Attenuation of Immune Suppression to Complement Glioma Immunotherapy

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Abstract

Glioblastoma Multiforme (GBM) is a malignant primary brain tumour with an extremely poor prognosis. Following surgical resection, radiotherapy, and concomitant and adjuvant chemotherapy, median survival is only 12-15 months. New therapeutic approaches are therefore desperately needed.

Accumulating evidence suggests that activated T cells are capable of selectively targeting and eliminating tumour cells, even in the brain, making vaccine-mediated immunotherapy a promising candidate for the treatment of brain cancers. However, cancer vaccination has generally been disappointing in the clinic, and is unlikely to bestow long-term survival unless suppressive mechanisms are overcome. Checkpoint blockade is a recent treatment modality that enhances naturally occurring T cell responses to cancer by relieving suppression mediated by immune checkpoints – molecular signals that prevent T cell function. While significant clinical responses are often seen, it is clear that most patients fail to respond to checkpoint blockade alone. Therefore, there is considerable interest in combining the different immunotherapeutic strategies, with vaccines providing an immunogenic stimulus to induce anti-tumour T cells, and checkpoint blockade to ensure T cell function is retained.

An orthotopic murine model of glioma was utilised to examine this form of combined treatment. Immune responses induced with a unique whole-cell vaccine that utilises the adjuvant properties of invariant natural killer T cells (iNKT cells) were able to resist tumour challenge, but failed to eradicate established tumours. When the vaccine was combined with blocking antibodies to the immune checkpoint molecule cytotoxic T lymphocyte antigen-4 (α-CTLA-4) regression of established intracranial tumours was observed, whereas α-CTLA-4 was ineffective as a monotherapy. In contrast, combining the vaccine with antibodies to programmed death-1 (α-PD-1) or lymphocyte activation gene-3 (LAG-3) failed to provide any survival advantage. This was despite α-PD-1 being effective against the same tumour implanted subcutaneously, suggesting efficacy in the

orthotopic setting was limited by poor access of α-PD-1 to effector T cells within the brain.

The effective combination of vaccine and α-CTLA-4 was associated with enhanced proliferation and accumulation of T cells in the lymphoid tissues without any obvious changes in the adjuvant function of iNKT cells or altered numbers of regulatory T cells, suggesting recently primed T cells were the targets of checkpoint inhibition. While tumours regressing under this combined treatment were highly infiltrated with a variety of leukocytes, tumour eradication was strictly dependent on $CD4^+$ T cells.

Further interrogation of the cell-types responsible for anti-tumour activity revealed that $CD11b^+$ cells were required for therapy, although it remains to be established whether these cells were involved in T cell priming or served as anti-tumour effectors in their own right, possibly under the influence of activated $CD4^+$ T cells. In addition, therapy was hampered, although not entirely eliminated, in hosts deficient in interferon-γ. Therapy was also reduced significantly, but not entirely, in hosts deficient in perforin. *In vitro* studies showed that restimulated splenocytes from animals that had received the combined therapy were able to kill glioma cells in a perforin and MHC-II dependent manner, suggesting that cytotoxic $CD4^+$ T cells were important effector cells.

Overall, these results demonstrate that immunotherapeutic vaccination can be combined effectively with checkpoint blockade to induce effective immune responses against glioma. The immune response induced in combination with CTLA-4 blockade differs from many other cancer models, with a strict dependence on $CD4^+$ T cells that can serve either as cytotoxic effector cells, or potentially as modulators of other accessory cells. Furthermore, the tumour location presents new challenges, with access of inhibitors to the brain, particularly important if immune checkpoints on intratumoural effector cells are to be targeted. In this context, strategies to improve access of checkpoint inhibitors like α-PD-1 and α-LAG3 to the brain warrant further investigation.

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Lindsay Ancelet and I worked together to examine antibody penetrance and immune responses to GL261 following checkpoint blockade. Lindsay contributed panel A of Figure 3.4 and panel A of Figure 3.6, following my checkpoint blockade administration schedules. Tumour measurements for panel B of Figure 3.8 were also performed by Lindsay. Experiments pertaining to Figures 4.2, 4.3, 4.6 and 4.7 were conceived together, performed by Lindsay and analysed together.

Histological sectioning and haematoxylin and eosin staining was performed by Jane Anderson of the Wellington School of Medical Research. Histopathological analysis of tissues was performed by Peter Ferguson of the Capital and Coast District Health Board. Magnetic Resonance Imaging was performed by Angela Slocombe of the Capital and Coast District Health Board. Fluorescence Activated Cell Sorting was performed by Alfonso Schmidt at the Malaghan Institute of Medical Research.

With the above exceptions, all flow cytometry data was collected and analysed myself, with samples stained with antibody panels I designed and optimised.

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

Publications

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*Equal first authorship

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α-GalCer	alpha-galactosylceramide
2 ME	2 Mercaptoethanol
AchR	Acetylcholine receptor
ADCC	Antibody dependent cell cytotoxicity
Akt/PKB	Protein kinase B (also known as PKB)
ANOVA	Analysis of variance
AP1	Activator protein-1
APC	Antigen presenting cell
ATP	Adenosine tri-phosphate
β 2M	beta 2 microglobulin
BBB	Blood brain barrier
BCG	Bacillus Calmette–Guérin
Bcl-2	B cell lymphoma-2
Bcl-XL	B cell lymphoma-XL
BCR	B cell receptor
BCRP	Breast cancer resistance protein
BIRC5	Baculoviral IAN repeat containing 5
BMDM	Bone marrow derived macrophage
Bp	Base pair
BTLA	B- and T-lymphocyte attenuator
CAR	Chimeric antigen receptor
CCL	Chemokine (C-C) motif ligand
CD	Cluster of differentiation molecule
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid

CSF-1	Colony stimulating factor-1
CSF-1R	Colony stimulating factor-1 receptor
Ct	Cycle threshold
CT	Cancer testis
CTLA-4	Cytotoxic T lymphocyte antigen-4
CVID	Common variable immune deficiency
CXCL	Chemokine (C-X-C) motif ligand
D011.10	H-2^{d} restricted T cell recognising $OVA_{323-339}$
DAMP	Danger associated molecular patterns
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR4	Death receptor 4
DR5	Death receptor 5
DT	Diphtheria toxin
DTH	Delayed type hypersensitivity
DTR	Diphtheria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
EAMG	Experimental autoimmune myasthenia gravis
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EGFR	Endothelial growth factor receptor
EGRFvIII	Endothelial growth factor receptor, variant III
EphA2	Erythropoietin-producing hepatocellular carcinoma A2
ER	Endoplasmic reticulum
Fab	Fragment antibody binding
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
Fc	Fragment crystallisable
FcγRIV	Fragment crystallisable gamma receptor IV

FCS	Flow Cytometry Standard
FDA	Food and Drug Administration
GBM	Glioblastoma multiforme
GITR	Glucocorticoid-induced TNFR family related gene
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Good manufacturing practice
gp100	Glycoprotein-100
HIF-1α	Hypoxia inducible factor-1 alpha
HMGB1	High mobility group box-1
HSP	Heat shock protein
HSV	Herpes simplex virus
HSV-TK	Herpes simplex virus thymidine kinase
ICAM-1	Intercellular adhesion molecule-1
ICOS	Inducible T cell costimulator
IDH-1	Isocitrate dehydrogenase 1
IDO	Indoleamine 2-3 dioxygenase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin-
IMDM	Iscove's modified Dulbecco's medium
iNKT	Invariant natural killer T cell
ISQ peptide	$OVA_{323-339}$ Amino acid sequence
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KLRG1	Killer cell lectin-like receptor G1
KO	Knockout
LAG-3	Lymphocyte-activation gene-3
LFA-1	Lymphocyte function-associated antigen-1
LLTC	Lewis lung carcinoma adapted for tissue culture
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MAFIA	Macrophage Fas-induced apoptosis

MBP	Myelin Basic Protein
MCA	Methylcholanthrene
MdLN	Mediastinal lymph nodes
MFI	Mean fluorescence intensity
MGMT	O-6-methylguanine-DNA methyltransferase
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MIP-1α	Macrophage inflammatory protein-1α
MMP	Matrix metalloprotein
MPEC	Memory precursor effector T cells
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis
NF1	Neurofibromatosis type 1
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NSCLC	Non small cell lung cancer
OT-II	H-2^b restricted T cell recognising $OVA_{323-339}$
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PD-L1	Programmed death 1 ligand 1
PD-L2	Programmed death ligand 2
PDGFR	Platelet-derived growth factor receptor
PFA	Paraformaldehyde

PI3K	Phosphoinositide 3-kinase
qPCR	Quantitative polymerase chain reaction
RAG2	Recombinase activating gene-2
RBC	Red blood cell
RECIST	Response evaluation criteria in solid tumours
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
RT-PCR	Reverse transcriptase-polymerase chain reactions
S1P	Sphingosine-1-phosphate
S1PR1	Sphingosine-1-phosphate receptor 1
SCLC	Small cell lung cancer
SEM	Standard error of the mean
SIGN-R1	Specific intercellular adhesion molecule-3-grabbing non-integrin-related protein 1
SLEC	Short lived effector T cells
SLP	Synthetic long peptide
SMA	Spontaneous murine astrocytoma
STAT-3	Signal transducer and activator of transcription 3
T-bet	T-box transcription factor TBX21
TAA	Tumour associated antigen
TAM	Tumour associated macrophage
TAP	Transporter associated with antigen processing
Tc1	Type 1 cytotoxic T cell
Tc2	Type 2 cytotoxic T cell
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
Tfh	T follicular helper cell
TGF-β	Transforming growth factor-beta
Th1	T helper 1 cell
Th17	T helper 17 cell

Th2	T helper 2 cell
TIGIT	T-cell immunoglobulin and ITIM domain
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TLR	Toll like receptor
TNF-α	Tumour necrosis factor-alpha
TNFR	Tumour necrosis factor receptor
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
TNFRSF14	Tumour necrosis factor receptor superfamily member 14
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T cell
Trm	Resident memory T cell
TRP-1	Tyrosinase related protein-1
TRP-2	Tyrosinase related protein-2
TSA	Tumour specific antigen
Tscm	Stem cell memory t cell
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
VEGF-C	Vascular endothelial growth factor-C
VISTA	V-domain Ig-containing suppressor of T-cell activation
VLA-1	Very late activation antigen-1
VLP	Virus-like particle
VSV	Vesicular stomatitis virus
WT	Wild type

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Chapter 1

1 Introduction

1.1 General Introduction

Cancer, in its various iterations, has been present throughout human existence. From its first description on Egyptian papyri, a diagnosis of "a tumour and abscess of the breast", through to present molecular methods to diagnose malignancies, the presence of both benign and malignant neoplasms seems to be an inherent consequence of our life as multicellular organisms. Cancer can be described as a group of diseases, resulting from a mutation at the DNA level, leading to abnormal cell growth and proliferation, with these cells having the potential to spread to other parts of the body. Treatments for cancer have varied throughout history. Initially radical debulking surgery was adopted. This method aimed to remove as much as diseased tissue as possible, as well as sites that it may have travelled to. As understanding of what caused disease deepened, more targeted therapies began to evolve. These included the use of chemotherapeutic agents aimed to interfere directly with cancer growth pathways. However, owing to the genetic instability of tumour cells, patients would often relapse, as cancer cells mutated to use different growth and survival pathways. Recently, the ability of the immune system to recognise tumours has come to the fore. This thesis aims to explore the use of immunotherapy for the treatment of cancer, using a murine model of glioma.

1.2 Glioblastoma Multiforme

Glioblastoma Multiforme (GBM) is highly malignant brain tumour and the most common occurring primary brain cancer in adults (1). With an incidence of 3.5 cases per 100,000 people in developed countries, prevalence of disease is relatively low (2). Similarly, NZ has a tumour incidence of 4.1 cases per 100,000 people (3). However, GBM is currently incurable as tumours readily invade local brain tissue, making full tumour resection difficult. Furthermore, the presence of the blood brain barrier (BBB) limits

chemotherapeutic penetrance into the brain and tumour site. Post-operative radiotherapy is an effective treatment and doubles median survival when compared to surgery alone but recurrence is unavoidable (4). In 2005 a clinical trial showed the benefit of concomitant temozolomide chemotherapy with radiotherapy following surgical resection. Temozolomide is a DNA alkylating agent which causes DNA damage within tumours cells, ultimately triggering cell death. However, despite the inclusion of temozolomide into standard treatment schedules, median patient survival still remains only 12-15 months following diagnosis; five-year survival is less than 10% and long term survival occurs only rarely (5).

GBM is thought to arise from glial cells or astrocytes within the brain, however recent evidence has shed doubt on the cell of origin, showing dedifferentiation of neurons by oncogenes can also drive tumourigenesis (6). The World Health Organisation classification system has GBM designated as a Grade IV astrocytoma (where Grade I is the least severe and Grade IV is the most severe) (7). As genomic techniques have developed, it is now clear that GBM tumour cells are genetically heterogeneous. This has allowed the discrimination of GBM tumours into four distinct subtypes; classical, proneural, mesenchymal and neural, based on their genetic mutation profile (8). Tumours within the classical subtype carry extra copies of the epidermal growth factor receptor (*EGFR*) gene, resulting in over expression of EGFR. Additionally, classical GBM tumours rarely have mutated p53 (9). In contrast, proneural tumours have a high mutation rates in the p53 protein as well as the genes for platelet-derived growth factor (*PDGFRA*) and isocitrate dehydrogenase-1 (*IDH-1*). Proneural tumours are typically associated with a younger age of onset and a slightly better overall prognosis (8). The mesenchymal signature is characterised by the expression of mesenchymal markers and a high mutation rate in neurofibromin 1 (*NF1*) but fewer mutations in the *EGFR* gene as well as reduced expression of EGFR compared to other subtypes. Finally, the neural subtype harbours mutations in many of the same genes as the other groups but is further classified by the expression of neuron markers which are typically expressed by non-transformed neuronal cells. Patients in the neuronal group are the oldest on average (8).

Importantly, as a consequence of the mutation profile, each of the GBM subtypes responds differently to therapy. Patient survival was examined following intensive therapy, defined as concurrent chemo- and radiotherapy or more than three subsequent cycles of chemotherapy, and compared to patients with non-concurrent treatment regimes, or short chemotherapy regimes. Here, aggressive treatment significantly reduced mortality in classical and mesenchymal subtypes. Some efficacy was suggested in neural subtypes but no survival advantage was observed within the proneural subtype (8). It is also important to note that while one of these subtypes can predominate in an individual tumour, a heterogeneous population of cells typically exists (10, 11), meaning that GBM can be viewed as being not simply one disease, but a complex combination of diseases, each potentially responding differently to therapy.

Treatment of GBM is difficult due to many complicating factors. The tumours are highly resistant to radiotherapy and chemotherapy due to the presence of resistant cells within the heterogeneous makeup. Recent studies have shown that these resistant cells have properties that overlap with stem cells (12). Such stem cell-like cells are identified based on the expression of CD133, and are capable of recapitulating the full tumour phenotype after serial passage *in vivo* with as few as 100 $CD133^+$ cells required, whereas other cells from the tumour mass are incapable of doing so unless greater than 10,000 $CD133^-$ cells were injected (13). A characteristic feature of stem cells is that they express high levels of expression of resistance genes such as *BCRP*, *MGMT*, *Bcl-2* and *Bcl-xL* (14) and can promote radio-resistance by preferential activation of the DNA damage response (15). In addition, tumours are known to contain zones of hypoxia within the tissue which are resistant to radiotherapy due to activation of the hypoxia-inducible factor (HIF-1α) mediated pathway. Under normoxic conditions, HIF-1α is rapidly degraded, however under hypoxic conditions, degradation of HIF-1α is prevented, resulting in activation of the hypoxia response element and upregulation of target genes to promote survival in low-oxygen conditions. These include glycolysis enzymes, permitting ATP synthesis in an oxygen independent manner, and vascular endothelial growth factor (VEGF) to promote angiogenesis (16). New approaches using trans sodium crocetinate, an oxygen diffusion

enhancing compound which can act as a radio-sensitiser, are currently being investigated (17).

While temozolomide has been shown to increase overall survival in GBM patients, expression of the O^6-methylguanine-DNA methyltransferase protein, driven by the *MGMT* gene (18, 19) within tumour cells can lead to DNA repair, reversing the DNA damage caused by chemotherapy (19). While tumours remain difficult to treat, the brain is susceptible to collateral damage due to radiotherapy including progressive brain volume loss (20). Following such damage, the brain has a very limited self-renewal capacity. Finally, many drugs cannot pass into the tumour due to the presence of the blood brain barrier (21).

As mentioned earlier, surgery is the first stage of treatment, with resected tissue used for pathological diagnosis, as well as reducing intracerebral pressure resulting from having a large mass press against the brain. The recent inclusion of the fluorescent dye 5-aminolevulinic acid has helped guide resection (22), and removal of over 98% of the tumour mass results in a significant increase in progression free survival (23). However, the highly infiltrative nature of glioma throughout the brain makes complete surgical resection difficult, resulting in inevitable tumour relapse either near the original tumour site, or at a distant site due to satellite lesions within the brain. To limit relapse, resection is followed by radiotherapy and concurrent temozolomide chemotherapy (5). However, the overall prognosis for patients is still bleak with median overall survival remaining at 12-15 months from initial diagnosis. Alternative therapeutic strategies with a good safety profile are desperately needed. For this reason immunotherapeutic approaches are now being investigated.

1.3 Immunotherapy

By the late 19th century, William Coley observed that some cancer patients who developed infections during their disease saw their tumours shrink. He used this knowledge to develop Coley's toxins, a mixed bacterial vaccine consisting of killed *Streptococcus pyogenes* and *Serratia marcescens* that was administered either intravenously, intramuscularly or directly into the tumour (24, 25). While Coley's treatments showed some spectacular

results (26), the treatment schedules were arduous (he maintained that high fevers should be induced at frequent intervals), and the agents he used often suffered from poor quality control. The development of radiotherapy, and then chemotherapy, ushered in a new era of cancer treatment, and immunotherapy was side-lined. A greater understanding of the nature of adaptive immunity in the 1980's and 1990's, particularly the pathways of antigen presentation to T cells, lead to renewed interest in cancer immunotherapy. An important step was in defining specific tumour-associated proteins that could serve as antigens for T cell attack (27). More recently, it is now recognised that many chemotherapeutics also have immune-modulatory function, with destroyed tumour tissue acting as a stimulus to prime anti-tumour immune responses (28). Stimulation of *de novo* immune responses, typically featuring T cells, remained the ultimate aim of immunotherapeutic research until a deeper understanding of T cell activation and regulation was determined, thus initiating the current era of "checkpoint blockade" immunotherapy where pre-existing weak responses are relieved from negative regulatory pathways.

This thesis will describe an investigation into inducing adaptive immune responses to glioma in an orthotopic mouse model which is commonly used to model human GBM. The research described is an investigation into how to maximise the therapeutic benefit of a vaccine that had been developed earlier by colleagues in my laboratory (29). As this vaccine was shown to be dependent on T cells for activity, the following sections in this introduction will provide an overview of T cell development, differentiation and function. It will also include a description of negative feedback mechanisms that control T cell responses, and how these molecular pathways can be exploited for therapy.

1.4 The Immune System

The immune system consists of a series of organs and cells, which serve to coordinate the discrimination between entities that pose a danger to the body and those that are otherwise benign. The innate immune system consists of phagocytic cells such as macrophages and neutrophils, which ingest and kill microbes via production of toxic chemicals and degradation enzymes. Innate immune recognition is enabled by receptors, which recognise conserved regions of pathogens, such as pathogen associated molecular patterns (PAMPs), or danger associated molecular patterns (DAMPS) released in

response to injury or insult. This facilitates a rapid, yet relatively non-specific response, with no lasting immunological memory.

Natural killer (NK) cells are a population of lymphocytes that do not undergo gene rearrangement to express diverse receptors. Rather, NK cell functions are controlled by a diverse array of germline encoded activating and inhibitory receptors, with activation determined by the balance of both stimulatory and inhibitory receptors. The expression of ligands for these receptors can be ubiquitous, or can become up-regulated or secreted in the presence of an infection or malignancy (30).

Conversely, the adaptive immune system is antigen specific and does confer immunological memory. The cells involved in adaptive immunity are lymphocytes, like NK cells, which are generated in large numbers in the bone marrow before moving into the lymphoid tissues. Whereas NK cell activation is non-antigen specific, adaptive antigen specificity is enabled due to antigen receptors on the cell surface, with each cell expressing multiple copies of a unique receptor generated through a random rearrangement process. The high degree of variability of these receptors allows the recognition of a wide variety of antigens; indeed a person's full repertoire contains over a billion lymphocytes (31) capable of recognising many foreign antigens. The two types of lymphocyte involved in adaptive immune responses are T cells and B cells, with each playing a distinct role in immunity. When an antigen binds to a B cell's antigen receptor (BCR), it will proliferate and differentiate into a plasma cell. In the process, the BCR becomes expressed as a soluble effector molecule, or antibody, which is released from plasma cells in large quantities. Thus the antigen responsible for activation of the B cell becomes the target of antibodies that recirculate throughout the body.

Antigen recognition via the T cell receptor (TCR) stimulates a T cell to proliferate and differentiate into an effector T cell. Effector T cells are capable of three distinct functions. Cytotoxic T cells have evolved to kill cells that present targeted antigens (the antigen presenting pathways will be discussed below), including infected cells or tumour cells. Helper T cells provide essential signals for the differentiation of cytotoxic T cells, and also provide signals to B cells to aid in antibody production and survival. The signals they

release include cytokines that can have effector functions in their own right, such as the interferons (IFNs) that serve to limit pathogen proliferation. Finally, regulatory T cells (Tregs) suppress the activity of cytotoxic and helper T cells, a mechanism needed to limit unwanted T cell function that could result in autoimmunity.

1.5 Antigens

The term antigen is used to describe any substance or entity that can be recognised and responded to by the immune system. Typically, proteins, polysaccharides and glycolipids of pathogens acts as antigens recognised by the immune system. Owing to the highly variable antigen receptors expressed on lymphocytes, many other structures can be recognised that are not pathogen-associated including drugs such as penicillin, plant products such as seeds and pollen and metals such as nickel. Furthermore, mutations in "self" proteins that are expressed on tumour cells allow the recognition of malignant tissue. These mutations can be patient specific, or can result in the overexpression of a protein across many patients.

As this thesis will focus primarily on T cell-mediated adaptive immune responses, it is important to note that, in general, T cells recognise peptide fragments derived from protein antigens; these sequences are commonly described as T cell epitopes. A defining feature of a peptide epitope is that it must be capable of being presented on the surface of a cell via major histocompatibility (MHC) molecules; indeed it is only in the context of presentation via MHC that a T cell can truly recognise a given antigen in order to become activated, or to exert its effector functions.

1.6 Tumour Antigens

Activated T cells isolated from cancer patients have been shown to have specific activity against autologous tumour cells *in vitro*. By analysing these T cell responses, gene products can be identified that serve as targets for T cell recognition. In addition, so-called "reverse immunology" can be applied to identify candidate antigens based on expression in the tumour relative to healthy tissue. Here, antigens are theoretically predicted based on protein sequences before subsequent high throughput screening *in silico*. Candidate antigens are synthesised and those with potential to bind MHC molecules are predicted

using computer based modelling and algorithms. Finally, *in vitro* and *in vivo* testing can be performed to verify which of the predicted antigens has physiological function. Strategies such as these have resulted in definition of a number of antigens (32) and could contribute to personalised treatment approaches in the future based on the "mutanome" of a given patient's cancer.

1.6.1 Tumour Specific Antigens

Unique peptide fragments spanning mutations found in tumour tissue, together with idiotype-specific peptides from neoplastic B or T cells, can be classified as tumour-specific antigens (TSAs). Such antigens may be those involved in the driving the neoplastic process. Mutations in the p53 protein commonly limit the regulatory function of this protein in cell cycle progression and therefore contribute towards tumourigenesis. Additionally, neoepitopes can arise from the tumour's genetic instability, reflecting an increase in both "driver" mutations that encourage tumour growth, or "passenger" mutations that have no such impact. Overall, if these mutations result in peptides that can be presented via MHC, they represent TSAs that are potentially unique to a patient's tumour, with the resulting immune responses likely to be patient-specific. The identification and incorporation of neoepitopes into vaccines has the potential to introduce a personalised approach for onco-immunotherapy.

Often seen in GBM is the amplification of the EFGR, bestowing these tumours a growth advantage. In a subset of cases, EGFR amplification is accompanied by gene rearrangement. The most common mutation observed is the EGFR variant III (EGFRvIII), which contains an in-frame deletion of 801 base pairs (bp). This causes in a loss of the ligand-binding domain of EGFR, resulting in ligand-independent constitutive phosphorylation of the receptor that is augmented by reduced internalisation and down-regulation. The in-frame deletion results in the loss of exons 2 to 7, resulting in the generation of a novel glycine residue at the junction of exons 1 and 8. This unconventional juxtaposition within the extracellular domain of the EGFR creates a tumour specific and immunogenic epitope (33). Currently, immunotherapies targeting EGFRvIII are under investigation (34).

1.6.2 Tumour Associated Antigens

Another group of antigens with abundant expression in tumours but restricted expression in healthy tissue are tumour-associated antigens (TAAs). Within this group, the cancer-testis (CT) antigens are the best characterised. CT antigens arise from epigenetic changes resulting in the differentiation of germ line cells into healthy adult testis, foetal ovary or placental trophoblasts. The expression of CT antigens in healthy people is typically restricted to "immune-privileged" sites such as the eyes and testicles, where physical barriers and reduced MHC expression protect the tissue from immune attack.

A second group of TAAs are the differentiation antigens, which are expressed in the tumour tissue and in the tissue of origin of the malignancy, ideally with very little expression elsewhere. As melanocytes and astrocytes are derived from the neural ectoderm, there is some overlap between antigens identified in both melanoma and GBM. These include, but are not limited to, gp100 and TRP-2, which are implicated in normal processes of melanin synthesis or melanosome biogenesis (35).

A third group of TAAs are the overexpressed antigens, which are expressed widely in normal tissues, but are significantly overexpressed in tumours. The anti-apoptotic protein survivin, also known as baculoviral IAN repeat containing 5 (BIRC5) functions to inhibit caspase activation, thereby leading to negative regulation of apoptosis. Survivin is expressed highly in human tumours and foetal tissue but is otherwise absent in terminally differentiated cells (36). Additionally, erythropoietin-producing hepatocellular carcinoma A2 (EphA2) is a receptor tyrosine kinase which is overexpressed in primary GBM compared to non-transformed tissues. Increased EphA2 expression is thought to be due to a decrease in the amount of ligand-mediated receptor degradation. As a consequence, receptor activation and subsequent degradation of EphA2 are decreased, producing a scenario whereby EphA2 is highly expressed yet significantly less activated (37). EphA2 mediates signal transduction pathways in cells including those controlling growth, migration and differentiation (38). The activation of EphA2 has been shown to negatively regulate integrin mediated adhesion, cell spreading and adhesion (39, 40). Therefore, when EphA2 is present in an inactive state, the cells on which it is expressed are more motile, faster growing and more invasive.

1.6.3 Immunotherapy in absence of defined tumour antigens

While the range of defined tumour antigens is increasing rapidly, there are still no validated targets for immunotherapy of GBM. In light of this paucity of information (which is also true for most cancers), immunotherapies have been developed which simply use the tumour itself as a source of antigens. This has taken many forms including killed tumours cells, tumour lysate or tumour derived RNA. The assumption in these therapies is that antigen-presenting cells acquire the tumour material after it is injected into patients, and then present it via MHC to T cells in the lymphoid tissues. As this form of "cell-based" immunotherapy is to be investigated in this thesis, the processes of antigen-presentation required will be reviewed here.

1.7 Antigen Presentation by MHC molecules

Due to the large diversity of TCRs, T cells have the ability to recognise almost all antigens a person will encounter in their lifetime. However, antigen cannot be recognised by T cells in its natural form. Rather, whole proteins are processed and "loaded" onto MHC molecules, a process known as antigen presentation. Even in a healthy state, proteins within a nucleated cell are constantly synthesised and degraded, with some peptide-derivatives being presented on the cell surface via MHC molecules. In the case of an infection, or neoplasia, this repertoire of presented peptides starts to include peptides that are pathogen- or tumour-derived.

1.8 Antigen Presenting Cells

Antigen-presenting cells (APCs) are cells that display foreign antigens in the context of MHC molecules to initiate an immune response. They can be classified as either non-professional or professional APCs depending on the constitutive expression of MHC class II (MHC-II); non-professional APCs do not constitutively express MHC-II. However, under inflammatory conditions, or in the presence of the cytokine interferon-γ (IFN-γ), MHC-II can be expressed. Professional APCs are efficient at internalising antigens either by phagocytosis, or receptor mediated endocytosis and displaying these on the cell surface to naïve T cells. In addition to MHC-II expression, professional APCs express the accessory molecules and factors that are needed induce T cell responses, once a T cell recognises peptide fragments in the context of MHC molecules. This includes

expression of adhesion molecules and costimultory molecules, and the ability to release cytokines to help drive T cell differentiation. Dendritic cells (DCs) have the broadest range of antigen presentation capabilities and are widely characterised as the most potent APCs due to their ability to activate naïve T cells.

1.9 Antigen Processing and Presentation on APCs

In order for APCs to induce an immune response, antigens must be appropriately processed into peptides of the right size and shape to be presented on MHC molecules. Two classical pathways exist that independently activate two different T cell populations; $CD4^+$ T cells and $CD8^+$ T cells. These are the MHC class I (MHC-I) presentation pathway, which presents antigen to $CD8^+$ T cells, and the MHC class II (MHC-II) presentation pathway, which presents antigen to $CD4^+$ T cells. More recently, an additional pathway of presentation to $CD8^+$ T cells has been defined, called the cross-presentation pathway, which can be broadly regarded as an exchange of antigen between the two classical pathways. The relevance of each pathway will be discussed below.

1.9.1 MHC-I antigen presentation pathway

Antigens presented by the MHC-I pathway are peptides derived from cytosolic proteins, degraded within the proteasome. All nucleated cells express MHC-I and in a resting state, proteins within a nucleated cell are constantly synthesised and degraded. Each MHC-I molecule on the surface of the cell displays a peptide fragment of a protein, and these can either be of host origin, or in the case of an infection or tumour, can be pathogen derived. The proteasome is a macromolecule consisting of 28 subunits which functions to degrade intracellular proteins into small peptides of 8-12 amino acids, which are then released into the cytosol. Processed peptides are then translocated from the cytosol to the endoplasmic reticulum (ER) where they are met by MHC-I. The molecule "transporter associated with antigen processing" (TAP) facilitates peptide translocation into the lumen of the ER. The MHC-I molecule is then loaded with peptides within the lumen of the ER. The peptide loading process involves several other molecules which form a large multimeric complex consisting of TAP, tapasin, calreticulin, calnexin and Erp57. MHC-I molecules are heterodimers consisting of two polypeptide chains, α and β2-microglobulin (β2m). Once synthesised, calnexin acts to stabilise the α chains of MHC-I prior to β2m

binding. After assembly of the MHC-I molecules, calnexin dissociates. However, in the absence of bound peptide, the MHC-I molecule is inherently unstable and still requires the chaperones calreticulin and Erp57. Tapasin binds the MHC-I molecule and acts as a link to the TAP proteins, facilitating peptide loading. Following peptide loading, the complex dissociates and leaves the ER via the secretory pathway to reach the cell surface (Fig 1.1 A).

1.9.2 MHC-II antigen presentation pathway

Antigens presented by the MHC-II pathway are usually derived from extracellular proteins that accumulate as a consequence of dead and dying cells in perturbed or infected tissues. As the exogenous protein antigens have to be internalised to be presented, the MHC-II pathway is restricted to cells that can efficiently acquire antigens via phagocytosis or receptor-mediated endocytosis; these cells are referred to as APCs, including DCs, macrophages and B cells, among others. With differing degrees of efficiency, APCs can initiate T cell responses, as well as being required in the target tissues to redirect effector activities to their targets.

Once internalised by APCs, antigenic proteins are degraded by peptidases into peptide fragments within the endosome, resulting in peptides that are between 12-24 amino acids in length. The MHC-II molecule is synthesised in the endoplasmic reticulum (ER), with the α and β chains complexed to a polypeptide called the invariant chain. The invariant chain acts as a chaperone and serves to stabilise the MHC-II complex and prevents MHC-II binding cellular peptides from the endogenous pathway. Binding of the invariant chain also facilitates export of MHC-II from the ER to the golgi apparatus, where fusion with the endosome containing peptide antigens occurs. The invariant chain is degraded by proteases called cathepsins, leaving a small fragment known as class II-associated invariant chain peptide (CLIP), which maintains blockage of the peptide binding groove. Finally, the CLIP fragment is removed, allowing the binding of peptides with higher affinities. The stable MHC-II molecule is then transported and presented on the cell surface (Fig 1.1 B).

1.9.3 *Cross-Presentation Pathway*

Mice immunised with exogenous antigens can induce $CD8^+$ T cell responses, suggesting overlap between the two classical antigen processing and presentation pathways (41). Cross-presentation refers to the ability of certain antigen presenting cells to internalise, process and present exogenous antigens on MHC-I molecules to $CD8^+$ T cells. Such a process is necessary for immunity against tumours and viruses which do not readily infect APCs, or impair normal APC function. Additionally, cross-presentation is required for induction of $CD8^+$ T cell-mediated immunity by vaccination with protein antigens. However, the classical antigen presentation pathways do not explain the mechanism underlying this phenomenon. It is thought that endocytosed proteins are transported out of the endosome and into the cytosol where they are processed by the proteasome into peptides, which are transported by TAP to the ER to facilitate MHC-I loading (42). Other studies have suggested that as the phagosome forms, it fuses with the ER to form an ER-phagosome compartment (43). From here, TAP proteins transport proteins out of the ER-phagosome compartment and into the cytosol for degradation, before transport back into the compartment to allow loading onto MHC-I (Fig 1.1 C).

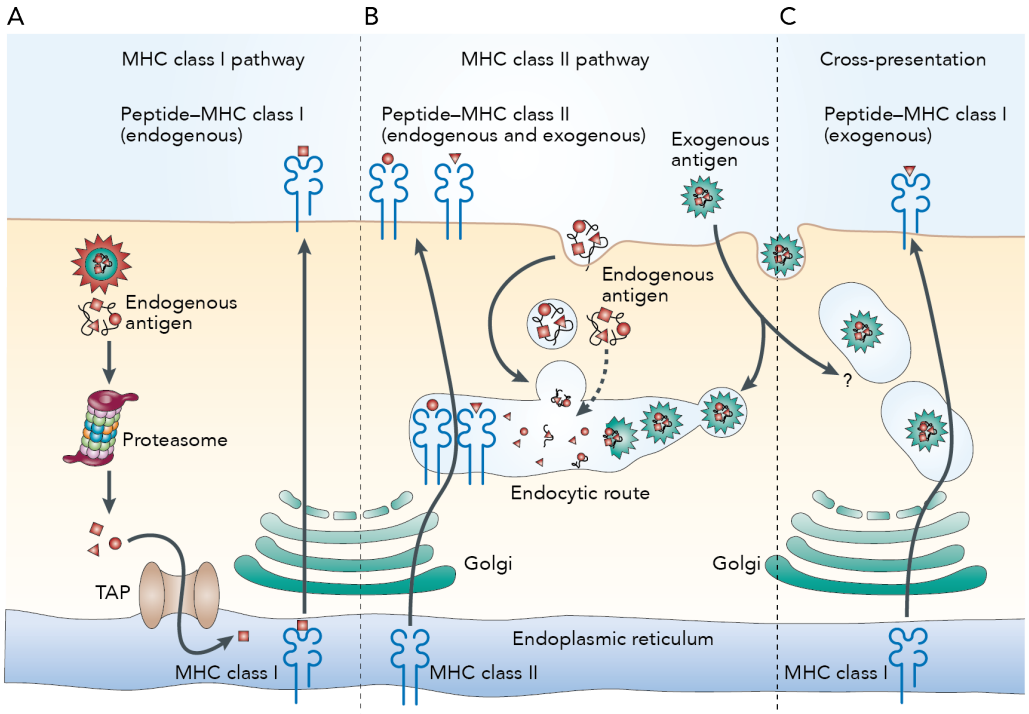


Figure 1.1 Antigen processing pathways in antigen presenting cells.
(A) MHC-I molecules present peptides dertived from endogenous proteins degraded within the cytosol. (B) MHC-II molecules acquire exogenous peptides which are generated by proteolytic cleavage in the endosomal compartments. (C) Cross-presentatation is the process whereby exogenous antigen is transferred to the endocytic pathway for presentation on MHC-I molecules. Figure adapted from Villadangos and Schnorrer (44).

1.10 Dendritic Cells

Dendritic cells are present in both the blood and tissues where they act as sentinels for the immune system. They are very efficient at sampling the local environment through macro-pinocytosis, phagocytosis and receptor mediated endocytosis, and can process acquired antigens to be presented onto both classes of MHC molecules so these can then be displayed to $CD4^+$ or $CD8^+$ T cells (45). DCs can be broadly separated into migratory DCs which reside in the tissues and migrate to the lymph nodes, and lymphoid resident DCs which develop from blood precursors but do not recirculate through the tissues (46). Both DCs can be further delineated into subsets based on cell surface markers and function. All DCs are capable of presenting exogenous antigens to prime $CD4^+$ T cells, however some DCs are particularly efficient at also cross-presenting exogenous antigen to $CD8^+$ T cells (47, 48). Cross-presenting DCs in mice typically express the T cell co-receptor CD8α, although the function of CD8α on these DCs is currently unknown (47). In addition to superior cross-presentation capacity, $CD8\alpha^+$ DCs also have higher expression of the C-type lectin domain (DEC205) compared to $CD8\alpha^-$ DCs (49). While the ligand for DEC205 is unknown, it has been shown to be involved in the uptake of dying cells (50). As the aim of most anti-cancer therapies is to elicit strong T cell responses, there is substantial interest in targeting antigens to cross-presenting DCs (51).

Interestingly, not all $CD8\alpha^+$ DCs are equally as efficient at cross-presenting exogenous antigen to $CD8^+$ T cells. Within the $CD8\alpha^+$ subset, expression of the C-type lectin langerin (CD207) defines a subset of DCs with superior cross-priming ability (52). Depletion of $langerin^+$ cells impairs the cross-priming of $CD8^+$ T cells following soluble OVA and adjuvant administration *in vivo* (52). Langerin binds carbohydrates found on the surface of fungi and viruses and has been shown to mediate the uptake of the measles virus by DCs (53), suggesting that in some instances, langerin can act as a pathogen

endocytosis receptor. While CD8α$^+$ langerin$^+$ DCs are effective at cross-presentation *in vitro* (52), cross presentation of soluble OVA by CD8α$^+$ DCs is not hampered in langerin-deficient mice (54), suggesting langerin is not required for antigen uptake or processing in this setting. Alternatively, the superior cross-presenting ability of these DCs may be as a result of their anatomical location. Langerin$^+$ DCs are found within the marginal zone of the spleen, and are optimally located to take up blood-borne antigens (55).

A similar organisation of DCs exist within the peripheral tissues. In murine skin, two main populations of migratory DCs are present within the dermis in a quiescent state and can be defined based on the expression of CD11b$^+$ and CD103$^+$, with the CD103$^+$ expressing subset efficient at cross-presenting skin-derived antigens within the draining lymph node (56). While CD8α$^+$ and CD103$^+$ DCs harbor the capacity for cross-presentation, they also express DEC205, Clec9A and langerin and are dependent on the transcription factor BatF3 for development, suggesting they share developmental pathways (57). The dominant CD11b$^+$ population within the skin is proposed to induce CD4$^+$ T cell activation and humoral immunity (58). Furthermore, it has been proposed CD11b$^+$ DCs play a role in peripheral tolerance as they are able to produce retinoic acid, promoting the conversion of naïve CD4$^+$ T cells to regulatory T cells (Tregs), and their ability to induce Tregs *in vitro* (59, 60). More recently, *in vivo* studies showed that Treg induction in the resting state was restricted to migratory langerin$^+$ Langerhans cells and dermal CD103$^+$ langerin$^+$ DC, suggesting that retinoic acid production may act in a paracrine loop to boost Treg induction by other migratory DC subsets (61).

1.11 Dendritic Cell Activation

The terms DC maturation, activation and licensing have been used to explain the phenotypic changes associated with a DC that acquires a greater stimulatory capacity. The term, "maturation" is often used to describe the process in which DCs respond to stimulation, typically by up-regulating MHC-II and costimulatory molecules. As this stimulation does not always result in cells with the full functional capability, the term "activation" is reserved for the process in which activatory stimuli convert resting DCs into effector DCs fully capable of priming T cells to both proliferate and differentiate into cytokine-producing effector T cells. Generally, maturation can be induced pro-

inflammatory cytokine exposure, whereas activation usually involves the ligation of receptors on DCs. These receptors recognise "danger" stimuli such as DAMPs (62) or PAMPs (63). DAMPs and PAMPs are released from damaged tissue or infectious pathogens respectively and can ligate the nucleotide-binding oligomerization domain (NOD) receptors and Toll like receptor (TLR) families, leading to a signalling cascade, which results in activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (64). Finally, "licensing" has been used to describe the process of DC activation whereby $CD4^+$ T cells provide help for the generation of primary $CD8^+$ T cell responses (65-67). This help occurs via the interaction of CD40L expressing $CD4^+$ T cells with CD40 expressing DCs. Here the term will be used for any T cell (not just $CD4^+$ T cells) that can provide positive feedback signals that improve the capacity of DCs to prime cytokine-producing effector T cells.

Importantly, DC activation is necessary for the induction of a T cell immune response. In the absence of danger stimuli or effective licensing, presentation of peptides results in immunological tolerance, with antigen specific T cells being rendered anergic, or depleted. Instead, inappropriately activated DCs can induce Treg function, where T cells have suppressive activity. When activated, migratory DCs migrate to the lymph nodes where they interact with T cells (68). Alternatively, lymphoid-resident DCs such as langerin cells within the spleen will interact with T cells. While DCs are professional antigen presenting cells with a high capacity to drive T cell responses, it should be noted that other immune cells such as B cells, macrophages and microglia can act as APCs under inflammatory conditions.

1.12 T Cell Development

The term "T cell" reflects the fact that these cells undergo a critical developmental stage in the thymus. They originate from haematopoietic stem cells in the bone marrow, and then seed the thymus to mature into T cells with a rearranged TCR. Only T cells that can recognise MHC molecules, but don't recognise self-antigens with high affinity, survive this process to leave the thymus and enter the lymphoid system. The common lymphoid precursors that enter the thymus are termed thymocytes and do not yet express a functional T cell receptor. Upon entering the thymus, cells express neither CD4 nor CD8

and are classed as double negative thymocytes. During this stage, the developing thymocyte expresses an invariant α-chain while the β chain of the TCR undergoes rearrangement. If the β-chain pairs successfully with the pre-TCR α-chain, rearrangement ceases. Due to the high level of rearrangement that occurs during this stage, a large number of β-chains are non-functional. If a non-functional β-chain is produced, it cannot pair with the invariant pre-TCR α-chain and the cell dies via apoptosis.

After β-selection, thymocytes progress through a double positive ($CD4^+$ $CD8^+$) state before undergoing thymic selection. While these thymocytes possess a TCR, many will be non-functional as effector cells due to an inability to bind MHC molecules. To ensure MHC binding, thymocytes undergo positive selection, where cells that interact with MHC-I and MHC-II molecules are retained, while those that are incapable of binding MHC die. The ability to bind MHC molecules is afforded by the TCR co-receptors CD4 and CD8, which bind to MHC-II and MHC-I respectively. In this process, MHC molecules loaded with self-antigens are presented by thymic cortical epithelial cells to double positive cells. Only those cells that interact with MHC appropriately, that is not too strongly or too weakly, receive a survival signal. Cells that bind either too strongly or too weakly will die by neglect. During positive selection, thymocyte fate is determined depending on which class of MHC it interacts with. Double positive cells which interact with MHC-I will downregulate expression of $CD4^+$ and become $CD8^+$ thymocytes, whereas interaction with MHC-II results in down-regulation of $CD8^+$, leaving single positive $CD4^+$ thymocytes.

The developing thymocyte then progresses to negative selection, the removal of auto-reactive thymocytes. Due to the random nature of gene rearrangement, there is the chance that thymocyte clones will develop that recognise self-antigens, resulting in autoimmunity. Negative selection selects thymocytes that are capable of strongly binding with MHC molecules presenting self-peptides for removal. If strong MHC binding occurs, cells receive an apoptotic signal, resulting in cell death. After negative selection, mature thymocytes exit the thymus as T cells and enter the circulation, entering peripheral lymph nodes in a naïve state. Selection prevents the formation of T cells that will recognise and

react to self-antigens, thereby preventing autoimmunity in the host. Negative selection however is not completely effective, with some auto-reactive T cells released into the periphery. To counter this, additional mechanisms exist to provide peripheral tolerance of self-antigens, including inducing an anergic phenotype in responding T cells, or deletion of these T cells from the repertoire after activation in the periphery. Also, some self-reactive T cells can be diverted into a suppressive function, and thus function as Tregs.

1.13 T Cell Activation and Proliferation

Following antigen encounter, DCs migrate to the lymph node draining the site of antigen uptake or, if DCs are resident within a lymphoid organ such as the spleen, they remain within the organ and present peptides to naïve T cells. DCs passage through the spleen or lymph node, contacting T cells and displaying processed peptides on MHC-I and MHC-II. Due to the "open" structure of MHC-II, longer peptides are presented to $CD4^+$ T cells, typically between 12 to 24 amino acids in length. Due to the "closed" confirmation of MHC-I, CD8 T cells will typically recognise "short" peptide antigens of 8 to 10 amino acids in length.

Following antigen specific MHC-TCR interaction, T cells require a second signal for full, functional activation. In the absence of signal two, a T cell may become anergic and fail to proliferate and differentiate. The second signal is non-antigen specific and is known as costimulation. Costimulatory molecules CD80 and CD86 are expressed upon activation of an APC and upregulation of these costimulatory molecules typically occurs via the processes of DC maturation and activation. The costimulatory receptor expressed on T cells is CD28 and upon binding to CD80 and CD86, allows T cells to respond to antigen. Importantly, CD28 costimulation is essential for the production of interleukin-2 (IL-2) as it leads to the activation of the phosphoinositide 3-kinase (PI3K) pathway, increasing the production of activator protein 1 (AP-1) and NF-κB. The activation of transcription factors NF-κB, AP-1 and nuclear factor of activated T cells (NFAT) enables nuclear translocation and binding to the promoter region of the *IL2* gene. The cytokine IL-2 is responsible for growth and proliferation of T cells and these transcription factors are essential for IL-2 production (69). The IL-2 receptor α subunit, CD25, has low affinity for IL-2. However, when complexed with the β and γ subunits, (CD122 and CD132

respectively), affinity for IL-2 is increased 100 fold, therefore heterodimerisation of the β and γ subunits of IL-2R is essential for T cell signalling. (69, 70). Additionally, PI3K activation aids in activation of the protein kinase Akt that promotes cell survival and growth, increasing the total production of IL-2 by activated T cells.

1.14 CD4+ T Cells

In addition to proliferation following costimulation, $CD4^+$ T cells differentiate from a naïve proliferating cell into a specialised functional cell. The third signal required for differentiation (after signal one, antigen, and two, costimulation, that are both required for activation and proliferation) is the provision of cytokines that influence T cell differentiation programmes. Cytokines, in addition to transcription factors within the cell aid in determining the lineage commitment of a T cell. Each subset has a unique set of functions and these effector subsets can be characterised based on different cytokine expression profiles and are termed Th1, Th2, Th17, Tfh and Treg. The most well defined cytokines in these scenarios are interleukin-12 (IL-12) and IFN-γ, which drive Th1 polarisation, and interleukin-4 (IL-4), which is responsible for Th2 polarisation (71).

Th1 differentiation occurs in the presence of IL-12 and IFN-γ and serves to activate both innate cells such as macrophages, as well as provide help to adaptive $CD8^+$ T cell responses (72). Additionally, Th1 cells have cytotoxic ability and may have direct tumouricidal ability via Fas-Fas Ligand (FasL) interactions (73). Expression of the master regulator transcription factor T-box transcription factor TBX21 (T-bet), encoded for by the gene *Tbx21,* drives Th1 polarisation (74). T-bet expression induces IFN-γ production through remodelling of the *Ifng* gene and by upregulation of IL-12Rβ2, thus promoting IFN-γ production and selective Th1 cell differentiation in response to IL-12.

Th2 responses are induced following presentation of antigens in the presence of IL-4 (75). These responses are essential for the clearance of helminth parasites and play a major role in allergic responses. GATA3 is the master regulator for Th2 differentiation. Additionally, GATA3 is critical for the development of $CD4^+$ T cells and can be detected at low levels in naïve $CD4^+$ T cells. Th2 differentiation is abolished both *in vitro* and *in vivo* in the

absence of GATA3, as demonstrated in mice harbouring *Gata3* deletion in peripheral T cells (76). Additionally, Th1 and Th2 cells counter-regulate each other, with IL-4 inhibiting IFN-γ and vice versa (77), resulting in a high Th2/Th1 cell ratio within the tumour microenvironment being suggested to be pro-tumourigenic (78-80).

Th17 cells have been recently described as responsible for clearance of fungal pathogens. Differentiated in the presence of transforming growth factor-β (TGF-β) and interleukin-6 (IL-6), interleukin-21 (IL-21) and interleukin-23 (IL-23), Th17 cells have also been implicated in playing a role in autoimmunity (81). Tfh cells are primarily localised to the B cell follicles in secondary lymphoid organs and play a role in antibody isotype switching and antibody production (82). The differentiation pathway for these cells is currently unknown, but it is distinct from aforementioned $CD4^+$ T cell subsets. Tfh can be defined based on the expression of CXCR5 and inducible T cell costimulator (ICOS) (83).

To maintain immune homeostasis, Tregs act to oppose T cell activation. Tregs are either thymus derived, and are referred to as natural Tregs, or differentiated in the periphery from naïve $CD4^+$ T cells in the presence of cytokines including TGF-β (85). Peripherally differentiated Tregs are referred to as induced Tregs. Distinguishing between the two populations is difficult as there is little consensus surrounding the best markers to use. While forkhead box P3 (FoxP3) is the key transcription factor for Tregs, there is some suggestion that Helios could be used to define induced Tregs (86). It now clear that $Helios^+$ and $Helios^-$ cells exist within the natural and induced $FoxP3^+$ populations (87).

As well as this capacity to differentiate into distinct effectors subsets, $CD4^+$ cells provide "help" to dendritic cells in the form of CD40 ligand, a process defined earlier as DC licencing. When APC expressed CD40 is ligated, the APC will upregulate the expression of CD80 and CD86 molecules as well as CD137L, allowing more potent costimulation (65-67). $CD4^+$ T cells also have the ability to directly promote $CD8^+$ activation via IL-2.

It is generally believed that effective immunotherapies for cancer will elicit $CD8^+$ T cell responses, supported by Th1 $CD4^+$ T cells. Production of IFN-γ by Th1 cells promotes

antigen presentation by upregulation of MHC class I and II on both immune and non-immune cells (88). Additionally, IFN-γ production and CD40-CD40L interactions enhance the ability of macrophages to kill tumours and intracellular infections (89-91). However, cytotoxic $CD4^+$ T cells have been described in a mouse model of melanoma. Here, $CD4^+$ T cells induced upregulation of MHC-II on melanoma cells by secreting IFN-γ before directly killing tumour cells via granzyme B (73). In a mouse model of glioma, $CD4^+$ T cells were required for prophylactic vaccine efficacy, as protection was lost following antibody-mediated depletion, or when experiments were performed MHC-$II^{-/-}$ mice (29).

1.15 CD8⁺ T Cells

$CD8^+$ T cells can differentiate into cytotoxic T cells, which are specialised in their capacity to kill tumour cells as well as virally infected cells and cells infected with intracellular bacteria. Once activated, $CD8^+$ T cells can kill target cells expressing as few as one peptide-MHC complex (92-95).

Similarly to $CD4^+$ T cells, IL-12 and IL-4 exposure during $CD8^+$ T cell priming generates effector $CD8^+$ T cells referred to as Tc1 and Tc2, based on the production of IFN-γ or IL-4 respectively. It is also notable that T-bet, which is required for Th1 differentiation, has only a minor effect on the generation of Tc1 cells (96). Regardless of the cytotoxic differentiation profile, both Tc1 and Tc2 cells are capable of eliciting tumour immunity by contrasting mechanims. Briefly, adoptive transfer of Tc1 and Tc2 cells into tumour bearing hosts resulted in endogenous T cell recruitment and type 1 immune responses, host memory CD8 T cell induction, with production of IFN-γ and tumour necrosis factor-α (TNF-α) and accumulation of both Tc1 and Th1 cells at sites of tumours growth (97).

Interest has been focused on $CD8^+$ T cells for their tumouricidal activity since cells isolated from melanoma patients were shown to be capable of killing autologous tumour cells *ex vivo* following a restimulation period with peptide pulsed dendritic cells (98). In the presented context, and for use throughout this thesis, the term "pulsed" refers to cells co-

incubated with either peptide or α-GalCer, with excess peptide or glycolipid washed away before downstream use. Indeed, persistence of the cells *in vivo* was observed following transfer, with T cell trafficking to the tumours. Additionally, responses could be measured in the blood and quantified with MHC tetramers (98). Analysis of tumour biopsies after T cell administration revealed a high concentration of adoptively transferred antigen specific T cells within sites of metastatic burden. However no clinical responses were observed which is perhaps not surprising as it is now recognised that tumours will typically appear larger on CT or MRI scans following immunotherapy which is a result of the influx of immune cells (98). Despite this, no complete responses were observed either (98).

1.16 T Cell Migration

Whereas B cells can differentiate into antibody-secreting plasma cells and mediate antigen clearance from a distance, T cells require cell contact to mediate cytotoxic function. Therefore, differentiated T cells must leave the secondary lymphoid organs following activation, proliferation and differentiation and traffic to peripheral sites to perform effector functions. When a DC migrates to the lymph node to present antigen to T cells, it will also imprint a signature on the T cell to allow the T cell to home to the antigenic target. In some instances, the expression of homing molecules is a reflection of the site of priming. T cells that undergo activation within the gut associated lymphoid tissues upregulate α4β7 integrin and CCR9 which are both important for homing to the small intestinal mucosa (99, 100). Similarly, T cells activated within the skin-draining inguinal lymph nodes upregulate E-selectin and P-selectin ligands as well as CCR4 which are associated with homing to the skin (101, 102). Conversely, T cells can be "educated" following transit through the lymphoid tissues. Adoptive transfer of *in vitro* activated T cells, specific for myelin basic protein (MBP) only acquire the ability to enter the central nervous system (CNS) and induce experimental autoimmune encephalomyelitis (EAE) after a period of transit through the lungs and mediastinal lymph node (103). Here, T cells up-regulated ninjurin 1, a molecule which participates in T cell intravascular crawling along cerebral blood vessels (103). Exit of effector T cells from the lymphoid tissue to sites of effector function is regulated by their responsiveness to the chemotactic lipid sphingosine 1-phosphate (S1P). Additionally, effector T cells will downregulate the expression of the lymph node homing marker L-selectin (CD62L), further permitting

egress from the tissue. The loss of CD62L is compensated by the increased synthesis and expression of the integrin α4β1 (VLA-4). VLA-4 binds to the adhesion molecule VCAM-1, which is expressed on endothelial cells.

1.17 Impact of Blood Brain Barrier on T cell migration

In the context of immunotherapy of glioma, it is necessary for T cells to migrate to the brain to eradicate tumour tissue. Medawar's seminal discovery in 1948, that skin (allo- or xeno-) grafts implanted into the brain were rejected more slowly than orthotopic control transplants, implied that the CNS is an immune-privileged site (104). However, autoimmune diseases of the CNS featuring T cells, such as multiple sclerosis (MS) indicate that cells of the immune system have the capacity to cross the blood barrier and mount an effector response (105). The limited rejection of transplanted tissue was mediated by the presence of the blood brain barrier (BBB), a highly selective permeable barrier that separates the central nervous system from circulating lymphocytes. The BBB is formed by endothelial cells with a unique complex of tight junctions. Surrounding the endothelial cells are pericytes which play a critical role in vesicle trafficking (106). Under homeostatic conditions, astrocytes maintain the integrity of the barrier. However, in inflammatory conditions such as EAE, the murine model of human MS, T cells are capable of crossing the blood brain barrier. This is facilitated by the expression of adhesion molecules and integrins that are capable of "unlocking" the BBB. In malignancies of the central nervous system, such as glioma, dedifferentiation of astrocytes is hypothesized to result in loss of integrity of the BBB, permitting the entrance of T cells from the circulation directly to the brain tissue. Alternatively, primary cultures derived from GBM can actively degrade tight junctions *in vitro*, possibly reflecting BBB disruption in invaded brain tissue (107). While detrimental in the case of EAE, breach of the BBB is imperative for T cells to clear tumour tissue. $CD4^+$ T cells play a critical role in pathologies of the CNS owing to their preferential expression of integrins, allowing transport across the blood brain barrier (108). Current evidence suggests T cells may gain the capacity to enter the CNS after a transition through the lung. From the lung, T cells migrate to the mediastinal lymph node before entering the blood and finally the CNS (103).

1.18 T cell cytotoxicity

Upon acquisition of effector function, T cells kill target cells expressing cognate antigen on MHC complexes in a contact dependent manner. When a specific TCR recognises the MHC-peptide complex, the T cell can induce cell death by apoptosis. Apoptosis is a form of regulated cellular suicide, which causes a cell to undergo nuclear blebbing, altered cellular morphology and eventual DNA fragmentation. Following DNA fragmentation, the target cell will shed its membrane as vesicles and continue to degrade itself from within. One of the hallmarks of apoptosis is the nuclease dependent fragmentation of DNA. Induction of apoptosis in a target cell by a cytotoxic T cell can involve release of cytotoxic granules, or via the interaction of FasL, expressed on activated T cells, with Fas on the target cell. In each case, an intracellular caspase cascade is induced that ultimately leads to the activation of nucleases responsible for DNA fragmentation (109).

In addition to Fas mediated cell death, cytotoxic T cells carry intracellular stores of proteins that can trigger apoptosis. These proteins remain in an active form within cytolytic granules and are released upon recognition of antigen on target cells. The first of these proteins is perforin, which perforates the cell membrane of the target cell (110). Also packaged with perforin are the granzymes, a family of serine proteases (111). Following the formation of a cytotoxic synapse between an effector and target cell and the release of perforin, granzymes are released into the cytosol of the target cell. From here, granzyme B will cleave caspase-3, resulting in the activation of the apoptosis pathway (112). Granzyme B can also cleave the protein Bid, resulting in a change in mitochondrial membrane permeability, leading to the release of cytochrome C (113). The formation of a cytolytic synapse ensures both perforin and granzymes are delivered to a specific target cell, and acts to limit collateral damage to bystander cells.

Finally, cytokines released by effector T cells can also induce cell death. TNF-α is a cytokine produced by many cell types including T cells and NK cells as well as macrophages and neurons (114-116). Secreted TNF-α binds to the TNF receptors TNFR1 and TNFR2, resulting in an intracellular signalling cascade, ultimately terminating in the recruitment of caspase-8. High concentrations of caspase-8 induce autoproteolytic

activation, leading to caspase mediated apoptosis (117). In addition to apoptosis induction, TNF-α release creates a pro-inflammatory environment, activating innate immune cells and also induces vascular permeability, easing immune cell extravasation (118, 119). The TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumour necrosis factor superfamily and is produced by most cells. Binding of TRAIL to the death receptors DR4 and DR5 results in caspase-8 mediated apoptosis, similarly to TNF-α (120). IFN-γ inhibits tumour angiogenesis and has immunomodulatory functions, including inducing the upregulation of MHC-I and MHC-II expression on tumour cells, thereby enhancing tumour cell susceptibility to T cell mediated cytotoxity (73, 121).

While $CD8^+$ T cells have been studied for their cytotoxic abilities, it is thought $CD4^+$ T cells can also perform these functions. Another mechanism by which T cells can serve as useful effector T cells in anti-tumour response is to activate innate immune cells. This involves the activation and differentiation of Th1 cells which can interact with macrophages through MHC-II and CD40-CD40L interactions (89, 90). Macrophages are recognised as playing a role in tumour regression or progression based on their phenotypic and functional characteristics. A functional spectrum of macrophages exists, with the populations at either pole described as "M1" and "M2". Classically activated macrophages, or M1 skewed macrophages arise following interactions with Th1 $CD4^+$ T cells, and exposure to TNF-α and IFN-γ in a pro-inflammatory environment (122-124). Alternatively activated macrophages, or M2 skewed macrophages have been shown to play a role in helminth infection clearance and differentiate under the influence of IL-4 and IL-13 produced by Th2 cells (125, 126). In addition to distinct differentiation pathways, M1 and M2 skewed macrophages are functionally distinct. M1 macrophages produce high levels of pro-inflammatory cytokines including TNF-α, IL-1, IL-6 and IL-12 and increase intracellular concentrations of nitrogen and oxygen radicals. Furthermore, M1 macrophages increase expression of MHC-I and class II to further activate T cells (127). Conversely, M2 macrophages express the mannose receptor and IL-10, which result in M2 macrophages playing a role in parasite clearance, tissue remodelling, immune modulation and tumour progression (127, 128). Tumour associated macrophages (TAMs) have been shown to be the predominant leukocyte population within solid tumours and

have both tumour promoting and anti-tumour roles (129, 130). In creating an inflammatory environment, macrophages promote angiogenesis, facilitating both tumour growth and metastatic potential (131, 132).

1.19 T Cell Inhibition via immune checkpoints

One of the characteristic features of a T cell response is that once T cells have undergone an intense period of proliferation after activation, there is then a significant reduction in T cell numbers as cells die by apoptosis, ultimately resulting in a small population of long lived memory cells. There are many mechanisms that are responsible for the reduction in cells numbers including extrinsic cell-receptor and caspase-dependent apoptosis, intrinsic mitochondria- and caspase-dependent apoptosis, or by caspase-independent cell death. Autonomous cell death occurs in the absence of survival signals such as cytokine deprivation or activated cell autonomous death (133). In addition, TCR engagement of effector T cells in the absence of appropriate costimulation leads to activation-induced cell death, a process where activated T cells undergo death following engagement of cell death receptors such as Fas (134, 135). The removal of activated T cells following clonal expansion and subsequent pathogen or tumour clearance is important to maintain homeostasis. Furthermore, it has recently been shown that activated T cells upregulate several proteins after activation that are involved in amendment of the costimulatory signal or negative regulation of T cell function. These inhibitory pathways are often referred as "immune checkpoints", and they limit T cell activity. Under normal physiological conditions, immune checkpoints are vital for the maintenance of self-tolerance (136). Additionally, they are required to protect tissues from damage during immune responses (137). Immune checkpoints provide a negative feedback loop to T cell activation, and are typically up-regulated 24-72 hours following TCR stimulation. In this capacity, checkpoint molecules can attenuate the activatory signal received by a T cell, resulting in a higher threshold for T cell activation. This limits unwanted T cell activity to self-antigens and maintains self-tolerance. The possibility of improving vaccine induced T cell response by blocking inhibitory checkpoints is a major focus of the research within this thesis. The key checkpoints investigated will be discussed below, with a brief review of the mechanism by which they function. Further mechanistic detail will be provided within the context of the research presented in later chapters of the thesis.

1.19.1 Cytotoxic T Lymphocyte Antigen 4 and Ipilimumab

After the 1987 cloning of cytotoxic T lymphocyte antigen-4 (CTLA-4) in mice, sequence similarities with CD28 were noticed, as well as the conservation of molecule expression in humans. While CD28 was identified as a T cell costimulatory molecule, early studies suggested CTLA-4 was also a costimulatory molecule (138) however subsequent studies identified CTLA-4 as a negative regulator of T cell activation (139, 140). CTLA-4 binds costimulatory molecules CD80/86 with up to 20 times more affinity than CD28 (85). This competition for CD80/86 binding results in an inhibitory signal to the T cell mediated by inhibition of TCR signalling as well as inhibition of Akt signalling resulting in inhibition of IL-2 production, ultimately causing cessation of T cell proliferation (141, 142). During immune quiescence, CTLA-4 is localised intracellularly within T cells where it is associated with clathrin-associated complexes (143, 144). Relocation of CTLA-4 to the cell surface occurs rapidly following T cell activation, followed by internalisation (145). Expression of CTLA-4 mRNA has been shown to peak between 24 to 48 hours after activation (138, 146) and functional effects in mice have been shown as early as 12 hours post activation (147). Consistent with the role for CTLA-4 in regulating immune priming, expression on T cells is typically restricted to the secondary lymphoid organs. Additionally, CD80/86 ligation by CTLA-4 results in signal transduction within the APC, resulting in upregulation of indoleamine 2,3-dioxygenase (IDO) (148). IDO is a catabolic enzyme which catalyses the breakdown of tryptophan to *N*-formylkynurine. T cell activation is then limited due to sequestration of the essential amino acid tryptophan, and also due to the immunosuppressive nature of *N*-formylkynurine. Krummel and Allison conceived the idea of CTLA-4 blockade as a means to enhance T cell activation, with initial studies showing CTLA-4 signalling inhibited T cell responses (139). From here, injection of antibodies aimed at blocking CTLA-4 signalling were performed which demonstrated that CTLA-4 blockade enhanced T cell responses in mice responding to vaccines (149). Application of these findings to tumour models were the first real validation that enhancement of the immune system could induce anti-tumour responses (150). The mechanism of action of these antibodies is currently under investigation and is likely to be multifactorial, with effector intrinsic (73, 151) and extrinsic (151, 152) mechanisms described in pre-clinical models.

Positive responses observed in preclinical testing promoted licensing of the patent for MDX-101 to Bristol Squibb Meyers where studies were advanced to clinical trials. The landmark study published in 2010 by Hodi and colleagues, which resulted in Food and Drug Administration (FDA) approval, aimed to compare ipilimumab combined with a vaccine to gp100, a melanocyte antigen, to each intervention as a lone agent. Here, efficacy was observed in the absence of gp100 vaccination, with 20% of patients having sustained anti-tumour responses as measured by overall survival (153).

As mentioned earlier, ipilimumab blockade acts in the lymph nodes, blocking CTLA-4 signalling to prevent attenuation of costimulation through CD28. Enhanced costimulation is thus received by T cells, promoting proliferation and effector function. Recent studies have examined the T cell repertoire in melanoma patients before and after ipilimumab therapy. Using tetramers to detect antigen specific $CD8^+$ T cells, Kvistborg and colleagues observed a broadening of the T cell response, suggesting that in the clinical setting, CTLA-4 blockade may lower the threshold for T cell activation, potentially permitting immune responses against antigens with lower MHC-I affinity which would otherwise drive anergy (154).

1.19.2 Programmed Death-1, Pembrolizumab and Nivolumab

Programmed death-1 (PD-1) was cloned in 1992 as a trans-membrane protein and has two known ligands – programmed death 1 ligand 1 and ligand 2 (PD-L1 and PD-L2). PD-L1 is constitutively expressed on a wide range of cells whereas PD-L2 is predominantly induced on APCs during activation. The expression of PD-1 can be detected 24 hours after activation, and is further up-regulated and maintained until at least 5 days following activation (155, 156). Treatment of α-CD3 stimulated T cells *in vitro* with PD-L1-Ig results in reduced T cell proliferation and IFN-γ production (137). Reduced T cell proliferation correlated with diminished IL-2 secretion, which could be rescued with the addition of exogenous IL-2 or α-CD28 antibodies (157).

In contrast to CTLA-4, the major role of PD-1 is to regulate T cell responses at within the tissues. Inflammatory cytokines within the tissues induce the expression of PD-L1 which downregulate the activity of T cells and therefore limit collateral damage in

response to either an infection or malignancy. The best-characterised signal for inducing PD-L1 expression is IFN-γ, which is produced by effector T cells. Several tumour cell lines have been shown to upregulate the expression of PD-L1 *in vitro* following treatment with IFN-γ (158, 159). When co-cultured with activated T cells, target tumour cells are poorly lysed. Conversely, *in vitro* cytotoxicity could be restored by administration of α-PD-L1 antibodies, or by genetic ablation of PD-1 in T cells. Many studies have revealed the expression of PD-L1 on a wide variety of tumour types, thus in the context of immunotherapy, the prevailing view is that PD-L1 expression on tumour cells engages PD-1 expressing T cells, suggesting the PD-1/PD-L1 axis is co-opted by tumour cells to prevent immune mediated killing (160, 161).

As CTLA-4 negatively regulates T cell responses and has shown success in an immunotherapeutic tumour setting, PD-1 has independently been investigated as a target for immunotherapy. Antibodies against both PD-1 and PD-L1 have been shown to have pre-clinical efficacy (162, 163), with two PD-1 antibodies currently FDA approved for clinical use (162). Pembrolizumab, formerly known as lambrolizumab, was the first FDA Approved α-PD-1 antibody. Interrogation of efficacy in patients with advanced melanoma included patients who had disease progression while receiving ipilimumab therapy. In this study, patient response rates were at 38% as evaluated by Response Evaluation Criteria in Solid Tumours (RECIST) criteria, with the best responses observed in the cohort receiving the highest dose of 10 mg/kg body weight every 2 weeks (162). Nivolumab is another FDA approved α-PD-1 antibody, which was compared to ipilimumab treatment, or as a combination treatment in metastatic melanoma patients. Both nivolumab as a monotherapy, and nivolumab combined with ipilimumab showed statistically improved progression free survival when compared to ipilimumab monotherapy (164). As these clinical trials were performed in late stage melanoma patients, overall responses were modest yet durable. As ipilimumab monotherapy results in durable response rates in 20% of patients, with some patients surviving greater than 10 years, there is interest in combining ipilimumab with other agents to increase efficacy. Recently, combination therapy with ipilimumab and nivolumab has been explored as a

means to increase both progression free survival and overall survival in lung cancer patients (165).

1.19.3 Late Activation Gene-3 preclinical and clinical studies

Late activation gene-3 (LAG-3) is currently being investigated for clinical application as a checkpoint molecule. The inhibitory role of LAG-3 was first shown *in vitro* with improved T cell proliferation observed in the presence of a blocking antibody (166). Further studies demonstrated that simultaneous signalling through the TCR and LAG-3 on activated T cells resulted in a lower calcium flux than signalling through the TCR only (167). Ligation of LAG-3 acts to negatively regulate cellular proliferation, activation and homeostasis of T cells, in a similar fashion to CTLA-4 and PD-1 (168, 169). Similarly, tumour lines transfected with LAG-3 have been used as cancer vaccines (170). Finally, overexpression of LAG-3 lead to impaired T cell proliferation in an antigen specific context (171). Whereas CTLA-4 and PD-1 are expressed on T cells, LAG-3 is expressed on T cells (172), NK cells (173), B cells (174) and plasmacytoid DCs (175). The ligand for LAG-3 is MHC-II to which it binds with a higher affinity than CD4 (176). Interestingly, whereas CTLA-4 and PD-1 are both membrane bound, a soluble form of LAG-3 exists (177). In mice, cross-linking of MHC-II on APCs inhibits DC activation (178) thus, engagement of MHC-II by LAG-3 expressing T cells in the presence of cognate antigen, inhibits CD86 upregulation and IL-12 secretion (178). In these studies, LAG-3 lacking the cytoplasmic tail was sufficient to confer regulatory activity to T cells, consistent with the notion that signalling through MHC-II on APCs, rather than direct signalling through the T cell, is responsible for inhibition of DC activation, limiting T cell activation (178). In addition to its role in T cell homeostasis, LAG-3 has reported to play a role in Treg suppressive function (175).

As a cancer therapy, two approaches are being explored. The first is the soluble form of LAG-3, aimed at increasing the activation of T cells via direct effects on DCs. Due to interactions between MHC-II and LAG-3 resulting in the down-regulation of $CD4^+$ T cell proliferation and cytokine secretion, as well as inhibition of human allo-responses, soluble LAG-3 was initially touted as an immunosuppressant (179). However, after Triebel *et al* successfully produced a soluble fusion protein in 1995, further studies demonstrated

soluble LAG-3 as a DC activator (180). As a clinical compound, soluble LAG-3 is being developed as IMP321 (181). Preclinical interrogation has revealed effectiveness of IMP321 both *in vitro* and *in vivo* in murine and human systems (182-186). The first phase I clinical trial was conducted in renal cell carcinoma patients as a dose escalation study with patients receiving up to 30 mg per injection per fortnight. As well as safety and efficacy, 8 patients exhibited significant $CD8^+$ T cell activation when administered IMP321 at 6 mg and 30 mg as measured by increased CD27 and CD28 expression (187). A dose escalation study has also been conducted in pancreatic cancer patients in combination with gemcitabine. The combination was found to be safe, however no differences were found when comparing pre and post-treatment levels of monocytes, DCs and T cells, with the lack of immune cell activation attributed to the relatively low dose of 2 mg administered to patients (188). Furthermore, a phase IIa clinical trial has been completed in metastatic breast cancer. In this study, patients received IMP321 one day following paclitaxel, with the investigators aiming to create a chemo-immunotherapeutic environment. Here, paclitaxel creates tumour debris, with IMP321 acting to further activate the DCs, which have taken up the debris. Interestingly, response rates were observed at the six-month follow up period according to RECIST criteria. In addition to decreased tumour burden, a sustained increase in numbers of NK cells, monocytes and $CD8^+$ T cells were observed in patients, suggesting immune cell activation (189). In line with current immunomodulators, are antibodies aimed at preventing negative regulation of T cell responses. A phase I dose escalation and safety and efficacy study is underway to assess BMS-986016 administered alone, and in combination with nivolumab (190).

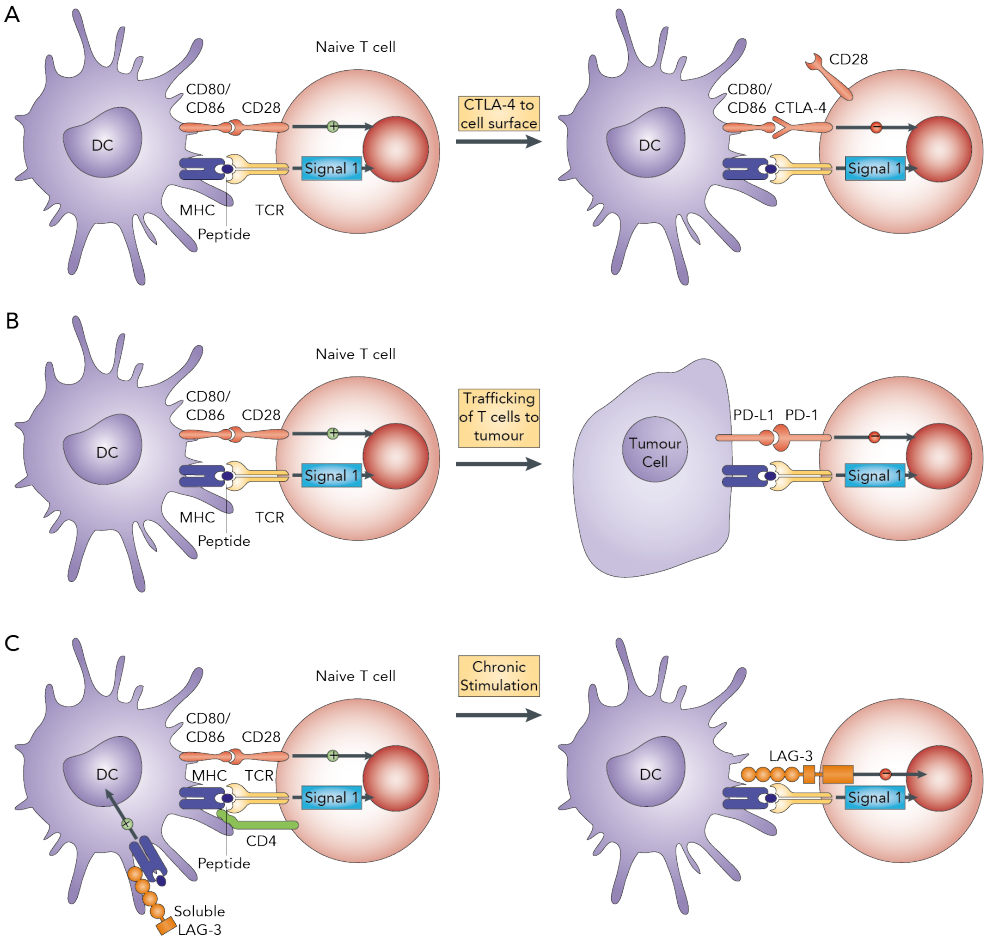


Figure 1.2 Immune checkpoints in cancer immunotherapy.
(A) The cytotoxic T-lymphocyte antigen-4 (CTLA-4)-mediated immune checkpoint is induced in T cells following initial exposure to antigen. The level of expression of CTLA-4 is dependent on the strength T cell receptor (TCR) signalling. High affinity ligands induce higher levels of CTLA-4 expression, which counteracts the initial TCR signalling. (B) Programmed cell death protein 1 (PD-1) acts to regulate T cell effector function in the peripheral tissues. Activated T cells upregulate PD-1 and maintain expression within peripheral tissue and tumour sites. Ligation of PD-1 acts to downregulate the activity of T cells and therefore limit collateral damage to healthy tissues. T cells frequently express PD-L1 as a means to limit effector T cell activity. (C) Differential outcomes of lymphocyte activation gene-3 (LAG-3) splice variants. Signalling through MHC-II on DCs following ligation by soluble LAG-3 results in dendritic cell activation. By contrast, signalling thorugh membrane bound LAG-3 on T cells following MHC-TCR interactions negatively regulates T cell activation. Figure adapted from Pardoll (191)

1.19.4 Future immune checkpoints

Currently, only two molecules have been approved for targeted therapy, however the clinical success of ipilimumab, pembrolizumab and nivolumab has invigorated the search for new molecular targets.

T-cell immunoglobulin domain and Mucin domain 3 (TIM-3) is expressed on activated T cells and has a role in regulating cytokine expression (192). Primary studies investigated the role of TIM-3 in the setting of autoimmunity. Here, antibody mediated blockade of TIM-3 worsened disease in EAE, providing the first indication that TIM-3 acts as an immune checkpoint (193). Further evidence in preclinical tumour models showed TIM-3 expressing $CD8^+$ T cells were the most dysfunctional population in both solid and haematologic malignancies (194, 195). Ligation of TIM-3 by galactin-9 on tumour cells induces aggregation and death of T cells *in vitro* and administration of galactin-9 *in vivo* results in selective loss of IFN-γ producing cells (196). B- and T-lymphocyte attenuator (BTLA) expression is induced following activation of T cells. Similarly to CTLA-4, BTLA interacts with a B7 homologue B7H4 (197). However, unlike CTLA-4, T cell inhibition occurs via interactions with tumour necrosis family receptors, notably tumour necrosis factor receptor superfamily member 14 (TNFRSF14) (198). Signalling through BTLA has been shown to inhibit the function of human $CD8^+$ specific cancer specific T cells (199). V-domain Ig-containing suppressor of T-cell activation (VISTA) is another negative checkpoint regulator which is expressed predominantly on myeloid and granulocytic cells, with weaker expression on T cells (200). Interestingly, T cells both express and respond to VISTA and administration of VISTA-Fc fusion protein is suppressive to T cell activation (201). The first indication of VISTA as an immune checkpoint were observed in a preclinical murine model of methylcholine 105-induced fibrosarcoma (196, 200). Intriguingly, immunotherapy targeting VISTA has been observed to be effective without detectable VISTA expression on tumour cells, and that VISTA blockade works even in the presence of high PD-L1 expression (202). This suggests VISTA may have a broad clinical application and is advantageous over PD-1 or PD-L1 blockade which may only be effective when expressed within the tumour microenvironment. T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT) is expressed on NK and T cells (203). Both genetic ablation and antibody blockade have

been shown to enhance NK cell killing and $CD4^+$ T cell priming both *in vitro* and *in vivo* (204-206). Additionally, the severity of $CD4^+$ T cell mediated diseases such as EAE can be exacerbated following TIGIT blockade *in vivo* (206).

As well as inhibitors of negative regulation, antibodies are being developed for stimulatory T cell molecules. Both CD27 and CD28 provide a stimulatory signal to T cells, there is interest in developing agonistic antibodies against these targets. In preclinical work, CDX-1127, an agonistic CD27 antibody has shown to be effective in T cell receptor stimulation (207). Similarly, CD28 has been targeted with a "superagonist" antibody TGN1412 (208). However, phase I trials resulted in hospitalisation of all six patients who received the drug, resulting from cytokine release syndrome (209). Glucocorticoid-induced TNFR family related gene (GITR) is expressed on Tregs and also plays a role in promoting T cell proliferation (210). Antibodies to GITR have been shown to promote anti-tumour immune reponses through the loss of Treg lineage stability (211). ICOS is a cell surface molecule expressed on activated T cells (212). Currently ICOS is being adapted as a pharmacodynamic biomarker for ipilimumab treatment (213). In addition, engagement of the ICOS pathway enhances the efficacy of CTLA-4 blockade in murine models of cancer (214). Tumour necrosis factor receptor superfamily member 9, also known as CD137 or 4-1BB is expressed on activated T cells as well as DCs, NK cells and granulocytes (215, 216). The best-characterised activity of CD137 is costimulatory signalling, resulting in enhanced T cell proliferation, IL-2 secretion and cytolytic activity (217, 218). Finally, OX40 is transiently expressed following TCR engagement and plays a role in the differentiation of effector and memory T cells (219, 220). Phase I clinical testing has revealed α-OX40 antibody blockade to have an acceptable toxicity profile and regression of at least one metastatic lesion in 12 out of 30 patients (221).

1.20 T cell memory

Following the resolution of a pathogen or tumour, 90-95% of the effector T cells will be eliminated as a means to return to homeostasis. Clearance of the effector T cell pool involves multiple pathways including Fas and FasL, TNF and TNFRI and TNFRII as well as CD40 and CD40L signalling. In addition, IL-2 withdrawal starves T cells of proliferative and survival signals (135, 222, 223). The 5-10% of cells that remain will

become long-lived memory T cells (224). These cells will activate genes responsible for cell survival such as *Bcl-2*. Memory T cells are more sensitive to antigen re-encounter than a naïve T cell experiencing antigen for the first time. As such, they respond more vigorously to produce key cytokines including IL-2, IFN-γ and TNF-α. Similar to naïve T cells, memory T cells rely on interleukin-7 (IL-7) and interleukin-15 (IL-15) for homeostatic maintenance (225).

Various populations of memory T cells exist, with distinct effector function and location. Effector memory T cells (Tem) patrol the tissues, typically at barrier sites, but also the blood and spleen. Upon antigen recognition, Tem rapidly mature to effector T cells and produce large amounts of IFN-γ early after antigen re-encounter. These cells lack CCR7 expression but express high levels of β integrins, suggesting they are specialised for rapid entry into inflamed tissue. Central memory T cells (Tcm) do express CCR7 and recirculate to lymphoid tissues. Upon antigen re-encounter, central memory T cells lose expression of CCR7 and differentiate into effector T cells (226, 227). Whereas Tem can rapidly respond to antigen re-encounter, Tcm cells require antigen processing and presentation (228). In this capacity, Tcm can supplement new immune responses within the lymph node.

Stem memory T cells (Tscm) have a naïve-like $CD44^{lo}$ $CD62L^{hi}$ phenotype and display stem cell-like properties but reside within the blood. It is thought these cells are the earliest and longest lasting developmental stage of memory T cells, exhibiting a gene profile which is considered to be a mixture of both naïve and Tcm cells (229, 230). In murine immunotherapy studies, Tscm have garnered interest due to their self-renewal capacities and superior anti-tumour responses, compared to other memory T cell subsets (231, 232). Tissue resident memory T cells (Trm) are another memory T cell subset which populate peripheral tissues without recirculating. In this capacity, they provide a rapid response against infections re-encountered at body surfaces such as the gastrointestinal and reproductive tracts. Indeed, Trm cells have been described within the brain following vesicular stomatitis virus (VSV) infection and their maintenance is dependent on the expression of the integrin αE (CD103). These T cells do not function or survive well

after dissociation from the tissue in which they reside, suggesting tissues specific adaptations, or that cells become dependent on factors within the environment to maintain survival (233).

The developmental pathway of memory T cells from effectors is uncertain, however, three models are currently assumed to describe how memory T cells are generated. The "linear" differentiation model suggests that T cells develop into effector T cells, which either die, or differentiate further following antigen clearance. Here, Tem are described as an intermediate phenotype between the effector and Tcm phenotypes i.e Naïve→ Effector → Tem → Tcm (234). The "divergent" model suggests that the fate of naïve T cells is predetermined, that is some cells are destined to die as terminally differentiated effector T cells, while others are destined to become memory T cells following priming (235). Additionally, the "bifurcative" differentiation model suggests primed T cells undergo asymmetrical cell division, with each daughter cell having a different fate to that of the mother cell (235).

It is likely that each of the models may be correct, as experiments which tracked the fate of naïve $CD8^+$ T cells revealed cells that had acquired effector function were still capable of differentiating into memory T cells (236). While it is likely that all cells must be initially activated to undergo memory differentiation, there is accumulating evidence that two distinct phenotypes emerge during the primary immune response. Short lived effector cells (SLEC) and memory precursor effector cells (MPEC) can be distinguished by the expression of the IL-7 receptor (CD127) and low expression of killer cell lectin-like receptor G1 (KLRG-1) (237).

While there is evidence of $CD4^+$ T cell memory, our current understanding is lacking compared to that of $CD8^+$ T cells. Both linear and divergent differentiation models have been proposed (238, 239). Upon restimulation, $CD4^+$ memory T cells have been observed to proliferate, however, this proliferation is less than a naïve T cell responding to antigen for the first time (240). The reduced proliferation was attributed to increased production of IFN-γ and decreased production of IL-2, therefore $CD4^+$ memory T cells have impaired proliferative capacity when compared to $CD8^+$ memory T cells (241). Protection

can be generated following vaccination with *Bacillus Calmette-Guérin* (BCG), resulting in cytokine producing memory $CD4^+$ T cells providing defense from subsequent disease caused by *Mycobacterium tuberculosis* (242, 243). Taken together, it suggests memory $CD4^+$ T cells may not be involved in the direct killing of pathogens, but act as catalysts to provide help to other cells.

The generation of T cell memory has considerable implications for tumour-immunotherapy where the goal of therapy is not only to generate effective effector responses, but also generate robust memory capable of preventing relapse (244).

1.21 The Cancer Immunoediting Hypothesis

The idea that the immune system can limit the growth of tumours originated in the 1900s and has been a topic of debate ever since. However, at the time so little was known about the immune system that it was difficult, if not impossible to challenge this hypothesis and validate this prediction. As our understanding of the immune system developed, and tumour associated antigens were identified, this idea again came to the fore. These advances lead to the formation of the cancer immune-surveillance hypothesis by Burnet and Thomas, which stipulated that the immune system was responsible for preventing tumour growth in immune-competent hosts (245). Subsequent studies however showed little support for this hypothesis. Experiments performed by Stutman showed that cancer susceptibility in immune-competent mice was comparable to the incidence seen in immune-compromised hosts (246). It was argued that tumour cells lacked the necessary DAMPs required for optimal APC activation, and therefore were silent to the immune system (247). Additionally, it was thought the immune system would not be able to discriminate between tumour cells and normal cells, as they would be too similar and hence drive a tolerogenic phenotype (248).

Later studies showed that the nude mice used in these experiments were not totally immune-compromised but still had low but detectable levels of T, NK and NKT cells (249, 250). As mouse models developed and improved, and pure genetic background strains were developed, the role of the immune system in the prevention of tumour outgrowth could be reassessed. Following the development of the recombination-

activating gene KO ($RAG2^{-/-}$) mouse (lacking functional T, NKT and B cells) (251), and the IFN-$\gamma^{-/-}$ mouse (252, 253), it was discovered that these mice were more susceptible to tumour formation (to both carcinogen induced and transplanted tumours) when compared to wild type (WT) controls (254).

As tumours from these mice were analysed, it became apparent that tumours that developed in immune-compromised hosts were more immunogenic when compared to tumours that had arisen in immune-competent hosts. A tumour mass does not exist as a homologous population of tumour cells, but rather a heterogeneous population of tumour cells of varying degrees of differentiation as well as local stromal and immune cells. Due to this complex relationship of distinct populations, there is a level of interplay that exists. This lead to the notion that the immune system not only plays a role in preventing tumour development, but also influences shaping tumours which do develop. From here, the cancer immune-editing hypothesis was formed. This hypothesis proposes that three phases of cancer development exist, elimination, equilibrium and escape (253, 255).

1.21.1 Elimination

Elimination postulates that both the innate and adaptive immune system are constantly surveying the body to detect the presence of developing tumours. The mechanisms that lead to immune cell activation *in vivo* in this setting are currently unknown, but could be attributed to Type 1 interferons released during the early transformation process (247), or to DAMPs released by either dying tumour cells such as high mobility group box 1 (HMGB1), or from damaged cells as tumours begin to invade nearby tissue (256). In addition, stress ligands expressed by tumour cells such as H60 and RAE-1 (mouse) or MICA/B (human) are expressed on the surface of tumour cells. These ligands bind to activating receptors on NK cells, leading to the release of pro-inflammatory cytokines, resulting in an environment that is permissive to an anti-tumour response (257). While innate immunity can be protect against the development of some tumours, experimental evidence suggests the both $CD4^+$ and $CD8^+$ T cells are required for the recognition of tumour antigens and thus effective tumour clearance. In this setting, the coordination of both arms of the immune system are required for effective tumour clearance, and the

effective clearance of a developing tumour before it has the chance to establish embodies the end of the elimination phase (Fig 1.3 A).

1.21.2 Equilibrium

Equilibrium can be observed in tumour variants that survive the elimination phase, but lack the capacity to "take off" and grow while being held in check by the immune system. During this phase, the immunogenicity of the tumour is sculpted in a constant back and forth between the possibilities of elimination and escape (258). This phase may be one of the longest of the cancer immune-editing process and can perhaps exist for the lifetime of the host. During this phase, the tumour can persist in a dormant state before regaining the capacity to grow or metastasise to a distant site. This was made evident by experiments performed in immune-competent mice that were treated with low dose methylcholanthrene (MCA). These mice harboured minor tumours that were non-apparent. Following administration of antibodies against T cells and IFN-γ, tumours developed swiftly at the original injection site (259). Deeper analysis revealed the requirement for IL-12, IFN-γ, $CD4^+$ and $CD8^+$ T cells in maintaining equilibrium. The necessity for adaptive immunity in this setting distinguishes elimination and equilibrium and suggests that, once a tumour has developed past the elimination phase, adaptive immunity is paramount in ensuring growth inhibition (Fig 1.3 B).

1.21.3 Escape

Escape is the final phase of cancer immune-editing, where a tumour has been sculpted by the equilibrium phase to favour expression of tumour antigens that are not recognised by the immune system. Additionally, immunosuppressive mechanisms can be co-opted and secreted by the tumour to limit immune cell activation. Now unrecognisable to the immune system, the developing tumour is free to proliferate unhindered (255, 260, 261). When a tumour accumulates enough mutations it will proliferate faster than it can be killed by the immune system. This abnormal turnover rate will result in the accumulation of further mutations, increasing the chance that the mutations will arise in key regulatory pathways.

Starting with the tumour, increased resistance to immune mediated cytotoxicity can occur via upregulation of anti-apoptotic pathways or the activation of oncogenic pathways. Antigen loss is the best described mechanism of tumour escape. The equilibrium phase can select for tumour cells that lack rejection antigens. Furthermore, tumour cells can downregulate or lose expression of MHC-I or MHC-II, limiting antigen presentation to T cells. In addition, tumour cells can be defective in both the TAP and MHC presentation pathways resulting in the inability to produce epitopes and load these onto MHC-I or MHC-II (255, 261). These changes are caused due to the genomic instability of tumour cells, resulting in a selection process for tumour cells that are invisible to the immune system. In addition to mutations in antigen processing pathways, favourable mutations can arise in pathways pertaining to cell death resistance. For example, overexpression of Bcl-2 and Bcl-XL promote cell survival, limiting the effectiveness of apoptosis induction (262). Additionally, down-regulation of Fas and other death receptors render tumour cells immune to contact dependent cytotoxicity (263).

Outside of the tumour, an immunosuppressive microenvironment can be generated due to the secretion of cytokines and enzymes including TGF-β, IL-10 and prostaglandin E_2 (PGE_2) and IDO (264-269). This leads to the generation of thymic derived Tregs, or the conversion of tumour infiltrating $CD4^+$ T cells into Tregs. In addition to Tregs, myeloid derived suppressor cells can also act to limit immune activation within the tumour microenvironment (270). Indoleamine 2,3 dioxygenase is an enzyme that catalyses the breakdown of tryptophan to *N*-formylkynurine. This acts both limit the amount of the essential amino acid tryptophan available to T cells, and generates a suppressive environment owing to the presence of *N*-formylkynurine. Similarly, secretion of IL-10 and TGF-β by tumour cells can create a suppressive environment through the conversion of tumour-associated macrophages (TAMs) from an M1 to an M2 phenotype. Macrophages that are M2 skewed can release more IL-10 as well as producing oxygen radicals, which are toxic to infiltrating lymphocytes (271). Finally, the ligation of the checkpoint molecule programmed death 1 (PD-1) can limit the activation of T cells (272). When the combination of suppressive mechanisms outcompete the cytotoxic ability of T cells, tumour outgrowth is achieved and tumour escape is complete (Fig 1.3 C).

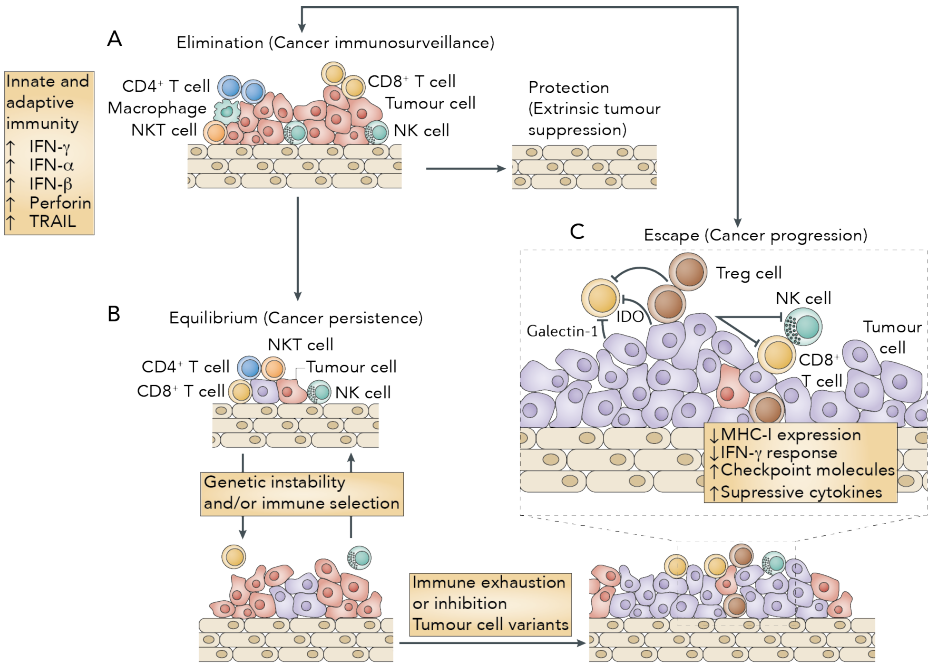


Figure 1.3 The cancer immunoediting process.
(A) The elimination phase consists of the recognition fo transformed cells by the innate and adaptive immune system, resulting in the destruction of these cells. (B) The equilibrium phase describes tumour persistence which is unable to expand due to immune pressure. (C) The escape phase beings when the equilibrium phase is shifted in favour of tumour outgrowth. This can occur as a result of immune exhaustion or as a result of tumour variants that are no longer recognised by the immune system. This phase concludes with the appearance of detectable, progressively growing tumours. Figure adapted from Dunn *et al* (273).

1.22 Immunotherapy for Glioma

Immunotherapeutic approaches under clinical development for GBM can broadly be classified into cellular therapies, monoclonal antibody therapy, checkpoint blockade and vaccine therapy. With T cells recognised for their anti-tumour capability, as evidenced by the immune-editing studies, they have been the focus of many studies. As tumours originate from otherwise benign cells, the recognition of T cell receptors to tumour antigens was thought to be low or impossible to detect. However some antigens are highly expressed such as those on haematologic cancers (274).

Chimeric Antigen Receptor (CAR) T cells are T cells that have been genetically modified to lose expression of the αβ TCR, with it being replaced with the fragment antibody-binding (Fab) region of an antibody to a defined tumour antigen. The Fab region is linked to an intracellular T cell signalling domain via a CD28 transmembrane portion. CAR T

cells are unique in their ability to recognise antigen, without the need for MHC presentation and to bypass traditional costimulation due to a genetically modified TCR. This facilitates the recognition of whole protein as opposed to processed peptide. To date, success using CAR T cells has been observed in the clinic with cells targeting CD19 on B cell lymphoma (275). While this results in the depletion of both malignant and benign cells, patients can be rescued with IgG replacement therapy (276). Whereas hematologic cancers have set the benchmark for CAR therapy, little success has been observed in solid malignancies. This may be due to the tumour antigen heterogeneity in solid tumours as well as the highly suppressive tumour microenvironment limiting both the T cell target, as well as their function (277). This approach may hold promise for GBM as 30% of patients harbour a mutation in the EGFR, resulting in constitutive activation of the receptor. This mutation, EGFRvIII, is currently being targeted by Novartis using CAR T cell therapy (277, 278).

In addition to cellular therapy, monoclonal antibodies can be utilised to target either tumour cell surface proteins, or factors, which facilitate tumour growth. Bevacizumab is an antibody that blocks angiogenesis by inhibiting VEGF-A and has been FDA approved for the treatment of GBM. While administration of Bevacizumab slows tumour growth, it does not affect overall survival in GBM patients (279). Indeed the limited efficacy observed in patients is thought to paradoxically be a result of the inability of bevacizumab to cross the BBB and limit angiogenesis within the tumour (280). Further, it is now thought that vascular normalisation, rather than anti-angiogenesis, is preferable as this permits the passage of chemotherapeutics to the tumour site (281, 282).

While checkpoint inhibitors have been approved for the treatment of metastatic melanoma, there is considerable interest in utilising these therapies for glioma. The CheckMate phase III trial aims to study the safety and efficacy of nivolumab compared to bevacizumab, and of nivolumab as a lone intervention, or combined with ipilimumab (283). A separate phase II trial is underway and aims to investigate the effectiveness of pembrolizumab as a lone therapy, or combined with bevacizumab in recurrent GBM patients (284). Finally, a new antibody targeting the PD-L1 pathway, currently referred to as MEDI4736, is being investigated in a phase II trial. Similar to the aforementioned trial,

MEDI4736 will be examined both as a lone therapy and also in combination with bevacizumab (285).

1.23 GBM vaccine immunotherapy

Thus far, the most commonly adopted approach for immunotherapy for GBM has been vaccine meditated. Several studies have been completed which have utilised the identification of GBM-specific antigens, such as the EGFRvIII, or have adopted a multi-antigen approach. This has typically been accomplished through the use of lysates generated from resected tumour tissue, or synthetic peptides which have been loaded onto autologous DCs isolated from patients (286). In the VICTORI phase 1 trial, EGFRvIII peptides were loaded onto DCs. Minimal adverse reactions were observed in patients and 60% of patients became sensitised to the peptides upon subsequent testing (287).

Whole tumour cell lysate as an antigen source has the benefit of providing a broad and patient specific repertoire of immunological targets. Generally, lysates are generated by culturing resected tumours *ex vivo* and isolating the surface proteins associated with MHC molecules. These can then be combined with autologous DCs and administered to patients. This method has been investigated in phase I clinical trials where vaccines were delivered subcutaneously over several weeks and patients were followed for up to 5 years (288, 289). Analysis of PBMCs revealed T cell reactivity against tumour cells, indicative of a systemic immune response. Furthermore, intratumoural T cell infiltration was observed in patients who required a second operation (289). As mentioned previously, GBM has been classified into four subtypes based on gene expression analysis (8). Interestingly, the mesenchymal subgroup has been identified as the subtype most likely to respond to tumour lysate vaccine therapy (290). There are currently several phase II and phase III trials underway to assess tumour lysate vaccines. One phase II trial currently underway aims to compare the efficacy of lysate-pulsed DCs with the TLR agonists resiquimod or poly-ICLC (291). Finally, the phase III trial is aiming to compare standard radiation and chemotherapy combined with tumour-lysate vaccination, to standard care plus placebo, with the option of crossover to the vaccine group if disease progresses (292). Similarly, a

phase II trial is underway in Vienna aiming to compare temozolomide and radiation to temozolomide, radiation and tumour-lysate vaccination (293).

A phase I clinical trial conducted in Wellington, NZ, aimed to assess the feasibility of treating patients who had previously been exposed to temozolomide chemotherapy with an autologous DC vaccine. While therapy was well tolerated, feasibility was marginal as insufficient vaccine was able to be produced in three of the fourteen patients (294). Here, the authors attributed the inability to generate enough vaccine to be a consequence of recent temozolomide exposure as previous reports have shown human monocytes are sensitive to temozolomide-induced apoptosis *in vitro* (295).

Different protocols for generating DCs for cancer vaccines may account for the varying differences in the efficacy of inducing T cell responses and ultimate induction of clinical responses in patients (296). Given the specialised cell culture techniques and *ex vivo* manipulation of PBMCs required for generation of DC vaccines, new approaches are being investigated which target a patients DCs, without having to culture cell *ex vivo*. Here, heat shock proteins (HSPs) have been investigated as they are known to bind receptors expressed by DCs, resulting in the delivery of a broad array of peptides (297). HSP-96 is a HSP expressed in GBM which has been investigated for use as a cancer vaccine (298). In a phase I study, patients were administered autologous tumour-derived HSP peptide complex intradermally. Eleven of the twelve patients recruited had specific peripheral immune responses as determined by *ex vivo* restimulation of PBMCs with vaccine and examining cytokine production and proliferation (298). Seven of the twelve patients also had post therapy tumour biopsies which revealed increased immune cell infiltrates (298). Based on these results, a phase II study has been completed in which patients received the HSP vaccine following standard treatment with radiation and temozolomide (299, 300).

To circumvent the identification of neoepitopes, computational analysis to predict MHC binding, and then peptide and vaccine synthesis, vaccines can be generated simply from resected tumour tissue (301, 302). To facilitate DC activation, tumour cells can be transduced to produce granulocyte monocyte-colony stimulating factor (GM-CSF) before

being irradiated and transferred back to the host (303). Irradiation renders the cells non-viable and can act to increase immunogenicity by inducing cell death (304, 305). In a myeloid leukaemia trial, co-injection of irradiated K562 cells, an immortalised myelogenous leukaemia line (306), transduced to produce GM-CSF, was observed to reduce tumour burden in patients on imatinib mesylate but with residual disease (307). Alternatively, resected tumour tissue can be fixed in paraformaldehyde to preserve antigen peptides and delivered in combination with an adjuvant (301). Each of these approaches are quick to manufacture and allow the entire epitope repertoire of a patient to be expressed without the need for exome sequencing. Conversely, as tumours arise from self-tissue and invade local and distant sites, any resected tumour will invariably contain a mixture of both self and transformed tissue. There is therefore the possibility that a vaccine generated this way may drive a response against healthy as well as transformed tumour tissues (308).

The induction of an effective anti-tumour response is reliant on both the efficient licensing of DCs as well as the cytotoxic capacity of T cells. Given the rarity of a specific TCR for a given antigen, whole cell based approaches are now being explored. Despite these pitfalls mentioned above, such an approach serves two purposes. First, a polyclonal population of T cells is activated, therefore increasing the population of cells with anti-tumour activity. Secondly, owing to the polyclonal T cell activation, the likelihood of tumour evasion of immunotherapy is decreased, compared to targeting therapy to a single antigen.

1.24 A cell-based vaccine for glioma based on the APC licensing function of NKT cells

Invariant NK T cells (iNKT) are innate T cells that share properties and receptors of both NK cells and T cells. iNKT cells develop in the thymus (309) but have a semi-invariant TCR, typically consisting of Vα14-Jα18 Vβ8.2 configuration in mice and Vα24-Jα18 Vβ11 in humans as opposed to the highly polymorphic αβ TCR expressed on conventional T cells. Whereas T cells recognise peptide antigens in the context of MHC class I and II, iNKT cells recognise the glycolipids presented by the MHC-like molecule CD1d (310). Upon administration of the glycolipid α-galactosylceramide (α-GalCer) to

mice, iNKT cells produce large amounts of IFN-γ and IL-4 (311). In addition to rapid cytokine release, iNKT cells are able to license DCs via CD40-CD40L interactions (Fig 1.4) (312). As described earlier, licensing is critical to ensure activation and potent costimulation ability of DCs to T cells. Increasing evidence suggests iNKT cells are required for clearing certain infectious diseases, as well as having roles in anti-tumour responses (313) and autoimmune diseases including EAE (314) and type 1 diabetes mellitus (315). As these are divergent diseases, it is likely that depending on the ligand dose and frequency, iNKT cells can enhance or regulate T cell activity in different diseases, most likely as a result of the cytokine milieu generated following activation.

One of the hurdles to DC based therapies has been the extensive *ex vivo* manipulation required to generate a suitable vaccine product. Additionally, patients undergoing chemo- and radiotherapy are often immune suppressed following treatment, resulting in fewer DCs with which to generate a vaccine (294). Due to these difficulties, considerable interest is being focused on targeting APCs within a patient with cancer vaccines. Resected tumour tissue is an attractive source of tumour specific antigens as mutations give rise to neoepitopes and other novel proteins, that can be targeted by T cells (316). The fundamental requirement of any vaccine strategy is the generation of systemic immune responses, resulting from the potent activation of APCs (317). Pulsing tumour cells with α-GalCer before irradiation is a simple yet elegant means to generate a vaccine with little manipulation of cells required. Incorporation of α-GalCer into a whole vaccine strategy, ensures both antigen and α-GalCer are delivered to the same APC, a phenomenon that has previously been shown to be essential for inducing effective immune responses (52).

This intravenous vaccine strategy has previously been shown to prevent tumour growth in several mouse models, including glioma (29). Vaccine efficacy is dependent on both α-GalCer and whole tumour cell antigen as neither irradiated cells in the absence of α-GalCer, nor free α-GalCer prevented tumour outgrowth in a subcutaneous setting (29). Additionally, $CD1d^{-/-}$ mice which lack NKT cells were unable to prevent tumour growth, suggesting NKT cell mediated licensing of DCs is required to induce anti-tumour

immune responses in this model (29). Induction of NKT cell activation could be observed by quantification of serum cytokines IL-4, IL-12p70 and IFN-γ following vaccine administration, with increased cytokine production not attributed to unpulsed vaccine cells, or free α-GalCer (29). Following activation, NKT cells enter a state of anergy and become unresponsive to further α-GalCer mediated activation, potentially restricting application in a prime-boost regime (318, 319). Indeed, previous exposure to free α-GalCer can abrogate response to a second α-GalCer-adjuvanted vaccine. Interestingly pre-treatment with a cell-based vaccine glioma vaccine loaded with α-GalCer only partially attenuated $CD8^+$ T cell expansion, suggesting that NKT cell anergy is somewhat averted by this vaccine strategy (29).

While considerable focus has been aimed at enhancing $CD8^+$ T cell responses for cancer immunotherapy, the α-GalCer-adjuvanted glioma vaccine efficacy was $CD4^+$ T cell mediated, as efficacy was lost in MHC class II-deficient mice, and also following $CD4^+$ T cell depletion in a prophylactic setting. While a large population of NKT cells reside in the spleen, and anti-tumour responses could be detected in this organ, it was surprising that anti-tumour responses could be induced in splenectomised animals. When examined further, it was revealed that anti-tumour immune responses could also be primed in the lung-draining lymph nodes, as revealed by enhanced overall cellularity, increased $CD11c^+$ MHC-II^+ DCs and increased expression of CD44 on $CD4^+$ T cells. Interestingly, this appeared to be specific to the route of administration as no difference was observed both in cellularity, or the expression of CD44 on $CD4^+$ T cells within the non-draining, inguinal lymph nodes (29). While prophylactic vaccine administration was effective at preventing tumour growth following either subcutaneous or intracranial tumour challenge, no anti-tumour effect was observed in a therapeutic setting unless Tregs were depleted before vaccination. The survival benefit was not a result of Treg depletion alone, as Tregs depletion in the absence of vaccine revealed no survival benefit (29).

To have translational relevance, this strategy requires NKT cells to be functional within GBM patients. Analysis of NKT cell number and function from the peripheral blood of histologically confirmed GBM patients revealed comparable numbers to age matched

healthy controls. Importantly, NKT cells could be expanded from the PBMCs of patients in response to α-GalCer *in vitro*, suggesting the NKT:DC axis is still functional in GBM patients. To confirm this further, patient derived primary tumour cell lines were pulsed with α-GalCer *in vitro* and were irradiated, washed and co-cultured with CFSE labelled PBMCs from a healthy donor. Proliferation of donor cells was measured as a loss of CFSE fluorescence on α-GalCer loaded CD1d tetramers, providing a proof-of-principle that a vaccine based on whole tumour cells and pulsed with α-GalCer can induce the proliferation of NKT cells in humans (29). Additionally, primary cell lines exposed to IFN-γ *in vitro* up-regulated MHC-II expression, suggesting that these cells could be susceptible to $CD4^+$ T cell targeted killing in patients (29).

Taken together, an α-GalCer pulsed autologous whole tumour cell vaccine generates anti-tumour responses within both the spleen and the mediastinal lymph node (MdLN). These responses are dependent on NKT cell licensing of DCs and induce $CD4^+$ T cell activation. Importantly, the NKT cell population appeared to be intact within the GBM patients sampled in this study, suggesting a vaccine approach described here could be an effective vaccine for humans (29). Thus far, no strategy exists to specifically deplete Tregs in human patients; however, checkpoint blockade is showing remarkable success in the clinic, albeit in a limited cohort of patients who respond to therapy. It is therefore conceivable that combining checkpoint blockade to unleash an underlying immune response could be enhanced with vaccination to induce anti-tumour immune responses.

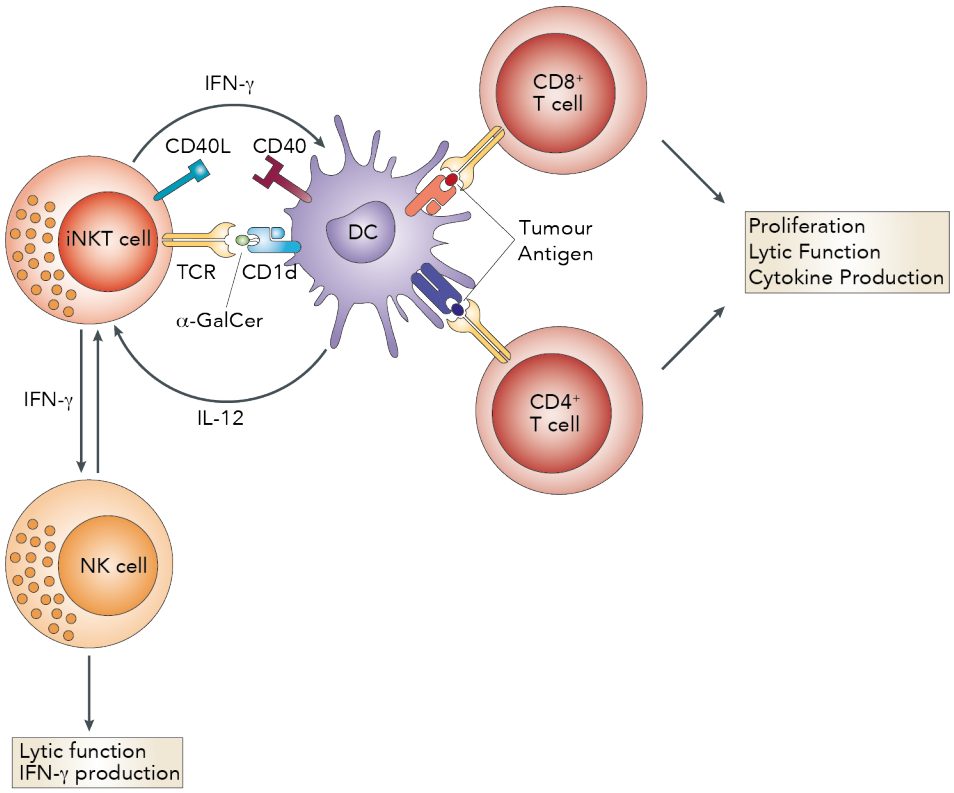


Figure 1.4 Immune cell modulation by activated iNKT cells.
(A) Ligation of CD40 on the surface of a DC by CD40L on an iNKT cell results in upregulation of costimulatory molecules such as CD86 and production of IL-12. This affords DCs stronger T cell stimulation capacity. iNKT cells also receive signals from DCs, promoting their activation. (B) Licensed DCs can present tumour antigenic peptides to $CD4^+$ and $CD8^+$ T cells. (C) Release of IFN-γ from iNKT cells acts to directly activate NK cells. Figure adapted from Cerundolo *et al* (320).

1.25 Gaps in knowledge

Given the dire outlook for GBM patients, immunotherapy represents a novel and promising treatment modality. While the CNS has traditionally been considered an immune privileged site, there is accumulating evidence that malignant tissue within the CNS can be targeted by T cells, giving the impetus to develop T cell-based immunotherapies. However, there is little consensus as to how to best achieve this. Research on vaccines to date have shown only limited efficacy in patients, and are often cumbersome to prepare. Recent clinical successes with checkpoint blockade antibodies in melanoma and non-small cell lung cancer (NSCLC) have shown that it is possible to unleashing immune responses in cancer patients. However, even in these diseases, only a

relatively small number of patients respond to this therapy (153). Given that checkpoint blockade is reliant on unleashing pre-existing immune responses, it is possible that those patients who fail to respond to therapy may have not induced an endogenous anti-tumour response in the first place. There is therefore accumulating interest in using therapeutic vaccines to initiate anti-tumour responses, which could be complemented with checkpoint blockade. Furthermore, the majority of the pre-clinical research in the checkpoint blockade field has focused on tumours within the periphery - little is know about the response to tumours behind the blood brain barrier. With this in mind, the work presented in this thesis aims to investigate checkpoint blockade combined with an autologous whole tumour cell vaccine in a mouse model of glioma. In particular, the work focuses on providing mechanistic insight into an effective combination therapy.

1.26 Hypothesis + aims

Administration of vaccines can be used to induce adaptive immune responses to tumour-associated antigens that have the capacity to limit tumour growth. Conversely, immune checkpoints pathways act as brakes for the immune system. However, following administration of checkpoint inhibitors, the immune system can again respond unhindered. The hypothesis underlying this thesis is:

"that the combination of vaccine-based immunotherapy and checkpoint blockade will result in improved immune-mediated tumour clearance."

Addressing the following aims will challenge this hypothesis.

1. To assess the efficacy of vaccination in combination with different checkpoint inhibitors
2. To assess the impact of the combination of efficacious combination therapy on the immune system
3. To determine the mechanism(s) of anti-tumour activity

Chapter 2

2 Materials and Methods

2.1 Labware

Table 2.1 Table of plasticware used throughout this thesis

Product	Supplier/Distributor
Acrodisc 13mm Syringe Filters with 0.2 μM membrane	PALL LifeSciences, Cornwall, UK
Axygen Microtubes 1.7 mL	Axygen Scientific Inc, Union City, CA, USA
BD 1 mL syringes BD 5 mL syringes BD 10 mL syringes BD 30 mL syringes	BD Biosciences, Bedford, MA, USA
Falcon tissue culture plates: 6-well plates 24-well plates 96-well round bottom plates 96-well U-bottom plates	BD Biosciences, Bedford, MA, USA
Falcon polypropylene conical tubes: 50 mL 15 mL	BD Biosciences, Bedford, MA, USA
Falcon tissue culture flasks: 75 cm^2 175 cm^2	BD Biosciences, Bedford, MA, USA
Falcon nylon cell strainers 70 μM	BD Biosciences, Bedford, MA, USA
Hamilton syringe: 31 gauge	Hamilton Laboratory Products, Reno, NV, USA
Iodine	Orion Las, Balcutta, WA, Australia
Minisart Syringe Filter	Sartorius
Precision Glide needles: 18 gauge 20 gauge 25 gauge 27 gauge	BD Biosciences, Bedford, MA, USA

2.2 Reagents and Buffers

Table 2.2 Table of reagents used throughout this thesis

Product	Supplier/Distributor
α-galactosylceramide	Produced by Ferrier Research Institute as previously described (321)
2 Mercaptoethanol (2 ME)	Gibco, Invitrogen, OR, USA. 2 ME was stored at 4°C
Bovine serum albumin (BSA)	ICP Biologicals, New Zealand
Brefeldin A	Sigma-Aldrich, MO, USA
CFSE	Molecular Probes, Invitrogen, OR, USA
Collagenase II	Gibco, Invitrogen, OR, USA
Dimethyl sulfoxide (DMSO)	Cryoserv, BionichePharma
Diphtheria Toxin	Sigma-Aldrich, MO, USA. Powdered diphtheria toxin was reconstituted in H_2O and stored at -20°C
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Invitrogen, OR, USA
DNAse	Roche, IN, USA. DNAse was stored at -20°C
Ultrapure 0.5 M, pH 8.0 Ethylenediaminetetraacetic acid (EDTA)	Invitrogen, Life Technologies, Auckland, New Zealand
Foetal Bovine Serum (FBS)	SAFC Biosciences Sigma-Aldrich, MO, USA. FBS was stored at -20°C
Glutamax	Gibco, Invitrogen, OR, USA
Glycine	Sigma-Aldrich, MO, USA
Iscove's Modified Mulbecco's Medium (IMDM)	Gibco, Invitrogen, OR, USA
Monensin	Sigma-Aldrich, MO, USA
Percoll	GE Healthcare Bioscience, Uppsala, Sweden
PBS	Gibco, Invitrogen, OR, USA
Red Blood Cell Lysis Buffer	Qiagen Sciences, MD, USA RBC Lysis Buffer stored at room temp
Roswell Park Memorial Institute (RPMI) 1640 Medium	Gibco, Invitrogen, OR, USA
Trypan Blue (0.4% v/v)	Gibco, Invitrogen, OR, USA
TrypLE	Gibco, Invitrogen, OR, USA
Tween 20	Bio-Rad Laboratories, CA, USA
Penicillin Streptomycin	Gibco, Invitrogen, OR, USA
Sodium Azide	Sigma-Aldrich, MO, USA

2.2.1 3% Hydrogen peroxide

PBS (40 mL), methanol (50 mL) and 30% hydrogen peroxide (10 mL)

2.2.2 Antibody purification buffers

Wash/Start Buffer: 1L of 20 mM di-Sodium Orthophosphate (Na_2HPO_4), pH 8.0

Add 2.48g to 1L dH_2O

Use HCl to bring pH to 8.0

Elution Buffer: 500 mL 0.1M glycine-HCl, pH 2.7

Add 3.75g to 500 mL dH_2O

Use HCl to bring pH to 2.7

Neutralising Buffer: 50 mL 1M Tris-HCl, pH 9.0

Add 6.06g to 50 mL dH_2O)

Use HCl to bring pH to 9.0

20% EtOH

Add 200 mL EtOH to 800 mL dH_2O

dH_2O

Filter sterilise 500 mL of dH_2O and leave overnight to degass

2.2.3 Citrate buffer

Citrate buffer was made fresh as needed by diluting 5.88g Sodium TriCitrate in 2L dH_2O before being pH adjusted to 6.0 with HCl. Tween 20 (1 mL) was added following pH adjustment.

2.2.4 Complete DMEM (cDMEM)

DMEM was supplemented with 20% FBS, 2mM Glutamax, 100 u/mL penicillin and 100 ug/mL streptomycin

2.2.5 Complete IMDM (cIMDM)

IMDM was supplemented with 10% FBS, 2mM Glutamax, 100 u/mL penicillin, 100 ug/mL streptomycin and 500 μL 2 ME

2.2.6 Complete IMDM for hybridomas

IMDM was supplemented with 5-20% FBS, 2mM Glutamax, 100 u/mL penicillin, 100 ug/mL streptomycin and 500 μL 2 ME

2.2.7 Complete RPMI (cRPMI)

RPMI was supplemented with 10% FBS, 2mM Glutamax, 100 u/mL penicillin, 100 ug/mL streptomycin and 500 μL 2 ME

2.2.8 Flow buffer

PBS containing 1% FBS, 0.01% NaN_3 and 2 mM EDTA was used for all flow cytometry experiments. Buffer was filter sterilised for FACS experiments

2.2.9 FoxP3 Fix/Perm Buffer Set

Purchased from BD Biosciences. Buffers were diluted in PBS prior to use

2.2.10 PBST

Tween 20 (500 μL) added to 1L PBS

2.2.11 PBS + EDTA

EDTA added to PBS to give final concentration of 10 mM

2.2.12 Percoll Diluent

Add 135 mL 10 X PBS to 145 mL dH_2O

2.2.13 37% Percoll

Combine 33.4% stock Percoll, 19.6% Percoll diluent and 47% 1 x PBS. Warm to room temperature before use

2.2.14 *Wurzburger buffer*

FBS, DNase and EDTA were added to PBS at final concentrations of 1%, 5 mM and 0.02 mg/mL, respectively. Buffer was stored at 4°C and used under sterile conditions.

2.3 Purified Antibodies

2.3.1 *Antibodies*

Hybridomas were grown in cIMDM, with FBS ranging in concentration from 5-20%. Antibodies were isolated from supernatants by fast pressure liquid chromatography using Hitrap protein G affinity columns used according to manufacturers instructions. After isolation into neutralising buffer, antibody was dialysed for 4 hours in 2L PBS using 10000 molecular-weight cut off cassettes. The PBS was changed and antibody was dialysed overnight. The next day, dialysed protein was extracted from the cassette and filter sterilised. Antibody concentration was determined by nanodrop absorption at 280 nm.

2.3.2 *α-CTLA-4*

α-CTLA-4 mAb (4F10, hamster IgG2a) was purified from hybridoma supernatant by protein G affinity columns. Mice were injected with 1 mg of antibody the day before vaccine administration, unless otherwise indicated. The 4F10 hybridoma was kindly provided by Dr Jeff Bluestone, UCSF, CA, USA.

2.3.3 *α-LAG-3*

α-LAG-3 mAb (C9B7W, rat IgG1) was purified from hybridoma supernatant by protein G affinity column. Mice were injected with 500 μg of antibody on days 7 and 250 μg, 10, 13 and 16 after vaccine administration, unless otherwise indicated. The RMPI-14 hybridoma was kindly provided by Mr Rohit Sinha, The University of Queensland Diamantina Institute, Australia.

2.3.4 *α-PD-1*

α-PD-1 mAb (RMPI-14, rat IgG2a) was purified from hybridoma supernatant by protein G affinity column. Mice were injected with 250 μg of antibody on days 3, 6 and 9 after

vaccine administration, unless otherwise indicated. The RMPI-14 hybridoma was kindly provided by Dr Hideo Yagata, Juntendo University School of Medicine, Toyko University, Japan.

2.3.5 IgG controls

The appropriate IgG controls were purchased from BioXCell and administered to mice in an identical dosing and timing schedule as the checkpoint blockade antibodies.

2.4 Cell Culture

Cell culture was performed under PC2 conditions at the Malaghan Institute of Medical Research, Wellington. Cell line work was performed in Class II biological safety cabinets with HEPA air filters (HERAsafe, Heraeus, Germany). Cells were incubated in humidified incubators (HERAcell incubator, Heraeus, Germany).

2.4.1 GL261

The murine glioma cell line GL261 was obtained from the DCTD Tumour Repository (NCI, Frederick, MD). Cells were grown in cDMEM at 37°C + 5% CO_2. When cells reached 80% confluency, they were harvested with TrypLE and passaged.

2.4.2 LLTC

The tissue culture adapted lewis lung carcinoma (LLTC) was kindly provided by Dr Graeme Finlay (Cancer Research Laboratories, Auckland, NZ). Cells were grown in cDMEM at 37°C + 5% CO_2. When cells reached 80% confluency, they were harvested with TrypLE and passaged.

2.4.3 Mycoplasma testing

Cultured cells were tested every 3-6 months for mycoplasma contamination using the Intron Mycoplasma PCR detection Kit, as per the manufacturers instructions. Cells were also tested for mycoplasma prior to freezing to ensure stocks were mycoplasma-free.

2.4.4 GL261 Cell Lysate

GL261 cells were bulked over the course of a week before media was removed and cells were harvested with TrypLE. Cells were washed three times with PBS by centrifugation at

400 x g for 4 mins. Cells were adjusted to 1 x 10^7 cells/mL in PBS and 1.5 mL aliquots were made into cryovials. Cells were lysed by repeated freeze/thaw cycles in liquid nitrogen and a 37°C waterbath. Cell death was verified using trypan blue exclusion and cells were triturated ten times with a 26G needle to burst dead cells. Large particles were removed by centrifugation at 1000 x g for 10 mins and lysate containing supernatant was transferred to a new tube. Lysate was then filter sterilised through a 0.2 μM syringe filter and protein concentration was determined by Nanodrop.

2.4.5 Protein Quantification

GL261 lysate concentration was determined using a NanoDrop ND-1000 Spectrophotometer at 280 nm, with ND-1000 v 3.8.1 Software.

2.5 Mice

2.5.1 Ethical Approval and Housing

All mice were bred and housed at the Biomedical Research Unit at the Malaghan Institute of Medical Research. Mice were age and sex matched where possible. All experiments were carried out within the requirements of the Animal Welfare Act of New Zealand and approved by the Victoria University of Wellington Animal Ethics Committee (2012R15M).

2.5.2 C57BL/6

The C57BL/6 inbred mouse strain was obtained from Jackson Laboratories (Bar Harbour, ME, USA).

Crossing C57BL/6 mice with SJL/J mice generated B6-Sj ptprca mice. These mice express the congenic marker CD45.1 (322). The B6-Sj ptprca mice were obtained from Jackson Laboratories (ME, USA)

2.5.3 B6Aa0

The B6Aa0 knockout strain was developed by targeting a mutation to the Aa gene in embryonic C57BL/6 stem cells. This mutation prevents the expression of MHC II,

preventing $CD4^+$ T cell positive selection (323). Mice were supplied by Dr H Bluethmann (Hoffmann-LaRoche, Basel, Switzerland).

2.5.4 *Tap1*$^{-/-}$

$TAP^{-/-}$ mice are defective in the stable assembly and intracellular transport of MHC class I molecules and are thus deficient in $CD8^+$ T cell responses (324). These mice were developed by Van Kaer and colleagues

2.5.5 *Pfp*$^{-/-}$

Mice lacking the perforin molecule have normal numbers of $CD8^+$ T cells and NK cells. These mice are unable to lyse virus-infected or allogeneic fibroblasts *in vitro* (325).

2.5.6 *Ifng*$^{-/-}$

Targeted disruption of the interferon-gamma gene results in mice which are normal in a resting state but display reduced macrophage function in response to pathogens (326).

2.5.7 *CD11b-DTR x C57Bl/6*

CD11b-DTR transgenic mice are on a BALB/c background and have a diphtheria toxin (DT) inducible system that transiently depletes macrophages in various tissues. The transgene insert contains a fusion product involving simian diphtheria toxin receptor and green fluorescent protein under the control of the human *ITGAM* (integrin alpha M) promoter (CD11b) (327). CD11b-DTR mice were crossed onto a C57BL/6 background for one generation before being used in experiments.

2.5.8 *OT-II*

A T cell hybridoma which was CD4 positive, MHC-II restricted and specific for chicken ovalbumin $(OVA)_{323-339}$. The α- and β-chain transgenic constructs were taken form the hybridoma and injected into fertilised B6 mouse eggs to generate OT-II mice. These mice harbour peripheral T cells which express the transgene and are therefore only receptive to MHC-II presenting $OVA_{323-339}$ peptide (328).

2.5.9 *Cd1d*$^{-/-}$

CD1d-deficient mice harbour a knock-out of the *CD1d1/CD1d2* locus, resulting in a lack of NKT cells (329).

2.6 Methods

2.6.1 Intracranial Surgery

For intracranial tumour implantation, from here on defined as tumour challenge, previously frozen cell aliquots were obtained from liquid nitrogen where they were stored at 2.5 x 10^6 cells/mL in 90% cDMEM20 + 10% DMSO. Cells were thawed by gentle agitation into 10 mL iDMEM. Cells were centrifuged at 400 x g for 4 mins. The cell pellet was resuspended in 200 μL sterile PBS. A 10 μL aliquot was taken before and after challenge to determine tumour cell viability. Ketamine and xylazine (Phoenix Phamraceuticals, Auckland, NZ) were used to anaesthetise mice (100 mg/kg and 10 mg/kg respectively). Buprenorphine (Renckitt Benckiser Pharmaceuticals, North Chesterfield, USA) and carprofen (Norbrook Laboratories, Corby, UK) were administered subcutaneously for perioperative analgesic pain management. Once under anaesthesia, the head was mounted onto a stereotactic frame (Harvard Apparatus, Holliston, MA, USA) and secured with ear bars. Lacrilube (Allergan, Parsippany, USA) was applied to prevent corneal desiccation and skin was prepped with povidone iodine solution (Betadine, Purdue Products, Stamford, CT, USA). A lateral incision was made from behind the eyes to the base of the neck to expose the skull. A 20 gauge needle (BD Biosciences) was used to make a burr hole 2 mm right of the bregma. Cells were drawn into a 10 μL Hamilton Syringe with a 32G needle (Hamilton Company, Reno, NV, USA). The needle was advanced to a depth of 4 mm from the surface of the brain then withdrawn 1 mm and 1 μL of cells deposited. The needle was then withdrawn another 1 mm and 1 μL cells deposited. The needle was withdrawn a further 1 mm and left in place for tumour cells to settle for 5 mins. The needle was then withdrawn completely and the hole sealed with sterile bone wax (Amtech Medical, Auckland, NZ). The wound was closed with two non-continuous 7/0 nylon sutures (Amtech Medical, Auckland, NZ). Mice were left to recover in cages on a heat pad.

2.6.2 Subcutaneous Challenge

For subcutaneous tumour challenge, GL261 cells were harvested during the logarithmic growth phase. The cell monolayer was washed with PBS and then cells were incubated with TrypLE at 37°C + 5% CO2 for 10 mins. TrypLE was quenched with the addition of cDMEM20 and cells were centrifuged at 400 x g for 4 mins. The cell pellet was resuspended in incomplete DMEM and counted before being resuspended at 1 x 10^7 cell/mL. Mice received 100 μL cells (1 x 10^6 cells total) in the subcutaneous left flank.

2.7 Therapy

2.7.1 Vaccine Generation

To generate vaccine, GL261 cells were grown in complete DMEM supplemented with 200 ng/mL α-GalCer for 24 hours. Media was discarded and the monolayer was washed three times with PBS to remove unbound α-GalCer. Cells were then lifted with TrypLE (Gibco) and resuspended in incomplete DMEM. Cells were then γ-irradiated (150 cGy) on ice and washed 3 times in PBS before freezing in 10% DMSO + 90% cDMEM.

2.7.2 Vaccine Administration

To administer vaccine, cells were thawed by gentle agitation into serum free incomplete DMEM. Cells were centrifuged at 400 x g for 4 mins and resuspended in PBS. Cells were adjusted to 5 x 10^5 cells/mL in PBS. Mice received 1 x 10^5 cells in 200 μL PBS, injected intravenously via the lateral tail vein.

2.7.3 Antibody administration

Checkpoint blockade antibodies, or appropriate isotype controls were administered intraperitoneally as indicated in the figure legends.

2.8 MRI

Magnetic Resonance Imaging (MRI) was conducted on anaesthetised animals using a clinical 1.5-T MR scanner (Philips Medical Systems) equipped with a wrist solenoid coil. T1 weighted images were acquired with the following parameters: TE = 20 milliseconds, TR = 800 milliseconds, thickness = 1 mm. Contrast was enhanced by intravenous

administration of 100 μl/mouse of gadolinium-DTPA (Magnevist, Bayer Schering Pharma). Image analysis was performed using Philips DICOM Viewer R2.5 Version 1.

2.9 Survival Studies

In some experiments, mice were intracranially challenged and treated with vaccine, checkpoint blockade antibodies or both. Mice were monitored for signs of weight loss or neurological deficit. When weight loss exceeded 10%, or mice exhibited signs of discomfort including decreased activity, hunched posture, ruffled fur or eye closure, mice were culled by cervical dislocation and tumour presence confirmed macroscopically.

2.10 Tissue Isolation

2.10.1 Brain

For analysis of infiltrates into tumours, brains were collected and dissociated with a scalpel before digestion in incomplete IMDM, supplemented with 4 μg/mL DNAse I and 2.4 mg/mL collagenase II. Samples were incubated at 37°C + 5% CO_2 for 30 mins before digested tissue was homogenized by passage through an 18G needle and filtered through a 70 μM filter and washed twice with PBS. Filtered tissue was centrifuged on a 37 % percoll gradient at 760 x g for 30 mins with no brake. Myelin and percoll was aspirated to allow cells to be recovered from the bottom of the gradient. Samples were washed with 10 mL PBS and centrifuged at 400 x g for 4 mins. Supernatant was discarded and cell pellets resuspended in 500 μL RBC lysis buffer and incubated at 37°C for 15 mins. RBC lysis was quenched with 1.5 mL FACS buffer and samples were transferred to FACS tubes for flow cytometric analysis.

2.10.2 Lymphocyte Isolation

Lymph nodes were isolated from mice using blunt forceps and transferred to 24 well plates containing gauze and 250 μL cDMEM. Lymph nodes were mechanically dissociated between gauze with the soft end of a syringe plunger. Wells were then washed with 1 mL media and total contents excluding gauze were transferred to 15 mL conical tubes.

2.10.3 Splenocyte Isolation

Spleens were isolated from mice using blunt forceps and transferred to 24 well plates containing gauze and 500 μL cDMEM. Spleens were mechanically dissociated between gauze with the hard end of a syringe plunger. Wells were then washed with 1 mL media and total contents excluding gauze were transferred to 15 mL conical tubes. Contents were centrifuged and supernatant discarded. The pellet was resuspended in 3mL RBC Lysis Buffer and incubated for 15 mins. Lysis buffer was quenched with 7 mL cDMEM and samples were filtered through 70 μM filter into a 50 mL conical tube.

2.10.4 Blood

Cardiac puncture was performed on euthanised mice and whole blood was transferred into a 1.5 mL eppendorf tube containing 500 μL PBS + EDTA. Samples were centrifuged at 700 x g for 4 mins in a Biofuge Fresco (Heraeus, ThermoFisher Scientific, New Zealand). Supernatant was aspirated before samples were washed with PBS. Samples were centrifuged again, supernatant aspirated and whole blood was resuspended in 1 mL RBC lysis buffer and incubated for 37°C + 5% CO_2 for 30 mins. White blood cells were pelleted by centrifugation at 700 x g for 4 mins and resuspended in 200 μL FACS buffer for flow cytometric analysis.

2.10.5 Serum

Cardiac puncture was performed on euthanised mice and whole blood was transferred into a 1.5 mL eppendorf tube containing 500 μL PBS + EDTA. Samples were stored at 4°C overnight and centrifuged at 13,250 x g for 10 min. The top serum layer within the tube after centrifugation was transferred into new tubes and stored at -20°C until analysis

2.10.6 Viable cell counts

To determine total cell counts, a 10 μL aliquot of cells was added to 190 μL 0.4% trypan blue and mixed. A 10 μL aliquot of this mixture was loaded onto a haemocytometer (source) and cells were counted on an inverted microscope. Viable cells were identified as white whereas dead cells were blue. The total cell number was calculated using the following formula:

Total cell number = (Average cell count) x (dultion factor) x (total volume) x 10^4

2.10.7 Restimulation with tumour lysate for intracellular flow cytometry

Splenocytes in cRPMI (2 x 10^6/mL) were plated into wells of a 24 well plate. GL261 cell (section 2.4.4) lysate was added to wells at a final concentration of 100 μg/mL. Final volume in cultures was brought to 2 mL. Cultures were incubated for 2 hours at 37°C + 5% CO_2. Monensin (20 μM) and brefeldin A (3 μg/mL) were added to cultures to prevent cytokine release and cultures were incubated for a further 4 hours. At the end of the culture, cells and supernatant were harvested into 5 mL FACS tubes. Wells were washed with 2 mL PBS + EDTA and residual cells were added to the respective FACS tubes. Cells were centrifuged at 400 x g for 4 mins and supernatant discarded. Samples were resuspended in residual volume and progressed to surface staining before staining for intracellular cytokines as described in section 2.12.2.

2.10.8 Restimulation with tumour lysate for in vitro killing assay

cRPMI10 media was supplemented with α-CD28 antibodies diluted 1/750 and GL261 tumour cell lysate (section 2.4.4) at a concentration of 100 μg/mL and 500 μL aliquots were made into individual wells of 24 well plates. Splenocytes were harvested from treated mice and processed into single cell suspension as described in section 2.10.3. Splenocytes were then resuspended in cRPMI10 at a concentration of 1 x 10^7 cells/mL and 500 μL aliquots were added to individual wells of a 24 well plate with supplemented media described above, to give a final dilution of 1/1500 α-CD28 and 50 μg/mL GL261 lysate. Cultures were incubated for 96 hours at 37°C + 5% CO_2.

2.10.9 Dynabead enrichment

Cells restimulated as described in section 2.10.8 were washed in Wurzburger buffer, counted and resuspended at 1 x 10^8 cells/mL. A Dynabead CD4 Positive Isolation Kit Invitrogen, Life Technologies) was then used to isolate $CD4^+$ cells following the manufacturers instructions. Cells were incubated for 20 min at 4°C with 25 μL $CD4^+$ Dynabeads/mL of sample and were intermittently mixed to prevent the beads from settling to the bottom of the tube. Sample tubes were placed in a magnetic column for 1 min and the negative unbound cells were transferred to a negative fraction tube. Bead bound cells were washed with Wurzburger buffer and placed back in the column for a further minute to remove any remaining unbound cells. Samples were then resuspended in 500 μL iIMDM and 10 μL DETACHaBEAD per 25 μL Dybabeads was added. Samples were incubated at room temperature for 45 mins with intermittent mixing. Sample tubes were placed in the magnetic column for 1 min and positive cells were removed from the tube and transferred to a positive fraction tube. This was repeated twice more before isolated positive and negative fractions were counted for downstream applications. A 100 μL aliquot from each of the positive and negative fractions was set aside for purity determination by flow cytometry, with samples stained as described in section 2.12.1.

2.10.10 In vitro T cell proliferation assay

Spleens were isolated from mice 18 hours following treatment and single cell suspensions were created as described in section 2.10.3. Cells were incubated with 10 μM $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) for 2 hours at 37°C + 5% CO_2. Cells were then fixed in 2% PFA and quenched with 0.1 mM glycine (Sigma-Aldrich). Quenched cells were resuspended in cIMDM and serially diluted across a 96-well plate. Cell suspensions created from spleens of OT-II mice were stained with 1 μM CFSE and 2 x 10^5 cells were added to the wells containing fixed splenocytes. Cultures were incubated for 6 days and CFSE dilution of OT-II cells, indicative of cell proliferation, was determined by flow cytometry.

2.10.11 Assessment of T cell proliferation in vivo

Single cell suspensions from B6-Sj ptprca mice were created as described in section 2.10.3. Cells were stained with 1 μM CFSE and 1 x 10^7 cells were adoptively transferred into naïve hosts. Hosts were treated the following day with α-CTLA-4, vaccination, combined treatment, or remained naïve. One week later, host mice were culled and spleens were harvested and processed as described in section 2.10.3. The proliferation of CD45.1 transferred splenocytes, indicated by CFSE dilution, was determined by flow cytometry following surface staining, as described in section 2.12.1.

2.10.12 In vitro killing assay

To assess the cytotoxic capacity of splenocytes following treatment, splenocytes were titrated into an *in vitro* killing assay. On day 3 after restimulation, 3 x 10^5 GL261 and LLTC were aliquoted into wells of a 24 well plate in cDMEM20. A final concentration of 1.88 μCi/mL tritiated ([^{3}H])-thymidine (PerkinElmer, MA, USA) was added to each well and cells were incubated overnight to allow the incorporation of [3H]-thymidine into the DNA of proliferating cells. On day 4, restimulated splenocytes were harvested and centrifuged at 400 x g for 4 mins. Cells were washed with cRMPI10 and readjusted to 5 x 10^6 cells/mL and 200 μL added to the first row of wells in a 96 well plate. A 2-fold serial dilution was made down the plate, with no cells added the final row. Target cells were harvested and washed in cRPMI10 and cells readjusted to 1 x 10^5 cells/mL and 100 μL added to each well of a 96 well plate. Cultures were incubated at 37°C + 5% CO_2 for 4 hours before being harvested onto filter paper (Walla, Turku, Finland) using a cell harvested (TOMTEC, Germany). Betaplate scintillation fluid (PerkinElmer) was added to the dried filter paper and the beta radioactivity was measured using a MICROBETA PLUS Liquid Scintillation Counter (WALLAC, PerkinElmer Inc) and Microbeta Windows Workstation Software.

To calculate the percentage of specific cell lysis per sample, counts per minute (CPM) the following formula was used:

% specific lysis = (spontaneous lysis – experimental lysis)/(spontaneous lysis x 100)

2.11 Serum Analysis

Serum samples were thawed at room temperature and cytokines IL-4, IFN-γ and IL-12p70 was assessed by cytokine bead arrays analysed on a Bio-Plex analyser (Bio-Rad Laboratories, CA, USA) according to manufacturers instructions. A 96-well filter plate on a vacuum manifold was pre-wet with 1 x Assay Buffer. The plate was removed from the manifold and 25 μL of the serially diluted standards was aliquoted into the allocated wells. Assay Buffer was added to the designated "blank" controls wells, before 25 μL of each serum samples was aliquoted into the designated sample wells. A volume of 25 μL of 1/40 Bead Mixture and 50 μL of 1/40 Biotin-Conjugate Mixture was added to each well. The plate was protected from light and incubated for 2 hours at room temperature on a plate shaker at 500 rpm. Wells were then washed twice with Assay Buffer using the vacuum manifold. The plate was removed from the vacuum and 100 μL Assay Buffer was added to each well in addition to 50 μL of 1/31.25 Streptavidin-PE solution. The plate was again protected from light and incubated for 1 hour at room temperature on a plate shaker at 500 rpm. The wells were washed twice with Assay Buffer using the vacuum manifold and the stained beads were resuspended in a 125 μL final volume of Assay Buffer before being read on the Bio-Plex analyser.

2.12 Flow Cytometry

2.12.1 Surface staining

Single cell suspensions from harvested organs were washed with flow buffer and resuspended in 1 mL flow buffer. The cell suspension was distributed to 5 mL FACS tubes for staining. Samples were centrifuged at 400 x g for 4 mins, and supernatants discarded. Pellets were resuspended in residual volume by vortexing before 100 μL of flow buffer containing FC receptor blocking 2.4G2 antibody at 10 μg/mL and Live/Dead fixable blue (1:1000) was added. Samples were incubated on ice for 20 mins. Cells were washed with 500 μL flow buffer before being centrifuged again, supernatants discarded and cells resuspended as described above. Cells were resuspended in 100 μL flow buffer containing fluorescently-conjugated antibodies at pre-determined optimal dilutions. Samples were incubated on ice for 20 mins. Cells were washed again with 500

μL flow buffer. Samples were resuspended in 200 μL of 2% PFA for analysis by flow cytometry or were progressed to intracellular staining.

2.12.2 *Intracellular staining*

Intracellular staining was performed using the FoxP3 staining kit (BD) according to the manufacturers instructions. Briefly, following extracellular staining described in section 2.12.1, cells were fixed by incubation in 500 μL Fix/Perm for 60 minutes at room temperature, in the dark. Samples were centrifuged at 400 x g for 4 mins and resuspended in 200 μL Perm buffer and incubated for 30 mins at room temp in the dark. Samples were washed once more and stained with the appropriate antibody diluted in 100 μL perm buffer for 30 mins at room temp, in the dark. Appropriate isotype control antibodies were used to stain extra cells or fluorescence minus one (FMO) controls were used to determine gating strategies.

2.12.3 *CFSE Staining*

Splenocytes isolated as described in section 2.10.3 were centrifuged at 400 x g for 4 mins and resuspended in PBS at 5 x 10^6 cells/mL. CFSE was diluted 1/100 from a stock concentration of 10 mM in PBS and 20 μL diluted CFSE was added per 1 mL of cells. Samples were vortexed immediately and incubated at 37°C + 5% CO_2 for 10 mins. Following incubation, CFSE was quenched with 5 volumes of cold FBS and centrifuged at 400 x g for 4 mins. Samples were washed with cIMDM and transferred to new tubes before being centrifuged at 400 x g for 4 mins for a total of 3 washes. Samples were then counted and resuspended at the appropriate concentration for *in vitro* or *in vivo* use.

2.13 Acquisition and Analysis

2.13.1 *BD LSR-II Special Order Product*

Samples were acquired on an LSR-II special order product (BD).

The LSR-II was equipped with the following lasers and detectors: Ultra violet laser (355 nm) UV740/35, UV450/50 and UV379/28. Violet laser (405 nm) V780/60, V720/40, V660/20, V605/40, V560/40, V525/50 and V450/50. Blue laser (488 nm) B705/70,

B515/20 and B488/10. Green laser (532 nm) G780/60, G610/20 and G575/26. Red laser (640 nm) R780/60, R710/50 and R670/14.

All fluorophores were detected using laser and detector combinations which best matched their excitation and emission profile.

2.13.2 BD Influx

Fluorescence Activated Cell Sorting (FACS) was performed using a BD Influx Cell Sorter. The influx was equipped with the following lasers and detectors: Ultra violet laser (355 nm) UV730/45, UV670/30, UV460/50 and UV379/34. Violet laser (405 nm) V780/60, V710/50, V660/20, V610/20, V520/35 and V425/26. Blue/Violet laser (445 nm) BV504/12. Blue Laser (488 nm), B692/40 and B520/35. Green laser (552 nm) G780/60, G710/50, G670/30, G610/20 and G575/26. Red laser (640 nm) R780/60, R720/40 and R670/30.

2.13.3 FlowJo

Flow Cytometry Standard (FCS) files were analysed using FlowJo software (Tree Star, San Carlos, CA, USA). Example flow plots in the results section were pre-gated on live, single cells based on FSC-A v FSC-H profile and negative staining for viability dye.

2.14 Antibodies

Table 2.3 Table of Flow Cytometry Antibodies used throughout this thesis

Specificity	Clone	Fluorophore	Manufacturer
CD3ε	17A2	BV421	Biolegend
	145-2C11	FITC	Biolegend
	145-2C11	PeCy7	eBioscience
CD4	RM4-5	eFluor450	eBioscience
	RM4-5	BV711	Biolegend
CD8α	53-6.7	Pacific Blue	BD Bioscience
	53-6.7	AlexaFluor 647	In house
CD11b	M1/70	ApcCy7	BD Bioscience
CD1d αGal-loaded tetramer		PE	NIH
CD11c	HL3	PeCy7	BD Bioscience
MHC-II	M5/114.15.1	PeCy7	Biolegend
NK1.1	PK136	PerCpCy5.5	BD Bioscience
B220	RA3-6B2	PerCpCy5.5	eBioscience
CD19	eBio1D3	eFluor450	eBioscience
	6D5	BV605	Biolegend
CD86	GL-1	BV650	Biolegend
	GL1	PE	eBioscience
CD44	1M7	Pacific Blue	eBioscience
	1M7	PerCpCy5.5	eBioscience
	1M7	APC-AF750	eBioscience
CD62L	MEL-14	Pacific Blue	eBioscience
	MEL-14	PeCy7	eBioscience
CD45	30-F11	APC	eBioscience
IFN-γ	XMG1.2	PeCy7	eBioscience
FoxP3	FJK-16S	PE	eBioscience
	FJK-16S	APC	eBioscience
TNF-α	MP6-XT22	FITC	eBioscience

All antibodies were titrated before first use to ensure the optimal signal to noise ratio.

2.15 Fluorophores

Table 2.4 Table of Flow Cytometry Fluorophores used throughout this thesis

Fluorophore	Manufacturer
DAPI	Invitrogen
Live Dead Fixable Blue (LDFB)	Invitrogen

2.16 RNA Analysis

2.16.1 RNA Extraction

Cells for RNA extraction were FACS sorted before being washed in PBS and centrifuged at 400 x g for 4 mins. Supernatant was removed and RNA extraction was performed using the NucleoSpin RNA kit (Machery-Nagel), as per the manufacturers instructions. Briefly, cells were lysed in Buffer RA1 and β-mercaptoethanol and lysate filtered through a NucleoSpin Filter by centrifugation at 11,000 x g for 1 min. The NucleoSpin Filter was then discarded, 70% ethanol was added to the lysate and mixed before both the lysate and ethanol were loaded onto a NucleoSpin RNA Column and centrifuged at 11,000 x g for 30 sec. The column was placed into a new tube and Membrane Desalting Buffer was added to the column membrane before centrifugation at 11,000 x g to dry the membrane. DNA was digested by the addition of DNase reaction mixture directly onto the silica membrane and samples were incubated at room temperature for 15 mins. Buffer RAW2 was then added to the column to inactivate rDNase and samples were centrifuged at 11,000 x g for 30 secs. Samples were washed twice with the addition of Buffer RA3 to the NucleoSpin RNA column and centrifugation at 11,000 x g for 30 secs. Finally, RNA was eluted from the column by addition of RNase-free water and centrifugation at 11,000 x g for 1 min. RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer at 280 nm, with ND-1000 v 3.8.1 Software.

2.16.2 Reverse Transcription PCR

The iScript cDNA synthesis protocol (Bio-Rad Laboratories, CA, USA) was used for reverse transcription as per the manufacturers instructions. Combined in an RNAse-free tube was 10 μL of 5 x iScript Reaction Mix, 1 μL of iScript Reverse Transcriptase, 30 ng of RNA and DNAse/RNAse-free water to make a total of 50 μL. The samples were placed in an Applied Biosystems Veriti Thermal Cycler with the parameters of 5 mins at 25°C, 30 mins at 42°C and 5 mins at 85°C for the primers to bind, the reverse transcriptase to transcribe RNA to cDNA and the inactivation of the reverse transcriptase enzyme. Samples were then held at 4°C.

2.16.3 Real-time quantitative PCR

Real-time quantitative PCR was performed in a 20 μL reaction volume 10 μL SYBR Green Master Mix, 3 μL primer, 2 μL sterile distilled water and 6 ng cDNA. Primers to 18s ribosomal RNA were used to normalise the amount of nuclear material. The 7500 Real Time PCR System with 7500 Software version 2.0.6 (Applied Biosystems, Life Technologies) was used for real-time PCR quantification. The parameters used were 1 cycle of 94°C for 15 mins to activate the DNA polymerase and denature the DNA; then 40 cycles of 94°C for 15 secs to separate the DNA strands, 55°C for 30 secs to allow the primers to anneal, and 72°C for 35 sec to allow extension of the DNA strands. The amount of DNA was measured sat the end of each cycle using SYBR green as the fluorescent detector.

2.16.4 Real-time quantitative PCR analysis

Microsoft Excel software was used to analyse the real-time quantitative PCR data. Cycle threshold (Ct) values were normalised by calculating the difference between the sample Ct value and the corresponding 18s CT value to give the ΔCT value. The difference in normalised ΔCT between the treated and untreated cell RNA was calculated, termed the ΔΔCT, and the fold change of the transcript determined using the equation:

$$\text{Fold change} = 2^{\Delta\Delta CT}$$

Table 2.5 Table of real-time quantitative PCR primers used throughout this thesis.
All primers were purchased from Qiagen

Gene	Name	Number	Reference sequence accession number
18s	18S ribosomal RNA	QT02448075	NR_003278
IL-1β	Interleukin 1 beta	QT01048355	NM_008361
CXCL2	Chemokine (C-X-C motif) ligand 3	QT00113253	NM_009140
CXCL5	Chemokine (C-X-C motif) ligand 5	QT01658146	NM_009141
CCL3	Chemokine (C-C motif) ligand 3	QT00248199	NM_013025
Arg1	Arginase	QT00134288	NM_007482
Relmα	Resistin like alpha	QT00254359	NM_020509
Ym1	Similar to Chitinase 3-like	QT02241722	XR_034276
CD206	Mannose receptor, C type 1	QT00103012	NM_008625
TNFα	Tumour Necrosis Factor	QT00104006	NM_013693
IL-6	Interleukin 6	QT00098875	NM_031168
iNOS	Inducible nitric oxide synthase	QT01547980	XM_001004823

2.17 IHC

Mice were tumour challenged intracranially and treated with α-CTLA-4, vaccination or a combination of both. On day 20 after challenge (13 days post therapy), mice were sacrificed by anaesthetic overdose and were perfused via the left ventricle with 10 mL ice cold PBS. Brains were harvested into 4% PFA (Sigma-Aldrich) for 72 hours before being transferred to 70% ethanol. Brains were processed into 10 μM sections using standard histological techniques by Jane Anderson of the Wellington School of Medicine.

2.17.1 Haemotoxylin and Eosin Staining

Was performed by Jane Anderson at the Wellington School of Medicine

2.17.2 CD3 staining

Slides were incubated at 60°C for 30 mins to melt wax and affix tissue to slides. Samples were then passaged through xylene and an ethanol rehydration gradient to remove

paraffin. Antigen retrieval was performed in citrate buffer for 10 mins at 90°C, with samples left for a further 30 mins to cool before being washed in PBS. Slides were permeabilised in 3% hydrogen peroxide buffer for 20 mins and washed in PBS, then PBST for 5 mins. Non-specific binding was prevented by incubation in serum (Vector Laboratories, USA) in a humidified chamber for 20 mins. Primary antibody (Abcam, New Zealand) was applied in PBS + 1% BSA and incubated for 1 hour at room temp before being washed three times in PBST. Secondary antibody (Vector Laboratories, USA) was applied for 10 mins at room temp. Samples were again washed in PBST for 5 mins before streptavidin-peroxidase complex (Vector Laboratories, USA) was added. Samples were incubated for 5 mins at room temp then washed in PBS. Two components of 3,3' Diaminobenzidine (DAB) substrate (Abcam, New Zealand) was mixed, applied to samples and left to develop for a minimum of 2 minutes. DAB was quenched with tap water for 3 mins before samples were counterstained with haematoxylin. Tissue was dehydrated by passage through an ethanol dehydration gradient before DPX glue (Scharlab, Sentmenat, Spain) and a coverslip were applied and left to dry.

2.18 Microscopy

Gross pathology photomicrographs were acquired using an Olympus SZX16 microscope with a 2 x objective lens equipped with an Olympus XC50 digital camera and Cell^{F} software.

IHC and microscopic photomicrographs were acquired using an Olympus BX51 microscope with a 40 x objective lens equipped with an Olympus DP70 digital camera and Cell^{F} software.

2.19 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.0 software (CA, USA). Between 3 or more groups, a one-way analysis of variance (ANOVA) with Bonferroni's post-test was used. When using two parameters with multiple groups, a two-way ANOVA with Bonferroni's post-test was used. P values of $P < 0.05$ were considered significant.

Chapter 3

3 Establishing an intracranial model of glioma and identifying a successful treatment regime

3.1 General Introduction

Historically, the CNS has been considered an immune privileged site owing to experiments conducted by Goldmann, showing compartmentalisation of the CNS from the circulatory system following trypan blue injection (330, 331). However, there is accumulating evidence that malignant tissue within the CNS can be targeted by T cells (332), giving impetus to develop vaccines for diseases like high-grade glioma for which current treatments are generally ineffective (5). However, the development of effective cancer vaccines faces numerous challenges, including a high level of tumour heterogeneity and a paucity of defined glioma-associated antigens (333). With surgical resection being a mainstay of glioma treatment, it is possible to use resected tumour material as a "personalized" source of antigens. Indeed intact irradiated tumour cells can act as a vehicle for the delivery of these antigens to APCs within the host. However, to best elicit an effective T cell response it is critical that these APCs also receive strong licensing signals. My colleagues have previously described a simple vaccine composed of irradiated autologous tumour cells pulsed with α-GalCer, an adjuvant that stimulates iNKT cells, which was shown to be an effective treatment against tumours in an orthotopic murine glioma model (29). The vaccine depends on the reciprocal activation of iNKT cells and APCs and elicits an adaptive immune response dominated by $CD4^+$ T cells that is tumour-specific and long lasting. Although evidence of cellular activation was observed in the spleen, the mediastinal lymph node (MdLN) was shown to be an important anatomical site of T-cell priming. Importantly, the principal effectors necessary for the

success of such a vaccine are present and functional in patients with high-grade glioma (29).

Significantly, it was found that the α-GalCer-adjuvanted vaccine could only prevent the development of tumours in a therapeutic setting if suppression mediated by Tregs was overcome (29). This is consistent with general observations in patients, where vaccine-mediated cancer treatment is generally insufficient to bestow long-term survival; most attribute this to a variety of different suppressive mechanisms driven by the tumour. In addition to tumour driven suppression, T cells are regulated by inhibitory signals from both APCs and peripheral tissues, which result in suppression of T cell responses. These signals are known as checkpoint molecules as they maintain self-tolerance and prevent the activation of autoreactive T cells. (136). Recent studies have shown that overriding these immune checkpoints with antibody blockade can result in sustained T cell responses, which induce clinical responses in patients (153, 162). While these treatments have validated the concept of enhancing T cell-mediated immune responses for clinical impact, it is clear that not all patients respond. There is therefore considerable interest in using vaccines to initiate anti-tumour responses (or boost weak ones), which could then potentially be enhanced through checkpoint blockade.

In order to develop treatments for glioma, it is important that relevant *in vivo* models are utilised. Tumours injected into mice subcutaneously form solid tumour masses with relative ease of injection and analysis, but fail to recapitulate many of the key features of tumours in the brain. Additionally, tissue specific cells and physiological barriers can affect both tumour growth, and the ability of a tumour to respond to therapies. Microglia, the brain-resident macrophages, are known to take on both pro- and anti-tumourigenic capacities depending on their activation status (334, 335). As these cells are present in the natural site of tumour development, it is likely that they will play a role in therapies aimed at activating the immune system (336, 337). Additionally, the BBB was thought to inhibit the trafficking of immune cells into the brain and CNS (104, 330, 331, 338). The recent discovery of lymphatic vessels within the dura mater and meninges of the brain has revealed a previously undocumented route of lymphatic drainage from the brain. Previously, antigen within the brain was observed to drain within the cerebrospinal fluid

(CSF), via the olfactory bulbs, along the cribriform palate and terminating in the superficial cervical lymph nodes. However, the identification of lymphatic vessels within the dura mater has revealed lymphatic drainage directly to the deep cervical lymph nodes, independent of the CSF (339, 340). It is now clear that there is a direct link between the immune system and the CNS, facilitating both a means for antigenic drainage as well as a conduit to permit lymphocyte entry to the brain.

In order to best test novel immunotherapeutic approaches, utilisation of an orthotopic model of glioma permits the best representation of the clinical manifestation of disease. The three main murine tumour models currently utilised for the examination of GBM involve the transplantation of murine glioma cells into syngeneic mice, transplantation of human glioma cells into immune-compromised mice and transgenic spontaneous models (341). Transplantable models are advantageous for testing therapeutic regimes as they are highly reproducible and have a predictable growth rate and are easily monitored as they are injected into a known location. Further, syngeneic models permit interactions between the immune system and the tumour which is prohibited in immune-compromised xenograft models. Additionally, transplantable models are best used for immunotherapy, as they are likely to have accumulated the neoantigens that are required for T cell recognition. Transgenic models have been developed whereby genetic mutations are generated, recapitulating those known to occur during tumour development (342-344). While recapitulating tumour development from the initial mutations through to full presentation of disease, spontaneous formation of tumours in these models can be unpredictable. The tumours which do form can range from low to high grade tumours and penetrance can be low, sometimes with only half of the mice developing tumours which resemble GBM (345). Additionally, the latency period taken for tumour development can take months, making these models more costly and time intensive to examine. While useful for studying the underlying determinants of disease and tumour progression, spontaneous models are less practical for the development and testing of novel treatment regimes.

Several syngeneic transgenic models exist which are either chemically induced, or were developed from spontaneously arising tumours. The GL261 cell line was created by the

injection of MCA pellets into the brains of immune-competent mice and maintained by serial passage of pieces of tumour tissue into the brains of recipient mice (346, 347). Another MCA induced model is the CT-2A brain tumour (348). CT-2A has been characterised and proposed as suitable for preclinical studies (349). Other transplant models utilised were derived from spontaneously derived glioma-like tumours. The 4C8 tumour arose in a transgenic mouse was transplanted into an F1 cross of C57BL/6 x DBA/2 mice and has been used for preclinical studies (350). Finally, the spontaneous murine astrocytoma (SMA) arose in the VM/Dk mouse strain (351). From here, the SMA-560 cell line was cloned from this tumour and has been used in preclinical studies (352, 353). Of these aforementioned models, GL261 is the most frequently used for preclinical studies and has been demonstrated to replicate human GBM both histopathologically and biologically (354, 355). To this end, intracranial injections of the murine cell line GL261 have been adopted for *in vivo* tumour challenge experiments in this thesis.

With the development of checkpoint blockade and other drugs aimed at targeting tumours to induce immunological cell death, it is imperative that these drugs are able to access the correct physiological site to exert their function. The BBB is a highly selective, permeable physical barrier, which serves to separate circulating blood from the brain extracellular fluid within the central nervous system (356). Formed by endothelial cells and maintained by astrocytes, the BBB permits the passage of water and lipid soluble molecules by passive diffusion, as well as the selective transport of molecules such as glucose and amino acids that are critical to neural function (357). In contrast to endothelia in the rest of the body, the endothelial cells of the BBB are devoid of fenestrations but have more extensive tight junctions to limit the paracellular flux of hydrophilic molecules. In addition to endothelial cells, the BBB is composed of the capillary basement membrane and pericytes with astrocytes ensheathing the vessels. Pericytes provide a key role in angiogenesis, structural integrity and differentiation of the barrier, as well as formation of the tight junctions (358). In serving its function, the BBB allows the passage of lipophilic substances such as oxygen and carbon dioxide (359) whereas nutrients including glucose and amino acids enter via transporters (357). Receptor mediated endocytosis is required for the uptake of larger molecules such as leptin and iron

transferrin (360). In maintaining its integrity, this highly selective barrier can also prevent the entry of antibodies as well as chemotherapeutics aimed at minimising tumour growth (361).

Antibodies to CTLA-4 and PD-1 were recently FDA approved (153, 162, 361) and are currently used for the treatment of metastatic melanoma. In addition to utility for melanoma, both human α-CTLA-4 (ipilimumab) and α-PD-1 (nivolumab and pembrolizumab) are being investigated for efficacy in other malignancies. Briefly, phase II trials have been conducted with ipilimumab for prostate and lung cancer (362, 363) in addition to trials underway in NSCLC, small-cell lung cancer (SCLC) (364), bladder cancer (365) and hormone-refractory prostate cancer (366). Importantly, ipilimumab, in combination with nivolumab is being investigated in GBM patients (283). As ipilimumab acts by unleashing immune responses, it is likely that patients who respond best will have cancers with high mutational loads (367).

As nivolumab acts via blockade of PD-1, antibody binding to T cells reverses the "exhausted" phenotype exhibited by intratumoural T cells. Again, while approved for the treatment of metastatic melanoma, nivolumab is also being investigated for cancers including lung cancer and Hodgkin's lymphoma. Similar to CTLA-4 blockade, PD-1 blockade is likely to be most efficacious in tumours with a high mutation load. As the ligand for PD-1, PD-L1, can be expressed on tumour tissue, tumour biopsies can be taken for histological identification of patients who are likely to respond to therapy (368). Nivolumab is currently in phase III clinical investigation, in combination with ipilimumab, for GBM (283). Also, pembrolizumab, a second FDA approved PD-1 monoclonal antibody is under phase II investigation for GBM both as a monotherapy, and in combination with bevacizumab, an antibody against VEGF-A (284). Antibodies to the ligand for PD-1, PD-L1, are in advanced clinical development and are also being explored for use in GBM (285).

In addition to these molecules, many more are under preclinical development. LAG-3 is a potential checkpoint, which could also be targeted for antibody mediated blockade (166, 167, 176). LAG-3 was recently discovered and is a $CD4^+$ T cell homologue, which binds

to MHC-II (176). While CTLA-4 and PD-1 are primarily limited to expression on T cells, LAG-3 has a broader expression profile, with expression detected on T cells, B cells, NK cells and DCs (172-175). Thus far, LAG-3 blockade has been examined in self- and tumour-tolerance systems *in vivo*, with enhanced accumulation of $CD8^+$ effector T cells (171). In this study, antibody mediated blockade of LAG-3, or genetic ablation of the *Lag-3* gene resulted in increased accumulation and effector function of $CD8^+$ T cells within tissues expressing the cognate antigen (171). In addition, the effects observed were $CD4^+$ T cell independent, which is intriguing as LAG-3 is a CD4 homologue (171). In another study, combination therapy of α-CTLA-4, α-PD-L1, α-GITR and α-LAG-3 was found to be ineffective as a therapy in an intracranial model of melanoma. However, the B16-F10 model is notoriously aggressive and this may have contributed towards the failure of therapy in this study (369).

Even if checkpoint blockade proves useful in glioma, it is likely that it will fail in many patients. Similarly vaccination alone is not likely to be efficacious, despite evidence that T cell responses can be generated. It is therefore possible that vaccination and checkpoint blockade will be useful to induce anti-tumour immune responses that can be further augmented by blocking negative regulation of T cell inhibitory pathways. Successful therapy assumes T cells can enter the brain and infiltrate the tumour, and may also require the checkpoint inhibitor to can also cross the blood brain barrier.

3.2 Aims

The experiments in this chapter were designed to evaluate the efficacy of vaccination combined with different checkpoint blockade antibodies in an intracranial glioma challenge model.

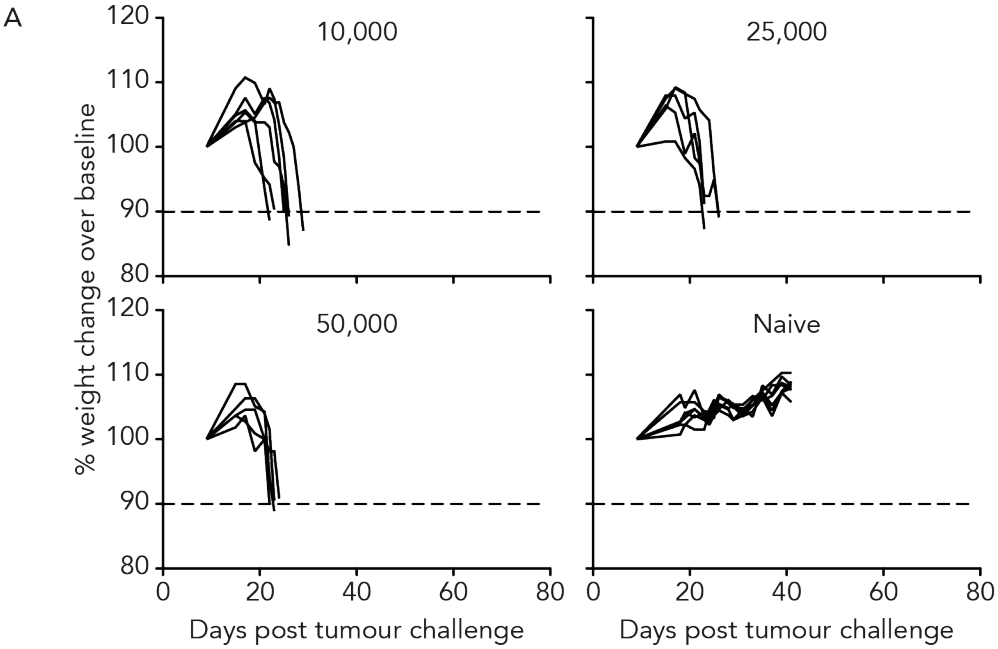
The specific aims were:

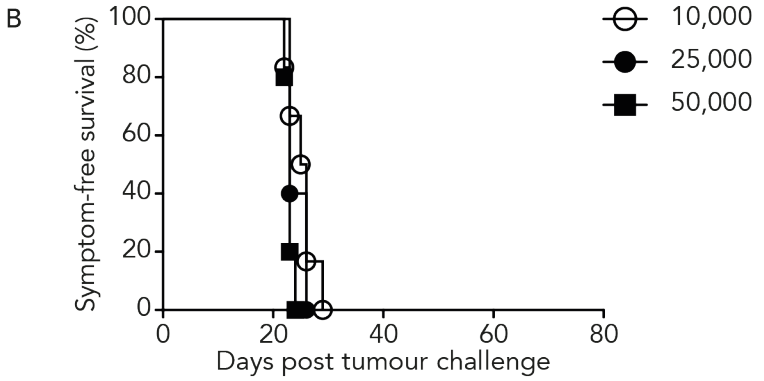
1. To develop a reproducible orthotopic GL261 challenge model
2. To evaluate the anti-tumour efficacy of combining the vaccine with checkpoint antibodies in a therapeutic setting

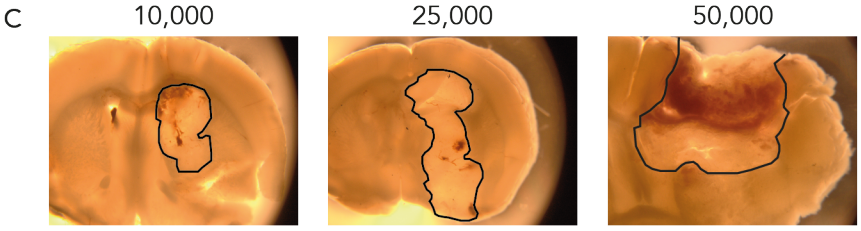
3.3 Results

3.3.1 Cryopreserved tumour cells implanted intracranially into immune-competent mice are lethal within 4 weeks

The GL261 tumour model used in this thesis were received from the National Cancer Institute (Frederick, MD) and subsequently authenticated genotypically (IDEXX 13512-2014). However, there have been previous reports of rapid phenotypic changes with passage with this model (370), which has been observed by colleagues in my laboratory. Therefore, in order to conduct reproducible glioma growth experiments for this thesis, it was decided to bulk up a large number of cells and cryopreserve them for use in multiple experiments. Initial experiments were conducted to determine the dose of frozen tumour cells to be delivered in an intracranial setting. Previous reports used 5,000 live cells, harvested during exponential growth, with untreated mice typically succumbing to tumour burden 3 weeks post challenge (29). Using this as a reference, tumours were thawed from storage in liquid nitrogen and resuspended in PBS at titrated doses. Cells were administered under general anaesthesia and mice were left to recover before being monitored for signs of weight loss or neurological deficit. When weight loss exceeded 10%, or mice exhibited signs of discomfort including decreased activity, hunched posture, ruffled fur or eye closure, mice were culled and presence of tumour confirmed macroscopically (Fig 3.1 A, B). To examine the gross tumour pathology, one mouse from each group was excluded from survival analysis on day 18 and the brain was harvested and fixed in 4% paraformaldehyde (PFA). Coronal sections were taken through the centre of the tumour mass for inspection. Challenge doses of 10,000 and 25,000 cells resulted in tumours that were still contained within the brain, however, an initial challenge dose of 50,000 cells the tumour mass breached the surface of the brain (Fig 3.1 C). Based on this experiment, a dose of 25,000 frozen cells typically lead to mice succumbing to tumour burden 3 weeks post challenge, and this dose was used in all future experiments as it allowed maximal vials of cells to be frozen, without resorting to the lowest dose. To assess the histopathological features associated with tumour growth, a brain was harvested from a tumour-bearing mouse on day 20 and fixed in PFA before being H&E stained. Histopathology revealed a dense tumour mass with a high mitotic rate and infiltration into local brain tissue (Fig 3.1 D).


A
10,000
25,000
50,000
Naive
% weight change over baseline
120
110
100
90
80
0
20
40
60
80
Days post tumour challenge
Days post tumour challenge


B
Symptom-free survival (%)
100
80
60
40
20
0
0
20
40
60
80
Days post tumour challenge
10,000
25,000
50,000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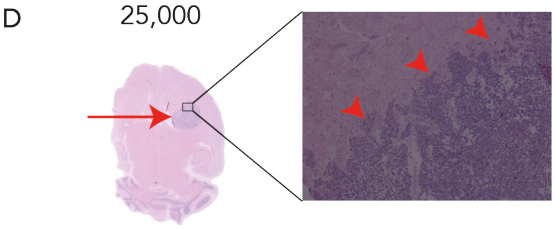
D
25,000

Figure 3.1 Intracranial implantation of cryopreserved tumour cells reproducibly results in tumour engraftment and growth.
(A) Individual percentage weight loss for mice with titrated doses of intracranial tumours. Mice were injected with indicated doses GL261 glioma cells from a cryopreserved source that were thawed immediately before injection. Cells were injected into the right striatum at a point 2 mm lateral to the bregma and at a depth of 3 mm using a stereotactic frame. Mice were monitored for weight loss as a symptom of tumour development. (B) Symptom-free survival curves for mice with titrated doses of intracranial tumours. Mice were euthanised when they lost weight loss reached >10% or showed signs of overt behavioural changes such as hunching or reduced activity (n = 5-6). (C) Mice were challenged with titrated tumour doses and were sacrificed on D18 for gross-pathological assessment. Tumour development was confirmed by visual inspection of coronal sections through the centre of the tumour mass. Tumour border is outlined in black. (D) Haematoxylin and eosin analysis of a formalin-fixed, paraffin-embedded tumour bearing brain 20 days following challenge with 25,000 cells. Tumour mass is indicated by an arrow. Arrowheads on the right image indicate the tumour infiltrative border.

3.3.2 Prophylactic administration of a cryopreserved vaccine prevents tumour growth

By using a tumour cell line, it is possible to generate vaccines on an as-required basis. However, given the same considerations as above, a frozen vaccine approach was devised. To generate the vaccine, GL261 cells were pulsed with α-GalCer overnight, before being irradiated. Cells were then frozen in 10% DMSO + 90% cDMEM until use. To test the efficacy of this frozen vaccine approach, mice were administered a vaccine which was cryopreserved, or the equivalent dose of a freshly made, but not frozen vaccine, in a prophylactic setting. One week later, mice were challenged intracranially and monitored for weight loss and neurological deficit. Both the fresh and frozen vaccines protected mice from intracranial challenge (Fig 3.2). From here on, the term vaccine will be used to describe the frozen vaccine unless otherwise stated.

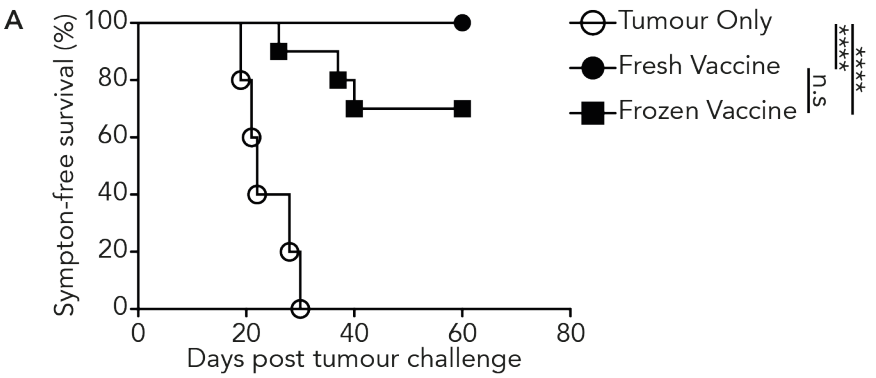

Figure 3.2 Prophylactic vaccination with cryopreserved vaccines impairs tumour development in an intracranial model of glioma.
Symptom-free survival curves for mice challenged with intracranial glioma one week following intravenous administration of vaccines consisting of 100,000 α-GalCer-pulsed and irradiated GL261 cells (n = 10). Vaccines were either prepared fresh, or had been cryopreserved and thawed immediately before use. Controls were challenged with tumour cells only (n = 5) **** $P < 0.001$ (Log-Rank (Mantel Cox) test). Results represent two combined experiments.

3.3.3 Therapeutic administration of cryopreserved vaccine does not prevent tumour growth

Having determined the α-GalCer pulsed, irradiated vaccine prevent mice succumbing to tumour burden, experiments were performed in a therapeutic setting to examine if vaccine administration was sufficient to induce rejection of intracranial glioma. In this setting, mice were challenged intracranially before receiving either the vaccine on day 7, or remaining as tumour only controls. Mice were monitored for weight loss and signs of neurological deficit. While vaccination provided a modest but statistically significant survival advantage, all mice eventually yielded to tumour burden and had to be sacrificed (Fig 3.3). The ability of the vaccine to prevent tumour growth in the prophylactic setting, yet prove relatively ineffective at controlling tumour growth in a therapeutic setting suggests that an immune response can be induced, but in the presence of a tumour, this immune response is suppressed. This is in keeping with an earlier report on this type of vaccine where suppression was overcome to a large extent by antibody-mediated depletion of Tregs, which improved vaccine responses significantly, with 40-60% of mice surviving past day 60 (29).

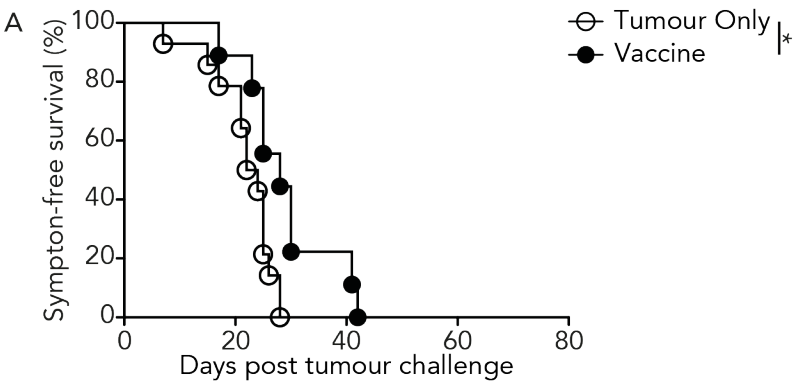

Figure 3.3 Therapeutic vaccination is ineffective at limiting intracranial tumour growth.
Symptom-free survival curves for mice (n = 9-14) with intracranial tumours treated with vaccine only on day 7 * $P < 0.05$ (Log-Rank (Mantel Cox) test). Results represent two combined experiments.

3.3.4 PD-1 blockade combined with therapeutic vaccination is an ineffective therapy for orthotopic glioma

It is now known that tumours subvert the immune system via manipulation of immune checkpoint molecules. For example, IFN-γ released from tumour infiltrating lymphocytes is known to induce the ligands for PD-1 in the tumour environment, preventing tumour rejection (158, 161). To test whether vaccination can be combined effectively with inhibiting PD-1, experiments were first conducted on established tumours that were implanted subcutaneously, thereby avoiding any confounding effects associated with accessing the brain. Thus, mice were challenged subcutaneously and allowed to form tumours before being treated with vaccine on day 7. The α-PD-1 antibodies were administered on days 10, 13 and 16 after challenge (representing days 3, 6 and 9 following vaccination). This dosing regime was adopted based on typical schedules from the literature (371, 372). In this setting, vaccination failed to eliminate established tumours. Interestingly, administration of α-PD-1 as a monotherapy on days 10, 13 and 16 was a very effective treatment for established subcutaneous tumours, and this could not be improved by combining with the vaccine (Fig 3.4 A). To determine if the therapeutic vaccination and α-PD-1 strategy could be applied to the orthotopic setting, mice were challenged intracranially before being treated with either vaccine immunotherapy alone one week later, α-PD-1 antibodies alone, or a combination of both as described for the

subcutaneous setting. Blockade of PD-1 both as a monotherapy and in combination with vaccination had no discernible effect on overall survival (Fig 3.4 B).

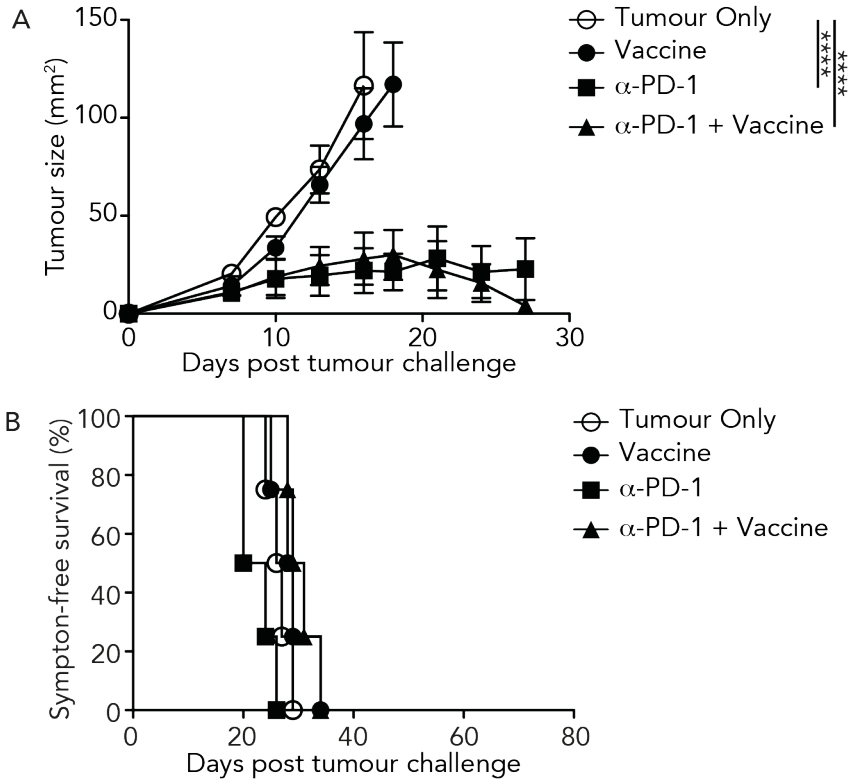

Figure 3.4 PD-1 blockade combined with vaccination is an ineffective therapy for intracranial glioma.

(A) Mean tumour size (± SEM) in groups of mice (n = 5) subcutaneously challenged with 10^6 GL261 cells and treated with vaccine on day 7, α-PD-1 on days 10, 13 and 16, or a combination of both **** $P < 0.001$ (Two-way ANOVA with Bonferroni post-test). Results are representative of three independent experiments. (B) Symptom-free survival curves for mice (n = 4) with intracranial tumours treated with vaccine alone on day 7, α-PD-1 on days 10, 13 and 16, or a combination of both. Results are representative of three independent experiments.

3.3.5 *LAG-3 blockade combined with therapeutic vaccination is an ineffective therapy for subcutaneous and orthotopic glioma*

Blocking antibodies to LAG-3 are currently in clinical trials for haematological malignancies (373) and combination therapy with PD-1 and LAG-3 blockade has shown preclinical efficacy (374-376) and are in clinical trials for solid tumours (190). Given this level of clinical development, checkpoint blockade with anti-murine LAG3 antibodies (α-LAG-3) was evaluated here. Initial experiments were performed on mice challenged subcutaneously. Therapy started on day 7 and consisted of vaccination, α-LAG-3 antibody administration, or combination therapy. For antibody treatment, mice received a 0.5 mg bolus dose of α-LAG-3 on day 7 after challenge, with 3 doses of 0.25 mg each administered every three days thereafter. In contrast to PD-1 blockade, no effect on subcutaneous tumour growth was observed (Fig 3.5 A). To test for efficacy in the orthotopic setting, mice were challenged intracranially and received the same treatment schedule as for subcutaneous tumours. Similar to PD-1 blockade, no survival advantage was observed when LAG-3 blockade was combined with therapeutic vaccination (Fig 3.5 B).

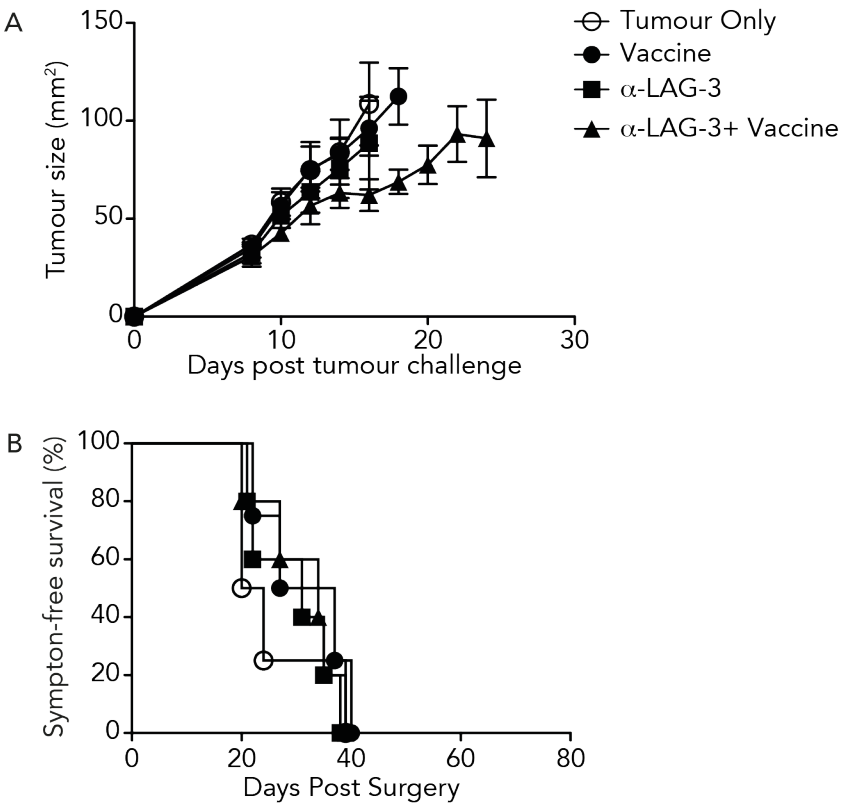

Figure 3.5 LAG-3 blockade combined with vaccination is an ineffective therapy for intracranial glioma.

(A) Mean tumour size (± SEM) in groups of mice (n = 5) subcutaneously challenged with 10^6 GL261 cells and treated with vaccine on day 7, α-LAG-3 on days 6, 9, 12 and 15, or a combination of both. Results are representative of two independent experiments. (B) Symptom-free survival curves for mice (n = 4-5) with intracranial tumours treated with vaccine alone on day 7, α-LAG-3 on days 6, 9, 12 and 15 or a combination of both. Results are representative of two independent experiments.

3.3.6 *CTLA-4 blockade combined with therapeutic vaccination confers long-term survival in mice with subcutaneous and orthotopic glioma*

In addition to PD-1, antibodies targeting the CTLA-4 pathway have received recent FDA approval. Again, initial experiments were performed in the subcutaneous setting. Mice were challenged subcutaneously and received a single dose of α-CTLA-4 administered by intraperitoneal injection one day prior to vaccination (day 6 after tumour challenge), or three days after vaccination (day 10 following challenge). This dosing schedule was based on a report showing increased accumulation and activation of adoptively transferred antigen specific T cells in the lymph nodes of mice immunised with peptide loaded DCs (377). Each of these treatment schedules induced complete tumour regression after an initial period of tumour growth (Figure 3.6 A), although reduced tumour growth, both in the overall size of the tumour, as well as the growth kinetic, was observed to a greater extent when α-CTLA-4 was delivered prior to vaccination. To examine how CTLA-4 blockade affects survival in the orthotopic setting, mice were challenged intracranially and treated with α-CTLA-4 antibody administration on day 6, vaccine immunotherapy on day 7, or received combination treatment. While no monotherapy was effective as a sole agent, the combination of CTLA-4 blockade and vaccine immunotherapy conferred a long-term survival advantage, with 80% of mice surviving past day 60 (Fig 3.6 B). Importantly, antibody administration was most effective when administered close to vaccination as delaying administration until 3 or 7 days post vaccination (day 10 or day 14 following challenge) significantly impaired survival, suggesting CTLA-4 blockade was most relevant when delivered close to immune priming (Fig 3.6 C).

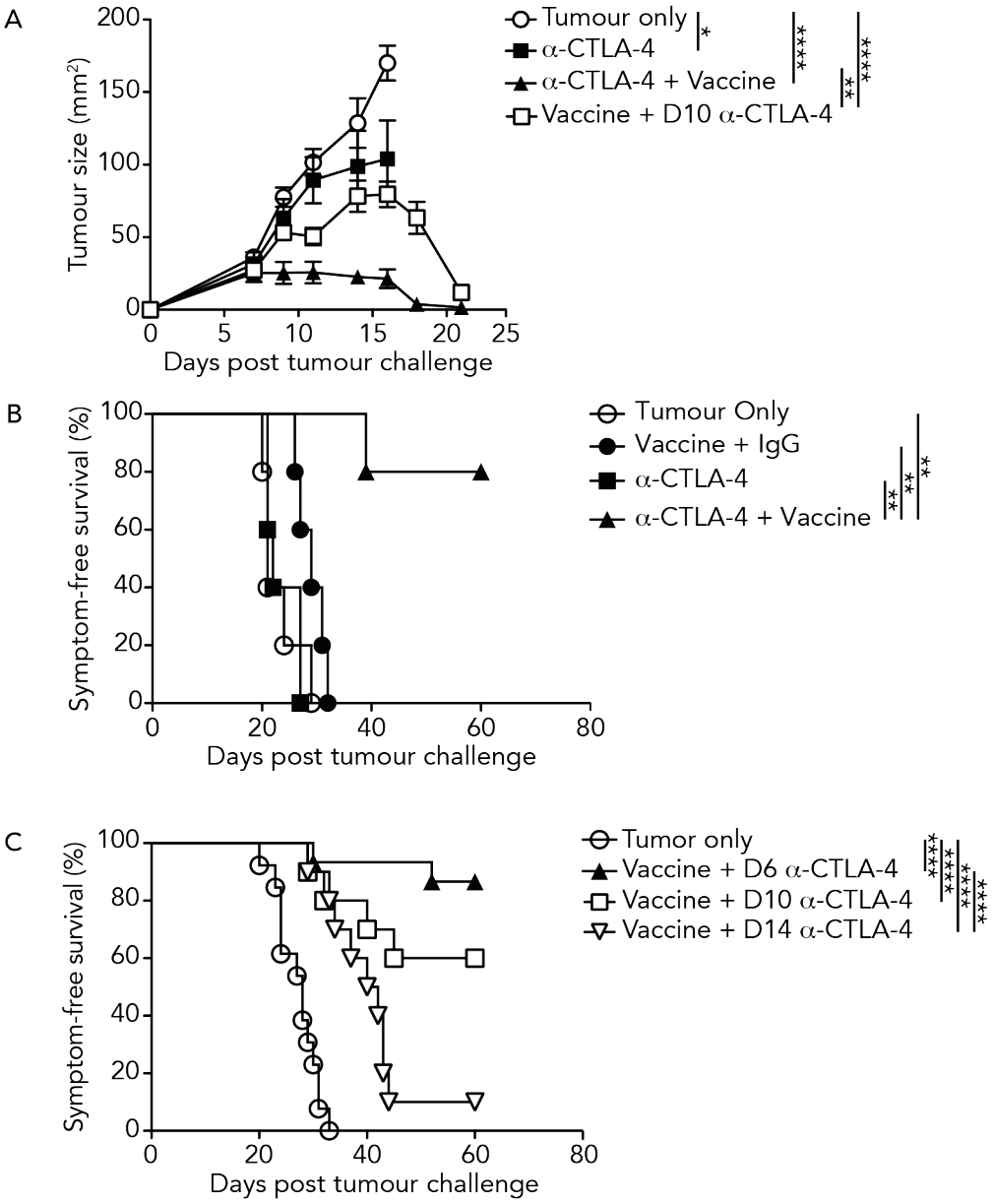

Figure 3.6 CTLA-4 blockade combined with vaccination is an effective therapy for intracranial glioma.

(A) Mean tumour size (± SEM) in groups of mice (n = 5) subcutaneously challenged with 10^6 GL261 cells and treated with vaccine on day 7, α-CTLA-4 on days 6 or 10, or received α-CTLA-4 on day 6 and vaccine on day 7 * $P < 0.05$, ** $P < 0.01$, **** $P < 0.001$ (Two-way ANOVA with Bonferroni post-test). Results are representative of three independent experiments. (B) Symptom-free survival curves for mice with intracranial tumours treated with either vaccine alone on day 7, α-CTLA-4 alone on day 6, or both ** $P <$ 0.01 (Log-Rank (Mantel Cox) test. Results are representative of three independent experiments. (C) Survival curves for mice (n = 10) with intracranial tumours treated with vaccine on day 7 together with α-CTLA-4 on either day 6, day 10 or day 14 **** $P < 0.0001$. Results represent combined data from two experiments.

3.3.7 *Blocking CTLA-4 signalling before vaccination can induce radiological regression*

To determine how CTLA-4 blockade and immunotherapeutic vaccination affects tumour progression, radiological analyses were performed. Mice were intracranially challenged and treated with α-CTLA-4 on day 6, vaccination on day 7, or combined therapy. Initial analysis by MRI on day 20-post implantation revealed gadolinium enhanced regions in all mice regardless of treatment (Fig 3.7 A). In agreement with previous experiments, all untreated mice, and those receiving monotherapy developed glioma-associated symptoms and were culled within days of this analysis. In contrast, four of the five mice receiving combination treatment remained symptom free and could be re-imaged on day 41. Of these, two now showed complete loss of enhanced regions, another was markedly decreased in size while the last increased in size only slightly (Fig 3.7 B).

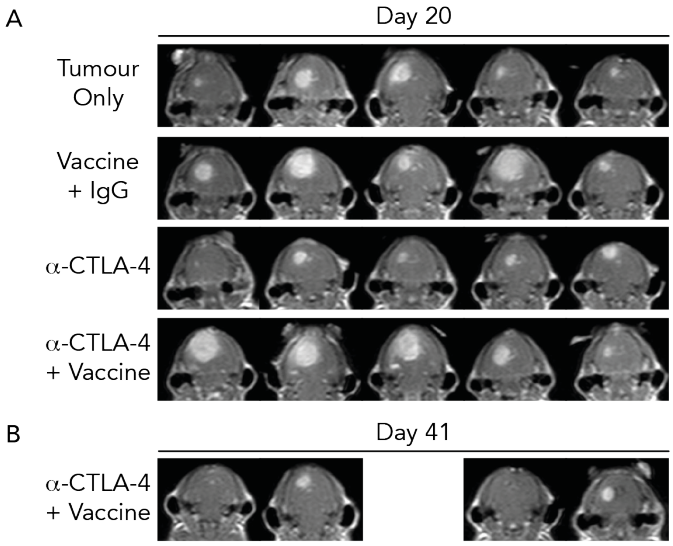

Figure 3.7 Combining vaccination with α-CTLA-4 induces radiologic regression of established intracranial tumours.

(A) Mice with intracranial tumours (n = 5) were treated with either vaccine alone on day 7, α-CTLA-4 on day 6, or received combination treatment. T1 weighted MR images of brains of mice were taken under general anaesthesia. Gadolinium was administered prior to imaging to enhance contrast and enhanced regions are revealed in white (B) Surviving mice underwent further MRI scans to assess tumour progression. Results are representative of one experiment.

3.3.8 Histolopathological analysis of tumours following immunotherapy

MRI analysis on day 20 revealed large enhanced regions within the brains of mice that received vaccination, both in the presence and absence of CTLA-4 blockade. To determine if the enhanced regions observed correlated with increased tumour burden at this time point, a separate experiment was performed where brains were harvested for histopathological analysis, 20 days following challenge (Fig 3.8 A). Here, mice receiving combination treatment had a significantly reduced tumour size compared to untreated animals (Fig 3.8 B). Mice treated with vaccine or α-CTLA-4 alone had hypercellular tumours with a high mitotic rate, whereas mice treated with both vaccination and α-CTLA-4 had relatively hypocellular tumours with low mitotic activity (Fig 3.8 C). Therefore, tumours had engrafted in all groups, but the combination treatment already showed some anti-tumour effect by day 20. The discrepancy in the combination treatment group between tumours appearing larger on MRI than the corresponding histology may be analogous to the phenomenon of pseudo-progression observed in human patients undergoing treatment for glioma as well as patients receiving checkpoint blockade therapy (378).

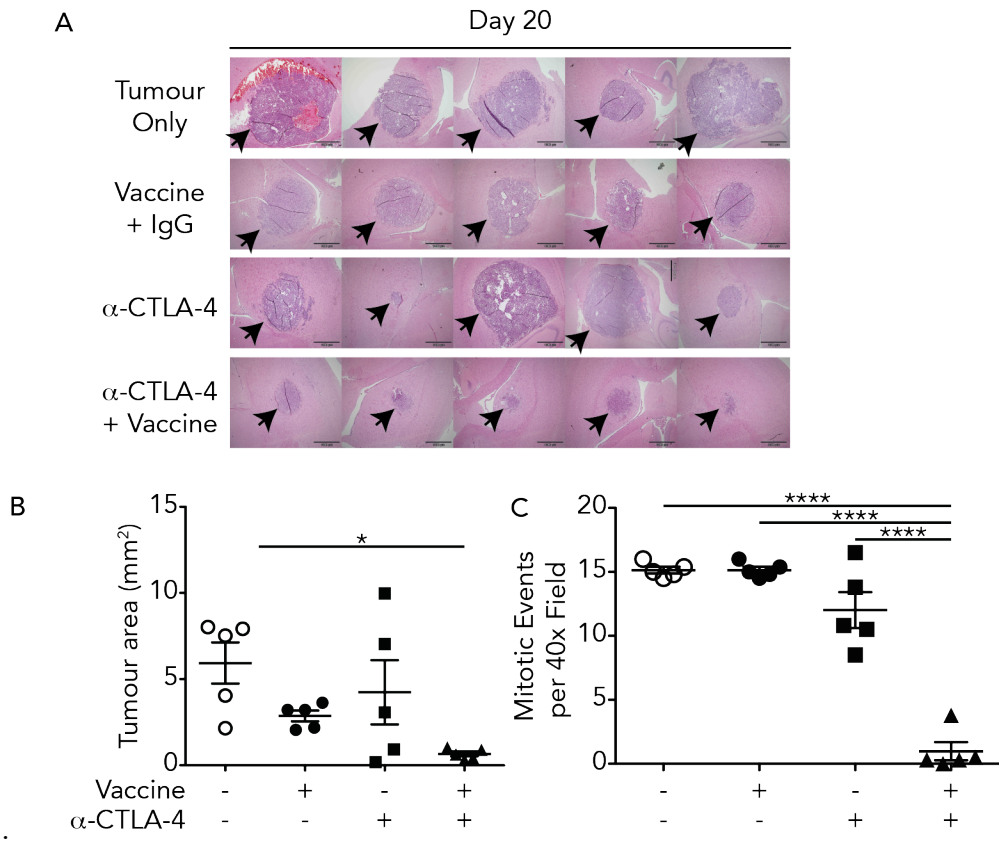

Figure 3.8 Histopathological characteristics of intracranial GL261 tumours following α-CTLA-4 and vaccine immunotherapy.

Mice with intracranial tumours (n = 5) were treated with α-CTLA-4 on day 6, vaccine on day 7, or both. Brains were harvested on day 20 following tumour challenge for histological analysis. (A) Photomicrographs of formalin-fixed paraffin-embedded coronal brain sections. Arrowheads indicate tumours. (B) Mean tumour area ± SEM (n = 5) was calculated per treatment group by transcribing the tumour perimeter for analysis by Cell^F software * $P < 0.05$ (One-way ANOVA with Bonferroni post-test). (C) Mean number of mitotic events per high power field ± SEM (n = 5) was calculated per treatment group by a histopathologist blinded to sample groups **** $P < 0.0001$ (One-way ANOVA with Bonferroni post-test). Results are representative of one experiment.

3.4 Discussion

In order to explore new therapeutic strategies for cancer, appropriate models must first be developed that allow the consistent growth of tumours while also properly recapitulating the natural site of development. Adoption of the GL261 cell line as an orthotopic model permits each of these parameters to be adhered to. Furthermore, the use of a cell line has additional advantages over spontaneous models, where mice have pre-disposing mutations (343), or inducible models where animals with pre-disposing mutations will characteristically develop a specific tumour after exposure to a given carcinogen, or cancer-causing agent (379, 380). Both spontaneous and inducible models typically rely on mutations in key tumour suppressor proteins (or are driven by transgenic oncogenes), resulting in tumours which develop much faster than a naturally occurring tumour would. Owing to this increased growth rate, tumour cells already have the required proliferative capacity required to outgrow the immune system. This rapid growth also limits the accumulation of mutations that can give rise to the neo-antigens typically targeted by the immune system. Transplantable models like the GL261 cell line used here are likely to have accumulated several neoantigens over the course of *in vitro* development. Indeed several transplantable tumours that have now been sequenced, including B16 melanoma, 4T1 breast cancer, and CT26 colon cancer, with each showing existence of neoantigens that could be targeted by T cells (381).

One of the goals of this chapter was to develop an intracranial tumour model that recapitulated much of the tumour biology seen in patients (382), but within a regular and reproducible timeframe. This was achieved by using a defined dose of frozen GL261 cells that were immediately thawed before injection. In contrast, spontaneous or inducible tumour models in the intracranial setting generally require a longer timeframes (several weeks to months), making confirmation of tumour development more difficult, and often requiring a luciferase reporter system or small animal MRI imaging systems for detection. Here, untreated GL261 tumours developed within 3 to 4 weeks, and became apparent as >10 % weight-loss, or overt behavioural including decreased activity, hunched posture, ruffled fur or eye closure. The presence of tumours was confirmed by histologic examination, and could easily be observed by visual inspection at necropsy.

Checkpoint blockade with monoclonal antibodies has ushered a new era in cancer therapy. Targeting the immune system and not the tumour directly has opened new avenues for therapeutic regimes, as well as new defined end points for treatment. While so far only approved for melanoma, both α-CTLA-4 and α-PD-1 are showing promise in other malignancies including NSCLC and SCLC (364), bladder cancer (365), metastatic hormone-refractory prostate cancer (366). Thus far, the only cerebral malignancies tested have been melanoma lesions which have metastasised to the brain. In general, therapy was well tolerated and some activity of ipilimumab was observed when melanoma metastases were small and asymptomatic (383-385). Given the dire prognosis for patients diagnosed with high grade glioma such as GBM, and the evidence (albeit limited) that immune therapy can alter the course of disease (386), it is promising that clinical trials are now underway to evaluate checkpoint blockade immunotherapy in combination with currently approved interventions (284, 285, 366).

However, while checkpoint blockade is showing remarkable success in metastatic melanoma compared to other treatments, overall response rates still remain low. As these antibodies are designed to amplify *de novo* immune responses that have developed in patients before treatment, the lack of responders may be a consequence of ineffective induction of T cell responses in the first place. There is therefore considerable interest in combining checkpoint blockade antibodies with vaccines to either initiate immune responses, or to boost weak responses. While a gp100 peptide vaccine was included in the clinical trial preceding FDA approval of ipilimumab, no increased efficacy was observed when the two therapies were combined. This highlights the necessity of integrating the most appropriate antigens into a vaccine strategy. The gp100 vaccine is based on generating responses to a commonly shared tumour-associated antigen. However, studies of patients who have responded to checkpoint blockade suggest that such shared antigens are not the predominant targets of T cell activity, and it is likely that patient-specific neoantigens are more relevant targets (316). Therefore, the field is currently focused on sequencing tumour samples to identify neoepitopes. As these neoepitopes are unique to a tumour specimen, they provide a patient-specific source of antigens, which must be considered for a vaccine strategy. Generation of patient-specific neoepitope vaccines is

being investigated using an RNA based vaccination approach (387, 388), and it is conceivable that a peptide-based approaches could be used in the future.

Conversely, whole tumour cells acquired from a patient can be used, circumventing the time intensive DNA sequencing and epitope binding predictions required for the aforementioned approaches. While there is some concern a tumour sample is not a homologous sample of cells but rather a heterologous sample, containing immune cells and tumour associated stroma in addition to a heterogeneous population of tumour cells, whole tumour cell vaccines have been combined with BCG in a phase III trials in stage II/III colon cancer patients (389, 390). In these studies, delayed type hypersensitivity (DTH) was used as a read out of tumour specific immunity and positive responses (>5mm) were observed in 97% of the 254 patients enrolled. In an intent to treat analysis, irradiated autologous tumour cell vaccination combined with BCG reduced the rate of disease recurrence by 44% but overall survival was not significantly affected (390). Additionally, irradiated autologous whole cell vaccines combined with BCG have been investigated in melanoma patients with stage III/IV disease (391). In patients with evidence of disease during the vaccination period, no clinical responses occurred. However, in patients with no detectable disease, the 5-year overall survival rate was superior compared to historical controls (391). Interestingly, the size of the DTH-reaction was observed to wane with repeated non-BCG-containing vaccines, suggesting booster vaccines require adjuvants for effective immune induction (391).

The vaccine generation protocol described in this chapter allows the rapid production of vaccine from the syngeneic cell line used to challenge mice. However, owing to its ease, it is possible that a similar approach could be adopted for the generation of a patient specific autologous, irradiated, whole cell vaccine from resected tumour material in a short timeframe, requiring only 24 hours of time in a good manufacturing practice (GMP) facility. Additionally, by cryopreserving the cells, the vaccine can remain frozen until required for administration. Current therapy for high grade glioma typically relies on traditional interventions consisting of surgical resection, followed by chemotherapy and radiotherapy to eliminate as much of the residual tumour as possible. Following this, vaccine immunotherapy could be applied to clear any residual cells which are either radio-

resistant or chemo-resistant. Of course, there is no guarantee that the sample obtained during resection will harbour the same antigens as those remaining following chemotherapy and radiotherapy however this is a caveat which exists for all forms of vaccine immunotherapy. For this reason, implementation of multivalent vaccine approaches will be an effective means to limit antigenic loss following therapy.

However, vaccination alone is unlikely to provide the therapeutic efficacy needed to eliminate significant residual tumour burden. In addition, while checkpoint blockade alone has resulted in remarkable success in rejuvenating the field of immunotherapy, overall response rates as a monotherapy are still modest. The meek response rates could be a result of inefficient or absent immune priming of the *de novo* immune responses that could otherwise be unleashed by checkpoint blockade. To this end, combination therapy, consisting of a vaccine strategy combined with checkpoint blockade represents an attractive means to achieve larger response rates, with the potential for application to a variety of different cancers. Here, data is presented showing that therapeutic vaccination can act to induce an immune response, which can be further improved by administration of α-CTLA-4 antibodies immune priming. Conversely, neither α-PD-1 nor α-LAG-3 were able to synergise with vaccination and increase survival in an intracranial challenge setting.

Survival benefit was not observed when α-CTLA-4 or α-PD-1 were used as monotherapies against established tumours in the intracranial setting. In agreement, a previous report showed that α-CTLA-4 as a monotherapy could not provide protection against intracranial GL261 tumours once established, but could induce limited protection when multiple treatments were started only three days after implantation. Effective treatment of established tumours could be achieved when α-CTLA-4 was combined with a vaccine comprised of GM-CSF-transfected glioma cells (392). Conversely, another study showed that α-CTLA alone could be used to treat SMA-560 glioma in VM/Dk mice (393). The authors attributed the activity of α-CTLA-4 in this model to enhancement of T cell-proliferative capacity, which was otherwise severely reduced in the presence of this tumour. While these reports adopted two distinct models, a common

feature of these studies is that α-CTLA-4 appears to mediate its effects on glioma through augmentation of immune cells in the secondary lymphoid organs. In contrast, α-PD-1 is generally thought to mediate its anti-tumour effect through preventing inhibition of T cells within the tumour (160, 394, 395). It is therefore likely that efficacy could be improved by enhancing access of blocking antibodies or other PD-1 inhibitors to effector cells in the brain, although strategies to improve penetrance of drugs and antibodies still remain in the early stages of development (396).

This is a highly relevant issue, as the clinical failure of bevacizumab, an antibody to VEGF-A, in glioma patients may also have been due to minimal antibody penetrance (280, 397, 398). It may be possible that the timing of antibody delivery with radiotherapy could improve access to brain tumour tissue. One study has demonstrated successful treatment of murine glioma when administration of α-PD-1 was delivered with stereotactic radiation (371). In agreement with this concept, increased uptake of gadolinium DPTA has been observed by MRI in glioma patients following radiotherapy, indicating increased permeability of the blood brain barrier (399).

For combination therapy to be effective, it is paramount that checkpoint blockade antibodies are able to bind their respective targets. CTLA-4 is expressed on T cells within secondary lymphoid organs following immune priming, thus systemic antibody administration has relatively unhindered access to bind. Conversely, while α-PD-1 and α-LAG-3 monoclonal antibodies are showing clinical and pre-clinical efficacy respectively, they were unable to promote a survival advantage in the intracranial model. As both membrane bound and soluble forms of LAG-3 exist, it is possible that administered antibodies bind soluble LAG-3, impeding APC activation (400), rather than enhancing T cell activation (400). If this is the case, a combination of soluble LAG-3 could be combined with CTLA-4 blockade, theoretically enhancing both APC activation and T cell effector function. Whether this has merit is yet to be investigated. Additionally, this could be due to both the dosing regime i.e, not enough antibody to drive tumour regression, or limited antibody penetrance due to physical barriers, such as the blood brain barrier. In the absence of further improvements to penetrating the blood brain barrier, inhibitors of

immune checkpoints that regulate immune priming in peripheral lymphoid organs, such as CTLA-4 and VISTA, will likely be of more therapeutic benefit in glioma than inhibitors that target immune checkpoints on intratumoural effector T cells such as PD-1 or TIM-3, unless these are combined with blood brain barrier disrupting therapies (191). Due to the failure of α-PD-1 and α-LAG-3 antibodies to exert efficacy when combined with vaccination, the combination of α-CTLA-4 with vaccination was taken further for characterisation.

3.5 Conclusions

An orthotopic GL261 model was developed as a reproducible model for intracranial glioma. Using this model, it was shown that an α-GalCer loaded, irradiated, whole cell vaccine was unable to prevent the growth of intracranially implanted GL261 unless combined with blocking antibodies to the immune checkpoint molecule CTLA-4. Blockade of CTLA-4 was ineffective as a monotherapy, suggesting that the combined activities of the two immunotherapies were required for tumour eradication. In contrast, blockade of the immune checkpoint molecules PD-1 or LAG-3 was ineffective, alone or in combination with vaccination. Efficacy with PD-1 blockade was observed in a subcutaneous model, both as a monotherapy and in combination with vaccination, suggesting antibody penetrance into the tumour site may be impaired by the blood brain barrier.

Chapter 4

4 Characterising the immune response induced by vaccination and CTLA-4 blockade

4.1 General Introduction

Cells of the innate immune system play an important role during in recognising malignant cells during the initial stages of transformation, and also play an essential role in transactivating cells of the adaptive immune system. Importantly though, the cancer immune-editing hypothesis states tumour clearance is critically dependent on T cells (401, 402). Indeed, it is now apparent that the extent of T cell infiltration into a tumour is indicative of how a patient is likely to respond to therapy (79). Thus, to generate an effective anti-tumour response, vaccination must induce a T cell response. Previous studies have shown α-GalCer loaded, irradiated whole tumour cell vaccines in a variety of different tumours can activate T cells (29, 403-406). Interestingly, in the GL261 glioma model anti-tumour efficacy was dependent on activation of $CD4^+$ T cells, provided that Tregs were depleted (29, 403). In the previous chapter, combination of this vaccine therapy with CTLA-4 blockade was also seen to provide therapeutic efficacy. In this chapter, the character of the immune responses elicited in this efficacious response is investigated.

While CTLA-4 functions to maintain immune homeostasis and attenuate T cell costimulation following antigen recognition, there is some controversy as to the mechanism of action of antibody-mediated blockade in an immunotherapeutic setting. Initial studies identified CTLA-4 as part of the family of costimulatory receptors on T cells, and it was thought to permit an additional activatory T cell signal (138, 407).

However, genetic ablation studies in mice revealed a fatal lymphoproliferative phenotype driven by $CD4^+$ T cells, resulting in multi-organ tissue destruction (136, 408). Indeed, a similar phenotype is observed with humans whereby mutations in the *CTLA-4* gene present as a disease referred to as common variable immunodeficiency (CVID) with symptoms including autoimmunity, granulatomous disease and other malignancies (409). Later studies revealed that CTLA-4 serves to compete for binding to CD80 and CD86, inhibiting the costimulatory signal afforded to T cells (139). As CTLA-4 binds with higher affinity to CD80 and CD86 than CD28, ligation of the CTLA-4 receptor activates an intracellular cascade, resulting in inhibition of IL-2 secretion and reduced T cell activation resulting in cell cycle arrest (147).

While the initial premise was that CTLA-4 blockade functioned via an effector cell intrinsic mechanism, with antibody mediated blockade of inhibitory signals resulting in enhanced effector T cell function, the demonstration that Tregs express high levels of CTLA-4 raised the suggestion that α-CTLA-4 antibodies could also have effector T cell extrinsic mechanisms (151). A principal feature of Tregs is the constitutive expression of CTLA-4 and thus, they remain a prime target for antibody binding, therefore, it is likely that α-CTLA-4 antibodies bind not only primed effector T cells but Tregs also.

Other suppressive mechanisms of CTLA-4 that are extrinsic to the activated T cell itself have been previously described. Dominant protection against fatal lymphoproliferation was observed by WT mixed chimerism following bone marrow transfer using $CTLA\text{-}4^{-/-}$ and WT donors, suggesting that the proliferative phenotype of T cells without CTLA-4 can be controlled by external factors from wild-type cells (410). It has also been shown that CTLA-4 blockade negates the protection afforded by Tregs in a colitis model where adoptive transfer of $CD4^+$ $CD45RB^{high}$ (naïve, wild-type) cells into immune-deficient hosts results in severe colitis (411, 412), indicating that Tregs are required for maintenance of T cell homeostasis and this is dependent on the expression of CTLA-4.

Each of these models is confounded by the inability to differentiate between the independent effects CTLA-4 blockade has on effector T cells and Tregs. To overcome this, a model system was developed whereby the extracellular domain of human CTLA-4

was combined with the mouse transmembrane and intracellular signalling domain (151). Human CTLA-4 is capable of interacting with mouse CD80/86 molecules, however antibody administration affects either mouse or human CTLA-4 independently, permitting uni-compartmental blockade of CTLA-4 on effector T cells or Tregs individually. When applying this system to a melanoma tumour model, it was shown that blockade of CTLA-4 on both effector T cells and Tregs, combined with therapeutic vaccination was required for effective anti-tumour activity (151). Indeed, earlier studies have shown ligation of CTLA-4 on Tregs results in TGF-β production, dampening effector $CD4^+$ T cell responses (411).

In addition to the effects on T cells, CTLA-4:CD86 binding also drives a signal within the APC, resulting in upregulation of IDO (413, 414). As a catabolic enzyme, IDO converts the essential amino acid tryptophan to *N*-formylkynurine, limiting immune responses in 2 distinct approaches (415). Firstly, in catabolising tryptophan, activated T cells are starved of an essential amino acid, required for rapid proliferation (415). Secondly, the accumulation of kynurenines within the local microenvironment acts to be directly immunosuppressive via apoptosis of T cells. Interestingly, apoptosis was unique to Th1 cells, as Th2 cells subjected to the same assay remained unaffected (416).

Further cell extrinsic mechanims relating to APC function include the trans-endocytosis of costimulatory molecules. Trans-endocytosis is the biological process whereby material from one cell undergoes endocytosis and enters another cell. In a resting state, activation of APCs is limited via trans-endocytosis of surface CD80 and CD86 from APCs into intracellular compartments within CTLA-4-expressing Tregs where they are degraded, preventing immune activation (417). Importantly, this phenomenon was observed in both murine and human systems. In accordance with the upregulation of CTLA-4 following T cell activation, TCR stimulated $CD4^+$ T cell blasts promoted trans-endocytosis of CD86 in this *in vitro* system (417). Finally, CD86 capture was observed *in vivo* using adoptive transfer of CD86-GFP transduced BALB/c $RAG2^{-/-}$ bone marrow into BALB/c $RAG2^{-/-}$ recipients, permitting the development of CD86-GFP APCs. $CD4^+$ T cells from CTLA-$4^{+/+}$ or CTLA-$4^{-/-}$ mice, specific for the H-2^d binding domain of OVA protein (DO11.10 cells) were then transferred and hosts were subsequently challenged with OVA protein.

Here, internalisation of CD86-GFP was only observed in mice harbouring CTLA-$4^{+/+}$ $CD4^+$ T cells which had been challenged with OVA peptide (417). Further studies have expanded on this concept, suggesting that for a T cell to become activated, the amount of ligand on the APC relative to the amount of CTLA-4 on T cells should dictate whether the threshold for CD28 costimulation is achieved (418).

Clinically, there is some evidence to suggest that melanoma patients receiving ipilimumab have a broadened T cell response. That is, the increased numbers of peripheral blood effector T cells are a result of a increased melanoma reactive T cell responses, with a varied TCR diversity, as opposed to an increase in the magnitude of a single TCR clone, suggesting that patients receiving ipilimumab have a lower threshold for T cell activation (154). By using tetramer technology to analyse specific T cell responses in patients pre- and post-therapy, Kvistborg and colleagues identified α-CTLA-4 therapy to selectively induce novel $CD8^+$ T cell responses (154). Importantly, the newly detected T cell responses were shown to have anti-tumour activity when co-cultured with tumour lines known to express the TCR relevant antigen (154).

A common observation between studies is the increase in the ratio of effector T cells to Treg cells within tumours, or within the peripheral blood of patients during clinical studies, with this expansion thought to be a result of preferential proliferation of effector T cells over Tregs (419-421). While the primary targets of blocking antibodies remain to be the surface receptors they are targeted against, receptor-mediated recognition of the fragment crystallisable region (Fc region) of the antibody can also occur. Macrophages express high levels of Fc receptors, facilitating antibody-dependent cell-mediated cytotoxicity (ADCC). Studies investigating the interplay between α-CTLA-4 antibodies and T cells within the tumour microenvironment have identified macrophage dependent depletion of Tregs (152). Here, effective depletion was reliant on the expression of FcγRIV on macrophages, with the clone and isotype of α-CTLA-4 antibody used important in mediating this effect (152).

Given the critical role of NKT cells in the mode of activity of the vaccines used in this thesis, it also important to note that NKT cells utilise, and are functionally constrained by, the same checkpoint receptors that regulate conventional T cells (311, 319, 422, 423). It is therefore possible that the efficacy of combining vaccines with checkpoint blockade may be through enhanced activity of iNKT cells. Furthermore, in addition to playing a role in initiating immune responses, iNKT cells also play a role in the regulation of host immunity (313, 424, 425). Following activation with α-GalCer, human iNKT cells produce IL-2, which, in the presence of allogeneic DCs has been observed to promote Treg proliferation (426). In this study, Tregs maintained immunosuppressive capacity and expressed high levels of intracellular CTLA-4 (426). Interestingly, the influence of activated NKT cells was limited to the proliferation, but not suppressive capacity of Tregs, as Tregs stimulated by DCs in the presence or absence of NKT cells demonstrated comparable levels of suppression (426). Further evidence of NKT:Treg interactions have been examined in a murine model of experimental autoimmune myasthenia gravis (EAMG) (427). Immunisation of C57BL/6 mice with the self-antigen acetylcholine receptor (AchR) induces EAMG (428) and development of disease is not impacted in the absence of NKT cells (429, 430). However, *in vivo* activation of NKT cells with α-GalCer protected mice from EAMG and this correlated with the expansion of peripheral Tregs (427). These Tregs expressed high levels of FoxP3 and Bcl-2 and suppressed proliferation and IFN-γ production of auto-reactive $CD4^+$ T cells (427). Further, Treg mediated control of disease following α-GalCer administration was demonstrated using α-CD25 depleting antibodies (427). It is therefore possible that vaccines incorporating α-GalCer induce Tregs as well as anti-tumour T cells, and that by combining with checkpoint blockade this regulatory pathway is blocked, leading to superior anti-tumour responses.

There is also a body of evidence describing Treg mediated regulation of NKT cell activation. While Vα14 iNKT cells have been shown to promote tumour surveillance in some models (431), one study has shown prophylactic immunisation of mice with plasmids encoding auto-antigens resulted in enhanced tumour growth, which was attributed to the induction of Tregs that suppressed the number and activity of NKT cells (432). In this scenario, it is possible that vaccines incorporating α-GalCer do not

maximally induce iNKT cell activation, and hence observe full adjuvant activity, unless Treg suppression is alleviated, which may be achieved by checkpoint blockade.

Altogether, combining blockade of CTLA-4 with an α-GalCer-adjuvanted vaccine could result in a multifaceted array of consequences, including modulating the activity of NKT cells, which are responsible for licensing APCs, or cell intrinsic and extrinsic mechanisms contributing to improved T cell function. To address this, the cellular responses induced following antibody-mediated CTLA-4 blockade combined with vaccination were examined in the murine model of glioma used in the previous chapter.

4.2 Aims

The experiments in this chapter were to designed to investigate the mechanism of anti-tumour activity of combination therapy with vaccination and CTLA-4 blockade, including adjuvant function of NKT cells and effector functions of T cells.

The specific aims were:

1. To characterise the iNKT cell response following vaccination and CTLA-4 blockade
2. To assess APC activation and stimulatory capacity as a T cell-extrinsic mechanism contributing to the efficacy of the combined treatment
3. To characterise the T cell response induced with vaccination combined with CTLA-4 blockade

4.3 Results

4.3.1 Vaccine induced activation of NKT cells is not affected by CTLA-4 blockade

Although previous studies with the α-GalCer-adjuvanted glioma vaccine showed complete dependence on NKT cell function, which was attributed a requirement for the NKT cells to licence vaccine-bearing APCs (29), it was possible that combination with α-CTLA-4 induced some activity that was independent of this function. To determine if iNKT cells were required for treatment efficacy, $CD1d^{-/-}$ mice were challenged and treated with α-CTLA-4 and vaccine combination therapy. Untreated C57BL/6 hosts served as tumour only controls, and treated C57BL/6 hosts served as positive controls for treatment efficacy. The combined therapy failed to avert tumour growth in $CD1d^{-/-}$ mice, suggesting that even in the presence of CTLA-4 blockade, the mechanism of activity of the vaccine was still reliant on iNKT cells, likely reflecting their capacity to license APCs (Fig 4.1 A).

It has previously been reported that activation of iNKT cells with free α-GalCer can induce anti-tumour immune responses in some models (433). To assess whether cell-associated α-GalCer can inhibit glioma growth, an α-GalCer pulsed, irradiated vaccine was generated using the irrelevant tumour line, Lewis lung carcinoma adapted for tissue culture (LLTC) which was cryopreserved in an identical fashion to the frozen vaccine described earlier. Mice were challenged intracranially and treated with CTLA-4 blockade on day 6. On day 7, they received either α-GalCer loaded and irradiated GL261 cells, or α-GalCer loaded and irradiated LLTC cells. Untreated mice served as tumour only controls. The LLTC vaccine failed to prevent tumour growth in this setting, suggesting that responses observed with the GL261 vaccine were not attributable to α-GalCer alone, but required antigen specific anti-tumour responses (Fig 4.1 B)

It was possible that α-CTLA-4 targeted CTLA-4 molecules on NKT cells and enhanced NKT cell activation by preventing signalling through this immune checkpoint. This in turn may have improved the capacity of NKT cells to license APCs, and thereby improve

the anti-tumour responses mediated by conventional T cells. To address this, the proportion, number and function of iNKT cells within the spleen was assessed at various times following vaccination and α-CTLA-4 administration. A large population of iNKT cells reside within the spleen, and are therefore exposed to α-GalCer following intravenous vaccine administration. Blockade of CTLA-4 had no impact on either the proportion of iNKT cells within in the spleen, nor their number when assessed throughout the course of one week (Fig 4.2 A, B).

As a read out of iNKT activation following therapy, IFN-γ production was assessed by intracellular cytokine staining and flow cytometry at various times following vaccination and CTLA-4 blockade. Again, no discernable difference was observed between the vaccine and combination treatment groups (Fig 4.3 A, B). Finally, previous reports have shown the α-GalCer adjuvanted whole cell vaccine can trigger NKT cell dependent release of IL-4 and IFN-γ into the serum, in addition to IL-12p70 which is likely derived from licensed APCs (29). Here, IL-4 is produced early and is derived directly from the activated NKT cells. While NKT cells also produce IFN-γ after activation, the bulk of the IFN-γ detected in the serum is derived from NK cells activated in a cytokine dependent manner (434, 435). No difference in the quantity or kinetics of cytokine accumulation in the serum were observed when α-CTLA-4 was combined with vaccination, further suggesting NKT cell activation was no further enhanced by blocking CTLA-4 signalling (Fig 4.3 C).

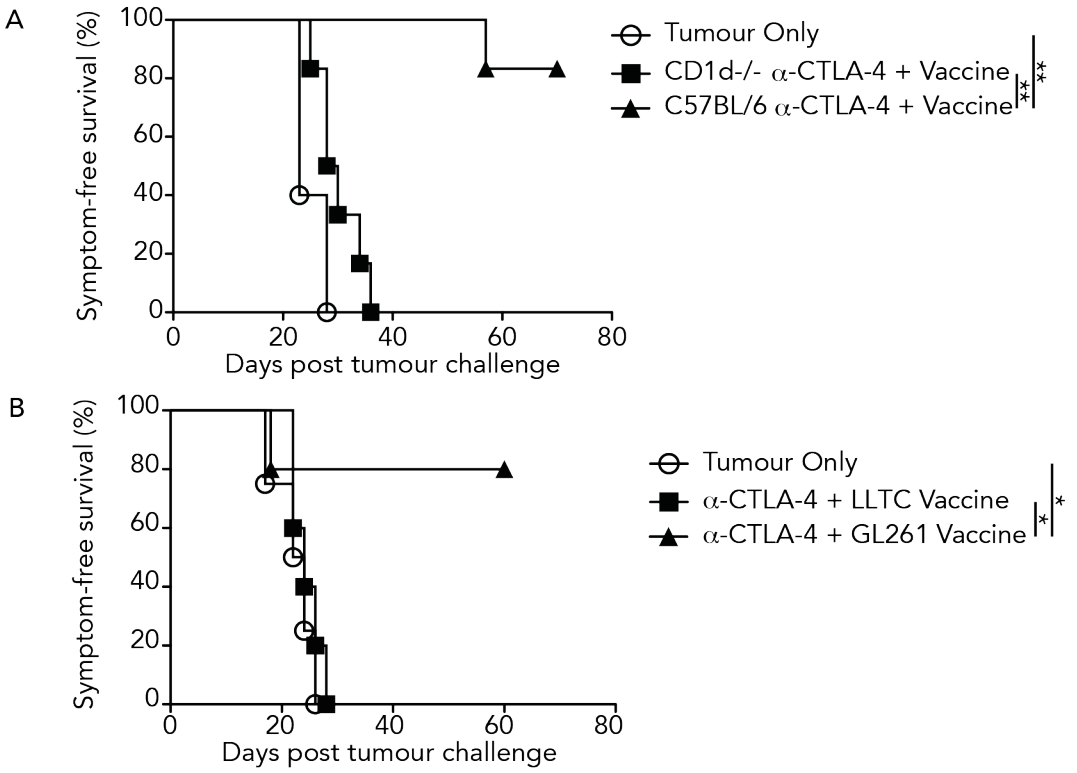

Figure 4.1 NKT cells and specific tumour antigens are required for effective combination therapy. Mice were intracranially challenged with GL261 and monitored for symptoms of tumour development, including > 10% weight loss and overt behavioural changes such as hunching or reduced activity. (A) Symptom-free survival curves for C57BL/6 and CD1d$^{-/-}$ mice (n = 6) with intracranial tumours treated with α-CTLA-4 on day 6 and vaccine on day 7. Untreated C57BL/6 mice (n = 5) served as tumour only controls. Results are representative of two independent experiments ** $P < 0.01$ (Log-Rank (Mantel Cox) test). (B) Symptom-free survival curves for C57BL/6 mice either untreated (n = 4) or treated with α-CTLA-4 and the GL261 vaccine or α-CTLA-4 and a lung carcinoma (LLTC) vaccine (n = 5 per group) * $P < 0.05$ (Log-Rank (Mantel Cox) test).

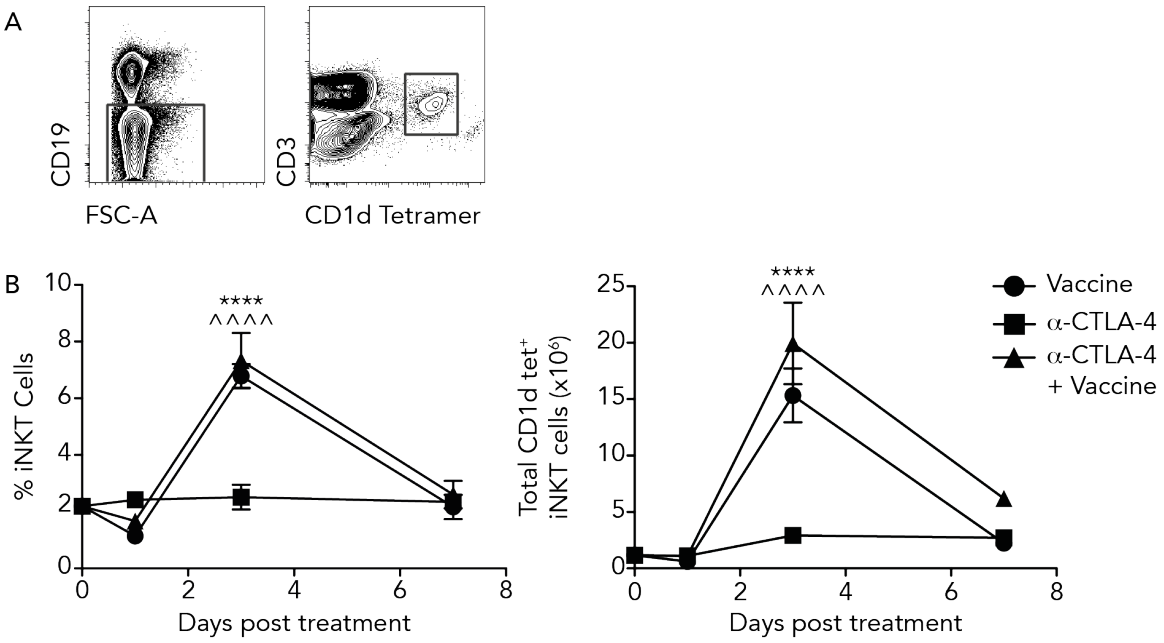

Figure 4.2 NKT cell numbers are not further increased following vaccine administration and CTLA-4 blockade.

Mice were treated with α-CTLA-4, vaccine or combination treatment and spleens were harvested at indicated times following therapy. (A) Gating strategy used to enumerate NKT cells in the spleen after treatment with α-CTLA-4 and vaccine. (B) Mean percentage and number (± SEM) of NKT cells per treatment group (n = 5) at indicated times **** $P < 0.001$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4) ^^^^ $P < 0.001$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4). Results are representative of two independent experiments.

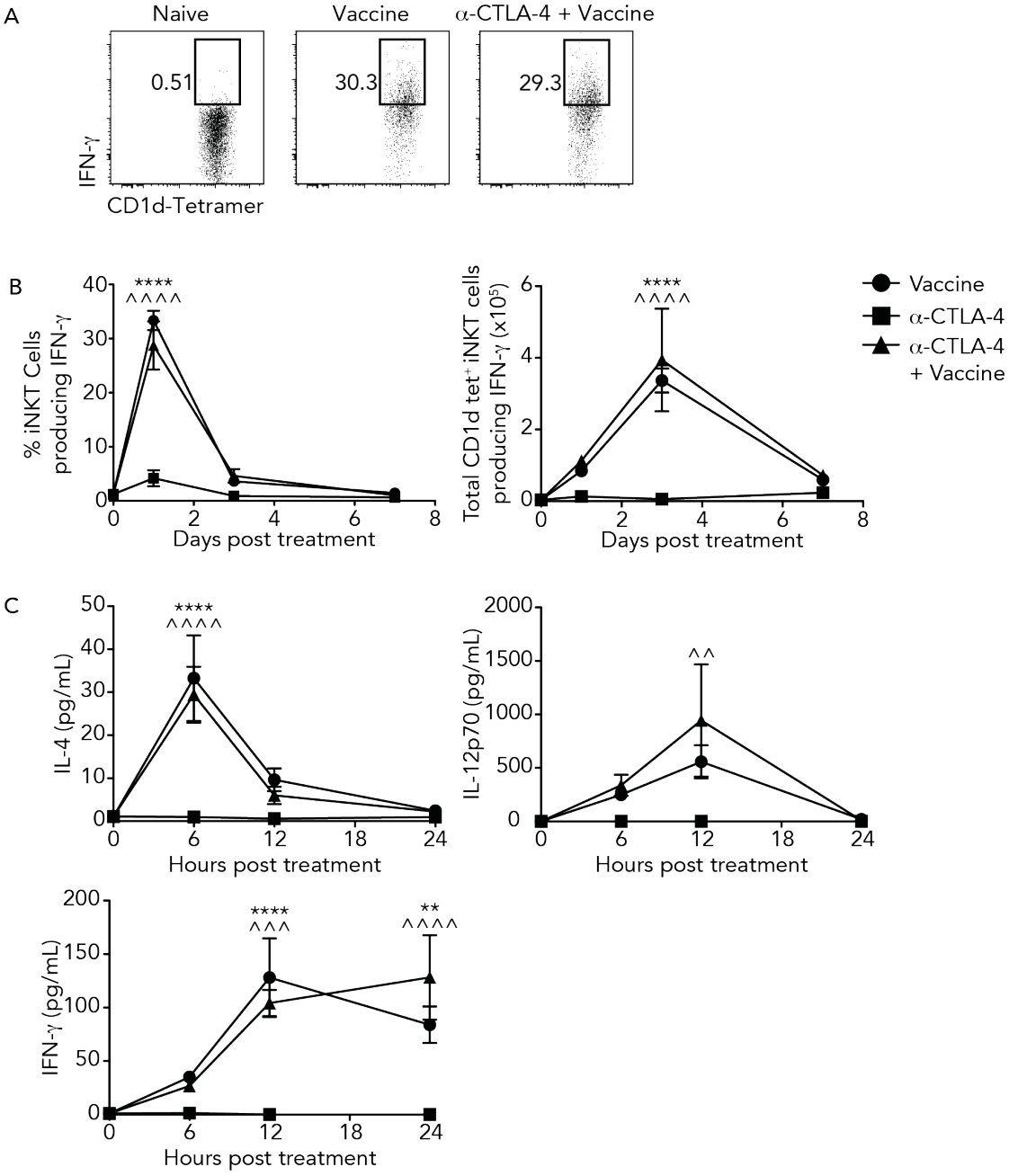
A
Naive
Vaccine
α-CTLA-4 + Vaccine
0.51
30.3
29.3
IFN-γ
CD1d-Tetramer
B
% iNKT Cells producing IFN-γ
Days post treatment
Total CD1d tet+ iNKT cells producing IFN-γ (x10^5)
Vaccine
α-CTLA-4
α-CTLA-4 + Vaccine
C
IL-4 (pg/mL)
Hours post treatment
IL-12p70 (pg/mL)
IFN-γ (pg/mL)

Figure 4.3 NKT cell activity is not enhanced following α-CTLA-4 and vaccine therapy.
Mice were treated with either α-CTLA-4, vaccination, or both and spleens were harvested at indicated times following therapy. (A) Gating strategy used to enumerate NKT cells and examine IFN-γ expression in the spleen following therapy. (B) Mean percentage and number (± SEM) of IFN-γ producing NKT cells per treatment group at indicated times (n = 5) **** $P < 0.001$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4) ^^^^ $P < 0.001$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4). Results are representative of two independent experiments. (C) Mice subjected to the same treatment were bled at the indicated times to determine the levels of cytokines IL-4, IL-12p70 and IFN-γ in the serum. Mean values per group (± SEM) are shown (n = 5) ** $P < 0.01$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4) **** $P < 0.001$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4) ^^ $P < 0.01$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4) ^^^ $P < 0.005$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4) ^^^^ $P < 0.001$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4). Results representative of two independent experiments.

4.3.2 *Blockade of CTLA-4 does not enhance expression of costimulatory molecules on APCs in vitro*

Trans-endocytosis of costimulatory molecules CD80 and CD86 by CTLA-4 expressing cells has been reported to maintain low activation status of APCs during immune quiescence. It was therefore possible that inhibition of CTLA-4 during immune priming compromised this process, enhancing the costimulatory ability of APCs involved in activation of T cells. To explore this, an *in vitro* experiment was conducted in which expression of CD86 was examined on splenocytes from animals with or with out prior α-CTLA-4 treatment, in co-cultures with titrated doses of the vaccine. While cultures containing the vaccine showed increased expression of CD86 on $CD11c^+$ $MHC\text{-}II^+$ DCs, reflecting the adjuvant activity afforded by iNKT cells within the culture, this was not increased when mice were pre-treated with α-CTLA-4 (Fig 4.4 A, B). Furthermore, no impact of combination with α-CTLA-4 was observed on other APC subsets including $B220^+$ cells in a dose dependent manner (Fig 4.4 C, D).

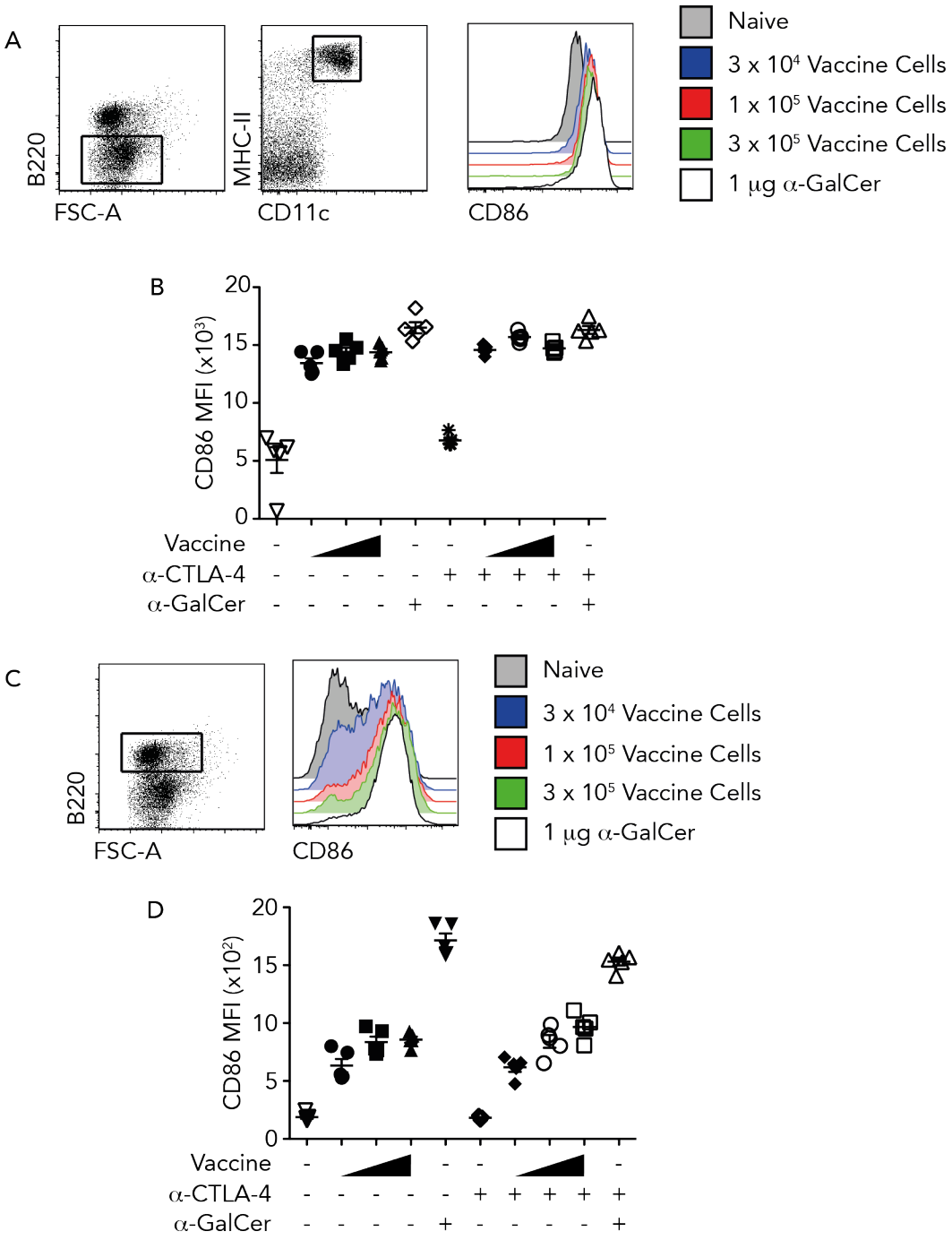

Figure 4.4 CTLA-4 blockade does not enhance costimulatory molecule expression on APCs *in vitro*.

Splenocytes from mice treated 24 hours earlier with α-CTLA-4 were incubated with titrated doses of vaccine *in vitro* or free α-GalCer. Expression of the activation marker CD86 was assessed 16 hours later on DCs, defined as $B220^{-}$ $CD11c^{+}$ $MHC\text{-}II^{+}$ cells. (A) Gating strategy used to identify DCs. (B) Mean Fluorescence Intensity (MFI) of CD86 (± SEM) at each vaccine dose (n = 5). Triangle indicates increasing dose of vaccine cells from left to right (1 x 10^5, 3.3 x 10^5, 1 x 10^6). (B) Gating strategy used to identify B Cells. (D) Mean Fluorescence Intensity (MFI) of CD86 (± SEM) at each vaccine dose (n = 5). Triangle indicates increasing dose of vaccine cells from left to right (1 x 10^5, 3.3 x 10^5, 1 x 10^6). Results are representative of three independent experiments.

4.3.3 *Blockade of CTLA-4 does not enhance expression of costimulatory molecules on APCs in vivo*

To accompany the previous *in vitro* results, activation of splenic APCs was assessed *in vivo* at various times following vaccination and α-CTLA-4 administration, or following α-CTLA-4 administration alone. Peak expression of CD80 and CD86 was observed on $CD11c^+$ DCs within the spleen 24 hours following vaccination, with the highest expression observed in vaccinated mice which did not receive α-CTLA-4 (Fig 4.5 A-B). Therefore, if anything, CTLA-4 blockade was actually detrimental to DC activation in this setting. Although it is not clear what role activated B cells play in the priming of T cell responses to α-GalCer adjuvanted vaccines, it was notable that CD86 expression was increased on these cells with vaccination as well, and α-CTLA-4 treatment did not alter this. Peak expression of CD86 was observed on B cells 24 hours following vaccination but no difference was seen when vaccination was combined with CTLA-4 blockade (Fig 4.5 C).

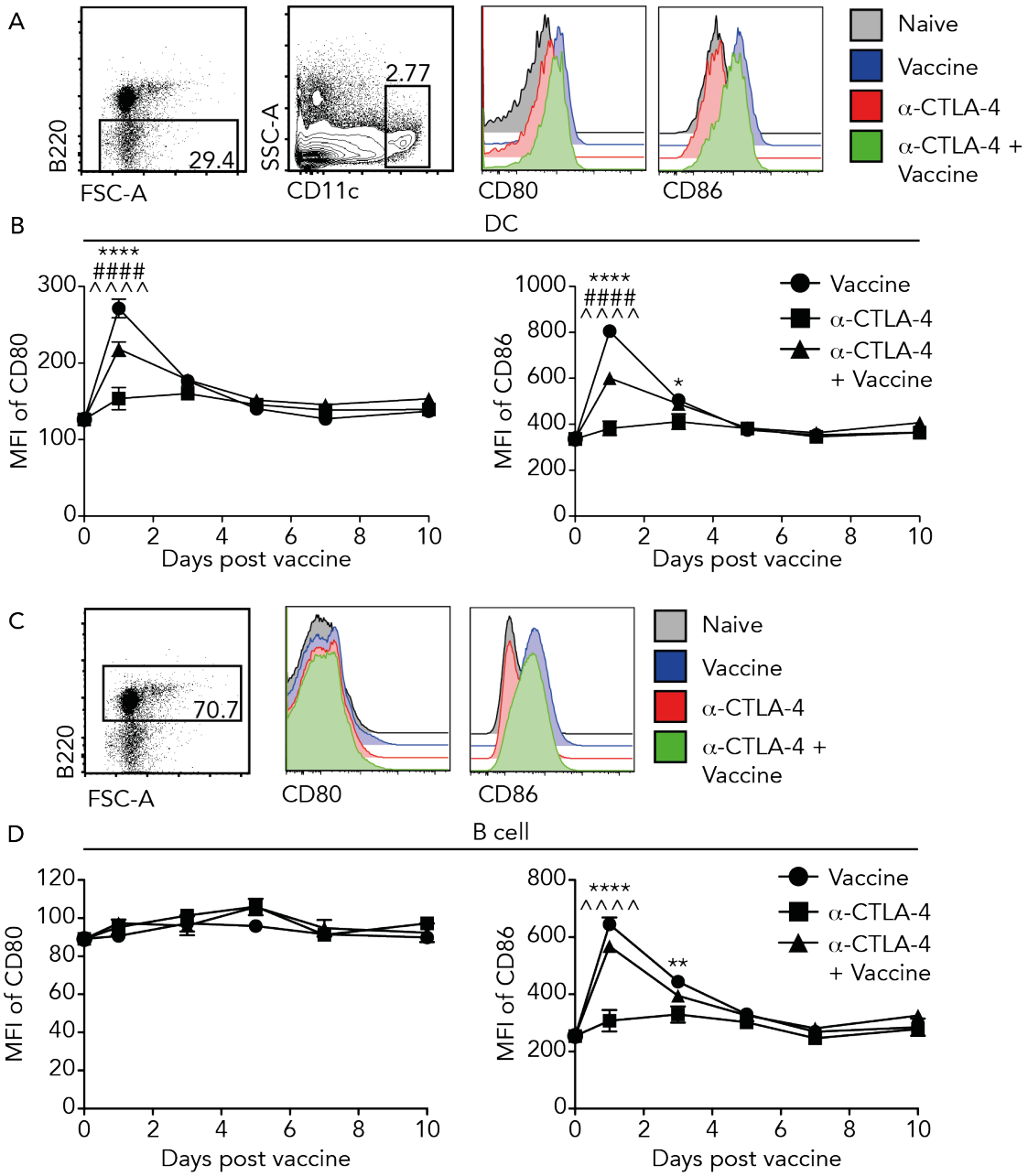
A
B220
FSC-A
29.4
SSC-A
CD11c
2.77
CD80
CD86
Naive
Vaccine
α-CTLA-4
α-CTLA-4 + Vaccine
B
DC
MFI of CD80
MFI of CD86
Days post vaccine
****
####
^^^^
*
Vaccine
α-CTLA-4
α-CTLA-4 + Vaccine
C
B220
FSC-A
70.7
CD80
CD86
Naive
Vaccine
α-CTLA-4
α-CTLA-4 + Vaccine
D
B cell
MFI of CD80
MFI of CD86
Days post vaccine
****
^^^^
**
Vaccine
α-CTLA-4
α-CTLA-4 + Vaccine

Figure 4.5 CTLA-4 blockade does not enhance costimulatory molecule expression on APCs *in vivo*.

Mice were treated with either α-CTLA-4, vaccination, or both and spleens were harvested at indicated times following therapy. Expression of the activation markers CD80 and CD86 was assessed on B cells, defined as $B220^+$ and DCs, defined as $B220^-$, $CD11c^+$ (A). Gating strategy used to identify DCs and assess CD80 and CD86 MFI. (B) Mean Fluorescence Intensity (MFI) of CD80 and CD86 (± SEM) (n = 3) at indicated times **** $P < 0.001$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4) #### $P < 0.001$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4 + Vaccine) ^^^^ $P < 0.001$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4). (C) Gating strategy used to identify B cells and assess CD80 and CD86 MFI. (D) Mean Fluorescence Intensity (MFI) of CD80 and CD86 (± SEM) (n = 3) ** $P < 0.01$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4) **** $P < 0.001$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4) ^^^^ $P < 0.001$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4). Results representative of two independent experiments.

4.3.4 *Blockade of CTLA-4 does not enhance the stimulatory function of APCs in vitro*

While no enhanced expression of costimulatory molecules was observed following vaccination with CTLA-4 blockade, further experiments were conducted to elucidate if α-CTLA-4 bestowed functional differences within the APC compartment. To this end, splenocytes were harvested from mice 18 hours following vaccination, α-CTLA-4 administration, or both, and pulsed with peptide corresponding to the $I\text{-}A^b$-binding epitope of chicken ovalbumin (ISQ peptide). They were then used as APCs to stimulate CFSE-labelled $CD4^+$ T cells from OT-II mice, which express a transgenic TCR specific for the ISQ epitope. The induction of T cell proliferation was determined by assessing CFSE fluorescence on the T cells by flow cytometry. As cells divide, the fluorescent signal is halved as CFSE is passed on to daughter cells, thus a reduction in fluorescence is indicative of proliferating cells. To define proliferating T cells, CFSE dilution was assessed relative to T cells plated in the absence of stimulator APCs (Fig 4.6A). While splenocytes from vaccinated mice induced increased proliferation of antigen specific T cells compared to splenocytes from untreated mice, or those treated with α-CTLA-4 alone, the proportion of proliferating T cells was not affected by *in vivo* administration of α-CTLA-4 (Fig 4.6 B). This data suggests that treatment with α-CTLA-4 prior to vaccination does not augment the stimulatory function of APCs in this *in vitro* setting.

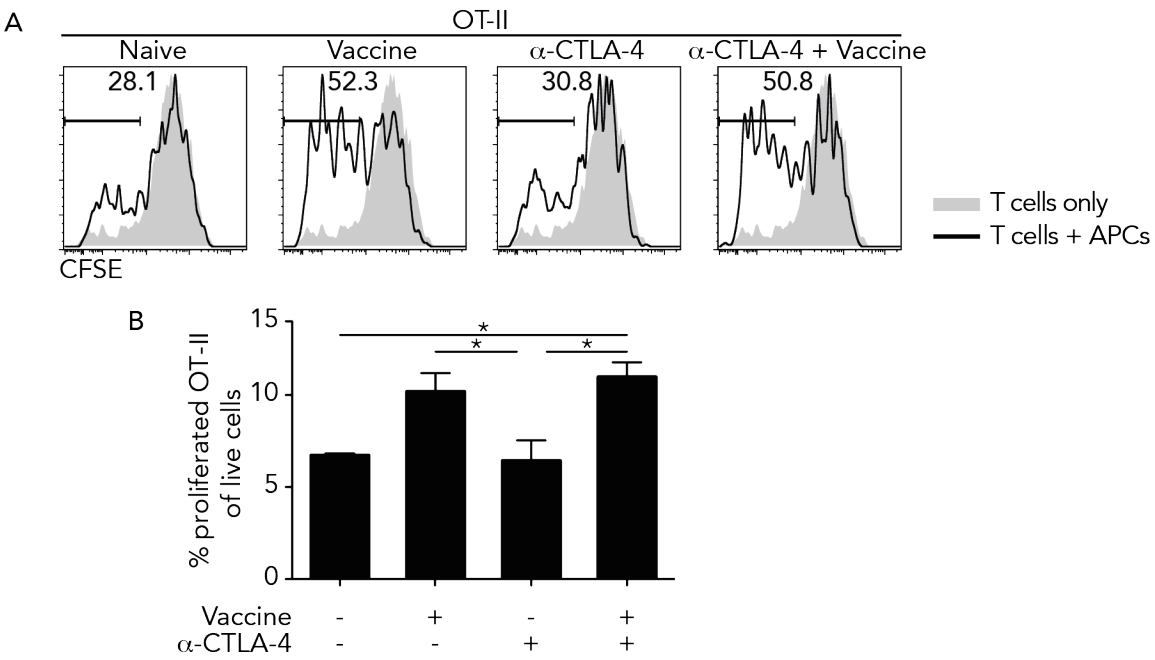

Figure 4.6 CTLA-4 blockade does not enhance APC function *ex vivo*.

Splenocytes from mice treated 24 hours earlier with α-CTLA-4, vaccine, or both, were pulsed with $OVA_{323-329}$ peptide for 2 hours and used to stimulate CFSE-labelled OT-II cells *in vitro* for 6 days. (A) The extent of proliferation induced was determined by examining CFSE dilution of T cells cultured with splenocytes (black line) relative to T cells cultured alone (shaded grey). (B) Mean percentage of proliferated cells (± SEM) treatment group ($n = 3$) are shown * $P < 0.05$ (One-way ANOVA with Bonferroni post-test). Results representative of two independent experiments.

4.3.5 *Enhanced proliferation and accumulation of T cells with CTLA-4 blockade and vaccination*

The expression of CTLA-4 is up-regulated early after T cell stimulation and signalling through this molecule is known to negatively impact on T cell proliferation in an effector T cell intrinsic manner. To examine if the blockade of CTLA-4 signalling affected the proliferation of vaccine stimulated T cells *in vivo*, CFSE labelled $CD45.1^+$ congenic splenocytes were adoptively transferred into $CD45.2^+$ hosts the day before treatment. Spleens were harvested from hosts one week later and the proliferation of transferred cells was assessed by flow cytometry. While vaccination alone resulted in a modest trend towards increased proliferation, the greatest response was observed in mice receiving both vaccination and α-CTLA-4 (Fig 4.7 A-D). Interestingly, significant proliferation was observed in both $CD4^+$ and $CD8^+$ T cells from mice receiving α-CTLA-4 as a monotherapy, suggesting inhibition of CTLA-4 is capable of releasing T cell proliferation in the absence of an obvious priming signal in this *in vivo* setting.

To determine if enhanced proliferation corresponded with amplified accumulation of T cells in the lymphoid organs of mice receiving vaccine and CTLA-4 blockade therapy, the number and proportion of T cells in the spleen were assessed on different days following therapy. Cellularity was significantly increased after three days in mice receiving vaccine alone compared to untreated controls and this was reduced to normal levels by day 7. The most pronounced increase was observed in mice receiving α-CTLA-4 combined with vaccine, with splenic cellularity remaining increased at day 7 (Fig 4.8 A). Within the T cell compartment, both $CD4^+$ and $CD8^+$ T cells were increased in the spleen following combination treatment, whereas no increase was observed in the number of Tregs (defined as $CD4^+$ $FoxP3^+$ cells) (Fig 4.8 B). Intriguingly, the increased in cellularity involved all cell types examined but was most pronounced within the B cell compartment (Fig 4.8 C). While the previous adoptive transfer experiments revealed significant proliferation of CFSE labelled cells in mice receiving CTLA-4 as a monotherapy, this observation was not reflected in an increased T cell accumulation in the spleen. Therefore, vaccine induced stimulation was required to drive the significant lymphocyte trapping observed with the combined treatment group, leading to increased cellularity.

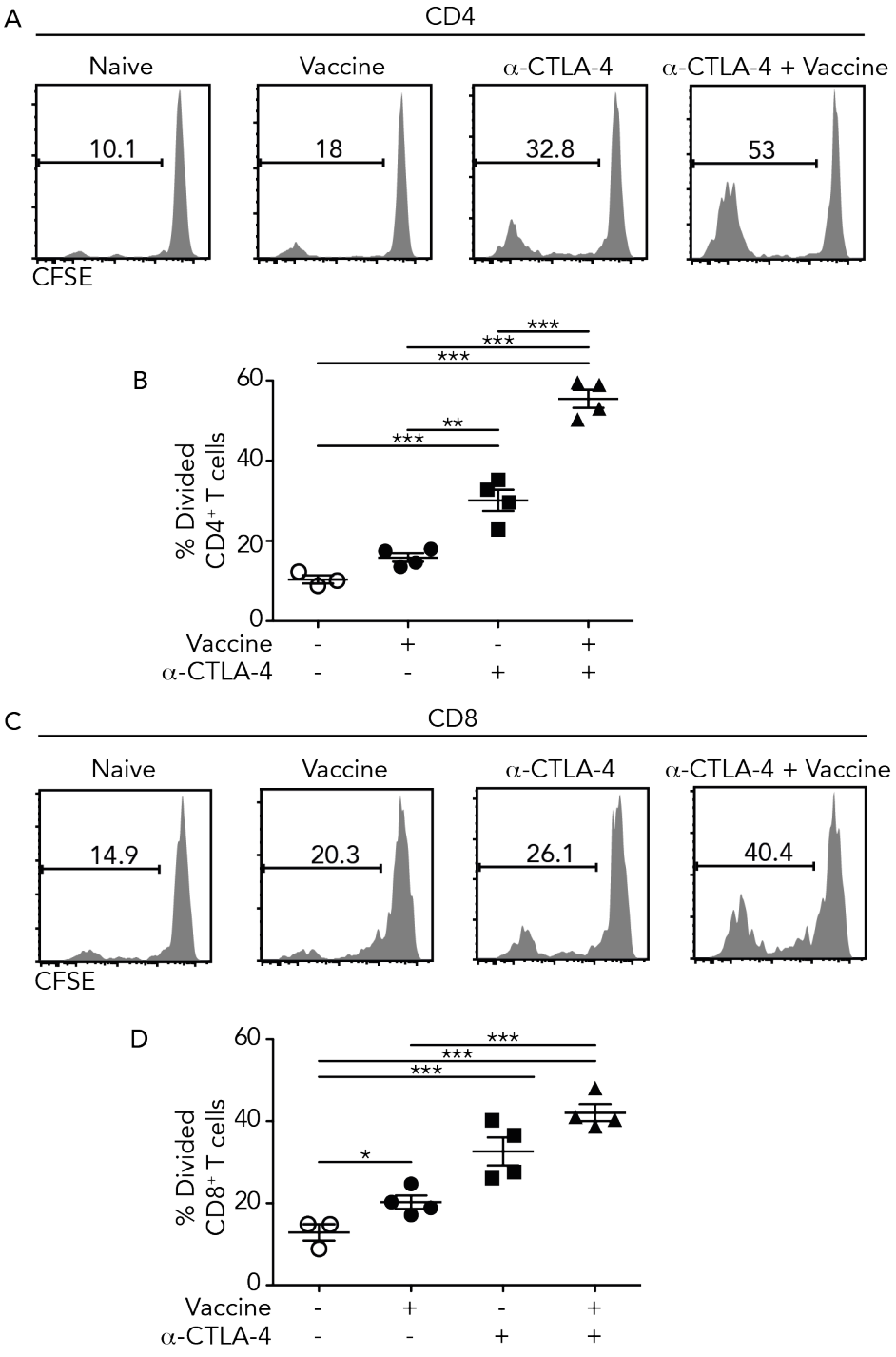
A
CD4
Naive
Vaccine
α-CTLA-4
α-CTLA-4 + Vaccine
10.1
18
32.8
53
CFSE
B
% Divided CD4+ T cells
60
40
20
0
***
***
***
**
***
Vaccine - + - +
α-CTLA-4 - - + +
C
CD8
Naive
Vaccine
α-CTLA-4
α-CTLA-4 + Vaccine
14.9
20.3
26.1
40.4
CFSE
D
% Divided CD8+ T cells
60
40
20
0
***
***
***
*
Vaccine - + - +
α-CTLA-4 - - + +

Figure 4.7 Combining vaccination with CTLA-4 blockade enhances T cell proliferation.
CFSE labelled, CD45.1$^+$ congenic splenocytes were adoptively transferred into CD45.2$^+$ hosts which were subsequently treated with α-CTLA-4 on day 6, vaccine on day 7, both or remained untreated. Proliferation was determined by examining CFSE dilution 7 days later. (A) Representative histograms of CFSE dilution in transferred CD4$^+$ T cells 7 days after vaccination. (B) Mean percentage (± SEM) of divided CD4$^+$ T cells on day 7 (n = 4 per treatment group) ** $P < 0.01$, *** $P < 0.005$ (One-way ANOVA with Bonferroni post-test). (C). Representative histograms of CFSE dilution in transferred CD8$^+$ T cells 7 days after vaccination. (D) Mean percentage (± SEM) of divided CD8$^+$ T cells on day 7 (n = 4 per treatment group). * $P < 0.05$, *** $P < 0.005$ (One-way ANOVA with Bonferroni post-test). Results are representative of two independent experiments.

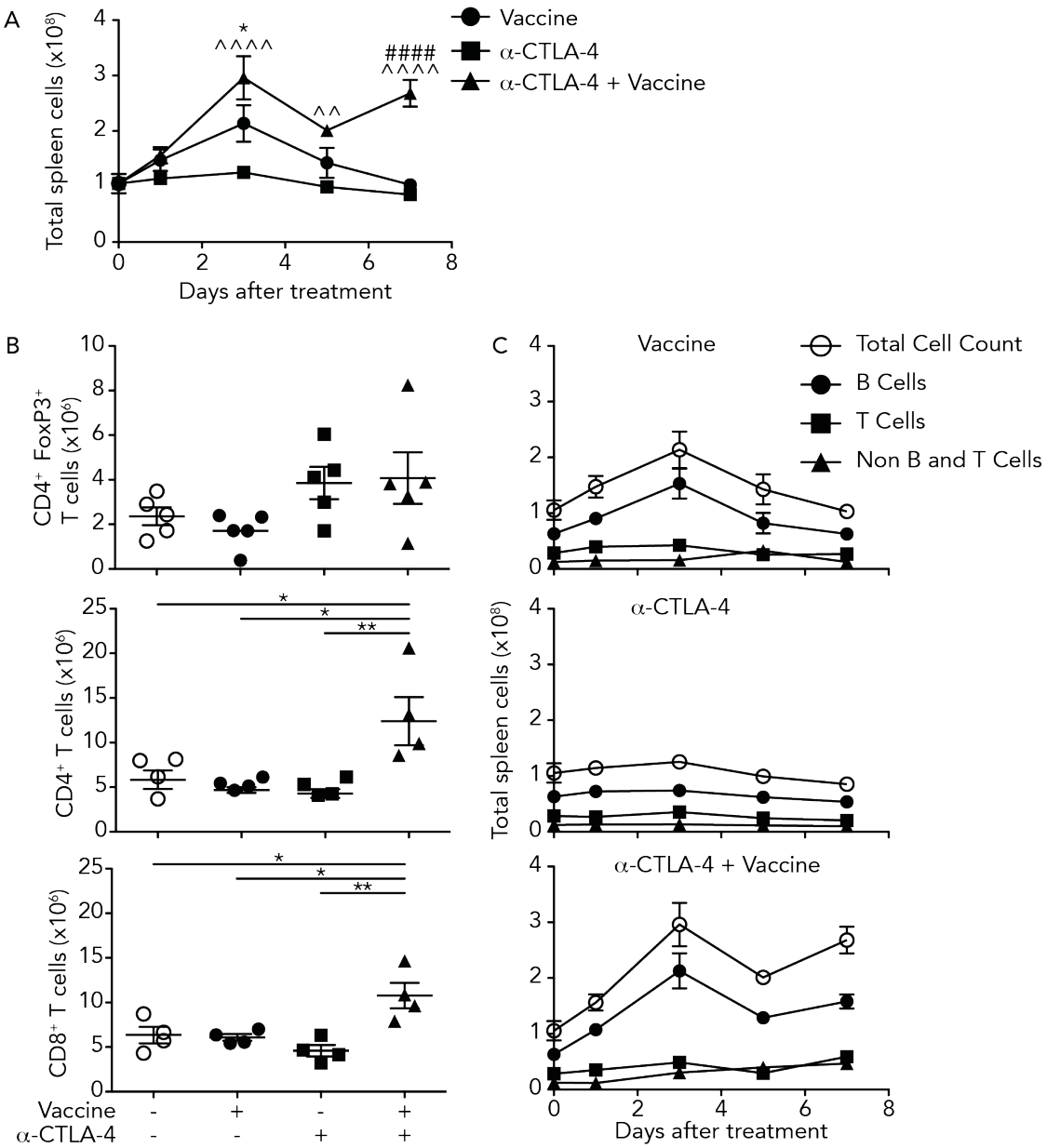

Figure 4.8 Combining vaccination with CTLA-4 blockade enhances leukocyte accumulation in the spleen.

Mice were treated with vaccine, α-CTLA-4, or both and spleens were harvested at indicated times. (A) Total cell count of splenocytes per treatment group (± SEM) (n = 5) * $P < 0.05$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4) ^^ $P < 0.01$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4) ^^^^ $P < 0.001$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4) #### $P < 0.001$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4 + Vaccine) Data are representative of two independent experiments. (B) Total cell count per treatment group (± SEM) of FoxP3⁺ CD4⁺ T cells, CD4⁺ T cells and CD8⁺ T cells were determined on day 7 in groups treated as in *A*, together with untreated controls. * $P < 0.05$, ** $P < 0.01$ (One-way ANOVA with Bonferroni post-test). (C) Total number of B cells, T cells and Non B and T cells (± SEM) in each treatment group (n = 5). Results are representative of two independent experiments.

4.3.6 *Increased T cell effector phenotype following vaccination and CTLA-4 blockade*

In order to elicit an effective immune response, T cells must not only proliferate, but also differentiate into useful effector T cells. As T cells were highly represented in the increased cellularity of the lymphoid organs observed in vaccinated mice, and their accumulation was further enhanced with CTLA-4 blockade, additional studies were performed to examine their effector phenotype. Mice were culled at various times following vaccine, α-CTLA-4 administration, or combination treatment and effector T cells were assessed based on the expression of CD44 and CD62L. The CD44 antigen is a glycoprotein involved in cell-cell interactions, cell adhesion and migration while CD62L is a "homing receptor" for lymphocytes to enter secondary lymphoid tissues. These markers were used because their expression can be used to discriminate naïve $CD44^{lo}$ $CD62L^{hi}$ cells from effector $CD44^{hi}$ $CD62L^{lo}$ T cells. A greater than 5 fold increase in effector $CD4^+$ T cells ($CD44^{hi}$ $CD62L^{lo}$) was observed in the spleens of mice treated with combination treatment compared to vaccine only or α-CTLA-4 monotherapy, with overall numbers continuing to increase until day 7 (Fig 4.9 A, B). The number of $CD8^+$ effector T cells were also increased in α-CTLA-4 and vaccine treated mice compared to monotherapy groups, with increased cell numbers occurring at day 3 and remaining sustained until day 7 (Fig 4.9 C, D). Notably, the number of effector $CD8^+$ T cells was lower than $CD4^+$ T cells.

While the glioma vaccine used is known to induce immune responses in the spleen (29), prophylactic vaccination in splenectomised mice still resulted in effective anti-tumour responses which was attributed to immune priming occurring in the lung draining lymph nodes (29). To explore whether such priming was occurring in combination with CTLA-4 blockade, the mediastinal lymph nodes were assessed 7 days following vaccination, α-CTLA-4, or combination therapy. Overall cellularity was increased in vaccinated mice in the presence and absence of α-CTLA-4 administration (Fig 4.10 A). Similarly to the spleen, the number of Tregs was comparable between treatment groups (Fig 4.10 B). To quantify effector T cells, the expression of CD44 on $CD4^+$ $FoxP3^-$ and $CD8^+$ T cells was used. Vaccination resulted in increased effector $CD4^+$ and $CD8^+$ T cells compared to

naïve mice, with vaccine only treated mice also having an increased effector $CD8^+$ T cells compared to α-CTLA-4 only mice. The most profound increase was observed in both $CD4^+$ and $CD8^+$ T cells in mice that received the combined treatemnt (Fig 4.10 B). As increased numbers of effector T cells could not account for the overall increase in cellularity within the MdLN, the remaining cells were quantified. As observed in the spleen, an increase was observed in all cell types examined, possibly reflecting trapping due to reduced egress (Fig 4.10).

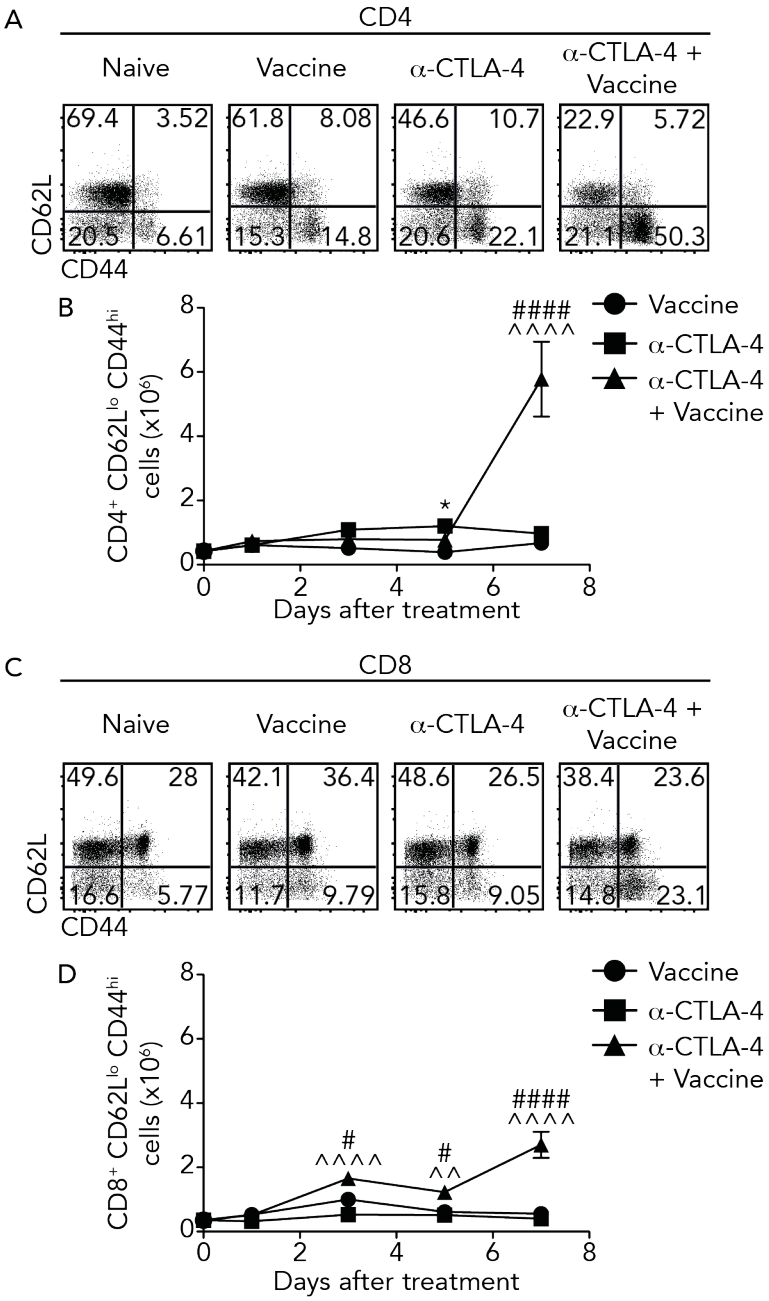

Figure 4.9 Vaccine and α-CTLA-4 therapy increases the accumulation of effector T cells in the spleen.

Mice were treated with vaccine, α-CTLA-4, or both and spleens were harvested at indicated times. Effector phenotype of T cells was assessed based on the expression of CD44 and CD62L. Effector T cells were defined as $CD44^+$ $CD62L^-$. (A) Gating strategy used to define and enumerate effector T cells on day 7 following therapy. Examples are pregated on $CD4^+$ T cells. (B) Total number (± SEM) of effector $CD4^+$ T cells per group ($n = 5$) at indicated times following therapy. Significance between vaccinated mice and mice treated with vaccine and α-CTLA-4 is shown * $P < 0.05$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4) #### $P < 0.001$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4 + Vaccine) ^^^^ $P < 0.001$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4). (C) Gating strategy used to define and enumerate effector T cells on day 7 following therapy. Examples are pregated on $CD8^+$ T cells. (D) Total number (± SEM) of effector $CD8^+$ T cells per group ($n = 5$) at indicated times following therapy # $P < 0.05$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4 + Vaccine) #### $P < 0.001$ (Two-way ANOVA with Bonferroni post-test Vaccine *cf.* α-CTLA-4 + Vaccine) ^^ $P < 0.01$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4) ^^^^ $P < 0.001$ (Two-way ANOVA with Bonferroni post-test α-CTLA-4 + Vaccine *cf.* α-CTLA-4) Results representative of two independent experiments.

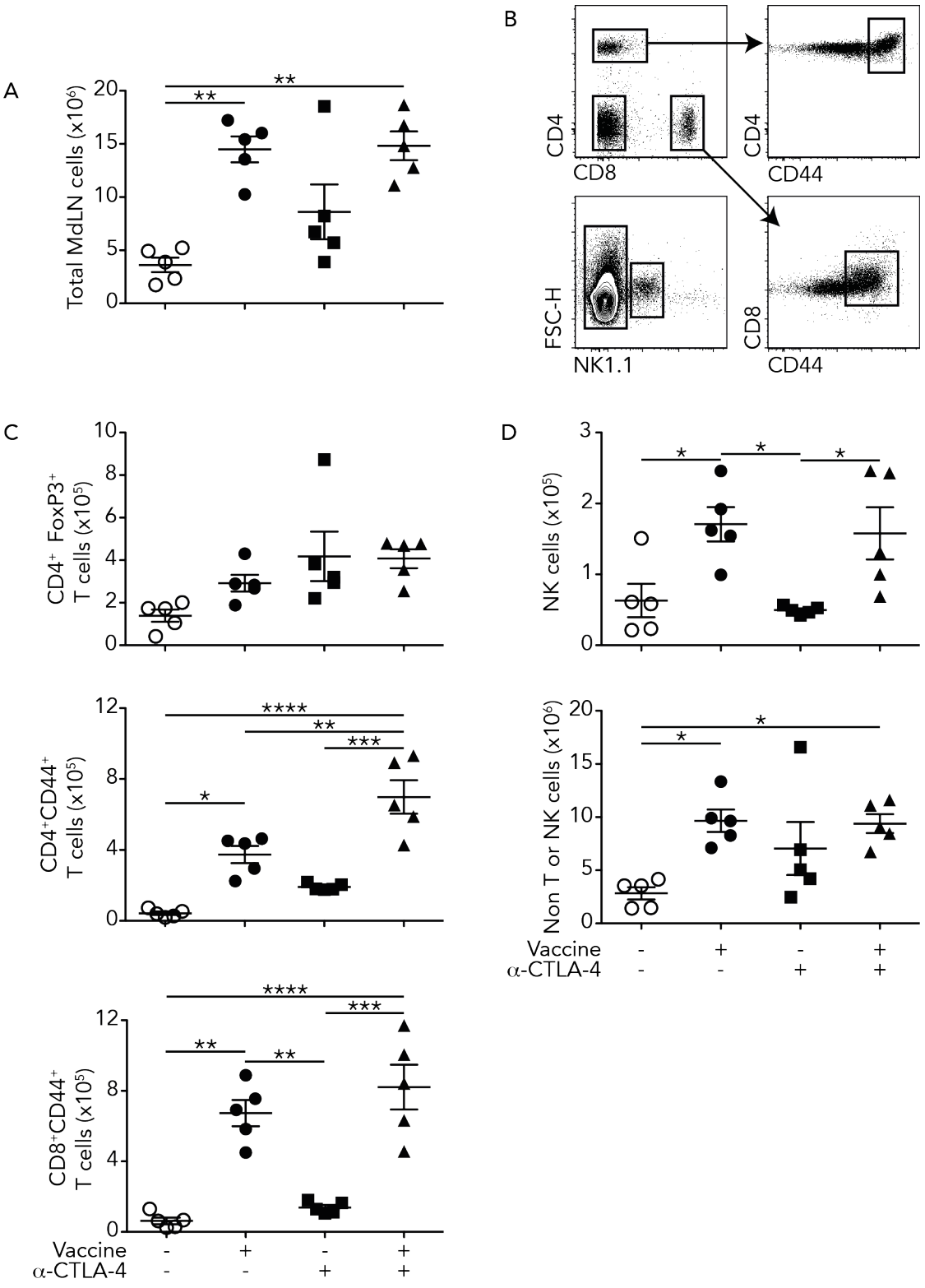
A
Total MdLN cells (x10⁶)
B
CD4
CD8
CD4
CD44
FSC-H
NK1.1
CD8
CD44
C
CD4⁺ FoxP3⁺ T cells (x10⁵)
CD4⁺CD44⁺ T cells (x10⁵)
CD8⁺CD44⁺ T cells (x10⁵)
D
NK cells (x10⁵)
Non T or NK cells (x10⁶)
Vaccine
α-CTLA-4

Figure 4.10 Vaccine-induced immune priming within the mediastinal lymph node is enhanced with CTLA-4 blockade

Mice were treated with vaccine, α-CTLA-4, combination treatment or remained untreated. Mediastinal lymph nodes were harvested one week following therapy and immune cells were assessed. (A) Total cell count (± SEM) within the MdLN (n = 5) ** $P < 0.01$ (One-way ANOVA with Bonferroni post-test). (B) Gating strategy used to identify effector CD4 and CD8 cells as well as NK cells. (C) Total cell count (± SEM) of $CD4^+$ $FoxP3^+$ T cells, $CD44^+$ effector $CD4^+$ T cells and $CD44^+$ effector $CD8^+$ T cells within the MdLN (n = 5) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$ (One-way ANOVA with Bonferroni post-test). (D) Total cell count (± SEM) of NK cells and all remaining cells within the MdLN (n = 5) * $P < 0.05$ (One-way ANOVA with Bonferroni post-test). Results representative of two independent experiments.

4.3.7 Enhanced T cell cytokine production following α-CTLA-4 and vaccination

To examine whether anti-tumour effector T cells were elicited following vaccination, and enhanced through combination with CTLA-4 blockade, splenocytes were restimulated with GL261 tumour cell lysate one week following therapy and assessed for cytokine production. In concert with the increased effector T cells induced within the spleen following α-CTLA-4 and vaccine therapy, both $CD4^+$ and $CD8^+$ T cells proportionally produced higher levels of IFN-γ and TNF-α compared to each intervention alone (Fig 4.11 A-D). While no defined tumour antigen could be evaluated using this system, the high proportion of T cells responding to tumour cell lysate suggests tumour-specific T cells were highly represented within the expanded population.

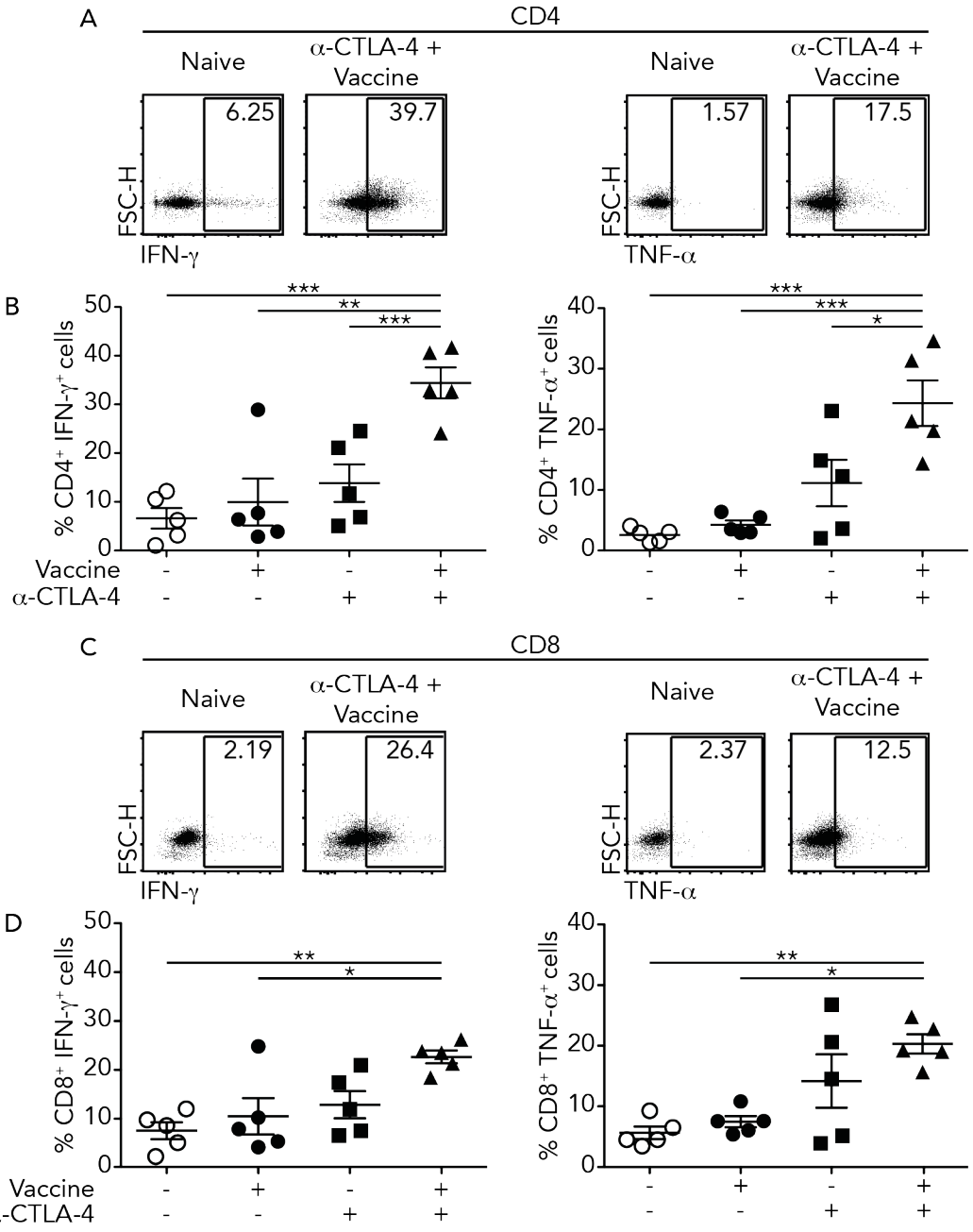

Figure 4.11 Combining vaccination with α-CTLA-4 enhances T cell cytokine production in the spleen.

Splenocytes were harvested from mice 7 days following vaccine, α-CTLA-4, combination treament, or untreated controls and restimulated *ex vivo* with tumour lysate for 6 hours in the presence of monensin to prevent cytokine release. Cytokines IFN-γ and TNF-α were assessed by intracellular cytokine staining. (A) Gating strategy used to determine cytokine production in restimulated $CD4^+$ T cells. Examples are pregated on $CD4^+$ T cells. (B) Proportion of $CD4^+$ T cells (± SEM) producing IFN-γ or TNF-α are shown ($n = 5$) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ (One-way ANOVA with Bonferroni post-test). (B) Gating strategy used to determine cytokine production in restimulated $CD8^+$ T cells. Examples are pregated on $CD8^+$ T cells. (D) Proportion of $CD8^+$ T cells (± SEM) producing IFN-γ or TNF-α are shown ($n = 5$) * $P < 0.05$, ** $P < 0.01$ (One-way ANOVA with Bonferroni post-test). Results are representative of two independent experiments.

4.4 Discussion

Targeting of CTLA-4 with blocking monoclonal antibodies has resulted in unprecedented durable clinical responses in cancer patients, most notably in advanced melanoma (436). In contrast, the induction of anti-tumour T cells through vaccination has been met with less clinical success, potentially because the induced responses are not potent or broad enough to generate a clinical response, or the T cells ultimately come under regulation, including through the same checkpoint pathways. Having observed synergy between CTLA-4 blockade and vaccination in a therapeutic model of glioma in the previous chapter, whereby 80% of mice consistently survived long term, studies were performed here to characterise the immune response induced following therapy.

Therapeutic efficacy was lost in $CD1d^{-/-}$ mice suggesting that even when combined with α-CTLA-4, therapy was still dependent on the ability of iNKT cells to license APCs. Through examination of the early immune cascade involved in APC activation and T cell priming, therapeutic benefit of vaccination with α-CTLA-4 could not be attributed to improved responses to the adjuvant used. Focusing on iNKT cells, no further increase in cell number or activation was observed when α-CTLA-4 was combined with vaccination, compared to vaccine only, with comparable levels of IFN-γ production observed both in the presence and absence of CTLA-4 blockade. Additionally, serum cytokines IL-4, IL-12p70 and IFN-γ, indicative of iNKT cell activation, were increased with vaccine administration (29), but α-CTLA-4 did not increase these further.

In contrast to other studies showing anti-tumour effects mediated by α-GalCer induced activation of iNKT cells (433), no therapeutic efficacy was observed here when an α-GalCer loaded, unrelated tumour cell line was used as a vaccine. This suggests that the anti-tumour effect was not a consequence of delivery of cell-associated α-GalCer driving NKT cells with direct anti-tumour activity. Rather it was an indirect effect, with NKT cell activation enhancing an antigen specific anti-tumour response.

Following intravenous vaccination, antigens will travel via the heart to the lungs where antigen can be transported to the MdLN (437, 438). In the lymph node, NKT cells

primarily reside within the paracortex, similar to $CD4^+$ T cells (439). Large antigens are retained by cells lining the subcapsular sinus and are therefore restricted from direct interactions with cells within the paracortex (440, 441). Within the subcapsular sinus, $CD169^+$ macrophages have been shown to mediate the retention of antigen (442) and to also induce NKT cell localisation (439). Intriguingly, depletion of $CD169^+$ macrophages by administration of clodronate liposomes abolished NKT cell proliferation and activation (439). In addition to inducing NKT cell arrest, $CD169^+$ macrophages within the subcapsular sinus were shown to present antigen to NKT cells to an extent similar to that of DCs *in vitro* (439). Importantly, when the ability of $CD169^+$ macrophages to present antigen was tested *in vivo*, it was found that the $CD169^+$ macrophages, but not DCs or B cells, permitted activation of iNKT cells as early as 2 hours following antigen administration (439). In this situation, it is conceivable that macrophages within the subcapsular sinus induce NKT cell activation at the initiation of an immune response providing an inflammatory environment whereby later DC:T cell interactions favour the development of a Th1 immune response. Additionally, targeting vaccines to the subcapsular macrophages may further enhance early immune cell activation.

The priming of immune responses within the lung draining lymph node is fascinating as recent evidence suggests T cells undergo gene-expression reprogramming within the lung. This was characterised as down-regulation of proliferation- and activation-associated genes and up-regulation of migration-associated molecules as well as chemokine and adhesion receptors including sphingosine 1-phosphate (S1P), integrin $\alpha 4$ and ninjurin 1 (103). With the recent discovery of lymphatic vessels within the meninges, draining to the cervical lymph nodes (340, 443), it is conceivable that an aerosol-based vaccine could be adopted for malignancies of the CNS. An aerosol vaccine could potentially target both the cervical lymph nodes via the nasal lymphatics, as well as migratory DCs within the lung parenchyma which could transport antigen to the mediastinal lymph node.

Vaccine antigens which are not trapped within the MdLN will continue to recirculate where they can accumulate in both the spleen and liver (444, 445). Arterial branches within the spleen terminate at he marginal sinus, allowing blood to pass through the marginal zone and into the red pulp (446). Such organisation allows the spleen to screen

blood borne antigens. Within the spleen, NKT cells are compartmentalised at the marginal zone and within the red pulp, where they are ideally positioned to respond to lipid antigen (447). Whereas NKT cells have been observed to arrest at the subcapsular sinus, splenic NKT cells arrest at the marginal zone 2 hours following antigen administration, suggesting rapid processing and presentation of antigen by APCs within the marginal zone permits the rapid induction of immune responses. Indeed, disruption of the marginal zone abrogated NKT cell responses to lipid antigen (447). Further interrogation of this phenomenon revealed SIGN-R1$^+$ macrophages were able to activate NKT cells *ex vivo*, however, activation was further enhanced when DCs were used to stimulate NKT cell hybridomas *ex vivo* (447). In the current study many of the downstream effector activities that were observed following vaccination in the MdLN were also seen in the spleen, including increased overall cellularity and increased numbers of activated T cells. Importantly, increases in anti-tumour T cells could be more readily examined in this organ, as well as changes in APC function.

The function of DCs can be manipulated with CTLA-4-Ig fusion proteins, where CTLA-4 binding to CD80 and CD86 has been show to limit the ability of DCs to stimulate T cells, an effect that has been attributed to induction of IDO production (148). Additionally, aggregation of Tregs, which express CTLA-4 constitutively, around DCs following TCR stimulation can occur in an LFA-1 dependent but CTLA-4 independent manner, leading to down-regulation of CD80 and CD86 expression on DCs. This down-regulation is dependent on CTLA-4 expression (448), with some suggesting a mechanism involving trans-endocytosis of surface CD80 and CD86 from APCs into intracellular compartments within Treg cells. Activation of both DCs and B cells *in vitro* was comparable, whether mice had been previously treated with α-CTLA-4 or not. Similarly, APC responses *in vivo* were not enhanced with the addition of α-CTLA-4. Taken together, these results suggest that improved APC licensing or function do not contribute towards the anti-tumour responses observed following α-CTLA-4 and vaccine immunotherapy. Perhaps this is unsurprising, as while blocking Treg:APC interactions is a plausible mechanism, supporting experimental evidence only exists in extremely artificial model systems (417, 418).

Having been unable to identify a role for increased APC licensing or APC function to contribute towards effective anti-tumour responses in mice receiving the combined treatment, attention was focused on the T cell response. A notable feature of the combined treatment was accumulation of lymphocytes in the spleen and MdLN, likely reflecting reduced cellular egress leading to accumulation of circulating cells within the secondary lymphoid organs. It is possible that the enhanced lymphocyte trapping that was a feature of the increased cellularity may improve responses to the vaccine by increasing the probability of APC:T cell contacts. The enlargement of secondary lymphoid organs in mice is a typical feature of antigen administration and is thought to occur through interactions between APCs and T cells recognising cognate antigen (449-451). In a murine model of herpes simplex virus (HSV) infection, $CD4^+$ T cells were found to be critical for controlling lymph node input (452). In this study, $CD4^+$ T cells were required for remodelling the LN afferent arteriole to a larger diameter. Additionally, draining lymph node enlargement following DC vaccination was dependent on MHC-II and CD40 signalling (452). While no difference in NKT cell activation was found when comparing vaccinated mice to mice receiving α-CTLA-4 and vaccination, the requirement for CD40 signalling for DC licensing and subsequent lymphocyte trapping could be a coordinated effort between both $CD4^+$ T cells and NKT cells in mice receiving α-CTLA-4 and vaccination.

The enlargement of lymphoid organs during an immune response has been attributed to vascular endothelial growth factor-C (VEGF-C) by activated APCs, which is required for lymphangiogenesis (453, 454). Increased lymphangiogenesis serves to build new lymphatic vessels, however lymphoid remodelling is also regulated by the fibroblast reticular cells (FRCs) which are found within the secondary lymphoid organs (455). The reticular network formed by FRCs acts to simultaneously provide strength to the tissue and space for movement while acting as a barrier for compartmentalisation of T and B cells (456). Additionally, the production of IL-7 by FRCs may provide survival signals to T, B and NKT cells while lymphocyte trapping is underway (457, 458). While $CD4^+$ T cells and FRCs have been shown to modulate cellular ingress into secondary lymphoid organs, expression of the S1P within efferent lymphatic vessels influences cellular egress.

As S1P induces transient desensitisation of the S1P receptor 1 (S1PR1) on lymphocytes; recent migrants that enter secondary lymphoid organs are relatively insensitive to the S1P gradient (459, 460). Taken together, it is conceivable that CTLA-4 blockade could have an effect on enhanced cellular ingress in a DC and $CD4^+$ T cell dependent manner and, reduced cellular egress as a result of continued insensitivity to S1P.

Some of the increased cellularity may be explained as a result of increased cellular proliferation following T cell priming. To address this, adoptive T cell transfer experiments were conducted which showed increased proliferation of donor cells following α-CTLA-4 administration, or α-CTLA-4 combined with vaccination. Interestingly, α-CTLA-4 treatment alone showed some activity in these assays, suggesting that the efficacy of the combined treatment may be the result of two activities; priming of anti-tumour T cells by the vaccine, and removal of CTLA-4-mediated checks on the proliferation of anti-tumour T cells. It remains to be established whether the same T cells were affected in each case. In agreement with previous studies, the $CD4^+$ T cell compartment was most affected with increased proliferation observed highlighting the role of CTLA-4 as an immune checkpoint (139, 461). Importantly, when phenotyped, the increased $CD4^+$ T cells were not $FoxP3^+$ Tregs.

Further characterisation of the T cells revealed an expansion of effector T cells, based on the expression of CD44 and CD62L, with these cells producing effector cytokines IFN-γ and TNF-α following *ex vivo* restimulation with tumour lysate. Due to the lack of a defined antigen in this system, it cannot be determined if the increased effector T cell pool within the spleens of α-CTLA-4 and vaccine treated mice is due to antigen specific expansion, however, the increased proportion of cells producing IFN-γ and TNF-α suggests that T cells recognise antigens within the tumour lysate.

4.5 Conclusions

Treatment with CTLA-4 blockade combined with vaccination induces a profound increase in cellularity in the lymphoid organs that is not achieved with either treatment alone. Within this response, there is clear evidence of an enhanced T cell response. This

may have resulted from two activities; priming of anti-tumour T cells by the vaccine, and removal of CTLA-4-mediated checks on the proliferation of anti-tumour T cells. Without knowing the antigens targeted, it is hard to determine whether the same T cells are affected in each case.

Chapter 5

5 Mechanisms underlying successful therapy with CTLA-4 blockade and vaccine immunotherapy

5.1 General Introduction

The results in the previous chapter had indicated an important role for T cells in the effective combination of vaccine and α-CTLA-4 to treat glioma. The ability of T cells to kill tumour cells is dependent on their intrinsic ability to perform effector functions that have a direct effect on tumour growth, or to recruit or activate additional cells, which are in turn capable of performing anti-tumour effector functions.

Activated T cells migrate towards effector sites by following chemokine gradients, with chemokine recognition driven by the expression of surface receptors on the cell surface (462). The expression of particular cell surface receptors facilitate the entry of T cells into specific tissues, and the site of immune priming can influence this receptor expression (463). The expression of VLA-4 (464) and LFA-1 (105) by T cells has previously been shown to be required for T cell adhesion and migration into the brain parenchyma. Alternatively, cells can undergo genetic reprogramming within the secondary lymphoid organs. For example, it has been shown that encephalitogenic T cells require passage through the lung draining lymph nodes, resulting in the upregulation of receptors to allow entry into the CNS (103).

Following migration to the effector site, T cells will encounter cognate antigen presented on MHC-I and MHC-II within the local environment, in the context of tumour cells themselves, or on the tumour stroma. If the cell has been appropriately activated within

the lymphoid organs, recognition of MHC-peptide complexes within the tissue results in the specific effector function (465). T cells induce contact dependent apoptosis, resulting in nuclear blebbing, altered cellular morphology and eventual DNA fragmentation. The target cell will shed its membrane as vesicles, and continue to degrade itself from within. Killing can be mediated by the interaction of FasL on an effector T cell with Fas on a target cell, which induces apoptosis, resulting in the induction of an intracellular caspase cascade that ultimately leads to the activation of nucleases responsible for DNA fragmentation (109). In addition, cytotoxic T cells carry intracellular stores of perforins and granzymes that can trigger apoptosis (110, 111). The formation of a cytolytic synapse ensures both perforin and granzymes are delivered to a specific target cell, and acts to limit collateral damage to bystander cells. Following the formation of a cytotoxic synapse between an effector and target cell and the release of perforin, granzymes are released into the cytosol of the target cell, resulting in caspase-3 cleavage and activation of the apoptosis pathway (112).

Furthermore, cytokines released by both effector T cells and other immune cells can induce cell death. Many cell types including T cells; NK cells, macrophages and neurons produce TNF-α. (114-116). Secreted TNF-α binds to the TNF receptors resulting in an intracellular signalling cascade, finally concluding in the recruitment of caspase-8, leading to caspase-mediated apoptosis (117). As well as inducing apoptosis, TNF-α release creates a pro-inflammatory environment; activating innate immune cells and also induces vascular permeability, easing immune cell extravasation (118, 119). TRAIL is a member of the tumour necrosis factor superfamily and is produced by most cells. Similarly to TNF-α, caspase-8 mediated apoptosis is induced when TRAIL binds receptors DR4 and DR5 (120). Similarly, IFN-γ is expressed by a diverse array of cell types and inhibits tumour angiogenesis and has immunomodulatory functions, including inducing the upregulation of MHC-I and MHC-II expression on tumour cells, thereby enhancing tumour cell susceptibility to T cell mediated cytotoxicity (73, 121).

While $CD8^+$ T cells have been studied for their tumouricidal abilities, it is thought $CD4^+$ T cells can also perform cytotoxic functions. The identification of a population of $CD4^+$

cytotoxic T cells was described using murine lymphocyte cultures in an *in vitro* MLR. Cells were observed to lyse both Fas^+ and Fas^- target cells and were seen to develop in Fas deficient mice. Conversely, $CD4^+$ T cells derived from perforin deficient mice had decreased lytic capacity, indicating a primarily perforin-dependent mechanism (466). In agreement, $CD4^+$ T cells with cytotoxic capacity have been isolated from human patients and have been observed to display a Th1 cytokine profile. Neutralising antibodies to Fas had no bearing on decreasing target cell lysis in an *in vitro* ^{51}Cr-release assay (467). Further analysis of Fas-deficient families revealed granule exocytosis, but not Fas-FasL interactions to be the main pathway of cytotoxicity by alloantigen-specific $CD4^+$ T cells (468). Intriguingly, $CD4^+$ T cells have been shown to readily infiltrate the CNS (469), and are therefore likely to be required for eliciting anti-tumour immune responses.

While T cells play a vital role in provoking anti-tumour immunity, successful tumour clearance relies on the dynamic coordination of both adaptive and innate immune cells. Th1 cells activate innate immune cells including macrophages recruited from the blood and microglia, a brain resident macrophage population, through MHC-II and CD40-CD40L interactions (89, 90). This is thought to be a reciprocal interaction, as microglia have been shown to present antigen to stimulate T cell responses, albeit weakly compared to thymocytes (470). In addition to lymphocytes, histological analysis of human glioma tissue samples has revealed infiltration of both macrophages and microglia (471-473). Tumour associated macrophages are associated with high tumour grade and commonly infiltrate glioma and while the numbers and distribution of these cells vary with treatment and tumour severity, both macrophages and microglia have been identified in 85-100% of glioma tumour samples (474). Recruitment of macrophages can occur via the chemokines CXCL2 and CXCL5, which are produced by monocytes and macrophages and act as a chemotactic signal for granulocytes (475-478). The production of CXCL5 can be increased in the presence of IL-1 or TNF-α (478). Further, CCL3 is also known as macrophage inflammatory protein 1α (MIP1α) and is involved in acute inflammation and the recruitment of polymorphonuclear leukocytes (475). The cytokine IL-1β is an important mediator inflammation and is involved in an array of cellular processes including proliferation, differentiation and apoptosis (479, 480).

Macrophages are recognised as playing a role in tumour regression or progression based on their phenotypic and functional characteristics. A functional spectrum of macrophages exists, with the populations at either pole described as "M1" and "M2". Classically activated macrophages, or M1 skewed macrophages arise following interactions with Th1 $CD4^+$ T cells, and exposure to TNF-α and IFN-γ in a pro-inflammatory environment (122-124) and can play an anti-tumour role. M1 macrophages also produce high levels of pro-inflammatory cytokines including TNF-α, IL-1, IL-6 and IL-12 and increase intracellular concentrations of nitrogen and oxygen radicals. As previously mentioned, TNF-α is an important mediator of apoptosis and vascular permeability (481, 482). Secretion of IL-6 by macrophages following trauma or tissue damage leads to inflammation (483) and induces the differentiation of monocytes from DCs to macrophages (484). In cancer, IL-6 has been shown to have pro-inflammatory and anti-inflammatory roles (485). Furthermore, M1 macrophages can elicit effector function through the production of nitric oxide (NO) from L-arginine and increase expression of MHC-I and MHC-II to further activate T cells (127).

Certainly, in the presence of a tumour, both macrophages and microglia can adopt an M2, or pro-tumourigenic phenotype and function, which limits the ability of T cells to exert anti-tumour responses. Similarly, the recruitment of macrophages from the blood into the tumour can play a role in shaping the immune response. Additionally, M2 skewed macrophages can play a pro-tumourigenic role, limiting inflammation by secretion of chemokines and cytokines including TGF-β as well as inducing immune cell death via secretion of oxygen free radicals (486). Arginase inhibits NO production and allows the production of ornithine, a precursor of hydroxyproline and polyamines. Consequently, arginase-producing macrophages have been associated with wound-healing (487, 488).

Furthermore, M2 macrophages express the mannose receptor and IL-10, which result in M2 macrophages playing a role in tissue remodelling, immune modulation and tumour progression (127, 128). The mannose receptor CD206 is a c-type lectin primarily found on the surface of macrophages. Depletion of CD206 expressing macrophages has been

shown to exacerbate endotoxemic lung injury in mice, suggesting CD206 expression defined a population of anti-inflammatory macrophages (489). The lectin Ym1 is expressed in an IL-13 dependent manner and has been shown to promote Th2 cytokine expression from $CD4^+$ T cells (488, 490). RELM-α expressing macrophages have been implicated in playing an immunoregulatory role within the lungs of mice treated with *Schistosoma*, with RELM-α deficient mice displaying exacerbated disease (491).

In creating an inflammatory environment, macrophages promote angiogenesis, facilitating both tumour growth and metastatic potential (131, 132). Indeed, inhibition of the macrophage colony-stimulating factor (CSF-1) receptor (CSF-1R) has been shown to block tumour growth and regress glioma in a spontaneous murine glioma model (492). Differentiation of bone marrow-derived macrophages (BMDMs) in the presence of glioma-conditioned media stimulates the expression of M2-associated genes and this expression is diminished when the CSF-1R is blocked (492).

Macrophages are also involved in phagocytosing blood borne antigens within the secondary lymphoid organs and inducing immune responses. It is therefore conceivable that macrophages play a role in both inducing adaptive immune responses, as well as co-ordinating immune response at effector sites. As innate cells, macrophages respond rapidly to insult or injury. Accordingly, recent evidence has suggested a role for macrophages in facilitating the entry of T cells into the brain (493, 494).

Following recruitment of T cells to the brain, T cells must transverse the BBB to enter the brain. The final physical barrier before complete cerebral penetrance is the glia limitans, which is biochemically distinct from the endothelial basement membrane. Here, the laminins α1 and α2 are expressed, in contrast to the α4 and α5 laminins of the endothelial cells (495). As previously mentioned, primary cell cultures from GBM patients can actively degrade tight junctions *in vitro*, potentiating the ability of T cells to enter the brain (107). Intriguingly, encepalitogenic T cells are unable to interact with laminins α1 and α2 (495), however penetration of these the brain by these cells during EAE correlates with sites of focal activity of matrix metalloproteinases (MMPs). During EAE disease

progression, MMP-2 and MMP-9 cleave β-dystroglycan, a cell surface receptor which anchors astrocytes to the basement membrane, compromising the integrity of the BBB (494). In support, overexpression of CCL2 within the CNS parenchyma induces immune cell recruitment across the BBB into the perivascular space, but induction of MMP-2 and MMP-9 is required to liberate lymphocytes from the perivascular space and into the CNS parenchyma (496, 497). Altogether, macrophages within the lymphoid organs may contribute to the induction of adaptive immune response, with perivascular macrophages ensuring effective migration of T cells into the brain parenchyma, permitting tumour clearance.

The role of T cells in anti-tumour immunity is therefore complex, and may involve direct tumour killing, or the coordination of killing function in other effector cells. To assess how T cells were involved in the effective combination of vaccine and α-CTLA-4 in the previous chapter, here the type of immune response induced following treatment was examined in some detail, including within the brain of tumour bearing mice.

5.2 Aims

The experiments in this chapter were designed to delineate the mechanism of action behind successful therapy with α-CTLA-4 and vaccine immunotherapy.

The specific aims were:

1. To examine the immune cells infiltrating intracranial tumours
2. To analyse the effector cells responsible for tumour clearance following α-CTLA-4 and vaccination
3. To determine the killing mechanism utilised by the effector cells

5.3 Results

5.3.1 Enhanced immune infiltrate featuring T cells in tumours of animals subject to vaccination with CTLA-4 blockade.

Characterisation of peripheral immune responses in non tumour-burdened mice revealed the induction of potent $CD4^+$ T cell responses. To explore the induction of adaptive immune responses in tumour burdened mice, an experiment was conducted to analyse the gross pathology of tumours in response to vaccine therapy, α-CTLA-4 administration, or combination therapy. Mice were intracranially challenged and treated with α-CTLA-4, vaccination, or combined therapy one week later. Brains were harvested one week following therapy and fixed in formalin before being subjected to immunohistological staining. Staining for CD3 revealed T cell infiltration throughout the tumour and surrounding stroma in all groups (Fig 5.1 A). Quantification of $CD3^+$ cells was then conducted and compared to mean tumour size to calculate T cell density. Here, α-CTLA-4 combined with vaccine therapy resulted in a significantly higher density of T cells per tumour compared to tumour only controls, as well as α-CTLA-4 and vaccination as monotherapies (Fig 5.1 B).

5.3.2 Increased macrophages, microglia and $CD4^+$ T cells within the brain of α-CTLA-4 and vaccine treated mice

To further explore the phenotype of infiltrating immune cells, the whole brain was assessed by flow cytometry seven days after therapy. No obvious increase in immune infiltration was observed following α-CTLA-4 treatment as a monotherapy, and a trend towards an increased number of $CD45^+$ immune cells was observed in brains of mice receiving vaccine treatment compared to tumour only controls. However statistically significant increases of $CD45^+$ immune cells were observed when α-CTLA-4 was combined with vaccine therapy. The immune infiltrate was predominantly comprised of macrophages ($CD45^+CD11b^+$), microglia ($CD45^{mid}$, $CD11b^+$) and lymphocytes ($CD45^+CD11b^-$) (Fig 5.2 A, B). Within the T cell compartment, $CD4^+$ T cells were mostly highly represented, accounting for 60-75% of all T cells within the brain (Fig 5.2 C). In

contrast, no differences in the numbers of $CD8^+$ T cells or Tregs was observed within any of the treatment regimes (Fig 5.2 C).

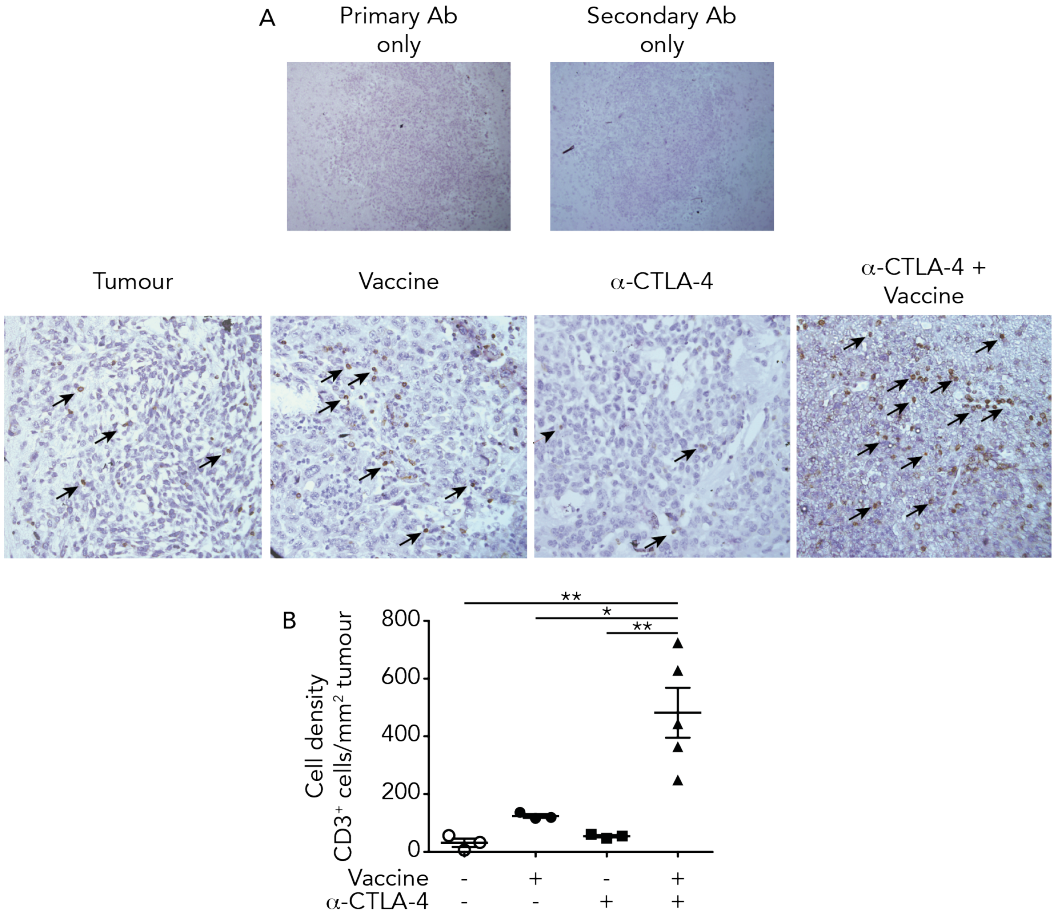

Figure 5.1 Increased intratumoural T cell infiltration following CTLA-4 blockade and vaccination. Mice were intracranially challenged with GL261 and treated with α-CLTA-4 on day 6, vaccine on day 7, or received combination treatment. On day 20, mice were culled and brains were harvested and fixed in 4% paraformaldehyde before being paraffin embedded for histological analysis. (A) Photomicrographs of formalin-fixed paraffin-embedded coronal brain sections. Arrowheads indicate $CD3^+$ cells. (B) Mean density of $CD3^+$ cells within the tumour ± SEM (n = 3-5) was calculated per treatment group * $P < 0.05$, ** $P < 0.01$ (One-way ANOVA with Bonferroni post-test). Results are representative of one experiment.

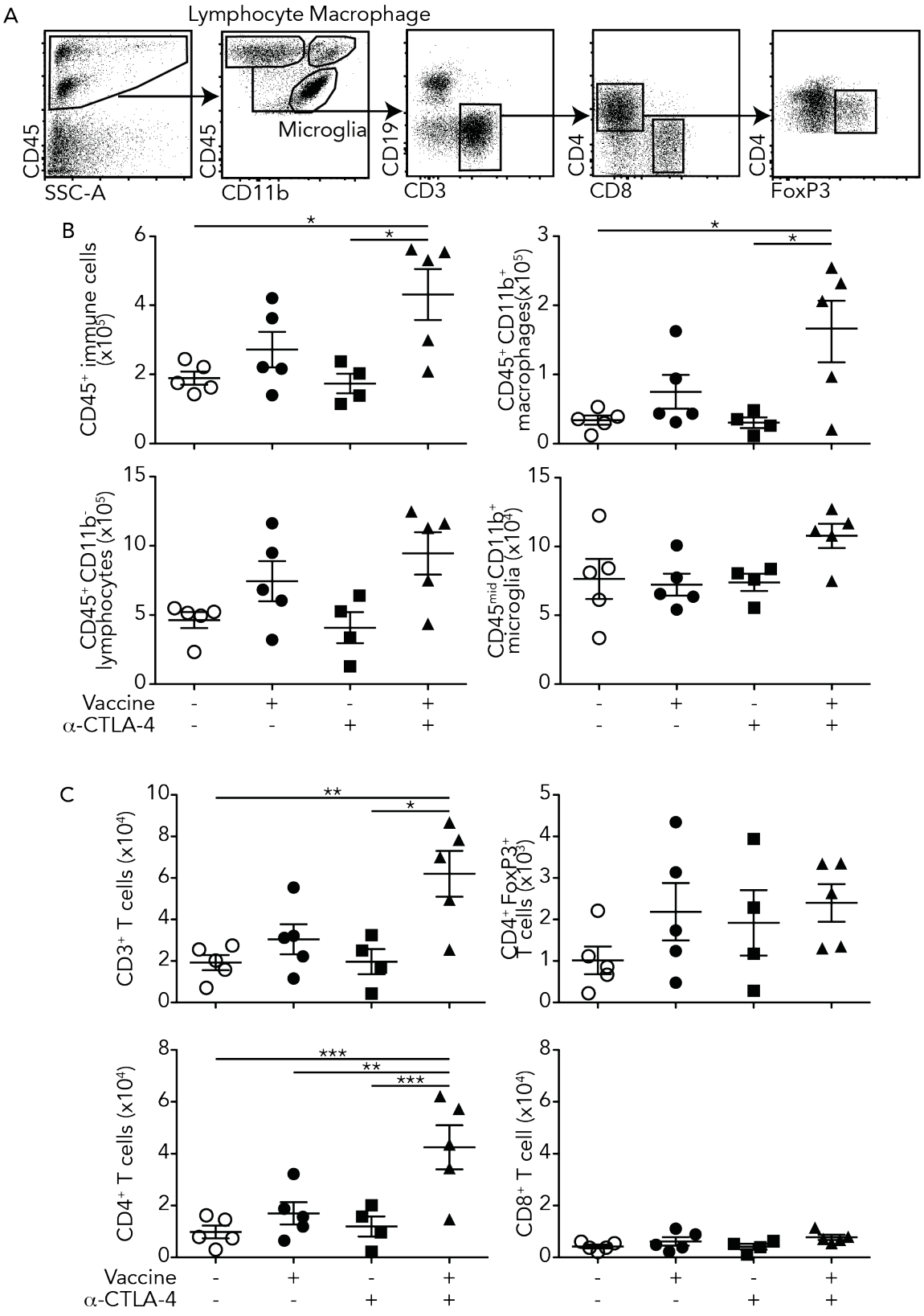
A
Lymphocyte Macrophage
CD45
SSC-A
CD45
CD11b
Microglia
CD19
CD3
CD4
CD8
CD4
FoxP3
B
CD45+ immune cells (x10^5)
CD45+ CD11b+ macrophages(x10^5)
CD45+ CD11b- lymphocytes (x10^5)
CD45mid CD11b+ microglia (x10^4)
Vaccine
α-CTLA-4
C
CD3+ T cells (x10^4)
CD4+ FoxP3+ T cells (x10^3)
CD4+ T cells (x10^4)
CD8+ T cell (x10^4)
Vaccine
α-CTLA-4

Figure 5.2 Therapeutic success of combining vaccination with CTLA-4 blockade is associated with accumulation of CD4+ T cells in brain.

Mice were intracranially challenged with GL261 and treated with α-CLTA-4 on day 6, vaccine on day 7, or received combination treatment. On day 14, mice were culled and brains were harvested for flow cytometry. (A) Gating strategy used to quantify brain-infiltrating lymphocytes. (B) Mean number ± SEM of $CD45^+$ immune cells, $CD45^+$ $CD11b^+$ macrophages and $CD45^{mid}$ $CD11b^+$ microglia ($n = 5$) was calculated per treatment group * $P < 0.05$ (One-way ANOVA with Bonferroni post-test). Results are representative of two independent experiments. (C) Mean number ± SEM of $CD3^+$ T cells, $CD4^+$ $FoxP3^+$ Tregs, $CD4^+$ and $CD8^+$ T cells ($n = 5$) was calculated per treatment group * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ (One-way ANOVA with Bonferroni post-test). Results are representative of two independent experiments.

5.3.3 *Therapeutic efficacy of vaccine and α-CTLA-4 is CD4+ T cell dependent*

Given the robust infiltration of T cells within the brain, the dependency on T cells for tumours clearance was next examined. Survival experiments were conducted in mice deficient in MHC-II or TAP, which are unable to mount $CD4^+$ or $CD8^+$ T cell responses respectively. Mice were challenged intracranially and treated one week later with α-CTLA-4 or α-CTLA-4 combined with vaccination. In the absence of $CD4^+$ T cells, therapeutic efficacy was lost, with mice treated with the combined therapy succumbing to disease at a rate similar to tumour only controls (Fig 5.3 A). Strikingly, in the absence of $CD8^+$ T cells, mice were able to mount an effective anti-tumour response (Fig 5.3 B). Taken together, these results suggest that, vaccination can be improved with CTLA-4 blockade, resulting in effective $CD4^+$ T cell mediated responses that are critical for the elimination of intracranial glioma.

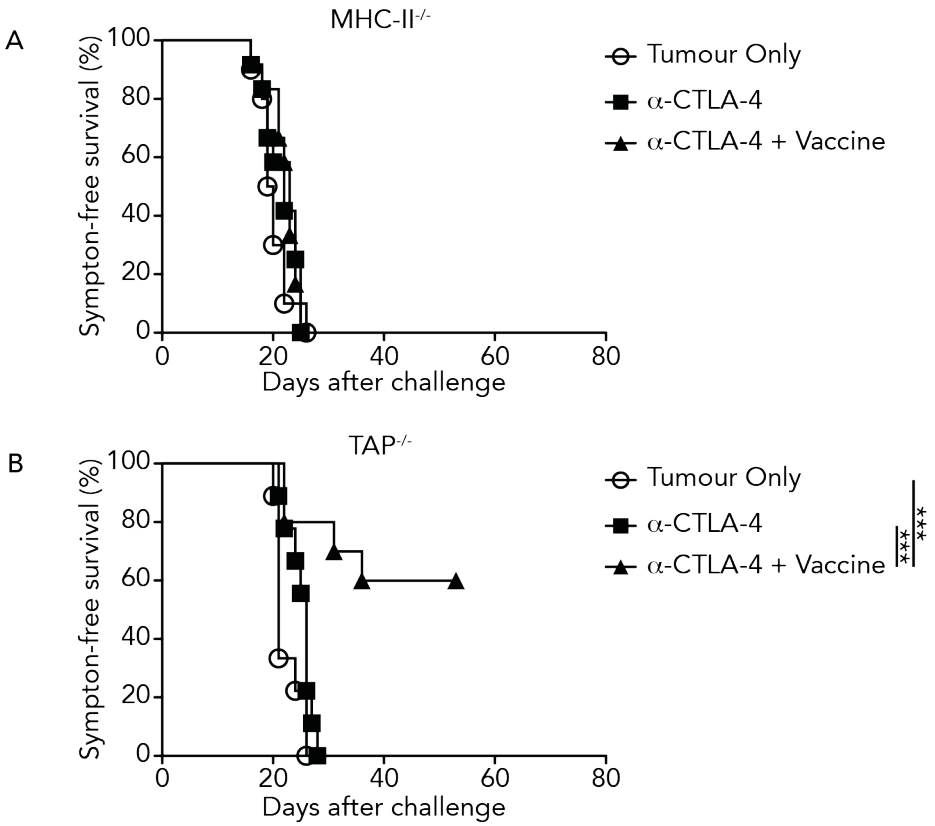

Figure 5.3 Successful therapeutic efficacy is dependent on CD4+ T cells.
Mice were intracranially challenged with GL261 and monitored for symptoms of tumour development, including > 10% weight loss and overt behavioural changes such as hunching or reduced activity. (A) Symptom-free survival curves for MHC-II$^{-/-}$ (n = 10 per group) with intracranial tumours treated with α-CTLA-4 on day 6, or α-CTLA-4 on day 6 and vaccine on day 7. Untreated MHC-II$^{-/-}$ (n = 10) served as tumour only controls. Results represent two combined experiments. (B) Symptom-free survival curves for TAP$^{-/-}$ mice (n = 10 per group) with intracranial tumours treated with α-CTLA-4 on day 6, or α-CTLA-4 on day 6 and vaccine on day 7. Untreated TAP$^{-/-}$ (n = 10) served as tumour only controls *** $P < 0.005$ (Log-Rank (Mantel Cox) test). Results represent two combined experiments.

5.3.4 *IFN-γ and perforin are required for effective α-CTLA-4 and vaccination therapy*

The ability of T cells to kill tumour cells is dependent on their intrinsic ability to perform effector functions, or to recruit or activate additional cells, which are in turn capable of performing effector functions. To assess the contribution of the effector molecules IFN-γ and perforin to α-CTLA-4 and vaccine therapeutic efficacy, C57BL/6 wild-type mice, or mice deficient for IFN-γ (IFN-γ KO) and perforin (Perforin KO) mice on a C57BL/6 background were challenged intracranially and treated with α-CTLA-4 on day 6 and vaccination on day 7. Additionally, some mice remained untreated to assess tumour development in the absence of IFN-γ or perforin. Mice which were challenged but remained untreated succumbed to disease at a similar rate to wild-type C57BL/6 mice (Fig 5.4 A). In the absence of either IFN-γ or perforin, the efficacy of combined vaccine and checkpoint blockade was less effective, with some animals succumbing to tumour growth early after treatment; only 40 and 44% of mice survived past day 60 in IFN-γ or perforin knockouts respectively, whereas, 90% of WT mice survived past day 60 (Fig 5.4 B). This suggests there is an additive effect between IFN-γ and perforin, with each effector mechanism responsible for the clearance of intracranial tumours, with the absence of either IFN-γ or perforin unable to completely eliminate the survival afforded by α-CTLA-4 and vaccination in C57BL/6 mice.

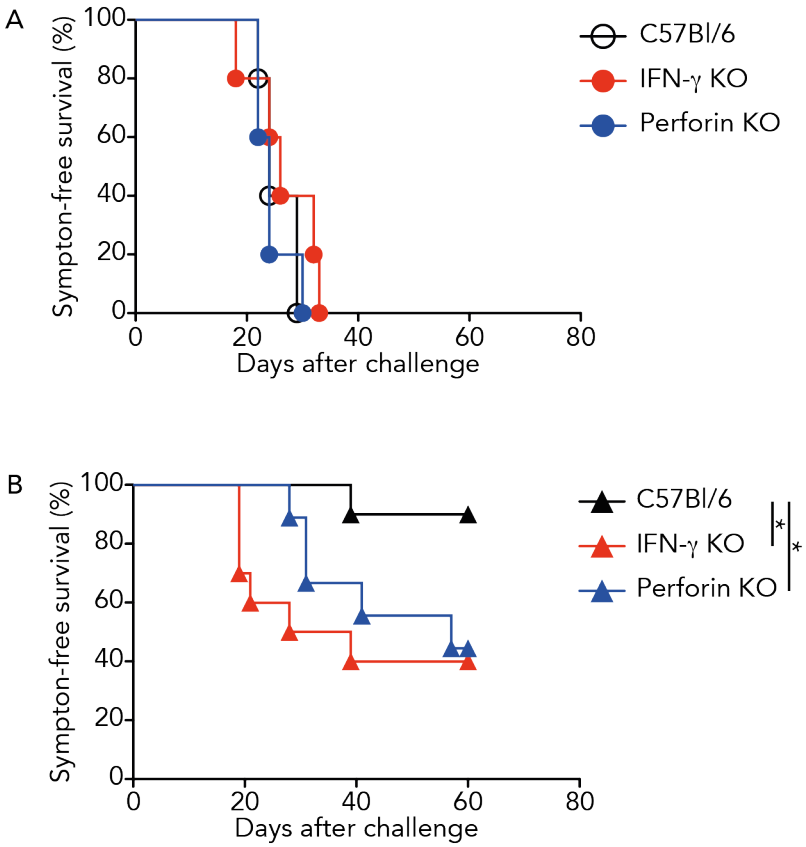

Figure 5.4 Therapeutic efficacy is partially dependent on IFN-γ and perforin.
Mice were intracranially challenged with GL261 and monitored for symptoms of tumour development, including > 10% weight loss and overt behavioural changes such as hunching or reduced activity. (A) Symptom-free survival curves for C57BL/6, IFN-$\gamma^{-/-}$ and $Pfp^{-/-}$ mice (n = 5 per group) with intracranial tumours. Results are representative of two independent experiments. (B) Symptom-free survival curves for C57BL/6, IFN-$\gamma^{-/-}$ and $Pfp^{-/-}$ mice (n = 10 per group) with intracranial tumours treated with α-CTLA-4 on day 6 and vaccine on day 7. Results are representative of two independent experiments * $P < 0.05$ (Log-Rank (Mantel Cox) test).

5.3.5 *Infiltration of T cells into brain in response to combined treatment is reduced in IFN-γ but not Perforin deficient mice*

As therapeutic efficacy was hindered in the absence of either IFN-γ or perforin, the induction of immune responses in response to the combined therapy was examined in each strain of mice and compared to WT control mice. Mice were challenged intracranially as described previously and received α-CTLA-4 and vaccine therapy on day 6 and 7 respectively. The splenocytes, as well as the tumour infiltrating lymphocytes, were examined one week following therapy. Reduced numbers of splenocytes were observed in $Pfp^{-/-}$ mice, compared to WT, while the number of splenocytes was trending towards a decrease in IFN-$\gamma^{-/-}$ mice (Fig 5.5 B). In addition, a reduction in the overall numbers of $CD4^+$ T cells was observed in IFN-$\gamma^{-/-}$ and $Pfp^{-/-}$ mice, compared to WT controls, with a similar trend observed within the $CD8^+$ compartment (Fig 5.5 B, C). Despite the overall reduction in $CD4^+$ T cell numbers, the number of Tregs was comparable between all groups. Conversely, the number of $CD44^+$ effector $CD4^+$ T cells was trending towards a decrease in the absence of IFN-γ or perforin, with a significant reduction of $CD8^+$ effector T cells observed in the absence of perforin (Fig 5.5 B, C). These results suggest immune priming was potentially compromised in the absence of IFN-γ or perforin following CTLA-4 blockade and vaccination, resulting the induction of a tolerogenic phenotype.

Within the brain, no obvious difference in the recruitment of immune cells to the brain could be observed, as the proportions and total number of live $CD45^+$ leukocytes was comparable between all groups (Fig 5.6 A, B). However, in the absence of IFN-γ, a reduced proportion of $CD45^+$ $CD11b^-$ lymphocytes was observed relative to WT, with a concurrent increase in the proportion of $CD45^+$ $CD11b^+$ macrophages. Despite this shift in the proportions of cells infiltrating the brain, no significant differences could be observed in the overall cellularity of these populations within the brain (Fig 5.6 A, B).

Within the lymphocyte population, proportionally fewer $CD4^+$ T cells were observed in the absence of IFN-γ, with trends towards a reduction in both Tregs and $CD8^+$ T cells,

suggestive of a failure to recruit T cells to the brain. No differences in the total number of $CD4^+$, Tregs nor $CD8^+$ T cells was observed however, nor were significant differences were seen in Pfp KO mice relative to WT. (Fig 5.7 A, B). These results, together with the results observed in the spleen suggest ineffective immune priming and subsequent induction of effector function may underlie the inability of IFN-$\gamma^{-/-}$ and $Pfp^{-/-}$ mice to clear tumours.

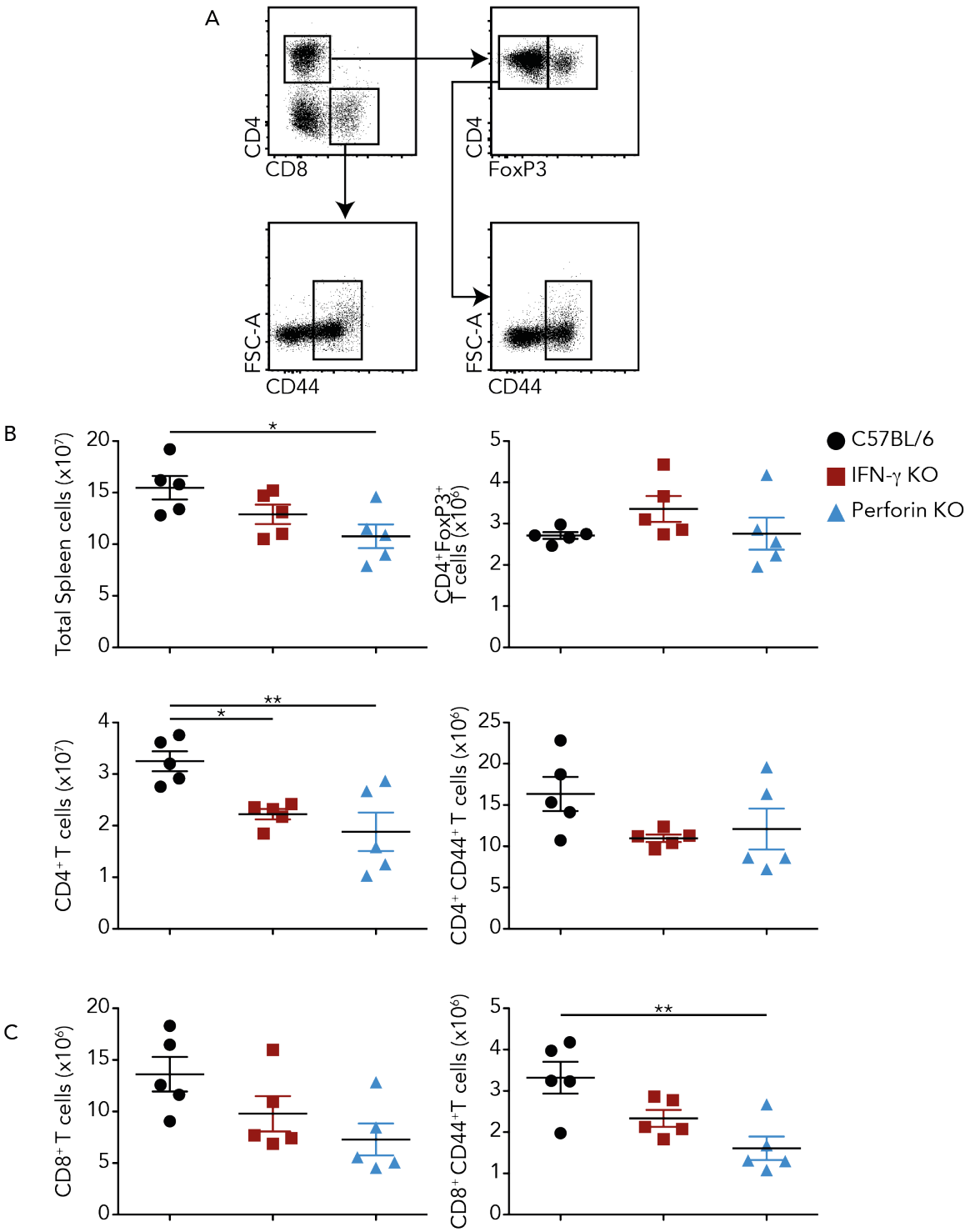
A
CD4
CD8
CD4
FoxP3
FSC-A
CD44
FSC-A
CD44
B
C57BL/6
IFN-γ KO
Perforin KO
Total Spleen cells (x10⁷)
CD4⁺FoxP3⁺ T cells (x10⁶)
CD4⁺ T cells (x10⁷)
CD4⁺ CD44⁺ T cells (x10⁶)
C
CD8⁺ T cells (x10⁶)
CD8⁺ CD44⁺T cells (x10⁶)

Figure 5.5 Assessment of immune priming within the spleen of tumour bearing mice treated with CTLA-4 blockade and vaccination.

C57BL/6, IFN-$\gamma^{-/-}$ and $Pfp^{-/-}$ mice were intracranially challenged with GL261 and treated with α-CLTA-4 on day 6 and vaccine on day 7. On day 14, spleens were harvested and cell populations analysed by flow cytometry. (A) Gating strategy used to quantify Tregs and effector T cells (B) Mean proportion ± SEM of $CD4^+$ T cells, $CD4^+$ $FoxP3^+$ Tregs, and $CD4^+$ $CD44^+$T cells (n = 5) was calculated per treatment group ** $P < 0.01$ (One-way ANOVA with Bonferroni post-test). Results are representative of two independent experiments. (C) Mean proportion ± SEM of $CD8^+$ T cells and $CD8^+$ $CD44^+$T cells (n = 5) was calculated per treatment group * $P < 0.05$ (One-way ANOVA with Bonferroni post-test). Results are representative of two independent experiments.

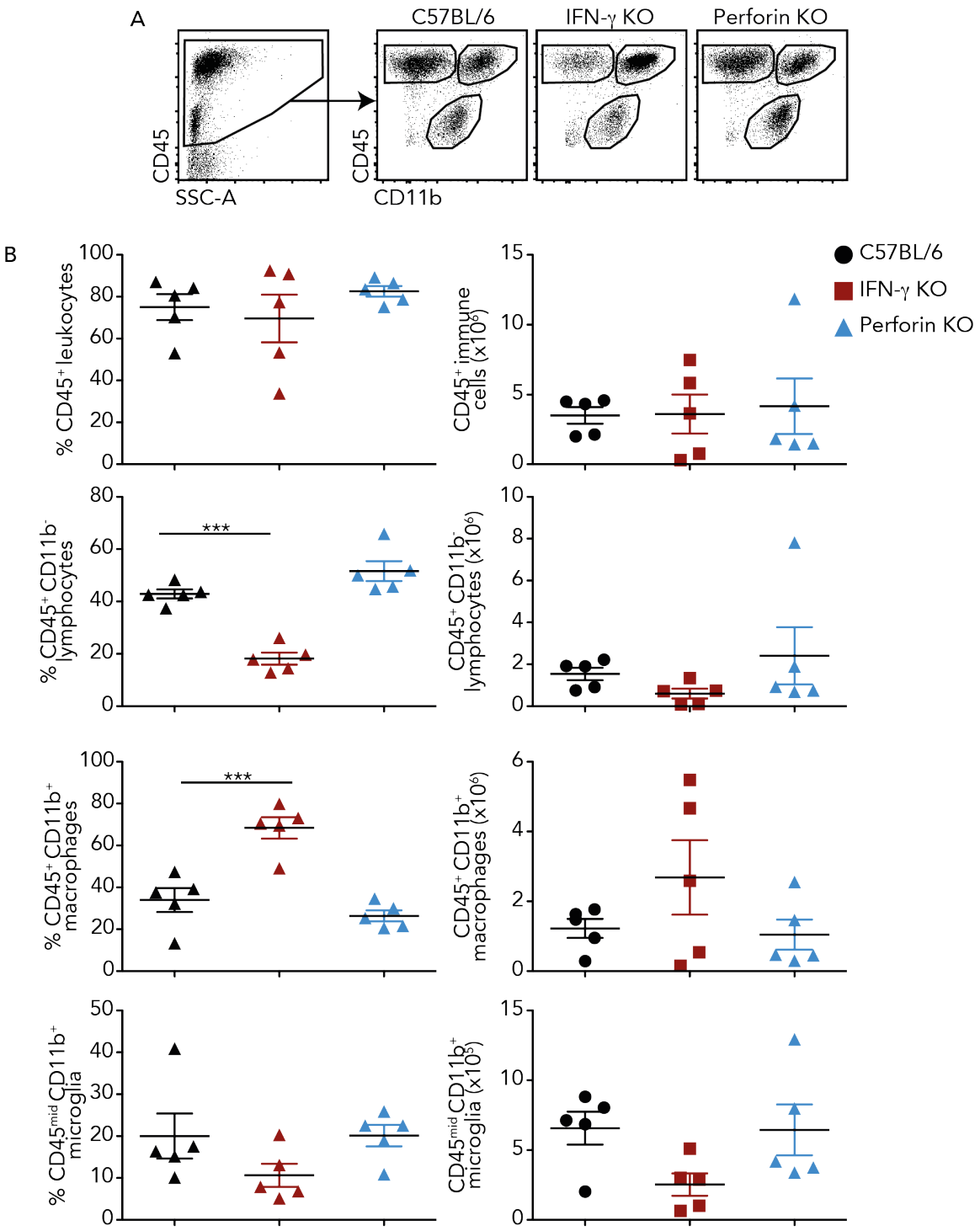

Figure 5.6 Assessment of brain infiltrating lymphocytes in IFN-γ and perforin deficient mice treated with vaccination and CTLA-4 blockade.

C57BL/6, IFN-$\gamma^{-/-}$ and $Pfp^{-/-}$ mice were intracranially challenged with GL261 and treated with α-CLTA-4 on day 6 and vaccine on day 7. On day 14, mice were culled and brains were harvested for flow cytometry. (A) Gating strategy used to quantify brain-infiltrating lymphocytes. (B) Mean proportion ± SEM of $CD45^+$ immune cells, $CD45^+$ $CD11b^+$ macrophages and $CD45^{mid}$ $CD11b^+$ microglia (n = 5) was calculated per treatment group *** $P < 0.005$ (One-way ANOVA with Bonferroni post-test). Results are representative of two independent experiments.

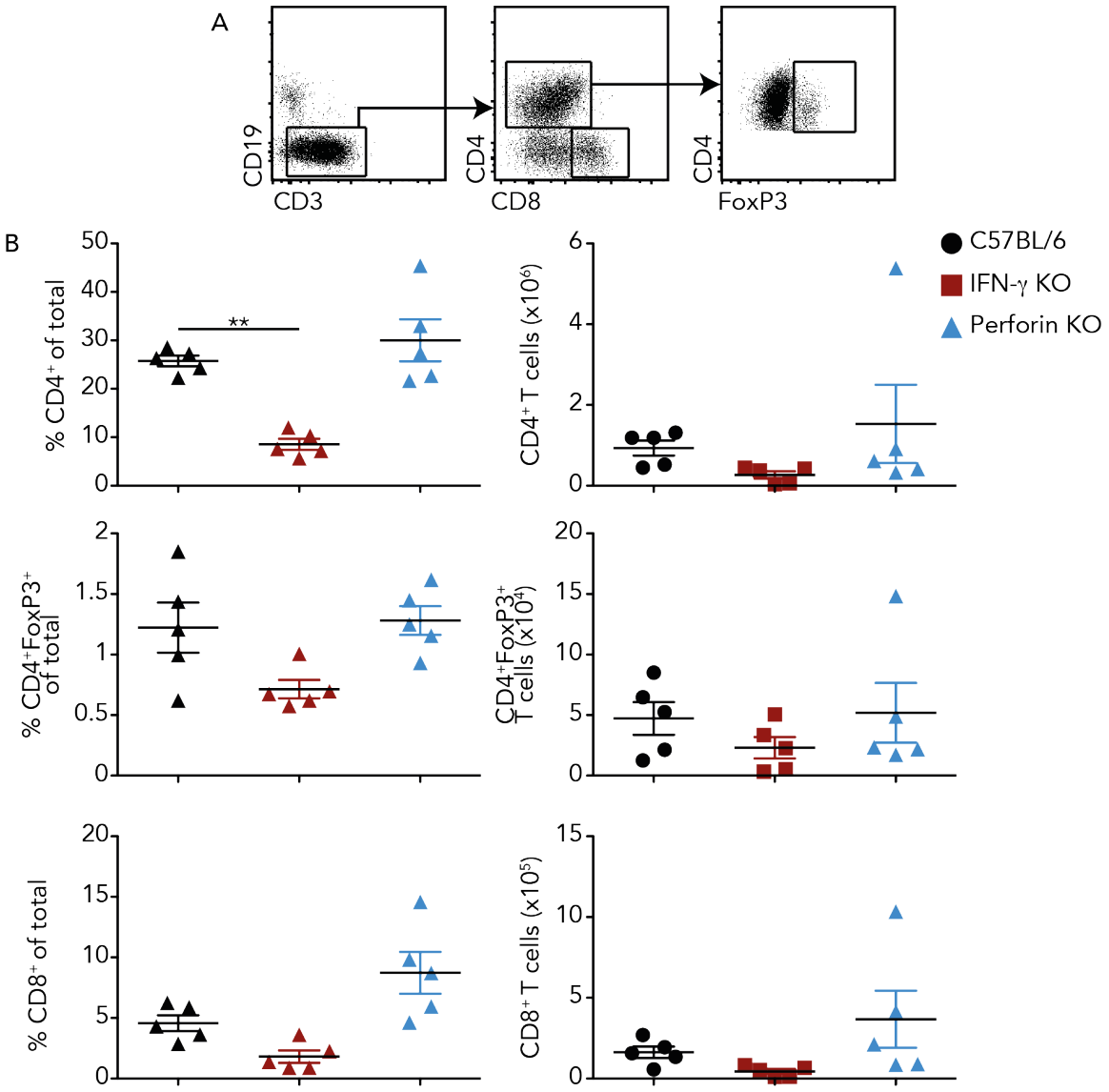

Figure 5.7 Assessment of brain infiltrating lymphocytes in IFN-γ and perforin deficient mice treated with vaccination and CTLA-4 blockade.

C57BL/6, IFN-γ$^{-/-}$ and Pfp$^{-/-}$ mice were intracranially challenged with GL261 and treated with α-CLTA-4 on day 6 and vaccine on day 7. On day 14, mice were culled and brains were harvested for flow cytometry. (A) Gating strategy used to quantify brain-infiltrating lymphocytes. Examples are pregated on CD45^{+} CD11b^{-} lymphocytes. (B) Mean proportion ± SEM of CD4^{+} T cells, CD4^{+} FoxP3^{+} Tregs, and CD8^{+} T cells (n = 5) was calculated per treatment group ** $P < 0.01$, *** $P < 0.005$ (One-way ANOVA with Bonferroni post-test). Results are representative of two independent experiments.

5.3.6 *Phenotype of the CD11b$^+$ cells within the brain shows both M1 and M2 skewed responses*

Tumour infiltrating macrophages and microglia were increased significantly in mice that had the combined treatment. It is known that these cells can be skewed towards a M2 phenotype, which is regarded as pro-tumourigenic, or perhaps in the presence of pro-inflammatory stimuli can acquire an anti-tumourigenic M1 phenotype. To assess the phenotypic changes associated with the macrophages and microglia, mice were challenged and treated with α-CTLA-4 and vaccination as previously described, or remained untreated. Brains were harvested 7 days following therapy and pooled before macrophages and microglia were sorted via fluorescence activated cell sorting (FACS) based on the expression of CD11b and CD45, allowing the discrimination between CD45^{hi} macrophages and CD45^{mid} microglia (498, 499). Purified cells were then subjected to RNA isolation and subsequent cDNA generation before genes associated with chemotaxis, M1 skewing or M2 skewing were assessed by quantitative-PCR. The relative expression of each gene was assessed relative to 18S control levels and then assessed as fold change over sham-challenged mice.

Macrophages isolated from tumour-bearing mice treated with α-CTLA-4 and vaccine had higher relative expression of the cytokine IL-1β, and the chemokines CXCL2 and CCL3, compared to tumour only mice, with a similar pattern of gene expression observed in tumour bearing mice treated with vaccine only. Additionally, vaccine only mice also had higher expression of CXCL5 compared to both tumour only and α-CTLA-4 and vaccine treated mice (Fig 5.8 A). The relative expression of the M2 markers Arg1, RELM-α, Ym1 and CD206 were decreased in α-CTLA-4 and vaccine treated mice compared to tumour only mice and mice treated with vaccine only. (Fig 5.8 B). Accordingly the relative expression of the M1 markers TNF-α, IL-6 and iNOS was increased in mice that received CTLA-4 blockade and vaccine therapy, or vaccine only over tumour only (Fig 5.8 C). Therefore, macrophage gene expression generally reflected a bias towards an M1 phenotype following therapy.

Interrogation of gene expression within the $CD45^{mid}$ microglia revealed increased levels of IL-1β, CXCL2, CXCL5 and CCL3 in α-CTLA-4 and vaccine treated mice, compared to vaccine only mice (Fig 5.9 A). Similarly, the relative expression of both Arg1 and RELM-α were decreased in α-CTLA-4 and vaccine treated mice compared to tumour only and vaccine only mice. Conversely, the relative expression of Ym1 and CD206 was increased, suggesting heterogeneity between M1 and M2 populations within the microglia of these mice. (Fig 5.9 B). Expression of TNF-α and IL-6 was increased in combination treated mice compared to vaccine only and tumour only mice, however, iNOS was only increased in α-CTLA-4 and vaccine compared to vaccine only mice (Fig 5.9 C). The distinct gene expression profiles of macrophages and microglia in this setting may be a reflection of the role each cell is playing throughout the course of anti-tumour immunity. While M1 macrophages are associated with tumour clearance, the heterogeneous gene expression revealed within microglia, consistent with M2 bias, could mean these cells act in parallel to limit overt local inflammation and to promote tissue repair (500, 501).

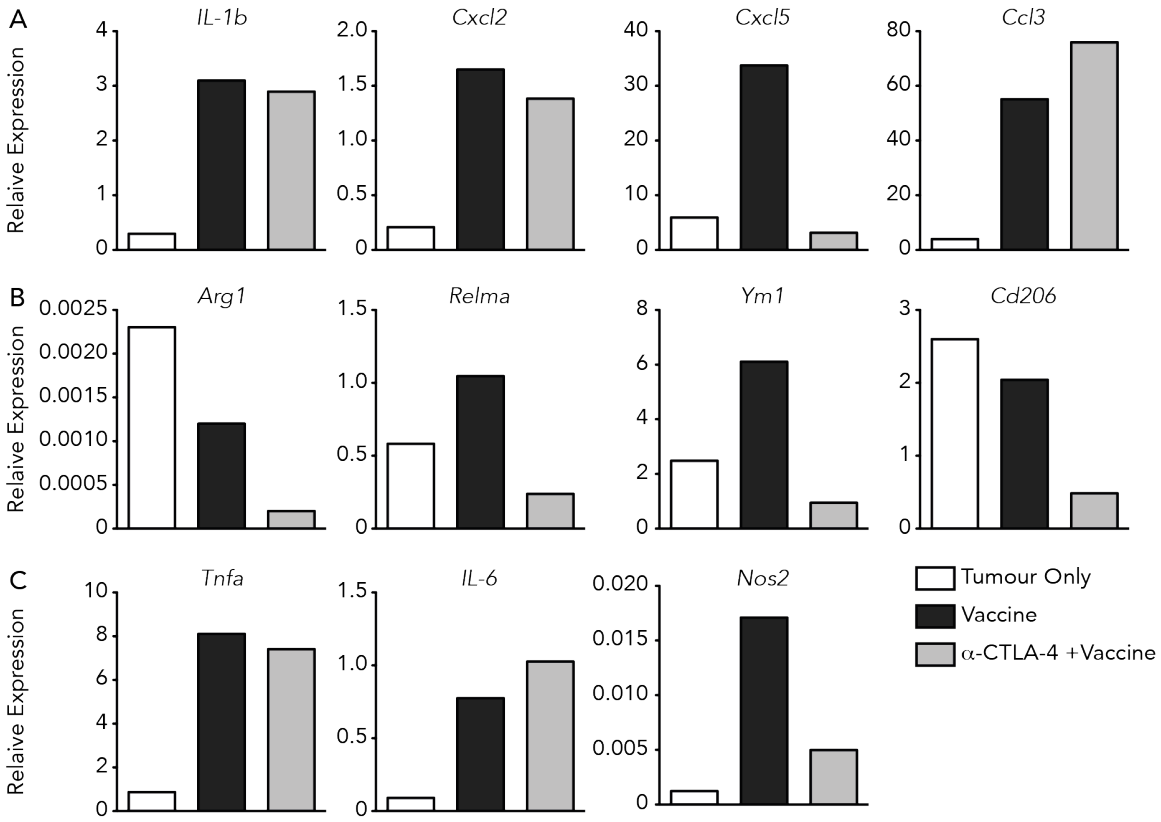

Figure 5.8 Increased M1 polarised macrophages within the brain following vaccine and CTLA-4 blockade.

Mice were intracranially challenged with GL261 and treated with vaccination on day 7, vaccination on day 7 combined with α-CTLA-4 on day 6, or remained untreated. On day 14, mice were culled and brains were harvested and samples were combined for FACS. As controls, sham injected mice received PBS in place of tumour challenge. RNA was isolated from $CD45^+$ $CD11b^+$ purified cells and analysed by q-PCR. (A) Relative expression of cytokines and chemokines from $CD45^+$ $CD11b^+$ macrophages. (B). Relative expression of markers associated with an M2 phenotype from $CD45^+$ $CD11b^+$ macrophages. (C). Relative expression of markers associated with an M1 phenotype from $CD45^+$ $CD11b^+$ macrophages.

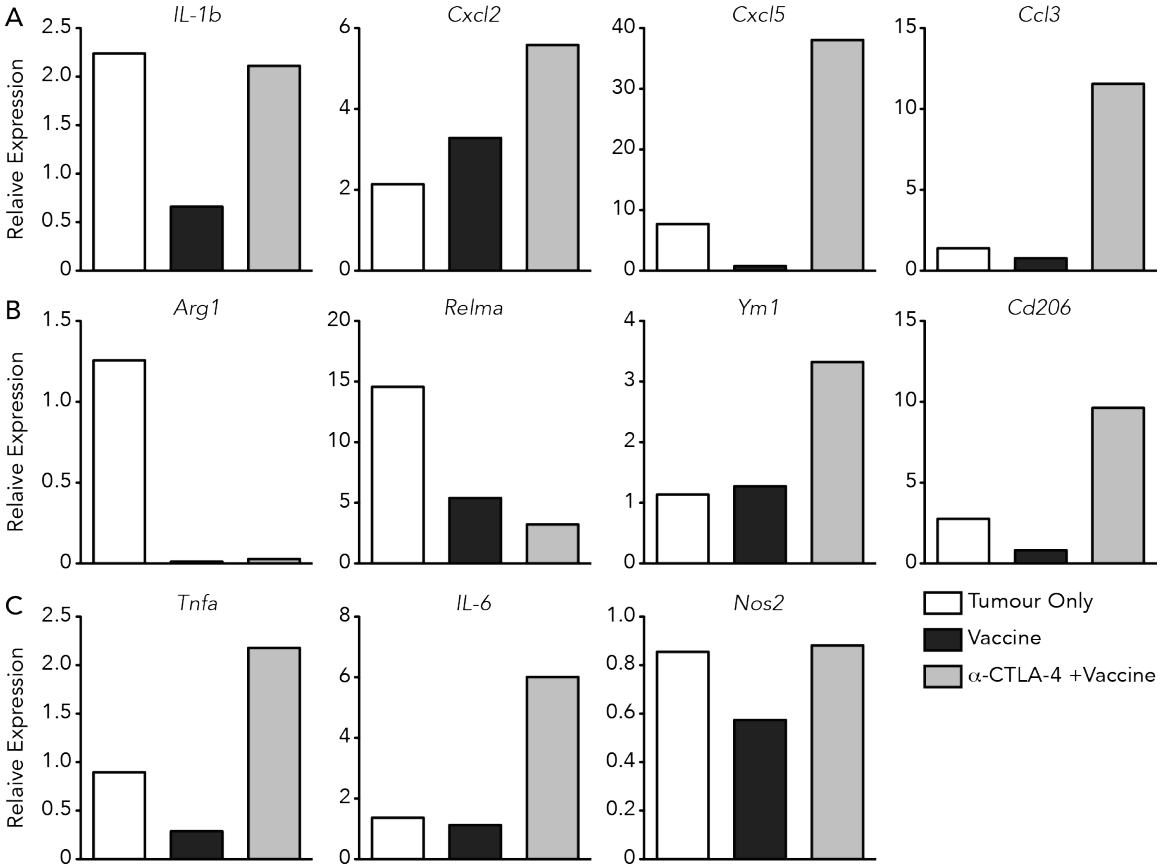

Figure 5.9 Increased M2 polarised microglia within the brain following α-CTLA-4 and vaccine.
Mice were intracranially challenged with GL261 and treated with vaccination on day 7, vaccination on day 7 combined with α-CTLA-4 on day 6, or remained untreated. On day 14, mice were culled and brains were harvested and samples were combined for FACS. As controls, sham injected mice received PBS in place of tumour challenge. RNA was isolated from $CD45^{mid}$ $CD11b^{+}$ purified cells and analysed by q-PCR. (A) Relative expression of cytokines and chemokines from $CD45^{mid}$ $CD11b^{+}$ microglia. (B). Relative expression of markers associated with an M2 phenotype from $CD45^{mid}$ $CD11b^{+}$ microglia. (C). Relative expression of markers associated with an M1 phenotype from $CD45^{mid}$ $CD11b^{+}$ microglia.

5.3.7 *CD11b$^+$ cells are required for α-CTLA-4 and vaccine therapeutic efficacy*

Macrophages and microglia have both been implicated in the pathogenesis of GBM. As both cell populations express CD11b, the contribution of CD11b$^+$ cells towards therapeutic efficacy was assessed. Mice expressing the human diphtheria toxin receptor under control of the CD11b promoter were crossed to a C57BL/6 background to generate CD11b-DTRxC57 mice. These mice were challenged intracranially and treated with α-CTLA-4 on day 6 and vaccine on day 7 as previously described. In addition to receiving combination therapy, one group of CD11b-DTRxC57 mice also received 750 ng diphtheria toxin (DT) every second day from day 8 to day 14 to deplete CD11b cells. Some CD11b-DTRxC57 mice were challenged intracranially and received no treatment, but received DT as above. As controls, C57BL/6 mice were challenged intracranially and treated with α-CTLA-4 and vaccine therapy in addition to DT as above, or remained untreated. Initial experiments confirmed the DT-mediated depletion of CD11b positive cells in the spleen and brain, with tissue samples taken on day 14 following tumour challenge (the day of the final DT treatment) (Fig 5.10 A). Interestingly, whereas CD11b-DTRxC57 mice without DT could mount effective responses, in animals that were treated with DT to remove CD11b$^+$ cells, this efficacy was lost. This was not an off-target effect of DT, as tumour growth was not affected by DT in untreated CD11b-DTRxC57 hosts, and C57BL/6 mice receiving combination therapy and DT exhibited the expected tumour clearance, with long-term survival past day 60 observed (Fig 5.10 B). These results suggest CD11b$^+$ cells are required for α-CTLA-4 and vaccine efficacy.

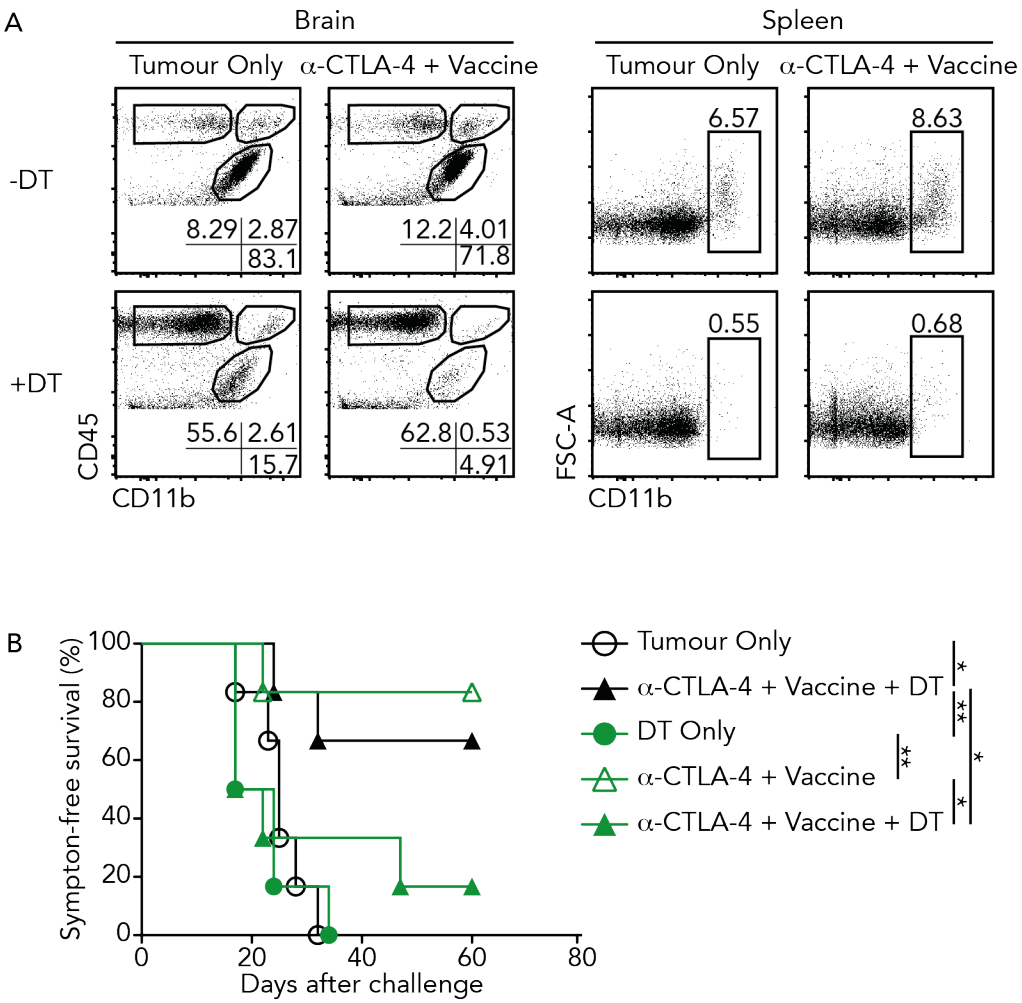

Figure 5.10 Successful therapy is CD11b dependent.
C57BL/6 and CD11b-DTRxC57 mice were intracranially challenged with GL261 and monitored for symptoms of tumour development, including > 10% weight loss and overt behavioural changes such as hunching or reduced activity. (A) Gating strategy used to check CD11b$^+$ cell depletion in CD11b-DTRxC57 mice (n = 1-2 per group) with intracranial tumours either untreated, treated with α-CTLA-4 on day 6 and vaccine on day 7 or treated with α-CTLA-4 on day 6, vaccine on day 7 and 750 ng DT administered on days 8, 10, 12, 14. Results represent one experiment. (B) Symptom-free survival curves for C57BL/6 and CD11b-DTRxC57 mice (n = 5-6 per group) with intracranial tumours either untreated, treated with α-CTLA-4 on day 6 and vaccine on day 7 or treated with α-CTLA-4 on day 6, vaccine on day 7 and 750 ng DT administered on days 8, 10, 12, 14. Black denotes C57BL/6 mice while green denotes CD11b-DTRxC57 mice * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, (Log-Rank (Mantel Cox) test). Results represent three independent experiments.

5.3.8 *Splenocytes from α-CTLA-4 and vaccine treated mice are cytotoxic in vitro*

Results from the previous chapter showed vaccination and α-CTLA-4 combination therapy induces a $CD4^+$ T cell response, with $CD4^+$ T cells capable of producing effector cytokines IFN-γ and TNF-α. In tumour-burdened mice, $CD4^+$ T cells are required for therapeutic efficacy. Also, IFN-γ and perforin are partially required for complete vaccine efficacy. To examine whether T cells with cytotoxic activity were generated, splenocytes were harvested from mice one week following treatment and were restimulated with GL261 tumour cell lysate and soluble α-CD28 antibody for 4 days before tumour specific killing was analysed using the JAM test. Here, [^{3}H]-thymidine-pulsed target cells were co-cultured with titrated doses of effector cells for 4 hours, and specific killing was measured as a loss of [^{3}H]-thymidine signal, reflecting DNA fragmentation in apoptotic target cells.

Splenocytes from α-CTLA-4 and vaccine treated mice induced specific lysis of GL261 target cells whereas LLTC target cells were not lysed (Fig 5.11 A). Tumour cell lysis was dependent on the combination treatment, as restimulated splenocytes from vaccine monotherapy treated mice were unable to lyse target cells. Furthermore, when the assay was performed with the addition of blocking antibodies to MHC-II, splenocytes from α-CTLA-4 and vaccine treated mice were unable to perform effector function, suggesting the lytic activity was mediated by $CD4^+$ T cells. Additionally, restimulated splenocytes from $Pfp^{-/-}$ mice were unable to lyse target cells (Fig 5.11 B). To further explore the role of $CD4^+$ T cells in killing tumour cells, whole splenocytes from α-CTLA-4 and vaccine treated mice were restimulated as above, and then $CD4^+$ T cells were purified from the culture and their killing ability was compared to the CD4 negative fraction. In the absence of $CD4^+$ T cells, splenocytes were unable to induce tumour cell lysis. Conversely, purified $CD4^+$ T cells were seen to induce tumour specific lysis, indicating a role for cytotoxic $CD4^+$ T cells in this model (Fig 5.11 C).

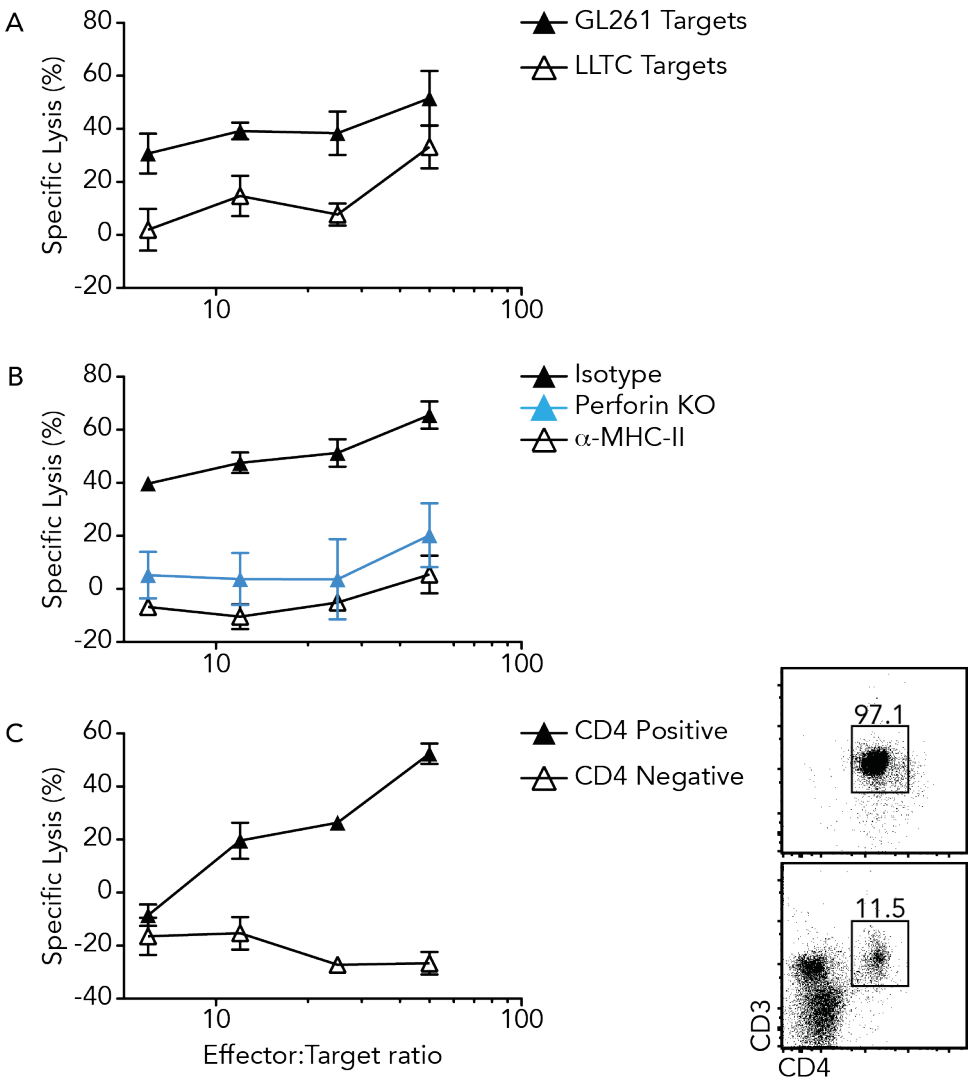

Figure 5.11 CD4⁺ T cells are cytotoxic *ex vivo*.
C57BL/6 and Pfp$^{-/-}$ mice were administered α-CTLA-4 and vaccine, or received vaccine only. Splenocytes were harvested one week later and restimulated with GL261 lysate and soluble α-CD28 antibody for 4 days. Cells were co-incubated with [3H]-thymidine pulsed target cells for 4 hours and cytotoxicity was measured as a loss of radioactivity. (A) Mean specific lysis (± SEM) of α-CTLA-4 and vaccine treated C57BL/6 mice (n = 5) co-incubated with GL261 or LLTC target cells. (B) Mean specific lysis (± SEM) of α-CTLA-4 and vaccine treated C57BL/6 or Pfp$^{-/-}$ mice (n = 5) co-incubated with GL261 target cells. MHC-II signalling was blocked within the assay by addition of α-MHC-II. (C) Following *ex vivo* restimulation, CD4⁺ cells were positively selected by magnetic Dynabead selection and purity checked by flow cytometry. Mean specific lysis (± SEM) of CD4⁺ positive fraction and CD4⁺ negative fraction (n = 5).

5.4 Discussion

Successful tumour clearance requires the intricate relationship between both the innate and adaptive arms of the immune system. This relationship not only exists during the initial stages of antigen acquisition and priming, but also during the effector phase to incite antigen clearance.

In the previous chapter, T cell responses were shown to be involved in successful therapy with vaccination and checkpoint blockade, with both $CD4^+$ and $CD8^+$ T cell responses induced in the lymphoid tissues. Here it was shown that $CD4^+$ T cells were highly represented within the brain infiltrating lymphocytes, suggesting they preferentially play a critical role in tumour clearance. Furthermore, the combined therapy was ineffective in MHC-II-deficient mice, further reinforcing the necessity for $CD4^+$ T cells in eliciting an anti-tumour response in this model. While these cells may play a role in co-ordinating effector functions in MHC-II positive cells in the tumour stroma, which may explain the loss of treatment efficacy in the absence of $CD11b^+$ cells, the data presented in Figure 5.12 point to a mechanism in which the $CD4^+$ T cells have direct anti-tumour activity through a cytotoxic mechanism. It remains possible that several mechanisms are involved, as indicated by only partial loss of activity when experiments were conducted in hosts deficient in perforin or IFN-γ.

While $CD4^+$ T cells were beneficial in this study, the results observed within the brain oppose findings in human samples whereby high $CD4^+$ T cell infiltration is associated with poor prognosis (502). Typically, human GBM is highly infiltrated with $CD4^+$ $FoxP3^+$ Tregs (503), consequently the ratio of intra-tumoural $CD8^+$ T cells to Tregs can be used as a predictor of clinical outcome (502). Intriguingly, enhanced IFN-γ and TNF-α production by $CD4^+$ T cells, compared to $CD8^+$ T cells, was observed in a case study of a patient who received a peptide vaccine in combination with temozolomide (504). Despite this finding, the levels of $CD8^+$ and Tregs within the blood of the patient were monitored during the course of treatment. As a case study, this was performed with $n = 1$ and therefore further studies are required before concrete conclusions can be formed.

The failure of vaccine alone to mediate anti-tumour activity may have several causes. Within the tumour microenvironment, the competition for energy substrates is high, and tumour cells have been shown to secrete IDO to deplete T cells of tryptophan (505, 506). Aerobic glycolysis has been shown to be required for optimal T cell effector function (507), but not for activation, proliferation or survival (508). Glioma is highly dependent on glycolysis for energy production (509, 510), thus, tumour infiltrating cells could experience a loss of function as a result of glucose restriction. Using a mouse model of regressing and progressing sarcoma, glucose uptake by tumour cells was observed to act as a metabolic checkpoint, dampening the ability of T cells to respond and elicit an effective anti-tumour immune response (511). Importantly in relation to the current study, glycolysis was regained in T cells of mice bearing progressor tumours when these mice were treated with α-CTLA-4, α-PD-1 or α-PD-L1 (316, 511). Given the brain is a highly metabolic organ, and this metabolic burden is likely to be enhanced in the presence of a tumour, it is possible that anti-tumour immunity is blunted in tumour bearing mice following vaccination as a monotherapy. However, when combined with CTLA-4 blockade, T cells have enhanced glycolysis and effector function, resulting in effective tumour clearance in immune-competent mice (316, 511).

While previous reports in various tumour models have demonstrated an indispensible role for IFN-γ in mediating tumour clearance (253), tumour clearance in the setting presented here is not solely dependent on IFN-γ. Rather, mice deficient for IFN-γ were able to induce an anti-tumour response, as evidenced by prevention of tumour growth in some animals, and some evidence of activation of T cells within the spleen with the combined treatment. However, overall the anti-tumour response was significantly weaker than that seen WT hosts. The most striking finding here was a proportional decrease in T cells in the brain, suggesting IFN-γ is required for trafficking of the T cells to the intracranial tumour.

While IFN-γ and TNF-α have direct cytotoxic effects, evidence suggests they may play a role in altering the BBB to enhance leukocyte migration. Endothelial cells of the BBB express TNFR1 (512, 513) and IFN-γ receptor (IFN-γR) (512, 514), with TNF-α

reported to increase IFN-γR expression (515). Both TNF-α and IFN-γ modulate the expression and secretion of chemokines from the BBB endothelial cells including CCL2, CCL3, CCL5, CXCL1, CXCL8, CXCL9, CXCL10 and CX3CL1 (513, 516, 517). These chemokines both augment adhesion of leukocytes to endothelial cells and permit the migration of leukocytes across the BBB (518-520). Stimulation of endothelia with both TNF-α and IFN-γ increases the expression of ICAM-1 (521) and VCAM-1 (522) as well as E- and P-selectins (523, 524). The interaction between VCAM-1 and ICAM-1 with VLA-4 and LFA-1 respectively on T cells is associated with the adhesion of $CD4^+$ T cells to the BBB, and crawling, polarisation and extravasation of T cells across the BBB (469). Thus, the expression of TNF-α and IFN-γ permit interactions between activated lymphocytes and the BBB under inflammatory conditions.

The requirement for $CD11b^+$ cells for effective therapy is multifactorial. As previously discussed, macrophages and $CD11b^+$ DCs within the lung can acquire glycolipid antigens and activate iNKT cells. Given the DT administration schedule, it is possible that depletion of $CD11b^+$ cells starting on one day after priming may have compromised the induction of effective anti-tumour immunity. Conversely, microglia express CD11b and are therefore able to be depleted following DT administration, as demonstrated in Fig 5.10. Microglia play a vital role in managing neuro-inflammation, and it is possible that in the absence of these cells, overt inflammation within the brain was ultimately fatal (500, 501). In other studies, the role of macrophages in glioma pathogenesis is somewhat discordant. One study demonstrated increased glioma growth in mice following macrophage depletion. Within syngeneic mice expressing the herpes simplex virus thymidine kinase (HSV-TK) under control of the CD11b promoter, macrophages could be depleted following administration of ganciclovir. When applied to the intracranial GL261 model, ganciclovir administration resulted in a 45% reduction in the number of macrophages within the tumour, subsequently leading to a 33% increase in glioma volume (525). Macrophage depletion also resulted in reduced TNF-α mRNA within the tumour and reduced numbers of T cells (525).

In a separate study, macrophage ablation reduced M2-skewed macrophage populations, limiting glioma growth in a macrophage Fas-induced apoptosis (MAFIA) model. In this model, the murine CSF-1R promoter induces the expression of enhanced green fluorescent protein (EGFP) and a suicide fusion gene (526). Administration of AP20187, a synthetic cell-permeable ligand, dimerizes the suicide protein, inducing Fas mediated apoptosis of CSF-1R expressing cells. (527). Within the brains of these mice, 60-80% of GFP^+ cells were $Arg1^+$, as examined by confocal microscopy, with few $iNOS^+$ cells detected (528). Depletion of CSF-1R expressing cells, from days 1-5 or days 6-10 following intracranial challenge resulted in decreased tumour volumes and this was attributed to depletion of M2-like macrophages, hindering tumour progression (528). In accordance with this study, inhibition of CSF-1R was seen to improve overall survival and decrease glioma malignancy in a PDG transgenic model of glioma (492). Rather than depleting macrophages, administration of a specific CSF-1R inhibitor was seen to "re-educate" macrophages, resulting in the reduction of genes associated with an M2 phenotype (492). Clinically, macrophage infiltration has been associated with tumour progression, with tumour-associated macrophages M2 polarised (529, 530). Proliferation of the human GBM cell line T98G has been shown to increase *in vitro* when co-cultured with M2 skewed monocyte-derived macrophages (531). Enhanced proliferation was dependent on direct cell contact and was due to enhanced tumour cell STAT-3 signalling (531).

It is yet to be determined if the observed phenotype of macrophages and microglia is a cause or consequence of T cell infiltration into the brain following CTLA-4 blockade and vaccine immunotherapy. Clearance of tumour tissue by infiltrating T cells could liberate antigen, which is phagocytosed by macrophages within the local microenvironment, with macrophages acting as APCs to further activate T cells and promote tumour clearance (532). Alternatively, $CD4^+$ T cells could be interacting with macrophages via MHC-II, imparting a M1 phenotype required for tumour clearance (533). The results above suggest $CD11b^+$ cells are required for vaccine and α-CTLA-4 therapeutic efficacy. Given that DT administration to deplete $CD11b^+$ cells was initiated a day after vaccination, it may have impeded immune priming, resulting in a decreased T cell response rather than depleting

$CD11b^+$ cells during the effector phase. A lack of availability of sufficient CD11b-DTRxC57 mice precluded further analysis.

The ability of $CD4^+$ T cells to have direct cytotoxicity may suggest that treatment failure in mice depleted of $CD11b^+$ cells was simply due to impaired immune priming. However given the abundance of evidence showing the importance of macrophages within the tumour microenvironment, and the loss of therapeutic efficacy following CTLA-4 blockade and vaccination, when $CD11b^+$ cells were depleted, the role of macrophages in this model cannot be completely excluded. With these considerations, targeting macrophages within the tumour microenvironment with drugs to modify the phenotype and function, or to transiently deplete cell populations may be a promising clinical approach, on conjunction with vaccines and checkpoint blockade antibodies to stimulate T cell responses. It is important that such inhibitors are tested in orthotopic models to determine the appropriate bioavailability behind the BBB (396).

In the absence of perforin, survival benefit was again blunted, suggesting direct cytotoxicity mediated by the perforin and granzyme pathway is important for mediating tumour clearance. In fact, the weakened anti-tumour activity in IFN-$\gamma^{-/-}$ hosts may also reflect a limited ability of these important cytotoxic effectors to access the brain and eliminate tumour tissue, given evidence of failure of T cells to migrate in these animals (Fig 5.8). The role of cytotoxic cells was further examined using the JAM test to detect antigen specific killing. Whereas WT $CD4^+$ T cells were capable of killing GL261 targets *in vitro*, this ability was lost in the absence of perforin.

Taken together, this suggests a prominent role for cytotoxic $CD4^+$ T cells in mediating tumour clearance in this model. Given the gene expression profile changes in macrophages and microglia seen in this model, it remains possible that a bias away from the M2-like macrophages contributes to the anti-tumour effect, while microglia polarised towards an M2-like phenotype may contribute to induce tissue repair and remodelling following tumour clearance.

5.5 Conclusions

CTLA-4 blockade combined with vaccination induces increased T cell infiltration into the tumour of mice bearing intracranial glioma. Within the infiltrating T cell population, $CD4^+$ T cells were seen to be the predominant population, with no increase in the number of Tregs. Macrophages were seen to be M1 skewed following combination treatment, and transient depletion of $CD11b^+$ cells impaired therapeutic efficacy, however the relative contributions of macrophages and microglia to tumour-clearance could not be determined in this setting and loss of efficacy may have arisen through impaired immune priming. Rather, therapeutic efficacy was observed to be dependent on the presence of $CD4^+$ T cells as well as the production of IFN-γ and perforin. Furthermore, $CD4^+$ T cells were shown to be capable of killing tumour cells *in vitro*, suggesting that anti-tumour efficacy of combined vaccination and checkpoint blockade may be through the direct killing of glioma cells by $CD4^+$ T cells.

Chapter 6

6 Discussion

Glioblastoma Multiforme is a rapidly fatal brain tumour with limited treatment options. While the inclusion of radiotherapy and temozoloimde chemotherapy following debulking surgery has extended overall survival to 12-15 months from diagnosis, patients ultimately succumb to disease. Given this dire prognosis, new treatment modalities are required. Immunotherapy is now recognised as a legitimate treatment option for melanoma, and is gaining traction as a therapy for many other cancers. The studies within this thesis challenged the principal hypothesis that the combination of vaccine-based immunotherapy and checkpoint blockade will result in improved immune-mediated tumour clearance.

Using a vaccine comprised of α-GalCer loaded, irradiated whole tumour cells, it has previously been shown that prophylactic vaccine administration prevents tumour growth in both GL261 subcutaneous and intracranial challenge settings. Therapeutic efficacy however, required the depletion of Tregs, thus vaccine efficacy in a therapeutic setting required immunosuppressive mechanisms to be overcome. In the intracranial challenge setting, combining vaccination with blockade of CTLA-4 provided robust long-term protection in 80-90% of mice and was not dependent on either intervention as a monotherapy. The timing of antibody administration was important for successful treatment as delaying antibody delivery until 3 or 7 days post-vaccine significantly impaired long-term survival.

The combination of α-CTLA-4 and vaccination induced an antigen specific anti-tumour response, which was dependent on the adjuvant activity of NKT cells. No enhancement in the phenotype or function of APCs could be ascertained with combination treatment, nor were Tregs depleted following antibody administration. Rather, CTLA-4 blockade combined with vaccination resulted in enhancement of effector cell intrinsic proliferation

and effector phenotype and function within the secondary lymphoid organs. In agreement with this, T cell infiltration into tumours was markedly increased following successful therapy. Within the brain, $CD4^+$ T cells the predominant cell within the infiltrating lymphoid population, and were also found to be indispensible for treatment. Additionally, $CD4^+$ T cells were found to be cytotoxic *in vitro*.

While many immunotherapies largely focus on activating cytotoxic $CD8^+$ T cells, there is growing acceptance of the cytotoxic capacity of $CD4^+$ T cells. $CD4^+$ T cell dependent anti-tumour responses have been described in both GL261 (534), as well as melanoma models (73, 535, 536). Transfer of $CD4^+$ T cells, specific for the melanoma antigen TRP1, into tumour-bearing, lympho-depleted hosts induced tumour regression, which was further enhanced with CTLA-4 blockade. In this study, adoptive transfer of TRP1 specific $CD4^+$ T cells and treatment with α-CTLA-4 resulted in an increased proportion of $TRP1^+$ $CD4^+$ T cells within the blood, with a decrease percentage of $TRP1^+$ $FoxP3^+$ cells. Additionally, concentrations of IFN-γ and TNF-α were increased in the serum of α-CTLA-4 treated mice, with secretion attributed to $CD4^+$ T cells (73).

In addition to creating a pro-inflammatory environment, IFN-γ is known to induce the upregulation of MHC-II on tumour cells. Cell surface expression of MHC-II on GL261 is absent *in vitro* but can be induced in the presence of IFN-γ. Similarly, resected GL261 cells that were implanted subcutaneously, express MHC-II *ex vivo*. Indeed, IFN-γ-dependent upregulation of MHC-II on tumour cells has been shown to prepare tumour cells for killing *in vivo* (73). While previous reports in various tumour models have demonstrated an indispensible role for IFN-γ in mediating tumour clearance (253), tumour clearance in this setting is not solely dependent on IFN-γ. The altered immune response induced in IFN-$\gamma^{-/-}$ mice is curious. Both IFN-γ and perforin were required for complete tumour clearance and analysis of the induction of anti-tumour immunity, as evidenced by the activation of T cells within the spleen of tumour bearing animals revealed relatively comparable responses, suggesting immune priming is intact in these mice, but effector function is impeded. The most striking finding here was a proportional decrease of lymphocytes, particularly $CD4^+$ T cells, and the reciprocal increase of $CD45^{hi}$

$CD11b^+$ macrophages within the brains of tumour bearing IFN-$\gamma^{-/-}$ mice treated with vaccination and CTLA-4 blockade.

While IFN-γ and TNF-α have direct cytotoxic effects, the expression of TNF-α and IFN-γ permit interactions between activated lymphocytes and the BBB under inflammatory conditions. Therefore, decreased T cell infiltration into the brains of IFN-$\gamma^{-/-}$ mice may be a consequence of reduced T cell trafficking and extravasation across the blood brain barrier (537). Using a gene-therapy approach to treat mice bearing intracranial GL26 tumours, administration of adenoviral vectors containing INF-γ or TNF-α directly to the tumour site offered improved overall survival over empty vector controls (538). Decreased tumour size correlated with increased $CD4^+$ and $CD8^+$ T cell infiltration, as well as increased MHC-I and MHC-II expression on tumour cells *in vivo* (538). Additionally, decreased FasL expression was observed on endothelial cells within tumours, bot not normal brain tissue (538), suggesting IFN-γ and TNF-α may facilitate immune cell penetration by allowing glioma-induced barriers to be overcome. Clinically, IFN-γ treatment, combined with low-dose cyclophosphamide has been examined in children bearing high-grade glioma (539). Following radiotherapy and chemotherapy, patients were treated daily with increasing doses of IFN-γ, with cyclophosphamide administered every 3 weeks. While acceptable toxicities were observed in patients, combination treatment of IFN-γ and cyclophosphamide offered no beneficial effect in terms of overall survival in these patients (539).

The exact role of $CD11b^+$ cells in therapy is yet to be determined and warrants further investigation. Indeed, this result may suggest the $CD11b^+$ DCs are an important subset for the induction of anti-tumour T cell responses in this model, which is in agreement with other studies showing the role of lung $CD11b^+$ APCs for the induction of anti-tumour immunity (439, 540, 541). To address the involvement of macrophages using these mice, alternate DT administration schedules, starting 3 or more days following immune priming may alleviate this problem. Otherwise, alternate approaches to specifically deplete macrophages, such as the administration of clodronate liposomes, or depleting antibodies could be used to dissect the relative contribution of macrophages to

overall anti-tumour efficacy. In either case it is likely that cells would need to be depleted from the periphery, before reaching the tumour site, as the presence of the BBB can limit drug and antibody penetrance (542). Depletion of macrophages from the periphery before they enter the tumour may also provide valuable insight into the relative contribution microglia within the CNS play in anti-tumour immunity following CTLA-4 blockade combined with vaccination.

Certainly, inducing allergic encephalopathy is a risk with vaccine strategies, and reports have documented in the induction of allergic encephalopathy in primates and guinea pigs following vaccination with human GBM in complete freund's adjuvant (543). Encouragingly, no signs of adverse immune related advents were observed in tumour bearing mice treated with vaccination, nor were they observed in combination with CTLA-4 blockade, suggesting that, at least in this setting, α-CTLA-4 and vaccination induces potent anti-tumour responses without induction of encephalomyeopathy. This is in agreement with another study using α-CD25 administration combined with DC vaccination in SMA-560 bearing VM/Dk mice (544). Intriguingly, α-CD25 administration did not deplete Tregs in this study but rather impaired their suppressive ability, which is in agreement with other reported studies examining α-CD25 administration (544, 545). Conversely, Treg depletion following α-CD25 administration has been observed, as measured by a decrease of the number of $CD4^{+}FoxP3^{+}$ T cells in the blood (29). Differences between these results are likely attributed to differences in mouse genetic background, antibody clones and isotypes used for depletion, and the methods used to detect Tregs (546, 547). While depletion or modification of Treg function provides valuable insight into the role of Tregs in tumour-burdened hosts, no clinical Treg depletion method exists yet.

In contrast to other studies, no survival benefit was observed when α-CTLA-4 and α-PD-1 were used as monotherapies against established tumours in the intracranial setting. In keeping with this, a previous report showed that α-CTLA-4 alone could not provide protection against intracranial GL261 tumours once established, but could induce limited protection when multiple treatments were started only three days after implantation.

Established tumours could be effectively treated when α-CTLA-4 was combined with a vaccine comprised of GM-CSF-transfected glioma cells (392). However, another study showed that α-CTLA alone could be used to treat SMA-560 glioma in VM/Dk mice (393). The authors attributed the activity of α-CTLA-4 in this model to enhancement of T cell-proliferative capacity, which was otherwise severely reduced in the presence of this tumour. A feature of these studies is that α-CTLA-4 appears to mediate its effects on glioma through alteration of immune cells in the lymphoid compartments. In contrast, α-PD-1 is generally thought to mediate its anti-tumour effect through preventing regulation of T cells in the tumour (160, 394, 395).

Further work within my research group has revealed that α-PD-1 can induce regression in subcutaneous GL261 tumours, even when they are quite large (> 100 mm in diameter, Lindsay Ancelet, personal communication), suggesting that this form of regulation is still of high relevance in glioma. It is therefore likely that efficacy could be improved by enhancing access of blocking antibodies or other PD-1 inhibitors to effector cells in the brain, although strategies to improve penetrance of drugs and antibodies still remain in the early stages of development (396). This is an issue of high relevance, as the clinical failure of antibodies to VEGF-A in glioma patients may also have been due to minimal antibody penetrance (280, 397, 398). It may be possible that the timing of antibody delivery with radiotherapy could improve access to brain tumour tissue. One study demonstrated successful treatment of murine glioma when administration of α-PD-1 was delivered with stereotactic radiation (371). In accordance with this concept, MRI of glioma patients following radiotherapy demonstrated increased uptake of gadolinium DPTA at the tumour site, indicative of increased vascular permeability (399). In the absence of further improvements to penetrating the blood-brain barrier, inhibitors of immune checkpoints that regulate immune priming in peripheral lymphoid organs, such as CTLA-4, OX40 and VISTA, will likely be of more therapeutic benefit in glioma than inhibitors that target immune checkpoints on intra-tumoural effector T cells such as PD-1 or TIM-3 (191).

It is worthwhile to note that murine tumour models do have their limitations. Although GL261 is used widely to assess immunotherapeutic regimes for glioma, and is described as the "gold standard" model for glioma (382), there are many ways in which GL261 differs from human GBM. Histologically, GL261 tumours are highly cellular, and vascular, with areas of necrosis (548). Conversely, human GBM is highly invasive and readily infiltrates local brain tissue whereas GL261 can only be considered moderately invasive (548). Histology examined in Chapter 3 revealed dense infiltrating tumours with tumour margins extending past the periphery of the main tumour mass, supporting the moderately invasive phenotype. Similarly to human GBM, GL261 harbours activating mutations of the K-ras oncogene as well as mutations of the p53 tumour suppressor gene (549-552). As a tumour of glial origin, glial fibrillary acidic protein (GFAP) and S-100 proteins can be detected by IHC staining (354). However, the expression of GFAP is undetected in some reports, or is only detected under special conditions (370, 553). Absence of GFAP expression in some research groups may be a consequence of serial passage number of tumour cells.

Immunologically, Tregs are recruited to GL261 tumours (265, 554), reflecting the clinical situation (555), where Tregs actively dampen immune responses. Additionally, macrophages and myeloid-derived suppressor cells are recruited from the periphery to the tumour site and have been associated with poor clinical outcome (556, 557). Partial immunogenicity is conferred due to the high expression of MHC-I on GL261. As mentioned above, expression of MHC-II can be observed *in vitro* after IFN-γ exposure, and can also be observed *ex vivo* from resected tumour cells (29) whereas expression of CD80 and CD86 is limited (549). Evidence of immunogenicity can be observed when mice are subcutaneously vaccinated with at least 10^6 irradiated cells before intracranial challenge, however protection is lost when mice are vaccinated on the day of, or 3-7 days following challenge (549). Many tumour associated antigens have been defined in GL261 including Trp-2, and gp100 (558), survivin (559), EphA2 (560) and GARC-1 (561). While these antigens are shared across many tumour types, the immunodominant antigens present within GL261 are under investigation as they are presently unknown.

The discrepancies in tumour response to therapy between the orthotopic implantation and subcutaneous challenge settings are highly important. The main disadvantage of the I.C model is the inability to monitor tumour development over time. Repeat MRI scanning is impractical and costly without a small animal-imaging platform and there is evidence anaesthetic drugs including ketamine are immunosuppressive (562). Transduction of GL261 to allow *in vivo* bioluminescence is possible but only provides a semi-quantitative measure and still requires mice to be anaesthetised (563). Indeed, in this study mice were monitored daily until they reached a moribund state suggesting bioluminescence imaging is a luxury but relying on other signs of neurologic deficit or weight loss still provide a robust readout of disease progression (563). Adopting a S.C route of administration provides an easy means of challenge but subverts many of the advantages of intracranial challenge. Whereas intracranial implantation resulted in 100% tumour penetrance in mice, subcutaneous tumour growth could be variable and exponential tumour growth was not seen. As mentioned previously, expression of MHC-I and MHC-II on GL261 *in vivo* render these cells moderately immunogenic. Regression of S.C tumours following α-PD-1 administration, in the absence of a vaccine stimulus suggests antigen recognition can occur and anti-tumour immunity is promoted when PD-1 signalling is blocked.

The difficulties in using a surgically implantable tumour model include the sterile inflammation induced following implantation, potentially inducing an innate immune response, which is exacerbated following treatment to promote anti-tumour immunity. In addition, utilisation of a cell line allows rapid tumour growth, limiting the reciprocal interactions between tumour cells, stromal cells and immune cells which would otherwise occur in human patients. Spontaneous murine models alleviate some of these concerns, as genetic mutations are introduced which correspond with the human counterpart, supposedly resulting in a more human-representative tumour than carcinogen induced models. As mutations are introduced into germline cells, the possibility for off target effects is present. Conditional models permit tissue specific deletion of tumour suppressor genes and evade some of the problems faced by spontaneous models, however these systems have practical limitations including higher costs, variable time to tumour appearance, and variations in tumour grade (564, 565). In a study comparing the

immunogenicity of murine tumour models, 4/31 (IFN-γ, PD-L1, VEGF and STAT3) intratumoural immune-related genes were found to be different between GL261, and a spontaneous brain tumour induced by intracerebral injection of oncogene containing plasmids into brains of neonatal mice (566). This study suggests the immune infiltrate and degree of immune-editing occurring within the tumour is relatively comparable between GL261 and a spontaneous model. Immunodeficient xenograft models are unsuitable for evaluating immunotherapy and while "humanised" mouse models can be useful for investigating some immunotherapies, recapitulation of the complete human immune system in a mouse is impossible, as this requires interactions with human stromal cells (567).

Recent evidence suggests carcinogen-induced tumours respond better to checkpoint blockade therapy, due to the higher mutation load generally resulting in increased neoepitopes available for immune priming (567-569). Adoption of appropriate tumour models relies heavily on the question being investigated and hypothesis being challenged. Genetic ablation models are likely more suitable for investigating the events and interactions between cells during early transformation (567). Similarly, conditionally induced models, featuring targeted mutations in particular pathways or tumour suppressor proteins may be ore appropriate for investigating targeted inhibitors. In light of these considerations, implantation of GL261 cells in an orthotopic setting remains a valid option for the development and characterisation of novel immunotherapy approaches. Indeed, based on the number of somatic mutations identified within tumour samples, GBM could be categorised as having an occasional likelihood of acquiring neoantigen formation (569, 570). In this case, the limited frequency of somatic mutation would limit neoantigen generation, thereby decreasing the probability of checkpoint antibodies generating anti-tumour response as monotherapies. Even in the case of melanoma, which has the highest frequency of somatic mutation, response rates to checkpoint blockade monotherapy remains relatively modest (153). Thus, the addition of vaccination strategies to checkpoint blockade schedules is paramount for the induction of anti-tumour immunity.

The ultimate goal of preclinical research is translational application. Vaccination of glioma patients with autologous DCs pulsed with tumour lysate has been performed in various clinical trials (286). One deterrent to using whole tumour cells is that they will contain self-antigens, and therefore present the risk of inducing autoimmunity. However, tumour lysate preparations also carry this danger and yet, DC vaccination approaches are the most commonly used form of anti-tumour vaccination (286). Until understanding of the immunodominant epitopes within GBM are known, whole tumour cell, or lysate preparations are the most attractive approach for preparing tumour vaccines. To circumvent the need to sequence GL261 and determine appropriate antigens for generation of a synthetic vaccine, whole tumour cells were loaded with α-GalCer and irradiated before being cryopreserved. Here, the entire antigenic repertoire of GL261 is expressed. In the absence of defined antigens, it is challenging to assess vaccine-induced responses.

Undoubtedly, the definition and validation of discrete biomarkers to measure vaccine responses precedes clinical application, as new therapies must first be tested for safety and efficacy. Recently, the percentage of $ICOS^+$ polyclonal T cells within the blood of patients has been suggested as a measure of vaccine efficacy (571). In the murine setting, an OVA-expressing GL261 cell line could be used (557, 572), permitting vaccine-specific responses to be tracked using OT-I and OT-II T cells. Similarly, peptides to known antigens could be incorporated into the vaccine for clinical applicability. Cytomegalovirus is a member of the Herpesviridae family, and latent infection is highly prevalent within the human population (573-575), therefore adopting CMV antigens into a whole vaccine preparation may allow for monitoring vaccine responses over time. Further work would need to be performed to ensure the incorporation of defined antigens would not affect vaccine efficacy. Additionally, it is possible the "indicator" antigens may be the dominant vaccine-induced response, rendering the vaccine otherwise incompetent. In this case, whole tumour cells could be combined with a library of overlapping synthetic long peptides (SLP) to glioma-associated antigens including EGFRvIII, survivin, gp100 or TRP-2 (576-578). While this would require the synthesis of peptides, negating the relative ease of vaccine generation described in this thesis, SLP vaccines covering the E6 and E7

proteins of HPV16 have shown to provide beneficial anti-tumour responses in patients with vulvar intraepithelial neoplasia (579).

Currently, there is a strong focus on identifying neoantigens within tumours to generate patient specific vaccines for immunotherapy. As neoantigens are tumour specific, they provide a unique therapeutic opportunity however the identification and functional testing of these can be difficult. In a recent study of the neoantigens within a mutagen-induced sarcoma, 62 predicted epitopes were screened before two were isolated based on functional analysis (316). Synthetic vaccines generated based on these peptides were found to be effective in a therapeutic setting, with 85% of mice showing complete rejection (316). It is remarkable that targeting just two epitopes could induce complete tumour rejection. Additionally, the nature of neoantigen peptide selection has thus far resulted in MHC-I binding epitopes being selected.

Recent work has revealed human $CD4^+$ T cells have the capacity to recognise mutated tumour neoantigens (580, 581). Whereas irradiated splenocytes have been used to perform functional analysis for MHC-I binding epitopes in murine studies (316), oncogene-immortalised autologous B cells can serve as APCs to allow for functional analysis of human $CD4^+$ T cells to be performed (581). While these are showing remarkable clinical success, peptide generation and screening is still relatively cumbersome. As generation of a patient specific mutanome requires sequencing of a tumour, mutations identified by exome-sequencing can be selected as vaccine targets and synthetic poly-neoepitope mRNA vaccines can be generated. Using this approach, tumour-bearing mice showed a reduction in growth when vaccines were delivered in a therapeutic setting, with responses dependent on $CD4^+$ T cells (381). It is not unreasonable to propose that the $CD4^+$ T cell responses observed within this thesis were resulting from recognition of MHC-II neoantigens within the whole cell vaccine.

Prior work has demonstrated vaccine toxicity when cells are delivered at high doses intravenously, with toxicity alleviated by heparin pre-treatment (Martin Hunn, personal communication). Toxicity in this setting was attributed to the pro-coagulant Tissue Factor (582, 583). To overcome this hurdle, alternate delivery methods should be examined such

as intradermal or subcutaneous vaccination routes. While these may overcome toxicity issues, the appeal of intravenous administration is that the large population of iNKT cells within the liver and spleen are targeted, promoting anti-tumour immunity. Secondly, priming of immune responses in the skin-draining lymph nodes may induce "inappropriate" responses, compared to targeting the secondary lymphoid organs where T cell priming may result in a CNS homing phenotype (103, 584, 585). In keeping with this, early work examining CTLA-4 blockade with different vaccine administration routes do not appear to offer superior, nor comparable protection, compared to intravenous administration (Lindsay Ancelet, personal communication), however this may be a result of antigen dose and higher doses may be required. As NKT cells were shown to be required for successful therapy in this thesis, it may be that alternate routes of administration do not afford iNKT:DC interactions to promote sufficient licensing. If this is shown to be the case, administration of α-CD40 antibodies, in combination with α-CTLA-4 and vaccination may overcome the requirement for iNKT cell licensing. Alternatively, the inclusion of ligands for other innate like T cells, including γδ T cells, or mucosal associated invariant T (MAIT) cells may permit DC licensing.

Alternatively, redirecting immune priming may allow NKT cell targeting without intravenous vaccine administration. An intranasal delivery system may represent a solution to overcome this. As the brain lymphatics drain to the deep and superficial cervical lymph nodes (340, 443), and the mediastinal lymph nodes are required for T cell reprogramming (103), an aerosolised vaccine could be inhaled, reaching both the tumour draining lymph nodes, as well as the lung draining MdLN to induce T cell priming. Virus-like particles (VLPs) can be coupled with α-GalCer to induce iNKT cell activation and DC licensing, resulting in antigen specific T cell responses (586). Using a similar system, RNA could be packaged inside a VLP, coated with α-GalCer and then administered as an aerosol. Combined with checkpoint blockade antibodies this may represent a novel approach for inducing anti-tumour immunity in patients. Whether this has any therapeutic feasibility remains to be investigated.

To conclude, checkpoint blockade has afforded significant advances for cancer patients and reinvigorated the field of immunotherapy. The potential for these successes to apply to GBM patients is of great interest. Combination treatment for GBM patients involving vaccination with irradiated α-GalCer loaded, autologous tumour cells, combined with checkpoint blockade, may represent the best application of a novel therapy for a cancer for which current treatment options are limited.

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