Mesenchymal Stem Cells in Inflammatory Bowel Disease and Cancer

Sarah Miller

This thesis is submitted to fulfil the requirements for the degree of

Masters by Research (Science)

Principal Supervisor: A/Prof. Kulmira Nurgali

Co-Supervisors: Prof. Vasso Apostolopoulos and Dr. Samy Sakkal

College of Health and Biomedicine

Victoria University, Melbourne, Australia

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Abstract

Inflammatory bowel disease (IBD) is comprised of two main intestinal diseases: ulcerative colitis and Crohn's disease. The major characteristic of IBD is chronic uncontrolled inflammation of the intestinal mucosa which can affect any part of the gastrointestinal tract. The link between inflammation and cancer is widely known and studied with those afflicted by chronic inflammatory conditions (such as IBD) more likely to develop cancer. Recently, mesenchymal stem cells (MSCs) have shown therapeutic potential in IBD; however whether MSCs promote or suppress tumour growth still remains contentious within the literature. A number of studies indicate that MSCs exert anti-tumour effects and suppress tumour growth whilst other studies report pro-tumour effects. The use of MSCs as a treatment for IBD has shown promise in both animal models and human trials. However, as MSC treatment is still novel the long term risks remain unknown.

This thesis aims to uncover how the immune system is affected by colorectal cancer (CRC) in an inflammatory environment *in vivo* and also whether treatment with MSCs has an anti or pro-tumour effect in the same *in vivo* model.

The kinetics of the immune response to CRC development and the key effector lymphocytes that are implicated in tumour immunity were studied in the murine orthotopic model of CRC induced by CT26 cells implanted into the caecum. This study demonstrated significant changes in the number of NK cells in mesenteric lymph nodes and significant changes in the number of $CD8^+$ T lymphocytes in Peyer's patches of mice with CRC. Immunohistochemical labelling revealed that γδT cells were depleted in the colon of mice with CRC and immune cells (eosinophils, $CD69^+$ T cells and $CD11b^+$cells) infiltrated to the tumour site. Cytokine analysis revealed that CT26 cells and infiltrating $CD45^+$ cells secreted IL-6 both *in vitro* and *in vivo*; $CD45^+$ cells also expressed TNFα, which further contributed to a pro-inflammatory tumour microenvironment.

This study also demonstrated that the *Winnie* mouse is a viable model of spontaneous chronic inflammation with immune responses similar to what is

noted in human IBD. The *Winnie* mouse is also a good model of colitis-associated CRC as injection of MC38 cells into the caecum resulted in a higher number of tumours compared to C57BL/6 mice. When CRC is induced by MC38 cells implanted into the caecum of *Winnie* mice, an increase in the percentage of M1 macrophages, T cells and decrease in M2 macrophages was shown, which is expected, as the immune system is attempting to eliminate the foreign cells.

MSCs have the ability to adapt to the environment in which they are placed, it has been theorised that in an inflammatory environment MSCs will have an anti-inflammatory response. However, the role of MSCs in the tumour microenvironment is still contentious in the literature. The results of this study demonstrated that MSC treatment after CRC induction in both C57BL/6 and *Winnie* mice induces an immune response promoting tumour growth and progression, whereas MSC treatment given before CRC induction in both C57BL/6 and *Winnie* mice leads to an immune response that promoting an anti-tumour environment.

In conclusion, this study has established that CT26 colorectal cancer cells in an orthotopic model contribute to a pro-inflammatory tumour microenvironment and has significant effects on the immune system. It demonstrated that *Winnie* mouse is a good model of colitis associated CRC. It also showed that MSC treatment given after CRC inductions leads to a pro-tumour environment and MSC treatment given before CRC induction leads to an anti-tumour environment.

Declaration

I, Sarah Miller, declare that the Master by Research thesis entitled “Mesenchymal stem cells in inflammatory bowel disease and cancer” is no more than 60,000 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.

Sarah Miller

15rd March 2017

Declaration of Contribution to Work

Under my direction the following people have made the stated contributions to this work:

Chapter 1:

Assistance in preparing the manuscript that contributed to this chapter was provided by M Prakash, S Sakkal and K Nurgali.

Chapter 2:

Cancer cells were grown by PV Senior. Assistance in flow cytometry experiments and analysis was given by S Sakkal and V Apostolopoulos. Technical assistance in performing orthotopic surgeries was provided by V Jovanovska and M Prakash. Assistance in preparing the manuscript for this chapter was provided by S Sakkal, V Apostolopoulos and K Nurgali.

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Publications

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Other Publications

ROBINSON AM, RAHMAN AA, **MILLER S**, STAVELY R, SAKKAL S, NURGALI K (2017) Neuroprotective effects of human bone marrow mesenchymal stem cells are dose-dependent in TNBS colitis. *Stem Cell Research & Therapy* (accepted 16-03-2017).

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ROBINSON AM**, MILLER S**, PAYNE N, BOYD R , SAKKAL S. & NURGALI K (2015) Neuroprotective Potential of Mesenchymal Stem Cell-Based Therapy in Acute Stages of TNBS-Induced Colitis in Guinea-Pigs. *PloS ONE,* 10**,** e0139023.

STAVELY R., ROBINSON AM, **MILLER S**, BOYD R, SAKKAL S. & NURGALI K (2015) Allogeneic guinea pig mesenchymal stem cells ameliorate neurological changes in experimental colitis. *Stem Cell Research & Therapy,* 6**,** 1-21.

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attenuate enteric neuropathy in the guinea-pig model of acute colitis. *Stem Cell Research & Therapy,* 6**,** 1-21.

ROBINSON, A. M., SAKKAL, S., PARK, A., JOVANOVSKA, V., PAYNE, N., CARBONE, S. E., **MILLER, S**., BORNSTEIN, J. C., BERNARD, C. & BOYD, R (2014) Mesenchymal stem cells and conditioned medium avert enteric neuropathy and colon dysfunction in guinea pig TNBS-induced colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 307**,** G1115-G1129.

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Presentations

Oral Presentations

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Miller S, Robinson AM, Sakkal S, Jovanovska V, Park A, Carbone SE, Boyd R, Bernard C, Nurgali K (2014) Treatment of TNBS-colitis with bone marrow mesenchymal stem cells and conditioned medium prevents enteric neuropathy in guinea-pigs. Australasian Neuroscience Society 34th Annual Meeting - Oral Presentation (ORAL-01-04).

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List of Abbreviations

APC – Adenomatous Polyposis Coli

APCs – Antigen Presenting Cells

bFGf – Basic Fibroblast Growth Factor

BM – Bone Marrow

CAC – Colitis Associated Cancer

CARD15 - Caspase Recruitment Domain-containing Protein 15

CCR – Chemokine Receptor

CD – Cluster Differentiation

CD – Crohn's Disease

CDAI – Crohn's Disease Activity Index

CLIP – Class II-associated Invariant Chain Peptide

CRC – Colorectal Cancer

CSF – Colony Stimulating Factor

CTLs – Cytotoxic T Lymphocytes

DNP-KLH – 2,4-Dinitrophenyl-Keyhole Limpet Hemocyanin

DSS – Dextran Sodium Sulfate

FGF – Fibroblast Growth Factor

GIT – Gastrointesitnal Tract

HLA – Human Leukocyte Antigen

HSCs – Hematopoietic Stem Cells

IBD – Inflammatory Bowel Disease

ICAM - Intercellular Adhesion Molecule

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

ILC1s – Group 1 Innate Lymphoid Cells

ILC2 – Group 2 Innate Lymphoid Cells

ILC3 – Group 3 Innate Lymphoid Cells

ILCs – Innate Lymphoid Cells

IR – Immunoreactive

ISCT – International Society for Cellular Therapy

LAK – Lymphokine-Activated Killer

MAIT – Mucosal Associated Invariant T

MCP – Monocyte Haemoattractant Protein

MDR – Multiple Drug Resistant

MHC – Major Histocompatibility Complex

MIP – Macrophage Inflammatory Protein

MLNs – Mesenteric Lymph Nodes

MMPs – Matrix Metalloproteinases

MSCs – Mesenchymal Stem Cells

Muc2 – Mucin 2

NF – Nuclear Factor

NK – Natural Killer

NKT – Natural Killer T

PAMPs – Pathogen Associated Molecular Patterns

PD-1 – Programmed Death Receptor-1

PDGFR – Platelet Derived Growth Factor Receptor

PD-L1 – Programmed Death Ligand-1

PRRs – Pattern Recognition Receptors

RANTES – Regulated on Activation, Normal T Cell Expressed and Secreted

RPMI – Roswell Park Memorial Institute

SCID – Severe Combined Immunodeficiency

STAT – Signal Transducer and Activator of Transcription

TAF – Tumour Associated Fibroblasts

TAMs – Tumour Associated Macrophages

TCR – T Cell Receptor

TGF – Transforming Growth Factor

Th – T Helper

TNF – Tumour Necrosis Factor

Tregs – T Regulatory Cells

TSLP – Thymic Stromal Lymphopoietin

UC – Ulcerative Colitis

VEGF – Vascular Endothelial Growth Factor

γδ – Gamma Delta

Chapter One: Literature Review

Parts of the material presented in this chapter is published and has been reproduced here with the permission of the publisher with minor alterations:

PRAKASH, M. D., **MILLER, S**., RANDALL-DEMLLO, S. & NURGALI, K. 2016. Mesenchymal Stem Cell Treatment of Inflammation-Induced Cancer. *Inflammatory Bowel Diseases,* 22, 2694-2703.

Cancer is frequently associated with chronic inflammation as this was based on observations dating back to 1863 where it was noted that cancers initiated at sites of existing inflammation (Virchow, 1863); an observation that has since been confirmed by epidemiological studies with strong correlations found between chronic inflammation and increased risk of cancer (Coussens and Werb, 2002, Balkwill and Mantovani, 2012).

Inflammatory bowel disease (IBD) is characterised by recurrent, idiopathic intestinal inflammation, the complications of which are potentially fatal. IBD is believed to be of multiple genetic origins and is a result of an uncontrolled immune reaction to gut bacteria (Becker et al., 2015). However due to it's poorly understood aetiology, current IBD therapies treat the symptoms rather than the disease and are limited by adverse side effects and toxicity, rendering them largely ineffective. For many years the cause of IBD has been contentious with the most prominent theories hypothesising infectious causes such as Mycobacteria paratuberculosis and measles, allergic and nutritional related causes and micro-particles. However these theories rely on the idea that there is increased intestinal permeability being the central defect leading to IBD. Rather than an excessive T cell driven process, Crohn's has been suggested to be an innate immune deficiency disease, leading to the use of colony stimulating factors to augment the intestinal barrier function and innate immunity. A variety of changes in the gut flora, ranging from a basic dysbiosis to the absence of helminths, have also been proposed as the root cause of IBD.

The risk of CRC for patients with IBD increases by 0.5 – 1% yearly, 8 – 10 years after diagnosis (Eaden et al., 2001). Although IBD patients only contribute to 1-2% to all cases of CRC, the mortality rate in patients with a diagnosis of CRC in the setting of IBD is higher than those afflicted with sporadic cases (Itzkowitz and Harpaz, 2004, Vagefi and Longo, 2005).

Recently mesenchymal stem cells (MSCs) have gained popularity due to their therapeutic potential in many diseases including IBD. MSCs are multipotent stem cells that can be derived from many adult tissues including bone marrow and fat tissue (Mosna et al., 2010). MSCs are currently being used to treat inflammatory disorders such as arthritis, multiple sclerosis and IBD (González et al., 2009,

Duijvestein et al., 2010, Freedman et al., 2010, Ciccocioppo et al., 2011, Connick et al., 2012, Lee et al., 2013, Forbes et al., 2014).

The long term effects of MSC treatment is still yet to be determined and a main concern of MSC treatment is whether MSCs actually suppress or promotes tumour development; in the absence of a consensus on this matter, therapeutics goods administration (TGA) and food and drugs administration (FDA) approval for clinical trials will be curtailed. (Lazennec and Jorgensen, 2008, Klopp et al., 2011). Many studies indicate MSCs exert anti-tumour effects and suppress tumour growth (Khakoo et al., 2006, Qiao et al., 2008b) while other studies report pro-tumourigenic effects (Karnoub et al., 2007, Zhu et al., 2009).

1.1 Inflammatory Bowel Disease (IBD)

IBD is comprised of two main chronic intestinal diseases: ulcerative colitis (UC) and Crohn's disease (CD). The major characteristic of IBD is chronic, uncontrolled inflammation of the intestinal mucosa which can affect any part of the gastrointestinal tract (Hanauer, 2006). Diagnosis of IBD is often based on the presence of architectural distortion in the gastrointestinal tract; however chronic inflammation without any diagnostic abnormality can also be a feature of a normal gut. IBD can be distinguished from a normal inflammatory reaction in that IBD patients have the inability to down regulate this response and therefore the mucosal immune system is chronically activated and the intestines remain chronically inflamed (Hanauer, 2006).

IBD incidence in Australia is 24.2 per 100,000 people with the peak onset age for CD being 20 to 30 years old and UC being 30 to 40 years, with some studies reporting a second onset peak of IBD being diagnosed between 50 and 70 years of age (Cosnes et al., 2011, Studd et al., 2016). Breakdowns of racial and ethnic subgroups indicate that higher rates of IBD occur in people of Caucasian and Ash-kenazic Jewish origin than individuals from other backgrounds. The distribution of IBD among ethnic and racial groups remains dynamic as it was originally thought that IBD occurred less frequently in ethnic and racial minority groups when compared to Caucasians. However this gap has been closing with increased incidence in African Americans and in second generation south Asians who have moved to developed countries (Andres and Friedman, 1999, Carr and Mayberry, 1999, Loftus, 2004).

IBD is most prevalent in developed countries such as the United States, United Kingdom and Australia (Loftus, 2004, Hanauer, 2006). The higher incidence of IBD is seen industrialised countries and the dramatic increase in cases during the 20th century support the theory that environmental factors contribute to disease development (Krishnan and Korzenik, 2002). Increases in incidence have been noted in southern countries, Asia and among migrants to first world countries. It is proposed that this may be a result of 'westernisation' lifestyle, including changes in diet, smoking, and variances in exposure to sunlight, pollution and industrial chemicals.

1.1.1 Environmental Risk Factors

The strongest environmental risk factor for IBD is smoking; the relationship between IBD and smoking is complex. Several case-control studies have shown that smoking is protective against UC with these results consistent across diverse geographic regions. However, the decreased risk for UC appears to be dose dependent. Conversely ex-smokers are 1.7 times more likely to develop UC than those who have never smoked (Lindberg et al., 1988, Loftus et al., 1996, van Erpecum et al., 1996). Ex-smokers also appear to have a poorer disease outcome with more frequent hospitalisations then current smokers and are more likely to require a colectomy than those who have never smoked. In contrast to UC, smoking is a significant risk factor for the development of CD. Smokers with CD have a poorer disease course than non-smokers, with higher disease recurrence, more frequent surgical interventions and a greater need for immunosuppressive agents (Lindberg et al., 1988, Krishnan and Korzenik, 2002).

Another risk factor is hygiene and sanitation given that IBD is perceived to be a disease of cleanliness. It has an inverse relationship with the degree of sanitation: i.e. poor sanitation appears to protect against IBD. It is suggested that good hygiene alters the intestinal flora by decreasing exposure to certain critical bacteria. This hypothesis is further supported by an increased frequency of UC and CD in higher socioeconomic groups (Farrell and LaMont, 2002, Krishnan and Korzenik, 2002).

Occupation plays a role in IBD susceptibility with both UC and CD more prevalent in white-collar occupations. Higher mortality from IBD has been noted in managers, clerical and sales positions, all of which typically involve sedentary, indoor type work.

Conversely, mortality resulting from IBD is low among farmers and construction workers (Sonnenberg, 1990). There is a theory that suggests employment outside involving outdoor activities is protective against IBD, whereas work indoors confers an increased risk for IBD. This theory could explain the higher risk for people living in northern climates given that they spend more time indoors and also explains the increased risk to immigrants moving to developed countries as well as varying rates among ethnic groups in different regions (Sonnenberg, 1990, Andres and Friedman, 1999).

Diet as a risk factor in IBD appears to be inconclusive with some studies showing a higher intake of fatty acids increases the risk for IBD (Sonnenberg, 1990, Krishnan and Korzenik, 2002). It has also been suggested that frequent fast food intake confers a 3 to 4 fold greater risk for IBD (Persson et al., 1992).

1.1.2 Genetic Risk Factors

Epidemiological studies show that genetic factors play role in predisposition to IBD, however IBD is a complex disease and cannot be explained, using a single genetic model (Satsangi et al., 2003). UC and CD are thought to be heterogeneous polygenic disorders sharing some but not all susceptibility loci. It is more likely that the disease phenotype is determined by several factors, including interaction between allelic variant at a number of loci, as well as genetic and environmental influences (Satsangi et al., 2003). There is an increased prevalence of IBD in first- and second degree relatives and higher risk among siblings. The higher risk of IBD in the Jewish populations suggests that genetic factors play a larger role in some genetic sub-groups. Families with a high incidence of IBD among first degree relatives , 75% of those are concordant for either UC or CD , whereas 25% are non-concordant with some family members having UC and others having CD. This is indicative of multiple, overlapping genetics factors that may contribute to disease pathogenesis. The strongest evidence of genetic factors contributing to susceptibility to IBD come from concordance studies in twins (Baumgart and Carding, 2007). These studies have shown that the concordance rate for UC is much lower than CD indicating that the genetic predisposition is much greater in CD.

Susceptibility regions on 12 chromosomes have been identified including regions on chromosomes 1, 3, 5, 6, 12, 14, 16, and 19 have been renamed as IBD1-9, respectively (Baumgart and Carding, 2007). A gene that has been identified as being linked to IBD is the *CARD15* (NOD2) gene on chromosome 16. NOD2 is a polymorphic gene, involved in the innate immune system. NOD2 is the first gene to be clearly associated with IBD, and more than 60 mutations have been linked to the development of CD (Hugot et al., 2001, Ogura et al., 2001). It is estimated that defects in the NOD2 gene account for 17 to 27% of CD cases (Hugot et al., 2001). The mechanism by which the NOD2 gene leads to the development of IBD remains unclear; however one effect of the NOD2 gene is the activation of the nuclear factor (NF)-κB, a transcription factor involved in cellular inflammatory responses and macrophage apoptosis (Ogura et al., 2001, Satsangi et al., 2003). Activation of NF-κB leads to the production of a wide variety of inflammatory factors including cytokines, growth factors and reactive oxygen species which all facilitate the inflammatory process and contribute to tissue destruction. NOD2 mutations reduce macrophage activation which leads to an increase in inflammation as seen in IBD. However the mechanistic basis of these findings remain ambiguous (Abreu, 2002). Microbial products that normally induce the production of chemokines via epithelial cells fail to maintain this function in the absence of NOD2 expression, thus leading to proliferation of bacteria and potential loss of mucosal barrier protection.

1.1.3 Immunoregulatory Defects in IBD

IBD is characterised by immunoregulatory effects in the mucosa, which appear to be associated with microbial exposure. Normally the relationship between commensal bacteria and the host is symbiotic as it is has been hypothesised that exposure to commensal bacteria in healthy individuals down-regulates inflammatory genes which leads to the inhibition of the NF-κB pathway and thus the immune response to the constant mirage of microbes and food antigens is inhibited (Donnenberg, 2000, Neish et al., 2000, Abreu, 2002, Sands, 2004). However in IBD this tolerance is lost and constant exposure to microbes and food antigens now triggers an inflammatory response subsequently causing a chronic destructive immune response. This has been demonstrated in mouse models by showing that colitis will not develop in

mutated strains of mice maintained in a germ free environments, conversely when a single commensal or mixed bacteria load is introduced, this results in rapid mucosal inflammation (Rath et al., 2001). IBD is also associated with increased permeability of the gut epithelial lining resulting in continuous stimulation of the mucosal immune system. In animal models it appears the most severe inflammation develops in the location of the permeability defect (Hanauer, 2006).

T helper cells are mediators of inflammation producing differential cytokine production that can drive one of several different inflammatory pathways (Hendrickson et al., 2002). Evidence suggests that IBD may follow 1 of 2 pathways: an excessive T helper 1 phenotype, which is associated with CD or an excessive T helper 2 phenotype which is linked to UC. It is important to note that this dichotomy is in some individuals, is oversimplified and it is possible to have a combination of Th1 and Th2 pathologies in IBD patients (Bamias et al., 2005).

Over production of IL-12, a macrophage-derived cytokine shifts the immune response in favour of a T helper 1 pathway. This response is characterised by increased production of interferon-γ, Tumour necrosis factor (TNF)-α, interleukin (IL) - 1β, IL-2 and IL-6 and results in a self-sustaining cycle of activation (Abreu, 2002, Hendrickson et al., 2002, Bouma and Strober, 2003, Fuss, 2003). An excessive T helper 2 response is associated with increased secretion of IL-4, IL-5, IL-10, and IL-13. T helper 2 cells also support the humoral immune response (Hendrickson et al., 2002). Mucosal inflammation may also result from a defect in the mature T cells, T helper 1 cells, suppressor cells that produce transforming growth factor (TGF)-β, IL-10, and other immune-inhibitory cytokines. Such a defect would accelerate a loss of tolerance to ordinary antigens in the mucosal microflora, resulting in proliferation and production of inflammatory cytokines. This theory is supported by experimental studies where IL-10 deficient mice develop colitis and delivery of TGF-β or IL-10 ameliorates the colitis (Abreu, 2002).

T helper 17 (Th17) cells have also been linked to IBD pathophysiology particularly CD. Th17 cells are a group of IL-17 producing T cells linked to autoimmunity (Brand, 2009). It was widely believed that chronic intestinal inflammation that is characteristic of IBD is the consequence of pathogenic Th1 CD4 cell responses against the luminal flora, which in turn is driven by pro-inflammatory cytokines such as IL-12 and TNF

(Sartor, 2006, Shih and Targan, 2008, Yadav and Liu, 2009). However recently evidence of high number of CD4 Th17 cells found in colonic lamina propria in mice and further analysis confirmed that commensal gut flora contribute to the expansion of these CD4 Th17 cells, leading to mucosal inflammation (Niess et al., 2008, Ivanov et al., 2009). IL-17 mRNA has been found to be highly expressed in inflamed mucosa from both UC and CD patients and it has also been shown that transcripts for Th17 related cytokines were increased in both UC and CD patients (Fujino et al., 2003). The significance of Th17 immunity in UC is further supported with studies showing that recombinant IL-23 actually enhanced IL-17 production by lamina propria CD4 T cells in UC, however had a lesser effect on CD4 T cells in CD. This could be due to the Th1 pathway which has been reported to antagonise the Th17 pathway via various mechanisms (Liu et al., 2009).

1.2 Current IBD Therapies

CD and UC have similar treatment regimes. Yet each treatment regime is specific to each individual as the physician sees fit. Currently there are five drug categories that may be prescribed to patients with IBD: anti-inflammatory, immunosuppressive, antibiotics, probiotics and biological therapy (Hanauer and Baert, 1994). Anti-inflammatory drugs are the most commonly prescribed treatments for both UC and CD. These include the drug mesalazine and a group of drugs known as corticosteroids (prednisolone, methylprednisolone, butesonide) (Pithadia and Jain, 2011). Mesalazine is only effective for mild-moderate sufferers of UC and shows no significant effect on CD (Camma et al., 1997). Few side-effects have been reported, however there is a risk of renal dysfunction. Of the patients prescribed corticosteroids, approximately one third failed to respond to this treatment in both UC and CD. This result could be explained by the differential expression of the glucocorticoid receptor (GR) promotor usage in T cells (Purton et al., 2004); not all T cells produce equivalent GR transcript and thus they may possess different susceptibility to glucocorticoid induced cell death, potentially accounting for why corticosteroid administration is ineffective.

IBD sufferers on corticosteroids also have the risk of several side-effects such as weight gain, thinning of the skin, glaucoma, diabetes and osteoporosis (Pithadia and Jain, 2011). Immunosuppressive drugs are also a commonly prescribed treatment for both UC and CD. This is due to the vast amount of evidence in support of an altered immune system playing a large role in the pathogenesis of IBD. General drug treatments that are used in IBD are azathioprine, methotrexate, cyclosporine, 6-mercaptopurine and tacrolimus (Pearson et al., 1995, Aberra et al., 2003). Azathioprine and 6-mercaptopurine both fail to precipitate a response to one third of the recipients and as a collective, this group of drugs can have severe side-effects such as renal dysfunction, hepatic injury, tremors, pancreatitis, hypertension, nausea and diarrhoea (Pithadia and Jain, 2011). The third category of prescribed drugs for IBD sufferers is antibiotics. Some common courses of antibiotics recommended are metronidazole, clarithromycin and ornidazole (Sutherland et al., 1991, Arnold et al., 2002). Antibiotics are only necessary if bacterial infection or a gastrointestinal tract (GIT) micro-flora imbalance is diagnosed (Sartor, 1995).

Probiotics are also used in the treatment of IBD; the main side-effects reported for this course of treatment are flatulence and bloating causing severe discomfort. Patients that are on an immunosuppressive therapy should not be prescribed probiotics due to a risk of causing a micro-flora imbalance (Pithadia and Jain, 2011). Probiotics are not commonly prescribed as optimisation of the probiotic; dose and patient population have not been conducted. Over the past decade IBD has emerged as one of the most studied conditions linked to gut microbiota (Swidsinski et al., 2002, Sartor, 2008). With some studies theorising that IBD pathogenesis may result from dysregulation of the mucosal immune system driving a pathogenic immune response against the commensal gut flora (Strober et al., 2007). Short-term treatment with enterically-coated antibodies dramatically reduced intestinal inflammation and has been demonstrated to have some efficacy in IBD (Casellas et al., 1998, Sartor, 2004). Studies have also consistently reported a decrease in alpha-diversity in IBD, a measure of the total number of species in a community. Reduced alpha-diversity in faecal microbiome has been shown in CD, specifically within the Firmicutes phylum (Kang et al., 2010b) and interestingly a reduced diversity has been shown in inflamed tissue versus non-inflamed tissue within the same patient. A major study recently analysed over 1000 patients-naïve samples, which were

collected from multiple concurrent gastrointestinal locations, from patients representing a variety of disease phenotypes with respect to location, severity, and behaviour. This study indicated that assessing the rectal mucosa-associated microbiome offers a potential for convenient and early diagnosis of CD (Gevers et al., 2014).

The last category of drugs prescribed for IBD patients is biologics. The most common biologic on the market is infliximab, a tumour necrosis factor alpha (TNF-α) blocker (Targan et al., 1997). TNF-α is a cytokine primarily involved in inflammation and if the receptor of TNF-α can be blocked, inflammation will then decrease. Side-effects of infliximab can include heart failure, malignancies, autoimmunity and opportunistic infection (Triantafillidis et al., 2011). There are also many new biologic therapies in clinical trials that are of interest to IBD research scientists. The challenge for these researchers are to find specific receptors and corresponding blockers for key cytokines involved in the inflammatory process in order to ameliorate inflammation (Cohen, 2010). The issue with these biologic therapies is that the majority of the IBD population are not-responsive as the treatment does not prevent relapsing of the disease (Cohen, 2010).

1.3 Mesenchymal Stem Cells (MSCs)

One emerging therapy for IBD is mesenchymal stem cells (MSCs). Cells from bone marrow are aspirated and cultured in plastic flasks, hematopoietic cells and hematopoietic stem cells (HSCs) do not adhere to plastic.Once removed the remaining adherent cells were originally called colony-forming unit fibroblasts now referred to as MSCs. Like HSCs, MSCs are rare in bone marrow, representing only 1 in 10,000 nucleated cells. MSCs are multi potent bone marrow cells able to differentiate in cells from mesenchymal origin such as adipose cells, bone cells, muscle cells and cartilage cells. MSCs provide the support for the growth and differentiation of hematopoietic progenitor cells in bone marrow microenvironments (Noort et al., 2002, Dalal et al., 2012). MSCs have been observed inhibiting T cell proliferation *in vitro* in 2002- 2003 by three independent investigators which opened the door for the use of MSCs for autoimmune disorders, first in animal models and then in humans (Bartholomew et al., 2002, Di Nicola et al., 2002, Le Blanc et al.,

2003, Dalal et al., 2012). In co-culture experiments with allogenic lymphocytes, MSCs do not induce lymphocyte proliferation, interferon-γ production, or upregulation of activation markers. Despite this *ex vivo* property, survival of infused allogenic MSCs in immunocompetent mice was estimated to be less than 40 days and when mice were rechallenged, survival of infused MSCs was less than 5 days. There was also evidence of immune memory induction by MSCs suggesting that MSCs cannot completely evade the immune response and are eventually rejected (Bartholomew et al., 2002, Zangi et al., 2009, Dalal et al., 2012). MSCs suppress proliferation of activated lymphocytes *in vitro* in dose-dependent, non-human leukocyte antigen-restricted manner. In a baboon skin-graft model, investigators showed that infusion of ex-vivo expanded donor-derived or third party cells prolonged the time to rejection of histoincompatible skin grafts. Furthermore, infused cells improve the outcome of acute renal, neural, and lung injury, possibly by promoting a shift from production of pro-inflammatory to anti-inflammatory cytokines at the site of injury (Ortiz et al., 2003, Tögel et al., 2005, Zappia et al., 2005).

Once administered MSCs can migrate through chemotaxis towards the site of inflammation, specifically targeting pathological manifestations. After homing to the site of inflammation, MSC can facilitate tissue regeneration through the secretion of pro-angiogenic and trophic factors, which have been shown to promote endogenous repair mechanisms (Stavely et al., 2014). MSCs appear to be immunomodulatory and secrete anti-inflammatory factors suppressing the immune response and inflammation.

1.3.1 MSC Treatment in Clinical Trials

Currently MSCs are being used in clinical trials for the treatment of CD fistulae and luminal inflammation have demonstrated that MSCs as a therapy in in IBD is shown to be effective and feasible. However, the MSCs are being used to treat fistulae caused by CD rather the CD itself. These trials have shown that MSCs used for the treatment of fistulising CD and have resulted in complete re-epithelisation of rectovaginal, enterocutaneous and complex perianal fistulae in most trial patients. The therapeutic outcome of MSC therapy in fistulising CD may be dose dependent with greater efficacy achieved by doses of $2x10^7$ or $4x10^7$MSCs/ml compared to $1x10^7$ MSCs/ml (Cho et al., 2013). Long term effects of these treatments have been

reported with CD and perianal activity index scores declining 12 months post treatment and furthermore sustained closure of fistulae was achieved in 88-100% of all patients 8-12 months after a course of MSC treatment; however these effects are relatively transient given that only 58% of subjects saw closure after 3 years. This suggests that repeated treatment may be required to maintain the therapeutic benefits of MSC therapy. While autologous MSCs have demonstrated efficacy in the healing of fistulae further evidence is needed to conclude the long term immune tolerance in patients with repeated MSC exposure. Bacterial contamination is also a major concern as it poses a problem in the expansion of autologous MSCs and has done so in the past, which in turn causes delays in the MSC treatment (García-Olmo et al., 2005, García-Olmo et al., 2010).

In the first human trial of systemic MSCs in CD patients, Onken et al. 2006 treated patients who had failed previous treatment with steroids and immunosuppressant's and had active disease; patients were randomised to receive either low (2 million cells/kg) or high dose (8 million cells/kg) by intravenous injection from a third party healthy human bone. The MSC treatments were done in 2 doses, 7 days apart. All patients had a decrease in CDAI score by day 28. In another phase I study of autologous bone marrow derived MSCs were used to treat luminal refractory CD. Patients were administered intravenously two doses of MSCs at 1-2x10^6 cells/kg body weight 7 days apart. All patients in this study had previously failed corticosteroids and at least to anti-TNF drugs and 9 out of 10 patients had also failed 2 immunosuppressive drugs. The results in this study show no clear efficacy and no remission was achieved in any patients, only 3 patients had a reduction in CDAI score with 4 patients requiring surgery or rescue medication within 14 weeks after cell treatment.

1.3.2 Mechanism of Action

While clinical trials have demonstrated the efficacy of MSCs in fistulising CD, the mechanism of the therapeutic effects of MSCS in IBD is less understood and needs to be explored. The intestinal epithelium creates a distinct barrier protecting the underlying tissues from pathogens in the gut lumen. Restoring the integrity of the epithelial lining ameliorates the excessive immune response in IBD, by preventing

interaction with foreign antigens (Okamoto, 2011, Fries et al., 2013). Gross morphological damage to the intestine is seen in IBD; however MSC treatment has shown to decrease this damage in experimental colitis models (Ando et al., 2008, Hayashi et al., 2008, Castelo-Branco et al., 2012). Histopathologically, MSCs appear to prevent the loss and discontinuity of column epithelial lining and derangement of the crypts and a protective effect on mucin secretion has also been observed This has been attributed to MSCs having a regenerative effect by promoting the proliferation of intestinal epithelium and the differentiation of intestinal stem cells (Tanaka et al., 2011, Fawzy et al., 2013). It has also been reported that MSCs stimulate endogenous mechanism of intestinal epithelial repair. It has also been shown that the tissue culture medium MSCs is grown in (referred to as conditioned medium), also has therapeutic effects. MSC conditioned medium has been shown to decrease epithelial damage in experimental models of colitis, this highlights the importance of the MSC secretome (Sémont et al., 2013, Watanabe et al., 2014).

1.4 Animal Models of IBD

Animal models are commonly used in IBD research, animal models are important in understanding the underlying genetic and environmental factors in IBD. Much of the recent progress in understanding mucosal immunity has been due to the study of new experimental models of intestinal inflammation (Elson et al., 2005). These models cannot replicate the complexity of IBD and do not replace studies with patient material, they are valuable tools for studying many important disease aspects that are difficult to address in humans, for example the pathophysiological mechanisms in early phases of colitis and the effect of emerging therapeutic options. The clinical appearance of human IBD is heterogeneous which is also reflected in the increasing number of transgenic or gene targeted mouse strains displaying IBD like intestinal alterations (Wirtz and Neurath, 2007). Animal models of IBD can be broadly characterised into 3 categories (Table 1.1) according to the defect in mucosal immunity: (1) defects in epithelial integrity/permeability, (2) defects in innate immune cells and (3) defects in cells of the adaptive immune system.

The intestinal epithelium is a physical and immunological barrier that prevents direct contact of the intestinal mucosa with luminal microbiota (Wirtz and Neurath, 2007). It

has been shown that some IBD patients have a compromised intestinal barrier and that may play a crucial role in the development of IBD by allowing luminal antigens and microorganisms into the mucosa and thus initiating an immune response (MacDonald and Monteleone, 2005). Some animal models of IBD that have defects in intestinal barrier include the following mouse models (summarised in Table 1.1) including, DSS colitis, TNBS/Oxazolone colitis, multiple drug resistant gene deficient mice and *Winnie* mice (MacDonald and Monteleone, 2005, Wirtz et al., 2007).

Other models of IBD can be grouped based on defects found within the innate immune system, as shown in Table 1.1. The innate immune cells recognise foreign organisms through receptors (such as toll like receptors) that recognise pathogen-associated molecular patterns (PAMPs). The interaction between PAMPs and pattern recognition receptors (PRRs) results in the activation of innate leukocytes leading to a cascade of events that are geared towards reducing the pathogen load and ultimately eliminating the infectious agent (Wirtz and Neurath, 2007). It has been shown that resident lamina propria macrophages in the healthy gut express innate response receptors and when triggered down-regulation of the production of pro-inflammatory cytokines occur, suggesting the status of 'inflammatory anergy' and could be a potential mechanism for the absence of inflammation in the normal intestinal mucosa despite proximity to commensal bacteria flora. Mice with specific disruption of the signal transducer and activator of transcription 3 (STAT3) gene in macrophages and neutrophils have been shown to develop enterocolitis (Takeda et al., 1999). STAT3 is a critical factor within the signal transduction pathway of IL-10; this suggests that the absence of an IL-10 mediated counter regulatory effect on colonic macrophages continuously subjected to stimulation by luminal bacterial or food antigen is sufficient for the development of chronic intestinal colitis. A20 deficient mice are also an example of a model of IBD with innate immune defects, A20 is an inducible and broadly expressed cytoplasmic protein that inhibits TNF-induced NF-κB activity. A20 deficient mice develop spontaneous inflammation, cachexia and premature death in part due to failure of A20 deficient cells to terminate TNF-induced NF-κB responses (Lee et al., 2000).

In addition to innate immune system defects, animal models that centre on adaptive immunity defects are also well characterised, as shown in Table 1.1. For example, it has been shown that CD4 T cells play a key role in the normal and

pathophysiological immune regulatory processes in the gastro intestinal tract, but different T cell subsets are also present and activated in CD vs UC indicating that the failure of regulation by T helper cells could be a major contributing factor to the pathogenesis of IBD (Neurath et al., 2002, Peluso et al., 2006). CD4 $CD45RB^{H}$ T cells isolated using fluoresce activated cell sorting from spleens of donor mice transferred to immunodeficient SCID mice cause a wasting syndrome with transmural inflammation primarily in the colon, starting 5-10 weeks after transfer. The role of IL-10 in this model was further emphasized by the fact that SCID mice administered both $CD45RB^{hi}$ and regulatory T cells together with anti-IL-10 receptor antibodies develop colitis (Asseman et al., 1999). Another model of IBD with an adaptive immune defect is STAT4 transgenic mice, STAT4 is a regulatory transcription factor specifically associated with IL-12/IL-23 receptor signalling (Kaplan et al., 1998), Mice over expressing STAT4 under control of a cytomegalovirus promoter system, which express highly elevated nuclear STAT4 levels in spleen and lamina propria CD4 T cells after systemic administration of the antigenic stimulus DNP-KLH, have been shown to develop severe transmural colitis (Wirtz et al., 1999).

Table 1.1 Animal models of IBD

Category	***Animal Model***	***Mechanism of IBD***
1. Defects in epithelial integrity/ permeability	DSS colitis	Is directly toxic to gut epithelial cells of the basal crypts and effects the integrity of the mucosal barrier (Dieleman et al., 1994, Wirtz and Neurath, 2007).
	TNBS/Oxazolone colitis	Are haptenating substances that are dissolved in ethanol. Ethanol breaks down the mucosal barrier and TNBS/Oxazolone haptenize colonic autologous or microbiota proteins making them immunogenic to the host immune system (Wirtz et al., 2007, Morris et al., 1989)
	DN N-cadherin transgenic mice	Have a dominant negative mutant of the cell adhesion molecule N-cadherin in intestinal epithelial cells along the crypt villus axis (Hermiston and Gordon, 1995, Wirtz and Neurath, 2007)
	Keratin $8^{-/-}$ mice	Keratin 8 deficient mice develop colonic hyperplasia and colitis due to primary epithelial rather than immune cell defects (Baribault et al., 1994)
	Multiple drug resistant (MDR1) gene deficient mice	Mdr1$\alpha^{-/-}$ mice display spontaneous bowel inflammation triggered by the bacterial flora (Panwala et al., 1998)
	IKK-γ (NEMO)/IKKαβ deficiency in intestinal epithelial cells	Activation of the transcription factor NFκB controls the inducible expression of most of the genes of inflammatory cytokines involved in the pathogenesis of IBD (Karban et al., 2004)
	SAMP1/YitFc (Samp) mice	Spontaneous inflammation occurs with severe inflammation in the terminal ileum (Matsumoto et al., 1998)
	Winnie Mice	*Winnie* mice carry a missense mutation in Muc2 which leads to severe endoplasmic reticulum stress in intestinal goblet cells and spontaneous chronic colitis (Eri et al., 2011, Heazlewood et al., 2008).

2. Defects in innate immune cells	STAT3 deficiency in myeloid cells	Mice with a specific disruption of the STAT3 gene in macrophages and neutrophils develop spontaneous enterocolitis (Takeda et al., 1999)
	A20 deficient mice	A20 is an inducible and broadly expressed cytoplasmic protein that inhibits TNF-induced NFκB activity. A20 deficient mice develop spontaneous inflammation (Lee et al., 2000)
	$TNF^{\Delta ARE}$ MICE	Over production of TNF leads to polyarthritis and chronic intestinal inflammation (Kontoyiannis et al., 1999, Wirtz and Neurath, 2007)
3. Defects in cells of the adaptive immune system	$CD45RB^{HI}$ transfer model	$CD4^{+}CD45RB^{HI}$ T cells transferred to immunodeficient mice cause a wasting syndrome with transmural intestinal inflammation primarily in the colon (Powrie et al., 1993, Powrie et al., 1994, Leach et al., 1996, Read et al., 2000)
	STAT4 transgenic mice	STAT4 is a regulatory transcription factor specifically associated with IL-12/IL-23 receptor signalling. Over expression of STAT4 leads to severe transmural colitis (Wirtz and Neurath, 2007)
	IL-10/CRF2-4 deficient mice	Mice with IL-10 gene deletion spontaneously develop chronic enterocolitis with massive infiltration of lymphocytes (Kühn et al., 1993)
	TCRα $chain^{-/-}$ mice	Mice deficient for the TCR-α chain (TCR-$\alpha^{-/-}$) spontaneously develop mucosal inflammation at 12–16 weeks of age with some characteristics similar to UC in humans (Mombaerts et al., 1993)
	MAIT cell depletion	When mucosal associated invariant T (MAIT) cells are depleted in mice it leads to the development of IBD (Ruijing et al., 2012) Fewer MAIT cells have also been found in the peripheral blood of IBD patients compared to non-IBD patients and the MAIT cells in IBD patients also exhibited pro-apoptotic features suggesting pathological involvement in IBD (Hiejima et al., 2015)

1.5 Chronic inflammation and cancer

The link between inflammation and cancer is widely known and studied with those afflicted by chronic inflammatory conditions (such as IBD) more likely to develop cancer. (Ferrante et al. 2006; Boland et al. 2010; Bergman et al. 2011; Rizzo et al. 2011). Inflammation arises as a response to tissue damage that can occur from infection, chemical irritation or trauma. First neutrophils arrive at the site of inflammation in response to signals produced by resident macrophages, mast cells or even epithelium. Other immune cells are then recruited to the site of inflammation by a network of signalling molecules that includes growth factors, cytokines and chemokines.

During chronic inflammation, inflammatory infiltrate predominantly consist of lymphocytes and macrophages. Macrophages are largely responsible for generating growth factors, cytokines and reactive oxygen and nitrogen species. Under normal conditions, these factors drive the inflammatory response, but during prolonged inflammation they may lead to continuous tissue damage, subsequent sustained cell proliferation and hence a predisposition to malignant transformation (Macarthur et al., 2004). The NF-κB transcription factor plays a key role in many physiological and pathophysiological processes and has an important part in mediating inflammatory signals. Although genetic alterations in NF-κB are rare in human tumours it has been found that in 40% of CRC tissues, NF-κB is constitutively activated (Kraus and Arber, 2009). IL-6 is a cytokine that binds to the IL-6 receptor (IL-6R) in the membrane of many immune cells like macrophages and T cells. The IL-6/IL-6R complex then binds to the signal transducer gp130 subunits and this promotes the dimerization and initiation of the intracellular signal transduction. In inflammation it is thought that IL-6 signalling plays a key role in the transition between innate and acquired immunity. IL-6 supresses neutrophil infiltration and promotes the accumulation of mononuclear leukocytes, which leads to the resolution of acute inflammation and the activation of acquired immunity. IL-6 has also been detected in multiple epithelial tumours and has been implicated in in cell proliferation, survival and metabolism. IL-6 has also

been implicated in tumourigenesis, however the nature of IL-6's involvement in cancer is quite controversial because IL-6 is both tumour-promoting and suppressive for example IL-6 has been linked to pro and anti-apoptotic activity in breast cancer cells (Schafer and Brugge, 2007). Reactive oxygen and nitrogen species may also contribute to DNA damage that can result in neoplasia (Coussens and Werb, 2002). Cancer development is a step-wise process whereby genetic changes confer a growth advantage, driving tumour development. Malignancy is characterised by several hallmarks: self-sufficiency of growth signals, resistance to anti-growth signals, escape from apoptosis, unregulated proliferation, enhanced angiogenesis and metastasis (Hanahan and Weinberg, 2000). To date, research into inflammation-induced cancer has largely focussed on chemokines, cytokines and their downstream targets. These inflammatory mediators may promote tumour growth, invasion, and metastasis and facilitate angiogenesis.

1.5.1 Cancer Immunology

The immune system has three primary roles in the prevention of tumours: 1) the immune system can protect the host from virus-induced tumours by eliminating or suppressing viral infections, 2) the timely elimination of pathogens and prompt resolution of inflammation can prevent the establishment of an inflammatory environment conducive to tumourigenesis and 3) the immune system can specifically identify and eliminate tumour cells on the basis of their expression of tumour-specific antigens or molecules induced by cellular stress. It has been well documented that tumours are generally infiltrated by immune cells (Whiteside, 2008, Balkwill and Mantovani, 2012) with many initially linking the presence of immune cells to an improved prognosis. However, recently studies have linked the presence of immune cells in tumours to a poorer prognosis depending on type, density and location of immune cells (Pagès et al., 2005, Galon et al., 2006, Galon et al., 2007, Whiteside, 2008). The immune system can react to cancer cells in two different ways

either by reacting against tumour specific antigens or tumour associated antigens (Finn, 2008). Immunity to carcinogen-induced tumours is normally directed at the products produced by unique mutations in normal cellular genes, these mutant proteins are known as tumour specific antigens (Finn, 2008). Numerous immune cells contribute to this reaction including macrophages, T lymphocytes, eosinophils and neutrophils.

In the tumour microenvironment mature macrophages express distinct functional properties, known as M1 and M2 macrophages. Classically activated M1 macrophages are induced by IFN-γ alone or in concert with microbial stimuli or cytokines, alternatively M2 macrophages are activated by cytokines IL-4, IL-13, IL-1 or IL-10. M1 macrophages have a high capacity to present antigens, high IL-12 and IL-23 production and consequent activation of type I T cell responses and M1 macrophages are also cytotoxic towards tumour cells as well as towards cells that have ingested intracellular micro-organisms. Whereas M2 macrophages have poor antigen presenting capabilities, have an IL-12^{low}, IL-10^{high} phenotype, suppress the inflammatory responses and Th1 adaptive immunity, actively scavenge debris, promote wound healing, angiogenesis and tissue remodelling (Mantovani, 1999, Biswas et al., 2006). Tumour associated macrophages (TAMs) are a major component of leukocyte infiltrate of tumours for cancer related inflammation and have the capabilities to express both pro and anti-tumour properties (De Palma et al., 2007, Mantovani et al., 2008, Pollard, 2009). TAMs recruitment is mediated by cytokines belonging to different classes including colony stimulating factor-1 (Joyce and Pollard, 2009), vascular endothelial growth factor and chemokines (Mantovani et al., 2010). The tumour micro-environment expresses signals that play a central role in the polarisation of the macrophages recruited thus taking the local immune system away from anti-tumour functions. Differentiated mature TAMs have the phenotype and similar functions to M2 macrophages (Allavena et al., 2008). Lin et al (2001) showed that macrophage colony stimulating factor-1 (CSF-1) promotes malignancy in mammary tumours by regulating infiltration and function of TAMs (Lin et al., 2001). Ong et al. (2012) investigated whether

macrophages primed T lymphocytes towards a type-1 inflammatory response in CRC. Macrophages were isolated from peripheral blood samples and it was found that tumour associated macrophages (TAMs), recruit T cells to the tumour site and promote a type-1 inflammatory response (Ong et al., 2012).

In addition, to the well characterised TAMs, neutrophils have recently emerged as new tumour infiltrating myeloid cell, playing an important role in tumour growth and progression; similarly to TAMs neutrophils also have both pro and anti-tumour roles. Neutrophil derived cytokines and proteins stored within granules may also play a dual role in tumour progression (Whiteside, 2008). For example neutrophil elastase was taken up by epithelial lung cancer cells and favoured tumour proliferation through the hydrolysis of insulin receptor substrate-1, which usually blocks PI3K activity and reduces platelet derived growth factor receptor (PDGFR) signalling (Houghton et al., 2010). However neutrophil elastase in breast cancer cells has been shown to promote tumour lysis by cleaving cyclin E in a truncated isoform, which is presented in the context of HLA-ABC and promotes T lymphocyte mediated lysis of tumour cells (Mittendorf et al., 2012). Neutrophils also express numerous angiogenic factors that are able to modulate tumour angiogenesis such as CXCL1/MIP-2 which recruits neutrophils that release a biologically active form of vascular endothelial growth factor (VEGF)-A (Scapini et al., 2004). Neutrophils have also been shown to be a source of anti-angiogenic mediators such as elastase which promotes the degradation of VEGF-A, basic fibroblast growth factor (bFGf) and α-defensins. Tumour infiltrating neutrophils and cancer prognosis is still unclear in the literature as tumour infiltrating neutrophils have been shown to lead to a poor prognosis in CRC, hepatocellular carcinoma, head and neck squamous cell carcinoma and bronchioloalvolar cancer. Conversely tumour infiltrating neutrophils have been associated with a better prognosis in gastric cancer (Galdiero et al., 2013).

Exogenous antigens secreted by tumour cells can enter a processing pathway known as cross presentation; this allows cytotoxic lymphocytes to respond to the antigens secreted (den Haan et al., 2000, Heath and

Carbone, 2001a, Heath and Carbone, 2001b). It is an efficient process and because it can induce either tolerance or immunity to antigens expressed in normal tissue, it is thought to have a role in the maintenance of self-tolerance as well as the rapid clearance of viruses (Heath and Carbone, 2001a). Adaptive immunity is defined by its capacity to elucidate an antigen-specific immune response by CD4 and CD8 T cells. This property is entirely based on the presentation of antigen in complex with the major histocompatibility complex (MHC) molecules (peptide-MHC complex) and its recognition by T cell receptor (Fehres et al., 2014). The loading of extracellular antigen in MHC-class II (MHC-II), recognised by CD4 T cells, occurs in a different intercellular compartment than the loading of antigen in MHC-class I (MHC-I), recognised by CD8 T cells. In the case of MHC-II, after its synthesis in the endoplasmic reticulum, complexes are formed with CD74 (known as the invariant chain) to allow proper folding, trafficking, and protection of the peptide binding groove. CD74 helps guiding the CD74-MHC-II complex move on to the endolysosomal pathway, where late endosomal proteases such as cathepsin S and L degrade CD74 (the CLIP peptide), which is later exchanged for an antigenic fragment with the help of chaperone HLA-DM (Rocha and Neefjes, 2008). The process leading to antigen presentation on MHC-I involves 6 basic steps: 1) acquisition of antigens, 2) tagging of antigenic peptide for destruction, 3) proteolysis, 4) transport of peptides to the endoplasmic reticulum, 5) loading of peptides to MHC-I molecules, 6) and the display of peptide-MHC-I complexes on the cell surface (Vyas et al., 2008).

Innate lymphoid cells (ILCs) is a broad term used to encompass many cells including lineage marker-negative ILCs, natural killer (NK) cells and lymphoid tissue-inducer cells. ILCs are cells that produce many Th cell-associated cytokines, but they do not express cell-surface markers that are associated with other immune cell lineages (Walker et al., 2013). As mentioned above ILCs include lineage marker-negative ILC subsets that do not express a T cell receptor and thus do not respond in an antigen-specific manner. ILCs can be divided into three major groups; Group 1

ILCs (ILC1s) comprise of NK cells and ILCs that produce type 1 cytokines, notably IFNγ and TNF-α. Group 2 ILCs (ILC2s) primarily produce type 2 cytokines such as IL-5, IL-9, IL-13 and small amounts of IL-4. Group 3 ILCs (ILC3s) express the NK cell activating receptor NKp46, however these cells bear little functional resemblance to conventional NK cells (Herberman et al., 1975, Kiessling et al., 1975, Satoh-Takayama et al., 2008, Luci et al., 2009, Sanos et al., 2009, Moro et al., 2010, Neill et al., 2010, Price et al., 2010, Spits et al., 2013, Walker et al., 2013)

ILC1, ILC2 and ILC3 have all been described in the gut under normal homeostatic conditions (see table 1.2), the first major population of ILCs that were comprehensively described in mucosal tissue was IL-22 producing NCR+(CD56+NKp44+) cells (Cella et al., 2009). These cells were later classified as NCR+ILC3; this observation highlighted a new population of innate lymphocytes present in mucosal barriers with distinct functions from conventional NK cells (Spits et al., 2013). ILCs have also been implicated in the development of IBD (see Table 1.2), with multiple studies showing ILC populations in different animal models of IBD, leading to the possibility that these cells could have a pathologically relevant role in IBD (Goldberg et al., 2015).

Table 1.2 Role of intestinal ILCs in IBD

Cell Type	Functions in a healthy gut	Implied role in IBD
ILC1	Resistance to bacterial infection (Klose et al., 2014)	Increased in patients with Crohn's disease and functionally implicated in preclinical models of colitis (Takayama et al., 2010, Vonarbourg et al., 2010, Bernink et al., 2013, Fuchs et al.,

		2013)
ILC2	Eosinophil recruitment, and involved in the immune response to helminths.(Neill et al., 2010, Sawa et al., 2011, Nussbaum et al., 2013)	Possible role in fibrosis in patients with Crohn's disease (Bailey et al., 2012)
ILC3	Involved in resistance to mucosal infections, epithelial integrity and possible role in antifungal immunity (Satoh-Takayama et al., 2008, Reynders et al., 2011, Gladiator et al., 2013, Diefenbach et al., 2014)	Possible role in protection from experimental colitis, increased innate IL-17 production patients with IBD (Geremia et al., 2011, Mielke et al., 2013, Qiu et al., 2013)

NK cells were initially identified as a lymphoid population representing the 10-20% of peripheral blood mononuclear cells, able to lyse MHC-I negative tumour and virus infected cells and to orchestrate innate immunity. Majority of NK cells are located in the peripheral blood, lymph nodes, spleen and bone marrow (Ferlazzo et al., 2004) but can be induced to migrate toward inflammation site by different chemoattractants (Robertson, 2002). NK cells constitutively express lytic machinery able to kill target cells independently from any other previous activation. NK cells express surface receptors that are both inhibitory and activatory (Bottino et al., 2004, Moretta et al., 2004), there are several inhibitory receptors with different molecular structures and specificities for different alleles of class I molecules, the two main groups being killer Ig-like receptors (Bottino et al., 2004), which bind HLA-ABC, and the heterodimeric receptors CD49-NKG2A/B, which recognise HLA-E. The lack of MHC-I allele, a frequent event in cancer cells, potentially marks them for NK cell killing , however

this is not always the case, many cancers can evade the immune system while maintaining MHC-I expression, such as the use of decoy proteins or changing cytokine expression (Bottino et al., 2004, Moretta et al., 2004). Upon cytokine stimulation NK cells become lymphokine-activated killer (LAK) cells that proliferate, produce cytokines and up regulate effector molecules such as adhesion molecules like ICAM-1 and ICAM-2, NKp4, perforin, granzymes, Fas ligand and TRAIL (Trinchieri, 1989, Jackson et al., 1992, Medvedev et al., 1997, Zamai et al., 1998, Johnsen et al., 1999, Trinchieri, 2003, Bottino et al., 2004, Mirandola et al., 2004, Moretta et al., 2004). LAK cells have enhanced ability to adhere and recognise target cells which in turn leads to a broader killing activity against tumour cells.

Eosinophils are pleiotropic granulocytes that are implicated in the pathogenesis of allergic disorders such as asthma, rhinitis, atopic dermatitis, and responsible for host defence against selected pathogens (Munitz and Levi-Schaffer, 2004). Eosinophils are present in low numbers in normal steady state conditions (i.e. 1-6% of all peripheral blood leukocytes); however they are increased in many conditions such as parasitic infections, asthma, cancer and responses to some viral infections (Legrand et al., 2010). The morphological features of eosinophils include a bi-lobed nucleus, and large acidophilic cytoplasmic granules. Eosinophils are a rich source of chemokines including eotaxin, interleukin-8, macrophage inflammatory protein and eosinophil-derived neurotoxin. Eosinophils also appear to be capable of modulating acute phase and innate inflammatory responses as well as acquired immunity associated with both Th1 and Th2 immune responses (Munitz and Levi-Schaffer, 2004). Tumour-associated tissue eosinophilia can be linked with a favourable prognosis, notably in CRC (Legrand et al., 2010). However, underlying mechanisms of eosinophil contribution to anti-tumour responses are poorly understood. Studies by Legrand et al. (2010) concluded that eosinophils can detect human CRC cells and may be valuable in the early detection of cancer.

1.6 Animal Models of Colorectal Cancer

Animal models are a valuable tool in cancer research and the selection of an appropriate animal model in which to assess novel drug therapies and cancer remains controversial. Mice are generally used as their tumour growth is very similar to human and mice are readily available and inexpensive (Flatmark et al., 2004). A widely used cell line for the CRC model in wild strains of mice research is CT26; Brattain et al. (1980) published one of the first studies on different CRC cell lines and determined which murine CRC cell lines behaved most like human CRC cells. Cell lines (CT26, CT36, and CT51) were cultured and the different properties were analysed including *in vitro* growth properties, tumourigenicity and formation of metastases. This study demonstrated that in cell culture CT26 cells were the most aggressive and their malignant attributes very similar to human CRC (Brattain et al., 1980).

Like animal CRC cell lines and animal models, human CRC cell lines are also a valuable tool used to study colorectal cancer, HT29 and SW480 are two popular cells lines used. Severe combined immunodeficiency (SCID) mice are a strain of mouse that lacks B and T lymphocytes and immunoglobulin's and are used in CRC research with human CRC cell lines (Schumacher et al., 2012). Another strain of mouse that is used is NU/NU nude mice (Fogh et al., 1977). Nude mice have a genetic mutation that causes severe deterioration of the thymus or an absent thymus which results in an altered immune system due to a greatly reduced number of T lymphocytes (Belizário, 2009). Both of these mouse strains are genetically modified organisms and need special facilities to be able to keep them in the lab, the nude mice especially as they can only be kept in PC4 labs under very sterile conditions.

Mouse models with a mutation of the adenomatous polyposis coli (APC) gene are also commonly used. Mutations in the in the APC gene can lead to the development of polyposis in the gut and eventually development of CRC. APC has multiple functions that include controlling the Wnt signal transduction pathway, cell adhesion, migration, apoptosis, and

chromosomal segregation at mitosis. All of these functions are potentially linked with cancer development (Fodde et al., 2001, Moossavi and Bishehsari, 2012). Robanus-Maandag et al. (2010) investigated a mouse model with a new mutation in the APC gene. In other animal models with APC mutations (Yamada and Mori, 2007, Sale et al., 2009, Murphy et al., 2011, Puppa et al., 2011) the cancer develops in the small intestine and then migrates to the colon. However in this new APC mutated animal model it was found that 95% of tumours develop in the small intestine and 5% in the colon (Robanus-Maandag et al., 2010). CRC in humans develops in the colon first and then metastasizes to the small intestine, so this model does not accurately replicate human CRC development.

Another common animal model used in CRC research is subcutaneous injection of CRC cells. This model is commonly used to show biological tumour properties and drug response. In the subcutaneous model of CRC, cancer cells are injected under the skin of the animal and left to form a tumour over a period of time. Tumour properties such as tumour size, vascularisation and cell density can be measured. However a major limitation of this model of is the lack of metastasis from the subcutaneous site, making this model inappropriate to measure the metastatic process in cancer (Flatmark et al., 2004). This model is also not orthotopic meaning that the cancer cells are not injected in the colon when CRC forms, so the tumour microenvironment and immune reaction may be different than in an orthotopic model.

Orthotopic transplantation of CRC tumours in mice has been around for many years. Usually CRC cells are injected into the colon or caecal wall or tumour fragments are sutured into the caecum or colon wall. A major advantage of the orthotopic animal model of CRC is the metastasis process; it is now clear that the process of metastasis is more efficient and closely mimics human metastasis (Killion et al., 1998, Bibby, 2004). However one of the most obvious advantages of orthotopic implantation is that the tumour cells are implanted at the site where the tumour would be found (i.e. CRC tumours in the colon) and therefore the tumour microenvironment and immune system would react in a way similar to

what is seen in humans with CRC (Bibby, 2004). Disadvantages to orthotopic implantation of CRC cells in an animal are endpoints for determining the effect of therapy are more complex than the normal tumour measurement used in the subcutaneous injection models and ensuring the animal is not suffering is kept to a minimum can also be quite difficult if there is no way to view the tumour growth *in vivo* (Bibby, 2004). However the orthotopic implant of tumour cell into an animal is still one of the most valuable models of cancer to research the tumour microenvironment and metastatic phenotype of CRC.

In most animal models of CRC the cancer cells are injected at one time point which does not reflect the slow nature of CRC development. A sporadic CRC mouse model has been developed (Czéh et al., 2010). This model eliminates the biased immune response shown in animal models exposed to a lot of cancer cells at once instead of slowly developing tumourigenesis and immune response (Czéh et al., 2010). Only mice heterogeneous for the that mutation that causes CRC were used and it was found that the tumour and cancer cells in this mouse were found to behave more like human CRC tumours and cancer cells. Therefore this model provides a biologically accurate of model of CRC (Czéh et al., 2010).

Mice are not the only animal models used in research. Robertson et al. (2008), studied whether BDIX rats would be appropriate *in vivo* models of early CRC liver metastases. In this study BDIX rats were injected with DHDK12 cancer cells into the right carotid artery, allowing the cancer to develop and metastasize to the liver. The authors found that BDIX rats provided a biologically accurate *in vivo* model of early CRC liver metastases (Robertson et al., 2008).

1.7 MSC Therapy for Cancer

Resident MSCs may have a critical role in maintaining homeostasis of injured tissue through immune modulatory effects of angiogenetic

stimulation by secreting bioactive molecules (Lazennec and Jorgensen, 2008, Uccelli et al., 2008). Within the body the actual population of MSCs appears to be quite small and therefore the behaviour of large quantities of experimentally administered MSCs is likely to behave very differently to the behaviour of the small number of resident MSCs (MSC like cells found within the body) therefore the role of administered MSCs in cancer development is still controversial. Various reports describe the ability of MSCs to promote tumour progression by enhancing metastatic potential (Karnoub et al., 2007). Conversely MSCs have also been reported to have tumour suppressive effects via modification of the Akt signalling shown by co-administration of MSCs and glioma cells resulting in a significant reduction in tumour volume and vascular density, this anti-tumour effect has also been shown in other cancers (Otsu et al., 2009, Dasari et al., 2010). These conflicting results may be due to variable experimental factors such as differences in cell source (bone marrow or fat tissue), different time points, methods of MSC administration, and timing. Therefore cultured MSCS should not be considered equivalent to MSCs under physiological conditions *in vivo* (Yagi and Kitagawa, 2013).

1.7.1 Pro-tumourigenic Properties of MSCs

MSCs are reported to promote tumour proliferation, fibroblast growth, angiogenesis and metastasis by providing immunosuppression, cytoprotection and vascular support largely by the production of paracrine factors, as summarised in Table 1.

Bone marrow-derived MSCs (BM-MSC) have been shown to promote the *in vivo* growth of colon cancer, lymphoma, and melanoma (Klopp et al., 2011). Table 2 summarises the key findings of studies to date that have used MSCs to treat inflammation-induced cancers, particularly CRC.

Foetal or adult MSCs co-injected subcutaneously with tumour cells favour tumour growth and metastasis in immunocompromised mice (Karnoub et al., 2007, Liu et al., 2011, Tsai et al., 2011, De Boeck et al., 2013, Huang et al., 2013, Mele et al., 2014, Widder et al., 2015); this is accompanied by

extensive necrosis and angiogenesis when compared to mice injected with tumour cells alone (Lazennec and Jorgensen, 2008). When melanoma cells were subcutaneously injected into an allogeneic recipient, melanoma cells only formed tumours in the presence of MSCs (Djouad et al., 2003). Interestingly, this occurred whether MSCs were co-injected at the tumour site or injected at a distance. In an orthotopic model of CRC, co-injection of MSCs resulted in increased tumour size and liver metastasis that was not observed at in mice injected with cancer cell alone (Shinagawa et al., 2010). MSCs can also promote tumour growth within the bone; multiple myeloma malignancy leads to the formation of osteolytic lesions in the bone and this is enhanced by interaction with MSCs (Lazennec and Jorgensen, 2008).

A few studies have now tried to identify the molecules produced by MSCs that enhance the growth of tumours. Co-culture or indirect interaction of MSCs with breast cancer and melanoma cells enhances tumour growth, indicating that soluble factors are involved (Sun et al., 2008, Pasanen et al., 2015). MSCs have been shown to produce IL-6, which promotes proliferation of multiple myeloma cells and CRC tumour formation (Chauhan et al., 1996, Tsai et al., 2011). Activation of the IL-6 pathway typically involves signal transduction via STAT3, which is frequently mutated in sporadic colorectal cancers. Dysregulation of STAT3 activation promotes colorectal tumour progression through transcriptional alteration of cell-cycle control genes cyclin-D1, c-Myc and metabolic regulator mTORC1. Signalling through STAT3, IL-6 is also capable of suppressing pro-apoptotic signals through the induction of Bcl2, Bcl-X_L and survivin.

Immunosuppression could explain the enhancement of tumour growth by MSCs. MSCs can modulate major immune cell populations when stimulated with a mitogenic signal and supress lymphocyte proliferation to both allogenic and xenogenic antigens (Sudres et al., 2006). The inhibitory effects of MSCs on B cell proliferation occurs via cell cycle arrest at the G_0/G_1 phase and not through apoptosis as previously thought (Plumas et al., 2005, Corcione et al., 2006). MSCs are also resistant to CTL-mediated lysis and are able to inhibit CTL cytotoxicity in a dose-dependent manner

when exposed at CTL priming (Rasmusson et al., 2007). Although originally believed to directly inhibit natural killer (NK) cell activation and proliferation, instead MSCs have been shown to inhibit the production of IFN-γ by NK cells (Spaggiari et al., 2008).

MSCs have also been shown to secrete factors involved in angiogenesis. During tumour progression, angiogenesis is an essential feature of cancer pathology, as without an adequate blood supply, a tumour cannot grow beyond 2–3 mm^3 (Folkman, 1971). *In vitro* experiments have shown that MSC-conditioned medium supports survival of cardiac myocytes and stimulated proliferation and migration of endothelial cells (Kinnaird et al., 2004b, Gnecchi et al., 2006). Secretion of specific angiogenic factors vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), hepatocyte growth factor, insulin-like growth factor 1, monocyte haemoattractant protein (MCP)-2, MCP-3, PDGF, SDF-1 and IL-8 have also been demonstrated (Kamihata et al., 2001, Chen et al., 2003, Kinnaird et al., 2004b, Nagaya et al., 2004, Tang et al., 2005, Potapova et al., 2007, Wang et al., 2015a). *In vivo*, subcutaneous tumours co-injected with MSC described above with increased tumour growth and metastasis have also exhibited increased vascularity in some cases (Mele et al., 2014, Huang et al., 2013).

Table 1.3 Pro-tumourigenic effects of MSCs

Pro-tumour effect	Reference
<u>Proliferation</u> • Co-injection of tumour cells with MSCs enhanced tumour growth	(Shinagawa et al., 2010)
Vascular support • MSCs can differentiate into pericytes and possibly endothelial cells	(Potapova et al., 2007, Zhu et al., 2009, Kang et al., 2010a, Portalska et

• MSCs secrete various factors that support vascular growth including VEGF, fibroblast derived growth factor, PDGF, and SDF-1	al., 2012, Nakagaki et al., 2015) (Kamihata et al., 2001, Chen et al., 2003, Kinnaird et al., 2004a, Nagaya et al., 2004, Tang et al., 2005, Potapova et al., 2007, Wang et al., 2015b)
<u>Fibroblast growth</u> • Tumour fibroblasts are derived from MSCs and therefore may be recruited from circulating populations • After tumour exposure MSCs acquire tumour-associated fibroblast (TAF) antigens, which are important to tumourigenesis	(Mishra et al., 2008, Spaeth et al., 2008) (Mishra et al., 2008, Spaeth et al., 2009)
<u>Paracrine factors</u> • MSCs secrete a variety of factors that induce tumour proliferation, migration, and angiogenesis. MSCs have also been shown to secrete exosomes and microparticles, that contain proteins or RNA that regulate intracellular signalling in adjacent cells	(Potapova et al., 2007, Salazar et al., 2009, Seib et al., 2009, Park et al., 2010, Lozito and Tuan, 2014)
<u>Immunosuppression</u> • MSC-mediated immunosuppression promotes tumour growth	(Djouad et al., 2003, Mele et al., 2014)

• MSCs can directly impair immune cell function, including T and B cells, dendritic cells, natural killer cells and macrophages • MSCs can supress T cell proliferation via multiple mechanisms including upregulation of B7-H1 and Stro-1+ expression • MSCs have been shown to switch macrophages from an M1 to M2 phenotype	(Di Nicola et al., 2002, Krampera et al., 2003, Plumas et al., 2005, Zappia et al., 2005, Corcione et al., 2006, Rutella et al., 2006, Sotiropoulou et al., 2006, Sheng et al., 2008, Nasef et al., 2009)
<u>Metastasis</u> • MSCs secrete several factors that promote metastasis e.g.CCL5/RANTES	(Karnoub et al., 2007)
<u>Cytoprotection</u> • MSCs have been shown to protect tumour cells from chemotherapeutic treatment; interactions with MSCs in the bone marrow can promote the survival of acute and chronic myeloid leukaemia • MSCs produce high levels of leptin, which has anti-apoptotic properties	(Konopleva et al., 2002) (Liu and Hwang, 2005)

<u>1.7.2 Anti-tumourigenic properties of MSCs</u>

In addition to the studies described above, there are numerous studies that describe either mixed or anti-tumour effects of MSCs in animal models. Maestroni and colleagues first observed tumour suppression by MSCs in models of Lewis lung carcinoma and B16 melanoma, showing that co-injection of tumour cells and MSCs inhibited primary tumour growth (Maestroni et al., 1999). The anti-tumour effects of MSCs have also been demonstrated in a model of colon carcinoma in rats (Ohlsson et al., 2003). MSCs inhibited the growth of rat colon carcinoma when co-implanted with

tumour cells. Increased macrophage and granulocyte infiltration was noted in tumours co-injected with MSCs, compared to no MSC control tumours, suggesting a pro-inflammatory effect. In fact, MSCs alone increased the engraftment of monocytes and granulocytes; however, this may have been due to the immunogenicity of MSCs transplanted across different rat strains.

Human foetal skin-derived MSCs can also inhibit the growth of human liver cancer cell lines. The cancer cells co-cultured with MSCs showed reduced proliferation, colony formation and oncogene expression; the same effects were shown when MSCs were co-injected with the same cancer cell lines *in vivo* (Hou et al., 2014). In addition, MSCs inhibited the growth of breast cancer cells *in vitro* and treatment with MSC-conditioned media also resulted in inhibition of cell growth (Qiao et al., 2008a).

MSCs have been shown to inhibit cancer growth by altering the cell cycle (Cousin et al., 2009). MSC co-culture *in vitro* with pancreatic tumour cells showed an increase G1-phase arrest in the tumour cells (Cousin et al., 2009). *In vivo* injection of adipose-derived MSCs into an established pancreatic cancer xenograft also inhibited tumour growth. In a similar approach, bone marrow-derived MSC were injected into an established model of subcutaneous melanoma, resulting in apoptosis and abrogation of tumour growth (Otsu et al., 2009). However, MSCs placed in a Matrigel insert in order for them to be exposed to soluble factors without cell to cell contact, no cytotoxic effects were noted (Otsu et al., 2009). This is in contrast to previous studies reported by Qiao and colleagues (Qiao et al., 2008a), and may reflect multiple mechanisms by which MSC exert their anti-tumour effects.

Furthermore, three other studies have noted that in colitis-associated colorectal cancer (CAC) models, MSC inhibit tumour initiation, and ameliorate inflammation, reducing damage to the colon (Chen et al., 2014, Nasuno et al., 2014, Tang et al., 2015). Nasuno *et al.* reported that the decreased tumour initiation was transforming growth factor beta (TGF-β)-dependent. It was not clear however, whether TGF-β was MSC-derived, although MSCs have been reported to produce and secrete TGF-β

(Stavely et al., 2015). In the normal colon, TGF-β regulates the growth and self-renewal of colonic epithelium along the crypt axis by opposing Wnt/β-catenin signalling (Reynolds et al., 2014). TGF-β signalling inhibits proliferation of epithelial cells, arresting cell cycle progression in G1 restitution (McKaig et al., 1999). Mutations in TGF-βRII are common in sporadic CRC (13%) (Grady et al., 1999). Only one group has reported anti-tumour effects of MSCs using a subcutaneous tumour model (Ohlsson et al., 2003), while numerous studies in subcutaneous tumour models have reported pro-tumourigenic effects. In addition, in two studies differential tumour growth in response to MSC treatment was observed for different CRC cell lines (Nakagaki et al., 2015, Rhyu et al., 2015). These cell line-specific responses could not be explained by host immune competency. Rhyu *et al.* observed that while expression of several genes were altered in MSC-treated A549 tumours, no genes were altered by MSC-treatment of HT-29 tumours (Rhyu et al., 2015). These conflicting studies reflect the different effects of MSCs in different types of cancer and in different models and must be carefully considered when drawing conclusions.

Table 1.4 Anti-tumourigenic effects of MSCs

Anti-tumour effect	Reference
Cytotoxicity • MSCs inhibit tumour initiation in a TGF β-dependent manner • MSCs induce apoptosis of tumour cells	(Nasuno et al., 2014, Otsu et al., 2009)
Immunomodulation • MSCs express toll like receptors which can switch MSCs from an immunosuppressive to pro-inflammatory phenotype	(Waterman et al., 2010)

Vascular Damage • Under certain conditions MSCs can inhibit capillary growth by producing reactive oxygen species and causing endothelial apoptosis	(Otsu et al., 2009)
Anti-proliferative • MSCs secrete anti-proliferative factors	(Zhu et al., 2009)

1.8 Conclusion

In summary, current treatment strategies for IBD largely combat the symptoms rather than the disease and are limited by adverse side effects, toxicity and inefficacy. In recent years, there has been much interest in the therapeutic potential of MSCs; however it is still debated in the literature whether MSCs are both efficacious and safe with the majority of experimental studies indicating MSCs exert an anti-tumour effect whilst others have reported a pro-tumour effect. MSC treatment in IBD has shown promising results in both animal models and human trials, however, they appear to be functionally different, depending on the type of cancer and animal model used. This may be due to the ability of MSCs to migrate and engraft to the inflamed tissue and also the ability to modulate and evade host immune responses. Understanding the role of MSCs in inflammatory and cancer environments could pave the way of new avenues for therapeutic targets.

Chapter Two: Leukocyte Populations and IL-6 in the Tumour Microenvironment of an Orthotopic Colorectal Cancer Model

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2.1 Background

Cancer of the colon and rectum is the third most common type of cancer in the world. Chronic inflammatory diseases such as Crohn's disease and ulcerative colitis lead to a high risk of developing colorectal cancer (CRC) (Boland and Goel, 2010, Saleh and Trinchieri, 2010, Bergman et al., 2011). Other risk factors for the development of CRC include an unhealthy diet, smoking, analgesics and genetic factors (Cappell, 2008, Erdelyi et al., 2009). Due to the asymptomatic nature of CRC, it is often diagnosed at the late stages when cancer has spread to other parts of the body (Orbell and West, 2010, Adelstein et al., 2011).

The immune system has many specialised cells that are involved in the detection and elimination of tumours. Dendritic cells and macrophages can detect tumour antigens and are involved in activating T cells. Natural killer (NK) lymphocytes and neutrophils, part of innate immunity, also play a role in the elimination of tumour cells and activation of T cells. γδT cells (highly abundant in gut mucosa) and NKT cells (share properties of both T cells and NK cells and recognise lipids and glycolipids in complex with CD1d molecules) bridge innate and adaptive responses, in that they can eliminate tumour cells without prior sensitisation (Waldhauer and Steinle, 2008). The immune system plays a vital role in the prevention of tumour growth including response to tumour-specific and tumour-associated antigens, however, the leukocyte-driven inflammatory responses can also play a role in the initiation of CRC (Smyth et al., 2001, Finn, 2008). Inflammatory responses are largely driven by $CD4^+$ T helper cell subsets including the canonical 'pro-inflammatory' Th1 responses: cytotoxic T lymphocytes (CTLs), NK and M1 macrophages and/or canonical 'anti-inflammatory' Th2 responses: M2 macrophages, eosinophils and activated B cells such as IgE-secreting plasma cells.

Th1 responses are generally associated with inducing cellular toxicity to tumour cells. Indeed, enhanced CTL responses are generally associated with a good prognosis for cancer patients (Yukihirofunada et al., 2003). Likewise, NK cells mediate cytotoxicity in many types of tumours (Wu and

Lanier, 2003) and macrophages (M1) have Fc receptors for opsonising antibodies and can clear tumour cells and tumour antigens (Sica et al., 2006). Pro-inflammatory Th1 cytokines such as IL-2, tumour necrosis factor (TNF)-α and interferon (IFN)-γ have been shown to lead to a better prognostic outcome in many cancers (Schreiber et al., 2011). In some cases IL-2 therapy is being used to treat cancers (Balkwill and Mantovani, 2012).

Th2 responses are generally linked to poor prognosis; excessive anti-inflammatory responses contribute to disease development and have been associated with tumour pathophysiology. The tumour microenvironment consists largely of tumour-associated macrophages (predominantly M2 macrophages) commonly found in tumour biopsies which typically secrete IL-10 (Erreni et al., 2011) which aids in the diversion of Th1 cytotoxic responses to Th2 responses. In addition, IL-6 has been linked to poor tumour outcomes (Chung and Chang, 2003) and IL-4 has been linked to colon cancer stem cells that elude cell death (Todaro et al., 2008). Understanding the tumour microenvironment allows the prediction of immune response outcomes and the design of treatments that will stimulate Th1 cytotoxic responses and subvert tumour growth and metastasis. The immune system has specialised organs to help combat gastrointestinal cancers including Peyer's patches and mesenteric lymph nodes (MLNs). Lymph nodes provide a gateway for CRC metastasis. Peyer's patches are lymphoid organs that are irregularly distributed along the anti-mesenteric side of the small intestine. In the distal ileum Peyer's patches are numerous and form a lymphoid ring. Peyer's patches serve as major sites for the generation of immunity to intestinal antigens. Their unique micro-organisation is crucial for the generation of an immune response.

Animal models of human CRC can provide insight into the mechanisms that underlie the development and pathogenesis of CRC. The ideal animal model should therefore replicate all aspects of tumour development, including the acquirement of genetic alterations with consequent changes

in cell behaviour and characteristic sensitivity to therapeutics. The tumour model should also be reliable i.e. tumour take should be predictable and constant, with a high incidence of affected animals in a narrow time frame. Currently, there are several models for implanting tumour cells into an animal. The easiest and most frequently used model is the subcutaneous injection/implantation model. The accessibility of subcutaneous tumours is extremely advantageous when monitoring tumour growth. However, a major disadvantage is that the subcutaneous microenvironment greatly differs from that of the colon or other organs. Interaction between the host environment and tumour graft determines tumour cell expression profiles, the levels of growth factors and nutrients as well as tumour angiogenesis and metastatic behaviour (Heijstek et al., 2005).

Alternatively, the orthotropic injection model, in which the tumour cells are injected into the caecal wall, closely mimics human CRC. This model has many advantages, including the microenvironment in which the tumour grows and metastasises to nearby areas of the colon and lymph nodes similar to that seen in humans. An obvious disadvantage of this model is technical side; the procedure is far more difficult than subcutaneous injection. It requires more technical skills and is more time-consuming. In addition, endpoints for determining the effects of therapy are more complex than normal tumour measurement in subcutaneous models (Bibby, 2004).

In this regard, an orthotropic model of tumour growth was characterised and the effects of CRC development in MLN and Peyer's Patches leukocyte populations are presented. In addition, cytokines within the tumour microenvironment were also analysed.

2.2 The Gaps

The orthotopic model of CRC closely mimics human CRC and allows for the tumour microenvironment to be studied in an animal model.

2.3 Aim

To characterise the orthotopic model of tumour growth in CRC and the development in the MLNs and Peyer's Patches leukocyte populations and cytokines in the tumour microenvironment.

2.4 Materials and Methods

2.4.1 Animal Model

Male BALB/c mice aged 5-8 weeks (n = 30) were purchased from the Animal Resources Centre (Canning Vale, WA, Australia) and housed in groups of three. Animals were kept on a 12-hour light and dark cycle at approximately 22 °C with free access to food and water. The mice were allowed to acclimatise for at least one week before undergoing surgery. This study was approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Code of Practise for the Care and Use of Animals for Scientific Purposes.

2.4.2 CT26 Colorectal Cancer Cell Culture

CT26 murine colorectal cancer cells were cultured at 37 °C in 5% CO_2 in tissue culture flasks in RPMI 1640 medium supplemented with 10% foetal calf serum, 200 mM L-glutamine, 1 M HEPES, 10,000 U/ml penicillin, 10 mg/ml streptomycin, 100 mM sodium pyruvate, and 25 µg/ml amphotericin B. All these reagents were from Sigma-Aldrich (Castle Hill, NSW, Australia). Cells were passaged when they reached 70%-90% confluency by treating with 1 ml of trypsin/EDTA (2.5 g trypsin and 0.2 g EDTA) (Sigma-Aldrich) for 3-5 min followed by inactivating the trypsin by adding complete media. CT26 cells were prepared for injection in Matrigel™ (BD Biosciences, Two Oak Park, Bedford, MA, USA) at a concentration of $4x10^7$ cells/ml.

2.4.3 Orthotropic Injection of CT26 Cells

Mice were assigned to either CRC-induced group or sham-operated group. Prior to surgery, mice were anaesthetised with an intraperitoneal injection comprising a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), the volume was calculated per animal body weight, the maximum volume did not exceed 200 μl. Mice had their eyes coated with Viscotears and during surgery the level of anaesthesia was monitored by the paw pinch response. The surgery was performed under aseptic conditions on a heat mat. The mice had their abdomen shaved and wiped with 70% ethanol before being covered with sterile film. A small incision was made along the midline of the abdomen, and the caecum was exteriorised. Cell suspension of viable tumour cells ($1x10^6$ CT26 cells) in 25 μl of the Matrigel™ was injected into the caecal wall. Sham-operated mice underwent the same surgery under the same conditions as the CRC-induced mice group, however instead of an injection of CT26 cells, the sham-operated group had the tip of a 27 gauge needle inserted to the caecum wall. After surgery, the abdominal muscle wall was closed using polygalactin 5.0 gut sutures. Surgical silk suture was used to close the skin and the wound area was then sterilised with iodine. Mice received a subcutaneous injection of an analgesic buprenorphine (0.05 mg/kg) calculated per animal body weight, the maximum volume did not exceed 200 μl (Sigma-Aldrich) and were placed in cages on heated mats to recover. Mice were closely monitored and regularly checked post-surgery. Mice were killed by cervical dislocation at three time points post-surgery (3, 7 and 14 days); the caecum, colon, Peyer's patches and MLNs were collected for immunohistochemistry and FACS analysis.

2.4.4 Cell Surface Labelling

FACS analysis was used to enumerate and phenotype the different leukocyte subpopulations in the MLNs and Peyer's patches. A minimum of 500,000 cells per sample was used for antibody staining. Cells were labelled with the monoclonal antibodies at 4 °C for 20 min to label specific types of immune cells. Antibodies used were as follows: rat anti-mouse

Gr-1 conjugated to FITC (clone RB6-8C5), rat anti-mouse CD11b conjugated to R-phycoerythrin and cyanine (clone M1/70), rat anti-mouse CD193 conjugated to Alexa Fluor 647 (clone J073E5), rat anti-mouse Ia/Ie conjugated to allophycocyanin (M5/114.15.2), rat anti-mouse F4/80 conjugated to R-phycoerythrin (clone BM8), rat anti-mouse CD11c conjugated to pacific blue (clone N418), rat anti-mouse CD49b conjugated to FITC (clone DX5), rat anti-mouse TCRβ conjugated allophycocyanin (clone H57-597), rat anti-mouse CD1δα Galcer loaded tetramer conjugated to R-phycoerythrin, hamster anti-mouse γδTCR conjugated to R-phycoerythrin and cyanine (clone GL3), rat anti-mouse $CD8^+$ conjugated to pacific blue (clone 53-6.7), and rat anti-mouse $CD4^+$ conjugated to pacific orange (clone GK1.5) . All these antibodies were from BioLegend (San Diego, USA).

To prevent non-specific Fc receptor binding, cells were also co-stained with rat anti-mouse CD16/CD32 hybridoma supernatant (2.4G2 clone; Developmental Studies Hybridoma Bank, Iowa City, USA). Samples were analysed using a BD LSRII FACS analyser (BD Biosciences). All data were compensated using BD FACSDIVA v6.0 and either analysed in FACSDIVA or exported as FCS3.0 file format for data analysis in FlowJo (Ashland, USA).

2.4.5 Cytokine Analysis

Cytokines of *in vitro* cultured CT26 and SW480 cells were analysed by FACS using the cytometric bead array (CBA) kit (BD Biosciences) (Morgan et al., 2004) according to the manufacturer's instructions. The cytokine concentration (pg/ml) was determined with calibration curves separately established using the CBA analysis software (BD Biosciences). Th1 and Th2 cytokines were measured in the supernatant of CT26 and SW480 cell cultures with the CBA mouse and human kits. Furthermore, tumour cells were isolated from the caecum at days 7 and 14, and made into single cell suspension using collagenase (0.01 mg/ml). Cells were labelled with surface marker CD45 (clone: 30-F11) and fixed using BD

cytofix/cytoperm kit according to the manufacturer's instructions. $CD45^+$ and $CD45^-$ (CT26) cells were stained for the expression of intracellular cytokines IL-6 (clone: MP5-20F3), IL-10 (clone: JES5-13E3), TGFβ (clone: TW7-16B4) and TNFα (clone: MP6-XT22).

2.4.6 Immunohistochemistry

Immunohistochemistry was performed in cross sections of the caecum and colon as described previously (Nurgali et al., 2011). Tissue sections were thawed and incubated for one hour at room temperature with 10% normal donkey serum (Chemicon, Temecula, CA, USA) followed by an overnight incubation with a primary antibody at 4°C. The secondary antibody was added and then incubated at room temperature for 2 h. Primary antibodies used were as follows: hamster anti-mouse γδTCR monoclonal antibody (clone: GL3) conjugated to FITC (BioLegend), rat anti-mouse CD11b monoclonal antibody (BioLegend), anti-mouse CD69 monoclonal antibody (BioLegend) and rabbit anti-mouse eosinophil derived neurotoxin (EDN) antibody (Novus Biologicals, Littleton, USA). Secondary antibodies used were as follows: donkey anti-rat Alexa Fluor 594 and anti-rabbit Alexa Fluor 594 (Jackson ImmunoResearch, West Grove, USA). Slides were cover slipped with fluorescent mounting medium (DAKO, North Sydney, NSW, Australia).

2.4.7 Imaging

After immunohistochemistry treatment, the three-dimensional (z-series) images of sections were taken and analysed by using an Olympus Fluo-View FV1000 confocal laser scanning microscope (OlympusTokyo, Japan). Fluorophores were visualised using excitation filters for Alexa 594 (excitation wavelength 559 nm) or Alexa 488 (excitation wavelength 473 nm). Z-series images were taken at a step size of 1.75 μm (1600 x 1200 pixels) using the confocal microscope.

2.4.8 Statistical Analysis

FACS data were analysed using a two way ANOVA followed by Bonferroni *post hoc* test for multiple group comparisons. Analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, USA). All data were presented as mean ±SEM. Data were considered statistically significant at $P<0.05$.

2.5 Results

2.5.1 The Effect of Colorectal Cancer on Immune Cell Counts in Mesenteric Lymph Nodes and Peyer's Patches

There was no significant difference between the sham and CRC groups at any time point (days 3, 7, and 14, n=5/group/time point) in either MLNs or Peyer's patches. However, the MLNs in mice with CRC had higher absolute cell counts at all days compared with the sham-operated mice (data not shown). Conversely there was an opposing trend in the Peyer's patches where the absolute counts in sham mice were higher than that in mice with CRC. This anomaly could be accounted for by differences in tumour load and therefore cell death in the Peyer's patches, thus any increase in cell subpopulations in the Peyer's patches is likely to be resulted from newly-activated lymphocytes generated in MLNs.

For accurate enumeration of natural killer (NK) cells, the presence of the pan-NK cell marker CD49b and the absence T cell receptor (TCR) were used for their identification. TCR is required to exclude $CD49b^+$ natural killer T (NKT) cells from our analysis. Prior to analysis, doublets were gated out based on their forward scatter (FSC) properties (FSC-height vs FSC-width). NK cells were gated on their presence of $CD49b^+$ and absence of TCR (**Fig. 2.1A, C**). No significant difference in the number of NK cells was seen at any time point in the Peyer's patches (**Fig. 2.1B**). However, in the MLNs the number of NK cells in the CRC group was significantly increased from 3 to 7 days with no difference in the sham group (**Fig. 2.1D**). In addition, on day 7, a significant increase in the number of NK cells ($P<0.05$) in the CRC group compared with the sham group was noted (**Fig. 2.1D**).

The expressions of CD8 and TCR were used to enumerate $CD8^+$ lymphocytes (**Fig. 2.2A, C**). A significant increase in the number of $CD8^+$ lymphocytes can be seen between the CRC and sham groups in Peyer's patches at days 3 and 7 (**Fig. 2.2B**, $P<0.05$ for both). No significant

difference was evident in the $CD8^+$ T cell number of MLN's groups (**Fig. 2.2D**).

One of the concerns with this study was the variability in our mesenteric lymph node size and immune cell composition. In an attempt to minimise this variation, 5 lymph nodes were pooled from each mouse. However as lymph nodes vary dramatically in size, we found it quite difficult to obtain lymph nodes of similar size, which resulted in high standard errors (**Figs. 2.1** and **2.2).**

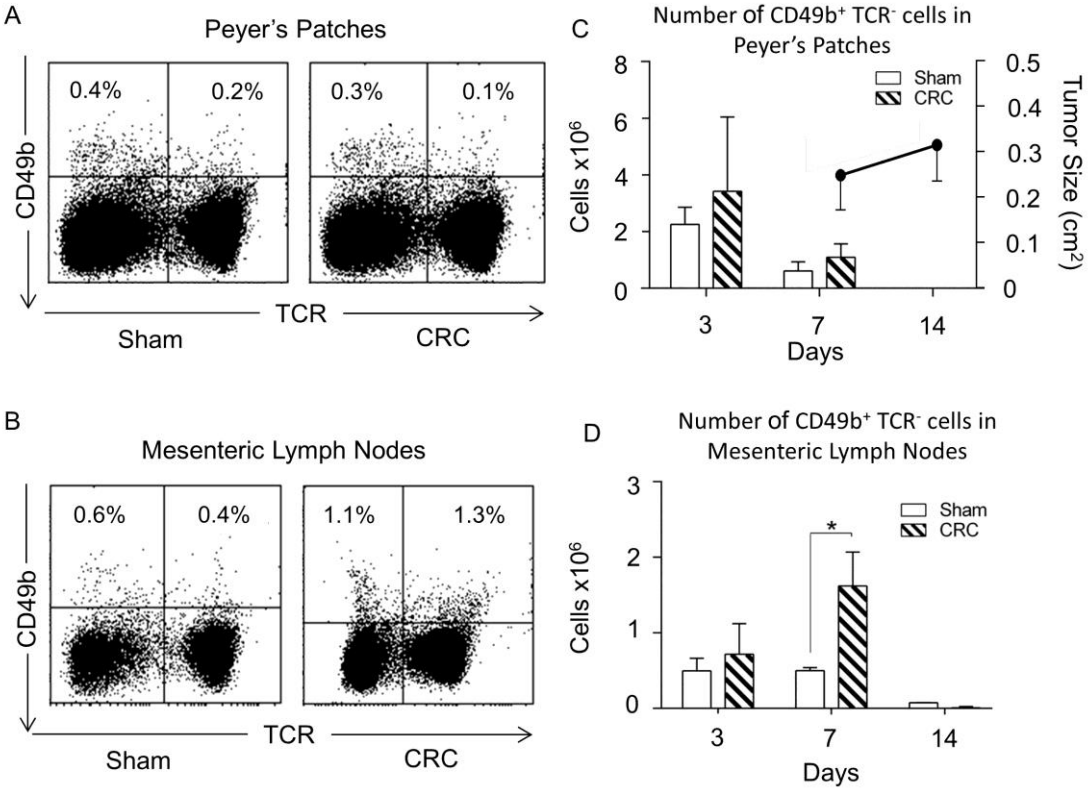


Figure 2.1: Effect of Colorectal Cancer on Natural Killer Cells in Peyer's Patches and Mesenteric Lymph Nodes.

FACS plots of natural killer cells at day 7 in (**A**) Peyer's patches and (**B**) mesenteric lymph nodes (MLNs) from sham surgery and CRC induced mice. Number of $CD49b^{+}TCR^{-}$ cells in (**C**) Peyer's patches and (**D**) MLNs from sham-operated and CRC-induced groups at days 3, 7 and 14. (**C**) Tumour size at days 7 and 14. A significant increase ($P<0.05$) in the number of $CD49b^{+}TCR^{-}$ cells is seen at day 7 in MLNs. Data are represented as the mean ± SEM and statistical significance is indicated by asterisks, $*P<0.05$.

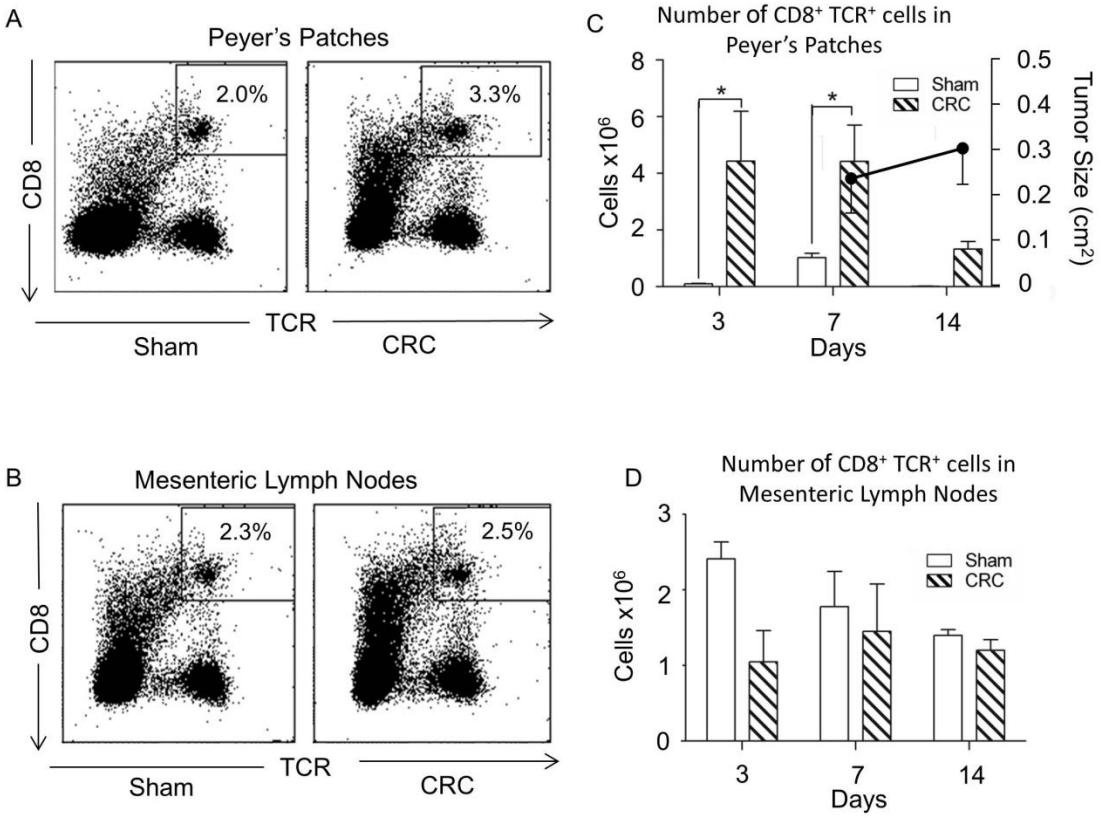


Figure 2.2: Effect of Colorectal Cancer on CD8+ T Cells in Peyer's patches and Mesenteric Lymph Nodes.

FACS plots of $CD8^+$ T cells at day 7 in (**A**) Peyer's patches and (**B**) mesenteric lymph nodes (MLNs) from sham surgery and CRC induced mice. Number of $CD8^+TCR^+$ cells in (**C**) Peyer's patches and (**D**) MLNs from sham-operated and CRC-induced groups at days 3, 7 and 14 post surgery. (**C**) Tumour size at days 7 and 14. Data are represented as the mean ± SEM and statistical significance is indicated by asterisks, **P*<0.05.

2.5.2 The Effect of Colorectal Cancer on Immune Cells in the Colon

Gamma delta (γδ) T cells are resident cells within the gastrointestinal tract which are known as intra-epithelial lymphocytes and play an important role in anti-tumour immunity (Lee et al., 2012). Immunohistochemical studies of the colon cross sections demonstrated that γδ T cells that normally are abundant in the epithelial layers of the gastrointestinal tract were absent in the colon sections from mice with CRC (**Fig. 2.3A**). Immunohistochemical studies of caecum cross sections (site of tumour induction) demonstrated that eosinophils, activated $CD69^{+}$ T cells and $CD11b^{+}$ cells infiltrated the tumour tissue (**Fig. 2.3B**).

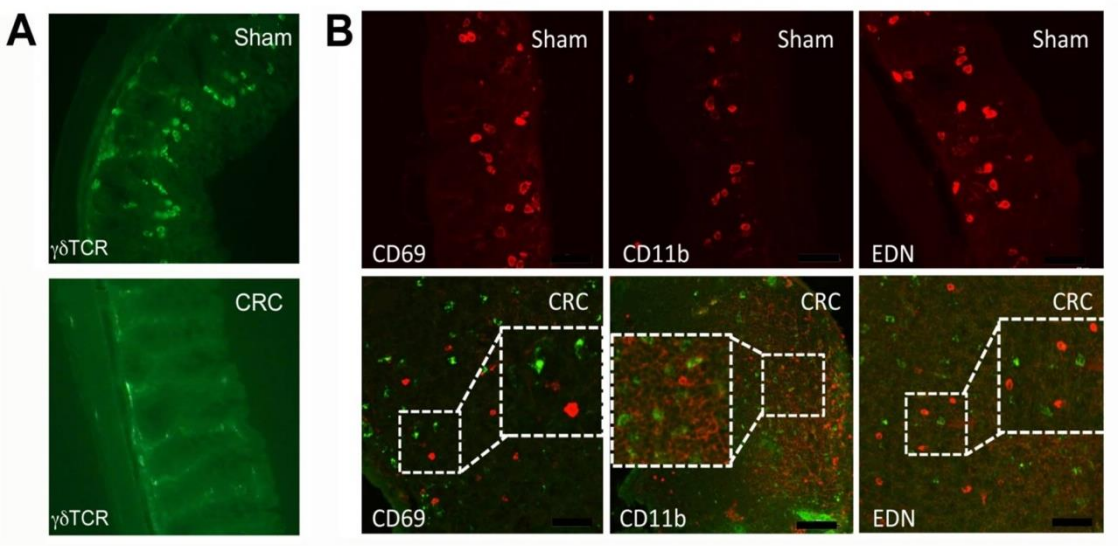


Figure 2.3: The Effect of Colorectal Cancer on γδT Cells in the Colon and Tumour Infiltrating Leukocytes in the Caecum.

(**A**) Immunohistological labelling of γδT cells in the colon. (**B**) Immunohistochemical labelling of tumour infiltrating leukocytes (red). $CD69^{+}$ T cells, $CD11b^{+}$ cells and eosinophils (EDN) were found within the tumours (green) in the caecum. Cross sections of caecum tissue without tumour cells (sham) were also immunohistochemically labelled to show normal level of immune cells.

2.5.3 Cytokine Profile of the Murine CT26 CRC Cell Line *in Vitro* and *In Vivo*

Cytokine analysis revealed that CT26 cells were negative for IL-2, IL-4, IL-10, IFN-γ, TNF-α and IL-17a secretion. However, CT26 cells secreted high level of IL-6 (61.76 pg/ml), while the human SW480 cell line secreted high level of IL-10 (164.97 pg/ml) (**Fig. 2.4A**). Likewise, flow cytometry analysis of tumour cells from mice with CRC demonstrated the expression of IL-6 by CT26 cells (**Fig. 2.4B-B′, C**). All other cytokines tested were negative (**Fig. 2.4C**). Of interest, tumours that infiltrate $CD45^+$ cells also expressed IL-6 and TNF-α (**Fig. 2.4C′**) within the tumour microenvironment.

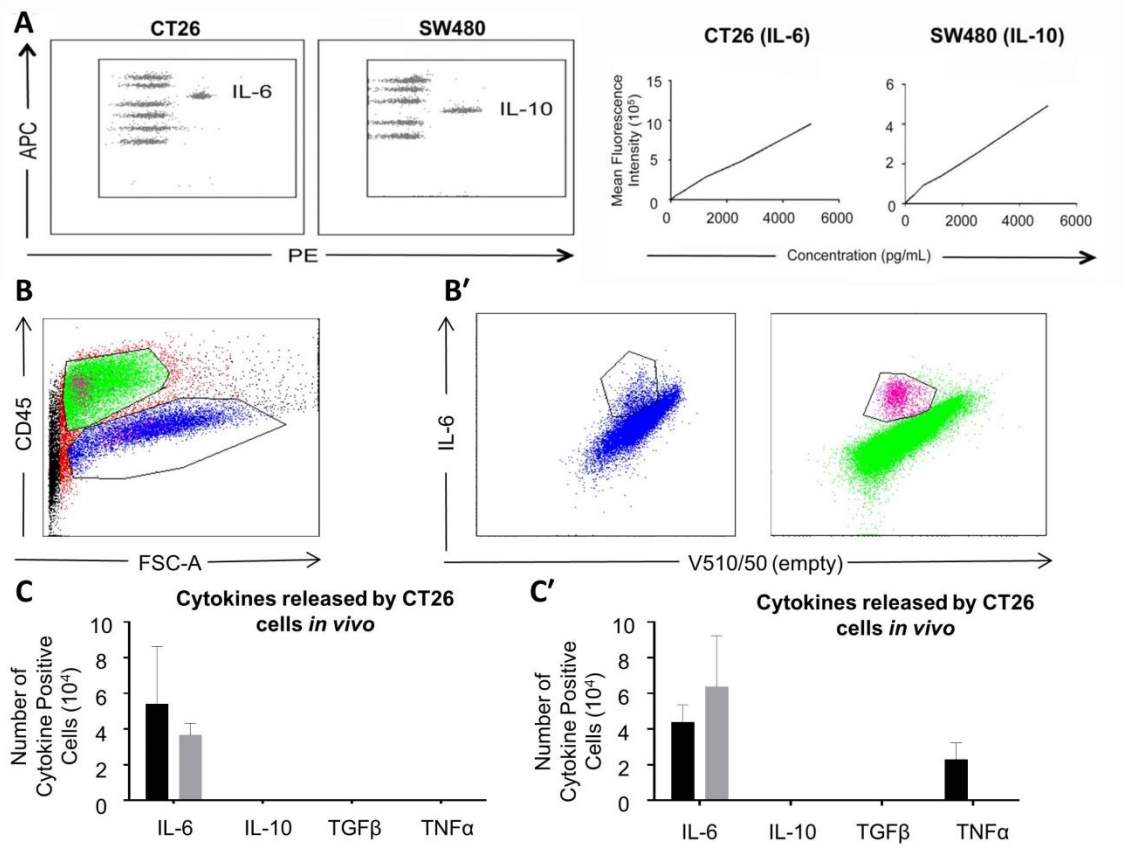


Figure 2.4: Cytokine Profile of the Murine CT26 and Human SW480 CRC Cell Line *In vitro* and *In vivo.*

(**A**) Cytokine FACS plots of murine CT26 and human SW480 colon cancer cells and concentration graphs for both CT26 and SW480 cell lines. Analysis of intracellular cytokines of *in vivo* CT26-induced tumours: (**B**) CD45 vs forward scatter (FSC-A) plot used for gating: (**B′ left panel**) Intracellular IL-6 expressed by CT26 cells *in vivo*: (**B ′right panel**) Intracellular IL-6 expressed by tumour-infiltrating $CD45^+$ cells. (**C**) Quantitative analysis of intracellular cytokines expressed by CT26 cells *in vivo* at Days 7 (black bar) and 14 (grey bar): (**C′**) Quantitative analysis of intracellular cytokines expressed by $CD45^+$ tumour-infiltrating cells *in vivo* at days 7 (black bar) and 14 (grey bar).

2.6 Discussion

CRC pathophysiology has been described as asymptomatic and can therefore go undiagnosed until a sufficient tumour mass has been established. In this study, an animal model of orthotopic CRC development was used.

IL-6 acts both as a pro-inflammatory and an anti-inflammatory cytokine. In cancer patients, IL-6 is highly elevated leading to poor prognosis (Bellone et al., 2006), and is abundant at the tumour microenvironment where it plays a role in cancer metastasis via down-regulation of E-cadherin (Miao et al., 2014). In mice, CT26 cells that express IL-6 exert tumour-promoting activities by activating growth and survival (Fisher et al., 2011). However, IL-6 tumour microenvironment also activates $CD8^+$ T cells to the tumour site (Fisher et al., 2011). It is likely that IL-6 produced by CT26 in our studies contributed to its metastatic ability from the caecum to the nearby colon. Conversely, human SW480 CRC cell line produced high amounts of IL-10. IL-10, a T helper type 2 cytokine, is known to possess many immunosuppressive activities including: the inhibition of T lymphocyte proliferation and T helper 1 type cytokine production, impairment of antigen presenting cells and blunting of cytotoxic responses. Many studies have demonstrated that IL-10 is a prognostic indicator in CRC (Szkaradkiewicz et al., 2009). It is clear that the murine CRC CT26 cell line produces tumours via an IL-10-independent manner.

The immune response to slow-forming cancers is complex because the kinetics of tumour antigen processing particularly the antigen presenting cells (APCs), as well as the T cell activation is unknown. APCs must travel from the site of the tumour growth to either the MLNs or Peyer's patches; a process that can take a significant amount of time because activated T cells could take several days to travel to the tumour site (Norian et al., 2009). Indeed, we demonstrated a detectable increase in the number of $CD8^+$ lymphocytes in Peyer's patches in the CRC group as early as day 3

and an increase in the number of NK cells in MLNs at day 7 post CRC induction. Once at the tumour site, activated T cells recognise tumour antigens in complex with MHC class I, and lyse tumour cells, however the success by which this can occur is largely influenced by the tumour microenvironment.

The tumour specificity of increased $CD8^+$ T cells observed in our study is evidenced by the fact that all experimental mice have only orthotopic cancer development and no other comorbidities (e.g. inflammation) which could stimulate $CD8^+$ T cell activation. Likewise, it was shown in another study that silencing MUC2 from CT26-promoted tumour growth by increasing IL-6 secretion was followed by an increase of CD8 T cells in the peritoneal cavity specific to CT26 cells (Shan et al., 2014). In cancer, $CD8^+$ T cells are activated to kill tumour cells and their presence results in better survival (Galon et al., 2006). In the orthotropic CT26 injection model used in our study, an increase in $CD8^+$ T cells and NK cells was noted as early as day 3 and day 7, which correlates with the pro-inflammatory cytokine IL-6 produced by these cells. An increase in $CD8^+$ T cells and NK cells leads to better prognosis. In fact, we demonstrated an inverse correlation between increased number of $CD8^+$ T cells and NK cells and decreased tumour growth in the CT26 CRC-injected groups. When the tumour load was high, there were less $CD8^+$ T cells and NK cells. In addition, we have also demonstrated that there was an inverse correlation between CTL precursor frequency and tumour size in other tumour models (Apostolopoulos et al., 1998, Pietersz et al., 1998).

Furthermore, the prognostic significance of NK cells in CRC was demonstrated in CRC patients, where patients with little NK cell infiltration had a significantly shorter survival rate than those who had extensive infiltration (Coca et al., 1997). In addition, patients with grade III CRC had significantly longer survival rates with high NK cell infiltration compared with those with low NK cell infiltration (Coca et al., 1997).

γδT cells are resident gastrointestinal lymphocytes and are vital to gastrointestinal immunity, as they recognise tumour-expressed ligands that other T lymphocytes cannot recognise (Moser, 2012). γδT cells are similar to NKT cells in that they share features of both the innate and adaptive lymphocytes due to their expression of invariant T cell receptors. Activated γδT cells can have strong cytotoxic effects via both the death receptor and death ligand pathways and the cytolytic granule pathways (Bonneville et al., 2010). In addition, γδT cells exhibit lytic activity against cancer cells in an MHC-unrestricted manner *in vitro*, suggesting their potential as anticancer therapy (Corvaisier et al., 2005, Todaro et al., 2009). Based on the IL-6 CT26 data (**Fig. 2.3D**) we have clearly shown that the mechanism in our murine model of CRC is different from human CRC (IL-10-dependent). The mechanism of how IL-6 signalling influences γδT cell viability or mobilisation is unclear. Further studies are needed to address this mechanism by using the neutralising anti-IL-6 receptor monoclonal antibody (Tocilizumab) (Scheller et al., 2011).

Immune cell infiltration within the tumour in the caecum demonstrated that $CD11b^+$ cells, $CD69^+$ T cells and eosinophils were present. Eosinophils have been found in a number of tumours, however, their role is still being debated (Davis and Rothenberg, 2014). In colorectal cancer however, their presence has been associated with a favourable prognosis (Legrand et al., 2010). $CD69^+$ cells are indicative of an activated T cell state. As there was an increase in $CD8^+$ T cells, it was not unexpected to detect T cells within tumour itself, and in fact, T cells were present in their activated state ($CD69^+$). This is in accord to the pro-inflammatory state of the CT26 tumour micro-environment. Moreover, $CD11b^+$ cells were also found within the tumour microenvironment.

2.7 Conclusion

In this study, we investigated the effect of the murine orthotropic CT26 CRC model on immune cells and revealed significant changes in the number of NK cells in mesenteric lymph nodes at day 7 and significant

changes in the number of $CD8^+$ T lymphocytes at days 3 and 7 in Peyer's patches. We also demonstrated that γδT cells were depleted in the colon in mice with CRC and immune cells (eosinophils, $CD69^+$ T cells and $CD11b^+$ cells) infiltrated into the tumour site. The cytokine analysis revealed that CT26 cells secreted IL-6 cytokine both *in vitro* and *in vivo*, which is in accord to the immune activation state noted. Recently, it was demonstrated that cancer cells and immune cells communicate via the presence of IL-6 in the tumour microenvironment which is secreted by both cancer cells and immune cells (Patel and Gooderham, 2015). Likewise, in the orthotopic CRC model presented here, both tumour cells and infiltrating $CD45^+$ cells expressed IL-6; $CD45^+$ cells also expressed TNFα which further contributed to a pro-inflammatory tumour microenvironment. Furthermore, it would be of interest to study the immune response to this orthotopic cancer cell model in inflammation-induced cancer which is more representative of human condition.

Chapter Three: Immune Cell Populations in an Animal Model of Spontaneous Chronic Colitis with or without Colorectal Cancer

3.1 Background

Inflammatory bowel disease (IBD) is comprised of two types of chronic intestinal diseases, Crohn's disease (CD) and ulcerative colitis (UC). IBD incidence in Australia is 24.2 per 100,000 people and its peak onset is 5 – 49 years of age (CCA, 2013, Studd et al., 2016). CD generally involves the ileum and colon, but can affect any region of the intestine, often discontinuously. UC however, involves the rectum and may affect part of the colon or the entire colon in an uninterrupted pattern with inflammation typically confined to the mucosa (Hanauer, 2006). In CD, inflammation is often transmural and is associated with intestinal granulomas, strictures and fistulas (Thoreson and Cullen, 2007). IBD incidence appears to be lower in developing countries, however as societies become more 'westernised' with changes in life style, diet and other environmental exposures, the incidence rates rise globally. There is also an alarming increase in the number of IBD cases in low incidence areas such as Asia (Ng, 2014). Accumulating evidence suggests that IBD results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host (Abraham and Cho, 2009). The exact causes of UC and CD remain elusive, but thus far, IBD is thought to be the result of an inappropriate and ongoing activation of the mucosal immune system driven by luminal flora in genetically susceptible hosts (Bouma and Strober, 2003).

Animal models of IBD contribute greatly to our understanding of the underlying mechanisms of inflammation and disease pathogenesis as well as treatment. In general, an appropriate or an optimal animal model should display certain key characteristics: morphological and functional alterations in the gut, inflammation, symptoms and signs, pathophysiology and a course similar or identical to that of human IBD (Mizoguchi and Mizoguchi, 2010). It is also recommended that the animal being used should have a well-defined genetic background, as well as a well characterised immune system (Jurjus et al., 2004). Animal models of IBD can be categorised into 5 groups: gene knockout models, transgenic

mouse and rat models, inducible colitis models, adoptive transfer models and spontaneous colitis models (Hibi et al., 2002).

(i) Genetic knockout models such as interleukin (IL)-10, IL-2 and T cell receptor knockouts greatly contribute to our understanding of the role of key immune-related molecules in the pathogenesis of chronic intestinal inflammation. These models have clearly allowed identification of key components involved in gut immune regulation (Pizarro et al., 2003). However, it is unlikely that the imposed genetic mutations represent the underlying defect in human IBD, limiting the utility of these models for understanding causative factors in both ulcerative colitis and Crohn's disease (Pizarro et al., 2003). (ii) Transgenic IBD animals such as IL-7 mice and HLA-B27 rats have contributed to our understanding of the immunopathology behind IBD. In fact high levels of IL-7 are found in the sera of ulcerative colitis patients which influences the differentiation and proliferation of T cells in the thymus (Hibi et al., 2002). In addition, HLA-B27 transgenic rats have been used to study the effects of resident intestinal bacteria in acute and chronic stages of gastrointestinal inflammation (Hibi et al., 2002). (iii) The most commonly used animal model in IBD is the inducible colitis model which involves a chemical inflammatory stimuli, such as acetic-acid, Iodoacetamide, indomethacin, 2,4,6-Trinitrobenzenesulfonic acid (TNBS), oxazolone, dextran sodium sulphate (DSS) and peptidoglycan–polysaccharide. (iv) The adoptive transfer model of IBD such as the T cell transfer model involves the transfer of $CD4^{+}CD45RB^{high}$ T cells (naive T cells) from healthy wild-type mice into syngeneic recipients that lack T and B cells. This transfer induces a pancolitis and small bowel inflammation at 5–8 weeks following T cell transfer (Ostanin et al., 2009).

However, all these animal models for IBD are quite artificial therefore making it difficult to obtain accurate results that could be translated in human clinical trials. Analyses of animal models generated by genetic manipulation and human disease, together with results from genome-wide association studies, have identified several types of defects that appear to contribute to intestinal inflammation. These include alterations in the

mucosal barrier, abnormalities of innate immunity and inappropriate specific immune responses, particularly activation of effector T cells and increased production of IL-17 in response to gut microbes culminating in chronic inflammation. (v) One of these models is the *Winnie* mouse model of spontaneous chronic colitis. In fact, *Winnie* mice (C57BL/6 background) carry a missense mutation in Muc2 which leads to severe endoplasmic reticulum stress in intestinal goblet cells and spontaneous chronic colitis (Heazlewood et al., 2008, Eri et al., 2011). In humans, expression of Muc2 is reduced or depleted in Crohn's disease (Buisine et al., 2001) and is still active in ulcerative colitis but its secretion is reduced (Van Klinken et al., 1999). Inflammation in the colon develops in *Winnie* mice from 6 weeks and results in severe chronic colitis by 16 weeks of age. *Winnie* mice also have periods of remission and relapse similar to that observed in humans with IBD. Since *Winnie* mice closely mimic human chronic colitis, this makes the *Winnie* mouse an excellent model of IBD.

The Winnie model offers a unique insight into the role of a primary intestinal epithelial defect leading to a progressively escalating immune response, where there is less secretion of the mucosal immune system conditioning factor thymic stromal lymphopoietin (TSLP), an accumulation of activated mucosal dendritic cells, elevated IL-17A, and IFN-γ production by the mucosal $CD4^+$ T lymphocytes and increased expression of Th17 genes at the sites of histological inflammation. In addition, leukocytes from the intestinal draining lymph nodes secrete multiple Th1, Th2, and Th17-type cytokines in a complex pattern as in IBD (Eri et al., 2011).

A consistent finding in both UC and CD is markedly elevated intestinal IL-1, which has been demonstrated the *Winnie* mouse model. Similarly, increased Th17 gene expression and increased IL-17A production by T cells is consistent with findings in IBD (Fujino et al., 2003, Yen et al., 2006, Kobayashi et al., 2008, Rovedatti et al., 2009). Although detailed cytokine analyses from colonic explant cultures, stimulated $CD11c^+$ APCs, and activated lymph node leukocytes indicated increases in many cytokines, the most highly elevated cytokines were IL-1, IL-17A, and IL-12/23p40, offering a clue toward a skewing to a IL-23 /Th17 response in *Winnie* mice

(Eri et al., 2011) Likewise, these cytokines are important in the pathogenesis of IBD (Eri et al., 2011). Of interest anti-IL-10 neutralising antibody injected in *Winnie* mice resulted in rapid exacerbation of endoplasmic reticulum stress and increased pro-inflammatory cytokine secretion by T cells (Hasnain et al., 2013). However, IL-17A deficient T cells failed to ameliorate disease in *Winnie* mice (Wang et al., 2015b).

It is estimated that underlying infection and inflammatory reactions are linked to 25 % of all cancer cases. There are well known associations between inflammatory processes and cancer, such as IBD and colorectal cancer (Coussens and Werb, 2002). Tissue injury whether physical, chemical or infectious, triggers a sequence of events leading to inflammation. Inflammation is an important mechanism that eliminates the agent responsible for injury and initiates tissue repair by a cascade of a coordinated immune response. The inflammatory mechanism involves both innate and adaptive immunity, which is characterised by coordinated delivery of cells and soluble mediators to injured tissues (Coussens and Werb, 2002, Eiró and Vizoso, 2012).

Tumour cells produce an array of various cytokines and chemokines that attract leukocytes, including neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes (Coussens and Werb, 2002). Infiltrating leukocytes secrete cytokines, cytotoxic mediators, reactive oxygen species, serine and cysteine proteases, matrix metalloproteinases (MMPs) and membrane-perforating agents (Wahl and Kleinman, 1998, Kuper et al., 2000). As discussed in Chapter 2, animal models of CRC such as the orthotopic injection of tumour cells into the caecum provide valuable insight into mechanisms that are involved in the development and pathogenesis of CRC.

Furthermore, the interaction between programmed death receptor-1 (PD-1) and programed death ligand-1 (PD-L1) has been of great interest in recent years, since the expression of PD-L1 strongly correlates with survival of cancer patients (Blank and Mackensen, 2007). PD-1 is

expressed on activated $CD8^+$ T cells and when interacting with PD-L1 expressed on epithelial cells, antigen presenting cells and tumour cells, inhibitory signals are triggered, which results in a reduction to $CD8^+$ T cells and apoptosis. In inflammatory disorders the expression of PD-L1 is reduced which leads to the activation of T cells, therefore, PD-L1 and PD-1 are important to immune responses in both cancer and inflammatory environments.

3.2 The Gaps

1. Although comprehensive immune cell populations and their cytokine products have been characterised in *Winnie* mice, the specific immune cell populations, such as macrophages (M1/M2) and neutrophils have not been described in the colon.
2. The immune cell populations of M1/M2 macrophages and neutrophils in *Winnie* mice with colorectal cancer are not known.
3. The level of expression of PD-L1 on inflamed intestinal tissues in *Winnie* mice compared to non-inflamed tissue is not known.
4. The expression of PD-1 on activated T cells has not been studied in *Winnie* mice, with or without CRC.

3.3 Aims

1. To determine specific immune cell populations in *Winnie* mice with spontaneous chronic colitis.
2. To determine specific immune populations in an orthotopic model of colorectal cancer in *Winnie* mice with spontaneous chronic colitis.

3.4 Materials and Methods

3.4.1 Animals

C57BL/6 and *Winnie* mice aged 12-24 weeks (n = 22) were obtained from the Monash Animal Services. Animals were kept on a 12-hour light and dark cycle at approximately 22 °C with free access to food and water. Mice were allowed to acclimatise for at least one week. This study was approved by Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Code of Practise for the Care and Use of Animals for Scientific Purposes.

3.4.2 Orthotopic Cancer Model

Orthotopic colorectal cancer induction was performed as described in chapter 2. Briefly, prior to surgery, mice were anesthetised with an intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). The volume was calculated per animal body weight and the maximum volume did not exceed 200 µl. Viscotears™ was used to coat mouse eyes during surgery. The level of anaesthesia during surgery was monitored by the paw pinch response. The surgery was performed under aseptic conditions on a heat mat. Mice had their abdomen shaved and wiped with 70 % ethanol before being covered with sterile film. A small incision was made along the midline of the abdomen and the caecum was exteriorised. Cell suspension of viable murine colorectal tumour MC38 cells (1×10^6 cells) in 25 µl of Matrigel™ was injected into the caecal wall. Matrigel™ is used as a vehicle to inject the cancer cells into the caecum as it provides nutrients to the cells, while tumours are establishing. After surgery, the abdominal muscle wall was closed using polygalactin 5.0 gut sutures. Surgical silk suture was used to close the skin and the wound area was then sterilized with iodine. Mice received a subcutaneous injection of an analgesic buprenorphine (0.05 mg/kg the maximum volume did not exceed 200 µl), and were placed in cages on heated mats to

recover. Mice were closely monitored and regularly checked post-surgery. Mice were killed by cervical dislocation 3 weeks post-surgery; spleen, colon, and caecum were collected for immunohistochemistry and fluorescence-activated cell sorting (FACS) analysis.

3.4.3 Single Cell Suspension

Colon was dissected out and smooth muscle was removed leaving only the mucosa. The mucosa was cut into fine pieces and left in collagenase (0.1 mg/10mL) for 2 hours (h) at 37 °C. Every 30 minutes the mucosa and collagenase were mixed using a pipette, for 2 h total after which the colon collagenase mixture was removed and filtered. Spleen was dissected out and made into a suspension using the glass slide method and red blood cells lysed. The samples were centrifuged (450 g for 5 minutes) and resuspended in 1 mL of FACS buffer (1xphosphate buffered solution, 1 % foetal bovine serum and 0.02 % sodium azide). The cell suspension was counted using a haemocytometer and viable cells were counted using trypan blue (dilution 1/1). The viability of cell suspensions used for experiments was between 65-75 %.

3.4.4 Cell Surface and Intracellular Labelling

FACS analysis was used to enumerate and phenotype the different leukocyte subpopulations in the colon of both the *Winnie* and C57BL/6 mice. A minimum of 500,000 cells per sample were used for antibody staining. Cells were labelled with monoclonal primary antibodies at 4 °C for 20 minutes to label specific immune cell types. Cytofix/cytoperm kit was used for intracellular labelling following manufacturer's instructions (BD Bioscience cat no. 554715). Antibodies: rat anti-mouse Ly6G (clone 1A8) conjugated to FITC, rat anti-mouse Gr-1 (clone RB6-8C5) conjugated to FITC, rat anti-mouse CD206 (clone 17A2) conjugated to Alexa Fluor 488, rat anti-mouse F4/80 (clone BM8) conjugated to PE, rat anti-mouse Ly6C (clone HK1.4) conjugated to PE, rat anti-mouse CD11b (clone M1/70) conjugated to PE/Cy7, rat anti-mouse CD11c (clone N418)

conjugated to APC/Cy7, rat anti-mouse TCRβ (clone H57-597) conjugated to APC, rat anti-mouse IL-6 (clone MP5-20F3) conjugated to APC, rat anti-mouse IL-10 (clone JES5-16E3) conjugated to APC, rat anti-mouse TNFα (clone MP6- XT22) conjugated to brilliant violet 421, rat anti-mouse TGFβ (clone TW7-16B4) conjugated to brilliant violet 421 and rat anti-mouse MHC II (clone M5/114.15.2) conjugated to brilliant violet 510 (all antibodies from Biolegend, USA). To prevent non-specific Fc receptor binding cells were co-stained with rat anti-mouse CD16/CD32 hybridoma supernatant (2.4G2 clone, Developmental Studies Hybridoma Bank, USA). Samples were analysed using a BD Canto FACS analyser (BD Biosciences, USA). All data were compensated using BD FACSDIVA v6.0 and analysed in FACSDIVA.

3.4.5 Immunohistochemistry

Immunohistochemistry was performed in cross sections of the caecum and colon as described previously (Nurgali et al., 2011). Frozen tissue sections were thawed and incubated for 1 h at room temperature with 10 % normal donkey serum (Chemicon, USA) followed by an overnight incubation with a primary antibody. Primary monoclonal antibodies rat anti-mouse PD-1 (clone J43.1) (dilution 1/500), rabbit anti-mouse and PD-L1 (ab58810) (dilution 1/500) (both from Abcam, Melbourne, Australia). Secondary antibody was added followed by incubation at room temperature for 2 h. Secondary antibodies: donkey anti-rat Alexa Fluor 488 and anti-rabbit Alexa Fluor 488 (both from Jackson ImmunoResearch, Pennsylvania, USA) and slides were cover slipped with fluorescent mounting medium (DAKO, Australia).

3.4.6 Imaging

Three dimensional (z-series) images of cross sections of colon/ caecum were taken using a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan). Fluorophores were visualised using excitation filters for Alexa 594 Red (excitation wavelength 559 nm), Alexa 488 (excitation wavelength 473

nm). Z-series images were taken at step size of 1.75 µm (1600 x 1200 pixels).

3.4.7 Morphometric Analysis of Tumours in the Caecum

The caecum was dissected out and opened, the numbers of tumours were counted by eye and an average was taken for each experimental group.

3.4.8 Quantitative Analysis

FACS data presented as percentages, the percentages of T cells ($TCR\beta^+$ cells), neutrophils ($Ly6G^+/Ly6C^+$ cells) and $CD11b^+$ cells were calculated from total number of viable cells in the colon or spleen suspension and M1 ($CD206^-$ cells) and M2 ($CD206^+$ cells) macrophage percentages were calculated from the number of $CD11b^+/CD45^+/F4/80^+/MHCII^+$ cells. FACS and morphometric analysis data were analysed using Students' *t*-test. Analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California USA). All data are presented as mean ± standard error of the mean (SEM).

Images were analysed using Image J software (National Institute of Health, Bethesda, MD, USA). PD-1 and PD-L1 expression was assessed by measuring the density of PD-1 or PD-L1-immunoreactive (IR) cells per area (average of 8 areas of 500 μm^2 per animal at × 20 magnification). Image J software was employed to adjust colour images from RGB to 8 bit, after which thresholding to a consistent value was applied to obtain the percentage area of PD-1 or PD-L1-immunoreactivity. In colon tissue PD-1-IR cells were counted in cross sections within a 2-mm^2 area randomly capturing 8 images per slide.

3.5 Results

3.5.1 Immune Cell Characterisation in *Winnie* Mice Compared to Controls

3.5.1.1 Immune Cell Infiltrates in the Spleen of *Winnie* Mice are Significantly Different to C57BL/6 Control Mice

Flow cytometry was used to analyse immune cells in the spleen, representative gating strategies are shown in **Figs 3.1, 3.2** and **3.3**. Analysis of the spleen revealed significant differences between *Winnie* mice and C57BL/6 (control) mice. Pan leukocyte marker CD45 was used to define leukocytes in the colon and immune populations are stated as percentage of $CD45^+$ cells. The percentage of T cells was determined using TCRβ and was significantly increased in *Winnie* mice (32.33 ± 1.07%) when compared to control mice (12.40 ± 0.36%) *(p*<0.0001) (**Fig 3.4**). Ly6C and Ly6G were used to determine the percentage of neutrophils in the spleen. Interestingly the percentage of neutrophils in *Winnie* mice (1.73 ± 0.30%) was significantly lower when compared to control mice (4.87 ± 0.62%) (*p*<0.05) (**Fig 3.5**). Furthermore the percentage of $CD11b^+$ cells in the spleen of *Winnie* mice (9.767 ± 0.3180%) was similar to the control group (10.27 ± 1.97%) (**Fig 3.6A**). However, the percentage of M1 ($CD206^-$ cells) macrophages was significantly lower in *Winnie* mice (53.63 ± 0.088%) when compared to control mice (71.37 ± 1.27, *p*<0.001) (**Fig 3.6B**) and M2 ($CD206^+$ cells) macrophages were significantly increased in *Winnie* mice (46.20 ± 0.12%) compared to control mice (26.27 ± 0.39%, *p*<0.0001) (**Fig 3.6C**). The percentage of M1/M2 macrophages are presented as a percentage of $CD11b^+/CD45^+/F4/80^+/MHCII^+$ cells.

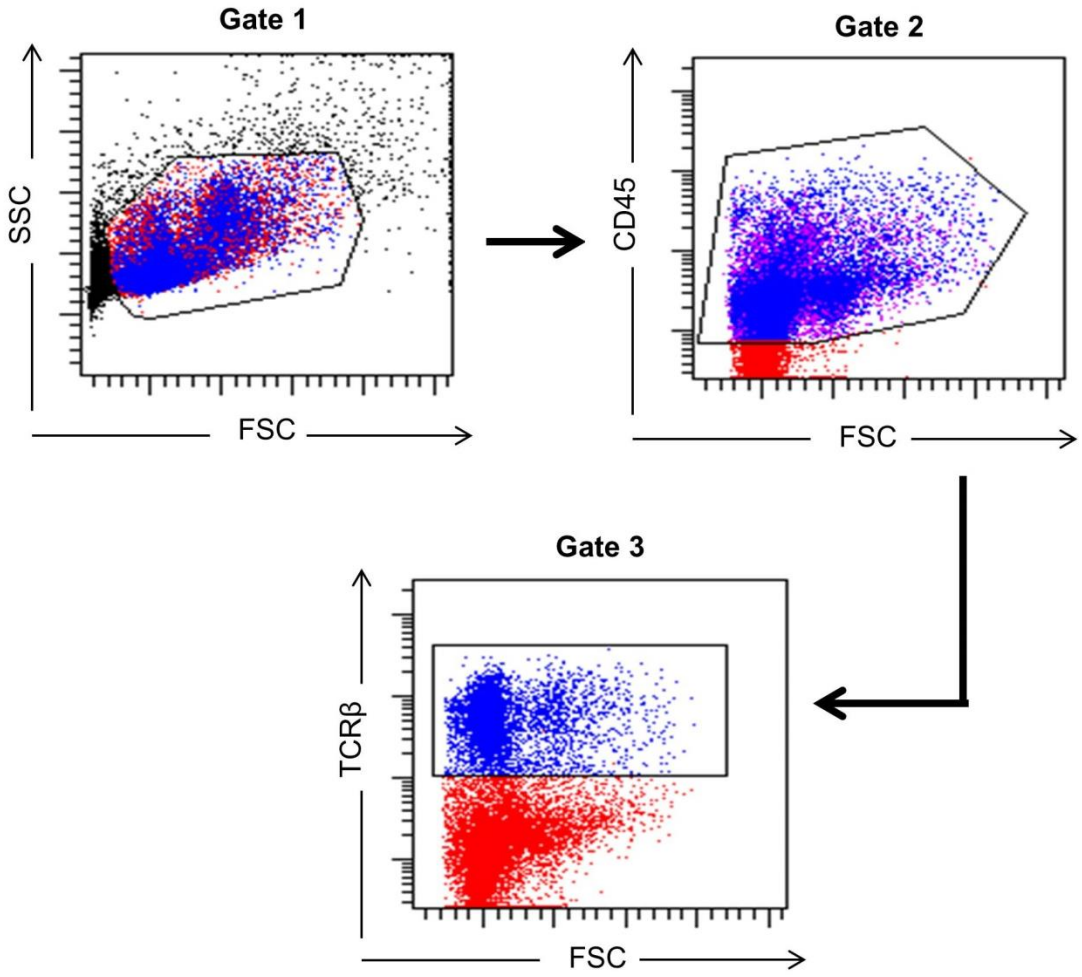


Figure 3.1: Gating Strategies used to Determine the Percentage of T cells (TCRβ$^+$ cells) in the Spleen.

Gate 1 – live cells based on FSC-SSC gating, Gate – 2 CD45$^+$ cells in the spleen and Gate 3 – TCRβ$^+$ cells in the spleen

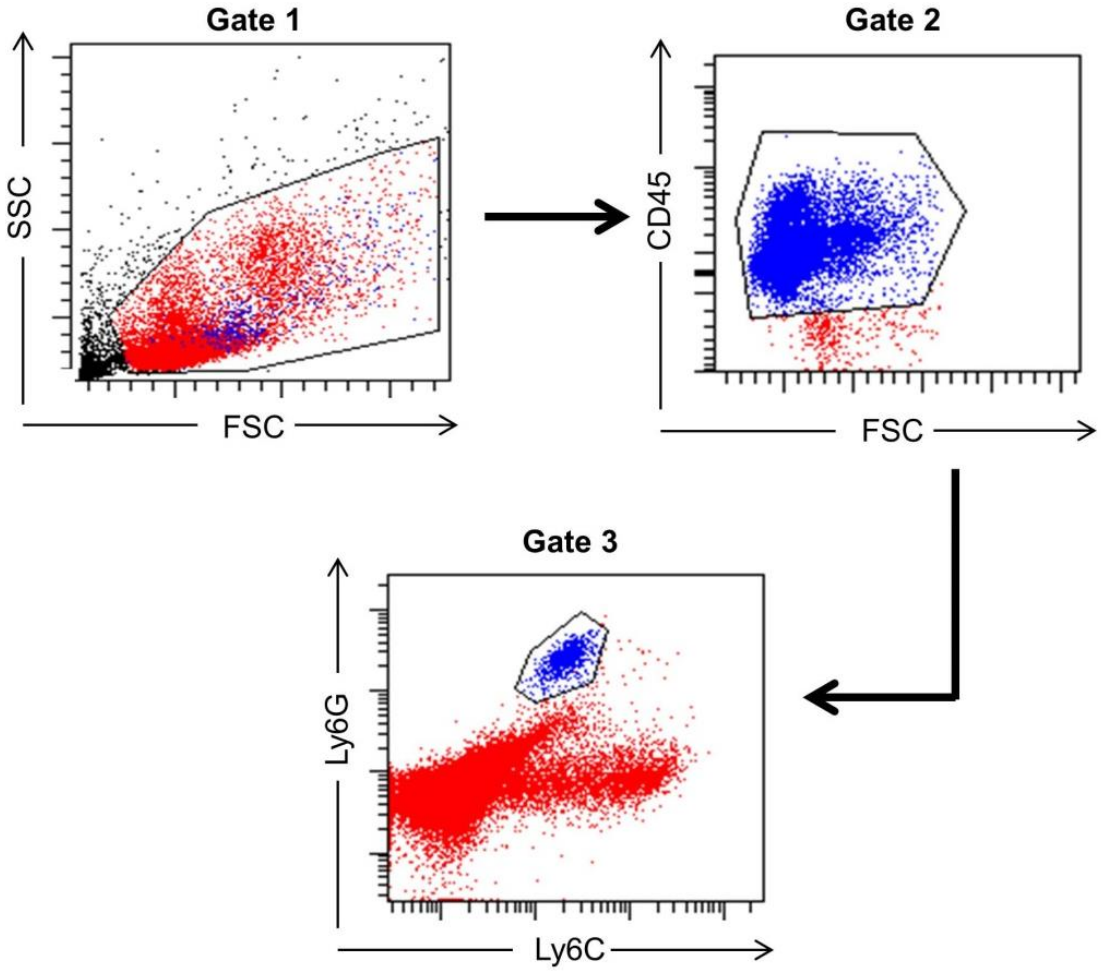


Figure 3.2: Gating Strategies used to Determine the Percentage of Neutrophils (Ly6G$^+$/Ly6C$^+$ Cells) in the Spleen.

Gate 1 – live cells based on FSC-SSC gating, Gate – 2 CD45$^+$ cells in the spleen and Gate 3 - Ly6G$^+$/Ly6C$^+$ cells (neutrophils).

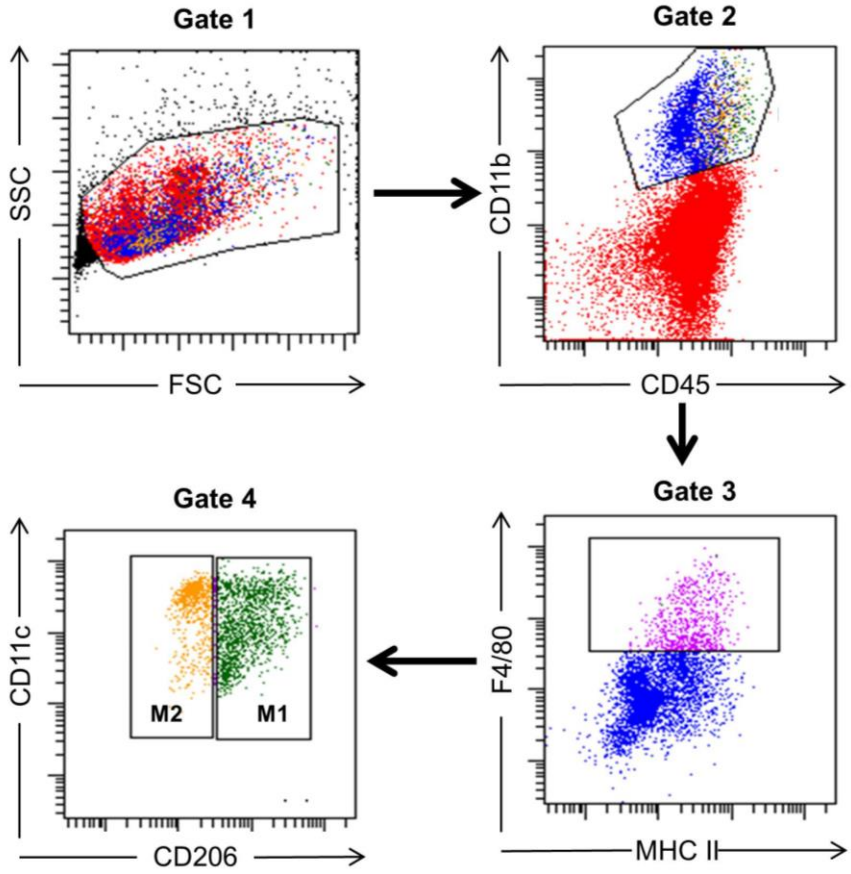


Figure 3.3: Gating Strategy used to Determine CD11b^{+}/CD45^{+} cells, M1 and M2 Macrophages in the Spleen.

Gate 1 – live cells based on FSC-SSC gating, Gate 2 – CD11b^{+}/CD45^{+} cells, Gate 3 – CD11b^{+}/CD45^{+}/F4/80^{+}/MHC II^{+} cells and Gate 4 - CD11c^{+}/CD206$^{+/-}$ cells, of gate 3 cells to distinguish M1 and M2 cells.

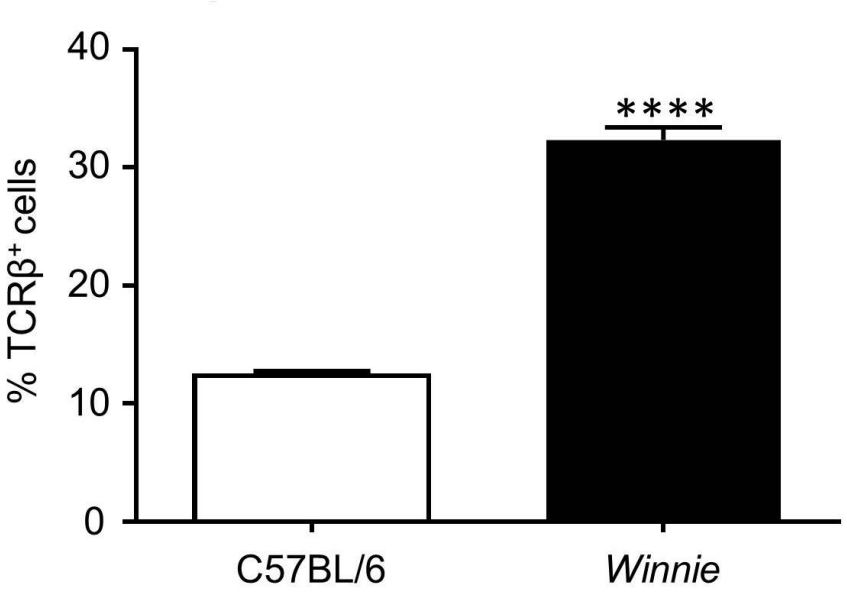


Figure 3.4: Percentage of T cells in the Spleen.

Quantitative analysis of the percentage of T cells (TCRβ$^+$ cells) in the spleen of *Winnie* mice and C57BL/6 (control) mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, ****p<0.0001, n=6 mice/group.

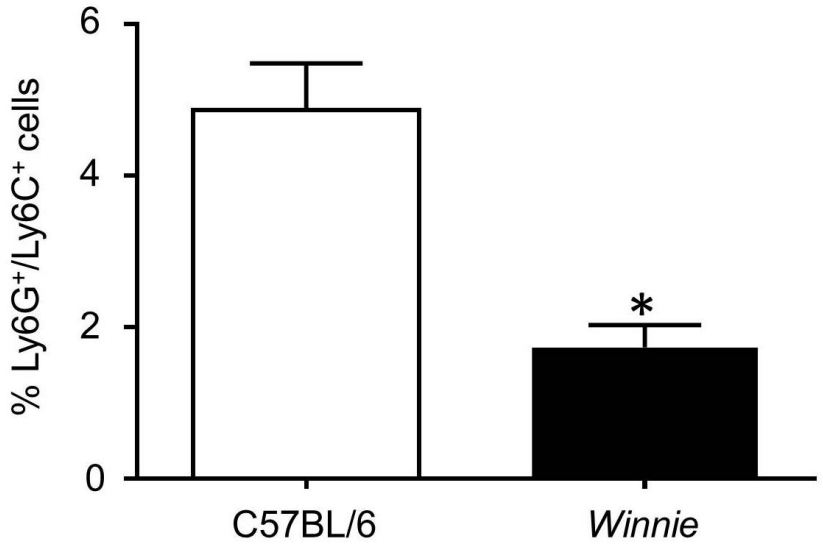


Figure 3.5: Percentage of Neutrophils in the Spleen.

Quantitative analysis of the percentage of neutrophils (Ly6G$^+$/Ly6C$^+$ cells) in the spleen of *Winnie* mice and C57BL/6 (control) mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, *p<0.05, n=6 mice/group.

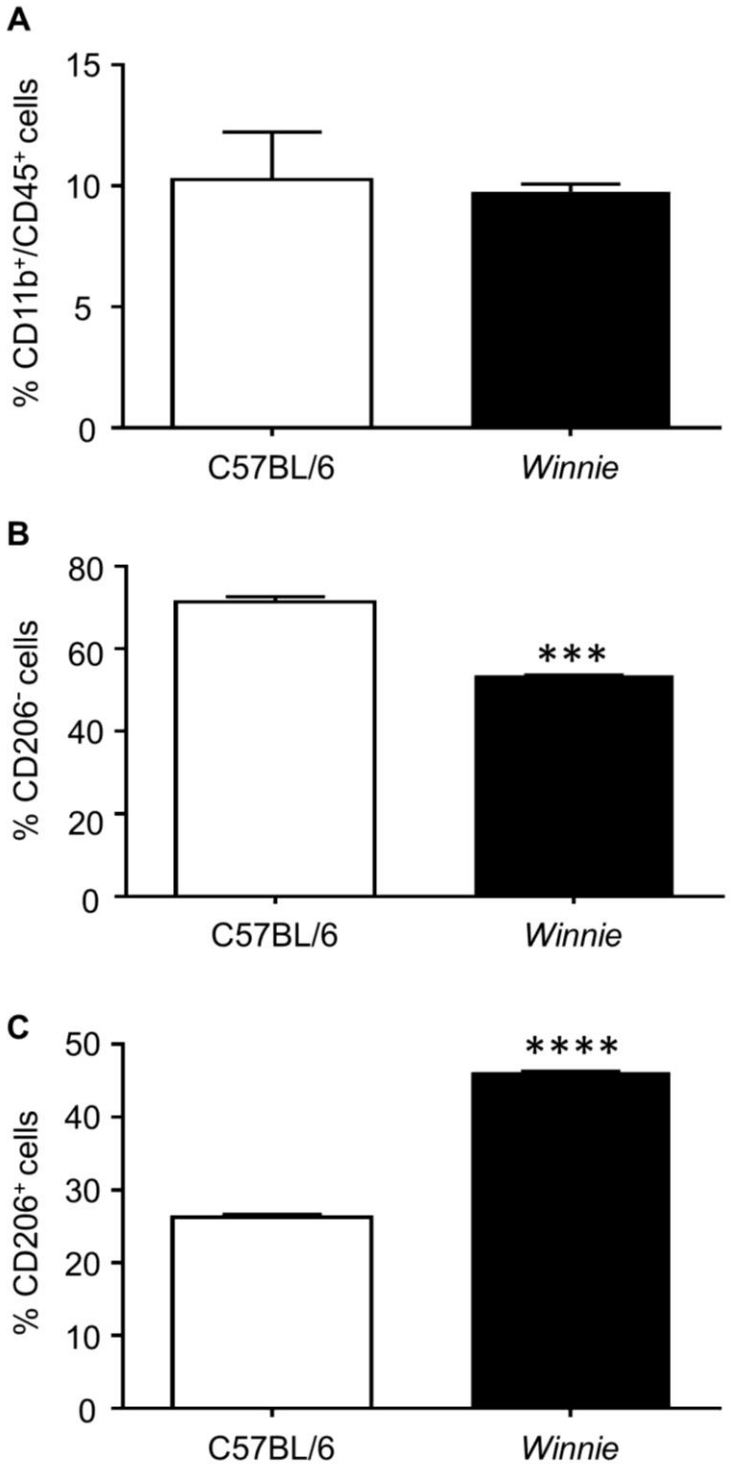


Figure 3.6: Percentage of CD11b^{+}/CD45^{+} Cells, M1 and M2 Macrophages in the Spleen.

Quantitative analysis of the percentage of CD11b^{+}/CD45^{+} cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the spleen of *Winnie* mice and C57BL/6 (control) mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, ***p<0.001, ****p<0.0001, n=6 mice/group.

3.5.1.2 Immune Cell Infiltrates in the Colon of *Winnie* Mice are Significantly Different to C57BL/6 Control Mice.

Flow cytometry was used to analyse immune cells in the colon, representative gating strategies are shown in **Figs 3.7, 3.8** and **3.9**. Analyses of colon immune cell infiltrates revealed significant differences between *Winnie* and control (C57BL/6) mice. Pan leukocyte marker CD45 was used to show total immune cell infiltration in the colon and immune populations are stated as a percentage of $CD45^+$ cells. The percentage of T cells labelled with TCRβ is significantly higher in *Winnie* mice (33.80 ± 2.94%) compared to the control mice (9.50 ± 1.61%, $p<0.01$) (**Fig 3.10**). To identify neutrophils, Ly6C and Ly6G was used; the percentage of neutrophils in *Winnie* mice (4.83 ± 0.18%) is comparable to control mice (6.75 ± 4.75%) (**Fig 3.11**). The percentage of $CD11b^+$ cells in *Winnie* mice (24.40 ± 2.77%) is significantly decreased when compared to control mice (40.37 ± 3.38%, $p<0.05$) (**Fig 3.12A**). Further, FACS analysis of colon cell infiltrates revealed the presence of M1 macrophages identified as positive for CD11b, CD45, F4/80 and negative for CD206. The percentage of M1 macrophages is significantly lower in *Winnie* mice (41.40 ± 1.20%) compared to control mice (67.20 ± 3.90%, $p<0.01$) (**Fig 3.12B**). However, the percentage of M2 macrophages ($CD206^+$ cells) in *Winnie* mice (47.17 ± 0.96%) is similar to control mice (42.55 ± 13.55%) (**Fig 3.12C**).

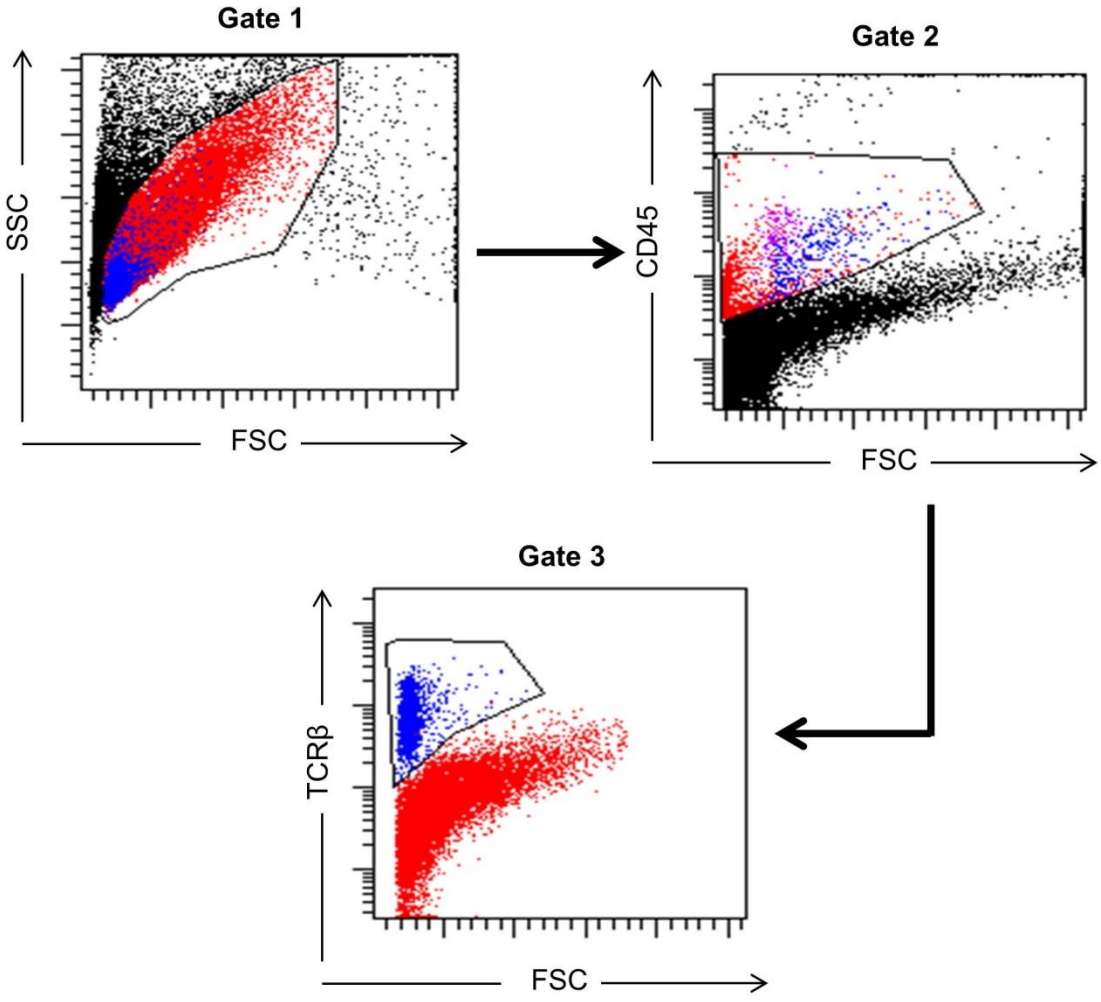


Figure 3.7: Gating Strategy used to Determine the Percentage of T cells (TCRβ^{+}cells) in the Colon.

Gate 1 – live cells based on FSC-SSC gating, Gate – 2 CD45^{+}cells in the colon and Gate 3 – TCRβ^{+}cells in the colon.

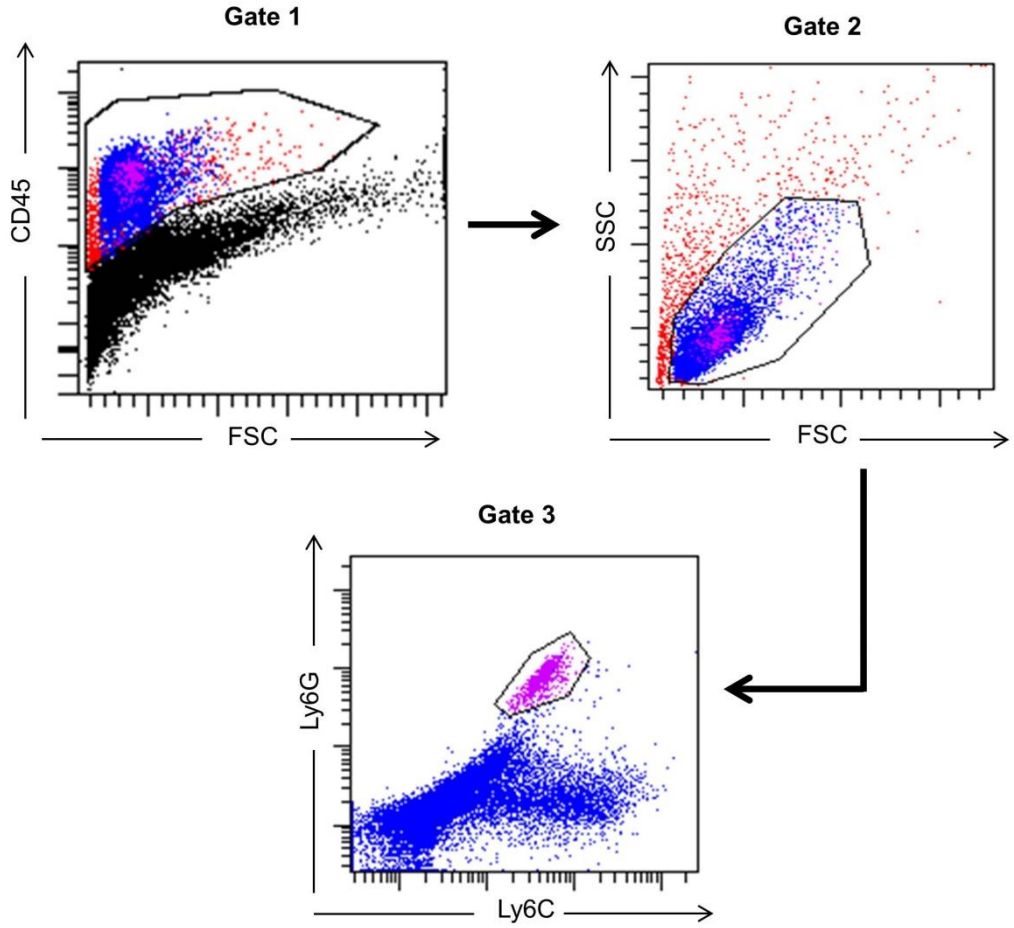


Figure 3.8: Gating Strategy used to Determine the Percentage of Neutrophils (Ly6G$^+$/Ly6C$^+$cells) in the Colon.

Gate – 1 CD45$^+$cells in the colon, Gate 2 – live cells based on FSC-SSC gating and Gate 3 - Ly6G$^+$/Ly6C$^+$cells (neutrophils).

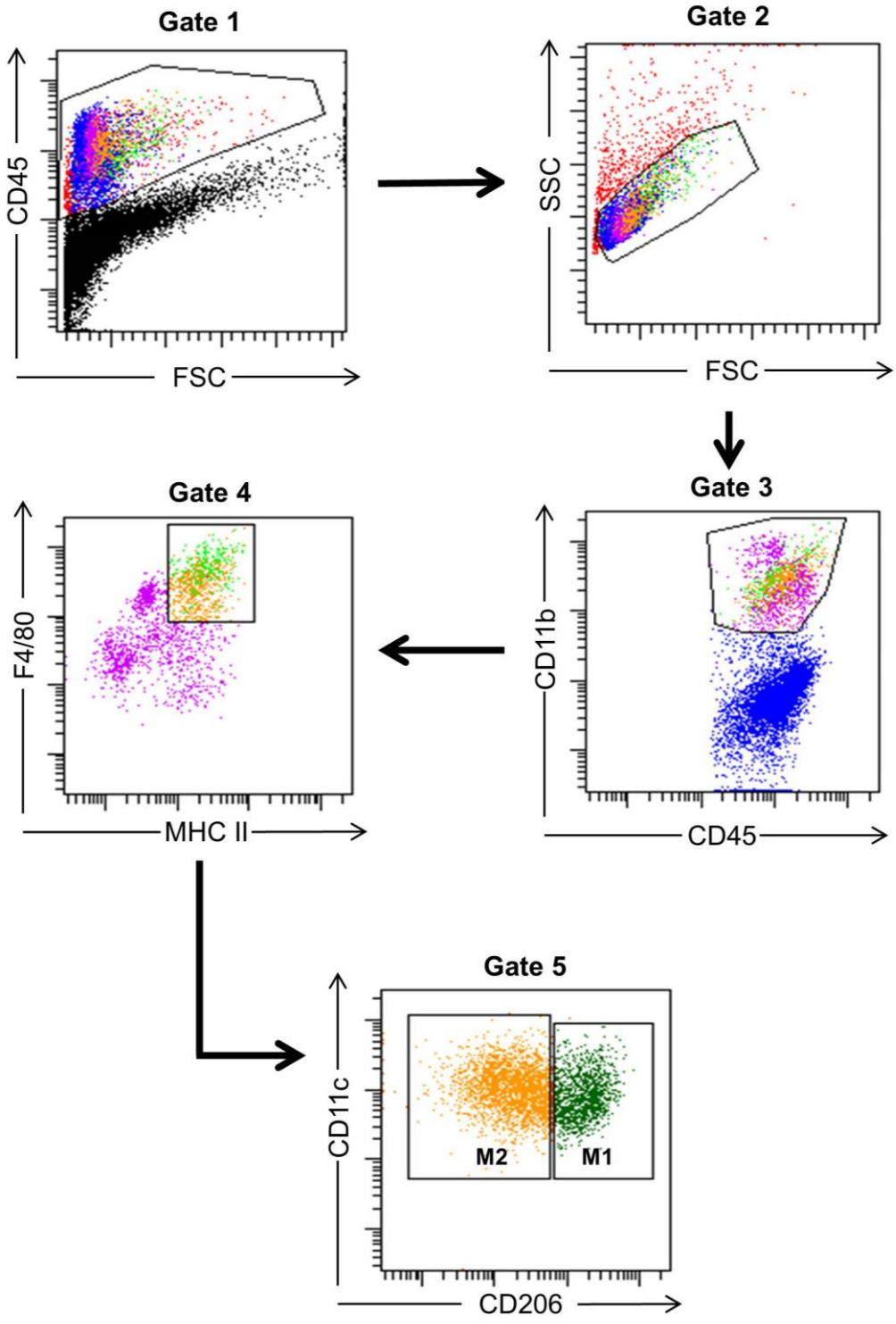


Figure 3.9: Gating Strategy used to Determine CD11b^{+}/CD45^{+} cells and M1/M2 Macrophages in the Colon.

Gate 1 – CD45^{+}cells, Gate 2 – live cells based on FSC-SSC gating, Gate 3 – CD11b^{+}/CD45^{+}cells, Gate 4 – F4/80^{+}/MHC II^{+} cells and Gate 5 - CD11c^{+}/CD206$^{+/-}$ cells.

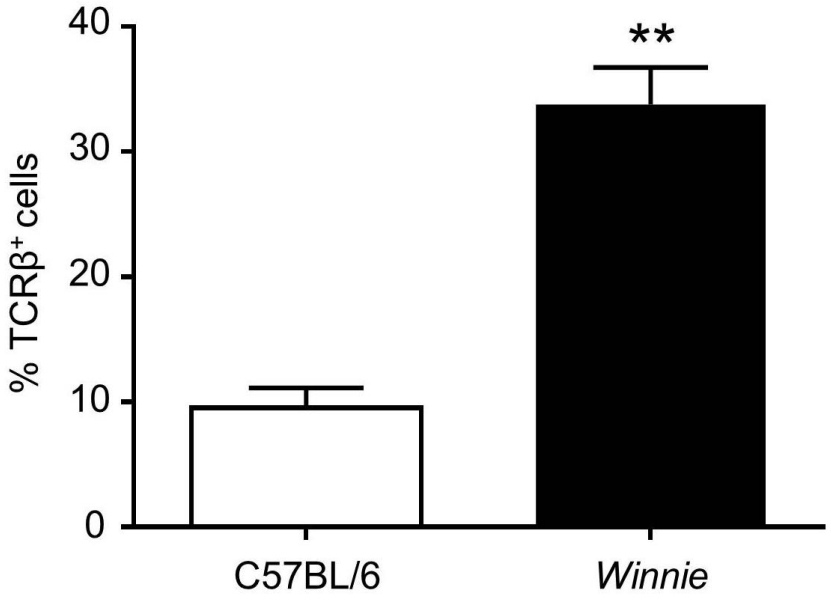


Figure 3.10: Percentage of T cells in the Colon.

Quantitative analysis of the percentage of T cells ($TCR\beta^+$cells) in the colon of *Winnie* mice and C57BL/6 (control) mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, $^{**}p<0.01$, n=6 mice/group.

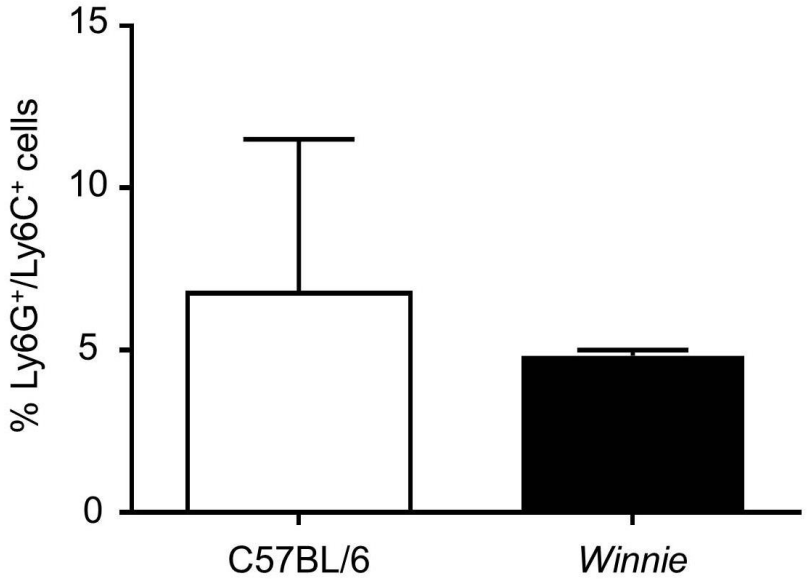


Figure 3.11: Percentage of Neutrophils in the Colon.

Quantitative analysis of the percentage of neutrophils ($Ly6G^+/Ly6C^+$cells) in the colon of *Winnie* mice and C57BL/6 (control) mice. Data represented as mean ± SEM, n=6 mice/group.

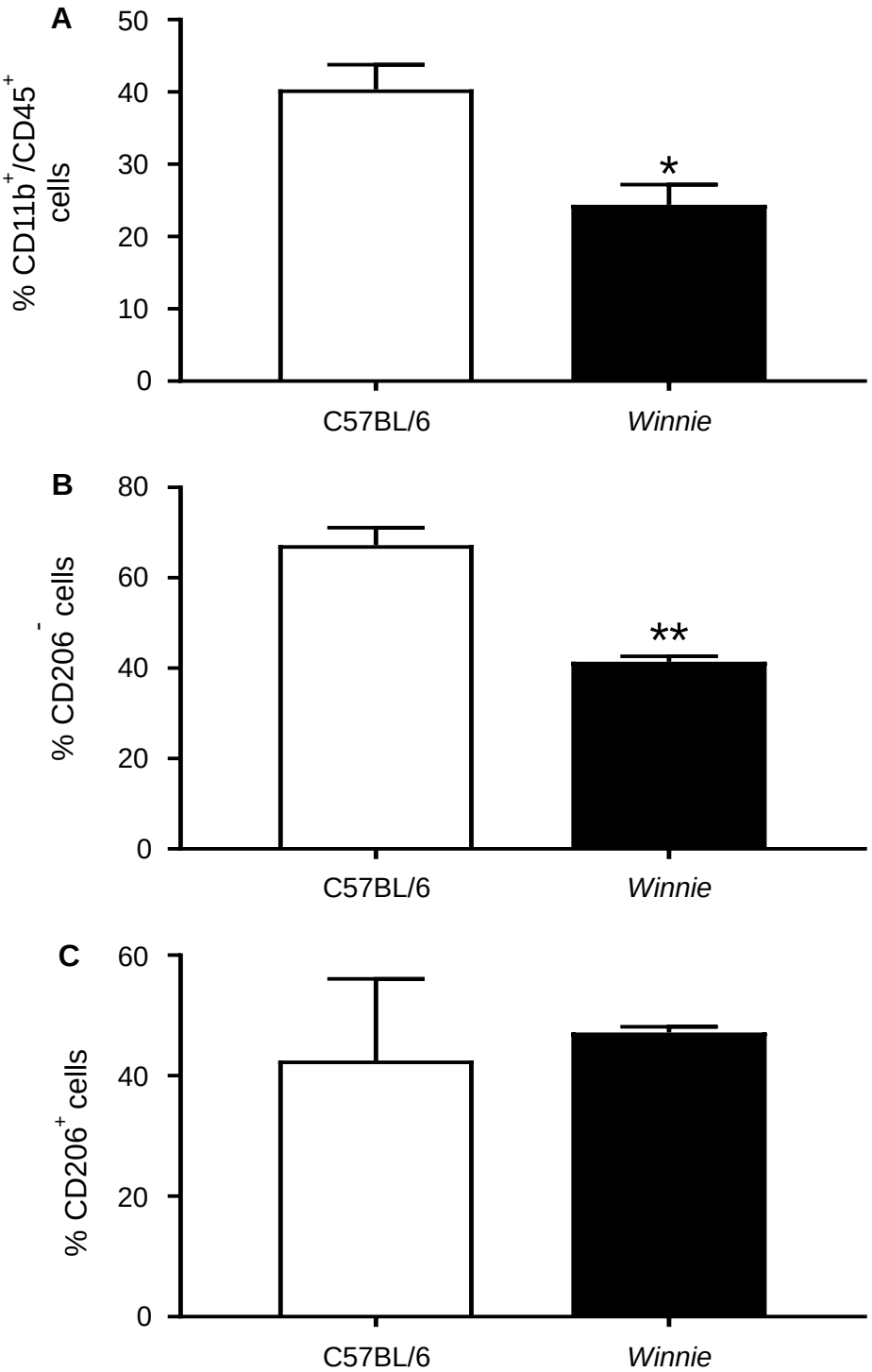


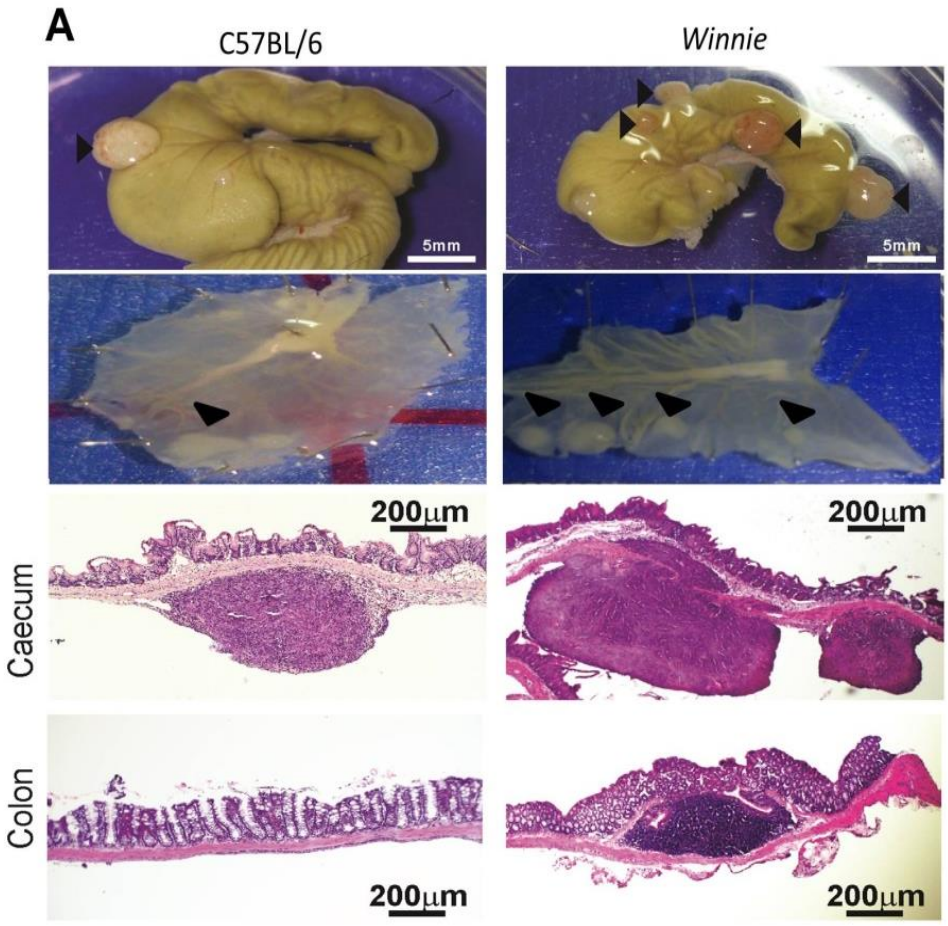
Figure 3.12: Percentage of CD11b^{-}/CD45^{+} Cells, M1 and M2 Macrophages in the Colon.

Quantitative analysis of the percentage of CD11b^{+}/CD45^{+} cells (**A**), M1 (**B**) and M2 macrophage (**C**) in the colon of *Winnie* mice and C57BL/6 (control) mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, **p*<0.05, ***p*<0.01, n=6 mice/group.

3.5.2 Characterisation of Immune Cells in an Orthotopic Cancer Model Induced in Control and *Winnie* Mice

3.5.2.1 Number of Tumours in The Caecum Following Orthotopic Injection of MC38 Cells.

MC38 cells ($1x10^6$ cells in 25 µL of matrigel™) were injected into the caecum of *Winnie* and C57BL/6 mice (control). The caecum was dissected out 3 weeks post-surgery and tumours counted. The number of tumours in the caecum was significantly increased in *Winnie* mice (*Winnie*+CRC group) (3.00 ± 0.58) when compared to the number of tumours in C57BL/6 control mice (C57BL/6+CRC group) (1.00±0.00, $p<0.01$). The volume of tumours was highly variable in *Winnie* mice (*Winnie*+CRC group) (8.39±7.06mm^3), therefore no significant differences were observed when compared to the C57BL/6 mice (C57BL/6+CRC group) (4.71±2.16mm^3) (**Fig 3.13**).



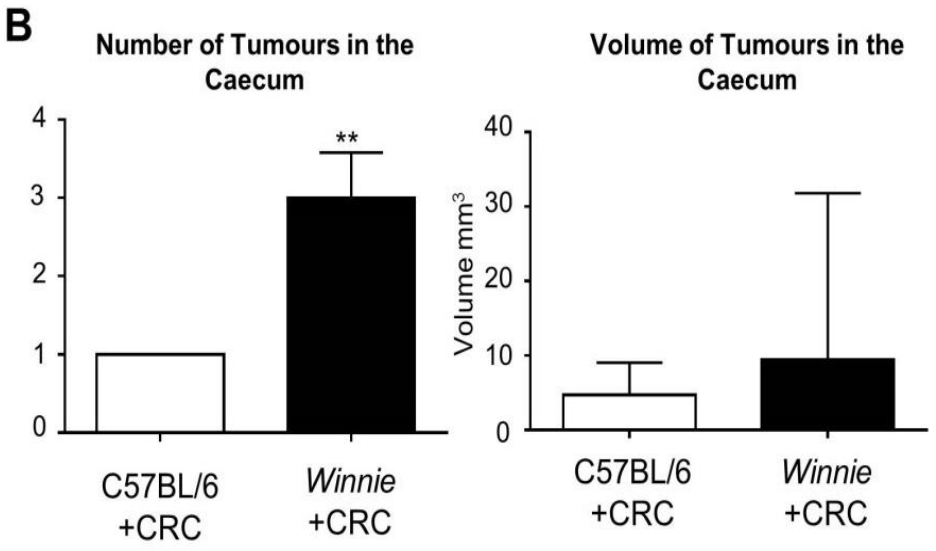


Figure 3.13: Number and Volume of Tumours in the Caecum

Images **(A)** and analysis of the number of tumours in the caecum of C57BL/6 and *Winnie* mice at 3 weeks post implantation of MC38 colorectal tumour cells **(B)**. Data represented as mean ± SEM and statistical significance is indicated by asterisk,* *p<0.01, n=5 mice/group.

3.5.2.2 Immune Cell Infiltrates in the Spleen of *Winnie* Mice are Different to Control Mice in an Orthotopic Colorectal Cancer Model

Analysis of immune cell populations in the spleen of mice with CRC showed significant differences between *Winnie* mice (*Winnie*+CRC) and control mice (C57BL/6+CRC). Pan leukocyte marker CD45 was used to define leukocytes in the colon and immune populations are stated as percentage of $CD45^+$ cells. Analysis of the spleen showed a significant increase in the percentage of T cells ($TCR\beta^+$ cells) in *Winnie*+CRC mice (25.33 ± 3.44%) compared to C57BL/6+CRC (control) mice (11.47 ± 1.99%, $p<0.05$) (**Fig 3.14**). A significant increase in percentage of neutrophils($Ly6G^+/Ly6C^+$ cells) was also noted in *Winnie*+CRC mice (2.67 ± 0.41%), when compared to C57BL/6+CRC mice (1.40 ± 0.06%, $p<0.05$) (**Fig 3.15**). However $CD11b^+$ cells in *Winnie*+CRC mice (8.13 ± 0.44%) are similar to C57BL/6+CRC mice (7.20 ± 0.49%) (**Fig 3.16A**). Further analysis also revealed no difference between *Winnie*+CRC mice and C57BL/6+CRC mice for M1 and M2 macrophages (**Figs 3.16B, C**).

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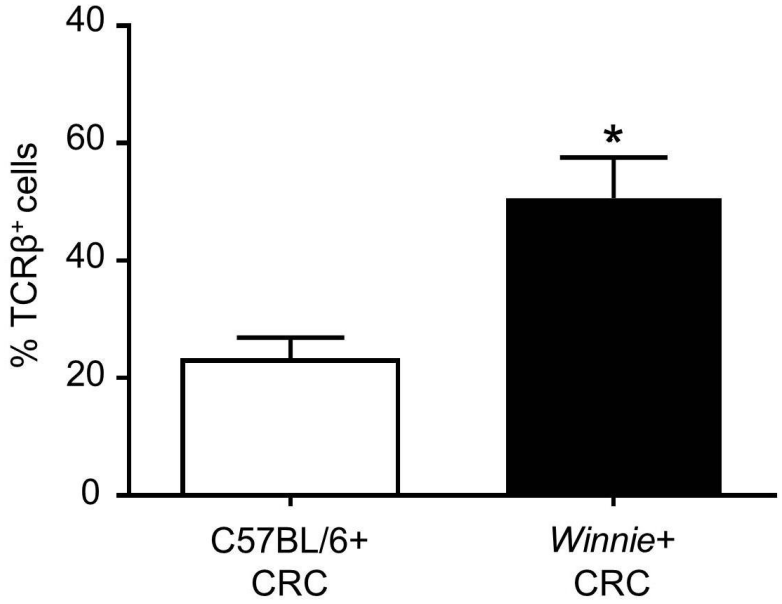

Figure 3.14: Percentage of T cells in the Spleen.

Quantitative analysis of the percentage of T cells ($TCR\beta^+$cells) in the spleen of *Winnie*+CRC mice and C57BL/6+CRC (control) mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, **p*<0.05, n=5 mice/group.

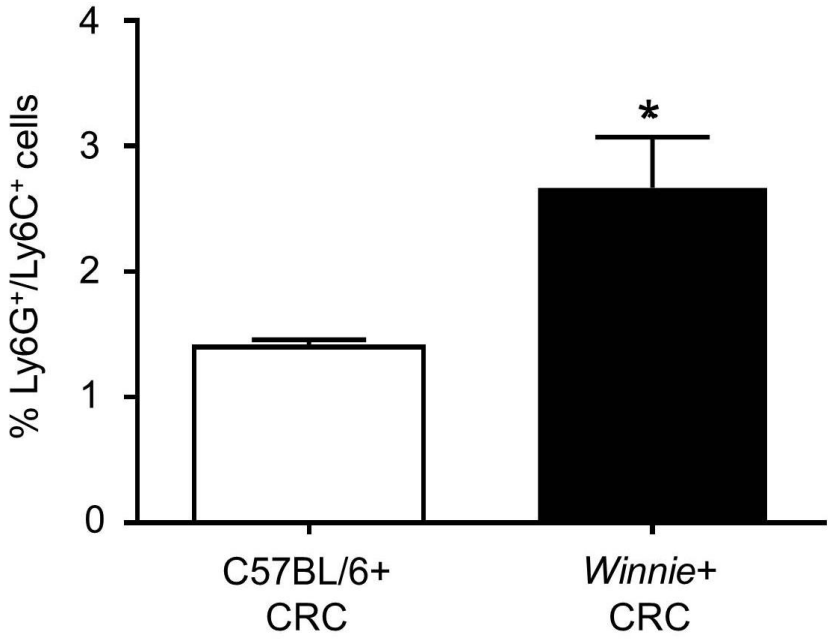

Figure 3.15: Percentage of Neutrophils in the Spleen.

Quantitative analysis of the percentage of neutrophils ($Ly6G^+/Ly6C^+$ cells) in the spleen of *Winnie*+CRC mice and C57BL/6+CRC (control) mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, **p*<0.05, n=5 mice/group.

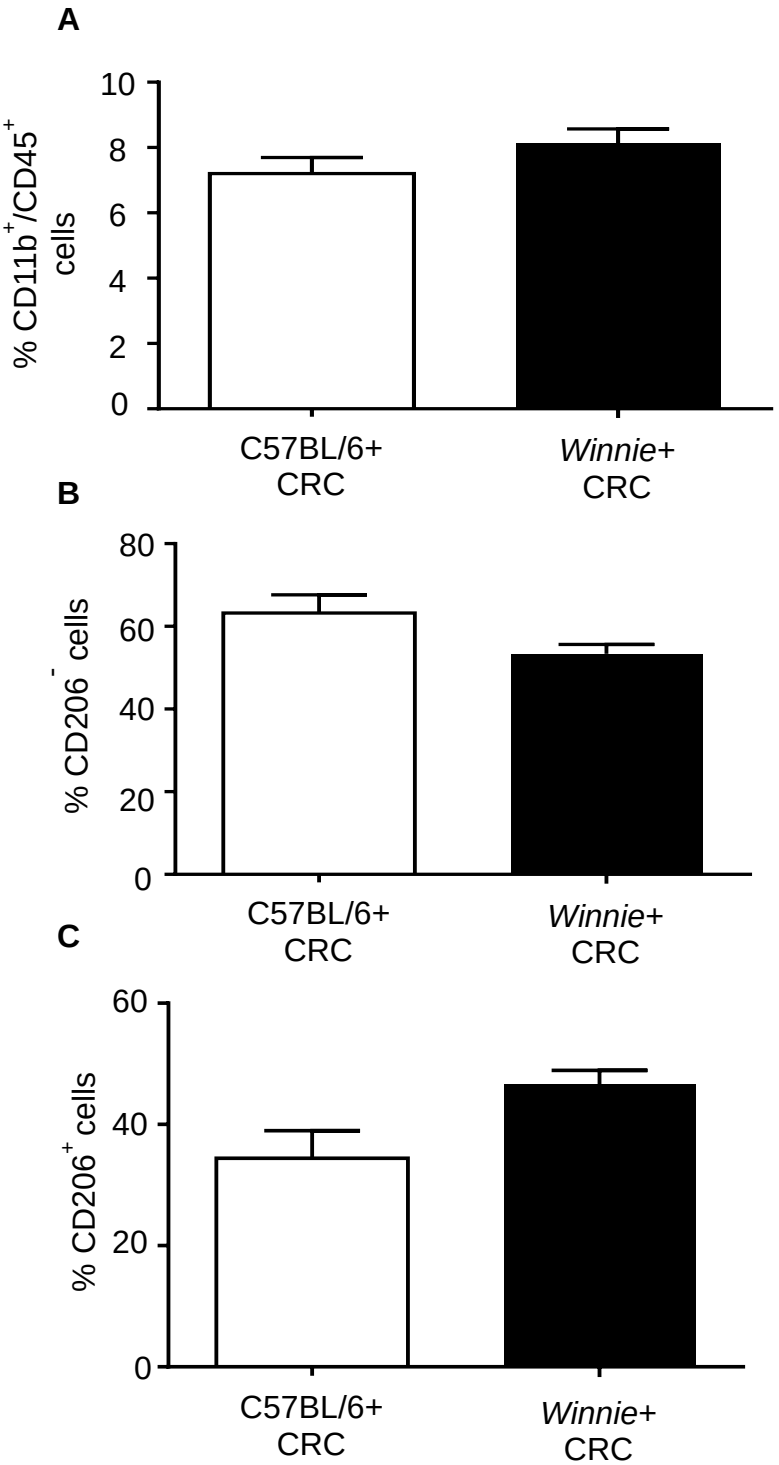

Figure 3.16: Percentage of CD11b^{+}/CD45^{+} Cells, M1 and M2 Macrophages in the Spleen.

Quantitative analysis of the percentage of CD11b^{+}/CD45^{+} cells (**A**), M1(**B**) and M2 macrophages (**C**) in the spleen of *Winnie*+CRC mice and C57BL/6+CRC (control) mice. Data represented as mean ± SEM, n=5 mice/group.

3.5.2.3 Immune Cell Populations in the Colon of *Winnie* Mice are Different to Control Mice in an Orthotopic Colorectal Cancer Model

Analysis of the colon from *Winnie* mice with CRC showed significant differences between *Winnie*+CRC mice when compared to C57BL/6+CRC (control) mice. Pan leukocyte marker CD45 was used to define leukocytes in the colon and immune populations are stated as percentage of $CD45^+$ cells. A significant increase of T cells was noted in *Winnie*+CRC mice (19.80 ± 1.95%) compared to the C57BL/6+CRC (control) mice (12.53 ± 0.39%, $p<0.05$) (**Fig 3.17**). Ly6C and Ly6G were used to enumerate neutrophils, which demonstrated that *Winnie*+CRC mice (3.50 ± 0.62%) tend to have increased percentage of neutrophils compared to C57BL/6+CRC mice (1.33 ± 1.33%) but this is not significant (**Fig 3.18**). This study also shows that in the colon of *Winnie*+CRC mice there is a higher percentage of $CD11b^+$ cells in (34.10 ± 4.86%) compared to C57BL/6+CRC mice (24.60 ± 1.55%) but this is not significant (**Fig 3.19A**). However there are increased percentage of M1 macrophages (61.23 ± 3.00% vs 46.47 ± 0.84%) and lower percentage of M2 macrophages (35.67 ± 2.95% vs 50.23 ± 1.04%) in *Winnie*+CRC mice compared to C57BL/6+CRC mice ($p<0.01$) respectively (**Figs 3.19 B, C**).

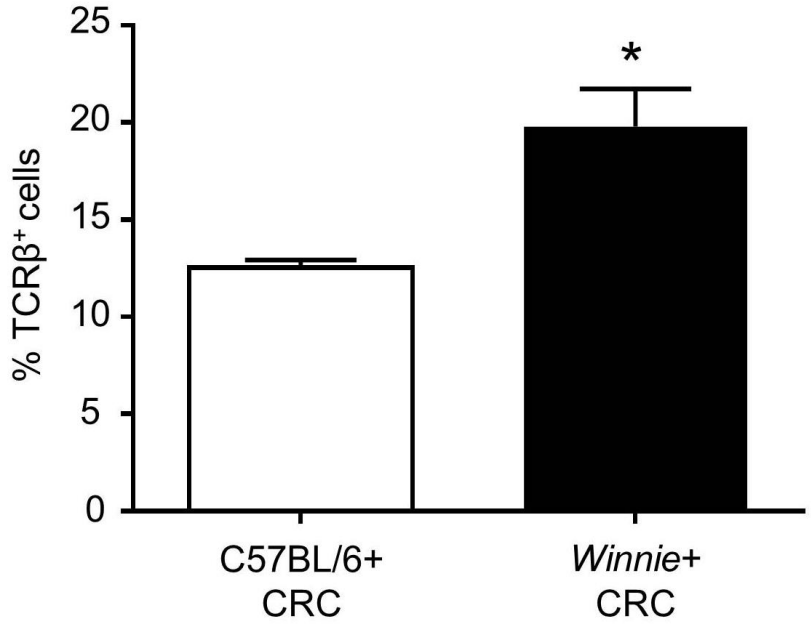

Figure 3.17: Percentage of T cells in the Colon.

Quantitative analysis of the percentage of T cells ($TCR\beta^+$ cells) in the colon of *Winnie*+CRC mice and C57BL/6+CRC (control) mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, *p<0.05, n=5 mice/group.

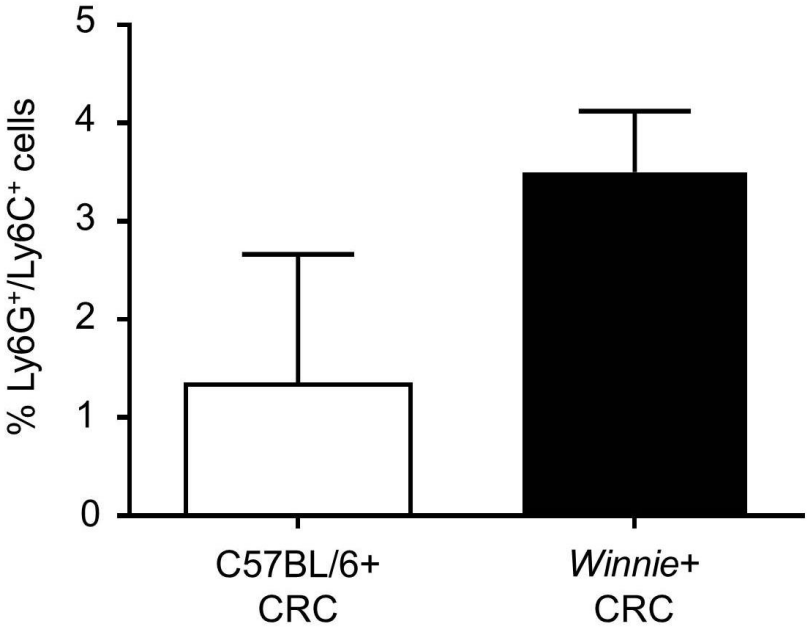

Figure 3.18: Percentage of Neutrophils in the Colon.

Quantitative analysis of the percentage of neutrophils ($Ly6G^+/Ly6C^+$ cells) in the colon of *Winnie*+CRC mice and C57BL/6+CRC (control) mice. Data represented as mean ± SEM, n=5 mice/group.

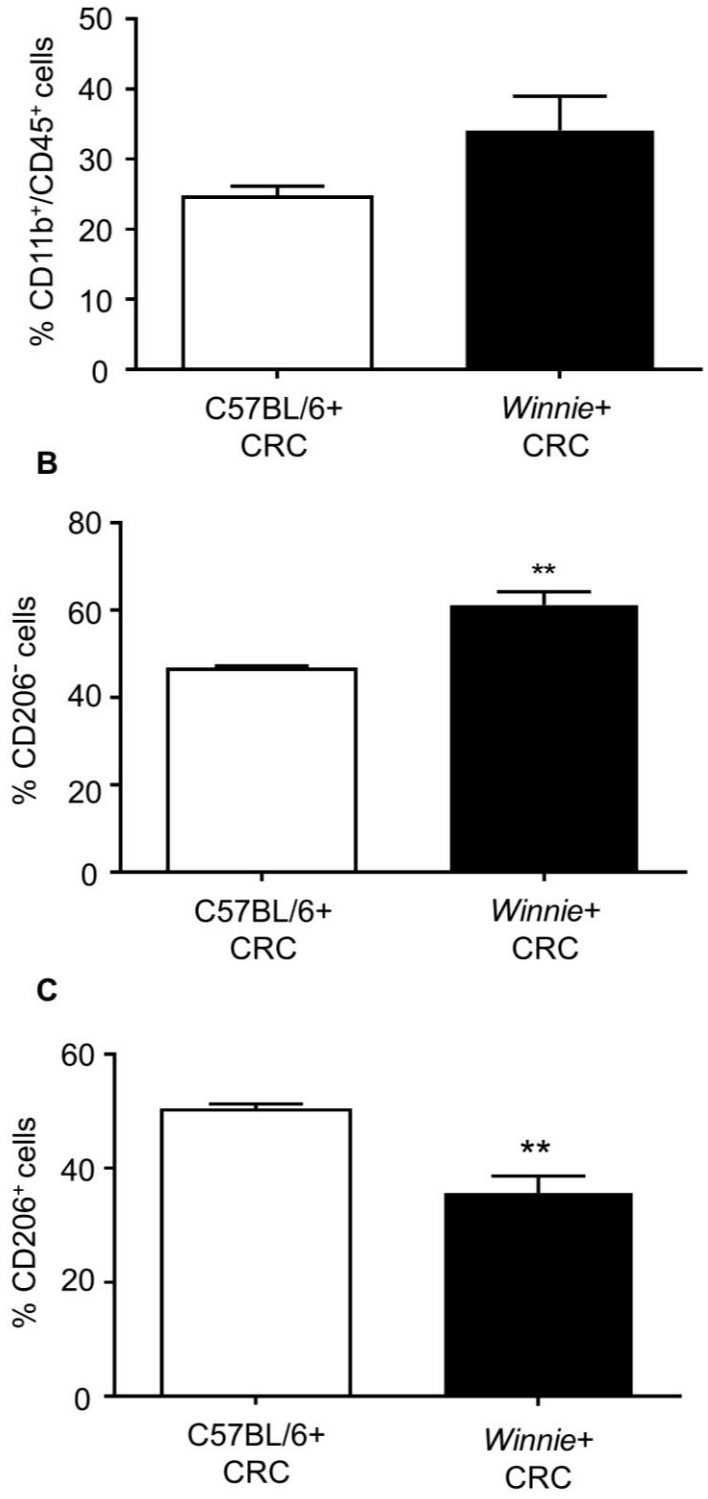

Figure 3.19: Percentage of CD11b$^+$/CD45$^+$ Cells, M1 and M2 Macrophages in the Colon.

Quantitative analysis of the percentage of CD11b$^+$/CD45$^+$ cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the colon of *Winnie*+CRC mice and C57BL/6+CRC (control) mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, ***p*<0.01, n=5 mice/group.

3.5.2.4 Immunohistochemical Analysis of PD-1 and PD-L1 in a Model of Cancer and Inflammation

In inflammatory disorders it has been noted that the expression of PD-L1 is reduced, leading to the activation of T cells ($CD8^+$), however in the tumour microenvironment the opposite is true. PD-L1 is expressed by tumour cells which upon binding of PD-1 expressed by activated T cells lead to inhibitory signals being triggered and a reduction in $CD8^+$ T cells and apoptosis. Therefore, the PD-1/PD-L1 interaction is an important regulator of the immune system in cancer and IBD. Tumour and colon tissues from *Winnie*+CRC mice were labelled for PD-1 and PD-L1 to determine the overall expression compared to C57BL/6+CRC mice. The expression of PD-L1 in the tumours of *Winnie*+CRC mice is significantly increased compared to C57BL/6+CRC mice (**Fig 3.20**). In addition, the expression of PD-1 within the tumour is significantly increased in *Winnie*+CRC mice compared the C57BL/6+CRC mice (**Fig 3.21**) and PD-1 expression in the colon of Winnie+CRC mice is also significantly increased compared to C57BL/6+CRC mice (**Fig 3.22**)

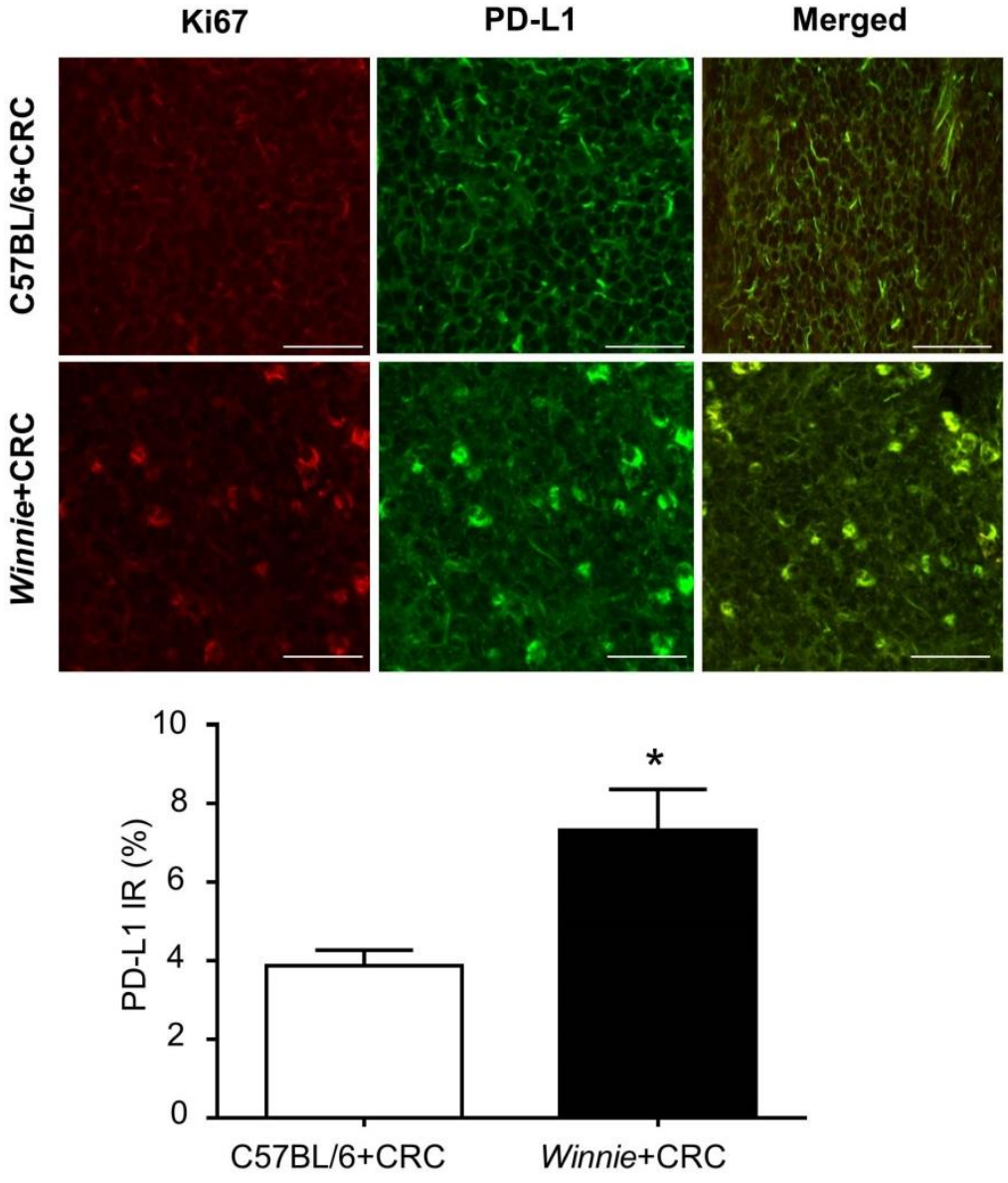

Figure 3.20: Immunohistological labelling of PD-L1 in Tumour Cross Sections. Immunohistological labelling of PD-L1 (Green) and Ki67 (Red) in the tumour cross sections in the caecum and quantitative analysis of PD-L1 in tumour sections found in the caecum of mice with CRC, Data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm.

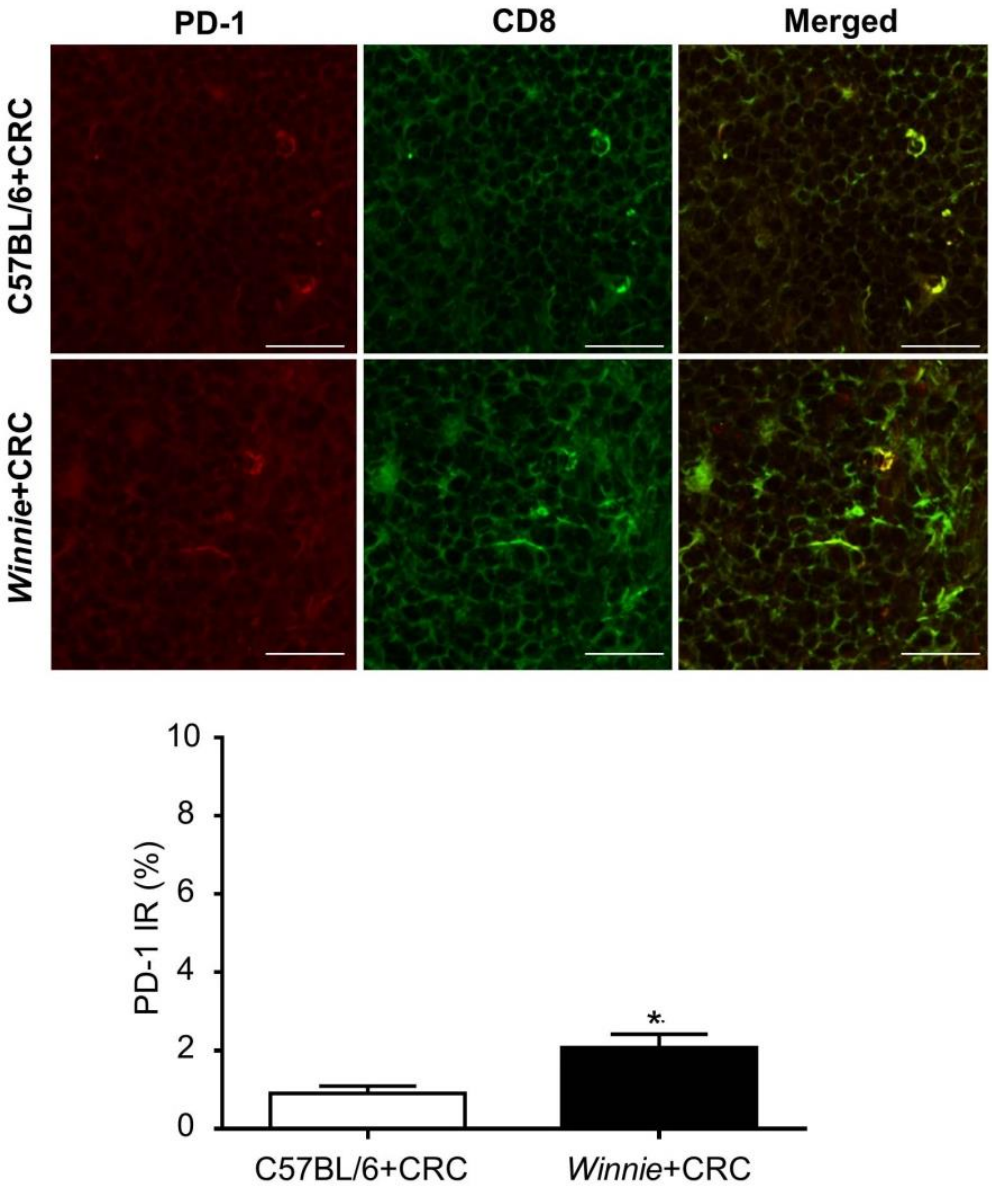

Figure 3.21: Immunohistological labelling of PD-L1 are in Tumor Cross Sections. Immunohistological labelling of CD8 (Green) and PD-1 (Red) in the tumor cross sections and in the caecum and quantitative analysis of PD-1 in tumor sections found in thecaecum of mice with CRC, Data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm

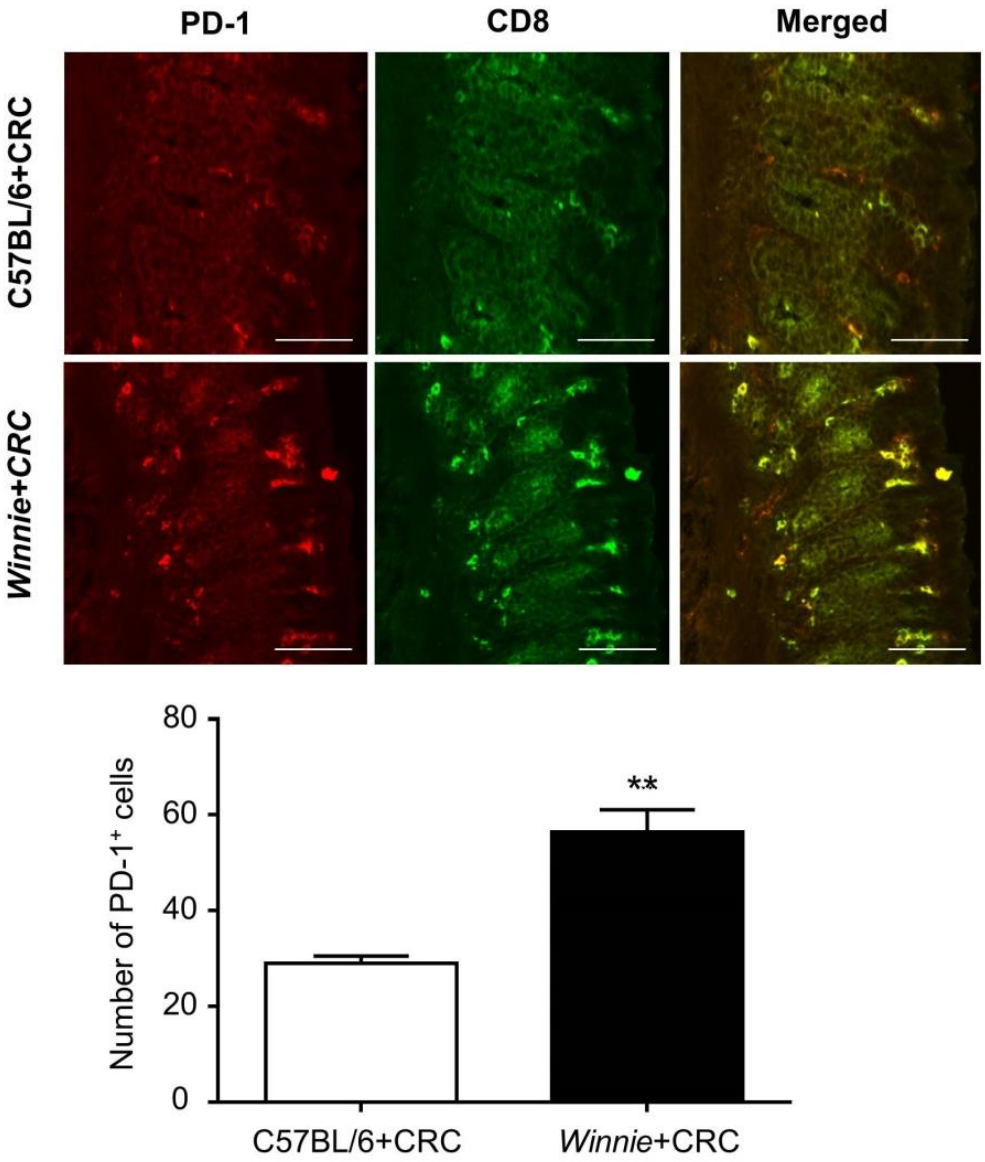

Figure 3.22: Immunohistological labelling of PD-1 in Colon Cross Sections. Immunohistological labelling of CD8 (Green) and PD-1 (Red) in the colon cross sections and quantitative analysis of PD-L1 in colon cross sections, Data represented as mean ± SEM and statistical significance is indicated by asterisk, $^{**}p<0.01$, n=5 mice/group. Scale bar is 100 µm

Table 3.1: Summary of immune cell populations in *Winnie* compared to C57BL/6 mice with or without colorectal cancer

	***Winnie* compared to C57BL/6**		***Winnie*+CRC compared to C57BL/6+CRC**		**Published Results**					**Reference**
	Spleen	**Colon**	**Spleen**	**Colon**	**Lamina propria mononuclear cells**	**Proximal colon**	**Distal colon**	**Spleen**	**Mesenteric lymph nodes**	
T cells	↑	↑	↑	↑	↑ CD4/↓CD8			↑ CD4	↔ CD4	(Eri et al., 2011)
Neutrophils	↓	↔	↑	↔						
CD11b^{+} cells	↔	↓	↔	↔	↔					(Eri et al., 2011)
M1 macrophages	↓	↓	↔	↑						
M2 macrophages	↑	↔	↔	↓						
PD-1				↑ (colon & caecum)						
PD-L1				↑ (caecum)						
B cells					↑					(Eri et al., 2011)
CD11c$^{=}$ cells					↑					(Eri et al., 2011)
Cytokines, chemokines and growth factors					↑IL-6, ↑IL-12/23p40, ↑MIP-1α, ↑ RANTES	↑ IL-1β, ↑ IL-12p40, ↓ TSLP, ↑ IL-17A, ↔ IL-17F, ↑ Tgfb1, ↔ Ccr6	↑ IL-1β, ↑ IL-12p40, ↓ TSLP, ↑ IL-17A, ↑ IL-17F, ↑ Tgfb1, ↑ Ccr6		↑TNFα. ↑IL-13, ↑IFNγ, ↑IL-17	(Eri et al., 2011, Hasnain et al., 2013)

Table 3.1 legend - ↑ Increase, ↓ Decrease, ↔ No change.

3.6 Discussion

This study investigated the immune cell populations in an animal model of spontaneous chronic colitis, closely related to human UC.

IBD in humans is associated with defects in both innate and adaptive immunity (Brown and Mayer, 2007). In humans, IBD involves defects in the mucosal barrier, in some individuals the overt inflammatory response may be an appropriate immune response to an excessively permeable intestinal barrier (Brown and Mayer, 2007). This barrier defect may be inherent or induced by intercurrent infection or nonsteroidal anti-inflammatory drugs (Brown and Mayer, 2007). Greater intestinal permeability has been reported in humans with IBD, this permeability is also seen in IL-10 deficient mice and in *Winnie* mice (Brown and Mayer, 2007, Eri et al., 2011). A number of T cell defects are associated with IBD. In fact, in IBD patients, excessive T helper (Th) 1 and Th17 responses are noted, in addition to defective T cell responses including faulty apoptosis, regulatory defects and function (Boirivant et al., 1999, Ina et al., 1999, Brown and Mayer, 2007). Similarly, *Winnie* mice have both excessive Th1 and Th17 responses and deficient anti-inflammatory responses. Herein, it was shown that in both the spleen and colon there is a significant increase in the percentage of T cells compared to controls.

The innate immune system is also part of the pathology of IBD. The importance of the innate immune system was highlighted by the role of the CARD15 gene, which is preferentially expressed by macrophages and dendritic cells (Brown and Mayer, 2007). Macrophages and dendritic cells play a major role in the development of Th-1 immune responses (Parronchi et al., 1997, Brown and Mayer, 2007). In addition, neutrophils play a role in IBD; they secrete nonspecific inflammatory mediators including reactive oxygen intermediates and neutrophil-mediated tissue damage also appears to be exacerbated in IBD (Nixon and Riddell, 1990, Carlson et al., 2002). Likewise, as shown in the spleen of *Winnie* mice the percentage neutrophils was decreased, however, there was an increase in the percentage of T cells. This shows a systemic response to the inflammatory status in the colon of these mice and indicates movement to the site of inflammation. In *Winnie* mice there is an increase in

the proportion of $CD11c^+$ cells, B cells and no change in $CD4^+$ T cells, natural killer cells and natural killer T cells immune populations *Winnie* mice show a similar percentage of $Ly6G^+/Ly6C^+$ cells (neutrophils) in the colon when compared to controls, however further investigation is needed to identify the cytokines secreted by neutrophils in the colon from *Winnie* mice.

Interestingly in Winnie mice there are fewer M1 macrophages but the percentage of M2 macrophages is similar to that of the C57BL/6 control mice. M1 macrophages participate in the inflammatory reaction by secreting pro-inflammatory cytokines with multiple downstream targets and effects, such as IL-23 a cytokine that promotes the expansion/maintenance of Th17 cells and also reduces IL-10 production (Hue et al., 2006, Yen et al., 2006, Elson et al., 2007, Zhu et al., 2014). M2 macrophages down regulate Th1 and Th17 responses by increasing $CD4^+$ $Foxp3^+$ Tregs in inflamed tissue (Sica and Bronte, 2007, Zhu et al., 2014). Therefore an increase in M1 macrophages is consistent with previous findings that show *Winnie* mice have an increase in Th17 responses, these findings also show that the greatest activation of Th17 responses was in the distal colon, where the maximal histological damage is seen (Eri et al., 2011).

It is well known that chronic inflammation such as that seen in IBD is a risk factor in developing colorectal cancer. Inflammation plays an important role in cancer progression; tumour cells can produce cytokines and growth factors to promote tumour growth (Coussens and Werb, 2002). The tumour microenvironment contains innate immune cells including macrophages, neutrophils, dendritic cells and adaptive immune cells including T and B cells (Mantovani et al., 2008, Grivennikov, 2013). These cells communicate with each other by means of direct contact or cytokine and chemokine secretion and act in an autocrine and paracrine manner to control tumour growth.

Colitis associated tumours are infiltrated by an array of immune cells of both the innate (including macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells) and adaptive immune systems (T and B cells) (Sica et al., 2006). T cells for instance are required for inflammation, tumour progression, as well as, anticancer activity (Terzić et al.,

2010). In colitis associated cancer, the immune system seems to have a mostly pro-tumourigenic role (Eiró and Vizoso, 2012, Grivennikov, 2013). In the orthoptopic colorectal cancer model induced in *Winnie* mice with chronic colitis the number of tumours and their volume is higher compared to C57BL/6 mice with colorectal cancer. This demonstrated that in an inflammatory milieu, such as spontaneous chronic colitis (*Winnie* mice) colorectal tumours grow more rapidly. An increase in macrophages and neutrophils is associated with poor prognosis in colorectal cancer (Terzić et al., 2010). Macrophages and neutrophils produce cytokines and growth factors that promote tumour growth and angiogenesis (Terzić et al., 2010). An increase in the percentage of M1 macrophages shows the immune system is reacting to the tumour growth as M1 macrophages have a Th1 phenotype and have an anti-tumour effect and the decrease in M2 macrophages also shows an anti-cancer effect as M2 macrophages have a Th2 phenotype and can promote tumour growth (Sica et al., 2006). Furthermore, the ensuing immune populations show a pro-inflammatory phenotype with increased T cells and neutrophils. This study also demonstrated an increase in T cells which can exert both tumour suppressive and tumour promoting properties as determined by their effector functions (Smyth et al., 2006, Langowski et al., 2007, DeNardo et al., 2009). Many T cell subsets found in solid tumours are involved in tumour promotion, progression, or metastasis by producing cytokines and growth factors in that help promote tumour growth.

Subtypes of T cells, cytotoxic $CD8^+$ T cells express PD-1 receptors, which play a pivotal role in the ability of tumour cells to evade the host immune system. PD-L1 is increased in tumours which leads to the apoptosis of PD-1 ($CD8^+$) T cells (Fife and Pauken, 2011, McDermott and Atkins, 2013). However in inflammatory environments this is reversed and PD-L1 is decreased which leads to an increase in PD-1, . *Winnie* mice have higher levels of PD-1 expression in colonic tissue further suggestive of an inflammatory response. However in the tumour tissue an increase in both PD-L1 and PD-1 is seen in *Winnie* mice indicative of the tumour evading the host immune system but also an inflammatory response.

3.7 Conclusion

This study shows that the *Winnie* mouse is a viable model of spontaneous inflammation similar to what is seen in human IBD. *Winnie* mice have increased percentage of T cells and decreased M1 macrophages in both spleen and colon; this is consistent with emerging findings that IBD has a Th17phenotype. *Winnie* mice injected with MC38 colorectal cancer cells showed an increase in the percentage of M1 macrophages and a decrease in the percentage of M2 macrophages which shows an anti-cancer effect and furthermore, *Winnie* mice have an increase in the percentage of T cells, which can exert both an anti- and pro-tumourigenic effect. In fact, some T cell subsets isolated from solid tumours such as, colorectal cancer are involved in tumour promotion, progression, or metastasis by producing cytokines and growth factors that aid in tumour growth. This study shows that in *Winnie* mice the immune system promotes a response similar to what is noted in humans with IBD, and, *Winnie* mice with cancer show both pro- and anti-tumour effects in the immune system.

Chapter Four: Effect of Mesenchymal Stem Cells on the Immune System in Animal Models of Inflammatory Bowel Disease and Colorectal Cancer

4.1 Background

Mesenchymal stem cells (MSCs) are multipotent stem cells that can be derived from many adult tissues including bone marrow and adipose tissue (Mosna et al., 2010). In recent years, MSCs have gained significant attention due to their therapeutic potential. MSCs are currently being tested in human trials against numerous disorders including arthritis, multiple sclerosis and inflammatory bowel disease (IBD) (González et al., 2009, Duijvestein et al., 2010, Freedman et al., 2010, Ciccocioppo et al., 2011, Connick et al., 2012, Lee et al., 2013, Forbes et al., 2014).

MSCs have unique biological characteristics that allow them to escape immune rejection and induce endogenous repair mechanisms (Uccelli et al., 2008). Indeed, in clinical trials administration of MSCs is safe and a feasible treatment option for complex perianal fistulas associated with Crohn's disease (CD) (Duijvestein et al., 2010, Ciccocioppo et al., 2011). MSCs are thought to be derived from fibroblasts, which are important in wound healing, regeneration and involved in the pathophysiology of disease. MSCs play pivotal roles in not only maintenance but also regeneration and replacement of connective and damaged tissues following inflammation, injury and destruction (Liechty et al., 2000, Burdon et al., 2010). These properties make MSCs a viable therapeutic option in managing IBD.

MSCs have shown promise in both animal models of IBD and in human trials (Duijvestein et al., 2010, Ciccocioppo et al., 2011, Forbes et al., 2014, Gonzalez-Rey and Delgado, 2014, Robinson et al., 2014). In a guinea-pig model of TNBS-induced colonic inflammation, human bone marrow MSCs applied locally to the site of inflammation persevered colonic architecture, reduced immune infiltrate in the colon, prevented neuronal loss and axonal damage in enteric neurons and alleviated inflammation-induced changes to gastrointestinal motility 7 days after treatment (Robinson et al., 2014). In a rat model of TNBS induced colonic inflammation, MSCs injected submucosally had reduced histopathological severity of colitis, increased the proliferation of colonic epithelium, decreased neutrophil infiltration in the colon and repaired colonic ulcers (Ando et al., 2008).

Similarly, Hayashi et al (2008) showed that submucosal injection of MSCs in a rat model of TNBS-induced colonic inflammation, lessened the clinical and histopathological severity of colitis, lowered the lesion size and expressed vascular endothelial growth factor and transforming growth factor-β1. Likewise, in a number of mouse models of TNBS-induced colonic inflammation, MSCs decrease the clinical and histopathological severity of colitis, inhibit neutrophil infiltration in the colon, decrease pro-inflammatory cytokines and Th17 marker RORyt (Chen et al., 2003, González et al., 2009, Liang et al., 2011, Anderson et al., 2012, Elinav et al., 2013, Kim et al., 2013). In the dextran sulfate sodium (DSS) mouse model of inflammation, MSCs decrease clinical and histopathological severity of the colitis and reduce pro-inflammatory cytokines and immune cells (González et al., 2009, Zhang et al., 2009, Anderson et al., 2012, He et al., 2012, Li et al., 2013, Kim et al., 2013, Wang et al., 2014)

MSCs are currently being tested in human trials and have shown varying levels of success in IBD patients. Clinical trials using MSCs for the treatment of CD fistulae and luminal inflammation have demonstrated that MSC therapy in IBD is both efficacious and feasible (García-Olmo et al., 2005, Garcia-Olmo et al., 2009a, Garcia-Olmo et al., 2009b, Cho et al., 2013, Lee et al., 2013). Most MSCs trials have focused on the treatment of fistulae caused by CD rather than CD manifestations as a whole. The use of MSCs in the treatment of IBD has resulted in the complete re-epithelisation of fistulae in the majority of subjects (Garcia-Olmo et al., 2009a, García-Olmo et al., 2010). One clinical trial demonstrated that *in vitro* expansion of MSCs is likely to be essential in harnessing the therapeutic potential of MSCs (Garcia-Olmo et al., 2009b). The therapeutic outcome of MSC therapy in fistulising CD may be dose dependent with greater efficacy achieved by doses of $2x10^7$ or $4x10^7$ MSC/ml compared to $1x10^7$ MSCs/ml (Garcia-Olmo et al., 2009b).

Chronic inflammation is a known risk factor in cancer development and hence IBD patients have a higher risk of developing cancer. Over 150 years ago, it was noted that cancer often occurred at inflammatory sites (Virchow, 1863), and, since then, numerous studies have shown that chronic inflammation is a risk associated with cancer (Balkwill and Mantovani, 2001, Coussens and Werb,

2002, Philip et al., 2004, Lu et al., 2006). Inflammation occurs in response to tissue damage that can result from infection, chemical irritation or trauma. In the early stages, neutrophils migrate to the inflammatory site in response to chemical signals produced by resident macrophages and mast cells. Other immune cells are subsequently recruited by a complex network of signalling molecules including growth factors, cytokines and chemokines. Anti-inflammatory molecules also exist to allow resolution of the inflammatory response, promoting tissue repair and the rapid programmed clearance of inflammatory cells via apoptosis and subsequent phagocytosis. However if this process is dysregulated, chronic inflammation can occur. IBD is characterised by immunoregulatory defects in the mucosa, which appears to be associated with microbial exposure. The relationship between commensal bacteria and the host is symbiotic and exposure to commensal bacteria in healthy individuals down regulate inflammatory genes and thus the immune response to microbes and food antigens is inhibited (Donnenberg, 2000, Neish et al., 2000, Abreu, 2002, Sands, 2004). In IBD this tolerance is lost and constant exposure to microbes and food antigens now triggers an inflammatory response causing chronic inflammation (Rath et al., 2001, Hanauer, 2006).

During chronic inflammation, inflammatory foci predominantly consist of lymphocytes, plasma cells and macrophages. Macrophages are largely responsible for generating growth factors, cytokines and reactive oxygen and nitrogen species (Macarthur et al., 2004). Under normal conditions, these factors drive the inflammatory response, but during prolonged inflammation may lead to continuous tissue damage, subsequent sustained cell proliferation and hence a predisposition to malignant transformation (Macarthur et al., 2004). Reactive oxygen and nitrogen species may also contribute to DNA damage that can result in neoplasia. Reactive oxygen and nitrogen species can be produced by immune cells to fight infections and are believed to possess mutagenic potential for tumour development (Coussens and Werb, 2002)

Cancer development is a step-wise process whereby genetic changes confer a growth advantage, driving tumour development. Malignancy is characterised by several hallmarks: self-sufficiency of growth signals, resistance to anti-growth

signals, and escape from apoptosis, unregulated proliferation, enhanced angiogenesis and metastasis (Hanahan and Weinberg, 2000).

To date, research into inflammation-induced cancer has largely focussed on chemokines, cytokines and their downstream targets (Elinav et al., 2013). These inflammatory mediators may promote tumour growth, invasion, and metastasis and may facilitate angiogenesis. (Elinav et al., 2013). MSCs have been reported to migrate to tumour sites and sites of injury to incorporate into tissue stroma (Klopp et al., 2011). The interaction between MSCs and tumour cells and the mechanisms underlying this interaction are unclear. Whether MSCs lead to tumour development or supress tumour development is still being debated (Lazennec and Jorgensen, 2008, Klopp et al., 2011). A number of studies indicate that MSCs have an anti-tumour effect (supress tumour growth) (Khakoo et al., 2006, Qiao et al., 2008b) whilst other studies report MSCs have a pro-tumour effect (promotes tumour growth) (Karnoub et al., 2007, Zhu et al., 2009).

4.2 The Gap

The effects of MSCs in an orthotopic model of colon cancer are still unclear in the literature. It was previously shown that in an orthotopic model of colon cancer, injection of MSCs together with tumour cells promotes cancer growth and metastasis (Shinagawa et al., 2010). However, injection of MSCs after the tumour has developed in an orthotopic model of colorectal cancer (CRC) has not being studied. It is unclear whether MSCs promote tumour growth or supress tumour growth. There are no animal studies with orthotopic CRC induced in *Winnie* mice with spontaneous chronic colitis.

4.3 Aim

To study the effects of MSCs on the immune response in spleen and colon in an animal model of spontaneous chronic colitis and an orthotropic model of colon cancer.

4.4 Materials and Methods

4.4.1 Animals

C57BL/6 and *Winnie* mice aged 12-24 weeks (n = 10) were obtained from Monash Animal Services. Animals were kept on a 12-hour light and dark cycle at approximately 22 °C with free access to food and water. Mice were allowed to acclimatise for at least one week. This study was approved by Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Code of Practise for the Care and Use of Animals for Scientific Purposes.

4.4.2 MSC Culture

Passage 4 human BM-MSC cell lines BM-7025 and BM-7081 (Tulane University) were characterised for their expression of surface antigens, differentiation potential, and colony forming ability as previously described (Robinson et al., 2014). All tests confirmed that MSCs used in this study met criteria for defining *in vitro* MSC cultures proposed by the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). Cells were plated at an initial density of 60 cells/cm^2 and incubated in complete culture medium; minimum essential medium (α-MEM) supplemented with 16.5% MSC qualified foetal bovine serum (FBS) (validated by Life Technologies and is tested to successfully support the differentiation and culture of human MSCs according to ISCT guidelines), 100 U/mL penicillin/streptomycin, and 100X GlutaMAX (all purchased from GIBCO, Life Technologies) at 37 °C. Medium was replenished every 48–72 hours for 10–14 days until the cells were 70–85 % confluent (maximum). MSCs were rinsed in 5 mL sterile phosphate buffered solution (PBS) (1X) prior to incubation with 3 mL trypsin/ethylenediaminetetraacetic acid

(EDTA) solution (TrypLE Select; GIBCO, Life Technologies) for 3 minutes at 37 °C to detach cells. Enzymatic activity was neutralized by 8mL of stop solution (α-MEM + 5 % FBS) and MSCs were collected and centrifuged at 450 g for 5 minutes at room temperature. Cells were then re-suspended in fresh culture medium and counted using a light microscope.

4.4.3 MSC Treatment

Mice were anesthetised using isoflurane (4 %); $1x10^6$ human bone marrow derived MSCs in 100 µL phosphate buffered solution were administered via enema either before or after CRC induction. The sham group received 100 µL of PBS via enema. Treatments were given twice a week for 2 weeks and then mice were killed 7 days after the last treatment to investigate the effects of MSCs.

4.4.4 Orthotopic Model of Colorectal Cancer

Orthotopic CRC induction was performed as described in Chapter 2. In this study we used C57BL/6 mice and therefore different a colorectal tumour cancer cell line (MC38). Prior to surgery, mice were anesthetised with an intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). The volume was calculated per animal body weight and the maximum volume did not exceed 200 µl. Viscotears™ was used to coat mouse eyes during surgery. The level of anaesthesia during surgery was monitored by the paw pinch response. The surgery was performed under aseptic conditions on a heat mat. Mice had their abdomen shaved and wiped with 70 % ethanol before being covered with sterile film. A small incision was made along the midline of the abdomen, and the caecum was exteriorised. Cell suspension of viable mouse colorectal tumour MC38 cells (1×10^6 cells) in 25 µl of the Matrigel™ was injected into the caecal wall. After surgery, the abdominal muscle wall was closed using polygalactin 5.0 gut sutures. Surgical silk suture was used to close the skin and the wound area was then sterilized with iodine. Mice received a subcutaneous injection of an analgesic buprenorphine (0.05 mg/kg the

maximum volume did not exceed 200 µl), and were placed in cages on heated mats to recover. Mice were closely monitored and regularly checked post-surgery. Mice were killed by cervical dislocation 3 weeks post-surgery; spleen, colon, and caecum were collected for immunohistochemistry and fluorescence-activated cell sorting (FACS) analysis.

4.4.5 Single Cell Suspension

Colon was dissected out and smooth muscle was removed leaving only the mucosa. The mucosa was cut into fine pieces and left in collagenase (0.1 mg/10mL) for 2 hours at 37 °C. Every 30 minutes the mucosa and collagenase were mixed using a pipette, for 2 hours total after which the colon collagenase mixture was removed and filtered. Spleen was dissected out and made into a suspension using the glass slide method and red blood cells lysed. The samples were centrifuged (450 g for 5 minutes) and resuspended in 1 mL of FACS buffer (1xphosphate buffered solution, 1% foetal bovine serum and 0.02 % sodium azide). The cell suspension was counted using a haemocytometer and viable cells were counted using trypan blue (dilution 1/1). The viability of cell suspensions used for experiments was between 65-75 %

4.4.6 Cell Surface and Intracellular Labelling

FACS analysis was used to enumerate and phenotype the different leukocyte subpopulations in the colon of both the *Winnie* and C57BL/6 mice. A minimum of $5x10^5$ cells per sample were used for antibody staining. Cells were labelled with monoclonal primary antibodies at 4 °C for 20 minutes to label specific immune cell types. Cytofix/cytoperm kit was used for intracellular labelling following manufacturer's instructions (BD Bioscience cat no. 554715).

Antibodies used were divided into 2 different cocktails and run on the spleen and colon tissue. Antibodies: rat anti-mouse Ly6G (clone 1A8) conjugated to FITC, rat anti-mouse Gr-1 (clone RB6-8C5) conjugated to FITC, rat anti-mouse CD206 (clone 17A2) conjugated to Alexa Fluor 488, rat anti-mouse F4/80 (clone BM8) conjugated to PE, rat anti-mouse Ly6C (clone HK1.4) conjugated to PE,

rat anti-mouse CD11b (clone M1/70) conjugated to PE/Cy7, rat anti-mouse CD11c (clone N418) conjugated to APC/Cy7, rat anti-mouse TCRβ (clone H57-597) conjugated to APC, rat anti-mouse IL-6 (clone MP5-20F3) conjugated to APC, rat anti-mouse IL-10 (clone JES5-16E3) conjugated to APC, rat anti-mouse TNFα (clone MP6- XT22) conjugated to brilliant violet 421, rat anti-mouse TGFβ (clone TW7-16B4) conjugated to brilliant violet 421 and rat anti-mouse MHC II (clone M5/114.15.2) conjugated to brilliant violet 510 (all antibodies from Biolegend, USA). To prevent non-specific Fc receptor binding cells were co-stained with rat anti-mouse CD16/CD32 hybridoma supernatant (2.4G2 clone, Developmental Studies Hybridoma Bank, USA). Samples were analysed using a BD Canto FACS analyser (BD Biosciences, USA). All data were compensated using BD FACSDIVA v6.0 and analysed in FACSDIVA.

4.4.7 Immunohistochemistry

Immunohistochemistry was performed in cross sections of the caecum and colon as described previously (Miller et al., 2016). Tissue sections were thawed and incubated for 1 hour at room temperature with 10 % normal donkey serum (Chemicon, USA) followed by an overnight incubation with a primary antibody. Primary antibodies used: rat anti-mouse PD-1 (dilution 1/500), rabbit anti-mouse and PD-L1 (dilution 1/500) (both from Abcam, Melbourne, Australia). Secondary antibody was added followed by incubation at room temperature for 2 hours. Secondary antibodies: donkey anti-rat Alexa Fluor 488 and anti-rabbit Alexa Fluor 488 (both from Jackson ImmunoResearch, Pennsylvania, USA) and slides were cover slipped with fluorescent mounting medium (DAKO, Australia).

4.4.8 Morphometric Analysis of Tumours in the Caecum

The caecum was dissected out and opened, the number of macroscopic tumours was counted and volumes were measured by callipers and an average was taken for each experimental group.

4.4.9 Imaging

Three dimensional (z-series) images of colon and caecum cross sections were taken using a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan). Fluorophores were visualised using excitation filters for Alexa 594 Red (excitation wavelength 559 nm), Alexa 488 (excitation wavelength 473 nm). Z-series images were taken at step size of 1.75 µm (1600 x 1200 pixels).

4.4.10 Quantitative Analysis

FACS data presented as percentages, the percentages of T cells (TCRβ$^+$ cells), neutrophils (Ly6G$^+$/Ly6C$^+$ cells) and CD11b$^+$ cells were calculated from total number of viable cells in the colon or spleen suspension and M1 (CD206$^-$ cells) and M2 (CD206$^+$ cells) macrophage percentages were calculated from the number of CD11b$^+$/CD45$^+$/F4/80$^+$/MHCII$^+$ cells. FACS and morphometric analysis data were analysed using Student's *t*-test. Analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California USA). All data are presented as mean ± standard error of the mean (SEM).

Images were analysed using Image J software (National Institute of Health, Bethesda, MD, USA). PD-1 and PD-L1 expression was assessed by measuring the density of PD-1 or PD-L1-immunoreactive (IR) cells per area (average of 8 areas of 500 µm^2 per animal at × 20 magnification). Image J software was employed to adjust colour images from RGB to 8 bit, after which thresholding to a consistent value was applied to obtain the percentage area of PD-1 or PD-L1-immunoreactivity. In colon tissue PD-1-IR cells were counted in cross sections within a 2-mm^2 area randomly capturing 8 images per slide.

4.5 Results

PART A: MSC treatment after CRC induction in C57BL/6 mice

4.5.1 Number and Volume of Tumours in the Caecum Following Orthoptopic Injection of MC38 Cells and MSC Treatment in C57BL/6 Mice

C57BL/6 mice were injected with MC38 cells into the caecum in an orthotopic colorectal cancer model and then underwent MSC treatment 7 days post-surgery (**Fig 4.1A**). The number of tumours in the caecum of MSC-treated mice was significantly increased (2.4±0.24, $p<0.05$) compared to the CRC only mice (1±0) (**Fig 4.1B**). The volume of tumours also had a tendency to be higher in the MSC-treated mice (7.70±5.51mm^3) compared to the CRC only mice (4.71±2.17mm^3) (**Fig 4.1B'**).

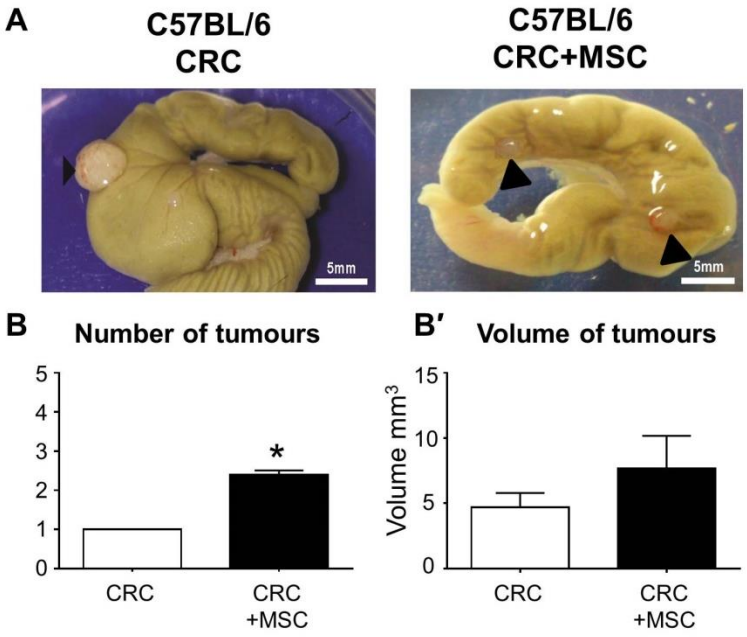

Figure 4.1: Number and Volume of Tumours in the Caecum from C57BL/6 mice

Images of caecum post CRC induction and MSC treatment after CRC induction from C57BL/6 mice **(A)**. Analysis of the number **(B)** and volume **(B′)** of tumours post CRC induction and MSC treatment after CRC induction in C57BL/6 mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk,*p<0.05, n=5 mice/group.

4.5.2 Immune cell Infiltrates in the Spleen of C57BL/6 Mice are Different Following MSC Treatment after CRC Induction

The immune system has a primary role in the prevention of tumours, it can do this in 3 different ways: 1) protection from virus-induced tumours by eliminating or suppressing viral infections, 2) elimination of pathogens and prompt resolution of inflammation to prevent an inflammatory environment conducive to tumourigenesis and 3) specifically identify and eliminate tumour cells on the basis of their expression of tumour-specific antigens. A number of immune cells contribute to this reaction including macrophages, T lymphocytes and neutrophils.

Flow cytometry was used to analyse the immune cells in the spleen and representative gating strategies are shown in **Figs 4.2-4.4**. MSCs were administered to mice after CRC induction and pan leukocyte marker CD45 was used to define leukocyte population in the spleen; all results are stated as percentage of $CD45^+$ cells. Analysis of spleens from mice with MSC treatment after CRC induction revealed T cells ($TCR\beta^+$ cells) are significantly increased in the MSC-treated mice (25.95±0.35%) when compared to the CRC only mice (11.47±1.99%, $p<0.05$) (**Fig 4.5**). Ly6C and Ly6G was used to determine the percentage of neutrophils in the spleen; MSC-treated mice (2.60±0.60%) have more neutrophils when compared to the CRC only mice (1.40±0.06%) (**Fig 4.6**), although not significant. In addition, the percentage of $CD11b^+$ cells in MSC-treated mice (8.43±1.13%) was similar to the CRC only mice (8.13±0.44) (**Fig 4.7A**). The percentage of M1/M2 macrophages are presented as a percentage of $CD11b^+/CD45^+/F4/80^+/MHCII^+$ cells. The percentage of M1 macrophages ($CD206^-$ cells) in MSC-treated mice (56.83±5.09%) was comparable to the CRC only mice (63.27±4.38%) (**Fig 4.7B**). The percentage of M2 macrophages ($CD206^+$ cells) in the MSC-treated mice (34.87±4.59%) was similar to that shown in the CRC only mice (34.40±4.56%) (**Fig 4.7C**).

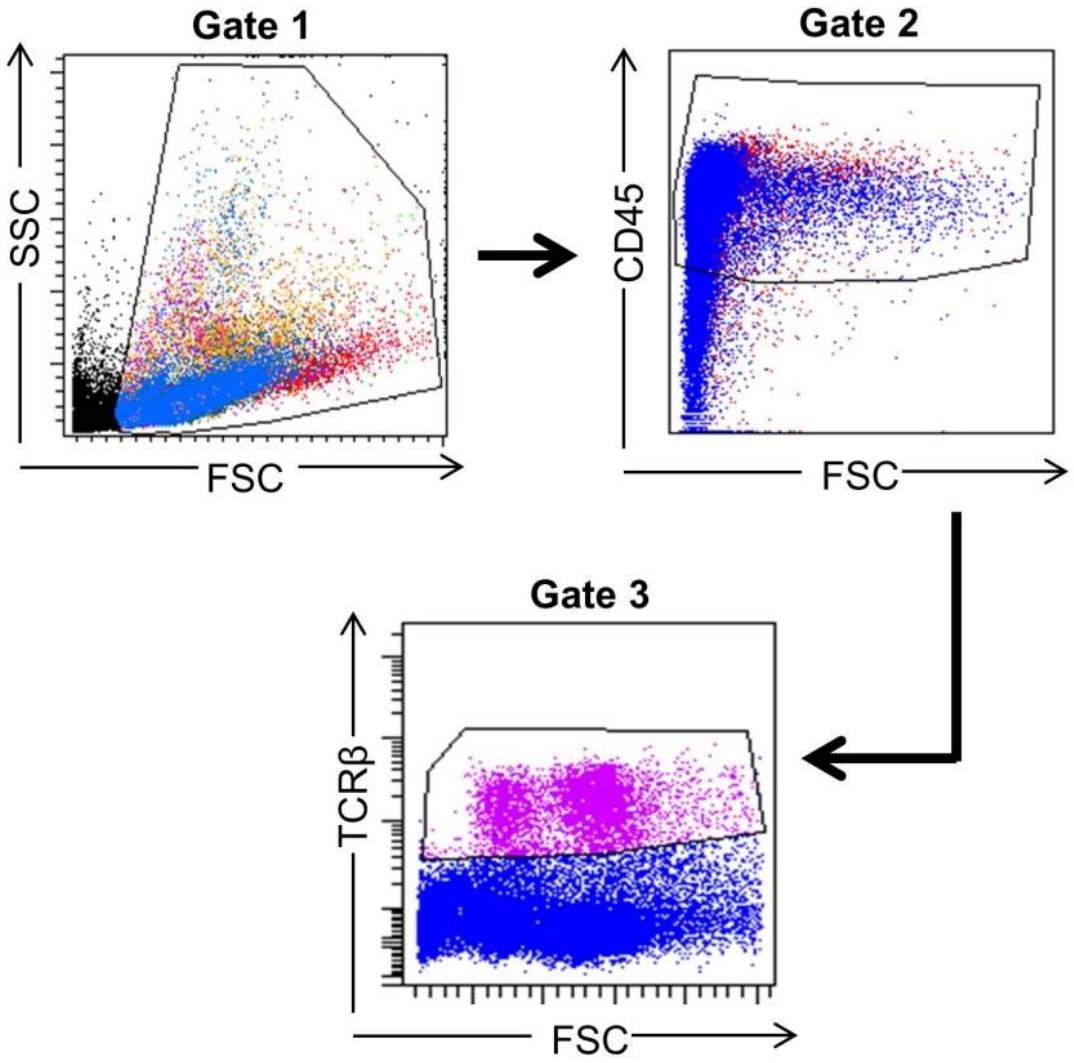

Figure 4.2: Gating Strategies used to determine the Percentage of T cells (TCRβ$^+$cells) in the Spleen.

Gate 1 – live cells based on FSC/SSC, Gate – 2 CD45$^+$ cells in the spleen, Gate 3 – TCRβ$^+$ cells in the spleen

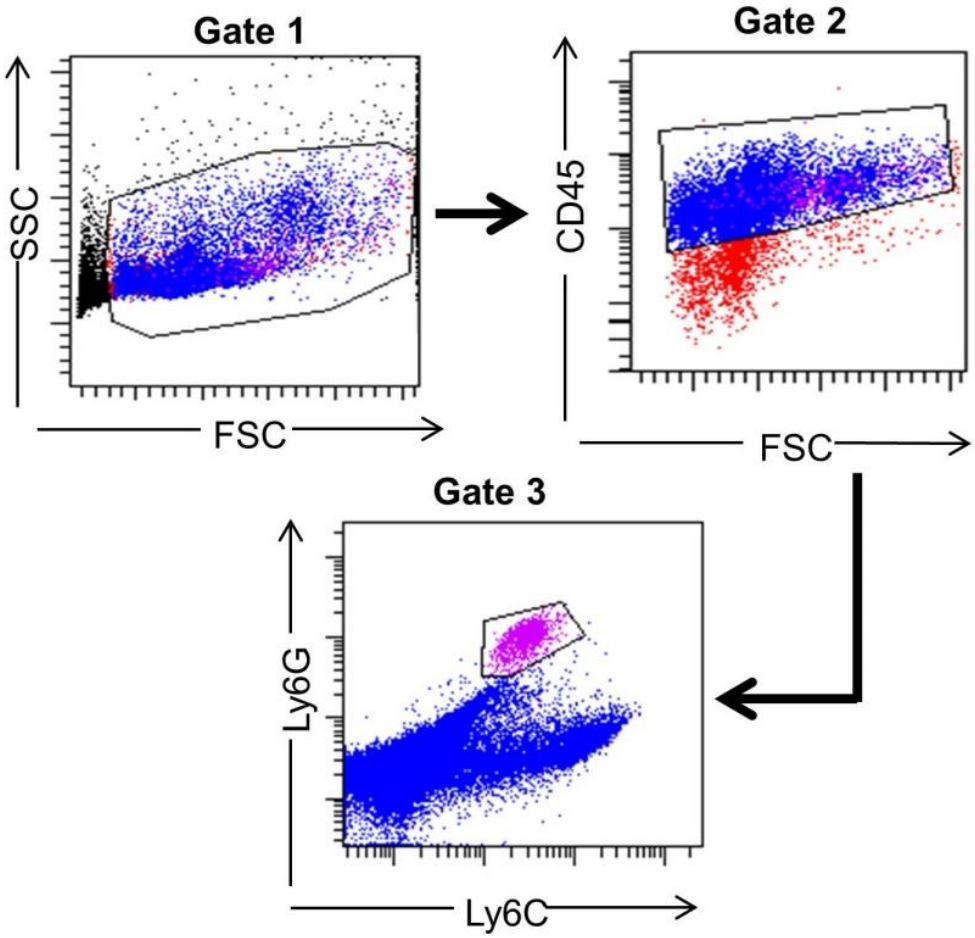

Figure 4.3: Gating Strategies used to determine the Percentage of Neutrophils (Ly6G$^+$/Ly6C$^+$cells) in the Spleen.

Gate 1 – live cells based on FSC/SSC, Gate – 2 CD45$^+$ cells in the spleen, Gate 3 - Ly6G$^+$/Ly6C$^+$ cells (granulocytes).

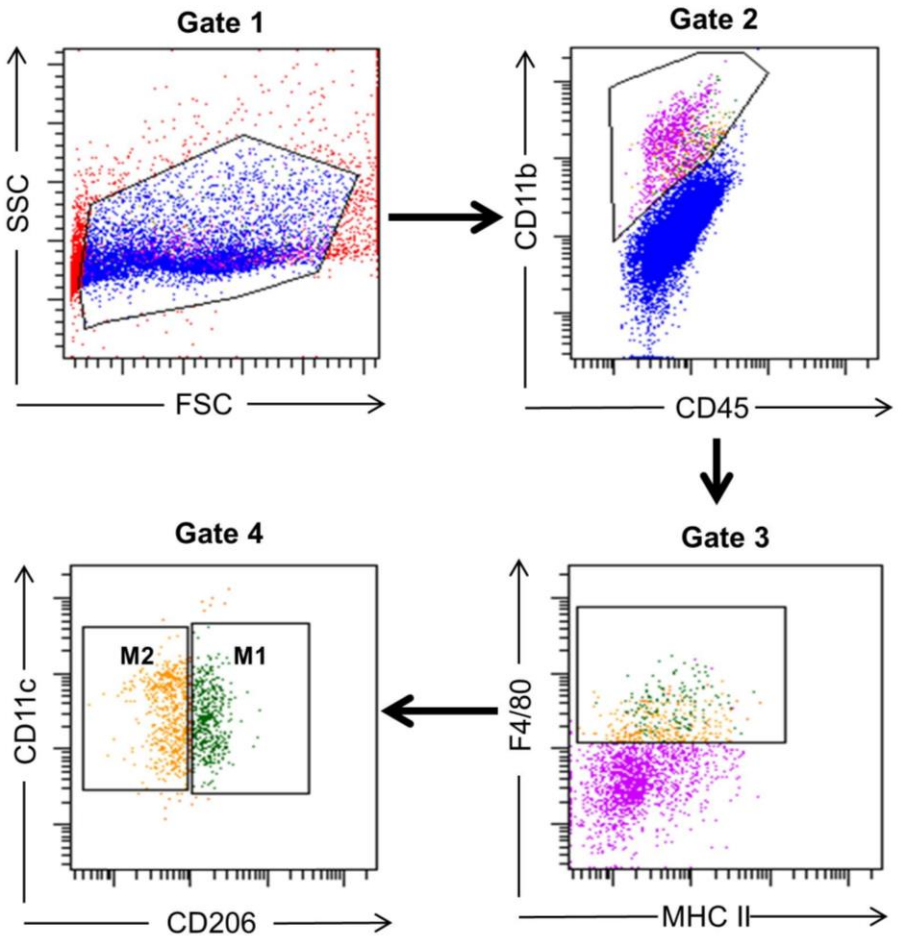

Figure 4.4: Gating Strategy used to Determine CD11b^{+}/CD45^{+} cells, M1 and M2 Macrophages in the Spleen.

Gate 1 – live cells based on FSC/SSC, Gate 2 – CD11b^{+}/CD45^{+} cells, Gate 3 – CD11b^{+}/CD45^{+}/F4/80^{+}/MHC II^{+} cells, Gate 4 - CD11c^{+}/CD206$^{+/-}$ cells, of gate 3 cells to distinguish M1 and M2 cells.

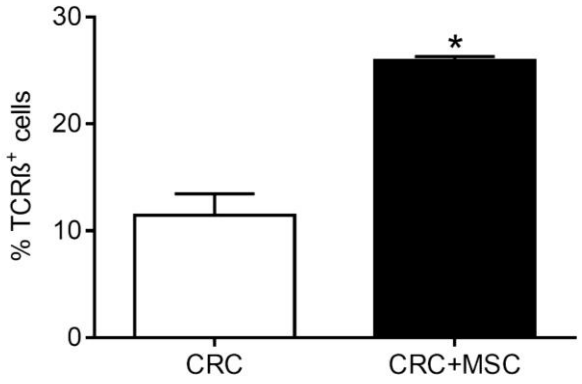

Figure 4.5: Percentage of T cells in the Spleen from C57BL/6 mice

Quantitative analysis of the percentage of T cells ($TCR\beta^+$ cells) in the spleen of mice treated with MSCs post CRC induction and CRC only mice. Data represented as mean ± SEM and statistical significance is indicated by asterisk, *p<0.05, n=5 mice/group.

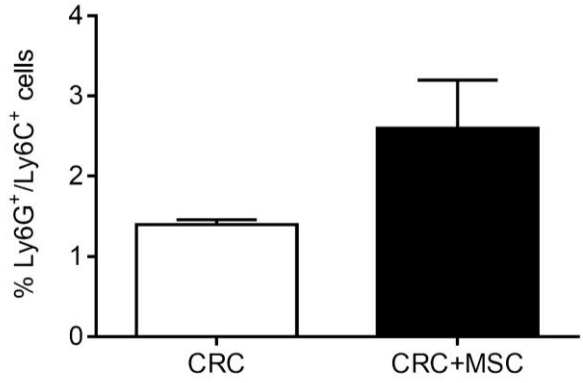

Figure 4.6: Percentage of Neutrophils in the Spleen from C57BL/6 mice

Quantitative analysis of the percentage of neutrophils ($Ly6G^+/Ly6C^+$ cells) in the spleen of mice treated with MSCs post CRC induction and CRC only mice. Data represented as mean ± SEM, n=5 mice/group.

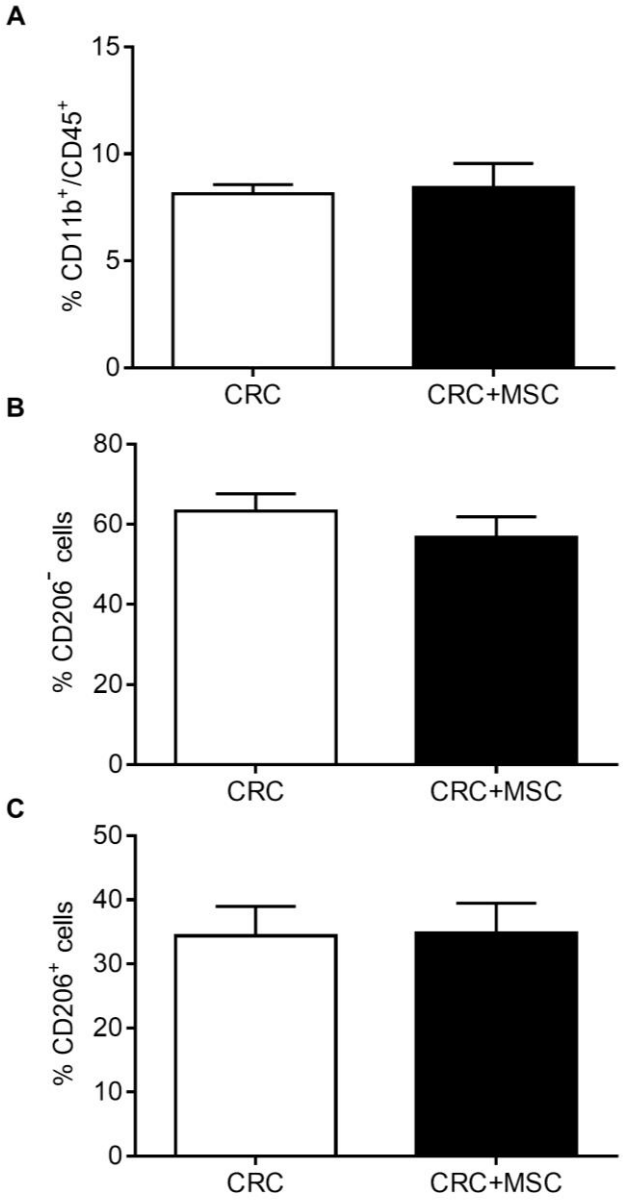

Figure 4.7: Percentage of $CD11b^+/CD45^+$ Cells, M1 and M2 Macrophages in the Spleen from C57BL/6 mice

Quantitative analysis of the percentage of $CD11b^+/CD45^+$ cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the spleen of mice treated with MSCs post CRC induction and CRC only mice. Data represented as mean ± SEM, n=5 mice/group.

4.5.3 Immune Infiltrates in the Colon of C57BL/6 Mice with Colorectal Cancer are Different after MSC Treatment

Analysis of immune cells in the colon using flow cytometry showed significant differences between C57BL/6 control mice and *Winnie* mice injected with MC38 colorectal cancer cells and then underwent MSC treatments. Representative gating is shown in **Figs 4.8-4.10**. Pan leukocyte marker CD45 was used to show total immune cell infiltration in the colon and all results are stated as a percentage of $CD45^+$ cells. The percentage of T cells ($TCR\beta^+$ cells) are increased in MSC-treated mice (16.87±2.58%) when compared to CRC only mice (12.53±0.39%), although not significant (**Fig 4.11**). Neutrophils were identified as positive for Ly6G and Ly6C, the percentage of neutrophils in MSC-treated mice (2.27±0.27%) is similar to CRC only mice (1.33±1.33%) (**Fig 4.12**). Furthermore, within the colon a significant decrease in $CD11b^+$ cells in MSC-treated mice (17.90±0.40%) compared CRC only mice (24.60±1.55%) were noted (**Fig 4.13A**). However, no changes in the percentage of M1 macrophages (40.95±8.15% vs 46.47±0.84%) (**Fig 4.13B**) and M2 macrophages (59.00±8.10% vs 50.23±1.04%) (**Fig 4.13C**) were found in MSC-treated mice compared to the CRC only mice.

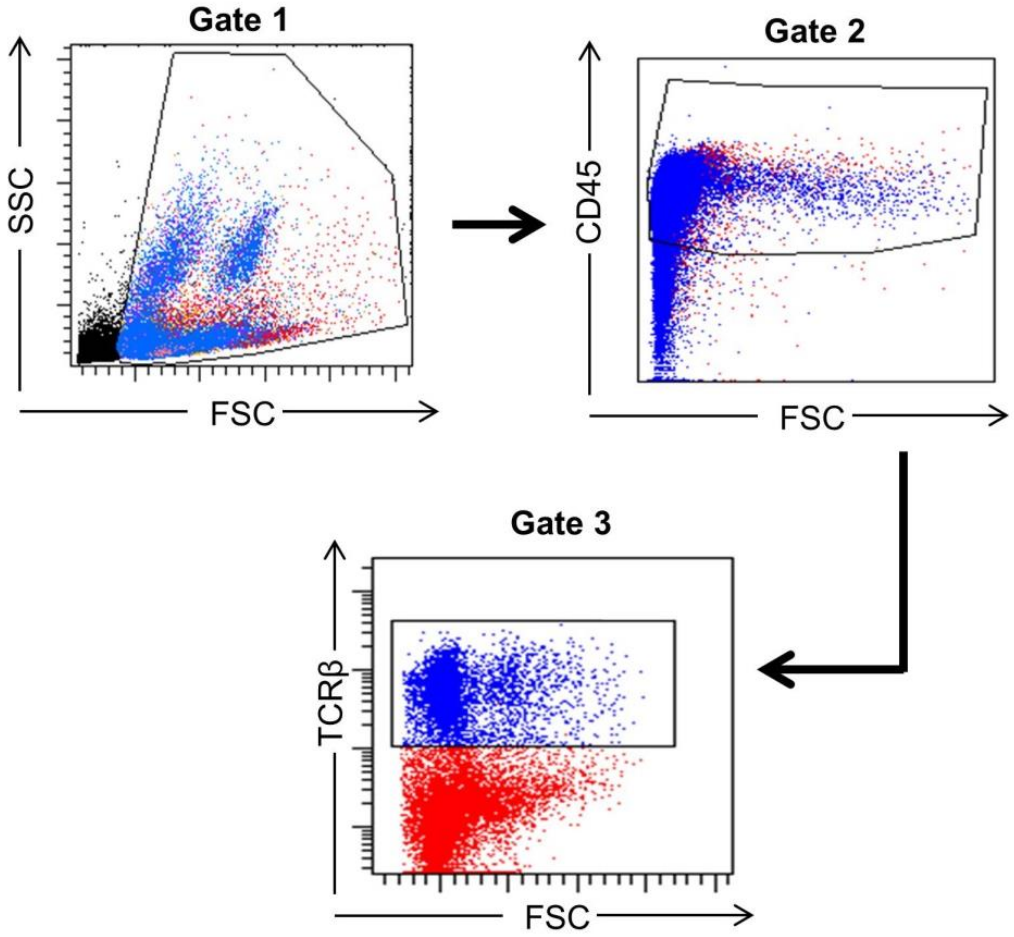

Figure 4.8: Gating Strategies used to determine the Percentage of T cells (TCRβ^{+}cells) in the Colon.

Gate 1 – live cells, Gate – 2 CD45 cells in the spleen and Gate 3 – TCRβ cells in the colon

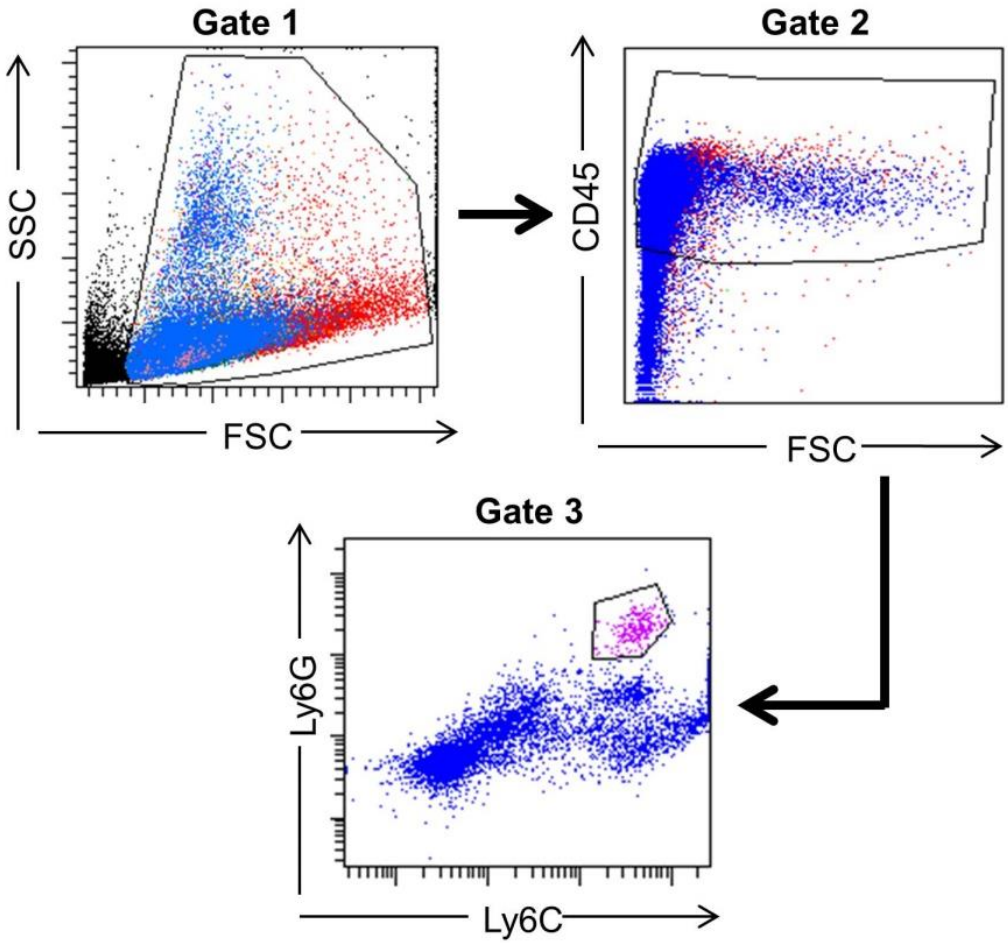

Figure 4.9: Gating Strategies used to determine the Percentage of Neutrophils (Ly6G/Ly6Ccells) in the Colon.

Gate 1 – live cells based on FSC/SSC, Gate – 2 CD45 cells in the colon, Gate 3 - Ly6G/Ly6C cells (granulocytes).

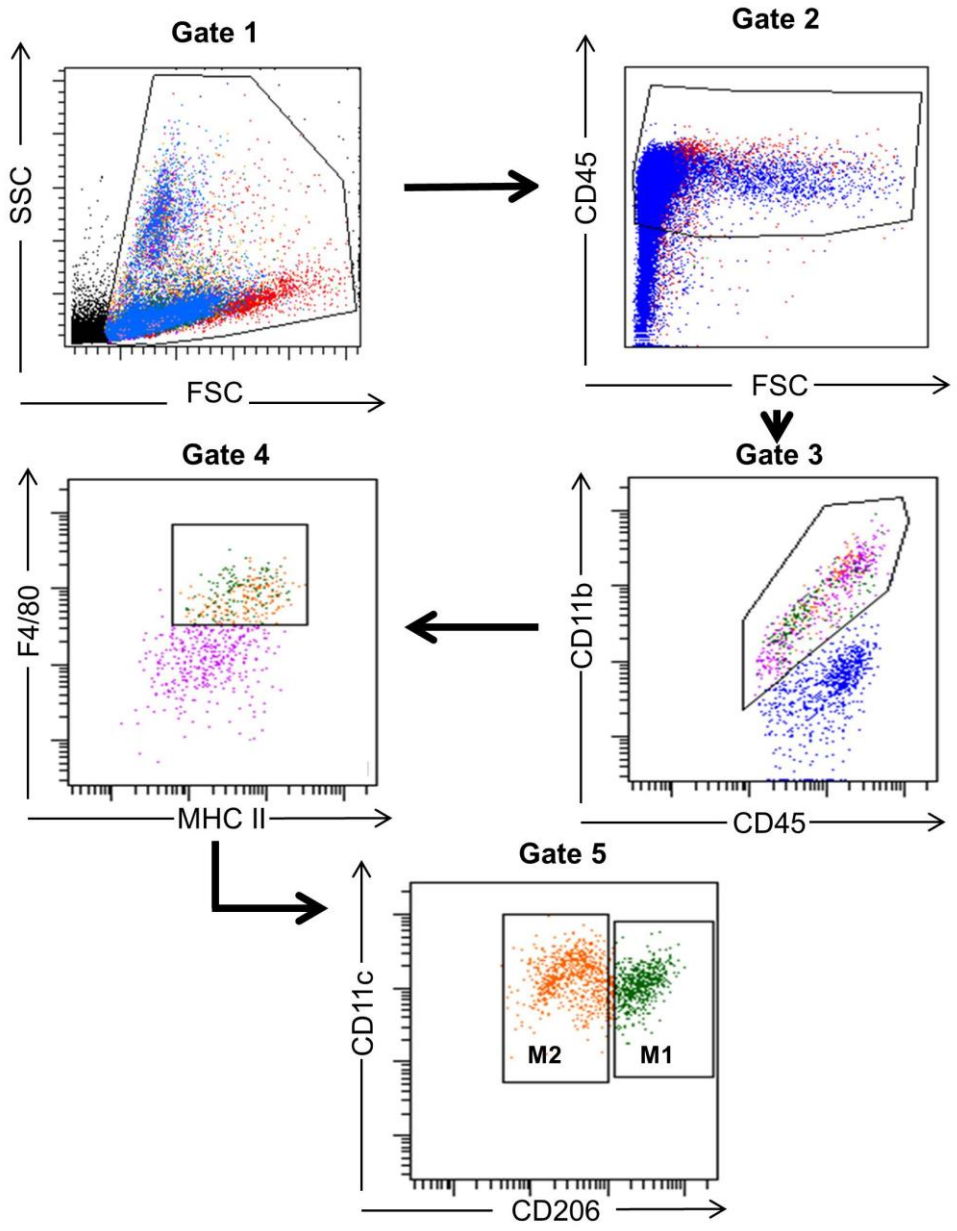

Figure 4.10: Gating Strategy used to Determine CD11b^{+}/CD45^{+} cells and M1/M2 Macrophages in the Colon.

Gate 1 – CD45^{+}cells based on FSC/SSC, Gate 2 – live cells, Gate 3 – CD11b^{+}/CD45^{+}cells, Gate 4 – F4/80^{+}/MHC II^{+} cells, Gate 5 - CD11c^{+}/CD206$^{+/-}$ cells.

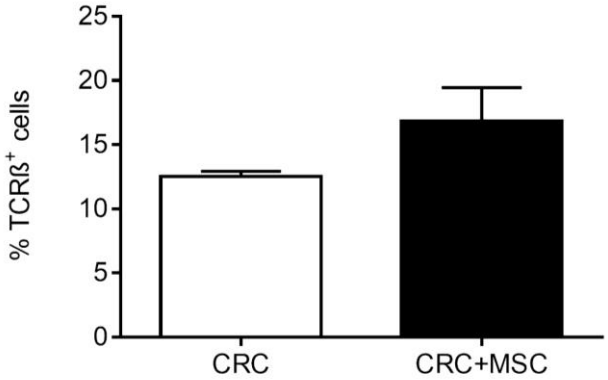

Figure 4.11: Percentage of T cells in the Colon from C57BL/6 mice

Quantitative analysis of the percentage of T cells ($TCR\beta^+$ cells) in the colon of mice with CRC only and mice treated with MSCs after CRC induction. Data represented as mean ± SEM, n=5 mice/group.

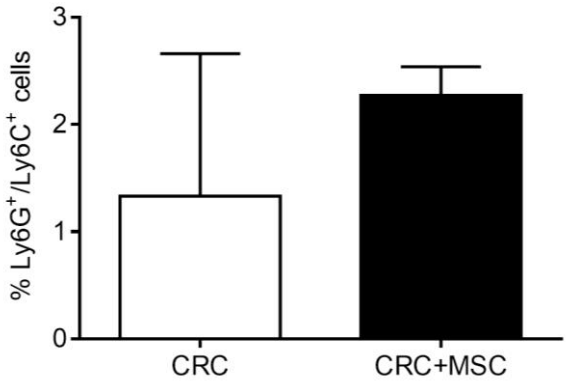

Figure 4.12: Percentage of Neutrophils in the Colon from C57BL/6 mice

Quantitative analysis of the percentage of neutrophils ($Ly6G^+/Ly6C^+$ cells) in the colon of mice with CRC only and mice treated with MSCs after CRC induction. Data represented as mean ± SEM, n=5 mice/group.

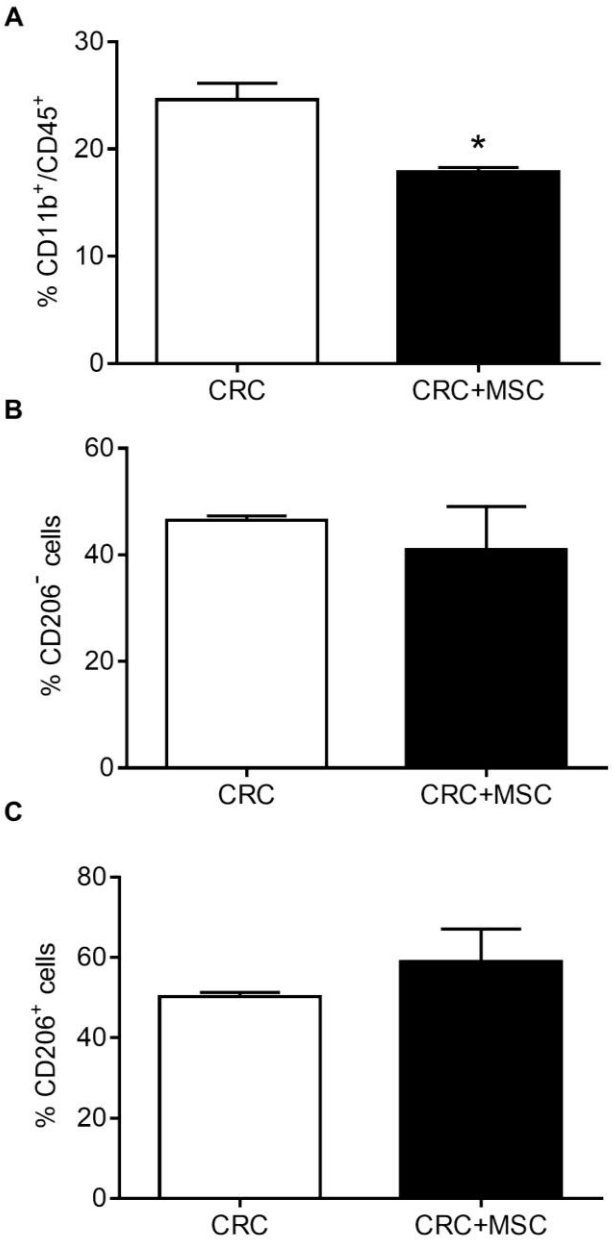

Figure 4.13: Percentage of CD11b$^+$/CD45$^+$ Cells, M1 and M2 Macrophages in the Colon from C57BL/6 mice

Quantitative analysis of the percentage of CD11b$^+$/CD45$^+$ cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the colon of mice with CRC only and mice treated with MSCs after CRC induction. Data represented as mean ± SEM and statistical significance is indicated by asterisk, *p<0.05, n=5 mice/group.

4.5.4 PD-1 and PD-L1 Expression in Mice with Colorectal Cancer and MSC Treatment

In the tumour microenvironment PD-L1 is expressed by tumour cells which upon binding of PD-1 expressed by activated T cells lead to inhibitory signals being triggered and a reduction in $CD8^+$ T cells and apoptosis. Therefore, the PD-1/PD-L1 interaction is an important regulator of the immune system in cancer and IBD. Tumour tissue from the caecum and colon tissues from MSC-treated mice were labelled for PD-1 and PD-L1 to determine the overall expression compared to untreated CRC only mice. The expression of PD-L1 in the tumours of MSC-treated (4.56±0.89%) mice had a tendency to be increased compared to the CRC only mice (3.88±0.39%) (**Fig 4.14**); PD-1 expression in the tumour tissue also appeared to be higher in MSC-treated mice (0.79±0.09%) compared to CRC only mice (0.54±0.08%) (**Fig 4.15**). PD-1 expression in the colon was similar in the MSC-treated mice (29.75±2.69) compared to CRC only mice (29±1.58) (**Fig 4.16**). The images also show the co-localisation of PD-1 and CD8 and PD-L1 and Ki67 was observed. The effect of MSC treatment on the immune responses in the spleen and colon is summarised on **Fig 4.17.**

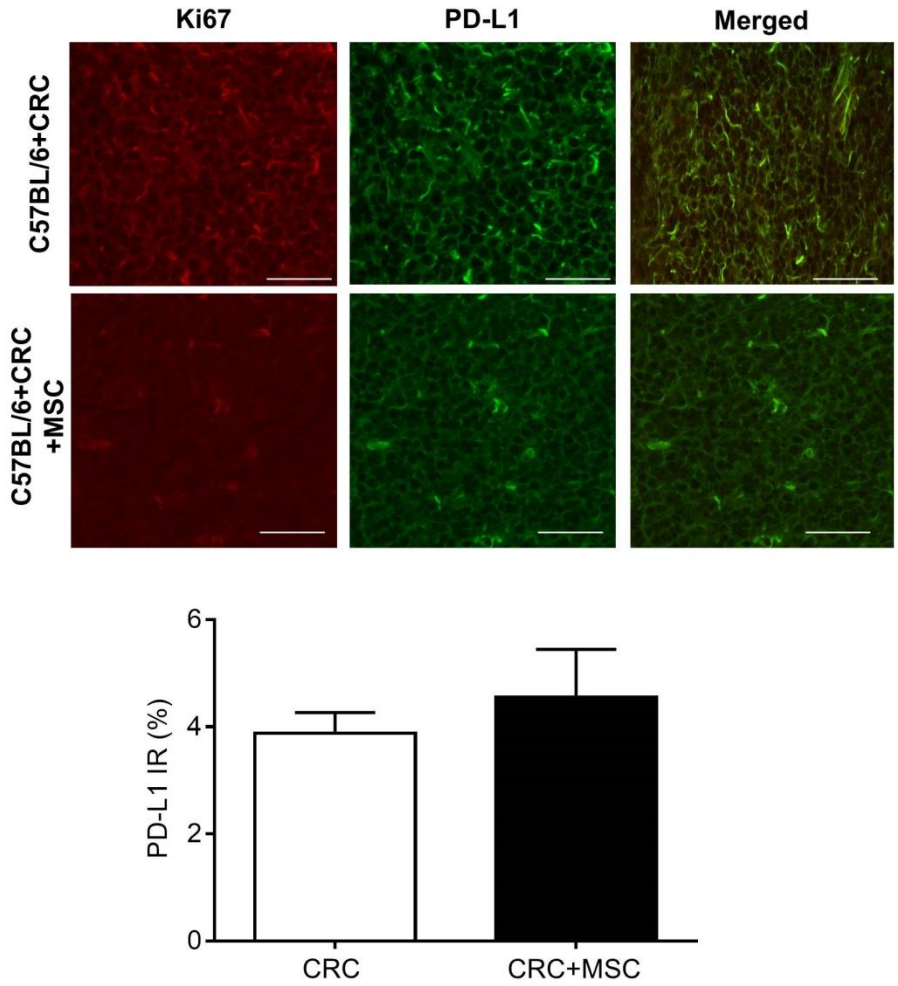

Figure 4.14: Immunohistological Labelling of PD-L1 in Tumour Cross Sections from C57BL/6 mice

Immunohistological labelling of PD-L1 (Green) and Ki67 (Red) and quantitative analysis of cells expressing PD-L1 in tumour sections found in the caecum of mice with MSC treatment after CRC induction. Data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm.

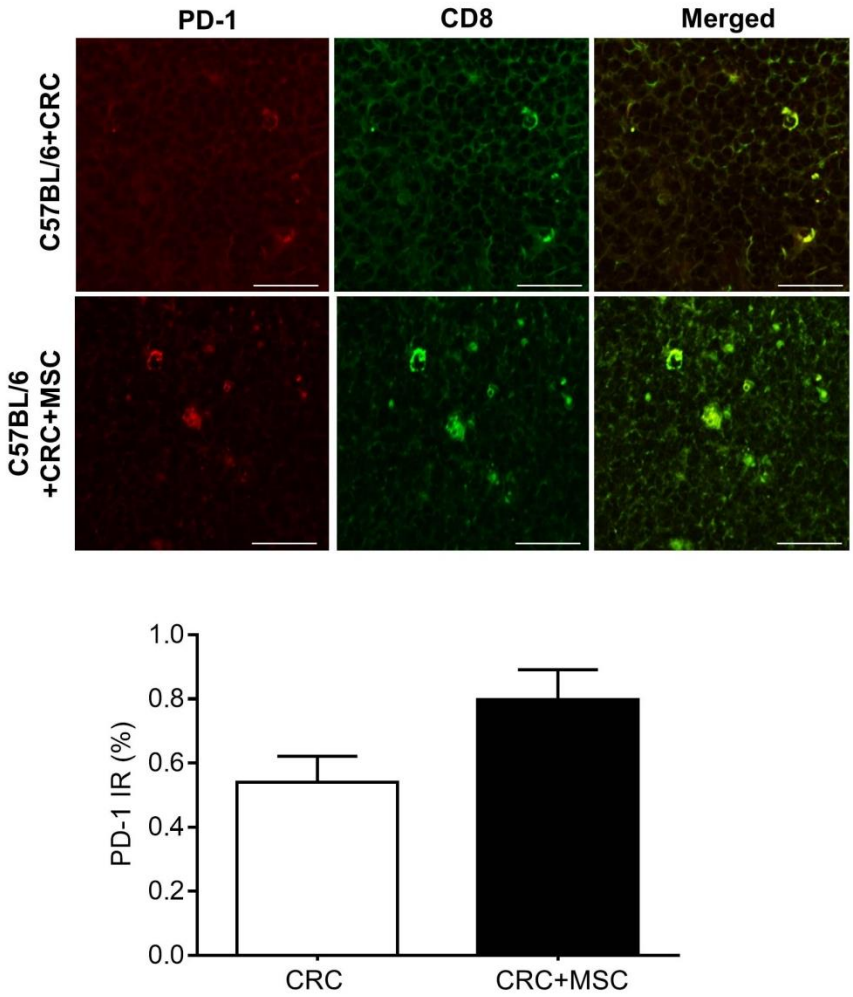

Figure 4.15: Immunohistological Labelling of PD-1 in Tumour Cross Sections from C57BL/6 mice

Immunohistological labelling of CD8 (Green) and PD-1 (Red) and quantitative analysis of $CD8^+$ cells expressing PD-1 in tumour cross sections found in the caecum of mice with CRC and MSC treatment after CRC induction. Data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm.

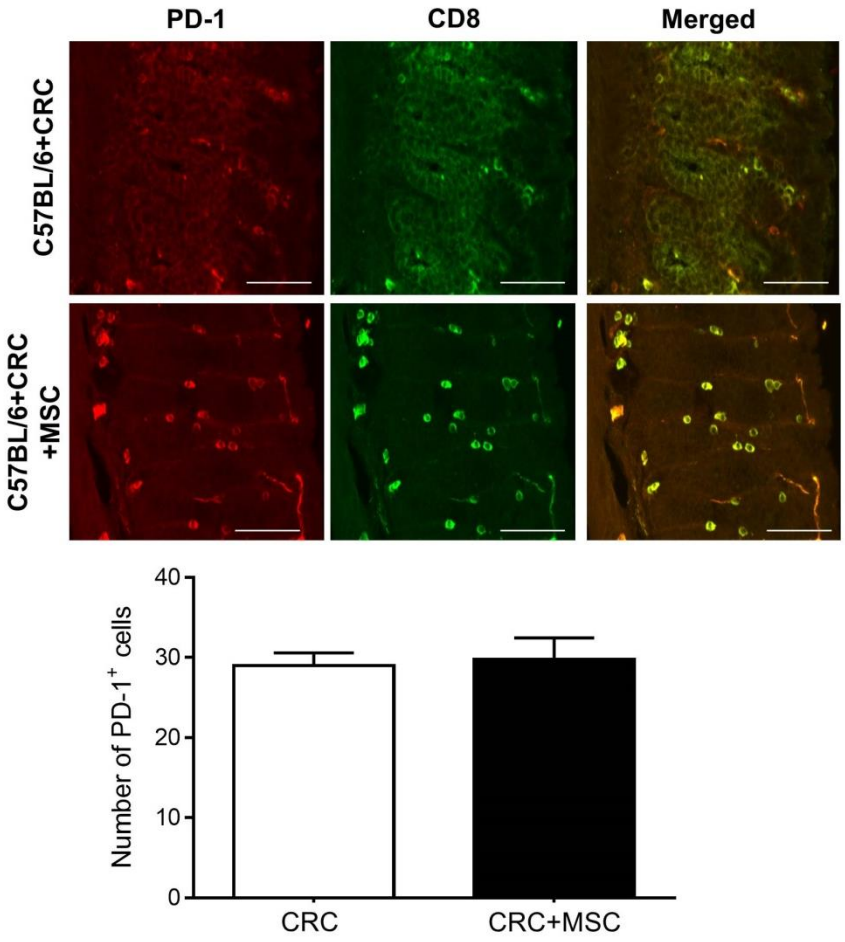

Figure 4.16: Immunohistological Labelling of PD-1 in Colon Cross Sections from C57BL/6 mice

Immunohistological labelling of CD8 (Green) and PD-1 (Red) and quantitative analysis of $CD8^+$ cells expressing PD-1 in colon cross sections from mice with MSC treatment after CRC induction. Data represented as mean ± SEM, n=5 mice/group. Scale bar is 100 µm.

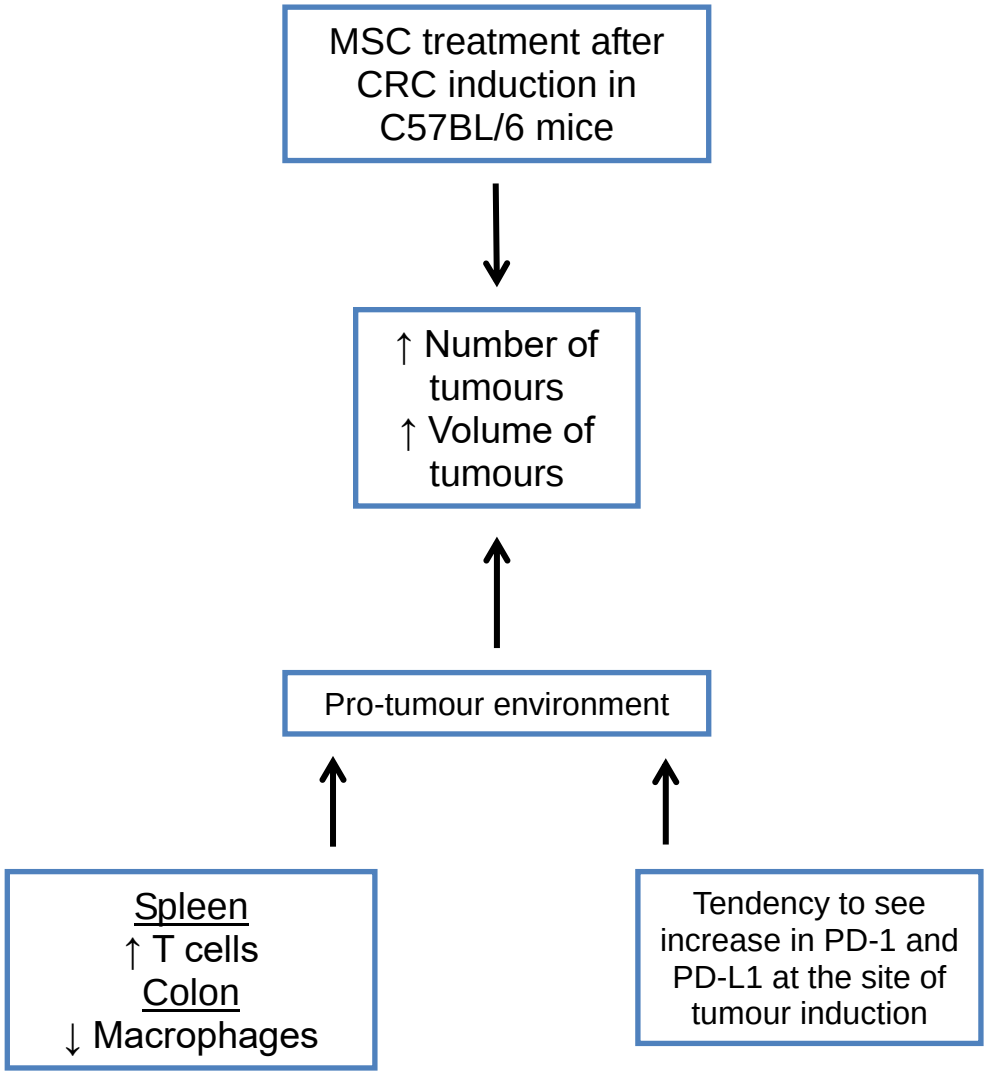

Figure 4.17: Summary of MSC treatment after CRC induction in C57BL/6 mice

PART B: MSC treatment before CRC induction in C57BL/6 mice

4.5.5 Number and Volume of Tumours in the Caecum of C57BL/6 Mice Treated with MSCs before Orthotopic Injection of MC38 Colorectal Cancer Cells

C57BL/6 mice were treated with MSCs before being injected with MC38 cells into the caecum in an orthotopic colorectal cancer model. The number of tumours in the caecum was similar in the MSC-treated mice (1.0±0.0) to the number of tumours in the CRC only mice (1.0±0.0) (**Fig 4.18**). The volume of tumours had a tendency to be lower in the MSC-treated mice (1.7±0.22mm^3) compared to the CRC only mice (4.71±2.17mm^3).

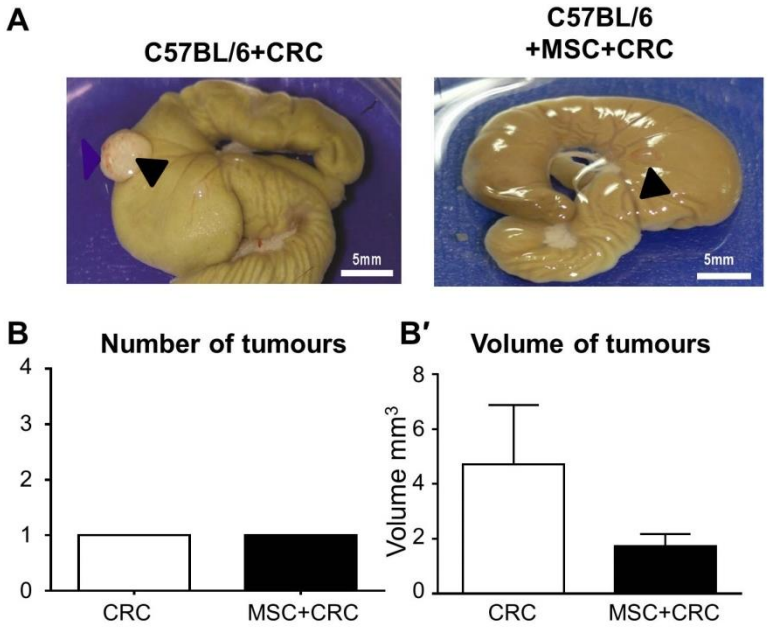

Figure 4.18: Number and Volume of Tumours in the Caecum from C57BL/6 mice

Images **(A)** and analysis of the number (**B**) and volume (**B′**) of tumours in the caecum of C57BL/6 mice at 3 weeks post implantation of MC38 colorectal tumour cells with MSC treatment before CRC induction. Data represented as mean ± SEM, n=5 mice/group.

4.5.6 MSC Treatment Prior to CRC Induction Reduces the Number of $CD206^-$ cells but not Other Cell Populations in the Spleen of C57BL/6 Mice

Flow cytometry analysis of the spleen showed minor differences between CRC only mice and mice treated with MSCs before orthotopic injection of MC38 CRC. Pan leukocyte marker CD45 was used to identify total immune cell infiltration in the spleen and all results are stated as a percentage of $CD45^+$ cells. The percentage of T cells ($TCR\beta^+$ cells) tend to increase in the spleen of MSC-treated mice (16.95±2.15%) compared to CRC only mice (11.47±1.99%) (**Fig 4.19**). Neutrophils were identified using Ly6G and Ly6C, the percentage of neutrophils tended to be increased in MSC-treated mice (3.56±1.06%) compared to CRC only mice (1.40±0.06%) (**Fig 4.20**), however, this difference was not significant. In addition, MSC-treated mice (9.40±0.96%) showed no change in the percentage of $CD11b^+$ cells compared to CRC only mice (8.13±0.44%) (**Fig 4.21A**). M1/M2 macrophages were defined as $CD45^+CD11b^+F4/80^+MHC\text{-}II^+CD11c^+CD206^{+/-}$; interestingly, the percentage of M1 macrophages in MSC-treated mice (9.40±0.96%) are significantly decreased compared to CRC only mice (63.26±4.38%, $p<0.05$) (**Fig 4.21B**) and the percentage of M2 macrophages showed a decreased trend in MSC-treated mice (27.27±3.90%) compared to CRC only mice (34.40±4.56%) (**Fig 4.21C**).

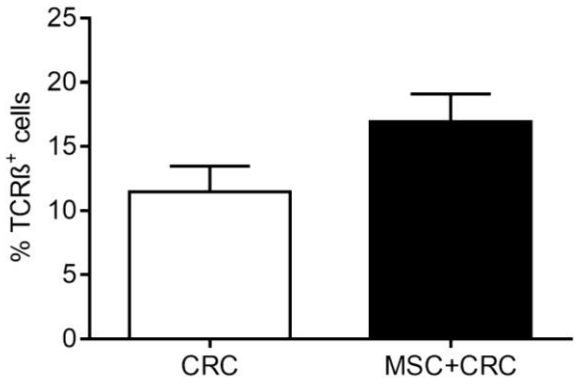

Figure 4.19: Percentage of T cells in the Spleen from C57BL/6 mice

Quantitative analysis of the percentage of T cells (TCRβ cells) in the spleen of mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM and n=5 mice/group.

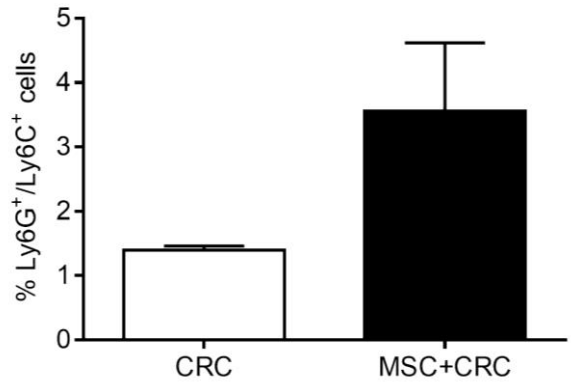

Figure 4.20: Percentage of Neutrophils in the Spleen from C57BL/6 mice

Quantitative analysis of the percentage of neutrophils (Ly6G/Ly6C cells) in the spleen of mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM and n=5 mice/group.

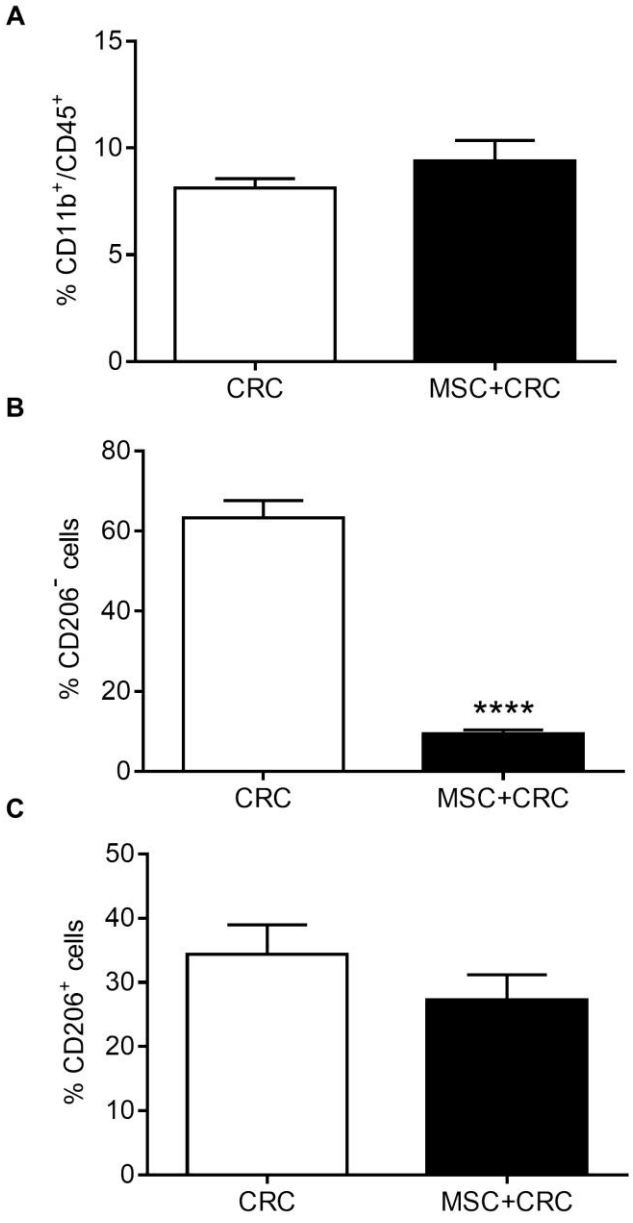

Figure 4.21: Percentage of $CD11b^{+}/CD45^{+}$ Cells, M1 and M2 Macrophages in the Spleen from C57BL/6 mice

Quantitative analysis of the percentage of $CD11b^{+}/CD45^{+}$ cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the spleen of mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM and statistical significance is indicated by asterisk, ****p<0.0001, n=5 mice/group.

4.5.7 MSC Treatment before Cancer Induction has No Significant Effect on Immune Cell Populations in the Colon of C57BL/6 Mice Compared to Controls

Analysis of the colon by flow cytometry showed no differences between CRC only mice and mice treated with MSCs before orthotopic injection of MC38 colorectal cancer cells. Pan leukocyte marker CD45 was used to show total immune cell infiltration within the colon and all data are stated as a percentage of $CD45^+$ cells. The percentage of T cells (TCR^+ cells) in MSC-treated mice (11.43±2.49%) was similar to that of CRC only group (12.53±0.39%) (**Fig 4.22**). Ly6G and Ly6C were used to enumerate neutrophils; MSC-treated mice had a lower percentage of neutrophils (0.70±0.20%) compared to the CRC only mice (1.33±1.33%) but this was not significant (**Fig 4.23**). The percentage of $CD11b^+$ cells in the colon of MSC-treated mice (23.28±1.00%) was similar to that of the CRC only mice (24.60±1.55%) (**Fig 4.24A**). No significant differences were noted in the percentage of M1 and M2 macrophages in MSC-treated mice (53.07±4.17% and 44.70±3.94%) compared to CRC only mice (46.47±0.84% and 50.23±1.04%) (**Fig 4.24B&C**).

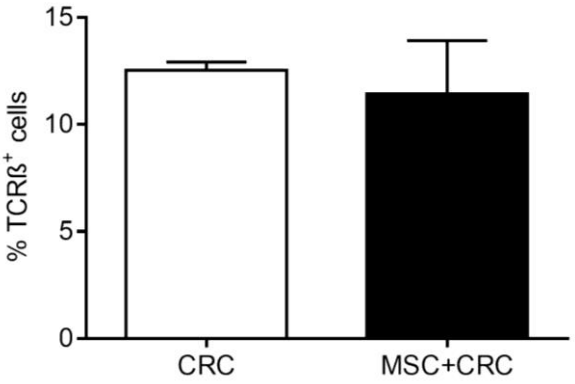

Figure 4.22: Percentage of T cells in the Colon from C57BL/6 mice

Quantitative analysis of the percentage of T cells ($TCR\beta^+$ cells) in the colon of mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM, n=5 mice/group.

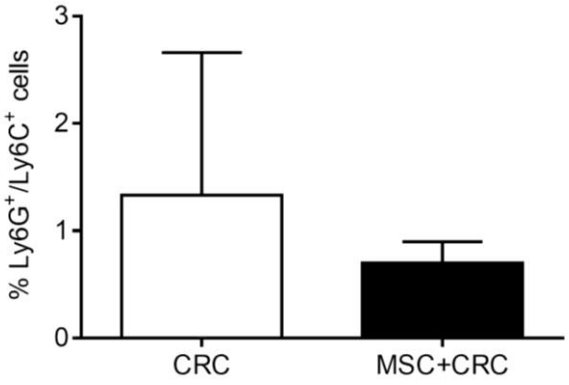

Figure 4.23: Percentage of Neutrophils in the Colon from C57BL/6 mice

Quantitative analysis of the percentage of neutrophils ($Ly6G^+/Ly6C^+$ cells) in the colon of mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM, n=5 mice/group.

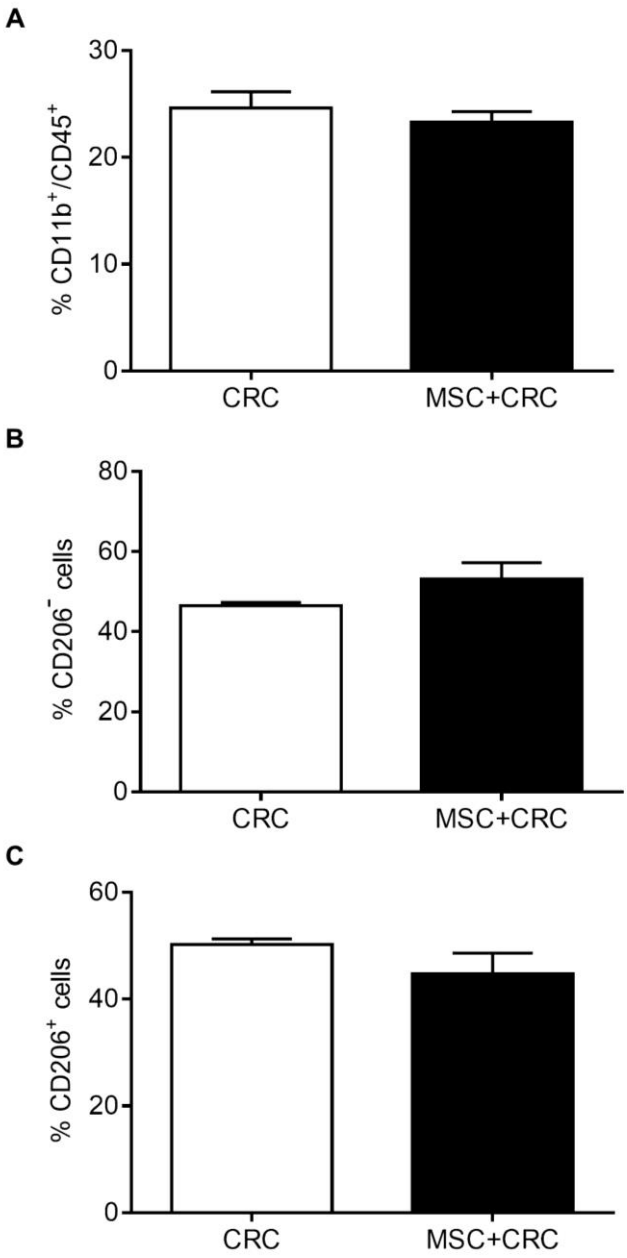

Figure 4.24: Percentage of CD11b$^+$/CD45$^+$ Cells, M1 and M2 Macrophages in the Colon from C57BL/6 mice

Quantitative analysis of the percentage of CD11b$^+$/CD45$^+$ cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the colon of mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM and statistical significance is indicated by asterisk, n=5 mice/group.

4.5.8 Immunohistochemical Analysis of PD-1 and PD-L1 in C57BL/6 Mice Treated with MSCs before Colorectal Cancer Induction

In the tumour microenvironment PD-L1 expression on tumour cells binding to PD-1 expressed on activated T cells leading to inhibitory signals being triggered and reduction in $CD8^+$ T cells and apoptosis. Therefore, the PD-1/PD-L1 interaction is an important regulator of the immune system in cancer and IBD. Tumour and colon tissues from MSC-treated mice were labelled for PD-1 and PD-L1 to determine the overall expression compared to CRC only mice. The expression of PD-L1 in the tumours of MSC-treated mice (4.97±0.93%) had a tendency to be increased compared to the CRC only mice (3.88±0.39%) (**Fig 4.25**), PD-1 expression in the tumour also showed a tendency to be higher in MSC-treated mice (0.63±0.12%) compared the CRC only mice (0.54±0.08%) (**Fig 4.26**) and PD-1 expression in the colon of MSC-treated mice (38.5±7.4) showed a trend to be higher compared to CRC only mice (29±1.58) (**Fig 4.27**). The effect of MSC treatment after CRC induction on the immune system is summarised in **Fig 4.28**.

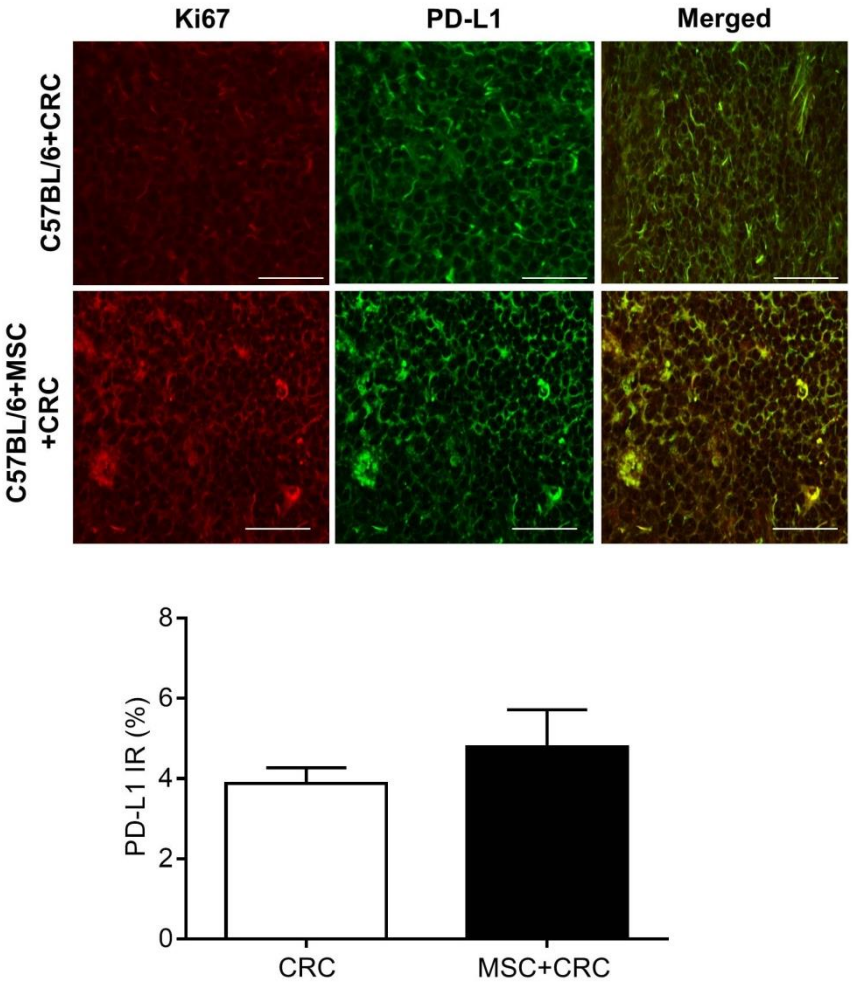

Figure 4.25: Immunohistological labelling of PD-L1 in Tumour Cross Sections from C57BL/6 mice

Immunohistological labelling of PD-L1 (Green) and Ki67 (Red) and quantitative analysis of $Ki67^{+}$ $PD\text{-}L1^{+}$ cells in tumour sections found in the caecum of mice with MSC treatment before CRC induction. Data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm.

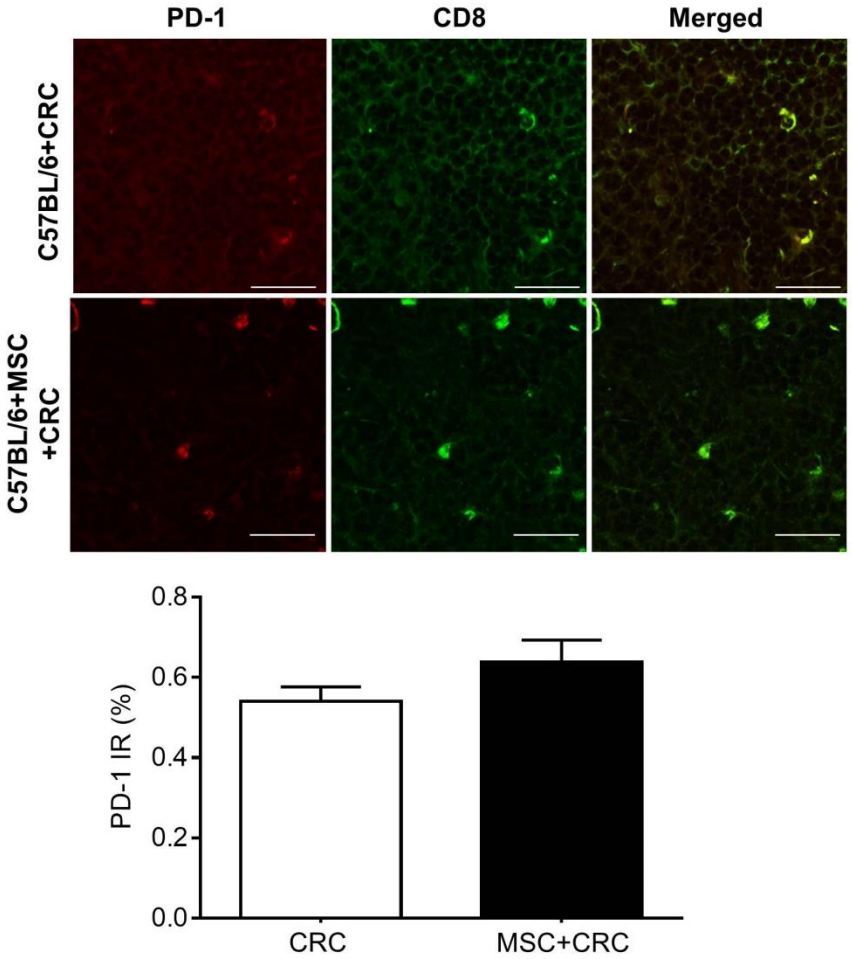

Figure 4.26: Immunohistological labelling of PD-1 in Tumour Cross Sections from C57BL/6 mice

Immunohistological labelling of CD8 (Green) and PD-1 (Red) quantitative analysis of $CD8^+$ $PD\text{-}1^+$ cells in tumour cross sections found in the caecum of mice with MSC treatment and CRC. Data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm.

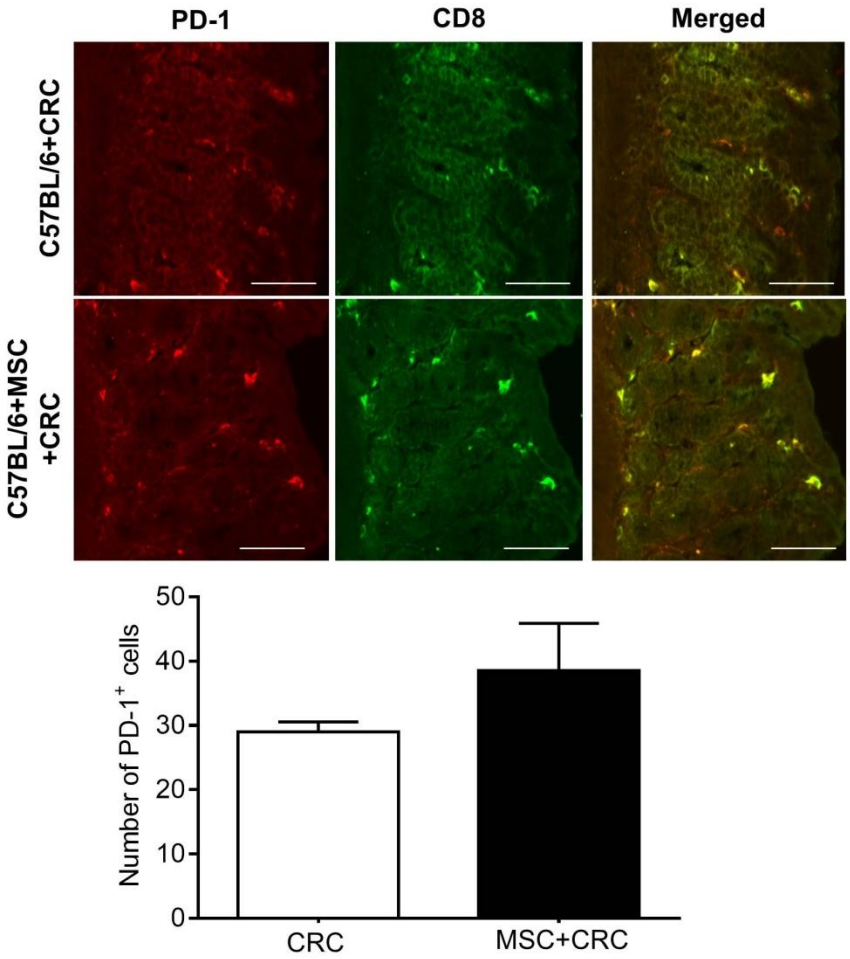

Figure 4.27: Immunohistological labelling of PD-1 in Colon Cross Sections from C57BL/6 mice

Immunohistological labelling of CD8 (Green) and PD-1 (Red) and quantitative analysis of $CD8^+$ $PD\text{-}1^+$ cells in colon cross sections of mice with MSC treatment before CRC induction. Data represented as mean ± SEM, n=5 mice/group. Scale bar is 100 µm.

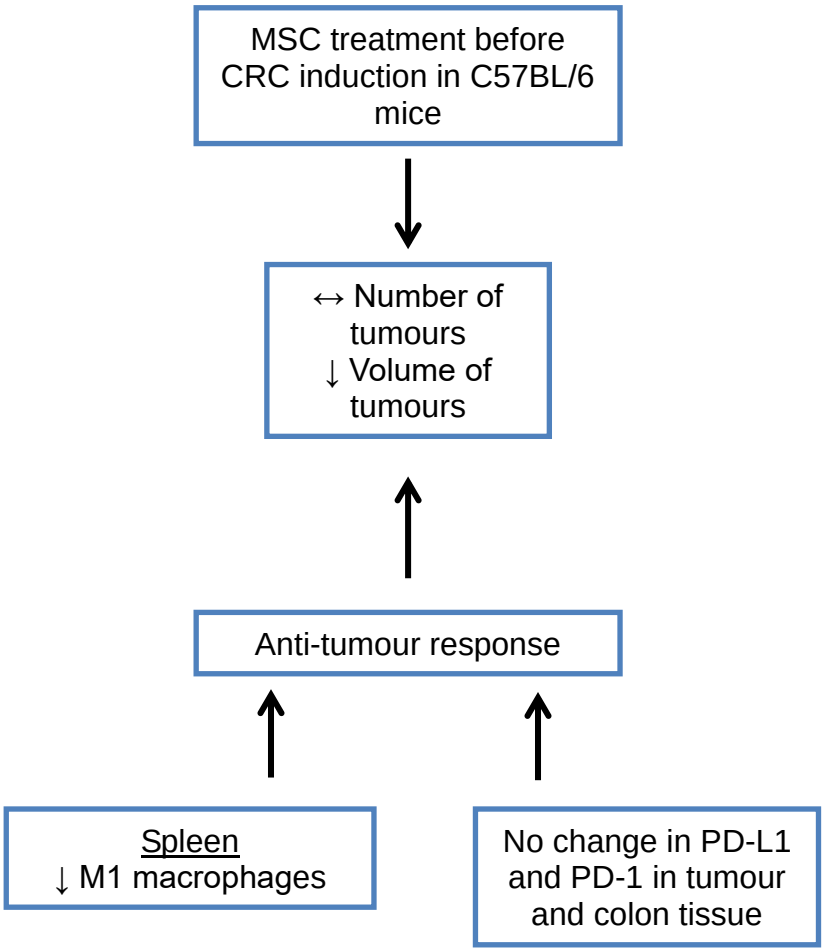

Figure 4.28: Summary of MSC treatment before CRC induction in C57BL/6 mice

PART C: MSC treatment after CRC induction in *Winnie* mice

4.5.9 Number and Volume of Tumours in *Winnie* Mice Treated With MSCs after CRC Induction

Winnie mice were treated with MSCs after being injected with MC38 cells into the caecum in an orthotopic colorectal cancer model (**Fig 4.29A**). The number of tumours in the caecum was similar in the MSC-treated mice (3.00±1.14) compared to the number of tumours in the CRC only mice (3.00±0.57) (**Fig 4.29B**). The volume of tumours had a tendency to be lower in the MSC-treated mice (2.87±1.05mm^3) compared to the CRC only mice (8.39±7.06mm^3) (**Fig 4.29B'**).

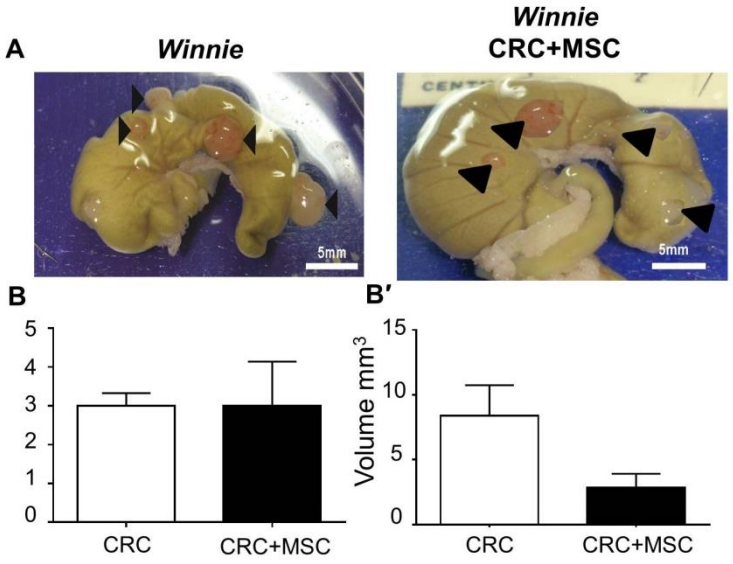

Figure 4.29: Number and Volume of Tumours in the Caecum from *Winnie* mice

Images **(A)** and analysis of the number (**B**) and volume (**B'**) of tumours in the caecum of *Winnie* mice at 3 weeks post implantation of MC38 colorectal tumour cells and with MSC treatment after CRC induction. Data represented as mean ± SEM, n=5 mice/group.

4.5.10 Immune Cell Infiltrate in the Spleen of *Winnie* mice treated with MSCs after CRC induction are Different compared to Controls

Flow cytometry analysis of the spleen showed significant differences between CRC only mice and the mice treated with MSCs after orthotopic injection of MC38 CRC. Pan leukocyte marker CD45 was used to identify total immune cell infiltration in the spleen and all results are stated as a percentage of $CD45^+$ cells. The percentage of T cells ($TCR\beta^+$ cells) shown an increased trend in the spleen of MSC-treated mice (30.57±2.57%) compared to CRC only mice (25.33±3.44%) although not significant (**Fig 4.30**). The percentage of neutrophils (defined as, $Ly6G^+$ and $Ly6C^+$ cells) also shown an increased trend in MSC-treated mice (3.76±0.52%) compared to CRC only mice (2.67±0.41%) (**Fig 4.31**). Interestingly, MSC-treated mice (12.37±0.70%) have a significantly higher percentage of $CD11b^+$ cells compared to CRC only mice (2.67±0.41%, $p<0.0001$) (**Fig 4.32A**). Further analysis of M1/M2 macrophages defined as $CD45^+CD11b^+F4/80^+MHC\text{-}II^+CD11c^+CD206^{+/-}$ showed the percentage of M1 macrophages in MSC-treated mice (73.77±0.74%) to be significantly higher compared to CRC only mice (53.36±2.45%, $p<0.0001$) (**Fig 4.32B**) and the percentage of M2 macrophages to be significantly decreased in MSC-treated mice (23.33±0.78%) compared to CRC only mice (46.60±2.31%, $p<0.0001$) (**Fig 4.32C**).

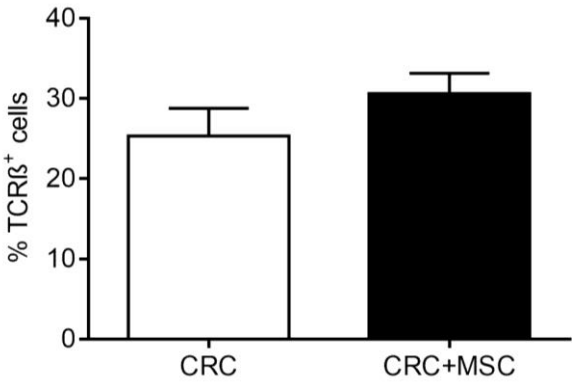

Figure 4.30: Percentage of T cells in the Spleen from *Winnie* mice

Quantitative analysis of the percentage of T cells (TCRβ$^+$ cells) in the spleen of *Winnie* mice with CRC only and mice treated with MSCs after CRC induction. Data represented as mean ± SEM, n=5 mice/group.

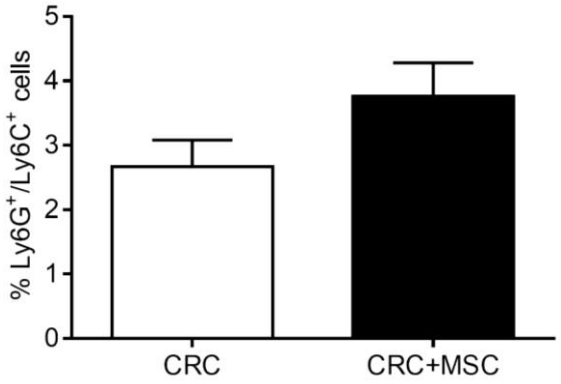

Figure 4.31: Percentage of Neutrophils in the Spleen from *Winnie* mice

Quantitative analysis of the percentage of neutrophils (Ly6G/Ly6C cells) in the spleen of *Winnie* mice with CRC only and mice treated with MSCs after CRC induction. Data represented as mean ± SEM, n=5 mice/group.

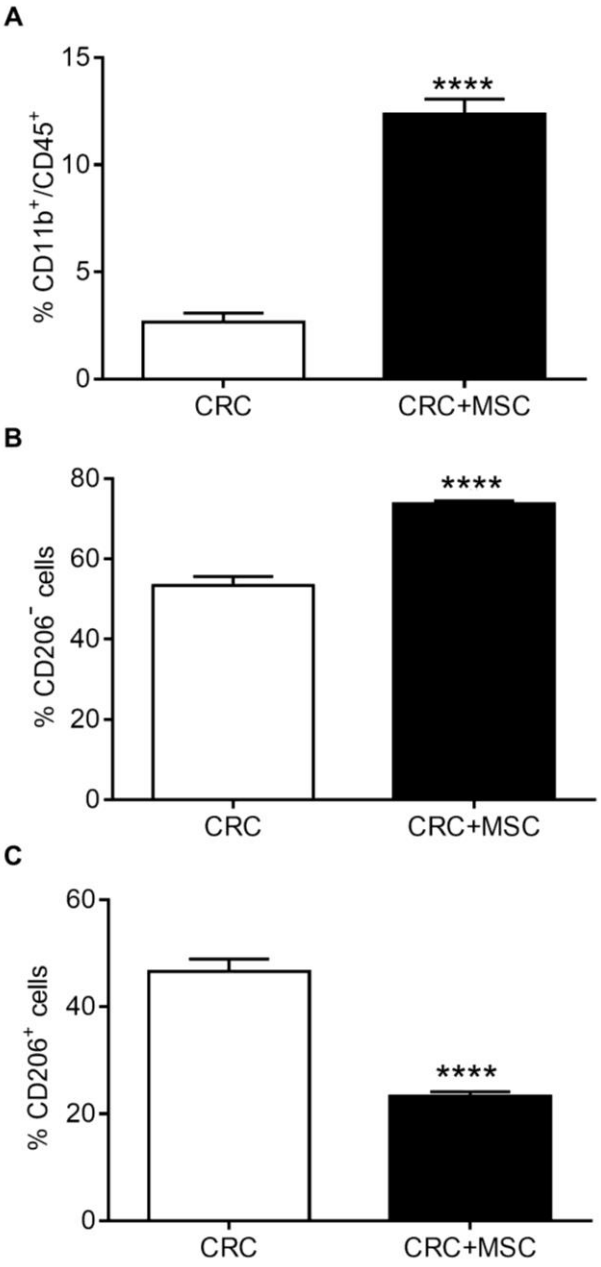

Figure 4.32: Percentage of CD11b^{+}/CD45^{+} Cells, M1 and M2 Macrophages in the Spleen from *Winnie* mice

Quantitative analysis of the percentage of CD11b^{+}/CD45^{+} cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the spleen of *Winnie* mice with CRC only and mice treated with MSCs after CRC induction. Data represented as mean ± SEM and statistical significance is indicated by asterisk, ****p<0.0001, n=5 mice/group.

4.5.11 Immune Cell Infiltrate in the Colon of *Winnie* mice with MSC treatment after CRC induction are No different to Controls

Flow cytometry analysis of the colon showed no differences in immune cell populations between CRC only mice and mice treated with MSCs following an orthotopic injection of MC38 colorectal cancer cells. Pan leukocyte marker CD45 was used to show total immune cell infiltration in the colon and all results are stated as a percentage of $CD45^+$ cells. The percentage of T cells (TCR^+ cells) in the MSC-treated mice (6.83±0.29%) tended to be lower compared to the CRC only group (11.41±10.68%), although not significant (**Fig 4.33**). Ly6G and Ly6C were used to enumerate neutrophils; MSC-treated mice had similar percentage of neutrophils (3.90±0.57%) compared to the CRC only mice (3.50±0.62%) (**Fig 4.34**). The percentage of $CD11b^+$ cells in the colon of MSC-treated mice (26.20±2.64%) show a lower trend compared to that of the CRC only mice (31.40±4.86%), although no significant (**Fig 4.35A**). No significant differences were noted in the percentage of M1 and M2 macrophages in MSC-treated mice (61.90±2.04% and 35.90±1.86%) compared to CRC only mice (61.23±3.00% and 35.67±2.95%) (**Fig 4.35B&C**).

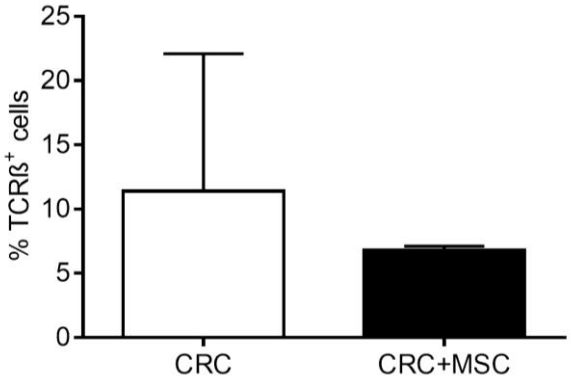

Figure 4.33: Percentage of T cells in the Colon from *Winnie* mice

Quantitative analysis of the percentage of T cells (TCRβ cells) in the colon of *Winnie* mice with CRC only and mice treated with MSCs after CRC induction. Data represented as mean ± SEM, n=5 mice/group.

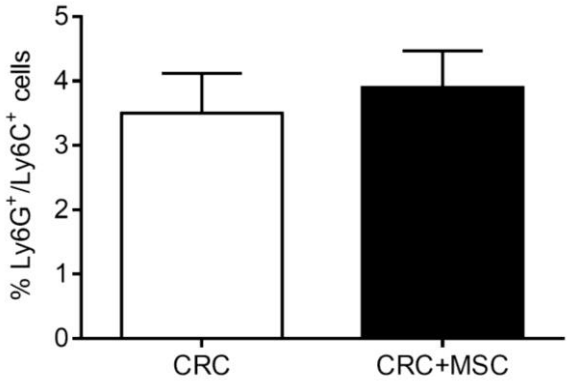

Figure 4.34: Percentage of Neutrophils in the Colon from *Winnie* mice

Quantitative analysis of the percentage of neutrophils ($Ly6G^+/Ly6C^+$ cells) in the colon of *Winnie* mice with CRC only and mice treated with MSCs after CRC induction. Data represented as mean ± SEM, n=5 mice/group.

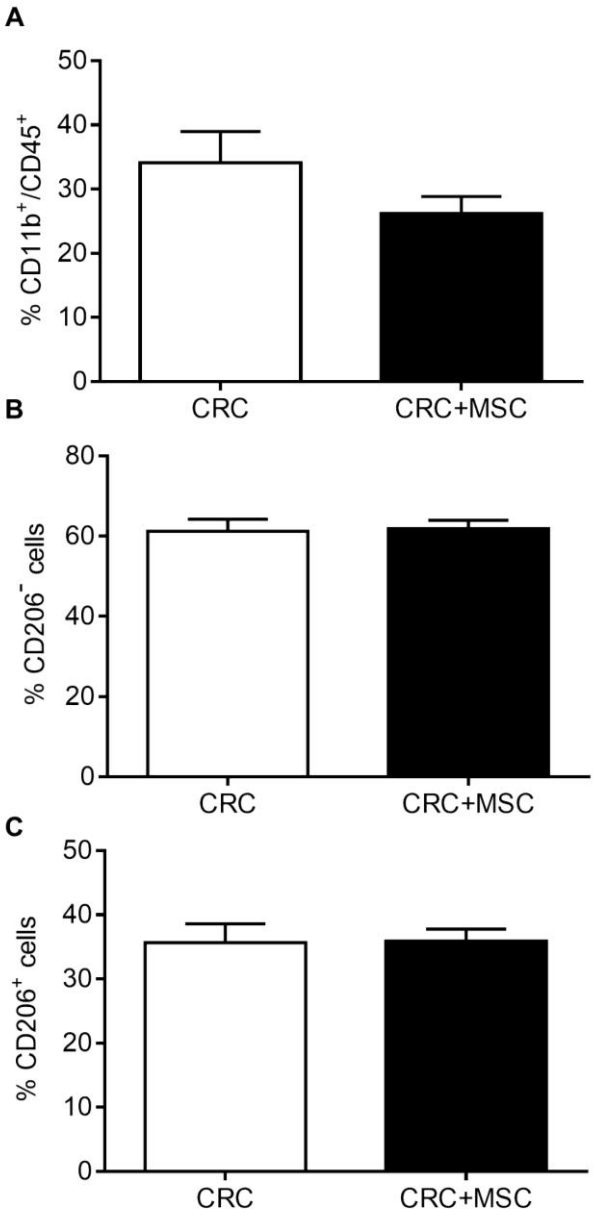

Figure 4.35: Percentage of CD11b^{+}/CD45^{+} Cells, M1 and M2 Macrophages in the Colon from *Winnie* mice

Quantitative analysis of the percentage of CD11b^{+}/CD45^{+} cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the colon of *Winnie* mice with CRC only and mice treated with MSCs after CRC induction. Data represented as mean ± SEM, n=5 mice/group.

4.5.12 PD-L1 and PD-1 Expression in Tumour and Colon Tissue of MSC-treated Mice after CRC Induction in *Winnie* Mice

The expression of PD-L1 in inflammatory disorders has been shown to be reduced, which leads to the activation of T cells ($CD8^+$). In the tumour microenvironment however the opposite is true with PD-L1 expression on tumour cells binding to PD-1 expressed on activated T cells leading to inhibitory signals being triggered and reduction in $CD8^+$ T cells and apoptosis. Therefore, the PD-1/PD-L1 interaction is an important regulator of the immune system in cancer and IBD. Tumour and colon tissues from MSC-treated mice were labelled for PD-1 and PD-L1 to determine the overall expression compared to CRC only mice. The expression of PD-L1 in the tumours of MSC-treated mice (4.30±0.6%) had a tendency to be decreased compared to the CRC only mice (6.32±2.03%) (**Fig 4.36**), PD-1 expression in the tumour showed a tendency to be higher in MSC-treated mice (0.94±0.24%) compared the CRC only mice (0.74±0.17%) (**Fig 4.37**) and PD-1 expression in the colon of MSC-treated mice (35.5±3.74%) was significantly lower compared to CRC only mice (56.5±4.57%) (**Fig 4.38**). The effect of MSC-treatment after CRC induction on the immune response in the spleen and colon of *Winnie* mice is summarised in **Fig 4.39**.

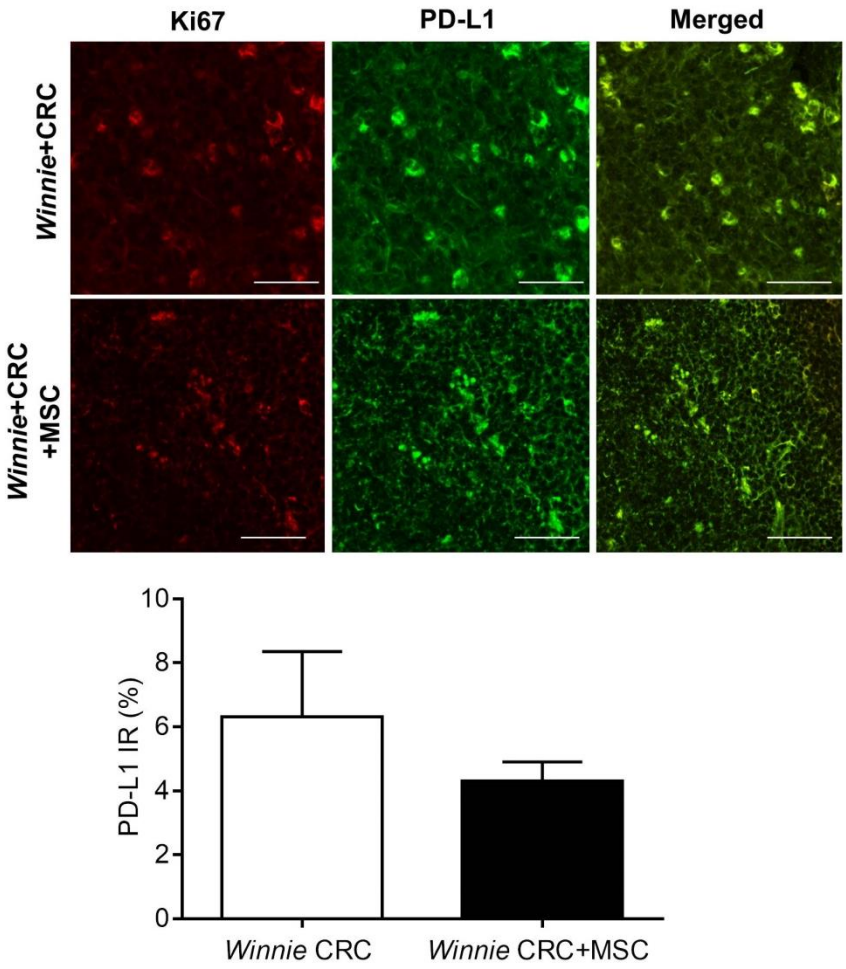

Figure 4.36: Immunohistological labelling of PD-L1 in Tumour Cross Sections from *Winnie* mice

Immunohistological labelling of PD-L1 (Green) and Ki67 (Red) and quantitative analysis of $Ki67^{+}$ $PD\text{-}L1^{+}$ cells in tumour sections found in the caecum of mice with CRC and MSC treatment after CRC induction, Data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm.

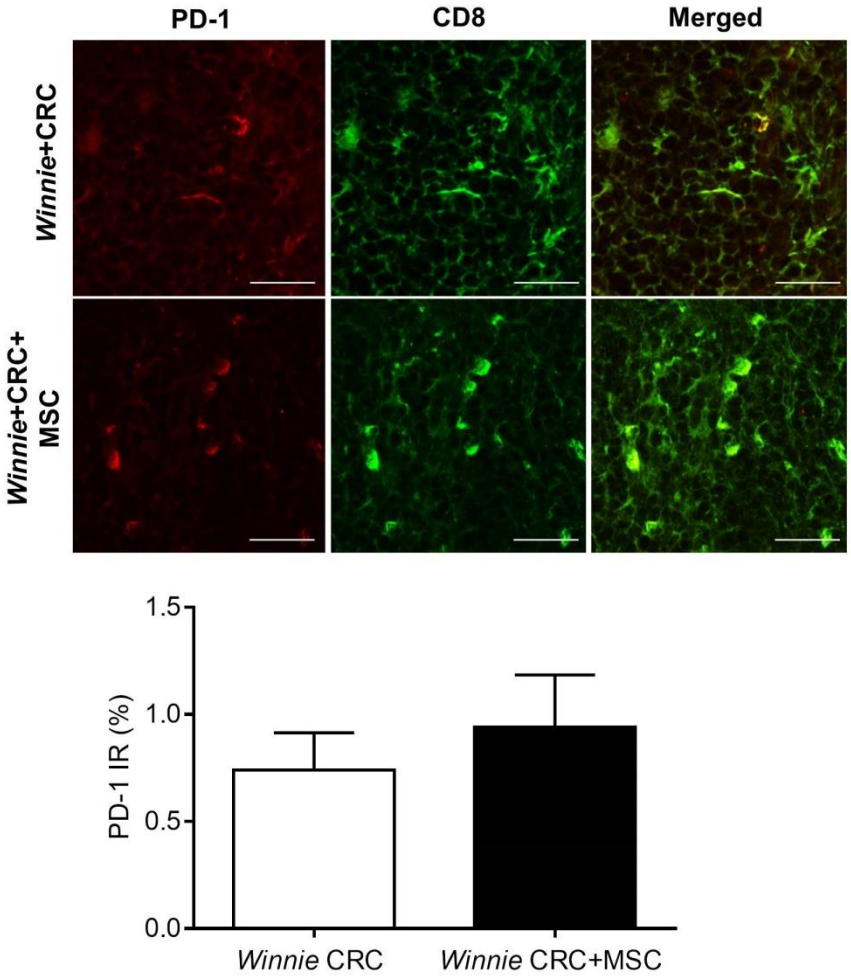

Figure 4.37: Immunohistological labelling of PD-1 in Tumour Cross Sections from *Winnie* mice

Immunohistological labelling of CD8 (Green) and PD-1 (Red) and quantitative analysis of $CD8^+$ $PD\text{-}1^+$ cells in tumour cross sections found in the caecum of mice with CRC and MSC treatment after CRC induction, data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm.

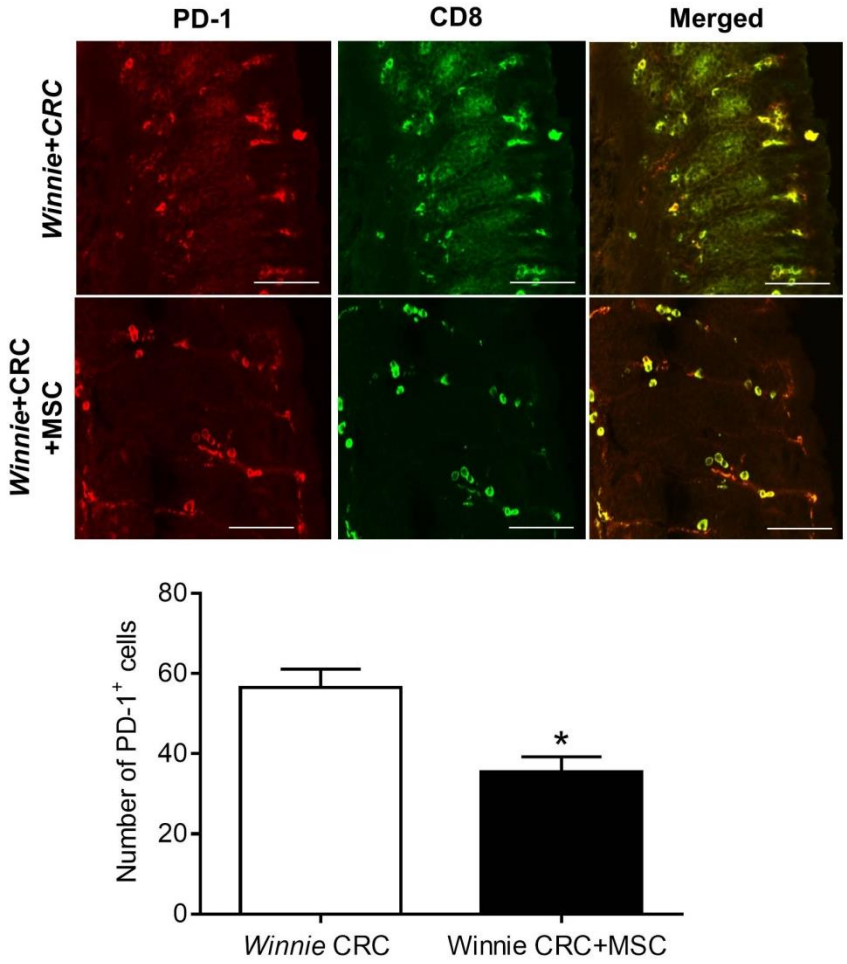

Figure 4.38: Immunohistological labelling of PD-1 in Colon Cross Sections from *Winnie* mice

Immunohistological labelling of CD8 (Green) and PD-1 (Red) and quantitative analysis of $CD8^+$ $PD\text{-}1^+$ cells in colon cross sections, Data represented as mean ± SEM and statistical significance is indicated by asterisk, *p<0.05, n=5 mice/group. Scale bar is 100 µm

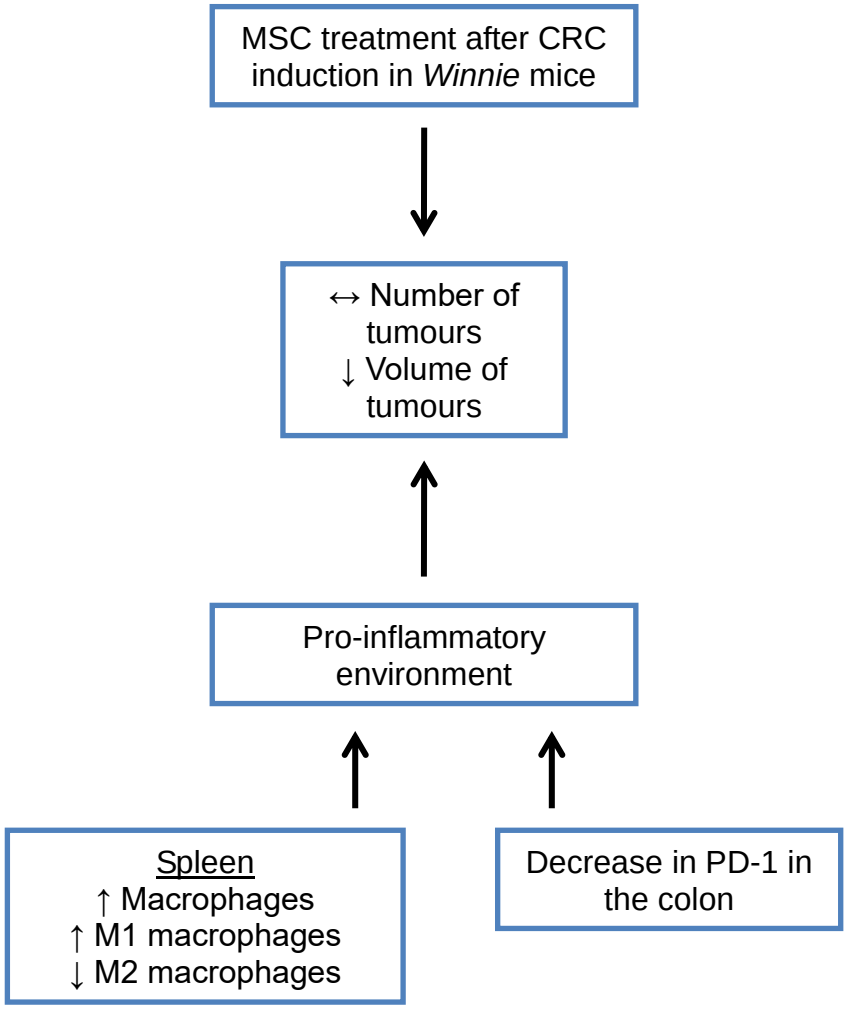

Figure 4.39: Summary of MSC treatment after CRC induction in *Winnie* mice

PART D: MSC treatment prior to CRC induction in *Winnie* mice

4.5.13 Number and Volume of Tumours in *Winnie* mice with MSC Treatment before CRC Induction

Winnie mice were treated with MSCs before being injected with MC38 cells into the caecum in an orthotopic colorectal cancer model (**Fig 4.40A**). The number of tumours in the caecum was tend to be lower in the MSC-treated mice (2.40±0.24) compared to the number of tumours in the CRC only mice but not significant (3.00±0.57) (**Fig 4.40B**). The volume of tumours also had a tendency to be lower in the MSC-treated mice (2.26±0.49mm^3) compared to the CRC only mice (8.39±7.06mm^3), but not significant (**Fig 4.40B'**).

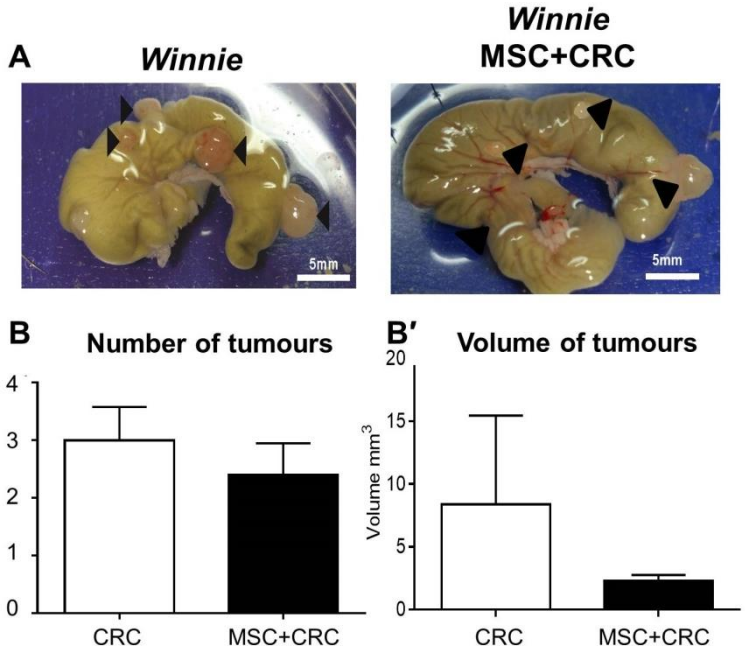

Figure 4.40: Number and Volume of Tumours in the Caecum from *Winnie* mice

Images **(A)** and analysis of the number (**B**) and volume (**B′**) of tumours in the caecum of *Winnie* mice at 3 weeks post implantation of MC38 colorectal tumour cells and with MSC treatment before CRC induction. Data represented as mean ± SEM, n=5 mice/group.

4.5.14 Immune Infiltrates in the Spleen of *Winnie* Mice with MSC Treatment before CRC Induction is Different Compared to Untreated Mice with CRC

Flow cytometry analysis of the spleen showed significant differences between CRC only mice and the mice treated with MSCs before orthotopic injection of MC38 CRC. Pan leukocyte marker CD45 was used to identify total immune cell infiltration in the spleen and all results are stated as a percentage of $CD45^+$ cells. The percentage of T cells ($TCR\beta^+$ cells) was significantly higher in the spleen of MSC-treated mice (35.30±2.10%) compared to CRC only mice (25.33±3.44%, $p<0.05$) (**Fig 4.41**). The percentage of neutrophils ($Ly6G^+$ and $Ly6C^+$ cells) in MSC-treated mice (2.45±0.05%) is similar compared to CRC only mice (2.67±0.41%) (**Fig 4.42**). Moreover MSC-treated mice (31.50±1.70%) have a significantly higher percentage of $CD11b^+$ cells compared to CRC only mice (8.13±0.44%, $p<0.05$) (**Fig 4.43A**). Further analysis showed no difference in the percentage of M1 macrophages in MSC-treated mice (62.03±6.40%) compared to CRC only mice (53.36±2.24%) (**Fig 4.43B**) and the percentage of M2 macrophages is similar in MSC-treated mice (38.05±11.15%) compared to CRC only mice (46.60±2.31%) (**Fig 4.43C**).

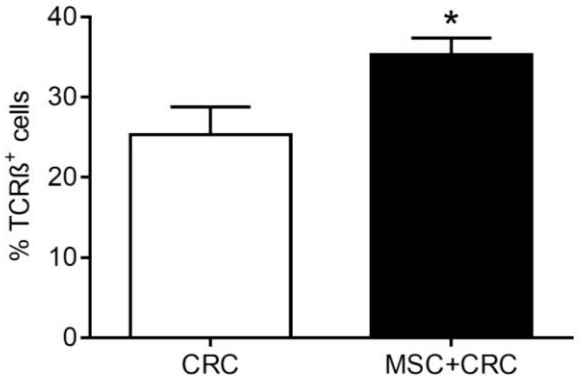

Figure 4.41: Percentage of T cells in the Spleen from *Winnie* mice

Quantitative analysis of the percentage of T cells ($TCR\beta^+$ cells) in the spleen of *Winnie* mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM and statistical significance is indicated by asterisk, **p*<0.05, n=5 mice/group.

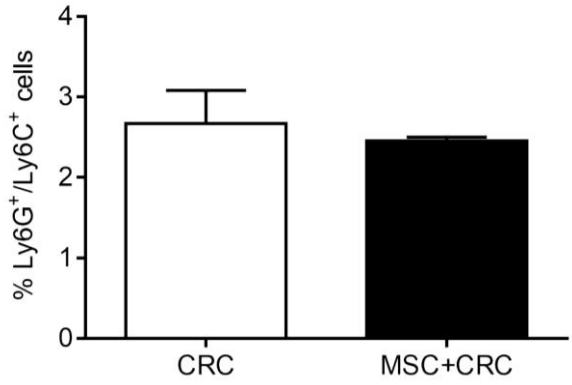

Figure 4.42: Percentage of Neutrophils in the Spleen from *Winnie* mice

Quantitative analysis of the percentage of neutrophils ($Ly6G^+/Ly6C^+$ cells) in the spleen of *Winnie* mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM, n=5 mice/group.

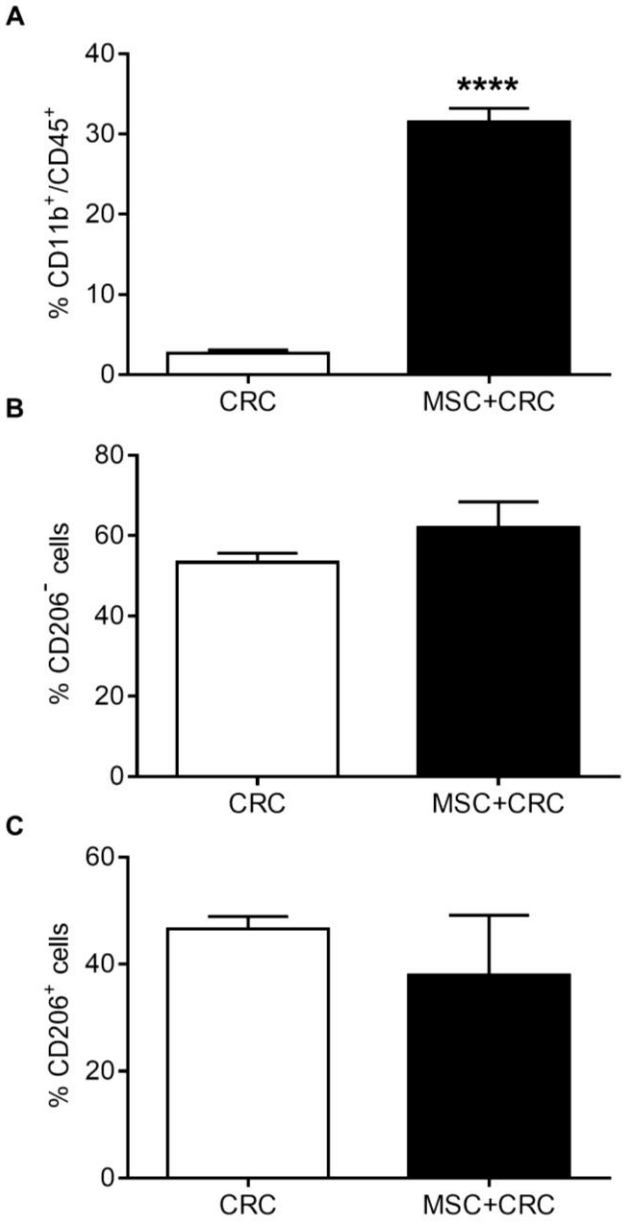

Figure 4.43: Percentage of CD11b$^+$/CD45$^+$ Cells, M1 and M2 Macrophages in the Spleen from *Winnie* mice

Quantitative analysis of the percentage of CD11b$^+$/CD45$^+$ cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the spleen of *Winnie* mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM and statistical significance is indicated by asterisk, ****p<0.0001, n=5 mice/group.

4.5.15 Immune Cell Infiltrates (M1/M2 macrophages) in the Colon of *Winnie* mice Treated with MSCs before CRC Induction are Different to Controls

Analysis of the colon using flow cytometry showed differences between the CRC only mice and mice treated with MSCs before orthotopic injection of MC38 colorectal cancer cells in particular M1/M2 macrophages defined as $CD45^{+}CD11b^{+}F4/80^{+}MHC\text{-}II^{+}CD11c^{+}CD206^{+/-}$. Pan leukocyte marker CD45 was used to show total immune cell infiltration in the colon and all results are stated as a percentage of $CD45^{+}$ cells. The percentage of T cells (TCR^{+} cells) in the MSC-treated mice (10.40±1.30%) was similar to the CRC only group (11.41±10.68%) (**Fig 4.44**). Ly6G and Ly6C were used to enumerate neutrophils; MSC-treated mice had a lower percentage of neutrophils (2.05±0.75%) compared to the CRC only mice (3.50±0.62%), but not significant (**Fig 4.45**). The percentage of $CD11b^{+}$ cells in the colon of MSC-treated mice (23.81±1.07%) was lower to that of the CRC only mice (31.40±4.86%), although not significant (**Fig 4.46A**). However, there was a significant decrease in the percentage of M1 macrophages in MSC-treated mice (43.25±2.35%) compared to the CRC only mice (61.23±3.00%) (**Fig 4.46B**). The percentage of M2 macrophages was significantly increased in MSC-treated mice (55.00±2.60%) compared the CRC only group (35.67±2.95%) (**Fig 4.46C**).

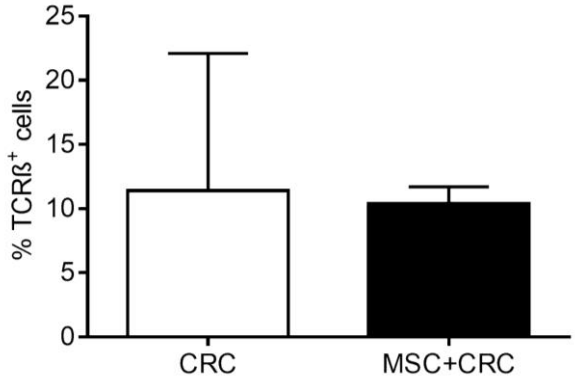

Figure 4.44: Percentage of T cells in the Colon from *Winnie* mice

Quantitative analysis of the percentage of T cells ($TCR\beta^+$ cells) in the colon of *Winnie* mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM, n=5 mice/group.

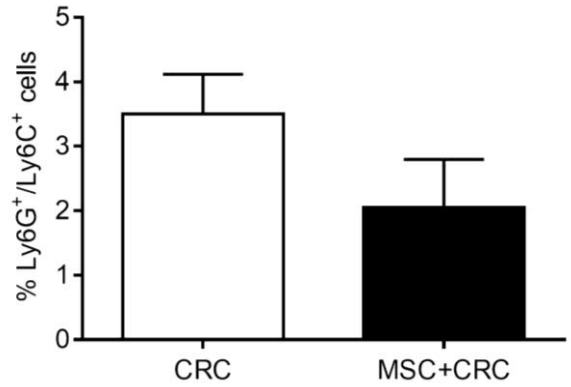

Figure 4.45: Percentage of Neutrophils in the Colon from *Winnie* mice

Quantitative analysis of the percentage of neutrophils ($Ly6G^+/Ly6C^+$ cells) in the colon of *Winnie* mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM, n=5 mice/group.

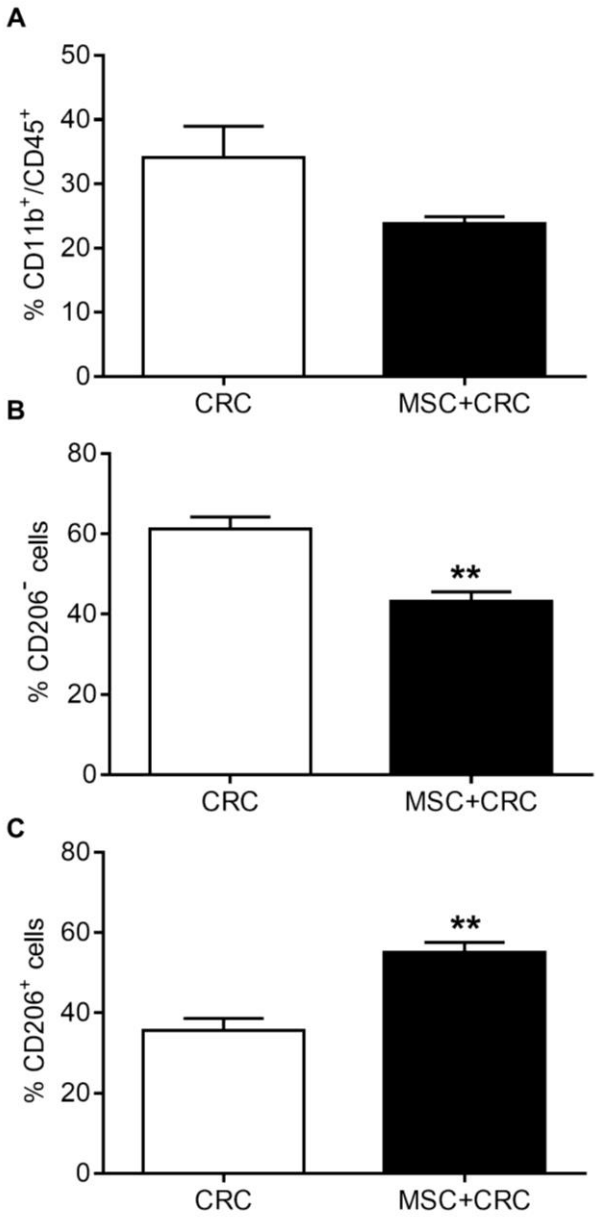

Figure 4.46: Percentage of CD11b^{+}/CD45^{+} Cells, M1 and M2 Macrophages in the Colon from *Winnie* mice

Quantitative analysis of the percentage of CD11b^{+}/CD45^{+} cells (**A**), M1 (**B**) and M2 macrophages (**C**) in the colon of *Winnie* mice with CRC only and mice treated with MSCs prior to CRC induction. Data represented as mean ± SEM and statistical significance is indicated by asterisk, ***p*<0.01, n=5 mice/group.

4.5.16 Expression of PD-L1 and PD-1 in the Tumour and Colon of *Winnie* Mice Treated With MSCs before CRC Induction

PD-L1 is expressed by tumour cells and upon binding to PD-1 (expressed on activated T cells) leads to inhibitory signals being triggered and a down regulation of $CD8^+$ T cells. Therefore, the PD-1/PD-L1 interaction is an important regulator of the immune system in cancer and IBD. Tumour tissue from the caecum and colon tissues from MSC-treated mice were labelled for PD-1 and PD-L1 to determine the overall expression compared to CRC only mice. The expression of PD-L1 in the tumours of MSC-treated *Winnie* mice (4.81±0.89%) had a tendency to be decreased compared to the CRC only mice (6.32±2.03%) (**Fig 4.47**). PD-1 expression in the tumour tissue also showed a tendency to be higher in MSC-treated mice (1±0.15%) compared to CRC only mice (0.74±0.17%) (**Fig 4.48**). PD-1 expression in the colon was significantly lower in the MSC-treated mice (26.75±1.68) compared to CRC only mice (56.5±4.57, $p<0.05$) (**Fig 4.49**). The images also show the co-localisation of PD-1 and CD8 and also PD-L1 and Ki67. The effect of MSC treatment after CRC induction on the immune response in the spleen and colon is summarised in **Fig 4.50**.

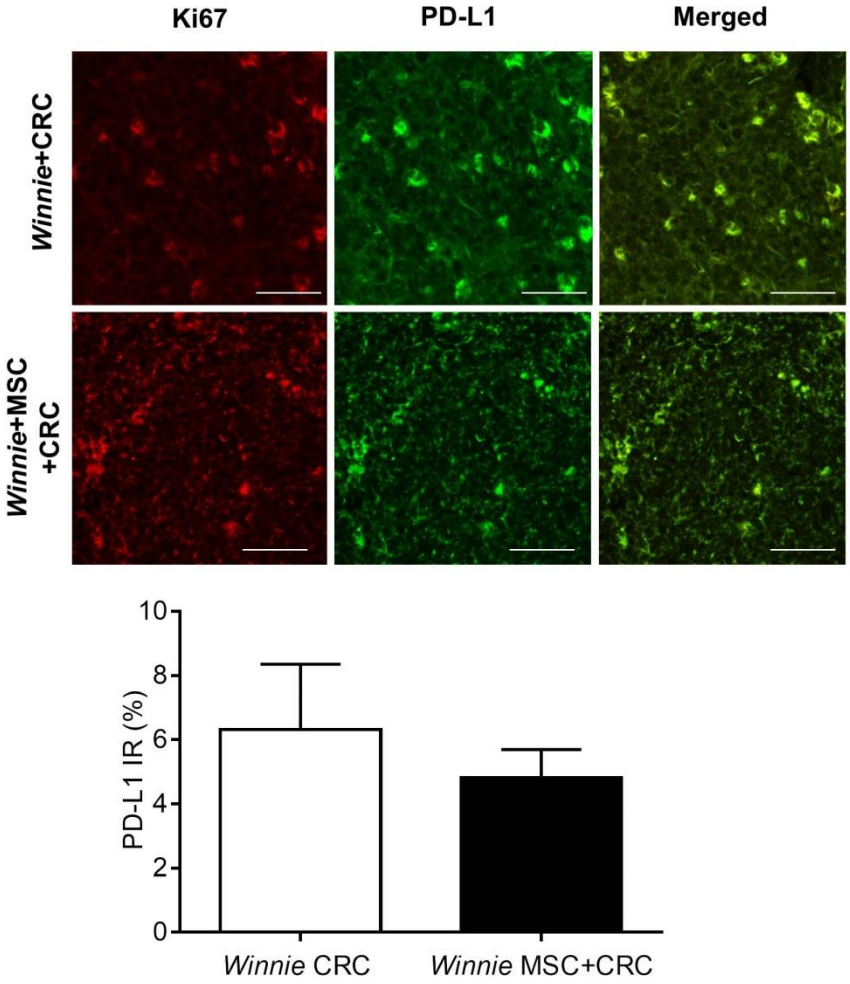

Figure 4.47: Immunohistological labelling of PD-L1 in Tumour Cross Sections from *Winnie* mice

Immunohistological labelling of PD-L1 (Green) and Ki67 (Red) and quantitative analysis of $Ki67^{+}$ $PD\text{-}L1^{+}$cells in tumour sections found in the caecum of mice with MSC treatment before CRC only induction. Data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm, IR - immunreactive.

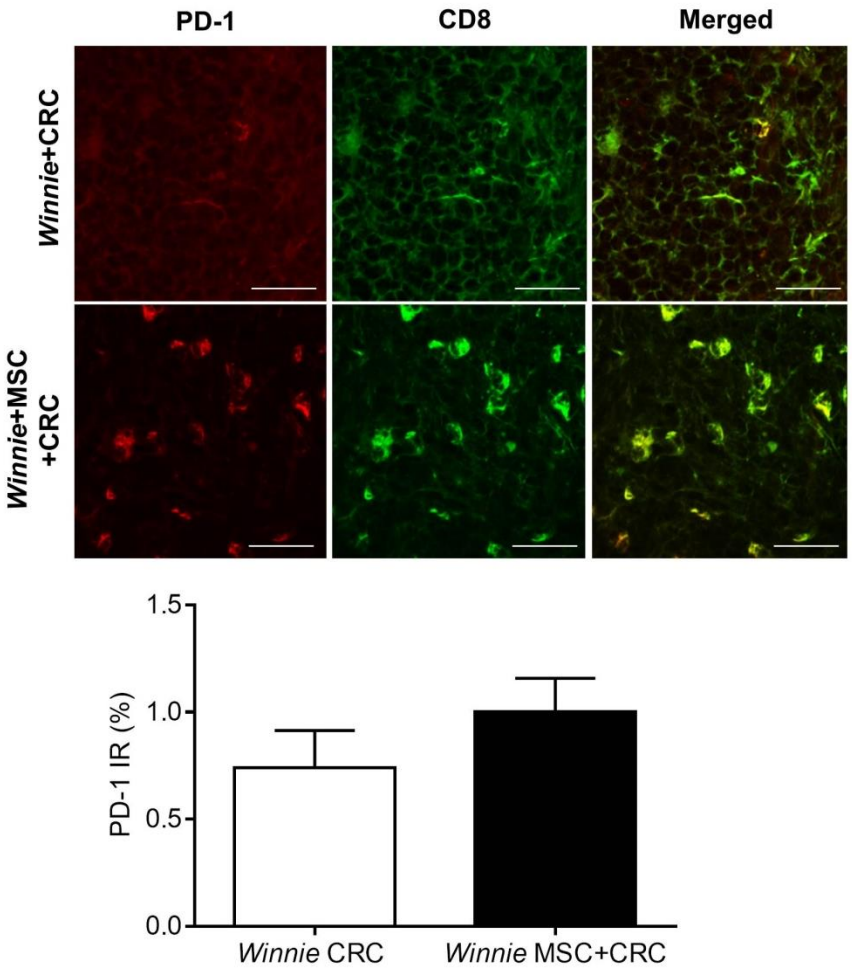

Figure 4.48: Immunohistological labelling of PD-1 in Tumour Cross Sections from *Winnie* mice

Immunohistological labelling of CD8 (Green) and PD-1 (Red) and quantitative analysis of $CD8^+$ $PD\text{-}1^+$ cells in tumour cross sections found in the caecum of mice with CRC only and MSC treatment before CRC induction. Data represented as mean ± SEM, n=5 mice/group. Scale bar is 50 µm.

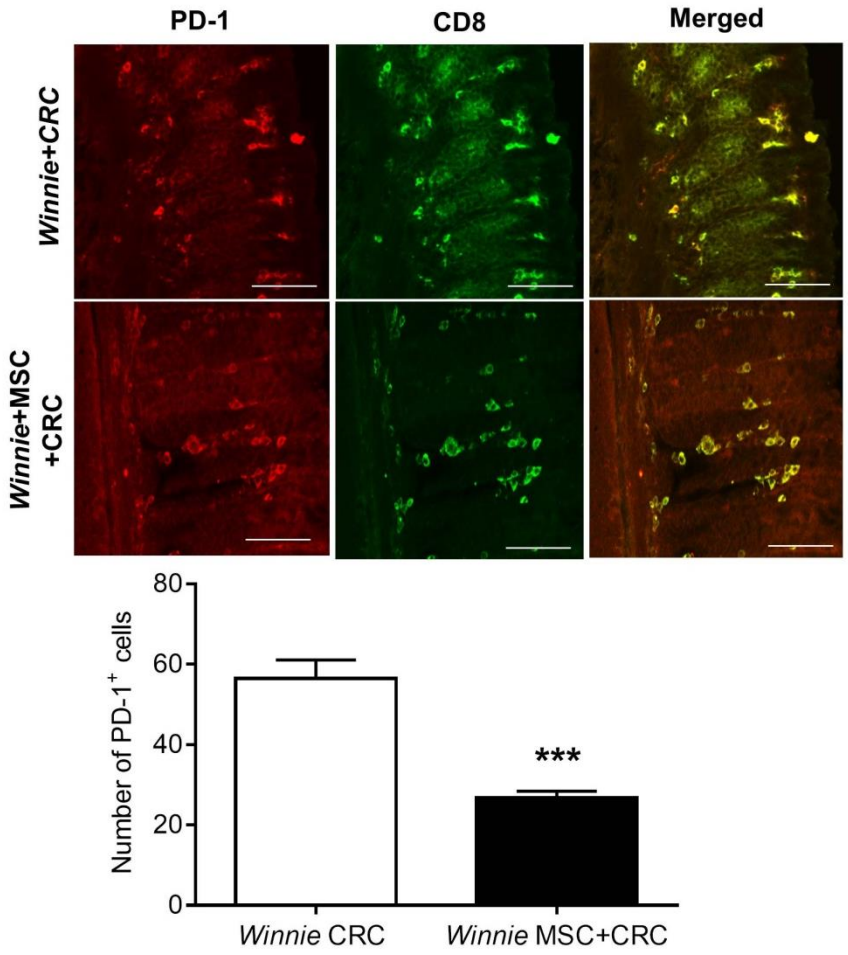

Figure 4.49: Immunohistological labelling of PD-1 in Colon Cross Sections from *Winnie* mice

Immunohistological labelling of CD8 (Green) and PD-1 (Red) and quantitative analysis of $CD8^+$ $PD\text{-}1^+$ cells in colon cross sections of mice with CRC only and MSC treatment before CRC induction. Data represented as mean ± SEM and statistical significance is indicated by asterisk, ***p<0.001, n=5 mice/group, Scale bar is 100 µm.

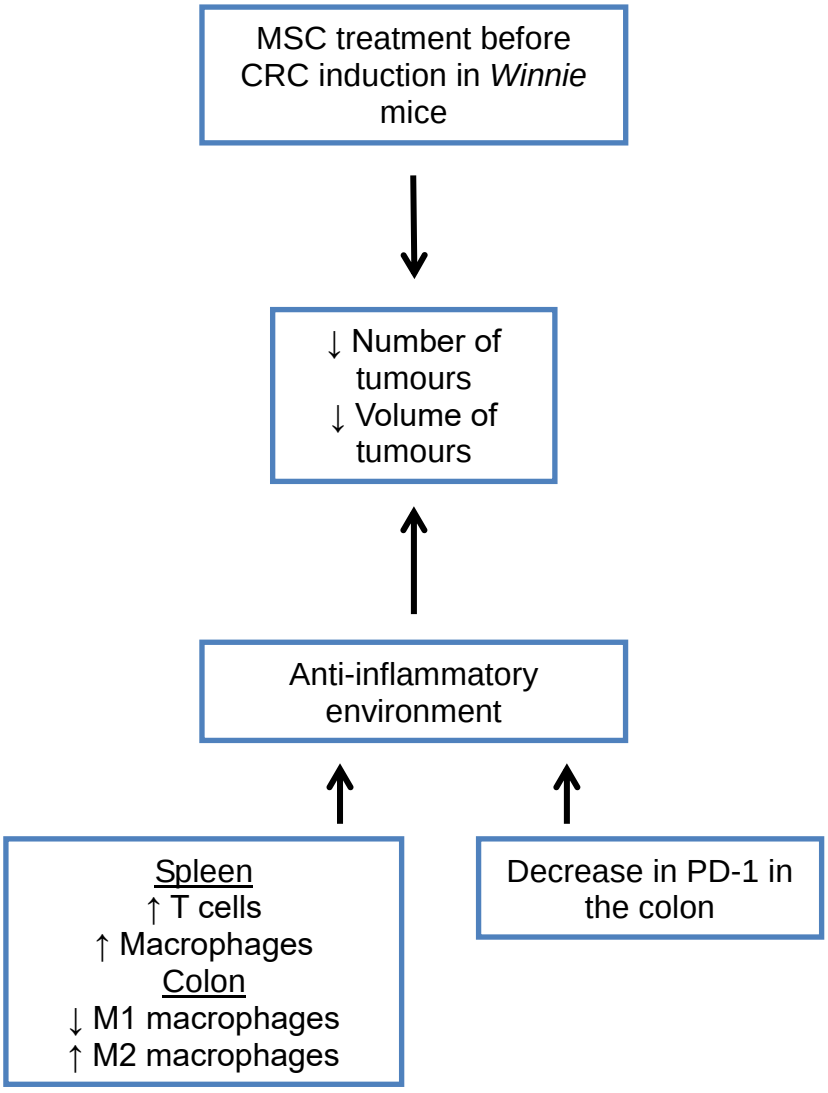

Figure 4.50: Summary of MSC treatment before CRC induction in *Winnie* mice

4.6 Discussion

Inflammation occurs in response to tissue damage that can result from infection, chemical irritation and trauma. In the early stages of inflammation, neutrophils migrate to the inflammatory site in response to chemical signals produced by resident macrophages and mast cells (Coussens and Werb, 2002). Other immune cells are then recruited by a complex network of signalling molecules including growth factors, cytokines and chemokines. Anti-inflammatory molecules also exist to allow resolution of inflammatory response, promoting tissue repair and the rapid programmed clearance of inflammatory cells via apoptosis and subsequent phagocytosis. When this process is dysregulated, chronic inflammation can occur. Lymphocytes and macrophages are the main immune cells in chronic inflammation. Macrophages are especially important in chronic inflammation as they are largely responsible for secreting growth factors, cytokines and reactive oxygen and nitrogen species, which during prolonged inflammation can lead to continuous tissue damage, subsequent sustained cell proliferation and hence the predisposition to tumour formation (Macarthur et al., 2004).

MSCs are an emerging therapy for IBD, they are derived from adult tissues including adipose and bone marrow and have the capacity to differentiate into osteoblasts, chondrocytes, adipocytes, myoblasts and neuronal like cells (Noort et al., 2002, Dalal et al., 2012). MSCs have immunosuppressive properties that are largely attributed to the number of anti-inflammatory cytokines they produce (Bartholomew et al., 2002, Di Nicola et al., 2002, Le Blanc et al., 2003, Dalal et al., 2012). MSCs have been reported to both promote tumour progression and tumour suppression. MSCs have been noted to promote tumour proliferation, fibroblast growth, angiogenesis and metastasis by providing immune suppression, cytoprotection, and vascular support largely by the production of paracrine factors and shown to promote the *in vivo* growth of colon cancer, lymphoma and melanoma (Klopp et al., 2011). MSCs have also been shown to promote anti-tumour effects; this has been noted in lung cancer, melanoma, colon carcinoma and human liver cancer cell lines (Maestroni et al., 1999). In this study when C57BL/6 mice were given MSC treatment after CRC induction

the number and volume of tumours was increased, however in the *Winnie* mice the number of tumours was very similar to what is seen in the control CRC only mice. MSC treatment before CRC induction resulted in a decrease in the number and volume of tumours in *Winnie* mice. In C57BL/6 mice MSC treatment before CRC induction showed no change in the number or volume of tumours. This suggests that MSCs provide a pro-tumourigenic environment when given after CRC induction that allows the tumour to grow, and when MSC treatment is given before CRC induction an anti-tumourigenic environment is seen with decreased tumour number and volume.

The immune system plays a vital role in tumour growth and suppression, hence having the ability to control the immune system is a major advantage for tumours. Tumours have been shown to have some immunosuppressive properties; numerous studies into cancer prognosis have been linked to the presence of immune cells (Pagès et al., 2005, Galon et al., 2006, Galon et al., 2007, Whiteside, 2008). MSCs have also been shown to have immunosuppressive properties; they have been shown to supress cytotoxic T cells (CD8) and T helper cells (CD4). Herein, MSC treatment after CRC induction showed in the *Winnie* mice a decrease in T cells, T cell suppression by MSCs has also been noted by Djouad et al (2012), where MSCs supressed T cells in a tumour environment. Both C57BL/6 and *Winnie* mice showed an increase in T cells with MSC treatment before CRC induction, which is indicative of an anti-tumour response.

Macrophages also play an important role in tumour growth or suppression; they are recruited to tumours by cytokines belonging to different classes including colony stimulating factor-1, vascular endothelial growth factor and chemokines (Mantovani, 1999, Biswas et al., 2006). In the tumour microenvironment mature macrophages express distinct functional properties, known as M1 and M2 macrophages. Classically, M1 macrophages are activated and induced by IFN-γ alone or in concert with microbial stimuli or cytokines, alternatively M2 macrophages are activated by IL-4, IL-13, IL-1 or IL-10 cytokines. M1 macrophages are cytotoxic towards tumour cells and M2 macrophages have the ability to supress inflammatory responses and Th1 adaptive immunity

(Mantovani et al., 2008). MSC treatment after CRC induction showed decrease in M1 macrophages in both the spleen and colon of C57BL/6 mice, however in the *Winnie* mice a significant increase in M1 macrophages was shown in the spleen. This indicates that, in a non-inflammatory environment like in the C57BL/6 mice, a decrease in M1 macrophages shows a pro-tumourigenic response, in an inflammatory environment such as that in *Winnie* mice, MSCs are promoting inflammation and the M1 macrophages do not appear to be having a cytotoxic effect towards tumour cells. As stated above, M2 macrophages have an anti-inflammatory phenotype, however tumour-associated macrophages also have an M2 phenotype and have been shown to promote tumour growth (Mantovani et al., 2002). MSC treatment after CRC induction in the C57BL/6 mice showed an increase in M2 macrophages in the colon; however in the *Winnie* mice a significant decrease in M2 macrophages was observed. In a non-inflammatory environment, such as the C57BL/6 mice, the increase in M2 macrophages is expected as they have been shown to promote tumour growth and progression. In an inflammatory environment such as the *Winnie* mice, M2 macrophages were significantly decreased, indicating that MSCs are supressing M2 macrophages and adapting to their environment, thus, MSCs appear to be promoting inflammation.

MSC treatment before CRC induction showed a significant decrease in percentage of M1 macrophages in the spleen of C57BL/6 mice. The decrease of M1 macrophages in the spleen could be indicative of a systemic response to the tumour. In the colon of C57BL/6 mice there is a trend of M1 macrophages increasing showing an anti-tumour response. In *Winnie* mice a significant decrease in M1 macrophages was found in the colon. In *Winnie* mice the significant decrease is more likely due to an anti-inflammatory response than an anti-tumour response; however decreasing the inflammatory environment can also affect tumour growth.

Neutrophils are also important in tumour development; similarly to macrophages neutrophils have both pro and anti-tumour roles (Treffers et al., 2016). Neutrophil-derived cytokines and proteins stored within granules may also play a dual role in tumour progression. Neutrophil elastase has been shown to be

taken up by epithelial lung cancer and favoured tumour proliferation (Sato et al., 2006). In our study a MSC treatment after CRC induction observed a trend where an increase in neutrophils was found in the spleen of both C57BL/6 and *Winnie* mice this could show an anti-tumour response, however as stated above neutrophils can have both pro and anti-tumuorigenic properties, this also shows that an inflammatory environment does not affect how neutrophils interact with tumour cells. MSC treatment before CRC induction showed a tendency for the percentage of neutrophils to decrease in the colon of both C57BL/6 and *Winnie* mice. The decrease of neutrophils could show an anti-tumour response as neutrophils and neutrophil granules can promote tumour growth.

Cytotoxic CD8 T cells express PD-1 receptors; these PD-1 receptors play a crucial role in the ability of tumour cells to evade the host immune system. PD-L1 (the ligand for PD-1) is found on tumour cells (Fife and Pauken, 2011, McDermott and Atkins, 2013). The interaction between PD-1 and PD-L1 plays an important role in the tumours ability to evade the immune system. If PD-L1 is increased this leads to a decrease in PD-1 and therefore a decrease in cytotoxic T cells. So therefore, in the cancer environment, where PD-L1 is increased cytotoxic T cells are decreased however, in the inflammatory environment this is reversed where PD-L1 is decreased and PD-1 is increased; this was shown is Chapter 3 in the *Winnie* mouse. MSC treatment before CRC induction showed a significant decrease in PD-1 in the colon in *Winnie* mice, this indicates a down-regulation of PD-1 in the colon and is consistent with the reduction of CD8 T cells in colon, however in tumour tissue in *Winnie* mice a decrease in PD-L1 and increase in PD-1 is shown, which indicates more CD8 T cells at the tumour site.

4.7 Conclusion

In conclusion this study demonstrated that MSC treatment after CRC induction displayed an increase in tumour number and volume in C57BL/6 mice and an

increase in tumour volume in *Winnie* mice. MSC treatment before CRC induction showed a no change in number of tumours and tumour volume in both C57BL/6 mice and *Winnie* mice. These results indicate that MSC treatment given after CRC induction leads to a pro-tumourigenic environment and MSC treatment given before CRC induction leads to an anti-tumourigenic environment. In terms of clinical applications, the use of MSCs to target cancer cells is not new, but this area is still inconclusive regarding MSCs therapeutic potential. The effect of MSCs appears to be mediated by the tumour microenvironment as shown above, which means the timing of MSC treatment and disease severity is very important to the clinical outcome. If MSCs are put into the tumour microenvironment they can have anti-cancer properties (Khakoo et al., 2006). Moreover, other studies have shown MSCs have the potential for use as an anti-cancer vehicle by exploiting their ability to migrate and proliferate at the tumour site (Serakinci et al., 2011). Although most studies have yielded positive results, the timing of MSC treatment when given to patients needs to be considered carefully. The results of my study show that the timing of the treatment is just as important as the treatment itself.

Chapter Five: Conclusion and Future Directions

5.1 Conclusion

IBD incidence in Australia is 24.2 per 100, 000 people and peak onset is 20 – 40 years of age (CCA, 2013, Studd et al., 2016). IBD is an idiopathic disease characterised by intestinal inflammation with potentially fatal complications.It is believed to be of multigenic origin and to occur as a result of an exaggerated immune response to gut bacteria (Becker et al., 2015). However, due to its unknown aetiology, current treatment strategies target the symptoms rather than the disease and are limited by factors including toxicity, inefficacy and adverse side-effects.

Cancer is often associated with chronic inflammation, as early in 1863, it was noticed the cancer often develops at inflammatory sites (Virchow, 1863). During chronic inflammation, macrophages are largely responsible for generating growth factors, cytokines and reactive oxygen and nitrogen species (Coussens and Werb, 2002). During prolonged inflammation these factors may lead to continuous tissue damage and subsequent sustained cell proliferation and hence the predisposition to cancer.

Mesenchymal stem cells are a new emerging therapy for IBD, current trials using MSCs have demonstrated that MSC therapy in IBD is both efficacious and feasible (García-Olmo et al., 2005, Garcia-Olmo et al., 2009a, Garcia-Olmo et al., 2009b, Cho et al., 2013, Lee et al., 2013). However, clinical trials have been focusing on the MSC treatment of fistulae caused by Crohn's disease (CD) rather than disease manifestations as a whole. The long term side-effects of MSC treatment use are still unknown. Many studies have shown that MSCs promote tumour progression but also can prevent tumour growth (Klopp et al., 2011).

The aims of this thesis were to investigate the effect of CT26 cells in an orthotopic model of CRC on immune cells in Peyer's patches and MLN and to identify cytokines produced by CT26 cells *in vitro* and *in vivo*; to determine specific immune cell populations in *Winnie* mice with spontaneous chronic colitis and to study the effects of CRC in the *Winnie* mice and lastly, to determine the effects of MSC treatment given before or after cancer induction in control and *Winnie* mice

5.1.1 Cytokines and immune populations in an orthotopic model of colorectal cancer

Chapter 2 of this thesis discussed the immune populations and cytokines in an orthotopic model of colorectal cancer and demonstrated an increase in IL-6 both *in vitro* and *in vivo.* IL-6 acts as both an anti-inflammatory and pro-inflammatory cytokine and in cancer patients elevated IL-6 leads to a poorer prognosis (Bellone et al., 2006). IL-6 is abundant in the tumour microenvironment where it plays a role in cancer metastasis via down regulation of E-cadherin and has also been shown to activate $CD8^+$ T cells to the tumour site (Fisher et al., 2011, Miao et al., 2014). It is likely that the IL-6 produced by CT26 cells in our studies contributed to its metastatic ability from the caecum to nearby colon. It has also been recently demonstrated that cancer cells and immune cells communicate via the presence of IL-6 in the tumour microenvironment which is secreted by both cancer cells and immune cells (Patel and Gooderham, 2015) and could explain the increase in immune cells noted in the mesenteric lymph nodes and Peyer's patches found in this study.

5.1.2 Immune populations in the *Winnie* mice with and without cancer

The *Winnie* mouse model of spontaneous chronic inflammation has a missense mutation in the Muc2 gene which leads to an intestinal barrier defect in these mice. A number of T cell defects are associated with IBD. In fact, in IBD patients excessive Th1 and Th17 responses are noted, as well as defective T cell responses (Boirivant et al., 1999, Ina et al., 1999, Brown and Mayer, 2007). Similarly, the results presented in Chapter 3 demonstrated that *Winnie* mice have both excessive Th1 and Th17 responses as shown by an increase in M1 macrophages.. Herein, it was shown that in both the spleen and colon there is a significant increase in the percentage of T cells compared to controls.

It is well known that chronic inflammation such as that seen in IBD is a risk factor in developing colorectal cancer. Inflammation also can play an important role in cancer progression which was explored in Chapter 3. by inducing cancer in *Winnie* mice. Colitis-associated cancers are generally infiltrated by an array

of immune cells from both the innate and adaptive systems. In colitis-associated cancer, the immune system seems to play a pro-tumourigenic role (Eiró and Vizoso, 2012, Grivennikov, 2013). However, in the *Winnie* mice with CRC an increase in M1 macrophages and a decrease in M2 macrophages in the colon was noted, indicating an anti-tumour response as M1 macropahge encourage tumour supression and M2 macrophages can promote tumour growth. This study also demonstrated an increase in T cells in the spleen and colon which can exert both tumour suppressive and tumour promoting properties as determined by their effector functions (Smyth et al., 2006, Langowski et al., 2007, DeNardo et al., 2009). Overal results in this thesis have demonstrated that CRC induction in *Winnie* mice is associated with an anti-tumour immune response. However, even though the immune system is trying to eliminate tumour cells, the environment was still conducive to tumour growth.

5.1.3 Effect of MSC treatment in orthotopic model of colorectal cancer in the *Winnie* mouse

MSCs have been shown to adapt to the environment in which they are placed, however the effect of MSC treatment on the tumour microenvironment is still contentious within the literature, with numerous studies pointing towards an anti-cancer effect and others leaning towards pro-tumuorigenic effect (Khakoo et al., 2006, Karnoub et al., 2007, Lazennec and Jorgensen, 2008, Qiao et al., 2008b, Zhu et al., 2009, Klopp et al., 2011). In Chapter 4 the effects of MSC treatment on immune populations in the cancer microenvironment in inflammatory (*Winnie* mice) and non-inflammatory conditions (C57BL/6 mice) were determined. MSC treatment after CRC induction revealed an increase in tumour number and volume in C57BL/6 mice. MSC treatment before CRC induction showed the tumour number and volume are similar in *Winnie* and C57BL/6 mice. Indicating that MSC treatment given after CRC induction leads to a pro-tumuorigenic environment and MSC treatment given before CRC induction leads to an anti-tumourigenic environment.

The tumour microenvironment can determine tumour progression and the immune system plays a vital role in this. MSC treatment after CRC induction

showed a decrease in M1 macrophages and increase in M2 macrophages in C57BL/6 and *Winnie* mice suggesting a pro-tumourigenic environment. Overall MSC treatment after CRC nduction in both C57BL/6 and *Winnie* mice promotes tumour growth and progression.

MSC treatment before CRC induction in C57BL/6 mice results in an increase in T cells and M1 macrophages indicating an anti-tumourigenic environment. In *Winnie* mice an increase in T cells and M2 macrophages and a decrease in M1 macrophages was found indicating an anti-inflammatory response, This suggests that overall MSC treatment given before CRC induction in both C57BL/6 and *Winnie* mice leads to an immune response that promotes an anti-tumour environment. This shows MSCs ability to adapt to the environment in which they are placed in is crucial in cancer development and if using MSCs to treat patients with chronic inflammation (such as IBD), timing of treatment and disease severity need to considered.

5.2 Future directions

To extend on the observations contained within this thesis, it is suggested that the following work in this area should be conducted:

5.2.1 The *Winnie* mouse as a model of IBD

The *Winnie* mouse model of spontaneous chronic inflammation is still quite a new animal model of IBD. The cause of IBD is still unknown, however accumulating evidence suggests that IBD results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host (Abraham and Cho, 2009). Studies by Eri et al (2011) have shown that the *Winnie* mice also have an abnormal mucosal barrier which causes spontaneous chronic inflammation making the *Winnie* mouse an optimal model of IBD. Further investigation into how IBD affects other systems and new treatments for IBD should be tested in *Winnie* mice as it is an optimal animal model of IBD.

5.2.2 Mesenchymal stem cells

The use of MSCs as a treatment for IBD is still quite a new therapy with promising effects being seen in human trials and animal models. This thesis has shown that MSCs have immunomodulatory effects and are able to change the immune environment in a therapeutic way which is consistent with other studies (Robinson et al., 2014).

Studies on the effect of MSCs over different time periods are very limited. It has been suggested that multiple MSC injections are necessary for an effect, but the duration of this effect can vary. Some trials reported that 88-100 % patients seeing effects 12 months post treatment but only 58 % patients reporting effects 3 years post treatment, suggesting that repeated treatment is required to maintain a therapeutic effect (Garcia-Olmo et al., 2009b, Lee et al., 2013). Although results presented in Chapter 4 demostrated that mutilple MSCs injections were effective, however results were obtained only from a short time period post treatment and further investigation into the long-term effects of MSC treatment is warranted.

MSCs have primarily been administered by local injection directly into the damaged or surrounding tissue. Systemic administration of MSCs has also been used in clinical trials treating luminal CD (Duijvestein et al., 2010, Forbes et al., 2014) and is the preferred route in studies of experimental colitis. Systemic administration may be the leading choice for MSC application as it is relatively non-invasive; however it may result in inefficient targeting of the pathological site of IBD. Even though MSCs have been shown to migrate to the site of inflammation, some studies in experimental colitis models have reported that systemically injected MSCs can accumulate in the lungs and other filtering organs (Hayashi et al., 2008, Liang et al., 2011). The efficacy of systemic treatment and its side-effects has not been studied in animal models of chronic colitis like *Winnie* mice.

As stated above MSCs have been shown to have immunomodulatory effects. As demonstrated in this thesis and many other studies, MSCs have a direct effect on cells in the immune system including T cells, macrophages, and neutrophils. MSCs have also been shown to secrete different factors, including cytokines, growth factors and chemokines. Our current understanding of the

interaction between immune cells and MSCs is that MSCs secrete factors activating immune cells and causing the immunodulatory effects seen after MSC treatment. However, whether this is the same in chronic inflammatory conditions still needs to be further determined.

5.2.3 Mesenchymal stem cells and cancer

As stated in this thesis, the effects of MSCs treatment in a tumour environment are still highly contentious in the literature. MSCs have been reported to migrate to tumour sites and sites of injury to incorporate into tissue stroma (Klopp et al., 2011). The interaction between MSCs and tumour cells and the mechanisms underlying this interaction are unclear. Whether MSCs lead to tumour development or supress it is still being debated (Lazennec and Jorgensen, 2008, Klopp et al., 2011). Many studies indicate that MSCs have an anti-tumour effect and supress tumour growth (Khakoo et al., 2006, Qiao et al., 2008b) while other studies report that MSCs have a pro-tumourigenic effect (Karnoub et al., 2007, Zhu et al., 2009). Chronic inflammation is a risk factor strongly linked to cancer (Coussens and Werb, 2002). MSCs have also been shown to secrete a variety of factors that are influenced by their environment (Bai et al., 2012).

Further studies into the mechanisms of MSC interaction in a cancer environment with or without inflammation is needed. Future studies should include using a higher dose of MSCs and replicate the experiments in Chapter 4 to see if the effects are different or heightened. Furthermore, longer time periods should also be investigated to see if the MSCs lose their effect in the cancer environment over time. MSCs should also be tested in different cancers not just colorectal cancer as they have been shown previously and in this thesis to adapt to their environment and may have different effects on different cancers.

5.3 General Conclusions

In conclusion the data presented in this thesis demonstrated that a chronic inflammatory environment such as that in IBD is a risk factor in the development of cancer shown in Chapter 3 when *Winnie* mice injected with CRC cells had

significantly increased number of tumours compared to the group without inflammation. As mentioned above MSCs adapt to the environment they are placed in and can secrete different factors. In an inflammatory environment MSCs have been shown to have an anti-inflammatory effect, however in a tumour microenvironment the role MSCs is contentious with many studies showing MSCs have an anti-tumour effect and others showing a pro-tumour effect (Karnoub et al., 2007, Otsu et al., 2009, Dasari et al., 2010). However, this thesis revealed that MSC treatment given after CRC induction, in both inflammatory and non-inflammatory environments stimulates the immune response promoting tumour growth. In contrary, when MSC treatment is given before CRC induction in both inflammatory and non-inflammatory environments the immune system suppresses tumour growth. Overall, the studies in this thesis show that while MSCs are effective at neutralising inflammation, other factors such as the timing of treatment and cancer risk factors need to be considered before the use of MSCs as a therapy for IBD.

Chapter Six: References

ABERRA, F. N., LEWIS, J. D., HASS, D., ROMBEAU, J. L., OSBORNE, B. & LICHTENSTEIN, G. R. 2003. Corticosteroids and immunomodulators: postoperative infectious complication risk in inflammatory bowel disease patients. *Gastroenterology,* 125**,** 320-327.

ABRAHAM, C. & CHO, J. H. 2009. Inflammatory Bowel Disease. *The New England Journal of Medicine,* 361**,** 2066-2078.

ABREU, M. T. 2002. The pathogenesis of inflammatory bowel disease: translational implications for clinicians. *Current Gastroenterology Reports,* 4**,** 481-489.

ADELSTEIN, B.-A., MACASKILL, P., CHAN, S. F., KATELARIS, P. H. & IRWIG, L. 2011. Most bowel cancer symptoms do not indicate colorectal cancer and polyps: a systematic review. *BMC Gastroenterology,* 11**,** 65.

ALLAVENA, P., SICA, A., SOLINAS, G., PORTA, C. & MANTOVANI, A. 2008. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Critical Reviews in Oncology/Hematology,* 66**,** 1-9.

ANDERSON, P., SOUZA-MOREIRA, L., MORELL, M., CARO, M., O'VALLE, F., GONZALEZ-REY, E. & DELGADO, M. 2012. Adipose-derived mesenchymal stromal cells induce immunomodulatory macrophages which protect from experimental colitis and sepsis. *Gut,* 63**,** 1131-1141.

ANDO, Y., INABA, M., SAKAGUCHI, Y., TSUDA, M., QUAN, G. K., OMAE, M., OKAZAKI, K. & IKEHARA, S. 2008. Subcutaneous adipose tissue–derived stem cells facilitate colonic mucosal recovery from 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)–induced colitis in rats. *Inflammatory Bowel Diseases,* 14**,** 826-838.

ANDRES, P. G. & FRIEDMAN, L. S. 1999. Epidemiology and the natural course of inflammatory bowel disease. *Gastroenterology Clinics of North America,* 28**,** 255-281.

APOSTOLOPOULOS, V., OSINSKI, C. & MCKENZIE, I. F. 1998. MUC1 cross-reactive Galα (l, 3) Gal antibodies in humans switch immune responses from cellular to humoral. *Nature Medicine,* 4**,** 315-320.

ARNOLD, G. L., BEAVES, M. R., PRYJDUN, V. O. & MOOK, W. J. 2002. Preliminary study of ciprofloxacin in active Crohn's disease. *Inflammatory Bowel Diseases,* 8**,** 10-15.

ASSEMAN, C., MAUZE, S., LEACH, M. W., COFFMAN, R. L. & POWRIE, F. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *The Journal of Experimental Medicine,* 190**,** 995-1004.

BAI, L., LENNON, D. P., CAPLAN, A. I., DECHANT, A., HECKER, J., KRANSO, J., ZAREMBA, A. & MILLER, R. H. 2012. Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. *Nature Neuroscience,* 15**,** 862-870.

BAILEY, J. R., BLAND, P. W., TARLTON, J. F., PETERS, I., MOORGHEN, M., SYLVESTER, P. A., PROBERT, C. S. & WHITING, C. V. 2012. IL-13 promotes collagen accumulation in Crohn's disease fibrosis by down-regulation of fibroblast MMP synthesis: A role for innate lymphoid cells? *PLoS One,* 7**,** e52332.

BALKWILL, F. & MANTOVANI, A. 2001. Inflammation and cancer: back to Virchow? *Lancet,* 357**,** 539-45.

BALKWILL, F. R. & MANTOVANI, A. Cancer-related inflammation: common themes and therapeutic opportunities. Seminars in Cancer Biology, 2012. Elsevier, 33-40.
BAMIAS, G., NYCE, M. R., SARAH, A. & COMINELLI, F. 2005. New concepts in the pathophysiology of inflammatory bowel disease. *Annals of Internal Medicine,* 143**,** 895-904.
BARIBAULT, H., PENNER, J., IOZZO, R. V. & WILSON-HEINER, M. 1994. Colorectal hyperplasia and inflammation in keratin 8-deficient FVB/N mice. *Genes & Development,* 8**,** 2964-2973.
BARTHOLOMEW, A., STURGEON, C., SIATSKAS, M., FERRER, K., MCINTOSH, K., PATIL, S., HARDY, W., DEVINE, S., UCKER, D. & DEANS, R. 2002. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Experimental Hematology,* 30**,** 42-48.
BAUMGART, D. C. & CARDING, S. R. 2007. Inflammatory bowel disease: cause and immunobiology. *The Lancet,* 369**,** 1627-1640.
BECKER, C., NEURATH, M. F. & WIRTZ, S. 2015. The intestinal microbiota in inflammatory bowel disease. *ILAR Journal,* 56**,** 192-204.
BELIZÁRIO, J. E. 2009. Immunodeficient mouse models: an overview. *Open Immunol J,* 2**,** 79-85.
BELLONE, G., SMIRNE, C., MAURI, F. A., TONEL, E., CARBONE, A., BUFFOLINO, A., DUGHERA, L., ROBECCHI, A., PIRISI, M. & EMANUELLI, G. 2006. Cytokine expression profile in human pancreatic carcinoma cells and in surgical specimens: implications for survival. *Cancer Immunology, Immunotherapy,* 55**,** 684-698.
BERGMAN, M., DJALDETTI, M., SALMAN, H. & BESSLER, H. 2011. Inflammation and colorectal cancer: does aspirin affect the interaction between cancer and immune cells? *Inflammation,* 34**,** 22-28.
BERNINK, J. H., PETERS, C. P., MUNNEKE, M., TE VELDE, A. A., MEIJER, S. L., WEIJER, K., HREGGVIDSDOTTIR, H. S., HEINSBROEK, S. E., LEGRAND, N. & BUSKENS, C. J. 2013. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nature Immunology,* 14**,** 221-229.
BIBBY, M. 2004. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. *European Journal of Cancer,* 40**,** 852-857.
BISWAS, S. K., GANGI, L., PAUL, S., SCHIOPPA, T., SACCANI, A., SIRONI, M., BOTTAZZI, B., DONI, A., VINCENZO, B. & PASQUALINI, F. 2006. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). *Blood,* 107**,** 2112-2122.
BLANK, C. & MACKENSEN, A. 2007. Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. *Cancer Immunology, Immunotherapy,* 56**,** 739-745.
BOIRIVANT, M., MARINI, M., DI FELICE, G., PRONIO, A. M., MONTESANI, C., TERSIGNI, R. & STROBER, W. 1999. Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis. *Gastroenterology,* 116**,** 557-565.
BOLAND, C. R. & GOEL, A. 2010. Microsatellite instability in colorectal cancer. *Gastroenterology,* 138**,** 2073-2087. e3.

BONNEVILLE, M., O'BRIEN, R. L. & BORN, W. K. 2010. γδ T cell effector functions: a blend of innate programming and acquired plasticity. *Nature Reviews Immunology,* 10**,** 467-478.
BOTTINO, C., MORETTA, L., PENDE, D., VITALE, M. & MORETTA, A. 2004. Learning how to discriminate between friends and enemies, a lesson from natural killer cells. *Molecular Immunology,* 41**,** 569-575.
BOUMA, G. & STROBER, W. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nature Reviews Immunology,* 3**,** 521-533.
BRAND, S. 2009. Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. *Gut,* 58**,** 1152-1167.
BRATTAIN, M. G., STROBEL-STEVENS, J., FINE, D., WEBB, M. & SARRIF, A. M. 1980. Establishment of mouse colonic carcinoma cell lines with different metastatic properties. *Cancer Research,* 40**,** 2142-2146.
BROWN, S. J. & MAYER, L. 2007. The immune response in inflammatory bowel disease. *The American Journal of Gastroenterology,* 102**,** 2058-2069.
BUISINE, M., DESREUMAUX, P., LETEURTRE, E., COPIN, M., COLOMBEL, J., PORCHET, N. & AUBERT, J. 2001. Mucin gene expression in intestinal epithelial cells in Crohn's disease. *Gut,* 49**,** 544-551.
BURDON, T. J., PAUL, A., NOISEUX, N., PRAKASH, S. & SHUM-TIM, D. 2010. Bone marrow stem cell derived paracrine factors for regenerative medicine: current perspectives and therapeutic potential. *Bone Marrow Research,* 2011.
CAMMA, C., GIUNTA, M., ROSSELLI, M. & COTTONE, M. 1997. Mesalamine in the maintenance treatment of Crohn's disease: a meta-analysis adjusted for confounding variables. *Gastroenterology,* 113**,** 1465-1473.
CAPPELL, M. S. 2008. Pathophysiology, clinical presentation, and management of colon cancer. *Gastroenterology Clinics of North America,* 37**,** 1-24.
CARLSON, M., RAAB, Y., SEVEUS, L., XU, S., HÄLLGREN, R. & VENGE, P. 2002. Human neutrophil lipocalin is a unique marker of neutrophil inflammation in ulcerative colitis and proctitis. *Gut,* 50**,** 501-506.
CARR, I. & MAYBERRY, J. F. 1999. The effects of migration on ulcerative colitis: a three-year prospective study among Europeans and first-and second-generation South Asians in Leicester (1991–1994). *The American Journal of Gastroenterology,* 94**,** 2918-2922.
CASELLAS, F., BORRUEL, N., PAPO, M., GUARNER, F., ANTOLIN, M., VIDELA, S. & MALAGELADA, J.-R. 1998. Antiinflammatory effects of enterically coated amoxicillin-clavulanic acid in active ulcerative colitis. *Inflammatory Bowel Diseases,* 4**,** 1-5.
CASTELO-BRANCO, M., SOARES, I., LOPES, D. V., BUONGUSTO, F., MARTINUSSO, C. A., DO ROSARIO JR, A., SOUZA, S., GUTFILEN, B., FONSECA, L. & ELIA, C. 2012. Intraperitoneal but not intravenous cryopreserved mesenchymal stromal cells home to the inflamed colon and ameliorate experimental colitis. *PLoS One,* 7**,** e33360.
CCA 2013. Improving Inflammatory Bowel Disease care across Australia Crohn's & Colitis Australia.
CELLA, M., FUCHS, A., VERMI, W., FACCHETTI, F., OTERO, K., LENNERZ, J. K., DOHERTY, J. M., MILLS, J. C. & COLONNA, M. 2009. A human

natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature,* 457**,** 722-725.
CHAUHAN, D., UCHIYAMA, H., AKBARALI, Y., URASHIMA, M., YAMAMOTO, K., LIBERMANN, T. A. & ANDERSON, K. C. 1996. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B. *Blood,* 87**,** 1104-12.
CHEN, J., LI, Y., KATAKOWSKI, M., CHEN, X., WANG, L., LU, D., LU, M., GAUTAM, S. C. & CHOPP, M. 2003. Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. *Journal of Neuroscience Research,* 73**,** 778-786.
CHEN, Z., HE, X., HE, X., CHEN, X., LIN, X., ZOU, Y., WU, X. & LAN, P. 2014. Bone marrow mesenchymal stem cells ameliorate colitis-associated tumorigenesis in mice. *Biochem Biophys Res Commun,* 450**,** 1402-8.
CHO, Y. B., LEE, W. Y., PARK, K. J., KIM, M., YOO, H.-W. & YU, C. S. 2013. Autologous adipose tissue-derived stem cells for the treatment of Crohn's fistula: a phase I clinical study. *Cell Transplantation,* 22**,** 279-285.
CHUNG, Y. C. & CHANG, Y. F. 2003. Serum interleukin-6 levels reflect the disease status of colorectal cancer. *Journal of Surgical Oncology,* 83**,** 222-226.
CICCOCIOPPO, R., BERNARDO, M. E., SGARELLA, A., MACCARIO, R., AVANZINI, M. A., UBEZIO, C., MINELLI, A., ALVISI, C., VANOLI, A. & CALLIADA, F. 2011. Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. *Gut,* 60**,** 788-798.
COCA, S., PEREZ-PIQUERAS, J., MARTINEZ, D., COLMENAREJO, A., SAEZ, M. A., VALLEJO, C., MARTOS, J. A. & MORENO, M. 1997. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer,* 79**,** 2320-2328.
COHEN, R. D. 2010. The pharmacoeconomics of biologic therapy for IBD. *Nature Reviews Gastroenterology and Hepatology,* 7**,** 103-109.
CONNICK, P., KOLAPPAN, M., CRAWLEY, C., WEBBER, D. J., PATANI, R., MICHELL, A. W., DU, M.-Q., LUAN, S.-L., ALTMANN, D. R. & THOMPSON, A. J. 2012. Autologous mesenchymal stem cells for the treatment of secondary progressive multiple sclerosis: an open-label phase 2a proof-of-concept study. *The Lancet Neurology,* 11**,** 150-156.
CORCIONE, A., BENVENUTO, F., FERRETTI, E., GIUNTI, D., CAPPIELLO, V., CAZZANTI, F., RISSO, M., GUALANDI, F., MANCARDI, G. L. & PISTOIA, V. 2006. Human mesenchymal stem cells modulate B-cell functions. *Blood,* 107**,** 367-372.
CORVAISIER, M., MOREAU-AUBRY, A., DIEZ, E., BENNOUNA, J., MOSNIER, J.-F., SCOTET, E., BONNEVILLE, M. & JOTEREAU, F. 2005. Vγ9Vδ2 T cell response to colon carcinoma cells. *The Journal of Immunology,* 175**,** 5481-5488.
COSNES, J., GOWER–ROUSSEAU, C., SEKSIK, P. & CORTOT, A. 2011. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology,* 140**,** 1785-1794. e4.
COUSIN, B., RAVET, E., POGLIO, S., DE TONI, F., BERTUZZI, M., LULKA, H., TOUIL, I., ANDRÉ, M., GROLLEAU, J.-L. & PÉRON, J.-M. 2009. Adult

stromal cells derived from human adipose tissue provoke pancreatic cancer cell death both in vitro and in vivo. *PLoS One,* 4**,** e6278.
COUSSENS, L. M. & WERB, Z. 2002. Inflammation and cancer. *Nature,* 420**,** 860-7.
CZÉH, M., LODDENKEMPER, C., SHALAPOUR, S., SCHÖN, C., ROBINE, S., GOLDSCHEID, E., STEIN, H., SCHÜLER, T., WILLIMSKY, G. & BLANKENSTEIN, T. 2010. The immune response to sporadic colorectal cancer in a novel mouse model. *Oncogene,* 29**,** 6591-6602.
DALAL, J., GANDY, K. & DOMEN, J. 2012. Role of mesenchymal stem cell therapy in Crohn's disease. *Pediatric Research,* 71**,** 445-451.
DASARI, V. R., KAUR, K., VELPULA, K. K., GUJRATI, M., FASSETT, D., KLOPFENSTEIN, J. D., DINH, D. H. & RAO, J. S. 2010. Upregulation of PTEN in glioma cells by cord blood mesenchymal stem cells inhibits migration via downregulation of the PI3K/Akt pathway. *PloS One,* 5**,** e10350.
DAVIS, B. P. & ROTHENBERG, M. E. 2014. Eosinophils and cancer. *Cancer Immunology Research,* 2**,** 1-8.
DE BOECK, A., PAUWELS, P., HENSEN, K., RUMMENS, J. L., WESTBROEK, W., HENDRIX, A., MAYNARD, D., DENYS, H., LAMBEIN, K., BRAEMS, G., GESPACH, C., BRACKE, M. & DE WEVER, O. 2013. Bone marrow-derived mesenchymal stem cells promote colorectal cancer progression through paracrine neuregulin 1/HER3 signalling. *Gut,* 62**,** 550-60.
DE PALMA, M., MURDOCH, C., VENNERI, M. A., NALDINI, L. & LEWIS, C. E. 2007. Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. *Trends in Immunology,* 28**,** 519-524.
DEN HAAN, J. M., LEHAR, S. M. & BEVAN, M. J. 2000. CD8+ but not CD8− dendritic cells cross-prime cytotoxic T cells in vivo. *The Journal of Experimental Medicine,* 192**,** 1685-1696.
DENARDO, D. G., BARRETO, J. B., ANDREU, P., VASQUEZ, L., TAWFIK, D., KOLHATKAR, N. & COUSSENS, L. M. 2009. CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell,* 16**,** 91-102.
DI NICOLA, M., CARLO-STELLA, C., MAGNI, M., MILANESI, M., LONGONI, P. D., MATTEUCCI, P., GRISANTI, S. & GIANNI, A. M. 2002. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood,* 99**,** 3838-3843.
DIEFENBACH, A., COLONNA, M. & KOYASU, S. 2014. Development, differentiation, and diversity of innate lymphoid cells. *Immunity,* 41**,** 354-365.
DIELEMAN, L. A., RIDWAN, B. U., TENNYSON, G. S., BEAGLEY, K. W., BUCY, R. P. & ELSON, C. O. 1994. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology-Orlando,* 107**,** 1643-1652.
DJOUAD, F., PLENCE, P., BONY, C., TROPEL, P., APPARAILLY, F., SANY, J., NOËL, D. & JORGENSEN, C. 2003. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood,* 102**,** 3837-3844.
DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. & HORWITZ, E. 2006. Minimal criteria for defining multipotent

mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy,* 8**,** 315-317.
DONNENBERG, M. S. 2000. Pathogenic strategies of enteric bacteria. *Nature,* 406**,** 768-774.
DUIJVESTEIN, M., VOS, A. C. W., ROELOFS, H., WILDENBERG, M. E., WENDRICH, B. B., VERSPAGET, H. W., KOOY-WINKELAAR, E. M., KONING, F., ZWAGINGA, J. J. & FIDDER, H. H. 2010. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut,* 59**,** 1662-1669.
EADEN, J., ABRAMS, K. & MAYBERRY, J. 2001. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut,* 48**,** 526-535.
EIRÓ, N. & VIZOSO, F. J. 2012. Inflammation and cancer. *World J Gastrointest Surg,* 4**,** 62-72.
ELINAV, E., NOWARSKI, R., THAISS, C. A., HU, B., JIN, C. & FLAVELL, R. A. 2013. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nature Reviews Cancer,* 13**,** 759-771.
ELSON, C. O., CONG, Y., MCCRACKEN, V. J., DIMMITT, R. A., LORENZ, R. G. & WEAVER, C. T. 2005. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunological Reviews,* 206**,** 260-276.
ELSON, C. O., CONG, Y., WEAVER, C. T., SCHOEB, T. R., MCCLANAHAN, T. K., FICK, R. B. & KASTELEIN, R. A. 2007. Monoclonal anti–interleukin 23 reverses active colitis in a T Cell–mediated model in mice. *Gastroenterology,* 132**,** 2359-2370.
ERDELYI, I., LEVENKOVA, N., LIN, E. Y., PINTO, J. T., LIPKIN, M., QUIMBY, F. W. & HOLT, P. R. 2009. Western-style diets induce oxidative stress and dysregulate immune responses in the colon in a mouse model of sporadic colon cancer. *The Journal of Nutrition,* 139**,** 2072-2078.
ERI, R., ADAMS, R., TRAN, T., TONG, H., DAS, I., ROCHE, D., OANCEA, I., PNG, C. W., JEFFERY, P. & RADFORD-SMITH, G. L. 2011. An intestinal epithelial defect conferring ER stress results in inflammation involving both innate and adaptive immunity. *Mucosal Immunology,* 4**,** 354-364.
ERRENI, M., MANTOVANI, A. & ALLAVENA, P. 2011. Tumor-associated macrophages (TAM) and inflammation in colorectal cancer. *Cancer Microenvironment,* 4**,** 141-154.
FARRELL, R. J. & LAMONT, J. T. 2002. Microbial factors in inflammatory bowel disease. *Gastroenterology Clinics of North America,* 31**,** 41-62.
FAWZY, S. A., ABO-ELNOU, R. K. E.-D. & EL, D. F. A.-E.-M. 2013. The possible role of mesenchymal stem cells therapy in the repair of experimentally induced colitis in male albino rats. *International Journal of Stem Cells,* 6**,** 92.
FEHRES, C. M., UNGER, W. W., GARCIA-VALLEJO, J. J. & VAN KOOYK, Y. 2014. Understanding the biology of antigen cross-presentation for the design of vaccines against cancer. *Frontiers in Immunology,* 5**,** 149.
FERLAZZO, G., THOMAS, D., LIN, S.-L., GOODMAN, K., MORANDI, B., MULLER, W. A., MORETTA, A. & MÜNZ, C. 2004. The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. *The Journal of Immunology,* 172**,** 1455-1462.

FIFE, B. T. & PAUKEN, K. E. 2011. The role of the PD-1 pathway in autoimmunity and peripheral tolerance. *Annals of the New York Academy of Sciences,* 1217**,** 45-59.
FINN, O. J. 2008. Cancer immunology. *New England Journal of Medicine,* 358**,** 2704-2715.
FISHER, D. T., CHEN, Q., SKITZKI, J. J., MUHITCH, J. B., ZHOU, L., APPENHEIMER, M. M., VARDAM, T. D., WEIS, E. L., PASSANESE, J. & WANG, W.-C. 2011. IL-6 trans-signaling licenses mouse and human tumor microvascular gateways for trafficking of cytotoxic T cells. *The Journal of Clinical Investigation,* 121**,** 3846-3859.
FLATMARK, K., MÆLANDSMO, G. M., MARTINSEN, M., RASMUSSEN, H. & FODSTAD, Ø. 2004. Twelve colorectal cancer cell lines exhibit highly variable growth and metastatic capacities in an orthotopic model in nude mice. *European Journal of Cancer,* 40**,** 1593-1598.
FODDE, R., KUIPERS, J., ROSENBERG, C., SMITS, R., KIELMAN, M., GASPAR, C., VAN ES, J. H., BREUKEL, C., WIEGANT, J. & GILES, R. H. 2001. Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nature Cell Biology,* 3**,** 433-438.
FOGH, J., FOGH, J. M. & ORFEO, T. 1977. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *Journal of the National Cancer Institute,* 59**,** 221-226.
FOLKMAN, J. 1971. Tumor angiogenesis: therapeutic implications. *N Engl J Med,* 285**,** 1182-6.
FORBES, G. M., STURM, M. J., LEONG, R. W., SPARROW, M. P., SEGARAJASINGAM, D., CUMMINS, A. G., PHILLIPS, M. & HERRMANN, R. P. 2014. A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clinical Gastroenterology and Hepatology,* 12**,** 64-71.
FREEDMAN, M. S., BAR-OR, A., ATKINS, H. L., KARUSSIS, D., FRASSONI, F., LAZARUS, H., SCOLDING, N., SLAVIN, S., LE BLANC, K. & UCCELLI, A. 2010. The therapeutic potential of mesenchymal stem cell transplantation as a treatment for multiple sclerosis: consensus report of the International MSCT Study Group. *Multiple Sclerosis,* 16**,** 503-510.
FRIES, W., BELVEDERE, A. & VETRANO, S. 2013. Sealing the broken barrier in IBD: intestinal permeability, epithelial cells and junctions. *Current Drug Targets,* 14**,** 1460-1470.
FUCHS, A., VERMI, W., LEE, J. S., LONARDI, S., GILFILLAN, S., NEWBERRY, R. D., CELLA, M. & COLONNA, M. 2013. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12-and IL-15-responsive IFN-γ-producing cells. *Immunity,* 38**,** 769-781.
FUJINO, S., ANDOH, A., BAMBA, S., OGAWA, A., HATA, K., ARAKI, Y., BAMBA, T. & FUJIYAMA, Y. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut,* 52**,** 65-70.
FUSS, I. J. 2003. Cytokine network in inflammatory bowel disease. *Current Drug Targets-Inflammation & Allergy,* 2**,** 101-112.
GALDIERO, M. R., BONAVITA, E., BARAJON, I., GARLANDA, C., MANTOVANI, A. & JAILLON, S. 2013. Tumor associated macrophages and neutrophils in cancer. *Immunobiology,* 218**,** 1402-1410.
GALON, J., COSTES, A., SANCHEZ-CABO, F., KIRILOVSKY, A., MLECNIK, B., LAGORCE-PAGÈS, C., TOSOLINI, M., CAMUS, M., BERGER, A. &

WIND, P. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science,* 313**,** 1960-1964.
GALON, J., FRIDMAN, W.-H. & PAGÈS, F. 2007. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. *Cancer Research,* 67**,** 1883-1886.
GARCÍA-OLMO, D., GARCÍA-ARRANZ, M., HERREROS, D., PASCUAL, I., PEIRO, C. & RODRÍGUEZ-MONTES, J. A. 2005. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. *Diseases of the Colon & Rectum,* 48**,** 1416-1423.
GARCÍA-OLMO, D., HERREROS, D., DE-LA-QUINTANA, P., GUADALAJARA, H., TRÉBOL, J., GEORGIEV-HRISTOV, T. & GARCÍA-ARRANZ, M. 2010. Adipose-derived stem cells in Crohn's rectovaginal fistula. *Case Reports in Medicine,* 2010**,** 1-3.
GARCIA-OLMO, D., HERREROS, D., PASCUAL, I., PASCUAL, J. A., DEL-VALLE, E., ZORRILLA, J., DE-LA-QUINTANA, P., GARCIA-ARRANZ, M. & PASCUAL, M. 2009a. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. *Diseases of the Colon & Rectum,* 52**,** 79-86.
GARCIA-OLMO, D., HERREROS, D., PASCUAL, M., PASCUAL, I., DE-LA-QUINTANA, P., TREBOL, J. & GARCIA-ARRANZ, M. 2009b. Treatment of enterocutaneous fistula in Crohn's disease with adipose-derived stem cells: a comparison of protocols with and without cell expansion. *International Journal of Colorectal Disease,* 24**,** 27-30.
GEREMIA, A., ARANCIBIA-CÁRCAMO, C. V., FLEMING, M. P., RUST, N., SINGH, B., MORTENSEN, N. J., TRAVIS, S. P. & POWRIE, F. 2011. IL-23–responsive innate lymphoid cells are increased in inflammatory bowel disease. *Journal of Experimental Medicine***,** jem. 20101712.
GEVERS, D., KUGATHASAN, S., DENSON, L. A., VÁZQUEZ-BAEZA, Y., VAN TREUREN, W., REN, B., SCHWAGER, E., KNIGHTS, D., SONG, S. J. & YASSOUR, M. 2014. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host & Microbe,* 15**,** 382-392.
GLADIATOR, A., WANGLER, N., TRAUTWEIN-WEIDNER, K. & LEIBUNDGUT-LANDMANN, S. 2013. Cutting edge: IL-17–secreting innate lymphoid cells are essential for host defense against fungal infection. *The Journal of Immunology,* 190**,** 521-525.
GNECCHI, M., HE, H., NOISEUX, N., LIANG, O. D., ZHANG, L., MORELLO, F., MU, H., MELO, L. G., PRATT, R. E., INGWALL, J. S. & DZAU, V. J. 2006. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *Faseb J,* 20**,** 661-9.
GOLDBERG, R., PRESCOTT, N., LORD, G. M., MACDONALD, T. T. & POWELL, N. 2015. The unusual suspects [mdash] innate lymphoid cells as novel therapeutic targets in IBD. *Nature Reviews Gastroenterology & Hepatology,* 12**,** 271-283.
GONZALEZ-REY, E. & DELGADO, M. 2014. Therapeutic Application of Mesenchymal Stromal Cells in Murine Models of Inflammatory Bowel Disease. *Animal Models for Stem Cell Therapy.*
GONZÁLEZ, M. A., GONZALEZ-REY, E., RICO, L., BÜSCHER, D. & DELGADO, M. 2009. Treatment of experimental arthritis by inducing

immune tolerance with human adipose-derived mesenchymal stem cells. *Arthritis & Rheumatism,* 60**,** 1006-1019.
GRADY, W. M., MYEROFF, L. L., SWINLER, S. E., RAJPUT, A., THIAGALINGAM, S., LUTTERBAUGH, J. D., NEUMANN, A., BRATTAIN, M. G., CHANG, J., KIM, S. J., KINZLER, K. W., VOGELSTEIN, B., WILLSON, J. K. & MARKOWITZ, S. 1999. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res,* 59**,** 320-4.
GRIVENNIKOV, S. I. Inflammation and colorectal cancer: colitis-associated neoplasia. Seminars in Immunopathology, 2013. Springer, 229-244.
HANAHAN, D. & WEINBERG, R. A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.
HANAUER, S. B. 2006. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflammatory Bowel Diseases,* 12**,** S3-S9.
HANAUER, S. B. & BAERT, F. 1994. Medical therapy of inflammatory bowel disease. *Medical Clinics of North America,* 78**,** 1413-1426.
HASNAIN, S. Z., TAURO, S., DAS, I., TONG, H., CHEN, A. C. H., JEFFERY, P. L., MCDONALD, V., FLORIN, T. H. & MCGUCKIN, M. A. 2013. IL-10 promotes production of intestinal mucus by suppressing protein misfolding and endoplasmic reticulum stress in goblet cells. *Gastroenterology,* 144**,** 357-368. e9.
HAYASHI, Y., TSUJI, S., TSUJII, M., NISHIDA, T., ISHII, S., IIJIMA, H., NAKAMURA, T., EGUCHI, H., MIYOSHI, E. & HAYASHI, N. 2008. Topical implantation of mesenchymal stem cells has beneficial effects on healing of experimental colitis in rats. *Journal of Pharmacology and Experimental Therapeutics,* 326**,** 523-531.
HE, X.-W., HE, X.-S., LIAN, L., WU, X.-J. & LAN, P. 2012. Systemic infusion of bone marrow-derived mesenchymal stem cells for treatment of experimental colitis in mice. *Digestive Diseases and Sciences,* 57**,** 3136-3144.
HEATH, W. R. & CARBONE, F. R. 2001a. Cross-presentation in viral immunity and self-tolerance. *Nature Reviews Immunology,* 1**,** 126-134.
HEATH, W. R. & CARBONE, F. R. 2001b. Cross-presentation, dendritic cells, tolerance and immunity. *Annual Review of Immunology,* 19**,** 47-64.
HEAZLEWOOD, C. K., COOK, M. C., ERI, R., PRICE, G. R., TAURO, S. B., TAUPIN, D., THORNTON, D. J., PNG, C. W., CROCKFORD, T. L. & CORNALL, R. J. 2008. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med,* 5**,** e54.
HEIJSTEK, M., KRANENBURG, O. & BOREL RINKES, I. 2005. Mouse models of colorectal cancer and liver metastases. *Digestive Surgery,* 22**,** 16-25.
HENDRICKSON, B. A., GOKHALE, R. & CHO, J. H. 2002. Clinical aspects and pathophysiology of inflammatory bowel disease. *Clinical Microbiology Reviews,* 15**,** 79-94.
HERBERMAN, R. B., NUNN, M. E., HOLDEN, H. T. & LAVRIN, D. H. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *International Journal of Cancer,* 16**,** 230-239.

HERMISTON, M. L. & GORDON, J. I. 1995. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science,* 270**,** 1203-1207.
HIBI, T., OGATA, H. & SAKURABA, A. 2002. Animal models of inflammatory bowel disease. *Journal of Gastroenterology,* 37**,** 409-417.
HIEJIMA, E., KAWAI, T., NAKASE, H., TSURUYAMA, T., MORIMOTO, T., YASUMI, T., TAGA, T., KANEGANE, H., HORI, M. & OHMORI, K. 2015. Reduced numbers and proapoptotic features of mucosal-associated invariant T cells as a characteristic finding in patients with inflammatory bowel disease. *Inflammatory Bowel Diseases,* 21**,** 1529-1540.
HOU, L., WANG, X., ZHOU, Y., MA, H., WANG, Z., HE, J., HU, H., GUAN, W. & MA, Y. 2014. Inhibitory effect and mechanism of mesenchymal stem cells on liver cancer cells. *Tumor Biology,* 35**,** 1239-1250.
HOUGHTON, A. M., RZYMKIEWICZ, D. M., JI, H., GREGORY, A. D., EGEA, E. E., METZ, H. E., STOLZ, D. B., LAND, S. R., MARCONCINI, L. A. & KLIMENT, C. R. 2010. Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. *Nature Medicine,* 16**,** 219-223.
HUANG, W. H., CHANG, M. C., TSAI, K. S., HUNG, M. C., CHEN, H. L. & HUNG, S. C. 2013. Mesenchymal stem cells promote growth and angiogenesis of tumors in mice. *Oncogene,* 32**,** 4343-54.
HUE, S., AHERN, P., BUONOCORE, S., KULLBERG, M. C., CUA, D. J., MCKENZIE, B. S., POWRIE, F. & MALOY, K. J. 2006. Interleukin-23 drives innate and T cell–mediated intestinal inflammation. *The Journal of Experimental Medicine,* 203**,** 2473-2483.
HUGOT, J.-P., CHAMAILLARD, M., ZOUALI, H., LESAGE, S., CÉZARD, J.-P., BELAICHE, J., ALMER, S., TYSK, C., O'MORAIN, C. A. & GASSULL, M. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature,* 411**,** 599-603.
INA, K., ITOH, J., FUKUSHIMA, K., KUSUGAMI, K., YAMAGUCHI, T., KYOKANE, K., IMADA, A., BINION, D. G., MUSSO, A. & WEST, G. A. 1999. Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance. *The Journal of Immunology,* 163**,** 1081-1090.
ITZKOWITZ, S. H. & HARPAZ, N. 2004. Diagnosis and management of dysplasia in patients with inflammatory bowel diseases. *Gastroenterology,* 126**,** 1634-1648.
IVANOV, I. I., ATARASHI, K., MANEL, N., BRODIE, E. L., SHIMA, T., KARAOZ, U., WEI, D., GOLDFARB, K. C., SANTEE, C. A. & LYNCH, S. V. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell,* 139**,** 485-498.
JACKSON, A., ALEXANDROV, A., PRESCOTT, S., JAMES, K. & CHISHOLM, G. 1992. Role of adhesion molecules in lymphokine-activated killer cell killing of bladder cancer cells: further evidence for a third ligand for leucocyte function-associated antigen-1. *Immunology,* 76**,** 286.
JOHNSEN, A.-C., HAUX, J., STEINKJER, B., NONSTAD, U., EGEBERG, K., SUNDAN, A., ASHKENAZI, A. & ESPEVIK, T. 1999. Regulation of APO-2 ligand/trail expression in NK cells—involvement in NK cell-mediated cytotoxicity. *Cytokine,* 11**,** 664-672.
JOYCE, J. A. & POLLARD, J. W. 2009. Microenvironmental regulation of metastasis. *Nature Reviews Cancer,* 9**,** 239-252.

JURJUS, A. R., KHOURY, N. N. & REIMUND, J.-M. 2004. Animal models of inflammatory bowel disease. *Journal of Pharmacological and Toxicological Methods,* 50**,** 81-92.

KAMIHATA, H., MATSUBARA, H., NISHIUE, T., FUJIYAMA, S., TSUTSUMI, Y., OZONO, R., MASAKI, H., MORI, Y., IBA, O., TATEISHI, E., KOSAKI, A., SHINTANI, S., MUROHARA, T., IMAIZUMI, T. & IWASAKA, T. 2001. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation,* 104**,** 1046-52.

KANG, S.-G., SHINOJIMA, N., HOSSAIN, A., GUMIN, J., YONG, R. L., COLMAN, H., MARINI, F., ANDREEFF, M. & LANG, F. F. 2010a. Isolation and perivascular localization of mesenchymal stem cells from mouse brain. *Neurosurgery,* 67**,** 711.

KANG, S., DENMAN, S. E., MORRISON, M., YU, Z., DORE, J., LECLERC, M. & MCSWEENEY, C. S. 2010b. Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. *Inflammatory Bowel Diseases,* 16**,** 2034-2042.

KAPLAN, M. H., WURSTER, A. L. & GRUSBY, M. J. 1998. A signal transducer and activator of transcription (Stat) 4-independent pathway for the development of T helper type 1 cells. *The Journal of Experimental Medicine,* 188**,** 1191-1196.

KARBAN, A. S., OKAZAKI, T., PANHUYSEN, C. I., GALLEGOS, T., POTTER, J. J., BAILEY-WILSON, J. E., SILVERBERG, M. S., DUERR, R. H., CHO, J. H. & GREGERSEN, P. K. 2004. Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis. *Human Molecular Genetics,* 13**,** 35-45.

KARNOUB, A. E., DASH, A. B., VO, A. P., SULLIVAN, A., BROOKS, M. W., BELL, G. W., RICHARDSON, A. L., POLYAK, K., TUBO, R. & WEINBERG, R. A. 2007. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature,* 449**,** 557-563.

KHAKOO, A. Y., PATI, S., ANDERSON, S. A., REID, W., ELSHAL, M. F., ROVIRA, I. I., NGUYEN, A. T., MALIDE, D., COMBS, C. A. & HALL, G. 2006. Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *The Journal of Experimental Medicine,* 203**,** 1235-1247.

KIESSLING, R., KLEIN, E. & WIGZELL, H. 1975. „Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *European journal of immunology,* 5**,** 112-117.

KILLION, J. J., RADINSKY, R. & FIDLER, I. J. 1998. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. *Cancer and Metastasis Reviews,* 17**,** 279-284.

KIM, H. S., SHIN, T. H., LEE, B. C., YU, K. R., SEO, Y., LEE, S., SEO, M. S., HONG, I. S., CHOI, S. W. & SEO, K. W. 2013. Human umbilical cord blood mesenchymal stem cells reduce colitis in mice by activating NOD2 signaling to COX2. *Gastroenterology,* 145**,** 1392-1403. e8.

KINNAIRD, T., STABILE, E., BURNETT, M., LEE, C., BARR, S., FUCHS, S. & EPSTEIN, S. 2004a. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro

and in vivo arteriogenesis through paracrine mechanisms. *Circulation Research,* 94**,** 678-685.
KINNAIRD, T., STABILE, E., BURNETT, M. S., LEE, C. W., BARR, S., FUCHS, S. & EPSTEIN, S. E. 2004b. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res,* 94**,** 678-85.
KLOPP, A. H., GUPTA, A., SPAETH, E., ANDREEFF, M. & MARINI, F. 2011. Concise review: Dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? *Stem Cells,* 29**,** 11-19.
KLOSE, C. S., FLACH, M., MÖHLE, L., ROGELL, L., HOYLER, T., EBERT, K., FABIUNKE, C., PFEIFER, D., SEXL, V. & FONSECA-PEREIRA, D. 2014. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell,* 157**,** 340-356.
KOBAYASHI, T., OKAMOTO, S., HISAMATSU, T., KAMADA, N., CHINEN, H., SAITO, R., KITAZUME, M. T., NAKAZAWA, A., SUGITA, A. & KOGANEI, K. 2008. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut,* 57**,** 1682-1689.
KONOPLEVA, M., KONOPLEV, S., HU, W., ZARITSKEY, A., AFANASIEV, B. & ANDREEFF, M. 2002. Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia,* 16**,** 1713-1724.
KONTOYIANNIS, D., PASPARAKIS, M., PIZARRO, T. T., COMINELLI, F. & KOLLIAS, G. 1999. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity,* 10**,** 387-398.
KRAMPERA, M., GLENNIE, S., DYSON, J., SCOTT, D., LAYLOR, R., SIMPSON, E. & DAZZI, F. 2003. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood,* 101**,** 3722-3729.
KRAUS, S. & ARBER, N. 2009. Inflammation and colorectal cancer. *Current Opinion in Pharmacology,* 9**,** 405-410.
KRISHNAN, A. & KORZENIK, J. R. 2002. Inflammatory bowel disease and environmental influences. *Gastroenterology Clinics of North America,* 31**,** 21-39.
KÜHN, R., LÖHLER, J., RENNICK, D., RAJEWSKY, K. & MÜLLER, W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell,* 75**,** 263-274.
KUPER, H., ADAMI, H. O. & TRICHOPOULOS, D. 2000. Infections as a major preventable cause of human cancer. *Journal of Internal Medicine,* 248**,** 171-183.
LANGOWSKI, J. L., KASTELEIN, R. A. & OFT, M. 2007. Swords into plowshares: IL-23 repurposes tumor immune surveillance. *Trends in Immunology,* 28**,** 207-212.
LAZENNEC, G. & JORGENSEN, C. 2008. Concise review: adult multipotent stromal cells and cancer: risk or benefit? *Stem Cells,* 26**,** 1387-1394.
LE BLANC, K., TAMMIK, L., SUNDBERG, B., HAYNESWORTH, S. & RINGDEN, O. 2003. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major

histocompatibility complex. *Scandinavian Journal of Immunology,* 57**,** 11-20.
LEACH, M. W., BEAN, A., MAUZE, S., COFFMAN, R. L. & POWRIE, F. 1996. Inflammatory bowel disease in CB-17 scid mice reconstituted with the CD45RBhigh subset of CD4+ T cells. *The American Journal of Pathology,* 148**,** 1503.
LEE, A.-J., KIM, S.-G., CHAE, H.-D., LEE, G. H. & SHIN, I.-H. 2012. γδ T cells are increased in the peripheral blood of patients with gastric cancer. *Clinica Chimica Acta,* 413**,** 1495-1499.
LEE, E. G., BOONE, D. L., CHAI, S., LIBBY, S. L., CHIEN, M., LODOLCE, J. P. & MA, A. 2000. Failure to regulate TNF-induced NF-κB and cell death responses in A20-deficient mice. *Science,* 289**,** 2350-2354.
LEE, W. Y., PARK, K. J., CHO, Y. B., YOON, S. N., SONG, K. H., KIM, D. S., JUNG, S. H., KIM, M., YOO, H. W. & KIM, I. 2013. Autologous Adipose Tissue-Derived Stem Cells Treatment Demonstrated Favorable and Sustainable Therapeutic Effect for Crohn's Fistula. *Stem Cells,* 31**,** 2575-2581.
LEGRAND, F., DRISS, V., DELBEKE, M., LOISEAU, S., HERMANN, E., DOMBROWICZ, D. & CAPRON, M. 2010. Human eosinophils exert TNF-α and granzyme A-mediated tumoricidal activity toward colon carcinoma cells. *The Journal of Immunology,* 185**,** 7443-7451.
LI, L., LIU, S., XU, Y., ZHANG, A., JIANG, J., TAN, W., XING, J., FENG, G., LIU, H. & HUO, F. 2013. Human umbilical cord-derived mesenchymal stem cells downregulate inflammatory responses by shifting the Treg/Th17 profile in experimental colitis. *Pharmacology,* 92**,** 257-264.
LIANG, L., DONG, C., CHEN, X., FANG, Z., XU, J., LIU, M., ZHANG, X., GU, D. S., WANG, D. & DU, W. 2011. Human umbilical cord mesenchymal stem cells ameliorate mice trinitrobenzene sulfonic acid (TNBS)-induced colitis. *Cell Transplantation,* 20**,** 1395-1408.
LIECHTY, K. W., MACKENZIE, T. C., SHAABAN, A. F., RADU, A., MOSELEY, A. B., DEANS, R., MARSHAK, D. R. & FLAKE, A. W. 2000. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. *Nature Medicine,* 6**,** 1282-1286.
LIN, E. Y., NGUYEN, A. V., RUSSELL, R. G. & POLLARD, J. W. 2001. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *The Journal of Experimental Medicine,* 193**,** 727-740.
LINDBERG, E., TYSK, C., ANDERSSON, K. & JÄRNEROT, G. 1988. Smoking and inflammatory bowel disease. A case control study. *Gut,* 29**,** 352-357.
LIU, C.-H. & HWANG, S.-M. 2005. Cytokine interactions in mesenchymal stem cells from cord blood. *Cytokine,* 32**,** 270-279.
LIU, Y., HAN, Z. P., ZHANG, S. S., JING, Y. Y., BU, X. X., WANG, C. Y., SUN, K., JIANG, G. C., ZHAO, X., LI, R., GAO, L., ZHAO, Q. D., WU, M. C. & WEI, L. X. 2011. Effects of inflammatory factors on mesenchymal stem cells and their role in the promotion of tumor angiogenesis in colon cancer. *J Biol Chem,* 286**,** 25007-15.
LIU, Z.-J., YADAV, P. K., SU, J.-L., WANG, J.-S. & FEI, K. 2009. Potential role of Th17 cells in the pathogenesis of inflammatory bowel disease. *World J Gastroenterol,* 15**,** 5784-5788.

LOFTUS, E., SANDBORN, W., TREMAINE, W., MAHONEY, D., ZINSMEISTER, A., OFFORD, K. & MELTON, L. 1996. Primary sclerosing cholangitis is associated with nonsmoking: a case-control study. *Gastroenterology,* 110**,** 1496-1502.

LOFTUS, E. V. 2004. Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology,* 126**,** 1504-1517.

LOZITO, T. P. & TUAN, R. S. 2014. Endothelial and cancer cells interact with mesenchymal stem cells via both microparticles and secreted factors. *Journal of Cellular and Molecular Medicine,* 18**,** 2372-2384.

LU, H., OUYANG, W. & HUANG, C. 2006. Inflammation, a key event in cancer development. *Mol Cancer Res,* 4**,** 221-33.

LUCI, C., REYNDERS, A., IVANOV, I. I., COGNET, C., CHICHE, L., CHASSON, L., HARDWIGSEN, J., ANGUIANO, E., BANCHEREAU, J. & CHAUSSABEL, D. 2009. Influence of the transcription factor RORγt on the development of NKp46+ cell populations in gut and skin. *Nature Immunology,* 10**,** 75-82.

MACARTHUR, M., HOLD, G. L. & EL-OMAR, E. M. 2004. Inflammation and Cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 286**,** G515-G520.

MACDONALD, T. T. & MONTELEONE, G. 2005. Immunity, inflammation, and allergy in the gut. *Science,* 307**,** 1920-1925.

MAESTRONI, G., HERTENS, E. & GALLI, P. 1999. Factor (s) from nonmacrophage bone marrow stromal cells inhibit Lewis lung carcinoma and B16 melanoma growth in mice. *Cellular and Molecular Life Sciences CMLS,* 55**,** 663-667.

MANTOVANI, A. 1999. The chemokine system: redundancy for robust outputs. *Immunology Today,* 20**,** 254-257.

MANTOVANI, A., ALLAVENA, P., SICA, A. & BALKWILL, F. 2008. Cancer-related inflammation. *Nature,* 454**,** 436-444.

MANTOVANI, A., SAVINO, B., LOCATI, M., ZAMMATARO, L., ALLAVENA, P. & BONECCHI, R. 2010. The chemokine system in cancer biology and therapy. *Cytokine & Growth Factor Reviews,* 21**,** 27-39.

MANTOVANI, A., SOZZANI, S., LOCATI, M., ALLAVENA, P. & SICA, A. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in Immunology,* 23**,** 549-555.

MATSUMOTO, S., OKABE, Y., SETOYAMA, H., TAKAYAMA, K., OHTSUKA, J., FUNAHASHI, H., IMAOKA, A., OKADA, Y. & UMESAKI, Y. 1998. Inflammatory bowel disease-like enteritis and caecitis in a senescence accelerated mouse P1/Yit strain. *Gut,* 43**,** 71-78.

MCDERMOTT, D. F. & ATKINS, M. B. 2013. PD-1 as a potential target in cancer therapy. *Cancer Medicine,* 2**,** 662-673.

MCKAIG, B. C., MAKH, S. S., HAWKEY, C. J., PODOLSKY, D. K. & MAHIDA, Y. R. 1999. Normal human colonic subepithelial myofibroblasts enhance epithelial migration (restitution) via TGF-beta3. *Am J Physiol,* 276**,** G1087-93.

MEDVEDEV, A. E., JOHNSEN, A.-C., HAUX, J., STEINKJER, B., EGEBERG, K., LYNCH, D. H., SUNDAN, A. & ESPEVIK, T. 1997. Regulation of Fas and Fas-ligand expression in NK cells by cytokines and the involvement of Fas-ligand in NK/LAK cell-mediated cytotoxicity. *Cytokine,* 9**,** 394-404.

MELE, V., MURARO, M. G., CALABRESE, D., PFAFF, D., AMATRUDA, N., AMICARELLA, F., KVINLAUG, B., BOCELLI-TYNDALL, C., MARTIN, I. & RESINK, T. J. 2014. Mesenchymal stromal cells induce epithelial-to-mesenchymal transition in human colorectal cancer cells through the expression of surface-bound TGF-β. *International Journal of Cancer,* 134**,** 2583-2594.

MIAO, J.-W., LIU, L.-J. & HUANG, J. 2014. Interleukin-6-induced epithelial-mesenchymal transition through signal transducer and activator of transcription 3 in human cervical carcinoma. *International Journal of Oncology,* 45**,** 165-176.

MIELKE, L. A., JONES, S. A., RAVERDEAU, M., HIGGS, R., STEFANSKA, A., GROOM, J. R., MISIAK, A., DUNGAN, L. S., SUTTON, C. E. & STREUBEL, G. 2013. Retinoic acid expression associates with enhanced IL-22 production by γδ T cells and innate lymphoid cells and attenuation of intestinal inflammation. *Journal of Experimental Medicine***,** jem. 20121588.

MILLER, S., SENIOR, P. V., PRAKASH, M., APOSTOLOPOULOS, V., SAKKAL, S. & NURGALI, K. 2016. Leukocyte populations and IL-6 in the tumor microenvironment of an orthotopic colorectal cancer model. *Acta Biochimica et Biophysica Sinica***,** 334-341.

MIRANDOLA, P., PONTI, C., GOBBI, G., SPONZILLI, I., VACCAREZZA, M., COCCO, L., ZAULI, G., SECCHIERO, P., MANZOLI, F. A. & VITALE, M. 2004. Activated human NK and CD8+ T cells express both TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors but are resistant to TRAIL-mediated cytotoxicity. *Blood,* 104**,** 2418-2424.

MISHRA, P. J., MISHRA, P. J., HUMENIUK, R., MEDINA, D. J., ALEXE, G., MESIROV, J. P., GANESAN, S., GLOD, J. W. & BANERJEE, D. 2008. Carcinoma-associated fibroblast–like differentiation of human mesenchymal stem cells. *Cancer Research,* 68**,** 4331-4339.

MITTENDORF, E. A., ALATRASH, G., QIAO, N., WU, Y., SUKHUMALCHANDRA, P., JOHN, L. S. S., PHILIPS, A. V., XIAO, H., ZHANG, M. & RUISAARD, K. 2012. Breast cancer cell uptake of the inflammatory mediator neutrophil elastase triggers an anticancer adaptive immune response. *Cancer Research,* 72**,** 3153-3162.

MIZOGUCHI, A. & MIZOGUCHI, E. 2010. Animal models of IBD: linkage to human disease. *Current Opinion in Pharmacology,* 10**,** 578-587.

MOMBAERTS, P., MIZOGUCHI, E., GRUSBY, M. J., GLIMCHER, L. H., BHAN, A. K. & TONEGAWA, S. 1993. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell,* 75**,** 275-282.

MOOSSAVI, S. & BISHEHSARI, F. 2012. Inflammation in sporadic colorectal cancer. *Archives of Iranian Medicine (AIM),* 15**,** 166-170.

MORETTA, L., BOTTINO, C., PENDE, D., VITALE, M., MINGARI, M. C. & MORETTA, A. 2004. Different checkpoints in human NK-cell activation. *Trends in Immunology,* 25**,** 670-676.

MORGAN, E., VARRO, R., SEPULVEDA, H., EMBER, J. A., APGAR, J., WILSON, J., LOWE, L., CHEN, R., SHIVRAJ, L. & AGADIR, A. 2004. Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clinical Immunology,* 110**,** 252-266.
MORO, K., YAMADA, T., TANABE, M., TAKEUCHI, T., IKAWA, T., KAWAMOTO, H., FURUSAWA, J.-I., OHTANI, M., FUJII, H. & KOYASU, S. 2010. Innate production of TH2 cytokines by adipose tissue-associated c-Kit+ Sca-1+ lymphoid cells. *Nature,* 463**,** 540-544.
MORRIS, G. P., BECK, P. L., HERRIDGE, M. S., DEPEW, W. T., SZEWCZUK, M. R. & WALLACE, J. L. 1989. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology,* 96**,** 795-803.
MOSER, B. 2012. Tumor-killing γδ-TCRs take center stage. *Blood,* 120**,** 5093-5094.
MOSNA, F., SENSEBÉ, L. & KRAMPERA, M. 2010. Human bone marrow and adipose tissue mesenchymal stem cells: a user's guide. *Stem Cells and Development,* 19**,** 1449-1470.
MUNITZ, A. & LEVI-SCHAFFER, F. 2004. Eosinophils:'new'roles for 'old'cells. *Allergy,* 59**,** 268-275.
MURPHY, E. A., DAVIS, J. M., MCCLELLAN, J. L. & CARMICHAEL, M. D. 2011. Quercetin's effects on intestinal polyp multiplicity and macrophage number in the Apc min/+ mouse. *Nutrition and Cancer,* 63**,** 421-426.
NAGAYA, N., FUJII, T., IWASE, T., OHGUSHI, H., ITOH, T., UEMATSU, M., YAMAGISHI, M., MORI, H., KANGAWA, K. & KITAMURA, S. 2004. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. *American Journal of Physiology-Heart and Circulatory Physiology,* 287**,** H2670-H2676.
NAKAGAKI, S., ARIMURA, Y., NAGAISHI, K., ISSHIKI, H., NASUNO, M., WATANABE, S., IDOGAWA, M., YAMASHITA, K., NAISHIRO, Y. & ADACHI, Y. 2015. Contextual niche signals towards colorectal tumor progression by mesenchymal stem cell in the mouse xenograft model. *Journal of Gastroenterology,* 50**,** 962-974.
NASEF, A., ZHANG, Y., MAZURIER, C., BOUCHET, S., BENSIDHOUM, M., FRANCOIS, S., GORIN, N. C., LOPEZ, M., THIERRY, D. & FOUILLARD, L. 2009. Selected Stro-1-enriched bone marrow stromal cells display a major suppressive effect on lymphocyte proliferation. *International Journal of Laboratory Hematology,* 31**,** 9-19.
NASUNO, M., ARIMURA, Y., NAGAISHI, K., ISSHIKI, H., ONODERA, K., NAKAGAKI, S., WATANABE, S., IDOGAWA, M., YAMASHITA, K. & NAISHIRO, Y. 2014. Mesenchymal Stem Cells Cancel Azoxymethane-Induced Tumor Initiation. *Stem Cells,* 32**,** 913-925.
NEILL, D. R., WONG, S. H., BELLOSI, A., FLYNN, R. J., DALY, M., LANGFORD, T. K., BUCKS, C., KANE, C. M., FALLON, P. G. & PANNELL, R. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature,* 464**,** 1367-1370.
NEISH, A. S., GEWIRTZ, A. T., ZENG, H., YOUNG, A. N., HOBERT, M. E., KARMALI, V., RAO, A. S. & MADARA, J. L. 2000. Prokaryotic regulation of epithelial responses by inhibition of IκB-α ubiquitination. *Science,* 289**,** 1560-1563.

NEURATH, M. F., FINOTTO, S. & GLIMCHER, L. H. 2002. The role of Th1/Th2 polarization in mucosal immunity. *Nature Medicine,* 8**,** 567-573.
NG, S. C. 2014. Epidemiology of inflammatory bowel disease: focus on Asia. *Best Practice & Research Clinical Gastroenterology,* 28**,** 363-372.
NIESS, J. H., LEITHÄUSER, F., ADLER, G. & REIMANN, J. 2008. Commensal gut flora drives the expansion of proinflammatory CD4 T cells in the colonic lamina propria under normal and inflammatory conditions. *The Journal of Immunology,* 180**,** 559-568.
NIXON, J. & RIDDELL, R. 1990. Histopathology in ulcerative colitis. *Inflammatory Bowel Disease. Edingburgh: Churchill Livingstone***,** 247-85.
NOORT, W. A., KRUISSELBRINK, A. B., IN'T ANKER, P. S., KRUGER, M., VAN BEZOOIJEN, R. L., DE PAUS, R. A., HEEMSKERK, M. H., LÖWIK, C. W., FALKENBURG, J. F. & WILLEMZE, R. 2002. Mesenchymal stem cells promote engraftment of human umbilical cord blood–derived CD34+ cells in NOD/SCID mice. *Experimental Hematology,* 30**,** 870-878.
NORIAN, L. A., RODRIGUEZ, P. C., O'MARA, L. A., ZABALETA, J., OCHOA, A. C., CELLA, M. & ALLEN, P. M. 2009. Tumor-infiltrating regulatory dendritic cells inhibit CD8+ T cell function via L-arginine metabolism. *Cancer Research,* 69**,** 3086-3094.
NURGALI, K., QU, Z., HUNNE, B., THACKER, M., PONTELL, L. & FURNESS, J. B. 2011. Morphological and functional changes in guinea-pig neurons projecting to the ileal mucosa at early stages after inflammatory damage. *The Journal of Physiology,* 589**,** 325-339.
NUSSBAUM, J. C., VAN DYKEN, S. J., VON MOLTKE, J., CHENG, L. E., MOHAPATRA, A., MOLOFSKY, A. B., THORNTON, E. E., KRUMMEL, M. F., CHAWLA, A. & LIANG, H.-E. 2013. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature,* 502**,** 245-248.
OGURA, Y., BONEN, D. K., INOHARA, N., NICOLAE, D. L., CHEN, F. F., RAMOS, R., BRITTON, H., MORAN, T., KARALIUSKAS, R. & DUERR, R. H. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature,* 411**,** 603-606.
OHLSSON, L. B., VARAS, L., KJELLMAN, C., EDVARDSEN, K. & LINDVALL, M. 2003. Mesenchymal progenitor cell-mediated inhibition of tumor growth in vivo and in vitro in gelatin matrix. *Experimental and Molecular Pathology,* 75**,** 248-255.
OKAMOTO, R. 2011. Epithelial regeneration in inflammatory bowel diseases. *Inflammation and Regeneration,* 31**,** 275-281.
ONG S, TAN Y, BERETTA O, JIANG D, YEAP W, TAI J, WONG W, YANG H, SCHWARZ H & LIM K 2012. Macrophages in human colorectal cancer are pro-inflammatory and prime T cells towards an anti-tumour type-1 inflammatory response. *European Journal Of Immunology,* 42**,** 89-100.
ONKEN, J., GALLUP, D., HANSON, J., PANDAK, M. & CUSTER, L. Successful outpatient treatment of refractory Crohn's disease using adult mesenchymal stem cells. American College of Gastroenterology Annual Meeting, 2006.
ORBELL, J. & WEST, N. J. 2010. Improving detection of colorectal cancer. *The Practitioner,* 254**,** 17-21, 2-3.
ORTIZ, L. A., GAMBELLI, F., MCBRIDE, C., GAUPP, D., BADDOO, M., KAMINSKI, N. & PHINNEY, D. G. 2003. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and

ameliorates its fibrotic effects. *Proceedings of the National Academy of Sciences,* 100**,** 8407-8411.
OSTANIN, D. V., BAO, J., KOBOZIEV, I., GRAY, L., ROBINSON-JACKSON, S. A., KOSLOSKI-DAVIDSON, M., PRICE, V. H. & GRISHAM, M. B. 2009. T cell transfer model of chronic colitis: concepts, considerations, and tricks of the trade. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 296**,** G135-G146.
OTSU, K., DAS, S., HOUSER, S. D., QUADRI, S. K., BHATTACHARYA, S. & BHATTACHARYA, J. 2009. Concentration-dependent inhibition of angiogenesis by mesenchymal stem cells. *Blood,* 113**,** 4197-4205.
PAGÈS, F., BERGER, A., CAMUS, M., SANCHEZ-CABO, F., COSTES, A., MOLIDOR, R., MLECNIK, B., KIRILOVSKY, A., NILSSON, M. & DAMOTTE, D. 2005. Effector memory T cells, early metastasis, and survival in colorectal cancer. *New England Journal of Medicine,* 353**,** 2654-2666.
PANWALA, C. M., JONES, J. C. & VINEY, J. L. 1998. A novel model of inflammatory bowel disease: mice deficient for the multiple drug resistance gene, mdr1a, spontaneously develop colitis. *The Journal of Immunology,* 161**,** 5733-5744.
PARK, K.-S., KIM, Y.-S., KIM, J.-H., CHOI, B., KIM, S.-H., TAN, A. H.-K., LEE, M.-S., LEE, M.-K., KWON, C.-H. & JOH, J.-W. 2010. Trophic molecules derived from human mesenchymal stem cells enhance survival, function, and angiogenesis of isolated islets after transplantation. *Transplantation,* 89**,** 509-517.
PARRONCHI, P., ROMAGNANI, P., ANNUNZIATO, F., SAMPOGNARO, S., BECCHIO, A., GIANNARINI, L., MAGGI, E., PUPILLI, C., TONELLI, F. & ROMAGNANI, S. 1997. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *The American Journal of Pathology,* 150**,** 823-832.
PASANEN, I., PIETILA, M., LEHTONEN, S., LEHTILAHTI, E., HAKKARAINEN, T., BLANCO SEQUEIROS, R., LEHENKARI, P. & KUVAJA, P. 2015. Mesenchymal stromal cells from female donors enhance breast cancer cell proliferation in vitro. *Oncology,* 88**,** 214-25.
PATEL, S. A. & GOODERHAM, N. J. 2015. IL6 mediates immune and colorectal cancer cell cross-talk via miR-21 and miR-29b. *Molecular Cancer Research,* 13**,** 1502-1508.
PEARSON, D. C., MAY, G. R., FICK, G. H. & SUTHERLAND, L. R. 1995. Azathioprine and 6-mercaptopurine in Crohn disease: a meta-analysis. *Annals of Internal Medicine,* 123**,** 132-142.
PELUSO, I., PALLONE, F. & MONTELEONE, G. 2006. Interleukin-12 and Th1 immune response in Crohn's disease: pathogenic relevance and therapeutic implication. *World J Gastroenterol,* 12**,** 5606-5610.
PERSSON, P.-G., AHLBOM, A. & HELLERS, G. 1992. Diet and inflammatory bowel disease: a case-control study. *Epidemiology,* 3**,** 47-52.
PHILIP, M., ROWLEY, D. A. & SCHREIBER, H. 2004. Inflammation as a tumor promoter in cancer induction. *Semin Cancer Biol,* 14**,** 433-9.
PIETERSZ, G. A., LI, W., POPOVSKI, V., CARUANA, J.-A., APOSTOLOPOULOS, V. & MCKENZIE, I. F. 1998. Parameters for using mannan-MUC1 fusion protein to induce cellular immunity. *Cancer Immunology, Immunotherapy,* 45**,** 321-326.

PITHADIA, A. B. & JAIN, S. 2011. Treatment of inflammatory bowel disease (IBD). *Pharmacological Reports,* 63**,** 629-642.
PIZARRO, T. T., ARSENEAU, K. O., BAMIAS, G. & COMINELLI, F. 2003. Mouse models for the study of Crohn's disease. *Trends in Molecular Medicine,* 9**,** 218-222.
PLUMAS, J., CHAPEROT, L., RICHARD, M.-J., MOLENS, J.-P., BENSA, J.-C. & FAVROT, M.-C. 2005. Mesenchymal stem cells induce apoptosis of activated T cells. *Leukemia,* 19**,** 1597-1604.
POLLARD, J. W. 2009. Trophic macrophages in development and disease. *Nature Reviews Immunology,* 9**,** 259-270.
PORTALSKA, K. J., LEFERINK, A., GROEN, N., FERNANDES, H., MORONI, L., VAN BLITTERSWIJK, C. & DE BOER, J. 2012. Endothelial differentiation of mesenchymal stromal cells. *PloS One,* 7**,** e46842.
POTAPOVA, I. A., GAUDETTE, G. R., BRINK, P. R., ROBINSON, R. B., ROSEN, M. R., COHEN, I. S. & DORONIN, S. V. 2007. Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro. *Stem Cells,* 25**,** 1761-1768.
POWRIE, F., LEACH, M. W., MAUZE, S., CADDIE, L. B. & COFFMAN, R. L. 1993. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *International Immunology,* 5**,** 1461-1471.
POWRIE, F., LEACH, M. W., MAUZE, S., MENON, S., CADDLE, L. B. & COFFMAN, R. L. 1994. Inhibition of Thl responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RB hi CD4+ T cells. *Immunity,* 1**,** 553-562.
PRICE, A. E., LIANG, H.-E., SULLIVAN, B. M., REINHARDT, R. L., EISLEY, C. J., ERLE, D. J. & LOCKSLEY, R. M. 2010. Systemically dispersed innate IL-13–expressing cells in type 2 immunity. *Proceedings of the National Academy of Sciences,* 107**,** 11489-11494.
PUPPA, M. J., WHITE, J. P., SATO, S., CAIRNS, M., BAYNES, J. W. & CARSON, J. A. 2011. Gut barrier dysfunction in the Apc Min/+ mouse model of colon cancer cachexia. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease,* 1812**,** 1601-1606.
PURTON, J. F., MONK, J. A., LIDDICOAT, D. R., KYPARISSOUDIS, K., SAKKAL, S., RICHARDSON, S. J., GODFREY, D. I. & COLE, T. J. 2004. Expression of the glucocorticoid receptor from the 1A promoter correlates with T lymphocyte sensitivity to glucocorticoid-induced cell death. *The Journal of Immunology,* 173**,** 3816-3824.
QIAO, L., XU, Z.-L., ZHAO, T.-J., YE, L.-H. & ZHANG, X.-D. 2008a. Dkk-1 secreted by mesenchymal stem cells inhibits growth of breast cancer cells< i> via</i> depression of Wnt signalling. *Cancer Letters,* 269**,** 67-77.
QIAO, L., XU, Z., ZHAO, T., ZHAO, Z., SHI, M., ZHAO, R. C., YE, L. & ZHANG, X. 2008b. Suppression of tumorigenesis by human mesenchymal stem cells in a hepatoma model. *Cell Research,* 18**,** 500-507.
QIU, J., GUO, X., ZONG-MING, E. C., HE, L., SONNENBERG, G. F., ARTIS, D., FU, Y.-X. & ZHOU, L. 2013. Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. *Immunity,* 39**,** 386-399.

RASMUSSON, I., UHLIN, M., LE BLANC, K. & LEVITSKY, V. 2007. Mesenchymal stem cells fail to trigger effector functions of cytotoxic T lymphocytes. *Journal of Leukocyte Biology,* 82**,** 887-893.

RATH, H. C., SCHULTZ, M., FREITAG, R., DIELEMAN, L. A., LI, F., LINDE, H.-J., SCHÖLMERICH, J. & SARTOR, R. B. 2001. Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. *Infection and Immunity,* 69**,** 2277-2285.

READ, S., MALMSTRÖM, V. & POWRIE, F. 2000. Cytotoxic T lymphocyte–associated antigen 4 plays an essential role in the function of CD25+ CD4+ regulatory cells that control intestinal inflammation. *The Journal of Experimental Medicine,* 192**,** 295-302.

REYNDERS, A., YESSAAD, N., MANH, T. P. V., DALOD, M., FENIS, A., AUBRY, C., NIKITAS, G., ESCALIÈRE, B., RENAULD, J. C. & DUSSURGET, O. 2011. Identity, regulation and in vivo function of gut NKp46+ RORγt+ and NKp46+ RORγt− lymphoid cells. *The EMBO Journal,* 30**,** 2934-2947.

REYNOLDS, A., WHARTON, N., PARRIS, A., MITCHELL, E., SOBOLEWSKI, A., KAM, C., BIGWOOD, L., EL HADI, A., MUNSTERBERG, A., LEWIS, M., SPEAKMAN, C., STEBBINGS, W., WHARTON, R., SARGEN, K., TIGHE, R., JAMIESON, C., HERNON, J., KAPUR, S., OUE, N., YASUI, W. & WILLIAMS, M. R. 2014. Canonical Wnt signals combined with suppressed TGFbeta/BMP pathways promote renewal of the native human colonic epithelium. *Gut,* 63**,** 610-21.

RHYU, J. J., YUN, J. W., KWON, E., CHE, J. H. & KANG, B. C. 2015. Dual effects of human adipose tissue-derived mesenchymal stem cells in human lung adenocarcinoma A549 xenografts and colorectal adenocarcinoma HT-29 xenografts in mice. *Oncol Rep,* 34**,** 1733-44.

ROBANUS-MAANDAG, E. C., KOELINK, P. J., BREUKEL, C., SALVATORI, D. C., JAGMOHAN-CHANGUR, S. C., BOSCH, C. A., VERSPAGET, H. W., DEVILEE, P., FODDE, R. & SMITS, R. 2010. A new conditional Apc-mutant mouse model for colorectal cancer. *Carcinogenesis,* 31**,** 946-952.

ROBERTSON, J. H., YANG, S. Y., IGA, A. M., SEIFALIAN, A. M. & WINSLET, M. C. 2008. An in vivo rat model for early development of colorectal cancer metastasis to liver. *International Journal of Experimental Pathology,* 89**,** 447-457.

ROBERTSON, M. J. 2002. Role of chemokines in the biology of natural killer cells. *Journal of Leukocyte Biology,* 71**,** 173-183.

ROBINSON, A. M., SAKKAL, S., PARK, A., JOVANOVSKA, V., PAYNE, N., CARBONE, S. E., MILLER, S., BORNSTEIN, J. C., BERNARD, C. & BOYD, R. 2014. Mesenchymal stem cells and conditioned medium avert enteric neuropathy and colon dysfunction in guinea pig TNBS-induced colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 307**,** G1115-G1129.

ROCHA, N. & NEEFJES, J. 2008. MHC class II molecules on the move for successful antigen presentation. *The EMBO Journal,* 27**,** 1-5.

ROVEDATTI, L., KUDO, T., BIANCHERI, P., SARRA, M., KNOWLES, C., RAMPTON, D. S., CORAZZA, G. R., MONTELEONE, G., DI SABATINO, A. & MACDONALD, T. T. 2009. Differential regulation of interleukin 17 and interferon γ production in inflammatory bowel disease. *Gut,* 58**,** 1629-1636.

RUIJING, X., MENGJUN, W., XIAOLING, Z., SHU, P., MEI, W., YINGCHENG, Z., YULING, H. & JINQUAN, T. 2012. Jα33+ MAIT cells play a protective role in TNBS induced intestinal inflammation. *Hepato-Gastroenterology,* 59**,** 762-767.

RUTELLA, S., DANESE, S. & LEONE, G. 2006. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood,* 108**,** 1435-1440.

SALAZAR, K. D., LANKFORD, S. M. & BRODY, A. R. 2009. Mesenchymal stem cells produce Wnt isoforms and TGF-β1 that mediate proliferation and procollagen expression by lung fibroblasts. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 297**,** L1002-L1011.

SALE, S., FONG, I. L., DE GIOVANNI, C., LANDUZZI, L., BROWN, K., STEWARD, W. P. & GESCHER, A. J. 2009. APC10. 1 cells as a model for assessing the efficacy of potential chemopreventive agents in the ApcMin mouse model in vivo. *European Journal of Cancer,* 45**,** 2731-2735.

SALEH, M. & TRINCHIERI, G. 2010. Innate immune mechanisms of colitis and colitis-associated colorectal cancer. *Nature Reviews Immunology,* 11**,** 9-20.

SANDS, B. E. 2004. From symptom to diagnosis: clinical distinctions among various forms of intestinal inflammation. *Gastroenterology,* 126**,** 1518-1532.

SANOS, S. L., BUI, V. L., MORTHA, A., OBERLE, K., HENERS, C., JOHNER, C. & DIEFENBACH, A. 2009. RORγt and commensal microflora are required for the differentiation of mucosal interleukin 22–producing NKp46+ cells. *Nature Immunology,* 10**,** 83-91.

SARTOR, R. B. 1995. Current concepts of the etiology and pathogenesis of ulcerative colitis and Crohn's disease. *Gastroenterology Clinics of North America,* 24**,** 475-507.

SARTOR, R. B. 2004. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology,* 126**,** 1620-1633.

SARTOR, R. B. 2006. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nature Clinical Practice Gastroenterology & Hepatology,* 3**,** 390-407.

SARTOR, R. B. 2008. Microbial influences in inflammatory bowel diseases. *Gastroenterology,* 134**,** 577-594.

SATO, T., TAKAHASHI, S., MIZUMOTO, T., HARAO, M., AKIZUKI, M., TAKASUGI, M., FUKUTOMI, T. & YAMASHITA, J.-I. 2006. Neutrophil elastase and cancer. *Surgical Oncology,* 15**,** 217-222.

SATOH-TAKAYAMA, N., VOSSHENRICH, C. A., LESJEAN-POTTIER, S., SAWA, S., LOCHNER, M., RATTIS, F., MENTION, J.-J., THIAM, K., CERF-BENSUSSAN, N. & MANDELBOIM, O. 2008. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity,* 29**,** 958-970.

SATSANGI, J., MORECROFT, J., SHAH, N. & NIMMO, E. 2003. Genetics of inflammatory bowel disease: scientific and clinical implications. *Best Practice & Research Clinical Gastroenterology,* 17**,** 3-18.

SAWA, S., LOCHNER, M., SATOH-TAKAYAMA, N., DULAUROY, S., BÉRARD, M., KLEINSCHEK, M., CUA, D., DI SANTO, J. P. & EBERL, G. 2011. ROR [gamma] t+ innate lymphoid cells regulate intestinal

homeostasis by integrating negative signals from the symbiotic microbiota. *Nature Immunology,* 12**,** 320-326.
SCAPINI, P., MORINI, M., TECCHIO, C., MINGHELLI, S., DI CARLO, E., TANGHETTI, E., ALBINI, A., LOWELL, C., BERTON, G. & NOONAN, D. M. 2004. CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. *The Journal of Immunology,* 172**,** 5034-5040.
SCHAFER, Z. T. & BRUGGE, J. S. 2007. IL-6 involvement in epithelial cancers. *The Journal of Clinical Investigation,* 117**,** 3660-3663.
SCHELLER, J., CHALARIS, A., SCHMIDT-ARRAS, D. & ROSE-JOHN, S. 2011. The pro-and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1813**,** 878-888.
SCHREIBER, R. D., OLD, L. J. & SMYTH, M. J. 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science,* 331**,** 1565-1570.
SCHUMACHER, U., NEHMANN, N., ADAM, E., MUKTHAR, D., SLOTKI, I. N., HORNY, H.-P., FLENS, M. J., SCHLEGELBERGER, B. & STEINEMANN, D. 2012. MDR-1-overexpression in HT 29 colon cancer cells grown in SCID mice. *Acta Histochemica,* 114**,** 594-602.
SEIB, F. P., PREWITZ, M., WERNER, C. & BORNHÄUSER, M. 2009. Matrix elasticity regulates the secretory profile of human bone marrow-derived multipotent mesenchymal stromal cells (MSCs). *Biochemical and Biophysical Research Communications,* 389**,** 663-667.
SÉMONT, A., DEMARQUAY, C., BESSOUT, R., DURAND, C., BENDERITTER, M. & MATHIEU, N. 2013. Mesenchymal stem cell therapy stimulates endogenous host progenitor cells to improve colonic epithelial regeneration. *PloS One,* 8**,** e70170.
SERAKINCI, N., CHRISTENSEN, R., FAHRIOGLU, U., SORENSEN, F. B., DAGNÆS-HANSEN, F., HAJEK, M., JENSEN, T. H., KOLVRAA, S. & KEITH, N. W. 2011. Mesenchymal stem cells as therapeutic delivery vehicles targeting tumor stroma. *Cancer Biotherapy and Radiopharmaceuticals,* 26**,** 767-773.
SHAN, Y.-S., HSU, H.-P., LAI, M.-D., YEN, M.-C., FANG, J.-H., WENG, T.-Y. & CHEN, Y.-L. 2014. Suppression of mucin 2 promotes interleukin-6 secretion and tumor growth in an orthotopic immune-competent colon cancer animal model. *Oncology Reports,* 32**,** 2335-2342.
SHENG, H., WANG, Y., JIN, Y., ZHANG, Q., ZHANG, Y., WANG, L., SHEN, B., YIN, S., LIU, W. & CUI, L. 2008. A critical role of IFNγ in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. *Cell Research,* 18**,** 846-857.
SHIH, D. Q. & TARGAN, S. R. 2008. Immunopathogenesis of inflammatory bowel disease. *World Journal of Gastroenterology: WJG,* 14**,** 390-400.
SHINAGAWA, K., KITADAI, Y., TANAKA, M., SUMIDA, T., KODAMA, M., HIGASHI, Y., TANAKA, S., YASUI, W. & CHAYAMA, K. 2010. Mesenchymal stem cells enhance growth and metastasis of colon cancer. *International Journal of Cancer,* 127**,** 2323-2333.
SICA, A. & BRONTE, V. 2007. Altered macrophage differentiation and immune dysfunction in tumor development. *The Journal of Clinical Investigation,* 117**,** 1155-1166.

SICA, A., SCHIOPPA, T., MANTOVANI, A. & ALLAVENA, P. 2006. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *European Journal of Cancer,* 42**,** 717-727.

SMYTH, M. J., DUNN, G. P. & SCHREIBER, R. D. 2006. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Advances in Immunology,* 90**,** 1-50.

SMYTH, M. J., GODFREY, D. I. & TRAPANI, J. A. 2001. A fresh look at tumor immunosurveillance and immunotherapy. *Nature Immunology,* 2**,** 293-299.

SONNENBERG, A. 1990. Occupational mortality of inflammatory bowel disease. *Digestion,* 46**,** 10-18.

SOTIROPOULOU, P. A., PEREZ, S. A., GRITZAPIS, A. D., BAXEVANIS, C. N. & PAPAMICHAIL, M. 2006. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells,* 24**,** 74-85.

SPAETH, E., KLOPP, A., DEMBINSKI, J., ANDREEFF, M. & MARINI, F. 2008. Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells. *Gene Therapy,* 15**,** 730-738.

SPAETH, E. L., DEMBINSKI, J. L., SASSER, A. K., WATSON, K., KLOPP, A., HALL, B., ANDREEFF, M. & MARINI, F. 2009. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PloS One,* 4**,** e4992.

SPAGGIARI, G. M., CAPOBIANCO, A., ABDELRAZIK, H., BECCHETTI, F., MINGARI, M. C. & MORETTA, L. 2008. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood,* 111**,** 1327-33.

SPITS, H., ARTIS, D., COLONNA, M., DIEFENBACH, A., DI SANTO, J. P., EBERL, G., KOYASU, S., LOCKSLEY, R. M., MCKENZIE, A. N. & MEBIUS, R. E. 2013. Innate lymphoid cells—a proposal for uniform nomenclature. *Nature Reviews Immunology,* 13**,** 145-149.

STAVELY, R., ROBINSON, A. M., MILLER, S., BOYD, R., SAKKAL, S. & NURGALI, K. 2015. Allogeneic guinea pig mesenchymal stem cells ameliorate neurological changes in experimental colitis. *Stem Cell Research & Therapy,* 6**,** 1-21.

STAVELY, R., SAKKAL, S., STOJANOVSKA, V. & NURGALI, K. 2014. Mesenchymal stem cells for the treatment of inflammatory bowel disease: from experimental models to clinical application. *Inflammation and Regeneration,* 34**,** 184-197.

STROBER, W., FUSS, I. & MANNON, P. 2007. The fundamental basis of inflammatory bowel disease. *The Journal of Clinical Investigation,* 117**,** 514-521.

STUDD, C., CAMERON, G., BESWICK, L., KNIGHT, R., HAIR, C., MCNEIL, J., DESMOND, P., WILSON, J., CONNELL, W. & BELL, S. 2016. Never underestimate inflammatory bowel disease: High prevalence rates and confirmation of high incidence rates in Australia. *Journal of Gastroenterology and Hepatology,* 31**,** 81-86.

SUDRES, M., NOROL, F., TRENADO, A., GREGOIRE, S., CHARLOTTE, F., LEVACHER, B., LATAILLADE, J. J., BOURIN, P., HOLY, X., VERNANT,

J. P., KLATZMANN, D. & COHEN, J. L. 2006. Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. *J Immunol,* 176**,** 7761-7.
SUN, T., SUN, B. C., NI, C. S., ZHAO, X. L., WANG, X. H., QIE, S., ZHANG, D. F., GU, Q., QI, H. & ZHAO, N. 2008. Pilot study on the interaction between B16 melanoma cell-line and bone-marrow derived mesenchymal stem cells. *Cancer Lett,* 263**,** 35-43.
SUTHERLAND, L., SINGLETON, J., SESSIONS, J., HANAUER, S., KRAWITT, E., RANKIN, G., SUMMERS, R., MEKHJIAN, H., GREENBERGER, N. & KELLY, M. 1991. Double blind, placebo controlled trial of metronidazole in Crohn's disease. *Gut,* 32**,** 1071-1075.
SWIDSINSKI, A., LADHOFF, A., PERNTHALER, A., SWIDSINSKI, S., LOENING–BAUCKE, V., ORTNER, M., WEBER, J., HOFFMANN, U., SCHREIBER, S. & DIETEL, M. 2002. Mucosal flora in inflammatory bowel disease. *Gastroenterology,* 122**,** 44-54.
SZKARADKIEWICZ, A., MARCINIAK, R., CHUDZICKA-STRUGAŁA, I., WASILEWSKA, A., DREWS, M., MAJEWSKI, P., KARPIŃSKI, T. & ZWOŹDZIAK, B. 2009. Proinflammatory cytokines and IL-10 in inflammatory bowel disease and colorectal cancer patients. *Archivum Immunologiae et Therapiae Experimentalis,* 57**,** 291-294.
TAKAYAMA, T., KAMADA, N., CHINEN, H., OKAMOTO, S., KITAZUME, M. T., CHANG, J., MATUZAKI, Y., SUZUKI, S., SUGITA, A. & KOGANEI, K. 2010. Imbalance of NKp44+ NKp46− and NKp44− NKp46+ natural killer cells in the intestinal mucosa of patients with Crohn's disease. *Gastroenterology,* 139**,** 882-892. e3.
TAKEDA, K., CLAUSEN, B. E., KAISHO, T., TSUJIMURA, T., TERADA, N., FÖRSTER, I. & AKIRA, S. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity,* 10**,** 39-49.
TANAKA, H., ARIMURA, Y., YABANA, T., GOTO, A., HOSOKAWA, M., NAGAISHI, K., YAMASHITA, K., YAMAMOTO, H., SASAKI, Y. & FUJIMIYA, M. 2011. Myogenic lineage differentiated mesenchymal stem cells enhance recovery from dextran sulfate sodium-induced colitis in the rat. *Journal of Gastroenterology,* 46**,** 143-152.
TANG, R. J., SHEN, S. N., ZHAO, X. Y., NIE, Y. Z., XU, Y. J., REN, J., LV, M. M., HOU, Y. Y. & WANG, T. T. 2015. Mesenchymal stem cells-regulated Treg cells suppress colitis-associated colorectal cancer. *Stem Cell Res Ther,* 6**,** 71.
TANG, Y. L., ZHAO, Q., QIN, X., SHEN, L., CHENG, L., GE, J. & PHILLIPS, M. I. 2005. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. *The Annals of Thoracic Surgery,* 80**,** 229-237.
TARGAN, S. R., HANAUER, S. B., VAN DEVENTER, S. J., MAYER, L., PRESENT, D. H., BRAAKMAN, T., DEWOODY, K. L., SCHAIBLE, T. F. & RUTGEERTS, P. J. 1997. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor α for Crohn's disease. *New England Journal of Medicine,* 337**,** 1029-1036.
TERZIĆ, J., GRIVENNIKOV, S., KARIN, E. & KARIN, M. 2010. Inflammation and colon cancer. *Gastroenterology,* 138**,** 2101-2114. e5.

THORESON, R. & CULLEN, J. J. 2007. Pathophysiology of inflammatory bowel disease: an overview. *Surgical Clinics of North America,* 87**,** 575-585.
TODARO, M., D'ASARO, M., CACCAMO, N., IOVINO, F., FRANCIPANE, M. G., MERAVIGLIA, S., ORLANDO, V., LA MENDOLA, C., GULOTTA, G. & SALERNO, A. 2009. Efficient killing of human colon cancer stem cells by γδ T lymphocytes. *The Journal of Immunology,* 182**,** 7287-7296.
TODARO, M., PEREZ ALEA, M., SCOPELLITI, A., MEDEMA, J. P. & STASSI, G. 2008. IL-4-mediated drug resistance in colon cancer stem cells. *Cell Cycle,* 7**,** 309-313.
TÖGEL, F., HU, Z., WEISS, K., ISAAC, J., LANGE, C. & WESTENFELDER, C. 2005. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *American Journal of Physiology-Renal Physiology,* 289**,** F31-F42.
TREFFERS, L. W., HIEMSTRA, I. H., KUIJPERS, T. W., BERG, T. K. & MATLUNG, H. L. 2016. Neutrophils in cancer. *Immunological Reviews,* 273**,** 312-328.
TRIANTAFILLIDIS, J. K., MERIKAS, E. & GEORGOPOULOS, F. 2011. Current and emerging drugs for the treatment of inflammatory bowel disease. *Drug Design, Development and Therapy,* 5**,** 185.
TRINCHIERI, G. 1989. Biology of natural killer cells. *Advances in Immunology,* 47**,** 187-376.
TRINCHIERI, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature Reviews Immunology,* 3**,** 133-146.
TSAI, K. S., YANG, S. H., LEI, Y. P., TSAI, C. C., CHEN, H. W., HSU, C. Y., CHEN, L. L., WANG, H. W., MILLER, S. A., CHIOU, S. H., HUNG, M. C. & HUNG, S. C. 2011. Mesenchymal stem cells promote formation of colorectal tumors in mice. *Gastroenterology,* 141**,** 1046-56.
UCCELLI, A., MORETTA, L. & PISTOIA, V. 2008. Mesenchymal stem cells in health and disease. *Nature Reviews Immunology,* 8**,** 726-736.
VAGEFI, P. A. & LONGO, W. E. 2005. Colorectal cancer in patients with inflammatory bowel disease. *Clinical Colorectal Cancer,* 4**,** 313-319.
VAN ERPECUM, K. J., SMITS, S., VAN DE MEEBERG, P. C., LINN, F., WOLFHAGEN, F. & ALGRA, A. 1996. Risk of primary sclerosing cholangitis is associated with nonsmoking behavior. *Gastroenterology,* 110**,** 1503-1506.
VAN KLINKEN, B. J., VAN DER WAL, J. G., EINERHAND, A., BÜLLER, H. & DEKKER, J. 1999. Sulphation and secretion of the predominant secretory human colonic mucin MUC2 in ulcerative colitis. *Gut,* 44**,** 387-393.
VIRCHOW, R. 1863. Cellular pathology as based upon physiological and pathological histology. Philadelphia: J.B. Lippincott and Co.
VONARBOURG, C., MORTHA, A., BUI, V. L., HERNANDEZ, P. P., KISS, E. A., HOYLER, T., FLACH, M., BENGSCH, B., THIMME, R. & HÖLSCHER, C. 2010. Regulated expression of nuclear receptor RORγt confers distinct functional fates to NK cell receptor-expressing RORγt+ innate lymphocytes. *Immunity,* 33**,** 736-751.
VYAS, J. M., VAN DER VEEN, A. G. & PLOEGH, H. L. 2008. The known unknowns of antigen processing and presentation. *Nature Reviews Immunology,* 8**,** 607-618.

WAHL, L. M. & KLEINMAN, H. K. 1998. Tumor-associated macrophages as targets for cancer therapy. *Journal of the National Cancer Institute,* 90**,** 1583-1584.
WALDHAUER, I. & STEINLE, A. 2008. NK cells and cancer immunosurveillance. *Oncogene,* 27**,** 5932-5943.
WALKER, J. A., BARLOW, J. L. & MCKENZIE, A. N. 2013. Innate lymphoid cells—how did we miss them? *Nature Reviews Immunology,* 13**,** 75-87.
WANG, C., CHEN, J., SUN, L. & LIU, Y. 2014. TGF-beta signaling-dependent alleviation of dextran sulfate sodium-induced colitis by mesenchymal stem cell transplantation. *Molecular Biology Reports,* 41**,** 4977-4983.
WANG, J., WANG, Y., WANG, S., CAI, J., SHI, J., SUI, X., CAO, Y., HUANG, W., CHEN, X., CAI, Z., LI, H., BARDEESI, A. S., ZHANG, B., LIU, M., SONG, W., WANG, M. & XIANG, A. P. 2015a. Bone marrow-derived mesenchymal stem cell-secreted IL-8 promotes the angiogenesis and growth of colorectal cancer. *Oncotarget,* 6**,** 42825–42837.
WANG, R., HASNAIN, S. Z., TONG, H., DAS, I., CHEN, A. C.-H., OANCEA, I., PROCTOR, M., FLORIN, T. H., ERI, R. D. & MCGUCKIN, M. A. 2015b. Neutralizing IL-23 is superior to blocking IL-17 in suppressing intestinal inflammation in a spontaneous murine colitis model. *Inflammatory Bowel Diseases,* 21**,** 973-984.
WATANABE, S., ARIMURA, Y., NAGAISHI, K., ISSHIKI, H., ONODERA, K., NASUNO, M., YAMASHITA, K., IDOGAWA, M., NAISHIRO, Y. & MURATA, M. 2014. Conditioned mesenchymal stem cells produce pleiotropic gut trophic factors. *Journal of Gastroenterology,* 49**,** 270-282.
WATERMAN, R. S., TOMCHUCK, S. L., HENKLE, S. L. & BETANCOURT, A. M. 2010. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. *PloS One,* 5**,** e10088.
WHITESIDE, T. 2008. The tumor microenvironment and its role in promoting tumor growth. *Oncogene,* 27**,** 5904-5912.
WIDDER, M., LUTZKENDORF, J., CAYSA, H., UNVERZAGT, S., WICKENHAUSER, C., BENNDORF, R. A., SCHMOLL, H. J., MULLER-TIDOW, C., MULLER, T. & MULLER, L. P. 2015. Multipotent mesenchymal stromal cells promote tumor growth in distinct colorectal cancer cells by a beta1-integrin-dependent mechanism. *Int J Cancer,* 138**,** 964–975.
WIRTZ, S., FINOTTO, S., KANZLER, S., LOHSE, A. W., BLESSING, M., LEHR, H. A., GALLE, P. R. & NEURATH, M. F. 1999. Cutting edge: chronic intestinal inflammation in STAT-4 transgenic mice: characterization of disease and adoptive transfer by TNF-plus IFN-γ-producing CD4+ T cells that respond to bacterial antigens. *The Journal of Immunology,* 162**,** 1884-1888.
WIRTZ, S., NEUFERT, C., WEIGMANN, B. & NEURATH, M. F. 2007. Chemically induced mouse models of intestinal inflammation. *Nature Protocols,* 2**,** 541-546.
WIRTZ, S. & NEURATH, M. F. 2007. Mouse models of inflammatory bowel disease. *Advanced Drug Delivery Reviews,* 59**,** 1073-1083.
WU, J. & LANIER, L. L. 2003. Natural killer cells and cancer. *Advances in Cancer Research,* 90**,** 127-156.

YADAV, P. K. & LIU, Z. 2009. Current strategies for the treatment of ulcerative colitis. *Recent Patents on Inflammation & Allergy Drug Discovery,* 3**,** 65-72.

YAGI, H. & KITAGAWA, Y. 2013. The role of mesenchymal stem cells in cancer development. *Frontiers in Genetics,* 4**,** 261.

YAMADA, Y. & MORI, H. 2007. Multistep carcinogenesis of the colon in ApcMin/+ mouse. *Cancer Science,* 98**,** 6-10.

YEN, D., CHEUNG, J., SCHEERENS, H., POULET, F., MCCLANAHAN, T., MCKENZIE, B., KLEINSCHEK, M. A., OWYANG, A., MATTSON, J. & BLUMENSCHEIN, W. 2006. IL-23 is essential for T cell–mediated colitis and promotes inflammation via IL-17 and IL-6. *The Journal of Clinical Investigation,* 116**,** 1310-1316.

YUKIHIROFUNADA, T., KIKUCHI, R., SHINSUKETAKENO, Y. & GABBERT, H. 2003. Prognostic significance of CD8+ T cell and macrophage peritumoral infiltration in colorectal cancer. *Oncology Reports,* 10**,** 309-313.

ZAMAI, L., AHMAD, M., BENNETT, I. M., AZZONI, L., ALNEMRI, E. S. & PERUSSIA, B. 1998. Natural killer (NK) cell–mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. *The Journal of Experimental Medicine,* 188**,** 2375-2380.

ZANGI, L., MARGALIT, R., REICH-ZELIGER, S., BACHAR-LUSTIG, E., BEILHACK, A., NEGRIN, R. & REISNER, Y. 2009. Direct imaging of immune rejection and memory induction by allogeneic mesenchymal stromal cells. *Stem Cells,* 27**,** 2865-2874.

ZAPPIA, E., CASAZZA, S., PEDEMONTE, E., BENVENUTO, F., BONANNI, I., GERDONI, E., GIUNTI, D., CERAVOLO, A., CAZZANTI, F. & FRASSONI, F. 2005. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood,* 106**,** 1755-1761.

ZHANG, Q., SHI, S., LIU, Y., UYANNE, J., SHI, Y., SHI, S. & LE, A. D. 2009. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. *The Journal of Immunology,* 183**,** 7787-7798.

ZHU, W., YU, J., NIE, Y., SHI, X., LIU, Y., LI, F. & ZHANG, X.-L. 2014. Disequilibrium of M1 and M2 macrophages correlates with the development of experimental inflammatory bowel diseases. *Immunological Investigations,* 43**,** 638-652.

ZHU, Y., SUN, Z., HAN, Q., LIAO, L., WANG, J., BIAN, C., LI, J., YAN, X., LIU, Y. & SHAO, C. 2009. Human mesenchymal stem cells inhibit cancer cell proliferation by secreting DKK-1. *Leukemia,* 23**,** 925-933.

Appendix A: Specificity for M1/M2 cells

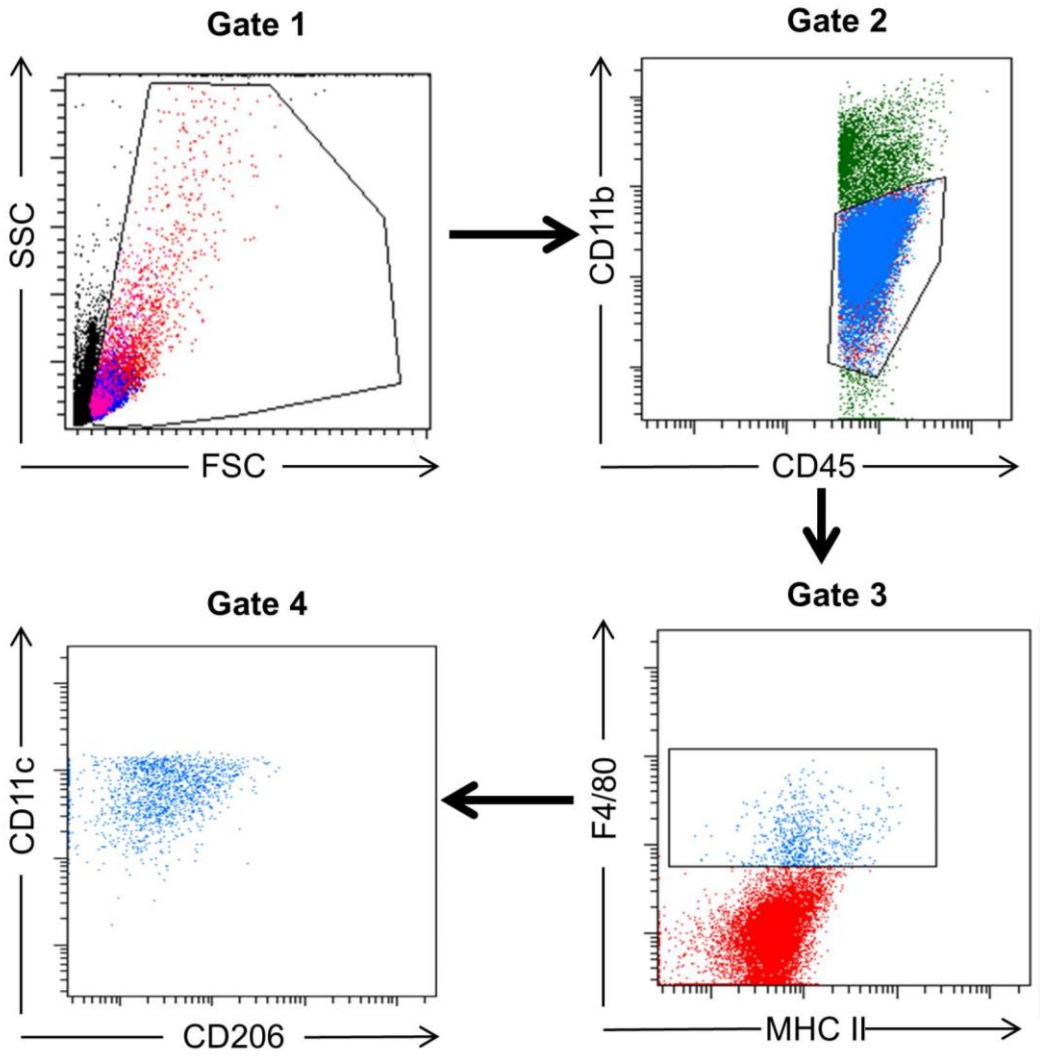

Figure S.1: Specificity for M1/M2 Macrophages in the Spleen.

Gate 1 – live cells based on FSC-SSC gating, Gate 2 – $CD11b^{-}/CD45^{+}$ cells, Gate 3 – $CD11b^{-}/CD45^{+}/F4/80^{+}$ cells and Gate 4 - $CD11c^{+}/CD206^{-}$ cells, of gate 3 cells to demostrate specificity of M1/M2 cells.

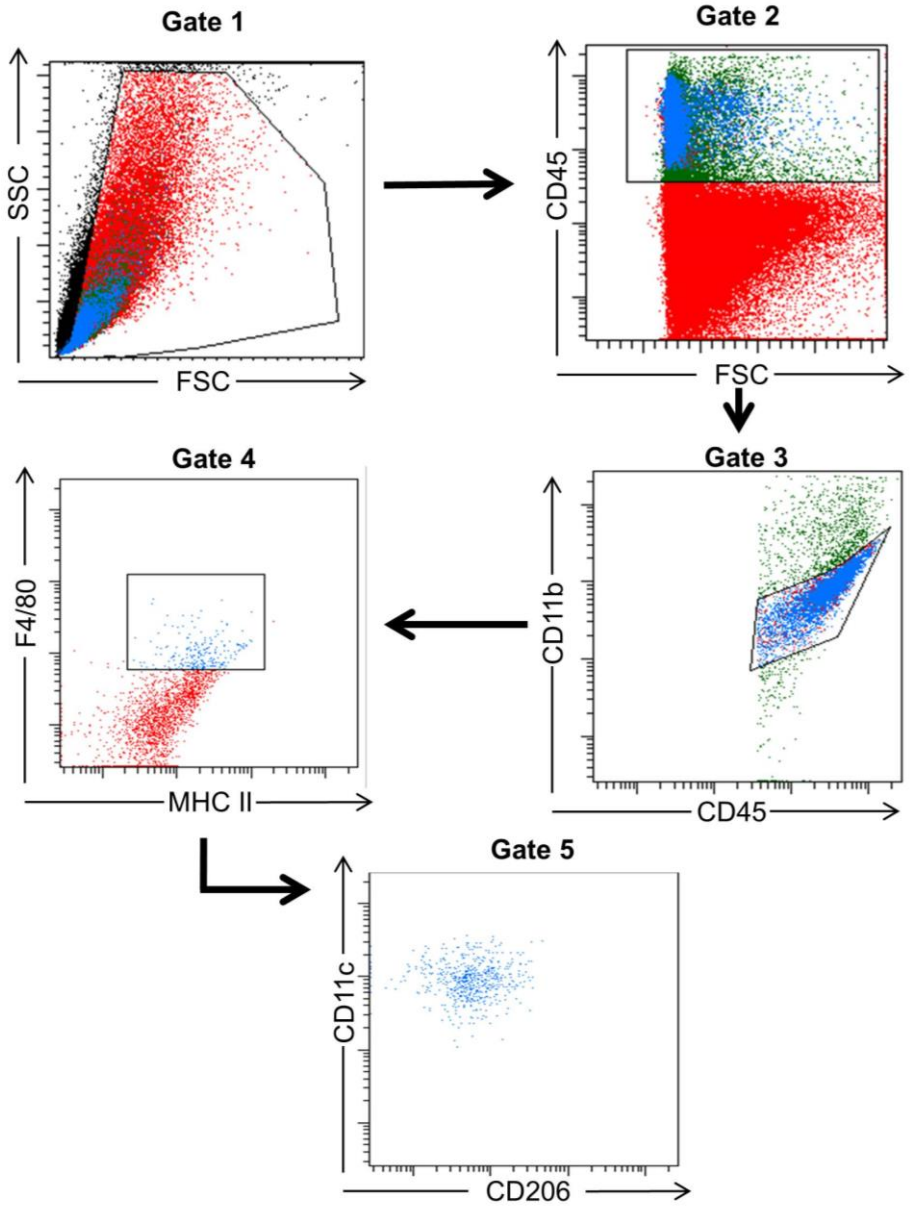

Figure S.2: Specificity for M1/M2 Macrophages in the Colon.

Gate 1 – live cells based on FSC-SSC gating, Gate 2 – $CD45^{+}$cells, Gate 3 – $CD11b^{-}/CD45^{+}$cells, Gate 4 – $F4/80^{+}$ cells and Gate 5 - $CD11c^{+}/CD206^{-}$ cells, of gate 3 cells to demostrate specificity of M1/M2 cells.

Appendix B: Publications Arising From This Thesis

Mesenchymal Stem Cell Treatment of Inflammation-Induced Cancer

Monica D. Prakash, PhD, Sarah Miller, BSc (Hon) (Biomedical Science),*
Sarron Randall-Demllo, BSc (Hon) (Biomedical Science),† and Kulmira Nurgali, PhD**

Abstract: Cancer development is often associated with chronic inflammation. To date, research into inflammation-induced cancer has largely focused on chemokines, cytokines, and their downstream targets. These inflammatory mediators may promote tumor growth, invasion, metastasis, and facilitate angiogenesis. However, the exact mechanisms by which inflammation promotes neoplasia remain unclear. Inflammatory bowel disease (IBD) is characterized by recurrent, idiopathic intestinal inflammation, the complications of which are potentially fatal. IBD incidence in Australia is 24.2 per 100,000 and its peak onset is in people aged 15 to 24 years. Symptoms include abdominal pain, cramps, bloody stool, and persistent diarrhoea or constipation and so seriously compromise quality of life. However, due to its unknown etiology, current treatment strategies combat the symptoms rather than the disease and are limited by inefficacy, toxicity, and adverse side-effects. IBD is also associated with an increased risk of colorectal cancer, for which treatment options are similarly limited. In recent years, there has been much interest in the therapeutic potential of mesenchymal stem cells (MSCs). However, whether MSCs suppress or promote tumor development is still contentious within the literature. Many studies indicate that MSCs exert anti-tumor effects and suppress tumor growth, whereas other studies report pro-tumor effects. Studies using MSCs as treatment for IBD have shown promising results in both animal models and human trials. However, as MSC treatment is still novel, the long-term risks remain unknown. This review aims to summarize the current literature on MSC treatment of inflammation-induced cancer, with a focus of colorectal cancer resulting from IBD.

(Inflamm Bowel Dis 2016;00:1–10)

Key Words: inflammation, cancer

Cancer development is often associated with chronic inflammation. As early as 1863, it was noticed that cancers often occurred at inflammatory sites.[1] Since that time, copious epidemiological data have been emerging to show that chronic inflammation is frequently associated with increased risk of cancer.[2–5] It was initially believed that reactive oxygen and nitrogen species produced by immune cells to combat infection may possess the mutagenic potential to form tumors.[6] However, more recently it has become clear that the development of cancer from inflammation is a complex, multifactorial process.[7]

Inflammatory bowel disease (IBD) is characterized by recurrent, idiopathic intestinal inflammation, the complications of which are potentially fatal. IBD is believed to be of multigenetic origin and to occur as a result of an exaggerated response to gut bacteria.[8] However, due to its unknown etiology, current treatment strategies combat the symptoms rather than the disease and are limited by inefficacy, toxicity, and adverse side-effects. IBD is also associated with an increased risk of colorectal cancer (CRC), for which treatment options are similarly limited.

In recent years, there has been much interest in the therapeutic potential of mesenchymal stem cells (MSCs). MSCs are multipotent stem cells that can be derived from many adult tissues, including bone marrow and adipose tissue.[9] MSCs are currently used to treat inflammatory diseases such as arthritis, multiple sclerosis, and IBD.[10–16] However, whether MSCs suppress or promote tumor development is still contentious within the literature.[17,18] Many studies indicate that MSCs exert antitumor effects and suppress tumor growth,[19,20] whereas other studies report pro-tumor effects.[21,22] Studies using MSCs as treatment for IBD have shown promising results in both animal models[23,24] and human trials.[11,13,16] However, as MSCs treatment is still novel the long-term risks remain unknown.

CHRONIC INFLAMMATION AND CANCER

Inflammation occurs in response to tissue damage that can result from infection, chemical irritation, or trauma. In the early stages, neutrophils migrate to the inflammatory site in response to chemical signals produced by resident macrophages and mast cells. Other immune cells are subsequently recruited by a complex network of signaling molecules, including growth factors, cytokines, and chemokines. Anti-inflammatory molecules also exist to

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From the *Enteric Neuropathy Laboratory, Western Centre for Health Research and Education, College of Health and Biomedicine, Victoria University, Melbourne, Victoria, Australia; and †School of Health Sciences, University of Tasmania, Launceston, Victoria, Australia.

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Address correspondence to Monica D. Prakash, PhD, Western Centre for Health Research and Education, College of Health and Biomedicine, Victoria University, Sunshine Hospital, 176 Furlong Road, St Albans, Melbourne, Victoria 3021, Australia (e-mail: monica.prakash@vu.edu.au).

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allow resolution of the inflammatory response, promoting tissue repair and the rapid programmed clearance of inflammatory cells through apoptosis and subsequent phagocytosis. However, if this process is dysregulated, chronic inflammation can occur.

During chronic inflammation, inflammatory foci predominantly consist of lymphocytes, plasma cells, and macrophages. Macrophages are largely responsible for generating growth factors, cytokines and reactive oxygen and nitrogen species. Under normal conditions, these factors drive the inflammatory response, but during prolonged inflammation may lead to continuous tissue damage, subsequent sustained cell proliferation and hence a predisposition to malignant transformation.[25] Reactive oxygen and nitrogen species may also contribute to DNA damage that can result in neoplasia.[3]

Cancer development is a step-wise process whereby genetic changes confer a growth advantage, driving tumor development. Malignancy is characterized by several hallmarks: self-sufficiency of growth signals, resistance to antigrowth signals, escape from apoptosis, unregulated proliferation, enhanced angiogenesis, and metastasis.[7]

To date, research into inflammation-induced cancer has largely focused on chemokines, cytokines, and their downstream targets. These inflammatory mediators may promote tumor growth, invasion, metastasis, and facilitate angiogenesis.

INFLAMMATORY BOWEL DISEASE (IBD)

IBD incidence in Australia is 24.2 per 100,000 and its peak onset is in people aged 15 to 24 years.[26] Symptoms include abdominal pain, cramps, bloody stool, and persistent diarrhoea or constipation and so seriously compromise quality of life. Potential complications such as perforation, excessive bleeding, bowel obstruction, and intestinal scarring can lead to fatality. IBD comprises 2 main pathologies: Crohn's disease (CD) and ulcerative colitis (UC). UC generally manifests in the rectum but may spread to part of or all of the colon in an uninterrupted pattern. The inflammation observed is typically confined to the mucosa. However, CD can affect any region of the intestine, though most commonly affects the ileum and colon, often discontinuously. Inflammation observed in CD is often transmural; occurring across the entire intestinal wall. CD is also associated with intestinal granuloma, strictures, and fistulas, however, these are not associated with UC. Though the underlying cause of IBD is unknown, numerous predisposing genes have been identified and ~30% of loci are overlapping for both CD and UC, suggesting similar etiology.

Accumulating evidence suggests that IBD results from an inappropriate inflammatory response to intestinal microorganisms in a genetically susceptible host.[27] The intestinal tract is a complex organ that has evolved in cooperation between the host and its microbiome. Its primary function is to provide nutrients and energy for survival and reproduction. A secondary function is to defend the host from potentially harmful ingested food antigens, bacteria, parasites, or toxins. The intestinal epithelium is the interface between the intestinal microbiome and the rest of the body. As only a single layer of epithelial cells separates the two, the gut has also evolved to be the largest immune organ of the body. The intestinal microbiota plays a critical role in shaping the intestinal immune system.[28] Alterations in microbiota have been observed in both animal models of IBD and human IBD patients.[8] IBD is also associated with dysregulation of pro-inflammatory cytokines interferon-gamma (IFN-γ), tumor necrosis factor-alpha, interleukins (IL) -6, -8, -17, and -23.

The intestinal barrier is maintained by interepithelial adhesive complexes known as tight junctions, secreted mucus, and immune cells. Intestinal permeability is the property that allows fluid and solute exchange between the lumen and the rest of the body through the bloodstream. Increased intestinal permeability refers to the nonmediated diffusion of large, normally restricted molecules from the intestinal lumen across the intestinal barrier to the blood and occurs during barrier dysfunction. This may be through direct epithelial damage, or the failure of tight junctions. Tight junctions are formed through interaction of specialized proteins on adjacent epithelial cells. These proteins include claudins, occludin, and ZO family proteins. Tight junction protein expression and localization can be regulated by immune cytokines such as IFN-γ, tumor necrosis factor-alpha, and IL-1β, -6, -10, and -17. Intestinal permeability is also affected by exogenous factors or changes in the immune environment. In IBD, the paracellular space has increased permeability and regulation of tight junctions is defective.[29,30]

CRC development in the chronically inflamed colon has been described as a colitis–dysplasia–carcinoma sequence.[31] Stepwise acquisition of key mutations produces hyperproliferative lesions, or dysplasia, which may eventually acquire the invasive properties of carcinoma. Although colitis-associated CRC exhibits similar molecular events to sporadic CRCs, the timing of these events is markedly different, particularly in the early stages of tumorigenesis. For example, sporadically occurring colorectal dysplasia often displays mutation of the adenomatous polyposis coli/β-catenin pathway, typically resulting in a polypoid outgrowth of the glandular epithelium (adenoma) readily detected endoscopically. Mutations in adenomatous polyposis coli are rare in the inflamed, nondysplastic mucosa, and uncommon in low-grade dysplasia or carcinoma.[32,33] Conversely, mutations of the *TP53* gene in patients with UC are frequent in inflamed but nondysplastic mucosa.[32,34,35] Although the connection between inflammation and carcinogenesis are well known, the mechanisms by which inflammation promotes neoplasia remain unclear.

Due to the idiopathic nature of IBD, current treatments focus on symptomatic relief rather than the cause of the disease. Biological treatments are largely immunosuppressive and so are associated with their own side effects. They are predominantly cytokine/receptor blockers, though there are also reports of treatment through blockade of leukocyte adhesion and costimulatory molecules to inhibit leukocyte trafficking and activation, respectively. Investigation into colitis-associated CRC has also focused attention on the pro-inflammatory leukocytes that migrate

to the inflamed mucosa, and the inflammatory mediators they produce.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are an emerging therapy for IBD. Though many reports show effective treatment of IBD, as MSCs are a novel treatment the long-term side effects of their use are still unknown. Furthermore, despite extensive research, the impact of unmodified MSCs on tumor progression remains unclear. Many studies have shown MSCs promote tumor progression and metastasis, whereas other studies report that MSCs suppress tumor growth.[38] There are many potential reasons for these discrepancies, including differences in tumor models, heterogeneity of MSCs, the dose or timing of MSCs injected, the animal model used, etc.

MSCs can be derived from many adult tissues, including adipose and bone marrow and have the capacity to differentiate into osteoblasts, chondrocytes, adipocytes, myoblasts, and neuronal-like cells. Characterized by their in vitro properties, the International Society for Cellular Therapy has defined specific criteria to classify a stem cell as a MSC: (1) MSCs must be plastic-adherent when maintained under standard culture conditions using tissue culture flasks; (2) ≥95% of the MSC population must express CD105, CD73, and CD90 as measured by flow cytometry and lack the expression (≤2% positive) of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA class II; (3) Cells must possess multipotent differentiation potential i.e. be able to differentiate into osteoblasts, adipocytes, and chondrocytes under standard in vitro differentiating conditions.[36]

Human MSCs are poorly immunogenic, so escape immune recognition and do not induce an immune response when administered into other species.[37] Mouse MSCs, however, do elicit an immune response. This is due to the low levels of the major histocompatibility complex class I (MHC-I) expressed on the surface of mouse MSCs. MHC molecules mediate interactions between immune cells and are categorized into 3 subgroups: classes I, II, and III. Both MHC-I and MHC-II enable discrimination between self- and non–self-antigens.[37] Human MSCs express low levels of MHC-I and also do not express any MHC-II molecules or costimulatory molecules such as CD40 & CD80. These costimulatory molecules are essential for T-cell activation; therefore MSCs have a very low incidence of immune rejection and are thus tolerated well, making them an ideal cellular therapy.[37]

MSCs also exhibit tropism for sites of inflammation and the tumor microenvironment; many of the same inflammatory mediators are found at both sites and are thought to be involved in attracting MSCs. MSC migration is dependent on a multitude of signals, including growth factors, chemokines, and cytokines and is similar to migration of immune cells that respond to injury and sites of inflammation. MSCs' response to tumors gives them the unique ability to function as cellular delivery vehicles for antitumor agents. MSCs are currently being used to deliver a vast array of antitumor agents, including interferon-β (IFN-β), tumor necrosis factor-related apoptosis inducing ligand, cytosine deaminase, and oncolytic viruses. These approaches are being explored and producing potent antitumor effects.

MSC THERAPY FOR IBD AND CANCER

MSCs have immunosuppressive properties that are largely attributed to the number of antiinflammatory cytokines they produce.[38] Emerging research has provided evidence that MSCs also have the ability to suppress $CD8^+$ cytotoxic T cell (CTL) and $CD4^+$ T helper cell activation and proliferation.[39] Conversely, MSCs can increase the number of T regulatory cells, which are responsible for modulating the inflammatory response and preserving tolerance to self-antigens.[39] IL-10 is essential for this function, as deletion of IL-10 in T regulatory cells results in the spontaneous development of a chronic colitis.[40] Furthermore, single nucleotide polymorphisms in IL10RA and IL10RB, the genes encoding the 2 IL-10 receptor subunits, have been linked to pediatric IBD.[41] IL-10 signaling in macrophages results in a STAT3-dependent reduction in proinflammatory cytokines IL-6 and tumor necrosis factor-alpha.[42,43]

MSCs are also neuroprotective. Studies using experimental models of multiple sclerosis have shown that MSC treatment decreases axonal damage and neuronal death by release of soluble factors.[44,45] These studies demonstrate that in the correct micro-environment MSCs can stop and prevent further neuronal damage and in some cases induce axonal regeneration.[46]

To date, studies applying the anti-inflammatory and neuroprotective properties of MSCs as a therapy for IBD are limited. In rats, MSCs reduced intestinal mucosal barrier destruction, ZO-1 downregulation, and tight junction disruption through a TNFα-mediated

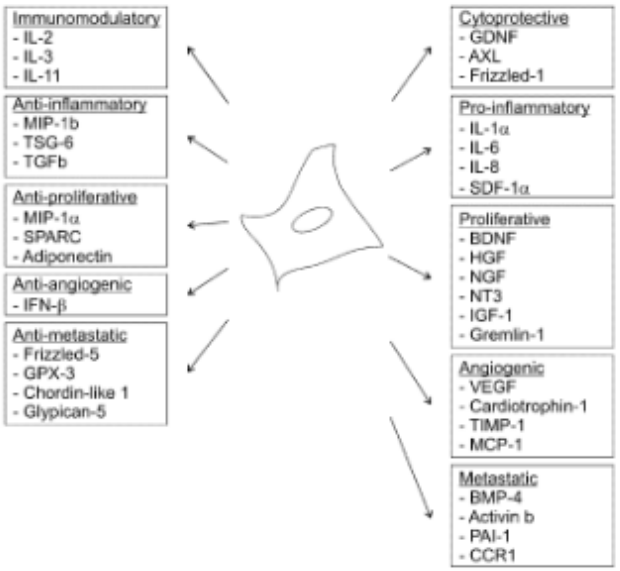

FIGURE 1. Factors secreted by MSCs that impact cancer development.

TABLE 1. Effects of MSCs on Inflammation-Induced Cancer

Key Observations	References
Tumor promoting	
Proliferation	
Tumor cells coinjected with MSCs show enhanced tumor growth	Shinagawa et al[48]
Metastasis	
MSCs secrete several factors that promote metastasis eg, CCL5/RANTES	Karnoub et al[21]; Shinagawa et al[48]
Immunosuppression	
MSC-mediated immunosuppression promotes tumor growth	Djouad et al[49]; Mele et al[50]; Li et al[51]; Di Nicola et al[52]; Krampera et al,[53] Plumas et al[54]; Zappia et al[55]; Corcione et al[56]; Rutella et al[57]; Sotiropoulou et al[58]; Sheng et al[59]; Nasef et al[60]
MSCs can directly impair immune cell function, including T and B cells, dendritic cells, natural killer cells, and macrophages	
MSCs can supress T-cell proliferation through multiple mechanisms, including upregulation of B7-H1 and Stro-1+ expression	
MSCs have been shown to switch macrophages from an M1 to M2 phenotype	
Paracrine factors	
MSCs secrete factors that induce tumor proliferation, migration, and angiogenesis. MSCs have also been shown to secrete exosomes and microparticles that contain proteins or RNA that regulate intracellular signaling in adjacent cells	Potapova et al[61]; Salazar et al[62]; Seib et al[63]; Park et al[64]; Lozito and Tuan[65]; Chen et al[66]
Cytoprotection	
MSCs have been shown to protect tumor cells from chemotherapeutic treatment; interactions with MSCs in the bone marrow can promote the survival of acute and chronic myeloid leukaemia	Konopleva et al[67]; Liu and Hwang[68]
MSCs produce high levels of leptin, which has antiapoptotic properties	
Vascular Support	
MSCs can differentiate into pericytes and endothelial cells	Kang et al[69]; Portalska et al[70]; Nakagaki et al[71]; Kamihata et al[72]; Chen et al[73]; Kinnaird et al[74]; Nagaya et al[75]; Tang et al[76]; Potapova et al[61]; Wang et al[77]
MSCs secrete factors that support vascular growth including VEGF, FGF, PDGF, SDF-1, hepatocyte growth factor, insulin-like growth factor 1, MCP-2, MCP-3, and IL-8.	
Fibroblast growth	
MSC-derived tumor fibroblasts may be recruited from circulating populations	Mishra et al[77]; Spaeth et al[78]; Mishra et al[77]; Spaeth et al[79]
MSCs acquire tumor-associated fibroblast antigens on tumor exposure which promote tumorigenesis	
Tumor suppressive	
Cytotoxicity	
MSCs inhibit tumor initiation in a TGFβ-dependent manner	Nasuno et al[80]; Otsu et al[82]
MSCs induce apoptosis of tumor cells	
Antiproliferative	
MSCs secrete antiproliferative factors	Zhu et al[22]
Immunomodulation	
MSCs express toll like receptors which can switch MSCs from a immunosuppressive (M2) to a pro-inflammatory (M1) phenotype	Waterman et al[81]
Vascular damage	
MSCs can inhibit capillary growth by producing reactive oxygen species that cause endothelial apoptosis	Otsu et al[82]

mechanism.[47] In humans, Ciccocioppo et al reported in 2011 that of 10 patients with fistulising Crohn's disease that were administered MSCs, 7 achieved complete fistula closure and 3 incomplete closure with an increase in rectal mucosa healing. But together, the poorly immunogenic, strong immunosuppressive, anti-inflammatory, and neuroprotective properties of MSCs are promising for the development of new cell-based therapies (Figure 1). However, it remains to be seen whether MSCs can also promote tumor growth and metastasis, which is vital to ensure the safety of their therapeutic applications.

TABLE 2. Key Findings From Studies Using MSC to Treat Inflammation-Induced Cancer

Study	MSC		Model	Key Findings
	Species	Tissue Origin		
Tumor promoting				
Li et al[51]	Human	Adipose	In vitro proliferation assay	MSC-conditioned media stimulated the proliferation of human LoVo CRC cells, and the replicative senescent (P30) MSCs had more obvious effects in comparison with that of early passage (P3) MSCs.
Wang et al[77]	Human	Bone marrow	Subcutaneous xenograft ± coinjected MSC	Tumors formed from CRC cells coinjected with MSCs were substantially larger than those formed from CRC cells alone. IL-8 secreted by MSCs is an important contributor to tumor growth.
Widder et al[54]	Human	Bone marrow	Subcutaneous xenograft ± coinjected MSC	MSC promoted growth of tumors derived from HCT8, but not DLD1 cells when compared with normal fibroblasts.
Mele et al[50]	Human	Bone marrow	Subcutaneous xenograft ± coinjected MSC or with tumor cells cocultured with MSC	MSC triggered epithelial to mesenchymal transition (EMT) in tumor cells in vitro, mediated by surface-bound TGF-β newly expressed on MSC on coculture with tumor cells. In vivo tumor masses formed by MSC-conditioned CRC cells were larger, more vascular, and cells displayed increased invasiveness in vitro and enhanced capacity to invade peripheral tissues in vivo. MSC did not affect tumor initiation but only tumor growth.
De Boeck et al[55]	Human	Bone marrow	Subcutaneous xenograft ± coinjected MSC	MSC or MSC-conditioned medium boosted tumor initiation and growth of CRC cells
Huang et al[56]	Human	Not specified	Subcutaneous xenograft ± coinjected MSC	Colorectal cancer cells, when mixed with otherwise nontumorigenic MSCs, increase the tumor growth rate and angiogenesis more than that when mixed with carcinoma-associated fibroblasts or normal colonic fibroblasts.
Liu et al[57]	Mouse	Bone marrow	Subcutaneous xenograft ± coinjected MSC	Relative to C26 colorectal cancer cells alone, C26 cells coinjected with MSCs resulted in enhanced tumor growth.
Tsai et al[58]	Human	Bone marrow	Subcutaneous xenograft ± coinjected MSC	MSC-derived IL-6 signals through STAT3 to increase the numbers of colorectal tumor-initiating cells and promote tumor formation.
Shinagawa et al[45]	Human	Bone marrow	Orthotopic colon cancer ± coinjected MSC	Orthotopic transplantation of KM12SM human tumor cells mixed with MSCs resulted in larger tumors than transplantation of KM12SM cells alone Liver metastasis was seen only in coinjected mice. Systemically injected MSCs migrated to the stroma of orthotopic colon tumors and metastatic liver tumors.
Tumor promoting and suppressive				
Nakagaki et al[71]	Rat	Bone marrow	Subcutaneous xenograft ± coinjected MSC	Differential tumor growth in response to MSC treatment was observed for each cell line examined. Cell-line specific responses could not be explained by host immune competency. MSCs injected with COLO 320 cells differentiated into pericytes that enhanced angiogenesis. MSCs injected with HT-29 cells conferred an antiproliferative property on HT-29 cells

TABLE 2 (*Continued*)

Study	MSC		Model	Key Findings
	Species	Tissue Origin		
Rhyu et al[55]	Human	Adipose	Subcutaneous xenograft ± intratumoral MSC	The growth of the A549 tumors was inhibited by hATMSCs, yet that of the HT-29 tumors was significantly promoted by hATMSCs in the in vivo xenograft models.
				Significant numbers of genes involved in biological processes were altered in the hATMSC-treated A549 tumors, whereas no biological process was regulated by treatment with hATMSCs in the HT-29 tumors, reflecting the different effects of hATMSCs in the different types of cancer.
Tumor Suppressive				
Chen et al[66]	Mouse	Bone marrow	CAC model ± MSC	MSCs ameliorated the severity of colitis-associated tumorigenesis compared with controls, with attenuated weight loss, longer colons and smaller spleens. Tumor number and tumor load were significantly less in the MSC-treated group whereas tumor size remained comparable.
				Histological assessment indicated that MSCs reduced colon damage.
				Decreased expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), and down-regulation of STAT3 phosphorylation in colon tissue were found after MSC treatment.
Tang et al[102]	Human	Cord blood	CAC model ± MSC	MSCs migrated from the vasculature to colon and suppressed colitis-related neoplasm.
				MSCs alleviated the pathology of inflammation in the colitis stage of CAC model and inhibited inflammatory cytokine production both in colon and serum.
Nasuno et al[30]	Rat	Bone marrow	CAC model ± MSC	MSCs inhibited AOM-induced tumor initiation but not tumor promotion.
				MSCs broadly affected the tumor cell-cycle machinery, potentially leading to G1 arrest in vivo
Ohlsson et al[59]	Rat	Bone marrow	Subcutaneous allograft ± MSC	MSCs inhibited the outgrowth of the rat colon carcinoma; complete inhibition was seen if the number of MSCs were at least equal to the number of tumor cells.
				Tumor cells coimplanted with MSCs induced greater monocyte and granulocyte infiltration than tumor cells or MSCs alone.

MSC PRO-TUMORIGENIC PROPERTIES

MSCs are reported to promote tumor proliferation, fibroblast growth, angiogenesis, and metastasis by providing immunosuppression, cytoprotection, and vascular support largely by the production of paracrine factors, as summarized in Table 1.

Bone marrow-derived MSCs (BM-MSC) have been shown to promote the in vivo growth of colon cancer, lymphoma, and melanoma.[18] Table 2 summarizes the key findings of studies to date that have used MSCs to treat inflammation-induced cancers, particularly CRC.

Foetal or adult MSCs coinjected subcutaneously with tumor cells favor tumor growth and metastasis in immunocompromised mice;[21,50,83–87] this is accompanied by extensive necrosis and angiogenesis when compared with mice injected with tumor cells alone.[17] When melanoma cells were subcutaneously injected into an allogeneic recipient, melanoma cells only formed tumors in the presence of MSCs.[49] Interestingly, this occurred whether MSCs were coinjected at the tumor site or injected at a distance. In an orthotopic model of CRC, coinjection of MSCs resulted in increased tumor size and liver metastasis that was not observed in mice injected with cancer cell alone.[48] MSCs can also promote tumor growth within the bone; multiple myeloma malignancy leads to the formation of osteolytic lesions in the bone and this is enhanced by interaction with MSCs.[17]

A few studies have now tried to identify the molecules produced by MSCs that enhance the growth of tumors. Coculture or indirect interaction of MSCs with breast cancer and melanoma cells enhances tumor growth, indicating that soluble factors are involved.[90,91] MSCs have been shown to produce IL-6, which promotes proliferation of multiple myeloma cells and CRC tumor formation.[87,92] Activation of the IL-6 pathway typically involves signal transduction through STAT3, which is frequently mutated in sporadic colorectal cancers. Dysregulation of STAT3 activation promotes colorectal tumor progression through transcriptional alteration of cell-cycle control genes cyclin-D1, c-Myc, and metabolic regulator mTORC1. Signaling through STAT3, IL-6 is also capable of suppressing pro-apoptotic signals through the induction of Bcl2, Bcl-X_L, and survivin.

Immunosuppression could explain the enhancement of tumor growth by MSCs. MSCs can modulate major immune cell populations when stimulated with a mitogenic signal and suppress lymphocyte proliferation to both allogenic and xenogenic antigens.[93] The inhibitory effects of MSCs on B cell proliferation occurs through cell cycle arrest at the G_0/G_1 phase and not through apoptosis as previously thought.[54,56] MSCs are also resistant to CTL-mediated lysis and are able to inhibit CTL cytotoxicity in a dose-dependent manner when exposed at CTL priming.[94] Although originally believed to directly inhibit natural killer (NK) cell activation and proliferation, MSCs have been shown to inhibit the production of IFN-γ by natural killer cells.[95]

MSCs have also been shown to secrete factors involved in angiogenesis. During tumor progression, angiogenesis is an essential feature of cancer pathology, as without an adequate blood supply, a tumor cannot grow beyond 2 to 3 mm^3.[96] In vitro experiments have shown that MSC-conditioned medium supports survival of cardiac myocytes and stimulated proliferation and migration of endothelial cells.[74,97] Secretion of specific angiogenic factors vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor, insulin-like growth factor 1, monocyte hemoattractant protein (MCP)-2, MCP-3, PDGF, SDF-1, and IL-8 have also been demonstrated.[61,72–77] In vivo, subcutaneous tumors coinjected with MSC described above with increased tumor growth and metastasis have also exhibited increased vascularity in some cases.[50,85]

MSC ANTITUMORIGENIC PROPERTIES

In addition to the studies described above, there are numerous studies that describe either mixed or antitumor effects of MSCs in animal models. Maestroni and colleagues first observed tumor suppression by MSCs in models of Lewis lung carcinoma and B16 melanoma, showing that coinjection of tumor cells and MSCs inhibited primary tumor growth.[98] The antitumor effects of MSCs have also been demonstrated in a model of colon carcinoma in rats.[89] MSCs inhibited the growth of rat colon carcinoma when coimplanted with tumor cells. Increased macrophage and granulocyte infiltration was noted in tumors coinjected with MSCs, compared with no MSC control tumors, suggesting a pro-inflammatory effect. In fact, MSCs alone increased the engraftment of monocytes and granulocytes; however, this may have been due to the immunogenicity of MSCs transplanted across different rat strains.

Human foetal skin-derived MSCs can also inhibit the growth of human liver cancer cell lines. The cancer cells cocultured with MSCs showed reduced proliferation, colony formation, and oncogene expression; the same effects were shown when MSCs were coinjected with the cancer cell lines in vivo.[99] These same MSCs also inhibited the growth of breast cancer cells in vitro and treatment with MSC-conditioned medium also resulted in inhibition of cell growth.[20]

MSCs have been shown to inhibit cancer growth by altering the cell cycle.[100] MSC coculture in vitro with pancreatic tumor cells showed an increase in G1-phase arrest in the tumor cells.[100] In vivo injection of adipose-derived MSCs into an established pancreatic cancer xenograft also inhibited tumor growth. In a similar approach, bone marrow-derived MSCs were injected into an established model of subcutaneous melanoma, resulting in apoptosis and abrogation of tumor growth.[82] However, when MSCs were placed in a Matrigel insert so that they were exposed to the soluble factors but no cell to cell contact, they seemed to have no cytotoxic effects.[82] This is in contradiction to the experiments previously reported by Qiao and colleagues,[20] and may reflect multiple mechanisms by which MSCs exert their antitumor effects.

As shown in Table 2, 3 recent studies have shown in colitis-associated CRC models that MSCs inhibited tumor initiation and ameliorated inflammation, reducing damage to the colon.[80,101,102] Nasuno et al reported that the decreased tumor initiation was transforming growth factor beta (TGF-β)-dependent. They did not specify whether the TGF-β was MSC-derived, however, MSCs have been reported to produce and secrete TGF-β. In the normal colon, TGF-β regulates the growth and self-renewal of colonic epithelium along the crypt axis by opposing Wnt/β-catenin signaling.[103] TGF-β signaling inhibits proliferation of epithelial cells, arresting cell cycle progression in G1 restitution.[104] Mutations in TGF-βRII are common in sporadic CRC (13%).[105] Only one group has reported antitumor effects of MSCs using a subcutaneous tumor model,[89] whereas numerous studies in subcutaneous tumor models have reported pro-tumorigenic effects. In addition, in 2 studies differential tumor growth in response to MSC treatment was observed for different CRC cell lines.[71,88] These cell line-specific responses could not be explained by host immune competency. Rhyu et al[88] observed that while expression of several genes were altered in MSC-treated A549 tumors, no genes were altered by MSC-treatment of HT-29 tumors. These conflicting studies reflect the different effects of MSCs in different types of cancer and in different models and must be carefully considered when drawing conclusions.

CONCLUDING REMARKS

Current treatment strategies for IBD and CRC largely combat the symptoms rather than the disease and are limited by

inefficacy, toxicity, and adverse side-effects. In recent years, there has been much interest in the therapeutic potential of mesenchymal stem cells (MSCs), however, it is still contentious whether they are responsible for harm or good. Many studies indicate that MSCs exert antitumor effects and suppress tumor growth, whereas other studies report pro-tumor tumor effects. Yet, studies using MSCs as treatment for IBD have shown promising results in both animal models and human trials. MSCs seem to behave differently in different types of cancer and in different models and this must be carefully considered is each case.

REFERENCES

1. Virchow R. *Cellular pathology as based upon physiological and pathological histology*. Philadelphia, PA: JB Lippincott, Co; 1863.
2. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet*. 2001;357:539–545.
3. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002;420:860–867.
4. Philip M, Rowley DA, Schreiber H. Inflammation as a tumor promoter in cancer induction. *Semin Cancer Biol*. 2004;14:433–439.
5. Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. *Mol Cancer Res*. 2006;4:221–233.
6. Sandhu JK, Birnboim HC. Mutagenicity and cytotoxicity of reactive oxygen and nitrogen species in the MN-11 murine tumor cell line. *Mutat Res*. 1997;379:241–252.
7. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646–674.
8. Becker C, Neurath MF, Wirtz S. The intestinal microbiota in inflammatory bowel disease. *Ilar J*. 2015;56:192–204.
9. Mosna F, Sensebé L, Krampera M. Human bone marrow and adipose tissue mesenchymal stem cells: a user's guide. *Stem Cells Dev*. 2010;19:1449–1470.
10. González MA, Gonzalez-Rey E, Rico L, et al. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. *Arthritis Rheum*. 2009;60:1006–1019.
11. Duijvestein M, Vos ACW, Roelofs H, et al. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut*. 2010;59:1662–1669.
12. Freedman MS, Bar-Or A, Atkins HL, et al. The therapeutic potential of mesenchymal stem cell transplantation as a treatment for multiple sclerosis: consensus report of the International MSCT Study Group. *Mult Scler*. 2010;16:503–510.
13. Ciccocioppo R, Bernardo ME, Sgarella A, et al. Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. *Gut*. 2011;60:788–798.
14. Connick P, Kolappan M, Crawley C, et al. Autologous mesenchymal stem cells for the treatment of secondary progressive multiple sclerosis: an open-label phase 2a proof-of-concept study. *Lancet Neurol*. 2012;11:150–156.
15. Lee WY, Park KJ, Cho YB, et al. Autologous adipose tissue-derived stem cells treatment demonstrated favorable and sustainable therapeutic effect for Crohn's fistula. *Stem Cells*. 2013;31:2575–2581.
16. Forbes GM, Sturm MJ, Leong RW, et al. A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol*. 2014;12:64–71.
17. Lazennec G, Jorgensen C. Concise review: adult multipotent stromal cells and cancer: risk or benefit? *Stem Cells*. 2008;26:1387–1394.
18. Klopp AH, Gupta A, Spaeth E, et al. Concise review: dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? *Stem Cells*. 2011;29:11–19.
19. Khakoo AY, Pati S, Anderson SA, et al. Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *J Exp Med*. 2006;203:1235–1247.
20. Qiao L, Xu Z, Zhao T, et al. Suppression of tumorigenesis by human mesenchymal stem cells in a hepatoma model. *Cell Research*. 2008;18:500–507.
21. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449:557–563.
22. Zhu Y, Sun Z, Han Q, et al. Human mesenchymal stem cells inhibit cancer cell proliferation by secreting DKK-1. *Leukemia*. 2009;23:925–933.
23. Gonzalez-Rey E, Delgado M. Therapeutic Application of Mesenchymal Stromal Cells in Murine Models of Inflammatory Bowel Disease.In: *Animal Models for Stem Cell Therapy*. New York: Springer; 2014:331–339.
24. Robinson AM, Sakkal S, Park A, et al. Mesenchymal stem cells and conditioned medium avert enteric neuropathy and colon dysfunction in Guinea pig TNBS-induced colitis. *Am J Physiol Gastrointest Liver Physiol*. 2014;307:G1115–G1129.
25. Macarthur M, Hold GL, El-Omar EM. Inflammation and Cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. *Am J Physiol Gastrointest Liver Physiol*. 2004;286:G515–G520.
26. Studd C, Cameron G, Beswick L, et al. Never underestimate inflammatory bowel disease: high prevalence rates and confirmation of high incidence rates in Australia. *J Gastroenterol Hepatol*. 2015;31:81–86.
27. Abraham C, Cho JH. Inflammatory bowel disease. *The New Engl Journal Medicine*. 2009;361:2066–2078.
28. Bäckhed F, Ley RE, Sonnenburg JL, et al. Host-bacterial mutualism in the human intestine. *Science*. 2005;307:1915–1920.
29. Eckburg PB, Relman DA. The role of microbes in Crohn's disease. *Clin Infect Dis*. 2007;44:256–262.
30. Frank DN, Amand ALS, Feldman RA, et al. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci*. 2007;104:13780–13785.
31. Ullman TA, Itzkowitz SH. Intestinal inflammation and cancer. *Gastroenterology*. 2011;140:1807–1816.
32. Redston MS, Papadopoulos N, Caldas C, et al. Common occurrence of APC and K-ras gene mutations in the spectrum of colitis-associated neoplasias. *Gastroenterology*. 1995;108:383–392.
33. Aust DE, Terdiman JP, Willenbucher RF, et al. The APC/beta-catenin pathway in ulcerative colitis-related colorectal carcinomas: a mutational analysis. *Cancer*. 2002;94:1421–1427.
34. Brentnall TA, Crispin DA, Rabinovitch PS, et al. Mutations in the p53 gene: an early marker of neoplastic progression in ulcerative colitis. *Gastroenterology*. 1994;107:369–378.
35. Hussain SP, Amstad P, Raja K, et al. Increased p3 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. *Cancer Res*. 2000;60:3333–3337.
36. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315–317.
37. Chen P-M, Yen M-L, Liu K-J, et al. Immunomodulatory properties of human adult and fetal multipotent mesenchymal stem cells. *J Biomed Sci*. 2011;18:49.
38. Lanzoni G, Roda G, Belluzzi A, et al. Inflamm Bowel Disease: moving toward a Stem Cell-based therapy. *World J Gastroenterol*. 2008;14:4616.
39. Newman RE, Yoo D, LeRoux MA, et al. Treatment of inflammatory diseases with mesenchymal stem cells. *Inflamm Allergy Drug Targets*. 2009;8:110–123.
40. Rubtsov YP, Rasmussen JP, Chi EY, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity*. 2008;28:546–558.
41. Moran CJ, Walters TD, Guo CH, et al. IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. *Inflamm Bowel Dis*. 2013;19:115–123.
42. Williams L, Bradley L, Smith A, et al. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol*. 2004;172:567–576.
43. Murray PJ. The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. *Proc Natl Acad Sci U S A*. 2005;102:8686–8691.
44. Payne N, Siatskas C, Bernard CC. The promise of stem cell and regenerative therapies for multiple sclerosis. *J Autoimmunity*. 2008;31:288–294.

45. Siatskas C, Payne NL, Short MA, et al. A consensus statement addressing mesenchymal stem cell transplantation for multiple sclerosis: it's time! *Stem Cell Rev Rep*. 2010;6:500–506.
46. Payne N, Siatskas C, Barnard A, et al. The prospect of stem cells as multi-faceted purveyors of immune modulation, repair and regeneration in multiple sclerosis. *Curr Stem Cell Research Therapy*. 2011;6:50–62.
47. Shen ZY, Zhang J, Song HL, et al. Bone-marrow mesenchymal stem cells reduce rat intestinal ischemia-reperfusion injury, ZO-1 downregulation and tight junction disruption via a TNF-alpha-regulated mechanism. *World J Gastroenterol*. 2013;19:3583–3595.
48. Shinagawa K, Kitadai Y, Tanaka M, et al. Mesenchymal stem cells enhance growth and metastasis of colon cancer. *Int J Cancer*. 2010; 127:2323–2333.
49. Djouad F, Plence P, Bony C, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood*. 2003;102:3837–3844.
50. Mele V, Muraro MG, Calabrese D, et al. Mesenchymal stromal cells induce epithelial-to-mesenchymal transition in human colorectal cancer cells through the expression of surface-bound TGF-beta. *Int J Cancer*. 2014;134:2583–2594.
51. Li Y, Xu X, Wang L, et al. Senescent mesenchymal stem cells promote colorectal cancer cells growth via galectin-3 expression. *Cell Biosci*. 2015;5:21.
52. Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood*. 2002;99:3838–3843.
53. Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood*. 2003;101:3722–3729.
54. Plumas J, Chaperot L, Richard M-J, et al. Mesenchymal stem cells induce apoptosis of activated T cells. *Leukemia*. 2005;19:1597–1604.
55. Zappia E, Casazza S, Pedemonte E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood*. 2005;106:1755–1761.
56. Corcione A, Benvenuto F, Ferretti E, et al. Human mesenchymal stem cells modulate B-cell functions. *Blood*. 2006;107:367–372.
57. Rutella S, Danese S, Leone G. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood*. 2006;108:1435–1440.
58. Sotiropoulou PA, Perez SA, Gritzapis AD, et al. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells*. 2006; 24:74–85.
59. Sheng H, Wang Y, Jin Y, et al. A critical role of IFNgamma in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. *Cell Res*. 2008;18:846–857.
60. Nasef A, Zhang YZ, Mazurier C, et al. Selected Stro-1-enriched bone marrow stromal cells display a major suppressive effect on lymphocyte proliferation. *Int J Lab Hematol*. 2009;31:9–19.
61. Potapova IA, Gaudette GR, Brink PR, et al. Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro. *Stem Cells*. 2007;25:1761–1768.
62. Salazar KD, Lankford SM, Brody AR. Mesenchymal stem cells produce Wnt isoforms and TGF-β1 that mediate proliferation and procollagen expression by lung fibroblasts. *Am J Physiol Cell Mol Physiol*. 2009; 297:L1002–L1011.
63. Seib FP, Prewitz M, Werner C, Bornhäuser M. Matrix elasticity regulates the secretory profile of human bone marrow-derived multipotent mesenchymal stromal cells (MSCs). *Biochem Biophys Res Commun*. 2009;389:663–667.
64. Park K-S, Kim Y-S, Kim J-H, et al. Trophic molecules derived from human mesenchymal stem cells enhance survival, function, and angiogenesis of isolated islets after transplantation. *Transplantation*. 2010;89: 509–517.
65. Lozito TP, Tuan RS. Endothelial and cancer cells interact with mesenchymal stem cells via both microparticles and secreted factors. *J Cell Mol Med*. 2014;18:2372–2384.
66. Chen Z, He X, He X, et al. Bone marrow mesenchymal stem cells ameliorate colitis-associated tumorigenesis in mice. *Biochem Biophys Res Commun*. 2014;450:1402–1408.
67. Konopleva M, Konoplev S, Hu W, et al. Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia*. 2002;16:1713–1724.
68. Liu C-H, Hwang S-M. Cytokine interactions in mesenchymal stem cells from cord blood. *Cytokine*. 2005;32:270–279.
69. Kang S-G, Shinojima N, Hossain A, et al. Isolation and perivascular localization of mesenchymal stem cells from mouse brain. *Neurosurgery*. 2010;67:711.
70. Portalska JK, Leferink A, Groen N, et al. Endothelial differentiation of mesenchymal stromal cells. *PLoS One*. 2012;7:e46842.
71. Nakagaki S, Arimura Y, Nagaishi K, et al. Contextual niche signals towards colorectal tumor progression by mesenchymal stem cell in the mouse xenograft model. *J Gastroenterol*. 2015;50:962–974.
72. Kamihata H, Matsubara H, Nishiue T, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation*. 2001;104:1046–1052.
73. Chen J, Li Y, Katakowski M, et al. Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. *J Neurosci Res*. 2003;73:778–786.
74. Kinnaird T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res*. 2004;94:678–685.
75. Nagaya N, Fujii T, Iwase T, et al. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. *Am J Physiol Heart Circ Physiol*. 2004;287:H2670–H2676.
76. Tang YL, Zhao Q, Qin X, et al. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. *Ann Thorac Surg*. 2005;80: 229–236; discussion 236–227.
77. Wang J, Wang Y, Wang S, et al. Bone marrow-derived mesenchymal stem cell-secreted IL-8 promotes the angiogenesis and growth of colorectal cancer. *Oncotarget*. 2015;40:42825–42837.
78. Mishra PJ, Mishra PJ, Humeniuk R, et al. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. *Cancer Res*. 2008;68:4331–4339.
79. Spaeth E, Klopp A, Dembinski J, et al. Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells. *Gene Ther*. 2008;15:730–738.
80. Spaeth EL, Dembinski JL, Sasser AK, et al. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PloS One*. 2009;4:e4992.
81. Nasuno M, Arimura Y, Nagaishi K, et al. Mesenchymal stem cells cancel azoxymethane-induced tumor initiation. *Stem Cells*. 2014;32:913–925.
82. Waterman RS, Tomchuck SL, Henkle SL, Betancourt AM. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. *PloS One*. 2010;5:e10088.
83. Otsu K, Das S, Houser SD, et al. Concentration-dependent inhibition of angiogenesis by mesenchymal stem cells. *Blood*. 2009;113:4197–4205.
84. Widder M, Lutzkendorf J, Caysa H, et al. Multipotent mesenchymal stromal cells promote tumor growth in distinct colorectal cancer cells by a beta1-integrin-dependent mechanism. *Int J Cancer*. 2015;138:964–975.
85. De Boeck A, Pauwels P, Hensen K, et al. Bone marrow-derived mesenchymal stem cells promote colorectal cancer progression through paracrine neuregulin 1/HER3 signalling. *Gut*. 2013;62:550–560.
86. Huang WH, Chang MC, Tsai KS, et al. Mesenchymal stem cells promote growth and angiogenesis of tumors in mice. *Oncogene*. 2013; 32:4343–4354.
87. Liu Y, Han ZP, Zhang SS, et al. Effects of inflammatory factors on mesenchymal stem cells and their role in the promotion of tumor angiogenesis in colon cancer. *J Biol Chem*. 2011;286:25007–25015.
88. Tsai KS, Yang SH, Lei YP, et al. Mesenchymal stem cells promote formation of colorectal tumors in mice. *Gastroenterology*. 2011;141:1046–1056.
89. Rhyu JJ, Yun JW, Kwon E, et al. Dual effects of human adipose tissue-derived mesenchymal stem cells in human lung adenocarcinoma A549 xenografts and colorectal adenocarcinoma HT-29 xenografts in mice. *Oncol Rep*. 2015;34:1733–1744.
90. Ohlsson LB, Varas L, Kjellman C, et al. Mesenchymal progenitor cell-mediated inhibition of tumor growth in vivo and in vitro in gelatin matrix. *Exp Molecular Pathology*. 2003;75:248–255.

91. Sun T, Sun BC, Ni CS, et al. study on the interaction between B16 melanoma cell-line and bone-marrow derived mesenchymal stem cells. *Cancer Lett.* 2008;263:35–43.
92. Pasanen I, Pietila M, Lehtonen S, et al. Mesenchymal stromal cells from female donors enhance breast cancer cell proliferation in vitro. *Oncology*. 2015;88:214–225.
93. Chauhan D, Uchiyama H, Akbarali Y, et al. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B. *Blood*. 1996;87:1104–1112.
94. Sudres M, Norol F, Trenado A, et al. Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. *J Immunol*. 2006;176:7761–7767.
95. Rasmusson I, Uhlin M, Le Blanc K, et al. Mesenchymal stem cells fail to trigger effector functions of cytotoxic T lymphocytes. *J Leukoc Biol*. 2007;82:887–893.
96. Spaggiari GM, Capobianco A, Abdelrazik H, et al. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood*. 2008;111:1327–1333.
97. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971;285:1182–1186.
98. Gnecchi M, He H, Noiseux N, et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *Faseb J*. 2006;20:661–669.
99. Maestroni G, Hertens E, Galli P. Factor(s) from nonmacrophage bone marrow stromal cells inhibit Lewis lung carcinoma and B16 melanoma growth in mice. *Cell Mol Life Sci CMLS*. 1999;55:663–667.
100. Hou L, Wang X, Zhou Y, et al. Inhibitory effect and mechanism of mesenchymal stem cells on liver cancer cells. *Tumour Biol*. 2014;35:1239–1250.
101. Cousin B, Ravet E, Poglio S, et al. Adult stromal cells derived from human adipose tissue provoke pancreatic cancer cell death both in vitro and in vivo. *PLoS One*. 2009;4:e6278.
102. Chen Z, He X, He X, et al. Bone marrow mesenchymal stem cells ameliorate colitis-associated tumorigenesis in mice. *Biochem Biophys Res Commun*. 2014;450:1402–1408.
103. Tang RJ, Shen SN, Zhao XY, et al. Mesenchymal stem cells-regulated Treg cells suppress colitis-associated colorectal cancer. *Stem Cell Res Ther*. 2015;6: 71.
104. Reynolds A, Wharton N, Parris A, et al. Canonical Wnt signals combined with suppressed TGFbeta/BMP pathways promote renewal of the native human colonic epithelium. *Gut*. 2014;63:610–621.
105. McKaig BC, Makh SS, Hawkey CJ, et al. Normal human colonic subepithelial myofibroblasts enhance epithelial migration (restitution) via TGF-beta3. *Am J Physiol*. 1999;276(5 pt 1):G1087–G1093.
106. Grady WM, Myeroff LL, Swinler SE, et al. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res*. 1999;59:320–324.

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Original Article

Original Article

Leukocyte populations and IL-6 in the tumor microenvironment of an orthotopic colorectal cancer model

Sarah Miller[1], Paul V. Senior[2], Monica Prakash[1], Vasso Apostolopoulos[1], Samy Sakkal[1], and Kulmira Nurgali[1,*]

[1]Centre for Chronic Diseases, College of Health and Biomedicine, Victoria University, Melbourne, Australia, and [2]North West Academic Centre, University of Melbourne and Western Health, Sunshine Hospital, St Albans, Australia

*Correspondence address. Tel: +61-3-83958223; E-mail: kulmira.nurgali@vu.edu.au

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Abstract

Colorectal cancer (CRC) is a major health problem worldwide. It is often diagnosed late due to its asymptomatic nature. As with all cancers, an immune reaction is involved; however, in CRC, it is unknown if this immune response is favorable or unfavorable for disease progression. In this study, the immune response in mesenteric lymph nodes (MLNs) and Peyer's patches was investigated during development of CRC in an orthotopic mouse model. CRC was induced by injecting CT26 cells into the cecum wall of BALB/c mice. Flow cytometry was used to analyze leukocyte populations involved in tumor immunity in MLNs and Peyer's patches. Cryostat sections for immunohistochemistry were prepared from the caecum and colon from CRC-induced and sham-operated animals. Cytokines produced by mouse CT26 cell line were measured *in vitro* and *in vivo*. Significant increases in the number of $CD8^+/TCR^+$ and $CD49b^+/TCR^-$ (natural killer) cells were found in MLNs and Peyer's patches in the CRC group. In addition, γδT cells were present in the lamina propria of the colon tissues from sham-operated mice, but absent in the colon tissues from mice with CRC. Immunohistochemical analysis of tumorous tissues showed eosinophil, $CD69^+$ T cell, and $CD11b^+$ cell infiltration. Both *in vitro* and *in vivo* CT26 tumor cells were interleukin (IL)-6 positive. In addition, tumor-infiltrating $CD45^+$ cells were also IL-6 positive. In summary, the kinetics of the immune response to CRC and the key effector lymphocytes that are implicated in tumor immunity are demonstrated. Furthermore, IL-6 is a key cytokine present within the tumor microenvironment.

Key words: colorectal cancer, immune system, mesenteric lymph nodes, Peyer's patches, IL-6

Introduction

Cancer of the colon and rectum is the third most common type of cancer in the world. Chronic inflammatory diseases such as Crohn's disease and ulcerative colitis lead to a high risk of developing colorectal cancer (CRC) [1–3]. Other risk factors for the development of CRC include an unhealthy diet, smoking, analgesics and genetic factors [4,5]. Due to the asymptomatic nature of CRC, it is often diagnosed at the late stages when cancer has spread to other parts of the body [6,7].

The immune system has many specialized cells that are involved in the detection and elimination of tumors. Dendritic cells and macrophages can detect tumor antigens and are involved in activating T cells. Natural killer (NK) lymphocytes and neutrophils, part of innate immunity, also play a role in the elimination of tumor cells and activation of T cells. γδT cells (highly abundant in gut mucosa) and NKT cells (share properties of both T cells and NK cells and recognize lipids and glycolipids in complex with CD1d molecules) bridge innate

and adaptive responses, in that they can eliminate tumor cells without prior sensitization [8]. The immune system plays a vital role in the prevention of tumor growth including response to tumor-specific and tumor-associated antigens; however, the leukocyte-driven inflammatory responses can also play a role in the initiation of CRC [9,10]. Inflammatory responses are largely driven by $CD4^+$ T helper cell subsets including the canonical 'pro-inflammatory' Th1 responses: cytotoxic T lymphocytes (CTLs), NK and M1 macrophages; and/or the canonical 'anti-inflammatory' Th2 responses: M2 macrophages, eosinophils and activated B cells such as IgE-secreting plasma cells.

Th1 responses are generally associated with inducing cellular toxicity to tumor cells. Indeed, enhanced CTL responses are generally associated with a good prognosis for cancer patients [11]. Likewise, NK cells mediate cytotoxicity in many types of tumors [12], and macrophages (M1) have Fc receptors for opsonizing antibodies and can clear tumor cells and tumor antigens [13]. Pro-inflammatory Th1 cytokines such as interleukin (IL)-2, tumor necrosis factor (TNF)-α and interferon (IFN)-γ have been shown to lead to a better prognostic outcome in many cancers [14]. In some cases, IL-2 therapy is being used to treat cancers [15].

Th2 responses are generally linked to poor prognosis; excessive anti-inflammatory responses contribute to disease development and have been associated with tumor pathophysiology. The tumor microenvironment consists largely of tumor-associated macrophages (predominantly M2 macrophages) commonly found in tumor biopsies which typically secrete IL-10 [16] which aids in the diversion of Th1 cytotoxic responses to Th2 responses. In addition, IL-6 has been linked to poor tumor outcomes [17] and IL-4 has been linked to colon cancer stem cells that elude cell death [18]. Understanding the tumor microenvironment allows the prediction of immune response outcomes and the design of treatments that will stimulate Th1 cytotoxic responses and subvert tumor growth and metastasis. The immune system has specialized organs to help combat gastrointestinal cancers including Peyer's patches and mesenteric lymph nodes (MLNs). Lymph nodes provide a gateway for CRC metastasis. Peyer's patches are lymphoid organs that are irregularly distributed along the antimesenteric side of the small intestine. In the distal ileum, Peyer's patches are numerous and form a lymphoid ring. Peyer's patches serve as major sites for the generation of immunity to intestinal antigens. Their unique micro-organization is crucial for the generation of an immune response.

Animal models of human CRC can provide insight into the mechanisms that underlie the development and pathogenesis of CRC. The ideal animal model should, therefore, replicate all aspects of tumor development, including the acquirement of genetic alterations with consequent changes in cell behavior and characteristic sensitivity to therapeutics [19]. The tumor model should also be reliable, i.e. tumor take should be predictable and constant, with a high incidence of affected animals in a narrow time frame. Currently, there are several models for implanting tumor cells into an animal. The easiest and most frequently used model is the subcutaneous injection/implantation model. The accessibility of subcutaneous tumors is extremely advantageous when monitoring tumor growth. However, a major disadvantage is that the subcutaneous microenvironment greatly differs from that of the colon or other organs. Interaction between the host environment and tumor graft determines tumor cell expression profiles, the levels of growth factors and nutrients, as well as tumor angiogenesis and metastatic behavior [19].

Alternatively, the orthotropic injection model, in which the tumor cells are injected into the cecal wall, closely mimics human CRC. This model has many advantages, including the microenvironment in which the tumor grows and metastasizes to nearby areas of the colon and lymph nodes similar to that seen in humans. An obvious disadvantage of this model is technical side, since the procedure is far more difficult than subcutaneous injection. It requires more technical skills and is more time consuming. In addition, end points for determining the effects of therapy are more complex than normal tumor measurement in subcutaneous models [20].

In this regard, we characterized an orthotropic model of tumor growth and presented the effects of CRC development in MLNs' and Peyer's patches' leukocyte populations. In addition, cytokines within the tumor microenvironment were also analyzed.

Materials and Methods

Animal model

Male BALB/c mice aged 5–8 weeks ($n = 30$) were purchased from the Animal Resources Centre (Canning Vale, Australia) and housed in groups of three. Animals were kept on a 12-h light and dark cycle at ~22°C with free access to food and water. The mice were allowed to acclimatize for at least 1 week before undergoing surgery. This study was approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes.

CT26 CRC cell culture

CT26 murine CRC cells were cultured at 37°C in 5% CO_2 in tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal calf serum, 200 mM L-glutamine, 1 M HEPES, 10,000 U/ml penicillin, 10 mg/ml streptomycin, 100 mM sodium pyruvate, and 25 μg/ml amphotericin B. All these reagents were from Sigma-Aldrich (Castle Hill, Australia). Cells were passaged when they reached 70%–90% confluency by treating with 1 ml of trypsin/ethylenediaminetetraacetic acid (EDTA) (2.5 g trypsin and 0.2 g EDTA) (Sigma-Aldrich) for 3–5 min followed by inactivating the trypsin by adding complete media. CT26 cells were prepared for injection in Matrigel™ (BD Biosciences, Bedford, USA) at a concentration of 4×10^7 cells/ml.

Orthotropic injection of CT26 cells

Mice were assigned to either CRC-induced group or sham-operated group. Prior to surgery, mice were anesthetized with an intraperitoneal injection comprising a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), the volume was calculated per animal body weight, and the maximum volume did not exceed 200 μl. Mice had their eyes coated with Viscotears, and during surgery, the level of anesthesia was monitored by the paw pinch response. The surgery was performed under aseptic conditions on a heat mat. The mice had their abdomen shaved and wiped with 70% ethanol before being covered with sterile film. A small incision was made along the midline of the abdomen, and the cecum was exteriorized. Cell suspension of viable tumor cells (1×10^6 CT26 cells) in 25 μl of the Matrigel™ was injected into the cecal wall. Sham-operated mice underwent the same surgery under the same conditions as the CRC-induced mice group; however, instead of an injection of CT26 cells, the sham-operated group had the tip of a 27-gauge needle inserted to the cecum wall. After surgery, the abdominal muscle wall was closed using polygalactone 5.0 gut sutures. Surgical silk suture was used to close the skin and the wound area was then sterilized with iodine. Mice received a subcutaneous injection of an analgesic buprenorphine (0.05 mg/kg) calculated per animal body weight, the maximum volume did not exceed 200 μl (Sigma-Aldrich), and were

placed in cages on heated mats to recover. Mice were closely monitored and regularly checked postsurgery. Mice were killed by cervical dislocation at three time points postsurgery (3, 7, and 14 days); the cecum, colon, Peyer's patches, and MLNs were collected for immunohistochemistry and fluorescence-activated cell sorting (FACS) analysis.

Cell surface labeling

FACS analysis was used to enumerate and phenotype the different leukocyte subpopulations in the MLNs and Peyer's patches. A minimum of 500,000 cells per sample was used for antibody staining. Cells were incubated with the monoclonal antibodies at 4°C for 20 min to label specific types of immune cells. Antibodies used were as follows: rat anti-mouse Gr-1 conjugated to fluorescein isothiocyanate (FITC; clone RB6-8C5), rat anti-mouse CD11b conjugated to R-phycoerythrin and cyanine (clone M1/70), rat anti-mouse CD193 conjugated to Alexa Fluor 647 (clone J073E5), rat anti-mouse Ia/Ie conjugated to allophycocyanin (M5/114.15.2), rat anti-mouse F4/80 conjugated to R-phycoerythrin (clone BM8), rat anti-mouse CD11c conjugated to pacific blue (clone N418), rat anti-mouse CD49b conjugated to FITC (clone DX5), rat anti-mouse TCRβ conjugated allophycocyanin (clone H57-597), rat anti-mouse CD1δα Galcer loaded tetramer conjugated to R-phycoerythrin, hamster anti-mouse γδTCR conjugated to R-phycoerythrin and cyanine (clone GL3), rat anti-mouse $CD8^+$ conjugated to pacific blue (clone 53-6.7), and rat anti-mouse $CD4^+$ conjugated to pacific orange (clone GK1.5). All these antibodies were from BioLegend (San Diego, USA).

To prevent nonspecific Fc receptor binding, cells were also costained with rat anti-mouse CD16/CD32 hybridoma supernatant (2.4G2 clone; Developmental Studies Hybridoma Bank, Iowa City, USA). Samples were analyzed using a BD LSRII FACS analyzer (BD Biosciences). All data were compensated using BD FACSDIVA v6.0 and either analyzed in FACSDIVA or exported as FCS3.0 file format for data analysis in FlowJo (Ashland, USA).

Cytokine analysis

Cytokines of *in vitro* cultured CT26 and SW480 cells were analyzed by FACS using the cytometric bead array (CBA) kit (BD Biosciences) [21] according to the manufacturer's instructions. The cytokine concentration (pg/ml) was determined with calibration curves separately established using the CBA analysis software (BD Biosciences). Th1 and Th2 cytokines were measured in the supernatant of CT26 and SW480 cell cultures with the CBA mouse and human kits. Furthermore, tumor cells were isolated from the cecum at days 7 and 14, and made into single cell suspension using collagenase (0.01 mg/ml). Cells were labeled with surface marker CD45 (clone 30-F11) and fixed using BD cytofix/cytoperm kit according to the manufacturer's instructions. $CD45^+$ and $CD45^-$ (CT26) cells were stained for the expressions of intracellular cytokines IL-6 (clone MP5-20F3), IL-10 (clone JES5-13E3), TGFβ (clone TW7-16B4), and TNFα (clone MP6-XT22).

Immunohistochemistry

Immunohistochemistry was performed in cross sections of the cecum and colon as described previously [22]. Tissue sections were thawed and incubated for 1 h at room temperature with 10% normal donkey serum (Chemicon, Temecula, USA) followed by an overnight incubation with a primary antibody at 4°C. The secondary antibody was added and then incubated at room temperature for 2 h. Primary antibodies used were as follows: hamster anti-mouse γδTCR monoclonal antibody (clone GL3) conjugated to FITC (BioLegend), rat anti-mouse CD11b monoclonal antibody (BioLegend), rat anti-mouse CD69 monoclonal antibody (BioLegend), and rabbit anti-mouse eosinophil-derived neurotoxin (EDN) antibody (Novus Biologicals, Littleton, USA). Secondary antibodies used were as follows: donkey anti-rat Alexa Fluor 594 and anti-rabbit Alexa Fluor 594 (Jackson ImmunoResearch, West Grove, USA). The same conjugate was used since only one antibody was employed for each sample at any one time. Slides were coverslipped with fluorescent mounting medium (DAKO, North Sydney, Australia).

Imaging

After immunohistochemistry treatment, the three-dimensional (z-series) images of sections were taken and analyzed using an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). Fluorophores were visualized using excitation filters for Alexa 594 (excitation wavelength 559 nm) or FITC (excitation wavelength 473 nm). z-series images were taken at a step size of 1.75 μm (1600 × 1200 pixels) using the confocal microscope.

Statistical analysis

FACS data were analyzed using a two-way analysis of variance followed by Bonferroni *post hoc* test for multiple group comparisons. Analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, USA). All data were presented as mean ± SEM. Differences were considered statistically significant at $P < 0.05$.

Results

The effect of CRC on immune cell counts in MLNs and Peyer's patches

There was no significant difference between the sham and CRC groups at any time point (days 3, 7, and 14, $n = 5$/group/time point) in either MLNs or Peyer's patches. However, the MLNs in mice with CRC had higher absolute cell counts at all days compared with the sham-operated mice (data not shown). Conversely, there was an opposing trend in the Peyer's patches where the absolute counts in sham mice were higher than that in mice with CRC. This anomaly could be accounted for by differences in tumor load and, therefore, cell death in the Peyer's patches; thus, any increase in cell subpopulations in the Peyer's patches is likely to result from newly activated lymphocytes generated in MLNs.

For accurate enumeration of NK cells, the presence of the pan-NK cell marker CD49b and the absence of TCR were used for their identification. TCR is required to exclude $CD49b^+$ NKT cells from our analysis. Prior to analysis, doublets were gated out based on their forward scatter (FSC) properties (FSC-height vs. FSC-width). NK cells were gated on their presence of CD49b and absence of TCR (Fig. 1A,C). No significant difference in the number of NK cells was seen at any time point in the Peyer's patches (Fig. 1B). However, in the MLNs, the number of NK cells in the CRC group was significantly increased from 3 to 7 days with no difference in the sham group (Fig. 1D). In addition, on day 7, a significant increase in the number of NK cells ($P < 0.05$) in the CRC group compared with the sham group was noted (Fig. 1D).

The expressions of CD8 and TCR were used to enumerate $CD8^+$ lymphocytes (Fig. 2A,C). A significant increase in the number of $CD8^+$ lymphocytes can be seen between the CRC and sham groups in Peyer's patches at days 3 and 7 (Fig. 2B, $P < 0.05$ for both). No significant change was evident in the $CD8^+$ T cell number of MLNs (Fig. 2D).

One of the concerns with this study was the variability in our MLN size and immune cell composition. In an attempt to minimize this

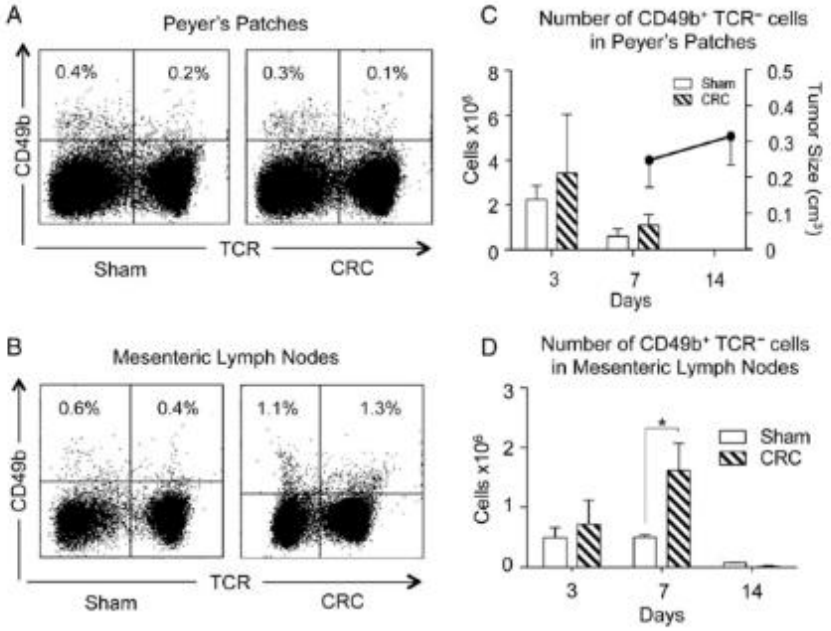

Figure 1. Effect of CRC on NK cells in Peyer's patches and MLNs FACS plots of NK cells at day 7 in (A) Peyer's patches and (B) MLNs from sham surgery and CRC-induced mice. Number of CD49b$^+$TCR$^-$ cells in (C) Peyer's patches and (D) MLNs from sham-operated and CRC-induced groups at days 3, 7, and 14. (C) Tumor size at days 7 and 14. A significant increase ($P < 0.05$) in the number of CD49b$^+$TCR$^-$ cells is seen at day 7 in MLNs. Data are represented as the mean ± SEM and statistical significance is indicated by asterisks, $^*P < 0.05$.

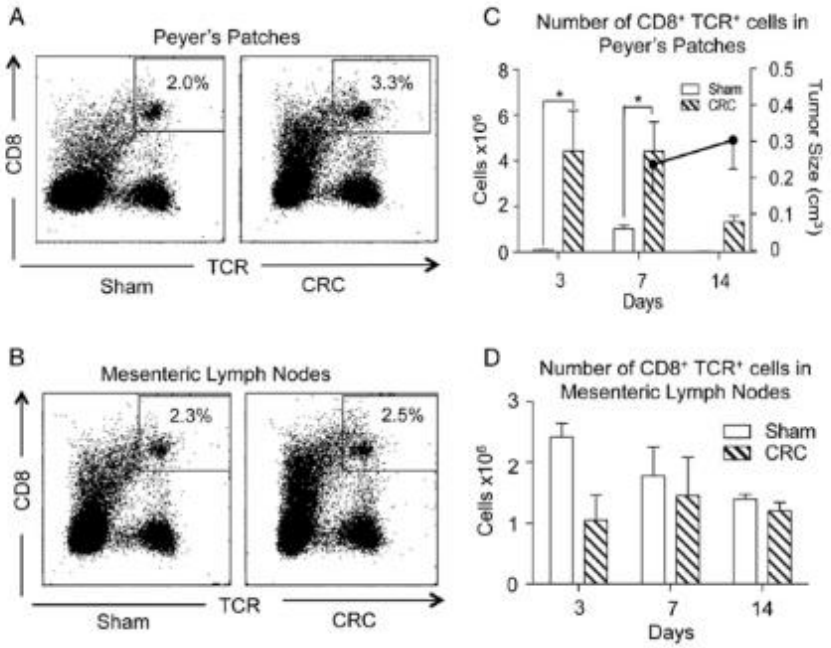

Figure 2. Effect of CRC on CD8$^+$ T cells in Peyer's patches and MLNs FACS plots of CD8$^+$ T cells at Day 7 in (A) Peyer's patches and (B) MLNs from sham surgery and CRC-induced mice. Number of CD8$^+$TCR$^+$ cells in (C) Peyer's patches and (D) MLNs from sham-operated and CRC-induced groups at Days 3, 7, and 14 post surgery. (C) Tumor size at Days 7 and 14. Data are represented as the mean ± SEM and statistical significance is indicated by asterisks, $^*P < 0.05$.

variation, five lymph nodes were pooled from each mouse. However, as lymph nodes vary dramatically in size, we found it quite difficult to obtain lymph nodes of similar size, which resulted in high standard errors (Figs. 1 and 2).

The effect of CRC on immune cells in the colon

γδT cells are resident cells within the gastrointestinal tract, which are known as intraepithelial lymphocytes and play an important role in antitumor immunity [23]. Immunohistochemical studies of the colon cross sections demonstrated that γδT cells that normally are abundant in the epithelial layers of the gastrointestinal tract were absent in the colon sections from mice with CRC (Fig. 3A). Immunohistochemical studies of cecum cross sections (site of tumor induction) demonstrated that eosinophils, activated $CD69^+$ T cells, and $CD11b^+$ cells infiltrated the tumor tissue (Fig. 3B).

Cytokine profile of the murine CT26 CRC cell line *in vitro* and *in vivo*

Cytokine analysis revealed that CT26 cells were negative for IL-2, IL-4, IL-10, IFN-γ, TNF-α, and IL-17a secretion. However, CT26 cells secreted high level of IL-6 (61.76 pg/ml), while the human SW480 cell line secreted high level of IL-10 (164.97 pg/ml) (Fig. 4A). Likewise, flow cytometry analysis of tumor cells from mice with CRC demonstrated the expression of IL-6 by CT26 cells (Fig. 4B,B′,C). All other cytokines tested were negative (Fig. 4C). Of interest, tumors that infiltrated $CD45^+$ cells also expressed IL-6 and TNFα (Fig. 4C′) within the tumor microenvironment.

Discussion

CRC pathophysiology has been described as asymptomatic and can, therefore, go undiagnosed until a sufficient tumor mass has been established. In this study, we used an animal model of orthotopic CRC development.

IL-6 acts both as a pro-inflammatory and an anti-inflammatory cytokine. In cancer patients, IL-6 is highly elevated leading to poor prognosis [24], and is abundant at the tumor microenvironment where it plays a role in cancer metastasis via downregulation of E-cadherin [25]. In mice, CT26 cells that express IL-6 exert tumor-promoting activities by activating growth and survival [26]. However, IL-6 tumor microenvironment also activates $CD8^+$ T cells to the tumor site [26]. It is likely that IL-6 produced by CT26 cells in our studies contributed to its metastatic ability from the cecum to the nearby colon. Conversely, human SW480 CRC cell line produced high amount of IL-10. IL-10, a T helper type 2 cytokine, is known to possess many immunosuppressive activities including the inhibition of T lymphocyte proliferation and Th1 type cytokine production, impairment of antigen-presenting cells (APCs), and blunting of cytotoxic responses. Many studies have demonstrated that IL-10 is a prognostic indicator in CRC [27]. It is clear that the murine CRC CT26 cell line produces tumors via an IL-10-independent manner.

The immune response to slow-forming cancers is complex because the kinetics of tumor antigen processing particularly by the APCs as well as the T-cell activation is unknown. APCs must travel from the site of the tumor growth to either the MLNs or Peyer's patches; a process that can take a significant amount of time because activated T cells could take several days to travel to the tumor site [28]. Indeed, we demonstrated a detectable increase in the number of $CD8^+$ lymphocytes in Peyer's patches in the CRC group as early as day 3 and an increase in the number of NK cells in MLNs at day 7 post CRC induction. Once at the tumor site, activated T cells recognize tumor antigens in complex with MHC class I, and lyse tumor cells; however, the success of this process is largely influenced by the tumor microenvironment.

Increases in $CD8^+$ T cell numbers observed in our study were specific to tumor development; this was evidenced by the fact that all experimental mice have only orthotopic cancer development and no other comorbidities (e.g. inflammation) which could stimulate $CD8^+$ T cell activation. Likewise, it was shown in another study that silencing MUC2 from CT26 cell-promoted tumor growth by increasing IL-6 secretion was followed by an increase of $CD8^+$ T cells in the peritoneal cavity specific to CT26 cells [29]. In cancer, $CD8^+$ T cells are activated to kill tumor cells and their presence results in better survival [30]. In the orthotropic CT26 cell injection model used in our study, an increase in $CD8^+$ T cells and NK cells was noted as early as days 3 and 7, which correlates with the pro-inflammatory cytokine IL-6

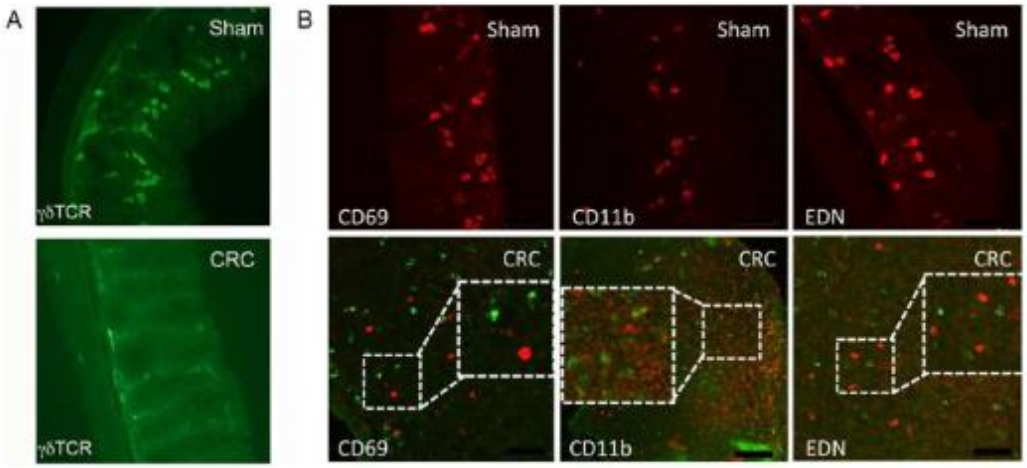

Figure 3. The effect of CRC on γδT cells in the colon and tumor-infiltrating leukocytes in the cecum (A) Immunohistological labeling of γδT cells in the colon. (B) Immunohistochemical labeling of tumor-infiltrating leukocytes (red). $CD69^+$ T cells, $CD11b^+$ cells, and eosinophils (EDN) were found within the tumors (green) in the cecum. Cross sections of cecum tissue without tumor cells (sham) were also immunohistochemically labeled to show normal level of immune cells.

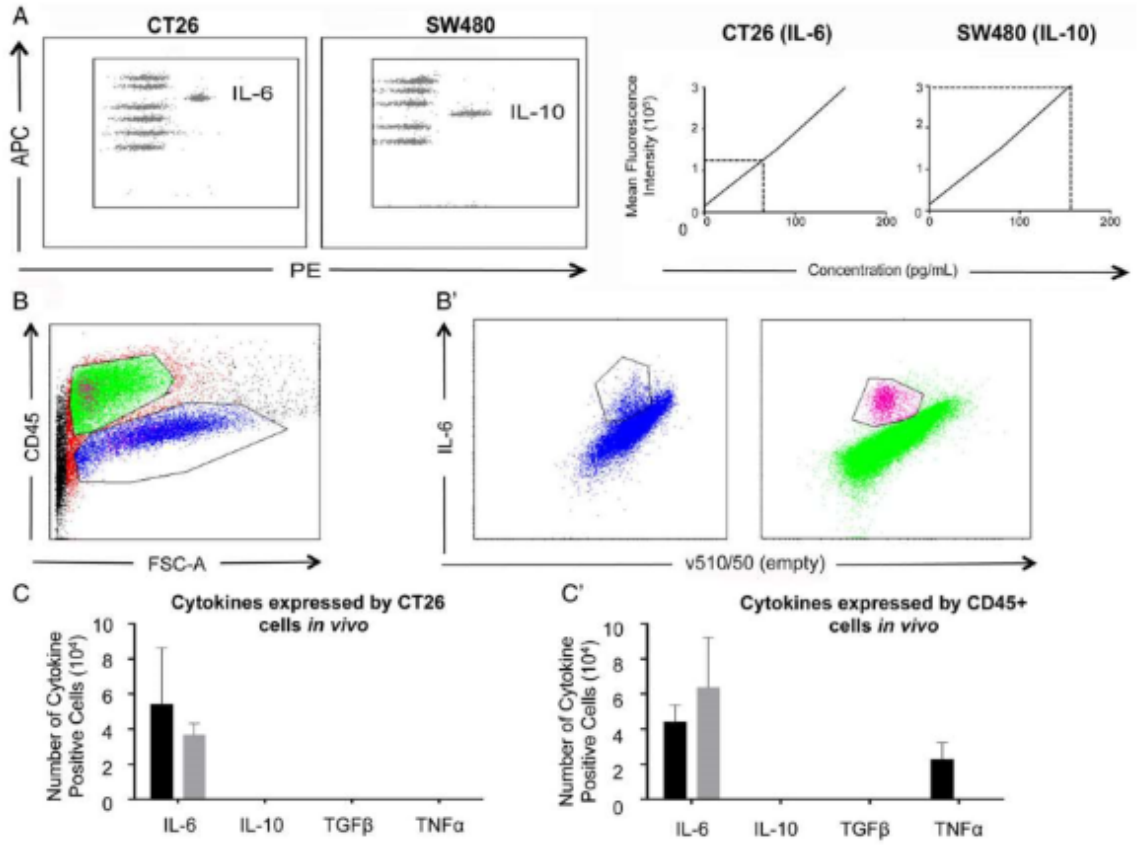

Figure 4. Cytokine profile of the murine CT26 and human SW480 CRC cell line *in vitro* and *in vivo* (A) Cytokine FACS plots of murine CT26 and human SW480 colon cancer cells and concentration graphs for both CT26 and SW480 cell lines. Analysis of intracellular cytokines of *in vivo* CT26-induced tumors: (B) CD45 vs. forward scatter (FSC-A) plot used for gating; (B', left panel) Intracellular IL-6 expressed by CT26 cells *in vivo*; (B', right panel) Intracellular IL-6 expressed by tumor-infiltrating CD45⁺ cells. (C) Quantitative analysis of intracellular cytokines expressed by CT26 cells *in vivo* at day 7 (black bar) and day 14 (gray bar). (C') Quantitative analysis of intracellular cytokines expressed by CD45⁺ tumor-infiltrating cells *in vivo* at day 7 (black bar) and day 14 (gray bar).

produced by these cells. An increase in CD8⁺ T cells and NK cells leads to better prognosis. In fact, we demonstrated an inverse correlation between increased number of CD8⁺ T cells and NK cells and decreased tumor growth in the CT26 cell-injected groups. When the tumor load was high, there were less CD8⁺ T cells and NK cells. In addition, we have also demonstrated that there was an inverse correlation between CTL precursor frequency and tumor size in other tumor models [31,32].

Furthermore, the prognostic significance of NK cells in CRC was demonstrated in CRC patients, where patients with little NK cell infiltration had a significantly shorter survival rate than those who had extensive infiltration [33]. In addition, patients with Grade III CRC had significantly longer survival rates with high NK cell infiltration compared with those with low NK cell infiltration [33].

γδT cells are resident gastrointestinal lymphocytes and are vital to gastrointestinal immunity, as they recognize tumor-expressed ligands that other T lymphocytes cannot recognize [34]. γδT cells are similar to NKT cells in that they share features of both the innate and adaptive lymphocytes due to their expression of invariant TCRs. Activated γδT cells can have strong cytotoxic effects via both the death receptor and death ligand pathways and the cytolytic granule pathways [35]. In addition, γδT cells exhibit lytic activity against cancer cells in an MHC-unrestricted manner *in vitro*, suggesting their potential as anticancer therapy [36,37]. Based on the IL-6 data (Fig. 3D), we have clearly shown that the mechanism in our murine model of CRC is different from human CRC (IL-10-dependent). The mechanism of how IL-6 signaling influences γδT cell viability or mobilization is unclear. Further studies are needed to address this mechanism by using the neutralizing anti-IL-6 receptor monoclonal antibody (Tocilizumab) [38].

Immune cell infiltration within the tumor in the cecum demonstrated that CD11b⁺ cells, CD69⁺ T cells, and eosinophils were present. Eosinophils have been found in a number of tumors; however, their role is still being debated [39]. In CRC, however, their presence has been associated with a favorable prognosis [40]. CD69⁺ cells are indicative of an activated T-cell state. As there was an increase in CD8⁺ T cells, it was not unexpected to detect T cells within tumor itself, and in fact, T cells were present in their activated state (CD69⁺). This is in accord to the pro-inflammatory state of the CT26 tumor microenvironment. Moreover, CD11b⁺ cells were also found within the tumor microenvironment.

Conclusion

In this study, we investigated the effect of the murine orthotropic CT26 CRC model on immune cells and revealed significant changes

in the number of NK cells in MLNs at day 7 and significant changes in the number of $CD8^+$ T lymphocytes at days 3 and 7 in Peyer's patches. We also demonstrated that γδT cells were depleted in the colon in mice with CRC and immune cells (eosinophils, $CD69^+$ T cells, and $CD11b^+$ cells) infiltrated into the tumor site. The cytokine analysis revealed that CT26 cells secreted IL-6 cytokine both *in vitro* and *in vivo*, which is in accord to the immune activation state noted. Recently, it was demonstrated that cancer cells and immune cells communicate via the presence of IL-6 in the tumor microenvironment which is secreted by both cancer cells and immune cells [41]. Likewise, in the orthotopic CRC model presented here, both tumor cells and infiltrating $CD45^+$ cells expressed IL-6; $CD45^+$ cells also expressed TNFα, which further contributed to a pro-inflammatory tumor microenvironment. Furthermore, it would be of interest to study the immune response to this orthotopic cancer cell model in inflammation-induced cancer, which is more representative of human condition.

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References

1. Boland CR, Goel A. Microsatellite instability in colorectal cancer. *Gastroenterology* 2010, 138: 2073–2087.e2073.
2. Bergman M, Djaldetti M, Salman H, Bessler H. Inflammation and colorectal cancer: does aspirin affect the interaction between cancer and immune cells? *Inflammation* 2011, 34: 22–28.
3. Saleh M, Trinchieri G. Innate immune mechanisms of colitis and colitis-associated colorectal cancer. *Nat Rev Immunol* 2010, 11: 9–20.
4. Cappell MS. Pathophysiology, clinical presentation, and management of colon cancer. *Gastroenterol Clin North Am* 2008, 37: 1–24.
5. Erdelyi I, Levenkova N, Lin EY, Pinto JT, Lipkin M, Quimby FW, Holt PR. Western-style diets induce oxidative stress and dysregulate immune responses in the colon in a mouse model of sporadic colon cancer. *J Nutr* 2009, 139: 2072–2078.
6. Orbell J, West NJ. Improving detection of colorectal cancer. *Practitioner* 2010, 254: 17–21, 12–13.
7. Adelstein B-A, Macaskill P, Chan SF, Katelaris PH, Irwig L. Most bowel cancer symptoms do not indicate colorectal cancer and polyps: a systematic review. *BMC Gastroenterol* 2011, 11: 65.
8. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. *Oncogene* 2008, 27: 5932–5943.
9. Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol* 2001, 2: 293–299.
10. Finn OJ. Cancer immunology. *N Engl J Med* 2008, 358: 2704–2715.
11. Funada Y, Noguchi T, Kikuchi R, Takeno S, Uchida Y, Gabbert HE. Prognostic significance of CD8+ T cell and macrophage peritumoral infiltration in colorectal cancer. *Oncol Rep* 2003, 10: 309–313.
12. Wu J, Lanier LL. Natural killer cells and cancer. *Adv Cancer Res* 2003, 90: 127–156.
13. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer* 2006, 42: 717–727.
14. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011, 331: 1565–1570.
15. Balkwill FR, Mantovani A. Cancer-related inflammation: common themes and therapeutic opportunities. *Semin Cancer Biol* 2012, 22: 33–40.
16. Erreni M, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) and inflammation in colorectal cancer. *Cancer Microenviron* 2011, 4: 141–154.
17. Chung YC, Chang YF. Serum interleukin-6 levels reflect the disease status of colorectal cancer. *J Surg Oncol* 2003, 83: 222–226.
18. Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, *et al.* Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 2007, 1: 389–402.
19. Heijstek MW, Kranenburg O, Borel Rinkes IH. Mouse models of colorectal cancer and liver metastases. *Dig Surg* 2005, 22: 16–25.
20. Bibby MC. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. *Eur J Cancer* 2004, 40: 852–857.
21. Morgan E, Varro R, Sepulveda H, Ember JA, Apgar J, Wilson J, Lowe L, *et al.* Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clin Immunol* 2004, 110: 252–266.
22. Nurgali K, Qu Z, Hunne B, Thacker M, Pontell L, Furness JB. Morphological and functional changes in guinea-pig neurons projecting to the ileal mucosa at early stages after inflammatory damage. *J Physiol* 2011, 589: 325–339.
23. Lee AJ, Kim SG, Chae HD, Lee GH, Shin IH. γδ T cells are increased in the peripheral blood of patients with gastric cancer. *Clin Chim Acta* 2012, 413: 1495–1499.
24. Bellone G, Smirne C, Mauri FA, Tonel E, Carbone A, Buffolino A, Dughera L, *et al.* Cytokine expression profile in human pancreatic carcinoma cells and in surgical specimens: implications for survival. *Cancer Immunol Immunother* 2006, 55: 684–698.
25. Miao JW, Liu LJ, Huang J. Interleukin-6-induced epithelial-mesenchymal transition through signal transducer and activator of transcription 3 in human cervical carcinoma. *Int J Oncol* 2014, 45: 165–176.
26. Fisher DT, Chen Q, Skitzki JJ, Muhitch JB, Zhou L, Appenheimer MM, Vardam TD, *et al.* IL-6 trans-signaling licenses mouse and human tumor microvascular gateways for trafficking of cytotoxic T cells. *J Clin Invest* 2011, 121: 3846–3859.
27. Szkaradkiewicz A, Marciniak R, Chudzicka-Strugala I, Wasilewska A, Drews M, Majewski P, Karpiński T, *et al.* Proinflammatory cytokines and IL-10 in inflammatory bowel disease and colorectal cancer patients. *Arch Immunol Ther Exp (Warsz)* 2009, 57: 291–294.
28. Norian LA, Rodriguez PC, O'Mara LA, Zabaleta J, Ochoa AC, Cella M, Allen PM. Tumor-infiltrating regulatory dendritic cells inhibit CD8 + T cell function via L-arginine metabolism. *Cancer Res* 2009, 69: 3086–3094.
29. Shan YS, Hsu HP, Lai MD, Yen MC, Fang JH, Weng TY, Chen YL. Suppression of mucin 2 promotes interleukin-6 secretion and tumor growth in an orthotopic immune-competent colon cancer animal model. *Oncol Rep* 2014, 32: 2335–2342.
30. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, Tosolini M, *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006, 313: 1960–1964.
31. Pietersz GA, Li W, Popovski V, Caruana JA, Apostolopoulos V, McKenzie IF. Parameters for using mannan-MUC1 fusion protein to induce cellular immunity. *Cancer Immunol Immunother* 1998, 45: 321–326.
32. Apostolopoulos V, Osinski C, Mckenzie IF. MUC1 cross-reactive Galα (1, 3) Gal antibodies in humans switch immune responses from cellular to humoral. *Nat Med* 1998, 4: 315–320.
33. Coca S, Perez-Piqueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C, Martos JA, *et al.* The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* 1997, 79: 2320–2328.
34. Moser B. Tumor-killing γδ-TCRs take center stage. *Blood* 2012, 120: 5093–5094.
35. Bonneville M, O'Brien RL, Born WK. γδ T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* 2010, 10: 467–478.
36. Corvaisier M, Moreau-Aubry A, Diez E, Bennouna J, Mosnier JF, Scotet E, Bonneville M, *et al.* V gamma 9V delta 2T cell response to colon carcinoma cells. *J Immunol* 2005, 175: 5481–5488.

37. Todaro M, D'Asaro M, Caccamo N, Iovino F, Francipane MG, Meraviglia S, Orlando V, *et al*. Efficient killing of human colon cancer stem cells by gammadelta T lymphocytes. *J Immunol* 2009, 182: 7287–7296.
38. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta* 2011, 1813: 878–888.
39. Davis BP, Rothenberg ME. Eosinophils and cancer. *Cancer Immunol Res* 2014, 2: 1–8.
40. Legrand F, Driss V, Delbeke M, Loiseau S, Hermann E, Dombrowicz D, Capron M. Human eosinophils exert TNF-α and granzyme A-mediated tumoricidal activity toward colon carcinoma cells. *J Immunol* 2010, 185: 7443–7451.
41. Patel SA, Gooderham NJ. IL-6 mediates immune and colorectal cancer cell cross-talk via miR-21 and miR-29b. *Mol Cancer Res* 2015, 13: 1502–1508.