INVESTIGATING THE EFFECTS OF CHRONIC LOW-GRADE INFLAMMATION ON CANCER IMMUNOTHERAPY

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Cancer is the leading cause of death in New Zealand and is responsible for approximately one third of all deaths. Due to various factors, including unhealthy life style choices and an aging population, the number of new cases is expected to rise by close to 70% over the next 20 years. While current cancer treatments are effective in most cases, in aggressive cases they are rarely curative, and their lack of specificity can lead to many unwanted side effects. These limitations have propelled research toward more targeted therapies, including immune system harnessing therapies such as cancer immunotherapies. Cancer immunotherapies, including cancer vaccines, such as virus-like particle (VLP) vaccines, have demonstrated effective improvements in disease-free survival in mouse models of melanoma, colorectal and breast cancer. However, such therapies have not been tested under conditions where the immune system is altered, as seen in chronic inflammation. With many conditions that have underlying chronic low-grade inflammation, such as metabolic disorders, on the rise, this may be an important aspect of cancer treatment to consider. In light of this, the aim of this project was to investigate the effect of chronic low-grade inflammation on the ability of VLP-peptide vaccines to induce anti-tumour immunity against melanoma, colorectal and breast cancer.

To investigate this, hyperuricemic and obese mouse strains as chronic inflammatory models were used. A pilot tumour growth kinetics study was conducted to assess differences in tumour growth rates in our inflamed mouse models. To assess differences in adaptive immune cell activation in response to the vaccines *in vivo*, mice were vaccinated, and antibody production and T cell cytotoxicity were evaluated. Lastly, bone marrow (BM) cells and BM derived dendritic cells (BMDCs) from wild type, hyperuricemic and obese murine models were analysed by flow cytometry, along with co-cultured T cells, to identify markers associated with activation, proliferative potential and effector function.
In vivo experiments revealed that breast cancer tumour growth decreased, and overall survival increased in the hyperuricemic and possibly the obese mice compared to the wild type mice, however no differences were seen in the other models. No difference was observed between the melanoma vaccine-induced cytotoxicity in the hyperuricemic mice compared to the wild type mice. However, lower antibody titres were detected in the blood of hyperuricemic mice in response to the breast and colorectal cancer vaccine as compared with the wild type mice. Analysis of immune cell populations in the BM did not reveal any differences between the frequencies of dendritic cells (DCs), B and T cells from the three mice models. However, the BM cells from the obese mice showed increased frequencies of myeloid and immunosuppressive cells. DCs from the BM of obese mice had decreased MHC class II (MHC II) expression when unstimulated and decreased activation marker expression following CpG stimulation. Lastly, DCs from the three mice models were all able to stimulate T cell proliferation following VLP stimulation, a trend indicates that DCs from the obese mice BM were able to generate the greatest level of proliferation.

In conclusion, this data suggests that the reduced breast cancer tumour growth rate in hyperuricemic mice may be due to the increased interleukin-10 (IL-10) in these animals. Additionally, the higher frequency of myeloid and immunosuppressive cells in the BM of obese mice and the decreased expression of activation markers and MHC II on DCs from obese mice may affect anti-tumour immunity downstream.

Future experiments will determine the impact of an altered immune system, as a result of chronic low-grade inflammation, in the obese and hyperuricemic mice on our VLP-peptide vaccine’s ability to generate an anti-tumour response against melanoma, colorectal and breast cancer.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATM</td>
<td>Adipose tissue macrophage-</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow derived dendritic cell</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Wild type</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CD11c</td>
<td>Classical dendritic cells</td>
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<tr>
<td>cIMDM</td>
<td>Complete Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>cPBS</td>
<td>Coupling Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>CPG</td>
<td>Cytosine-phosphate-guanosine</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet-induced obesity</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HTRU</td>
<td>Hercus Taieri Research Unit</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>lb/lb</td>
<td>Obese</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>mGM-CSF</td>
<td>Murine recombinant Granulocyte-Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>MHC I/II</td>
<td>MHC class I/II</td>
</tr>
<tr>
<td>MoDC</td>
<td>Monocyte-derived dendritic cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-B</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<tr>
<td>PD-1</td>
<td>Programmed death-1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed death ligand-1</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cells</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RHDV</td>
<td>Rabbit haemorrhagic disease virus</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>SUA</td>
<td>Serum uric acid</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellites</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour associated antigen</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt; cell</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour-necrosis factor-alpha</td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt; cell</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>Urah&lt;sup&gt;Plt2/Plt2&lt;/sup&gt;</td>
<td>Hyperuricemic</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>VLP-surv</td>
<td>VLP-survivin</td>
</tr>
<tr>
<td>VLP-surv.MUC1</td>
<td>VLP-survivin+mucin1</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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1 INTRODUCTION

1.1 CANCER AND CANCER IMMUNOTHERAPY

1.1.1 CANCER AND CURRENT TREATMENTS

Cancer remains one of the leading causes of morbidity and mortality worldwide, with the number of new cases predicted to rise by approximately 70% over the next 20 years (1). Carcinogenesis, the formation of cancer, is a process with many influencers including genetic, environmental and lifestyle factors which all contribute to the development and malignant progression of the disease (1). The World Health Organisation (WHO) estimates that unhealthy lifestyles, cause around 30% of current cancer deaths (1).

Current cancer treatments, such as surgical resection, radiotherapy and chemotherapy although usually effective, struggle to remain curative in cancers with high tumour burden or with a high degree of metastasis and are often only able to slow cancer progression in such cases. Alongside this, due to their lack of specificity these treatments can cause various unwanted side effects. The limitations of these current treatments have accelerated research into more specific therapies to increase the chance of complete eradication of cancer cells and to decrease the number of side effects.

Since many of the treatments involve the activation of the immune system, a brief overview of relevant immune cell populations and their principal mechanisms of action will be provided before immunotherapies are discussed.
1.2 Anti-Tumour Immunity

1.2.1 Tumour Associated Antigens

Tumour-associated antigens (TAA) are proteins generated as the result of genetic mutations and dysregulated epigenetic modification in cancerous cells resulting in varying expression of TAAs. These antigens are recognised by the immune system as foreign as a result of either: mutations altering the normal structure of the protein, overexpression of proteins usually expressed in small quantities, proteins normally isolated in immune privileged sites being expressed elsewhere or the expression of proteins not ordinarily expressed in adult tissues (2). Therefore, expression of these specific antigens by cancerous cells differentiates them from the normal cells from which they were derived, allowing specific recognition and destruction of these cells by the immune system (2). These antigenic proteins can be expressed on major histocompatibility complex (MHC) proteins on the surface of the tumour cells or released as proteins from necrosing tumour cells, which are taken up by antigen-presenting cells (APC) to be processed and presented on both surface MHC class I and II (MHC I and MHC II) (2).

1.2.2 Dendritic Cells

Dendritic cells (DCs) are APCs whose role is to detect and take up dangerous or foreign cells and present their antigens to cells of the adaptive immune system (3). DCs occupy various tissues that are in contact with the external environment, in order to constantly sample these changing surroundings. They can be found in tissues such as the skin, lungs, stomach and intestines, as well as circulating the blood in an immature state (3).

DCs exist as various developmentally and functionally defined subsets that differ in their location, phenotypes, pattern recognition receptor (PRR) expression, cytokine secretion and their ability to regulate T cell functions (4). These subsets are important for the initiation of
different immune responses during infections, autoimmunity, vaccination and cancer therapy (4). Various DC subsets exist, such as classical DCs (cDCs) and plasmacytoid DCs (pDCs) which are present in steady-state and monocyte-derived DCs (MoDCs) which are present during inflammation (4, 5).

cDCs can be divided into two genetically and functionally separate lineages, cDC1 and cDC2. The cDC1 lineage is important for combating intracellular pathogens as it is specialised in antigen cross-presentation, which is important for the induction of cytotoxic T lymphocyte (CTL) responses (6, 7). The cDC2 lineage is specialised for CD4+ T cells and promotes differentiation of T helper (T_h) cell subsets, primarily towards a T_h2 phenotype, making it important in initiating responses to extracellular pathogens (6, 7). pDCs represent a DC subset whose morphology resembles plasma cells, however, upon viral stimulation they secrete large quantities of type 1 interferons and differentiate to prime T cells against viral antigens (4, 7). Lastly, MoDCs unlike the other DC subtypes are not present during steady-state and develop following inflammation and rapidly differentiate from monocytes and infiltrate tissues to primarily induce T_h1 and T_h17 responses (7).

While all DC subsets are important in normal immunity, cDC1s, pDCs and MoDCs are the primary subsets crucial for activating cytotoxic anti-tumour responses in steady state and inflammatory conditions (see Section 1.2.5).

1.2.3 Recognition and Phagocytosis of Tumour-Associated Antigens

Following necrosis of tumour cells, TAAs and danger associated molecular patterns (DAMPs) are released, which can be detected by DCs via PRRs such as Toll-like receptors (TLR).
Stimulation of PRR leads to the activation of DCs that take up TAAs, which are processed into a presentable peptides (3).

**1.2.4 Activation and Priming of T cells by DCs**

Once activated, DCs migrate to the lymph node where they present tumour peptides on MHC I and II to various adaptive immune cells (3). Cells including naïve T cells, which via its T cell receptor (TCR) recognises specific proteins presented on MHC. Binding of the TCR to the antigen-MHC complex results in partial activation of the naïve T cell (3). To fully activate the naïve T cell, the DC provides co-stimulation via the CD80/86 receptors, which bind to the CD28 protein on T cells (3). This results in the release of cytokines, such as IL-12 from the DCs and interferon-gamma (IFN-γ) from T cells, that drive the differentiation of T cells (3). Together, these three signals result in activation and proliferation, leading to the clonal expansion of tumour-specific effector T cells. These T cells then begin to migrate from of the lymph node, where they encounter other APCs presenting the same TAA on MHC II, which can further activate these effector T cells via binding of the CD40 ligand (CD40L) on the T cell to the CD40 receptor (8).

**1.2.5 T cells and Tumour Associated Antigens**

There are two main types of effector T cells: CD4+ T\textsubscript{H} cells and CD8+ CTLs which differ in the way recognise antigen and how they execute their anti-tumour effects.

**1.2.5.1 T Helper Cells**

CD4+ T\textsubscript{H} cells recognise exogenous or extracellular antigens presented on MHC II (3). MHC II are present usually only on professional APCs such as DCs, macrophages and B cells (9). There are various T\textsubscript{H} cell subsets each with different functions, however in the context of anti-tumour
responses \( T_{h1} \) cells are the most important subset. \( T_{h1} \) cells are usually not cytotoxic and therefore do not directly target the tumour, however they generate anti-tumour responses by regulating other immune cells via the release of various cytokines. During inflammation MoDCs rapidly activate naïve T cells, secreting cytokines such as IL-12 to induce a \( T_{h1} \) phenotype (4). Once activated, via antigen presentation and co-stimulation by DCs, the tumour-specific \( T_{h1} \) cells exit the lymph node and at the tumour site release cytokines such as IL-2, IFN-\( \gamma \) and tumour-necrosis factor-alpha (TNF-\( \alpha \)), which are required for the complete activation and proliferation of tumour-specific CTLs (3). The secretion of type 1 IFNs by pDCs and their subsequent activation helps promote this \( T_{h1} \) cell differentiation, by polarising the large population of \( T_{h1} \) cells which are antigen-specific but unpolarised, following the normal immune response induced by cDCs via IL-12 secretion (10, 11). This is required to generate a more robust anti-tumour response and increase tumour cell destruction by CTLs. Once in the periphery, as MHC II are not usually present on cancerous cells, with the exception of certain cancers, including some cervical cancers, \( T_{h1} \) cells cannot directly be secondarily activated by tumour cells, and require further activation via other APCs (12).

1.2.5.2 Cytotoxic T Lymphocytes

CD8\(^+\) CTLs are another type of effector T cell, which recognise endogenous or intracellular antigens presented on MHC I (3). MHC I are present on all normal tissues as well as malignant tissues, with the exclusion of red blood cells (RBCs) (9). However, before they move to the periphery CTLs must be activated in the lymph node via cross-presentation of tumour peptides on MHC I on DCs (13). While exogenous antigens are usually restricted to presentation on MHC II, certain APCs, such as DCs, are able to take up external antigens and display them on MHC I proteins, in a process known as cross-presentation (13). Antigen cross-presentation and co-stimulation by DCs, primarily cDC1s, results in tumour specific CTL activation and proliferation. The CTL exit the lymph node and migrate to the tumour site to induce destruction
of tumour cells expressing the TAAs via the release of cytotoxic molecules including perforin, granulysin and granzymes to induce apoptosis (14). CTLs can also recognise tumour peptides displayed by the tumour cells on MHC I molecules, however, occasionally cancerous cells will downregulate their MHC I, preventing recognition of TAAs by CTLs (15).

1.2.5.3 Memory T Cells

The development of memory T cells is crucial for the sustained protection against recurring cancers. The generation of an immunological memory is the underlying mechanism behind prophylactic vaccination. Furthermore, it is also implicated in the control of metastatic spread and prevention of cancer recurrence via the generation of tumour-specific memory T cells. Although, memory T cells were originally thought to arise from a small proportion of surviving effector cells, such as CTLs following pathogen or tumour clearance, recent evidence indicates a more developmental model is likely dictating the generation of memory cells (16). According to this developmental model, long-lived memory T cells are generated first from activated tumour-specific naïve T cells and then can transition into short-lived effector T cells following stimulation from the inflammatory environment (16). These effector T cells now terminally differentiated cannot give rise to memory T cells and instead only more effector cells (16). However, memory T cells are capable of maintaining themselves as a memory population and are also able to give rise to differentiated progeny (16). Then, following tumour cell eradication, the short-lived effector cells die off, leaving only the self-sustaining long-lived memory T cell population. This remaining population is able to rapidly respond to recurring tumours, following the induction of an inflammatory anti-tumour response, and rapidly generate differentiated tumour-specific effector T cells (16).
1.2.6 THE ROLE OF B CELLS IN T\textsubscript{H} CELL ACTIVATION

B cells are immune cells which play essential roles in both antigen presentation and effector functions. As APCs, B cells detect, capture, process and present antigen to T\textsubscript{H} cells (17). B cells detect dangerous or foreign cells via immunoglobulin (Ig) receptors present on their cell surface, and PRR like TLRs, resulting in TAA uptake from necrosing tumour cells, processing and presentation onto MHC II (17). Engagement of the MHC-antigen complex to the TCR results in partial activation of the T\textsubscript{H} cell, full activation occurs following binding of the costimulatory molecule CD40 on B cells to CD40L on T\textsubscript{H} cells (17). This interaction is required for maximal antigen-specific T\textsubscript{H} cell expansion, cytokine production and memory cell formation (17, 18). This interaction also subsequently results in the differentiation of activated B cells into short or long-lived plasma cells or long-lived memory B cells. Activation of B cells by T\textsubscript{H} cells generates antibody-producing plasma cells, while short-lived cells rapidly produce mainly low-affinity antibodies such as IgM, long-lived cells undergo a process termed class switching, resulting in their production of large amounts of high-affinity antibodies such as IgG, as occurs in the case of anti-tumour responses, following IFN-\(\gamma\) and TNF-\(\alpha\) cytokine release by T\textsubscript{H}1 cells (17). Memory B cells arise during this process, they express higher levels of MHC II and co-stimulatory molecules than naïve cells and although they express high-affinity, class-switched antibodies on their cell surface, but they do not secrete them (19). Memory B cells persist in the blood, lymph and spleen and rapidly generate antibody-producing plasma cells when TAAs are re-encountered (19). The antibodies generated by the various B cells are specific to the TAAs expressed on the tumour cells they were generated from and are capable of binding to such tumour cells to ‘tag’ them for destruction by other immune cells (18). Following this activation, B cells upregulate their expression of CD80/86 receptors which bind to CD28 receptors on peripheral T cells, causing them to divide and produce various cytokines needed for T cell differentiation (20). B cells are crucial in the development of a robust anti-tumour response via their antigen presentation to T cells, activation of T cells via
co-stimulation, memory response generation and release of antibodies specific to TAAs inducing antibody-dependent cell-mediated cytotoxicity (ADCC) (18, 21).

Figure 1 Overview of the anti-tumour immune response.

(1) TAAs released from necrosing tumour cells are internalised by antigen presenting cells (APCs), such as DCs and B cells. (2) DCs mature and become activated following internalisation and (3) DCs present antigen on MHC I and II to naïve CD8+ and CD4+ T cells. (4) T cells differentiate into T H cells CTLs and undergo clonal expansion prior to migrating to the tumour site. B cells present antigen to activated T H cells via MHC II and differentiate into plasma or memory cells. (5) CTLs release cytotoxins to kill the tumour cells with cytokine ‘help’ from T H1 cells via IL-2 and IFN-γ. B cells secrete tumour specific antibodies, targeting tumours for immune cell killing.

1.2.7 CHRONIC INFLAMMATION AND MODULATION OF THE ANTI-TUMOUR IMMUNE RESPONSE

This anti-tumour response can be modulated by various external and internal factors, including chronic inflammation. Chronic inflammation refers to inflammation which persists beyond an initial stimulus causing damage to tissues, organs and even promoting certain conditions such as asthma and cancer (22, 23). Chronic inflammation affects innate immune cells, which are polarised towards an immune-suppressive phenotype through inflammatory cytokines and chemokines.
Constant secretion of pro-inflammatory cytokines including IL-1β, TNF-α, and IL-6 results in the induction of immunosuppressive cell populations such as myeloid-derived suppressor cells (MDSCs) (24). MDSCs are a heterogeneous cell population that expands during chronic inflammation and suppress T cell responses (25). This population is comprised of myeloid cell progenitors which, as a result of inflammation, were unable to differentiate into mature myeloid cells such as DCs, macrophages and neutrophils and instead remaining an immature population of immune cells (25). Activation of these cells leads to an upregulation of immune suppressive factors such as arginase, an increase in nitric oxide (NO) production, inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS) (25, 26). These suppressive factors lead to downstream inhibition of T cell proliferation, function, activation and responsiveness to antigen-specific stimulation (25). MDSCs can be further categorised into two subsets: granulocytic MDSCs (gMDCSs) which express high ROS and low NO levels and monocytic MDSCs (mMDSCs) which express high NO levels and low ROS levels (27). It has also been suggested that MDSCs are involved in regulatory T (T_{reg}) cell differentiation, these cells are suppressive cells which inhibit effector T cells via cytokine production and cell-cell interactions (25).

This generates an environment that is immune suppressive, resulting in immune cells that cannot be activated or expanded, making them unable to carry out their anti-tumour effect, leading to malignant disease.

1.3 CANCER IMMUNOTHERAPIES

Immunotherapies are an emerging therapeutic approach toward the treatment of cancer, which aims to exploit the adaptive immune system’s ability to recognise and eliminate cancerous cells. These therapies act by initiating or enhancing the body’s endogenous anti-tumour response
against TAAs. Compared to current standard of care, this cancer therapeutic is specifically targeted towards cancerous cells via TAAs, enabling effective elimination of tumours with minimal impact on normal cells, minimising overall toxicity. Alongside its increased specificity, immunotherapy is an attractive treatment option as it is capable of generating an immunological memory that is specific that tumour and its associated TAAs and therefore prevent recurrence of the disease. Over recent years many immune therapies such as monoclonal antibodies, adoptive cell transfers, immune checkpoint inhibitors and cancer vaccines have been developed and have shown unprecedented success in cancer treatment (28).

1.3.1 CANCER VACCINES

Cancer vaccines are an emerging form of cancer immunotherapy, which have shown promise in both murine models and some clinical trials, with the US Food and Drug Administration (FDA) approving the first therapeutic cancer vaccine in 2010 (29). Following this, a broad spectrum of other cancer vaccine types, targeted to a diverse selection of TAA, are currently being tested in pre-clinical and clinical trials. The therapeutic approach of all cancer vaccines is to provide an effective method of TAA delivery in the presence of a vaccine adjuvant in order to elicit or amplify the immune system’s anti-tumour response (30).

1.3.1.1 Recombinant Virus Vaccines

Recombinant viruses or micro-organisms are utilised as immunogenic viral vectors, which express TAAs. The co-delivery of TAA with a foreign viral vector activates PRRs, such as TLRs on APCs, and generates a more robust immune response against the antigen. An example of this is PROSTVAC or vaccinia-PSA, a cancer vaccine used to treat castration-resistant metastatic prostate cancer. It is made up of a vaccinia virus, which contains DNA that encodes for the TAA PSA (31). Clinical trials of this immunotherapy have shown that PROSTVAC
increased the median overall survival by 8.5 months, although progression-free survival observed no difference between the control and the PROSTVAC groups (32).

Virus-like particles (VLPs) are another form of cancer vaccine that have shown promising results in mice, as an immune-stimulatory delivery system, against various cancers such as, colorectal cancer and melanoma (33, 34). VLPs are assembled from viral capsid proteins but contain no genetic information from the original virus making them not only stable and safe but also highly immunogenic and able to generate a strong immune response (35). VLPs can display repetitive high density TAAs such as tumour peptides and deliver them directly to APCs. This increases the immunogenicity of these antigens and consequently the activation of tumour-specific immune cells (36).

However, one major downfall for all recombinant viral vector vaccines is that they can be rendered ineffective if a pre-existing immune response is already present against that virus. This results in immediate destruction of the vector via an immune memory response before an immune response can be generated against the TAA is contains (36). This can be circumvented by using viruses that do not infect humans. For example, alternative viruses such as rabbit haemorrhagic disease virus (RHDV), have been brought forward to eliminate this outcome as humans do not have pre-existing immunity to them (37).

Our lab has generated RHDV VLP-peptide vaccines, containing cytosine-phosphate-guanosine (CpG) as an adjuvant, displaying the tumour peptides gp100, survivin and survivin with mucin-1 to combat melanoma, colorectal cancer and breast cancer, respectively. These vaccines showed significant anti-tumour responses and prolonged tumour rejection in these cancer models in wild type (C57BL/6) mice (33, 38, 39).
1.3.2 **CHECKPOINT INHIBITORS**

Cancer vaccines have demonstrated increased efficacy and therapeutic success when given in combination with other immunotherapies such as checkpoint inhibitors (40). Checkpoint inhibitors target immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), present on T_{reg} cells and activated T cells, programmed death-1 (PD-1) on antigen-experienced T cells and programmed death ligand-1 (PD-L1) on tumour cells and bind to them thus preventing their activation (40). CTLA-4 is a checkpoint molecule that is recruited to the cell surface of T cells following activation and binds to co-stimulatory molecules such as CD80/86, preventing their interaction with CD28 thus inhibiting further activation or proliferation (41). CTLA-4 is also constitutively present on T_{reg} cells and is important for their immunosuppressive function, preventing T cell activation and dampening T cell responses (41). PD-1 is present on antigen-experienced T cells and binds to PD-L1 on target cells, such as tumour cells, which often upregulate this ligand, resulting in T cell ‘exhaustion’ and thus rendering the T cells unresponsive (40, 41). PD-L1 expression has been observed in a variety of tumours including melanoma, glioblastoma, colorectal and breast cancer (41-43). A strong correlation has also been observed between PD-L1 expression by tumour cells and poorer prognosis in multiple cancers (41). This led to the generation of monoclonal antibodies targeted to these immune checkpoints, termed checkpoint inhibitors, which bind to these molecules and prevent their immune suppression, permit T cell activation and increase anti-tumour T cell responses (41). The first checkpoint inhibitor approved by the FDA in 2011 for use in melanoma was an anti-CTLA-4 monoclonal antibody, since then, due to their success a variety of antibodies targeted to CTLA-4, PD-1 and PD-L1 have been approved by the FDA for use in a variety of different cancers (41, 44).
1.3.3 Efficiency of Immunotherapies

However, a majority of immunotherapies do not proceed beyond pre-clinical and clinical trials, failing to successfully treat cancer and often result in multiple severe and life threatening toxicities. This discrepancy between pre-clinical and clinical results may be due to unsuitable pre-clinical models, where common morbidities in the human population, are not present. It is suspected that metabolic conditions, that are characterised by chronic inflammation, negatively impact the efficacy of cancer immunotherapies, however, they are rarely represented in pre-clinical models. Chronic inflammation, characterised by a persistent inflammatory environment as a result of ongoing pro-inflammatory signalling, alters immune cell function and impairs the immune system’s anti-tumour response, allowing cancer cells to evade immune surveillance. This immune suppressive environment is likely to impact and be detrimental to the success of immunotherapies.

1.4 Metabolic Disorders and Inflammation

Metabolic disorders, defined as conditions where the normal biochemical processes in the body are disrupted, such as obesity, hyperuricemia and type 2 diabetes mellitus (T2DM), are often caused by unhealthy lifestyle habits. Studies have revealed that chronic low-grade inflammation is an underlying component of many metabolic disorders (45, 46). This metabolic dysregulation leads to changes in cytokines, hormones and immune cell populations, and are suspected to impair immune responses.

1.4.1 Adipokines

Similar to immune cells, adipocytes release various signalling molecules known as adipokines. These are hormones and cytokines which exert various effects on cell proliferation, angiogenesis and metabolism, and are often altered in metabolic disorders (47, 48).
1.4.1.1 Leptin

Leptin is a adipokine, which is produced in higher amounts in individuals with metabolic conditions compared to those without (48, 49). Leptin is a regulator of energy balance and exerts its effects by inhibiting hunger, however, many obese individuals develop resistance to this adipokine (50). Leptin is a driver of inflammation via the induction of immune cells known as mast cells, which produce large amounts of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6, which can drive macrophage phenotype switching (see section 1.4.3.1, (51)). Increased leptin is also associated with immune system dysfunction as it has been shown to drive impairment of cytotoxicity by natural killer (NK) cells, altering DC maturation and function and inhibiting neutrophil infiltration (51). Lastly, elevated leptin plays a role in immune suppression, as increased leptin is associated increased MDSCs accumulation and increased suppression by T_{reg} cells during obesity (51, 52).

1.4.1.2 Adiponectin

Circulating adiponectin concentration has been found to be decreased in metabolic disorders including obesity, insulin resistance, T2DM and hyperuricemia (48, 53). Decreased adiponectin is proposed to be associated with chronic inflammatory conditions due to the decrease of its immune inhibitory roles. Adiponectin is an adipokine that has been shown to have anti-inflammatory effects via its induction of anti-inflammatory cytokines, such as IL-10 and IL-1RA, in primary human monocytes, DCs and monocyte derived macrophages, which causes inhibition of pro-inflammatory cytokines, such as IL-1, IL-2, IL-6, IFN-γ, and TNF-α (54). When adiponectin is decreased the production of pro-inflammatory cytokines is no longer inhibited, leading to an increase of systemic inflammation, as well as further inhibition of adiponectin production (55).
1.4.2 Soluble Factors

Elevated soluble factors such as soluble uric acid (SUA) is associated with various metabolic disorders including obesity, T2DM, metabolic syndrome, hyperuricemia and insulin resistance (48). Increased SUA has been proposed to mediate, in part, the chronic low-grade inflammation observed in these conditions via its pro-inflammatory properties (48). SUA is capable of entering cells, primarily vascular smooth muscle cells, and activating mitogen-activated protein (MAP) kinases, stimulating nuclear factor kappa-B (NF-κB) and inducing the expression of inflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1) and c-reactive protein (CRP) (56, 57). Previous studies have also found that SUA stimulates mononuclear cells to produce pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (58, 59).

1.4.3 Immune Cell Populations

Alongside alterations in hormone production and soluble compounds, metabolic disorders can cause various changes amongst immune cell populations. Generating an environment that impairs the immune system’s ability to eliminate cancerous cells. While these changes can be present in many metabolic conditions, the presence of obesity is usually required to alter immune cells as stated below.

1.4.3.1 Adipose Tissue Macrophages

Various immune cell populations reside within fat deposits, amongst them are macrophages, known as adipose tissue macrophages (ATMs) (60). In normal, healthy fat deposits these ATMs are favoured toward the M2 anti-inflammatory phenotype, however, during obesity, the M1 pro-inflammatory phenotype becomes increasingly dominant (61, 62). Pro-inflammatory ATMs continuously secrete pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β, as
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well as chemokines including MCP-1 and MIP-1. Increased levels of these cytokines have been found in obese individuals (60).

1.4.3.2 Dendritic Cells

The immune response impairment observed in obesity has, in part, been attributed to the impaired ability of APCs, such as DCs, to stimulate T cells (63). One aspect of this appears to be the decreased ability or decreased potency of T cell stimulation by DCs (64, 65).

Studies have shown that splenic DC frequency is elevated in obese compared to lean mice. However, these DCs have a reduced ability to stimulate naïve T cell proliferation despite identical expression of MHC and co-stimulatory molecules (64, 65). Impaired T cell proliferation is speculated to be the result of impaired antigen presentation and co-stimulation by DC. Limited production of IL-12 by DCs has been attributed to the lower levels of IL-12 found in the lymph nodes of obese mice. Consequently, activation of CTLs is impaired as IL-12 is required to augment cytolytic activity and production of IFN-γ by T\textsubscript{H} cells (3, 66). However, other studies have attributed the impaired ability of DCs to be the result of transforming growth factor-beta (TGF-β) secretion, exerting immunosuppressive effects, as no changes in IL-12 were observed (65, 67). Moreover, DCs have been found to inhibit effector T cell expansion at the tumour site, with an increase in suppressive DCs in this environment, thus compromising their conventional functions (64). Although it has yet to be determined exactly what causes this DC impairment, it is proposed that increased systemic lipid availability and elevated factors in the serum may negatively impact their function (64). For example, IL-6 which is overproduced in chronically inflamed individuals, has been implicated in switching DC progenitor commitment toward a phenotype that cannot present antigen and instead only phagocytose it (68). Evidently, the impaired functioning of DC has the potential to disrupt the
formation of an adaptive immune response and impair anti-tumour immune responses in obese individuals.

### 1.4.3.3 Cytotoxic T Lymphocytes

Lastly, obesity impacts effector T cells, which, although they are fully functional demonstrate a marked reduction in their activity and proliferation rate (64, 65). CTL numbers are decreased in obese patients, additionally there is also a decreased influx of them to the tumour site (64, 65). Although the mechanism by which this occurs is undefined, decreased numbers of naïve T cells as a result of thymic involution and DC impairment are likely to be contributing factors. Reduced CTL activation and infiltration into the tumour site impedes the ability of CTL to develop and effectuate an anti-tumour immune response.

### 1.4.3.4 Thymic Involution

Thymic involution or shrinking of the thymus is an age-related process facilitated by adipose tissue accumulation in the thymus that is accelerated in obesity, compromising immune function (69). The thymus is a primary lymphoid organ where thymocytes mature into T cells and then move to the periphery to contribute to many aspects of the adaptive immune system. Studies have demonstrated that obesity accelerated age-related thymic involution is associated with increased apoptosis of developing thymocytes (69). This results in a reduced naïve T cell production which restricts TCR repertoire diversity (69). As a result of this impaired T cell response there is an increased risk of infection and decreased immune surveillance in obese individuals.
1.4.4 Metabolic Disorders and Their Impact on Cancer Immunotherapy

Although increased research into the link between metabolic disorders and inflammation has answered many questions it has also begun to raise some questions surrounding the efficacy of cancer immunotherapy in these patients. As these treatments rely entirely on the ability of the immune system to generate an immune response, an impaired immune system, as seen during metabolic dysfunction, may render this therapeutic ineffective.

Studies have shown that the prophylactic vaccine immune response is diminished in obese compared to normal-weight individuals. For example, the influenza vaccine when administered to obese individuals resulted in decreased antibody levels and defective CTL responses compared to normal-weight individuals (70). The impaired immune response to the influenza vaccine results in the decreased efficacy of the prophylaxis, meaning these individuals may not be fully protected from influenza. Furthermore, the memory T cell response against influenza has also been shown to be impaired in obese mice due to their inability to generate and maintain functioning influenza memory T cells (71).

Similar issues were observed in obese mice receiving a DC-based immunotherapy treatment for renal cell carcinoma, where the treatment was found to be mostly ineffective in the obese mice while it managed to control outgrowth of the tumour in normal-weight mice (64). The loss of efficacy observed coincided with an increase in the amount of suppressive DCs in the tumour-bearing kidneys of the obese mice, as well as a decreased influx of CTL into the tumour site compared to the normal-weight mice (64).
Although studies in humans with metabolic disorders are lacking, these first results from chronic inflammatory animal models may explain the lack of efficiency of some cancer immunotherapies in diverse patient populations.

1.5 AIMS AND RATIONALE

Previous research in our lab has demonstrated the efficacy of VLP-peptide vaccine therapies against melanoma, colorectal and breast cancer in wild type mice. These results have shown that these vaccines are able to cause CTL specific lysis of target cells and increase overall survival.

Recent evidence indicates that the chronic low-grade inflammation observed in metabolic disorders, such as obesity and hyperuricemia, leads to altered immune cell function, generating an immunosuppressive environment. In light of this evidence, the aim of this project was to investigate the effect of chronic low-grade inflammation on VLP-peptide cancer vaccines against melanoma, colorectal and breast cancer. Specifically, the aims of this research were to (i) determine if the tumour growth rates of obese and hyperuricemic mice differ from wild type mice, (ii) examine how obese and hyperuricemic mice respond to effective VLP-peptide treatment compared to wild type mice, and (iii), to investigate the underlying mechanisms of chronic inflammation mediated impaired anti-tumour immunity in obese and hyperuricemic mice.

It was hypothesised that VLP-peptide vaccines against melanoma, colorectal and breast cancer will be less effective in obese and hyperuricemic mice as compared to wild type mice.
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2 MATERIALS AND METHODS

2.1 ANIMALS AND CELL CULTURE

2.1.1 MICE

Specific pathogen-free (SPF) 2-6-month-old female wild type (C57BL/6), female and male hyperuricemic (Urah<sup>Prl2/Prl2</sup>) and obese (lb/lb) mice were acquired from the Hercus Taieri Research Unit (HTRU), University of Otago, New Zealand (72, 73). Pmel-1 transgenic mice, expressing T cells specific to the MHC I restricted gp100<sub>25-33</sub> epitope aged 5-6 months old were supplied by The Jackson Laboratory (Bar Harbour, ME, USA) (74). Each experimental protocol was approved prior to commencement by the University of Otago Animal Ethics Committee.

2.1.2 PREPARATION OF MURINE BONE MARROW CELLS

Following euthanasia, C57BL/6, Urah<sup>Prl2/Prl2</sup> and lb/lb mice were sprayed with 70% ethanol then both the femur and tibia were removed. As much of the surrounding muscle and tissue was removed and the bones were placed in a petri dish containing complete Iscove’s modified Dulbecco’s medium (cIMDM, Gibco, Invitrogen, San Diego, CA, USA, Appendix)+5% fetal calf serum (FCS). Femur and tibia were split at the knee. The bone ends of the tibia were removed, and the bone marrow was washed out into the petri dish with cIMDM+5% FCS using a 25-gauge needle until all bone marrow was removed. This was repeated with the femur. The contents of the petri dish were then pipetted through a cell strainer into a 50mL Falcon tube including a wash step with fresh media. The tubes were spun at 250 x g for 7 minutes. The supernatant was removed, the cells were resuspended in 3mL RBC Lysis buffer (Appendix) and left in a 37°C water bath for 1.5 minutes. Following this 20mL of cIMDM+5% FCS were added to the cells and the tube was spun again at 250 x g for 7 minutes. The supernatant was discarded, and the cells were resuspended in 10mL cIMDM+5% FCS supplemented with 20ng/mL murine recombinant Granulocyte-Macrophage Colony-Stimulating Factor (mGM-
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CSF, ProSpec, East Brunswick, NJ, USA) and the cell number was obtained using a haemocytometer (trypan blue exclusion method, Appendix). An aliquot of cells was stained with fluorophore-conjugated anti-mouse B220, CD11b, CD11c, CD101, CD3, Ly6C and Ly6G, the antibodies used along with their manufacturer are listed in Table 1. Fluorescence intensity of each fluorophore was analysed by flow cytometry (refer to section 2.4).

2.1.3 Generation of Bone Marrow Derived Dendritic Cells (BMDCs)

To generate DCs the bone marrow cells were resuspended in cIMDM+5% FCS+mGM-CSF at 0.5x10^6 cells/mL and 4mL were plated in separate 6 well plates. Cells were left to incubate at 37°C with 5% CO. On days 3 and 5, the cells were fed by removing 2mL of old media from each well and replacing this with 3mL of fresh cIMDM+5% FCS+mGM-CSF. On day 6, the immature BMDCs were harvested by collecting the non-adherent cells into a 50mL falcon tube. This was centrifuged for 7 minutes at 250 x g and the cells were resuspended in 10mL of cIMDM+5% FCS+mGM-CSF. The cell number was calculated using a haemocytometer and the cells were resuspended at 1x10^6 cells/mL in cIMDM+5% FCS+mGM-CSF.

2.1.4 Activation of BMDCs

To assess the expression of activation and cell-specific markers on the BMDCs, the harvested immature BMDCs were plated into a round bottom 24-well plate with 500µL per well and treated with 0.25nmol/mL CpG (Integrated DNA Technologies, Skokie, Illinois, USA), or left untreated in cIMDM+5% FCS+mGM-CSF for 24 hours. Following this incubation, the BMDCs were stained with fluorophore-conjugated anti-mouse CD11c, CD40, CD86, MHCII and CD80 monoclonal antibodies (Table 1). Fluorescence intensity of each fluorophore was analysed by flow cytometry (refer to section 2.4).
2.1.5 Maturation and Peptide-pulsing of BMDCs

BMDC from harvested on day 6 of culture were resuspended at 2x10^5 cells/mL and 50µL were plated in a 96-well round bottom plate. The cells were pulsed with 2.5µg/mL gp100.VLP, 0.307µg/mL gp100_{25-33} peptide alone or left untreated, then 50µL cIMDM+5% FCS+mGM-CSF were added to the cells. The cells were incubated for 24 hours at 37°C, 5% CO2.

2.1.6 Preparation of Splenocytes

CD8^+ T cells were obtained from the spleens of female pmel-1 mice. The mice were euthanised, sprayed with 70% ethanol and the spleens removed using sterilised scissors and tweezers. The spleens were placed in petri dishes containing Dulbecco’s Phosphate-Buffered Saline (DPBS, Appendix) and homologised by gently pushing them through a 70µm cell strainer using the plunger of a 5mL syringe and rinsing with cIMDM until cells were no longer visible. These cell suspensions were centrifuged for 5 minutes at 300 x g. The supernatant was discarded and the RBCs were lysed by resuspending the cells in 10mL warm RBC lysis buffer for 3 minutes in a 37°C water bath. The lysis buffer was neutralised with 15mL cIMDM, the cells were centrifuged for 5 minutes at 300 x g and resuspended in 10mL cIMDM+5% FCS. The cell number was determined using a haemocytometer, centrifuged a final time for 5 minutes at 300 x g. The cells were resuspended in 90µL of magnetic-activated cell sorting (MACS) buffer (Appendix) per 10^7 total cells. Then 10µL of CD8a (Ly-2) MicroBeads was added per 10^7 total cells, mixed and left to incubate for 10 minutes at 4°C. The cells were washed with 15mL MACS buffer, centrifuged at 300 x g for 5min and resuspended in 1mL MACS buffer per 1x10^8 cells. The cells underwent magnetic cell separation using an AutoMACs (Miltenyi Biotech, Bergisch Gladbach, Germany) under the positive selection programme. Cells were counted, topped up with Phosphate-Buffered Saline (PBS) to 15mL and centrifuged for 5 minutes at 300 x g. The supernatant was discarded and the cells were resuspended at 1 x 10^8 cells/mL in PBS for CFSE staining. The cells were added to 500µL of 10µM CFSE and left to incubate for 5
minutes at room temperature while the tube was inverted intermittently. The reaction was quenched with 500\(\mu\)L FCS. The cells were washed three times with 30mL PBS+5% FCS and centrifuged for 5 minutes at 300 x g. Finally, the cells were resuspended in 1mL T cell media, Roswell Park Memorial Institute medium (RPMI), 5 % FCS, 10 ng/mL mIL-7, and staining was analysed on the flow cytometer (refer to section 2.4).

2.1.7 CO-culture of T-cells and BMDCs

The CD8\(^{+}\) sorted, CFSE stained T cells from the pmel-1 mice were resuspended at 1x10\(^6\) cells/mL in T-cell media and 100\(\mu\)L were co-cultured with the matured 1x10\(^4\)cells/mL BMDCs previously pulsed with VLP-gp100, gp100\(_{25-33}\) peptide or untreated controls. T cell controls were cultured in the absence of BMDCs with VLP-gp100, gp100\(_{25-33}\) peptide or left untreated. Co-cultured were incubated at 37°C, 5% CO2 for 72 hours. Following this incubation, the T-cells were stained with fluorophore-conjugated anti-mouse CD8 monoclonal antibody (Table 1) and the fluorescence intensity was analysed by flow cytometry (refer to section 2.4).

2.1.8 PD-L1 Expression in B16, MC-38 and C57mg.MUC1 Cells

All cancer cell lines were cultured in T75 flasks with 19mL media, RPMI (Gibco, Invitrogen, San Diego, CA, USA)+10%FCS for B16, Dulbecco's Modified Eagle's medium (DMEM, Gibco, Invitrogen, San Diego, CA, USA) +10% FCS for MC-38 and C57.mgMUC1. When B16, MC-38 and C57.mgMUC1 cells reached around 70% confluency all media was removed and the flask was washed with 5mL DPBS. Following this, 5mL of warm 1x ethylenediaminetetraacetic acid (EDTA) was added and left to incubate for 5 minutes at 37°C. The flask was lightly tapped on the side and detachment of the cells was confirmed via light microscope. Next, 3mL of DMEM+10% FCS (MC-38 and C57.mgMUC1 cells) or RPMI+10% FCS (B16 cells) was added and the cells were transferred into three separate 50mL falcon tubes.
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Cells were counted using a haemocytometer and then resuspended at 7.5x10^4 cells/mL. The cells were transferred into three separate 6 well plates with 4mL of cells in each well. Five hours later IFN-γ was added to 5 of the 6 wells at a concentration of 5ng/mL, while the remaining well was left untreated.

Following a 48 hour incubation, all media was removed and the wells were washed with 5 mL DPBS. Then, 500μL EDTA was then added and left to incubate for 5 minutes at 37°C, detachment was confirmed via a light microscope. 1mL of DMEM+10% FCS (MC-38 and C57.mgMUC1 cells) or RPMI+10% FCS (B16 cells) was added to each well and then cells were transferred into FACS tubes. The cells were stained with Live/Dead Zombie Yellow™ and fluorophore-conjugated anti-mouse PD-L1 for all cell types (Table 1). Fluorescence intensity was measured by flow cytometry (refer to section 2.4).

2.2 **In Vivo Cytotoxicity**

Female C57BL/6 and Urah^Pkp2/Plt2^ mice were allocated into 4 treatment groups of 6 mice per group. Vaccines were administered into the left flank subcutaneously on day 0 and day 21. These vaccines consisted of coupling PBS (cPBS), or 100μg of VLP expressing gp100 (gp100.2L.VLP), survivin (surv.VLP) or survivin+mucin1 (surv.VLP-SS-MUC1) in cPBS, each with 25μg CpG. On day 28, donor cells were isolated and prepared from 12 naive C57BL/6 mouse splens. A single cell suspension was prepared by passing the spleens through a 70μm cell strainer with DPBS containing 5% FCS into three 50mL falcon tubes. The tubes were centrifuged at 300 x g for 10 minutes at 4°C and supernatant was discarded. Following this 12mL of warm RBC lysis buffer was added to each tube and incubated for 3 minutes. Then approximately 40mL of DPBS supplemented with 5% FCS was added to each tube, the contents of each tube were filtered through a 70μm cell strainer and the tubes were centrifuged at 300 x
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g for 10 minutes at 4°C. The cells were combined and resuspended in 30mL cIMDM containing 5% FCS buffer. Cells were counted using a haemocytometer, resuspended at 2x10⁷ cells/mL and distributed equally into 4 tubes. Each tube was pulsed with either 10µM survivin, 10µM MUC1, 10µM gp100 or left unpulsed and left to incubate at 37°C + 5% CO₂ for 2 hours.

Following incubation, DPBS was added to each tube to a total volume of 50mL and spun at 300 x g for 10 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 50mL DPBS and centrifuged at 300 x g at 10 minutes at 4°C. The cells of each tube were counted and resuspended in DPBS at 2x10⁶ cells/mL. Each cell suspension was stained with either 50µM CFSE (survivin), 4µM VPD (survivin+mucin1), 50µM CFSE + 4µM VPD (gp100) or 5µM CFSE (PBS control) and left at room temperature for 7 minutes in the dark and quenched with FCS for 1 minute. The cells were washed three times with DPBS at 300 x g for 10 minutes at 4°C, using a new 50mL falcon tube after each wash. The cells were resuspended at 1x10⁸ cells/mL and 100µL of the target cells were injected intravenously into the vaccinated mice.

On day 30 the mice were culled, blood was collected via cardiac punctures and spleens were harvested. Splenocytes were prepared by homogenising spleens through a 70µm cell strainer rinsed with DPBS containing 5% FCS into separate 50mL falcon tubes. The cells were spun at 300 x g for 5 minutes at 4°C and supernatant was discarded. Then, 3mL warm RBC lysis buffer was added to each tube and left for 3 minutes then each tube was topped up with approximately 50mL DPBS and spun for 350 x g for 5 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 1mL DPBS. 250µL of each cell suspension was filtered through gauze into fluorescence activated cell sorting (FACS) tubes. Cells were stained with 0.5µL Near-IR L/D stain for 15 minutes at 4°C (Table 1) and fixed in 2% paraformaldehyde (PFA,
Materials and Methods

Sigma-Aldrich, St. Louis, MO, USA, Appendix). All samples were analysed by flow cytometry (refer to section 2.4) by acquiring 2 million cells in two 1 million runs, followed by merging the data. Specific lysis (%) was calculated using the formula below:

\[
\% \text{ specific lysis} = \left[ 1 - \frac{\left( \frac{\text{target cell #}}{\text{control cell #}} \right)_{\text{vaccinated}}}{\left( \frac{\text{target cell #}}{\text{control cell #}} \right)_{\text{PBS}}} \right] \times 100
\]

2.2.1 ELISA FOR VP60-SPECIFIC ANTIBODIES

ELISA plates were coated with 100µL/well of RHDV dissociated VP60 subunits diluted to 20µg/mL in carbonate buffer (Appendix) and then incubated for 1 hour at 37°C. Following this the plates were washed six times in wash buffer (Appendix) and 200µL/well of blocking buffer (Appendix) was added and left to incubate for 1 hour at 37°C. The plates were washed six times in wash buffer. Serum samples were serially diluted in blocking buffer and 100µL/well of 10^{-2}-10^{-7} dilutions was added to the plate with 100µL/well of blocking buffer as a negative control. The plates were left to incubate for 2 hours at 37°C. The plates were washed seven times in wash buffer and 100µL/well of horseradish peroxidase (HRP)-conjugated antibody diluted 1:60,000 in blocking buffer was added and left to incubate for 1 hour at 37°C. The plates were washed seven times in wash buffer. To develop the signal, 100µl TMB substrate was added to each well and the reaction was stopped with 100µl 1NH₂SO₄ once sufficient colour development was observed. The absorbance was read at 450nm within the 1 hour using the xyz plate reader.
2.3 TUMOUR GROWTH KINETICS

MC-38 cells were injected into the right flank of male Urah^{Plt2/Plt2} mice at 5x10^4, 1x10^5 or 5x10^5 cells in 100µL DPBS. B16 cells were injected into the right flank of male Urah^{Plt2/Plt2} and lb/lb mice at 5x10^3, 1x10^4 or 5x10^4 cells in 100µL PBS. C57.mgMUC1 cells were injected into the mammary fat pad of female Urah^{Plt2/Plt2} mice at 5x10^4, 1x10^5, 5x10^5 or 1x10^6 cells in 20µL PBS. Lastly, C57.mg cells were injected into the mammary fat pad of female lb/lb mice at 1x10^5 or 5x10^5 in 20µL PBS. To inject into the mammary fat pad, mice were anesthetised using a ketamine/domitor/atropine combination, except for the lb/lb mice which, due to their extreme weights were not anesthetised and instead restrained for injections. Fur was subsequently shaved and hair removal cream was applied around the 2nd thoracic nipple where tumour cells were injected. For domitor reversal mice were then injected with antisedan.

Following injection all mice were monitored, with weight and tumour growth measured daily for the first 2 weeks and then every 2 days following this. In accordance with ethical guidelines mice that lost >10% body weight in a single day or those whose tumours began to ulcerate or reached a size of 150mm^2 were culled. All remaining mice were culled on day 42.

2.4 ANTIBODY STAINING AND DATA ANALYSIS

2.4.1 ANTIBODY STAINING

Antibodies used in experiments and their manufacturers are listed in Table 1. For all antibody staining, cells were transferred into FACs tubes and centrifuged for 5 minutes at 300 x g at 21°C. Cells were then washed in 2mL FACS buffer (Appendix) and centrifuged for 5 minutes at 300 x g at 21°C. Cells were stained with Zombie Yellow™ viability dye (Biolegend) diluted in 50µL DPBS for 15 minutes in the dark at room temperature. The cells were then washed in 2mL FACS buffer and centrifuged for 5 minutes at 300 x g at 21°C. In order to prevent non-
specific binding of antibodies, Fc block (BD Biosciences) diluted in 50µL FACS buffer was added to each sample and incubated for 10 minutes at 4°C. To stain for cell surface markers, fluorophore-conjugated monoclonal antibodies diluted in 50µL FACS buffer were added to the cells and incubated for 10 minutes in the dark at 4°C. Cells were then washed twice in 2mL FACS buffer and centrifuged for 5 minutes at 300 x g at 21°C. Cells were then either resuspended in 200µL FACS buffer or fixed with 2% PFA. To fix the cells they were resuspended in 200µL PFA fixation buffer (Appendix), incubated for 15 minutes in the dark at room temperature and centrifuged for 5 minutes at 300 x g at 21°C and resuspended in 200µL FACS buffer. All samples were analysed by flow cytometry.

2.4.2 Flow Cytometric Analysis

Flow cytometry data was acquired using a Gallios™ flow cytometer (Beckman Coulter, Brea, CA, USA) and analysed using Kaluza analysis software (Backman Coulter, Brea, CA, USA), version 1.3.

2.4.3 Statistical Analysis

Statistically significant differences between mean values were determined by unpaired Student’s t tests, unpaired Mann-Whitney test, or Mantel-Cox test using Prism 7.0 statistical software (Graphpad Software Inc., La Jolla, CA, USA). A p value of <0.05 was deemed statistically significant.


<table>
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<th>Antibodies</th>
<th>Concentration</th>
<th>Dilution</th>
<th>Clone</th>
<th>Fluorophore(s)</th>
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CHAPTER 3 – RESULTS
3 RESULTS

3.1 TUMOUR GROWTH KINETICS

To determine the cell number needed to establish consistent tumours for later tumour trials and to investigate whether tumour growth differed between the three mice strains, a tumour titration was conducted. All C57BL/6 mice results were conducted previously by other lab members and used here to compare with results observed in our lb/lb and Urah\(^{Ph2/Ph2}\) mouse models.

3.1.1 HYPERURICEMIA SLOWS TUMOUR GROWTH AND INCREASES OVERALL SURVIVAL IN MURINE BREAST CANCER

Urah\(^{Ph2/Ph2}\) and lb/lb mice were injected into the mammary fat pad with different amounts of C57.mg.MUC1 cells to assess tumour growth kinetics of breast cancer cells. Mice were monitored for wellbeing and tumours were measured daily. These results were compared to tumour titrations conducted in C57BL/6 mice previously by Katrin Kramer, with the same numbers of C57.mgMUC1 cells used.

Analysis of C57.mgMUC1 tumour growth in the Urah\(^{Ph2/Ph2}\) mice compared to C57BL/6 mice revealed a trend towards slowed tumour growth and decreased tumour size in the Urah\(^{Ph2/Ph2}\) mice, independent of the number of tumour cells injected (Figure 2A). All tumours were small compared to the C57BL/6 mice injected with 5x10^4, 1x10^5, 5x10^5 cells and none of the mice had to be culled prior to the 42-day cut off due to large tumours. Analysis of C57.mgMUC1 tumour growth in the lb/lb mice compared to C57BL/6 mice revealed no obvious differences between tumour growth (Figure 2A). Interestingly, the different cell numbers of 1x10^5 and 5x10^5 cells in the lb/lb mice showed little observable differences in tumour growth.
C57BL/6 mice injected with $5 \times 10^4$, $1 \times 10^5$, $5 \times 10^5$ cells had a median survival of 35 days ($p=0.0246$), 32 days ($p=0.0998$) and 25 days ($p=0.0246$), whereas the size of tumours of Urah$^{Plt2/Plt2}$ mice did not reach the pre-defined endpoint, thus no median survival could be calculated (Figure 2B). Urah$^{Plt2/Plt2}$ mice injected with $1 \times 10^6$ cells had a significantly longer ($p=0.0224$) median survival of 33 days compared to 20 days observed in the C57BL/6 mice (Figure 2B). No differences were observed between the median survival of the C57BL/6 mice compared to the lb/lb mice injected with $1 \times 10^5$ cells, with median survivals of 32 and 31 days, respectively (Figure 2B). However, lb/lb mice injected with $5 \times 10^5$ cells had significantly increased overall survival ($p=0.0246$) compared to the C57BL/6 mice, with a median survival of 37 days compared to 25 days.
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Figure 2 Tumour growth kinetics and overall survival of C57BL/6, Urah<sup>Pt2/Pt2</sup> lb/lb mice with C57.<sup>mg</sup>MUC1 tumours.

C57BL/6 and Urah<sup>Pt2/Pt2</sup> mice (n = 3 mice/condition) were injected into the mammary fat pad with 5x10<sup>4</sup>, 1x10<sup>5</sup>, 5x10<sup>5</sup>, or 1x10<sup>6</sup> C57.<sup>mg</sup>MUC1 tumour cells. lb/lb mice were injected into the mammary fat pad with 1x10<sup>6</sup> and 5x10<sup>5</sup> C57.<sup>mg</sup>MUC1 tumour cells. Following injection, the tumour growth was monitored over 42 days and mice were sacrificed when the tumour reached a size of 150 mm<sup>2</sup>. (A) Tumour size of individual mice is displayed and (B) shows the overall survival curves. Statistically significant differences were determined using the Mantel-Cox test. * p < 0.05.
3.1.2 **Hyperuricemia and Obesity do not Impact Tumour Growth or Overall Survival in Melanoma in Mice**

Tumour growth kinetics of melanoma cells (B16) were assessed in C57BL/6 Urah\textsuperscript{Plt2/Plt2}, lb/lb mice. Urah\textsuperscript{Plt2/Plt2} and lb/lb mice were injected subcutaneously with different amounts of B16 cells and tumours were measured daily. These results were compared to tumour titrations conducted previously by Kunyu Li in C57BL/6 mice injected with $2 \times 10^4$, $5 \times 10^4$ and $1 \times 10^5$ B16-OVA cells. Unfortunately, some mice were injected intraperitoneally instead of subcutaneously leading to 4 mice (3 lb/lb mice, 1 Urah\textsuperscript{Plt2/Plt2} mouse) being excluded from these results. In compliance with ethical guidelines all remaining mice were culled shortly after this discovery, on day 24, in case they too had intraperitoneal tumours.

Visualisation of B16 tumour growth in the Urah\textsuperscript{Plt2/Plt2} and lb/lb mice injected correctly compared to the C57BL/6 mice revealed no obvious differences in tumour growth rates (Figure 3A).

C57BL/6 mice injected with $5 \times 10^4$ cells had a median survival of 17 days which was not significantly different from the median survival of 17 and 13 days seen in Urah\textsuperscript{Plt2/Plt2} and lb/lb mice respectively (Figure 3B).
Figure 3 Tumour growth kinetics and overall survival of C57BL/6 and Urah\textsuperscript{Plt2/Plt2} mice with B16 tumours.

C57BL/6 mice (n=5) were injected subcutaneously with 5x10\(^3\) B16-OVA tumour cells and Urah\textsuperscript{Plt2/Plt2} (n=2 for 5x10\(^3\) cells, n = 3 for 1x10\(^4\) and 5x10\(^4\) cells) and lb/lb mice (n=3 for 5x10\(^3\) cells, n = 2 for 1x10\(^4\) cells and n=1 for 5x10\(^4\) cells) were injected subcutaneously with 5x10\(^3\), 1x10\(^4\), 5x10\(^4\) B16 tumour cells and the tumour growth was monitored over 42 days. Mice were sacrificed when the tumour reached a size of 150 mm\(^2\). (A) Tumour size of individual mice is displayed and (B) shows the overall survival curves. Statistically significant differences were determined using the Mantel-Cox test.
3.1.3 Hyperuricemia does not impact tumour growth or overall survival in colorectal cancer in mice

Tumour growth kinetics of colorectal cancer cells (MC-38) were assessed in C57BL/6 and Urah<sup>Plt2/Plt2</sup> mice. Urah<sup>Plt2/Plt2</sup> were injected subcutaneously with different amounts of MC-38 cells and tumours were measured daily. These results were compared to tumour titrations conducted previously by Nick Shields in C57BL/6 mice injected with 1x10<sup>5</sup>, 5x10<sup>5</sup> and 1x10<sup>6</sup> MC-38 cells.

Visualisation of MC-38 tumour growth in the Urah<sup>Plt2/Plt2</sup> mice compared to the C57BL/6 mice revealed no obvious differences in tumour growth rates (Figure 4A).

C57BL/6 mice injected with 1x10<sup>5</sup> cells had and median survival of 23 days, which was not significantly different from the median survival of 27 days seen in Urah<sup>Plt2/Plt2</sup>. C57BL/6 mice injected with 5x10<sup>5</sup> cells had and median survival of 18 days which was not significantly different and to the median survival of 27 days seen in Urah<sup>Plt2/Plt2</sup> (Figure 4B).
Figure 4 Tumour growth kinetics and overall survival of C57BL/6 and Urah<sup>P2O/P2</sup> mice injected with MC-38 tumours.

C57BL/6 (n=8-10) were injected subcutaneously with 1x10<sup>5</sup>, 5x10<sup>5</sup> MC-38 tumour cells, Urah<sup>P2O/P2</sup> mice (n = 3 mice) were injected subcutaneously with 5x10<sup>4</sup>, 1x10<sup>5</sup>, 5x10<sup>5</sup> MC-38 tumour cells and the tumour growth was monitored over 42 days. Mice were sacrificed when the tumour reached a size of 150 mm<sup>2</sup>. (A) Tumour size of individual mice is displayed and (B) shows the overall survival curves. Statistically significant differences were determined using the Mantel-Cox test.
3.2 Assessment of PD-L1 Expression in Cancer Cell Lines

PD-L1 can be expressed by cancer cells in the tumour microenvironment and contributes to immune suppression. As therapy with PD-L1 blocking antibodies has been shown to synergise with cancer immunotherapies we intended to administer VLP-peptide constructs together with PD-L1 blockade to reverse immune-suppression. To assess whether PD-L1 is expressed by B16, MC-38 and C57.mgMUC1 cancer cell lines, cells were treated with IFN-γ or left untreated and PD-L1 expression was measured using flow cytometry (Figure 5A). Analysis of all flow cytometric experiments was performed in Kaluza, which utilises a different algorithm than FlowJo to calculate MFI values. The values presented here multiplied by 256 to equal the values calculated in FlowJo.

PD-L1 expression was significantly increased in MC-38 cells \((p=0.0017)\) following IFN-γ treatment with a median fluorescence intensity (MFI) of 18 compared to the MFI of the untreated cells, 1.9 (Figure 5B). Similar results were observed in the B16 cells, where PD-L1 expression was significantly increased \((p=0.0472)\) following IFN-γ treatment with a mean MFI of 27.9 compared to the MFI of 2.5 seen in the untreated cells (Figure 5B). However, in the C57.mgMUC1 cells, no significant difference in PD-L1 expression of the IFN-γ treated compared to the untreated cells was observed, with mean MFI values of 7.9 and 5.7, respectively (Figure 5B).
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Figure 5 Expression of PD-L1 in MC-38, B16 and C57.mgMUC1 tumour cells.

MC-38, B16 and C57.mgMUC1 cells were plated and treated with 5ng/mL IFN-γ or left untreated for 48 hours. Following this incubation cells were stained with fluorophore-conjugated antibodies against PD-L1. (A) MC-38, B16 and C57.mgMUC1 cells were analysed by flow cytometry gating on size (forward scatter) and granularity (side scatter) patterns for doublet cell exclusion and Zombie Yellow Live/Dead viability staining for dead cell exclusion. Cells were labelled with fluorophore-conjugated antibodies against the cell surface marker PD-L1. (B) Expression levels of PD-L1 shown as mean fluorescence intensity (MFI). The results represent the mean (+SEM) of three independent experiments for each cell line, with the IFN-γ treated group repeated in triplicate. Statistically significant differences were determined by using an unpaired Student’s t-test. ** p ≤ 0.01; * p < 0.05.
Results

3.3 ASSESSMENT OF ADAPTIVE IMMUNE RESPONSE TO VLP-PEPTIDE VACCINES

3.3.1 HYPERURICEMIA DOES NOT IMPACT ANTIGEN-SPECIFIC KILLING IN RESPONSE TO VLP-PEPTIDE VACCINES

VLP vaccines have previously been shown to induce antigen-specific killing in C57BL/6 mice \textit{in vivo}. Thus, to assess the impact of hyperuricemia on antigen-specific killing, VLP-peptide vaccines were injected into Urah\textsuperscript{Plt2/Plt2} mice to compare the level of antigen-specific killing to that of C57BL/6 mice. C57BL/6 and Urah\textsuperscript{Plt2/Plt2} mice were vaccinated with VLP-peptide vaccines containing either the gp100, survivin or survivin+mucin1 peptide and boosted with the same vaccine on day 21 (Figure 6A). On day 28, mice were injected with peptide-pulsed, dye-stained target cells and specific killing of target cells was determined 40h later by comparing the number of unpulsed cells to peptide-pulsed cells (Figure 6A, 6B).

In the case of gp100-pulsed target cell lysis, Urah\textsuperscript{Plt2/Plt2} mice vaccinated with VLP-gp100 generated a high rate of specific lysis with a median of 73.2\% (Figure 6C). However, this was not significantly different from the C57BL/6 mice receiving the same vaccine, which generated a median specific lysis rate of 68.2\%. C57BL/6 mice vaccinated with VLP-survivin (VLP-surv) achieved a mean rate of specific lysis of survivin-pulsed target cells of 10.6\%, whereas no specific lysis of the target cells was observed in the Urah\textsuperscript{Plt2/Plt2} mice (Figure 6C). No specific lysis was observed in the C57BL/6 or the Urah\textsuperscript{Plt2/Plt2} mice vaccinated with VLP-survivin+mucin1 (VLP-surv.MUC1) (Figure 6C).
Figure 6 Induction of target-specific immunity with VLP-peptide vaccines in Urah<sup>Ph2/Ph2</sup> and C57BL/6 mice. (A) C57BL/6 and Urah<sup>Ph2/Ph2</sup> mice were vaccinated with either VLP-gp100, VLP-surv, VLP-surv,MUC1 or PBS and boosted with same vaccine 3 weeks later. On week 4, all mice were injected IV with gp100-pulsed-VPD<sup>High</sup>, CFSE<sup>High</sup>, survivin-pulsed-CFSE<sup>High</sup>, survivin+mucin1-pulsed-VPD<sup>High</sup> or unpulsed-CFSE<sup>Low</sup> splenocytes from naive C57BL/6 mice. (B) Peptide-pulsed splenocytes were analysed by flow cytometry gating on size (forward scatter) and granularity (forward scatter) patterns for doublet cell exclusion, Zombie Yellow Live/Dead viability staining for dead cell exclusion. (C) 48 hours following IV injection the mice were culled and analysed for % specific lysis of target cells. The results represent the median (±SEM) of 2 individual experiments (n = 6 mice/group) each comprised of 24 mice. Statistically significant differences were determined using an unpaired Mann-Whitney tests.
3.3.2 Hyperuricemia decreases generation of VLP-peptide vaccine specific antibodies

To compare the level of antibody production against the VLP vaccine in vivo, C57BL/6 and Urah\textsuperscript{Plt2/Plt2} mice were vaccinated with VLP-peptide vaccines containing gp100, survivin or survivin+mucin1. Blood was collected 30 days following vaccination and IgG antibody generation specific to VP60, the viral subunits that make up the VLP-peptide vaccine was measured by ELISA. Initial titrations were conducted and determined that a dilution of $10^{-4}$ was the most stable and likely to reveal differences (Figure. 7A). A dilution of $10^{-4}$ was used for all future experiments to statically compare antibody titres in the blood of C57BL/6 and Urah\textsuperscript{Plt2/Plt2}.

In the case of antibody production against the VLP-surv. vaccine, C57BL/6 mice generated significantly increased amounts ($p = 0.0001$) following the removal of two outliers (1 from each mouse) of VP60 specific IgG antibodies with a mean absorbance of 0.203 compared to the absorbance of 0.128 generated by the Urah\textsuperscript{Plt2/Plt2} mice (Figure 7B). In the case of antibody production against the VLP-surv.MUC1 vaccine, C57BL/6 mice generated significantly greater proportions ($p = 0.0015$) of VP60 specific IgG antibodies with a mean absorbance of 0.229 compared to the absorbance of 0.135 measured in the Urah\textsuperscript{Plt2/Plt2} mice (Figure 7B). In the case of antibody production against the VLP-gp100 vaccine, C57BL/6 mice generated more VP60 specific IgG antibodies with a mean absorbance of 0.266 compared to the absorbance of 0.229 generated by the Urah\textsuperscript{Plt2/Plt2} mice, however this difference was not significant (Figure 7B).
Figure 7 Production of VP60 specific IgG antibodies following VLP-peptide vaccine administration in Urah<sup>Plt2/Plt2</sup> and C57BL/6 mice. C57BL/6 and Urah<sup>Plt2/Plt2</sup> mice were vaccinated with either VLP-gp100, VLP-surv, VLP-surv-MUC1 or PBS and boosted with same vaccine 3 weeks later. On week 4, all mice were injected IV with gp100-pulsed-CFSE<sup>High</sup>, survivin-pulsed-CFSE<sup>High</sup>, survivin+MUC1-pulsed-CFSE<sup>High</sup> or unpulsed-CFSE<sup>Low</sup> splenocytes from naïve C57BL/6 mice. (A) 48 hours following IV injection blood was collected via cardiac puncture and VP60 specific IgG antibody production was determined using a VP60 specific ELISA. The results represent the mean (±SEM) of 2 individual experiments (n = 4-6 mice/group, 2 outliers removed from VP-survivin group) each comprised of 24 mice. Statistically significant differences were determined using an unpaired Student’s t-tests. *** p ≤ 0.001; ** p ≤ 0.01.
3.4 Bone Marrow Immune Cell Populations in Obese Mice Have Increased Frequencies of Myeloid and Immunosuppressive Cells

Initial experiments aimed to characterise phenotypic differences in BM cells between lb/lb, Urah\textsuperscript{Plt2/Plt2} and C57BL/6 mice. Flow cytometry was used to determine the expression of the cell surface markers B220, CD11b, CD11c, CD3, Ly6C and Ly6G on BM cells commonly used to identify B cells, DCs, T cells, neutrophils and MDSCs (Figure 8A).

Cells in the BM of lb/lb mice contained a significantly higher frequency (Figure x, \(p=0.0082\)) of CD11b\textsuperscript{+} cells as compared to cells in the BM of C57BL/6, with mean frequencies of 49.6\% and 33.5\%, respectively (Figure 8B). However, the frequency of CD11b\textsuperscript{+} cells in the BM of Urah\textsuperscript{Plt2/Plt2} mice was not significantly different from that of C57BL/6 mice, with a mean frequency of 40.6\%.

Compared to cells in the BM of C57BL/6 mice, a significantly higher frequency (\(p=0.0012\)) of cells in the BM of lb/lb mice were characterised as CD11b\textsuperscript{+} Ly6C\textsuperscript{-}, Ly6G\textsuperscript{-} (also known as mMDSCs) with a mean frequency of 11.3\% as compared with 9.0\% for BM cells of C57BL/6 mice (Figure 8B). Comparably, the frequency of CD11b\textsuperscript{+} Ly6C\textsuperscript{-}, Ly6G\textsuperscript{-} cells was not significantly different in cells in the BM of Urah\textsuperscript{Plt2/Plt2} and C57BL/6 mice, with a mean frequency of 9.4\% and 9.0\%, respectively. Cells in the BM of lb/lb mice contained a significantly higher frequency (\(p=0.0172\)) of CD11b\textsuperscript{+} Ly6C\textsuperscript{+}, Ly6G\textsuperscript{+} cells (also known as gMDSCs) compared to the cells in the BM of C57BL/6 mice with mean frequencies of 27.5\% and 19.1\%, respectively (Figure 8B). Comparably, the frequency of cells in the BM of Urah\textsuperscript{Plt2/Plt2} mice characterised as Ly6C\textsuperscript{+}, Ly6G\textsuperscript{+} (gMDSCs) did not significantly differ from frequencies observed in C57BL/6 mice, with a mean frequency of 23.1\%. 

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The frequency of CD3+ cells in the BM of C57BL/6 mice, lb/lb and Urah<sup>Plt2/Plt2</sup> mice did not differ significantly with mean frequencies of 4.4%, 4.4% and 3.4%, respectively (Figure 8C). There were no differences in the frequencies of B220+ cells in the BM of C57BL/6 lb/lb and Urah<sup>Plt2/Plt2</sup> mice with mean frequencies of 27.3% compared to 14.9% and 20.5%, respectively, however this indicates a trend of C57BL/6 mice BM cells having increased B220+ cells compared to the lb/lb and Urah<sup>Plt2/Plt2</sup> mice (Figure 8C). Lastly, cells in the BM of all the three different types of mice, contained low frequencies of CD11c+ cells, with mean frequencies of 1.2% for C57BL/6, 1.3% for lb/lb and 1.2% for Urah<sup>Plt2/Plt2</sup>, these frequencies were not significantly different from one another (Figure 8C).
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Figure 8 Phenotype of BM cells isolated from lb/lb, C57BL/6 and UrahPlt2/Plt2 mice.

(A) BM cells were analysed by flow cytometry gating on size (forward scatter) and granularity (side scatter) patterns for doublet cell exclusion and Zombie Yellow Live/Dead viability staining for dead cell exclusion and cells were labelled with fluorophore-conjugated antibodies against the cell surface markers B220, CD11b, CD11c, CD3, Ly6C and Ly6G. (B and C) Expression levels of phenotypic markers shown as percentage of live cells. These results represent the mean (+SEM) of four independent experiments with a total number of four mice/model. Statistically significant were determined using an unpaired Student's t-test. **, p < 0.01, *, p < 0.05.
3.5 Phenotypic Analysis of Bone Marrow-Derived Dendritic Cells Generated from Wild Type, Obese and Hyperuricemic Mice

3.5.1 BMDC Populations from Obese Mice Have Increased Frequencies of MHC II Negative BMDCs

To identify phenotypic differences in BMDCs from lb/lb, Urah<sup>Plt2/Plt2</sup> and C57BL/6 mice, BM cells were cultured for 6 days with GM-CSF and analysed via flow cytometry. Based on their use in the literature and high expression frequency, the cell surface marker CD11c was used to identify BMDCs (Figure 9A) (75). Following this CD11c<sup>+</sup> DCs were further analysed for their expression of MHC II and three populations were defined CD11c<sup>+</sup> MHC II<sup>-</sup>, CD11c<sup>+</sup> MHC II<sup>mid</sup> and CD11c<sup>+</sup> MHC II<sup>high</sup> cells (Figure 9A).

The percentage of CD11c<sup>+</sup> MHC II<sup>high</sup> cells was not significantly different in BMDCs derived from lb/lb, C57BL/6 and Urah<sup>Plt2/Plt2</sup> mice with mean frequencies of 19.2%, 21.8% and 20.0%, respectively (Figure 9B). Similarly, the frequency of CD11c<sup>+</sup> MHC II<sup>mid</sup> cells was also not significantly different in BMDCs from lb/lb, C57BL/6 and Urah<sup>Plt2/Plt2</sup> mice with mean frequencies of 23.2%, 25.3% and 24.4%, respectively (Figure 9B). However, the percentage of CD11c<sup>+</sup> MHC II cells was significantly increased in cultures from lb/lb mice compared to C57BL/6 mice (p=0.0018), with mean frequencies of 29.7% and 22.9%, respectively (Figure 9B). In comparison, no significant differences in the percentage of CD11c<sup>+</sup> MHC II cells was observed in the BMDCs from Urah<sup>Plt2/Plt2</sup> (27.2%), when compared to the DCs from C57BL/6 mice.
Figure 9 Phenotype of BMDCs isolated from lb/lb, C57BL/6 and Urah<sup>Plt2/Plt2</sup> mice.

(A) BMDCs were analysed by flow cytometry gating on size (forward scatter) and granularity (side scatter) patterns for doublet cell exclusion, Zombie Yellow Live/Dead viability staining for dead cell exclusion and expression of the cell surface marker CD11c was used for dendritic cell selection. Cells were labelled with fluorophore-conjugated antibodies against the cell surface marker MHC II. (B) Expression levels of activation markers shown as the percentage CD11c<sup>+</sup> live cells. These results represent the mean (+SEM) of two independent experiments with a total number of two mice/model. Statistically significant were determined using an unpaired Student’s t-test. * p < 0.05.
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3.5.2 BMDC POPULATIONS FROM OBESE MICE HAVE DECREASED EXPRESSION OF ACTIVATION MARKERS

To characterise differences in activation markers on BMDCs from lb/lb, Urah^{Plt2/Plt2} and C57BL/6 mice, BMDCs were left unstimulated or stimulated with CpG (0.25nmol/mL) and analysed by flow cytometry 24 hours later (Figure 10A). The cell surface marker CD11c was used to identify BMDCs (Figure 10A). Following this, CD11c^{+} DCs were analysed for their expression of MHC II and the costimulatory molecules CD40, CD80 and CD86 (Figure 10A).

In BMDCs from C57BL/6 mice, the percentage of CD11c^{+} cells that stained positive for MHC II after CpG stimulation did not significantly differ from unstimulated DCs (Figure 10B). However, the percentage of MHC II^{+} BMDCs from lb/lb and Urah^{Plt2/Plt2} mice was significantly upregulated following CpG stimulation compared to the unstimulated BMDCs (p= 0.0001 and 0.0301, respectively). The percentage of BMDCs expressing MHC II was lower in lb/lb as compared to C57BL/6 mice in response to CpG treatment (p=0.0109), with a mean frequency of 61.8% compared to 69.6%. BMDCs generated from Urah^{Plt2/Plt2} mice had similar levels of CD11c^{+} MHCII^{+} cells as the C57BL/6 BMDCs (66.1%).

Expression of CD40 in BMDCs from lb/lb, C57BL/6 and Urah^{Plt2/Plt2} mice was significantly upregulated following CpG stimulation compared to the unstimulated BMDCs (p<0.0001, for all groups, Figure 10B). BMDCs from C57BL/6 mice stimulated with CpG showed a significantly higher expression (p= 0.0394) of CD40 as compared to BMDCs from lb/lb mice, with mean frequencies of 55.1% and 48.7%, respectively. The percentage of CD11c^{+} cells staining positive for CD40 was not significantly different in CpG-stimulated BMDCs from Urah^{Plt2/Plt2} mice at a mean frequency of 47.4%.
CD80 expression in BMDCs from lb/lb, C57BL/6 and Urah$^{Plt2/Plt2}$ mice was upregulated in response to CpG stimulation compared to the unstimulated DCs ($p<0.0001$, for all groups, Figure 10B). However, there were no significant differences in CD80 expression following CpG stimulation observed between the BMDCs from lb/lb, C57BL/6 and Urah$^{Plt2/Plt2}$ mice, with mean frequencies of 85.2%, 86.6% and 85.8%, respectively.

Lastly, in the BMDCs from lb/lb, C57BL/6 and Urah$^{Plt2/Plt2}$ mice, CpG stimulation lead to significantly increased CD86 expression levels ($p<0.0001$, for all groups, Figure 10B). BMDCs from C57BL/6 mice expressed significantly higher levels of CD86 when un-stimulated in comparison to the BMDCs from lb/lb mice ($p=0.0161$), with mean frequencies of 31.8% and 26.8%, respectively. This significant increase was also observed in the CpG stimulated BMDCs from C57BL/6 mice ($p=0.0217$), at mean frequency of 63.7%, compared to the mean frequency of 55.0% seen in the BMDCs from lb/lb mice. In comparison, CD86 expression was not significantly different in the BMDCs from Urah$^{Plt2/Plt2}$ mice compared to the BMDCs from C57BL/6 mice, with mean frequencies of 29.8% (un-stimulated) 58.67% (CpG stimulated).
Figure 10 Expression of activation markers in BMDCs following treatment with CpG.

BMDCs stimulated with CpG (0.25nmol/mL) or left untreated for 24 hours at 37°C. (A) BMDCs were analysed by flow cytometry gating on size (forward scatter) and granularity (side scatter) patterns for doublet cell exclusion, Zombie Yellow Live/Dead viability staining for dead cell exclusion and expression of the cell surface marker CD11c was used for dendritic cell selection. Cells were labelled with fluorophore-conjugated antibodies against the cell surface markers MHCII, CD40, CD80 and CD86. (B) Expression levels of activation markers shown as the percentage CD11c+ live cells, following no stimulation or stimulation with CpG. These results represent the mean (+SEM) of two independent experiments performed in triplicate, with a total number of two mice/model. Statistically significant were determined using an unpaired Student’s t-test. ****p<0.0001; * p < 0.05.
To further confirm the differences observed between activation marker expression, MFI values of the activation markers were compared in the different BMDC cultures (Figure 11A). Analysis of all flow cytometric experiments was performed in Kaluza, which utilises a different algorithm than FlowJo to calculate MFI values. The values presented here multiplied by 256 to equal the values calculated in FlowJo.

In the BMDCs from the lb/lb, C57BL/6 and Urah<sup>Plt2/Plt2</sup> mice, MHC II expression analysis revealed that CpG stimulation lead to significantly increased expression levels compared to the unstimulated DCs ($p=0.0001$, $p<0.0001$ and $p=0.0035$, respectively, Figure 11B). However, there were no significant differences in MHC II expression following CpG stimulation observed between the BMDCs from lb/lb, C57BL/6 and Urah<sup>Plt2/Plt2</sup>, with mean MFI values of 56.2, 79.8 and 61.8, respectively.

Expression of CD40 was significantly upregulated in the BMDCs from lb/lb, C57BL/6 and Urah<sup>Plt2/Plt2</sup> mice following CpG stimulation compared to the unstimulated DCs ($p=0.0493$, $p=0.0101$ and $p=0.0101$, respectively, Figure 11B). BMDCs from C57BL/6 mice expressed significantly higher levels of CD40 following CpG stimulation compared to the BMDCs from the lb/lb mouse ($p=0.0493$), with mean MFI values of 1.5 and 1, respectively. In comparison, CD40 expression was not significantly different following CpG stimulation in the BMDCs from Urah<sup>Plt2/Plt2</sup> mice compared to the BMDCs from C57BL/6 mice, with a mean MFI of 1.5.

In the BMDCs from the lb/lb, C57BL/6 and Urah<sup>Plt2/Plt2</sup> mice, CD80 expression analysis revealed that CpG stimulation lead to significantly increased expression levels compared to the unstimulated DCs ($p<0.0001$, $p<0.0001$ and $p=0.0001$, respectively, Figure 11B). However, there were no significant differences in CD80 expression following CpG stimulation observed
between the BMDCs from lb/lb, C57BL/6 and Urah^Plt2/Plt2^ mice, with mean MFI values of 3.8, 4.2 and 3.5, respectively.

Lastly, expression of CD86 was significantly upregulated in the BMDCs from lb/lb, C57BL/6 and Urah^Plt2/Plt2^ mice following CpG treatment compared to the unstimulated BMDCs (p<0.0001, p<0.0001 and 0.0056, respectively, Figure 11B). CD86 expression was significantly increased in the unstimulated BMDCs from C57BL/6 mice compared to the BMDCs from lb/lb mice (p=0.0005), with mean MFI values of 1.8 and 1, respectively. However, there were no significant differences in CD86 expression observed between the unstimulated BMDCs from the Urah^Plt2/Plt2^ mice compared to the BMDCs from the C57BL/6 mice, with a mean MFI value of 1.5. Following CpG stimulation CD86 was significantly increased in the BMDCs from the C57BL/6 mice compared to the BMDCs from the lb/lb mice (p=0.0482), with MFI values of 16.5 and 10.8, respectively. However, there were no significant differences in CD86 expression following CpG stimulation in the BMDCs from Urah^Plt2/Plt2^ mice compared to the BMDCs from C57BL/6 mice, with a mean MFI value of 14.

The results from sections 3.4 and 3.5 are summarised on Table 1.
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Figure 11 Expression of activation markers BMDCs following treatment with CpG.

BMDCs stimulated with CpG (0.25nmol/mL) or left untreated for 24 hours at 37°C. BMDC cells were analysed by flow cytometry gating on size (forward scatter) and granularity (side scatter) patterns for doublet cell exclusion, Zombie Yellow Live/Dead viability staining for dead cell exclusion and expression of the cell surface marker CD11c was used for dendritic cell selection. Cells were labelled with fluorophore-conjugated antibodies against the cell surface markers MHCII, CD40, CD80 and CD86. (A) Expression levels of activation markers shown as mean fluorescence intensity (MFI), following no stimulation or stimulation with CpG. These results represent the mean (+SEM) of two independent experiments performed in triplicate, with a total number of two mice/model. Statistically significant were determined using an unpaired Student’s t-test. **** p<0.0001; *** p≤0.001; ** p≤0.01; * p < 0.05.
Overview of results from the BM and BMDC phenotypic analysis experiments. Where ↑ refers to an increase compared to wild type, ↓ refers to a decrease compared to wild type and — indicates no change compared to wild type.

<table>
<thead>
<tr>
<th>Cells</th>
<th>lb/lb Mice</th>
<th>Urah&lt;sup&gt;Plt2/Plt2&lt;/sup&gt; Mice</th>
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<tr>
<td><strong>BM Cells</strong></td>
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</tr>
<tr>
<td>CD11b&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>mMDSCs</td>
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<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;</td>
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<td><strong>BMDCs</strong></td>
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<tr>
<td>CD11c&lt;sup&gt;+&lt;/sup&gt; MHC II&lt;sup&gt;High&lt;/sup&gt;</td>
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<td><strong>Stimulated BMDCs</strong></td>
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<td>CD11c&lt;sup&gt;+&lt;/sup&gt; MHC II&lt;sup&gt;+&lt;/sup&gt;</td>
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3.6 BMDCs from Hyperuricemic Mice May Induce Greater T Cell Proliferation

As we observed differences in BMDC populations between the three different mouse models, we next assessed the ability of BMDCs from the lb/lb, C57BL/6 and Urah\textsuperscript{Plt2/Plt2} mice to stimulate T cell responses. BMDCs were co-cultured with T cells, isolated from the spleen of pmel-1 mice, which express a T-cell receptor capable of recognising the gp100 peptide. BMDCs were pre-incubated for 24 hours with either VLP-gp100 constructs, the peptide alone or left untreated. T cells were isolated from pmel-1 mice, stained with CFSE (5\textmu M) and added to the BMDCs in a 1:10 ratio (BMDCs: T cells). T cell proliferation was analysed via flow cytometry after 72 hours (Figure 12A). Statistical analysis could not be performed as only one biological repeat has been conducted thus far.

No differences were observed in the proliferation of the T cells co-cultured with no antigen and DCs from the from lb/lb, C57BL/6 and Urah\textsuperscript{Plt2/Plt2} mice, with mean proliferation percentages of 18.5\%, 17.8\% and 17.3\%, respectively (Figure 12B). There appears to be a trend towards T cell proliferation being increased following co-culture with VLP-peptide stimulated DCs from the lb/lb, C57BL/6 and Urah\textsuperscript{Plt2/Plt2} mice (Figure 12B). There were also no differences seen in the proliferation of the T cells co-cultured with VLP-gp100 and DCs from the from lb/lb, C57BL/6 and Urah\textsuperscript{Plt2/Plt2} mice, with mean proliferation percentages of 91.8\%, 86.9\% and 88.7\%, respectively (Figure 12B). However, there is a slight trend towards increased proliferation in the T cells co-cultured with DCs from the from lb/lb mice compared to the C57BL/6 mice. Lastly, there appeared to be no differences observed in the proliferation percentages between the T cells co-cultured with gp-100\textsubscript{25-33} peptide and DCs from lb/lb, C57BL/6 or Urah\textsuperscript{Plt2/Plt2} mice, with mean proliferation percentages of 97.97\%, 98.38\% and 98.17\%, respectively (Figure 12B).
Figure 12 Proliferation of T cells co-cultured with BMDCs from lb/lb, C57BL/6 and Urah^{Plt2/Plt2} mice.

(A) T cells were analysed by flow cytometry gating on size (forward scatter) and granularity (side scatter) patterns for doublet cell exclusion and Zombie Yellow Live/Dead viability staining for dead cell exclusion and cells were labelled with fluorophore-conjugated antibodies against the cell surface marker CD8, percent proliferation of T cells was determined by CFSE dilution of proliferation peaks. (B) Percentage of proliferation of live T cells following incubation with BMDCs and no antigen, VLP-gp100 or gp100_{25-33} peptide. These results represent the mean (+SEM) of one independent experiment performed in triplicate, with a total number of one mouse/model.
CHAPTER 4 – DISCUSSION
4 DISCUSSION

Previous studies conducted in the Young lab have demonstrated the efficacy of VLP-peptide cancer vaccines in slowing tumour growth and increasing survival in melanoma, colorectal and breast cancer mouse models. However, this therapeutic approach has only been investigated in young and lean wild type mice, and therefore the impact of co-morbidities on this vaccine have not been addressed. Due to the inflammation present in metabolic disorders, the fact that many cancer patients have co-morbidities and previous studies indicating decreased efficacy of immunotherapies in obese mice, this may be a vital step to ensuring the efficacy of this vaccine in a representative population. In light of this, the impact of chronic low-grade inflammation, as seen in obesity and hyperuricemia, on these VLP-peptide cancer vaccines was investigated.

Initial experiments intended to determine the tumour growth kinetics of the three different cancers in hyperuricemic and obese mice compared to wild type mice. Following this, the presence of PD-L1 on each of the three cancer cell lines was determined, to discern if checkpoint blockade would be appropriate in a future tumour trial. Further experiments assessed the ability of T cells to kill antigen-pulsed target cells and measured titres of VP60 IgG antibodies in response to vaccination with VLP-peptide vaccines in wild type and hyperuricemic mice. Flow cytometric analysis was conducted to determine the expression of cell surface molecules of BM cells from obese, wild type and hyperuricemic mice to investigate whether frequencies of various immune cell populations varied amongst these mice. Flow cytometry was also used to determine the expression of molecules associated with DC and T cell dysfunction, proliferative potential and effector function in BMDCs generated from BM precursors from the three mouse models.
4.1 Identifying Tumour Growth Kinetics in Hyperuricemic and Obese Mice

Initial in vivo experiments set out to determine the optimal number of melanoma, colorectal and breast cancer cells to be injected subcutaneously or into the mammary fat pad of obese and hyperuricemic mice to result in tumour formation for a future tumour trial.

No differences in the colorectal cancer tumour growth rates or overall survival were found between the hyperuricemic mice and the wild type mice. While metabolic syndrome, a condition which hyperuricemia is a common component of, has been associated with increased colorectal cancer incidence and mortality, the role of hyperuricemia in this has not yet been ascertained (76). However, based on our results hyperuricemia does not alter colorectal tumour growth or mortality rate in this murine model.

Similarly, no differences in the melanoma tumour growth rates or overall survival were found between the obese and hyperuricemic mice compared to the wild type mice. These results are similar to what is observed in humans, where obesity and hyperuricemia has not been reported to impact melanoma incidence, tumour growth or mortality. However, as four of the mice had to be removed from the study due to intraperitoneally injected tumours and the remaining mice had to be culled as a result of this, it is hard to confidently draw conclusions from this experiment. Thus, these experiments would have to be repeated to correctly determine differences between the inflamed mice and the wild type mice. These results were also compared to wild type mice that were injected with B16-OVA cells as opposed to the B16 cells the obese and hyperuricemic mice received. This could have impacted these results as the presence of ovalbumin (OVA), an egg white protein, makes the tumours more immunogenic as
it is a foreign antigen, potentially resulting in a greater anti-tumour response, again making it difficult to compare these results and draw conclusions.

In comparison, the breast cancer cells grew slower in the hyperuricemic mice and significantly increased overall survival at multiple cell numbers. This was not expected as research has indicated that increased SUA, as seen in hyperuricemia, is associated with increased cancer site incidence and mortality in humans (48). This is possibly due to the increased IL-10 levels observed in the hyperuricemic mouse model (77). While the increased IL-10 levels secreted by monocytes in this mouse model was previously observed following LPS stimulation, necrosing tumour cells in this study may have caused similar immune stimulation leading to increased IL-10 (77). Research into the effect of IL-10 on tumours is controversial, indicating pleiotropic abilities of IL-10 to influence various immune and cancer cells in positive or negative ways in different models (78, 79). However, research has indicated that in some murine breast cancer models IL-10 predominately exerts inhibitory action on tumour progression and growth. This was mediated via NK cell activation, synergistic CTL activation, increased MHC protein expression on cancer cells, enhanced immune cell tumour infiltration and inhibition of metalloproteinases leading to alterations in angiogenesis and invasiveness (78, 80). It has also been proposed that IL-10 inhibits the release of pro-inflammatory cytokines from macrophages thereby decreasing their tumour growth promoting activities (80). Therefore, it is possible that in these mice, the increased IL-10 levels are inducing some of the protective effects seen against the breast cancer cells. To establish whether differences in tumour growth kinetics in these mice are the result of an altered immune system, further studies need to be conducted. This could for example include the administration of an anti-IL-10 antibody to assess if blockage of IL-10 would reverse the observed delay in tumour progression. Furthermore, it remains to be determined if observed changes in tumour growth kinetics would alter the efficacy of VLP vaccines when administered to tumour-burdened mice. Due to the slower growth of these
tumours in the hyperuricemic mice a higher number of cells would be used in a tumour trial compared to what has been previously used in the wild type mice, to ensure sufficient tumours are induced. The highest number of cells, $1 \times 10^6$, was able to produce large tumours and thus will likely be the number injected to investigate the effect of the vaccine on these tumours in the hyperuricemic mice.

Unexpectedly, overall survival in response to breast cancer tumours was also increased in the obese mice compared to the wild type mice. Again, this was not expected as obesity in humans has been linked to an increased incidence and mortality of breast cancer (81). Unlike the hyperuricemic murine model, this obese mouse model has not been shown to have increased IL-10, thus this is unlikely to be a possible cause of this result (82). Unfortunately, due to complications surrounding breeding, not enough obese mice were available in time thus, only two different numbers of breast cancer cells investigated in these mice. And as increased survival in the obese mice compared to the wild type mice was only seen at one of these cell numbers, further experiments would need to be conducted to ascertain if this trend is still present when the mice are injected with different cell numbers and conclude if obesity does increase overall survival in a murine breast cancer model. Due to the different cell numbers resulting in similar levels of tumour growth this indicates that $1 \times 10^5$ cells were enough to induce adequately sized tumours and a higher number of cells is not needed as tumour growth would remain similar. However, due to the location of these tumours and the increased adipose tissue in these mice the tumours were difficult to measure as they did not protrude outward initially, as seen in the leaner mouse models. Thus, it is possible the similar tumour growth rates in the first 2 weeks, before the tumours were large enough to measure accurately, were the result of this and this would need to be considered when conducting similar experiments.
Unfortunately, again due to not having enough obese mice available in time, the tumour growth kinetics experiments of the colorectal cancer cells in the obese mice was not possible to investigate. It would be interesting to investigate the colorectal cancer tumour growth and overall survival in these mice as obesity in humans is associated with an increased incidence and mortality of colorectal cancer (76).

4.2 Assessment of PD-L1 Expression in Cancer Cell Lines

*In vitro* cancer cell line experiments were conducted to determine the presence of the immunosuppressive PD-L1 protein on the cancer cell lines used in the *in vivo* studies. These initial experiments were performed to determine if the use of anti-PD-L1 checkpoint inhibitors in future tumour trials would be effective and able to potentially enhance the anti-tumour effect of the cancer vaccines.

The experiments revealed that all three cancer cell lines expressed PD-L1, however the melanoma and colorectal cell lines only expressed PD-L1 following IFN-γ exposure, indicating induced PD-L1 expression. These results align with previous studies, which found that in B16 cells PD-L1 was induced following IFN-γ secretion by T<sub>H</sub> cells (83). Similar results were reported from studies with MC-38 cancer cells that did not express PD-L1 until treated with IFN-γ (84). It has also been demonstrated that MC-38 tumour cells respond effectively to PD-L1 treatment, causing a decrease in tumour burden in tumour bearing mice (84). In comparison, the breast cancer cell line expressed PD-L1 regardless of IFN-γ exposure, indicating constitutive PD-L1 expression. This is corroborated in the literature, as multiple studies have found that most breast cancer cell lines constitutively express high levels of PD-L1 (42). As PD-L1 is present in all cancer cell lines, this confirms that it would be appropriate to administer anti-PD-L1 checkpoint inhibitors in a future tumour trial, alongside the VLP-peptide vaccines.
While anti-PD-L1 checkpoint inhibitors will take effect upon IFN-\(\gamma\) exposure in the inducible cell lines, e.g. as released by T\(_h\) cells necessary to generate tumour specific CTLs, it will take effect immediately in the constitutively expressed cell lines. This is in line with previous research, which has shown that combining cancer vaccines and checkpoint inhibitors augments the anti-tumour response, mediated by blocking the immunosuppressive function of these molecules on the tumour cells and thereby increasing the efficacy of the vaccine (40).

### 4.3 Assessment of In Vivo Cytotoxicity in Wild Type and Hyperuricemic Mice

A major challenge for vaccines is the inability to generate an appropriate immune response due to a dysfunctional immune system. Thus, it is important to determine if altered immune responses, as seen in chronic low-grade inflammation, would impact the level of CTL targeted cell killing induced by cancer vaccines.

Unfortunately, in response to the VLP-surv and VLP-surv.MUC1 vaccines minimal CTL specific lysis occurred, indicating no targeted cytotoxic immune response was generated. To investigate possible reasons for the failure of these vaccines to generate a targeted T cell response, particle formation was examined by transmission electron microscope (TEM) confirming that the VLPs were still able to form particles. Alongside this the VP60 was run on an SDS page gel confirming survivin was still attached, this was also confirmed via mass spectrometry. Lastly, it was confirmed that the conjugate bond that attaches mucin1 to the VLP is still present. Thus, further tests must be conducted to determine why these vaccines did not generate T cell responses in either mouse model prior to their use in future animal studies to ensure the most usable data can be generated to minimise the number of animals used overall.
In contrast, the VLP-gp100 vaccine generated a high percentage of specific lysis in the wild type mice as a result of vaccine induced gp100-specific CTLs. Surprisingly, similar levels of cytotoxicity were observed in the hyperuricemic mice, indicating that CTL induction in response to the vaccine does not differ between the two mouse models. However, to fully ascertain the cytotoxic potential of tumour specific immune cells a tumour trial would need to be conducted that determines the effect of these CTLs on the tumour cells themselves. Unfortunately, again due to slow breeding, not enough obese mice could be generated in time to conduct the same assay in these mice. It would be interesting to investigate this in the obese mice as previous research has shown that CTL activation and proliferation is reduced in these mice, indicating that induction of CTL specific lysis would be reduced following VLP-peptide vaccination in these animals compared to wild mice (65).

4.4 ASSESSMENT OF ANTIBODY PRODUCTION IN WILD TYPE HYPERURICEMIC MICE

Although B cells themselves are unable to kill tumour cells directly, their role in antigen presentation, T cell activation, and their subsequent release of tumour specific antibodies following activation is vital for generating a robust anti-tumour response. Therefore, it is important to determine if chronic low-grade inflammation impacts the level of VP60 IgG antibody production in response to our VLP vaccine. This will help to understand its impact on B cell functioning and the downstream effects this may have on the anti-tumour response generated.

This revealed that although the VLP-surv and the VLP-surv.MUC1 vaccine were unable to generate a cytotoxic immune response, VP60 specific IgG antibody titres were generated in response to these vaccines in both the hyperuricemic and wild type mice. Interestingly, the
hyperuricemic mice had decreased antibody titres compared to the wild type mice following VLP-surv.MUC1 vaccination. Similar results were seen in response to the VLP-surv vaccine, where again the hyperuricemic mice generated less antibodies compared to the wild type mice. Decreased antibody titres indicate decreased B cell activation or functioning. As B cells are activated via antigen presentation and activation of T\textsubscript{H} cells, this could also indicate decreased activation of TAA-specific T\textsubscript{H} cells. Decreased T\textsubscript{H} cell activation would impede complete activation and proliferation of tumour specific CTLs downstream. Alongside this, reduction in tumour specific antibodies would subsequently reduce antibody-dependent cell-mediated cytotoxicity. As these were IgG antibodies, which are high-affinity antibodies released by long-lived plasma cells, this indicates that both the initial and memory responses generated by these vaccines may have been impeded (17, 19). This suggests a decreased anti-tumour response and memory response generated by the VLP-surv and VLP-surv.MUC1 vaccines in the hyperuricemic mice which could result in decreased initial and recurrent tumour killing initiated by the vaccine. However, this will have to be confirmed in a future tumour trial, where mice are rechallenged with tumour cells following initial tumour rejection.

In comparison, the hyperuricemic and wild type mice produced relatively similar antibody titres in response to the VLP-gp100 vaccine. This suggests that B cell responses were not altered in response to this vaccine, indicating hyperuricemia did not impair B cell activation or B cell-induced T\textsubscript{H} cell activation in response to this vaccine in these mice.

Again, due to a lack of mice this experiment could not be conducted in obese mice, thus clearly this would need to be conducted in these animals in the future. This would be interesting to investigate in obese mice as studies have found that antibody titres generated in response to vaccines is significantly decreased in obese compared to normal-weight humans (85).
4.5 \textbf{PHENOTYPIC CHARACTERISTICS OF BONE MARROW IMMUNE CELL POPULATIONS IN OBESE, WILD TYPE AND HYPERURICEMIC MICE}

Phenotypic analysis of BM cells from each of the three mouse models revealed key differences, which suggest that obese BM cells contain a higher frequency of immunosuppressive cells.

Firstly, obese mice have a higher proportion of CD11b\textsuperscript{+} cells compared to wild type mice. CD11b\textsuperscript{+} expression is commonly used as a marker to identify cells of the myeloid lineage, which give rise to monocytes, macrophages, neutrophils and other blood cells (86, 87). In healthy individuals, myeloid cells mature in the bone marrow, however, various cytokines released during inflammatory conditions, for example infections and cancer, can block the differentiation of immature myeloid precursors into effector cells (DCs, macrophages, neutrophils etc.) (86, 87). Immature myeloid precursors released from the BM are immune-suppressive and their increased frequency in these mice implies a more immunosuppressive environment, which may impact the efficacy of our VLP-peptide vaccine (86, 87).

MDSC expansion is associated with chronic inflammation and results in a decrease in differentiated DCs and macrophages (88). MDSCs have also been implicated in suppressing T cell responses and inducing CTL tolerance to cancer cells via the upregulation of immune suppressive factors arginase, NO, iNOS and ROS (88). In addition, premature monocytic cells have been found to be increased in obese mouse models as a result of heightened inflammation and increased secretion of MCP-1 expression (89). In this study, cells expressing CD11b\textsuperscript{+} Ly6C\textsuperscript{+}, Ly6G\textsuperscript{-} were present in higher frequencies in the BM of obese mice. CD11b\textsuperscript{+} Ly6C\textsuperscript{+}, Ly6G\textsuperscript{-} cells are often regarded as monocytic MDSCs (mMDSCs) that deprive the tumour environment of L-arginine and L-cysteine thereby starving T cells (27). Higher frequencies of BM cells expressing CD11b\textsuperscript{+} Ly6C\textsuperscript{+}, Ly6G\textsuperscript{+} cells were observed in the BM of obese mice.
compared to wild type mice. Cells expressing this phenotype are usually characterised as granulocytic MDSCs (gMDSCs), which suppress T-cell responses via increased ROS production (27). The observed increase in the proportion of MDSCs indicates a more immunosuppressive environment in the obese mice compared to the wild type mice. Notably, studies have revealed that these cells contribute to the failure of immunotherapies, indicating an increase of these cells in the obese mice could impact the ability of our VLP-peptide vaccine to generate an anti-tumour response (90, 91).

However, use of these markers to identify MDSCs is controversial, with debate over whether they accurately detect suppressive cells or conventional monocytes and neutrophils (92). Therefore, to accurately conclude if these cells are suppressive, a T cell suppression assay must be conducted.

Interestingly, none of the cells involved in the conventional adaptive immune cell response were altered in the BM cells from obese or hyperuricemic mice compared to the wild type cells. Cells expressing B220, a marker used to characterise B cells, cells expressing CD3, a marker used to indicate T cells, and CD11c+ cells, a marker used to characterise DCs, were not significantly different between the BM cells from the three mouse models (75, 93, 94). This may suggest that the changes that can occur to these cells during chronic inflammation induced by metabolic disorders may not be the result of changes to their frequencies in the BM. Instead, it is possible that alterations, to DCs, T cells and B cells, occurs outside of the bone marrow as a result of interaction with various immune cells and immunosuppressive factors in the environment.
4.6 Phenotypic Characteristics of BMDCs from Obese, Wild Type and Hyperuricemic Mice

To investigate if observed changes in immature myeloid cell populations would impact the generation of fully functional DCs, the phenotype of BMDCs was investigated.

Phenotypic analysis of the unstimulated BMDCs generated from the BM from the each of the three mouse models revealed an increased proportion of DCs not expressing MHC II (MHC II\textsuperscript{-}) in the obese mice compared to the wild type mice. However, no differences were observed in the proportion of DCs expressing intermediate (MHC II\textsuperscript{mid}) or high levels of MHC II (MHC II\textsuperscript{high}).

Some studies have found that BMDC populations from GM-CSF cultures are heterogeneous and are comprised of not only dendritic cells but macrophages as well (95). MHC II has been used to identify the different cell types making up these heterogeneous populations with cells expressing MHC II\textsuperscript{mid} characterised as macrophage-like cells and cells expressing MHC II\textsuperscript{high} characterised as DCs (95). In the paper of Helft et al, macrophage-like cells were found to produce more pro-inflammatory cytokines following stimulation however they were less able to present antigen and less motile than DCs (95). In comparison, the DCs produced less pro-inflammatory cytokines by were more able to present antigens (95). It remains to be determined if CD11c\textsuperscript{+} MHC II\textsuperscript{-} cells, found to be increased in BMDC cultures from obese mice, are DCs or other cell types as CD11c is also expressed by pDCs, activated monocytes, macrophages, and some NK cells (96).
4.7 ASSESSMENT OF ACTIVATION MARKER EXPRESSION ON BMDCs FROM OBESE, WILD TYPE AND HYPERURICEMIC MICE

DCs are crucial in forming a sustained and robust anti-tumour immune response in response to VLP vaccines, as they are cells which take up the VLP, present TAAs to T cells and stimulate the activation and proliferation of these tumour specific T cells. Therefore, the ability of DCs to present antigens and effectively stimulate T cell activation and proliferation is vital to the success of VLP vaccines against cancer. To investigate these factors, BMDCs from obese, hyperuricemic and wild type mice were analysed and flow cytometric analysis revealed some key differences in their expression of various activation markers.

Phenotypic analysis revealed that MHC II expression is decreased in obese BMDCs compared to wild type BMDCs, following stimulation with the immune adjuvant CpG, the adjuvant administered with our VLP vaccines. MHC upregulation is an important response following antigen detection and it is vital to ensure increased antigen presentation following immune activation. Previous studies have found that MHC expression in DCs from obese mice does not differ from that of wild type mice, however these were following no stimulation or LPS stimulation, another immune adjuvant (64, 65). Decreased MHC II expression may lead to impaired antigen presentation, decreasing induction of T cell activation.

CD40, a co-stimulatory molecule expressed on APCs, was found to have decreased expression in BMDCs from obese mice compared to BMDCs from wild type mice following CpG stimulation. CD40 functions by binding to CD40L on licensed T cells, resulting in cytokine production by DCs, induction of co-stimulatory molecule cell surface expression and it promotes antigen cross-presentation (97). This DC ‘maturation’ is required for effective T cell
differentiation and activation (97). Decreased expression of CD40 may result in impaired DC maturation leading to a downstream impact of T cell responses in these mice.

Comparatively, expression of the co-stimulatory molecule CD80 was not altered between the obese or hyperuricemic BMDCs compared to the wild type BMDCs. CD80 is expressed by activated APCs, including DCs, and binds to the CD28 protein, expressed on the cell surface of T cells, during DC-T cell interactions (98). Engaging of CD28 results in activation of signalling cascades leading to the amplification of TCR mediated proliferation of T cells (98). CD28 signalling is crucial for TCR signal amplification to effectively prime naïve T cells (98). A lack of CD28 stimulation reduces T cell activation and proliferation and compromises T cell responses (98). Therefore, as CD80 expression was not altered in the inflamed mice, T cell proliferation is unlikely to be impeded in this way. Activated APCs also express another co-stimulatory molecule, CD86, which can also bind to CD28 on T cells and induce T cell proliferation (98). CD86 expression was decreased in unstimulated and CpG stimulated BMDCs from obese mice compared to wild type mice BMDCs. This suggests that the ability of the BMDCs from the obese mice to stimulate CD28 signalling in T cells is impaired compared to that of the BMDCs from the wild type mice.

Studies in CD80 and CD86 knockout mice propose that during T cell activation CD86 is the initial ligand for CD28, due to its rapid and high expression on APCs (99). Whereas, in terms of activation CD80 appears to be the more potent ligand for CD28 (99). Thus, decreased CD86 but not CD80 may imply that DCs from the obese mice may have impaired initial T cell co-stimulation, however potent co-stimulation may not be impacted.
CpG stimulation successfully induced upregulation of expression of the three co-stimulatory molecules measured in the obese, hyperuricemic and wild type mice. This confirms CpG’s adjuvanticity, indicating its use as an adjuvant with our VLP-peptide vaccine is appropriate and is capable of eliciting responses in under different conditions in different mice models.

To further confirm differences in these activation markers between the obese, wild type and hyperuricemic mice BMDCs the MFI values were also compared. These MFI results confirmed the differences observed in CD40, CD80 and CD86 activation markers between the DCs from the different mice determined by the percentage of live cells. However, MHC II expression was different when comparing the MFI to the percentage of MHC II⁺-gated cells. MHC II expression was upregulated by BMDCs from all mice following CpG stimulation and no differences were observed in the expression of MHC II between the BMDCs of the different mice.

Unfortunately, due to complications surrounding expired media supplements, only two biological repeats of these experiments have been performed so far. Therefore, to confirm these differences at least one further repeat must be conducted and will be carried out post-thesis submission.

### 4.8 Ability of BMDCs from Obese, Wild Type and Hyperuricemic Mice to Stimulate T Cell Proliferation

As detailed above, in order to induce a robust anti-tumour immune response from our VLP-peptide vaccines, DCs must be able to successfully present antigens to and activate T cells. Although the expression of various activation markers was already discerned, BMDCs from each mice model were co-cultured with T cells specific to the gp100\(_{25,33}\) epitope to investigate their ability to directly induce T cell proliferation.
The BMDCs from all mice types were able to induce proliferation following stimulation with VLP-gp100 compared to when no antigen was present. This indicates that the BMDCs from each mouse type exhibited the ability to induce proliferation in the presence of a foreign antigen, indicating proliferative potential was not impeded by either obesity or hyperuricemia. Interestingly, there was a trend towards increased T proliferation in the T cells co-cultured with BMDCs from the obese mice following VLP-gp100 stimulation compared to the BMDCs from the wild type mice, indicating they may be more capable of activating T cells. This was unexpected as the phenotypic analysis revealed that co-stimulatory molecule expression was decreased in the BMDCs from the obese mice compared to those from the wild type mice, indicating their T cell activation may be impaired. This also contradicts previous research which has shown that the ability and potency of DCs to stimulate T cell activation and proliferation is decreased in obese mice, moreover, these DCs have also been found to inhibit T cell expansion (64, 65). However, these results are preliminary and statistical analysis could not be performed yet as they were generated from results from a single experiment due to complications surrounding our previous BMDC experiments. To draw meaningful conclusions from this experiment and identify true differences in the ability of the BMDCs from the different mice models to induce proliferation, at least two more repeats must be conducted, and will be carried out following thesis submission.

It is possible that no differences were observed in the proliferation of T cells cultured with BMDCs from the obese mice because it was an isolated experiment without other immune cells present. The increased proportion of immature myeloid cells and MDSCs in the BM of the obese mice likely contribute to the impaired APC and effector cell function seen in obese animals. However, as this experiment lacks these cells it is possible that no changes in T cell proliferation is the result of suppressive cells not being present to interact with and impact T
cell activation and proliferation. Thus, further *in vivo* studies must also be conducted to ascertain the true impact chronic inflammation has on T cell proliferation.

No differences were observed in the proliferation of the T cells co-cultured with gp100\textsubscript{25-33} peptide stimulated BMDCs from any of the three mice groups. This was expected as peptides do not always need to be taken up and processed by the BMDCs and instead ‘stick’ to their MHC molecules and therefore these were used as a positive control.

### 4.9 Limitations and Future Directions

Although *in vitro* phenotypic analysis provides useful insight into cell populations and their functionality it is only an indication and may not accurately reflect the phenotype and function of these immune cells *in vivo*. Primarily, in a model of chronic inflammation where suppressive cells interact with effector immune cells to impede their function, an isolated *in vitro* experiment may not accurately represent how these cells are impacted in such an environment. Therefore, to accurately determine the ability of these immune cells to carry out anti-tumour responses, more *in vivo* experiments would need to be conducted. The ideal experiment to investigate the impact of chronic low-grade inflammation on VLP-peptide vaccines in these mouse models would be a tumour trial. This experiment would involve challenging naïve obese and hyperuricemic mice with B16, MC-38 and C57.mgMUC1 tumour cell lines at cell numbers determined from our tumour growth kinetics experiments. Once the tumour is palpable the mice will be vaccinated with the VLP-peptide vaccine which contains the peptide associated with their specific cancer cell. Following this, if the mice have been tumour free for 80 days they will be re-challenged with the same tumour cells. Tumour growth, survival of the mice and recurrent tumour rejection would enable the evaluation of the impact chronic low-grade inflammation has on the ability of the vaccines to generate a specific, effective and lasting anti-
tumour response directly. This will provide a greater insight into whether these treatments would be effective under such conditions.

Beyond this, many different avenues could be embarked on to investigate the impact of chronic low-grade inflammation on cancer vaccines further. For example, different mice models could be used, such as diet-induced obesity (DIO) mice, which represent a more realistic, clinically relevant model of human obesity with slower development and increased production of leptin compared to other models, as well as systemic inflammation as present in obese humans (64). Another interesting model would be geriatric mice, as one of the main characteristics of aging is chronic low-grade inflammation (100). Age is a risk factor for developing cancer and with our population aging, a large proportion of elderly individuals are likely to receive cancer immunotherapies in the future (100). Previous studies have revealed that elderly people display poor responsiveness to various vaccines, such as the influenza vaccine, thus suggesting that age may be an important condition to test our VLP-peptide vaccine under (101).

Alongside investigating the impact of administering these cancer vaccines in combination with checkpoint inhibitors, the impact of attempting to alleviate chronic inflammation with anti-inflammatory drugs prior to vaccine administration could also be investigated. Previous research has found that anti-inflammatory drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) reduces colon cancer risk and that other anti-inflammatory drugs enhance responses to chemotherapy and radiotherapy (102). Studies have also shown increased efficacy of cancer vaccines against melanoma in mice when administered alongside a NSAID, via the reduction of immune suppression caused by suppressive cell populations potentiating the anti-tumour response induced by the vaccine (103).
4.10 Conclusion

Cancer is a complex disease that is challenging to treat effectively primarily due to its ability to adapt to its surrounding environment and therapy. Although immunotherapies are an attractive and promising treatment option, the majority of therapies have not succeeded beyond pre-clinical and clinical trials. The reasons surrounding why certain patients might respond to immunotherapy while others do not is not well understood and could be due to a variety of different factors. However, it has been postulated that this failure may be the result of unrepresentative pre-clinical mouse models, where co-morbidities present in normal populations, such as metabolic disorders, are absent. With conditions such as these on the rise, research must move in a way that accounts for this, to ensure findings are applicable to the general population. The preliminary findings from this study demonstrate that there appears to be a difference in the immune cells of the obese mice, proposing a more immunosuppressive environment in these animals. It has also revealed that the immune environment in the hyperuricemic mice may impair vaccine activation of some immune cells, specifically B cells and alter breast cancer tumour growth. These differences have the potential to affect the efficacy of our VLP-peptide vaccine and thus further investigation into the impact of chronic low-grade inflammation on such immunotherapies must be conducted.
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APPENDIX
APPENDIX

BLOCKING BUFFER
- 1x PBS
- 1% BSA (Gibco)
- Store at -20°C between uses

CARBONATE BUFFER
- 0.1M NaHCO₃ in dH₂O
- pH 8.2

COMPLETE ISCOVE’S MODIFIED DULBECCO’S MEDIUM (CIMDM)
- 500mL Iscove’s Modified Dulbecco’s Medium (IMDM)
- 5mL Penicillin-streptomycin (100µg/mL penicillin and 100µg/mL streptomycin, Gibco)
- 0.5mL 2-Mercaptoethanol

DULBECCO’S PHOSPHATE-BUFFERED SALINE (DPBS)
- 10g Dulbecco’s PBS powder (Gibco)
- 1L Milli-Q deionised water
- Sterile filtered

ELISA WASH BUFFER
- 1x PBS
- 0.5% Tween-20

FLUORESCENCE ACTIVATED CELL SORTING (FACS) BUFFER
- 1g Bovine serum albumin (BSA, 0.1%)
- 0.1g NaN₃ (0.01%)
- 1L 1x PBS
- Sterile filtered

MAGNETIC-ACTIVATED CELL SORTING (MACS) BUFFER
- 1x DPBS
- 0.5% bovine serum albumin (BSA)
- 2 mM EDTA
- Filter sterilize
- Store at 4°C
**4x PFA Fixation Buffer**
- 8g paraformaldehyde (PFA, 8%)
- 100mL FACS buffer

**10x Phosphate-Buffered Saline (PBS)**
- 160g NaCl
- 22.7g Na₂HPO₄
- 4g KH₂PO₄
- 4g KCl
- 2L Milli-Q deionised water
- Sterile filtered

**Red Blood Cell Lysis Buffer**
- 4.15g NH₄Cl
- 0.5g KHCO₃
- 0.02g EDTA
- 500mL Milli-Q deionised water
- Sterile filtered
- Store at 4°C

**Trypan Blue Solution**
- 0.25g Trypan blue powder (0.25%, Sigma-Aldrich)
- 100mL 1x PBS solution
- Sterile filtered