Assessment of combined vaccination and immune modulation as an anti-tumour therapy

Emma Victoria Petley

A thesis submitted to Victoria University of Wellington in fulfilment of the requirements for the degree of Master of Biomedical Science

December 2016
Abstract

Glioblastoma multiforme (GBM) is a common and lethal type of brain cancer, with a very poor prognosis. Current therapy consisting of surgical resection, radiation and chemotherapy produces a median survival of only 12-15 months. Therefore, there is a need to develop new therapeutic approaches for the treatment of GBM.

This thesis investigates a new series of synthetic cancer vaccines, conjugating tumour-associated antigens (TAAs) to an isomer of α-Galactosylceramide (α-GalCer), a potent invariant natural killer T (iNKT) cell agonist with documented adjuvant activity. Upon antigen encounter, activated iNKT cells are capable of licensing dendritic cells (DCs) through CD40:CD40L interactions and cytokine production. The licensed DCs subsequently stimulate potent CD8⁺ T cell responses, capable of killing cancerous tissue. Conjugation of α-GalCer to the TAA-derived peptide was achieved via an enzymatically cleavable linker sensitive to cathepsin B activity. This strategy allows co-delivery of the active components, with the rationale that the same DC will be able to co-present both α-GalCer for iNKT cell activation, and peptide to induce an enhanced CD8⁺ T cell responses.

The conjugate vaccines assessed in this thesis were able to induce iNKT cell activation and produce CD8⁺ T cell cytoxicity. However, this did not correlate with in vivo anti-tumour activity, as the vaccine that incorporated the TAA survivin, produced minimal cytotoxicity but potent anti-tumour responses against an implantable model of glioma.

Enhancing T cell-mediated immune responses has been validated by immune checkpoint inhibition for the treatment of cancer. However, many patients do not respond to the therapy. It is thought that this subset of patients may lack pre-existing T cell responses, which are required for the efficacy of checkpoint inhibition. Therefore, there is considerable interest in whether the use of vaccines that stimulate
T cell activation can improve responses to checkpoint blockade and other immune modulating drugs. The survivin vaccine was combined with the immune checkpoint blockade inhibitors α-PD-1, α-CTLA-4 and α-LAG-3, the co-stimulatory agent α-4-1BB, or administered with T regulatory cell (TREG) depletion, to reveal the immunogenicity of the vaccine.

This research revealed that combining the survivin vaccine with the immune checkpoint inhibitor α-CTLA-4 improved overall survival of mice, compared to the vaccine alone. This finding suggests that this combined therapy may be a useful immunotherapeutic strategy for the treatment of GBM.
Acknowledgements

To my supervisor, Prof. Ian Hermans, thank you for taking a chance on me as your first masters student. You have pushed me to critically think and ask the right questions. I greatly value these traits and know they will benefit me in the next chapter of my life. To Prof. Graham Le Gros, thank you for crafting the Malaghan into the incredible institute it is today. It has been an honour to study here.

To the Vaccine Research Group, thank you all for teaching me how to be a scientist. Taryn, thank you for your endless patience, teaching, laughter and advice. Ching-Wen thank you for teaching me how to be a thorough scientist. Kathryn, thank you for always being willing to help me with my experiments. Josh and Liv, thank you for rescuing me and helping with my experiments in the final days. All of your help has never gone unnoticed. Lindsay, thank you for continuing to inspire me and having faith in my abilities as a scientist. Your mentorship has helped me to continue my journey in science, and I am incredibly thankful for your teaching, love and encouragement.

To the Ferrier Research Institute, in particular Prof. Gavin Painter, thank you for being incredibly patient when explaining chemistry, and supplying vaccines on demand.

To Dr. Elizabeth Forbes-Blom, thank you for your mentorship over the last two years. I appreciate all the advice you’ve given me, the times you’ve listened to me complain and the times you’ve given me a well-needed dose of tough love! Thank you for showing me what a kick-ass woman in science looks like.
To the HGCC, thank you for maintaining the machines and allowing us to carry out our science. In particular Sally, thank you for your patience and endless help on the cytometers when everything is coming crashing down. To the BRU, thank you for the work you do. It doesn’t go unnoticed! Ian, you run an incredible unit and I could not ask to work in a better facility. Emma, thank you for the banter, laughs and looking after my mice! A special thanks to Lee, you’ve always managed to put a smile on my face no matter my mood. Thank you for the biscuits.

To the PhD club, past and present, I could not have done this without your support. In particular Cam and Alanna, my father and mother in science, thank you for the support and guidance from NZ and half-way across the globe. Cat thank you for letting me know 3pm beers on a Wednesday is totally ok. Connie, I am forever privileged that I can now call you one of my closest friends. Your guidance academically and spiritually has carried me through the last couple of years. I am blessed to have you in my life and incredibly excited for what God has instore for the next chapter of your life. Anna, Ellie, Jodie, Josh, Kirsty, Liv, Ruby and Sophie thank you for the beers, coffees, cuddles, laughter and love. Most of all thank you for being my second family. As your social co-ordinator, I know you will all be lost without me.

To the 289 homies, thanks for providing an amazing place to come home to every night. You guys have kept me sane!

To the FGs, thank you for the love, general banter, encouragement and trips to the Horn that have kept me going through some dark times. You guys continue to inspire me as the dirty, sassy, alcoholic, feminists you are. Stay nasty.

To the LLL dancers, thank you for always providing such an amazing positive environment full of laughter and love. Knowing that I finish each week dancing with you lot, has made this journey achievable. Thanks for helping me achieve illusions.
To Laura and Hadley, you guys have been there since the beginning and will continue to be there as I embark on the next chapter of my life. I am incredibly lucky to have you in my life and am forever excited about what your future holds.

To Granny and Dennis, thank you for all the love from the other side of the globe. I could not have done this without your love and support.

To my family, Juliet, James, Scarlett and Lily thank you for always being supportive and proud of me. You have been the most incredible addition to my life and I am incredibly grateful to call you family. To Sophie, my ride or die, you’ve been the most incredible support throughout this journey. Thank you for always being there for me and trying to understand what I do. You’ve provided invaluable times of laughter, love and have frequently let me vent. Thank you for being my biggest hype girl. Love ya.

To my Mum and Dad, thank you for never failing to believe in me. I could not have done this without you, and this thesis is dedicated to you. Your endless love, support, guidance and laughter have gotten me through this. Thank you for your spiritual guidance throughout my life. I love you.

To my best friend and favourite person on the planet, Mark, I couldn’t have done this without you. Thank you for keeping me sane, loving me and making me a better person.

And finally, to the rock in my life, you have carried me throughout this journey, your endless and unfailing love is constantly overwhelming. You have bought me this far and will continue to take me even further. For I can do all things through Christ, who strengthens me.
Disclosure Statement

Some of the experiments performed during the course of my Masters have been in the context of collaborative efforts of my laboratory.

Dr Lindsay Ancelet performed and supplied the data for the prophylactic vaccine experiments, which I subsequently analysed.

Dr Colin Hayman performed, supplied and analysed the data for the mass spectrometry analysis.

With these disclosures, I declare that the content of this document is my own work.
# Table of contents

Abstract.......................................................................................................................... iii  
Acknowledgements......................................................................................................... v  
Disclosure Statement....................................................................................................... viii  
Table of contents ........................................................................................................... ix  
List of figures .................................................................................................................. xiii  
List of tables .................................................................................................................... xv  
List of abbreviations ....................................................................................................... xvi  

1 General Introduction .................................................................................................... 21  
  1.1 Cancer and the immune system ............................................................................. 2  
    1.1.1 History of cancer therapies ........................................................................... 3  
    1.1.2 Glioblastoma multiforme .............................................................................. 4  
    1.1.3 Overview of the immune system .................................................................. 5  
  1.2 Antigens .................................................................................................................. 7  
    1.2.1 Antigen-presenting cells (APCs) .................................................................. 8  
    1.2.2 Pathways of antigen presentation .................................................................. 8  
  1.3 Dendritic cells (DCs) ............................................................................................. 11  
    1.3.1 Dendritic cell activation .............................................................................. 12  
    1.3.2 Invariant natural killer T (iNKT) cells can activate dendritic cells ............ 13  
  1.4 T cell immunity ..................................................................................................... 16  
    1.4.1 T cell development and specificity ............................................................... 16  
    1.4.2 T cell effector mechanisms ......................................................................... 18  
    1.4.3 CTL-mediated killing of cancer cells ........................................................... 21  
  1.5 T cell-mediated immunotherapy ........................................................................... 22  
    1.5.1 Immune surveillance and tumour suppression ............................................. 22  
    1.5.2 Vaccination and modulating the immune system ........................................ 23  
    1.5.3 Peptide vaccination can direct CTL immune responses ............................ 25  
    1.5.4 Design of synthetic conjugate peptide vaccines to stimulate CTLs .......... 26  
    1.5.5 Peptides from TAAs for vaccine design ..................................................... 28
1.5.6 Immune modulation with monoclonal antibodies ........................................... 30
1.6 Rationale for thesis: ............................................................................................ 38
1.7 Aims and Hypothesis ........................................................................................ 39
    1.7.1 Specific Aims .............................................................................................. 39
2 Material and Methods ......................................................................................... 41
    2.1 Materials ........................................................................................................ 42
        2.1.1 Labware .................................................................................................. 42
        2.1.2 Reagents and Buffers ............................................................................. 43
        2.1.3 Cell lines ................................................................................................. 49
        2.1.4 Mice ........................................................................................................ 50
    2.2 Methods .......................................................................................................... 52
        2.2.1 Tissue isolation ....................................................................................... 52
        2.2.2 *In vitro* T cell assays ............................................................................. 52
        2.2.3 *In vivo* T cell assay .............................................................................. 55
        2.2.4 Serum analysis ...................................................................................... 55
        2.2.5 Flow cytometry ...................................................................................... 56
        2.2.6 Tumour challenge ................................................................................... 57
        2.2.7 Therapy .................................................................................................. 58
        2.2.8 Statistical analysis .................................................................................. 59
3 Evaluating the immune response induced by conjugate vaccine ....................... 41
    3.1 Introduction ..................................................................................................... 62
    3.2 Aims ................................................................................................................ 63
    3.3 Results ............................................................................................................. 64
        3.3.1 Vaccines containing glycolipid conjugated to peptide do not bind CD1d
directly and activate iNKT cells *in vitro* ............................................................ 64
        3.3.2 Conjugate vaccines are capable of stimulating iNKT cells *in vivo* .......... 66
        3.3.3 Assessment of antigen-specific cytotoxicity of conjugate vaccines against
target cells ............................................................................................................. 68
3.3.4 Prophylactic survivin vaccination *in vivo* delays SC GL261 tumour growth ................................................................. 70
3.3.5 Therapeutic survivin vaccination *in vivo* delays SC GL261 tumour growth ......................................................... 72
3.3.6 Oxidation status of the survivin peptide .............................................................................................................. 74
3.3.7 Survivin peptide efficiently binds H-2Kb, and is further enhanced with TCEP treatment ................................................................. 78
3.3.8 Antigen-specific cytotoxicity is improved when treating peptide-loaded target cells with TCEP ....................... 80
3.3.9 CD8+ T cells do not produce IFN-γ following survivin vaccination .......... 82
3.3.10 The anti-tumour response of survivin vaccine depends on CD4+ T cells .. 84
3.3.11 CD4+ T cells do not produce IFN-γ following survivin vaccination ....... 86
3.4 Discussion .................................................................................................................................................. 88
3.4.1 Conclusion ............................................................................................................................................. 92
4 Assessment of survivin vaccine in combination with immune modulators .......... 95
4.1 Introduction .............................................................................................................................................. 96
4.2 Aims .......................................................................................................................................................... 98
4.3 Results ................................................................................................................................................... 99
4.3.1 Anti-tumour effects of α-PD-1 were not enhanced by vaccination .......... 99
4.3.2 Anti-tumour effects of lower dose of α-PD-1 were not enhanced by vaccination .................................................. 101
4.3.3 Anti-tumour effects of α-LAG-3 were not enhanced by vaccination ...... 103
4.3.4 Combining survivin vaccine with α-4-1BB improved anti-tumour response ................................................................................. 105
4.3.5 Anti-tumour effects of inactivation of TREGS were not enhanced by vaccination ................................................................. 107
4.3.6 The survivin vaccine combines with α-CTLA-4 to improve anti-tumour response ................................................................. 109
4.3.7 Vaccine induced activation of iNKT cells is enhanced with CTLA-4 blockade .............................................................................. 111
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4 Discussion</td>
<td>113</td>
</tr>
<tr>
<td>4.4.1 Conclusion</td>
<td>118</td>
</tr>
<tr>
<td>5 General Discussion</td>
<td>121</td>
</tr>
<tr>
<td>5.1 Summary of results</td>
<td>122</td>
</tr>
<tr>
<td>5.2 The survivin vaccine is dependent on CD4⁺ T cells</td>
<td>123</td>
</tr>
<tr>
<td>5.3 Combinational therapy with α-CTLA-4 enhanced iNKT and NK cell activation</td>
<td>126</td>
</tr>
<tr>
<td>5.4 The future of peptide vaccination</td>
<td>129</td>
</tr>
<tr>
<td>5.5 Conclusion</td>
<td>129</td>
</tr>
<tr>
<td>6 References</td>
<td>131</td>
</tr>
</tbody>
</table>
List of figures

Figure 1.1: Antigen processing pathways of APCs .................................................. 11
Figure 1.2: The use of iNKT cells as cellular adjuvants ........................................ 15
Figure 1.3: Structure of functional α-GalCer .......................................................... 27
Figure 1.4: Vaccine design and in vivo processing ................................................ 28
Figure 1.5: Regulatory receptors of CTL activation ............................................... 31
Figure 3.1: Conjugate vaccines require cleavage by DCs to present α-GalCer to iNKT cells ................................................................. 65
Figure 3.2: Comparison of the ability of conjugate vaccines delivered IV, SC or IM to activate iNKT cells ................................................................. 67
Figure 3.3: Route of administration of conjugate vaccines alters antigen-specific killing ................................................................. 69
Figure 3.4: Prophylactic survivin vaccination delays GL261 SC tumour growth ...... 71
Figure 3.5: Tumour growth is delayed with therapeutic survivin vaccination .......... 73
Figure 3.6: Potential oxidation of residues in the CD8+ epitope of survivin peptide .. 74
Figure 3.7: HPLC-MS analysis of survivin peptide ............................................... 76
Figure 3.8: HPLC-MS analysis of TCEP-treated survivin peptide ......................... 77
Figure 3.9: Survivin peptide does not require TCEP treatment to bind H-2Kb .......... 79
Figure 3.10: Antigen-specific killing is improved when treating survivin targets with TCEP ................................................................................. 81
Figure 3.11: Following survivin vaccination, CD8+ T cells do not produce IFN-γ ....... 83
Figure 3.12: Survivin vaccine efficacy is lost in MHC class II+ ................................ 85
Figure 3.13: Following survivin vaccination, CD4+ T cells do not produce IFN-γ ....... 87
Figure 4.1: PD-1 inhibition with checkpoint inhibitor is sufficient for GL261 SC tumour clearance ................................................................. 100
Figure 4.2: Low dose α-PD-1 does not inhibit tumour growth .............................. 102
Figure 4.3 Combination of survivin vaccine and α-LAG-3 did not improve anti-tumour responses ................................................................. 104
Figure 4.4: Combined survivin vaccine and α-4-1BB may enhance anti-tumour response ................................................................................. 106
Figure 4.5: Depletion of T_{REGS} with α-CD25 causes anti-tumour response against GL261 SC tumours. ................................................................. 108
Figure 4.6: Improved anti-tumour response when combining survivin vaccine with α-CTLA-4 .................................................................................. 110
Figure 4.7: iNKT cell activity is enhanced with combined survivin vaccine and α-CTLA-4 .................................................................................. 112
Figure 5.1: Alternative cellular mechanisms of anti-tumour activity induced by the survivin vaccine ................................................................. 128
List of tables

Table 2.1: List of labware ........................................................................................................ 42
Table 2.2: List of reagents .......................................................................................................... 43
Table 2.3: Flow cytometry antibodies ...................................................................................... 48
Table 2.4: Viability dyes ............................................................................................................ 49
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α- (prefix)</td>
<td>Anti-</td>
</tr>
<tr>
<td>α-GalCer</td>
<td>alpha-Galactosylceramide</td>
</tr>
<tr>
<td>β2m</td>
<td>beta-2-microglobulin</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>4-1-BB (CD137)</td>
<td>Tumour necrosis factor receptor superfamily member 9</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplant</td>
</tr>
<tr>
<td>CD-</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>cIMDM</td>
<td>Complete Iscove’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>CLIP</td>
<td>Class II-associated invariant chain peptide</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte associated protein 4</td>
</tr>
<tr>
<td>CTO</td>
<td>Cell Tracker Orange CMTMR</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-linked immunoSpot</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>gp100</td>
<td>Glycoprotein-100</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>iI</td>
<td>Invariant chain</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin-</td>
</tr>
<tr>
<td>ILT</td>
<td>Innate-like T cells</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant natural killer T cell</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>iTCR</td>
<td>Invariant T cell receptor</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte-activation gene-3</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid derived suppressor cell</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
</tbody>
</table>
MPL  Monophosphoryl lipid A
MHC  Major histocompatibility complex
NaN3 Sodium azide
NK  Natural killer cell
NKT  Natural killer T cell
OS  Overall-survival
PAMP  Pathogen associated molecular pattern
PBS  Phosphate buffered saline
PD-1  Programmed cell death protein 1
PD-L1  Programmed death ligand 1
PD-L2  Programmed death ligand 2
PE  Phycoerythrin
PerCP  Peridinin chlorophyll protein
PFA  Paraformaldehyde
PI  Propidium Iodide
PRR  Pattern recognition receptor
RBC  Red blood cell
RPMI  Roswell Park Memorial Institute 1640 medium
SA  Streptavidin
SC  Subcutaneous
SEM  Standard error of the mean
SFC  Spot forming cell
SIINFEKL  Ovalbumin MHC class I peptide
sLAG-3  Soluble lymphocyte-activation gene-3
SSC  Side scatter
TAA  Tumour associated antigen
TAP  Transporter associated with antigen processing
Tc  Cytotoxic T cell
TCEP  Tris(2-carboxyethyl)phosphine)
TCR  T cell receptor
TGFβ  Transforming growth factor beta
T H  T helper cell
TIL  Tumour infiltrating lymphocyte
TLR  Toll-like receptor
TME  Tumour microenvironment
TNFα  Tumour necrosis factor alpha
T REG  Regulatory T cell
TRP-2  Tyrosine-related protein-2
WBC  White blood cell
WT  Wild type
1 General Introduction
1.1 Cancer and the immune system

Cancer is defined as an uncontrolled, abnormal growth of cells and is one of the world’s leading causes of death\(^1\). Genetic alterations that arise through mutations and epigenetic changes lead to an unregulated proliferation of cells with the potential to invade surrounding tissues or metastasise to other organs\(^2\). Not all tumours are cancerous, some are benign and do not spread to other areas of the body\(^1\). Malignant tumours, however, are genetically unstable, harmful tumours capable of spreading\(^3\). Tumours that become malignant share similar characteristics referred to as the ‘ten hallmarks of cancer’. These hallmarks include resistance to cell death, genomic instability and mutation, establishment of new blood vessels, activation of metastases, tumour promoting inflammation, replicative immortality, evading growth suppression, sustaining proliferative signalling, deregulating cellular energetics, and of particular relevance to this study, avoiding immune destruction\(^4\).

Through a series of steps known as the immune response, the immune system is capable of co-ordinating appropriate responses for the removal of harmful substances, including a cell-based arm that is able to recognise and eliminate cancerous cells\(^5\). However, to succeed it is necessary for these immune effector cells to bypass the numerous mechanisms cancer cells use to avoid immune destruction. In recent years, new immunotherapeutic drugs have been developed that relieve this immunosuppression, and remarkable clinical responses have been achieved. Nonetheless, clinical responders are still a minority, and further work is needed for immunotherapy to achieve sustainable responses in a larger range of patients. This thesis aims to explore the use of vaccine-based immunotherapy to enhance targeted immune responses against cancer cells, and combining the vaccines with other immunotherapies to improve the treatment of cancer.
1.1.1 History of cancer therapies

The earliest known description of cancer appears in several papyri from Ancient Egypt. The Edwin Smith Papyrus, thought to be written around 1600 BC, contains not only a description of cancer, but also a procedure to remove breast tumour by cauterisation. The origin of the word cancer is credited to Hippocrates, the “Father of Medicine”, who documented descriptions of tumours between 460-370 BC. It was not until mid 1800s, when William Halsted started exploring radical mastectomy, that any treatment with reproducible efficacy was recorded. This involved removal of the entire breast, surrounding lymph nodes and chest muscle, and successfully reduced recurrence of disease. Surgery remains a treatment option for many solid cancers, with continuous refinements and improvements being developed. In 1903, five years after Marie Curie discovered radium, doctors reported the first successful use of implanting radioactive elements inside or next to cancer to deliver radiation at a close range. Refined radiotherapy is used as a treatment option today to target cancerous cells more precisely.

A major breakthrough with chemotherapeutic agents ushered the next age of cancer treatment. Following results of clinical trials in 1949, the first chemotherapy drug approved by the US Food and Drug Administration (FDA) was for treatment of Hodgkin’s lymphoma. Nitrogen mustard or ‘mustard gas’, used as a weapon in World War II, kills cancerous cells by modifying DNA through alkylation. This discovery led to rapid advancements in chemotherapy, and drugs based on this mode of activity are still used today. Although partially successful in treating cancer, many chemotherapies simply target dividing cells, and do not differentiate between healthy and cancerous cells. They can therefore be associated with significant negative side effects. The development of molecularly targeted chemotherapies, which target pathways involved in the neoplastic process, have been a significant advance, although some toxicities still occur.
Overall, surgery, radiotherapy, chemotherapy and targeted drugs are effective to some degree but are often associated with morbidity and development of resistance. Therefore, there is a great need to develop improved cancer therapies. Recently, focus has been on investigating immunotherapeutic drugs, including strategies to initiate or boost anti-tumour responses, such as vaccination or adoptive cell therapy, or drugs that relieve tumour-induced immunosuppression, such as immune checkpoint inhibitors\(^\text{12}\). The term immunotherapy can cover a range of strategies that involve immune cells, but will be used here to describe any therapy where cells of the immune system, specifically T cells, become the effectors that cause elimination of cancerous tissue. Although off-target activities causing autoimmunity are a genuine concern with this type of therapy, and are routinely observed with checkpoint inhibitors, it is possible that like chemotherapy, immunotherapy will move into more targeted age. The peptide-based vaccines to be described in this thesis aim to provide a more specific and safer therapeutic alternative.

### 1.1.2 Glioblastoma multiforme

Much of the early work on cancer immunotherapy has been in so-called “immunogenic” cancers such as melanoma, driven initially by anecdotes of spontaneous tumour regression and some early successes with primitive immunotherapies. In recent years, it has been suggested that part of the reason these cancers may respond to immunotherapy is a high mutation load that generates novel immune responses. Perhaps surprisingly, glioblastoma multiforme (GBM), a highly malignant, incurable brain cancer common in adults\(^\text{13}\), has also received considerable attention as a target for immunotherapy. In this case, momentum is largely driven by the dismal failure of all other therapies. The tumours are extremely invasive, making removal with surgery difficult. Radiotherapy is relatively effective initially, but recurrence is unavoidable\(^\text{14}\). Furthermore, due to the blood-brain barrier (BBB) treatment of tumours with chemotherapy is difficult. The current therapeutic strategy
for GBM involves resection of the tumour followed by radiation and chemotherapy with the alkylating agent temozolomide. However, this aggressive treatment strategy merely provides overall-survival for patients of 12-15 months\textsuperscript{15}.

In this thesis, therapies for GBM were tested \textit{in vivo} in an implantable murine glioma (GL261) model\textsuperscript{16}. The cell line was implanted subcutaneous (SC) to avoid undertaking timely and difficult intracranial (IC) procedures. Although this does mean that the impact of the BBB and the unique environment of the brain are no longer considered, this approach can be used to answer some basic immunologic questions, and guide research to be conducted later in an intracranial model; this approach has been successfully used in our laboratory to develop and characterise a cell-based vaccine for glioma therapy\textsuperscript{17}. It should also be noted that the tumour cell line is highly immunogenic and occasionally prone to natural regression\textsuperscript{16}. Nonetheless, investigating therapies for GBM using GL261 is commonly practiced\textsuperscript{18,19}, due to a lack of other easily established animal models.

\section*{1.1.3 Overview of the immune system}

The body is protected from infectious agents and harmful toxins by a network of cells and molecular structures that together make the immune system\textsuperscript{20}. Two arms of the immune system exist, the innate immune system and the adaptive immune system. Cells of the innate system identify pathogens in a non-specific way, through a limited number of receptors that recognise conserved structural patterns common to many pathogens. Innate immunity provides immediate defence against infection as most pathogens are detected and eliminated in minutes to hours\textsuperscript{21}. A major function of the innate system is the recruitment of immune cells to sites of infections via production of chemical mediators called cytokines. The innate system provides initial defences against pathogens, but is also critical for initiating the adaptive immune response\textsuperscript{22}. The adaptive immune system is composed of lymphocytes that have the capacity, once
activated, to recognise pathogens with a much higher level of specificity than cells of the innate system. This is because somatic gene rearrangement events provide each lymphocyte with a unique antigen receptor, and across the entire lymphocyte population there will be cells that can recognise antigens that are entirely unique to a given pathogen. An effective adaptive response takes several days to develop, as pathogen-specific lymphocytes must undergo clonal expansion to provide sufficient numbers of cells to have any impact on pathogen growth and spread. Two broad lymphocyte populations direct the adaptive immune response, with B lymphocytes producing antibodies that constitute “humoral” immunity, and T lymphocytes responsible for “cell-mediated” immunity.

To initiate a humoral immune response, B lymphocytes must recognise antigens via their antigen receptor, a membrane-bound immunoglobulin (Ig) also known as a B-cell receptor (BCR). These antigen-specific Igs can be secreted as antibodies that neutralise pathogens and toxins, activate complement and promote opsonisation to encourage phagocytosis and pathogen elimination. The cellular components of the adaptive immune response are T lymphocytes, which recognise antigens via membrane-bound T-cell receptors (TCRs) and can be further categorised into subgroups based on function. Cytotoxic T lymphocytes (CTL), which are generally activated CD8+ T cells, are capable of directly killing infected or neoplastic cells through the release of cytotoxic granules, perforin, granzymes and granulysin, or through Fas:Fas ligand (FasL) mediated pathway of apoptosis or cell death. CTLs can also produce a range of cytokines, including interferon-gamma (IFN-γ), which can block viral replication or eliminate viruses. Helper T cells (T_H), which are activated CD4+ T cells, play critical roles in mediating an adaptive immune response, including helping CD8+ T cell differentiate into CTLs, and helping B cells make antibodies. They can also act as effector cells themselves through the production of multiple cytokines, and have recently been shown to be able to acquire cytotoxic properties, including cytotoxic granules and Fas:FasL interactions. T_H cells can also recognise antigens
acquired and presented by macrophages, and deliver signals that trigger different macrophage responses to the pathogen\textsuperscript{28}. Activated $T_H$ cells also play a pathogenic role in autoimmunity and allergic responses. These different functions of $T_H$ cells are achieved through differentiation of naïve CD4$^+$ T cells when stimulated with the cognate antigen\textsuperscript{29}, discussed in more detail in later section.

Importantly, it is now clear that some CD4$^+$ and CD8$^+$ T lymphocytes can also differentiate into regulatory T cells (T\textsubscript{REGS}) necessary for suppressing conventional lymphocytes in order to inhibit an excessive immune response, to prevent collateral damage and autoimmunity\textsuperscript{30}. The ratio between, and function of, these activated effector CD4$^+$ and CD8$^+$ T lymphocytes, and the properties of the pathogen, determine the course and response of the adaptive immune system.

### 1.2 Antigens

Any substance recognised and responded to by the immune system is referred to as an antigen. Antigens can be molecularly diverse, including proteins, polysaccharides and lipids. This thesis will largely focus on T cells, which generally recognise proteins, although there are some notable exceptions, such as the Natural Killer-like T cells (NKT cells) to be discussed later, which recognise glycolipids. Importantly T cells do not generally recognise intact antigens, but rather fragments that are presented on the cell surface by Major Histocompatibility Complex (MHC) molecules\textsuperscript{31}. The process by which the proteins are processed into peptides and presented via MHC, which occurs in all nucleated cells, is called antigen presentation, and is critical for the priming and effector phases of a T cell-mediated immune response.

Antigens that are unique to cancer cells, or are over-expressed relative to healthy tissue are known as tumour-associated antigens (TAAs). This thesis focuses on targeting
peptides from three TAAs, overexpressed in a range of tumour types including glioma$^{32,33}$.

### 1.2.1 Antigen-presenting cells (APCs)

APCs are cells that acquire and display foreign antigens in the context of MHC molecules to initiate an immune response. By constantly sampling the local environment they are able to acquire potentially harmful foreign antigens and present them to CD4$^+$ and CD8$^+$ T cells in the lymphoid tissue. Antigen uptake can occur through a variety of processes including phagocytosis, pinocytosis and receptor-mediated endocytosis. Phagocytosis in Greek means to “devour cells” and is the process by which APCs engulf large solid particles. Similarly, pinocytosis, or ‘cell-drinking’, is the engulfment of smaller particles. APCs can also acquire antigens through external receptor recognition in a process known as receptor-mediated endocytosis$^{34}$. Presentation involves processing of the antigen into peptides, with some fragments displayed on the APC surface via MHC molecules. Any T cell bearing a TCR that recognises antigen-derived peptide within the binding groove of an MHC molecule, can potentially be activated by the presenting APC. However, the APC must receive other ‘danger signals’ from the environment to efficiently present peptide to the T cell. Activated T cells undergo significant clonal proliferation and acquire effector capabilities, such as the ability to lyse cancerous cells. It is therefore the role of APCs to orchestrate a successful adaptive immune response.

### 1.2.2 Pathways of antigen presentation

There are two ‘classical’ pathways of antigen presentation operating in APCs, with antigen either presented via MHC class I molecules to CD8$^+$ T cells, or via MHC class II molecules to CD4$^+$ T cells. The MHC class I presentation pathway is involved in presenting internal, or ‘endogenous’ antigens (including antigens from intracellular
pathogens or TAAs) and is active in all nucleated cells. The MHC class II presentation pathway is involved in presenting acquired, or “exogenous” antigens, and is unique to APCs. There is an additional pathway, called cross-presentation, in which exogenous antigens are diverted into the MHC class I presentation pathway, which appears to have enhanced activity in some APCs.\(^{35}\)

Endogenous antigens are derived from cytosolic proteins and processed by the proteasome into peptides 8-12 amino acids long. The peptides are then transported into the endoplasmic reticulum (ER) from the cytosol via the transporter associated with antigen processing (TAP). The processed peptide is loaded onto MHC class I molecules, which consists of two polypeptide chains, a MHC class I alpha(\(\alpha\))-chain and Beta(\(\beta\))-2-microglobulin (\(\beta2m\)). The \(\alpha\)-chain is stabilised by calnexin prior to dimerising with \(\beta2m\). After assembly of the chains, calnexin dissociates. Once the MHC class I molecule is loaded with peptide, the complex leaves the ER via the secretory pathway to reach the cell surface. The array of peptide/MHC class I complexes on the surface gives an external ‘inventory’ of the protein content of a cell, enabling pathogenic changes to be identified. This recognition involves interaction with specific TCRs on CD8\(^+\) T cells. In this way, an infected APC is able to activate CTLs.\(^{36}\) It is also the process by which infected or neoplastic cells are identified and killed by activated CTLs (Figure 1.1 A).

Exogenous antigens can be acquired by APCs via macropinocytosis or phagocytosis, or engulfed by receptor-mediated endocytosis. Once internalised, the protein is degraded into peptides by peptidases, resulting in peptides between 12-24 amino acids in length. The MHC class II molecule consists of \(\alpha\)- and \(\beta\)-chains complexed to a polypeptide chain called the invariant chain (\(\text{Ii}\)), that serves to block binding of peptides and misfolded peptides. Ii is cleaved in an acidified endosome, leaving a short peptide known as class II-associated invariant chain peptide (CLIP) blocking the peptide groove. Endocytosed antigens are processed into peptides in endosomes,
which merge with other endosomes that contain the MHC class II molecule. An MHC class II-like molecule called H-2M in mice (HLA-DM in humans) binds to the MHC class II molecule, releasing CLIP and allowing for peptides to bind\textsuperscript{37}. The MHC class II molecule is stable again once peptide is bound, and is transported and presented on the APC cell surface. Peptide and MHC class II molecule complexes can interact with specific TCRs on CD4\textsuperscript{+} T cells, and therefore activate T\textsubscript{H} cells (Figure 1.1 B)\textsuperscript{38}.

These two classical pathways alone cannot provide the mechanism by which vaccine therapies can induce a CTL response, as vaccines deliver exogenous antigens that would be presented on MHC class II molecules and therefore only activate CD4\textsuperscript{+} T cells. However, as vaccines have successfully activated CD8\textsuperscript{+} T cells, another functional pathway of antigen presentation exists. Cross-presentation involves certain APCs processing and presenting exogenous antigens on MHC class I molecules to CD8\textsuperscript{+} T cells\textsuperscript{35}. The exact mechanism of cross-presentation remains poorly understood, although it is likely that it involves release of antigens from endocytic vesicles into the cytoplasm of the cell, where the MHC class I molecule presentation process is initiated. Alternatively, it may involve merging of endocytic vesicles with other vesicles involved in recycling MHC molecules to the cell surface\textsuperscript{39}. Regardless of the mechanism of cross-presentation, a vaccine that exploits cross-presenting APCs and activates CTLs is a desirable cancer therapy (Figure 1.1 C).
Figure 1.1: Antigen processing pathways of APCs. (A) Peptides derived from endogenous protein and degraded in the cytosol are presented on MHC class I molecules. (B) Acquired exogenous proteins are processed in endosomes and presented on MHC class II molecules. (C) Exogenous antigen is transferred to the endocytic pathway and presented on MHC class I in a process called cross-presentation. Adapted from Villadangos and Schnorrer.

1.3 Dendritic cells (DCs)

DCs are referred to as ‘professional’ APCs due to their superior ability to co-ordinate adaptive immune responses compared to other APCs, such as monocytes and B cells. Immature DCs, which have not been exposed to any signals that indicate danger to the host, are very efficient at antigen uptake through phagocytosis, pinocytosis and receptor-mediated uptake mechanisms. Immature DCs express only low levels of MHC molecules and other costimulatory molecules required for T cell activation, and
therefore cannot readily activate T cells; they may even induce differentiation of T cells into T<sub>REGS</sub> that serve to suppress immune responses.

When an immature DC acquires antigen in the context of stimuli from damaged or infected tissue, it matures, marked by decreased antigen uptake and upregulation of MHC and costimulatory molecules, which are important for signal transduction and the development of an effective immune response. This phenotypic change is also associated with migration to the appropriate regions of the lymphoid tissue. Only activated DCs can provide the signals required for differentiation of T cells, with the appropriate antigen-specific TCR, into effector cells<sup>41</sup>.

### 1.3.1 Dendritic cell activation

As activation of DCs is critical for stimulating adaptive immune responses, there has been considerable attention given to defining the manner in which this can be achieved. Activation can be triggered by stimulation of pattern recognition receptors (PRRs), which recognise a diverse array of pathogen- or danger-associated molecular patterns (PAMPs and DAMPs respectively). These are molecular structures associated with microbial pathogens, such as lipid structures from bacterial cell walls or viral nucleic acid structures, or ‘danger’ molecules released from cells under stress, such as adenosine triphosphate (ATP) or uric acid.<sup>42</sup> Accordingly, many of these structures have been used to deliberately mature DCs in vaccination strategies, such as the use of monophosphoryl lipid A (MPL) as a mimic of lipopolysaccharide (LPS) found in bacteria cell wall structures. These DC-maturing compounds, which improve responses to vaccination, are termed immune adjuvants as they enhance the body’s immune response to an antigen.

Driving T cell responses that differentiate into useful effector cells also commonly requires DCs to be stimulated via the costimulatory molecule CD40. Many effective
CD8+ T cell responses require this form of “help” from CD4+ T cells, which usually occurs when the antigen recognised by the CTL is from a pathogen that does not cause initial inflammation. In these situations, CD4+ T cell help is required to activate DCs to cause a competent CD8+ T cell response, in a process known as ‘licensing’. Licensing involves the induction of CD40 on the DC, interacting with CD40-ligand (CD40L) expressed by activated T cells. This interaction is regarded as a ‘positive feedback’ signal that increases the function of DCs once an immune response has been initiated. The binding of CD40 on DCs induces enhanced activation associated with further upregulation of other costimulatory molecules, CD80 and CD86 (CD80/86), and increased release of the inflammatory cytokine, interleukin(IL)-12, which potentiates differentiation of antigen specific CD4+ and CD8+ T cells into potent effector cells. DC activation can directly and indirectly enhance stronger CTL responses through both CD40:CD40L interaction and cytokine production.

1.3.2 Invariant natural killer T (iNKT) cells can activate dendritic cells

The vaccines used in this thesis are based on the novel observation that there are subpopulation of T cells with shared TCR structures that are able to provide a rapid and potent source of CD40 signals to DCs. As noted earlier, a feature of T lymphocytes, is the diversity of antigen receptors generated through gene rearrangement. There are however, some subsets of T lymphocytes that show limited diversity, and are notable in that they exhibit features more akin to cells of the innate immune system, such as ability to produce cytokines rapidly to respond efficiently to pathogens. These cells are therefore known as innate-like T cells (ILT)47.

A subset of ILTs, are iNKT cells, which possess an invariant TCR α-chain, Vα24Jα18 in humans and Vα14Jα18 in mice, where they are abundant in the spleen, bone
marrow, thymus and liver\textsuperscript{48}. While conventional T cells have a TCR that recognises peptides bound to MHC, the invariant TCR (iTCR) of iNKT cells recognises glycolipids bound to an MHC class I-like complex, CD1d\textsuperscript{49}. The invariant nature of both the iTCR and CD1d molecule ensures that the same glycolipid antigens can be recognised by a large number of iNKT cells in all individuals in a population. In fact, the same glycolipids can be recognised by iNKT cells in different mammalian species\textsuperscript{50}.

When DCs present a glycolipid on CD1d to the iTCR, iNKT cells upregulate CD40L and produce inflammatory cytokines, IL-4 and IFN-\(\gamma\). Binding of CD40L to CD40, along with inflammatory cytokines, enhances activation of DCs. Experiments in animals have shown that inclusion of glycolipids for iNKT cell activation in vaccines is sufficient to drive this very potent process, resulting in strong conventional T cell responses to the antigen in the vaccine\textsuperscript{51-54}. Such iNKT ligands can therefore be considered as useful immune adjuvants\textsuperscript{56} (Figure 1.2).
Figure 1.2: The use of iNKT cells as cellular adjuvants. iNKT cells can provide costimulatory molecules (CD40L) and cytokines (IL-4, IFN-γ) that increase DC activation. Activated DCs upregulate CD80/86 and produce IL-12 that further enhances CTL activation and effector function. Adapted from Thaiss et al., 201156.
1.4 T cell immunity

The common lymphoid progenitor in the bone marrow gives rise to the antigen-specific B and T lymphocytes of the adaptive immune system. This thesis specifically investigates T cell-mediated immunity, which is overviewed below. Antibodies were used in the research to be presented, but simply as injected reagents with defined targets. The process of B cell development, activation and antibody production will therefore not be covered in detail in this introduction.

1.4.1 T cell development and specificity

Lymphocytes are a subset of white blood cells (WBCs) that circulate through the lymphatic system into the bloodstream and, as the name suggests, are found in large numbers in the lymphoid organs. The central or primary lymphoid organs, comprising of the thymus and bone marrow, are where lymphocytes are generated and mature. The peripheral or secondary lymphoid organs, including the spleen and lymph nodes, maintain mature naïve lymphocytes and initiate an adaptive immune response. The bone marrow is the site where B and T cell progenitors are generated. Maturation of B lymphocytes continues in the bone marrow, while T cell progenitors migrate to the thymus for maturation. Mature lymphocytes enter circulation and accumulate in peripheral lymphoid tissue as ‘naïve’ lymphocytes that have yet to be exposed to antigen. An adaptive immune response occurs when a lymphocyte interacts with, and recognises, a specific antigen in the context of other signals of ‘danger’ to the host, the latter generally detected first by cells of the innate immune system\(^57\).

Every T lymphocyte has multiple copies of a single randomly generated TCR, which is formed from combination of \(\alpha\)- and \(\beta\)-chains, or gamma-(\(\gamma\)) and delta-(\(\delta\)) chains. In \(\alpha\beta\) TCRs, found on the majority of T cells including CD4\(^+\) and CD8\(^+\) T cells, the vast diversity of recognition is achieved through random somatic rearrangements of the
diversity (D), joining (J), variable (V) and constant (C) gene segments. D-to-J recombination in the β-chain occurs first, and is followed by Vβ-Dβ-Jβ gene rearrangement. The α-chain of the TCR is then rearranged, where V-to-J rearrangement takes place prior to addition of the C chain.\(^{58}\) Assembly of the rearranged α- and β-chain results in novel amino acid sequences in antigen binding regions, allowing for greater receptor diversity (~10\(^{18}\)). The vast majority of T cells respond to short amino acid sequences within the structure of a protein, which must be unfolded and processed into peptide fragments, or epitopes, to be recognised within the context of MHC molecules.\(^ {59}\) Collectively these T cells respond to a great range of antigens due to variations of the antigen-binding site that occurs via gene rearrangement.

T cells express various unique cell surface molecules useful for immunophenotyping. In the thymus, precursor T lymphocytes do not express CD4 or CD8 molecules and are classed as double-negative (DN, CD4\(^{-}\)CD8\(^{-}\)) cells. As they progress through development in the thymus they become double-positive (DP, CD4\(^{+}\)CD8\(^{+}\)) thymocytes. However, mature T lymphocytes are generally either CD4\(^{+}\) or CD8\(^{+}\) T cells. A central theory to T cell development is that DP thymocytes terminate the transcription of one co-receptor molecule, either CD4 or CD8, to become a single positive (SP, CD4\(^{+}\)CD8\(^{-}\) or CD4\(^{-}\)CD8\(^{+}\)) T lymphocyte. A process termed positive selection selects for T cells capable of interacting with MHC molecules. It is the type of MHC molecule the T cell interacts with that determines which co-receptor transcription will be terminated. DP cells capable of interacting with MHC class II molecules mature into CD4\(^{+}\) T cells, and DP capable of interacting with MHC class I molecules mature into CD8\(^{+}\) T cells.\(^ {60}\) However, it has recently been suggested that DP thymocytes initially terminate CD8 co-receptor transcription even when differentiating into CD8\(^{+}\) T lymphocytes. Thymocytes that successfully terminate CD8 transcription can be rescued via IL-7, to reinitiate CD8 transcription and terminate CD4 transcription. This event has been termed ‘co-receptor reversal’ and has challenged the current theory for the mechanism
of CD4/CD8 lineage determination. A second process termed negative selection eliminates T cells capable of binding strongly to self-peptides, to prevent potentially harmful activation when the self-peptide is later encountered in the peripheral tissues. Positive and negative selection are both equally important processes for development of functional T lymphocytes to generate appropriate immune responses.

Mature naïve T cells enter the circulation and migrate to peripheral lymphoid tissues. When an antigen-specific T cell response is initiated, T cells with the appropriate peptide-specific TCR undergo clonal proliferation and differentiation, creating an army of genetically identical antigen-specific T cells that can eliminate cells expressing the targeted antigen. These effector T cells leave the lymphoid tissue to circulate back to sites where the target antigen is found, guided by other signals of infection, or signals associated with tissue perturbation that is often observed in tumours. Recognition at this site is again via MHC molecules presenting peptides from the antigen on the surface of the target cells. This allows for the T cells to precisely recognise, bind and exert effector functions towards target cells.

**1.4.2 T cell effector mechanisms**

Different types of effector T cells have specialised effector functions aimed at eliminating pathogens or directly killing infected or cancerous cells. Activated CD8+ T cells can differentiate into CTLs, which are capable of directly killing tumour cells. However, under some conditions they can differentiate in CD8+ TREGS that play a role in suppressing the immune system. The principle mechanism of CTL killing is controlled by the release of cytotoxic granules stored in lysosomes. These cytotoxic granules include perforin, granzymes and granulysin. Perforin aids the delivery of granules to the target cell, granzymes activate apoptosis once in the cytoplasm of the target cell and granulysin is an antimicrobial protein that also induces apoptosis. CTLs are able to selectively kill target cells displaying a specific antigen without killing
themselves or neighbouring cells$^{66}$. Once a CTL recognises a target cell, it will rearrange its secretory apparatus towards the target cell, ensuring focused killing$^{67}$. Not only is the killing direct, but also rapid as the cytotoxic granules are pre-stored in an inactive environment inside the lysosome. This allows for a single CTL to kill a series of targets in succession. CTLs can also kill cells through Fas:FasL mediated apoptosis pathway. Fas contains a death domain in its cytoplasmic domain and when bound by FasL induces death by apoptosis in the target cell$^{68}$. Cells that undergo apoptosis are ingested by phagocytic cells, broken down and completely digested$^{69}$. Therefore, apoptosis is considered a ‘quiet’ process that does not cause an influx of immune cells or an immune response. CTLs are also capable of releasing cytokines such as IFN-$\gamma$, which recruits other effector cells and can induce upregulation of MHC class I molecules$^{70}$ to increase antigen recognition.

Activated CD4$^+$ T cells differentiate down distinct pathways to a wider repertoire of subsets, each with different effector mechanisms. The main subsets are Th1, Th2, Th17 and regulatory T cells (TREGS). Th cells produce cytokines capable of killing cancerous cells and also controlling pathogens. Th1 cells produce cytokines, such as IFN-$\gamma$ and IL-12, that activate macrophages, enabling them to control intracellular bacterial infections$^{71}$. Th2 cells produce cytokines, including IL-4, IL-5 and IL-13, that recruit and activate eosinophils, basophils and mast cells, to control parasitic infections$^{72}$. Th17 cells produce IL-17 that induces epithelial cells to produce chemokines that recruit neutrophils to the site of infection$^{73}$. These effector cells work to clear pathogens from the body, but their role in anti-tumour immunity, perhaps even killing cancer cells, is controversial. It is clear that appropriately differentiated Th cells play important “helper” roles, such as helping activate B cells to release antibodies and macrophages to destroy ingested pathogens. Perhaps more important to this thesis, they supply help to CTLs via CD40:CD40L interactions and inflammatory cytokines to increase their activation and capacity to kill cancerous target cells.
Both CD4+ and CD8+ T cells can differentiate into T\textsubscript{REGS}, which serve to limit activation and effector functions of conventional T cells. Regulating an immune response is necessary for preventing autoimmune responses and collateral damage. Two main groups of T\textsubscript{REGS} exist, natural T regulatory cells (nT\textsubscript{REGS}) and induced T regulatory cells (iT\textsubscript{REGS}). Differentiation of T\textsubscript{REGS} that occurs in the thymus produces nT\textsubscript{REGS}, while differentiation of T cells caused by environmental factors in the periphery produces iT\textsubscript{REGS}. The suppressive effects of T\textsubscript{REGS} are mediated through release of IL-10 and transforming growth factor-β (TGF-β), upregulation of inhibitory molecules and inhibition of costimulatory molecules\textsuperscript{74}. Although T\textsubscript{REGS} serve an important function for opposing an immune response, they can be problematic in cancer settings as they provide protection to tumours by suppressing conventional T cell effector mechanisms in the tumour microenvironment (TME)\textsuperscript{75}.

As previously mentioned, the cytokine IFN-γ plays a role in effector functions of T cells. Extrinsic activities of IFN-γ include increased survival and activation\textsuperscript{76}, proliferation\textsuperscript{77} and effector function\textsuperscript{78,79} of both CD4+ and CD8+ T cells. The cytokine has also been shown to improve activation and cross-presentation of DCs\textsuperscript{80} and suppress myeloid-derived suppressor cells (MDSC)\textsuperscript{81} and T\textsubscript{REGS}\textsuperscript{82-84}. It is important to note that IFN-γ can also act directly on tumour cells to upregulate MHC class I molecules\textsuperscript{85-88} and improve antigen expression\textsuperscript{89}.

Since potent T cell effector functions can cause such destruction, it is important that they are controlled. In this context, inhibitory molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1) serve as inhibitory checkpoints and are upregulated on the surface of a T cell after activation, preventing T cell proliferation and avoiding further stimulation. These molecules are topics of this thesis, and will be discussed in more detail later.
Many argue that CTLs are more important for cancer control than T\textsubscript{H} cells, as they can directly kill tumour cells\textsuperscript{28,63}. It is for this reason that induction of a CTL response has been the focus of vaccine development leading up to this study. However, analysis of immune responses in patients that have responded to immunotherapies, such as checkpoint blockade, have indicated that CD4\textsuperscript{+} T cells play a greater role than originally thought, and should be considered in any new immunotherapy.

### 1.4.3 CTL-mediated killing of cancer cells

The concept that T cells can control cancer has been validated through a variety of clinical procedures, including bone marrow transplantation (BMT), adoptive T cell transfer and immune checkpoint blockade. BMT replaces the immune system with a replete immune system that has capacity to offer a chance of cure or long-term remission, due largely to the donor T cells provoking an allogeneic response to the host tumour. Survival rates from this therapy have been gradually improving with increased understanding of the mechanisms involved\textsuperscript{90}. Adoptive T cell transfer involves isolation and \textit{ex vivo} expansion of tumour-specific T cells (ie. T cells that have infiltrated the tumour) that are then injected back into the patient. This technique has been used to treat patients with advanced melanoma and colorectal carcinoma\textsuperscript{91}. More recently, immune checkpoint blockade has been shown to be a remarkably effective cancer therapy in diseases that have not seen improvements in treatment for many decades, such as advanced melanoma. Inhibitory checkpoint molecules, such as CTLA-4 and PD-1 on T cells and there cognate ligands, are necessary for dampening down effector responses of activated T cells to prevent autoimmunity. Immune checkpoint inhibitors are antibodies or small molecules that block signalling through inhibitory receptors, preventing the inhibition of T cell function. Currently, there are FDA approved immune checkpoint blockade drugs used for the treatment of melanoma and lung cancer\textsuperscript{92}. 

1.5 T cell-mediated immunotherapy

Harnessing a potent T cell response capable of exerting anti-tumour response is a desirable therapeutic strategy. However, with the exception of Provenge – a vaccine for castration-resistant prostate cancer\textsuperscript{93} - no vaccines have been approved for routine clinical use due to poor clinical trial results\textsuperscript{94-97}. It is clear that vaccination as a monotherapy will not be a panacea for cancer. Instead, researchers are investigating combinational therapies, where vaccination stimulates a T cell response that is enhanced with the addition of other therapies, such as immune modulators\textsuperscript{98}.

1.5.1 Immune surveillance and tumour suppression

In the 1950s, Burnet and Thomas postulated the ‘Immune Surveillance’ hypothesis that the immune system can remove malignant cells. This hypothesis was further refined by Schreiber to the ‘Immunoediting’ hypothesis, which involves three phases of the immune system to detect and destroy tumour cells. The elimination phase is the first phase, where tumours that arise in a tissue can be recognised and eliminated by a variety of immune cells, including CTLs. If elimination is unsuccessful, the tumour cells can undergo mutations during the second phase or equilibrium phase, becoming more resistant to cell death. In the final phase, the escape phase, the cancer cells have accumulated adequate mutations and modified the TME such that they escape immune-mediated killing mechanisms. The altered TME in this phase tends to favour recruitment of suppressive T\textsubscript{REGS} and other cells with regulatory qualities such as immature myeloid cells that function as MDSCs\textsuperscript{99}.

In an immunocompetent individual, the immune system is generally capable of eliminating immune cells or maintaining equilibrium, although the tendency for this to fail increases with age. In an immunodeficient person, this equilibrium is in jeopardy. It has been shown that some recipients of organ transplants have developed
tumours of donor origin. In this scenario, the donor can sustain equilibrium over the cancer cells in the organ, but once engrafted into the recipient, who will be on immunosuppressive drugs for the treatment, equilibrium fails\(^\text{100}\).

Tumours acquire numerous mechanisms to avoid immune recognition by T cells, including being seen by the immune system as ‘weakly immunogenic’. This is achieved by downregulating MHC molecules expressing peptide, the antigen itself, or other molecules critical for the T cell to recognise and exert effector functions against the tumour. Additionally, tumour antigens taken up and presented by APCs in absence of costimulation, will tolerise any antigen-specific T cell rather than activate it\(^\text{101}\). Furthermore, established tumours are capable of releasing factors that suppress T cells and even induce T\(\text{REGS}\), such as TGF-\(\beta\), IL-10 and indolamine 2,3-dioxygenase (IDO)\(^\text{75}\). Therefore, successful T cell based therapies have to counteract this suppression by stimulating potent T cell responses that overcome suppressive mechanisms induced by the tumour and its microenvironment.

### 1.5.2 Vaccination and modulating the immune system

Many vaccine strategies have been employed to create strong T cell responses against tumours. Irradiated whole tumour cell vaccines are designed to deliver the entire cellular content of resected tumour tissue cells to the immune system in a safe but immunogenic manner. These vaccines are advantageous as they avoid the long and difficult task of determining the presence of TAAs on the surface of the tumour. Unfortunately, the production process and quality control of these vaccines is complex\(^\text{102}\). Immunogenicity can be increased by loading the cellular material onto DCs. However, preparing DC vaccines is costly, timely and complex as each vaccine is customised to the patient\(^\text{103}\). An alternative is to develop synthetic peptides vaccines that target defined TAAs on the surface of the tumour cells. These vaccines that encode TAAs are relatively cheap and easy to manufacture and store, as well as
reproducible\textsuperscript{104}. Peptide vaccines can engineer highly targeted immune responses against a selected target, and therefore avoid off-target responses\textsuperscript{105}.

As already noted, there have been spectacular successes in the clinic with checkpoint blockade. These inhibitors aim to unleash T cell responses that are otherwise constrained by checkpoint molecules. Antagonistic antibodies prevent interactions between checkpoint inhibitory receptors on T cells, and their ligands on the tumour cells or APCs, thereby maintaining an active T cell response\textsuperscript{106-108}. Although checkpoint inhibition has confirmed the concept of stimulating a potent T cell response in some patients, it is clear that not all patients respond. Checkpoint inhibitors are only effective in unleashing anti-tumour responses if activated tumour-specific T cells are present in patients in the first place. For immunogenic cancers like melanoma, it has long been known that T cell responses can arise naturally in some patients, which have been observed as tumour-infiltrating lymphocytes (TILs). It is possible patients that fail to respond to checkpoint blockade do not have existing responses that can be unleashed. Stimulating a T cell response in patients with vaccination may overcome this limitation of checkpoint inhibition.

While checkpoint blockade is leading the charge of new therapies to the clinic, there are other targets being considered that modulate the immune response to improve T cell responses. These include agonistic antibodies that bind costimulatory molecules and enhance activation of T cells\textsuperscript{109}. Modulation of the immune system can also be achieved with monoclonal antibodies that deplete T\textsubscript{REGS} and therefore remove suppressive effects associated with them.

Vaccination, checkpoint inhibitors, costimulatory agents and the depletion of T\textsubscript{REGS} all have the same end goal to stimulate potent T cell responses to target tumour tissue. Vaccines that initiate T cell responses or improve weak responses could potentially combine synergistically with these agents. A commonly used analogy for vaccines and
checkpoint inhibitors is that vaccines ‘push the accelerator’ while checkpoint inhibitor antibodies ‘take the foot off the brake’. Costimulatory agents can be seen as an addition of a ‘brick on the accelerator’, while depleting T_{REGS} is ‘further release of the brake’. Combining these therapies may lead to a beneficial, synergistic anti-tumour response, compared to a single therapy alone.

1.5.3 Peptide vaccination can direct CTL immune responses

Vaccination works best when administering antigenic material in the presence of an adjuvant that can improve antigen uptake and/or increase the activation status of APCs\textsuperscript{110}. Vaccination with defined TAAs can limit non-specific, off-target killing and reduce unintended collateral damage, as the generated T cell response is specific for the tumour. However, there has been limited evidence of clinical benefit using current vaccine strategies.

Early studies in a murine model of infection with lymphocytic choriomeningitis virus (LCMV), a peptide-specific CTL response following vaccination prevented chronic viral infection\textsuperscript{111,112}, validating the use of vaccination to induce CTLs. The discovery that CTLs recognise small peptides of around 8-12 amino acids in length bound to MHC class I molecules\textsuperscript{113,114}, and the development of methods to identify ‘optimal’ antigenic peptides\textsuperscript{115}, greatly facilitated the use of peptide vaccines to induce CTL responses. A major obstacle found in early vaccination development, was that administrating peptide alone failed to generate a strong immune response\textsuperscript{116-119}. Animal studies overcame this limitation with the addition of adjuvants to boost the CTL immune response, particularly those that improved APC function\textsuperscript{116,120-122}. 
1.5.4 Design of synthetic conjugate peptide vaccines to stimulate CTLs

Previously, our laboratory designed a iNKT-cell dependent glycolipid-peptide conjugate vaccine, that increased iNKT cell activation and suppressed Th2-derived allergic airway inflammation. It was also observed that co-delivery of both components to the same DC allowed for the glycolipid adjuvant to activate iNKT cells, causing increased CD40:CD40L interaction and inflammatory cytokine production. In turn, this enhanced the activation of the cross-presenting DCs leading to increased CD80/86 expression and a stronger antigen-specific CTL response. These conjugate vaccines were shown to activate fewer iNKT cells than free glycolipid adjuvant in the unconjugated admixed controls. Free glycolipid from the admixed controls has the ability to readily bind CD1d and activate iNKT cells whereas the vaccines must first be cleaved in order to release the adjuvant. This additional step of vaccine processing may explain reduced iNKT cell activation with the conjugate vaccines.

Following the success of the conjugate vaccine in an allergy model, the iNKT-cell dependent glycolipid-peptide construct was used to design a series of therapeutic conjugate vaccines that expand specific populations of anti-tumour T cells. These refined conjugated vaccines showed increased CD8+ T cell responses and potent therapeutic activity in an aggressive model of melanoma.

This thesis investigates three novel iNKT-cell dependent glycolipid-peptide conjugate vaccines, for the treatment of GBM. The vaccines consist of three components; an adjuvant, a linker and a defined peptide from a known TAA. The adjuvant, alpha-Galactosylceramide (α-GalCer; Figure 1.3) is a known ligand for iNKT cells. This glycolipid has been shown to be a powerful adjuvant when simply admixed with antigen, which has successfully demonstrated an increase in immunogenicity in a variety of studies. For these studies, the possibility that α-GalCer is
internalised and presented on CD1d molecules within the same APC that acquires the peptide antigen is left to chance. Alternatively, our conjugate vaccines aim to co-deliver antigenic peptide and α-GalCer to the same APC, by conjugating the components together. This increases the likelihood that peptide presented by an APC has received sufficient stimulatory signals from the adjuvant, and thereby adopts the necessary phenotype for stimulating T cells.

Conjugation of α-GalCer to the peptide is achieved with protease-sensitive valine–citrulline–p-amino-benzyl (VC–PAB) carbamates, which focus the vaccines to splenic CD8α+ DCs, that efficiently cross-present antigen to stimulate CTLs. Delivery of the vaccine to cross-presenting DCs is preferable, as this allows for the peptide portion of the vaccine to be presented on MHC class I to CD8+ T cells. The protease sensitive linker can be cleaved by cathepsin B, a lysosomal cysteine protease that is involved in intracellular proteolysis. Cleavage of the self-immolating linker occurs in phagolysosomal compartments inside DCs, resulting in simultaneous presentation of α-GalCer and peptide from the same cell.

The VC-PAB linker conjugates the peptide of interest to a functionally inactive form of α-GalCer, that prevents premature iNKT cell activation before DC cleavage. The α-GalCer ‘prodrug’ has a nitrogen to oxygen acyl migration induced under acidic conditions. The nitrogen molecule is then capped with the enzymatically cleavable linker, providing a construct with an inactive form of α-GalCer (Figure 1.4 A). Once
internalised by a cross-presenting DC, and cleaved by cathepsin B, reverse oxygen to nitrogen migration reverts α-GalCer to an active state, capable of inducing iNKT cell activation (Figure 1.4 C). The peptide portion of the vaccine is also released following cleavage of the linker, and contains another cleavage site that requires further enzymatic processing to provide an epitope that can be presented on MHC class I molecules (Figure 1.4 B). The peptides incorporated into the vaccines are discussed in more detail below.

**Figure 1.4: Vaccine design and in vivo processing.** (A) Chemical structure of vaccine containing inactive form of α-GalCer. (B) Antigenic peptide cleaved from A is further processed to release defined peptide sequence. (C) Prodrug is released from A and rearranged to form active α-GalCer. Adapted from Anderson et al., 2015.

### 1.5.5 Peptides from TAAs for vaccine design

It is well understood that peptide epitopes from TAAs can be recognised by CTLs through MHC molecules, making TAAs appropriate targets for the vaccines. It has become widely accepted that TAA expression is heterogeneous among tumours from different patients and origins. Therefore, it is important to identify TAAs, which are expressed in a number of different cancers and restricted on healthy tissue. The novel conjugate vaccines target three antigens present in normal tissues but overexpressed in tumours; tyrosinase-related protein 2 (TRP-2), glycoprotein 100 (gp100) and survivin. The melanoma differentiation antigens, TRP-2 and gp100, are highly expressed on both melanoma and glioma cells, as melanocytes and glial cells are both derived from embryonic neural ectoderm. The apoptosis inhibitor
protein, survivin, is generally found in embryonic tissues\textsuperscript{129}, and is abundantly expressed in the thymus, testis and proliferating cells\textsuperscript{130}. However, survivin is overexpressed in many cancers, including gastrointestinal carcinoma\textsuperscript{131}, oesophageal cancer\textsuperscript{132}, lung carcinoma\textsuperscript{133}, breast carcinoma\textsuperscript{134}, prostate carcinoma\textsuperscript{135}, leukaemia\textsuperscript{136} and glioma\textsuperscript{129}, and when inhibited results in increased apoptosis\textsuperscript{33}. Incorporating TAAs expressed on multiple tumour types, increases the breadth of murine models and potentially widens clinical application of these conjugate vaccines, although the focus of this project is on glioma.

Numerous studies have previously shown vaccine-mediated anti-tumour responses when targeting TRP-2, gp100 and survivin in glioma and melanoma murine models. Potent anti-tumour CTL responses against subcutaneous (SC), intravenous (IV) and intracerebral (IC) glioma tumours have been found with a DNA vaccine expressing TRP-2\textsuperscript{137}. Furthermore, in murine melanoma models, TRP-2 antigen delivered via either cationic lipid (R)-DOTAP\textsuperscript{138} or lipid-calcium-phosphate (LCP) nanoparticles that preferentially deliver TRP-2 to DCs\textsuperscript{139}, were able to increase antigen-specific CTL responses and produce significant anti-tumour activity\textsuperscript{138}. Vaccines targeting gp100 have preferentially been explored in melanoma models, including potent CTL-mediated anti-tumour protection with DNA vaccines expressing gp100 alone\textsuperscript{140} or gp100 and granulocyte-macrophage colony-stimulating factor (GM-CSF)\textsuperscript{141}.

Research using the survivin peptide in a therapeutic peptide-loaded DC vaccine was able to induce potent CD8\textsuperscript{+} and CD4\textsuperscript{+} survivin-specific T cell responses\textsuperscript{142}, and increase survival against GL261 IC tumours\textsuperscript{143}. Furthermore, a survivin DNA vaccine slowed tumour growth and prolonger survival in murine pancreatic and lymphoma models\textsuperscript{144}.
1.5.6 Immune modulation with monoclonal antibodies

T cells possess numerous activatory and inhibitory receptors in order to control T cell mediated immune responses. When activatory receptors are engaged, the positive signals provide ‘positive feedback’ and amplify effector response. Conversely, when inhibitory receptors are engaged, ‘negative feedback’ is signalled to the T cells to diminish the immune response. The use of monoclonal antibodies that bind and signal activatory receptors, or alternatively interfere with the signalling of inhibitory receptors, can be applied to the immune system in an attempt to create enhanced T cell responses. Monoclonal antibodies can also bind receptors highly expressed on TREGS and effectively deplete them and remove their suppressive effects.

An aim of this thesis was to combine the vaccines with a range of monoclonal antibodies in an attempt to increase the immune response against glioma. The immune checkpoint inhibitors used include α-PD-1, α-CTLA-4 and α-LAG-3, which prevent inhibitory receptor activation on T cells. Conversely, the costimulatory agent, α-4-1BB, binds an activatory receptor and provides positive feedback to the T cell. Vaccination was also used under TREG depleted conditions (Figure 1.5). An overview of these immunomodulatory treatments follows.
Figure 1.5: Regulatory receptors of CTL activation. Inhibitory receptors (red) send negative signals to the T cells to dampen activity. Activatory receptors (green) amplify T cell activation. Monoclonal antibodies (grey) disrupt interactions of inhibitory receptors with ligands, or signal activatory receptors.

1.5.6.1 Immune checkpoint inhibitors

The immune checkpoint inhibitor CTLA-4 was the first to be targeted in the clinic. It is expressed on CD4+ and CD8+ T cells and regulates priming of these cells at early stages of activation. Upon recognition of an antigen and MHC molecule by a TCR, the costimulatory molecule CD80/86 expressed on APCs binds CD28 and amplifies T cell activation. After T cell activation, CTLA-4 is mobilised from intracellular stores to the surface of the T cell within an hour after antigen engagement, providing rapid negative feedback to diminish a prolonged immune response. The checkpoint molecule CTLA-4 opposes the activity of the T cell co-receptor CD28 by binding CD80/86 at a higher affinity than CD28. This interaction with CTLA-4 inhibits the T cells by preventing activatory signals from CD28, and also delivering inhibitory signals to the T cell. Engagement of CTLA-4 inhibits accumulation of the T cell.
growth factor IL-2, and upregulation of the IL-2 receptor alpha chain (IL-2Rα) is inhibited\textsuperscript{148}.

Mice deficient in CTLA-4 exhibit lymphoproliferation and multi-organ lymphocyte infiltration, which is fatal within 4 weeks after birth\textsuperscript{149-151}. These early studies provided evidence that CTLA-4 is a negative regulator of T cell-mediated immune responses. As the ligand for CTLA-4 is on APCs without tumour specificity, the application of the checkpoint inhibitor α-CTLA-4 was originally questioned, as there is potential for lethal systemic autoimmunity\textsuperscript{92}. The major physiological role of α-CTLA-4 involves modulating CD4\textsuperscript{+} T cells by downregulating Th activity and activating immunosuppressive activity of T\textsubscript{REGS}\textsuperscript{152}. Preclinical models demonstrated that a partial block of CTLA-4 could indeed lead to anti-tumour responses\textsuperscript{153}. These preclinical findings led to the production of the FDA approved, fully humanised monoclonal antibody ipilimumab (α-CTLA-4). Ipilimumab began clinical trials in the late 1990s. Phase I/II trials have shown anti-tumour responses in a range of tumour types including melanoma\textsuperscript{154}, renal cell carcinoma\textsuperscript{155}, urothelial carcinoma\textsuperscript{156}, ovarian cancer\textsuperscript{157} and prostate cancer\textsuperscript{158}. Following these results, phase III clinical trials for treating advanced metastatic melanoma with α-CTLA-4 were conducted. In a randomised phase III clinical trial for patients with advanced melanoma, patients received either a peptide vaccine targeting gp100, ipilimumab or a combination of both therapies. Patients who received ipilimumab had a 3.5 month increase in survival, irrespective of administration of the vaccine. Furthermore, 18% of patients who received ipilimumab survived beyond two years, compared with only 5% of patients who received the vaccine alone\textsuperscript{106}. Following this success, a phase III study of ipilimumab plus dacarbazine, a chemotherapeutic drug, was conducted in patients with previously untreated metastatic melanoma. Overall survival was significantly longer in patients with the combined treatment (11.2 months), compared to dacarbazine alone (9.1 months)\textsuperscript{159}. More recently, a phase II randomised trial investigating ipilimumab plus sargramostim, a granulocyte-macrophage colony-
General Introduction

stimulating factor used as an immune stimulator, found improved overall survival of the combined treatment (17.5 months) compared to ipilimumab alone (12.7 months)\textsuperscript{160}. These trials demonstrate the effectiveness of $\alpha$-CTLA-4 as a therapy for advanced metastatic melanoma. Further investigation into combinational therapy with $\alpha$-CTLA-4 and the conjugate vaccines may provide improved anti-tumour responses against glioma tumours.

The major role of PD-1 is to limit T cell activity in the periphery at the site of infection or cancerous tissue, and limit autoimmunity\textsuperscript{161}. PD-1 is upregulated on T cells after activation and when engaged by its ligands, kinases involved in T cell activation through phosphatase SHP2 are inhibited\textsuperscript{162}, thereby dampening the cells activity. PD-1 is expressed on CD4$^+$ and CD8$^+$ T cells and highly expressed on T$_{REUGS}$, with a large proportion of CD4$^+$PD-1$^+$ tumour-infiltrating lymphocytes (TILs) representing these T$_{REUGS}$. Conversely, CD8$^+$PD-1$^+$ T cells generally reflect an exhausted state, with reduced effector function and cytokine production\textsuperscript{163}. PD-1 is more broadly expressed than CTLA-4, and is induced on non-T lymphocytes such as NK\textsuperscript{164} and B cells\textsuperscript{165}. Blockade of PD-1 has been shown to enhance NK cell function in multiple myeloma\textsuperscript{166}, and may also have an effect on B cells. PD-1 has two known ligands, PD-L1 and PD-L2, which are expressed on APCs, tumour cells and myeloid cells in tumour microenvironment (TME). Not only is PD-1 expressed on TILs in many different cancers, but PD-L1/L2 are also upregulated on many different tumour cells\textsuperscript{167} such as epithelial cells, and serve as an evasion mechanism in the TME.

Unlike CTLA-4$^+$ mice, PD-1 deficient mice appear to develop and grow normally, but shown significant splenomegaly\textsuperscript{168}. The relatively mild phenotypes of these knockout mice suggest that blockade of PD-1 would result in less toxicity than blocking CTLA-4. Administration of $\alpha$-PD-1 has shown significant anti-tumour effects in B16 melanoma and CT26 colon carcinoma murine models\textsuperscript{169,170} as well as intracranial gliomas\textsuperscript{171}. Antibodies against PD-1 have undergone clinical trials and been FDA
approved for the treatment of melanoma. A phase I clinical trial with MK-3475, an α-PD-1 antibody, for advanced melanoma showed response in 38% of patients\textsuperscript{172}. This finding led to the FDA approval of pembrolizumab (MK-3475) in September 2014 for treatment of melanoma. Following approval, Phase II trials with pembrolizumab have shown improved progression-free survival in 34-38% of the patients, compared to 16% in patients treated with chemotherapy\textsuperscript{173}. Nivolumab, another antibody against PD-1, also underwent a phase I trial for melanoma, non-small-cell lung cancer, castration-resistant prostate cancer, renal-cell cancer and colorectal cancer and found antitumour responses across the cancers\textsuperscript{174}. A phase III trial of nivolumab against advanced melanoma showed promise with an overall survival rate of 72.9% patients at 1 year, leading to FDA approval in December 2014 for treatment of melanoma\textsuperscript{175}. Two phase III trials comparing the efficacy of ipilimumab (α-CTLA-4) with pembrolizumab (α-PD-1) in patients with melanoma, found that blocking PD-1 improved responses compared to blocking α-CTLA-4\textsuperscript{176}. However, long-term durability of PD-1 blockade compared to CTLA-4 remains to be investigated. Combinational therapy of PD-1 and CTLA-4 blockade has significantly improved objective response rates in patients with melanoma\textsuperscript{177,178}, and phase III trials are currently being investigated in a range of tumours.

Lymphocyte activation gene 3 protein (LAG-3) shares 20% identity with the human CD4 gene and unsurprisingly binds MHC class II with high affinity\textsuperscript{179}. LAG-3 is up-regulated on T cells several days after T cell activation\textsuperscript{180} and is highly expressed on T_{REGS}\textsuperscript{181}. The inhibitory receptor is also expressed on NK cells, B cells and plasmacytoid DCs (pDC)\textsuperscript{145}. LAG-3\textsuperscript{−/−} mice have been shown to have a defect in downregulation of T cell responses, implying a role of LAG-3 in inhibiting T cells\textsuperscript{182}. The LAG-3 receptor has dual roles dependent on the cell that expresses it. It has been previously shown that T_{REGS} in mice that lack LAG-3 have reduced regulatory activity\textsuperscript{181}. Another study showed that α-LAG-3 enhanced effector functions of CD8\textsuperscript{+} T cells, suggesting that inhibition of LAG-3 may be a potential treatment for cancer\textsuperscript{183}. Furthermore, LAG-3
expression on conventional T cells has been shown to increase susceptibility to suppression from T\textsubscript{REGS} and also skew to a T\textsubscript{H1} response\textsuperscript{184}. Exhausted T cells also up-regulate LAG-3\textsuperscript{185}, which is commonly co-expressed with PD-1. Blockade of LAG-3 alone was found to be insufficient at restoring the T cell response, however, when combined with blocking PD-1 a stronger T cell response was found than with PD-1 blockade alone\textsuperscript{186}. This suggests that targeting TILs that co-express LAG-3 and PD-1 may be a more effective anti-tumour strategy.

LAG-3 encodes a spliced variant that is translated into a soluble form of LAG-3 (sLAG-3), which exhibits immune adjuvant activity. sLAG-3 also binds MHC class II molecules, however only if the molecules are present in lipid raft microdomains on minor sets of APCs\textsuperscript{187}. There is no known role of sLAG-3 inhibiting T cells, however, it is possible that sLAG-3 affects interactions of LAG-3 with MHC II and therefore indirectly affects T cell function. It is important to note that clinical application of α-LAG-3 does not aim to target sLAG-3, but instead membrane-bound LAG-3 on the surface of T cells.

Although currently not FDA approved, the clinical relevance of α-LAG-3 has been investigated. Phase I trials began in 2007, where α-LAG-3 (IMP321) was administrated with the standard influenza vaccine. No toxicity or increased humoral response was observed, however, an increase in T\textsubscript{H1} cells was found\textsuperscript{188}. In 2010 a phase I/II trial used α-LAG-3Ig fusion protein combined with paclitaxel, a chemotherapeutic agent, against metastatic breast carcinoma and found an objective tumour response rate of 50\%\textsuperscript{189}. With this preliminary success, there is potential for α-LAG-3 to be available in the clinic in due course.
1.5.6.2 Costimulatory agents

While the previously mentioned antagonistic antibodies work to interrupt inhibitory receptor binding, agonistic antibodies bind activatory receptors and generate positive feedback to the T cells. These agonistic antibodies, or costimulatory agents, aim to further enhance T cell activation leading to increased immune responses. The costimulatory marker 4-1BB (also known as CD137) is a member of the tumour necrosis factor receptor family and is a promising target for anti-tumour immune responses. The molecule 4-1BB is rapidly expressed after activation on CD4+ and CD8+ T cells, TREGS, DCs and natural killer (NK) cells.

It has been observed 4-1BB+/− mice had decreased cytokine production and CTL response from CD8+ T cells. Further studies have shown that when 4-1BB binds 4-1BB ligand (4-1BBL) on APCs, costimulation is provided to CD4+ and CD8+ T cells through activation of NF-κB, c-Jun and p38 downstream pathways that prevent apoptosis. However, studies have provided conflicting data for the role of 4-1BB on both expanding and inhibiting TREGS. Importantly, as 4-1BB+/− mice develop autoimmunity, this suggests that 4-1BB is involved in regulating immune homeostasis. Consistent with results from knockout mice, blockade of 4-1BB inhibited tumour growth and increased CTL activity in sarcoma and mastocytoma models. Furthermore α-4-1BB has shown increased survival to 78% in mice with breast carcinoma, increased cure rates in fibrosarcoma and prolonged survival in 40% of mice with intracranial glioma. These studies have provided evidence that α-4-1-BB, provides costimulatory signals to CD4+ and CD8+ T cells, increasing cytolytic T cell activity, proliferation, resistance to apoptosis and IFN-γ secretion.

Following success in murine models, α-4-1BB was tested clinically. Phase I trials with α-4-1BB (urelumab), have shown increased T cell and NK proliferation and activity, however, phase II trials with urelumab were terminated early due to hepatotoxicity.
Phase I/II trials with another α-4-1-BB therapy (BMS-666513) for treatment of melanoma, renal cell carcinoma and ovarian cancer has shown a fairly well-tolerated response with promising outcomes\textsuperscript{109}.

1.5.6.3 Depletion of Tregs

Vaccine induced T cell responses may be limited by the suppressive effects of T\textsubscript{REGS} in lymphoid organs and the TME. Removal of suppression, by depleting T\textsubscript{REGS}, eliminates factors that impede the anti-tumour effects of conventional T cells. Before the discovery of FoxP3, T\textsubscript{REGS} were identified by CD25 expression, an IL-2 receptor-alpha (IL-2R\textalpha) subunit\textsuperscript{200}. Depletion of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells can be achieved by targeting the IL-2 receptor with α-CD25, and has been accepted and employed by a number of groups\textsuperscript{201-203}.

Controversial studies have suggested that α-CD25 causes down-regulation or shredding of CD25, implying that the therapy causes functional inactivation instead of depletion\textsuperscript{204}. However, this highly contentious paper was rebutted in subsequent papers illustrating T\textsubscript{REG} depletion with α-CD25\textsuperscript{205,206}. It was also found that α-CD25 does not affect IL-2 binding or IL-2-induced cell proliferation\textsuperscript{207} and therefore should not interfere with T\textsubscript{REG} generation or maintenance. One study in particular showed that α-CD25 did not completely remove T\textsubscript{REGS}, as T\textsubscript{REGS} with low or no CD25 expression were not affected by the monoclonal antibody. This murine study also discovered that α-CD25 induced antibody-dependent cellular phagocytosis (ADCP), a mechanism macrophages use to devour cells bound with antibody. Blocking of macrophage F\textsubscript{C}γRIII, a receptor that mediates ADCP, was found to inhibit T\textsubscript{REG} depletion, suggesting a role of macrophages in mediating α-CD25 T\textsubscript{REG} depletion\textsuperscript{208}.

Development of Denileukin Diftitox (DD), a fusion protein of IL-2 and diphtheria toxin, was shown to target T\textsubscript{REGS} and enhance antigen-specific T cell responses in
mice. The IL-2R that is highly expressed on T REGS is also overexpressed in leukaemias and lymphomas, and many clinical trials have used DD to target these tumour cells. Phase I/II clinical trials with DD, for cutaneous T-cell lymphoma (CTCL) and non-Hodgkin’s lymphoma, were well-tolerated and produced anti-tumour effects in patients. Following successive results, DD received FDA approval in 2008 for the treatment of CD25+ CTCL. Phase III trials with DD in CTCL patients, produced objective responses in over 30% of patients and overall response rates in 49.1% of patients. These clinical trials provide evidence that targeting IL-2R with diphtheria toxin can produce anti-tumour responses. Although there is less focus of DD therapy to deplete T REGS, the use of α-CD25 to target IL-2R on T REGS may also have clinical benefit in cancers such as glioma.

1.6 Rationale for thesis:

Collectively, the literature presented so far demonstrates that although CTLs can directly eradicate cancer tissue, further improvements for T cell immunotherapies are needed. Vaccination with peptides is potentially a scalable technology that could be readily adopted in the clinic, and has been shown to stimulate antigen-specific T cell responses in a variety of diseases including cancer, although these responses have generally been disappointingy weak. This led researchers in our group to design synthetic conjugate peptide vaccines that link α-GalCer to specific TAAs, in the attempt to stimulate a potent antigen-specific anti-tumour T cell response. Most of this early work has been conducted on tumours expressing model antigens. Here the aim was to test a new series of vaccines that target over-expressed TAAs in an in vivo model of GBM.

Various preclinical studies have shown that improvement of T cell function in a cancer setting can be achieved with checkpoint blockade inhibitors, costimulatory agents, and depletion of T REGS. In the case of checkpoint blockade, this treatment alone can induce
significant clinical responses, and even cures in some patients. Nonetheless, a large portion of patients do not respond. Hence a second aim was to determine whether vaccination can be combined with these treatments to improve anti-tumour T cell responses. This work was also conducted in the GBM model.

1.7 Aims and Hypothesis

In this thesis two hypotheses will be tested:

Firstly, I hypothesise that iNKT-cell dependent glycolipid-peptide vaccines will induce potent CD8+ T cell immune responses capable of producing significant anti-tumour responses \textit{in vivo}, against a GBM model.

Secondly, I hypothesise that combination of the conjugate vaccines with immune modulators, such as checkpoint blockade, costimulatory agents and depletion of T\textsubscript{REGS}, will result in improved anti-tumour response.

1.7.1 Specific Aims

To address the hypotheses stated above, the research undertaken in this thesis seeks to pursue the following aims:

- To investigate iNKT and T cell stimulation by a series of conjugate iNKT-cell dependent glycolipid-peptide vaccines.
- To assess the therapeutic potential of the conjugate vaccines in a GL261 glioma cancer model.
- To improve anti-tumour response with combination of the conjugate vaccines and immune modulators.
2 Material and Methods
2.1 Materials

2.1.1 Labware

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axygen Microtubes 1.7 ml</td>
<td>Axygen Scientific Inc, Union City, CA, USA</td>
</tr>
<tr>
<td>BD 1 ml syringes</td>
<td>BD Biosciences, MA, USA</td>
</tr>
<tr>
<td>BD 5 ml syringes</td>
<td></td>
</tr>
<tr>
<td>BD 10 ml syringes</td>
<td></td>
</tr>
<tr>
<td>BD 30 ml syringes</td>
<td></td>
</tr>
<tr>
<td>BD Falcon polypropylene conical tubes:</td>
<td></td>
</tr>
<tr>
<td>15 ml</td>
<td></td>
</tr>
<tr>
<td>50 ml</td>
<td></td>
</tr>
<tr>
<td>BD Falcon polystyrene tissue culture flasks:</td>
<td></td>
</tr>
<tr>
<td>25 cm²</td>
<td></td>
</tr>
<tr>
<td>75 cm²</td>
<td></td>
</tr>
<tr>
<td>175 cm²</td>
<td></td>
</tr>
<tr>
<td>BD Falcon tissue culture plates:</td>
<td></td>
</tr>
<tr>
<td>6-well plates</td>
<td></td>
</tr>
<tr>
<td>24-well plates</td>
<td></td>
</tr>
<tr>
<td>96-well round bottom plates</td>
<td></td>
</tr>
<tr>
<td>96-well U-bottom plates</td>
<td></td>
</tr>
<tr>
<td>BD Tuberculin Syringe (1, 3, 5, 10 ml)</td>
<td></td>
</tr>
<tr>
<td>Corning Costar Stripette serological pipettes (2, 5, 10, 25 ml)</td>
<td>Sigma-Aldrich, MO, USA</td>
</tr>
</tbody>
</table>
Materials and Methods

2.1.2 Reagents and Buffers

2.1.2.1 Cell culture reagents and buffers

<table>
<thead>
<tr>
<th>Table 2.2: List of reagents</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product</strong></td>
<td><strong>Supplier/Distributor</strong></td>
</tr>
<tr>
<td>2-mercaptoethanol (2-ME) 55 mM solution</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE)</td>
<td>Molecular Probes, Invitrogen, OR, USA</td>
</tr>
<tr>
<td>α-Galactosylceramide</td>
<td>Ferrier Research Institute, Wellington, New Zealand</td>
</tr>
<tr>
<td>Cell Tracker Orange CMTMR (CTO)</td>
<td>Invitrogen, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma-Aldrich, MO, USA</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle Medium (DMEM)</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>Material</td>
<td>Supplier</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>Dulbecco’s Phosphate Buffered Saline (PBS)</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>Fetal Calf Serum (FCS)</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>Geneticin® Selective Antibiotic (G418)</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>GlutaMAX™</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>gp100 vaccine (migrated α-GalCer-para-benzol-amino-alcohol-tri-C3-FFRK-AVGALEGPRNQDWLGVPRQL)</td>
<td>Ferrier Research Institute, Wellington, New Zealand</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>Sigma-Aldrich, MO, USA</td>
</tr>
<tr>
<td>Iscove’s Modified Dulbecco’s Medium (IMDM)</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>Liberase TL Research Grade</td>
<td>Roche, Mannheim, Germany</td>
</tr>
<tr>
<td>Mouse IFN-γ ELISPOT kit</td>
<td>BD Bioscience, San Diego</td>
</tr>
<tr>
<td>Mouse IL-2 ELISA MAX Deluxe Set</td>
<td>BioLegend, CA, USA</td>
</tr>
<tr>
<td>OneComp eBeads</td>
<td>eBioscience, CA, USA</td>
</tr>
<tr>
<td>Ovalbumin peptide (SIINFEKL)</td>
<td>GenScript, NJ, USA</td>
</tr>
<tr>
<td>Penicillin Streptomycin (Pen/Strep)</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>Phorbol myristate acetate (PMA)</td>
<td>Sigma-Aldrich, MO, USA</td>
</tr>
<tr>
<td>Red Blood Cell (RBC) lysis buffer</td>
<td>Qiagen, CA, USA</td>
</tr>
<tr>
<td>Roswell Park Memorial Institute (RPMI) medium</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>Sodium Azide (NaN₃)</td>
<td>Sigma-Aldrich, MO, USA</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>Ferrier Research Institute, Wellington, New Zealand</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>Survivin vaccine (migrated $\alpha$-GalCer-para-benzol-amino-alcohol-tri-C3-FFRK-DLAQMFFCFKELEGW)</td>
<td>Ferrier Research Institute, Wellington, New Zealand</td>
</tr>
<tr>
<td>Tris(2-carboxyethyl)phosphine (TCEP)</td>
<td>Sigma-Aldrich, MO, USA</td>
</tr>
<tr>
<td>TRP-2 vaccine (migrated $\alpha$-GalCer-para-benzol-amino-alcohol-tri-C3-FFRK-SVYDFFVWLKFFHRTCKCTGNFA)</td>
<td>Ferrier Research Institute, Wellington, New Zealand</td>
</tr>
<tr>
<td>Trypan blue (0.4% v/v)</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>TrypLE Select 1x</td>
<td>GIBCO, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>UltraPure 0.5 M, pH 8.0 Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Invitrogen, Life Technologies, Auckland, New Zealand</td>
</tr>
<tr>
<td>Vybrant carboxyfluorescein diacetate succinimidyl ester (CFDA-SE ) Cell Tracer Kit</td>
<td>Invitrogen, Life Technologies, Auckland, New Zealand</td>
</tr>
</tbody>
</table>

**Complete IMDM (cIMDM)**

IMDM was supplemented with 5% FCS, 2mM Glutamax, 100 u/ml penicillin, 100 $\mu$g/ml streptomycin and 500 $\mu$l 2-ME.

**Complete DMEM (cDMEM)**

DMEM was supplemented with 20% FCS, 2mM Glutamax, 100 u/ml penicillin and 100 $\mu$g/ml streptomycin.
Complete RPMI (cRPMI)
RPMI was supplemented with 10% FCS, 2mM Glutamax, 100 u/ml penicillin, 100 µg/ml streptomycin and 500 µl 2-ME.

**Fluorescent-activating cell sorting buffer (flow buffer)**
PBS containing 1% FCS, 0.01% NaN₃ and 2 mM EDTA was used for all flow cytometry experiments.

### 2.1.2.2 Peptides and antigens

**α-Galactosylceramide (α-GalCer)**
The iNKT cell ligand α-GalCer was manufactured by the Ferrier Research Institute (Wellington) and reconstituted in sterile dH₂O at 0.5 mg/ml. Reconstituted α-GalCer was stored at 4°C.

**Ovalbumin peptide (SIINFEKL)**
The ovalbumin peptide (OVA\textsubscript{257-264}; SIINFEKL) was manufactured by GenScript (USA) and reconstituted to 5 mg/ml in DMSO. Reconstituted ovalbumin peptide was stored at -20°C.

**Human Papillomavirus type 16 E7 peptide (E7)**
The E7 peptide (E7\textsubscript{49-57}; RAHYNIVTF) was manufactured by GenScript (USA) and reconstituted to 5 mg/ml in DMSO. Reconstituted ovalbumin peptide was stored at -20°C.

**Tyrosinase-related protein 2 peptides (TRP-2)**
The long TRP-2 peptide (modified TRP-2\textsubscript{180-188,89-102}; 4-pentynoyl(232211)-FFRK-SVYDFVWLKKFFHRTCKCTGNFA) and short TRP-2 peptide (TRP-2\textsubscript{180-188}; SVYDFVWL) were manufacture by the Ferrier Research Institute and reconstituted
to 5 mg/ml in DMSO. Reconstituted TRP-2 peptides were stored at -20°C and further diluted in PBS for injection.

**Glycoprotein 100 peptides (gp100)**

The long gp100 peptide (gp100<sub>19-38/P<sub>26; 4-pentynoyl-FFRK-AVGALEGPRNQDWLGVPRQL) and short gp100 peptide (gp100<sub>25-33; EGPRNQDWL) were manufactured by the Ferrier Research Institute and reconstituted to 5 mg/ml in DMSO. Reconstituted gp100 peptides were stored at -20°C and further diluted in PBS for injection.

**Survivin peptides**

The long survivin peptide (modified SVN<sub>53-67/M<sub>57; 4-pentynoyl-FFRK DLADMFFCFKELEGW) and short survivin peptide (SVN<sub>57-64/M<sub>57; MFFCFKEL) were manufactured by the Ferrier Research Institute and reconstituted to 5 mg/ml in DMSO. Reconstituted survivin peptides were stored at -20°C and further diluted in PBS for injection.

**2.1.2.3 Purified antibodies**

**α-CTLA-4**

Anti-CTLA-4 (4F10, hamster IgG2a) was affinity purified from hybridoma culture supernatants using protein G affinity columns. Mice received 500 µg IP of antibody the day prior to vaccination.

**α-4-1BB**

Anti-4-1BB (3H3, rat IgG2) was affinity purified from hybridoma culture supernatants using protein G affinity columns. Mice received 100 µg IP of antibody 1 and 4 days prior to vaccination.
\textit{\textalpha{}-LAG-3}

Anti-LAG-3 (C9B7W, rat IgG1) was affinity purified from hybridoma culture supernatants using protein G affinity columns. Mice received 200 \( \mu g \) IP of antibody 2 days post vaccination.

\textit{\textalpha{}-PD-1}

Anti-PD-1 (RMPI-14, rat IgG2a) was affinity purified from hybridoma culture supernatants using protein G affinity columns. Mice received 250 \( \mu g \) IP of antibody 3 days post vaccination.

\textit{\textalpha{}-CD25}

Anti-CD25 (PC61, rat IgG1) was affinity purified from hybridoma culture supernatants using protein G affinity columns. Mice received 125 \( \mu g \) IP of antibody 7 days prior to vaccination.

\textit{2.1.2.4 Murine flow cytometry antibodies}

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220</td>
<td>FITC</td>
<td>RA3-6b2</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD86</td>
<td>PE</td>
<td>GL1</td>
<td>eBioscience</td>
</tr>
<tr>
<td>H2-D\textsuperscript{b}</td>
<td>FITC</td>
<td>KH95</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>H2-K\textsuperscript{b}</td>
<td>APC</td>
<td>AF6-88.5</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>
2.1.2.5 Flow viability dyes

Table 2.4: Viability dyes

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>4',6-diamidino-2-phenylindole (DAPI)</td>
<td>Invitrogen, New Zealand</td>
</tr>
<tr>
<td>Propidium Iodide (PI)</td>
<td>Sigma-Aldrich, St. Louis, MO, USA</td>
</tr>
</tbody>
</table>

2.1.3 Cell lines

Cells were cultured under PC2 laboratory guidelines, incubated in humidified incubators, and stored in liquid nitrogen. When adhesive cell lines reached 80% confluency they were harvested with TrypLE solution (GIBCO Life Technologies). Live cells were counted on a haemocytometer after a 1:10 or 1:20 dilution with 0.4% v/v trypan blue solution (GIBCO Life Technologies). Trypan blue is a vital stain used to selectively colour dead tissues or cells. Live cells with intact membranes are not coloured.

2.1.3.1 Murine tumour cell lines

*B16-OVA, B16-GP33*

The melanoma cell lines B16-OVA, which stably expresses chicken ovalbumin, and B16-GP33, which express glycoprotein epitope amino acid 33-41 (GP33) were obtained from frozen stocks at the Malaghan Institute of Medical Research. Cells were cultured in cIMDM5 at 37°C and 5% CO₂, with B16-OVA cells also under 0.5mg/ml G418 selection (GIBCO Life Technologies), which blocks polypeptide synthesis of dividing cells that do not have the neomycin resistance gene.
GL261
The glioma cell line GL261 was obtained from frozen stocks at the Malaghan Institute of Medical Research. Cells were grown in cDMEM at 37°C and 5% CO₂.

RMA-S
The Rausher murine leukemia virus (MuLV) induced murine lymphoma RMA-S was obtained from frozen stocks at the Malaghan Institute of Medical Research. Cells were cultured in cRPMI at either 37°C or 26°C and 5% CO₂. The RMA-S cells have defective TAP machinery and therefore express low levels of MHC class I molecules. When incubated with exogenous peptides capable of binding, the MHC molecules become stabilised²¹⁶.

2.1.3.2 iNKT cell line

DN32.D3
The Vα14⁺ CD1d-specific iNKT hybridoma DN32.D3 cell line was obtained from frozen stocks at the Malaghan Institute of Medical Research. Cells were cultured in cIMDM at 37°C and 5% CO₂.

2.1.4 Mice

2.1.4.1 Maintenance and ethical approvals

All mice were bred and maintained in the Biomedical Research Unit at the Malaghan Institute of Medical Research. Mice were age and sex matched where possible. Experimental procedures were performed within the requirements of the Animal Welfare Act of New Zealand approval of the Victoria University Animal Ethics Committee (under 2012R27M until 2015 and under 22311 from then until present).
Materials and Methods

2.1.4.2 Mouse breeds

C57BL/6
Breeding pairs of C57BL/6 mice were originally obtained from the Jackson Laboratory (Bar Harbour, ME, USA).

B6Aa0/Aa0
The B6Aa0/Aa0 (B6Aa0) mice lack the expression of MHC class II molecules, preventing CD4+ T cell positive selection. Targeting a mutation to the Aa gene in embryonic C57BL/6 stem cells developed this knockout strain. The B6Aa0 mice were obtained from Biological Research Laboratories Ltd, Wolferstrasse, Switzerland.

TAP−
The TAP− mice are unable to successfully transport MHC class I molecules to the cell surface and therefore are deficient in CD8+ T cell responses. The TAP+ mice were obtained from University of Melbourne, Victoria, Australia.
2.2 Methods

2.2.1 Tissue isolation

2.2.1.1 Blood

Blood samples were collected in 200 µl of 10 mM EDTA-PBS, to prevent cells aggregating. Cells were pelleted at 700 x g for 4 min in a microcentrifuge and the supernatant discarded. Samples were incubated with 700 µl of red blood cell (RBC) lysis buffer for 15 min at 37°C in order to yield a pellet of pure white blood cells.

2.2.1.2 Spleen

Spleens from C57BL/6 mice were collected in IMDM and processed to a single cell suspension through either 70 µm cell strainer, or mashed between two pieces of gauze. Samples were pelleted at 600 x g for 4 min in a centrifuge and supernatants discarded. Pellets were resuspended in 1 ml of RBC lysis buffer and incubated for 1 min to remove RBCs from the samples.

2.2.2 In vitro T cell assays

2.2.2.1 iNKT cell proliferation assay

An ELISA flat-bottom 96 well plate was coated with 5 µg/ml murine CD1d monomer in 100 µl of PBS for 24 h at 4°C. The remaining wells did not contain CD1d monomer, only PBS, and were used as a control. Supernatants were gently removed, and the plate was washed with PBS to remove any unbound CD1d monomer. Wells received 50 ng/ml of α-GalCer and molar equivalents of conjugate vaccines, or PBS as a control, and were incubated for 1 h at 37°C. Supernatants were removed and any excess conjugate or glycolipid was washed away with PBS and then IMDM. Each well received 3 x 10^4 DN32.D3 cells and the plate was incubated for 18 h at 37°C. The plate
was centrifuged for 2 min at 600 x g, supernatants were gently removed and stored at -20°C for subsequent analysis by ELISA, discussed below in 2.2.2.2.

2.2.2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The ability of the vaccines to stimulate iNKT cells to release IL-2, without APC processing, was assessed by a mouse IL-2 ELISA MAX Deluxe Set, according to the manufacturer’s instructions. Briefly, a mouse IL-2 monoclonal antibody was coated onto a flat-bottom 96 well plate for 18 h at 4°C. A volume of 100 µl of standards and supernatant samples, from the in vitro iNKT cell proliferation assay, were added to each well and incubated at room temperature for 2 h. The plate-bound antibody captured any IL-2 present in the supernatant samples. The wells were washed three times with ELISA wash buffer (PBS + 0.05% tween20). A biotinylated anti-mouse IL-2 detection antibody was added and incubated at room temperature for 1 h. This also bound IL-2, creating an antibody-antigen-antibody complex. The wells were washed three times with ELISA wash buffer to remove excess biotinylated antibody, and incubated with avidin-HRP (horseradish-peroxidase) antibody at room temperature for 30 min. Excess antibody was washed three times with wash buffer. A TMB (3, 3′, 5, 5′-Tetramethylbenzidine) Substrate Solution was added to produce a blue colour proportional to the concentration of IL-2 present in each sample. Once a visible colour change was detected across the standard gradient wells, a stop solution of H₂SO₄ was added to stop the enzymatic colour-change reaction. A microplate reader took absorbance readings at 450 nm.
2.2.2.3 Enzyme-Linked ImmunoSpot (ELISPOT)

A mouse IFN-γ ELISPOT kit was used to assess the contribution of IFN-γ responsible for the antitumour response by the conjugate treatment. Similar to the sandwich ELISA (2.2.2.2), this ELISPOT assay measures the frequency of cells that produce and secrete IFN-γ. Briefly, 5 µg/ml of peptide in 100 µl of cRPMI was added to an ELISPOT plate pre-coated with detection antibody. Spleens were harvested from previously vaccinated mice, and 100µl of 5 x 10⁶ splenocytes were added and incubated for 18 h at 37°C 5% CO₂ in the dark. Mice receiving PBS injections and stimulated without peptides were used as negative controls. A PMA-ionomycin positive control was included, as well as a biological positive control where mice received ovalbumin vaccine and splenocytes were stimulated with ovalbumin peptide (SIINFEKL) known to induce IFN-γ release. The cell suspensions were washed twice with deionised water, allowing for 3-5 min of soaking. Wash buffer (20x) was diluted to 1x with deionised water, and the wells were washed three times with 200 µl/well. Detection antibody solution, prepared at 2 µg/ml in assay buffer, was transferred at 100 µl/well and incubated at room temperature for 2 h. Any unbound detection antibody was discarded and the plate was washed three times with wash buffer, allowing for 1-2 min of soaking. Avidin-HRP solution was prepared at 10 µg/ml and 100 µl was added per well and incubated for 1 h at room temperature. Streptavidin-HRP solution was discarded and wells were washed four times with 200 µl/well of wash buffer, allowing 1-2 min of soaking at each wash. Wells were washed twice with 200 µl/well of prepared PBS. Prepared 3-amino-9-ethyl-carbazole (AEC) substrate solution was added to each well at 100 µl/well, and spot development was monitored for 5 min. The substrate reaction was stopped by washing the plate five times with deionised water. The plate was left overnight to dry and spots were counted on an ELISpot plate reader.
2.2.4 H-2K\textsuperscript{b} binding assessment

To assess survivin peptide binding to H-2K\textsuperscript{b}, the RMA-S cell line that only expresses H-2K\textsuperscript{b} when peptide is bound, was used. The RMA-S cells were cultured in cRPMI at 37°C 5% CO\textsubscript{2}, and 100 \( \mu \)l of 1x10\textsuperscript{5} cells/well were added to a 96-well U-bottom plate. Survivin peptide was prepared at 100 \( \mu \)M and control peptides (ovalbumin, E7 peptide) at 50 \( \mu \)M, and 100 \( \mu \)l of appropriate peptide was added to cells. The plate containing cells and peptides were incubated for 2 h at 37°C and stained with an H-2K\textsuperscript{b} antibody, as described in 2.2.5.1, that indicated bound peptide. Samples were analysed by flow cytometry, as described in 2.2.4.

2.2.3 In vivo T cell assay
2.2.3.1 B cell activation

Up-regulation of CD86 on peripheral B cells was assessed as an indicator of iNKT cell activation. Blood samples were collected a day after priming, processed as described in 2.2.1.1, aliquoted into a 96-well U-bottom plate, pelleted at 700 \( \times \) g for 2 min in a megafuge, and supernatants were removed. Samples were resuspended in 200 \( \mu \)l flow buffer and stained with antibody mix, described in 2.2.5.1, and incubated at 4°C for 10 min. Samples were analysed by flow cytometry to assess activation status of peripheral B cells.

2.2.4 Serum analysis

Analysis of IL-2, IL-4, IL-12p70 and IFN-\( \gamma \) was assessed using a Bio-Plex Pro Mouse Cytokine Th1/Th2 Panel, 8-plex kit analysed on a Bio-Plex analyser (Bio-Rad Laboratories, CA, USA) according to manufacturer’s instructions. A 96-well filter plate on a vacuum manifold was pre-wet with 1 x Assay Buffer, and 25 \( \mu \)l of serially diluted standard and samples were added to the appropriate wells. Assay Buffer was used as
a ‘blank’ for control wells. A volume of 25 µl of 1:40 Bead Mixture and 50 µl of 1:40 Biotin-Conjugate Mixture was added to the wells. The plate was incubated for 2 h at room temperature in the dark on a plate shaker at 850 rpm. Wells were washed twice with wash buffer using the vacuum manifold. The plate was removed from the vacuum and 100 µl assay buffer was added to each well in 50 µl of 1/31.25 Streptavidin-PE solution. The plate was incubated for 1 h at room temperature in the dark on a plate shaker at 850 rpm. The wells were washed twice with Assay Buffer using the vacuum manifold and the beads were resuspended in 125 µl of Assay Buffer before being read on the Bio-Plex analyser. The data was analysed using the FlowCytomixPro software (eBioscience).

2.2.5 Flow cytometry

2.2.5.1 Cell surface staining

All flow cytometry antibodies were purchased from eBioscience, BD Pharmingen and Biolegend, and titrated for optimal performance prior to being used in experiments. Single cell suspensions of lymphocytes were processed, washed once in flow buffer and resuspended in 200 µl flow buffer. The cell suspensions were distributed to a 96-well U-bottom plate and incubated with anti-CD32/16 (clone 2.4G2) in flow buffer for 5 min to block FcyRII/III and prevent non-specific binding. Samples were centrifuged at 400 x g for 4 min, and supernatants were discarded from plates. Lymphocytes were incubated with 50 µl of flow buffer containing cell surface antibodies 4°C for 10 min. Cells were washed with flow buffer and pelleted at 400 x g for 4 min. After staining, cells were washed once and resuspended in 200 µl flow buffer. A further 200 µl of flow buffer containing the viability dyes DAPI or PI, was added to each sample to ensure only live cells were included in the final analysis. Resuspended cells were collected using a BD FACSCalibur, BD LSRII or BD LSRRFortessa instrument and analysed using FlowJo software version 9.4 (TreeStar, Inc., OR, USA).
### 2.2.5.2 In vitro VITAL assay

A VITAL assay\(^{217}\) was used to assess cytotoxicity against fluorescent-labelled spleen cell targets administrated IV into mice. Splenocytes were pulsed with three different concentrations of either the short TRP-2 peptide (400, 40 or 4 \(\mu\)M), short gp100 peptide (2, 0.2 or 0.02 \(\mu\)M), short survivin peptide (400, 40 or 4 \(\mu\)M) or no peptide (control) and incubated for 2 h at 37°C. Unpulsed splenocytes were washed three times with cIMDM and resuspended in 5 ml of warm cIMDM. These cells were stained with 10 \(\mu\)M CTO in cIMDM and incubated for 20 min at 37°C. Cells were pelleted and resuspended in warm cIMDM, incubated for a further 15 min, then washed twice in cIMDM and once in IMDM. Peptide-pulsed splenocytes were washed twice in IMDM and once in PBS. Dilutions of 7, 2 and 0.2 \(\mu\)M CFSE were made in PBS and added to the high, medium and low peptide-loaded cells, respectively. The cells were incubated in the dark for 6 min at room temperature. To stop the reaction, FCS was added and the cells were washed twice in cIMDM and once in IMDM. The target cells were combined with equal proportions of peptide-loaded cells labelled with CFSE and the control population labelled with CTO. These peptide-loaded fluorescent target cells were resuspended in IMDM and injected IV at 6 \(\times\) 10^6 cells per mouse seven days after priming with conjugate vaccines. Blood samples were taken 18 h later and analysed by flow cytometry to assess in vivo cytotoxicity compared to internal control populations.

### 2.2.6 Tumour challenge

#### 2.2.6.1 Subcutaneous tumour challenge

C57BL/6 male mice were injected SC with 1 \(\times\) 10^6 GL261 glioma cells or B16-OVA melanoma cells in the left flank. Tumours became palpable after five days and were measured every two-three days. When tumours ulcerated or reached 200 mm^2, mice were sacrificed.
2.2.6.2 Lung tumour challenge

C57BL/6 mice were injected intravenously (IV) with $3 \times 10^5$ B16-GP33 cells. Tumours formed in the lung tissue and mice were monitored and weighed daily. Mice were sacrificed 15 days after tumour challenge and lungs were harvested and weighed as a measure of tumour burden.

2.2.7 Therapy

2.2.7.1 Vaccine generation

Freeze-dried TRP-2 vaccine (migrated \(\alpha\)-GalCer-para-benzol-amino-alcohol-tri-C3-FFRK-SVYDFFWLKFHTCTGFWNA) containing the defined CD8 epitope TRP-2\(^{180-188}\) and the defined CD4 epitope TRP-2\(^{289-102/102}\); gp100 vaccine (migrated \(\alpha\)-GalCer-para-benzol-amino-alcohol-tri-C3-FFRK-AVGALEGPRNQDWLGVPRQL) containing the defined CD8 epitope gp100\(^{25-33}\); and survivin vaccine (migrated \(\alpha\)-GalCer-para-benzol-amino-alcohol-tri-C3-FFRK-DLAQMFFCFKELEGW) containing the defined CD8 epitope SVN\(^{57-64/57}\), were formulated in sucrose, tween and histidine\(^{53}\) and obtained from the Ferrier Research Institute. Conjugates were reconstituted to 0.5 mg/ml in sterile dH\(_2\)O and stored at -20°C. Vaccines were further diluted in PBS prior to injection.

2.2.7.2 Solubilisation and administration of compounds to mice

The peptide admixes, consisting of free \(\alpha\)-GalCer mixed with peptide, and conjugate vaccines were further diluted in PBS before administration into mice. Mice were injected with either peptide admix or conjugate vaccine either IV, SC or intramuscular (IM). In tumour models, mice injected with conjugate vaccines received a molar equivalent of 445 ng of \(\alpha\)-GalCer. The varied weights of compounds were used to
ensure conjugates and free α-GalCer admixed with peptide were equimolar.

### 2.2.7.3 Antibody administration

Checkpoint blockade antibodies were administered IP to mice as stated in figure legends. Timing and amount administrated depended on the checkpoint targeted.

### 2.2.8 Statistical analysis

Statistical analysis and graphing of data was performed using Prism software (GraphPad Prism Version 5.0 for MacIntosh, GraphPad Software Incorporated, USA). Figure legends state the statistical analysis used for each experiment. Student’s unpaired, two-tailed t-test was used to calculate significance between 2 groups. When analysing three or more samples, one-way analysis of variance (ANOVA) with Bonferroni’s pose test was used. For analysing two parameters with multiple groups, two-way ANOVA with Bonferroni’s post test was used. Error bars represent the mean data ± the standard error of mean (SEM). P values of *p<0.05, were considered significant, and **p<0.01, ***p<0.001, ****p<0.0001 were used. Survival curves were analysed using Log-Rank (Mantel Cox) test with Bonferroni correction threshold applied. The Bonferroni corrected threshold was calculated by taking the significance level of p<0.05 and dividing it by the number of comparisons made to give a new P value. If a P value is less than the Bonferroni-corrected threshold, then the comparison can be said to be statistically significant.
3 Evaluating the immune response induced by conjugate vaccine
3.1 Introduction

Our laboratory has previously shown that conjugate vaccines incorporating a prodrug form of α-GalCer conjugated to an antigen-derived peptide via a protease-sensitive VC-PAB linker, are capable of activating iNKT cells, leading to improved CD8⁺ T cell cytotoxic responses. Furthermore, this conjugate vaccine construct has been shown to induce significant anti-tumour responses in vivo against B16.OVA melanoma, an aggressive tumour model. The three conjugate vaccines assessed in this thesis were made to the same overall design, with the aim of targeting the antigens, TRP-2, gp100 and survivin, that have been reported to be expressed by GL261 glioma tumours.

The peptides used from these TAA contained longer sequences than just the minimal MHC-binding epitopes. This is because longer peptides are more likely to be used in future clinical applications, as it is unlikely that specific epitopes will be defined for all individuals in a given population. It is also likely that longer peptides will contain MHC class II-binding epitopes, so that vaccination can also initiate CD4⁺ T cell responses. The TRP-2 vaccine contains the peptide sequence TRP-2₁₈₀-₂₀₂ (SVYDFVWLKFFHRTCKCTGNFA). Contained within this peptide is H-2Kb-restricted CD8⁺ T cell epitope (SVYDFVWL) and the H-2-A₅-restricted CD4⁺ T cell epitope (KFFHRTCKCTGNFA). The gp100 vaccine contains the peptide sequence gp100₁₉-₃₈/P₂₆ (AVGALEGPRNQDWLGVPRQL). Contained within this peptide is the H-2Db-restricted CD8⁺ T cell epitope (EGPRNQDWL). Studies have shown that substituting the serine (S) at position 26 to proline (P), induced robust CTL responses compared to the natural sequence. The survivin vaccine contains the modified peptide sequence SVN₅₃-₆₄/M₅₇ (DLAQMFFCFKELEGW) with a defined CD8⁺ T cell epitope (MFFCFKEL), and a putative CD4⁺ epitope. The murine survivin protein SVN₅₃-₆₄ is homologous to the corresponding human survivin molecule, but is weakly immunogenic in humans. Studies have shown when substitution of the cysteine (C)
residue at position 57 to a methionine (M) residue, provides enhanced anti-tumour immune responses against glioma\textsuperscript{143}. For this reason, the survivin vaccine incorporates a methionine residue at position 57. Additionally, between the linker and each peptide in the conjugate vaccines is another proteolytic cleavage sequence (FFRK)\textsuperscript{223}. This sequence, which can be considered a universal part of the linker, has been shown to promote cleavage after the lysine, thereby ensuring that any epitope that is contiguous with the linker is released.

### 3.2 Aims

The experiments described in this chapter aim to investigate the anti-tumour efficacy of iNKT-dependent glycolipid-peptide vaccines against defined TAA that have been reported to be expressed in GL261 glioma.

Specific aims were to:

1. Assess whether the vaccines induce iNKT cellular activation required for vaccine activity.
2. Measure the T cell responses induced following conjugate vaccination.
3. Evaluate the therapeutic potential of the conjugate vaccines in against established GL261 tumours.
3.3 Results

3.3.1 Vaccines containing glycolipid conjugated to peptide do not bind CD1d directly and activate iNKT cells in vitro

The conjugate vaccine design is reliant on delivering the adjuvant α-GalCer and antigen-derived peptide to the same APCs in vivo. Ideally, the components must not be released and become active until enzymatic cleavage takes place within APCs. To test this, the ability of the vaccines to stimulate iNKT cells were assessed in vitro in an APC-free presentation assay where the conjugates were incubated with plate-bound CD1d monomers, before iNKT cells from a hybridoma cells line (DN32.D3) were added. The ability of the vaccines to activate iNKT cells was compared to free α-GalCer, which can bind to CD1d directly and stimulate iNKT cells without any need for enzymatic processing. Production of IL-2 was utilised as a quantitative measure of iNKT cell activation. Any activity from the conjugate vaccines in this assay would suggest that the compounds were chemically unstable, or that they have been prematurely cleaved by proteases produced by iNKT cells.

As expected, α-GalCer was able to directly bind CD1d monomer to activate iNKT cells, as shown by the large production of IL-2 from activated cells (Figure 3.1 A). This activation of iNKT cells was CD1d-dependent, as there was little to no iNKT cell activation observed in the absence of CD1d monomer (Figure 3.1 B). In contrast to free α-GalCer, the conjugate vaccines were unable to directly bind CD1d and activate iNKT cells (Figure 3.1 A).
Figure 3.1: Conjugate vaccines require cleavage by DCs to present α-GalCer to iNKT cells. A tissue culture plate was coated with CD1d monomer before 50 ng of α-GalCer or equimolar amounts of the indicated compounds were added. The plate was washed extensively and DN32.D3 cells were added to each well and cultured for 18 h. (A) Assessment of IL-2 levels produced by activated iNKT cells, measured by an ELISA. (B) As in A, except in the absence of plate-bound CD1d monomer. Statistical analysis was performed using One-way ANOVA with Bonferroni post test, showing mean ± SEM of three technical replicates.
3.3.2 Conjugate vaccines are capable of stimulating iNKT cells \textit{in vivo}.

Next, the \textit{in vivo} capacity of the conjugate vaccines to stimulate iNKT cells was assessed. It is known that iNKT cell activation can result in release of activated B cells into the blood\textsuperscript{224}, therefore circulating activated B cells were assessed as a proxy measure for activated iNKT cells. The route of administration of a vaccine can dictate the immune response elicited, dependent on the APCs encountered\textsuperscript{225,226}. As IV administration is not a favoured route for vaccination in the clinic, and very little information has been acquired on conjugate vaccines administered by other routes, B cell activation was assessed following SC and intramuscular (IM) administration.

Blood was sampled 24 hours following IV, SC or IM vaccine administration, to assess B cell activation, with activation measured by examining upregulation of the costimulatory molecule CD86 on the surface of B220\textsuperscript{+} B cells using flow cytometry (\textit{Figure 3.2 A, B}). The B220\textsuperscript{+} population with a higher forward scatter (FSC) most likely represents plasmacytoid DCs (pDCs)\textsuperscript{227} and therefore were excluded from the gate.

Administration of the conjugate vaccines IV caused increased iNKT cell activation compared to conjugate vaccines administered SC and IM (\textit{Figure 3.2 C-K}). Administration of the gp100 vaccine SC, and all three conjugate vaccines IM, caused activation of B cells, indicative of some iNKT cell activation (\textit{Figure 3.2 E, G H, K}). However, the greatest level of B cell activation was achieved through IV administration, with all three vaccines inducing marked upregulation of CD86. This experiment therefore did show that each of the vaccines could activate iNKT cells (without the need to cull the animals), and although a somewhat indirect measure of activity, did suggest that the IV route elicited a greater response.
Evaluating the immune response induced by conjugate vaccine

Figure 3.2: Comparison of the ability of conjugate vaccines delivered IV, SC or IM to activate iNKT cells. (A) Experimental outline for the assessment of B cell activation. C57BL/6 mice were treated with PBS, or conjugate vaccines either IV, SC or IM. B cell activation was analysed 24 h later by flow cytometry. (B) Representative flow cytometry plots showing B220⁺ B cell activation via CD86 upregulation. Gating strategies were used to identify total live cells (gate I), lymphocytes (gate II) and B220⁺ B cells (gate III). (C-K) Bar graphs of the mean fluorescence intensity (MFI) from the flow cytometry data shown in B. (C-E) C57BL/6 mice received either PBS IV or TRP-2 vaccine. The TRP-2 vaccine was administrated either IV, SC or IM. (F-H) As with C-E with gp100 vaccine. (I-K) As with C-E with survivin vaccine. Data shown is n = 5 per group. (C, F, I) Bar graphs are representative of two independent experiments. (D-E, G-H, J-K) Bar graphs are representative of one independent experiment. Statistical analysis was performed using Student’s t-test, showing mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant.
3.3.3 Assessment of antigen-specific cytotoxicity of conjugate vaccines against target cells

The capacity of the vaccines to induce antigen-specific CD8+ T cell-mediated killing was assessed with a VITAL assay\textsuperscript{217}. In this assay splenocytes from donor mice were pulsed with peptides and injected into vaccinated mice to serve as targets of cytotoxic activity. Four populations of targets were administered; three loaded with different doses of peptide and labelled with different concentrations of fluorescent dye (CFSE), and a fourth population without peptide that was labelled with cell tracker orange (CTO) and used as a control. Cytotoxicity was assessed by examining relative killing of peptide-loaded target cells compared to control cells by flow cytometry.

The TRP-2 vaccine delivered IV induced potent antigen-specific killing, while SC administration failed to induce any cytotoxicity, and IM delivery stimulated minimal killing (Figure 3.3 C-E). The gp100 vaccine delivered IV and SC, produced a potent cytotoxic response against gp100 targets at the highest concentration (100 nM), while marginal killing was observed with lower target concentrations and IM delivery (Figure 3.3 F-H). Although statistically insignificant, the survivin vaccine delivered IV produced minimal CD8+ cytotoxicity against target cells, and failed to produce significant cytotoxicity when delivered SC or IM (Figure 3.3 I-K).

Overall, the data suggest that vaccine administration IV is the most suitable route for the vaccines, although some weak activity can be induced by the other routes. The IV route was chosen for further investigations.
Figure 3.3: Route of administration of conjugate vaccines alters antigen-specific killing. (A) Experimental outline for the assessment of antigen-specific killing. C57BL/6 mice were treated with conjugate vaccines either IV, SC or IM. Three splenocyte populations labelled with different amounts of CFSE and loaded with titrated doses of peptide, and a control population with no peptide labelled with CTO, were adoptively transferred on day seven. Cytotoxic activity was assessed on day eight by flow cytometry. (B) Representative flow cytometry plots showing antigen-specific killing of peptide–pulsed target cells. Gating strategies were used to identify lymphocytes (gate I) and populations of peptide-pulsed target cells at different concentrations and the control population (gates II, III, IV and V). (C-K) Bar graphs of the flow cytometry data shown in (B). (C-E) Mice received either PBS IV or TRP-2 vaccine administrated IV, SC or IM. (F-H) As with (C-E) except with gp100 vaccine. (I-K) As with (C-E) except with survivin vaccine. Data shown is \( n = 5 \) per group. Bar graphs are representative of either two independent experiments (C, F, I), or one independent experiment (D-E, G-H, J-K). Statistical analysis was performed using Two-Way ANOVA with Bonferroni’s post test, showing mean ± SEM *\( p<0.05 \), ***\( p<0.001 \), ****\( p<0.0001 \).
3.3.4 Prophylactic survivin vaccination *in vivo* delays SC GL261 tumour growth

To evaluate the anti-tumour activity of the vaccines it was decided to utilise a SC model of GL261 to avoid difficult IC procedures. To assess anti-tumour activity a single dose of vaccine was given prior to tumour challenge, to assess prevention of GL261 tumour formation. Mice were vaccinated, or treated with PBS, seven days before $5 \times 10^5$ GL261 tumours were implanted SC. Tumours were monitored and measured every two-three days, and mice were sacrificed when tumours ulcerated or exceeded 200 mm$^2$.

In this prophylactic setting, the TRP-2 and gp100 vaccine failed to cause an anti-tumour response (*Figure 3.4 B, C*), despite evidence of antigen-specific killing (*Figure 3.3 C, F*). However, a significant delay in tumour growth was observed with the survivin vaccine (*Figure 3.4 D*). This initial tumour study was conducted by Dr Lindsay Ancelet, as indicated in figure legend. Based on this result, it was decided to focus primarily on the survivin vaccine in this glioma model, while the gp100 and TRP-2 vaccines continued to be analysed by other members of the laboratory in a melanoma model.
Figure 3.4: Prophylactic survivin vaccination delays GL261 SC tumour growth. (A) Experimental outline for the treatment of SC GL261 tumours with survivin vaccine. C57BL/6 mice were vaccinated seven days prior to receiving $5 \times 10^5$ GL261 cells SC. Tumour sizes were monitored and recorded and mice were culled when tumours reached 200 mm$^2$ in size. Tumour growth curves for mice vaccinated with PBS and either (B) TRP-2 vaccine, (C) gp100 vaccine or (D) survivin vaccine IV. Statistical analysis was performed using Two-Way ANOVA with Bonferroni post-test, showing mean ± SEM at the indicated time points **$p<0.01$, ****$p<0.0001$, ns = not significant. Experiment conducted by Dr Lindsay Ancelet.
3.3.5 Therapeutic survivin vaccination \textit{in vivo} delays SC GL261
tumour growth

As the survivin vaccine induced anti-tumour responses in a prophylactic setting, the
efficacy of the vaccine in a therapeutic setting was assessed, where induction of anti-
tumour response is typically more challenging\textsuperscript{228}. Mice received $1 \times 10^6$ GL261 tumour
cells SC and seven days later received either PBS or survivin vaccine.

The survivin vaccine improved the period of survival in mice (Figure 3.5 D). However,
it should be noted that the vaccine alone was unable to completely resolve tumours,
but instead delayed tumour growth (Figure 3.5 C).

Overall, both prophylactic and therapeutic vaccination against survivin produced an
anti-tumour response. It was therefore perhaps surprising that only a minimal CD8$^+$
CTL response was detected. This may reflect a lack of sensitivity in the cytotoxicity
assay, or it is possible that the survivin vaccine may operate through different
mechanisms than the proposed CD8$^+$ T cell cytotoxicity assumed. The following data
in this chapter explores additional anti-tumour mechanisms the survivin vaccine may
employ.
Evaluating the immune response induced by conjugate vaccine

Figure 3.5: Tumour growth is delayed with therapeutic survivin vaccination. (A) Experimental outline for the treatment of SC GL261 tumours with survivin vaccine. C57BL/6 mice were injected SC with 1 x 10^6 GL261 cells. Tumour sizes were monitored and recorded and mice were culled when tumours reached 200 mm^2 in size. Individual tumour growth for mice with SC tumours treated with either (B) PBS or (C) vaccine IV on day seven. Data shown is n = 7 per group. (D) Survival curves are two combined experiments, representative of three independent experiments. Statistical analysis of survival curves was performed using Log-Rank (Mantel Cox) test **p<0.01.
3.3.6 Oxidation status of the survivin peptide

The minimal CTL response induced by the survivin vaccine may be due to the survivin peptide not efficiently binding H-2K\(^b\) when assessed via the VITAL assay\(^{217}\). The survivin peptide contains the CD8\(^+\) peptide epitope (MFFCFKEL), which incorporates methionine (M) and cysteine (C) residues that are both susceptible to oxidation\(^{229}\). An oxidised state may alter the peptide and sterically hinder binding to the H-2K\(^b\) peptide groove, thus affecting presentation to CD8\(^+\) T cells and accounting for the minimal cytotoxicity (Figure 3.3 1-K). For example, the thioether in methionine can be oxidised to sulfoxide or further to sulphone (Figure 3.6  A). The cysteine residues can also be oxidised to produce intermolecular disulphides (Figure 3.6  B). Tris(2-carboxyethyl)phosphine (TCEP) is a reducing agent frequently used in biochemistry. The survivin peptide was treated with TCEP to assess reduction of the potentially oxidised compound. For these reasons an analysis of the peptide samples was commissioned with the Ferrier Research Institute, utilising high-performance liquid chromatography (HPLC) coupled to mass spectrometry (collectively HPLC-MS).

![Potential oxidation of residues in the CD8\(^+\) epitope of survivin peptide](image)

Figure 3.6: Potential oxidation of residues in the CD8\(^+\) epitope of survivin peptide. (A) Oxidation of the thioether in methionine to sulfoxide and sulphone. (B) Oxidation of the cysteine residue forming internal disulfide bonds.
Samples were analysed on a C18 reverse phase column utilising MeOH-H2O gradient elution with 0.01% trifluoroacetic acid (TFA). UV detection (214 nm) and mass spectral (MS) detection were used in tandem, where the UV signal was used for quantification and MS for identification of the chemical components. Analysis of the survivin peptide utilising UV showed two signals at 7.1 and 7.6 minutes (Figure 3.7 A), indicating different chemical structures. The mass spectrometer was operated in single ion monitoring (SIM) mode, to detect specific ions that relate to the chemical structures of the parent, un-oxidised peptide or its oxidised derivatives. This data confirmed the major peak to be the un-oxidised survivin peptide (7.1 min, 81% relative, Figure 3.7 B) whereas the slower eluting peak (7.6 min, 19% relative intensity) was confirmed to be disulfide (Figure 3.7 D). No sulfone was detected by mass spectroscopy (data not shown), however, a signal consistent with a trace amount of sulfoxide was observed by SIM (Figure 3.7 C). Importantly, after treatment with the reductant TCEP, the signal in the mass spectrometry for disulfide bonds was substantially reduced (Figure 3.8 A) (Dr. Hayman, personal communication).
Figure 3.7: HPLC-MS analysis of survivin peptide. A solution of the peptide at 0.2 mg/ml was analysed by HPLC on a C18 column under reverse phase conditions using gradient elution of water and methanol each containing 0.01% TFA. The gradient was 5-90% methanol over nine min. Expansion of the survivin peptide region of the chromatograms are shown with relevant peaks shaded showing retention time and integral values. Detection by UV at 214 nm was undertaken in series with mass spectroscopic detection running in SIM mode for selected mass-to-charge ratio (m/z) values. (A) UV (214nm) chromatogram. (B) SIM chromatogram monitoring for the survivin peptide charge states m/z 532.7 ([M+2H]^2+) and 1064.5 ([M+H]+). (C) SIM chromatogram monitoring for the survivin peptide sulfoxide charge states m/z 540.7 ([M+2H]^2+) and 1080.5 ([M+H]+). (D) SIM chromatogram monitoring for the survivin peptide disulfide charge states m/z 426.0 ([M+5H]^5+) and 709.3 ([M+3H]^3+). Data provided by Dr Colin Hayman, Ferrier Research Institute.
Evaluating the immune response induced by conjugate vaccine

Figure 3.8: HPLC-MS analysis of TCEP-treated survivin peptide. A solution of the peptide treated with TCEP for 1 h at a final peptide concentration of 3.9 mg/ml in 83:17 DMSO/water was analysed by HPLC on a C18 column under reverse phase conditions using gradient elution of water and methanol each containing 0.01% TFA. The gradient was 5–100% methanol over 10 min. Expansion of the survivin peptide region of the chromatograms are shown with relevant peaks shaded showing retention time and integral values. Detection by UV at 214 nm was undertaken in series with mass spectroscopic detection running in SIM mode for selected m/z values. (A) UV (214 nm) chromatogram. (B) SIM chromatogram monitoring for the survivin peptide charge states m/z 532.7 ([M+2H]^2+) and 1064.5 ([M+H]^+). (C) SIM chromatogram monitoring for the survivin peptide sulfoxide charge states m/z 540.7 ([M+2H]^2+) and 1080.5 ([M+H]^+). (D) SIM chromatogram monitoring for the survivin peptide disulfide charge states m/z 426.0 ([M+5H]^5+) and 709.3 ([M+3H]^3+). Data provided by Dr Colin Hayman, Ferrier Research Institute.
3.3.7 Survivin peptide efficiently binds H-2K\(^b\), and is further enhanced with TCEP treatment

To assess the impact of oxidation of the peptide on binding MHC, the reducing agent TCEP was added to the survivin peptide, and incubated for an hour before binding to H-2K\(^b\) was measured. The cell line RMA-S, which are deficient in transporter associated with antigen processing (TAP) machinery and consequently present few surface MHC class I molecules\(^{230}\), can only stably express H-2K\(^b\) molecules when peptide is bound, making it possible to assess the survivin binding to H-2K\(^b\) by flow cytometry with α-H-2K\(^b\). Included in the assay were cells incubated without peptide, and cells incubated with the E7 peptide (RAHYNIVTF), a negative control that binds H-2D\(^b\) instead of H-2K\(^b\). As expected, these cells were unable to stabilise the H-2K\(^b\) molecule. Also included were cells incubated with the ovalbumin peptide (SIINFEKL), which served as a positive control as it readily binds H-2K\(^b\) and stabilises the molecule. Histograms from flow cytometry are presented in Figure 3.9 A, and summarised from replicates in Figure 3.9 B.

The survivin peptide was able to bind H-2K\(^b\) and cause stabilisation with or without TCEP-treatment, albeit at lower levels than the ovalbumin peptide. When treated with TCEP, binding to H-2K\(^b\) was improved. This suggests that the oxidised cysteine residues prevent maximum survivin peptide binding to H-2K\(^b\), and may contribute to the limited cytotoxicity observed in the VITAL assay (Figure 3.3).
Figure 3.9: Survivin peptide does not require TCEP treatment to bind H-2Kb. RMA-S cells were incubated with either no peptide, E7 (RAHYNIVTF), ovalbumin (SIINFEKL), survivin (MFFCFKEL) or TCEP-treated survivin (MFFCFKEL) peptides for 2 h, and H-2Kb expression was analysed by flow cytometry. (A) Representative flow cytometry plots showing peptide-stabilised H-2Kb expression on RMA-S cells. Gating strategies were used to identify lymphocytes (gate I) and total live cells (gate II). (B) Bar graphs of the flow cytometry data shown in (A). Results are displayed as n = 3 per group and is representative of three independent experiments. Statistical analysis was performed using Student's t-test, between survivin peptide and TCEP-treated survivin peptide, showing mean ± SEM, **p<0.01.
3.3.8 Antigen-specific cytotoxicity is improved when treating peptide-loaded target cells with TCEP

Although it appears that untreated survivin peptide can bind H-2Kb, a VITAL assay\textsuperscript{217} was conducted one week after vaccination with the survivin conjugate, this time with TCEP-treated peptide loaded onto the target cells in an attempt to improve the sensitivity of detecting a cytotoxic response.

Significant antigen-specific killing (21.6\%) was detected against targets loaded with the highest peptide concentration (Figure 3.10). This was an improvement on the level detected with non-TCEP treated targets (12.3\%) in the previous experiment (Figure 3.3 I), which did not reach statistical significance. These data therefore suggest that the vaccine can indeed induce CD8\textsuperscript{+} T cell-mediated cytotoxic killing, although the level detected was still low compared to the other vaccines tested.
Evaluating the immune response induced by conjugate vaccine

Figure 3.10: Antigen-specific killing is improved when treating survivin targets with TCEP. (A) C57BL/6 mice were treated with PBS or survivin vaccine IV. Three splenocyte populations labelled with different concentrations of CFSE and loaded with titrated doses of TCEP-treated peptide, as indicated, and a control population with no peptide labelled with CTO, were adoptively transferred on day seven. Cytotoxic activity was assessed on day eight by flow cytometry. (B) C57BL/6 mice received either PBS or survivin vaccine IV. Data shown is n = 5 per group. Bar graphs are representative of two independent experiments. Statistical analysis was performed using Two-Way ANOVA with Bonferroni’s post test, showing mean ± SEM **p<0.01.
3.3.9 CD8\(^+\) T cells do not produce IFN-\(\gamma\) following survivin vaccination

CD8\(^+\) T cell effector functions are not limited to antigen-specific killing, as the cells can produce cytokines with anti-tumour properties, such as IFN-\(\gamma\). To investigate the involvement of IFN-\(\gamma\) produced by CD8\(^+\) T cells as an anti-tumour mechanism of the survivin vaccine, an ELISPOT was performed. Mice were vaccinated with survivin conjugate, or an irrelevant vaccine containing peptide derived from chicken ovalbumin. Control mice received PBS only. One week later, spleens were collected and reactivity to MHC class I-binding peptides from survivin or ovalbumin were assessed by ELISpot, an assay used to detect IFN-\(\gamma\) producing cells.

As expected from earlier published work\(^{53}\), mice vaccinated with the ovalbumin vaccine and then restimulated with the ovalbumin peptide (SIINFEKL) produced significant levels of IFN-\(\gamma\)-producing cells (Figure 3.11 A). However, mice primed with survivin vaccine and re-stimulated with survivin peptide (MFFCFKEL) did not (Figure 3.11 A).
Figure 3.11: Following survivin vaccination, CD8+ T cells do not produce IFN-γ. C57BL/6 mice were vaccinated on day 0 with PBS, survivin vaccine or ovalbumin vaccine and one week later splenocytes were isolated and restimulated with survivin peptide (MFFCFKEL), ovalbumin (SIINFEKL) or no peptide (control) for 18 h in vitro. ELISPOT assays were performed using an IFN-γ ELISPOT kit to detect IFN-γ producing T cells. (A) Bar graphs showing IFN-γ production from mice injected with PBS, survivin vaccine or ovalbumin vaccine IV and restimulated with peptide. Data shown is n = 3 per group. Bar graphs are representative of three independent experiments. Statistical analysis was performed using Two-Way ANOVA with Bonferroni’s post test, showing mean ± SEM ****p<0.0001.
3.3.10 The anti-tumour response of survivin vaccine depends on CD4\(^+\) T cells

The involvement of CD8\(^+\) T cells in the anti-tumour response to the survivin conjugate vaccine remained equivocal, with some evidence of cytotoxicity, but no evidence of CD8\(^+\) T cell-mediated IFN-\(\gamma\) production. It was also possible that the response was mediated by CD4\(^+\) T cells, as the peptide used in the vaccine has been reported to have a putative CD4\(^+\) T cell epitope, but this has not been clearly defined\(^{142}\). To test this possibility, the efficacy of the survivin vaccine was tested in MHC class II\(^{-}\) mice, which are devoid of CD4\(^+\) T cells due lack of positive selection of thymocytes on MHC class II molecules in the thymus. If the vaccine does not rely on CD4\(^+\) T cells responses, the vaccine is expected to have similar efficacy in C57BL/6 and MHC class II\(^{-}\) mice. Mice were given GL261 cells SC and treated on day seven with the survivin vaccine IV.

Survivin vaccinated C57BL/6 mice, which contain functional MHC class I and II, delayed tumour growth as expected. Surprisingly, the anti-tumour response was completely abolished in MHC class II\(^{-}\) mice (Figure 3.12), suggesting that the delayed tumour growth mediated by the survivin vaccine was dependent on CD4\(^+\) T cells.
Figure 3.12: Survivin vaccine efficacy is lost in MHC class II⁻. (A) Experimental outline for the treatment of SC GL261 tumours with survivin vaccine. C57BL/6 and MHC class II⁻ mice, were injected SC with 1 x 10⁶ GL261 cells. Tumour sizes were monitored and recorded. Individual tumour growth for C57BL/6 mice with SC tumours treated with either (B) PBS or (C) survivin vaccine IV on day seven, or MHC class⁻ mice with SC tumours treated with either (D) PBS or (E) survivin vaccine IV on day seven. Data shown is n = 7. (F) Survival curves are two combined independent experiments. Statistical analysis of survival curves was performed using Log-Rank (Mantel Cox) test using a Bonferroni correction threshold of p<0.0016.
3.3.11 CD4⁺ T cells do not produce IFN-γ following survivin vaccination

It is known that CD4⁺ T cells can also produce cytokines with anti-tumour properties, such as IFN-γ, which may mediate the anti-tumour response of the survivin vaccine. To further investigate the CD4⁺ T cell response an ELISPOT assay was used to detect IFN-γ production in response to the long peptide incorporated into the vaccine, which should include the putative MHC class II-binding sequence. Mice were primed with PBS or the survivin vaccine and seven days later splenocytes were stimulated with either the short (SVN57-63) or long (SVN53-67) survivin peptide before the IFN-γ ELISPOT assay was performed. Unspecific stimulation of the T cells was achieved with PMA/Ionomycin, and served as a positive control that showed the cells were viable and capable of cytokine production. No IFN-γ-producing cells were detected in response to restimulation with either peptide (Figure 3.13). This suggests that the anti-tumour activity induced by CD4⁺ T cells may not be mediated through release of IFN-γ.
Figure 3.13: Following survivin vaccination, CD4+ T cells do not produce IFN-γ. C57BL/6 mice were vaccinated on day 0 and one week later splenocytes were isolated and restimulated with short survivin peptide (SVN57-64), long survivin peptide (SVN53-67) or no peptide (control) for 18 h in vitro. ELISPOT assays were performed using an IFN-γ ELISPOT kit to detect IFN-γ producing T cells. (A) Bar graphs showing IFN-γ production from mice injected with PBS or survivin vaccine IV and restimulated with peptide. PMA/ionomycin was added to naïve splenocytes and used as a positive control. Data shown is n = 3 per group and is representative of two independent experiments. Statistical analysis was performed using Two-Way ANOVA with Bonferroni’s post test, showing mean ± SEM.
3.4 Discussion

In this chapter, the mechanism of activity for three TAA-targeted iNKT-dependent glycolipid-peptide conjugate vaccines were investigated. In each case, the vaccines were shown to be stable in vitro, and required processing to activate iNKT cells in vivo. All three vaccines induced CD8+ T cell-mediated cytotoxic responses, although the activity induced with the survivin vaccine was considerably weaker. Despite this, the survivin vaccine was the only one to show significant anti-tumour activity against the murine glioma GL261. Further evaluation of the survivin vaccine showed that the anti-tumour activity could not be attributed to induction of IFN-γ-producing cells. In addition, activity of the vaccine was lost in MHC class II deficient hosts, suggesting CD4+ T cells are critically involved.

It was observed that the α-GalCer component of the conjugate vaccines was unable to freely bind CD1d and activate iNKT cells in the absence of APCs. The vaccines were therefore chemically stable and free α-GalCer is not being released extracellularly. However, the vaccines were able to stimulate iNKT cell in vivo. This implies that the conjugate vaccines require uptake and processing by DCs to present α-GalCer before stimulating iNKT cells. The slight IL-2 response from α-GalCer in the in vitro assay in the absence of CD1d likely reflects α-GalCer excess adhering to the surface of the plate, not being washed away, and causing weak iNKT cell activation. Our lab has previously shown cathepsin B-mediated cleavage of the VC-PAB linker\textsuperscript{54}, which remains to be investigated for the TRP-2, gp100 and survivin vaccines.

Strong iNKT cell activation was observed when the TRP-2, gp100 and survivin vaccines were administered IV, as this route allows for systemic delivery of α-GalCer, permitting access to iNKT cell-rich regions such as the spleen and liver\textsuperscript{50}. Conversely, SC and IM immunisation delivers antigens from the site of entry to the draining lymph nodes via direct drainage or cellular transport\textsuperscript{231,232}. Antigen that is drained to the
lymph nodes is less likely to circulate to the spleen and liver and activate iNKT cells, and this may account for the observed decrease in iNKT cell activation when delivering vaccines SC or IM. Furthermore, the survivin vaccine IV induced the strongest activation of iNKT cells, compared to the TRP-2 and gp100 vaccines. It is possible that the structure of the peptide attached to α-GalCer may alter factors such as solubility, cellular uptake, chemical stability or biodistribution of α-GalCer, leading to differences in iNKT cell activation, although these phenomena require further investigation.

Overall, improved cytotoxic CD8⁺ T cell responses were observed when the conjugate vaccines were delivered IV, compared to SC and IM. This may reflect the greatest increase of iNKT cell activation with IV delivered vaccines, causing subsequent cytotoxic CD8⁺ T cell immune responses capable of efficiently killing the target cells. Although antigen-specific CD8⁺ cytotoxic responses were induced with the TRP-2 vaccine IV and the gp100 vaccines IV and SC, these vaccines were unable to cause any \textit{in vivo} anti-tumour response. As previously mentioned, studies have shown vaccine-mediated anti-tumour responses when targeting TRP-2 and gp100 in glioma models\textsuperscript{137,233,234}. Additionally, effective anti-tumour responses have been observed with our conjugate vaccine constructs\textsuperscript{54}, confirming the efficacy of the vaccine design. Furthermore, RT-PCR confirmed that TRP-2, gp100 and survivin are all expressed on GL261 cells (Farrand \textit{et al}, unpublished data). Another possibility is that tumour-mediated immune suppression is interfering with the ability of the TRP-2 and gp100 vaccines to activate T cells \textit{in vivo}, or exert their effector function at the tumour site. Without tumours, the vaccines may be able to exert more killing mechanisms in an environment free of tumour-derived immunosuppressive factors. Due to time constraints, this study could not investigate the impact of alleviating tumour-mediated immune suppression with immune modulators to improve anti-tumour responses of the TRP-2 and gp100 vaccines.
Although the survivin vaccine delivered IV was able to induce iNKT cell activation, the conjugate vaccine produced weak survivin-specific killing, despite containing a defined CD8+ epitope. Previous studies using a survivin-loaded DC vaccine induced potent CD8+ survivin-specific T cell responses, and prolong survival in mice with GL261 IC tumours. As our survivin vaccine was unable to induce a strong CD8+ cytotoxic response, it was predicted that the survivin vaccine would not induce an anti-tumour response in vivo. Interestingly, the survivin vaccine significantly delayed tumour growth in a GL261 SC model, suggesting additional anti-tumour mechanisms to the minimal CD8+ cytotoxic response observed.

It was anticipated that the minimal antigen-specific killing observed with the survivin vaccine could be further improved with the addition of TCEP-treated peptide on the target cells. In fact, TCEP-treatment of the peptide loaded target cells did improve CD8+ T cell killing, and likely reflects the improved H-2Kb binding of the reduced peptide. Further treatment of the survivin vaccine, in addition to the peptide-loaded target cells, before administration to the mice may further increase antigen-specific killing. However, the effect of TCEP reduction on α-GalCer and the linker in the survivin vaccine may affect the structure and efficacy of the vaccine, although this remains unknown. Supplementary investigation to improve the CTL response with additional vaccine TCEP treatment was unable to be explored, but could potentially enhance antigen-specific CD8+ killing. It is plausible that oxidation of the survivin peptide portion of the conjugate vaccine, prior to injection, is processed or reduced in vivo. This would allow for the peptide to readily bind H-2Kb in vivo and induce CD8+ T cell killing against the tumour cells.

Inducing apoptosis in a target cell is the main way by which cytotoxic CD8+ T cells eliminate cancerous tissue. Other effector mechanisms of CD8+ T cells exist, including release cytokines such as IFN-γ, which have anti-tumour properties. Upon investigation, IFN-γ production was not detected in mice following survivin
Evaluating the immune response induced by conjugate vaccine

vaccination. Therefore, IFN-γ produced by CD8+ T cells is unlikely to be responsible for anti-tumour responses seen in mice treated with the survivin vaccine. However, additional experiments that investigate IFN-γ release from T\(_{H1}\) cells should be undertaken. It is possible that other cytokines produced by CD8+ T cells, such as tumour necrosis factor alpha (TNFα) and lymphotoxin-α (LT-α)\(^{236}\), may mediate the anti-tumour response and require further evaluation as potential effector mechanisms of the survivin vaccine.

With only minimal CD8+ cytotoxic responses detected from the survivin vaccine, CD4+ T cells were investigated as potential contributors to the anti-tumour response, as the survivin peptide contains a putative CD4+ epitope shown to stimulate CD4+ T cells\(^ {142}\). It was observed that in mice lacking MHC class II molecules, the anti-tumour response of the survivin vaccine was completely abolished, suggesting CD4+ T cells are required for the anti-tumour efficacy of the survivin vaccine. However, with further investigation of the CD4+ T cell response, the cells did not produce IFN-γ, suggesting that the anti-tumour response is not mediated through IFN-γ release from T\(_{H1}\) cells. Again, other cytokines may be involved.

It has been shown that CD4+ T cells are capable of acquiring cytotoxic abilities and exerting effector mechanisms on cancerous tissue\(^ {237-240}\). Perhaps the putative CD4+ epitope in the survivin vaccine stimulates cytotoxic CD4+ T cells (CD4+ CTLs) that mediate anti-tumour activity. Investigation of CD4+ CTLs induced by the survivin vaccine may identify an additional anti-tumour mechanism. If the efficacy of the survivin vaccine relies on CD4+ CTLs, in addition to CD8+ CTLs, this may explain the induction of only minimal antigen-specific cytotoxicity against CD8+ targets cells, as CD4+ T cells may mediate the majority of anti-tumour activity. A VITAL assay against target cells pulsed with the survivin peptide containing the putative CD4+ epitope, may produce potent antigen-specific killing. Unfortunately, due to time constraints cytotoxic CD4+ T cells induced by the survivin vaccine could not be explored.
A study conducted by Semmling et al, found that help provided by CD4+ T cells and iNKT cells, are qualitatively different\textsuperscript{241}, suggesting that both of these cells can provide help to the same CTL. Perhaps CD4+ T cells are still required for the induction of a potent CTL response. Further studies utilising TAP\textsuperscript{-} mice, which lack the ability to activate CD8+ T cells, may indicate the requirement for CD8+ T cells for the survivin vaccine-mediated anti-tumour response.

Alternative explanations for the anti-tumour response of the survivin vaccine could be attributed solely to \(\alpha\)-GalCer stimulating potent iNKT cell responses, or the linker portion of the vaccine causing additional, unknown immune responses. As both TRP-2 and gp100 vaccines contain the same \(\alpha\)-GalCer prodrug and linker as the survivin vaccine, but neither show efficacy \textit{in vivo}, it is unlikely that either \(\alpha\)-GalCer or the linker contribute to the anti-tumour response of the survivin vaccine.

Although anti-tumour mechanisms produced by the survivin vaccine remain unclear, it is clear that the vaccine is capable of causing a significant response against GL261 tumour cells. It is unlikely that CD8+ cytotoxicity is the sole anti-tumour mediator of the survivin vaccine, as only minimal killing was observed. Irrespective of the mechanism of anti-tumour activity, the survivin vaccine may be further improved with combinational therapies that enhance CD8+ T cell response.

\textbf{3.4.1 Conclusion}

In this chapter, I have demonstrated that the conjugate vaccines induce T cell responses to the TAA they encode. While the TRP-2 and gp100 vaccines were capable of causing a potent CTL response \textit{in vitro}, they failed to cause significant anti-tumour responses \textit{in vivo} against established GL261 tumours. Conversely, the survivin vaccine, which induced a weak CD8+ T cell-mediated cytotoxic response did delay tumour
growth. The anti-tumour activity of the survivin vaccine was shown to be dependent on CD4+ T cells, although the exact involvement of these cells remains unclear.

These findings do not unequivocally support the initial hypothesis that the iNKT-cell dependent glycolipid-peptide vaccines induce potent CD8+ T cell responses that mediate anti-tumour activity, although this data provide some evidence in that direction. Further evaluation of the involvement of CD4+ T cell responses, which have often been overlooked in cancer vaccines studies, are warranted.

Nonetheless, some significant anti-tumour activity was observed with the survivin vaccine, although full resolution of the tumour was not achieved in most cases. Therefore, the impact of immunomodulation on vaccine-induced outcome is explored in the next chapter.
4 Assessment of survivin vaccine in combination with immune modulators
4.1 Introduction

While the success of immune checkpoint inhibitors for cancer treatment validates the concept of enhancing T cell-mediated immune responses to eliminate malignant tumours, it is clear that not all patients respond. As checkpoint inhibitors unleash T cell-mediated anti-tumour responses that develop naturally in patients as cancers evolve, it is thought that perhaps patients who fail to respond to this form of immunotherapy have not initiated a process of T cell priming in the first place. Therefore, there is considerable interest in utilising vaccination to prime an effective T cell response in patients that lack a pre-existing one, to improve response to checkpoint blockade. Furthermore, vaccine-mediated T cell responses may be enhanced with other immune modulators, such as costimulatory agents or the depletion of TREGS, leading to improved anti-tumour responses.

T cell activation can be modified by positive and negative immunological checkpoints, that provide signals to control T cell activation. Positive checkpoints are controlled by costimulatory receptors, such as 4-1BB, which enhance T cell activation, while negative checkpoints are provided by inhibitory receptors, such as PD-1, LAG-3 and CTLA-4, which dampen T cell activity. The inhibitory receptors regulate T cell activation at different stages of an immune response. An important checkpoint molecule, CTLA-4, inhibits T cell activity by binding CD80/86 on APCs and delivering negative signals to the T cell, which must be overcome for activation of the cell. In the periphery, PD-1 and LAG-3, inhibit T cell activation to prevent excessive autoimmunity. Checkpoint blockade interferes with these normal signals that regulate T cell activation, by binding inhibitory receptors and preventing negative signals that oppose T cell activation. Therefore, T cells bound with antagonistic antibodies remain activated and can exert anti-tumour effector functions. Checkpoint inhibitors such as ipilimumab (α-CTLA-4), pembrolizumab and nivolumab (both α-PD-1), are used in the clinic and are promising anti-tumour therapies for patients with advanced cancers.
The success of these checkpoint inhibitors has sparked interest for the development of next-generation monoclonal antibodies that modulate the immune response.

The activity of T cells can also be enhanced through targeting activatory receptors. Costimulatory receptors, such as 4-1BB, regulate T cell response by increasing T cell activation\textsuperscript{109}. The agonistic antibody, α-4-1BB, binds the activatory receptor and enhances T cell activity\textsuperscript{198}. Vaccine-induced T cell responses that are further activated with α-4-1BB, produce potent responses capable of killing cancerous tissue\textsuperscript{196,242}.

Furthermore, activation of T cells can be inhibited by T\textsubscript{REGS} in the TME. Cumulative evidence supports the existence of increased numbers of T\textsubscript{REGS} in solid tumours\textsuperscript{243-245} and haematological malignancies\textsuperscript{246-248}, compared to healthy tissue. The monoclonal antibody, α-CD25, depletes T\textsubscript{REGS} and alleviates suppression in the TME and enhances activation of vaccine-stimulated T cells\textsuperscript{249}.

As demonstrated in the previous chapter, the survivin conjugate vaccine is capable of delaying growth of established GL261 tumours implanted SC, although eventually the mice succumb to the tumour. Here, the possibility that the stimulated T cell response of the survivin vaccine can be further enhanced with the addition of immune modulators was examined. As previously noted, immune modulation with checkpoint inhibitors can produce durable responses, but not in all patients. Therefore, combining vaccination with immune modulators may increase the number of patients who respond to therapy. However, it remains unclear as to which combination of cancer therapies will produce durable anti-tumour responses in the majority of patients, and which of these patients will respond. If the conjugate vaccines studied here are to reach the clinic in a cancer setting, this information will be important for the clinical plan for commercial development, especially as vaccination alone is unlikely to have clinical impact without some other form of immunomodulation in patients with advanced disease.
4.2 Aims

This chapter investigates the anti-tumour activity of the survivin conjugate vaccine in combination with immune modulators, including checkpoint inhibitors, costimulatory agents, or the depletion of T\textsuperscript{REGS}.

Specific aims were to:

1. Assess the anti-tumour response of the survivin vaccine combined with immune checkpoint inhibitors \(\alpha\)-PD-1, \(\alpha\)-LAG-3 or \(\alpha\)-CTLA-4.

2. Assess the anti-tumour effect of combining the survivin vaccine with the costimulatory agent, \(\alpha\)-4-BB.

3. Assess the anti-tumour response of the survivin vaccine in the absence of T\textsuperscript{REGS}. 
4.3 Results

4.3.1 Anti-tumour effects of α-PD-1 were not enhanced by vaccination

As previously mentioned, PD-1 receptors work to inhibit T cell activity in the periphery to avoid excessive autoimmunity. The immune checkpoint inhibitor, α-PD-1, prevents PD-1 on activated T cells binding PD-L1/L2 on APCs or tumour cells. As PD-L1/L2 are expressed on tumour cells, and a high proportion of TILs are PD-1+, it was decided to test the combination with vaccination by administering α-PD-1 after survivin vaccination had been used to initiate an anti-tumour response. The aim was to prevent inhibition of activated T cells in the periphery and TME. Mice received 1 x 10⁶ GL261 tumour cells SC and seven days later received either PBS or survivin vaccine. Three days following vaccination mice were treated with either PBS control or 250 µg α-PD-1 intraperitoneally (IP) (Figure 4.1 A).

Administration of α-PD-1 alone produced durable tumour regression in the majority of mice (80%) with SC GL261 tumours (Figure 4.1). The survivin vaccine alone was able to induce regression in this experiment, although 60% of animals still ultimately succumbed. The combination of vaccine and checkpoint inhibitor produced tumour regression in 80% of the mice. Therefore, there was no evidence that the combined therapy had improved activity over either therapy alone.
Figure 4.1: PD-1 inhibition with checkpoint inhibitor is sufficient for GL261 SC tumour clearance. (A) Experimental outline for the treatment of SC GL261 tumours with survivin vaccine and α-PD-1. C57BL/6 mice were injected SC with 1 x 10⁶ GL261 cells. Tumour sizes were monitored and recorded and mice were culled when tumours reached 200 mm² in size. Individual tumour growth for mice with SC tumours treated with either (B) PBS, (C) vaccine alone on day seven, (D) 250 µg α-PD-1 on day 10 or (E) both. Data shown is n = 5 per group. (F) Survival curves are two combined experiments. Statistical analysis of survival curves was performed using Log-Rank (Mantel Cox) test using a Bonferroni correction threshold of p<0.008.
4.3.2 Anti-tumour effects of lower dose of α-PD-1 were not enhanced by vaccination

Due to notable clearance of tumours with α-PD-1 monotherapy, interpreting the combined effect of survivin vaccine and α-PD-1 is difficult. To investigate the contribution of the vaccine to the anti-tumour response in the combined therapy, a lower dose of α-PD-1 was assessed. In this experiment (Figure 4.2), mice were inoculated with $1 \times 10^6$ GL261 SC tumours and vaccinated IV one week later. Three days post-vaccination, mice received PBS control or 50 µg of α-PD-1 IP (one fifth of the dose used in the previous experiment).

Here, surprisingly, the survivin vaccine failed to produce an anti-tumour response (Figure 4.2 C, F). Therefore, interpreting the combined therapy is difficult since the survivin vaccine may not be functioning optimally. However, it is important to note that α-PD-1 monotherapy at this lower dose was unable to prevent tumour growth in the mice, suggesting the anti-tumour effect of α-PD-1 requires a higher dose. Further experiments with a dose-titration of α-PD-1 will be useful to discern potential synergy of the vaccine and α-PD-1 in the combined therapy. However, due to time restraints these experiments could not be performed.
Assessment of survivin vaccine in combination with immune modulators

Figure 4.2: Low dose α-PD-1 does not inhibit tumour growth. (A) Experimental outline for comparing monotherapy of the survivin vaccine or low dose α-PD-1 to combinational therapy. C57BL/6 mice were injected SC with 1 x 10^6 GL261 cells. Tumour sizes were monitored and recorded and mice were culled when tumours reached 200 mm^2 in size. Individual tumour growth for mice with SC tumours treated with either (B) PBS, (C) vaccine alone on day seven, (D) 50 µg α-PD-1 on day 10 or (E) both. Data shown is n = 6 - 7 per group. (F) Survival curves are representative of one experiment. Statistical analysis of survival curves was performed using Log-Rank (Mantel Cox) test using a Bonferroni correction threshold of p<0.008.
4.3.3 Anti-tumour effects of α-LAG-3 were not enhanced by vaccination

The checkpoint molecule LAG-3, inhibits T cell activity to prevent an excessive immune response. As LAG-3 is upregulated on T cells several days after activation, α-LAG-3 was administered after survivin vaccination. Mice were inoculated with 1 x 10^6 GL261 cells SC and treated on day seven with either PBS or the survivin vaccine. Mice received PBS control or 200 µg α-LAG-3 IP two days post-vaccination (Figure 4.3 A).

As previously seen in Figure 4.2, the unreliability of the survivin vaccine confounded analysis, as it failed to produce an anti-tumour response as a single agent (Figure 4.3 C, F). Interpreting the combined therapy of survivin vaccine and α-LAG-3 is therefore difficult. The data indicate that monotherapy of α-LAG-3 was effective in 40% of mice, and this was not improved by vaccination. In fact, no animals survived in the combined group. It is important to note that a mouse in the PBS treatment also cleared the tumour, suggesting endogenous anti-tumour responses can be mounted in the absence of therapy. Due to time constraints, this experiment was only conducted once, and needs to be repeated before strong conclusions can be made. Nonetheless, there does not appear to be any advantage of combining vaccination with α-LAG-3.
Figure 4.3 Combination of survivin vaccine and α-LAG-3 did not improve anti-tumour responses. (A) Experimental outline for comparing monotherapy of the survivin vaccine or α-LAG-3 to combinational therapy. C57BL/6 mice were injected SC with 1 x 10^6 GL261 cells. Tumour sizes were monitored and recorded and mice were culled when tumours reached 200 mm² in size. Individual tumour growth for mice with SC tumours treated with either (B) PBS, (C) vaccine alone on day seven, (D) 200 µg α-LAG-3 on day nine or (E) both. Data shown is n = 5 per group. (F) Survival curves are representative of one experiment. Statistical analysis of survival curves was performed using Log-Rank (Mantel Cox) test using a Bonferroni correction threshold of p<0.008.
4.3.4 Combining survivin vaccine with α-4-1BB improved anti-tumour response

The aforementioned antagonistic antibodies, α-PD-1 and α-LAG-3, work to inhibit and prevent interactions with corresponding ligands. Conversely, the agonistic antibody α-4-1BB, provides costimulatory signals to CD4+ and CD8+ T cells to further enhance activation. Administration of α-4-1BB was given twice prior to vaccination to amplify T cell activity. Mice were inoculated with 1 x 10^6 GL261 cells SC and treated with PBS control or 100 µg α-4-1BB IP on day three and day six. Mice were subsequently vaccinated with PBS or survivin vaccine on day seven (Figure 4.4 A).

In this experiment, again there was evidence of an endogenous anti-tumour response in the PBS treated group. Nonetheless the survivin vaccine produced some further delay in tumour growth, although this did not reach significance on survival (Figure 4.4 C). Tumour regression of a single mouse was observed with a monotherapy of α-4-1BB, but there was no significant difference in survival outcome (Figure 4.4 D, F). When the vaccine was combined with α-4-1BB, tumour regression was found in 50% of the mice, and survival was improved compared to vaccine alone (Figure 4.4 E, F).
Figure 4.4: Combined survivin vaccine and α-4-1BB may enhance anti-tumour response. (A) Experimental outline for comparing monotherapy of the survivin vaccine or α-4-1BB to combinational therapy. C57BL/6 mice were injected SC with 1 x 10^6 GL261 cells. Tumour sizes were monitored and recorded and mice were culled when tumours reached 200 mm² in size. Individual tumour growth for mice with SC tumours treated with either (B) PBS, (C) vaccine alone on day seven, (D) 100 µg α-4-1BB alone on day 3 and day 6, or (E) both. Data shown is n = 6-7 per group. (F) Survival curves are two combined experiments. Statistical analysis of survival curves was performed using Log-Rank (Mantel Cox) test using a Bonferroni correction threshold of p<0.008.
4.3.5 Anti-tumour effects of inactivation of T\textsubscript{REGS} were not enhanced by vaccination

Removing T\textsubscript{REG} suppression encourages an unrestricted immune response directed by conventional T cells. While administration of antibodies to the IL-2R\alpha (CD25) is able to limit T\textsubscript{REG} function, this receptor is necessary on all activated T cells as IL-2 promotes differentiation of T cells into effector and memory T cells\textsuperscript{251}. Therefore, α-CD25 was administered early, at the time of tumour inoculation, so that there was less circulating antibody at the time of vaccination to avoid depleting vaccine-induced effector T cells. This regimen had been shown to work in combination with a cellular vaccine in the GL261 model in our laboratory\textsuperscript{249}. Therefore, mice were inoculated with $1 \times 10^6$ GL261 cells SC and also treated with PBS control or 125 µg of α-CD25 IP at the time of tumour challenge. One week later mice were vaccinated with either PBS or survivin vaccine IV (Figure 4.1 A).

Administration of the survivin vaccine alone delayed tumour growth, but did not induce regression (Figure 4.5 C, F). Administering α-CD25 alone was sufficient at clearing tumours in 71% of the mice (Figure 4.5 D). However, this tumour clearance was not further improved with the addition of the survivin vaccine.
Figure 4.5: Depletion of T_{REGS} with α-CD25 causes anti-tumour response against GL261 SC tumours.
(A) Experimental outline for comparing monotherapy of the survivin vaccine or α-CD25 to combinational therapy. C57BL/6 mice were injected SC with 1 x 10^6 GL261 cells. Tumour sizes were monitored and recorded and mice were culled when tumours reached 200 mm² in size. Individual tumour growth for mice with SC tumours treated with either (B) PBS, (C) vaccine alone on day seven, (D) 125 µg α-CD25 alone on day zero, or (E) both. Data shown is n = 6-7 per group. (F) Survival curves are representative of one experiment. Statistical analysis of survival curves was performed using Log-Rank (Mantel Cox) test using a Bonferroni correction threshold of p<0.008.
4.3.6 The survivin vaccine combines with α-CTLA-4 to improve anti-tumour response

The checkpoint molecule, CTLA-4, is expressed early on T cells after activation, and interacts with ligands CD80/86, which are expressed on APCs, and generally not on tumour cells. It is therefore thought that CTLA-4-mediated inhibition of T cell function occurs in secondary lymphoid organs and not the TME. Based on this consideration, it was decided to administer α-CTLA-4 near the time of vaccination. Mice were inoculated with 1 x 10^6 GL261 cells SC and received PBS control or 500 µg of α-CTLA-4 IP six days later. Mice were vaccinated with either PBS or survivin vaccine on day seven (Figure 4.6 A).

As previously seen in some earlier experiments, the survivin vaccine alone was unable to delay tumour growth (Figure 4.6 C). Nonetheless, improved anti-tumour responses were observed when combining the survivin vaccine with α-CTLA-4, where 58% of mice were able to completely eradicate tumours (Figure 4.6 E). In fact, this treatment was the only combination to show statistically significant anti-tumour activity over the PBS group; α-CTLA-4 monotherapy, while inducing some tumour regression, did not reach this level of statistical significance.
Figure 4.6: Improved anti-tumour response when combining survivin vaccine with α-CTLA-4. (A) Experimental outline for comparing monotherapy of the survivin vaccine or α-CTLA-4 to combinational therapy. C57BL/6 mice were injected SC with 1 x 10⁶ GL261 cells. Tumour sizes were monitored and recorded and mice were culled when tumours reached 200 mm² in size. Individual tumour growth for mice with SC tumours treated with either (B) PBS, (C) vaccine alone on day seven, (D) 500 µg α-CTLA-4 alone on day six, or (E) both. Data shown is n = 5-7 per group. (F) Survival curves are two combined experiments. Statistical analysis of survival curves was performed using Log-Rank (Mantel Cox) test using a Bonferroni correction threshold of p<0.008.
4.3.7 Vaccine induced activation of iNKT cells is enhanced with CTLA-4 blockade

Because iNKT cells can express CTLA-4 it is plausible that the checkpoint inhibitor α-CTLA-4 enhanced α-GalCer-mediated activation of iNKT cells in the combined therapy, leading to improved anti-tumour response. Release of various cytokines in the serum can be used to indirectly measure iNKT cell activation. After activation with a strong agonist like α-GalCer, iNKT cells produce a burst of IL-4, followed by a subsequent burst of IFN-γ. Activated iNKT cells then feedback to DCs via CD40, which release IL-12p70. These events then transactivate NK cells, which induce a prolonged period of IFN-γ release, providing the majority of IFN-γ in the serum over the following 24-48 h. To test whether blockade of CTLA-4 signalling had an effect on iNKT cell activation in response to the survivin conjugate vaccine, serum was collected at 3, 6 and 21 h after the vaccine was administered with or without α-CTLA-4.

Administration of α-CTLA-4 alone did not generate a cytokine response that could be detected in serum. As expected, IL-4, IFN-γ and IL-12p70 could be detected after vaccine alone, reflecting release of α-GalCer from the conjugate. Interestingly, when the vaccine was combined with α-CTLA-4, increased IL-4 production was observed at 6 h, and increased IFN-γ production was observed at 21 h, suggesting that the impact of iNKT cell activation was altered. However, no significant impact was observed on IL-12p70 (Figure 4.7).
Figure 4.7: iNKT cell activity is enhanced with combined survivin vaccine and α-CTLA-4. C57BL/6 mice were injected SC with 1 x 10⁶ GL261 cells and treated with 500 µg α-CTLA-4 alone on day six, vaccine alone on day seven or both. Mice were bled at the indicated times to determine the levels of the cytokines (A) IL-4, (B) IL-12p70 and (C) IFN-γ in the serum, by a Bio-Plex kit. Data shown is n = 7. Statistical analysis was performed using One-Way ANOVA with Bonferroni post-test, showing mean ± SEM between survivin vaccine and combined therapy at the indicated time points. *p<0.05, **p<0.01, ns = not significant.
4.4 Discussion

It was hypothesised that the addition of immune modulating monoclonal antibodies would synergise with vaccine-induced T cell responses to survivin, and produce superior anti-tumour activity compared to either monotherapy alone. It was observed that α-PD-1 and α-CD25 monotherapies produced anti-tumour responses that were not improved with the vaccine. Additionally, preliminary data did not show an improved response with the survivin vaccine and α-LAG-3. An improvement in anti-tumour response was observed when combining the vaccine with α-4-1BB, however due to time constraints this mechanism could not be further explored. Interestingly, the vaccine-induced anti-tumour response was significantly improved with α-CTLA-4 therapy. Furthermore, higher levels of cytokines were observed in the serum with this combination, suggesting some iNKT cell-induced downstream activities were enhanced.

Karyampudi et al., demonstrated an enhanced response when combining a multi-peptide vaccine with α-PD-1, that led to improved survival and antigen-specific responses in a murine model of breast cancer. Other vaccine strategies also effectively combine with α-PD-1, such as irradiated tumour cell vaccines, which have shown potent anti-tumour responses with combined therapies. Collectively, the literature suggests that α-PD-1 therapy combined with vaccination can improve anti-tumour responses. However, as α-PD-1 monotherapy was sufficient at clearing tumours in 80% of mice in the model used here, it was difficult to show improved outcome with the addition of the survivin vaccine. To further investigate this, the dose of α-PD-1 was lowered to 50 µg per mouse. This decreased dosage, chosen without earlier experimentation, completely abolished α-PD-1-induced anti-tumour responses, and an additive or synergistic response between the therapies could not be observed. Further investigation with a suitable dose of α-PD-1, capable of inducing
some impact on tumour growth without causing full eradication, will be required to fully investigate the potential of the vaccine combined with α-PD-1.

The checkpoint molecule, α-LAG-3, was combined with the survivin vaccine in an attempt to enhance conventional T cell response. It has previously been shown that α-LAG-3 in combination with anti-tumour vaccination results in increased accumulation of effector CD8+ T cells in the tumour. Although combining vaccines with α-LAG-3 is less explored, studies have shown synergistic anti-tumour effects with tumour cell vaccines and sLAG-3, against renal cell carcinoma and mammary adenocarcinoma. Furthermore, Cappello et al., found a synergistic response when combining sLAG-3 with a DNA vaccine that encoded a protein for HER-2/neu oncogene against mammary carcinoma. Although only performed once, the combined therapy of the survivin vaccine and α-LAG-3 did not improve anti-tumour response compared to either therapy alone.

An increase in survival was observed with the survivin vaccine combined with α-4-1BB. Previous studies have shown increased anti-tumour responses with vaccination and α-4-1BB. DC vaccination combined with α-4-1BB, producing potent anti-tumour responses against mammary carcinoma and prostatic carcinoma. Given its role as a costimulatory molecule, it is possible that the survivin vaccine’s impact on iNKT cells is enhanced with α-4-1BB leading to increased anti-tumour response. Additional costimulatory agents, such as α-CD40, may have similar impact, and should also be investigated.

Studies investigating vaccination and depletion of TREGs, have provided some evidence of improved anti-tumour response. A peptide vaccine targeting AH1, a CD8+ epitope from CT26 colon carcinoma cells, showed enhanced anti-tumour responses with the addition α-CD25 before vaccination. Furthermore, DC-based vaccines in combination with α-CD25 have shown improved tumour-free survival against colon
carcinoma decreased numbers of melanoma pulmonary metastases, and enhanced protective tumour immunity against melanoma. It is important to note that α-CD25 does not completely deplete TREGS, as cells with low or no CD25 are not affected by the antibody. Some have argued that the antibody inactivates, rather than depletes TREGS. In the model assessed here, the depletion of TREGS alone with α-CD25 was capable of inducing tumour regression in 71% of the animals, suggesting that there were endogenous anti-tumour T cells in the TME capable of killing the cancerous cells. This endogenous response was also evident in some PBS-treated groups, where regression was seen sporadically. However, combining the survivin vaccine with depleted TREGS did not provide any further improvement of anti-tumour response. Perhaps some TREGS remain in the TME, or are even induced by the vaccine, and exert immunosuppressive effects on vaccine-induced effector T cells.

The only statistically significant improvement in anti-tumour activity of the survivin vaccine with immunomodulation was when the vaccine was combined with α-CTLA-4. Similarly improved vaccine-induced anti-tumour responses have been previously been reported, notably when α-CTLA-4 was combined with a GM-CSF-expressing tumour cell vaccine in a murine model of melanoma. Another study combined an irradiated tumour cell vaccine with α-CTLA-4, and found lower tumour grades and increased inflammatory cells in prostate carcinoma. Gregor et al., showed improved tumour free survival when combining a DNA vaccine encoding TRP-2 and α-CTLA-4, compared to either monotherapy. More recently, a whole glioma cell vaccine combined with α-CTLA-4 produced prolonged survival in mice, compared to either monotherapy.

As the survivin vaccine alone was unable to consistently delay tumour growth, the improved anti-tumour response with the α-CTLA-4 combined therapy was perhaps unexpected. Analysis of cytokines in serum showed that administration of survivin vaccine with α-CTLA-4 led to increased IFN-γ, perhaps reflecting improved iNKT cell
activation and subsequent transactivation of NK cells. There are limited studies investigating the effects of checkpoint inhibitors on iNKT cells. In studies on human cells Kamata et al., (2016) showed that α-PD-L1 and α-GalCer pulsed APCs enhanced iNKT cell mediated anti-tumour response, suggesting checkpoint blockade augmented iNKT cell activation. This study also showed increased iNKT cell IFN-γ production, which led to subsequent NK cell activation\textsuperscript{277}. Although α-PD-L1 was not investigated here, α-PD-1 did not combine effectively with the survivin vaccine, so if there was some relief from PD-1 signalling in iNKT cells, it did not have significant clinical impact in the GL261 model. However, blockade of CTLA-4 did have some impact, perhaps by having a greater capacity to enhance iNKT cell activation and increase transactivation of NK cells. An increase in IFN-γ was observed, a cytokine that can have a direct anti-tumour responses through blockade of angiogenesis\textsuperscript{70}. Both iNKT cell and NK cells can also have direct anti-tumour activity through release of cytotoxic granules. Future experiments investigating iNKT cell and NK cell activation, such as \textit{in vitro} killing assays with NK cells, or \textit{in vivo} depletion of NK cells, may demonstrate an additional anti-tumour mechanism of the survivin vaccine.

Future work investigating the survivin vaccine combined with multiple immune modulators may unveil novel therapeutic combinations that increase anti-tumour activity. The potential to improve anti-tumour therapies by combining multiple checkpoint inhibitors that target different pathways, has been investigated in clinical trials. A phase I study investigating nivolumab and ipilimumab for the treatment of melanoma found an overall response of 61% in the combined group, compared to 11% for ipilimumab alone\textsuperscript{178}. However, this trial did not contain a nivolumab monotherapy group. A phase III study investigating combined nivolumab and ipilimumab for the treatment of melanoma found the median progression-free survival was increased with the combined therapy (11.5 months), compared to ipilimumab (2.9 months) and nivolumab (6.9 months)\textsuperscript{278}. 
A major limitation of this study is the variable anti-tumour responses from the survivin vaccine, making interpretation of the combined therapies difficult. Reasons for this variation may include batch to batch variability of the survivin vaccine, storage time affecting stability or oxidation state prior to injection, or time the vaccine spent reconstituted before freezing. These factors have the potential to alter the release of the active components in vivo, and impair anti-tumour activity of the survivin vaccine. To minimise the potential inhibition of mechanistic activity, new vaccines were made up from solid material prior to injection for each experiment. While the variable response of the survivin vaccine made interpretation difficult, it was possible to enhance the anti-tumour response with α-CTLA-4. In fact, this effect may have been overlooked if the survivin vaccine alone produced potent anti-tumour responses in these combined experiments. A second limitation to this study is that the GL261 tumours proved immunogenic and naturally regressed in some mice in the control groups, which may have skewed results in both control and treatment groups. For this reason, tumour groups were increased to seven in the latter part of the study, in order to minimise this effect and improve statistical significance.

This chapter illustrated that some immunomodulators can combine with vaccination to improve anti-tumour responses, while others were sufficient as monotherapies in this particular glioma model. In the clinic, it is apparent that not all patients’ respond to cancer immunotherapies such as checkpoint blockade, and succumb to disease. Therefore, there is a critical need to develop methods to identify the most suitable cancer therapy for any given patient. Predicting the best therapy for each patient is essential for increasing the number of responders to treatment. The results in this chapter suggest that some, but not all, combinations provided a beneficially anti-tumour response.
4.4.1 Conclusion

In this chapter I have combined the survivin vaccine with an assortment of immune modulators in an attempt to improve anti-tumour response. In this particular model α-PD-1 and α-CD25 alone were sufficient at clearing GL261 SC tumours and therefore did not require additional T cell stimulation with the vaccine. Preliminary data investigating the combined effects of α-LAG-3 and the survivin vaccine were not promising, and were not further investigated. Combining the survivin vaccine with α-4-1BB improved anti-tumour responses, although this was not further explored due to time constraints. The data demonstrate that combining the survivin vaccine with α-CTLA-4 improved tumour clearance, compared to the survivin vaccine alone. It is possible that administration of α-CTLA-4 prior to vaccination boosted vaccine-induced immune responses through enhanced iNKT cell and subsequent NK cell activation.
5 General Discussion
5.1 Summary of results

The aim of this thesis was to investigate the ability of iNKT cell-dependent glycolipid-peptide vaccines to stimulate anti-tumour CD8\(^+\) T cell immune responses, and assess whether responses could be further enhanced by the addition of immune modulators in a murine model of glioma.

Vaccines encompassing epitopes to TRP-2 and gp100 showed significant antigen-specific CTL responses, but surprisingly did not limit tumour growth. The lack of anti-tumour response may be due to tumour-mediated suppression that interferes with the vaccines ability to activate effector T cells \textit{in vivo}. It is possible that immune suppression could be overcome with the use of immune modulators, however, alleviating suppression for these vaccines was unable to be investigated within the scope of this thesis.

Conversely, I demonstrated that a vaccine comprising the survivin peptide produced only minimal antigen-specific CD8\(^+\) T cell responses, but slowed tumour growth in a SC model. Therefore, it was hypothesised that the anti-tumour response of the survivin vaccine was unlikely to be solely mediated by CD8\(^+\) cytotoxicity, and so additional mechanisms of activity were investigated. Since the survivin peptide contains a putative CD4\(^+\) T cell epitope, the induction of CD4\(^+\) T cell responses was assessed. The efficacy of the survivin vaccine was lost in MHC class II\(^+\) mice, suggesting that the anti-tumour response was dependent on CD4\(^+\) T cells. Upon further investigation, IFN-\(\gamma\) release was not detected, suggesting the CD4\(^+\) T cell response was not reliant on T\(_{H1}\) cells.

An overarching aim of this thesis was to improve vaccine-mediated anti-tumour responses with the addition of immune modulating monoclonal antibodies. Treatment with either \(\alpha\)-PD-1, or the depletion of T\(_{REGS}\), was successful at producing potent anti-
tumour responses; however, it was observed that these therapies do not require further combination with the survivin vaccine to be effective in a GL261 SC model. Preliminary data indicated that combination treated with the vaccine and α-LAG-3 did not enhance tumour regression. An increase in anti-tumour activity when the vaccine was combined with both α-4-1BB and α-CTLA-4 was observed. Further investigation revealed that the survivin vaccine combined with α-CTLA-4 enhanced iNKT cell activation and transactivation of NK cells.

5.2 The survivin vaccine is dependent on CD4⁺ T cells

Although there has been great interest in the design of vaccines that induce potent CD8⁺ T cell responses that can directly kill cancer cells, it is now clear that CD4⁺ T cells also play critical roles in anti-tumour response. Older studies have previously demonstrated that peptide vaccines incorporating MHC class II-restricted peptides augment anti-tumour immunity, but protection is dependent on CD8⁺ CTLs²⁷⁹-²⁸³. More recently a study demonstrated that the majority of immunogenic mutations on murine melanoma, colon carcinoma and mammary carcinoma models were recognised by CD4⁺ T cells, and vaccination targeting these mutations produced strong anti-tumour responses²⁸⁴.

The efficacy of the survivin vaccine was lost in MHC class II⁻/⁻ mice, demonstrating a requirement for CD4⁺ T cells to generate anti-tumour responses. It was predicted that CD4⁺ T cell help, along with iNKT cell help, was necessary for the induction of CD8⁺ T cell-mediated anti-tumour response. Studies have suggested that both Th1 and Th2 cells mediate anti-tumour activity, although Th1 cells may be more potent²⁸⁵,²⁸⁶. IFN-γ release from Th1 cells enhances priming and expansion of CD8⁺ T cells, and recruits NK cells and macrophages to the tumour, where they can contribute to tumour
eradication. However, IFN-\(\gamma\) release from \(\text{T}_{\text{H}}1\) cell was not detected, suggesting the CD4\(^+\) dependent anti-tumour response did not rely on \(\text{T}_{\text{H}}1\) cells.

It has been hypothesised that the survivin vaccine’s dependence on CD4\(^+\) T cells may be due to the induction of cytotoxic CD4\(^+\) T cells (CD4\(^+\) CTLs), which are capable of directly killing tumour cells. Several mouse\(^ {290}\) and human\(^ {291-293}\) studies have reported cytotoxic capabilities of CD4\(^+\) T cells. Originally CD4\(^+\) CTLs were observed in cell lines and CD4\(^+\) T cell clones generated \textit{in vitro}, and thought to be artefacts\(^ {294-297}\). Studies have since provided unambiguous evidence of antigen-specific cytotoxic CD4\(^+\) T cells under conditions of viral infection. The presence of these cells has been observed in peripheral blood of subjects with acute influenza virus\(^ {298}\), but are more often associated with chronic infections, including cytomegalovirus (CMV)\(^ {299}\), Epstein-Barr Virus (EBV)\(^ {300}\) and Human Immune deficiency Virus (HIV)\(^ {301}\). These studies argue that persistent antigen exposure may drive differentiation of CD4\(^+\) T cells into CTLs. Furthermore, it has been reported that persistent antigen exposure can terminate a \(\text{T}_{\text{H}}\) lineage transcription factor and reactivate the CTL pathway in CD4\(^+\) T cells\(^ {302,303}\).

During thymic development, thymocytes commit to either a \(\text{T}_{\text{H}}\) or CTL lineage fate. This dichotomy is tightly controlled by the zinc finger transcription factor T helper-inducing POZ-Kruppel-like factor (ThPOK) and Runt related transcription factor 3 (Runx3). ThPOK actively suppresses the cytolytic program in MHC class II CD4\(^+\) thymocytes\(^ {304}\), while Runx3 opposes ThPOK in MHC class I CD8\(^+\) thymocytes, promoting CTL lineage commitment\(^ {305}\). The potential of post-thymic differentiation of CD4\(^+\) T cells into CTLs raises the possibility that they may be induced to eliminate cancerous cells in certain situations.

More recently, the cytotoxic ability of CD4\(^+\) T cells against tumour cells has been studied. In Burkitt’s lymphoma cell lines, EBV-specific CD4\(^+\) CTLs were shown to directly target cancerous cells\(^ {238}\). In transgenic mouse models, CD4\(^+\) T cells protected against B cell lymphoma\(^ {306}\), and eradicated established B16 melanoma tumours in the
absence of CD8$^+$ T cells$^{307,308}$. Furthermore, naïve CD4$^+$ T cells, adoptively transferred into melanoma-bearing mice, developed a cytotoxic phenotype and anti-tumour responses were dependent on MHC class II restricted CD4$^+$ CTLs$^{239}$.

Interestingly, the mechanisms of direct CD4$^+$ CTL-killing of virally-infected and cancerous cells have been hotly debated. It was reported that the Fas:FasL apoptosis pathway was a major mechanism of cell death induced by cytotoxic CD4$^+$ T cells with virally-infected B cells$^{309}$ and LCMV-infected target cells$^{310}$. Although CD4$^+$ T cell-mediated lysis has been reported to be Fas:FasL-induced, it is likely that other pathways are involved$^{309}$. More recently, studies have provided evidence that CD4$^+$ CTLs directly kill cancerous tissue through the perforin-granzyme pathway$^{311-313}$. Consistent with this data, analysis of CD4$^+$ CTLs from patients with B-cell chronic lymphocytic leukaemia (B-CLL) indicate the presence of lytic granules that contain granzymes and perforin, and that lytic activity is MHC class II-restricted$^{237}$.

Since CD4$^+$ CTLs have been shown to be capable of exerting effector mechanisms on cancerous tissue, vaccines that induce cytotoxic CD4$^+$ T cells represent an attractive approach to tumour therapy. Recently, there have been two reports demonstrating vaccine-induced cytotoxic CD4$^+$ T cells via a TLR-4 agonist and a recombinant protein antigen$^{314}$, and complete Freud’s adjuvant (CFA) and viral mouse cytomegalovirus (MCMV)$^{315}$. Interestingly, the first study found that killing required expression of CD40L on the CD4$^+$ CTL and CD40 on the target cells, with no role for granzyme and perforin, or FasL-mediated killing$^{314}$. The second study found that granzyme was expressed on the vaccine-induced CD4$^+$ CTLs, but the mechanisms of vaccine-induced killing were not determined$^{315}$.

The growing evidence for the involvement of CD4$^+$ CTLs in killing cancer cells, and the induction of cytotoxic CD4$^+$ T cells through vaccination, supports the theory that the anti-tumour response observed \textit{in vivo} with the survivin vaccine may be mediated
by cytotoxic CD4+ T cells (Figure 5.1 A). Indeed, a CD4+ CTL-mediated anti-tumour response might explain the minimal cytotoxicity observed when targeting the CD8+ epitope in the VITAL assay. Conducting a VITAL assay that targets CD4+ epitopes, or assessing the expression of ThPOK may address the unresolved involvement of CD4+ CTLs.

5.3 Combinational therapy with α-CTLA-4 enhanced iNKT and NK cell activation

The endogenous immune responses to GL261 unleashed with both α-PD-1 and α-CD25 administration, as well as spontaneous regression in some mice, suggests that the immune system is capable of killing GL261 cells, but requires additional help to overcome immune suppression. Conversely, the preliminary data with α-LAG-3 was not successful at slowing tumour growth, suggesting that the endogenous response against GL261 is controlled to a lesser extent through LAG-3. This endogenous immune response was also observed in control groups, where the highly immunogenic tumour regressed in some instances.

Improved survival when α-4-1BB administration was combined with the survivin vaccine was observed, however this combination was not further explored. Perhaps more promising was the improved anti-tumour response observed with the survivin vaccine and α-CTLA-4 combined therapy. As ipilimumab is in the clinic, this combination was further explored. Although only performed once, investigation of systemic cytokine production found greater IL-4 release with the combined therapy, compared to the vaccine alone, suggesting increased iNKT cell activation with the addition of α-CTLA-4 to the vaccine. iNKT cells have been shown to express CTLA-4252,316,317, therefore it is possible that α-CTLA-4 further enhanced activation of these
cells and subsequently increased anti-tumour response, compared to the vaccine alone.

It has previously been reported that activation of iNKT cells with free α-GalCer can induce anti-tumour immune responses in a melanoma model\textsuperscript{318}. GL261 tumour cells do not express CD1d\textsuperscript{249} and therefore iNKT cells cannot directly recognise the tumour. Instead, iNKT cell-mediated anti-tumour response must be facilitated by downstream effects on other cells, rather than direct lysis by iNKT cells. Following activation with α-GalCer presented by CD1d-expressing APCs, a burst of IL-4 from iNKT cells is detected, followed by a prolonged burst of IFN-γ by iNKT cells and transactivated NK cells, as well as IL-12 production from DCs\textsuperscript{253,254}. Increased IFN-γ production was observed 21 h after vaccination in the combined therapy, suggesting enhanced NK cell activation. Thus, a potential mechanism of the anti-tumour activity induced by the survivin vaccine may be augmentation of NK-mediated tumour cell killing.

NK cells have the capacity to distinguish stressed cells, such as tumour cells, from healthy tissue\textsuperscript{319}. The activation of these cells is controlled by changes in the composition of activatory and inhibitory receptor ligands on the surface of target cells. Inhibitory receptors on NK cells bind MHC class I on normal tissue and therefore do not kill healthy cells. In an attempt to avoid recognition by CD8+ T cells, tumours can decrease expression of MHC class I, however, this activates NK cells that are no longer held in check by the inhibitory receptors\textsuperscript{320,321}. In addition, ‘stressed’ cells, such as tumour cells, can upregulate activating ligands for NK cells\textsuperscript{322,323}. NK cells recognise and kill directly through the release of cytotoxic granules and pro-inflammatory cytokines such as IFN-γ\textsuperscript{324}. The increased IL-4 and IFN-γ production observed with the survivin vaccine and α-CTLA-4 combined therapy, suggest that enhanced iNKT cell activation led to increased NK cell activation that may orchestrate the anti-tumour response of this therapy (Figure 5.1 B).
Figure 5.1: Alternative cellular mechanisms of anti-tumour activity induced by the survivin vaccine. (A) iNKT cells enhance activation of DCs through CD40:CD40L interaction and cytokine production, which activates cytotoxic CD4+ T cells. CD4+ CTLs subsequently kill tumour cells either directly with Fas:FasL or indirectly with perforin and granzyme release. Furthermore, α-CTLA-4 may enhance activation of CD4+ CTLs. (B) CD1d+ DCs activate iNKT cells, which produce IFN-γ and promote NK cell activation. NK cells kill tumour cells either directly through activatory receptor engagement or indirectly through cytokine and cytotoxic mediator release. iNKT cells that express CTLA-4 molecules may be further stimulated by the addition of α-CTLA-4.
5.4 The future of peptide vaccination

The concept that the immune system can be harnessed by vaccination to kill tumours has been repeatedly demonstrated in murine models, but limited success in clinical trials has questioned efficacy in humans. Anti-tumour immunisation is a complex process, and although tremendous progress has been made in the last few years, we are still trying to understand the antigens and vaccine strategies that are optimal. Synthetic peptide-vaccines incorporating defined TAAs are more suitable for reproducibility and large-scale production compared to whole-tumour cell or DC vaccines. However, selection of the appropriate TAA to target is vital for mounting an effective immune response.

Without pre-screening patients, it is unknown if the targeted TAA is present on the tumour cells. Cancerous cells are often genetically unstable and harbour multiple mutations that lead to new epitopes called neo-antigens. T cells that recognise these neo-antigens as foreign can subsequently mount anti-tumour responses against the cancer. Sequencing the whole exome of each individual’s tumour, coupled with epitope prediction, may identify novel neo-antigens that can be targeted by personalised vaccines. The development of neo-antigen targeting peptide vaccines offers the promise of high specificity that defined TAA peptide vaccines cannot. Although hindered by cost, personalised immunotherapy represents an attractive approach to produce potent anti-tumour responses in patients for a broad range of cancers.

5.5 Conclusion

Current treatment options for patients with GBM are insufficient, therefore there is a need for the development of alternative therapies. Immunotherapy is becoming
recognised as a viable therapy for the treatment of melanoma, and may be a promising new treatment option for GBM. This thesis investigated the use of iNKT-dependent glycolipid-peptide vaccines to stimulate an anti-tumour response against glioma, which could be further enhanced with the addition of immune modulators.

The results presented here have shown that the α-GalCer portion of the vaccines is capable of activating iNKT cells. The TRP-2 and gp100 vaccines produced potent CD8+ T cell responses, but failed to prolong survival. Conversely, the survivin vaccine, which produced a minimal CD8+ T cell response, was able to increase survival. The in vivo response of the survivin vaccine was found to be dependent on CD4+ T cell, although the exact mechanism remains unknown. As the Th1 cytokine IFN-γ was not found, it was hypothesised that the CD4+ T cell response of the vaccine may be cytotoxic and able to directly kill tumours. CD4+ CTLs may mediate the anti-tumour response alongside CD8+ CTLs.

Importantly, a new combinational therapy with the survivin vaccine and α-CTLA-4 was found to improve anti-tumour responses, compared to the vaccine alone. This combinational treatment enhanced iNKT cell activation, which subsequently may increase transactivation of NK cells. It was postulated that the anti-tumour response of the survivin vaccine was mediated by NK cells, and further enhanced with α-CTLA-4 combined therapy.

While further work is required to elucidate the mechanism of an anti-tumour response produced by the survivin vaccine and α-CTLA-4, the combination treatment may represent a novel therapeutic strategy for the treatment of GBM.
6 References


15. Stupp R, Hegi ME, Mason WP, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in


58. Petrie HT, Livak F, Schatz DG, Strasser A, Crispe IN, Shortman K. Multiple


98. Antonarakis ES. Combining active immunotherapy with immune checkpoint


113. Townsend ARM, Rothbard J, Gotch FM, et al. The Epitopes of Influenza Nucleoprotein Recognized by Cytotoxic T Lymphocytes Can Be Defined with


151. Chambers CA, Sullivan TJ, Allison JP. Lymphoproliferation in CTLA-4-deficient mice is mediated by costimulation-dependent activation of CD4+ T


Foss F. Clinical Experience With Denileukin Diftitox (ONTAK). Seminars in Oncology. 2006;33:11-16.


265. Sharma S, Luciadomingues A, Lustgarten J. Aging affect the anti-tumor potential of dendritic cell vaccination, but it can be overcome by co-stimulation with anti-OX40 or anti-4-1BB. *Experimental Gerontology*. 


287. Li K, Baird M, Yang J, Jackson C, Ronchese F, Young S. Conditions for the


156


326. Hacohen N, Fritsch EF, Carter TA, Lander ES, Wu CJ. Getting Personal with
