# The Effects of Vitamin B6 and B12 On Inflammation And Cancer

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# Abstract

We are only just beginning to understand the intricate relationship between nutrition, immune health, inflammation, and cancer. Epidemiological studies have demonstrated a clear association between inflammation and cancer development. Both undernutrition and overnutrition (malnutrition) have been shown to have a significant impact on immune health and function. Even in countries where food is plentiful, a diet high in processed food can be high in calories whilst being nutritionally deficient. The emergence of B vitamins as anti-inflammatory and anti-cancer agents is an area which in recent years has gained interest within the scientific community and as the development of genetic and epigenetic investigative techniques becomes more available to a greater number of researchers, there is ongoing investigation occurring concerning how nutrition affects gene expression. Low blood serum vitamin B6 is frequently noted in patients with high inflammatory markers and vitamin B6 supplementation has previously been shown to downregulate inflammation and oxidative stress in both inflammation and as an anti-cancer mechanism. In contrast, the effects of vitamin B12 supplementation have been shown within the literature to be ambiguous with links both to cancer progression and pro-inflammatory actions versus tumour regression and anti-inflammatory properties. The purpose of this thesis was to ascertain, with greater clarity, the mechanisms of action of high dose vitamin B6 and B12 on inflammation and cancer. This was achieved by conducting studies on both cancer and immune cells and using protein and gene studies to ascertain the effects of high-dose vitamin B supplementation. It was found that high dose vitamin B6 was shown to have an anti-proliferative effect on promonocytic lymphoma cells, likely due to a downregulation of the mevalonate pathway (MVP) whereby vitamin B6 acted in a ‘steroid-like' fashion to reduce MVP, restoring mutant p53 function and re-establishing the G1/S checkpoint. Vitamin B6

was also shown to have a broad-spectrum, anti-inflammatory effect on key inflammatory pathways in lipopolysaccharide (LPS) stimulated monocytes. In contrast, vitamin B12 supplementation produced an upregulation in key inflammatory gene expressions and showed a dose-dependent effect on inflammation. The important and novel findings from this thesis conclude, that high dose vitamin B6 may prove to be an important nutraceutical agent in both inflammatory and oncological medicine and that B12 over-supplementation may potentially contribute to inflammation and tumourigenesis so caution should be taken when supplementing in dosages above the recommended daily intake.

# Doctor of Philosophy Declaration

"I, Kathleen Mikkelsen, declare that the PhD thesis entitled
**THE EFFECTS OF VITAMIN B6 AND B12 ON INFLAMMATION AND CANCER** is no more than 80,000 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".

"I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures.

Signature [redacted] Date 07/01/2022

# Research publications

1. **Mikkelsen, K**., Prakash, M., Kuol, N., Apostolopoulos, V. Anti-Tumour Effects of Vitamin B2, B6 and B9 in Promonocytic Lymphoma Cells. *International Journal of Molecular Sciences* 20(15):3763, 2019. [IF=5.542, Q1]

# Conference Abstracts

2. **Mikkelsen, K.,** Dose-dependent effects of vitamin B12 on promonocytic lymphoma cells**.** *11th Annual Victorian ASMR Student Research Symposium*. Melbourne November 2020
3. **Mikkelsen, K.,** Dose-dependent effects of vitamin B12 on promonocytic lymphoma cells**.** *IHeS student conference Victoria University*. Melbourne December 2020

# Poster presentations

4. **Mikkelsen, K.,** Dose-dependent effects of vitamin B12 on promonocytic lymphoma cells**.** *11th Annual Victorian ASMR Student Research Symposium*. Melbourne November 2020
5.. **Mikkelsen, K.,** Dose-dependent effects of vitamin B12 on promonocytic lymphoma cells**.** *IHeS student conference Victoria University*. Melbourne December 2020

# Conference presentations

6. **Mikkelsen, K.,** Dose-dependent effects of vitamin B12 on promonocytic lymphoma cells**.** *IHeS student conference Victoria University*. Melbourne December 2020

# Awards

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I know how proud you would be of me for achieving this doctorate. Sometimes I like to imagine my graduation day. You would fly down from Brisbane and stay with me for a few days. I would pick you up from the airport, you would be standing at our pickup point dressed in multiple colourful layers with your hot pink suitcase, shoulders raised against the cold as you tried to acclimatize to the cooler Melbourne weather. I would hug you and throw your ever overpacked suitcase into the back. You'd sit beside me in the passenger seat smelling of perfume and hand cream and we'd catch up on months of separation, what were the kids doing? how was dad? what was happening in the village? how cute was the newest grandchild! We'd stop for coffee somewhere maybe share a snack and then talk about the pandemic and lockdowns and the talent school that has been part of both of our lives for nearly fifty years shutting down. I'd take you back to the house, you'd reprimand me on how untidy my kitchen was and immediately start cleaning it. Later we'd all have a sit-down family dinner, and you'd catch up with the three kids and James, there would be teasing, and laughter and you would probably have a present for Lila. The next day we'd have the graduation ceremony and I'd take a photo with you in my floppy hat that I'd eventually get framed and display somewhere around the house. We'd go to our favourite wine bar

after the ceremony, some of my friends might come along as well. It would be a happy time celebrating a lifetime achievement for me but a time that will only ever exist in my imagination because cancer took you away from us much too soon. Just like it has taken countless other mothers fathers sisters brothers' children, robbing families of these precious moments and leaving gaping holes in the lives of their loved ones.

My grief is but a drop in the ocean of the global grief caused by cancer.

Mum this thesis is dedicated to you.

Margaret Ann Mikkelsen 1949-2018.

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# List of Abbreviations

| | |
|---|---|
| 5-MTHFR | 5- methyl tetrahydrofolate |
| 3-MST | cysteine aminotransferase 3-mercaptopyruvate sulfurtransferase |
| AGES | Advanced glycation end products |
| AKT | AK strain transforming |
| ANOVA | Analysis of variance |
| AOM | Azoxymethane |
| AOM | Azoxymethane |
| BFGF | Basic fibroblast growth factor |
| Caco2 | Human colorectal adenocarcinoma |
| Casp1 | Cysteine-aspartic acid protease 1 |
| CBS | Cystathionine beta-synthase |
| CCL5 | Chemokine (C-C motif) ligand 5 |
| CCL5 | Chemokine ligand 5 |
| CDK | Cyclin dependent kinase |
| CDKN1A | Cyclin-dependent kinase inhibitor 1 |
| cDNA | Complementary DNA |
| CLR | C-type lectin receptors |
| CRP | C reactive protein |
| CSE | Cystathionine γ-lyase |
| CSF-1 | Colony-stimulating factor 1 |
| CXCL10/IP10 | CXC motif chemokine ligand 10 |
| CXCL16 | CXC motif chemokine ligand 16 |
| DAMPS | Damage-Associated-Molecular-Patterns |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DC | Dendritic cells |
| DDX58 | DExD/H-Box helicase 58 |
| DEGs | Differentially expressed genes |
| DNMT's | DNA methyltransferases |

| | |
|---|---|
| E2F | E2 transcription factor |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ERK | Extracellular signal-regulated kinase |
| FCS | Fetal Calf Serum |
| FITC | Fluorescein isothiocyanate) |
| FOXP3 | Forkhead box protein P3 |
| Fura | 5-fluorouracil |
| G2 phase | Second gap phase |
| GABA | γ-amino butyric acid |
| GO | Gene ontology |
| GO phase | Gap 0 phase |
| GOI | Genes of interest |
| GTP | Guanosine triphosphate |
| $H_2S$ | Hydrogen sulphide |
| HC | Haptocorrin |
| Hcy | Homocysteine |
| HEK293T | Human embryonic kidney cell line |
| HepG2 | Human hepatoma cell line |
| HGF | Hepatocyte growth factor |
| HLA | Human Leukocyte antigens |
| HLA-E | Major Histocompatibility complex, Class I,E |
| HMGB | High mobility group Box |
| HO-1 | Heme oxygenase 1 |
| HSP70 | 70 kilodalton heat shock protein |
| HT29 | Human colorectal cancer cell line |
| IFN | Interferon |
| IGIF | IFNγ inducing factor |
| IL | Interleukin |
| IRF | Interferon regulatory factor |
| ITGAM | Integrin Alpha M |

| | |
|---|---|
| LAG-3 | Lymphocyte activation gene 3 |
| LoVo | Human colon adenocarcinoma cell line |
| LPS | Lipopolysaccharides |
| M phase | Mitotic phase |
| MAPK | Mitogen-activated protein kinase |
| MAT2A | Methyl-adenosyl transferase 2A |
| Met | Methionine |
| MHC | Major histocompatibility |
| MHCI | Major histocompatibility class I |
| MHCII | Major histocompatibility class II |
| M-MDSCs | Monocyte related myeloid-derived suppressor cells |
| MMP-9 | Matrix metallopeptidase 9 |
| MPO | Myeloperoxidase |
| mRNA | Messenger RNA |
| mTOR | Mammalian target of rapamycin |
| MTT | (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MUT | Methyl-malonyl CoA mutase |
| mutp53 | Mutant p53 |
| MVP | Mevalonate-5-phosphate |
| MX1 | MX Dynamin like GTPase 1 |
| NaOH | Sodium hydroxide |
| NF-κB | Nuclear Factor kappa-light-chain-enhancer of activated B cells. |
| NGF | Nerve growth factor |
| NGS | Next-generation sequencing |
| NK | Natural killer |
| NLRP | Nod-like receptor protein |
| NLRP3 | NOD-LRR-and pyrin domain-containing protein 3 |
| NOD | Nucleotide-binding oligomerisation domain |
| NOS | Nitric oxide synthase |
| NSAIDS | Non-steroidal anti-inflammatory drugs |
| PAMPs | Pathogen-Associated-Molecular-Patterns |
| PBS | Phosphate buffered saline |

| | |
|---|---|
| PD1 | Programmed cell death protein |
| PDGF | Platelet-derived growth factor |
| PDL-1 | Programmed death-ligand |
| PDXK | Pyridoxal kinase |
| PL | Pyridoxal |
| PLP | pyridoxol-5′-phosphate |
| PM | Pyridoxamine |
| PMP | Pyridoxamine-5′-phosphate |
| PMT | Photomultiplier tube |
| PN | Pyridoxine |
| PNP | pyridoxin-5'-phosphate |
| pRb/Rb | Retinoblastoma protein |
| PRR | Pattern recognition receptor |
| PS | Phospholipid phosphatidylserine |
| QC | Quality control |
| RAGEs | Receptor AGEs |
| RB1 | Retinoblastoma tumour suppressor protein 1 |
| RDI | Recommended daily intake |
| REVIGO | Reduce and Visualize Gene Ontology |
| RIN | RNA integrity number |
| RLR | RIG-1 like receptors |
| RNA-seq | RNA sequencing |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park memorial Institute (media) |
| S phase | Synthesis phase |
| S1P | Sphingosine-1-phosphate |
| S1PL | Sphingosine 1-phosphate lyase |
| SAC | Spindle assembly checkpoint |
| SAM | s-adenosyl methionine |
| SPT | Serine palmitoyl-transferase |
| STAR | Spliced Transcripts Alignment to a Reference |
| STAT | Signal transducer and activator of transcription |

| | |
|---|---|
| STING | Stimulator of interferon genes |
| TAFs | Tumour-associated factors |
| TAMS | Tumour-associated macrophages |
| TGF-β1 | Transforming growth factor-beta 1 |
| Th | Helper T |
| THF | Tetrahydrofolate |
| TICAM | TLR adaptor molecule 1 |
| TIM3 | T cell immunoglobulin and mucin-domain containing-3 |
| TL | Telomere length |
| TLRs | Toll-like receptors |
| TME | Tumour micro-environment |
| TNF | Tumour necrosis factor |
| TP53/p53 | Tumour protein 53 |
| Treg | Regulator T |
| VEGF | Vascular endothelial growth factor |
| VITAL | Vitamins and lifestyle |

# Preface

After a successful study on the effects of yeast-based spreads (rich in vitamin B) on mental health in 2017, I wanted to further explore the effects of B vitamins on cells.

During my BSc honours year, the effects on cancer cell proliferation and apoptosis of all B vitamins were evaluated. These included vitamin B1 (thiamine), B2 (riboflavin), B3 (niacin), B4 (choline), B5 (pantothenic acid), B6 (pyridoxine), B7 biotin), B9 (folate), B12 (cobalamin). It was clear that vitamins B2, B6, B9, and B12 were effective in decreasing cellular proliferation; B3, B4, and B5 did not affect proliferation whilst B1 increased proliferation. I wanted to explore this effect further in my PhD.

Early experiments within the first year of my PhD. using vitamins B2, B6, B9, and B12 involved repeat cell proliferation assays, in addition to cell migration, apoptosis, expression of checkpoint markers PD-L1, cytokine secretion, and cell surface marker expression. These experiments showed that vitamin B6 appeared to display anti-tumour properties by decreasing proliferation, migration, PD-L1 expression as well as downregulating inflammatory mediators. (Chapter 2). Vitamin B12 however seemed to exert dose-dependent effects on proliferation migration and inflammatory mediators (Chapter 3).

To gain more insight into the anti-proliferative action of high dose B6 (as this anti-proliferative effect was consistent in my B6 experiments and I was needing to adjust for the smaller number of cells when designing other experiments) I employed Next Generation Sequencing for detailed gene analysis to gain insights into the mechanistic effects of vitamin B6 on U937 pro-monocytic lymphoma cells. Studies were limited to vitamin B6 as NGS is expensive, and this vitamin was showing the most interesting effects. U937 cells were treated with an IC-50 dosage of vitamin B6 (calculated from data from proliferation assays)

over 24 hrs and 6 days and genome changes were assessed. These gene experiments were able to uncover novel mechanistic pathways that explained the anti-proliferative effect of B6 on pro-monocytic cancer cells (Chapter 4).

To understand in more depth, the mechanisms behind vitamin B6's anti-inflammatory effects, and vitamin B12's dose-dependent and ambiguous effects on inflammation, I sought to investigate further the direct effect of vitamin B on LPS stimulated immune monocytes. The U937 cell line is a useful cell line to study the effects of inflammation as the cell line can be differentiated into monocyte/macrophage cells by incubating the cells with vitamin D3. Several experiments were conducted using differentiated cells, treating them with LPS to induce an inflammatory profile and observing the effects of vitamin B6 and vitamin B12 on inflammation using an inflammatory gene arrays panel. Once again, I found that vitamin B6 consistently and extensively displayed anti-inflammatory properties by decreasing inflammatory gene expression (Chapter 5) while B12 showed a decrease in inflammatory gene expression for some genes but vastly upregulated gene expression in some other genes (Chapter 6).

Overall, it is concluded that vitamin B6 may exert anti-tumour effects in high doses via its ability to act as a broad-spectrum anti-inflammatory agent along with its ability to decrease proliferation by downregulating the mevalonate pathway and restoring the p53 pathway. This property may give it some clinical potential in high doses as both a broad-spectrum anti-inflammatory to assist other anti-inflammatory medications in severe inflammatory conditions such as sepsis or cytokine storm in COVID-19 patients and as a potential anticancer agent in oncological therapies (Chapter 7).

Vitamin B12 may promote tumourigenesis in high doses by contributing to inflammation (Chapter 6). Some studies have suggested that high doses of vitamin B12 could contribute

to tumourigenesis by altering gene expression and DNA methylation. Future studies of vitamin B12 on gene expression should be performed to provide evidence to either confirm or deny this. It seems by the data in chapters 3 and 5 however, that vitamin B12 may a narrow therapeutic index. This means that both deficiency and over-supplementation could cause adverse effects.

This study was very much an exploratory study with an open-ended question "what mechanistic effects does vitamin B6/12 have?". This type of exploratory research, whilst important in the initial stages of investigating a problem that is not clearly defined, can be somewhat unstructured and dependent on results to direct the next stage of exploration. Some PhD. studies are set up to investigate and answer specific questions that have been raised by the exploratory research that has come before. In some regards, this research has uncovered as many questions as it has answered and clarified the need for further investigation into the effects of high dose vitamin B6 and B12.

# Chapter One

## Literature Review: The Mechanistic Effects of Vitamins B6 and B12 on Inflammation and Cancer

## Abstract

High dose vitamin B6 offers potential nutraceutical action as an anti-cancer and anti-inflammatory agent via various mechanisms, whereas high dose vitamin B12 may conversely contribute to tumorigenesis and inflammation. Previous studies have elucidated the effects of vitamin B6 and B12 deficiency but less is known about the potential therapeutic effects of these vitamins above the recommended daily intake and currently, more research is required to further clarify the mechanistic effects of high dose B6 and B12 on both cancer and immune cells. This review will examine current knowledge of links between vitamin B6 and B12 status and discuss some of the mechanistic effects with which vitamins B6 and B12 may directly influence inflammation and cancer.


## 1.1 Introduction

The human immune system is imperfect. We know this only too well in these days of COVID-19 where the world has shut down, not just from fear of the virus itself, but the fear of how our own immune systems may respond to this tiny invader. Patients with fatal COVID-19 have immune responses ranging from reduced function to overactivation and in all patients who contract COVID-19, the outcome of the disease is determined by both the host's immune response and viral factors (Zhou & Ye, 2021).

We are only just beginning to understand the intricate relationship between nutrition, immune health, inflammation, and cancer. The recent advances in genomic and epigenetic medicine allow us to trace a path from that which occurs at a macro level in relation to diet, lifestyle choices, nutrition etc. to the micro-level of genetic and epigenetic differential expression. It is well established that there is a direct relationship between immune response,

inflammatory process, and dietary habits. Both undernutrition and overnutrition (malnutrition) have been shown to have a significant impact on immune health and function. A substantial percentage of the global population does not consume the recommended daily intake of nutrients (Beal et al., 2017). Even in countries where food is plentiful, a diet high in processed food can be high in calories whilst being nutritionally deficient. Marginal deficiencies can progress to chronic disease states due to an insufficient intake of essential micronutrients (Beal et al., 2017). Vitamin B plays a crucial role in the healthy balance of the immune system and vitamin B deficiencies are often found in diets high in over-processed foods (Kennedy, 2016).

The state of nutrition can directly affect immune response, development, maintenance, and function. As vitamins are essential for health and proper human function it is important that the daily intake is enough to continue homeostasis. Humans are unable to synthesize many of the vitamins that are required daily to continue normal function including, vitamins A, most of the vitamin B group, vitamins C, E and K (Drouin et al., 2011). For this reason, dietary sources are essential and especially important for the water-soluble B and C vitamin families as these are not stored in the body like the fat-soluble vitamins.

Vitamin B is synthesized by plants, yeasts, and bacteria. Human beings are capable of synthesizing vitamin B from bacteria residing within their own gut but typically this is synthesized within the colon and the absorption of vitamin B generally occurs above the colon within the small intestine (Yoshii et al., 2019). Consequently, many of the B vitamins within the human diet are obtained from bioaccumulation in animal tissue and are ingested as meat, dairy, and egg products (Herbert, 1988; Rizzo et al., 2016). Other forms of vitamin B come from vegetable sources such as legumes, beans, and leafy green vegetables.

Inadequate levels of B vitamins cause deleterious changes in the regulation of the immune response by affecting nucleic acid production, protein synthesis, immune cell inhibition, and metabolic interference (Mikkelsen, Stojanovska, Prakash, et al., 2017). Processes such as methylation are disrupted and can result in hyperhomocysteinemia which in turn can lead to vascular and systemic inflammation. Normally inflammation is self-limiting due to the dual-type nature of the immune system which follows inflammatory reactions with anti-inflammatory action, but when inflammation becomes chronic, it can cause oxidative stress which in turn can damage DNA and inhibit DNA repair. Genomic instability and cellular mutation can then trigger tumour initiation and promotion, increased rate of cell division, neovascularization and angiogenesis; all of these are hallmarks of cancer (Bird, 2018) (Eime et al., 2013) (Mikkelsen, Stojanovska, Polenakovic, et al., 2017).

The emergence of B vitamins as anti-inflammatory and anti-cancer agents is an area that is just beginning to gain more interest within the scientific community and as the development of genetic and epigenetic investigative techniques becomes more available to a greater number of researchers, there is ongoing investigation occurring in relation to how nutrition effects gene expression. Vitamin B6 and vitamin B12 have both recently become nutrients of interest in relation to anti-inflammatory medicine and cancer risk reduction agents. The active form of vitamin B6, Pyridoxal phosphate (PLP), has shown to be consistently low in inflammatory conditions and is inversely associated with many inflammatory markers (Ueland et al., 2017) and cancer risk (Mocellin et al., 2017), whilst vitamin B12 deficiencies have a high correlation with disturbances in immune cell function which can be restored with B12 supplementation (Erkurt et al., 2008; Tamura et al., 1999). New evidence is required to clarify the roles of B6 and B12 as anticancer agents and a deeper insight is needed into the functional effects of vitamins B6 and B12 on inflammation and cancer pathways and mechanisms at a molecular and genetic level. This review will examine current

knowledge of links between vitamin B6 and B12 status and discuss some of the mechanistic effects with which vitamins B6 and B12 may directly influence inflammation and cancer.

## 1.2. The immune system

### *1.2.1 General overview of the immune system*

The immune system is made up of a highly complex array of specialised and highly distributed, defence cells and proteins. The purpose of our body's immune response is protection against environmental agents, and preservation of the integrity of the body (Schultz & Grieder, 1987).

Innate immunity or non-specific immunity is the body's first defence against any intruder. The skin and mucous linings provide physical barriers whilst chemical barriers such as lysosomes or stomach acid can also prevent the entry of pathogens. The body provides its own normal flora or microbial community which can also act as a barrier in addition to phagocytes which contain antimicrobial proteins and attack cells (Schultz & Grieder, 1987).

When the body's barrier is breached, whether, by a physical injury or invasion of a pathogen, inflammation produced by mast cells in the form of a histamine reaction attracts leucocytes such as neutrophils, macrophages, natural killer (NK) and dendritic cells (DC) to the infiltrated area to destroy any foreign bodies. Dendritic cells act as the link between the innate and adaptive immune systems as they consume the pathogen and share information regarding the pathogen to the adaptive immune system in the form of antigens. When T cells receive information that an infection has occurred, they produce a cell-mediated immune response. B cells produce a humoral immune response when infiltration has occurred, but disease has not yet occurred. T cells that receive signals from antigen-presenting cells,

namely DCs and macrophages, are termed helper T (Th) cells (CD4+) and their key tasks are to produce effector T cells and memory T cells which record the antigen type and store the information for future reference. Cytotoxic T cells (CD8+) are also produced which destroy cells to overcome infection. B cells produce antibodies which are chemicals that fit onto the antigens of the pathogens in a lock and key type arrangement. These antibodies act like tags that signal to macrophages to destroy the tagged pathogens. Memory B cells also keep a record of encountered pathogens and together with T memory cells can increase the body's immune response to previously encountered infections (Chen et al., 2018; Nguyen & Soulika, 2019; Thangam et al., 2018).

The pressure of natural selection is such that organisms with the ability to survive infectious diseases, epidemics, and pandemics, are those whose immune systems have the capacity to mount the fittest or "right" immune response. The 'right" immune response is one that can clear infection without producing excessive inflammation, which can be harmful. For this reason, each person develops their own unique immune system which acknowledges only itself. Everything that is not recognised is potentially a threat, therefore an effective immune system must be able to not only distinguish between self and non-self, but also between non-self, presenting as a threat, and non-self which is harmless (Goodman, 2020).

This ability to distinguish between self and non-self is achieved by a system called human leukocyte antigens or HLA complex (Medzhitov & Janeway, 2000). This gene complex encodes MHC (major histocompatibility) proteins which are located on the surface of all cells and are responsible for the regulation of the immune system. HLA remains unique to the individual and is important for distinguishing self-cells from non-self-cells. MHC class I (MHC-I) proteins are expressed on most nucleated cells in the form of functional receptors whilst MHC class II (MHC-II) proteins are expressed only on antigen-presenting cells (monocytes, macrophages, dendritic cells), B cells and on activated T cells (Choo, 2007).

The primary function of MHC-I and MHC-II is the presentation of antigens in the form of short peptides to the cell surface. These antigens are then recognised by CD8+ (cytotoxic) or CD4+ (helper) T cells respectively. This then allows these cells to monitor what is occurring inside the cell for detection of infection or tumourigenesis (Wieczorek et al., 2017).

### *1.2.2 When the immune system goes wrong*

The immune system is complex and highly developed with a mission to protect the body and seek out and destroy pathogens. It requires an enormous quantity of resources and produces many cells to ensure its successful function. At times, however, excessive immune responses can harm the very system it is designed to protect when the differentiation system that discriminates between self and non-self fails. Total body surveillance is an important factor when ensuring the efficient functioning of the immune system. It is essential that there is no part of the body that can evade the immune response but because of this precision, when immune function goes awry, many systems can be negatively affected.

#### *1.2.2.1 Acute versus chronic inflammation*

An acute immune response is an immediate and short-lived response with limited specificity that is caused by stimuli such as infection or tissue damage. It is characterised by the production of cytokines, chemokines and acute phase proteins which promote the migration of leukocytes, and erythrocytes to the site of injury. Vasodilation, increased blood flow, capillary permeability and neutrophil migration are also hallmarks of acute inflammation.

If inflammation is not resolved, the acute inflammatory process can lead to chronic inflammation typified by lymphocyte and macrophage tissue infiltration, production of pro-inflammatory cytokines, growth factors and enzymes which contribute to the progression of tissue damage. A critical balance between pro- and anti-inflammatory cytokines dictates the overall effect of the inflammatory response. Th1 cells secrete pro-inflammatory cytokines, such as interleukin (IL)-1β IL-2, IL-6, IL-12, tumour necrosis factor (TNF)-αand interferon-gamma (IFN-γ; in addition to activation of transcription factors STAT1 and STAT4. The differentiation of Th1 cells is mediated by IL-2, IL-12, IL-18, IL-27, and IFN-γ.

Th2 cells are involved in the activation and maintenance of the humoral (antibody) mediated immune response and secrete anti-inflammatory cytokines including, IL-4, IL-5, IL-6, IL-9, IL-13, IL-17E, IL-31 and IL-33). IL-4, IL-5, and IL-13 signals STAT6 which upregulates the expression of GATA3 which in turn allows Th2 cell differentiation; in addition to, IL-2, IL-6, IL-27, IL-17E, IL-31, IL-33 which are also involved in differentiation. In recent years, Th17 cells (which secrete IL-17, IL-21, IL-22) have been found to promote inflammation and to be involved in autoimmunity and cancer. To maintain homeostasis and self-tolerance, regulatory (Treg) cells are required which inhibit the proliferation of T cells and the production of cytokines. If the process between Th1 and Th2 becomes dysregulated and chronic inflammation results, this leads to chronic illnesses including, allergies, atherosclerosis, arthritis, autoimmune disorders, Alzheimer's disease, and cancer. Although acute inflammation is well understood there is still much required to understand chronic inflammation and its associated molecular and cellular pathways (Medzhitov & Janeway, 2000; Mikkelsen, Stojanovska, Prakash, et al., 2017; Saghazadeh et al., 2019; Schultz & Grieder, 1987).

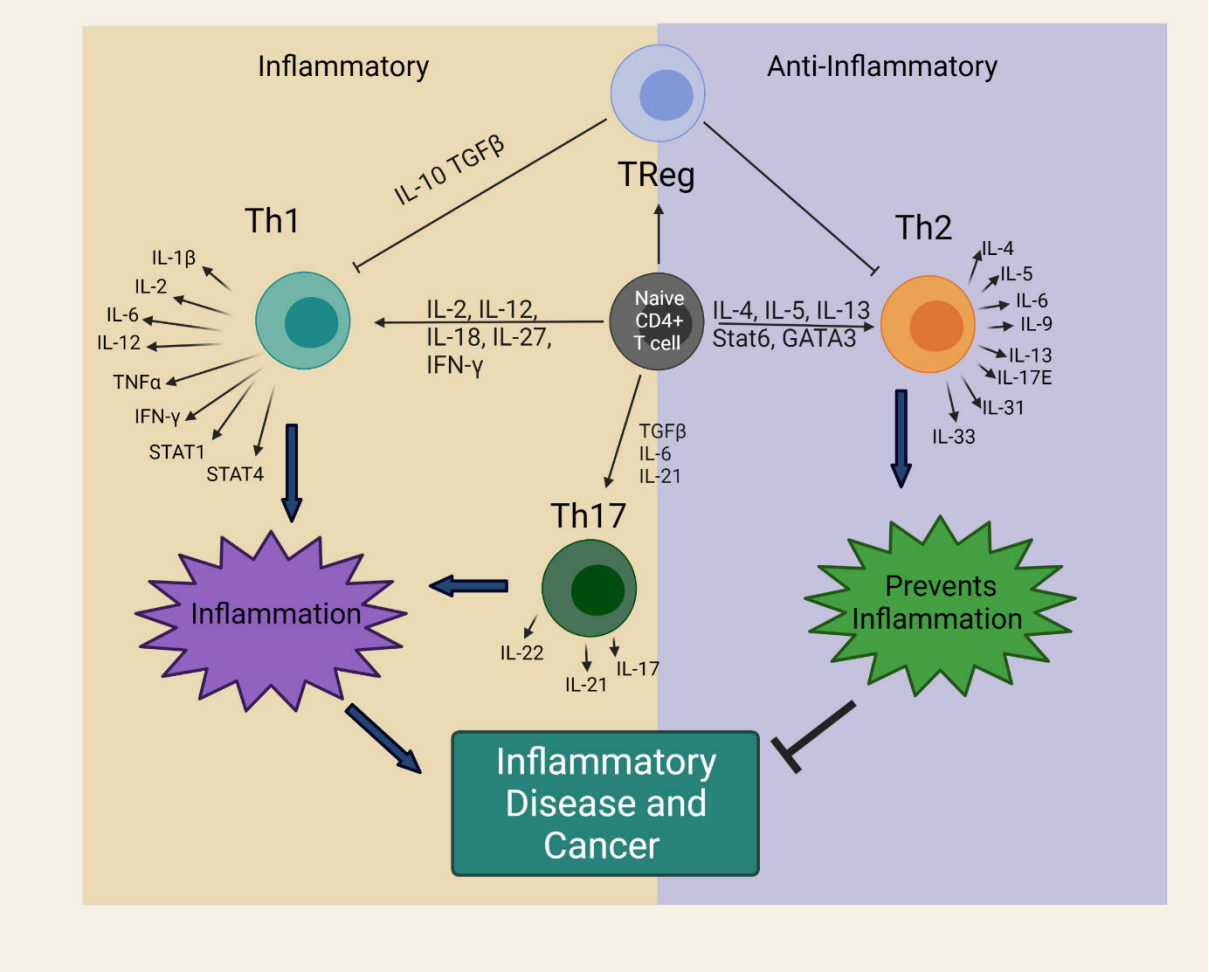


**Figure 1. A schematic diagram showing CD4+ T cell differentiation in inflammation and cancer.**

## 1.3. The link between inflammation and cancer

### *1.3.1 What is cancer*

Cancer is one of the most devastating diseases, affecting all ages, genders, and races worldwide and increasing every year in incidence and mortality. Identifying methods to treat cancer is the primary goal for researchers and scientific institutions globally, however, cancer prevention is proving to be just as significant an endeavour with 30-50 % of cancers being preventable through existing evidence-based strategies and early detection (Organization, 2021). A healthy diet, and maintaining a healthy weight combined with regular physical activity are known to reduce the risk of developing cancer, second to that after cessation of tobacco smoking (Byers et al., 2002).

Cancer is an uncontrolled division and proliferation of abnormal cells within the body that fail to respond appropriately to signals which regulate normal cellular behaviour. This unregulated behaviour affects several different cell types and can therefore give rise to different types of cancer. Cancer is essentially a genetic disease that is caused by an accumulation of unfavourable mutations in the genome over time. The classification of cancer is based according to the tissue of origin. Adenocarcinomas arise from epithelial cells, such as prostate, breast, colon, and liver cancer. Squamous epithelium gives rise to squamous cell carcinomas which include skin cancers, bladder, oesophagus, and some lung cancers, whilst sarcomas arise from connective tissues. Leukaemias are types of haematological cancers originating in the bone marrow whereas lymphomas originate from the lymphatic system (Carbone, 2020).

Cancer development is a multi-step process beginning with hyperplasia where irregular cells begin to proliferate abnormally. Dysplasia follows where cells start to become visually different with abnormal nuclei and shape. As dysplasia progresses and cancer cells start to invade it is termed metastasis. The metastatic cascade has five separated stages of which the first is invasion of the basement membrane and subsequent migration through the stroma of the tumour. Vasculature intravasation follows and the tumour will need to survive within the circulation whilst evading immune surveillance. The tumour can then penetrate the endothelial barrier of a distant organ and establish itself within a metastatic target. (Hapach et al., 2019).

### *1.3.2 Cellular hallmarks of cancer*

In the year 2000, six biological capabilities that cells acquire to enable them to become tumourigenic were proposed. This was updated in 2011 to reflect new information and today ten cellular hallmarks are recognised to be useful to framework an understanding of tumour pathogenesis (Hanahan & Weinberg, 2011) (Figure 2). The cellular hallmarks are useful for directing research towards specific molecular functions and biological processes when developing anti-cancer treatments. Currently, the ten hallmarks of cancer are 1. Genome instability and mutation. 2. resisting cell death, 3. deregulating cellular energetics (altered metabolism), 4. sustaining proliferative signalling, 5. evading growth suppressors, 6. avoiding immune destruction, 7. enabling replicative immortality, 8. tumour-promoting inflammation, 9. activating invasion and metastasis, and 10. inducing angiogenesis.

#### *1.3.2.1 Genome instability and mutation*

The highly proliferative nature of cancer cells increases their likelihood of mutations and carcinogenic adaptations. This may result in a direct change in DNA through genomic rearrangement and deletions or via epigenetic modifications affecting protein expression levels. Normal cells can stop the cell cycle when a mutation is detected, repair the mutation, and then re-enter the cell cycle. Genes involved in this process are termed tumour suppressor genes. Cancer cells however possess an ability to continue mitosis even when bearing these mutations. Oncogenes are genes that are mutationally activated and overexpressed in cancer and can cause cells to proliferate uncontrollably. Oncogenes and tumour suppressor genes both feature in the initiation and progression of many different types of cancers. The main difference between the two is that oncogenes can contribute to cancer when they are activated, (switched on) whilst tumour suppressor genes contribute to cancer when they are inactivated (switched off) (Negrini et al., 2010).

#### *1.3.2.2 Resisting cell death*

Normal cells demonstrate a capability for initiating apoptosis (cell death), as a normal response to mutation and a natural barrier to cancer development. Cancer cells can prevent apoptotic signalling as a strategy to limit or circumvent apoptosis allowing them to survive longer. Targeting apoptotic pathways is a promising focus for anti-cancer therapy (Pfeffer & Singh, 2018).

#### *1.3.2.3 Deregulating cellular energetics (altered metabolism)*

The ability of cancer cells to reprogram metabolic functions and alter their metabolism is an advantageous adaptation that supports its increased energy needs due to sustained and uncontrolled growth and proliferation. Metabolic pathways that are upregulated in cancer cells include aerobic glycolysis, glutaminolysis and mitochondrial biogenesis (Cazzaniga & Bonanni, 2015). Emphasis may be placed on these pathways as possible therapeutic targets for anti-cancer research.

#### *1.3.2.4 Sustaining proliferative signalling*

Normal cell growth requires a tightly regulated cell cycle to sustain proliferation and maintain homeostasis. Tumour suppressor genes such as p53, stop the cell from cycling to repair damaged DNA or signal the apoptotic process to destroy cells that have been damaged beyond repair. This cell cycling process is disrupted in cancer cell growth. Cancer cells overcome the requirement for external growth factors and instead respond to their own growth factors such as epidermal growth factor (EGF) which activates the oncogene RAS and promotes cancer development. The three main signalling pathways that drive this self-sufficiency in cell proliferation are AK strain transforming (AKT), Mitogen-activated protein kinase/Extracellular signal-regulated kinase (MAPK/ERK) and mTOR (Hanahan & Weinberg, 2011).

#### *1.3.2.5 Evading growth suppressors*

Cancer cells can resist external growth inhibitory signalling which would otherwise stop cancer progression. Mitotic division in normal cells has vital checkpoints where anti-growth signals block cellular proliferation. Most cancer cells can circumvent these signals, particularly in the G1 phase of the cell cycle. The major pathways affected that help cancer cells evade growth suppression include the autophagy and apoptosis pathways. Gate keeper genes such as tumour protein (TP53, P53) or retinoblastoma protein (pRb, Rb) are lost or mutated in many cancer cell types allowing for the cell cycle to progress despite DNA damage or other cellular stresses which would typically kill a normal cell (Amin et al., 2015).

#### *1.3.2.6 Avoiding immune destruction*

Some cancer cells can evade immune surveillance and avoid destruction by deactivating components of the immune system that have been despatched to eradicate them. Hijacking immune checkpoint control and modulating innate immune response are two strategies employed by cancer cells. A key intermediary of innate immunity STING (stimulator of interferon genes) is one example of a pathway hijacked by cancer cells to avoid immune destruction whereas immune checkpoint targets include Programmed cell death protein/ Programmed death ligand (PD1/PD-L1), T cell immunoglobulin and mucin-domain containing-3 (TIM3) and Lymphocyte activation gene 3 (LAG3) (Finn, 2012).

#### *1.3.2.7 Enabling replicative immortality*

Malignant cell populations can undergo continuous proliferation with unlimited replicative potential. This feature allows subsequent populations to accumulate sequential abnormalities which further increase autonomous growth and invasiveness. Dysfunctional telomerase activity and oncogene expression prevent normal terminal cues which would otherwise induce senescence and apoptosis. Targeting therapeutic agents which can successfully disrupt replicative immortality will be an important element in the combinatorial approach to cancer treatments (Yaswen et al., 2015).

#### *1.3.2.8 Tumour promoting inflammation*

Cancer cells can commandeer the inflammatory response to support their own growth and survival. Within the tumour microenvironment, immune cells which would normally seek out and destroy malignant cells are subverted into tumour promoting cells that support mechanisms such as growth and metastasis, migration, and anti-body detection. Some important pro-tumour pathways and molecules involved in this response include the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway, inflammasome signalling, and immune checkpoint signalling (Crusz & Balkwill, 2015).

#### *1.3.2.9 Activating invasion and metastasis*

An important factor in cancer prognosis and cancer morbidity are the processes of invasion and metastasis which describe the ability of the cancer cell to expand into nearby tissues and form secondary tumours in distant organs. Epithelial to mesenchymal transition and metastasis both involve changes to cell-cell and cell-matrix interactions which facilitate invasion and migration. The adherence junction signalling pathway involves the adhesion molecule E-cadherin which is responsible for forming adherent junctions with adjacent epithelial cells and assembling epithelial cell sheets. Cancer cells have a reduced expression and occasional mutational inactivation of this molecule which renders them unable to maintain stabilisation of cell-cell adhesion and regulation of order amongst cells and tissue homeostasis (Hanahan & Weinberg, 2011).

#### *1.3.2.10 Inducing angiogenesis*

Cancer cells require their own vascular network to provide them with an ample supply of oxygen and nutrients and waste exchange to enable them to carry out proliferation and metastatic spread. This is achieved by both angiogenesis and lymphangiogenesis. Angiogenesis regulation is achieved by both activator and inhibitor molecules and requires the interaction of many different proteins. Antiangiogenic therapies are still being trailed and show promise in the future treatment of cancer (Zuazo-Gaztelu & Casanovas, 2018).

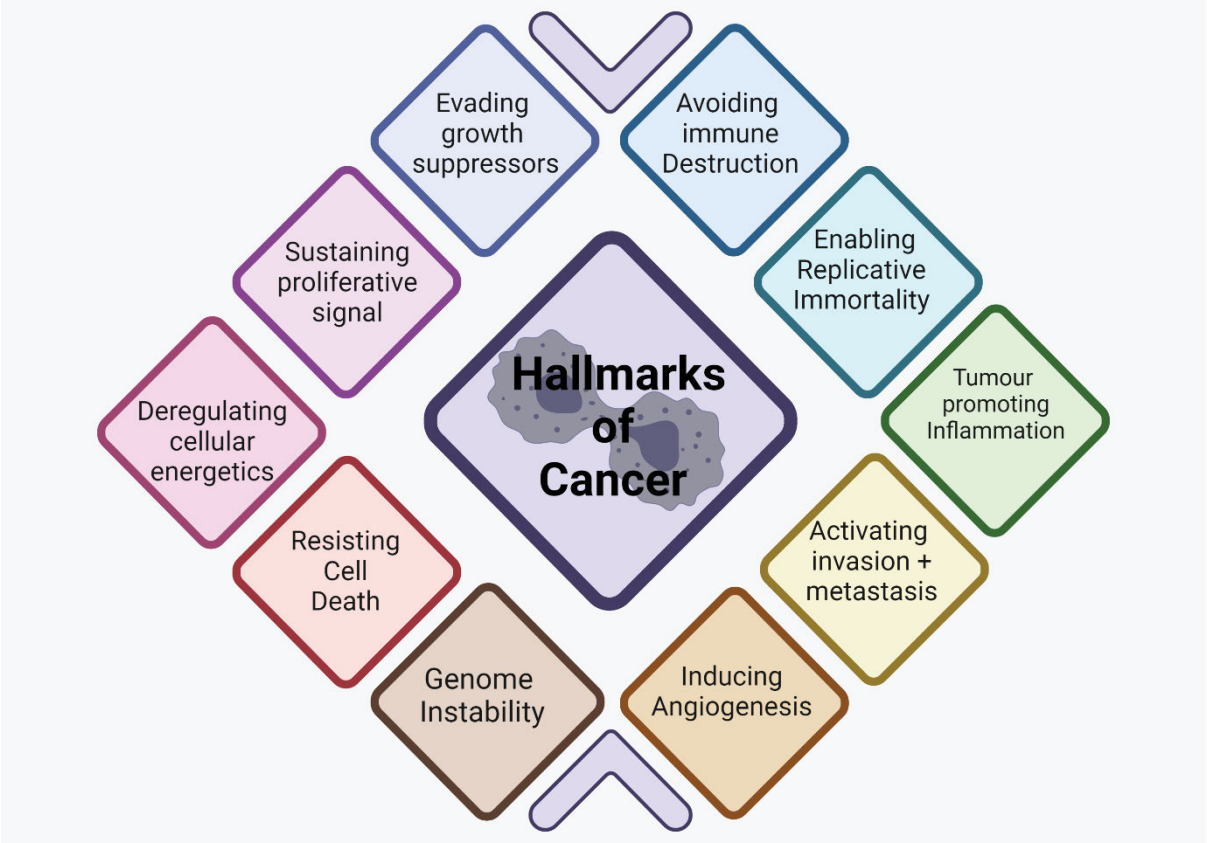


**Figure 2. Cellular hallmarks of cancer**

### *1.3.3 From inflammation to cancer*

Cellular hallmarks of cancer demonstrate the close alignment between cancer and the immune system. Epidemiological data indicate that over one-quarter of known cancers relate to chronic and unresolved inflammation (Vendramini-Costa & Carvalho, 2012). Chronic inflammation can increase the risk of cancer through the promotion of many of the cancer hallmarks.

Cancer formation and progression is controlled by genetic and epigenetic change within cancer cells and the interaction and dynamic cross-talk of these cells with the tumour micro-

environment (TME). The TME has an alliance with stromal cells such as fibroblasts and endothelial cells, immune cells including tumour associated macrophages (TAMS), mast cells, dendritic cells, natural killer cells, neutrophils, eosinophils, and lymphocytes as well as non-cellular factors like extracellular matrix components, exosomes, circulating free DNA, and apoptotic bodies (Pitt et al., 2016). Immune cells within the TME provide a source of intracellular communication which promote tumourigenesis via a complexity of chemicals including cytokines, chemokines, growth factors, and other cytotoxic and inflammatory mediators and matrix remodelling enzymes (Baghban et al., 2020). Immune cells can activate or be activated by transcription factors like NF-κB and STAT3 (Whiteside, 2008; Yu et al., 2009). These oncogenes control tumour growth and progression and can alter cancer cell physiology through their effects on immune and inflammatory cells in the tumour microenvironment. Similarly, tumour cells utilize immune cells, immune cell products and other cellular and non-cellular components of the TME to assist their growth and proliferation in a hostile environment.

### *1.3.4 Immune cells in the tumour microenvironment*

The TME comprises immune cells from both the innate and adaptive immune response and includes macrophages, neutrophils, eosinophils, natural killer cells, T cells, dendritic cells and B cells (Barriga et al., 2019). However, as this thesis is focused primarily on monocyte/macrophage lineage cells, only tumour-associated macrophages (TAMs) are described.

### *1.3.4.1 Tumour Associated Macrophages (TAMs)*

Macrophages are highly plastic cells, differentiated from monocytes, which can polarise into either M1 or M2 macrophages. The classically activated, pro-inflammatory M1 macrophages induced by lipopolysaccharides (LPS) and IFN-γ, are promoted to polarisation by Th1 cytokines including IL-12 and IL-18 and Toll-like receptors (TLRs) (Orecchioni et al., 2019). During carcinogenesis, M1 macrophages attract and activate adaptive immune cells and play a role in the elimination of immunogenic cancer cells by direct cytotoxicity, or antibody-dependent cell-mediated cytotoxicity M1 macrophages express nitric oxide synthase (iNOS), reactive oxygen species (ROS) as well as cytokines IL-1, IL-18, IL-1β, IL-23 and arginase1. M2, alternatively activated, anti-inflammatory macrophages are polarised by IL-4, IL-10 and Il-13 and promote tumour cell proliferation and invasion, angiogenesis, and tissue reconstruction (Pan et al., 2020). M2 macrophages can be further induced into four distinct subsets M2a, M2b, M2c and M2d. M2a is induced by IL-4 or IL-13, expresses IL-10, and TGF-β and are responsible for type II inflammation and Th2 response. M2b macrophages are induced by immune complexes combined with IL-1β or LPS, express IL-10, IL-1β, IL-6, and TNF-α and participate in Th2 activation and immunoregulation. M2c macrophages are induced by IL-10, TGF-β, and glucocorticoid hormones, express IL-10, and TGF-β, and are involved in immunoregulation, matrix deposition and tissue remodelling. M2d subgroup is induced by TLR agonists, adenosine and/or tumour associated factors (TAFs) and expresses IL-12, IL-10, Vascular endothelial growth factor (VEGF), Chemokine (C-C motif) ligand 5 (CCL5), CXC motif chemokine ligand 10 (CXCL10) and CXC motif chemokine ligand 16 (CXCL16) and have an immunosuppressive and pro-tumour effect (Barriga et al., 2019; Chen et al., 2019; Pan et al., 2020; Yao et al., 2019).

Tumour-associated macrophages (TAMs) differ in phenotype depending on tumour type and location within the tumour microenvironment and play an important role in connecting inflammation with cancer (Figure 3). They are the most abundant leukocyte to be found within tumours and are present in a wide variety of different tumour types. TAMs are generally known to be pro-tumourigenic with an M2 macrophage-like profile and have both immunosuppressive effects whilst contributing to angiogenesis lymphangiogenesis, tumour proliferation and epithelial to mesenchymal transition and drug resistance (Barriga et al., 2019; Lin et al., 2019). The accumulation of TAMs within the tumour micro-environment can originate from bone marrow monocytes as well as blood monocytes. Monocyte related myeloid-derived suppressor cells (M-MDSCs) and tissue-resident macrophages are also known to be precursors for TAMs (Kumar et al., 2016; Pan et al., 2020; Tcyganov et al., 2018). TAMs can reprogram the immunosuppressive microenvironment via the promotion of proliferation, invasion, and metastasis of tumour cells and inhibit antitumour immune responses mediated by T cells and dendritic cells. Tams promote metastasis by directly producing soluble factors and destroying matrix membranes of endothelial cells via matrix metallopeptidase 9 (MMP-9) and colony-stimulating factor 1 (CSF1) secretion, this helps the migration of tumour cells and tumour stromal cells. TAMS will directly stimulate cancer cell proliferation via the secretion of epidermal growth factor (EGF), platelet-derived growth factor (PDGF) epidermal growth factor receptor (EGFR), transforming growth factor-beta 1 (TGF-β1), hepatocyte growth factor (HGF), and basic fibroblast growth factor (BFGF). TAMs also promote tumour angiogenesis through the secretion of proangiogenic factors such as Basic fibroblast growth factor (BFGF), VEGF, IL-1, NO, TNF-α, MMP-9 and MMp-2 and stimulate invasion by the production of metalloproteinases MMP-2, MMP-3, MMP-7, MMP-9, as well as EGF and CSF1 (Barriga et al., 2019; Ge & Ding, 2020; Malekghasemi et al., 2020; Orecchioni et al., 2019; Wei et al., 2019; Zhang et al., 2010).

The TAM population is in a constant state of transition between M1 and M2 phenotypes with the balance being tipped either way due to the plastic nature of macrophages and the constant interplay of pro/anti-inflammatory factors within the TME (Chen et al., 2019; Lin et al., 2019; Zhou et al., 2020). The correlation between inflammation and cancer is further evidenced by data associating prolonged use of anti-inflammatory medication such as non-steroidal anti-inflammatory drugs (NSAIDs) and Aspirin with decreased cancer risk in some forms of cancer (Macfarlane et al., 2014; Verdoodt et al., 2016; Vidal et al., 2015; Wong, 2019). It is not beyond reasonable conjecture that bioactive dietary components like vitamin B, which affect the inflammatory process, may also have the potential to impact tumourigenesis and cancer progression (Mikkelsen, Stojanovska, Prakash, et al., 2017; Mikkelsen K., 2019; Tatina T. Todorova, 2017).

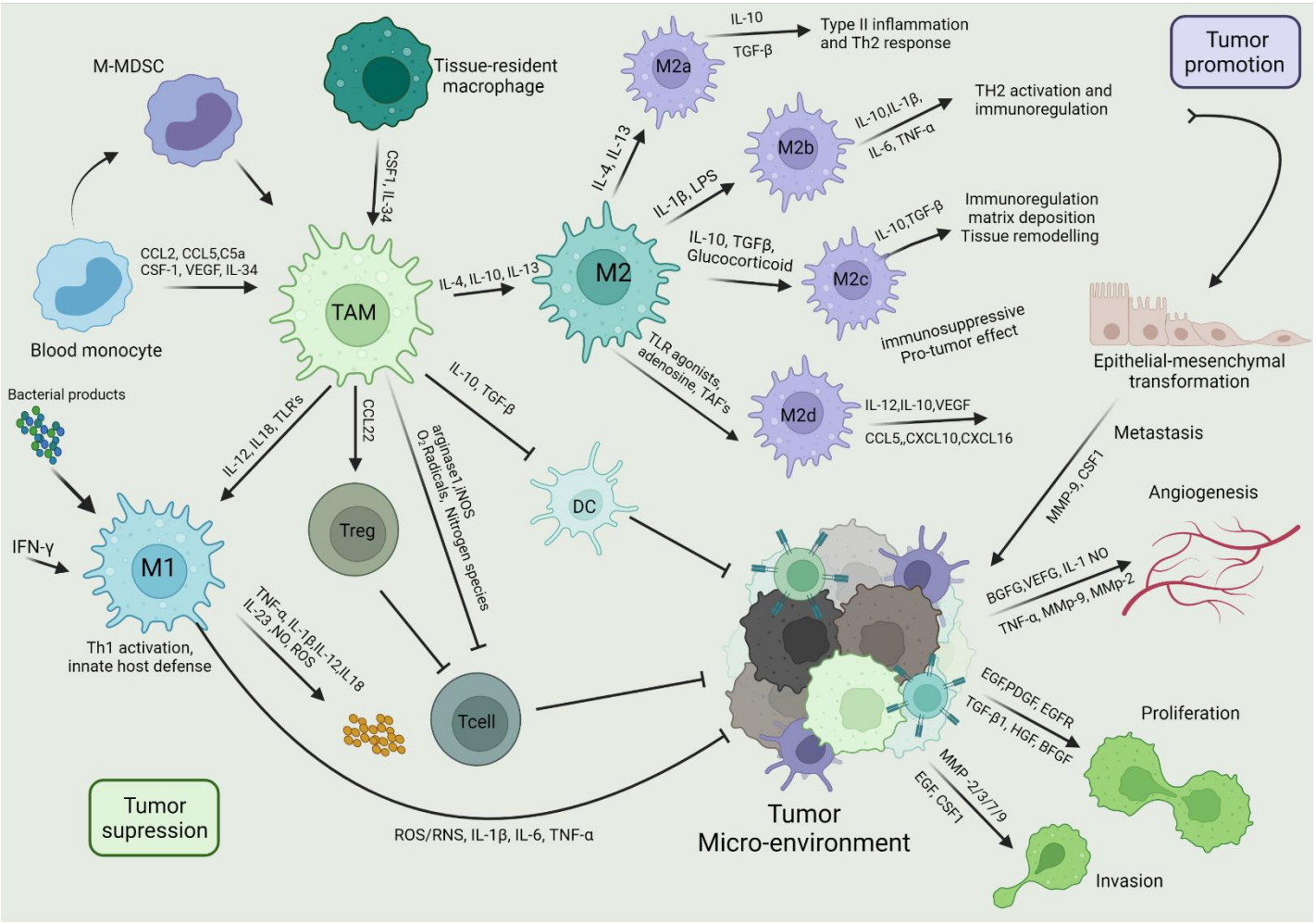


**Figure 3. The complexity of tumour-associated macrophages in the tumour micro-environment.**

## 1.4. Vitamin B6

Vitamin B6 or pyridoxine (PN) is an essential micronutrient in human health with molecular capabilities central to cell survival in living organisms. Vitamin B6 mediates a diverse range of biochemical reactions and its role as a co-enzyme means it participates in more than 150 enzymatic reactions affecting cellular processes, these include regulating metabolism and synthesizing carbohydrates, lipids, proteins, heme, and other crucial bioactive metabolites, neurotransmitter synthesis, folate metabolism, gluconeogenesis, synthesis of coenzyme Q and contributes to the amino acid trans-sulfuration pathway which oversees the degradation of homocysteine (Hcy) into taurine and hypo taurine. (Mooney et al., 2009). The antioxidant and anti-inflammatory properties of vitamin B6 and its vitamers have been shown to have an impact on inflammation-related chronic illness and cancer progression. Currently, much of the vitamin B6 research is focused on deficiency but perhaps a more important area of research is on the intake above the current recommended dietary intake (RDI) and the use of higher doses of vitamin B6 as a therapeutic measure. Exploring the effect of vitamin B6 on the many molecular pathways to which it contributes, however, is key to understanding its role in inflammation and carcinogenesis (Bird, 2018; Havaux et al., 2009; Parra et al., 2018; Saghazadeh et al., 2019; Ueland et al., 2017).

Vitamin B6 is a generalised term for a group of chemically similar compounds all characterised by a centralised pyridine ring. Synthesis of pyridoxine occurs in plants as a protective mechanism from ultra violet rays and an aid to photosynthesis (Parra et al., 2018). Animals are unable to synthesise any of the B6 vitamers and therefore are required to obtain B6 from plants or bioaccumulated within the flesh of other animals. Bacteria within the human gut microbiome are capable of pyridoxine synthesis but generally not in high enough

quantities to benefit the host. The conversion of pyridoxine within humans to its associated vitamers is a complex process. Pyridoxal kinase (PDXK) can phosphorylate pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM) to generate pyridoxin-5'-phosphate (PNP), pyridoxal-5′-phosphate (PLP) and pyridoxamine-5′-phosphate (PMP) (Figure 4). Once phosphorylated they can then function as co-factors. PLP is the biologically active form of vitamin B6 and along with PN, both forms have the greatest impact on cellular processes (Peterson et al., 2020). Vitamin B6 absorption occurs via carrier-mediated transport in the gut and then enters the blood where it is converted back to PLP or PMP (Yoshii et al., 2019). Vitamin B6 deficiency is less common in a healthy population but there are certain factors including age, obesity, malabsorption, and inflammation associated with chronic disease as well as lifestyle factors such as continuous alcohol consumption, poor diet, and certain drugs which can make this more likely (Bird, 2018).

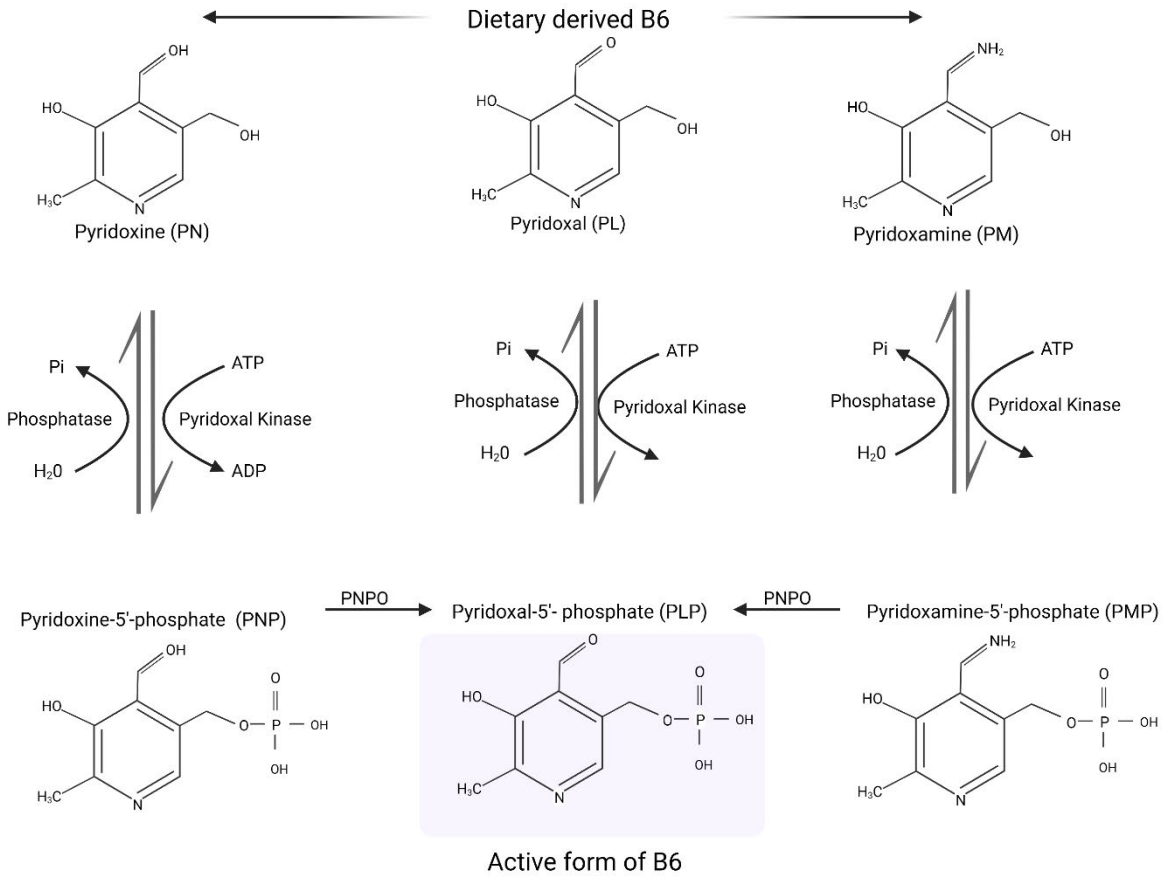


**Figure 4**. **The chemical structures of vitamin B6 vitamers**

### *1.4.1 The mechanistic role of vitamin B6 in inflammation and cancer*

Vitamin B6 deficiency is well established as a causative factor for increased inflammation, and patients with high inflammatory markers are often shown to have significantly lower blood levels of vitamin B6 (Sakakeeny et al., 2012). Low vitamin B6 status is associated with many inflammatory diseases including atherosclerosis and cardiovascular disease (Ji et al., 2021; Kumrungsee et al., 2019; Lotto et al., 2011; Pusceddu et al., 2020; Waly et al., 2016), rheumatoid arthritis (Sakakeeny et al., 2012; Sande et al., 2019), inflammatory bowel disease (Hwang et al., 2012; Saibeni et al., 2003; Selhub et al., 2013; Vavricka & Rogler, 2012; Weisshof & Chermesh, 2015), diabetes (Fields et al., 2021; Marzio et al., 2014; Merigliano, Mascolo, Burla, et al., 2018; Merigliano, Mascolo, La Torre, et al., 2018; Nix et al., 2015; Oxenkrug et al., 2013; Oxenkrug, 2015), non-alcoholic fatty liver disease (Abe et al., 2021; Kobayashi et al., 2021) and cancer (Bird, 2018; Crusz & Balkwill, 2015; Merigliano, Mascolo, Burla, et al., 2018; Mocellin et al., 2017; Peterson et al., 2020; Pusceddu et al., 2020; Waly et al., 2016). Supplementation of vitamin B6 in deficiency states has been shown in many studies to reverse these inflammatory effects (Cappuccilli et al., 2020; Dakshinamurti & Dakshinamurti, 2015; Du et al., 2020; Lee et al., 2018; Mikkelsen et al., 2019; Zhong et al., 2017). High dose vitamin B6 supplementation as a therapeutic agent or as an adjunct in anti-inflammatory medicine is an exciting new area just beginning to be explored (Figure 5).

#### *1.4.1.1 Inflammatory mediators and inflammasome pathways*

Emerging evidence indicates that inflammatory cytokine/chemokine production resulting from chronic illness or infection may be downregulated by high dose supplementation of vitamin B6. In a single-blind co-intervention study in humans, it was shown that high dose vitamin B6 supplementation (100 mg per day) could suppress pro-inflammatory cytokines IL-6 and TNF-α in patients with rheumatoid arthritis (Huang et al., 2012). Similarly, high dose vitamin B6 combined with compound amino acids reduced the expression of IL-6, TNF-α and IL-1β cytokines as well as high mobility group box (HMGB)/TLR4/NF-κB in a rabbit model and cellular model in traumatic coagulopathy (Yi et al., 2020). In another study, vitamin B6 downregulated inflammation (decreased, IL-1β, IL-6, TNF-α), delayed death and increased the survival rate in high dose lipopolysaccharide (LPS) treated mice compared to controls. Likewise, bone marrow-derived macrophages treated with pyridoxal and stimulated with LPS reduced IL-1β, TNF-α and IL-6 (Du et al., 2020). In mice, vitamin B6 was able to suppress IL-1β production by inhibiting nod like receptor protein (NLRP) inflammasome activation and protect mice from LPS-induced endotoxic effects (P. Zhang et al., 2016).

#### *1.4.1.2 Oxidative stress pathways*

Oxidative stress occurs when levels of reactive oxygen species (ROS) outweigh antioxidant mechanisms, thus causing an imbalance that can lead to inflammation and inflammatory-related diseases. Vitamin B6 deficiency contributes to oxidative stress via oxidative lipid peroxidation, generation of oxygen radicals and increase of Hcy levels caused by ineffective

methylation (Bird, 2018; Hsu et al., 2015). In a non-deficiency state, vitamin B6 (pyridoxine) can perform as a highly effective antioxidant due to its hydroxyl group on the 3-position in the pyridine structure. Several studies have determined the effects of vitamin B6 and vitamers pyridoxamine, pyridoxine and pyridoxal phosphate supplementation on endothelial cells, to have antioxidant activity and decreased superoxides and lipid peroxides induced by hydrogen peroxide (Mahfouz et al., 2009). In addition, acute treatment of vitamin B6 was shown to diminish oxidative markers in the livers of septic rats (Giustina et al., 2019), as well as decrease liver oxidative stress in neonatal rats undergoing hyperoxia therapy (Lee et al., 2018). The addition of vitamin B6 to neuronal cells *in vitro* treated with hydrogen peroxide displayed antioxidative abilities via xanthine oxidase inhibition (Danielyan & Simonyan, 2017). In humans, vitamin B6 treatment suppressed oxidative stress and inflammation in cardiovascular disease by increasing cardiac levels of imidazole dipeptides, histamine, and γ-amino butyric acid (GABA) and suppressing P2X7 receptor-mediated NLRP3 inflammasome thus, implying that vitamin B6 may offer cardioprotective properties (Kumrungsee et al., 2019; Kumrungsee et al., 2021). Furthermore, high dose vitamin B6 supplementation over 6 months improved blood biomarkers of oxidative stress by increasing oxidative metabolism in healthy adults (Ford et al., 2018).

#### *1.4.1.3 Vitamin B6 and advanced glycation end products (AGEs)*

Advanced glycation end products (AGEs) also known as glycotoxins, are established contributors to oxidative stress and inflammation and have pathogenic significance in several diseases such as diabetes, obesity, non-alcoholic fatty liver, cardiovascular disease neurological disorders and cancer (Koike et al., 2021; Merigliano, Mascolo, Burla, et al.,

2018; Pereira et al., 2017; Pereira et al., 2020; Ramis et al., 2019; Wetzels et al., 2018; P. Zhang et al., 2016). AGEs are formed through the reduction of sugars and free amino groups of proteins, lipids, or nucleic acids. This non-enzymatic chemical reaction, also known as the Maillard or browning reaction, is a normal part of metabolism but can be problematic in the modern diet where a high consumption of sugar and fat and certain cooking techniques can greatly increase AGEs formation (Uribarri et al., 2010). Vitamin B6 has been shown in some studies to act as an effective inhibitor of AGEs. As such, pyridoxamine (PM) was shown to form stable complexes with metal ions and react with carbonyl compounds in oxidative reactions in protein glycation. More specifically PM could trap free methylthaizolo (OCH3) radicals in both aqueous and lipid media demonstrating its antioxidant capabilities and explaining its inhibitory role in the glycation process (Ramis et al., 2019).

The effects of PM have been studied widely in diabetes. A recent study noted that PM supplementation decreased AGEs and attenuated intervertebral disc generation in diabetic rats (Glaeser et al., 2020). In a similar study PM could alleviate diabetic neuropathic pain partially by suppressing spinal receptor activity in AGEs' NF-κB/extracellular signal-regulated kinase signalling pathway. It also served to decrease AGEs and modify low-density lipoprotein oxidase, low-density lipoprotein and IL-1β levels in the serum of diabetic rats (Zhang, Xu, et al., 2020). In two earlier diabetes studies the combination treatment of PM and sulforaphane normalised endothelial dysfunction associated with type 2 diabetes in a rat model (Pereira et al., 2017), whist PLP was shown to have a protective role against DNA damage by inhibiting AGEs and chromosome aberrations in a drosophila model of type 2 diabetes (Merigliano, Mascolo, La Torre, et al., 2018). Finally, PM supplementation protects against the early stages of diet-induced kidney disfunction by counteracting an

increase in AGEs and RAGEs (receptor AGEs) NF-κB and Rho/ROCK overactivated pathways (Chiazza et al., 2017).

Pyridoxamine was able to modulate oxidative stress and AGEs/TNF-α transcript levels and improve metabolic disturbances in non-alcoholic fatty liver disease (Pereira et al., 2020). It is also touted as a viable candidate to treat obesity and obesity-induced inflammation after it was shown to suppress weight increase and macrophage-1 polarisation and increase Glo-1 expression through the RAGE pathway in high-fat diet-induced obesity in rats (Oh et al., 2019). In addition, PM treatment prevented an increase in AGEs, improved survival, and limited cardiac dysfunction after myocardial infarction in a rat model (Deluyker et al., 2017), and reduced glycation and quenched ROS, thereby reducing DNA oxidative damage (Abdullah et al., 2018). Vitamin B6 has also been shown to have a neurologic effect on AGEs. It was recently shown that vitamin B6 deficient Glo1-KO mice had a greater accumulation of carbonyl proteins in the brain compared to C57BL/6J wild type B6 sufficient mice (Koike et al., 2021). AGEs have also been shown to be increased in an animal model of multiple sclerosis, but PM treatment could not inhibit experimental autoimmune encephalitis induced AGEs production or affect the disease progression (Wetzels et al., 2018).

#### *1.4.1.4 Hydrogen sulphide, trans-sulphuration, and vitamin B6 relationship*

Methionine and cysteine metabolism within body tissues determines the concentration of key metabolites including Hcy and hydrogen sulphide ($H_2S$). $H_2S$ is produced by either enzymatic synthesis, nonenzymatic synthesis, or production within the microbiome (Shackelford RE, 2021). The transsulfuration pathway mediates cysteine production from

Hcy catabolism and contributes to the endogenous production of gasotransmitter $H_2S$. $H_2S$ is significantly regulated by vitamin B6 status and is produced mainly by two PLP-dependent metabolic pathways in mammalian cells; cystathionine beta-synthase (CBS) and cystathionine γ-lyase (CSE) although a third pathway, sequential activity of cysteine aminotransferase 3-mercaptopyruvate sulfurtransferase (3-MST), is used to a lesser extent (Du et al., 2020). $H_2S$ is involved in biological processes such as mitochondrial energy metabolism, post-translational modifications of proteins by s-Sulfhydratin in the cardiovascular system, lipid metabolism, attenuation of inflammation, tissue repair and glucose regulation (Bird, 2018). The biological activity of $H_2S$ is dependent on how much is produced and the concentration at target tissues, it has both anti- and pro-inflammatory actions and participates in vasodilation, angiogenesis, neurotransmission, apoptosis, cardio protection, and insulin release. $H_2S$ can enhance tumour growth by stimulating cellular bioenergetics and can contribute to proliferation, angiogenesis, migration, and invasion (Shackelford RE, 2021), and enhances tumourigenesis by protecting mitochondrial biogenesis (Hellmich & Szabo, 2015; Marutani & Ichinose, 2020).

The diversity in $H_2S$ activity means that its effects are sometimes contradictory in tumourigenesis. Human hepatoma cells cultured in vitamin B6 deficient media reduce the production of $H_2S$ and its associated biomarkers via the transsulfuration pathway (DeRatt et al., 2014). There is reasonable evidence to suggest that the endogenous production of $H_2S$ could contribute to the growth and proliferation of colon and ovarian cancer cells but not melanoma, whilst in glioma, the silencing of CBS accelerated tumour cell proliferation. In fact, different cancer types utilize different $H_2S$ dependent pathways. Although endogenous production of $H_2S$ could contribute to cancer promotion increasing $H_2S$ production by exogenous means contributes to anti-tumour effects (Hellmich & Szabo, 2015). Five years later Zhang et al conducted experiments supporting this theory whereby, $H_2S$ treatment

showed significant anti-cancer effects in SNU398 hepatocellular carcinoma cells. They attributed this exogenously produced anti-cancer effect to the ability of the hepatocellular carcinoma cells to metabolically reprogram sulphur metabolism(Zhang, Chen, et al., 2020). This capacity for metabolic change is one of the cellular hallmarks of cancer. In addition, endogenous production of $H_2S$ where gasotransmitter levels are at their optimal concentration level supports colon cancer cell proliferation whereas exogenous production of $H_2S$ which keeps it above optimal concentration levels decrease colon cancer cell proliferation (Olah et al., 2018). Currently, there are no studies which directly determine the effect of high dose vitamin B6 treatment on $H_2S$ production in cancer cells but given the current evidence, it may be plausible that high dose vitamin B6 may increase $H_2S$ above optimal concentration levels and contribute to a decrease in cancer cell proliferation.

#### *1.4.1.5 Sphingosine-1-phosphate and vitamin B6*

Sphingolipids are essential components in mammalian cell membrane structure. Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite that contributes to a variety of cellular functions including growth, migration, immune cell trafficking cell survival, and angiogenesis. Increased S1P has been associated with inflammation and cancer progression (Hait & Maiti, 2017). NF-κB, MAPK, and STAT3 are all inflammatory pathways that are upregulated in response to excessive S1P levels (Du et al., 2020). S1P synthesis requires the PLP dependent enzyme, serine palmitoyl-transferase (SPT) (Ueland et al., 2017) and degradation of S1P is also PLP dependent, via the enzyme Sphingosine 1-phosphate lyase (S1PL). A few studies have examined the role of vitamin B6 supplementation on inflammation via S1P pathways. Of interest, vitamin B6 supplementation was shown to

prevent excessive inflammation by reducing the accumulation of S1P in an S1PL dependent manner (Du et al., 2020). Likewise, vitamin B6 supplementation could be a potential therapy for patients suffering from S1PL insufficiency syndrome, a rare metabolic disorder that results in deficiency of the enzyme S1PL which is required to break down S1P thus, resulting in S1P accumulation (Zhao et al., 2020). Further research however is required to determine the effects of vitamin B6 supplementation on the S1P pathway in inflammation and cancer.

#### *1.4.1.6 Telomere length and vitamin B6*

Recent attention has been focused on the association between shortened telomere length, inflammation, and chronic disease (Ding et al., 2018; Duckworth et al., 2021; Fragkiadaki et al., 2020; Margaritis et al., 2017; Mazidi et al., 2017; Ningarhari et al., 2021) with some association with the involvement of vitamin B6 as a key player. Mazidi et al (2017) correlated dietary components and patterns with telomere length (TL). Assessing dietary intake and TL from 10,568 participants from 1999-to 2001 in the US, it was shown that vitamin and mineral consumption (vitamin B6, magnesium, iron, copper vitamin C) are associated with longer telomeres in an adult population (Mazidi et al., 2017). In addition, leukocyte telomere length is inversely proportional to Hcy, thus suggesting that vitamin B6 status may be important given its role in one-carbon metabolism and hyperhomocysteinemia (Rane et al., 2015). Further, the relationship between Hcy, vitamin B6, inflammation and relative telomere length in humans over 9.9 years was assessed and concluded that subjects with the longest telomeres had a significantly higher concentration of vitamin B6 and lower concentration of Hcy and inflammatory markers, IL-6 and high sensitivity CRP (Pusceddu et al., 2020).

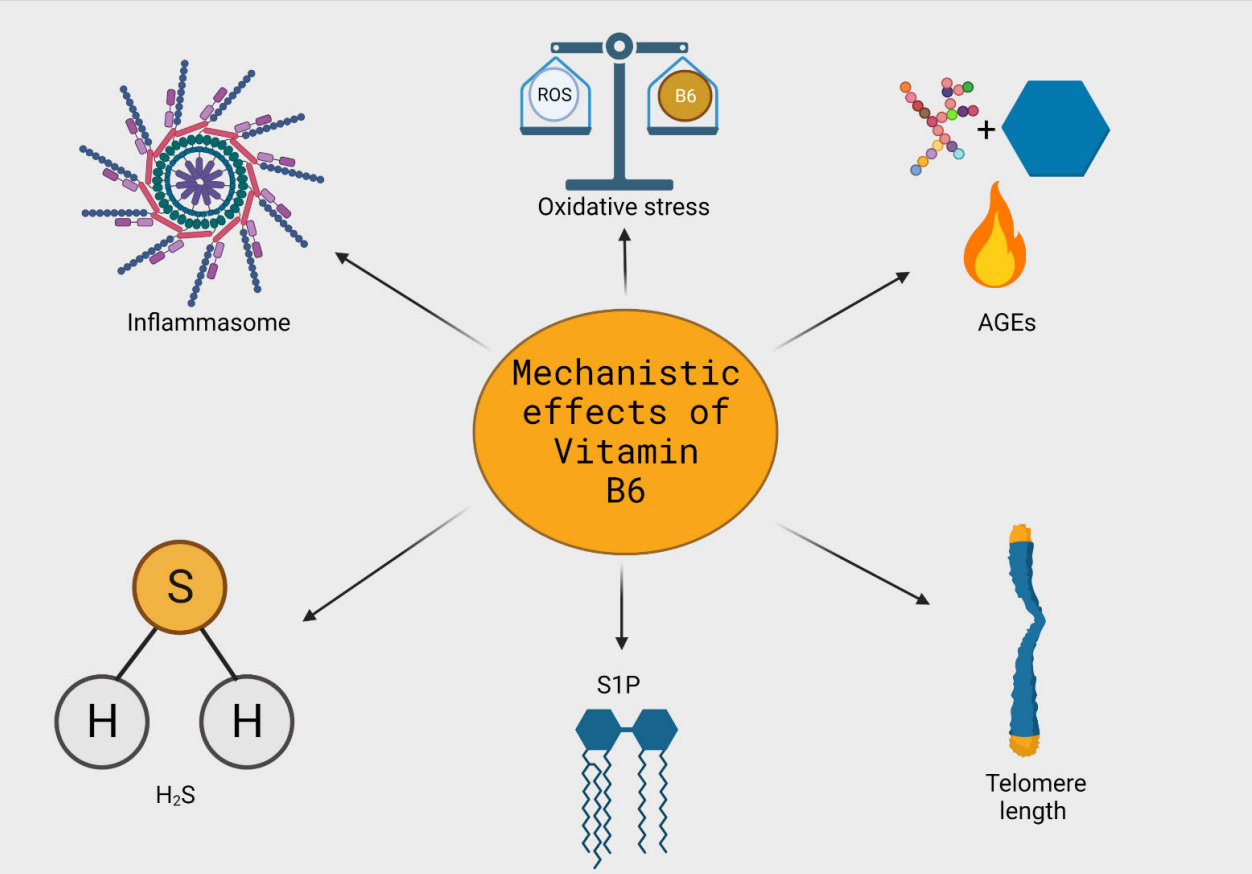


**Figure 5. The mechanistic effects of vitamin B6 in inflammation and cancer.**

### *1.4.2 Vitamin B6 as an immunomodulatory agent in cancer*

Although studies show vitamin B6 supplementation may help decrease inflammation it is unclear what role vitamin B6 supplementation plays as a therapeutic agent in cancer. Vitamin B6 deficiency can contribute to cancer pathogenesis via increasing inflammation and inflammatory mechanisms, however, the effects of vitamin B6 once cancer is established are not clear, including the potential to downregulate key pathways if supplemented above the accepted recommended daily intake (RDI).

There have been numerous murine studies where vitamin B6 supplementation has been used to treat cancer or tumourigenesis. In one model, vitamin B6 supplementation was able to suppress protein expression for proliferation-related genes c-myc and c-fas and reduced oxidative stress in azoxymethane (AOM) treated mice (Komatsu et al., 2002). In repeated experiments under different parameters vitamin B6 supplementation suppressed colon tumourigenesis by reducing cell proliferation, oxidative stress, nitric oxide production and angiogenesis in AOM treated mice (Komatsu et al., 2002; Matsubara et al., 2003). In rats, vitamin B6 supplementation was effective in suppressing 1,2-dimethylhydrazine induced colon damage, the proliferation of epithelial cells and expression of 70 kilodalton heat shock protein (HSP70) and HO-1 (targets for anti-tumour agents) (Kayashima et al., 2011). Similarly, vitamin B6 supplementation downregulated Cd8a and CCL8 mRNA expression in AOM treated mice using DNA microanalysis, suggesting that vitamin B6 may be protective against colon cancer development (Toya et al., 2012). In another study in APC1638N mice, a model for familial adenomatous polyposis-associated desmoid tumours and cutaneous cysts, maternal vitamin B supplementation (B2, B6, B9, B12) suppressed, while deficiency promoted, intestinal tumourigenesis in mouse offspring (Ciappio et al., 2011). Rats fed a high-fat diet and supplemented with vitamin B6 markedly reduced the ratio of lithocholic acid to deoxycholic acid. Lithocholic acid is a toxic secondary bile acid and a risk factor for colon cancer. The study also noted that vitamin B6 increased faecal mucin levels, which is a marker of intestinal barrier function, in a dose-dependent manner (Okazaki et al., 2012). In a very recent murine study, Wu et al (2021), confirmed that a combination of vitamin B6 and curcumin supplementation, was superior to either agent alone in preventing obesity promoted colorectal carcinogenesis in mice by augmenting suppression of pro-inflammatory cytokines and signalling pathways (Wu et al., 2021).

Similar effects of vitamin B6 on tumourigenesis have been reported in studies involving humans. In a meta-analysis study, it was identified that higher serum levels of vitamins B2, B6, B12 and folic acid through supplementation, lowered the risk of renal cell carcinoma (Mao et al., 2015). Additionally, a study in patients who had undergone tumour resection for hepatocellular carcinoma showed that supplementation of vitamin B6 reduced plasma Hcy concentrations and potentially had antioxidant capacity (although the study did not directly measure this) (Cheng et al., 2016). Further, vitamin B6 supplementation in humans was shown to be inversely related to carcinogenesis of the oesophagus whereas B12 showed an increased risk (Ma et al., 2018). Of relevance, vitamin B6 intake is statistically inversely associated with breast cancer risk in middle-aged women with low to no alcohol intake but not with high alcohol consumption (Egnell et al., 2017). A recent pilot study of high dose vitamin B6 supplementation was able to enhance the anti-tumour effect of 5-fluorouracil (FUra) and folinic acid, a treatment currently used for patients with colorectal, pancreatic, and gastric carcinomas (Machover et al., 2021). We also demonstrated the antitumour effect of vitamin B6 in promonocytic lymphoma cells. It was noted that high dose vitamin B6, decreased proliferation, migration, and modulated cytokine production and PD-L1 expression (Mikkelsen et al., 2019). In contrast to these findings, long term use of supplemental B vitamins in patients linked to a cancer registry and vitamins and lifestyle (VITAL) cohort. They reported that although supplemental B6 was not associated with lung cancer in women, it was associated with a 30-40% increase in lung cancer in men. Interestingly the same authors published a study three years later using data from women's health initiative and concluded that post-menopausal women who supplemented with $\geq$50 mg/day of vitamin B6, had a 16% reduced cancer risk than those who did not supplement at all (Brasky et al., 2020).

## 1.5. Vitamin B12

Vitamin B12 is the largest, most structurally complex of the B vitamins and describes a group of compounds, often referred to as cobalamins, which contain a cobalt centred corrin nucleus. Cyanocobalamin is the metabolically active form of B12 however other forms are naturally found in biological systems. Vitamin B12 is an essential requirement and important for many processes such as nervous system maintenance including myelination, cell synthesis, catabolism of fatty acids and proteins, red blood cell formation, DNA synthesis and cell division and acts as a co-factor in many metabolic processes including methylation (Mikkelsen, Stojanovska, & Apostolopoulos, 2016; Mikkelsen K., 2019)

Vitamin B12 is synthesised by bacteria in the human gut but this is largely unavailable for use within the body, and consequently, the main source of B12 comes from animal origin biomagnified through the food chain (Rizzo et al., 2016). B12 is bound to protein in food and released by haptocorrin (HC) which is found in saliva and gastric fluids. Vitamin B12 is released from HC once it reaches the duodenum by pancreatic proteases (Lyon et al., 2020). For absorption to occur into the body, B12 must be combined with intrinsic factor before being absorbed into the ileum. Following absorption, vitamin B12 is released into the circulation and is transferred into the cell via a plasma transporter, transcobalamin II. Once inside the cell the transcobalamin II-B12 complex is then degraded by lysosomal activity and free B12 is released into the cytoplasm (Figure 6) (Mikkelsen, Stojanovska, & Apostolopoulos, 2016; Mikkelsen K., 2019).

It has long been documented that vitamin B12 deficiency can contribute to hematologic and neurological symptoms such as pernicious anaemia, and cognitive impairment, (Ankar & Kumar, 2021; Balk et al., 2007; Dayon et al., 2017; Mikkelsen, Stojanovska, & Apostolopoulos, 2016; Mikkelsen, Stojanovska, Tangalakis, et al., 2016; Sengul et al., 2014;

Seppala et al., 2013; Syed et al., 2013; Wolffenbuttel et al., 2019). Vitamin B12 deficiency can also impact the immune system compromising both cell-mediated and humoral immunity by inhibiting the activity of immune cells, affecting nucleic acid production and protein synthesis, and interfering with methylation which can lead to hyperHcymia and contribute to systemic and vascular inflammation (Mikkelsen K., 2019). This in turn can contribute to the pathogenesis of many different inflammatory-related diseases. Vitamin B12 deficiency can stem from dietary deficiencies such as those found in vegetarian and vegan diets, or malabsorption. There are many reasons that vitamin B12 absorption may be impaired. Reduced production of intrinsic factor, long term use of certain drugs like metformin and acid-reducing medication, bacterial overgrowth where bacteria compete for vitamin B12 leading to vitamin B12 deficiency, surgical intervention including total or partial gastrectomy which serves to eliminate site of intrinsic factor production, infection of some parasites such as Diphyllobothrium latum with utilizes luminal B12, or *helicobacter pylori* infection. Pancreatic insufficiency can cause failure to inactivate cobalamin binding proteins. Malignancy of pancreas or bowel, transcobalamin II deficiency which causes impairment in transmembrane transport of B12 (Ankar & Kumar, 2021; Choi, 1999; Friso & Choi, 2005; Lyon et al., 2020; Mikkelsen K., 2019). Vitamin B12 deficiency can contribute to many pathological conditions, many of these occur due to increased inflammation from ineffective methylation in one-carbon metabolism (Lyon et al., 2020).

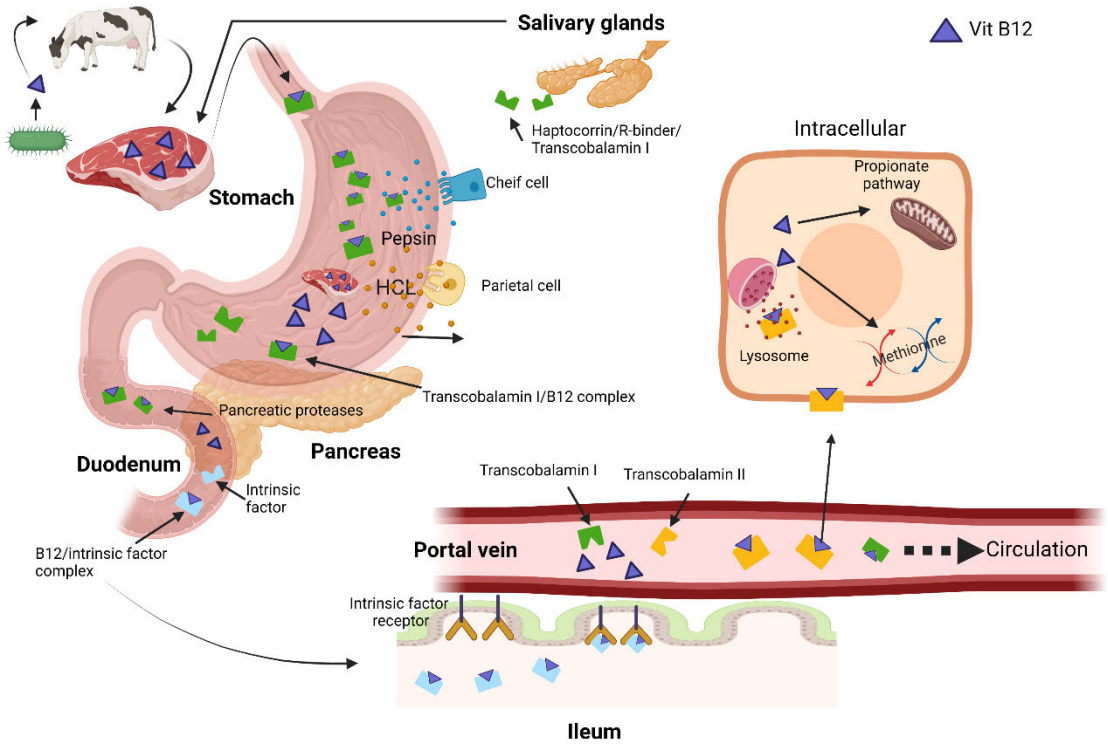


**Figure 6. Digestion and absorption of vitamin B12.**

### *1.5.1 Vitamin B12 and one-carbon metabolism*

Methylation is a crucial metabolic process involving the transfer of a methyl group onto amino acids, enzymes, proteins, or DNA. Many chemical reactions within the body involve methylation reactions which contribute to activities such as regulation of the healing process, production of energy, genetic expression, neurological function, detoxification, and efficient immune function. The two cycles of events in which methylation takes place are the S-adenosyl methionine (SAM) cycle which is vitamin B12 and B6 dependent and the folate cycle which is B9 and B12 dependent.

Methionine (Met) is converted to SAM within the methionine cycle, by an enzyme called methyl-adenosyl transferase 2A (MAT2A). SAM acts as a substrate for a variety of methylation reactions of which Hcy (Hcy) is a by-product. The synthesis of Hcy back into

Met uses B12 as a cofactor (Nuru et al., 2018). Similarly, B6 can be used as a co-factor to convert Hcy into cysteine via the transsulfuration pathway during glutathione synthesis. B12 is also an important co-factor in propionate metabolism where branched-chain amino acids and odd chain fatty acids along with cholesterol are broken down to be utilised within the TCA cycle within mitochondria. Methyl-malonyl CoA mutase (MUT) utilizes B12 in the form of adenosyl cobalamin, to convert L-methylmalonyl-CoA into succinyl-CoA. It is in this form that it can then enter the TCA cycle (Figure 7) (Lyon et al., 2020).

Within the Folate cycle methyltransferase utilizes vitamin B12 as a cofactor to transfer a methyl group from 5- methyl tetrahydrofolate (5-MTHFR) toHcyHcy. This generates tetrahydrofolate (THF) and methionine for use within the methionine cycle (Figure 8) (Mikkelsen, Stojanovska, & Apostolopoulos, 2016). Vitamins B6 and B12 (and other B vitamins) deficiencies can lead to ineffective methylation increasing the by-product, HcyHcy. Elevated HcyHcyor hyperHcymia, is known to contribute to inflammation, oxidative stress, and subsequent cancer progression (Locke et al., 2019; Mikeska & Craig, 2014; Wu & Wu, 2002). B vitamins deficiencies not only contribute to elevated Hcy levels which can progress to inflammation but can also affect the methylation of DNA by affecting the function of the genes and modifying gene expression. DNA demethylation involves the removal of a methyl group from DNA. DNA demethylation is a process that involves the epigenetic reprogramming of genes and is an important factor when looking at disease mechanisms such as inflammation and tumour progression (Chen & Riggs, 2011)

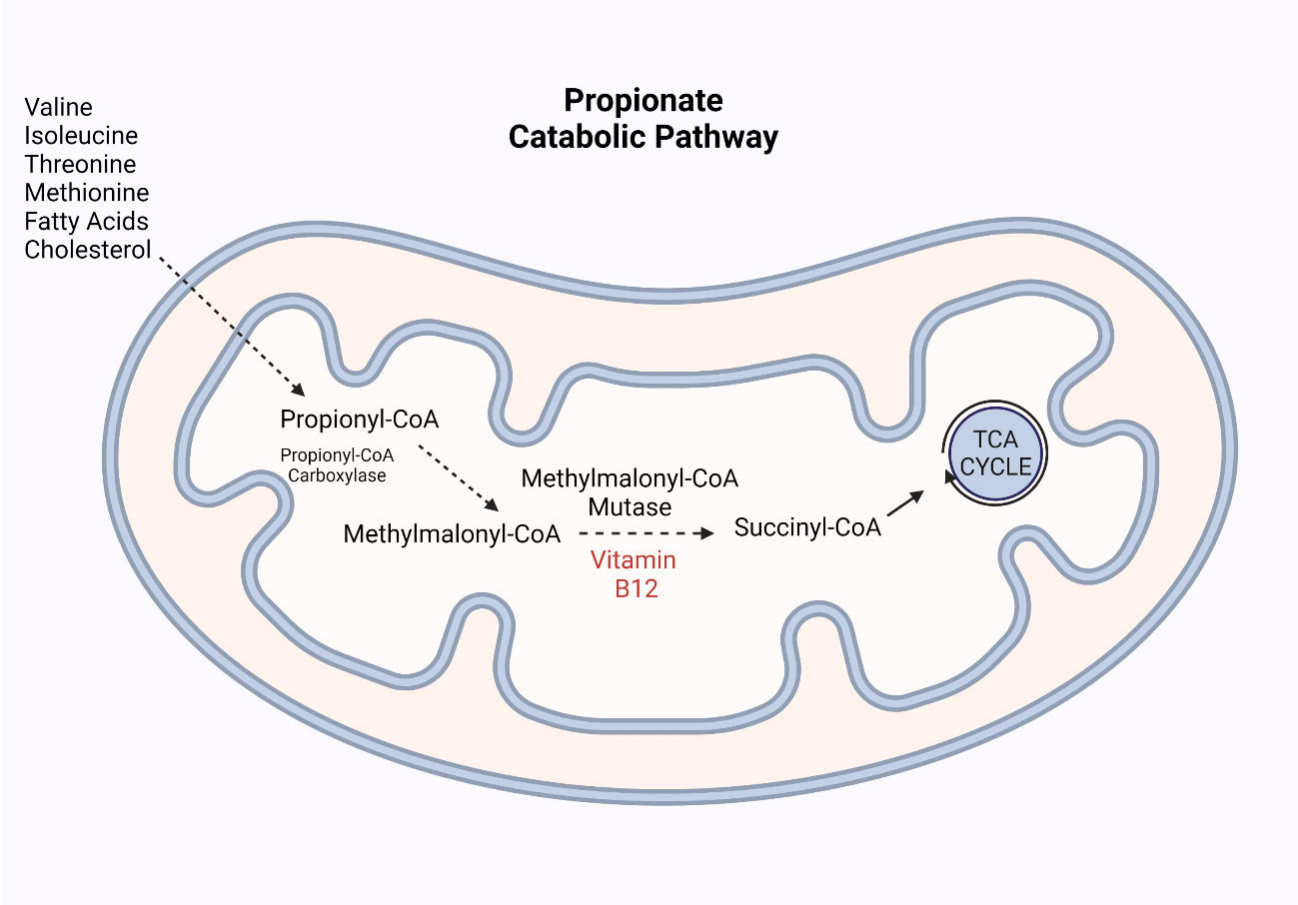


**Figure 7. Propionate Catabolic Pathway.**

Methyl-malonyl CoA mutase (MUT) utilizes B12 in the form of adenosyl cobalamin, to convert methylmalonyl-CoA into succinyl-CoA. It is in this form that it can then enter the TCA cycle

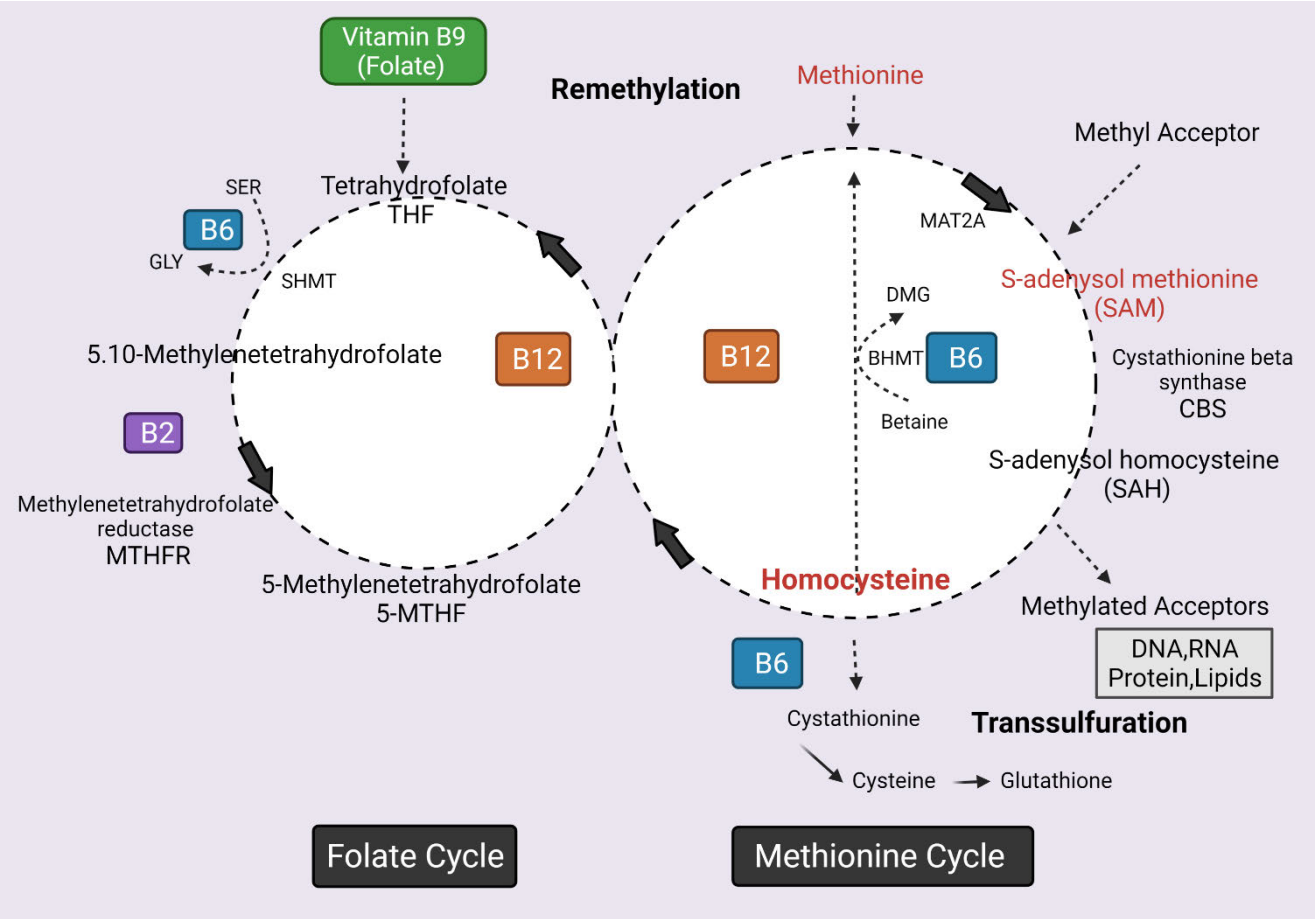


**Figure 8. Folate and methionine methylation cycles.**

Within the Folate cycle methyltransferase utilizes vitamin B12 as a cofactor to transfer a methyl group from 5- methyl tetrahydrofolate (5-MTHFR) to Hcy. This generates tetrahydrofolate (THF) and methionine for use within the methionine cycle. Methionine (Met) is converted to SAM within the methionine cycle, by an enzyme called methyl-adenosyl transferase 2A (MAT2A). SAM acts as a substrate for a variety of methylation reactions of which (Hcy) is a by-product. The synthesis of Hcy back into Met uses B12 as a cofactor

### *1.5.2 Vitamin B12 status on inflammation and cancer*

Numerous studies have linked vitamin B12 deficiency with inflammatory-related diseases such as colorectal cancer (Hanley et al., 2020; Padmanabhan et al., 2019), inflammatory bowel disease (Hwang et al., 2012; Pan et al., 2017; Park et al., 2021), non-alcoholic fatty liver (Ankar & Kumar, 2021; Boachie et al., 2021), chronic kidney disease (Lemoine et al., 2019; Shevchuk et al., 2019), diabetes (Bherwani et al., 2017; Liu et al., 2020), multiple sclerosis (Mahfouz et al., 2009; Nemazannikova et al., 2018), and systemic lupus erythematosus (Tsai et al., 2021). Vitamin B12 deficiency has also been linked to cancer risk in cervical (Pathak et al., 2014; Silva et al., 2020), breast (Wu et al., 1999; Zhang et al., 2003), gastric (Miranti et al., 2017; Murphy et al., 2015), liver (Brunaud et al., 2003), oesophageal (Khairan et al., 2021) and colorectal cancers (Dahlin et al., 2008). Within the last ten years, however, there has been great interest in the association between high vitamin B12 levels and an increase in cancer risk and the idea of vitamin B12 as an onco-protective factor is now being questioned.

Critically ill patients with high serum vitamin B12 upon hospital admission, have higher mortality after 90 days than those with lower B12 levels (Sviri et al., 2012). Similarly, elevated vitamin B12 and total Hcy concentrations in women over 85 years of age were associated with an increased risk of cardio-vascular disease and all-cause mortality (Mendonca et al., 2018). A cohort study in Denmark examined data from patients on medical registries with B12 measurements greater than the lower reference limit ($\geq$200 pmol/l) during the period 1998-to 2009. The data was compared to the data on cancer incidence and noted elevated vitamin B12 serum levels to be associated with a subsequent cancer diagnosis, particularly for haematological, alcohol and smoking-related cancers (Arendt et al., 2013). In lung cancer, however, vitamin B supplementation (B6 and B12) are not chemo-

preventive and may be harmful (Brasky et al., 2017), and high vitamin B12 status increase the risk of lung cancer (Fanidi et al., 2019). Vitamin B12 and B9 supplementation with an increased risk of colorectal cancer (Oliai Araghi et al., 2019) whilst a more recent study in 2020 by Urbanski et al confirmed that elevated B12 levels were associated with solid cancers and metastases (Urbanski et al., 2020). Interestingly, elevated serum B12 correlated with a risk of haematological cancer one year after follow up, when compared to all other cancer types (Arendt et al., 2019). This relationship between high serum B12 and haematological malignancies is not new, in fact, it was reported in 1954 that some cases of leukemia were associated with vitamin B12 serum concentrations greatly above the normal range (Beard, Pitney, & Sanneman, 1954; Beard, Pitney, Sanneman, et al., 1954). There is some conjecture that high serum B12 may occur partly because of high Hcy produced by cancer cells which causes B12 to bind to Hcy within the blood serum which in turn decreases cellular B12 uptake, this theory postulates that high serum B12 is actually a result of cancer rather than a cause of it and that in fact in this regard, cancer patients may actually suffer from B12 deficiency due to lower cellular uptake (Gimsing, 1995; Lyon et al., 2020). Considering this information, it seems that vitamin B12 status has a dose-response association with cancer whereby both deficiency and surplus states can affect cancer initiation and progression (Figure 9).

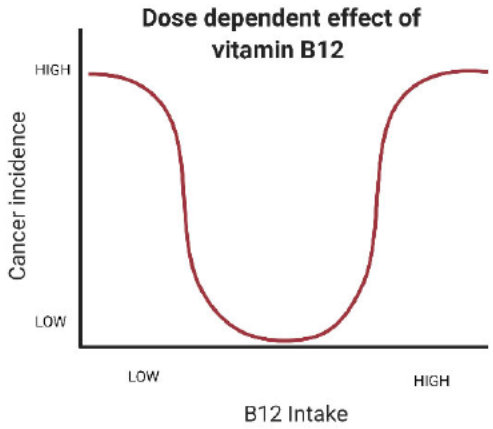


**Figure 9. Dose dependent effect of vitamin B12.**

Vitamin B12 deficiency is related to dysfunction in one-carbon metabolism which can affect DNA synthesis, increase oxidative stress, Hcy production and genomic instability and decrease methylation potential. The effects of high levels of B12 on pro-tumour mechanisms are less understood but increasing evidence suggests that factors may involve an increase in one-carbon metabolism which could increase nucleic acid synthesis and assist the rapid turnover of cancer cells and illicit other changes in DNA methylation (Lyon et al., 2020).

### *1.5.3 Involvement of vitamin B12 in DNA methylation and cancer*

Vitamin B12 plays a fundamental role in DNA synthesis and DNA methylation and demethylation. In normal cells, methylation (switching genes off) and demethylation (switching genes on) processes are balanced and contribute to the normal function of the cell, however in cancer cells, DNA methylation and demethylation are dysregulated(Moore et al., 2013; Wajed et al., 2001). DNA methylation which occurs at promoter regions in the genome is responsible for gene silencing and these promoter regions are mostly responsible for regulating gene transcription. Methylation of DNA is carried out by a group of enzymes called DNA methyltransferases or DNMTs, and a substrate called S-Adenosyl methionine (SAM), of which production is vitamin B12 dependent provides the methyl group for transfer.

(Cytosine, guanine) sites are specific regions in which a cytosine nucleotide, connected by a phosphodiester bond, is followed by a guanine in a 5'-3' direction and they are found widely distributed in DNA. Areas with high CpG frequency are referred to as CpG islands (CGIs). Most CpG sites are methylated but CGIs are largely unmethylated. In cancer cells, hypermethylation of promoter CGIs are associated with tumour suppressor gene

inactivation, in contrast to this cancer cells also experience widespread hypomethylation across the genome. This bimodal deregulation of the epigenetic landscape is found widely in all cancer cells (Locke et al., 2019; Mikeska & Craig, 2014; Moore et al., 2013). There has been some conjecture that high dose vitamin B12 may directly affect methylation and demethylation processes in cancer cells and potentially contribute to carcinogenesis. One idea may be that high doses of B12 could increase SAM levels and contribute to hypermethylation at promoter CGIs (Fig 10) In fact, vitamin B12 supplements influence the regulation of several genes as well as 589 differentially methylated CpGs and 2892 differentially methylated regions (Boughanem et al., 2020). This study claimed that there was an inverse association between DNA methylation and vitamin B12 in colorectal cancer.

High concentrations of serum B9/B12 levels are associated with the risk of promoter methylation in tumour specific genes, and this relationship can be modified by MTHFR C677T genotypes (Mokarram et al., 2008). Meanwhile, vitamin B9/B12 levels are significantly correlated with DNA methylation of tumour suppressor genes P16, MutL homolog 1 (MLH) and O-6-Methylguanine-DNA Methyltransferase (MGMT). (Sanchez et al., 2017). Another study by Zeng et al (2019) showed that dysregulation of mTOR signalling and DNA methylation were both closely linked to tumour progression (Zeng et al., 2019). It was proposed that serine and one-carbon metabolism may serve as a bridge linking mTor and DNA methylation in tumour promotion. In addition, MTHFR and C677T genotypes correlate with serum vitamin B2 and B12 levels and are associated with oesophageal pre-cancerous lesions and oesophageal squamous cell carcinoma risk via aberrant DNA methylation of tumour suppressor gene P6 or P53 (Pan et al., 2019). Furthermore, methyl donors, L-methionine, choline (vitamin B4), folic acid (vitamin B9) and vitamin B12 on breast and lung cancer cell lines, significantly reduced the proliferation, and upregulation of pro-apoptotic proteins Bak and Box (Kiss et al., 2021). Additionally, p-

p53 (Thr55) was downregulated by treatment. It was concluded that methyl donors may promote apoptosis by protecting p53 function via the downregulation of MAPK/ERK and AKT pathways in these cell lines (Kiss et al., 2021).

This relationship between vitamin B12 and methylation may in part explain why some studies have correlated high dosage vitamin B12 supplementation with cancer progression and initiation. Further research is required to ascertain the strength of this relationship.

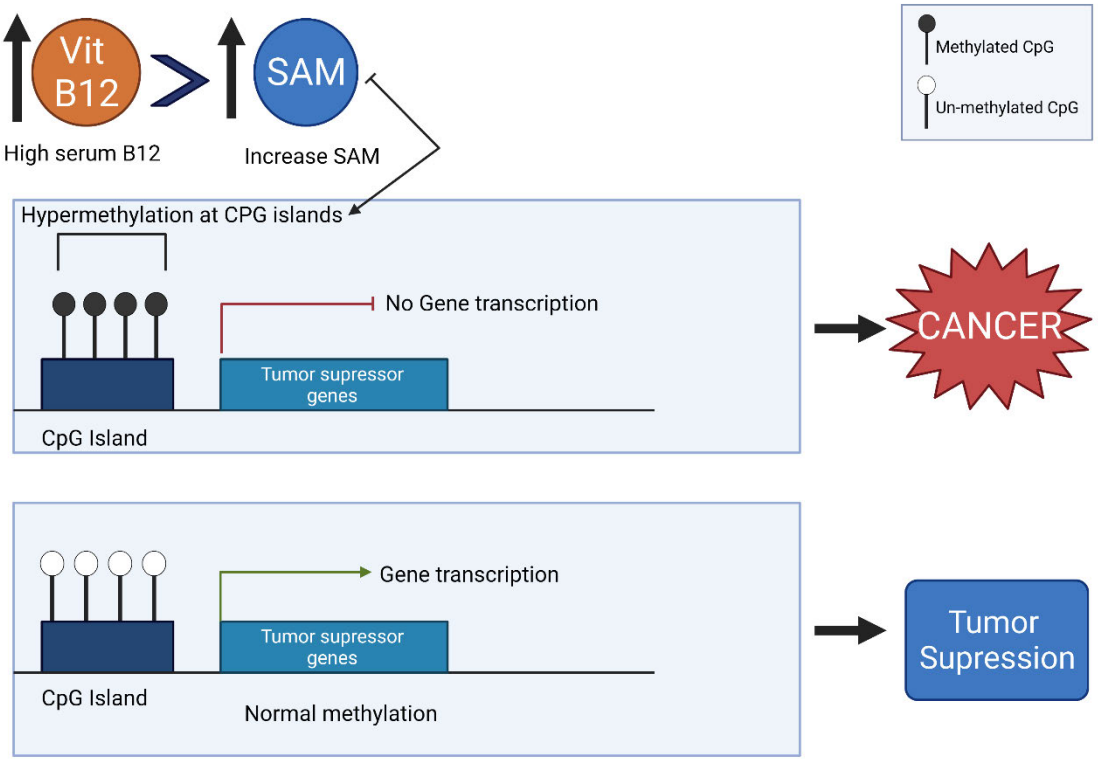


**Figure 10. High dose vitamin B12 may contribute to hypermethylation at CPG islands.**
High dose vitamin B12 can cause hypermethylation to occur at CpG islands resulting in suppression of gene transcription of tumour suppressor genes.

## 1.7. Conclusion

Nutrition, inflammation, and cancer have a two-sided and complex association. Adequate nutrition has been reported to increase the effectiveness of the immune system and prevent cancer development, conversely undernutrition or overnutrition have been linked with inflammation and malignancy. Vitamin B6 and cobalamin (vitamin B12) play a crucial role in the healthy balance of the immune system and inadequate levels of vitamin B6 and B12 can drastically alter the immune response. HyperHcymia, an increase in oxidative stress and changes to immune function can occur due to a deficiency of vitamin B6 and B12, in turn, this causes systemic and vascular inflammation contributing to the pathogenesis of many diseases such as cardiovascular, kidney, and neurovascular diseases, osteoporosis, and cancer. Adequate dietary levels of vitamin B6 and B12 can act as preventative measures for inflammation, immune dysfunction, and disease and cancer progression and dietary supplementation in deficiency states can rectify pathogenesis. High dose vitamin B6 above RDI may be onco-protective via its ability to downregulate inflammation and oxidative stress, decrease inflammation causing AGEs, downregulate H2S and S1P levels and prevent telomere shortening. It also has proven to be an effective immunomodulatory agent in inflammatory pathology and inflammatory carcinogenesis. High dose B12 intake may conversely contribute to tumourigenesis by increasing one-carbon metabolism. This can increase nucleic acid synthesis and cause changes in DNA methylation which contribute to tumorigenesis. Further research is required to clarify the mechanistic effects of vitamin B6 and B12 on cancer and immune cells.

# Chapter Two

# Anti-tumour effects of vitamin B2, B6 and B9

## Abstract

We determined the effects of vitamin B2 (riboflavin) B6, (pyridoxine), and B9 (folic acid) on un-differentiated pro-monocytic lymphoma cells regarding their ability to alter, proliferation, migration, apoptosis, cytokines, and expression levels of programmed death-ligand 1 (PD-L1). We demonstrated that vitamin B2, B6 and B9, on undifferentiated pro-monocytic lymphoma cells exerted an anti-tumourigenic effect. This data could form the basis for future studies in using vitamin B supplementation to reduce cancer cell growth *in vivo*.


## 2.1 Introduction

Chronic inflammation can lead to cancer via epigenetic change. Infection and irritation instigating chronic inflammatory states can produce environments that promote genomic lesions and tumour initiation, activate oncogenes and cause dysfunction of tumour suppressors leading to cancer development (Rakoff-Nahoum, 2006). Genetic change as a result of inflammation effectively provides cancer cells with a mechanistic means of survival. Current scientific thought on inflammation and cancer progression centres on finding a means to dampen inflammation to prevent cancer development and provide a possible means of treatment (Coussens & Werb, 2002).

There is a clear association between inflammation and cancer development demonstrated by numerous clinical and epidemiological studies. Patients who suffer from inflammatory bowel diseases such as ulcerative colitis and Crohn's disease are ten times more likely to develop colorectal cancer (Chang et al., 2015; Itzkowitz & Yio, 2004; Moody et al., 1996). Whilst cancers of the gastrointestinal tract, prostate and liver have been shown to originate from sites of chronic inflammation (Allavena et al., 2008; Block et al., 2003; Haverkamp et al., 2008).

There are also several studies in the literature describing the role of vitamin B in the regulation of immune responses and inflammation. Inadequate levels of certain B vitamins can drastically alter immune response by affecting the production of nucleic acid and protein synthesis, inhibiting the activity of immune cells, and interfering with metabolic processes including methylation, serine, glycine, and purine cycles. Inefficient methylation can lead to HHCY which causes systemic and vascular inflammation contributing to the pathophysiology of other diseases (Cianciolo et al., 2017; Clarke et al., 1998; Henry et al., 2017; L., 2014). Many of the mechanistic effects of vitamin B deficiency correlate with the properties of cancer development.

### *2.1.1 The link between B vitamins, Inflammation and Cancer*

Current knowledge of B vitamins and inflammation predominantly revolves around the systemic effects of vitamin B in deficiency states and the associated pathology of these deficiencies. Much evidence exists establishing the crucial role of B vitamins in immunity and it is well recognised that poor nutrition impacts significantly on immune cells. Globally, chronic malnutrition presents as the main cause of immune deficiency and chronic diseases result from even marginal deficiencies (Schaible & Kaufmann, 2007). Vitamins B2, B6, and B9 are some of the B complex vitamins which are important contributors of nutritional support to the immune system. The deficiency of these can alter immune function considerably by disturbing nucleic acid production and protein synthesis, impeding immune cell activity, interfering with metabolic processes such as methylation and contributing to oxidative stress. HyperHcymia is a result of ineffective methylation which triggers systemic and vascular inflammation and contributes to disease pathogenesis. Immune dysfunction at a cellular level can present as improper antigen presentation, disturbed cytokine production, unmodulated autoimmune responses, disruptions in immune cell function, and ineffective viral clearance (Bayer & Fraker, 2017). Furthermore, vitamin B2 also plays a crucial role

within the immune system because of its association with mucosal-associated invariant T cells (MAIT) cells which play a significant role in autoimmune, inflammatory diseases and cancer (Kumar & Ahmad, 2018).

The roles of B2, B6 and B9 in DNA and protein synthesis, cell maintenance and proliferation are vital factors when looking at how deficiency states can affect the immune system. Both cell-mediated and humoral immunity is compromised in B vitamin deficiency states and the effects on immune cells are varied and numerous. Supplementation of B vitamins has been shown to improve immune response in both animal and human models in many studies (Adhikari et al., 2016; Au-Yeung et al., 2006; Fukuda et al., 2015; Gross et al., 1975; Huang et al., 2015; Lewicki et al., 2014; Scalabrino et al., 2002), however, some studies have also reported that over-supplementation can also adversely affect immune function (Adhikari et al., 2016; Henry et al., 2017; Meadows et al., 2015).

### *2.1.2 B vitamin deficiency, methylation, and inflammation*

There is a link between vitamin B2, B6 and B9 deficiencies and ineffective methylation resulting in increased Hcy levels and cancer. Numerous studies within the literature verify links between vitamin B deficiency and cancer through inflammatory processes. Vitamin B9 levels were found to be significantly lower and mean Hcy levels significantly higher in newly diagnosed cancer patients than in aged match controls in one study, indicating that high Hcy and low folate could be associated with lung cancer, although additional studies would be required to support these findings (Tastekin et al., 2015). Another study involving breast cancer hypothesised that elevated Hcy levels may influence the methylation of some specific genes that control breast cancer initiation and progression. Breast cancer cell lines MCF-7 and MDA-MB-231 showed epigenetic modulations of RASS-F1 and BRACA1, as a result of elevated Hcy levels. Elevated plasma Hcy levels are also correlated with increased risk of colorectal cancer (Miller et al., 2013) whilst elevated levels of enzyme, PDXK, which

facilitates the conversion of pyridoxine (a vitamin B6 precursor) into pyridoxal-5'-phosphate the bioactive form of vitamin B6, indicated a good prognostic marker in patients suffering from non-small cell lung carcinoma (Galluzzi et al., 2013).

In contrast, however, some studies have associated an over-supplementation of some B vitamins with cancer progression. More recently it was noted that long term supplementation of vitamin B12 in a total of 77,118 patients was associated with a 40% increased risk of lung cancer in males but not females in comparison to those who were not supplemented with B12. The study showed that B12 supplementation may be harmful rather than chemo-preventative for lung cancer (Brasky et al., 2017). Another study noted that high levels of B12 are associated with a threefold risk of prostate cancer (Hultdin et al., 2005). However, a pooled data-based meta-analysis of vitamin B supplementation effects on the incidence of cancer showed that vitamin B supplementation did not affect cancer incidence (S. L. Zhang et al., 2016). Conflicting evidence on B supplementation and cancer leads us to a justification of this project's proposition that B vitamin status and dose-response is an important factor to explore.

### *2.1.3 B vitamin deficiency increases oxidative stress and inflammation*

B vitamin deficiencies can increase Hcy levels which in turn can lead to oxidative stress because of auto-oxidation of thiol groups in extra-cellular fluid and plasma. This auto-oxidation mechanism causes the generation of hydrogen peroxide and reactive radical oxygen species, superoxide and hydroxyl radical (Domagala et al., 1998). Several studies have shown this to occur in cultured smooth muscle cells (Heinecke et al., 1987) and aortic endothelial cells (Upchurch et al., 1997). This increased oxidative stress alters the availability and action of nitric oxide and an influx of leucocyte recruiting, and adhesion molecules which can result in endothelial dysfunction (De la Fuente et al., 2005). Within the cell cytoplasm, oxidative stress is more likely to occur from calcium influx as a result of

excitotoxic activation of NMDA receptors by Hcy (McCully, 2009). Calcium hydroxylapatite, an insoluble salt, is formed which inhibits oxidative phosphorylation, thus depleting phosphate levels and causing failure of ATP synthesis and the accumulation of oxygen radicals and oxidative stress. It is the task of antioxidants to reduce and control free radicals however, a deficiency of available antioxidants leaves cells and tissues unprotected and open to aberrant destruction. Oxidative stress has implications for the normal ageing process, but it can also feature in the pathogenesis of chronic disease states.

Immune dysfunction arising from oxidative stress can cause impairment in the production and function of key immune cells and an imbalance in inflammatory mediators leading to cancer progression (Khanna RD, 2014). Some B vitamins such as vitamin B6 have been indicated as antioxidant nutrients and as such, exert a protective role against inflammation and cancer progression.

The relationship between inflammation, carcinogenesis and vitamin B status is not completely understood and many of the mechanistic and molecular processes involved in this association are yet unresolved. The principal goal of this study was to determine the effect of supplementing undifferentiated pro-monocytic lymphoma cells with vitamin B2, B6 or B9 and we note altered effects on cell proliferation, migration, apoptosis, cytokines, and inflammatory marker expression, as well as the expression of PD-L1.

## 2.2 Materials and Methods

### *2.2.1 Cell culture*

The U937 cell line was isolated from a 37-year-old male patient with histiocytic lymphoma. U937 cells are commonly used to study the behaviour and differentiation of monocytes. They can be differentiated into monocyte/macrophages following stimulation with certain stimulants such as vitamin D3 (Chanput, 2015). The U937 cell line was purchased from

ATCC (https://www.atcc.org) /by the Monash University Department of Immunology. U937 cells are immortal and allow for multiple passages without transformation, however, we ensured to keep the number of passages to a minimum. U937 cells were cultured in RPMI 1640 media supplemented with 2 nm. L-glutamine (Sigma-Aldrich, St Louis, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 10 % heat-inactivated foetal bovine serum (FBS; Sigma-Aldrich) at 37$^{o}$C and 5 % $CO_2$. Culture media was changed every 3-4 days and cells were passaged accordingly. Once 80-90 % confluent, cells were used in experiments.

### *2.2.2 Preparation of vitamin B stocks*

A stock solution of each Vitamin B was freshly prepared according to the manufacturer's instructions (Sigma, VIC Australia) in either phosphate-buffered saline (PBS) (Vitamin B6) or Sodium Hydroxide (NaOH – Vitamin B2, B9). The corresponding concentration of vehicle control NaOH was included. Each vitamin stock was filtered using 0.2 -micron filters and further dilutions were made to 2 mg/ml in RPMI-1640 media, to allow for serial dilution of the working concentrations.

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### *2.2.3 Proliferation of pro-monocytic cells using MTT*

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,) a tetrazole forms purple formazan in the mitochondria of living cells in the reduction reaction, which takes place only when mitochondrial enzymes are active, and therefore conversion can be directly related to the number of viable cells (Riss et al., 2004). The absorbance of the purple solution can be quantified by measuring a wavelength by a spectrophotometer. The amount of purple formazan produced by cells treated with vitamin B, (vitamin B2, B6, B9) with the amount of formazan produced by untreated cells or control solvent, to determine the amount

of cell death. A higher number of viable cells results in a greater amount of MTT formazan formation which triggers an increase in absorbance. U bottom 96-well plates (1 plate for each day of incubation) were seeded with 100 μL of titrated concentrations of vitamin B (1000 μg/ml, 500 μg/ml, 250 μg/ml, 125 μg/ml, 62.5 μg/ml, 31.25 μg/ml, 15.6 μg/ml and untreated control 0 μg/ml) and 100 μL of cell suspension (at density of $1x10^4$ cells/well) in triplicate for vitamins B6 and B9 and 1 μg/ml, 0.5 μg/ml, 0.25 μg/ml, 0.125 μg/ml, 0.62 μg/ml, 0.31 μg/ml, 0.15 μg/ml and untreated control 0 μg/ml for vitamin B2). Untreated cells and vehicle control wells corresponding to each dose of NaOH in the vitamin B2 and B9 wells were also used. The plates were incubated at 37 °C and 5 % $CO_2$ for three days. On day three of incubation half the well volume was removed and replaced with fresh media/Vitamin B. MTT assay was performed on days 3, 4, 5 and 6 of incubation to assess cellular proliferation.

Cellular proliferation was assessed using a spectrophotometer (Bio-Rad microplate reader, 6.0) using a wavelength of 570 nm. Three-five independent experiments were conducted in triplicates. Supernatants from each day were transferred to cryovials and stored at -80 °C for later use of cytokine analysis using the Bio-Plex system.

### *2.2.4 Visual assessment of cell proliferation*

On incubation days 3, 4, 5 and 6 plates were viewed under an IX81 Olympus microscope and photos were taken of one triplicate of each concentration at 4x magnification. Photos were then collated for each vitamin, day and concentration using Microsoft PowerPoint.

### *2.2.5 Data analysis using Excel and Prism software*

Statistical analysis of MTT proliferation assays was performed using 2way ANOVA and Tukey's multiple comparison test. The mean of triplicate wells was considered significantly different when compared to vehicle control groups if $p \leq 0.05$ in proliferation assays.

## *2.2.6 Cell migration assays*

Cells were treated with vitamin B2 0.125 µg/ml, B6 125 µg/ml and 250 µg/ml or B9 125 µg/ml, (corresponding with IC-50 amounts for 3 days after which fresh media and vitamin B were added and allowed to incubate a further 3 days. Controls included untreated and NaOH at corresponding doses to the B vitamins dissolved in NaOH. Cell migration was performed using the Boyden chamber assay 8-µm pore size membrane filter inserts in 24-well tissue culture plates. The cells were trypsinised and re-suspended in serum-free RPMI-1640 media at the density of $2\times10^5$ cells/ml. A total of 200 µl of cell suspension was seeded in the upper chamber of the trans wells, and 600 µl of media into the lower chamber. The chambers were incubated at 37 °C in a humid atmosphere with 5% $CO_2$. After 20-22 hrs, the non-migrating cells on the upper surface of the insert were removed and the cells that migrated to the underside of the membrane were counted using a light microscope. Excel and Prism excel (Graph Pad Software, La Jolla, CA, USA) was used to aid in the statistical analysis using the student's T-test and $p\leq0.05$ was considered significant.

## *2.2.7 Apoptosis assay*

### *2.2.7.1 Annexin V FITC/PI*

Changes in the plasma membrane and loss of membrane asymmetry are amongst the earliest indications of apoptosis. The translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer membrane exposes PS to the external cellular environment. Annexin V, having a high affinity for PS binds to PS presenting cells and signals the beginning of membrane degradation seen in the last stages of cell death caused by apoptosis or necrosis (Demchenko, 2013). Using an accompanying dye such as Fluorescein isothiocyanate (FITC) helps to signal cells that are in the early stages of apoptosis. Viable non-apoptotic cells are Annexin V negative and PI negative. Cell dead by

apoptosis are Annexin V positive and PI-positive. Cells in early apoptosis are Annexin V positive and Propidium Iodide negative while cells dead by either apoptosis or necrosis are Annexin V negative and PI-positive.

#### *2.2.7.2 Sample preparation and flow cytometry*

U937 cells (100 µl) at $1x10^6$ cells/ml were co-cultured with 100 µl of vitamin B/RPMI media in 96 well plates for 3 days and 1 ml of U937 cells in 9 ml of vitamin B/RPMI media for 6 days at the following concentrations. Vitamin B2-0.250 µg/ml and *0.95 µg/ml, B6-250 µg/ml and *190 µg/ml, B9-250 µg/ml and *120 µg/ml, NaOH-6.2 mM and 250 µM vehicle controls corresponding to the amount of NaOH within the vitamin B well) or culture media alone. * Amounts with asterisk coincided with IC-50 amounts calculated from the previous MTT assay.

U937 cells were harvested by centrifugation at 1200 rpm for 5 min at room temperature. The supernatant was discarded, and the cells were washed twice in 200 µl of FACS buffer (Phosphate buffered saline (PBS), 1 mM Ethylenediaminetetraacetic acid (EDTA), 2 % Foetal Calf Serum (FCS) and 0.1 % sodium azide) by pipetting. Cells were then re-suspended in 100 µl per well of Annexin V binding buffer with FITC Annexin V (Bio Legend USA) at 1:1000 and 0.5 µg/ml of PI. Wells containing untreated, FITC and PI, PI alone, FITC alone were set up as controls. Samples were transferred to FACS tubes and data was collected using BD FACS canto™ II cell analyser at a medium setting and 30,000 events were collected. Cells were analysed using BD FACS Diva software. Results were displayed as percentages in quadrant boxes corresponding to the data in (Figure 13.)

### *2.2.8 Bio-Plex cytokine assay*

The Bio-Plex human cytokine immunoassay is a highly sensitive and reproducible magnetic bead-based assay that allows accurate measurement of low levels of human cytokines. The Bio-Plex cytokine assay uses 8 μm magnetic beads coated with antibodies against an array of cytokines. Cytokine assays were performed using a bead-based multiplex immunoassay (MIA, 9 Bio-Plex Panel B, Bio-Rad Laboratories Inc. VIC Australia) that included the cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFNγ, TNFα, Granulocyte-macrophage colony-stimulating factor (GM-CSF). Supernatant samples from day 6 of vitamin B culture were run neat (day 6 was chosen as this is the day that showed the best effect on anti-proliferation). In addition, 24 hrs-time points were used to collect supernatants for differentiated U937 cells stimulated with vitamin B and cytokines assessed. Standard low photomultiplier tube (PMT) settings were prepared with "blank" negative controls in duplicate. Ninety-six-well plates were coated with beads, followed by the addition of samples and standards and detection antibodies, then streptavidin-phytoerythrin as per the manufacturer's instructions. The beads were re-suspended, and the fluorescence output was read and calculated on the Bio-Plex array reader (Bio-Rad, VIC Australia). Statistical analysis of data included the mean and standard deviation (SD), as well as a Two-Way ANOVA followed by a Sidak's multiple comparison test using GraphPad Prism (GraphPad Software, USA). Significance was defined as $p \leq 0.05$.

### *2.2.9 PD-L1 expression and confocal microscopy*

PD-L1 is expressed by cancer cells as a way to escape T cell immune responses. Recently, it was shown that there is a relationship between the expression of PD-L1 with proliferation and invasion of cells (Xue et al., 2017), hence, the expression of PD-L1 on U937 cells with or without vitamin B treatment was determined. Confocal microscopy was used to visually determine its expression on U937 cells. U937 cells were treated with vitamin B2

(0.125 μg/ml), B6 (125 μg/ml and 250 μg/ml), B9 (125 μg/ml) for 6 days. Controls included untreated cells and NaOH at corresponding doses to the B vitamins dissolved in NaOH. U937 treated cells (100 μl) at $5x10^5$ cells/ml were added to 96 well round bottom plates and washed 2x 5 minutes with PBS. Cells were fixed with 4% paraformaldehyde for 10 minutes. Cells were permeabilised for 15 minutes in 0.1% Triton X-100/PBS. Non-specific binding was blocked using 10% donkey serum for 1 hour at room temperature. Plates were then washed twice for 4 minutes with 0.1% Triton/PBS. Cells were labelled with primary monoclonal antibody PD-L1 (Abcam, ab210931) at 1:500 and incubated for 2 hrs at room temperature. Cells were washed twice in 0.1% Triton X-100/PBS and incubated at room temperature in the dark for 2 hrs. Secondary antibody Alexa Fluor 647-conjugated donkey anti-mouse at 1:250 dilution (Abacus, JI715605150). Following 2 washes with 0.1% Triton X-100/PBS, cells were incubated for 2 minutes with 4′,6-diamidino-2-phenylindole (DAPI) washed and 2 small drops of cells were placed onto slides and left to dry. Slides were mounted with fluorescence mounting media, a coverslip was applied over the cells and clear nail polish was applied around the perimeter of the coverslip. Slides were labelled and left to dry wrapped in tin foil in the dark overnight.

Images were captured on a Nikon Eclipse Ti multichannel confocal laser scanning system (Nikon, Japan). Z-series images were acquired at a nominal thickness of 0.5 μm (512x512 pixels). The numbers of U937 cells expressing PD-L1 markers were counted within eight randomly captured images (total area size 2 $mm^2$) per preparation at x20 magnification. Image J software (National Institute of Health, Bethesda, MD, USA) was employed to convert images from RGB to greyscale 8 bit and then to binary; particles were then analysed to obtain the percentage area of immunoreactivity. Excel and Prism excel (Graph Pad Software, La Jolla, CA, USA) was used to aid in the statistical analysis and $p \leq 0.05$ was considered significant.

## 2.3 Results

### *2.3.1 Vitamins B2, B6, and B9 inhibit pro-monocytic U937 cell proliferation*

The effects of vitamin B2, B6 and B9 on cellular proliferation of U937 pro-monocytic cells were assessed using an MTT proliferation assay. All experiments were repeated at least 5 times in triplicate wells. Doses of 0.062 μg/ml, 0.031μg/ml and 0.015 μg/ml of vitamin B2 (riboflavin), cell proliferation was similar to that of control cells whereas 0.125 μg/ml inhibition of cell proliferation was noted ($p \leq 0.05$). Significant anti-proliferative effects were noted at doses 0.250 μg/ml - 1.0 μg/ml ($p \leq 0.0001$) (Figure 11). These findings were verified by well photos (Figure 11)

Incubation of vitamin B6 (pyridoxine) with U937 cells showed no anti-proliferative effects on day 3 however on days 4-6 the anti-proliferative effects increased significantly in a dose-dependent manner. On day 6, 1000 μg/ml, 500 μg/ml, and 250 μg/ml showed the most inhibition ($p \leq 0.0001$), followed by less but significant inhibition at 125 μg/ml ($p \leq 0.01$). No anti-proliferative effects of B6 at 15-62 μg/ml were noted. (Figure 11C, 11D)

At high doses of vitamin B9 (folic acid; 250 - 1000 μg/ml), significant inhibition of cell proliferation was noted on days 4 ($p \leq 0.01$) and days 5 and 6 ($p \leq 0.0001$). Although there was a trend of lower proliferation on day 3, this was not significant (Figure 11E). At 125 μg/ml of folic acid concentration, there was less proliferation, but significant anti-proliferative effects were noted on days 3-6 ($p \leq 0.05$). The anti-proliferative effects were specific to folic acid as the corresponding NaOH vehicle control concentrations did not influence cell proliferation (Figure 11E, 11G) These findings were confirmed by well images (Figures 11F, 1H).

### *2.3.2 Vitamin B2, B6, and B9 inhibit cell migration of pro-monocytic cells.*

Cell migration is evaluated via several different techniques such as microfluidic assays, scratch assays and cell-exclusion zone assays. However, the Boyden chamber assay is the most widely accepted cell migration assay (Chen, 2005). U937 pro-monocytic lymphoma cells were added inside the chamber and allowed to migrate through the porous membrane for 20-22 hrs. The number of cells that had migrated through the membrane were stained and counted using a light microscope (Chen, 2005). Vitamin B2 (0.125μg/ml), significantly reduced the number of cells migrating through the membrane ($p \leq 0.5$). Similarly, B6 (125 μg/ml, $p \leq 0.05$) (250 μg/ml, $p \leq 0.05$), and B9 (125 μg/ml, $p \leq 0.05$), showed inhibition of cell migration (Figure 12). These data correspond to the anti-proliferative effects exhibited by vitamin B2, B6 and B9

### *2.3.3 Vitamin B does not induce apoptosis or cell death*

To determine whether the anti-proliferative and anti-migratory effects of vitamin B2, B6 and B9 were due to apoptosis or cell death, the annexin-v assay was used which utilizes flow cytometry assay. Quadrants were set based on untreated control cells with either PI or FITC alone or PI/FITC control staining (Figure 13). Q1 corresponds to early apoptosis (Annexin V $FITC^{+}/PI^{-}$) Q2 corresponds to dead cells by apoptosis (Annexin V $FITC^{+}/PI^{+}$), Q3 corresponds to live cells and non-apoptotic (Annexin V $FITC^{-}/PI^{-}$), Q4 demonstrates dead cells by necrosis or apoptosis (Annexin V $FITC^{-}/PI^{+}$). Control non-vitamin B treated cells were mostly viable 93 %) and showed background levels of dead cells (Figure 13). The addition of vitamin B2, B6 and B9 at 250 μg/ml showed similar live/dead cell distribution as control, hence no evidence of apoptosis or death by necrosis is noted (Figure 13). Likewise, vitamin B2 and its vehicle control NaOH showed a similar % of cell populations

in each quadrant. Data for the 3-day vitamin B treatment is shown; treatment for 6 days showed similar effects (not shown).

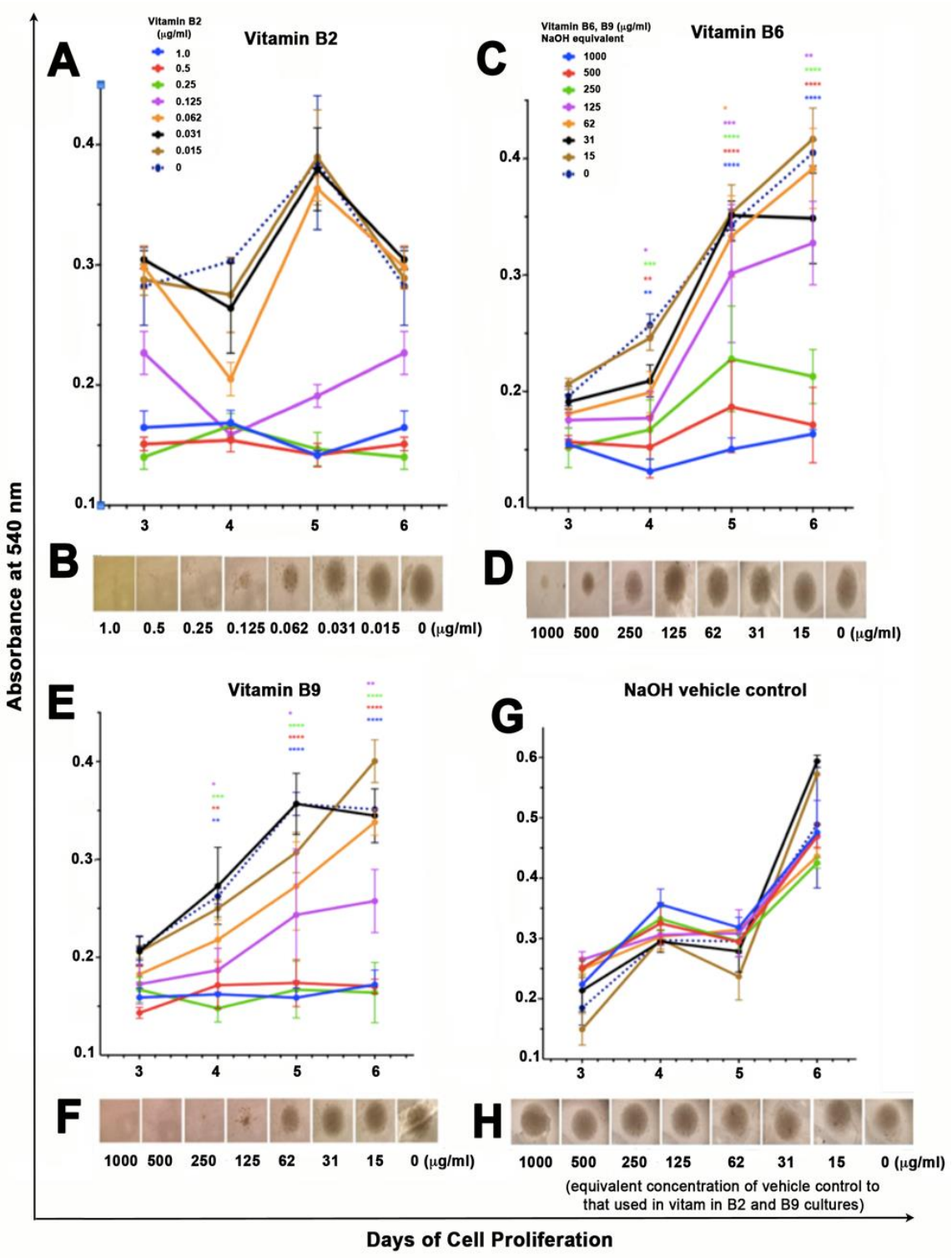



**Figure 11. Proliferation Assays**

(A, B), Effect of vitamin B2 (riboflavin) (C, D), vitamin B6 (pyridoxine) (E, F), vitamin B9 (folic acid) (G, H), NaOH control on U937 cell proliferation. Cells were incubated with increasing doses of vitamin B for 6 days in 96 well U bottom plates and analyzed by MTT assay. Absorbance readings were taken at 540 nm to assess for cellular proliferation compared to control well (0 µg/ml). Significance was established at P, 0.05, two-way Anova followed by Tukey's multiple comparisons test and marked with asterisk (* p≤0.05, ** p≤ 0.01, *** p≤0.001, **** p≤0.0001). Cells were viewed under an IX81 Olympus microscope at 4x magnification and photos taken at each concentration and control NaOH on day 6 of culture

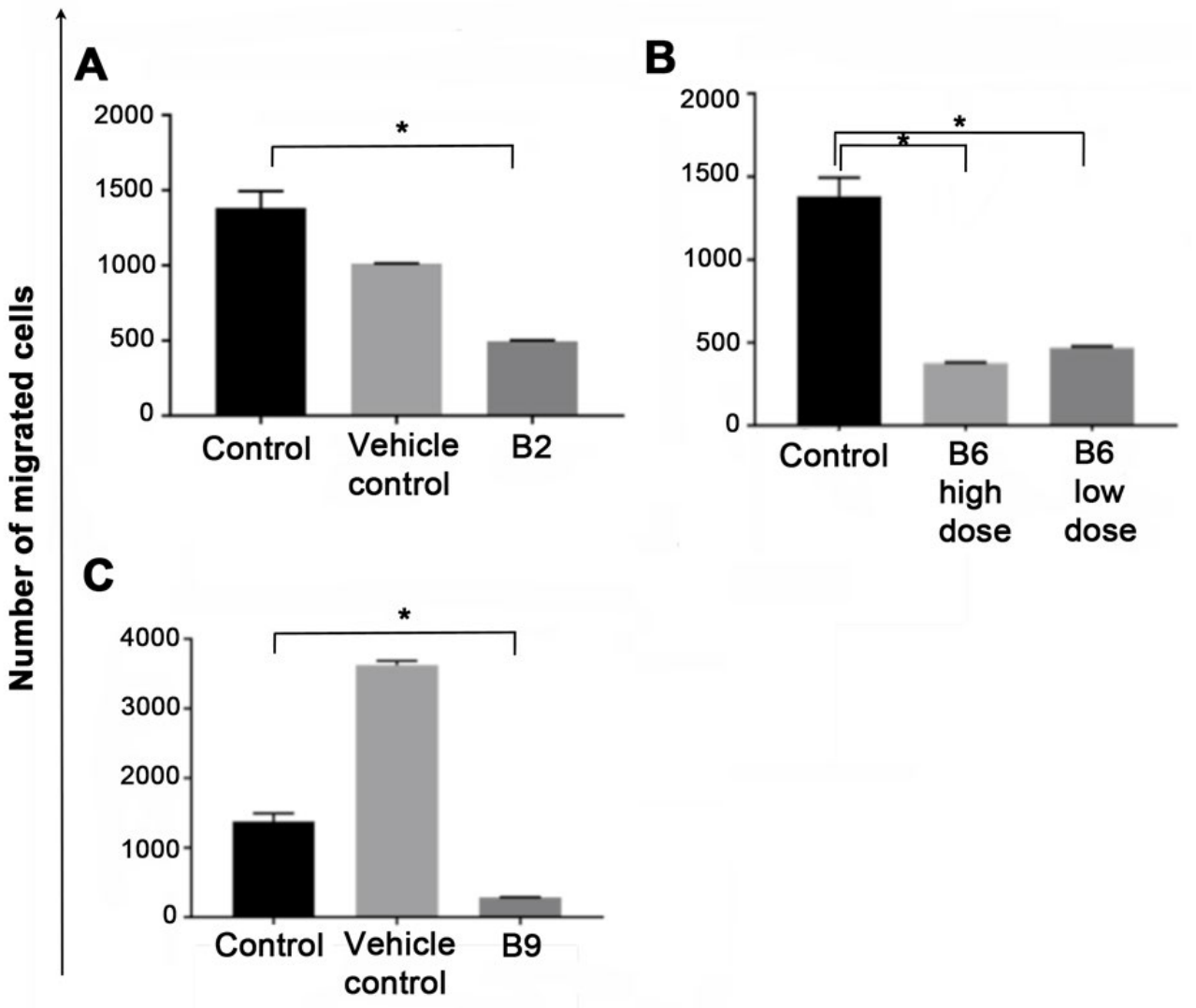



**Figure 12**. **Migration Assays.**

Effect on cell migration of pro-monocytic cells in the presence of (A) vitamin B2 (riboflavin), (B) vitamin B6 (pyridoxine) and (C) vitamin B9 (folic acid) using Boyden chamber assay. Data presented as mean +/- standard error of the mean of duplicate wells. Excel and Prism excel (Graph Pad Software, La Jolla, CA, USA) were used to aid in the statistical analysis using students T-Test and * $p \leq 0.05$ was considered significant.

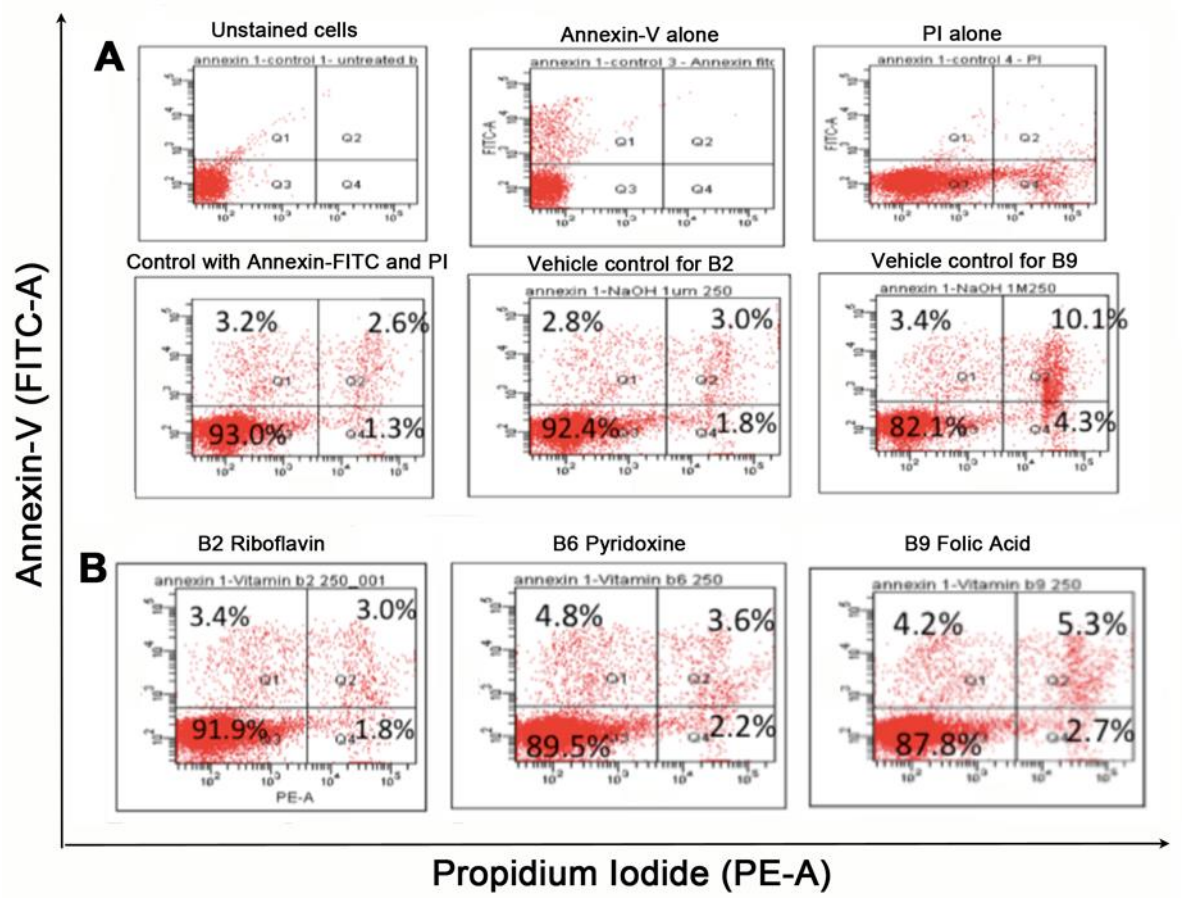



**Figure 13. Apoptosis Assays.**

Annexin V-FITC/PI staining of undifferentiated U937 cells incubated with vitamin B. 1x106 of U937 cells treated with 0.25 µg/ml of B2 and 250 µg/ml of vitamin B6 and B9 for 72 hours were used for analysis. Resuspended cells were incubated with Annexin V-FITC at 1:1000 for 15 minutes in the dark. Proprium Iodide (PI) at 0.5 µg/ml was used as a counterstain to differentiate necrotic/dead cells from apoptotic cells. Shown in the figure are (A) controls, (B) vitamin B samples.

### *2.3.4 Vitamin B2, B6 and B9 induce an anti-tumorigenic cytokine profile*

Magnetic bead Bio-Plex assay was used to evaluate the effect of vitamin B (B2, B6 and B9) on cytokine secretion of promonocytic lymphoma U937 cells. Results are shown for vitamin B2, B6 and B9 as they showed significant anti-proliferative and migratory effects. Vitamin B2 significantly increased the secretion of IL-10, IL-6, and GM-CSF ($p \leq 0.05$) (Table 1, Figure 14.) Vitamin B6 also induced high levels of IL-10 ($p \leq 0.001$) and IL-8 ($p \leq 0.001$), and significant lower levels of IL-1β ($p \leq 0.001$) (Table 1, Figure 14). Vitamin B9 (folic acid) showed higher levels of IL-10 compared to control ($p \leq 0.05$) and significant increased IL-8 secretion ($p \leq 0.01$). No other cytokines measured significance. It is clear that B2, B6 and B9 significantly increase IL-10 and IL-8 cytokine secretion by U937 cells. B6 also decreases IL-1β and B2 increases GM-CSF levels (Table 1, Figure 14).

**Table 1. Summary of cytokine secretion by promonocytic U937 cell line.**

| Vitamin | Cytokine | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | IL-1β | IL-2 | IL4 | IL-6 | IL-8 | IL-10 | IFNγ | TNFα | GM-CSF |
| **B2** | - | - | - | - | increase | increase | - | - | increase |
| **B6** | decrease | - | - | - | increase | increase | - | - | - |
| **B9** | - | - | - | - | increase | increase | - | - | - |

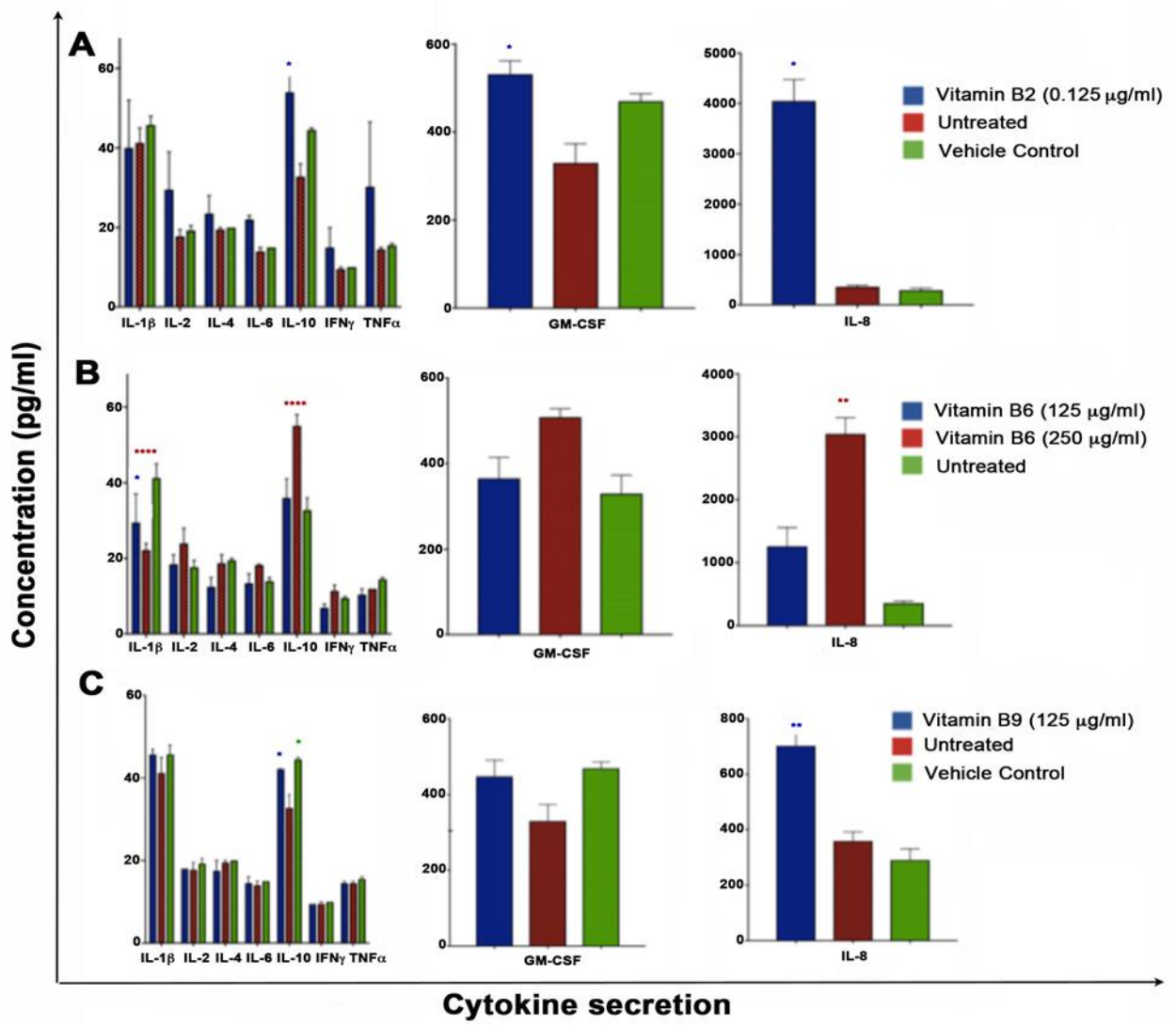



**Figure 14. Cytokine Assays.**

U937 cells were treated with (A) 0.125 μg/ml of vitamin B2 and NaOH vehicle control, (B) 125 μg/ml and 250 μg/ml of vitamin B6 and (C) 125 μg/ml of vitamin B9 and NaOH vehicle control for 3 days. Media and fresh vitamin B were replaced and cultured for a further 3 days. Supernatants were collected and cytokine secretion was analysed by Bio-Plex. Significance to untreated control is indicated by Asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Bio Plex data for IL-1β, IL-2, Il-4, IL-6, Il-8, Il-10, IFNγ, TNFα and GM-CSF are shown.

### *2.3.5 Vitamin B2, B6 and B9 decrease the expression of PD-L1*

PD-L is an important checkpoint marker expressed by monocytes, macrophages, epithelial cells and cancer cells (Kuol et al., 2017). The expression of PD-L1 on cancer cells has an immune suppression mechanism where cancer cells escape lysis by activated CD8+ T cells. PD-L1 is commonly overexpressed on tumour cells and aids in their invasiveness (Xue et al., 2017). Confocal microscopy was used to assess the effect of B vitamins on PD-L1 expression. U937 cells cultured with vitamin B2, B6 or B9 showed reduced expression of PD-L1 compared to control ($p \leq 0.05$) and to NaOH vehicle control ($p \leq 0.0001$) (Figure 15). The number of tumour cells expressing PD-L1 were counted and fluorescence in arbitrary units was measured. Excel and Prism excel (Graph Pad Software, La Jolla, CA, USA) was used to aid in the statistical analysis and $p \leq 0.05$ was considered significant.

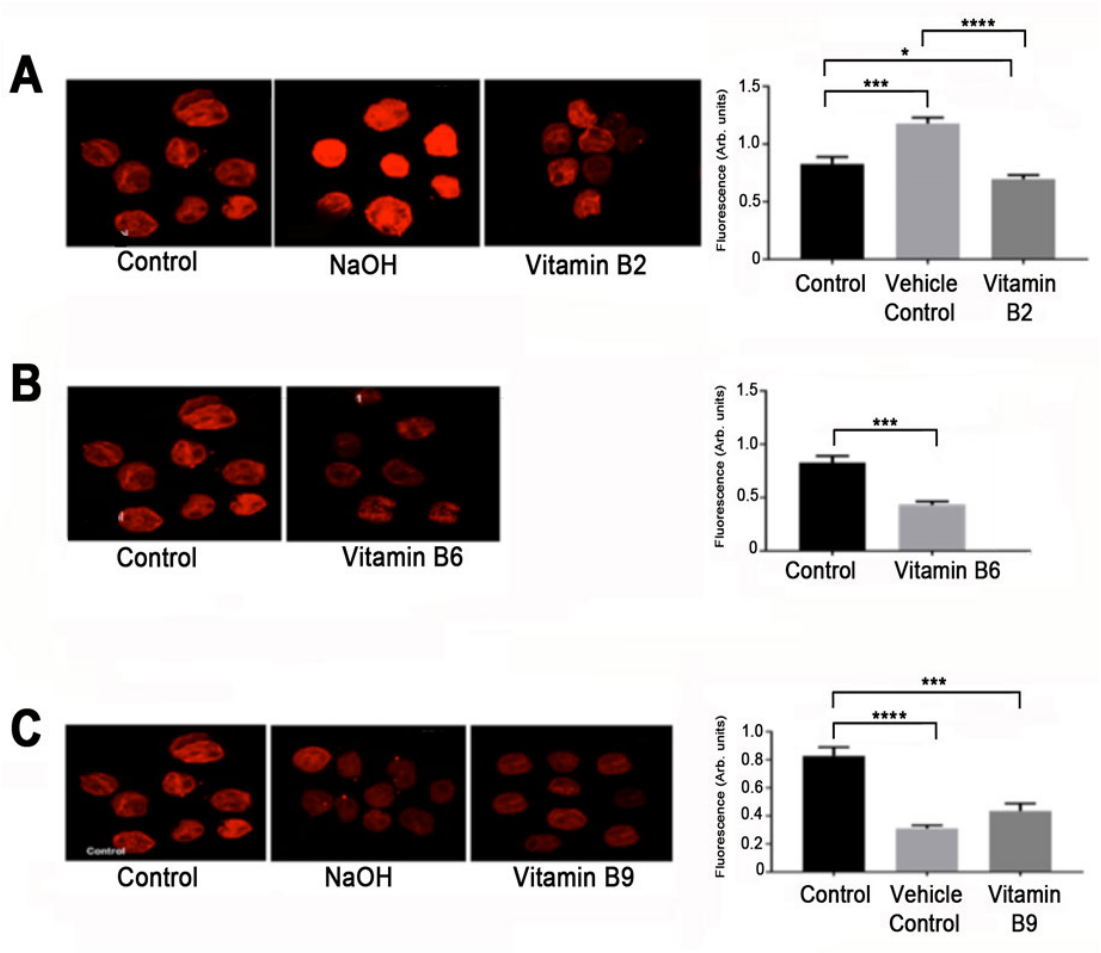



**Figure 15. PD-L1 Assays.**

Expression of PD-L1 as assessed by confocal imaging on U937 cells in the presence of (A) vitamin B2 at 0.125 µg/ml and vitamin B2 vehicle control (NaOH) and untreated control. (B) Vitamin B6 at 125 µg/ml and untreated control, and (C) vitamin B9 at 125 µg/ml and untreated control.

## 2.4 Discussion

Monocytes are circulatory blood cells, which differentiate into macrophages or dendritic cells at tissue sites. Macrophages and dendritic cells are specialised antigen-presenting cells and process and present antigens to T cells and secrete cytokines (Chanput, 2015). Histiocytic lymphoma is an aggressive non-Hodgkin's lymphoma, a type of cancer that originates from cells of the immune system, specifically of monocyte pro-monocytic blast origin. The effects of vitamin B2, B6 and B9 on the proliferation and migration capacity of U937 cells were determined and some insights into the mechanisms involved in altered proliferation were further determined.

Vitamin B2 B6 and B9) exert anti-proliferative and anti-migratory properties to U937 cells in a dose-dependent manner. The cells grown in the presence of either vitamin B2, B6 and B9 are healthy and viable, with only fewer cells growing under normal culture conditions. In fact, in apoptosis assays using annexin-V / PI staining, none of the B vitamins (B2, B6 and B9) induced apoptosis or cell death, suggesting that these B vitamins exert anti-proliferative/migratory properties. Further research is required to understand the mechanism behind these properties. Vitamin B2 is associated with energy production, antioxidant protection and Hcy metabolism. Vitamin B2 deficiency is associated with growth retardation, anaemia, neurodegeneration, and even certain cancers (Thakur et al., 2016). Riboflavin deficiency in HepG2 cells was recently shown to promote cell proliferation and reduce cell viability (Liu et al., 2017). In addition, vitamin B2 deficiency exacerbates iron deficiency and increases gastrointestinal cell crypt proliferation (Werner et al., 2005). Conversely, the addition of riboflavin to lung cancer cell lines leads to increased cell proliferation suggesting that riboflavin may promote the progression of lung cancer (Chen et al., 2013). A vitamin B6 analogue, B6PR, at low doses cultured with HUT78 cancer cell line has been shown to suppress cell proliferation and at high doses induce selective cell

death (Kesel et al., 1999). Likewise, pyridoxal phosphate (a bioactive form of pyridoxine) inhibits rat pituitary adenoma cell lines even though the anti-proliferative effects were due to apoptosis of cells (Frasure-Smith & Lesperance, 2006). In mice bearing colorectal cancer, supplementation of vitamin B6 reduces the number of tumours, and cell proliferation and induces the expression of *c-myc* and *c-fos* proteins (Komatsu et al., 2001). Similar to our findings, pyridoxine did not induce apoptosis of colon cancer cells, thus pyridoxine suppresses colon tumourigenesis by reducing cell proliferation (Komatsu et al., 2001). Conversely, vitamin B6 supplementation in mice did not inhibit cell proliferation of glial cells but rather promoted cell proliferation (Yoo et al., 2011). Furthermore, the culture of colon cancer cell line, COLO-205, with folic acid (vitamin B9) inhibited cell proliferation via G0/G1 cell cycle arrest and through activation of c-SRC mediated pathway and increased levels of cyclin-dependent kinase inhibitor 1A, 1B and tumour protein p53 (Kuo et al., 2015). In addition, vitamin B9 supplementation to HCT116 and Caco-2 colon cancer cell lines reduced TGFβ secretion, induced cancer cell proliferation, and reduced tyrosine kinase activity and epidermal growth factor receptor expression (Jaszewski et al., 1999). The anti-proliferative and anti-migratory properties noted herein with vitamin B2, B6 and B9 are in accord with previously published studies.

The potential mechanisms underlying the anti-proliferative and anti-migratory effects of vitamin B2, B6 and B9 may include, angiogenesis, altered cytokine secretion, altered PD-L1 expression, oxidative stress, and nitric oxide synthesis. Vitamin B2, B6 and B9 all increased the secretion of IL-8 and IL-10 by U937 cells compared to controls, with B2 additionally increasing GM-CSF and B6 decreasing IL-1β. IL-8 an immune chemotaxis cytokine/chemokine correlates with angiogenesis, tumorigenicity, cell proliferation, invasiveness, metastasis, and its expression on cancer cells is linked to poor prognosis (Seaton et al., 2008; Waugh & Wilson, 2008). Indeed, it's been shown that IL-8 increases

the proliferation of pancreatic and breast cancer cell lines (Chen et al., 2014; Singh et al., 2013). Based on this information, it is not clear why IL-8 is upregulated by U937 cells in the presence of vitamins B2, B6, and B9. However, given that IL-8 is highly secreted by activated macrophages, it may be likely that the undifferentiated U937 cells are behaving more like macrophage cells rather than pro-monocytic blast cell lymphoma and hence indicating an activation state of macrophages. Furthermore, IL-10 was also upregulated in the presence of vitamins B2, B6 and B9. IL-10 is a potent anti-inflammatory (deactivates macrophages and monocytes) and immunosuppressive (inhibits antigen-presenting cells and T cells) cytokine. On the other hand, IL-10 also exerts immuno-stimulatory effects, in particular on B cells, NK cells and CD8+ T cells (Goldman et al., 1997). Administration of recombinant (r), rIL-10 into cancer cells *in vivo* results in tumour rejection (Goldman et al., 1997), mainly due to its ability to stimulate CD8+ T cells. Indeed, PEGylated rIL-10 (PEG-rIL-10; AM0010) increases tumour infiltrating CD8+ T cells by 4-fold *in vivo*, and results in tumour rejection and long-term protection (Chanput, 2015). As such, rIL-10 has recently surfaced as a treatment for intra-tumoural injection in cancer patients. In a dose-escalation phase, 1/1b trial of AM0010 in 14 different cancer types rIL-10 activated immune cells (CD8+ T cells), increased pro-inflammatory cytokines, decreased TGFβ, and induced partial and complete clinical responses (Naing et al., 2016). At the 12$^{th}$ European International Kidney Cancer Symposium in Germany (April 21-22, 2017) and ARMO Biosciences' press release, it was announced that AM0010 in combination with checkpoint inhibitors induces better objective response rates and disease control of renal cell carcinoma patients in a phase 2 trial (Meloa, 2017). A phase 3 clinical trial (ClinicalTrials.gov identifier: NCT02923921) in combination with FOLFOX chemotherapy is being conducted in patients with metastatic pancreatic carcinoma. Given the anti-tumour properties of rIL-10, the increased secretion of IL-10 by U937 cells in the presence of vitamin B2, B6 and B9 is likely to be one of the

contributing factors to the anti-proliferative / anti-migratory effects noted herein. Moreover, vitamin B2 in addition increased the secretion of GM-CSF. GM-CSF is involved in the proliferation, differentiation, and function of myeloid-derived cells. Although GM-CSF has growth-promoting properties, it was noted over 30 years ago, that GM-CSF inhibited the proliferation of U937 cells due to the activation of TNFα (Cannistra et al., 1987). Consequently, rGM-CSF has been shown to increase the number of type-1 dendritic cells and is used in patients with cancer either on its own as an immune stimulant or as a vaccine adjuvant leading to anti-tumour responses (Arellano & Lonial, 2008; Yan et al., 2017). Hence, the activation of GM-CSF by U937 cells in the presence of vitamin B2 is likely to contribute to the anti-proliferative effects noted. Finally, vitamin B6 reduced expression levels of IL-1β by U937 cells. IL-1β is known to promote tumour proliferation, angiogenesis, and metastasis, to several cancers (Lewis et al., 2006). In fact, in IL-1 knockout mice, the growth of murine melanoma cell line was significantly reduced with no lung metastasis compared to wild-type mice (Voronov et al., 2003). As such, anti-IL-1β monoclonal antibodies (i.e., canakinumab) and IL-1β blockers (i.e., rilonacept) have been developed and show reduced cell proliferation, angiogenesis, and metastasis of cancer cells in mice and humans (de Mooij et al., 2017; Dinarello, 2010). Given the tumour promoting properties of IL-1β, the consequent reduced expression levels IL-1β by U937 cells in the presence of vitamin B6, is suggestive that this decrease may be a contributing factor to the anti-proliferative effects noted by vitamin B6.

To further gain insights into the mechanisms involved in the anti-proliferative and anti-migratory effects of vitamin B2, B6 and B9, the expression levels of vascular endothelial growth factor-A (VEGF-A) and PD-L1 were determined. VEGF-A is a signal protein produced by cells to promote the formation of new blood vessels. Cancer cells express VEGF to grow (proliferate) and metastasize (Mahecha & Wang, 2017). However, there was

no significant alteration in intracellular expression levels of VEGF-A by U937 cells in the presence of vitamin B2, B6 and B9 (data not shown). PD-L1 however, was significantly downregulated in the presence of vitamin B2, B6 and B9. PD-L1 is a protein expressed on the surface of immune and non-immune cells. It serves as an immune checkpoint marker where it binds to its receptor PD-1 expressed on activated T cells leading to immune suppression (Kuol et al., 2017). Its increased expression on tumour cells leads to tumour progression (proliferation) and metastasis, and its expression is dependent on TNFα, IL-1β, and IFNγ via toll-like receptors (TLRs) (Brogden et al., 2016). Ovarian (ID8) and melanoma (B16) cell lines were transformed to express high levels of PD-L1 or PD-L1 was silenced; PD-L1 promoted cell proliferation *in vitro* and *in vivo*, whereas the silenced PD-L1 tumour cells resulted in slower proliferation rates *in vitro* and tumour load in mice (Clark et al., 2016). Thus, the decreased expression levels of PD-L1 in the presence of vitamin B2, B6 and B9 correspond to the reduced proliferation of U937 cells noted.

## 2.5 Conclusion

Overall, these results indicate that vitamin B2, B6 and B9 inhibit pro-monocytic cell proliferation and migration which is not due to apoptosis or cell death. It is not clear what role IL-8 plays in the anti-proliferative and anti-migratory effects, as it is generally believed that IL-8 is associated with increased proliferation, migration, and angiogenesis of cancer cells; the mechanism associated with increased levels of IL-8 needs to be further elucidated. However, collectively, vitamin B2, B6 and B9 increased IL-10, GM-CSF, and decreased IL-1β cytokine levels which are in accord with reduced cell proliferation and anti-tumourigenic properties based on other studies in the literature. In addition, the decreased expression levels of PD-L1 support the reduced proliferation and migration of U937 cells. The data gives insights into the anti-tumourigenic properties of vitamin B2, B6 and B9 and could

form the basis for future studies in using vitamin B supplementation to reduce cancer cell growth *in vivo* in animal models and humans. Results of this study suggest that B2 B6 and B9 could be recognised as potential anti-tumorigenic nutrients, although subsequent studies would need to be completed to gain more insight into the mechanisms behind these anti-tumour effects. Future studies may further illuminate some of these mechanisms by observing changes in gene expression of key immune cells.

# Chapter 3

# Dose-dependent effects of vitamin B12 on pro-monocytic lymphoma cells

## Abstract:

Vitamin B12 can be preventative for cancer due to its role in maintaining healthy function of the immune system and moderation of Hcy levels, yet the role of vitamin B12 in tumorigenesis is unclear. Many studies are indicating that increased serum B12 is associated and prognostic with many different types of cancers. It is not clear whether high serum vitamin B12 levels cause cancer or cancer causes high serum B12 levels? Is there a dosage spectrum effect of vitamin B12 intake, whereby higher doses are harmful, but lower doses are necessary to prevent illness? Herein, we determined the effects of vitamin B12 (cobalamin) supplementation (high and low dose) on pro-monocytic lymphoma cells, to ascertain whether dosage could exert changes to cellular processes. The results of this study showed that B12 (low dose) inhibits pro-monocytic cell proliferation and migration which is not due to apoptosis or cell death. Conversely, vitamin B12 (high dose) promoted U937 cell proliferation and increased PD-L1 expression suggesting a pro-tumorigenic mechanism.


## 3.1 Introduction

Vitamin B12 supplementation is available to the public from chemists, health food shops and supermarket shelves. Self-prescribed vitamin B12 intake is popular amongst people wanting to build muscle or suffering from fatigue, and information is freely available on the internet and social media claiming the benefits of B12 supplementation for fatigue and muscle building. Some members of the community are more at risk to suffer from vitamin B12 deficiencies' including those following vegan or vegetarian diets, the elderly or people with digestive and absorption impairment problems and are justified in B12 supplementation to avoid deficiencies. Adequate vitamin B12 intake is vitally important for wellbeing, and deficiency can cause significant impairment of health and lead to many conditions including

anaemia (Wolffenbuttel et al., 2019), neuropsychiatric disorders (Balk et al., 2007; Dayon et al., 2017; Jerneren et al., 2015; Sengul et al., 2014), and inflammation due to elevated Hcy levels, which in turn can lead to chronic health conditions (Mikkelsen et al., 2019; Mikkelsen, Stojanovska, & Apostolopoulos, 2016; Mikkelsen, Stojanovska, Prakash, et al., 2017; Mikkelsen, Stojanovska, Tangalakis, et al., 2016; Nemazannikova et al., 2018).

The actions of B12 assist brain and nervous system function along with cell synthesis and catabolism of fatty and amino acids. B12 and related compounds (cyanocobalamin, methyl-cobalamin, cob(I)alamin, and 5”-deoxyadenosylcobalamin) are united by a cobalt centred corrin nucleus. Highly reactive C-Co bonds allow B12 to participate in isomerase and methyltransferase reactions, for energy extraction from proteins and fats. They also participate in methylation reactions. Vitamin B12 deficiency has been linked to immune system impairment and inflammation with subsequent progression to tumourigenesis. It is well known within the scientific community, that chronic inflammation is a key trigger for carcinogenesis. Cancer is characterised by an overgrowth of cells, which have become insensitive to anti-proliferative and apoptotic signals. It is thought that this insensitivity is due to genomic change and instability within cells, as a result of inflammation and oxidative stress. Many endogenous and exogenous factors can trigger these responses including vitamin B12 deficiency, but it is still unclear whether vitamin B12 consumption above the recommended dietary guidelines, can also contribute to a pro-cancer environment. Whilst vitamin B12 deficiency is problematic, and often routinely tested in prognostic evaluations, there is evidence that elevated levels of Vitamin B12, are associated with short term increased cancer risk (Arendt et al., 2016; Arendt & Nexo, 2012), an increased risk of lung cancer (Brasky et al., 2017; Fanidi et al., 2019), haematological malignancies (Andres et al., 2013) and other solid cancers (Arendt et al., 2016; Bailey & van Wijngaarden, 2015; Collin et al., 2010; Urbanski et al., 2020). The question remains as to why increased B12 levels are

associated with cancer risk (Arendt & Nexo, 2012), does elevated B12 cause cancer which brings into question over-supplementation or does cancer cause elevated B12 and if so is elevated B12 prognostic for cancer (Arendt et al., 2016; Miranti et al., 2017; Oh et al., 2018).

Numerous theories have been proposed as to why elevated B12 is associated with cancer. Some authors have suggested that in fact, inflammation caused by the anti-tumour response may contribute to elevated B12 levels (Andres et al., 2013; Arendt et al., 2016) or that cancer itself may affect B12 metabolism causing a reduced uptake of B12 into the cells and therefore indicating high serum B12 (Arendt et al., 2016). There is some evidence at this point to say that B12 over-supplementation may be directly linked to cancer progression. A study on postmenopausal women found that a daily vitamin B12 uptake of 6ug, which is higher than the current RDA of 2.4ug, was sufficient to ensure a steady serum level of cobalamin for those whose metabolic uptake of B12 was not impaired by abnormal gastric production (Bor et al., 2006). It is possible though that dosage considerably higher than this could have negative effects on carcinogenesis and that B12 intake has a dose-dependent effect on gene expression in relation to tumorigenesis.

Ordinarily, intake of B vitamins that meet RDI is deemed beneficial for cancer prevention as deficiency can influence gene expression and cause aberrant methylation. For example, one study reported that micronutrients involved in one-carbon metabolism like B12 enhance tumour suppression in head and neck cancers by enhancing DNA methylation (when a gene is methylated, the expression of that gene is turned off). However, another recent study on lung cancer, claimed that male smokers who consumed high individual B12 doses of vitamin supplements of more than 55 mg/day over a 10-year period, were 4 times more likely to develop lung cancer than male smokers who did not supplement with B12 (Brasky et al., 2017). This discrepancy in research findings on vitamin B12 regarding tumourigenesis suggests that the question of dosage of vitamin B12 needs to be further explored.

Considering these findings, the dose-dependent effect of B12 on lymphoma cells was conducted and the effects on cell proliferation, migration, apoptosis, cytokine secretion and expression of PD-L1 were determined. These results suggest that the effect of B12 on these cells was dose-dependent with high vitamin B12 supplementation inducing a pro tumourigenic effect whilst at lower B12 dosage there were anti-proliferative effects.

## 3.2 Materials and Methods

### *3.2.1 Cell culture and reagents*

The U937 cell line was purchased from ATCC (https://www.atcc.org) by the Monash University Department of Immunology. We ensured the number of passages was kept to a minimum, where multiple stocks were frozen and freshly thawed cells were used for each experiment. U937 cells were cultured in RPMI 1640 media supplemented with 2 mM L-glutamine (Sigma-Aldrich, St Louis, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 10 % heat-inactivated foetal bovine serum (FBS; Sigma-Aldrich) at 37 ºC and 5 % CO2. Culture media was changed every 3-4 days and cells were passaged accordingly. Once 80-90 % confluent, cells were used in experiments.

A stock solution of vitamin B12 was freshly prepared according to the manufacturer's instructions (Sigma, VIC Australia) in phosphate-buffered saline (PBS). Vitamin B12 stock was filtered using 0.2-micron filters and further dilutions were made to 2 mg/ml in RPMI-1640 media, to allow for serial dilution of the working concentrations.

### *3.2.2 Cell proliferation*

The rapid colourimetric assay for cellular growth and survival, Thiazolyl Blue Tetrazolium Bromide (MTT) assay was used for cell proliferation. The MTT assay is a robust and reliable

method that acquires the signal of mitochondrial performances, and thus measures cellular viability. Other methods to measure cell proliferation, such as $^{3}$H-thymidine or BrdU test, assess DNA damage in response to treatment (i.e., DNA damage vs mitochondrial enzymatic performance). In these studies, however, it was decided to use the MTT assay as a readout for cell proliferation. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) a tetrazole, forms purple formazan in the mitochondria of living cells in the reduction reaction, which takes place only when mitochondrial enzymes are active, and therefore conversion can be directly related to the number of viable cells (Riss et al., 2004). The absorbance of the purple solution can be quantified by measuring a wavelength by a spectrophotometer. The amount of purple formazan produced by cells treated with vitamin B12 with the amount of formazan produced by untreated cells or control solvent, to determine the amount of cell death. A higher number of viable cells results in a greater amount of MTT formazan formation which triggers an increase in absorbance. U bottom 96-well plates (1 plate for each day of incubation) were seeded with 100 μL of titrated concentrations of vitamin B12 (0-1,000 μg/ml) and 100 μL of cell suspension (at a density of $1x10^{4}$ cells/well) in triplicate. The plates were incubated at 37 $^{o}$C and 5 % $CO_2$ for three days after which half the well volume was removed and replaced with fresh media/vitamin B12. MTT assay was performed on days 3-6 of incubation to assess cellular proliferation. Cellular proliferation was measured using a spectrophotometer (Bio-Rad microplate reader, 6.0) using a wavelength of 570 nm. Three-five independent experiments were conducted in triplicates. Supernatants from each day were transferred to cryovials and stored at -80 $^{o}$C for later use for cytokine secretion measurements.

### *3.2.3 Cell migration*

Cells were treated with vitamin B12 at 125 µg/ml and 1,000 µg/ml for 3 days after which fresh media and vitamin B were added and allowed to incubate for a further 3 days. Control included untreated cells. Cell migration was performed using the Boyden chamber assay 8 µm pore size membrane filter inserts in 24-well tissue culture plates. The cells were trypsinised and resuspended in serum-free RPMI-1640 media at the density of $2\times10^5$ cells/ml. A total of 200 µl of cell suspension was seeded in the upper chamber of the trans wells, and 600 µl of media into the lower chamber. The chambers were incubated at 37 °C in a humid atmosphere with 5% $CO_2$. After 20-22 hrs, the non-migrating cells on the upper surface of the insert were removed and the cells that migrated to the underside of the membrane were counted using a light microscope. Excel and Prism excel (Graph Pad Software, La Jolla, CA, USA) was used to aid in the statistical analysis using the student's T-test and $p\leq0.05$ was considered significant.

### *3.2.4 Apoptosis assay*

Changes in the plasma membrane and loss of membrane asymmetry are amongst the earliest indications of apoptosis. The translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer membrane exposes PS to the external cellular environment. Annexin V, having a high affinity for PS binds to PS presenting cells and signals the beginning of membrane degradation seen in the last stages of cell death caused by apoptosis or necrosis (Demchenko, 2013). Using an accompanying dye such as FITC helps to signal cells that are in the early stages of apoptosis. Viable non-apoptotic cells are Annexin V negative and PI negative. Cell dead by apoptosis are Annexin V positive and PI-positive. Cells in early apoptosis are Annexin V positive and Propidium Iodide negative

while cells dead by either apoptosis or necrosis are Annexin V negative and PI-positive. U937 cells (100 µL) at $1 \times 10^6$ cells/mL were co-cultured with 100 µL of vitamin B12 (125 µg/mL)/RPMI media in 96 well plates for 3 days and 1 mL of U937 cells for 6 days; culture media alone was used as a control. Cells were harvested by centrifugation at 1,200 rpm for 5 min at room temperature. The supernatant was discarded, and the cells were washed twice in 200 µL of FACS buffer (PBS, 1 mM EDTA, 2% foetal bovine serum and 0.1% sodium azide) by pipetting. Cells were then re-suspended in 100 µL per well of Annexin V binding buffer with FITC Annexin V (Bio Legend, San Diego, CA, USA) at 1:1000 and 0.5 µg/mL of PI. Wells containing untreated, FITC and PI, PI alone, FITC alone were set up as controls. Samples were transferred to FACS tubes and data were collected using BD FACS cantoTM II cell analyser at a medium setting; 30,000 events were collected. Cells were analysed using BD FACS Diva software. Results are displayed as percentages in quadrant boxes.

### *3.2.5 Cytokine assay*

The Bio-Plex human cytokine immunoassay is a highly sensitive and reproducible magnetic bead-based assay that allows accurate measurement of low levels of human cytokines. The Bio-Plex cytokine assay uses 8 µm magnetic beads coated with antibodies against an array of cytokines. Cytokine assays were performed using a bead-based multiplex immunoassay (MIA, 9 Bio-Plex Panel B, Bio Rad Laboratories Inc., Melbourne, VIC, Australia) that included the cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFNγ, TNFα, GM-CSF. Supernatant samples from day 6 of vitamin B culture were run neat (day 6 was chosen as this is the day that showed the most effect of anti-proliferation). In addition, 24 h-time point was used to collect supernatants for differentiated U937 cells stimulated with vitamin B and cytokines assessed. Standard low photomultiplier tube settings were prepared with "blank" negative controls in duplicate. Ninety-six-well plates were coated with beads, followed by the addition of samples and standards and detection antibodies, then streptavidin-

phytoerythrin as per the manufacturer's instructions. The beads were re-suspended, and the fluorescence output was read and calculated on the Bio-Plex array reader (Bio-Rad, Melbourne, VIC, Australia). Statistical analysis of data included the mean and standard deviation (SD), as well as a two-way analysis of variance (ANOVA) followed by a Sidak's multiple comparison test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Significance was defined as $p \leq 0.05$.

### *3.2.6 Program death-ligand 1 expression on U937 cells*

PD-L1 is expressed by cancer cells as a way to escape T cell immune responses. Recently, it was shown that there is a relationship between the expression of PD-L1 with proliferation and invasion of cells (Xue et al., 2017), hence, the expression of PD-L1 on U937 cells with or without vitamin B treatment was determined. Confocal microscopy was used to visually determine its expression on U937 cells. U937 cells were treated with Vitamin B12 (125 μg/ml and 1,000 μg/ml) for 6 days. U937 treated cells (100 μL) at $5x10^5$ cells/ml were added to 96 well round bottom plates and washed twice at 5 minutes each time with PBS. Cells were fixed with 4% paraformaldehyde for 10 minutes. Cells were permeabilised for 15 minutes in 0.1% Triton X-100/PBS. Non-specific binding was blocked using 10% donkey serum for 1 hour at room temperature. Plates were then washed twice for 4 minutes with 0.1% Triton/PBS. Cells were labelled with primary monoclonal antibody PD-L1 (Abcam, ab210931) at 1:500 and incubated for 2 hrs at room temperature. Cells were washed twice in 0.1% Triton X-100/PBS and incubated at room temperature in the dark for 2 hrs. Secondary antibody Alexa Fluor 647-conjugated donkey anti-mouse at 1:250 dilution (Abacus, JI715605150). Following 2 washes with 0.1% Triton X-100/PBS, cells were incubated for 2 minutes with DAPI washed and 2 small drops of cells were placed onto slides and left to dry. Slides were mounted with fluorescence mounting media, a coverslip

was applied, over the cells and clear nail polish was applied around the perimeter of the coverslip. Slides were labelled and left to dry wrapped in tin foil in the dark overnight.

Images were captured using a Nikon Eclipse Ti multichannel confocal laser scanning system (Nikon, Japan). Z-series images were acquired at a nominal thickness of 0.5 μm (512x512 pixels). The numbers of U937 cells expressing PD-L1 markers were counted within eight randomly captured images (total area size 2 $mm^2$) per preparation at x20 magnification. Image J software (National Institute of Health, Bethesda, MD, USA) was employed to convert images from RGB to greyscale 8 bit and then to binary; particles were then analysed to obtain the percentage area of immunoreactivity. Excel and Prism excel (Graph Pad Software, La Jolla, CA, USA) were used to aid in the statistical analysis and $p \leq 0.05$ was considered significant.

### *3.2.7 Statistical analysis*

Statistical analyses were performed using 2-way ANOVA and Tukey's in excel and prism programs. The mean of triplicate wells was considered significantly different when compared to vehicle control groups if $p \leq 0.05$.

## 3.3 Results

### *3.3.1 Dose-dependent effects of vitamin B12 on cell proliferation*

The effects of vitamin B12 on cell proliferation of pro-monocytic lymphoma cells were assessed using the (MTT) assay. Significant inhibition of proliferation was noted on day 6 for the lower doses of vitamin B12 of 15 μg/ml ($p \leq 0.001$), 31 μg/ml ($p \leq 0001$), and 62 μg/ml ($p \leq 0.01$) (Figure 16). There was no difference in vitamin B12 at 125 μg/ml at all time points compared to control. At higher levels (250, 500 and 1,000 μg/ml) of vitamin B12, increased cell proliferation was noted on days 4-6. Cell proliferation was significantly increased for

250 μg/ml vitamin B12 dose on day 5 ($p \leq 0.001$) and at 500 μg/ml on days 5 and 6 ($p \leq 0.0001$). On days 4, 5, and 6 significant increases in proliferation were noted for 1,000 μg/ml ($p \leq 0.001$, $p \leq 0.01$, $p \leq 0.0001$ respectively). These findings suggest that B12 exerts dose-dependent effects on cell proliferation.

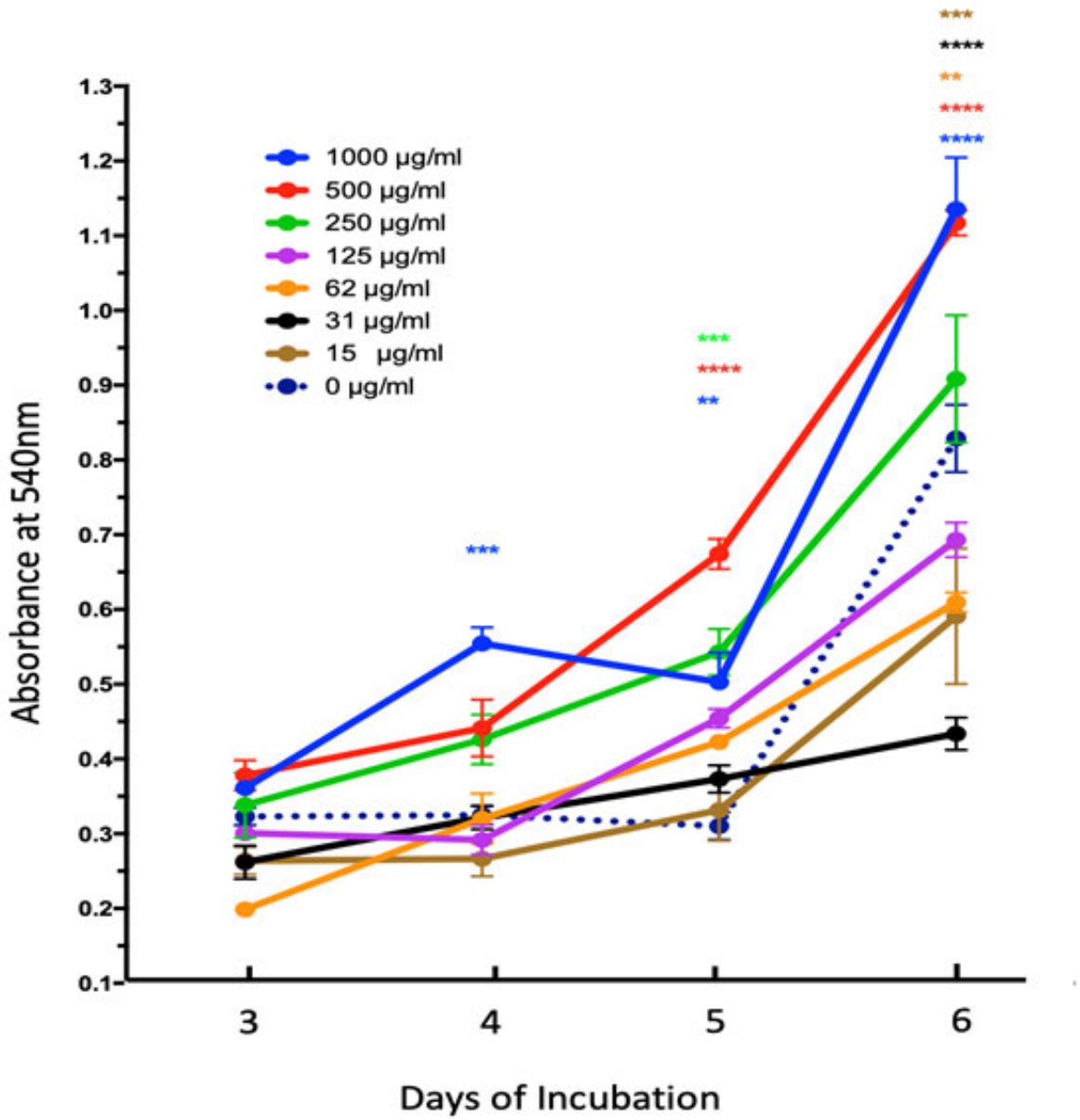



**Figure 16. Effect of vitamin B12 on proliferation of U937 pro-monocytic lymphoma cells.**

Cells were incubated with increasing doses of vitamin B12 for 6 days in 96 well U bottom plates and analysed by MTT assay. Absorbance readings were taken at 540 nm to assess for cellular proliferation and compared to control wells (0 mg/ml). Significance was established at $p \leq 0.05$ two-way Anova followed by Tukey's multiple comparisons test and marked with a sterisk (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). All experiments were repeated at least 3 times in triplicate wells.

### *3.3.2 Low dose of vitamin B12 inhibits cell migration of pro-monocytic cells.*

Cell migration is evaluated via several different techniques such as microfluidic assays, scratch assays and cell-exclusion zone assays. However, the Boyden chamber assay is the most widely accepted cell migration assay (Chen, 2005). U937 pro-monocytic lymphoma cells were added inside the chamber and allowed to migrate through the porous membrane for 20-22 hrs. The number of cells that had migrated through the membrane were stained and counted using a light microscope (Chen, 2005). Vitamin B12 low dose (125 μg/ml, $p \leq 0.05$) and B12 high dose (1000 μg/ml, no significant effects compared to control) showed inhibition of cell migration (Figure 17). These data correspond to the anti-proliferative effects exhibited by vitamin B12 (low dose).

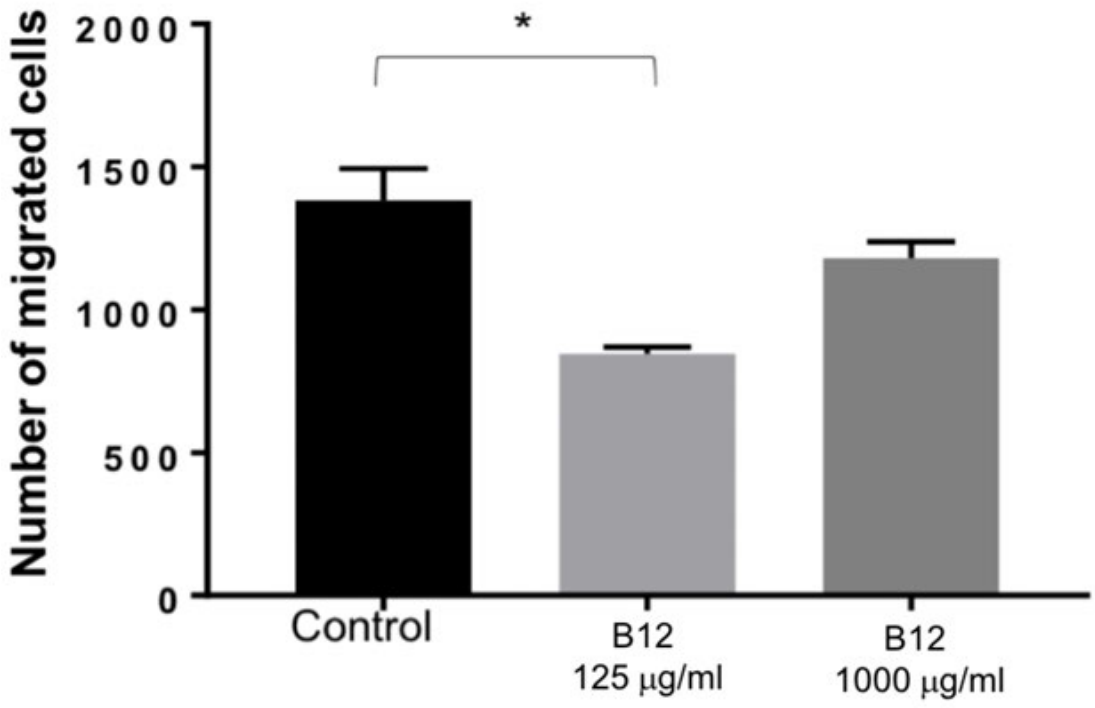



**Figure 17. B12 cell migration assay.**
Effect on cell migration of pro-monocytic cells in the presence of vitamin B12 (low 125 mg/ml, and high 1,000 mg/ml dose) using Boyden chamber assay. Data presented as mean + standard error of the mean of duplicate wells. Excel and Prism excel (Graph Pad Software, La Jolla, CA, USA) were used to aid in the statistical analysis using students T-test and $p \leq 0.05$ was considered significant.

### *3.3.3 Vitamin B12 does not induce apoptosis or cell death*

Cell migration is evaluated via several different techniques such as microfluidic assays, scratch assays and cell-exclusion zone assays. However, the Boyden chamber assay is the most widely accepted cell migration assay (Chen, 2005). U937 pro-monocytic lymphoma cells were added inside the chamber and allowed to migrate through the porous membrane for 20-22 hrs. The number of cells that had migrated through the membrane were stained and counted using a light microscope (Chen, 2005). Vitamin B12 low dose (125 μg/ml, $p \leq 0.05$) and B12 high dose (1000 μg/ml, no significant effects compared to control) showed inhibition of cell migration (Figure 18). These data correspond to the anti-proliferative effects exhibited by vitamin B12 (low dose).

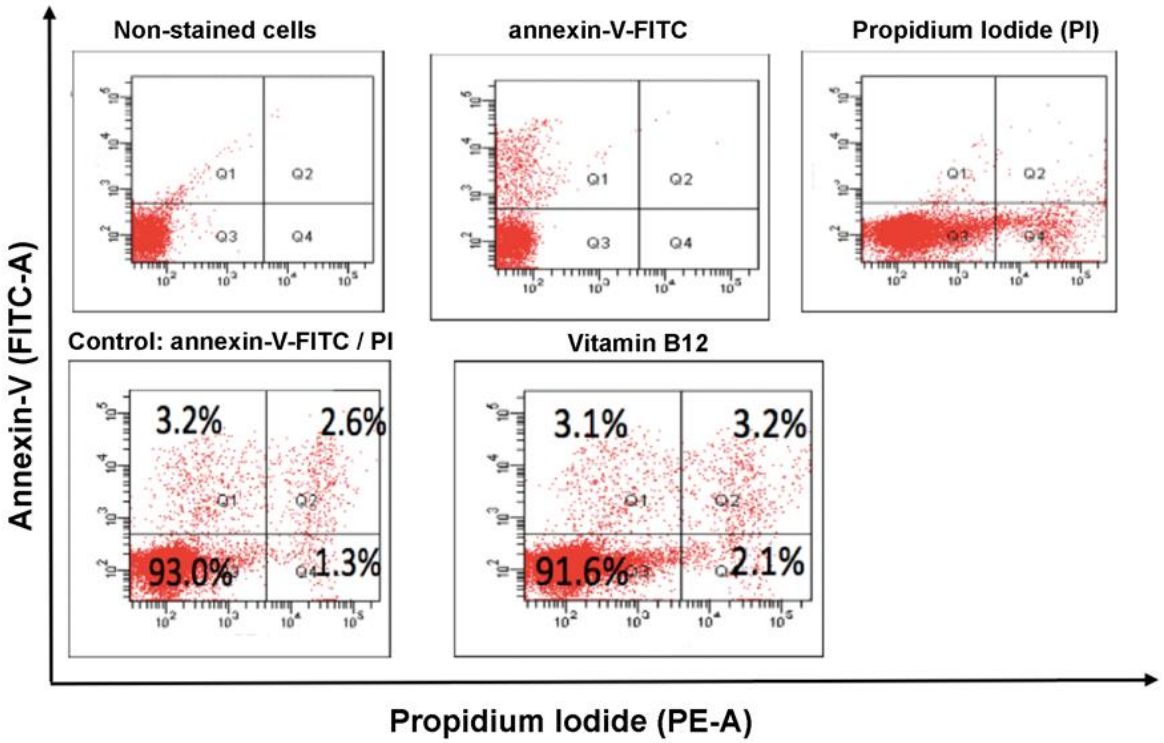



**Figure 18. Apoptosis assay.**

Annexin V-FITC/PI staining of pro-monocytic U937 cells incubated with vitamin B 12. Cells were incubated with annexin-V-FITC at 1:1,000 for 15 minutes in the dark. Proprium Iodide (PI) at 0.5 mg/ml was used as a counterstain to differentiate necrotic/dead cells from apoptotic cells. Controls include non-stained cells, annexin-V-FITC alone, PI alone and annexin-FITC/PI and were used to set the quadrant gates. Vitamin B12 was compared to annexin-V-FITC/PI control.

### *3.2.4 Low dose of vitamin B12 increases IL-8 and IL-10 cytokine profile*

Cytokine Bio-Plex assay was used to evaluate the effect of vitamin B12 on cytokine secretion of pro-monocytic U937 cells. Vitamin B12 at a low dose (125 μg/ml) showed significant stimulation of IL-10 ($p \leq 0.05$) and IL-8 ($p \leq 0.05$), which was not evident at high vitamin B12 dose (1,000 μg/ml) (Figure 19). All other cytokines (IL-1, IL-2, IL-4, IL-6, IFN-γ, TNF-α, GM-CSF) showed no significance above control (not shown).

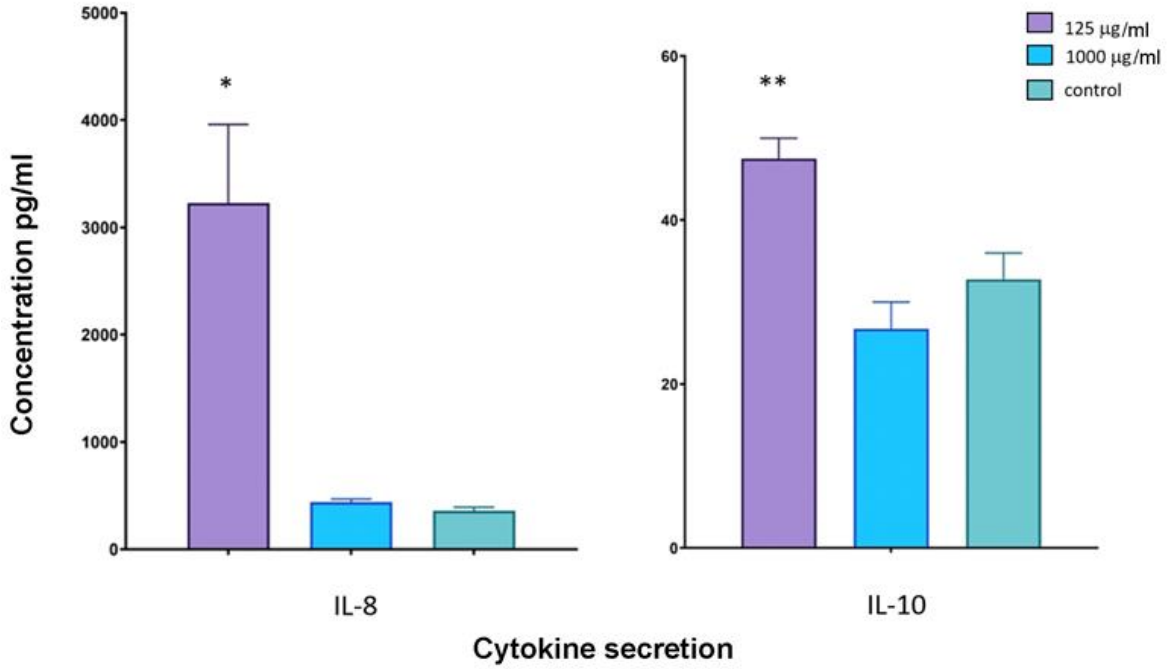



**Figure 19. Cytokine assay.**
U937 cells were treated with 125 mg/ml or 1,000 mg/ml vitamin B12 for 3 days. Media and fresh vitamin B12 were replaced and cultured for a further 3 days. Supernatants were collected and cytokine secretion analyzed by bioplex. Significance in relation to control is indicated as *$p \leq 0.05$, **$p \leq 0.01$.

### *3.2.5 Vitamin B12 increases the expression of PD-L1*

PD-L1 is an important checkpoint marker expressed by monocytes, macrophages, epithelial cells and cancer cells (Kuol et al., 2017). The expression of PD-L1 on cancer cells has an immune suppression mechanism where cancer cells escape lysis by activated $CD8^+$ T cells. PD-L1 is commonly overexpressed on cancer cells and aids in their invasiveness (Xue et al., 2017). Confocal microscopy was used to assess the effect of vitamin B12 on PD-L1 expression. Vitamin B12 co-cultured with pro-monocytic lymphoma cells resulted in increased expression of PD-L1 ($p \leq 0.01$) (Figure 20). The number of cells expressing PD-L1 were counted and fluorescence in arbitrary units was measured. Excel and Prism excel (Graph Pad Software, La Jolla, CA, USA) were used to aid in the statistical analysis and $p \leq 0.05$ was considered significant.

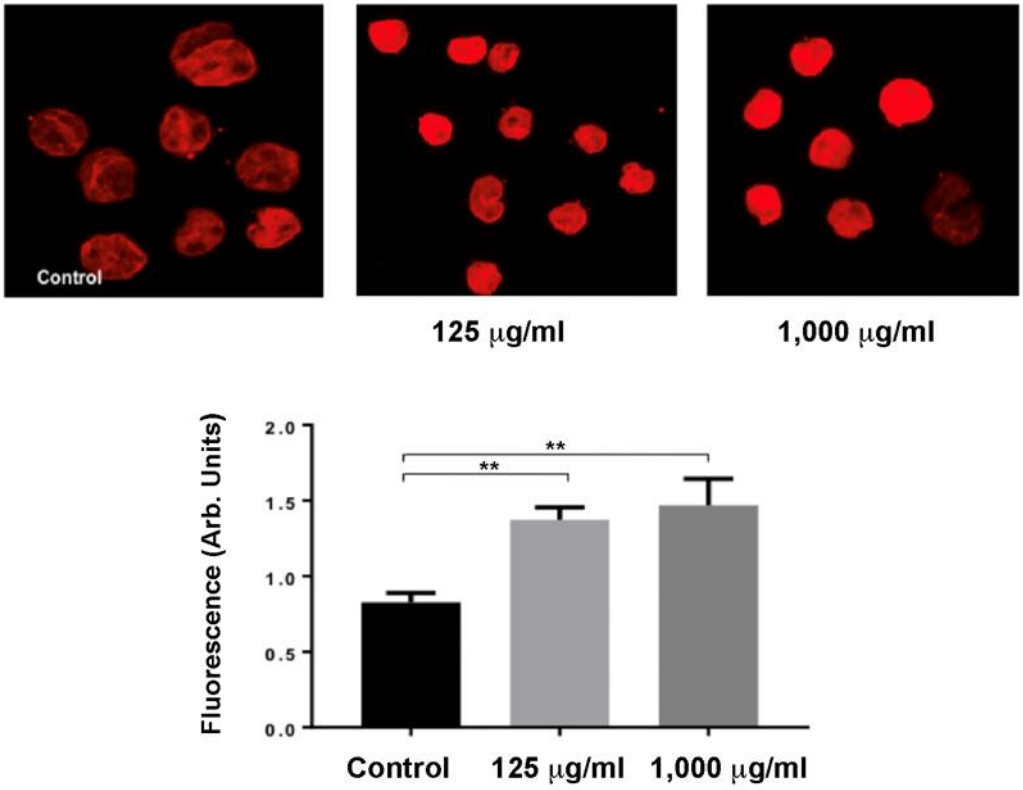



**Figure 20. Expression of PD-L1.**
As assessed by confocal imaging of pro-monocytic U937 cells in the presence of vitamin B12 at 125 μg/ml and 1,000 μg/ml concentration. Significance in relation to control is at ** $p \leq 0.01$.

## 3.4 Discussion

Monocytes are circulatory blood cells, which differentiate into macrophages or dendritic cells in tissues. These cells are phagocytic, process and present antigens and secrete cytokines (Chanput, 2015). Histiocytic lymphoma is an aggressive non-Hodgkin's lymphoma, a type of cancer that originates from cells of the immune system, specifically of pro-monocytic blast origin. U937 cells, isolated from a patient with histiocytic lymphoma, are a pro-monocytic cell line, that can be differentiated into different types of macrophages or dendritic cells (Chanput, 2015). When non-differentiated, U937 cells proliferate and have a cancer cell growth nature (Strefford et al., 2001). As such, the U937 cell line was used to determine the effects of vitamin B12.

The dose-dependent effects of vitamin B12 were significantly noted in proliferation and migration assays, PD-L1 and cytokine expression. The potential mechanisms underlying the anti-proliferative and anti-migratory effects of low dose vitamin B12 (B12low) are not fully understood. B12 (low) increased the secretion of IL-8 and IL-10 by U937 cells compared to controls. IL-8 is a cytokine secreted by macrophages and is an important mediator of the immune reaction in the innate immune response. IL-8 correlates with angiogenesis, tumorigenicity, cell proliferation, invasiveness, metastasis, and its expression on cancer cells is associated with poor prognosis (Seaton et al., 2008; Waugh & Wilson, 2008). Indeed, it has been shown that IL-8 increases the proliferation of pancreatic and breast cancer cell lines (Chen et al., 2014; Singh et al., 2013). Based on this information, it is not clear why IL-8 is upregulated by U937 cells in the presence of vitamin B12 (low) where anti-proliferative and anti-migratory effects were noted. However, given that IL-8 is highly secreted by monocytes and macrophages, and U937 cells are pro-monocytic in nature, secretion of IL-8 may suggest an activation state of the cells. In addition, IL-10 was also upregulated in the presence of vitamin B12 (low). IL-10 is a potent anti-inflammatory (deactivates macrophages and

monocytes) and immunosuppressive cytokine. IL-10 also exerts immuno-stimulatory effects, in particular on B cells, NK cells and CD8+ T cells (Goldman et al., 1997). Administration of recombinant (r), rIL-10 into cancer cells *in vivo* results in tumour rejection (Goldman et al., 1997), mainly due to its stimulatory effects on CD8+ T cells. Indeed, PEGylated rIL-10 (PEG-rIL-10; AM0010) increases tumour infiltrating CD8+ T cells by 4-fold and in mice, complete tumour rejection and long-term protection (Chanput, 2015). As such, rIL-10 has surfaced as a treatment for intra-tumoural injection in cancer patients. In a dose-escalation phase, a 1/1b trial of AM0010 in 14 different cancer types led to systemic immune activation, increased pro-inflammatory cytokines, decreased TGF-beta, increased number of systemic and tumour infiltrating CD8+ T cells, and induced partial and complete clinical responses (Naing et al., 2016). At the 12th European International Kidney Cancer Symposium in Germany (April 21-22, 2017) and ARMO Biosciences' press release, it was announced that AM0010 in combination with checkpoint inhibitors induces better objective response rates and disease control of renal cell carcinoma patients in a phase 2 trial (Meloa, 2017). Currently, a phase 3 clinical trial (ClinicalTrials.gov identifier: NCT02923921) in combination with FOLFOX chemotherapy is being conducted in patients with metastatic pancreatic carcinoma. Given the anti-cancer properties of rIL-10, the increased secretion of IL-10 by U937 cells in the presence of vitamin B12 (low) is likely one of the contributing factors to the anti-proliferative / anti-migratory effects noted herein.

To further gain insights into the mechanisms involved in the anti-proliferative and anti-migratory effects of vitamin B12 (low), the expression levels of VEGF and PD-L1 were determined. VEGF-A is a signal protein produced by cells to promote the formation of new blood vessels. Cancer cells express VEGF to grow (proliferate) and metastasize (Mahecha & Wang, 2017). There was no significant alteration in intracellular expression levels of VEGF-A by U937 cells in the presence of vitamin B12 (low) (not shown). PD-L1 however,

was significantly upregulated in the presence of vitamin B12 (low) and vitamin B12 (high). PD-L1 is a protein expressed on the surface of immune and non-immune cells. It serves as an immune checkpoint marker where it binds to its receptor PD-1 expressed on activated T cells leading to immune suppression (Barriga et al., 2019; Kuol et al., 2017, 2018). Its increased expression on cancer cells leads to progression (proliferation) and metastasis, and its expression is dependent on TNF-α, IL-1beta, and IFN-γ via toll-like receptors (Brogden et al., 2016). In fact, ovarian (ID8) and melanoma (B16) cell lines were developed to express high levels of PD-L1 or PD-L1 was knockdown; PD-L1 promoted cell proliferation *in vitro* and *in vivo*, whereas the knockdown on cancer cells resulted in slower proliferation rates *in vitro* and tumour load in mice (Clark et al., 2016). This being said, it may explain vitamin B12 (high) with increased levels of PD-L1 showing increased U937 cell proliferation. However, it is not clear what the role of increased PD-L1 on U937 cells is in the presence of vitamin B12 (low). It is known that IL-10 promotes the expression of PD-L1, and high levels of IL-10 were noted for vitamin B12 (low). Targeting IL-10 and PD-L1 is the subject of targeted therapy for cancer. Further research is required to understand the mechanisms of vitamin B12 (low and high) and its effects on cancer cells.

Vitamin B12 high dose (1,000 μg/ml) behaved differently from vitamin B12 low dose (125 μg/ml). With B12 (high) incubated cells, significant upregulation of cell proliferation was noted, with no significantly reduced migration effects. No significant changes were noted to all cytokines tested from B12 (high) cultured U937 cells (IL-1beta, IL-2, IL-4, IL-6, IL-10, IFN-γ, IL-8, and GM-CSF). Similarly, to B12 (low), there was increased expression of PD-L1, but this is in accordance with the increase of cellular proliferation produced by B12 (high). This data suggests that high concentrations of B12 may influence cellular processes which affect tumour promotion. B12 influences metabolism in cells and plays a pivotal role in cell growth.

The deficiency of B12 can vastly affect the body's immune defences against cancer which involve lymphocytes, CD8+ and NK cells as well as cytokines such as IL-6 and TNF-α. The biological actions of vitamin B12 were shown in newly diagnosed vitamin B12 deficient patients whereby significant reduction of CD8+ and NK cells were noted, which could be augmented by methyl-B12 treatment (43). Likewise, abnormally high CD4/CD8 ratio and low NK activity in patients with pernicious anaemia could be a result of vitamin B12 deficiency (44). In addition, the production of TNF-α is amplified in the spinal cord of vitamin B12 deficient rats (43), and in mice, vitamin B12 deficiency up-regulates TNF-α synthesis by macrophages (45). Furthermore, IL-6 is decreased in cobalamin-deficient rats, as a result of dysregulation of gp130. Gp130 is a transmembrane glycoprotein and the founding member of the class of all cytokine receptors, which promotes demyelination in the mammalian central nervous system. Both IL-6 and TNF-α deviations were corrected with vitamin B12 supplementation (46).

The dose-dependent response of B12 on cellular processes may in part, explain why over-supplementation of vitamin B12 has been linked to lung cancer progression (Brasky et al., 2017) whereas vitamin B12 deficiency is linked to breast cancer (Choi, 1999), gastric cancer (Miranti et al., 2017) and cervical cancer (Hernandez et al., 2003). Other studies have made correlations between high B12 serum levels with pancreatic, colon/rectum, lung, prostate, urothelium, and bone and liver metastases, as well as myeloproliferative disorders (Arendt & Nexo, 2012; Chiche et al., 2008; Collin et al., 2010; Norredam et al., 1983; Price et al., 2016; Urbanski et al., 2020) but these studies, did not claim that over-supplementation was the reason for the increased serum B12 levels. It is likely that the noted increased B12 serum levels are a physiological result of cancer due to impairment of tissue and cellular uptake of the vitamin rather than a causative factor. Further research is therefore required to

understand the discrepancy amongst these studies and the role vitamin B12 plays in cancer regression versus cancer promotion.

## 3.5 Conclusion

The results in this chapter indicate that B12 (low) inhibits pro-monocytic cell proliferation and migration which is not due to apoptosis or cell death. It is not clear, however, what role IL-8 plays in the anti-proliferative and anti-migratory effects, as it is generally believed that IL-8 is associated with increased proliferation, migration, and angiogenesis of cancer cells; the mechanism associated with increased levels of IL-8 needs to be further elucidated. However, vitamin B12 (low) increased IL-10, GM-CSF and decreased IL-1beta cytokine levels which are in accord with reduced cell proliferation and anti-tumourigenic properties based on other studies in the literature. Conversely, the increased expression levels of PD-L1 on B12 (low) do not support the reduced proliferation and migration of U937 cells. In addition, vitamin B12 (high) promoted U937 cell proliferation and increased PD-L1 expression suggesting a pro-tumorigenic mechanism. The role of B12 in cancer progression is ambiguous, with research claiming both pro-tumour and anti-tumour effects. We propose that this role is likely to be dose-dependent, and although the correlation between vitamin B12 deficiency and inflammation to cancer progression is strong, there is also a possibility, in light of our research, that over-supplementation may play a role in cancer progression by changing the mechanistic effects of B12 at a cellular level. Studies that correlate high vitamin B12 serum levels and cancer without vitamin B12 over-supplementation may be elucidating the physiological effects that cancer can exert on the body regarding impaired cellular absorption which could likewise pose as vitamin B12 deficiency and contribute to

cancer advancement. This data gives insights into the dose-dependent properties of vitamin B12 at a cellular level and could form the basis for future studies to gain insights into the mechanistic effects of vitamin B12 in cancer cells.

# Chapter Four

# High dose vitamin B6 downregulates the mevalonate pathway and re-establishes the G1/S checkpoint in a promonocytic lymphoma cell line

## Abstract

Vitamin B6 is an essential nutrient for human health and is required for over 150 enzymatic reactions affecting cellular processes in humans and other living organisms. The inflammatory and carcinogenic effects of vitamin B6 deficiency have been the main focus of research in recent times. However, attention is now shifting towards determining the effects of high dose (above RDI) vitamin B6 intake as an anti-cancer therapeutic agent. In this chapter, the anti-proliferative effects of high dose vitamin B6 (pyridoxine) on U937 promonocytic lymphoma cell line with a Tp53 gene mutation using Next Generation Sequencing were evaluated. The resulting change in transcriptomic expression indicated that vitamin B6 is able to re-establish Tp53 expression and restore G1/S checkpoint signalling. Further to this, vitamin B6 also downregulated genes related to the mevalonate pathway.


## 4.1. Introduction

Vitamin B6 is an essential micronutrient for human health. Vitamin B6 participates in over 150 enzymatic reactions and facilities many other biochemical responses vital for cellular function including regulation of metabolism, synthesis of carbohydrates, lipids, proteins, heme, and many other bioactive metabolites (Mooney et al., 2009). Vitamin B6 is widely established as a nutrient of interest in both anti-inflammatory medicine and cancer research. New evidence is required, however, to clarify the mechanistic effects of vitamin B6 as an anti-cancer agent and to understand its effects on cancer pathways and mechanisms at a molecular level. Vitamin B6 deficiency is known to contribute to cancer initiation and progression in several cancers via various mechanisms (Bird, 2018; Crusz & Balkwill, 2015; Merigliano, Mascolo, Burla, et al., 2018; Mocellin et al., 2017; Peterson et al., 2020;

Pusceddu et al., 2020; Waly et al., 2016). These include an increase in inflammation (Bird, 2018; Huang et al., 2010) and oxidative stress (Giustina et al., 2019) due to a disruption in immune response and a decrease in the production of key proteins and nucleic acids which inhibit immune cell function and interfere with cellular metabolism. Vitamin B6 deficiency also contributes to disorders in one-carbon metabolism which can lead to hyperhomocysteinemia (Lyon et al., 2020), and dysregulated DNA methylation and synthesis which increases genomic instability (Wu & Lu, 2012). Although it has been well documented that low vitamin B6 status may contribute to carcinogenesis, it is less clear what effect vitamin B6 supplementation, in dosages above the accepted recommended daily intake (RDI), can have on cancer cells. In animal models and human cancer cell lines, high dose vitamin B6 supplementation has been correlated with a reduction in cancer via anti-proliferative, anti-oxidative and anti-inflammatory effects (Ciappio et al., 2011; Kayashima et al., 2011; Komatsu et al., 2002; Mao et al., 2015; Matsubara et al., 2003; Okazaki et al., 2012; Toya et al., 2012; Wu et al., 1999). For example, in murine studies, vitamin B6 supplementation was able to suppress protein expression for proliferation-related genes c-myc and c-fas and reduced oxidative stress in azoxymethane treated mice (Komatsu et al., 2002). Recently it was demonstrated that the combination of vitamin B6/curcumin supplementation suppressed pro-inflammatory cytokines and signalling pathways in colorectal carcinogenesis (Wu et al., 2021) and vitamin B6 supplementation is inversely related to carcinogenesis of the oesophagus (Ma et al., 2018) and breast cancer risk in middle-aged women (Egnell et al., 2017). Further to this, B6 supplementation was shown to diminish oxidative markers in the liver of septic rats (Giustina et al., 2019) and neonatal rats undergoing hyperoxia therapy (Lee et al., 2018). B6 supplementation has also been shown to prevent excessive inflammation by reducing the accumulation of sphingosine-1-phosphate (Du et al., 2020).

We previously demonstrated that high dose vitamin B6 in the form of pyridoxine (PN) in culture for 6 days, displayed anti-tumour properties via its ability to downregulate cell proliferation and migration, downregulate the expression of programmed death-ligand 1 (PD-L1) checkpoint maker and reduce inflammatory cytokine secretion in U937 promonocytic lymphoma cell line (Mikkelsen et al., 2019) (Chapter 2). In this regard, the mechanisms behind the effects of vitamin B6 were determined using Next Generation Sequencing (NGS). Through analysis of significantly altered genetic pathways via NGS, it is clear that high dose supplementation of vitamin B6 for 6 days, reduces proliferation by resurrecting a mutant p53 pathway in U937 cells and restores the G1/S checkpoint. This occurs via the upregulation of interleukin (IL)-10, altered ribosome biogenesis, or a combination of both. In addition, pathways relating to p21 expression are also upregulated which may be occurring as a result of the upregulated p53 pathway having downstream effects on p21 or via the upregulation of RUNX3 and nerve growth factor pathways which are also upregulated in gene analysis by high dose vitamin B6. Furthermore, incubation of vitamin B6 for 24 hrs identified downregulation of the mevalonate pathway which may be contributing to the degradation of mutant p53 found inherently in the U937 cell line and the gradual restoration of wild type p53. Vitamin B6 has clear anti-cancer properties and further work is required *in vitro* on other cell lines as well as in p53 knockout and/or transgenic mouse models. This data gives evidence that vitamin B6 may be applied as an adjunct to cancer treatments in the future.

## 4.2 Material and methods

### *4.2.1 Cell culture and reagents*

#### *4.2.1.1 Culture of U937 cells*

The U937 myeloid cell line was isolated from a 37-year-old male patient with histiocytic lymphoma. U937 cells are commonly used to study the behaviour and differentiation of monocytes. They can be differentiated into monocytes and macrophages following stimulation with certain stimulants such as vitamin $D_3$ (Chanput, 2015). U937 cells have a p53 mutation which causes them to be highly proliferative, a characteristic that makes them such a successful cell line for experimentation (Sugimoto et al., 1992). The U937 cell line was purchased from ATCC (https://www.atcc.org) by the Monash University Department of Immunology. U937 cells are immortal and allow for multiple passages without transformation, however, it was ensured that the number of passages was kept to a minimum. U937 cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 2 mM $_L$-glutamine (Sigma-Aldrich), 100 mg/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) at 37$^o$ C and 5 % $CO_2$. Culture media was changed every 3-4 days and cells were passaged accordingly. Once 80-90 % confluent, cells were used in experiments.

#### *4.2.1.2 Preparation of vitamin B6 stock*

A stock solution of vitamin B6 in the form of pyridoxine hydrochloride (PN) (P6280-100G) was freshly prepared according to the manufacturer's instructions (Sigma, VIC Australia) in phosphate-buffered saline (PBS). Vitamin B6 stock was filtered using a 0.2-micron filter and used fresh for each experiment.

#### *4.2.1.3 Treatment of cells with vitamin B6.*

Cells were treated with 250 μg/ml vitamin B6 (corresponding to IC-50 concentration. for (A) 24 hrs and (B) 3 days, after which fresh media and vitamin B6 were added and then allowed to incubate a further 3 days (6 days in total). Control included cells not treated with vitamin B6.

## *4.2.2 Proliferation and cytokine analysis*

#### *4.2.2.1 Vitamin B6 proliferation assay using MTT*

The rapid colourimetric assay for cellular growth and survival, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used for cell proliferation. The MTT assay is a robust and reliable method that acquires the signal of mitochondrial performances, hence cellular viability. Other methods to measure cell proliferation, such as $^{3}$H-thymidine or BrdU test, assess DNA damage in response to treatment (i.e., DNA damage vs mitochondrial enzymatic performance). In these studies, however, it was decided to use the MTT assay as a readout for cell proliferation.

MTT forms purple formazan in the mitochondria of living cells in the reduction reaction, which takes place only when mitochondrial enzymes are active, and therefore conversion can be directly related to the number of viable cells (Riss et al., 2004). The absorbance of the purple solution can be quantified by measuring the wavelength via a spectrophotometer. The amount of purple formazan produced by cells treated with vitamin B6 with the amount of formazan produced by untreated cells or control solvent, to determine the amount of cell death. A higher number of viable cells results in a greater amount of MTT formazan formation which triggers an increase in absorbance. U bottom 96-well plates were seeded

with 100 µL of 0-1,000 µg/ml vitamin B6 or untreated control and 100 µL of cell suspension (at a density of $1x10^4$ cells/well) in triplicate. The plates were incubated at 37 $^oC$ and 5 % $CO_2$ for 6 days, with fresh media/vitamin B6 added on day 3. Cellular proliferation was assessed using a spectrophotometer (Bio-Rad microplate reader, 6.0) using a wavelength of 570 nm. Three-five independent experiments were conducted in triplicates. Supernatants on day 0 and day 6 were collected and stored at -80 $^oC$ for later use for Bio-Plex cytokine analysis. On incubation, plates were viewed under an IX81 Olympus microscope and photos were taken at 4x magnification on day 6.

#### *4.2.2.2 Assessment of Interleukin-10 production*

The Bio-Plex human cytokine immunoassay is a highly sensitive and reproducible magnetic bead-based assay that allows accurate measurement of low levels of human cytokines. The Bio-Plex cytokine assay uses 8 µm magnetic beads coated with antibodies against an array of cytokines. Cytokine assay was performed using a bead-based multiplex immunoassay (MIA, 9 Bio-Plex Panel B, Bio Rad Laboratories Inc., Melbourne, VIC, Australia) for IL-10. Supernatant samples from day 6 of vitamin B6 proliferation assay culture were used. Standard low photomultiplier tube settings were prepared with "blank" negative controls in duplicate. Ninety-six-well plates were coated with beads, followed by the addition of samples and standards and detection antibodies, then streptavidin-phytoerythrin as per the manufacturer's instructions. The beads were re-suspended, and the fluorescence output was read and calculated on the Bio-Plex array reader (Bio-Rad, Melbourne, VIC, Australia). Statistical analysis of data included the mean and standard deviation (SD), as well as a two-way analysis of variance (ANOVA) followed by a Sidak's multiple comparison test using GraphPad Prism ™ (GraphPad Software, San Diego, CA, USA). Significance was defined as $p < 0.05$.

### *4.2.3 Gene analysis*

Typically, the protocol for RNA-seq experiments involves RNA isolation from treated cells or cells of interest, conversion of RNA to cDNA, preparation of sequencing library, and running the sequence through an NGS platform (Haque et al., 2017; Kukurba & Montgomery, 2015). RNA-seq data analysis is then performed by analysing and visualising differential gene expression and identifying statistically differentially expressed genes using false discovery rate (FDR) $\leq 0.05$, $\log_2$ fold change >1 (abs log FC+2). RNA extracted from vitamin B6 treated cells with RNA integrity (RIN) score of no less than 9.5 were sent to Monash University, Clayton or processing by Micromon Genomics Microbial Biotechnology and Diagnostic Unit for NGS (See Figure 21).

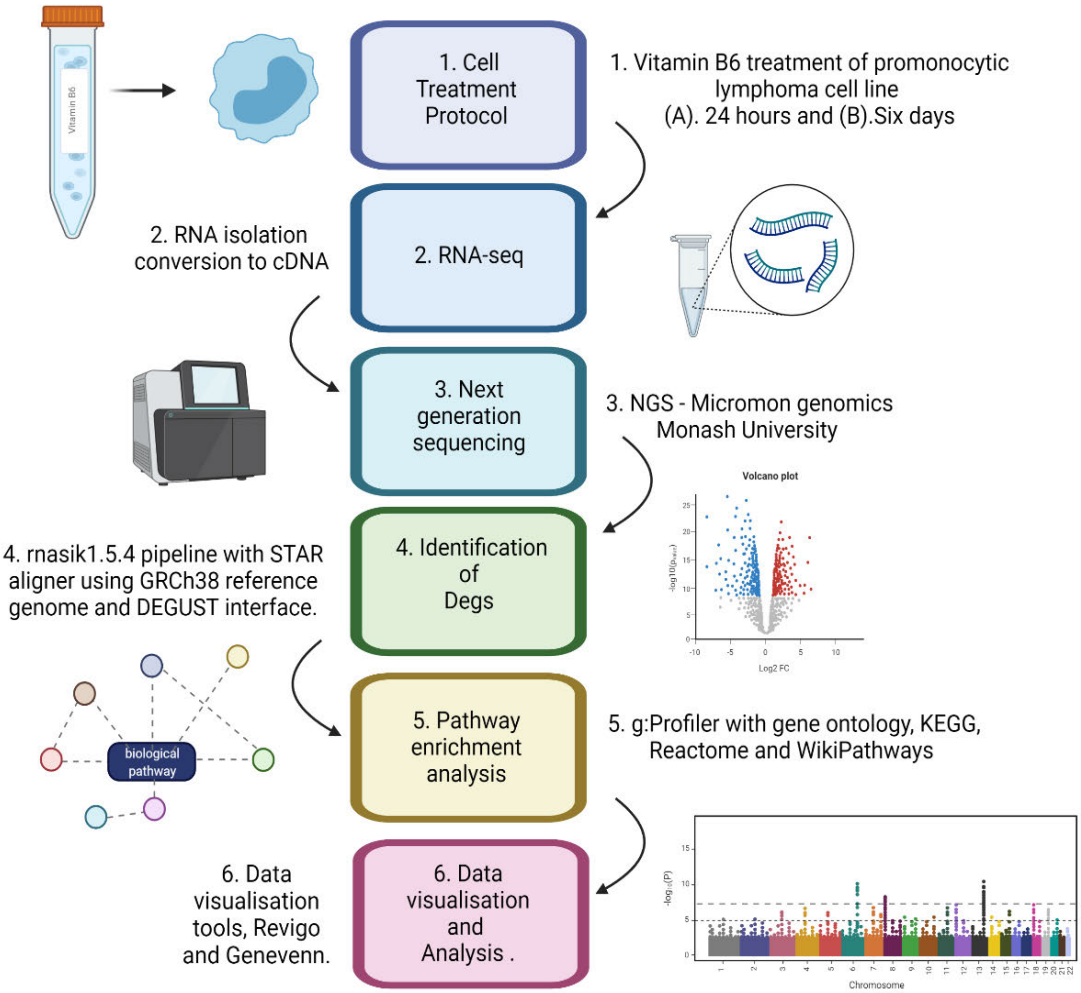



**Figure 21. Workflow outline of genetic component of present study including initial treatment protocols and use of bioinformatic strategies.**

#### *4.2.3.1 RNA extraction from U937 cells*

Extraction of RNA from treated cells was performed using an RNeasy1 mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Messenger RNA (mRNA) from treated cells, was extracted from each cell pellet by disrupting cells with lysis buffer/β–mercaptoethanol mix. Cells were lysed with RLN buffer (Qiagen, Hilden, Germany) and the lysate was placed in the supplied Qia-shredder columns for homogenisation and then transferred to RNeasy mini-spin columns. DNase was used to eliminate any genomic DNA contamination with the RNase-free DNase set (Qiagen, Hilden, Germany). Samples were then tested for RIN using the Agilent 2100. Samples with a RIN >9.5 were included for the next part of the study.

#### *4.2.3.2 RNA analysis / RNAseq*

Samples were taken to Monash University for processing by Micromon Genomics for Next-Generation Sequencing. Bulk RNAseq was used to evaluate the effect of vitamin B6 on U937 promonocytic lymphoma cells following culture with or without vitamin B6 at 24 hrs (n=3) and 6 days (n=3). Samples were measured using the Invitrogen Qubit (according to the manufacturer's instructions) using 2 μL of sample and assayed with the RNA HS assay (according to manufacturer's instructions). Samples were sized and measured for RNA integrity using the Agilent Bioanalyser 2100 and RNA Nano assay kit (according to the manufacturer's instructions). Samples were then processed using an MGI RNA directional Library preparation set V2 (according to the manufacturer's instructions) with the following modifications/options: RNA fragmented at 87° C for 6 minutes to target insert size 200-400 base pairs; Adapters were diluted 1/5; The libraries were amplified with 13 cycles of PCR and then pooled in equimolar concentrations sequenced using an MGIDNBSEQ-2000RS with reagent chemistry V3.1.

**Multi QC report** ~94% aligned, ~70% assigned. **Differential gene expression**: Statistically differently expressed genes identified using a maximum FDR ≤ 0.05, log2 fold change>1 (abs log FC=2)). **Analysis** was performed using RNAsik 1.5.4 pipeline with STAR aligner, using the GRCh38 (*Homo sapiens*) reference genome (Tsyganov. K, 2018). Data presented using Degust. Degust is an interactive interface for RNA-seq data exploration, analysis, and visualisation, and includes QC metrics (see Figure 24).

## 4.3. Results

### *4.3.1 Vitamin B6 decreases the proliferation of U937 cells*

The effects of vitamin B6 on the cellular proliferation of pro-monocytic U937 lymphoma cells were assessed using an MTT proliferation assay. All experiments were repeated at least 3 times in triplicate wells. Inhibition of cell proliferation was noted in a dose-dependent manner on days 4-6 (chapter 2) (Mikkelsen et al., 2019) with 1,000 μg/ml ($p \leq 0.001$), 500 μg/ml ($p \leq 0.01$) and 250 μg/ml ($p \leq 0.01$) showing significant inhibition (Figure 22A).

### *4.3.2 Vitamin B6 significantly increases the expression of interleukin-10*

Magnetic bead Bio-Plex assay was used to evaluate the effect of 125 μg/ml or 250 μg/ml of vitamin B6 on IL-10 cytokine secretion of U937 promonocytic lymphoma cells. Vitamin B6 (250 μg/ml) was shown to significantly increase the secretion of IL-10 ($p \leq 0.0001$) (Figure 22B).

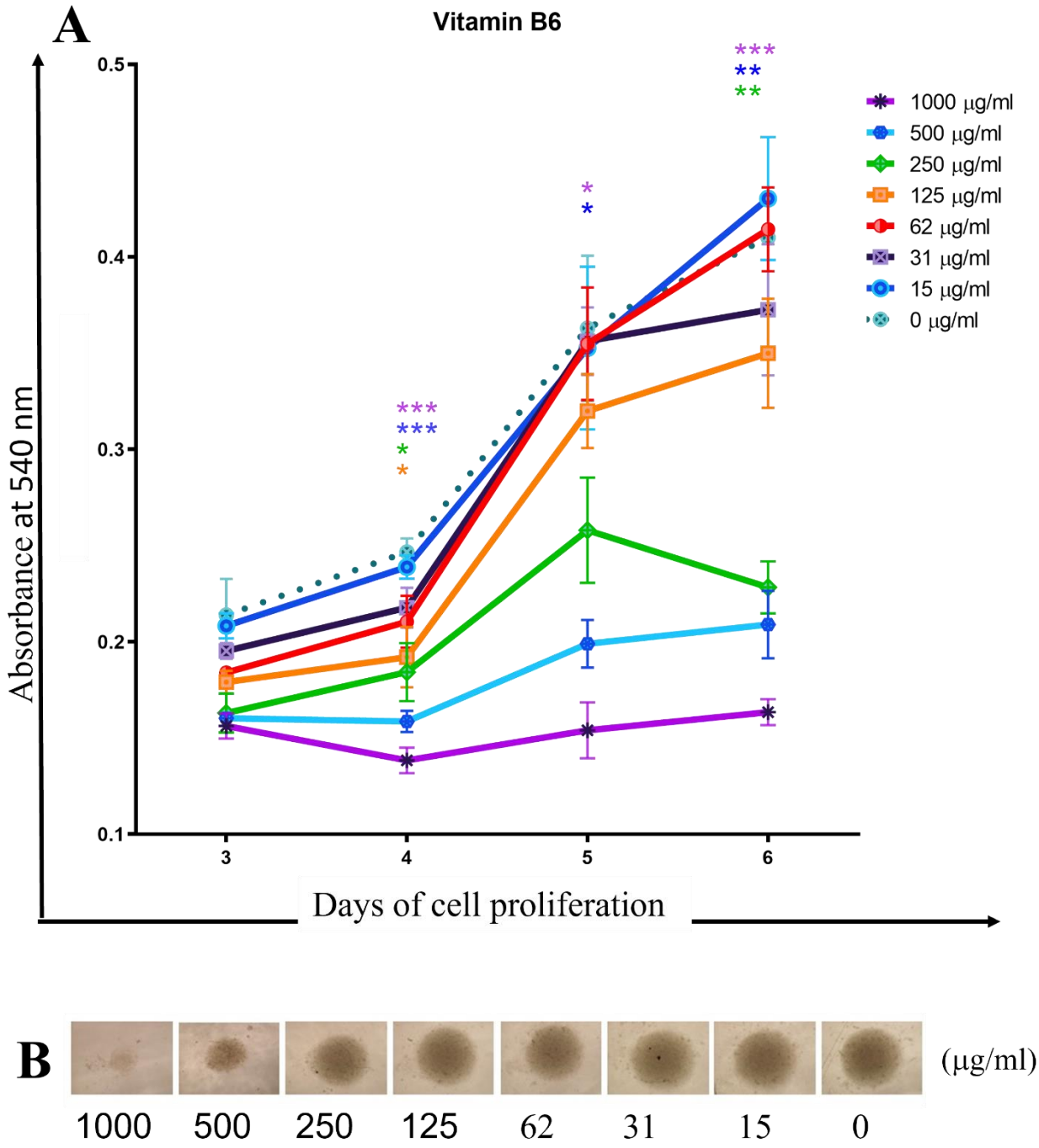



**Figure 22. Vitamin B6 downregulates cellular proliferation in promonocytic lymphoma U937 cells**.

(A). Cells were incubated with increasing doses of vitamin B6 for 6 days in 96 well U bottom plates and analysed by MTT assay. OD readings were taken at 540nm to assess for cellular proliferation compared to control well (0mg/ml). Significance was established at $P < 0.05$ two-way Anova followed by Sidak's multiple comparisons test and marked with asterisk (*P<0.05 ** P<0.01 *** P<0.001). (B). Cells were viewed under a IX81 Olympus microscope at 4x magnification and photos taken of each concentration and control at day 6.

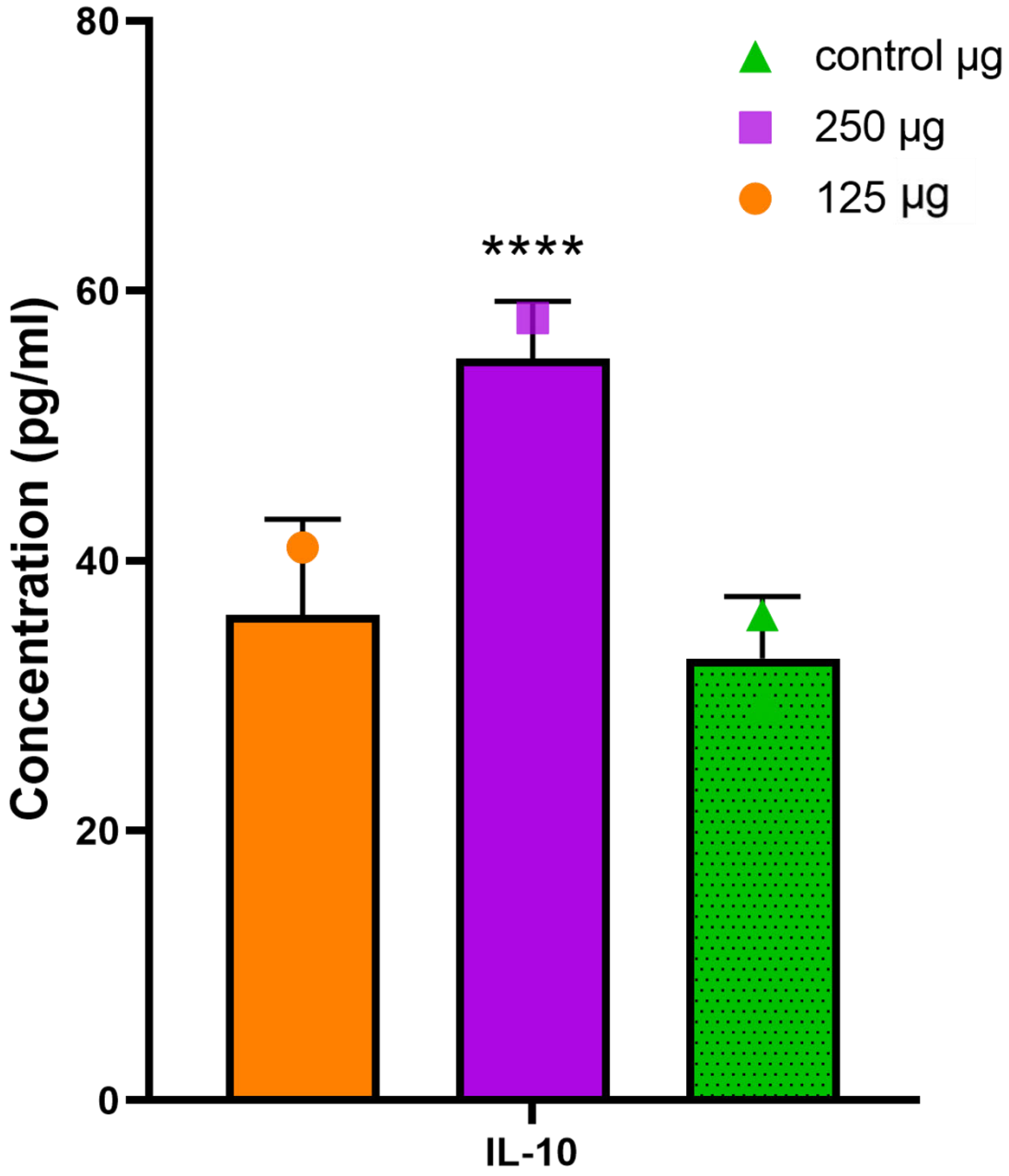



**Figure 23. Vitamin B6 increases the secretion of IL-10.**

U937 cells were cultured with 125 mg/ml and 250 mg/ml vitamin B6 for 3 days. Media and fresh vitamin B6 were replaced, and cells were cultured for a further 3 days. Supernatants were collected and cytokine secretion of IL-10 detected by bioplex. Significance in relation to untreated control is indicated as **** $p < 0.0001$.

### *4.3.3 Gene Analysis*

Cellular information is stored within DNA, transcribed into RNA, and translated into proteins; this is the central dogma of molecular biology. The transcriptional genetic subsets, both coding and non-coding, are collectively described as the transcriptome and give insights into the molecular functioning of a group of cells (Kukurba & Montgomery, 2015).

#### *4.3.3.1 Next Generation Sequencing*

Next-generation sequencing (NGS) is a revolutionary, high-throughput method of RNA analysis through the sequencing of cDNA (complementary DNA) which has allowed a greater understanding of the complexity and ever-changing nature of the transcriptome as it responds to physiological and pathological conditions (Kukurba & Montgomery, 2015). RNA sequencing (RNA-Seq) provides a quantitative vision of gene expression for transcriptome analyses.

#### *4.3.3.2 Quality control metrics using multiQC*

Quality control (QC) metrics are important for use in NGS data to ensure accurate measurements and avoid confounding batch effects (Leek et al., 2010). This project used a tool called MultiQC which presents the visual output of data from multiple common bioinformatic tools and gathers it into a single report (Figures 25 + 26) (Ewels et al., 2016). All samples from both experiments passed quality control measurements ( Figure 24). Batch effects can occur when experiments are done at different times so this is dealt with by normalization procedures. STAR (Spliced transcripts Alignment to a Reference) Aligner determines which location on the genome that the reads originated from. STAR Alignment

scores show a percentage of uniquely mapped reads, higher percentages are indicative of high-quality samples. Samples dropping below 60% indicate low-quality samples and require further troubleshooting (Dobin et al., 2013) (Figure 24). FeatureCounts is a program that was designed to aid read summarisation in NGS data by efficiently comparing the mapping location of base pairs in a read with a genomic region (Liao et al., 2014) (Figure 24).

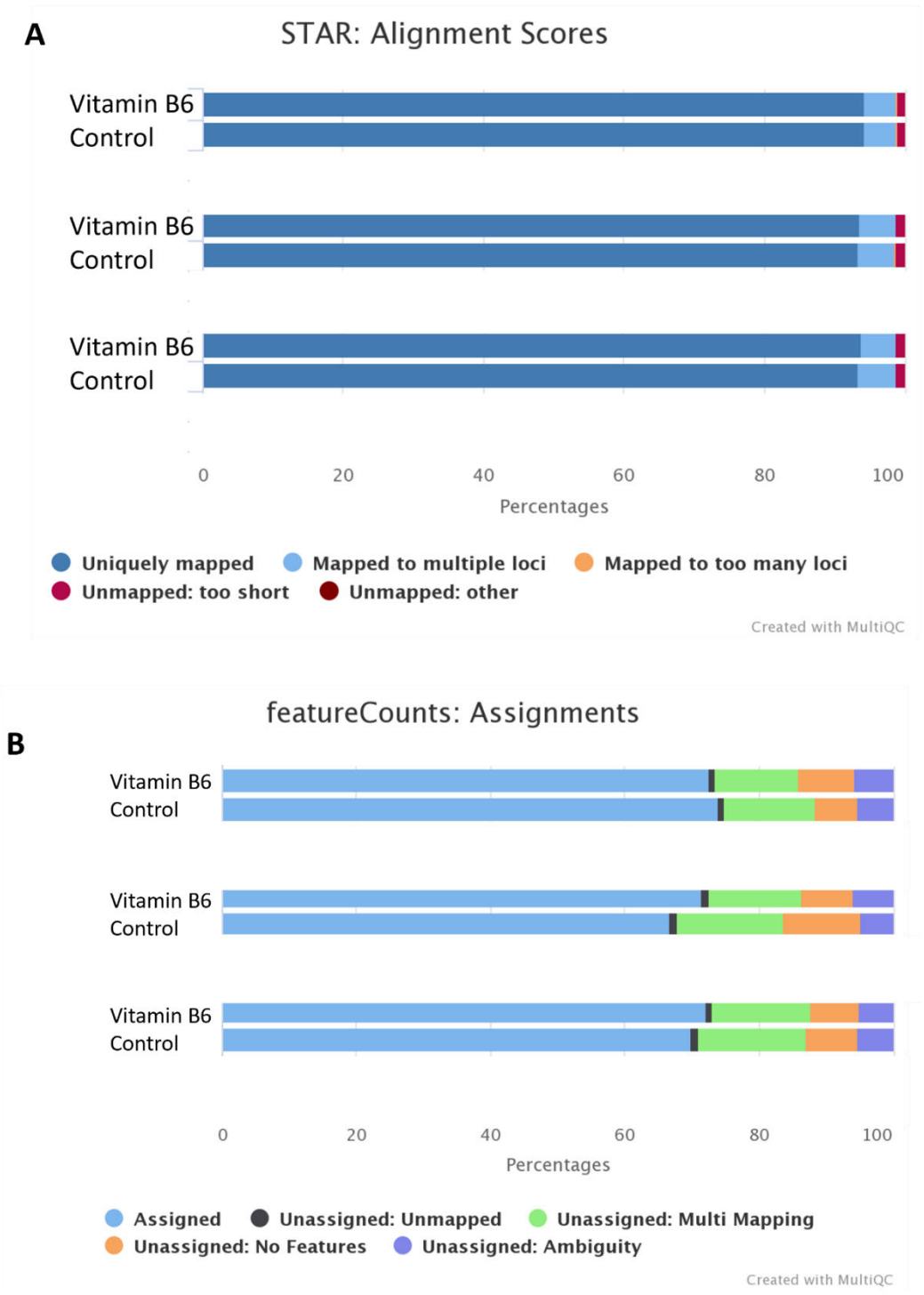



**Figure 24. MultiQC metrics using STAR alignment and feature Counts assignments, Quality control (QC) metrics using MultiQC.**

(A). STAR Alignment scores showing 94 % of reads uniquely mapped, this is indicative of high-quality samples. Scores dropping lower than 60% indicate low quality samples and require trouble shooting (Dobin et al., 2013). (B). Feature counts data showing 70% successfully assigned reads (Liao et al., 2014).

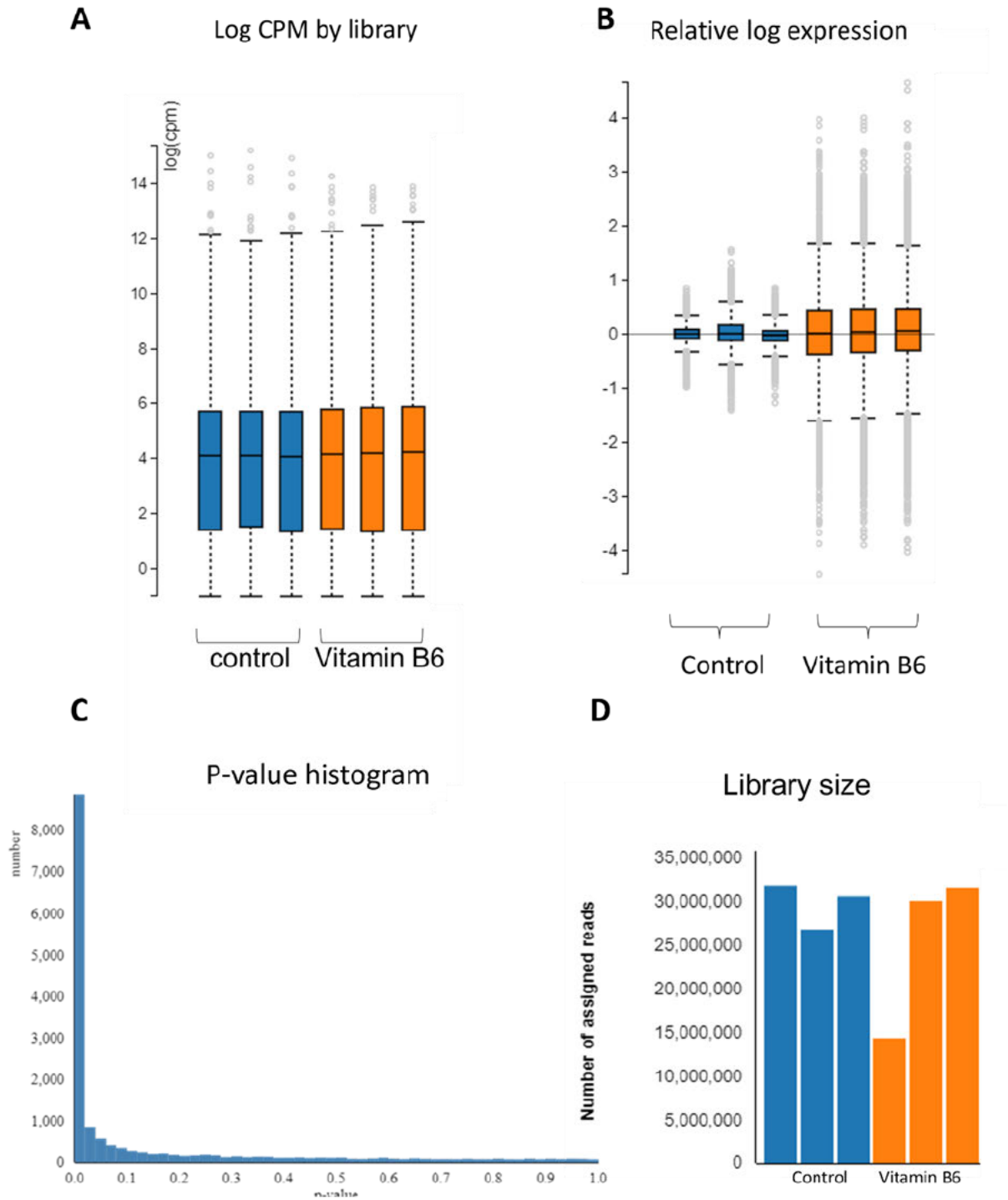



**Figure 25. Degust session QC - Vitamin B6 versus control at time = 6 days**

cDNA library quality assessment. (A). Normalised expression intensity. (B). Relative Log expression showing variation within samples. (C). P value histogram showing distribution of p values throughout samples. (D). Sequencing Library size distribution.

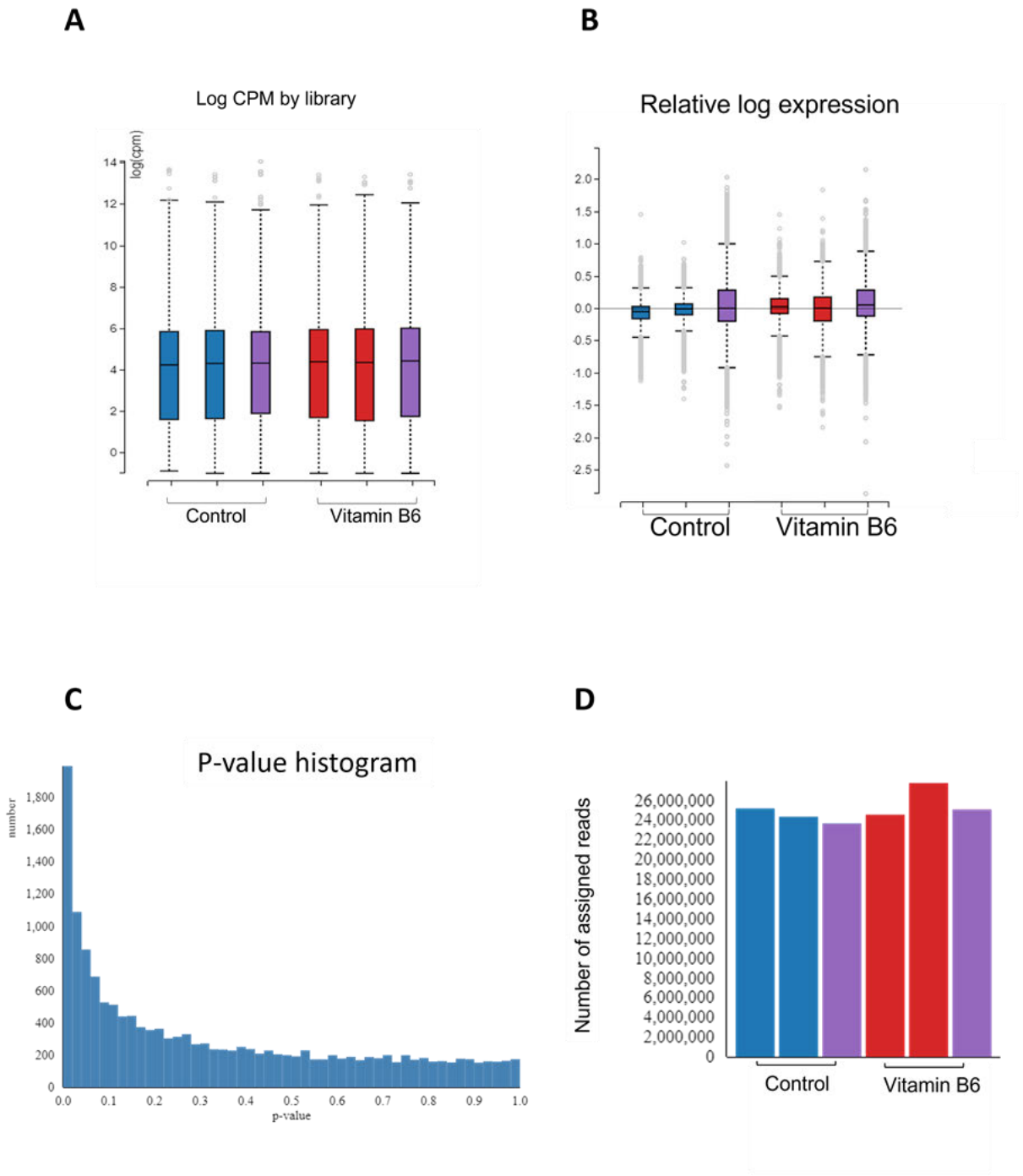



**Figure 26. Degust session QC- Vitamin B6 versus control at 24 hours.**

cDNA library quality assessment. (A). Normalised expression intensity with batch effect. (B). Relative Log expression showing variation within samples and batch effect. (C). P value histogram showing distribution of p values throughout samples. (D). Sequencing Library size distribution with batch effect. These samples were collected over multiple experimental days and some batch effect was observed and corrected for.

#### *4.3.3.3 Identification of statistically differentially expressed genes*

Data were analyzed in this study using the rnasik1.5-4 pipeline with STAR aligner, using the GRCh38 (*Homo sapiens*) reference genome and utilizing DEGUST, an interactive interface for RNA-seq data exploration, analysis, and visualisation. For each experimental group (A) 24-hour vitamin B6 treatment versus control (Figure 27) and (B) 6-day vitamin B6 treatment versus control (Figures 28-29) are shown and statistically differentially expressed genes (DEGs) were identified using a minimum false discovery rate (FDR) $\leq 0.05$, $\log_2$ fold change >1 absolute Fold change (abs FC=2). DEGs were then analysed through multiple enrichment pathway programs in 3 different groups. (i) Upregulated DEGs, (ii) downregulated DEGs, and (iii) up and downregulated DEGs combined, to capture pathways that are involved in both up and down-regulated genes.

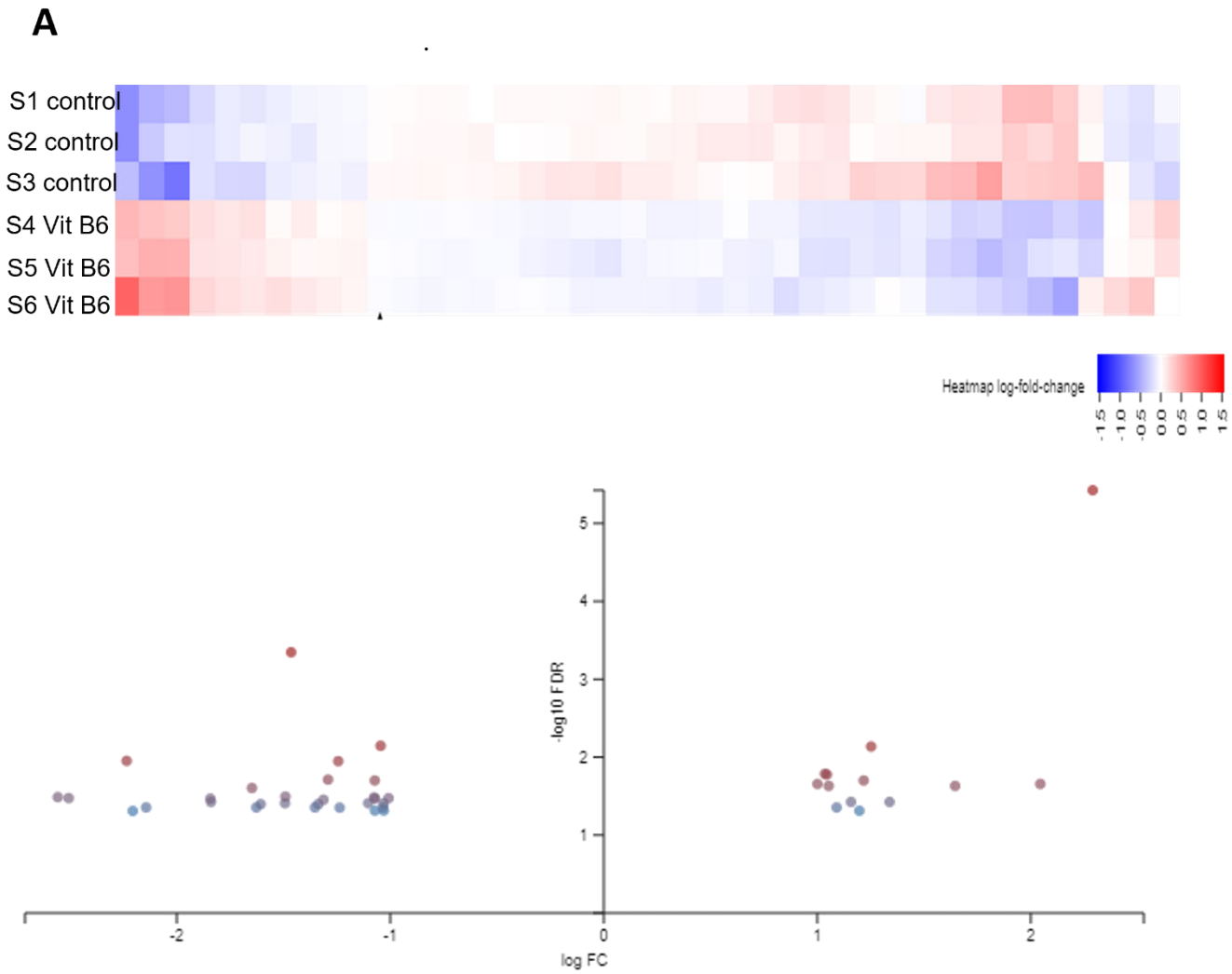



**Figure 27. Degust session, vitamin B6 versus control at time point = 24 hours: Identification of statistically differentially expressed genes**

Genes were identified as statistically differentially expressed (DEGs) using a minimum FDR $\leq$ 0.05, log2 fold change >1 (abs log FC=2). Using these parameters 42 DEGs were revealed of which 13 were upregulated and 29 were downregulated. (A) Heat map showing log fold change for each gene. Each vertical strip corresponds to a gene, with blue representing downregulated genes and red representing upregulated genes. (B) Volcano plot displaying statistical significance FDR $\leq$ 0.05 versus magnitude of fold change.

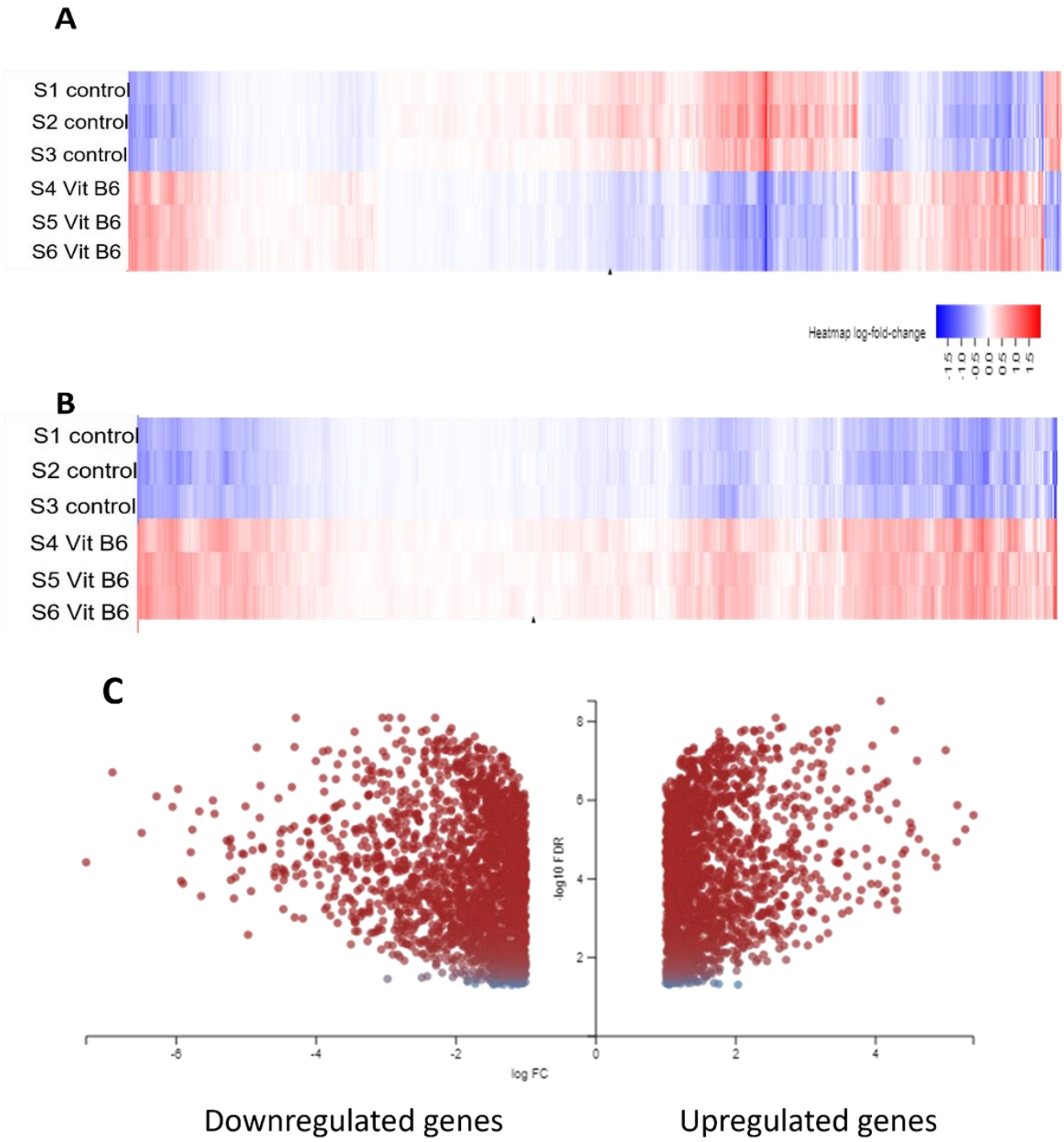



**Figure 28. Degust session, vitamin B6 versus control at time point = 6 days**:

Identification of statistically differentially expressed genes. Genes were identified as statistically differentially expressed (DEGs) using a minimum FDR < 0.05, log2 fold change>1 (abs log FC=2). Using these parameters 3996 DEGs were revealed, 1866 of these were upregulated whereas 2130 were downregulated. (A) Heat maps showing log fold change for all genes. Each vertical strip corresponds to a gene. Blue represents downregulated genes whilst red represents upregulated genes. (B) Heat map showing log fold change for upregulated genes. Each vertical strip corresponds to a gene. Blue represents downregulated genes whilst red represents upregulated genes. (C) Volcano plot displaying statistical significance FDR < 0.05, versus magnitude of fold change

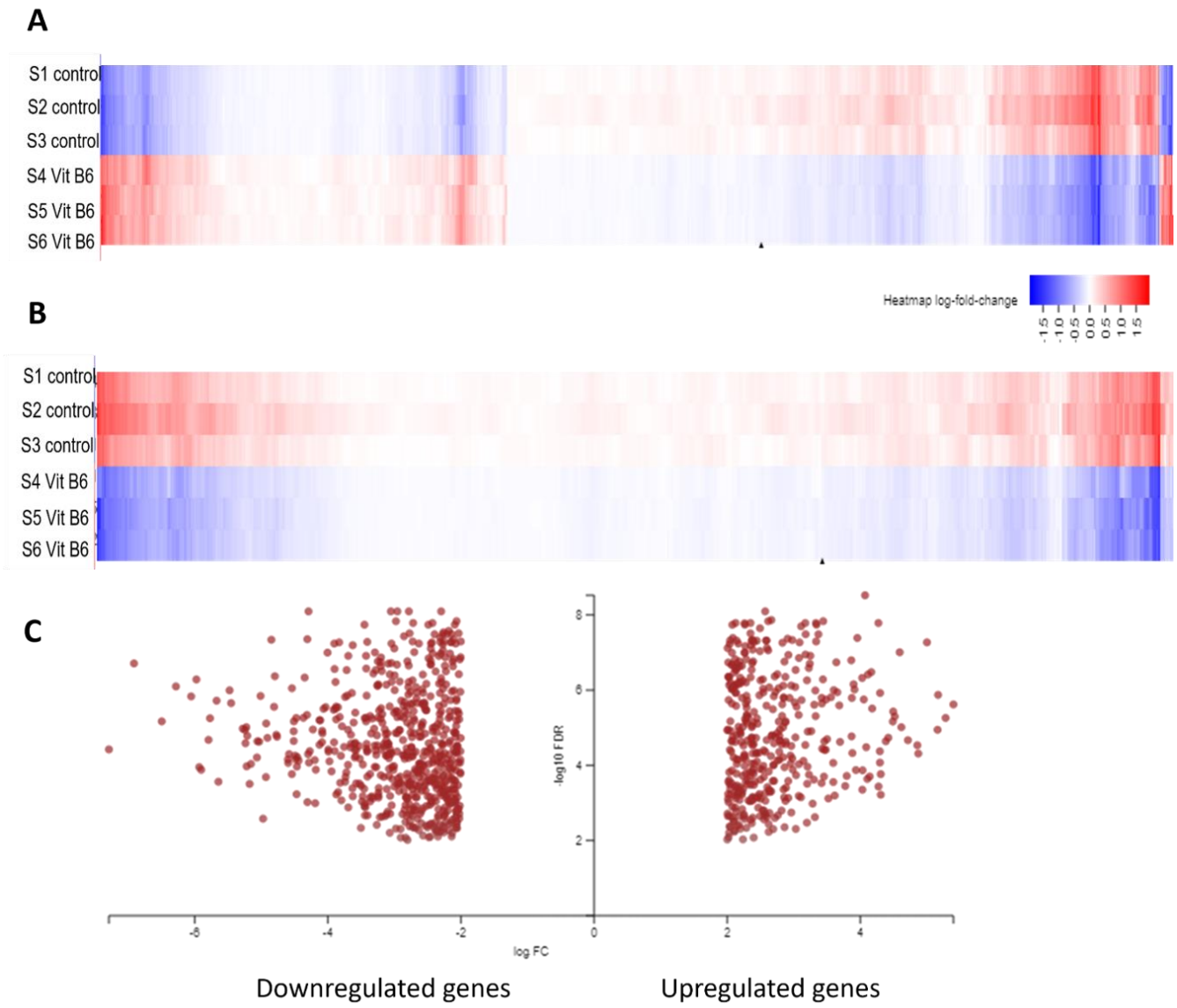



**Figure 29. Degust session, vitamin B6 versus control at time point = 6 days: Identification of statistically differentially expressed genes at higher statistical profile.**

Genes were identified as statistically differentially expressed (DEGs) using a minimum FDR $\leq$ 0.01, log2 fold change>2 (abs log FC = 4). Using these parameters 1042 DEGs were revealed, 409 of these were upregulated whereas 633 were downregulated. (A) Heat map showing log fold change for all genes. Each vertical strip corresponds to a gene. Blue represents downregulated genes whilst red represents upregulated genes. (B) Heat map depicting log fold change for up-regulated genes. Each vertical strip corresponds to a gene, with blue representing downregulated genes and red representing upregulated genes. (C) Volcano plot displaying statistical significance FDR $\leq$ 0.01 versus magnitude of fold change.

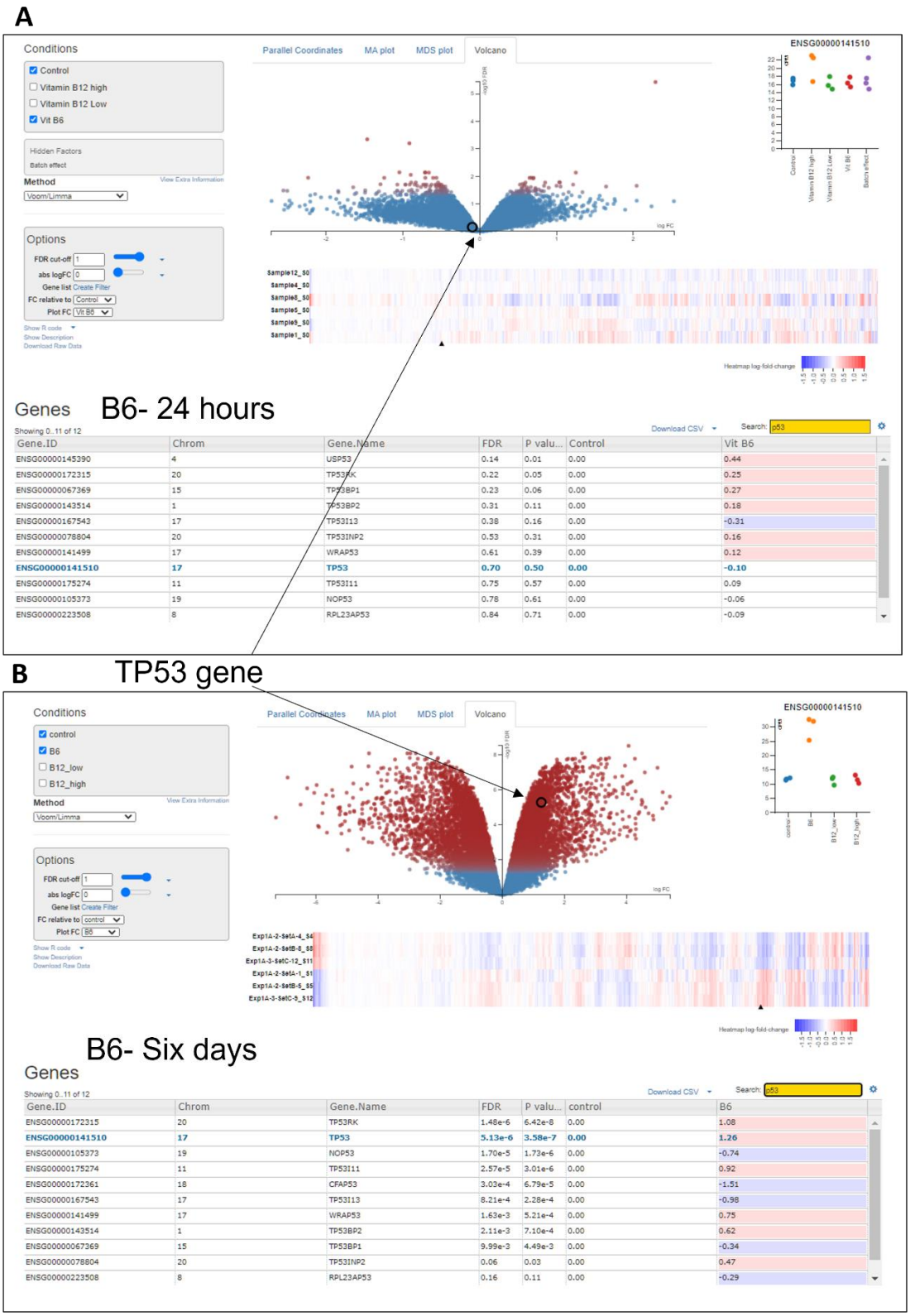



**Figure 30. Single gene evaluation of TP53 gene comparing 24-hour sample with 6-day sample**

Results indicate the significant increase in TP53 expression from 24- hour treatment with vitamin B6 compared to six-day treatment with vitamin B6.

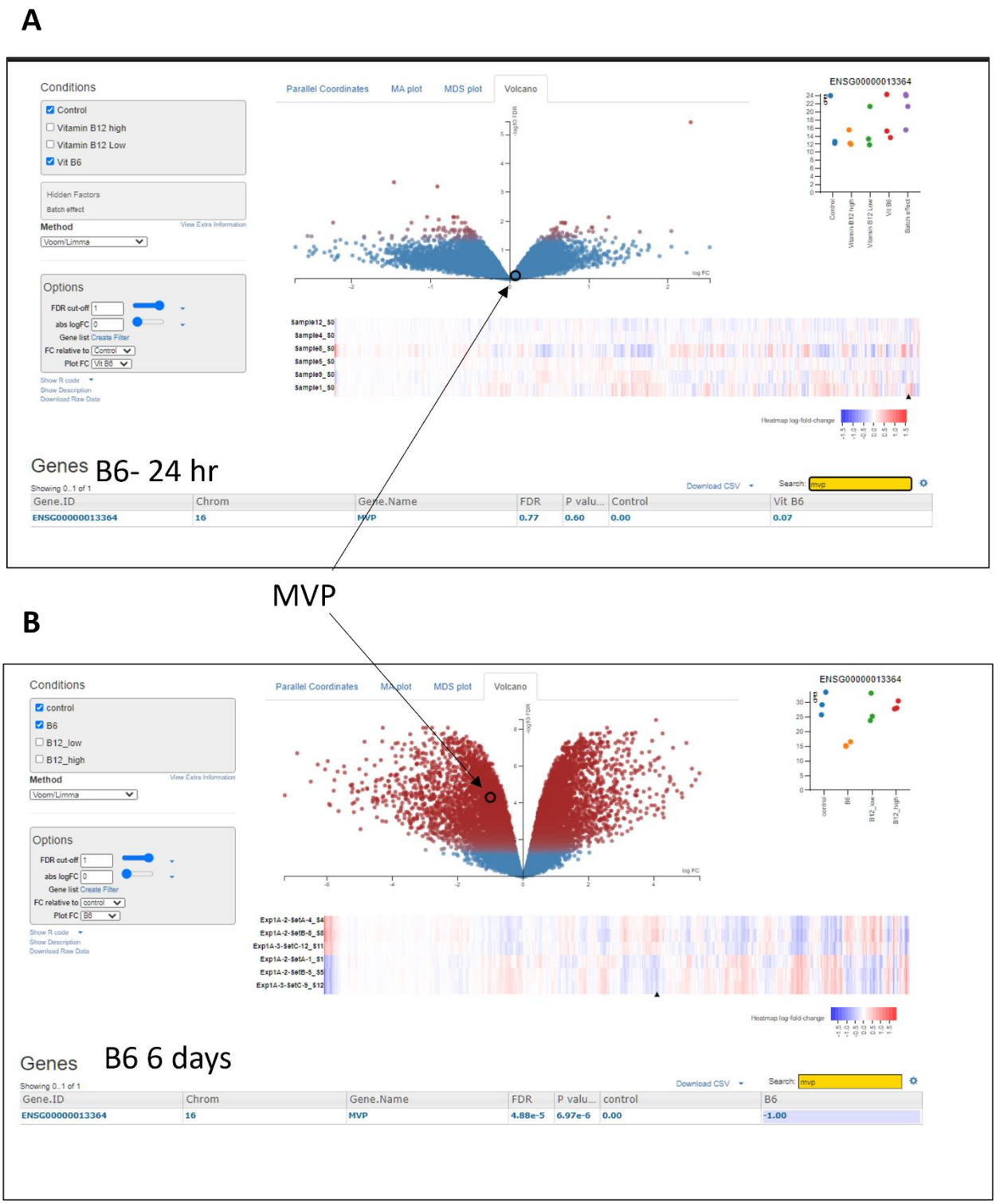



**Figure 31. Single gene evaluation of MVP gene comparing 24-hour sample with 6-day sample.**
Results indicate a significant change in the downregulation of MVP expression when comparing 24-hour vitamin B6 treatment to six-day vitamin B6 treatment.

### *4.3.4 Pathway enrichment analysis*

Pathway enrichment analysis identifies biological pathways in a specific gene list that are enriched more than would be expected by chance. The interface used for the interpretation of the gene lists generated by this research was g: Profiler.

G: Profiler provides data from multiple sources including Gene Ontology (GO); GO molecular function, describing the genes product functions, GO biological process, describing the biological process in which the gene participates, GO cellular component, describing the cellular compartment the gene resides in. Other biological pathways that were utilised through this program were KEGG, Reactome and WikiPathways. Additional pathway enrichment tools, including Reactome, ToppGene, GOrilla, EnRichr, David, Panther and String DB, were utilised in the early stages of data analysis (See appendix), g: Profiler, however, proved to be the most stringent of the pathway enrichment analysis tools as it provided multiple choices for data settings suitable to this study. These choices include recognition of Ensemble gene IDs, ordered query option which allows genes to be placed in a biologically meaningful order and identifies more specific functional terms associated with more dramatic changes, 'only annotated' versus the whole gene for the elimination of less verified genes that may produce more spurious results. g: Profiler also utilizes the g: SCS algorithm which is more sophisticated than the Bonferroni algorithm in computing multiple testing corrections for p-values gained from GO and pathway enrichment analysis in all tests the adjusted p-value of $\leq 0.05$ was used. The final justification for using this program is that g: Profiler streamlines data from multiple biological sources into easy-to-read interactive Manhattan graphs with accompanying tables, for visual clarity and homogeneity of data presentation (Reimand et al., 2019).

Gene ontology is frequently used to evaluate large-scale genomic or transcriptomic data. Up and downregulated genes were identified as statistically differentially expressed (DEGs),

using a minimum FDR $\leq$ 0.05, $\log_2$ fold change>1 (abs log FC=2). Total gene enrichment analysis was sought by comparing expression levels of (A). Both upregulated and downregulated genes together (B). Upregulated genes and (C) Downregulated genes. This was to ensure the capture of all pathways that may be relevant to the parameters of the experiment which was to examine all effects that vitamin B6 exerted on gene expression in cells with a carcinogenic profile. The resulting 3 gene lists were run through g: Profilers, g: Gost functional profiling tool using the following settings: Ensemble gene list of both up and downregulated genes, whitespace-separated. Organism option - *Homo sapiens*, following option-Ordered query. Advanced options: statistical domain scope option - only annotated genes, significance threshold option- g: SCS threshold, user threshold option- 0.05, numeric IDs treated as – ENTREZGENE_ACC. Data sources checked: Go molecular function, GO cellular component, GO biological process, Kegg, Reactome, and WikiPathways. This process was used for both day-6- and 24-hour incubation samples.

#### *4.3.4.1 Gene ontology GO molecular function (GO: MF)*

GO molecular function can encompass broad functional descriptions such as "Catalytic activity", or more precise functional descriptions such as adenylate cyclase activity. GO molecular function describes activities such as 'catalyses or 'transport', which occur within the cell at a molecular level and are often performed by individual gene products, some activities are also performed by multiple gene products.

Using the settings described above for the use of the g: Gost functional profiling tool, two significantly enriched GO: MF pathways were uncovered for all DEGs (6-day incubation), ubiquitin-*protein ligase binding GO:0031625*, *ubiquitin-like protein ligase*

*bindingGO:0044389 (*Figure 32, Table 2) One pathway was uncovered for GO: MF (24-hour) -*Acyltransferase activityGO:0016746* (Figure 33, Table 7).

For Upregulated DEGs (six-day) the following GO: MF pathways were deemed significant- Catalytic activity, acting on RNA GO:0140098, RNA methyltransferase activity GO:0008173, Methyltransferase activity GO:0008168, Exonuclease activity GO:0004527, Transferase activity, transferring one-carbon groups GO:001674,1 DNA replication origin binding GO:0003688, tRNA methyltransferase activity GO:0008175, tRNA (adenine) methyltransferase activity GO:0016426. (Figure 32 Table 4). For upregulated DEGs (24-hour) one significant pathway was upregulated –piRNA binding GO:0034584 (Figure 33, Table 8).

There were no significant GO: MF pathways detected for downregulated DEGs (6-day incubation) (Figure 32, Table 6) or GO: MF (24-hour) (Figure 33, Table 9).

### *4.3.4.2 Gene ontology Go Biological process (GO: BP)*

GO Biological process encompasses larger cellular processes that deal with multiple molecular activities such as 'regulation of cell cycle'. An example of a more specific process is 'regulation transcription involved in G1/S transition of mitotic cell cycle'. Using the settings described in 3.4., thirty-one significant pathways for uncovered for All DEGs (day-6) The GO:BP pathways uncovered involved- *ribosome biogenesisGO:0042254, leukocyte degranulation GO:0043299, granulocyte activation GO:0036230, myeloid leukocyte mediated immunity GO:0002444, neutrophil activation GO:0042119, T cell proliferation GO:0042098, leukocyte activation involved in immune response GO:0002366, neutrophil activation involved in immune response GO:0002283, cell activation involved in immune response GO:0002263, lymphocyte proliferation*

*GO:0046651, neutrophil-mediated immunity GO:0002446, myeloid cell activation involved in immune response GO:0002275, ribonucleoprotein complex biogenesis GO:0022613, ncRNA processing GO:0034470, mononuclear cell proliferation GO:0032943, neutrophil degranulation GO:0043312, regulation of protein localization to nucleolus GO:1904749, regulation of lymphocyte proliferation GO:0050670, leukocyte mediated immunity GO:0002443* (Figure 32, Table 2). GO: BP pathways uncovered for all DEGs at 24-hrs include - *Cholesterol biosynthetic process GO:0006695, Secondary alcohol biosynthetic process GO:1902653, Sterol biosynthetic process GO:0016126* (Figure 33, Table 7).

Upregulated DEGs (day-6) revealed 30 significant GO biological process pathways these included- NcRNA processing GO:0034470, Ribonucleoprotein complex biogenesis GO:0022613, NcRNA metabolic process GO:0034660, Ribosome biogenesis GO:0042254, rRNA processing GO:0006364, RRNA metabolic process GO:0016072, Ribosomal large subunit biogenesis GO:0042273, DNA replication GO:0006260, Endonucleolytic cleavage involved in rRNA processing GO:0000478, Endonucleolytic cleavage of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) GO:0000479, Ribosomal small subunit biogenesis GO:0042274, Regulation of cell cycle phase transition GO:1901987, Cell cycle phase transition GO:0044770, Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) GO:0000462, Negative regulation of cell cycle phase transition GO:1901988, Negative regulation of cell cycle process GO:0010948, RNA methylation GO:0001510, tRNA processing GO:0008033, Negative regulation of cell cycle GO:0045786, Regulation of mitotic cell cycle GO:0007346, Cleavage involved in rRNA processing GO:0000469, Regulation of cell cycle process GO:0010564, Maturation of SSU-rRNA GO:0030490, DNA-dependent DNA replication GO:0006261, Regulation of DNA topoisomerase (ATP-hydrolyzing) activity GO:2000371, Positive regulation of DNA topoisomerase (ATP-hydrolyzing) activity GO:2000373, Myeloid leukocyte mediated

immunity GO:0002444, G1/S transition of mitotic cell cycle GO:0000082, RNA phosphodiester bond hydrolysis, endonucleolytic GO:0090502 (Figure 32 Table 4). Upregulated DEGs (24-hour) showed no significantly upregulated pathways (Figure 32, Table 8).

There were no significant pathways found for downregulated DEGs (6-day) (Figure 32, Table 6). Downregulated DEGs (24-hour) showed 4 significant pathways - *cholesterol biosynthetic process GO:0006695, secondary alcohol biosynthetic process GO:1902653, sterol biosynthetic process GO:0016126, alcohol biosynthetic process GO:0046165* (Figure 33, Table 9).

#### *4.3.4.3 Gene ontology Go Cellular compartment (GO: CC)*

GO cellular component refers to the locations relative to the cellular anatomy in which the gene product performs a function, e.g., ribosome, mitochondria, nucleus. Adhering to the settings described in 3.4., four significant GO: CCs were uncovered for all DEGs (day-6), these were – *preribosome GO:0030684, nucleolus GO:0005730, ficolin-1-rich granule GO:0101002, ficolin-1-rich granule membrane GO:0101003* (Figure 32, Table 2). No significant GO: CC was detected for all DEGs (24-hrs) (Figure 33, Table 7).

For upregulated DEGs (day-6) there were 8 significant GO: CC pathways uncovered including Nucleolus GO:0005730, Preribosome GO:0030684, Ficolin-1-rich granule GO:0101002, CMG complex GO:0071162, Ribonucleoprotein complex GO:1990904, Small-subunit processome GO:0032040, 90S preribosome GO:0030686, Cyclin E1-CDK2 complex GO:0097134, Preribosome, small subunit precursor GO:0030688, DNA replication preinitiation complex GO:0031261 (Figure 32 Table 4). One significant GO: CC

was detected for up-regulated DEGS (24-hour time point) –cytosol GO:0005829 (Figure 33, Table 8).

Two significant GO: CCs were uncovered for downregulated DEGs (six-day) these were *Basement membrane GO:0005604, Cytoplasmic vesicle membrane GO:0030659* (Figure 32, Table 6). No significant GO: CC was detected for Down-regulated DEGs (24-hrs) (Figure 33, Table 9).

#### *4.3.4.4 Reactome, Kegg and WikiPathways*

g: Profiler in addition to Gene Ontology, also sources databases for biological pathways, including Reactome, KEGG and WikiPathways. Reactome is a curated pathways and reactions database which defines "reactions" as a biological event resulting in a change of state of a biological molecule. Reactome pathway visualisations highlight parent-child relationships and processes shared between pathways in a circular burst centred around nodes which represent the uppermost level of the Reactome hierarchy and become more specific as it branches outwards (Fabregat et al., 2018). Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology is a database of molecular functions represented as KEGG pathways maps dedicated to helping interpret gene function in large scale molecular datasets (Kanehisa & Goto, 2000).

#### *4.3.4.5 Reactome*

When analysing all DEGs, (six-day), the Reactome pathways uncovered included- TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle ArrestREAC: R-HSA-6804116, Aberrant regulation of mitotic G1/S transition in cancer due to RB1 defects, REAC: R-HSA-9659787, Defective binding of RB1 mutants to E2F1, (E2F2, E2F3) REAC:

R-HSA-9661069, (Figure 32, Table 2). All DEGs (24-hrs) revealed one Reactome pathway-Cholesterol biosynthesis REAC: R-HSA-191273 (Figure 33, Table 7).

Upregulated DEGs (day-6), via Reactome uncovered pathways which included-Defective binding of RB1 mutants to E2F1,(E2F2, E2F3) REAC: R-HSA-9661069, Aberrant regulation of mitotic G1/S transition in cancer due to RB1 defects, REAC: R-HSA-9659787, rRNA modification in the nucleus and cytosol REAC: R-HSA-6790901, Mitotic G1 phase and G1/S transition REAC: R-HSA-453279, TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest REAC: R-HSA-6804116, G1/S Transition REAC: R-HSA-69206, Major pathway of rRNA processing in the nucleolus and cytosol REAC: R-HSA-6791226, G1/S-Specific Transcription REAC: R-HSA-69205, rRNA processing REAC: R-HSA-72312, Aberrant regulation of mitotic cell cycle due to RB1 defects REAC: R-HSA-9687139 (Figure 33, Table 4). No significant pathways were detected for Upregulated DEGs (24-hrs) (Figure 33, Table 8).

Downregulated DEGs (six-day) did not uncover any Reactome pathways (Figure 33, Table 6). Downregulated DEGs (24-hrs) uncovered one pathway – *Cholesterol biosynthesis REAC: R-HSA-191273* (Figure 33, Table 9).

#### *4.3.4.6 WikiPathways*

WikiPathways is a curated database of biological pathways which supports data analysis and enables the visualisation of the myriad of interactions involved in biological processes (Slenter et al., 2018). In all DEGs (day-6), WikiPathways uncovered - *Nucleotide Metabolism WP: WP404* (Figure 32, Table 2). All DEGs (24-hrs) uncovered 2 pathways - Cholesterol *biosynthesis pathway WP: WP197, Mevalonate pathway WP: WP3963* (Figure 33, Table 7).

Upregulated DEGs (day-6), WikiPathways uncovered - TNF *related weak inducer of apoptosis (TWEAK) Signalling Pathway WP: WP2036* (Figure 32, Table 4). No significant pathways were uncovered for upregulated DEGs (24-hrs) (Figure 33, Table 8). Likewise, no significant pathways were uncovered for downregulated DEGs (day 6), (Figure 33, Table 6). Three significant pathways were uncovered for downregulated DEGs (24-hrs)- *Cholesterol Biosynthesis Pathway WP: WP197, Mevalonate pathway WP: WP3963, Cholesterol metabolism (includes both Bloch and Kandutsch-Russell pathways) WP: WP4718* (Figure 33, Table 9).

#### *4.3.4.7 KEGG*

All DEGs (24-hrs) uncovered one significant KEGG pathway - *Terpenoid backbone biosynthesis KEGG:00900* (Figure 33, Table 7). Downregulated DEGs (24-hrs) uncovered 2 KEGG pathways - *Terpenoid backbone biosynthesis KEGG:00900, Synthesis and degradation of ketone bodies KEGG:00072* (Figure 33, Table 9). No other significant KEGG pathways were revealed.

#### *4.3.4.8 Further analysis of significant Reactome pathways using DEGs with a higher statistical profile*

Due to the number of statistically significant pathways generated from the original analysis of DEGs (day 6), using a minimum FDR $\leq$ 0.05, $\log_2$ fold change >1 (abs log FC=2), it was decided to further refine this information by increasing the statistical profile of the original analysis. This was done by running the gene list through the DEGUST software using a minimum FDR $\leq$ 0.01, $\log_2$ fold change >2 (abs log FC=4) (Figure 28). Using these new parameters 1042 DEGs were revealed, 409 of these were upregulated whereas 633 were downregulated (see Figure 29). These DEGs were then run through REACTOME software and subsequent data were summarised in Table 3 and Table 6.

All DEGs (day-6), at this higher statistical profile uncovered the following significant REACTOME pathways -Interleukin-10 signalling, Dissolution of Fibrin Clot, RUNX3 regulates CDKN1A transcription, NGF-stimulated transcription, Regulation of TP53 Expression, ATF4 activates genes in response to endoplasmic reticulum stress, Interleukin-4 and Interleukin-13 signalling, TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest, Phosphorylation of proteins involved in G1/S transition by active Cyclin E:Cdk2 complexes, TFAP2 (AP-2) family regulates transcription of cell cycle factors, Transcriptional activation of p53 responsive genes, Transcriptional activation of cell cycle inhibitor p21, Cross-presentation of particulate exogenous antigens (phagosomes), Loss of Function of TGFBR1 in Cancer, PERK regulates gene expression, Acetylcholine inhibits contraction of outer hair cells, RUNX1 regulates expression of components of tight junctions, SDK interactions, Signalling by activated point mutants of FGFR3, Defective binding of RB1 mutants to E2F1,(E2F2, E2F3) (Table 3).

Upregulated DEGS (day-6), at the higher statistical profile uncovered the following significant REACTOME pathways, 1/S-Specific Transcription, NGF-stimulated transcription, Nuclear Events (kinase and transcription factor activation), G1/S Transition, Transcription of E2F targets under negative control by DREAM complex, Mitotic G1 phase and G1/S transition, Interleukin-10 signalling, Regulation of TP53 Expression, rRNA modification in the nucleus and cytosol, G0 and Early G1, Unwinding of DNA, TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest, Transcriptional activation of p53 responsive genes, Transcriptional activation of cell cycle inhibitor p21, Defective binding of RB1 mutants to E2F1, (E2F2, E2F3), Aberrant regulation of mitotic G1/S transition in cancer due to RB1 defects, Microtubule-dependent trafficking of connexons from Golgi to the plasma membrane, Transport of connexons to the plasma

membrane, HSP90 chaperone cycle for steroid hormone receptors (SHR) in the presence of ligand, Defective RIPK1-mediated regulated necrosis (Table 5).

The REACTOME pathways uncovered at the higher statistical profile (FDR $\leq$ 0.01, $\log_2$ fold change>2 (abs log FC=4)) profile were very similar to those uncovered by the lower statistical profile (FDR $\leq$ 0.05, $\log_2$ fold change>1 (abs log FC=2)) thus ascertaining that the effects of vitamin B6 on U937 lymphoma cells were scientifically robust.

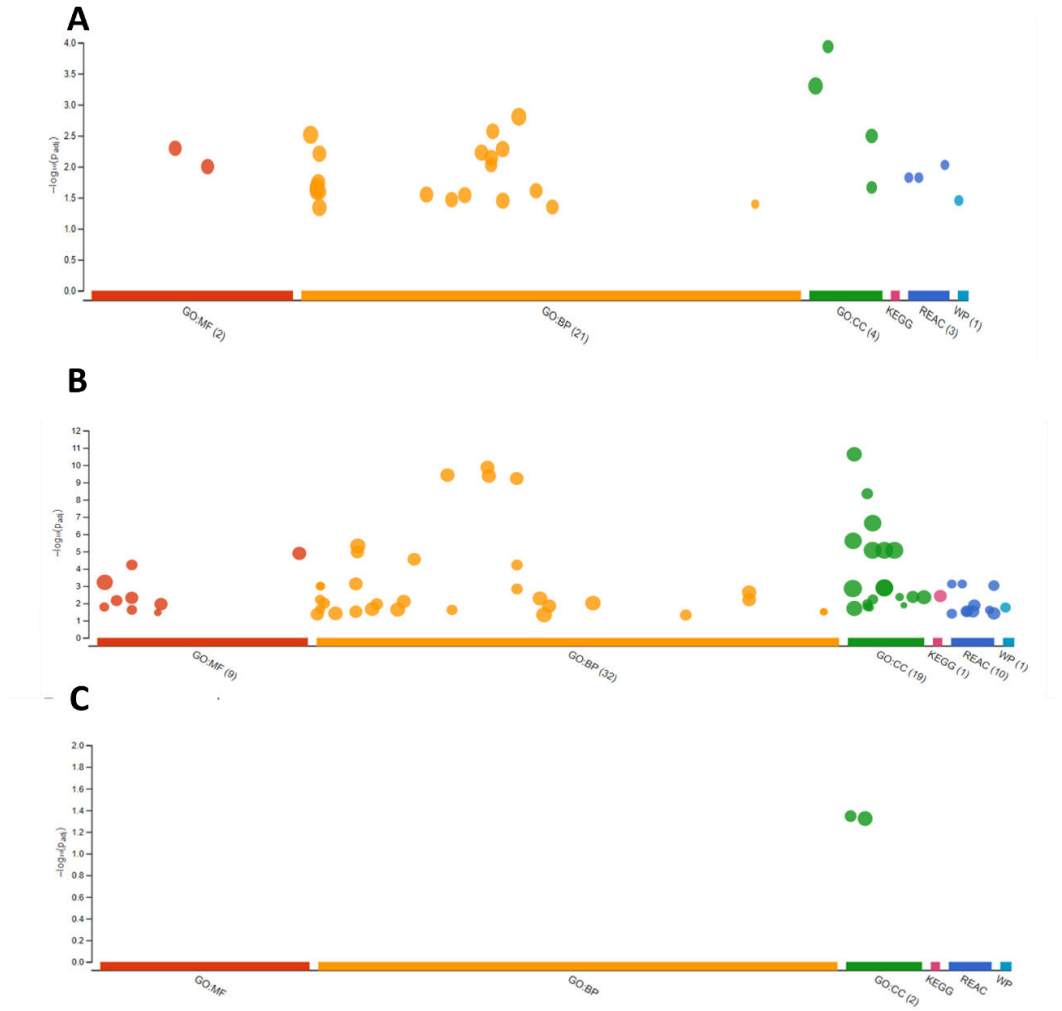



**Figure 32. DEGs - Significant pathways via g: Profiler tool. 6-day incubation.**

Promonocytic lymphoma cells were treated with vitamin B6 (250 µg/ml) for 6-days versus untreated. RNA was extracted and RNA-Seq was performed using an NGS platform at Micromon genomics (Monash University, Australia). MultiQC analysis ensured quality control. Data were analysed using the rnasik1.5-4 pipeline with STAR aligner, using the GRCh38 (Homo sapiens) reference genome and utilising DEGUST interface. Up and downregulated genes identified as statistically differentially expressed using a minimum FDR < 0.05, log2 fold change>1 (abs log FC=2) were analysed for pathway enrichment using G: profiler online interface encompassing GO MF, GO BP, GO CC, Reactome, Kegg and Wiki-pathways within a Manhattan plot illustrating enrichment analysis. X-axis = functional term names. Y-axis = adjusted enrichment p values in negative log scale. Circles sizes correspond to term size. (A). All DEGs. Corresponds to Table 1. (B). Upregulated DEGs. Corresponds to table 3. (C). Downregulated DEGs. (Corresponds to Table 5).

**Table 2 All DEGs (6-day) significant pathways via g:Profiler too**

| Source | Term name | Term_id | Adjusted_p_value |
|---|---|---|---|
| GO:MF | **ubiquitin protein ligase binding** | GO:0031625 | 0.005062 |
| GO:MF | **ubiquitin-like protein ligase binding** | GO:0044389 | 0.009999 |
| GO: BP | **ribosome biogenesis** | GO:0042254 | 0.002692 |
| GO: BP | **leukocyte degranulation** | GO:0043299 | 0.005222 |
| GO: BP | **granulocyte activation** | GO:0036230 | 0.005945 |
| GO: BP | **myeloid leukocyte mediated immunity** | GO:0002444 | 0.006192 |
| GO: BP | **neutrophil activation** | GO:0042119 | 0.007225 |
| GO: BP | **T cell proliferation** | GO:0042098 | 0.009402 |
| GO: BP | **leukocyte activation involved in immune response** | GO:0002366 | 0.018042 |
| GO: BP | **neutrophil activation involved in immune response** | GO:0002283 | 0.020397 |
| GO: BP | **cell activation involved in immune response** | GO:0002263 | 0.022774 |
| GO: BP | **lymphocyte proliferation** | GO:0046651 | 0.024576 |
| GO: BP | **neutrophil mediated immunity** | GO:0002446 | 0.025413 |
| GO: BP | **myeloid cell activation involved in immune response** | GO:0002275 | 0.025421 |
| GO: BP | **ribonucleoprotein complex biogenesis** | GO:0022613 | 0.028182 |
| GO: BP | **ncRNA processing** | GO:0034470 | 0.028875 |
| GO: BP | **mononuclear cell proliferation** | GO:0032943 | 0.034049 |
| GO: BP | **neutrophil degranulation** | GO:0043312 | 0.035414 |

**Table 3. Reactome pathways, all DEGs (6-day) at higher statistical profile.**

| Pathway name | pValue | FDR |
|---|---|---|
| **Interleukin-10 signalling** | 1.42E-03 | 9.58E-01 |
| **Dissolution of Fibrin Clot** | 2.45E-02 | 9.58E-01 |
| **RUNX3 regulates CDKN1A transcription** | 2.50E-02 | 9.58E-01 |
| **NGF-stimulated transcription** | 3.28E-02 | 9.58E-01 |
| **Regulation of TP53 Expression** | 3.91E-02 | 9.58E-01 |
| **ATF4 activates genes in response to endoplasmic reticulum stress** | 5.13E-02 | 9.58E-01 |
| **Interleukin-4 and Interleukin-13 signalling** | 6.79E-02 | 9.58E-01 |
| **TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest** | 7.18E-02 | 9.58E-01 |
| **Phosphorylation of proteins involved in G1/S transition by active Cyclin E: Cdk2 complexes** | 7.96E-02 | 9.58E-01 |
| **TFAP2 (AP-2) family regulates transcription of cell cycle factors** | 7.96E-02 | 9.58E-01 |
| **Transcriptional activation of p53 responsive genes** | 7.96E-02 | 9.58E-01 |
| **Transcriptional activation of cell cycle inhibitor p21** | 7.96E-02 | 9.58E-01 |
| **Cross-presentation of particulate exogenous antigens (phagosomes)** | 8.14E-02 | 9.58E-01 |
| **Loss of Function of TGFBR1 in Cancer** | 1.03E-01 | 9.58E-01 |
| **PERK regulates gene expression** | 1.11E-01 | 9.58E-01 |
| **Acetylcholine inhibits contraction of outer hair cells** | 1.28E-01 | 9.58E-01 |
| **RUNX1 regulates expression of components of tight junctions** | 1.28E-01 | 9.58E-01 |
| **SDK interactions** | 1.43E-01 | 9.58E-01 |
| **Signalling by activated point mutants of FGFR3** | 1.47E-01 | 9.58E-01 |
| **Defective binding of RB1 mutants to E2F1, (E2F2, E2F3)** | 1.47E-01 | 9.58E-01 |

**Table 4. Upregulated DEGs (6-day) significant pathways via g: Profiler tool.**

| Source | Term name | Term_id | Adjusted_p_value |
|---|---|---|---|
| GO:MF | Catalytic activity, acting on RNA | GO:0140098 | 1.28E-05 |
| GO:MF | RNA methyltransferase activity | GO:0008173 | 5.98E-05 |
| GO:MF | Methyltransferase activity | GO:0008168 | 0.004818148 |
| GO:MF | Exonuclease activity | GO:0004527 | 0.006948602 |
| GO:MF | Transferase activity, transferring one-carbon groups | GO:0016741 | 0.011135896 |
| GO:MF | DNA replication origin binding | GO:0003688 | 0.016374106 |
| GO:MF | Trna methyltransferase activity | GO:0008175 | 0.024047656 |
| GO:MF | Trna (adenine) methyltransferase activity | GO:0016426 | 0.034303039 |
| GO: BP | NcRNA processing | GO:0034470 | 1.35E-10 |
| GO: BP | Ribonucleoprotein complex biogenesis | GO:0022613 | 3.77E-10 |
| GO: BP | NcRNA metabolic process | GO:0034660 | 4.18E-10 |
| GO: BP | Ribosome biogenesis | GO:0042254 | 5.91E-10 |
| GO: BP | RRNA processing | GO:0006364 | 1.07E-05 |
| GO: BP | RRNA metabolic process | GO:0016072 | 2.81E-05 |
| GO: BP | Ribosomal large subunit biogenesis | GO:0042273 | 6.04E-05 |
| GO: BP | DNA replication | GO:0006260 | 0.000746421 |
| GO: BP | Endonucleolytic cleavage involved in rRNA processing | GO:0000478 | 0.001012775 |
| GO: BP | Endonucleolytic cleavage of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) | GO:0000479 | 0.001012775 |
| GO: BP | Ribosomal small subunit biogenesis | GO:0042274 | 0.001478816 |
| GO: BP | Regulation of cell cycle phase transition | GO:1901987 | 0.002286409 |

| | | | |
|---|---|---|---|
| GO: BP | Cell cycle phase transition | GO:0044770 | 0.005251461 |
| GO: BP | Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) | GO:0000462 | 0.006164824 |
| GO: BP | Negative regulation of cell cycle phase transition | GO:1901988 | 0.006286035 |
| GO: BP | Negative regulation of cell cycle process | GO:0010948 | 0.007748847 |
| GO: BP | RNA methylation | GO:0001510 | 0.009951804 |
| GO: BP | Trna processing | GO:0008033 | 0.011013806 |
| GO: BP | Negative regulation of cell cycle | GO:0045786 | 0.014406984 |
| GO: BP | Regulation of mitotic cell cycle | GO:0007346 | 0.021425263 |
| GO: BP | Cleavage involved in rRNA processing | GO:0000469 | 0.02166199 |
| GO: BP | Regulation of cell cycle process | GO:0010564 | 0.022879642 |
| GO: BP | Maturation of SSU-rRNA | GO:0030490 | 0.024181081 |
| GO: BP | DNA-dependent DNA replication | GO:0006261 | 0.030569425 |
| GO: BP | Regulation of DNA topoisomerase (ATP-hydrolyzing) activity | GO:2000371 | 0.030785415 |
| GO: BP | Positive regulation of DNA topoisomerase (ATP-hydrolyzing) activity | GO:2000373 | 0.030785415 |
| GO: BP | Myeloid leukocyte mediated immunity | GO:0002444 | 0.038211449 |
| GO: BP | G1/S transition of mitotic cell cycle | GO:0000082 | 0.042711854 |
| GO: BP | RNA phosphodiester bond hydrolysis, endonucleolytic | GO:0090502 | 0.047620551 |
| GO:CC | Nucleolus | GO:0005730 | 2.34E-11 |
| GO:CC | Preribosome | GO:0030684 | 4.49E-09 |
| GO:CC | Ficolin-1-rich granule | GO:0101002 | 0.004256258 |
| GO:CC | CMG complex | GO:0071162 | 0.004321813 |
| GO:CC | Ribonucleoprotein complex | GO:1990904 | 0.004431086 |
| GO:CC | Small subunit processome | GO:0032040 | 0.005979163 |
| GO:CC | 90S preribosome | GO:0030686 | 0.010856685 |
| GO:CC | Cyclin E1-CDK2 complex | GO:0097134 | 0.012953704 |
| GO:CC | Preribosome, small subunit precursor | GO:0030688 | 0.015832844 |

| | | | |
|---|---|---|---|
| **GO:CC** | **DNA replication preinitiation complex** | **GO:0031261** | **0.017278998** |
| **KEGG** | **Epstein-Barr virus infection** | **KEGG:05169** | **0.003780273** |
| **REAC** | **Defective binding of RB1 mutants to E2F1,(E2F2, E2F3)** | **REAC: R-HSA-9661069** | **0.000763627** |
| **REAC** | **Aberrant regulation of mitotic G1/S transition in cancer due to RB1 defects** | **REAC: R-HSA-9659787** | **0.000763627** |
| **REAC** | **rRNA modification in the nucleus and cytosol** | **REAC: R-HSA-6790901** | **0.000941414** |
| **REAC** | **Mitotic G1 phase and G1/S transition** | **REAC: R-HSA-453279** | **0.013146328** |
| **REAC** | **TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest** | **REAC: R-HSA-6804116** | **0.02440989** |
| **REAC** | **G1/S Transition** | **REAC: R-HSA-69206** | **0.029155431** |
| **REAC** | **Major pathway of rRNA processing in the nucleolus and cytosol** | **REAC: R-HSA-6791226** | **0.029223774** |
| **REAC** | **G1/S-Specific Transcription** | **REAC: R-HSA-69205** | **0.030537768** |
| **REAC** | **RRNA processing** | **REAC: R-HSA-72312** | **0.038325352** |
| **REAC** | **Aberrant regulation of mitotic cell cycle due to RB1 defects** | **REAC: R-HSA-9687139** | **0.039953204** |
| **WP** | **TNF related weak inducer of apoptosis (TWEAK) Signaling Pathway** | **WP: WP2036** | **0.017530835** |

**Table 5. Reactome pathways using upregulated DEGs (6-day) at higher statistical profile.**

| Pathway name | pValue | FDR |
|---|---|---|
| G1/S-Specific Transcription | 1.70E-05 | 2.79E-02 |
| NGF-stimulated transcription | 4.81E-04 | 2.81E-01 |
| Nuclear Events (kinase and transcription factor activation) | 5.47E-04 | 2.81E-01 |
| G1/S Transition | 8.86E-04 | 2.81E-01 |
| Transcription of E2F targets under negative control by DREAM complex | 9.83E-04 | 2.81E-01 |
| Mitotic G1 phase and G1/S transition | 1.03E-03 | 2.81E-01 |
| Interleukin-10 signalling | 1.43E-03 | 3.36E-01 |
| Regulation of TP53 Expression | 2.60E-03 | 5.32E-01 |
| rRNA modification in the nucleus and cytosol | 3.06E-03 | 5.35E-01 |
| G0 and Early G1 | 3.27E-03 | 5.35E-01 |
| Unwinding of DNA | 7.44E-03 | 9.39E-01 |
| TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest | 7.95E-03 | 9.39E-01 |
| Transcriptional activation of p53 responsive genes | 1.06E-02 | 9.39E-01 |
| Transcriptional activation of cell cycle inhibitor p21 | 1.06E-02 | 9.39E-01 |
| Defective binding of RB1 mutants to E2F1, (E2F2, E2F3) | 1.09E-02 | 9.39E-01 |
| Aberrant regulation of mitotic G1/S transition in cancer due to RB1 defects | 1.09E-02 | 9.39E-01 |
| Microtubule-dependent trafficking of connexons from Golgi to the plasma membrane | 1.34E-02 | 9.39E-01 |
| Transport of connexons to the plasma membrane | 1.70E-02 | 9.39E-01 |
| HSP90 chaperone cycle for steroid hormone receptors (SHR) in the presence of ligand | 2.78E-02 | 9.39E-01 |
| Defective RIPK1-mediated regulated necrosis | 3.39E-02 | 9.39E-01 |

**Table 6. Reactome pathways using downregulated DEGs (6-day).**

| Source | Term name | Term ID | Adjusted_p_value |
|---|---|---|---|
| GO:CC | **Basement membrane** | GO:0005604 | 0.045080204 |
| GO: CC | **Cytoplasmic vesicle membrane** | GO:0030659 | 0.047478226 |

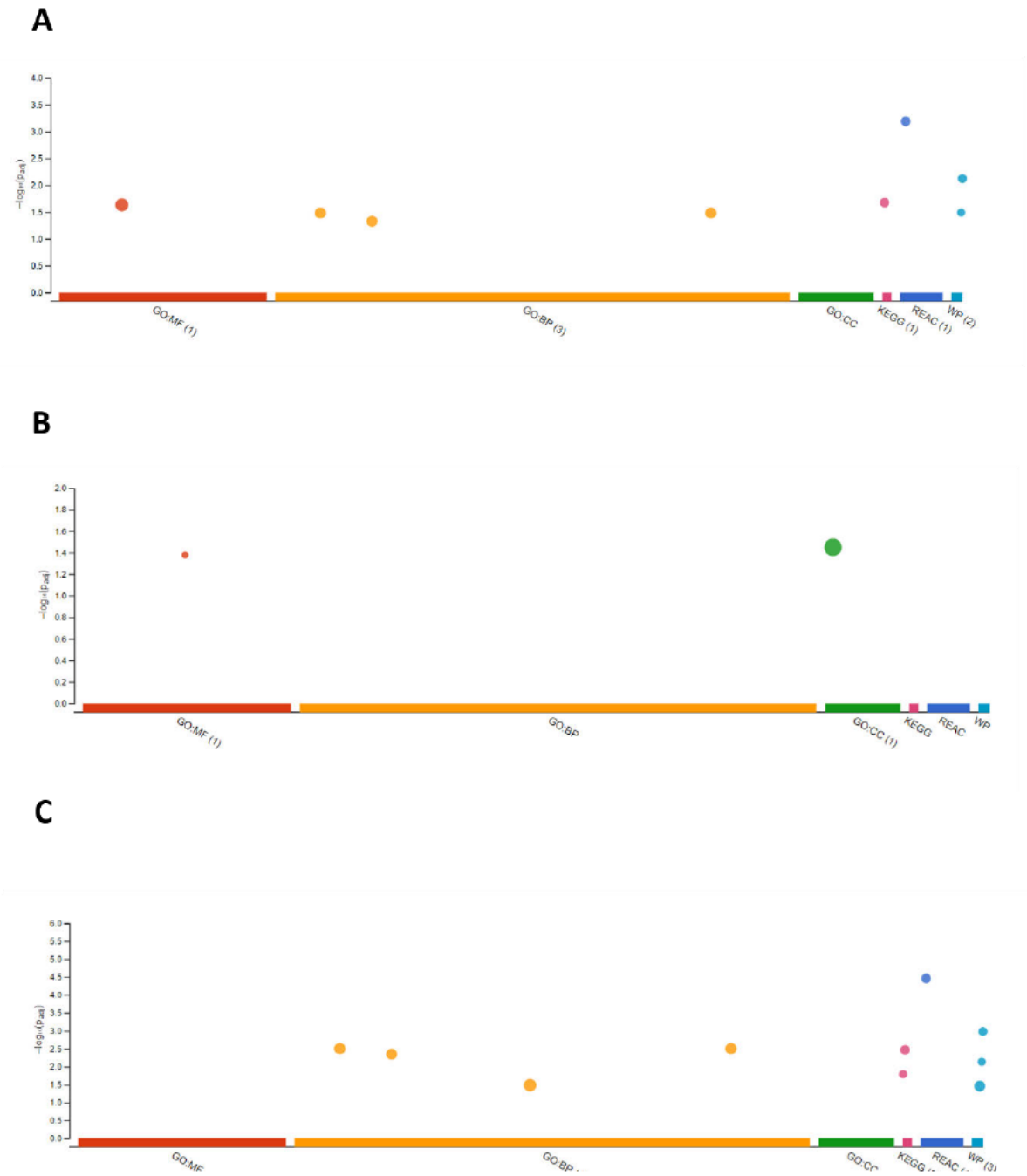



**Figure 33. All Genes - Significant pathways via g: Profiler tool at 24-hours.**

Promonocytic lymphoma cells were treated with vitamin B6 (250ug/ml) for 24-hours versus untreated. RNA was extracted and RNA-Seq performed using an NGS platform at Micromon genomics (Monash University). MultiQC analysis ensured quality control. Data was analyzed using the nasik1.5-4 pipeline with STAR aligner, using the GRCh38 (Homo sapiens) reference genome and utilising DEGUST interface. Up and downregulated genes identified as statistically differentially expressed using a minimum FDR $\leq 0.05$, log2 fold change>1 (abs log FC=2) was analysed for pathway enrichment using G: profiler online interface encompassing GO MF, GO BP, GO CC, Reactome, Kegg and Wiki-pathways within a Manhattan plot illustrating enrichment analysis. X axis = functional term names. Y axis = adjusted enrichment p values in negative log scale. Circles sizes correspond to term size. **(A)** All DEGs – corresponding to Table 6. **(B)** Upregulated DEGs corresponding to table 7 **and (C)** Downregulated DEGs – corresponding Table 8.

**Table 7. All DEGs at 24 hrs.**

| Source | Term name | Term_ID | Adjusted_p_value |
|---|---|---|---|
| GO:MF | **Acyltransferase activity** | GO:0016746 | 0.023220177 |
| GO: BP | **Cholesterol biosynthetic process** | GO:0006695 | 0.032794776 |
| GO: BP | **Secondary alcohol biosynthetic process** | GO:1902653 | 0.032794776 |
| GO: BP | **Sterol biosynthetic process** | GO:0016126 | 0.046863994 |
| KEGG | **Terpenoid backbone biosynthesis** | KEGG:00900 | 0.021041918 |
| REAC | **Cholesterol biosynthesis** | REAC: R-HSA-191273 | 0.000645034 |
| WP | **Cholesterol biosynthesis pathway** | WP: WP197 | 0.007528312 |
| WP | **Mevalonate pathway** | WP: WP3963 | 0.032116523 |

**Table 8. Up-regulated DEGs at 24 hours.**

| Source | Term name | Term_id | Adjusted_p_value |
|---|---|---|---|
| GO:MF | **piRNA binding** | GO:0034584 | 0.041972268 |
| GO:CC | **cytosol** | GO:0005829 | 0.03558133 |

**Table 9. Down-regulated DEGs 24 hrs.**

| Source | Term name | Term_id | Adjusted_p_value |
|---|---|---|---|
| GO: BP | **cholesterol biosynthetic process** | GO:0006695 | 0.003128354 |
| GO: BP | **secondary alcohol biosynthetic process** | GO:1902653 | 0.003128354 |
| GO: BP | **sterol biosynthetic process** | GO:0016126 | 0.004489381 |
| GO: BP | **alcohol biosynthetic process** | GO:0046165 | 0.032799072 |
| KEGG | **Terpenoid backbone biosynthesis** | KEGG:00900 | 0.00339371 |
| KEGG | **Synthesis and degradation of ketone bodies** | KEGG:00072 | 0.016096404 |
| REAC | **Cholesterol biosynthesis** | REAC: R-HSA-191273 | 3.49E-05 |
| WP | **Cholesterol Biosynthesis Pathway** | WP: WP197 | 0.001047125 |
| WP | **Mevalonate pathway** | WP: WP3963 | 0.007343958 |
| WP | **Cholesterol metabolism (includes both Bloch and Kandutsch-Russell pathways)** | WP: WP4718 | 0.034799298 |

### *4.3.5 REVIGO pathway visualisation for Gene ontology*

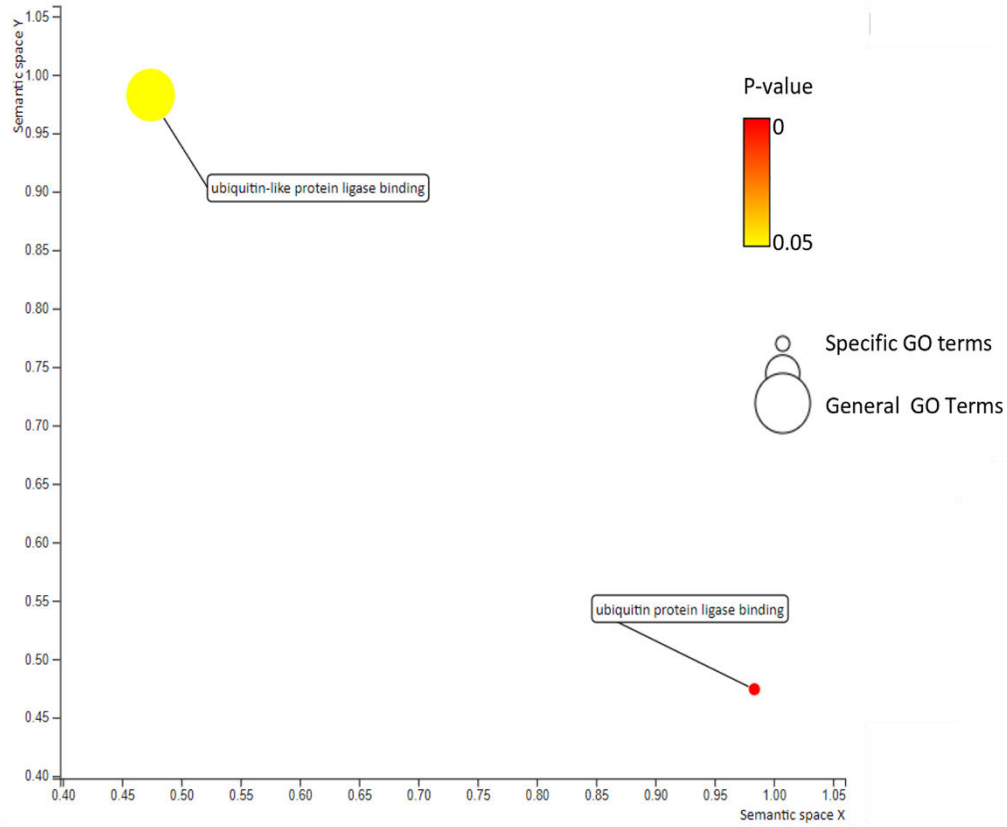
Due to the volume of information produced by NGS, it can often be a challenging process to summarize and interpret the results of genome-scale experiments. GO pathway enrichment analysis results are generated as a flat list of overrepresented GO terms which fails to capture the intricacy of hierarchical structure (Supek. Fran, 2017). REVIGO (Reduce and visualize gene ontology) is a visualisation tool that assists in the interpretation of large lists of GO terms generated by RNA-seq, by providing additional visual elements such as scatterplots, treemaps, and interactive graphs using values that describe the GO term in a meaningful way. For instance, the value may be inputted as P-value, fold change, enrichment etc.

Many genes were up or down-regulated as a response to the addition of vitamin B6 in cell culture and categorised into GO pathways relating to molecular function, biological process, and cellular compartment. These pathways were imported from g: Profiler and run through the REVIGO tool as two lists. List one was the GO term Id and list two was its corresponding P-Value that had previously been generated by g: profiler. Default settings of REVIGO tool were used. The scatterplot view visualizes the GO terms in a "semantic space" where the more similar terms are positioned closer together. The colour of the bubble reflects the p-value obtained in the g: Profiler analysis. The size of the bubble reflects the specificity of GO terms whereby smaller bubbles represent more specific terms and larger bubbles are more general terms. The treemap displays clustered terms visualised by coloured tiles. The interactive graph connects GO terms based on the structure of GO hierarchy (Interactive graph function was not included in the figures below).

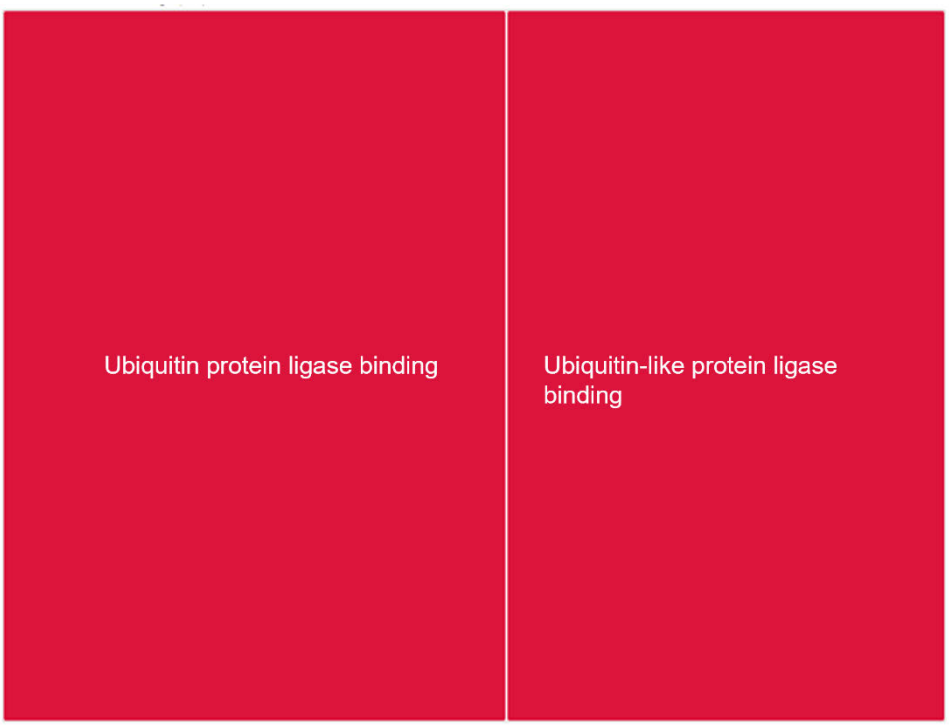
Significant pathways were visualised for **(1)** All DEGs, 6-days incubation of GO: BP, GO: MF, GO: CC (Figure 34); **(2)** Upregulated DEGs 6-days incubation of GO: BP, GO: MF, GO: CC (Figure 35); **(3)** Downregulated pathways 6-days incubation were deemed not relevant for analysis and these have been moved to appendix 1; **(4)** All DEGs 24-hrs incubation GO: BP

(Figure 36) (GO: MF and GO: CC not visualised due to lack of data) (**5**); Upregulated DEGs 24 hrs incubation pathways were deemed not relevant for analysis due to lack of data and these have been moved to appendix 1, and, **(6)** Downregulated DEGs 24-hrs incubation GO: BP (Figure 36) GO: MF and GO: CC not visualised due to lack of data).

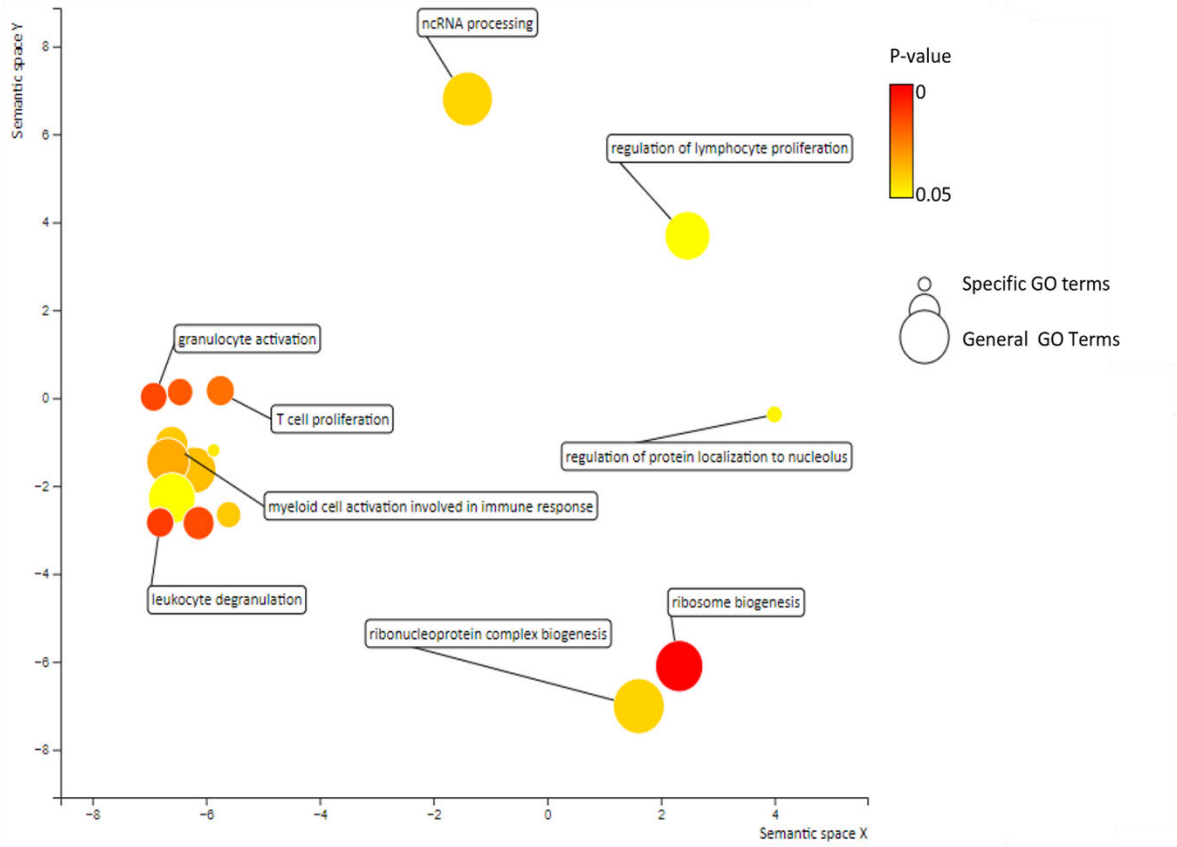
A


Semantic space Y
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
ubiquitin-like protein ligase binding
ubiquitin protein ligase binding
P-value
0
0.05
Specific GO terms
General GO Terms
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0.75
0.80
0.85
0.90
0.95
1.00
1.05
Semantic space X

B


Ubiquitin protein ligase binding
Ubiquitin-like protein ligase binding

C


ncRNA processing
regulation of lymphocyte proliferation
granulocyte activation
T cell proliferation
regulation of protein localization to nucleolus
myeloid cell activation involved in immune response
leukocyte degranulation
ribosome biogenesis
ribonucleoprotein complex biogenesis
P-value
0
0.05
Specific GO terms
General GO Terms
Semantic space Y
Semantic space X

D


Ribosome biogenesis
Leukocyte degranulation
Neutrophil activation
Neutrophil Mediated immunity
Leukocyte activation Involved in Immune response
Granulocyte Activation
ncRNA processing
Ribonucleoprotein Complex biogenesis
T cell proliferation
Cell activation Involved in Immune response
Neutrophil degranulation
Regulation of Lymphocyte proliferation
Regulation of protein Localization to nucleolus
Myeloid leukocyte mediated Immunity
Leukocyte mediated Immunity
Myeloid cell activation Involved in immune response

.

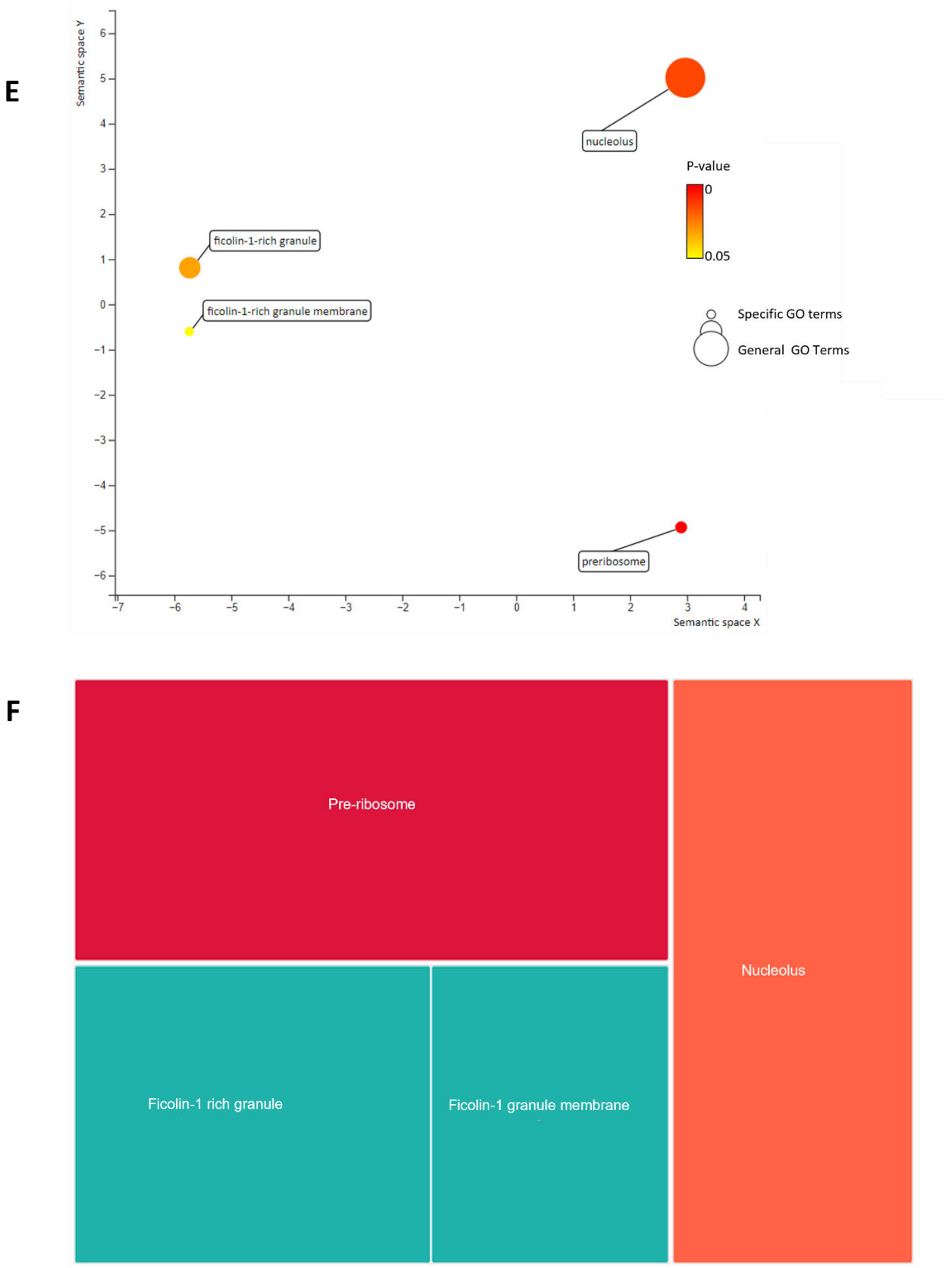
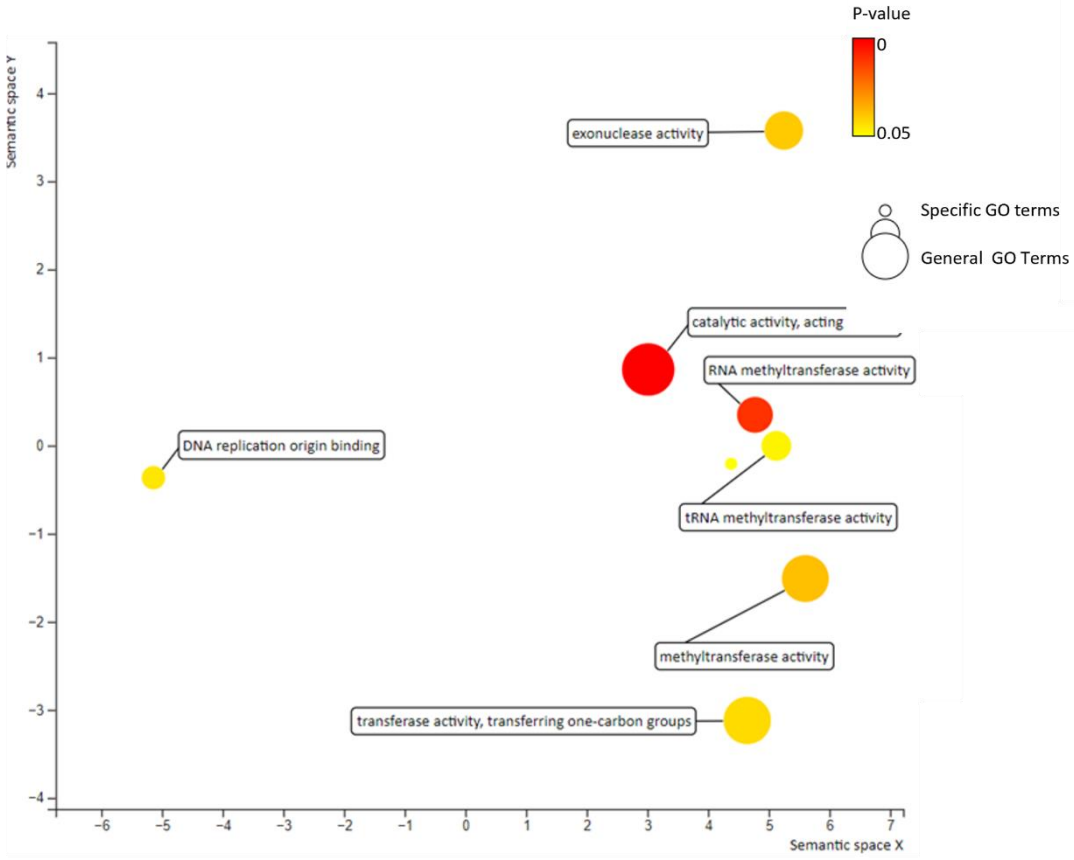



**Figure 34. REVIGO Visualisations: All DEGs, 6-days incubation.**

Visualisations of **(A-B).** Molecular function **(C-D).** Biological Process and **(E-F).** Cellular compartment GO annotations using REVIGO: scatterplot view, and REVIGO: Tree map. The dataset used was imported from g: Profiler. Default settings of REVIGO tool were used. The scatterplot view visualizes the GO terms in a "semantic space" where the more similar terms are positioned closer together. The color of the bubble reflects the p-value obtained in the g: Profiler analysis. The size of the bubble reflects specificity of GO terms whereby smaller bubbles represent more specific terms and larger bubbles more general terms. The tree map displays clustered terms visualised by coloured tiles. The interactive graph connects GO terms based on structure of GO hierarchy

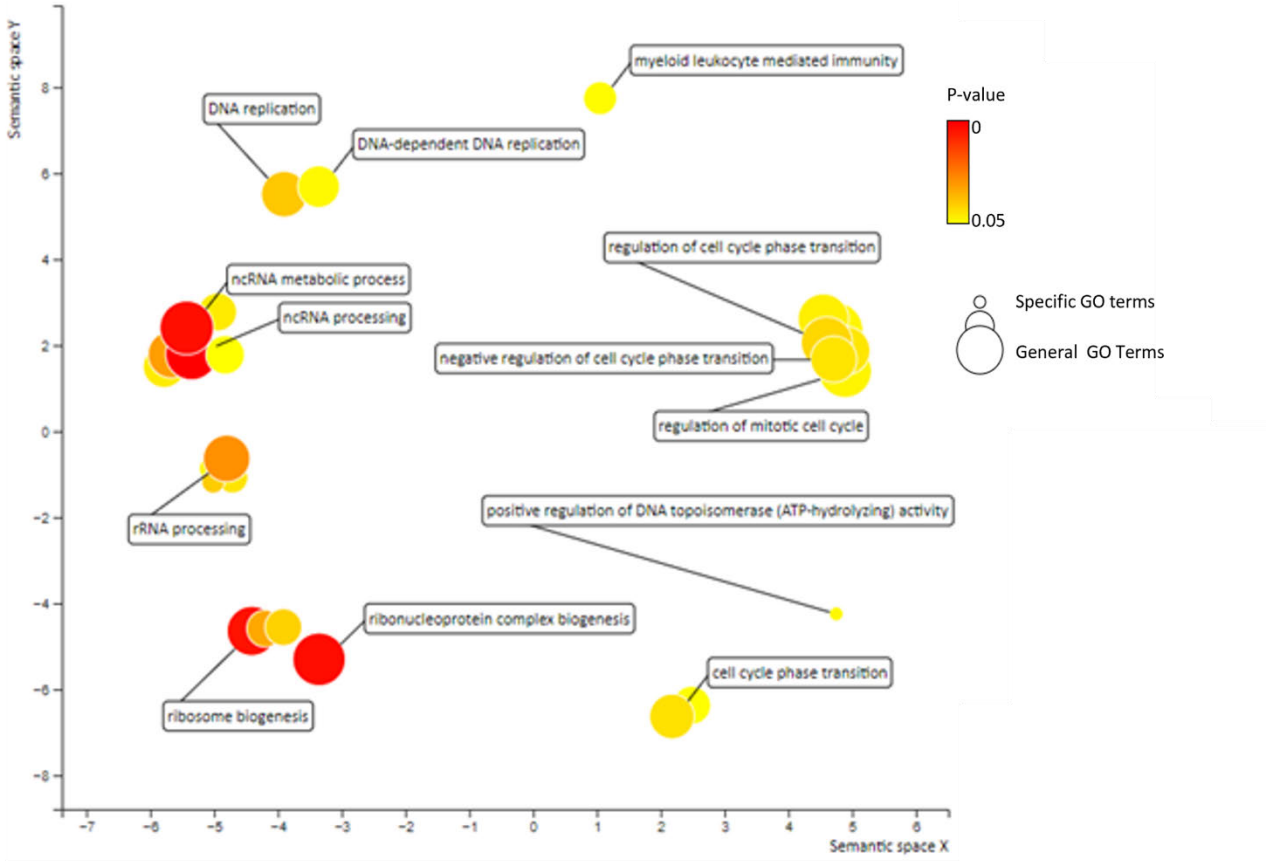
**A**


P-value
0
0.05
Specific GO terms
General GO Terms
exonuclease activity
catalytic activity, acting
RNA methyltransferase activity
DNA replication origin binding
tRNA methyltransferase activity
methyltransferase activity
transferase activity, transferring one-carbon groups
Semantic space Y
Semantic space X

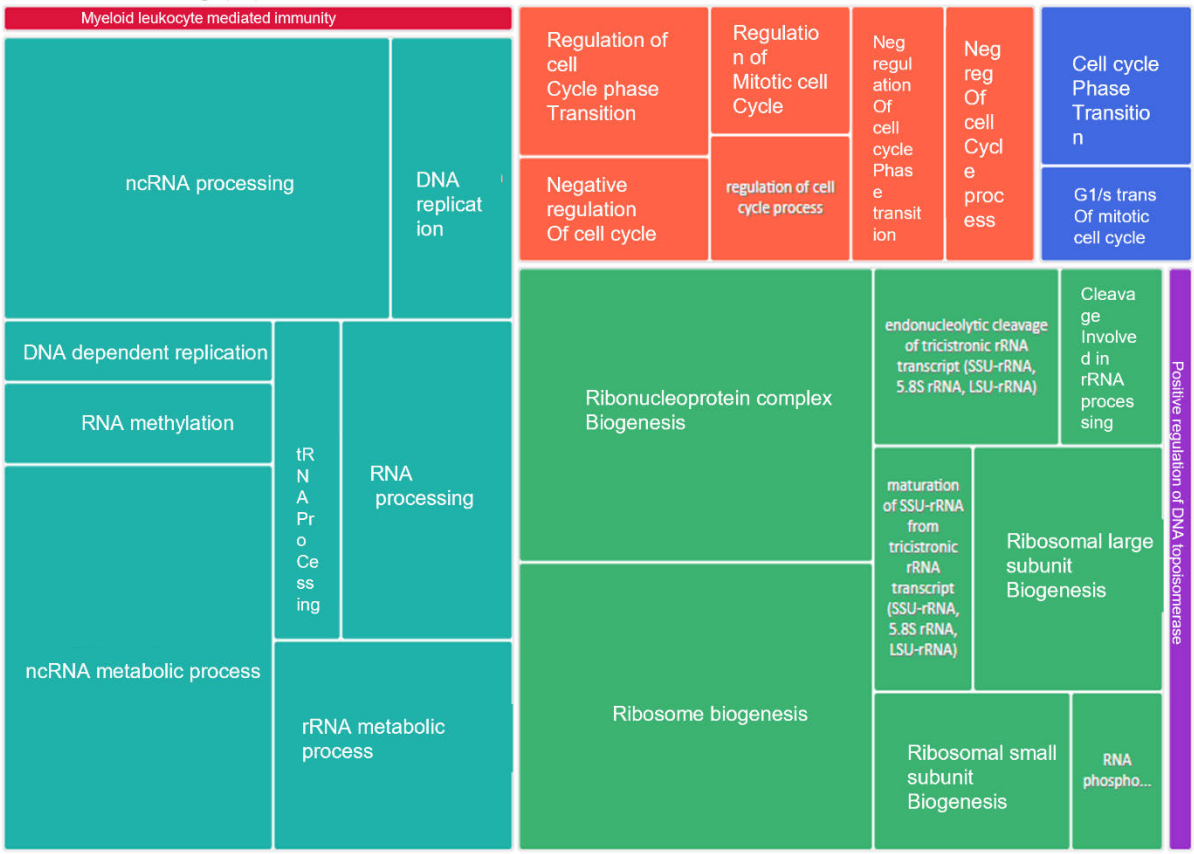
**B**


DNA replication origin binding
Catalytic activity , acting on RNA
Transferase activity ,transferring one-carbon groups
tRNA (adenine methyltransferase activity
Methyltransferase activity
RNA methyltransferase activity
Exonuclease activity
tRNA methyltransferase activity

C


myeloid leukocyte mediated immunity
DNA replication
DNA-dependent DNA replication
regulation of cell cycle phase transition
ncRNA metabolic process
ncRNA processing
negative regulation of cell cycle phase transition
regulation of mitotic cell cycle
rRNA processing
positive regulation of DNA topoisomerase (ATP-hydrolyzing) activity
ribonucleoprotein complex biogenesis
cell cycle phase transition
ribosome biogenesis
P-value
0
0.05
Specific GO terms
General GO Terms
Semantic space Y
Semantic space X

D


Myeloid leukocyte mediated immunity
ncRNA processing
DNA replicat ion
DNA dependent replication
RNA methylation
ncRNA metabolic process
tR N A Pr o Ce ss ing
RNA processing
rRNA metabolic process
Regulation of cell Cycle phase Transition
Regulatio n of Mitotic cell Cycle
Neg regul ation Of cell cycle Phas e transit ion
Neg reg Of cell Cycl e proc ess
Cell cycle Phase Transitio n
Negative regulation Of cell cycle
regulation of cell cycle process
G1/s trans Of mitotic cell cycle
Ribonucleoprotein complex Biogenesis
endonucleolytic cleavage of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
Cleava ge Involve d in rRNA proces sing
maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
Ribosomal large subunit Biogenesis
Ribosome biogenesis
Ribosomal small subunit Biogenesis
RNA phospho...
Positive regulation of DNA topoisomerase

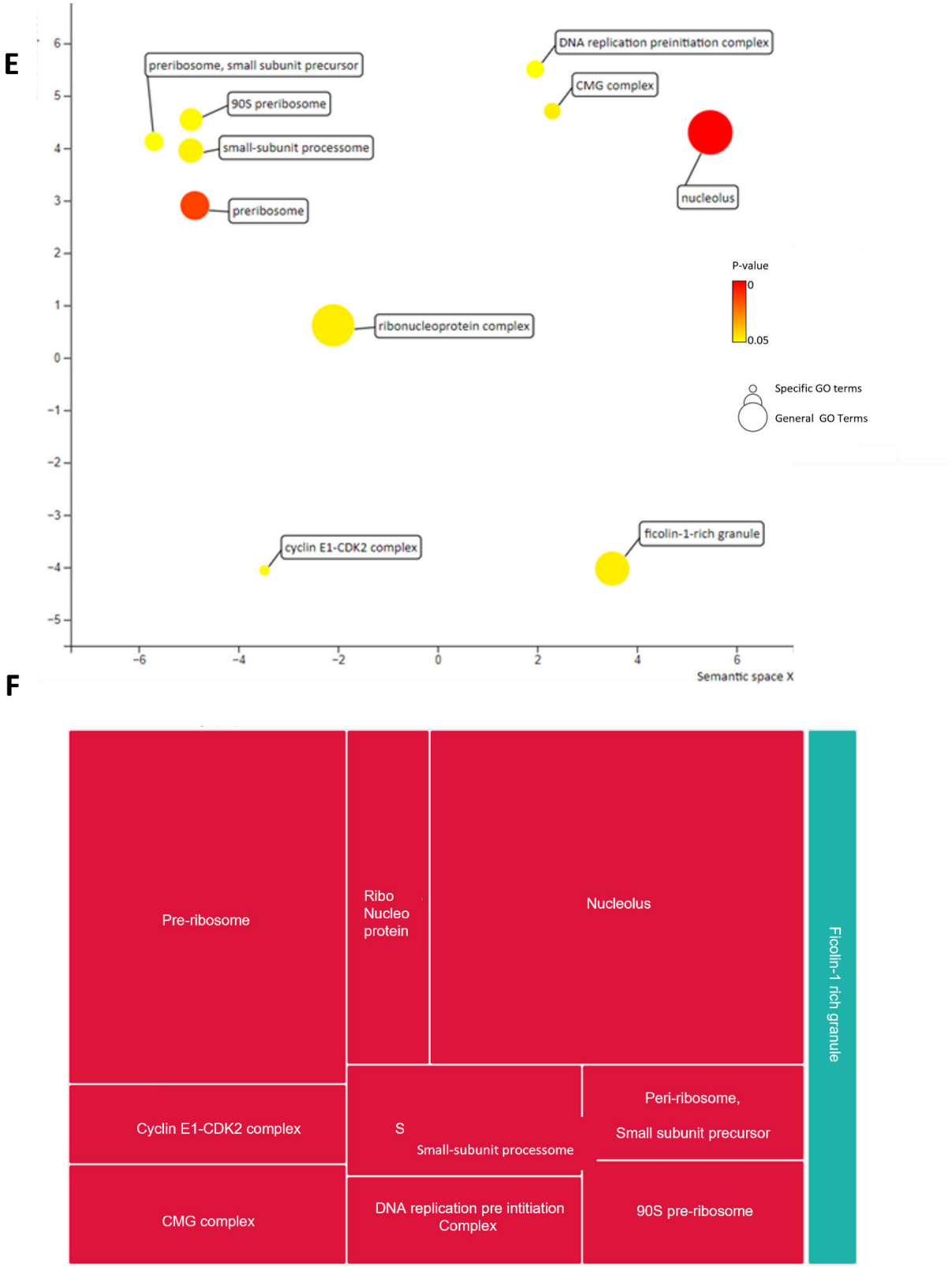
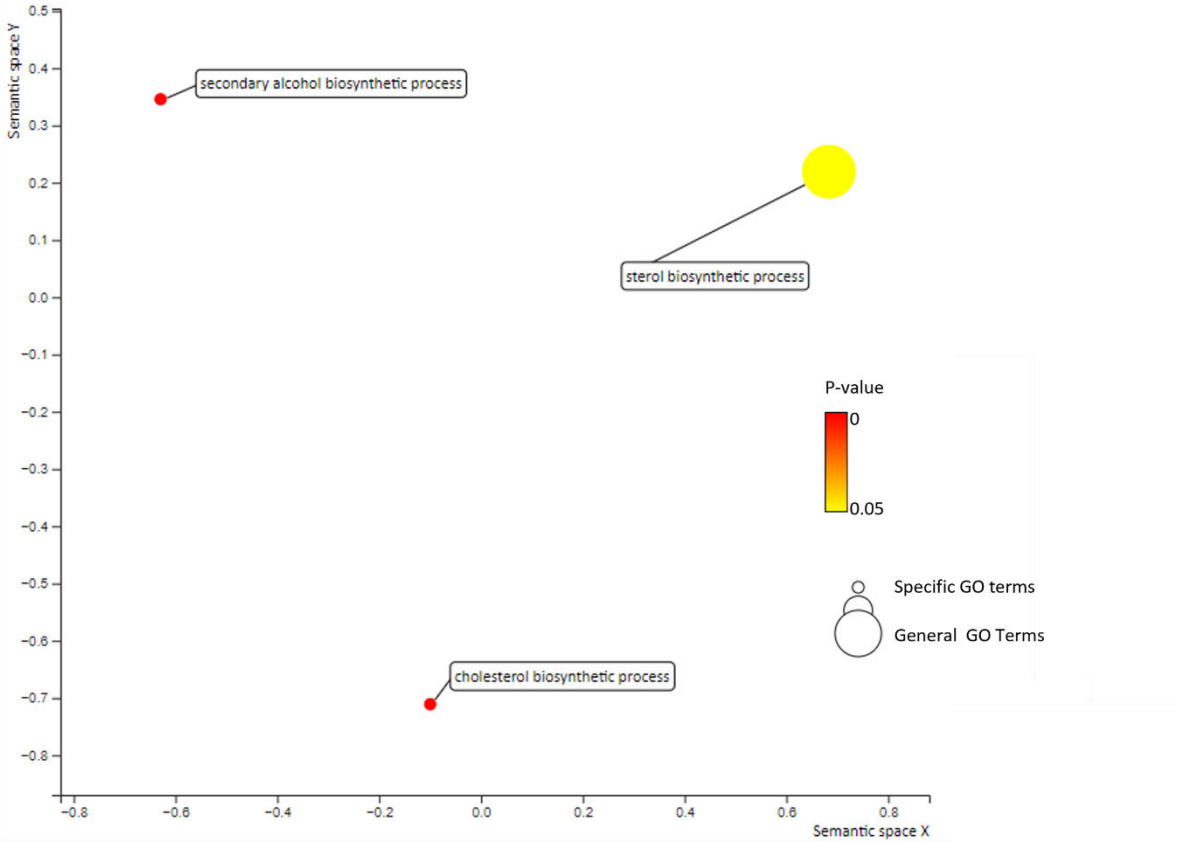



**Figure 35. REVIGO Visualisations: Upregulated DEGs, 6-days incubation.**

Visualisations of **(A-B).** Molecular function **(C-D).** Biological Process and **(E-F).** Cellular compartment GO annotations using REVIGO: scatterplot view, and REVIGO: Tree map. The dataset used was imported from g: Profiler. Default settings of REVIGO tool were used. The scatterplot view visualizes the GO terms in a "semantic space" where the more similar terms are positioned closer together. The color of the bubble reflects the p-value obtained in the g: Profiler analysis. The size of the bubble reflects specificity of GO terms whereby smaller bubbles represent more specific terms and larger bubbles more general terms. The tree map displays clustered terms visualised by coloured tiles. The interactive graph connects GO terms based on structure of GO hierarchy.

**A**


Semantic space Y
Semantic space X
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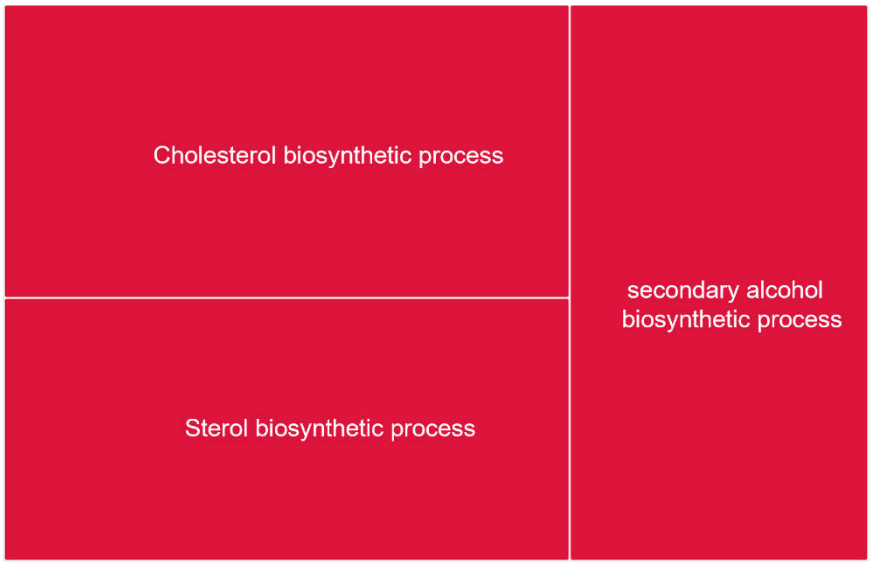
sterol biosynthetic process
cholesterol biosynthetic process
P-value
0
0.05
Specific GO terms
General GO Terms

**B**


Cholesterol biosynthetic process
Sterol biosynthetic process
secondary alcohol biosynthetic process

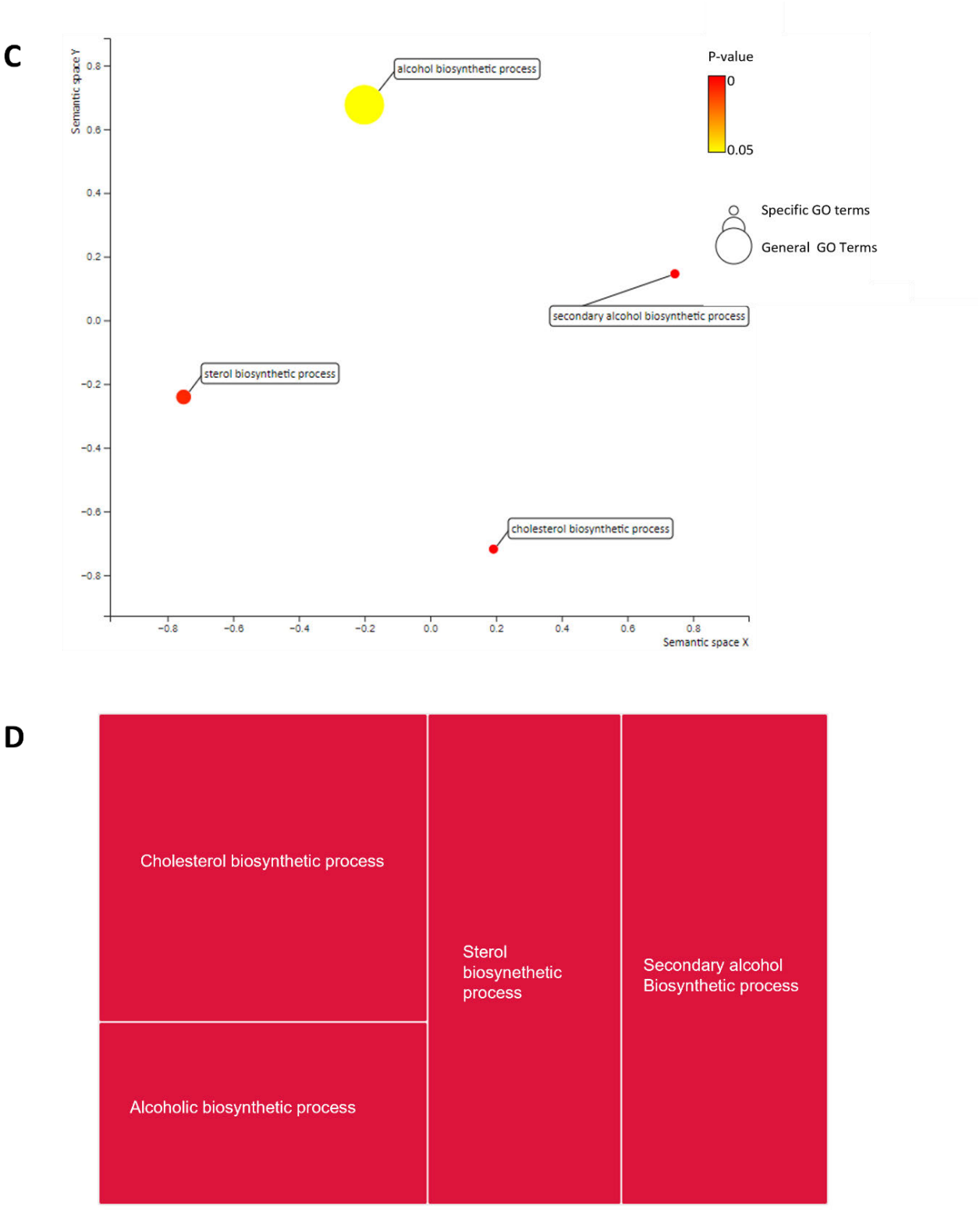



**Figure 36. REVIGO: ALL and Upregulated DEGs, 24 hours incubation.**

Visualisations of **(A-B).** All DEGs GO:BP **(C-D).** Downregulated DEGs GO:BP using REVIGO: scatterplot view, and REVIGO: Tree map. The dataset used was imported from g: Profiler. Default settings of REVIGO tool were used. The scatterplot view visualizes the GO terms in a "semantic space" where the more similar terms are positioned closer together. The color of the bubble reflects the p-value obtained in the g: Profiler analysis. The size of the bubble reflects specificity of GO terms whereby smaller bubbles represent more specific terms and larger bubbles more general terms. The tree map displays clustered terms visualised by coloured tiles. The interactive graph connects GO terms based on structure of GO hierarchy.

## 4.4. Discussion

In this chapter, the anti-proliferative effects of high dose vitamin B6 on promonocytic lymphoma cells were evaluated. NGS data gave insights into the mechanistic effects/pathways in which vitamin B6 exerts its anti-proliferative and anti-inflammatory properties. The data suggest that high dose vitamin B6 may show an anti-tumorigenic profile via several mechanisms. The transcriptomic profile of B6 treated cells demonstrated enrichment in areas that align with anti-proliferative effects. One of these areas is related to changes in the cell cycle.

The cell cycle describes the cellular process of DNA replication for the purpose of producing a new cell. It is a tightly regulated process relying on two main control mechanisms. The first involves a series of phosphorylations by a family of cyclin-dependent kinases (CDK)'s, that propel the cell through each stage of the cell cycle. The activity of CDKs is finely tuned by phosphorylation and dephosphorylation processes which ensures the smooth transition of the cell through each stage of the cell cycle. The second process ensuring successful cellular replication involves a set of supervisory checkpoint controls to monitor the achievement of crucial cell cycle events and halt progression to the next stage if there is any detection of flaws in the replication of DNA or chromosomal segregation. Damaged or under replicated DNA triggers the activation of these checkpoints which then prompts the cell cycle to stop until the damage is repaired, or cell death is initiated (Collins et al., 1997; Dang et al., 2021; Matthews et al., 2021).

There are four main phases in the cell cycle. (1) The first gap phase, otherwise known as G1, corresponds to a period of cell growth and the duplication of cellular contents ready for future division. In this phase cyclin, D1/CDK4/6 complex enables the partial phosphorylation of Retinoblastoma tumour suppressor protein 1 (RB1). This facilitates the release of E2 factor

(E2F) transcription factors which in turn allows the expression of a collection of genes that mediate the progression of the cell cycle (Dimova & Dyson, 2005). Upon completion of the G1 phase, the accumulation of cyclin E triggers CDK2, promoting full phosphorylation of RB1 and the transition from G1 to the S phase of the cell cycle (Bertoli et al., 2013). (2) During the synthesis phase, (S phase), a complete copy of DNA and duplication of the centrosome is achieved. This is initiated by Cyclin A/CDK2. Cyclin A/CDK, later in the S phase prepares the cell for entry into the mitotic phase (M phase) (Bertoli et al., 2013). (3) Second gap phase (G2 phase), corresponds to a period whereby the cell increases in size, produces new proteins and prepares for mitosis. (4) During the mitotic phase cell growth and protein production cease and the cells divide their copied DNA and cellular components during a multifaceted and organised division into two daughter cells. The regulation of chromatin separation and progression of mitosis is controlled by Cyclin B/CDK1 (Dang et al., 2021). A final phase called the Gap 0 or GO phase is a quiescent stage occurring outside of the cell cycle where cells will stop dividing or preparing for division and enter a temporary (or sometimes more permanent) resting stage (diagram 2) (Dang et al., 2021).

Further to these four main phases in the cell cycle, three cell checkpoints ensure the progression of the cell cycle, certifying that the cell undertakes appropriate growth, chromosomal replication, and segregation (Barnum & O'Connell, 2014) These are termed the G1/S, G2-M DNA damage checkpoint and the spindle assembly checkpoint (Lawrence et al., 2015).

Cell cycle checkpoints are responsible for delaying cell cycle progression and repairing DNA damage or in instances where damage cannot be repaired then cell cycle exit, or cell death is induced (Otto & Sicinski, 2017). Cancer causes cellular mutations which disrupt cell cycle control, bypassing checkpoints and triggering continuous cell division and an inability for the cell to exit the cell cycle(Otto & Sicinski, 2017). Nuclear transcription factor and tumour suppressor gene p53 is a pro-apoptotic gene that plays a critical role in regulating proteins

responsible for growth arrest and apoptosis. P53 is involved with both the G1/s checkpoint and the G2/M checkpoint and is considered the guardian of the genome through its ability to suppress tumours by inhibiting cell growth and activating apoptotic machinery (Chen, 2016). When activated by DNA damage, p53 will move into the nucleus and initiate transcription of downstream targets. One of these targets is Cyclin-dependent kinase inhibitor 1 (CDKN1A) which encodes the protein $p21^{Cip1}$ (alternatively $p21^{WAF1}$ or just p21), a cyclin-dependent kinase inhibitor that achieves cell cycle arrest through the inhibition of Cyclin-CDK complexes. These complexes contribute to the phosphorylation of a particular group of cell cycle proteins which facilitate the passage from G1 to S (Hyun & Jang, 2015). The inhibition by p21, causes the retinoblastoma protein pRB to remain hyperphosphorylated, E2F remains bound to pRB and arrest occurs at the G1/S boundary (Figure 38) (Senturk & Manfredi, 2013).

NGS pathway analysis showed that vitamin B6 affected Reactome pathways concerned with the regulation of cell cycle phase transition within the G1/S phase, the transcriptional activation of P53 and P21, phosphorylation of proteins involved in G1/S transition by active cyclin E1-CDK2 complexes and was also involved with defective binding of RB1 mutants to E2F1, (E2F2, E2F3) (Tables 2-4, Figure 35 C-D and E-F). The upregulation of these particular pathways indicates that high dose vitamin B6 plays an important role within the cell cycle. The U937 cell line has a notable mutant p53 gene with a 46-base deletion caused by an abnormal splicing event (Bairoch, 2018; Rivlin et al., 2011; Sugimoto et al., 1992) which gives it proliferative advantages and plays an important part in the performance of the cell line *in vitro* providing it has optimal cell culture conditions. A p53 mutation is often found in cancer cells and it not only causes the cells to lose their tumour suppressor abilities but increases oncogenic cell function contributing to growth and survival (Rivlin et al., 2011). Vitamin B6 appears to be restoring checkpoint controls by re-establishing/upregulating p53 expression. The resulting downstream effect of this is likely an upregulation of p21 expression which inhibits cyclin

CDK complexes. RB remains unphosphorylated and bound to E2F and cell cycle arrest occurs at the G1/S checkpoint. This mechanism explains why high dose vitamin B6 is effective at decreasing cellular proliferation (Figure 38).

The effect of vitamin B6 activating the expression of p53 in other cell lines has also been reported by Zhang et al (2014). Vitamin B6 in the active form pyridoxal (PL), was shown to increase phosphorylated p53 protein levels in whole-cell lysates and cell nuclei of human colorectal cancer (HT29), human colon adenocarcinoma (LoVo), and human hepatoma (HepG2) cell lines. Further to this, it was also noted that PL increased p21 mRNA expression in HT29, human colorectal adenocarcinoma (Caco2), LoVo, human embryonic kidney (HEK293T) and human hepatoma (HepG2) cells. Interestingly, in contrast to this study, no changes to p53 or p21 in these cancer cell lines with the use of (PN) or any of the other B6 vitamers. An earlier large prospective cohort study correlated folate and vitamin B6 deficiency with an increase of p53-over expressing in colon cancer but not wild type cancers (Schernhammer et al., 2008) but folate and B6 deficiency was deemed to not affect p53 expression in esophageal adenocarcinogenesis (Balbuena & Casson, 2010)

Further to the aforementioned upregulated pathways, analysis of all DEGs at the higher statistical profile (Table 3) unveiled Reactome pathway terms, '*RUNX3 regulates CDKN1A transcription R-HSA-8941855 '* and '*NGF-stimulated transcription R-HSA-9031628 '*. Both pathways have been demonstrated to directly affect p21 expression. Chi et al (2005) and Mabuchi et al (2010) showed that RUNX3 could induce the upregulation of p21 gene transcription in response to TGF-beta (TGFB1) signalling (Chi et al., 2005; Mabuchi et al., 2010). Interestingly TGFB1 was also noted to be involved following Reactome pathway analysis *'Loss of Function of TGFBR1 in Cancer R-HSA-3656534 '* (Table 3). Decker (1995) originally found that NGF was capable of inducing growth arrest and induction of p21 in an NIH-3T3 cell line (Decker, 1995), similarly Yan et al (1997) and Marampon et al (2008) found

that Nerve growth factor (NGF) was able to induce transcription of p21 and cyclin D1 genes within the PC12 pheochromocytoma cell line (Marampon et al., 2008; Yan & Ziff, 1997). Considering the inhibiting effect of p21 on cell cycle progression, it is also likely that these two pathways may contribute to the anti-proliferative effects of vitamin B6.

At this point, it may be judicious to also question why or how vitamin B6 can restore p53 expression in the U937cell line (Bairoch, 2018; Senturk & Manfredi, 2013; Sugimoto et al., 1992). The clues might lie in some of the other important pathways that were uncovered during pathway analysis of significantly changed genes. Interleukin-10 (IL-10, IL-10A) signalling is highlighted in Reactome pathways in both 'all' and 'upregulated' DEGs at the higher statistical profile (Tables 2 and 4). IL-10 is a pleiotropic cytokine produced primarily by monocytes, which affects immunoregulation and inflammation. IL-10 is normally associated with potent anti-inflammatory activity (Iyer & Cheng, 2012) and along with TGFβ is capable of suppressing Th1 inflammatory responses (Couper et al., 2008). IL-10 has a controversial role in cancer as it has been implicated in both its pathogenesis and development (Mannino et al., 2015), as well as having anti-tumour effects in other studies, via the enhancement of vaccine-induced T cell responses (Ni et al., 2020). More recently (2020), IL-10 is associated with the induction of cellular senescence via the upregulation of p53 expression and initiation of cell cycle arrest (Huang et al., 2020). We demonstrated (Figure 23), that high dose vitamin B6 can increase protein expression of IL-10 in the U937 cell line. It is possible that elevated IL-10 expression stimulated by vitamin B6 could contribute to the upregulation of p53.

The second group of pathways highlighted by 'all DEGs" and 'upregulated DEGs" as significant, centre around ribosomal biogenesis and include *ribosome biogenesis GO:0042254*, *Ribonucleoprotein complex biogenesis GO:0022613*, *Preribosome GO:0030684* (Table 2, Figure 34 C, D, E, F*) Ribosomal large subunit biogenesis GO:0042273, Ribosomal small subunit biogenesis GO:0042274, Small-subunit processomeGO:0032040, 90S preribosome*

*GO:0030686, Preribosome, small subunit precursor GO:0030688* (table 4, Figure 35 C, D, E, F). Ribosome biogenesis is a highly regulated activity overseeing protein synthesis. Cancer cells are characterised by increased ribosomal production which is required to maintain the high turnover of cell division and proliferation (Pecoraro et al., 2021). In fact, a heightened requirement for protein synthesis requires major metabolic effort within a proliferating cancer cell and that effort is concentrated around the G1 phase where adequate protein synthesis is required for the cell to progress through the G1-S transition. Research has found that defects in ribosome biogenesis can cause stabilization of p53 and subsequent inhibition of cell proliferation (Burger & Eick, 2013; Donati et al., 2012). Given the number of significantly upregulated pathways concerned with ribosome biogenesis highlighted by this study, it is relevant that altered ribosome biogenesis may also be contributing to the restoration of p53.

The effects of 24-hour incubation of high dose vitamin B6 were also examined on the U937 promonocytic cell line. The result of this shorter incubation period resulted in highlighting significant pathway changes in the following pathways. All DEG's- *Acyltransferase activity GO:0016746, Cholesterol biosynthetic process GO:0006695, Secondary alcohol biosynthetic process GO:1902653, Sterol biosynthetic process GO:0016126, Terpenoid backbone biosynthesis KEGG:00900, Cholesterol biosynthesis REAC: R-HSA-191273, Cholesterol biosynthesis pathway WP: WP197, Mevalonate pathway WP: WP3963 cholesterol biosynthetic process GO:0006695, secondary alcohol biosynthetic process GO:1902653, sterol biosynthetic process GO:0016126, alcohol biosynthetic process GO:0046165* (Figure 33, Tables 8 and 9). These pathways indicate that vitamin B6 at 24-hrs is downregulating the cholesterol biosynthesis/mevalonate pathway

Interestingly, the downregulation of these pathways may relate to the upregulation of p53 seen in the six-day samples. As the U937 cell line has mutant p53 it is expected that the p53 gene expression will be low. When comparing single gene analysis through the DEGUST tool (as

opposed to pathway analysis) it is clear to see that p53 is greatly upregulated in 6-day samples compared to 24-hour samples (Figure 30). Literature searches have correlated cholesterol synthesis/ mevalonate pathway with mutant p53 activity and cancer (Borini Etichetti et al., 2020; Gobel et al., 2020; Moon et al., 2019; Parrales & Iwakuma, 2016; Parrales et al., 2018). Highly proliferative cancer cells require an increased demand for fatty acids and cholesterol which provides for their energy needs and contributes to membrane synthesis and lipid signalling (Yang et al., 2020). The mevalonate pathway is fundamental in cholesterol synthesis in cellular metabolism and is often hijacked for use by cancer cells (Guerra et al., 2021; Parrales et al., 2018).

Inhibition of the mevalonate pathway has been shown to reduce the malignant properties of cancer cells via the degradation of mutant p53 (mutp53) (Borini Etichetti et al., 2020; Moon et al., 2019; Parrales & Iwakuma, 2016; Parrales et al., 2018). Statin drugs are effective in inhibiting enzymes within the mevalonate pathway and subsequent MVP levels which effectively stabilised mutp53 (Parrales et al., 2016). It was shown that mutp53 degradation occurs via a metabolic intermediate in the mevalonate pathway called mevalonate-5-phosphate (MVP). Decreased MVP inhibits mutp53's ability to bind to a molecular chaperone of the HSP40 family DNAJA1 leading to CHIP ubiquitin ligase-mediated mutp53 degradation (Parrales et al., 2018). Vitamin B6 pathway analysis within this study also revealed pathways that relate to this concept; *HSP90 chaperone cycle for steroid hormone receptors in the presence of ligand R-HSA-3371497* (Table 5) *ubiquitin-protein ligase binding GO:0031625, ubiquitin-like protein ligase binding GO:0044389* (Table 2). Further to this single gene analysis shows that MVP gene expression is significantly downregulated in 6-day samples compared to 24 -hour samples (Figure 31) whereas p53 gene expression in the single-gene analysis is significantly upregulated in 6-day samples compared to 24- hour samples (Figure 30).

Interestingly a very recent study by Zhang et al (2021) has found that B6 supplementation was able to ameliorate high-fat diet-induced hepatic lipid accumulation by inhibiting fatty acid and cholesterol synthesis. This study was conducted with the aim of finding effective means of treating hyperlipidemia via the administration of nutrition supplements in place of current treatments such as statins which can cause damaging side effects on liver and kidney function (Zhang et al., 2021). Given all these factors it is likely that vitamin B6, may be acting in a "statin" like way, to inhibit the mevalonate pathway by downregulating MVP and triggering the degradation of mutp53 (Figure 37).

If this mutp53 degradation is occurring in 24-hour samples then it could be speculated that this may be a contributing factor in the restoration of wild type p53 seen in 6-day samples which would explain B6's ability to restore checkpoint controls and exert an anti-proliferative effect on promonocytic lymphoma U937 cell line.

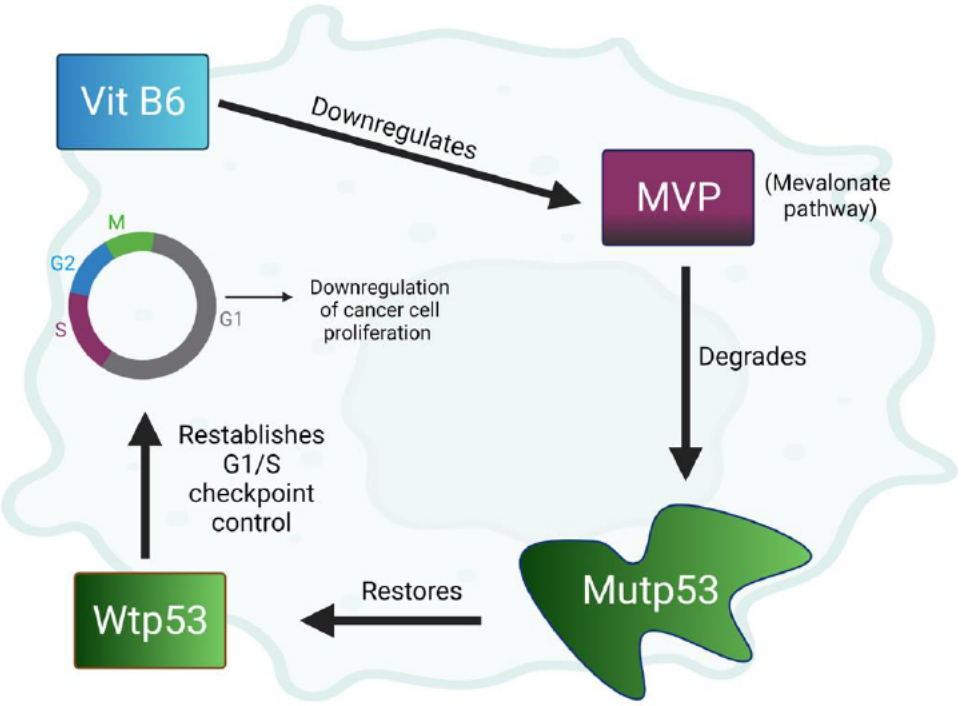



**Figure 37. Vitamin B6 downregulates mevalonate pathway restoring mutant p53 and re-establishing G1/S checkpoint control to downregulate proliferation in pro-monocytic lymphoma cell line U937.**

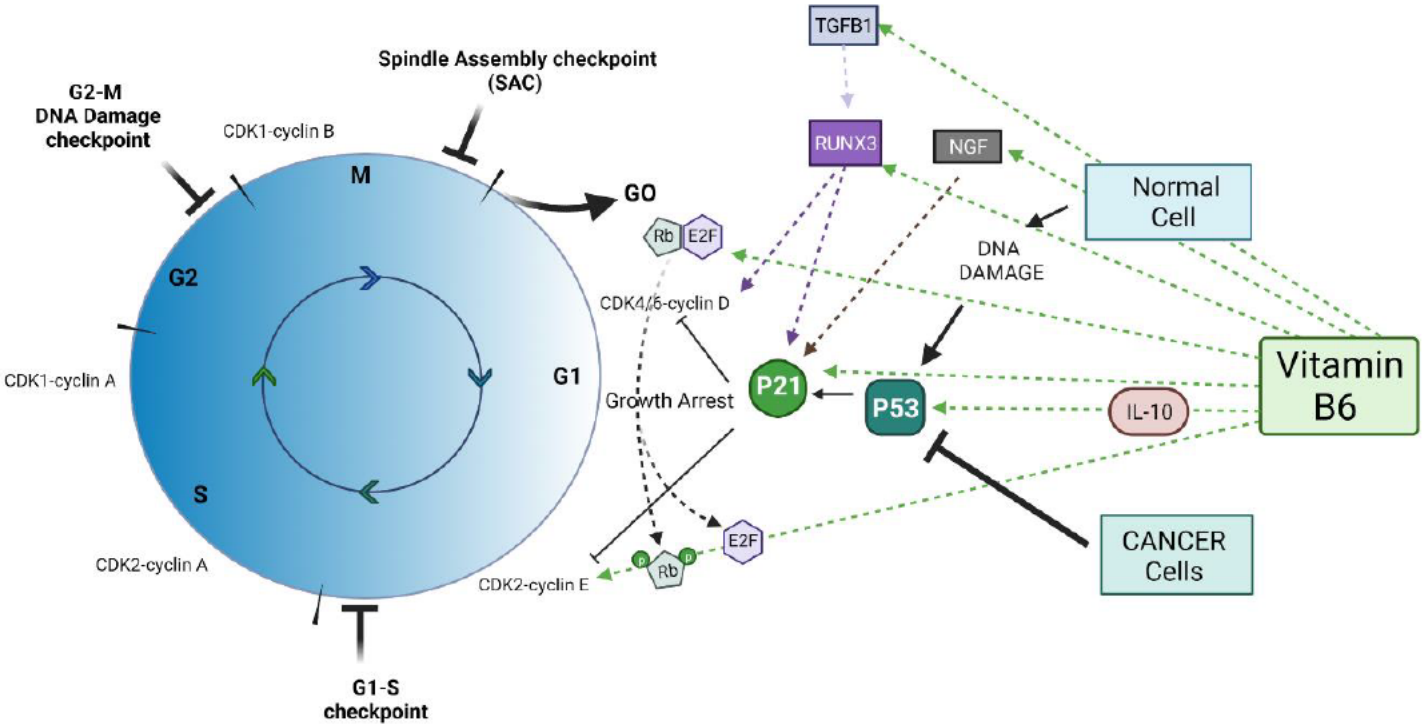



**Figure 38. The effect of vitamin B6 on cell cycle in U937 promonocytic lymphoma**

## 4.5. Conclusion

The use of high dose vitamin B6 as a therapeutic agent is an area of research gaining momentum within the cancer field. In this study, it was shown that high dose vitamin B6 in the vitamer form of pyridoxine (PN) significantly down-regulated proliferation of U937 promonocytic lymphoma cell line in a dose-dependent manner and significantly upregulated protein expression of IL-10. Genetic studies via Next Generation Sequencing also discovered that vitamin B6 at 24-hour incubation succeeded in downregulation of the mevalonate pathway which may have had the effect of restoring mutant p53 function. At 6-day incubation, vitamin B6 was able to upregulate p53 pathways and restore G1/S checkpoint control and thus inhibit U937 cancer cell proliferation. Considering that p53 mutations are implicated in many types of human cancers (Mantovani et al., 2019) these effects of vitamin B6 on downregulation of the mevalonate pathway and the restoration of the p53 pathway are important findings unique to this study and elevate vitamin B6 further as a molecule of interest in anticancer therapies. Much research needs to be done to provide a clearer understanding of these anti-tumour effects of vitamin B6 but the potential of high dose B6 as a significant contributor to oncological medicine remains intriguing.

# Chapter Five

# High dose vitamin B6 (pyridoxine) displays strong anti-inflammatory properties in LPS stimulated monocytes

## Abstract

Vitamin B6 has been shown to have anti-inflammatory properties which makes it an interesting nutraceutical agent. Vitamin B6 deficiencey is well established as a contributor to inflammatory-related conditions whilst B6 supplementation can reverse these inflammatory effects. There is less information available regarding the effects of high-dose vitamin B6 supplementation (above RDI) as a therapeutic agent. This study set out to examine the effects of high dose vitamin B6 on an LPS stimulated monocyte/macrophage cell population via an analysis of protein and gene expression using an RT2 profiler PCR array for Human Innate and Adaptive Immune responses. It was identified that high dose vitamin B6 has a global anti-inflammatory effect on LPS induced inflammation in monocyte/macrophage cells by downregulating the key broad-spectrum inflammatory mediators, CCL2, CCL5, CXCL2, CXCL8, CXCL10, CCR4, CCR5, CXCR3, IL-1β, IL-5, IL-6, IL-10, IL-18, IL-23-a, TNF-α, CSF2, DDX58, NLRP3, NOD1, NOD2, TLR-1 -2 -4 -5 -7 -8 -9, MYD88, C3, FOXP3, STAT1, STAT3, STAT6, LYZ, CASP-1, CD4, HLA-E, MAPK1, MAPK8 MPO, MX-1, NF-kB, NF-kB1A, CD14, CD40, CD40LG, CD86, Ly96, ICAM1, IRF3, ITGAM, IFCAM2 . The outcomes of this study show promise regarding vitamin B6 within the context of a potent broad-spectrum anti-inflammatory mediator and could prove useful as an adjunct treatment for inflammatory-related diseases.


## 5.1. Introduction

Vitamin B6 (pyridoxine) is establishing a reputation in the world of immunology as a molecule of interest due to its potent anti-inflammatory and antioxidant properties. These properties have been shown to have an impact on inflammatory-related conditions and chronic illness. It is well established that vitamin B6 deficiency contributes to inflammation and inflammatory disease

whilst vitamin B6 supplementation, in deficiency states, can reverse these effects. Less is known, however, about the effect of high dose vitamin B6 as a therapeutic agent and its potential to be developed as a broad-spectrum anti-inflammatory, in conditions such as sepsis, or cytokine storm as is seen in COVID-19 patients.

Low blood serum B6 is frequently noted in patients with high inflammatory markers (Ueland et al., 2017). In fact, numerous inflammatory diseases have been correlated with vitamin B6 deficiency including atherosclerosis and cardiovascular disease (Ji et al., 2021; Kumrungsee et al., 2019; Lotto et al., 2011; Pusceddu et al., 2020; Waly et al., 2016), rheumatoid arthritis (Sakakeeny et al., 2012; Sande et al., 2019), inflammatory bowel disease (Hwang et al., 2012; Saibeni et al., 2003; Selhub et al., 2013; Vavricka & Rogler, 2012; Weisshof & Chermesh, 2015), type-2 diabetes (Fields et al., 2021; Marzio et al., 2014; Merigliano, Mascolo, Burla, et al., 2018; Merigliano, Mascolo, La Torre, et al., 2018; Nix et al., 2015; Oxenkrug et al., 2013; Oxenkrug, 2015), non-alcoholic fatty liver disease (Abe et al., 2021; Kobayashi et al., 2021) and cancer (Bird, 2018; Crusz & Balkwill, 2015; Merigliano, Mascolo, Burla, et al., 2018; Mocellin et al., 2017; Peterson et al., 2020; Pusceddu et al., 2020; Waly et al., 2016). Vitamin B6 deficiency can disrupt the immune response by decreasing the production of protein and nucleic acids, inhibiting immune cell function, and interfering with the metabolic machinery of cells (Mikkelsen, Stojanovska, Prakash, et al., 2017). Further to this, vitamin B6, as a key player in one-carbon metabolism, is involved in methylation processes which when disrupted as occurs frequently in deficiency states, can cause an increase in Hcy resulting in vascular and systemic inflammation. Inflammation is normally self-limiting with pro-inflammatory reactions followed by anti-inflammatory action creating a balance within the process of resolving the initial inflammatory trigger , but chronic inflammation is often harder to resolve with the inflammatory microenvironment becoming more complex as the inflammation progresses (Qu et al., 2018).

Monocytes and macrophages are key players in the inflammatory process and are the first line of defence against infection caused by bacteria, viruses, and other microorganisms. Monocytes are particularly sensitive to lipopolysaccharides (LPS) which is the major component of gram-negative bacteria cell walls. LPS is useful for studying the effects of inflammation in monocytes and macrophages due to the profusion of inflammatory mediators triggered via LPS stimulation.

This study sought to investigate the effects of high dose vitamin B6 on the inflammatory process in LPS stimulated monocytes, via the analysis of protein and gene expression. It was found that a high dose of vitamin B6 in cell culture, exhibited a broad mediation of many inflammatory cytokines, chemokines, pattern recognition receptors, cell surface markers and other genes involved in inflammatory and defence responses. In particular, inflammatory mediators implicated in hyperinflammatory conditions related to LPS involvement such as 'cytokine storm' in patients with sepsis, and/or COVID-19 were significantly downregulated in vitamin B6 cultured samples. These include interleukin (IL)-6, IL-1β, TNF-α, NF-κB, toll-like receptor (TLR)-4. The evidence from this study suggests that high dose vitamin B6 may be useful as a broad-spectrum anti-inflammatory agent and a possible adjunct to current anti-inflammatory treatments.

## 5.2. Materials and methods

### *5.2.1 Cell culture and reagents*

#### *5.2.1.1 Culture of U937 cells*

The U937 cell line, isolated from a 37-year-old male patient with histiocytic lymphoma is commonly used to study the behaviour and differentiation of monocytes. They can be differentiated into monocyte/macrophage cells following culture with certain stimulants such as phorbol 12-myristate 13 acetate or vitamin $D_3$ (Chanput, 2015). The U937 cell line was purchased from ATCC (https://www.atcc.org) by the Monash University Department of Immunology. U937 cells are immortal and allow for multiple passages without transformation, however, we ensured to keep the number of passages to a minimum. U937 cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 100 mg/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) at 37$^{o}$ C and 5 % $CO_2$. Culture media was changed every 3-4 days and cells were passaged accordingly. Once 80-90 % confluent, cells were used in experiments.

#### *5.2.1.2 Preparation of reagents and treatment of cells*

A stock solution of Pyridoxine Hydrochloride (Vitamin B6 - P6280-100G) was freshly prepared according to the manufacturer's instructions (Sigma, VIC Australia) in phosphate-buffered saline (PBS). Vitamin B6 stock was filtered using a 0.2-micron filter and used in culture at a final concentration of 250 µg/ml. Lipopolysaccharide was dissolved in PBS and used at a final concentration of 1 µg/ml overnight to induce inflammation of the monocyte/macrophage cells.

### *5.2.2 Cell surface marker expression by flow cytometry*

Immune cells express several cell surface markers which each play a different role. Markers expressed on monocytes/macrophages were evaluated on differentiated U937 cells to determine whether vitamin B6 modulates the inflammatory response induced by LPS. U937 cells were seeded at a density of $1x10^6$ cells/ml in tissue culture flasks and stimulated with 100 nM $VitD_3$ for a total of 72 hrs (hrs). Differentiated U937 cells (monocyte/macrophage cells) were stimulated with 1 μg/ml LPS $\pm$ 250 μg/ml vitamin B6 for 24 hrs. Differentiated monocyte/macrophage cells were used for cell surface marker expression and supernatants were collected for cytokine secretion analysis using Bio-Plex (Bio-Rad, VIC Australia). Flow cytometry was used to determine the expression of cell surface markers, cluster differentiation (CD)11b, CD14, CD16, CD86, CD206 and MHC-I. U bottom 96 well plates were seeded with 100 μl of treated cells and controls at $5x10^5$ cells per/well and incubated with Fc block (1:100 dilution) for 30 minutes on ice. After washing, cells were labelled with cell surface antibody cocktails and isotype controls linked to fluorochrome and incubated on ice for 30 minutes in the dark. The antibodies were diluted in FACS buffer at the following dilutions according to the manufacturer's recommendations (CD11b-PE 1:400; CD14-BV421 1:200; CD86-Alexafluor 488 1:400; CD16-PE 1:400; CD206-PE/Cy7 1:200; MHCI-BV510 1:200) (Bio-Legend and BD Life Sciences Victoria Australia). Cells were washed and re-suspended in 300 μl FACS buffer and transferred to FACS tubes. Cells were collected using the BD FACS Canto II, using the Cell Quest program (BD, Victoria Australia), and % cell surface marker expression was analysed using BD FACS Diva software.

### *5.2.3 Bio-Plex cytokine assay*

The Bio-Plex human cytokine immunoassay is a highly sensitive and reproducible magnetic bead-based assay that allows accurate measurement of low levels of human cytokines. The Bio-

Plex cytokine assay uses 8 μm magnetic beads coated with antibodies against an array of cytokines. Cytokine assays were performed using a bead-based multiplex immunoassay (MIA, 9 Bio-Plex Panel B, Bio-Rad Laboratories Inc. VIC Australia) that included the cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17α, IFN-γ, TNF-α. U937 cells were seeded at an appropriate density in tissue culture flasks and stimulated with 100 nm $VitD_3$ for a total of 72 hrs. Differentiated U937 cells were then stimulated with (A) 1 μg/ml LPS, (B) 1 μg/ml LPS + 250 μg/ml vitamin B6, or (C) untreated control. A B and C were performed in triplicate. Cells were cultured for 1 hr, 3 hrs, 6hrs, 12 hrs, 24 hrs, 72 hrs and 144 hrs to assess the time-course expression of cytokines. Supernatants were collected at the end of each time period and placed immediately at -80$^{o}$ C to prevent cytokine degradation. Standard low photomultiplier tube (PMT) settings were prepared with "blank" negative controls in duplicate. Ninety-six-well plates were coated with beads, followed by the addition of samples and standards and detection antibodies, then streptavidin-phytoerythrin as per the manufacturer's instructions. The beads were re-suspended, and the fluorescence output was read and calculated on the Bio-Plex array reader (Bio-Rad, VIC Australia). Statistical analysis of data included the mean and standard deviation (SD), as well as a Two-Way ANOVA followed by Sidak's multiple comparison test using GraphPad Prism (GraphPad Software, USA). Significance was defined as $p \leq 0.05$.

### *5.2.4 RT$^2$ profiler PCR array for Human Innate and Adaptive Immune responses*

The RT$^2$ profiler polymerase chain reaction (PCR) array kit for pathway expression profiling was used to assess the role of vitamin B6 in modulating monocyte/macrophage inflammatory responses to that induced by LPS. LPS is widely used as a powerful activator of monocytes

and macrophages and induces the production of key inflammatory mediators (Tucureanu et al., 2018). The human Innate and adaptive Immune responses PCR array consists of 84 genes relating to IL-1R and toll-like receptor (TLR) signalling pathways involved in pathogen detection and host defence against bacteria, acute phase response, complement activation inflammatory response and antibacterial humoral response, as well as genes involved in innate immune response and septic shock. U937 cells were seeded at an appropriate density in tissue culture flasks and differentiated with 100 nm. $VitD_3$ for 72 hrs. Differentiated U937 cells (monocyte/macrophage cells) were stimulated with either (A) 1 μg/ml LPS or (B) 1 μg/ml LPS + 250 μg/ml vitamin B6. Both A and B were performed in triplicates. Cells were cultured for 24 hrs and both adherent and non-adherent cells were removed from the flask, washed, and the pellet snap-frozen in liquid nitrogen and stored immediately at -80$^{o}$C until used.

#### *5.2.4.1 RNA extraction from cells*

Extraction of RNA from vitamin B6 cultured cells was performed using an RNeasy1 mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Messenger RNA (mRNA) from treated cells, was extracted from each cell pellet by disrupting cells with lysis buffer/mercaptoethanol mix. Cells were lysed, and the lysate was placed in the supplied Qia-shredder columns for homogenization and then transferred to RNeasy mini-spin columns. DNase was used to eliminate any genomic DNA contamination with the RNase-free DNase set (Qiagen, Hilden, Germany). Samples were evaluated for RNA integrity number (RIN) using the Agilent 2100 Bioanalyser and Agilent RNA 6000 nano kit (Agilent Technologies, Santa Clara, CA, USA). All samples tested and used had a RIN higher than the cut-off point of 8.

### *5.2.4.2 Assessing change in gene expression.*

RNA was reverse transcribed using an $RT^2$ first strand kit (Qiagen, Hilden, Germany) and resultant cDNA was used on the real-time $RT^2$ Profiler PCR Array for Innate and adaptive immunity (QIAGEN, Cat. no. PAHS-052Z) in combination with $RT^2$ SYBR® Green qPCR Mastermix (Cat. no. 330529) for evaluation of gene expression. LPS alone and LPS with 250 µg/ml vitamin B6 were analysed in comparison to untreated (no LPS or vitamin B6) cells using Qiagen web-based software for calculation of fold change and results compared using criteria of >2.0-fold increase/decrease in gene expression as biologically meaningful.

### *5.2.4.3 Analysis of Data*

Fold-Change (2^ (-Delta Delta CT)) is the normalised gene expression (2^ (- Delta CT)) in the Test Samples (LPs alone and LPS + vitamin B6) divided by the normalised gene expression (2^ (- Delta C)) in the Control Sample (Untreated). $C_T$ values for each group were exported into Excel to create a Table of $C_T$ values which was then uploaded to the data analysis web portal (http://www.qiagen.com/geneglobe). Samples were categorised into control and test groups. $C_T$ values were normalised based on manual selection of reference genes. Reference genes are endogenous genes employed as a reference for certifying the authenticity of RT-PCR results. The basis for this internal referencing presumes that the less variation in endogenous gene expression, the better the experimental outcome (Fu et al., 2006). The data analysis web portal calculates fold change/regulation using the delta-delta $C_T$ method, in which delta $C_T$ is calculated between the gene of interest (GOI) and an average of reference genes (HKG), followed by delta-delta CT calculations (delta CT (Test Group)-delta CT (Control Group)).

Fold Change is then calculated using the 2^ (delta-delta CT) formula. Fold change data was graphed using prism software whereby fold change of the two treatment groups (LPS and B6+LPS) was plotted against the control group (untreated) represented as 0 on the X-axis. Further analysis was performed using LPS as control, and the difference in fold change between B6+LPS and LPS was plotted on the same graphs along with relevant p values between the two conditions.

#### *5.2.4.4 Statistical Analysis*

Calculation of P values was performed using the student's t-test of the triplicate 2^ (- Delta CT) [(2^-ΔCT)] values for each gene in the control group versus the treatment groups. P values less than 0.05 were considered significant. The p-value calculation used is based on parametric, unpaired, two-sample equal variance, and two-tailed distribution. Genes were considered significantly altered and included in the analysis if they displayed a greater than >2.0-fold change up/down and a p-value of $P \leq 0.05$.

## 5.3. Results

LPS is the main component of the outer membrane of gram-negative bacteria and acts as a powerful activator of cells concerning cytokines, chemokines, and cell surface markers, making them pro-inflammatory. LPS is widely used to study inflammation and was used in all experiments to induce an inflammatory profile to determine the effects of vitamin B6 in an inflammatory environment.

### *5.3.1 Vitamin B6 changes cell surface marker expression in LPS stimulated monocytes*

Monocytes and macrophages express cell surface markers that determine their activation state. CD14 is expressed by monocytes, macrophages, and dendritic cells, and acts as a co-receptor (together with TLR-4) for detection and activation by bacterial LPS. CD40, CD80 and CD86 are primarily involved in determining the activation state of mature antigen-presenting cells such as dendritic cells, monocytes, and macrophages. Expression of CD40, CD80, or CD86 on mature antigen-presenting cells is required for stimulation of T cells. CD206 (or mannose receptor) a pathogen recognition receptor that binds microbial antigens is expressed on macrophages and immature dendritic cells. CD206 is considered a marker of the M2 macrophage phenotype (Tsuchiya et al., 2019). CD209 activates phagocytosis in macrophages by binding to pathogen-associated molecular patterns (PAMPS) found in bacteria, viruses and fungi (McGreal et al., 2005). Expression levels of CD14, CD40, CD80, CD86, CD206 and CD209 on differentiated U937 monocyte/macrophages were determined. LPS was used to induce an inflammatory profile and vitamin B6 was added to determine its effects on cell surface markers.

An increase or decrease change of 2-2.5-fold was considered to be of interest when determining the effect of vitamin B6+LPS compared to LPS alone. In this regard, CD14 was increased from 26.5% to 55.5% in the presence of LPS which was further increased to 70% in LPS+vitamin B6, suggesting that B6 synergistically with LPS increases the expression of CD14 stimulating the cells (Fig 39). Similarly, CD40, CD80 and CD86 were increased in the presence of LPS and were further increased in LPS+vitamin B6, indicating that vitamin B6 stimulates monocyte/macrophage cells; there were no major differences in the expression of CD206 and CD209.

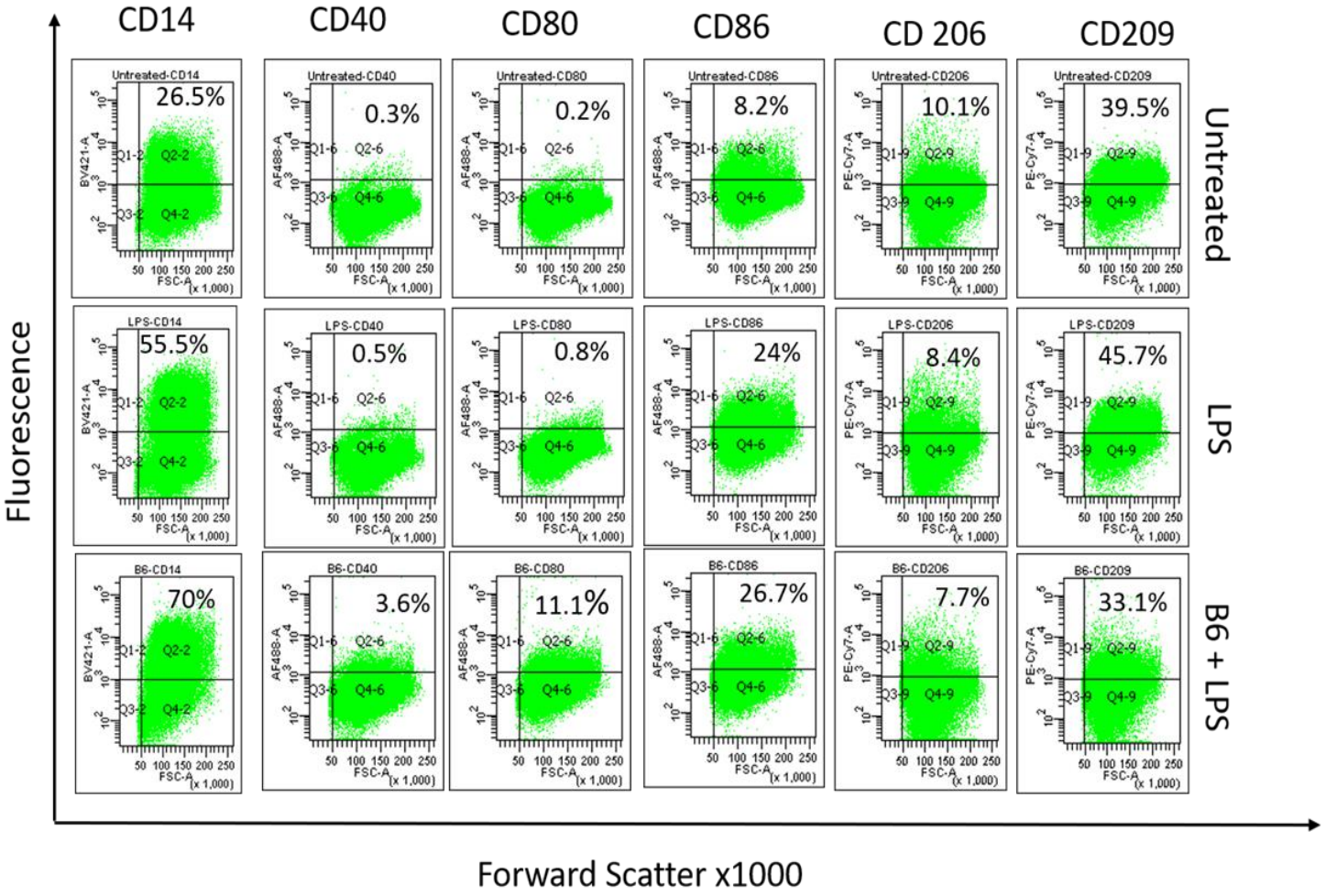



**Figure 39. Cell surface marker expression of monocytes cultured with LPS ± vitamin B6.**
Percentage expressions are represented as dot plots. U937 cells were differentiated into monocyte/macrophage cells followed by stimulation with LPS ± vitamin B6 for 24 hours. Quadrants were determined based on isotype controls. Top right quadrant represents cells which are surface marker positive.

### *5.3.2 Vitamin B6 decreases secretion of IL-1β, IL-6, IL-10, and TNF-α in LPS stimulated monocytes*

A time-course Bio-Plex immunoassay was used to study the effects of vitamin B6 on LPS stimulated monocyte/macrophage cells. LPS was shown to induce an activated inflammatory profile by stimulating the secretion of IL-6, IL-10, TNF-α and IL-1β compared to non-stimulated control (Figure 40). Two-way ANOVA and Sidak's multiple comparison test were used to analyse data for P-value significance, using Prism graph pad software. The addition of vitamin B6 to LPS stimulated cells significantly decreased the expression of IL-6 at 12 hrs and 72 hrs ($p \leq 0.05$), TNF-α at 6 hrs ($p \leq 0.00005$) and 12 hrs ($p \leq 0.005$), IL-10 at 12 hrs ($p \leq 0.005$) and 24 hrs ($p \leq 0.05$), and IL-1β at 72 hrs, ($p \leq 0.00005$) and 144 hrs ($p \leq 0.05$). Vitamin B6 did not alter the secretion of other cytokines (IL-2, IL-4, IL-8, IL-17α, IFN-γ) measured (not shown).

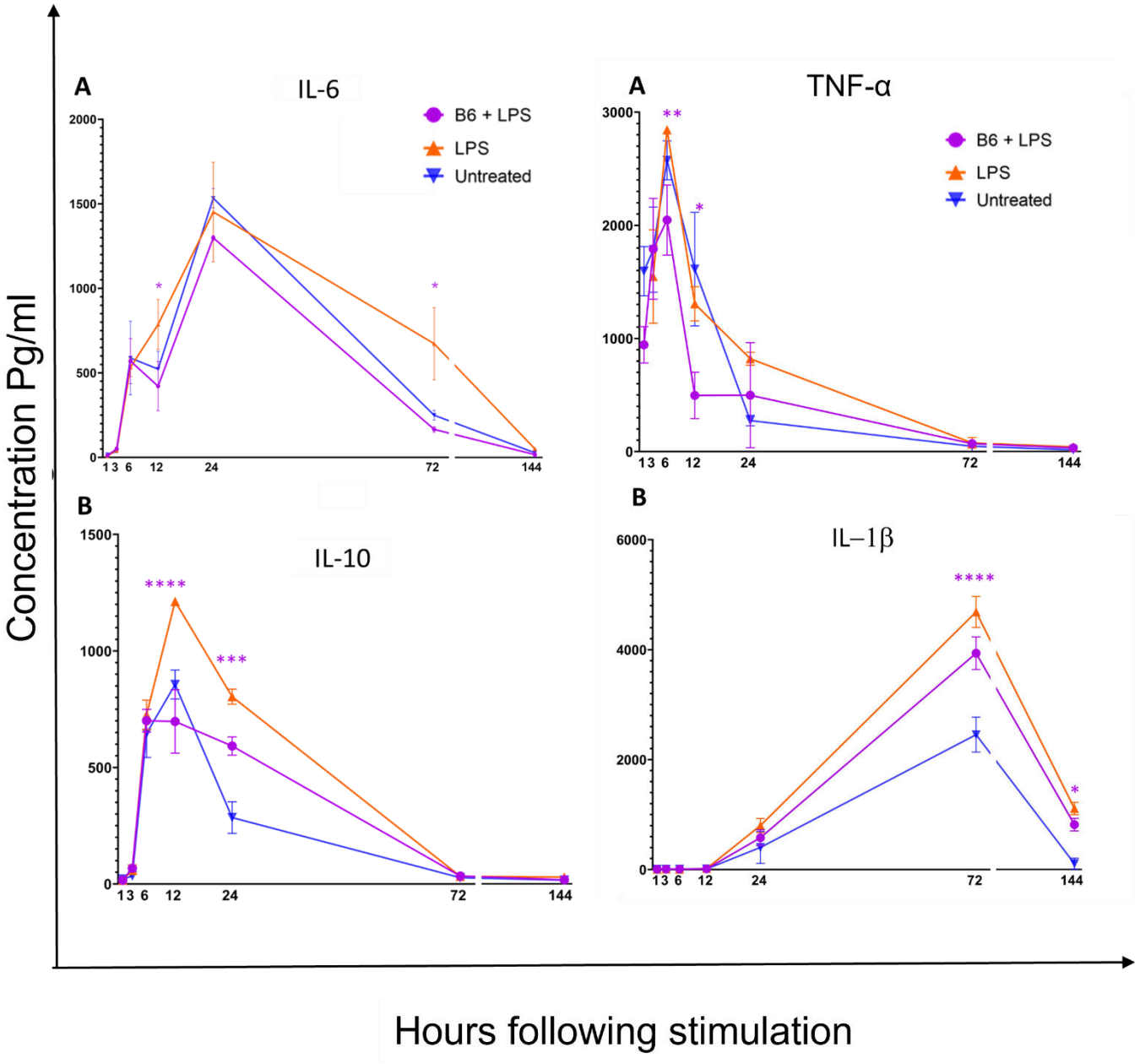



**Figure 40. The effect of vitamin B6 on cytokine expression in LPS stimulated monocytes.**

U937 cells were differentiated into monocyte cells and incubated with LPS to trigger inflammatory response (orange lines). Vitamin B6 was added to LPS stimulated cultures to determine its modulatory effects. Supernatants of the cultures were collected over 1-144 hours and (A) IL-6 (B) IL-10 (C) IL-1β and (D) TNF-α cytokines measured using bioplex kit. Two-way Anova and Sidak's multiple comparison test was used to analyse data for P value significance, using Prism graph pad software. Significance was demonstrated as *P<0.05, **P< 0.05, ****P<0.00005).

### *5.3.3 Vitamin B6 decreases inflammatory gene expression in LPS stimulated monocyte/macrophage cells*

Pathway expression profiling, using $RT^2$ profiler PCR arrays for human innate and adaptive immune responses, was used to assess the effects of LPS $\pm$ vitamin B6 stimulated monocyte/macrophage cells against non-stimulated control cells. Vitamin B6 showed clear modulation by downregulating 83 genes that LPS had upregulated compared to untreated control, 54 of these genes showed a fold change greater than 2 which were deemed biologically relevant. Significance between the two conditions (LPS) and (vitamin B6+LPS) and control (untreated) is indicated by P values ($p \leq 0.05$). The difference in fold change between (B6+LPS) and (LPS) was plotted on the same graphs along with relevant P values between the two conditions.

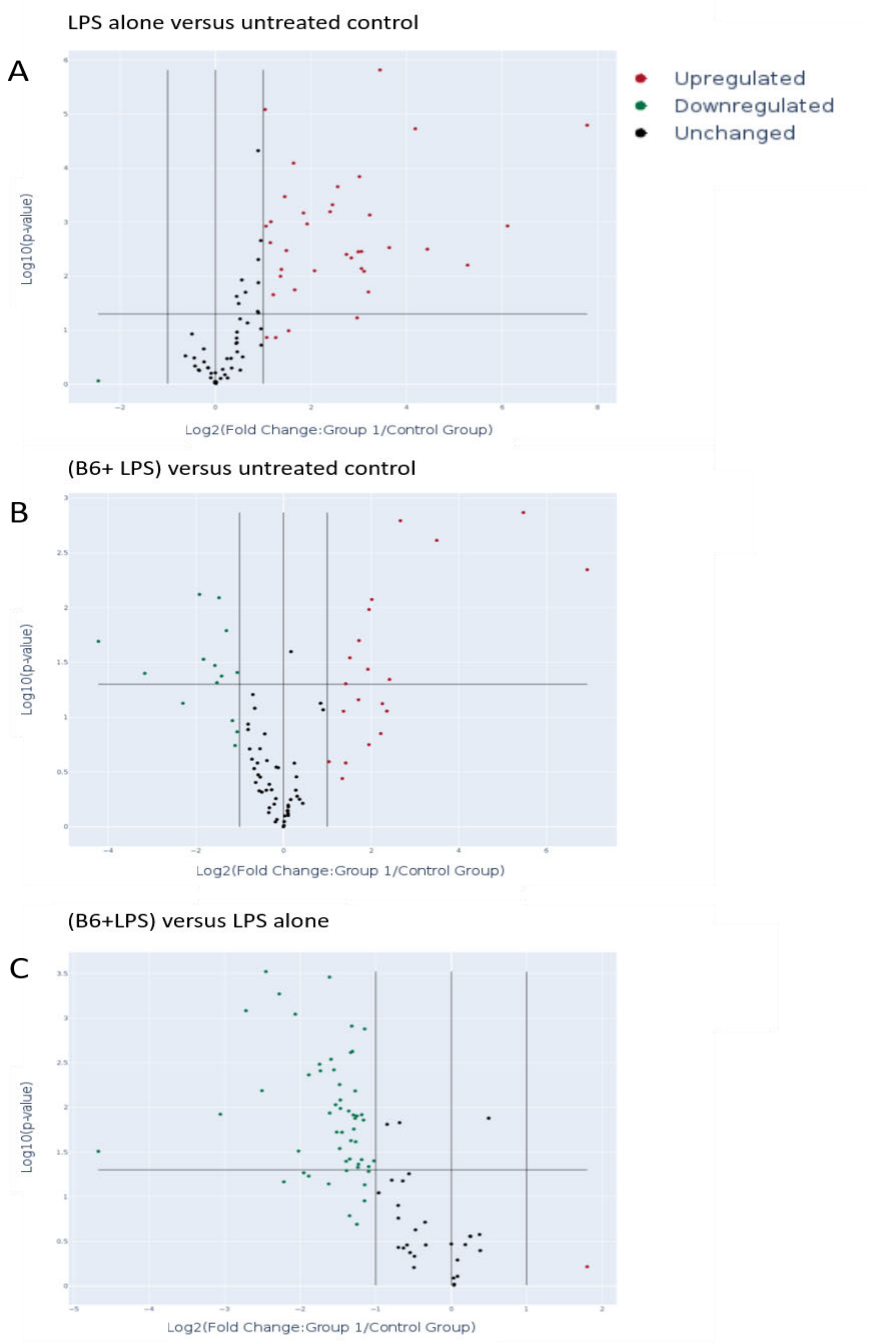



**Figure 41. Vitamin B6 down regulates the inflammatory effect induced by LPS on monocyte/macrophage cells.**

Volcano plots identifying significant changes in gene expression. X-axis = $Log_2$ fold change in gene expression. Y-axis = statistical significance. Centre vertical line = unchanged gene expression. Outer vertical lines = selected fold regulation threshold. Horizontal line indicates P value threshold. Far upper left quadrant = down regulated genes of significance. Far upper right quadrant = upregulated genes of significance. U937 cells were differentiated into monocytes/macrophages and co-cultured with LPS ± vitamin B6 compared to control for 24 hours (n=3). Cells were collected and trypsinised to ensure collection of all cells including adherent cells. mRNA was extracted and genes were profiled with $RT^2$ gene profiler for innate and adaptive immune response to determine fold change in normalised gene expression compared to those that were not treated.

### *5.3.3.1 Vitamin B6 down-regulates expression of cytokine genes*

Cytokines are secreted by monocyte/macrophage cells in both the innate and adaptive immune systems as a response to pathogenic infection. Cytokines released by innate immune cells aid the front line of defence by controlling opportunistic invasion from microbial pathogens including bacteria and viruses. Within the adaptive immune system, cytokines mediate growth and differentiation and activate different types of effector cells for microbial elimination. Cytokines enable cells to regulate immune response and pro-inflammatory cytokines activate immune cells and instigate the release of additional cytokines. Chemokines are a group of chemoattractant cytokines that function to induce chemotaxis in proximal responsive cells. This study shows that vitamin B6 can significantly modulate cytokine responses, in LPS induced monocyte/macrophage cells.

Chemokine Ligand-5 (CCL5/RANTES) functions as a ligand for chemokine receptors CCR1, CCR3 and CCR5 and is a chemotactic cytokine for macrophages and T cells. CCL5 also facilitates the recruitment of leukocytes to sites of inflammation (Marques et al., 2013; Ness et al., 2004). CCL5 was downregulated by vitamin B6 2.8-fold from 3.8-fold (LPS) to 1-fold (B6+LPS) ($p \leq 0.005$) (Fig 42A).

IL-18, an IFN-γ inducing factor (IGIF) is a strong inducer of inflammatory cytokines including IFN-γ , and its biological activity is mediated via its binding to the IL-18 receptor complex and activation of the NF-κB pathway (E.N. Benveniste, 2014,; Rex et al., 2020). Vitamin B6 downregulated IL-18 expression by 3-fold from 1.4-fold (LPS) to -1.5-fold (B6+LPS) ($p \leq 0.05$) (Fig 42A). IL-5 is frequently associated with eosinophils and asthma (Pelaia et al., 2019; Takatsu, 2011), but has also been shown in some animal studies to polarise monocytes towards an anti-inflammatory phenotype (Merriwether et al., 2021). IL-5 expression was

downregulated by vitamin B6, 3.6-fold from 1.4-fold (LPS) to -2.2-fold (B6+LPS) ($p \leq 0.05$) (Fig 42A).

The colony-stimulating factor 2 (CSF2) gene encodes the cytokine GMSF-2 (Granulocyte-Macrophage colony-stimulating factor). GMSF-2 is expressed by myeloid cells and controls the production, differentiation, and function of granulocytes and macrophages, and plays a role in tissue inflammation (Hamilton, 2019). LPS+B6 v LPS alone reduces CSF2 expression 6.8-fold, from 18.1-fold (LPS) to 11.3-fold (B6+LPS) ($p \leq 0.05$) (Fig 42B).

Interleukin 10 (IL-10) is secreted by monocytes/macrophage cells (and other immune cells in certain contexts) (Iyer & Cheng, 2012). Its ability to limit host immune response to pathogens renders it a potent anti-inflammatory cytokine. IL-10 produced by monocytes to alternatively activate macrophages IL-10 can also downregulate the expression of other pro-inflammatory cytokines produced by activated macrophages such as TNF-a, IL-6 and IL-1 (Williams et al., 2004). LPS upregulated the expression of IL-10 9.2-fold which was reduced to 3.9-fold in the presence of vitamin B6, a change in expression of 5.3- fold ($p \leq 0.05$) (Fig 4B).

C-C Motif Chemokine Ligand 2 (CCL2) is a chemokine ligand also referred to as monocyte chemoattractant protein-$_1$ (MCP-$_1$). It is a chemotactic protein for myeloid and lymphoid cells and reacts synergistically, with other inflammatory stimuli, to pathological and physiological circumstances during immune defence, it is responsible for recruitment, regulation and polarisation of macrophages during inflammation (Gschwandtner et al., 2019). CCL2 was downregulated by vitamin B6 5-fold from 8.3 (LPS) to 3.3 (B6+LPS) ($p \leq 0.05$) (Fig 42B).

C-X-C Motif Chemokine Ligand 10 (CXCL10) (IP-10) encodes an anti-microbial chemokine that stimulates monocytes upon binding of the receptor CXCR3. The CXCR3 receptor also reacts with the chemokines CXCL9 (MIG), CXCL11 (I-TAC/IP-9) and CXCL4, all of which function as immune chemo-attractants associated with interferon-induced inflammatory

responses (Kuo et al., 2018). CXCL10 was downregulated by vitamin B6 34-fold from 38.8 (LPS) to 4.7-fold (B6+LPS). ($p \leq 0.05$) (Fig 42C). CXCL8 (IL-8) is a chemoattractant cytokine, released in response to inflammation by monocytes and macrophages, targeting neutrophils, basophils and T cells involved in inflammation and chemotaxis (Kathleen Brennan, 2007). It is considered a major mediator of inflammatory response and is rapidly produced in response to bacterial and viral infiltration. LPS + B6 v LPS reduced gene expression by 25-fold from 69-fold (LPS) to 44.4-fold (B6+LPS) ($p \leq 0.05$) (Fig 42C).

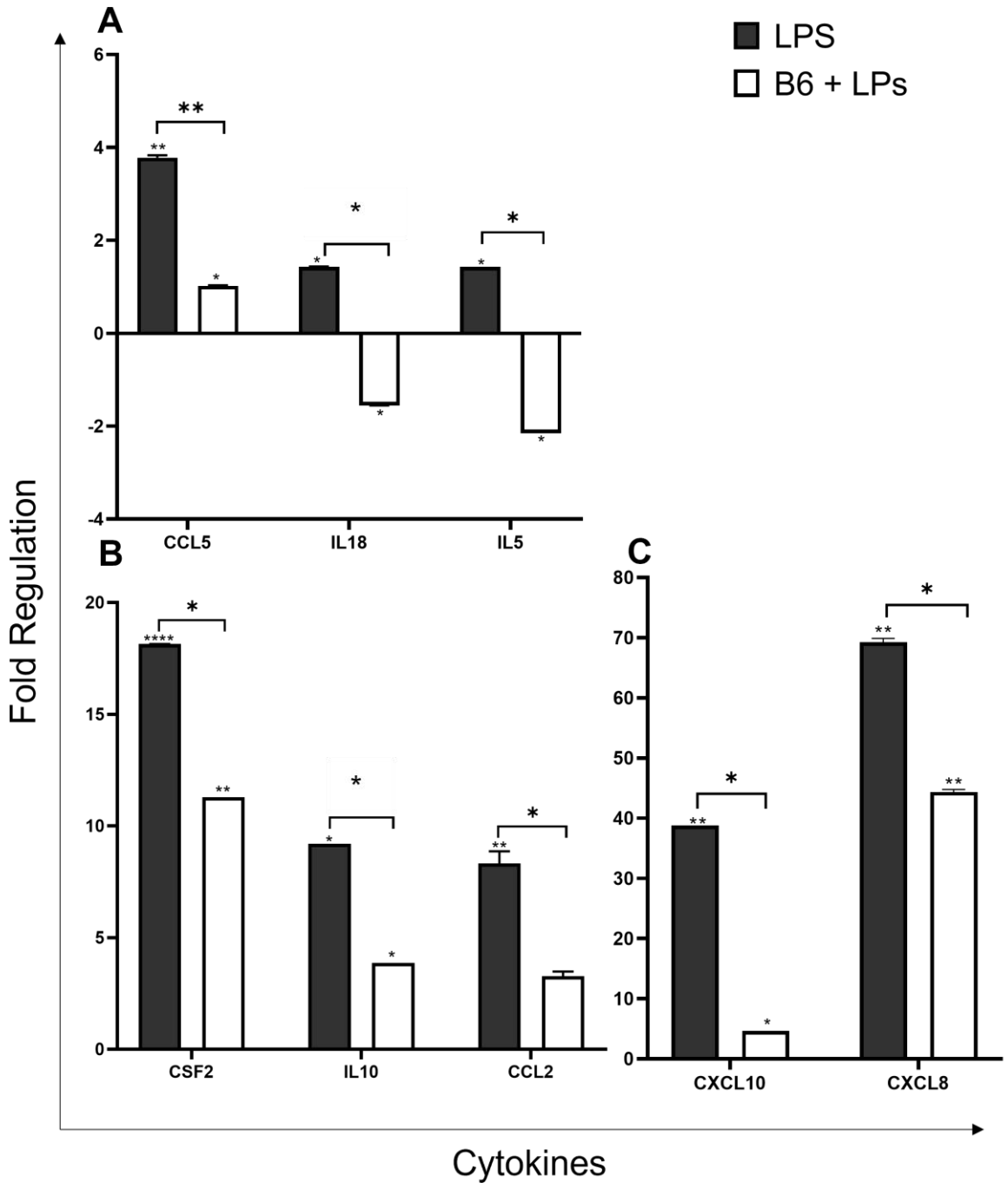



**Figure 42. Vitamin B6 downregulates the expression of cytokines in LPS stimulated monocyte/macrophage cells.**

U937 cells were differentiated into monocytes/macrophages and cultured with LPS + vitamin B6 or no treatment control for 24 hrs (n=3). Cells were trypsinised and mRNA was extracted, and genes were profiled with RT$^2$ gene profiler for Innate and adaptive immune response to determine fold change in normalised gene expression compared to untreated (Control cells indicated as 0 on X-axis). P values were calculated using the Student's T-test where significance was determined as *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

#### *5.3.3.2 Vitamin B6 downregulates expression of inflammatory and defence response genes*

Regulation and control of cellular defence in humans is vital for protection from environmental stresses, ranging from microbial infections to aging, diet and lifestyle choices. Defence response involves the activation of specific pathways which utilize cellular machinery to create physiological and behavioural cell responses, often by triggering transcriptional activation of inflammatory gene sets (Drew, 2012). Inflammatory response to harmful stimuli such as LPS initiates transcriptional activation of a variety of genes aimed at maintaining host defence and developing acquired immunity (Ahmed et al., 2015).

Pattern recognition receptor (PPR) genes, encode proteins for pattern recognition receptors which are part of the innate immune system and play a pivotal role in their ability to recognise molecules that are frequently found in pathogens. Pathogen-Associated Molecular-Patterns (PAMPs) are commonly found in pathogens such as viruses or bacteria but not in eukaryotic organisms (Amarante-Mendes et al., 2018). DAMPS Damage-associated Molecular-Patterns (DAMPs) are molecules released by damaged cells. LPS is an example of a PAMP and the cell's response to LPS is a microbiocidal and pro-inflammatory reaction due to the signal engagement of PRRs (Amarante-Mendes et al., 2018). PRRs are divided into four main subgroups. Toll-like receptors (TLR) extracellular signalling receptors, C-type lectin receptors (CLR); extracellular receptors, NLR or nucleotide-binding oligomerisation domain-like receptors (NOD); intracellular receptors, RIG-1 like receptors (RLR); cytoplasmic receptors. Of these, both TLR and NLR recognise LPS (Zaru, 2021). Gene array results indicated that vitamin B6 was able to downregulate the pro-inflammatory effect of LPS on PRR genes.

NOD- LRR- and pyrin domain-containing protein 3 (NLRP3) is an intracellular sensor for microbial detection which forms the NLRP3 inflammasome triggering the release of

inflammatory cytokines via the caspase-1 dependent pathway (Swanson et al., 2019). Vitamin B6 decreased expression of NLRP3, 3.8-fold, from 1.4 (LPS) to -2.5 (LPs+B6) ($p \leq 0.005$) (Fig 43A). DExD/H-Box Helicase 58 (DDX58) encodes for the protein RIG-1 an antiviral innate immune response receptor that, in response to viral nucleic acids, initiates a pro-inflammatory signalling cascade. This gene may also stimulate the production of granulocytes, bacterial phagocytosis, cell migration regulation and cellular differentiation (Shi et al., 2017). DDX58 expression was downregulated by vitamin B6 2.5-fold from 3.6-fold (LPS) to 1.1 (B6+LPS) ($p \leq 0.005$) (Figure 43).

Nucleotide-binding Oligomerisation Domain-like receptors (NOD1 and NOD 2), are intracellular receptors that specialise in detecting microbial pathogens with the ability to invade and multiply intracellularly. Once these receptors have been activated by pathogens, they activate signalling pathways which trigger transcriptional responses leading to the expression of a pro-inflammatory gene set (Moreira & Zamboni, 2012). Gene array results show that vitamin B6 downregulates the pro-inflammatory effect of LPS on bacterial defence genes NOD1, 3.9-fold from 1-fold (LPS) to -2.5-fold (B6+LPS) ($p \leq 0.005$), and NOD2, by 1.9-fold from 3.2-fold (LPS) to 02.4-fold (B6+LPS). (Figure 43A).

Toll-like receptors (TLRs) have emerged as an important part of the antimicrobial host defence response within the adaptive immune system as they recognize PAMPS from numerous microbes and are responsible for the activation of transcription factors, NF-kB and IRFs (Iwasaki & Medzhitov, 2004). Cell surface TLRs are responsible for the recognition of microbial membrane components such as proteins, lipoproteins, and lipids. These include TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10. Intracellular TLRs recognise bacterial and viral nucleic acids and self-nucleic acids in autoimmunity, these include TLR3, TLR7, TLR8, TLR11 and TLR12. TLR-4 recognises LPS and uses both MYD88 and TRIF dependent signalling pathways and selectively recruits TRAM adapter protein (Manicassamy &

Pulendran, 2009). Vitamin B6 downregulated TLR-4 expression by 3.2-fold from 1.5-fold (LPS) to -1.75-fold (B6+LPS) ($p \leq 0.05$) (Fig. 43A). TLR-9 binds bacterial DNA and activates NF-κB via the MYD88 and TRAF6 pathways. Vitamin B6 downregulated TLR-9 expression by 4.2-fold from 1.2 (LPS) to -2.8-fold (B6+LPS) ($p \leq 0.05$) (Fig. 43A). The following TLR genes were also downregulated by vitamin B6; TLR-1 down by -3-fold from 9.4-fold (LPS) to 6.4-fold (B6+LPS) ($p \leq 0.05$), TLR-2 down by 4.6-fold from 8.6 (LPS) to 4-fold (B6+LPS) ($p \leq 0.05$), TLR-5 – 3.7- fold from 1.1 (LPS) to -2.7 (B6+LPS) ($p \leq 0.05$), TLR-7 – 4.8 fold from 5.9-fold (LPS) to 1.1-fold (B6+LPS) ($p \leq 0.0005$), TLR-8 – 4.4 fold from 5.5-fold (LPS) to 1.1-fold (B6+LPS) ($p \leq 0.0005$) (Fig 43A).

The MYD88 gene provides instructions for making a protein (myeloid differentiation primary response protein 88) involved in cross membrane signalling within immune cells. MYD88 is used by all TLRs, it responds to inflammatory response via cytokine secretion and activates NF-κB via IRAK1, IRAK2, IRF7, and TRAF6 signalling pathways (Kawai et al., 2004; Yamamoto et al., 2014), as well as activating the MAPK signalling pathway (Campbell et al., 2021). The presence of vitamin B6 in monocyte/macrophage LPS stimulated cells was shown to downregulate MYD88 expression by 3.2-fold, from 1.4-fold (LPS) to -1.8-fold ($p \leq 0.05$) (Fig. 43D).

Cytokines, IL-1-beta (IL-1β) and IL-1α (IL-1α) are equally potent pro-inflammatory cytokines and produced in response to inflammation caused by infections and microbial endotoxins and other inflammatory agents. IL-1β is known to be responsible for contributing to further damage during chronic disease (Lopez-Castejon & Brough, 2011) and is implicated in many inflammatory conditions such as sepsis, inflammatory bowel disease and rheumatoid arthritis. LPS+B6 compared to LPS alone reduced IL-1β expression by 97-fold from 219.5-fold (LPS) to 121.8-fold ($p \leq 0.05$) (Fig 43B). IL-6 is known to respond with broad-ranging effects to

infection, and tissue injury, and contribute to host defence (Tanaka et al., 2014). IL-6 has pleiotropic effects, being both anti- and pro-inflammatory (Del Giudice & Gangestad, 2018; Takatsu, 2011) and works both to inhibit Th1 polarisation and promote Th2 differentiation (Diehl & Rincon, 2002). Excessive IL-6 synthesis is implicated in several disease pathologies including COVID-19 (Magro, 2020; Tanaka et al., 2014). Vitamin B6 significantly downregulate IL-6 by 17.8-fold from 21.6-fold (LPS) to 3.8-fold (B6+LPS) ($p \leq 0.005$) (Fig. 43C). IL-23-α is produced by macrophages and DCs and is an important part of the inflammatory response in peripheral tissues. IL-23-α expression is substantially increased in several human cancers (Langowski et al., 2006) leading to its tumour promoting properties. In addition, IL-23 producing macrophages are involved in inflammatory responses (Wang et al., 2019). The addition of vitamin B6 in LPS stimulated macrophage/monocyte cells significantly downregulated IL-23A expression by 3.4-fold from 1.9-fold (LPS) to -1.5-fold ($p \leq 0.05$) (Fig. 43D), suggesting vitamin B6 to be anti-inflammatory. Tumour necrosis factor-alpha (TNF-α) is an inflammatory cytokine secreted mainly by macrophages which assist in the regulation of proliferation and differentiation as well as apoptosis, coagulation, and lipid metabolism (Parameswaran & Patial, 2010). LPS+B6 v LPS alone reduce TNF expression by 1.6-fold, from 2.8-fold (LPS) to 1.2-fold (B6+LPS) ($p \leq 0.005$) (Fig. 43D). It is clear that vitamin B6 is anti-inflammatory by decreasing IL-1, IL-6, IL-23, and TNF-α expression on monocyte/macrophage cells.

LYZ is a gene encoding human lysozyme that acts as an antibacterial enzymatic agent and is found in leucocytes within human milk, spleen, lungs, plasma, saliva, and tears. Lysozyme is considered one of the most vital anti-bacterial agents in human and animal immunity. A recent study has demonstrated the anti-inflammatory action of lysozyme via gene regulation involving the TNF-α/IL-1β pathways in monocytes (Bergamo et al., 2019; Yanai et al., 2018). Vitamin

B6 was shown to downregulate LYZ expression by 4.9-fold, from 1.1-fold (LPS) to -3.8-fold ($p \leq 0.0005$) (Fig. 43D).

Complement component 3 (C3) is a key activator of the complement system involving both classical and alternative complement pathways. Pathogenic invasion triggers the cleaving of the C3 protein into two segments, C3a and C3b. C3a, known also as C3a anaphylatoxin is a modulator of inflammation and demonstrates antimicrobial activity. C3b acts as a regulating protein in complement system response (Bajic et al., 2013; Tausk & Gigli, 1990). Vitamin B6 was shown to downregulate C3 expression by 5.4-fold, from 8.1-fold (LPS) to -1.3-fold ($p \leq 0.005$) (Fig. 43D).

Forkhead box protein P3 (FOXP3), is a transcription factor that regulates immune control and is crucial in aiding the regulatory activity of regulatory T cells (Tregs) and is responsible for much of the cell's ability to suppress immune function (Lu et al., 2017). FOXP3 has also been shown to have a suppressive function and promote tumour growth. (Leavy, 2011). FOXP3+ macrophages can inhibit CD4+ T cells, and, inhibiting FOXP3 expression by small interfering RNA knockout abrogated their T cell suppressive ability (Leavy, 2011). Vitamin B6 was shown to downregulate FOXP3 expression on monocyte/macrophage cells by 2.9-fold, from 1.2-fold (LPS) to -1.7-fold ($p \leq 0.05$) (Fig 43D), suggesting that vitamin B6 may restore T cell functionality *in vivo*, as it decreases FOXP3 expression on monocyte/macrophage cells.

Signal transducer and activator of transcription 3 (STAT3) is important for its role in the maturation of immune cells, and immune response to bacteria and fungi (Gao & Ward, 2007). High levels of STAT-3 in the tumour microenvironment especially expressed by monocyte/macrophage cells result in a poorer prognosis, i.e., promote an immunosuppressive phenotype (Andersen et al., 2019). Herein, it was noted that STAT-3 expression was

downregulated by vitamin B6 by 3.2-fold from 1.9-fold (LPS) to -1.2-fold (B6+LPS) ($p \leq 0.005$) (Fig 43D), indicating that this lowered expression would promote anti-cancer properties *in vivo*.

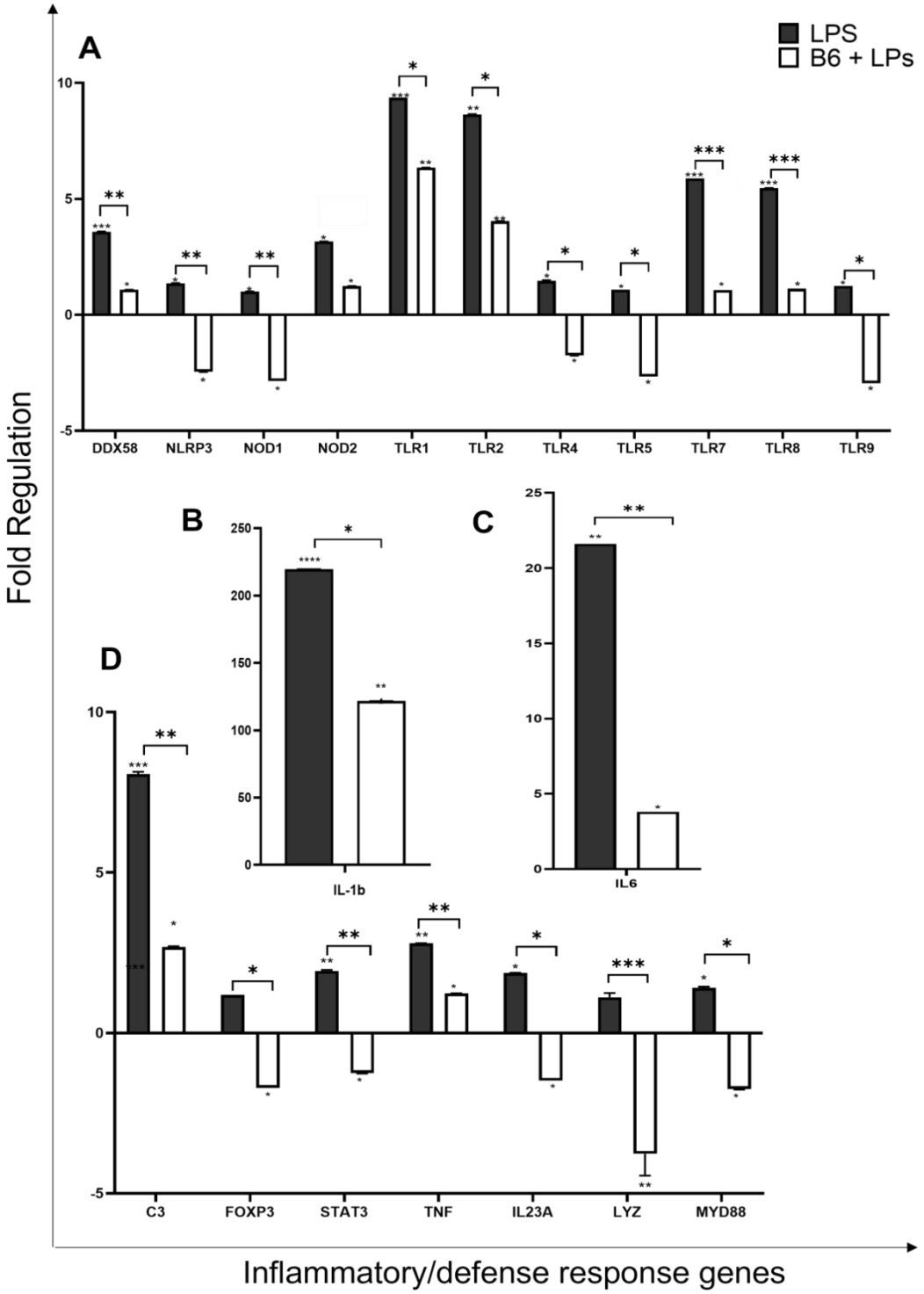



**Figure 43. Vitamin B6 decreases the expression of inflammatory/defense response genes in LPS stimulated monocyte/macrophage cells.**

U937 cells were differentiated into monocytes/macrophages and co-cultured with LPS + vitamin B6 for 24 hours. No treatment was used as a negative control (n=3). Cells were trypsinised and collected and mRNA was extracted, and genes profiled with $RT^2$ gene profiler for innate and adaptive immune response to determine fold change in normalised gene expression compared to untreated; control cells indicated as 0 on X axis. P values were calculated using Student's T test and significance determined as * P<0.05, ** P<0.01, *** P<0.001.

### *5.3.3.3 Vitamin B6 downregulates the expression of other genes in the Innate immune system*

The caspase 1 (Casp1) gene encodes a protein of the caspase (Cysteine-aspartic acid protease) family. Caspase 1 plays a role in innate immunity by responding to cytosolic signalling, by various inflammasomes, and initiating a two-fold response. The first deals with the activation and secretion of the pro-inflammatory cytokines IL-1β and IL-18, and the second response involves the triggering of pyroptosis, a form of lytic cell death forming part of the antimicrobial response (Jorgensen & Miao, 2015). Vitamin B6 was shown to downregulate Casp1 expression by 3.5-fold, from 2.2 (LPS) to -1.3-fold ($p \leq 0.005$) (Fig 44A).

Cluster differentiation 4 (CD4) molecule is expressed by T helper cells, but also by monocytes and macrophages. It is a membrane glycoprotein that performs an essential role in the immune response by assisting the T cell receptor (TCR) and amplifying TCR signalling. CD4 directly interacts with MHC class II molecules on antigen-presenting cells and is a primary receptor for the entry of HIV into host cells (Owen et al., 2018). Vitamin B6 was shown to downregulate CD4 expression by 3.5-fold, from 2.1-fold (LPS) to -1.5-fold ($p \leq 0.05$) (Fig 44A).

Major Histocompatibility complex class E (HLA-E) molecules are present in both innate and adaptive immune function modulating either activation or inhibition of natural killer (NK) cytotoxicity and cytokine production. HLA-E also plays a part in viral or bacterial peptide presentation provoking T-cell response (Camilli, 2016). In monocytes, HLA-E is upregulated during monocyte-macrophage differentiation (Camilli, 2016). Vitamin B6 was shown to downregulate HLA-E expression by 3-fold, from 1.8-fold (LPS) to -1.2-fold ($p \leq 0.05$) (Fig 44A).

Mitogen-Activated Protein Kinase 1 (MAPK1/ERK2) and MAPK3/ERK1 are prototypic MAP kinases that function primarily in mitogen-activated signal transduction pathways (Guan, 1994). They both play an important role in the MAPK/ERK cascade. The protein encoded by the MAPK1 gene is a component of the MAP kinase family. MAP kinases or Extracellular signal-regulated kinases (ERKs) are diverse in function, mediating processes such as cell differentiation, growth, and adhesion. They also play a role in the regulation of transcription, translation and arrangement of the cytoskeleton (Hommes et al., 2003). Vitamin B6 was shown to downregulate MAPK1 expression by 3-fold, from 1.5-fold (LPS) to -1.4-fold ($p \leq 0.05$) (Fig 44A).

Myeloperoxidase (MPO) is a heme-containing peroxidase that is synthesised and expressed mainly by myeloid cells during myeloid differentiation. It is mostly found in neutrophils and monocytes, released into the extracellular fluid during inflammation (Oyenuga et al., 2018). Vitamin B6 was shown to downregulate MPO expression by 3.1-fold, from 1.5-fold (LPS) to -1.6-fold ($p \leq 0.05$)( (Fig 44A).

MX Dynamin Like GTPase 1 (MX1) gene encodes a guanosine triphosphate (GTP) metabolising protein, induced by type I and type II interferons, that mounts an antiviral response against a variety of RNA, and some DNA viruses. Vitamin B6 was shown to downregulate MX1 expression by 3-fold, from 1.4-fold (LPS) to -1.6-fold ($p \leq 0.005$)( (Fig. 44A).

Nuclear Factor Kappa B Subunit 1 (NF-κB1) plays an important role in the regulation of immune response to infections and stimuli such as pro-inflammatory cytokines, chemokines, adhesion molecules and enzymes. NF-kB1 is involved in biological processes which include inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis (Best et al., 2019). Vitamin B6 was shown to downregulate NF-kB1 expression by 3-fold, from 1.9-fold

(LPS) to -1.3-fold ($p \leq 0.005$)(Fig 44A). NF-κB inhibitor alpha gene (NF-κBIA) encodes for the NF-κBIA protein. This protein enables NF-κB to remain bound within a protein complex termed IKK. NF-κBIA responds to signalling which allows NF-κB to be released from this complex and move into the nucleus to bind to DNA. NF-κB is responsible for the regulation of many genes that mediate inflammatory responses. One study showed that NF-κBIA mutation causes defective NF-κB signalling leading to hyper IL-1β secretion in macrophages (Tan et al., 2020). Vitamin B6 was shown to significantly downregulate NF-κBIA expression by 4.6-fold, from 7.9-fold (LPS) to 3.3-fold ($p \leq 0.05$) (Fig 44B).

Signal Transducer and Activator of Transcription 1 (STAT1) encodes a protein that drives multiple, complex, and contrasting transcriptional functions. It is involved in the inhibition of the IL-17 inflammatory pathway (Boisson-Dupuis et al., 2012), promotion of interferon-alpha/beta signalling pathways involved in viral defences and interferon-gamma signalling pathways important for bacterial defence (Boisson-Dupuis et al., 2012; Ramana et al., 2000). Vitamin B6 was shown to downregulate STAT1 expression by 3.1-fold, from 1.9-fold (LPS) to -1.3-fold ($p \leq 0.05$)( (Fig 44A). STAT6 is predominantly stimulated by IL-4 and IL-13 and is involved in allergic inflammatory disease (Montaser Shaheen, 2018,). It is known to drive M2 macrophage polarisation (Yu et al., 2019). STAT-6 expression was downregulated by vitamin B6 by 3.4-fold from 2.3-fold (LPS) to -1.1-fold (B6 + LPS) ($p \leq 0.05$) (Fig 44A).

The cell surface receptor, CD14 forms a multi-receptor complex along with TLR-4 and LYS96 which plays a significant role in LPS recognition and innate immune response. Monocytes and macrophages strongly express CD14 whilst promonocytes and monoblasts show only weak expression, hence CD14 is often viewed as a monocyte differentiation marker (Zanoni & Granucci, 2013). Vitamin B6 was shown to significantly downregulate CD14 gene expression by 3.5-fold, from 12.4-fold (LPS) to 2.7-fold ($p \leq 0.005$) (Fig 44B). Additionally, CD80 and

CD86 cell surface markers expressed by activated monocytes/macrophages have a differential role in the regulation of inflammation, especially in the innate immune response to sepsis. Upregulation of CD80 and loss of CD86 expression correlate with increased illness severity and inflammation in humans (Nolan et al., 2009). Decrease expression of CD86 is also involved in decreased T cell stimulation. CD80 expression was downregulated by vitamin B6 by 3.2-fold from 8.3-fold (LPS) to 5.1-fold (B6+LPS), although this change was not significant p=0.37 (not shown). CD86 expression, however, was significantly downregulated by vitamin B6, by 6.1-fold from 10.9-fold (LPS) to 4.8-fold (B6+LPS) ($p \leq 0.05$) (Fig 44B).

C-C motif chemokine receptor 4 and C-C motif chemokine receptor 5 (CCR4, CD194) and (CCR5, RANTES) are G protein-coupled chemokine receptors. Chemokines comprise a group of molecules that play a fundamental role in the regulation of leukocyte trafficking as well as the development, and homeostasis of the immune system. CCR4 regulations inflammatory macrophage function in many inflammatory disorders including multiple sclerosis; CCR4 knockout mice show delayed disease progression (Forde et al., 2011) CCR4 expression on monocyte/macrophage cells was upregulated by 1.1-fold in the presence of LPS but was downregulated by 4.7-fold ($p \leq 0.05$) in LPS+vitamin B6 cultures (Fig 44C). In addition, vitamin B6 significantly downregulated CCR5 (CD195) expression by 7.6-fold ($p \leq 0.005$) (Fig 44C). The expression of CCR5 on monocyte/macrophage cells is involved in inflammatory responses to infection, hence, its downregulation in the presence of vitamin B6 suggests its anti-inflammatory properties. Furthermore, intracellular adhesion molecule (ICAM) is greatly increased in immune cells as a response to inflammatory stimulation. It is best known for its role in directing leukocytes from the circulation to sites of inflammation (Bui et al., 2020). Vitamin B6 was shown to downregulate ICAM expression by 3.5-fold, from 5.3 (LPS) to 1.8-fold ($p \leq 0.005$) (Fig 44C). Moreover, C-X-X Motif Chemokine Receptor 3 (CCXCR3, GPR9, CD 183) is an interferon-inducible chemokine-receptor expressed on monocytes and other cell

types which is involved in cytoskeletal changes, chemotactic migration, and activation of integrin (Jinquan et al., 2000). CXCR3 expression was downregulated in the presence of vitamin B6 from LPS by 3.4-fold from 1.9-fold (LPS) to -1.4-fold (B6 + LPS) ($p \leq 0.05$) (Fig 44C)

Signal transducer and activator of transcription 6 (STAT6) is predominantly stimulated by IL-4 and IL-13 and is involved in allergic inflammatory disease (Montaser Shaheen, 2018,). STAT-6 expression was downregulated by vitamin B6 by 3.4-fold from 2.3-fold (LPS) to -1.1-fold (B6 + LPS) ($p \leq 0.05$) (Fig 44C).

Interferon Regulatory Factor (IRF3) is a transcriptional regulator of type I IFN, involved in response to pathogenic infection in the innate immune system (Yanai et al., 2018). Vitamin B6 was shown to downregulate IRF3 expression by 3.8-fold, from -1-fold (LPS) to -2.8-fold. ($p \leq 0.005$) (Fig 44C)

Integrin alpha M (ITGAM) also known as CD11b or CR3 (complement receptor 3), is a gene encoding integrin adhesion molecule M. It is important for various adhesive interactions involving monocytes, macrophages and granulocytes and the phagocytosis of complement coated particles. ITGAM is also involved in leukocyte migration in the presence of CD18 (DiScipio et al., 1998). (Yanai et al., 2018). Vitamin B6 was shown to downregulate ITGAM expression by 2-fold, from 3.1-fold (LPS) to 1.1-fold ($p \leq 0.05$) (Fig 44C). TLR adaptor molecule 1 (TICAM) is an adapter molecule within the innate immune system which responds to pathogenic invasion. TICAM facilitates protein to protein interactions between TLRs, especially TLR3 (Takashima et al., 2018), to mediate NF-κB and IRF (interferon regulatory factor) activation during an antiviral immune response (Seya et al., 2012). Vitamin B6 was shown to downregulate TICAM expression by 3.9-fold, from 2.7-fold (LPS) to -1.1-fold ($p \leq 0.05$) (Fig 44C).

Mitogen-activated protein kinase 8 (MAPK8) also known as JNK1, is a member of the MAP kinase family. MAP kinases oversee a broad range of cellular processes including proliferation, differentiation, transcription regulation and development. MAPK8 mediates early gene expression in response to cellular stimuli. MAPK8 is involved in TNF-α induced apoptosis, it is also thought to be involved in the cytochrome c-mediated cell death pathway via ultraviolet radiation-induced apoptosis (Gaestel et al., 2009; Sabio & Davis, 2014; Tournier et al., 2000) Vitamin B6 was shown to downregulate MAPK8 expression by 3.3-fold, from 1.2-fold (LPS) to -2.1-fold ($p \leq 0.05$) (Fig 44C).

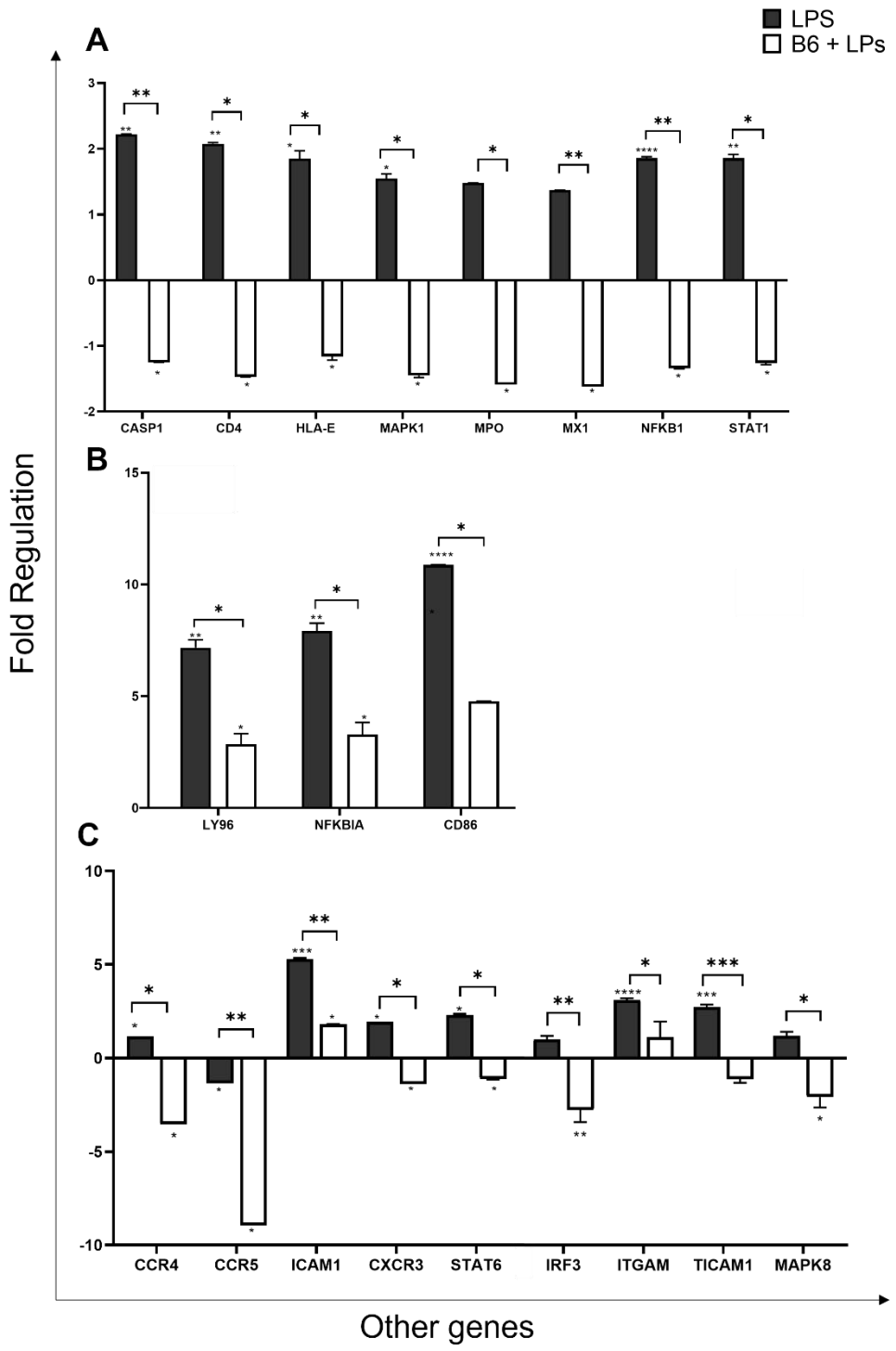



**Figure 44. Vitamin B6 downregulates the inflammatory response of other genes in LPS stimulated monocyte/macrophage cells.**

U937 cells were differentiated into monocytes/macrophages and co-cultured with LPS + vitamin B6 for 24 hrs. No treatment was used as a negative control (n=3). Cells were collected and mRNA was extracted, and genes were profiled with $RT^2$ gene profiler for Innate and adaptive immune response to determine fold change in normalised gene expression compared to untreated. Control cells are indicated as 0 on X-axis. P values were calculated using Student's T-test and significance determined as *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, +P>0.05.

## 5.4. Discussion

This is the first study to demonstrate the extensive broad array of anti-inflammatory effects of high dose vitamin B6 on an LPS activated monocytes/macrophage cell line. Vitamin B6 was shown to downregulate genes associated with inflammatory responses and defence responses to bacteria. These include inflammatory cytokines and chemokines, pattern recognition receptors, and cell surface markers. LPS is a key component of the outer membrane of gram-negative bacteria and is a potent activator of the highly LPS sensitive monocytes and macrophages with an ability to upregulate the production of key inflammatory mediators such as cytokines, chemokines, and other inflammatory-related proteins (Guha & Mackman, 2001; Plevin et al., 2016). LPS monocyte stimulation induces an array of genes that express inflammatory mediators and the addition of vitamin B6 to the cell cultures, was shown to downregulate these responses. The ability of vitamin B6 to downregulate inflammatory cytokine production has previously been reported. Supplementation of vitamin B6 was shown to suppress TNF-α and IL-6 levels in patients with rheumatoid arthritis (Huang et al., 2010). Likewise, bone marrow-derived macrophages treated with pyridoxal and stimulated with LPS reduced IL-1β, TNF-α and IL-6 (Du et al., 2020). In another study, vitamin B6 downregulated inflammation (decreased, IL-1β, IL-6, TNF-α), delayed death and increased the survival rate in high dose LPS treated mice compared to controls (Du et al., 2020). Vitamin B6 has also been found to protect mice from toxic effects induced by LPS by preventing IL-1B protein production via the inhibition of NLRP3 inflammasome activation (P. Zhang et al., 2016) and also inhibits activation of NF-κB in LPS stimulated mouse macrophages (Yanaka et al., 2005). Further, high-dose B6 combined with compound amino acid was shown to prevent inflammation by inhibiting the HMNGB1/TLR4/NF-κB signalling pathway (Yi et al., 2020).

Interestingly we note that vitamin B6 significantly downregulated NF-κB IL-6, IL-1β, TNF-α and NLPR3 which aligns with these previous studies.

Monocytes are crucial players in the innate immune system and as such, rely on PRRs to detect pathogens and initiate host defence responses. Amongst these are TLRs which play a vital part in host defence response and activation, direct microbiocidal activity and activate inflammatory pathways. TLRs on the surface of cells recognize microbial membrane components whilst intracellular TLRs recognize bacterial and viral nucleic acids. TLR4 recognizes LPS and utilizes MYD88 and TRIF (TICAM) dependent signalling pathways (Kawai et al., 2004; Manicassamy & Pulendran, 2009; Monguio-Tortajada et al., 2018). Herein, we note that vitamin B6 was also able to downregulate both cell surface and intracellular TLRs as well as MYD88 and TICAM gene expression. Other genes (STAT1, STAT3, C3, LYZ, L96, CCL5) relating to the inflammatory response to bacteria were also shown to be downregulated in the presence of vitamin B6.

Although it is interesting to consider the downregulation of inflammatory response gene by gene, the value of this study lies in the overall context of the data. Vitamin B6 appears to be effective in mediating an overall dampening of pro-inflammatory responses induced by LPS stimulation. The importance of these findings lies in the potential therapeutic use of vitamin B6 in the context of hyperinflammatory immune disorders. Gram-negative sepsis is associated with significant morbidity and mortality in both adults and children worldwide (Rudd KE, 2020; Tan et al., 2019). Sepsis occurs when the immune system succumbs in the battle against severe infection. Immune cell activation by LPS from the cell wall of infecting bacteria during sepsis can cause the production of inflammatory cytokines such as IL-6, IL-1β and TNF-α (Nedeva et al., 2019). If the immune system is overwhelmed and dysregulated by bacterial load a “cytokine storm “ can ensue producing catastrophic inflammation leading ultimately to organ failure and death (Nedeva et al., 2019). Currently, there are no drugs specifically

approved to treat sepsis although some drugs have been (unsuccessfully) trialled that target particular inflammatory pathways, for example, Cytofab a drug made by AstraZeneca targeting TNF-α (Rice et al., 2006).

A small percentage of patients who contract lethal SARS-CoV-2 virus display a similar dysfunctional immune response whereby a 'cytokine storm' can lead to acute respiratory distress syndrome and multiple organ failure (Ragab et al., 2020). These patients also exhibit high levels of inflammatory factors such as TNF-α, IL-1β, IL-6, IL-10, CXCL8, and IFN-γ (Chen et al., 2021). It appears that many of these inflammatory pathways that are upregulated in cytokine storm in both patients with sepsis or COVID-19 are the same pathways downregulated by vitamin B6 in LPS stimulated monocytes. It is possible that the use of a natural immunosuppressant such as high dose vitamin B6 as a monotherapy, or in combination with other drugs, may be useful in targeting the inflammation in cytokine storms associated with these hyper-inflammatory conditions. High dose intravenous vitamin C monotherapy has been shown to possess some benefits in critically ill sepsis patients by lowering inflammatory markers, as has high-dose vitamin C (up to 10,000 mg/day) in combination with hydrocortisone (Wald et al., 2021), but to date, vitamin B6 has not been studied for use in humans for the treatment of cytokine storm in sepsis or COVID-19 patients although one study has shown that high dose B6 can successfully reduce oxidative stress and exert anti-inflammatory effects in peripheral organs of cecal ligation puncture induced infection in adult male Wistar rats (Giustina et al., 2019).

Although the potential of these findings is exciting, a note of caution is due here given the limitations of this study. The main limitation is that monocyte and macrophages were studied in isolation, when in fact the inflammatory microenvironment includes numerous other immune cells and the crosstalk between them could greatly affect the data, we note *in vitro,* to than when studied *in vivo*. Furthermore, only one dosage and time point were examined and

protein studies done in this study over different time points indicate that cytokines have their own timelines, and this may be an important factor in the administration of dosages in therapy situations.

## 5.5. Conclusion

In conclusion, this study has identified that high dose vitamin B6 has a global anti-inflammatory effect on LPS induced inflammation in monocyte/macrophage cells by downregulating the key broad-spectrum inflammatory mediators, CCL2, CCL5, CXCL2, CXCL8, CXCL10, CCR4, CCR5, CXCR3, IL-1β, IL-5, IL-6, IL-10, IL-18, IL-23-a, TNF-α, CSF2, DDX58, NLRP3, NOD1, NOD2, TLR-1 -2 -4 -5 -7 -8 -9, MYD88, C3, FOXP3, STAT1, STAT3, STAT6, LYZ, CASP-1, CD4, HLA-E, MAPK1, MAPK8 MPO, MX-1, NF-kB, NF-kB1A, CD14, CD40, CD40LG, CD86, Ly96, ICAM1, IRF3, ITGAM, IFCAM2. Although further studies are required to understand whether these effects can be translated in *in vivo* animal models and amongst cross talk from other immune cells in the complex inflammatory micro-environment, the findings herein show promise regarding vitamin B6 within the context of a potent broad-spectrum anti-inflammatory mediator and could be useful as an adjunct treatment for inflammatory-related diseases.

# Chapter Six

# High dose vitamin B12 increases the expression of inflammatory markers in LPS stimulated monocytes

## Abstract

Vitamin B12 is an essential nutrient required for many cellular processes in humans. Vitamin B12 deficiency can cause inflammation and leads to the pathogenesis of inflammatory disease which can be reversed by supplementation. It is unclear, however, what the effects are of B12 supplementation above the recommended daily intake (RDI) or whether high dose vitamin B12 has any therapeutic effects on inflammatory processes. In fact, some studies have correlated high serum vitamin B12 with increased mortality. This study set out to examine the effects of high dose vitamin B12 on LPS stimulated monocytes to ascertain a potential role in the inflammatory process in these cells. It was noted that vitamin B12 did not ameliorate the inflammatory effects of LPS but instead, contributed to the inflammatory effects of LPS by further upregulating key inflammatory mediators.


## 6.1. Introduction

Vitamin B12 belongs to a group of compounds known as cobalamins, as they entail cobalt centred corrin nucleus. Vitamin B12 is the largest and most complex of the B vitamins of which cyanocobalamin is the metabolically active form. Vitamin B12 is synthesised by bacteria and sources of it are found in the human diet primarily of animal origin or biomagnified through the food chain (Rizzo et al., 2016). Vitamin B12 is bound to protein in food and released by haptocorrin (HC) found in saliva and gastric fluids. Vitamin B12 is released from HC once it reaches the duodenum by pancreatic proteases (Lyon et al., 2020). For absorption to occur into the body, vitamin B12 must be combined with intrinsic factor prior to it being absorbed into the ileum. Following absorption, vitamin B12 is released into the circulation and transferred into cells via a plasma transporter, transcobalamin II. Once inside cells the transcobalamin II-B12 complex is then degraded by lysosomal activity and free vitamin B12 is released into the

cytoplasm (Figure 45) (Mikkelsen, Stojanovska, & Apostolopoulos, 2016; Mikkelsen K., 2019).

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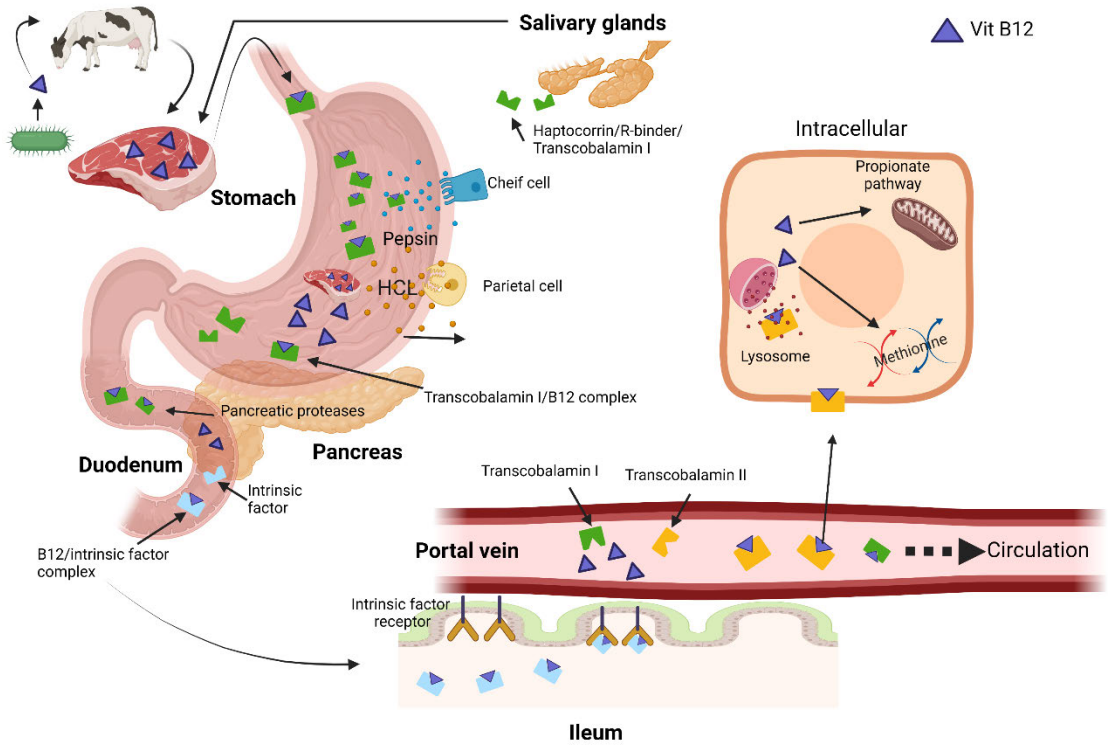


**Figure 45. Digestion and absorption of vitamin B12.**

Vitamin B12 is an essential nutrient required for many processes in humans. These include maintenance of the nervous system including myelination, cell division and synthesis, energy extraction from proteins and fats via methyltransferase reactions, cell synthesis, red blood cell formation, fatty acid and protein catabolism, DNA synthesis, cell division and as a co-factor in many metabolic processes including methylation and synthesis of neurotransmitters serotonin and dopamine (Ankar & Kumar, 2021; Mikkelsen, Stojanovska, & Apostolopoulos, 2016; Mikkelsen, Stojanovska, Prakash, et al., 2017; Mikkelsen, Stojanovska, Tangalakis, et al., 2016). Vitamin B12 requirements in humans are very small compared to other essential nutrients including other B vitamins. The recommended daily intake (RDI) proposed by the National Health and Medical Research Council (Australian dietary guidelines) as well as the

National Institute of Health USA is only 2.4 μg per day (and is much lower for < 14-year-olds) compared to, vitamin B6, which is 1.3 mg/day (Government, 2018). Vitamin B12 deficiency can occur via dietary deficiency as is sometimes noted in vegetarian/vegan diets, or malabsorption caused by various factors including reduced production of intrinsic factor, long term use of certain drugs, bacterial overgrowth, surgical intervention, malignancy of pancreas or bowel and aging.

Vitamin B12 deficiency contributes to many pathological conditions, many of these resulting from increased inflammation caused by ineffective methylation in one-carbon metabolism, resulting in hyperhomocysteinemia and contributing to systemic and vascular inflammation (Lyon et al., 2020). Vitamin B12 is crucial for the production of red blood cells which ensure adequate delivery of oxygen to body tissues and for the regulation of cellular immunity in particular in the number and function of NK and CD8+ T cells (Tamura et al., 1999). In fact, in pernicious anaemia, a decreased number of CD4 and CD8 T cells are noted as well as reduced NK cell activity (Erkurt et al., 2008) but not serum antibody levels.

Vitamin B12 deficiencies can also impact immune system function by compromising both cell-mediated and humoral immunity (Funada et al., 2000) and inhibit immune cell activities such as nucleic acid production and protein synthesis. Vitamin B12 deficiency also leads to inflammation which can be reversed by B12 supplementation (Azadibakhsh et al., 2009; Dierkes et al., 1999). An interesting area of research that has not yet fully been explored is what effect high dose vitamin B12 supplementation may have on pre-existing inflammation not caused by B12 deficiency.

The advantages of the therapeutic use of high dose vitamin B12 are ambiguous, with some studies noting its beneficial actions as an anti-inflammatory/anti-oxidant (Birch et al., 2009; Manzanares & Hardy, 2010; Wheatley, 2006) whilst other studies associate high dose serum

B12 levels with increased inflammation and poor prognosis in critically ill patients (Sviri et al., 2012). Elevated serum B12 is often found in and prognostic for, myeloid and solid cancers, metastases of the pancreas, colon/rectum, lungs, urothelium, bone and liver (Ermens et al., 2003; Urbanski et al., 2020). It appears that illness itself can lead to elevated serum B12 levels due to organ inefficiency and decreased cellular uptake. Andres et al., stated that three essential pathophysiological mechanisms account for elevated B12 levels, these being (1) Dietary excess or direct plasma increase by liberation from an internal reservoir, (2) An increase in transcobalamin via excess production or lack of clearance and (3) Quantitative deficiency or lack of affinity of transcobalamin for vitamin B12 (Andres et al., 2013). Paradoxically high serum B12 can be reflective of a functional deficiency related to defects in cellular uptake and accompanied by clinical signs of B12 deficiency. These include an increase in serum Hcy and methylmalonic acid which contribute to an increase in inflammation (Andres et al., 2013).

Monocytes are circulatory blood cells, which differentiate into macrophages or dendritic cells in tissues. These cells are phagocytic, process and present antigens and secrete cytokines (Chanput, 2015). The U937 cell line is a promonocytic cell line that can be differentiated into monocytes and macrophages with vitamin $D_3$ and presents a viable model to study inflammation. As such, in this chapter, the effects of high dose vitamin B12 on lipopolysaccharide (LPS) stimulated monocyte/macrophage cells were studied to ascertain whether vitamin B12 could modulate inflammation. Interestingly, high dose vitamin B12 did not resolve the pro-inflammatory effects of LPS as assessed using gene arrays, but in many genes, it increased the inflammatory effects even further. Within the protein studies, however, B12 was shown to downregulate cytokine production of IL-1β, IL-6 and IL-10 at 24 hrs.

## 6.2. Materials and methods

### *6.2.1 Cell culture and reagents6.2.1.1 Culture of U937 cells*

The U937 cell line was originally isolated from a patient with histiocytic lymphoma. U937 cells are frequently used to examine the performance and differentiation of monocytes. Treating U937 cells with vitamin $D_3$ initiates differentiation into monocyte/macrophages (Chanput, 2015). The U937 cell line was purchased from ATCC (https://www.atcc.org) by the Monash University Department of Immunology. U937 cells allow for multiple passages without transformation, however, we ensured to keep the number of passages to a minimum. U937 cells were cultured in RPMI 1640 media supplemented with 2 mM L-glutamine (Sigma-Aldrich, St Louis, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 10 % heat-inactivated foetal bovine serum (FBS; Sigma-Aldrich) at 37$^{o}$C and 5 % $CO_2$. RPMI 1640 media was replaced every 3-4 days and cells were passaged accordingly. Once 80-90 % confluent, cells were utilised in experiments.

#### *6.2.1.2 Preparation and treatment with vitamin B12*

Vitamin B12 (Cyanocobalamin-V6629, Sigma, VIC Australia) was dissolved in phosphate-buffered saline (PBS) and filtered using a 0.2-micron filter to ensure it was free from microbial pathogens at the beginning of each new experiment. A dosage of 250 µg/ml of vitamin B12 was used in this study. This dosage was based on data from previous proliferation studies performed assessing dose-dependent effects of vitamin B12 on undifferentiated U937 cells. The dosage used correlated with the lowest dose whereby vitamin B12 increased proliferation above control (Figure 46)).

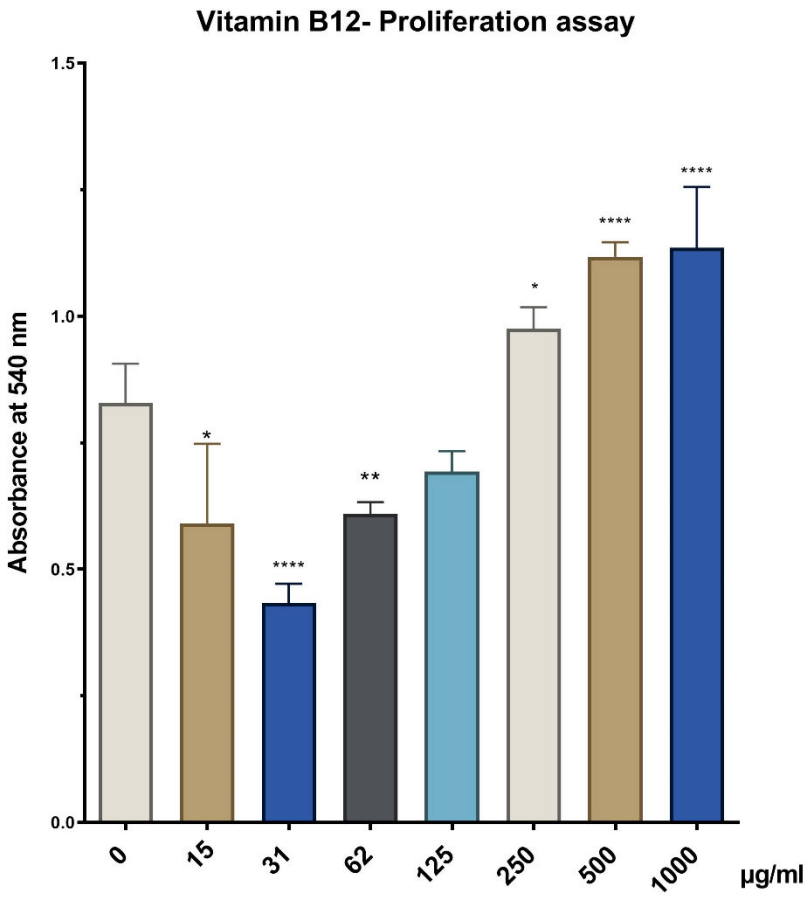


**Figure 46. Results of day 6 vitamin B12 proliferation assay.**
Cells were incubated with increasing doses of vitamin B12 for 6 days in 96 well U bottom plates and analysed by MTT assay. Absorbance readings were taken at 540 nm to assess for cellular proliferation and compared to control wells (0 mg/ml). Significance was established at $p \leq 0.05$ two-way Anova followed by Tukey's multiple comparisons test and marked with asterisk (* $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$). All experiments were repeated at least 3 times in triplicate wells.

## *6.2.2 Cell surface marker expression by flow cytometry*

Cell surface markers are displayed on the surface of immune cells, and each contributes in a different way to the function of the cell. Markers expressed on U937 differentiated monocyte/macrophages were assessed following LPS stimulation and the addition of vitamin

B12. Cells were seeded at densities of $1x10^6$ cells/ml in tissue culture flasks and treated with 100 nM $VitD_3$ for a total of 72 hrs (hrs). Differentiated U937 cells were stimulated with 1 μg/ml LPS with or without 250 μg/ml vitamin B12. Cells were cultured for a further 24 hrs. Cells were surface labelled for cluster differentiation (CD)14, CD40, CD80, CD86, CD206 and CD209 markers and supernatants were collected for cytokine secretion analysis using Bio-Plex (Bio-Rad, VIC Australia). U bottom 96 well plates were seeded with 100 μl of vitamin B12 treated monocytes and controls at $5x10^5$ cells per/well and incubated with Fc block (1:100 dilution) for 30 minutes on ice. After washing with PBS, cells were labelled with surface antibody cocktails and isotype controls linked to a fluorochrome (made up in FACS buffer (PBS, 2-5 % FBS, 0.5 mM EDT and incubated on ice for 30 minutes in the dark. The antibodies were diluted in FACS buffer at the following dilutions according to the manufacturer's recommendations (CD14-BV421 1:200; CD40+CD80-FITC (A488) 1:400, CD86-Alexafluor 488 1:400; CD206 + CD209 -PE/Cy7). Cells were washed and re-suspended in 300 μl of FACS buffer and transferred to FACS tubes. Cells were collected using the BD FACS Canto II, using the Cell Quest program (BD, VIC Australia), and % cell surface marker expression was analysed using BD FACS Diva software. Background quadrants were set up using the percentage of expressed markers; isotype antibody controls (Biolegend and BD Life Sciences).

### *6.2.3 Bio-Plex cytokine assay*

The Bio-Plex human cytokine immunoassay is a highly sensitive and reproducible magnetic bead-based assay that allows accurate measurement of low levels of human cytokines. The Bio-Plex cytokine assay uses 8 μm magnetic beads coated with antibodies against an array of

cytokines. Cytokine assays were performed using a bead-based multiplex immunoassay (MIA, 9 Bio-Plex Panel B, Bio-Rad Laboratories Inc. VIC Australia) that included the cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17α, IFN-γ, TNF-α. U937 cells were seeded at an appropriate density in tissue culture flasks and stimulated with 100 nm $VitD_3$ for a total of 72 hrs. Differentiated U937 monocyte/macrophage cells were then stimulated in triplicates with 1 μg/ml LPS, with or without 250 μg/ml vitamin B12; untreated cells were used as controls. Cells were cultured for 1 hr, 3 hrs, 6 hrs, 12 hrs, 24 hrs, 72 hrs and 144 hrs to assess the time course secretion of cytokines. Supernatants were collected at the end of each time period and placed immediately at -80 °C to prevent cytokine degradation. Standard low photomultiplier tube (PMT) settings were prepared with "blank" negative controls in duplicate. Ninety-six-well plates were coated with beads, followed by the addition of samples and standards and detection antibodies, then streptavidin-phytoerythrin as per the manufacturer's instructions. The beads were re-suspended, and the fluorescence output was read and calculated on the Bio-Plex array reader (Bio-Rad, VIC Australia). Statistical analysis of data included the mean and standard deviation (SD), as well as a Two-Way ANOVA followed by Sidak's multiple comparison test using GraphPad Prism (GraphPad Software, USA). Significance was defined as $p \leq 0.05$.

### *6.2.4 RT² profiler PCR array for Human Innate and Adaptive Immune responses*

The RT² profiler polymerase chain reaction (PCR) array kit for pathway expression profiling was used to assess the role of vitamin B12 in modulating monocyte/macrophage immune responses to LPS. LPS is widely used as a powerful activator of monocytes and macrophages and induces the production of key pro-inflammatory mediators (Tucureanu et al., 2018). The

human innate and adaptive immune responses PCR array assess 84 genes relating to IL-1R and toll-like receptor (TLR) signalling pathways involved in pathogen detection and host defence against bacteria, acute phase response, complement activation inflammatory response and antibacterial humoral response, as well as genes involved in innate immune response and septic shock. U937 cells were seeded at an appropriate density in tissue culture flasks and stimulated with 100 nM $VitD_3$ for a total of 72 hrs. Differentiated U937 monocyte/macrophage cells were stimulated in triplicates with 1 μg/ml LPS with or without 250 μg/ml vitamin B12. Cells were cultured for an additional 24 hrs and then removed from the flask and used. The leftover adherent cell population (macrophages) were removed from the flask using 1 mg/ml of trypsin and then washed twice with PBS and centrifuged. Media was removed and cell pellets were snap-frozen in liquid nitrogen and stored immediately at -80 °C.

#### *6.2.4.1 RNA extraction from immune cells*

Extraction of RNA from treated cells was performed using an RNeasy1 mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. RNA from treated cells was extracted from each cell pellet by disrupting cells with lysis buffer/mercaptoethanol mix. Cells were lysed with buffer RLN (a buffer containing non-ionic detergent), and the lysate was placed in the supplied Qia-shredder columns for homogenization and then transferred to RNeasy mini-spin columns. DNase was used to eliminate any genomic DNA contamination with the RNase-free DNase set (Qiagen, Hilden, Germany). Samples were then tested for RNA Integrity (RIN) using the Agilent 2100 Bioanalyser and Agilent RNA 6000 nano kit (Agilent Technologies, Santa Clara, CA, USA). All samples tested and used had an RNA integrity number (RIN) higher than the cut-off point of 8.

### *6.2.4.2 Assessing change in gene expression.*

RNA was reverse transcribed using an $RT^2$ first strand kit (Qiagen, Hilden, Germany) and resultant cDNA was used on the real-time $RT^2$ Profiler PCR Array for Innate and adaptive immunity (QIAGEN, Cat. no. PAHS-052Z) in combination with $RT^2$ SYBR® Green qPCR Mastermix (Cat. no. 330529) for evaluation of gene expression. LPS alone and LPS with vitamin B12, 250 μg/ml were analysed in comparison to untreated (no LPS or vitamin B12) cells using Qiagen web-based software for calculation of fold change and results compared using criteria of >2.0-fold increase/decrease in gene expression as biologically meaningful.

### *6.2.4.3 Analysis of Data*

Fold-Change (2^ (-Delta Delta CT)) is the normalised gene expression (2^ (- Delta CT)) in the Test Samples (LPS alone and LPS+vitamin B12) divided by the normalised gene expression (2^ (- Delta C)) in the control sample (Untreated). $C_T$ values for each group were exported into Excel to create a table of $C_T$ values which was then uploaded to the data analysis web portal (http://www.qiagen.com/geneglobe). Samples were categorised into control and test groups. $C_T$ values were normalised based on Manual Selection of reference genes. Reference genes are endogenous genes employed as a reference for certifying the authenticity of RT-PCR results. The basis for this internal referencing presumes that the less variation in endogenous gene expression, the better the experimental outcome (Fu et al., 2006). The data analysis web portal calculates fold change/regulation using the delta-delta $C_T$ method, in which delta $C_T$ is calculated between the gene of interest (GOI) and an average of reference genes (HKG), followed by delta-delta $C_T$ calculations (delta $C_T$ (Test Group)-delta $C_T$ (Control Group)). Fold Change is then calculated using the 2^ (-delta-delta $C_T$) formula. Fold change data was graphed

using prism software whereby fold change of the two treatment groups (LPS alone and LPS+ vitamin B12) was plotted against the control group (untreated) represented as 0 on the X-axis.

#### *6.2.4.4 Statistical Analysis*

Calculation of values was performed using the student's t-test of the triplicate 2^ (- Delta CT) [(2^-ΔCT)] values for each gene in the control group versus the treatment groups. P values less than 0.05 were considered significant. The p-value calculation used is based on parametric, unpaired, two-sample equal variance, and two-tailed distribution. Genes were considered significantly altered and included in the analysis if they displayed a greater than >2.0-fold change up/down and a p-value of $P \leq 0.05$.

## 6.3. Results

### *6.3.1 Vitamin B12 alters the cell surface expression of CD14*

Monocytes and macrophages express cell surface markers that determine their activation state. CD14 is expressed by monocytes, macrophages, and dendritic cells, and acts as a co-receptor (together with TLR-4) for detection and activation by bacterial LPS. CD40, CD80, and CD86 are primarily involved in determining the activation state of mature antigen-presenting cells such as dendritic cells, macrophages, and monocytes; they are present on activated antigen-presenting cells. Expression of CD40, CD80, or CD86 on mature antigen-presenting cells is required for stimulation of T cells. CD206 (or mannose receptor) a pathogen recognition receptor that binds microbial antigens is expressed on immature dendritic cells and not

monocytes. Therefore, the expression levels of CD14, CD40, CD80, CD86, CD206 and CD209 were determined on differentiated U937 monocyte/macrophage cells. LPS was used to induce an inflammatory profile of cells and vitamin B12 was added to determine its effects on cell surface markers expression. Vitamin B12 showed an increase in expression of CD14, 81.5 % (LPS+vitamin B12) versus 55.5 % (LPS), no significant changes were noted for CD40, CD80, CD86 CD206 or CD209 (Figure 47).

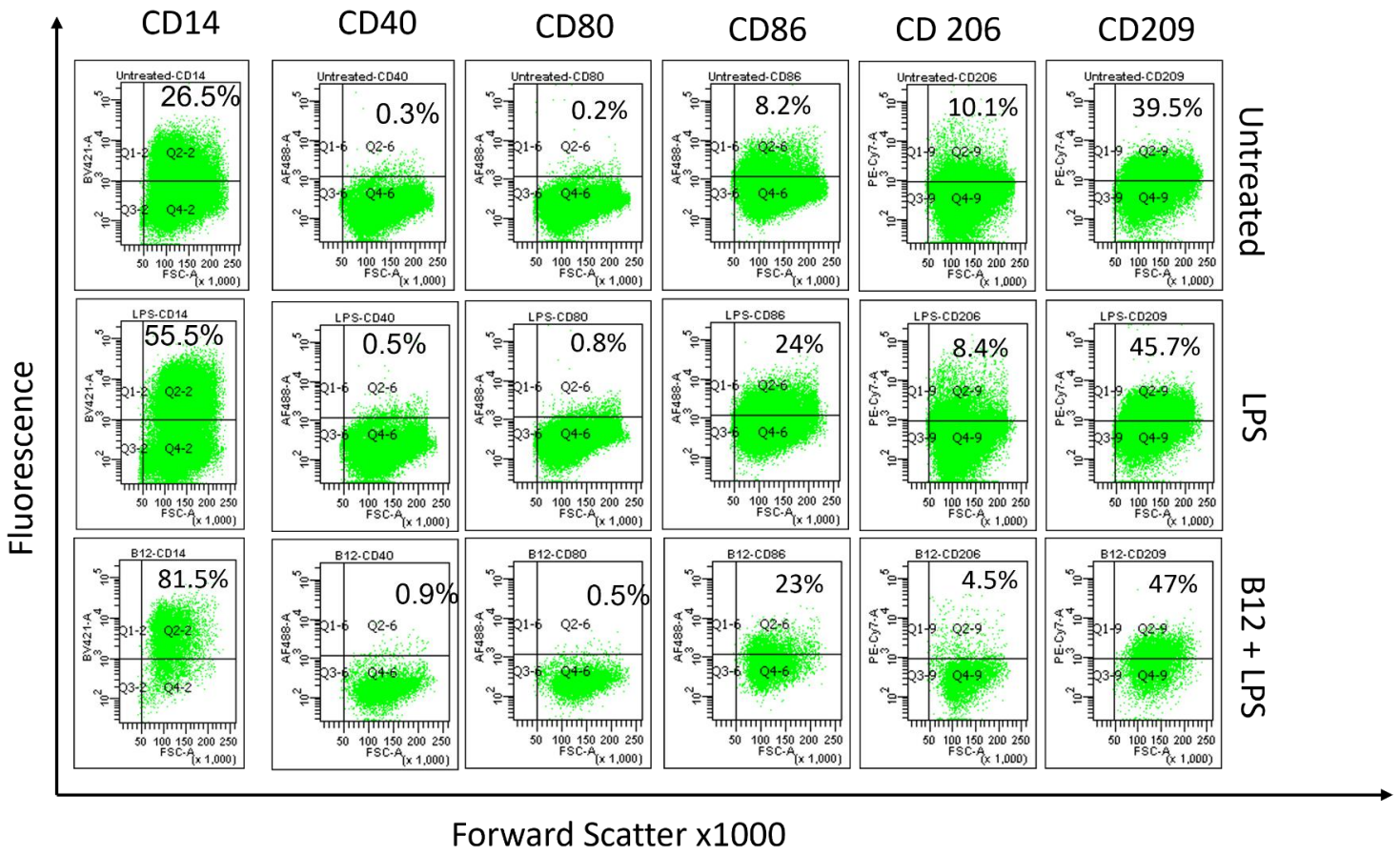


**Figure 47. Vitamin B12 increases CD14 inflammatory marker expression in monocyte/macrophage population.**

Percentage expressions are represented as dot plots. U937 cells were differentiated into monocyte/macrophage cells for 72 hours followed by stimulation with LPS for 24 hours with or without vitamin B12. Top right quadrant represents cells which are surface marker positive. Cells were co-cultured 250 µg vitamin B12 + 1 µg/ml LPS or 1 µg/ml LPS alone. LPS stimulated cells increased the surface expression of CD14 compared to untreated which was further enhanced in the presence of vitamin B12 (B12 + LPS). *There were no significant changes in expression of CD40, CD80, CD86, CD206 and CD209 with the addition of vitamin B12.*

### *6.3.2 Vitamin B12 significantly alters IL-1β, IL-6, IL-10 and TNF-α secretion*

Cytokine production in monocytes and macrophages can be stimulated using LPS, which is the main component of the outer membrane in gram-negative bacteria. LPS is widely utilised for the study of inflammation and responds via the stimulus of key cytokines and chemokines. A time course, bio-plex, cytokine, and immunoassay were employed to ascertain the effects of vitamin B12 on an LPS stimulated monocyte cell population at different time points.

IL-6 was upregulated between 12-72 hrs in the presence of LPS which was down-regulated in the presence of vitamin B12 at 12 hrs ($P \leq 0.01$) even lower than untreated cells; at 24 hrs there was a trend of downregulation, but this was not significant (Figure 48A). Similarly, high levels of IL-10 were secreted by LPS stimulated cells by 12-24 hrs which was significantly downregulated in the presence of vitamin B12, to the levels of untreated cells ($P \leq 0.00001$) (Figure 48B). Vitamin B12 also downregulated the levels of IL-1β secretion at 24 hrs (Figure 48D) ($P \leq 0.01$), There was an upsurge of TNF-α secretion within 3 hrs ($P \leq 0.00001$) in the presence of vitamin B12 however, by 12-24 hrs the levels were significantly decreased compared to LPS treated cells ($P \leq 0.01$) (Figure 48C). There were no differences in the secretion levels of other cytokines (IL-2, IL-4, IL-8, IL-17α, IFN-γ) of LPS compared to LPS+vitamin B12 (not shown).

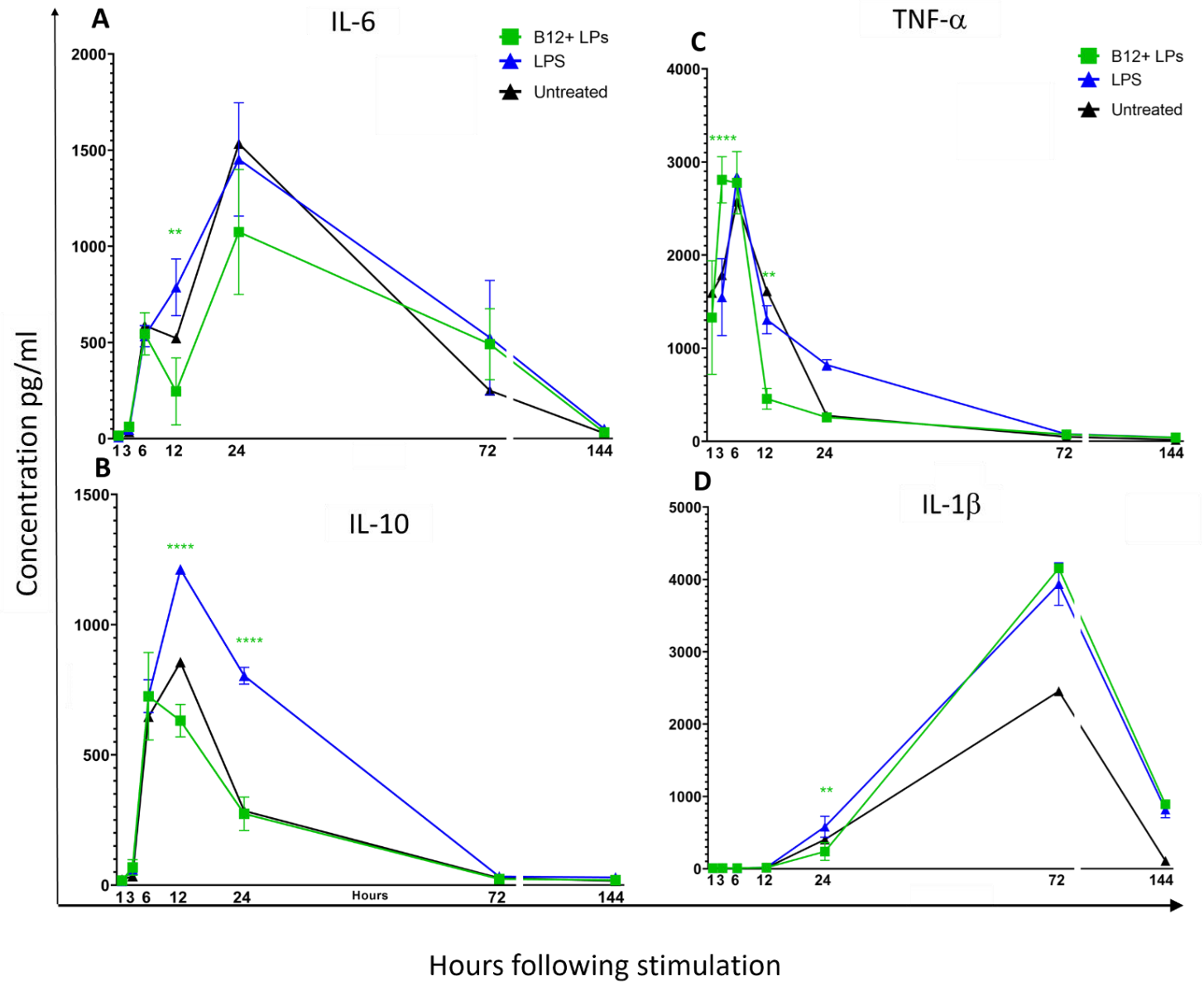


**Figure 48. The effect of B12 on cytokine expression on monocytes.**

U937 cells were differentiated into monocytes and were incubated with 1 μg/ml LPS to trigger an inflammatory response, and 250 μg of vitamin B12 added for up to 144 hours. Supernatants were collected and seeded in a bioplex plate testing for cytokine expression. Two-way Anova and Sidak's multiple comparison test was used to analyse data for p value significance, using Prism graph pad software. Significance was demonstrated using the following: (*P<0.05 ** P< 0.01 ***P<0.001****P<0.00001).

### *6.3.3 Vitamin B12 increases inflammatory genes in LPS stimulated cells*

Pathway expression profiling, using $RT^2$ profiler PCR arrays for Human Innate and Adaptive Immune responses was used to evaluate changes in differential gene expression, as a response to treatment of monocyte/macrophage cells with LPS vs LPS+vitamin B12, compared to control (cells not stimulated by LPS nor treated with vitamin B12). This gave insights into the mechanistic and immune-modulating effects of vitamin B12 in an LPS activated monocyte/macrophage population. A total of 84 genes were evaluated for changes in gene expression, with 42 genes being upregulated by LPS compared to control with a fold change greater than 2 which was deemed biologically relevant (Figure 49A). Vitamin B12 was shown to further upregulate the response from LPS in 14 genes and down-regulate 6 genes to that of LPS with a fold change of greater than 2 (Figures 49B). Of these 20 genes, 6 genes (CSF2, CCL2, CD14, IL-1β, IL-6 and CXCL10) were statistically significant ($P \leq 0.05$) (Figure 50).

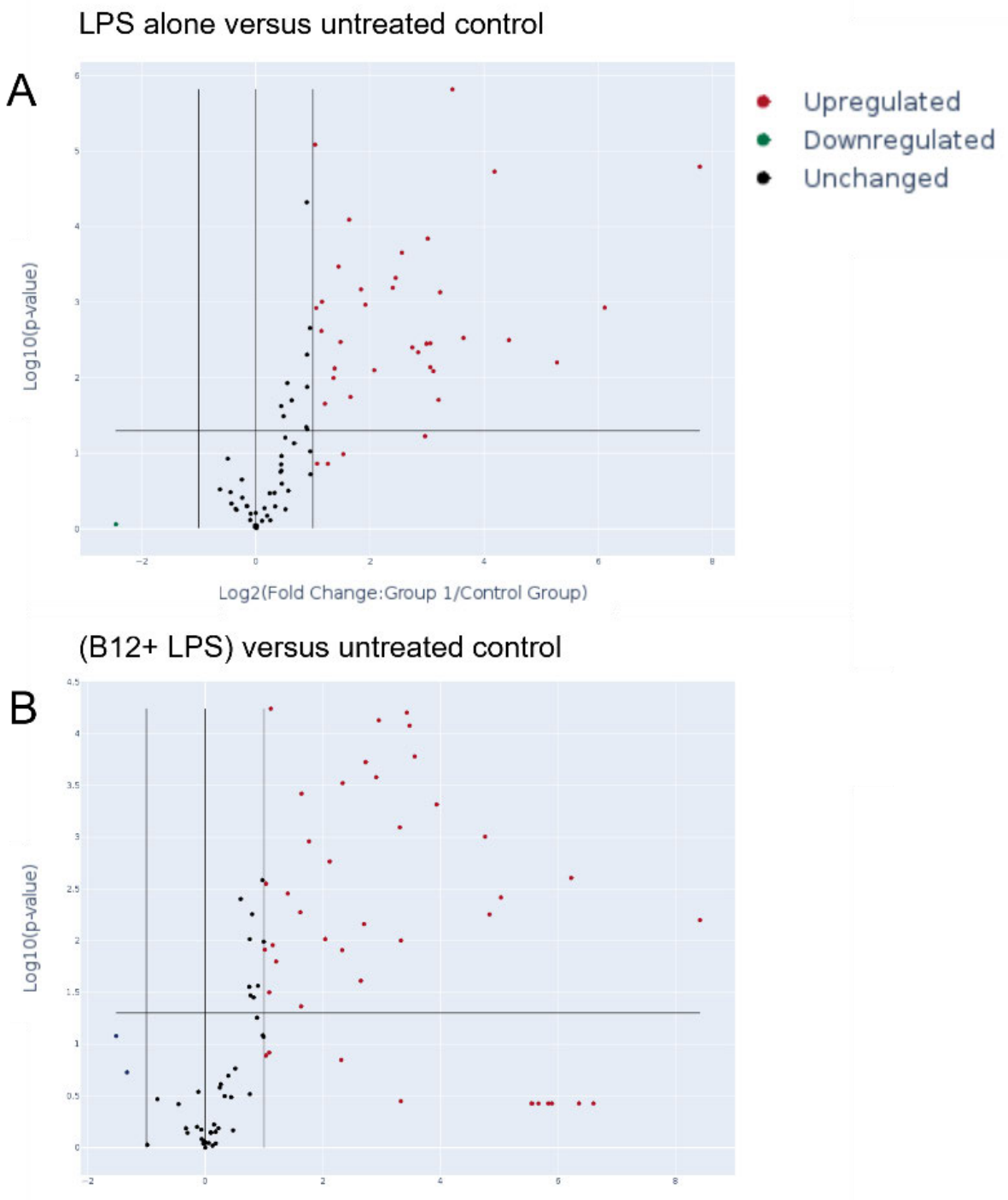


**Figure 49. Vitamin B12 modulates the inflammatory effect of LPS in monocyte/macrophage cells.**

Volcano plots identifying significant changes in gene expression of (A) LPS treated cells compared to untreated control, (B) vitamin B12 + LPS treated cells compared to untreated control. (X-axis = $Log_2$ fold change in gene expression. Y-axis = Statistical significance. Centre vertical line = unchanged gene expression. Outer vertical lines = selected fold regulation threshold. Horizontal line indicates P value threshold. Far upper left quadrant = down regulated genes of significance. Far upper right quadrant = upregulated genes of significance. U937 cells were differentiated into monocytes/macrophages with vitamin $D_3$ for 72 hours after which they were co-cultured with 1 μg/ml LPS with or without 250 μg/ml vitamin B12 for 24 hours; no treatment cells were used as controls (n=3). Cells were collected and trypsinised to ensure collection of adherent macrophages. mRNA was extracted and genes were profiled with $RT^2$ gene profiler for innate and adaptive immune response to determine fold change in normalised gene expression compared to untreated.

### *6.3.3.1 Vitamin B12 upregulates CSF2, IL-6, CCL2, IL-1β, and CD14 and downregulates CXCL10 gene expression*

The regulation of immune response is important in cell defence when encountering an attack from microbial pathogens. Cytokines and chemokines enable cells to regulate this immune response by inducing chemotaxis and mediating growth, differentiation, and activation of different types of effector cells for microbial elimination. It is clear that vitamin B12 contributes in part, to an upregulation of inflammatory gene expression. These conclusions were drawn from observing gene expressions that were both biologically relevant (fold change greater than 2) and statistically significant (fold change greater than 2 and $p \leq 0.05$).

#### *Statistically significant gene expression*

The colony-stimulating factor 2 (CSF2) gene encodes the cytokine granulocyte-macrophage colony-stimulating factor (GMCSF-2). GMCSF-2 is an inflammatory cytokine expressed by many cells but mainly expressed by myeloid cell populations. GM-CSF is responsible for the production, differentiation and function of granulocytes and macrophages, and plays a role in tissue inflammation. LPS+vitamin B12 vs LPS alone increases CSF2 expression 10.4-fold, from 18.1-fold (LPS) ($p \leq 0.0005$) to 28.6-fold (B12+LPS) ($p \leq 0.05$) (Fig. 50).

C-X-C motif chemokine ligand 10 (CXCL10) (IP-10) encodes an anti-microbial chemokine that stimulates monocytes upon binding of the receptor CXCR3. The CXCR3 receptor also reacts with the chemokines CXCL9 (MIG), CXCL11 (I-TAC/IP-9) and CXCL4, all of which function as immune chemo-attractants associated with interferon-induced inflammatory

responses (Kuo et al., 2018). CXCL10 was downregulated by vitamin B12 from 38.8-fold (LPS) ($p \leq 0.05$) to 27.1-fold (B12+LPS) ($p \leq 0.005$) (Fig 50).

IL-6 has broad-ranging effects on infection, and tissue injury, and contributes to host defence (Tanaka et al., 2014). IL-6 works both to inhibit Th1 polarisation and promote Th2 differentiation (Diehl & Rincon, 2002), and is considered to play a role in both inflammatory and anti-inflammatory pathways (Del Giudice & Gangestad, 2018; Takatsu, 2011). Excessive IL-6 synthesis is implicated in several disease pathologies such as COVID-19 (Magro, 2020; Tanaka et al., 2014), sepsis (Song et al., 2019) and inflammatory bowel disease (Atreya & Neurath, 2005). LPS+vitamin B12 vs LPS alone increased IL-6 expression from 21.6-fold (LPS) ($p \leq 0.05$) to 32.7-fold (B12+LPS) ($p \leq 0.05$) (Fig 50).

C-C motif chemokine ligand 2 (CCL2) is a chemokine ligand also referred to as monocyte chemoattractant protein-1 (MCP-1/CCL2). It is a chemotactic protein for myeloid and lymphoid cells and reacts synergistically, with other inflammatory stimuli, to pathological and physiological circumstances during immune defence, it is responsible for recruitment, regulation and polarisation of macrophages during inflammation (Gschwandtner et al., 2019). CCL2 was upregulated by vitamin B12 from 8.3-fold (LPS) ($p \leq 0.05$) to 11.8-fold (B12+LPS) ($p \leq 0.005$) (Fig 50).

IL-1 family of cytokines are associated strongly with the innate immune system and its ability to boost nonspecific resistance to infection and develop the immune response to foreign antigens. IL-1 cytokines are linked to the toll-like receptor (TLR) family and share similar functions (Dinarello, 2018). IL-1$\alpha$ and IL-1$\beta$ are equally potent pro-inflammatory cytokines and produced in response to inflammation caused by infections and microbial endotoxins and other inflammatory agents. IL-1$\beta$ is known to be responsible for contributing to further damage during chronic disease (Lopez-Castejon & Brough, 2011) and is implicated in many

inflammatory conditions such as sepsis, inflammatory bowel disease and rheumatoid arthritis. LPS+B12 vs LPS alone increases IL-1β expression 122-fold ($p \leq 0.05$) from 219.5-fold (LPS) to 341.7-fold ($p \leq 0.0005$) (Fig 50).

In line with the results from the protein studies (Figure 47), vitamin B12 was also shown to upregulate the gene expression of CD14. LPS+B12 increases CD14 expression 15.3-fold ($p \leq 0.05$) from 13.4-fold (LPS) ($p \leq 0.005$) (Fig 50).

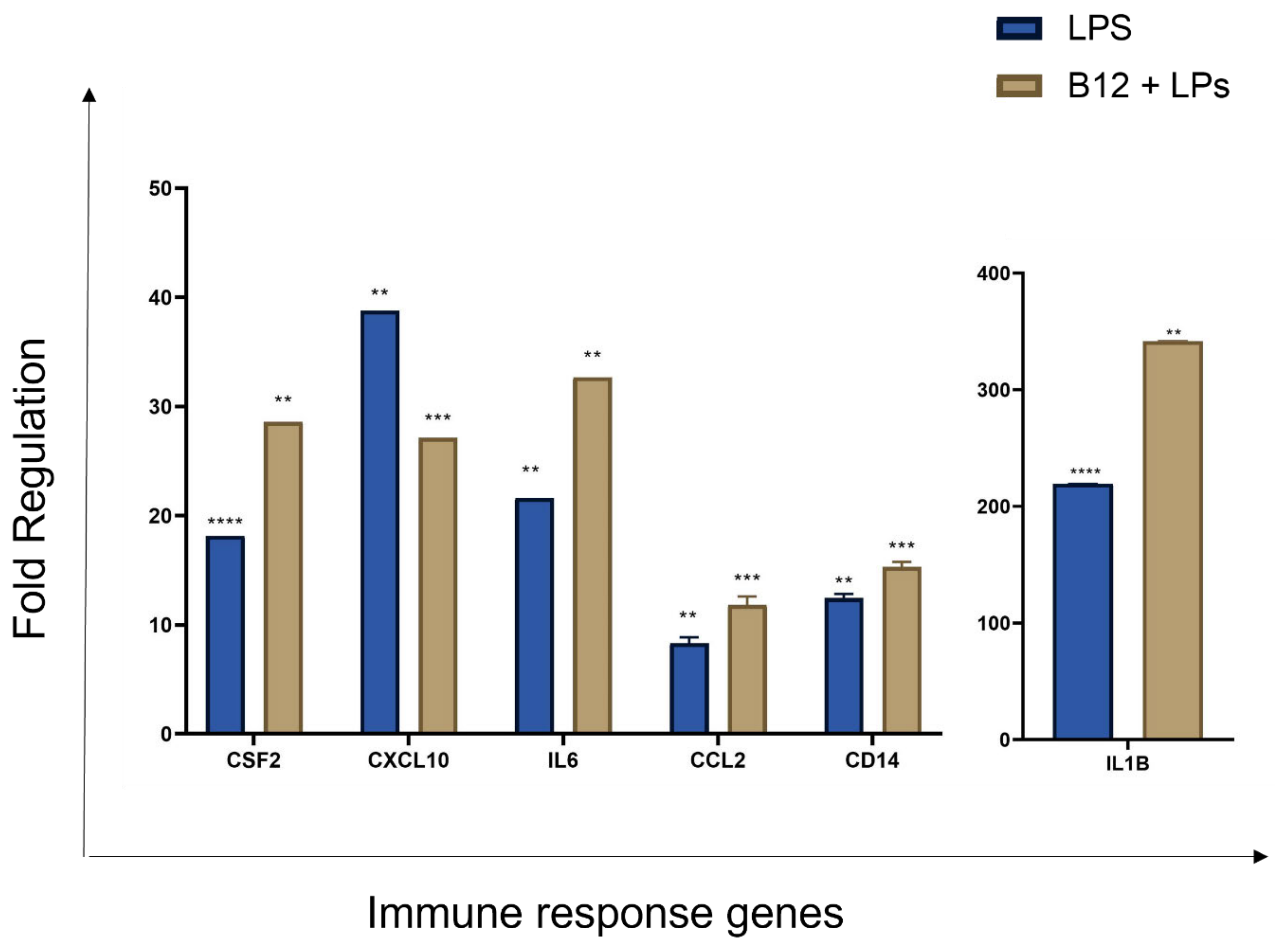


**Figure 50 . Statistically significant gene changes comparing (LPS) to (LPS+B12). Untreated cells are displayed as zero on X axis.**

U937 differentiated into monocytes/macrophage cells were co-cultured with (1) 1 μg/ml LPS, (2) 1 μg/ml LPS + 250μg/ml vitamin B12 or (3) No treatment control for 24 hours (n=3). Cells were collected and trypsinised to ensure collection of adherent macrophages. mRNA was extracted and genes were profiled with RT$^2$ gene profiler for innate and adaptive immune response to determine fold change in normalised gene expression compared to untreated (control cells indicated as 0 on X axis). P values were calculated using Student's T test where *P<0.05 **P<0.01 ***P<0.001 ****P<0.0001. *Vitamin B12 increases log fold change of inflammatory cytokine genes CSF2, IL-6, CCL2, CD14, IL-1β and decreases log fold change of CXCL10.*

***Biologically relevant gene expression***

As p values were not significant within this gene set (Figures 51-52) it is with some caution that the results are reported in this section. Non-significant p values indicate that the results between the n=3 samples showed greater variance than the significant n=3 samples (Figure 51). However, considering some of the fold changes in gene expression in this set of genes were so high, these results cannot go unreported as they may have some biological relevance for future studies. Vitamin B12 showed an upregulation in gene expression of thirteen biologically relevant genes; TBX21, IL-4, IL-1β, IL-17α, IFNG, FASLG, CSF2, CRP, CCR8, CCL2, TRAF6, CD80, SLC11A1 and downregulation of 6 biologically relevant genes; CXCL10, IL-13, IL-10, TLR7, TLR1, NFKBIA.

Seven of these genes showed an upregulation of gene expression by B12 that wasn't replicated by LPS only samples indicating that vitamin B12 is likely able to cause an upregulation of these genes that do not respond to LPS; TBX21, IL-4, IL-17α, IFNG, FASLG, CRP, CCR8 (Figure 52).

The gene with perhaps the most biological relevance in this gene set however is C-reactive protein (CRP). CRP gene encodes for the CRP protein, a member of the pentraxin family which is involved in complement activation and amplification. CRP's ability to recognise cellular damage and pathogenic threat deems it an important molecule in host defence and acute phase response to tissue injury. CRP is well recognised as an acute marker of inflammation and circulatory CRP is elevated during inflammatory occurrences. During bacterial infection, CRP levels increase in response to IL-1 and IL-6. CRP can activate both the classical and alternative complement pathways which facilitate the immune system to activate phagocytosis (Sproston & Ashworth, 2018). CRP also has a relationship with monocyte chemoattractant protein-1

(MCP-1/CCL2) and TNF-α, via monocyte/macrophage stimulation in inflammation (Han et al., 2004). CRP gene expression in LPS compared to control was -1.2-fold whereas LPS+B12 was reported at 47.3-fold a change of 48-fold (Figure 51). This indicates that vitamin B12 may be responsible for the high change in expression of CRP.

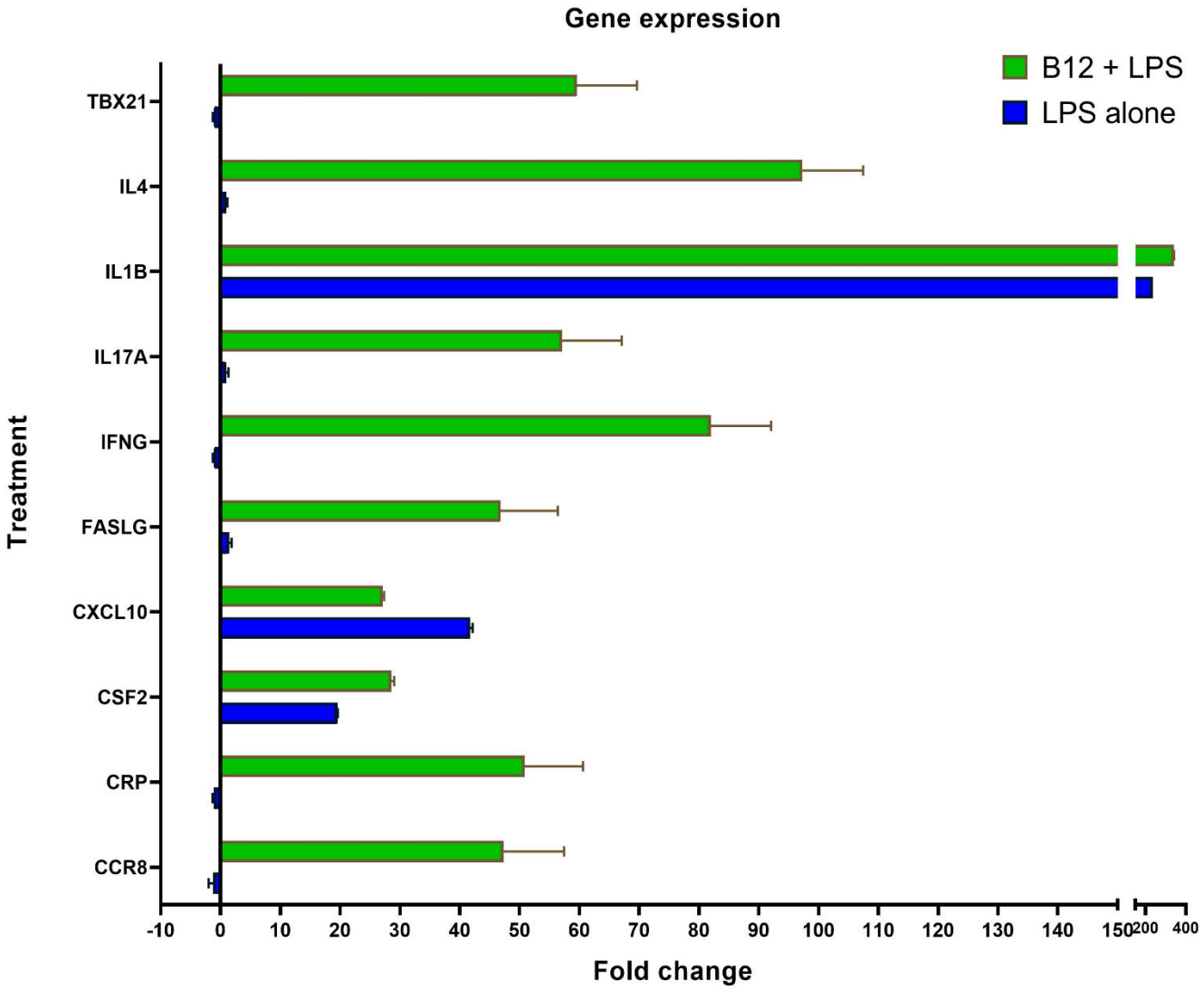


**Figure 51. Biologically significant gene changes comparing (LPS) to (LPS+B12).**

Untreated cells are displayed as zero on X axis U937 differentiated into monocytes/macrophage cells were co-cultured with (1) 1 μg/ml LPS, (2) 1 μg/ml LPS + 250 μg/ml vitamin B12 or (3) No treatment control for 24 hours (n=3). Cells were collected and trypsinised to ensure collection of adherent macrophages. mRNA was extracted and genes were profiled with $RT^2$ gene profiler for innate and adaptive immune response to determine fold change in normalised gene expression compared to untreated (control cells indicated as 0 on X axis). Vitamin B12 increases log fold change of genes TBX21, IL-4, IL-1β, IL-17α, IFNG, FASLG, CSF2, CRP, CCR8, CCL2, TRAF6, CD80, SLC11A1 and decreased log fold change of CXCL10.

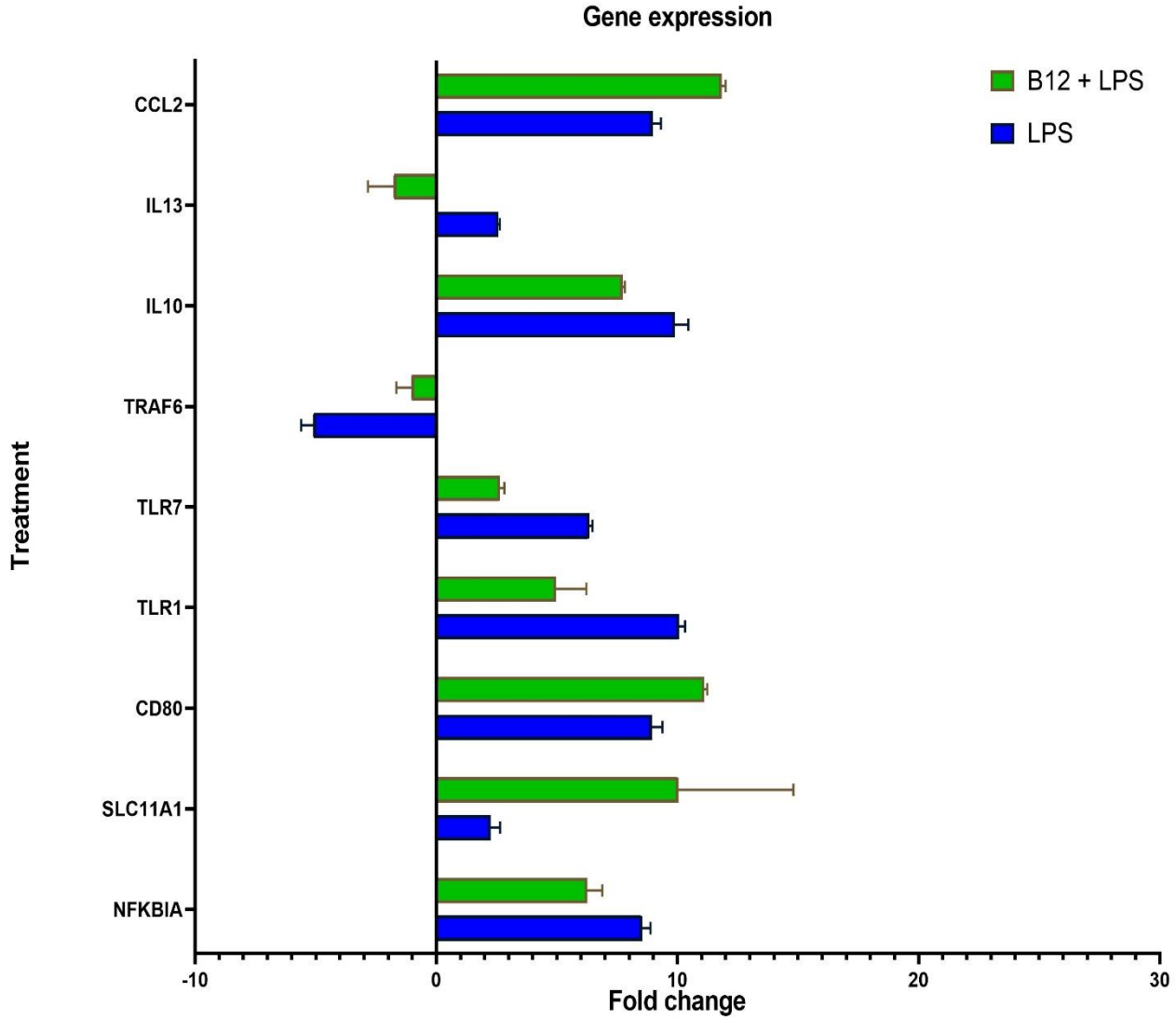


**Figure 52. Biologically significant gene changes comparing (LPS) to (LPS+B12).**

Untreated cells are displayed as zero on X axis U937 differentiated into monocytes/macrophage cells were co-cultured with (1) 1 µg/ml LPS, (2) 1 µg/ml LPS + 250 µg/ml vitamin B12 or (3) No treatment control for 24 hours (n=3). Cells were collected and trypsinised to ensure collection of adherent macrophages. mRNA was extracted and genes were profiled with $RT^2$ gene profiler for innate and adaptive immune response to determine fold change in normalised gene expression compared to untreated (control cells indicated as 0 on X axis). Vitamin B12 increases log fold change of inflammatory genes ***CCL2, CD-80, SLC11A1,*** and decreased log fold change of genes ***IL-13, IL-10, TRAF6, TLR7, TLR1, NFKBIA***

## 6.4. Discussion

Natural sources of anti-inflammatory medications for treating chronic and degenerative inflammatory pain can be effective in avoiding long term side effects and safety concerns of non-steroidal drugs and commonly prescribed pharmaceutical medication. Plant and animal-derived nutraceutical products, such as B vitamins, have garnered much interest in health promotion and as such, much research has focussed on their potential therapeutic effects on inflammation and autoimmunity, mental health and cancer (Nasri et al., 2014).

Due to its ambiguous role in inflammation, this study determined the effects of high dose vitamin B12 on LPS stimulated monocytes to ascertain whether vitamin B12 exerts anti-or pro-inflammatory properties. Stimulation of monocytes with LPS upregulates several pro-inflammatory mediators and interestingly, the data herein implies that high dose vitamin B12 may exert further pro-inflammatory effects on monocyte/macrophage cells. This was indicated by a statistically significant increase in inflammatory gene expression of key inflammatory mediators IL-6, IL-1β, CSF2 and CCL2 and cell surface marker CD14 combined with a further 14 inflammatory genes upregulated that were deemed biologically relevant (Fig 50). In contrast, vitamin B12 downregulated gene expression of six genes (CXCL10, IL-13, IL-10, TLR7, TLR1, NFKBIA) (Fig 50). Although gene expression showed an upregulation of IL-6 and IL-1β, this was not followed by the same change in cytokine secretion; in fact, cytokine levels of IL-6 and IL-1β were decreased compared to LPS alone. This rather contradictory result may be due to the fact that a change in mRNA expression is not always followed by the same change in protein expression and often attempts to correlate the two can have variable success (Greenbaum et al., 2003).

An interesting aspect of this study is the observation of the effect of B12 on genes that were not upregulated from control samples by LPS. These include IL-4, IL-17α, IFNG, FASLG, CRP and CCR8 (Fig 51). These genes were not shown to be induced by LPS alone, but they

were strongly induced by LPS+vitamin B12. It seems possible, therefore, that these results are due solely to the action of high dose B12 on this monocyte population. An interesting further study design would be to test the effects of high dose vitamin B12 on monocytes without the addition of LPS. This would enable a more accurate assessment of the effects of high dose vitamin B12 as a pro-inflammatory agent.

Perhaps the most interesting gene change within this gene set was that of CRP. Although LPS was not directly shown to induce CRP gene expression by monocytes in this study, the stimulation of CRP by LPS+vitamin B12 was significantly upregulated. Of relevance, vitamin B12 has previously been shown to correlate with CRP as a predictive factor of mortality in palliative care cancer patients (Geissbuhler et al., 2000; Kelly et al., 2007; Sviri et al., 2012). Geissbuhler et al (2000), developed what is now known as the B12/CRP index (BCI) (Geissbuhler et al., 2000). They noted that there was a relationship between vitamin B12 levels and survival in a study group of 161 terminally ill cancer patients, whereby the length of survival decreased with an increase in serum B12. They also showed that CRP, in multivariate analysis, was the most important prognostic factor for predicting survival within this study group. It was subsequently determined that vitamin B12 levels were predictive of mortality independent of CRP and a new prognostic index was created whereby vitamin B12 serum levels were multiplied by CRP to ascertain varying levels of mortality risk at three months. The BCI was verified in a confirmatory study a few years later and found to be robust (Kelly et al., 2007). The authors of that study linked elevated vitamin B12 in neoplastic or inflammatory disease with transcobalamin metabolism and hepatic involvement. They further hypothesised independence between pathophysiological processes of elevated vitamin B12 and CRP in predicting survival. Given the results of our study, it may be plausible to suggest that elevated vitamin B12 and elevated CRP are not independent pathophysiological processes, and they may indeed have a closer affiliation than previously thought.

Although it is interesting to consider the change in inflammatory response gene by gene, it may be relevant to evaluate the overall context of the results. It is clear in the current study that vitamin B12 increases the expression of inflammatory genes above that of LPS alone. The importance of these findings lies in recognising the potential negative effects that high dose vitamin B12 may have by adding to inflammation and that caution should be taken if administering in dosages outside the normal range of requirements. Currently, there seem to be no studies advocating the benefit of using high dosage vitamin B12 as an anti-inflammatory agent, although a clinical trial is currently underway whereby 30 septic shock patients are to receive either a single 5-gram dose of intravenous vitamin B12 or placebo in addition to standard care vasopressor doses at 3 hrs; the trial is due to be completed in 2023 (Patel, 2020). As the amount of vitamin B12 needed daily for optimal health and normal human function is very small (2.4 μg/day), vitamin B12 likely has a narrow therapeutic range, and for supplementation to have any benefit, beyond correcting deficiencies, the 'right' dosage needs to be determined. In this regard, extensive studies should be conducted to evaluate the effects of different dosages of vitamin B12 on immune cell activity to establish what this dosage may be if it exists at all. Based on the results of this study, however, it seems that high dose vitamin B12 does not offer any therapeutic potential as an anti-inflammatory and that care should be taken when consuming in quantities outside the currently accepted RDI.

## 6.5. Conclusion

Natural sources of anti-inflammatory medications can be beneficial for avoiding long term side effects of non-steroidal anti-inflammatory drugs (NSAIDs) and pharmaceutical medication. The use of vitamin B12 as a nutraceutical anti-inflammatory medication or immune modulator has not yet been fully explored and reports on the effects of high dose vitamin B12 within the

literature have previously been ambiguous. It was the goal of this study to ascertain what effects vitamin B12 has on LPS stimulated immune monocyte/macrophage cells. Interestingly, vitamin B12 did not mediate the anti-inflammatory effects, but rather further upregulated pro-inflammatory genes induced by LPS stimulated cells. Further to this, vitamin B12 upregulated other inflammatory-related genes that were not upregulated by LPS indicating that vitamin B12 may express its own pro-inflammatory mechanisms. Based on this study it seems that vitamin B12 does not offer a viable option as a nutraceutical anti-inflammatory agent although further research needs to be undertaken to ascertain the dose-dependent effects of vitamin B12 on immune cells. Until this research has been conducted, we propose that the use of vitamin B12 outside its currently RDI should be undertaken with caution.

# Chapter Seven

# General discussion and implications

## 7.1 General comments and key findings

The purpose of this body of work was to ascertain, with greater clarity, the mechanisms of action of vitamin B6 and B12 on inflammation and cancer. This was achieved by conducting studies on two different types of cell sets. The first was undifferentiated U937 cells which represented a model of malignant lymphoma derived from a histio-monocytic lineage (Sundstrom & Nilsson, 1976). The U937 cell line possesses a mutant p53 gene which gives it proliferative advantages and plays an important part in its suitability as a malignant cell line (Rivlin et al., 2011). The second cell set used was differentiated U937 cells. The U937 cell line, once differentiated using vitamin $D_3$, exhibits many of the characteristics of monocyte/macrophage cells and is an easy, widely used, and uniform experimental monocyte model. As cell lines always have a slightly malignant background there is always a risk of experimental bias and the sensitivity in response to experimental conditions *in vitro* has to be taken into consideration when comparing to monocytes within their natural environment (Chanput, 2015). Despite these limitations, the U937 cell line was deemed an appropriate and consistent model for the highly experimental and exploratory nature of this work. The third phase of work initially planned for this PhD was to repeat experiments using (a) peripheral blood mononuclear cells (PBMC) derived from human buffy coats to repeat the experiments on the effects of vitamin B6 and B12 on inflammation; and (b) other cancer cell lines including breast, lung, and bowel cancer to determine if the effects noted on U937 cells are also represented in other cancer cell types. Due to the effects of the COVID-19 pandemic shutting down university labs over two years during this PhD, this work was not undertaken but would provide an interesting area to pursue further studies.

The experiments undertaken on these two different cell sets were aimed at uncovering some of the mechanistic actions of vitamin B6 and B12 on immune and cancer cells. Vitamins B6 and B12 have recently become nutrients of interest in both inflammatory and oncogenic medicine. Much investigation has already been undertaken in ascertaining their mechanistic effects in deficiency states and supplementation as a correction of deficiency, but there has been far less inquiry into their roles as potential therapeutic agents in dosages above their normal RDI. In summary, and with a cautionary note it seems that preliminary results suggest that high dose vitamin B6 possesses a greater anti-inflammatory scope than what may have previously been reported in the literature (chapter 5) and that it may also possess anti-cancer properties via the broad downregulation of inflammation (chapter 5) and its ability to reduce proliferation (Chapter 2) by restoring p53 function and cell cycle checkpoints (Chapter 4). In this regard, high dose vitamin B6 should be further investigated as a potential anti-inflammatory agent for use in conditions where patients encounter cytokine storms such as is seen in sepsis and COVID-19. In cancer, the role of high dose vitamin B6 could be explored in other cancers possessing a p53 mutation to ascertain its ability to restore function to this gene. Furthermore, the role of high dose vitamin B6 on the mevalonate pathway within cancer also needs to be further investigated as results from this study suggest that B6 may be acting in a 'statin' like way to inhibit the mevalonate pathway, downregulate MVP and trigger the degradation of mutp53 (Chapter 4). In contrast, high dose vitamin B12 may be contributing to inflammation via upregulation of key inflammatory genes. This may also suggest that it may contribute to cancer initiation and progression (in high doses) by acting as a pro-inflammatory agent. As the RDI of vitamin B12 in humans is quite small, these results may indicate that caution needs to be taken when consuming vitamin B12 in dosages higher than RDI and that B12 may indeed be highly dosage-sensitive

### *7.1.2 High dose vitamin B6 as an anti-cancer agent.*

Within the literature, vitamin B6 supplementation has been shown to reduce tumourigenesis in a few murine studies via suppression of proliferation or proliferation-related genes (Ciappio et al., 2011; Komatsu et al., 2002; Matsubara et al., 2003), or reduce cancer via anti-inflammatory/anti-oxidative mechanisms (Cheng et al., 2016; Toya et al., 2012). High serum vitamin B6 levels have also been associated with lower cancer risk in renal cell carcinoma (Mao et al., 2015), oesophageal cancer (Ma et al., 2018), breast cancer (Egnell et al., 2017) and enhanced anti-tumour effects of cancer treatment in colorectal, pancreatic and gastric carcinoma (Machover et al., 2021). Furthermore, anti-cancer mechanisms of vitamin B6 that have been previously noted include downregulation of inflammation and oxidative stress. Initial studies undertaken for this PhD showed that high dose vitamin B6 could downregulate the proliferation of pro-monocytic lymphoma cells. This was not caused by apoptosis or cell death. B6 also prevented cellular migration, lowered PD-L1 levels and IL-1β expression and increased the expression of IL-10. These results were consistent with anti-cancer mechanisms as described in cellular hallmarks of cancer (Fig 1.3.2) and anti-proliferative effects of vitamin B6 as previously described in the literature. The anti-proliferative effects of B6 were perhaps the most compelling results of this early study and next-generation sequencing (NGS) studies were performed to determine the anti-proliferative mechanisms of high dose vitamin B6 (chapter 4). The NGS study revealed that the mechanistic effect behind the ability of B6 to downregulate inflammation was likely due to its capacity to resurrect the p53 pathway and reinstate the G1/S checkpoint resulting in the anti-proliferative effects on this, normally highly proliferative, cell line. This is the first study to unveil this mechanism of high dose vitamin B6 and future studies could be focused on determining whether this effect translates to other cancers with mutant and non-mutant p53 genes.

### *7.1.3 High dose vitamin B6 as a powerful global anti-inflammatory agent*

Low blood serum vitamin B6 is frequently noted in patients with high inflammatory markers (Ueland et al., 2017) and the link between B6 deficiency and inflammatory disease (including cancer) is well established (Chapters 1 and 5). Although the effect of high dose B6 has been shown in some studies to downregulate inflammatory cytokine production, this is the first study to show that high dose vitamin B6 exerts a broad-spectrum anti-inflammatory effect on LPS induced inflammation in monocytes by downregulating key inflammatory pathways including the inflammasome pathway, NF-κB and IFN response via CD14/TLR4 interaction (Chapter 5). These results are interesting in that they offer new insights into the potential role of high dose vitamin B6 in targeting inflammation as seen in cytokine storms or other hyper-inflammatory conditions. Furthermore, it strengthens the notion of vitamin B6 as an anti-cancer agent via its role as an anti-inflammatory agent.

### *7.1.4 Pro-inflammatory indications and dose-dependent effects of high dose vitamin B12.*

The effects of B12 supplementation have been shown within the literature to be ambiguous with links both to cancer progression and pro-inflammatory action (Arendt & Nexo, 2012; Brasky et al., 2017; Chiche et al., 2008; Collin et al., 2010; Norredam et al., 1983; Price et al., 2016; Urbanski et al., 2020), versus tumour regression and anti-inflammatory action (Birch et al., 2009; Manzanares & Hardy, 2010; Wheatley, 2006). It was also noted in this study of some ambiguous effects of vitamin B12. Firstly, on promonocytic lymphoma cells, vitamin B12 displayed a dose-dependent effect on proliferation whereby higher doses increased proliferation and lower doses decreased proliferation. Both low and high dose B12 were not

shown to induce apoptosis or cell death. Low dose vitamin B12 showed anti-migratory effects whereas high dose B12 did not. Interestingly both high and low dose B12 increased the expression of PD-L1 (Chapter 3). These results indicate that dosage is an important factor in the mechanistic effects of vitamin B12. This may be related to the fact that B12 RDI in humans is very small and consequently the therapeutic range of B12 may be narrow. It was further shown that when differentiated LPS stimulated monocytes were incubated with high dose vitamin B12, key inflammatory genes were upregulated. High dose vitamin B12 was also shown to upregulate genes that LPS had not upregulated including C-reactive protein (CRP), indicating that vitamin B12 possessed its own inflammatory mechanisms (Chapter 6). These results may support the findings within the literature that vitamin B12 supplementation may contribute to or is associated with tumorigenesis. Moreover, this knowledge could have relevance from a dietary/lifestyle perspective for people who follow diets high in B12, for example, the carnivore diet (O'Hearn, 2020), where high quantities of red meat are consumed or in the high dosages often found in protein shakes and supplements commonly consumed by bodybuilders (Karimian & Esfahani, 2011) or energy drinks (Takahashi et al., 2013).

## 7.2 Implications of this research

The results from this study relate to and agree with previous research on vitamin B6 and B12 as discussed in the literature review (Chapter one). This research however is built upon this new data and provides clearer and novel insights into the mechanistic effects of high dose B6 and B12. There are two main implications to arise from this research. 1. Vitamin B6 may prove to be an important nutraceutical agent in both inflammatory and oncological medicine. 2. Over supplementation of vitamin B12 may contribute to inflammation and tumorigenesis.

## 7.3 Limitations of this study and recommendations for further research.

The generalizability of the results is limited by a few main factors. Firstly, the study was only conducted on one cell line. This was due in part to a lack of access to the lab due to Covid lockdowns and restrictions. In future studies, different types of immune cells might be used. Natural killer cells, dendritic cells, T-cells, B-cells. PBMCs from human blood and immune cells are incubated together *in vitro* so that cross talk can be factored into the results. This would yield a greater understanding of what high dose vitamin B6 and B12 do within this environment which is closer to an *in vivo* model. A second limitation of this study is that changes within the gene studies were not always followed by the same change within the protein studies. This is not an unusual occurrence when comparing gene and protein change, it would judicious however to follow up the gene studies with secondary studies to ascertain the robustness of the data.

Within the cancer studies, other blood cancer cell lines might be used, or other cell lines with known p53 mutations could be used to ascertain whether this effect crosses over to other cancer types. Similarly, other studies need to be conducted to verify that B6 is changing p53 expression at a protein level to verify this mechanism. Exploring various concentrations of vitamin B6 and B12 at different time points also needs to be investigated as the dose-dependent effects could have pharmacological relevance. NGS studies on B12 would also help to give insight into the mechanistic effects of B12 at a gene level. Studies could also be undertaken looking at the combined effect of high dose B6, B9 and B12 on both immune and cancer cells as these vitamins work synergistically within the methylation cycle. Progression from cell lines to animal studies, especially for B6, needs to occur to ascertain effects on "cytokine storm' like

inflammation. Studies on the effect of vitamin B6 on lung alveolar macrophages may be important for determining the effect of COVID-19 respiratory inflammation.

## 7.4 Conclusion

In this thesis high dose vitamin B6 was shown to have an anti-proliferative effect on promonocytic lymphoma cells. The mechanism causing this was likely due to the downregulation of the mevalonate pathway whereby vitamin B6 acted in a 'steroid-like fashion to reduce MVP, restoring mutant p53 function and re-establishing the G1/S checkpoint. B6 was also shown to have a broad-spectrum, anti-inflammatory effect on key inflammatory pathways in LPS stimulated monocytes. It was further shown that LPS stimulated monocytes incubated with high dose vitamin B12 displayed an upregulation of key inflammatory genes. High dose vitamin B12 was also shown to upregulate genes that LPS had not upregulated indicating that vitamin B12 possessed its own inflammatory mechanisms. It was concluded from this study that dosage is an important factor in the mechanistic effects of vitamin B12.

The important and novel findings from this thesis conclude that high dose vitamin B6 may prove to be an important nutraceutical agent in both inflammatory and oncological medicine. In addition, vitamin B12 over-supplementation may potentially contribute to inflammation and tumourigenesis thus, caution should be taken when supplementing in dosages above RDI. The data presented in this thesis has provided important insights into the mechanisms of vitamins B6 and B12 and validation for continued investigation into the therapeutic use of these nutrients.

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