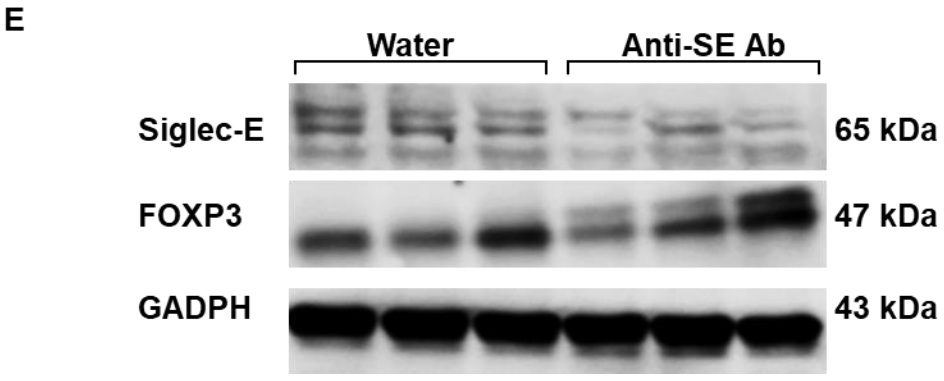
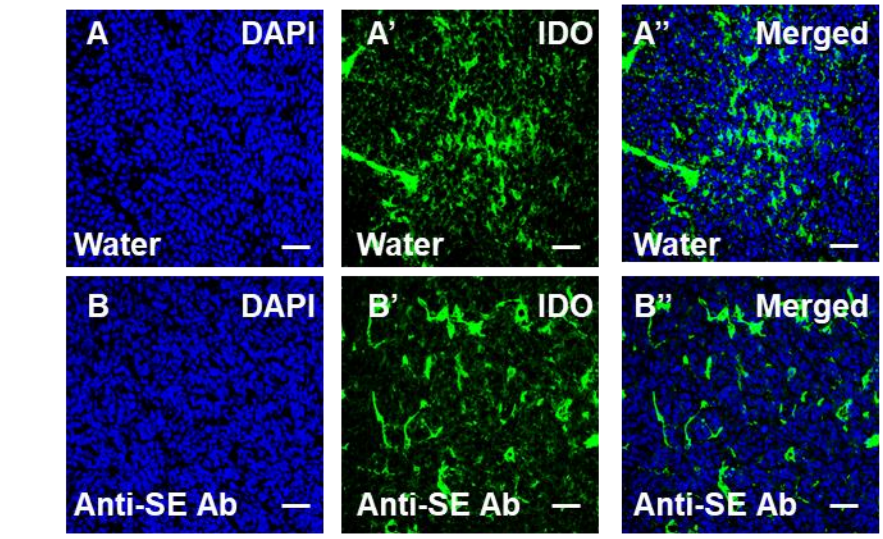
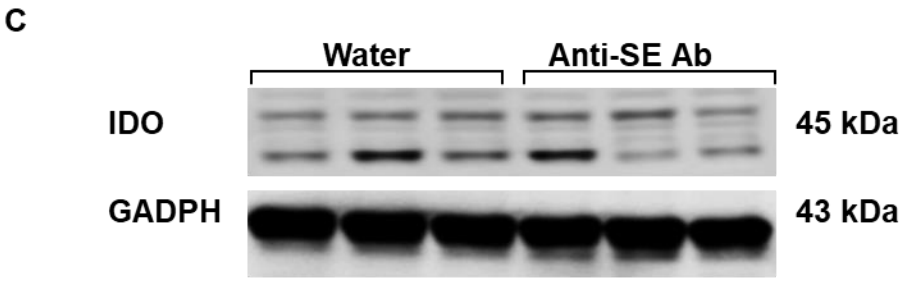

E
Water
Anti-SE Ab
Siglec-E
65 kDa
FOXP3
47 kDa
GADPH
43 kDa

Figure 6.10. Effect of a mouse anti-siglec-E antibody on the expression IDO *in vivo*

IDO expression in tumour samples from mice bearing-CT-26 cell-induced CRC treated with sterile water (**A-A''**) and mouse anti-siglec-E antibody (**B-B''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), IDO (green; **A'-B'**) and all markers merged (**A''-B''**). Scale bar represents 50µm. Western blot bands for IDO expression (**C**) in tumour samples from sterile water-treated and anti-siglec-E antibody-treated mice. Bar graphs displaying the IDO mean fluorescence intensity (**D**) and western blot expression level (**E**) in tumour samples from sterile water-treated and anti-siglec-E antibody-treated groups. Data presented as mean ± SEM, n=8 mice per group. Student's *t-test*. Anti-SE Ab, anti-siglec-E antibody.


A
DAPI
Water
A'
IDO
Water
A''
Merged
Water
B
DAPI
Anti-SE Ab
B'
IDO
Anti-SE Ab
B''
Merged
Anti-SE Ab


C
Water
Anti-SE Ab
IDO
45 kDa
GADPH
43 kDa

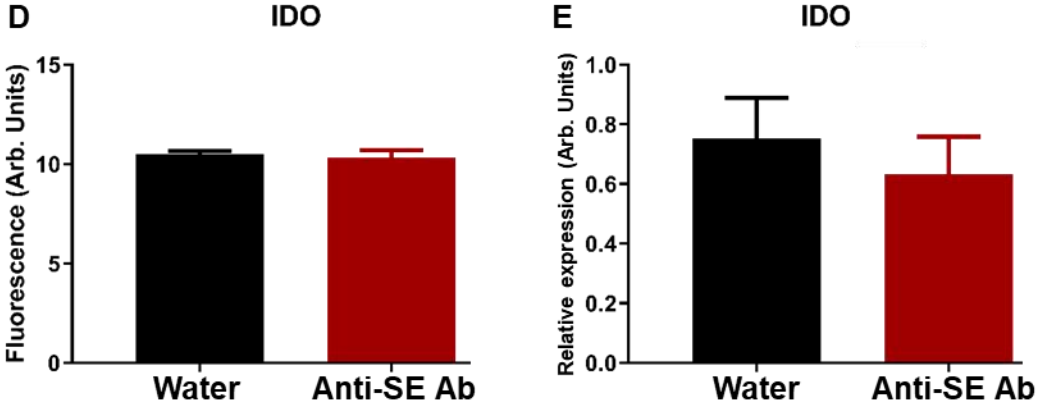
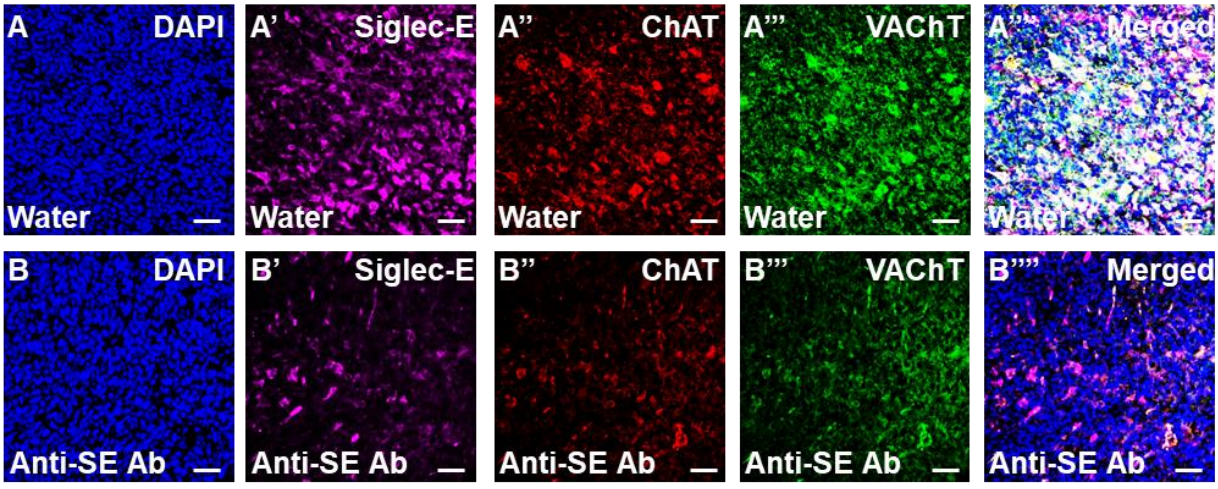

D
IDO
Fluorescence (Arb. Units)
15
10
5
0
Water
Anti-SE Ab
E
IDO
Relative expression (Arb. Units)
1.0
0.8
0.6
0.4
0.2
0.0
Water
Anti-SE Ab

Figure 6.11. Correlation of siglec-E expression with cholinergic markers in tumour samples from mice bearing CT-26 cell-induced CRC

Expression of siglec-E and cholinergic markers (ChAT and VAChT) in tumour samples from mice bearing CT-26 cell-induced CRC treated with sterile water (**A-A''**) and mouse anti-siglec-E antibody (**B-B''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), siglec-E (magenta; **A'-B'**), ChAT (red; **A''-B''**), VAChT (green; **A'''-B'''**) and all markers merged (**A''''-B''''**). Scale bar represents 50µm. Bar graphs displaying the mean fluorescence siglec-E (**C**), ChAT (**D**) and VAChT (**E**) in tumour samples from sterile water-treated and anti-siglec-E antibody-treated mice. Data presented as mean ± SEM, n=8 mice per group. Student's *t-test, **p<0.01, ***p<0.001, ****p<0.0001*. Anti-SE Ab, anti-siglec-E antibody.


A
DAPI
Water
A'
Siglec-E
Water
A''
ChAT
Water
A'''
VAChT
Water
A''''
Merged
Water
B
DAPI
Anti-SE Ab
B'
Siglec-E
Anti-SE Ab
B''
ChAT
Anti-SE Ab
B'''
VAChT
Anti-SE Ab
B''''
Merged
Anti-SE Ab

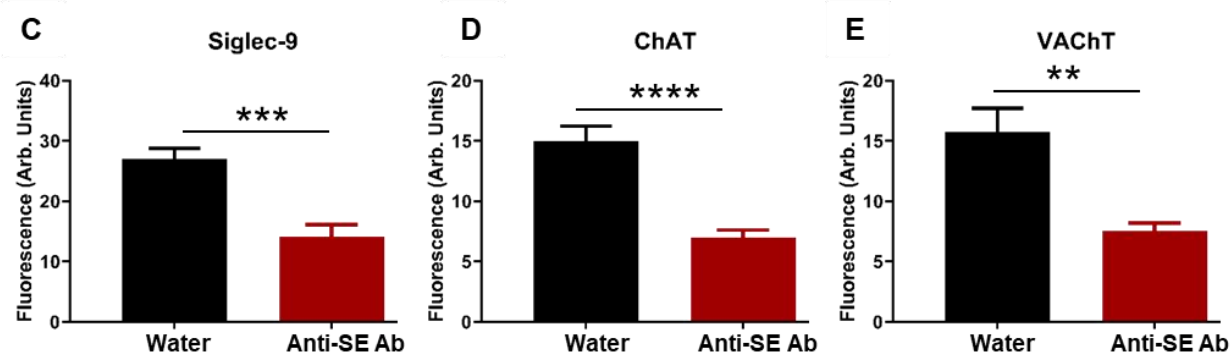
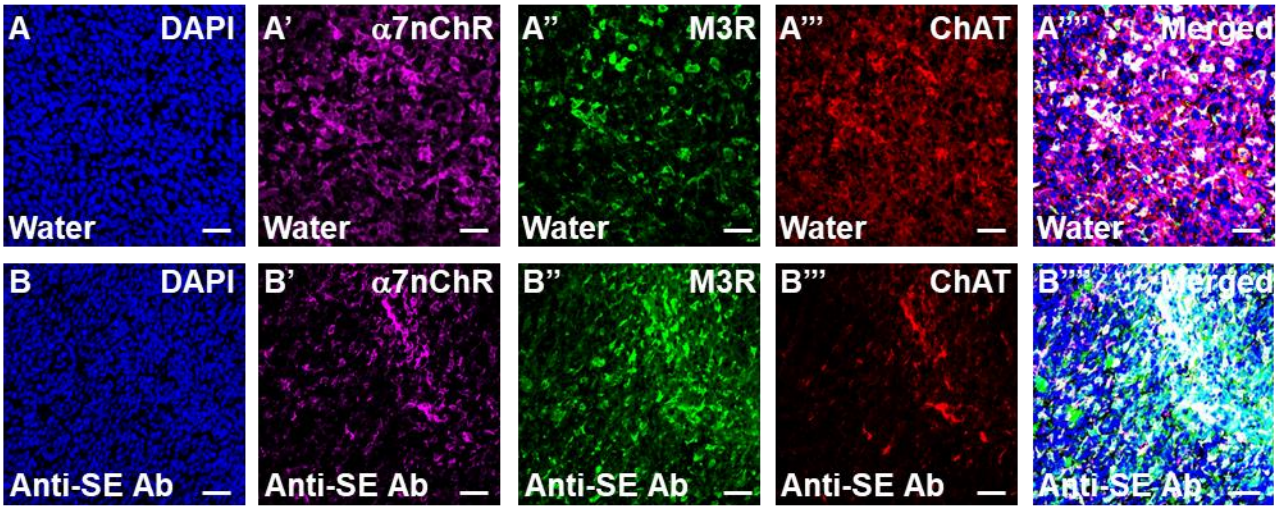
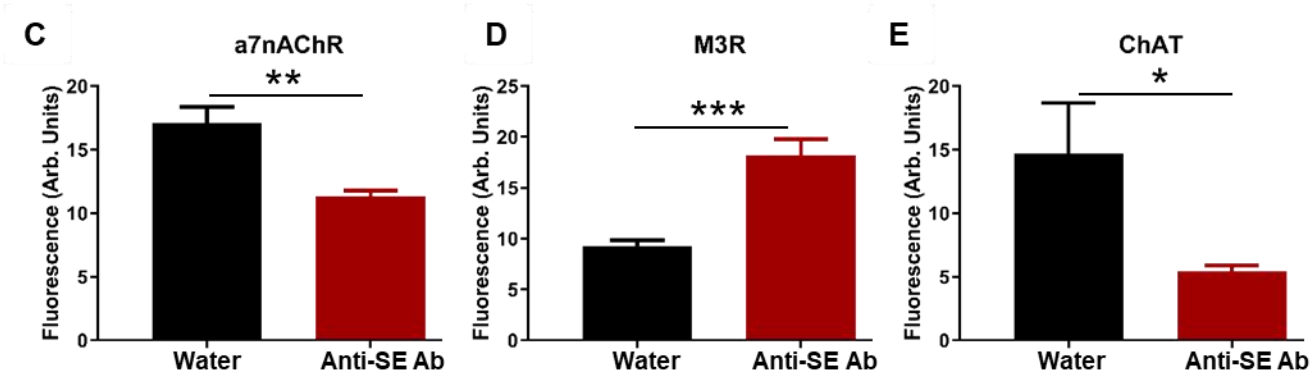
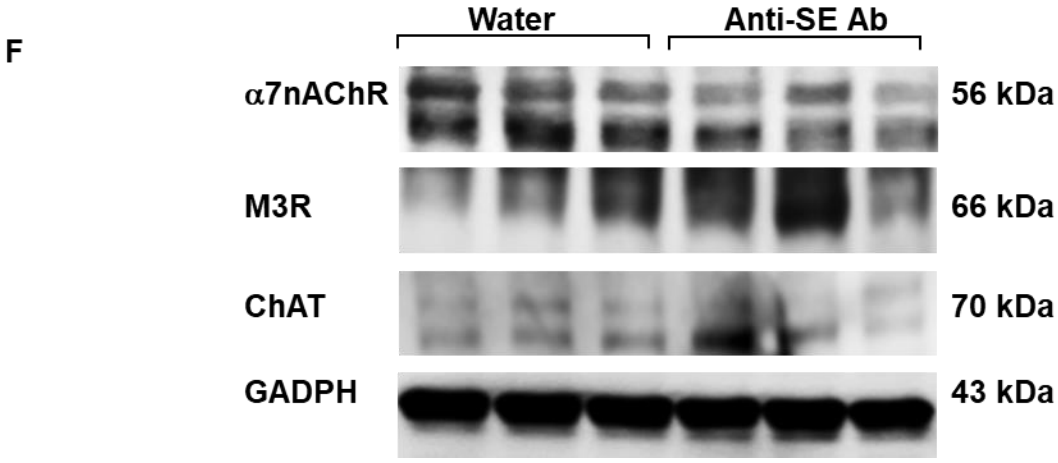

C
Siglec-9
***
Fluorescence (Arb. Units)
40
30
20
10
0
Water
Anti-SE Ab
D
ChAT
****
Fluorescence (Arb. Units)
20
15
10
5
0
Water
Anti-SE Ab
E
VAChT
**
Fluorescence (Arb. Units)
20
15
10
5
0
Water
Anti-SE Ab

Figure 6.12. Expression of cholinergic markers in tumour samples from mice bearing CT-26 cell-induced CRC

Expression of cholinergic markers in tumour samples from mice bearing CT-26 cell-induced CRC treated with sterile water (**A-A''**) and a mouse anti-siglec-E antibody (**B-B''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), α7nAChR (red; **A'-B'**), M3R (magenta; **A''-B''**), ChAT (green; **A'''-B'''**) and all markers merged (**A''''-B''''**). Scale bar represents 50µm. Bar graphs displaying the mean fluorescence α7nAChR (**C**), M3R (**D**), ChAT (**E**) and western blot bands (**F**) in tumour samples from sterile water-treated and anti-siglec-E antibody-treated mice. Data presented as mean ± SEM, n=8 mice per group. Student's *t-test, *p<0.05, **p<0.01, ***p<0.001*. Anti-SE Ab, anti-siglec-E antibody.


A
DAPI
Water
A'
α7nChR
Water
A''
M3R
Water
A'''
ChAT
Water
A''''
Merged
Water
B
DAPI
Anti-SE Ab
B'
α7nChR
Anti-SE Ab
B''
M3R
Anti-SE Ab
B'''
ChAT
Anti-SE Ab
B''''
Merged
Anti-SE Ab


C
a7nAChR
**
Fluorescence (Arb. Units)
0
5
10
15
20
Water
Anti-SE Ab
D
M3R
***
Fluorescence (Arb. Units)
0
5
10
15
20
25
Water
Anti-SE Ab
E
ChAT
*
Fluorescence (Arb. Units)
0
5
10
15
20
Water
Anti-SE Ab


F
Water
Anti-SE Ab
α7nAChR
56 kDa
M3R
66 kDa
ChAT
70 kDa
GADPH
43 kDa

6.3.8. Effect of blocking siglec-E on tumour-infiltrating immune cells

To evaluate the effect of mouse anti-siglec-E antibody on the tumour-infiltrating immune cells within the tumour microenvironment, fresh tumours were collected into RPMI media. Tumours were mechanically dissected into small pieces and incubated at 37˚C for 1hr with collagenase before commencing labelling with antibodies of interested as described in the Materials and Methods. To characterise leukocyte populations in tumour samples from sterile water-treated and anti-siglec-E antibody-treated mice, flow cytometry was used. From the scatter plot, only viable cells were analysed, and compensation was performed (when applicable) to prevent false-positive/false-negative results (**Figure 6.13A-B**). The gating strategy for CD45+ cells was defined from single cell doublets (**Figure 6.13C-D**). CD4+, CD8+ T lymphocyte populations were gated from CD45+ cells (**Figure 6.13E-F**; **G-H**). No significant differences were observed in CD45+ cells in tumours from both sterile water-treated and anti-siglec-E antibody-treated groups (**Figure 6.14A**, **Δ:** 0.44±4.04, *p=0.9158*). However, administration of a mouse anti-siglec-E antibody significantly increased proportion of CD4+ (**Figure 6.14B**, **Δ:** 11.48±3.91, *p<0.01*) and CD8+ (**Figure 6.14C**, **Δ:** 7.06±2.55, *p<0.01*) T cells compared to sterile water-treated group. On the other hand, no significant differences were noted in the ratio of CD4+/CD8+ T cells (**Figure 6.14D**, **Δ:** 2.49±1.52, *p=0.1392*) and γδ T cells infiltration (**Figure 6.14E**, **Δ:** -0.10±0.06, *p=0.1368*) between anti-siglec-E antibody-treated and sterile water-treated groups.

In addition, there was no significant difference observed in the infiltration of B cells between mice treated with sterile water and anti-siglec-E antibody (**Figure 6.15A**). However, eosinophil infiltration was significantly reduced in tumours from anti-siglec-E antibody-treated mice compared to tumours from sterile water-treated group (**Figure 6.15B**). No statistical differences observed in the infiltration of all macrophages (presented as M0) between sterile water-treated and anti-siglec-E antibody-treated groups, but M2 phenotype macrophages were slightly increased in anti-siglec-E antibody-treated group. However, infiltrating M2 macrophages presented only a small fraction of all M0 macrophages in both groups (**Figure 6.15C**). This suggests that most of the M0 macrophages are in fact M1 phenotype, which have an anti-tumour effect. As there are

no specific markers to distinguish between N1 and N2 neutrophils, overall neutrophil infiltration was evaluated. Neutrophil infiltration was augmented in tumours from anti-siglec-E antibody-treated mice compared to sterile water-treated group (**Figure 6.15D**), suggesting that infiltrated neutrophils might be of N1 phenotype as they exert an anti-tumour effect.

Figure 6.13. Gating strategy for leukocytes in tumour samples from tumour-bearing mice

Gating strategy for debris exclusion (**A**), viable cells (**B**), single cell doublets discrimination (**C**), single cells (**D**), CD45-positive cells from sterile water-treated group (**E**) and T lymphocytes from sterile water-treated group (**F**), CD45-positive cells from anti-siglec-E antibody-treated group (**G**) and T lymphocytes from anti-siglec-E antibody-treated group (**H**). Anti-SE Ab, anti-siglec-E antibody.

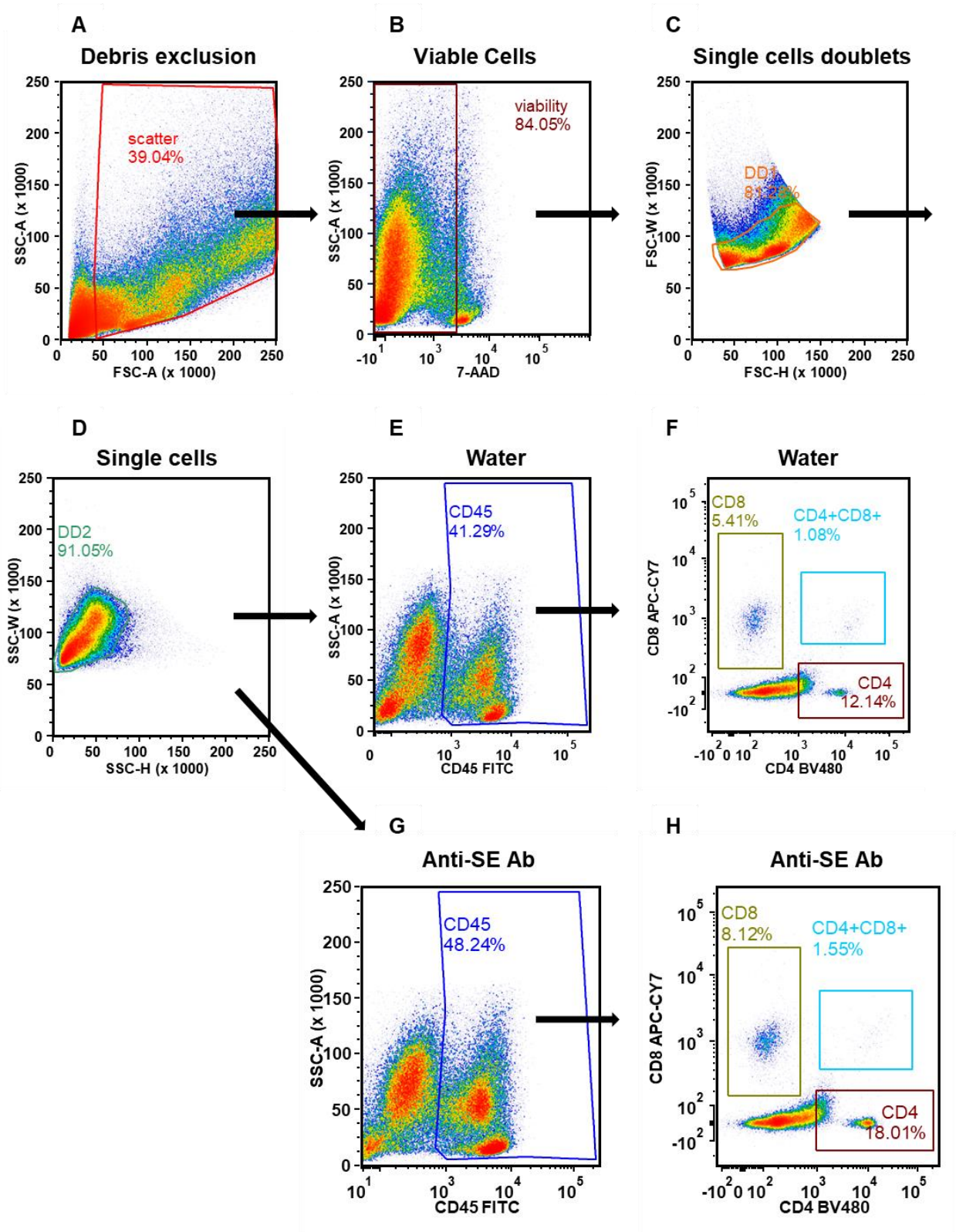

A
Debris exclusion
scatter
39.04%
SSC-A (x 1000)
FSC-A (x 1000)
B
Viable Cells
viability
84.05%
SSC-A (x 1000)
7-AAD
C
Single cells doublets
DD1
81.28%
FSC-W (x 1000)
FSC-H (x 1000)
D
Single cells
DD2
91.05%
SSC-W (x 1000)
SSC-H (x 1000)
E
Water
CD45
41.29%
SSC-A (x 1000)
CD45 FITC
F
Water
CD8
5.41%
CD4+CD8+
1.08%
CD4
12.14%
CD8 APC-CY7
CD4 BV480
G
Anti-SE Ab
CD45
48.24%
SSC-A (x 1000)
CD45 FITC
H
Anti-SE Ab
CD8
8.12%
CD4+CD8+
1.55%
CD4
18.01%
CD8 APC-CY7
CD4 BV480

Figure 6.14. Flow cytometry analysis of CD45+ cells and T lymphocytes in tumours

Proportion of the CD45+ cells (**A**), CD4+ T cells (**B**), CD8+ T cells (**C**), CD4+/CD8+ T cells ratio (**D**) and γδ T cells (**E**). Data presented as mean ± SEM, sterile water-treated, anti-siglec-E antibody-treated group Student's *t-test,* n=7 mice per group, ***p<0.01*. Anti-SE Ab, anti-siglec-E antibody.

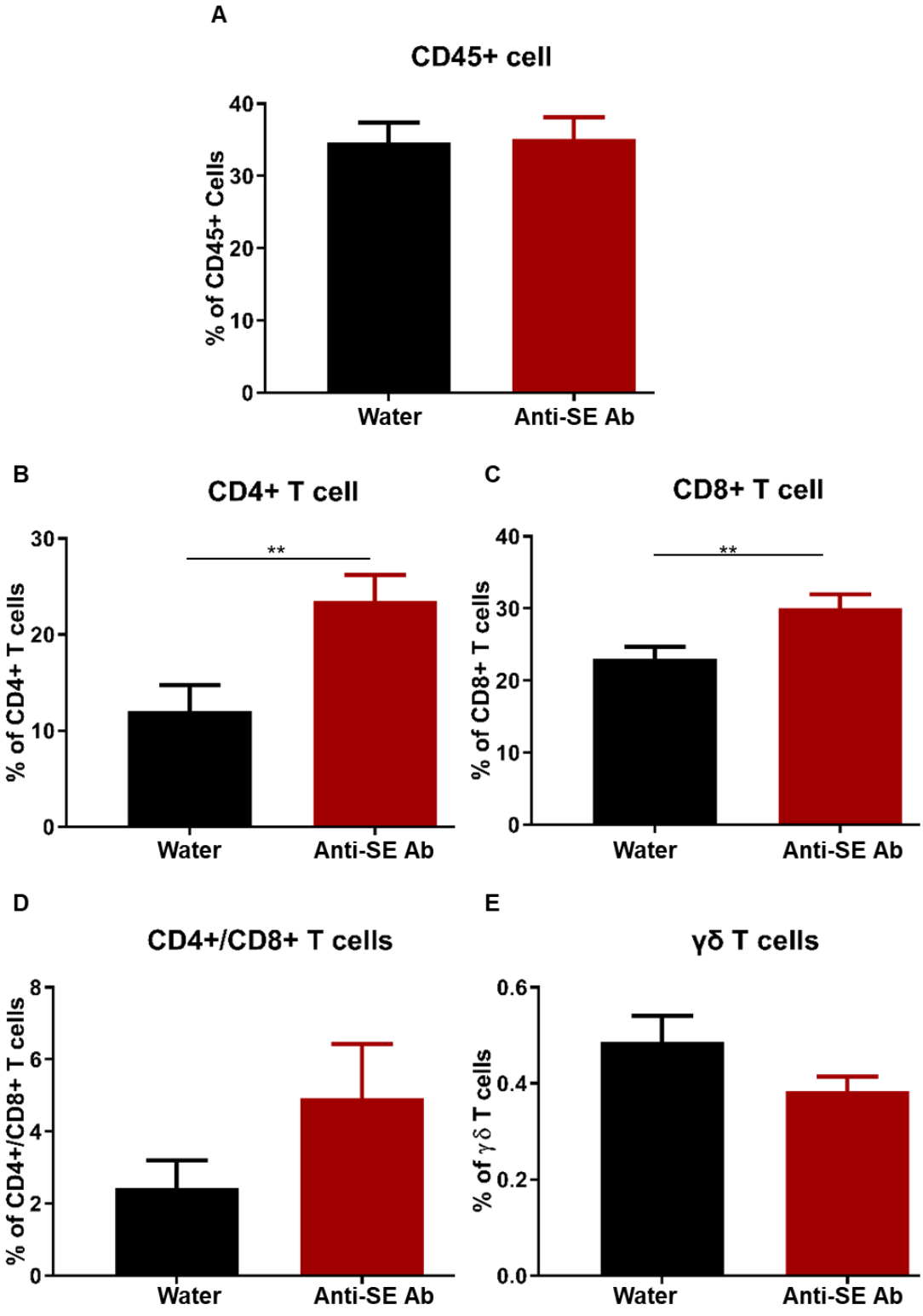
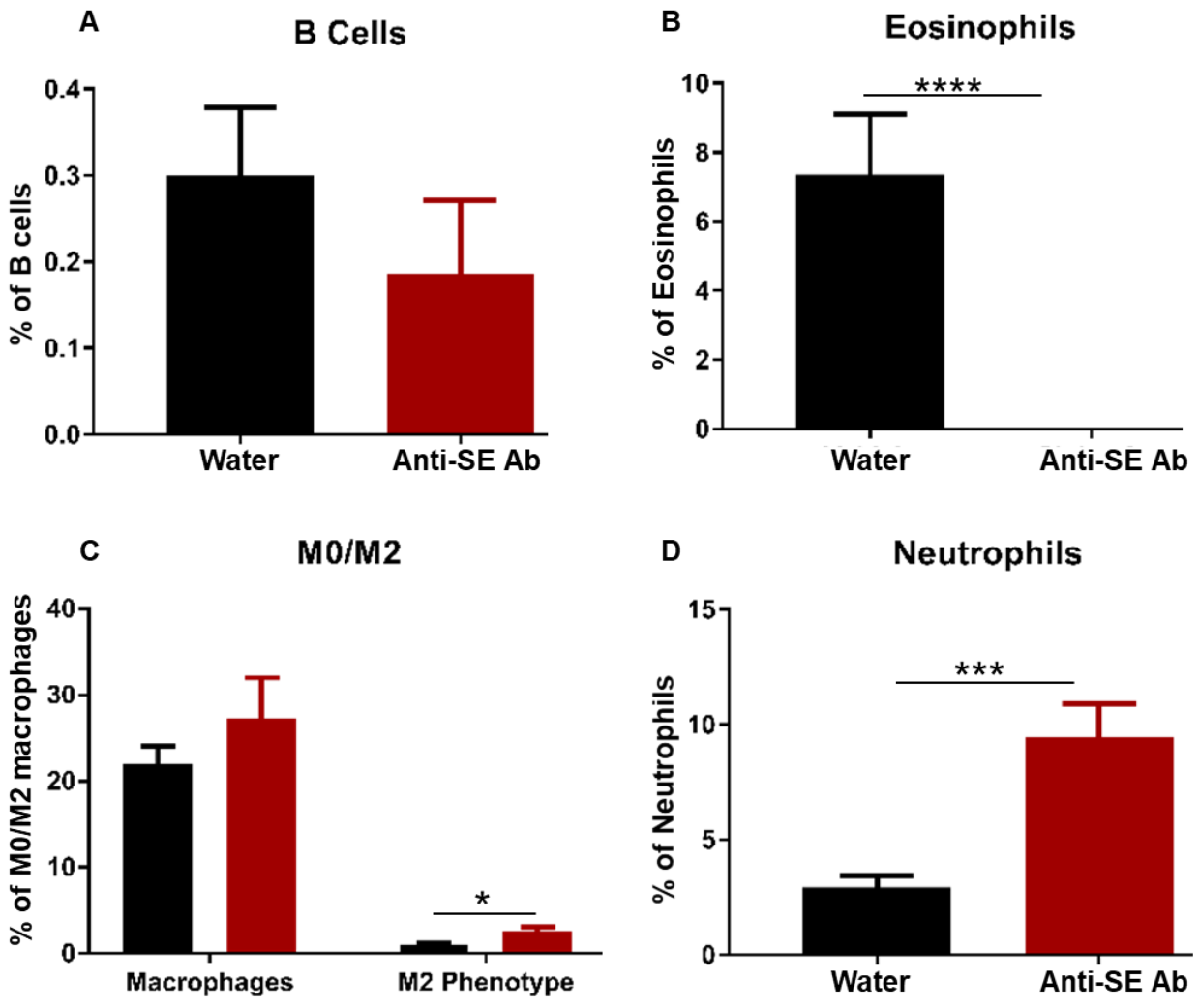

A
CD45+ cell
% of CD45+ Cells
40
30
20
10
0
Water
Anti-SE Ab
B
CD4+ T cell
% of CD4+ T cells
30
20
10
0
**
Water
Anti-SE Ab
C
CD8+ T cell
% of CD8+ T cells
40
30
20
10
0
**
Water
Anti-SE Ab
D
CD4+/CD8+ T cells
% of CD4+/CD8+ T cells
8
6
4
2
0
Water
Anti-SE Ab
E
γδ T cells
% of γδ T cells
0.6
0.4
0.2
0.0
Water
Anti-SE Ab

Figure 6.15. Flow cytometry analysis of tumour-associated immune cells

Proportion of B cells (**A**), eosinophils (**B**), macrophages (**C**) and neutrophils (**D**) in tumours from sterile water-treated and mouse anti-siglec-E antibody-treated groups. Data presented as mean ± SEM, Student's *t-test,* n=7 mice per group, **p<0.05, ***p<0.001, ****p<0.0001*. Anti-SE Ab, anti-siglec-E antibody.


A
B Cells
% of B cells
0.4
0.3
0.2
0.1
0.0
Water
Anti-SE Ab
B
Eosinophils
****
% of Eosinophils
10
8
6
4
2
0
Water
Anti-SE Ab
C
M0/M2
% of M0/M2 macrophages
40
30
20
10
0
*
Macrophages
M2 Phenotype
D
Neutrophils
***
% of Neutrophils
15
10
5
0
Water
Anti-SE Ab

6.3.9. Effect of blocking siglec-E on tumour angiogenesis

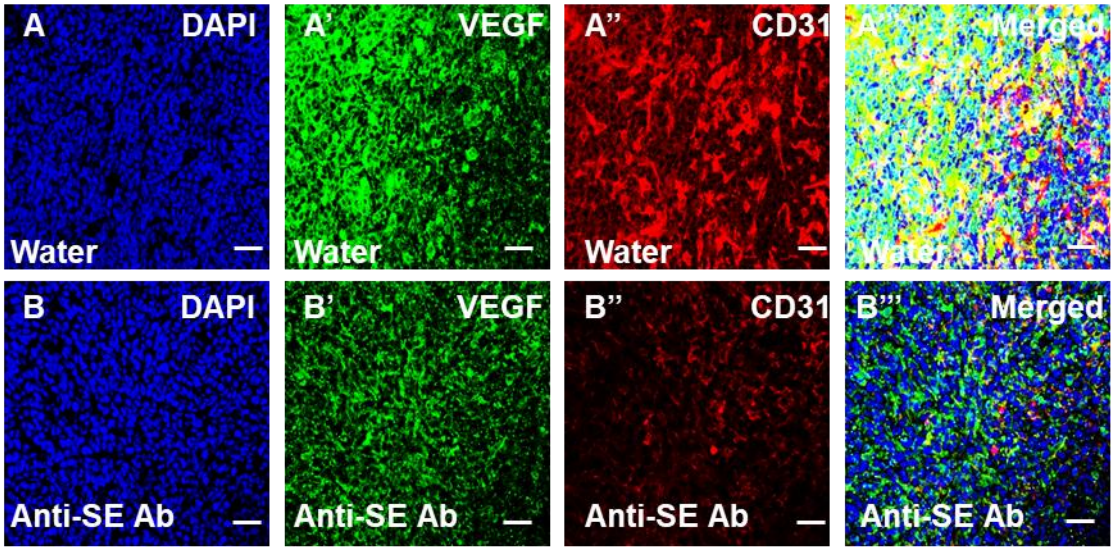
To evaluate the effect of blocking siglec-E on the tumour angiogenesis, tumour sections were labelled with angiogenic markers, CD31 and VEGF. The results demonstrated that administration of a mouse anti-siglec-E antibody attenuated expression of both VEGF (**Figure 6.16A' B', C** and **E**, Δ: -12.62±1.88, *p<0.0001*) and CD31 (**Figure 6.16A'', B'', D**, Δ: -8.28±1.11, *p<0.0001*) compared to sterile water-treated group. In addition, expression levels of tyrosine kinases in tissue lysates were evaluated using mouse Phospho-RTK Array in pooled samples from each group. The results show that tumours from the sterile water-treated group overexpressed platelet-derived growth factor receptor α (PDGFRα) and VEGF receptor 3 (VEGFR3) (**Figure 6.17A**), both markers are involved in tumour angiogenesis. Mouse anti-siglec-E antibody treatment had no significant effects on the expression of PDGFRα but abolished VEGFR3 expression (**Figure 6.17B**). Taken together, these findings suggest that siglec-E or human counterpart, siglec-9, might play an important role in tumour angiogenesis and targeting it could be a potential therapeutic for angiogenesis.

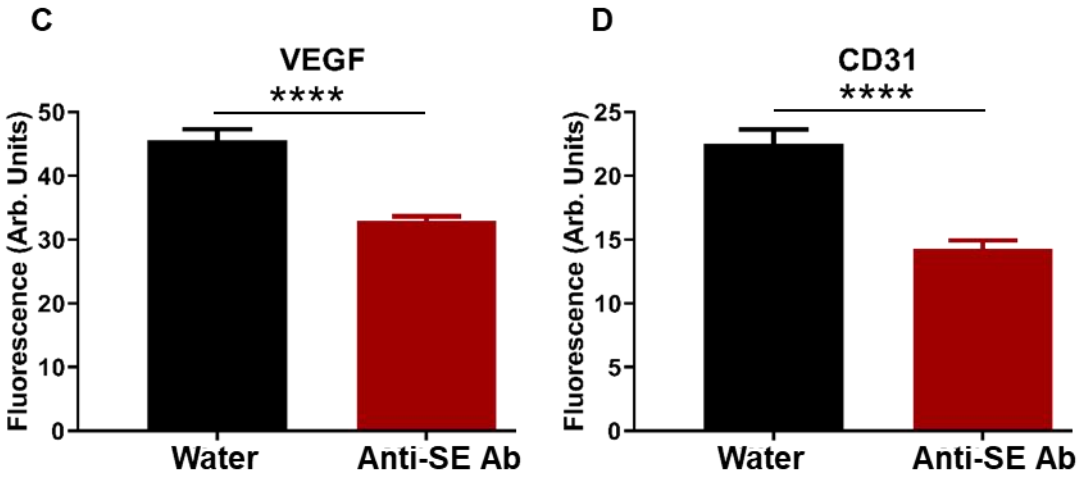
6.3.10. Effect of blocking siglec-E on phosphorylation of AKT and ERK in *in vivo* model

In Chapter 5, it was demonstrated that blocking siglec-E exhibits its effects via suppressing ERK/STAT3 signalling pathways. In this chapter, *in vitro* data showed that inhibitory effects of anti-siglec-E antibody occur through suppression of EGFR/AKT/ERK signalling pathways. To determine the effect of *in vivo* anti-siglec-E treatment on the phosphorylation of EGFR, STAT3, AKT and ERK in tumour samples, western blot was used. The results demonstrated that mouse anti-siglec-E antibody treatment significantly inhibited the phosphorylation of ERK compared to sterile water treatment (**Figure 6.18A**, **B**, Δ**:** -0.24±0.06, p<*0.05*). No significant difference was observed in pAKT expression between sterile water-treated and anti-siglec-E antibody-treated groups (**Figure 6.18A**, **C**, Δ**:** 0.32 ± 0.29, *p=0.6315*). However, EGFR and pSTAT3 were not detected; this could be due to fast degradability of these proteins.

Figure 6.16. Effect of a mouse anti-siglec-E antibody treatment on the expression of VEGF and CD31 *in vivo*

VEGF and CD31 expression in tumour samples from mice bearing CT-26 cell-induced CRC treated with sterile water (**A-A'''**) and a mouse anti-siglec-E antibody (**B-B'''**). Tumours were labelled with the nuclei marker DAPI (blue; **A-B**), VEGF (green; **A'-B'**), CD31 (red; **A''-B''**) and all markers merged (yellow; **A'''-B'''**). Scale bar represents 50µm. Bar graphs displaying the mean fluorescence level of VEGF (**C**), CD31 (**D**) and image of VEGF western blot bands (**E**) in tumour samples from sterile water-treated and anti-siglec-E antibody-treated mice. Data presented as mean ± SEM, n=8 mice per group. Student's *t-test, ****p<0.0001*. Anti-SE Ab, anti-siglec-E antibody.


A
DAPI
Water
A'
VEGF
Water
A''
CD31
Water
A'''
Merged
Water
B
DAPI
Anti-SE Ab
B'
VEGF
Anti-SE Ab
B''
CD31
Anti-SE Ab
B'''
Merged
Anti-SE Ab


C
VEGF
****
Fluorescence (Arb. Units)
50
40
30
20
10
0
Water
Anti-SE Ab
D
CD31
****
Fluorescence (Arb. Units)
25
20
15
10
5
0
Water
Anti-SE Ab

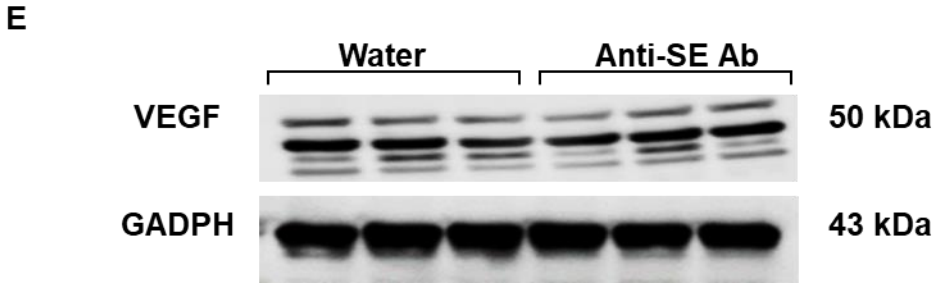

E
Water
Anti-SE Ab
VEGF
50 kDa
GADPH
43 kDa

Figure 6.17. Effect of a mouse anti-siglec-E antibody on the expression of phospho kinases *in vivo*

Mouse Phospho-RTK Array measuring phospho-RTK activity in tumours from mice with CT-26-induced CRC treated with sterile water (**A**) and a mouse anti-siglec-E antibody (**B**). n=8 mice per group. Anti-SE Ab, anti-siglec-E antibody.

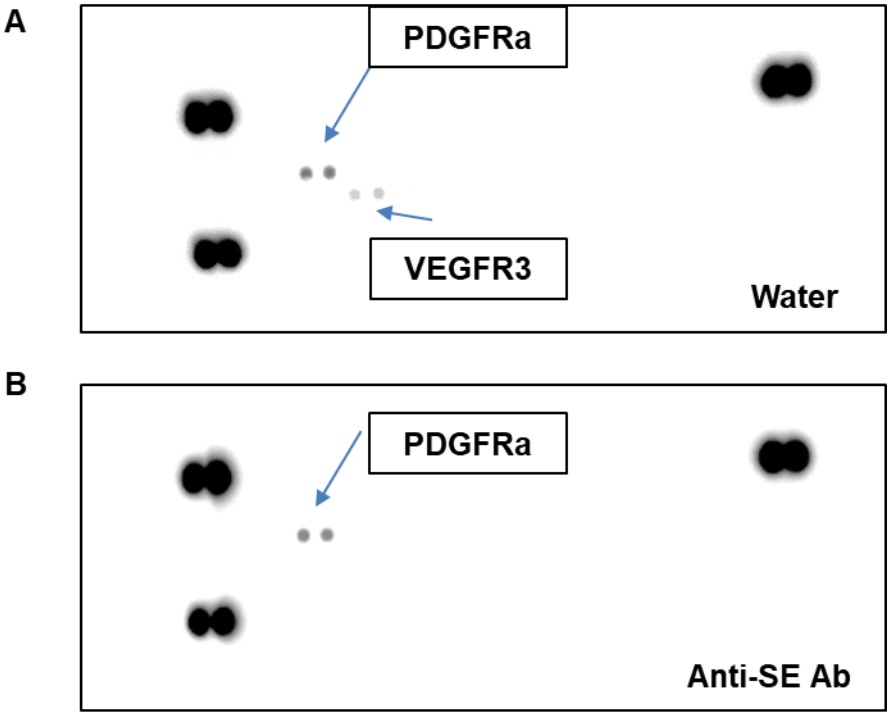
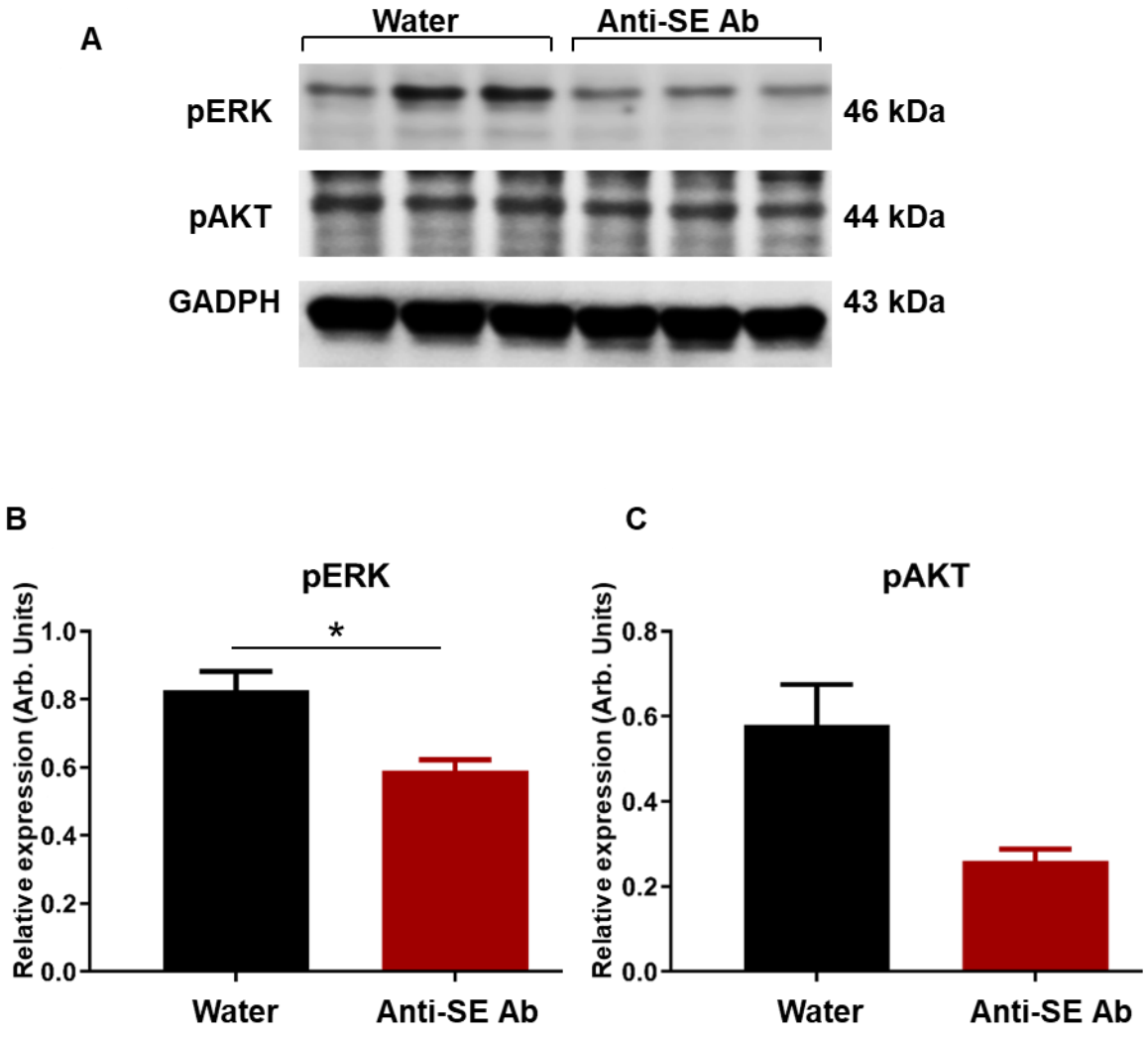

A
PDGFRa
VEGFR3
Water
B
PDGFRa
Anti-SE Ab

Figure 6.18. Effect of a mouse anti-siglec-E antibody on the expression of protein kinases *in vivo*

Western blot bands for pERK and pAKT expression in tumour samples from mice bearing CT-26 cell-induced CRC treated with sterile water and a mouse anti-siglec-E antibody (**A**). Bar graphs displaying the mean intensity of pERK (**B**) and pAKT (**C**) expression in tumour samples from mice treated with sterile water and anti-siglec-E antibody. Data presented as mean ± SEM, Student's *t-test, *p<0.05*. Anti-SE Ab, anti-siglec-E antibody.


A
Water
Anti-SE Ab
pERK
46 kDa
pAKT
44 kDa
GADPH
43 kDa
B
pERK
*
Relative expression (Arb. Units)
1.0
0.8
0.6
0.4
0.2
0.0
Water
Anti-SE Ab
C
pAKT
Relative expression (Arb. Units)
0.8
0.6
0.4
0.2
0.0
Water
Anti-SE Ab

6.4. Discussion

The immune system consists of a complex array of cells which work together to protect the body against invading pathogens, eliminates mutated cells and keeps an immune balance to prevent an autoimmune attack. Cancer cells have evolved to evade the host's immune system by upregulating immunosuppressive markers (siglec-9 or siglec-E, IDO, PD-L1 and PD-L2). In addition, cholinergic signalling has been shown to enhanced tumour growth in many cancers. Although there are current therapies targeting some of these immunosuppressive molecules, they have shortcomings such as causing adverse events. Hence, it is crucial to understand the role of these immunosuppressive molecules and cholinergic signalling during tumorigenesis in order to develop appropriate therapeutic strategies in cancer patients. Currently, there are no studies investigating the interaction between the expression of siglec-9, IDO and cholinergic markers in cancer. In Chapter 5, we have evaluated the expression of siglec-9 and IDO in human samples and the effect of blocking siglec-9 on the expression of immunosuppressive and cholinergic markers in normal epithelial and human colon cancer cell lines.

In the present study, the effect of recombinant mouse siglec-E on the expression of immunosuppressive, cholinergic and angiogenic markers *in vitro* and *in vivo* was determined. It was hypothesised that the blocking of siglec-E might influence cholinergic signalling. Indeed, *in vitro* results demonstrated that CT-26 cells expressed siglec-E and cholinergic markers (M3R and ChAT), which were attenuated by recombinant mouse siglec-E treatment through EGFR/AKT/ERK pathway. To further evaluate whether the *in vitro* effect of a mouse anti-siglec-E antibody can be confirmed *in vivo*, mice bearing CT-26 cell-induced CRC were injected daily with sterile water or a mouse anti-siglec-E antibody for 3 weeks. The results demonstrated that treatment with a mouse anti-siglec-E antibody reduced tumour weight, volume and size when compared to vehicle group treated with sterile water. Furthermore, anti-siglec-E antibody treatment significantly attenuated siglec-E expression; however, there was no significant difference observed in the expression of IDO in tumours from sterile water-treated and anti-siglec-E antibody-treated groups. In addition, administration of a mouse anti-siglec-E antibody significantly

decreased cholinergic and angiogenic markers when compared to sterile water-treated group. Moreover, mouse anti-siglec-E antibody treatment significantly augmented anti-tumour immune response through increased infiltration of CD4+ and CD8+ T lymphocytes.

Overexpression of siglec-9 creates a protective mechanism for the tumour cells from NK cells and in turn, leads to the growth of the tumour cells [101, 629]. Siglec-9 and its murine orthologous protein siglec-E have been implicated in myeloid cell-mediated cancer progression [94, 644]. It has been demonstrated that T cells expressing siglec-9 can be co-expressed with some of the inhibitory receptors such as PD-1 [645]. In fact, siglec-9 binding to MUC1 on macrophages was found to induce infiltration of tumours with tumour-associated macrophages (TAM) phenotype and increased the expression of IDO and PD-L1 through the induction of a calcium flux leading to activation of the MEK-ERK pathway [646]. Furthermore, siglec-E-deficient mice showed an improved immunosurveillance against tumour cells via lacking siglec-E expressed on neutrophils [94]. This concurs with our findings demonstrating that blocking of siglec-E resulted in an improved immune response against cancer and increased expression of neutrophils. Although studies have insinuated that siglec-9/siglec-E plays a role in tumour angiogenesis and metastasis. The data presented in this Chapter demonstrated that siglec-9/siglec-E might play a significant role in tumour angiogenesis and metastasis as blocking of siglec-E resulted in decreased angiogenic markers and metastasis. In an experimental metastasis assay, expression of siglec-9/siglec-E binding to tumour-associated ligands inhibits neutrophils and enhances lung colonisation [94]. Taken together, these findings suggest that siglec-9/siglec-E plays a functional role in tumour angiogenesis and metastasis, which might be dependent on tumour microenvironment constituents.

In Chapter 5, it was demonstrated that siglec-9 and IDO are co-localised and previous studies have shown that expression of siglec-9 influences IDO and PD-L1 expression [646]. Expression of IDO in the tumour microenvironment plays a key role in tumour progression. Studies on the expression of IDO have been shown to be linked with stage III of CRC and associated with poor prognosis [624]. However, other studies suggest that the prognostic value of IDO expression depends on the type of tissue affected [93]. This

implies that tumour microenvironment constituents can influence the prognostic value of IDO. In fact, several cells within tumour microenvironment such as regulatory T cells (Tregs), dendritic cells, macrophages and cancer-associated fibroblasts can express IDO [640]. In this study, IDO expression was not detected in CT-26 cells *in vitro* but in tumour tissues *in vivo*, reinforcing that IDO expression can be influenced by tumour microenvironment constituents such as the presence of IFN-γ, TNF-α and tumour-associated immune cells.

Although the role of IDO in tumour escape mechanisms has been previously demonstrated [514, 647], emerging evidence suggests that IDO expression may play a functional role in tumour angiogenesis. In fact, in breast cancer, increased expression of IDO was correlated with high microvessel density as determined by the expression of CD31 and CD105 [648]. The data presented in this Chapter demonstrated that blocking siglec-E with a mouse anti-siglec-E antibody significantly decreased CD31 expression. Similarly, overexpression of IDO in ovarian cancer was shown to promote new blood vessel formation through inhibition of NK cells [649]. Furthermore, *in vitro* study of 2LL Lewis lung cancer cells, IDO expression was shown to promote migration, attachment, invasion and angiogenesis via the JAK2/STAT3 pathway [650]. In this study, administration of a recombinant mouse anti-siglec-E antibody significantly attenuated angiogenic markers, suggesting that siglec-9/siglec-E blockers might hold potential as anti-angiogenic therapy.

The findings presented in this chapter suggest that the expression of immunosuppressive markers and ACh receptors play a significant role in tumour progression. To the best of our knowledge, there are no data currently available examining the effect of siglec-9/siglec-E on the expression of cholinergic markers and cancer cell growth. In fact, *in vitro* study on human colon cancer cells presented in Chapter 5 and murine CT-26 cells in this Chapter demonstrated that siglec-9/siglec-E expression can crosstalk with cholinergic signalling. To further evaluate this crosstalk *in vivo*, mice bearing CT-26 cell-induced CRC were treated with sterile water or a recombinant mouse anti-siglec-E antibody for 3 weeks. Blocking siglec-E inhibited cholinergic signalling via inhibition of ACh production by reducing the amount of ChAT, an enzyme crucial for ACh synthesis, VAChT, essential

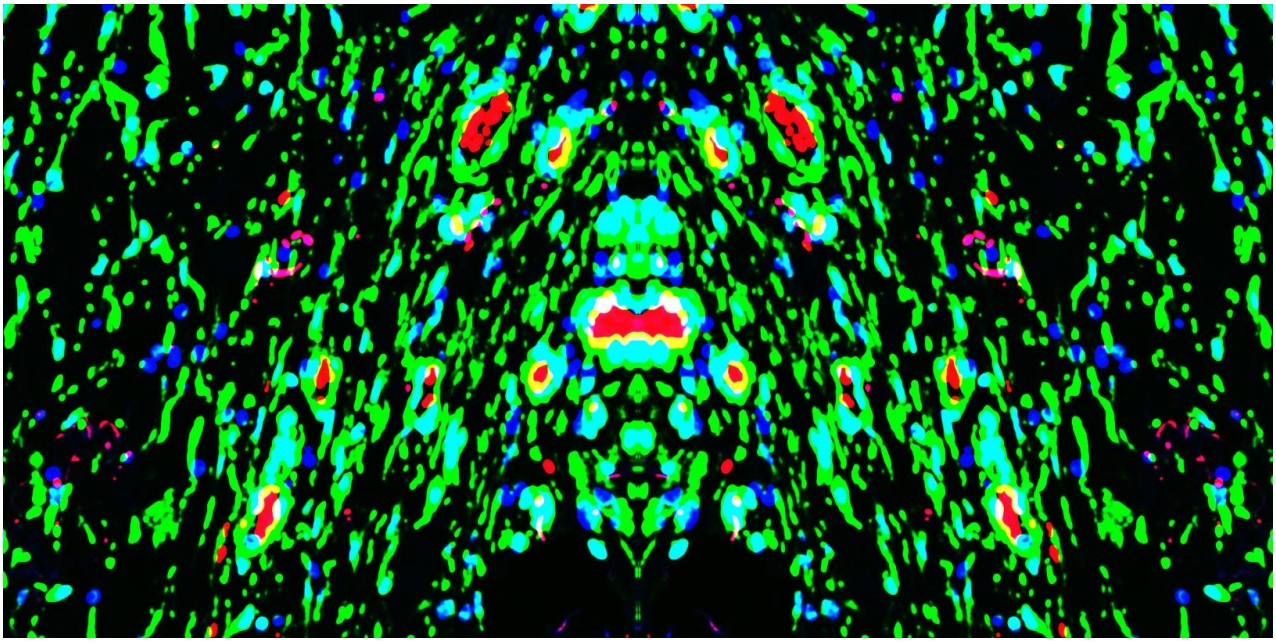
for transporting of ACh, and excitatory receptor, α7AChR. Moreover, the anti-siglec-E treatment augmented M3R expression, suggesting that anti-siglec-E might exerts its effects through ACh production and α7AChR but not M3R.

6.5. Conclusion

Cancer cells have evolved to create an environment, which favours their growth by expressing immunosuppressive and cholinergic markers, enabling them to avoid the host's immune response. In this Chapter, findings suggest that siglec-E might influence tumour progression and metastasis by modulating the expression of IDO, cholinergic signalling and angiogenesis as well as tumour-associated immune cells. Cancer cells have evolved to create an environment, which favours their growth by expressing immunosuppressive and cholinergic markers, enabling them to avoid the host's immune response. In this Chapter, findings suggest that siglec-E might influence tumour progression and metastasis by modulating the expression of IDO, cholinergic signalling and angiogenesis as well as tumour-associated immune cells. Inhibition of siglec-E has significant anti-cancer effects evidenced in both *in vitro* and *in vitro* models. The results of our studies demonstrated that these effects are exerted via several mechanisms including enhancement of anti-tumour immune response, suppression of angiogenesis via inhibition of cholinergic signalling through reducing ACh production, transport and cholinergic receptor, α7nAChR via inhibition of EGFR/AKT/ERK signalling pathway. These are important findings that provide basis for understanding the interaction between the immunosuppressive molecules and cholinergic signalling and hold great potential in revolutionising the current therapies targeting colorectal cancer and many other solid tumours.

CHAPTER SEVEN

General Discussion and Conclusions



7.1. General comments

Cancer cells can influence their microenvironment and bi-directionally communicate with other systems such as the immune and nervous systems. The immune system plays a key role in the eradication of cancer cells. Studies have shown that multiple mechanisms are responsible for the suppression of the immune system in cancer, one of which being the expression of immune checkpoint inhibitors such as programmed death 1 (PD-1), PD-L1, programmed death-ligand 1 and 2 (PD-L1, PD-L2), sialic acid-binding lectins 9 (siglec-9) and indoleamine-2,3-dioxygenase (DO) [509, 514]. These molecules function by inhibiting the anti-tumour effects of T cell-mediated immune responses. Although current therapies are targeting some of these molecules, they have shortcomings such as causing adverse events. Therefore, it is crucial to understand the possible underlying mechanisms involved in the complex interaction between these molecules with cholinergic signalling within the tumour microenvironment

Several lines of evidence have implicated the nervous and immune systems to play essential roles in cancer progression. Dysfunction of the nervous system may influence cancer progression by inhibiting functions of the immune system and vice versa. The sympathetic and parasympathetic (cholinergic) nerves are involved in the control of inflammation, which is believed to be a driving force for cancer progression [622].

Deciphering mechanisms by which immune and nervous systems stimulate tumour development may open new avenues for understanding cancer progression, identification of new biomarkers for cancer diagnosis and prognosis, and, defining novel targets for therapeutic interventions.

Currently, there are no data available in determining the interaction between the expression of immunosuppressive and cholinergic markers in colorectal cancer (CRC). The studies presented in this thesis provide novel insight into the interaction between the expression of immunosuppressive and cholinergic markers and mechanisms involved in this interaction. The findings of this work have important clinical relevance by revealing new therapeutic targets for the treatment of CRC.

7.2. Effects of cholinergic signalling on tumour microenvironment

The nervous system bi-directionally communicates with the immune system via neurotransmitters and neuropeptides, common receptors, tumour-associated immune cells and cytokines as discussed in **Chapter 1**. The release of neurotransmitters by sympathetic and parasympathetic nerve fibers has been shown to influence tumour microenvironment to promote tumour growth and progression via the expression of cytokines and tumour-associated immune cells. However, crosstalk between the nervous and immune systems is highly multifaceted, and numerous variations are possible according to the type of cancer involved.

Tumour cells use multiple mechanisms to escape recognition by immune cells and to downregulate the hosts' immune system by expressing immunosuppressive molecules, mPD-L1 and PD-L2, which interact with PD-1 receptor on tumour-infiltrating lymphocytes [525]. The presence of PD-L1 and PD-L2 on the surface of tumours functions as an immune resistance mechanism allowing tumours to go undetected, leading to cancer cell proliferation and progression of tumour growth. However, the nervous system has emerged to play a significant role in promoting cancer development and progression. The results of studies presented in this thesis have provided new data that the expression of immunosuppressive and cholinergic markers may play an important role in influencing each other, leading to tumour cells avoiding immune system detection and cancer progression.

Although several studies have evaluated the expression of PD-L1 and PD-L2 in CRC [528, 569, 651, 652], to the best of our knowledge, the data presented in this thesis are the first to determine the correlation between immunosuppressive and cholinergic markers at different stages of CRC. The results presented in **Chapter 2** (**Figure 2.1-2, Table 2.3**) showed that high expression of PD-L1 mainly in the mucosa and muscularis mucosa layers correlated with advanced stages of CRC. High PD-L1 expression was associated with tumour node metastasis, poor prognosis and shorter survival in CRC patients [62, 527]. In addition, upregulation of PD-L1 by cancer cells results in cancer

invasion and correlates with poor prognostic outcomes in breast, gastric, meningioma, non-small cell lung carcinoma (NSCLC) and soft tissue sarcoma patients [54, 653-656].

Although, our findings did not correlate PD-L1 expression with tumour metastasis, however, there was a strong association with a higher risk of CRC and shorter survival in CRC patients, concurring with these studies. The findings suggest that the prognostic value of PD-L1 expression could be dependent on the subset of CRCs as well as the presence of infiltrating immune cells.

Similarly, PD-L2 was highly expressed at stages II, III and IV compared to stage I and was predominantly expressed in mucosa with exception to stage IV where it was expressed in the mucosa and muscularis mucosa layers (**Chapter 2, Figure 2.1-2, Table 2.4**). High levels of PD-L2 expression was associated with a higher risk of CRC and poor patients' survival outcomes. Studies have reported that PD-L2 correlates with tumour stages, lymph node metastasis and poor survival in CRC patients [569, 657]. It has been proposed that depending on the molecules present in the tumour microenvironment, the expression of PD-L2 can be augmented on immune as well as non-immune cells [539].

Cholinergic signalling in CRC has emerged as one of the important hallmarks of tumour progression. Studies that precisely focus on the expression of alpha 7 nicotinic receptor (α7nAChR) in CRC are limited. Human colon cancer cells, HT-29, overexpress α7nAChR, which facilitates cell proliferation, tumour angiogenesis [463, 547] and metastasis [201, 522, 548-550]. Data presented in Chapter 2, no significant difference in the expression of α7nAChR in tumours from patients at all stages of CRC demonstrate (**Chapter 2, Figure 2.4, Table 2.5**). Similarly, no association between α7nAChR expression and patients' risk of CRC and survival outcome was observed.

There are limited studies associating the expression of muscarinic receptor 3 (M3R) and choline acetyltransferase (ChAT) with different stages of CRC and clinical parameters. Studies regarding the role of M3R and ChAT in CRC are mainly performed in the cell lines or animal models. Only few studies have reported that M3R and ChAT are overexpressed in human colon cancer tissues compared to normal samples. M3R was found to be expressed in 60% of colon cancer cell lines [551, 553] and 8-fold increased expression

of M3R in 62% of colon cancers when compared to normal adjacent and normal colon epithelium [552]. Similarly, ChAT was found to be upregulated in NSCLC while cholinesterase enzymes are downregulated, leading to an increased ACh in tumour tissues [572, 573]. Data presented in Chapter 2 show elevated levels of M3R and ChAT expression at stages III and IV compared to early stages (**Chapter 2**, **Figure 2.4)**. High levels of M3R and ChAT were associated with a high risk of CRC and poor patients' survival outcomes.

Overall, the contradiction in findings regarding PD-L1 and PD-L2 expression in CRC might be in part due to the expression of cholinergic or other immunosuppressive markers as we found the link between the expression of immunosuppressive and cholinergic markers. Taken together, these findings suggest that there is a crosstalk between immunosuppressive and cholinergic markers. In fact, our findings are supported by a recent study by Kamiya et al (2019) in breast cancer patients demonstrating decreased parasympathetic nerve density, determined by vesicular acetylcholine transporter (VAChT) expression, was associated with poor clinical outcomes and elevated levels of immune checkpoint molecules [558].

To explore this further, the effect of blocking muscarinic receptors with atropine and selective M3R blocker 1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) on the expression of immunosuppressive and cholinergic markers was evaluated in normal epithelial (T4056) and human colon cancer cell lines (LIM-2405 and HT-29). Results presented in **Chapter 3** are amongst the first studies to determine the interaction between immunosuppressive markers and ACh acting on muscarinic receptors. The results showed that PD-L1, PD-L2 and M3R were significantly elevated in human colon cancer cells compared to normal epithelial cells and this effect was abolished by 4-DAMP and atropine treatment via the modulation of EGFR/ERK/STAT3 pathway. *In vitro* results demonstrated that CT-26 murine colon cancer cells expressed PD-L1 and PD-L2, M3R and ChAT, which were attenuated by atropine and 4-DAMP treatment via EGFR/AKT/ERK signalling pathway (**Chapter 4)**. To evaluate whether the effect of 4-DAMP *in vitro* can be confirmed *in vivo*, mice bearing CT-26 cell-induced CRC were injected daily with (0.1% dimethyl sulfoxide) DMSO or 4-DAMP for 3 weeks. Treatment

with 4-DAMP significantly decreased tumour weight, volume and size when compared to vehicle group treated with DMSO. Enhanced expression of PD-L1, M3R, ChAT and angiogenic markers were attenuated by 4-DAMP treatments *in vivo* through inhibition of AKT and ERK phosphorylation, leading to an enhanced immune response against cancer. The results presented in **Chapters, 2-4**, suggest that cholinergic signalling not only stimulates the expression of immunosuppressive markers but could explain the inconsistency in prognostic value.

7.3. Effects of immunosuppressive markers on tumour microenvironment

Data presented in **Chapters, 2-4** demonstrated that dysfunction in cholinergic signalling influences the expression of immunosuppressive markers, PD-L1 and PD-L2. In Chapter 5 and 6, the expression of siglec-9 and IDO expression was evaluated in human specimens, and the effects of blocking siglec-9/siglec-E on the expression of cholinergic markers as well as signalling pathways involved in these effects were investigated *in vitro* and *in vivo*. Currently, there are no studies investigating the interaction between the expression of siglec-9 and cholinergic markers in cancer. It was hypothesised that the blocking of siglec-9 might influence cholinergic markers.

Data presented in **Chapter 5** demonstrated high expression of siglec-9 and IDO at stages III and IV compared to early stages, I and II, of CRC (**Chapter 5**, **Figure 5.1**). Siglec-9 and IDO expression were associated with a higher risk of CRC and poor patients' survival outcomes (**Chapter 5**, **Figure 5.2**). However, there was no significant correlation between the expression of these markers and patient's gender, age and metastasis. Interestingly, overall expression of siglec-9 was correlated with M3R expression, suggesting crosstalk between siglec-9 and M3R. Studies have demonstrated that T cells expressing siglec-9 can be co-localised with other immunosuppressive markers such as PD-1, PD-L1 and IDO [645, 646]. In fact, findings of this study showed that siglec-9 was co-expressed with IDO in tumour tissues from CRC patients at different stages (**Chapter 5**, **Figure 5.2**). It can be speculated that siglec-9 can also be co-expressed with cholinergic markers and treatment with human anti-siglec-9 antibody can influence the expression of cholinergic markers. *In vitro* data presented in **Chapter 5** showed that blocking siglec-9 with human

anti-siglec-9 antibody significantly inhibited cell proliferation and choline production. It also significantly decreased IDO expression in HT-29 but had no effects on T4056 and LIM-2405 cells. Human anti-siglec-9 antibody had no effects on T4056 but significantly reduced siglec-9 expression in human colon cancer cells, LIM-2405 and HT-29. Interestingly, blocking siglec-9 decreased M3R expression in T4056 but not human colon cancer cells and had no effects on ChAT expression, proposing that siglec-9 effects on cholinergic markers might be influenced by the tumour microenvironment. Taken together, human siglec-9 antibody was found to unveil its effects in a normal epithelial cell via suppressing EGFR/ERK/STAT3 signalling pathway, while in LIM-2405 human colon cancer cells, it acts by inhibiting ERK/STAT3 signalling pathway.

To further investigate the effects of blocking siglec-9 and the influence of tumour microenvironment, mice bearing CT-26 cell-induced were injected with mouse anti-siglec-E antibody, a human siglec-9 counterpart. Similar to human colon cancer cells, mouse anti-siglec-E antibody significantly inhibited the expression of siglec-9 and cholinergic markers in CT-26 cells via inhibiting EGFR/AKT/ERK signalling pathway (**Chapter 6**, **Figure 6.3-5**). Similarly, mouse anti-siglec-E antibody significantly reduced tumour weight, volume and size, and inhibited the expression of siglec-9, cholinergic and angiogenic markers *in vivo* through inhibition of EGFR/AKT/ERK signalling pathway. *In vivo* findings confirmed our hypothesis that siglec-9 might influence cholinergic signalling as siglec-E was co-expressed with cholinergic markers which was reduced by anti-siglec-E antibody treatment (**Chapter 6**, **Figure 6.11**). Moreover, mouse anti-siglec-E antibody treatment significantly augmented anti-tumour response through increased infiltration of CD4+ and CD8+ T cells. These findings are in line with studies demonstrating that siglec-E deficient mice showed an improved immunosurveillance against tumour cells via blocking siglec-9 expressed on neutrophils [94]. Our findings demonstrated blocking of siglec-E resulted in an improved immune response against cancer and increased expression of neutrophils **(Chapter 6**, **Figure 6.15D**).

Though the overall survival of patients with metastatic CRC has increased over the past decade as a result of improvements and implementation of new therapies, the 5-year survival of patients remains poor, and cancer metastasis remains as one of the leading

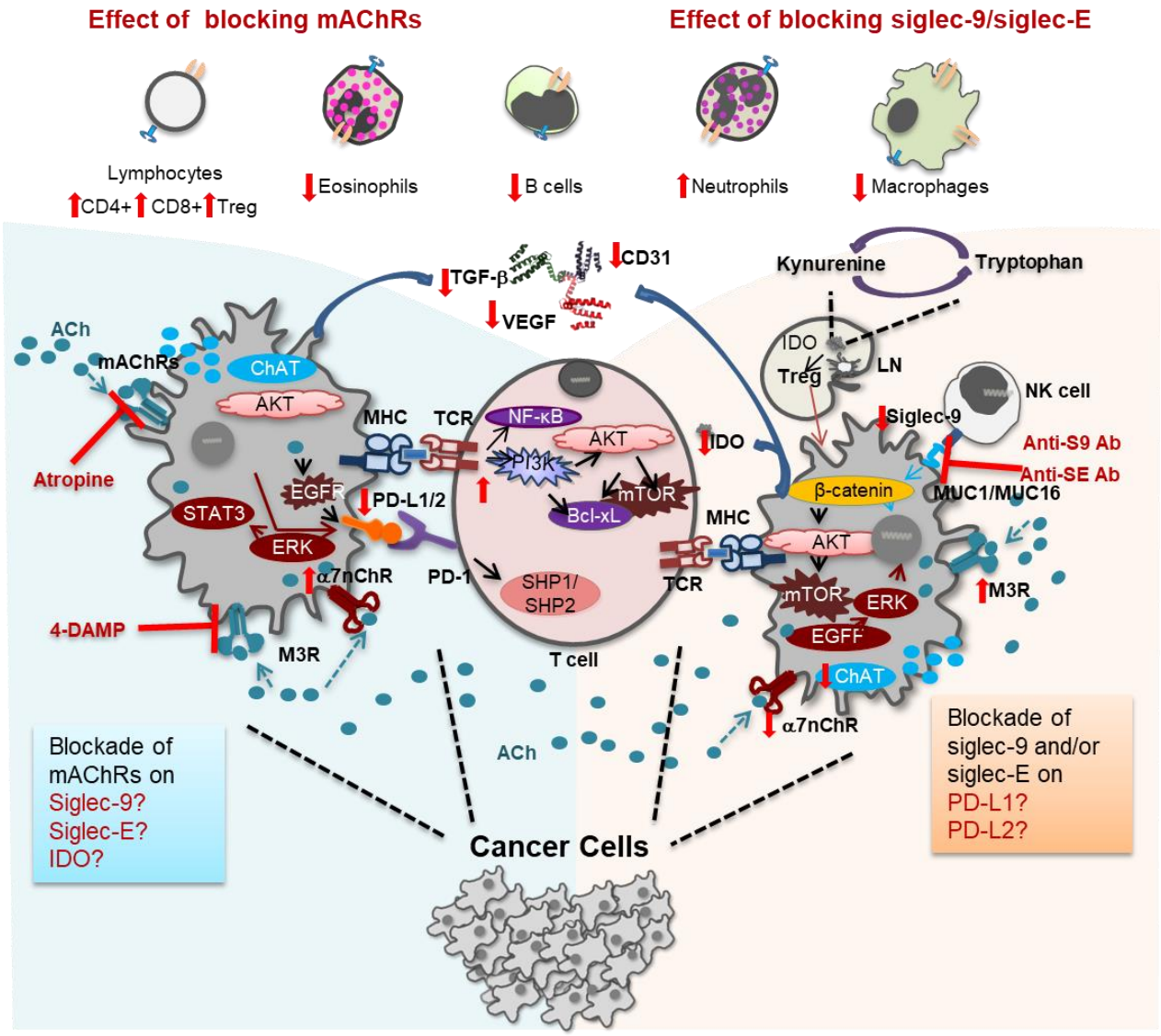
causes of cancer-related death [578, 658, 659]. In experimental metastasis assay, expression of siglec-9/siglec-E binding to tumour-associated ligands inhibits neutrophils and enhances lung colonisation [94]. This insinuates that siglec-9 or siglec-E may play a role in tumour metastasis. The data presented in **Chapter 6** confirmed that siglec-9/siglec-E plays an important role in tumour angiogenesis and metastasis as treatment with anti-siglec-E antibody significantly inhibited metastasis and reduced angiogenic markers (**Chapter 6**, **Figure 6.8** and **Figure 6.15**).

Taken together, these findings suggest that siglec-9/siglec-E might influence the expression of IDO, cholinergic and angiogenic markers as well as tumour-associated immune cells.

Figure 7.1. Schematic diagram illustrating the effects of blocking muscarinic receptors and siglec-9/siglec-E on tumour microenvironment

T cell activation entails antigen recognition in complex with MHC class I (CD8+ T cells) or MHC class II (CD4+ T cells), followed by further support from the co-stimulatory signal that determines whether the T cell will be switched on or off in response to the antigenic peptide. Tumour cells have evolved to display mechanisms that generate an immunosuppressive environment enabling them to avoid this safeguard mechanism. These mechanisms encompass the alteration of antigen presentation components (such as downregulation of MHC class I) and upregulation of immunosuppressive (PD-L1, PD-L2, IDO, and siglec-9) and angiogenic (VEGF, CD31 and TGF-β) markers. In addition, tumour cells utilise cholinergic signalling through their receptors, leading to growth, proliferation and migration of tumours. Understanding the regulation of these mechanisms might contribute to disabling the tumour immunosuppressive microenvironment. Blockade of muscarinic receptors with atropine and M3R with 4-DAMP, significantly reduced immunosuppressive, cholinergic and angiogenic marker as well as tumour-associated immune cells. Similarly, blockade of siglec-9/siglec-E with anti-S9/anti-SE Ab significantly attenuated immunosuppressive molecules, cholinergic and angiogenic marker as well as tumour-associated immune cells.

Abbreviations: 4-DAMP, 1-dimethyl-4-diphenylacetoxypiperidinium iodide; α7nAChR, alpha 7 nicotinic receptor; AKT, serine/threonine kinase or protein kinase B; Anti-S9 Ab, anti-siglec-9 antibody; Anti-SE Ab, anti-siglec-E antibody; Bcl-xL, B-cell lymphoma-extra large; EGFR, epidermal growth factor receptor; IDO, indoleamine-2,3-dioxygenase; JAK, Janus kinase; MHC, major histocompatibility complex; mTOR, mammalian/mechanistic target of rapamycin; NF-kB, nuclear factor kappa B; MUC, mucin; NK, natural killer; PI3K, phosphoinositide 3-kinase; PD-1, programmed death 1; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2; PI3K, phosphoinositide 3-kinase; SHP1/SHP2, Src-homology domain-containing protein tyrosine phosphatases; TCR, T cell receptor; Treg, regulatory T cell; Siglec-9, sialic acid-binding lectins 9; Siglec-E, murine sialic acid-binding lectins; STAT3, signal transducer and activator of transcription 3; TGF-β, tumour growth factor-beta.



Apoptosis

Proliferation

Angiogenesis

Invasion

Migration

Metastasis

7.4. Limitations and Further Directions

PD-1/PD-L1 expression has been shown to play protective role in autoimmune, neurological diseases and stroke but exacerbate cancer [513]. This may be due to the nervous system releasing neuromodulators that desensitize PD-1/PD-L1 to inhibit the activation of PD-1/PD-L1 signalling. In cancer, their expression generally results in disease acceleration, supporting the notion that cancer cells secrete factors that desensitize the nervous system leading to alteration of the immune system response to attack the cancer cells. Although this work is amongst the few studies that provide evidence of the interaction between the expression of immunosuppressive and cholinergic markers in CRC supporting this notion, there are some limitations that need to be address in the future studies. Firstly, in **Chapters 2, 4-6**, the expression of immunosuppressive and cholinergic markers was evaluated in small pieces of tumours from human specimens and tumours from mice bearing-CT-26 cell. Evaluating whole tumours would have given more information on the overall expression of these markers. Secondly, it not clear whether the expression of these markers was from tumour cells or tumour microenvironment constituents as they are able to express these markers. Thirdly, the location of the expression was not clear; this can be addressed by taking images at higher magnifications. Finally, some of the immunosuppressive markers that rely on IFN-γ for expression such as PD-L1, PD-L2 and IDO [660, 661] could have their expression increased in a similar way with ACh. This link could have therapeutic implications with targeting the production of ACh and essentially alleviating effects on several immunosuppressive markers.

7.5. General conclusion

Although immunotherapies have shown great efficacy in many solid tumours, there is still a need to develop better therapies with less side-effects. The results presented in this thesis suggest that blocking of M3R and siglec-9/siglec-E have the potential to be used in conjunction with current immune checkpoint inhibitors or traditional cancer therapeutics to increase the efficacy of anti-cancer treatment. In conclusion, it is important to evaluate the expression status of some or all these immunosuppressive molecules and cholinergic markers for cancer prognosis and the development of appropriate therapeutic strategies

in cancer patients. The findings of this work have important clinical relevance and might create a new therapeutic avenue, which could target both immunosuppressive and cholinergic markers that might be beneficial for the treatment of CRC patients.

CHAPTER EIGHT

8.1. References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F: **Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012**. *International journal of cancer* 2015, **136**(5):E359-386.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A: **Global cancer statistics, 2012**. *CA: a cancer journal for clinicians* 2015, **65**(2):87-108.
3. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, Gong Z, Zhang S, Zhou J, Cao K *et al*: **Role of tumor microenvironment in tumorigenesis**. *J Cancer* 2017, **8**(5):761-773.
4. Place AE, Jin Huh S, Polyak K: **The microenvironment in breast cancer progression: biology and implications for treatment**. *Breast Cancer Res* 2011, **13**(6):227.
5. Mravec B, Gidron Y, Hulin I: **Neurobiology of cancer: Interactions between nervous, endocrine and immune systems as a base for monitoring and modulating the tumorigenesis by the brain**. *Seminars in cancer biology* 2008, **18**(3):150-163.
6. Holliday DL, Brouilette KT, Markert A, Gordon LA, Jones JL: **Novel multicellular organotypic models of normal and malignant breast: tools for dissecting the role of the microenvironment in breast cancer progression**. *Breast cancer research : BCR* 2009, **11**(1):R3-R3.
7. Quail DF, Joyce JA: **Microenvironmental regulation of tumor progression and metastasis**. *Nature medicine* 2013, **19**(11):1423-1437.
8. Garcia-Martinez E, Gil GL, Benito AC, Gonzalez-Billalabeitia E, Conesa MA, Garcia Garcia T, Garcia-Garre E, Vicente V, Ayala de la Pena F: **Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer**. *Breast cancer research : BCR* 2014, **16**(6):488.
9. Huang D, Su S, Cui X, Shen X, Zeng Y, Wu W, Chen J, Chen F, He C, Liu J *et al*: **Nerve fibers in breast cancer tissues indicate aggressive tumor progression**. *Medicine* 2014, **93**(27):e172.
10. Sloan EK, Priceman SJ, Cox BF, Yu S, Pimentel MA, Tangkanangnukul V, Arevalo JM, Morizono K, Karanikolas BD, Wu L *et al*: **The sympathetic nervous system induces a metastatic switch in primary breast cancer**. *Cancer research* 2010, **70**(18):7042-7052.
11. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M: **Angiogenesis in cancer**. *Vascular health and risk management* 2006, **2**(3):213-219.

12. Gupta GP, Massague J: **Cancer metastasis: building a framework**. *Cell* 2006, **127**(4):679-695.
13. Weis SM, Cheresh DA: **Tumor angiogenesis: molecular pathways and therapeutic targets**. *Nature medicine* 2011, **17**(11):1359-1370.
14. Jobling P, Pundavela J, Oliveira SM, Roselli S, Walker MM, Hondermarck H: **Nerve-Cancer Cell Cross-talk: A Novel Promoter of Tumor Progression**. *Cancer research* 2015, **75**(9):1777-1781.
15. Ondicova K, Mravec B: **Role of nervous system in cancer aetiopathogenesis**. *The Lancet Oncology* 2010, **11**(6):596-601.
16. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A: **Global cancer statistics, 2012**. *CA: A Cancer Journal for Clinicians* 2015, **65**(2):87-108.
17. Haggar FA, Boushey RP: **Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors**. *Clinics in colon and rectal surgery* 2009, **22**(04):191-197.
18. Sameer ASS: **Colorectal cancer: molecular mutations and polymorphisms**. *Frontiers in oncology* 2013, **3**:114.
19. Jan-Sing H, Shiu-Ru L, Mei-Yin C, Fang-Ming C, Chien-Yu L, Tsung-Jen H, Yu-Sheng H, Che-Jen H, Jaw-Yuan W: **APC, K-ras, and p53 gene mutations in colorectal cancer patients: correlation to clinicopathologic features and postoperative surveillance**. *The American Surgeon* 2005, **71**(4):336-343.
20. Nazemalhosseini Mojarad E, Kuppen PJ, Aghdaei HA, Zali MR: **The CpG island methylator phenotype (CIMP) in colorectal cancer**. *Gastroenterol Hepatol Bed Bench* 2013, **6**(3):120-128.
21. Bardhan K, Liu K: **Epigenetics and colorectal cancer pathogenesis**. *Cancers* 2013, **5**(2):676-713.
22. Koopman M, Kortman GA, Mekenkamp L, Ligtenberg MJ, Hoogerbrugge N, Antonini NF, Punt CJ, van Krieken JH: **Deficient mismatch repair system in patients with sporadic advanced colorectal cancer**. *Br J Cancer* 2009, **100**(2):266-273.
23. Llosa NJ, Cruise M, Tam A, Wicks EC, Hechenbleikner EM, Taube JM, Blosser RL, Fan H, Wang H, Luber BS *et al*: **The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints**. *Cancer Discov* 2015, **5**(1):43-51.
24. Xiao Y, Freeman GJ: **The microsatellite instable subset of colorectal cancer is a particularly good candidate for checkpoint blockade immunotherapy**. *Cancer Discov* 2015, **5**(1):16-18.
25. Gatalica Z, Snyder CL, Yeatts K, Xiao N, Holterman D, Lynch HT: **Programmed death 1 (PD-1) lymphocytes and ligand (PD-L1) in colorectal cancer and their relationship to microsatellite instability status**. *Journal of Clinical Oncology* 2014, **32**(15_suppl):3625-3625.
26. Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C, Marini FC: **Tumor-associated stromal cells as key contributors to the tumor microenvironment**. *Breast Cancer Research* 2016, **18**(1):84.
27. Karwacz K, Bricogne C, MacDonald D, Arce F, Bennett CL, Collins M, Escors D: **PD-L1 co-stimulation contributes to ligand-induced T cell receptor down-modulation on CD8(+) T cells**. *EMBO Molecular Medicine* 2011, **3**(10):581-592.

28. Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen L: **B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells**. *Blood* 2008, **111**(7):3635-3643.
29. Ceeraz S, Nowak EC, Noelle RJ: **B7 family checkpoint regulators in immune regulation and disease**. *Trends Immunol* 2013, **34**(11):556-563.
30. Swanson MS, Sinha UK: **Rationale for combined blockade of PD-1 and CTLA-4 in advanced head and neck squamous cell cancer–review of current data**. *Oral oncology* 2015, **51**(1):12-15.
31. Janakiram M, Abadi YM, Sparano JA, Zang X: **T cell coinhibition and immunotherapy in human breast cancer**. *Discovery medicine* 2012, **14**(77):229.
32. Yu J, Du W, Yan F, Wang Y, Li H, Cao S, Yu W, Shen C, Liu J, Ren X: **Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer**. *Journal of immunology (Baltimore, Md : 1950)* 2013, **190**(7):3783-3797.
33. OuYang LY, Wu XJ, Ye SB, Zhang RX, Li ZL, Liao W, Pan ZZ, Zheng LM, Zhang XS, Wang Z *et al*: **Tumor-induced myeloid-derived suppressor cells promote tumor progression through oxidative metabolism in human colorectal cancer**. *Journal of translational medicine* 2015, **13**:47.
34. Levano KS, Jung EH, Kenny PA: **Breast cancer subtypes express distinct receptor repertoires for tumor-associated macrophage derived cytokines**. *Biochemical and Biophysical Research Communications* 2011, **411**(1):107-110.
35. Li T, Yang Y, Hua X, Wang G, Liu W, Jia C, Tai Y, Zhang Q, Chen G: **Hepatocellular carcinoma-associated fibroblasts trigger NK cell dysfunction via PGE2 and IDO**. *Cancer letters* 2012, **318**(2):154-161.
36. Wei T, Zhang J, Qin Y, Wu Y, Zhu L, Lu L, Tang G, Shen Q: **Increased expression of immunosuppressive molecules on intratumoral and circulating regulatory T cells in non-small-cell lung cancer patients**. *American journal of cancer research* 2015, **5**(7):2190-2201.
37. Ritprajak P, Azuma M: **Intrinsic and extrinsic control of expression of the immunoregulatory molecule PD-L1 in epithelial cells and squamous cell carcinoma**. *Oral Oncol* 2015, **51**(3):221-228.
38. Chen J, Shao R, Chen C: **Possible therapeutic implication of PD-L1/PD-1 axis in endometrial cancer**. *Journal of Solid Tumors* 2015, **5**(1).
39. Rosell R, Gonzalez-Cao M, Karachaliou N, Viteri S, Morales Espinosa D, Teixidó C, Sanchez Ruiz J, Molina-Vila MA, Santarpia M: **Targeting PD-1/PD-L1 in lung cancer: current perspectives**. *Lung Cancer: Targets and Therapy* 2015:55.
40. Cedrés S, Ponce-Aix S, Zugazagoitia J, Sansano I, Enguita A, Navarro-Mendivil A, Martinez-Marti A, Martinez P, Felip E: **Analysis of Expression of Programmed Cell Death 1 Ligand 1 (PD-L1) in Malignant Pleural Mesothelioma (MPM)**. *PLoS ONE* 2015, **10**(3):1-12.
41. D'Angelo SP, Shoushtari AN, Agaram NP, Kuk D, Qin L-X, Carvajal RD, Dickson MA, Gounder M, Keohan ML, Schwartz GK: **Prevalence of tumor-infiltrating lymphocytes and PD-L1 expression in the soft tissue sarcoma microenvironment**. *Human pathology* 2015, **46**(3):357-365.

42. He J, Hu Y, Hu M, Li B: **Development of PD-1/PD-L1 Pathway in Tumor Immune Microenvironment and Treatment for Non-Small Cell Lung Cancer**. *Scientific reports* 2015, **5**.
43. Mittendorf EA, Philips AV, Meric-Bernstam F, Qiao N, Wu Y, Harrington S, Su X, Wang Y, Gonzalez-Angulo AM, Akcakanat A *et al*: **PD-L1 expression in triple-negative breast cancer**. *Cancer Immunol Res* 2014, **2**(4):361-370.
44. Jilaveanu LB, Shuch B, Zito CR, Parisi F, Barr M, Kluger Y, Chen L, Kluger HM: **PD-L1 Expression in Clear Cell Renal Cell Carcinoma: An Analysis of Nephrectomy and Sites of Metastases**. *J Cancer* 2014, **5**(3):166-172.
45. Zandberg DP, Strome SE: **The role of the PD-L1:PD-1 pathway in squamous cell carcinoma of the head and neck**. *Oral Oncol* 2014, **50**(7):627-632.
46. D'Incecco A, Andreozzi M, Ludovini V, Rossi E, Capodanno A, Landi L, Tibaldi C, Minuti G, Salvini J, Coppi E *et al*: **PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients**. *British Journal of Cancer* 2014, **112**(1):95-102.
47. Guo Z, Wang X, Cheng D, Xia Z, Luan M, Zhang S: **PD-1 Blockade and OX40 Triggering Synergistically Protects against Tumor Growth in a Murine Model of Ovarian Cancer**. *PLoS ONE* 2014, **9**(2):1-10.
48. Mu CY, Huang JA, Chen Y, Chen C, Zhang XG: **High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation**. *Med Oncol* 2011, **28**(3):682-688.
49. Alsuliman A, Colak D, Al-Harazi O, Fitwi H, Tulbah A, Al-Tweigeri T, Al-Alwan M, Ghebeh H: **Bidirectional crosstalk between PD-L1 expression and epithelial to mesenchymal transition: Significance in claudin-low breast cancer cells**. *Molecular cancer* 2015, **14**(1):149.
50. Ghebeh H, Barhoush E, Tulbah A, Elkum N, Al-Tweigeri T, Dermime S: **FOXP3+ Tregs and B7-H1+/PD-1+ T lymphocytes co-infiltrate the tumor tissues of high-risk breast cancer patients: Implication for immunotherapy**. *BMC Cancer* 2008, **8**:57.
51. Ghebeh H, Tulbah A, Mohammed S, Elkum N, Bin Amer SM, Al-Tweigeri T, Dermime S: **Expression of B7-H1 in breast cancer patients is strongly associated with high proliferative Ki-67-expressing tumor cells**. *International journal of cancer* 2007, **121**(4):751-758.
52. Isabelle Gingras, Hatem A. Azim Jr, Michail Ignatiadis, Sotiriou C: **Immunology and Breast Cancer: Toward a New Way of Understanding Breast Cancer and Developing Novel Therapeutic Strategies**. *Clinical Advances in Hematology & Oncology* 2015, **13**(6).
53. Karasar P, Esendagli G: **T helper responses are maintained by basal-like breast cancer cells and confer to immune modulation via upregulation of PD-1 ligands**. *Breast Cancer Res Treat* 2014, **145**(3):605-614.
54. Muenst S, Schaerli A, Gao F, Däster S, Trella E, Droeser R, Muraro M, Zajac P, Zanetti R, Gillanders W: **Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer**. *Breast cancer research and treatment* 2014, **146**(1):15-24.

55. Muenst S, Soysal SD, Gao F, Obermann EC, Oertli D, Gillanders WE: **The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer**. *Breast Cancer Res Treat* 2013, **139**(3):667-676.
56. Sabatier R, Finetti P, Mamessier E, Adelaide J, Chaffanet M, Ali HR, Viens P, Caldas C, Birnbaum D, Bertucci F: **Prognostic and predictive value of PDL1 expression in breast cancer**. *Oncotarget* 2014, **6**(7).
57. Schalper KA: **PD-L1 expression and tumor-infiltrating lymphocytes: Revisiting the antitumor immune response potential in breast cancer**. *Oncoimmunology* 2014, **3**:e29288.
58. Soliman H, Khalil F, Antonia S: **PD-L1 Expression Is Increased in a Subset of Basal Type Breast Cancer Cells**. *PLoS ONE* 2014, **9**(2).
59. Sun S, Fei X, Mao Y, Wang X, Garfield D, Huang O, Wang J, Yuan F, Sun L, Yu Q *et al*: **PD-1 immune cell infiltration inversely correlates with survival of operable breast cancer patients**. *Cancer Immunology, Immunotherapy* 2014, **63**(4):395-406.
60. Tymoszuk P, Charoentong P, Hackl H, Spilka R, Müller-Holzner E, Zlatko T, Obrist P, Revillion F, Peyrat J-P, Fiegl H: **High STAT1 mRNA levels but not its tyrosine phosphorylation are associated with macrophage infiltration and bad prognosis in breast cancer**. *BMC Cancer* 2014, **14**(1):1-27.
61. Wimberly H, Brown JR, Schalper K, Haack H, Silver MR, Nixon C, Bossuyt V, Pusztai L, Lannin DR, Rimm DL: **PD-L1 Expression Correlates with Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy in Breast Cancer**. *Cancer Immunol Res* 2015, **3**(4):326-332.
62. Shi SJ, Wang LJ, Wang GD, Guo ZY, Wei M, Meng YL, Yang AG, Wen WH: **B7-H1 expression is associated with poor prognosis in colorectal carcinoma and regulates the proliferation and invasion of HCT116 colorectal cancer cells**. *PLoS One* 2013, **8**(10):e76012.
63. Zhao LW, Li C, Zhang RL, Xue HG, Zhang FX, Zhang F, Gai XD: **B7-H1 and B7-H4 expression in colorectal carcinoma: correlation with tumor FOXP3(+) regulatory T-cell infiltration**. *Acta histochemica* 2014, **116**(7):1163-1168.
64. Noguchi T, Ward JP, Gubin MM, Arthur CD, Lee SH, Hundal J, Selby MJ, Graziano RF, Mardis ER, Korman AJ *et al*: **Temporally Distinct PD-L1 Expression by Tumor and Host Cells Contributes to Immune Escape**. *Cancer Immunology Research* 2017.
65. Ritprajak P, Azuma M: **Intrinsic and extrinsic control of expression of the immunoregulatory molecule PD-L1 in epithelial cells and squamous cell carcinoma**. *Oral Oncology* 2015, **51**(3):221-228.
66. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, Sharpe AH: **PD-L1 regulates the development, maintenance, and function of induced regulatory T cells**. *The Journal of Experimental Medicine* 2009, **206**(13):3015-3029.
67. Ghebeh H, Mohammed S, Al-Omair A, Qattan A, Lehe C, Al-Qudaihi G, Elkum N, Alshabanah M, Amer SB, Tulbah A *et al*: **The B7-H1 (PD-L1)T Lymphocyte-Inhibitory Molecule Is Expressed in Breast Cancer Patients with Infiltrating**

Ductal Carcinoma: Correlation with Important High-Risk Prognostic Factors. *Neoplasia (New York, NY)* 2006, **8**(3):190-198.
68. Ali HR, Glont SE, Blows FM, Provenzano E, Dawson SJ, Liu B, Hiller L, Dunn J, Poole CJ, Bowden S *et al*: **PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes**. *Annals of oncology : official journal of the European Society for Medical Oncology* 2015, **26**(7):1488-1493.
69. Masugi Y, Nishihara R, Yang J, Mima K, da Silva A, Shi Y, Inamura K, Cao Y, Song M, Nowak JA *et al*: **Tumour CD274 (PD-L1) expression and T cells in colorectal cancer**. *Gut* 2016.
70. Yang CY, Lin MW, Chang YL, Wu CT, Yang PC: **Programmed cell death-ligand 1 expression in surgically resected stage I pulmonary adenocarcinoma and its correlation with driver mutations and clinical outcomes**. *Eur J Cancer* 2014, **50**(7):1361-1369.
71. Baptista MZ, Sarian LO, Derchain SF, Pinto GA, Vassallo J: **Prognostic significance of PD-L1 and PD-L2 in breast cancer**. *Hum Pathol* 2016, **47**(1):78-84.
72. Garcia-Lora A, Algarra I, Garrido F: **MHC class I antigens, immune surveillance, and tumor immune escape**. *Journal of cellular physiology* 2003, **195**(3):346-355.
73. Masuda K, Hiraki A, Fujii N, Watanabe T, Tanaka M, Matsue K, Ogama Y, Ouchida M, Shimizu K, Ikeda K: **Loss or down-regulation of HLA class I expression at the allelic level in freshly isolated leukemic blasts**. *Cancer science* 2007, **98**(1):102-108.
74. Ogino T, Shigyo H, Ishii H, Katayama A, Miyokawa N, Harabuchi Y, Ferrone S: **HLA class I antigen down-regulation in primary laryngeal squamous cell carcinoma lesions as a poor prognostic marker**. *Cancer research* 2006, **66**(18):9281-9289.
75. Kaneko K, Ishigami S, Kijima Y, Funasako Y, Hirata M, Okumura H, Shinchi H, Koriyama C, Ueno S, Yoshinaka H *et al*: **Clinical implication of HLA class I expression in breast cancer**. *BMC Cancer* 2011, **11**(1):1-6.
76. Inoue M, Mimura K, Izawa S, Shiraishi K, Inoue A, Shiba S, Watanabe M, Maruyama T, Kawaguchi Y, Inoue S: **Expression of MHC Class I on breast cancer cells correlates inversely with HER2 expression**. *Oncoimmunology* 2012, **1**(7):1104-1110.
77. Simpson JA, Al-Attar A, Watson NF, Scholefield JH, Ilyas M, Durrant LG: **Intratumoral T cell infiltration, MHC class I and STAT1 as biomarkers of good prognosis in colorectal cancer**. *Gut* 2010, **59**(7):926-933.
78. Yazdi MT, van Riet S, van Schadewijk A, Fiocco M, van Hall T, Taube C, Hiemstra PS: **The positive prognostic effect of stromal CD8+ tumor-infiltrating T cells is restrained by the expression of HLA-E in non-small cell lung carcinoma**. *Oncotarget* 2016, **7**(3):3477.
79. Andersson E, Poschke I, Villabona L, Carlson JW, Lundqvist A, Kiessling R, Seliger B, Masucci GV: **Non-classical HLA-class I expression in serous ovarian carcinoma: Correlation with the HLA-genotype, tumor infiltrating immune cells and prognosis**. *Oncoimmunology* 2016, **5**(1):e1052213.

80. Angell TE, Lechner MG, Jang JK, LoPresti JS, Epstein AL: **MHC class I loss is a frequent mechanism of immune escape in papillary thyroid cancer that is reversed by interferon and selumetinib treatment in vitro**. *Clinical Cancer Research* 2014, **20**(23):6034-6044.
81. Lees CJ, Smorodinsky N, Horn G, Wreschner DH, McKenzie IF, Pietersz G, Stojanovska L, Apostolopoulos V: **MUC1 immunotherapy against a metastatic mammary adenocarcinoma model: Importance of IFN-gamma**. *Prilozi* 2016, **37**(1):15-25.
82. Chen J-Y, Li C-F, Kuo C-C, Tsai KK, Hou M-F, Hung W-C: **Cancer/stroma interplay via cyclooxygenase-2 and indoleamine 2, 3-dioxygenase promotes breast cancer progression**. *Breast Cancer Research* 2014, **16**(4):1.
83. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, Boon T, Van den Eynde BJ: **Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase**. *Nature medicine* 2003, **9**(10):1269-1274.
84. Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vacca C, Orabona C, Bianchi R, Belladonna ML, Volpi C *et al*: **The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells**. *Journal of immunology (Baltimore, Md : 1950)* 2006, **176**(11):6752-6761.
85. Smith C, Chang MY, Parker KH, Beury DW, DuHadaway JB, Flick HE, Boulden J, Sutanto-Ward E, Soler AP, Laury-Kleintop LD: **IDO is a nodal pathogenic driver of lung cancer and metastasis development**. *Cancer discovery* 2012, **2**(8):722-735.
86. Della Chiesa M, Carlomagno S, Frumento G, Balsamo M, Cantoni C, Conte R, Moretta L, Moretta A, Vitale M: **The tryptophan catabolite L-kynurenine inhibits the surface expression of NKp46-and NKG2D-activating receptors and regulates NK-cell function**. *Blood* 2006, **108**(13):4118-4125.
87. Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, Belladonna ML, Fioretti MC, Alegre M-L, Puccetti P: **Modulation of tryptophan catabolism by regulatory T cells**. *Nature immunology* 2003, **4**(12):1206-1212.
88. Frumento G, Rotondo R, Tonetti M, Damonte G, Benatti U, Ferrara GB: **Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2, 3-dioxygenase**. *The Journal of Experimental Medicine* 2002, **196**(4):459-468.
89. Brandacher G, Perathoner A, Ladurner R, Schneeberger S, Obrist P, Winkler C, Werner ER, Werner-Felmayer G, Weiss HG, Gobel G *et al*: **Prognostic value of indoleamine 2,3-dioxygenase expression in colorectal cancer: effect on tumor-infiltrating T cells**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2006, **12**(4):1144-1151.
90. Wainwright DA, Balyasnikova IV, Chang AL, Ahmed AU, Moon K-S, Auffinger B, Tobias AL, Han Y, Lesniak MS: **IDO expression in brain tumors increases the recruitment of regulatory T cells and negatively impacts survival**. *Clinical cancer research* 2012, **18**(22):6110-6121.

91. Hanagiri T, Fukumoto M, Koyanagi Y, Furutani Y, Tanaka F: **Indoleamine2, 3-Dioxygenase as A Prognostic Factor in Patients with Non-Small Cell Lung Cancer**. *J Clin Cell Immunol* 2014, **5**(260):2.
92. Soliman H, Rawal B, Fulp J, Lee JH, Lopez A, Bui MM, Khalil F, Antonia S, Yfantis HG, Lee DH *et al*: **Analysis of indoleamine 2-3 dioxygenase (IDO1) expression in breast cancer tissue by immunohistochemistry**. *Cancer immunology, immunotherapy : CII* 2013, **62**(5):829-837.
93. Jacquemier J, Bertucci F, Finetti P, Esterni B, Charafe-Jauffret E, Thibult ML, Houvenaeghel G, Van den Eynde B, Birnbaum D, Olive D *et al*: **High expression of indoleamine 2,3-dioxygenase in the tumour is associated with medullary features and favourable outcome in basal-like breast carcinoma**. *International journal of cancer* 2012, **130**(1):96-104.
94. Läubli H, Pearce OMT, Schwarz F, Siddiqui SS, Deng L, Stanczak MA, Deng L, Verhagen A, Secrest P, Lusk C *et al*: **Engagement of myelomonocytic Siglecs by tumor-associated ligands modulates the innate immune response to cancer**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**(39):14211-14216.
95. Chugh S, Gnanapragassam VS, Jain M, Rachagani S, Ponnusamy MP, Batra SK: **Pathobiological implications of mucin glycans in cancer: Sweet poison and novel targets**. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 2015, **1856**(2):211-225.
96. Apostolopoulos V, Stojanovska L, Gargosky SE: **MUC1 (CD227): a multi-tasked molecule**. *Cellular and molecular life sciences : CMLS* 2015, **72**(23):4475-4500.
97. Das S, Batra SK: **Understanding the Unique Attributes of MUC16 (CA125): Potential Implications in Targeted Therapy**. *Cancer research* 2015, **75**(22):4669-4674.
98. Yin BW, Lloyd KO: **Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16**. *The Journal of biological chemistry* 2001, **276**(29):27371-27375.
99. Jandus C, Boligan KF, Chijioke O, Liu H, Dahlhaus M, Démoulins T, Schneider C, Wehrli M, Hunger RE, Baerlocher GM: **Interactions between Siglec-7/9 receptors and ligands influence NK cell–dependent tumor immunosurveillance**. *The Journal of clinical investigation* 2014, **124**(4):1810-1820.
100. Belisle JA, Horibata S, Jennifer GAA, Petrie S, Kapur A, André S, Gabius HJ, Rancourt C, Connor J, Paulson JC *et al*: **Identification of Siglec-9 as the receptor for MUC16 on human NK cells, B cells, and monocytes**. *Mol Cancer* 2010, **9**:118.
101. Tanida S, Akita K, Ishida A, Mori Y, Toda M, Inoue M, Ohta M, Yashiro M, Sawada T, Hirakawa K: **Binding of the sialic acid-binding lectin, Siglec-9, to the membrane mucin, MUC1, induces recruitment of β-catenin and subsequent cell growth**. *Journal of Biological Chemistry* 2013, **288**(44):31842-31852.
102. Sun Z, Fourcade J, Pagliano O, Chauvin J-M, Sander C, Kirkwood JM, Zarour HM: **Interleukin-10 and programmed death-1 cooperate to regulate tumor**

antigen-specific CD8+ T cells in melanoma patients. *Cancer research* 2015, **75**(15 Supplement):4046-4046.
103. Jana D, Sarkar DK, Ganguly S, Saha S, Sa G, Manna AK, Banerjee A, Mandal S: **Role of Cyclooxygenase 2 (COX-2) in Prognosis of Breast Cancer**. *Indian Journal of Surgical Oncology* 2014, **5**(1):59-65.
104. Wu CT, Chang YH, Lin WY, Chen WC, Chen MF: **TGF Beta1 Expression Correlates with Survival and Tumor Aggressiveness of Prostate Cancer**. *Annals of surgical oncology* 2015, **22 Suppl 3**:S1587-1593.
105. Yang C, He L, He P, Liu Y, Wang W, He Y, Du Y, Gao F: **Increased drug resistance in breast cancer by tumor-associated macrophages through IL-10/STAT3/bcl-2 signaling pathway**. *Medical Oncology* 2015, **32**(2):1-8.
106. Taga K, Tosato G: **IL-10 inhibits human T cell proliferation and IL-2 production**. *Journal of immunology (Baltimore, Md : 1950)* 1992, **148**(4):1143-1148.
107. Groux H, Bigler M, de Vries JE, Roncarolo M-G: **Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells**. *The Journal of Immunology* 1998, **160**(7):3188-3193.
108. Chen CJ, Sung WW, Su TC, Chen MK, Wu PR, Yeh KT, Ko JL, Lee H: **High expression of interleukin 10 might predict poor prognosis in early stage oral squamous cell carcinoma patients**. *Clinica chimica acta; international journal of clinical chemistry* 2013, **415**:25-30.
109. Itakura E, Huang R-R, Wen D-R, Paul E, Wünsch PH, Cochran AJ: **IL-10 expression by primary tumor cells correlates with melanoma progression from radial to vertical growth phase and development of metastatic competence**. *Modern Pathology* 2011, **24**(6):801-809.
110. Yue FY, Dummer R, Geertsen R, Hofbauer G, Laine E, Manolio S, Burg G: **Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-I, HLA class-II and ICAM-1 molecules**. *International journal of cancer* 1997, **71**(4):630-637.
111. Xiu B, Lin Y, Grote D, Ziesmer S, Gustafson M, Maas M, Zhang Z, Dietz A, Porrata L, Novak A: **IL-10 induces the development of immunosuppressive CD14+ HLA-DRlow/− monocytes in B-cell non-Hodgkin lymphoma**. *Blood cancer journal* 2015, **5**(7):e328.
112. Liu C, Liu J, Wang J, Liu Y, Zhang F, Lin W, Gao A, Sun M, Wang Y, Sun Y: **B7-H3 expression in ductal and lobular breast cancer and its association with IL-10**. *Molecular medicine reports* 2013, **7**(1):134-138.
113. Pockaj BA, Basu GD, Pathangey LB, Gray RJ, Hernandez JL, Gendler SJ, Mukherjee P: **Reduced T-cell and dendritic cell function is related to cyclooxygenase-2 overexpression and prostaglandin E2 secretion in patients with breast cancer**. *Annals of surgical oncology* 2004, **11**(3):328-339.
114. Holt DM, Ma X, Kundu N, Collin PD, Fulton AM: **Modulation of host natural killer cell functions in breast cancer via prostaglandin E2 receptors EP2 and EP4**. *Journal of immunotherapy (Hagerstown, Md : 1997)* 2012, **35**(2):179-188.

115. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S: **Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells**. *Cancer research* 2007, **67**(9):4507-4513.
116. Derynck R, Akhurst RJ, Balmain A: **TGF-[beta] signaling in tumor suppression and cancer progression**. *Nat Genet* 2001, **29**(2):117-129.
117. Maeda H, Shiraishi A: **TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice**. *Journal of immunology (Baltimore, Md : 1950)* 1996, **156**(1):73-78.
118. Wang Y, Deng B, Tang W, Liu T, Shen X: **TGF-beta1 secreted by hepatocellular carcinoma induces the expression of the Foxp3 gene and suppresses antitumor immunity in the tumor microenvironment**. *Digestive diseases and sciences* 2013, **58**(6):1644-1652.
119. Javle M, Li Y, Tan D, Dong X, Chang P, Kar S, Li D: **Biomarkers of TGF-β signaling pathway and prognosis of pancreatic cancer**. *PloS one* 2014, **9**(1):e85942.
120. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limón P: **The polarization of immune cells in the tumour environment by TGFβ**. *Nature reviews Immunology* 2010, **10**(8).
121. Thomas DA, Massague J: **TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance**. *Cancer cell* 2005, **8**(5):369-380.
122. Stephen TL, Rutkowski MR, Allegrezza MJ, Perales-Puchalt A, Tesone AJ, Svoronos N, Nguyen JM, Sarmin F, Borowsky ME, Tchou J: **Transforming growth factor β-mediated suppression of antitumor T cells requires FoxP1 transcription factor expression**. *Immunity* 2014, **41**(3):427-439.
123. Ghiringhelli F, Puig PE, Roux S, Parcellier A, Schmitt E, Solary E, Kroemer G, Martin F, Chauffert B, Zitvogel L: **Tumor cells convert immature myeloid dendritic cells into TGF-β–secreting cells inducing CD4(+)CD25(+) regulatory T cell proliferation**. *The Journal of Experimental Medicine* 2005, **202**(7):919-929.
124. Hanks BA, Holtzhausen A, Evans KS, Jamieson R, Gimpel P, Campbell OM, Hector-Greene M, Sun L, Tewari A, George A *et al*: **Type III TGF-beta receptor downregulation generates an immunotolerant tumor microenvironment**. *J Clin Invest* 2013, **123**(9):3925-3940.
125. Zhao Y, Hu J, Li R, Song J, Kang Y, Liu S, Zhang D: **Enhanced NK cell adoptive antitumor effects against breast cancer in vitro via blockade of the transforming growth factor-β signaling pathway**. *OncoTargets and therapy* 2015, **8**:1553-1559.
126. Kitamura T, Qian B-ZZ, Pollard JW: **Immune cell promotion of metastasis**. *Nature reviews Immunology* 2015, **15**.
127. Kitamura T, Qian B-ZZ, Soong D, Cassetta L, Noy R, Sugano G: **CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages**. *The Journal of Experimental Medicine* 2015, **212**.

128. McAllister SS, Weinberg RA: **The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis**. *Nature cell biology* 2014, **16**(8):717-727.
129. Sousa S, Brion R, Lintunen M, Kronqvist P, Sandholm J, Monkkonen J, Kellokumpu-Lehtinen PL, Lauttia S, Tynninen O, Joensuu H *et al*: **Human breast cancer cells educate macrophages toward the M2 activation status**. *Breast cancer research : BCR* 2015, **17**:101.
130. Grugan KD, McCabe FL, Kinder M, Greenplate AR, Harman BC, Ekert JE, van Rooijen N, Anderson GM, Nemeth JA, Strohl WR: **Tumor-associated macrophages promote invasion while retaining Fc-dependent anti-tumor function**. *The Journal of Immunology* 2012, **189**(11):5457-5466.
131. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A: **Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes**. *Trends Immunol* 2002, **23**.
132. Lewis C, Murdoch C: **Macrophage Responses to Hypoxia: Implications for Tumor Progression and Anti-Cancer Therapies**. *The American Journal of Pathology* 2005, **167**(3):627-635.
133. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CM, Pryer N, Daniel D, Hwang ES, Rugo HS, Coussens LM: **Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells**. *Cancer cell* 2014, **26**(5):623-637.
134. Wang R, Lu M, Zhang J, Chen S, Luo X, Qin Y, Chen H: **Increased IL-10 mRNA expression in tumor-associated macrophage correlated with late stage of lung cancer**. *Journal of experimental & clinical cancer research : CR* 2011, **30**:62.
135. Daurkin I, Eruslanov E, Stoffs T, Perrin GQ, Algood C, Gilbert SM, Rosser CJ, Su LM, Vieweg J, Kusmartsev S: **Tumor-associated macrophages mediate immunosuppression in the renal cancer microenvironment by activating the 15-lipoxygenase-2 pathway**. *Cancer research* 2011, **71**(20):6400-6409.
136. Bloch O, Crane CA, Kaur R, Safaee M, Rutkowski MJ, Parsa AT: **Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2013, **19**(12):3165-3175.
137. He R, Yang X, Shi Y-H, Li B, Liu W, Yin W, Dang Y, Chu Y, Fan J: **FAP promotes immunosuppression by cancer-associated fibroblasts in the tumor microenvironment via STAT3-CCL2 signaling**. *Cancer research* 2016:canres. 2973.2015.
138. Kato T, Noma K, Katsura Y, Kashima H, Ninomiya T, Ohara T, Tazawa H, Kagawa S, Shirakawa Y, Fujiwara T: **Prognostic correlation of tumor-infiltrating lymphocytes (TILs) and cancer associated fibroblasts (CAFs) in patients with human esophageal carcinoma**. *Cancer research* 2016, **76**(14 Supplement):4160-4160.
139. Kubo N, Araki K, Kuwano H, Shirabe K: **Cancer-associated fibroblasts in hepatocellular carcinoma**. *World journal of gastroenterology* 2016, **22**(30):6841-6850.

140. Takahashi H, Sakakura K, Kawabata-Iwakawa R, Rokudai S, Toyoda M, Nishiyama M, Chikamatsu K: **Immunosuppressive activity of cancer-associated fibroblasts in head and neck squamous cell carcinoma**. *Cancer immunology, immunotherapy : CII* 2015, **64**(11):1407-1417.
141. Herrera M, Herrera A, Dominguez G, Silva J, Garcia V, Garcia JM, Gomez I, Soldevilla B, Munoz C, Provencio M *et al*: **Cancer-associated fibroblast and M2 macrophage markers together predict outcome in colorectal cancer patients**. *Cancer Sci* 2013, **104**(4):437-444.
142. Powell DR, Huttenlocher A: **Neutrophils in the Tumor Microenvironment**. *Trends in Immunology* 2016, **37**(1):41-52.
143. García-Mendoza MG, Inman DR, Ponik SM, Jeffery JJ, Sheerar DS, Van Doorn RR, Keely PJ: **Neutrophils drive accelerated tumor progression in the collagen-dense mammary tumor microenvironment**. *Breast Cancer Research* 2016, **18**(1):49.
144. Wang J, Jia Y, Wang N, Zhang X, Tan B, Zhang G, Cheng Y: **The clinical significance of tumor-infiltrating neutrophils and neutrophil-to-CD8+ lymphocyte ratio in patients with resectable esophageal squamous cell carcinoma**. *Journal of translational medicine* 2014, **12**:7.
145. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, von der Maase H: **Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma**. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2009, **27**(28):4709-4717.
146. Li YW, Qiu SJ, Fan J, Zhou J, Gao Q, Xiao YS, Xu YF: **Intratumoral neutrophils: a poor prognostic factor for hepatocellular carcinoma following resection**. *Journal of hepatology* 2011, **54**(3):497-505.
147. Rao HL, Chen JW, Li M, Xiao YB, Fu J, Zeng YX, Cai MY, Xie D: **Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis**. *PLoS One* 2012, **7**(1):e30806.
148. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM: **Polarization of Tumor-Associated Neutrophil Phenotype by TGF-β: "N1" versus "N2" TAN**. *Cancer cell* 2009, **16**(3):183-194.
149. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S: **Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model**. *J Clin Invest* 2010, **120**(4):1151-1164.
150. Tabariès S, Ouellet V, Hsu BE, Annis MG, Rose AA, Meunier L, Carmona E, Tam CE, Mes-Masson A-M, Siegel PM: **Granulocytic immune infiltrates are essential for the efficient formation of breast cancer liver metastases**. *Breast Cancer Research* 2015, **17**(1):45.
151. Galdiero MR, Bianchi P, Grizzi F, Di Caro G, Basso G, Ponzetta A, Bonavita E, Barbagallo M, Tartari S, Polentarutti N: **Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer**. *International journal of cancer* 2016, **139**(2):446-456.
152. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, Akimova T, Vachani A, Litzky L, Hancock WW: **Tumor-**

associated neutrophils stimulate T cell responses in early-stage human lung cancer. *The Journal of clinical investigation* 2014, **124**(12):5466-5480.
153. Mishalian I, Bayuh R, Levy L, Zolotarov L, Michaeli J, Fridlender ZG: **Tumor-associated neutrophils (TAN) develop pro-tumorigenic properties during tumor progression**. *Cancer immunology, immunotherapy : CII* 2013, **62**.
154. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S *et al*: **Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells**. *International immunology* 2004, **16**(11):1643-1656.
155. Campbell DJ, Ziegler SF: **FOXP3 modifies the phenotypic and functional properties of regulatory T cells**. *Nature reviews Immunology* 2007, **7**(4):305-310.
156. Fontenot JD, Gavin MA, Rudensky AY: **Foxp3 programs the development and function of CD4+CD25+ regulatory T cells**. *Nat Immunol* 2003, **4**(4):330-336.
157. Schreiber TH: **The use of FoxP3 as a biomarker and prognostic factor for malignant human tumors**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2007, **16**(10):1931-1934.
158. Scurr M, Ladell K, Besneux M, Christian A, Hockey T, Smart K, Bridgeman H, Hargest R, Phillips S, Davies M *et al*: **Highly prevalent colorectal cancer-infiltrating LAP(+) Foxp3(-) T cells exhibit more potent immunosuppressive activity than Foxp3(+) regulatory T cells**. *Mucosal immunology* 2014, **7**(2):428-439.
159. Li Z, Dong P, Ren M, Song Y, Qian X, Yang Y, Li S, Zhang X, Liu F: **PD-L1 Expression Is Associated with Tumor FOXP3(+) Regulatory T-Cell Infiltration of Breast Cancer and Poor Prognosis of Patient**. *J Cancer* 2016, **7**(7):784-793.
160. Jie HB, Gildener-Leapman N, Li J, Srivastava RM, Gibson SP, Whiteside TL, Ferris RL: **Intratumoral regulatory T cells upregulate immunosuppressive molecules in head and neck cancer patients**. *Br J Cancer* 2013, **109**(10):2629-2635.
161. Kindlund B, Sjoling A, Yakkala C, Adamsson J, Janzon A, Hansson LE, Hermansson M, Janson P, Winqvist O, Lundin SB: **CD4+ regulatory T cells in gastric cancer mucosa are proliferating and express high levels of IL-10 but little TGF-beta**. 2016.
162. Kong YY, Fuchsberger M, Xiang SD, Apostolopoulos V, Plebanski M: **Myeloid derived suppressor cells and their role in diseases**. *Current medicinal chemistry* 2013, **20**(11):1437-1444.
163. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V: **Coordinated regulation of myeloid cells by tumours**. *Nature reviews Immunology* 2012, **12**.
164. Ostrand-Rosenberg S, Sinha P, Beury DW, Clements VK: **Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression**. *Seminars in cancer biology* 2012, **22**(4):275-281.
165. Gabrilovich DI, Nagaraj S: **Myeloid-derived suppressor cells as regulators of the immune system**. *Nature reviews Immunology* 2009, **9**.

166. Heuvers ME, Muskens F, Bezemer K, Lambers M, Dingemans AM, Groen HJ, Smit EF, Hoogsteden HC, Hegmans JP, Aerts JG: **Arginase-1 mRNA expression correlates with myeloid-derived suppressor cell levels in peripheral blood of NSCLC patients**. *Lung cancer (Amsterdam, Netherlands)* 2013, **81**(3):468-474.
167. Holmgaard RB, Zamarin D, Li Y, Gasmi B, Munn DH, Allison JP, Merghoub T, Wolchok JD: **Tumor-Expressed IDO Recruits and Activates MDSCs in a Treg-Dependent Manner**. *Cell reports* 2015, **13**(2):412-424.
168. Folkman J: **Angiogenesis: an organizing principle for drug discovery?** *Nature Reviews Drug Discovery* 2007, **6**(4):273-286.
169. Zhao Y, Adjei AA: **Targeting Angiogenesis in Cancer Therapy: Moving Beyond Vascular Endothelial Growth Factor**. *The Oncologist* 2015, **20**(6):660-673.
170. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M: **Angiogenesis in cancer**. *Vascular Health and Risk Management* 2006, **2**(3):213.
171. McMahon G: **VEGF receptor signaling in tumor angiogenesis**. *The Oncologist* 2000, **5**(Supplement 1):3-10.
172. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, Jain RK: **Normalization of the vasculature for treatment of cancer and other diseases**. *Physiological Reviews* 2011, **91**(3):1071-1121.
173. Zibara K, Awada Z, Dib L, El-Saghir J, Al-Ghadban S, Ibrik A, El-Zein N, El-Sabban M: **Anti-angiogenesis therapy and gap junction inhibition reduce MDA-MB-231 breast cancer cell invasion and metastasis in vitro and in vivo**. *Scientific Reports* 2015, **5**.
174. Rusckowski M, Wang Y, Blankenberg FG, Levashova Z, Backer MV, Backer JM: **Targeted scVEGF/177Lu radiopharmaceutical inhibits growth of metastases and can be effectively combined with chemotherapy**. *EJNMMI Research* 2016, **6**(1):1-9.
175. Vasudev NS, Reynolds AR: **Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions**. *Angiogenesis* 2014, **17**(3):471-494.
176. Quail DF, Joyce JA: **Microenvironmental regulation of tumor progression and metastasis**. *Nature Medicine* 2013, **19**(11):1423-1437.
177. Gupta GP, Massagué J: **Cancer metastasis: building a framework**. *Cell* 2006, **127**(4):679-695.
178. Weis SM, Cheresh DA: **Tumor angiogenesis: molecular pathways and therapeutic targets**. *Nature Medicine* 2011, **17**(11):1359-1370.
179. Jobling P, Pundavela J, Oliveira SM, Roselli S, Walker MM, Hondermarck H: **Nerve–cancer cell cross-talk: A novel promoter of tumor progression**. *Cancer research* 2015, **75**(9):1777-1781.
180. Ondicova K, Mravec B: **Role of nervous system in cancer aetiopathogenesis**. *The Lancet Oncology* 2010, **11**(6):596-601.
181. Sarkar C, Chakroborty D, Basu S: **Neurotransmitters as regulators of tumor angiogenesis and immunity: the role of catecholamines**. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* 2013, **8**(1):7-14.

182. Chakroborty D, Sarkar C, Basu B, Dasgupta PS, Basu S: **Catecholamines regulate tumor angiogenesis**. *Cancer research* 2009, **69**(9):3727-3730.
183. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, Jennings NB, Armaiz-Pena G, Bankson JA, Ravoori M: **Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma**. *Nature medicine* 2006, **12**(8):939-944.
184. Liu J, Deng G-H, Zhang J, Wang Y, Xia X-Y, Luo X-M, Deng Y-T, He S-S, Mao Y-Y, Peng X-C: **The effect of chronic stress on anti-angiogenesis of sunitinib in colorectal cancer models**. *Psychoneuroendocrinology* 2015, **52**:130-142.
185. Xie H, Li C, He Y, Griffin R, Ye Q, Li L: **Chronic stress promotes oral cancer growth and angiogenesis with increased circulating catecholamine and glucocorticoid levels in a mouse model**. *Oral Oncology* 2015, **51**(11):991-997.
186. Sloan EK, Priceman SJ, Cox BF, Yu S, Pimentel MA, Tangkanangnukul V, Arevalo JM, Morizono K, Karanikolas BD, Wu L: **The sympathetic nervous system induces a metastatic switch in primary breast cancer**. *Cancer research* 2010, **70**(18):7042-7052.
187. Madden KS, Szpunar MJ, Brown EB: **beta-Adrenergic receptors (beta-AR) regulate VEGF and IL-6 production by divergent pathways in high beta-AR-expressing breast cancer cell lines**. *Breast Cancer Res Treat* 2011, **130**(3):747-758.
188. Reedijk M, Odorcic S, Chang L, Zhang H, Miller N, McCready DR, Lockwood G, Egan SE: **High-level coexpression of Jag1 and Notch1 is observed in human breast cancer and is associated with poor overall survival**. *Cancer research* 2005, **65**(18):8530-8537.
189. Dickson BC, Mulligan AM, Zhang H, Lockwood G, O'Malley FP, Egan SE, Reedijk M: **High-level JAG1 mRNA and protein predict poor outcome in breast cancer**. *Modern Pathology* 2007, **20**(6):685-693.
190. Chen H, Liu D, Yang Z, Sun L, Deng Q, Yang S, Qian L, Guo L, Yu M, Hu M *et al*: **Adrenergic signaling promotes angiogenesis through endothelial cell-tumor cell crosstalk**. *Endocrine-related cancer* 2014, **21**(5):783-795.
191. Basu S, Nagy JA, Pal S, Vasile E, Eckelhoefer IA, Bliss VS, Manseau EJ, Dasgupta PS, Dvorak HF, Mukhopadhyay D: **The neurotransmitter dopamine inhibits angiogenesis induced by vascular permeability factor/vascular endothelial growth factor**. *Nature medicine* 2001, **7**(5):569-574.
192. Tilan J, Kitlinska J: **Sympathetic neurotransmitters and tumor angiogenesis-link between stress and cancer progression**. *Journal of oncology* 2010, **2010**:539706.
193. Sarkar C, Chakroborty D, Chowdhury UR, Dasgupta PS, Basu S: **Dopamine increases the efficacy of anticancer drugs in breast and colon cancer preclinical models**. *Clinical Cancer Research* 2008, **14**(8):2502-2510.
194. Chakroborty D, Sarkar C, Mitra RB, Banerjee S, Dasgupta PS, Basu S: **Depleted dopamine in gastric cancer tissues: dopamine treatment retards growth of gastric cancer by inhibiting angiogenesis**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2004, **10**(13):4349-4356.

195. Moreno-Smith M, Lee SJ, Lu C, Nagaraja AS, He G, Rupaimoole R, Han HD, Jennings NB, Roh J-W, Nishimura M: **Biologic effects of dopamine on tumor vasculature in ovarian carcinoma**. *Neoplasia (New York, NY)* 2013, **15**(5):502-IN515.
196. Schuller HM: **Is cancer triggered by altered signalling of nicotinic acetylcholine receptors?** *Nature Reviews Cancer* 2009, **9**(3):195-205.
197. Egleton RD, Brown KC, Dasgupta P: **Angiogenic activity of nicotinic acetylcholine receptors: Implications in tobacco-related vascular diseases**. *Pharmacology & Therapeutics* 2009, **121**(2):205-223.
198. Cucina A, Sapienza P, Corvino V, Borrelli V, Mariani V, Randone B, Santoro D'Angelo L, Cavallaro A: **Nicotine-induced smooth muscle cell proliferation is mediated through bFGF and TGF-beta 1**. *Surgery* 2000, **127**(3):316-322.
199. Conklin BS, Zhao W, Zhong DS, Chen C: **Nicotine and cotinine up-regulate vascular endothelial growth factor expression in endothelial cells**. *Am J Pathol* 2002, **160**(2):413-418.
200. Brown KC, Lau JK, Dom AM, Witte TR, Luo H, Crabtree CM, Shah YH, Shiflett BS, Marcelo AJ, Proper NA *et al*: **MG624, an alpha7-nAChR antagonist, inhibits angiogenesis via the Egr-1/FGF2 pathway**. *Angiogenesis* 2012, **15**(1):99-114.
201. Davis R, Rizwani W, Banerjee S, Kovacs M, Haura E, Coppola D, Chellappan S: **Nicotine promotes tumor growth and metastasis in mouse models of lung cancer**. *PloS One* 2009, **4**(10):e7524.
202. Lee CH, Huang CS, Chen CS, Tu SH, Wang YJ, Chang YJ, Tam KW, Wei PL, Cheng TC, Chu JS *et al*: **Overexpression and activation of the alpha9-nicotinic receptor during tumorigenesis in human breast epithelial cells**. *Journal of the National Cancer Institute* 2010, **102**(17):1322-1335.
203. Wu C-H, Lee C-H, Ho Y-S: **Nicotinic acetylcholine receptor-based blockade: applications of molecular targets for cancer therapy**. *Clinical Cancer Research* 2011, **17**(11):3533-3541.
204. Wong HPS, Yu L, Lam EKY, Tai EKK, Wu WKK, Cho C-H: **Nicotine promotes colon tumor growth and angiogenesis through β-adrenergic activation**. *Toxicological Sciences* 2007, **97**(2):279-287.
205. Dasgupta P, Chellappan SP: **Nicotine-mediated cell proliferation and angiogenesis: new twists to an old story**. *Cell Cycle* 2006, **5**(20):2324-2328.
206. Lombardi Mí G, Negroni M, Pelegrina LT, Castro Mí E, Fiszman GL, Azar Mí E, Morgado CC, Sales Mí E: **Autoantibodies against muscarinic receptors in breast cancer: their role in tumor angiogenesis**. *PLoS One* 2013, **8**(2).
207. de la Torre E, Davel L, Jasnis MA, Gotoh T, de Lustig ES, Sales ME: **Muscarinic receptors participation in angiogenic response induced by macrophages from mammary adenocarcinoma-bearing mice**. *Breast cancer research : BCR* 2005, **7**(3):R345-352.
208. Fiszman GL, Middonno MC, de la Torre E, Farina M, Español AJ, Sales ME: **Activation of muscarinic cholinergic receptors induces MCF-7 cells proliferation and angiogenesis by stimulating nitric oxide synthase activity**. *Cancer Biology & Therapy* 2007, **6**(7):1106-1113.

209. Fava G, Marucci L, Glaser S, Francis H, De Morrow S, Benedetti A, Alvaro D, Venter J, Meininger C, Patel T: **γ-Aminobutyric Acid Inhibits Cholangiocarcinoma Growth by Cyclic AMP–Dependent Regulation of the Protein Kinase A/Extracellular Signal-Regulated Kinase 1/2 Pathway**. *Cancer research* 2005, **65**(24):11437-11446.
210. Medeiros PJ, Jackson DN: **Neuropeptide Y Y5-receptor activation on breast cancer cells acts as a paracrine system that stimulates VEGF expression and secretion to promote angiogenesis**. *Peptides* 2013, **48**:106-113.
211. Li L, Kilbourn RG, Adams J, Fidler IJ: **Role of nitric oxide in lysis of tumor cells by cytokine-activated endothelial cells**. *Cancer research* 1991, **51**(10):2531-2535.
212. Cianchi F, Cortesini C, Fantappiè O, Messerini L, Schiavone N, Vannacci A, Nistri S, Sardi I, Baroni G, Marzocca C *et al*: **Inducible nitric oxide synthase expression in human colorectal cancer : Correlation with tumor angiogenesis**. *Am J Pathol* 2003, **162**(3):793-801.
213. Fukumura D, Kashiwagi S, Jain RK: **The role of nitric oxide in tumour progression**. *Nature Reviews Cancer* 2006, **6**(7):521-534.
214. Ridnour LA, Thomas DD, Donzelli S, Espey MG, Roberts DD, Wink DA, Isenberg JS: **The biphasic nature of nitric oxide responses in tumor biology**. *Antioxidants & Redox Signaling* 2006, **8**(7-8):1329-1337.
215. Andrade SP, Hart IR, Piper PJ: **Inhibitors of nitric oxide synthase selectively reduce flow in tumour-associated neovasculature**. *British Journal of Pharmacology* 1992, **107**(4):1092-1095.
216. Chu SC, Marks-Konczalik J, Wu H-P, Banks TC, Moss J: **Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: characterization of differences between human and mouse iNOS promoters**. *Biochemical and Biophysical Research Communications* 1998, **248**(3):871-878.
217. Nomelini RS, Ribeiro LCdA, Tavares-Murta BM, Adad SJ, Murta EFC: **Production of nitric oxide and expression of inducible nitric oxide synthase in ovarian cystic tumors**. *Mediators of Inflammation* 2009, **2008**.
218. Vahora H, Khan MA, Alalami U, Hussain A: **The potential role of nitric oxide in halting cancer progression through chemoprevention**. *Journal of Cancer Prevention* 2016, **21**(1):1.
219. Nakamura Y, Yasuoka H, Tsujimoto M, Yoshidome K, Nakahara M, Nakao K, Nakamura M, Kakudo K: **Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis**. *Clinical Cancer Research* 2006, **12**(4):1201-1207.
220. Wang L, Shi GG, Yao JC, Gong W, Wei D, Wu TT, Ajani JA, Huang S, Xie K: **Expression of endothelial nitric oxide synthase correlates with the angiogenic phenotype of and predicts poor prognosis in human gastric cancer**. *Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association* 2005, **8**(1):18-28.
221. Radisky ES, Radisky DC: **Matrix metalloproteinases as breast cancer drivers and therapeutic targets**. *Frontiers in bioscience (Landmark edition)* 2015, **20**:1144-1163.

222. Nocito A, Dahm F, Jochum W, Jang JH, Georgiev P, Bader M, Graf R, Clavien P-A: **Serotonin regulates macrophage-mediated angiogenesis in a mouse model of colon cancer allografts**. *Cancer research* 2008, **68**(13):5152-5158.
223. Speyer CL, Hachem AH, Assi A, DeVries JA, Gorski DH: **Metabotropic glutamate receptor-1 as a novel target for the anti-angiogenic treatment of breast cancer**. *Cancer research* 2013, **73**(8 Supplement):3895.
224. Wangari-Talbot J, Wall BA, Goydos JS, Chen S: **Functional effects of GRM1 suppression in human melanoma cells**. *Molecular cancer research : MCR* 2012, **10**(11):1440-1450.
225. Wen Y, Li J, Koo J, Shin SS, Lin Y, Jeong BS, Mehnert JM, Chen S, Cohen-Sola KA, Goydos JS: **Activation of the glutamate receptor GRM1 enhances angiogenic signaling to drive melanoma progression**. *Cancer research* 2014, **74**(9):2499-2509.
226. Xia H, Zhao YN, Yu CH, Zhao YL, Liu Y: **Inhibition of metabotropic glutamate receptor 1 suppresses tumor growth and angiogenesis in experimental non-small cell lung cancer**. *European journal of pharmacology* 2016, **783**:103-111.
227. Deng G-H, Liu J, Zhang J, Wang Y, Peng X-C, Wei Y-Q, Jiang Y: **Exogenous norepinephrine attenuates the efficacy of sunitinib in a mouse cancer model**. *Journal of Experimental & Clinical Cancer Research* 2014, **33**(1):1-12.
228. Moreno-Smith M, Lu C, Shahzad MM, Pena GNA, Allen JK, Stone RL, Mangala LS, Han HD, Kim HS, Farley D: **Dopamine blocks stress-mediated ovarian carcinoma growth**. *Clinical Cancer Research* 2011, **17**(11):3649-3659.
229. Hoeppner LH, Wang Y, Sharma A, Javeed N, Van Keulen VP, Wang E, Yang P, Roden AC, Peikert T, Molina JR: **Dopamine D2 receptor agonists inhibit lung cancer progression by reducing angiogenesis and tumor infiltrating myeloid derived suppressor cells**. *Molecular Oncology* 2015, **9**(1):270-281.
230. Park SH, Kim B-R, Lee JH, Park ST, Lee S-H, Dong SM, Rho SB: **GABARBP down-regulates HIF-1α expression through the VEGFR-2 and PI3K/mTOR/4E-BP1 pathways**. *Cellular Signalling* 2014, **26**(7):1506-1513.
231. Zhang Q, Tang X, Zhang Z-F, Velikina R, Shi S, Le AD: **Nicotine induces hypoxia-inducible factor-1α expression in human lung cancer cells via nicotinic acetylcholine receptor–mediated signaling pathways**. *Clinical Cancer Research* 2007, **13**(16):4686-4694.
232. Medeiros PJ, Al-Khazraji BK, Novielli NM, Postovit LM, Chambers AF, Jackson DN: **Neuropeptide Y stimulates proliferation and migration in the 4T1 breast cancer cell line**. *International journal of cancer* 2012, **131**(2):276-286.
233. Alasvand M, Rashidi B, Javanmard SH, Akhavan MM, Khazaei M: **Effect of blocking of neuropeptide Y Y2 receptor on tumor angiogenesis and progression in normal and diet-induced obese C57BL/6 Mice**. *Global journal of health science* 2015, **7**(7):46883.
234. Lu C, Everhart L, Tilan J, Kuo L, Sun CJ, Munivenkatappa RB, Jönsson-Rylander A-C, Sun J, Kuan-Celarier A, Li L: **Neuropeptide Y and its Y2 receptor: potential targets in neuroblastoma therapy**. *Oncogene* 2010, **29**(41):5630-5642.

235. Romon R, Adriaenssens E, Lagadec C, Germain E, Hondermarck H, Le Bourhis X: **Nerve growth factor promotes breast cancer angiogenesis by activating multiple pathways**. *breast cancer* 2010, **9**:11.
236. Walsh EM, Kim R, Del Valle L, Weaver M, Sheffield J, Lazarovici P, Marcinkiewicz C: **Importance of interaction between nerve growth factor and α9β1 integrin in glial tumor angiogenesis**. *Neuro-Oncology* 2012, **14**(7):890-901.
237. Staniszewska I, Zaveri S, Del Valle L, Oliva I, Rothman VL, Croul SE, Roberts DD, Mosher DF, Tuszynski GP, Marcinkiewicz C: **Interaction of α9β1 integrin with thrombospondin-1 promotes angiogenesis**. *Circulation Research* 2007, **100**(9):1308-1316.
238. Vlahakis NE, Young BA, Atakilit A, Hawkridge AE, Issaka RB, Boudreau N, Sheppard D: **Integrin α9β1 directly binds to vascular endothelial growth factor (VEGF)-A and contributes to VEGF-A-induced angiogenesis**. *Journal of Biological Chemistry* 2007, **282**(20):15187-15196.
239. Staniszewska I, Sariyer IK, Lecht S, Brown MC, Walsh EM, Tuszynski GP, Safak M, Lazarovici P, Marcinkiewicz C: **Integrin α9β1 is a receptor for nerve growth factor and other neurotrophins**. *Journal of Cell Science* 2008, **121**(4):504-513.
240. Lin C-Y, Hung S-Y, Chen H-T, Tsou H-K, Fong Y-C, Wang S-W, Tang C-H: **Brain-derived neurotrophic factor increases vascular endothelial growth factor expression and enhances angiogenesis in human chondrosarcoma cells**. *Biochemical Pharmacology* 2014, **91**(4):522-533.
241. Nilsson UW, Abrahamsson A, Dabrosin C: **Angiogenin regulation by estradiol in breast tissue: tamoxifen inhibits angiogenin nuclear translocation and antiangiogenin therapy reduces breast cancer growth in vivo**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2010, **16**(14):3659-3669.
242. Katona TM, Neubauer BL, Iversen PW, Zhang S, Baldridge LA, Cheng L: **Elevated expression of angiogenin in prostate cancer and its precursors**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2005, **11**(23):8358-8363.
243. Hisai H, Kato J, Kobune M, Murakami T, Miyanishi K, Takahashi M, Yoshizaki N, Takimoto R, Terui T, Niitsu Y: **Increased expression of angiogenin in hepatocellular carcinoma in correlation with tumor vascularity**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2003, **9**(13):4852-4859.
244. Miyake H, Hara I, Yamanaka K, Gohji K, Arakawa S, Kamidono S: **Increased angiogenin expression in the tumor tissue and serum of urothelial carcinoma patients is related to disease progression and recurrence**. *Cancer* 1999, **86**(2):316-324.
245. Gao X, Xu Z: **Mechanisms of action of angiogenin**. *Acta Biochimica et Biophysica Sinica* 2008, **40**(7):619-624.
246. He T, Qi F, Jia L, Wang S, Wang C, Song N, Fu Y, Li L, Luo Y: **Tumor cell-secreted angiogenin induces angiogenic activity of endothelial cells by suppressing miR-542-3p**. *Cancer letters* 2015, **368**(1):115-125.

247. Miyake M, Goodison S, Lawton A, Gomes-Giacoia E, Rosser C: **Angiogenin promotes tumoral growth and angiogenesis by regulating matrix metallopeptidase-2 expression via the ERK1/2 pathway**. *Oncogene* 2015, **34**(7):890-901.
248. Shu J, Huang M, Tian Q, Shui Q, Zhou Y, Chen J: **Downregulation of angiogenin inhibits the growth and induces apoptosis in human bladder cancer cells through regulating AKT/mTOR signaling pathway**. *Journal of Molecular Histology* 2015, **46**(2):157-171.
249. Larrieu-Lahargue F, Welm AL, Bouchecareilh M, Alitalo K, Li DY, Bikfalvi A, Auguste P: **Blocking fibroblast growth factor receptor signaling inhibits tumor growth, lymphangiogenesis, and metastasis**. *PLoS One* 2012, **7**(6):e39540.
250. de Aguiar RB, Parise CB, Souza CRT, Braggion C, Quintilio W, Moro AM, Navarro Marques FL, Buchpiguel CA, Chammas R, de Moraes JZ: **Blocking FGF2 with a new specific monoclonal antibody impairs angiogenesis and experimental metastatic melanoma, suggesting a potential role in adjuvant settings**. *Cancer letters* 2016, **371**(2):151-160.
251. Sasi SP, Yan X, Enderling H, Park D, Gilbert H-Y, Curry C, Coleman C, Hlatky L, Qin G, Kishore R: **Breaking the 'harmony'of TNF-α signaling for cancer treatment**. *Oncogene* 2012, **31**(37):4117-4127.
252. Lai KC, Liu CJ, Lin TJ, Mar AC, Wang HH, Chen CW, Hong ZX, Lee TC: **Blocking TNF-alpha inhibits angiogenesis and growth of IFIT2-depleted metastatic oral squamous cell carcinoma cells**. *Cancer letters* 2016, **370**(2):207-215.
253. Geng L, Chaudhuri A, Talmon G, Wisecarver JL, Wang J: **TGF-Beta suppresses VEGFA-mediated angiogenesis in colon cancer metastasis**. *PloS One* 2013, **8**(3):e59918.
254. Sugie S, Mukai S, Yamasaki K, Kamibeppu T, Tsukino H, Kamoto T: **Plasma macrophage-stimulating protein and hepatocyte growth factor levels are associated with prostate cancer progression**. *Human Cell* 2016, **29**(1):22-29.
255. Kawaguchi M, Kataoka H: **Mechanisms of hepatocyte growth factor activation in cancer tissues**. *Cancers* 2014, **6**(4):1890-1904.
256. Kataoka H, Miyata S, Uchinokura S, Itoh H: **Roles of hepatocyte growth factor (HGF) activator and HGF activator inhibitor in the pericellular activation of HGF/scatter factor**. *Cancer metastasis reviews* 2003, **22**(2-3):223-236.
257. Ren Y, Cao B, Law S, Xie Y, Lee PY, Cheung L, Chen Y, Huang X, Chan HM, Zhao P: **Hepatocyte growth factor promotes cancer cell migration and angiogenic factors expression: a prognostic marker of human esophageal squamous cell carcinomas**. *Clinical Cancer Research* 2005, **11**(17):6190-6197.
258. Wang W-M, Zhao Z-L, Ma S-R, Yu G-T, Liu B, Zhang L, Zhang W-F, Kulkarni AB, Sun Z-J, Zhao Y-F: **Epidermal growth factor receptor inhibition reduces angiogenesis via hypoxia-inducible factor-1α and notch1 in head neck squamous cell carcinoma**. *PloS One* 2015, **10**(2).
259. Ellis LM: **The role of neuropilins in cancer**. *Molecular cancer therapeutics* 2006, **5**(5):1099-1107.

260. Djordjevic S, Driscoll PC: **Targeting VEGF signalling via the neuropilin co-receptor**. *Drug Discovery Today* 2013, **18**(9–10):447-455.
261. Li L, Jiang X, Zhang Q, Dong X, Gao Y, He Y, Qiao H, Xie F, Xie X, Sun X: **Neuropilin-1 is associated with clinicopathology of gastric cancer and contributes to cell proliferation and migration as multifunctional co-receptors**. *Journal of experimental & clinical cancer research : CR* 2016, **35**(1):16.
262. Douma S, van Laar T, Zevenhoven J, Meuwissen R, van Garderen E, Peeper DS: **Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB**. *Nature* 2004, **430**:1034.
263. Ishihara K, Kamata M, Hayashi I, Yamashina S, Majima M: **Roles of bradykinin in vascular permeability and angiogenesis in solid tumor**. *International immunopharmacology* 2002, **2**(4):499-509.
264. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F: **The role of substance P in inflammatory disease**. *J Cell Physiol* 2004, **201**(2):167-180.
265. Celià-Terrassa T, Kang Y: **Distinctive properties of metastasis-initiating cells**. *Genes & Development* 2016, **30**(8):892-908.
266. MacDougall JR, Matrisian LM: **Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis**. *Cancer metastasis reviews* 1995, **14**(4):351-362.
267. Deryugina EI, Quigley JP: **Matrix metalloproteinases and tumor metastasis**. *Cancer metastasis reviews* 2006, **25**(1):9-34.
268. Okada Y, Eibl G, Guha S, Duffy JP, Reber HA, Hines OJ: **Nerve growth factor stimulates MMP-2 expression and activity and increases invasion by human pancreatic cancer cells**. *Clinical & experimental metastasis* 2004, **21**(4):285-292.
269. Okada Y, Eibl G, Duffy JP, Reber HA, Hines OJ: **Glial cell-derived neurotrophic factor upregulates the expression and activation of matrix metalloproteinase-9 in human pancreatic cancer**. *Surgery* 2003, **134**(2):293-299.
270. Yang EV, Sood AK, Chen M, Li Y, Eubank TD, Marsh CB, Jewell S, Flavahan NA, Morrison C, Yeh PE *et al*: **Norepinephrine up-regulates the expression of vascular endothelial growth factor, matrix metalloproteinase (MMP)-2, and MMP-9 in nasopharyngeal carcinoma tumor cells**. *Cancer research* 2006, **66**(21):10357-10364.
271. Simpson CD, Anyiwe K, Schimmer AD: **Anoikis resistance and tumor metastasis**. *Cancer letters* 2008, **272**(2):177-185.
272. Paoli P, Giannoni E, Chiarugi P: **Anoikis molecular pathways and its role in cancer progression**. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 2013, **1833**(12):3481-3498.
273. Fan M, Sun J, Wang W, Fan J, Wang L, Zhang X, Yang A, Wang W, Zhang R, Li J: **Tropomyosin-related kinase B promotes distant metastasis of colorectal cancer through protein kinase B-mediated anoikis suppression and correlates with poor prognosis**. *Apoptosis* 2014, **19**(5):860-870.

274. Okugawa Y, Tanaka K, Inoue Y, Kawamura M, Kawamoto A, Hiro J, Saigusa S, Toiyama Y, Ohi M, Uchida K *et al*: **Brain-derived neurotrophic factor/tropomyosin-related kinase B pathway in gastric cancer**. *British Journal Of Cancer* 2012, **108**:121.
275. Dasgupta P, Rizwani W, Pillai S, Kinkade R, Kovacs M, Rastogi S, Banerjee S, Carless M, Kim E, Coppola D *et al*: **Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines**. *International journal of cancer Journal international du cancer* 2009, **124**(1):36-45.
276. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M *et al*: **The epithelial-mesenchymal transition generates cells with properties of stem cells**. *Cell* 2008, **133**(4):704-715.
277. Kalluri R, Weinberg RA: **The basics of epithelial-mesenchymal transition**. *J Clin Invest* 2009, **119**(6):1420-1428.
278. Polyak K, Weinberg RA: **Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits**. *Nature reviews Cancer* 2009, **9**(4):265-273.
279. Bao W, Qiu H, Yang T, Luo X, Zhang H, Wan X: **Upregulation of TrkB Promotes Epithelial-Mesenchymal Transition and Anoikis Resistance in Endometrial Carcinoma**. *PLOS ONE* 2013, **8**(7):e70616.
280. Park SY, Kang JH, Jeong KJ, Lee J, Han JW, Choi WS, Kim YK, Kang J, Park CG, Lee HY: **Norepinephrine induces VEGF expression and angiogenesis by a hypoxia-inducible factor-1alpha protein-dependent mechanism**. *International journal of cancer* 2011, **128**(10):2306-2316.
281. Toda M, Suzuki T, Hosono K, Hayashi I, Hashiba S, Onuma Y, Amano H, Kurihara Y, Kurihara H, Okamoto H *et al*: **Neuronal system-dependent facilitation of tumor angiogenesis and tumor growth by calcitonin gene-related peptide**. *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105**(36):13550-13555.
282. Lee EW, Michalkiewicz M, Kitlinska J, Kalezic I, Switalska H, Yoo P, Sangkharat A, Ji H, Li L, Michalkiewicz T *et al*: **Neuropeptide Y induces ischemic angiogenesis and restores function of ischemic skeletal muscles**. *J Clin Invest* 2003, **111**(12):1853-1862.
283. Lutgendorf SK, Cole S, Costanzo E, Bradley S, Coffin J, Jabbari S, Rainwater K, Ritchie JM, Yang M, Sood AK: **Stress-related mediators stimulate vascular endothelial growth factor secretion by two ovarian cancer cell lines**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2003, **9**(12):4514-4521.
284. Kuol N, Stojanovska L, Apostolopoulos V, Nurgali K: **Crosstalk between cancer and the neuro-immune system**. *Journal of Neuroimmunology* 2017.
285. Tracey KJ: **Reflex control of immunity**. *Nature Reviews Immunology* 2009, **9**(6):418-428.
286. Gidron Y, Perry H, Glennie M: **Does the vagus nerve inform the brain about preclinical tumours and modulate them?** *The Lancet Oncology* 2005, **6**(4):245-248.

287. Erin N, Boyer PJ, Bonneau RH, Clawson GA, Welch DR: **Capsaicin-mediated denervation of sensory neurons promotes mammary tumor metastasis to lung and heart**. *Anticancer research* 2004, **24**(2B):1003-1010.
288. Cole SW, Sood AK: **Molecular pathways: beta-adrenergic signaling in cancer**. *Clinical Cancer Research* 2012, **18**(5):1201-1206.
289. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, Gallagher WM, Wadhwani N, Keil SD, Junaid SA: **Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy**. *Cancer Discovery* 2011, **1**(1):54-67.
290. DeNardo DG, Coussens LM: **Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression**. *Breast cancer research : BCR* 2007, **9**(4):212.
291. Mantovani A, Allavena P, Sica A, Balkwill F: **Cancer-related inflammation**. *Nature* 2008, **454**(7203):436-444.
292. Bapat AA, Hostetter G, Von Hoff DD, Han H: **Perineural invasion and associated pain in pancreatic cancer**. *Nature Reviews Cancer* 2011, **11**(10):695-707.
293. Matsushita Y, Yanamoto S, Takahashi H, Yamada S, Naruse T, Sakamoto Y, Ikeda H, Shiraishi T, Fujita S, Ikeda T *et al*: **A clinicopathological study of perineural invasion and vascular invasion in oral tongue squamous cell carcinoma**. *International Journal of Oral and Maxillofacial Surgery* 2015, **44**(5):543-548.
294. Zhou Y, Wang H, Gong H, Cao M, Zhang G, Wang Y: **Clinical significance of perineural invasion in stages II and III colorectal cancer**. *Pathology-Research and Practice* 2015, **211**(11):839-844.
295. Zhou Z-H, Xu G-F, Zhang W-J, Zhao H-B, Wu Y-Y: **Reevaluating significance of perineural invasion in gastric cancer based on double immunohistochemical staining**. *Archives of Pathology and Laboratory Medicine* 2014, **138**(2):229-234.
296. Shen WR, Wang YP, Chang JYF, Yu SY, Chen HM, Chiang CP: **Perineural invasion and expression of nerve growth factor can predict the progression and prognosis of oral tongue squamous cell carcinoma**. *Journal of Oral Pathology & Medicine* 2014, **43**(4):258-264.
297. Yu EH, Lui MT, Tu HF, Wu CH, Lo WL, Yang CC, Chang KW, Kao SY: **Oral carcinoma with perineural invasion has higher nerve growth factor expression and worse prognosis**. *Oral diseases* 2014, **20**(3):268-274.
298. Karak SG, Quatrano N, Buckley J, Ricci A, Jr.: **Prevalence and significance of perineural invasion in invasive breast carcinoma**. *Connecticut medicine* 2010, **74**(1):17-21.
299. Marchesi F, Piemonti L, Mantovani A, Allavena P: **Molecular mechanisms of perineural invasion, a forgotten pathway of dissemination and metastasis**. *Cytokine & Growth Factor Reviews* 2010, **21**(1):77-82.
300. Liebig C, Ayala G, Wilks J, Verstovsek G, Liu H, Agarwal N, Berger DH, Albo D: **Perineural invasion is an independent predictor of outcome in colorectal cancer**. *Journal of Clinical Oncology* 2009, **27**(31):5131-5137.

301. Duraker N, Caynak Z, Türköz K: **Perineural invasion has no prognostic value in patients with invasive breast carcinoma**. *The Breast* 2006, **15**(5):629-634.
302. He S, Chen C-H, Chernichenko N, He S, Bakst RL, Barajas F, Deborde S, Allen PJ, Vakiani E, Yu Z: **GFRα1 released by nerves enhances cancer cell perineural invasion through GDNF-RET signaling**. *Proceedings of the National Academy of Sciences* 2014, **111**(19):E2008-E2017.
303. Ma J, Jiang Y, Jiang Y, Sun Y, Zhao X: **Expression of nerve growth factor and tyrosine kinase receptor A and correlation with perineural invasion in pancreatic cancer**. *Journal of Gastroenterology and Hepatology* 2008, **23**(12):1852-1859.
304. Kolokythas A, Cox DP, Dekker N, Schmidt BL: **Nerve growth factor and tyrosine kinase A receptor in oral squamous cell carcinoma: is there an association with perineural invasion?** *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons* 2010, **68**(6):1290-1295.
305. Zhang J, Wang L-S, Ye S-L, Luo P, Wang B-L: **Blockage of tropomyosin receptor kinase a (TrkA) enhances chemo-sensitivity in breast cancer cells and inhibits metastasis in vivo**. *International Journal of Clinical and Experimental Medicine* 2015, **8**(1):634.
306. Drell IV T, Joseph J, Lang K, Niggemann B, Zaenker K, Entschladen F: **Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells**. *Breast Cancer Research and Treatment* 2003, **80**(1):63-70.
307. Lagadec C, Meignan S, Adriaenssens E, Foveau B, Vanhecke E, Romon R, Toillon R-A, Oxombre B, Hondermarck H, Le Bourhis X: **TrkA overexpression enhances growth and metastasis of breast cancer cells**. *Oncogene* 2009, **28**(18):1960-1970.
308. Urabe K, Murakami Y, Kondo N, Uemura K, Hashimoto Y, Nakagawa N, Sasaki H, Hiyama E, Takahashi S, Sueda T: **Nerve growth factor expression is not associated with perineural invasion in extrahepatic cholangiocarcinoma**. *Digestive diseases and sciences* 2015:1-11.
309. Gil Z, Cavel O, Kelly K, Brader P, Rein A, Gao SP, Carlson DL, Shah JP, Fong Y, Wong RJ: **Paracrine regulation of pancreatic cancer cell invasion by peripheral nerves**. *Journal of the National Cancer Institute* 2010, **102**(2):107-118.
310. Gattelli A, Nalvarte I, Boulay A, Roloff TC, Schreiber M, Carragher N, Macleod KK, Schlederer M, Lienhard S, Kenner L *et al*: **Ret inhibition decreases growth and metastatic potential of estrogen receptor positive breast cancer cells**. *EMBO Molecular Medicine* 2013, **5**(9):1335-1350.
311. Kim MS, Lee WS, Jin W: **TrkB Promotes Breast Cancer Metastasis via Suppression of Runx3 and Keap1 Expression**. *Molecules and Cells* 2016, **39**(3):258-265.
312. Tanaka K, Okugawa Y, Toiyama Y, Inoue Y, Saigusa S, Kawamura M, Araki T, Uchida K, Mohri Y, Kusunoki M: **Brain-derived neurotrophic factor (BDNF)-induced tropomyosin-related kinase B (Trk B) signaling is a potential**

therapeutic target for peritoneal carcinomatosis arising from colorectal cancer. *PLoS One* 2014, **9**(5):e96410.
313. Sasahira T, Ueda N, Kurihara M, Matsushima S, Ohmori H, Fujii K, Bhawal UK, Yamamoto K, Kirita T, Kuniyasu H: **Tropomyosin receptor kinases B and C are tumor progressive and metastatic marker in colorectal carcinoma**. *Human Pathology* 2013, **44**(6):1098-1106.
314. De la Cruz-Morcillo MA, Berger J, Sánchez-Prieto R, Saada S, Naves T, Guillaudeau A, Perraud A, Sindou P, Lacroix A, Descazeaud A *et al*: **p75 neurotrophin receptor and pro-BDNF promote cell survival and migration in clear cell renal cell carcinoma**. *Oncotarget* 2016, **7**(23):34480-34497.
315. Zhang S, Guo D, Luo W, Zhang Q, Zhang Y, Li C, Lu Y, Cui Z, Qiu X: **TrkB is highly expressed in NSCLC and mediates BDNF-induced the activation of Pyk2 signaling and the invasion of A549 cells**. *BMC Cancer* 2010, **10**:43.
316. Jiffar T, Yilmaz T, Lee J, Miller Y, Feng L, El-Naggar A, Kupferman ME: **Brain derived neutrophic factor (BDNF) coordinates lympho-vascular metastasis through a fibroblast-governed paracrine axis in the tumor microenvironment**. *Cancer cell & microenvironment* 2017, **4**(2):e1566.
317. Truzzi F, Marconi A, Lotti R, Dallaglio K, French LE, Hempstead BL, Pincelli C: **Neurotrophins and their receptors stimulate melanoma cell proliferation and migration**. *The Journal of investigative dermatology* 2008, **128**(8):2031-2040.
318. Louie E, Chen XF, Coomes A, Ji K, Tsirka S, Chen EI: **Neurotrophin-3 modulates breast cancer cells and the microenvironment to promote the growth of breast cancer brain metastasis**. *Oncogene* 2013, **32**(35):4064-4077.
319. Lu R, Fan C, Shangguan W, Liu Y, Li Y, Shang Y, Yin D, Zhang S, Huang Q, Li X *et al*: **Neurons generated from carcinoma stem cells support cancer progression**. 2017, **2**:16036.
320. Ayala GE, Dai H, Powell M, Li R, Ding Y, Wheeler TM, Shine D, Kadmon D, Thompson T, Miles BJ *et al*: **Cancer-related axonogenesis and neurogenesis in prostate cancer**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2008, **14**(23):7593-7603.
321. Zhao CM, Hayakawa Y, Kodama Y, Muthupalani S, Westphalen CB, Andersen GT, Flatberg A, Johannessen H, Friedman RA, Renz BW *et al*: **Denervation suppresses gastric tumorigenesis**. *Science translational medicine* 2014, **6**(250):250ra115.
322. Albo D, Akay CL, Marshall CL, Wilks JA, Verstovsek G, Liu H, Agarwal N, Berger DH, Ayala GE: **Neurogenesis in colorectal cancer is a marker of aggressive tumor behavior and poor outcomes**. *Cancer* 2011, **117**(21):4834-4845.
323. Zhao Q, Yang Y, Liang X, Du G, Liu L, Lu L, Dong J, Han H, Zhang G: **The clinicopathological significance of neurogenesis in breast cancer**. *BMC Cancer* 2014, **14**:484.
324. Pundavela J, Roselli S, Faulkner S, Attia J, Scott RJ, Thorne RF, Forbes JF, Bradshaw RA, Walker MM, Jobling P *et al*: **Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer**. *Mol Oncol* 2015, **9**(8):1626-1635.

325. Magnon C, Hall SJ, Lin J, Xue X, Gerber L, Freedland SJ, Frenette PS: **Autonomic nerve development contributes to prostate cancer progression**. *Science* 2013, **341**(6142):1236361.
326. Dobrenis K, Gauthier LR, Barroca V, Magnon C: **Granulocyte colony-stimulating factor off-target effect on nerve outgrowth promotes prostate cancer development**. *International journal of cancer* 2015, **136**(4):982-988.
327. Pundavela J, Demont Y, Jobling P, Lincz LF, Roselli S, Thorne RF, Bond D, Bradshaw RA, Walker MM, Hondermarck H: **ProNGF correlates with Gleason score and is a potential driver of nerve infiltration in prostate cancer**. *Am J Pathol* 2014, **184**(12):3156-3162.
328. De Couck M, Gidron Y: **Norms of vagal nerve activity, indexed by Heart Rate Variability, in cancer patients**. *Cancer Epidemiology* 2013, **37**(5):737-741.
329. Ohira H, Matsunaga M, Osumi T, Fukuyama S, Shinoda J, Yamada J, Gidron Y: **Vagal nerve activity as a moderator of brain–immune relationships**. *Journal of Neuroimmunology* 2013, **260**(1):28-36.
330. De Couck M, Maréchal R, Moorthamers S, Van Laethem J-L, Gidron Y: **Vagal nerve activity predicts overall survival in metastatic pancreatic cancer, mediated by inflammation**. *Cancer Epidemiology* 2016, **40**:47-51.
331. Erin N, Korcum AF, Tanriover G, Kale S, Demir N, Koksoy S: **Activation of neuroimmune pathways increases therapeutic effects of radiotherapy on poorly differentiated breast carcinoma**. *Brain Behav Immun* 2015, **48**:174-185.
332. Erin N, Barkan GA, Harms JF, Clawson GA: **Vagotomy enhances experimental metastases of 4THMpc breast cancer cells and alters substance P level**. *Regulatory Peptides* 2008, **151**(1):35-42.
333. Erin N, Zhao W, Bylander J, Chase G, Clawson G: **Capsaicin-induced inactivation of sensory neurons promotes a more aggressive gene expression phenotype in breast cancer cells**. *Breast Cancer Research and Treatment* 2006, **99**(3):351-364.
334. Magnon C: **Role of the autonomic nervous system in tumorigenesis and metastasis**. *Molecular and Cellular Oncology* 2015, **2**(2):e975643.
335. Rodriguez PL, Jiang S, Fu Y, Avraham S, Avraham HK: **The proinflammatory peptide substance P promotes blood–brain barrier breaching by breast cancer cells through changes in microvascular endothelial cell tight junctions**. *International journal of cancer* 2014, **134**(5):1034-1044.
336. Luthy IA, Bruzzone A, Piñero CP, Castillo L, Chiesa I, Vazquez S, Sarappa M: **Adrenoceptors: non conventional target for breast cancer?** *Current medicinal chemistry* 2009, **16**(15):1850-1862.
337. Garcia-Recio S, Fuster G, Fernandez-Nogueira P, Pastor-Arroyo EM, Park SY, Mayordomo C, Ametller E, Mancino M, Gonzalez-Farre X, Russnes HG: **Substance P autocrine signaling contributes to persistent HER2 activation that drives malignant progression and drug resistance in breast cancer**. *Cancer research* 2013, **73**(21):6424-6434.
338. Zhang D, Li X, Yao Z, Wei C, Ning N, Li J: **GABAergic signaling facilitates breast cancer metastasis by promoting ERK 1/2-dependent phosphorylation**. *Cancer letters* 2014, **348**(1):100-108.

339. Gumireddy K, Li A, Kossenkov AV, Sakurai M, Yan J, Li Y, Xu H, Wang J, Zhang PJ, Zhang L: **The mRNA-edited form of GABRA3 suppresses GABRA3-mediated Akt activation and breast cancer metastasis**. *Nature Communications* 2016, **7**.
340. Munoz M, Coveñas R: **Involvement of substance P and the NK-1 receptor in cancer progression**. *Peptides* 2013, **48**:1-9.
341. Yang T, He W, Cui F, Xia J, Zhou R, Wu Z, Zhao Y, Shi M: **MACC1 mediates acetylcholine-induced invasion and migration by human gastric cancer cells**. *Oncotarget* 2016, **7**(14):18085-18094.
342. Hanaki T, Horikoshi Y, Nakaso K, Nakasone M, Kitagawa Y, Amisaki M, Arai Y, Tokuyasu N, Sakamoto T, Honjo S *et al*: **Nicotine enhances the malignant potential of human pancreatic cancer cells via activation of atypical protein kinase C**. *Biochimica et Biophysica Acta (BBA) - General Subjects* 2016, **1860**(11):2404-2415.
343. Xiang T, Fei R, Wang Z, Shen Z, Qian J, Chen W: **Nicotine enhances invasion and metastasis of human colorectal cancer cells through the nicotinic acetylcholine receptor downstream p38 MAPK signaling pathway**. *Oncology Reports* 2016, **35**(1):205-210.
344. Powe DG, Entschladen F: **Targeted therapies: Using β-blockers to inhibit breast cancer progression**. *Nature Reviews Clinical Oncology* 2011, **8**(9):511-512.
345. Powe DG, Voss MJ, Zänker KS, Habashy HO, Green AR, Ellis IO, Entschladen F: **Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival**. *Oncotarget* 2010, **1**(7):628-638.
346. Lutgendorf SK, DeGeest K, Dahmoush L, Farley D, Penedo F, Bender D, Goodheart M, Buekers TE, Mendez L, Krueger G: **Social isolation is associated with elevated tumor norepinephrine in ovarian carcinoma patients**. *Brain, Behavior, and Immunity* 2011, **25**(2):250-255.
347. Hassan S, Karpova Y, Baiz D, Yancey D, Pullikuth A, Flores A, Register T, Cline JM, D'Agostino Jr R, Danial N: **Behavioral stress accelerates prostate cancer development in mice**. *The Journal of Clinical Investigation* 2013, **123**(2):874.
348. Szpunar MJ, Burke KA, Dawes RP, Brown EB, Madden KS: **The antidepressant desipramine and α2-adrenergic receptor activation promote breast tumor progression in association with altered collagen structure**. *Cancer Prevention Research* 2013, **6**(12):1262-1272.
349. Lamkin DM, Sung HY, Yang GS, David JM, Ma JC, Cole SW, Sloan EK: **α 2-adrenergic blockade mimics the enhancing effect of chronic stress on breast cancer progression**. *Psychoneuroendocrinology* 2015, **51**:262-270.
350. Lu M, Li J, Luo Z, Zhang S, Xue S, Wang K, Shi Y, Zhang C, Chen H, Li Z: **Roles of dopamine receptors and their antagonist thioridazine in hepatoma metastasis**. *OncoTargets and therapy* 2015, **8**:1543-1552.
351. Flamand V, Zhao H, Peehl DM: **Targeting monoamine oxidase A in advanced prostate cancer**. *Journal of cancer research and clinical oncology* 2010, **136**(11):1761-1771.

352. Kimura R, Kasamatsu A, Koyama T, Fukumoto C, Kouzu Y, Higo M, Endo-Sakamoto Y, Ogawara K, Shiiba M, Tanzawa H: **Glutamate acid decarboxylase 1 promotes metastasis of human oral cancer by β-catenin translocation and MMP7 activation**. *BMC Cancer* 2013, **13**(1):555.
353. Liu G, Zhu J, Yu M, Cai C, Zhou Y, Yu M, Fu Z, Gong Y, Yang B, Li Y *et al*: **Glutamate dehydrogenase is a novel prognostic marker and predicts metastases in colorectal cancer patients**. *Journal of translational medicine* 2015, **13**.
354. Wu JB, Shao C, Li X, Li Q, Hu P, Shi C, Li Y, Chen YT, Yin F, Liao CP *et al*: **Monoamine oxidase A mediates prostate tumorigenesis and cancer metastasis**. *J Clin Invest* 2014, **124**(7):2891-2908.
355. Li J, Yang X-M, Wang Y-H, Feng M-X, Liu X-J, Zhang Y-L, Huang S, Wu Z, Xue F, Qin W-X *et al*: **Monoamine oxidase A suppresses hepatocellular carcinoma metastasis by inhibiting the adrenergic system and its transactivation of EGFR signaling**. *Journal of hepatology* 2014, **60**(6):1225-1234.
356. Satram-Maharaj T, Nyarko JN, Kuski K, Fehr K, Pennington PR, Truitt L, Freywald A, Lukong KE, Anderson DH, Mousseau DD: **The monoamine oxidase-A inhibitor clorgyline promotes a mesenchymal-to-epithelial transition in the MDA-MB-231 breast cancer cell line**. *Cellular Signalling* 2014, **26**(12):2621-2632.
357. Owens DF, Kriegstein AR: **Is there more to GABA than synaptic inhibition?** *Nature Reviews Neuroscience* 2002, **3**(9):715-727.
358. Chen Z-a, Bao M-y, Xu Y-f, Zha R-p, Shi H-b, Chen T-y, He X-h: **Suppression of human liver cancer cell migration and invasion via the GABAA receptor**. *Cancer Biology & Medicine* 2012, **9**(2):90.
359. Neman J, Termini J, Wilczynski S, Vaidehi N, Choy C, Kowolik CM, Li H, Hambrecht AC, Roberts E, Jandial R: **Human breast cancer metastases to the brain display GABAergic properties in the neural niche**. *Proceedings of the National Academy of Sciences* 2014, **111**(3):984-989.
360. Zhou H, Huang S: **Role of mTOR signaling in tumor cell motility, invasion and metastasis**. *Current Protein & Peptide Science* 2011, **12**(1):30.
361. Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E: **PI3K/AKT signaling pathway and cancer: an updated review**. *Annals of Medicine* 2014, **46**(6):372-383.
362. Lodewyks C, Rodriguez J, Yan J, Lerner B, Lipschitz J, Nfon C, Rempel JD, Uhanova J, Minuk GY: **GABA-B receptor activation inhibits the in vitro migration of malignant hepatocytes**. *Canadian Journal of Physiology and Pharmacology* 2011, **89**(6):393-400.
363. Nie H, Cao Q, Zhu L, Gong Y, Gu J, He Z: **Acetylcholine acts on androgen receptor to promote the migration and invasion but inhibit the apoptosis of human hepatocarcinoma**. *PloS One* 2013, **8**(4):e61678.
364. Hung C-S, Peng Y-J, Wei P-L, Lee C-H, Su H-Y, Ho Y-S, Lin S-Y, Wu C-H, Chang Y-J: **The alpha9 Nicotinic Acetylcholine Receptor is the Key Mediator in Nicotine-enhanced Cancer Metastasis in Breast Cancer Cells**. *Journal of Experimental & Clinical Medicine* 2011, **3**(6):283-292.

365. Momi N, Ponnusamy MP, Kaur S, Rachagani S, Kunigal SS, Chellappan S, Ouellette MM, Batra SK: **Nicotine/cigarette-smoke promotes metastasis of pancreatic cancer through α7nAchR-mediated MUC4 up-regulation**. *Oncogene* 2013, **32**(11):1384-1395.
366. Lin G, Sun L, Wang R, Guo Y, Xie C: **Overexpression of muscarinic receptor 3 promotes metastasis and predicts poor prognosis in non–small-cell lung cancer**. *Journal of Thoracic Oncology* 2014, **9**(2):170-178.
367. Xu R, Shang C, Zhao J, Han Y, Liu J, Chen K, Shi W: **Activation of M3 muscarinic receptor by acetylcholine promotes non-small cell lung cancer cell proliferation and invasion via EGFR/PI3K/AKT pathway**. *Tumor Biology* 2015, **36**(6):4091-4100.
368. Belo A, Cheng K, Chahdi A, Shant J, Xie G, Khurana S, Raufman J-P: **Muscarinic receptor agonists stimulate human colon cancer cell migration and invasion**. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2011, **300**(5):G749-G760.
369. Xie G, Cheng K, Shant J, Raufman J-P: **Acetylcholine-induced activation of M3 muscarinic receptors stimulates robust matrix metalloproteinase gene expression in human colon cancer cells**. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2009, **296**(4):G755-G763.
370. Pfitzinger P, Fangmann L, Wang K, Demir E, Gürlevik E, Fleischmann-Mundt B, Brooks J, D'Haese J, Teller S, Hecker A *et al*: **Indirect cholinergic activation slows down pancreatic cancer growth and tumor-associated inflammation**. In.: Research Square; 2020.
371. Renz BW, Tanaka T, Sunagawa M, Takahashi R, Jiang Z, Macchini M, Dantes Z, Valenti G, White RA, Middelhoff MA *et al*: **Cholinergic Signaling via Muscarinic Receptors Directly and Indirectly Suppresses Pancreatic Tumorigenesis and Cancer Stemness**. *Cancer Discov* 2018, **8**(11):1458-1473.
372. Partecke LI, Käding A, Trung DN, Diedrich S, Sendler M, Weiss F, Kühn JP, Mayerle J, Beyer K, von Bernstorff W *et al*: **Subdiaphragmatic vagotomy promotes tumor growth and reduces survival via TNFα in a murine pancreatic cancer model**. *Oncotarget* 2017, **8**(14):22501-22512.
373. Erin N, Duymuş O, Oztürk S, Demir N: **Activation of vagus nerve by semapimod alters substance P levels and decreases breast cancer metastasis**. *Regul Pept* 2012, **179**(1-3):101-108.
374. De Couck M, Mravec B, Gidron Y: **You may need the vagus nerve to understand pathophysiology and to treat diseases**. *Clin Sci (Lond)* 2012, **122**(7):323-328.
375. Seckl MJ, Higgins T, Widmer F, Rozengurt E: **[D-Arg1, D-Trp5, 7, 9, Leu11] substance P: a novel potent inhibitor of signal transduction and growth in vitro and in vivo in small cell lung cancer cells**. *Cancer research* 1997, **57**(1):51-54.
376. Guha S, Eibl G, Kisfalvi K, Fan RS, Burdick M, Reber H, Hines OJ, Strieter R, Rozengurt E: **Broad-spectrum G protein–coupled receptor antagonist,[D-Arg1, D-Trp5, 7, 9, Leu11] SP: a dual inhibitor of growth and angiogenesis in pancreatic cancer**. *Cancer research* 2005, **65**(7):2738-2745.

377. Chen XY, Ru GQ, Ma YY, Xie J, Chen WY, Wang HJ, Wang SB, Li L, Jin KT, He XL *et al*: **High expression of substance P and its receptor neurokinin-1 receptor in colorectal cancer is associated with tumor progression and prognosis**. *OncoTargets and therapy* 2016, **9**:3595-3602.
378. Lang K, Bastian P: **Neurotransmitter effects on tumor cells and leukocytes**. *Progress in experimental tumor research* 2007, **39**:99-121.
379. Entschladen F, Lang K, Drell TL, Joseph J, Zaenker KS: **Neurotransmitters are regulators for the migration of tumor cells and leukocytes**. *Cancer Immunology, Immunotherapy* 2002, **51**(9):467-482.
380. Bigioni M, Benzo A, Irrissuto C, Maggi CA, Goso C: **Role of NK-1 and NK-2 tachykinin receptor antagonism on the growth of human breast carcinoma cell line MDA-MB-231**. *Anti-Cancer Drugs* 2005, **16**(10):1083-1089.
381. Li X, Ma G, Ma Q, Li W, Liu J, Han L, Duan W, Xu Q, Liu H, Wang Z: **Neurotransmitter substance P mediates pancreatic cancer perineural invasion via NK-1R in cancer cells**. *Molecular Cancer Research* 2013, **11**(3):294-302.
382. Movafagh S, Hobson JP, Spiegel S, Kleinman HK, Zukowska Z: **Neuropeptide Y induces migration, proliferation, and tube formation of endothelial cells bimodally via Y1, Y2, and Y5 receptors**. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2006, **20**(11):1924-1926.
383. Tilan JU, Krailo M, Barkauskas DA, Galli S, Mtaweh H, Long J, Wang H, Hawkins K, Lu C, Jeha D *et al*: **Systemic levels of neuropeptide Y and dipeptidyl peptidase activity in patients with Ewing sarcoma--associations with tumor phenotype and survival**. *Cancer* 2015, **121**(5):697-707.
384. Hong SH, Tilan JU, Galli S, Izycka-Swieszewska E, Polk T, Horton M, Mahajan A, Christian D, Jenkins S, Acree R *et al*: **High neuropeptide Y release associates with Ewing sarcoma bone dissemination - in vivo model of site-specific metastases**. *Oncotarget* 2015, **6**(9):7151-7165.
385. Dupouy S, Viardot-Foucault V, Alifano M, Souazé F, Plu-Bureau G, Chaouat M, Lavaur A, Hugol D, Gespach C, Gompel A *et al*: **The Neurotensin Receptor-1 Pathway Contributes to Human Ductal Breast Cancer Progression**. *PLoS ONE* 2009, **4**(1):e4223.
386. Stock AM, Powe DG, Hahn SA, Troost G, Niggemann B, Zanker KS, Entschladen F: **Norepinephrine inhibits the migratory activity of pancreatic cancer cells**. *Exp Cell Res* 2013, **319**.
387. Palm D, Lang K, Niggemann B, Drell TL, Masur K, Zaenker KS, Entschladen F: **The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers**. *International journal of cancer* 2006, **118**.
388. Barbieri A, Bimonte S, Palma G, Luciano A, Rea D, Giudice A, Scognamiglio G, La Mantia E, Franco R, Perdona S *et al*: **The stress hormone norepinephrine increases migration of prostate cancer cells in vitro and in vivo**. *International journal of oncology* 2015, **47**(2):527-534.
389. Azuma H, Inamoto T, Sakamoto T, Kiyama S, Ubai T, Shinohara Y, Maemura K, Tsuji M, Segawa N, Masuda H: **γ-Aminobutyric acid as a promoting factor of**

cancer metastasis; induction of matrix metalloproteinase production is potentially its underlying mechanism. *Cancer research* 2003, **63**(23):8090-8096.
390. Hao J, Shi F-D, Abdelwahab M, Shi SX, Simard A, Whiteaker P, Lukas R, Zhou Q: **Nicotinic receptor β2 determines NK cell-dependent metastasis in a murine model of metastatic lung cancer**. *PloS One* 2013, **8**(2):e57495.
391. Esteban F, Muñoz M, González-Moles M, Rosso M: **A role for substance P in cancer promotion and progression: a mechanism to counteract intracellular death signals following oncogene activation or DNA damage**. *Cancer and Metastasis Reviews* 2006, **25**(1):137-145.
392. Kuol N, Stojanovska L, Nurgali K, Apostolopoulos V: **The mechanisms tumor cells utilize to evade the host's immune system**. *Maturitas* 2017.
393. Mravec B, Gidron Y, Hulin I: **Neurobiology of cancer: Interactions between nervous, endocrine and immune systems as a base for monitoring and modulating the tumorigenesis by the brain**. In: *Seminars in cancer biology: 2008*: Elsevier; 2008: 150-163.
394. Markwell SM, Weed SA: **Tumor and Stromal-Based Contributions to Head and Neck Squamous Cell Carcinoma Invasion**. *Cancers* 2015, **7**(1):382-406.
395. Liguori M, Solinas G, Germano G, Mantovani A, Allavena P: **Tumor-associated macrophages as incessant builders and destroyers of the cancer stroma**. *Cancers* 2011, **3**(4):3740-3761.
396. Sun Y: **Translational horizons in the tumor microenvironment: harnessing breakthroughs and targeting cures**. *Medicinal Research Reviews* 2015, **35**(2).
397. Hanahan D, Coussens LM: **Accessories to the crime: functions of cells recruited to the tumor microenvironment**. *Cancer cell* 2012, **21**(3):309-322.
398. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation**. *Cell* 2011, **144**(5):646-674.
399. Joyce JA, Pollard JW: **Microenvironmental regulation of metastasis**. *Nature Reviews Cancer* 2009, **9**(4):239-252.
400. Schmid MC, Varner JA: **Myeloid cells in the tumor microenvironment: modulation of tumor angiogenesis and tumor inflammation**. *Journal of oncology* 2010, **2010**.
401. Mancino M, Ametller E, Gascón P, Almendro V: **The neuronal influence on tumor progression**. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 2011, **1816**(2):105-118.
402. Grivennikov SI, Greten FR, Karin M: **Immunity, Inflammation, and Cancer**. *Cell* 2010, **140**(6):883-899.
403. ThyagaRajan S, Priyanka HP: **Bidirectional communication between the neuroendocrine system and the immune system: relevance to health and diseases**. *Annals of neurosciences* 2012, **19**(1):40-46.
404. Stojanovska V, Sakkal S, Nurgali K: **Platinum-based chemotherapy: gastrointestinal immunomodulation and enteric nervous system toxicity**. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2015, **308**(4):G223-G232.
405. Matteoli G, Boeckxstaens GE: **The vagal innervation of the gut and immune homeostasis**. *Gut* 2013, **62**(8):1214-1222.

406. Cortez-Retamozo V, Etzrodt M, Newton A, Rauch PJ, Chudnovskiy A, Berger C, Ryan RJ, Iwamoto Y, Marinelli B, Gorbatov R *et al*: **Origins of tumor-associated macrophages and neutrophils**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(7):2491-2496.
407. Zhang BC, Gao J, Wang J, Rao ZG, Wang BC, Gao JF: **Tumor-associated macrophages infiltration is associated with peritumoral lymphangiogenesis and poor prognosis in lung adenocarcinoma**. *Med Oncol* 2011, **28**(4):1447-1452.
408. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, Figueiredo J-L, Kohler RH, Chudnovskiy A, Waterman P: **Identification of splenic reservoir monocytes and their deployment to inflammatory sites**. *Science* 2009, **325**(5940):612-616.
409. Nance DM, Sanders VM: **Autonomic Innervation and Regulation of the Immune System (1987-2007)**. *Brain Behav Immun* 2007, **21**(6):736–745.
410. Ng YH, Chalasani G: **Role of secondary lymphoid tissues in primary and memory T-cell responses to a transplanted organ**. *Transplantation reviews (Orlando, Fla)* 2010, **24**(1):32-41.
411. Buettner M, Bode U: **Lymph node dissection–understanding the immunological function of lymph nodes**. *Clinical & Experimental Immunology* 2012, **169**(3):205-212.
412. Koning JJ, Mebius RE: **Interdependence of stromal and immune cells for lymph node function**. *Trends in Immunology* 2012, **33**(6):264-270.
413. Swerdlin N, Cohen IR, Harel D: **The lymph node B cell immune response: Dynamic analysis in-silico**. *Proceedings of the IEEE* 2008, **96**(8):1421-1443.
414. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES: **The sympathetic nerve—an integrative interface between two supersystems: the brain and the immune system**. *Pharmacological Reviews* 2000, **52**(4):595-638.
415. St-Pierre S, Jiang W, Roy P, Champigny C, LeBlanc É, Morley BJ, Hao J, Simard AR: **Nicotinic Acetylcholine Receptors Modulate Bone Marrow-Derived Pro-Inflammatory Monocyte Production and Survival**. *PloS One* 2016, **11**(2):e0150230.
416. Hao J, Shi FD, Abdelwahab M, Shi SX, Simard A, Whiteaker P, Lukas R, Zhou Q: **Nicotinic receptor beta2 determines NK cell-dependent metastasis in a murine model of metastatic lung cancer**. *PLoS One* 2013, **8**(2):e57495.
417. Saussez S, Laumbacher B, Chantrain G, Rodriguez A, Gu S, Wank R, Levite M: **Towards neuroimmunotherapy for cancer: the neurotransmitters glutamate, dopamine and GnRH-II augment substantially the ability of T cells of few head and neck cancer patients to perform spontaneous migration, chemotactic migration and migration towards the autologous tumor, and also elevate markedly the expression of CD3zeta and CD3epsilon TCR-associated chains**. *Journal of neural transmission (Vienna, Austria : 1996)* 2014, **121**(8):1007-1027.
418. Wu J, Zhang R, Tang N, Gong Z, Zhou J, Chen Y, Chen K, Cai W: **Dopamine inhibits the function of Gr-1+CD115+ myeloid-derived suppressor cells through D1-like receptors and enhances anti-tumor immunity**. *Journal of leukocyte biology* 2015, **97**(1):191-200.

419. Liu H, Leak RK, Hu X: **Neurotransmitter receptors on microglia**. *Stroke and Vascular Neurology* 2016, **1**(2):52-58.
420. Dolma S, Selvadurai HJ, Lan X, Lee L, Kushida M, Voisin V, Whetstone H, So M, Aviv T, Park N *et al*: **Inhibition of Dopamine Receptor D4 Impedes Autophagic Flux, Proliferation, and Survival of Glioblastoma Stem Cells**. *Cancer cell* 2016, **29**(6):859-873.
421. Stepulak A, Luksch H, Gebhardt C, Uckermann O, Marzahn J, Sifringer M, Rzeski W, Staufner C, Brocke KS, Turski L *et al*: **Expression of glutamate receptor subunits in human cancers**. *Histochemistry and cell biology* 2009, **132**(4):435-445.
422. Banda M, Speyer CL, Semma SN, Osuala KO, Kounalakis N, Torres Torres KE, Barnard NJ, Kim HJ, Sloane BF, Miller FR *et al*: **Metabotropic Glutamate Receptor-1 Contributes to Progression in Triple Negative Breast Cancer**. *PLOS ONE* 2014, **9**(1):e81126.
423. Di Giovangiulio M, Verheijden S, Bosmans G, Stakenborg N, Boeckxstaens GE, Matteoli G: **The Neuromodulation of the Intestinal Immune System and Its Relevance in Inflammatory Bowel Disease**. *Frontiers in Immunology* 2015, **6**(590).
424. Erin N, Barkan GA, Clawson GA: **Vagus nerve regulates breast cancer metastasis to the adrenal gland**. *Anticancer research* 2013, **33**(9):3675-3682.
425. Poulopoulou C, Davaki P, Koliaraki V, Kolovou D, Markakis I, Vassilopoulos D: **Reduced expression of metabotropic glutamate receptor 2mRNA in T cells of ALS patients**. *Annals of neurology* 2005, **58**(6):946-949.
426. Ganor Y, Levite M: **The neurotransmitter glutamate and human T cells: glutamate receptors and glutamate-induced direct and potent effects on normal human T cells, cancerous human leukemia and lymphoma T cells, and autoimmune human T cells**. *Journal of neural transmission (Vienna, Austria : 1996)* 2014, **121**(8):983-1006.
427. Young SZ, Bordey A: **GABA's Control of Stem and Cancer Cell Proliferation in Adult Neural and Peripheral Niches**. *Physiology (Bethesda, Md)* 2009, **24**:171-185.
428. Jiang X, Su L, Zhang Q, He C, Zhang Z, Yi P, Liu J: **GABA(B) Receptor Complex as a Potential Target for Tumor Therapy**. *Journal of Histochemistry and Cytochemistry* 2012, **60**(4):269-279.
429. Thaker PH, Yokoi K, Jennings NB, Li Y, Rebhun RB, Rousseau DL, Jr., Fan D, Sood AK: **Inhibition of experimental colon cancer metastasis by the GABA-receptor agonist nembutal**. *Cancer Biol Ther* 2005, **4**(7):753-758.
430. Jin Z, Mendu SK, Birnir B: **GABA is an effective immunomodulatory molecule**. *Amino Acids* 2013, **45**(1):87-94.
431. Shajib MS, Khan WI: **The role of serotonin and its receptors in activation of immune responses and inflammation**. *Acta physiologica (Oxford, England)* 2015, **213**(3):561-574.
432. Grygier B, Arteta B, Kubera M, Basta-Kaim A, Budziszewska B, Leskiewicz M, Curzytek K, Duda W, Lason W, Maes M: **Inhibitory effect of antidepressants on B16F10 melanoma tumor growth**. *Pharmacological reports : PR* 2013, **65**(3):672-681.

433. Saha B, Mondal AC, Basu S, Dasgupta PS: **Circulating dopamine level, in lung carcinoma patients, inhibits proliferation and cytotoxicity of CD4+ and CD8+ T cells by D1 dopamine receptors: an in vitro analysis**. *International immunopharmacology* 2001, **1**(7):1363-1374.
434. Qin T, Wang C, Chen X, Duan C, Zhang X, Zhang J, Chai H, Tang T, Chen H, Yue J *et al*: **Dopamine induces growth inhibition and vascular normalization through reprogramming M2-polarized macrophages in rat C6 glioma**. *Toxicology and applied pharmacology* 2015, **286**(2):112-123.
435. Figueroa C, Galvez-Cancino F, Oyarce C, Contreras F, Prado C, Valeria C, Cruz S, Lladser A, Pacheco R: **Inhibition of dopamine receptor D3 signaling in dendritic cells increases antigen cross-presentation to CD8+ T-cells favoring anti-tumor immunity**. *J Neuroimmunol* 2017, **303**:99-107.
436. Levite M: **Dopamine and T cells: dopamine receptors and potent effects on T cells, dopamine production in T cells, and abnormalities in the dopaminergic system in T cells in autoimmune, neurological and psychiatric diseases**. *Acta physiologica (Oxford, England)* 2016, **216**(1):42-89.
437. Watanabe M, Maemura K, Oki K, Shiraishi N, Shibayama Y, Katsu K: **Gamma-aminobutyric acid (GABA) and cell proliferation: focus on cancer cells**. *Histology and histopathology* 2006, **21**(10):1135-1141.
438. Cole SW: **Nervous system regulation of the cancer genome**. *Brain Behav Immun* 2013, **30 Suppl**:S10-18.
439. Chanmee T, Ontong P, Konno K, Itano N: **Tumor-associated macrophages as major players in the tumor microenvironment**. *Cancers* 2014, **6**(3):1670-1690.
440. Laoui D, Movahedi K, Van Overmeire E, Van den Bossche J, Schouppe E, Mommer C, Nikolaou A, Morias Y, De Baetselier P, Van Ginderachter JA: **Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions**. *The International journal of developmental biology* 2011, **55**(7-9):861-867.
441. Entschladen F, Drell TL, Lang K, Joseph J, Zaenker KS: **Tumour-cell migration, invasion, and metastasis: navigation by neurotransmitters**. *The Lancet Oncology* 2004, **5**(4):254-258.
442. Nilsson MB, Armaiz-Pena G, Takahashi R, Lin YG, Trevino J, Li Y, Jennings N, Arevalo J, Lutgendorf SK, Gallick GE: **Stress hormones regulate interleukin-6 expression by human ovarian carcinoma cells through a Src-dependent mechanism**. *Journal of Biological Chemistry* 2007, **282**(41):29919-29926.
443. Cole SW, Arevalo JM, Takahashi R, Sloan EK, Lutgendorf SK, Sood AK, Sheridan JF, Seeman TE: **Computational identification of gene–social environment interaction at the human IL6 locus**. *Proceedings of the National Academy of Sciences* 2010, **107**(12):5681-5686.
444. Shahzad MM, Arevalo JM, Armaiz-Pena GN, Lu C, Stone RL, Moreno-Smith M, Nishimura M, Lee J-W, Jennings NB, Bottsford-Miller J: **Stress effects on FosB- and interleukin-8 (IL8)-driven ovarian cancer growth and metastasis**. *Journal of Biological Chemistry* 2010, **285**(46):35462-35470.
445. Yang EV, Sood AK, Chen M, Li Y, Eubank TD, Marsh CB, Jewell S, Flavahan NA, Morrison C, Yeh P-E: **Norepinephrine up-regulates the expression of**

vascular endothelial growth factor, matrix metalloproteinase (MMP)-2, and MMP-9 in nasopharyngeal carcinoma tumor cells. *Cancer research* 2006, **66**(21):10357-10364.
446. Landen CN, Jr., Lin YG, Armaiz Pena GN, Das PD, Arevalo JM, Kamat AA, Han LY, Jennings NB, Spannuth WA, Thaker PH *et al*: **Neuroendocrine modulation of signal transducer and activator of transcription-3 in ovarian cancer**. *Cancer research* 2007, **67**(21):10389-10396.
447. Palm D, Lang K, Niggemann B, Drell TLt, Masur K, Zaenker KS, Entschladen F: **The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers**. *International journal of cancer* 2006, **118**(11):2744-2749.
448. Lang K, Drell TLt, Lindecke A, Niggemann B, Kaltschmidt C, Zaenker KS, Entschladen F: **Induction of a metastatogenic tumor cell type by neurotransmitters and its pharmacological inhibition by established drugs**. *International journal of cancer* 2004, **112**(2):231-238.
449. Inbar S, Neeman E, Avraham R, Benish M, Rosenne E, Ben-Eliyahu S: **Do stress responses promote leukemia progression? An animal study suggesting a role for epinephrine and prostaglandin-E2 through reduced NK activity**. *PLoS One* 2011, **6**(4):e19246.
450. Collado-Hidalgo A, Sung C, Cole S: **Adrenergic inhibition of innate anti-viral response: PKA blockade of Type I interferon gene transcription mediates catecholamine support for HIV-1 replication**. *Brain Behav Immun* 2006, **20**(6):552-563.
451. Busquets S, Figueras MT, Fuster G, Almendro V, Moore-Carrasco R, Ametller E, Argiles JM, Lopez-Soriano FJ: **Anticachectic effects of formoterol: a drug for potential treatment of muscle wasting**. *Cancer research* 2004, **64**(18):6725-6731.
452. Shi M, Liu D, Yang Z, Guo N: **Central and peripheral nervous systems: master controllers in cancer metastasis**. *Cancer metastasis reviews* 2013, **32**(3-4):603-621.
453. Bellinger DL, Millar BA, Perez S, Carter J, Wood C, ThyagaRajan S, Molinaro C, Lubahn C, Lorton D: **Sympathetic modulation of immunity: relevance to disease**. *Cellular Immunology* 2008, **252**(1):27-56.
454. Forsgren S, Alfredson H, Bjur D, Rantapää-Dahlqvist S: **Novel information on the non-neuronal cholinergic system in orthopedics provides new possible treatment strategies for inflammatory and degenerative diseases**. *Orthopedic Reviews* 2009, **1**(1).
455. Tracey KJ: **The inflammatory reflex**. *Nature* 2002, **420**(6917):853-859.
456. Huston JM, Ochani M, Rosas-Ballina M, Liao H, Ochani K, Pavlov VA, Gallowitsch-Puerta M, Ashok M, Czura CJ, Foxwell B: **Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis**. *The Journal of Experimental Medicine* 2006, **203**(7):1623-1628.
457. Wang H, Yu M, Ochani M, Amella C, Tanovic M, Susaria S, Hua LJ, Wang H, Yang H: **Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation**. *Nature* 2003, **421**:384-388.

458. Rosas-Ballina M, Olofsson PS, Ochani M, Valdés-Ferrer SI, Levine YA, Reardon C, Tusche MW, Pavlov VA, Andersson U, Chavan S: **Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit**. *Science* 2011, **334**(6052):98-101.
459. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ: **Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin**. *Nature* 2000, **405**(6785):458-462.
460. Song P, Spindel ER: **Basic and clinical aspects of non-neuronal acetylcholine: expression of non-neuronal acetylcholine in lung cancer provides a new target for cancer therapy**. *Journal of Pharmacological Sciences* 2008, **106**(2):180-185.
461. Paleari L, Grozio A, Cesario A, Russo P: **The cholinergic system and cancer**. In: *Seminars in cancer biology: 2008*: Elsevier; 2008: 211-217.
462. Song P, Sekhon HS, Lu A, Arredondo J, Sauer D, Gravett C, Mark GP, Grando SA, Spindel ER: **M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion**. *Cancer research* 2007, **67**(8):3936-3944.
463. Wong HP, Yu L, Lam EK, Tai EK, Wu WK, Cho CH: **Nicotine promotes cell proliferation via alpha7-nicotinic acetylcholine receptor and catecholamine-synthesizing enzymes-mediated pathway in human colon adenocarcinoma HT-29 cells**. *Toxicology and applied pharmacology* 2007, **221**(3):261-267.
464. Pettersson A, Nilsson L, Nylund G, Khorram-Manesh A, Nordgren S, Delbro DS: **Is acetylcholine an autocrine/paracrine growth factor via the nicotinic α7-receptor subtype in the human colon cancer cell line HT-29?** *European journal of pharmacology* 2009, **609**(1):27-33.
465. Chen R-J, Ho Y-S, Guo H-R, Wang Y-J: **Rapid activation of Stat3 and ERK1/2 by nicotine modulates cell proliferation in human bladder cancer cells**. *Toxicological Sciences* 2008, **104**(2):283-293.
466. Wei P-L, Chang Y-J, Ho Y-S, Lee C-H, Yang Y-Y, An J, Lin S-Y: **Tobacco-specific carcinogen enhances colon cancer cell migration through α7-nicotinic acetylcholine receptor**. *Annals of Surgery* 2009, **249**(6):978-985.
467. Chen C-S, Lee C-H, Hsieh C-D, Ho C-T, Pan M-H, Huang C-S, Tu S-H, Wang Y-J, Chen L-C, Chang Y-J: **Nicotine-induced human breast cancer cell proliferation attenuated by garcinol through down-regulation of the nicotinic receptor and cyclin D3 proteins**. *Breast Cancer Research and Treatment* 2011, **125**(1):73-87.
468. Lee C-H, Chang Y-C, Chen C-S, Tu S-H, Wang Y-J, Chen L-C, Chang Y-J, Wei P-L, Chang H-W, Chang C-H: **Crosstalk between nicotine and estrogen-induced estrogen receptor activation induces α9-nicotinic acetylcholine receptor expression in human breast cancer cells**. *Breast Cancer Research and Treatment* 2011, **129**(2):331-345.
469. de la Torre E, Davel L, Jasnis Mí A, Gotoh T, de Lustig ES, Sales Mí E: **Muscarinic receptors participation in angiogenic response induced by macrophages from mammary adenocarcinoma-bearing mice**. *Breast cancer research : BCR* 2005, **7**(3):R345-352.

470. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN: **Involvement of chemokine receptors in breast cancer metastasis**. *Nature* 2001, **410**(6824):50-56.
471. Kim SJ, Shin JY, Lee KD, Bae YK, Sung KW, Nam SJ, Chun KH: **MicroRNA let-7a suppresses breast cancer cell migration and invasion through downregulation of C-C chemokine receptor type 7**. *Breast cancer research : BCR* 2012, **14**(1):R14.
472. Lewis CE, Pollard JW: **Distinct role of macrophages in different tumor microenvironments**. *Cancer research* 2006, **66**(2):605-612.
473. Dirkx AE, Oude Egbrink MG, Wagstaff J, Griffioen AW: **Monocyte/macrophage infiltration in tumors: modulators of angiogenesis**. *Journal of leukocyte biology* 2006, **80**(6):1183-1196.
474. Roland CL, Dineen SP, Lynn KD, Sullivan LA, Dellinger MT, Sadegh L, Sullivan JP, Shames DS, Brekken RA: **Inhibition of vascular endothelial growth factor reduces angiogenesis and modulates immune cell infiltration of orthotopic breast cancer xenografts**. *Molecular cancer therapeutics* 2009, **8**(7):1761-1771.
475. Lin EY, Nguyen AV, Russell RG, Pollard JW: **Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy**. *The Journal of Experimental Medicine* 2001, **193**(6):727-740.
476. Oosterling SJ, van der Bij GJ, Meijer GA, Tuk CW, van Garderen E, van Rooijen N, Meijer S, van der Sijp JR, Beelen RH, van Egmond M: **Macrophages direct tumour histology and clinical outcome in a colon cancer model**. *The Journal of pathology* 2005, **207**(2):147-155.
477. Ley S, Weigert A, Brüne B: **Neuromediators in inflammation—a macrophage/nerve connection**. *Immunobiology* 2010, **215**(9):674-684.
478. Campbell MJ, Tonlaar NY, Garwood ER, Huo D, Moore DH, Khramtsov AI, Au A, Baehner F, Chen Y, Malaka DO: **Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome**. *Breast Cancer Research and Treatment* 2011, **128**(3):703-711.
479. Mukhtar RA, Nseyo O, Campbell MJ, Esserman LJ: **Tumor-associated macrophages in breast cancer as potential biomarkers for new treatments and diagnostics**. *Expert review of molecular diagnostics* 2011, **11**(1):91-100.
480. Buchsbaum RJ, Oh SY: **Breast Cancer-Associated Fibroblasts: Where We Are and Where We Need to Go**. *Cancers* 2016, **8**(2).
481. Smith HA, Kang Y: **The metastasis-promoting roles of tumor-associated immune cells**. *Journal of Molecular Medicine* 2013, **91**(4):411-429.
482. Liao D, Luo Y, Markowitz D, Xiang R, Reisfeld RA: **Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model**. *PloS One* 2009, **4**(11):e7965.
483. Yu Y, Xiao C, Tan L, Wang Q, Li X, Feng Y: **Cancer-associated fibroblasts induce epithelial–mesenchymal transition of breast cancer cells through paracrine TGF-β signalling**. *British Journal of Cancer* 2014, **110**(3):724-732.
484. Erez N, Truitt M, Olson P, Hanahan D: **Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting**

Inflammation in an NF-κB-Dependent Manner. *Cancer cell* 2010, **17**(2):135-147.
485. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, Kellokumpu-Lehtinen P-L, Bono P, Kataja V, Desmedt C: **Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial**. *Annals of Oncology* 2014, **25**(8):1544-1550.
486. Ali H, Provenzano E, Dawson S-J, Blows F, Liu B, Shah M, Earl H, Poole C, Hiller L, Dunn J: **Association between CD8+ T-cell infiltration and breast cancer survival in 12 439 patients**. *Annals of Oncology* 2014:mdu191.
487. Pretscher D, Distel LV, Grabenbauer GG, Wittlinger M, Buettner M, Niedobitek G: **Distribution of immune cells in head and neck cancer: CD8+ T-cells and CD20+ B-cells in metastatic lymph nodes are associated with favourable outcome in patients with oro-and hypopharyngeal carcinoma**. *BMC Cancer* 2009, **9**(1):1.
488. Hu S, Li L, Yeh S, Cui Y, Li X, Chang H-C, Jin J, Chang C: **Infiltrating T cells promote prostate cancer metastasis via modulation of FGF11→ miRNA-541→ androgen receptor (AR)→ MMP9 signaling**. *Molecular Oncology* 2015, **9**(1):44-57.
489. Olkhanud PB, Damdinsuren B, Bodogai M, Gress RE, Sen R, Wejksza K, Malchinkhuu E, Wersto RP, Biragyn A: **Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T regulatory cells**. *Cancer research* 2011, **71**(10):3505-3515.
490. Sica A, Porta C, Riboldi E, Locati M: **Convergent pathways of macrophage polarization: The role of B cells**. *European journal of immunology* 2010, **40**(8):2131-2133.
491. Iglesia MD, Vincent BG, Parker JS, Hoadley KA, Carey LA, Perou CM, Serody JS: **Prognostic B-cell signatures using mRNA-seq in patients with subtype-specific breast and ovarian cancer**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2014, **20**(14):3818-3829.
492. Morgan RK, Kingham PJ, Walsh MT, Curran DC, Durcan N, McLean WG, Costello RW: **Eosinophil adhesion to cholinergic IMR-32 cells protects against induced neuronal apoptosis**. *The Journal of Immunology* 2004, **173**(10):5963-5970.
493. Ishibashi S, Ohashi Y, Suzuki T, Miyazaki S, Moriya T, Satomi S, Sasano H: **Tumor-associated tissue eosinophilia in human esophageal squamous cell carcinoma**. *Anticancer research* 2006, **26**(2B):1419-1424.
494. von Wasielewski R, Seth S, Franklin J, Fischer R, Hübner K, Hansmann ML, Diehl V, Georgii A: **Tissue eosinophilia correlates strongly with poor prognosis in nodular sclerosing Hodgkin's disease, allowing for known prognostic factors**. *Blood* 2000, **95**(4):1207-1213.
495. Sakkal S, Miller S, Apostolopoulos V, Nurgali K: **Eosinophils in Cancer: Favourable or Unfavourable?** *Current medicinal chemistry* 2016, **23**(7):650-666.
496. Harbaum L, Pollheimer MJ, Kornprat P, Lindtner RA, Bokemeyer C, Langner C: **Peritumoral eosinophils predict recurrence in colorectal cancer**. *Modern*

pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 2015, **28**(3):403-413.
497. Mattes J, Hulett M, Xie W, Hogan S, Rothenberg ME, Foster P, Parish C: **Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process**. *The Journal of Experimental Medicine* 2003, **197**(3):387-393.
498. Minton K: **Granulocytes: Eosinophils enable the antitumour T cell response**. *Nature reviews Immunology* 2015, **15**(6):333.
499. Cormier SA, Taranova AG, Bedient C, Nguyen T, Protheroe C, Pero R, Dimina D, Ochkur SI, O'Neill K, Colbert D *et al*: **Pivotal Advance: eosinophil infiltration of solid tumors is an early and persistent inflammatory host response**. *Journal of leukocyte biology* 2006, **79**(6):1131-1139.
500. Davis BP, Rothenberg ME: **Eosinophils and cancer**. *Cancer Immunol Res* 2014, **2**(1):1-8.
501. Stockmann C, Schadendorf D, Klose R, Helfrich I: **The impact of the immune system on tumor: angiogenesis and vascular remodeling**. *Frontiers in Oncology* 2014, **4**.
502. Szalayova G, Ogrodnik A, Spencer B, Wade J, Bunn J, Ambaye A, James T, Rincon M: **Human breast cancer biopsies induce eosinophil recruitment and enhance adjacent cancer cell proliferation**. *Breast Cancer Research and Treatment* 2016, **157**(3):461-474.
503. Amini R-M, Aaltonen K, Nevanlinna H, Carvalho R, Salonen L, Heikkilä P, Blomqvist C: **Mast cells and eosinophils in invasive breast carcinoma**. *BMC Cancer* 2007, **7**(1):165.
504. Howson LJ, Salio M, Cerundolo V: **MR1-restricted mucosal-associated invariant T cells and their activation during infectious diseases**. *Name: Frontiers in Immunology* 2015, **6**:303.
505. Serriari NE, Eoche M, Lamotte L, Lion J, Fumery M, Marcelo P, Chatelain D, Barre A, Nguyen-Khac E, Lantz O *et al*: **Innate mucosal-associated invariant T (MAIT) cells are activated in inflammatory bowel diseases**. *Clinical and experimental immunology* 2014, **176**(2):266-274.
506. Ussher JE, Klenerman P, Willberg CB: **Mucosal-associated invariant T-cells: new players in anti-bacterial immunity**. *Frontiers in Immunology* 2014, **5**.
507. Peterfalvi A, Gomori E, Magyarlaki T, Pal J, Banati M, Javorhazy A, Szekeres-Bartho J, Szereday L, Illes Z: **Invariant Vα7. 2-Jα33 TCR is expressed in human kidney and brain tumors indicating infiltration by mucosal-associated invariant T (MAIT) cells**. *International immunology* 2008, **20**(12):1517-1525.
508. Zabijak L, Attencourt C, Guignant C, Chatelain D, Marcelo P, Marolleau J-P, Treiner E: **Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients**. *Cancer Immunology, Immunotherapy* 2015, **64**(12):1601-1608.
509. Barriga V, Kuol N, Nurgali K, Apostolopoulos V: **The Complex Interaction between the Tumor Micro-Environment and Immune Checkpoints in Breast Cancer**. *Cancers* 2019, **11**(8).

510. Kuol N, Stojanovska L, Apostolopoulos V, Nurgali K: **Role of the Nervous System in Tumor Angiogenesis**. *Cancer microenvironment : official journal of the International Cancer Microenvironment Society* 2018, **11**(1):1-11.
511. Kuol N, Stojanovska L, Apostolopoulos V, Nurgali K: **Role of the nervous system in cancer metastasis**. *Journal of experimental & clinical cancer research : CR* 2018, **37**(1):5.
512. Kuol N, Stojanovska L, Apostolopoulos V, Nurgali K: **Crosstalk between cancer and the neuro-immune system**. *J Neuroimmunol* 2018, **315**:15-23.
513. Kuol N, Stojanovska L, Nurgali K, Apostolopoulos V: **PD-1/PD-L1 in disease**. *Immunotherapy* 2018, **10**(2):149-160.
514. Kuol N, Stojanovska L, Nurgali K, Apostolopoulos V: **The mechanisms tumor cells utilize to evade the host's immune system**. *Maturitas* 2017, **105**:8-15.
515. Pardoll DM: **The blockade of immune checkpoints in cancer immunotherapy**. *Nature reviews Cancer* 2012, **12**(4):252-264.
516. Wang X, Teng F, Kong L, Yu J: **PD-L1 expression in human cancers and its association with clinical outcomes**. *OncoTargets and therapy* 2016, **9**:5023.
517. Ghiotto M, Gauthier L, Serriari N, Pastor S, Truneh A, Nunes JA, Olive D: **PD-L1 and PD-L2 differ in their molecular mechanisms of interaction with PD-1**. *International immunology* 2010, **22**(8):651-660.
518. Yu H, Xia H, Tang Q, Xu H, Wei G, Chen Y, Dai X, Gong Q, Bi F: **Acetylcholine acts through M3 muscarinic receptor to activate the EGFR signaling and promotes gastric cancer cell proliferation**. *Scientific reports* 2017, **7**:40802-40802.
519. Friedman JR, Richbart SD, Merritt JC, Brown KC, Nolan NA, Akers AT, Lau JK, Robateau ZR, Miles SL, Dasgupta P: **Acetylcholine signaling system in progression of lung cancers**. *Pharmacol Ther* 2019, **194**:222-254.
520. Cheng K, Shang AC, Drachenberg CB, Zhan M, Raufman J-P: **Differential expression of M3 muscarinic receptors in progressive colon neoplasia and metastasis**. *Oncotarget* 2017, **8**(13):21106-21114.
521. Cheng K, Samimi R, Xie G, Shant J, Drachenberg C, Wade M, Davis RJ, Nomikos G, Raufman JP: **Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation**. *American journal of physiology Gastrointestinal and liver physiology* 2008, **295**(3):G591-597.
522. Chen RJ, Ho YS, Guo HR, Wang YJ: **Rapid activation of Stat3 and ERK1/2 by nicotine modulates cell proliferation in human bladder cancer cells**. *Toxicological sciences : an official journal of the Society of Toxicology* 2008, **104**(2):283-293.
523. Lin G, Sun L, Wang R, Guo Y, Xie C: **Overexpression of muscarinic receptor 3 promotes metastasis and predicts poor prognosis in non-small-cell lung cancer**. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* 2014, **9**(2):170-178.
524. Stavely R, Robinson AM, Miller S, Boyd R, Sakkal S, Nurgali K: **Human adult stem cells derived from adipose tissue and bone marrow attenuate enteric neuropathy in the guinea-pig model of acute colitis**. *Stem Cell Research & Therapy* 2015, **6**:244.

525. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K *et al*: **Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion**. *Nature medicine* 2002, **8**(8):793-800.
526. Kim HR, Ha SJ, Hong MH, Heo SJ, Koh YW, Choi EC, Kim EK, Pyo KH, Jung I, Seo D *et al*: **PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients**. *Sci Rep* 2016, **6**:36956.
527. Zhao LW, Li C, Zhang RL, Xue HG, Zhang FX, Zhang F, Gai XD: **B7-H1 and B7-H4 expression in colorectal carcinoma: correlation with tumor FOXP3(+) regulatory T-cell infiltration**. *Acta Histochemica* 2014, **116**(7):1163-1168.
528. Droeser RA, Hirt C, Viehl CT, Frey DM, Nebiker C, Huber X, Zlobec I, Eppenberger-Castori S, Tzankov A, Rosso R *et al*: **Clinical impact of programmed cell death ligand 1 expression in colorectal cancer**. *Eur J Cancer* 2013, **49**(9):2233-2242.
529. Wang H, Yao H, Li C, Liang L, Zhang Y, Shi H, Zhou C, Chen Y, Fang J-Y, Xu J: **PD-L2 expression in colorectal cancer: Independent prognostic effect and targetability by deglycosylation**. *OncoImmunology* 2017:e1327494.
530. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, Koulmanda M, Freeman GJ, Sayegh MH, Sharpe AH: **Tissue expression of PD-L1 mediates peripheral T cell tolerance**. *The Journal of Experimental Medicine* 2006, **203**(4):883-895.
531. Rozali EN, Hato SV, Robinson BW, Lake RA, Lesterhuis WJ: **Programmed death ligand 2 in cancer-induced immune suppression**. *Clin Dev Immunol* 2012, **2012**:656340-656340.
532. Leng C, Li Y, Qin J, Ma J, Liu X, Cui Y, Sun H, Wang Z, Hua X, Yu Y *et al*: **Relationship between expression of PD-L1 and PD-L2 on esophageal squamous cell carcinoma and the antitumor effects of CD8(+) T cells**. *Oncology Reports* 2016, **35**(2):699-708.
533. Ohigashi Y, Sho M, Yamada Y, Tsurui Y, Hamada K, Ikeda N, Mizuno T, Yoriki R, Kashizuka H, Yane K *et al*: **Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2005, **11**(8):2947-2953.
534. Shin SJ, Jeon YK, Kim PJ, Cho YM, Koh J, Chung DH, Go H: **Clinicopathologic Analysis of PD-L1 and PD-L2 Expression in Renal Cell Carcinoma: Association with Oncogenic Proteins Status**. *Annals of surgical oncology* 2016, **23**(2):694-702.
535. Gao Q, Wang XY, Qiu SJ, Yamato I, Sho M, Nakajima Y, Zhou J, Li BZ, Shi YH, Xiao YS *et al*: **Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2009, **15**(3):971-979.
536. Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, Nakamura S, Enomoto K, Yagita H, Azuma M *et al*: **Clinical Significance and Therapeutic**

Potential of the Programmed Death-1 Ligand/Programmed Death-1 Pathway in Human Pancreatic Cancer. *Clinical Cancer Research* 2007, **13**(7):2151.
537. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, Higuchi T, Yagi H, Takakura K, Minato N *et al*: **Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer**. *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**(9):3360-3365.
538. Derks S, Nason KS, Liao X, Stachler MD, Liu KX, Liu JB, Sicinska E, Goldberg MS, Freeman GJ, Rodig SJ *et al*: **Epithelial PD-L2 expression marks Barrett's Esophagus and Esophageal Adenocarcinoma**. *Cancer Immunol Res* 2015, **3**(10):1123-1129.
539. Ghiotto M, Gauthier L, Serriari N, Pastor S, Truneh A, Nunès J, Olive D: **PD-L1 and PD-L2 differ in their molecular mechanisms of interaction with PD-1**. *International immunology* 2010, **22**(8):651-660.
540. Mousa SA, Arias HR, Davis PJ: **Role of Non-neuronal Nicotinic Acetylcholine Receptors in Angiogenesis Modulation**. In: *Angiogenesis Modulations in Health and Disease: Practical Applications of Pro- and Anti-angiogenesis Targets.* edn. Edited by Mousa SA, Davis PJ. Dordrecht: Springer Netherlands; 2013: 55-75.
541. Arias HR, Richards VE, Ng D, Ghafoori ME, Le V, Mousa SA: **Role of non-neuronal nicotinic acetylcholine receptors in angiogenesis**. *The international journal of biochemistry & cell biology* 2009, **41**(7):1441-1451.
542. Egleton RD, Brown KC, Dasgupta P: **Nicotinic acetylcholine receptors in cancer: multiple roles in proliferation and inhibition of apoptosis**. *Trends in pharmacological sciences* 2008, **29**(3):151-158.
543. Schuller HM: **Regulatory role of the alpha7nAChR in cancer**. *Current drug targets* 2012, **13**(5):680-687.
544. Zhao Y: **The Oncogenic Functions of Nicotinic Acetylcholine Receptors**. *Journal of oncology* 2016, **2016**:9.
545. Pelissier-Rota M, Lainé M, Ducarouge B, Bonaz B, Jacquier-Sarlin M: **Role of cholinergic receptors in colorectal cancer: potential therapeutic implications of vagus nerve stimulation?** *Journal of cancer therapy* 2013, **4**:1116-1131.
546. Zheng Y, Ritzenthaler JD, Roman J, Han S: **Nicotine stimulates human lung cancer cell growth by inducing fibronectin expression**. *American journal of respiratory cell and molecular biology* 2007, **37**(6):681-690.
547. Pettersson A, Nilsson L, Nylund G, Khorram-Manesh A, Nordgren S, Delbro DS: **Is acetylcholine an autocrine/paracrine growth factor via the nicotinic alpha7-receptor subtype in the human colon cancer cell line HT-29?** *European journal of pharmacology* 2009, **609**(1-3):27-33.
548. Chen CS, Lee CH, Hsieh CD, Ho CT, Pan MH, Huang CS, Tu SH, Wang YJ, Chen LC, Chang YJ *et al*: **Nicotine-induced human breast cancer cell proliferation attenuated by garcinol through down-regulation of the nicotinic receptor and cyclin D3 proteins**. *Breast Cancer Res Treat* 2011, **125**(1):73-87.

549. Wei PL, Chang YJ, Ho YS, Lee CH, Yang YY, An J, Lin SY: **Tobacco-specific carcinogen enhances colon cancer cell migration through alpha7-nicotinic acetylcholine receptor**. *Ann Surg* 2009, **249**(6):978-985.
550. Lee CH, Chang YC, Chen CS, Tu SH, Wang YJ, Chen LC, Chang YJ, Wei PL, Chang HW, Chang CH *et al*: **Crosstalk between nicotine and estrogen-induced estrogen receptor activation induces alpha9-nicotinic acetylcholine receptor expression in human breast cancer cells**. *Breast Cancer Res Treat* 2011, **129**(2):331-345.
551. Frucht H, Gazdar AF, Park JA, Oie H, Jensen RT: **Characterization of functional receptors for gastrointestinal hormones on human colon cancer cells**. *Cancer research* 1992, **52**(5):1114-1122.
552. Yang WL, Frucht H: **Cholinergic receptor up-regulates COX-2 expression and prostaglandin E(2) production in colon cancer cells**. *Carcinogenesis* 2000, **21**(10):1789-1793.
553. Frucht H, Jensen RT, Dexter D, Yang WL, Xiao Y: **Human colon cancer cell proliferation mediated by the M3 muscarinic cholinergic receptor**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 1999, **5**(9):2532-2539.
554. Spindel ER: **Muscarinic receptor agonists and antagonists: effects on cancer**. *Handbook of experimental pharmacology* 2012(208):451-468.
555. Spindel ER: **Cholinergic Targets in Lung Cancer**. *Current pharmaceutical design* 2016, **22**(14):2152-2159.
556. Kruse AC, Kobilka BK, Gautam D, Sexton PM, Christopoulos A, Wess J: **Muscarinic acetylcholine receptors: novel opportunities for drug development**. *Nature reviews Drug discovery* 2014, **13**(7):549-560.
557. Song P, Sekhon HS, Fu XW, Maier M, Jia Y, Duan J, Proskosil BJ, Gravett C, Lindstrom J, Mark GP *et al*: **Activated cholinergic signaling provides a target in squamous cell lung carcinoma**. *Cancer research* 2008, **68**(12):4693-4700.
558. Kamiya A, Hayama Y, Kato S, Shimomura A, Shimomura T, Irie K, Kaneko R, Yanagawa Y, Kobayashi K, Ochiya T: **Genetic manipulation of autonomic nerve fiber innervation and activity and its effect on breast cancer progression**. *Nature neuroscience* 2019, **22**(8):1289-1305.
559. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K *et al*: **Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion**. *Nature Medicine* 2002, **8**(8):793.
560. Lastwika KJ, Wilson W, Li QK, Norris J, Xu H, Ghazarian SR, Kitagawa H, Kawabata S, Taube JM, Yao S: **Control of PD-L1 Expression by Oncogenic Activation of the AKT–mTOR Pathway in Non–Small Cell Lung Cancer**. *Cancer research* 2016, **76**(2):227-238.
561. Liu J, Hamrouni A, Wolowiec D, Coiteux V, Kuliczkowski K, Hetuin D, Saudemont A, Quesnel B: **Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-γ and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway**. *Blood* 2007, **110**(1):296-304.

562. Marzec M, Zhang Q, Goradia A, Raghunath PN, Liu X, Paessler M, Wang HY, Wysocka M, Cheng M, Ruggeri BA: **Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1)**. *Proceedings of the National Academy of Sciences* 2008, **105**(52):20852-20857.
563. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, Chen S, Klein AP, Pardoll DM, Topalian SL: **Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape**. *Science translational medicine* 2012, **4**(127):127ra137-127ra137.
564. Cheng K, Shang AC, Drachenberg CB, Zhan M, Raufman JP: **Differential expression of M3 muscarinic receptors in progressive colon neoplasia and metastasis**. *Oncotarget* 2017, **8**(13):21106-21114.
565. Novotny A, Ryberg K, Heiman Ullmark J, Nilsson L, Khorram-Manesh A, Nordgren S, Delbro DS, Nylund G: **Is acetylcholine a signaling molecule for human colon cancer progression?** *Scandinavian journal of gastroenterology* 2011, **46**(4):446-455.
566. Song P, Sekhon HS, Jia Y, Keller JA, Blusztajn JK, Mark GP, Spindel ER: **Acetylcholine is synthesized by and acts as an autocrine growth factor for small cell lung carcinoma**. *Cancer research* 2003, **63**(1):214-221.
567. Rosenbaum MW, Bledsoe JR, Morales-Oyarvide V, Huynh TG, Mino-Kenudson M: **PD-L1 expression in colorectal cancer is associated with microsatellite instability, BRAF mutation, medullary morphology and cytotoxic tumor-infiltrating lymphocytes**. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2016, **29**(9):1104-1112.
568. Wang H, Yao H, Li C, Liang L, Zhang Y, Shi H, Zhou C, Chen Y, Fang J-Y, Xu J: **PD-L2 expression in colorectal cancer: Independent prognostic effect and targetability by deglycosylation**. *OncoImmunology* 2017, **6**(7):e1327494.
569. Guo P-D, Sun Z-W, Lai H-J, Yang J, Wu P-P, Guo Y-D, Sun J: **Clinicopathological analysis of PD-L2 expression in colorectal cancer**. *OncoTargets and therapy* 2018, **11**:7635-7642.
570. Qiu Y, Peng Y, Wang J: **Immunoregulatory role of neurotransmitters**. *Advances in neuroimmunology* 1996, **6**(3):223-231.
571. Razani-Boroujerdi S, Behl M, Hahn FF, Pena-Philippides JC, Hutt J, Sopori ML: **Role of muscarinic receptors in the regulation of immune and inflammatory responses**. *Journal of neuroimmunology* 2008, **194**(1-2):83-88.
572. Song P, Sekhon HS, Fu XW, Maier M, Jia Y, Duan J, Proskosil BJ, Gravett C, Lindstrom J, Mark GP *et al*: **Activated cholinergic signaling provides a target in squamous cell lung carcinoma**. *Cancer Res* 2008, **68**(12):4693-4700.
573. Martinez-Moreno P, Nieto-Ceron S, Torres-Lanzas J, Ruiz-Espejo F, Tovar-Zapata I, Martinez-Hernandez P, Rodriguez-Lopez JN, Vidal CJ, Cabezas-Herrera J: **Cholinesterase activity of human lung tumours varies according to their histological classification**. *Carcinogenesis* 2006, **27**(3):429-436.
574. Lee SY, Lee A-R, Ahn J-Y, Ko JH, Lee L, Han J, Kim Y-C, Kim Y-H: **Proteomic analysis of muscarinic acetylcholine receptor-mediated proliferation in HT-**

29 human colon cancer cells. *Molecular & Cellular Toxicology* 2018, **14**(2):155-162.
575. Mino-Kenudson M: **Programmed cell death ligand-1 (PD-L1) expression by immunohistochemistry: could it be predictive and/or prognostic in non-small cell lung cancer?** *Cancer Biology & Medicine* 2016, **13**(2):157-170.
576. Haggar FA, Boushey RP: **Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors**. *Clinics in colon and rectal surgery* 2009, **22**(4):191-197.
577. Farhood B, Raei B, Malekzadeh R, Shirvani M, Najafi M, Mortezazadeh T: **A review of incidence and mortality of colorectal, lung, liver, thyroid, and bladder cancers in Iran and compared to other countries**. *Contemp Oncol (Pozn)* 2019, **23**(1):7-15.
578. Weitz J, Koch M, Debus J, Hohler T, Galle PR, Buchler MW: **Colorectal cancer**. *Lancet (London, England)* 2005, **365**(9454):153-165.
579. Shen Z, Gu L, Mao D, Chen M, Jin R: **Clinicopathological and prognostic significance of PD-L1 expression in colorectal cancer: a systematic review and meta-analysis**. *World J Surg Oncol* 2019, **17**(1):4-4.
580. Kuol N, Stojanovska L, Apostolopoulos V, Nurgali K: **Role of the nervous system in cancer metastasis**. *Journal of Experimental & Clinical Cancer Research* 2018, **37**(1):5.
581. Mandai M: **PD-1/PD-L1 blockage in cancer treatment-from basic research to clinical application**. *International journal of clinical oncology* 2016, **21**(3):447.
582. Rosenbaum MW, Bledsoe JR, Morales-Oyarvide V, Huynh TG, Mino-Kenudson M: **PD-L1 expression in colorectal cancer is associated with microsatellite instability, BRAF mutation, medullary morphology and cytotoxic tumor-infiltrating lymphocytes**. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2016.
583. Yang CY, Lin MW, Chang YL, Wu CT, Yang PC: **Programmed cell death-ligand 1 expression in surgically resected stage I pulmonary adenocarcinoma and its correlation with driver mutations and clinical outcomes**. *European Journal of Cancer* 2014, **50**(7):1361-1369.
584. Baptista MZ, Sarian LO, Derchain SF, Pinto GA, Vassallo J: **Prognostic significance of PD-L1 and PD-L2 in breast cancer**. *Human Pathology* 2016, **47**(1):78-84.
585. Mancino M, Ametller E, Gascon P, Almendro V: **The neuronal influence on tumor progression**. *Biochimica et biophysica acta* 2011, **1816**(2):105-118.
586. Mancino M, Ametller E, Gascón P, Almendro V: **The neuronal influence on tumor progression**. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 2011, **1816**(2):105-118.
587. Yu H, Xia H, Tang Q, Xu H, Wei G, Chen Y, Dai X, Gong Q, Bi F: **Acetylcholine acts through M3 muscarinic receptor to activate the EGFR signaling and promotes gastric cancer cell proliferation**. *Scientific reports* 2017, **7**:40802.
588. Xu R, Shang C, Zhao J, Han Y, Liu J, Chen K, Shi W: **Activation of M3 muscarinic receptor by acetylcholine promotes non-small cell lung cancer cell proliferation and invasion via EGFR/PI3K/AKT pathway**. *Tumour biology*

: the journal of the International Society for Oncodevelopmental Biology and Medicine 2015, **36**(6):4091-4100.
589. Yang YH, Li DL, Bi XY, Sun L, Yu XJ, Fang HL, Miao Y, Zhao M, He X, Liu JJ *et al*: **Acetylcholine Inhibits LPS-Induced MMP-9 Production and Cell Migration via the alpha7 nAChR-JAK2/STAT3 Pathway in RAW264.7 Cells**. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2015, **36**(5):2025-2038.
590. Shao J-X, Wang B, Yao Y-N, Pan Z-J, Shen Q, Zhou J-Y: **Autonomic nervous infiltration positively correlates with pathological risk grading and poor prognosis in patients with lung adenocarcinoma**. *Thorac Cancer* 2016, **7**(5):588-598.
591. Stavely R, Robinson AM, Miller S, Boyd R, Sakkal S, Nurgali K: **Human adult stem cells derived from adipose tissue and bone marrow attenuate enteric neuropathy in the guinea-pig model of acute colitis**. *Stem Cell Research & Therapy* 2015, **6**.
592. Belo A, Cheng K, Chahdi A, Shant J, Xie G, Khurana S, Raufman J-P: **Muscarinic receptor agonists stimulate human colon cancer cell migration and invasion**. *Am J Physiol Gastrointest Liver Physiol* 2011, **300**(5):G749-G760.
593. Xie G, Cheng K, Shant J, Raufman JP: **Acetylcholine-induced activation of M3 muscarinic receptors stimulates robust matrix metalloproteinase gene expression in human colon cancer cells**. *American journal of physiology Gastrointestinal and liver physiology* 2009, **296**(4):G755-763.
594. Cheng K, Samimi R, Xie G, Shant J, Drachenberg C, Wade M, Davis RJ, Nomikos G, Raufman J-P: **Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation**. *American journal of physiology Gastrointestinal and liver physiology* 2008, **295**(3):G591-G597.
595. Tseng W, Leong X, Engleman E: **Orthotopic mouse model of colorectal cancer**. *J Vis Exp* 2007(10):484-484.
596. Kim M, Grimmig T, Grimm M, Lazariotou M, Meier E, Rosenwald A, Tsaur I, Blaheta R, Heemann U, Germer CT *et al*: **Expression of Foxp3 in colorectal cancer but not in Treg cells correlates with disease progression in patients with colorectal cancer**. *PLoS One* 2013, **8**(1):e53630.
597. Bielenberg DR, Zetter BR: **The Contribution of Angiogenesis to the Process of Metastasis**. *Cancer J* 2015, **21**(4):267-273.
598. Calon A, Lonardo E, Berenguer-Llergo A, Espinet E, Hernando-Momblona X, Iglesias M, Sevillano M, Palomo-Ponce S, Tauriello DV, Byrom D *et al*: **Stromal gene expression defines poor-prognosis subtypes in colorectal cancer**. *Nat Genet* 2015, **47**(4):320-329.
599. Calon A, Espinet E, Palomo-Ponce S, Tauriello DV, Iglesias M, Cespedes MV, Sevillano M, Nadal C, Jung P, Zhang XH *et al*: **Dependency of colorectal cancer on a TGF-beta-driven program in stromal cells for metastasis initiation**. *Cancer cell* 2012, **22**(5):571-584.
600. Konishi M, Hayakawa Y, Koike K: **Role of Muscarinic Acetylcholine Signaling in Gastrointestinal Cancers**. *Biomedicines* 2019, **7**(3).
601. Wang L, Zhi X, Zhang Q, Wei S, Li Z, Zhou J, Jiang J, Zhu Y, Yang L, Xu H *et al*: **Muscarinic receptor M3 mediates cell proliferation induced by**

acetylcholine and contributes to apoptosis in gastric cancer. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2016, **37**(2):2105-2117.
602. Fukumura D, Kloepper J, Amoozgar Z, Duda DG, Jain RK: **Enhancing cancer immunotherapy using antiangiogenics: opportunities and challenges**. *Nature reviews Clinical oncology* 2018, **15**(5):325-340.
603. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, Gong Z, Zhang S, Zhou J, Cao K *et al*: **Role of tumor microenvironment in tumorigenesis**. *Journal of Cancer* 2017, **8**(5):761-773.
604. Magnon C: **Role of the autonomic nervous system in tumorigenesis and metastasis**. *Molecular & cellular oncology* 2015, **2**(2):e975643.
605. Goon PK, Lip GY, Boos CJ, Stonelake PS, Blann AD: **Circulating endothelial cells, endothelial progenitor cells, and endothelial microparticles in cancer**. *Neoplasia (New York, NY)* 2006, **8**(2):79-88.
606. Samame Perez-Vargas JC, Biondani P, Maggi C, Gariboldi M, Gloghini A, Inno A, Volpi CC, Gualeni AV, di Bartolomeo M, de Braud F *et al*: **Role of cMET in the development and progression of colorectal cancer**. *International journal of molecular sciences* 2013, **14**(9):18056-18077.
607. De Smedt L, Lemahieu J, Palmans S, Govaere O, Tousseyn T, Van Cutsem E, Prenen H, Tejpar S, Spaepen M, Matthijs G *et al*: **Microsatellite instable vs stable colon carcinomas: analysis of tumour heterogeneity, inflammation and angiogenesis**. *Br J Cancer* 2015, **113**(3):500-509.
608. Albini A, Bruno A, Noonan DM, Mortara L: **Contribution to Tumor Angiogenesis From Innate Immune Cells Within the Tumor Microenvironment: Implications for Immunotherapy**. *Frontiers in immunology* 2018, **9**:527-527.
609. Xue S, Hu M, Li P, Ma J, Xie L, Teng F, Zhu Y, Fan B, Mu D, Yu J: **Relationship between expression of PD-L1 and tumor angiogenesis, proliferation, and invasion in glioma**. *Oncotarget* 2017, **8**(30):49702-49712.
610. Mohamed SY, Mohammed HL, Ibrahim HM, Mohamed EM, Salah M: **Role of VEGF, CD105, and CD31 in the Prognosis of Colorectal Cancer Cases**. *Journal of gastrointestinal cancer* 2019, **50**(1):23-34.
611. Wang R, Ma Y, Zhan S, Zhang G, Cao L, Zhang X, Shi T, Chen W: **B7-H3 promotes colorectal cancer angiogenesis through activating the NF-kappaB pathway to induce VEGFA expression**. *Cell death & disease* 2020, **11**(1):55.
612. Lebrun JJ: **The Dual Role of TGFbeta in Human Cancer: From Tumor Suppression to Cancer Metastasis**. *ISRN molecular biology* 2012, **2012**:381428.
613. Pak KH, Park KC, Cheong JH: **VEGF-C induced by TGF- beta1 signaling in gastric cancer enhances tumor-induced lymphangiogenesis**. *BMC Cancer* 2019, **19**(1):799.
614. Wehler TC, Frerichs K, Graf C, Drescher D, Schimanski K, Biesterfeld S, Berger MR, Kanzler S, Junginger T, Galle PR *et al*: **PDGFRalpha/beta expression correlates with the metastatic behavior of human colorectal cancer: a**

possible rationale for a molecular targeting strategy. *Oncology Reports* 2008, **19**(3):697-704.
615. Schimanski C, Wehler T, Galle P, Gockel I, Moehler M: **PDGFR-α/β expression correlates with the metastatic behavior of human colorectal cancer—A rationale for a molecular targeting strategy?** *Journal of Clinical Oncology* 2008, **26**(15_suppl):22019-22019.
616. Carvalho I, Milanezi F, Martins A, Reis RM, Schmitt F: **Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression**. *Breast cancer research : BCR* 2005, **7**(5):R788-795.
617. Zhu C, Wei Y, Wei X: **AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications**. *Mol Cancer* 2019, **18**(1):153.
618. Ruan GX, Kazlauskas A: **Axl is essential for VEGF-A-dependent activation of PI3K/Akt**. *The EMBO journal* 2012, **31**(7):1692-1703.
619. Tanaka M, Siemann DW: **Axl signaling is an important mediator of tumor angiogenesis**. *Oncotarget* 2019, **10**(30):2887-2898.
620. Soliman H, Mediavilla-Varela M, Antonia S: **Indoleamine 2,3-Dioxygenase: Is It an Immune Suppressor?** *Cancer J* 2010, **16**(4):10.1097/PPO.1090b1013e3181eb3343.
621. Haas Q, Boligan KF, Jandus C, Schneider C, Simillion C, Stanczak MA, Haubitz M, Seyed Jafari SM, Zippelius A, Baerlocher GM *et al*: **Siglec-9 Regulates an Effector Memory CD8⁺ T-cell Subset That Congregates in the Melanoma Tumor Microenvironment**. *Cancer Immunology Research* 2019, **7**(5):707-718.
622. Kuol N, Stojanovska L, Apostolopoulos V, Nurgali K: **Role of the nervous system in cancer metastasis**. *J Exp Clin Cancer Res* 2018, **37**(1):5-5.
623. Drell TLt, Joseph J, Lang K, Niggemann B, Zaenker KS, Entschladen F: **Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells**. *Breast Cancer Res Treat* 2003, **80**(1):63-70.
624. Brandacher G, Perathoner A, Ladurner R, Schneeberger S, Obrist P, Winkler C, Werner ER, Werner-Felmayer G, Weiss HG, Georg G: **Prognostic value of indoleamine 2, 3-dioxygenase expression in colorectal cancer: effect on tumor-infiltrating T cells**. *Clinical cancer research* 2006, **12**(4):1144-1151.
625. Brandacher G, Perathoner A, Ladurner R, Schneeberger S, Obrist P, Winkler C, Werner ER, Werner-Felmayer G, Weiss HG, Gobel G *et al*: **Prognostic value of indoleamine 2,3-dioxygenase expression in colorectal cancer: effect on tumor-infiltrating T cells**. *Clinical Cancer Research* 2006, **12**(4):1144-1151.
626. Jacquemier J, Bertucci F, Finetti P, Esterni B, Charafe-Jauffret E, Thibult ML, Houvenaeghel G, Van den Eynde B, Birnbaum D, Olive D: **High expression of indoleamine 2, 3-dioxygenase in the tumour is associated with medullary features and favourable outcome in basal-like breast carcinoma**. *International journal of cancer* 2012, **130**(1):96-104.
627. Apostolopoulos V, McKenzie IF: **Cellular mucins: targets for immunotherapy**. *Critical reviews in immunology* 1994, **14**(3-4):293-309.
628. Betge J, Schneider NI, Harbaum L, Pollheimer MJ, Lindtner RA, Kornprat P, Ebert MP, Langner C: **MUC1, MUC2, MUC5AC, and MUC6 in colorectal**

cancer: expression profiles and clinical significance. *Virchows Archiv* 2016, **469**(3):255-265.
629. Jandus C, Boligan KF, Chijioke O, Liu H, Dahlhaus M, xE, moulins T, Schneider C, Wehrli M, Hunger RE *et al*: **Interactions between Siglec-7/9 receptors and ligands influence NK cell–dependent tumor immunosurveillance**. *The Journal of Clinical Investigation* 2014, **124**(4):1810-1820.
630. Macauley MS, Crocker PR, Paulson JC: **Siglec-mediated regulation of immune cell function in disease**. *Nature reviews Immunology* 2014, **14**(10):653-666.
631. Cheong KA, Chang YS, Roh JY, Kim BJ, Kim MN, Park YM, Park HJ, Kim ND, Lee CH, Lee AY: **A Novel Function of Siglec-9 A391C Polymorphism on T Cell Receptor Signaling**. *International Archives of Allergy and Immunology* 2011, **154(**2):111-118.
632. Zhang JQ, Nicoll G, Jones C, Crocker PR: **Siglec-9, a novel sialic acid binding member of the immunoglobulin superfamily expressed broadly on human blood leukocytes**. *The Journal of biological chemistry* 2000, **275**(29):22121-22126.
633. Tourdot BE, Brenner MK, Keough KC, Holyst T, Newman PJ, Newman DK: **Immunoreceptor tyrosine-based inhibitory motif (ITIM)-mediated inhibitory signaling is regulated by sequential phosphorylation mediated by distinct nonreceptor tyrosine kinases: a case study involving PECAM-1**. *Biochemistry* 2013, **52**(15):2597-2608.
634. Billadeau DD, Leibson PJ: **ITAMs versus ITIMs: striking a balance during cell regulation**. *J Clin Invest* 2002, **109**(2):161-168.
635. Crocker PR, Varki A: **Siglecs in the immune system**. *Immunology* 2001, **103**(2):137-145.
636. Ando M, Tu W, Nishijima K, Iijima S: **Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs**. *Biochem Biophys Res Commun* 2008, **369(**3):878-883.
637. Matsubara K, Matsushita Y, Sakai K, Kano F, Kondo M, Noda M, Hashimoto N, Imagama S, Ishiguro N, Suzumura A *et al*: **Secreted ectodomain of sialic acid-binding Ig-like lectin-9 and monocyte chemoattractant protein-1 promote recovery after rat spinal cord injury by altering macrophage polarity**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2015, **35**(6):2452-2464.
638. Banzola I, Mengus C, Wyler S, Hudolin T, Manzella G, Chiarugi A, Boldorini R, Sais G, Schmidli TS, Chiffi G *et al*: **Expression of Indoleamine 2,3-Dioxygenase Induced by IFN-gamma and TNF-alpha as Potential Biomarker of Prostate Cancer Progression**. *Front Immunol* 2018, **9**:1051.
639. Takamatsu M, Hirata A, Ohtaki H, Hoshi M, Ando T, Ito H, Hatano Y, Tomita H, Kuno T, Saito K *et al*: **Inhibition of indoleamine 2,3-dioxygenase 1 expression alters immune response in colon tumor microenvironment in mice**. *Cancer Sci* 2015, **106**(8):1008-1015.
640. Munn DH, Mellor AL: **IDO in the Tumor Microenvironment: Inflammation, Counter-Regulation, and Tolerance**. *Trends in immunology* 2016, **37**(3):193-207.

641. Spindel ER: **Cholinergic Targets in Lung Cancer**. *Curr Pharm Des* 2016, **22**(14):2152-2159.
642. Matsumoto T, Takahashi N, Kojima T, Yoshioka Y, Ishikawa J, Furukawa K, Ono K, Sawada M, Ishiguro N, Yamamoto A: **Soluble Siglec-9 suppresses arthritis in a collagen-induced arthritis mouse model and inhibits M1 activation of RAW264.7 macrophages**. *Arthritis research & therapy* 2016, **18**(1):133.
643. Tseng W, Leong X, Engleman E: **Orthotopic mouse model of colorectal cancer**. *Journal of visualized experiments : JoVE* 2007(10):484.
644. Laubli H, Alisson-Silva F, Stanczak MA, Siddiqui SS, Deng L, Verhagen A, Varki N, Varki A: **Lectin galactoside-binding soluble 3 binding protein (LGALS3BP) is a tumor-associated immunomodulatory ligand for CD33-related Siglecs**. *The Journal of biological chemistry* 2014, **289**(48):33481-33491.
645. Stanczak MA, Siddiqui SS, Trefny MP, Thommen DS, Boligan KF, von Gunten S, Tzankov A, Tietze L, Lardinois D, Heinzelmann-Schwarz V *et al*: **Self-associated molecular patterns mediate cancer immune evasion by engaging Siglecs on T cells**. *J Clin Invest* 2018, **128**(11):4912-4923.
646. Beatson R, Tajadura-Ortega V, Achkova D, Picco G, Tsourouktsoglou TD, Klausing S, Hillier M, Maher J, Noll T, Crocker PR *et al*: **The mucin MUC1 modulates the tumor immunological microenvironment through engagement of the lectin Siglec-9**. *Nat Immunol* 2016, **17**(11):1273-1281.
647. Muller AJ, DuHadaway JB, Jaller D, Curtis P, Metz R, Prendergast GC: **Immunotherapeutic suppression of indoleamine 2,3-dioxygenase and tumor growth with ethyl pyruvate**. *Cancer research* 2010, **70**(5):1845-1853.
648. Wei L, Zhu S, Li M, Li F, Wei F, Liu J, Ren X: **High Indoleamine 2,3-Dioxygenase Is Correlated With Microvessel Density and Worse Prognosis in Breast Cancer**. *Frontiers in Immunology* 2018, **9**(724).
649. Nonaka H, Saga Y, Fujiwara H, Akimoto H, Yamada A, Kagawa S, Takei Y, Machida S, Takikawa O, Suzuki M: **Indoleamine 2,3-dioxygenase promotes peritoneal dissemination of ovarian cancer through inhibition of natural killercell function and angiogenesis promotion**. *International journal of oncology* 2011, **38**(1):113-120.
650. Su C, Zhang P, Liu J, Cao Y: **Erianin inhibits indoleamine 2, 3-dioxygenase – induced tumor angiogenesis**. *Biomedicine & Pharmacotherapy* 2017, **88**:521-528.
651. Li Y, Liang L, Dai W, Cai G, Xu Y, Li X, Li Q, Cai S: **Prognostic impact of programed cell death-1 (PD-1) and PD-ligand 1 (PD-L1) expression in cancer cells and tumor infiltrating lymphocytes in colorectal cancer**. *Mol Cancer* 2016, **15**(1):55.
652. Kollmann D, Schweiger T, Schwarz S, Ignatova D, Chang YT, Lewik G, Schoppmann SF, Hoetzenecker W, Klepetko W, Guenova E *et al*: **PD1-positive tumor-infiltrating lymphocytes are associated with poor clinical outcome after pulmonary metastasectomy for colorectal cancer**. *Oncoimmunology* 2017, **6**(9):e1331194.
653. Anagnostou VK, Brahmer JR: **Cancer immunotherapy: a future paradigm shift in the treatment of non–small cell lung cancer**. *Clinical Cancer Research* 2015, **21**(5):976-984.

654. Du Z, Abedalthagafi M, Aizer AA, McHenry AR, Sun HH, Bray M-A, Viramontes O, Machaidze R, Brastianos PK, Reardon DA: **Increased expression of the immune modulatory molecule PD-L1 (CD274) in anaplastic meningioma**. *Oncotarget* 2015, **6**(7):4704-4716.
655. Kim JR, Moon YJ, Kwon KS, Bae JS, Wagle S, Kim KM, Park HS, Lee H, Moon WS, Chung MJ: **Tumor infiltrating PD1-positive lymphocytes and the expression of PD-L1 predict poor prognosis of soft tissue sarcomas**. 2013.
656. Qing Y, Li Q, Ren T, Xia W, Peng Y, Liu G-L, Luo H, Yang Y-X, Dai X-Y, Zhou S-F: **Upregulation of PD-L1 and APE1 is associated with tumorigenesis and poor prognosis of gastric cancer**. *Drug design, development and therapy* 2015, **9**:901.
657. Wang H, Yao H, Li C, Liang L, Zhang Y, Shi H, Zhou C, Chen Y, Fang J-Y, Xu J: **PD-L2 expression in colorectal cancer: Independent prognostic effect and targetability by deglycosylation**. *Oncoimmunology* 2017, **6**(7):e1327494-e1327494.
658. Brody H: **Colorectal cancer**. *Nature* 2015, **521**(7551):S1.
659. Shen Z, Gu L, Mao D, Chen M, Jin R: **Clinicopathological and prognostic significance of PD-L1 expression in colorectal cancer: a systematic review and meta-analysis**. *World journal of surgical oncology* 2019, **17**(1):4.
660. Munn DH, Mellor AL: **Indoleamine 2,3-dioxygenase and tumor-induced tolerance**. *The Journal of clinical investigation* 2007, **117**(5):1147-1154.
661. Mimura K, Teh JL, Okayama H, Shiraishi K, Kua LF, Koh V, Smoot DT, Ashktorab H, Oike T, Suzuki Y *et al*: **PD-L1 expression is mainly regulated by interferon gamma associated with JAK-STAT pathway in gastric cancer**. *Cancer science* 2018, **109**(1):43-53.

Appendix A

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Review

The mechanisms tumor cells utilize to evade the host's immune system

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ABSTRACT

The immune system plays an essential role in the tumor progression; not only can it inhibit tumor growth but it can also promote tumor growth by establishing a favorable environment. Tumor cells utilize several strategies to evade the host's immune system, including expression of immunosuppressive molecules such as PD-L1, IDO and siglec-9. In addition, tumor cells not only regulate the recruitment and development of immunosuppressive forces to influence the tumor microenvironment but also shift the phenotype and function of normal immune cells from a possibly anti-tumor state to a pro-tumor state. As a result, tumor cells evade the host's immune system, leading to metastasis and/or recurrent disease.

1. Introduction

The "survival of the fittest" theory generally describes how tumor cells are able to adapt to host immune surveillance and invade the host. Tumorigenic cells can adapt host immunity via upregulating the expression of molecules such as programmed death ligand 1 (PD-L1), indoleamine-2,3-dioxygenase (IDO), siglec-9, and downregulating other molecules, including the major histocompatibility complex (MHC) class I. In addition to these immunosuppressive molecules, tumor cells can recruit and educate immune cells to promote immune evasion [1]. PD-L1, a transmembrane protein plays a crucial role in suppressing the immune system. T cells express the receptor PD-1 and upon interaction with PD-L1, inhibitory signals are triggered resulting in apoptosis of cytotoxic T lymphocytes (CTLs, $CD8^+$ T cells) [2]. Interestingly, PD-L1 serves as an anti-apoptotic factor on tumor cells, leading to their resistance to cytolysis by CTLs as well as drug-induced apoptosis [3]. Moreover, in a functional immune system, T cells are activated by interacting with MHC expressed on antigen-presenting cells [4,5]. In addition to the interaction between co-inhibitory and co-stimulatory receptors, these interactions prevent the host against auto-immune reactivity. The balanced interaction between co-inhibitory and co-stimulatory receptors determines whether T cells are stimulated or whether they become anergic to a specific antigen displayed on the MHC. The balance of co-stimulation and co-inhibition appears to be skewed by cancer cells toward co-inhibition due to dysregulation of several cell surface markers, such as MHC class I, B7 and CD28 [6]. Furthermore, tumor-associated immune (TAI) cells play an intriguing role in immune evasion. In fact, the presence of TAI cells within the tumor microenvironment correlates with poor prognosis as noted in several cancers [7,8]. TAI cells are capable of expressing immunosuppressive factors, such as IL-10, tumor growth factor beta (TGF-β), and prostaglandin E_2 (PGE_2) to exhibit their effects on T cell inhibition [9–11]. These factors play a crucial role in supporting tumor immune evasion by regulating TAI cells or suppressing systemic immune cell function, particularly T cells, which are responsible for immunosurveillance. Herein, we focus on the role of immunosuppressive molecules PD-L1, IDO, siglec-9, downregulation of MHC class I, infiltration of TAI cell and their secreted factors that promote immune evasion, leading to metastasis and/or disease recurrence in patients with cancer.

2. Methodology

We used PubMed searches with the following key terms: cancer AND programmed death ligand 1, cancer AND PD-L1, cancer AND MHC class I, cancer AND indoleamine-2,3-dioxygenase, cancer AND IDO, cancer AND sialic acid-binding lectins, cancer AND siglec-9, cancer AND immunosuppression, cancer AND IL-10, cancer AND PGE2, cancer AND COX-2, cancer AND fibroblasts, cancer AND neutrophils, cancer AND macrophage, cancer AND TAMs, cancer and MDSC, cancer AND myeloid derived dendritic cells, cancer AND regulatory T cells, cancer AND Tregs. In particular, publications in the last 10 years, 2007–2017 are mostly cited.

3. Mechanisms of tumor cell escape from immune detection

3.1. Upregulation of programmed death ligand 1

The expression of PD-L1 by tumor cells plays an essential role in the

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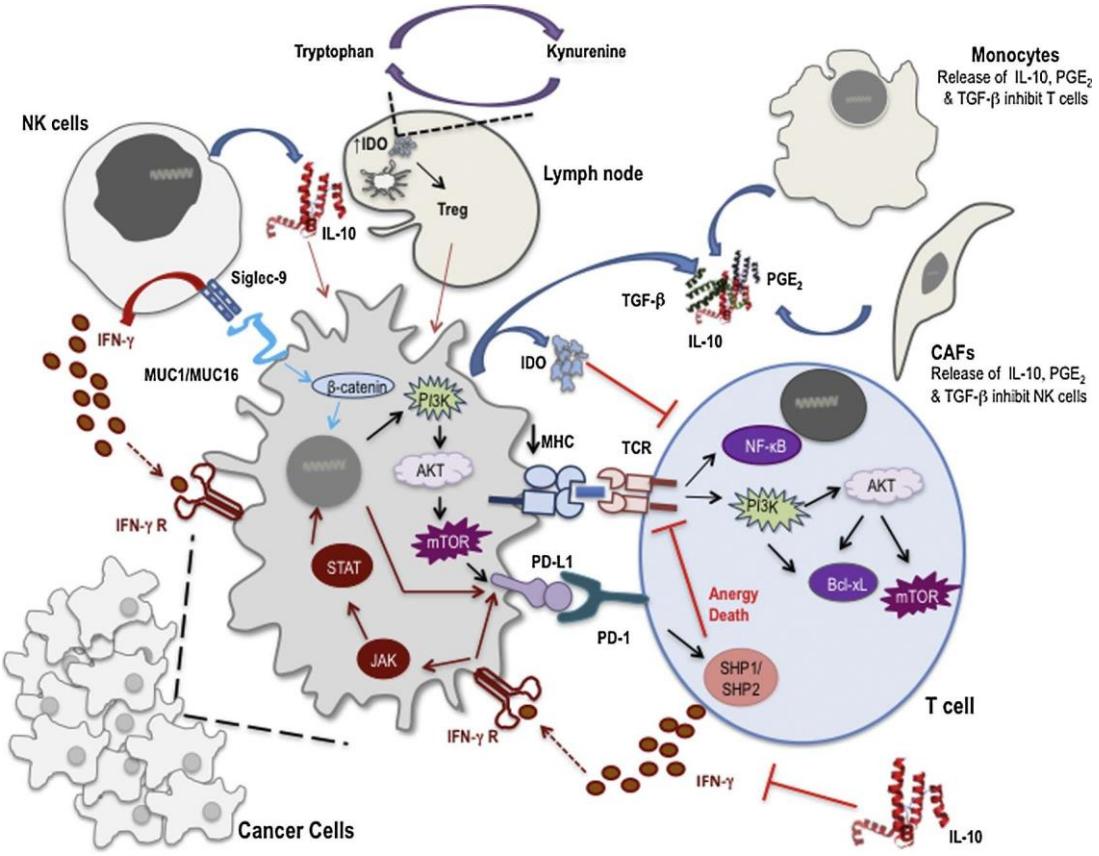

Fig. 1. Immunosuppressive factors involved in tumor escape mechanisms from host immunity. There are several mechanisms that are involved in the tumor evasion, however this schematic diagram demonstrates mechanisms discussed in this paper. Tumorigenic cells exhibit various immunosuppressive mechanisms to evade host immune responses, either to circumvent immune recognition or to immobilize effector T cells. These comprise modification of components of the antigen presentation machinery (such as downregulation of MHC class I) and secretion of immunosuppressive factors, including PD-L1, IDO, siglec-9, IL-10, PGE_2 and TGF-β. These mechanisms assist cancer to suppress the ability of the host immune system to restrain from tumor evasion. Understanding the regulation of these mechanisms might contribute to overcoming the tumor immunosuppressive microenvironment. Bcl-xL, B-cell lymphoma-extra large; CAFs, cancer-associated fibroblasts; IDO, indoleamine-2,3-dioxygenase; IFN-γ, interferon gamma; IFN-γR, interferon gamma receptor; IL-10, Interleukin; JAK, Janus kinase; MHC, major histocompatibility complex; mTOR, mammalian/mechanistic target of rapamycin; MUC, mucins; NK, natural killer; NF-κB, nuclear factor-kappaB; PI3K, phosphoinositide 3-kinase; PD-1, programmed death-1; PD-L1, programmed death-ligand1; PGE_2, prostaglandins; Treg, regulatory T cell; AKT, serine/threonine kinase or protein kinase B; Siglec-9, sialic acid-binding lectins 9; STAT, signal transducer and activator of transcription; SHP, Src homology protein-tyrosine phosphatase; TCR, T cell receptor; TGF-β, tumor growth factor-beta.

establishment of an immunosuppressive force that facilitates tumor cells escape from immune. Indeed, the expression of PD-L1 in head and neck squamous cell carcinoma, carcinomas of the lung, ovary, breast, endometrium, and melanoma, contribute significantly to evading the immune system [5,6,12–36]. Higher PD-L1 expression is associated with tumor node metastasis, poor prognosis and shorter survival in patients with colorectal cancer [37,38]. The expression of PD-L1 by tumor cells is dependent on interferon gamma (IFN-γ) production by tumor-infiltrating immune cells [39]. In addition, downstream signaling molecules such as, nuclear factor-kappaB (NF-κB), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), mammalian/mechanistic target of rapamycin (mTOR) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) which act via toll-like receptors and IFN-γ receptor, regulate the nuclear translocation of transcription factors to the PD-L1 promoter leading to PD-L1 induction [12]. PD-L1 upregulation enhances regulatory T cells (Tregs) via AKT and mTOR phosphorylation leading to immunosuppression [40]. Interestingly, the expression of PD-L1 by tumor-associated macrophages (TAMs) also mediates immunosuppression and is important for tumor escape from immune response. Furthermore, the expression of PD-1 on tumor infiltrating lymphocytes (TILs) correlates with aggressive features of breast cancer cells, including lack of estrogen receptor expression, higher TIL counts, high tumor grade, and, the triple-negative breast cancer subtype [25,29,36,41]. In addition, PD-1 on TILs is associated with poor survival of luminal B and basal-like carcinomas [30] and is highly noted in non-small cell lung cancer (TILs and Treg cells) [42]. Similarly, PD-L1 expression in stromal or tumor cells inversely correlates with $Foxp3^+$ cell density (Treg cells) in colorectal cancer, further reinforcing the fundamental role of Treg cells in the tumor microenvironment [43]. In contrast, there are studies suggesting that the expression of PD-L1 is associated with better prognosis in breast cancer patients [42]. PD-L1 expression correlated with elevated TIL infiltration and longer recurrence-free survival in breast cancer and in pulmonary adenocarcinoma patients [32,44,45]. The inconsistencies in findings warrant further research into the mechanisms of action of PD-L1 on cancer cells. It is possible that the expression levels of PD-L1 on cancer cells and the co-infiltration of TILs, TAMs and Tregs within the tumor microenvironment vary according to the different stages of disease, to prognosis and ability of tumor cells to evade the host immune system.

3.2. Downregulation of MHC class I

The MHC class I also known as human leukocyte antigen (HLA)-A, -B, -C in humans is present on all nucleated cells and presents small processed antigenic peptides on its surface to $CD8^+$ T cells to activate the adaptive immune response [46]. MHC class I downregulation on tumor cells is a well documented mechanism used by tumors to escape host immune detection [47]. In laryngeal squamous cell carcinoma, downregulation of MHC class I correlates with decreased $CD8^+$ T cell

infiltration which associates with poor survival [48]. Similarly, in breast cancer, MHC class I downregulation associates with lymphatic invasion, lymph node metastasis and venous invasion [49]. It has been shown that MAPK signaling adversely regulates the expression of MHC class I in MDA-MB-231 breast cancer cell lines providing mechanistic insights [50]. High intratumoral T cell infiltration and MHC class I expression associates with better survival via nuclear STAT1 stimulation in colorectal cancer patients; downregulation of these markers signified tumor escaping immunosurveilance [51]. Likewise, tumors expressing high levels of MHC class I associate with good prognosis in non-small cell lung carcinoma patients; however, the effects of $CD8^+$ T cells are abolished in tumors expressing non-classical HLA-E [52]. Consistent with these findings, expression of non-classical HLA-E and HLA-G (key modulators of immune responses interfering with $CD8^+$ T cells and natural killer (NK) cell action) correlates with poor prognosis in serous ovarian carcinoma patients, suggesting that therapies targeting HLA-E and HLA-G hold potential benefit [53]. In papillary thyroid cancer, MHC class I expression associates with lower levels of $Foxp3^+$ Treg cells and $CD16^+$, $CD3^+$ and $CD8^+$ tumor-associated immune effector cells [54]. Administration of IFN-γ or selumetinib MEK1/2 inhibitor increases HLA-A, -B, -C expression in papillary thyroid cancer cell lines (BCPAP, TPC-1 and K-1) in vitro [54]. Likewise, we demonstrated that murine mammary adenocarcinoma cell line with low levels of MHC class I and ability to grow in mice without being rejected, was reversed by IFN-γ stimulation which upregulated MHC class I and resulted in tumor inhibition in mice [55]. Hence, tumor cells have evolved into ways to escape from the immune system by downregulating the expression of MHC class I molecules. Thus, it is important to check the expression of MHC class I on cancer tissues in order to develop appropriate treatment modalities for cancer patients (Figs. 1 and 2).

3.3. Overexpression of the enzyme INDOLEAMINE-2,3-DIOXYGENASE

Indoleamine-2,3-dioxygenase is an enzyme in which itsoverexpression leads to increased degradation of the essential amino acid L-tryptophan along the kynurenine pathway resulting in T cell inhibition, hence, promoting a mechanism of tumor escape from host immune detection [56]. IDO provokes L-tryptophan deficiency which impairs T cell proliferation in the tumor microenvironment by inducing apoptosis [57]. In addition, L-tryptophan deficiency impairs $CD8^+$ T cell function via downregulation of the T cell receptor ζ-chain [58]. IDO not only exhibits its effect on T cells but also on other immune cells including NK cells and supports the activity and generation of TAI cells such as, Treg cells and myeloid-derived suppressor cells (MDSC) [59–61]. IDO can inhibit NK cells and $CD4^+$ and $CD8^+$ T cell proliferation, however, has no effect on B cells [62]. Overexpression of IDO by a number of cancer cells holds poor prognostic value as noted in colorectal cancer [63], breast cancer [56], glioma [64] and non-small cell lung carcinoma [65]. In colorectal cancer patients, the expression of IDO by tumor cells is associated with liver metastases and inversely correlates with infiltrating T cells as well as clinical outcome [63]. Similarly to the expression of PD-L1, the expression of IDO is dependent on IFN-γ. Moreover, cancer-associated fibroblasts (CAFs) expressing IDO are linked to stage III and poor prognosis in breast cancer patients as well as enhanced invasiveness of breast cancer cells in vivo in mice [56]. IDO expression is associated with estrogen receptor but not progesterone receptor or epithelial receptor 2 status. For example, low IDO expression correlates with estrogen receptor negative breast cancers and higher neoangiogenesis [66]. IDO expression correlates with increased Foxp3 Treg cells and is associated with lower five year survival rate in non-small cell lung carcinoma patients [65]. Likewise, higher IDO expression in glioma patients associates with poor prognosis and high grade; and in orthotopic GL261 bearing mice models, IDO expression increases the recruitment of Treg whilst simultaneously

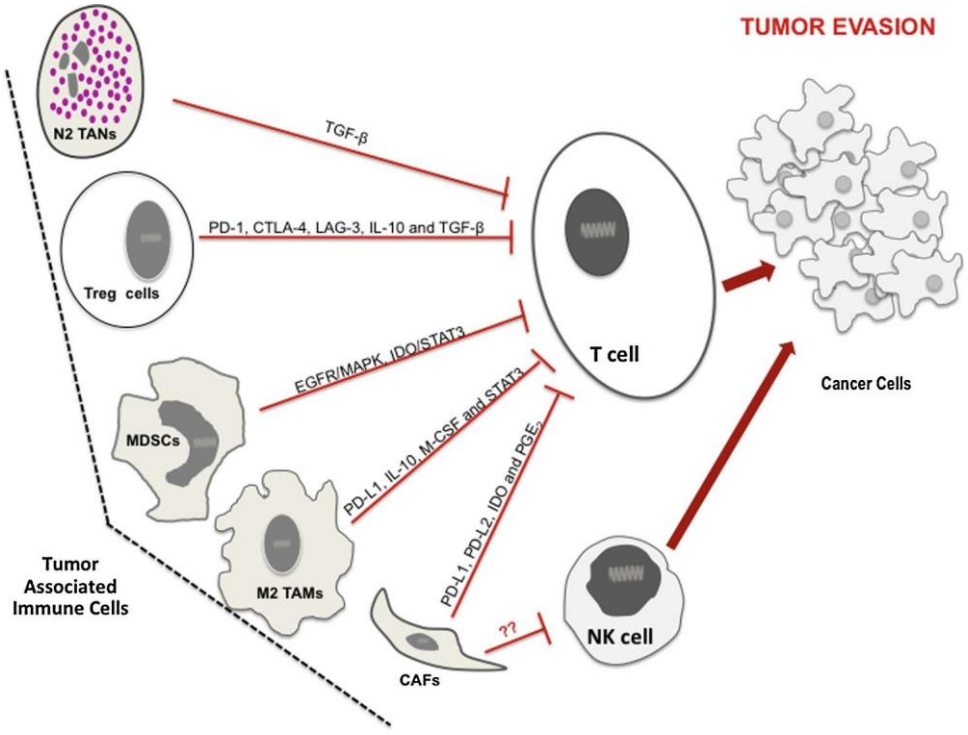

Fig. 2. The role of tumor associated immune cells inducing immunosuppression. This schematic diagram illustrates tumor-associated immune cells that enhance tumor evasion discussed in this paper. Tumorigenic cells can utilize host immune cells to promote tumor progression via the expression of immunosuppressive factors. CAFs, cancer-associated fibroblasts; CTLA-4, cytoxic T lymphocyte-associated antigen-4; EGFR, epidermal growth factor receptor; IDO, indoleamine-2,3-dioxygenase; IL-10, interleukin; LAG-3, lymphocytes activation genes-3; MAPK, mitogen-activated protein kinase; MDSCs, myeloid-derived suppressor cells; NK, natural killer; PD-1, programmed death-1; PD-L1, programmed death-ligand1; PD-L2, programmed death-ligand 2; PGE_2, prostaglandins; Treg, regulatory T cell; STAT3, signal transducer and activator of transcription 3; TAI, tumor-associated immune cells; TANs, tumor-associated neutrophils; TAMs, tumor-associated macrophages; TGF-β, tumor growth factor-beta.

decreasing CD8+ T cells [64]. In contrary, high expression of IDO has an independent good prognostic value in basal-like breast carcinomas [67]; high IDO expression associates with estrogen receptor positive breast cancers and better overall survival [66]. Whether these findings are based on tumor specificity warrants further studies into mechanisms and pathways contributing to immunosuppression.

3.4. Sialic acid-binding lectin-9 and tumor growth

Sialic acid-binding lectins, or siglecs, play an important role in modulating the immune response. Siglecs are expressed by some immune cells such as macrophages, monocytes, neutrophils, B cells, dendritic cells and NK cells [68]. In particular, siglec-9, which is expressed on the surface of immune cells such as, NK cells, B cells and monocytes, has been shown to interact with transmembrane epithelial mucins (MUC), MUC1 and MUC16 [69]. MUC1 (CD227) is over-expressed on adenocarcinomas and hematological cancers [70] whilst MUC16 (CA125) is primarily overexpressed on ovarian cancer cells although studies show that it is expressed on a number of cancers [71,72]. Siglec-9 enhances the chemotactic potential and mature phenotype of NK cells and cytokine secretion (tumor necrosis factor alpha (TNF-α), IFN-γ and macrophage inflammatory protein-1β (MIP-1β)) in neuraminidase-treated K562 cell line [73]. Interestingly, enhanced expression of siglec-9 is noted in melanomas, chronic lymphocytic leukemias and acute myeloid leukemias; however, in the peripheral blood of these patients, siglec-9 positive NK cell population is decreased [73]. Expression of siglec-7 and siglec-9 protects tumor cells from NK cell lysis in vitro (K562, A375, LAU2106, and HCT116 cell lines) and in huNSG mouse model, suggesting an immunosuppressive mechanism by tumor cells [73]. The interaction of siglec-9 with MUC16 has been shown to inhibit immune cell (NK and T cells) priming as noted in OVCAR-3 cell line leading to tumor cell evasion [74]. In human breast and colon cancer tissues, siglec-9 positive cells associate with the MUC1 positive cells suggesting siglec-9 to be a counterreceptor for MUC1 [75]. In addition, in vitro binding of siglec-9 to MUC1 expressed on HCT116 human colon cancer cell line, results in β-catenin recruitment in tumor cells where it is transported to the nucleus, leading to cell growth [75]. Inhibition of TAMs via siglec-9 leads to M1 polarization and reduced growth promoting inflammation within the tumor microenvironment [68]. In addition, blocking of siglec-9 enhances neutrophil activity against tumor cells; likewise, siglec-E, equivalent to siglec-9, deficient mice show increased immunosurveillance against tumor cells [68]. However, this outcome is dependent on the stage of tumor and the microenvironment. These findings suggest that the expression of siglec-9 on immune cells and its interaction with MUC1 or MUC16 on tumor cells may be involved in tumor growth, however, the nature of this interaction as well as the cellular framework in vivo remains to be defined.

4. Immunosuppressive factors secreted by tumor cells

Cancer cells induce immunosuppression resulting in escape mechanism from the host immune system by secreting factors such as interleukin (IL)-10, prostaglandins, cyclooxygenase and TGF-β. These factors are secreted within tumor microenvironment and are associated with poor prognosis and overall survival of cancer patients [76–79].

4.1. Interleukin-10

The cytokine IL-10 (also known as cytokine synthesis inhibitory factor) is an anti-inflammatory cytokine primarily secreted by monocytes, T helper (Th)-2 cells and Treg cells. IL-10 downregulates Th1 cytokines and blocks NF-κB activity. Interestingly, tumor cells utilize IL-10 to suppress T cell function [80]. In fact, high expression of IL-10 at the tumor site associates with poor prognosis [76,81,82]. Cancer cells and TAI cells such as, TAMs, secrete IL-10 into the tumor microenvironment resulting in tumor growth [76,79]. It is likely that IL-10 induces immunosuppression by downregulating MHC class I expression on cancer cells, resulting in tumor escape from the host [83]. In addition, elevated levels of IL-10 in the serum of cancer patients is associated with increased peripheral monocytes correlating with poor prognosis in lymphoma patients [84]. Enhanced expression of IL-10 receptor on tumor cells and its interaction with PD-1, regulates CD8+ T cells of advanced melanoma patients [76]. Consistent with this finding, high expression of IL-10 positively correlates with B7-H3 (CD276) resulting in lymph node metastasis, advanced disease stage II-IV and large tumors [85]. Furthermore, IL-10 mediates immunosuppressive effects via suppressing T cell expansion through inhibition of IL-2 and IFN-γ secretion [86]. Upregulation of IL-10 is associated with HER-2/neu positive breast cancers, however, there is no correlation with age, estrogen receptor or progesterone receptor status in ductal and lobular breast cancer tissues [85]. It is clear that IL-10 aids in tumor escape from the host immune system leading to metastasis or recurrence.

4.2. Prostaglandin E_2 and cyclooxygenase-2

Cyclooxygenase 2 (COX-2) is an enzyme responsible for the production of prostanoids, including prostaglandins (PGE_2). COX-2 is expressed by several malignancies including breast cancer which associates with an aggressive tumor phenotype, contributing to the high metastatic capacity of cancer cells [77]. COX-2 suppresses NK cells, dendritic cells (DCs) and T cells leading to tumor escape from host immune detection. In fact, enhanced expression of PGE_2 and COX-2 inhibits T cells and DC function in breast cancer patients [87]. PGE_2 mediates cancer growth via stimulation of a family of G-protein coupled receptors. Tumor-bearing mammary adenocarcinoma cells escape immune detection as PGE_2 inhibits the function of NK cells to migrate, secrete IFN-γ and exert cytotoxic effects [88]. In fact, inhibition of PGE_2 reduces breast cancer metastasis in mice [88]. Furthermore, it was noted that prostaglandins in 4T1 breast cancer tumor-bearing mice, results in tumor escape mechanism via inducing myeloid-derived suppressor cells (MDSCs) which leads to CD4+ T cells suppression and to some extent CD8+ T cells [89]. These findings concur with studies using prostaglandin E_2 receptor 2 (EP2) knockout mice which show a decrease in MDSC accumulation and impede tumor growth, suggesting immunosuppression [89]. In addition, PGE_2 mediates immunosuppression by enhancing IDO expression by CAFs via STAT3 and EP4/signal transducer signaling pathways as noted in MCF-7 and MDA-MB-231 tumor-bearing mouse model [56].

4.3. Tumor growth factor-beta

Tumor growth factor-beta is produced by a number of immune cells including macrophages. Its increased expression often correlates with malignancy of cancer cells. TGF-β is an immunosuppressive cytokine leading to tumor growth and progression [90]. It is known that TGF-β supports CD4+ T cell polarization to Th2 rather than Th1 cells reducing anti-tumor immune responses [91]. In addition, TGF-β regulates the differentiation and expansion of NK cells, macrophages (M2 pro-tumor phenotype instead of M1 anti-tumor phenotype), DCs and CD4+ T cells. Several studies have determined the mechanisms of how TGF-β impedes with anti-tumor immunity. For example, enhanced levels of TGF-β correlates with an aggressive tumor phenotype and is a good indicator of poor prognosis in several cancers [78,92,93]. Furthermore, TGF-β suppresses NK cell cytolytic activity via NKG2D receptor activation, further enhancing poor anti-tumor response [94]. In addition, inhibiting several cytolytic gene expression molecules including FAS ligand, IFN-γ, and, granzyme A and B, TGF-β is able to suppress tumor cell lysis by CD8+ T cells [95]. The presence of TGF-β at the tumor site signifies immunosuppression via stimulation of signaling pathways including IL-6/STAT3, PI-3/AKT pathways. In fact, in C57BL/6 mice, TGF-β requires Foxp3 to inhibit CD8+ T cell responses via

stimulating the translocation og downstream molecules Smad 2 and Smad 3 [96]. Overexpression of TGF-β is also associated with enhanced Treg cells and tumor associated neutrophils (TAN) in mice [78]. Interestingly, tumor cells stimulate DCs to release TGF-β which promotes the expansion of Treg cells and indirectly inhibits T cell effectors [97]. Similarly, in mice-bearing melanoma or breast cancer cells, reduced expression of type III TGF-β receptor (TGFBR3) enhanced TGF-β signaling which correlate with elevated Foxp3 Treg cells and reduced $CD8^+$ T cells within the tumor microenvironment [98]. In addition, TGF-β regulates IDO expression [98] and blocking of TGF-β in vitro using DNTβRII plasmid, improves the anti-tumor effects of NK cells to MDA-MB-231 and T47D breast cancer cell lines [99]. It is clear that, TGF-β mediates immunosuppression via regulating Treg cells, TAN and reduces $CD8^+$ T cells, resulting in a pro-tumor phenotype for enhanced metastasis and/or recurrent disease. Hence, anti-TGF-β therapy may be a viable treatment strategy for cancer patients.

5. Immunosuppressive effects of tumor-associated immune cells

Tumor-associated immune cells such as TAMs, CAFs, TILs (particular Tregs), MDSCs and TANs, are key immunosuppressive cells that promote tumor progression via their ability to suppress host anti-tumor responses and stimulate tumor angiogenesis [100–102].

5.1. Tumor-associated macrophages

Tumor-associated macrophages particularly of the M2 phenotype are associated with poor prognosis in several cancers including breast cancer [103]. However, what triggers TAMs to differentiate into M2 pro-tumor phenotype and not M1 anti-tumor phenotype? TAMs exposed to tumor microenvironment stimuli such as, TGF-β, IL-10, monocyte colony stimulating factor (M-CSF) and other immunosuppressive factors, induce M2 differentiation [9,104,105]. Furthermore, the presence of TAMs in hypoxic (avascular) environment modifies their gene expression promoting M2 pro-tumor phenotype [106]. In addition, TAMs inhibit $CD8^+$ T cell proliferation as well as TAM-derived IL-10 suppresses IL-12 secretion by intratumoral DCs as noted in an animal model of breast cancer [107]. Likewise, overexpression of IL-10 by TAMs correlates with advanced stages of disease and poor prognosis in non-small cell lung carcinoma patients [108]. TAMs isolated from renal cell carcinoma cells induce Foxp3 Treg cells and IL-10 derived from T cells leading to immune evasion via 15-lipoxygenase-2 pathway activation [109]. Upregulation of TAMs expressing B7-H1 mediates immunosuppression of glioma cells via autocrine/paracrine IL-10 signaling modulation [110]. These studies clearly demonstrate the fundamental role TAMs play in the tumor microenvironment leading to tumor escape mechanisms.

5.2. Cancer-associated fibroblasts

Cancer-associated fibroblasts are the main stromal components which play an essential role within the tumor microenvironment resulting in modulation of tumor growth. CAFs mediate immunosuppression via promoting several other factors including immune infiltrating cells, factors secreted by tumor cells (cytokines/chemokines) and immunosuppressive molecules including IDO [56,111–114]. CAFs are overexpressed in esophageal carcinomas and correlate with poor prognosis [112]. High expression of CAFs and M2 correlate with clinical outcome of colorectal cancer patients [115]. CAFs inhibit NK cells function creating nourished environment for tumor growth, however, these effects are reduced following administration of IDO and PGE_2 inhibitors in a murine model of hepatocellular carcinoma, suggesting that CAFs posses immunosuppressive abilities [10]. CAFs suppress T cell proliferation by promoting the expression of PD-L1 and PD-L2 by cancer cells [114].

5.3. Tumor-associated neutrophils

Tumor-associated neutrophils play an essential role in tumor evasion and are often present within the tumor microenvironment; however their role in immunosuppression has recently surfaced [116,117]. Neutrophils orchestrate innate and adaptive immunity during inflammation. In fact, TANs at the tumor site signify tumor evasion and in most malignant tumors including colorectal cancer, enhanced expressions of TANs are associated with poor prognosis [118–121]. TGF-β regulates the expression of N2 pro-tumor phenotype and reduces $CD8^+$ T cell stimulation [122], whilst other studies demonstrate that N1 anti-tumor phenotype is regulated by IFN-β [123]. Elevated levels of intratumoral neutrophils correlate with advanced stage, lymph node metastasis and poor patient survival in esophageal squamous cell carcinoma [118]. In 4T1 tumor-bearing mice, neutrophils (N2 pro-tumor phenotype) are noted to enhance tumor progression and metastasis [124]. However, contrastingly, high TAN density is associated with better prognosis in advanced colorectal cancer patients [125]. Similarly, findings in early stages of lung cancer patients demonstrate that infiltration of TANs enhances $CD4^+$ and $CD8^+$ T cell proliferation rather than inducing immunosuppression [126]. TANs at early stage of disease secrete high levels of nitric oxide, TNF-α and H_2O_2 and exhibit cytotoxicity toward tumor cells, as noted in Lewis lung carcinoma and mesothelioma models [127]. Thus, what triggers TANs to become pro-tumorigenic in advanced stages of lung cancer but not in colorectal cancer? This could be due to cancer specific signaling pathways activated or combination of other immunosuppressive molecules secreted within the tumor microenvironment. These inconsistencies in findings warrant further studies to better understand the role of TAN in tumor growth, metastasis and recurrent disease.

5.4. Regulatory T cells

Regulatory T cells are distinct $CD4^+$ Th cell subset defined by the $CD25^+CD4^+$ phenotype which suppress effector T cells, believed to be dependent on IL-10 and/or TGF-β [128]. Tregs are regulated by Foxp3 and hold prognostic value in several cancers [128–132]. Tregs inhibit anti-tumor responses mediating tumor escape mechanisms through the secretion of well-known immunosuppressive factors, PD-1, cytoxic T lymphocyte-associated antigen-4 (CTLA-4), lymphocyte activation genes-3 (LAG-3), IL-10 and TGF-β [11]. In fact, enhanced $Foxp3^+$ Treg infiltrates with elevated PD-L1 expression correlate with high grade, basal-like subtype, and negative estrogen receptor and progesterone receptor status [133]. This suggests that $Foxp3^+$ Tregs work synergistically with PD-L1 to endorse immune evasion in breast cancer. In peripheral blood of non-small cell lung carcinoma patients, enhanced expression of $CD4^+CD25^+Foxp3^+$ Tregs was co-expressed with immunosuppressive molecules CTLA-4, PD-1 and LAG-3 [11]. Similarly, in colorectal cancer, enhanced expression of intratumoral $CD4^+Foxp3^+$ Tregs associates with suppressive markers, CTLA-4 and ectonucleotidase CD39; whilst $CD4^+Foxp3^-$ Tregs associate with regulatory markers including LAG-3, latency-associated peptide (LAP) and CD25 [132]. Enhanced intratumoral expression of $Foxp3^+$ Tregs expressing LAP and CD39 is noted in head and neck carcinomas [134]. Enhanced expression of $CD4^+CD25^{high}Foxp3^+$ T cells expressing elevated IL-10 and decreased TGF-β and IFN-γ is reported in gastric cancer patients, challenging the theory that $CD4^+CD25^{high}$ T cells are the main makers of TGF-β [135]. These findings suggest that CD39 and CTLA-4 are commonly co-expressed on several $CD4^+Foxp3^+$ Tregs, suggesting that these markers may play an essential role in regulatory functions of Tregs in situ.

5.5. Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid progenitor cells that contribute to the develop-

ment of tumor and chronic inflammation [136]. The presence of MDSCs within the tumor microenvironment induces immunosuppression as noted in several cancers including breast, colon, pancreatic and non-small cell lung cancer [7,137–140]. MDSCs utilize several mechanisms to influence innate and adaptive immune responses, such as, inducing PD-L1 expression on tumors leading to $CD8^+$ T cell inhibition. MDSCs crosstalk with other immunosuppressive factors such as IDO and Treg cells. For instance, IDO inhibition or Treg exhaustion results in decreased MDSCs, thus reversing immunosuppression in B16 melanoma cell bearing mice [141]. In breast cancer patients, MDSCs mediates immunosuppression via upregulating IDO expression dependent on STAT3 phosphorylation [7]. Hence, MDSCs play a significant immunosuppressive role within the tumor microenvironment leading to tumor escape from host immunity.

6. Conclusion and future prospects

The immune system plays a crucial role in eliminating invading pathogens, eliminating mutated cells before becoming invasive carcinomas, and, in rejecting transplanted organs. Cancer cells however, have evolved enabling them to evade the host's immune system. Cancer cells have been shown to upregulate immunosuppressive markers (IDO, siglec-9, PD-L1) and downregulate MHC class I which aids in their invasion, metastasis and/or recurrent disease. In addition, immune cells (neutrophils, fibroblasts, macrophages, Tregs, MDSCs) infiltrate the tumor microenvironment further contributing to an immunosuppressive milieu, promoting tumor growth. Furthermore, immunosuppressive molecules and cytokines such as, IL-10, TGF-β, PGE_2 and COX-2 play a role in promoting an immunosuppressive environment. It is important to understand the role of these immunosuppressive molecules during tumorigenesis in order to develop appropriate therapeutic strategies in cancer patients. Hence, a comprehensive analysis of immunosuppressive markers during cancer diagnosis will aid in better treatment options and strategies to prevent metastasis and/or recurrent disease.

Contributors

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Conflict of interest

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References

[1] K.M. Bussard, L. Mutkus, K. Stumpf, C. Gomez-Manzano, F.C. Marini, Tumor-associated stromal cells as key contributors to the tumor microenvironment, Breast Cancer Res. 18 (2016) 84.
[2] K. Karwacz, C. Bricogne, D. MacDonald, F. Arce, C.L. Bennett, M. Collins, et al., PD-L1 co-stimulation contributes to ligand-induced T cell receptor down-modulation on CD8(+) T cells, EMBO Mol. Med. 3 (2011) 581–592.
[3] T. Azuma, S. Yao, G. Zhu, A.S. Flies, S.J. Flies, L. Chen, B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells, Blood 111 (2008) 3635–3643.
[4] S. Ceeraz, E.C. Nowak, R.J. Noelle, B7 family checkpoint regulators in immune regulation and disease, Trends Immunol. 34 (2013) 556–563.
[5] M.S. Swanson, U.K. Sinha, Rationale for combined blockade of PD-1 and CTLA-4 in advanced head and neck squamous cell cancer – review of current data, Oral Oncol. 51 (2015) 12–15.
[6] M. Janakiram, Y.M. Abadi, J.A. Sparano, X. Zang, T cell coinhibition and immunotherapy in human breast cancer, Discov. Med. 14 (2012) 229.
[7] J. Yu, W. Du, F. Yan, Y. Wang, H. Li, S. Cao, et al., Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer, J. Immunol. 190 (2013) 3783–3797.
[8] L.Y. OuYang, X.J. Wu, S.B. Ye, R.X. Zhang, Z.L. Li, W. Liao, et al., Tumor-induced myeloid-derived suppressor cells promote tumor progression through oxidative metabolism in human colorectal cancer, J. Transl. Med. 13 (2015) 47.
[9] K.S. Levano, E.H. Jung, P.A. Kenny, Breast cancer subtypes express distinct receptor repertoires for tumor-associated macrophage derived cytokines, Biochem. Biophys. Res. Commun. 411 (2011) 107–110.
[10] T. Li, Y. Yang, X. Hua, G. Wang, W. Liu, C. Jia, et al., Hepatocellular carcinoma-associated fibroblasts trigger NK cell dysfunction via PGE2 and IDO, Cancer Lett. 318 (2012) 154–161.
[11] T. Wei, J. Zhang, Y. Qin, Y. Wu, L. Zhu, L. Lu, et al., Increased expression of immunosuppressive molecules on intratumoral and circulating regulatory T cells in non-small-cell lung cancer patients, Am. J. Cancer Res. 5 (2015) 2190–2201.
[12] P. Ritprajak, M. Azuma, Intrinsic and extrinsic control of expression of the immunoregulatory molecule PD-L1 in epithelial cells and squamous cell carcinoma, Oral Oncol. 51 (2015) 221–228.
[13] J. Chen, R. Shao, C. Chen, Possible therapeutic implication of PD-L1/PD-1 axis in endometrial cancer, J. Solid Tumors (2015) 5.
[14] R. Rosell, M. Gonzalez-Cao, N. Karachaliou, S. Viteri, D. Morales Espinosa, C. Teixidó, et al., Targeting PD-1/PD-L1 in lung cancer: current perspectives, Lung Cancer Targets Therapy (2015) 55.
[15] S. Cedrés, S. Ponce-Aix, J. Zugazagoitia, I. Sansano, A. Enguita, A. Navarro-Mendivil, et al., Analysis of expression of programmed cell death 1 ligand 1 (PD-L1) in malignant pleural mesothelioma (MPM), PLoS ONE 10 (2015) 1–12.
[16] S.P. D'Angelo, A.N. Shoushtari, N.P. Agaram, D. Kuk, L.-X. Qin, R.D. Carvajal, et al., Prevalence of tumor-infiltrating lymphocytes and PD-L1 expression in the soft tissue sarcoma microenvironment, Hum. Pathol. 46 (2015) 357–365.
[17] J. He, Y. Hu, M. Hu, B. Li, Development of PD-1/PD-L1 pathway in tumor immune microenvironment and treatment for non-small cell lung cancer, Scientific Rep. (2015) 5.
[18] E.A. Mittendorf, A.V. Philips, F. Meric-Bernstam, N. Qiao, Y. Wu, S. Harrington, et al., PD-L1 expression in triple-negative breast cancer, Cancer Immunol. Res. 2 (2014) 361–370.
[19] L.B. Jilaveanu, B. Shuch, C.R. Zito, F. Parisi, M. Barr, Y. Kluger, et al., PD-L1 expression in clear cell renal cell carcinoma: an analysis of nephrectomy and sites of metastases, J. Cancer 5 (2014) 166–172.
[20] D.P. Zandberg, S.E. Strome, The role of the PD-L1:PD-1 pathway in squamous cell carcinoma of the head and neck, Oral Oncol. 50 (2014) 627–632.
[21] A. D'Incecco, M. Andreozzi, V. Ludovini, E. Rossi, A. Capodanno, L. Landi, et al., PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients, Br. J. Cancer 112 (2014) 95–102.
[22] Z. Guo, X. Wang, D. Cheng, Z. Xia, M. Luan, S. Zhang, PD-1 blockade and OX40 triggering synergistically protects against tumor growth in a murine model of ovarian cancer, PLoS ONE 9 (2014) 1–10.
[23] C.Y. Mu, J.A. Huang, Y. Chen, C. Chen, X.G. Zhang, High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation, Med. Oncol. 28 (2011) 682–688.
[24] A. Alsuliman, D. Colak, O. Al-Harazi, H. Fitwi, A. Tulbah, T. Al-Tweigeri, et al., Bidirectional crosstalk between PD-L1 expression and epithelial to mesenchymal transition: significance in claudin-low breast cancer cells, Mol. Cancer 14 (2015) 149.
[25] H. Ghebeh, E. Barhoush, A. Tulbah, N. Elkum, T. Al-Tweigeri, S. Dermime, FOXP3+ Tregs and B7-H1+/PD-1+ T lymphocytes co-infiltrate the tumor tissues of high-risk breast cancer patients: implication for immunotherapy, BMC Cancer 8 (2008) 57.
[26] H. Ghebeh, A. Tulbah, S. Mohammed, N. Elkum, S.M. Bin Amer, T. Al-Tweigeri, et al., Expression of B7-H1 in breast cancer patients is strongly associated with high proliferative Ki-67-expressing tumor cells, Int. J. Cancer 121 (2007) 751–758.
[27] G. Isabelle, Hatem A. Azim Jr., I. Michail, C. Sotiriou, Immunology and breast cancer: toward a new way of understanding breast cancer and developing novel therapeutic strategies, Clin. Adv. Hematol. Oncol. (2015) 13.
[28] P. Karasar, G. Esendagli, T helper responses are maintained by basal-like breast cancer cells and confer to immune modulation via upregulation of PD-1 ligands, Breast Cancer Res. Treat. 145 (2014) 605–614.
[29] S. Muenst, A. Schaerli, F. Gao, S. Däster, E. Trella, R. Droeser, et al., Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer, Breast Cancer Res. Treat. 146 (2014) 15–24.
[30] S. Muenst, S.D. Soysal, F. Gao, E.C. Obermann, D. Oertli, W.E. Gillanders, The

presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer, Breast Cancer Res. Treat. 139 (2013) 667–676.
[31] R. Sabatier, P. Finetti, E. Mamessier, J. Adelaide, M. Chaffanet, H.R. Ali, et al., Prognostic and predictive value of PDL1 expression in breast cancer, Oncotarget (2014) 6.
[32] K.A. Schalper, PD-L1 expression and tumor-infiltrating lymphocytes: revisiting the antitumor immune response potential in breast cancer, Oncoimmunology 3 (2014) e29288.
[33] H. Soliman, F. Khalil, S. Antonia, PD-L1 expression is increased in a subset of basal type breast cancer cells, PLoS ONE (2014) 9.
[34] S. Sun, X. Fei, Y. Mao, X. Wang, D. Garfield, O. Huang, et al., PD-1 immune cell infiltration inversely correlates with survival of operable breast cancer patients, Cancer Immunol. Immunother. 63 (2014) 395–406.
[35] P. Tymoszuk, P. Charoentong, H. Hackl, R. Spilka, E. Müller-Holzner, T. Zlatko, et al., High STAT1 mRNA levels but not its tyrosine phosphorylation are associated with macrophage infiltration and bad prognosis in breast cancer, BMC Cancer 14 (2014) 1–27.
[36] H. Wimberly, J.R. Brown, K. Schalper, H. Haack, M.R. Silver, C. Nixon, et al., PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer, Cancer Immunol. Res. 3 (2015) 326–332.
[37] S.J. Shi, L.J. Wang, G.D. Wang, Z.Y. Guo, M. Wei, Y.L. Meng, et al., B7-H1 expression is associated with poor prognosis in colorectal carcinoma and regulates the proliferation and invasion of HCT116 colorectal cancer cells, PLoS ONE 8 (2013) e76012.
[38] L.W. Zhao, C. Li, R.L. Zhang, H.G. Xue, F.X. Zhang, F. Zhang, et al., B7-H1 and B7-H4 expression in colorectal carcinoma: correlation with tumor FOXP3(+) regulatory T-cell infiltration, Acta Histochem. 116 (2014) 1163–1168.
[39] T. Noguchi, J.P. Ward, M.M. Gubin, C.D. Arthur, S.H. Lee, J. Hundal, et al., Temporally distinct PD-L1 expression by tumor and host cells contributes to immune escape, Cancer Immunol. Res. 5 (2017) 106–117.
[40] L.M. Francisco, V.H. Salinas, K.E. Brown, V.K. Vanguri, G.J. Freeman, V.K. Kuchroo, et al., PD-L1 regulates the development, maintenance, and function of induced regulatory T cells, J. Exp. Med. 206 (2009) 3015–3029.
[41] H. Ghebeh, S. Mohammed, A. Al-Omair, A. Qattan, C. Lehe, G. Al-Qudaihi, et al., The B7-H1 (PD-L1)T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors, Neoplasia 8 (2006) 190–198.
[42] H.R. Ali, S.E. Glont, F.M. Blows, E. Provenzano, S.J. Dawson, B. Liu, et al., PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes, Ann. Oncol. 26 (2015) 1488–1493.
[43] Y. Masugi, R. Nishihara, J. Yang, K. Mima, A. da Silva, Y. Shi, et al., Tumour CD274 (PD-L1) expression and T cells in colorectal cancer, Gut (2016) [Epub ahead of print].
[44] C.Y. Yang, M.W. Lin, Y.L. Chang, C.T. Wu, P.C. Yang, Programmed cell death-ligand 1 expression in surgically resected stage I pulmonary adenocarcinoma and its correlation with driver mutations and clinical outcomes, Eur. J. Cancer 50 (2014) 1361–1369.
[45] M.Z. Baptista, L.O. Sarian, S.F. Derchain, G.A. Pinto, J. Vassallo, Prognostic significance of PD-L1 and PD-L2 in breast cancer, Hum. Pathol. 47 (2016) 78–84.
[46] A. Garcia-Lora, I. Algarra, F. Garrido, MHC class I antigens, immune surveillance, and tumor immune escape, J. Cell Physiol. 195 (2003) 346–355.
[47] K. Masuda, A. Hiraki, N. Fujii, T. Watanabe, M. Tanaka, K. Matsue, et al., Loss or down-regulation of HLA class I expression at the allelic level in freshly isolated leukemic blasts, Cancer Sci. 98 (2007) 102–108.
[48] T. Ogino, H. Shigyo, H. Ishii, A. Katayama, N. Miyokawa, Y. Harabuchi, et al., HLA class I antigen down-regulation in primary laryngeal squamous cell carcinoma lesions as a poor prognostic marker, Cancer Res. 66 (2006) 9281–9289.
[49] K. Kaneko, S. Ishigami, Y. Kijima, Y. Funasako, M. Hirata, H. Okumura, et al., Clinical implication of HLA class I expression in breast cancer, BMC Cancer 11 (2011) 1–6.
[50] M. Inoue, K. Mimura, S. Izawa, K. Shiraishi, A. Inoue, S. Shiba, et al., Expression of MHC Class I on breast cancer cells correlates inversely with HER2 expression, Oncoimmunology 1 (2012) 1104–1110.
[51] J.A. Simpson, A. Al-Attar, N.F. Watson, J.H. Scholefield, M. Ilyas, L.G. Durrant, Intratumoral T cell infiltration, MHC class I and STAT1 as biomarkers of good prognosis in colorectal cancer, Gut 59 (2010) 926–933.
[52] M.T. Yazdi, S. van Riet, A. van Schadewijk, M. Fiocco, T. van Hall, C. Taube, et al., The positive prognostic effect of stromal CD8+ tumor-infiltrating T cells is restrained by the expression of HLA-E in non-small cell lung carcinoma, Oncotarget 7 (2016) 3477.
[53] E. Andersson, I. Poschke, L. Villabona, J.W. Carlson, A. Lundqvist, R. Kiessling, et al., Non-classical HLA-class I expression in serous ovarian carcinoma: correlation with the HLA-genotype, tumor infiltrating immune cells and prognosis, Oncoimmunology 5 (2016) e1052213.
[54] T.E. Angell, M.G. Lechner, J.K. Jang, J.S. LoPresti, A.L. Epstein, MHC class I loss is a frequent mechanism of immune escape in papillary thyroid cancer that is reversed by interferon and selumetinib treatment in vitro, Clin. Cancer Res. 20 (2014) 6034–6044.
[55] C.J. Lees, N. Smorodinsky, G. Horn, D.H. Wreschner, I.F. McKenzie, G. Pietersz, et al., MUC1 immunotherapy against a metastatic mammary adenocarcinoma model: importance of IFN-gamma, Prilozi 37 (2016) 15–25.
[56] J.-Y. Chen, C.-F. Li, C.-C. Kuo, K.K. Tsai, M.-F. Hou, W.-C. Hung, Cancer/stroma interplay via cyclooxygenase-2 and indoleamine 2,3-dioxygenase promotes breast cancer progression, Breast Cancer Res. 16 (2014) 1.
[57] C. Uyttenhove, L. Pilotte, I. Theate, V. Stroobant, D. Colau, N. Parmentier, et al., Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase, Nat. Med. 9 (2003) 1269–1274.
[58] F. Fallarino, U. Grohmann, S. You, B.C. McGrath, D.R. Careener, C. Vacca, et al., The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells, J. Immunol. 176 (2006) 6752–6761.
[59] C. Smith, M.Y. Chang, K.H. Parker, D.W. Beury, J.B. DuHadaway, H.E. Flick, et al., IDO is a nodal pathogenic driver of lung cancer and metastasis development, Cancer Discov. 2 (2012) 722–735.
[60] M. Della Chiesa, S. Carlomagno, G. Frumento, M. Balsamo, C. Cantoni, R. Conte, et al., The tryptophan catabolite L-kynurenine inhibits the surface expression of NKp46-and NKG2D-activating receptors and regulates NK-cell function, Blood 108 (2006) 4118–4125.
[61] F. Fallarino, U. Grohmann, K.W. Hwang, C. Orabona, C. Vacca, R. Bianchi, et al., Modulation of tryptophan catabolism by regulatory T cells, Nat. Immunol. 4 (2003) 1206–1212.
[62] G. Frumento, R. Rotondo, M. Tonetti, G. Damonte, U. Benatti, G.B. Ferrara, Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase, J. Exp. Med. 196 (2002) 459–468.
[63] G. Brandacher, A. Perathoner, R. Ladurner, S. Schneeberger, P. Obrist, C. Winkler, et al., Prognostic value of indoleamine 2,3-dioxygenase expression in colorectal cancer: effect on tumor-infiltrating T cells, Clin. Cancer Res. 12 (2006) 1144–1151.
[64] D.A. Wainwright, I.V. Balyasnikova, A.L. Chang, A.U. Ahmed, K.-S. Moon, B. Auffinger, et al., IDO expression in brain tumors increases the recruitment of regulatory T cells and negatively impacts survival, Clin. Cancer Res. 18 (2012) 6110–6121.
[65] T. Hanagiri, M. Fukumoto, Y. Koyanagi, Y. Furutani, F. Tanaka, Indoleamine 2,3-dioxygenase as a prognostic factor in patients with non-small cell lung cancer, J. Clin. Cell Immunol. 5 (2014) 2.
[66] H. Soliman, B. Rawal, J. Fulp, J.H. Lee, A. Lopez, M.M. Bui, et al., Analysis of indoleamine 2-3 dioxygenase (IDO1) expression in breast cancer tissue by immunohistochemistry, Cancer Immunol. Immunother. 62 (2013) 829–837.
[67] J. Jacquemier, F. Bertucci, P. Finetti, B. Esterni, E. Charafe-Jauffret, M.L. Thibult, et al., High expression of indoleamine 2,3-dioxygenase in the tumour is associated with medullary features and favourable outcome in basal-like breast carcinoma, Int. J. Cancer 130 (2012) 96–104.
[68] H. Läubli, O.M.T. Pearce, F. Schwarz, S.S. Siddiqui, L. Deng, M.A. Stanczak, et al., Engagement of myelomonocytic Siglecs by tumor-associated ligands modulates the innate immune response to cancer, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 14211–14216.
[69] S. Chugh, V.S. Gnanapragassam, M. Jain, S. Rachagani, M.P. Ponnusamy, S.K. Batra, Pathobiological implications of mucin glycans in cancer: sweet poison and novel targets, Biochim. Biophys. Acta (BBA)-Rev. Cancer 1856 (2015) 211–225.
[70] V. Apostolopoulos, L. Stojanovska, S.E. Gargosky, MUC1 (CD227): a multi-tasked molecule, Cell Mol. Life Sci.: CMLS 72 (2015) 4475–4500.
[71] S. Das, S.K. Batra, Understanding the unique attributes of MUC16 (CA125): potential implications in targeted therapy, Cancer Res. 75 (2015) 4669–4674.
[72] B.W. Yin, K.O. Lloyd, Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16, J. Biol. Chem. 276 (2001) 27371–27375.
[73] C. Jandus, K.F. Boligan, O. Chijioke, H. Liu, M. Dahlhaus, T. Démoulins, et al., Interactions between Siglec-7/9 receptors and ligands influence NK cell-dependent tumor immunosurveillance, J. Clin. Invest. 124 (2014) 1810–1820.
[74] J.A. Belisle, S. Horibata, G.A.A. Jennifer, S. Petrie, A. Kapur, S. André, et al., Identification of Siglec-9 as the receptor for MUC16 on human NK cells, B cells, and monocytes, Mol. Cancer 9 (2010) 118.
[75] S. Tanida, K. Akita, A. Ishida, Y. Mori, M. Toda, M. Inoue, et al., Binding of the sialic acid-binding lectin, Siglec-9, to the membrane mucin, MUC1, induces recruitment of β-catenin and subsequent cell growth, J. Biol. Chem. 288 (2013) 31842–31852.
[76] Z. Sun, J. Fourcade, O. Pagliano, J.-M. Chauvin, C. Sander, J.M. Kirkwood, et al., Interleukin-10 and programmed death-1 cooperate to regulate tumor antigen-specific CD8+ T cells in melanoma patients, Cancer Res. 75 (2015) 4046–4050.
[77] D. Jana, D.K. Sarkar, S. Ganguly, S. Saha, G. Sa, A.K. Manna, et al., Role of cyclooxygenase 2 (COX-2) in prognosis of breast cancer, Indian J.Surg. Oncol. 5 (2014) 59–65.
[78] C.T. Wu, Y.H. Chang, W.Y. Lin, W.C. Chen, M.F. Chen, TGF Beta1 expression correlates with survival and tumor aggressiveness of prostate cancer, Ann. Surg. Oncol. 22 (Suppl. 3) (2015) S1587–S1593.
[79] C. Yang, L. He, P. He, Y. Liu, W. Wang, Y. He, et al., Increased drug resistance in breast cancer by tumor-associated macrophages through IL-10/STAT3/bcl-2 signaling pathway, Med. Oncol. 32 (2015) 1–8.
[80] H. Groux, M. Bigler, J.E. de Vries, M.-G. Roncarolo, Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells, J. Immunol. 160 (1998) 3188–3193.
[81] C.J. Chen, W.W. Sung, T.C. Su, M.K. Chen, P.R. Wu, K.T. Yeh, et al., High expression of interleukin 10 might predict poor prognosis in early stage oral squamous cell carcinoma patients, Clin. Chim. Acta 415 (2013) 25–30.
[82] E. Itakura, R.-R. Huang, D.-R. Wen, E. Paul, P.H. Wünsch, A.J. Cochran, IL-10 expression by primary tumor cells correlates with melanoma progression from radial to vertical growth phase and development of metastatic competence, Modern Pathol. 24 (2011) 801–809.
[83] F.Y. Yue, R. Dummer, R. Geertsen, G. Hofbauer, E. Laine, S. Manolio, et al., Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-I, HLA class-II and ICAM-1 molecules, Int. J. Cancer 71 (1997) 630–637.
[84] B. Xiu, Y. Lin, D. Grote, S. Ziesmer, M. Gustafson, M. Maas, et al., IL-10 induces the development of immunosuppressive CD14+ HLA-DRlow/— monocytes in B-cell non-Hodgkin lymphoma, Blood Cancer J. 5 (2015) e328.
[85] C. Liu, J. Liu, J. Wang, Y. Liu, F. Zhang, W. Lin, et al., B7-H3 expression in ductal and lobular breast cancer and its association with IL-10, Mol. Med. Rep. 7 (2013) 134–138.
[86] K. Taga, G. Tosato, IL-10 inhibits human T cell proliferation and IL-2 production, J. Immunol. 148 (1992) 1143–1148.
[87] B.A. Pockaj, G.D. Basu, L.B. Pathangey, R.J. Gray, J.L. Hernandez, S.J. Gendler,

et al., Reduced T-cell and dendritic cell function is related to cyclooxygenase-2 overexpression and prostaglandin E2 secretion in patients with breast cancer, Ann. Surg. Oncol. 11 (2004) 328–339.
[88] D.M. Holt, X. Ma, N. Kundu, P.D. Collin, A.M. Fulton, Modulation of host natural killer cell functions in breast cancer via prostaglandin E2 receptors EP2 and EP4, J. Immunother. 35 (2012) 179–188.
[89] P. Sinha, V.K. Clements, A.M. Fulton, S. Ostrand-Rosenberg, Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells, Cancer Res. 67 (2007) 4507–4513.
[90] R. Derynck, R.J. Akhurst, A. Balmain, TGF-[beta] signaling in tumor suppression and cancer progression, Nat. Genet. 29 (2001) 117–129.
[91] H. Maeda, A. Shiraishi, TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice, J. Immunol. 156 (1996) 73–78.
[92] Y. Wang, B. Deng, W. Tang, T. Liu, X. Shen, TGF-beta1 secreted by hepatocellular carcinoma induces the expression of the Foxp3 gene and suppresses antitumor immunity in the tumor microenvironment, Dig. Dis. Sci. 58 (2013) 1644–1652.
[93] M. Javle, Y. Li, D. Tan, X. Dong, P. Chang, S. Kar, et al., Biomarkers of TGF-β signaling pathway and prognosis of pancreatic cancer, PLoS ONE 9 (2014) e85942.
[94] R.A. Flavell, S. Sanjabi, S.H. Wrzesinski, P. Licona-Limón, The polarization of immune cells in the tumour environment by TGFβ, Nat. Rev. Immunol. (2010) 10.
[95] D.A. Thomas, J. Massague, TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance, Cancer Cell 8 (2005) 369–380.
[96] T.L. Stephen, M.R. Rutkowski, M.J. Allegrezza, A. Perales-Puchalt, A.J. Tesone, N. Svoronos, et al., Transforming growth factor β-mediated suppression of antitumor T cells requires FoxP1 transcription factor expression, Immunity 41 (2014) 427–439.
[97] F. Ghiringhelli, P.E. Puig, S. Roux, A. Parcellier, E. Schmitt, E. Solary, et al., Tumor cells convert immature myeloid dendritic cells into TGF-β-secreting cells inducing CD4(+)CD25(+) regulatory T cell proliferation, J. Exp. Med. 202 (2005) 919–929.
[98] B.A. Hanks, A. Holtzhausen, K.S. Evans, R. Jamieson, P. Gimpel, O.M. Campbell, et al., Type III TGF-beta receptor downregulation generates an immunotolerant tumor microenvironment, J. Clin. Invest. 123 (2013) 3925–3940.
[99] Y. Zhao, J. Hu, R. Li, J. Song, Y. Kang, S. Liu, et al., Enhanced NK cell adoptive antitumor effects against breast cancer in vitro via blockade of the transforming growth factor-β signaling pathway, Onco. Targets Ther. 8 (2015) 1553–1559.
[100] T. Kitamura, Qian B-ZZ, J.W. Pollard, Immune cell promotion of metastasis, Nat. Rev. Immunol. (2015) 15.
[101] T. Kitamura, Qian B-ZZ, D. Soong, L. Cassetta, R. Noy, G. Sugano, CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages, J. Exp. Med. (2015) 212.
[102] S.S. McAllister, R.A. Weinberg, The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis, Nat. Cell Biol. 16 (2014) 717–727.
[103] S. Sousa, R. Brion, M. Lintunen, P. Kronqvist, J. Sandholm, J. Monkkonen, et al., Human breast cancer cells educate macrophages toward the M2 activation status, Breast Cancer Res. 17 (2015) 101.
[104] K.D. Grugan, F.L. McCabe, M. Kinder, A.R. Greenplate, B.C. Harman, J.E. Ekert, et al., Tumor-associated macrophages promote invasion while retaining Fc-dependent anti-tumor function, J. Immunol. 189 (2012) 5457–5466.
[105] A. Mantovani, S. Sozzani, M. Locati, P. Allavena, A. Sica, Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes, Trends Immunol. (2002) 23.
[106] C. Lewis, C. Murdoch, Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies, Amer. J. Pathol. 167 (2005) 627–635.
[107] B. Ruffell, D. Chang-Strachan, V. Chan, A. Rosenbusch, C.M. Ho, N. Pryer, et al., Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells, Cancer Cell 26 (2014) 623–637.
[108] R. Wang, M. Lu, J. Zhang, S. Chen, X. Luo, Y. Qin, et al., Increased IL-10 mRNA expression in tumor-associated macrophage correlated with late stage of lung cancer, J. Exp. Clin. Cancer Res. 30 (2011) 62.
[109] I. Daurkin, E. Eruslanov, T. Stoffs, G.Q. Perrin, C. Algood, S.M. Gilbert, et al., Tumor-associated macrophages mediate immunosuppression in the renal cancer microenvironment by activating the 15-lipoxygenase-2 pathway, Cancer Res. 71 (2011) 6400–6409.
[110] O. Bloch, C.A. Crane, R. Kaur, M. Safaee, M.J. Rutkowski, A.T. Parsa, Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages, Clin. Cancer Res. 19 (2013) 3165–3175.
[111] R. He, X. Yang, Y.-H. Shi, B. Li, W. Liu, W. Yin, et al., FAP promotes immunosuppression by cancer-associated fibroblasts in the tumor microenvironment via STAT3-CCL2 signaling, Cancer Res. (2016) canres.2973.015.
[112] T. Kato, K. Noma, Y. Katsura, H. Kashima, T. Ninomiya, T. Ohara, et al., Prognostic correlation of tumor-infiltrating lymphocytes (TILs) and cancer associated fibroblasts (CAFs) in patients with human esophageal carcinoma, Cancer Res. 76 (2016) 4160–4170.
[113] N. Kubo, K. Araki, H. Kuwano, K. Shirabe, Cancer-associated fibroblasts in hepatocellular carcinoma, World J. Gastroenterol. 22 (2016) 6841–6850.
[114] H. Takahashi, K. Sakakura, R. Kawabata-Iwakawa, S. Rokudai, M. Toyoda, M. Nishiyama, et al., Immunosuppressive activity of cancer-associated fibroblasts in head and neck squamous cell carcinoma, Cancer Immunol. Immunother. 64 (2015) 1407–1417.
[115] M. Herrera, A. Herrera, G. Dominguez, J. Silva, V. Garcia, J.M. Garcia, et al., Cancer-associated fibroblast and M2 macrophage markers together predict outcome in colorectal cancer patients, Cancer Sci. 104 (2013) 437–444.
[116] D.R. Powell, A. Huttenlocher, Neutrophils in the tumor microenvironment, Trends Immunol. 37 (2016) 41–52.
[117] M.G. García-Mendoza, D.R. Inman, S.M. Ponik, J.J. Jeffery, D.S. Sheerar, R.R. Van Doorn, et al., Neutrophils drive accelerated tumor progression in the collagen-dense mammary tumor microenvironment, Breast Cancer Res. 18 (2016) 49.
[118] J. Wang, Y. Jia, N. Wang, X. Zhang, B. Tan, G. Zhang, et al., The clinical significance of tumor-infiltrating neutrophils and neutrophil-to-CD8+ lymphocyte ratio in patients with resectable esophageal squamous cell carcinoma, J. Transl. Med. 12 (2014) 7.
[119] H.K. Jensen, F. Donskov, N. Marcussen, M. Nordsmark, F. Lundbeck, H. von der Maase, Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma, J. Clin. Oncol. 27 (2009) 4709–4717.
[120] Y.W. Li, S.J. Qiu, J. Fan, J. Zhou, Q. Gao, Y.S. Xiao, et al., Intratumoral neutrophils: a poor prognostic factor for hepatocellular carcinoma following resection, J. Hepatol. 54 (2011) 497–505.
[121] H.L. Rao, J.W. Chen, M. Li, Y.B. Xiao, J. Fu, Y.X. Zeng, et al., Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis, PLoS ONE 7 (2012) e30806.
[122] Z.G. Fridlender, J. Sun, S. Kim, V. Kapoor, G. Cheng, L. Ling, et al., Polarization of tumor-associated neutrophil phenotype by TGF-β: "N1" versus "N2" TAN, Cancer Cell 16 (2009) 183–194.
[123] J. Jablonska, S. Leschner, K. Westphal, S. Lienenklaus, S. Weiss, Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model, J. Clin. Invest. 120 (2010) 1151–1164.
[124] S. Tabariès, V. Ouellet, B.E. Hsu, M.G. Annis, A.A. Rose, L. Meunier, et al., Granulocytic immune infiltrates are essential for the efficient formation of breast cancer liver metastases, Breast Cancer Res. 17 (2015) 45.
[125] M.R. Galdiero, P. Bianchi, F. Grizzi, G. Di Caro, G. Basso, A. Ponzetta, et al., Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer, Int. J. Cancer 139 (2016) 446–456.
[126] E.B. Eruslanov, P.S. Bhojnagarwala, J.G. Quatromoni, T.L. Stephen, A. Ranganathan, C. Deshpande, et al., Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer, J. Clin. Invest. 124 (2014) 5466–5480.
[127] I. Mishalian, R. Bayuh, L. Levy, L. Zolotarov, J. Michaeli, Z.G. Fridlender, Tumor-associated neutrophils (TAN) develop pro-tumorigenic properties during tumor progression, Cancer Immunol. Immunother. (2013) 62.
[128] H. Yagi, T. Nomura, K. Nakamura, S. Yamazaki, T. Kitawaki, S. Hori, et al., Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells, Int. Immunol. 16 (2004) 1643–1656.
[129] D.J. Campbell, S.F. Ziegler, FOXP3 modifies the phenotypic and functional properties of regulatory T cells, Nat. Rev. Immunol. 7 (2007) 305–310.
[130] J.D. Fontenot, M.A. Gavin, A.Y. Rudensky, Foxp3 programs the development and function of CD4+CD25+ regulatory T cells, Nat. Immunol. 4 (2003) 330–336.
[131] T.H. Schreiber, The use of FoxP3 as a biomarker and prognostic factor for malignant human tumors, Cancer Epidemiol. Biomarkers Prev. 16 (2007) 1931–1934.
[132] M. Scurr, K. Ladell, M. Besneux, A. Christian, T. Hockey, K. Smart, et al., Highly prevalent colorectal cancer-infiltrating LAP(+) Foxp3(−) T cells exhibit more potent immunosuppressive activity than Foxp3(+) regulatory T cells, Mucosal Immunol. 7 (2014) 428–439.
[133] Z. Li, P. Dong, M. Ren, Y. Song, X. Qian, Y. Yang, et al., PD-L1 expression is associated with tumor FOXP3(+) regulatory T-cell infiltration of breast cancer and poor prognosis of patient, J. Cancer 7 (2016) 784–793.
[134] H.B. Jie, N. Gildener-Leapman, J. Li, R.M. Srivastava, S.P. Gibson, T.L. Whiteside, et al., Intratumoral regulatory T cells upregulate immunosuppressive molecules in head and neck cancer patients, Br. J. Cancer 109 (2013) 2629–2635.
[135] B. Kindlund, A. Sjoling, C. Yakkala, J. Adamsson, A. Janzon, L.E. Hansson, et al., CD4+ regulatory T cells in gastric cancer mucosa are proliferating and express high levels of IL-10 but little TGF-beta, Gastric Cancer 20 (2017) 116–125.
[136] Y.Y. Kong, M. Fuchsberger, S.D. Xiang, V. Apostolopoulos, M. Plebanski, Myeloid derived suppressor cells and their role in diseases, Curr. Med. Chem. 20 (2013) 1437–1444.
[137] D.I. Gabrilovich, S. Ostrand-Rosenberg, V. Bronte, Coordinated regulation of myeloid cells by tumours, Nat. Rev. Immunol. (2012) 12.
[138] S. Ostrand-Rosenberg, P. Sinha, D.W. Beury, V.K. Clements, Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression, Semin. Cancer Biol. 22 (2012) 275–281.
[139] D.I. Gabrilovich, S. Nagaraj, Myeloid-derived suppressor cells as regulators of the immune system, Nat. Rev. Immunol. (2009) 9.
[140] M.E. Heuvers, F. Muskens, K. Bezemer, M. Lambers, A.M. Dingemans, H.J. Groen, et al., Arginase-1 mRNA expression correlates with myeloid-derived suppressor cell levels in peripheral blood of NSCLC patients, Lung Cancer 81 (2013) 468–474.
[141] R.B. Holmgaard, D. Zamarin, Y. Li, B. Gasmi, D.H. Munn, J.P. Allison, et al., Tumor-expressed IDO recruits and activates MDSCs in a Treg-dependent manner, Cell Rep. 13 (2015) 412–424.

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REVIEW

Role of the Nervous System in Tumor Angiogenesis

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Abstract

The development of cancer involves an intricate process, wherein many identified and unidentified factors play a role. Tumor angiogenesis, growth of new blood vessels, is one of the major prerequisites for tumor growth as tumor cells rely on adequate oxygen and nutrient supply as well as the removal of waste products. Growth factors including VEGF orchestrate the development of angiogenesis. In addition, nervous system via the release of neurotransmitters contributes to tumor angiogenesis. The nervous system governs functional activities of many organs, and, as tumors are not independent organs within an organism, this system is integrally involved in tumor growth and progression via regulating tumor angiogenesis. Various neurotransmitters have been reported to play an important role in tumor angiogenesis.

Keywords Nervous system · Neurotransmitters · Neuropeptides · Neuro-cancer interaction · Angiogenesis · Cancer

Introduction

New growth in the vascular network (angiogenesis) is a normal physiological phenomenon that tumors utilize to aid in their growth, proliferation and metastatic spread. Angiogenesis involves migration and division of endothelial cells, generation of new basement membrane, arrangement into tubular structures and coverage by pericytes. Angiogenesis is regulated by a plethora of pro- and anti-angiogenic molecules such as, interleukin (IL)-8, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-α, TGF-β, angiogenin, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [1, 2]. The level of angiogenic factors in tissues reflects the aggressiveness of tumor cells which play a significant role in prognostic outcomes [3, 4]. In cancer, the balance between pro- and anti-angiogenic factors is lost, resulting in uncontrolled angiogenesis with irregular blood vessels lacking a clear hierarchal arrangement [1, 5]. As a consequence, anti-angiogenic therapies (in particular anti-VEGF) have been approved for cancer treatment [4, 6–8]. The interaction between VEGF with its receptor, VEGFR2, is responsible for the majority of the angiogenic stimulatory signals in vivo, however, their therapeutic value for long-term patient survival is relatively modest [3].

In addition to these factors, the impact of the tumor microenvironment in tumor angiogenesis has attracted much interest in recent years as another regulator of angiogenesis [9–12]. Furthermore, the role of the nervous system has also surfaced as one of the major contributors to cancer progression through the regulation of tumor angiogenesis via release of neurotransmitters. The nervous system governs functional activities of many organs, and, as tumors are not independent organs within an organism, this system is integrally involved in tumor growth and progression [13, 14]. Here we present an overview of the nervous system role in tumor angiogenesis.

Neurotransmitters Influencing Tumor Angiogenesis

Neurotransmitters are group of neurological chemical messengers synthesized by neurons and secreted at nerve terminals where they transmit signals to target cells through binding to their receptors. Studies have demonstrated that various cancers express receptors for different neurotransmitters which have been identified to play essential role in the control of tumor angiogenesis (Table 1, Fig. 1).

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Table 1 Neurotransmitters influencing tumor angiogenesis

Neurotransmitters	Receptor	Type of cancer	Model	Mechanism/pathway	Ref.
NE	β2-AR	Breast cancer	MCF-7, MDA-453, and MDA-231 cell lines, subcutaneous injection of 4 T1 cells in BALB/c mice	β2-AR expression is elevated in MDA-453, decreased in MCF-7 and intermediate in MDA-231 cells. Administration of β-AR agonist, isoproterenol upregulates Jagged 1 expression and enhances tumor microvessel density via NE-induced β2-AR/PKA/mTOR pathway in vivo.	[15]
		Colorectal cancer	HT-29 and CT26 cells in vitro and subcutaneous injection of HT-29 cells in nude miceand CT26 cells in BALB/c mice	Activation of β2-AR by NE enhances expression of VEGF, IL-8 and IL-6 in vitro and in vivo → stimulation of tumor angiogenesis via β-AR -cAMP-PKA signaling pathway.	[16]
		Melanoma	B16-F1 cells in vitro and subcutaneous injection in the flanks of C57BL/6 mice		[17]
		Lung adenocarcinoma	A549 cells in vitro		
DA	DR1 & DR2	Ovarian cancer	SKOV3p 1, HeyA8 cells in vitro and intraperitoneal injection of these cells in a chronic stress C57BL/6 mouse model	Activation of DR2 mediates inhibitory effect of DA on tumor angiogenesis cAMP-PKA signaling pathway.	[18, 19]
		Gastric cancer	Human gastric cancer tissues, subcutaneous injection of Hs746T cells in nude mice, MNNG-induced gastric cancer in rats	DA suppresses gastric cancer growth by inhibition of VEGF-stimulated angiogenesis. In both human gastric cancer and MNNG-induced animal model DA is depleted. Suppression of VEGFR-2 phosphorylation in endothelial cell → inhibition of angiogenesis.	[20]
		Lung cancer	Orthotopic injection of LLC1 cells in C57BL/6 mice and A549 cells in SCID mice	Administration of DR2 agonists inhibits in vivo lung tumor progression via suppressing angiogenesis and reducing myeloid-derived suppressor cells infiltration.	[21]
GABA	GABA	Cholangiocarcinoma	H-69, Mz-ChA-1, HuH28, and TFK-1 cells, subcutaneous injection of Mz-ChA-1 cells in BALB/c mice	$GABA_A$, $GABA_B$, and $GABA_C$ receptors were expressed by cells in vitro which inhibit cell growth and proliferation via IP3 /cAMP, PKA phosphorylation, and ERK1/2 dephosphorylation. GABA ↓ tumor size and VEGF-A/C expression in vivo.	[22]
	$GABA_A$	Ovarian cancer	OVCAR-3 cells in vitro	↑ Level of GABARBP inhibited VEGF expression and ↓ HIF-1α protein via PI3K-mTOR-4E-BP1 signaling pathway in vitro.	[23]
5-HT	5-HT receptor	Colon cancer	Subcutaneous injection of MC-38 cells in *Tph1*$^{-/-}$ mice	5-HT regulates angiogenesis by reducing MMP12 expression in TAMs, thus affecting the production of circulating angiostatin.	[24]
Glu	mGluR1 on endothelial cells	Breast cancer	4 T1 cells injected into the mammary fat pads of BALB/c mice	↓mGluR1 expression results in ↓ angiogenesis in vivo.	[25]
	GRM1	Melanoma	UACC903-G2, UACC903-G4, C8161-G21, C81–61-G6, and C81–61-G7 cells, subcutaneous injection of these cells into each flank of nude mice	In vitro ↑ expression of GRM1 →↑ expression of IL-8 and VEGF via the AKT-mTOR-HIF1 signaling pathway activation. In vivo ↑ expression of GRM1 → larger melanoma tumors.	[26]
ACh	α7-nAChRs	Lung cancer	Human NSCLC A549 and H157 cell lines	Nicotine increases HIF-1 & VEGF expression. Nicotine mediates tumor angiogenesis through PI3K/Akt and ERK1/2 signalling pathway.	[27]
		Colon cancer	Subcutaneous injection of HT-29 cells in BALB/c mice	Administration of nicotine ↑ VEGF expression →↑ microvessel densities and angiogenesis via stimulation of β2-AR.	[28]
	mAChR	Breast cancer			[29]

Table 1 (continued)

Neurotransmitters	Receptor	Type of cancer	Model	Mechanism/pathway	Ref.
			Intradermal injection of MCF-7 and MCF-10A cells in nude mice, intradermal injection of LMM3 cells in BALB/c mice	mAChR activation promotes VEGF-A production and neovascularisation in breast cancer models.	
		Mammary adenocarcinoma	Subcutaneous injection of LMM3 cells in BALB/c mice	↑ Expression of VEGF by activation of M1 and M2 mAChRs via arginine metabolic pathway.	[30]
NPY	Y5R	Breast cancer	4 T1 cell line	Activation of NPY ↑ the expression and secretion of VEGF → angiogenesis.	[31, 32]
	Y2R	Melanoma	Subcutaneous injection of B16F10 cells into C57BL/6 mice	Blockade of the Y2R inhibited tumor growth by ↓ tumor angiogenesis.	[33]
		Neuroblastomas	Human tissue	Y2R expression is observed in both tumor and endothelial cells.	[34]
NO		Breast cancer	MDAMB-231cell and human invasive breast cancer tissues	NO induces the expression of VEGF-C in both breast cancer cell line and human tissues.	[35]
		Ovarian cancer	Cystic fluid samples and human tissues	The expression of iNOS correlates with the degree of tumor differentiation; level of intracystic NO metabolite correlates with tumor stage.	[36]
		Gastric cancer	Human tissues (all stages)	NOS III protein is ↑ in both primary gastric tumors and lymph node metastases.	[37]

α7nAChR, α7 nicotinic acetylcholine receptor; ACh, acetylcholine; β2-AR, β_2-adrenergic receptor; cAMP, cyclic adenosine monophosphate; AKT, serine/threonine kinase or protein kinase B; DA, dopamine; DR1 & DR2, dopamine receptor 1 & 2; $ERK_{1/2}$, extracellular signal-regulated kinase; GABA, gamma-aminobutyric acid; GABARBP, $GABA_A$ receptor-binding protein; $GABA_{A,B\&C}$, gamma-aminobutyric acid receptor $_{A,B\&C}$; Glu, glutamate; GRM1, glutamate receptor metabotropic 1; HIF-1α, hypoxia inducible factor-1alpha; 5-HT, 5-hydroxytryptamine (serotonin); iNOS, inducible nitric oxide synthase; IL-6, interleukin 6; IL-8, interleukin 8; mGluR1, metabotropic glutamate receptor 1; mAChRs, muscarinic acetylcholine receptors; M1 & M2, muscarinic 1 & 2 receptors; MMP12, matrix metallopeptidase 12; MNNG, N-methyl N′-nitro-N-nitrosoguanidine; mTOR, mammalian/mechanistic target of rapamycin; NE, norepinephrine; NO, nitric oxide; NOS, nitric oxide synthase; NPY, neuropeptide Y; NSCLC, non-small cell lung carcinoma; PI3K, phosphoinositide 3-kinase; 4E–BP1, phosphorylated 4E binding protein 1; PKA, protein kinase A; TAMs, tumor-infiltrating macrophages; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; Y2R & Y5R, neuropeptide Y receptor 2 & 5

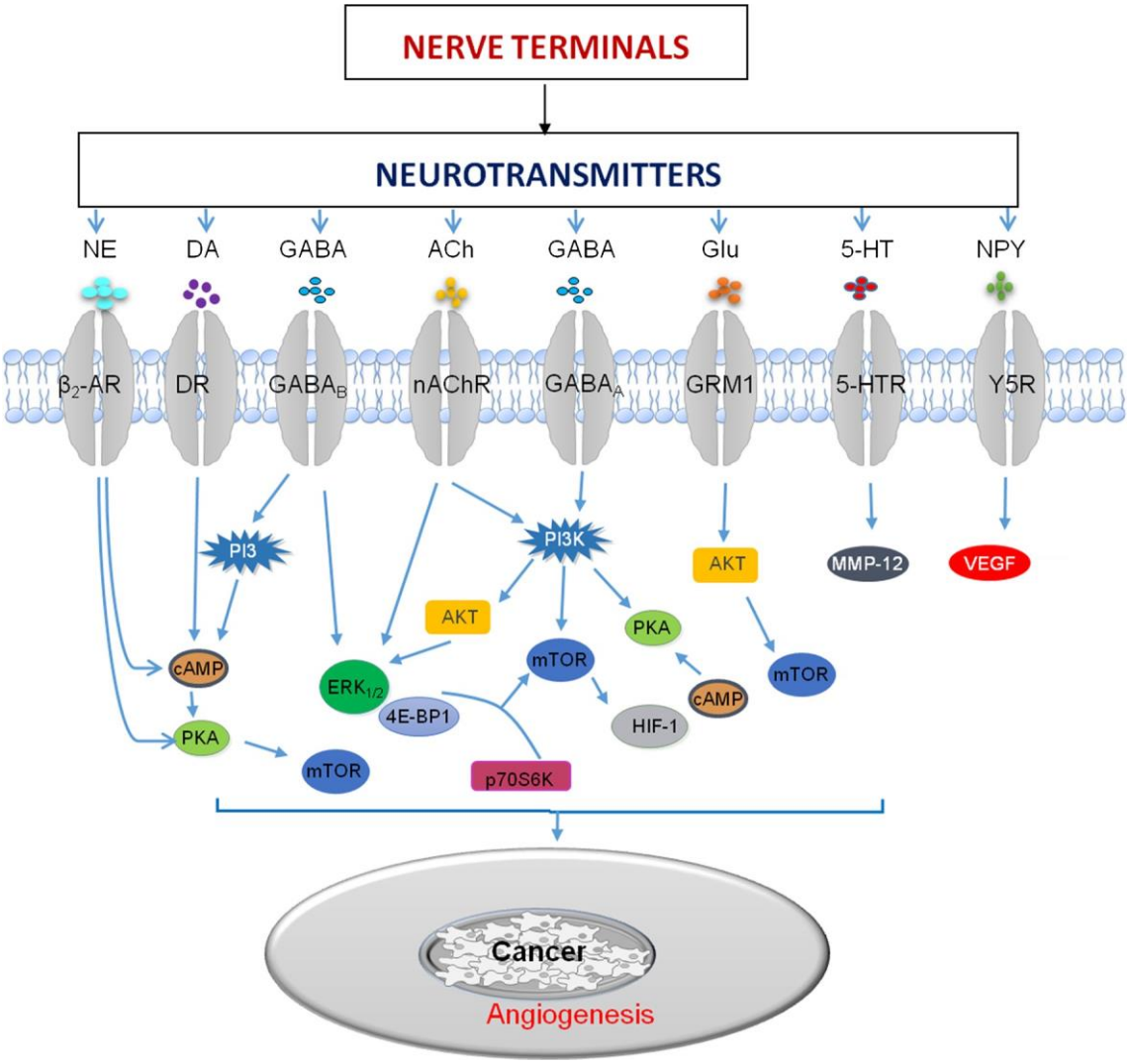

Fig. 1 Neurotransmitter signalling pathways in cancer angiogenesis. Neuro-cancer communication is through the release of neurotransmitters activating different signalling kinases which promote cancer progression via angiogenesis. ACh, acetylcholine; β2-AR, β_2-adrenergic receptor; cAMP, cyclic adenosine monophosphate; AKT, serine/threonine kinase or protein kinase B; DA, dopamine; DR, dopamine receptor; $ERK_{1/2}$, extracellular signal-regulated kinase; GABA, gamma-aminobutyric acid; $GABA_{A\&B}$, gamma-aminobutyric acid $receptor_{A\&B}$; Glu, glutamate; GRM1, glutamate receptor metabotropic 1; HIF-1, hypoxia inducible factor 1; 5-HT, 5-hydroxytryptamine (serotonin); 5-HTR, 5-hydroxytryptamine receptor (serotonin); MMP12, matrix metallopeptidase 12; mTOR, mammalian/mechanistic target of rapamycin; nAChR, nicotinic acetylcholine receptor; NE, norepinephrine; NPY, neuropeptide Y; PI3, phosphoinositide 3; PI3K, phosphoinositide 3-kinase; 4E–BP1, phosphorylated 4E binding protein 1; PKA, protein kinase A; p70S6K, serine/threonine kinase; VEGF, vascular endothelial growth factor; Y5R, neuropeptide receptor

Catecholamines are a group of neurotransmitters that are synthesized from amino acid tyrosine. These neurotransmitters are intricately involved in the normal physiological response of fight or flight response during stress [38, 39]. Epinephrine and norepinephrine released during chronic stress play an important role in tumorigenesis via regulation of angiogenesis through β-adrenergic signaling. The β-adrenergic signaling pathway is involved in regulation of cancer initiating factors such as apoptosis, DNA damage repair, inflammation, cellular immune response, angiogenesis and epithelial-mesenchymal transition. Numerous in vitro and animal studies have demonstrated that epinephrine and norepinephrine acting on their receptors expressed on tumor cells, stimulate angiogenesis via increased VEGF synthesis [16, 38–41] through the cAMP-PKA signaling pathway [40]. In fact, activation of the β-adrenergic signaling pathway in primary mammary tumors has been shown to elevate tumor-associated macrophages (TAMs) expressing *vegf* gene which enhances angiogenesis [42]. Moreover, in some breast cancer cell lines, direct activation of β-adrenergic signaling can amplify expression of VEGF and cytokines, IL-6, and IL-8 that stimulate tumor angiogenesis [43]. Jagged 1 is essential factor mediating Notch signaling which regulates tumor angiogenesis through β2-AR-PKA-mTOR pathway.

Upregulation of Jagged 1 in breast cancer patients correlates with poor prognosis [44, 45]. Knockdown of Jagged 1 by siRNA in MDA-231 breast cancer cells inhibits Notch signaling in endothelial cells and impairs tumor angiogenesis induced by norepinephrine [15].

In contrary, dopamine inhibits angiogenesis by downregulation of VEGFR-2-mediated signaling pathway in both tumor endothelial and endothelial progenitor cells through D_2 dopamine receptors (DR2) [38, 39, 46, 47]. Furthermore, in mouse models of breast cancer induced by MCF-7 cell line and colon cancer induced by HT29 cell line, dopamine administration in combination with anticancer drugs (eg. doxorubicin and 5-fluorouracil) impairs tumor growth and improves survival outcome [48]. However, dopamine effect was found to have no direct impact on tumor growth and survival but by inhibiting tumor endothelial cell proliferation and migration via the suppression of VEGFR-2 and mitogen-activated protein kinase as demonstrated in vitro [48]. In tissues from gastric cancer patients and in rats with chemically-induced as well as mice with Hs746T cell-induced gastric cancer, administration of dopamine decelerates tumor growth by suppressing angiogenesis via inhibition of VEGFR-2 phosphorylation in endothelial cells [20]. This concurs with results obtained in ovarian cancer mouse models induced by systemic injection of SKOV3ip1 and HeyA8 cells in which exogenous administration of dopamine inhibits angiogenesis by a stimulation of DR2, however stimulation of DR1 stabilizes tumor blood vessels via cAMP-PKA signaling pathway [18].

Acetylcholine and Nicotine Nicotinic acetylcholine receptors (nAChRs) can have either stimulatory or inhibitory effect on the production and release of angiogenic factors [49]. Indeed, the expression of VEGF, TGF-β, FGF and PDGF in endothelial cells is increased by nicotine [50–53]. Nicotine-mediated angiogenesis via activation of α7 and α9-nAChRs is cell-type specific, e.g. in lung cancer cells angiogenesis is promoted via activation of α7-nAChRs [53, 54], whereas in breast tumors overexpression of α9-nAChRs [55] stimulates release of pro-angiogenic factors [56]. In colon tumor tissues from HT-29 cell-bearing BALB/c mice, VEGF expression is elevated by nicotine which correlates with enhanced microvessel density [28]. The molecular pathways of nicotine-induced angiogenesis have been extensively reviewed [57]. The role of muscarinic acetylcholine receptors (mAChRs) in tumor angiogenesis is not well understood, however administration of autoantibodies against mAChRs in mouse models of breast cancer (Table 1) mediates tumor angiogenesis via activation of mAChRs through release of VEGF-A [29]. In addition, in BALB/c mice bearing LMM3 mammary adenocarcinoma cells, administration of muscarinic agonist, carbachol, in the presence or absence of various muscarinic antagonists shows an increase in VEGF expression [30, 58]. Furthermore, tumor macrophages stimulate angiogenesis via activation of M1 and M2 mAChRs which trigger arginine metabolic pathway [30].

Y-Aminobutyric Acid (GABA), Neuropeptide Y (NPY), Nitric Oxide (NO) and Serotonin have varying effects on angiogenesis and tumor progression. In a mouse model of cholangiocarcinoma, GABA inhibits VEGF-A/C, decreases cell proliferation and tumor mass [22]. NPY enhances the expression of VEGF and its secretion promoting angiogenesis and breast cancer progression [31]. The suggested mechanism by which NPY induces angiogenesis is by its influence on endothelial cells dependent on endothelial nitric oxide synthase (eNOS) activation and partly on VEGF signaling pathway The release of NO results in endothelial activation inducing tumor cells lysis [59], although NO can also promote tumor growth and metastasis by enhancing angiogenesis [36, 59–65]. For instance, NO increases VEGF-C and nitrite/nitrate production in MDA-MB-231 breast cancer cells and high levels of nitrotyrosine correlate with increased VEGF-C, lymph node metastasis, reduced disease-free and overall survival in invasive breast carcinoma [35]. The expression of iNOS and VEGF in colorectal cancer correlates with enhanced intratumor micro-vessel density suggesting that NO can promote tumor angiogenesis [60]. In gastric cancer, overexpression of NOS III via abnormal activation of sequence-specific DNA-binding protein (Sp1) correlates with enhanced micro-vessel density and poor survival [37]. Serotonin has also been implicated in tumor angiogenesis. In C57BL/6 mice bearing MC-38-induced tumors, serotonin regulates angiogenesis by plummeting matrix metalloproteinase 12 (MMP12) expression (eg. [66]) in macrophages infiltrating the tumor, as well as reducing angiostatin (an endogenous inhibitor of angiogenesis) levels [24].

Glutamate is an excitatory neurotransmitter that regulates synaptic and cellular activity via binding to its receptors including metabotropic glutamate receptors (mGluRs). The expression of mGluRs has been implicated in tumor angiogenesis as noted in mouse models of melanoma and breast cancer [25, 26, 67]. As such, decreased activity of mGluR1 inhibits angiogenesis in an orthotopic breast cancer (4 T1) model suggesting that mGluR1 acts is a pro-angiogenic and pro-tumorigenic factor [25]. Likewise, in an experimental non-small cell lung cancer in A549-bearing nude mice, inhibition of mGlu1 receptor with BAY36–7620 led to suppression of angiogenesis via inhibiting AKT/HIF-1α/VEGF signaling pathway [68]. Similarly, high expression of glutamate receptor GRM1 in several human melanoma cell lines (Table 1) leads to increased expression of IL-8 and VEGF via activation of the AKT/mTOR/HIF1 signaling pathway [26].

Table 2 Other factors influencing tumor angiogenesis

Factors	Type of cancer	Model	Mechanism/pathway	Ref.
ANG	Breast cancer	Human tissues	The level of ANG correlates with clinical progression. ANG derived from tumors activates angiogenesis via suppression of miR-543-2p.	[69]
	Bladder cancer	Human tissues, T24, UROtsa and HeLa cells subcutaneously injected in athymic BALB/c (nu/nu) mice	↑ ANG expression correlates with high grade, and muscle-invasive tumors via ERK 1/2 and MMP2. Downregulation of ANG inhibits tumor angiogenesis via AKT/GSK3β/ mTOR pathways.	[70, 71]
TNF-α	Lung cancer	LLC1 cells subcutaneously injected in wild type, p75 knockout (KO) and double p55KO/p75KO mouse xenograft models	Tumor growth ↓ in both LLC and B16 p75KO mice. Decreased tumor growth correlates with ↓ VEGF expression and capillary density via TNFR2/p75.	[72]
	Melanoma	B16 cell subcutaneously injected in C57BL/6 mice. Wild type, p75 knockout (KO) and double p55KO/p75KO mouse tumor xenograft models		
TGF-β	Colon cancer	Human tissues, FETα/DNRII cell	TGF-β signaling is inversely correlates with the expression of VEGF-A in tissues. TGF-β ↓ VEGF-A expression via ubiquitination and deterioration in a PKA- and Smad3-dependent and Smad2-independent pathways in vitro.	[73]
BDNF	Chondrosarcoma	JJ012 cell line, JJ012 cells subcutaneously injected in CB17-SCID mice	The expression of BDNF and VEGF correlates with tumor grade. BDNF knockdown ↓ angiogenesis and tumor growth in mouse model. BDNF ↑ expression of VEGF and stimulates angiogenesis via the TrkB receptor, PKCα, PLCγ and HIF-1α signaling pathways.	[74]
FGF	Mammary cancer	Mouse 66c14 mammary carcinoma and inguinal mammary fat pad injection in BALB/c mice	In tumor cells suppression of FGFR signaling inhibits expression of VEGF-C and induces VEGFR-3, netrin-1, prox1 and integrin α9 expression.	[75]
	Glioma	Rat C6 glioma cancer cells injected subcutaneously into rats		
EGFR	HNSCC	Human tissues, CAL27 cells subcutaneously injected in nude mice	In human tissues, ↑ EGFR correlates with ↑ HIF-1α and microvessel density. EGFR inhibitors ↓ the regulation of HIF-1α & Notch1 →↓ angiogenesis and tumor size.	[76]
NGF	Breast cancer	MDA-MB-231 cells subcutaneously injected into SCID mice	NGF ↑ the release of VEGF in breast cancer cells and mediates angiogenic effect via the activation of PI3K-Akt, ERK, MMP2 and NO synthase pathways.	[77]
HGF	ESCC	Serum samples, human tissues, HKESC-1, HKESC-2 and SLMT cells	In tissues, ↑ level of HGF correlates with tumor metastasis and poorer survival. In serum samples, ↑ HGF level correlated with expression of VEGF and IL-8. HGF stimulates cells to express VEGF and IL-8 in vitro via extracellular signal-regulated kinase signaling pathways.	[78]
	Prostate cancer	Castration-resistant prostate cancer blood samples and PC3 cell line	HGF levels ↑ in both blood samples and cell line.	[79]

AKT, serine/threonine kinase or protein kinase B; ANG, angiogenin; BDNF, brain-derived neurotrophic factor; EGFR, epidermal growth factor receptor; ESCC, esophageal squamous cell carcinoma; $ERK_{1/2}$, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GSK3β, glycogen synthase kinase 3β; HNSCC, head and neck squamous cell carcinoma; HGF, hepatocyte growth factor; HIF-1α, hypoxia inducible factor 1α; IL-8, interleukin-8; MMP2, matrix metalloprotease 2; mTOR, mammalian/mechanistic target of rapamycin; NGF, nerve growth factor; NO, nitric oxide; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKCα, protein kinase C alpha; PLCγ, phospholipase Cγ; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; TNFR2/p75, tumor necrosis factor receptor 2/neurotrophin receptor; TrkB, tropomyosin related kinase B; VEGF, vascular endothelial growth factor

Hence, these studies clearly demonstrate involvement of neurotransmitters in tumor angiogenesis; however, most of the studies have been performed mainly in animal models and cell lines. Understanding their relevance to human pathology may aid in the development of better anti-angiogenic therapies.

Other Factors Influencing Tumor Angiogenesis

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), angiogenin (ANG), FGF, TNF-α, TGF-β, hepatocyte growth factor (HGF) and epidermal growth factor receptor (EGF) are important signaling molecules promoting angiogenesis (Table 2, Fig. 2). NGF is a neurotrophic factor that is upregulated in tumor microenvironment of various cancers including breast cancer [77]. NGF, secreted by MDA-MB-231 breast cancer cells, stimulates angiogenesis in vivo after injection of these cells subcutaneously to immunodeficient mice and enhances endothelial cell proliferation, invasion, migration and tubule formation in vitro [77]. Furthermore, NGF enhances secretion of VEGF by breast cancer cells; in vivo administration of anti-VEGF antibody inhibits its angiogenic capacity [77]. In human glioma microvascular endothelial cells, NGF mediates tumor angiogenesis by interaction with α9β1 integrin [80–83]. Another neurotrophic factor, BDNF has been shown to play a role in tumor angiogenesis. For instance, in chondrosarcoma patients, BDNF and VEGF protein expression is significantly higher which is correlated with

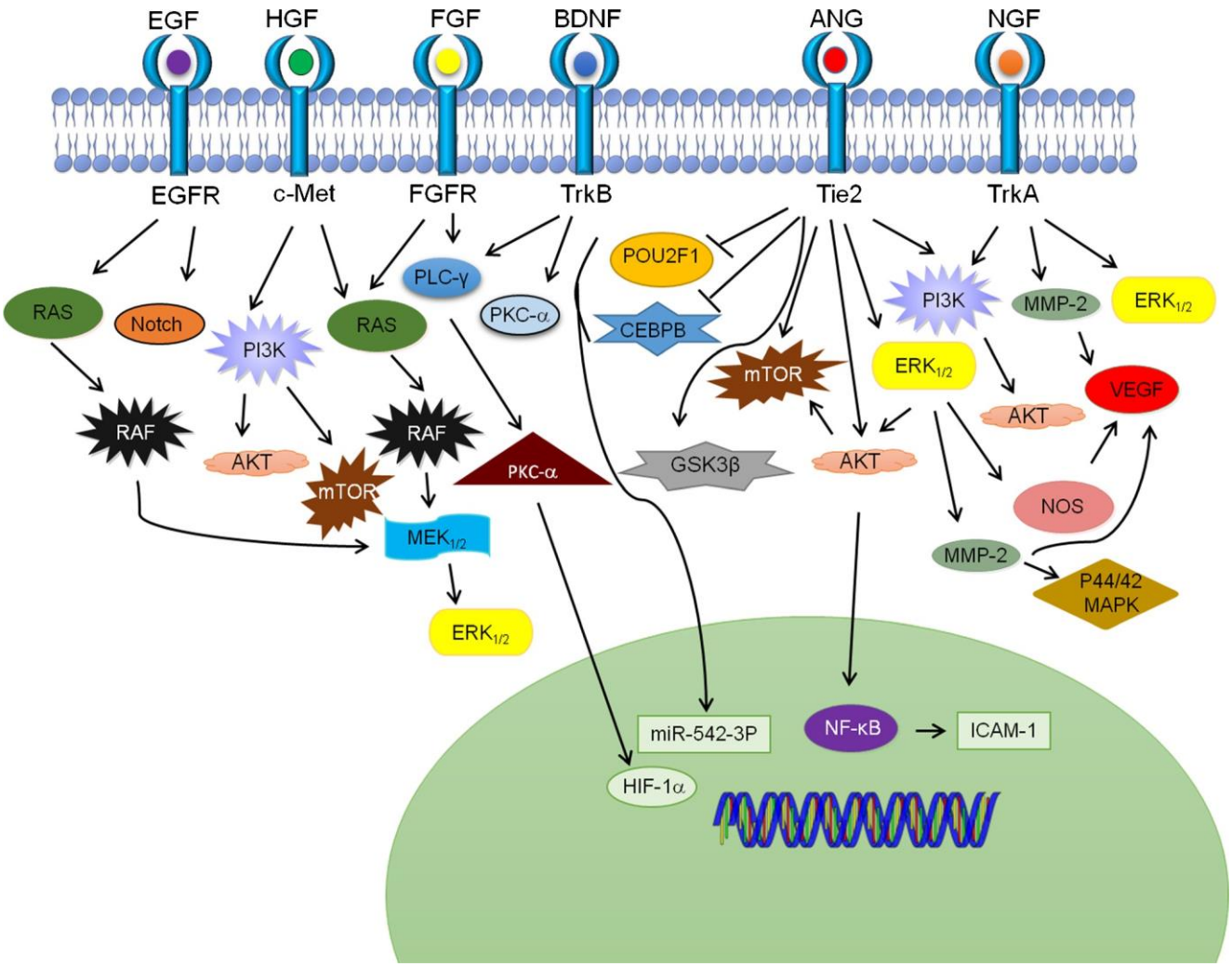

Fig. 2 Growth factors intracellular signalling pathways in cancer angiogenesis. The binding of growth factors to their respective receptors (eg, EGF to EGFR) activates multiple kinase pathways which are involved in cancer angiogenesis. AKT, serine/threonine kinase or protein kinase B; ANG, angiogenin; BDNF, brain-derived neurotrophic factor; CEBPB, CCAAT/enhancer-binding protein beta; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; $ERK_{1/2}$, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GSK3β, glycogen synthase kinase 3 beta; HGF, hepatocyte growth factor; c-Met, hepatocyte growth factor receptor; HIF-1α, hypoxia inducible factor 1 alpha; ICAM-1, intercellular adhesion molecule-1; MAPK, mitogen activated protein kinase; $MEK_{1/2}$, MAPK/ERK kinase; MMP2, matrix metallopeptidase 2; mTOR, mammalian/mechanistic target of rapamycin; NGF, nerve growth factor; NF-kB, nuclear factor-kappa B; NOS, nitric oxide synthase; PI3K, phosphoinositide 3-kinase; PKC-α, protein kinase C alpha; PLC-γ, phospholipase C-gamma; POU2F1, POU domain class 2 transcription factor 1; RAF, mitogen activated protein kinase; RAS, mitogen activated protein kinase; Tie2, angiopoietin receptor 2; TrkA, tropomyosin related kinase A; TrkB, tropomyosin related kinase B; VEGF, vascular endothelial growth factor

tumor stage [74]. Furthermore, BDNF knockdown decreases the expression of VEGF and abolishes angiogenesis in in vitro studies and animal models of chondrosarcoma [74].

In addition to neurotrophic factors, angiogenic factor ANG is upregulated in number of cancers [84–86] and is associated with worse clinical prognosis in urothelial carcinoma patients [87]. ANG regulates tumor angiogenesis via activation of endothelial and smooth muscle cells triggering various molecular pathways involved in the initiation of angiogenesis (Fig. 2) [69–71, 88]. Elevated expression of ANG associates with high grade and muscle-invasive human bladder tumors involving increase p-ERK1/2 and MMP2 expression [70]. Similarly, downregulation of ANG inhibits tumor angiogenesis via AKT/GSK3β/ mTOR pathways [71]. FGF is involved in angiogenesis by suppressing VEGF-C expression and stimulating expression of pro-lymphangiogenic factors including integrin α9, VEGFR-3, prox1 and netrin-1 [75]. In fact, blocking of FGF2 with anti-FGF2 monoclonal antibody results in impaired angiogenesis of B16-F10 cell induced melanoma in mice [89]. In addition, TNF-α binding to TNFR1/p55 and TNFR2/p57 receptors has been implicated in the secretion of cytokines and pro-angiogenic factors [72]. For example, blocking p75 by short-hairpin RNA in cultured Lewis lung carcinoma cells results in decreased TNF-mediated expression of VEGF, placental growth factor and HGF, suggesting that p75 is essential factor for tumor angiogenesis [72]. Similarly, blocking TNF-α inhibits angiogenesis in metastatic oral squamous cell carcinoma cells (sh-IFIT2 meta cell) in NOD/SCID mice [90]. TGF-β negatively regulates VEGF-A expression via a PKA- and Smad2-independent and Smad3-dependent pathways as demonstrated in FETα/DNRII colon cancer cell lines [73]. HGF is an angiogenic factor secreted predominantly by fibroblasts; it stimulates invasiveness of cancer cells via c-Met receptor tyrosine kinase activation [79, 91, 92]. In fact, high HGF serum levels is correlated with VEGF and IL-8 expression, advanced tumor stage and poor survival of esophageal squamous cell carcinoma (ESCC) patients [78]. High expression of another pro-angiogenic factor, EGFR correlates with increased microvessel density resulting in enhanced tumor angiogenesis via the HIF-1α and Notch1 pathways in tissues from head and neck squamous cell carcinoma patients [76]. Neuropilin is a transmembrane glycoprotein which serves as a receptors or co-receptor for multiple ligands including VEGF, HGF, EGF and FGF which are involved in tumor angiogenesis [93, 94]. In gastric cancer, high expression of neuropilin correlates with advanced clinical stages (III and IV) [95]. Depletion of neuropilin-1 inhibits the activation of EGF/EGFR, VEGF/VEGFR2 and HGF/c-Met angiogenic pathways activated by recombinant human VEGF-165, HGF and EGF proteins [91, 95]. Thus, the role of neurotrophic factors such as NGF, BDNF and their molecular pathways should be considered in the development of anti-angiogenic therapies.

Concluding Remarks

Despite the increasing interest to the role of the nervous system in cancer development and progression, the knowledge in this area is scarce. Most neurotransmitters released by nerve fibers promote tumor angiogenesis, however, some neurotransmitters induce anti-cancer effects. Whether these effects are cancer type or receptor dependent need further elucidation.

To date, most studies investigating the role of the nervous system in modulation of tumor angiogenesis have been performed in cell lines and animal models. Limited studies are available from cancer patients and at different stages of disease. Understanding molecular mechanisms by which nervous system modulates tumor angiogenesis may open new avenues for understanding mechanisms of tumor angiogenesis, identification of new biomarkers for cancer diagnosis and prognosis, and, defining novel targets for therapeutic interventions.

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Compliance with Ethical Standards

Conflict of Interest The authors confirm that this article content has not conflict of interest.

References

1. Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 6(4):273–286
2. Zhao Y, Adjei AA (2015) Targeting angiogenesis in cancer therapy: moving beyond vascular endothelial growth factor. Oncologist 20(6):660–673
3. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M (2006) Angiogenesis in cancer. Vasc Health Risk Manag 2(3):213–219
4. McMahon G (2000) VEGF receptor signaling in tumor angiogenesis. Oncologist 5(Supplement 1):3–10
5. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, Jain RK (2011) Normalization of the vasculature for treatment of cancer and other diseases. Physiol Rev 91(3):1071–1121
6. Zibara K, Awada Z, Dib L, El-Saghir J, Al-Ghadban S, Ibrik A, El-Zein N, El-Sabban M (2015) Anti-angiogenesis therapy and gap junction inhibition reduce MDA-MB-231 breast cancer cell invasion and metastasis in vitro and in vivo. Sci Rep 5:12598
7. Rusckowski M, Wang Y, Blankenberg FG, Levashova Z, Backer MV, Backer JM (2016) Targeted scVEGF/177Lu radiopharmaceutical inhibits growth of metastases and can be effectively combined with chemotherapy. EJNMMI Res 6(1):1–9
8. Vasudev NS, Reynolds AR (2014) Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions. Angiogenesis 17(3):471–494
9. Quail DF, Joyce JA (2013) Microenvironmental regulation of tumor progression and metastasis. Nat Med 19(11):1423–1437
10. Gupta GP, Massagué J (2006) Cancer Metastasis: building a framework. Cell 127(4):679–695

11. Place AE, Jin Huh S, Polyak K (2011) The microenvironment in breast cancer progression: biology and implications for treatment. Breast Cancer Res 13(6):227
12. Weis SM, Cheresh DA (2011) Tumor angiogenesis: molecular pathways and therapeutic targets. Nat Med 17(11):1359–1370
13. Jobling P, Pundavela J, Oliveira SM, Roselli S, Walker MM, Hondermarck H (2015) Nerve–cancer cell cross-talk: a novel promoter of tumor progression. Cancer Res 75(9):1777–1781
14. Ondicova K, Mravec B (2010) Role of nervous system in cancer aetiopathogenesis. Lancet Oncol 11(6):596–601
15. Chen H, Liu D, Yang Z, Sun L, Deng Q, Yang S, Qian L, Guo L, Yu M, Hu M, Shi M, Guo N (2014) Adrenergic signaling promotes angiogenesis through endothelial cell-tumor cell crosstalk. Endocr Relat Cancer 21(5):783–795
16. Liu J, Deng G-H, Zhang J, Wang Y, Xia X-Y, Luo X-M, Deng Y-T, He S-S, Mao Y-Y, Peng X-C (2015) The effect of chronic stress on anti-angiogenesis of sunitinib in colorectal cancer models. Psychoneuroendocrinology 52:130–142
17. Deng G-H, Liu J, Zhang J, Wang Y, Peng X-C, Wei Y-Q, Jiang Y (2014) Exogenous norepinephrine attenuates the efficacy of sunitinib in a mouse cancer model. J Exp Clin Cancer Res 33(1):1–12
18. Moreno-Smith M, Lee SJ, Lu C, Nagaraja AS, He G, Rupaimoole R, Han HD, Jennings NB, Roh J-W, Nishimura M (2013) Biologic effects of dopamine on tumor vasculature in ovarian carcinoma. Neoplasia 15(5):502–IN515
19. Moreno-Smith M, Lu C, Shahzad MM, Pena GNA, Allen JK, Stone RL, Mangala LS, Han HD, Kim HS, Farley D (2011) Dopamine blocks stress-mediated ovarian carcinoma growth. Clin Cancer Res 17(11):3649–3659
20. Chakroborty D, Sarkar C, Mitra RB, Banerjee S, Dasgupta PS, Basu S (2004) Depleted dopamine in gastric cancer tissues: dopamine treatment retards growth of gastric cancer by inhibiting angiogenesis. Clin Cancer Res 10(13):4349–4356
21. Hoeppner LH, Wang Y, Sharma A, Javeed N, Van Keulen VP, Wang E, Yang P, Roden AC, Peikert T, Molina JR (2015) Dopamine D2 receptor agonists inhibit lung cancer progression by reducing angiogenesis and tumor infiltrating myeloid derived suppressor cells. Mol Oncol 9(1):270–281
22. Fava G, Marucci L, Glaser S, Francis H, De Morrow S, Benedetti A, Alvaro D, Venter J, Meininger C, Patel T (2005) γ-aminobutyric acid inhibits cholangiocarcinoma growth by cyclic AMP–dependent regulation of the protein kinase a/extracellular signal-regulated kinase 1/2 pathway. Cancer Res 65(24):11437–11446
23. Park SH, Kim B-R, Lee JH, Park ST, Lee S-H, Dong SM, Rho SB (2014) GABARBP down-regulates HIF-1α expression through the VEGFR-2 and PI3K/mTOR/4E-BP1 pathways. Cell Signal 26(7): 1506–1513
24. Nocito A, Dahm F, Jochum W, Jang JH, Georgiev P, Bader M, Graf R, Clavien P-A (2008) Serotonin regulates macrophage-mediated angiogenesis in a mouse model of colon cancer allografts. Cancer Res 68(13):5152–5158
25. Speyer CL, Hachem AH, Assi A, DeVries JA, Gorski DH (2013) Metabotropic glutamate receptor-1 as a novel target for the anti-angiogenic treatment of breast cancer. Cancer Res 73(8 Supplement):3895
26. Wen Y, Li J, Koo J, Shin SS, Lin Y, Jeong BS, Mehnert JM, Chen S, Cohen-Sola KA, Goydos JS (2014) Activation of the glutamate receptor GRM1 enhances angiogenic signaling to drive melanoma progression. Cancer Res 74(9):2499–2509
27. Zhang Q, Tang X, Zhang Z-F, Velikina R, Shi S, Le AD (2007) Nicotine induces hypoxia-inducible factor-1α expression in human lung cancer cells via nicotinic acetylcholine receptor–mediated signaling pathways. Clin Cancer Res 13(16):4686–4694
28. Wong HPS, Yu L, Lam EKY, Tai EKK, Wu WKK, Cho C-H (2007) Nicotine promotes colon tumor growth and angiogenesis through β-adrenergic activation. Toxicol Sci 97(2):279–287
29. Lombardi Mí G, Negroni M, Pelegrina LT, Castro Mí E, Fiszman GL, Azar Mí E, Morgado CC, Sales Mí E (2013) Autoantibodies against muscarinic receptors in breast cancer: their role in tumor angiogenesis. PLoS One 8(2):e57572
30. de la Torre E, Davel L, Jasnis MA, Gotoh T, de Lustig ES, Sales ME (2005) Muscarinic receptors participation in angiogenic response induced by macrophages from mammary adenocarcinoma-bearing mice. Breast Cancer Res 7(3):R345–R352
31. Medeiros PJ, Jackson DN (2013) Neuropeptide Y Y5-receptor activation on breast cancer cells acts as a paracrine system that stimulates VEGF expression and secretion to promote angiogenesis. Peptides 48:106–113
32. Medeiros PJ, Al-Khazraji BK, Novielli NM, Postovit LM, Chambers AF, Jackson DN, Neuropeptide Y (2012) Stimulates proliferation and migration in the 4T1 breast cancer cell line. Int J Cancer J Int du Cancer 131(2):276–286
33. Alasvand M, Rashidi B, Javanmard SH, Akhavan MM, Khazaei M (2015) Effect of blocking of neuropeptide Y Y2 receptor on tumor angiogenesis and progression in normal and diet-induced obese C57BL/6 mice. Glob J Health Sci 7(7):46883
34. Lu C, Everhart L, Tilan J, Kuo L, Sun CJ, Munivenkatappa RB, Jönsson-Rylander A-C, Sun J, Kuan-Celarier A, Li L (2010) Neuropeptide Y and its Y2 receptor: potential targets in neuroblastoma therapy. Oncogene 29(41):5630–5642
35. Nakamura Y, Yasuoka H, Tsujimoto M, Yoshidome K, Nakahara M, Nakao K, Nakamura M, Kakudo K (2006) Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis. Clin Cancer Res 12(4): 1201–1207
36. Nomelini RS, Ribeiro LCDA, Tavares-Murta BM, Adad SJ, Murta EFC (2009) Production of nitric oxide and expression of inducible nitric oxide synthase in ovarian cystic tumors. Mediators Inflamm 2008:186584
37. Wang L, Shi GG, Yao JC, Gong W, Wei D, Wu TT, Ajani JA, Huang S, Xie K (2005) Expression of endothelial nitric oxide synthase correlates with the angiogenic phenotype of and predicts poor prognosis in human gastric cancer. Gastric Cancer 8(1):18–28
38. Sarkar C, Chakroborty D, Basu S (2013) Neurotransmitters as regulators of tumor angiogenesis and immunity: the role of catecholamines. J Neuroimmune Pharmacol Off J Soc NeuroImmune Pharmacol 8(1):7–14
39. Chakroborty D, Sarkar C, Basu B, Dasgupta PS, Basu S (2009) Catecholamines regulate tumor angiogenesis. Cancer Res 69(9): 3727–3730
40. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, Jennings NB, Armaiz-Pena G, Bankson JA, Ravoori M (2006) Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nat Med 12(8):939–944
41. Xie H, Li C, He Y, Griffin R, Ye Q, Li L (2015) Chronic stress promotes oral cancer growth and angiogenesis with increased circulating catecholamine and glucocorticoid levels in a mouse model. Oral Oncol 51(11):991–997
42. Sloan EK, Priceman SJ, Cox BF, Yu S, Pimentel MA, Tangkanangnukul V, Arevalo JM, Morizono K, Karanikolas BD, Wu L (2010) The sympathetic nervous system induces a metastatic switch in primary breast cancer. Cancer Res 70(18):7042–7052
43. Madden KS, Szpunar MJ, Brown EB (2011) Beta-adrenergic receptors (beta-AR) regulate VEGF and IL-6 production by divergent pathways in high beta-AR-expressing breast cancer cell lines. Breast Cancer Res Treat 130(3):747–758
44. Reedijk M, Odorcic S, Chang L, Zhang H, Miller N, McCready DR, Lockwood G, Egan SE (2005) High-level coexpression of Jag1 and Notch1 is observed in human breast cancer and is associated with poor overall survival. Cancer Res 65(18):8530–8537

45. Dickson BC, Mulligan AM, Zhang H, Lockwood G, O'Malley FP, Egan SE, Reedijk M (2007) High-level JAG1 mRNA and protein predict poor outcome in breast cancer. Mod Pathol 20(6):685–693
46. Basu S, Nagy JA, Pal S, Vasile E, Eckelhoefer IA, Bliss VS, Manseau EJ, Dasgupta PS, Dvorak HF, Mukhopadhyay D (2001) The neurotransmitter dopamine inhibits angiogenesis induced by vascular permeability factor/vascular endothelial growth factor. Nat Med 7(5):569–574
47. Tilan J, Kitlinska J (2010) Sympathetic neurotransmitters and tumor angiogenesis-link between stress and cancer progression. J Oncol 2010:539706
48. Sarkar C, Chakroborty D, Chowdhury UR, Dasgupta PS, Basu S (2008) Dopamine increases the efficacy of anticancer drugs in breast and colon cancer preclinical models. Clin Cancer Res 14(8):2502–2510
49. Schuller HM (2009) Is cancer triggered by altered signalling of nicotinic acetylcholine receptors? Nat Rev Cancer 9(3):195–205
50. Egleton RD, Brown KC, Dasgupta P (2009) Angiogenic activity of nicotinic acetylcholine receptors: implications in tobacco-related vascular diseases. Pharmacol Ther 121(2):205–223
51. Cucina A, Sapienza P, Corvino V, Borrelli V, Mariani V, Randone B, Santoro D'Angelo L, Cavallaro A (2000) Nicotine-induced smooth muscle cell proliferation is mediated through bFGF and TGF-beta 1. Surgery 127(3):316–322
52. Conklin BS, Zhao W, Zhong DS, Chen C (2002) Nicotine and cotinine up-regulate vascular endothelial growth factor expression in endothelial cells. Am J Pathol 160(2):413–418
53. Brown KC, Lau JK, Dom AM, Witte TR, Luo H, Crabtree CM, Shah YH, Shiflett BS, Marcelo AJ, Proper NA, Hardman WE, Egleton RD, Chen YC, Mangiarua EI, Dasgupta P (2012) MG624, an alpha7-nAChR antagonist, inhibits angiogenesis via the Egr-1/FGF2 pathway. Angiogenesis 15(1):99–114
54. Davis R, Rizwani W, Banerjee S, Kovacs M, Haura E, Coppola D, Chellappan S (2009) Nicotine promotes tumor growth and metastasis in mouse models of lung cancer. PLoS One 4(10):e7524
55. Lee CH, Huang CS, Chen CS, Tu SH, Wang YJ, Chang YJ, Tam KW, Wei PL, Cheng TC, Chu JS, Chen LC, Wu CH, Ho YS (2010) Overexpression and activation of the alpha9-nicotinic receptor during tumorigenesis in human breast epithelial cells. J Natl Cancer Inst 102(17):1322–1335
56. Wu C-H, Lee C-H, Ho Y-S (2011) Nicotinic acetylcholine receptor-based blockade: applications of molecular targets for cancer therapy. Clin Cancer Res 17(11):3533–3541
57. Dasgupta P, Chellappan SP (2006) Nicotine-mediated cell proliferation and angiogenesis: new twists to an old story. Cell Cycle 5(20): 2324–2328
58. Fiszman GL, Middonno MC, de la Torre E, Farina M, Español AJ, Sales ME (2007) Activation of muscarinic cholinergic receptors induces MCF-7 cells proliferation and angiogenesis by stimulating nitric oxide synthase activity. Cancer Biol Ther 6(7):1106–1113
59. Li L, Kilbourn RG, Adams J, Fidler IJ (1991) Role of nitric oxide in lysis of tumor cells by cytokine-activated endothelial cells. Cancer Res 51(10):2531–2535
60. Cianchi F, Cortesini C, Fantappiè O, Messerini L, Schiavone N, Vannacci A, Nistri S, Sardi I, Baroni G, Marzocca C, Perna F, Mazzanti R, Bechi P, Masini E (2003) Inducible nitric oxide synthase expression in human colorectal cancer: correlation with tumor angiogenesis. Am J Pathol 162(3):793–801
61. Fukumura D, Kashiwagi S, Jain RK (2006) The role of nitric oxide in tumour progression. Nat Rev Cancer 6(7):521–534
62. Ridnour LA, Thomas DD, Donzelli S, Espey MG, Roberts DD, Wink DA, Isenberg JS (2006) The biphasic nature of nitric oxide responses in tumor biology. Antioxid Redox Signal 8(7–8):1329–1337
63. Andrade SP, Hart IR, Piper PJ (1992) Inhibitors of nitric oxide synthase selectively reduce flow in tumour-associated neovasculature. Br J Pharmacol 107(4):1092–1095
64. Chu SC, Marks-Konczalik J, Wu H-P, Banks TC, Moss J (1998) Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: characterization of differences between human and mouse iNOS promoters. Biochem Biophys Res Commun 248(3):871–878
65. Vahora H, Khan MA, Alalami U, Hussain A (2016) The potential role of nitric oxide in halting cancer progression through chemoprevention. J Cancer Prev 21(1):1–12
66. Radisky ES, Radisky DC (2015) Matrix metalloproteinases as breast cancer drivers and therapeutic targets. Front Biosci (Landmark ed) 20:1144–1163
67. Wangari-Talbot J, Wall BA, Goydos JS, Chen S (2012) Functional effects of GRM1 suppression in human melanoma cells. Mol Cancer Res 10(11):1440–1450
68. Xia H, Zhao YN, Yu CH, Zhao YL, Liu Y (2016) Inhibition of metabotropic glutamate receptor 1 suppresses tumor growth and angiogenesis in experimental non-small cell lung cancer. Eur J Pharmacol 783:103–111
69. He T, Qi F, Jia L, Wang S, Wang C, Song N, Fu Y, Li L, Luo Y (2015) Tumor cell-secreted angiogenin induces angiogenic activity of endothelial cells by suppressing miR-542-3p. Cancer Lett 368(1):115–125
70. Miyake M, Goodison S, Lawton A, Gomes-Giacoia E, Rosser C (2015) Angiogenin promotes tumoral growth and angiogenesis by regulating matrix metallopeptidase-2 expression via the ERK1/2 pathway. Oncogene 34(7):890–901
71. Shu J, Huang M, Tian Q, Shui Q, Zhou Y, Chen J (2015) Downregulation of angiogenin inhibits the growth and induces apoptosis in human bladder cancer cells through regulating AKT/mTOR signaling pathway. J Mol Histol 46(2):157–171
72. Sasi SP, Yan X, Enderling H, Park D, Gilbert H-Y, Curry C, Coleman C, Hlatky L, Qin G, Kishore R (2012) Breaking the 'harmony'of TNF-α signaling for cancer treatment. Oncogene 31(37):4117–4127
73. Geng L, Chaudhuri A, Talmon G, Wisecarver JL, Wang J (2013) TGF-Beta suppresses VEGFA-mediated angiogenesis in colon cancer metastasis. PLoS One 8(3):e59918
74. Lin C-Y, Hung S-Y, Chen H-T, Tsou H-K, Fong Y-C, Wang S-W, Tang C-H (2014) Brain-derived neurotrophic factor increases vascular endothelial growth factor expression and enhances angiogenesis in human chondrosarcoma cells. Biochem Pharmacol 91(4): 522–533
75. Larrieu-Lahargue F, Welm AL, Bouchecareilh M, Alitalo K, Li DY, Bikfalvi A, Auguste P (2012) Blocking fibroblast growth factor receptor signaling inhibits tumor growth, lymphangiogenesis, and metastasis. PLoS One 7(6):e39540
76. Wang W-M, Zhao Z-L, Ma S-R, Yu G-T, Liu B, Zhang L, Zhang W-F, Kulkarni AB, Sun Z-J, Zhao Y-F (2015) Epidermal growth factor receptor inhibition reduces angiogenesis via hypoxia-inducible factor-1α and notch1 in head neck squamous cell carcinoma. PLoS One 10(2):e0119723
77. Romon R, Adriaenssens E, Lagadec C, Germain E, Hondermarck H, Le Bourhis X (2010) Nerve growth factor promotes breast cancer angiogenesis by activating multiple pathways. Breast Cancer 9: 11
78. Ren Y, Cao B, Law S, Xie Y, Lee PY, Cheung L, Chen Y, Huang X, Chan HM, Zhao P (2005) Hepatocyte growth factor promotes cancer cell migration and angiogenic factors expression: a prognostic marker of human esophageal squamous cell carcinomas. Clin Cancer Res 11(17):6190–6197
79. Sugie S, Mukai S, Yamasaki K, Kamibeppu T, Tsukino H, Kamoto T (2016) Plasma macrophage-stimulating protein and hepatocyte

growth factor levels are associated with prostate cancer progression. Hum Cell 29(1):22–29
80. Walsh EM, Kim R, Del Valle L, Weaver M, Sheffield J, Lazarovici P, Marcinkiewicz C (2012) Importance of interaction between nerve growth factor and α9β1 integrin in glial tumor angiogenesis. Neuro-Oncology 14(7):890–901
81. Staniszewska I, Zaveri S, Del Valle L, Oliva I, Rothman VL, Croul SE, Roberts DD, Mosher DF, Tuszynski GP, Marcinkiewicz C (2007) Interaction of α9β1 integrin with thrombospondin-1 promotes angiogenesis. Circ Res 100(9):1308–1316
82. Vlahakis NE, Young BA, Atakilit A, Hawkridge AE, Issaka RB, Boudreau N, Sheppard D (2007) Integrin α9β1 directly binds to vascular endothelial growth factor (VEGF)-a and contributes to VEGF-A-induced angiogenesis. J Biol Chem 282(20):15187–15196
83. Staniszewska I, Sariyer IK, Lecht S, Brown MC, Walsh EM, Tuszynski GP, Safak M, Lazarovici P, Marcinkiewicz C (2008) Integrin α9β1 is a receptor for nerve growth factor and other neurotrophins. J Cell Sci 121(4):504–513
84. Nilsson UW, Abrahamsson A, Dabrosin C (2010) Angiogenin regulation by estradiol in breast tissue: tamoxifen inhibits angiogenin nuclear translocation and antiangiogenin therapy reduces breast cancer growth in vivo. Clin Cancer Res 16(14):3659–3669
85. Katona TM, Neubauer BL, Iversen PW, Zhang S, Baldridge LA, Cheng L (2005) Elevated expression of angiogenin in prostate cancer and its precursors. Clin Cancer Res 11(23):8358–8363
86. Hisai H, Kato J, Kobune M, Murakami T, Miyanishi K, Takahashi M, Yoshizaki N, Takimoto R, Terui T, Niitsu Y (2003) Increased expression of angiogenin in hepatocellular carcinoma in correlation with tumor vascularity. Clin Cancer Res 9(13):4852–4859
87. Miyake H, Hara I, Yamanaka K, Gohji K, Arakawa S, Kamidono S (1999) Increased angiogenin expression in the tumor tissue and serum of urothelial carcinoma patients is related to disease progression and recurrence. Cancer 86(2):316–324
88. Gao X, Xu Z (2008) Mechanisms of action of angiogenin. Acta Biochim Biophys Sin 40(7):619–624
89. de Aguiar RB, Parise CB, Souza CRT, Braggion C, Quintilio W, Moro AM, Navarro Marques FL, Buchpiguel CA, Chammas R, de Moraes JZ (2016) Blocking FGF2 with a new specific monoclonal antibody impairs angiogenesis and experimental metastatic melanoma, suggesting a potential role in adjuvant settings. Cancer Lett 371(2):151–160
90. Lai KC, Liu CJ, Lin TJ, Mar AC, Wang HH, Chen CW, Hong ZX, Lee TC (2016) Blocking TNF-alpha inhibits angiogenesis and growth of IFIT2-depleted metastatic oral squamous cell carcinoma cells. Cancer Lett 370(2):207–215
91. Kawaguchi M, Kataoka H (2014) Mechanisms of hepatocyte growth factor activation in cancer tissues. Cancers 6(4):1890–1904
92. Kataoka H, Miyata S, Uchinokura S, Itoh H (2003) Roles of hepatocyte growth factor (HGF) activator and HGF activator inhibitor in the pericellular activation of HGF/scatter factor. Cancer Metastasis Rev 22(2–3):223–236
93. Ellis LM (2006) The role of neuropilins in cancer. Mol Cancer Ther 5(5):1099–1107
94. Djordjevic S, Driscoll PC (2013) Targeting VEGF signalling via the neuropilin co-receptor. Drug Discov Today 18(9–10):447–455
95. Li L, Jiang X, Zhang Q, Dong X, Gao Y, He Y, Qiao H, Xie F, Xie X, Sun X (2016) Neuropilin-1 is associated with clinicopathology of gastric cancer and contributes to cell proliferation and migration as multifunctional co-receptors. J Exp Clin Cancer Res 35(1):16

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Role of the nervous system in cancer metastasis

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Abstract

Cancer remains as one of the leading cause of death worldwide. The development of cancer involves an intricate process, wherein many identified and unidentified factors play a role. Although most studies have focused on the genetic abnormalities which initiate and promote cancer, there is overwhelming evidence that tumors interact within their environment by direct cell-to-cell contact and with signaling molecules, suggesting that cancer cells can influence their microenvironment and bidirectionally communicate with other systems. However, only in recent years the role of the nervous system has been recognized as a major contributor to cancer development and metastasis. The nervous system governs functional activities of many organs, and, as tumors are not independent organs within an organism, this system is integrally involved in tumor growth and progression.

Keywords: Neurotransmitters, Neuropeptides, Neuro-cancer interaction, Metastasis, Cancer

Background

Cancer is the leading cause of death worldwide due to the aging population and unhealthy lifestyle [1]. Although it is highly treatable when localized, metastatic or recurrent cancer has a poor prognosis. Metastasis involves a complex series of steps including proliferation, angiogenesis, embolization, dissemination, evasion of immune system surveillance and surviving in ectopic organs [2–5]. However, despite significant advances in understanding metastasis and its mechanisms, the prognosis remains poor. In the past decades, research has focused on identifying and characterising genes and gene products that manipulate the metastatic processes [6–9]. More recently, the impact of the tumor microenvironment on tumor cell invasion and metastasis has attracted extensive attention (see ref. [10] for detailed review) [2, 10–13]. Multiple cellular and extracellular components within the tumor microenvironment, such as immune cells, endothelial cells, mesenchymal stromal cells (fibroblasts and myofibroblasts), and their secretory products, exert active functions to modulate gene expression patterns of tumor cells and to alter biological behavior of tumor cells [14–16]. Invariable crosstalk amongst these components within the tumor microenvironment triggers pro-survival, invasion, and metastatic pathways of tumor cells [17–20]. Several studies, both clinical and in vitro, reinforce the concept of the nervous system involvement in cancer metastasis [5, 21–26]. Nerve fibers present in and around the tumor could release neurotransmitters and neuropeptides directly acting on receptors expressed by cancer cells. The findings, primarily in cancer cell lines and animal models, indicate that there is a bi-directional correlation between the neural factors released and cancer progression and metastasis. Understanding the complex neurotransmitter-cancer interaction is important for the development of new avenues for targeted therapeutic intervention. This review presents an overview of the role of the nervous system in cancer metastasis.

The role of the nervous system in metastatic cascade

Studies have demonstrated that the nervous system facilitates development of tumor metastasis by modulating metastatic cascades through the release of neural-related factors from nerve endings such as neurotrophins, neurotransmitters and neuropeptides [27–29]. The process of metastasis formation involves tumor cells breaking away from the primary tumor and overcoming the

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obstacles of primary tissue inhibition (initiation and clonal expansion), anoikis inhibition (evasion from apoptosis), breakdown of base membranes (epithelial-mesenchymal transition (EMT) and invasion), extravasation and colonization, angiogenesis, evasion of immune response and establishment of tumor microenvironment.

Initiation and clonal expansion

Tumor metastasis initiation and clonal expansion is a complex process where contributing factors are not well understood. It is believed that metastasis process is initiated when genetically unstable tumor cells adjust to a secondary site microenvironment [11]. This process involves selecting traits that are beneficial to tumor cells and affiliated recruitment of traits in the tumor stroma that accommodate invasion by metastatic cells. Metastasis-initiating cells possess these traits and can hijack some of the normal stem cell pathways to increase cellular plasticity and stemness [30]. Proteolytic enzymes such as matrix metalloproteinases (MMPs) facilitate this process by degrading the surrounding normal tissues. MMPs are regulated by neural-related factors and neurotransmitters and are overexpressed in tumors [31–35]. Hence, nervous system modulates the initiation and clonal expansion via the expression of MMPs and the stimulation of metastasis-initiating cells.

Evasion from apoptosis

Anoikis is a programmed cell death induced upon cell detachment from extracellular matrix, acting as a critical mechanism in preventing adherent-independent cell growth and attachment to unsuitable matrix, thus avoiding colonizing of distant organs [36, 37]. For tumor metastasis to progress, tumor cells must be resistant to anoikis. Tumor cell resistance to anoikis is attributed to alteration in integrins' repertoire, overexpression of growth factor receptor, activation of oncogene, activation of pro-survival signals, or upregulation/mutation of key enzymes involved in integrin or growth factor receptor signaling [37]. Neurotransmitters and neurotrophins play a role in tumor evasion from anoikis. Increased expression of brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) induces anoikis inhibition in rat intestinal epithelial cells [27]. Similarly, TrkB overexpression induces anoikis inhibition protecting colorectal cancer cells [38]. Application of recombinant human BDNF to gastric cancer cells inhibited anoikis and stimulated cellular proliferation, invasion and migration [39]. Nicotine exposure promotes anchorage-independent growth of A549, MDA-MB-468 and MCF-7 cell lines by downregulation of anoikis [40]. Furthermore, tumor microenvironment contributes to anoikis resistance of cancer cells by producing pro-survival soluble factors, triggering EMT, enhancing oxidative stress, regulating matrix stiffness, as well as leading to metabolic deregulations of cancer cells [37]. These events assist tumor cells to prevent the apoptosis mechanism and sustain pro-survival signals after detachment, counteracting anoikis.

EMT and invasion

EMT is a fundamental process for tumor progression by increasing invasiveness and resistance to anoikis and significantly elevating the production of extracellular matrix constituents leading to metastasis [41–43]. EMT development results in the degradation of basement membrane and formation of mesenchymal-like cells [42]. Studies have demonstrated that nervous system regulates EMT development via the release of neurotransmitters and neurotrophins [40, 44]. The overexpression of TrkB or activation by BDNF in human endometrial cancer cell lines results in altered expression of EMT molecular mediators [44]. Nicotine treatment induces changes in gene expression associated with EMT in lung and breast cancer cells [40].

Extravasation and colonization

Nervous system modulates the function of vascular system which is essential for tumor cell extravasation and colonization. It has been found that neuropeptides such as substance P (SP) and bradykinin enhance vascular permeability promoting tumor cell extravasation and colonization [28, 29]. In a mouse model bearing sarcoma 180 cells, bradykinin enhances tumor-associated vascular permeability [28]. SP regulates physiological functions of vascular system including smooth muscle contractility, and vascular permeability [29]. Cell extravasation and colonization are prerequisite for angiogenesis which is a crucial step in the development of cancer metastasis.

Angiogenesis

Development of tumor angiogenesis is essential for tumor growth and progression. Vascular endothelial growth factor (VEGF) plays significant role in tumor angiogenesis, leading to metastasis [45–47]. Studies have demonstrated the important role of neurotransmitters and neuropeptides in regulating angiogenesis. In the xenograft models of ovarian cancer, chronic stress mediates the vascularization of intraperitoneal metastasis and enhances tumor angiogenesis via increasing VEGF expression [48, 49]. In breast cancer cell lines, direct activation of β-adrenergic signaling can amplify expression of VEGF and cytokines, interleukin (IL)-6, and IL-8 that stimulate tumor angiogenesis [50]. In colon tumor tissues from HT-29 cell-bearing BALB/c mice, VEGF expression is elevated by nicotine which correlates with enhanced microvessel density [51]. Neuropeptide Y (NPY) enhances the expression of VEGF and its

secretion promoting angiogenesis and breast cancer progression [52].

Evasion of immune response

The nervous system plays a fundamental role in regulating immune responses [53]. Inflammatory mediators can activate sensory nerves that send signals regarding inflammation to the central nervous system, which in turn leads to the release of neuromediators modulating local inflammation and influencing immune cells [54]. Since inflammatory signals are important for tumor progression in both the early and late stages, the anti-inflammatory role of the vagus nerve may play an important role in cancer metastasis [55]. β-adrenergic receptor agonist suppressed natural killer (NK) cell activity resulting in increased lung metastasis in murine metastatic mammary adenocarcinoma [56]. In addition, pharmacological or stress-associated β-adrenergic stimulation results in increased macro- phage infiltration and cancer metastasis in breast can- cer model [57].

Tumor microenvironment

Tumor microenvironment (mainly contain stromal cells and signal molecules) plays essential role in the formation of cancer metastasis. Stromal cells produce neural-related factors and express β-adrenergic receptor that facilitated tumor cell proliferation and survival in the primary site and secondary organ [10, 24]. Tumor-associated macrophages play a role in β-adrenergic signaling pathways, by accelerating angiogenesis, chemokine secretion to attract tumor cells, secretion of pro-inflammatory cytokines (IL-1, IL-6, IL-8, and tumor necrosis factor (TNF)-α) and escape of anti-tumor responses [58–60]. Hence, tumor microenviron- ment creates a feedback loop with the nervous system en- abling the growth of primary and secondary tumors. Overall, these studies have demonstrated that the nervous system modulates each step of cancer metastasis through the release of neural-related factors.

Role of perineural invasion in cancer metastasis

Perineural invasion (PNI) also known as neurotropic carcinomatous spread is a process mainly categorized by neoplastic invasion of the nerves. PNI is defined as the presence of cancer cells in the perineurium; it is believed to be a common route for cancer metastasis can cause cancer-related pain [61–68]. The presence of PNI is mostly associated with poor prognosis and high recurrence in colorectal [69], gastric [64], oral tongue squamous cell carcinoma (OTSCC) [62], and pancreatic [61] cancers. In stage II and III colorectal cancer patients, the presence of PNI is associated with tumor grade, metastasis to lymph nodes and poor patient survival [63]. However, in invasive breast carcinoma the presence of PNI has been demonstrated to have no prognostic value [67, 70].

PNI is influenced by the interaction between the nerve microenvironment and neurotrophic molecules expressed by cancer cells such as nerve growth factor (NGF), BDNF, glial cell line-derived neurotrophic factor (GDNF) and their receptors [61, 68, 71]. A number of studies demonstrated correlation between the presence of PNI with high expression of NGF and its receptor tropomyosin related kinase A (TrkA) [61, 72, 73]. It is speculated that neurotrophins released by neural tissue act as chemotactic factors, and in cancer cells where Trks are overexpressed, they provide mechanism to in- vade the perineural space. High expression of NGF or TrkA and $P75^{NTR}$ receptors is associated with lymph node metastasis in a mouse model of breast cancer [74]. In OTSCC patients [73], the presence of PNI and NGF is associated with larger tumor size and lymph node metastasis, suggesting that its presence can be a valuable marker to predict the disease progression and prognosis [65]. Overexpression of TrkA associates with enhanced growth, invasion and migration of breast cancer cells in vitro as well as enhanced metastasis in xenografted immunodeficient mice via the PI3K-AKT and ERK/P38 MAP kinases [75]. Conversely, immuno-histochemical evaluation of tissues from patients with extrahepatic cholangiocarcinoma shows that intra-tumoral NGF expression does not correlate with PNI, absence of disease recurrence and overall patient survival [76]. GDNF has been demonstrated to induce cancer cells migration. In human pancreatic adenocarcinoma tissues and MiaPaCa-2 cell lines, binding of GDNF to its receptor GFRα1 stimulates PNI via GDNF-(Ret proto-oncogene) RET signaling pathway [71]. Activation of GDNF-GFRα1-RET signaling triggers the MAPK signaling pathway leading to pancreatic cancer cell migration toward nerves in both in vitro and animal models of PNI [77]. Cancer-nerve interaction studied in in vitro co-cultures of DRG and MiaPaCa-2 pancreatic cancer cells demonstrated that GFRα1 facilitates migration of cancer cells along neurites toward the center of the DRG [71]. Furthermore, decreased release of soluble GFRα1 from DRG inhibits migration of cancer cells towards nerves in vivo providing further evidence that GFRα1 expression is important in facilitating PNI [71]. In a metastatic breast cancer model, in vivo inhibition of Ret suppresses tumour outgrowth and metastatic potential [78].

BDNF facilitates cancer metastasis via binding to its receptors, TrkB/ TrkC and/or p75NTR as demonstrated in breast [79], colorectal [80, 81], clear cell renal cell carcinoma [82] and non-small cell lung cancer (NSCLC) [83]. The expression of TrkB associates with nodal metastasis and peritoneal metastasis; whereas, TrkC expression associates with liver metastasis in colorectal cancer

patients [81]. BDNF-TrkB signaling pathway mediates metastatic effect through modulation of cancer-associated fibroblasts (CAFs) as demonstrated in mouse model co-injected with OSC19-Luc transfected cell line and CAFs [84]. In melanoma, neurotrophin (NT)-3, NT-4, and NGF induce cell migration, with a stronger effect on metastatic cell lines via binding to p75NTR coreceptor sortilin [85]. In breast cancer, NT-3 enhances breast cancer metastasis in the brain via promoting the mesenchymal–epithelial transition of breast cancer cells to a more epithelial-like phenotype and via increasing the ability of these cells to proliferate in the brain [86].

Collectively, these studies demonstrate that neurotrophins and their receptors play crucial role in PNI. These studies also suggest that the presence of PNI could be an effective predictor of metastatic potential and patient survival.

Tumor innervation influencing cancer metastasis

Tumor innervation

Cancer-related neurogenesis (tumor innervation) is attributed to the ability of cancer cells to attract normal nerve fibers via the secretion of signalling molecules and neurotrophic factor. However, recent study has demonstrated that cancer stem cells are capable of directly initiating tumor neurogenesis [87]. Cancer stem cells derived from human gastric and colorectal cancer patients generate neurons including sympathetic and parasympathetic neurons which promote tumor progression [87]. Knocking down their neural cell generating abilities inhibit tumor growth in human xenograft mouse model. Neurogenesis and its putative regulatory mechanisms have been reported in prostate [88], gastric [89], colorectal [90] and breast [91] cancers. There is a correlation between the expression of a pan-neuronal marker protein gene product 9.5 with clinicopathological characteristics of breast cancer [91]. In fact, neurogenesis is associated with aggressive features including tumor grade, poor survival as well as angiogenesis, especially in estrogen receptor-negative and node-negative breast cancer subtypes [91, 92]. In prostate cancer, infiltration of the tumor microenvironment by nerve fibers associates with poor clinical outcomes [93] and is driven by the expression of granulocyte colony-stimulating factor (G-CSF) [94] and proNGF [95]. Similarly, in orthotopic PC3-luc xenografts model of prostate cancer, neurogenesis and axonogenesis correlate with aggressive features including metastatic spread which is attributed to the neo-cholinergic parasympathetic nerve fiber [94]. These findings indicate that neurogenesis, like angio- genesis, is also a trait of cancer invasion and can alter tumor behaviour.

Tumor denervation

On the other hand, disruption of tissue innervation might cause accelerated tumor growth and metastasis [56, 96–101]. For instance, in humans, decreased vagal nerve activity correlates with advanced stages of cancer [96–98]. Similarly, modulation of vagal nerve activity enhances metastasis of breast cancer in mice [99, 100]. In addition, capsaicin-induced inactivation of sensory neurons enhances metastasis of breast cancer cells [56, 101]. On contrary, pharmacological or surgical denervation supresses the tumor progression as noted in three independent mice models of gastric cancer [89]. Thus, these findings suggest that there might be differences in the effects of local tumor innervation and extrinsic innervation on cancer progression.

Neurotransmitters influencing cancer metastasis

Tumor innervation influences metastasis as the ingrown nerve endings release neurotransmitters (such as norepinephrine, dopamine and substance P), which enhance metastatic spread [102]. To date, several neurotransmitters and neuropeptides involved in tumor metastasis have been identified (Table 1 and Fig. 1). In fact, several cancer cells express receptors for a number of neuropeptides and neurotransmitters, like norepinephrine, epinephrine, dopamine, GABA, acetylcholine, SP and NPY which have stimulatory effects on migration of cancer cells [103–112].

Catecholamines

The increased expression of β-adrenergic receptor for catecholamines is associated with poor prognosis in breast cancer [113]. Stress stimulation leads to macrophage infiltration to the tumor site which activates β-adrenergic signaling pathways leading to increased metastasis in an orthotopic breast cancer model in BALB/c mice [57]. In this model, administration of β-adrenergic antagonist, propranolol, decreases breast cancer metastasis [57]. Similarly, the use of β-blockers in breast cancer patients inhibits metastasis and disease recurrence as well as improving survival of patients [113, 114]. In ovarian cancer patients, the grade and stage of tumors correlate with higher tumor norepinephrine levels associated with stress [115]. In an orthotopic mouse model of ovarian cancer, chronic stress elevates tumor noradrenaline levels and increases the aggressiveness of tumor growth [49]. In prostate cancer C42 xenografts in nude mice and Hi-Myc mice with prostate cancer, plasma adrenaline promotes carcinogenesis via β_2 adrenergic receptor/protein kinase A/BCL2-associated death protein anti-apoptotic signaling pathway [116]. Hence, stimulation of catecholamines plays a major role in activation of signals for breast cancer metastasis. Therefore,

Table 1 Neurotransmitters influencing tumor metastasis

Neurotransmitters	Receptor	Type of cancer	Model	Mechanism/pathway	Ref.
NE	β2-AR	Pancreatic cancer	CFPAC1, MiaPaCa2 Panc1, and IMIM-PC2 cells	NE treatment reduces migratory activity of pancreatic cancer cells. NE mediates inhibitory effect via imbalanced activation of PKC/PLC signaling pathway → to activation of anti-migratory cAMP/PKA signalling.	[155]
		Prostate cancer	Subcutaneous injection of PC-3 cells in BALB/c nude mice	↑ NE leads to lumbar lymph node metastasis in an animal model.	[156, 157]
DA	DR1 & DR5	HCC	Tumor and non-tumor adjacent tissues from patients; LM3, Huh7 and SNU449 cells; subcutaneous injection of LM3 cells in BALB/c nude mice	DR5 is upregulated in tumor tissue and DR1 is upregulated in non-tumor human tissues. Dopamine ↑ cell proliferation in SNU449 cells. Administration of DR antagonist (thioridazine) inhibits cell proliferation in vitro and *in* and cell migration through EMT →↓ tumor metastasis	[120]
GABA	$GABA_A$	HCC	Human primary and adjacent non-tumor tissues, and Orthotopic inoculation of SMMC-7721 cells into the liver of BALB/c nude mice	$GABA_A$ receptor subunit ε1 expression is lower in human HCC tissues than in non-tumor liver tissues. GABA inhibits invasion and migration of human liver cancer cells in vitro. In mice, inoculation of SMMC-7721 cells pretreated with GABA ↓ tumor metastasis.	[128]
	$GABA_B$		PLC/PRF/5 and Huh cells	Administration of $GABA_B$ agonist (baclofen) ↓ cell migration associated with ↓ in intracellular cAMP levels.	[132]
		Breast cancer	Human tissues, 4 T1 and MCF-7 cells	Administration of $GABA_B$ agonist (baclofen) promotes invasion and migration of breast cancer cells in vitro and metastasis in vivo via $ERK_{1/2}$ and MMP-2signaling pathway.	[107]
		Prostate cancer	Human prostate and lymph node tissues, C4–2 cells	↑ Expression of GABA → cell invasion in vitro and lymph node metastasis in patients mediated by activation of MMPs signalling.	[158]
		HCC	Human primary and adjacent non-tumor tissues	The mRNA levels of $GABA_B$ R1.2 and $GABA_B$ R1.4 are higher in HCC tissues than in non-tumor liver tissues	[128]
ACh	AR	HCC	SNU-449 cells	ACh activates AR receptors → ↑ invasion and migration of SNU-449 cells via activation of AKT and STAT3 signaling pathways.	[133]
	α7-nAChR	Pancreatic cancer	CD18/HPAF, Capan1, FG/Colo357 cells in vitro and orthotopically implanted CD18/HPAF cells in immunodeficient mice	Nicotine treatment stimulates the expression of α7-nAChR and MUC4 in vitro. In the in vivo model, exposure to low and high cigarette smoking increases the tumor metastasis and MUC4 expression compared to sham controls. Nicotine induces tumor metastasis by upregulating MUC4 via α7-nAChR-mediated JAK2/STAT3 signaling in collaboration with Ras/Raf/MEK/$ERK_{1/2}$ signalling pathway.	[135]
		Lung cancer	Line 1 cells in vitro, and subcutaneous injection of Line 1 cells in BALB/c mice	Intraperitoneal injection of nicotine ↑ tumor growth and metastasis through change in gene expression via nAChR signalling pathway.	[159]
	nAChR β2	Lung cancer	B16 cells intravenous injection in C57BL/6 mice	↑ Nicotine exposure → activation of nAChR β2 on NK cells mediates metastasis	[160]
	α9-nAChR	Breast cancer	MDA-MB-231 and MCF-7 cells	Nicotine treatment enhances the migratory abilities of both cells by activating α9-nAChR through elevated expression of EMT markers	[134]
	mAChR	Colon cancer	Hh508 and SNU-C4 cells		[138, 139]

Table 1 Neurotransmitters influencing tumor metastasis *(Continued)*

Neurotransmitters	Receptor	Type of cancer	Model	Mechanism/pathway	Ref.
				Administration of muscarinic inhibitor (atropine) →↓ cell invasion and migration. ACh binding to M3R mediates cell migration via the activation of post-ERBB1, ERK and PI3K-dependent RhoA pathway.	
		NSCLC	Human tissues, micA549, PC9, SPC-A1, GLC82, L78 and HLF cells	M3R expression correlates with clinical stage and poor survival in patients. M3R stimulation by ACh enhances in vitro cell invasion and migration via PI3K/AKt pathway.	[136, 137]
		Prostate cancer	Human tissues, Hi-Myc transgenic mice-bearing PC-3	Presences of cholinergic nerve fibers associate with poor clinical outcome in human patients. Pharmacological blockade or genetic disruption of the M1R inhibit metastasis leading to improved survival of the mice	[93]
SP	NK-1R	Pancreatic cancer	MiaPaCa-2, BxPC-3, CFPAC-1, HAPC, Panc-1, and SW1990 cells	Binding of SP to NK-1R promotes cell invasion and migratory potential which is mediated by expression of MMP-2. SP also increases cell migration and neurite outgrowth toward DRG demonstrating important role in metastasis and PNI.	[146, 161]
NPY		Ewing sarcoma	Human serum, SCID/beige mice bearing SK-ES1 cells	Enhanced level of systemic NPY associate with metastatic tumors. In the xenograft model, NPY expression associate with bone metastases.	[149, 150]
	Y5	Breast cancer	4 T1 cell line	NPY mediates metastatic effect via the activation of Y5 receptor.	[148]
Neurotensin	NTSR1	Breast cancer	Human tissues	The expression of NTSR1 associates with lymph node metastasis.	[151]

Ach acetylcholine, *AR* androgen receptor, *β2-AR* β_2-adrenergic receptor, *cAMP* cyclic adenosine monophosphate, *DA* dopamine, *DR* dopamine receptor, *DRG* dorsal root ganglia, *ERBB1* epidermal growth factor receptor 1, *EMT* epithelial–mesenchymal transition, $ERK_{1/2}$ extracellular signal-regulated kinase, *GABA* gamma-aminobutyric acid, $GABA_{A\&B}$ gamma-aminobutyric acid receptor $_{A\&B}$, *HCC*, hepatocellular carcinoma, *JAK2* janus kinase 2, *MEK* MAPK/ERK kinase, *MMP* matrix metallopeptidase, *RAF* mitogen activated protein kinase, *RAS* mitogen activated protein kinase, *MUC4* mucin 4, *mAChRs* muscarinic acetylcholine receptors, *M3R* muscarinic receptors 3, *NK* natural killer cells, *NK-1R* neurokinin-1 receptor, *nAChR* nicotinic acetylcholine receptor, *NSCLC* non-small cell lung cancer, *NE* norepinephrine, *PNI* perineural invasion, *PLC* phospholipase C, *PI3K* phosphoinositide 3-kinase, *PKA* protein kinase A, *PKC* protein kinase C, *RhoA* Ras homolog gene family member A, *AKT* serine/threonine kinase or protein kinase B, *STAT3* signal transducer and activator of transcription 3, *SP* substance P

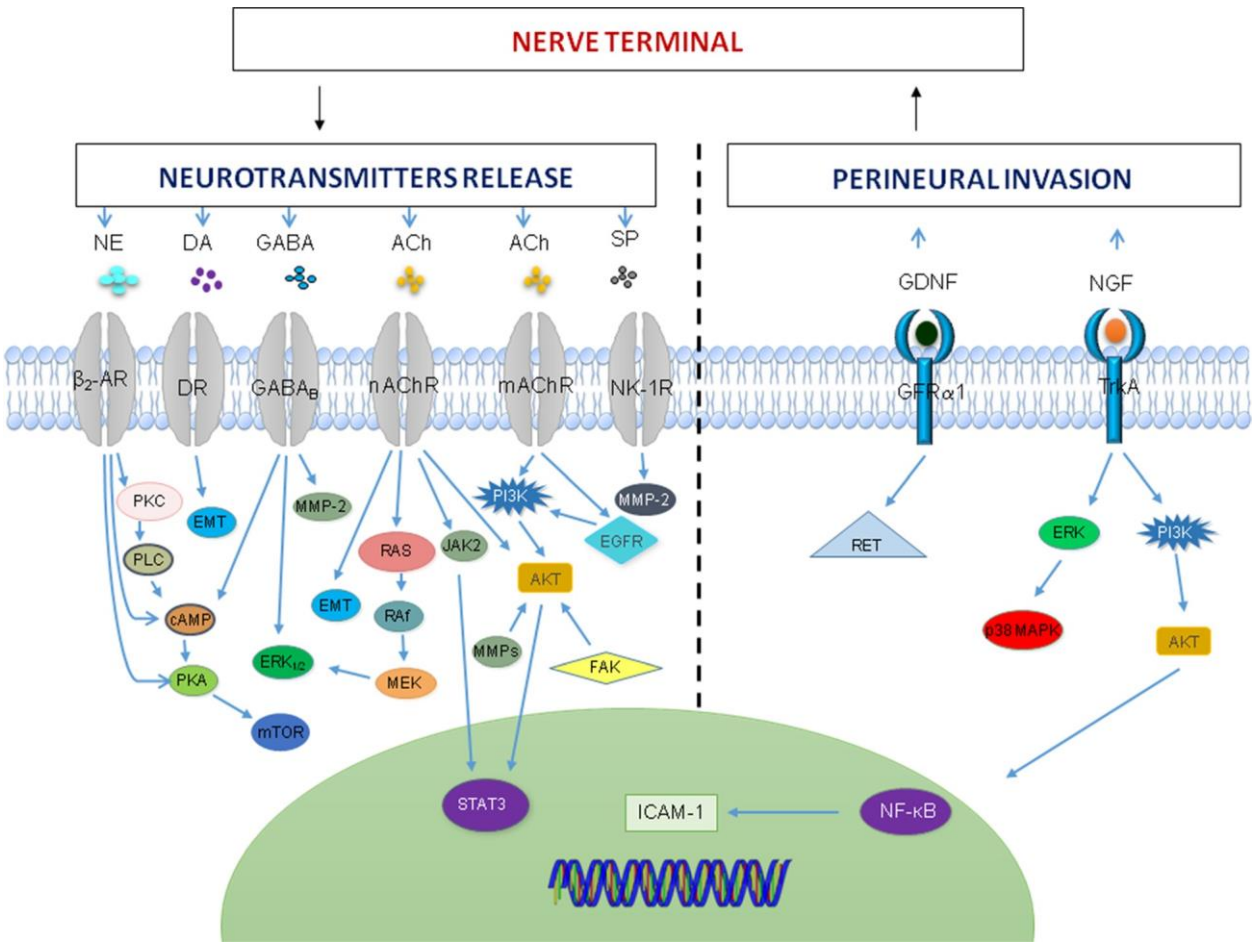

Fig. 1 Neurotransmitters signalling pathways in cancer. Cancer neuro-immune communication is through the release of neurotransmitters using different signalling kinases which promote cancer progression via metastasis. Perineural invasion mediate cancer metastasis through the release of the NGF and GDNF via the activation of different signaling pathway. Ach, acetylcholine; β2-AR, β_2-adrenergic receptor;cAMP, cyclic adenosine monophosphate; DA, dopamine; DR, dopamine receptor; EGFR, epidermal growth factor receptor;EMT,epithelial–mesenchymal transition; $ERK_{1/2}$, extracellular signal-regulated kinase;FAK, focal adhesion kinase; GABA, gamma-aminobutyric acid; $GABA_B$,gamma-aminobutyric acid $receptor_B$;GDNF, glial cell line-derived neurotrophic factor; GFRα, glial cell line-derived neurotrophic factor receptor 1;ICAM-1, intercellular adhesion molecule-1; JAK2,janus kinase 2;MEK, MAPK/ERK kinase;mTOR, mammalian/mechanistic target of rapamycin;MMP, matrix metallopeptidase;MAPK,-mitogen-activated protein kinases;RAF, mitogen activated protein kinase;RAS, mitogen activated protein kinase;mAChRs, muscarinic acetylcholine receptors;NK-1R, neurokinin-1 receptor; NGF, nerve growth factor;nAChR, nicotinic acetylcholine receptor;NE, norepinephrine;NF-kB, nuclear factor-kappa B;PLC, phospholipase C; PI3K, phosphoinositide 3-kinase;PKA, protein kinase A;PKC, protein kinase C;RET, proto-oncogene;AKT, serine/threonine kinase or protein kinase B;STAT3,signal transducer and activator of transcription 3; SP,substance P;TrkA,tropomyosin related kinase A

inhibition of the sympathetic nervous system signaling pathways with β-blockers holds great promise in preventing metastasis of various tumors including breast cancer. On the other hand, involvement of α-adrenergic receptors in cancer metastasis is not well understood. In the murine model of metastatic mammary adenocarcinoma induced by 4 T1 cells in BALB/c mice, activation of α_2-adrenergic receptors increases tumor growth rate and the number of metastasis [117]. In contrast, blockade of α-adrenergic receptors in the absence of stress increases distant metastasis in the orthotopic model of mammary adenocarcinoma induced by MDA-MB-231HM cell line in nude mice [118].

The role of dopamine in cancer metastasis is not clear. Low levels of dopamine have been reported in stressed mice with ovarian carcinoma [119]. In contrary, in hepatocellular carcinoma (HCC) patients dopamine levels are elevated in the blood samples compared to healthy individuals [120]. Moreover, enzymes such as monoamine oxidase A (MAOA) degrading catecholamines and serotonin [121] may also play an important role in influencing cancer metastasis [122–124]. Studies have demonstrated that

MAOA expression is decreased in HCC patients; it suppresses HCC cell metastasis by inhibiting adrenergic and epidermal growth factor receptor (EGFR) signaling pathways [125]. Inhibition of MAOA stimulates malignant behavior in MDA-MB-231 breast cancer cells [126]. On the other hand, high expression of MAOA in human tissues correlates with poor prognostic in prostate cancer patients and increased tumor metastasis in xenograft mouse model of prostate cancer via HIF1-α/VEGF-A/FOXO1/TWIST1 signaling pathway [124]. These limited studies on the role of MAOA in cancer metastasis are controversial.

γ-Aminobutyric acid (GABA)

Plays a role in cancer metastasis via activation of ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors [127]. It has been demonstrated that GABA mediates its inhibitory effect through $GABA_A$ receptor. For example, HCC cell lines and human adjacent non-tumor liver tissues, express $GABA_A$ receptor. GABA inhibits HCC cell migration through the activation of $GABA_A$ receptor [128]. However, there are studies demonstrating that $GABA_A$ receptor enhances metastasis. The activation of $GABA_A$ receptors upregulates brain metastasis of breast cancer patients [129]. Expression of the $GABA_A$ receptor subunit, Gabra3, which is normally not present in breast epithelial cells, is increased in human metastatic breast cancer which correlated with poorer patients survival [108]. Gabra3 overexpression promotes migration and metastasis of breast cancer cells via activating serine/threonine kinase or protein kinase B (AKT) signaling pathway demonstrated in a mouse orthotopic model induced by MCF7 and MDA-MB-436 breast cancer cell lines [108]. Mechanistically, the activation of AKT signaling pathway enhances metastasis via downstream molecules such as focal adhesion kinase and MMPs [130, 131]. Therefore, it could be speculated that the effect of $GABA_A$ receptor depends on the activated downstream molecules and signalling pathways. Murine (4 T1) and human (MCF7) breast cancer cell lines and human breast cancer tissues express $GABA_B$ receptor [107]. In mice, $GABA_B$ receptor mediates 4 T1 cell invasion and pulmonary metastasis via $ERK_{1/2}$ signaling [107]. $GABA_B$ activation inhibits migration of PLC/PRF/5 and Huh 7 malignant hepatocyte cell lines in vitro [132].

Acetylcholine (ACh)

Plays a functional role in cellular proliferation, differentiation and apoptosis. In HCC, the release of ACh acting on androgen receptor promotes SNU-449 cell invasion and migration via activation of AKT and signal transducer and activator of transcription 3 (STAT3) signaling pathways [133]. Nicotine stimulation of nicotinic acetylcholine receptor (nAChRs) enhances SW620 and LOVO colorectal cancer cell invasion and metastasis in vitro via the activation of p38 mitogen-activated protein kinases (MAPK) signaling pathway [112]. Similarly, nicotine pretreatment stimulates the activation of α9-nAChR which mediates MCF-7 and MDA-MB-231 breast cancer cell migration via the expression of epithelial mesenchymal transition markers [134]. Furthermore, implantation of CD18/HPAF pancreatic cancer cells into immuno-deficient mice, demonstrates that nicotine treatment activates α7-nAChR and mediates tumor metastasis via Janus kinase 2 (JAK2)/STAT3 signaling in synergy with mitogen activated protein kinase (Ras/Raf/MEK/$ERK_{1/2}$) signalling pathway [135]. ACh promoted cancer metastasis and associate with poor clinical outcomes in prostate adenocarcinoma via M1R; and pharmacological blockade or genetic disruption of the M1R inhibit tumor invasion and metastasis leading to improved survival of the mice-bearing PC-3 prostate tumor xenografts [93]. In addition, ACh acting on M3 muscarinic receptor (M3R) associates with metastasis and low survival rate of NSCLC patients [136]. M3R activation increases invasion and migration of NSCLC cells and increased release of interleukin (IL)-8 via the activation of EGFR/PI3K/AKT pathway [137]. In human SNU-C4 and H508 colon cancer cell lines, administration of muscarinic receptor inhibitor, atropine, abolished SNU-C4 cell migration, however, H508 cell migration requires the activation of MMP7 [138, 139].

Neuropeptides

Expression of SP is shown to exert functional effects on small cell lung cancer [140], pancreatic [141], colon [142], prostate [143, 144] and breast cancer [145] cells. SP acting on neurokinin-1 (NK-1) receptors enhances pancreatic cancer cell migration and perineural invasion to the dorsal root ganglia (DRG) mediated by MMP-2 demonstrating its essential role in metastasis [146]. Enhanced expression of SP correlated with lymph node metastasis and poor prognosis in colorectal cancer patients [142]. NPY modulates cell proliferation, differentiation and survival via acting on its G protein-coupled receptors designated Y1R–Y5R leading to the development of metastasis [147, 148]. High levels of systemic NPY associates with metastatic tumors as noted in Ewing sarcoma patients [149]. Similarly, in the SK-ES1 xenograft model, elevated levels of NPY associates with bone in- vasion and metastases [150]. NPY mediates 4 T1 cell proliferation and migration via the activation of NPY Y5 receptor [148]. Neurotensin mediates metastasis by binding to neurotensin receptors 1 (NTSR1). In breast cancer, the expression of NTSR1 correlates with lymph node metastasis [151]. These studies demonstrate the important role of neuropeptide signaling in cancer metastasis.

Concluding remarks and future directions

Metastasis continues to be the main cause of cancer-related death. Although genetic compartments that influence metastasis have been identified, there are still needs to conduct comprehensive evaluation of the factors that contribute to cancer metastasis. This review demonstrates that the nervous system influences cancer metastasis through the release of neurotransmitters and neuropeptides leading to metastasis. However, sensory nerve fibres have been given less attention. Sensory stimuli activate pain transmission pathways which result in acute or chronic pain depending on the intensity and the nature of the stimulus [152, 153]. Cancer-related pain is linked to accelerating cancer progression and metastasis. Sensory nerves can innervate primary tumors and metastases, thus contributing to tumor-associated pain as demonstrated in pancreatic [61] and prostate cancer [154]. Therefore, a possible involvement of sensory fibers in tumor progression and metastasis, although not well demonstrated at this stage, cannot be excluded.

In conclusion, cancer cells can transduce neurotransmitter-mediated intracellular signaling pathways which lead to their activation, growth and metastasis. The findings reported here are primarily done in cancer cell lines and animal models. Therefore, better understanding the interaction between these signaling molecules and tumor cells in human cancers would enhance our knowledge on pathways promoting cancer metastasis.

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Authors' contributions

NK wrote the manuscript. LS, VA and KN revised and corrected the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors confirm that this article content has not competing interests.

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References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87–108.
2. Fisher E, Fisher B. Recent observations on concepts of metastasis. Arch Pathol. 1967;83(4):321.
3. Fidler IJ. The pathogenesis of cancer metastasis: the'seed and soil'hypothesis revisited. Nat Rev Cancer. 2003;3(6):453–8.
4. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov. 2007;6(4):273–86.
5. Moreno-Smith M, Lutgendorf SK, Sood AK. Impact of stress on cancer metastasis. Future Oncol. 2010;6(12):1863–81.
6. Hynes RO. Metastatic potential: generic predisposition of the primary tumor or rare, metastatic variants—or both? Cell. 2003;113(7):821–3.
7. Nguyen DX, Massagué J. Genetic determinants of cancer metastasis. Nat Rev Genet. 2007;8(5):341–52.
8. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. Nat Genet. 2003;33(1):49–54.
9. Hynes RO. Metastatic potential: generic predisposition of the primary tumor or rare, metastatic variants or both? Cell. 2003;113(7):821–3.
10. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423–37.
11. Gupta GP, Massagué J. Cancer Metastasis: building a framework. Cell. 2006; 127(4):679–95.
12. Place AE, Jin Huh S, Polyak K. The microenvironment in breast cancer progression: biology and implications for treatment. Breast Cancer Res. 2011;13(6):227.
13. Miller S, Senior PV, Prakash M, Apostolopoulos V, Sakkal S, Nurgali K. Leukocyte populations and IL-6 in the tumor microenvironment of an orthotopic colorectal cancer model. Acta Biochim Biophys Sin. 2016:48(4): 334-41.
14. Markwell SM, Weed SA. Tumor and Stromal-based contributions to head and neck Squamous cell carcinoma invasion. Cancers. 2015;7(1):382–406.
15. Liguori M, Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages as incessant builders and destroyers of the cancer stroma. Cancers. 2011;3(4):3740–61.
16. Sun Y. Translational horizons in the tumor microenvironment: harnessing breakthroughs and targeting cures. Med Res Rev. 2015;35(2):408-36.
17. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell. 2012;21(3):309–22.
18. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.
19. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2009;9(4):239–52.
20. Schmid MC, Varner JA. Myeloid cells in the tumor microenvironment: modulation of tumor angiogenesis and tumor inflammation. J Oncol. 2010;2010
21. Li S, Sun Y, Gao D. Role of the nervous system in cancer metastasis (review). Oncol Lett. 2013;5(4):1101–11.
22. Voss MJ, Entschladen F. Tumor interactions with soluble factors and the nervous system. Cell Commun Signal. 2010;8:21.
23. Chang A, Kim-Fuchs C, Le CP, Hollande F, Sloan EK. Neural regulation of pancreatic cancer: a novel target for intervention. Cancers. 2015;7(3):1292–312.
24. Cole SW, Sood AK. Molecular pathways: beta-adrenergic signaling in cancer. Clin Cancer Res. 2012;18(5):1201–6.
25. Ondicova K, Mravec B. Role of nervous system in cancer aetiopathogenesis. Lancet Oncol. 2010;11(6):596–601.
26. Mravec B, Gidron Y, Hulin I. Neurobiology of cancer: interactions between nervous, endocrine and immune systems as a base for monitoring and modulating the tumorigenesis by the brain. Semin Cancer Biol. 2008;18(3): 150-63.
27. Douma S, van Laar T, Zevenhoven J, Meuwissen R, van Garderen E, Peeper DS. Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB. Nature. 2004;430:1034.
28. Ishihara K, Kamata M, Hayashi I, Yamashina S, Majima M. Roles of bradykinin in vascular permeability and angiogenesis in solid tumor. Int Immunopharmacol. 2002;2(4):499–509.
29. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. J Cell Physiol. 2004; 201(2):167–80.
30. Celià-Terrassa T, Kang Y. Distinctive properties of metastasis-initiating cells. Genes Dev. 2016;30(8):892–908.
31. MacDougall JR, Matrisian LM. Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. Cancer Metastasis Rev. 1995;14(4):351–62.
32. Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev. 2006;25(1):9–34.
33. Okada Y, Eibl G, Guha S, Duffy JP, Reber HA, Hines OJ. Nerve growth factor stimulates MMP-2 expression and activity and increases invasion by human pancreatic cancer cells. Clin Exp Metastasis. 2004;21(4):285–92.
34. Okada Y, Eibl G, Duffy JP, Reber HA, Hines OJ. Glial cell-derived neurotrophic factor upregulates the expression and activation of matrix metalloproteinase-9 in human pancreatic cancer. Surgery. 2003;134(2):293–9.

35. Yang EV, Sood AK, Chen M, Li Y, Eubank TD, Marsh CB, Jewell S, Flavahan NA, Morrison C, Yeh PE, et al. Norepinephrine up-regulates the expression of vascular endothelial growth factor, matrix metalloproteinase (MMP)-2, and MMP-9 in nasopharyngeal carcinoma tumor cells. Cancer Res. 2006; 66(21):10357–64.
36. Simpson CD, Anyiwe K, Schimmer AD. Anoikis resistance and tumor metastasis. Cancer Lett. 2008;272(2):177–85.
37. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. Biochim Biophys Acta (BBA) - Mol Cell Res. 2013; 1833(12):3481–98.
38. Fan M, Sun J, Wang W, Fan J, Wang L, Zhang X, Yang A, Wang W, Zhang R, Li J. Tropomyosin-related kinase B promotes distant metastasis of colorectal cancer through protein kinase B-mediated anoikis suppression and correlates with poor prognosis. Apoptosis. 2014;19(5):860–70.
39. Okugawa Y, Tanaka K, Inoue Y, Kawamura M, Kawamoto A, Hiro J, Saigusa S, Toiyama Y, Ohi M, Uchida K, et al. Brain-derived neurotrophic factor/ tropomyosin-related kinase B pathway in gastric cancer. Br J Cancer. 2012;108:121.
40. Dasgupta P, Rizwani W, Pillai S, Kinkade R, Kovacs M, Rastogi S, Banerjee S, Carless M, Kim E, Coppola D, et al. Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines. Int J Cancer. 2009;124(1):36–45.
41. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133(4):704–15.
42. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420–8.
43. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer. 2009; 9(4):265–73.
44. Bao W, Qiu H, Yang T, Luo X, Zhang H, Wan X. Upregulation of TrkB promotes epithelial-Mesenchymal transition and Anoikis resistance in endometrial carcinoma. PLoS One. 2013;8(7):e70616.
45. Park SY, Kang JH, Jeong KJ, Lee J, Han JW, Choi WS, Kim YK, Kang J, Park CG, Lee HY. Norepinephrine induces VEGF expression and angiogenesis by a hypoxia-inducible factor-1alpha protein-dependent mechanism. Int J Cancer. 2011;128(10):2306–16.
46. Toda M, Suzuki T, Hosono K, Hayashi I, Hashiba S, Onuma Y, Amano H, Kurihara Y, Kurihara H, Okamoto H, et al. Neuronal system-dependent facilitation of tumor angiogenesis and tumor growth by calcitonin gene-related peptide. Proc Natl Acad Sci U S A. 2008;105(36):13550–5.
47. Lee EW, Michalkiewicz M, Kitlinska J, Kalezic I, Switalska H, Yoo P, Sangkharat A, Ji H, Li L, Michalkiewicz T, et al. Neuropeptide Y induces ischemic angiogenesis and restores function of ischemic skeletal muscles. J Clin Invest. 2003;111(12): 1853–62.
48. Lutgendorf SK, Cole S, Costanzo E, Bradley S, Coffin J, Jabbari S, Rainwater K, Ritchie JM, Yang M, Sood AK. Stress-related mediators stimulate vascular endothelial growth factor secretion by two ovarian cancer cell lines. Clin Cancer Res. 2003;9(12):4514–21.
49. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, Jennings NB, Armaiz-Pena G, Bankson JA, Ravoori M. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nat Med. 2006;12(8):939–44.
50. Madden KS, Szpunar MJ, Brown EB. Beta-adrenergic receptors (beta-AR) regulate VEGF and IL-6 production by divergent pathways in high beta- AR-expressing breast cancer cell lines. Breast Cancer Res Treat. 2011; 130(3):747–58.
51. Wong HPS, Yu L, Lam EKY, Tai EKK, Wu WKK, Cho C-H. Nicotine promotes colon tumor growth and angiogenesis through β-adrenergic activation. Toxicol Sci. 2007;97(2):279–87.
52. Medeiros PJ, Jackson DN. Neuropeptide Y Y5-receptor activation on breast cancer cells acts as a paracrine system that stimulates VEGF expression and secretion to promote angiogenesis. Peptides. 2013;48:106–13.
53. Kuol N, Stojanovska L, Apostolopoulos V, Nurgali K. Crosstalk between cancer and the neuro-immune system. J Neuroimmunol. 2017;315:15-23.
54. Tracey KJ. Reflex control of immunity. Nat Rev Immunol. 2009;9(6):418–28.
55. Gidron Y, Perry H, Glennie M. Does the vagus nerve inform the brain about preclinical tumours and modulate them? Lancet Oncol. 2005;6(4):245–8.
56. Erin N, Boyer PJ, Bonneau RH, Clawson GA, Welch DR. Capsaicin-mediated denervation of sensory neurons promotes mammary tumor metastasis to lung and heart. Anticancer Res. 2004;24(2B):1003–10.
57. Sloan EK, Priceman SJ, Cox BF, Yu S, Pimentel MA, Tangkanangnukul V, Arevalo JM, Morizono K, Karanikolas BD, Wu L. The sympathetic nervous system induces a metastatic switch in primary breast cancer. Cancer Res. 2010;70(18):7042–52.
58. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, Gallagher WM, Wadhwani N, Keil SD, Junaid SA. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discov. 2011;1(1):54–67.
59. DeNardo DG, Coussens LM. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. Breast Cancer Res. 2007;9(4):212.
60. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454(7203):436–44.
61. Bapat AA, Hostetter G, Von Hoff DD, Han H. Perineural invasion and associated pain in pancreatic cancer. Nat Rev Cancer. 2011;11(10):695–707.
62. Matsushita Y, Yanamoto S, Takahashi H, Yamada S, Naruse T, Sakamoto Y, Ikeda H, Shiraishi T, Fujita S, Ikeda T, et al. A clinicopathological study of perineural invasion and vascular invasion in oral tongue squamous cell carcinoma. Int J Oral Maxillofac Surg. 2015;44(5):543–8.
63. Zhou Y, Wang H, Gong H, Cao M, Zhang G, Wang Y. Clinical significance of perineural invasion in stages II and III colorectal cancer. Pathol-Res Pract. 2015;211(11):839–44.
64. Zhou Z-H, Xu G-F, Zhang W-J, Zhao H-B, Wu Y-Y. Reevaluating significance of perineural invasion in gastric cancer based on double immunohistochemical staining. Arch Pathol Lab Med. 2014;138(2):229–34.
65. Shen WR, Wang YP, Chang JYF, Yu SY, Chen HM, Chiang CP. Perineural invasion and expression of nerve growth factor can predict the progression and prognosis of oral tongue squamous cell carcinoma. J Oral Pathol Med. 2014;43(4):258–64.
66. Yu EH, Lui MT, Tu HF, Wu CH, Lo WL, Yang CC, Chang KW, Kao SY. Oral carcinoma with perineural invasion has higher nerve growth factor expression and worse prognosis. Oral Dis. 2014;20(3):268–74.
67. Karak SG, Quatrano N, Buckley J, Ricci A Jr. Prevalence and significance of perineural invasion in invasive breast carcinoma. Conn Med. 2010;74(1):17–21.
68. Marchesi F, Piemonti L, Mantovani A, Allavena P. Molecular mechanisms of perineural invasion, a forgotten pathway of dissemination and metastasis. Cytokine Growth Factor Rev. 2010;21(1):77–82.
69. Liebig C, Ayala G, Wilks J, Verstovsek G, Liu H, Agarwal N, Berger DH, Albo D. Perineural invasion is an independent predictor of outcome in colorectal cancer. J Clin Oncol. 2009;27(31):5131–7.
70. Duraker N, Caynak Z, Türköz K. Perineural invasion has no prognostic value in patients with invasive breast carcinoma. Breast. 2006;15(5):629–34.
71. He S, Chen C-H, Chernichenko N, He S, Bakst RL, Barajas F, Deborde S, Allen PJ, Vakiani E, Yu Z. GFRα1 released by nerves enhances cancer cell perineural invasion through GDNF-RET signaling. Proc Natl Acad Sci. 2014; 111(19):E2008–17.
72. Ma J, Jiang Y, Jiang Y, Sun Y, Zhao X. Expression of nerve growth factor and tyrosine kinase receptor a and correlation with perineural invasion in pancreatic cancer. J Gastroenterol Hepatol. 2008;23(12):1852–9.
73. Kolokythas A, Cox DP, Dekker N, Schmidt BL. Nerve growth factor and tyrosine kinase a receptor in oral squamous cell carcinoma: is there an association with perineural invasion? J Oral Maxillofac Surg. 2010; 68(6):1290–5.
74. Zhang J, Wang L-S, Ye S-L, Luo P, Wang B-L. Blockage of tropomyosin receptor kinase a (TrkA) enhances chemo-sensitivity in breast cancer cells and inhibits metastasis in vivo. Int J Clin Exp Med. 2015;8(1):634.
75. Lagadec C, Meignan S, Adriaenssens E, Foveau B, Vanhecke E, Romon R, Toillon R-A, Oxombre B, Hondermarck H, Le Bourhis X. TrkA overexpression enhances growth and metastasis of breast cancer cells. Oncogene. 2009; 28(18):1960–70.
76. Urabe K, Murakami Y, Kondo N, Uemura K, Hashimoto Y, Nakagawa N, Sasaki H, Hiyama E, Takahashi S, Sueda T. Nerve growth factor expression is not associated with perineural invasion in extrahepatic cholangiocarcinoma. Dig Dis Sci. 2015:1–11.
77. Gil Z, Cavel O, Kelly K, Brader P, Rein A, Gao SP, Carlson DL, Shah JP, Fong Y, Wong RJ. Paracrine regulation of pancreatic cancer cell invasion by peripheral nerves. J Natl Cancer Inst. 2010;102(2):107–18.
78. Gattelli A, Nalvarte I, Boulay A, Roloff TC, Schreiber M, Carragher N, Macleod KK, Schlederer M, Lienhard S, Kenner L, et al. Ret inhibition decreases growth and metastatic potential of estrogen receptor positive breast cancer cells. EMBO Mol Med. 2013;5(9):1335–50.

79. Kim MS, Lee WS, Jin W. TrkB promotes breast cancer metastasis via suppression of Runx3 and Keap1 expression. Mol Cells. 2016;39(3):258–65.
80. Tanaka K, Okugawa Y, Toiyama Y, Inoue Y, Saigusa S, Kawamura M, Araki T, Uchida K, Mohri Y, Kusunoki M. Brain-derived neurotrophic factor (BDNF)-induced tropomyosin-related kinase B (Trk B) signaling is a potential therapeutic target for peritoneal carcinomatosis arising from colorectal cancer. PLoS One. 2014;9(5):e96410.
81. Sasahira T, Ueda N, Kurihara M, Matsushima S, Ohmori H, Fujii K, Bhawal UK, Yamamoto K, Kirita T, Kuniyasu H. Tropomyosin receptor kinases B and C are tumor progressive and metastatic marker in colorectal carcinoma. Hum Pathol. 2013;44(6):1098–106.
82. De la Cruz-Morcillo MA, Berger J, Sánchez-Prieto R, Saada S, Naves T, Guillaudeau A, Perraud A, Sindou P, Lacroix A, Descazeaud A, et al. p75 neurotrophin receptor and pro-BDNF promote cell survival and migration in clear cell renal cell carcinoma. Oncotarget. 2016;7(23):34480–97.
83. Zhang S, Guo D, Luo W, Zhang Q, Zhang Y, Li C, Lu Y, Cui Z, Qiu X. TrkB is highly expressed in NSCLC and mediates BDNF-induced the activation of Pyk2 signaling and the invasion of A549 cells. BMC Cancer. 2010;10:43.
84. Jiffar T, Yilmaz T, Lee J, Miller Y, Feng L, El-Naggar A, Kupferman ME. Brain derived neutrophic factor (BDNF) coordinates lympho-vascular metastasis through a fibroblast-governed paracrine axis in the tumor microenvironment. Cancer Cell Microenvironment. 2017;4(2):e1566.
85. Truzzi F, Marconi A, Lotti R, Dallaglio K, French LE, Hempstead BL, Pincelli C. Neurotrophins and their receptors stimulate melanoma cell proliferation and migration. J Invest Dermatol. 2008;128(8):2031–40.
86. Louie E, Chen XF, Coomes A, Ji K, Tsirka S, Chen EI. Neurotrophin-3 modulates breast cancer cells and the microenvironment to promote the growth of breast cancer brain metastasis. Oncogene. 2013;32(35):4064–77.
87. Lu R, Fan C, Shangguan W, Liu Y, Li Y, Shang Y, Yin D, Zhang S, Huang Q, Li X, et al. Neurons generated from carcinoma stem cells support cancer progression. Signal Transduct Target Ther. 2017;2:16036.
88. Ayala GE, Dai H, Powell M, Li R, Ding Y, Wheeler TM, Shine D, Kadmon D, Thompson T, Miles BJ, et al. Cancer-related axonogenesis and neurogenesis in prostate cancer. Clin Cancer Res. 2008;14(23):7593–603.
89. Zhao CM, Hayakawa Y, Kodama Y, Muthupalani S, Westphalen CB, Andersen GT, Flatberg A, Johannessen H, Friedman RA, Renz BW, et al. Denervation suppresses gastric tumorigenesis. Sci Transl Med. 2014;6(250):250ra115.
90. Albo D, Akay CL, Marshall CL, Wilks JA, Verstovsek G, Liu H, Agarwal N, Berger DH, Ayala GE. Neurogenesis in colorectal cancer is a marker of aggressive tumor behavior and poor outcomes. Cancer. 2011;117(21):4834–45.
91. Zhao Q, Yang Y, Liang X, Du G, Liu L, Lu L, Dong J, Han H, Zhang G. The clinicopathological significance of neurogenesis in breast cancer. BMC Cancer. 2014;14:484.
92. Pundavela J, Roselli S, Faulkner S, Attia J, Scott RJ, Thorne RF, Forbes JF, Bradshaw RA, Walker MM, Jobling P, et al. Nerve fibers infiltrate the tumor microenvironment and are associated with nerve growth factor production and lymph node invasion in breast cancer. Mol Oncol. 2015;9(8):1626–35.
93. Magnon C, Hall SJ, Lin J, Xue X, Gerber L, Freedland SJ, Frenette PS. Autonomic nerve development contributes to prostate cancer progression. Science (New York, NY). 2013;341(6142):1236361.
94. Dobrenis K, Gauthier LR, Barroca V, Magnon C. Granulocyte colony-stimulating factor off-target effect on nerve outgrowth promotes prostate cancer development. Int J Cancer. 2015;136(4):982–8.
95. Pundavela J, Demont Y, Jobling P, Lincz LF, Roselli S, Thorne RF, Bond D, Bradshaw RA, Walker MM, Hondermarck H. ProNGF correlates with Gleason score and is a potential driver of nerve infiltration in prostate cancer. Am J Pathol. 2014;184(12):3156–62.
96. De Couck M, Gidron Y. Norms of vagal nerve activity, indexed by heart rate variability, in cancer patients. Cancer Epidemiol. 2013;37(5):737–41.
97. Ohira H, Matsunaga M, Osumi T, Fukuyama S, Shinoda J, Yamada J, Gidron Y. Vagal nerve activity as a moderator of brain–immune relationships. J Neuroimmunol. 2013;260(1):28–36.
98. De Couck M, Maréchal R, Moorthamers S, Van Laethem J-L, Gidron Y. Vagal nerve activity predicts overall survival in metastatic pancreatic cancer, mediated by inflammation. Cancer Epidemiol. 2016;40:47–51.
99. Erin N, Korcum AF, Tanriover G, Kale S, Demir N, Koksoy S. Activation of neuroimmune pathways increases therapeutic effects of radiotherapy on poorly differentiated breast carcinoma. Brain Behav Immun. 2015;48:174–85.
100. Erin N, Barkan GA, Harms JF, Clawson GA. Vagotomy enhances experimental metastases of 4THMpc breast cancer cells and alters substance P level. Regul Pept. 2008;151(1):35–42.
101. Erin N, Zhao W, Bylander J, Chase G, Clawson G. Capsaicin-induced inactivation of sensory neurons promotes a more aggressive gene expression phenotype in breast cancer cells. Breast Cancer Res Treat. 2006; 99(3):351–64.
102. Magnon C. Role of the autonomic nervous system in tumorigenesis and metastasis. Mol Cell Oncol. 2015;2(2):e975643.
103. Drell T IV, Joseph J, Lang K, Niggemann B, Zaenker K, Entschladen F. Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells. Breast Cancer Res Treat. 2003;80(1):63–70.
104. Rodriguez PL, Jiang S, Fu Y, Avraham S, Avraham HK. The proinflammatory peptide substance P promotes blood–brain barrier breaching by breast cancer cells through changes in microvascular endothelial cell tight junctions. Int J Cancer. 2014;134(5):1034–44.
105. Luthy IA, Bruzzone A, Piñero CP, Castillo L, Chiesa I, Vazquez S, Sarappa M. Adrenoceptors: non conventional target for breast cancer? Curr Med Chem. 2009;16(15):1850–62.
106. Garcia-Recio S, Fuster G, Fernandez-Nogueira P, Pastor-Arroyo EM, Park SY, Mayordomo C, Ametller E, Mancino M, Gonzalez-Farre X, Russnes HG. Substance P autocrine signaling contributes to persistent HER2 activation that drives malignant progression and drug resistance in breast cancer. Cancer Res. 2013;73(21):6424–34.
107. Zhang D, Li X, Yao Z, Wei C, Ning N, Li J. GABAergic signaling facilitates breast cancer metastasis by promoting ERK 1/2-dependent phosphorylation. Cancer Lett. 2014;348(1):100–8.
108. Gumireddy K, Li A, Kossenkov AV, Sakurai M, Yan J, Li Y, Xu H, Wang J, Zhang PJ, Zhang L. The mRNA-edited form of GABRA3 suppresses GABRA3-mediated Akt activation and breast cancer metastasis. Nat Commun. 2016;7
109. Munoz M, Coveñas R. Involvement of substance P and the NK-1 receptor in cancer progression. Peptides. 2013;48:1–9.
110. Yang T, He W, Cui F, Xia J, Zhou R, Wu Z, Zhao Y, Shi M. MACC1 mediates acetylcholine-induced invasion and migration by human gastric cancer cells. Oncotarget. 2016;7(14):18085–94.
111. Hanaki T, Horikoshi Y, Nakaso K, Nakasone M, Kitagawa Y, Amisaki M, Arai Y, Tokuyasu N, Sakamoto T, Honjo S, et al. Nicotine enhances the malignant potential of human pancreatic cancer cells via activation of atypical protein kinase C. Biochim Biophys Acta Gen Subj. 2016;1860(11):2404–15.
112. Xiang T, Fei R, Wang Z, Shen Z, Qian J, Chen W. Nicotine enhances invasion and metastasis of human colorectal cancer cells through the nicotinic acetylcholine receptor downstream p38 MAPK signaling pathway. Oncol Rep. 2016;35(1):205–10.
113. Powe DG, Entschladen F. Targeted therapies: using β-blockers to inhibit breast cancer progression. Nat Rev Clin Oncol. 2011;8(9):511–2.
114. Powe DG, Voss MJ, Zänker KS, Habashy HO, Green AR, Ellis IO, Entschladen F. Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival. Oncotarget. 2010;1(7):628–38.
115. Lutgendorf SK, DeGeest K, Dahmoush L, Farley D, Penedo F, Bender D, Goodheart M, Buekers TE, Mendez L, Krueger G. Social isolation is associated with elevated tumor norepinephrine in ovarian carcinoma patients. Brain Behav Immun. 2011;25(2):250–5.
116. Hassan S, Karpova Y, Baiz D, Yancey D, Pullikuth A, Flores A, Register T, Cline JM, D'Agostino R Jr, Danial N. Behavioral stress accelerates prostate cancer development in mice. J Clin Invest. 2013;123(2):874.
117. Szpunar MJ, Burke KA, Dawes RP, Brown EB, Madden KS. The antidepressant desipramine and α2-adrenergic receptor activation promote breast tumor progression in association with altered collagen structure. Cancer Prev Res. 2013;6(12):1262–72.
118. Lamkin DM, Sung HY, Yang GS, David JM, Ma JC, Cole SW, Sloan EK. α2-adrenergic blockade mimics the enhancing effect of chronic stress on breast cancer progression. Psychoneuroendocrinology. 2015;51:262–70.
119. Moreno-Smith M, Lu C, Shahzad MM, Pena GNA, Allen JK, Stone RL, Mangala LS, Han HD, Kim HS, Farley D. Dopamine blocks stress-mediated ovarian carcinoma growth. Clin Cancer Res. 2011;17(11):3649–59.
120. Lu M, Li J, Luo Z, Zhang S, Xue S, Wang K, Shi Y, Zhang C, Chen H, Li Z. Roles of dopamine receptors and their antagonist thioridazine in hepatoma metastasis. OncoTargets Ther. 2015;8:1543–52.
121. Flamand V, Zhao H, Peehl DM. Targeting monoamine oxidase a in advanced prostate cancer. J Cancer Res Clin Oncol. 2010;136(11):1761–71.
122. Kimura R, Kasamatsu A, Koyama T, Fukumoto C, Kouzu Y, Higo M, Endo-Sakamoto Y, Ogawara K, Shiiba M, Tanzawa H. Glutamate acid decarboxylase 1 promotes metastasis of human oral cancer by β-catenin translocation and MMP7 activation. BMC Cancer. 2013;13(1):555.

123. Liu G, Zhu J, Yu M, Cai C, Zhou Y, Yu M, Fu Z, Gong Y, Yang B, Li Y, et al. Glutamate dehydrogenase is a novel prognostic marker and predicts metastases in colorectal cancer patients. J Transl Med. 2015;13
124. Wu JB, Shao C, Li X, Li Q, Hu P, Shi C, Li Y, Chen YT, Yin F, Liao CP, et al. Monoamine oxidase a mediates prostate tumorigenesis and cancer metastasis. J Clin Invest. 2014;124(7):2891–908.
125. Li J, Yang X-M, Wang Y-H, Feng M-X, Liu X-J, Zhang Y-L, Huang S, Wu Z, Xue F, Qin W-X, et al. Monoamine oxidase a suppresses hepatocellular carcinoma metastasis by inhibiting the adrenergic system and its transactivation of EGFR signaling. J Hepatol. 2014;60(6):1225–34.
126. Satram-Maharaj T, Nyarko JN, Kuski K, Fehr K, Pennington PR, Truitt L, Freywald A, Lukong KE, Anderson DH, Mousseau DD. The monoamine oxidase-a inhibitor clorgyline promotes a mesenchymal-to-epithelial transition in the MDA-MB-231 breast cancer cell line. Cell Signal. 2014; 26(12):2621–32.
127. Owens DF, Kriegstein AR. Is there more to GABA than synaptic inhibition? Nat Rev Neurosci. 2002;3(9):715–27.
128. Z-a C, Bao M-y, Xu Y-f, R-p Z, H-b S, T-y C, He X-h. Suppression of human liver cancer cell migration and invasion via the GABAA receptor. Cancer Biol Med. 2012;9(2):90.
129. Neman J, Termini J, Wilczynski S, Vaidehi N, Choy C, Kowolik CM, Li H, Hambrecht AC, Roberts E, Jandial R. Human breast cancer metastases to the brain display GABAergic properties in the neural niche. Proc Natl Acad Sci. 2014;111(3):984–9.
130. Zhou H, Huang S. Role of mTOR signaling in tumor cell motility, invasion and metastasis. Curr Protein Pept Sci. 2011;12(1):30.
131. Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E. PI3K/AKT signaling pathway and cancer: an updated review. Ann Med. 2014;46(6):372–83.
132. Lodewyks C, Rodriguez J, Yan J, Lerner B, Lipschitz J, Nfon C, Rempel JD, Uhanova J, Minuk GY. GABA-B receptor activation inhibits the in vitro migration of malignant hepatocytes. Can J Physiol Pharmacol. 2011;89(6): 393–400.
133. Nie H, Cao Q, Zhu L, Gong Y, Gu J, He Z. Acetylcholine acts on androgen receptor to promote the migration and invasion but inhibit the apoptosis of human hepatocarcinoma. PLoS One. 2013;8(4):e61678.
134. Hung C-S, Peng Y-J, Wei P-L, Lee C-H, Su H-Y, Ho Y-S, Lin S-Y, Wu C-H, Chang Y-J. The alpha9 nicotinic acetylcholine receptor is the key mediator in nicotine-enhanced cancer metastasis in breast cancer cells. J Exp Clin Med. 2011;3(6):283–92.
135. Momi N, Ponnusamy MP, Kaur S, Rachagani S, Kunigal SS, Chellappan S, Ouellette MM, Batra SK. Nicotine/cigarette-smoke promotes metastasis of pancreatic cancer through α7nAchR-mediated MUC4 up-regulation. Oncogene. 2013;32(11):1384–95.
136. Lin G, Sun L, Wang R, Guo Y, Xie C. Overexpression of muscarinic receptor 3 promotes metastasis and predicts poor prognosis in non–small-cell lung cancer. J Thorac Oncol. 2014;9(2):170–8.
137. Xu R, Shang C, Zhao J, Han Y, Liu J, Chen K, Shi W. Activation of M3 muscarinic receptor by acetylcholine promotes non-small cell lung cancer cell proliferation and invasion via EGFR/PI3K/AKT pathway. Tumor Biol. 2015; 36(6):4091–100.
138. Belo A, Cheng K, Chahdi A, Shant J, Xie G, Khurana S, Raufman J-P. Muscarinic receptor agonists stimulate human colon cancer cell migration and invasion. Am J Physiol Gastrointest Liver Physiol. 2011;300(5):G749–60.
139. Xie G, Cheng K, Shant J, Raufman J-P. Acetylcholine-induced activation of M3 muscarinic receptors stimulates robust matrix metalloproteinase gene expression in human colon cancer cells. Am J Physiol Gastrointest Liver Physiol. 2009;296(4):G755–63.
140. Seckl MJ, Higgins T, Widmer F, Rozengurt E. [D-Arg1, D-Trp5, 7, 9, Leu11] substance P: a novel potent inhibitor of signal transduction and growth in vitro and in vivo in small cell lung cancer cells. Cancer Res. 1997; 57(1):51–4.
141. Guha S, Eibl G, Kisfalvi K, Fan RS, Burdick M, Reber H, Hines OJ, Strieter R, Rozengurt E. Broad-spectrum G protein–coupled receptor antagonist,[D-Arg1, D-Trp5, 7, 9, Leu11] SP: a dual inhibitor of growth and angiogenesis in pancreatic cancer. Cancer Res. 2005;65(7):2738–45.
142. Chen XY, Ru GQ, Ma YY, Xie J, Chen WY, Wang HJ, Wang SB, Li L, Jin KT, He XL, et al. High expression of substance P and its receptor neurokinin-1 receptor in colorectal cancer is associated with tumor progression and prognosis. OncoTargets Ther. 2016;9:3595–602.
143. Lang K, Bastian P. Neurotransmitter effects on tumor cells and leukocytes. Prog Exp Tumor Res. 2007;39:99–121.
144. Entschladen F, Lang K, Drell TL, Joseph J, Zaenker KS. Neurotransmitters are regulators for the migration of tumor cells and leukocytes. Cancer Immunol Immunother. 2002;51(9):467–82.
145. Bigioni M, Benzo A, Irrissuto C, Maggi CA, Goso C. Role of NK-1 and NK-2 tachykinin receptor antagonism on the growth of human breast carcinoma cell line MDA-MB-231. Anti-Cancer Drugs. 2005;16(10):1083–9.
146. Li X, Ma G, Ma Q, Li W, Liu J, Han L, Duan W, Xu Q, Liu H, Wang Z. Neurotransmitter substance P mediates pancreatic cancer perineural invasion via NK-1R in cancer cells. Mol Cancer Res. 2013;11(3):294–302.
147. Movafagh S, Hobson JP, Spiegel S, Kleinman HK, Zukowska Z. Neuropeptide Y induces migration, proliferation, and tube formation of endothelial cells bimodally via Y1, Y2, and Y5 receptors. FASEB J. 2006;20(11):1924–6.
148. Medeiros PJ, Al-Khazraji BK, Novielli NM, Postovit LM, Chambers AF, Jackson DN. Neuropeptide Y stimulates proliferation and migration in the 4T1 breast cancer cell line. Int J Cancer. 2012;131(2):276–86.
149. Tilan JU, Krailo M, Barkauskas DA, Galli S, Mtaweh H, Long J, Wang H, Hawkins K, Lu C, Jeha D, et al. Systemic levels of neuropeptide Y and dipeptidyl peptidase activity in patients with Ewing sarcoma–associations with tumor phenotype and survival. Cancer. 2015;121(5):697–707.
150. Hong SH, Tilan JU, Galli S, Izycka-Swieszewska E, Polk T, Horton M, Mahajan A, Christian D, Jenkins S, Acree R, et al. High neuropeptide Y release associates with Ewing sarcoma bone dissemination - in vivo model of site-specific metastases. Oncotarget. 2015;6(9):7151–65.
151. Dupouy S, Viardot-Foucault V, Alifano M, Souazé F, Plu-Bureau G, Chaouat M, Lavaur A, Hugol D, Gespach C, Gompel A, et al. The Neurotensin Receptor-1 pathway contributes to human Ductal breast cancer progression. PLoS One. 2009;4(1):e4223.
152. Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. Cell. 2009;139(2):267–84.
153. Ossipov MH. The perception and endogenous modulation of pain. Scientifica. 2012;2012
154. Jimenez-Andrade JM, Bloom AP, Stake JI, Mantyh WG, Taylor RN, Freeman KT, Ghilardi JR, Kuskowski MA, Mantyh PW. Pathological sprouting of adult nociceptors in chronic prostate cancer-induced bone pain. J Neurosci. 2010; 30(44):14649–56.
155. Stock AM, Powe DG, Hahn SA, Troost G, Niggemann B, Zanker KS, Entschladen F. Norepinephrine inhibits the migratory activity of pancreatic cancer cells. Exp Cell Res. 2013;319
156. Palm D, Lang K, Niggemann B, Drell TL, Masur K, Zaenker KS, Entschladen F. The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers. Int J Cancer. 2006;118
157. Barbieri A, Bimonte S, Palma G, Luciano A, Rea D, Giudice A, Scognamiglio G, La Mantia E, Franco R, Perdona S, et al. The stress hormone norepinephrine increases migration of prostate cancer cells in vitro and in vivo. Int J Oncol. 2015;47(2):527–34.
158. Azuma H, Inamoto T, Sakamoto T, Kiyama S, Ubai T, Shinohara Y, Maemura K, Tsuji M, Segawa N, Masuda H. γ-Aminobutyric acid as a promoting factor of cancer metastasis; induction of matrix metalloproteinase production is potentially its underlying mechanism. Cancer Res. 2003;63(23):8090–6.
159. Davis R, Rizwani W, Banerjee S, Kovacs M, Haura E, Coppola D, Chellappan S. Nicotine promotes tumor growth and metastasis in mouse models of lung cancer. PLoS One. 2009;4(10):e7524.
160. Hao J, Shi F-D, Abdelwahab M, Shi SX, Simard A, Whiteaker P, Lukas R, Zhou Q. Nicotinic receptor β2 determines NK cell-dependent metastasis in a murine model of metastatic lung cancer. PLoS One. 2013;8(2):e57495.
161. Esteban F, Muñoz M, González-Moles M, Rosso M. A role for substance P in cancer promotion and progression: a mechanism to counteract intracellular death signals following oncogene activation or DNA damage. Cancer Metastasis Rev. 2006;25(1):137–45.

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Review Article

Crosstalk between cancer and the neuro-immune system

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ABSTRACT

In the last decade, understanding of cancer initiation and progression has been given much attention with studies mainly focusing on genetic abnormalities. Importantly, cancer cells can influence their microenvironment and bi-directionally communicate with other systems such as the immune system. The nervous system plays a fundamental role in regulating immune responses to a range of disease states including cancer. Its dysfunction influences the progression of cancer. The role of the immune system in tumor progression is of relevance to the nervous system since they can bi-directionally communicate via neurotransmitters and neuropeptides, common receptors, and, cytokines. However, cross-talk between these cells is highly complex in nature, and numerous variations are possible according to the type of cancer involved. The neuro-immune interaction is essential in influencing cancer development and progression.

1. Introduction

Cancer is the major health related cause of death worldwide due to unhealthy lifestyle and other factors (Torre et al., 2015). Although the mechanisms of cancer progression have been extensively studied in the last decades, these have been predominantly focused on cellular pathways of proto-oncogene, tumor suppressor gene mutations and mechanisms by which immune cells can eliminate cancer cells (Kuol et al., 2017; Mravec et al., 2008a; Mravec et al., 2008b; Place et al., 2011). More recently, the impact of the tumor microenvironment in tumor cell invasion has attracted much interest (Place et al., 2011; Quail and Joyce, 2013). Multiple cellular and extracellular components within the tumor microenvironment, such as, immune cells, endothelial cells, mesenchymal stromal cells (fibroblasts and myofibroblasts), and their secretory products, exert active functions to modulate gene expression patterns of tumor cells which have an impact on their biological behavior (Markwell and Weed, 2015; Liguori et al., 2011; Sun, 2015). Invariable crosstalk among these components within the tumor microenvironment triggers pro-survival, invasion and metastatic spread of tumor cells (Hanahan and Coussens, 2012; Hanahan and Weinberg, 2011; Joyce and Pollard, 2009; Schmid and Varner, 2010). In addition, tumor cells interact with other cells to form organ-like structures that drive and promote cancer growth (Mravec et al., 2008a; Place et al., 2011). The interaction between the tumor microenvironment and the complex immune system plays a major role in tumor progression and as a result, is of concern in cancer treatment (Mravec et al., 2008a). However, it is only in recent years that the role of the neuro-immune network has surfaced as a major contributor to cancer progression. The mechanisms by which neuro-immune signaling in cancer influences its progression are not clear.

The nervous system plays a fundamental role in regulating immune responses to a range of disease states (Mancino et al., 2011). Its dysfunction influences the progression of disease outcomes including cancer cell growth. The role of the nervous system in tumor progression is of relevance to the immune system since they can bi-directionally communicate via neurotransmitters and neuropeptides, common receptors and cytokines (Lang and Bastian, 2007; Grivennikov et al., 2010). However, the crosstalk between these cells is highly complex in nature, and numerous variations are possible according to the type of cancer involved (Erin et al., 2015). The interaction of the nervous system in modulating immune responses, innervation of lymphoid organs, affects various neurotransmitters influencing cancer. This review presents an overview of the neuro-immune interaction in cancer progression: lymphoid organs innervation; neurotransmitters and immune

Abbreviations: Ach, acetylcholine; beta2-AR, beta2-adrenergic receptor; CAF, cancer associated fibroblasts; CNS, central nervous system; cAMP, cyclic adenosine monophosphate; DC, dendritic cells; DA, dopamine; DR, dopamine receptor; IL, interleukin; IFN-gamma, interferon gamma; LN, lymph node; MMP, matrix metalloproteinase; MCP-1, monocyte chemoattractant protein-1; MAIT, mucosal associated invariant T; mAChRs, muscarinic acetylcholine receptors; MDSCs, myeloid-derived suppressor cells; NK, natural killer cells; nAChRs, nicotinic acetylcholine receptors; PNS, peripheral nervous system; SP, substance P; TAMs, tumor-associated macrophages; TIL, tumor infiltrating lymphocytes; TNF-alpha, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor

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Table 1
Modulation of immune cells by neurotransmitters.

Neurotransmitter	Cancer type	Model	Function on immune cells	Ref.
Acetylcholine	Melanoma	B16 melanoma cells in vitro and intravenous injection of B16 cells in C57BL/6 mice	Nicotine inhibits NK cells capability to release cytokines and kill target cells via nAChR β2	(Hao et al., 2013)
Dopamine	Lung cancer	Human patients, in vitro dopamine concentration	Plasma levels of dopamine is elevated in lung carcinoma patients Dopamine inhibits the cytotoxicity and proliferation of T cells via the activation of dopamine receptor 1 mediated by intracellular cAMP	(Saha et al., 2001)
		Human blood from lung cancer patients (stage I-IV); Lewis lung carcinoma and B16 melanoma cells in vitro and their subcutaneous injection in C57BL/6 mice	Dopamine administration inhibits the suppressive function of $Gr\text{-}1^+$ $CD115^+$ MDSC on T cell proliferation via the activation of DR1 both in human blood in vitro and in vivo	(Wu et al., 2015)
	HNC	Human patients	Dopamine increases spontaneous migration of peripheral T cells in HNC patients	(Saussez et al., 2014)
Epinephrine	Leukemia	CRNK-16 leukemia cells in vitro and intravenous injection of CRNK-16 cells in F344 rats	Administration of epinephrine reduces NK activity	(Inbar et al., 2011)
Glutamate	HNC	Human patients	Glutamate increases spontaneous migration of peripheral T cells in HNC patients	(Saussez et al., 2014)
Norepinephrine	Breast cancer	66c14 mammary adenocarcinoma cell injected into mammary fat pad of BALB/c mice	NE acts on β2-AR enhancing $CD11b^+F4/80^+$ macrophage and $CD11b^+Gr^{int}Ly6Chi$ myeloid-derived suppressor cell infiltration	(Sloan et al., 2010)
Substance P	Breast cancer	4TBM cells in vitro and orthotopic injection of 4TBM cells in BALB/c mice	SP increases $CD4^+CD25$ cells in draining LNs Prevents tumor-induced degeneration of sensory nerve endings Alters CAFs releasing of angiogenic factors Enhances lymphokine-activated killer cell cytotoxicity, NK cell cytotoxicity, TNF-alpha, IL-10 and IL-12 secretion by macrophages Decreases tumor-infiltrating myeloid-derived suppressor T cells	(Erin et al., 2015)

DR1, dopamine receptor 1; NE, norepinephrine; SP, substance P; cAMP, cyclic adenosine monophosphate; nAChR, nicotinic acetylcholine receptor; IL-10, interleukin 10; IL-12, interleukin 12; CAFs, cancer associated fibroblasts; TNF-alpha, tumor necrosis factor-α; NK, natural killer cells; LN, lymph node; beta2-AR, beta2-adrenergic receptor; HNC, head and neck cancer.

cells in cancer, tumor associated immune cells and the nervous system.

2. Innervation of lymphoid organs

The link between the nervous and immune systems is via direct innervation of lymphoid organs. In particular, sympathetic noradrenergic fibers innervate primary (thymus and bone marrow) and secondary (lymph nodes and spleen) lymphoid organs (ThyagaRajan and Priyanka, 2012). In lymphoid organs, the immune responses against pathogens or tissue damage are altered by the release of neuropeptides and neurotransmitters such as, neuropeptide Y, substance P (SP), norepinephrine and dopamine from nerve endings (ThyagaRajan and Priyanka, 2012; Stojanovska et al., 2015). Dysregulation of this interaction promotes pathogenesis and progression of many diseases including cancer (ThyagaRajan and Priyanka, 2012). The spleen plays an important role in response to pathogens or tissue damage; however, its response to cancer has been less empathized. In systemic inflammation, the vagal afferents activate the central nervous system (CNS) which triggers the efferent via the celiac ganglion and, as a result, activates immune cells in the spleen (as reviewed by (Matteoli and Boeckxstaens, 2013)). As a consequence, the activation of adrenergic fibers innervating the spleen results in the release of norepinephrine leading to the activation of T cells secreting acetylcholine.

The spleen accumulates monocytic and granulocytic precursors that directly replenish tumor-associated macrophages (TAMs) and neutrophils, as noted in lung adenocarcinoma (Cortez-Retamozo et al., 2012; Zhang et al., 2011). Moreover, the cords of the splenic subcapsule red pulp contain a reservoir of monocyte subsets (e.g. Ly-$6C^{high}$ and Ly-$6C^{low}$) that are promptly released in the bloodstream following acute injury (Swirski et al., 2009). Therefore, it can be speculated that the spleen would detect cancer as a pathogen and respond to it in a similar manner. However, cancer invades tissues without the spleen influencing it, in the same way as viruses invade target tissues by inactivating immune responses. In fact, stress or central inflammatory stimulation of the sympathetic nervous system (SNS) inhibits splenic macrophage function, thus, beta-adrenergic mechanisms influence splenic macrophages (Nance and Sanders, 2007). This supports the speculation that the spleen's response to pathogens is via catecholamine release which acts on beta-adrenergic receptors to inhibit splenic macrophage activity. However, specific mechanisms of this action in cancer are not clear.

Detailed neuro-anatomical description of lymph node (LN) innervation is scarce (ThyagaRajan and Priyanka, 2012), however, sympathetic fibers in LNs have been reported (Nance and Sanders, 2007). In LNs, immune responses to antigens are initiated (Nance and Sanders, 2007; Ng and Chalasani, 2010). During antigen detection, immune cells (dendritic cells (DCs), T cells, etc.) are recruited into regional LNs which activate immune responses against the antigen. The decision process within LNs to either induce an active immune response or be tolerant is not clear, although in most instances an active immune response is initiated (Buettner and Bode, 2012; Koning and Mebius, 2012; Swerdlin et al., 2008). Just like other foreign antigens, cancer cells can escape LN surveillance. It is suggested that the lack of LNs innervation may be a contributing factor to cancer escaping immune surveillance. Thus, information of LN innervation could aid in the understanding of the decision process within LNs to induce protective responses and its lack of response in cancer initiation. Furthermore, understanding the interaction between LN and cancer may aid therapeutic modalities at the early stages of disease.

The SNS regulates bone marrow function (Nance and Sanders, 2007). Innervation within the bone marrow is also scarce and likely due to the fact that there is close contact with surrounding mineralized bone which receives sympathetic and sensory innervation (Elenkov et al., 2000). However, sensory fibers containing SP and calcitonin gene-related peptide together with noradrenergic sympathetic fibers and veins are distributed throughout the bone marrow and surrounding bone.

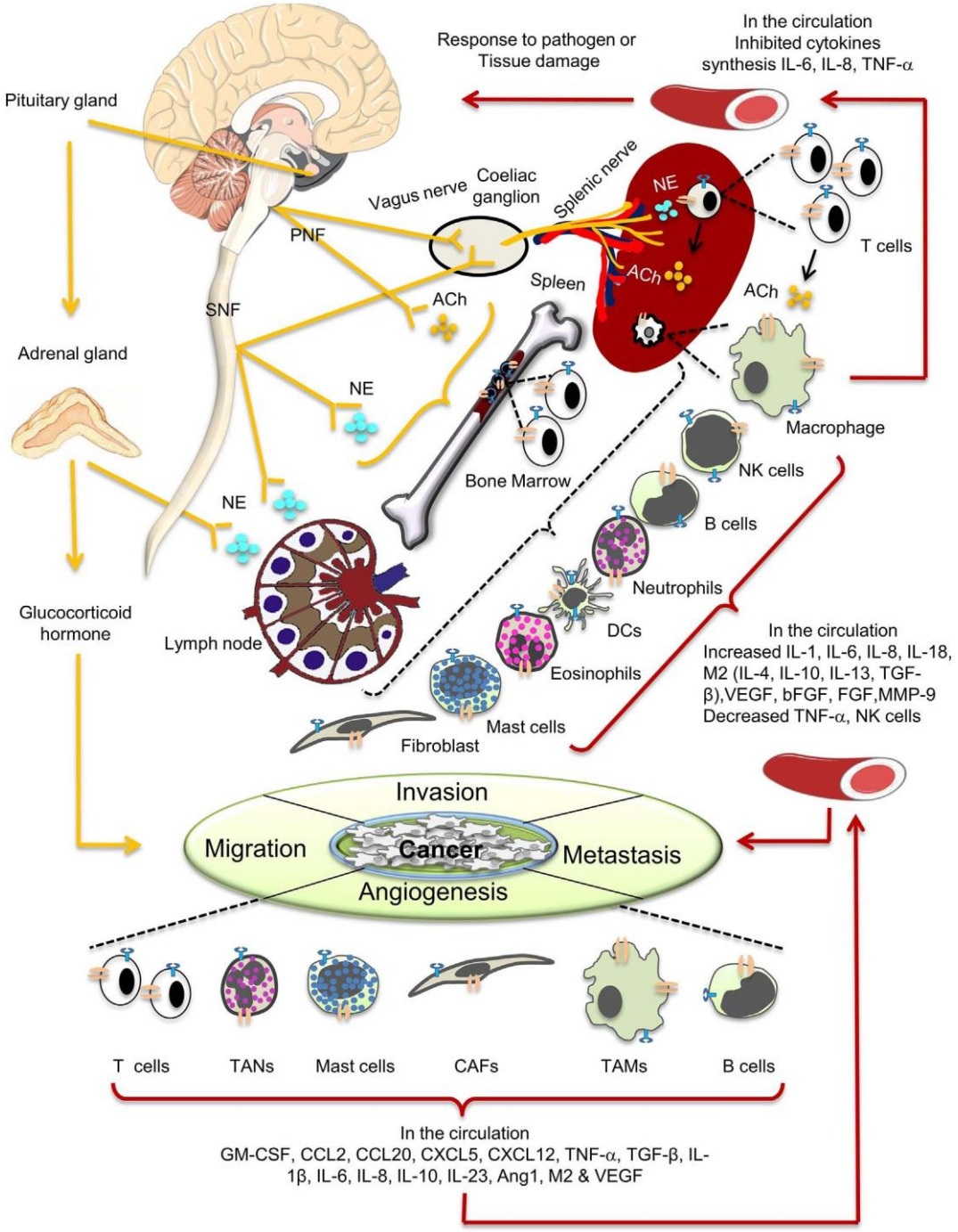

Fig. 1. Schematic diagram highlighting the critical function of the nervous system in modulating immune responses to cancer. ACh released from vagus nerve in macrophages binds to α7 nicotinic receptors on tissue macrophages and inhibits the release of pro-inflammatory cytokines. In the functional immune response to pathogen invasion or tissue damage these are recognized by macrophages within the spleen which triggers secretion of pro-inflammatory cytokines. Stress initiates a cascade of responsive neural pathways in the central nervous system, leading to the activation of sympathetic nervous systems and HPA axis. The stress response results in release of catecholamines (principally norepinephrine and epinephrine) and glucocorticoids from sympathetic nerve fibers located within organs and the adrenal medulla. Prolonged exposure to catecholamines under chronic stress importantly affects the process of tumor development. Glucocorticoids are associated with a decreased immune response, which further enhances tumor progression. Most immune cells and cancer cells express adrenergic and cholinergic receptors. Through these receptors the nervous system is able to communicate with cancer cells via the release of neurotransmitters, cytokines and chemokines from both ends which eventually influences tumor growth. Ach, acetylcholine; HPA, hypothalamic-pituitary-adrenal; IL, interleukin; NK, natural killer cells; NE, norepinephrine; PNF, peripheral nerve fibers; SNF, sympathetic nerve fibers; TNF-alpha, tumor necrosis factor-alpha; Ang1, angiopoietin 1; bFGF, basic fibroblast growth factor.

Distinguishing between innervated bone and bone marrow is not clear (ThyagaRajan and Priyanka, 2012), even though, in rodents bone marrow innervation occurs late in fetal life, just prior to hemopoietic activity. Understanding innervation of the bone marrow will enhance our knowledge of bone marrow cancers including but not limited to, lymphoma, leukemia, and myeloma.

3. Neurotransmitters and immune cells in cancer

Neurotransmitters play an essential role in the modulation of immunity. A number of immune cells such as, T cells, DCs, natural killer (NK) cells, microglia and myeloid-derived suppressor cells (MDSCs) express cell surface neurotransmitter receptors including substance P

(SP), glutamate, gamma-aminobutyric acid (GABA), serotonin, dopamine, epinephrine, norepinephrine and acetylcholine (Table 1, Fig.1) (St-Pierre et al., 2016; Hao et al., 2013; Saussez et al., 2014; Wu et al., 2015; Liu et al., 2016). Furthermore, studies have shown that various cancers express receptors for different neurotransmitters which play an essential role in the control of tumor progression (Hao et al., 2013; Saussez et al., 2014; Dolma et al., 2016; Stepulak et al., 2009; Banda et al., 2014).

3.1. Substance P

Substance P (SP) is expressed in both the central and peripheral nervous systems (PNS) and plays an essential role in the neuroimmune system crosstalk. Of the sensory neuropeptides, SP is distributed widely and regulates immune functions, including that of B and T cells (Esteban et al., 2006) and cytokine secretion by monocytes (Erin et al., 2015) and macrophages. Binding of SP to its receptor NK1 triggers activation of intracellular pathways including cAMP, MEK, ERK1/2, mTOR and NF-kB resulting in proinflammatory cytokine production (Di Giovangiulio et al., 2015). In addition, SP enhances lymphocyte proliferation and lymphokine-activated killer cell cytotoxicity, NK cell cytotoxicity, augments tumor necrosis factor alpha (TNF-alpha), interleukin (IL)-10 and IL-12 secretion by macrophages, and, decreases the number of tumor-infiltrating MDSCs (Erin et al., 2015). The effects of chronic administration of low dose SP to the brain in a murine model of metastatic breast cancer co-treated with radiation treatment, increased the antigenicity of cancer cells (Erin et al., 2013). Hence, SP through neuro-immune modulation can avert an immunosuppressive tumor microenvironment and consequently inhibiting metastatic growth.

3.2. Glutamatergic, GABAergic and serotonergic signaling

Glutamate is the principal excitatory neurotransmitter that regulates synaptic and cellular activity in the CNS via binding to its receptors including metabotropic glutamate receptors (mGluRs) or ionotropic glutamate receptors (iGluRs). In addition, glutamate also plays a fundamental role in the neuroimmune system crosstalk and it modulates immune cell functions via the expression of its functional receptors on immune cells (Poulopoulou et al., 2005). Furthermore, immune cells such as T cells, DC, monocytes and macrophages release glutamate where they act in both an autocrine and paracrine fashion (Ganor and Levite, 2014). Although the role of glutamate and its receptors is well-established in neurological disorders and neuroprotection, it has become evident that glutamate plays a functional role in cancer via regulating immune cells as noted in head and neck, glioma, melanoma, gastric, prostate, squamous cell carcinoma, colorectal and breast cancers (Saussez et al., 2014; Stepulak et al., 2009; Banda et al., 2014). For instance, in head and neck cancer patients, elevated levels of glutamate increase spontaneous migration of peripheral T cells (Saussez et al., 2014).

GABA is the main inhibitory neurotransmitter in the CNS that exerts its physiologic effects in non-neuronal peripheral tissues and organs via the activation of ionotropic ($GABA_A$ or $GABA_C$) and metabotropic ($GABA_B$) receptors (Young and Bordey, 2009). GABA plays a functional role in the proliferation, migration and differentiation of cells including tumorigenic cells (Jiang et al., 2012). It has been noted that GABA mediates its inhibitory effect through $GABA_A$ receptor. For instance, GABA inhibits hepatocellular carcinoma cell migration through the activation of $GABA_A$ receptor (Z-a et al., 2012). In addition, administration of GABA agonist Nembutal suppresses tumor metastasis in colon cancer (Thaker et al., 2005). However, there are studies demonstrating that $GABA_A$ receptor enhances metastasis. The activation of $GABA_A$ receptors upregulates brain metastasis of breast cancer patients (Neman et al., 2014). It is speculated that since GABA mediates it functional effect on T lymphocytes and DC through the activation of GABA-A (Jin et al., 2013), this may explain the inconsistency in findings.

5-hydroxytryptamine (5-HT), also known as serotonin, is a monoamine neurotransmitter synthesized in the serotonergic neurons in the brain and it plays an essential role in the modulation of immune response. 90% of the body's 5-HT is secreted by enterochromaffin cells of the gut mucosa. 5-HT regulates a wide range of behavioral, cognitive and physiological functions in pathological disease including cancer (Shajib and Khan, 2015). In mouse models of melanoma, administration of selective serotonin reuptake inhibitors decreases tumor growth via enhancing mitogen-induced T cell proliferation, IL-1beta production, and by inhibiting IFN-gamma and IL-10 production (Grygier et al., 2013). Furthermore, in a mouse model of colon cancer allografts, serotonin regulates macrophages-mediated tumor angiogenesis (Nocito et al., 2008). These findings demonstrate the essential role of glutamate, GABA and serotonin in regulating tumor growth; however, further mechanistic studies are required.

3.3. Dopaminergic signaling

Dopamine is an important monoamine neurotransmitter in the CNS, however, it also plays a role in immune modulation. Elevated levels of dopamine increase spontaneous migration of peripheral T cells in head and neck cancer patients (Saussez et al., 2014). Dopamine inhibits cytotoxicity and proliferation of T cells via the activation of dopamine receptor 1 (DR1) mediated by intracellular cAMP in lung cancer (Saha et al., 2001). Dopamine treatment induces M2 (pro-tumor phenotype) shift to M1 (anti-tumor phenotype) of RAW264.7 cells and mouse peritoneal macrophage in rat C6 glioma (Qin et al., 2015). Similarly, in human blood samples from lung cancer patients (stage I-IV) and mouse models using Lewis lung carcinoma and B16 melanoma cell lines, application of dopamine inhibits the effects of MDSC on T cell proliferation via the activation of DR1 (Wu et al., 2015), suggesting a possible mechanism of inhibition by dopamine. Moreover, inhibition of DR3 signaling in DCs enhances antigen cross-presentation to CD8 + T cells favoring anti-tumor immunity (Figueroa et al., 2017). Dopamine acting on DRD4 causes impairment in the endolysosomal system, a block in autophagic flux, and eventual cell death in glioblastoma (Dolma et al., 2016). It has been shown that CD8 + T cells express functional dopamine receptors DR1-DR5 in both humans and mice, and dopamine plays a significant role in migration and homing of naive CD8 + T cells via DR3 (Levite, 2016; Watanabe et al., 2006). Moreover, dopamine activates resting effector T cells (Teffs) and suppresses regulatory T cells (Tregs) (Watanabe et al., 2006). Hence, it can be speculated that dopamine inhibits tumor growth via regulating DC antigen presentation to CD8 + T cells. Furthermore, screening cancer patients that present with elevated levels of dopamine for DCs and CD8 + T cells could aid in delivering an effective targeted therapy.

3.4. Beta-adrenergic signaling

SNS activation regulates an array of cancer-related molecular pathways via the stimulation of beta-adrenergic signaling and its receptors expressed by tumor cells, immune and vascular cells (Cole, 2013; Cole and Sood, 2012). beta-adrenergic receptors mediate a range of catecholamine effects on target cells and immune cells, as well as cancer cells, i.e. breast cancer cells (Lee et al., 2010; Chanmee et al., 2014; Laoui et al., 2011). Several cellular and molecular processes (such as inflammation, angiogenesis, epithelial mesenchymal transition and apoptosis) mediate beta-adrenergic influences on tumor progression (Cole and Sood, 2012) and recruitment of macrophages into primary tumors (Entschladen et al., 2004; Sloan et al., 2010). Moreover, beta-adrenergic signaling influences the secretion of pro-inflammatory cytokines (IL-1, IL-6 and IL-8) by immune cells (Entschladen et al., 2004; Nilsson et al., 2007; Cole et al., 2010; Shahzad et al., 2010), upregulation of vascular endothelial growth factor (VEGF) resulting in increased angiogenesis (Yang et al., 2006), matrix metalloproteinase (MMP) related increase of tissue invasion (Yang et al., 2006; Landen Jr.

et al., 2007), tumor cell assembly and motility (Palm et al., 2006; Lang et al., 2004). Furthermore, beta-adrenergic signaling suppresses CD8 T cell and NK cell responses (Inbar et al., 2011) and inhibits the expression of type I interferons (Sloan et al., 2010; Collado-Hidalgo et al., 2006). In fact, in murine metastatic mammary adenocarcinoma, beta-adrenergic receptor agonist suppressed NK cell activity resulting in increased lung metastasis (Erin et al., 2004). In addition, either stress or pharmacological beta-adrenergic stimulation results in increased macrophage infiltration and cancer metastasis which can be prevented by injection of a beta-adrenergic antagonist, propranolol (Sloan et al., 2010). Furthermore, the use of beta2-adrenergic agonist in experimental animals reverse muscle wasting (cachexia) associated with cancer (Busquets et al., 2004). Catecholamines can induce apoptosis of lymphocytes, alter the distribution of NK cells and suppress NK cell activity, which are all required for anti-tumor immunity (Shi et al., 2013), leading to tumor cell escape mechanisms. Thus, persistent release of neurotransmitters from nerve terminals may promote tumor growth and metastasis via modulation of the immune system.

3.5. Cholinergic SIGNALING

Modulation of the immune system by the sympathetic nervous system (SNS) has been extensively studied (Matteoli and Boeckxstaens, 2013; Bellinger et al., 2008; Forsgren et al., 2009). However, the role of the parasympathetic nervous system has gained attention only recently (Tracey, 2002). Inflammatory mediators can activate sensory nerves that send signals regarding inflammation to the CNS, which in turn leads to the release of neuromediators modulating local inflammation and influencing immune cells (Tracey, 2009). Consequently, the nervous system can regulate immune responses in peripheral tissues and restore local immune homeostasis (Huston et al., 2006). Since inflammatory signals are important for tumor progression in both the early and late stages, the anti-inflammatory role of the vagus nerve may play an important role in tumorigenesis (Gidron et al., 2005).

It has been established that acetylcholine (ACh) acting on α7 nicotinic receptors (nAChRs) modulates splenic macrophages and inhibits TNF-alpha production in the spleen (Wang et al., 2003; Rosas-Ballina et al., 2011). In addition, vagus nerve activation stimulates ACh synthesis by splenic T lymphocytes leading to inhibition of cytokine production (Rosas-Ballina et al., 2011). In lipopolysaccharide-induced inflammation in C56BL/6J mice, activation of α7 and α9 nAChRs expressed by bone marrow cells stimulates secretion of anti-inflammatory cytokines (IL-10 and transforming growth factor beta (TGF)-beta) and inhibits production of pro-inflammatory cytokines (TNF-alpha, IL-1beta and IL-12) (St-Pierre et al., 2016). Similarly, secretion of TNF-alpha, IL-1beta, IL-6 and IL-18 induced by endotoxin was significantly inhibited by ACh and nicotine in human macrophage cultures (Borovikova et al., 2000). ACh receptors including both muscarinic (mAChRs) and nAChRs are functionally expressed by cancer cells (Song and Spindel, 2008; Paleari et al., 2008; Song et al., 2007). Moreover, cancer cells synthesize and secrete ACh (Song et al., 2007). In a mouse bearing B16 melanoma cells, administration of nicotine inhibits the release of cytokines and cell killing by NK cells via nAChR β2 (Hao et al., 2013). Overexpression of α7 nAChRs by cancer cells (i.e. human colon cancer cell line HT-29) promotes cancer angiogenesis (Wong et al., 2007; Pettersson et al., 2009), cell proliferation and metastasis (Davis et al., 2009; Chen et al., 2008; Wei et al., 2009; Chen et al., 2011; Lee et al., 2011). α9 nAChRs are reported to play a crucial role in breast cancer development; the correlation between expression levels of α9 nAChR mRNA and disease outcome was found in breast cancer patients (Lee et al., 2010). On the other hand, it has been demonstrated that mAChRs antagonists inhibit small cell lung carcinoma growth both in vitro and in vivo via inhibiting MAPK pathway (Song et al., 2007). In BALB/c mice bearing LMM3 mammary adenocarcinoma cells, tumor macrophages express M1 and M2 mAChRs which trigger arginine metabolic pathway leading to tumor angiogenesis (de la Torre et al., 2005). Understanding the principal mechanisms of cholinergic signaling in regulating the immune system may highlight the significance of ACh inhibitors in cancer therapy.

4. Tumor associated immune cells and the nervous system

The role of nervous system in modulating tumor-associated immune (TAI) cells is not well understood. However, various TAI cells within tumor microenvironment play essential role in promoting tumor growth. It could be speculated that nervous system modulates TAI cells in its original form as normal immune cells.

4.1. TUMOR-ASSOCIATED MACROPHAGES

Tumor associated macrophages (TAMs) play a role in beta-adrenergic signaling pathways, by accelerating angiogenesis, chemokine secretion to attract immune and tumor cells, secretion of pro-inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-alpha) and escape of anti-tumor responses (DeNardo et al., 2011; DeNardo and Coussens, 2007; Mantovani et al., 2008). Hence, TAMs are sensitive to sympathetic signaling and raise the likelihood that stress-response pathways influence macrophage infiltration within the tumor microenvironment and, as a result, enhance metastasis. In the early or regression stages of tumors, TAMs, in particular, M1 macrophages (pro-inflammatory; releasing IL-1β, IL-6, IL-12, TNF-alpha, monocyte chemoattractant protein-1 (MCP-1)) inhibit angiogenesis and activate an anti-tumor immune response. In contrast, TAMs shift to a M2 phenotype (anti-inflammatory, releasing IL-1 receptor antagonist, TGF-beta, IL-4, IL-10, IL-13) which enhance tumor angiogenesis in advanced tumors (Müller et al., 2001; Kim et al., 2012; Lewis and Pollard, 2006; Dirkx et al., 2006; Roland et al., 2009), tumor growth (Lewis and Pollard, 2006), invasion, migration (Lin et al., 2001), metastatic spread (Oosterling et al., 2005) and possess immunosuppressive activities which are regulated by neuromediators (Ley et al., 2010). In breast cancer, infiltrating TAMs correlate with higher tumor and vascular grade (Laoui et al., 2011) and increased necrosis (Campbell et al., 2011) leading to poor prognosis (Laoui et al., 2011; DeNardo et al., 2011; Mukhtar et al., 2011). In fact, eliminating macrophages from the tumor site, either genetically or therapeutically, results in reduced tumor progression in breast cancer (Laoui et al., 2011). However, detailed understanding of the neuro-immune interaction influencing TAMs in human breast cancer needs further elucidation.

4.2. CANCER-ASSOCIATED FIBROBLASTS

The role of nervous system in modulating cancer associated fibroblasts (CAFs) remains scare. To understand how nervous system might modulate CAFs, studies need to understand the origin of CAFs. It is believe that CAFs originated from bone marrow-derived mesenchymal stem cells, fibroblasts or cancer cells that undergo endothelial - or epithelial -mesenchymal transition (Buchsbaum and SY, 2016). Therefore, it is possible that nervous system may regulate CAFs via modulating bone marrow-derived mesenchymal stem cells or fibroblasts. CAFs are the key constituent cells within the tumor microenvironment which interact with cancer cells promoting tumor growth and metastasis (Smith and Kang, 2013). For example, in the tumor microenvironment of 4T1 metastatic breast cancer model, in vivo abolition of CAFs causes Th2 shift to Th1 polarization which is characterized by increased expression of IL-2 and IL-17, suppressed TAMs, T regulatory cells, MDSCs and decreased angiogenesis (Liao et al., 2009). In addition, CAFs enhanced the aggressive phenotype of T47D, MCF-7 and MDA-MB-231 breast cancer cells via epithelial mesenchymal transition induced through paracrine TGF-beta signaling (Yu et al., 2014). Similarly, in human sample of squamous cell carcinoma, CAFs mediate angiogenesis and inflammation via employing macrophages and stimulating angiogenesis, consequently enhancing tumor growth (Erez

et al., 2010). These findings demonstrate significant importance of CAFs in mediating tumor progression. Understanding the origin of CAFs could lead to better understanding of how nervous system modulates it, resulting in better therapies design.

4.3. Tumor-infiltrating lymphocytes

Nervous system plays essential role in the modulation of T cell. T cell expressed adrenergic and cholinergic receptors creating a communication loop with the nervous system. Tumor infiltrating lymphocytes (TILs) particularly CD8 + T cells are associated with positive prognostic relevance in various tumors. For example, in a prospective-retrospective study of a primary triple-negative breast cancer demonstrate elevated levels of TILs present at diagnosis were considerably associated with reduced distant recurrence rates (Loi et al., 2014; Ali et al., 2014). Similar findings are reported in patients with oro- and hypopharyngeal carcinoma showing increased expression of intraepithelial CD8 + TIL in metastatic tumors to be associated with favorable outcome (Pretscher et al., 2009). In prostate cancer, infiltration of CD4 + T cells enhances LNCaP, CWR22RV1 and C4-2 cell invasion and metastasis via fibroblast growth factor 11 → miRNA-541 → androgen receptor → matrix metalloproteinase 9 signaling (Hu et al., 2015). In addition to the presence of T lymphocytes at the tumor site, B lymphocyte infiltration also plays a role within the tumor microenvironment. Infiltration of B cell subset called tumor evoked Bregs (B regulatory) plays a crucial role in lung metastasis by converting CD4 + T cell to Foxp3 + Treg cells through induction of TGF-beta-dependent which promote immune escape in the 4T1 tumor-bearing mouse model of breast cancer (Olkhanud et al., 2011). Similarly, B cell infiltration facilitates the switch of M1 macrophages to a pro-tumoral M2 phenotype via IL-10 secretion (Sica et al., 2010). On the contrary, elevated expression of peritumoral B-cells in lymph node metastases in patients with oro- and hypopharyngeal carcinoma is associated with favorable outcome (Pretscher et al., 2009). Correspondingly, tumor-infiltrating B cells correlate with improved survival outcome in the immunoreactive ovarian cancer subtype and HER2-enriched and basal-like breast cancer subtypes (Iglesia et al., 2014). Although B cells normally do play active roles in anti-tumor immunity; these studies have demonstrated the capacity of the tumor microenvironment to modify immune function to promote tumor progression.

4.4. Eosinophils

Eosinophils release an array of cytokines, including IL-1beta, TNF-alpha, and interferon gamma and eosinophil derived neurotoxin that are potentially toxic to nerve cells. Eosinophils localize to nerves (eosinophil-nerve interaction) and are associated with enhanced nerve activity (Morgan et al., 2004). In addition, eosinophils infiltrate cancer cells leading to either favorable or unfavorable prognosis (Ishibashi et al., 2006). For instance, in Hodgkin's lymphoma, eosinophils infiltration correlate with an unfavorable prognosis (von Wasielewski et al., 2000) whereas in colon cancer the presence of eosinophils leads to a favorable prognosis (Sakkal et al., 2016; Harbaum et al., 2015). However, the role of eosinophils and nerve interactions in cancer aetiology is not clear. The presence of eosinophils in necrotic regions of the tumor suggests that they may have anti-tumor effects associated with a favorable prognosis (Mattes et al., 2003; Minton, 2015). Conversely, it has been noted that eosinophils may contribute to tumor invasion via activation of gelatinase (Harbaum et al., 2015; Mattes et al., 2003; Cormier et al., 2006; Davis and Rothenberg, 2014). Furthermore, eosinophils at the tumor site can influence angiogenesis via VEGF secretion (Stockmann et al., 2014). Moreover, TNF-alpha-stimulated eosinophils release pro-angiogenic factors such as, basic fibroblast growth factor, IL-6, IL-8, platelet-derived growth factor and MMP-9 (Cormier et al., 2006). However, pro-angiogenic factors such as IL-15 and TNF-alpha-stimulated eosinophils have only been noted, and theirs role in tumors is not clear (Schmid and Varner, 2010). Secretion of eosinophilic granular proteins has been noted in breast cancer (Szalayova et al., 2016) and is associated with increased survival. However, Amini and colleagues reported lack of eosinophils in breast cancer (Amini et al., 2007) which warrants further research into eosinophil infiltration in breast cancer.

4.5. Mucosal-associated invariant T cells

The role of nervous system in regulating mucosal associated invariant (MAIT) cells is not clear. However, since MAIT cells are subset of T cells, it may be in a similar manner of how T cells get modulated, that MAIT cells may be regulated. MAIT cells have anti-microbial specificity (Howson et al., 2015; Serriari et al., 2014; Ussher et al., 2014) and are present in a number of cancers (Peterfalvi et al., 2008). Their presence correlates with the level of pro-inflammatory cytokines within the tumor microenvironment (Peterfalvi et al., 2008), suggesting they have anti-cancer functions. However, enhanced expression of tumor-associated MAIT cells associates with poor prognosis in colorectal cancer contradicting norm that MAIT cells may have anti-tumor effects (Zabijak et al., 2015). In fact, tumor-associated MALT cells are increased while circulating CD8 + MAIT cells decreased in advanced colorectal cancer patients (Ling et al., 2016). Co-culture of HCT116 cells with MAIT cells stimulated with phorbol 12-myristate 13-acetate results in enhanced TNF-alpha, IFN-gamma and IL-17 expression and reduced HCT116 cells feasibility, suggesting MAIT cells may contribute to colorectal cancer immunosurveilance (Ling et al., 2016). Whether this effects of MAIT cells is cancer type specific, warrant further research. Thus, considering the key role of MAIT cells in response to infections, understanding their potential in cancer would aid in a better understanding of the cancer microenvironment.

5. Conclusion

The release of neurotransmitters by sympathetic nerve fibers as a result of chronic stress assists the tumor microenvironment to promote tumor growth and progression via the expression of cytokines and tumor-associated immune cells. Moreover, nervous system regulation of metastasis emphasizes the significance of determining metastatic tumor features in a physiological context. To date, most studies in determining the role of the nervous system in the modulation of cancer cell development and metastasis either use cell lines or animal models. Despite the increasing interest in the role the nervous system plays in cancer development and progression, the knowledge in this area is scarce. Limited studies are available from cancer patients at different stages of disease. Understanding molecular mechanisms by which the nervous system modulates tumor growth and progression holds a great prospect. Revealing the interplay between the nervous and immune systems in cancer may open new avenues for understanding mechanisms of tumor development and progression, identification of new biomarkers for cancer diagnosis and prognosis, and defining novel targets for therapeutic interventions.

Conflict of interest

The authors confirm no conflict of interest.

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References

Ali, H., Provenzano, E., Dawson, S.-J., Blows, F., Liu, B., Shah, M., Earl, H., Poole, C., Hiller, L., Dunn, J., 2014. Association between CD8 + T-cell infiltration and breast cancer survival in 12 439 patients. Ann. Oncol. 25 (8), 1536–1543 (mdu191).

Amini, R.-M., Aaltonen, K., Nevanlinna, H., Carvalho, R., Salonen, L., Heikkilä, P., Blomqvist, C., 2007. Mast cells and eosinophils in invasive breast carcinoma. BMC Cancer 7 (1), 165.

Banda, M., Speyer, C.L., Semma, S.N., Osuala, K.O., Kounalakis, N., Torres Torres, K.E., Barnard, N.J., Kim, H.J., Sloane, B.F., Miller, F.R., et al., 2014. Metabotropic glutamate receptor-1 contributes to progression in triple negative breast cancer. PLoS One 9 (1), e81126.

Bellinger, D.L., Millar, B.A., Perez, S., Carter, J., Wood, C., ThyagaRajan, S., Molinaro, C., Lubahn, C., Lorton, D., 2008. Sympathetic modulation of immunity: relevance to disease. Cell. Immunol. 252 (1), 27–56.

Borovikova, L.V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R., Wang, H., Abumrad, N., Eaton, J.W., Tracey, K.J., 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405 (6785), 458–462.

Buchsbaum, R.J., SY, Oh., 2016. Breast cancer-associated fibroblasts: where we are and where we need to go. Cancer 8 (2).

Buettner, M., Bode, U., 2012. Lymph node dissection–understanding the immunological function of lymph nodes. Clin. Exp. Immunol. 169 (3), 205–212.

Busquets, S., Figueras, M.T., Fuster, G., Almendro, V., Moore-Carrasco, R., Ametller, E., Argiles, J.M., Lopez-Soriano, F.J., 2004. Anticachectic effects of formoterol: a drug for potential treatment of muscle wasting. Cancer Res. 64 (18), 6725–6731.

Campbell, M.J., Tonlaar, N.Y., Garwood, E.R., Huo, D., Moore, D.H., Khramtsov, A.I., Au, A., Baehner, F., Chen, Y., Malaka, D.O., 2011. Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. Breast Cancer Res. Treat. 128 (3), 703–711.

Chanmee, T., Ontong, P., Konno, K., Itano, N., 2014. Tumor-associated macrophages as major players in the tumor microenvironment. Cancer 6 (3), 1670–1690.

Chen, R.-J., Ho, Y.-S., Guo, H.-R., Wang, Y.-J., 2008. Rapid activation of Stat3 and ERK1/2 by nicotine modulates cell proliferation in human bladder cancer cells. Toxicol. Sci. 104 (2), 283–293.

Chen, C.-S., Lee, C.-H., Hsieh, C.-D., Ho, C.-T., Pan, M.-H., Huang, C.-S., S-H, Tu, Wang, Y.-J., Chen, L.-C., Chang, Y.-J., 2011. Nicotine-induced human breast cancer cell proliferation attenuated by garcinol through down-regulation of the nicotinic receptor and cyclin D3 proteins. Breast Cancer Res. Treat. 125 (1), 73–87.

Cole, S.W., 2013. Nervous system regulation of the cancer genome. Brain Behav. Immun. 30 (Suppl), S10–18.

Cole, S.W., Sood, A.K., 2012. Molecular pathways: beta-adrenergic signaling in cancer. Clin. Cancer Res. 18 (5), 1201–1206.

Cole, S.W., Arevalo, J.M., Takahashi, R., Sloan, E.K., Lutgendorf, S.K., Sood, A.K., Sheridan, J.F., Seeman, T.E., 2010. Computational identification of gene–social environment interaction at the human IL6 locus. Proc. Natl. Acad. Sci. 107 (12), 5681–5686.

Collado-Hidalgo, A., Sung, C., Cole, S., 2006. Adrenergic inhibition of innate anti-viral response: PKA blockade of type I interferon gene transcription mediates catecholamine support for HIV-1 replication. Brain Behav. Immun. 20 (6), 552–563.

Cormier, S.A., Taranova, A.G., Bedient, C., Nguyen, T., Protheroe, C., Pero, R., Dimina, D., Ochkur, S.I., O'Neill, K., Colbert, D., et al., 2006. Pivotal advance: eosinophil infiltration of solid tumors is an early and persistent inflammatory host response. J. Leukoc. Biol. 79 (6), 1131–1139.

Cortez-Retamozo, V., Etzrodt, M., Newton, A., Rauch, P.J., Chudnovskiy, A., Berger, C., Ryan, R.J., Iwamoto, Y., Marinelli, B., Gorbatov, R., et al., 2012. Origins of tumor-associated macrophages and neutrophils. Proc. Natl. Acad. Sci. U. S. A. 109 (7), 2491–2496.

Davis, B.P., Rothenberg, M.E., 2014. Eosinophils and cancer. Cancer Immunol. Res. 2 (1), 1–8.

Davis, R., Rizwani, W., Banerjee, S., Kovacs, M., Haura, E., Coppola, D., Chellappan, S., 2009. Nicotine promotes tumor growth and metastasis in mouse models of lung cancer. PLoS One 4 (10), e7524.

DeNardo, D.G., Coussens, L.M., 2007. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. Breast Cancer Res. 9 (4), 212.

DeNardo, D.G., Brennan, D.J., Rexhepaj, E., Ruffell, B., Shiao, S.L., Madden, S.F., Gallagher, W.M., Wadhwani, N., Keil, S.D., Junaid, S.A., 2011. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discov. 1 (1), 54–67.

Di Giovangiulio, M., Verheijden, S., Bosmans, G., Stakenborg, N., Boeckxstaens, G.E., Matteoli, G., 2015. The neuromodulation of the intestinal immune system and its relevance in inflammatory bowel disease. Front. Immunol. 6 (590).

Dirkx, A.E., Oude Egbrink, M.G., Wagstaff, J., Griffioen, A.W., 2006. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. J. Leukoc. Biol. 80 (6), 1183–1196.

Dolma, S., Selvadurai, H.J., Lan, X., Lee, L., Kushida, M., Voisin, V., Whetstone, H., So, M., Aviv, T., Park, N., et al., 2016. Inhibition of dopamine receptor D4 impedes autophagic flux, proliferation, and survival of glioblastoma stem cells. Cancer Cell 29 (6), 859–873.

Elenkov, I.J., Wilder, R.L., Chrousos, G.P., Vizi, E.S., 2000. The sympathetic nerve—an integrative interface between two supersystems: the brain and the immune system. Pharmacol. Rev. 52 (4), 595–638.

Entschladen, F., Drell, T.L., Lang, K., Joseph, J., Zaenker, K.S., 2004. Tumor-cell migration, invasion, and metastasis: navigation by neurotransmitters. Lancet Oncol. 5 (4), 254–258.

Erez, N., Truitt, M., Olson, P., Hanahan, D., 2010. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-κB-dependent manner. Cancer Cell 17 (2), 135–147.

Erin, N., Boyer, P.J., Bonneau, R.H., Clawson, G.A., Welch, D.R., 2004. Capsaicin-mediated denervation of sensory neurons promotes mammary tumor metastasis to lung and heart. Anticancer Res. 24 (2B), 1003–1010.

Erin, N., Barkan, G.A., Clawson, G.A., 2013. Vagus nerve regulates breast cancer metastasis to the adrenal gland. Anticancer Res. 33 (9), 3675–3682.

Erin, N., Korcum, A.F., Tanriover, G., Kale, S., Demir, N., Koksoy, S., 2015. Activation of neuroimmune pathways increases therapeutic effects of radiotherapy on poorly differentiated breast carcinoma. Brain Behav. Immun. 48, 174–185.

Esteban, F., Muñoz, M., González-Moles, M., Rosso, M., 2006. A role for substance P in cancer promotion and progression: a mechanism to counteract intracellular death signals following oncogene activation or DNA damage. Cancer Metastasis Rev. 25 (1), 137–145.

Figueroa, C., Galvez-Cancino, F., Oyarce, C., Contreras, F., Prado, C., Valeria, C., Cruz, S., Lladser, A., Pacheco, R., 2017. Inhibition of dopamine receptor D3 signaling in dendritic cells increases antigen cross-presentation to CD8 + T-cells favoring anti-tumor immunity. J. Neuroimmunol. 303, 99–107.

Forsgren, S., Alfredson, H., Bjur, D., Rantapää-Dahlqvist, S., 2009. Novel information on the non-neuronal cholinergic system in orthopedics provides new possible treatment strategies for inflammatory and degenerative diseases. Orthop. Rev. 1 (1).

Ganor, Y., Levite, M., 2014. The neurotransmitter glutamate and human T cells: glutamate receptors and glutamate-induced direct and potent effects on normal human T cells, cancerous human leukemia and lymphoma T cells, and autoimmune human T cells. J. Neural Transm. 121 (8), 983–1006.

Gidron, Y., Perry, H., Glennie, M., 2005. Does the vagus nerve inform the brain about preclinical tumors and modulate them? Lancet Oncol. 6 (4), 245–248.

Grivennikov, S.I., Greten, F.R., Karin, M., 2010. Immunity, inflammation, and cancer. Cell 140 (6), 883–899.

Grygier, B., Arteta, B., Kubera, M., Basta-Kaim, A., Budziszewska, B., Leskiewicz, M., Curzytek, K., Duda, W., Lason, W., Maes, M., 2013. Inhibitory effect of antidepressants on B16F10 melanoma tumor growth. Pharmacol. Rep. 65 (3), 672–681.

Hanahan, D., Coussens, L.M., 2012. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21 (3), 309–322.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144 (5), 646–674.

Hao, J., Shi, F.D., Abdelwahab, M., Shi, S.X., Simard, A., Whiteaker, P., Lukas, R., Zhou, Q., 2013. Nicotinic receptor beta2 determines NK cell-dependent metastasis in a murine model of metastatic lung cancer. PLoS One 8 (2), e57495.

Harbaum, L., Pollheimer, M.J., Kornprat, P., Lindtner, R.A., Bokemeyer, C., Langner, C., 2015. Peritumoral eosinophils predict recurrence in colorectal cancer. Mod. Pathol. 28 (3), 403–413.

Howson, L.J., Salio, M., Cerundolo, V., 2015. MR1-restricted mucosal-associated invariant T cells and their activation during infectious diseases. Front. Immunol. 6, 303.

Hu, S., Li, L., Yeh, S., Cui, Y., Li, X., Chang, H.-C., Jin, J., Chang, C., 2015. Infiltrating T cells promote prostate cancer metastasis via modulation of FGF11 → miRNA-541 → androgen receptor (AR) → MMP9 signaling. Mol. Oncol. 9 (1), 44–57.

Huston, J.M., Ochani, M., Rosas-Ballina, M., Liao, H., Ochani, K., Pavlov, V.A., Gallowitsch-Puerta, M., Ashok, M., Czura, C.J., Foxwell, B., 2006. Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. J. Exp. Med. 203 (7), 1623–1628.

Iglesia, M.D., Vincent, B.G., Parker, J.S., Hoadley, K.A., Carey, L.A., Perou, C.M., Serody, J.S., 2014. Prognostic B-cell signatures using mRNA-seq in patients with subtype-specific breast and ovarian cancer. Clin. Cancer Res. 20 (14), 3818–3829.

Inbar, S., Neeman, E., Avraham, R., Benish, M., Rosenne, E., Ben-Eliyahu, S., 2011. Do stress responses promote leukemia progression? An animal study suggesting a role for epinephrine and prostaglandin-E2 through reduced NK activity. PLoS One 6 (4), e19246.

Ishibashi, S., Ohashi, Y., Suzuki, T., Miyazaki, S., Moriya, T., Satomi, S., Sasano, H., 2006. Tumor-associated tissue eosinophilia in human esophageal squamous cell carcinoma. Anticancer Res. 26 (2B), 1419–1424.

Jiang, X., Su, L., Zhang, Q., He, C., Zhang, Z., Yi, P., Liu, J., 2012. GABA(B) receptor complex as a potential target for tumor therapy. J. Histochem. Cytochem. 60 (4), 269–279.

Jin, Z., Mendu, S.K., Birnir, B., 2013. GABA is an effective immunomodulatory molecule. Amino Acids 45 (1), 87–94.

Joyce, J.A., Pollard, J.W., 2009. Microenvironmental regulation of metastasis. Nat. Rev. Cancer 9 (4), 239–252.

Kim, S.J., Shin, J.Y., Lee, K.D., Bae, Y.K., Sung, K.W., Nam, S.J., Chun, K.H., 2012. MicroRNA let-7a suppresses breast cancer cell migration and invasion through downregulation of C-C chemokine receptor type 7. Breast Cancer Res. 14 (1), R14.

Koning, J.J., Mebius, R.E., 2012. Interdependence of stromal and immune cells for lymph node function. Trends Immunol. 33 (6), 264–270.

Kuol, N., Stojanovska, L., Nurgali, K., Apostolopoulos, V., 2017. The mechanisms tumor cells utilize to evade the host's immune system. Maturitas 105, 8–15.

Landen Jr., C.N., Lin, Y.G., Armaiz Pena, G.N., Das, P.D., Arevalo, J.M., Kamat, A.A., Han, L.Y., Jennings, N.B., Spannuth, W.A., Thaker, P.H., et al., 2007. Neuroendocrine modulation of signal transducer and activator of transcription-3 in ovarian cancer. Cancer Res. 67 (21), 10389–10396.

Lang, K., Bastian, P., 2007. Neurotransmitter effects on tumor cells and leukocytes. Prog. Exp. Tumor Res. 39, 99–121.

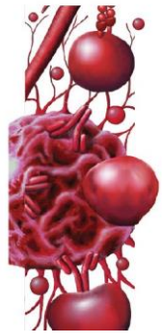
Lang, K., TLt, Drell, Lindecke, A., Niggemann, B., Kaltschmidt, C., Zaenker, K.S., Entschladen, F., 2004. Induction of a metastatogenic tumor cell type by neurotransmitters and its pharmacological inhibition by established drugs. Int. J. Cancer 112 (2), 231–238.

Laoui, D., Movahedi, K., Van Overmeire, E., Van den Bossche, J., Schouppe, E., Mommer, C., Nikolaou, A., Morias, Y., De Baetselier, P., Van Ginderachter, J.A., 2011. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. Int. J. Dev. Biol. 55 (7–9), 861–867.
Lee, C.H., Huang, C.S., Chen, C.S., Tu, S.H., Wang, Y.J., Chang, Y.J., Tam, K.W., Wei, P.L., Cheng, T.C., Chu, J.S., et al., 2010. Overexpression and activation of the alpha9-nicotinic receptor during tumorigenesis in human breast epithelial cells. J. Natl. Cancer Inst. 102 (17), 1322–1335.
Lee, C.-H., Chang, Y.-C., Chen, C.-S., S-H, Tu, Wang, Y.-J., Chen, L.-C., Chang, Y.-J., Wei, P.-L., Chang, H.-W., Chang, C.-H., 2011. Crosstalk between nicotine and estrogen-induced estrogen receptor activation induces α9-nicotinic acetylcholine receptor expression in human breast cancer cells. Breast Cancer Res. Treat. 129 (2), 331–345.
Levite, M., 2016. Dopamine and T cells: dopamine receptors and potent effects on T cells, dopamine production in T cells, and abnormalities in the dopaminergic system in T cells in autoimmune, neurological and psychiatric diseases. Acta Physiol. 216 (1), 42–89.
Lewis, C.E., Pollard, J.W., 2006. Distinct role of macrophages in different tumor microenvironments. Cancer Res. 66 (2), 605–612.
Ley, S., Weigert, A., Brüne, B., 2010. Neuromediators in inflammation—a macrophage/nerve connection. Immunobiology 215 (9), 674–684.
Liao, D., Luo, Y., Markowitz, D., Xiang, R., Reisfeld, R.A., 2009. Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model. PLoS One 4 (11), e7965.
Liguori, M., Solinas, G., Germano, G., Mantovani, A., Allavena, P., 2011. Tumor-associated macrophages as incessant builders and destroyers of the cancer stroma. Cancer 3 (4), 3740–3761.
Lin, E.Y., Nguyen, A.V., Russell, R.G., Pollard, J.W., 2001. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J. Exp. Med. 193 (6), 727–740.
Ling, L., Lin, Y., Zheng, W., Hong, S., Tang, X., Zhao, P., Li, M., Ni, J., Li, C., Wang, L., 2016. Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. Sci. Rep. 6.
Liu, H., Leak, R.K., Hu, X., 2016. Neurotransmitter receptors on microglia. Stroke Vasc. Neurol. 1 (2), 52–58.
Loi, S., Michiels, S., Salgado, R., Sirtaine, N., Jose, V., Fumagalli, D., Kellokumpu-Lehtinen, P.-L., Bono, P., Kataja, V., Desmedt, C., 2014. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann. Oncol. 25 (8), 1544–1550.
Mancino, M., Ametller, E., Gascón, P., Almendro, V., 2011. The neuronal influence on tumor progression. Biochimica et Biophysica Acta (BBA)-Rev. Cancer 1816 (2), 105–118.
Mantovani, A., Allavena, P., Sica, A., Balkwill, F., 2008. Cancer-related inflammation. Nature 454 (7203), 436–444.
Markwell, S.M., Weed, S.A., 2015. Tumor and stromal-based contributions to head and neck squamous cell carcinoma invasion. Cancer 7 (1), 382–406.
Matteoli, G., Boeckxstaens, G.E., 2013. The vagal innervation of the gut and immune homeostasis. Gut 62 (8), 1214–1222.
Mattes, J., Hulett, M., Xie, W., Hogan, S., Rothenberg, M.E., Foster, P., Parish, C., 2003. Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. J. Exp. Med. 197 (3), 387–393.
Minton, K., 2015. Granulocytes: eosinophils enable the antitumor T cell response. Nat. Rev. Immunol. 15 (6), 333.
Morgan, R.K., Kingham, P.J., Walsh, M.T., Curran, D.C., Durcan, N., McLean, W.G., Costello, R.W., 2004. Eosinophil adhesion to cholinergic IMR-32 cells protects against induced neuronal apoptosis. J. Immunol. 173 (10), 5963–5970.
Mravec, B., Gidron, Y., Hulin, I., 2008a. Neurobiology of cancer: interactions between nervous, endocrine and immune systems as a base for monitoring and modulating the tumorigenesis by the brain. Semin. Cancer Biol. 150–163.
Mravec, B., Gidron, Y., Hulin, I., 2008b. Neurobiology of cancer: interactions between nervous, endocrine and immune systems as a base for monitoring and modulating the tumorigenesis by the brain. Semin. Cancer Biol. 18 (3), 150–163.
Mukhtar, R.A., Nseyo, O., Campbell, M.J., Esserman, L.J., 2011. Tumor-associated macrophages in breast cancer as potential biomarkers for new treatments and diagnostics. Expert. Rev. Mol. Diagn. 11 (1), 91–100.
Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., 2001. Involvement of chemokine receptors in breast cancer metastasis. Nature 410 (6824), 50–56.
Nance, D.M., Sanders, V.M., 2007. Autonomic innervation and regulation of the immune system (1987-2007). Brain Behav. Immun. 21 (6), 736–745.
Neman, J., Termini, J., Wilczynski, S., Vaidehi, N., Choy, C., Kowolik, C.M., Li, H., Hambrecht, A.C., Roberts, E., Jandial, R., 2014. Human breast cancer metastases to the brain display GABAergic properties in the neural niche. Proc. Natl. Acad. Sci. 111 (3), 984–989.
Ng, Y.H., Chalasani, G., 2010. Role of secondary lymphoid tissues in primary and memory T-cell responses to a transplanted organ. Transplant. Rev. (Orlando) 24 (1), 32–41.
Nilsson, M.B., Armaiz-Pena, G., Takahashi, R., Lin, Y.G., Trevino, J., Li, Y., Jennings, N., Arevalo, J., Lutgendorf, S.K., Gallick, G.E., 2007. Stress hormones regulate interleukin-6 expression by human ovarian carcinoma cells through a Src-dependent mechanism. J. Biol. Chem. 282 (41), 29919–29926.
Nocito, A., Dahm, F., Jochum, W., Jang, J.H., Georgiev, P., Bader, M., Graf, R., Clavien, P.-A., 2008. Serotonin regulates macrophage-mediated angiogenesis in a mouse model of colon cancer allografts. Cancer Res. 68 (13), 5152–5158.
Olkhanud, P.B., Damdinsuren, B., Bodogai, M., Gress, R.E., Sen, R., Wejksza, K., Malchinkhuu, E., Wersto, R.P., Biragyn, A., 2011. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T regulatory cells. Cancer Res. 71 (10), 3505–3515.
Oosterling, S.J., van der Bij, G.J., Meijer, G.A., Tuk, C.W., van Garderen, E., van Rooijen, N., Meijer, S., van der Sijp, J.R., Beelen, R.H., van Egmond, M., 2005. Macrophages direct tumor histology and clinical outcome in a colon cancer model. J. Pathol. 207 (2), 147–155.
Paleari, L., Grozio, A., Cesario, A., Russo, P., 2008. The cholinergic system and cancer. Semin. Cancer Biol. 211–217.
Palm, D., Lang, K., Niggemann, B., TLt, Drell, Masur, K., Zaenker, K.S., Entschladen, F., 2006. The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers. Int. J. Cancer 118 (11), 2744–2749.
Peterfalvi, A., Gomori, E., Magyarlaki, T., Pal, J., Banati, M., Javorhazy, A., Szekeres-Bartho, J., Szereday, L., Illes, Z., 2008. Invariant Vα7. 2-Jα33 TCR is expressed in human kidney and brain tumors indicating infiltration by mucosal-associated invariant T (MAIT) cells. Int. Immunol. 20 (12), 1517–1525.
Pettersson, A., Nilsson, L., Nylund, G., Khorram-Manesh, A., Nordgren, S., Delbro, D.S., 2009. Is acetylcholine an autocrine/paracrine growth factor via the nicotinic α7-receptor subtype in the human colon cancer cell line HT-29? Eur. J. Pharmacol. 609 (1), 27–33.
Place, A.E., Jin Huh, S., Polyak, K., 2011. The microenvironment in breast cancer progression: biology and implications for treatment. Breast Cancer Res. 13 (6), 227.
Poulopoulou, C., Davaki, P., Koliaraki, V., Kolovou, D., Markakis, I., Vassilopoulos, D., 2005. Reduced expression of metabotropic glutamate receptor 2mRNA in T cells of ALS patients. Ann. Neurol. 58 (6), 946–949.
Pretscher, D., Distel, L.V., Grabenbauer, G.G., Wittlinger, M., Buettner, M., Niedobitek, G., 2009. Distribution of immune cells in head and neck cancer: CD8 + T-cells and CD20+ B-cells in metastatic lymph nodes are associated with favourable outcome in patients with oro-and hypopharyngeal carcinoma. BMC Cancer 9 (1), 1.
Qin, T., Wang, C., Chen, X., Duan, C., Zhang, X., Zhang, J., Chai, H., Tang, T., Chen, H., Yue, J., et al., 2015. Dopamine induces growth inhibition and vascular normalization through reprogramming M2-polarized macrophages in rat C6 glioma. Toxicol. Appl. Pharmacol. 286 (2), 112–123.
Quail, D.F., Joyce, J.A., 2013. Microenvironmental regulation of tumor progression and metastasis. Nat. Med. 19 (11), 1423–1437.
Roland, C.L., Dineen, S.P., Lynn, K.D., Sullivan, L.A., Dellinger, M.T., Sadegh, L.,Sullivan, J.P., Shames, D.S., Brekken, R.A., 2009. Inhibition of vascular endothelial growth factor reduces angiogenesis and modulates immune cell infiltration of orthotopic breast cancer xenografts. Mol. Cancer Ther. 8 (7), 1761–1771.
Rosas-Ballina, M., Olofsson, P.S., Ochani, M., Valdés-Ferrer, S.I., Levine, Y.A., Reardon, C., Tusche, M.W., Pavlov, V.A., Andersson, U., Chavan, S., Acetylcholine-synthesizing, T., 2011. Cells relay neural signals in a vagus nerve circuit. Science 334 (6052), 98–101.
Saha, B., Mondal, A.C., Basu, S., Dasgupta, P.S., 2001. Circulating dopamine level, in lung carcinoma patients, inhibits proliferation and cytotoxicity of CD4 + and CD8 + T cells by D1 dopamine receptors: an in vitro analysis. Int. Immunopharmacol. 1 (7), 1363–1374.
Sakkal, S., Miller, S., Apostolopoulos, V., Nurgali, K., 2016. Eosinophils in cancer: favourable or unfavourable? Curr. Med. Chem. 23 (7), 650–666.
Saussez, S., Laumbacher, B., Chantrain, G., Rodriguez, A., Gu, S., Wank, R., Levite, M., 2014. Towards neuroimmunotherapy for cancer: the neurotransmitters glutamate, dopamine and GnRH-II augment substantially the ability of T cells of few head and neck cancer patients to perform spontaneous migration, chemotactic migration and migration towards the autologous tumor, and also elevate markedly the expression of CD3zeta and CD3epsilon TCR-associated chains. J. Neural Transm.(Vienna) 121 (8), 1007–1027.
Schmid, M.C., Varner, J.A., 2010. Myeloid cells in the tumor microenvironment: modulation of tumor angiogenesis and tumor inflammation. J. Oncol. 2010.
Serriari, N.E., Eoche, M., Lamotte, L., Lion, J., Fumery, M., Marcelo, P., Chatelain, D., Barre, A., Nguyen-Khac, E., Lantz, O., et al., 2014. Innate mucosal-associated invariant T (MAIT) cells are activated in inflammatory bowel diseases. Clin. Exp. Immunol. 176 (2), 266–274.
Shahzad, M.M., Arevalo, J.M., Armaiz-Pena, G.N., Lu, C., Stone, R.L., Moreno-Smith, M., Nishimura, M., Lee, J.-W., Jennings, N.B., Bottsford-Miller, J., 2010. Stress effects on FosB-and interleukin-8 (IL8)-driven ovarian cancer growth and metastasis. J. Biol. Chem. 285 (46), 35462–35470.
Shajib, M.S., Khan, W.I., 2015. The role of serotonin and its receptors in activation of immune responses and inflammation. Acta Physiol. 213 (3), 561–574.
Shi, M., Liu, D., Yang, Z., Guo, N., 2013. Central and peripheral nervous systems: master controllers in cancer metastasis. Cancer Metastasis Rev. 32 (3–4), 603–621.
Sica, A., Porta, C., Riboldi, E., Locati, M., 2010. Convergent pathways of macrophage polarization: the role of B cells. Eur. J. Immunol. 40 (8), 2131–2133.
Sloan, E.K., Priceman, S.J., Cox, B.F., Yu, S., Pimentel, M.A., Tangkanangnukul, V., Arevalo, J.M., Morizono, K., Karanikolas, B.D., Wu, L., 2010. The sympathetic nervous system induces a metastatic switch in primary breast cancer. Cancer Res. 70 (18), 7042–7052.
Smith, H.A., Kang, Y., 2013. The metastasis-promoting roles of tumor-associated immune cells. J. Mol. Med. 91 (4), 411–429.
Song, P., Spindel, E.R., 2008. Basic and clinical aspects of non-neuronal acetylcholine: expression of non-neuronal acetylcholine in lung cancer provides a new target for cancer therapy. J. Pharmacol. Sci. 106 (2), 180–185.
Song, P., Sekhon, H.S., Lu, A., Arredondo, J., Sauer, D., Gravett, C., Mark, G.P., Grando, S.A., Spindel, E.R., 2007. M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion. Cancer Res. 67 (8), 3936–3944.
Stepulak, A., Luksch, H., Gebhardt, C., Uckermann, O., Marzahn, J., Sifringer, M., Rzeski, W., Staufner, C., Brocke, K.S., Turski, L., et al., 2009. Expression of glutamate

receptor subunits in human cancers. Histochem. Cell Biol. 132 (4), 435–445.
Stockmann, C., Schadendorf, D., Klose, R., Helfrich, I., 2014. The impact of the immune system on tumor: angiogenesis and vascular remodeling. Front. Oncol. 4.
Stojanovska, V., Sakkal, S., Nurgali, K., 2015. Platinum-based chemotherapy: gastrointestinal immunomodulation and enteric nervous system toxicity. Am. J. Physiol.-Gastrointest. Liver Physiol. 308 (4), G223–G232.
St-Pierre, S., Jiang, W., Roy, P., Champigny, C., LeBlanc, É., Morley, B.J., Hao, J., Simard, A.R., 2016. Nicotinic acetylcholine receptors modulate bone marrow-derived pro-inflammatory monocyte production and survival. PLoS One 11 (2), e0150230.
Sun, Y., 2015. Translational horizons in the tumor microenvironment: harnessing breakthroughs and targeting cures. Med. Res. Rev. 35 (2).
Swerdlin, N., Cohen, I.R., Harel, D., 2008. The lymph node B cell immune response: dynamic analysis in-silico. Proc. IEEE 96 (8), 1421–1443.
Swirski, F.K., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Retamozo, V., Panizzi, P., Figueiredo, J.-L., Kohler, R.H., Chudnovskiy, A., Waterman, P., 2009. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. Science 325 (5940), 612–616.
Szalayova, G., Ogrodnik, A., Spencer, B., Wade, J., Bunn, J., Ambaye, A., James, T., Rincon, M., 2016. Human breast cancer biopsies induce eosinophil recruitment and enhance adjacent cancer cell proliferation. Breast Cancer Res. Treat. 157 (3), 461–474.
Thaker, P.H., Yokoi, K., Jennings, N.B., Li, Y., Rebhun, R.B., Rousseau Jr., D.L., Fan, D., Sood, A.K., 2005. Inhibition of experimental colon cancer metastasis by the GABA-receptor agonist nembutal. Cancer Biol. Ther. 4 (7), 753–758.
ThyagaRajan, S., Priyanka, H.P., 2012. Bidirectional communication between the neuroendocrine system and the immune system: relevance to health and diseases. Ann. Neurosci. 19 (1), 40–46.
de la Torre, E., Davel, L., Jasnis Mí, A., Gotoh, T., de Lustig, E.S., Sales Mí, E., 2005. Muscarinic receptors participation in angiogenic response induced by macrophages from mammary adenocarcinoma-bearing mice. Breast Cancer Res. 7 (3), R345–352.
Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., Jemal, A., 2015. Global cancer statistics, 2012. CA Cancer J. Clin. 65 (2), 87–108.
Tracey, K.J., 2002. The inflammatory reflex. Nature 420 (6917), 853–859.
Tracey, K.J., 2009. Reflex control of immunity. Nat. Rev. Immunol. 9 (6), 418–428.
Ussher, J.E., Klenerman, P., Willberg, C.B., 2014. Mucosal-associated invariant T-cells: new players in anti-bacterial immunity. Front. Immunol. 5.
Wang, H., Yu, M., Ochani, M., Amella, C., Tanovic, M., Susaria, S., Hua, L.J., Wang, H., Yang, H., 2003. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature 421, 384–388.
von Wasielewski, R., Seth, S., Franklin, J., Fischer, R., Hübner, K., Hansmann, M.L., Diehl, V., Georgii, A., 2000. Tissue eosinophilia correlates strongly with poor prognosis in nodular sclerosing Hodgkin's disease, allowing for known prognostic factors. Blood 95 (4), 1207–1213.
Watanabe, M., Maemura, K., Oki, K., Shiraishi, N., Shibayama, Y., Katsu, K., 2006. Gamma-aminobutyric acid (GABA) and cell proliferation: focus on cancer cells. Histol. Histopathol. 21 (10), 1135–1141.
Wei, P.-L., Chang, Y.-J., Ho, Y.-S., Lee, C.-H., Yang, Y.-Y., An, J., Lin, S.-Y., 2009. Tobacco-specific carcinogen enhances colon cancer cell migration through α7-nicotinic acetylcholine receptor. Ann. Surg. 249 (6), 978–985.
Wong, H.P., Yu, L., Lam, E.K., Tai, E.K., Wu, W.K., Cho, C.H., 2007. Nicotine promotes cell proliferation via alpha7-nicotinic acetylcholine receptor and catecholamine-synthesizing enzymes-mediated pathway in human colon adenocarcinoma HT-29 cells. Toxicol. Appl. Pharmacol. 221 (3), 261–267.
Wu, J., Zhang, R., Tang, N., Gong, Z., Zhou, J., Chen, Y., Chen, K., Cai, W., 2015. Dopamine inhibits the function of Gr-1+CD115+ myeloid-derived suppressor cells through D1-like receptors and enhances anti-tumor immunity. J. Leukoc. Biol. 97 (1), 191–200.
Yang, E.V., Sood, A.K., Chen, M., Li, Y., Eubank, T.D., Marsh, C.B., Jewell, S., Flavahan, N.A., Morrison, C., Yeh, P.-E., 2006. Norepinephrine up-regulates the expression of vascular endothelial growth factor, matrix metalloproteinase (MMP)-2, and MMP-9 in nasopharyngeal carcinoma tumor cells. Cancer Res. 66 (21), 10357–10364.
Young, S.Z., Bordey, A., 2009. GABA's control of stem and cancer cell proliferation in adult neural and peripheral niches. Physiology (Bethesda) 24, 171–185.
Yu, Y., Xiao, C., Tan, L., Wang, Q., Li, X., Feng, Y., 2014. Cancer-associated fibroblasts induce epithelial–mesenchymal transition of breast cancer cells through paracrine TGF-β signalling. Br. J. Cancer 110 (3), 724–732.
Z-a, Chen, Bao, M.-y., Xu, Y.-f., R-p, Zha, H-b, Shi, T-y, Chen, He, X.-h., 2012. Suppression of human liver cancer cell migration and invasion via the GABAA receptor. Cancer Biol. Med. 9 (2), 90.
Zabijak, L., Attencourt, C., Guignant, C., Chatelain, D., Marcelo, P., Marolleau, J.-P., Treiner, E., 2015. Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients. Cancer Immunol. Immunother. 64 (12), 1601–1608.
Zhang, B.C., Gao, J., Wang, J., Rao, Z.G., Wang, B.C., Gao, J.F., 2011. Tumor-associated macrophages infiltration is associated with peritumoral lymphangiogenesis and poor prognosis in lung adenocarcinoma. Med. Oncol. 28 (4), 1447–1452.

Review

Immunotherapy



PD-1/PD-L1 in disease

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Aim: Expression of PD-1 on T/B cells regulates peripheral tolerance and autoimmunity. Binding of PD-1 to its ligand, PD-L1, leads to protection against self-reactivity. In contrary, tumor cells have evolved immune escape mechanisms whereby overexpression of PD-L1 induces anergy and/or apoptosis of PD-1 positive T cells by interfering with T cell receptor signal transduction. PD-L1 and PD-1 blockade using antibodies are in human clinical trials as an alternative cancer treatment modality. **Areas covered:** We describe the role of PD-1/PD-L1 in disease in the context of autoimmunity, neurological disorders, stroke and cancer. **Conclusion:** For immunotherapy/vaccines to be successful, the expression of PD-L1/PD-1 on immune cells should be considered, and the combination of checkpoint inhibitors and vaccines may pave the way for successful outcomes to disease.

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The immune system is complex, yet so simple in its ability to induce nonspecific (innate) immunity and specific immune responses against pathogens, including, bacteria, viruses and parasites. However, for cancer prevention, the elimination or inactivation of mutated cells is debated on whether this is a prime function of the immune system. Hence, the concept of 'immune surveillance' was introduced 50 years ago by Thomas and Burnet [1–4]. Their theory concurred with studies by Doherty and Zinkernagel, where they demonstrated that the immune system plays an essential role in immune surveillance by recognizing small peptide epitopes in conjunction with the MHC-I presented on the surface of virus infected cells [5]. Consequently, it was shown by others that tumor cells also expressed MHC-I and presented short tumor-associated peptides to immune cells [6]. However, tumor cells can evade host's immune surveillance using a number of protective mechanisms, including downregulation of MHC-I molecules, secretion of anti-inflammatory cytokines, in other words, TGF-β and IL-10, secretion of immunosuppressive factors, VEGF, upregulation of PD-L1 and downregulation of co-stimulatory molecules thereby preventing activation of T cells, resulting in cancer invasion.

PD-L1 (also known as CD274 or B7-homolog 1 [B7-H1]) is a transmembrane protein involved in the immune system suppression. The expression of PD-L1 on cells, including lymphoid and non-lymphoid tissues, antigen-presenting cells (APC), dendritic cells (DCs), macrophages, activated monocytes, natural killer (NK) cells, T cells, B cells, epithelial cells, vascular endothelial cells, glial cells and tumor cells is upregulated by IFN-γ [7]. In addition, PD-L2 (also known as, CD273, PDCD1LG2, B7-DC), is predominantly expressed on DCs and some macrophages [8,9]. On the other hand, activated immune cells such as, natural killer T (NKT) cells, myeloid cells, B cells and T cells, express the ligand, programmed death 1 (PD-1; CD279), which plays a significant function in immune tolerance [10]. The binding of T cells expressing PD-1 and tumor cells expressing PD-L1 initiates an array of inhibitory signals resulting in reduced function and/or apoptosis of T cells [8,11] providing a mechanism for tumor cell evasion of host's immune surveillance [12–14]. In fact, cancer cells, in particular renal and breast cells express high levels of PD-L1 leading to poor patient survival [15,16].

PD-1 and PD-L1 inhibitory signaling is an essential mechanism behind immune regulation of disease states, such as autoimmunity, cancer and neurodegenerative diseases (Figure 1). In fact, PD-1 deficiency results in spontaneous autoimmunity in murine models of Type 1 diabetes and systemic lupus erythematosus [14,17–25]. Furthermore, upregulation of PD-L1 by cancer cells results in cancer invasion and correlates with poor prognostic outcomes in breast, gastric, meningioma, non-small-cell lung carcinoma (NSCLC) and soft-tissue sarcoma patients [9,26–29].

Future Medicine

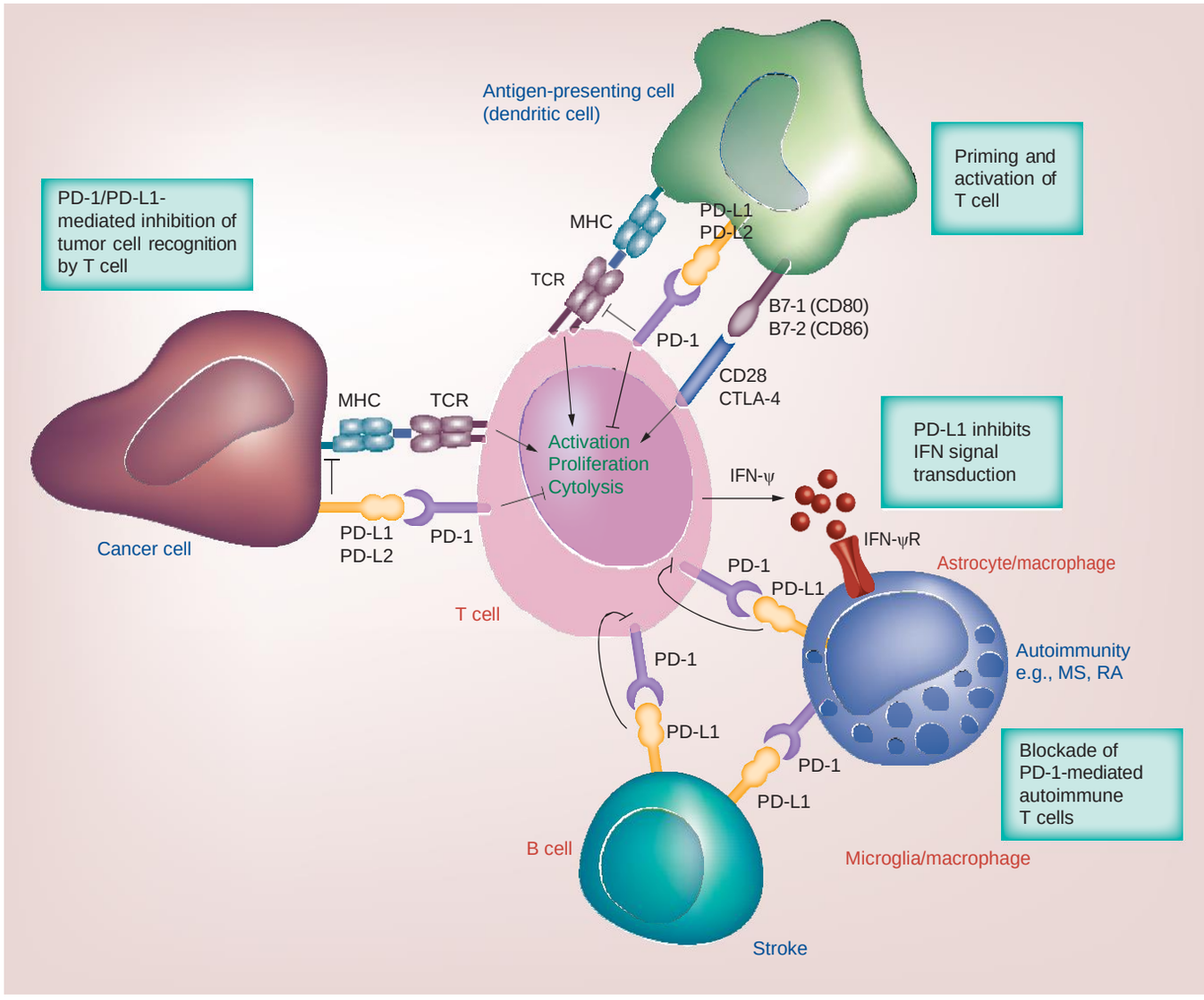

Figure 1. PD-1/PD-L1 interaction in disease. T cell activation requires antigen recognition, followed by extra support from the co-stimulatory signal that determines whether the T cell will be switched on or off in response to the antigenic peptide. Cancer cells can express high levels of PD-L1, which by binding to PD-1 expressed by T cells initiates an inhibitory signaling network that switches off activated T cells and results in T cell suppression (designated as red line in T cell). The interaction between T cells and dendritic cells also leads to T cell suppression. On the other hand, PD-1/PD-L1 interaction has protective effects in stroke and autoimmunity diseases such as multiple sclerosis and rheumatoid arthritis. DC: Dendritic cell; MHC: Major histocompatibility complex; MS: Multiple sclerosis; RA: Rheumatoid arthritis; TCR: T cell receptor.

Hence, overexpression of PD-L1 by tumor cells suppresses the host immune response by interfering with T cell receptor signal transduction resulting in cytotoxic T cells inhibition [30]. As a result, several anticancer drugs have been developed to block PD-1 or its major ligand PD-L1. Here we present an overview of the effects of PD-1/PD-L1 interaction in disease states.

Association between PD-1/PD-L1 & disease

Autoimmunity

The role of PD-1/PD-L1 in autoimmunity was demonstrated in PD-1 knockout mice where breakdown of peripheral tolerance resulted in negative regulation of lymphocyte activation leading to autoimmune features, depending on the genetic background [31]. PD-1 knockout mice (C57BL/6 background) develop lupus-like IgG3 deposition glomerulonephritis and destructive arthritis [23]; mutation of H-2L^{d} of H-2$^{d/b}$ background

mice results in graft versus host like symptoms. Conversely, PD-1 knockout mice (BALB/c background) develop autoimmune IgG antibody-mediated cardiomyopathy and sudden death [24]. PD-1/PD-L1 signaling is important in the pathogenesis of autoimmune diseases and understanding their mechanism of action is crucial in the prevention and/or development of vaccines. It is possible that a combination of vaccines together with methods to increase the expression of PD-1 or PD-L1 could aid in improved therapeutics against autoimmune diseases.

Autoimmune diabetes

Treatment with anti-PD-1 or anti-PD-L1 antibody in nonobese diabetic (NOD) mice precipitated diabetes suggest that PD-1/PD-L1 plays a crucial role in regulating autoimmune diabetes [17]. Upregulation of PD-L1 on pancreatic beta cells in NOD mice significantly decreased insulitis and disease onset [32]. NOD/PD-1 knockout mice enhance $CD4^+$ T-helper (Th)-1 cell infiltration within islet cells, increase chemokine receptor CXCR3 expression, enhance destructive insulitis and enhance the onset of diabetes [33,34]. Hence, altered PD-1/PD-L1 signaling associates with diabetes in NOD mice; PD-1 regulates autoimmunity by suppressing T cell proliferation and infiltration in the pancreas limiting diabetes. Recently, in a Japanese cohort of autoimmune diabetic patients (Type 1A), a significant decrease in PD-1 expression was noted on $CD4^+$ T cells compared with Type 1 (fulminant Type 1), Type 2 diabetic or healthy control subjects [35]. Thus, decreased expression of PD-1 on $CD4^+$ T cells contributes to Type 1A autoimmune diabetes via T cell activation. However, the mechanism of action is not clear.

Multiple sclerosis

The interaction between PD-1 on activated T cells and PD-L1 suppresses T cell responses in the central nervous system (CNS). Within the brain, PD-L1 is upregulated on endothelial cells and PD-1, PD-L1 and PD-L2 are expressed on autoimmune T cells [36]. In an animal model of multiple sclerosis (MS), known as, experimental autoimmune encephalomyelitis (EAE), increased PD-L1 and PD-1 expression (but not PD-L2) is evident within the CNS [25]. PD-1 inhibition results in enhanced level of autoimmune T cells and antibodies resulting in accelerated EAE symptoms [25]. Indeed, EAE symptoms in PD-1 and PD-L1 knockout mice are more severe with higher proinflammatory Th-1 cytokines (IL-6, IL-17, TNF-α and IFN-γ) compared with PD-L2 knockout or control mice [37]. Estrogen stimulates PD-1 expression on T cells and APCs [10]. In fact, estrogen suppresses EAE through enhanced PD-1 expression on regulatory T cells (T_{reg}) and decreased secretion of IL-17 [38]. Hence, PD-1/PD-L1 signaling plays an essential part in EAE progression. PD-1 expression is enhanced on antimyelin basic protein $CD4^+$ and $CD8^+$ T cells in stable MS patients compared with T cells from acute remitting relapsing disease [39]. Interestingly, in MS lesions PD-L1 is elevated, however, PD-1 is not expressed by $CD8^+$ T cells in such lesions, and is therefore insensitive to PD-L1 interaction. Strategies for enhancing the expression of PD-1 on $CD8^+$ T cells is of interest, given that its ligand, PD-L1 is already increased on target organs. As the PD-1/PD-L1 signaling is important in MS pathogenesis, therapeutic strategies blocking PD-1/PD-L1 pathway show great potential to be developed against MS.

Inflammatory bowel disease

In mouse models of chronic colitis and in humans with inflammatory bowel disease, PD-1 is highly expressed on T cells and PD-L1 expression is elevated on macrophages, DCs, T cells, B cells and in inflamed colon tissues [40]. Injection of anti-PD-L1 antibody but not anti-PD-L2 antibody, reduces Th1 $CD4^+$ T cells (IFN-γ and TNF-α) but not Th2 $CD4^+$ (IL-4, IL-10 producing) T cells in inflamed tissues, suggesting that PD-1/PD-L1 may be involved in inflammatory bowel disease [40]. Inactivation of PD-1/PD-L1 signaling pathway by either transfer of PD-1 knockout T cells or anti-PD-L1 antibody induces a substantial increase of $CD8^+$ T cells producing high Th1 cytokines [41]. Hence, inactivation of PD-1/PD-L1 interaction disrupts $CD8^+$ T cell tolerance to self-intestinal antigen resulting in intestinal autoimmunity. In addition, PD-L1 knockout mice are highly susceptible to trinitrobenzenesulfonic acid or dextran sulfate sodium induced intestinal injury [42]. This results in high morbidity and mortality, which are associated with severe pathological features including overgrowth of commensal bacteria and loss of epithelial integrity. Expression of PD-L1 reduces intestinal inflammation, with low TNF-α and high IL-22 cytokines from CD11c+CD11b+ lamina propria cells [42]. In addition, injection of adenovirus expressing Fc-PD-L1 in dextran sulfate sodium-treated mice reduces colitis. Recently, it was noted that APCs from intestinal tissues of Crohn's disease patients do not express PD-L1, although it is expressed on APCs from ulcerative colitis patients [43]. These findings suggest that in Crohn's disease intestinal antigen uptake by APCs is presented without PD-L1, hence, affecting tolerogenic signaling which might contribute to disease initiation.

Rheumatoid arthritis

It has been proposed that PD-1/PD-L1 pathway plays a function in the pathogenesis of rheumatoid arthritis (RA). In RA patients, overexpression of both synovial fluid and plasma soluble PD-1 is significantly correlated with joint counts and autoantibody production, suggesting a possible role in the pathogenesis [44]. Soluble PD-1 is induced by IFN-γ, IL-17A and TNF-α [45]. Similarly, PD-1 expressed on synovial fluid-derived $CD4^+$ T cells is elevated compared with $CD4^+$ T cells from peripheral blood of RA patients [46]. Moreover, PD-L1 protein expression is elevated on synovial fluid myeloid DCs compared with peripheral blood myeloid DCs and correlates with T cell hyporesponsiveness. Furthermore, stimulation of IL-7 and PD-1 blockade significantly enhances T cell proliferation [46]. In addition, PD-1/PD-L1 signaling is overexpressed in macrophages and synovial T cells in RA patients as compared with controls [47]. Especially, level of soluble PD-1 expression significantly correlates with TNF-α level in RA patient's synovial fluid. PD-1 (−/−) mice demonstrated enhanced occurrence and greater severity of collagen-induced arthritis which correlates with elevated T cell proliferation and enhanced secretion of cytokines (IFN-γ and IL-17) in response to type II collagen [48]. These findings provide evidence that PD-1/PD-L1 pathway plays a role in the pathogenesis of RA warranting further studies elucidating possible mechanisms.

Neurological disorders

PD-1/PD-L1 pathway may play a role in immune regulation of neurological disorders, including ischemic stroke, MS and Alzheimer's disease [49,50]. The immunity against CNS infection, neurodegeneration or injury involves infiltration of immune cells and glial cells [50]. A plethora of neurochemical mediators and cascades of signal transduction molecules, including inhibitory signaling via PD-1/PD-L1 pathway regulate immune cells in the CNS as the mechanism of avoiding inflammatory destruction to the compromised brain [50]. In Alzheimer's disease patients and in patients with mild cognitive impairment, the expression of PD-1 on $CD4^+$ T cells and expression of PD-L1 on $CD14^+$ macrophages/monocytes are decreased [51]. Impairment in PD-1/PD-L1 signaling correlates with decreased IL-10 secretion [49]. IL-10, an anti-inflammatory cytokine, is known to ameliorate Alzheimer's disease pathology in animal models [49,52]. However, more studies are essential to elaborate the molecular and cellular mechanisms of PD-1/PD-L1 interaction in Alzheimer's disease and their influence on immune cell in the CNS which may aid in the design of improved immunotherapeutics against Alzheimer's disease.

Stroke

The PD-1/PD-L pathway plays a role in poststroke inflammation via negative regulation of cell–cell interaction [53]. Interestingly, PD-L1 or PD-L2 expression on B cells prevents the activation of effector T cells, microglia or macrophages, thus, reducing ischemic brain inflammation [50]. In addition, administration of T_{regs} isolated from PD-L1 deficient mice or T_{regs} pretreated with anti-PD-L1 antibodies failed to inhibit MMP-9 secretion by neutrophils in an acute phase after stroke [53]. This clearly demonstrates that one possible mechanism by which PD-L1 serves as neuroprotective factor is through mediating the suppressive effect of T_{regs} on neutrophil-acquired MMP-9. Moreover, the experimental model of stroke shows significantly elevated expression of PD-L1 and PD-L2 on B cells from CNS, blood and spleen, 4 days post-transient middle cerebral artery occlusion [54]. In contrast, PD-L1 or PD-L2 knockout mice play an adverse role in stroke outcomes and exacerbate poststroke inflammation. It has been suggested that PD-1 exhibits protective effects and inhibits inflammatory responses by other effector immune cells via the expression of PD-1 on B cells. However, the detrimental effects of PD-L1 may depend on the prevention of $CD8^+$ $CD122^+$ suppressor T cell migration from the spleen into the ischemic brain [18,39,55–61]. Thus, understanding the molecular mechanisms of PD-1/PD-L1 signaling in the ischemic brain could lead to better treatment options.

Role of PD-1/PD-L1 in cancer

Immunity against cancer cells and their eradication is dependent on the induction of CD8+ T cells and their differentiation into cytolytic cells which relies on two signals from the APCs. One signal is produced by the interaction of the antigenic peptide (from the tumor) presented on the MHC to T cells [62]. The other are the costimulatory signals, B7 (B7–1 [CD80] and B7–2 [CD86]) on APCs which bind to CD28 (CD152 or CTLA-4) on T cells [63,64]. Cancer cells, however, can escape host's immune response which in many cases directly involves these two signals. Such evasion mechanisms include reduced (or no) expression of costimulatory molecules (CD80, CD86), adhesion molecules or Fas ligand on cancer cells, downregulation of MHC class I expression and antigen processing defects [65–67]. Furthermore, it has been demonstrated recently that not only can PD-L1 protect cancer

cells from a direct attack of cytotoxic T cells but also plays a significant role in overcoming type I mediated cytotoxicity [68]. In addition, cancer cells have additional escape mechanisms, one of which is by expressing PD-L1 and/or PD-L2 on their surface, which upon binding to PD-1 expressed by activated $CD8^+$ T cells leads to their anergy and/or apoptosis [69]. More recently, Aranza *et al.*, 2017 have demonstrated comprehensive review on PD-1 signal transduction pathways involved in T cell activation (see [30] for detailed information on T cell activation). Interestingly, PD-L1 serves as an antiapoptotic factor on cancer cells, leading to resistance of lysis by $CD8^+$ T cells as well as apoptosis induced by drugs [70]. Though, PD-1 signaling provides a protective outcome for autoimmunity, tumorigenic cells have exploited it to escape immune-mediated toxicity [71]. Furthermore, PD-1 expression on tumor-infiltrating lymphocytes (TILs) correlates with aggressive features [71] and is correlated with poor patient outcome.

There has been an upsurge in the number of studies demonstrating that tumor cells express PD-L1 which inhibits the immune microenvironment, as shown in but not limited to, head and neck squamous cell, lung, breast, melanoma and endometrium cancers. In many malignancies including melanoma, the expression of PD-L1 is associated with the presence of TILs and IFN-γ expression. It is imperative to highlight that PD-L1 expression occurs along a spectrum of heterogeneous within tumors as demonstrated in melanoma [68,72,73]. Similarly, there is significant upregulation of PD-L1 in advanced NSCLC patients compared with healthy individuals [74]. In breast cancer for example, PD-L1 is highly expressed on primary cancer cells which associates with estrogen- and progesterone-negative expression status and histological grade III type, and with highly proliferative Ki-67-expressing tumor cells [71], large tumors and correlates with poor prognosis [9]. Triple-negative breast cancers highly express PD-L1 suggesting that such cancer types may benefit from immune checkpoint therapy [75]. In addition, PD-L1 is highly expressed on blood circulating metastatic cells which could be used as a marker in patients undergoing immune checkpoint blockade [76]. Further, transgenic expression of PD-L1 in mouse tumor cell lines such as mastocytoma, melanoma and myeloma/plasmocytomas aids in their escape from the host T cells and markedly enhances their invasiveness *in vivo*. These studies demonstrate that the expression of PD-L1 is an independent negative prognostic factor in cancer.

Conversely, other studies indicate that the expression of PD-L1 associates with good disease outcome. PD-L1 expression in primary breast and lung cancer tissues is linked to increased TILs which associates with longer recurrence-free survival [77–79]. Likewise, NSCLC patients with overexpression of PD-L1 have longer overall survival that is independent of age, stage and histology. Moreover in melanoma, melanocytic lesions co-localize with PD-L1 and TILs leading to better prognosis [80]. In fact PD-L1-positive metastatic melanoma has delayed progression compared with PD-L1 negative metastatic melanomas patients [80]. These findings imply that PD-1/PD-L1 expression holds better prognosis value when co-expressed with TILs.

In cancer, it is still not clear whether PD-L1 expression leads to better or worse prognosis. The role of PD-L2 in cancer has not been elucidated. However, it is known that tumor cells can stimulate PD-L1 expression via multiple oncogenic signaling pathways such NF-kB, MAPK, mTOR, MEK/ERK/STAT1, PI3K and JAK/STAT mediated by IFN-γ produced by infiltrating immune cells [80–85] (Figure 2). For instance, blockade of the MyD88/TRAF6 or MEK/ERK pathway inhibits PD-L1 expression induced by toll-like receptor ligands and IFN-γ in plasma cells from a myeloma patient [83]. In addition, PTEN/PI3K signaling is increased in MDA-MB-468 breast cancer cell line; PI3K inhibition with mTOR inhibitor rapamycin and AKT inhibitor MK-2206 resulted in decreased PD-L1 expression [86]. The oncogenic signalings activated and the tumor type may influence these mechanisms.

These findings demonstrated the importance of PD-L1 expression in cancer; hence the detection methods of PD-L1 within or around the tumor need precision. PD-L1 expression undergoes modification in the tumor microenvironment, making immunohistochemical detection cautionary. For instance, VEGF has been reported to downregulate PD-L1 expression whereas TNF-α and IFN-γ upregulate PD-L1 expression in tumor [81,87,88]. There are no precise criteria to define PD-L1 positivity by immunohistochemical and misinterpretation may arise due to heterogeneous expression within or between tumor lesions. Noninvasive *in vivo* imaging with radiolabeled anti-PD-L1 antibodies can overcome some of the limitations associated with immunohistochemical analysis of PD-L1 expression in tumor biopsies. Radiolabeled anti-PD-L1 antibodies *in vivo* imaging allows accurate detection of PD-L1 expression of whole tumors and their metastases, thus avoiding sampling errors; hence, misinterpretation due to intratumoral and interlesional heterogeneity [89–93]. Radiolabeled anti-PD-L1 antibodies *in vivo* imaging may hold potential valuble as biomarker to select patients for PD-1/PDL1-targeted therapy.

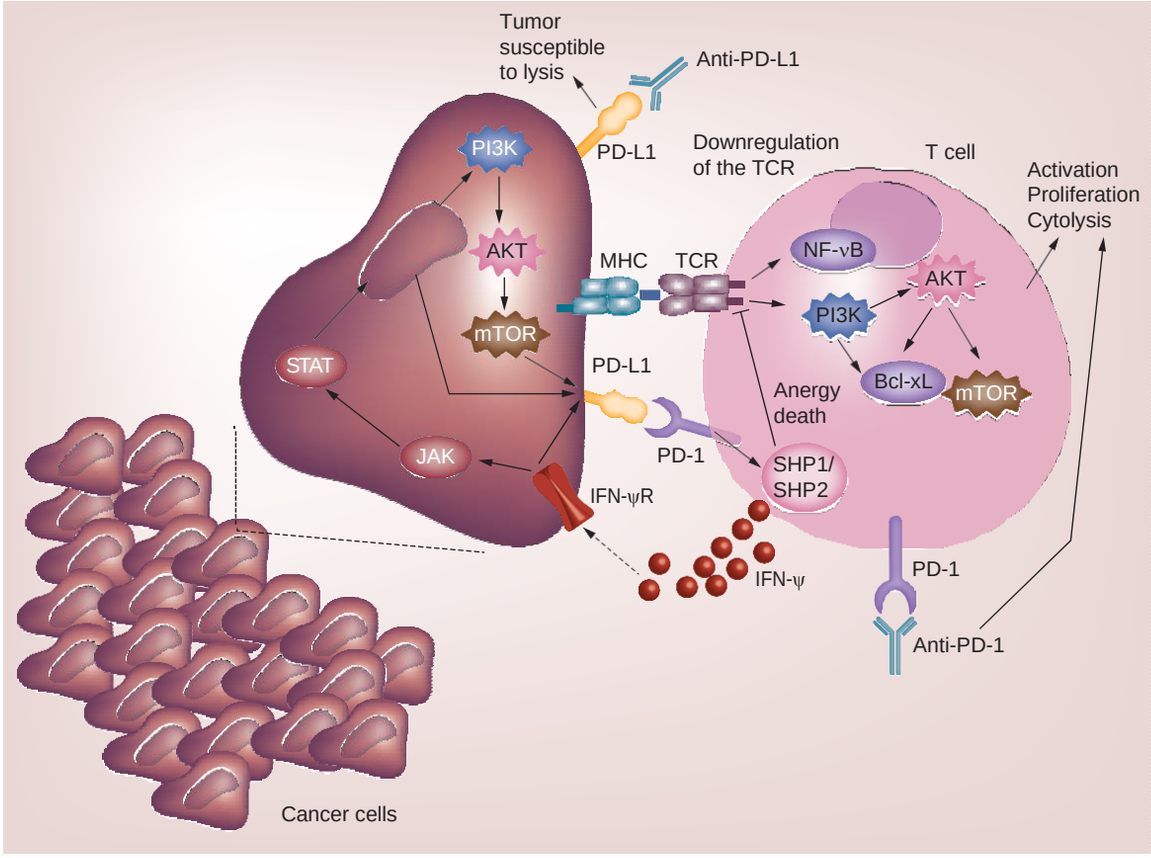

Figure 2. PD-1/PD-L1 mechanism between tumor cells and T cells. T cell activation requires antigen recognition in complex with MHC class I ($CD8^+$ T cells) or MHC class II ($CD4^+$ T cells). This is followed by extra support from the co-stimulatory signal that determines whether the T cell will be switched on or off in response to the antigenic peptide. Tumor cells can express high levels of PD-L1 which by binding to PD-1 initiates an inhibitory signaling network via SHP1/SHP2 that switches off activated T cells and results in T cell suppression (designated as red line in T cell). Mechanistically, the expression of PD-L1 by tumor cells is upregulated following IFN-γ secreted by T cells binding to IFN-γ receptor on tumor cells activating JAK and STAT signaling pathway resulting in activation of PD-L1. In addition, tumor cells use other signaling pathways including NF-κB, mTOR and PI3K. These mechanisms may be influenced by the tumor type and other oncogenic signaling pathways that are activated in the tumor cell. Checkpoint inhibitors have been designed to block the effects of PD-L1, the effects of PD-1 on T cells and the effects of PD-L1/PD-1 interaction.

Immune checkpoint inhibitors

Immune checkpoint inhibitors which block PD-1 receptor, PD-L1 and anti-CTLA-4 are the current ground-breaking first-line treatment options in several cancers including melanoma, lung cancer and gastric cancer [72,73,94,95]. Injection of anti-PD-L1 antibodies decreases CT26 colon carcinoma cell growth and B16 melanoma cell growth in mice and decreased pancreatic carcinoma cell line growth in mice [96]. Numerous clinical trials are currently in progress to determine the effects of PD-1 inhibitors (Table 1). PD-1 inhibitors (including nivolumab and pembrolizumab), were approved by the US FDA for treatment of advanced melanoma [97–99]. Pembrolizumab was also recently approved for treating patients with advanced NSCLC whose tumor expresses PD-L1 [95]. A recent interim report from patients with NSCLC or melanoma with brain metastasis indicates that brain metastasis response was achieved in 4/18 melanoma patients and in 6/18 NSCLC patients [100]. Although, high-grade adverse events are rare in these therapies, there has been some adverse events reported which can be controlled with standard anti-inflammatory agents. It has been reported that pembrolizumab induces grade 3–4 adverse events such as colitis, pneumonitis, fatigue, hyperkalemia, acute kidney injury, transient cognitive dysfunction and seizures [100]. In a rare case, the patient undergoing pembrolizumab treatment developed autoimmune diabetes, possibly as a result of PD-1 inhibition [101]. Furthermore, nivolumab, the first approved inhibitor for urological cancer, presents

Table 1. Checkpoint inhibitors in human clincial trials.

Name	Cancer type	Effects	Ref.
Anti-PD-1 monoclonal antibodies			
Pembrolizumab	Hodgkin's lymphoma Stage IV	After 6 months of treatment, the patient developed cutaneous sarcoidosis, acute iritis, dyspnea and adenopathy	[111]
Pembrolizumab + anti-CD40 – (Ipilimumab)	Advanced melanoma	Stable disease in 3/9 patients Median overall survival 8 months No grade 3/4 adverse events	[112]
Pembrolizumab	SCC of thymus with multiple lung metastases	Lung metastases dissapeared Complete remission No toxicity other than grade I rash	[113]
Pembrolizumab	Metastatic uveal melanoma	Side effects, blurred vision Uveitis, stopped treatment	[114]
Nivolumab	Hodgkin's lymphoma	53/80 objective response Fatigue, rash, neutropenia, pyrexia in some (4–20%) of patients)	[115]
Nivolumab	Leiomyosarcoma with lung, bone, skin metastases	Metastases regressed, skin lesions almost completely dissapeared Regression for 6 months No side effects	[116]
Nivolumab	Metastatic	Acute kidney transplant rejection Melanoma (kidney translpant recipient 14 years prior)	[117]
Anti-PD-L1 monoclonal antibodies			
Avelumab	Chemotherapy-refractory metastatic Merkel cell carcinoma	Phase II trial, well tolerated, 28/88 patients achieved an objective response, 8/88 complete response 20/88 partial response, no grade 4 adverse effects	[118]
Avelumab	Refractory metastatic urothelial carcinoma	Phase IB study, Objective response in 18.2% of patients, 5/44 complete responses, fatigue/asthenia, infusion related reaction, nausea, 3/44 grade, 3–4 adverse events	[119]
Atezolizumab	Platinum-treated locally advanced or metastatic urothelial carcinoma	Single-arm study, patients who continued treatment beyond initial injection, showed prolonged clinical benefit	[120]
Atezolizumab	Previously treated NSCLC	Phase III trial, 13.8 vs 9.6 months improved survival in treated group Generally well tolerated	[121]
Atezolizumab	Advanced NSCLC	Phase II trial, n = 268. Significant objective responses, progression-free survival and overall survival Well tolerated	

NSCLC: Non-small-cell lung carcinoma; SCC: Squamous cell carcinoma.

with immune-mediated side effects including nephritis, colitis, diarrhea, pneumonitis and hyperthyroidism [102]. However, overall side effects of these therapies are less frequent compared with chemo and radiotherapy. More recently, the first PD-L1 inhibitor (atezolizumab; Tecentriq™) has been approved to be used as a second-line therapy for urothelial cancers and submission for approval has been done for NSCLC [103]. Thus, an enhanced knowledge of the mechanisms and signaling pathways involved in PD-1/PD-L1 induction would aid in better therapeutic options. The combination of checkpoint inhibitors and vaccines may be a viable option for improved clinical outcomes in cancer patients.

Conclusion

The immune system consists of a complex array of cells which work together to protect the body against invading pathogens, eliminates mutated cells and keeps an immune balance to prevent autoimmune attack. PD-1 present on B cells and T cells maintains peripheral tolerance and prevents autoimmune disorders. The interaction between PD-1 with PD-L1 leads to protection against self-reactivity. As discussed herein, the breakdown of the balance

between PD-1 and PD-L1 leads to disease. PD-1 is downregulated in autoimmunity, however, on cancer cells, there is upregulation of PD-L1 and the interaction of PD-L1 on cancer cells with PD-1 on T cells results in anergy of T cells. It is important to understand the mechanisms by which the balance of PD-1/PD-L1 are altered and methods to overcome this breakdown in order for immunotherapy/vaccines to be successful.

Expert commentary

PD-1/PD-L1 expression plays protective role in autoimmune, neurological diseases and stroke; however, in cancer, its expression generally results in disease acceleration. Studies thus far have established implications of PD-1/PD-L1 interaction in tumor immunity; however, many issues still remain unexplored. For instance, the mechanisms by which PD-1/PD-L1 pathway act as protective in autoimmune diseases and stroke but cause exacerbation in cancer. Therefore, understanding the mechanisms engaged in the activation of PD-1/PD-L1 pathway and the role of the nervous system in its activation could lead to designing better PD-L1/PD-1 checkpoint inhibitor drugs. In addition, several preclinical and clinical studies have shown that combining vaccines and immunotherapeutic associate with improved T cell functionality, resulting in improved patient outcomes [104–106]. For instance, activation of PD-L1-specific T cells modulates immunogenicity of DC vaccines [107]. In addition, blocking of PD-1 or PD-L1 restores antitumor efficacy of DNA vaccine immunization [106]. In subcutaneous and metastatic tumors induced by TL-1 and SiHa cells, antitumor activity was significantly enhanced with anti-PD-L1 monoclonal antibody + Lm-LLO-E6 vaccine compared with anti-PD-L1 monoclonal antibody or Lm-LLO-E6 alone [108]. Hence, immunotherapy/vaccines to be successful, the expression of PD-L1/PD-1 should be considered, and the combination of checkpoint inhibitors and vaccines may pave the way for successful outcomes of immunotherapeutic approaches to many diseases.

Future perspective

In autoimmune disorders methods to increase the expression of PD-1 on T and B cells is important to reverse the effects of autoimmunity. Hence, understanding the mechanisms by which immune cells downregulate the expression of PD-1 will lead to methods of upregulating the expression of PD-1 on immune cells for the effective treatment of autoimmune disorders. Cancer cells have evolved to suppress the immune system leading to evasion of the host. In addition to the upregulation of PD-L1 [109], cancer cells also upregulate other immunosuppressive markers including IDO and Siglec-9. Furthermore, cancer cells downregulate MHC class I which aids in their invasion, metastasis and/or recurrent disease. Moreover, myeloid-derived suppressor cells, regulatory T cells and tumor-associated neutrophils, fibroblasts, macrophages and immune and secretory molecules (e.g., IL-10, TGF-β and prostaglandins) results in an immunosuppressive tumor microenvironment [110]. Together, this allows cancer cells to evade the host immune system. Hence, it is important to determine the expression of a combination of immunosuppressive markers on cancer cells and within the tumor microenvironment before any tumor immunotherapeutics/vaccines can be effective. We are in a good position for research efforts to be put toward understanding the role of PD-1/PD-L1 in disease and the next 5 years will shed light into the mechanisms and will aid in newly improved immunotherapeutics against a range of diseases.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1 Burnet F. The concept of immunological surveillance. 13, 1–27 (1970).

2 Burnet M. Cancer – a biological approach: I. The processes of control. II. The significance of somatic mutation. *British Med. J.* 1(5022), 779 (1957).

3 Thomas L. On immunosurveillance in human cancer. *Yale J. Biol. Med.* 55(3–4), 329 (1982).

4 Thomas L, Lawrence H. *Cellular and Humoral Aspects of the Hypersensitive States.* Hoeber-Harper, NY, USA 529–532 (1959).

5 Doherty PC, Zinkernagel RM. H-2 compatibility is required for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. *J. Exp. Med.* 141(2), 502–507 (1975).

Executive summary

- PD-L1 is a transmembrane protein expressed on lymphoid and nonlymphoid tissues, antigen presenting cells and immune cells.
- Activated immune cells such as, NKT cells, myeloid cells, B cells and T cells, express PD-1, which plays a significant function in immune tolerance.
- PD-1 and PD-L1 inhibitory signaling is an essential mechanism behind immune regulation of disease states, such as autoimmunity, cancer and neurodegenerative diseases.

  PD-1 deficiency results in autoimmunity including Type 1 diabetes, multiple sclerosis and rheumatoid arthritis.
- In inflammatory bowel disorders, PD-L1 is upregulated on macrophages, dendritic cells, T cells, B cells; injection of anti-PD-L1 antibody reduces Th1 CD4+ T cells. In Crohn's disease patients, PD-L1 is downregulated in intestinal tissues which may contribute to its pathogenesis.
- PD-1/PD-L1 pathway may play a role in immune regulation of neurological disorders, including ischemic stroke, multiple sclerosis and Alzheimer's disease.

  The PD-1/PD-L pathway plays a role in poststroke inflammation via negative regulation of cell–cell interaction.
- Upregulation of PD-L1 on cancer cells suppresses the host–immune responses to avoid immune detection. Immune checkpoint inhibitors that either block PD-1 or PD-L1 hold promise for the treatment of cancer.

6 Coulie PG, Van Den Eynde BJ, Van Der Bruggen P, Boon T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat. Rev. Cancer* 14(2), 135–146 (2014).

7 Flies DB, Chen L. The new B7s: playing a pivotal role in tumor immunity. *J. Immunother.* 30(3), 251–260 (2007).

8 Ishida M, Iwai Y, Tanaka Y *et al.* Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. *Immunol. Lett.* 84(1), 57–62 (2002).

9 Muenst S, Schaerli A, Gao F *et al.* Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. *Breast Cancer Res. Treat.* 146(1), 15–24 (2014).

10 Polanczyk MJ, Hopke C, Vandenbark AA, Offner H. Estrogen-mediated immunomodulation involves reduced activation of effector T cells, potentiation of Treg cells, and enhanced expression of the PD-1 costimulatory pathway. *J. Neurosci. Res.* 84(2), 370–378 (2006).

11 Yamazaki T, Akiba H, Iwai H *et al.* Expression of programmed death 1 ligands by murine T cells and APC. *J. Immunol.* 169(10), 5538–5545 (2002).

12 Ferris R. PD-1 targeting in cancer immunotherapy. *Cancer* 119(23), E1–E3 (2013).

13 Nishimura H, Minato N, Nakano T, Honjo T. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int. Immunol.* 10(10), 1563–1572 (1998).

14 Sui X, Ma J, Han W *et al.* The anticancer immune response of anti-PD-1/PD-L1 and the genetic determinants of response to anti-PD-1/PD-L1 antibodies in cancer patients. *Oncotarget* 6(23), 19393–19404 (2015).

15 Li Z, Dong P, Ren M *et al.* PD-L1 expression is associated with tumor FOXP3(+) regulatory T-cell infiltration of breast cancer and poor prognosis of patient. *J. Cancer* 7(7), 784–793 (2016).

16 Shin S-J, Jeon YK, Kim P-J *et al.* Clinicopathologic analysis of PD-L1 and PD-L2 expression in renal cell carcinoma: association with oncogenic proteins status. *Ann. Surg. Oncol.* 23(2), 694–702 (2016).

- **A nice study demonstrating the expression of programmed death ligand 1 (PD-L1) and programmed death ligand 2 on renal cell carcinomas.**

17 Ansari MJI, Salama AD, Chitnis T *et al.* The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J. Exp. Med.* 198(1), 63–69 (2003).

- **The role of programmed death 1 (PD-1) in autoimmune diabetes in nonobese diabetic mice.**

18 Bodhankar S, Wang C, Vandenbark AA, Offner H. Estrogen-induced protection against experimental autoimmune encephalomyelitis is abrogated in the absence of B cells. *Eur. J. Immunol.* 41(4), 1165–1175 (2011).

19 Chen L, Pai V, Levinson R *et al.* Constitutive neuronal expression of the immune regulator, programmed death 1 (PD-1), identified during experimental autoimmune uveitis. *Ocul. Immunol. Inflamm.* 17(1), 47–55 (2009).

20 Dai S, Jia R, Zhang X, Fang Q, Huang L. The PD-1/PD-Ls pathway and autoimmune diseases. *J. Clin. Cell Immunol.* 290(1), 72–79 (2014).

- **The role of PD-1/PD-L1 in autoimmune disorders is discussed.**

21 Eppihimer MJ, Gunn J, Freeman GJ *et al.* Expression and regulation of the PD-L1 immunoinhibitory molecule on microvascular endothelial cells. *Microcirculation* 9(2), 133–145 (2002).

22 Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol. Rev.* 236, 219–242 (2010).

•• **A comprehensive review of PD-1 and autoimmunity.**

23 Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11(2), 141–151 (1999).

24 Nishimura H, Okazaki T, Tanaka Y *et al.* Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291(5502), 319–322 (2001).

25 Salama AD, Chitnis T, Imitola J *et al.* Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J. Exp. Med.* 198(1), 71–78 (2003).

26 Anagnostou VK, Brahmer JR. Cancer immunotherapy: a future paradigm shift in the treatment of non-small cell lung cancer. *Clin. Cancer Res.* 21(5), 976–984 (2015).

• **A nice study demonstrating the expression of PD-L1 in anaplastic meningioma.**

27 Du Z, Abedalthagafi M, Aizer AA *et al.* Increased expression of the immune modulatory molecule PD-L1 (CD274) in anaplastic meningioma. *Oncotarget* 6(7), 4704–4716 (2015).

28 Kim JR, Moon YJ, Kwon KS *et al.* Tumor infiltrating PD1-positive lymphocytes and the expression of PD-L1 predict poor prognosis of soft tissue sarcomas. 8(12), e82870 (2013).

29 Qing Y, Li Q, Ren T *et al.* Upregulation of PD-L1 and APE1 is associated with tumorigenesis and poor prognosis of gastric cancer. *Drug Des. Devel. Ther.* 9, 901 (2015).

30 Arasanz H, Gato-Cañas M, Zuazo M *et al.* PD1 signal transduction pathways in T cells. *Oncotarget* 8(31), 51936–51945 (2017).

31 Keir ME, Liang SC, Guleria I *et al.* Tissue expression of PD-L1 mediates peripheral T-cell tolerance. *J. Exp. Med.* 203(4), 883–895 (2006).

32 Wang C-J, Chou F-C, Chu C-H *et al.* Protective role of programmed death 1 ligand 1 (PD-L1) in nonobese diabetic mice the paradox in transgenic models. *Diabetes* 57(7), 1861–1869 (2008).

33 Pauken KE, Jenkins MK, Azuma M, Fife BT. PD-1, but not PD-L1, expressed by islet-reactive CD4+ T cells suppresses infiltration of the pancreas during Type 1 diabetes. *Diabetes* 62(8), 2859–2869 (2013).

• **The role of PD-1 on CD4$^+$ T cells is shown in Type 1 diabetes.**

34 Wang J, Yoshida T, Nakaki F, Hiai H, Okazaki T, Honjo T. Establishment of NOD-Pdcd1$^{-/-}$ mice as an efficient animal model of Type I diabetes. *Proc. Natl Acad. Sci. USA* 102(33), 11823–11828 (2005).

35 Fujisawa R, Haseda F, Tsutsumi C *et al.* Low programmed cell death-1 (PD-1) expression in peripheral CD4(+) T cells in Japanese patients with autoimmune Type 1 diabetes. *Clin. Exp. Immunol.* 180(3), 452–457 (2015).

36 Liang SC, Latchman YE, Buhlmann JE *et al.* Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur. J. Immunol.* 33(10), 2706–2716 (2003).

37 Carter LL, Leach MW, Azoitei ML *et al.* PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 182(1–2), 124–134 (2007).

38 Wang C, Dehghani B, Li Y, Kaler LJ, Vandenbark AA, Offner H. Oestrogen modulates experimental autoimmune encephalomyelitis and interleukin-17 production via programmed death 1. *J. Immunol.* 126(3), 329–335 (2009).

39 Trabattoni D, Saresella M, Pacei M *et al.* Costimulatory pathways in multiple sclerosis: distinctive expression of PD-1 and PD-L1 in patients with different patterns of disease. *J. Immunol.* 183(8), 4984–4993 (2009).

40 Kanai T, Totsuka T, Uraushihara K *et al.* Blockade of B7-H1 suppresses the development of chronic intestinal inflammation. *J. Immunol.* 171(8), 4156–4163 (2003).

41 Reynoso ED, Elpek KG, Francisco L *et al.* Intestinal tolerance is converted to autoimmune enteritis upon PD-1 ligand blockade. *J. Immunol.* 182(4), 2102–2112 (2009).

42 Scandiuzzi L, Ghosh K, Hofmeyer KA *et al.* Tissue-expressed B7-H1 critically controls intestinal inflammation. *Cell reports* 6(4), 625–632 (2014).

43 Robertson J, Haas CT, Pele LC *et al.* Intestinal APCs of the endogenous nanomineral pathway fail to express PD-L1 in Crohn's disease. *Sci. Rep.* 6, 26747 (2016).

44 Hassan WA, Baraka EA, Fouad NA. Clinical significance of soluble programmed death-1 (sPD-1) in rheumatoid arthritis patients: relation to disease activity and functional status. *Egypt. Rheumatol.* 37(4), 165–169 (2015).

45 Liu C, Jiang J, Gao L *et al.* Soluble PD-1 aggravates progression of collagen-induced arthritis through Th1 and Th17 pathways. *Arthritis Res. Ther.* 17(1), 340 (2015).

46 Moret FM, Van Der Wurff-Jacobs KM, Bijlsma JW, Lafeber FP, Van Roon JA. Synovial T-cell hyporesponsiveness to myeloid dendritic cells is reversed by preventing PD-1/PD-L1 interactions. *Arthritis Res. Ther.* 16(6), 497 (2014).

• **A nice study demonstrating the role of PD-1/PD-L1 in rheumatoid arthritis.**

47 Wan B, Nie H, Liu A *et al.* Aberrant regulation of synovial T-cell activation by soluble costimulatory molecules in rheumatoid arthritis. *J. Immunol.* 177(12), 8844–8850 (2006).

48 Raptopoulou AP, Bertsias G, Makrygiannakis D *et al.* The programmed death 1/programmed death ligand 1 inhibitory pathway is up-regulated in rheumatoid synovium and regulates peripheral T-cell responses in human and murine arthritis. *Arthritis Rheum.* 62(7), 1870–1880 (2010).

49 Koronyo-Hamaoui M, Ko MK, Koronyo Y *et al.* Attenuation of AD-like neuropathology by harnessing peripheral immune cells: local elevation of IL-10 and MMP-9. *J. Neurochem.* 111(6), 1409–1424 (2009).

50 Zhao S, Li F, Leak RK, Chen J, Hu X. Regulation of neuroinflammation through programed death-1/programed death ligand signaling in neurological disorders. *Front. Cell Neurosci.* 8, 271 (2014).

51 Saresella M, Calabrese E, Marventano I *et al.* A potential role for the PD1/PD-L1 pathway in the neuroinflammation of Alzheimer's disease. *Neurobiol. Aging* 33(3), 624.e611–624.e622 (2012).

52 Saresella M, Calabrese E, Marventano I *et al.* PD1 negative and PD1 positive CD4+ T regulatory cells in mild cognitive impairment and Alzheimer's disease. *J. Alzheimer's Dis.* 21(3), 927 (2010).

53 Li P, Mao L, Liu X *et al.* Essential role of program death 1-ligand 1 in regulatory T-cell–afforded protection against blood–brain barrier damage after stroke. *Stroke* 45(3), 857–864 (2014).

54 Ren X, Akiyoshi K, Vandenbark AA, Hurn PD, Offner H. Programmed death-1 pathway limits central nervous system inflammation and neurologic deficits in murine experimental stroke. *Stroke* 42(9), 2578–2583 (2011).

55 Bodhankar S, Chen Y, Vandenbark AA, Murphy SJ, Offner H. PD-L1 enhances CNS inflammation and infarct volume following experimental stroke in mice in opposition to PD-1. *J. Neuroinflammation* 10(111.10), 1186 (2013).

56 Bodhankar S, Vandenbark AA, Offner H. Oestrogen treatment of experimental autoimmune encephalomyelitis requires 17β-oestradiol-receptor-positive B cells that up-regulate PD-1 on CD4+ Foxp3+ regulatory T cells. *Immunology* 137(4), 282–293 (2012).

57 Brandl C, Ortler S, Herrmann T, Cardell S, Lutz MB, Wiendl H. B7-H1-deficiency enhances the potential of tolerogenic dendritic cells by activating CD1d-restricted type II NKT cells. *PloS One* 5(5), e10800–e10800 (2010).

58 Chang W-S, Kim J-Y, Kim Y-J *et al.* Cutting edge: programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT-cell anergy. *J. Immunol.* 181(10), 6707–6710 (2008).

59 Joller N, Peters A, Anderson AC, Kuchroo VK. Immune checkpoints in central nervous system autoimmunity. *Immunol. Rev.* 248(1), 122–139 (2012).

60 Latchman Y, Wood CR, Chernova T *et al.* PD-L2 is a second ligand for PD-1 and inhibits T-cell activation. *Nat. Immunol.* 2(3), 261–268 (2001).

- **The importance of programmed death ligand 1 in inhibiting T-cell activation.**

61 Schreiner B, Bailey SL, Shin T, Chen L, Miller SD. PD-1 ligands expressed on myeloid-derived APC in the CNS regulate T-cell responses in EAE. *Eur. J. Immunol.* 38(10), 2706–2717 (2008).

62 Ceeraz S, Nowak EC, Noelle RJ. B7 family checkpoint regulators in immune regulation and disease. *Trends Immunol.* 34(11), 556–563 (2013).

63 Leung HT, Linsley PS. The CD28 costimulatory pathway. *Ther. Immunol.* 1(4), 217–228 (1994).

64 Leung J, Suh WK. The CD28-B7 family in anti-tumor immunity: emerging concepts in cancer immunotherapy. *Immune Netw.* 14(6), 265–276 (2014).

65 Garrido F, Cabrera T, Concha A, Glew S, Ruiz-Cabello F, Stern PL. Natural history of HLA expression during tumour development. *Immunol. Today* 14(10), 491–499 (1993).

66 Garrido F, Romero I, Aptsiauri N, Garcia-Lora AM. Generation of MHC class I diversity in primary tumors and selection of the malignant phenotype. *Int. J. Cancer* 138(2), 271–280 (2016).

67 Nawrocki S, Mackiewicz A. Genetically modified tumour vaccines – where we are today. *Cancer Treat. Rev.* 25(1), 29–46 (1999).

68 Gato-Canas M, Zuazo M, Arasanz H *et al.* PDL1 signals through conserved sequence motifs to overcome interferon-mediated cytotoxicity. *Cell. Rep.* 20(8), 1818–1829 (2017).

69 Mandai M. PD-1/PD-L1 blockage in cancer treatment-from basic research to clinical application. *Int. J. Clin. Oncol.* 21(3), 447 (2016).

70 Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen L. B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. *Blood* 111(7), 3635–3643 (2008).

71 Ghebeh H, Tulbah A, Mohammed S *et al.* Expression of B7-H1 in breast cancer patients is strongly associated with high proliferative Ki-67-expressing tumor cells. *Int. J. Cancer* 121(4), 751–758 (2007).

72 Shin DS, Zaretsky JM, Escuin-Ordinas H *et al.* Primary resistance to PD-1 blockade mediated by JAK1/2 mutations. *Cancer Discov.* 7(2), 188–201 (2017).

73 Zaretsky JM, Garcia-Diaz A, Shin DS *et al.* Mutations associated with acquired resistance to PD-1 blockade in melanoma. *N. Engl. J. Med.* 375(9), 819–829 (2016).

74 Zhang J, Gao J, Li Y *et al.* Circulating PD-L1 in NSCLC patients and the correlation between the level of PD-L1 expression and the clinical characteristics. *Thoracic Cancer* 6(4), 534–538 (2015).

75 Wimberly H, Brown JR, Schalper K *et al.* PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer. *Cancer Immunol. Res.* 3(4), 326–332 (2015).

76 Mazel M, Jacot W, Pantel K *et al.* Frequent expression of PD-L1 on circulating breast cancer cells. *Mol. Oncol.* 9(9), 1773–1782 (2015).

77 Baptista MZ, Sarian LO, Derchain SF, Pinto GA, Vassallo J. Prognostic significance of PD-L1 and PD-L2 in breast cancer. *Hum. Pathol.* 47(1), 78–84 (2016).

78 Schalper KA. PD-L1 expression and tumor-infiltrating lymphocytes: revisiting the antitumor immune response potential in breast cancer. *Oncoimmunology* 3, e29288 (2014).

79 Yang CY, Lin MW, Chang YL, Wu CT, Yang PC. Programmed cell death-ligand 1 expression in surgically resected stage I pulmonary adenocarcinoma and its correlation with driver mutations and clinical outcomes. *Eur. J. Cancer* 50(7), 1361–1369 (2014).

80 Taube JM, Anders RA, Young GD *et al.* Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci. Transl. Med.* 4(127), 127ra137–127ra137 (2012).

81 Dong H, Strome SE, Salomao DR *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat. Med.* 8(8), 793 (2002).

82 Lastwika KJ, Wilson W, Li QK *et al.* Control of PD-L1 expression by oncogenic activation of the AKT–mTOR pathway in non-small cell lung Cancer. *Cancer Res.* 76(2), 227–238 (2016).

83 Liu J, Hamrouni A, Wolowiec D *et al.* Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-γ and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood* 110(1), 296–304 (2007).

84 Marzec M, Zhang Q, Goradia A *et al.* Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). *Proc. Natl Acad. Sci. USA* 105(52), 20852–20857 (2008).

85 Ritprajak P, Azuma M. Intrinsic and extrinsic control of expression of the immunoregulatory molecule PD-L1 in epithelial cells and squamous cell carcinoma. *Oral Oncol.* 51(3), 221–228 (2015).

86 Mittendorf EA, Philips AV, Meric-Bernstam F *et al.* PD-L1 expression in triple-negative breast cancer. *Cancer Immunol. Res.* 2(4), 361–370 (2014).

87 Joseph RW, Parasramka M, Eckel-Passow JE *et al.* Inverse association between programmed death ligand 1 and genes in the VEGF pathway in primary clear cell renal cell carcinoma. *Cancer Immunol. Res.* 1(6), 378–385 (2013).

88 Kondo A, Yamashita T, Tamura H *et al.* Interferon-γ and tumor necrosis factor-α induce an immunoinhibitory molecule, B7-H1, via nuclear factor-κB activation in blasts in myelodysplastic syndromes. *Blood* 116(7), 1124–1131 (2010).

89 Heskamp S, Hobo W, Molkenboer-Kuenen JDM *et al.* Noninvasive imaging of tumor PD-L1 expression using radiolabeled Anti–PD-L1 antibodies. *Cancer Res.* 75(14), 2928–2936 (2015).

90 Chatterjee S, Lesniak WG, Gabrielson M *et al.* A humanized antibody for imaging immune checkpoint ligand PD-L1 expression in tumors. *Oncotarget* 7(9), 10215–10227 (2016).

91 Josefsson A, Nedrow JR, Park S *et al.* Imaging, biodistribution, and dosimetry of radionuclide-labeled PD-L1 Antibody in an immunocompetent mouse model of breast cancer. *Cancer Res.* 76(2), 472–479 (2016).

92 Lesniak WG, Chatterjee S, Gabrielson M *et al.* PD-L1 detection in tumors using [(64)Cu]atezolizumab with PET. *Bioconjug. Chem.* 27(9), 2103–2110 (2016).

93 Broos K, Keyaerts M, Lecocq Q *et al.* Non-invasive assessment of murine PD-L1 levels in syngeneic tumor models by nuclear imaging with nanobody tracers. *Oncotarget* 8(26), 41932–41946 (2017).

94 Le DT, Bendell JC, Calvo E *et al.* Safety and activity of nivolumab monotherapy in advanced and metastatic (A/M) gastric or gastroesophageal junction cancer (GC/GEC): results from the CheckMate-032 study. *J. Clin. Oncol.* 34(4 Suppl), 6–6 (2016).

95 Sul J, Blumenthal GM, Jiang X, He K, Keegan P, Pazdur R. FDA approval summary: pembrolizumab for the treatment of patients with metastatic non-small cell lung cancer whose tumors express programmed death-ligand 1. *Oncologist* 21(5), 643–650 (2016).

96 Nomi T, Sho M, Akahori T *et al.* Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin. Cancer Res.* 13(7), 2151–2157 (2007).

97 Ascierto PA, Marincola FM. 2015: the year of anti-PD-1/PD-L1s against melanoma and beyond. *EBioMedicine* 2(2), 92–93 (2015).

98 Mahoney KM, Sun H, Liao X *et al.* PD-L1 Antibodies to its cytoplasmic domain most clearly delineate cell membranes in immunohistochemical staining of tumor cells. *Cancer Immunol. Res.* 3(12), 1308–1315 (2015).

99 Weber JS, D'angelo SP, Minor D *et al.* Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, Phase III trial. *Lancet Oncol.* 16(4), 375–384 (2015).

100 Goldberg SB, Gettinger SN, Mahajan A *et al.* Pembrolizumab for patients with melanoma or non-small-cell lung cancer and untreated brain metastases: early analysis of a non-randomised, open-label, Phase 2 trial. *Lancet Oncol.* 17(7), 976–983 (2016).

•• Checkpoint inhibitors in non-small-cell lung cancer patients.

101 Martin-Liberal J, Furness AJ, Joshi K, Peggs KS, Quezada SA, Larkin J. Anti-programmed cell death-1 therapy and insulin-dependent diabetes: a case report. *Cancer Immunol. Immunother.* 64(6), 765–767 (2015).

102 Oppel-Heuchel H, Grimm MO. Therapy monitoring and management of adverse events in PD-1/PD-L1 immune checkpoint inhibition. *Urologe A* 55(5), 677–690 (2016).

103 Markham A. Atezolizumab: first global approval. *Drugs* 76(12), 1227–1232 (2016).

104 Pen JJ, Keersmaecker BD, Heirman C *et al.* Interference with PD-L1/PD-1 co-stimulation during antigen presentation enhances the multifunctionality of antigen-specific T cells. *Gene Ther.* 21(3), 262–271 (2014).

105 Soares KC, Rucki AA, Wu AA *et al.* PD-1/PD-L1 blockade together with vaccine therapy facilitates effector T-cell infiltration into pancreatic tumors. *Int. J. Immunother. Cancer Res.* (Hagerstown, MD, USA 1997) 38(1), 1–11 (2015).

106 Rekoske BT, Smith HA, Olson BM, Maricque BB, Mcneel DG. PD-1 or PD-L1 blockade restores antitumor efficacy following SSX2 Epitope-Modified DNA vaccine immunization. *Cancer Immunol. Res.* 3(8), 946–955 (2015).

107 Munir Ahmad S, Martinenaite E, Hansen M *et al.* PD-L1 peptide co-stimulation increases immunogenicity of a dendritic cell-based cancer vaccine. *Oncoimmunology* 5(8), e1202391 (2016).

108 Lin PL, Cheng YM, Wu DW *et al.* A combination of anti-PD-L1 mAb plus Lm-LLO-E6 vaccine efficiently suppresses tumor growth and metastasis in HPV-infected cancers. *Cancer Med.* 6(9), 2052–2062 (2017).

109 Kuol N, Senior PV, Stojanovska L, Nurgali K, Apostolopoulos V. PD-L1 and Siglec 9 are significantly upregulated in stage III compared to stage II colorectal cancer: implications of tumor escape mechanisms. *Maturitas* (2017). (In Press).

•• **Demonstrates that PD-L1 and Siglec-9 are upregulated in colorectal cancer tissues.**

110 Kuol N, Stojanovska L, Nurgali K, Apostolopoulos V. The mechanisms tumor cells utilize to evade the host's immune system. *Maturitas* 105, 8–15 (2017).

•• **A comprehensive review on tumor escape mechanisms from host immunity.**

111 Cotliar J, Querfeld C, Boswell WJ, Raja N, Raz D, Chen R. Pembrolizumab-associated sarcoidosis. *JAAD Case Rep.* 2(4), 290–293 (2016).

112 Kirchberger MC, Hauschild A, Schuler G, Heinzerling L. Combined low-dose ipilimumab and pembrolizumab after sequential ipilimumab and pembrolizumab failure in advanced melanoma. *Eur. J. Cancer* 65, 182–184 (2016).

• **Together with [111], the use of checkpoint inhibitors in melanoma patients.**

113 Yang Y, Ding L, Wang P. Dramatic response to anti-PD-1 therapy in a patient of squamous cell carcinoma of thymus with multiple lung metastases. *J. Thorac. Dis.* 8(7), E535–E537 (2016).

• **Checkpoint inhibitors in squamous cell carcinoma patients.**

114 Aaberg MT, Aaberg TM Jr. Pembrolizumab administration associated with posterior uveitis. *Retin. Cases Brief Rep.* 11(4), 348–351 (2016).

115 Younes A, Santoro A, Shipp M *et al.* Nivolumab for classical Hodgkin's lymphoma after failure of both autologous stem-cell transplantation and brentuximab vedotin: a multicentre, multicohort, single-arm Phase II trial. *Lancet Oncol.* 17(9), 1283–1294 (2016).

• **A Phase II clinical trial using checkpoint inhibitors in Hodgkin's lymphoma.**

116 Heine A, Kristiansen G, Schild HH, Brossart P. Successful treatment of refractory leiomyosarcoma with the PD-1 inhibitor nivolumab. *Annal. Oncol.* 27(9), 1813–1814 (2016).

• **Checkpoint inhibitors for the treatment of refractory leiomyosarcoma.**

117 Spain L, Higgins R, Gopalakrishnan K, Turajlic S, Gore M, Larkin J. Acute renal allograft rejection after immune checkpoint inhibitor therapy for metastatic melanoma. *Ann. Oncol.* 27(6), 1135–1137 (2016).

118 Kaufman HL, Russell J, Hamid O *et al.* Avelumab in patients with chemotherapy-refractory metastatic Merkel cell carcinoma: a multicentre, single-group, open-label, Phase II trial. *Lancet Oncol.* 17(10), 1374–1385 (2016).

119 Apolo AB, Infante JR, Balmanoukian A *et al.* Avelumab, an anti-programmed death-ligand 1 antibody, in patients with refractory metastatic urothelial carcinoma: results from a multicenter, Phase IB study. *J. Clin. Oncol.* 35(19), 2117–2124 (2017).

120 Necchi A, Joseph RW, Loriot Y *et al.* Atezolizumab in platinum-treated locally advanced or metastatic urothelial carcinoma: post-progression outcomes from the Phase II IMvigor210 study. *Ann. Oncol.* 35(6), 323(2017).

121 Rittmeyer A, Barlesi F, Waterkamp D *et al.* Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. *Lancet* 389(10066), 255–265 (2017).