Determining the best combination of TLR Agonist and Tumour Peptide for Cancer Vaccination

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A thesis submitted for the degree of Bachelor of Biomedical Science Honours at the University of Otago Dunedin, New Zealand October 2017
Abstract

Immunotherapy has revolutionised the treatment of cancer in recent years; significantly improving patient response and long-term survival. Though many immunotherapies focus on increasing the effector function of immune cells, the ability to generate and stimulate new tumour-specific immune cells has become an important topic for patients who do not respond to first line therapy. Previous work in our laboratory identified that intracellularly reversible conjugation of mode antigen ‘Ovalbumin’ (OVA) to CpG B adjuvant improves the tumour specific response both in terms of immune cell activation, proliferation, and cytokine release, leading to complete tumour clearance in mice. This year I aimed to repeat this using the clinically significant melanoma antigen ‘gp100’ instead of OVA and to compare the use of CpG B adjuvant to CpG C adjuvant, which stimulates additional cytokine release, in the conjugate vaccine model. Using a combination of reversed phase high performance liquid chromatography (RP-HPLC) and cell culture, conjugates were produced and tested on their ability to activate Dendritic cells, induce their production of pro-inflammatory cytokines and induce T cell response in co-culture. Purification of gp100 conjugates was unsuccessful via RP-HPLC and testing reverted to the OVA model when comparing CpG conjugates. In antigen presenting cells, both conjugates induced similar levels of activation and antigen presentation but had unique cytokine profiles, with both conjugates trending towards higher levels of IL-1β and IL-12p70. Both CpG B-OVA and CpG C-OVA conjugates also induced a strong tumour-specific response with increased CD4+ and CD8+ T cell proliferation and significantly increased CD8+ T cell IFN-γ secretion. With these results in mind, both conjugates appear as strong candidates for therapeutic vaccination trials as either a monotherapy or a combined therapy with Checkpoint Blockade or Adoptive T
Cell Therapy. *In vivo* testing using the CpG C construct is needed to assess its efficacy over CpG B.

**Acknowledgements**

I would first like to give a special thanks to Associate Professor Sarah Young for all of her help and support with my project this year. Even though not everything went to plan, I thank you for your enthusiasm of my results and for teaching me the importance of time management.

Secondly, I would like to thank Dr Katrin Kramer for all her day to day help. Honestly, you are one of the most patient people I’ve ever known and how on earth you put up with me for this long, I will never know. Thank you for all your advice, light-heartedness, and expert trouble-shooting, without you I don’t know how I would have survived this year.

Thirdly, I would like to thank Katie Young for all her assistance throughout the year. You have immensely helpful in my understanding of Flow cytometry and have taught me the importance of order things way in advance.

I would also like to thank Dr Greg Walker and Dr Pummy Krittaphol from the School of Pharmacy for all your help and advice in terms of HPLC methodology and chemistry concepts. As someone who started the year knowing nothing about HPLC, you guys have taught me a lot and I truly appreciate it.

I would also like to give a wider thanks to everyone in the Young Laboratory including Nick Shields, Braeden Donaldson, Estelle Peyroux, Silke Neumann and Yasmin Sadrolodabai for your support and for keeping me relatively sane and on task each day.

Finally, I would like to thank my parents, Delphi and Steve, for your endless love and support and your sound advice during difficult times.
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Chapter 1

Introduction
1.1 Chapter 1 – Introduction

1.1.1 Cancer overview

Cancer is one of the leading causes of death in the developed world. With over 14.1 million new cases in 2012 alone, the search for new and effective treatment options remains a challenge for oncologists and researchers alike (1). Cancer is a genetic and often age-related disease in which the accumulation of mutations within a cell enable it to grow and behave abnormally in its residing tissue (2-4). As cells increase in number, they form a benign mass or ‘tumour’ that is encapsulated in a fibrous membrane. Continual growth and mutation can eventually lead to new cell characteristics that enable tumours to invade the surrounding tissue causing different pathologies. These invasive/malignant tumours are commonly known as ‘cancer’. Designing new treatments for malignant tumours is an ongoing challenge as they are present in multiple tissues, originating from healthy cells. Similarities to healthy cell markers and receptors limit treatment as ‘selective toxicity’ cannot easily be obtained (5). Treatments such as chemotherapy can produce side effects associated with healthy, as well as malignant, cell death (6). These side effects negatively affect patient quality of life and treatment outcomes. Until 2011, few treatments were available for cancers such as melanoma and those available offered little survival benefit. Since the introduction of targeted molecular therapies to treat cancer, there have been significant increases in patient survival and remission rates (7-9). Immunotherapies such as Ipilimumab and Pembrolizumab, (immune checkpoint inhibitors) have also revolutionised metastatic melanoma treatment. Immune checkpoint inhibitor approval has also renewed interest in other immune based therapies including therapeutic vaccines. This article will review the current and future advancements in this field for the treatment of melanoma, compared to conventional
treatments and other immunotherapies. I will also describe the immune response to cancer and the aim of my research; to determine the best combination of gp100 peptide and CpG adjuvant to develop an effective cancer vaccine.

1.1.2 Melanoma

Melanoma is described as the benign or malignant growth of melanocytes that reside in the skin. Whilst not as common as other skin cancers, melanoma has the highest mortality rate at 65%, killing around 50,000 people worldwide each year (10). Identification of melanoma occurs through microscopic analysis of patient biopsies. This is followed by a more thorough diagnosis including B- rapidly accelerated fibrosarcoma (BRAF) mutation status, tumour staging and tumour grading to determine the therapeutic regime (11). Tumours are staged according to their thickness in millimetres, degree of spread to local lymph nodes and metastatic spread to other tissues. Grading of tumours depends on their appearance and behaviour compared to normal cells in the tissue, with grade-I tumours being slow growing and similar to healthy cells and grade-IV tumours being poorly differentiated and displaying rapid growth. Most benign melanomas are low-stage and grade and thus can be removed surgically with few complications. Despite the 99% cure rate of benign tumours in stage I-III via surgical resection, invasive stage III and IV can often be fatal, with a 5-year survival rate of less than 10% (10, 12-14). At this point, surgical removal is redundant as the tumour is present in multiple tissue layers and no longer has a well-defined border from which the surgery can be performed (15).
1.1.3 Conventional treatments for Metastatic Melanoma

Conventional treatments for malignant melanomas include radiation therapy, chemotherapy, and immunotherapy. These will be discussed further below.

1.1.3.1 Radiation therapy

Radiation therapy has had limited success in the treatment of metastatic melanoma, yet remains a treatment option; given its ability to reach tumours inaccessible by surgery. Used in 1-6% of melanoma cases, it is often used as a palliative measure, offering little survival benefit in practical terms. Despite this, radiation therapy has been shown to have an abscopal effect on distant tumours, suggested to be immune mediated (16, 17). For this reason, combination with immunotherapies has been considered. A study by Safwat et al showed a response rate of 71% in combination with IL-2 (18, 19). Ipilimumab combination has also been trialled and more than tripled the median survival time; compared to patients treated with radiosurgery alone (20).

1.1.3.2 Chemotherapy

For over 40 years, chemotherapy has been the standard treatment for metastatic melanoma. Until 2011, the alkylating agent, Dacarbazine was the main treatment for most cases; producing response rates of 10-20% and poor overall survival (21-24). Since 2011, the approval of targeted molecular therapies, such as BRAF inhibitors, Vemurafenib and Dabrafenib, has successfully increased patient response and survival (7, 14). BRAF inhibitors act by targeting a mutated protein involved in the signalling
pathway that mediates tumour growth. BRAF mutations are present in over 50% of melanoma cases and so are an ideal molecular target for therapy (3, 7, 14, 25). Though BRAF inhibitors can improve progression-free survival, (PFS), in the short term, resistance mechanisms, which develop within tumours, limit their long-term efficacy. The use of mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitors, such as Trametinib in addition to BRAF inhibition, have overcome this problem (3, 26). These inhibit resistance mechanisms developing downstream in the BRAF signalling pathway (25). Combined BRAF and MEK inhibitor therapies have successfully increased PFS (8, 27). Although chemotherapy has had poor success in the past, newer targeted therapies, have greatly increased patient survival; particularly when used in combination.

1.1.3.3 Immunotherapy

Immunotherapies for cancer have focused on enhancing the number and activity of effector T cells. This is achieved through: the administration of cytokines to the tumour site, which directly act on T cells, blockade of known T cell inhibitors, as well as production of vaccines that aim to enhance antigen presenting cell (APC) activation and their presentation of tumour peptides. One first-line treatment for metastatic melanoma involves the administration of intra-lesional IL-2 into the tumour site (28). Although this cytokine induces both CD8+ and CD4+ T cell proliferation, response rates for IL-2 have been low, with some suggesting that IL-2 administration may enhance suppressive T regulatory cell activity; which dampens the effector response (29). Severe side effects such as vascular leakage also limit its use. Despite this, IL-2 administration has shown some promise when used in combination with other immunotherapies such as IFN-α (30,
The most successful immunotherapy to date is Immune Checkpoint Blockade. This involves the use of monoclonal antibodies that target suppressive mechanisms that limit effector T-cell responses. Ipilimumab, an anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) antibody and Pembrolizumab, an anti-programmed cell death protein 1, (PD1) are examples of these. In combination, these therapies have produced response rates as high as 60% and increased patient survival (9, 32).

1.2 Immune response to cancer

1.2.1 Tumour associated antigens

The key step in generating anti-tumour immune responses is the recognition of tumour-associated antigens (TAAs). These are tumour-expressed compounds that potentially activate the immune system. Immunotherapy design for cancer has traditionally focused on identifying TAAs and facilitating their uptake by APCs. APCs play an important role in immunity by processing TAAs and presenting them to TAA-specific effector T cells (33). These then migrate from the lymph node to wider tissue cells; terminating cells expressing these TAAs. Melanoma is extensively modelled in immune-based cancer research due to the identification of several TAAs and their unique immunogenicity, compared to other cancers. As such, many studies have shown some melanoma patients have pre-existing TAA specific T cells and antibodies prior to treatment (34, 35).
1.2.2 Tumour antigen uptake and presentation

Cancers originate from healthy cells that accumulate mutations and behave abnormally in their tissue of origin. The first step in generating a tumour specific response is detection of these abnormal cells by the immune system. The release of stress signals and the expression of mutated self-proteins by tumours enables this detection. APCs within the tissue, such as macrophages and dendritic cells (DCs), are the first to encounter these proteins through phagocytosis, receptor-mediated endocytosis and macropinocytosis of material from their external environment (36-38). Peptides are deemed immunogenic if they, or compounds associated with them, stimulate pattern recognition receptors (PRRs) which activate a cascade of intracellular signals. The best described PRRs are the Toll-like receptors, (TLRs), which are expressed on the cell surface and within endocytic vesicles (39). Products such as Heat Shock Proteins (HSP) produced by necrotically dying cells, under-methylated bacterial DNA (CpG) and Lipopolysaccharide, all activate TLRs (40). Signalling of TLRs leads to APC activation including production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, upregulation of cell surface proteins such as major histocompatibility complex (MHC), and expression of costimulatory molecules CD80, CD86 and CD40 (39, 41-43). After intracellular uptake by APCs, TAAs are broken down into peptides in the endo-lysosomal compartments by a combination of low pH and degradative enzymes such as cathepsins (36, 38, 44, 45). These TAAs are then loaded onto MHC-II molecules and presented on the cell surface (46, 47). TAAs within the cytoplasm can be presented onto MHC-I molecules after ubiquitination. This process tags them for degradation by the proteasome, a multicatalytic complex that degrades intracellular proteins (48). Exogenous TAAs may also be fed into this MHC-I pathway, through a process called cross-presentation which is crucial in activating a CD8+ response. This occurs through the vacuolar and cytosolic
pathways and enables APCs to present antigen on both MHC-I and II, to effector T cells. Peptides in the vacuolar pathway are produced in lysosomes, mediated by the enzymatic activity of cathepsins. They are then loaded onto MHC-I that enters during receptor recycling and endosomal MHC-I transport to the cell membrane (49, 50). The cytosolic pathway involves transport of peptides into the cytosol, mediated by the proteins SEC61 and p97. These peptides are then degraded by the proteasome and loaded on MHC-I within the endoplasmic reticulum (ER) (51). Alternatively, this may involve the delivery of ER proteins including; transporter associated with antigen processing (TAP) to the phagosome by protein Sec22B. This then facilitates proteasome-processed peptide transport back into the phagosomes, before loading onto MHC-I (52, 53).

1.2.3 T cell activation and anti-tumour activity

To activate a T cell, APCs must produce three unique signals. Together these drive T cell proliferation and polarisation to certain phenotypes, each with unique activity (54). After TAA acquisition, DCs with peptide-loaded MHC migrate to the draining lymph node where they present antigen on MHC-II to CD4+ T cells and MHC-I to CD8+ T cells. This antigen-specific process produces the first signal for T cell activation; full activation is not achieved until co-stimulatory molecules also bind (55). T cell ligands such as cell surface protein CD28 bind to CD80 and CD86 on APCs producing a co-stimulatory signal leading to T cell clonal expansion (signal 2). As these cells differentiate, they form a small memory T cell population that protects against secondary tumour challenge and an effector cell population. Pro-inflammatory signals from the APC also act on the T cell influencing its polarisation to phenotypes such as Th1, Th2 and Th17 (signal 3). In cancer, the cytokines IL-12 and IL-18 released from APCs, act together to cause a Th1 based phenotype in CD4+ cells (56-59). Th1 cells are crucial for tumour clearance due
to their ability to produce IFN-γ and tumour necrosis factor (TNF)-α which enhance CD8+ T cell cytotoxicity (60). In addition to CD4+ T cells, APCs may activate CD8+ T cells through peptide presentation on MHC-I molecules, in conjunction with co-stimulatory proteins and cytokines similar to CD4+ activation (54, 61). CD8+ T cell activation leads to differentiation into effector cells that use both indirect and direct methods to kill tumours. Indirect killing involves the release of TNF-α and IFN-γ at the tumour site which can induce cellular apoptosis (62, 63). There are two methods of direct killing; firstly, recognition of the foreign peptide on tumour MHC-I can induce secretion of perforin and granzyme molecules that create pores in the cell membrane and activate intracellular caspases, that initiate apoptosis. Secondly, effector CD8+ cells may induce cell death through expression of their CD95L protein, which binds to the CD95 receptors on tumour cells also initiating apoptosis (64, 65). Following activation, both effector T cells express suppressive receptors such as CTLA 4, which competes with CD28 for costimulatory molecule binding, and PD-1 that can induce apoptosis when it binds to PD-L1. Expression of these molecules increases overtime in tumour sites with chronic inflammation and their inhibition has had significant promise in treating cancer (66, 67).
Figure 1: The three steps in generating an anti-tumour response 1) Antigen presenting cells take up tumour antigens, activate and migrate to the lymph node. 2) APCs process these and present them on MHC-I to CD8+ T cells and on MHC-II to CD4+ T cells, activating them in addition to co-stimulatory signals 3) Activated T cells clonally expand and migrate to the tumour site eliciting anti-tumour activity via cytokine release and induction of tumour cell apoptosis.
1.3 Other Effector Immune cells

1.3.1 B cells

B-lymphocytes (B cells) also play a role in anti-tumour immunity. As adaptive immune cells, B cell responses are important in tumour clearance through their ability to act as an APC, and their production of TAA specific polyclonal antibodies (68). TAA-specific polyclonal antibodies play a key role in anti-tumour immunity through two mechanisms. Firstly, these antibodies, specifically Immunoglobulin M (IgM), are potent stimulators of the classical pathway of the complement cascade. This cascade consists of a series of innate catalytic proteins that can act as pro-inflammatory mediators or directly lyse cells through formation of a membrane attack complex on their surface (69, 70). IFN-γ produced by T cells also stimulates B cells, inducing isotype switching to production of IgG2 titres. As well as complement activation, polyclonal antibodies also induce Natural killer cell (NK) mediated cell death. NK cells are lymphoid cells that play a key role in the clearance of tumours that do not express MHC. This occurs through a process called antibody dependant cell mediated cytotoxicity (ADCC), in which antibodies bound on a cell’s surface induce secretion of perforin and granzyme by NK cells leading to cell lysis (71). This plays a pivotal role in the efficacy of several monoclonal antibody based treatments and in the cytotoxic effects of some chemotherapies (72, 73).

1.3.2 Natural Killer cells

NK cells play a crucial role in tumour surveillance and clearance, without the need for APC stimulation, and patrol the tissue and blood searching for cellular markers of stress and infection. This is indicated by a downregulation of MHC-I on the cell surface, a
characteristic feature of virally infected cells and an important resistance mechanism used by tumour cells to avoid CD8+ killing (74). NK cells that recognise stressed/malignant cells activate and induce apoptosis using perforin and granzyme or CD95L like CD8+ cells (75). Activation is also triggered by pro-inflammatory cytokine release such as IL-12, IL-18, IL-15 and IFN-β from APCs and epithelial cells (58, 72, 76, 77). These cells may also release chemokines, which signal and recruit NK cells to the tumour site. Tumour cell killing by NK cells is also triggered by the presence of antibodies bound to the tumour via ADCC (see above) (72). Some tumour cells may express ligands on their surface such as ULBP1 and MICA/B which are recognised by NK receptors such as NKG2D leading to tumour cell killing.

1.4 Cancer vaccines

Cancer vaccines have shown significant promise in the treatment of melanoma; both in combination and as a monotherapy. They are formulations of tumour peptide that are delivered to APCs to increase their activation of T cells and resulting number of tumour infiltrating lymphocytes (TILs), a strong determinant of patient prognosis (78, 79). Used in conjunction with other therapies including intra-lesional IL-2 and checkpoint inhibitors, vaccines have been shown to augment effective long-term memory responses whilst improving patient outcome. In the case of melanoma, several vaccines have been trialled each using different tumour antigens. One phase 1 study by Slingluff et al, which used the melanoma glycoprotein ‘gp-100’(280-288) in Montanide, an oil in water emulsion, showed an overall survival rate of 75% at 5 years (80). The use of differentiation antigens like gp100 show promise for vaccine treatment given their expression on 90% of melanomas (81). Studies using melanoma antigen recognised by
T cells 1 (MART-1) and gp100 in oil emulsion showed similar response rates (82). Vaccines use in the treatment of malignancy continues to be investigated, with the aim of improving efficacy; these include addition of TLR agonists, bio-conjugation strategies and nanoparticle delivery, discussed below.

1.4.1 TLR agonists

TLR agonists have long been proposed as a mechanism to improve vaccine efficacy. Activation of TLRs enhances T-cell responses by inducing upregulation of co-stimulatory molecules on APCs and release of pro-inflammatory cytokines (83, 84). TLR agonists such as Imiquimod and CpG oligodeoxynucleotides (ODN) have been involved in several cancer-based therapies, often as an addition to tumour-associated peptides (85, 86). Imiquimod in particular, has been used extensively in murine trials. As a TLR7/8 agonist, Imiquimod stimulates production of type 1 interferons (IFN-α/β) by DCs, leading to Th1 polarisation and a robust CD8+ response. When administered both topically and/or intradermally along-side peptide vaccines, Imiquimod led to significant increases in TILs and mononuclear cells at the tumour site; compared to controls (85). CpG ODNs have also shown promise mediating tumour-specific responses with CpG Class B, characterized by its stimulation of B cells, NK cells and CD8+ T cells; currently being used in several treatments (86). As a method to enhance therapeutic potential, CpG has also been co-delivered to DCs; alongside tumour antigens. This strategy has shown to increase both tumour specific CD8+ and NK cell numbers more than independent delivery (87, 88). Recent studies propose the use of Class C CpG adjuvants; given their ability to stimulate production of both B cell-acting cytokines such as IL-6 as well as type 1 interferons similar to Imiquimod. The administration of CpG Class C in conjunction with radiation produced significant increases in DC number and activation,
in a study by Cerkovnik et al (89). Another study by Yang L et al showed similar results with effective tumour rejection when injected to the draining lymph nodes of mice with breast cancer. This effect was greater than Class B CpG ODNs (90). In sum, the use of TLRs effectively boosts anti-tumour responses in a variety of cancer models. In designing vaccines, one should consider co-delivery with TAAs, as pre-clinical studies have shown this can further enhance the immune response.

1.4.2 Co-delivery mechanisms

Although TLR agonists have produced promising results when administered singularly and as a mixture with TAAs, more effective co-delivery mechanisms have been proposed. These, including bio-conjugates and nanoparticles facilitate delivery of both TAA and adjuvant to the same DC; inducing full activation, enhanced cross-presentation, and a greater T cell response, discussed below.

1.4.3 Bio-conjugation

Bio-conjugation strategies have extensively been used to co-deliver TAAs and adjuvants to the same APC. These often involve direct and stable conjugation to proteins or antibodies; this has had mixed results in murine models.

The majority of bio-conjugations involve reacting primary amine groups on proteins/peptides with N-Hydroxysuccinimide (NHS) ester groups on other molecules, which form a stable amide bond, resistant to hydrolysis (91, 92). The popularity of amine-based conjugation stems from its location within proteins, on both the N-terminus and lysine residues, which are accessible due the positive charge of the amino group at neutral
pH. As a result, conjugation can be performed whilst still maintaining protein structure and biological activity.

Thiol chemistry has also been utilised for bio-conjugation, though it is not as popular as amine modification. The limited availability of cysteine groups in biological molecules of interest does however enable more selective conjugation (93). Despite this, thiol groups, particularly disulphide bonds, are key components of many proteins and modification can disrupt normal biological function. This limits their application, particularly in conjugations with proteins such as antibodies, as it may alter their binding specificity and solubility. Functional groups including amines can be modified into thiols increasing their number for conjugation and avoiding functional disruption.

Aldehyde functional groups can also be used in bio-conjugation. Their absence from most biological compounds however, limits their utilisation in direct conjugation strategies. Despite this, aldehyde groups are often used in linker molecules introduced to biological molecules via modified amine groups etc. An example of this is succinimidyl 4-formylbenzoate, which contains both an NHS ester and an aromatic aldehyde. This NHS ester conjugates onto biological molecules making the aromatic aldehyde available for additional linkages. Aldehyde groups can be reacted with free amino groups forming unstable Schiff’s bases requiring additional reductions to stabilise the linkage (94). More commonly though, aldehydes are reacted with hydrazine molecules to form stable hydrazone bonds. Innovative linker technologies at Solulink have further improved these conjugates by producing linkers with aromatic aldehydes and hydrazines, which when combined, form a highly stable bis-arylhydrazone bond. These conjugated products can also be quantified due to the colorimetric nature of this bond (95).
Recently, the utilisation of reversible conjugates has been investigated in vaccine formulation. These conjugates are designed to cleave intracellularly due to differences in reductive environment or pH \((96, 97)\). Reversible conjugates offer several advantages over stable formulations and non-conjugated antigen-adjuvant mixtures. Firstly, their sensitivity to intracellular conditions often provides them with stability extracellularly, where conditions do not favour cleavage. Additionally, reversibility within the cell enables each constituent to dissociate and activate their respective receptors; with less hindrance than stable conjugates. This is particularly evident when each receptor lies in different cellular compartments. Immunologically, reversible conjugates may also facilitate cross-presentation of antigen to MHC-I. Studies by Nembrini et al., Hirosue et al. and Flannery et al., demonstrated that OVA reversibly conjugated to nanoparticles and polymeric carriers respectively, caused significant increases in MHC-I presentation and CD8+ T cell responses; compared to stable conjugates \((96, 98, 99)\). Research in our laboratory using soluble Glutathione (GSH) sensitive OVA-CpG conjugates also showed an increased CD8+ response and enhanced anti-tumour immunity in mice challenged with OVA-expressing tumour cells \((100)\)(see figure 2).
Figure 2: Effect of reversible GSH sensitive conjugate on APC. After cellular uptake, the higher concentration of glutathione intracellularly leads to reduction and cleavage of the linker. As the linker is cleaved the tumour antigen is released, processed and presented onto MHC. Simultaneously, the TLR agonist activates TLRs in the endosome leading to increased expression of co-stimulatory molecules (CD86, CD40) and pro-inflammatory cytokines.

1.4.4 Nanoparticles

Though bio-conjugates remain a viable strategy for co-delivery of TAA and adjuvant into the same cell, they rely heavily on the stability of each component and its ability to resist degradation in the extracellular environment. Nanoparticles (NPs) are one solution to this challenge and are employed to protect sensitive products targeted for intracellular uptake. This APC uptake is also significantly greater than soluble vaccines (101).

Many compounds are conjugated directly onto nanoparticles for this very reason as well as the variety of substances NPs can be made from. NPs describe a broad category of
substances smaller than 1000nm including virus-like particles (VLPs) and polymeric NPs; VLPs being the most well-known \(102\). As self-assembled virus shells, VLPs are popular due to their ease of production and the variety of options for presenting TAAs of interest. Antigens can be conjugated onto the VLP, packaged inside the VLP or the antigen sequence can be added to the VLP sequence and be expressed alongside their capsid proteins. As well as this, the highly repetitive structure of VLPs has shown to induce humoral immune responses \(103\). VLPs have been extensively used in several vaccine trials in combination with TAAs, generating strong CD8+ responses and Th1-associated IgG2 production \(104\).

Polymeric NPs are another delivery mechanism commonly used, with a reputation for superior biodegradability and biocompatibility. These structures are made from a variety of compounds including poly (g-glutamic acid) and poly (D, L-lactic-coglycolic acid). Polymeric NPs have been promising, with studies showing the induction of a strong humoral and cell mediated immune responses when antigen is conjugated onto them \(96, 105\). A study by Silva et al showed that encapsulation of TAAs in combination with TLR agonists also induces a strong anti-tumour response, with delivery of CpG and gp100 \(209-217\) enhancing in vivo immunity to melanoma \(106\). Current polymeric NP vaccines aim at further improving delivery through targeted localisation to the draining lymph nodes, a key site for APC-T cell interactions and thus induction of an adaptive anti-tumour response. A promising new nanoparticle is the ISCOMATRIX adjuvant composed of a mixture of phospholipid, \textit{Quillaia saponaria} extract and cholesterol that form a cage-like structure. Pre-clinical tests using OVA and TLR agonists with ISCOMATRIX have produced strong CD8+ T cell responses with associated IFN-\(\gamma\) release \(107\).
1.5 Current research and aim

This introduction has aimed to give insight into the current treatments for melanoma and the potential of therapeutic vaccination as an effective immune therapy. With current challenges such as improving immunogenicity and delivery of soluble cancer vaccines acknowledged, this study aims to test new soluble vaccine strategies using clinically relevant TAAs. Though current soluble vaccines have significantly less uptake than other strategies, such as nanoparticles, delivery mechanisms can be improved. For example the use of CpG conjugates enhances cellular uptake 50-fold compared to mixtures, due to its interaction with the DEC205 receptor on DCs (100, 108).

Research in our laboratory has previously demonstrated that intracellular cleavable conjugates of OVA and Class B CpG ODN 1668 elicit strong anti-tumour responses in mice (100). I aim to assess the efficacy of this strategy when using the TAA ‘gp100’ as opposed to OVA. I also aim to determine the optimal amino acid length of gp100 for use in this conjugate by comparing two peptides, gp100 (25-33) which requires no additional processing and gp100 (19-39) which does. Class B CpG ODN 1668 will also be brought under question as the optimal CpG class for this strategy, and will be compared to Class C CpG ODN 2395, which induces production of IFN-α in addition to the cytokines induced by 1668.

To understand the mechanism of these conjugates’ anti-tumour effect, I will measure the expression of MHC-II, CD40 and CD86 and cytokine release (IL-12, IL-1β, IL-6, TNF-α, IFN-α) after pulsing DCs with either stable or reversible conjugates. This will give an indication of the level of activation within these cells and therefore their propensity to activate T cells. Pulsed DCs will then be co-cultured with T cells for 72 hrs and T cell proliferation and IFN-γ secretion will be measured giving a complete view of the Immunostimulatory effect of each conjugate. As well as this, I will measure the cytotoxic
function of CD8+ T cells after co-culture using an *in vivo* cytotoxicity assay. I hypothesize that reversible conjugation of Class C CpG ODN 2395 to gp100 (25-33) will elicit the strongest immune response characterized by greater BMDC activation and cytokine release and greater CD4+ and CD8+ T cell proliferation and IFN-γ release.

To conclude, research into therapeutic cancer vaccination remains ongoing. Although currently not a first line treatment for cancer, its potential in combination with other therapies may ensure it becomes a viable option in the future.
Chapter 2

Materials and Methods
2.1 Production of CpG-Gp100 conjugates

2.1.1 Acquisition of CpG oligonucleotides and Gp100 peptides

CpG oligonucleotides were purchased and custom synthesised by GeneWorks Pty Ltd, Hindmarsh, AUS (CpG 1668) and Integrated DNA Technologies Inc., Iowa, USA (CpG 2395). Phosphorothioated (PT) CpG 1668 (5’ TCCATGACGTTTCTGATGCT 3’) was modified with a 3’ amino (NH₂) group whilst PT CpG 2395 (5’ TCGTCGTTTTCGGCGCGCGC 3’) was modified with a 5’ amino group. Both oligonucleotides were bought in lyophilised form and stored at 4 °C. At time of use, CpG ODNs were suspended in Modification buffer (0.1 M Na₂HPO₄, 0.15 M NaCl pH 8.0) and OD₂₆₀/µl measured using the Nanodrop 1000 (NanoDrop, Version 3.7.1, Thermo Fisher Scientific, Waltham, MA, USA). Approximately 30-50 nmol of each CpG was used per conjugation for modification steps. Gp100 peptides 25-33 (KVPRNQDWL) and 19-39 (CAVGALKVPRNQDWLGVPRQL) were purchased in lyophilized form from Mimotopes Pty Ltd, Victoria, AUS. Each was resuspended in modification buffer and separated into 30 µl aliquots at approx 3.07 mg/ml and 6.89 mg/ml respectively. Aliquots were stored at -20 °C until use.

2.1.2 Solulink two-linker strategy

Conjugation of gp100 to CpG involved a two-linker strategy designed by Solulink. Firstly, aromatic aldehyde linkers succinimidyl-4 formylbenzoate (S-4FB, Solulink Inc., San Diego, CA, USA) or succinimidyl-SS-4 formylbenzoate (SS-4FB, Solulink) are mixed with CpGs containing either a 5’ or 3’ amino group. NHS ester groups within each
linker, react with the NH$_2$ group to form a stable amide bond. After reaction, and removal of excess linker, the rate of modification is measured through addition of 2-Hydrazinopyridine solution (2-HP, Solulink). This reacts with the aromatic aldehyde and enables colorimetric analysis of each linker based on its absorbance at 360 nm. This measurement is then combined with the OD260/µl of the modified CpG in the equation below, to give the molar substitution ratio (MSR) of the reaction. MSRs of 0.8-1.2 indicate a successful reaction. The modified CpG was then reacted with succinimidyl 6-hydrazinonicotinate acetone hydrazone (S-HyNic, Solulink). This linker contains an aromatic hydrazine which reacts with the aromatic aldehyde of S/SS-4FB to form a stable bis-aryl hydrazone bond. This can then be quantified based in its absorbance at 345 nm. Finally, gp100 is added to the construct. Primary amine groups on the N terminus of gp100 and on lysine residues react with the NHS ester group present on the S-HyNic of the modified CpG. This then forms another amide bond and completes the conjugation. A detailed method of each steps is provided below.

\[
MSR = \frac{A_{360} \times \varepsilon(CpG) \times \text{total volume in assay}}{24500 \times \left(\frac{OD_{260}}{\mu l}\right) \times \text{CpG in assay} \times 1000}
\]
Figure 3: Schematic of CpG reversible conjugation to gp100 peptide. Firstly, CpG is reacted with SS-4FB linker, followed by addition of S-HyNic and finally addition of gp100.
2.1.3 Modification of CpG Oligodeoxynucleotides

Concentrations of CpG were measured by their UV absorbance at 260 nm using Nanodrop 1000 and each suspended into 1 x Modification buffer. CpG was modified by adding 4 molar equivalents of 20 mg/ml S-4FB (Solulink) or SS-4FB (Solulink) linker dissolved in dimethyl sulphoxide (DMSO, Thermo Fisher Scientific) at volumes supplied by the peptide/oligo calculator on the Solulink website. At least 30 nmol of CpG was used for each modification to ensure a sufficient product yield for further conjugation steps. This also ensured sufficient linker modification. CpGs were then left to react at room temperature for 2 hrs in Eppendorf tubes. The resulting products were then exchanged into conjugation buffer (0.1 M Na₂HPO₄, 0.15 M NaCl pH 6.0) and centrifuged (Heraeus™ Fresco™ 17 Microcentrifuge, Thermo Fisher Scientific) five times at 13000 x g 4 °C for 15 mins using 3 KDa molecular weight cut off (MWCO) spin filters (Amicon Ultra - 0.5 ml Centrifugal Filters, Regenerated Cellulose 3000 NMWL, Millipore, Cork, IRL) to remove excess linker. The samples were then reverse spun at 1500 x g for 2 mins 4 °C by inverting the filter and placing into a new Eppendorf tube.

The Molar Substitution Ratios (MSR) were then measured by adding 2 μl of each modified CpG to separate tubes containing 18 μl of 0.5 mM 2-HP solution (Solulink) in 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer. This was incubated for 30 minutes at 37 °C followed by A360 nm measurement on the Nanodrop 1000. 2-HP allows colorimetric analysis of the aromatic aldehyde present on the S-4FB and after removal of excess linker via centrifugation, gives an indication of the level of CpG modification. MSR between 0.8 - 1.2 CpG to S-4FB and SS-4FB indicated that the modifications were sufficient.
2.1.4 S-HyNic Modification

SS-4FB and S-4FB-modified CpG oligonucleotides were treated with a 40-fold molar excess of S-HyNic (Solulink) dissolved in DMSO at a concentration of 20 mg/ml. Volume added was determined by the peptide/oligo calculator on the Solulink website. Solution was briefly vortexed and then left to react for 2hrs at room temperature. Following reaction, the sample was transferred to Amicon 500 spin filters, exchanged in conjugation buffer, and centrifuged five times at 13000 x g for 15 mins at 4 °C to remove any unconjugated S-HyNic. Gp100 peptides were then added to individual modified CpG tubes at a four-fold molar excess along with turbolink buffer (0.1 M Na$_2$HPO$_4$, 0.15 M NaCl, 0.1 M aniline pH 6.0) at a volume determine on the Solulink calculator (~5-10 μl) to catalyse the reaction. This was then left to react for 2 hrs. The final product was then spun again five times at 13000 x g for 15 mins at 4 °C in conjugation buffer to remove any unreacted gp100 peptide.

2.1.5 Analysis of gp100 peptide conjugates

Gp100 peptide conjugates were analysed using Reverse Phase High Performance Liquid Chromatography (RP-HPLC) on an Agilent Technologies 1290 Infinity Chromatograph (Agilent Technologies, Santa Clara, CA, USA). Firstly, 10 μl of each sample was added to individual HPLC vials with 20 μl of Phosphate Buffered Saline (PBS) (Appendix 1). Individual Gp100 peptides were tested at concentrations of 50, 100, 250 and 500 μg/ml and CpG oligonucleotides at 0.5 μM, 2 μM, 4 μM, 8 μM, 10 μM and 100 μM. Conjugates were tested at concentrations similar to the gp100 added for the final conjugation step.
This ranged from 0.3 mg/ml to 1.3 mg/ml. Before samples were run, the HPLC was equilibrated overnight using 65% acetonitrile (ACN). The column, (Thermo Scientific™ Hypersil™ BDS C18 100 x 4.6mm, 3 μm), was then loaded onto the machine and UV-lamp set to measure 210 nm, 214nm, 220 nm and 280 nm. The column was then equilibrated in 50% ACN + 50% MilliQ water for 2 hrs before sample loading. Samples were run on the HPLC for 45 mins at a flow rate of 0.5 ml/min, with an injection volume of 10 μl and a column temperature of 25 °C. Lines were periodically washed every 15 mins with seal wash (10% isopropyl alcohol). Mobile phase A was 0.1% Trifluoroacetic acid (TFA) in MilliQ and mobile phase B was 0.1% TFA in 100% ACN (see Mobile phase elution gradient table 1). 3D spectral analysis was then performed on each sample using Agilent ChemStation (Agilent Technologies) software.
Table 1: Initial RP-HPLC Elution gradient

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Mobile phase A</th>
<th>% Mobile phase B</th>
<th>Flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
<td>0.5 ml/min</td>
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<tr>
<td>20</td>
<td>56</td>
<td>44</td>
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<tr>
<td>45</td>
<td>90</td>
<td>10</td>
<td>0.5 ml/min</td>
</tr>
</tbody>
</table>

Following initial testing, the run time was adjusted to 35 minutes (see table 2 for elution gradient).

Table 2: Modified RP-HPLC Elution gradient

<table>
<thead>
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<th>Time (min)</th>
<th>% Mobile phase A</th>
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<th>Flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
<td>0.5 ml/min</td>
</tr>
<tr>
<td>20</td>
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<td>0.5 ml/min</td>
</tr>
<tr>
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<td>50</td>
<td>50</td>
<td>0.5 ml/min</td>
</tr>
<tr>
<td>35</td>
<td>90</td>
<td>10</td>
<td>0.5 ml/min</td>
</tr>
</tbody>
</table>
2.1.6 Purification of OVA

Lyophilised Chicken Egg Ovalbumin (OVA) (purity ≥98 %, Sigma-Aldrich) was first weighed and dissolved in modification buffer (Appendix 1) via both vortexing and sonication. OVA, at approximately 15 mg/ml, was then purified using size exclusion chromatography (SEC) via Shimadzu High Performance Liquid Chromatography System (HPLC, Shimadzu, Columbia, MD, USA) using a Superdex™ 200 10/300 GL SEC column (GE Healthcare Bio-Sciences, Uppsala, SE). Modification buffer (Appendix 1) was used as the mobile phase after 2 hrs equilibration in the column and the flow rate was set to 0.5 ml/min. Each sample was loaded onto the column at an injection volume of 500 μl at room temperature. Purified OVA was then identified via its absorbance at 280 nm on the UV-Vis detector and collected at an elution time of 35 minutes. OVA was then concentrated using Vivaspin® 6 spin filtration (3 kDa MWCO, GE Healthcare UK Ltd., Little Chalfont Buckinghamshire, UK) in a fixed angle swing bucket centrifuge (5415R centrifuge, Eppendorf AG) at 3500 x g for 30 mins to a volume of 300 μl. A280 was then measured in triplicate and mean averaged using Nanodrop 1000 at a molar absorption coefficient of 7.2 to give a concentration in mg/ml of the purified product.

2.1.7 OVA Modification

Modification of OVA was performed similar to CpG modification, with the exception that S-HyNic was used as the linker molecule at 30 molar equivalents to OVA. The MSR was measured by the addition of 18 μl 0.5 mM 2- sulpho benzaldehyde solution (2-SBA,
Solulink) in 0.1 M MES buffer to 2 μl of the S-HyNic modified OVA. Colourimetric analysis of the aromatic hydrazine on the S-HyNic molecule was then performed via A360 nm measurement on the Nanodrop 1000.

2.1.8 OVA-CpG Conjugation

Modified CpG was reacted at a 4 molar excess with modified OVA. SS-4FB modified CpG and S-HyNic modified OVA were mixed at concentrations supplied by Solulink, to ensure sufficient product was formed, and Turbolink buffer (Appendix 1) was added to catalyse the reaction. This was left to react for 2 hrs at room temperature. Conjugates were then purified from any unreacted products via SEC via the Superdex™ 200 10/300 GL SEC column (GE Healthcare) at an elution time of ~ 27 minutes using PBS as the mobile phase. This was then concentrated using Vivaspin® 6 similar to OVA purification (see above).

2.1.9 Quantification of conjugates

Conjugate concentration was measured using a combination of A345 nm measurement and Quant-iT assay. Following reaction and concentration via Vivaspin® 6, A345 nm was measured for each conjugate using the Nanodrop 1000. This measures the presence of hydrazone bonds between SS-4FB-modified CpG and S-HyNic-modified OVA, and indicates if the reaction was successful. OVA concentration within the conjugate samples was measured using Quant-iT Protein Assay Kit (Thermo Fisher Scientific) via manufacturer's instruction. Briefly, Quant-iT reagent with diluted 1/200 in Quant-iT
buffer and 200 μl was added to 18 wells of a black 96 well microplate. Standard concentrations of Bovine serum albumin (BSA) (0, 0.25, 0.50, 100, 200, 300, 400 and 500 ng/μl) (10 μl) and each conjugate (5 μl) were then added to separate wells in duplicate and mixed by pipetting up and down. The plate was then run on a POLARStar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at a wavelength of 490/545 nm. Results were plotted on excel to form a standard curve and from this, the concentration of OVA in each conjugate was estimated in mg/ml. Measurements from both A345 nm and Quant-iT were then combined to determine the conjugation ratio of CpG to OVA using the formula below.

\[ \text{Molar ratio} = \frac{\Delta A_{345 \text{ nm}}}{29000 \times 1} \times \frac{\text{MW conjugate}}{\text{mg/ml conjugate}} \]

### 2.1.10 Analytical Size Exclusion Chromatography of CpG-OVA conjugates

Prior to sample loading, an Agilent Technologies 1290 Infinity Chromatograph was briefly purged using 10% Methanol solution to remove any residual salts. Lines were then washed for 2 hrs in MilliQ water. A Yarra 2000 SEC column (3 μm, 145 Å pore size, 300 x 4.6 mm; Phenomenex Inc. Torrance, CA, USA) was then loaded onto the HPLC and equilibrated for 2 hrs in MilliQ, followed by 2 hrs equilibration in PBS. Approximately 10 μl of each CpG-OVA conjugate diluted 1 in 6 in PBS was injected into the column and run for 15 mins at a flow rate of 0.35 ml/min at 25 °C. UV detection was set to 260 nm and 280 nm. 3D spectral analysis was then performed using Agilent ChemStation (Agilent Technologies) software.
2.2 Immunological tests

2.2.1 Animal acquisition

Six to eight-week old male C57BL/6, OT-I and OT-II mice were sourced from the Hercus Taieri Research Unit (HTRU), University of Otago. All mice were specific pathogen free (SPF) and ethical approval was obtained for all mouse experiments by the University of Otago Animal Ethics Committee.

2.2.2 Isolation of Bone marrow derived dendritic cells (BMDC)

Femurs and tibiae of 6-8-week-old male C57BL/6 mice were extracted, stripped of surrounding tissue, and placed into a petri dish containing Dulbecco’s PBS (DPBS, Gibco by Life Technologies Corp, Grand Island, NY, USA) before transfer into a biological safety cabinet. Bones were then immersed in 70% ethanol on ice for 2 mins before being washed twice in DPBS in a six well plate. After washing, the epiphyses of each bone were cut using sterile scissors and shafts were flushed with DPBS + 5% Foetal Bovine Serum (FBS, Moregate Biotech, Bulimba, Australia) into a separate well. The bone marrow was collected and transferred to a 50 ml Falcon tube and spun at 250 x g for 7 mins at 4 °C. Supernatant was then poured off and red blood cells were lysed with 3 ml ammonium chloride red blood cell (RBC) lysis buffer for 2 mins after resuspension. Cells were then washed in 30 ml DPBS + FBS to neutralise the lysis buffer and filtered through a 70 μm cell strainer (Corning Inc., Tewksbury, MA) into a new falcon tube. The cells were once again centrifuged at 250 x g for 7 mins at 20 °C. Supernatant was then poured off and cells were resuspended in 2 ml warm complete
Iscove's Modified Dulbecco's Medium (cIMDM) (Gibco) + 5% FBS + 20 ng/ml
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF; ProSpec, East Brunswick, NJ, USA) solution. Cells were then manually counted using a hemocytometer by adding 10 µl of cells to 90 µl of trypan blue and visualizing under a microscope at the 20x objective lens. Cells were then resuspended at 0.5 x 10^6 cells/ml in 24 ml and transferred to a six-well plate, 4 ml per well. This was incubated at 37 °C 5% CO2. On day 3 cells were fed by replacing 2 ml of media with 2.5 ml of warm fresh media (cIMDM + 5% FBS + GM-CSF).

### 2.2.3 Activation of bone marrow derived dendritic cells

On day 6 of culture, BMDCs were collected by gently pipetting the media up and down, so as not to dislodge any adherent macrophages, and transferred to a 50 ml falcon tube. They were then centrifuged at 250 x g for 7 mins at 20 °C, supernatant discarded, and resuspended in 2 ml of cIMDM + 5% FBS + GM-CSF before counting via trypan blue method. Cells were then plated onto a U-bottom 96-well plate, at a concentration of 1 x 10^6 cells, 200 µl per well for BMDCs or 1 x 10^5 cells, 100 µl per well for T cell co-cultures, in triplicate per treatment. Cells were then pulsed with either OVA (3.5 µg/ml), CpG B (0.425 µM), CpG C (0.53 µM), OVA + CpG B, OVA + CpG C, CpG B conjugate (3.5 µg/ml), CpG C conjugate (3.5 µg/ml) or Lipopolysaccharide (LPS) (1 µg/ml). Cells were incubated for either 24 hrs (BMDC activation) or 72 hrs (T cell assays) at 37 °C 5% CO2.
2.2.4 Bone Marrow Dendritic Cell Flow Cytometry preparation

After 24 hrs incubation, cells were spun down at 350 x g for 7 mins at 4 °C and 100 μl of supernatant from each well was transferred to a new 96-well plate and frozen at -20 °C for use in cytokine assays. Remaining cells were resuspended by pipetting up and down and transferred into separate Fluorescent Assisted Cell Sorting (FACS) tubes. They were then topped up with 1 ml of PBS before being spun at 350 x g for 7 mins at 20 °C. After spinning, supernatant was removed using a glass pipette via suction and cells were resuspended in remaining PBS and stained with 50 μl of fixable live/dead NIR dye on ice, for 15 mins in the dark. Following staining, the tubes were topped up with 1 ml of FACS buffer and spun at 350 x g for 7 mins at 20 °C. Supernatant was sucked off and cells were stained with 50 μl anti-CD16/32 Fc Block for 5 mins on ice followed by staining with 50 μl of CD86-PE, CD40-PE-Cy7, CD11c APC (as a dendritic cell marker) and MHC-II FITC master mix on ice for 15 mins in the dark. Dilutions of these antibodies is supplied in table 3. Following staining, cells were topped up with 1 ml of FACS buffer and spun at 350 x g for 7 mins at 20 °C. After removal of supernatant, 200 μl of FACS buffer was added to each tube and tubes vortexed to resuspend the cells. The cells were run on the Gallios flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analysed using Kaluza version 1.6 software (Beckman Coulter).
2.2.5 T cell isolation and co-culture

Spleens isolated from OT-I, OT-II male mice were prepared into a single cell suspension by gently pushing them through a 70 μm cell strainer using the plunger of a sterile 5 ml syringe. The strainer was then rinsed using DPBS + 5% FBS to collect all cells in a 50 ml Falcon tube before centrifugation at 300 x g for 7 mins at 4 °C. Supernatants were then poured off and cells were treated with 7 ml of ammonium chloride RBC lysis buffer before neutralization in 30 ml DPBS + 5% FBS. Cells were centrifuged (300 x g for 7 mins at 4 °C), resuspended in 10 ml of MACs buffer (DPBS + 0.5% BSA + 25μM EDTA), and counted using trypan blue method. After cell count cells were centrifuged again at 300 x g for 7 mins at 4 °C before resuspension in 90 μl of MACS buffer per 10^7 cells. Cells were then treated with 10 μl CD8α+, CD4+ micro beads (Miltenyi Biotech, Bergisch Gladbach, Germany) per 10^7 cells and incubated for 15 mins in an ice block. Every 5 mins, cells were inverted to ensure good mixing and interaction with the microbeads. After 15 mins, CD4+/CD8+ bead treated cells were washed in 15 ml MACS buffer and spun at 300 x g for 7 mins at 4 °C. Supernatant was then poured off and cells resuspended in 1 ml MACS buffer per 10^8 cells. Cells were then separated using the AutoMACS pro (Miltenyi Biotech) on the Possel-s setting with MACS running buffer (see appendix 1). Following cell sorting, the positive fraction was collected and counted using trypan blue. Cells were topped up with warm cIMDM + 5% FBS and centrifuged at 300 x g for 7 mins at 20 °C. Cells were then resuspended at 1 x 10^6 cells/ml and 3.6 ml of this was then added at 100 μl/well (single co-culture) or 50 μl/well (double co-culture) to BMDCs for use in cytokine assays Remaining cells were then spun at 300 x g for 7 mins at 4 °C and resuspended in cIMDM + 5% FBS. They were treated with 20 μM carboxyfluorescein succinimidyl ester (CFSE) (CellTrace™ CFSE Cell Proliferation Kit,
Life Technologies Corp) solution and left to react in the dark for 8 mins at room temperature. FBS was then added at equal amount to the cell suspension to quench the reaction and cells were washed three times in DPBS + 1% FBS spun at 300 x g for 7 mins at 20 °C. Cells were counted using trypan blue method and resuspended at 1 x 10^6 cells/ml in cIMDM + 5% FBS. From this suspension, 100 μl was added to each well of a BMDC culture. This was then incubated for 72 hrs at 37 °C 5% CO2.

2.2.6 T cell Flow cytometry preparation

After 72 hrs incubation, non-CFSE stained co-cultures were spun down at 350 x g for 7 mins at 4 °C and 100 μl/ well of supernatant was transferred to a new 96-well plate and frozen at -20 °C for future use in cytokine assays. CFSE cells were suspended in their supernatant by pipetting up and down and then transferred into separate FACS tubes. They were then topped up with 1 ml of PBS before being spun at 350 x g for 7 mins at 4 °C. After spinning, supernatant was poured off and cells resuspended in remaining PBS before staining with 50 μl of fixable live/dead zombie yellow stain on ice, for 15 mins in the dark. Cells were then topped up with 1 ml of PBS and spun again at 350 x g for 7 mins at 4 °C. Supernatant was then poured off, and cells were then stained with 50 μl anti-CD16/32 Fc Block for 5 mins on ice followed by staining with 50 μl of CD3-PE Dazzle, CD8 APC and CD4 APC/CY7 also on ice for 15 mins in the dark. Dilutions of these antibodies is supplied in table 3. Stained cells were topped up with 1 ml of FACS buffer and spun at 350 x g for 7 mins at 4 °C, after removal of supernatant. FACS buffer was added to each tube (200 μl) and each was vortexed to resuspend the cells. These were then analysed on the Gallios flow cytometer (see BMDC FACS prep).
**Table 3: FACS antibodies and dilutions**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Clone</th>
<th>Manufacturer</th>
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</thead>
<tbody>
<tr>
<td>Live/Dead NIR violet Dye</td>
<td>1:1000</td>
<td>-</td>
<td>Life technologies</td>
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<td>Live/Dead Zombie Yellow Dye</td>
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<td>CD8α APC</td>
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<td>53-6.7</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD11c APC</td>
<td>1:800</td>
<td>N418</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD40 PE/Cy7</td>
<td>1:200</td>
<td>3/23</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD86 PE</td>
<td>1:400</td>
<td>GL-1</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD335 PE/Cy7</td>
<td>1:100</td>
<td>29A1.4</td>
<td>Biolegend</td>
</tr>
<tr>
<td>MHC-II FITC</td>
<td>1:800</td>
<td>M5/114.15.2</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Fc block – anti CD16/32</td>
<td>1: 250</td>
<td>93</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>
2.2.7 Interferon gamma (IFN-γ) Enzyme Linked Immunosorbent Assay (ELISA)

Wells of a 96-well ELISA plate were first coated with 50 μl of purified anti-mouse IFN-γ primary antibody at a 1/250 dilution in coating buffer (0.1M NaHCO₃ pH9.5) and incubated for 1 hr at 37°C. The buffer was then briefly flicked out and the plate washed by immersing in wash buffer (appendix 1) to fill the wells and then vigorously flicking out the contents into a sink. This was repeated 6 times before blotting on a paper towel to remove residual buffer. Plates were then treated with blocking buffer (PBS + 1% BSA) at 200 μl per well and incubated for 2 hrs at 37°C. Solution was then flicked off and the plate washed 6 times in wash buffer. Samples were then added to wells at 50 μl/well in triplicate. IFN-Y was added in duplicate to separate wells at 15 ng/ml followed by a two-fold dilution series at concentrations of 10 ng/ml - 0 ng/ml. The plate was then incubated overnight at 4°C. The next day the plate was washed 6 times and 50 μl/well of biotinylated anti-IFN-γ antibody was added to each well at a 1/250 dilution in blocking buffer before incubation for 30 mins at 37°C. Following incubation, the plate was washed 6 times and 100 μl/well of streptavidin-horse radish peroxidase (HRP) was added to each well at a 1/3000 dilution in blocking buffer. The plate was incubated at 37°C for a further 20 mins, washed 6 times and 100 μl/well of TMB substrate was added. The plate was then covered in aluminium foil to protect from light and left to react for 8-10 mins with colour development checked regularly. Once a clear colour change was visible in the standard wells, the reaction was stopped by adding 100 μl/well of 1N H₂SO₄ solution. The plate was then read at 450 nm on a microplate reader (Perkin Elmer) and standard curve extrapolated using Excel spreadsheet to determine the concentration of IFN-γ.
2.2.8 Interferon alpha (IFN-α) ELISA

Pre-coated IFN-α primary antibody ELISA 96-well plate supplied by Invitrogen was first washed twice by immersing each well in wash buffer for 15 seconds before flicking out the contents into a sink. The plate was then blotted dry using a paper towel and 50 μl of IFN-α Assay buffer was added to each well. Standards at concentrations of 2000, 1000, 500, 250, 125, 62.5 and 31.3 pg/ml externally diluted in calibrator diluent were then added to 14 wells, 50 μl each. Calibrator diluent (50 μl) was also added to 2 wells to act as a blank. Diluted biotin-conjugate was then added at 50 μl to all well including the blanks and the plate was covered in adhesive film and left to incubate for 2 hrs at room temperature on a microplate shaker set at 400 rpm. Film was then removed, and plate washed 4 times as previously described. Streptavidin-HRP was then added at 100 μl per well to each of the wells before the plate was covered in film and placed on a microplate shaker for 1 hr. Film was then removed, plate washed 4 times and 100 μl of TMB substrate solution was added to each well to start the colour changing reaction. This was left to react for 15 mins under aluminium foil, to protect it from light, before being stopped via the addition of 100 μl of stop solution. The plate was then read at 450 nm using a microplate reader (Perkin Elmer) and concentration of IFN-α in each sample extrapolated from the standard curve produced.

2.2.9 LegendPlex cytokine assays

LegendPlex bead samples (85 μl) (TNF-α, IL-1β, IL-12p70 and IL-6) (Biolegend) were added to 1860 μl of Assay buffer (Biolegend) to form a master mix of beads, each at a 1 in 2 dilution. Assay buffer was then individually added at 25 μl per well to 73 wells of a
96-well V-bottom plate. LegendPlex standard was then added at 25 μl per well to 14 wells to form the standard concentrations after a four-fold series dilution. Samples (25 μl) were then added to the remaining wells. Finally, 25 μl of Legendplex bead master mix was then added to all wells and the plate was covered in tin foil and placed on a shaker for 2 hrs at room temperature at 800 rpm. Following incubation, the plate was centrifuged at 250 x g for 5 mins. The supernatant was then removed by flicking the plate over a sink in one fluid motion. The plate was then blotted dry and washed by adding 200 μl of wash buffer to each well and placing on the shaker for 1-2 mins at 800 rpm. The plate was then centrifuged and washed once more before addition of 25 μl of detection antibodies at a ½ dilution. The plate was sealed, covered in aluminium foil and placed back on the shaker for 1 hr at room temperature, set to 800 rpm. After 1 hr, 25 μl of fluorescent antibody was added to each well and the plate was returned to the shaker, covered in aluminium foil for a further 1 hr. The plate was then removed and well contents transferred to individual FACS tubes for analysis on the flow cytometer.

2.2.10 Statistical analysis

All statistical analyses were performed using Graphpad prism software version 7.03 (GraphPad Software, La Jolla, CA, USA). A one-way analysis of variance (ANOVA) with Tukey post hoc test was used in each immunological assay to compare the means of each treatment group. Error bars represent the standard error of the mean (SEM) and a p value of <0.05 was deemed statistically significant.
Chapter 3

Results
3.1 Synthesis and characterization of conjugates

3.1.1 Generation of Gp100-CpG conjugates

CpG oligonucleotides were successfully modified with either S-4FB or SS-4FB, with each producing MSRs between 0.8-1.2 (table 4). After addition of S-HyNic and gp100, the A345 nm was then measured using Nanodrop 1000. A345 nm readings were not detectable for any conjugate. Therefore, RP-HPLC, initially a method to purify the conjugates, also became a method to determine the success of this reaction.

Table 4: Molar substitution rates of S-4FB/SS-4FB modified CpGs used for each conjugate

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>MSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG B-S-gp100 (25-33)</td>
<td>1.06</td>
</tr>
<tr>
<td>CpG B-SS-gp100 (25-33)</td>
<td>0.84</td>
</tr>
<tr>
<td>CpG B-S-gp100 (19-39)</td>
<td>0.81</td>
</tr>
<tr>
<td>CpG B-SS-gp100 (19-39)</td>
<td>0.93</td>
</tr>
<tr>
<td>CpG C-S-gp100 (25-33)</td>
<td>1.18</td>
</tr>
<tr>
<td>CpG C-SS-gp100 (25-33)</td>
<td>1.19</td>
</tr>
<tr>
<td>CpG C-S-gp100 (19-39)</td>
<td>1.04</td>
</tr>
<tr>
<td>CpG C-SS-gp100 (19-39)</td>
<td>0.95</td>
</tr>
</tbody>
</table>
3.1.2 RP-HPLC analysis of individual gp100 peptides and CpGs

Before RP-HPLC analysis of each conjugate, it was clear that the ability to distinguish Gp100 peptides and CpG B/C ODNs was necessary to accurately identify each conjugate. Thus initially, gp100 peptides and CpGs were run through the C18 column at increasing concentrations (see methods 2.1.5) for 45 minutes. Both gp100 peptides had UV absorbance (~2-10 mAU) detectable at both 210, 214, 220 and 280 nm. At the lowest concentration of 50 µg/ml, gp100 peptides eluted at 13.8 mins (25-33) and 16.8 mins (19-39) respectively (figure 4a). Prominent peaks which distinguished gp100 peptides from surrounding products within the 280 nm absorbance range was not observed until a concentration 100 µg/ml. Higher concentrations of gp100 presented with greater absorbance (~14 mAU) (See supplementary). CpG oligonucleotides were not identified until a concentration of 10 µM eluting at approximately 12.8 minutes (CpG B) and 7.58 (CpG C) based on consistent absorbance readings at 214, 220 and 280 nm (figure 4b).
Figure 4: RHPLC analysis of individual Gp100 and CpG oligonucleotides. (A) 50 µg/ml Gp100 peptides (25-33) (top) and (19-39) (bottom) were run through the HPLC at a flow rate of 0.5 ml/min for 25 mins. UV detection was set to 280 nm. Products eluted at approx. 13.8 mins and 16.8 mins respectively. (B) 10 µM CpG B (top) and CpG C (bottom) RP HPLC elution profiles. CpG B eluted at approx. 12.5 mins whilst CpG C eluted at 7.5 mins.
3.1.3 RP-HPLC analysis of Gp100 conjugates

Gp100 conjugates were each loaded onto the HPLC based off the concentration of gp100 used during the final reaction step. Each combination of CpG ODN, linker type and gp100 peptide produced an abundance of absorbance readings at elution times ranging from 5 to 20 minutes on the HPLC making gp100 conjugates unable to be identified accurately. Individual gp100 peptides were identifiable in most conjugate samples at an elution time of between 13.3-13.8 mins alongside other products with unique elution times but similar UV absorbance at 220 and 280 nm. The figure below shows the elution profiles of CpG B-SS-gp100 (25-33) (figure 5a) and CpG B-SS-gp100 (19-39) (figure 5b) at 260 and 280 nm.
3.1.4 Spin filtration effectively removes unconjugated products

To visualise the final reaction, linkage of gp100 to modified CpG, a time course was performed using RP-HPLC. Samples of ~10 µl were taken from modified CpG B upon introduction of Gp100 (25-33) at 1 min, 2 hrs and at 2 hrs after 5 rounds of spin filtration at 13,000 x g for 15 mins at 4°C. All samples were then analysed on the HPLC to identify any changes that indicate the reaction had occurred. Based on both absorbance and 3D spectral analysis, each sample had little change. Absorbance readings pre-and post-spin filtration showed a ten-fold reduction in absorbance of almost all products after spin filtration. Several products, which had eluted at 5.1 mins, 18.8 mins and 31.1 mins with shared absorbance at 220 and 280 nm, were also removed.

Figure 5: RP HPLC profiles of CpG B reversible gp100 conjugates: Each conjugate was loaded onto the HPLC at a flow rate of 0.5 ml/min for 35 mins. UV absorbance was measured at 210, 214, 220 and 280 nm. (A) Elution profiles of reversible CpG B linked to gp100 (19-39) at 220 nm and 280 nm. (B) Elution profiles of reversible CpG B linked to gp100 (25-33) at 220 nm and 280 nm.
Figure 6: RP HPLC elution profiles of CpG B-SS-gp100 (25-33) at 260 nm, flow rate 0.5 ml/min. (A) Elution profiles at 1 min after addition of gp100 (B) Elution profile 2 hrs after gp100 addition, before spin filtration. (C) Elution profile 2 hrs post gp100 addition, after spin filtration
3.1.5 Switch to OVA system

The inability to accurately identify gp100-CpG conjugates in each sample via RP-HPLC meant that purification and thus, use for immunological assays, was not possible. Although further methodologies could have been tested, due to time constraints, this study switched to using the OVA system that has previously been characterized in our laboratory for CpG B(100). Ovalbumin is a 44 kDa chicken egg protein that is often used in as a model antigen in immunological tests due its T cell-dependant immunogenicity in mice and well established transgenic mice strains. Additional tests would now aim to produce reversible OVA conjugates linked to either CpG B or CpG C and compare their Immunostimulatory ability on BMDCs and CD4+ and CD8+ T cells.
3.1.6 Purification of OVA

With gp100 conjugates unsuccessful, experiments switched to using model antigen ‘OVA’ to compare CpG B/C immunogenicity within reversible conjugates. Though stable in lyophilised form, dissolved OVA is prone to aggregation and formation of complexes of variable molecular weight (109). To ensure all OVA used in conjugation and immunological assays was of consistent size, SEC was performed to purify the protein. Using the Superdex™ 200 10/300 GL SEC column (Ge Healthcare) on a Shimadzu high performance liquid chromatograph, OVA monomer was successfully purified at an elution time of 27-32.5 mins based off its absorbance at 280 nm (Figure 7) with higher molecular weight aggregates eluting at 7.5-17.5 mins and 20-26 mins. Pure OVA was pooled into a collection tube at a volume of 5 ml.

Figure 7: Purification of Ovalbumin: Dissolved OVA was run through a Superdex™ 200 10/300 GL SEC column for 45 mins at UV detection of 280 nm in Modification buffer. Monomeric OVA eluted between 27-32.5 mins with aggregates eluting 20-26 mins. This representative chromatogram is the result of two independent experiments.
3.1.7 Production of reversible OVA-CpG conjugates

After purification, OVA monomer (2.75 mg/ml) was first modified with S-HyNic linker and its MSR measured via Nanodrop. At an MSR of 1.2, the reaction was deemed sufficient for further modification. CpG oligonucleotides were modified with SS-4FB, producing an MSR of 1.1 each. With both constituents at sufficient MSRs, modified CpGs were added to modified OVA in the presence of turbolink buffer and left to react overnight. The presence of aniline in the buffer acts as a catalyst for hydrazine bond formation. Given a molecular weight difference of over ~6 kDa between single OVA and each conjugate, the resulting product was then purified using SEC. Both conjugates eluted at 25-30 mins detected at 260 nm, with unreacted products eluting at 30-35 mins. Approximately 3 ml of each pure conjugate was collected in a 15 ml Falcon tube upon elution. CpG C-SS-OVA elution profile is provided in supplementary figure 3.

Figure 8: Purification of CpG B-SS-OVA conjugate: CpG B-SS-OVA was injected at a volume of 500 µl and run for 45 mins in PBS on a Superdex™ 200 10/300 GL SEC column. UV detection was set to 260 nm. The resulting product eluted at 25-30 mins with unreacted CpG B eluting at 32-37 minutes.
3.1.8 Analysis of reversible OVA-CpG conjugates

Though conjugates had been purified using preparative size exclusion chromatography, their characterization required additional testing using analytical SEC. This would allow analysis of each conjugates’ absorbance profile at both 260 nm, (peak absorbance of CpG) and 280 nm (peak absorbance of OVA). For this reason, each conjugate was diluted 1 in 6 in PBS and run individually through the Yarra 2000 SEC column. Figure 9 below shows the result of both conjugates. CpG B-SS-OVA eluted at 7-9 mins with absorbance at both 260 and 280 nm. CpG C-SS-OVA eluted between 6-8.5 mins indicative of its larger molecular weight difference ~0.6 kDa compared to the CpG B conjugate. CpG C-SS-OVA showed similar absorbance profile to CpG B-SS-OVA at 260 and 280 nm and on 3D spectral analysis. In both samples, residual modified CpG was seen, eluting at ~9.9 mins.

![Graph showing absorbance profiles of CpG B-SS-OVA and CpG C-SS-OVA](attachment:image.png)
Figure 9: Analytical SEC and 3D spectral analysis of CpG, OVA and CpG-OVA conjugates (A) SEC of CpG B (0.47 µM)(top) and OVA (1 mg/ml)(bottom) at 260 nm. (B) SEC of CpG B-SS-OVA at 260 and 280 nm. (C) SEC of CpG C-SS-OVA at 260 and 280 nm. (D) 3D spectral analysis of CpG B-SS-OVA (top) and CpG C-SS-OVA (bottom).
### 3.1.9 Characterisation of CpG-OVA conjugates

Upon purification, the conjugation ratio of CpG-OVA conjugates was determined using a combination of A345 nm measurement via Nanodrop 1000 and QuantiT assay. A345 nm measurement of each conjugate was used to visualise the presence of bis-aryl hydrazone as described for gp100 conjugation. QuantiT assay was then used to determine the concentration of OVA within each conjugate based on extrapolation from a standard curve of BSA protein standards. Both values were then combined to assess the number of CpG oligonucleotides per OVA protein. For CpG B-SS-OVA this value was 4.25, whilst CpG C-SS-OVA produced a ratio of 5.3. The concentration of individual CpGs for BMDC and T cell activation, proliferation and cytokine assays were matched to control for this difference.
3.2 Immunological assays

3.2.1 BMDC activation by CpG-OVA conjugates

BMDCs were pulsed with either OVA, individual CpG B/C, OVA + CpG B/C mixtures, CpG B/C conjugates or LPS for 24 hrs at 37 °C and then stained and analysed using flow cytometry. Live, CD11c+ cells were deemed BMDCs. BMDCs did not show any significant difference in their expression of MHC-II, CD86 or CD40 when pulsed with any of the treatments compared to untreated.
Figure 10: BMDC activation: BMDCs were pulsed for 24 hrs with either OVA, CpG B/C, OVA + CpG B/C mixtures, CpG B/C-SS-OVA, LPS 1 µg/ml or left untreated. Mean fluorescence intensity (MFI) of cell surface markers (A) MHC-II, CD86 and (B) CD40 was measured using flow cytometry. Graphs represent the result of three independent experiments.

Figure 11: Representative-gating strategy for BMDC flow cytometry analysis: 1x10⁶ BMDCs were sorted for live, single cells and stained for Near IR live/dead, CD11c, CD40, MHC-II and CD86.
3.2.2 BMDCs cytokine production by CpG-OVA conjugates

Using LegendPlex (Biolegend) and ELISA, cell culture supernatants from 24 hrs pulsed DCs were measured for production of pro-inflammatory cytokines TNF-α, IL-12p70, IL-1β, IL-6 and IFN-α. Both CpG B-SS-OVA and CpG C-SS-OVA induced production of all cytokines however there was no significant difference compared to OVA + CpG B/C mixtures. LPS treatment showed a significant difference between all treatments for both TNFα and IL-1β (figure 12).
Figure 12: BMDC cytokine production: BDMCs were pulsed with either OVA, OVA + CpG B/C, CpG B/C, CpG B/C conjugate or LPS 1 µg/ml for 24 hrs at 37°C. (A) TNF-α (left), IL-12p70 (right), (B) IL-1β, (left) IL-6 (right) and (C) IFN-α cytokines were then measured using a LegendPlex assay or ELISA (IFN-α) on cell culture supernatants. Graphs are each the result of three independent experiments * p < 0.05 compared to untreated control.
3.2.3 CD4+ T cell proliferation and IFN-γ production after BMDC co-culture

Treated BMDCs were co-cultured with CD4+ T cells for 72 hrs and analysed on the flow cytometer. T cells were sorted based on size and then gated for live, single cells which were CD3+CD4+. CFSE+ CD4+ cells were then analysed to determine percentage proliferation. Both conjugates induced CD4+ T cell proliferation, which was statistically different from the untreated group. IFN-γ production was measured via ELISA, both conjugates induced significantly greater IFN-γ production than untreated and individual CpG B and CpG C treatments. In addition, CpG C-SS-OVA also showed significantly greater proliferation than CpG C + OVA and OVA treatments. In both CD4+ proliferation and IFN-γ assays there was no statistical difference between individual conjugates.

Figure 13: CD4+ T cell proliferation and IFN-γ production: (A) Percentage of CD4+ proliferation in each T cell treatment group after 72hrs BMDC co-culture (B) Measurement of IFN-γ in CD4+ T cell culture supernatants after 72 hrs BMDC co-culture. Graphs are each the result of three independent experiments. Statistical significance p = <0.05. Error bars = Standard error of the mean.
Figure 14: Representative-gating strategy for CD4+ T cells: T cells were stained with zombie yellow live dead dye, CD4 APC-Cy7 and CD3 PE dazzle and analysed on the Gallios flow cytometer.
3.2.4 CD8+ T cell proliferation and IFN-γ production after BMDC co-culture

After 72 hrs of co-culture, T cells were sorted based on size and then gated for live, single cells, which were CD3+CD8+. CFSE+ CD8+ cells were then analysed to determine percentage proliferation. CD8+ T cells had significant proliferation in both conjugates, OVA, OVA + CpG B/C and SIINFEKL treatments compared to untreated and CpG B/C treatments. IFN-γ production was significantly greater in both conjugate treatments and SIINFEKL than all other treatments. There was no significant difference between conjugate and SIINFEKL treatments in either assay.

**Figure 15**: CD8+ T cell proliferation and IFN-γ production: (left) Percentage of CD8+ proliferation in each T cell treatment group after 72hrs BMDC co-culture (right) Measurement of IFN-γ in CD8+ T cell culture supernatants after 72 hrs BMDC co-culture. Graphs are representative of three independent experiments. Statistical significance* p <0.05, ****p <0.0001, Error bars = Standard error of the mean
Figure 16: Representative-gating strategy for CD8+ T cells: T cells were stained with zombie yellow live dead dye, CD8α APC and CD3 PE dazzle and analysed on the Gallios flow cytometer.
3.2.5 T cell proliferation and IFN-γ production after CD8+/CD4+/BMDC co-culture

Anti-tumour immune response require interaction between multiple T cell types. In many adoptive cell therapies, it has been shown that CD4+ and CD8+ cultured at a 1:1 ratio elicit strong cytotoxic responses to tumours. Therefore, to investigate how each T cell population interacts to elicit anti-tumour immune response to each conjugate; CD4+ and CD8+ T cells were both co-cultured with dendritic cells at a 5:5:1 ratio. T cells were then sorted using flow cytometry, gating for live, single cells which were CD3+ and either CD4+ or CD8+ respectively. Analysis revealed that CD8+ T cells dominated the culture after 72 hrs with greater proliferation than CD4+ T cells. There was no significant difference in CD4+ T cell proliferation between any treatments. CD8+ T cells showed significantly greater proliferation in OVA, OVA + CpG B/C, and both conjugate treatments compared to untreated and individual CpG B/C. There was no statistical difference between these treatments groups however. CpG B and C conjugates, as well as SIINFEKL, all produced significantly greater IFN-γ than individual CpG and OVA treatments and mixtures. No statistical difference was observed between each conjugate and SIINFEKL, although a trend towards greater IFN-γ production was seen in both conjugates.
Figure 17: CD4+ and CD8+ T cell proliferation and IFN-γ production after CD8+/CD4+/BMDC co-culture: (A) Percentage of CD4+ proliferation (left) Percentage of CD8+ proliferation (right)(B) IFN-γ levels in co-culture supernatants. Graphs are representative of three independent experiments. Statistical significance p <0.05. Error bars = Standard error of the mean
Figure 18: Representative-gating strategy for CD8+ and CD4+ T cells: T cells were sorted for live, single cells, which were CD3 positive, and then either CD4 or CD8 positive. CFSE positive CD4+ and CD8+ cells were then plotted on individual histograms and proliferation peaks analysed.
Chapter 4

Discussion
4.1 Discussion

The initial aims of this study were to utilise a novel conjugation strategy to link clinically relevant, gp100 melanoma peptides (25-33) and (19-39) to TLR agonists CpG B and CpG C and measure their ability to stimulate anti-tumour responses. Conjugates were first produced using a solution chemistry-based two-linker strategy designed by Solulink. Resulting conjugates were then analysed using RP-HPLC to determine the success of the reactions and purify the resulting conjugate products. Though this study was successful in producing gp100 conjugates, they were unable to be purified using RP-HPLC. Therefore, experiments were switched to using the model antigen ‘OVA’ in conjugates, which already had a well-established purification protocol using SEC. This enabled the immunostimulatory effects of each CpG in a reversible vaccine construct to be compared. Using a combination of cell culture, flow cytometry, LegendPlex and ELISA, results demonstrated that both CpG B and CpG C reversible OVA conjugates stimulate comparable anti-tumour responses in dendritic cells and both CD4+ and CD8+ T cell populations. Specifically, upregulation of co-stimulatory markers and pro-inflammatory cytokines in BMDCs as well as increased proliferation and IFN-γ production in T cell co-cultures were observed. More importantly, both conjugates stimulated a greater immune response than CpG B/C + OVA mixtures; which model a variety of formulations currently trialled in cancer vaccine based immunotherapy. Each conjugate treatment significantly increased T cell IFN-γ production which is characteristic of effector function, and plays a key role in anti-tumour immunity. This highlighted the role of reversible formulations in increasing effector responses and validated the use of CpG oligonucleotides as effective anti-tumour vaccine adjuvants. To fully compare the ability of each conjugate to elicit a desirable anti-tumour immune response, in vivo testing is required.
4.1.1 Difficulties with RP-HPLC purification of Gp100 peptide conjugates

RP-HPLC is used in the purification of many chemical compounds including those involved in drug and vaccine production (110). Though individual gp100 peptides and CpG oligonucleotides were identified using RP-HPLC, its use to identify gp100 conjugates was not sensitive enough to distinguish them from other potential products in the sample. These may include degraded peptide variants, unconjugated CpG-S-HyNic and CpG-SS-HyNic. Samples were each analysed using UV absorbance, their elution time relative to the individual unreacted CpG and gp100 counterparts, and their 3D spectral absorbance. Though each sample had many peaks, or suspected conjugates, it was unclear which were the true conjugates. Many samples showed a strong absorbance at 280 nm indicative of gp100 peptide presence, though they had an absence of 260 nm absorbance, characteristic of CpG. Many samples also had absorbance at 260-300 nm, which were indistinguishable from each other, making it difficult to draw solid conclusions as to their structure. The same could also be seen for absorbance at 345 nm, the indicator of bis-aryl hydrazone bond formation, which links gp100 to modified CpG. It is likely that excess/residual aromatic hydrazine (S-HyNic) and aldehyde (S-4FB, SS-4FB) linkers may have affected absorbance readings, along with the sensitivity of the UV detector on the Agilent system. As well the presence of background peptides, it may be possible that the sample/conjugate itself was unstable and degraded before detection. Aggregation of samples may have also affected absorbance readings and these could also explain the variation in elution times of structures with similar 260 nm and 280 nm absorbance.

Interestingly at a higher concentration, CpG B oligonucleotide (500 µM) revealed an elution time of 9.3 minutes, significantly different from the suspected time of 12.5
minutes seen at lower concentrations (figure 1b). As this concentration was only measured once in this study, it is likely that this was a technical error within the column, which may be due to blockage of the column with air bubbles or other products. The CpG B reading was also undetectable in all conjugate samples supporting this. However, another suggestion as to why it wasn’t detectable could simply be its low concentration within conjugate structures due to low free amino group (NH$_2$) availability in each gp100 (2 amino groups per peptide). As well as this, the higher concentration CpG B favoured absorbance at 280 nm over 260 nm. Its reference as pure CpG therefore made identification of each conjugate based on 260 nm absorbance challenging as this reading was not significantly different from gp100 peptide absorbance at 280 nm. In future experiments individual CpG B and CpG C oligonucleotides should be analysed in triplicate to ensure accurate elution times for reference in conjugate samples. The similarities in each conjugates RP-HPLC result also suggested that the reaction may not have occurred. Given the susceptibility of the NHS-ester groups to hydrolysis, I suggested that the addition of S-HyNic and gp100 steps be combined to reduce any S-HyNic hydrolysis that may be preventing efficient conjugation. This way, once S-HyNic bound to modified CpG, it could almost instantaneously bind to gp100. This would however, lead to new products such as CpG-HyNic from unmodified CpG residing in the sample and SS-4FB-modified gp100 from residual aldehydes in solution. In a repeated experiment, using CpG B with reversible SS-4FB linker, samples were run through the HPLC at 2 mins after the addition of gp100 and S-HyNic, then after 2 hrs reaction time, before spin filtration and then once more after spin filtration. There was insignificant difference between the two earliest time points, suggesting that the reaction either did not occur, or occurred rapidly. It is likely that a variety of factors may have influenced this result, including the time it took to prepare each sample for RP-HPLC analysis. Any
reaction occurring during preparation would therefore not be recorded. As well as this, samples taken after the 2 mins of gp100 addition were frozen at -20°C as a method to stop the reaction until analysis. It is highly likely that the reaction may have completed before the sample was fully frozen or resumed and completed after thawing. In future tests, the sample should be immediately analysed on the HPLC instead of freezing and running each sample at the same time. Despite failure to observe the reaction, comparison of samples before and after spin filtration did highlight the importance of this step in the product purification. Spin filtration removed/reduced many background products in the sample (excess linker and degraded peptides etc). Despite this, there were still too many candidates which could have been the true conjugates, with almost identical absorbance at 260 and 280 nm. In future experiments, liquid chromatography/mass spectrometry (LC/MS) or MALDI-TOF mass spectroscopy could be used to fully identify each conjugate, as done for several other conjugates mentioned in the literature (111-113). Conjugates could then be run on RP-HPLC to purify at a later stage. Alternatively, RP-HPLC may also not be the best method to purify the conjugates and alternatives such as Native polyacrylamide gel electrophoresis (Native PAGE) could be investigated. Native PAGE separates products based on their relative electrical charge and may be more effective if the conjugates have significantly different net charges to their individual counterparts. Ultimately, given the efficiency in terms of time of RP-HPLC, a combination of both Native PAGE and RP-HPLC could be used to purify the conjugates with PAGE as the initial purification, followed by RP-HPLC to identify them singularly and their elution times. RP-HPLC could then be solely used for purification of each conjugate in future/repeated experiments.

With RP-HPLC purification unsuccessful, studies switched to using model antigen ‘OVA’ as opposed to gp100. Previous work on production of OVA-CpG B conjugates
had already been studied in our laboratory using SEC. Therefore, pre-existing methodology could be used and altered for the production of our reversible OVA-CpG B and OVA-CpG C conjugates.

4.1.2 CpG B/C-SS-OVA conjugates requires additional characterization

Although conjugation between CpG B/C and OVA was successful, quantification and determination of the conjugation ratio produced unexpected results. Both modification of CpG and OVA produced MSRs well within ranges deemed successful (between 0.8-1.2). However, measurement of OVA concentration within conjugates using Quant-It, combined with A345 nm readings led to conjugation ratios much higher than expected. The MSR of OVA indicated that the maximum conjugation ratio possible for both conjugates was 1.4. Interestingly results from conjugation ratio calculation revealed ratios of 4.6 and 5.3 for CpG B and CpG C respectively. In immunological assays this was controlled for, although it remains unclear if this was the true conjugation ratio. It is likely that operator errors during the assay may have played a role in this finding and led to overestimation of the conjugation ratio. In future experiments, Quant-iT tests should be repeated to minimise this. Additionally, the use of OVA standards at differing concentrations instead of the BSA standards provided could strengthen the use of this assay by directly comparing concentrations of the protein of interest. Results from SEC support this, as conjugation ratios as high as 5.3 would produce conjugates of a much greater molecular weight and thus earlier elution times. Both conjugates analysed through the Yarra2000 eluted at similar times to previous studies which had a conjugation ratio around 2. These results may have also impacted immunological results as higher
CpG B/C concentrations in individual and OVA mixtures may mask the significance of each conjugates immunostimulatory effect.

With OVA-CpG conjugates produced and purified, the next steps in this study aimed to assess their Immunostimulatory effects on BMDCs and T cells. Therefore, to achieve this, experiments shifted to cell culture.

### 4.1.3 Immunostimulatory effects of CpG-OVA conjugates on BMDCs

MHC and co-stimulatory molecules such as CD80/86 and CD40 play a key role in coordinating both tumour-specific T cell activation and proliferation (33, 36, 55, 114, 115). It is well documented that upregulation of these proteins occurs when antigen presenting cells such as DCs are pulsed with toll-like receptor agonists such as CpG (114, 116). This study observed no significant difference between each treatment; which supports previous studies using CpG B conjugates (100). Pro-inflammatory cytokine production was also not significantly different although both CpG conjugates showed a trend to higher levels of IL-1β and IL-12p70 than other treatments excluding LPS. It is important to note that IL-12p70 is known to induce differentiation and proliferation of CD8+ T cells and direct CD4+ T cells to a Th1 phenotype (60, 115, 117, 118). This cytokine also increases T cell and NK cell production of IFN-γ (57, 77). Though IL-1β does not directly stimulate T cells, it is an important inducer of inflammation and has effects on many cells including endothelial cells, macrophages and other DCs. Therefore, full assessment of the influence of this IL-1β production on the anti-tumour immune response would require in vivo testing.

Remarkably, LPS in this study did not stimulate significant increases in IL-1β, IL-12p70 and co-stimulatory molecule expression compared to untreated samples. This contradicts
the literature and suggests that there may be issues in cell culture preparation (42, 119).

It is possible that our BMDCs cultured for 7 days were not sufficiently matured and thus less sensitive to LPS stimulation. It is likely that operator errors during the LegendPlex assay may also explain this, including insufficient washing of residual beads and short incubation times. These should therefore, all be repeated in future experiments.

Interestingly, there was also no significant change in IFN-α, a cytokine that should have been higher in both LPS and CpG C treatments based off previous literature (114, 120). One suggestion is that the IFN-α was already degraded by the time the ELISA was performed (supernatants were frozen at -20°C until use) or rapidly taken up by other BMDCs before detection. Measurements may have become inconsistent as supernatant storage time varied and all samples were measured on the same ELISA plate at one time. This suggests that simple experimental errors could explain the result including premature stoppage of the colour reaction; however, standard dilutions were measured successfully indicating the ELISA kit itself was not at fault. Another likely reason could simply be the culturing conditions. BMDCs cultured with GM-CSF may favour differentiation into the conventional DC (cDC) phenotype, which functions more in antigen presentation than cytokine production, especially in the case of IFN-α. Further experiments should direct attention to pulsing DC cultures containing Plasmacytoid DCs (pDC), the chief producers of type 1 interferon, as well as cDCs to assess the effect of CpG C conjugates. This can be done by either pulsing splenic DCs, which consist of all DC subsets including pDCs, or addition of Fms-related tyrosine kinase 3 ligand (Flt3L) into the BMDC culture media to promote generation of DCs that mimic that of the murine spleen (121-123). With this said, GM-CSF media may stimulate pDC growth, so initial
experiments should aim to identify these in culture, perhaps via their exclusive expression of cell marker B220 and low CD11c expression compared to cDCs (124).

### 4.1.4 Effects of conjugate pulsed BMDCs on CD4+ T cells

CD4+ T cells are crucial in co-ordinating anti-tumour immune responses. In particular, they play a key role in the production of IFN-γ which promotes MHC expression on tumours, activates tissue-resident macrophages and promotes CD4+ T cell polarisation to T helper 1 phenotype. CD4+ T cells are also chief producers of IL-2, which induces proliferation in CD4+ T cells, CD8+ T cells and Natural Killer cells, all of which contribute to the anti-tumour immune response. The presence of IL-2 and IFN-γ within patient serum is often linked to positive disease outcome and therefore the ability to stimulate CD4+ T cell proliferation and their cytokine production, is of great interest in immunotherapy (125-127).

In this study, CD4+ T cells co-cultured with BMDCs showed antigen-specific expansion characterised by the greater percentage of proliferation in treatments containing OVA in both full protein and peptide forms. Interestingly, the results of this study did not support previous findings, with conjugates not showing significant differences in CD4+ IFN-γ production compared to CpG + OVA mixtures(100). It is however, important to consider that this study had conjugation ratios over double that of previous work. As well as this, CpG concentrations were matched to account for this difference. It is likely that the higher CpG concentrations in mixtures may have resulted in increased overall uptake and thus increased activation of BMDCs. Uptake of the conjugates was not affected as it was administered in terms of the OVA concentration of 3.5 µg/ml.
With no difference observed in MHC-II expression within BMDC cultures, it is likely that both co-stimulatory molecules and pro-inflammatory cytokines expressed by BMDCs also played a key role in this result. It is however important to consider that CD86 expression was also not statistically different between any of the treatments tested. Therefore, other co-stimulatory markers not measured in this study may be responsible for this result such as CD80. To fully characterise any differences in MHC-II expression, measurements of specific OVA peptide 323-339 presentation on MHC-II complexes would need to be performed, perhaps via anti-MHC-II-OVA323-339 fluorescent antibodies, which could then be analysed using flow cytometry. It is also possible that the OVA within conjugates had increased loading onto MHC-II, whilst other treatments favoured the loading of endogenous self-peptides, which did not stimulate the OVA-specific T cells, onto MHC-II.

Pro-inflammatory cytokines such as IL-12p70 could also explain the increased T cell numbers. IL-12p70 is well documented as a potent activator of CD4+ T cells and inducer of their proliferation. In addition, IL-12p70 is also known to enhance CD4+ T cell IFN-\(\gamma\) production. Despite this, it is unlikely that IL-12p70 was the sole contributor to this response. Other cytokines not measured in this study such as IL-18, are also expressed by CpG stimulated BMDCs and polarise CD4+ T cells to a Th1-IFN-\(\gamma\) producing phenotype. IL-18 is also known to synergize with IL-12p70 to induce CD4+ IFN-\(\gamma\) production in an antigen-independent manner in humans (128). This result shows that reversible OVA conjugates can effectively stimulate CD4+ T cells in vitro. In relation to the original aims of this study it seems likely that this conjugation strategy may also stimulate gp100-specific CD4+ T cell responses.
4.1.5 Effects of conjugate pulsed BMDCs on CD8+ T cells

CD8+ T cells play a key role in anti-tumour immunity through various mechanisms including death receptor ligation and IFN-γ release (60, 78, 129). Results from this study show that both conjugates induce similar levels of T cell proliferation and IFN-γ release, regardless of CpG adjuvant used. Curiously, CD8+ T cells had both higher proliferation percentages than CD4+ T cells and produced a greater volume of IFN-γ, indicative of effector function. One explanation for this result is that the conjugates may favour presentation of OVA peptides onto MHC-I molecules, either by inducing trafficking of OVA to the cytoplasm or via upregulation of proteins involved in cross-presentation such as TAP. This is unlikely, as co-cultures of DCs with both CD4+ and CD8+ T cells induced similar proliferation levels in treatments with OVA 323-339 which only loads onto MHC-II. Future tests could examine the levels of MHC-I loaded with SIINFEKL peptide on dendritic cells determine its contribution to this result. This could be measured using fluorescent antibodies analysed via flow cytometry. Similar to CD4+ T cells, IL-12p70 could also explain the increase in CD8+ T cell numbers. Other cytokines such as IL-18 are also known to synergise with IL-12p70, as previously mentioned (115, 118). Further experiments to measure these cytokines would clarify this result. Intriguingly, in co-cultures containing both CD4+ and CD8+ T cells, CD8+ cell numbers dominated the culture at 72 hrs and had greater proliferation than their CD4+ counterparts despite a lower ratio of each T cell relative to BMDCs. This supports the idea of MHC-I favourability, though it is just as likely that stimulation with IL-2 produced by activated CD4+ T cells, in addition to IL-12p70, drove this proliferation and differentiation.
4.1.6 Effects of conjugates on CD4+ and CD8+ T cells in co-culture

Generating an effective anti-tumour immune response requires multiple immune cell populations. It has already been highlighted that CD4+ T cells play a crucial role in enhancing CD8+ T cell effector function in many studies. Although typical T cell co-cultures use at least a 1:2 ratio of CD4+ to CD8+ T cells, many adoptive cell therapies have used CD4+ and CD8+ T cell at a 1:1 ratio and shown enhanced T cell cytotoxic function\((130, 131)\). To investigate if this effect translates in each conjugate, CD4+ and CD8+ T cells were co-cultured with BMDCs at a ratio of 5:5:1. Results showed a significant expansion of CD8+ T cells within the culture at 72 hrs. Interestingly, CD4+ T cells showed little change in proliferation, even in treatments groups which favoured MHC-II presentation such as OVA 323-339. It is well documented that CD4+ T cells enhance CD8+ T cell and NK cell proliferation through production of IL-2 and this is likely the cause of the CD8+ T cell expansion \((28, 132)\). The supernatants of conjugate treated cultures also showed greatly increased IFN-\(\gamma\) compared to OVA + CpG B/C mixtures. This is likely a result of both initial CD4+ production and CD8+ production after differentiation into CTLs brought on by BMDC cytokines including IL-12p70. In addition, early IFN-\(\gamma\) production by CD4+ T cells may have enhanced MHC-I expression on BMDCs leading to increase CD8+ stimulation.

In summary, both conjugates appear as strong inducers of T cell proliferation and IFN-\(\gamma\) production. With suspected increases in MHC loading of OVA peptides and synergy between pro-inflammatory cytokines including IL-12p70 and IL-18, it is likely that this enhanced response is the result of both antigen-dependant and anti-independent processes working in tandem.
4.1.7 Future immunological tests

Results from this study have demonstrated that both CpG B and CpG C use, within reversible vaccine constructs, elicits a robust peptide-specific T cell response in vitro. Though this is a powerful predictor of the vaccines’ efficacy, in vivo studies should be investigated to fully compare each conjugate. These may include testing the ability of T cells to kill target cells using an in vivo cytotoxicity assay, or full tumour trial in a mouse model. Additional in vitro tests could be performed to investigate the effect of these conjugates on other immune cells such as Natural Killer cells, which are known to be activated by IFN-α as well as IL-12p70 (77). NK cells are also a source of IFN-γ and thus have great therapeutic potential for the treatment of tumours. The effect of conjugates could also be assessed on their ability to stimulate B cells, another adaptive immune cell which acts in both antigen presentation and antibody production. B cells give an alternative method of antigen presentation, which may synergize with presentation by cDCs. Their production of polyclonal antibodies can also act to ‘tag’ tumours for cell-mediated killing by Natural Killer cells. Finally, the in vitro results gathered in this study indicate that both conjugates have immense potential as therapeutic cancer vaccines, as either a monotherapy or a combined therapy. Therefore testing in combination with either Checkpoint Blockade or Adoptive Cell Therapy could further characterise its Immunostimulatory and therapeutic effects.

4.1.8 Conclusion

Cancer remains an ongoing challenge for clinicians and researchers alike. Though many immunotherapies have shown promise in treating cancer, it is unlikely that cancer vaccines alone will be effective enough to cure malignancy. Therapeutic cancer vaccines
do however; remain a useful treatment option, which can be combined with other treatments to enhance their therapeutic effect. Its ability to boost tumour-specific T cell responses make it a great candidate for combinational therapy with Checkpoint Blockade as well as Adoptive Cell Therapy. Overall, improvements in cancer vaccine design and testing through the choice of immune adjuvant and tumour peptide seem set to increase its importance in cancer treatment, especially in the realm of combinational immunotherapy. In sum, this study illustrated the numerous challenges present in production and testing of soluble cancer vaccines. Although purification of the gp100 conjugates was unsuccessful, experiments using OVA successfully compared each CpG type within reversible conjugates. Results showed a similar level of immune stimulation with significantly increased T cell responses compared to individual OVA protein and CpGs. Given the importance of T cell responses in anti-tumour immunity and the clinical relevance of gp100 as a melanoma-associated antigen, it remains of high interest to investigate the effect of these CpGs within gp100 constructs. Both CpG classes appear, as strong candidates for use in cancer vaccines and with careful experimental design and consideration of these challenges in both purification and immunological assays, the production and testing of these conjugates seems highly achievable in the future.
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Appendices
Appendix 1: Buffers and Recipes

Modification buffer
14.2 g Na$_2$HPO$_4$
8.76 g NaCl
pH 8.0

Conjugation buffer
14.2 g Na$_2$HPO$_4$
8.76 g NaCl
pH 6.0

MES buffer
0.96 g MES
250 ml MilliQ

2-HP solution
0.00045 g 2-HP
50 ml MES buffer
Protect from light
2-SBA solution

0.00052 g 2-SBA

50 ml MES buffer

Protect from light

Turbolink catalyst buffer

15 ml conjugation buffer

139.65 µl aniline

pH 6.0

10 x Phosphate Buffered Saline (PBS)

80 g NaCl

11.35 g Na₂HPO₄

2 g KCl

2 g KH₂PO₄

1L MilliQ

Red Cell Lysis Buffer

4.15 g NH₄Cl

0.5 g KHCO₃

0.0186 g EDTA

500 ml MilliQ

pH 7.2-7.4
**10 x FACS buffer**

100 ml 10 x PBS
10 g BSA
1g NaN3
7.44g EDTA
1L MilliQ

**MACS buffer**

292.24 mg EDTA
5 g DPBS
2.5 mg BSA
Top up with MilliQ to 500 ml
pH 7.2

**MACS running buffer**

292.24 mg EDTA
5 g DPBS
0.5 mg BSA
Top up with MilliQ to 500 ml
pH 7.2
**ELISA Coating buffer**

0.42 g NaHCO₃

50 ml MilliQ

**ELISA Wash buffer**

200 ml 10 X PBS

1 ml Tween 20

MilliQ 1.8L

**ELISA Blocking buffer**

100 ml PBS

1 g BSA

**LegendPlex Wash buffer**

25 ml of 20 X Legendplex wash buffer (Biolegend)

475 ml MilliQ
Figure 19 Supplementary 1: RP-HPLC analysis of CpG B stable conjugates. (A) Analysis of CpG B-S-gp100 (25-33) at 220 nm (top) and 280 nm (bottom) (B) Analysis of CpG B-S-gp100 (19-39) at 220 nm (top) and 280 nm (bottom)
Figure 20 Supplementary 2: RP-HPLC analysis of CpG C reversible and stable gp100 conjugates. (A) Analysis of CpG C-SS-gp100 (19-39) at 220 nm (top) and 280 nm (bottom) (B) Analysis of CpG C-SS-gp100 (25-33) at 220 nm (top) and 280 nm (bottom) (C) Analysis of CpG C-S-gp100 (19-39) at 220 nm (top) and 280 nm (bottom) (D) Analysis of CpG C-S-gp100 (25-33) at 220 nm (top) and 280 nm (bottom).
Figure 21 Supplementary 3: Purification of CpG C-SS-OVA conjugate: CpG C-SS-OVA was injected at a volume of 500 µl and run for 45 mins in PBS on a Superdex™ 200 10/300 GL SEC column. UV detection was set to 260 nm. The resulting product eluted at 25-30 mins with unreacted CpG C eluting at 32-37 minutes.
Figure 22: BMDC Unstained control gating: Untreated BMDCs were harvested from cell culture supernatants and directly run on the Galios flow cytometer. This formed the basis of the BMDC gating strategy used on stained samples.