IMPROVING IMMUNOTHERAPY FOR HIGH GRADE GLIOMA

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Abstract

Glioblastoma multiforme (GBM) is a malignant primary brain tumour that is almost always fatal. Conventional treatment modalities are limited by toxicity. T cell-based immunotherapy is a promising alternative that has the potential to specifically target tumour cells.

The author of this thesis was a principal investigator for a recently completed Phase I clinical trial in which patients with recurrent GBM were treated with surgery, dendritic cell-based immunotherapy and chemotherapy. In addition to conducting the trial in collaboration with others, the author used peripheral blood mononuclear cells from trial participants to assess anti-tumour immune responses before and after treatment. A broad correlation was observed between clinical outcome and anti-tumour immunity, with sustained progression-free survival occurring in two patients with baseline responses that persisted or increased after treatment. However, the overall clinical benefit was modest. For progress to be made, there is a need to develop a more potent vaccine.

With this in mind, a novel “Glioma-Gal” vaccine was devised and tested in an orthotopic mouse model of glioma. This simple vaccine consisted of irradiated autologous tumour cells pulsed with the glycolipid alpha-galactosylceramide, an immunoadjuvant that induces invariant Natural Killer T cells to licence endogenous dendritic cells. The vaccine was shown to be effective in a therapeutic setting when accompanied by depletion of regulatory T cells. Mechanistically, vaccine efficacy was dependent on CD4 T cells and the mediastinal lymph node was shown to be an important site of T cell priming. It was further shown that components of the immune system necessary for the vaccine to work were present and competent in a cohort of GBM patients.

The final chapters explore the idea of enhancing the therapeutic benefit of this vaccine by targeting certain tumour cell subsets or phenotypes. Cancer stem cells (CSC) are proposed to be a subset of tumour cells with a unique capacity for initiating and maintaining tumours. Eliminating these cells may therefore be both necessary and sufficient to achieve cure. Using

the same mouse model, a variety of methods were assessed for their ability to isolate or enrich for a CSC subset. Of these, culture in serum-free medium in the presence of certain growth factors was shown to enrich for a more stem cell-like phenotype. However, a vaccine constructed from these stem cell-like cells was not more effective than the standard vaccine. Next, the author tested the hypothesis that a vaccine manipulated to target chemoresistant cells would be more effective than standard vaccine when used in combination with chemotherapy. However, the modified vaccine showed no advantage over standard vaccine in this model. In the course of these experiments, synergy was observed between the vaccine and the chemotherapy agent doxorubicin. The mechanism responsible for this supra-additive effect remains undetermined but is most likely due to an immunomodulatory effect of low dose doxorubicin.

The Glioma-Gal vaccine design holds promise but more studies are needed to realise the full potential of this approach. The data presented in the thesis did not support targeting CSC or chemoresistant cells as ways of achieving this. In contrast, combining the vaccine with immunomodulation was effective and merits further exploration.

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List of Abbreviations

α -GalCer	Alpha-galactosylceramide
2-DOG	2-deoxyglucose
ABC	ATP-binding cassette (transporter protein)
ACT	Adoptive cell transfer
ADCC	Antibody-dependent cell-mediated cytotoxicity
AE	Adverse event
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ATC	Autologous tumour cell
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BCG	Bacille Camille Guérin
BCRP-1	Breast cancer resistance protein-1
bFGF	Basic fibroblast growth factor
BMDC	Bone marrow-derived dendritic cell
BMP	Bone morphogenetic protein
CAM	Classically-activated macrophage
CAR	Chimeric antigen receptor
CCL, CXCL	Chemokine receptor ligand
CCR, CXCR	Chemokine receptor
CD	Cluster of differentiation molecule
CD4 T cell	CD4 ⁺ T helper cell
CD8 T cell	CD8 ⁺ T cell
CD40L	CD40 ligand
cDNA	Complementary DNA
CEF	CMV, EBV, Influenza [peptide pool]
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CMV	Cytomegalovirus
CNS	Central nervous system
CR	Complete response
CSC	Cancer stem cell
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CT	Computerised tomography
CTL	Cytotoxic CD8 T cell
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DTEP	Drug-tolerant expanding persister
DTH	Delayed-type hypersensitivity
EAE	Experimental allergic encephalomyelitis
ECOG	Eastern Oncology Cooperative Group
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor, variant III
ELISpot	Enzyme-linked immunosorbent spot (assay)
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FBC	Full blood count
FC	Flow cytometry
FCS	Foetal calf serum
FNSC	Foetal neural stem cell
GAA	Glioma-associated antigen
GFP	Green fluorescent protein
GBM	Glioblastoma multiforme
GM-CSF	Granulocyte Macrophage colony-stimulating factor
gp100	Glycoprotein 100
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HMGB1	High mobility group box 1
i.c.	Intracranial
i.d.	Intradermal
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM-1	Intercellular adhesion molecule-1
IDH1	Isocitrate dehydrogenase 1
IDO	Indoleamine 2,3-dioxygenase 1
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin-
IMDM	Iscoe's modified Dulbecco's medium
iNKT	Invariant Natural Killer T [cell]
iNOS	Inducible Nitric Oxide synthase
iTreg	Induced regulatory T cell
IQR	Interquartile range

KLH	Keyhole limpet haemocyanin
LAK	Lymphokine-activated killer [cell]
LC	Langerhans cell
LLC	Lewis Lung Carcinoma
LN	Lymph node
mAb	Monoclonal antibody
MdLN	Mediastinal lymph node
MDR-1	Multidrug resistance protein - 1
MDSC	Myeloid-derived suppressor cell
MGMT	O ⁶ -methylguanine-DNA methyltransferase
MHC	Major histocompatibility molecule
MPEC	Memory precursor effector cell
MR	Mannose receptor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
Msi-1	Musashi-1
MTD	Maximally tolerated dose
NCI CTC	National Cancer Institute Common Terminology Criteria
NF1	Neurofibromatosis type 1
NK	Natural Killer [cell]
NKT	Natural Killer T [cell]
NO	Nitric oxide
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
NSC	Neural stem cell
nTreg	Natural regulatory T cell
OS	Overall survival
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Progressive disease
PD-1	Programmed cell death protein 1
PD-L1	B7 family member, ligand for PD-1
pDC	Plasmacytoid dendritic cell
PDGR	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PEG-Dox	Pegylated liposomal doxorubicin
PFS	Progression-free survival
PFS _{6MO}	Progression-free survival at 6 months
PGE ₂	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase

PR	Partial response
PRR	Pattern-recognition receptor
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinyl difluoride
RBC	Red blood cell
rhX	Recombinant human X
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Recursive partitioning analysis
RT-PCR	Reverse transcription polymerase chain reaction
s.c.	Subcutaneous
SC	Stem cell
SD	Stable disease
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SLCC	Stem-like cancer cell
SLEC	Short-lived effector cell
SLP	Synthetic long peptide
SOX2	Sex determining region Y-box 2
SP	Side population
STAT-3	Signal transducer and activator of transcription-3
TAA	Tumour-associated antigen
TAM	Tumour associated macrophage
TAP	Transporter associated with antigen processing
T _{CM}	Central memory T cell
TCR	T cell receptor
T _{EM}	Effector memory T cell
T _{FH}	Follicular helper T cell
TGF- β	Transforming growth factor-beta
T _H	T helper [cell]
TIDC	Tumour-infiltrating dendritic cell
TIL	Tumour-infiltrating leucocyte
TLR	Toll-like receptor
TMZ	Temozolomide
TNF- α	Tumour Necrosis Factor-alpha
Topo II	Type 2 topoisomerase
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T cell
TRP-2	Tyrosinase-related protein-2
VEGF	Vascular endothelial growth factor
WT	Wild type
WHO	World Health Organisation
YB-1	Y-box binding protein 1

CHAPTER 1: GENERAL INTRODUCTION

1.1 THE CLINICAL PROBLEM: GLIOBLASTOMA MULTIFORME

Glioblastoma multiforme (GBM) is a highly malignant primary brain tumour that is almost universally fatal. It is the most common form of primary brain tumour in adults. A study of patients referred to the Wellington neurosurgery unit found an incidence of 4.1 new cases per year per 100,000 population that was similar for Maori and non-Maori when adjusted for age (Alexander et al. 2010). The median age at first presentation was 62 years with males outnumbering females by a ratio of 1.5 to 1. These tumours almost always arise in the deep white matter of the cerebral hemispheres. Patients present with symptoms of raised intracranial pressure, seizures or focal neurological deficits. Imaging with Magnetic Resonance Imaging (MRI) or Computerised Tomography (CT) typically shows a mass lesion surrounded by a zone of oedema that exerts pressure on the surrounding brain and, after administration of an intravenous contrast agent, tumours typically have necrotic centre surrounded by a contrast-enhancing rim.

GBM tumours are locally invasive. Location in a critical organ combined with a poorly-defined infiltrative border means that surgery alone is never curative. Until recently, standard management consisted of maximum safe surgical debulking followed by external beam radiotherapy. Post-operative radiotherapy is effective and doubles median survival compared with surgery alone, but recurrence is inevitable (M. D. Walker et al. 1979). In a landmark paper published in 2005, Stupp and colleagues reported the results of a Phase III randomised controlled trial showing a statistically significant survival benefit when the alkylating agent temozolomide (TMZ) is added to surgery and radiotherapy, and this combined regimen has now been adopted as the standard management for newly-presenting patients (Stupp et al. 2005). Although TMZ is the most important advance in the

treatment of GBM for more than 50 years, median survival is still only 15 months, five year survival is less than 10 %, and long term survival remains an extremely rare event (Stupp et al. 2009).

In clinical practice the diagnosis of GBM is histopathological, and requires the presence of atypical pleomorphic cells of astrocytic morphology, evidence of mitotic activity, and either areas of necrosis or vascular proliferation (D. N. Louis et al. 2007). It is now widely accepted that these microscopic appearances are a common morphology shared by a set of tumours that are different at a molecular level. Under the World Health System (WHO) for classifying central nervous system (CNS) tumours, GBM is classified as the most aggressive form of diffuse astrocytoma (“Grade IV”), with the cell of origin presumed to be an oncologically-transformed astrocyte. (Note: The commonly used term “high grade glioma” encompasses both GBM and the less common anaplastic astrocytoma, itself a synonym for WHO Grade III Diffuse Astrocytoma). However, there is some support for the idea that neural stem cells or early progenitor cells may be the cells of origin of at least some GBM (The concept of “cancer stem cells” is discussed in detail in Chapter 5).

The aetiology of most GBM tumours is unknown. Some inherited mutations increase the risk of high grade glioma (reviewed in Schwartzbaum et al. 2006) but these account for only a small minority of patients. The only proven environmental risk factor is high dose radiation (Schwartzbaum et al. 2006). Several studies have suggested an association with exposure to petrochemicals, pesticides, and other chemicals but this has not been a consistent finding (reviewed in Schwartzbaum et al. 2006). Studies on the effect of cell phone usage on the incidence of brain tumours have also reached conflicting conclusions (Corle et al. 2012). A causal link with infection is unproven. Although human cytomegalovirus (CMV) is detectable in over 90 % of GBM tumours and in the peripheral blood of 80 % of patients with newly diagnosed GBM, it remains unclear whether this is due to reactivation of latent infection due to tumour-induced immunosuppression or whether the virus plays a role in oncogenesis (Mitchell et al. 2008b). The possibility of exploiting CMV

infection of glioma tumour cells by targeting CMV antigens with immunotherapy is under investigation (Mitchell et al. 2015b; Prins et al. 2008) Interestingly, an inverse correlation between the incidence of GBM and infection by the Herpes virus Varicella-Zoster has been reported (Wrensch et al. 2005).

Clinically, most patients with GBM present with a rapidly progressive illness and their resected tumours satisfy the histological criteria for GBM at this first presentation (“de novo” or primary GBM). Others evolve over time from a lower grade astrocytoma (secondary GBM). These two clinical subtypes of GBM are associated with different patterns of genetic abnormality. Primary GBM commonly have gain-of-function epidermal growth factor receptor (EGFR) mutations or amplifications or deletion of phosphatase and tensin homolog (PTEN), while secondary tumours commonly lack EGFR mutations but have deletions of the tumour suppressor gene p53 (Ohgaki and Kleihues 2007; Schwartzbaum et al. 2006). Although these differences demonstrate different pathways to the formation of tumours with identical morphology, a simple “two-pathway” model does not account for the significant proportion of GBM tumours that do not fit into either of these two patterns (Schwartzbaum et al. 2006).

More recently, sub-classifications based on gene expression “signatures” (Freije et al. 2004; Nutt et al. 2003; H. S. Phillips et al. 2006) or a combination of gene expression and patterns of genetic mutation (Verhaak et al. 2009) have been described that propose the existence of three or four molecular subgroups of GBM. Although these studies have reached slightly different conclusions, this kind of molecular classification holds promise for more accurate prognostic stratification and refinement of treatment selection. A “proneural” tumour signature is characterised by p53 mutation, platelet-derived growth factor receptor (PDGFR) amplification, isocitrate dehydrogenase-1 (IDH-1) mutations, and expression of oligodendroglial (Verhaak et al. 2009) or neuronal (H. S. Phillips et al. 2006) markers, and is clinically associated with younger age, secondary tumours, and a slightly better overall prognosis. A “mesenchymal” signature is characterised by the

expression of mesenchymal markers and genes associated with angiogenesis such as vascular endothelial growth factor (VEGF), as well as mutations or deletion of neurofibromatosis type 1 (NF1). Another group of tumours termed “proliferative” (H. S. Phillips et al. 2006) or “classical” (Verhaak et al. 2009) harbour mutations or amplifications of EGFR and deletions of PTEN, leading to constitutive overactivity of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway that drives proliferation. The potential usefulness of this kind of molecular classification to immunotherapists is illustrated by the recent report of a clinical trial of dendritic cell vaccination in which prolonged survival was seen only in the group of patients with tumours of the “mesenchymal” type (Prins et al. 2011).

There is no standard treatment for recurrent GBM. The range of treatment options includes further surgical debulking, second-line chemotherapy regimens and additional focused irradiation. However, these treatment modalities are often restricted by local or systemic toxicity or a risk of unacceptable neurological deficits. Survival benefit is limited and the emphasis of management is on symptom palliation. Median survival from time of progression or recurrence is just 6.9 months (Stupp et al. 2005). Motivating this thesis is the clear need to develop more effective treatments for this lethal disease. One promising alternative approach is to exploit the power of the patient’s own immune system. Immunotherapies have the potential to specifically eliminate cancer cells without the unacceptable collateral damage associated with standard modalities (Palucka et al. 2011; Pardoll 2012). Furthermore, by inducing long-lived memory cells, vaccines can potentially have anti-tumour effects that persist long after the completion of treatment. In this chapter I will start by introducing the immune system and cancer immunotherapy in general and then focus on the particular field of therapeutic vaccination for glioma.

1.2 INTRODUCTION TO THE IMMUNE SYSTEM: REQUIREMENTS FOR ANTI-TUMOUR IMMUNE RESPONSES

1.2.1 The innate and adaptive immune systems

The immune system has evolved primarily to provide protection against pathogenic microorganisms (K. Murphy 2008). The innate and adaptive immune systems work in an interconnected way to achieve this purpose. The innate system is a first line of defence that responds rapidly to pathogens that have succeeded in breaching epithelial barriers. An innate response is triggered by the recognition of non-specific signals that alert the immune system to the presence of foreign microorganisms and/or tissue damage. Innate effector cells include phagocytic cells such as macrophages and neutrophils, and Natural Killer (NK) cells that are specialised to recognise “altered self”. The innate system also includes a range of soluble factors elaborated by non-immune cells such as antibacterial defensin proteins secreted by mucosal cells of the gut and the components of the complement system produced by the liver.

In some cases the innate system alone is capable of clearing a pathogen but, failing this, the innate system serves to control the infection until a more potent and pathogen-specific adaptive immune response has time to fully develop. The key effectors of this second line of defence are T cells and B cells, responsible for cell-mediated and antibody-mediated responses respectively. With few exceptions, adaptive responses are controlled by dendritic cells (DC), specialised antigen-presenting cells (APC) that take up and present antigen to T cells. The adaptive system is also responsible for the phenomenon of immunological memory, whereby subsequent encounters with the same pathogen elicit a faster and more potent response. The two arms of the immune system, innate and adaptive, are closely integrated. For example, cells of the adaptive system may exert some of their effector functions indirectly through the actions of innate cells. Conversely, the innate system has an important role in determining whether the threshold for triggering an adaptive response is reached.

In addition to its primary role of providing protection against infections, the immune system is also capable of mediating anti-tumour effects. Whereas cells of

the innate immune system are rapidly activated by the recognition of generic pathogen- or danger-associated molecules, the lymphocytes of the adaptive system provide a more specific line of defence that is also more potent. B-lymphocytes are responsible for humoral immunity, namely the production and secretion of soluble antibodies. Antibodies are primarily concerned with binding to extracellular antigens, such as extracellular pathogens and toxins. However, antibodies can also bind to antigens on cancer cell surfaces, thereby opsonising cells for phagocytosis by macrophages, triggering antibody-dependent cellular cytotoxicity (ADCC), or blocking the activity of a surface molecule that promotes tumour progression (Heo et al. 2012). Circulating antibodies to tumour-associated antigens (TAA) have been widely reported in patients with cancer (reviewed in Heo et al. 2012) and have proved a useful means of identifying potential antigen targets for immunotherapy (Ueda et al. 2004). However, although the presence of TAA-specific antibodies proves that an adaptive response exists, the importance of endogenous humoral responses to effective anti-tumour immunity is unclear (Heo et al. 2012). In contrast, animal experiments have firmly established a role for T cells anti-tumour immunity (K. Murphy 2008) and generating tumour-specific T cells has been the goal of most cancer vaccines.

1.2.2 T cells in anti-cancer immune responses

1.2.2.1 T cell development: diversity and clonal selection

T cells recognise peptide antigens using T cell receptors (TCR) on the cell surface. Unlike antibodies, TCR do not bind to free antigen but instead recognise a complex of peptide bound to a Major Histocompatibility Complex (MHC) molecule on the surface of another cell. There are two main T cell subsets. CD8 T cells recognise peptides of 8-10 amino acids in length that are bound to MHC class I molecules. CD4 T cells recognise peptides of 12-16 amino acids presented by MHC class II. The specificity of each TCR (*i.e.* the specific peptide-MHC complex it will recognise) is determined by its variable region, the region of the TCR that binds to the peptide-

MHC complex. Immature T lymphocytes are produced in the bone marrow throughout life and travel via the circulation to the thymus to complete differentiation. In the thymus, genes encoding the amino acid sequence of the variable region undergo a process of rearrangement that in turn generates T cells with different TCR variable regions and hence diverse specificities. The purpose of this diversity is to generate T cells capable of recognising a wide range of foreign antigens. However, before leaving the thymus, T cells undergo a process of negative and positive selection. The essentially random process of gene rearrangement inevitably produces cells that are of no use (because they bind too weakly to MHC) or are too dangerous (because they bind too strongly to MHC complexed to “self” peptides derived from normal tissues). These cells are deleted. The deletion of strongly self-reactive T cells is termed “central tolerance”. In order to be positively selected, T cells must receive a survival signal through the TCR when it successfully binds to a peptide-MHC complex expressed by specialised thymic epithelial cells. It is pertinent to note that the peptides presented to T cells in the thymus are derived from normal tissues – thus all T cells that survive clonal selection are to some extent self-reactive. This is reassuring for tumour immunotherapy, where the aim is usually to target over-expressed self-antigens. However, it also means that “central tolerance” must be backed up by another set of “peripheral tolerance” mechanisms to prevent auto-reactivity (described below). Mature T cells that have survived the process of clonal selection leave the thymus to join a pool of naïve lymphocytes that continuously circulate through the blood stream and secondary lymphoid organs (lymph nodes and spleen) searching for their “cognate” antigen, namely a peptide-MHC combination that binds strongly enough to the TCR to trigger downstream signalling.

1.2.2.2 CD8 T cell effector functions

Naïve CD8 T cells are primed in secondary lymphoid organs when they encounter cognate antigen on the surface of a DC along with other activating signals. Primed CD8 T cells (also termed “Cytotoxic T cell” or CTL) re-enter the circulation and home

to the target site guided by chemokines, chemokine receptors and adhesion molecules. CTL that encounter cognate antigen at the target site are capable of direct killing in a number of ways. The release of lytic granules containing perforin (which creates pores in the plasma membrane of the target cell) and granzyme B, a protease that enters the target cell and initiates an enzyme cascade within the cytosol that leads to apoptosis (or programmed cell death). An alternative, perforin-independent mechanism of inducing apoptosis involves the binding of Fas ligand on the CTL surface with the “death receptor” Fas (CD95) on the target cell. Tumour Necrosis Factor-related apoptosis-inducing ligand (TRAIL) is another death receptor ligand that has been shown, for example, to be required for the graft-*versus*-leukaemia effect of CTL in mouse models of allogeneic bone marrow transplant (Schmaltz et al. 2002). CTL also release the cytokines Interferon- γ (IFN- γ) and Tumour Necrosis Factor- α (TNF- α). IFN- γ inhibits tumour angiogenesis and has a wide range of immunomodulatory effects including upregulation of MHC class I expression by target cells and enhanced macrophage function (Kane and Yang 2010). In addition to a number of pro-inflammatory effects, TNF- α was named for its ability to directly induce apoptosis in tumour cell lines. However, the ability of TNF- α to directly kill tumour cells *in vivo* seems to be limited to certain tumour types, notably sarcoma (Starnes 1992). In the clinic, recombinant TNF- α has been used most successfully to improve delivery of chemotherapeutic agents by increasing vascular permeability or for its property of selectively inducing endothelial necrosis in tumour neo-vasculature (Lejeune et al. 2006; van Horssen et al. 2006).

1.2.2.3 CD4 T cell helper and effector functions

All cells (except erythrocytes in humans) express MHC class I and are therefore potential targets for CTL. In contrast, CD4 T cells recognise cognate antigen bound to MHC class II molecules (they are thus MHC class II-restricted). In the steady state, MHC class II is expressed at low levels by most cells. The exceptions are the antigen-

presenting cells: DC, macrophages and B cells. Thus, CD4 T cells mainly interact with these cell types.

The term T cell help was first used to describe the process by which CD4 T cells promote B cell responses. Subsequently, it was discovered that CD4 T cells also provide “help” to CD8 T cell responses (Husmann and Bevan 1988) by providing activation signals to DC that licence the DC to prime CD8 T cells. This process (discussed below in more detail) requires the CD4 T cell to recognise its cognate antigen bound to MHC class molecules on the DC cell surface and is predominantly mediated by CD40 ligand (CD40L) on T cells binding to CD40 on DC. CD4 T cells not only provide help to DC but may be reciprocally activated to develop effector functions. Depending on the local cytokine milieu at the time of priming, naïve CD4 T cells have an ability to differentiate into a number of different effector phenotypes (J. Zhu and Paul 2008; Mosmann et al. 1986; K. M. Murphy and Reiner 2002).

Interleukin 12 (IL-12) released by DC drives expression of the transcription factor T-bet which induces a type 1 T-helper (T_H1) phenotype. T_H1 cells secrete IL-2, TNF- α and IFN- γ (W. E. Paul and Seder 1994). T_H1 cells that traffic to the site of inflammation can provide help to local innate cells. IFN- γ and CD40-CD40L interactions boost the ability of macrophages to kill intracellular pathogens and tumour cells (Corthay et al. 2005; Grewal and Flavell 1996; Mumberg et al. 1999; Y. Suzuki et al. 1988). IFN- γ also promotes antigen presentation by upregulating MHC class I and class II expression on immune and non-immune cells (reviewed in Kane and Yang 2010). T_H1 cells are also able to kill immune cells directly via Fas-FasL interactions, which provides another way of killing infected cells and is an important regulatory mechanism for dampening immune responses (Janssens et al. 2003).

T_H2 cell differentiation is promoted by IL-4 and the transcription factor GATA-3 (K. M. Murphy and Reiner 2002; J. Zhu and Paul 2008). T_H2 cells secrete IL-4, IL-5, IL-10 and IL-13 and support certain humoral immune responses, particularly IgE-mediated responses directed at extracellular parasites (W. E. Paul and Seder 1994; J.

Zhu and Paul 2008). T_H2 cells are major mediators of asthma and allergic responses. T_H1 and T_H2 cells counter-regulate each other so that IL-4 inhibits IFN- γ signalling and vice versa (W. E. Paul and Seder 1994). T_{FH} (follicular helper) cells have a specialised role in supporting humoral responses in germinal centres (Vinuesa et al. 2005). T_H17 cells develop in the combined presence of transforming growth factor- β (TGF- β) and IL-6 (at least in mice), and secrete IL-17 and other cytokines and chemokines that promote tissue inflammation and recruit neutrophils and other immune cells (Bettelli et al. 2006; Zou and Restifo 2010). T_H17 cells are believed to be important in responses directed at extracellular bacteria and fungi and to play a major role in some auto-immune diseases such as rheumatoid arthritis and the mouse model of multiple sclerosis, experimental autoimmune encephalitis (EAE) (A. Kimura and Kishimoto 2010; Zou and Restifo 2010). Exposure to TGF- β alone, or to TGF- β and retinoic acid, drives expression of the transcription factor FoxP3 and can induce naïve CD4 T cells to convert to a regulatory phenotype (induced regulatory T cell or iTreg) that suppresses T cell responses (W. Chen et al. 2003; C.-M. Sun et al. 2007).

It is generally believed that the aim of cancer vaccines should be to elicit a CD8 CTL response supported by a T_H1-dominated CD4 T cell response. Because T_H2 cytokines inhibit T_H1 differentiation and antagonise T_H1 cytokines, it follows that T_H2 responses are generally considered to be undesirable in this context (M. Kobayashi et al. 1998; Shimato et al. 2012). Furthermore, T_H2 cells secrete IL-13, that induces macrophages and other myeloid cells to secrete the immunosuppressive cytokine TGF- β (Berzofsky and Terabe 2008), and IL-10, that suppresses macrophage and DC function and induces IL-10-producing suppressive T cells (Groux et al. 1997; Yamaguchi et al. 2011). It is therefore interesting that several studies have found an inverse correlation between the incidence of GBM and atopic asthma, a disease characterised by T_H2 responses (reviewed in C. S. Curran and Bertics 2012; Schwartzbaum et al. 2006). Together with studies in animal models showing that IL-4 at the tumour site can have anti-tumour effects that are dependent on a local accumulation of IL-12-producing dendritic cells (Eguchi et al. 2005) or an influx of

eosinophils (Tepper et al. 1992), this raises the intriguing possibility that T_H2 responses might be protective against GBM.

In addition to the helper functions described above, there is some evidence that activated CD4 T cells can also directly lyse tumour cells using the Fas-FasL pathway to directly kill Fas-expressing tumour cells (D. M. Brown 2010; Stalder et al. 1994). However, many human tumour cells downregulate Fas or even subvert signalling via Fas to promote tumour cell growth (L. Chen et al. 2010). Recently, “cytotoxic” CD4 T cells were demonstrated in a mouse model of melanoma (Quezada et al. 2010). In this model, CD4 T cells induced upregulation of MHC class II expression on the target cells by secreting IFN- γ before directly killing tumour cells in an MHC class II- and granzyme B-dependent manner.

1.2.3 Dendritic Cells and the control of T cell responses

1.2.3.1 Overview of DC biology

T cell responses are controlled by APC that are equipped to take up, process, and present antigen in the form of peptide-MHC complexes along with co-stimulatory molecules and cytokines. Although macrophages and B cells also have this capacity, DC are the most potent APC and are specialised for this purpose (leading to the term “professional” APC). DC are named for their characteristic dendritic morphology, the purpose of which is to encourage DC-T cell interactions, and are part of the mononuclear phagocyte system that also includes monocytes and macrophages (Collin et al. 2013).

In the steady state, DC develop from a common DC precursor that travels from the bone marrow via the circulation to peripheral tissues and secondary lymphoid organs, where development into DC is completed. Migratory DC take up antigen in the periphery and travel via the lymphatic system to draining lymph nodes (LNs) to present antigen to T cells. Migratory DC make up approximately half of the DC

population in LNs in the steady state (Randolph et al. 2005). Lymphoid organ-resident DC in the LN and spleen take up and present antigens arriving in lymph and blood respectively. In disease states, “inflammatory” DC may also develop from circulating monocytes (Collin et al. 2013).

1.2.3.2 Plasmacytoid DC

DC can be classified at a high level into “conventional” DC and plasmacytoid DC (pDC). Although they develop from the same precursor cell, the precise role of pDC is still unclear (Reizis et al. 2011). In contrast to conventional DC, pDC are found predominantly in the circulation in the steady state and are named for their plasma cell-like appearance. Plasmacytoid DC express receptors that recognise pathogen-associated molecules and, in inflammatory situations, migrate to lymph nodes where they produce large quantities of Type 1 interferons that are pro-inflammatory and inhibit viral replication (Cella et al. 1999). Once activated, pDC adopt a dendritic morphology but they are unable to take up exogenous antigen by phagocytosis or macropinocytosis, and their capacity to prime naïve T cells in vivo is controversial (Young et al. 2008). Pertinent to the subject of this thesis, there is evidence that pDC can be immunosuppressive in a tumour context. In a study of patients with breast cancer, the presence of tumour-infiltrating pDC strongly correlated with shorter survival (Treilleux et al. 2004). Subsequent studies have shown an ability of pDC to promote regulatory T cell (Treg)-mediated immunosuppression (M. D. Sharma et al. 2007; Sisirak et al. 2013). In apparent contradiction, activated pDC were recently reported to be capable of exerting direct TRAIL- and Granzyme B-mediated tumour cytotoxicity in a mouse model of melanoma (Drobits et al. 2012). In summary, the effect of pDC in the context of cancer is complex. The remainder of this discussion will focus on conventional DC.

1.2.3.3 Antigen Processing and Presentation

Steady state DC continuously sample their surrounding environment by macropinocytosis. DC also express a large number of receptors that mediate endocytosis of a wide range of potential sources of antigen including microbes, dead or dying cells, exosomes, cellular debris, and soluble antigens (reviewed in Heath et al. 2004; Petersen et al. 2010a; Trombetta and Mellman 2005). Acquisition of antigen from living cells has also been described (Heath et al. 2004). Once inside the cell, soluble proteins and cellular debris are enzymatically digested within endosomes into peptides (Watts 2004). The endosomal pH progressively falls as early endosomes evolve into late endosomes, favouring increased protease activity. MHC class II molecules, synthesized in the endoplasmic reticulum (ER), are transported to late endosomes where they are loaded with 12-16 mer peptides. The peptide-MHC complexes are then transported to the cell surface. This process is known as the “exogenous pathway” of antigen presentation. An “endogenous pathway” is used to present peptides from proteins synthesized within the DC itself, whether these are normal “self” proteins or, in virally-infected DC, the translated products of viral nucleic acid (Trombetta and Mellman 2005). Endogenous proteins in the cytosol and nucleus are continuously cleaved into peptides by large tubular protease complexes called proteasomes. Most of the resulting peptides are rapidly destroyed by cytosolic peptidases but some are transported into the ER by Transporter associated with Antigen Presentation (TAP) molecules where 8-10 mer peptides are loaded on to MHC class I molecules. The peptide-MHC complexes are then transported to the surface for presentation.

There are exceptions to these general arrangements. Exogenous proteins, usually presented on MHC class II to CD4 T cells, can be diverted to be presented on MHC class I to CD8 T cells, a process known as “cross-presentation” (Trombetta and Mellman 2005). Cross-presentation is required for CD8 T cell mediated immunity against viruses that do not infect APCs directly or against tumour antigens that are not endogenously expressed by DCs (Burgdorf et al. 2007). A number of different

pathways for cross-presentation have been described but the relative importance of each of these pathways *in vivo* is controversial (reviewed in Petersen et al. 2010a). Common to most of these pathways is a mechanism by which exogenous protein is transferred from endosomes to the cytosol where it can enter the endogenous processing pathway (Norbury et al. 1995; Ackerman et al. 2005; Rodriguez et al. 1999). Similarly, endogenous proteins that access endosomal compartments (for example as a result of autophagy, or endosomal recycling of membrane proteins) can be loaded onto MHC class II, thereby allowing the presentation of endogenous antigens to CD4 T cells (Crotzer and Blum 2010; Strawbridge and Blum 2007; Villadangos and Schnorrer 2007; N. S. Wilson et al. 2004).

1.2.3.4 DC activation, CTL priming and CD4 T cell help

Naïve T cells express the integrin CD62L (also known as L-selectin) and the chemokine receptor CCR7. These surface molecules allow naïve T cells arriving at lymph nodes via the circulation to migrate across the endothelium of high endothelial venules and reach the T cell areas of the lymph node. Intravital imaging studies show that in the first 8 hours after entering the lymph node T cells form brief contacts with multiple DC (Mempel et al. 2004). If cognate antigen is present, a second phase occurs in which T cells form long-lasting contacts with DC over the next 12 hours followed by a third phase of proliferation and high motility. It has been proposed that, in the first phase, T cells integrate signals from multiple DC contacts before reaching a threshold for the prolonged immunological synapse formation necessary for full activation. Antigen dose has been shown to be a crucial factor in determining whether the threshold for long-lasting DC-T cell contacts is reached (Henrickson et al. 2008). The third phase of “frantic” migration is believed to represent a search for an exit into efferent lymphatics.

The outcome of DC-T cell interactions in secondary lymphoid organs is crucially dependent on the activation state of DC (Behrens et al. 2004; Bevan 2004; Curtsinger et al. 2003). Fully activated DC are licensed to deliver all the necessary

signals to prime a fully-competent CD8 T cell response (Curtsinger et al. 2003). Antigen presentation by DC that are not activated can induce T cell anergy (Schwartz 2003) or conversion to FoxP3⁺ Treg (Yamazaki et al. 2008). In the steady state, where the stimuli for DC activation are not present, migratory DC play an important role in maintaining peripheral tolerance (R M Steinman et al. 2000; Ralph M Steinman et al. 2003). Tolerising DC may also play an important role during infections by counteracting potential auto-reactivity caused by fully activated DC presenting both foreign and self-antigens in an immunogenic manner (Joffre et al. 2009). Activating stimuli for DC include “pathogen-associated molecular patterns” (PAMPs), “danger-associated molecular patterns” (DAMPs), pro-inflammatory cytokines (released by stromal or immune cells), and help from CD4 T cells.

PAMPs are molecular motifs conserved across a wide range of bacterial and viral pathogens. PAMPs are detected by surface receptors termed pattern recognition receptors (PRRs). The best studied class of PRR are the Toll-like receptors (TLRs) of which there are currently ten known members (reviewed in Iwasaki and Medzhitov 2004). Resting or “immature” DC stimulated by TLRs undergo changes rendering them competent to prime naïve T cells. These changes include reduced uptake of further antigen, translocation of peptide-MHC class II complexes from endosomes to the cell surface, increased expression of T cell co-stimulatory molecules, secretion of pro-inflammatory cytokines, upregulation of the DC activating receptor CD40, increased expression of NK activating ligands, secretion of chemokines to attract T cells, and, in the case of migratory DC, expression of chemokine receptors (notably CCR7) to promote trafficking to LNs (R M Steinman et al. 2000). TLR agonists are already in clinical use for certain skin cancers (Drobits et al. 2012) and are attracting attention as adjuvants in vaccines, including therapeutic vaccines for glioma (Prins et al. 2011; Xiong and Ohlfest 2011).

DC can also be activated in the absence of pathogen-derived molecules by endogenous “danger signals” (Gallucci et al. 1999; Matzinger 2002). DAMPs consist mainly of molecules released or exposed by necrotic cells that provide a signal to the

immune system that pathological death of host cells is underway (as opposed to orderly programmed cell death that is efficiently cleared by phagocytic cells). DAMP-mediated DC activation is believed to explain spontaneous endogenous anti-tumour immune responses and is probably also responsible for autoimmune conditions that arise in situations of tissue damage or inflammation (Gallucci et al. 1999). A variety of DAMPs with diverse actions have been described. Calreticulin translocates from the ER to the surface of dying cells where it has a role in enhancing phagocytosis of the dying cell by DC (Obeid et al. 2007). High Mobility Group Box Protein1 (HMGB1) is a nuclear chromatin-binding protein that, when released by post-apoptotic cells, becomes a soluble “alarmin” that signals through TLR4 to promote cross-presentation (Apetoh et al. 2007). Other DAMPs include uric acid (Hu et al. 2004), heat shock proteins (Binder and Srivastava 2005) and adenosine triphosphate (ATP), which binds to purinergic receptors on DC to activate the NLR3P inflammasome and induce secretion of pro-inflammatory IL-1 β (Ghiringhelli et al. 2009).

DC can also be activated by pro-inflammatory cytokines such as TNF- α and Type 1 interferons released by other immune cells or stromal cells. This “indirect” activation is exploited in DC-based immunotherapies to activate antigen-loaded DC prior to adoptive transfer. However, it is controversial whether this kind of activation alone is sufficient for DC to prime fully competent T cell responses (Joffre et al. 2009). This is discussed further below.

CD4 T cells that encounter cognate antigen presented by DC upregulate CD40L surface expression. CD40L binding to CD40 on the surface of DC provides a strong activation signal that licenses DC and stimulates the release of chemokines such as CCR5 ligands that attract naïve CD8 T cells (Bennett et al. 1998; Ridge et al. 1998; Schoenberger et al. 1998). CD4 T cell help has been described as a form of immunological “second opinion” because licensing to prime CTL responses against MHC class I-restricted epitopes requires recognition by CD4 T cells of MHC class II-restricted epitopes (Kurts et al. 2010). This form of help to CTL responses requires

CD4 and CD8 T cells to both interact with the same DC (Bevan 2004), a point of critical importance to vaccine design. It is possible that CD4 T cells also provide help directly to CD8 T cells by paracrine release of cytokines or via CD40 expressed on CD8 T cells but this remains controversial (Bevan 2004).

Naïve CD8 T cells require three signals from DC to differentiate into competent cytolytic cells: cognate antigen bound to MHC class I (signal 1), costimulation (signal 2), and a third signal (Curtsinger et al. 2003; Clint S Schmidt and Mescher 2002). Signals 1 and 2 can be sufficient to initiate weak expansion but T cells lack cytolytic function and fail to proliferate on restimulation (Curtsinger et al. 2003; C S Schmidt and Mescher 1999). IL-12 released by activated DC can provide the necessary third signal for CTL differentiation (Clint S Schmidt and Mescher 2002) but is not the only possible signal because competent CTL responses can be primed in IL-12-deficient mice (C S Schmidt and Mescher 1999).

CD40-CD40L interactions, provided by CD4 T cells, are capable of licensing DC to provide the third signal (Bennett et al. 1998; Ridge et al. 1998; Schoenberger et al. 1998). However, PAMPs signalling through TLRs can also be sufficient to license DC, independent of CD4 T cell help (Buller et al. 1987; Rahemtulla et al. 1991). Thus the importance of T cell help to CTL responses depends on the strength of other danger signals (Bevan 2004). There is, however, strong evidence that all CTL responses require at least some CD4 T cell help for the formation of long-lived competent memory cells (discussed below).

The induction of fully activated, licensed DC is fundamental to the success of any immunotherapy aiming to elicit a fully competent and long-lasting adaptive immune response. In active specific immunotherapies this is commonly achieved by providing antigen in association with a PAMP in the form of a live attenuated organism, microbial fragments, or synthetic TLR ligands (reviewed in Olive 2012; Steinhagen et al. 2011). Alternatively, or in addition, DC licensing by CD4 T cells can be encouraged by providing MHC class II as well as MHC class I epitopes in the vaccine. In DC-based therapies, a commonly used approach is to induce DC

maturation by exposure to a cytokine “cocktail” (Feuerstein et al. 2000). The term “DC maturation” describes the increased expression of MHC class II and co-stimulatory molecules in response to an activating stimulus. However, as discussed above, the ability to provide signals 1 and 2 is necessary but not sufficient to prime a fully competent effector T cell response (Joffre et al. 2009; Reis e Sousa 2006; Spörri and Reis e Sousa 2005). In fact, migratory DC naturally undergo maturation as they traffic to lymph nodes where, in the steady state, they serve a tolerising function (Albert et al. 2001). The common practice of using surface marker expression as a surrogate for DC immunogenicity may therefore be misleading and the optimum method of activating DC for adoptive transfer remains controversial (Joffre et al. 2009).

1.2.3.5 T cell memory

In a primary adaptive immune response, the activated T cell population rapidly expands, reaching a peak in about 7 days. Then, providing the antigen is efficiently cleared, 90-95 % of these cells die over the next few weeks, leaving a small pool of long-lived antigen-experienced cells (Harty and Badovinac 2008). These long-lived cells are responsible for the phenomenon of immunological memory, in which subsequent exposure to the same antigen elicits a stronger and more rapid response.

T cells that survive beyond the contraction phase are termed memory cells. Whereas naïve lymphocytes have a finite lifespan and require constant replacement, memory T cells are long-lived, with T cells activated in the first year of life capable of surviving for the lifetime of the host organism (Bevan 2011). Activated naïve T cells downregulate receptors for the cytokines IL-7 (CD127) and IL-15 (CD122), required for survival and homeostatic proliferation respectively. A distinguishing feature of memory T cells is the early re-expression of these receptors, in turn leading to increased expression of anti-apoptotic molecules (Kaech et al. 2003; van

Leeuwen et al. 2009). This permits the survival of an expanded population of antigen-specific cells without a need for TCR stimulation.

Two distinct memory cell phenotypes have been described (Sallusto et al. 1999). Effector memory T cells (T_{EM}) patrol the tissues, particularly at barrier sites such as the skin, lung and gut, but also blood and spleen. They are equipped to rapidly exhibit effector functions (such as the ability to secrete IFN- γ) as soon as they encounter cognate antigen, without needing co-stimulation from APC. Central memory T cells (T_{CM}) express the lymph node-homing molecules CCR7 and CD62L and circulate through secondary lymphoid organs. T_{CM} do not have the ability to immediately perform effector functions. Instead, cognate antigen recognition by T_{CM} triggers IL-2 secretion and proliferation, with differentiation to an effector phenotype occurring later (Pepper and Jenkins 2011). Thus T_{CM} provide a second line of defence that can expand the ranks of effector cells at the infected site or deal with a systemic infection that has managed to breach barrier defences (Sallusto et al. 1999). Which memory subset is most important for protection thus depends on the location of the rechallenge and the nature of the pathogen (Obar and Lefrançois 2010). The timing of the rechallenge may also be relevant in this respect. In one study, the relative importance of T_{CM} in mediating protection after an acute infection increased over time due to a progressive decrease in the proportion of T_{EM} to T_{CM} and a decrease in the proliferative potential of T_{EM} cells (Roberts et al. 2005).

How memory cells develop from naïve cells is uncertain and a full discussion is beyond the scope of this introduction. Briefly, the “linear differentiation” model (Wherry et al. 2003) proposes that naïve CD8 T cells develop into effector cells which then either die or differentiate into memory cells as the antigen is cleared. According to this model T_{EM} are an intermediate phenotype in the transition from effector to memory cell *i.e.* Naïve $\rightarrow$ Effector $\rightarrow T_{EM} \rightarrow T_{CM}$. The alternative “divergent” model holds that the fate of a naïve cell is determined from the outset, with some cells programmed to become effector cells and others to become long-lived memory cells at the time of priming. In fact, both models may be correct.

Experiments in which the fate of naïve CD8 T cells was tracked *in vivo* using a granzyme B reporter system have shown that cells that have acquired at least some effector function can still retain the capacity to differentiate into memory cells (Bannard et al. 2009). On the other hand, although it seems likely that all activated CD8 T cells pass through an early effector phase, there is now strong evidence that two distinct phenotypes emerge early in the course of a primary response. Short-lived effector cells (SLEC) and Memory precursor effector cells (MPEC) can be distinguished by the early re-expression by MPEC of the alpha chain of the IL-7 receptor (CD127) (Kaeck et al. 2003). The relative proportion of these subsets is determined by the prevailing conditions at the time of priming (Lees and Farber 2010). IL-12, for example, drives expression of the transcription factor T-bet (the same “master transcription factor” of T_H1 CD4 T cell responses) and promotes SLEC differentiation (Joshi et al. 2007). A third “hierarchical” model was recently proposed in which stimulation of a naïve T cell leads to expansion of a stem cell-like population, that can then progressively differentiate into T_{CM}, T_{EM} or terminally-differentiated effectors as needed, *i.e.* Naive → T_{CM} → T_{EM} → Effector cell (Gattinoni et al. 2011).

Lineage controversy aside, a consistent observation is that terminally-differentiated CD8 T cells have a reduced capacity for survival and proliferation than memory cells. This is important in designing immunotherapies for cancer, particularly in regard to the adoptive cell transfer (ACT) of T cells. Clinical responses in melanoma patients receiving *in vitro*-activated CD8 T cells are strongly correlated with survival and persistence of the transferred cells (Perret and Ronchese 2008). The optimal conditions for generating long-lived functional T cells for ACT are still contentious but evidence from mouse models has shown that repeated antigen exposure and extended culture in IL-2 *in vitro* drives T cells towards a terminally-differentiated senescent effector phenotype that survives poorly and is suboptimal for treatment (Gattinoni et al. 2005a). The data regarding the relative efficacy of T_{CM} compared with T_{EM} in adoptive T cell transfer for tumour immunotherapy is conflicting although, in most models, T_{CM} appear to be superior (reviewed in Perret and

Ronchese 2008). Reasons for this may be superior survival and proliferation potential, and the ability to home to secondary lymph nodes for restimulation by resident APC (Klebanoff et al. 2005b).

Our understanding of CD4 T cell memory is less advanced than for CD8 T cells. With respect to origin, there is evidence for both linear and divergent models, with a degree of plasticity between CD4 memory cell subsets. For example, T_{EM} derived from T_H1 effector cells can produce T_H2 cytokines depending on conditions at the time of restimulation and vice versa (Pepper and Jenkins 2011). CD4 T cell memory decays more quickly than CD8 over time, possibly related to lower IL-15R expression (Pepper and Jenkins 2011; van Leeuwen et al. 2009).

States of persistent antigen (such as some chronic viral infections and cancer) are associated with defective memory formation (Klebanoff et al. 2006). Conversely, early pathogen clearance accelerates memory formation (Badovinac and Harty 2007). In chronic infection in mice, effector CD8 T cells fail to upregulate the receptors for IL-7 and IL-15, leaving them dependent (or “addicted”) on TCR stimulation for survival, and unable to survive even when transferred into naïve mice (Shin and Wherry 2007). This process is closely linked to the phenomenon of T cell exhaustion (discussed further below) (Shin and Wherry 2007; Wherry 2011).

Importantly, there is evidence that CD4 T cell help at the time of priming is required to “program” CD8 T cells with the capacity to develop into memory cells (reviewed in Bevan 2004). The exact mechanism is uncertain. There is also evidence that CD4 T cells supports CD8 memory in a way that is not antigen-specific, possibly by providing cytokines that support the survival of memory cells (J. C. Sun et al. 2004). This fact has important implications for active vaccination strategies for established cancer, where the goal is to generate not only a potent effector response but also long-lived memory cells capable of preventing relapse (Palucka et al. 2011).

1.2.3.6 DC subsets

Surface markers and gene profiling have identified distinct DC subsets that differ in anatomical location, migratory behaviour, the nature of the antigens they take up and the T cell populations they stimulate. DC can be broadly separated into migratory DC that reside in tissues and have the capacity to migrate to lymph nodes, and lymphoid organ resident DC that develop from blood precursors without trafficking through tissues (Collin et al. 2013). DC of both kinds can be further resolved into subsets based on function and surface markers. Whereas all DC can present exogenous antigen to CD4 T cells (Bedoui et al. 2009), some DC are also capable of cross-priming CD8 T cells. In the secondary lymphoid organs of mice these cross-presenting DC reside in a CD8 α^+ fraction (Heath et al. 2004) (although the function of this T cell co-receptor molecule on DC is unknown). CD8 α^+ DCs are more efficient at phagocytosing dead cells than CD8 α^- DC (den Haan et al. 2000; Iyoda et al. 2002; Schulz and Reis e Sousa 2002). This is thought to be mainly due to the differential expression of receptors that recognise ligands on dead cells, because all DC subsets are able to phagocytose latex beads and bacteria (Iyoda et al. 2002; Schulz and Reis e Sousa 2002; Valdez et al. 2002). One such receptor is DEC205, a plasma membrane-bound molecule with an external C-type lectin domain that is differentially expressed on CD8 α^+ DC relative to CD8 α^- DC (Idoyaga et al. 2011). Although the ligand for DEC205 is still unknown, it has been shown to be involved in the uptake of dying cells (Shrimpton et al. 2009).

There are also differences downstream of antigen uptake. CD8 α^+ DC are much more efficient at cross-presenting exogenous antigen on MHC class I than CD8 α^- DC, even when antigen uptake is equivalent (Schnorrer et al. 2006). This can be partly explained by the finding that CD8 α^+ DC express higher levels of molecules involved in MHC class I presentation and lower levels of molecules involved in MHC class II presentation when compared with CD8 α^- DC (Dudziak et al. 2007). In addition, the processing pathway into which an antigen is directed can depend on the way in which it enters the DC, which can differ because of different endocytic receptor expression by the two subsets. For example, the mannose receptor (MR) is a C-type

lectin receptor that mediates the endocytosis of chicken ovalbumin (OVA) (Burgdorf et al. 2006). OVA taken up by this mechanism is directed from early endosomes into a cross-presentation pathway (Burgdorf et al. 2007). OVA taken up by pinocytosis follows the normal exogenous pathway to be presented on MHC class II. Whereas all DC subsets are capable of pinocytosis, only the CD8 α^+ DC subset express MR. Thus CD8 α^+ DC can present peptides from soluble OVA on MHC class I and class II, whereas CD8 α^- DC can present only on MHC class II (Burgdorf et al. 2007).

Recently it has been shown that CD8 α^+ DC differentially express Clec9A, a C-type lectin that is required for the cross-presentation of dead cell-associated antigens (Sancho et al. 2008; Sancho et al. 2009). Clec9A recognises an undefined ligand in normal host cells that becomes exposed when plasma membrane integrity is lost. It is not required for the uptake of necrotic cell fragments but appears to act as a sensor for diverting the ingested necrotic cell-associated material to the cross-presentation pathway.

In summary, in the lymphoid organs of mice, whereas CD8 α^- DC are capable of presenting exogenous antigen to CD4 T cells, CD8 α^+ DC are equipped to present antigen to both CD4 and CD8 T cells, and are particularly specialised at presenting antigen associated with dead or dying cells. Because the aim of most anti-cancer vaccines is to elicit strong CTL responses, there is considerable interest in trying to target antigens to cross-presenting DC (Kreutz et al. 2013).

CD8 α^+ DC are not all equally efficient at cross-priming CD8 T cells. It has recently been shown in mice that a subset of CD8 α^+ DC express the C-type lectin langerin (CD207) and that depletion of langerin $^+$ cells severely impairs the cross-priming of CD8 T cells in response to soluble OVA co-administered with adjuvant *in vivo* (Farrand et al. 2009). The reasons for this are still under investigation. Langerin binds to carbohydrate molecules found on the surface of certain fungi and viruses and has recently been shown to mediate the uptake of the measles virus by DC (Stoitzner and Romani 2011) so that in some circumstances langerin acts as a pathogen/antigen endocytosis receptor. Langerin $^+$ CD8 α^+ DC are more efficient than

langerin⁻ DC at cross-presenting soluble OVA *in vitro* (Farrand et al. 2009) but cross-presentation of soluble OVA by CD8 α ⁺ DC is not impaired in langerin-deficient mice (Kissenpfennig et al. 2005), suggesting that langerin itself is not required for antigen uptake or cross-presentation in this model. Other possible explanations for the superior cross-priming ability of langerin⁺ DC include the anatomical location of these cells (in the marginal zone of the spleen, so that langerin⁺ DC are optimally situated to take up blood-borne antigens) (Idoyaga et al. 2009) or some other intrinsic property such as increased IL-12 or chemokine production (Farrand et al. 2009). Interestingly, a recent study in which antigen was targeted to DC *in vivo* by conjugation to antibodies to DEC205, Clec9A or langerin found no difference between groups in the magnitude of antigen-specific CD8 T cell responses (Idoyaga et al. 2011).

A similar arrangement of DC subsets exists in tissues. In murine skin, for example, two main populations of migratory DC are present in the deep dermis in the resting state: a CD103⁺ minority and a CD11b⁺ majority (CD103 is integrin α E, the main ligand for which is E-cadherin expressed on epithelial cells). CD103⁺ DC are efficient at cross-presenting skin-derived antigens in draining LNs and support T_H1 responses (Henri et al. 2010; Igyártó et al. 2011). In addition to a shared capacity for efficient cross-presentation, migratory CD103⁺ DC and lymphoid organ-resident cross-presenting CD8 α ⁺ DC also share expression of the surface receptors DEC205, Clec9A and langerin, and are absent in mice deficient in the transcription factor Batf3, suggesting a close developmental relationship between these two subsets (Edelson et al. 2010). The larger CD11b⁺ fraction of migratory DC is more heterogeneous and less well studied. Proposed roles for this DC subset include specialisation in CD4 T cell activation, humoral immunity and responses to extracellular parasites (Guilliams et al. 2010). A role for CD11b⁺ DC in maintaining peripheral tolerance has been proposed based on their ability to produce retinoic acid (that promotes conversion of naïve T cells to iTreg) and to induce Treg *in vitro* (Guilliams et al. 2010; Vitali et al. 2012). However, a recent study using an *in vivo* model found that Treg induction in the resting state was restricted to migratory

langerin⁺ Langerhans cells (LC) and CD103⁺ langerin⁺ dermal DC, leading to the suggestion that the retinoic acid produced by CD11b⁺ DC might act in paracrine fashion to boost Treg induction by other migratory DC subsets (Idoyaga et al. 2013).

Other DC subsets found in tissues include tissue-specific DC such as LC and “inflammatory” DC. LC uniquely reside in the epidermis. Although long thought to be important sentinels of pathogen invasion in the epidermis, the physiological role of LC is controversial. Recent evidence suggests LC do not cross-present antigen *in vivo* (Igyártó et al. 2011). It has been proposed that LC may therefore act primarily as antigen transporters and/or have a major role in maintaining tolerance to commensal skin flora. “Inflammatory” DC, found in tissues and lymphoid organs, are believed to differentiate from circulating monocytes in response to PAMPs and/or cytokines and may act to locally amplify or tune down T cell responses depending on the time point of the immune response (Alonso et al. 2011; Karman et al. 2006; Ziegler-Heitbrock et al. 2010).

Tumour-infiltrating DC (TIDC) are present in murine (Norian et al. 2009) and human (Karthaus et al. 2012) tumours but the presence of these cells is not necessarily conducive to tumour immunity. In mouse tumour models, TIDC have been found to lack efficient antigen-presentation capacity (Stoitzner et al. 2008) or even to suppress T cell function (Norian et al. 2009). However, these TIDC can regain the capacity to prime naïve T cells if removed from the tumour environment (Preynat-Seauve et al. 2006) and, in animal models of glioma, intra-tumoural administration of DC (Pellegatta et al. 2010; Tsugawa et al. 2004) or activation of TIDC *in situ* (Mineharu et al. 2011) can promote tumour immunity.

Human and murine DC subsets share a considerable degree of functional homology. Three main DC subsets are currently recognised in human blood: pDC and two classical DC subsets identified by expression of CD1c (recognised by the blood DC antibody BDCA-1) and CD141 (recognised by BDCA-3), being the functional equivalents of the CD11b⁺ and CD103⁺ / CD8α⁺ murine subsets respectively (Collin et al. 2013). Although blood DC are developmentally less mature, corresponding DC

subsets have also been described in human lymphoid organs and tissues (Ziegler-Heitbrock et al. 2010) with an additional CD14⁺ DC subset also found in tissues (Collin et al. 2013; Haniffa et al. 2012). Importantly, CD141⁺ human DC are superior at cross-presenting soluble antigen compared with CD1c⁺ or CD14⁺ DC and share similar expression of PRRs (particularly TLR3), chemokine receptors (XCR1) and endocytosis receptors (Clec9A) with their CD8 α ⁺ / CD103⁺ murine counterparts (Guilliams et al. 2010; Haniffa et al. 2012).

DC subsets differ in their expression of PRRs consistent with their specialised roles. Plasmacytoid DC are activated by single-stranded RNA and CpG-containing DNA through TLR7 and TLR9 respectively, consistent with their proposed role in innate responses to extracellular viral material through release of type 1 Interferon (Kaisho 2012). With respect to classical DC, whereas CD11b⁺ migratory DC and CD8 α ⁺ lymphoid organ-resident DC express a wide range of TLRs, subsets that specialise in cross-presentation of exogenous antigen to CD8 T cells and driving T_H1 differentiation (and hence are equipped to mount effective responses against intracellular viral infections) express a more restricted range of TLRs including TLR3 that recognises double-stranded RNA (Schreibelt et al. 2010). *Ex vivo* generated monocyte-derived DC uniquely express high levels of TLR4 (Schreibelt et al. 2010). These differences have clear implications for the design of immunotherapies.

1.2.4 Innate-like T cells

1.2.4.1 Natural Killer T cells

Natural Killer T (NKT) cells are a T cell subset that also express NK markers and differ from conventional T cells in a number of ways. The defining characteristic of NKT cells is the expression of a T cell receptor of limited diversity that recognises antigens presented on the non-classical MHC class I-like molecule CD1d. The α - and β -chains that together form the TCR are specified by VDJ gene regions. Because of genetic recombination during T cell development in the thymus, TCR diversity in

conventional T cells is highly diverse. In contrast the Type 1 NKT cell TCR α -chain is invariably specified in mice by V α 14-J α 18 and the β -chain semi-invariably by one of V β 2, V β 7 or V β 8 (Cerundolo et al. 2009). Cells bearing this TCR are accordingly termed “invariant” NKT cells (iNKT). In human iNKT cells, gene usage is limited to V α 24-J α 18 and V β 11 (Cerundolo et al. 2009). Type 2 NKT cells are also CD1d-restricted but have different VDJ gene usage and different properties and will be discussed separately later.

The proportion of $\alpha\beta$ T cells that are iNKT cells varies according to tissue. In mice, iNKT cells account for 2-3 % of T cells in the spleen, 1 % in blood, 0.5 % in lymph nodes, and 30 % in the liver (Albert Bendelac et al. 2007). The tissue distribution in humans is less studied. In peripheral blood, the proportion of T cells that are iNKT cells is highly variable and age-dependent (Weinkove et al. 2013), with a mean of 0.3 % in young adults falling to 0.1 % in the elderly (DelaRosa et al. 2002).

Although NKT cells were named for the fact that they also express markers associated with NK cells, this is not true of all iNKT cells. In C57BL/6 mice, iNKT cells acquire the NK marker NK1.1 as they mature. However, some mature iNKT cells remain NK1.1⁻ (Benlagha et al. 2005). In mice, iNKT cells are CD8⁻ but may be CD4⁺ or CD4⁻. In humans there is an additional subset that is CD8⁺ CD4⁻. Like all T cells, iNKT cells undergo selection in the thymus with the important difference that positive selection is dependent on CD1d rather than the classical MHC molecules (A Bendelac 1995). Thus CD1d-deficient mice lack iNKT cells.

1.2.4.2 Antigen recognition by iNKT cells

CD1 molecules are transmembrane proteins that share significant homology with MHC molecules and, like MHC class I, associate non-covalently with β 2-microglobulin (Porcelli et al. 1989). CD1 molecules perform a similar function to MHC in that they are expressed at high levels on APC and present antigen to T cells but present lipid antigens instead of peptides. Five CD1 isoforms have been

described in humans (CD1a-e) but only CD1d is expressed in mice. CD1b-d present a range of foreign and self-lipid molecules. CD1e is not expressed on the cell surface but is located in soluble form in phagolysosomes where it appears to act as a lipid transfer molecule, facilitating the loading of ingested lipid antigens onto other members of the CD1 family (reviewed in D. C. Barral and Brenner 2007).

The existence of a CD1-restricted subset of human $\alpha\beta$ T cells was first reported by Porcelli and colleagues in 1989 (Porcelli et al. 1989). The existence of a subset of NK1.1⁺ T cells that express a semi-invariant $\alpha\beta$ TCR was reported in 1994 (Lantz and Bendelac 1994). In 1995, a screening exercise for bioactive substances identified a novel compound in a marine sponge, alpha-galactosylceramide (α -GalCer) that had potent anti-tumour effects in mouse models (E. Kobayashi et al. 1995). A synthetic version of α -GalCer was subsequently shown to be a CD1d-restricted ligand for the iNKT cell TCR (Kawano et al. 1997). Several analogues of α -GalCer have been synthesized in an effort to maximise and direct iNKT responses (reviewed in Cerundolo et al. 2009) but α -GalCer remains one of the most potent known iNKT cell antigens.

The natural ligands for the iNKT TCR (and hence the physiological role of iNKT cells) remains uncertain. Alpha-galactosylceramide is a glycosphingolipid consisting of a sugar “head” and a bifid “tail” that has lipid and sphingosine components. The alpha link between the head and tail regions does not occur naturally in mammals and is crucial to the immunogenicity of the molecule as β -GalCer is much less potent (Kawano et al. 1997). Alpha-linked glycolipid antigens derived from *Borrelia burgdorferi* (the microbe responsible for the CNS infection Lyme Disease) (Kinjo et al. 2006) and from *Sphingomonas* (a bacterium of low virulence in humans) (Kinjo et al. 2005; Mattner et al. 2005) are recognised by iNKT cells, which raises the possibility that iNKT cells may serve a general purpose in responding to foreign α -linked glycolipids. However, because iNKT cell activation occurs in many situations where microbial lipid antigens do not appear to be present, it is widely believed that naturally-occurring endogenous iNKT cell ligands must exist. A recent study

(Brennan et al. 2011) screened a panel of naturally-occurring glycosphingolipids eluted from CD1d molecules and found that β -D-glucopyranosylceramide (β -GlcCer) potently activates iNKT cells in mice and humans. Because this molecule accumulates during infection, the authors propose that β -GlcCer serves as an endogenously-generated “danger” signal that recruits iNKT cells to immune responses during infections. Several TCR-independent mechanisms of iNKT cell activation have also been described including exposure to cytokines such as IL-12 (Nagarajan and Kronenberg 2007) and engagement of iNKT cell costimulatory molecule receptors, Fc receptors, NK cell activating receptors, and PRRs (Luc Van Kaer et al. 2013).

1.2.4.3 iNKT cell effector functions

In disease states, iNKT cells may stimulate or inhibit immunity depending on the context. Mice lacking iNKT cells experience more severe pathology after infection with *Borellia burgdorferi*, a known source of ligands for the iNKT cell TCR (Kinjo et al. 2006). Infections with *Streptococcus pneumoniae* (Kawakami et al. 2003) and Influenza virus (De Santo et al. 2008), organisms that do not furnish ligands for iNKT cells, are also prolonged or more severe in these mice. iNKT cells also appear to have a role in cancer immunosurveillance. Carcinogen-induced cancers occur earlier in iNKT cell-deficient mice than in wild type (WT) mice (Crowe et al. 2005; M J Smyth et al. 2000). iNKT cells are also implicated in dysfunctional immune states. Although the role of iNKT cells in human asthma is highly controversial, they are important mediators in a mouse model of asthma, allergic airway hyperresponsiveness, in an IL-4 and IL-13 –dependent manner (Akbari et al. 2003). Whereas production of these T_H2 cytokines by iNKT cells is harmful in some contexts, it may be protective in others. Nude Obese Diabetic (NOD) mice spontaneously develop diabetes due to autoimmune pancreatic islet destruction. NOD mice lack T- and B-cells but retain some iNKT cells. Diabetes develops more quickly in these mice if iNKT cells are depleted (B. Wang et al. 2001) suggesting a protective role for iNKT cells. In undepleted NOD mice, the protective

function of iNKT cells is enhanced by administering α -GalCer (S. Hong et al. 2001; Sharif et al. 2001; B. Wang et al. 2001) and even more enhanced by ligands that skew iNKT cells towards secretion of T_H2 cytokines (Mizuno et al. 2004). A protective effect of T_H2 -biased iNKT cell cytokine secretion has also been observed in EAE, a murine model of multiple sclerosis that is mediated by T_H1 and T_H17 cells (Miyamoto et al. 2001; Oki et al. 2004).

The complex and seemingly contradictory behaviour of iNKT cells in disease states is mirrored by diverse and complex effector functions. Firstly, several studies have shown that mouse and human iNKT cells are capable of directly inducing apoptosis in tumour cell lines and DC *in vitro* (Fais et al. 2004; Kawano et al. 1998; Nicol et al. 2000a; Takahashi et al. 2000). Some of these studies showed that blocking perforin secretion abrogated cytotoxicity, and that CD1d-expression by the target cell was not necessarily required. However, it has subsequently been suggested that these observations were due to contamination of the cell cultures by NK cells (Wingender et al. 2010). More recently it has been shown that α -GalCer-pulsed splenocytes and B16 melanoma cells are lysed *in vivo* in a way that requires iNKT cells and CD1d expression by the target cells, and that this was abrogated by blocking the Fas-FasL pathway (Wingender et al. 2010). This study would therefore suggest that direct iNKT cell killing in the setting of cancer would require the target cells to express both Fas and CD1d. At the present time, the relevance of iNKT cytotoxicity is unclear and other iNKT cell properties to be described below are probably more important. Direct iNKT cell killing of CD1d-expressing dendritic cells may be an important regulatory mechanism for limiting immune responses (Nicol et al. 2000b).

A hallmark of iNKT cells is the capacity of naïve cells to rapidly secrete large amounts of cytokine upon antigen encounter. Similar to innate cells such as NK cells, this property is due to the presence of preformed mRNA, and iNKT cells are often referred to as “innate-like”. Activated iNKT cells secrete a broad range of cytokines including IFN- γ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, granulocyte macrophage colony stimulating factor (GM-CSF), TNF- α and TGF- β (Coquet et al.

2008). Thus, confusingly, iNKT cells secrete both T_H1 and T_H2 cytokines that have very different effects and counter-regulate each other, as well as both pro-inflammatory and immunosuppressive cytokines. Cytokine secretion by iNKT cells depends to an extent on the ligand, with some synthetic analogues of α -GalCer eliciting a more T_H1 - or T_H2 -skewed cytokine profile than α -GalCer itself (reviewed in Cerundolo et al. 2009). The dominant effect of the cytokines secreted by iNKT cells probably depends on multiple factors including the ligand, the anatomical site, and the context in which activation is occurring. Further complexity is introduced by the fact that iNKT responses are sensitive to the time course of α -GalCer exposure. Thus iNKT cell cytokine secretion profile differs after single exposure to α -GalCer compared with repeated exposure (T_H1 - and T_H2 -skewed respectively) (Parekh et al. 2005).

A third important action of iNKT cells is the activation of other cells of the innate and adaptive immune systems. Interaction between iNKT cells and APC presenting α -GalCer on CD1d leads to iNKT activation and cytokine release but also causes iNKT cells to upregulate CD40L expression and provide an activation signal to DC (Fujii et al. 2003; Hermans et al. 2003b). In this way, iNKT cells are able to licence DC to initiate T cell responses, and provide help to B cells, in a manner similar to conventional CD4 T cells. DC activated by α -GalCer / iNKT cells are also induced to secrete significant quantities of IL-12 (Kitamura et al. 1999; Tomura et al. 1999). As discussed above, IL-12 provides a third signal to CD8 T cells during priming and promotes T_H1 polarisation of CD4 T cells. In addition, IL-12 enhances NK cytotoxicity and induces NK cells to release large quantities of IFN- γ (Carnaud et al. 1999; Mark J Smyth et al. 2002). Thus the iNKT cell–DC interactions induced by α -GalCer lead to a number of downstream effects that are conducive to anti-tumour immunity. For this reason, there is considerable interest in using iNKT cell agonists as adjuvants in vaccines. Vaccines that deliver antigen with α -GalCer (as coadministered free α -GalCer, pulsed on to DC, or pulsed on to tumour cells) are capable of inducing effective tumour-specific adaptive immune responses in mice (discussed in greater detail in Chapter 3) but this approach has not yet been tested

in humans. Adding to the interest in exploiting iNKT cells in cancer vaccines, iNKT cells have also been shown to be capable of negatively regulating several immunosuppressive cell types including tumour-associated macrophages (TAM) (Song et al. 2009), myeloid-derived suppressor cells (MDSC) (De Santo et al. 2008) and Type II NKT cells (Ambrosino et al. 2007).

Several studies have shown that DC in the spleen and Kupffer cells in the liver play major roles in presenting blood-borne lipid antigens to iNKT cells (Fujii et al. 2003; Schmiege et al. 2005). Less attention has been given to events in LN because of the lower number of iNKT cells in this location. However, it has now been shown that α -GalCer delivered intradermally (i.d.) with antigen is capable of inducing antigen-specific T cell responses in the draining LN (Bontkes et al. 2010; Dondji et al. 2008; Tripp et al. 2009). Vaccination via this route is particularly effective against skin tumours, possibly because priming in cutaneous LNs imprints responding T cells with a skin-homing phenotype (Mullins et al. 2003). Alpha-galactosylceramide has also been used successfully as an adjuvant in mouse models when administered orally, intraperitoneally (i.p.), or intranasally (Courtney et al. 2009; Courtney et al. 2011). When administered intranasally, α -GalCer-induced T cell priming has been shown to take place in the mediastinal LN (Courtney et al. 2011). Although some systemic activation of iNKT cells occurs when α -GalCer is given via these routes (presumably because some α -GalCer finds its way into the circulation), antigen-specific T cell responses are largely restricted to the draining LNs, consistent with the idea that efficient T cell priming requires presentation of α -GalCer and antigen by the same APC. When α -GalCer is administered systemically with soluble antigen, T cell priming in peripheral LNs has been reported to be either absent (Tripp et al. 2009) or modest (Bontkes et al. 2010).

A study of iNKT cell behaviour in intact lymph nodes showed that iNKT cells randomly patrol the paracortical region searching for cognate antigen in the same manner as conventional CD4⁺ T cells (P. Barral et al. 2010). When particulate α -GalCer is administered subcutaneously (s.c.) it quickly reaches the subcapsular

sinus of the draining LN where it is taken up by CD169⁺ macrophages. Within 6 hours of α -GalCer administration iNKT cells are observed to form long-lasting contacts with these CD169⁺ macrophages, which is dependent on CD1d (P. Barral et al. 2010). This suggests that, at least in the very early stages of an immune response, macrophages in draining lymph nodes may play an important role in taking up and presenting glycolipid antigen to induce early activation of iNKT cells. The authors of this study speculate that these macrophage-activated iNKT cells are then available to enhance adaptive immune responses by interacting with DC and B cells in the same lymph node. Consistent with this, others have shown that, although both skin-resident migratory DC and LN-resident DC express CD1d and are capable of presenting α -GalCer to iNKT cells *in vitro*, skin-resident DC are dispensable for the adjuvant effects of i.d. α -GalCer *in vivo* (Tripp et al. 2009).

1.2.4.4 iNKT cell anergy

After systemic administration of α -GalCer in mice, iNKT cells become undetectable in spleen, liver and blood within a few hours leading to the proposal that strong stimulation of iNKT cells causes antigen-induced cell death. Subsequently, it has been shown that this phenomenon is due to transient downregulation of NK1.1 and of the invariant TCR (Crowe et al. 2003; M. T. Wilson et al. 2003). In fact, surface expression of the TCR begins to return after 48 hours and the surviving cells proliferate, with peak expansion occurring at around Day 3 (greater than 10 fold in spleen) before contraction to baseline levels occurs (Parekh et al. 2005). However, iNKT cells typically exhibit a suppressed response to a second exposure to α -GalCer for at least 4-6 weeks, with a reduction in iNKT cell capacity to secrete cytokines, proliferate, activate DC, and provide tumour protection (Cerundolo et al. 2009; Parekh et al. 2005). The phenomenon of iNKT cell anergy is of vital importance to the design of vaccination strategies incorporating α -GalCer as an adjuvant.

Suppression of cytokine secretion is dose-dependent (Parekh et al. 2005) and does not affect all cytokines equally, with IFN- γ secretion being more profoundly suppressed than IL-4 (Parekh et al. 2005; N. Singh et al. 1999). At doses that induce a similar level of DC activation (as measured by IL-12 production), free α -GalCer is more suppressive than α -GalCer pulsed onto DC (Fujii et al. 2002), an effect that has been attributed to presentation of α -GalCer by non-professional APC (Fujii et al. 2002) such as B cells that do not provide equivalent co-stimulation to iNKT cells (Courtney et al. 2011; Parekh et al. 2005; Thapa et al. 2009). However, the idea that iNKT cell anergy is due to a lack of co-stimulation is not universally supported (Iyoda et al. 2010). Anergy may also depend on the route of administration, with lower levels of anergy reported after i.d. (Bontkes et al. 2010) and intranasal (Courtney et al. 2011) delivery of α -GalCer compared with intravenous (i.v.). The molecular events underlying iNKT cell anergy are not well understood. Upregulation of the co-inhibitory molecule programmed cell death protein 1 (PD-1) on the surface of activated iNKT cells may be at least partially responsible but, again, the evidence for this is inconsistent (Iyoda et al. 2010; Parekh et al. 2009). Chronic α -GalCer administration leads to a different form of tolerance in which there is depletion of existing iNKT cells and repopulation from thymus with iNKT cells expressing high levels of inhibitory receptors (Hayakawa et al. 2004).

In an early study in human cancer patients, free α -GalCer administered i.v. led to a sustained fall in iNKT cell numbers to undetectable levels, as measured using flow cytometry and antibodies to CD3 and the invariant TCR (Giaccone et al. 2002). Several subsequent studies using α -GalCer-pulsed DC administered i.v. have reported rises in iNKT cell numbers (D. H. Chang et al. 2005; A. Ishikawa et al. 2005; T. Uchida et al. 2008) or falls that were only transient (Nieda et al. 2004). In some patients, IFN- γ secretion (Nieda et al. 2004; T. Uchida et al. 2008) or iNKT cell proliferation (D. H. Chang et al. 2005; A. Ishikawa et al. 2005) was observed only after the second vaccine or was boosted by the second vaccine. Although it is difficult to reach firm conclusions from these studies, the data appear to suggest

that, in humans, α -GalCer pulsed-DC can be administered repeatedly at 1-4 week intervals without necessarily inducing systemic iNKT cell anergy.

1.2.4.5 iNKT cell subsets

Recently, evidence has been accumulating to support the existence of distinct iNKT cell subsets that diverge during thymic development. NKT1, NKT2, and NKT17 subsets are characterised by the secretion of T_H1 cytokines, T_H2 cytokines and IL-17 respectively (reviewed in Buechel et al. 2015). Two other putative subsets have been described but it is less clear whether these are distinct lineages or develop from other iNKT cell subsets. NKT_{FH} (follicular helper) cells appear to play an important role in supporting humoral responses to infections (P.-P. Chang et al. 2011b; King et al. 2012). NKT10 cells secrete the immunosuppressive cytokine IL-10 and can be detected in mice after strong stimulation with α -GalCer (Sag et al. 2014). The existence of distinct subsets with different effector functions would provide a plausible explanation for the apparently diverse effects of iNKT cells in different contexts and disease states.

1.2.4.6 Type 2 NKT cells

Type 2 NKT cells are also CD1d-restricted but have diverse $\alpha\beta$ TCRs and do not recognise α -GalCer (Masaki Terabe et al. 2005). Among the identified ligands for Type 2 NKT cell TCRs are phospholipid antigens derived from *Mycobacterium tuberculosis* and other bacteria suggesting a protective role for Type II NKT cells against some infections (Tatituri et al. 2013). Another well-known Type 2 NKT cell ligand is sulfatide, a major constituent of myelin, prompting speculation that these cells might have a natural regulatory role in the CNS (Blomqvist et al. 2009). In tumour models these cells act in an immunosuppressive manner by secreting IL-13 that, in turn, induces myeloid cells to produce TGF- β (M Terabe et al. 2000; Masaki

Terabe et al. 2003). Activated Type 2 cells also inhibit the tumour protective effect of Type 1 NKT cells (Ambrosino et al. 2007). Interestingly, one study reported that nearly half of all T cells infiltrating GBM tumours were also positive for the NK marker CD56 but were not iNKT cells (Waziri et al. 2008).

1.2.5 Anti-tumour responses mediated by innate cells

1.2.5.1 NK cells

Natural Killer cells are lymphocytes whose main purpose is to detect and kill tumour cells and host cells infected with pathogens (Hayakawa and Smyth 2006; J. C. Sun and Lanier 2011) but use a very different recognition system to T and B cells. NK cells develop in the bone marrow and reside principally in the blood, spleen, liver and lung. Although the majority of resting NK cells express the LN homing integrin L-selectin (CD62L), they are present at low frequency in LN in the steady state (J. C. Sun and Lanier 2011). When activated, NK cells proliferate, secrete cytokines such as IFN- γ and TNF- α , and induce apoptosis in target cells by releasing perforin and granzymes (J. C. Sun and Lanier 2011).

NK cell activation is determined by the integration of signals received from multiple activating and inhibitory receptors on the cell surface (Bryceson and Ljunggren 2008). The inhibitory receptors recognise host MHC class I molecules, so that cells expressing MHC class I are protected from NK cell attack (Karlhofer et al. 1992).

All nucleated cells continuously cleave cytosolic proteins into peptides and display these on the cell surface. In this way the adaptive immune system is alerted to the presence of foreign, mutant or overexpressed proteins inside the cell. As previously described, these peptides must be presented in a complex with MHC class I molecules in order to be recognised by the T cell receptor. Downregulation of MHC class I is a common immune evasion mechanism employed by many viruses and

tumours to evade the adaptive immune system. One of the main roles of NK cells is therefore to eliminate potentially dangerous cells that lack MHC class I expression.

The best characterised activating NK receptor is NKG2D. The ligands for this receptor are induced by cellular stress and/or DNA damage and are frequently expressed by tumours and infected cells (Hayakawa and Smyth 2006; Ljunggren 2008). Blocking the NKG2D activating receptor in mice increases susceptibility to chemically-induced tumours (Mark J Smyth et al. 2005). NK cells can also be activated by DC by direct cell-to-cell contact through activating receptors or by soluble cytokines such as IL-12 and type 1 interferon (J. C. Sun and Lanier 2011).

Although NK cells do not undergo gene rearrangement during development (and therefore do not have the degree of receptor diversity exhibited by T cells), it has been shown in mice that after a primary challenge with certain antigens or viruses, NK cells transferred to naïve hosts are able to protect against challenge, and that this protection is antigen-specific and persists for at least several months (Paust and Andrian 2011). Thus, although considered to be innate cells, NK cells are capable of exhibiting a form of antigen-specific immunological memory, at least in mice. The mechanisms underlying antigen-specific “NK memory” are unclear (Paust and Andrian 2011). NK cells activated by cytokines alone can also exhibit memory-like properties in that, for at least 3 weeks, they produce significantly more IFN- γ when restimulated (Cooper et al. 2009).

NK cells are able to lyse a wide range of tumour cells *in vitro* and many independent studies demonstrate a role for NK cells in tumour immune surveillance in mouse models *in vivo* (Hayakawa and Smyth 2006; Mark J Smyth et al. 2005; Vivier et al. 2008). In the clinic, allogeneic haematopoietic stem cell transplantation for acute and chronic myeloid leukaemia results in a graft-*versus*-leukaemia effect that is often curative. There is strong evidence in human patients and in mice that the graft-*versus*-leukaemia effect is at least partly due to lysis of HLA-mismatched leukaemic cells by donor NK (Barrett 2008; Ruggeri et al. 1999). However, whether

this phenomenon can be successfully extended to non-haematopoietic solid tumours is not yet clear (Vivier et al. 2008).

1.2.5.2 Macrophages

Macrophages are phagocytic cells of myeloid lineage found in almost all tissues and in secondary lymphoid organs (Murray and Wynn 2011). In the steady state, when danger signals are absent, macrophages play a central role in normal tissue homeostasis by removing apoptotic cells and cellular debris. The recognition of apoptotic cells via “scavenger” receptors (such as for phosphatidyl serine) does not trigger cytokine gene transduction (Mosser and Edwards 2008) but does stimulate release of TGF- β (Mosser and Edwards 2008) and IL-10 (Murray and Wynn 2011). Thus, the default setting of macrophages in the steady state is to suppress inflammation (Mosser and Edwards 2008; Murray and Wynn 2011).

Macrophages are important players in the innate response to invading pathogens. Invading bacteria are recognised by cell surface receptors, taken up by phagocytosis and then killed within phagosomes by several mechanisms including acidification, fusion with lysosomes, and exposure to nitric oxide (NO) and reactive oxygen species (ROS). Ligation of PRRs also causes activated macrophages to release cytokines and chemokines that recruit other immune cells to the site of infection (Murray and Wynn 2011).

Macrophages also play important roles in adaptive immune responses, including anti-tumoural responses. “Classically-activated macrophages” (CAM, also known as M1 macrophages) develop in response to PAMPs and/or pro-inflammatory cytokines IFN- γ and TNF- α . CAM themselves promote inflammation by secreting cytokines (such as TNF- α , IL-1, IL-6, and IL-23), chemokines and proteases, and by increasing production of toxic NO and ROS (Mosser and Edwards 2008). CAM also upregulate expression of MHC class II and co-stimulatory molecules and secrete IL-12, thereby restimulating arriving T cells and reinforcing T_H1 differentiation.

Although the initial source of IFN- γ required to generate CAMs can come from innate cells such as NK cells, sustained generation of CAMs requires IFN- γ from T_H1 or CTL. IFN- γ and TNF- α released by the arriving T cells in turn promotes phagocytic function and a “respiratory burst” in M1 macrophages that enhances the cells ability to kill intracellular pathogens and release tumouricidal free radicals (Miguel et al. 2010). Macrophages also elicit help from CD4 T cells by displaying antigen-MHC class II complexes and CD40 on their surface. Thus M1 macrophages and T cells are interdependent. Macrophages, that kill indiscriminantly, are dependent for sustained activation on antigen-specific T cells. Conversely, the tumouricidal properties of T cells may be mediated indirectly by boosting the effector functions of macrophages (Hicks et al. 2006; Mace et al. 1988). Contact-dependent mechanisms of tumour cell killing by macrophages have also been described (Hicks et al. 2006; Tsung et al. 2002). In keeping with this, depletion of macrophages has been shown to promote tumour growth in a murine glioma model (Galarneau et al. 2007). However, these tumouricidal functions of macrophages become subverted as tumours progress and immunosuppressive phenotypes predominate by the time patients present with symptomatic tumours (Mosser and Edwards 2008). This probably explains why the presence of TAM has been associated with a poorer prognosis in breast cancer (X. Tang 2013) and GBM (Nagai et al. 2009). This will be discussed in greater detail below.

1.3 IMMUNOTHERAPY FOR GBM

1.3.1 Spontaneous anti-tumour immune responses

The immunosurveillance hypothesis, according to which the immune system destroys tumour cells as they arise, is supported by animal models in which immunodeficient mice are more susceptible to tumour development than WT mice (Shankaran et al. 2001; Street et al. 2001; van den Broek et al. 1996). In human patients, all detectable tumours have, by definition, evaded elimination. However,

there is abundant evidence that anti-tumour immune responses exist in cancer-bearing patients and have useful anti-tumour effects (Apetoh et al. 2007; DuPage et al. 2011; reviewed in Fridman et al. 2012). In glioma patients, several studies have reported finding spontaneous antibody responses to tumour-associated antigens (Pallasch et al. 2005; Ueda et al. 2004) (Schmits et al. 2002) that, at least in one report, correlated with improved outcome (Pallasch et al. 2005). Reports of spontaneous cellular immunity to specific antigens also exist (Yajima et al. 2005) but are less common (Dunn et al. 2012). Tumour-infiltrating lymphocytes (TIL) extracted from glioma tumours and expanded *ex vivo* in IL-2 are capable of lysing autologous tumour cells *in vitro* (Sawamura et al. 1989) and, in one study, PBMC from four of four GBM patients released IFN- γ after stimulation with autologous tumour (J. Tang et al. 2005). In many other cancer types, the presence of TIL is a good prognostic factor (Mihm et al. 1996; Ohtani 2007; Sato et al. 2005; Fridman et al. 2012). In glioma, studies on the effect of the presence of TIL on prognosis have yielded conflicting results. Most studies have reported a beneficial impact (Böker et al. 1984; Kmiecik et al. 2013; Lohr et al. 2011; Palma et al. 1978; I. Yang et al. 2010). Two studies have reported no association between survival and infiltration with small round cells (Rutledge et al. 2013) or with CD4⁺ or CD8⁺ T cells (Rossi et al. 1989) and one found the presence of small round cells had a negative impact on survival but did not further phenotype these cells (Safdari et al. 1985). Recent studies that have, at least to some extent, characterised the infiltrating cells have reported positive correlations between survival and tumour infiltration by CD8⁺ T cells (I. Yang et al. 2010), by CD8⁺ or CD8⁻ effector T cells (Lohr et al. 2011), and by CD3⁺ or CD8⁺ cells (Kmiecik et al. 2013).

A notable feature of GBM is the fact that, although extensive local invasion is invariable, metastasis outside the CNS is extremely rare, with approximately ten reported cases of spontaneous metastasis of unresected GBM over 50 years (Volovitz et al. 2011). Strong evidence that this is due to immunological factors rather than to a barrier to tumour cells entering the circulation is provided by the observation that immunosuppressed patients receiving organ transplants from

donors with high grade glioma have a 31 % chance of developing a GBM metastasis in the transplanted organ (Buell et al. 2003). This suggests that, in many patients at least, spontaneous endogenous immune responses exist and are able to keep systemic metastases in check while failing to control the primary tumour.

Taken together, these data show that tumour-specific immune responses can develop spontaneously in glioma-bearing patients and that these responses are capable of exerting meaningful anti-tumour activity.

In the next section, progress in the field of immunotherapy for cancer is briefly reviewed. Although the focus is on glioma, reference is also made to landmark studies in other cancer types.

1.3.2 Immunotherapy for cancer

1.3.2.1 Cytokine therapy

Some of the earliest cancer immunotherapies have consisted of attempts to non-specifically stimulate or boost endogenous anti-tumoural immune responses. Based on the observation of spontaneous remissions of soft tissue sarcoma after streptococcal skin infection, a New York surgeon William Coley administered heat-killed bacteria, repeatedly eliciting high fever and rigors over a period of weeks or months. Remarkably, durable remissions occurred in approximately one half of patients with soft tissue sarcoma (Starnes 1992). The mechanism of action of “Coley’s toxins” is not known with certainty but is likely to involve release of pro-inflammatory cytokines such as TNF (Brouckaert et al. 1992). The advent of recombination technology has allowed more precise approaches. Systemic or intra-tumoural administration of pro-inflammatory cytokines has been highly effective in animal models but toxicity has hampered this approach in patients.

IL-2 stimulates NK cells and promotes T cell proliferation. In metastatic melanoma patients, the systemic administration of IL-2 achieved a 16 % objective response

rate in 8 clinical trials, including some durable complete responses (Atkins et al. 1999). Systemic treatment was associated with a high incidence of serious toxicity consisting mainly of transient hypotension and a 2.2 % incidence of death due to sepsis, thought to be due to an effect of IL-2 on neutrophil chemotaxis. In a recent report, repeated injections of IL-2 directly into metastatic melanoma lesions resulted in complete response lasting at least 6 months in 70 % of injected lesions with no serious toxicity (Weide et al. 2010). No effect on uninjected lesions was observed in these studies. In glioma, IL-2 has most notably been used as an adjunct to adoptive cell transfer as described below.

IL-12 stimulates NK cells to release cytokines, notably IFN- γ , and promotes T_H1 CD4 T cell differentiation. Systemic IL-12 administration has also been associated with severe toxicity, including death in some patients (Leonard et al. 1997). Local intracranial delivery of IL-12 by various means is highly effective in rodent models of glioma both as single agent (Dimeco et al. 2000; Ehtesham et al. 2002; Y. Liu et al. 2002) or in combination with systemic CTLA-4 blockade (Johannes Vom Berg, oral presentation, Keystone Symposium, Vancouver 2013) but has yet to be tested in patients.

A number of other cytokines (reviewed in Butowski 2010; Kane and Yang 2010) and PAMPs (Butowski 2010; Carpentier et al. 2010; Rosenfeld et al. 2010; Apostolis Stathopoulos et al. 2012) have also been tested in glioma models and early phase clinical trials but appear more likely to find a role as adjuvants or in combination with other immunomodulators than as single agents.

1.3.2.2 Monoclonal antibodies targeting tumour cells

Monoclonal antibodies are now firmly incorporated into the standard treatment for a number of haematological and solid cancers. Rituximab, a humanised monoclonal antibody (mAb) specific for the B cell marker CD20, has become a backbone of the treatment for chronic lymphocytic leukaemia and other B cell malignancies (Jain

and O'Brien 2013). Other FDA-approved mAbs that directly target tumour cells include trastuzumab for Her 2-positive breast cancer and EGFR-specific mAbs for colon and head and neck cancer (reviewed in Bhutani and Vaishampayan 2013). These mAbs are believed to act by blocking pathways that promote tumour cell survival and proliferation, and by mediating antibody-dependent and complement-mediated cytotoxicity (Bhutani and Vaishampayan 2013). Although EGFR is overexpressed in 40-50 % of GBM, early phase trials of the anti-EGFR mAbs cetuximab and nimotuzumab suggest modest efficacy (Bode et al. 2012; and reviewed in Mitra et al. 2010).

The anti-VEGF antibody bevacizumab targets tumour vasculature by binding and neutralising circulating VEGF. Early phase trials in patients with recurrent GBM revealed striking radiological response rates but modest or no effect on overall survival relative to historical controls (Friedman et al. 2009; Morris 2012; Norden et al. 2009; Vredenburgh et al. 2007b; Vredenburgh et al. 2007a). This phenomenon has led some to question whether the radiological response in these patients is due to an effect on the permeability of blood vessels to gadolinium rather than a real anti-tumour effect and has prompted the development of new methods for assessing clinical response in clinical trials in glioma patients (Quant and Wen 2011). An international Phase III multicentre trial of bevacizumab in combination with standard therapy for newly presenting GBM is currently underway.

Antibodies can also be used to deliver cytotoxic agents. An antibody to the extracellular matrix protein tenascin-C, expressed in most gliomas but not in normal brain, has been used to deliver a radioactive isotope to the tumour resection cavity in GBM patients (Reardon et al. 2008). Promising results in early phase trials have led to a randomised Phase III trial that is currently in progress (Mitra et al. 2010).

Bi-specific antibodies are a promising new approach. These bivalent antibodies typically bind both a defined tumour cell surface molecule and CD3, thereby combining high affinity binding of the target molecule with the cytotoxic machinery

of T cells irrespective of their TCR specificity (Withoff et al. 2001). As for anti-EGFR mAbs, this approach requires stable surface expression of the target molecule.

1.3.2.3 Adoptive cell transfer (ACT)

In ACT, autologous T lymphocytes with anti-tumour activity are expanded *ex vivo* and reinfused into the patient. Considerable success has been achieved with this approach in patients with melanoma. Using TIL expanded *in vitro* with IL-2, Rosenberg and colleagues reported objective tumour responses in 50 % of patients with metastatic melanoma, providing patients were lymphodepleted prior to adoptive transfer (summarised in Steven A Rosenberg et al. 2008). Although extraction of TIL from GBM tumours is feasible (Quattrocchi et al. 1999), most clinical studies of adoptive immunotherapy for glioma have involved “lymphokine-activated killer (LAK) cells”, a mixture of T lymphocytes and NK cells expanded from autologous peripheral blood mononuclear cells (PBMC) in the presence of IL-2. Systemic administration of LAK cells was pioneered in cancer patients by Rosenberg and colleagues (S A Rosenberg et al. 1985) but, in most glioma studies, LAK cells have been co-administered with IL-2 directly into the tumour resection cavity. Initial clinical trials in patients with recurrent GBM were disappointing and associated with local IL-2-related toxicity (Barba et al. 1989; Merchant et al. 1988). In several subsequent studies, long-lasting objective responses have been observed but the effect on overall survival relative to historical or contemporaneous controls has been variable (Boiardi et al. 1994; Robert O Dillman et al. 2004; Hayes et al. 1995; Hayes et al. 2001; Lillehei et al. 1991; Sankhla et al. 1996). Most recently, Dillman and colleagues (Robert Owen Dillman et al. 2009) tested this approach in the setting of newly presenting GBM, with direct surgical administration of LAK cells and IL-2 into the resection cavity following standard treatment. Considering that patients who progressed during standard treatment were excluded, the observed median survival of 20 months indicates a modest clinical benefit at best.

Allogeneic Haematopoietic Stem Cell Transplantation, a highly successful form of ACT for haematopoietic cancers that exploits a graft *versus* tumour effect, has also been used for solid cancers with some durable objective responses observed in patients with renal cell cancer (Childs et al. 2000) and metastatic breast cancer (Ueno et al. 2008) but is associated with a treatment-related mortality of up to 29 % (Ueno et al. 2008) and has not found a place in the standard treatment of solid cancers to date. Recently, autologous T cells engineered to express a TCR specific for a selected epitope or a chimeric antigen receptor (CAR) that combines the specificity of an antibody with the intracellular machinery of a T cell receptor are attracting considerable attention and early studies are showing particular promise in B cell lymphomas that express CD19 or CD20 (T. S. Park et al. 2011; Till et al. 2012). However, like antibody-based immunotherapies, this monovalent approach requires stable and ubiquitous expression of the target antigen by tumour cells.

1.3.2.4 Antigens for cancer vaccines

Active specific immunotherapy, or vaccination, can be defined as the administration of antigen along with a “danger” signal, or adjuvant, with the aim of eliciting a long-lasting antigen-specific adaptive immune response. While the efficacy of prophylactic vaccination against pathogenic microorganisms is firmly established, therapeutic vaccination against established cancer has proven to be much more difficult proposition. The nature of the target antigens is a major factor. Pathogen-derived “foreign” proteins furnish epitopes that bind T cell receptors with high affinity and provide strong T cell stimulation. Theoretically, the ideal antigens for cancer immunotherapy are also tumour-specific mutant proteins not previously encountered by the host immune system (Gilboa 1999; Neller et al. 2008). In practice, the targets in cancer vaccines are often “self” antigens that are over-expressed, termed “tumour-associated antigens” (TAA) or “shared” antigens. Thymic selection ensures that T cells recognising these antigens are of low avidity. In addition, peripheral tolerance mechanisms act in the steady state to continuously anergise self-reactive T cells. Despite this, tolerance to self-antigens can be “broken”

if the vaccine incorporates an adjuvant capable of stimulating APC to deliver the signals required to fully prime naïve T cells (Cordaro et al. 2002; Daniels et al. 2004; Kottke et al. 2011; Morgan et al. 1998). The quantity of antigen delivered to APC (and hence the density of peptide-MHC complexes on the APC surface) and the duration of antigen presentation are also important in determining whether T cells reach a threshold for priming (Henrickson et al. 2008). Assuming tolerance can be broken, a constant concern is the threat of “on-target” auto-immunity. In certain situations, an absence of inflammation in tissues expressing the target antigen protects them from immune attack (Kottke et al. 2011). In others, expression of the target antigen is restricted to expendable tissues such as melanocytes or prostate gland, so that auto-immunity, when it occurs, is clinically acceptable. These are special cases that do not apply to the GBM setting. However, auto-immunity does not inevitably accompany effective anti-tumour immunity even if the targeted TAA are also expressed by normal cells within the inflamed organ harbouring the tumour. How this occurs is not clear. One possibility is that degranulation of CTL requires a threshold density of peptide-MHC complexes on the target cell surface that is reached by tumour cells but not normal cells (Morgan et al. 1998; Weijtens et al. 2000).

The antigen component of a cancer vaccine may take many forms. Autologous tumour cells constitute a simple antigen source that is personalised and can therefore be applied to all patients regardless of HLA type. The provision of a wide range of protein antigens has the theoretical potential to provide epitopes for both CD4 and CD8 T cells and to induce a polyclonal response that reduces the risk of immune escape. Because the targeted antigens are personalised and not pre-determined, the possibility exists of targeting novel immunogenic mutant proteins particular to that patient’s tumour. The most commonly used vaccination platforms used to deliver whole tumour antigens in patients have been whole tumour cells or their lysates, and antigen-pulsed DC. DC vaccines in particular have been a workhorse in the field for several years due to excellent safety and applicability to a whole tumour antigen approach. The development of DC vaccines was in part driven

by the idea that APC activation could be enhanced by removing the APC from the immunosuppressive environment of the tumour-bearing patient (D. Gabrilovich 2004; Ralph M Steinman and Banchereau 2007). Recently, a landmark series of Phase III trials demonstrated a four month increase in median survival in patients with advanced prostate cancer who received Sipuleucel-T, a product consisting of autologous PBMC pulsed with a fusion protein combining Prostate Specific Antigen and GM-CSF (Higano et al. 2009; Kantoff et al. 2010). Most clinical trials of DC vaccines have used DC that are first differentiated from PBMC in the presence of GM-CSF or a combination of GM-CSF and IL-4, followed by antigen loading. Most protocols also include a maturation step using various combinations of pro-inflammatory cytokines although, as discussed above, the optimum phenotype for the infused DC remains uncertain. DC vaccines have most commonly been administered i.d. on the assumption that the injected DC will migrate and present antigen in draining LNs, although in some trials DC have been injected directly into LN or into the tumour site. Recently, it has even been questioned whether the infused DC actually present antigen or act mainly as couriers that transfer their antigen cargo to endogenous DC (Kleindienst and Brocker 2003; Petersen et al. 2011; Yewdall et al. 2010).

A disadvantage of any approach using whole tumour antigens is the inclusion of many irrelevant normal tissue proteins, raising concerns about auto-immunity and diluting the concentration of TAA. In addition, personalised cell-based vaccines introduce practical issues such as consistency of product, safety, expense, and the need to satisfy stringent regulations.

Peptide-based vaccines therefore appeal as “off-the-shelf” platforms. Because the target is known, responses can be more readily assessed *in vitro*. On-target toxicity is theoretically more predictable. A drawback of short peptide vaccines is that they are limited to patients of the appropriate Human Leucocyte Antigen (HLA) type. Also, since the epitope in single peptide vaccines is usually MHC Class I-restricted, CD4 T cell help is not recruited. Furthermore, a short peptide can be loaded directly

onto MHC molecules of non-APC, including cells with access to secondary lymphoid organs such as T cells. Thus so-called “exact” peptide vaccines can be weak or even tolerogenic (Melief and van der Burg 2008). To address some of these problems, Melief and colleagues developed synthetic long peptides (SLP) that require processing before presentation and provide epitopes for both CD4 and CD8 T cells (Melief and van der Burg 2008). Peptides and SLPs have been used in vaccines as free molecules, loaded onto DC, and in a wide variety of other delivery systems. Free nucleic acid as a source of antigen is poorly immunogenic but has been successfully used in DC-based vaccines. Viruses can also be manipulated to encode cancer antigens but prime-boost regimens are hampered by the development of neutralising humoral responses to the viral vector.

Another potential drawback of using peptide vaccines to treat heterogeneous cancer cell populations is that a narrow target can lead to immune escape. In a recently reported Phase III trial of systemic IL-2 *versus* systemic IL-2 plus a gp100 peptide vaccine in patients with advanced melanoma, the objective response rate was higher in patients who received the peptide vaccine but long term survival was no different (Schwartzentruber et al. 2011). Prins and colleagues (Prins et al. 2013) recently compared the results of two separate but concurrent trials of DC vaccination in patients with newly-diagnosed GBM in which DC were loaded with autologous tumour lysate or with a panel of four synthetic glioma-associated antigen (GAA) peptides. Median survival was 34 months in the autologous tumour group compared with 14 months in the GAA group. These findings need to be treated with caution because of small patient numbers and differences in the DC maturation protocols in the two trials. Nevertheless, the authors speculated that a diverse immune response may yield better long term results than targeting one or a few antigens. In support of this, Schmidt and colleagues (Neller et al. 2008) have analysed the results of 75 published trials of various cancer vaccines involving 1601 patients with metastatic melanoma and found an objective response rate of 12.7 % to immunotherapies directed at undefined antigens derived from whole tumour or tumour extracts *versus* 6.7 % to those directed at molecularly defined antigens ($P < 0.001$).

Interestingly, they found no significant difference in the response rates to antigens derived from autologous and allogeneic tumour.

1.3.2.5 Immunomodulatory antibodies

Cytotoxic Lymphocyte Antigen 4 (CTLA-4) is a receptor for the costimulatory molecules CD80 and CD86 but, unlike CD28, delivers an inhibitory signal that downregulates T cell function and proliferation (Krummel and Allison 1995). CTLA-4 is not expressed by resting T cells but is progressively upregulated after activation and is constitutively expressed by FoxP3⁺ Treg. Binding of the CTLA-4 receptor to its ligand not only delivers an inhibitory signal to T cells but also results in “capture” of the ligand by the CTLA-4-expressing cell, thereby reducing the number of CD80/86 molecules present on the surface of APC (Qureshi et al. 2011). This system represents an important “checkpoint” that prevents tissue damage due to excessive T cell overactivity (Sharpe and Freeman 2002). Antibodies to CTLA-4 can augment anti-tumour T cell responses both by blocking this CTLA-4-mediated “checkpoint” and by reducing the number of intra-tumoural Treg via a mechanism that depends on the Fc portion of the antibody (Selby et al. 2013). In a randomised Phase III trial, the humanised anti-CTLA-4 antibody ipilimumab resulted in objective responses in 11 % of patients with advanced melanoma, with stable disease in a further 17 % and improved overall survival compared with a peptide vaccine (median survival 10 months and 6 months respectively) (Hodi et al. 2010). A clinical trial of a CTLA-4 antibody in patients with GBM is currently in development (S. Tanaka et al. 2013).

Another “checkpoint” for T cell responses is mediated by the progressive upregulation of the PD-1 receptor on activated T cells. PD-L1 is a ligand for PD-1 and is expressed on activated immune cells and on stromal cells in response to IFN- γ (Zou and Chen 2008). PD-L1 is also expressed by tumour cells in a range of cancers (reviewed in Zou and Chen 2008) including glioma (Parsa et al. 2007; Wintterle et al. 2003). Engagement of PD-1 inhibits T cell function and promotes apoptosis

(Dong et al. 2002; Freeman et al. 2000). Tumour-infiltrating CD8 T cells in established human tumours are often found to lack effector functions and express high levels of PD-1 and other inhibitory receptors (Haymaker et al. 2012). A similar phenomenon in chronic viral infections, termed T cell “exhaustion”, has been attributed to chronic stimulation (Wherry et al. 2011). In the tumour setting it has been proposed that T cell dysfunction is due predominantly to the susceptibility of activated PD-1⁺ T cells to regulation by PD-1 ligands rather than “exhaustion” (Haymaker et al. 2012). In animal tumour models this dysfunctional state can be reversed by antibodies that block PD-1 and other “exhaustion” markers (Duraismamy et al. 2013; Sakuishi et al. 2010). In a recent study, a PD-1 antibody combined with focussed irradiation was effective in a murine glioma model, whereas neither treatment alone was effective (Zeng et al. 2013). Antibodies that block the PD-1 system have been tested in phase I trials in patients with melanoma and other solid cancers and are showing promising results (Brahmer et al. 2012; Topalian et al. 2012). Recently, it was reported that concomitant administration of anti-CTLA-4 and anti-PD-1 antibodies achieved tumour regression of more than 80 % in 53 % of patients with advanced melanoma (Wolchok et al. 2013).

1.3.3. Therapeutic vaccination for GBM

1.3.3.1 DC-based vaccines

The first case report of DC vaccination in a patient with high grade glioma was published in 2000 (L M Liao et al. 2000). Since then, at least 29 clinical trials of DC-based immunotherapy have been reported in this patient group (Ardon et al. 2010a; Ardon et al. 2010b; Ardon et al. 2012; Caruso et al. 2004; C.-N. Chang et al. 2011a; De Vleeschouwer et al. 2008; Camilo E Fadul et al. 2011b; Jie et al. 2012; T Kikuchi et al. 2001; Tetsuro Kikuchi et al. 2004; T. Kobayashi et al. 2003; Linda M Liao et al. 2005; Mitchell et al. 2015a; H Okada et al. 2001; Hideho Okada et al. 2011; Pellegatta et al. 2013; Phuphanich et al. 2012; Prins et al. 2011; Prins et al. 2013; Rutkowski et al.

2004; Sampson et al. 2009; Vik-Mo et al. 2013; D. G. Walker et al. 2008; Wheeler et al. 2008; R Yamanaka et al. 2003; Ryuya Yamanaka et al. 2005b; Ryuya Yamanaka et al. 2005a; J S Yu et al. 2001; John S Yu et al. 2004). Almost all of these trials have used monocyte-derived DC differentiated from autologous PBMC in IL-4 and GM-CSF and pulsed *in vitro* with unselected autologous tumour antigens in various forms including irradiated tumour cells (D. G. Walker et al. 2008), tumour lysates (De Vleeschouwer et al. 2008; Rutkowski et al. 2004; Wheeler et al. 2008; R Yamanaka et al. 2003; Ryuya Yamanaka et al. 2005b; John S Yu et al. 2004), peptides acid-eluted from tumour cells (Linda M Liao et al. 2005; J S Yu et al. 2001), whole tumour cell RNA (Caruso et al. 2004; T. Kobayashi et al. 2003; Vik-Mo et al. 2013), and defined antigen RNA (Mitchell et al. 2015a). Kikuchi and colleagues have uniquely reported the use of cellular fusions of tumour cells and DC (T Kikuchi et al. 2001; Tetsuro Kikuchi et al. 2004). In several trials DC were loaded with defined peptides, targeting either epidermal growth factor receptor, variant III (EGFRvIII) (Sampson et al. 2009) or a panel of selected GAA (Hideho Okada et al. 2011; Phuphanich et al. 2012; Prins et al. 2013). DC vaccines have been administered i.d. or s.c. in almost all of these trials although direct intratumoural injection (R Yamanaka et al. 2003), intranodal injection (Camilo E Fadul et al. 2011b) and i.v. infusion (Caruso et al. 2004) have also been reported. Vaccine design has evolved over time to include the incorporation of a dedicated DC maturation step using various cytokine combinations (De Vleeschouwer et al. 2008; Prins et al. 2013; Rutkowski et al. 2004) or TLR ligands (Hideho Okada et al. 2011; Ryuya Yamanaka et al. 2005b) and the integration of immunotherapy with standard chemo-radiotherapy in patients with newly presenting disease (Ardon et al. 2012; Phuphanich et al. 2012; Prins et al. 2013). Efforts to further enhance adjuvanticity have included the local application of a TLR ligand (Prins et al. 2011) or tetanus toxoid (Mitchell et al. 2015a) at the vaccination site and systemic administration of IL-12 (Tetsuro Kikuchi et al. 2004). One early trial uniquely co-administered tumour-lysate pulsed DC with fibroblasts engineered to secrete IL-4 but encountered major problems with feasibility (H Okada et al. 2001).

The results of most of these trials have been comprehensively reviewed (W. Kim and Liao 2010; Vauleon et al. 2010; X. Xu et al. 2012). Feasibility and safety have been conclusively demonstrated in patients with newly presenting and recurrent GBM. One trial reported NCI CTC Grade IV toxicity in one participant consisting of cerebral oedema in a patient with significant residual tumour bulk (De Vleeschouwer et al. 2008). Otherwise toxicity has typically been limited to Grade I and II toxicity such as flu-like symptoms, injection site reactions, lymphadenopathy and rashes 24-48 hours after vaccination (Prins et al. 2013). Seizures (Grade III) are also not uncommonly reported but, very reasonably, are usually attributed to disease progression (Prins et al. 2013). Auto-immune disease has not been reported in any of these trials.

With respect to outcome, many trials involved very small numbers of patients and/or included heterogeneous patient groups, and there has been no standardised approach in assessing vaccine-induced responses, making comparisons of efficacy difficult. Briefly, the larger and more recent trials have all reported objective radiological responses and/or improved survival relative to historical or contemporaneous controls and all have shown some evidence of vaccine bioactivity. To illustrate this, three recent studies conducted by groups with a longstanding interest in this field are described in more detail below.

Liao and colleagues reported a Phase I trial of autologous tumour lysate-pulsed DC vaccination in 28 patients with either newly diagnosed or recurrent GBM (Prins et al. 2011). In the newly diagnosed patients median overall survival (OS) was 35.9 months. The authors compared this with median OS of 15 months in patients undergoing standard treatment in the landmark study by Stupp and colleagues (Stupp et al. 2005). The main assessment of immune response in this study was measurement of serum cytokine levels 14 days after vaccination. Rises in TNF- α and IL-6 were observed in some patients but this did not correlate with clinical outcome. This study has provoked considerable interest by finding a significantly better OS in

patients with tumours with a mesenchymal gene expression profile, suggesting that gene expression profiling might be useful in predicting response to immunotherapy.

De Vleeschouwer and colleagues (De Vleeschouwer et al. 2008) treated 56 patients with recurrent GBM with 3 different vaccination dosing schedules using DC pulsed with autologous tumour lysate. Improved progression-free survival (PFS) and OS relative to historical controls was observed in some subgroups. Post-vaccine delayed-type hypersensitivity (DTH) tests were positive in nine of 22 patients tested but were known to be negative prior to vaccination in only two of these nine, making interpretation difficult. There was no correlation between clinical outcome and the DTH test results. This group subsequently undertook a pilot study (Ardon et al. 2010b) in which DC vaccinations were integrated into standard radiation and chemotherapy of eight newly diagnosed GBM patients. Median OS was 24 months. ELISpot was used to enumerate the proportion of PBMC secreting IFN- γ after stimulation with autologous tumour lysate. A two-fold increase in IFN- γ -secreting cells was seen in five of the eight patients. DTH tests were also performed. Only one became positive after vaccination. A further two were positive at baseline. The proportion of circulating CD8 T cells that were activated (defined as CD25⁺) increased in six of seven tested patients. In this small study, the authors found no correlation between the immunological assessments and clinical outcome. Recently, the same group reported the results of a Phase II study in 77 patients using a similar protocol (Ardon et al. 2012). Median OS in a subgroup of patients with better prognostic factors was 39 months compared with 18.7 months in the equivalent subgroup from the Stupp trial (Stupp et al. 2009). The authors concluded that the treatment was possibly beneficial and a randomised Phase IIb trial is currently underway. No immune monitoring was undertaken in this study.

Finally, Phuphanich and colleagues (Phuphanich et al. 2012) conducted a Phase I trial in which HLA-A1 or HLA-A2 glioma patients were vaccinated with DC pulsed with six synthetic peptide epitopes from known GAA (HER2, TRP-2, gp100, MAGE-1, IL13R α 2, and AIM-2). In the 17 newly diagnosed GBM patients, median OS was 38.4

months. Vaccine-induced responses were assessed by intracellular cytokine staining. A 1.5 fold increase in the proportion of CD8 T cells staining for IFN- γ after *in vitro* stimulation with the peptide pool occurred in 33 %. The authors found a trend toward increased progression-free survival in the responders but this was not significant and there was no correlation with overall survival.

1.3.3.2 Peptide and protein vaccines

EGFRvIII is a constitutively-active mutant form of EGFR expressed in approximately one third of GBM tumours (Heimberger et al. 2005). The in-frame deletion creates a novel amino acid sequence in the extracellular portion of the EGFR protein. Because the genetic mutation is not present in normal cells, EGFRvIII has the potential to be exploited as a tumour-specific antigen. Moscatello and colleagues (Moscatello et al. 1997) conjugated a short peptide spanning the novel region of EGFRvIII to keyhole limpet haemocyanin, (KLH) a carrier protein known to elicit CD4 T cell responses (J. R. Harris and Markl 1999) and showed efficacy of the conjugate as a vaccine in rodents against EGFRvIII-expressing tumours.

After initially testing the peptide conjugate in a DC-based vaccine, Sampson, Heimberger and colleagues moved to vaccinating directly with peptide (coadministered i.d. with GM-CSF) to reduce cost and variability (Kanaly et al. 2010) and have completed two Phase II trials. In the first trial (Sampson et al. 2010), which preceded the advent of the Stupp protocol as standard treatment, 21 patients with newly diagnosed EGFRvIII⁺ GBM underwent > 95 % debulking and concomitant radiation/TMZ followed by a priming course of three vaccinations. Monthly vaccinations continued indefinitely until progression. Three patients were retrospectively excluded from analysis because debulking was less than 95 % and progression during concomitant treatment was an exclusion criterion. Median OS was 26.0 months. At relapse, 11 tumours were resampled and 82 % were found to have lost expression of EGFRvIII. Perhaps surprisingly, no spontaneous immune responses to EGFRvIII were detected prior to vaccination. Post-vaccination, 43 % of

patients developed a humoral response and three of 17 assessed patients developed a positive DTH test. Median OS in immunological responders was markedly better than non-responders.

The second trial (Sampson et al. 2011) used the same vaccination schedule but the monthly booster vaccinations alternated with adjuvant cycles of TMZ. Two different TMZ dosing schedules were assessed with 12 patients receiving adjuvant TMZ according to the standard Stupp protocol and 10 patients treated according to a “dose-intensified” protocol of 100 mg/m² for 21 days every 28 days. The median OS for all patients combined was 23.6 months. 11 of 12 resampled tumours were found to be EGFRvIII negative. All 22 patients developed a *de novo* humoral response to EGFRvIII and seven of 13 tested patients developed a positive DTH test. Interestingly, antibody titres were higher in the dose-intensified group, and all seven positive DTH tests were in this group, even though TMZ-induced lymphopaenia was more severe.

These trials clearly demonstrated the feasibility of inducing an adaptive immune response to a tumour-specific antigen in GBM patients. These findings also strongly suggest that the effector arms of this response were capable of gaining access to the tumour site and killing EGFRvIII⁺ cells. However, the fact that most patients had relapsed at the time of publication suggests that a potent response against a single epitope may not be sufficient to achieve longterm remission. An international multicentre randomised Phase III trial of this peptide conjugate vaccine is currently underway.

Included in this discussion of peptide vaccines is the approach taken by Parsa and colleagues in which unselected peptides complexed to the “chaperone” heat shock protein gp-96 are extracted from GBM tumours and administered s.c. as a personalised vaccine without additional adjuvant (Crane et al. 2013). This design is based on the finding that DC take up heat shock protein-peptide complexes via the surface receptor CD91 (Binder and Srivastava 2004). This phase I trial in 12 patients reported encouraging efficacy data and evidence of a systemic immune

response to the vaccine in 11 of the 12 patients as assessed by IFN- γ gene expression in PBMC stimulated *in vitro* with the vaccine.

Taking a different approach, Yajima and colleagues (Yajima et al. 2005) selected patients for peptide vaccination based on the existence of spontaneous immune responses. 25 HLA-A24 or HLA-A2 patients with high grade glioma were screened for cell-mediated or humoral responses to a panel of peptides derived from 16 previously-described cancer-associated antigens. Patients were then vaccinated s.c. with up to four peptides emulsified in Montanide. In the 21 patients who completed the planned initial course of six vaccinations, 15 exhibited an augmented antigen-specific cell-mediated or humoral response to at least one of the peptides. Peptide-specific antibodies were also detected in CSF or the tumour cavity in three of three tested patients who had objective responses. Median OS in patients with recurrent GBM was 622 days, with a trend to improved survival in immunologically-responding patients that was not statistically significant.

Izumoto and colleagues (Izumoto et al. 2008) used a peptide vaccine to target the product of the *WT1* Wilms' tumour oncogene with a peptide vaccine. 37 patients with recurrent GBM were all found to have tumours expressing *WT1*. The 21 HLA-A2402⁺ patients then received at least 12 s.c. vaccinations with a 9-mer peptide derived from WT1 protein emulsified in Montanide. There were two partial responses (PR) and 10 patients with stable disease (SD), with a PFS at six months (PFS_{6MO}) of 33.3 %. Using tetramer staining, the frequency of WT1-specific CTL was higher in pre-vaccination GBM patients than normal controls but did not increase after vaccination, leading the authors to speculate that the vaccine may have acted by inducing a qualitative change in antigen-specific CTL. The important observation was also made that the longest survivors did not respond clinically until near the end of the 12 week vaccination regimen and never met the objective criteria for PR.

1.3.3.3 Autologous tumour cell (ATC) vaccines

Nine clinical trials and two case reports of vaccination with ATC in 101 patients with high grade glioma have been reported to date (Andrews et al. 2001; Clavreul et al. 2010; Fakhrai et al. 2006; E. Ishikawa et al. 2007; Muragaki et al. 2011; Hideho Okada et al. 2003; Hideho Okada et al. 2007; Parney et al. 2006; Schneider et al. 2001; Sobol et al. 1995; Steiner et al. 2004). In most studies tumour cells were irradiated prior to administration. In some, tumour cells were inactivated by exposure to chemotherapy or formalin-fixation (Muragaki et al. 2011). The ATC vaccines were administered s.c or i.d. with one exception in which irradiated ATC were enclosed in semi-permeable diffusion chambers implanted temporarily in the rectus sheath (Andrews et al. 2001). Immunogenicity of the injected tumour cells has been enhanced in a variety of ways including admixture with fibroblasts engineered to release IL-2 (Sobol et al. 1995) or IL-4 (Hideho Okada et al. 2007), continuous infusion of GM-CSF at the vaccination site (Clavreul et al. 2010), co-administration with BCG (Muragaki et al. 2011), infection of the tumour cells with Newcastle virus (Schneider et al. 2001; Steiner et al. 2004) and engineering of tumour cells to express pro-inflammatory cytokines and co-stimulatory molecules (Parney et al. 2006), downregulate expression of the immunosuppressant cytokine TGF- β 2 (Fakhrai et al. 2006) or downregulate expression of Insulin-like Growth Receptor-1 which in turn induces tumour cell apoptosis (Andrews et al. 2001). The vaccines were well tolerated and the results of these studies have been broadly similar to DC studies with most reporting objective responses in a minority of patients and/or a survival benefit in comparison to historical controls. Vaccine-induced immune responses were observed in many of these studies using a variety of assessment methods. Lethal allergic encephalomyelitis has been reported in rats vaccinated with human GBM tumour cells (Bigner et al. 1981). However, no adverse events attributable to auto-immunity were reported in these studies, including one study that specifically assessed for humoral responses to myelin basic protein or evidence of demyelination on MRI (Clavreul et al. 2010). Importantly, significant

feasibility issues were encountered in some of these studies (Clavreul et al. 2010; Hideho Okada et al. 2007).

In the largest and most recently reported study of an ATC vaccine (Muragaki et al. 2011), 22 patients with newly diagnosed GBM undergoing post-operative radiation were vaccinated concomitantly with formalin-fixed ATC mixed with BCG and tuberculin administered i.d. Most patients received TMZ at relapse (the study was conducted in 2005-2007 before TMZ became standard treatment for newly-diagnosed disease). Median OS was 21 months compared with 12 months in comparable historical controls. DTH tests, all negative at baseline, ranged from 1 mm to 47 mm post-vaccination. A DTH test ≥ 12 mm was strongly associated with prolonged PFS but not OS.

In a very early study in 1983, Mahaley and colleagues (Mahaley et al. 1983b; Mahaley et al. 1983a) vaccinated 20 patients with allogeneic glioma cells mixed with BCG cell wall. The vaccine was well tolerated and OS was improved relative to historical controls. Proof of principle for this interesting approach has subsequently been provided in a rodent model (Apostolos Stathopoulos et al. 2008).

1.3.4 Barriers to effective glioma immunotherapy

1.3.4.1 CNS immunology and Immunoprivilege

The concept of immunoprivilege arose from early studies showing that allogeneic and xenogeneic tissues were rejected after s.c. implantation but not when implanted in the anterior chamber of the eye or in the brain (Medawar 1948; J. B. Murphy and Sturm 1923). Factors contributing to relative immunoprivilege in the CNS are the lack of a conventional lymphatic system, the lack of conventional tissue-resident APC, the blood-brain barrier (BBB), and a low level of MHC expression by normal cells in the CNS (Kanaly et al. 2010; Lampson and Hickey 1986).

Despite the lack of a conventional lymphatic system, the outflow of antigen and immune cells from the steady state CNS has been demonstrated in animal models. Cserr and colleagues showed that 14 – 47 % of radiolabelled albumin injected into the brain parenchyma or cerebrospinal fluid (CSF) eventually entered the lymphatic system (Cserr et al. 1992). In a rat model, indian ink injected into CSF was shown to drain directly through the cribriform plate into the nasal submucosa and reached the deep cervical LNs within 30 minutes (Kida et al. 1993). Lumbar para-aortic LN were stained within six hours, presumably by draining along spinal nerve roots. Drainage from CSF to blood through arachnoid villi has also been proposed (Cserr et al. 1992). Lymphocytes and DC injected into CSF are able to reach secondary lymphoid organs (Hatterer et al. 2006; Seabrook et al. 1998). In disease states, T cell-mediated responses appear to be initiated in a manner similar to other tissues. Karman and colleagues injected Green Fluorescence Protein (GFP)-labelled DC pulsed with OVA into the brain parenchyma and detected these cells in cervical LN (Karman et al. 2004). OVA-specific CD8 T cells subsequently accumulated in the brain, a process that required the injected DC to be alive. The importance of the deep cervical LN as a major site of priming in responses to CNS-derived antigens has been supported by several other studies (de Vos et al. 2002; M. J. Phillips et al. 1997). However, this is not the only site where priming occurs. Exploiting the phenomenon of epitope spreading in an animal model of multiple sclerosis, de Vos and colleagues showed that, in an inflammatory state, priming of responses to CNS self-antigens could be observed in deep cervical LN and also in spleen (but not axillary or inguinal LN) (de Vos et al. 2002).

Whether the site of priming determines the capacity of activated T cells to home to the tumour site is clearly a point of vital interest to vaccine design. In the study by Karman et al referred to above (Karman et al. 2004), the OVA-pulsed DC failed to induce trafficking of antigen-specific T cells to the brain when injected i.v. even when the brain was physically disrupted with an injection of media. The authors pointed out that inflammation caused by injection into parenchyma may have altered the activation state of the brain-injected DC. However, the other possibility is

that brain-emigrant DC “imprint” primed T cells to specifically home to the CNS. Several studies have shown that the majority of T cells primed in secondary lymphoid organs in the skin and gut are induced to express adhesion molecules that encourage trafficking to skin and gut respectively (Ferguson and Engelhard 2010; Mora et al. 2003). There is no evidence of a phenotype that homes specifically to the CNS (Hickey 2001). T cells primed in the spleen and mediastinal LNs dominantly express integrin $\alpha 4\beta 1$, a phenotype associated with homing to multiple inflamed sites (Ferguson and Engelhard 2010). In a brain tumour model, expression of $\alpha 4\beta 1$ integrin (also known as Very Late Antigen-4, VLA-4), which binds Vascular Cell Adhesion Molecule 1 (VCAM-1), was shown to be critical for CTL homing to the tumour site (Sasaki et al. 2007). Expression of the chemokine CXCL10 at the brain tumour site has also been found to be critical (X. Zhu et al. 2010). CXCR3, the receptor for CXCL10, is expressed by a subset of activated T cells regardless of priming site (Ferguson and Engelhard 2010). Recently the lung was shown to be a site where adoptively transferred T cells can become competent to enter the CNS, a property dependent on the expression of CXCR3, VLA-4, and, in the EAE model employed in that study, the adhesion molecule Ninjurin-1 (Odoardi et al. 2012). Together these data suggest that glioma vaccines might be more effective if designed to induce priming in lymphoid organs that filter blood or in deep cervical lymph nodes rather than in skin draining lymph nodes. Inefficient homing to the tumour site may also help explain the poor correlation between systemic immune responses and clinical outcomes in many glioma vaccine trials, most of which have been administered i.d. or s.c. To add further complexity, a recent study used an orthotopic OVA-transfected glioma model to compare the outcome of cutaneous vaccination in the hindlimb, forelimb and neck and found that the magnitude of the OVA-specific CTL response at the tumour site increased as the distance from the tumour site increased (Ohlfest et al. 2013).

Formed by a combination of tight junctions between endothelial cells and active transport mechanisms, the BBB (along with the Blood-CSF barrier in the choroid plexus) regulates the entry of macromolecules and immune cells into the CNS.

Resting lymphocytes do enter the steady state CNS but to an extent that is quantitatively greatly reduced in comparison with other tissues (Pachter et al. 2003). Beyond the BBB, T cells encounter a hostile environment in which proliferation is inhibited by TGF- β and apoptosis promoted by an uncertain mechanism (J. Bauer et al. 1998; Hickey 2001). However, as described above, activated lymphocytes are more readily able to cross the BBB and local inflammation also encourages T cell entry. Pro-inflammatory cytokines increase BBB permeability and upregulate expression of intercellular adhesion molecules (Hickey 2001; Pachter et al. 2003).

The BBB presents a significant barrier to antibody-based treatments. In the steady state, IgG (but not IgA or IgM) is detectable in CSF at much lower concentrations than in serum (Mitchell et al. 2008a). In breast cancer patients treated with the anti-herceptin mAb trastuzumab, the ratio of antibody in CSF compared to serum was 1:420 (Stemmler et al. 2007), improving to 1:76 after whole brain radiotherapy. Accordingly, trastuzumab does not delay CNS metastases in these patients (Burstein et al. 2005). However, clinically useful concentrations of antibody may be achievable when the BBB is perturbed by the presence of a glioma and/or when the antibody is tumour-specific, encouraging gradual accumulation at the tumour site (Scott et al. 2007). Alternatively, convection-enhanced delivery has been used to deliver large molecules to the brain parenchyma (Bobo et al. 1994).

A lack of resident DC has also long been thought to contribute to CNS immunoprivilege. However, microglial cells, the resident macrophages of the brain parenchyma, appear to be capable of serving as APC (Kaur et al. 2010). Microglial cells arise from myeloid precursors in the yolk sac and populate the brain during early CNS development (Kierdorf et al. 2013). They are maintained in adult life predominantly by self-renewal (Ajami et al. 2007) although bone marrow precursors may contribute in inflammatory states (Soulet and Rivest 2008). Like conventional DC, microglia continuously sample their surroundings (Nimmerjahn et al. 2005) and a subset of microglia express the DC marker CD11c (Bulloch et al.

2008). Microglia are poor APC under resting conditions but readily upregulate MHC class II and co-stimulatory molecules (Aloisi et al. 1998; Gottfried-Blackmore et al. 2009) and are capable of processing and presenting exogenous antigen to CD4 and CD8 T cells (Aloisi et al. 1998; Beauvillain et al. 2008). Aloisi and colleagues (Aloisi et al. 1999) showed that IFN- γ -activated microglia were able to prime naïve CD4 T cells with an efficiency that was intermediate between DC and B cells. The ability of microglia to traffic to regional lymph nodes has yet to be definitively demonstrated (D'Agostino et al. 2012). In addition to parenchymal microglia, antigen presentation is likely to be performed also by perivascular macrophages (Fabriek et al. 2005) and myeloid cells in the choroid plexus (Nataf et al. 2006). Astrocytes can also serve as weak APC, being unable to prime naïve T cells but capable of reactivating primed T cells (Aloisi et al. 1999).

1.3.4.2 Immuno-evasion and immunosuppression

According to the cancer immunoediting hypothesis, the relationship between tumours and the immune system evolves from a phase of immunosurveillance to one of immunoequilibrium and finally to immunoescape (Dunn et al. 2002). Glioma immunotherapy faces the formidable opponent of an established tumour that has already overwhelmed the immune system's defences. The underlying mechanisms are many and varied. One strategy, shared with some viral infections, is to escape detection downregulating MHC class I expression (Zagzag et al. 2005). GBM tumour cells also elaborate a range of surface, intracellular and secreted molecules that directly inhibit the immune system. For example, GBM tumour cells have been reported to express high surface levels of programmed death receptor ligand -1 (PD-L1, also known as B7-H1) (Parsa et al. 2007; Wintterle et al. 2003). Interaction of PD-L1 with PD-1 expressed by activated T cells can induce anergy or apoptosis (Tsushima et al. 2007). Indoleamine 2,3-dioxygenase 1 (IDO) is an intracellular enzyme that catabolises the essential amino acid tryptophan. Local depletion of tryptophan directly inhibits effector T cell function (Choi et al. 2012). Other effects of IDO are discussed below. Soluble factors with immunosuppressive properties

reported to be secreted by GBM tumour cells include prostaglandin E2 (PGE₂) (Fujita et al. 2011), TGF- β (Crane et al. 2012; B. Qiu et al. 2011), IL-10 (both human and CMV-derived forms) (Dziurzynski et al. 2011), and a range of chemokines. Many of these act indirectly by inducing the accumulation of suppressive immune cells, some of which are now described in more detail.

1.3.4.3 Regulatory T cells

Treg are a distinct and functionally mature CD4 T cell subset that act to maintain self tolerance and to limit tissue damage in inflammatory conditions by suppressing immune responses (Yamaguchi et al. 2011). A hallmark of Treg is expression of the transcription factor FoxP3 (Yamaguchi et al. 2011). The importance of Treg in maintaining self-tolerance is demonstrated by the fact that FoxP3 deficiency leads to severe autoimmune disease in mice and humans. Under normal conditions, Treg account for approximately 10 % of CD4 T cells in mice and 5-10 % in humans (Sakaguchi 2005; Yamaguchi et al. 2011). The majority of Treg are “natural” Treg (nTreg) that develop in the thymus but conventional CD4 T cells may also be converted into “induced” Treg (iTreg) in the periphery by antigen recognition in the presence of TGF- β (Nishikawa and Sakaguchi 2010; Yamaguchi et al. 2001; Yamaguchi et al. 2011). In the steady state, Treg induction occurs notably in the gut (where tolerisation to abundant foreign microbial antigens is necessary to prevent excessive inflammation), a process that here requires the production of TGF- β and retinoic acid by DC (del Rio et al. 2010). Expression of Helios protein (Thornton et al. 2010) and the surface receptor neuropilin-1 (Weiss et al. 2012; Yadav et al. 2012) have been identified as markers that can be used to distinguish nTreg from iTreg. In the steady state, 70 % of FoxP3⁺ CD4 T cells are nTreg (Thornton et al. 2010). Treg constitutively express CTLA-4 and most also express the IL-2R α chain CD25. Immunosuppressive FoxP3⁺ CD8 T cells (Chaput et al. 2009), FoxP3⁺ iNKT cells (Monteiro et al. 2010) and other regulatory T cell populations (Nishikawa and Sakaguchi 2010) have been described but the importance of these populations in the context of cancer is unclear at present.

Treg have been described as “anergic” because they do not proliferate in response to cognate self-antigen alone *in vitro*. However, expansion in response to cognate antigen readily occurs *in vivo*. This may be explained by the finding that TGF- β effectively acts as a co-stimulator for Treg (Ghiringhelli et al. 2005). Although TCR-independent mechanisms of Treg activation have been described, it is generally believed that activation of Treg *in vivo* is antigen-specific (Sakaguchi et al. 2010). Once activated, Treg may act directly on conventional T cells or indirectly by suppressing the function of APC, and, in the context of tumour immunology, may act both at the tumour site and in secondary lymphoid organs (Nishikawa and Sakaguchi 2010; Yamaguchi et al. 2011). A large number of Treg suppressive mechanisms have been described *in vitro* but the relative importance of these mechanisms *in vivo* remains unclear and may depend on location and context (reviewed in Shevach 2009; D. Vignali 2008; D. A. A. Vignali et al. 2008; Yamaguchi et al. 2011). A consistent observation from *in vitro* studies is that Treg are unable to exert suppressive effects when separated from their target cells by a semi-permeable membrane (Shevach 2009). This has been interpreted as evidence that direct cell-to-cell contact is required. However, an alternative explanation is that close proximity is required to achieve sufficiently high local concentrations of soluble factors (D. A. A. Vignali et al. 2008). Sakaguchi has proposed that the predominant mode of action by which Treg maintain self-tolerance in the steady state is via CTLA-4 expression leading to a denial of co-stimulation for conventional T cells but that in inflammatory states, where more potent suppression is required, other mechanisms are also recruited, such as the secretion of soluble factors including TGF- β , that are not only directly suppressive but act in a positive feedback loop to expand and enhance Treg function (Yamaguchi et al. 2011). Sharma and colleagues (M. D. Sharma et al. 2007) found that the main mechanism of Treg-mediated immunosuppression in an animal cancer model was by upregulating DC expression of ligands for PD-1.

There is abundant evidence that Treg expand in tumour-bearing mice and humans and exert immunosuppressive effects that impair endogenous anti-tumour

immunity and constitute a major impediment to therapeutic immunotherapies. Studies in murine models of glioma (Cantini et al. 2011; Grauer et al. 2007; Wainwright et al. 2011) show that Tregs progressively accumulate at the tumour site after implantation, eventually accounting for 40 % of tumour-infiltrating CD4 T cells in one study (Grauer et al. 2007). The importance of Treg in suppressing endogenous immune responses is supported by the consistent observation that depletion of Treg alone is sufficient to confer protection or improve survival in many animal tumour models (Onizuka et al. 1999) including glioma (Curtin et al. 2008; El Andaloussi et al. 2006; Fecci et al. 2006b; Grauer et al. 2008; Wim Maes et al. 2009b).

Tumour-infiltrating CD4⁺ FoxP3⁺ T cells and expansion of circulating Treg have been described in many human cancers (Wolf et al. 2003). In glioma, several studies have reported a correlation between tumour FoxP3 expression (El Andaloussi and Lesniak 2007) or the presence of glioma-infiltrating FoxP3⁺ cells (Heimberger et al. 2008; Jacobs et al. 2010; Lohr et al. 2011) and tumour grade. Crane and colleagues (Crane et al. 2012) found that FoxP3⁺ CD25⁺ Treg were present disproportionately in the tumour tissue of 20 GBM patients, constituting 38.5 % of tumour-infiltrating CD4⁺ T cells compared with 12 % in peripheral blood. Findings with respect to the impact of tumour-infiltrating Treg on prognosis have been discordant (Heimberger et al. 2008; Jacobs et al. 2010; Lohr et al. 2011).

In the peripheral blood of glioma-bearing patients Fecci and colleagues (Fecci et al. 2006a) found a reduction in the absolute number of both conventional and regulatory CD4 T cells but in five of eight GBM patients there was a 2-2.5 fold increase in the proportion that were Treg. In these five there was a marked reduction in CD4 T cell proliferation *in vitro* which returned to normal when Treg were depleted. Crane and colleagues (Crane et al. 2012) found a modest increase in the proportion of Treg in peripheral blood (12 % of CD4 T cells compared with 5-10 % in control patients). This group also found that the proportion of Treg in

peripheral blood fell to normal levels after GBM resection in seven patients, but returned to pre-surgery levels at time of recurrence.

Human GBM-conditioned medium can induce conversion of conventional T cells to suppressive FoxP3⁺ T cells in vitro (Crane et al. 2012). However, a recent study showed that the great majority (90 %) of tumour-infiltrating Treg in murine glioma models and in human GBM tumour tissue samples are Helios⁺ nTreg (Wainwright et al. 2011). The origin of tumour-infiltrating Treg has important implications for immunotherapy because if they are recruited from the periphery then systemic Treg depletion should also reduce Treg at the tumour site. In support of this, in an orthotopic murine glioma model, a single i.p. dose of an anti-CD25 antibody reduced tumour-infiltrating Treg to the same level as tumours in thymectomised mice (Wainwright et al. 2011).

Several mechanisms have been proposed to explain the presence of Treg in glioma tumours. Circulating CCR4⁺ Treg may be recruited by chemokines CCL2 and/or CCL22 secreted by tumour cells (Crane et al. 2012; Jacobs et al. 2010; Jordan et al. 2008). TGF- β secreted by tumour cells and/or tumour-infiltrating immune cells may promote Treg survival and expansion (Ueda et al. 2009). IDO is attracting considerable interest as a potential “master controller” of Treg tumour-infiltration. IDO is expressed by glioma tumour cells, particularly after exposure to IFN- γ , and IDO expression correlates inversely with survival (Wainwright et al. 2012). As described above, depletion of tryptophan from the tumour microenvironment directly suppresses effector T cell function. The degradation of tryptophan also produces metabolites that lead to the accumulation of Treg at the tumour site. The precise mechanism remains unclear but one possibility is that a tryptophan catabolite induces CCL22 secretion by tumour cells or immune cells (Wainwright et al. 2013). Although a study in an implantable glioma model found that IDO expression by tumour cells, rather than host cells, was primarily responsible for Treg accumulation, it is possible that IDO expressed in immune cells is also relevant. Sharma and colleagues (M. D. Sharma et al. 2007) showed that pDC in the tumour-

draining LNs in mice bearing melanoma tumours promoted Treg suppressor function in an IDO-dependent manner. Clinical trials of IDO inhibitors in patients with a range of solid tumours are currently underway (reviewed in Wainwright et al. 2013).

Attenuation of Treg-mediated immunosuppression is likely to be a cornerstone of successful therapeutic immunotherapy for glioma and many strategies have been proposed or are already in use (reviewed in Byrne et al. 2011). Evidence is emerging that the success of anti-CTLA-4 treatment for melanoma may be partly due to a reduction in the number of tumour-infiltrating Treg, a process that is dependent on the Fc portion of the antibody (James Allison, oral presentation, Keystone Symposium, Vancouver 2012). Depletion of Treg is also one of the main mechanisms underlying the immunopotentiating effects of lymphodepleting chemotherapy prior to adoptive cell transfer (discussed in detail in Chapter 7). In animal models, including models of glioma (Fecci et al. 2006b; Grauer et al. 2008; Wim Maes et al. 2009b; Mitchell et al. 2011) depletion of Treg using anti-CD25 antibodies or using transgenic mice that permit the conditional depletion of FoxP3⁺ cells (Klages et al. 2010) has been shown to enhance the efficacy of active specific immunotherapies without inducing overt auto-immunity. One of the limitations of using CD25 to target Treg is that activated T cells also upregulate CD25 so that timing is crucial to avoid compromising the effector response (Curtin et al. 2008). In human trials, an anti-CD25 antibody conjugated to Diphtheria toxin induced a transient depletion of Treg at best, lasting less than three weeks (reviewed in Rech et al. 2012). A humanised anti-CD25 monoclonal antibody, daclizumab, looks more promising. A single dose of daclizumab in patients with metastatic breast cancer, co-administered with a peptide vaccine, resulted in a marked and prolonged (>5 weeks) decrease in the number of circulating Treg and was associated with successful induction of peptide-specific T cell responses without auto-immunity (Rech and Vonderheide 2009). Similar findings were reported in a pilot study of six patients with newly diagnosed GBM undergoing Stupp protocol treatment. Administration of a single dose of daclizumab resulted in 34 % mean fall in the

absolute number of circulating FoxP3⁺ CD4⁺ cells that was sustained for at least 50 days and did not prevent the expansion of antigen-specific T cells in response to a co-administered DC-based vaccine (Mitchell et al. 2011). *In vitro* studies suggest that the antibody works not just by reducing Treg numbers but also by inducing a downregulation in Treg suppressor function (Rech et al. 2012).

1.3.4.4 Tumour-associated macrophages and myeloid derived suppressor cells.

Several studies have reported that high grade glioma tumours are often infiltrated by large numbers of cells of the monocyte/macrophage lineage (Morantz et al. 1979; Dziurzynski et al. 2011; Hussain 2006; Parney et al. 2009; Shinonaga et al. 1988). Precise characterisation of these cells has been hampered by a lack of markers that clearly distinguish brain-resident microglia from cells derived from circulating monocytes. Recent studies in mice have used flow cytometry to distinguish CD11b⁺ CD45^{hi} and CD11b⁺ CD45^{dim} populations that are considered to represent tumour-infiltrating monocytes/macrophages and microglia respectively (Ford et al. 1995). Applying the same technique to human tissues Parney and colleagues assessed single cell suspensions from nine newly diagnosed high grade gliomas and found microglial cells and monocytes/macrophages to make up 1.6 % and 6 % (range 1-15 %) of cells respectively (Parney et al. 2009). Dziurzynski and colleagues reported equivalent figures of 3 % and 24 % albeit in a single patient (Dziurzynski et al. 2011). In rodent models of glioma, studies of the kinetics of immune cell infiltrates have shown that, whereas microglia account for almost all CD45⁺ cells in normal brain, macrophages come to dominate the immune cell infiltrate in the brains of terminally-ill glioma-bearing mice (Kennedy et al. 2009; Tran Thang et al. 2010). Interestingly, both these studies showed that peripheral expansion and tumour-infiltration by regulatory T cells preceded the final expansion of TAM.

Whereas M1-polarised macrophages are pro-inflammatory, antimicrobial, tumouricidal, and drive T_H1 responses, the role of “alternatively-activated” (or M2) macrophages is to promote tissue healing by releasing growth factors (Biswas and

Mantovani 2010; Murray and Wynn 2011). M2 macrophages also release TGF- β , IL-10 and arginase-1 that dampen T cell-mediated immune responses (Riccadonna 2012). Thus M2 polarisation, a normal event in the transition from inflammation to healing, can be a disadvantage in the context of cancer, both suppressing anti-tumour immunity and directly driving tumour growth. In GBM tumours, as in most established human tumours (Mantovani et al. 2011) the majority of TAM have an M2 phenotype (Komohara et al. 2008). The mechanisms responsible for M2 polarisation are diverse and context-specific (Mantovani et al. 2011). In glioma, tumour cell-derived IL-10 (Dziurzynski et al. 2011) and M-CSF (Komohara et al. 2008) have been implicated in this process. M2 macrophages in turn secrete IL-10 and TGF- β that promote Treg differentiation and sustain M2 differentiation in a positive feedback loop. However, the plasticity of macrophage phenotypes opens the door to possible therapeutic intervention. Conversion of TAM from an M2- to an M1-phenotype *in vivo* has been demonstrated in animal cancer models in response to IFN- γ (Duluc et al. 2009) and an agonistic anti-CD40 antibody (Beatty et al. 2011).

The extent to which TAM are recruited from circulating monocytes or derived from the expansion and differentiation of tissue-resident microglial cells remains clear (Mantovani et al. 2011) but there is evidence for a scenario in which growth factors and chemokines (notably CCL2) released by tumour cells act systemically to expand and release monocytes and immature myeloid cells from the bone marrow that are then recruited to the tumour site (Mantovani et al. 2011; Qian et al. 2011; Serbina and Pamer 2006). Amongst these are MDSC, a phenotypically heterogeneous mixture of cells with granulocytic or monocytic morphology but lacking the surface markers of fully differentiated cells (D. I. Gabrilovich and Nagaraj 2009).

Immature myeloid cells normally account for 20-30 % of bone marrow cells, 2-4 % of splenocytes and 0.5 % of PBMC (D. I. Gabrilovich and Nagaraj 2009). These immature myeloid cells become expanded by a factor of up to ten in the spleen of tumour-bearing mice and the peripheral blood of cancer patients and acquire immunosuppressive properties (D. I. Gabrilovich and Nagaraj 2009). Similar to Treg,

MDSC exert their effects both at the tumour site and in secondary lymphoid organs and multiple immunosuppressive mechanisms have been described. These include expression of high levels of arginase (which depletes L-arginine, in turn inhibiting T cell proliferation), iNOS (which also catabolises arginine and generates NO which in turn directly suppresses T cell function), ROS, and peroxynitrite (which results in the nitration of the TCR and renders T cells unresponsive to antigen) (Nagaraj et al. 2007). MDSC require close cell proximity to exert their suppressive effects on T cells (D. I. Gabrilovich and Nagaraj 2009). Although MDSC can suppress T cells non-specifically *in vitro*, *in vivo* suppression in one study appeared to be mainly antigen-specific, with suppression requiring MDSC to present TAA to CD8 T cells in an MHC class I-restricted manner (Kusmartsev and Gabrilovich 2002).

In murine studies, MDSC have been defined by the co-expression of the myeloid lineage marker Gr1 and CD11b, with further subclassification into monocytic and granulocytic subsets (D. I. Gabrilovich and Nagaraj 2009). Although both subsets are expanded in tumour models in mice, granulocytic MDSC are expanded to a greater extent (D. I. Gabrilovich and Nagaraj 2009). Functional differences in the suppressive mechanisms used by the two subsets have been described (Youn et al. 2008). In the presence of appropriate cytokines, up to 30 % of MDSC have the ability to differentiate *in vitro* into mature macrophages or DC, this ability being restricted to the monocytic subset (D. I. Gabrilovich and Nagaraj 2009). Thus MDSC are closely related to immunosuppressive TAM and may be their precursors (Mosser and Edwards 2008; Movahedi et al. 2008).

The relationship between Treg, TAM and MDSC in glioma is unclear. Some studies have shown that MDSC can participate in the expansion of Treg (Ghiringhelli et al. 2005; Ostrand-Rosenberg and Sinha 2009). However, Kennedy and colleagues studied the kinetics of immune cell populations in an implantable PDGF-driven murine glioma model and found an increase in the proportion of Tregs in the spleen preceded the expansion of CD11b⁺ CD45⁺ cells at the tumour site (Kennedy et al. 2009). Tran Thang and colleagues (Tran Thang et al. 2010) also studied the kinetics

of TIL in a transgenic mouse model of spontaneous glioma and found a progressive local expansion of Treg and TAM with expansion of CD11b⁺ Gr1⁺ MDSC occurring later.

Strategies to target MDSC that have been explored or proposed include differentiating MDSC into mature non-suppressive myeloid cells (Shirota et al. 2012), blocking tumour-derived growth factors that drive peripheral expansion of MDSC (Kao et al. 2011), inhibiting trafficking of MDSC to the tumour site (X. Zhu et al. 2011), inhibiting MDSC suppressive functions (Meyer et al. 2011), and direct elimination of MDSC (Ko et al. 2007). Of concern, some standard chemotherapy regimes may actually promote MDSC expansion (Diaz-Montero et al. 2009). Tempering this discussion is the fact that, while MDSC have been described in several rodent models of glioma (Fujita et al. 2011; Kennedy et al. 2009; Tran Thang et al. 2010) and circulating MDSC have been described in the PBMC of GBM patients (Raychaudhuri et al. 2011; Rodrigues et al. 2010), MDSC have not been conclusively demonstrated in human GBM tumour tissue to date (Riccadonna 2012).

STAT-3, a member of the Signal Transducer and Activation of Transcription family of signalling proteins, appears to play a role in the control of many immunosuppressive mechanisms operating in the tumour setting. STAT-3 phosphorylation downstream of IL-6 and IL-10 signalling inhibits the function of macrophages, DC and T cells (Kubo et al. 2003) and STAT-3 activation is required for MDSC to inhibit effector T cells (Mao et al. 2013). STAT-3 is also activated in GBM tumour cells and has been shown to control the release by tumour cells of soluble factors that induce M2 differentiation (A Wu et al. 2010). Accordingly, systemic treatment of glioma-bearing mice with a STAT-3 inhibitor improved survival in WT but not athymic mice, and synergized with adoptive CTL transfer (Fujita et al. 2008).

1.4 AIMS OF THE THESIS

This thesis starts by reporting the results of a recently completed clinical trial in which patients with recurrent GBM were treated with a DC-based vaccine combined with chemotherapy. As will be seen, a major conclusion of this Phase I trial was that the feasibility of making vaccines from autologous monocyte-derived DC in this patient group is marginal. An alternative immunotherapeutic strategy that could improve feasibility in the clinical arena would be to deliver antigen to endogenous DC along with an effective adjuvant. Accordingly, in the next chapter a novel a vaccine consisting of irradiated tumour cells pulsed with an adjuvant that potently stimulates iNKT cells is tested in a murine model of glioma. When coupled with attenuation of regulatory T cell-mediated immunosuppression, the vaccine is shown to be capable of eradicating established intracranial glioma tumours in 43 % of mice. To try and improve therapeutic outcome even further, the subsequent chapters explore the idea of modifying the whole tumour cell-based vaccine to target particular tumour cell subsets or phenotypes. The aims of the thesis are therefore:

Clinical study:

- Contribute to the conduct of a Phase 1 clinical trial of dendritic cell vaccination combined with temozolomide retreatment in patients with recurrent GBM.
- Establish methodology and undertake assessment of vaccine-induced immune responses in trial participants.
- Analyse and report the trial findings.

Pre-clinical studies:

- Establish a murine model of glioma

- Assess the feasibility, efficacy and mechanism of action of a vaccine consisting of irradiated tumour cells combined with an adjuvant that stimulates iNKT cells.
- Assess the feasibility and efficacy of selectively targeting cancer stem cells or chemotherapy-resistant cells with this vaccine.

CHAPTER TWO: MATERIALS AND METHODS

Explanatory note

Chapter 3 describes a clinical trial performed in collaboration with several colleagues. For ease of reading, the methods used in the trial are included in the body of that chapter. This chapter describes the materials and methods used in the rest of the thesis. In all chapters, the contributions of others are clearly acknowledged in the text and/or figure legends.

2.1 MICE

The inbred strain C57BL/6 was obtained from the Animal Resource Centre, Canning Vale, Western Australia. Also used were CD1d^{-/-} mice (Y. H. Chen et al. 1997), MHC class II-deficient B6Aa⁰/Aa⁰ mice (Köntgen et al. 1993), TAP1-deficient mice (L Van Kaer et al. 1992), *langerin*-DTREGFP mice that express the human Diphtheria toxin receptor under control of the langerin gene (Kissenpfennig et al. 2005), and FoxP3-GFP reporter mice that have the enhanced GFP coding sequence inserted in-frame into the first coding exon of the FoxP3 gene (Fontenot et al. 2005). For one experiment, cells from OT-I x B6.SJL-Ptprca^aPepcb^b/BoyJ mice were transferred into C57BL/6 recipients. OT-1 mice are transgenic for a TCR recognising an MHC class I-restricted epitope from OVA (Hogquist et al. 1994). B6.SJL-Ptprca^aPepcb^b/BoyJ mice express the congenic marker CD45.1 so that crossing these two strains allows discrimination of cells adoptively transferred into CD45.2⁺ WT recipients. All mice were maintained by the Biomedical Research Unit, Malaghan Institute of Medical Research, Wellington, New Zealand. Experimental protocols were approved by Victoria University Animal Ethics Committee and performed according to their guidelines. Mice were 6-10 weeks of age and matched for age and gender.

2.2 CELL CULTURE

2.2.1 Plasticware

Falcon and Nunc tissue culture flasks and plates were from BD Biosciences (San Jose, CA) and Thermo Fisher Scientific (Auckland, NZ) respectively.

2.2.2 Cell lines

The murine glioma cell line GL261 was obtained from the DCTD Tumour Repository (National Cancer Institute, Frederick, MD) and, unless stated, was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Auckland, NZ) supplemented with 20 % Foetal Calf Serum (FCS) (Sigma-Aldrich, St Louis, MO), 2 mM glutamax, 100 U/mL penicillin and 100 µg/mL streptomycin (all Life Technologies). Cultures were incubated at 37 °C in 5 % CO₂ until 80-90 % confluent then harvested with TrypLE (Life Technologies) and passaged. Cultures were discarded after approximately 8 weeks. The B16-F10 melanoma cell line (Fidler 1975) was obtained from the American Type Culture Collection (Manassas, VA). The Lewis Lung Carcinoma cell line (LLC) (Finlay et al. 1987) was kindly provided by Dr. Graeme Finlay (Cancer Research Laboratories, Auckland, NZ). B16 and LLC were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies) supplemented with 5 % FCS (Sigma-Aldrich), 2 mM glutamax, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µM 2-mercaptoethanol (all Life Technologies) at 37 °C in 5 % CO₂. A methylcholanthrene-induced fibrosarcoma cell line raised in a CD1d^{-/-} mouse, and a CD1d-transfected C1R cell line, both used as controls in flow cytometry experiments, were gifts from Prof. Mark Smyth (Peter MacCallum Cancer Institute, University of Melbourne, Melbourne, Australia) and Prof. Vincenzo Cerundolo (Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK) respectively. The DC2114 murine dendritic cell line (Fuertes Marraco et al. 2012) was kindly provided by Hans Acha-Orbea (Department of Biochemistry, University of Lausanne, Switzerland) and was cultured in IMDM supplemented with

10% FCS, 2 mM glutamax, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µM 2-mercaptoethanol at 37 °C in 5 % CO₂. DC2114 cultures were harvested with Trypsin-EDTA (Life Technologies).

2.2.3 Culture in Stem Cell Medium

To induce stem cell-like tumour cells, GL261 was cultured in serum-free “stem cell medium” (SC medium) consisting of DMEM/F12 with B27 supplement (Life Technologies), heparin 2 µg/mL, recombinant human epidermal growth factor (EGF) 20 ng/mL, and basic fibroblast growth factor (bFGF) 10 ng/mL (all Stem Cell Technologies, Victoria, Australia). When non-adherent spheroidal cell aggregations (spheres) reached approximately 100-150 µm in diameter, the spheres were harvested by collecting the culture supernatant, washed, mechanically dissociated by trituration 10 times through a 26 gauge needle, counted, and used to reseed new cultures in fresh SC medium.

2.2.4 Culture under hypoxic conditions

GL261 cultures were seeded in complete media and incubated in an hypoxic chamber (Pro-ox Model C21, Biospherix, Lacone, NY) placed inside a standard incubator at 37 °C with a CO₂ level of 5 %. O₂ concentration was lowered to 0.5 % by adjusting the level of N₂.

2.2.5 Testing for mycoplasma contamination

Contamination of the GL261 cell line by mycoplasma infection was excluded by polymerase chain reaction (PCR) using a commercially available kit (e-Myco, Intron BioTechnology, Seoul, South Korea) according to the manufacturer’s instructions.

2.3 *IN VIVO* TUMOUR MODELS

2.3.1 Subcutaneous implantation

GL261 cells were harvested, washed, and resuspended in DMEM. 5×10^5 cells in 100 μL of DMEM were injected subcutaneously in the left flank. Mice were considered to harbour tumours when any two perpendicular diameters were both at least 2 mm.

2.3.2 Intracranial implantation

GL261 cells were harvested, washed, resuspended in phosphate-buffered saline (PBS) (Life Technologies), and kept on ice until injection. General anaesthesia was induced by i.p. injection of 100 mg/kg ketamine and 10 mg/kg xylazine (both Phoenix Pharm, Auckland, New Zealand) and buprenorphine (Renckitt Benckiser Pharmaceuticals, Richmond, VA) 0.1 mg/kg was administered s.c. for analgesia. Mice were positioned within a small animal stereotactic frame (Harvard Apparatus, Holliston, MA) using ear bars to stabilise the head. Lacrilube ointment (Allergan, Irvine, CA) was applied to the cornea to prevent dessication. After preparing the skin with povidone-iodine (Betadine, Purdue Products, Stamford, CT), a burrhole was made 2.1 mm lateral to the bregma on the right side using the tip of a 22 gauge needle. The single cell suspension was drawn up into a 10 μL Hamilton syringe (Hamilton Company, Reno, NV) and 5×10^3 cells in 2 μL PBS were injected via a 32 gauge needle into the right striatum at a depth of 3 mm. The needle was left in position for 1 minute before withdrawal. The burrhole was sealed with bone wax (Ethicon, Amtech Medical, Auckland, NZ) and the skin closed with interrupted 7-0 nylon sutures (Ethicon, Amtech Medical). Carprofen (Norbrook, Auckland, NZ) 5 mg/kg was administered s.c. on days 1 and 2 post-surgery. Time to symptom appearance was defined as time to weight loss $\geq 10\%$ or overt behavioural symptoms (reduced activity, hunching). The presence of brain tumours in

symptomatic mice was confirmed by necropsy, including histological examination in some experiments.

2.4 MANUFACTURE AND ADMINISTRATION OF VACCINES

2.4.1 Whole tumour vaccine (Glioma-Gal)

The *i*NKT cell ligand α -galactosylceramide (α -GalCer) was manufactured by Industrial Research Laboratories (Lower Hutt, New Zealand) as previously described (A. Lee et al. 2006a). To generate Glioma-Gal vaccines, GL261 cells were incubated in complete media supplemented with 200 ng/mL α -GalCer for 24 h, gamma-irradiated (Gammacell 3000 Elan irradiator, Best Theratronics, Ottawa, Canada) to 150 Gy on ice, washed three times in PBS, and resuspended in PBS for injection. The method for Glioma-Gal-Dox vaccines was identical except that 0.05 μ M doxorubicin (Ebewe Pharm, Unterach, Austria) was also added to the final 24 hours of culture. Unless otherwise stated, mice received 1×10^5 cells in 200 μ L PBS, injected i.v. into a lateral tail vein. To mitigate toxicity when more than 10^5 cells were injected, mice were pre-treated 10 minutes before vaccination with 2 IU heparin i.v. (Pfizer, NY, NY). To deplete regulatory T cells, mice received 125 μ g of anti-CD25 antibody i.p. (clone PC61, prepared in-house from hybridoma supernatant), a dose that was shown to deplete approximately 50 % of FoxP3⁺ CD4⁺ T cells in naïve mice (Appendix B, Fig B2.1), consistent with a previous study in this laboratory (Ataera et al. 2011).

2.4.2 Lysate vaccine (Lysate-Gal)

Tumour cells were harvested with TrypLE, washed three times in PBS, and resuspended in PBS in a cryovial at 10×10^6 live cells/mL. Tumour cell lysis was achieved by 6 cycles of freezing by immersion in liquid N₂ alternating with thawing

in a waterbath at 37 °C. 100 % cell death was confirmed by staining with Trypan Blue (Life Technologies). The lysate was triturated ten times through a 26 gauge needle to disrupt dead and dying cells. Particulate matter was removed by centrifugation at 1000 g for 10 min. The supernatant was passed through 0.8 and 0.2 µm filters. The protein concentration of each lysate was measured using a colourimetric protein assay (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Auckland, NZ). Absorbance at wavelength 750 nm was read on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). Lysates were stored at -80 °C until required. To prepare vaccines, lysates were diluted with PBS to achieve the desired concentration and then α-GalCer was added to the lysate to achieve a dose of 200 ng per animal. The lysate + α-GalCer mixture was injected into a lateral tail vein, typically at a volume of 200 µL.

2.5 *IN VIVO* OT-1 CROSS-PRIMING ASSAY

Spleens and lymph nodes from OT-I x B6.SJL- Ptpcr^a Pepc^b/BoyJ mice (CD45.1⁺) were strained through a 70 µm filter, then CD8 cells positively selected using magnetic beads (Dynabead FlowComp, Life Technologies) and analyzed by flow cytometry to check purity. OT-1 cells (10⁴) were transferred into C57BL/6 mice by i.v. injection and 1 day later recipient mice were injected i.v. with 200 µg endotoxin-free OVA (Profos AG, Regensburg, Germany) and 200 ng α-GalCer. Seven days later mice were bled from a lateral tail vein and leukocytes stained directly *ex vivo* with antibodies for TCR Vα2, CD8, and CD45.1. Flow cytometry was used to enumerate OT-1 cells as a proportion of total CD8⁺ lymphocytes.

2.6 *IN VIVO* DEPLETION STUDIES

CD8α⁺ cells were depleted with antibody from clone 2.43, CD4⁺ cells with clone GK1.5, and NK1.1⁺ cells with clone PK136, all prepared in-house from hybridoma

supernatants. Appropriate i.p. dosing schedules were developed to achieve > 95 % depletion over the course of a given experiment (Appendix B, Figs B2.2, 2.3, 2.4). Depletion of langerin⁺ cells in *langerin*-DTREGFP recipients was achieved with 350 ng of Diphtheria toxin (Sigma-Aldrich) given 48 and 24 hours before vaccination, and one and two days after vaccination. In some experiments, antigen-presentation in spleen was prevented entirely by splenectomy performed 7 days before vaccination. Splenectomies were conducted under general anaesthesia using the method described by Reeves and colleagues (Reeves et al. 2001). Mice were allowed to recover for 7 days before experiments.

2.7 IN-HOUSE PREPARATION OF DEPLETING ANTIBODIES FROM HYBRIDOMA SUPERNATANTS

Hybridomas stored in the vapour phase of liquid N₂ were thawed and briefly cultured in IMDM supplemented with 5% FCS. The cell compartment of a double chamber cell culture flask (CELLine CL-1000, Integra Biosciences AG, Zizers, Switzerland) was seeded with 30 x 10⁶ cells at 2 x 10⁶ cells/mL in IMDM supplemented with 20 % FCS. The nutrient chamber was filled with 500 ml of IMDM supplemented with 1 % FCS. Cells were cultured at 37 °C, 5 % CO₂. Every few days, the cell culture chamber was emptied and reseeded with 150 x 10⁶ cells at 10 x 10⁶ cells/mL. Supernatant from the cell compartment was sterilised with 0.01% NaN₃ (Sigma-Aldrich) and stored at 4 °C until purification.

To purify IgG, supernatants were passed through a 0.2 µm filter and then run through a Protein G column (HiTrap Protein G HP, GE Healthcare, Auckland, NZ) on a low pressure liquid chromatography system (BioLogic LP, Bio-Rad Laboratories). Bound IgG was eluted at pH 2.7 while monitoring absorbance at a wavelength of 280 nm. Eluted fractions were collected using a BioLogic Biofrac Fraction Collector (Bio-Rad Laboratories). IgG content of collected fractions was quantified by spectrophotometry (Nanodrop ND-1000 with software V3.5.2, Thermo Fisher

Scientific). Fractions containing IgG were pooled and dialysed to exchange the buffer solution using a dialysis cassette (Slide-A-Lyzer Cassette with 3500 Da cut-off, Pierce, Thermo Fisher Scientific) placed overnight in 1.5 L PBS at 4 °C. The final product was passed through a 0.2 µm filter, aliquoted and stored at -80 °C until use.

Elution buffer (0.1 M glycine-HCl, pH 2.7): dH₂O 500 mL, glycine 3.75 g, HCl to bring pH to 2.7

2.8 SAMPLE COLLECTION

2.8.1 Collection and preparation of murine leucocytes

Blood was obtained by nicking a lateral tail vein with a scalpel blade and collecting 5-8 drops in 1.7 mL tubes containing 200 µL PBS and 10 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich). Red blood cells (RBC) were removed by adding 1 mL RBC lysis buffer (Qiagen, Germantown, MA) and incubating for 30 min at 37 °C. After centrifugation at 664 g for 5 min, the supernatant was discarded and the pellet resuspended in appropriate medium or buffer.

Spleens and lymph nodes were harvested and placed into IMDM on ice. Tissue was mechanically dissociated by passage through a 70 µm filter. RBC were removed from spleen samples with RBC lysis buffer as above. Cells were washed twice in IMDM and resuspended in appropriate medium or buffer.

For liver lymphocytes, mice were sacrificed by cervical dislocation and the livers perfused with ice cold PBS via the portal vein before harvesting. Livers were cut into small pieces, incubated in IMDM containing 2.4 mg/mL collagenase II (Life Technologies) and 0.2 mg/mL DNase I (Roche, Mannheim, Germany) for 60 min at 37 °C then passed through a 70 µm filter. Cells were washed and resuspended in IMDM. To enrich for lymphocytes, 7 mL of the single cell suspension was underlaid with 3 mL of Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). After centrifugation

at 840 g for 20 min at room temperature, cells at the sample-medium interface were collected. Cells were washed in IMDM and resuspended.

2.8.2 Patient sample collection

Peripheral blood and tumour tissue had previously been collected from GBM patients with their informed consent under a Central Regional Ethics Committee of New Zealand-approved protocol that allowed for future unspecified research. Venous blood was drawn into CPT cell preparation tubes (BD Biosciences) and the buffy coat collected after centrifugation at room temperature according to the manufacturer's instructions. PBMC were washed and cryopreserved in the vapour phase of liquid N₂ in 70 % RPMI (Life Technologies), 20 % FCS and 10 % Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) until use. Patient blood samples were predominantly processed by Malaghan Institute research officers although the author also processed some samples. Absolute differential blood counts were determined by separate venesection within 24 hours of PBMC collection by Wellington Hospital laboratory staff using a Sysmex XE 2100 analyser (Roche Diagnostics, Auckland, New Zealand). PBMC from 35 healthy age-matched controls were collected and stored by Dr R. Weinkove according to a similar protocol.

Tumour tissue collected at surgery was placed immediately into PBS supplemented with 0.5 % glucose and transported to the Malaghan Institute on ice. Tissue was mechanically dissociated to create a single cell suspension and primary GBM cultures were generated in RPMI (Life Technologies) supplemented with 10 % FCS, 100 U/mL penicillin, 100 µg/mL streptomycin. Processing and primary culture of the human tumour samples was performed predominantly by K. Broadley.

2.9 FLOW CYTOMETRY

2.9.1 Flow cytometric analysis of murine cells

Table 2.1 Monoclonal antibodies used for murine studies

Specificity	Clone	Fluorophore	Supplier
B220	RA3-6B2	PerCP	BD Biosciences
B220	RA3-6B2	PerCPCy5.5	eBioscience
CD1d	1B1	PE	Invitrogen
CD3ε	145-2C11	FITC	Invitrogen
CD3ε	145-2C11	PeCy7	eBioscience
CD4	GK1.5	PE	Invitrogen
CD4	GK1.5	APC	Invitrogen
CD4	RM4-5	Pac Blue	eBioscience
CD8α	53-6.7	FITC	Invitrogen
CD8α	53-6.7	BV650	Biolegend
CD11b	M1/70	APCCy7	BD Pharmingen
CD11c	HL3	APC	BD Biosciences
CD11c	N418	APC	Biolegend
CD15 (SSEA-1)	MC480	PE	BD Pharmingen
CD25	PC61	PE	Biolegend
CD40	3/23	PE	BD Biosciences
CD44	IM7	PE	BD Pharmingen
CD44	IM7	APC	BD Biosciences
CD45.1	A20	PE	Invitrogen
CD45.2	104	Alexa 488	Invitrogen*
CD69	H1.2F3	FITC	BD Biosciences
CD86	GL1	PE	eBioscience
CD133 (Prominin 1)	MB9-3G8	APC	Miltenyi Biotec
CD200	OX90	Alexa 488	eBioscience*
Fas	Jo2	FITC	BD Pharmingen
Gr-1	RB6-8C5	APC	BD Pharmingen
MHC class I (H-2K ^b)	AF6-88.5.5.3	APC	eBioscience
MHC class II (I-A/I-E)	M5/114.15.2	PE	eBioscience
MHC class II (I-A/I-E)	M5/114.15.2	Alexa 488	eBioscience*
MHC class II (I-Ab)	AF6-120.1	PE	BD Pharmingen
NK1.1	PK136	Alexa 488	BD Biosciences*
TCR Vα2	B20.1	Alexa 647	eBioscience*
TCRβ	H57-597	FITC	BD Biosciences
TCRβ	H57-597	PE	BD Biosciences
Isotype controls:			
Mouse IgG2a,κ	MOPC-173	FITC	Biolegend
Mouse IgG2a,κ	eBM2a	PE	eBioscience
Mouse IgG2a,κ	eBM2a	Alexa A488	eBioscience*
Mouse IgG2a,κ	eBM2a	APC	eBioscience
Mouse IgG2b,κ	149/10H5	PE	eBioscience
Mouse IgM,κ	G155-228	PE	BD Pharmingen
Rat IgG1,κ	R3-34	APC	BD Biosciences
Rat IgG2a	R35-95	PE	BD Biosciences
Rat IgG2b	eB149/10H5	PE	eBioscience
Hamster IgG	HTK888	FITC	Biolegend

* purified antibodies conjugated to fluorophores in-house

All antibody staining of murine cells was performed for 10 minutes at 4 °C in PBS supplemented with 1 % FCS, 0.05 % sodium azide, and 2 mM EDTA. Non-specific Fc receptor-mediated binding was blocked by incubation for 10 min with anti-CD16/32 (clone 24G2, prepared in-house from hybridoma supernatant). Propidium iodide (Sigma-Aldrich) was used to exclude dead cells. Flow cytometry was performed on a FACSCalibur (BD Biosciences) with data analysis using FlowJo software (TreeStar Inc, Ashland, OR). Compensation was performed with single-stained positive control cells or with fluorophore-labeled antibodies bound to beads coated with anti-rat Ig (BD Compbeads, BD Biosciences). Monoclonal antibodies were purchased from eBioscience (San Diego, CA), BD Biosciences, Miltenyi Biotec (Bergisch Gladbach, Germany) or Biolegend (San Diego, CA) and are listed in Table 2.1. Some purified antibodies were conjugated in-house to Alexa-A488 or Alexa-A647 (Life Technologies). A PE-labelled CD1d tetramer (Proimmune, Oxford, UK) loaded with α -GalCer according to the manufacturer's instructions was used to discriminate iNKT cells. All antibodies were used at the lowest concentration at which binding site saturation occurred. This concentration was determined by performing a titration study using splenocytes or a tumour cell line. A titration study was performed for each newly acquired antibody by a member of the author's research group, including some by the author.

2.9.2 Flow cytometric analysis of human PBMC samples

PBMC were thawed, washed, and resuspended in PBS. Antibody staining was conducted at 4 °C for 20 min in PBS. Fc receptors were blocked by incubation with 2 mg/mL polyclonal human IgG (Intragam P, CSL Limited, Australia) for 15 min. Dead cells were excluded using LIVE/DEAD Fixable Blue (Molecular Probes, Eugene, OR), 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes), or propidium iodide. Data were acquired on an LSRII flow cytometer or FACS Calibur apparatus (both BD Biosciences) and analyzed using FlowJo software. Compensation was performed using fluorophore-labeled antibodies bound to anti-mouse Ig-coated particles (BD

Compbeads, BD Biosciences). For non-antibody stains, compensation was performed using stained and unstained populations of positive control cells. The fluorescent monoclonal antibodies used in these studies are listed in Table 2.2. Human iNKT cells were detected using CD1d monomers (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA) that had been loaded with α -GalCer and tetramerized with streptavidin-PE (BD Biosciences). The antibody panels for FC of human PBMC samples described in Chapter 4 were designed and optimised by Dr R. Weinkove.

Table 2.2 Monoclonal antibodies used for flow cytometry of human PBMC

Specificity	Clone	Fluorophore	Supplier
CD1d	CD1d42	PE	BD Biosciences
CD3	SK7	APC-H7	Biolegend
CD11c	Bul5	APC	Biolegend
CD14	M ϕ P9	APC	BD Biosciences
CD16	3G8	FITC	Biolegend
CD19	SJ25C1	APC	BD Biosciences
HLA-DR	L243	PerCP	Biolegend
Lineage-1*		FITC	BD Biosciences

*CD3, CD14, CD16, CD19, CD20, CD56

2.10 ANALYSIS OF CYTOKINE RELEASE

Blood was collected from the lateral tail vein and allowed to clot at room temperature. Serum was collected after centrifugation. Levels of the cytokines IL-4, IL-12p70 and IFN- γ were assessed by cytokine bead arrays analysed on a Bio-Plex analyzer (Bio-Rad Laboratories) according to the manufacturer's instructions. For analysis of cytokine release in spleen, liver and lymph node post-vaccination, tissue was removed from euthanized animals and mechanically dissociated. Enrichment for liver lymphocytes was performed using a Lymphoprep gradient as described

above. Cells were washed and incubated at 37 °C in IMDM with 5 % FBS, 2 mM glutamax, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-mercaptoethanol. Culture supernatants were collected after 48 hours for analysis.

2.11 MAGNETIC RESONANCE IMAGING (MRI)

MRI was performed on anesthetized animals using a clinical 1.5 Tesla MR scanner (Philips Medical Systems, Eindhoven, The Netherlands) equipped with a wrist solenoid coil. T1-weighted images were acquired with the following parameters: TE= 21.4 ms, TR = 800 ms, pixel size = 300 mm x 300 mm, thickness = 1 mm, 3 averages. Contrast was enhanced by i.v. administration of 100 µL gadolinium-DTPA (Magnevist, Bayer Schering Pharma, Berlin, Germany). T2-weighted spin-echo images were acquired with the parameters: TE = 54 ms, TR = 2000 ms.

2.12 *IN VITRO* iNKT CELL PROLIFERATION ASSAY

PBMCs were thawed, washed, and plated (5×10^5 PBMCs per well in a 96-well plate) in IMDM supplemented with 5 % human AB serum (Life Technologies), and either 100 ng/mL α -GalCer or vehicle. After 24 hours, 50 U/mL recombinant human IL-2 (Chiron Corporation, Emeryville, CA) was added to each well. After 7 days, live cells were counted with Trypan Blue exclusion, and iNKT cells enumerated by flow cytometry.

2.13 IMPACT OF α -GALCER-LOADED TUMOUR CELLS ON iNKT CELL PROLIFERATION AND ALLORESPONSE

Primary GBM cell cultures from five patients were either pulsed overnight with 200 ng/mL α -GalCer or left untreated, and then γ -irradiated on ice (150 Gy). After

washing three times to remove any free α -GalCer, 10^5 irradiated tumour cells were co-cultured with 5×10^5 carboxyfluorescein succinimidyl ester (CFSE)-labelled allogeneic PBMC in 96-well plates. After 5 days, CFSE dilution was assessed by flow cytometry. CFSE was from Sigma-Aldrich.

2.14 ASSESSMENT OF MHC CLASS II EXPRESSION BY GBM TUMOUR CELLS

Primary GBM cell lines were cultured as above for 48 hours with and without 100 U/mL recombinant human IFN- γ (PeproTech, Rocky Hill, NJ). Cells were assessed for HLA-DR expression by flow cytometry as described above.

2.15 HARVEST AND ENRICHMENT OF FOETAL NEURAL STEM CELLS (FNSC)

Mating of C57BL6 mice was timed and E14 pregnant dams were sacrificed by cervical dislocation. Under sterile conditions, fetuses were harvested, decapitated, and heads placed in ice cold PBS. Under a laminar flow hood with HEPA filter, and using 10X magnification, skin and dura were removed as previously described (Chojnacki and Weiss 2008). Foetal brains were mechanically dissociated by straining through 70 and 40 μ m filters followed, if necessary, by gentle trituration to create a single cell suspension. Cells were washed, counted and used to seed cultures at a concentration of 5×10^4 /mL in NeuroCult Mouse Basal Medium supplemented with 10 % Neurocult Mouse Supplement, 10 ng/mL bFGF, 20 ng/mL EGF, 2 μ g/mL heparin (all Stem Cell Technologies), a medium shown to support and enrich for normal neural stem cells (Galli et al. 2008; S. A. Louis et al. 2008). Because settling debris was likely to prevent adherence of cells of interest (Chojnacki and Weiss 2008), only non-adherent cells were harvested at the first passage . Thereafter both floating cells and the adherent layer of “attached spheres” were serially passaged.

2.16 SIDE POPULATION ASSAY AND FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

Tumour cells at 70–80 % confluency were harvested with TrypLE and washed twice in Hanks Buffered Salt Solution (HBSS) (Life Technologies) with 2 % FCS. Cells were resuspended at 1×10^6 cells/mL in HBSS supplemented with 2 % FCS warmed to 37 °C, then incubated at 37 °C with 5 µg/mL Hoechst 33342 (Life Technologies) and 15 mM sodium azide. 2-deoxyglucose (2-DOG) (Sigma-Aldrich) which inhibits ATP-binding cassette transporters, was added to some cells prior to Hoechst 33342 as a negative control. Cells were shielded from ambient light and incubated for 90 min, with mixing every 30 min. Staining was halted by rinsing in ice-cold HBSS/2% FCS. Propidium iodide was added at 4 µM for viability assessment. Cells were resuspended at $2\text{--}4 \times 10^6$ cells/mL in HBSS/2% FCS and sorted on a BD FACS Vantage DiVa (BD Biosciences) by Ms Kylie Price. Cells were excited with a UV laser (150 mW on the FACS Vantage DiVa). The emission was detected through 250/50 nm (“Hoechst Blue”) and 620 LP (“Hoechst Red”) filters with signal separated by a 610-nm short pass dichroic mirror. Live cells were displayed on a Hoechst Blue *versus* Hoechst Red dot plot to visualize the SP. SP cells were sorted at 20 psi sheath pressure, with a 100- µm nozzle and approximately 37,500 Hz drop drive frequency, at a rate of approximately 2,000 cells per second. All data were collected in linear mode and analyzed using FlowJo.

2.17 ASSESSMENT OF GENE EXPRESSION BY QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Cells were washed in PBS and frozen at -80 °C until required. Cells were lysed and total RNA extracted using the Mini RNA Isolation II kit (Zymo Research, Irvine, CA, <http://www.zymoresearch.com>) according to the manufacturer’s instructions. Care was taken to prevent contamination with extraneous nucleic acid throughout the process. RNA concentration was measured by spectrophotometry (Nanodrop ND-

1000, Software V3.5.2, Thermo Fisher Scientific). cDNA was synthesized using commercially-available reverse transcriptase and reagents (iScript, Bio-Rad Laboratories) and an automated thermal cycler (Biorad iCycler, Bio-Rad Laboratories) programmed to heat to 25 °C for 5 min, 42 °C for 30 min, 85 °C degrees for 5 min. The resulting cDNA was pipetted into 96 well plates and mixed with pre-designed commercially-available forward and reverse primers for murine genes of interest (*CD133*, *Musashi-1*, *SOX2*, *SOX6*, *IL-13Ra2*, *gp100*, *TRP-2*, *survivin*, *nestin*, *Oct4*, *ABCB1*, *ABCC1*, *ABCG2*) or 18s ribosomal RNA (QuantiTect primer assays, Qiagen) and a commercially-available RT-PCR mixture that includes a DNA polymerase, deoxynucleotide triphosphates, a green dye that binds to dsDNA, and buffer components (SYBR green-based RT-PCR kit, Qiagen). RT-PCR was performed on the ABI 7500 RT-PCR platform (Applied Biosystems, Life Technologies) using 40 cycles of: 94 °C for 15 seconds, 55 °C for 30 seconds, 72 °C for 35 seconds. The exponential phase of the amplification curve was used to determine the cycle threshold (Ct) for each gene and Ct values were normalized to the Ct value for 18S rRNA (Δ Ct). Amplification efficiency of QuantiTect primer assays is equivalent (<http://www.qiagen.com/Products/PCR/QuantiTect/PrimerAssays>) so the $\Delta\Delta$ Ct method was used to determine fold change from parental cells.

2.18 GENERATION OF DOXORUBICIN-RESISTANT TUMOUR CELLS

GL261 cultures were set up as normal in complete media. After 1-2 days doxorubicin was added directly to the media to achieve a final concentration of 0.01 μ M. Extensive cell death was observed but a small fraction of cells remained viable (termed “drug-tolerant persisters”). After several days of growth arrest surviving cells eventually began to proliferate (termed “drug-tolerant expanding persisters” or DTEP). On reaching 80 -90 % confluency cells were harvested and reseeded in media containing 0.1 μ M doxorubicin. After several passages the doubling time of these cells stabilised at level close to parental culture. These cells were thereafter termed “DTEP 0.01”. Some DTEP 0.01 cells were reseeded in media containing

progressively increasing concentrations of doxorubicin. In this way stable DTEP cultures were established at a range of different doxorubicin concentrations although the doubling time of stable cultures progressively increased with increasing drug concentration.

2.19 ASSESSMENT OF TUMOUR CELL PROLIFERATION BY MEASUREMENT OF ³H-THYMIDINE INCORPORATION

GL261 parental and DTEP cultures were harvested and resuspended in complete media. 1×10^4 cells in 100 μ L media were added to wells of a round-bottomed 96 well plate. A further 100 μ L of media containing doxorubicin at different concentrations was added to each well to achieve final concentrations ranging from 0.001 – 100 μ M. Each cell line was assessed at each concentration in triplicate. Cells were incubated overnight at 37 °C, 5 % CO₂. The following day 0.25 μ Ci ³H-thymidine (MP Biomedicals, Santa Ana, CA) were added to each well. The plate was incubated at 37 °C for a further 16 hours and then thymidine uptake assessed by liquid scintillation counting. Briefly, the contents of the wells were transferred to a glassfibre filtermat (Wallac, PerkinElmer, Waltham, MA) using a cell harvester (TomTec, Unterschleisheim, Germany) and beta-emission read on a liquid scintillation counter (Wallac 1450 microbeta PLUS, PerkinElmer).

2.20 DOXORUBICIN TREATMENT OF MICE *IN VIVO*

Doxorubicin supplied by the manufacturer in soluble form at 2 mg/mL (Ebewe Pharm) was diluted in PBS shortly before use and administered to mice by slow injection into a lateral tail vein in a volume of 200 μ L. No obvious toxicity was observed in C57BL/6 mice at any of the doses tested, *i.e.* up to 8 mg/kg.

2.21 ASSESSMENT OF DC MATURATION IN RESPONSE TO PULSING WITH TUMOUR LYSATE OR TO DOXORUBICIN EXPOSURE *IN VITRO*

Cells of the DC2114 cell line were harvested, resuspended in complete IMDM with 10% FCS and each well of a 24 well flat bottom plate seeded with 2.5×10^5 cells in 1 mL medium. Plates were incubated for 4 hours at 37 °C to allow cells to adhere. In doxorubicin experiments, doxorubicin was then added at concentrations ranging from 1 µM to 0.001 µM. In some experiments, GL261 tumour cell lysates created by the freeze/thaw method described in Section 1.4.2 were added to wells at concentrations of 10 µg/mL or 100 µg/mL. In one experiment, GL261 tumour cells were harvested, washed, and resuspended in complete media at 2.5×10^5 /mL in cryovials. Cryovials were sonicated in a benchtop ultrasonic cleaner (Unisonics Model FXP8M, Unisonics Australia, Brookvale, NSW, Australia) until all cells were Trypan Blue positive (typically 90 min). Sonicate was then added to wells at a ratio of 1 DC : 1 sonicated tumour cell. Poly I:C (Invivogen, San Diego, CA) was added to some wells at 100 µg/mL as a positive control and all experimental conditions were assessed in triplicate wells. After incubation for 16 hours at 37 °C, 5 % CO₂ cells were harvested and expression of CD40 and CD86 assessed by antibody staining and flow cytometry, gating on live GFP⁺ cells.

2.22 WESTERN BLOT ANALYSIS OF TRP-2 PROTEIN IN CELL LYSATES

Cells were lysed by freeze/thaw and mechanical disruption as described for production of lysate vaccines. A protease inhibitor was not used during lysate preparation. Insoluble debris was removed by centrifugation with collection of supernatant. Protein concentration was quantified using the Lowry method (Bio-Rad DC Protein Assay, Bio-Rad Laboratories). Absorbance at wavelength 750 nm was measured on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). Protein was diluted in sodium dodecyl sulfate (SDS)-loading buffer and denatured at 96 °C for 5 min. A 10 % acrylamide resolving gel was prepared, cast between

parallel glass plates (Miniprotean III system, Bio-Rad Laboratories), overlaid with a stacking gel, left to set, and stored overnight at 4 °C. The gel was loaded with 10 µg of sample proteins, immersed in running buffer and electrophoresis applied at a constant voltage of 190 V for 1 hour (Bio-Rad PowerPack 3000, Bio-Rad Laboratories). To estimate protein size, side lanes were loaded with protein standards (Precision Plus All Blue Protein Standards, Bio-Rad Laboratories). Protein was transferred to a polyvinylidene difluoride (PVDF) membrane by electrophoresis at a constant current of 300 mA for 2 hours in transfer buffer. Non-specific protein binding was blocked with 3 % weight/volume non-fat milk in PBS with 0.01 % Tween 20 (Sigma-Aldrich) then the membrane was probed overnight at 4 °C with rabbit anti-mouse TRP-2 antibody (Santa Cruz Biotechnology, Dallas, TX) at 1:600. The following day the membrane was washed in PBS with 0.01 % Tween20, blocked, and secondary labeling performed using goat anti-rabbit antibody conjugated to peroxidase (Santa Cruz Biotechnology) at 1:2000. Signal was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Detection of β -actin loading control was performed the following day using mouse anti-mouse β -actin antibody (Sigma-Aldrich) at 1:200,000 and goat anti-mouse antibody conjugated to peroxidase (Santa Cruz Biotechnology). The fully developed membrane was imaged by autoradiography (X-Omat, Kodak, Los Angeles, CA).

Loading Buffer: 1 M Tris.HCl (pH 6.8), 20 % glycerol, 40 mg/mL SDS, 2 % 2-mercaptoethanol, 0.01 mg/mL bromophenol blue.

Resolving Gel: dH₂O, 10 % Acrylamide, 10 % SDS, 10 % APS, TEMED (5 µL per 10 mL).

Stacking Gel: dH₂O, 5 % Acrylamide, 0.5 M Tris.HCl (pH 6.8), 10 % SDS, 10 % APS, TEMED (5 µL per 4 mL).

Running Buffer (1 L): dH₂O, Tris base 3 g, Glycine 14.4 g, SDS 1 g, pH adjusted to 8.3

Transfer Buffer (1 L): dH₂O, pure methanol 100 mL (20 % v/v), Glycine 14.4 g, Tris base 3.02 g, pH adjusted to 8.3

2.23 HISTOLOGY

After cervical dislocation, mouse brains were removed whole, fixed overnight in 10 % formalin (Sigma-Aldrich) and then cut into 2 mm coronal sections. Selected sections were sent in formalin to the Department of Pathology and Molecular Medicine, University of Otago, Wellington. Under the supervision of Dr Rose Miller or Dr Peter Ferguson, the tissue provided was embedded in paraffin, cut into 4 μ m sections mounted on glass slides, and stained with Harris' haematoxylin and eosin stain. Slides were photographed at 20X magnification.

2.24 STATISTICAL ANALYSES

Unless stated otherwise, bars and error bars depict means and standard error of the mean except in graphs of observational patient data where they depict medians and quartiles. Non-parametric tests were used to assess statistical significance. For comparisons of one variable, the Mann Whitney test was used for unpaired data, the Wilcoxon matched pairs test for paired data, and the Kruskal Wallis one-way analysis of variance (ANOVA) for experiments comparing more than two groups, with Dunn's post-test to determine statistical significance between two individual groups. For comparing group means over time in animal experiments, non-parametric analysis was performed at each time point using a Mann Whitney test (for 2 groups) or Kruskal Wallis test with Dunn's post-test (for more than 2 groups). A log-rank test was used to determine statistical significance between Kaplan-Meier survival curves. In some experiments, comparisons of two variables was performed using a two way ANOVA test with Bonferroni's post-test. All statistical analyses were done with Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA), with *P* values of < 0.05 considered significant (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). In studies comparing patient groups, all subjects with complete data for that particular study were included. Age matching was performed by alternately removing the oldest or youngest subject from each group until the difference between the mean ages of the two groups was no more than 2.5 years.

CHAPTER THREE: ASSESSING VACCINE-INDUCED IMMUNE RESPONSES IN A CLINICAL TRIAL OF DENDRITIC CELL VACCINATION COMBINED WITH TEMOZOLOMIDE FOR PATIENTS WITH RECURRENT GLIOBLASTOMA MULTIFORME

Explanatory note: Author's contribution

A Phase I Clinical Trial of DC Vaccination and TMZ for patients with recurrent GBM was recently conducted at Wellington Hospital. The author of this thesis was one of three principal investigators for the trial that was jointly conducted by the Malaghan Institute, Wellington Hospital, and Cancer Trials New Zealand. The trial opened for recruitment in January 2009 and closed in December 2011. Thus, the trial was conducted within the time frame of this PhD. As the principal investigator with responsibility for neurosurgical aspects, the author had a major role in study design and obtaining funding and regulatory approval, and was heavily involved in the day-to-day conduct of the trial including taking the lead role in recruitment and consent, performing the great majority of the surgical procedures, sitting on the Trial Management Committee, and participating in the frequent consensus decision-making required for a trial of this kind. In addition, the author independently undertook an assessment of vaccine-induced immune responses in trial participants using patient PBMC stimulated by autologous tumour-pulsed DC or tumour lysate in an interferon- γ ELISpot assay, and led an assessment of immune cell populations in PBMC before and after vaccination, having primary responsibility for designing and interpreting the flow cytometry studies. Finally, the author was primarily responsible for collating the data and for writing this chapter although, as this work has subsequently formed the basis of a publication, others have also had some input. For ease of reading, the whole of the trial data is presented here together as it might appear in a published manuscript, with the contributions of others to laboratory-based aspects of the study clearly indicated in the text.

3.1 INTRODUCTION

GBM is a highly malignant primary brain tumour that almost invariably recurs after standard therapy. TMZ is a firmly established component of standard treatment for newly-presenting disease (Stupp et al. 2005). The relative tolerability of TMZ makes it an attractive option for use in the recurrent setting as well (C. Chen et al. 2013; Perry et al. 2010). However, there are few reports of retreating GBM patients with TMZ using the same maximally tolerated dose (MTD) regimen that is currently used in the adjuvant phase of standard treatment (Franceschi et al. 2005; A. Wick et al. 2009). While retreatment with MTD TMZ is clearly capable of achieving objective responses, the overall results are modest, with a PFS_{6mo} of 28.6 % in the largest series of 15 patients (A. Wick et al. 2009). The disappointing results of salvage chemotherapies in general have driven interest in novel treatments for GBM such as immunotherapies. DC are potent antigen-presenting cells and can be differentiated *in vitro* from patients' own PBMC. Vaccination with autologous monocyte-derived DC pulsed with unselected antigens derived from whole tumour cells has been the most widely tested active immunotherapy in GBM patients to date. Although well tolerated, the clinical benefit of DC-based vaccination in the recurrent setting has generally been modest (reviewed in Vauleon et al. 2010; X. Xu et al. 2012).

Interestingly, GBM patients who had previously participated in clinical trials of autologous tumour lysate-pulsed DC-based vaccines were observed anecdotally to respond unusually well to subsequent salvage chemotherapy (Wheeler et al. 2004) and similar observations have been reported in other cancers (reviewed in Ramakrishnan et al. 2008). To potentially explain this, there is evidence that cytokines released by T cells can enhance the tumouricidal activity of chemotherapeutic agents *in vitro* (Natsume et al. 2005) and *in vivo* (Radfar et al. 2009), including TMZ. Antigen-specific mechanisms have also been proposed. Tyrosinase-related protein-2 (TRP-2) is a melanoma-associated protein upregulated in many GBM tumours (Chi et al. 1997; G. Liu et al. 2003) that provides protection against apoptosis (Chu et al. 2000; Nishioka et al. 1999; Pak et al. 2001). TRP-2-

specific CTL responses have been detected in GBM patients vaccinated with autologous tumour lysate-pulsed DC, an event associated with editing of TRP2^{hi} tumour cells and a heightened sensitivity of resampled tumours to chemotherapy agents *in vitro* including TMZ (G. Liu et al. 2005). Survivin (Chakravarti et al. 2002; Ciesielski et al. 2010; Preusser et al. 2005) and the mutant epidermal growth factor receptor EGFRvIII (Choi et al. 2009) are two other glioma-associated antigens also implicated in mediating resistance of GBM to standard treatments. Thus, while relative overexpression of certain proteins confers chemoresistance, this may also render cells more susceptible to antigen-specific immune attack. We hypothesised that immunotherapy could be used to overcome resistance to a given chemotherapeutic agent after failure of primary treatment. In GBM, one way of achieving this could be to manufacture DC vaccines pulsed with tumour tissue from recurrent lesions; this tissue would already have been exposed to TMZ, and therefore likely to be enriched in proteins mediating resistance to that agent, potentially skewing the vaccine-induced response towards chemoresistant cells. Prior to assessing this possibility directly, we conducted a phase I study to determine whether manufacturing such DC vaccines is feasible in GBM patients recurring after standard treatment, and the overall safety of combining DC vaccination with TMZ retreatment.

3.2 MATERIALS AND METHODS

3.2.1 Patient population

Participants in this trial had MRI scans consistent with relapsing tumour following previous treatment with radiotherapy and TMZ for histologically-proven WHO grade 4 diffuse astrocytoma. There was a two-stage enrolment process, with final confirmation of eligibility after histological confirmation of recurrent GBM. Other inclusion criteria were: age ≥ 18 , ECOG performance status ≤ 2 ; prior completion of at least three cycles of adjuvant TMZ according to the Stupp protocol (Stupp et al.

2005); at least 1 cm³ of surgically-accessible tumour tissue; and ability to give informed consent. Exclusion criteria were: pregnancy or breastfeeding; diagnosis of another malignancy within 5 years or presence of other serious unstable medical conditions; serology indicating active infection with hepatitis B or C, or HIV; uncontrolled or unstable auto-immune disease; recent immunosuppressive therapy other than dexamethasone for management of GBM; previous treatment with a chemotherapeutic agent other than TMZ since first diagnosis with GBM; haemoglobin < 100 g/L; platelet count < 100 x 10⁹/L; neutrophil count < 1.5x10⁹/L; liver function tests or creatinine > 2 x upper limit normal; unfit for general anaesthesia; significant dysphasia or other neurological deficit precluding reliable communication. All participants gave written informed consent. The study was approved by the Central Regional Ethics Committee of New Zealand (CEN/07/12/086) and registered with the Australian and New Zealand Clinical Trials Registry (ACTRN12611000029998).

3.2.2 Treatment plan

Patients underwent leukapheresis to harvest PBMC followed a few days later by craniotomy for maximum safe tumour resection. Three weeks after surgery patients received a priming course of three rounds of DC-based vaccination administered i.d. at 2 week intervals. The intended dose for priming vaccinations was 4 x 10⁶ DC. One week after the third vaccine, patients began treatment with TMZ 150-200 mg/m² orally for 5 consecutive days, every 28 days, for up to 6 cycles. Dose reductions and treatment delays of up to two weeks were allowed for recovery of haematological parameters. A booster vaccination of 1 x 10⁶ DC was administered 2 weeks after each cycle of TMZ. Corticosteroid was reduced to the lowest possible dose tolerated by the patient.

3.2.3 Vaccine production

Vaccine production was carried out in a dedicated Good Manufacturing Practice facility. Fresh tumour tissue was disaggregated to create a single cell suspension and γ -irradiated to a dose of 200 Gy. PBMC were obtained by leukapheresis and the plastic-adherent fraction cultured for 5 days in RPMI-1640 (Invitrogen, Life Technologies, Auckland, NZ) supplemented with 1 % autologous serum, 1000 U/ml rhGM-CSF (Bayer Health Care, Lynnwood, WA) and 1000 U/ml rhIL-4 (CellGenix, Freiburg, Germany) to induce DC differentiation as previously described (O'Rourke et al. 2003; Romani et al. 1996). Non-adherent cells were harvested and pulsed for two days with autologous irradiated tumour cells. DC were matured in monocyte-conditioned medium obtained by culturing autologous monocytes on petri dishes pre-coated with human immunoglobulin (O'Rourke et al. 2003). The final product was cryopreserved until required in RPMI-1640 supplemented with 10 % DMSO (OriGen Biomedical, TX, USA) and 20 % autologous serum. Sterility was confirmed by negative cultures and endotoxin < 0.5 EU/ml in the final supernatant of DC culture. The phenotype of thawed cells was determined by flow cytometry, using FITC-labeled and PE-labeled monoclonal antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). Vaccine production and assessment of products was performed by trained GMP technicians (Evelyn Bauer, Brigitta Mester and Olivier Gasser).

3.2.4 Monitoring

Clinical examination and assessment of full blood count (FBC), serum biochemistry, and autoantibodies was undertaken before the first vaccination, one week after the third priming vaccination, and then every 8 weeks. MRI scans were performed at the same time points and within 48 hours of surgery. An additional FBC was performed prior to each cycle of TMZ. Adverse events (AE) were defined and graded according

to the National Cancer Institute Common Terminology Criteria Version 3 (<http://ctep.cancer.gov/>).

3.2.5 Endpoints

Primary endpoints were: (1) feasibility of using resected tumour tissue from recurrent TMZ-treated GBM patients to generate sufficient DC-based vaccines to complete immunotherapy (measured as the percentage of patients providing 3 'priming' doses of vaccine and 6 'booster' doses); (2) feasibility of completing a minimum course of combined treatment (measured as the percentage of patients completing the second cycle of TMZ); and (3) safety (measured as the percentage of patients undergoing therapy without serious adverse events requiring withdrawal).

Secondary endpoints were: (1) radiological response rate and PFS_{6mo} relative to retreatment with TMZ alone in historical controls; (2) rate of vaccine-induced immune responses; and (3) tolerability of treatment.

3.2.6 Clinical assessment

Tumour size was determined by an independent neuroradiologist and defined as the largest product of two perpendicular diameters on axial or coronal gadolinium-enhanced T1-weighted views (Shah et al. 2006). Classification of responses was based on the WHO UICC system (Miller et al. 1981) *i.e.* complete response (CR), complete disappearance of all lesions for ≥ 4 weeks; partial response (PR), reduction in tumour size ≥ 50 % for ≥ 4 weeks; progressive disease (PD), increase in tumour size ≥ 25 % or appearance of new lesions; stable disease (SD), not PD, PR or CR in comparison with the preceding scan. For CR and PR, scans were compared with maximum tumour size at any point. For PD, scans were compared with the post-operative scan. If patients experienced a CR, PR or SD, subsequent disease progression was determined by comparison with minimum tumour size reached

during the period of CR/PR/SD. New enhancement at the pre-vaccination time point (3 weeks following surgery) was interpreted as reactive change. Otherwise, new enhancement was considered to represent tumour growth. Some patients progressed during the priming vaccination phase but subsequently stabilised or responded after commencing TMZ. Since the aim of the trial was to assess the combined effect of both treatments, time to progression in these patients was defined as the day on which progression next occurred. Treatment was discontinued at time of progression. Tolerability was assessed with a validated quality of life assessment questionnaire (EORTC QLQ-C30 version 3, Aaronson et al. 1993).

3.2.7 Immunological assessment

Vaccine induced immune responses were assessed by DTH responses and by an IFN- γ Enzyme-Linked ImmunoSpot (ELISpot) assay. For DTH tests, 3×10^5 DC loaded with irradiated tumour tissue (*i.e.* an aliquot of the DC vaccine) were injected i.d. 5 days after the initial priming course of three vaccinations, just before the administration of the first cycle of TMZ. Unloaded DC were also injected as a negative control. Tests were read 24 and 48 h later. A positive test was defined as a diameter of induration or erythema > 5mm.

For IFN- γ ELISpot tests, whole blood samples were collected at enrolment, after the first and third vaccinations, and then every four weeks. PBMC were isolated by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway), and cryopreserved in RPMI 1640, supplemented with 20 % foetal calf serum (Sigma-Aldrich, St Louis, MO) and 10 % DMSO until required. All time points from an individual patient were tested on the same day. The PBMC were thawed and rested overnight at 37 °C in RPMI 1640 supplemented with 5 % foetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin (both Invitrogen), and then counted manually using Trypan Blue (Invitrogen) exclusion. Stimulation was with one of the following: autologous tumour cell-pulsed DC; autologous tumour lysate; or pools of

15-mer long peptides covering the full length of TRP-2, gp100, survivin, tyrosinase, and SOX2 proteins (peptides&elephants GmbH, Potsdam, Germany). Earlier studies had found that each of these antigens was expressed in at least one of a panel of five primary GBM cell lines (data not shown).

Tests were conducted using either pre-coated IFN- γ ELISpot 96 well plates (Mabtech, Nacka Strand, Sweden) or PVDF 96 well plates (Merk Ltd., Palmerston North, New Zealand) coated with anti-human IFN- γ (Mabtech) according to the manufacturer's instructions. Blocking was performed with RPMI supplemented with 10 % foetal calf serum, and wells were seeded with $0.5 - 2 \times 10^5$ viable PBMC. For stimulation with tumour cell-pulsed DC, aliquots of vaccine were thawed, rested for 2 hours, and added to wells at different responder cell to stimulator cell ratios. Unloaded DCs were used as controls for non-specific IFN- γ release. For tumour lysate-stimulated wells, irradiated single cell tumour suspensions were washed in PBS and subjected to six freeze-thaw cycles. Lysates equivalent to 1.5×10^5 tumour cells were added to each well. Pooled peptides were added at a concentration of 15 mg/mL. For tests using tumour lysate or peptides, background activity was assessed on wells with PBMC alone. Positive controls were wells of PBMC supplemented with 1 μ g/mL agonistic anti-CD3 mAb, or a pool of MHC class I-restricted viral peptides known to elicit recall responses in 90 % of normal adults (Currier et al. 2002). In this study seven of nine tested patients (78 %) responded to the viral peptides, in line with expectations. Plates were incubated at 37 °C for 48 hours and developed according to the manufacturer's instructions. Spots were counted using an automated ELISpot plate reader (Autoimmun Diagnostika, Strassberg, Germany) subject to visual audit, and spot counts were calculated by subtracting the mean of background wells from the mean of test wells. A positive response to *in vitro* stimulation was defined as a mean spot count greater than the mean + 2 standard deviations (SD) of background control wells, with this count also having to be greater than 50 tumour-specific spots per 10^6 PBMC (Slota et al. 2011). An alteration in a specific immune response over the course of therapy was defined as a greater than two-fold difference from baseline (after subtraction of background

activity) for any antigen, which was sustained over two or more consecutive time points. Background activity was generally low in wells containing PBMC only (median 4 spot forming units/ 10^6 PBMC) and somewhat higher in wells containing PBMC + unloaded DC (median 40). Unusually high background activity was observed in two assays, neither of which met the criteria for the presence of a tumour-specific response. ELISpot assays using antigen-loaded DC or tumour lysate for stimulation were performed by the author. ELISpot assays using peptides for stimulation were performed by Olivier Gasser.

Immune cell populations in the PBMC of trial participants were enumerated before and after vaccination using flow cytometry. PBMCs were labelled with the following anti-human antibodies: CD56-BV605 (HCD56), CD33-APC (WM53), NKG2D-PeCy7 (1D11), CD11b-PE (ICRF44), HLA-DR-PerCP (L243), CD45RA-FITC (HI100), CD3-Alexa Fluor 700 (UCHT1), CD4-BV510 (OKT4), CD8 α -PerCP/Cy5.5 (HIT8a), CCR7-BV605 (G043H7) and CD19-BV510 (HIB19) (all BioLegend, San Diego, CA). FoxP3 staining was performed using an anti-human FoxP3-PE Flow Kit (BioLegend) according to the manufacturer's instructions. Live cells were discriminated using a LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Molecular Probes, Eugene, OR). Data were acquired on an LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ). The author was responsible for experiment design and data analysis. Staining and flow cytometry was performed by Brigitta Mester.

3.2.8 Analysis of MGMT expression

Total RNA was isolated from single cell suspensions of resected GBM tumours using a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany), and cDNA was synthesized with iScript (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Real-time PCR was performed with using *MGMT* and *18s rRNA* primers (QuantiTect primer assay, Qiagen, Hilden, Germany), and SensiMix SYBR Low-ROX kit (Bioline, London, UK) on the ABI 7500 platform (Applied Biosystems, Foster City,

CA). The exponential phase of the amplification curves were used to calculate cycle threshold and each Ct was normalized to 18s rRNA (Δ Ct). Efficiency of amplification of each QuantiTect primer assay is equivalent, so the $\Delta\Delta$ Ct method was used to determine fold change from control. The median MGMT mRNA expression of 29 tumour samples was used as the threshold value to define high and low MGMT mRNA expression. This analysis was performed by Lindsay Ancelet.

3.2.9 Statistics

Feasibility and clinical outcomes are presented as descriptive data with 95 % confidence intervals.

3.3 RESULTS

3.3.1 Feasibility and safety

Fourteen patients were provisionally enrolled over 36 months from February 2009. The entry requirement for prior completion of at least three cycles of adjuvant TMZ was intended to avoid enrolling patients with pseudo-progression (Bartholomae et al. 2004; Chamberlain et al. 2007). One patient (A11) who had just completed the 6th cycle of adjuvant TMZ was found to have a ring-enhancing lesion on MRI typical of recurrence but histology revealed necrosis and a cellular infiltrate dominated by macrophages. This patient was withdrawn from the trial and is not included in the remainder of the analysis. The remaining 13 patients were confirmed histologically to have recurrent GBM. Demographic data and details of previous treatment are summarised in Table 3.1.

Although validation exercises in healthy volunteers suggested that a single leukapheresis procedure should suffice, DC yields were highly variable and often not

Table 3.1 Trial participants: demographic data and previous treatment

Patient	Age	Sex	Histology at first presentation	ECOG performance status at relapse	Cycles of TMZ completed at time of relapse	Time between last TMZ and relapse (months)	Location of recurrent tumour	Histology at re-operation
A01	39	M	GBM	1	6	1.5	R Frontal	GBM
A02	36	F	GBM	1	6	18	R Temp-Par	GBM
A03	64	M	GBM	1	6	0.5	R Temp	GBM
A04	69	M	GBM	1	5	0.5	R Insula	GBM
A05	57	F	GBM	1	6	3.5	R Fronto-Par	GBM
A06	39	M	GBM	2	6	6.5	L Frontal	GBM
A07	52	M	GBM	2	5	<1	R Frontal	GBM
A08	40	F	GBM	2	4	0.5	R Frontal	GBM
A09	30	F	GBM	0	6	0.5	R Frontal	GBM
A10	33	F	GBM	2	6	1	R Temp/BG	GBM
A11	50	F	GBM	2	6	<1	R Temp-Par	Necrosis*
A12	35	M	GBM	1	6	22	L Frontal/CC	GBM
A13	52	F	GBM	2	6	2	R Frontal	GBM
A14	65	M	GBM	2	6	3	L Frontal	GBM

* patient withdrawn from trial

sufficient for the intended regimen. This was addressed either by reducing the dose of the priming vaccinations and/or by harvesting additional PBMC by peripheral venesection. The second PBMC harvest was performed before the first dose of TMZ and was only undertaken when a single venesection was realistically expected to make up the full complement of vaccines. Detail on vaccine yield is shown in Table 3.2.

In three cases (A03, A04, A07) insufficient vaccine was produced after the first PBMC harvest to warrant venesection and these patients were withdrawn. In two cases (A03, A04), age and corticosteroid exposure may have been factors. Although every effort was made to reduce dexamethasone prior to leukapheresis, it proved difficult to wean the drug off completely and these two patients were both still taking 8 mg per day. In the third case (A07), very recent exposure to TMZ was a possible factor. In the remaining 10 patients there was sufficient vaccine to proceed with vaccination, albeit at a reduced dose in two. Three of these required an additional venesection to allow the full course of six booster vaccines to be made. In four cases it was necessary to deviate from protocol and perform urgent surgical debulking before leukapheresis. This did not appear to disadvantage vaccine production (Table 3.2). In summary, accepting that two patients received priming vaccines at lower doses than intended, the proportion of patients in whom vaccine was successfully produced was 77 % (95 % CI: 46 – 95 %). The majority of cells in the final vaccine expressed the dendritic cell marker CD209 (median 89.2 %, IQR 10.3) and were typically CD86⁺ CD83⁺ with little to no expression of CD14. Median viability of the thawed vaccine assessed by Trypan Blue staining in a post-hoc analysis was 82.5 % (IQR 26.6).

In one case (A10), the tumour progressed rapidly after enrolment, with invasion into the basal ganglia detected on MRI on the day of surgery. Only 50 % of the tumour mass could be safely removed. The patient's clinical state continued to rapidly deteriorate after surgery and treatment was discontinued after the first

Table 3.2 Leukapheresis yield and vaccine production

Patient	Age	Months since TMZ*	Dexamethasone		Peripheral blood counts* (x 10 ⁹)		Leukapheresis		Leukapheresis Yield		Venesection Yield	
			Dose* (mg)	Duration [#] (months)	Mono cyte	Lympho cyte	Before Surgery	Access	PBMC (x 10 ⁶)	DCV (x 10 ⁶)	PBMC (x 10 ⁶)	DCV (x 10 ⁶)
A01	39	2.75	0	-	0.46	1.8	Y	Periph	2820	18		
A02	36	18	8	0.75	0.59	1.18	Y	Periph	1308	24		
A03	64	1.5	8	1	0.9	1.3	Y	Periph	3750	0		(a)
A04	69	1	4	0.25	0.52	1.71	Y	Periph	2538	5		(b)
A05	57	4.5	0	0.5	0.42	1.57	Y	Periph	3726	19		
A06	39	7.5	6	16	0.55	0.72	Y	Periph	2502	11	54	9
A07	52	<1	4	0.25	0.7	0.55	Y	Fem V	2528	1		(c)
A08	40	1.5	1	7	0.81	1.33	N	Fem V	2452	24		(d)
A09	30	1.5	2	0.5	1.0	2.0	Y	Periph	3414	33		
A10	33	1.5	4	0.5	0.5	0.7	Y	Fem V	976	14		(e)
A12	35	22	6	0.25	0.57	0.58	N	Fem V	1652	14	366	28
A13	52	2	4	11	0.97	0.93	N	Fem V	2080	47		
A14	65	3	0	-	0.98	0.87	N	Fem V	3300	16	225	10

(a) Tendency of leukapheresis product to clot. Low yield of viable monocyte-derived DC from PBMC. Withdrawn from trial.

(b) Low yield of viable monocyte-derived DC from PBMC. Withdrawn from trial.

(c) Difficult venous access at venesection: low volume harvested

(d) Low yield of viable monocyte-derived DC from PBMC. Withdrawn from trial.

(e) Fragile monocytes, loss of viable cells after washing

* at time of leukapheresis

total duration of steroid treatment prior to leukapheresis, any dose

Laboratory normal ranges: Monocytes 0.1 – 0.8, lymphocytes 0.9 – 3.6

priming vaccination. Thus nine patients completed the defined minimum combined treatment ($9/13 = 69\%$; 95 % CI: 39 – 91 %), hereafter termed “treated patients”.

AE are listed in Table 3.3. There were no grade IV toxicities, premature withdrawals due to treatment-related toxicity, or AE directly attributable to vaccination. Three patients experienced TMZ-related thrombocytopenia requiring dose reductions or delays. One patient with an insular tumour experienced worsening of pre-existing upper limb weakness after the surgical procedure. Self-assessed general health and overall quality of life remained generally stable in the treated patients (Fig 3.1). In summary, other than an acceptable and predictable rate of moderate toxicity related to chemotherapy and surgery, the combined treatment was safe and well tolerated.

Table 3.3: Adverse Events

Adverse Event	Grade	<i>n</i>
Bilateral cataracts secondary to corticosteroid	III	1
Syncopal event	III	1
Thrombocytopenia requiring dose delay	II	1
Thrombocytopenia requiring dose delay	I	1
Thrombocytopenia requiring dose reduction	I	1
Worsening of existing deficit after surgery	II	1
Seizure	II	3
Headache	II	1
Headache	I	1
Vomiting	II	1
Nausea	I	1
Total events in 14 participants		13

Fig 3.1 Responses of treated patients to questions 29 and 30 of the EORTC QLQ-C30 version 3 self-assessment questionnaire. Patients rated their general health (A) and overall quality of life (B) on a scale of 1-7. Shown are individual patients over time (solid lines) and group means (dotted line).

3.3.2 Clinical outcome

A summary of treatment and clinical outcome is presented in Table 3.4. Two patients had a PR (A08, A12) yielding an objective response rate in the nine treated patients of 22 % (95 % CI: 0 - 49 %). In both cases the radiological response occurred after starting retreatment with TMZ. One patient (A01) had a long period of SD lasting 31.5 months. Four additional patients (44 %) experienced brief periods of SD lasting 2-3 months. After priming vaccinations but before TMZ, four patients had SD and five had progressed. The PFS_{6mo} for all enrolled patients with GBM was

Table 3.4 Treatment and clinical outcome in patients who did (top) and did not (bottom) receive combined treatment with vaccination and at least 2 cycles of TMZ

Patient	Extent of resection at reoperation	Vaccine dose (x 10° DC)			Cycles of TMZ received	Radiological Response		Time to Progression (months)	Overall survival from relapse (months)	Overall survival from 1 st diagnosis (months)
		Prime	Boost	Vaccinations Received (Prime+Boost)		DCV priming phase	Combined DCV + TMZ phase			
A01	Total	4	1	3 + 6	6	SD	SD then PD	31.5	40	52
A02	≥ 90 %	4	1	3 + 3	4	PD	SD then PD	5	7	34
A05	≥ 90 %	4	1	3 + 3	4	PD	PD	2	7	20
A06	≥ 90 %	2.6	1	3 + 5	4	PD	SD then PD	6	10	26.5
A08	≥ 90 %	4	1	3 + 6	6	SD	PR then PD	12.3	14	21
A09	Total	4	1	3 + 2	2	SD	PD	3	7.8	17.8
A12	≥ 90 %	4	1	3 + 3	3	PD	PR then PD	5.3	6	36
A13	≥ 90 %	4	1	3 + 2	2	PD	PD	2	7.8	18.8
A14	≥ 90 %	4	1	3 + 1	2	SD	PD	3.5	10.5	23
A03	≥ 90 %			0 + 0					6.5	16.5
A04	≥ 90 %			0 + 0					5	13.8
A07	Total			0 + 0					6.5	27
A10	50 %	3.6	1	1 + 0					2	12

15 % and for the nine treated patients was 22 % (95 % CI: 0 – 49 %). Prolonged progression-free survival was observed in two patients, A01 and A08, one of who also experienced a radiological response (Table 3.4; Figs 3.2 and 3.3). Median survival from the original diagnosis of GBM for the treated patients was 23 months.

Figure 3.2. Magnetic resonance imaging of Trial Participant A01. Gadolinium-enhanced T1-weighted axial MRI head scan at enrolment, immediately after surgery, at pre-vaccination baseline, and at the indicated times after enrolment. After complete resection of enhancing tissue, a region of new enhancement was seen 3 weeks later on the pre-vaccination baseline scan (arrow) that resolved over time leaving an area of gliosis. The possibility that this enhancement was due to reactive change could not be excluded and the patient was categorised as having stable disease.

Figure 3.3. Magnetic resonance imaging of Trial Participant A08. Gadolinium-enhanced T1-weighted coronal MRI head scan at enrolment, pre-vaccination baseline, and 5 months after enrolment, showing partial response after combined treatment. Arrows indicate measureable disease at pre-vaccination baseline.

3.3.3 Immunological assessments

DTH tests were negative in all the treated patients. The IFN- γ ELISpot results are presented in Table 3.5. All patients were tested for responses to tumour-loaded DC and to the panel of peptides from glioma-associated antigens. Insufficient material meant that not all patients could be tested for responses to autologous tumour lysate. Baseline immune responses to antigens in tumour material were observed prior to vaccination in 2 patients [A01, A08]. The magnitude of these responses was higher at some subsequent time points but fell short of reaching the strict criteria for a treatment-induced response (Table 3.5; Figs 3.4 and 3.5). Limited material restricted the number of time points that could be assessed in this way. One of these patients [A01] developed *de novo* responses to the selected tumour-associated antigens, with responses to TRP-2, survivin and SOX-2 being sustained over at least two consecutive time points.

Table 3.5 ELISpot data^a

Patient	Time ^b	Whole tumour ^c		Selected antigens - overlapping peptides ^d				
		Vaccine	Lysate	TRP-2	gp100	Survivin	Tyrosinase	SOX2
A01	0	55*		0	0	0	0	0
	5			120*	0	100*	0	0
	8	0		160**	85*	160**	70*	0
	12			0	0	0	0	20
	20	120*		0	0	30	0	45
	48			0	0	0	0	55*
	80			0	0	100*	0	215**
	100			0	0	0	0	35
A02	0	0		0	0	0	0	0
	5			0	0	0	0	0
	8	0		0	0	0	0	0
	16	5						
	20	5						
A05	0	0		30	0	0	0	0
	5			40	20	0	0	0
	8	0		0	20	0	10	0
	12	0						
	16	0		0	0	0	0	0
	20	0						
A06	0	50		0	0	0	0	0
	5			0	0	0	0	0
	8	10		0	0	0	0	0
	16			0	0	0	0	0
A08	0	0	145*	0	0	0	0	0
	5			0	0	0	0	0
	8	0	270*	0	0	0	0	0
	24			0	0	0	0	0
	32	0	335*					
	44			0	0	0	0	0
A09	0	0	0	0	0	50	0	0
	5			0	30	140*	40	0
	8	0	0	0	0	130**	0	0
A12	0	0	0	0	0	0	0	0
	5			20	0	0	0	0
	8	0	70*	60*	0	0	0	0
	12	0						
	16	0						
	20	0						
A13 [#]	0	0		0	0	0	0	0
	5			0	0	0	0	0
	8	15		0	0	0	0	0
	12			0	0	0	0	0
A14	0	0	0	0	0	120*	0	150*
	5			0	0	0	0	0
	8	0	0	0	0	0	0	0
	12	0		0	0	0	0	0
	16			0	0	0	0	0

^a Mean counts per 10⁶ cells after subtraction of mean count of background wells. Blank = not determined.

^b Time in weeks after initiation of treatment.

^c Stimulation with whole tumour in the form of antigen-loaded DCs (vaccine) or freeze-thawed crude lysate.

^d Stimulation with overlapping 15-mer peptides from selected antigens.

* Meets criteria for anti-tumour immune response.

** Meets criteria for treatment-induced immune response.

[#] Stimulation with lysate assessed but results considered uninterpretable due to wide variation in replicate wells.

Figure 3.4. ELISpot results for trial patient A01. PBMC were stimulated with unloaded DC (in triplicate) or with DC pulsed with autologous tumour cells (single wells per time point due to limited patient material). 2×10^5 PBMC and 6×10^3 DC per well. Positive control wells were stimulated with a viral peptide pool (CEF) or with an agonistic anti-CD3 antibody. The response to loaded DC at baseline is $>$ mean of the unloaded DC control wells $+ 2$ SD. Although the spot count (after subtraction of background) is higher at the 5 month time point, this did not meet the criteria for a treatment-induced response.

Figure 3.5. ELISpot results for trial patient A08. 2×10^5 PBMC were stimulated in duplicate with crude tumour lysate at a ratio of 4 PBMC:3 tumour cells (equivalent). Positive control wells as for Fig 3.4. The response to tumour lysate at baseline is $>$ mean of the PBMC only control wells $+ 2$ SD. Although the spot count (after subtraction of background) is higher at subsequent time points, this did not meet the criteria for a treatment-induced response.

Patients A01 and A08 both experienced long periods of disease stability, despite the fact that both patients expressed high levels of MGMT mRNA in tumour tissue (Fig 3.6). One additional patient, A09, developed a response to survivin over the course of therapy but exhibited only transient SD over the priming phase of immunotherapy. Thus 2 of 9 patients (22 %) met the strict criteria for a treatment-induced immune response (Table 3.5; Fig 3.7).

Fig.3.6. Relative expression of MGMT mRNA in recurrent tumours at time of enrolment. The median MGMT mRNA expression of 29 tumour samples, indicated by line, was used as the threshold value to define high and low MGMT mRNA expression.

Flow cytometric analysis was used to assess whether the presence of suppressive immune cell types at baseline correlated with response to vaccine. In addition to FoxP3⁺ Treg, we also assessed a population of immature myeloid cells associated with reduced responses to a peptide vaccine in patients with premalignant colonic lesions (T. Kimura et al. 2013). Although small sample size did not allow meaningful statistical analysis, it is noteworthy that the two patients who met the criteria for vaccine-induced response had the lowest level of Treg at baseline (as a proportion of CD4⁺ T cells) and one of these also had the lowest level of putative MDSC (Fig 3.8). We also examined other immune cell types over time for evidence of treatment

effects. Interestingly, the two patients with the most favourable clinical course (defined arbitrarily as PFS > 6 months) had a comparatively high proportion of CD8⁺ T cells with effector phenotype at baseline that persisted at subsequent time points (Fig 3.9), a finding consistent with the ELISpot results in these two patients. One of these (A08) also had an unusually high proportion of CD4⁺ effector cells at baseline (14.2 % of total CD4⁺ T cells; mean of other treated patients 2.2 %, range 0.2 – 4.3 %). No consistent trends were observed for the number of total CD4 cells, total CD8 cells, B cells or NK cells, or for selected NK cell activation markers (Fig 3.9; Appendix A, Fig A1; and data not shown).

Fig.3.7. Combined clinical and immunological data. (A) Radiological response, as determined by experienced independent neuroradiologist, and time to progression. (B) Immune response determined by IFN- γ ELISpot, where "pre" is positive ELISpot at baseline, "post" is retention of pre-existing response at at least one time point after treatment, and "induced" meets criteria for treatment-induced response (greater than two-fold difference from baseline for any antigen that was sustained over two or more consecutive time points).

Figure 3.8. Analysis of proportion of Treg and putative MDSC in PBMC of trial participants at the baseline pre-vaccination time point. A, Flow cytometric gating strategy for enumerating regulatory T cells. Left: Representative plot of live CD3⁺ lymphocytes from a trial participant. For each participant the gate for FoxP3⁺ cells was set using the patients' own CD4⁻ population as negative control. Right: The gating strategy was validated by staining PBMC from a healthy volunteer with the same panel minus FoxP3. B, Flow cytometric gating strategy for a subset of CD11b⁺ CD33^{+/int} HLA-DR⁻ putative MDSC. Representative plots from one trial participant. Plot on left was gated first on live PBMC. For each participant the gate for HLA-DR⁻ cells was set using the patient's own HLA-DR⁻ PBMC as negative control. C, Treg and putative MDSC populations in the PBMC of trial participants at the baseline pre-vaccination time-point. Regulatory T cells defined as FoxP3⁺ CD4⁺ CD3⁺ lymphocytes are plotted according to the left hand Y-axis. CD11b⁺ CD33^{+/int} HLA-DR⁻ putative MDSC are plotted according to the right hand Y-axis. Open symbols show the two patients that met the criteria for a vaccine-induced response. Small subgroup size precluded statistical analysis.

Figure 3.9. Analysis of CD4⁺ and CD8⁺ T cell phenotype in PBMC of trial participants. A. Gating strategy for enumerating T cell subsets in PBMC of trial participants. Shown are representative plots of CD4⁺ (left) and CD8⁺ (right) live CD3⁺ lymphocytes. Naïve, effector, effector memory and central memory cells were defined as CCR7⁺CD45RA⁺, CCR7⁺CD45RA⁺, CCR7⁺CD45RA⁻ and CCR7⁺CD45RA⁻ respectively. B. Left: Proportion of CD8⁺ T cells with CCR7⁺CD45RA⁺ effector phenotype. The two “best clinical responders” (defined as progression free survival > 6 months) are compared with the other 7 vaccinated participants. Bars show mean and range. The small subgroup size precluded statistical analysis. Right: Proportion of live CD3⁺ lymphocytes that were CD8⁺ in the same subgroups as A. Bars show means and range.

3.4 DISCUSSION

This phase I trial has shown that retreatment previously TMZ-exposed GBM patients with a combination of surgery, a DC-based vaccine and TMZ is safe and well-

tolerated. Previous studies have demonstrated the safety and feasibility of DC-based immunotherapies for recurrent GBM (reviewed in X. Xu et al. 2012) but this is the first study to explore the feasibility of manufacturing autologous monocyte-derived DC-based vaccines in GBM patients in which previous exposure to TMZ was an entry criterion. Many of the trial participants underwent monocyte harvest within a few weeks of TMZ exposure (Table 3.1). Feasibility of this treatment regimen proved to be marginal. In three cases we were unable to produce sufficient vaccine to proceed with treatment. The reasons for this are not clear but recent exposure to TMZ may have been an important factor. It has been shown that human monocytes, but not DC, are highly sensitive to TMZ-induced apoptosis *in vitro* (M. Bauer et al. 2012). Other possible factors include older age and treatment with dexamethasone at doses greater than 4 mg/day. Glucocorticoids have been shown to induce apoptosis in monocytes *in vitro* in a time and dose-dependent manner (M. Schmidt et al. 1999).

A unique feature of this trial protocol was that leukapheresis was performed prior to surgical debulking. The reason for this was to minimize delay in starting treatment after surgery so that participants could be exposed to both components of the combined regimen when tumor burden was still minimal. In practice, nearly half the treated patients underwent surgery before leukapheresis because of clinical necessity. We saw no statistically significant differences in DC yield or phenotype between these two groups (data not shown). Nevertheless, we cannot rule out the possibility that harvesting monocytes prior to debulking might have compromised optimum yield or function of the final product (Ogden et al. 2006).

Objective responses were observed in 22 % of treated patients with a period of SD in another 56 %. PFS_{6mo} was 22 %. These results are similar to those of Wick and colleagues (A. Wick et al. 2009) who reported objective responses in 17.2 % of patients, SD in 51.7 % and 28.6 % PFS_{6mo} in 15 GBM patients retreated with an MTD regimen of TMZ. Patients with high grade glioma can be prognostically stratified at first presentation using recursive partitioning analysis (RPA) (W. J. Curran et al. 1993) which was recently updated to reflect contemporary practice (Paravati et al.

2011). According to this revised system, all patients in our study were retrospectively assigned to RPA class III at first presentation, a group with a median survival of 21.3 months in that study. Median overall survival for treated patients in our study was 23 months. Thus, although this trial was not designed to assess efficacy as a primary endpoint, the combination of TMZ and DCV does not appear to be more promising than retreatment with TMZ alone.

Despite this disappointing result for the group as a whole, we did find a broad correlation between clinical outcome and anti-tumour immunity. Sustained progression-free survival in two patients was associated with a baseline response and a treatment-induced response in one [A01] and a baseline response that trended upwards in the other [A08]. The fact that these two patients had progressed on, or immediately after, standard TMZ treatment, coupled with relatively high MGMT expression in their tumour tissue, makes it highly unlikely that retreatment with TMZ alone accounted for their better clinical course. It is therefore possible that the vaccine had a chemo-potentiating effect, consistent with the hypothesis underpinning this study. However, when treatments are combined it becomes difficult to assess the contribution of individual components. It is also possible, for example, that the improved outcomes in these two patients were due to the vaccine alone. Both were relatively young, had good performance status and had undergone complete or near-complete radiological resection, factors known to be associated with favourable clinical course after DCV for recurrent GBM (De Vleeschouwer et al. 2012; Linda M Liaw et al. 2005). Other possibilities are that TMZ had a potentiating effect on the booster vaccines (C.-H. Kim et al. 2007; Sampson et al. 2011) or, since an anti-tumour immune response was detected prior to vaccination in both cases, surgical debulking alone may have allowed endogenous responses to become more effective by attenuating tumour-induced immunosuppression.

In one patient, A14, responses to two selected tumour antigens were present at baseline, but became undetectable at subsequent time points, raising the disconcerting possibility that the vaccine had a tolerising effect in this patient.

However, the phenotype of the infused DC in this patient did not reveal any obvious differences in maturation state (levels of CD83 and CD86) compared to the other vaccines generated in the trial. An alternative possibility is that this endogenous response was ultimately overwhelmed by tumour-induced immunosuppressive mechanisms.

To our knowledge this is the first report of a vaccine-induced response to SOX2 in a glioma patient. Normally expressed in developing brain and neural stem cells, this transcription factor is reportedly overexpressed by putative cancer stem cells (CSC) in GBM tumours (Gangemi et al. 2009; Y.-P. Yang et al. 2012). We speculate that the prolonged period of SD observed in patient A01 may have been due to vaccine-induced immune responses targeting the aggressive CSC phenotype.

In conclusion, treating GBM patients relapsing after TMZ with a combination of surgery, autologous DC-based vaccines and further TMZ is safe but is hampered by uncertainty in achieving the required vaccine yield. In contrast, several recent studies have demonstrated the feasibility of integrating DC-based vaccination into standard treatment for newly presenting patients (Ardon et al. 2012; Phuphanich et al. 2012; Prins et al. 2013). Although reported efficacy remains modest, recent advances in our capacity to attenuate tumour-induced immunosuppression and target T cell checkpoint molecules has the potential to considerably improve the efficacy of DC-based vaccines (Sabado and Bhardwaj 2013). Although the findings reported here offer some encouragement for the idea of using immunotherapy to attenuate resistance of GBM tumours to retreatment with TMZ, DC-based approaches seem more likely to achieve their full potential in the newly-presenting setting.

In the recurrent setting, a better strategy might be to use a vaccine that delivers antigen and adjuvant to the patients' endogenous DC. Ideally, such a vaccine would retain many of the theoretical advantages of whole tumour antigen-pulsed DC vaccines including the provision of a broad range of personalised antigens and

applicability to patients of all HLA types. A novel vaccine with these attributes is described in the next chapter.

CHAPTER FOUR: THERAPEUTIC VACCINATION FOR MURINE GLIOMA USING AN ADJUVANT THAT STIMULATES NKT CELLS

4.1 INTRODUCTION

The aim of therapeutic cancer vaccines is to generate a potent systemic anti-tumour immune response with long-lasting memory. Unless APC such as DC are properly licensed to initiate effector T cell responses, vaccination will be ineffective or even risk inducing a state of immunological tolerance (R M Steinman et al. 2000). One way to ensure optimal activity of DCs is to include a potent immune adjuvant in the vaccine. The glycolipid α -GalCer has been shown to specifically stimulate invariant iNKT cells, a population of T cells with innate-like function found in high numbers in spleen, liver and bone marrow (MacDonald 1995). In addition to expressing a variety of cell surface markers typically observed on NK cells, iNKT cells express a semi-invariant $\alpha\beta$ T cell receptor that recognizes glycolipid antigens (including α -GalCer) bound to the non-polymorphic MHC I-like molecule CD1d. CD1d is expressed at high levels on APC including DC, so that inclusion of an iNKT cell ligand such as α -GalCer in a vaccine can induce interactions between DC and iNKT cells that enhance innate and adaptive immune responses (Cerundolo et al. 2009; Fujii et al. 2003; Hermans et al. 2003b).

Anti-cancer vaccines exploiting the adjuvant function of activating iNKT cells with α -GalCer have been shown to be highly effective in animal studies, with long-lasting immunity induced in response to co-administration of tumour antigens and α -GalCer (Chung et al. 2007; C. Hong et al. 2006; Petersen et al. 2010b; Shimizu et al. 2007a). This approach has yet to be tested in a glioma model or in cancer patients. One potential obstacle to clinical translation is the observation that iNKT cell numbers appear to be reduced in many cancer patients (Crough et al. 2004; Giaccone et al. 2002).

Aims of the study:

1. Establish a murine model of glioma
2. Assess the efficacy of a vaccine consisting of irradiated tumour cells pulsed with α -GalCer
3. Characterise the effector arms of the vaccine-induced immune response
4. Assess whether the effectors required for the success of α -GalCer-based vaccines are present and competent in a cohort of typical GBM patients.

4.2 THE GL261 MOUSE MODEL OF GLIOMA

The first aim of this project was to establish a murine model of glioma suitable for assessing active immunotherapies *in vivo*. GL261 is a commercially-available methylcholanthrene-induced murine glioma cell line that can be surgically implanted into the brain of syngeneic, immunocompetent C57BL/6 hosts (Ausman et al. 1970; Wim Maes and Van Gool 2010; Seligman et al. 1939; Szatmari et al. 2006). GL261 is also capable of forming tumours after s.c. implantation. Although genetically-engineered autochthonous models may represent some aspects of human glioma biology more accurately (de Vries et al. 2009; Dranoff 2011; Huszthy et al. 2012), implantable models are more practical and allow progress to be made quickly.

GL261 is considered to be moderately immunogenic (Wim Maes and Van Gool 2010). Vaccines containing peptides derived from the melanoma-associated antigens TRP-2 and gp100 (Prins et al. 2003), or from survivin (Ciesielski et al. 2008), have been shown to have some activity against GL261 but the immunodominant antigens mediating the anti-tumour activity of whole tumour antigen-based vaccines have not been characterised (Grauer et al. 2008).

Figure 4.1. The GL261 mouse model of glioma. A, Photomicrograph of the GL261 cell line growing as an adherent monolayer in standard culture medium. B, Photomicrograph of haematoxylin and eosin-stained section of formalin-fixed paraffin-embedded brain tissue harvested from a C57BL/6 mouse 3 weeks after intracranial implantation of 5×10^3 GL261 tumour cells. Tumour is at left, normal brain parenchyma at right. C, Dose finding study. Mice were implanted intracranially with different numbers of GL261 tumour cells and weighed every 1-2 days. 1 mouse per group. Those mice implanted with 10,000 or 20,000 cells became symptomatic and were euthanased on day 23. The remaining mice were sacrificed on day 24 when they were still symptom-free. D, Coronal sections of brains from same mice depicted in C.

GL261 is customarily cultured in DMEM supplemented with FCS and antibiotics and grows as an adherent monolayer (Fig 4.1A). Intracranial (i.c.) implantation is performed by injecting a single cell suspension into the right striatum of anaesthetised mice using a syringe mounted on a stereotactic frame. On histological examination, i.c. tumours are highly cellular, highly pleomorphic and may contain areas of necrosis (Fig 4.1B). Tumours typically have a well demarcated border and do not exhibit the endothelial proliferation typical of human GBM tumours (Fig 4.1B, see also Fig 5.3E). After sectioning, brain tumours are usually visible to the naked eye (Fig 4.1D).

After obtaining consent from the VUW Animal Ethics Committee, titration studies were performed (Fig 4.1C). Injection of 5000 tumour cells was selected for i.c. implantation experiments because this number led to tumour formation with almost 100 % penetrance in 3-4 weeks, creating a suitable window for testing therapeutic vaccination. Mice were weighed and inspected daily and culled as soon as they developed unequivocal symptoms according to an Ethics Committee-approved grading system. In practice, the first objective symptom in the great majority of mice was 10 % weight loss, so that mice were euthanased before the appearance of other overt symptoms (Fig 4.1C). Subcutaneous implantation was used for some experiments in this study, particularly for large experiments with multiple groups.

4.3 RESULTS

4.3.1 Prophylactic treatment with an α -GalCer-loaded whole tumour vaccine prevents glioma growth in a mouse model.

To generate a vaccine, tumour cells were pulsed overnight with α -GalCer, irradiated, and then washed to remove excess α -GalCer (hereafter termed “Glioma-Gal” vaccine). Because iNKT cells are found predominantly in spleen and liver, the vaccine was delivered i.v. to provide access to these populations. When used in a prophylactic setting, the Glioma-Gal vaccine conferred significant protection against

an s.c. challenge with GL261 tumour cells (Fig. 4.2A). Injection of free α -GalCer, or irradiated tumour cells alone, did not confer any significant protection, suggesting that activation of iNKT cells with α -GalCer co-ordinates an improved response to the tumour antigens in the Glioma-Gal vaccine (Fig. 4.2A and B). The chemotherapeutic agent doxorubicin has been reported to increase the immunogenicity of dying tumour cells (Casares et al. 2005). To exploit this phenomenon, the α -GalCer-pulsed tumour cells were treated with doxorubicin prior to vaccination. While there was a consistent trend for this “Glioma-Gal-Dox” vaccine to provide improved protection (Fig. 4.2C), this did not reach statistical significance over the already highly effective Glioma-Gal vaccine. Regardless, the doxorubicin treated version of the vaccine was used in all i.c. tumour experiments. Accordingly, the Glioma-Gal-Dox vaccine was shown to confer protection against subsequent i.c. tumour challenge (Fig. 1D).

4.3.2 The α -GalCer-loaded whole tumour vaccine is dependent on the adjuvant function of activated iNKT cells.

The activation of iNKT cells was required for the Glioma-Gal vaccine to confer tumour immunity, as protection from tumour challenge was not observed, or was severely attenuated, in CD1d^{-/-} mice that do not have iNKT cells (Fig 4.3A). It has been shown that after i.v. treatment with free α -GalCer, the glycolipid is acquired by resident APCs and then presented via CD1d to activate iNKT cells (Kitamura et al. 1999; Tomura et al. 1999). Activation of iNKT cells provokes an early release of cytokines including IL-4, IL-13 and IFN- γ into the serum. The interaction with APCs is reciprocal, with the activated iNKT cells in turn stimulating the APCs to release IL-12p70. This interaction, which involves CD40/CD40L interactions, also promotes cross-presentation and stimulation of conventional T cells (Fujii et al. 2003; Hermans et al. 2003b). To examine whether a similar profile of intercellular interactions takes place after i.v. administration of Glioma-Gal, serum was collected at different time points after vaccination to analyse cytokine secretion, and

Figure 4.2. Glioma-Gal vaccine prevents glioma growth in a mouse model. A, Mice were vaccinated i.v. with Glioma-Gal cells, treated i.v. with unpulsed irradiated tumour cells or not treated, and then challenged with subcutaneous GL261 7 days later. 4-5 mice per group. One of two experiments with similar results is shown. In WT mice receiving prophylactic vaccination followed by s.c. challenge, mean long term tumour-free survival was 78% over 18 experiments, range 60 - 100% (excepting one outlying experiment in which long term survival was 20%). B, Mice were treated or not treated with 200 ng free α -GalCer i.v. and then challenged with subcutaneous GL261 7 days later. One of two experiments with similar results is shown. C, Mice were vaccinated with Glioma-Gal cells, Glioma-Gal-Dox cells, or not treated, and then challenged with subcutaneous GL261 7 days later. 5 mice per group. One of two experiments with similar results is shown. Two additional s.c. challenge experiments included a prophylactic Glioma-Gal-Dox vaccine arm. Mean long term tumour-free survival over the four experiments was 95%, range 80 - 100%. D, Mice were vaccinated with 5×10^5 Glioma-Gal-Dox cells or not treated and then challenged intracranially with GL261 7 days later. 5 mice per group. One of two independent experiments with similar results is shown.

compared to animals that received free α -GalCer (Fig. 4.3B). As expected, in wild-type mice administered free α -GalCer, high levels of IL-4, IL-12p70 and IFN- γ were detected that peaked at 2, 12 and approximately 24 hours respectively. In contrast, after infusion of Glioma-Gal the systemic release of these cytokines was much lower, and peaked later in the case of IL-4 and IL-12p70. Similar observations have been made by others who have compared the serum cytokine profiles of free *versus* cell-associated α -GalCer (Fujii et al. 2002). Notably, cytokine secretion after Glioma-Gal infusion was absent in serum in CD1d $^{-/-}$ mice, confirming that iNKT activation triggered the cytokine release observed in WT animals (Fig. 4.3C). Although

Figure 4.3. The α -GalCer-loaded whole tumour vaccine is dependent on the adjuvant function of activated iNKT cells. A, CD1^{-/-} mice or their CD1^{+/-} littermates were vaccinated with Glioma-Gal and challenged with GL261 s.c. 7 days later. 5 mice per group. One of 3 independent experiments with similar results is shown. Considering these three experiments together, long term survival was observed in 13/15 (87 %) CD1d-sufficient mice compared with 2/15 (13 %) CD1d-deficient mice ($p < 0.0001$, Fisher's exact test). B, Serum cytokine levels after i.v. administration of 200 ng free α -GalCer, 1×10^5 Glioma-Gal cells, or 1×10^5 irradiated tumour cells; 5 mice per group. Statistical analysis was with the Kruskal Wallis test at each time point, with Dunn's post-test. Results of comparisons with the Irradiated Cells group are shown. C, Serum cytokine levels after 5×10^5 Glioma-Gal cells were administered i.v. to WT or CD1^{-/-} mice; 3-5 mice per group. Statistical analysis was with the Mann Whitney test at each time point. D, Splenocytes were harvested from WT mice 7 days after i.v. infusion of Glioma-Gal vaccine, unpulsed irradiated tumour cells or 200 ng of α -GalCer and replated in 96-well plates in complete media without restimulation. Cytokine levels in the supernatant of splenocyte cultures were assessed 48 hours later; 3 mice per group. Statistical analysis with the Kruskal Wallis test showed no significant differences between groups. E, CD1d surface expression by cultured GL261 tumour cells assessed by flow cytometry. Positive and negative controls are WT CD11c⁺ splenocytes and a fibrosarcoma cell line raised in a CD1^{-/-} mouse. Solid grey: Isotype control. One of two experiments with similar results is shown.

the levels of IL-12p70 were lower after Glioma-Gal compared to free α -GalCer (Fig. 4.3B), the ratio of peak IL-12p70 to IL-4 was higher (approximately 100:1 compared to 3:1), possibly indicating more efficient vaccine-induced APC activation with proportionately lower systemic iNKT cell activation. Cytokines were still being secreted in splenic tissue isolated from mice one week after vaccination, which was not the case in mice that had received free α -GalCer (Fig. 4.3D). All of these observations suggest that α -GalCer is presented in a qualitatively different manner *in vivo* when incorporated into the vaccine. As GL261 tumour cells do not express CD1d (Fig. 4.3E), and are therefore incapable of stimulating iNKT cells directly, other CD1d⁺ cell types must have acquired α -GalCer from the vaccine for presentation to iNKT cells.

4.3.3 The α -GalCer-loaded whole tumour vaccine elicits a CD4⁺ T cell-mediated immune response.

Next, the important effector arms responsible for protection from tumour challenge were defined. Depletion of NK cells after vaccination in the host with antibody to NK1.1 did not abrogate protection (Fig. 4.4A) suggesting that these cells, which are typically activated immediately downstream of iNKT cell stimulation, were not important anti-tumour effectors. Transfer of serum to naïve mice from vaccinated mice that had survived a tumour challenge did not confer protection, indicating that antibodies were not likely to be playing a major role in protection (Fig 4.4B). The Glioma-Gal vaccine was not capable of protecting against challenge with the unrelated Lewis Lung Carcinoma cell line (Fig. 4.5A), indicating that vaccine-induced immunity was tumour-specific. Experiments conducted in animals depleted of specific T cell subsets showed that protection was lost in the absence of CD4⁺ T cells (Fig 4.5B), but retained in the absence of CD8⁺ T cells (Fig. 4.5C). This was confirmed in knockout mice, with protection lost in MHC class II^{-/-} hosts which lack CD4⁺ T cells, and retained in TAP1^{-/-} hosts which lack CD8⁺ T cells (Fig. 4.5D). It was independently shown that iNKT cells are present and able to respond to α -GalCer in

Figure 4.4. Tumour protection after Glioma-Gal vaccination is not mediated by NK cells or circulating antibodies. A. Mice vaccinated with Glioma-Gal on Day 0 were challenged on Day 7 with GL261 s.c. Some mice were also treated with NK1.1-depleting antibody on Days 4, 6, and then every 3-4 days until the completion of the experiment. 5 mice per group. One of two independent experiments with similar results is shown. B. 200 μ L of serum from naïve mice or from Glioma-Gal vaccinated mice that had survived a tumour challenge 7 weeks previously were injected i.v into naïve mice that were challenged the following day with GL261 s.c. 5 mice per group. One of 2 independent experiments with similar results is shown.

these MHC II^{-/-} mice (Appendix B, Fig B3.1). As further supporting evidence for a lack of significant involvement of CD8⁺ T cells in this vaccination strategy, protection was retained in the absence of langerin⁺ antigen-presenting cells (Fig. 4.5E), a cell type that has recently been shown to be critically involved in cross-presentation of blood-borne antigens to CD8⁺ T cells (Farrand et al. 2009; C.-H. Qiu et al. 2009).

Given the requirement for CD4⁺ T cells in conferring protection, it is notable that expression of MHC class II molecules by GL261 was negligible in the steady state, but induced after a brief exposure to IFN- γ (Fig. 4.5F). It is therefore at least possible that CD4⁺ T cells directly target tumour cells via MHC class II, as has recently been suggested (Quezada et al. 2010).

Figure 4.5. The α -GalCer-loaded whole tumour vaccine elicits a CD4⁺ T cell-mediated immune response. A, Glioma-Gal vaccinated mice were challenged s.c. 7 days later with 1×10^6 Lewis Lung Carcinoma cells. One of three independent experiments with similar results is shown. B, Mice vaccinated with Glioma-Gal on Day 0 were challenged s.c. on Day 7 with GL261. Some mice were also treated with CD4-depleting antibody on Days 2 and 4 and then every 3-4 days. 5 mice per group. One of two independent experiments with similar results is shown. Over two experiments, long term tumour-free survival was observed in 1/10 depleted mice compared with 9/10 undepleted mice ($p < 0.01$, Fisher's exact test). See Appendix B, Fig B2.2 for validation of depletion method. C, Mice vaccinated with Glioma-Gal on Day 0 were challenged s.c. on Day 7 with GL261. Some mice were also treated with CD8-depleting antibody on Days -2 and -1 and then every 3-4 days until the completion of the experiment. 5 mice per group. One of three independent experiments with similar results is shown. Over three experiments, long term survival was observed in 10/15 (67 %) mice in both depleted and undepleted groups. See Appendix B, Fig B2.3 for validation of depletion method. D, WT, MHC class II-deficient, or TAP1-deficient mice were challenged s.c. 7 days after Glioma-Gal vaccination. 5 mice per group. The ability of GL261 to form tumours in naive TAP1-deficient mice was confirmed separately (data not shown). E, Langerin-DTR mice were depleted of Langerin⁺ cells by administering Diphtheria toxin (DT) on Days -2, -1, 1 and 3, and were challenged s.c. on Day 7. Some mice were vaccinated with Glioma-Gal on Day 0. One of two independent experiments with similar results is shown. F, Surface expression of MHC class II by GL261 *in vitro*. Solid grey: expression after culture in normal media; Solid line: after 48 hours exposure to IFN- γ ; Broken line: isotype staining of IFN- γ treated cells. One of two independent experiments with similar results is shown.

4.3.4 The α -GalCer-loaded whole tumour vaccine primes adaptive responses in the lung-draining lymph nodes.

Interestingly, the Glioma-Gal vaccine was fully effective in conferring protection against tumour challenge after splenectomy (Fig. 4.6A). Thus, this lymphoid organ was not required for effective immunity. Different anatomic sites were therefore screened for cytokine production to identify possible alternative locations of antigen-presenting cells mediating the protective immune response. Significant levels of cytokine production were detected in the mediastinal lymph nodes (MdLNs) of vaccinated mice, but not in inguinal LN or liver (Figs. 4.6B and C). There was an increase in the overall size of MdLNs and a significant increase in the proportion of CD4⁺ T cells expressing the activation marker CD44 in the MdLNs of immunized mice that was not seen in inguinal LN (Fig. 4.6D and E). In addition, we observed an increase in the proportion of MHC II⁺ CD11c⁺ cells in the MdLNs of vaccinated mice (Fig. 4.6F). It is possible, therefore, that the infused tumour cells are filtered by the lung capillary bed where they are taken up by other lung-resident or circulating cells that traffic to lung-draining LN.

4.3.5 The α -GalCer-loaded whole tumour vaccine is effective against established intracranial tumours but requires depletion of regulatory T cells.

The efficacy of the Glioma-Gal vaccine was tested in a therapeutic setting where tumours were implanted i.c. and mice vaccinated six days later. Whereas the vaccine provided effective protection against i.c. tumours in a prophylactic model (see Fig. 4.2D), in a therapeutic setting only minimal protection, or no protection, was seen after vaccine alone (Figs. 4.7A and B). However, in line with several previous studies of glioma immunotherapy in mice (Fecci et al. 2006b; Grauer et al. 2008; Wim Maes et al. 2009b), we found that depletion of Treg at the time of vaccination resulted in effective treatment, with up to 60 % long-term survival after a single vaccination (Figs 4.7A-C). The survival benefit could not be explained by Treg depletion alone

Figure 4.6. The α -GalCer-loaded whole tumour vaccine primes adaptive responses in the lung-draining lymph nodes. A, Splenectomised mice were vaccinated with Glioma-Gal, or mice were not vaccinated, and challenged s.c. 7 days later with GL261. 5 mice per group. B, *In vitro* cytokine secretion without further stimulation by cells harvested from mice vaccinated 7 days previously, or from naive mice, as in Fig 4.3D. MdLN = mediastinal lymph node; IngLN = inguinal lymph nodes; Liver = liver lymphocytes. Shown are means (+ SEM) of results from 3 vaccinated and 3 naive mice. Statistical comparison of vaccinated and naive groups was performed at each site using the Mann Whitney test. No significant differences were observed. C, Experiment similar to B. *In vitro* cytokine secretion by cells harvested from the MdLN and inguinal LN of mice vaccinated with 5×10^5 Glioma-Gal 7 days

previously, or from naïve mice. 4-5 mice per group. Statistical analysis at each site was with the Mann Whitney test. D, Absolute number of live cells in MdLN and inguinal LN from naïve mice and from mice vaccinated 7 days previously with 5×10^5 Glioma-Gal. Plots show individual mice. Pooled data from 4 experiments all showing the same trend. Statistical analysis at each site was with the Mann-Whitney test. D, CD44 expression by $CD3^+CD4^+$ cells in MdLN and inguinal LN from naïve mice or from mice vaccinated 7 days previously with Glioma-Gal, assessed by flow cytometry. C. Representative histograms (left) and summary of 4-5 mice per group (right). Statistical analysis was with the Kruskal Wallis test with Dunn's post-test. One of 2 independent experiments with similar results is shown. E, Flow cytometry of MdLN cells from naïve mice and from mice vaccinated 7 days previously with Glioma-Gal, gated on live $B220^-$ cells. Complete gating strategy is shown in Appendix A, Figure A3. Concatenated plots (above) and summary of 3 mice per group (below). Statistical analysis was with the Mann Whitney test. One of two independent experiments with similar results is shown.

Figure 4.7. The α -GalCer-loaded whole tumour vaccine is effective against established intracranial tumours but requires depletion of regulatory T cells. A-D. Mice were implanted intracranially with 5×10^3 GL261 cells and vaccinated 6 days later with 5×10^5 Glioma-Gal-Dox cells (Vaccine). Some mice also received PC61, an anti-CD25 depleting antibody, i.p. on the day of tumour implantation (Vaccine + Treg depletion). Some mice were only treated with PC61 (Treg depletion). 4 independent experiments are shown. 4-7 mice per group. Over 3 experiments (A,B,C) mean long-term tumour-free survival was 43%. E, Gadolinium-enhanced T1-weighted MRI scans of a vaccinated mouse from the experiment depicted in C at 20 days (left) and 81 days (right) post-implantation.

(Figs. 4.7B and D). MRI scans at 20 days post-implantation (14 days post-vaccination) showed enhancing mass lesions in most vaccinated mice that were radiologically similar to, but smaller than, those seen in unvaccinated mice (Fig. 4.8), and complete resolution of the enhancing lesion was documented in one vaccinated mouse that exhibited long-term survival (Fig. 4.7E). To confirm that the radiological abnormalities in the vaccinated mice were tumours, some vaccinated mice were sacrificed on Day 20 for histological examination (Fig. 4.8). Overall, these data confirm that the vaccine is effective in prolonging survival and, in some cases, eradicating established i.c. tumours.

4.3.6 Patients with GBM have functional iNKT cells that can respond to α -GalCer-loaded GBM tumour cells in vitro.

To succeed in the clinic, this vaccination strategy would require iNKT cells to be functional and present in sufficient numbers in glioma patients. Invariant NKT number and function was therefore assessed in the peripheral blood of 38 histologically-confirmed GBM patients. Of these, 84 % were newly-presenting and 95 % were taking the corticosteroid dexamethasone at the time of blood donation (mean daily dose 13.7 mg). No patients had received any form of chemotherapy within six months. Because iNKT cell counts decline with age (Peralbo et al. 2007) the analysis in GBM patients was compared to age-matched healthy controls. Flow cytometry with fluorescent α -GalCer-loaded CD1d tetramers showed that the proportion of circulating T cells that were iNKT cells was actually slightly increased in the patient group (Fig. 4.9A). However, as previously described (Brooks et al. 1977), the GBM patients were lymphopaenic relative to healthy controls (Fig 4.9B), so that the absolute iNKT cell counts were not significantly different between the two groups (Fig 4.9C). In this analysis, it was also possible to examine CD1d expression in some of the major antigen-presenting cell populations in the blood that are capable of presenting α -GalCer to iNKT cells. Patients had reduced numbers of circulating myeloid DCs (Fig 4.9D) but monocyte numbers were preserved (Fig

Fig 4.8. Therapeutic Glioma-Gal vaccination prolongs survival by eradicating established tumours or by suppressing tumour growth. A, Mice were implanted i.c. with 5×10^3 GL261 cells and vaccinated 6 days later with 5×10^5 Glioma-Gal-Dox cells. Anti-CD25 depleting antibody (PC61) was administered i.p. on the day of tumour implantation. Symptom-free survival for this experiment is shown in Fig 4.7C. T1-weighted (post-gadolinium) and T2-weighted MR images were obtained on Day 20 post-implantation (14 days post-vaccination). Mouse #7 is the same as that depicted in Fig 4.7E. B, In a separate experiment, mice underwent intracranial tumour implantation followed by therapeutic PC61 + Glioma-Gal-Dox vaccination as in A. Two asymptomatic mice with enhancing mass lesions on Day 20 MRI scans were culled on the same day as the scan. A representative haematoxylin and eosin-stained section of formalin-fixed, paraffin-embedded brain tissue is shown, confirming the presence of tumour.

Figure 4.9. The cell populations required for α -GalCer to be an effective adjuvant are preserved in GBM patients. A, Flow cytometry of peripheral blood from patients with histologically-proven GBM and from an age-matched population of healthy volunteers, showing iNKT cells as a percent of total CD3⁺CD19⁺ lymphocytes. Mean ages of the patient and control groups were 56.1 years (range 44-74) and 58.6 years (range 27-78) respectively. Gating strategy is shown in Appendix A, Figure A4. B, Automated lymphocyte counts in the peripheral blood of the same subjects as in A. C, Absolute iNKT cell number in GBM patients and an age-matched population of healthy volunteers, estimated by multiplying % iNKT of total lymphocytes (on flow cytometry) and a contemporaneous automated peripheral blood lymphocyte count. Mean ages of the patient and control groups were 59.8 (range 44-74) and 61.1 (range 48-70) years respectively. D, Absolute number of myeloid dendritic cells (mDC) in peripheral blood from GBM patients and healthy volunteers (all subjects with sufficient PBMC available for FC), estimated by multiplying % mDC of total live PBMC (on flow cytometry) and a contemporaneous automated peripheral blood count of lymphocytes + monocytes. Myeloid DC were defined as lineage marker low, CD11c high, HLA-DR high cells. Mean ages of the patient and control groups were 58 and 64 years respectively. Gating strategy is shown in Appendix A, Figure A5. E, Automated monocyte counts in the peripheral blood of the same subjects as in A. F, CD1d expression on circulating mDC and CD14⁺⁺ monocytes in the peripheral blood of a subgroup of GBM patients and healthy volunteers, assessed by flow cytometry. Gating strategy for identifying CD14⁺⁺

monocytes is shown in Appendix A, Figure A6. E, PBMC from GBM patients and healthy volunteers were cultured *in vitro* in the presence of α -GalCer for 7 days. Absolute iNKT cell number before and after co-culture (left and centre); fold change in absolute iNKT cell number (right). In G, statistical analysis was performed using a Wilcoxon matched pairs test. A Mann Whitney test was used for all other comparisons in this figure.

4.9E) and CD1d expression by both cell types was equivalent to cells from healthy controls (Fig. 4.9F). Furthermore, populations of iNKT cells could be expanded from the PBMC of both patients and healthy controls in response to stimulation with α -GalCer *in vitro*, albeit to a slightly reduced level in patients (Fig. 4.9G). Thus the iNKT/DC axis appears to be sufficiently intact in GBM patients to allow an α -GalCer-based vaccine to be effective.

To confirm this, and to examine whether the action of the Glioma-Gal vaccine could be modelled with human cells *in vitro*, α -GalCer was loaded onto primary tumour cell lines derived from five GBM patients, which were then irradiated, washed, and co-cultured for 5 days with CFSE-labeled PBMC from a healthy donor. Proliferation of iNKT cells was observed in all cases, as represented by a loss of fluorescence of CFSE on cells identified with α -GalCer-loaded CD1d tetramers (Fig. 4.10A and B). Proliferation of tetramer-negative cells was also observed, which occurs in this assay because of HLA-mismatch between tumour cells and donor PBMC. In one case this allo-response was significantly enhanced when the tumour cells had been pulsed with α -GalCer, providing “proof-of-principle” that a vaccine based on α -GalCer-loaded tumour cells has the potential to enhance proliferation of non-CD1d restricted T cells as well as iNKT cells (Fig. 4.10A and B). The ability of α -GalCer-pulsed tumour cells to stimulate iNKT cells in these assays did not require the tumour to express CD1d because, like the murine GL261 line, no surface staining of CD1d was seen on any of the primary GBM lines tested (Fig 4.10C).

Finally, it was shown that the capacity of GL261 to express MHC II molecules after exposure to IFN- γ was shared by human primary GBM cell lines (Fig. 4.10D) which may therefore also be susceptible to direct targeting by CD4 T cells.

Figure 4.10. Invariant NKT cells can respond *in vitro* to α -GalCer-pulsed GBM tumour cells. A, GBM cell lines were pulsed or not pulsed overnight with α -GalCer, then irradiated, washed and co-cultured for 5 days with CFSE-labelled PBMC from an allogeneic healthy volunteer. As a measure of proliferation, % CFSE cells of total cells was measured by flow cytometry. Shown are means and SEM of triplicate wells for iNKT cells (above) and tetramer-negative T cells (below). Asterisks show the results of statistical analysis using 2 way ANOVA with Bonferroni multiple comparison post-tests. B, Representative flow cytometry plot of cell line 10/05 from experiment in A, gated on live CD3⁺CD19⁻ lymphocytes. C, CD1d surface-staining by GBM cell lines assessed by flow cytometry. Solid grey: Isotype; solid line: CD1d antibody. C1R-CD1d = CD1d-transfected cell line for positive control. D, MHC class II staining by GBM cell lines. Solid grey: expression after culture in normal media; Solid line: after 48 hrs exposure to IFN- γ ; Broken line: isotype staining of IFN- γ treated cells.

4.4 DISCUSSION

This study has shown that a simple vaccine composed of irradiated autologous tumour cells pulsed with α -GalCer, an adjuvant that stimulates iNKT cells, is an effective treatment against tumours in a murine glioma model. 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. While evidence of cellular activation was observed in the spleen, the mediastinal lymph node was shown to be an important anatomical site of T cell priming. It has also been shown that the principal effector arms necessary for the success of such a vaccine are present and functional in a typical cohort of patients with high-grade glioma.

In some animal models α -GalCer has been shown to have significant anti-tumour effects when injected intravenously as a sole agent (E. Kobayashi et al. 1995). These effects were shown to be largely due to stimulation of the innate immune system, particularly NK cells (Hayakawa et al. 2002), but enhancement of spontaneous adaptive responses were also observed in some tumour-bearing animals (Carnaud et al. 1999; Nishimura et al. 2000). However, most clinical trials in human cancer patients, using either free α -GalCer or α -GalCer loaded onto DC, have achieved limited clinical benefit despite evidence of iNKT activation (Cerundolo et al. 2009; Richter et al. 2013; Schneiders et al. 2011; van den Heuvel et al. 2011). In all of these clinical trials α -GalCer has been used without co-administration of antigen. Thus the adjuvant function of stimulating iNKT cells has yet to be tested directly in patients.

In animal models it has been reported that simple co-administration of α -GalCer with microbial products (Gonzalez-Aseguinolaza et al. 2002) or soluble antigens (Fujii et al. 2003; Hermans et al. 2003b) can significantly enhance antigen-specific T cell- and antibody-mediated responses. There have also been previous reports that injection of α -GalCer with whole tumour cells can provoke effective anti-tumour responses, although different effector arms were invoked depending on the model studied. As reported here for glioma, a previous report of an α -GalCer-pulsed

lymphoma cell vaccine showed that efficacy was dependent on CD4⁺ T cells, and did not require CD8⁺ T cells (Chung et al. 2007). Similarly, CD4⁺ T cells could independently mediate full immunity to a vaccine consisting of irradiated B16 melanoma cells administered i.p. with free α -GalCer, although CD8⁺ T cells were important effectors in fully T cell-replete animals (C. Hong et al. 2006). In contrast, it was reported that the anti-tumour activity of an α -GalCer-pulsed live B16 tumour cell vaccine was abrogated in both MHC class I^{-/-} and MHC class II^{-/-} animals (Shimizu et al. 2007a). These contradictory findings could be reconciled if the type of response induced is a consequence of the tumour antigens presented and/or the timing and manner in which the infused tumour cells die. Shimizu and colleagues' mechanistic analysis of their live tumour cell vaccine showed that the injected α -GalCer-loaded tumour cells were killed by iNKT cells and NK cells, thereby providing a reservoir of antigens for uptake by resident antigen-presenting cells, with CD8 α ⁺ DCs in spleen being important for cross-presentation (Shimizu et al. 2007a; Shimizu et al. 2007b). This mode of antigen delivery could be enhanced by transducing the tumour cells to express higher levels of CD1d, thereby making the injected cells more susceptible to attack by iNKT cells. In contrast, in the glioma study presented here, the tumour cells in the Glioma-Gal vaccine were CD1d-negative and, as a consequence of γ -irradiation, were likely to already be in the process of dying. These different pathways to cell death are likely to result in differences in the expression of danger-associated molecular patterns and ligands for phagocytosis receptors, and these in turn may determine the location, subset and maturation state of the antigen-presenting cells that take up the tumour cells and shape the immune response.

The manner in which α -GalCer is acquired from the vaccine and presented to iNKT cells *in vivo* may also have a significant bearing on the outcome of vaccination. Irradiated whole tumour cells pulsed with α -GalCer may be an efficient way of delivering "antigen plus adjuvant" to the same phagocytic cell. It has previously been shown that a vaccine composed of DC pulsed with antigen and α -GalCer remains effective even when the infused DC are CD1d-deficient, demonstrating that the

glycolipid must be transferred to host cells (Petersen et al. 2011). As tumour cells in the Glioma-Gal vaccine do not express CD1d, transfer of α -GalCer to host APC must also occur. Quite how α -GalCer becomes physically associated with tumour cells in the Glioma-Gal vaccine is not clear. One possibility is that the lipophilic sphingosine tail of the molecule adheres to, or becomes integrated into, the plasma membrane (Moody et al. 2002). While only modest amounts of glycolipid are likely to be incorporated, analysis of the cytokines released *in vivo* following vaccination suggests that, consistent with a previous report comparing free α -GalCer with α -GalCer pulsed onto DC (Fujii et al. 2002), stimulation of iNKT cells by tumour cell-associated α -GalCer is sustained over many days, perhaps suggesting slow release of vaccine material from an anatomical or cellular site of sequestration.

Because the Glioma-Gal vaccine was infused into the circulation, a role for antigen-presenting cells in the spleen, and for CD8 α^+ DC in particular, was anticipated as these APC have been shown to have a selective capacity to take up and present antigen from circulating dying cells (Iyoda et al. 2002; Shimizu et al. 2007a). Surprisingly, in the model used in this study splenic DC were redundant. Instead the evidence suggested that the MdLN was an important site of T cell priming. Two major CD11c $^+$ DC subsets have been described in the lung with recent studies suggesting the two populations have distinct functional roles (Jakubzick et al. 2008). CD11b $^{\text{lo}}$ CD103 $^+$ DCs, which are also langerin $^+$, are specialized in presenting antigen to CD8 $^+$ T cells. In contrast, CD11b $^{\text{hi}}$ CD103 $^-$ DCs preferentially induce proliferation of CD4 $^+$ T cells (del Rio et al. 2007; Jakubzick et al. 2008). Given that anti-tumour responses to Glioma-Gal were not abrogated when langerin $^+$ cells were depleted, we speculate that apoptotic tumour cells of the vaccine lodging in the pulmonary capillary bed might be preferentially taken up by CD11b $^{\text{hi}}$ CD103 $^-$ DCs that in turn generate the predominantly CD4 $^+$ T cell-mediated response observed. However, a role for other cell types of the monocyte/macrophage/DC lineage, either circulating in the blood or resident in the MdLNs, cannot be excluded. It was recently shown that CD169 $^+$ macrophages in the subcapsular sinus of LN are able to take up α -GalCer and activate iNKT cells (P. Barral et al. 2010). It has also been shown that

these cells have the capacity to phagocytose dead tumour cells arriving via afferent lymphatics, and act as antigen-presenting cells to initiate anti-tumour immune responses (Asano et al. 2011).

The CD4⁺ T-cell-mediated mechanisms responsible for anti-tumour immunity remain to be established. It is likely that CD4⁺ T cells act at the tumour site, either indirectly by secreting cytokines and promoting the anti-tumour effects of innate cells, or by a direct granzyme B-dependent cytolytic mechanism, as recently described (Quezada et al. 2010). With respect to the latter possibility, MHC class II expression can be induced by IFN- γ in GL261 cells (and in human GBM cell lines), thereby providing a plausible mechanism by which “cytotoxic CD4 T cells” might recognize tumour cells.

There is evidence that Treg play a major role in mediating glioma-induced immunosuppression, both in human patients and in mouse models (Dix et al. 1999; Fecci et al. 2006a; Fecci et al. 2006b; Raychaudhuri et al. 2011). In our model, established tumours were resistant to therapeutic vaccination unless supported by treatment with an anti-CD25 depleting antibody. Attenuation of immunosuppressive mechanisms is likely to be a vital component of successful glioma immunotherapies in the future.

Whole tumour preparation-based vaccines have some theoretical advantages over vaccines directed at one, or a few, predetermined antigens. There is still a paucity of identified glioma-associated antigens that are consistently expressed and/or capable of eliciting strong anti-tumour responses. For this reason, vaccines based on whole autologous tumour extracts have been the most common immunotherapeutic strategy tested in glioma patients. Other theoretical benefits of personalized whole tumour-based vaccines include applicability to all HLA types, the provision of both MHC class I and II epitopes, and the stimulation of a broad polyclonal response that reduces the risk of immune-escape. Most human studies of whole tumour-based glioma vaccines have involved loading antigen onto autologous monocyte-derived DC. However, as in the clinical trial described in Chapter 3, generating DC from

patients with advanced disease can be problematic, and there are many uncertainties surrounding the optimum phenotype of the infused DC (Melief 2008). There is also increasing evidence that antigen-pulsed DC administered as a vaccine may simply serve as couriers that deliver antigen to endogenous APC (Kleindienst and Brocker 2003; Petersen et al. 2011). Thus, a vaccine that aims to deliver whole tumour-derived antigens to endogenous DC may be a better approach than classical DC-based therapy, and intact autologous tumour cells constitute a ready-made vehicle for this purpose.

The use of irradiated whole tumour cells in anti-cancer vaccines raises some theoretical concerns such as the possibility of inducing auto-immunity, particularly if the treatment also includes deliberate attenuation of self-tolerance. However, immune cell infiltration was not seen in the normal brain parenchyma of treated mice in this study (data not shown). Another concern is the risk of acute toxicity associated with the i.v. infusion of apoptotic tumour cells. Toxicity encountered in this study at higher doses of the vaccine was effectively abrogated by pre-treating mice with i.v. heparin, suggesting that tissue factor or another procoagulant factor was probably responsible (Bastida et al. 1984; Fernandes et al. 2006).

Reduced numbers of iNKT cells and functional iNKT cell defects have been reported in patients with advanced solid cancers (Crough et al. 2004; Giaccone et al. 2002; Tahir et al. 2001) that could impact on the utility of α -GalCer as a useful immune adjuvant. However, GBM appears to be an exception in this regard. A small study of iNKT cells in the blood of nine patients with gliomas of various grades (Kavita M Dhodapkar et al. 2004) reported no difference in iNKT cell function or number (expressed as percentage of total lymphocytes) compared to control subjects. Here a much larger and more homogeneous group of patients was examined, with iNKT cell number and function compared to age-matched healthy controls. All patients had a histological diagnosis of GBM, and the great majority was taking moderate to high doses of lymphotoxic corticosteroid. Importantly, we found no difference in the absolute iNKT cell count in peripheral blood in patients compared to controls, even

though the patients were relatively lymphopaenic. The relative sparing of iNKT cells in patients might reflect a greater resistance of this cell type to corticosteroid. Some deficiencies in the iNKT/DC axis were detected, with mild impairment of iNKT cell expansion and reduced numbers of circulating myeloid DC. However, circulating CD14⁺ monocytes, known to be capable of differentiating into mature DC (Auffray et al. 2009; L. J. Zhou and Tedder 1996), are present in normal quantities in these patients and express normal levels of CD1d. Taken together, these data suggest there are no major defects in the iNKT/DC axis in patients with high-grade glioma and that α -GalCer-pulsed irradiated autologous glioma cells could be an effective vaccine in human patients.

CHAPTER FIVE: TARGETING CANCER STEM CELLS TO IMPROVE IMMUNOTHERAPY FOR GLIOMA

5.1 INTRODUCTION AND AIMS

5.1.1 Introduction to Cancer Stem Cells

In recent decades, major improvements in outcome have been realised for some forms of cancer. For patients with GBM, the prognosis remains bleak. One of the reasons for relapse after cancer treatment is that tumours are heterogeneous, consisting of diverse tumour cell phenotypes with differing susceptibilities to treatment (Marusyk et al. 2012). The “clonal selection” theory attributes this heterogeneity to random genetic and epigenetic events (Nowell 1976). The “cancer stem cell” (CSC) theory offers a fundamentally different explanation for tumour heterogeneity that has attracted intense interest as a plausible explanation for the failure of conventional therapy and a framework for the design of new treatments (Dick 2008; J. C. Y. Wang and Dick 2005).

The CSC model was first described in the context of haematological malignancies and is still most firmly established in this field. Dick and colleagues (Bonnet and Dick 1997; Lapidot et al. 1994), sorted peripheral blood leucocytes from patients with acute myeloid leukaemia and showed that only a small subset of leukaemic cells was able to re-establish the human leukaemia when transplanted into immunodeficient mice. Limiting dilution studies showed the frequency of the leukaemia-initiating cell to be 1 in 250,000 (Lapidot et al. 1994). These cells resided within a CD34⁺⁺CD38⁻ population that constituted less than 0.2 % of leukaemic cells (Bonnet and Dick 1997). After transplanting purified CD34⁺⁺CD38⁻ cells into mice, cells with this surface marker phenotype made up only 2 % of the reconstituted human leukaemia cells in the recipients, indicating an ability of these cells to give rise to the more differentiated progeny making up the bulk of the leukaemic population, while at the same time maintaining their own numbers. Because

leukaemic blasts making up the bulk tumour population have limited proliferative capacity, further evidence of the ability of these “leukaemia stem cells” to “self-renew” was provided by the ability to serially transplant the leukaemia to secondary recipient mice (Bonnet and Dick 1997). These findings together implied the existence of a hierarchy within the leukaemic cell population reminiscent of normal haematopoiesis, in which a small subset of oncologically-transformed self-sustaining stem cell (SC)-like cells have the capacity to give rise to a large number of more differentiated cells with a finite capacity for proliferation. Following the initial description of CSC in leukaemia, putative CSC have subsequently been described in several solid tumours including breast cancer (Al-Hajj et al. 2003), glioma (S. K. Singh et al. 2003), colon cancer (O'Brien et al. 2007), prostate cancer (Collins et al. 2005) and melanoma (Monzani et al. 2007; Schatton et al. 2008). There is also evidence that CSC are particularly resistant to chemotherapy and radiotherapy (Bao et al. 2006a; J. Chen et al. 2012). The concept of a small subset of quiescent tumorigenic cells that are particularly resistant to treatment provides an attractive explanation for late relapse in cancer patients, and implies that progress towards better outcomes will require an understanding of CSC biology rather than measuring the success of novel treatments by the extent to which they reduce the bulk tumour population (Reya et al. 2001).

5.1.2 Evidence for CSC in glioma

In 1992 Reynolds and Weiss cultured adult murine brain cells in specialised media that was serum-free and supplemented with EGF, bFGF and other factors that together were intended to promote proliferation but inhibit differentiation (B. A. Reynolds and Weiss 1992b). The surviving cells lacked lineage markers and grew as non-adherent spheroidal aggregations that they termed neurospheres. When placed back into media containing serum, these sphere cells gave rise to more differentiated cells expressing neuronal and glial markers. This study showed for the first time that multipotent neural stem cells (NSC) persist in the adult mouse

brain and the “neurosphere assay” has become widely adopted as a method of detecting, selecting, and enriching for neural stem cells. In 2004 Sanai and colleagues identified NSC in the subventricular zone and hippocampus of the adult human brain (Sanai et al. 2004).

In 1997, Miraglia et al discovered CD133, a surface protein with a unique structure consisting of 5 transmembrane domains that was found to be expressed on CD34⁺ haematopoietic stem cells and on some leukaemic cells (Miraglia et al. 1997). Uchida and colleagues subsequently used an antibody to CD133 to directly isolate neural stem cells from human foetal brain tissue (N. Uchida et al. 2000).

In 2003, Peter Dirks and colleagues first reported the presence of “Brain Tumour Stem Cells” in human primary brain tumours (S. K. Singh et al. 2003). Using a serum-free media supplemented with growth factors similar to that described by Weiss and Reynolds, they found that dissociated human primary brain tumour cells grew as non-adherent aggregations resembling neurospheres. The sphere cells expressed CD133 and nestin, an intermediate filament transiently expressed by NSC in the developing CNS (Lendahl et al. 1990). When placed into serum-containing media, the cells grew as an adherent monolayer, lost expression of CD133 and nestin, and began to express glial or neuronal markers depending on the original tumour type. After magnetic bead sorting, only the CD133⁺ cells were able to form spheres *in vitro*. Thus culture in SC media selected for, or induced, a more stem cell-like tumour cell. In 2004, two other groups reported that sphere cells derived from human GBM formed tumours when injected orthotopically into immunodeficient mice (Galli et al. 2004; Yuan et al. 2004) whereas adherent cells grown in standard media containing foetal bovine serum did not (Yuan et al. 2004). These early studies represent the beginning of the common practice of equating the neurosphere assay with the “isolation” of CSC.

Dirks’ group subsequently extended their *in vitro* findings to show that as few as 100 CD133⁺ human GBM cells were able to form tumours when injected orthotopically into immunodeficient NOD/SCID mice, whereas 1×10^5 CD133⁻ cells

engrafted but did not form tumours (S. K. Singh et al. 2004). By showing that fresh tumour tissue could be directly sorted into tumourigenic and non-tumourigenic fractions using a SC-associated surface marker, Dirks and colleagues provided compelling evidence in support of the existence of CSC in human glioma.

Following this landmark study, CD133 expression has been widely used to identify and isolate putative CSC from primary tumour tissue in glioma, colon cancer (O'Brien et al. 2007), prostate cancer (Collins et al. 2005) and melanoma (Monzani et al. 2007). Many studies (Colman et al. 2010; Metellus et al. 2011; Rebetz et al. 2008; Zeppernick et al. 2008; M. Zhang et al. 2008), though not all (K.-J. Kim et al. 2011; Pallini et al. 2011), have found a correlation between CD133 expression and worse outcome in patients with high grade glioma and, consistent with the idea that treatment-resistant CSC are the cause of relapse, CD133 expression has been reported to be increased in recurrent GBM compared with the original tumour (G. Liu et al. 2006; Pallini et al. 2011).

5.1.3 CSC in glioma: complexities and controversies

After this promising start to the CSC story, it has subsequently become clear that the situation is far more complex than first thought and controversy reigns in almost every area of the field. Many studies have now reported that CD133⁺ GBM cells are capable of tumour-initiation in xenotransplantation assays (D. Beier et al. 2007; Clément et al. 2009; Joo et al. 2008; Nishide et al. 2009; Ogden et al. 2008; Piccirillo et al. 2009; Jian Wang et al. 2008). Also, when antibodies are used to determine the proportion of CD133⁺ cells in GBM primary tumour samples, the proportion has variously been reported to be 19-29 % (S. K. Singh et al. 2004), 10-70 % (G. Liu et al. 2006), 0.01-46 % (Joo et al. 2008), 0.01-1.0 % (Clément et al. 2009), and 0 % (Osmond et al. 2010). The reason for these widely differing results is not clear. One possibility is that these differences reflect technical difficulties in detecting the CD133 protein. The commonly used antibodies AC133 and AC144 are sensitive to

the glycosylation state of the CD133 molecule which itself is sensitive to exposure of cells to serum and trypsin (Campos and Herold-Mende 2011; Wan et al. 2010). Also, it has also been shown that CD133 is often intracellular and that, due to alternative splicing, several variants of the CD133 protein exist that are not all recognised by commonly-used commercially-available antibodies (Osmond et al. 2010; Wan et al. 2010) so that immunoreactivity does not necessarily reflect CD133 protein levels. An alternative explanation is that some CSC are indeed CD133⁻ and that the CSC phenotype of a given tumour may depend on the cell-of-origin and/or molecular subtype of the tumour (D. Beier et al. 2007; Günther et al. 2008; Lottaz et al. 2010). Adding to the uncertainty about the reliability of CD133 as a marker of glioma CSC is the fact that the physiological function of CD133 remains unknown (Campos and Herold-Mende 2011; Wan et al. 2010). One study found that CD133 expression by melanoma and colon cancer cells was dependent on DNA content and was therefore a marker of actively cycling cells (Jaksch et al. 2008). Griguer and colleagues found that CD133 expression by a glioma cell line was reversibly induced by severe hypoxia or depletion of mitochondria and suggested that CD133 is a marker of bio-energetic stress (Griguer et al. 2008). To my knowledge, no study has yet reported failure of CD133⁺ GBM cells to form tumours in immunodeficient mice. Thus, while CD133 surface expression appears to be sufficient for tumourigenesis in xenotransplantation assays, it is not absolutely required and therefore does not, by itself, define CSC in glioma.

Uncertainty also surrounds the neurosphere assay as a method for selecting CSC. The proportion of CSC-like cells in glioma spheres is said to be 3-20 % (Wan et al. 2010) so that the neurosphere assay would not appear to be an ideal method for purifying these cells. However, there are more fundamental concerns than this. Sphere formation in SC media is not unique to NSC and CNS tumours but has now been described in several other solid tumours (Liao et al. 2007; Ricci-Vitiani et al. 2007). It has been proposed that the neurosphere assay does not select stem cells *per se*, but rather cells that express EGFR (Pastrana et al. 2011). It is possible that the superior tumourigenicity of sphere cells in xenotransplantation assays

compared to tumour cells grown in standard media is an artefact of exposure to potent mitogenic factors in the SC media that promote tumour cell survival and proliferation (Kelly et al. 2009). Furthermore, it has been reported that culture in SC media preserves the genotype and phenotype of the original tumour more faithfully, and should be used for this purpose, whereas culture in serum-containing media induces a loss of tumourigenicity that is not representative of the original tumour (Fael Al-Mayhany et al. 2009; J. Lee et al. 2006b). For all these reasons, it may be hazardous to assume that culture in SC medium is a way of selecting a CSC subset.

Another challenge to the CSC model comes from reports that the SC-like phenotype is not a fixed intrinsic property of certain glioma tumour cells but can be induced by the tumour microenvironment (Rosen and Jordan 2009). As already mentioned above, a CD133⁻ GBM cell line can reversibly become CD133⁺ in response to hypoxia *in vitro* (Griguer et al. 2008). In other studies, CD133⁻ GBM cells exposed to hypoxia *in vitro* upregulated SC-associated genes and exhibited an increased ability to form spheres (Heddleston et al. 2009), and CD133⁺ GBM cells cultured in 7 % O₂ increased their expression of neural stem cell markers CD133 and nestin and stem cell markers OCT4 and SOX2, these changes mostly reversing when the cells were restored to 20 % O₂ (McCord et al. 2009b). In an *in vivo* study, Wang and colleagues reported that CD133⁻ cells implanted into immunodeficient mice formed tumours that eventually contained CD133⁺ cells (Jian Wang et al. 2008). This plasticity, where tumour cells appear to be able to move between stem-like and non stem-like states in response to their environment, has similarities with the phenomenon of Epithelial-Mesenchymal Transition (EMT), a process central to embryogenesis and believed to play a major role in invasion and metastasis in some solid tumours (Gupta et al. 2009). Mani and colleagues (Mani et al. 2008) showed that experimental induction of EMT in an immortal human mammary epithelium cell line was associated with acquisition of a CD44^{hi}CD24^{lo} surface marker phenotype and the ability to form mammospheres, both features of putative CSC in breast cancer. Also pertinent is the recent report of Gupta and colleagues (Gupta et al. 2011) in which sorted cell populations from human breast tumours were transplanted into

immunodeficient mice along with irradiated “feeder” cells to promote tumourigenesis. As predicted by the CSC model, CD44⁺CD24^{lo} “CSC” cells formed tumours that also included other non-CSC tumour cell phenotypes. Less expected was the finding that tumours forming after implantation of purified “non-CSC” subsets were found to include a subset of cells with the “CSC” phenotype, with the same proportion of CSC to non-CSC cells as the CSC-derived tumours. This suggests the existence of an equilibrium between CSC and non-CSC phenotypes within tumour cell populations *in vivo*. At the present time, it is unclear whether heterogeneity in GBM tumours is the result of stochastic events, hierarchical events, or both. Until this is resolved, it may be hazardous to focus therapeutic efforts entirely on the putative CSC compartment.

One of the most attractive aspects of the CSC model is the concept that properties of CSC render them particularly resistant to radiation and chemotherapy thereby explaining the failure of standard treatments. Putative glioma CSC have been reported to express higher levels of anti-apoptotic molecules (G. Liu et al. 2006), reduced levels of caspase-8 (Capper et al. 2009) and exhibit enhanced DNA damage repair (Bao et al. 2006a), conferring a general resistance to radiation and chemotherapeutic agents. A feature of normal haematopoietic stem cells is their relative chemoresistance which is thought to be at least partly due to the rapid expulsion of xenobiotics by membrane-bound transporter proteins (Reya et al. 2001). Increased expression of these ATP-binding cassette (ABC) transporters proteins has also been reported to be a feature of putative CSC in several solid cancers including GBM (Bleau et al. 2009b; G. Liu et al. 2006). However, this does not explain the failure of chemotherapy to cure patients with GBM because TMZ, currently the standard chemotherapy agent for this condition, is not a substrate for ABC transporters (Bleau et al. 2009b). Instead, TMZ resistance is closely associated with expression of the DNA repair enzyme MGMT. Silencing of MGMT expression by promoter methylation is associated with improved outcome after standard treatment (Weller et al. 2010). Consistent with the idea that glioma CSC are more resistant to TMZ, Liu and colleagues found that CD133⁺ cells from primary GBM cell

lines contained higher levels of MGMT mRNA than CD133⁻ cells (G. Liu et al. 2006). However, the evidence for this is contradictory, with some studies reporting that CSC in glioma are actually more susceptible to TMZ (D. Beier et al. 2008) and that GBM sphere cells exhibit higher levels of MGMT promoter methylation (Sciусcio et al. 2011). Similarly, whereas one influential study showed that sorted CD133⁺ GBM cells were more resistant to radiation (Bao et al. 2006a), a contradictory study reported that GBM sphere cells were more sensitive to ionising radiation (McCord et al. 2009a). Thus, treatment resistance of putative glioma CSC remains controversial.

5.1.4 Definition of Cancer Stem Cells (CSC) and Stem-like Cancer Cells (SLCC)

In 2006 an expert panel defined CSC as “*a cell within a tumour that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumour*” and stated “*cancer stem cells can thus only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumour*” (Clarke et al. 2006). More recently, an authoritative review described CSC as “*a biologically distinct subset within the total malignant cell population*” that have “*the ability to give rise to progeny with limited proliferative capacity*” and stated that “*this model precludes the concept of reversibility between intrinsically determined states of unrestricted and limited proliferative potential*” (Nguyen et al. 2012). To put this slightly differently, in the practical clinical context of established tumours in patients, the defining characteristic of putative CSC is the exclusive ability to maintain the existing tumour. The definition of CSC above implies both *heterogeneity* and *hierarchy*, requiring the presence of at least one other tumour cell phenotype that does *not* having tumour-maintaining capacity or the capacity to acquire tumour-maintaining ability. The capacity to indefinitely maintain tumours in patients also requires the CSC compartment to indefinitely maintain itself or “self-renew”. Defined in this way, it follows that elimination of the CSC compartment should be both necessary and sufficient to achieve cure (Nguyen et al. 2012). These definitions also recognise the practical need to use tumour-

initiation in animal models as a surrogate for tumour-maintenance in human patients. In practice, an improved capacity for tumour initiation, rather than an exclusive capacity, is often accepted on the grounds that small numbers of CSC may contaminate non-CSC populations during sorting.

It is generally accepted that many features of the original CSC model described in leukaemia may not necessarily apply to solid tumours. As discussed above, cells with tumour-initiating ability in glioma tumours are not necessarily rare or quiescent (S. K. Singh et al. 2004), do not necessarily express normal stem cell markers (D. Beier et al. 2007; Read et al. 2009) and have not been proven to arise from transformed neural stem cells. Although proof-of-principle studies have shown that oncogenically-transformed NSC are capable of initiating tumours in experimental systems (Jacques et al. 2010; Y. Zhu et al. 2005), the cell-of-origin of GBM still remains uncertain (Shackleton et al. 2009). However, the definition of CSC does not require an origin from transformed normal stem cells (Clarke et al. 2006; Dick 2008; Gupta et al. 2009). Although importance is often placed on the ability of CSC to recapitulate the morphology of the original tumour, this may be too stringent a requirement for xenotransplantation assays where the recipient stroma is very different to the original host. Also, although the term CSC implies an ability to give rise to at least one other more differentiated phenotype with reduced tumourigenic capacity, the capacity for multilineage differentiation is not a mandatory part of the definition (Finocchiaro and Pellegatta 2011; Vescovi et al. 2006).

Although the exclusive ability to initiate tumours is regarded as the cardinal requirement for the operational definition of CSC, a persistent concern remains over the need to infer this property from behaviour in immunodeficient mice. In studies on melanoma, the size of the tumourigenic fraction depended on the degree of immunodeficiency of the recipient mouse strain (Quintana et al. 2010), raising the possibility that transplantation into commonly-used NOD/SCID mice (that still have NK cells and other innate immune effector cells) may underestimate the true size of the tumourigenic population (Shackleton et al. 2009). Also, interactions between

tumour cells and stromal cells are critical in determining behaviour *in vivo* (Bonavia et al. 2011; Charles et al. 2011). In the study of breast cancer cell subsets by Gupta and colleagues mentioned above (Gupta et al. 2011), whereas only CD44⁺CD24^{lo} putative CSC were tumourigenic when transplanted alone into immunodeficient mice, all subsets were tumourigenic when co-transplanted with irradiated stromal cells. Calabrese and colleagues also reported that the tumourigenicity of medulloblastoma cells implanted into mice was enhanced by the co-transplantation of human endothelial cells (Calabrese et al. 2007). Thus, it is possible that the proportion of GBM cells considered to be “tumourigenic” would increase substantially if yet-to-be-defined human stromal cells and/or human growth factors were included in these xenotransplantation models (Bonavia et al. 2011). If the bulk tumour population is in fact capable of tumourigenesis in its original syngeneic host, the CSC model cannot be said to apply. The term CSC should be used cautiously while this concern still exists.

As a minimum, a tumour-initiating cell should not be called a CSC without also demonstrating heterogeneity and hierarchy. This general principle is often violated in the literature, where cells expressing stem cell markers or exhibiting stem cell-like behaviours such as sphere formation or multipotency, are often referred to as CSC without confirming the existence of a mixed population of tumourigenic and non-tumourigenic cells. If these “stem cell-like cancer cells” (SLCC) are not a subset of a heterogeneous tumour cell population that otherwise consists of their non-tumourigenic progeny, they are not CSC by definition. However, SLCC may still be legitimate targets for novel therapies. The literature suggests that almost every adverse characteristic of malignant glioma is amplified in these more stem cell-like tumour cells including invasion (Ye et al. 2012), induction of angiogenesis (Bao et al. 2006b; Folkens et al. 2009; Heddlestone et al. 2009; Ricci-Vitiani et al. 2010; R. Wang et al. 2010b), resistance to radiation (Bao et al. 2006a) and chemotherapy (G. Liu et al. 2006), and immunosuppression (Wei et al. 2010; Wei et al. 2011). Thus treatments that specifically target SLCC may improve survival by suppressing a particularly aggressive and therapy-resistant phenotype.

5.1.5 Targeting Glioma CSC/SLCC with immunotherapy

Already, diverse ways of targeting SLCC and putative CSC in patients with high grade glioma and other solid tumours have been tested in animal models and some of these are now reaching the clinic. This topic is fully discussed in recent reviews (Alison et al. 2012; Frosina 2011). Conceptually, CSC-directed treatments aim to either eliminate CSC directly, to increase the sensitivity of CSC to standard treatments, or to attenuate some property of CSC that is driving tumour progression. For example, inhibition of the Notch pathway has been proposed both as a way of reducing the proliferative and invasive properties of CSC and as a way of sensitising CSC to radiation treatment (Fan et al. 2010; Jialiang Wang et al. 2010a; Jianpeng Wang et al. 2012). Bevacizumab, an anti-VEGF monoclonal antibody currently being evaluated in patients with GBM, is considered by some to be a form of CSC-targeted treatment (Li et al. 2012) on the grounds that glioma CSC are claimed to induce angiogenesis through VEGF secretion (Bao et al. 2006b) that in turn induces the formation of a perivascular niche that supports maintenance of a stem cell-like state (Calabrese et al. 2007). Some groups have explored the idea of combining an SLCC-targeted therapy with a therapy targeting the non-SLCC compartment. For example, using breast cancer cell lines, Hirsch and colleagues showed that the additive effects of metformin and doxorubicin were due to selective targeting of the CD44^{hi}CD24^{lo} CSC and CD44^{lo}CD24^{hi} non-CSC compartments respectively (Hirsch et al. 2009). Support for this approach was also provided by a recent study in a genetically engineered mouse model of glioma in which treatment with TMZ and conditional ablation of nestin-expressing cells had an additive effect (J. Chen et al. 2012).

The potential for improving the efficacy of glioma immunotherapy by targeting the putative CSC subset has been quickly recognised. In 2006 Pellegatta and colleagues reported that the GL261 murine glioma cell line grew as unattached spheres when cultured in SC media and that mice implanted orthotopically with sphere cells became symptomatic more quickly than mice implanted with GL261 grown in

standard media (Pellegatta et al. 2006b). In this study, vaccination with DC pulsed with a sphere cell lysate was more effective against intracranial tumours than DC pulsed with parental tumour lysate. In 2009 Xu and colleagues reported similar results in the 9L rat glioma model using DC loaded with peptides acid-eluted from sphere cells or cells cultured in standard media (Q. Xu et al. 2009). This study also reported that a range of known glioma-associated antigens were expressed at higher levels in human GBM sphere cell lines relative to tumour cells cultured in standard media, and that human DC pulsed with peptides acid-eluted from a human GBM sphere cell line elicited stronger T cell responses to these antigens *in vitro* than DC pulsed with peptides eluted from the same cell line cultured in standard media. The authors further reported that this was not due to differences in DC maturation or antigen uptake. However, some of the TAA identified in this study as being overexpressed by sphere cells relative to standard cells, such as the melanoma-associated differentiation antigen TRP-2, have no known association with a stem-like phenotype, suggesting the superior performance of the sphere cells in this study may have been due to some unexplained general effect of the SC medium on TAA expression. These studies in immunocompetent rodents suggest that sphere cells may be a superior source of antigens for DC vaccines but it is not clear that the vaccines necessarily acted by selectively targeting CSC. As previously mentioned, it has been proposed that culturing human glioma tissue in SC medium on laminin-coated plates preserves the phenotype and genotype of the original tumour more faithfully than culture in standard conditions (J. Lee et al. 2006b). If confirmed, this would suggest that sphere cells might supply antigens that align more closely with the antigens presented by the target tumour than cell lines raised in standard culture conditions. Vik-Mo and colleagues have recently published the results of a Phase I trial in which newly-presenting GBM patients were vaccinated with DC loaded with RNA extracted from autologous tumour sphere cultures (Vik-Mo et al. 2013). Progression-free survival in 7 treated patients was reported to be prolonged compared with matched historical controls.

Several preclinical studies have assessed the feasibility of targeting glioma CSC in patients with immunotherapy. Bertrand and colleagues (Bertrand et al. 2009) used the U87 immortal human glioma cell line to show that CD133⁺ cells were more resistant to Fas-mediated apoptosis than CD133⁻ cells. However, Brown and colleagues (C. E. Brown et al. 2009) compared sphere cells derived from human GBM tumours with their serum-cultured counterparts and found both were competent to process and present endogenous antigen and detected no differences in MHC class I expression, ICAM-1 expression, susceptibility to perforin, or ability to trigger degranulation of CTL. They also showed that CTL adoptively transferred into immunodeficient mice were able to eliminate human “CSC” *in vivo*. Wu and colleagues (Anhua Wu et al. 2007b) compared CD133⁺ and CD133⁻ tumour cells from two GBM tumours and found low expression of MHC class I molecules and NK activating receptor ligands by both cell types that could be significantly upregulated by treatment with IFN- γ *in vitro* and IFN- γ -treatment restored susceptibility of CD133⁺ cell to NK cell lysis. Avril and colleagues (Avril et al. 2011) showed that SLCC from GBM tumours were more sensitive to lysis by IL-2-activated NK cells and CTL than serum-cultured cells. Castriconi and colleagues (Castriconi et al. 2009) also showed that GBM SLCC were sensitive to lysis by IL-2-activated NK cells. Di Tomaso and colleagues (Di Tomaso et al. 2010) compared GBM-derived sphere cell lines with their respective serum-cultured cell lines and found defects in antigen-processing machinery and low expression of ligands for NK cell activating receptors that were similar for both cell types. MHC class I expression tended to be lower in sphere cells but could be induced by IFN- γ and T cell lines could be raised against autologous sphere cell lines from patient PBMC. They concluded that sphere cells were poorly immunogenic but no more so than serum-cultured cells. Wei and colleagues (Wei et al. 2010) found that GBM sphere cells expressed normal levels of MHC class I. Overall, preclinical studies using primary cell lines from human GBM tumour tissue have not found striking differences in susceptibility to lysis by competent effector cells between SC-like and non SC-like tumour cells.

T cell antigens that are differentially expressed by putative CSC and that could potentially be used to selectively target these cells have been described in glioma and other cancers. Schmitz and colleagues (Schmitz et al. 2007) used microarray data to compare GBM tumours with normal brain tissue and found that the transcription factor SOX2, normally only expressed in the developing brain, was overexpressed in 9 of 10 tumours. Using epitope prediction software, they created SOX2 peptides that were able to elicit T cell responses from the PBMC of healthy volunteers that in turn were capable of lysing HLA-compatible tumour cells *in vitro*. Ueda and colleagues (Ueda et al. 2004) identified SOX6, also a transcription factor normally expressed transiently in the developing brain, as another potential glioma-associated antigen by screening the serum of GBM patients for antibodies against a testis cDNA library. Vaccination of C57BL/6 mice against full-length SOX6 protein provided protective and therapeutic immunity against GL261 murine glioma (Ueda et al. 2008) and SOX6 peptide epitopes were capable of expanding SOX6-specific CTL from the PBMC of healthy volunteers and patients that were able to lyse HLA-compatible tumour cells *in vitro*, including a “CSC” line (Ueda et al. 2010). Dhodapkar and colleagues (K. M Dhodapkar et al. 2010) detected T cells specific for the protein product of the normal stem cell-associated gene *OCT4* in the serum of healthy volunteers and observed an increase in OCT4-specific T cells in the serum of patients with germ cell tumours after treatment with chemotherapy. As mentioned above, one group identified antigens (Her2/Neu, AIM-2 and TRP-2) that were overexpressed by GBM cells grown in SC medium relative to cells grown in standard media and termed these “Cancer Stem-like Cell-associated antigens” (Q. Xu et al. 2009). Subsequently, this group has reported the results of a clinical trial in which newly-presenting GBM patients were vaccinated with DC loaded with HLA A2-restricted peptides derived from these antigens along with three other known glioma-associated antigens (Phuphanich et al. 2012). The results of this Phase 1 trial were described in Chapter 1. Briefly, vaccine-induced CD8 T cell responses were detected in a third of patients. In the few patients who underwent further surgery after vaccination, loss of CD133 expression was observed in 4 of 4 patients who had

CD133⁺ tumours prior to vaccination. This may indicate editing of SLCC although peptide-specific CD8 T cell responses were not seen in some of these patients.

The use of monoclonal antibodies to target putative CSC surface markers has been explored in preclinical models of non-CNS malignancies. Dick and colleagues (Jin et al. 2006) found that an anti-CD44 mAb prevented human acute myeloid leukaemia CSC from engrafting in NOD/SCID mice at least in part by interfering with leukaemic stem cell trafficking to supportive niches. An anti-CD133 mAb conjugated to a cytotoxic drug potently induced apoptosis in a human CD133⁺ hepatocellular cancer cell line and delayed tumour growth in immunodeficient mice (Smith et al. 2008). This approach has not yet been tested in glioma patients.

Although the *in vitro* studies described above suggest CSC should be susceptible to T cell-mediated cytotoxicity, a potential problem for immunotherapies targeting CSC *in vivo* is raised by reports that these cells are particularly immunosuppressive. Heimberger and colleagues (Wei et al. 2010) found that sphere cell lines derived from human GBM tumours suppressed T cell proliferation and cytokine elaboration, induced T cell apoptosis, and induced regulatory T cells *in vitro*. These effects were mediated by direct cell-to-cell contact via the co-inhibitory factor PD-L1 (B7-H1) and by the secretion of soluble factors TGF- β 1, PGE₂ and CCL-2, a chemoattractant for Treg. In other studies, this group has also shown that sphere cells recruit macrophages and polarise macrophages and microglial cells towards an immunosuppressive M2 phenotype by secreting soluble Colony-Stimulating Factor, TGF- β 1 and Macrophage Inhibitory Factor-1 (A Wu et al. 2010). The immunosuppressive properties of sphere cells were regulated by expression of STAT-3 and potentiated by hypoxia (Wei et al. 2011). Di Tomaso and colleagues (Di Tomaso et al. 2010) also found that sphere cells, but not the corresponding serum-cultured lines, secreted soluble factors that inhibited allogeneic T cell proliferation. Contrary to Heimberger's study, this effect did not appear to be due to TGF- β 1, or to TGF- β 2 or IL-10. Wolpert and colleagues (Wolpert et al. 2012) reported that sphere cell lines express significantly higher levels of the atypical HLA molecule HLA-E, a

ligand for inhibitory receptors on NK cells, than GBM tumour cells cultured in serum-containing media.

5.1.6 Hypothesis and Aims

Two groups have reported the use of active immunotherapy to selectively target CSC in rodent models of glioma. Both studies used a vaccine consisting of DC loaded with unselected antigens derived from whole sphere cells (Pellegatta et al. 2006b; Q. Xu et al. 2009). DC-based vaccines require complex laboratory production steps and the optimum phenotype of the infused DC is uncertain. The Phase 1 clinical trial described in Chapter 3 showed that feasibility of DC-based immunotherapy in the recurrent setting is marginal. In Chapter 4 it was shown that a relatively simple vaccine consisting of irradiated autologous whole tumour cells pulsed with an adjuvant that stimulates iNKT cells was effective in a glioma model. The work in this chapter tested the hypothesis that the Glioma-Gal vaccine would be even more effective if modified to selectively target CSC or SLCC.

Reports from the literature suggested that the syngeneic immunocompetent GL261 murine glioma cell line would be an appropriate model for this investigation. As described above, Pellegatta and colleagues have shown that spheres can be grown in SC media and that sphere cells are more tumourigenic in mice than GL261 grown in standard media (Pellegatta et al. 2006b). Wu and colleagues reported that GL261 contained a small 0.5 % CD133⁺ fraction that can be isolated by FACS and that this subset was tumourigenic at titrations 100 times smaller than CD133⁻ cells when implanted in mice, suggesting the preservation of a true CSC cell sub-population within this cell line (Anhua Wu et al. 2008). In a different rodent glioma model, Kondo and colleagues (Kondo et al. 2004) reported that ABC transporter activity could be used to distinguish a subset of cells within the C6 rat glioma line that was more efficient at initiating tumours in nude mice. These studies together support the

intuitively unlikely possibility that an immortalised tumour cell line maintained for many years in serum-containing media can retain a true CSC subset.

Aims of the study:

1. Assess the feasibility of isolating, enriching for, or inducing CSC (or SLCC) from the GL261 murine glioma cell line
2. Characterise the cell populations obtained in (1)
3. Compare the efficacy of a whole tumour cell vaccine made from CSC (or SLCC) with the standard vaccine *in vivo*

5.2 RESULTS

5.2.1 Surface markers do not identify a discrete population of stem-like cells within GL261 cell line cultures.

A theoretically attractive way of isolating CSC in the clinic would be to sort fresh tumour tissue using a surface marker expressed uniquely by CSC. CD133 is an obvious candidate marker for glioma CSC. However, in contrast to the report by Wu et al (Anhua Wu et al. 2008), I could not detect surface expression of CD133 on GL261 cells cultured in standard media (Fig 5.1A). The B16 melanoma cell line, also reported to have a CD133⁺ subset (Dou et al. 2007), was used as a positive control (Fig 5.1A). It was possible that long-term culture *in vitro* had induced a CD133⁺ phenotype in tumour cells capable of CD133 expression. CD133 expression was therefore assessed under different conditions. Culture in serum-free media supplemented with EGF and bFGF (SC medium) resulted in growth of GL261 as

Figure 5.1. Surface expression of putative CSC surface markers by GL261 assessed by antibody staining and flow cytometry. A, CD133 expression by *in vitro* cultures of the GL261 and the B16 murine melanoma cell lines. Figures are percentages of total cells. B, CD133 expression by GL261 spheres cultured in SC media, by GL261 cells retrieved from subcutaneous tumours, and from GL261 cultured *in vitro* under hypoxic conditions. C, CD15 expression by *in vitro* cultured GL261. D, CD44 expression by *in vitro* cultured GL261. E, Left panel: CD200 expression by *in vitro* cultured GL261. Middle panel: CD200 expression by GL261 spheres cultured in SC media. Using the Kolmogorov-Smirnov method, the probability of the CD200+ and CD200- populations not being drawn from the same population was 0%. Expression of each marker was assessed at least twice.

detached floating spheres as previously described (Pellegatta et al. 2006b) but did not induce CD133 expression (Fig 5.1B). Next, it was hypothesised that tumour cells growing *in vivo* might encounter niches conducive to the induction of an SLCC phenotype. However, I saw no significant CD133 expression in single cell suspensions freshly prepared from subcutaneous tumours (Fig 5.1B). It has been

shown that CD133 expression is induced when tumour cells are subjected to bioenergetic stress such as hypoxia (Griguer et al. 2008). Others have reported that hypoxia promotes glycosylation of CD133 epitopes that in turn increases detection by anti-CD133 antibodies (Bidlemaier et al. 2008). In accordance with this, culture of GL261 under hypoxic conditions did induce some CD133 surface expression but only in 0.16 % of cells (Fig 5.1B). It was felt that this proportion was too low to realistically conduct immunotherapy experiments in mice using a Glioma-Gal vaccine manufactured from tumours cells purified by FACS.

Expression by GL261 cells of a number of other putative markers of glioma CSC was also assessed. CD15 surface expression has been shown to distinguish the only cells capable of initiating tumours in an implantable murine model of medulloblastoma (Read et al. 2009; Ward et al. 2009). GL261 did not express CD15 under standard culture conditions (Fig 5.1C). CD44 is a well-established marker of tumour-initiating cells in breast cancer models and has been proposed as a glioma CSC marker in GBM tumours (G. Liu et al. 2006; Q. Liu et al. 2009). CD44 was highly expressed by virtually all GL261 tumour cells (Fig 5.1D), a finding that was interesting but excluded CD44 as a way of discriminating CSC in this model. CD200 is a “don’t eat me” membrane-bound protein related to the B7 family that is normally expressed on lymphocytes, neurons and endothelial cells and delivers an inhibitory signal to cells of the macrophage lineage (Kretz-Rommel et al. 2007). It is also expressed by several human tumour types and an inverse correlation between CD200 expression and prognosis has been reported, suggesting tumours may exploit this molecule to evade the immune system (B. T. Kawasaki et al. 2007; Kretz-Rommel et al. 2007). Co-expression of CD200 and putative CSC-markers has been observed in human cancers including glioma, leading to the suggestion that CD200 could be a marker for CSC (B. T. Kawasaki et al. 2007; B. Kawasaki and Farrar 2008). In this study, a small proportion (0.4 - 0.6 %) of GL261 cells cultured in standard media were CD200⁺ (Fig 5.1E) but this finding was inconsistent (data not shown). Similar, inconsistent, results were obtained when cells were cultured in SC medium (Fig 5.1E and data not shown). An expert opinion (Kylie Price, Hugh Green Flow Cytometry

Fellow, MIMR) was sought regarding the feasibility of sorting these CD200⁺ cells. Because the CD200⁺ cells were not a distinct population, but located within the upper part of a normal distribution, and because of the small proportion of CD200⁺ cells, magnetic or fluorescence-activated sorting was felt unlikely to yield sufficient numbers of cells of acceptable purity to feasibly manufacture whole tumour cell-based vaccines. Coupled with the finding that CD200⁺ cells were not consistently present in all cultures, it was decided not to pursue enrichment of CD200⁺ cells as a strategy for making vaccines targeting CSC. In summary, the surface markers tested here did not identify a distinct tumour cell subset or, if a positive population was seen, it was too small and/or insufficiently demarcated from the bulk population to be of practical use in the manufacture of a whole tumour cell vaccine.

5.2.2 Side-population cells isolated from GL261 are less tumourigenic than non-SP cells

ABC proteins are a family of membrane-associated proteins that actively transport certain molecules out of the cell, including a range of chemotherapeutic agents (M A Goodell et al. 1996). The fluorescent lipophilic dye Hoechst 33342, which passively diffuses into all cells, is a substrate for some ABC family members so that cells expressing these transporters can be identified using flow cytometry as a population of dye-negative cells termed the “side-population” (SP) (Margaret A Goodell 2005). The SP phenotype is strongly, but not exclusively, associated with expression of the ABC family member ABCG2, also known as Breast Cancer Resistance Protein-1 (BCRP-1) (Bleau et al. 2009b; Hirschmann-Jax et al. 2004; Kondo et al. 2004; Patrawala et al. 2005; S. Zhou et al. 2001). In 1996, Goodell and colleagues discovered that the SP contains the vast majority of haematopoietic stem cells in murine bone marrow (M A Goodell et al. 1996). Subsequently, an SP has been demonstrated in some human solid cancers and several studies have reported that these SP cells are more tumourigenic when transplanted into immunodeficient mice

Figure 5.2. GL261 tumour cells have a side population (SP) that is less tumourigenic in vivo than the non-SP. A, Cultured GL261 tumour cells were incubated with Hoechst dye and the dye-negative “side population” isolated using fluorescence-activated cell sorting. The SP gate was set using cells also treated with 2-deoxyglucose (2-DOG), which blocks ABC transporters, as a negative control. Purity of the sorted SP and non-SP fractions assessed by flow cytometry was 96% and 99% respectively (data not shown). One experiment by the author. At least three additional experiments by K. Broadley showed similar results with a mean SP in parental cultures of 1.9%. B, 5×10^4 GL261 SP cells and non-SP cells from same experiment shown in A, and unsorted tumour cells, were injected s.c. into C57BL/6 mice. 10 mice per group, one experiment. C, GL261 cells were cultured as floating spheres in SC media and passaged nine times before flow cytometry to assess for the presence of a side population. One experiment, performed in collaboration with K. Broadley. At least three additional experiments by K. Broadley showed similar results with a mean SP in GL261 sphere cultures of 0.23%.

than non-SP cells (Hirschmann-Jax et al. 2004; Ho et al. 2007; Patrawala et al. 2005). Similar findings have been reported in some rodent glioma models (Bleau et al. 2009b; M. A. Harris et al. 2008; Kondo et al. 2004). Kondo and colleagues (Kondo et al. 2004) found a 0.4 % SP to be present within the C6 rat glioma line that was more efficient at initiating tumours in nude mice. Although SP cells could give rise to both

SP and non-SP cells, purified non-SP cells could not reform SP cells, suggesting the existence of a hierarchy. It has therefore been proposed that the ability to exclude Hoechst dye and/or expression of ABCG2 could be used as a marker of glioma CSC and a possible explanation for the chemoresistance of these cells (Bleau et al. 2009a; Hirschmann-Jax et al. 2004). An SP has also been reported to exist within the GL261 glioma cell line but the capacity of these cells to initiate tumours was not tested in that study (Anhwa Wu et al. 2008).

Colleagues at the Malaghan Institute have previously established methods for undertaking SP sorting by FACS and have shown that GL261 grown under standard culture conditions consistently has a small SP of 0.01 – 4 % that is not seen when cells are also treated with 2-deoxyglucose (2-DOG), an inhibitor of ABC transporters (Broadley et al. 2011). In this study, GL261 cells grown under standard culture conditions were sorted by FACS into SP and non-SP fractions (Fig 5.2A) and implanted s.c. into mice. Whereas non-SP cells formed tumours in all mice, SP cells did not always form tumours and the time to tumour appearance was delayed compared to non-SP cells or unsorted tumour cells (Fig 5.2B). This finding suggested that the GL261 SP is not enriched for tumour-initiating cells. Furthermore, culture of GL261 in SC media, expected to enrich for cells with a SLCC phenotype, did not increase the SP size (Fig 5.2C). Along with supportive data obtained by colleagues from human GBM tumour tissue (Broadley et al. 2011), these findings discouraged further investigation into using SP cells as the basis of a vaccine targeting CSC/SLCC.

5.2.3 Characterisation of GL261 spheres

In standard medium containing 20 % foetal calf serum, GL261 grows as an adherent monolayer (Fig 5.3A). As discussed above, culture of GBM tumour cells or rodent glioma cell lines in SC medium in uncoated flasks leads to the formation of detached

Figure 5.3. Tumour-initiating capacity of GL261 cultured in “stem cell medium” is similar to parental cells. A, Light microscopy of GL261 growing as an adherent monolayer in standard medium (“parental” cells). B, Light microscopy of GL261 growing in SC medium as floating spheroidal aggregations (“sphere” cells). C, 5×10^3 sphere or parental cells were implanted intracranially into the right striatum of C57BL/6 mice. Tumour-free survival was defined as time to symptom appearance. 4-5 mice per group. D, Experiment as in C. 5-6 mice per group. P-values in both figures indicate the result of a Log-rank test. E, F, G, Brain tissue was collected from symptomatic mice at the completion of the experiments depicted in C and D. Light microscopy of formalin-fixed, paraffin-embedded, haematoxylin and eosin-stained sections showing margins of intracranial tumours initiated by GL261 parental cells (E) and sphere cells (F). Arrows indicate tumour cells infiltrating brain parenchyma. G, Pathologists independently categorised the margin of each tumour as infiltrative or well demarcated. Infiltrative borders were poorly defined, with cords, clusters and individual tumour cells extending haphazardly into adjacent brain tissue. Well demarcated margins had a rounded, “pushing” appearance with a clearly defined boundary between tumour and adjacent brain. Shown are the pooled data from experiments in C and D. Statistical significance was assessed using a two-tailed Fisher’s exact test.

spherical aggregations closely resembling the “neurospheres” that form when cells from the striatum of adult murine brain are cultured in similar media (Reynolds and Weiss 1992a). The SC medium used in my study consisted of a commercially available serum-free medium augmented with a nutritional supplement and the growth factors EGF and bFGF. This same SC medium has been consistently reported to promote sphere formation in human and rodent glioma studies (Ali Jourabchi Ghods et al. 2007; Pellegatta et al. 2006b; Yuan et al. 2004). Still searching for a way to target CSC or SLCC with the Glioma-Gal vaccine, I decided to explore this approach as a possible way of enriching for, or inducing, a CSC/SLCC phenotype. When resuspended in SC medium, GL261 readily formed spheres as expected (Fig 5.3B). These sphere cells could be propagated indefinitely and reverted to an adherent phenotype when placed back into standard medium (data not shown). In addition to floating spheres, some cells in the SC medium grew on the bottom of the culture flask as a patchy adherent layer. However, this pattern of growth did not appear to be a fixed intrinsic property of individual cells because these adherent cells, as well as purified sphere cells, gave rise to both cell types when reseeded into SC medium (data not shown). Furthermore, the proportion of cells that grew in this adherent way proved to be dependent on the supplier of the tissue culture flasks.

As discussed above, the cardinal property of CSC is an increased ability to initiate tumours *in vivo* in comparison to bulk tumour cell populations. The ability of parental and sphere cells to initiate intracranial tumours was therefore compared in syngeneic immunocompetent C57BL/6 hosts. Both cell types were capable of forming tumours when 5000 cells were implanted i.c. (Figs 5.3C, D). There was no significant difference between sphere and parental cells with respect to the rate at which symptomatic tumours formed, a finding at odds with the previous report of Pellegatta and colleagues (Pellegatta et al. 2006b). Histology did not reveal any obvious differences in the morphology of individual tumour cells but differences in the pattern of tumour growth were observed, with tumours derived from parental cells tending to be well demarcated and sphere cell tumours more likely to exhibit

an infiltrative growth pattern (Figs 5.3E - G). This invasive pattern of growth more closely resembles the behaviour of human GBM.

To further assess the “stemness” of sphere cells and ASC, the expression of a panel of stem cell-associated genes was assessed. These genes were selected because of published studies describing an association between expression and pluripotent stem cells, normal neural stem cells, or glioma CSC (Table 5.1). To validate these

Table 5.1 Stem cell-associated gene panel

Gene	Function of protein	Gene expression associated with:			References
		Pluripotent SC	Neural SC	Glioma CSC	
SOX2	Transcription factor. Normally expressed in developing brain.	✓	✓	✓	{Takahashi, 2006 #1333}{Kim, 2008 #549} {Brazel, 2005 #703}{Episkopou, 2005 #880}{Kim, 2008 #549} {Gangemi, 2009 #259}
Musashi-1	RNA-binding protein. Promotes cell division while maintaining stem cell state in NSC		✓	✓	{Okano, 2002 #289} {Chong, 2009 #820}{Hong, 2012 #1195}
Nestin	Intermediate filament transiently expressed in developing CNS.		✓	✓	{Lendahl, 1990 #1222} {Singh, 2003 #442}
CD133	Unknown.		✓	✓	{Uchida, 2000 #1233} {Singh, 2003 #442}{Singh, 2004 #214}
BMPR2	Receptor for BMPs that have multiple actions including instructing astroglial differentiation of neural stem cells.		✓	✓	{Lim, 2000 #1274} {Piccirillo, 2006 #938}
SOX6	Transcription factor. Normally expressed in developing brain.		✓	✓	{Ueda, 2004 #694} {Ueda, 2010 #893}

genes as markers of neural stem cell-like cells, gene expression was assessed in foetal mouse brain cells cultured in a commercially-supplied serum-free media reported to enrich for normal neural stem cells (Galli et al. 2008; S. A. Louis et al. 2008). A little surprisingly, the cells grew as distinctive “attached spheres” rather than the classical floating spheres described in the landmark study of Reynolds and Weiss (Reynolds and Weiss 1992a) (Fig 5.4A). When mRNA was quantitatively assessed by real time PCR there was a progressive increase in expression over time of all six genes tested (Fig 5.4B), supporting an association between the selected

Figure 5.4. Expression of stemness genes by GL261 cultured in stem cell medium. A, Foetal brain tissue from C57BL/6 mice was cultured in medium that supports the survival of normal neural stem cells (NSC). Light microscopy showing cells growing as compact spheroidal aggregations attached to the floor of the tissue culture flask. B, Expression of a panel of NSC- and putative glioma CSC-associated genes in foetal NSC cultures assessed by measuring RNA using real-time reverse transcription polymerase chain reaction (RT-PCR). Results are depicted as fold change on Days 11 and 28 of culture relative to baseline expression on Day 0 (Day 0 = 1). Graph at right shows results for Musashi-1 at different scale. Single experiment. C, Ct values relative to 18S rRNA (Δ Ct) in uncultured foetal brain cells and Day 28 foetal NSC, compared with the mean Δ Ct of several experiments assessing expression in GL261 cultured in standard medium. D, Expression of the SC gene panel (except CD133) in GL261 sphere cells relative to GL261 cultured in standard medium. Top: Pooled results of six experiments by author in which some, or all, of the panel were assessed. Spheres were assessed after 1 - 6 passages in SC medium. No correlation between fold change and the number of passages was observed (data not shown). Bottom: Results of similar experiments by K.Farrand (with permission). Bars indicate means and SEM. E, Differences in gene expression between parental and sphere cells (in experiments performed by author) were analysed statistically by comparing Δ Ct values and applying a Wilcoxon matched pairs signed rank test. No statistically significant differences were observed for BMPR2, Nestin or SOX6 (not shown).

genes and “stemness”. The panel was then used to compare stemness of sphere cells relative to parental tumour cells. With respect to CD133, expression of this gene was found to be either close to, or below, the limit of detection in both GL261 cell types in three independent experiments (Fig 5.4C and data not shown), a finding consistent with the absence of anti-CD133 antibody staining by parental and sphere cells (Figs 5.1A and B). CD133 expression was therefore not assessed in subsequent experiments. With respect to the other genes on the panel, no significant differences were detected between parental and sphere cells with the exception of Musashi-1 expression which consistently increased in all sphere lines relative to parental cells, with a mean fold increase of 2.5 (Fig 5.4D, E). In support of this finding, contemporaneous experiments by a colleague also found a consistent rise in Musashi-1 expression by GL261 sphere cells of a similar magnitude (Fig 5.4D).

In summary, culture of GL261 cells in SC medium did not result in an increased capacity to initiate tumours *in vivo*. By not meeting this key criterion, sphere cells did not meet the definition for CSC. However, culture in SC medium did induce sphere formation and upregulation of Musashi-1, a gene strongly associated with neural stem cells, indicating at least a shift towards a more neural stem cell-like phenotype and justifying the term SLCC. Sphere cells also gave rise to i.c. tumours with an infiltrative morphology more typical of human high grade glioma. Culture in SC medium thus emerged from these studies as a potentially useful way of enriching for antigens associated with stem-like glioma cells.

5.2.4 Designing a vaccine targeting unselected antigens from whole tumour cells suitable for assessing the effect of qualitative changes in the antigen component of the vaccine.

Given that the aim of this study was to test whether the efficacy of the Glioma-Gal vaccine could be enhanced by qualitatively modifying the antigen component of the vaccine, it was important to compare Glioma-Gal vaccines that did not differ in the

total quantity of antigen or in adjuvanticity. To try and keep the quantity of protein in the two vaccines constant, I first tried injecting the same number of morphologically-intact cells, whether they were Trypan Blue negative or positive. In a pilot experiment, a Glioma-Gal vaccine made from sphere cells was less effective than the parental vaccine (data not shown). This result was unexpected and contrary to previous DC vaccine studies using sphere cells as the antigen source. However, experience accrued during the preparation of Glioma-Gal vaccines suggested several possible confounding factors. It was noted that live sphere cells in single cell suspensions after harvesting were smaller than their live parental counterparts, so that the *per cell* quantity of antigen and adjuvant may have been lower for sphere cells. One way of standardizing the protein content of the vaccines might have been to lyse an aliquot of the cellular vaccine and measure the protein content of a known quantity of tumour cells. The number of cells in each vaccine could then be adjusted to deliver an equivalent amount of protein. However, this would still not ensure equivalent delivery of adjuvant. The mechanism by which α -GalCer becomes physically associated with GL261 cells in culture is unknown. It is possible that differences in cell phenotype or the culture medium might affect this process. Also, it was consistently observed that the live-to-dead cell ratio of single cell suspensions after harvesting was variable, both between cell types and between experiments. Whether a tumour cell is live, dead or dying may affect its capacity to associate with α -GalCer. For these reasons, even if the protein content could be standardised, it was not possible to safely assume that the ratio of α -GalCer-to-protein would be constant between vaccines. It was therefore necessary to develop an alternative vaccine design to address the experimental aims.

Accordingly, a simple vaccine was designed consisting of tumour cell lysate co-administered intravenously with soluble α -GalCer (Fig 5.5A). Lysates were created using a freeze/thaw technique as described in Chapter 2. The protein concentrations of the lysates were measured and volume adjusted to provide vaccines of equal protein content. The α -GalCer dose of 200 ng per mouse was

Figure 5.5. A lysate-based vaccine suitable for assessing the effect of changing antigen content without altering adjuvanticity. A, Diagram showing method for making lysate vaccines (Lysate-Gal). B, Wild type and MHC class II-deficient mice were vaccinated i.v. with Lysate-Gal (25 µg lysate) and challenged s.c. with 1×10^6 GL261 tumour cells 7 days later. 3-5 mice per group, one experiment. C, The effect of tumour lysate on DC maturation was assessed *in vitro* using the DC2114 dendritic cell line. DC were pulsed for 16 hours in triplicate with 10 µg/mL or 100 µg/mL of parental or sphere cell tumour lysate. Some DC were pulsed with 100 µg/mL Poly I:C as a positive control and to model the effect of a strong adjuvant. CD40 and CD86 expression by DC was assessed by flow cytometry. Graphs show means and SEM of experimental triplicates. Statistical analysis was performed with a Kruskal-Wallis test with Dunn's multiple comparison post-test. One of two experiments with similar results is shown.

selected based on previous titration studies done by others at this institution showing that this is the threshold dose for inducing maximal DC maturation (data not shown, I. Hermans). Because the main purpose of the lysate vaccine experiments was ultimately to inform the design of an improved Glioma-Gal vaccine, it was important to confirm that the lysate vaccine acted by similar mechanisms and elicited adaptive immunity against protein antigens. Accordingly, it was shown that tumour protection was abrogated in MHC class II-deficient mice (Fig 5.5B). The vaccine design assumes that the lysate component does not materially affect the adjuvanticity of the vaccine. To confirm this, expression of DC maturation markers was assessed after pulsing with sphere or parental cell lysates. No effect on DC maturation was observed with either lysate type, in contrast to Poly I:C, a TLR3 ligand that served as a positive control (Fig 5.5C).

5.2.5 A lysate vaccine made from glioma spheres does not provide improved anti-tumour immunity.

Using the lysate vaccine design, vaccines made from sphere cells or parental cells were now assessed for their ability to protect against tumour challenge. A lysate dose of 100 µg was selected for preliminary experiments based on published experience with other tumour lysate vaccines (Hermans et al. 2003a; Jouanneau et al. 2006; Anhua Wu et al. 2007a). At this dose, both vaccines provided complete protection against i.c. (Fig 5.6A) and s.c. (Fig 5.6B) challenge. To establish an optimal dose for comparing the relative potency of the vaccines, a titration study was performed using smaller quantities of lysate. For practical reasons, these experiments were done using a s.c. challenge. As expected, reduction in the dose of lysate resulted in reduced protection but no differences in the level of protection mediated by the two vaccine types were observed (Fig 5.6C). The 5 µg dose provided an intermediate level of protection that was suitable for detecting differences between vaccines. This experiment was therefore repeated twice at the

Figure 5.6. Vaccines made from lysates of GL261 cells grown in stem cell media are not more effective than a vaccine made from parental cells. A,B, Mice were vaccinated with 200 ng α -GalCer co-administered i.v. with 100 µg of parental or sphere tumour cell lysate and then challenged 7 days later with 5×10^3 parental tumour cells i.c. (A) or with 1×10^5 cells s.c. (B). 5 mice per group. C, Mice were vaccinated with parental or sphere Lysate-Gal vaccines containing different quantities of lysate and challenged 7 days later s.c. with 5×10^5 parental tumour cells. 5 mice per group. The No Vaccine control group in the three graphs is the same. D, Mice were vaccinated with parental or sphere Lysate-Gal vaccines containing 5 µg lysate and challenged 7 days later s.c. with parental tumour cells. Two different parental-sphere lysate pairs were assessed in this experiment, each manufactured independently from parallel parental and sphere cultures seeded from the same original parental culture. Sphere cell lysates were made after 3-4 passages in SC media. The GL261-91 lysates are the same as those used in C. The No Vaccine control group in both graphs is the same. 5 mice per group.

5 µg dose (Fig 5.6D). The two vaccine types provided an equivalent level of protection at this dose in all three experiments.

5.3 DISCUSSION AND CONCLUSIONS

This study did not succeed in identifying a surface marker or functional property that could be used to isolate a subset of more stem-like or more tumourigenic cells from within the parental GL261 cell line. The finding that GL261 under routine culture conditions lacked a CD133⁺ subset contradicts the findings of an earlier study. Using antibody from the same clone as this study, Wu and colleagues (Anhua Wu et al. 2008) reported CD133 surface expression by 0.5 % of GL261 tumour cells. In contrast to most studies of human glioma tumours, in which the CD133⁺ fraction is depicted as a distinct and normally-distributed population (Griguer et al. 2008; Joo et al. 2008; Q. Liu et al. 2009; Lottaz et al. 2010; S. K. Singh et al. 2003), the flow cytometry plot in the report of Wu and colleagues depicts a population that is widely scattered both in CD133 positivity and side scatter, perhaps raising some doubt as to whether this is a genuine subset with a consistent phenotype. Recently, another group also reported finding a CD133⁺ subset in GL261 using flow cytometry that could be sorted by FACS but did not include the flow plot in their report (Ye et al. 2012). Two other studies have reported CD133 expression by GL261 to be “low” (Pellegatta et al. 2006b) or absent (Ali J Ghods et al. 2013) as assessed by RT-PCR and immunofluorescence staining respectively. One possible explanation for these apparently discordant findings is that labelling by anti-CD133 antibodies has been shown to be sensitive to the length of the CD133 protein and the glycosylation state of the epitope (Lehnus et al. 2013; Osmond et al. 2010). It is possible that the phenotype of GL261 in this respect has diverged over the course of longterm culture in different laboratories.

Several studies have reported that SP sorting of human glioma cell lines (Chua et al. 2008; Hirschmann-Jax et al. 2004; Patrawala et al. 2005) or rodent glioma tumours

(Bleau et al. 2009b; M. A. Harris et al. 2008; Kondo et al. 2004) enriches for a subset of tumour cells that are more tumourigenic. In almost all of these studies tumourigenicity was assessed in immunodeficient animals. An exception was Bleau and colleagues (Bleau et al. 2009b) who used a transgenic PDGF-driven glioma mouse model and showed enhanced tumourigenicity of SP cells when implanted into the cortex of syngeneic newborn pups. In the studies reported here, an SP was detected in GL261 as previously described (Anhua Wu et al. 2008) but SP cells were actually less tumourigenic than non-SP or bulk cells. The converse finding in other studies could conceivably be explained by the fact that Hoescht dye is toxic to cells, particularly when exposed to UV light (Golebiewska et al. 2011). Thus the tumourigenicity of non-SP cells, that cannot efflux dye, may be sensitive to differences in SP methodology such as shielding from ambient light. The study of Bleau and colleagues tried to address this concern by demonstrating equivalent viability of SP and non-SP populations ten days after sorting, but this does not exclude a transient effect on the tumour cells' ability to implant *in vivo* soon after sorting. Other work done in collaboration with colleagues showed that a panel of stem cell-associated genes was not upregulated in the SP of GL261 (Broadley et al. 2011). Taken together, these findings did not support the SP assay as a way of isolating a CSC or SLCC subset in GL261. Several other reports have also questioned the overlap of the SP and the CSC compartment in a range of solid human cancers (reviewed in Broadley et al. 2011) including GBM (Golebiewska et al. 2013).

Having failed to isolate a CSC or SLCC subset within GL261 tumour cell populations cultured in standard medium, this study next evaluated the feasibility of using antigens derived from GL261 cells cultured in SC medium. Although this technique enriches for a more stem cell-like cell, it remains unclear whether this is achieved by selecting a subset that is intrinsically more stem-like or by stochastically inducing this phenotype in a population of cells that are all capable of upregulating stem cell-like characteristics in response to appropriate stimuli. Unexpectedly, the lysate sphere vaccine showed no benefit over the parental vaccine, a finding at odds with two previous studies of DC vaccines in rodent glioma models (Pellegatta et al.

2006b; Q. Xu et al. 2009) including one that used the GL261 cell line (Pellegatta et al. 2006b). This discordance with a study using the same cell line deserves careful scrutiny.

It is appropriate to first consider the possibility that the cell lines used in the two studies were not the same. For the studies reported here, the GL261 cell line was obtained from a respected tumour and cell line repository of international standing, the Division of Cancer Treatment and Diagnosis Tumor Repository, located at the National Cancer Institute in Maryland, USA. To rule out the possibility of contamination or genetic drift over time, our cell line was sent for genotyping by a commercial laboratory that specializes in cell line validation. Two specimens were sent for analysis: a sample of the original stock obtained in 2008 and stored since then in the vapour phase of liquid nitrogen, and a sample from a GL261 culture in progress in 2012 at the time when many of the key experiments in this chapter were being performed. The two samples matched identically to each other and to the genetic profile established for the GL261 cell line at that facility (Appendix C) confirming the identity of our cell line beyond any reasonable doubt. It is difficult to be quite as certain about the provenance of the cell line described in the study of Pellegatta and colleagues because the source of their cell line is not stated in their published account (Pellegatta et al. 2006b), or in an earlier paper by the same group (Pellegatta et al. 2006a). Since it is not possible to resolve this matter, the discussion will move on to consider other explanations.

There were a number of notable differences between the vaccines used in the two studies, one of which was the method used to make the tumour cell lysate. In the other study, the lysate used to pulse DC was created by sonication of tumour cells. The lysate was then used without any further manipulation to pulse bone marrow-derived DC (BMDC) *in vitro*. One possibility is that the sonication method used in that study, but not the vaccine design used in my study, created parental and sphere vaccines that differed in their antigen content or adjuvanticity. I considered trying to test this hypothesis by reproducing the methods used by the other group but some

details for the sonication method were not clearly described in the manuscript or in the quoted references. However, some preliminary work along these lines yielded some interesting observations. Relevant to this discussion, in my hands, tumour cells suspended in a cryovial in complete media and sonicated in a water bath eventually became Trypan Blue positive but appeared otherwise morphologically intact. Secondly, sonication in a water bath for 60 minutes generated considerable heat. Thirdly, after 60 minutes of sonication a large proportion of tumour cells were still Trypan Blue negative. It is not possible to say from the information provided whether Pellegatta and colleagues controlled the temperature during sonication or checked cell viability at the end of the stated 60 minute period of sonication.

In my study, the lysate was created by cycles of freeze/thaw followed by removal of particulate matter larger than 0.2 μm using centrifugation and filtration. This process may have resulted in a relative depletion of membrane-associated proteins compared with the sonication method. If sphere cells and parental cells differ in their relative expression of an important antigen or DAMP that exists predominantly in membrane-bound form then the comparative advantage of the sphere cell lysate could be lost if the lysates are depleted of membrane fragments. I considered the possibility of treating the freeze/thaw lysate with a detergent to increase the availability of membrane-associated molecules but rejected this idea because of the possible toxicity of an i.v. lysate contaminated with detergent.

Another important difference in vaccine design is that the Lysate-Gal vaccines incorporated a potent adjuvant whereas, in the study by Pellegatta and colleagues, the only stimulus for DC maturation was delivered by the lysate itself. It has been shown here that the freeze/thaw lysates used in the Lysate-Gal vaccines had negligible effect on DC maturation *in vitro*. Subtle differences in adjuvanticity between lysates likely become irrelevant when the vaccine incorporates a potent adjuvant like α -GalCer. In contrast, Pellegatta and colleagues provide data to show that pulsing with the sonicated lysate was sufficient to induce BMDC to increase surface expression of MHC class I, MHC class II, CD80 and CD86 (Pellegatta et al. 2006b).

Using two different GL261 parental cell lines, I also found that sonicated GL261 tumour cells have the capacity to induce DC maturation (Appendix B, Fig B5.2). This maturation effect was weak in comparison to a TLR ligand but could perhaps become important when no other adjuvant is included in the system. Unfortunately, and perhaps surprisingly, Pellegatta and colleagues do not appear to have compared the level of DC maturation induced by the two sonicates. This raises the possibility that the sphere cell sonicate used in that study had a more potent effect on DC maturation than parental cell sonicate. Similar comments apply to the study of Xu and colleagues (Q. Xu et al. 2009) who, using the 9L rat glioma model, also reported superior induction of anti-tumour immunity by BMDC pulsed with peptides acid-eluted from sphere cells compared with DC pulsed with antigens eluted from tumour cells cultured in standard media, but did not report maturation status of the DC in the two vaccines.

Another potentially relevant difference between the two GL261 studies is that, in contrast to the freeze/thaw method, the sonication method of producing lysates has the capacity to generate significant heat. Sublethal heat shock induces the synthesis of heat shock proteins that can form complexes with proteins and act as “chaperones” to promote uptake and presentation of peptide antigens by DC (Crane et al. 2013). To speculate, it is possible that sphere cells become a better source of antigens for DC pulsing after exposure to heat because some component of the SC medium, such as insulin for example (Ting et al. 1989), promotes the synthesis of heat shock proteins (HSP), a difference that might not come into play when the freeze/thaw technique is used.

Another way of considering the apparent discordance of the two studies is to re-examine the strength of the conclusions reached by the earlier study. To begin, it seems possible that the results of some experiments in the earlier study could have been affected by contamination of the DC-based vaccines by tumour cells. As mentioned above, the lysates used in that study appear to have been created by 60 minutes of sonication but no steps to assess cell viability after sonication are

stipulated in the methods. My own preliminary observations showed that 60 minutes of sonication are not necessarily sufficient to render all tumour cells Trypan Blue positive. Pellegatta and colleagues provide data showing increased expression of MHC class II and co-stimulatory molecules by sphere cells relative to adherent cells and suggested that sonicated sphere cells might therefore be more immunogenic. If live or dying whole tumour cells can act as APC then, assuming at least some tumour cells could survive co-culture with BMDC for 2-3 hours, contamination of the DC vaccines by immunogenic tumour cells could conceivably explain the different performance of the two vaccines in that study.

Another factor to consider is variability in the behaviour of the GL261 model between experiments. In my view, the key experiment in the paper of Pellegatta and colleagues was that which showed the sphere-pulsed DC vaccine was more effective than the adherent (parental) cell-pulsed vaccine against established adherent cell tumours. In fact, both vaccines had a therapeutic benefit that was statistically significant compared with untreated controls, with long term survival of 80 % and 50 % for the sphere and adherent cell vaccines respectively*. The magnitude of this difference is not dissimilar to the level of variation seen in studies described earlier in this thesis using parental cell-based vaccines. It was shown in Chapter 4 that the level of protection against a s.c. tumour challenge afforded by the Glioma-Gal vaccine ranged from 60 to 100 % (Fig 4.2A). Similarly, therapeutic Glioma-Gal-based immunotherapy resulted in long term survival over three independent experiments in 29 to 60 % of i.c. tumour-bearing mice (Fig 4.7). Although several possible mechanisms could explain this variability from experiment to experiment, one that cannot be excluded is a level of variability in the cultured tumours cells used to construct the vaccines. Particular caution is therefore required when drawing conclusions about differences between vaccines derived from different GL261 cultures.

* No direct statistical comparison of the two vaccinated groups is provided in this unrepeatable experiment.

To illustrate this point, in the course of this thesis it was observed that single cell suspensions of harvested tumour cells always contained a significant proportion of tumour cells that were Trypan Blue-positive but otherwise intact. In the study by Pellegatta and colleagues, BMDC appear to have been pulsed with a defined number of sonicated cells. Although not stated, this may have been determined by counting live cells. Since the cell suspensions almost certainly included a variable number of dead cells, sonicates derived from separate cell cultures could contain substantially different quantities of protein. Similarly, in the study of Xu and colleagues (Q. Xu et al. 2009) peptide antigens were acid-eluted from sphere and “daughter” cells but it is not clear what steps were taken to standardize the quantity of antigen used to pulse the DC during vaccine production. The potential for antigen quantity to influence the magnitude of a vaccine-induced response is illustrated by the results of the titration experiment depicted in Fig 5.6C. It is also possible that adjuvanticity of sonicated tumour cells varies from culture to culture regardless of whether the sonicated cells are from sphere or parental cultures (Appendix B, Fig B5.2). Thus, whereas the Lysate-Gal vaccine was designed specifically to create vaccines of equivalent adjuvanticity and antigen quantity, the same cannot necessarily be said for the vaccines used in these two studies.

To complete this re-examination of the earlier GL261 study, and with the greatest respect for the pioneering work of this group, I question the emphasis placed on the finding that the sphere-pulsed DC vaccine outperformed the adherent (parental) cell-derived vaccine when used to treat implanted sphere cell tumours. The importance the authors attach to this is partly due to their findings that sphere cell tumours were more infiltrative, more rapidly lethal, and more closely resembled human gliomas, thus implying the sphere cell to be of greater clinical relevance. However, this experiment could be interpreted as simply showing that whole tumour cell-based vaccines are more likely to be protective when the cells used to make the vaccine and those used to seed the target tumour are the same, so that the antigens targeted by the vaccine most closely align with those expressed by the target cells. In my opinion, the more relevant experiment is that which tested the

two vaccines against the parental cell tumour. Consistent superiority of the sphere-pulsed vaccine in this context would have provided some support for the authors' conclusion that *"targeting of GL261 [sphere] cells depletes the tumor of its hierarchically and most relevant population: Cancer Stem Cells"*.

In summary, the work presented in this chapter has shown that, when adjuvanticity and antigen quantity are held constant, a GL261 sphere cell-based vaccine does not provide superior protection against a tumour challenge than a parental cell-based vaccine. While this conclusion at first glance appears to be discordant with that reached by a previous similar study, there were significant methodological differences between the two studies and, in my opinion, some of the conclusions reached by the earlier study may not have been justified. The two studies did find common ground in observing that culture of GL261 in SC medium induces a more stem cell-like phenotype that gives rise to more infiltrative tumours.

One of the most attractive aspects of the CSC hypothesis is the proposed existence of a subset of cells with the unique capacity to initiate and maintain tumours. If true, targeting these cells with treatment becomes necessary but also sufficient. The failure of the sphere lysate vaccine to elicit superior protective immunity against implanted GL261 tumours in this study raises the distinct possibility that there is no CSC/SLCC subset to target in this cell line. Published reports supporting the existence of a small subset of CSC in the GL261 cell line (Anhwa Wu et al. 2008) and other rodent glioma cell lines (Kondo et al. 2004) were the basis for selecting the GL261 model for this project. However, it seems intuitively unlikely that a cell line cultured *in vitro* for several decades as an adherent monolayer in the presence of serum would retain a CSC subset (Zheng et al. 2007). The findings presented here have shown that GL261 cells grown in standard culture media are universally CD44⁺ CD133⁻ and possess potent tumour initiation ability, with the capacity to initiate tumours after intracranial implantation of as few as 500 cells. GL261 is also by definition capable of self-renewal because it can be serially transplanted and maintained indefinitely in culture (Szatmari et al. 2006). A CD44⁺ CD133⁻

phenotype has been proposed for CSC in human GBM of mesenchymal type (Lottaz et al. 2010). Thus GL261 could be seen as a homogeneous population of CSC-like cells. Similar conclusions have been reached by others with respect to another rodent glioma cell line. Whereas Kondo and colleagues had previously reported finding a small subset of exclusively tumourigenic cells in the C6 glioma cell line (Kondo et al. 2004), Zheng and colleagues found in the same cell line that 100 % of single cells formed colonies *in vitro* that in turn all formed brain tumours in immunodeficient mice on implantation (Zheng et al. 2007). Unless the target tumour is heterogeneous, the concept of selectively targeting a CSC subset becomes invalid.

On reflection, a whole tumour cell-based vaccine may not be the optimal way of selectively targeting a particular tumour cell subset or phenotype. The immunodominant antigens responsible for the efficacy of vaccines targeting unselected antigens derived from whole GL261 tumour cells have not been characterized (Grauer et al. 2008). Although culturing cells in SC medium may well have resulted in a lysate skewed towards proteins expressed by CSC or SLCC, the proportion of protein in the lysate that was specifically CSC- or SLCC-associated may have been too small to materially alter the resulting immune response. Put another way, the lysates may all have included a highly dominant non-CSC-associated antigen common to all tumour cell types that negated any benefit of increasing the proportion of CSC-associated antigens. Theoretically, a vaccine that only contained CSC-specific antigens would selectively target CSC more efficiently but such antigens, if they exist, have yet to be defined in GL261. As discussed above, SOX2 and SOX6 are candidate CSC-associated antigens for human GBM tumours. It is interesting to speculate that Musashi-1, an RNA-binding protein expressed in NSC and shown here to be transcriptionally upregulated by GL261 sphere cells, could also potentially be a CSC-associated target for glioma immunotherapy.

Although this study did not produce “proof-of-principle” data to support the strategy of targeting CSC or SLCC with immunotherapy, this may have been due to the glioma model and/or the vaccine design. The possibility that this approach

might have merit in the clinic has not been excluded. Much of the literature on this topic is confused by incorrect assumptions and poorly-defined terms but there is, at least, strong evidence that GBM tumours include some cells that are more stem-like than others and that these could be a legitimate target for novel therapies. It will be interesting to see what unfolds in this field. Other putative glioma CSC surface markers continue to be described including CD90 (He et al. 2012), A2B5 (Ogden et al. 2008), Aldehyde dehydrogenase 1 (ALDH1) (Rasper et al. 2010) and integrin alpha-6 (Lathia et al. 2010), and autofluorescence has also been proposed as a marker-independent method of isolating glioma CSC (Clément et al. 2010).

Regardless of whether the CSC model proves to be applicable to GBM tumours, there may be advantages to culturing autologous tumour cells in SC media before incorporation into DC-based vaccines to more faithfully preserve the phenotype and genotype of the original tumour (Fael Al-Mayhany et al. 2009; J. Lee et al. 2006b). With respect to cancer in general, the prospect of finding cancer's "Achille's heel" has at least galvanised research into many aspects of tumour cell biology and, in particular, has brought the idea of tumour cell heterogeneity sharply into focus, including the concept that combinations of treatments may be required to comprehensively target all tumourigenic cell populations.

CHAPTER SIX: TARGETING CHEMORESISTANT TUMOUR CELLS TO IMPROVE IMMUNOTHERAPY FOR GLIOMA

6.1 INTRODUCTION AND OBJECTIVES

6.1.1 Combining immunotherapy and chemotherapy for cancer

High grade glioma tumour cell populations are highly heterogeneous in their morphology, genetics and biological behaviour (Bonavia et al. 2011). This heterogeneity can arise from the inherent genetic instability of cancer cells or from epigenetic changes induced by the tumour microenvironment. Phenotypic differences contribute to variability in the susceptibility of tumour cells to different treatments such as radiotherapy, chemotherapy and immunotherapies. Single modality treatment often leads to the selection of resistant clones and clinical recurrence. Because of this, there is growing interest in combining different treatment modalities to exploit additive and/or synergistic effects (Lake and Robinson 2005).

The standard treatment for GBM consists of maximum safe surgical resection followed by a combination of external beam radiotherapy and systemic chemotherapy. TMZ is administered concomitantly with radiotherapy for 6 weeks, followed by an adjuvant phase of 6 monthly cycles of TMZ alone. This regimen was designed in part to exploit complementary effects of radiotherapy and TMZ observed *in vitro* (Stupp et al. 2005; Wedge et al. 1997; W. Wick et al. 2002). The adoption of the “Stupp Protocol” as the standard of care has improved outcomes but 5 year survival for GBM patients is still under 10 % (Stupp et al. 2009). There is therefore still a pressing need to find a better treatment.

There are several reasons to consider a combination of chemotherapy and immunotherapy for the treatment of GBM. Firstly, there is the practical consideration that chemotherapy is already an established part of standard

treatment for newly presenting patients. It has recently been shown that combining DC-based immunotherapy with the adjuvant phase of cyclical TMZ is feasible (Ardon et al. 2010b). Secondly, in the recurrent setting, when radiotherapy is no longer a treatment option, chemotherapies and immunotherapies in their current form have been generally disappointing as stand-alone treatments. It is therefore logical to explore novel combinations of these two modalities. Thirdly, although combining cytotoxic chemotherapy with T cell-based immunotherapy is perhaps counter-intuitive, there is already incontrovertible evidence that these two modalities can have synergistic effects (reviewed in Lake and Robinson 2005; Schlom et al. 2007; Schlom 2012).

When there is significant tumour burden, one role for chemotherapy can be simply to reduce surgically inaccessible tumour bulk prior to vaccination. It is generally accepted that immunotherapy is more likely to succeed in a setting of minimal disease burden, probably because of the many immunosuppressive mechanisms associated with established tumours (Bilusic and Gulley 2012; Gulley et al. 2011). Another way in which the direct tumouricidal action of chemotherapy can augment anti-tumour immunity is by creating a large number of dying tumour cells that serve as a source of antigen combined with “danger” signals capable of priming or boosting anti-tumour immune responses. Nowak and colleagues first demonstrated that the apoptosis-inducing nucleoside analog gemcitabine could induce cross-presentation of a model tumour-associated antigen *in vivo* (Nowak et al. 2003). Subsequently, Zitvogel and others have shown that the key to chemotherapy-induced immunogenic tumour cell death is exposure or release of DAMPs including calreticulin (Obeid et al. 2007), High Mobility Group Box Protein1 (HMGB1) (Apetoh et al. 2007), uric acid (Hu et al. 2004), heat shock proteins (Binder and Srivastava 2005) and ATP (Ghiringhelli et al. 2009).

This ability of chemotherapy to induce immunogenic cell death may be a major reason for the therapeutic benefit of some anti-cancer agents in the clinic (Apetoh et al. 2008). Mihich showed in 1969 that the ability of chemotherapy agents to

eradicate tumours in several mouse models was abrogated by thymectomy or whole body irradiation (Mihich 1969). In humans, patients with breast cancer who have a loss-of-function allele at the loci encoding TLR4 or the P2X₇ purinergic receptor relapse more quickly after chemotherapy (Apetoh et al. 2007; Ghiringhelli et al. 2009) consistent with a requirement for immune cell activation in order for chemotherapy to be fully effective. The quantity of antigen released from dying cells in response to chemotherapy appears to be important. Hengst and colleagues found in an animal model that cyclophosphamide was effective against established plasmacytomas *in vivo*, an effect that required the presence of lymphocytes, but was not effective against small non-palpable tumours (Hengst et al. 1981).

Applying the phenomenon of immunogenic cell death to the design of anti-cancer vaccines, Zitvogel and colleagues showed that tumour cells exposed to lethal doses of doxorubicin *in vitro* are effective at protecting against tumour challenge when administered as a vaccine without any additional adjuvant (Casares et al. 2005). Mechanistically, the authors showed that the chemotherapy-treated cells, but not cells killed by freeze-thaw, were efficiently phagocytosed by DC *in vitro*, and tumour protection *in vivo* was dependent on CD8⁺ T cells. Importantly, the induction of immunogenic cell death is not a universal property of all cytotoxic agents. Although doxorubicin and γ -irradiation have this effect, other DNA-damaging agents such as mitomycin C and etoposide do not (Casares et al. 2005; Obeid et al. 2007).

Chemotherapy does not necessarily need to induce cell death to render tumour cells more immunogenic. It has recently been shown that exposure *in vitro* to very low non-cytotoxic levels of doxorubicin or paclitaxel increases the immunogenicity of human colon cancer cells when loaded onto DC (Kaneno et al. 2011). There is also abundant evidence that sublethal chemotherapy may increase tumour cell susceptibility to CTL-mediated killing in a number of ways. Tumour cells surviving exposure to doxorubicin *in vitro* have been shown to upregulate MHC class I expression and this in turn increased susceptibility to CTL-mediated lysis (Fisk and Ioannides 1998). Treatment with paclitaxel, cisplatin or doxorubicin upregulates

mannose-6-phosphate receptor expression by tumour cells that in turn make them more permeable to granzyme B (Ramakrishnan et al. 2010). In a mouse model of lung cancer, exposure to a sublethal dose of two platinum-based agents caused tumour cells to downregulate expression of anti-apoptotic survival genes and increase Fas expression, and rendered the cells more susceptible to Fas-dependent CTL lysis *in vitro* (Gameiro et al. 2011). Doxorubicin and an anti-Fas mAb have been shown to have synergistic cytotoxic effects against bladder carcinoma cells (Mizutani et al. 1997) and renal carcinoma cell lines (X. X. Wu et al. 2000). Mechanistically, it was shown that doxorubicin increased tumour cell expression of Fas and p53, and increased the activity of caspase 3 (X. X. Wu et al. 2000). Synergism with the anti-Fas mAb was not seen with other kinds of chemotherapy, suggesting this mechanism may be specific to anthracyclines.

In addition to direct effects on tumour cells, chemotherapy can also potentiate immunotherapies by modulating the immune system. The most striking example of this comes from the field of adoptive cell transfer. In mice, it has long been known that prior lymphodepletion with cyclophosphamide enhances the survival and therapeutic effect of adoptively transferred tumour-reactive lymphocytes (Berenson et al. 1975; Greenberg and Cheever 1984; Proietti et al. 1998). In human patients, the transfer of large numbers of *ex vivo*-expanded tumour-reactive T cells is capable of inducing regression of large solid tumours (Steven A Rosenberg et al. 2008) and this treatment is more effective when patients are lymphodepleted prior to transfer with either cyclophosphamide or total body irradiation. In a series of studies using different lymphodepletion regimes (reviewed in Steven A Rosenberg and Dudley 2009), the longevity of the transferred cells and the clinical response rate increased in proportion to the degree of lymphodepletion. The cause of these effects is believed to be a homeostatic increase in the levels of lymphocyte survival cytokines IL-7 and IL-15 and reduced competition for these cytokines (Gameiro et al. 2011; Gattinoni et al. 2005b), along with the elimination of immunosuppressive elements such as Treg (Klebanoff et al. 2005a; North 1982).

Pre-treatment with chemotherapy has also been shown to augment active specific immunotherapies in mice (Gameiro et al. 2011; Ghiringhelli et al. 2004; J.-Y. Liu et al. 2007; Machiels et al. 2001; Ramakrishnan et al. 2010) and in some human studies (Bass and Mastrangelo 1998; Berd et al. 1986). As for adoptive cell transfer, a major mechanism of action of cyclophosphamide in this context is believed to be a selective reduction in the number (Ghiringhelli et al. 2004; J.-Y. Liu et al. 2007) and function (Ghiringhelli et al. 2004) of Treg. With respect to TMZ, currently the first line chemotherapy agent for GBM, the effects on Treg are complex. In a rat glioma model, Banissi and colleagues found that a regimen designed to mimic the adjuvant phase of treatment (maximum tolerated dose delivered over 5 days) did not alter the Treg-to-CD4 T cell ratio but the ratio was reduced by a metronomic regimen (very low dose TMZ given daily for 3 weeks) (Banissi et al. 2009). Contrary to this, Fadul and colleagues assessed PBMC in 10 patients after the concomitant phase of TMZ and radiotherapy and found that although the absolute number of CD4 T cells decreased as expected, the absolute number of Treg was unchanged, resulting in a higher Treg-to-CD4 T cell ratio (C. E Fadul et al. 2011a). In a clinical trial of an EGFRvIII-specific peptide vaccine for GBM, in which vaccination was alternated with adjuvant cycles of TMZ, lymphopaenia was observed as expected but, when assessed after the 6th cycle of TMZ, there was no change in the proportion of T cells that were Treg in patients receiving standard adjuvant TMZ (200mg/m² for 5 days every 28 days). Patients randomly assigned to receive vaccine plus an alternative “dose-intensified” regime (100 mg/m² for 21 days every 28 days) experienced a more severe degree of lymphopaenia and the proportion of T cells that were Treg increased from 6.1 % to 8.7 % (Sampson et al. 2011). Despite this, peptide-specific humoral and cell-mediated responses were of greater magnitude in the dose-intensified group suggesting that the advantages of chemotherapy-induced lymphopaenia outweighed the disadvantage of an adverse Treg-to-Effector T cell ratio. In addition, TMZ has been reported to inhibit the suppressive effects of Treg in vitro (Banissi et al. 2009) and to inhibit the secretion by GBM cell lines of CCL2, a chemokine thought to play a major role in recruiting Treg to the tumour site (Jordan et al. 2008).

Chemotherapy can modulate the immune system to enhance subsequent immunotherapy in a number of other ways including the selective sparing of memory T cells (Hermans et al. 2003a; Turtle et al. 2009), the recruitment of tumour-infiltrating $\gamma\delta$ T cells that in turn recruit CTL (Ma et al. 2011), and direct effects on DC such as upregulation of antigen-processing machinery, expression of co-stimulatory molecules and IL-12 production (Shurin et al. 2009). The effects of chemotherapy on circulating MDSC in cancer patients are agent-specific. Whereas gemcitabine has been reported to selectively deplete MDSC (E. Suzuki et al. 2005), a cyclophosphamide/doxorubicin regimen has actually been shown to increase MDSC in patients with advanced cancer (Diaz-Montero et al. 2009).

This discussion has so far focused on immune-potential by chemotherapy, a mechanism termed “immunocentric chemoimmunotherapy” by Radfar and colleagues (Radfar et al. 2009). There has been less emphasis on the inverse effect, namely the potentiation of chemotherapy by prior immunotherapy (“chemocentric chemoimmunotherapy”). There are reports from a range of different cancer settings of better than expected responses to salvage chemotherapy in patients who have relapsed after previous immunotherapies (reviewed in Ramakrishnan et al. 2008). In patients with GBM who had previously participated in clinical trials of autologous tumour lysate-pulsed DC vaccination, it was observed that some patients responded unusually well to salvage chemotherapies, and survival of patients receiving DC vaccination and chemotherapy was longer than patients treated with DC vaccination or chemotherapy alone (Wheeler et al. 2004). Several groups have observed chemopotentiating effects of immunotherapy in animal models. Machiels and colleagues (Machiels et al. 2001) used a model of a tumour-associated self-antigen in which transgenic mice ubiquitously expressing Her2/neu were implanted with a tumour also expressing Her2/neu. In this model, vaccination against the “self”-antigen was ineffective. Doxorubicin was effective but only when given after the vaccine, at a dose that was ineffective as a stand-alone treatment. Hermans and colleagues (Hermans et al. 2003a) used a B16 melanoma model in which the tumour cells were modified to express an influenza peptide. Cyclophosphamide or a vaccine targeting

the influenza epitope were ineffective alone but cyclophosphamide became effective when given after the vaccine. One possible explanation for the efficacy of chemotherapy in these two studies, both of which were performed in a therapeutic setting in tumour-bearing animals, is that immunogenic tumour cell death induced by the chemotherapy agent acted to boost a weak vaccine-induced primary response. Radfar and colleagues (Radfar et al. 2009) found that pre-infusing non-specifically activated polyclonal CD4 T cells dramatically enhanced the efficacy of a number of chemotherapy agents including TMZ. This effect was found to be dependent on soluble factors secreted by the T cells including, but not solely explained by, IFN- γ . The authors speculated that the synergy between these two treatments might reflect a combination of their pro-apoptotic effects but also drew attention to work by others showing that inflammatory cytokines can inhibit DNA repair mechanisms. For example, Type 1 interferon has been shown to downregulate expression of the DNA repair enzyme MGMT and thereby sensitize glioma cell lines to TMZ *in vitro* (Natsume et al. 2005).

6.1.2 Immunotherapy targeting chemoresistant glioma cells

“Immunocentric” and “chemocentric” combinations use one treatment to augment the effect of another. Another model for combination treatment is the separate targeting of non-overlapping subsets within a heterogeneous tumour. The idea that the immunotherapy component of a combined chemoimmunotherapy regimen could be used to specifically target the chemoresistant compartment was proposed by Fisk and colleagues (Fisk and Ioannides 1998). This group first exposed tumour cells to doxorubicin *in vitro* to develop a chemoresistant cell line. After treating tumour cells with polyclonal CTL *in vitro*, they found that the residual “immuno-edited” cells were more chemosensitive than before CTL treatment. They observed that chemoresistant cells expressed higher levels of MHC class I that may have rendered them more vulnerable to CTL-mediated lysis in this model. Liu and colleagues extended this concept by proposing that immunotherapy could

selectively delete chemoresistant cells by directly targeting the same molecules that confer chemoresistance (G. Liu et al. 2005). This idea arose from their observation that vaccination of GBM patients with tumour lysate-pulsed DC elicited T cell responses specific for TRP-2, a protein implicated in mediating resistance to chemotherapy agents. TRP-2 is a melanoma-associated antigen that is also expressed by many GBM tumours, and can be naturally processed and presented on MHC by glioma cells (G. Liu et al. 2003; G. Liu et al. 2005). The mechanism by which increased TRP-2 expression increases drug-resistance is unclear. It does not appear to act indirectly by altering the levels of other drug-resistance proteins (G. Liu et al. 2005). Because TRP-2 transfection confers a broad resistance to both DNA-damaging drugs and radiation, it has been proposed that it may act by providing a general protection against apoptosis (Chu et al. 2000; Nishioka et al. 1999; Pak et al. 2001). Wheeler and colleagues provided evidence that in some GBM patients undergoing DC vaccination the development of a TRP-2-specific CTL response was associated with immunoediting of TRP-2^{hi} tumour cells, with tumour cells sampled after vaccination expressing lower levels of TRP-2 RNA and being more sensitive to chemotherapy *in vitro* than GBM cell lines derived from pre-vaccination specimens from the same patients (G. Liu et al. 2005). They further showed that transfection of a glioma cell line with TRP-2 increased resistance to TMZ and carboplatin and, when this TRP-2-transfected glioma cell line was subjected to lysis *in vitro* by a TRP-2-specific CTL line, the surviving “immunoselected” cells were more sensitive to TMZ and carboplatin than unselected cells.

A number of other proteins are known to be both glioma-associated antigens and implicated in mediating resistance to various chemotherapy agents. In theory, immunotherapies targeting these proteins have the potential to synergise particularly well with chemotherapy agents. EGFRvIII is a constitutively active mutant form of EGFR that is expressed by approximately one third of GBM tumours (Heimberger et al. 2005). A clinical trial of TMZ combined with an EGFRvIII peptide vaccine recently reported improved outcomes compared with historical controls treated with TMZ alone (Sampson et al. 2011). Although a specific role for EGFRvIII

in conferring resistance to TMZ has not been described, EGFRvIII has been implicated in mediating resistance to other chemotherapy agents (Montgomery et al. 2000). This raises the possibility that the improved outcomes in this trial were due to depletion by the vaccine of the most chemoresistant cells.

Survivin is a protein with anti-apoptotic activity that is selectively expressed in tumours (Trabulo et al. 2011) including almost all GBM tumours (Chakravarti et al. 2002; Preusser et al. 2005). Survivin is also implicated in mediating chemoresistance. Knockdown of survivin expression increases the sensitivity of cancer cell lines (Trabulo et al. 2011) including GBM cell lines (George et al. 2010) to chemotherapy agents *in vitro*, and reverses doxorubicin resistance (Trabulo et al. 2011). Survivin inhibitors are currently being assessed in clinical trials (Pennati et al. 2008) but there is evidence that survivin may also serve as a target for immunotherapy. CTL specific for survivin-derived epitopes have been detected in patients with various different cancer types (M. H. Andersen et al. 2001a; M. H. Andersen et al. 2001b). In a mouse model, DC pulsed with a survivin-derived long peptide induced a CTL and CD4 T cell response that was therapeutically-effective against survivin-expressing GL261 intracranial tumours (Ciesielski et al. 2008). Survivin-specific vaccines have already been tested in combination with chemotherapy in animal models. Kim and colleagues (C.-H. Kim et al. 2007) found that treatment with a DC vaccine loaded with recombinant survivin protein combined with low dose TMZ was effective against established intracranial GL26 tumours in mice, when both treatments showed minimal or no effect when used alone. More recently Zhang and colleagues (H. Zhang et al. 2012) reported synergism between carboplatin and a vaccine targeting survivin in a murine melanoma model.

6.1.3 Doxorubicin and GBM

As will be seen, the experiments described in this chapter involved the use of doxorubicin. For this reason, a brief introduction to this chemotherapeutic agent follows. There is considerable interest in doxorubicin as a potential treatment for high grade glioma. Doxorubicin is one of the anthracycline group of drugs (also known as adriamycin) that binds to DNA and inhibits the enzyme topoisomerase II (Topo II). The role of Topo II is to induce temporary double strand DNA breaks that help “unwind” the double helix during DNA replication and RNA transcription. Anthracyclines stabilise the intermediate “cleavage complex” between DNA breakage and reunion, thereby resulting in persistent double strand DNA breaks (Hortobágyi 1997; Nielsen et al. 1996; Tewey et al. 1984). This in turn leads to G2-M phase arrest and subsequently to caspase-dependent apoptosis (Opel et al. 2008; Supiot et al. 2005). Doxorubicin is commonly used in the clinic as a treatment for breast cancer and is therefore widely available. Although GBM tumours are usually highly sensitive to doxorubicin *in vitro*, the drug does not cross the blood-brain barrier and no clinical benefit has been reported when doxorubicin has been added to the standard treatment for patients with newly diagnosed GBM (Ananda et al. 2011; C. P. Beier et al. 2009). Efforts to improve access to the CNS are ongoing, including novel formulations of doxorubicin (Cheng et al. 2012; Sirova et al. 2007; Wohlfart et al. 2011) or direct delivery to the tumour site (Boiardi et al. 2003; Kang et al. 2011).

The ability to induce immunogenic tumour cell death makes doxorubicin a promising candidate for inclusion in combined chemo-immunotherapy approaches. However, the design of combined regimens need to take into account myelosuppressive effects, with dose-limiting myelosuppression and cardiac toxicity being the most common side-effects of doxorubicin at standard doses. At lower doses, doxorubicin has immunomodulating effects that have been shown in animal models to synergise with active specific immunotherapies (Eralp et al. 2004; D. Kim et al. 2008; Machiels et al. 2001) and with IL-2 or TNF- α cytokine therapy (Ewens et

al. 2006; Gautam et al. 1991; Mihich and Ehrke 2000) to evoke tumour-specific T cell responses. The underlying mechanisms for this synergism are not entirely clear. In some cases, it is possible that sublethal but pro-apoptotic effects of doxorubicin combine with signals from death receptors delivered by cytokines or immune cells to reach a threshold for tumour cell death. Doxorubicin has been shown to synergise with anti-Fas antibodies to kill human cancer cell lines *in vitro* (Mizutani et al. 1997; X. X. Wu et al. 2000), including GBM (Herrmann et al. 2008). Alternatively, doxorubicin can directly modulate the immune system, in ways that appear to be distinct from other immunomodulating chemotherapies such as cyclophosphamide. It is reported, for example, that doxorubicin does not significantly reduce Treg number or function, even at doses that induce transient lymphopenia (J. Y. Park et al. 2009; Skak et al. 2009). Mihich and colleagues (Mihich and Ehrke 2000) reported that doxorubicin promotes the tumouricidal capacity of macrophages.

Several different mechanisms for resistance to doxorubicin have been described *in vitro* but so far only one has been proven to be clinically relevant – namely that mediated by p-Glycoprotein, also known as ABCB1 or multidrug resistance protein 1 (MDR1) (de Jonge-Peeters et al. 2007). Breast cancer that responds initially to doxorubicin is typically multi-drug resistant at recurrence due to upregulation of this protein (Bargou et al. 1997). ABCB1 is a membrane-bound transporter protein that confers multi-drug resistance by expelling chemotherapy agents from the cell, including doxorubicin. Doxorubicin is also a substrate for two other members of this transporter family, ABCC1 (also called multidrug resistance-associated protein, MRP) and ABCG2 (also called breast cancer resistance protein, BCRP), both of which have also been identified in tumour cells (de Jonge-Peeters et al. 2007; Nielsen et al. 1996). There is already some evidence that these molecules can provide immunogenic epitopes. Niethammer and colleagues (Niethammer et al. 2005) developed a DNA vaccine encoding ABCB1 that conferred protective CD8 T cell-mediated immunity against multi-drug resistant tumours in mice. Perrin and colleagues (Perrin et al. 2007) demonstrated that a vaccine incorporating ABCB1 and an adjuvant raised anti-ABCB1 antibodies in mice and serum from the

immunized mice was able to restore chemo-sensitivity of multi-drug resistant cell lines *in vitro*.

6.1.4 Hypothesis and Aims

In summary, there are good theoretical and practical reasons for combining immunotherapy and chemotherapy for the treatment of GBM. As previously discussed, the Glioma-Gal vaccine design holds promise as a relatively simple, personalised vaccine that has the potential to provide a broad range of MHC class I- and class II-restricted antigens, and can be used in all patients regardless of HLA type. The experiments in this chapter explore the possibility of using a modified version of this vaccine to specifically target chemotherapy-resistant tumour cells. In a combined regimen with chemotherapy, such a vaccine might be more effective than the standard vaccine because the vaccine and the chemotherapy agent may selectively target different cell subsets (chemoresistant and chemosensitive cells respectively).

Although the ultimate aim of this work was to find ways of maximizing the therapeutic benefit of the Glioma-Gal vaccine, in this Chapter the experiments used the same Lysate-Gal design described in Chapter 5 in order to more accurately assess the effect of varying the antigen content of the vaccines. To target chemoresistant cells, the chosen method was to make a Lysate-Gal vaccine using an *in vitro*-generated chemoresistant tumour cell line. This approach assumes that cells are drug-resistant *in vitro* because they (a) express or upregulate certain proteins, (b) that the same proteins that mediate drug-resistance *in vitro* are also responsible for drug resistance *in vivo*, and that (c) these proteins can serve as antigenic targets for an anti-tumour adaptive immune response when over-expressed on tumour cells. Although TMZ is the established first line chemotherapy agent for GBM, preliminary studies in our laboratory showed that the GL261 line was resistant to TMZ *in vitro* (data not shown) making it unsuitable for use in this model system.

GBM tumour cells and GL261 are both sensitive *in vitro* to doxorubicin and this agent continues to attract interest as a possible treatment for GBM patients. Thus doxorubicin was chosen as the chemotherapy agent for this study.

To address the research aims, it was necessary to use a model suitable for assessing the combination of chemotherapy and antigen-specific immunotherapy in glioma-bearing mice. GL261, a murine glioma cell line that can be implanted i.c. or s.c. in syngeneic immunocompetent hosts, was felt to be an appropriate model system for this study. Because the combined treatment would necessarily be tested in a therapeutic setting, I elected to first test the combinations against s.c. tumours so that tumour size could be tracked over time.

Hypothesis:

That a whole tumour cell-based vaccine targeting chemoresistant tumour cells will be more effective than standard vaccine when used in a combined treatment regimen with chemotherapy.

Aims:

1. Generate and characterise a doxorubicin-resistant GL261 cell line.
2. Construct a whole tumour cell-based vaccine from doxorubicin-resistant tumour cells.
3. Compare the efficacy of this vaccine with the standard vaccine *in vivo* when used in a combination treatment with doxorubicin.

6.2 RESULTS

6.2.1 Stable doxorubicin-resistant GL261 cell lines can be generated and expanded *in vitro*.

In order to construct a whole tumour cell-based vaccine from chemotherapy-resistant tumour cells it was first necessary to generate doxorubicin-resistant GL261 cells *in vitro*. A single exposure method was tried initially, with doxorubicin added to the culture media for 16 hours, after which cells were harvested, washed, replated in normal media, and allowed to recover. Titration experiments established an appropriate dose (0.1 μM) that resulted in the survival of a small proportion of cells that slowly recovered in fresh drug-free media to form a confluent culture over 7-10 days. When exposed to doxorubicin a second time, the “recovered” cells were able to tolerate a higher dose, confirming that the method had indeed induced, or selected for, chemoresistance. However, this increased resistance was not sustained over time, being quickly lost after 1 or 2 passages in drug-free media (data not shown). Thus, this technique did not generate a stable population of chemoresistant cells in sufficient quantity to make the Lysate-Gal vaccine.

To achieve this objective, cell cultures were instead exposed continuously to doxorubicin at low concentrations that were gradually increased over time. Cells that survive and resume proliferating in drug-containing media have been termed “drug-tolerant expanding persisters” or DTEP (S. V. Sharma et al. 2010).

Doxorubicin is known to induce G2-M cell cycle arrest. In line with this, after adding 0.01 μM doxorubicin to the media of adherent GL261 cultures, an initial period of complete growth arrest occurred followed by a phase of cell death and detachment. Eventually, after 2 weeks, a small number of surviving cells began to proliferate albeit at a slower rate than untreated parental cultures. After reaching confluence, cells were harvested and replated in media containing 0.05 μM doxorubicin. This process was repeated using progressively higher concentrations of drug. In this way, a cell line with the ability to grow in the constant presence of 0.1 μM doxorubicin was established (hereafter termed DTEP 0.1). It was possible to generate DTEP cell

Figure 6.1. Characterisation of drug-tolerant expanding persister (DTEP) GL261 cell lines generated by chronic exposure to doxorubicin *in vitro*. A, Proliferation rate of GL261 DTEP cell lines in media containing doxorubicin at the concentration appropriate for each cell line, as assessed by thymidine incorporation. One experiment. c.p.m. = counts per minute. B, Proliferation rate of GL261 DTEP 0.1 and the parental cell line in media containing varying concentrations of doxorubicin, as assessed by thymidine incorporation. Statistical analysis was undertaken by performing a Mann-Whitney test at each time point. One of three experiments with similar results is shown. C, Expression of ABC genes in DTEP 0.1 and DTEP 5 cell lines compared to parental GL261 tumour cells, assessed by real-time reverse transcription polymerase chain reaction (RT-PCR). Fold change of gene expression in DTEP cells relative to parental cells was calculated from the means of triplicate samples. Results from one of two separate assays performed on the same samples with similar results is shown. D, Expression of a panel of stem cell-associated genes and selected tumour-associated antigens in DTEP 0.1 cells compared to parental cells, assessed by RT-PCR in triplicate. Results of two independent experiments using different DTEP 0.1 lines are shown.

lines capable of growing in even higher concentrations but the rate of growth progressively declined with increasing drug concentration, making it impractical to generate sufficient quantities of tumour cells to make vaccines in a reasonable timeframe (Fig 6.1A). Untreated parental cells that had not undergone this process of incremental exposure did not survive continuous exposure to 0.1 μ M doxorubicin, demonstrating that DTEP 0.1 had indeed acquired chemoresistance. To confirm chemoresistance, parental and DTEP cell lines were exposed to different concentrations of doxorubicin and proliferation measured using thymidine incorporation over 48 hours. This confirmed the DTEP cell lines to be more resistant to doxorubicin than the parental line, with the DTEP 0.1 cell line having an IC₅₀ (the concentration of drug at which proliferation is reduced by 50%) approximately ten-fold higher than parental cells (Fig 6.1B).

Previous studies have reported a strong association between cellular resistance to doxorubicin and expression of the ABC transporter ABCB1 (de Jonge-Peeters et al. 2007). Accordingly, expression of ABCB1 was higher in DTEP cells compared with parental cells, a pattern not consistently observed for ABCC1 or ABCG2 (Fig 6.1C).

Because of reports that CSC are more chemoresistant than bulk tumour cells (G. Liu et al. 2006), expression of genes associated with normal neural stem cells was also assessed to see if induction of chemoresistance had induced a more stem cell-like phenotype. A striking increase in the expression of Musashi-1 was observed but no general upregulation of other “stemness” genes was seen (Fig 6.1D). Interestingly, this pattern was similar to that seen in GL261 spheres grown in serum-free media with EGF and bFGF (see Chapter 5). Expression of the GL261 tumour-associated antigens TRP-2 and gp100 was also assessed. Expression of TRP-2 was increased in the DTEP 0.1 cell line (Fig 6.1D). This finding is consistent with the claim by Liu and colleagues (G. Liu et al. 2005) that TRP-2 also mediates chemoresistance.

In summary, the DTEP 0.1 cell line was shown to be a stable and practical substrate for constructing a vaccine targeting antigens derived from tumour cells that are

chemoresistant relative to parental cells and that have upregulated genes implicated in mediating drug-resistance.

6.2.2 Prophylactic GL261 DTEP vaccine is not more effective than unmodified parental vaccine.

Lysate vaccines were made from parental and DTEP 0.1 cell lines as previously described (Fig 5.5A). The hypothesis under examination was that the DTEP lysate vaccine would be more effective against chemoresistant cells than the unmodified vaccine because the lysate would be enriched in proteins mediating chemoresistance. The DTEP vaccine should therefore be more effective than parental vaccine when combined with doxorubicin. However, there were several reasons why DTEP vaccine might have been a more effective treatment even as monotherapy. Firstly, doxorubicin-induced tumour cell death has been shown to be immunogenic (Casares et al. 2005). Although the lysates used to construct the vaccines in this study were made from a live tumour cell population, and not from dying cells, it was nevertheless possible that the expression of DAMPs might be higher in the DTEP lysate vaccine. Secondly, expression of a known tumour-associated antigen (TRP-2) was increased in the DTEP 0.1 line relative to the parental line, at least at the transcriptional level. Thirdly, the chemo-exposed cells had upregulated at least one stem cell-associated gene. If the DTEP vaccine was enriched in proteins expressed by CSC it might target this putative subset more effectively than the parental lysate vaccine. To compare the performance of the two vaccines as monotherapies, the vaccines were assessed for their capacity to protect against parental tumour challenge. It has already been shown that Lysate-Gal vaccines containing 100 µg protein per mouse provided potent protection against s.c. tumour challenge (Fig 5.6B). The protein content of was therefore reduced to make it possible to detect differences between the DTEP and parental vaccines. In one experiment, vaccines containing 10 µg and 5 µg of protein conferred similar levels of protection, with the parental vaccine performing better at 1 µg (Fig 6.2A).

Figure 6.2. GL261 DTEP vaccine is not more effective than an unmodified parental vaccine.

A, Mice were treated i.v. with parental or DTEP Lysate-Gal vaccines containing different quantities of lysate and challenged s.c. with 1×10^6 GL261 cells 7 days later. 5 mice per group in the 10 μ g arms, 10 mice per group in the other arms. The same No Vaccine control group of 10 mice is shown in each graph. Asterisk indicates comparison between parental and DTEP vaccine groups using the Log-rank test. B, Mice were treated i.v. with Lysate-Gal vaccines containing 10 μ g of parental or DTEP lysate and challenged s.c. with 1×10^6 GL261 7 days later. 10 mice per group. Asterisk indicates comparison between parental and DTEP vaccine groups using the Log-rank test.

In a repeat experiment at the 10 μ g dose, the parental vaccine provided better protection (Fig 6.2B). Thus, the DTEP vaccine was less effective than the parental vaccine, albeit inconsistently across the two experiments. It seems reasonable to at least conclude that the DTEP vaccine was not more effective.

6.2.3 Establishing a model of combined treatment with lysate vaccine and doxorubicin.

Although the DTEP vaccine was not more effective against the parental tumour, this did not exclude the possibility that the DTEP lysate vaccine could be more effective against chemoresistant tumour cells. Theoretically, a simple way of testing this *in vivo* would be to administer prophylactic vaccination followed by challenge with the chemoresistant cell line. However, the DTEP 0.1 cells did not reliably form subcutaneous tumours in mice, even when high numbers of cells were implanted (data not shown). The reasons for this are not clear. One possibility is that the doxorubicin-treated cells were more immunogenic and primed an endogenous anti-tumour immune response that eradicated the tumour before it reached a detectable size.

Unable to test the hypothesis in this way, a combined treatment regimen was designed in which tumour-bearing mice were treated first with doxorubicin and then with the vaccine (Fig 6.3A). For the purposes of the model, the optimal timing for therapeutic vaccination was felt to be no later than one week post-implantation on the grounds that vaccine-induced immune response was not likely to peak for another 7 days (2 weeks post-implantation). Since tumours in some untreated control mice can reach the maximum allowable size within 2-3 weeks, delaying the therapeutic vaccination longer than one week post-implantation would risk needing to cull control animals on ethical grounds before the result of the experiment was clear. Consistent with previous findings for the Glioma-Gal vaccine, the Lysate-Gal vaccine delivered on Day 7 post-implantation was effective in the therapeutic setting only when preceded by depletion of Treg (data not shown and Fig 6.3B). Thus anti-CD25 depleting antibody was administered one week prior to vaccination, on the same day as tumour implantation (Fig 6.3A).

With respect to the chemotherapy component of treatment, there are no previous reports on the use of doxorubicin to treat GL261 tumours *in vivo*. Preliminary studies were therefore required to determine the optimum dosage and timing of

D

Figure 6.3. Validation of a model to test the combined effect of Lysate-Gal Vaccine and Doxorubicin. A, Model schema. Regulatory T cell depletion was achieved by i.p administration of 100 µg of the CD25-depleting antibody PC61. Mice were implanted s.c. with 1×10^6 GL261 cells. B, C, Experiments performed as in A. Groups treated as indicated by legend. 5 (in B) or 6 (in C) mice per group. Shown are group means and SEM. Statistical analysis was performed at time points where there was a complete data set for all groups using a Kruskal-Wallis test with Dunn's multiple comparison post-test. In the experiment represented in B, measurements of a group were continued until one mouse reached an experimental or ethical end point. In the experiment represented in C, in some groups (all except PC61 + Dox + Par Vac) one mouse progressed rapidly and was euthanased early in the experiment. To better reflect the outcome of the experiment, at subsequent time points these mice continued to be allocated their tumour size at time of culling and measurements for that group continued until a second mouse reached an end point. D, Tumour size of individual mice over time, pooled results of both experiments (blue and purple lines show experiments in B and C respectively).

doxorubicin. These studies suggested that a dose of 2.5 mg/kg on Day 3 was just above the threshold for observable effects on tumour growth when given as monotherapy (data not shown and Figs 6.3B-D). This dose of doxorubicin was next tested in combination with the parental lysate vaccine. Immunotherapy alone (Lysate-Gal preceded by Treg depletion) and low dose doxorubicin alone were partially effective (Figs 6.3C,D). The effect of combining the two treatments was at least additive, with permanent regression of established tumours in all mice (Figs 6.3B-D). The performance of the combination still left room for improvement however, with some tumours reaching a diameter of 8-9 mm before regression occurred. Thus the model was confirmed as being suitable for addressing the research aims.

Figure 6.4. GL261 parental and DTEP vaccines have similar efficacy when used in combination with doxorubicin to treat established s.c. tumours. A, B, Experiments performed as in Fig 6.3A. Some mice were vaccinated on Day 7 with 200 ng α -GalCer co-administered with 10 μ g of tumour lysate derived either from unmodified tumour cells (Par Vac) or from the Doxorubicin-resistant DTEP 0.1 cell line (DTEP Vac). These mice were also treated with PC61 antibody on the day of implantation and with Doxorubicin on Day 3. 5 (in A) or 6 (in B) mice per group. In B, three of the experimental groups were shared with the experiment depicted in Fig 6.3C. Shown are group means and SEM. For statistical analysis a Mann Whitney test comparing the two vaccinated groups was performed at each time point.

6.2.4 The GL261 DTEP vaccine is not more effective than parental vaccine when used in combination with doxorubicin to treat established s.c. tumours.

The model was next used to compare the performance of the DTEP and parental lysate vaccines in combination with doxorubicin. In both groups, all established tumours underwent rapid and permanent regression, with no major differences in the time course or rate of regression (Figs 6.4A, B). No statistically significant differences were seen between the mean tumour sizes of the two vaccinated groups in two independent experiments (Figs 6.4A, B). Thus, these experiments did not provide evidence to support the hypothesis that the DTEP vaccine would be more effective against tumours recently exposed to doxorubicin.

6.2.5 Low dose doxorubicin exerts immunomodulatory effects that may potentiate Lysate-Gal immunotherapy.

Regardless of the lysate vaccine used, doxorubicin and vaccination (with prior Treg depletion) were highly effective when used sequentially in that order, and the benefits of the two treatments combined were supra-additive (Appendix B, Fig B5.3). This was the case even though doxorubicin alone at a dose of 2.5 mg/kg does not appear to slow tumour growth in around half of the treated mice relative to untreated control mice (Fig 6.3D). One possibility is that a sublethal dose of doxorubicin may leave tumour cells more vulnerable to subsequent immune system-mediated apoptosis (Mizutani et al. 1997; X. X. Wu et al. 2000). To assess this possibility, GL261 cells were exposed *in vitro* to doxorubicin at different concentrations (Fig 6.5). Using Propidium Iodide staining as a measure of cell viability, a concentration of 0.001 μM was established as a sublethal dose just below the threshold for cytotoxic effects, a finding consistent with earlier observations on the effect of doxorubicin on GL261 cell cultures. Untreated GL261 cells already expressed high levels of the MHC class I molecule H-2K^b and this did not change after doxorubicin exposure (Fig 6.5). It has been shown previously that surface

Figure 6.5. Doxorubicin effect on GL261 tumour cells *in vitro*. A, GL261 cells were exposed to Doxorubicin for 16 hours at different concentrations in triplicate wells and then assessed by flow cytometry. Progressive loss of viability was observed at concentrations above 0.001 μM . B, Tumour cells exposed to the sublethal 0.001 μM dose of Doxorubicin were analysed further for expression of surface molecules that might render the cells more vulnerable to T cell-mediated immunotherapy. Representative histograms (left) and summary of the median fluorescence intensity (median FI) of experimental replicates (right). Blue line: Untreated GL261; Red line: Doxorubicin-treated GL261; Solid grey: Doxorubicin-treated GL261 stained with isotype control antibody. A slight increase in non-specific binding after Doxorubicin exposure was observed for the MHC class II antibody isotype. To adjust for this, the summary graph for MHC class II expression shows median FI relative to median FI of isotype control. No significant differences were observed between groups using a Mann Whitney test. One of two independent experiments with similar results is shown.

expression of MHC class II is not detectable on GL261 cells in culture unless stimulated with IFN- γ (Fig 4.5F). In this analysis, exposure to a sublethal concentration of doxorubicin did not induce surface expression of MHC class II or Fas (Fig 6.5). Thus, no immunopotentiating effect of low dose doxorubicin directly on tumour cells was identified.

An alternative mechanism for the immunopotentiating effect of doxorubicin could be modulation of one or more components of the immune system. Shurin and colleagues have reported that exposure to chemotherapy agents at low doses, including doxorubicin, induces changes in bone marrow-derived DC that enhance antigen presentation to CD8⁺ T cells including increased expression of maturation markers (Shurin et al. 2009). To assess whether this was likely to be occurring in this model, cells of the DC2114 dendritic cell line were exposed to doxorubicin *in vitro* at different concentrations. As for GL261 cells, cytotoxicity was observed at concentrations above 0.001 μ M (Fig 6.6A). Exposure to doxorubicin at this sub-cytotoxic dose did not alter CD40 or CD86 expression by DC2114 cells (Fig 6.6B).

It has been shown previously in this thesis that the Glioma-Gal and Lysate-Gal vaccines require prior depletion of Treg in order to be effective in a therapeutic setting. However, treatment with PC61 antibody depletes only 50 % of FoxP3⁺ CD4⁺ T cells (Appendix B, Fig B2.1 and Ataera et al. 2011). To investigate whether doxorubicin might be acting by more completely depleting FoxP3⁺ Treg, reporter mice that express GFP in FoxP3⁺ cells were treated i.v. with doxorubicin 2.5 mg/kg and splenocytes analysed by flow cytometry 24 hours later. Other selected immune cell populations were also included in this analysis. There was no change in the total number of splenocytes (Fig 6.7B). Surprisingly, the proportion of CD4⁺ T cells that were Treg actually increased in the spleen after low dose doxorubicin (Fig 6.7C and Appendix B, Fig B5.4). Absolute numbers of Treg also increased but this did not consistently reach statistical significance (Fig 6.7C and Appendix B, Fig B5.4).

Statistically significant differences were not observed for CD4⁺ T cells, CD8⁺ T cells, iNKT cells or B cells (Fig 6.7C and data not shown). MDSC are Gr1⁺CD11b⁺

Figure 6.6 Low dose Doxorubicin does not induce DC maturation. A, DC2114 cells were exposed *in vitro* to Doxorubicin for 16 hours at the concentrations shown and then assessed by flow cytometry. Cell viability progressively decreased at concentrations above 0.001 μM . B, Expression of maturation markers by DC2114 cells after exposure to Doxorubicin at the sublethal dose of 0.001 μM . Poly I:C 100 $\mu\text{g/ml}$ was added to some wells as a positive control. Representative histograms (left) and summary of experimental replicates (right). Red line: Dox-treated; Blue line: Medium only; Black line: Poly I:C; Solid grey: Isotype control for Dox-treated cells. A Mann Whitney test was used to compare Dox-treated cells with Medium only. No significant differences were seen. One of two experiments with similar results is shown.

immature myeloid cells that can suppress immune responses in disease states including in tumour-bearing mice (Youn and Gabrilovich 2010). Complete identification of MDSC requires demonstration of a functional capacity for immunosuppression and it is arguable whether MDSC even exist in the resting non-tumour bearing state (Youn and Gabrilovich 2010). Nevertheless, Gr1⁺CD11b⁺ splenocytes were included in this analysis to see whether there was a general susceptibility of this phenotype to doxorubicin. No statistically significant changes were seen in the absolute number or proportion of these cells (Fig 6.7C).

The main conclusion from these findings is that doxorubicin at the dose used in these experiments unequivocally has some immunomodulating effect because the proportion of CD4⁺ T cells that are Treg is increased. However, it is not immediately apparent how this might potentiate the immunotherapy component of the combined treatment. General lymphodepletion can be excluded as a mechanism by the lack of change in the absolute number of total splenocytes or effector lymphocyte subsets. The possible causes and implications of the observed changes in Treg cell numbers will be discussed below.

6.2.6 A combined chemo-immunotherapy regimen that is effective against subcutaneous tumours is not effective in the intracranial setting.

The combination of Treg depletion, low dose doxorubicin and the Lysate-Gal vaccine was highly effective against established s.c. tumours. This same regimen was now tested against established i.c. tumours. Disappointingly, the combined treatment provided no protection or only weak protection (Figs 6.8 and 6.9). One possible explanation for this is that the Blood-Brain Barrier prevented doxorubicin reaching a high enough concentration in the CNS. Although it has been shown above that doxorubicin has some immunomodulatory activity in secondary lymphoid organs, the importance of this is unclear. It remains possible that the immunopotentiating

Figure 6.7. Effects of low dose Doxorubicin on immune cell populations. FoxP3-GFP reporter mice were treated with Doxorubicin 2.5 mg/kg i.v. (Dox) or not treated (Control). Splenocytes were assessed by flow cytometry 24 hours later. A, Gating strategy. B, Total live splenocyte counts. C, Proportions (left) and absolute numbers (right) of selected immune cell types. 5 mice per group. The Mann Whitney test was used for statistical analysis. One of two independent experiments with similar results is shown (see text and Appendix B, Figure B5.4).

Figure 6.8 The combination treatment is less effective against intracranial tumours. Mice were implanted intracranially with 5×10^3 GL261 cells and treated with the same combined immunotherapy and chemotherapy regimen as the experiments depicted in Figs. 6.3 and 6.4. Immunotherapy = Treg depletion with PC61 antibody 100 μ g i.p. on day of implantation followed by Lysate-Gal vaccine (incorporating 10 μ g of parental cell lysate) i.v. on Day 7. Dox = Doxorubicin 2.5 mg/kg i.v. on Day 3. Two independent experiments are shown.

effect of doxorubicin is exerted at the tumour site. It has been argued above that a direct cytotoxic effect on tumour cells seems unlikely but this leaves open the possibility of some immunomodulatory effect of doxorubicin at the tumour site. For example, it has been reported that doxorubicin can enhance the tumouricidal functions of macrophages (Mihich and Ehrke 2000), a mechanism that seems plausible here given that the immunotherapy component is probably mediated primarily by CD4⁺ effector T cells. To investigate whether improved access of doxorubicin to the tumour site would improve efficacy of the combined treatment, the chemotherapy component was changed to a form of doxorubicin that achieves higher concentrations in the CNS and in i.c. tumours.

Figure 6.9. A pegylated liposomal formulation of Doxorubicin did not confer any advantage over the standard formulation. As in Fig 6.8, mice were implanted i.c. with 5×10^3 GL261 cells and then treated with combination immunotherapy and chemotherapy. Doxorubicin 2.5 mg/kg was administered on Day 3 using either standard formulation or a pegylated liposomal formulation (PEG Dox). Two independent experiments were performed. No significant differences between groups were observed.

Liposomal doxorubicin is an alternative formulation of doxorubicin in which the drug is encapsulated within a lipid membrane. Liposomes may also be coated with polyethylene glycol to prolong half-life. Pegylated liposomal doxorubicin (PEG-Dox) persists longer in the circulation and has been shown to reach higher concentrations in intracranial solid tumours than free doxorubicin at equivalent doses (Siegal et al. 1995). PEG-Dox has an established role in the management of breast cancer and ovarian cancer (Ananda et al. 2011; Pisano et al. 2013). In glioma patients, radio-labelled PEG-Dox administered i.v. has been shown to reach a concentration in

tumours 13-19 times higher than in normal brain tissue (Koukourakis et al. 2000). One of the main clinical advantages of PEG-Dox is a reduction in the main dose-limiting toxicities associated with standard doxorubicin such as cardiomyopathy and myelosuppression (Safra et al. 2000; Waterhouse et al. 2001). Thus, substituting PEG-Dox for standard doxorubicin in this model offered a potential way of increasing the concentration of doxorubicin at the tumour site without increasing extra-CNS effects. However, in two independent experiments, combining immunotherapy with PEG-Dox was ineffective against established intracranial tumours (Fig 6.9).

6.3 DISCUSSION AND CONCLUSIONS

The hypothesis tested in this chapter was (a) that a whole tumour cell-based vaccine incorporating antigens derived from doxorubicin-resistant tumour cells would better target doxorubicin-resistant tumour cells than a vaccine made from chemotherapy-naïve tumour cells and (b) that this vaccine would in turn be more effective when used in a combination regimen with doxorubicin. The experiments reported here did not provide support for that hypothesis. The DTEP vaccine offered no advantage over the standard vaccine when used in combination with doxorubicin to treat established GL261 s.c. tumours.

One possible reason for this is that the DTEP vaccine did not, in fact, target doxorubicin-exposed cells any better than the standard vaccine. Unfortunately, it was not possible to directly test the two vaccines against doxorubicin-resistant target cells because DTEP cells would not reliably form tumours after s.c. implantation. The tumour cells used to make the two vaccines undoubtedly differed in their resistance to doxorubicin and their pattern of gene expression but the extent to which this was reflected in the protein content of the two lysates was not fully assessed. A strong vaccine-potentiating effect of doxorubicin could have masked the impact of differences between the antigen content of the vaccines.

Alternatively, the dose of doxorubicin used in this model of combined treatment may have been too low to have a direct effect on tumour cells. Unless doxorubicin directly modified the target tumour cell population in some way to render it more susceptible to a DTEP vaccine-induced immune response, there would be no reason to expect any advantage from the DTEP vaccine. Designing and optimizing a model of combination treatment proved difficult. The rapid growth of GL261 s.c. tumours left a narrow therapeutic window in which to administer the combination of treatments. A number of preliminary studies were needed to titrate both components of the treatment so that each contributed to the therapeutic outcome yet were not so effective together that the capacity to detect differences was lost. In the model finally selected, doxorubicin was administered i.v. three days post-implantation at a relatively low dose of 2.5 mg/kg. Doses higher than this, at this time point, prevented tumours from growing (data not shown) suggesting that the dose of 2.5 mg/kg was close to the threshold for direct tumour cytotoxicity. Consistent with this, there was a non-significant trend towards slower tumour growth in mice treated with doxorubicin alone (Figs 6.3B-D). However, these data fall short of proving direct tumour cytotoxicity. Some spontaneous tumour regressions were observed after s.c. implantation in untreated control mice (Fig 6.3D) consistent with the induction of a spontaneous endogenous anti-tumour immune response. The weak therapeutic benefit of low dose doxorubicin alone could therefore have been due to an immunomodulating effect that promoted the endogenous anti-tumour response, rather than to direct tumour cytotoxicity. Also arguing against a tumouricidal effect of doxorubicin in this model is that some tumours in mice treated with doxorubicin alone continued to grow exponentially in a manner similar to untreated controls (Fig 6.3D). Conversely, the combination of doxorubicin and Lysate-Gal (with Treg depletion) was curative in 100 % of mice treated, suggesting direct tumour cell killing by doxorubicin was not required for the synergistic effect of the combination. If the predominant action of low dose doxorubicin in this model was on immune cells rather than tumour cells then one would not expect any advantage from a vaccine that was designed to target tumour cells modified *in vivo* by exposure to doxorubicin.

On reflection, the method used to generate a vaccine intended to target chemoresistant cells may have been optimistic. As discussed above, significant differences in the DTEP and parental cells must have existed to account for the difference in doxorubicin resistance. However, these differences may not have been reflected in the protein content of the two lysates. Changes in gene expression do not necessarily translate to proportional changes in protein synthesis (Appendix B, Fig B5.5). It is possible that differences in chemoresistance could occur because of differences in the location of a certain protein within an individual cell rather than in the total quantity of that protein per cell. For example, ABCB1 is likely to mediate chemoresistance most efficiently when located on the cell surface. Even if the total quantity of a particular protein such as ABCB1, for example, was increased in DTEP cells, the method used to make the lysates in this study may have led to an under-representation of membrane-bound molecules. Assuming ABC transporters and other proteins mediating doxorubicin resistance were indeed increased in the DTEP lysate as intended, the amount of such protein as a proportion of the total lysate may have been too small to skew the vaccine-induced response towards these targets. And finally, it is not known whether the mechanisms conferring resistance of chemotherapy-naïve tumour cells to a single dose of doxorubicin *in vivo* are necessarily the same as those upregulated by tumour cells chronically exposed to the drug *in vitro* (Ades et al. 2006) raising the possibility that the DTEP lysate may have been enriched for the wrong proteins in this context.

To conclude this part of the discussion, this study did not provide support for the simple idea of exposing tumour cells to chemotherapy *in vitro* before incorporating them into a whole tumour cell-based vaccine for use in combination with the same chemotherapy agent. A similar idea underpinned the clinical trial described in Chapter 3 except that the tumour cells incorporated into the DC vaccine in the trial study had been exposed to TMZ *in vivo* during the course of treatment. The approach is clinically attractive because it is simple, does not require prior knowledge of the particular proteins mediating chemoresistance, and retains all of the advantages of whole tumour cell-based vaccines including the potential to target

multiple chemoresistance pathways at the same time. However, the approach may be too imprecise to achieve its aim. A vaccine with a defined target might be a better strategy. Additive effects have been reported in animal models of immunotherapy-chemotherapy regimens in which the immunotherapy targeted a single protein implicated in mediating chemoresistance (C.-H. Kim et al. 2007; H. Zhang et al. 2012) (although, similar to the study reported here, these studies did not provide any evidence that the vaccine selectively targeted chemoresistant cells). There is evidence *in vitro* that the immunoediting of TRP-2⁺ (G. Liu et al. 2005) and ABCB1⁺ (Perrin et al. 2007) cells can restore chemosensitivity of tumour cell populations. However, in human cancer patients, targeting a single antigen runs the risk of immune-escape or of selecting tumour cells that resist chemotherapy by a different mechanism. To speculate, the optimum approach may be to target several or a few defined chemoresistance-related TAA, accepting that this would require prior definition of the important chemoresistance mechanisms operating in a particular clinical context.

Although the main aim of the study described in this chapter was not achieved, an interesting and potentially valuable finding was that the combination of low dose doxorubicin and the Lysate-Gal vaccine (with prior Treg depletion) had a supra-additive effect that eradicated established s.c. tumours in all treated mice. In contrast, the combination treatment was ineffective, or only weakly effective, against i.c. tumours. This should not discourage interest in the synergistic effect. It should be remembered that the lysate vaccines used in this model were purposefully attenuated to facilitate comparison of the DTEP and parental lysates. It has been shown here, and in the preceding chapter, that the level of tumour protection afforded by the lysate vaccines is sensitive to the quantity of lysate. It is therefore possible that a similar synergistic anti-tumour effect would have been observed in the i.c. tumour experiments if the vaccine had been used at a higher dose.

Initial efforts to elucidate the mechanism underlying the supra-additive effect were inconclusive. The inability of the combination treatment to eradicate i.c. tumours raises the possibility that low dose doxorubicin in this model requires access to the tumour site that is prevented by poor penetration of doxorubicin into the CNS. However, as discussed above, the data reported here suggests that the efficacy of the combined treatment may not have been dependent on a direct tumouricidal effect of doxorubicin. Furthermore, changing doxorubicin to an alternative formulation known to substantially increase CNS tissue concentration without increasing systemic toxicity did not restore efficacy against i.c. tumours. The possibility that low dose doxorubicin might have some sublethal immune-potentiating effect on tumour cells was considered. No change in MHC molecule or Fas expression was detected in this study. Although this does not comprehensively exclude all the ways in which sublethal chemotherapy might render tumour cells more sensitive to killing by CTLs (Hodge et al. 2013), temporal considerations make this an unlikely mechanism in this model. Tumours that were impalpable at the time of doxorubicin treatment continued to grow for 10-14 days and reached a size of at least 20 mm² before eventually regressing. It seems unlikely that changes in tumour cells induced by a single, brief, non-toxic exposure to doxorubicin would have persisted for this length of time and after multiple rounds of division.

Although evidence for a direct effect on tumour cells was lacking, this does not exclude the possibility that doxorubicin had some other important effect at the tumour site such as, for example, depletion or functional suppression of an immunosuppressive cell population or activation of an effector cell type. However, the same objection raised in opposition to a direct effect on tumour cells also applies here - it seems unlikely that a single brief exposure to doxorubicin would continue to exert a major influence on immune cells in tumours that continue to develop and expand for 10-14 days after treatment before regressing. From this temporal perspective, a more likely explanation would seem to be that low dose doxorubicin has an immunomodulating action that impacts on the priming phase of the vaccine-induced immune response. As a starting point, evidence for this possibility was

sought by exposing naïve animals to low dose doxorubicin and assessing immune cell populations in the spleen. Doxorubicin at this dose did not deplete lymphocytes in general or Treg in particular, consistent with a previous report that assessed lymphocytes in peripheral blood (J. Y. Park et al. 2009). In fact, doxorubicin unexpectedly increased the proportion of Treg in the spleen, demonstrating at least some effect on immune cells. One scenario that seems plausible is that doxorubicin administered i.v. is transiently toxic to some host cells, possibly circulating leucocytes, with filtration of dying cells in the spleen leading to increased presentation of self-antigens by splenic APC, leading in turn to a relative accumulation of Treg in the spleen as they recognize cognate antigen. Along with this, it is possible that exposure to DAMPs released by the dying doxorubicin-exposed cells could have an activating effect on splenic DC. In the combined treatment model, the increase in Treg would likely be largely abrogated by the prior administration of Treg-depleting antibody, leaving DC activation as the dominant effect. Further studies to assess the effects of doxorubicin on DC *in vivo* and on the magnitude of the vaccine-induced response in this model would be informative.

A scenario in which doxorubicin acts indirectly to activate APC by immunogenically killing host cells is consistent with the finding that exposure to sublethal doxorubicin *in vitro* did not directly induce maturation of DC2114, a murine DC cell line that has been shown to retain all the major features of wild-type CD8 α ⁺ splenic DC (Fuertes Marraco et al. 2012). Similar findings have been reported using another murine DC cell line (J. Y. Park et al. 2009). Caution is required, however, because the effects of doxorubicin may depend on the type of DC. Shurin and colleagues (Shurin et al. 2009) reported increased expression of CD40, CD80, and MHC class II by murine BMDC following exposure for 48 hours *in vitro* to a non-cytotoxic 2 nM dose of doxorubicin, although the magnitude of these statistically-significant differences was relatively small (the proportion of CD11c⁺ cells that were CD40⁺, for example, increased from 9 % to 11 %). In another study, exposure of murine BMDC to sub-cytotoxic doxorubicin for 24 hours induced increased expression of CD40 but not CD80, CD86, or MHC class II (H. Tanaka et al. 2009). Similar *in vitro* studies have

been performed using human DC. Exposure of human monocyte-derived DC to a non-toxic 10 nM dose of doxorubicin induced increased expression of CD83 but not CD40, CD80, CD86 or MHC class II and did not enhance antigen presentation in a mixed lymphocyte reaction (Kaneno et al. 2009). Another study exposed a DC subset isolated from human PBMC to the same 10 nM dose of doxorubicin and found no evidence of DC maturation. These studies, considered together with my own findings, do not provide strong support for direct activation of DC by low dose doxorubicin being a likely mechanism in the combined treatment model.

The fact that low dose doxorubicin did not alter CD4 or CD8 T cell numbers in the spleen or deplete Treg does not exclude the possibility that doxorubicin modulated the function of these cell types. Kim and colleagues (D. Kim et al. 2008) found that pretreatment with doxorubicin at a higher dose of 5 mg/kg i.v. enhanced the response to a vaccine designed to induce a CD4 T cell response but inhibited a vaccine designed to induce CD8 T cell responses. The same group subsequently showed that, whereas CD4 and CD8 T cells both upregulated CD40L and FasL after doxorubicin exposure *in vitro*, only CD4 T cells upregulated the co-stimulatory receptor 4-1BB (J. Y. Park et al. 2009). These authors concluded that pretreatment with doxorubicin may be particularly useful in augmenting vaccines mediated predominantly by CD4 T cells.

In summary, a vaccine constructed from chemotherapy-resistant cells, used in conjunction with chemotherapy, provided no advantage over a standard vaccine. However, this finding may be specific to this experimental model and does not necessarily invalidate the concept in general. A vaccine that targets chemoresistant cells more efficiently could warrant further investigation. This study did highlight a synergy between the vaccine and low dose doxorubicin. More work is needed to characterize the underlying mechanism but these initial studies point towards doxorubicin having some systemic vaccine-potentiating effect. A more precise understanding of this phenomenon could make a valuable contribution towards

improving the therapeutic benefit of the Glioma-Gal vaccine in particular, and of cancer vaccines in general.

CHAPTER 7: FINAL DISCUSSION

This thesis was motivated by the need to develop a better treatment for patients with GBM. Although immunotherapy has yet to make a major impact in neuro-oncology, lessons learned from clinical trials such as the one described in Chapter 3, together with data obtained from animal models, will hopefully lead to the development of more effective immunotherapy strategies in the future.

The main finding of the Phase I trial was that the combined treatment regimen was safe but that feasibility of the DC-based immunotherapy in the recurrent setting was marginal. The main reason for this was a catastrophic failure of DC vaccine production in three patients. It seems very possible that previous exposure to TMZ compromised the ability of monocytes to differentiate and survive *in vitro*. Age and corticosteroid use may have been factors. An adverse impact of harvesting PBMC (and/or the autologous serum used in cell culture) before tumour debulking surgery also cannot be excluded.

Although the trial was not designed to assess clinical efficacy or immunological outcomes as primary endpoints, improved clinical outcome broadly correlated with the presence of an anti-tumour immune response at the time of TMZ retreatment. Nevertheless, marginal feasibility discourages pursuing this approach in the recurrent setting. To further support this conclusion, very few patients in the trial could be weaned off corticosteroids before vaccination, despite the fact that $\geq 90\%$ tumour debulking was achieved in 13 of the 14 enrolled patients. In contrast, it is common for newly presenting patients to be successfully weaned off steroids completely and to experience a window of general good health after standard treatment. This would seem to be the optimum time to introduce immunotherapies.

In accordance with the trial protocol, treatment was discontinued as soon as radiological progression was detected. This was done to give participants the

opportunity to explore other treatment options if they wished. However, there is a strengthening viewpoint that radiological progression, widely used to define the failure of chemotherapy agents, may not be an appropriate endpoint for immunotherapies that are capable of conferring survival benefits without ever meeting commonly-used criteria for objective response (Hoos et al. 2010; Izumoto et al. 2008; Wolchok et al. 2009). Furthermore, the survival benefits of immunotherapies may only become apparent after several months of treatment (Bilusic and Gulley 2012; Hoos et al. 2010; Izumoto et al. 2008). In future trials, vaccination should probably continue for at least 12 months. In fact, if overall survival is selected as the primary clinical endpoint, an argument can be made for continuing treatment even in the face of radiological progression as long as the patient remains clinically well, particularly while effective salvage treatment options are lacking.

The assessments of vaccine-induced immune responses in trial patients were more difficult to perform and to interpret than anticipated. ELISpot tests using vaccine or tumour lysate for stimulation suffer from an inability to precisely control for non-specific activity induced by dead and dying autologous tumour cells and the possibility that innate cells, such as NK cells, were a source of IFN- γ in these assays cannot be excluded. These tests were also hampered by a shortage of patient material, a situation exacerbated further by the need to prioritise patient treatment. Recovery of viable PBMC from frozen samples also declined as the number of cycles of TMZ received by the patient increased (data not shown). These problems led the investigators to use the limited stock of PBMC in a different way, by assessing responsiveness to pools of overlapping long peptides. Although the results of ELISpot tests done in this way are intrinsically cleaner and more reliable, a method that tests only a few selected antigens clearly has the potential to underestimate the proportion of vaccine-responders. This could be addressed to some extent by expanding the size of the antigen panel but important responses to unique neo-antigens would still be missed. The two forms of stimulus are therefore

complementary and, with some adjustments to trial protocol, should both be retained in future similar trials if possible.

No single immunological test of vaccine-induced response currently correlates with clinical benefit in cancer vaccine studies (Hoos et al. 2010), a fact that appears to be discouraging some groups from even attempting the assessment (Ardon et al. 2012). However, until we have a clear understanding of how cancer vaccines work, the optimum approach in my view is to assess cell-mediated anti-tumour immune responses in as many different ways as practicably possible and to include also assessments of humoral and innate cell responses. Where possible, immune parameters should be assessed at the tumour site as well as in peripheral blood. Treating newly presenting patients would make this more feasible because many patients currently undergo re-operation at relapse as part of their usual treatment.

Although median PFS_{6mo} for the treated patients in the trial was no better than published reports of TMZ alone, there was encouraging evidence of treatment-induced immune responses in some patients. However, these cases were few and all patients ultimately progressed. The fact that all the trial participants have now died serves to emphasise the devastating nature of this disease. The challenge facing this field is how to increase the proportion of responding patients and to increase the amplitude and duration of clinical and immunological responses.

One way of doing this is to develop a more potent vaccine. In Chapter 4, it was shown that the iNKT cell agonist α -GalCer provides a strong adjuvant effect when incorporated into a simple vaccine based on irradiated tumour cells, and that iNKT cells are both present and functional in GBM patients. However, the adjuvanticity of α -GalCer in patients remains untested. A crucial question is whether some patients simply have too few iNKT cells to benefit from the adjuvant effect of α -GalCer. Adoptive transfer of an expanded iNKT cell population (East et al. 2012) prior to vaccination could conceivably circumvent this problem. Alternatively, the Glioma-Gal vaccine could be modified to increase DAMP expression (B. M. Andersen and Ohlfest 2012) or incorporate additional adjuvants to promote DC licensing through

multiple pathways.

On a cautionary note, this thesis showed that irradiated whole glioma cells injected i.v. into mice are toxic at high doses. Toxicity was largely, but not completely, mitigated by pre-treatment with heparin, suggesting that pro-coagulant Tissue Factor may have been the cause (Bastida et al. 1984; Fernandes et al. 2006). Any move to translate the Glioma-Gal vaccine to the clinic would require prior *in vitro* assessment of the effects of the vaccine on human blood components followed by a careful Phase I dose escalation study performed in an ICU setting. It is very likely that toxicity could be substantially reduced by administering the vaccine s.c. or i.d. but this might impact adversely on efficacy by reducing access to iNKT cells and/or by leading to priming in skin-draining lymph nodes rather than in those secondary lymphoid organs where T cell priming may induce a homing phenotype more conducive to entering the CNS (Ferguson and Engelhard 2010; Odoardi et al. 2012; Sasaki et al. 2007).

Both the vaccine designs used in this thesis were capable of eliciting anti-tumour immune responses *in vivo*. However, one of the potential advantages of the Glioma-Gal vaccine over the simpler Lysate-Gal design is that the Glioma-Gal vaccine may be less prone to inducing iNKT cell anergy. Fujii and colleagues showed in mice that an injection of DC pulsed with α -GalCer did not prevent iNKT cells from responding to a second injection of α -GalCer-pulsed DC 30 days later (Fujii et al. 2002). A single dose of Glioma-Gal vaccine also did not completely abrogate the capacity of a second α -GalCer-mediated vaccine to elicit an immune response 2 weeks later (albeit in a single experiment: Appendix B, Fig B5.1). Thus, it may be feasible to use the Glioma-Gal vaccine in a prime-boost regimen. Further studies are needed to test this. Also relevant to the potential use of Glioma-Gal in prime-boost regimens is the recent discovery that repeated i.v. administration of α -GalCer in mice can induce or expand a population of immunoregulatory IL-10-secreting iNKT cells (Sag et al. 2014). The dose of free α -GalCer used in that study, 4 μ g, was 20 times greater than that used in the Lysate-Gal experiments described in this thesis. Nevertheless, it would be

important to assess whether tumour cell-associated α -GalCer has the same effect at doses required for therapeutic benefit.

These concerns aside, the Glioma-Gal vaccine has much to recommend it including the practical advantage of avoiding the need to generate autologous DC *ex vivo*, application to patients of all HLA types, the provision of epitopes for both CD4 and CD8 T cells and the provision of a broad range of personalised antigens. This last point may be particularly important. Evidence in the literature favours a broad multivalent approach in cancer vaccines over targeting one or a few selected antigens (Neller et al. 2008). In addition, a personalised vaccine incorporating whole tumour antigens has the potential to target unique neo-antigens. Recent studies suggest that a major reason for the striking success of checkpoint inhibitors may be the promotion of responses to tumour-specific neo-antigens rather than to over-expressed shared antigens (Kvistborg et al. 2014; Snyder et al. 2014). It seems likely that significant progress in defining the optimum target antigens for cancer vaccines will be made in the coming years. Mass spectrometry and automated data analysis have been used to characterise entire “peptidomes” eluted from MHC molecules of GBM tumour cells leading to the discovery of antigens currently being tested in clinical trials (Dutoit et al. 2012). The development of high throughput gene sequencing coupled with advances in bioinformatics means it is becoming feasible to define “mutanomes” of tumours from individual patients in a rapid time frame, potentially allowing the design of personalised vaccines targeting neo-epitopes (Boisguérin et al. 2014). In the meantime, autologous tumour cells remain a readily available source of personalised antigens.

Unexpectedly, the anti-tumour effect of the Glioma-Gal vaccine did not require CD8 T cells or NK cells but was dependent on CD4 T cells. Two other groups have also described anti-tumour immune responses in GL261-bearing mice that were dependent on CD4, but not CD8, T cells (Belcaid et al. 2014; Leach et al. 1996). Although many studies have focused on the role of CD8 T cells in mediating anti-

cancer immune responses, the potential for exploiting CD4 T cells is attracting increasing interest (Tran et al. 2014).

CD4 T cells are capable of exerting anti-tumour effects in a number of ways that are independent of CTL including the promotion of effector functions of innate cells at the tumour site, anti-angiogenic effects of IFN- γ and TNF- α , direct tumour cell cytotoxicity, and provision of help to B cells (Corthay et al. 2005; Flynn and Stockinger 2003; H.-J. Kim and Cantor 2014; Mumberg et al. 1999; Quezada et al. 2010). Recently, it has been shown that the presence of tumour-infiltrating T_{FH} CD4 T cells is associated with an improved prognosis in patients with breast cancer (Gu-Trantien et al. 2013). In this study, the adoptive transfer of serum from Glioma-Gal vaccinated mice did not confer tumour protection, arguing against a role for humoral immunity in this model. However, a mechanism that involves B cell help should perhaps not be completely excluded because the anti-tumour effect of circulating antibodies might also be dependent on some effect of the vaccine on Fc receptor⁺ cells, such as tumour-associated macrophages for example.

As mentioned earlier in this thesis, one of the theoretical advantages of personalised whole tumour cell-based vaccines is the possibility of targeting unique neo-antigens. Recently, it has been shown that human CD4 T cells have the capacity to recognise mutated tumour neo-antigens (Linnemann et al. 2015; Tran et al. 2014). It is interesting to speculate that the immunodominant antigens targeted by the Glioma-Gal vaccine might be MHC class II-restricted epitopes derived from mutated proteins.

Although the ability of whole tumour cell-based vaccines to target a broad range of personalised antigens is a theoretical advantage, a weakness of this type of vaccine is the fact that the targets are undefined, making it much more difficult to accurately assess vaccine-induced responses than when the specificity of the vaccine is predetermined. A way must be found to reconcile these two competing needs because the ability to accurately measure the magnitude and specificity of immune responses induced by cancer vaccines in clinical trials is fundamental to making

progress in this field. As mentioned already above, one way to improve assessment of Glioma-Gal vaccine-induced immune responses in a clinical trial setting might be to expand the pool of TAA tested for T cell-reactivity. However, this approach will always tend to underestimate vaccine-induced responses. An alternative, but complementary, approach might be to “spike” the Glioma-Gal vaccine with a defined antigen. Incorporation of a control antigen could conceivably be done using techniques such as retroviral transfection or electroporation but this would negate one of the main advantages of Glioma-Gal, namely the lack of complex *in vitro* steps required for manufacture. A different way of achieving this might therefore be to pulse tumour cells with a conjugate of α -GalCer and a control protein, similar to conjugates recently described by Hermans and colleagues (Anderson et al. 2014), thereby exploiting the same unidentified mechanism by which α -GalCer associates with tumour cells and preserving the simple overnight pulsing method used to make the Glioma-Gal vaccine. Validation studies would be needed to check that this did not impact adversely on the adjuvant effect of α -GalCer. If a full length protein antigen, or perhaps a pool of overlapping SLPs covering the full protein, could be incorporated in this way then the vaccine would remain applicable to patients of all HLA-types thereby preserving another of the other attractive properties of Glioma-Gal. In studies of DC-based vaccines, antigens used as controls have included MHC class I-restricted viral peptide antigens and the large protein molecule keyhole limpet haemocyanin (Aarntzen et al. 2013; Banchereau et al. 2001; J. R. Harris and Markl 1999). A concern with this approach is the possibility that the “marker” antigen might come to dominate the vaccine-induced response. This could be addressed to some extent by using an “on-target” glioma-associated antigen.

Chapters 5 and 6 explored the possibility of extracting additional therapeutic benefit from the Glioma-Gal platform by modifying the vaccine to target a putative tumour cell subset or phenotype. In Chapter 5, it was shown that growing GL261 tumour cells in stem cell medium prior to incorporation into vaccine did not improve the efficacy of a lysate-based vaccine. However, this negative finding in the GL261 model cannot be said to invalidate the approach in general because an immortalised cell

line does not necessarily accurately represent the biology of heterogeneous tumour cell populations in human patients. The CSC hypothesis continues to attract intense interest. As previously mentioned, Vik-Mo and colleagues recently reported the results of a Phase I clinical trial in which 7 GBM patients vaccinated with DC pulsed with neurospheres cultured from autologous GBM tumour tissue survived longer than matched historical controls (Vik-Mo et al. 2013). While encouraging, the study was not designed to show whether or not the vaccine actually targeted CSC. There are other reasons why such a vaccine might be more effective than DC pulsed with tumour cells cultured in serum, such as, for example, a more faithful preservation of the genotype and phenotype of the original tumour (Fael Al-Mayhany et al. 2009; J. Lee et al. 2006b). A number of other trials based on the concept of glioma CSC are either planned or in progress (Esparza et al. 2015).

In Chapter 6, modifying the lysate vaccine with the intention of targeting chemoresistant tumour cells did not improve therapeutic benefit when used in combination with doxorubicin. Again, failure to show proof-of-principle in this particular model clearly does not invalidate the concept of targeting chemoresistance with immunotherapy altogether but does at least discourage pursuing this aim with the Glioma-Gal vaccine design. An interesting observation made in Chapters 5 and 6 was that Musashi-1 gene expression was upregulated both by culture in SC media and by doxorubicin exposure. Musashi-1 is an RNA-binding protein that binds to and represses translation of certain mRNAs. In normal neural stem cells it inhibits differentiation but also activates Notch and Wnt pathway signalling, thereby promoting progression through the cell-cycle while at the same time maintaining the stem cell-state (Glazer et al. 2008; Okano et al. 2002). Expression is required for normal nervous system development in mammals (Glazer et al. 2008). In adults, Musashi-1 expression in the CNS is negligible except in normal neural stem cells (Okano et al. 2002). It is also expressed in stem cells and early progenitors in some other tissues such as breast and colon (Vo et al. 2012). It is aberrantly over-expressed by astrocytic tumours in humans (Kanemura et al.

2001; Thon et al. 2010; Vo et al. 2012) and the level of expression correlates with tumour grade (Dahlrot et al. 2013; Kanemura et al. 2001; Thon et al. 2010).

It is not clear whether the doxorubicin-induced increase in Musashi-1 expression was directly involved in the acquired chemoresistance of tumour cells or merely an epiphenomenon. Johannessen and colleagues (Johannessen et al. 2009) showed that inhibition of PI3K/Akt signalling in GBM sphere cells reduced Musashi-1 expression and increased sensitivity to doxorubicin. Since the PI3K/Akt pathway lies downstream of EGFR (T. T. Huang et al. 2009), it seems likely that, in the case of sphere cells, the increase in Musashi-1 expression was stimulated by EGF in SC medium. In DTEP cells, it is conceivable that Musashi-1 expression was also due to PI3K/Akt signalling. PI3K/Akt pathway activity is upregulated by exposure to certain chemotherapy agents including doxorubicin and this increased activity is strongly associated with chemoresistance (reviewed in W.-C. Huang and Hung 2009). Thus upregulation of PI3K/Akt activity in DTEP cells in response to doxorubicin could potentially explain both an increase in Musashi-1 expression and the acquisition of chemoresistance.

There is also a line of evidence linking expression of Musashi-1 with ABCB1 expression. Doxorubicin exposure induced nuclear localisation of the transcription/translation factor Y-box binding protein-1 (YB-1) and the upregulation of ABCB1 in a human breast cancer cell line (Bargou et al. 1997). Forced expression of YB-1 in a drug-sensitive breast epithelial cell line induced resistance to doxorubicin (Bargou et al. 1997). Silencing YB-1 reduced the protein level of Musashi-1, SOX2 and Nestin in a human GBM cell line (Fotovati et al. 2011). Thus doxorubicin-induced nuclear translocation of YB-1 is a plausible explanation at the molecular level for the increased expression of both Musashi-1 and ABCB1 in DTEP cells.

Neither of these two speculative scenarios imply a direct role for Musashi-1 in mediating chemoresistance. I am not aware of any studies that have directly assessed this. However, it seems possible that Musashi-1 itself could confer a

general resistance to apoptosis by way its known property of inducing a downstream increase in Notch pathway activity (Artavanis-Tsakonas et al. 1999; Fassl et al. 2012; McAuliffe et al. 2012). In Chapter 5, I speculated that Musashi-1 could be a potential antigen for selectively targeting SCLC. To speculate further, if Musashi-1 is a mediator of chemoresistance in tumour cells then a vaccine targeting this protein could potentially perform particularly well in a combination regimen with chemotherapy.

It is pertinent to reflect here on the strengths and weaknesses of the animal model used in Chapters 4-6. Created in 1939 by the intracerebral implantation of carcinogenic methylcholanthrene pellets (Ausman et al. 1970; Seligman et al. 1939), the GL261 cell line forms lethal tumours after implantation into the brain of the syngeneic immunocompetent C57BL/6 mouse with almost 100 % penetrance making it a reliable and relatively inexpensive model for studies of glioma immunotherapy (Wim Maes and Van Gool 2010). Intracranial GL261 tumours have several features in common with their human high grade glioma counterparts. Histologically, they are highly cellular and pleomorphic and areas of necrosis are often present (Doblas et al. 2010). GL261 is reported to exhibit point mutations of the K-ras oncogene and p53 tumour suppressor gene (Wim Maes and Van Gool 2010; Szatmari et al. 2006) and express high levels of VEGF (Doblas et al. 2010). PDGF signalling has been shown to play an important role in GL261 tumour progression (Y. Huang et al. 2014). Immunologically, GL261 tumours exhibit a number of features relevant to the human setting. Treg are progressively recruited to the i.c. tumour site from an early stage (Grauer et al. 2007) and play an important role in both preventing rejection (Onizuka et al. 1999; Suttmüller et al. 2001) and in suppressing experimental immunotherapies (Grauer et al. 2007). TGF- β has also been implicated in suppressing anti-tumour immune responses in this model (Hülper et al. 2011; Ueda et al. 2009). Microglia, macrophages, and immature myeloid cells accumulate within GL261 tumours (Gabrasiewicz et al. 2011) and have been implicated in mediating invasion (Ye et al. 2012) and immunosuppression (Chae et al. 2014). Shown in this thesis and by others (Akbasak

et al. 1991; Wim Maes and Van Gool 2010), MHC class I and class II expression is constitutive or inducible, rendering tumour cells susceptible to T cell recognition. A number of TAA have been described for GL261, including TRP-2 and gp100 (Prins et al. 2003), survivin (Ciesielski et al. 2008), AN2 (A. K. Paul et al. 2009), GARC-1 (Iizuka et al. 2006), and EphA2 (Ueda et al. 2009) but the immunodominant antigens mediating responses to whole tumour antigen-based vaccines are unknown (Grauer et al. 2008). The model has been used extensively to assess active immunotherapies for glioma and has been described as the “gold standard” for this purpose (Wim Maes and Van Gool 2010).

There are, however, several ways in which GL261 tumours differ from human GBM. Although considered “moderately” invasive by some (Doblas et al. 2010), in this study parental tumours had relatively distinct borders on histological sections and on MRI. GL261 tumours are angiogenic (Doblas et al. 2010) but tumour vessels do not have the striking endothelial proliferation typical of human GBM. Individual cells have well-defined plasma membranes, a morphology not typical for the high grade astrocytic tumours most commonly found in humans and considered by some to more closely resemble ependymoblastoma (Ausman et al. 1970). Unlike most GBM tumours, GL261 does not express the glial differentiation marker GFAP (Pellegatta et al. 2006a; Szatmari et al. 2006) except perhaps under special conditions (Pellegatta et al. 2006b; Anhua Wu et al. 2008). Consistent with this, no GFAP protein was detected in GL261 tumour cells cultured in standard medium in our laboratory (M. McConnell, data not shown).

A feature of this model seems to be variability in reported behavior between laboratories. In a review of the literature, Maes and colleagues found a two log difference in the number of cells required for full penetrance after i.c. implantation (Wim Maes and Van Gool 2010). These authors speculated that differences between research groups might be due, in part, to variation in immunological features of the cell line during longterm culture. Adding further weight to this possibility is that, whereas GL261 was initially maintained by serial passage *in vivo*, it appears a

number of GL261 cell lines capable of immortal growth *in vitro* were established in parallel by separate research groups in the 1990s (Szatmari et al. 2006). As discussed in Chapter 5, this might explain why some of the findings in this thesis were discordant with some of the literature.

A valid criticism of implantable models is that the implantation procedure itself induces local inflammation that can boost anti-tumour immune responses. Another is that rapid tumour growth, while convenient, does not allow the extensive interactions between tumour cells, stromal cells, and immune cells that has taken place in symptomatic human tumours (Sughrue et al. 2009). Autochthonous models, in which brain tumours arise spontaneously in genetically modified rodents, circumvent some of these concerns. Genetically modified mouse models commonly involve introducing genetic alterations known to be present in the corresponding human tumour of interest and are therefore theoretically more similar to their human counterparts than carcinogen-induced tumours. A serious concern with germline genetic modification is the possibility of off target effects, such as effects on the immune cells. Conditional models, that allow genetic modifications to be tissue-specific and/or induced in a time-controlled manner, have been developed to try and address this (Dranoff 2011; Huszthy et al. 2012). However, genetically modified mouse models have significant practical limitations including higher cost, variable time to tumour appearance (creating a need for some way of detecting tumours before they are too advanced) and variation in tumour histology. Another concern with many genetically engineered models is the incorporation of viral protein that may itself be immunogenic (Sughrue et al. 2009). Immunodeficient xenograft models are not suitable for testing active immunotherapies. “Humanised” rodent models can be useful in certain circumstances, such as, for example, testing adoptively transferred genetically-engineered human T cells against human tumours, but it is not yet possible to recapitulate the entirety of a human immune response in mouse since this involves interactions with stromal cells (Dranoff 2011).

Although genetically engineered models theoretically recapitulate the biology of their human counterparts more faithfully (de Vries et al. 2009; Dranoff 2011; Huszthy et al. 2012), it remains to be seen whether they are better at predicting the performance of novel treatments in human patients. While far from perfect, implantable models are nevertheless capable of providing proof-of-principle and mechanistic insights. Studies in implantable models have paved the way for some of the most significant recent advances in clinical cancer immunotherapy including checkpoint inhibition (Dranoff 2011). Indeed, it has recently been suggested that the higher mutational load generally present in carcinogen-induced tumours might make them better models for testing immune checkpoint blockade than genetically engineered tumour models (Robert Schreiber, oral presentation, Keystone Symposium, Banff 2015). In my opinion, a rational approach is to tailor the choice of model to the particular research question, taking into account practical considerations such as time and cost. Large rodent models may be more suitable for experiments that involve post-implantation surgical interventions at the tumour site such as, for example, intra-tumoural administration of drugs, cytokines or cells. Genetically modified autochthonous models are probably optimal for studying some aspects of tumour biology, including tumour initiation, progression and invasion, angiogenesis, and immunoediting. Models based on known genetic modifications with predictable molecular downstream effects may also be highly suitable for testing treatments targeting specific molecules or pathways. Orthotopic models involving implantation of immortal cell lines into wild-type immunocompetent mice, while far from perfect, remain a valid tool for investigating many aspects of tumour immunotherapy. Thus, in this thesis, GL261 proved useful in developing and characterising a therapeutic cancer vaccine but, in hindsight, may have been a flawed model for investigating cancer stem cells.

The capacity of GL261 to form tumours after i.c. or s.c. implantation proved to be both an advantage and a potential hazard. One of the great disadvantages of the i.c. model is an inability to track tumour size over time, leaving time to symptom appearance as the only readout in therapeutic experiments. Serial MRI scanning

would have been impractical and costly and there is evidence that Ketamine and other general anaesthetic drugs can be immunosuppressive (F.-L. Liu et al. 2012). GL261 can be modified to permit *in vivo* bioluminescence imaging (W Maes et al. 2009a) but the technique is only semi-quantitative and susceptible to errors (W Maes et al. 2009a). Reports by colleagues of low sensitivity in detecting i.c. tumours (Lai-Ming Ching, personal communication) and difficulties in achieving stable luciferase expression (David Walker, personal communication) dissuaded us from this approach. Another drawback of i.c. implantation is that it is relatively demanding and time-consuming, making it difficult to ensure homogeneous tumour challenge in large experiments. The ability of GL261 to form s.c. tumours offered a way around these two problems. However, the experience reported here has emphasised important differences in behaviour between s.c. and i.c. tumours. After i.c. implantation, tumours formed in almost 100 % of untreated mice at a consistent rate, whereas s.c. tumours often did not grow exponentially and sometimes regressed spontaneously or even failed to form, even though the number of implanted cells was 100 times greater. Variable behaviour after s.c. implantation of GL261 leading to wide error bars is evident in other studies (Hülper et al. 2011). The most likely explanation for spontaneous regression is that s.c. implantation elicits an endogenous immune response. GL261 s.c. tumours grow more rapidly and in an exponential manner when implanted in immunodeficient mice (data not shown and Hülper et al. 2011). Volovitz and colleagues (Volovitz et al. 2011) coined the term “split immunity” to describe their observation in two implantable rat glioma models that i.c. tumours were invariably lethal but s.c. tumours all spontaneously regressed, an event that conferred protection against subsequent i.c. challenge. Although s.c. GL261 is clearly not as immunogenic as these cell lines, the findings reported here do point to quantitative and/or qualitative differences in the endogenous immune response according to site of implantation. In Chapter 6, a therapy effective against established s.c. tumours was not effective against tumours in the brain. It may be that the magnitude of the systemic therapy-induced anti-tumour immune response was greater when tumours were s.c. because a spontaneous response to implanted tumour cells was boosted by the

immunotherapy whereas, when tumours were implanted into the relatively immune-privileged CNS, the magnitude of the systemic immune response was determined solely by the immunotherapy. Alternatively, or perhaps in addition, the systemic immune response may have been more effective at the s.c. tumour site because of easier access for effector cells or a lower level of immunosuppression than at the i.c. site. Elucidating the reasons for “split immunity” has the potential to yield valuable insight into mechanisms hampering immunotherapies directed at tumours situated in the brain. In the meantime, although experiments involving s.c. tumour challenge served a useful purpose in this thesis, the data presented here illustrate a need for caution in extrapolating findings to the orthotopic setting.

Common to both the Glioma-Gal and Lysate-Gal vaccines was a need for prior depletion of Treg to obtain therapeutic benefit. Success in the clinic also seems unlikely unless GBM-induced immunosuppression can also be attenuated. This study and others have shown that Treg are a major source of immunosuppression in mouse models of glioma and there are strong grounds to believe that this is also the case in patients with GBM tumours (discussed in detail in Chapter 1, Section 1.3.4.3). The relative importance of other immunosuppressive mechanisms in the GBM setting is still not entirely clear. TAM, abundant in GBM, almost certainly contribute to local immunosuppression (Section 1.3.4.4) but the role of MDSC and plasmacytoid DC, both strongly implicated as suppressor cells in animal models or other cancer types in humans, requires further study. Rather than targeting certain cell types, a more efficient strategy might be to target molecules that have broad immunosuppressive effects such TGF- β or IDO. There is still much to be done to understand the many immunosuppressive mechanisms in play, how they interact with each other, and how to block these mechanisms in the most effective and efficient way.

Checkpoint-inhibiting antibodies directed at CTLA-4, PD-1 and PD-L1 are now achieving striking results in patients with metastatic melanoma (Brahmer et al. 2012; Hodi et al. 2010; Topalian et al. 2012). Importantly, these studies are

powerfully validating the concept of immunotherapy for cancer. However, melanoma is a relatively immunogenic tumour characterised by high levels of tumour-infiltrating lymphocytes and associated with occasional spontaneous remissions (Kalialis et al. 2009). In GBM patients, the potential of checkpoint inhibitors seems more likely to be fully realised if used in combination with a treatment that generates increased numbers of effector cells, such as a vaccine. Anti-CTLA-4 antibodies may prove to be a particularly useful in the GBM setting because they have been shown to act both by checkpoint inhibition and by depleting Treg at the tumour site (Peggs et al. 2009; Simpson et al. 2013). In support of this, colleagues at the Malaghan Institute have recently extended the work reported in this thesis to show that the Glioma-Gal vaccine combined with an anti-CTLA-4 antibody can eradicate 80-100 % of established intracranial GL261 tumours in mice (Cameron Field, personal communication).

The studies in Chapter 6 demonstrated marked efficacy of therapeutic vaccination preceded by anti-CD25 depleting antibody and an immunomodulatory dose of doxorubicin. Other combinations of immunomodulatory treatments such as anti-CTLA-4 and anti-PD-1 antibodies (Wolchok et al. 2013) or anti-CTLA-4 antibody and an IDO inhibitor (Holmgaard et al. 2013) are also showing promise in patients and animal models. In this context, TMZ is firmly established as a standard treatment for patients newly presenting with GBM, making it important to understand and leverage any immunomodulatory properties this agent may have. As previously discussed, TMZ-induced lymphopenia appeared to promote the efficacy of a peptide vaccination in GBM patients (Sampson et al. 2011) but TMZ can also unhelpfully increase the proportion of Treg (C. E Fadul et al. 2011a; Mitchell et al. 2011). Recent studies have shown treatment with an anti-CD25 antibody during TMZ-induced lymphopenia selectively depletes Tregs in mice and GBM patients (Mitchell et al. 2011; Sampson et al. 2012) and the two treatments synergistically enhanced the efficacy of a vaccine in a mouse model of melanoma (Mitchell et al. 2011). Combinations of complementary immunomodulatory agents may be a feature of successful immunotherapeutic strategies in the future.

To conclude, a next-generation immunotherapy for GBM could conceivably consist of a modified Glioma-Gal vaccine combined with checkpoint inhibition and Treg depletion. Used in the newly presenting setting, this complex combination therapy would need to be designed around standard treatment to maximize the amplitude of the vaccine-induced response. The goal of developing an effective treatment for patients with GBM remains a daunting one but, in light of recent advances in the field of cancer immunotherapy, the tools for making very significant progress may already exist.

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APPENDIX A: GATING STRATEGIES

Figure A1. Flow cytometry gating strategy for enumerating B cells, NK cells and selected NK cell activation markers in the PBMC of clinical trial participants. B cells were defined as CD19⁺ lymphocytes (second row, right). NK cells were defined as CD56⁺CD3⁺ lymphocytes (third row, left). Fluorescence Minus One panels in one subject were used as negative controls to guide the setting of NK cell gates (third row, right). Bottom row shows results for Patient A08 at pre-vaccination baseline (black lines) and 3 months post-vaccination (red lines). Solid grey histograms show NK cells stained with FMO panels (for CD16 and NKG2D) or CD3⁺ lymphocytes stained with the full panel (for CD56).

Figure A2. Flow cytometry gating strategy for *in vitro* cultured GL261 tumour cells.

Figure A3. Flow cytometry gating strategy for identifying CD4 T cells (A) and CD11c⁺MHC class II⁺ cells (B) in the mediastinal lymph nodes of C57BL/6 mice.

Figure A4. Flow cytometry gating strategy for enumerating iNKT cells in the PBMC of GBM patients. Unloaded CD1d Tetramer was used as a control for non-specific binding (bottom right).

Figure A5. Flow cytometry gating strategy for enumeration of myeloid DC in PBMC of GBM patients. Myeloid DC were defined as Lineage⁻CD11c^{hi}HLA-DR^{hi} cells. Histogram at bottom shows CD1d surface staining of mDC from a representative patient (black line) compared to fluorescence after staining with the same panel minus the anti-CD1d antibody (solid grey).

Figure A6. Flow cytometry gating strategy for assessment of CD1d expression on CD14⁺⁺ monocytes in PBMC of GBM patients. Histogram at bottom right shows result from a representative patient (black line) compared with staining of the same PBMC with the panel minus the anti-CD1d antibody (solid grey).

APPENDIX B: SUPPLEMENTARY FIGURES

Contents of this appendix

- Figure B1 Validation of ELISpot methods used in Chapter 3
- Figure B2 Validation of antibody depletion methods used in Chapter 4
- Figure B3 Characterisation of B6Ao0 mouse strain used in Chapter 4
- Figure B4 Characterisation of DC2114 dendritic cell line used in Chapters 5 - 6
- Figure B5 Additional experiments relating to Chapters 4 – 6.

Figure B1.1 IFN- γ ELISpot assay validation #1. PBMC from a patient who had participated in a clinical trial of a DC vaccine for metastatic melanoma were thawed, rested overnight, and then plated in a 96 well ELISpot plate precoated with anti-IFN- γ antibody. 2×10^5 PBMC were plated per well in RPMI supplemented with 10% heat-treated FCS. An agonistic anti-CD3 antibody was added to some wells. The plate was developed 48 hrs later. This experiment confirmed the capacity of the assay to detect IFN- γ and demonstrated low background activity from unstimulated PBMC in the selected medium.

Figure B1.2 IFN- γ ELISpot assay validation #2. PBMC from same patient as in Figure B1.1 above. PBMC were thawed and cultured for 5 days in the presence of autologous tumour-pulsed DC (from an aliquot of stored DC vaccine) and 50 U/ml recombinant human IL-2 prior to the ELISpot assay. 1×10^5 PBMC per well, except CD3-stimulated wells which were 3×10^4 per well. 2×10^4 autologous tumour-pulsed DC were added some wells. A marked increase in background activity was seen after pre-stimulation. Interestingly, this experiment also showed an increase in DC vaccine-specific PBMC in the post-vaccination sample. In view of the high background activity induced by pre-stimulation, it was decided to use direct ELISpot assays for subsequent experiments.

Figure B1.3. IFN- γ ELISpot assay validation #3. PBMC from same melanoma patient and from the same time points as in Figure B1.2. 1×10^5 PBMC per well, except CD3-stimulated wells which were 5×10^4 . Some wells were stimulated with 3×10^3 autologous tumour-pulsed DC (DCV), with a commercially-available pool of viral peptide antigens (CEF), or with an agonistic anti-CD3 antibody (CD3 mAb). This experiment confirmed low background activity from unstimulated PBMC. Responses to the CEF peptide pool demonstrated the capacity of assay to detect antigen-specific IFN- γ release by CD8 T cells. As in Figure B1.2, this experiment showed an increase in DCV-specific PBMC after vaccination, demonstrating consistency in the detection of DCV-elicited responses. In contrast, the number of virus-specific PBMC remained constant.

Figure B1.4. IFN- γ ELISpot assay validation #4. PBMC from a participant in the GBM clinical trial. Surplus PBMC from the pre-vaccination time point were thawed, rested overnight, and then assessed in a direct IFN- γ ELISpot assay. 2×10^5 PBMC per well. Some wells were stimulated with the CEF viral peptide pool. Unique to the trial study, this patient was found to have a relatively high background spot count, a finding subsequently reproduced during actual testing for tumour-specific responses. This preliminary experiment demonstrated the capacity of the assay to detect antigen-specific CD8 $^+$ T cell responses in the PBMC of a trial participant. Similar results across replicate wells demonstrated consistent assay performance.

Figure B2.1 Regulatory T-cell depletion. FoxP3-GFP mice were treated i.p. with 125 μ g of PC61 antibody. Peripheral blood was harvested 2 days later and analysed by flow cytometry. Shown are FC plots of live CD3⁺ CD4⁺ lymphocytes (left column) and live CD4⁺ lymphocytes (right column) of a treated mouse (bottom row) and an untreated control mouse (top row). Plots are representative of 3 treated mice and 2 controls.

Fig B2.2 CD4⁺ T cell depletion. WT C57BL/6 mice were treated i.p. with a single dose of GK1.5 antibody. Peripheral blood was harvested 13 days later and analysed by FC. Plots are gated on live CD3⁺ lymphocytes and are representative of 2 mice per group. Mice that received 250 µg were still fully depleted at Day 13. In depletion experiments mice were vaccinated with Glioma-Gal on Day 0 and challenged with subcutaneous tumours on Day 7. For depletion of CD4⁺ T cells, mice were treated i.p. with 125 µg GK1.5 on Days 2,4 and then every 3-4 days until completion of the experiment.

Figure B2.3 CD8⁺ T cell depletion validation. A, WT C57BL/6 mice were treated or not treated i.p. with 500 μ g 2.43 antibody and peripheral blood assessed by FC 4 days later. Gated on live CD3⁺ lymphocytes, 1 mouse per group. Repeated once to assess a different batch of antibody with similar results. B, C, For depletion experiments, mice were vaccinated with Glioma-Gal on Day 0 and challenged with s.c. tumours on Day 7. To deplete CD8⁺ T cells in these experiments, mice were treated i.p. with 500 μ g 2.43 antibody on Days -2 and -1 and then every 3-4 days until the end of the experiment. In one experiment, 15 days after tumour challenge peripheral blood was harvested from 3 of the 5 depleted mice and from 3 naive mice and analysed by FC (3 mice per group). B, Kaplan-Meier survival curves for the complete experiment showing the time point at which blood was collected for FC. At this time point the difference between the No Vaccine control group and the Vaccine + Depletion group was already statistically significant ($p < 0.01$). C, Representative FC plots gated on live TCRβ⁺ lymphocytes (right) and summary graph (left) showing the proportion of T cells that are CD4⁺.

Figure B2.4 NK cell depletion validation. C57BL/6 mice were treated or not treated with PK136 antibody 300 µg i.p. on Days 0 and 2. Peripheral blood was harvested on Day 7 and analysed by FC . Shown are plots gated on live lymphocytes representing the results in 3 depleted mice and 2 control mice. In depletion experiments, mice were vaccinated on Day 0 and challenged s.c. with tumours on Day 7. For depletion of NK cells, but to avoid depleting iNKT cells in the early stages of priming, mice were treated with PK136 300 µg i.p on Days 4 and 6 and then every 3-4 days until the end of the experiment.

Figure B3.1. Characterisation of the MHC class II-deficient B6Aa0 mouse strain. A, Mice were treated i.v. with 200 ng α -GalCer. Serum IL-4 was measured 2 hours later as a measure of iNKT cell responsiveness. 1-3 mice per group. B, Naive splenocytes were assessed for the presence of iNKT cells and CD8⁺ T cells in B6Aa0 and WT mice by flow cytometry. A fluorescent α -GalCer-loaded CD1d tetramer was used to enumerate iNKT cells. Shown are representative plots from one of 2 mice per group with similar findings.

Figure B4.1. The DC2114 dendritic cell line. A, Gating strategy for identifying DC2114 cells. B, CD11c expression by DC2114 (blue line) in comparison with WT C57BL/6 splenocytes (red line). C, MHC class II (I-Ab) expression by DC2114 (blue) compared with CD11c⁺ WT splenocytes (red).

Figure B5.1. Glioma-Gal Vaccine does not induce complete iNKT cell anergy. A, Mice were treated with 1×10^5 Glioma-Gal cells (GGVac) i.v. or with 200 ng free α -GalCer i.v. or were not treated. Two weeks later all mice were treated i.v. with 200 ng free α -GalCer. Serum IL-4 was measured 2 hours later as a measure of iNKT cell responsiveness. B, Mice were treated i.v. on Day 0 with 1×10^5 Glioma-Gal cells or received no treatment. On Day 13 1×10^4 OVA-specific CD8⁺ T cells (CD45.2⁺V α 2⁺ OT-1 cells) were transferred into all mice. On Day 14 some mice were vaccinated i.v. with 200 μ g OVA protein and 200 μ g of free α -GalCer. 5 mice per group. Single experiment. Graph shows individual mice and group means ($\pm$ SEM), with representative FC plots from each group above. The three groups receiving Ova + α -GalCer on Day 14 were compared using a Kruskal Wallis test with Dunn's post-test as shown.

Figure B5.2. Effect of sonicated tumour cells on DC maturation. Two GL261 parental tumour cell lines stored in the vapour phase of liquid N₂ were thawed, washed, resuspended in incomplete medium, and sonicated for 120 minutes until all cells were Trypan Blue positive. Cells of the DC2114 dendritic cell line were then pulsed with sonicated tumour cells at a ratio of 1:1 for 16 hours in triplicate wells. CD40 and CD86 expression by DC were assessed by flow cytometry. Some DC2114 cells were treated instead with Poly I:C 100 µg/mL as a positive control. Representative histograms (left), representative isotype controls (centre) and summary graphs showing means and SEM of experimental replicates (right). Statistical analysis was with a Kruskal Wallis test with Dunn's post-tests comparing all columns to all other columns.

Group	Survival	Effect on survival: Improvement over No Treatment	Peak Tumour Size < 75 mm ²	Effect on tumour burden: Improvement over No Treatment
No treatment	3/11		3/11	
Doxorubicin	4/11	1/11	4/11	1/11
Immunotherapy	8/11	5/11	5/11	2/11
Combination	11/11	8/11	11/11	8/11

Figure B5.3 Synergistic effect of combining doxorubicin with immunotherapy. Summary of pooled results from experiments shown in Figure 6.3. The effect of the combined treatment on survival and tumour burden was greater than the sum of the effects of the two treatments as monotherapies. Survival was defined as no tumour, or a regressing tumour, at the conclusion of the experiment. Size < 75 mm² was arbitrarily selected as a discriminator of tumour burden.

Figure B5.4. Effects of low dose Doxorubicin on immune cell populations. Proportion (left) and absolute number (right) of Treg in spleens of FoxP3-GFP reporter mice treated with Doxorubicin 2.5 mg/kg i.v. (Dox) or not treated (Control), as in Figure 6.7. Splenocytes were assessed by flow cytometry 24 hours after doxorubicin treatment. Gating strategy as in Figure 6.7A. 4 - 5 mice per group. The Mann Whitney test was used for statistical analysis. No statistically significant differences were seen between the total number of splenocytes in the two groups, or in the proportions or absolute numbers of CD4⁺ T cells, CD8⁺ T cells, iNKT cells, B cells or Gr1⁺CD11b⁺ cells (data not shown).

Figure B5.5. Discordance between TRP-2 gene expression and protein level in tumour lysates. Western Blot analysis of TRP-2 protein concentration in freeze/thaw lysates of GL261 parental tumour cells and the Doxorubicin-tolerant DTEP 0.05 tumour cell line (left). Gene expression in the same tumour cell lines assessed by RT-PCR (right).

APPENDIX C: GL261 CELL LINE VALIDATION

DNA was extracted from GL261 tumour cells and sent to a commercial provider (IDEXX BioResearch, Columbia, MO) for genotyping. Two samples were sent for testing: a sample of the original stock obtained from the DCTD Tumour Repository in 2008 and a sample from a culture in use in late 2012 when the majority of the experiments in Chapter 5 were being performed. The report from IDEXX BioResearch is reproduced below.

IDEXX BioResearch Case # 13512-2014

Received: 5/15/2014
Completed: 5/22/2014

Submitted By

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Specimen Description

Species: mouse
Description: DNA
Number of Specimens/Animals: 2

Purchase Order #: 111011

Client ID	Cell Line	Species
GL261 2008	GL261	mouse
GL261 2012	GL261	mouse

Services/Tests Performed: Cell Check

Summary: Cell Check: The samples were confirmed to be of mouse origin and no mammalian interspecies contamination was detected. A genetic profile was generated for the samples by using a panel of microsatellite markers for genotyping. This data is presented in the table as allele sizes in bp. The sample profiles match identically to each other and to the genetic profile established for this cell line. Please note that a 1 base pair difference in allele sizes between the samples and the comparison profile represents run-to-run variability only and sizes 2 base pairs or greater represent different allele sizes between the sample and the comparison profile. Please see the report for details.

CELL CHECK

Species-specific PCR Evaluation

Species	GL261 2008	GL261 2012
mouse	+	+
rat	-	-
human	-	-
Chinese hamster	-	-
African green monkey	-	-

Marker Analysis

Marker Name	GL261 2008	GL261 2012	GL261
	Allele Size	Allele Size	Allele Size
4	156, 158	156, 158	156, 158
5	113	113	113
136	150	150	150
78	197, 199	197, 199	197, 199
134	104	103	104
14	95	95	95
94	113	113	113
16	136, 140	136, 140	136, 140
139	125, 127	125, 127	125, 127
144	193	193	193
25	136, 141	136, 141	136, 141
163	219	219	219
133	75	75	75
138	191, 195	191, 195	191, 195
27	150	150	150
39	174	174	174
141	95	95	95
165	197	197	197
74	119	119	119
111	148	148	148
20	153	153	153
31	201	201	201
137	204	204	204
143	132	132	132
53	95, 97	95, 98	95, 97
171	210	210	210
47	113	113	113

APPENDIX D: PUBLICATIONS

Publications predominantly based on work presented in this thesis:

Hunn, M.K., Farrand, K.J., Broadley, K.W.R., Weinkove, R., Ferguson, P., Miller, R.J., Field, C.S., Petersen, T., McConnell, M.J., and Hermans, I.F. (2012) Vaccination with irradiated tumor cells pulsed with an adjuvant that stimulates NKT cells is an effective treatment for glioma. *Clin Cancer Research* 18: 6446-59.

Hunn, M.K., Hermans, I.F. Exploiting invariant NKT cells to promote T-cell responses to cancer vaccines. (2013) *Oncoimmunology* 2, e23789.

Hunn, M. K., Bauer, E., Wood, C.E., Gasser, O., Dzhelali, M., Ancelet, L.R, Mester, B., Sharples, K.J., Findlay, M.P., Hamilton, D.A., Hermans, I.F. (2015) Dendritic cell vaccination combined with temozolomide retreatment: results of a phase I trial in patients with recurrent glioblastoma multiforme. *J Neurooncol* 121(2): 319-29. doi: 10.1007/s11060-014-1635-7. Epub 2014 Oct 31.

Publications including work by the author that is presented in this thesis:

Broadley, K.W.R., **Hunn, M.K.**, Farrand, K.J., Price, K.M., Grasso, C., Miller, R.J., Hermans, I.F., and McConnell, M.J. (2011) Side population is not necessary or sufficient for a cancer stem cell phenotype in glioblastoma multiforme. *Stem Cells* 29: 452-61.

Publications contributed to by the author during the course of this thesis:

Authier A., Farrand K.J., Broadley K.W., Ancelet L.R., **Hunn M.K.**, Stone S., McConnell M.J., Hermans I.F. (2015) Enhanced immunosuppression by therapy-exposed glioblastoma multiforme tumor cells. *Int J Cancer* 136(11): 2566-78. doi: 10.1002/ijc.29309. Epub 2014 Nov 12