TARGETING TUMOUR ASSOCIATED ANTIGENS

TARGETING THE TUMOUR ASSOCIATED ANTIGENS PLAC1 AND GP100 WITH ONCOLYTIC CANCER VACCINES

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements of the Degree Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2013)

Hamilton, Ontario

TITLE:	Targeting the Tumour Associated Antigens Plac1 and Gp100 with Oncoloytic Cancer Vaccines
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NUMBER OF PAGES:	xvii, 118

ABSTRACT

In spite of the tremendous body of cancer research, cancer remains a significant health issue requiring development of better therapeutics. The elucidation of the relationship between cancer and the immune system and the identification of tumour associated antigens together suggest that novel therapeutics using the immune system to target cancer is a promising avenue of research. Since immunological tolerance is a barrier to generating immune responses to self antigen, strategies to circumvent tolerance need to be investigated to target given antigens.

Plac1 is a novel tumour associated antigen with expression restricted to placenta, testis and many tumour cells. Initial reports concerning the expression, immunogenicity and potential tumourigenic function of Plac1 suggest that it would be an ideal tumour antigen. Initial experiments in mice indicated that generating an immune response against the murine Plac1 would be difficult and the subsequent work sought to employ strategies to facilitate anti murine Plac1 immune responses and anti tumour efficacy in Plac1 expressing tumours.

Another more studied tumour associated antigen is gp100. Unlike Plac1, immune responses against the murine gp100 can be generated through vaccination. These responses are unable to demonstrate any anti tumour activity in gp100 expressing cells. The bulk of the gp100 studies described here sought to modify the immune:tumour interaction such that the anti tumour activity of the anti gp100 responses could be improved.

While the specific barriers to Plac1 vaccination and efficacy and gp100 vaccination and efficacy are different, they have in common that they represent likely issues in using therapeutic cancer vaccines clinically. In both cases investigating how these barriers can be overcome is important and relevant to the understanding of these barriers to success when they appear in the clinic.

ACKNOWLEDGMENTS

The work described herein would not have been possible without the support and direction from my supervisor Dr. Brian Lichty and the rest of my supervisory committee: Drs. Karen Mossman, Martin Stampfli and Yonghan Wan.

The collegial atmosphere at the MIRC that facilitates the teamwork of sharing expertise has also been critical. Specifically, I need to recognize Jonathon Pol and Byram Bridle for their help with many immune analysis aspects of experiments. Other members of the Lichty Lab: Kyle Stephenson and Frances Lai have provided invaluable support both in the lab and out.

I'd like to thank the graduate student association of McMaster for providing me the opportunities to recognize my true passion for business that I hope to combine with the science from my PhD and lead to a rewarding career.

To my family: Dad - thank you for all all your support throughout my academic career and especially during the period of thesis writing. Mom - I can only apologize that we as researchers weren't able to come up with something fast enough for you. You remain a source of inspiration and will be missed always.

Finally I need to recognize the new members of my family that weren't there at the beginning of my degree. Katie - you have been able to bring me back down to earth when "daddy's busy writing". Last but definitely not the least is my beautiful wife Melissa. These past few years have presented many challenges as we both continue our academic careers with a toddler at home while living on our island in Woodstock. Thanks for putting up with my "I have one step left of a reaction so I can leave in 15 min=1 1/2 hours". I hope I can return the favour to help you get through residency. I know I don't say it enough but I am thankful for your support and I love you.

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List of Symbols and Abbreviations

α	Alpha
β	Beta
γ	Gamma
μ	Micro
L	Litre
AIRE	Autoimmune regulator
Ad	Adenovirus
AML	Acute myloblastic leukemia
APC	Antigen presenting cell
B16-F10	Mouse melanoma cell line
BCR	B cell receptor
BLAST	Basic local alignment search tool
CD-	Cluster of differentiation -
cDNA	Complimentary deoxyribonucleic acid
CPA	Cyclophosphamide
CT26	Mouse colorectal cancer cell line
CTLA-4	Cytotoxic T lymphocyte associated protein 4
DC	Dendritic cell
DCT	Dopachrome tautomerase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E1/E3 Ad	Early1/3 Adenovirus Genes
EBV	Ebstein-Barr Virus
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbant assay
FoxP3	Forkhead box protein 3
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Good manufacturing practices
gp100	Glycoprotein 100
HBV	Hepatitis B virus
HDACi	Histone deacetylase inhibitor
HER2	Human estrogen receptor 2
hgp100	Human glycoprotein 100
HLA	Human leukocyte antigen
hPlac1	Human placenta-specific protein one
HPV	Human papilloma virus
HSV-1	Herpes simplex virus one

ICCS	Intracellular cytokine staining
IDO	Indolamine-2,3-dioxygenase
IFN	Interferon
IL-	Interleukin-
IV	Intravenous
Mar	Maraba
MEF	Murine embryonic fibroblasts
MHC	Major histocompatibility complex
mgp100	Murine glycoprotein 100
mPlac1	Murine placenta-specific protein
mRNA	Messenger ribonucleic acid
mTEC	Medullary thymus epithelial cell
MUC1	Mucin-1
NCBI	National Center for Biotechnology Information
NK	Natural killer cell
PAMP	Pathogen associated membrane protein
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PFU	Plaque forming unit
Plac1	Placenta-specific protein 1
PRR	Pattern recognition receptor
raPlac1	Rat placenta-specific protein 1
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
siRNA	Small interfering ribonucleic acid
TAA	Tumour associated antigen
TAP	Transporter associated with antigen processing
TBS	Tris buffered saline
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper cell
TIL	Tumour infiltrating lymphocyte
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	T regulatory cell
U2OS	Human osteosarcoma epithelial cell line
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus

- CHAPTER 1 -

Introduction

1.0 Cancer

Cancer is a widespread disease, or group of diseases, that has impacted many individuals in society. It is the subject of many fundraising efforts to help support the vast amount of research into the etiology, novel treatments and attempts at improving outcomes of those suffering from this disease. A simple pubmed search of 'cancer' yields over 2.8 million articles. Despite the 42 years since United States president Richard Nixon declared the 'war on cancer' and the countless research dollars spent, cancer remains a significant health issue which contributed to 1 in 8 of all deaths worldwide in 2008 and is predicted to cause thirteen million deaths a year worldwide by 2030[1].

Cancer is a disease where cell growth becomes sequentially unregulated through the loss or dysfunction of regulatory pathways or aberrant activation of growth signaling pathways[2]. These categories of changes have been enumerated in the often cited, landmark review by Hanahan and Weinberg and include: evading apoptosis, the ability to divide in the absence of external growth signals, the insensitivity to anti-growth signals, metastatic and tissue invasive properties, the ability for unlimited replication, and the ability to promote blood vessel formation[3]. These changes are the result of altered gene expression caused by DNA damage or viral transformation. DNA damage can happen as a chance mutation or can be caused by exposure to DNA damaging agents such as chemicals in cigarette smoke or ultraviolet radiation from the sun. Presumably, many mutations caused by these agents will be harmless but increased exposure increases the number of mutations thereby increasing the chance of affecting a critical area of genetic

information such as a tumour suppressor such as p53 or leading to chromosomal translocation. In addition to physical/chemical cancer induction, cancers can be caused by viruses. Expression of some viral proteins can promote cell division/growth or prevent regulated cell death. The best known example is the link between human papilloma viruses and cervical cancer, another common example can be seen with Epstein-Barr virus in nasopharangeal carcinoma and Burkitt's Lymphoma[4-6].

Typical cancer therapies involve surgery, radiation and chemotherapy. The latter is a class of drugs that targets the most notable differences between cancer and normal cells: the rapid, uncontrolled cell division. One common example is paclitaxel (Taxol) which inhibits microtubule depolymerization, targeting the ability of microtubules to successfully separate chromatids during mitosis[7]. The typical side effects of chemotherapy can be traced back to the lack of specificity of many chemotherapeutics. Other, normal, fast dividing cells such as those of the immune system, hair cells, and lining of the gastrointestinal tract are also targeted by these drugs. While some specific small molecule drugs exist such as Gleevec (Imatinib) for acute myelogenous leukemia (AML), there is a desperate need for new therapeutics that are more specific and more effective[8].

2.0 Cancer and the Immune System

2.1 Overview of the Immune System

The vertebrate immune system is primarily a defense mechanism responsible for fending off infection from nefarious foreign invaders such as viruses and bacteria. It is often divided into two sets of components: innate and adaptive. The innate immune system is the primary line of defense to microorganisms that are encountered on a daily basis, most of which are detected and destroyed with in minutes to hours[9]. The power of the innate response is that it is quick and non-specific. The innate immune system includes components that range from the basic protection provided by epithelial surfaces to cellular components such as neutrophils, macrophages, and natural killer (NK) cells. These cells rely on pattern recognition inherited in the genome that recognizes a broad class of pathogens and destroy them[10]. Neutrophils are short lived, polymorphonuclear cells of myeloid lineage that are present predominantly in the bone marrow[11]. Their release from the bone marrow is triggered by the G-CSF (granulocyte colony stimulation factor) produced and secreted by endothelia or other immune cells in inflammatory states[12]. Neutrophils circulating in the bloodstream are recruited to sites of damage or infection by chemoattractants such IL-8 that are produced as a result of inflammation. Once at the site of damage or infection, neutrophils can recognize pathogens via their pathogen associated molecular pattern (PAMPs) receptors such as the toll-like receptors (TLRs)[13]. TLRs detect features that are common to many bacterial or viral pathogens but not to normal cells such as the presence of lipopolysaccharide (LPS), unmethylated CpG

DNA or double stranded RNA. Upon pathogen recognition, neutrophils will phagocytose the foreign elements in addition to producing and secreting antimicrobial peptides and reactive oxygen species. Macrophages represent another innate immune cell of myeloid lineage who share the ability to phagocytose and destroy pathogens. In response to IFN-γ, their phagocytic ability is enhanced and MHC class II molecule expression is increased[14]. Through MHC-II molecules, macrophages present antigen to the CD4 T cells, providing a link between the innate and adaptive arms of the immune response. Another important cell in the innate immune response is the natural killer (NK) cell. Unlike neutrophils and macrophages, NK cells are from the lymphoid lineage. They kill target cells by secreting granules containing cytotoxic perforin and granzyme. NK cell killing can be activated by direct binding of some viral antigen such as influenza hemagglutinin or by binding Fc receptors on antibody coated cells through antibody dependent cellular cytotoxicity (ADCC)[15]. Alternatively, NK cells may be activated by the inability of the inhibitory receptors to bind MHC class I on the cell[15]. This down-regulation of MHC-I on the cell surface is a strategy employed by some viruses to prevent or delay detection by cells of the adaptive immune response[16]. By detecting cells that have ceased expressing appropriate MHC-I molecules, NK cells serve to prevent the circumvention of the adaptive immune response by pathogens.

The adaptive immune system can be divided into two parts: the cell mediated response and the humoral response. The cell mediated response consists of immune effector cells such as CD8 T cells, which target antigens in the intracellular space. Naive

T cells, generated in the thymus, circulate between peripheral tissues and lymph nodes[17]. They encounter professional antigen presenting cells (APCs), such as dendritic cells, along the way, where the TCR is tested against MHC molecules on the surface of these APCs. T cells that encounter dendritic cells presenting their specific antigen are retained in the lymph node where they are activated, resulting in their expansion and differentiation[18]. This activation is thought to require 3 signals from the presenting cell. The first signal is the binding of the MHC:peptide complex with the TCR[19]. The second signal is the co-stimulation resulting from the ligating of CD28 on the T cell with B7-1/ B7-2 on the APC[20,21]. In the absence of this second co-stimulatory signal, T cells can enter into a state of anergy from which they are refractory to subsequent activation in the context of normally sufficient co-stimulation[22]. In the case of CD8 T cell activation, CD4 T cell help is often required. If the APC is unable to provide adequate co-stimulation, CD40 ligand on a CD4 T cell can engage CD40 on the APC inducing higher expression of the co-stimulatory B7. The third signal involves the production of cytokines from the APC that bind receptors on the T cell resulting in IL-2 production and proliferation. Once the CD8 T cells have undergone activation, proliferation and differentiation, they leave the lymph node to travel back to the peripheral tissues as cytotoxic lymphocytes (CTLs) in search of cells presenting their target peptide on MHC class I molecules. When the CD8 T cells encounter cells presenting their cognate MHC:peptide, that cell is targeted for killing. The lymphocyte accomplishes this through the release of perforin and granzymes toward the targeted cell[23]. The perforin provides holes in the plasma

membrane of the targeted cell to facilitate the entry of granzymes. The granzymes are serine proteases that commence a cascade leading to programmed cell death known as apoptosis[23]. In addition to secreting granules to kill target cells, CD8 T cells also produce and secrete cytokines to aid in the response. IFNγ secretion acts both on other potential target cells to increase MHC I expression, increasing the chances of detection and on macrophages, recruiting them to the site of infection[24,25].

Unlike for CD8 T cells, signal three provides CD4 T cells with a variety of fates. Naive CD4 T cells are known to differentiate into at least four effector subtypes: Th1, Th2, Th17 and the regulatory T cells (Treg). Th1 cells secrete macrophage activating effector molecules such as GM-CSF, TNF-α and IFN-γ[26]. These cytokines allow for macrophage growth, direct them towards infection and stimulate them to destroy engulfed bacteria. Th2 cells stimulate the proliferation and differentiation of B cells[27]. Th17 cells are partially responsible for neutrophil recruitment[28]. Treg cells suppress T-cell activity to help prevent the development of autoimmunity. They do so by secreting suppressive cytokines such as IL-10 and TGF-β. Tregs also express the transcription factor, FoxP3, which interferes with the transcription of IL-2 thus decreasing the ability of T cells to proliferate[29].

The humoral response is tasked with detecting pathogens in the extracellular space. B lymphocytes express a B cell receptor on their surface that interacts with protein targets[30]. These targets are then internalized, degraded and presented on the B cell surface via MHC class II molecules[31]. This MHC II:peptide complex can bind

complimentary T cell receptors on Th2 CD4 cells. This successful binding constitutes the first signal in B cell activation[32]. The required second signal comes from the binding of CD40 on the B cell with CD40L on the T cell[33]. The Th2 cell then produces cytokines such as IL-4 that signal to the B cell to activate and differentiate into an antibody producing plasma cell. Some B cell clones will become further tuned to the antigen when traveling through lymph nodes. In the germinal centre of the lymph node, they will undergo several rounds of mutation of the B cell receptor[32]. The cells with the highest affinity for the target antigen are selected for, resulting in B cells that can differentiate into antibody producing plasma cells producing the highest affinity antibodies. The antibodies then go on to directly neutralize the target or mark a target for complement opsonization[34]. Complement bound targets are then endocytosed by phagocytes and thus are eliminate or can cause lysis through the membrane-attack complex directly[34,35].

2.2 Immunologic tolerance, differentiating self from non-self

The immune system has a number of mechanisms that discriminate between self and non self, preventing recognition of healthy tissue. The first of these strategies is known as central tolerance. Immature thymocytes from the bone marrow undergo most of their development in the thymus. The double positive (CD4+/CD8+) T cells with their varying T cell receptors (TCRs) are subjected to cortical thymic epithelial cells (cTECs) in the cortex of the thymus presenting self peptide loaded onto MHC class I or MHC class II molecules. Positive selection promotes the development of immature T cells

expressing TCRs that are able to interact with self peptide:MHC complexes. TCRs that are unable to interact with self MHC are useless and cells expressing them fail to receive a survival signal and undergo apoptosis[36-38]. The surviving double positive T cells now become single positive, dependent on which MHC they were able to engage. In the medulla of the thymus, these single positive T cells are exposed to medullary thymic epithelial cells (mTEC) and dendritic cells (DCs) which present a repertoire of self peptides to identify developing thymocytes that interact too strongly with the self peptide:MHC complex[39-41]. The mTECs express Autoimmune Regulator (AIRE), a promiscuous transcription factor that can express tissue specific protein out of their natural context[42,43]. T cells interacting too strongly with the self peptide:MHC complexes are eliminated through apoptosis in a process known as negative selection[44,45]. The result is the production of CD4 or CD8 positive T cells with a repertoire of TCRs that can interact with self MHC but not too strongly in the context of self peptides.

While CD8 and CD4 T cells that react too strongly with self peptides are eliminated, the escape from the thymus of weakly auto-reactive T cells necessitates a second level of immune control. Peripheral tolerance can be mediated by a subset of CD4+ T cells (Tregs) which can be produced directly in the thymus or can be induced in the periphery upon antigenic stimulation in the presence of TGF-β[46]. A notable feature of these cells is the expression of the FoxP3 transcription factor. FoxP3 leads to increased CTLA-4 expression and reduced IL-2 and IFNγ expression upon TLR activation[29]. These changes help the Tregs contribute to an immunosuppresive local environment.

2.3 Relationship between cancer and the immune system

The relationship between cancer and the immune system has been an area with increased volumes of research in recent years. The immunosurveillance hypothesis, whereby spontaneous tumours are constantly arising and being eliminated by the immune system was initially proposed in the early 1900s by Paul Ehrlich and again in the mid 1950s[47]. This hypothesis was largely discounted by others until the late 1990s when, with the help of improved techniques and a greater understanding of immunology, several studies were conducted to decipher the role of the immune system in immunosurveillance of cancer[48,49]. Most notably was the identification of the role of NK and NK T cells where mice lacking each of these were more susceptible to chemical induction of cancers[50]. These reports clearly demonstrate that the immune system has a role to play in detecting new tumours and getting rid of them. The model for immunosurveillance has now expanded to include equilibrium and escape phases of immune system:tumour interaction. Collectively, these phases have been described as immunoediting[51]. The equilibrium phase consists of components of the adaptive immune system keeping tumour growth in check[52]. The escape phase is characterized by selection of tumour cells with reduced immunogenicity that grow out despite the immune system.

The ability of a tumour to avoid immune destruction was deemed so critical that in a 2011 follow-up to their landmark paper describing the hallmarks of cancer, Hanahan and Weinberg added it as one of two new hallmarks. In the face of efforts by the immune system to detect and eliminate tumours, tumours have developed several mechanisms to

evade this immune system restricted growth. Many tumours down-regulate MHC-1 in order to hide from CD8 T cell recognition[53]. This down-regulation of MHC-1 necessitates other immunosuppressive strategies as these cells now become targets for NK cells. To achieve this, in part, tumour cells have been shown to produce the immunosuppressive cytokine TGF-β, which functions to alter the phenotype of many immune cells such as CD8 T cells, CD4 T cells, NK cells limiting their ability to clear the tumour[54,55]. Tumours also express indolamine-2,3-dioxygenase (IDO) which is an enzyme that depletes tryptophan stores, preventing T lymphocyte proliferation[56,57]. Lastly, tumour cells have been shown to express PD-1 ligand. PD-1 ligand interacts with the inhibitory co-receptor PD-1 on T lymphocytes. PD-1 expression is thought to be used to help cells evade immune detection not only in tumours but also in feto-maternal tolerance[58,59].

Many of these mechanisms employed by tumours to evade the immune system present targets whereby we can modify the tumour microenvironment in order to promote immune recognition of the tumour. Antibodies to PD-1 have been used in mouse models and more importantly in clinic[60-62]. IDO inhibition has also been employed in attempts to reinstall the normal immune function within the tumour microenvironment[63]. The immunosuppressive nature of the tumour can also be targeted with antibodies to CTLA-4 and 41BB. CTLA-4 is a glycoprotein that binds B7, preventing CD28 binding due to its higher affinity for B7 molecules[64]. It is constitutively expressed on the immunosuppressive CD4/CD25 regulatory T cells found within a tumour and on effector

T cells as well[65]. Blockade of the CTLA-4 pathway reinstates the normal T cell activation pathways[66,67]. Anti-41BB (anti-CD137) is an agonist antibody that can provide costimulation to T lymphocytes[68]. Each of anti-PD-1, IDO inhibition, anti-CTLA-4 and anti-41BB helps restore normal immune activity in a tumour microenvironment, with potential to detect and eliminate the tumour.

3.0 Tumour Associated Antigens

Tumour associated antigens (TAAs) are generally proteins in the tumour that are over-expressed, are expressed in mutated forms or are of viral origin[69]. Some antigens can be targeted using passive immunotherapeutics such as monoclonal antibodies or active therapies such as vaccines. An ideal tumour antigen is one whose expression is restricted to tumours and is not present at high levels in normal tissues. Clearly, targeting an antigen on normal tissues may lead to undesired autoimmune problems. The timing of when a tumour antigen is expressed by a tumour is also important. Graziano and Finn[69] argue that targeting antigens that are expressed early in the malignant process have a higher likelihood of success because targeting can happen early in the disease process before the immunosuppressive effects of the tumour are fully realized. While this concept seems reasonable, patients often don't present until disease has significantly progressed. The function of a target in the malignant process is also an important consideration when selecting a tumour antigen. Targets that actively promote tumourigenesis are less likely to be the subject of immune evasion following

immunotherapeutic targeting. In the event that cells do escape the immune targeting, they are likely to have less malignant potential as they will lack one of the tumourigenic factors present in the original tumour. In the case of active immunotherapeutics, an ideal tumour antigen is one that is immunogenic, where immune responses to the target antigen can be generated.

3.1 Tumour associated antigens as immunotherapy targets

Passive therapy takes advantage of antibodies specific for the tumour antigen that are produced ex vivo and delivered to the patient. These antibodies can both serve to inhibit the function of their targets and to target the expressing cell for complement activation and cell dependent lysis[70]. Examples of such immunotherapeutics include: Herceptin (tratuzumab), Avastin (bevacizumab) and Erbitux (cetuximab). Herceptin is a monoclonal antibody targeting the tumour associated antigen HER2 found in estrogen-responsive breast cancers[71]. Avastin is a monoclonal antibody targeting vascular endothelial growth factor (VEGF). VEGF is important in the recruitment of new blood vessels into tumour tissue, a process known as angiogenesis. Avastin not only targets VEGF expressing tumour cells, but can also target VEGF from other tumour stroma cells[72,73]. Avastin has also been shown to have direct effects on tumour cells, inducing their apoptosis[74]. Erbitux (cetuximab) is a monoclonal antibody targeting the epidermal growth factor receptor (EGFR), a tumour antigen that is part of a signaling pathway that is often upregulated in non-small cell lung cancer[75]. Efficacy with the single agent in

clinical trials has been limited thus far, but investigation continues with multiple different treatment regimens and reagent combinations[76].

3.2 Tumour associated antigens as vaccine targets

An active strategy to target tumour antigens involves the use of vaccines. Some current cancer vaccines are used prophylactically, however, these vaccines offer protection against the cancer causing viruses such as HPV and HBV. Targeting non-viral, tumour associated antigens in a therapeutic setting is more challenging. One of the most significant impediments to vaccinating against self, or modified self, antigens is immunological tolerance. The vast majority of T cells that can react strongly with self antigen are deleted through central tolerance. Others that escape central tolerance can be subject to peripheral tolerance. Despite the mechanisms of immune tolerance, the presence of weakly autoreactive T cells in the periphery is a tantalizing target for therapeutic cancer vaccines. Indeed, many strategies to generate vaccines targeting many tumour antigens are being developed. One example of such a strategy is the use of NY-ESO-1 vaccines. NY-ESO-1 is a tumour associated antigen of the cancer/testis family that is a reasonably immunogenic target expressed at a high level in epithelial ovarian cancer[77]. While numerous methods of targeting NY-ESO-1 expression are being pursued, the studies using virus vaccine vectors are most striking. Vaccinia and Fowlpox viruses expressing NY-ESO-1 have shown safety and efficacy in patients with melanoma or ovarian cancer[78,79]. These vaccines were also successful at inducing T cell responses and

antibody responses against the tumour antigen[78,80]. Positive reports such as these are fueling the ongoing search for new targets to add to the growing list of tumour associated antigens.

3.3 Plac1

Placentae and tumours share many common traits. These include the ability to promote new blood vessel formation, the ability to invade other tissues and the ability to down regulate immune responses[81]. This observation prompted a search for placenta-specific genes that may be re-expressed in tumour cells. Silva *et al* identified Plac1 as such. Plac1 is a protein 212 amino acids in length in humans and 173 amino acids in mice. It is predicted to be a transmembrane protein with a single transmembrane domain at the extreme amino terminus with the majority of the protein found on the extracellular face of the membrane[82]. It's specific function in placental development in unknown but it's expression in mice correlates to placental development and Plac1 knockout mice have decreased viability[83,84]. Plac1 is expressed in placenta, and to a lesser degree testis but not in other normal tissues[81]. Plac1 expression, albeit at a lower level than found in placenta, has also been reported in several tumour cell lines and in tumours of patients with breast, lung, liver, gastric and colorectal cancers[81,85-88]. Interestingly, some of the patients with Plac1 positive tumours had both serum antibodies and T cell responses[81,87,88]. Serum antibodies have not been detected in healthy males but were detected in a small number of healthy females, although information on the pregnancy

status of their subjects was not discussed[81]. It is unclear whether Plac1 possesses a tumour promoting function, but there is one observation that either knockdown by siRNA or blocking by neutralizing antibody can inhibit breast cancer cell line invasiveness in vitro[82]. Taken together, these observations suggest that Plac1 would be a good tumour antigen target as it appears to be spontaneously immunogenic, has expression restricted to tumours and may promote tumourgenesis.

3.4 Gp100

Gp100 is a melanocyte differentiation antigen involved in melanin synthesis[89]. Its expression is mostly limited to cells of the melanocyte lineage even though transcripts for it can be detected in a number of normal tissues[90,91]. This restricted expression has resulted in its used as a target for immunotherapies in mice and in patients. In patients, most gp100 studies use peptide vaccination in adjuvant or peptide pulsed dendritic cells[92-95]. It has also been used in combination with anti CTLA-4 antibodies with some success[96]. Some of the issues noted in gp100 therapies include immune related side effects of the anti CTLA-4 (ipilimumab) treatement, failure of T cells to track to the tumour and the lack of correlation between the presence of gp100 T cells and progression of disease. The immune side effects related to the ipilimumab treatment can be mitigated through steroid use[97]. The issue with the T cells remaining in the location of the peptide and adjuvant injection can be addressed by limiting the persistence of the antigen[92]. The third issue, where the presence of T cells fails to result in disease stabilization or

regression has not been completely resolved. This observation is also apparent in mice, where large numbers of gp100 specific CD8 T cells can be generated but tumours fail to regress[98]. Despite the issues encountered in patients using gp100 as an immunotherapeutic, there have been documented successes[95]. These observations suggest that by confronting the barriers to successful use of gp100 targeting therapies, we can expand the clinical benefits of such therapies.

4.0 Oncolytic Viruses

The association of viral infection with tumour regression dates back to the late 1800s and the early 1900s when several anecdotal reports were made of patients going into remission with leukemia following viral infections[99]. Since then, marked improvements in the understanding of viruses and cancer at the molecular level have facilitated an expansion in the number of viruses actively being studied as oncolytic therapeutics. Several oncolytic viruses are now in multiple stages of clinical trials[100]. The basic premise of how oncolytic viruses work is that genetic changes that allow the tumour to focus on rapid division render them sensitive to virus infection. One such pathway that can be exploited by oncolytic viruses is the innate antiviral response. In the absence of viral infection, there is no consequence to the tumour cells in proliferating these defects. Oncolytic virus therapy exploits this difference between normal and tumour cells by selectively infecting and lysing tumour cells while leaving normal cells with functional antiviral programming unscathed. This specificity can be enhanced by

attenuating the oncolytic virus, further limiting its ability to replicate in normal cells while maintaining its ability to infect and kill tumour cells[101]. Two examples of oncolytic viruses that have been used in the clinic are Newcastle disease virus (NDV) and adenovirus. The paramyxovirus, NDV, normallly infects birds and as such is attenuated in normal human cells[102]. One strain of NDV, PV701 has been successfully used in a phase I trial where it demonstrated limited toxicity[100,103]. Adenoviruses have also been used as oncolytics in the clinic. The ONYX-015 adenovirus has an E1B deletion that prevents the virus from inactivating p53, severely attenuating the virus in normal cells[104]. The mutation of p53 in many tumour cells allows ONYX-015 to replicate in and kill many tumours while leaving normal cells unharmed[104]. While the E1B:p53 interaction was the rationale for the adenoviral oncolytic potential, the actual mechanism through which this virus displays its oncolytic abilty is unclear[105]. Like NDV, Onyx-015 has been shown to be safe in several phase I trials[106]. These naturally attenuated or genetically attenuated oncolytic viruses represent the first generation of oncolytic viruses to be used in the clinic.

While the simplistic model of how oncolytic viruses work is they directly kill tumour cells, an equally important consideration is the induction of immune response against tumour antigens to aid in the clearance of uninfected cells and prevent disease recurrence. This effect has been described for a number of oncolytic viruses. In a subcutaneous B16-HSV thymidine kinase tumour model in C57BL/6 mice, oncolytic reovirus (Reolysin) results in anti tumour specific T cells to the immunodominant epitope

of DCT (dopachrome tautomerase), a melanoma associated antigen[107]. Secondly, in melanoma patients treated with talimogene laherparepvec (T-Vec, formerly Oncovex), an HSV-1 virus expressing GM-CSF, results in melanoma associated antigen specific T cells and some objective responses in a phase II trial[108]. In each of these two cases the role of the oncolytic virus expands beyond the simple tumour lysis and results in effects on the immune environment faced by the tumour.

The importance of anti tumour immune responses has been explored in the context of an accidental tumour vaccine. Cramer et al tried to explain the observation that patients who had previously had an active mumps infection were less likely to have ovarian cancer. They hypothesized that the hypoglycosylated form of MUC1 (a tumour associated antigen from the mucin family of genes) that is expressed in the salivary glands during a mumps viral infection primed an immune response to the same form that is often expressed in ovarian cancer[109]. They were able to detect higher serum levels of anti MUC1 antibodies in patients who had just had an active mumps infection, confirming the plausibility of their hypothesis.

4.1 Oncolytic viruses as vaccine vectors

Combining the effects of therapeutic cancer vaccines and oncolytic viruses has been the focus of a recent trend toward creation of the next generation of oncolytic viruses. There are several reports of incorporation of immunogenic artificial tumour antigens such as ovalbumin or beta-galactosidase into oncolytic vectors to target tumour

cells engineered to express those same antigens[110-112]. Work in our group has used this strategy to target the endogenous tumour antigen, DCT[113]. This strategy aims to take advantage of the cancer seeking and initial destruction of the tumour by the oncolytic virus before allowing the restimulated immune system to detect remaining and future recurring tumour cells. The inclusion of a specific tumour antigen builds on the passively generated immune response from the dying infected tumour cells.

Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV) is a bullet-shaped helical virus with a negative sense single stranded RNA genome coding 5 genes. Its sensitivity to innate antiviral pathways, and type I IFN signaling specifically makes it an excellent candidate as an oncolytic virus[114]. In addition to its capacity as an oncolytic virus, VSV has also been used as a vaccine vector by a number of groups[115,116]. We intend to exploit both capabilities of VSV in the context of this body of work.

Adenovirus

In constrast VSV, adenoviruses are non-enveloped with icosohedral capsid structure surrounding a linear non-segmented dsDNA genome[117]. While Adenoviruses can be oncolytic agents, they are perhaps more well known for the use in gene therapy and development as vaccine vectors for a wide range of infectious diseases including infuenza, rabies and HIV[118,119]. There suitability as gene therapy vectors stems from,

among other characteristics, the ability to package large amounts of foreign DNA[120]. It is in the capacity as vaccine vectors that they are used in the subsequent study.

5.0 Research Objectives

The Plac1 data in the literature concerning its restricted expression, immunogenicity and potential role in tumourigenesis led us to **hypothesize that we could generate cell mediated and humoral immune responses to Plac1 and the differential expression of Plac1 between tumour cells and normal cells could be exploited using oncolytic therapeutic vaccines expressing Plac1**. Additionally, the ability to generate anti mgp100 T cell responses led us to the **hypothesis that the effective immune response following vaccination against gp100 can be improved through boosting of primary responses, pharmacological manipulation, or combination with another vaccine transgene to demonstrate anti tumour efficacy**.

Objective 1: Develop Plac1 vaccine vectors capable of eliciting anti Plac1 immune responses.

Objective 2: Evaluate the ability of the Plac1 vaccine vectors to elicit anti tumour responses in mice.

Objective 3: Modify the gp100 vaccination methods to permit the anti gp100 response to have anti tumour activity in the B16F10 tumour model.

- CHAPTER 2 -

Materials and Methods

Cell lines

U2OS, CT26, A549, MEFs and Vero cells were grown in alpha modified Eagle's medium with 10% FCS , 2mM L-Glutamine, 100 U/mL each penicillin and streptomycin. B16F10 cells were maintained as with the addition of 50μM β mercaptoethanol (Gibco), sodium pyruvate, vitamins and non essential amino acids (Gibco). 293T cells were grown in Dulbecco's minimal essential medium with 10% FCS, 2mM L-Glutamine, 100 U/mL each penicillin and streptomycin. All cells were grown in 5% CO2 at 37 °C.

Viruses

Transgenes for recombinant VSVs were generated using Phusion PCR with XhoI/ NheI appended primers and cloned into the pJet cloning vector. Insert positive colonies were checked for correct sequence (Mobix) and subcloned into pVSV-XN. Recombinant VSVs were cloned using the pVSV-XN backbone using the XhoI and NheI restriction sites. Murine Plac1 (mPlac1) transgene was generated from CT26 cDNA. Human Plac1 (hPlac1) was generated from A549 cDNA. Rat Plac1 was generated from cDNA from a hybridoma cell line kindly provided from Dr. Dawn Bowdish. The codon optimized hmPlac1 fusion transgene was synthesized by GeneArt.

Recombinant VSVs were rescued as previously described[121]. A549 cells in 60mm dishes were infected with vaccinia virus expressing T7 RNA polymerase and transfected with genome plasmid and 3 helper plasmids, pBS-N, pBS-L and pBS-P using Lipofectamine 2000. Two days after transfection, the supernatants were filtered through .

22 um filter to remove residual vaccinia virus and passaged onto Vero cells. Successful rescues were identified by confirming cytopathic effect (CPE) two days later. Supernatants from successfully rescued wells were plaque-purified three times. The expansion of the final plaque took place on 293T cells. VSVs were expanded on 293T cells infected at a multiplicity of infection (MOI) of 0.01, with supernatants harvested 24-48 hours later when maximum CPE was observed. The collected supernatants were filtered through .22 um filter and pelleted at 15000xg for 90 minutes. The pellets were carefully resuspended and layered on a 5-40% sucrose (in phosphate buffered saline - PBS) gradient sitting on a 75% sucrose cushion. The gradients were spun at 24000rpm in a SW41 Ti rotor for 30 minutes. The band corresponding to monomeric VSV particles was retrieved and dialyzed against PBS to remove sucrose and stored in aliquots at -80 °C.

Recombinant Adenoviruses were rescued as previously described[122]. Plasmids harboring the transgenes described above were used as templates in a Phusion PCR to append EcoRI and HindIII restriction sites. These PCR products were once again cloned into pJet vectors and insert positive colonies screened for correct sequence. The transgenes were then subcloned into the pDC316 shuttle vector. The shuttle vector plasmids were combined with the pBHG adenovirus backbone and transfected using calcium phosphate. The AdmPlac1 and AdhPlac1 were rescued by Carole Evelegh in Dr. Jonathan Bramson's lab. Successful recombinants were plaque-purified and expanded by the Fitzhenry Vector facility and stored at -80 °C.

RT-PCR

RNA from murine cell lines was obtained using the Qiagen RNA extraction kit and used as template in a reverse transcriptase reaction using the Superscript RT kit from Life Technologies. Intron spanning primers for mPlac1 were CAGCTGCCAGAAGGAGAATC & CGAGCACAGCACATTCACTT.

Patient sample RNA was obtained from frozen samples provided by Dr. Rebecca Auer at the Ottawa Hospital using the QiaShredder and Qiagen RNA extraction kits. cDNA was generated using the SuperScript RT kit from Life Technologies. The hPlac1 primers were 5'-AAATTTGGCAGCTGCCTTCAC-3' & 5'-TGATGCCACATTCAGTAACAC-3'. The GAPDH primers were 5'-ACCCAGAAGACTGTGGATGG & 5'-CCCTGTTGCTGTAGCCAAAT-3'

Western Blotting

293T cells transfected with the plasmid to be tested, B16F10 or CT26 cells were lysed in 1X SDS PAGE loading buffer and passed through a 23G syringe >5 times to shear genomic DNA. Equivalent proportions of equally confluent wells were loaded on 15% acrylamide gels. Proteins were transfered onto nitrocellulose membranes which were subsequently blocked for 1 hours at room temperature or overnight at 4 °C. Rabbit anti FLAG primary (Rockland) was used at 1:3000. Goat anti mPlac1 (Santa Cruz) was used at 1:50. Chicken anti goat or anti rabbit secondary antibodies 680 conjugated antibodies were used at 1:2500 and blots visualized using the LiCor Odyssey scanner.

Immunofluorescence

U20S cells were plated on coverslips in 6 well plates. They were infected with and MOI of 10 with Ad mPlac1 or Ad BHG. At 48 hours post infection, cells were fixed with 4% paraformaldehyde and permeabilized with 1:1 Acetone:Methanol. Goat anti mPlac1 was used at 1:50. The chicken anti Goat-594 was used at 1:1000. The slides were observed at the McMaster Biophotonics facility.

Animal Studies

Animal experiments were conducted under our Animal Use Protocol (AUP) approved by McMaster University's Animal Research Ethics Board. Male or Female, 6 to 8 wk old BALB/c or C57BL/6 mice were purchased from Charles River Laboratories. Adenovirus for vaccination was prepared at 2 x 10^9 PFU/mL in sterile saline. 50 µL of virus suspension was injected into each quadricep of the anaesthatized mice. VSVs were prepared at 5 x 10^9 PFU/mL in sterile saline. Mice received 200 µL of suspension intravenously in the tail vein.

For prophylactic tumour studies, 1 x 10^5 B16F10 or CT26 tumour cells in 100 µL sterile saline were injected subcutaneously on the left flank of each mouse. Mice were shaved and were monitored for palpable tumours.

For therapeutic studies, 2.5 x 10^5 cells in 200µL sterile saline were injected intravenously in to the tail vein of restrained mice.

For studies evaluating pharmacological manipulation of the immune response, CPA was administered through the intraperitoneal route at 1mg/mouse the day prior to adenovirus prime. MS-275 was administered intraperitoneally at a dose of 100μg/mouse each of five consecutive days beginning on the day of the VSV boost.

Immune analysis

Serum from vaccinated mice was obtained by performing a terminal bleed via cardiac puncture. Blood was allowed to clot at room temperature for >2 hrs. The clot was then remove centrifugation at 12000 rpm for 15 minutes in a benchtop centrifuge. Serum was stored at -20 °C.

Peripheral Blood Mononuclear cells were isolated from blood drawn at intervals indicated after each vaccination using ACK lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1M Na_2EDTA, pH 7.4). Peptides (see tables 1, 2 &3 for sequences) were used to restimulate PBMCs for 5 hours. After 1 hour of stimulation, GolgiPlug (BD Biosciences) was added to prevent secretion of cytokines. After 4 hours of stimulation in the presence of GolgiPlug, cells were stained for CD4 or CD8, then fixed and permeabilized with CytoFix/CytoPerm and stained for IFNγ and TNFα. Staining was analyzed using the FACS Canto and FlowJo was used to analyze the flow data.

Table 1: Human Plac1 Peptides

Peptide	Sequence	Peptide	Sequence
1	MKVFKFIGLMILLTS	26	SSKGTPSKFVIPVSC
2	KFIGLMILLTSAFSA	27	TPSKFVIPVSCAAPQ
3	LMILLTSAFSAGSGQ	28	FVIPVSCAAPQKSPW
4	LTSAFSAGSGQSPMT	29	VSCAAPQKSPWLTKP
5	FSAGSGQSPMTVLCS	30	APQKSPWLTKPCSMR
6	SGQSPMTVLCSIDWF	31	SPWLTKPCSMRVASK
7	PMTVLCSIDWFMVTV	32	TKPCSMRVASKSRAT
8	LCSIDWFMVTVHPFM	33	SMRVASKSRATAQKD
9	DWFMVTVHPFMLNND	34	ASKSRATAQKDEKCY
10	VTVHPFMLNNDVCVH	35	RATAQKDEKCYEVFS
11	PFMLNNDVCVHFHEL	36	QKDEKCYEVFSLSQS
12	NNDVCVHFHELHLGL	37	KCYEVFSLSQSSQRP
13	CVHFHELHLGLGCPP	38	VFSLSQSSQRPNCDC
14	HELHLGLGCPPNHVQ	39	SQSSQRPNCDCPPCV
15	LGLGCPPNHVQPHAY	40	QRPNCDCPPCVFSEE
16	CPPNHVQPHAYQFTY	41	CDCPPCVFSEEEHTQ
17	HVQPHAYQFTYRVTE	42	PCVFSEEEHTQVPCH
18	HAYQFTYRVTECGIR	43	SEEEHTQVPCHQAGA
19	FTYRVTECGIRAKAV	44	HTQVPCHQAGAQEAQ
20	VTECGIRAKAVSQDM	45	PCHQAGAQEAQPLQP
21	GIRAKAVSQDMVIYS	46	AGAQEAQPLQPSHFL
22	KAVSQDMVIYSTEIH	47	EAQPLQPSHFLDISE
23	QDMVIYSTEIHYSSK	48	LQPSHFLDISEDWSL
24	IYSTEIHYSSKGTPS	49	HFLDISEDWSLHTDD
25	EIHYSSKGTPSKFVI	50	ISEDWSLHTDDMIGSM

Table 2: Mouse Plac1 Peptides

Peptide	Sequence	Peptide	Sequence
51	MNLRKFLGGTVLVAF	72	KAVSPDVVIYSSEIH
52	KFLGGTVLVAFMLFS	73	PDVVIYSSEIHYASK
53	GTVLVAFMLFSYSEQ	74	IYSSEIHYASKGSST
54	VAFMLFSYSEQNQVN	75	EIHYASKGSSTKYVI
55	LFSYSEQNQVNVLCS	76	ASKGSSTKYVIPVSC
56	SEQNQVNVLCSTDWF	77	SSTKYVIPVSCAAPR
57	QVNVLCSTDWFMVTV	78	YVIPVSCAAPRRSPW
58	LCSTDWFMVTVHPFL	79	VSCAAPRRSPWLTKP
59	DWFMVTVHPFLLNND	80	APRRSPWLTKPYSAK
60	VTVHPFLLNNDVYVH	81	SPWLTKPYSAKAPSN
61	PFLLNNDVYVHFYEV	82	TKPYSAKAPSNNMGA
62	NNDVYVHFYEVHLGL	83	SAKAPSNNMGATPKN
63	YVHFYEVHLGLGCPP	84	PSNNMGATPKNDTSY
64	YEVHLGLGCPPNHVH	85	MGATPKNDTSYHVFT
65	LGLGCPPNHVHPHFY	86	PKNDTSYHVFTLPEP
66	CPPNHVHPHFYQFHY	87	TSYHVFTLPEPSEQP
67	HVHPHFYQFHYRVTE	88	VFTLPEPSEQPNCSC
68	HFYQFHYRVTECGIR	89	PEPSEQPNCSCPPYV
69	FHYRVTECGIRIKAV	90	EQPNCSCPPYVYNQK
70	VTECGIRIKAVSPDV	91	CSCPPYVYNQKSM
71	GIRIKAVSPDVVIYS		

Table 3: Other peptides used in intracellular cytokine staining

Target	Peptide	Sequence
VSV	RGY	RGYVYQGL
VSV	MPY	MPYLIDFGL
DCT	SVY	SVYDFFVWL
P15E	P15E	KSPWFTTL
Adenovirus	FAL	FALSNAEDL
gp100	$hgp100_{(25-33)}$	KVPRNQDWL
gp100	$mgp100_{(25-33)}$	EGSRNQDWL

Plac1 Antibody ELISA

Twenty 150mm plates of Vero cells were infected with VSVhPlac1-flag at an MOI of 3. Cell were scraped into lysis buffer provided with the FLAG-IP kit (Sigma) FLAG (DYKDDDDYK) tagged protein was isolated according to the manufacturers instructions. ELISA plates were coated with Rabbit anti FLAG antibody (Rockland) at 1ug/mL in 100ul/well. FLAG lysate was used at 100µL of 1:2 dilution of lysate retrieved from IP kit. Serial dilutions of mouse serum were incubated on the ELISA plate for 2 hours. An automatic ELISA plate washer was used to wash 4 x with PBST (phosphate buffered saline + 0.05% TWEEN 20) between each step. Bound mouse anti hPlac1 antibody was detected with anti mouse IgG1 biotin conjugated antibody (Biolegend) at 1:10000. Streptavidin Alkaline Phosphatase was used at 1:200 in 100ul/well for 30 minutes in the dark. Solutions A & B (R&D) were mixed 1:1 in 100ul/well in the dark for 30 minutes. H_2SO_4 was used as stop solution and absorbance was read at 450nm.

- CHAPTER 3 -

The effectiveness of targeting the Plac1 tumour antigen using mouse tumour models

1.0 Introduction

Plac1 is a recently described tumour associated antigen whose overexpression has been reported in a number of human tumour cell lines[81]. Overexpression of Plac1 mRNA and protein have also been detected in the tumours of patients with hepatocellular carcinoma[88], non small cell lung[81] and colorectal cancer[87]. Interestingly, all three of these reports describe spontaneous antibody responses to Plac1 suggesting that it is immunogenic and perhaps a useful vaccine target. In addition to antibody responses, Liu *et al* demonstrated a correlation between survival and the presence of anti Plac1 CD8 T cell responses in a subset of patients having detectable responses. This suggested that the generation of these T cells would be a useful endeavor and prompted efforts to study the targeting of Plac1.

Our chosen platform to target Plac1 through vaccination was the Adenovirus prime followed by vesicular stomatitis virus (VSV) boost. Previous reports have demonstrated the utility of using an oncolytic vaccine vector[113,123]. By priming a response to a tumour antigen, the response to the boosting vector is diminished while focussing the response on the transgene. The strategy is thought to help overcome the issue that viral vector components will be far more immunogenic than the autoantigen and that immune responses in the absence of the prime would predominantly be directed at the vector[124].

2.0 Results

2.1 Plac1 expression

Plac1 expression in human tumours

We first sought to recapitulate the observations of Plac1 expression in patients. In collaboration with Dr. Rebecca Auer at the Ottawa Health Research Institute, we obtained tumour and normal tissue samples from colorectal cancer patients. These samples were processed to generate cDNA which we used to evaluate Plac1 mRNA expression. Table 4 shows results for 15 patients, where 5/15 (33%) of the tumour samples tested positive for Plac1 mRNA and 0/15 of the normal tissues tested positive. This frequency is in line with those reported in literature[81,87,88].

Table 4: Summary of RT-PCR data on normal and tumour samples from 15 patients

		Plac1 mRNA	
Patient	**Sample**	**Normal**	**Tumour**
1	rectum	-	-
2	colon	-	+
3	rectum	-	-
4	rectum	-	+
5	sigmoid	-	+
6	colon	-	+
7	colon-mets. liver	-	-
8	rectum	-	-
9	rectum	-	-
10	colon	-	+
11	colon	-	-
12	rectum	-	-
13	sigmoid	-	-
14	colon	-	-
15	colon	-	-

As part of a quality control for another study taking place with Dr. Jamie Millar in Dr Jonathan Bramson's group, efforts were made to isolate tumour infiltrating lymphocytes (TILs) from patient tumours. The TILs recovered were expanded in culture in the presence of artificial antigen presenting cells to generate sufficient numbers for downstream assays. In one sample, the expanded TILs were tested for their ability be restimulated with the pooled hPlac1 peptide library versus the DMSO vehicle control. The TILs were then analyzed by intracellular cytokine staining for the ability to produce IFNγ, TNFα and IL-2. Following in vitro expansion of the TILs, approximately 0.7% of the CD8 T cells were IFNγ positive following Plac1 pooled peptide restimulation (Figure 1). The majority of these were also TNFα positive but IL-2 negative. The presence of Plac1 responsive CD8 T cells in a patient tumour further reinforced our view that Plac1 was a worthwhile target of new therapeutic vaccine strategies.

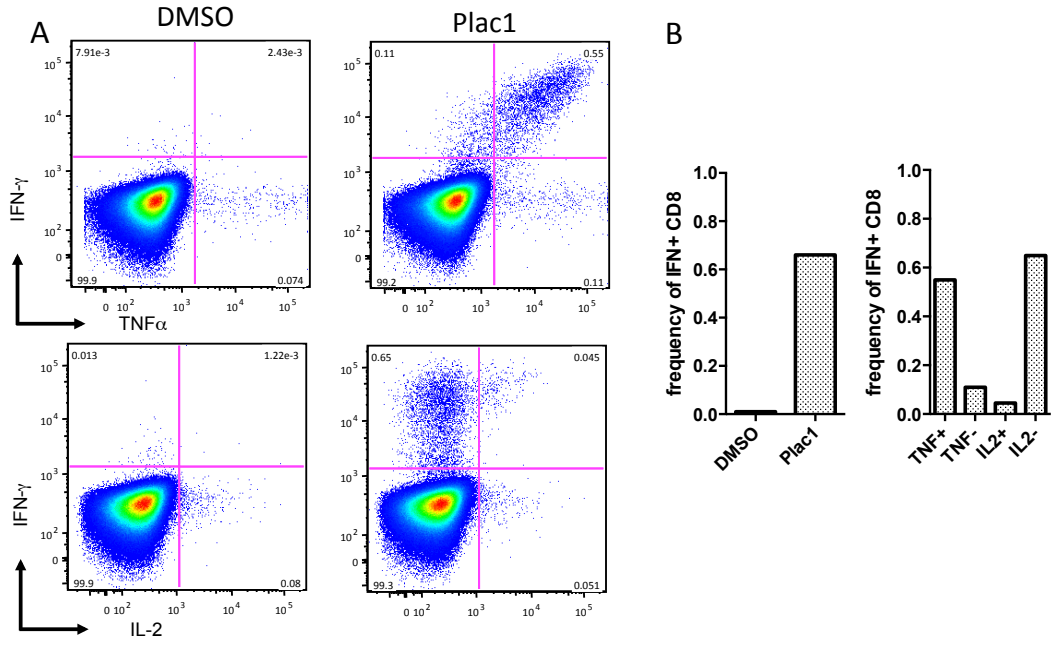

Figure 1: Plac1 responsive CD8 T cells isolated from a patient. **A**. Dot plots of flow cytometry results following intracellular cytokine staining showing staining for IFNγ, TNFα and IL-2 following restimulation with DMSO (left 2 panels) or Plac1 peptides (right 2 panels). **B**. Summary of flow data showing frequency of CD8 T cells that are IFNγ positive following DMSO or Plac1 peptide (left). Right panel shows the TNFα and IL-2 positive staining of the IFNγ positive CD8 T cells following restimulation with Plac1 peptides. *Data/Figure from Dr. Jamie Millar*

Is Plac1 expressed in mouse tumours?

The reported data with respect to Plac1 expression in human tumours, in addition to our RT-PCR analysis of patient samples, led us to investigate whether the murine homolog is expressed in mouse tumours as well. RT-PCR analysis of the B16-F10 and CT26 cell lines indicated that mPlac1 was in fact expressed at the level of mRNA in these two commonly used cell lines (Figure 2A). Attempts to detect mPlac1 protein in these cell lines were unsuccessful. However, this absence of detection was deemed to be the

result of limited sensitivity of the few available detection reagents. This assumption is supported by the observation that detection of overexpressed Plac1 by these same antibodies is weak as well (Figure 2B).

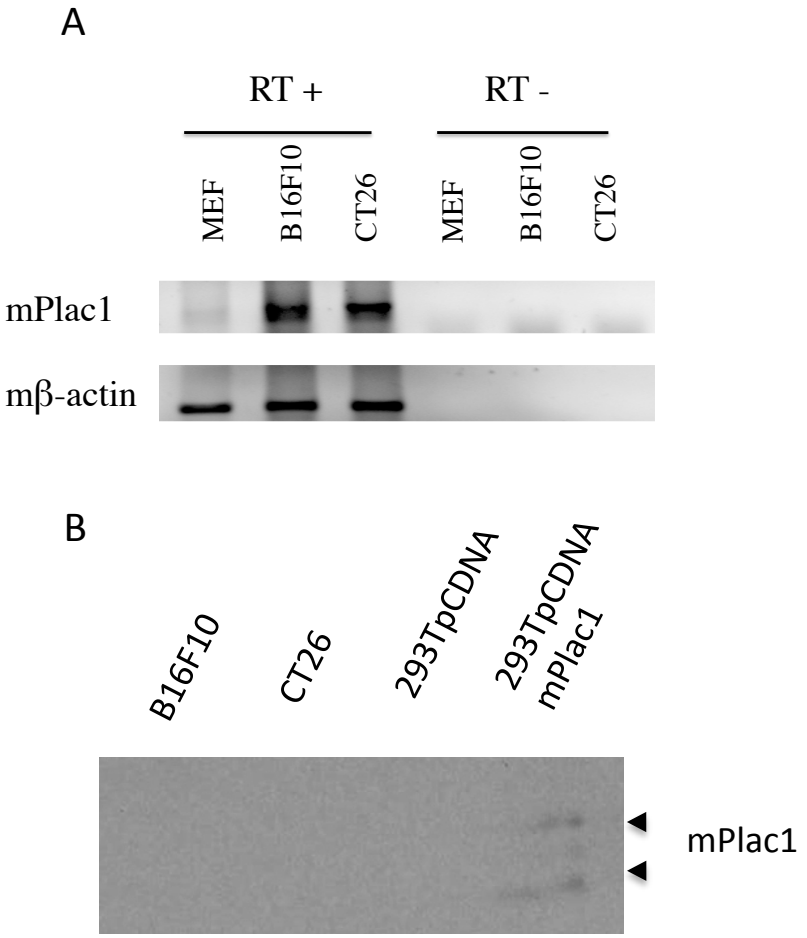

Figure 2. mPlac1 expression in mouse cell lines. A. RNA from murine embryonic fibroblasts (MEFs), B16-F10 and CT26 cells were analyzed by RT-PCR for the presence of mPlac1 mRNA (top) and mbeta actin mRNA bottom in the presence (left) or absence (right) of reverse transcriptase (RT). B. Western Blot of lysates from B16-F10 and CT26 as well as 293T cells transfected with empty pCDNA3 or pCDNA3-mPlac1.

2.2 Can an anti murine Plac1 immune response be generated?

Working on the assumption that mPlac1 protein is expressed in these tumour cell lines, if only at low levels, the next step in targeting it in the tumour model was to generate an anti mPlac1 immune response. Due to Plac1's restricted expression and the observation in humans of its immunogenic nature[81,87,88], we hypothesized that mPlac1 may be sufficiently immunogenic to use in vaccine vectors.

Can we vaccinate directly with mPlac1 vaccines?

We generated an adenovirus expressing murine Plac1 using an E1/E3-deleted replication incompetent Ad5 recombinant rescue system[120,125]. Expression of the transgene was confirmed by immunofluorescence shown in Figure 3a, where the left panel has U2OS cells infected with the empty vector and the right panel contains cells infected with AdmPlac1. We also generated a VSV expressing mPlac1 to use as an oncolytic boosting vector. Recombinant VSV was rescued as previously described [121]. Expression of mPlac1 from the recombinant virus was confirmed via western blot as shown in Figure 3b.

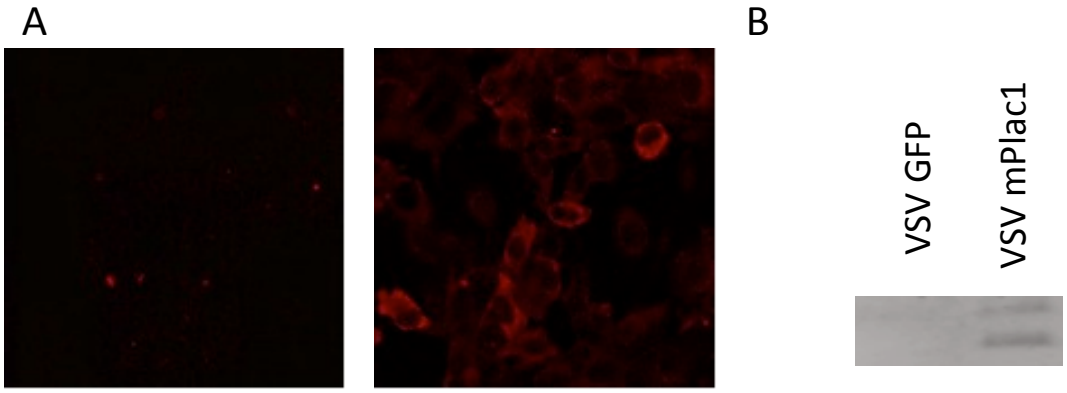

Figure 3. Expression of mPlac1 from vaccine vectors. **A.** Immunofluorescence of U2OS cells infected with AdBHG (left) or AdmPlac1 (right), stained with anti mPlac1 antibody and Alexafluor 594 chicken anti-goat secondary antibody.
B. Western blot analysis of lysates of 293T cells infected with VSV GFP (left) or VSVmPlac1 (right).

The AdmPlac1 was used to vaccinate female C57BL/6 mice using 1 x 10^8 PFU in 50μL saline in each quadricep (2 x 10^8 PFU in 100μL/mouse). The VSV mPlac1 was used as a boosting vaccine with 1 x 10^9PFU in 200μL saline given intravenously 14 days later. T cell responses were measured by performing intracellular cytokine staining on splenocytes harvested 5 days post boosting vaccination. The graphs in Figure 4 represent the frequency of IFNγ positive T cells as a percent of CD8 (top) or CD4 (bottom) T cells following in vitro stimulation with individual peptides from an overlapping library of Plac1 15mer peptides. None of the mPlac1 peptides, numbered 51-91 was able to stimulate IFNγ production in either CD8 or CD4 T cells. The assay appeared to work properly as the previously identified peptides corresponding to the immunodominant

epitopes in adenovirus (FAL) and VSV (RGY) were able to induce IFNγ production in CD8 T cells.

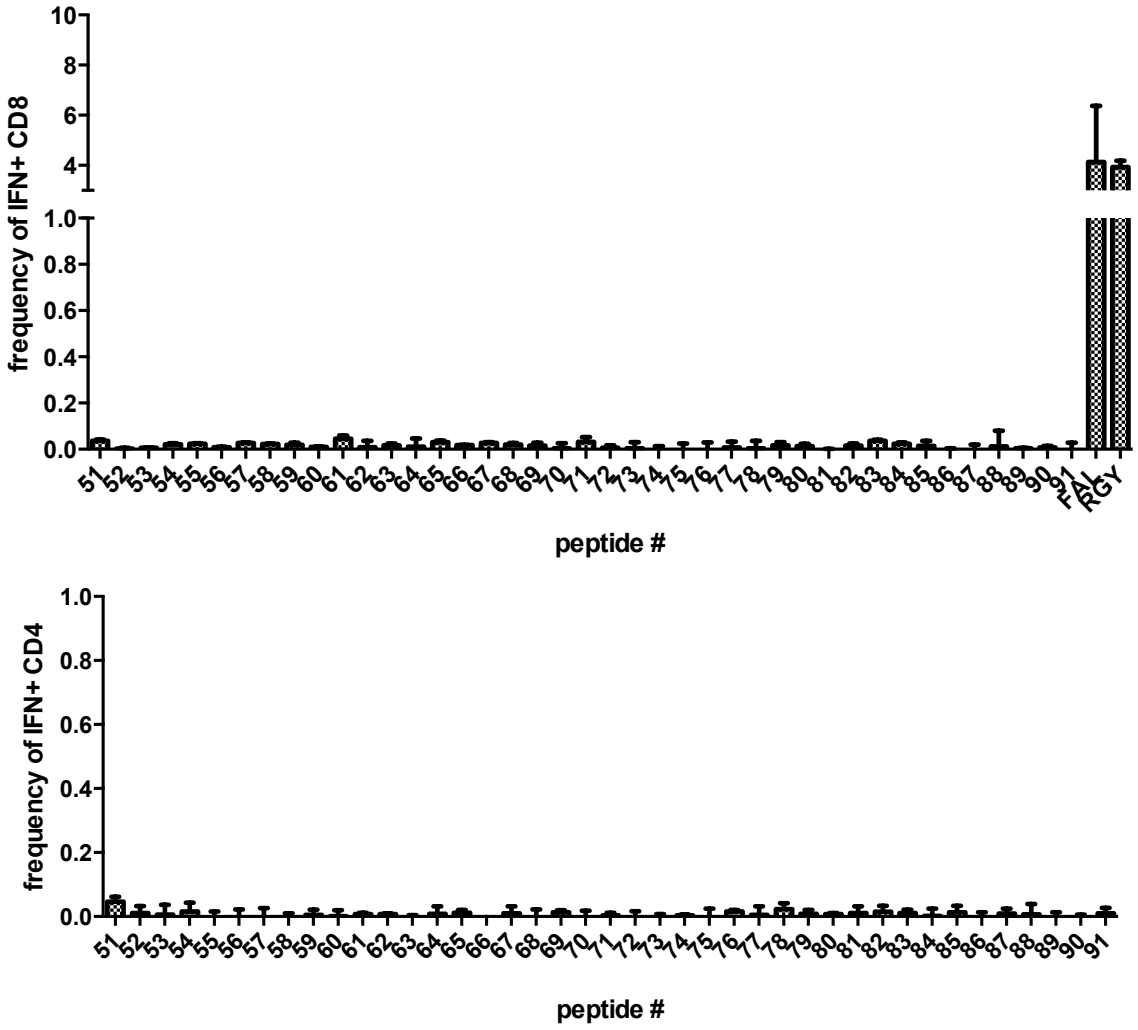

Figure 4. Epitope map of mPlac1 in C57BL/6. Frequency of CD8 T cell (top) and CD4 T cell (bottom) producing IFNγ in response to stimulation with individual peptides from the overlapping mPlac1 peptide library or the FAL and RGY peptide.

The assay was repeated using female BALB/c mice under the same vaccination protocol. Again, none of the Plac1 peptides was able to induce IFNγ production in CD8 or CD4 T cells. The peptide corresponding to the immunodominant VSV CD8 epitope (MPY) in BALB/c mice was able to induce IFNγ production in CD8 T cells (Figure 5). Despite our hope that the restricted expression and potential escape from central tolerance of mPlac1 might allow the generation of anti-mPlac1 immune responses by simply vaccinating with mPlac1 expressing vectors, it appears as though more complex vaccine protocols and modifications are required.

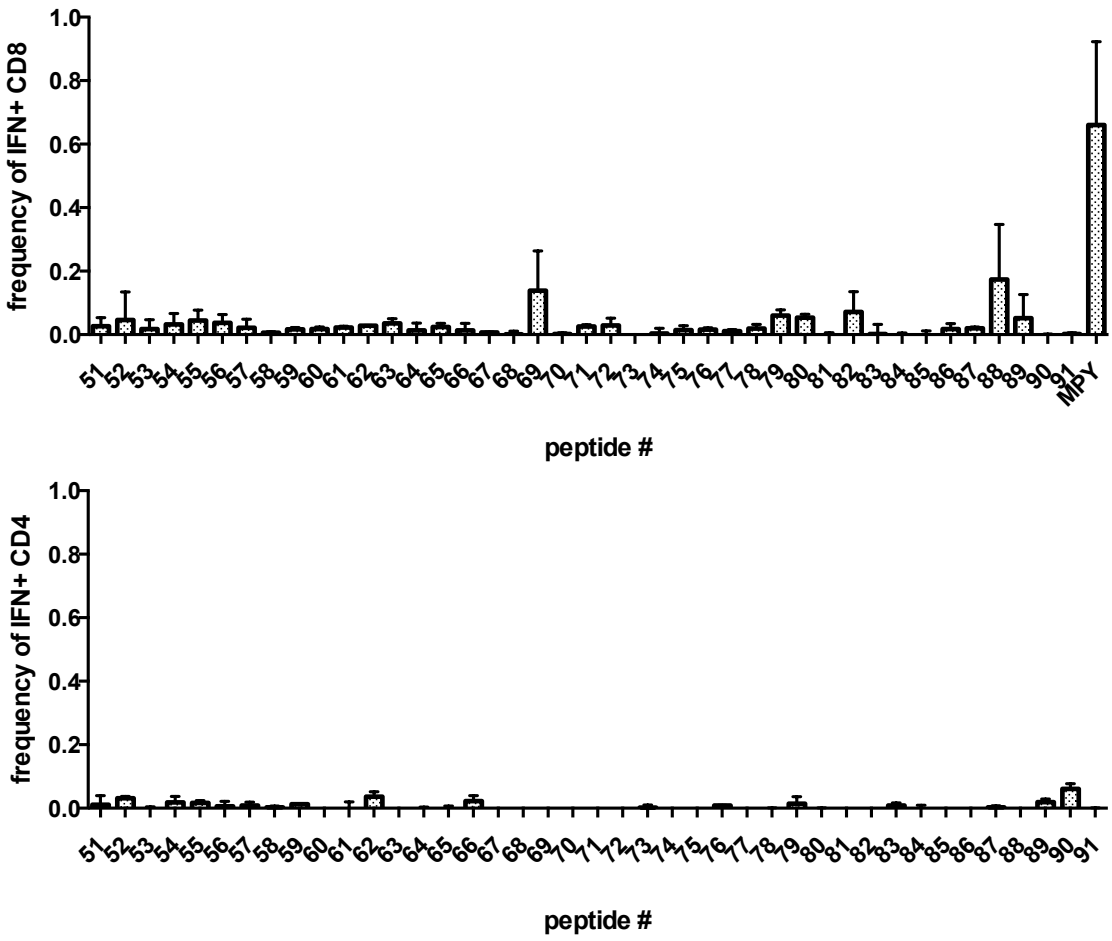

Figure 5. Epitope map of mPlac1 in BALB/c. Frequency of CD8 T cell (top) and CD4 T cell (bottom) producing IFNγ in response to stimulation with individual peptides from the overlapping mPlac1 peptide library or the MPY peptide.

Is there a difference in the ability to vaccinate male and female mice with mPlac1 expressing vectors?

We hypothesized that vaccinating male mice with mPlac1 vaccines may result in a greater anti mPlac1 immune response as a result of the difference in expression of mPlac1 between males and females coupled with the recent report of activating estrogen receptor

elements in the Plac1 promoter. Given that the Plac1 gene is located on the X chromosome[126], there are two copies per cell in females but only one in males. This led us to wonder if there would be a gender difference in the efficiency of negative selection of maturing thymocytes as part of central tolerance. It may be possible that mPlac1 responsive T cells are less likely to be eliminated in males since mPlac1 may be less likely to be expressed by promiscuous transcription factor AIRE in the thymus. To test for a sex specific difference, the experiments above were repeated in both BALB/c and C57BL/6 strains of male mice. In male C57BL/6 mice, while the FAL (Ad) and RGY (VSV) control peptides for the adenoviral and VSV epitopes were able to elicit IFNγ production in CD8 T cells, responses against the mPlac1 were virtually non existent with peptide #83 perhaps containing a very weak epitope that is barely distinguishable from background (Figure 6). No CD4 responses were detected.

In male BALB/c mice, we were initially excited to discover a CD8 epitope (Figure 7) contained within peptide #82 and perhaps a CD4 epitope contained at peptide #58-59. Attempts to confirm these epitopes in subsequent studies revealed that these responses were variable and that no difference between the ability to vaccinate males versus females exists in BALB/c. The weakness of CD8 responses to a CD8 epitope contained within peptide #82 suggested once again that further manipulation of the vaccine protocol was required to generate meaningful responses to mPlac1. It is unclear whether the initial response detected at peptide #82 was the result of variability of response or was the result of technical issues. With respect to the weak CD4 epitope initially identified in one of the

two male mice, this response was not reproduced in subsequent studies suggesting that peptide #59 did not contain a bona fide CD4 epitope.

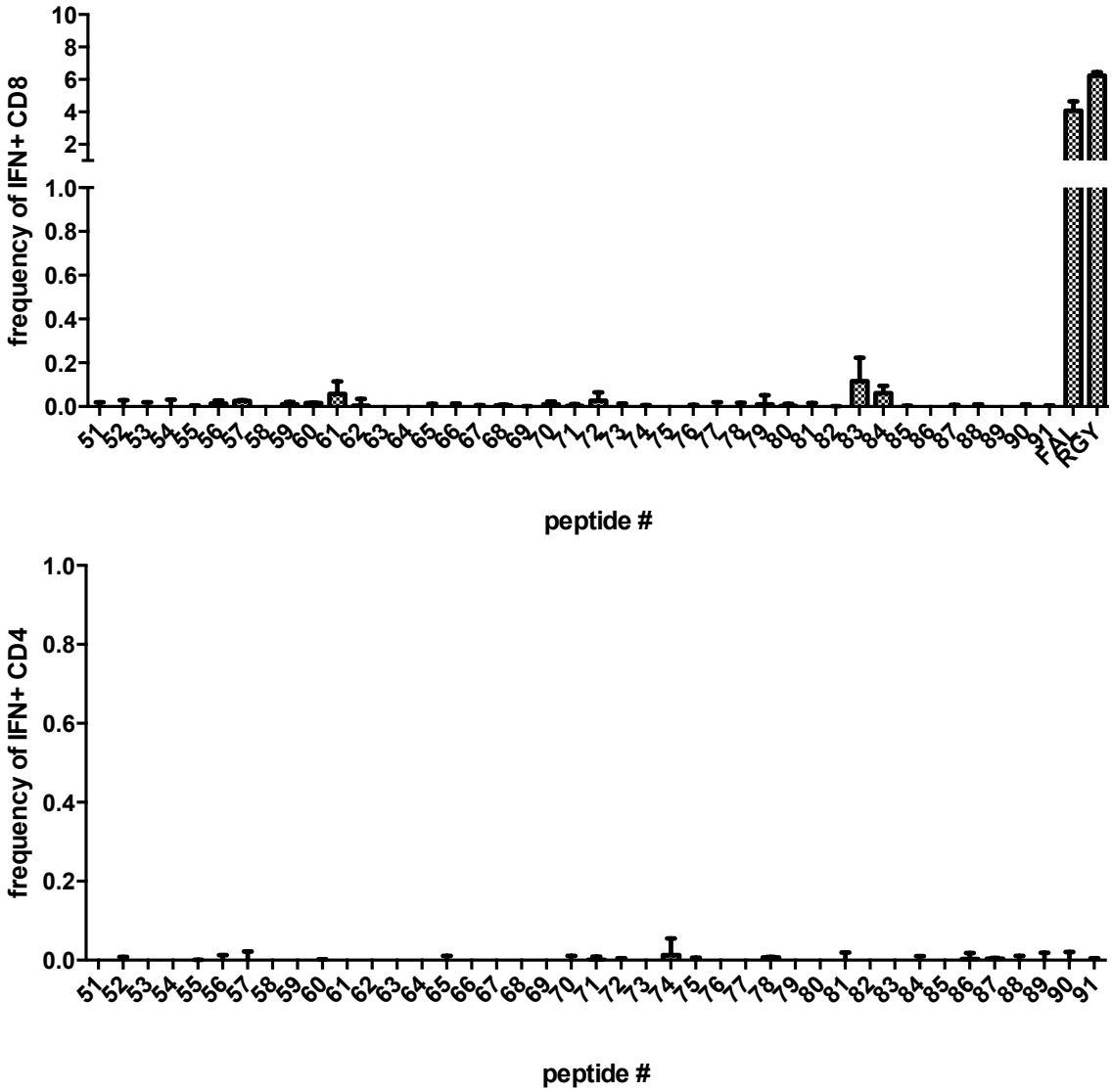

Figure 6. Epitope map of mPlac1 in male C57BL/6. Frequency of CD8 T cell (top) and CD4 T cell (bottom) producing IFNγ in response to stimulation with individual peptides from the overlapping mPlac1 peptide library or the FAL (Ad) and RGY (VSV) peptide.

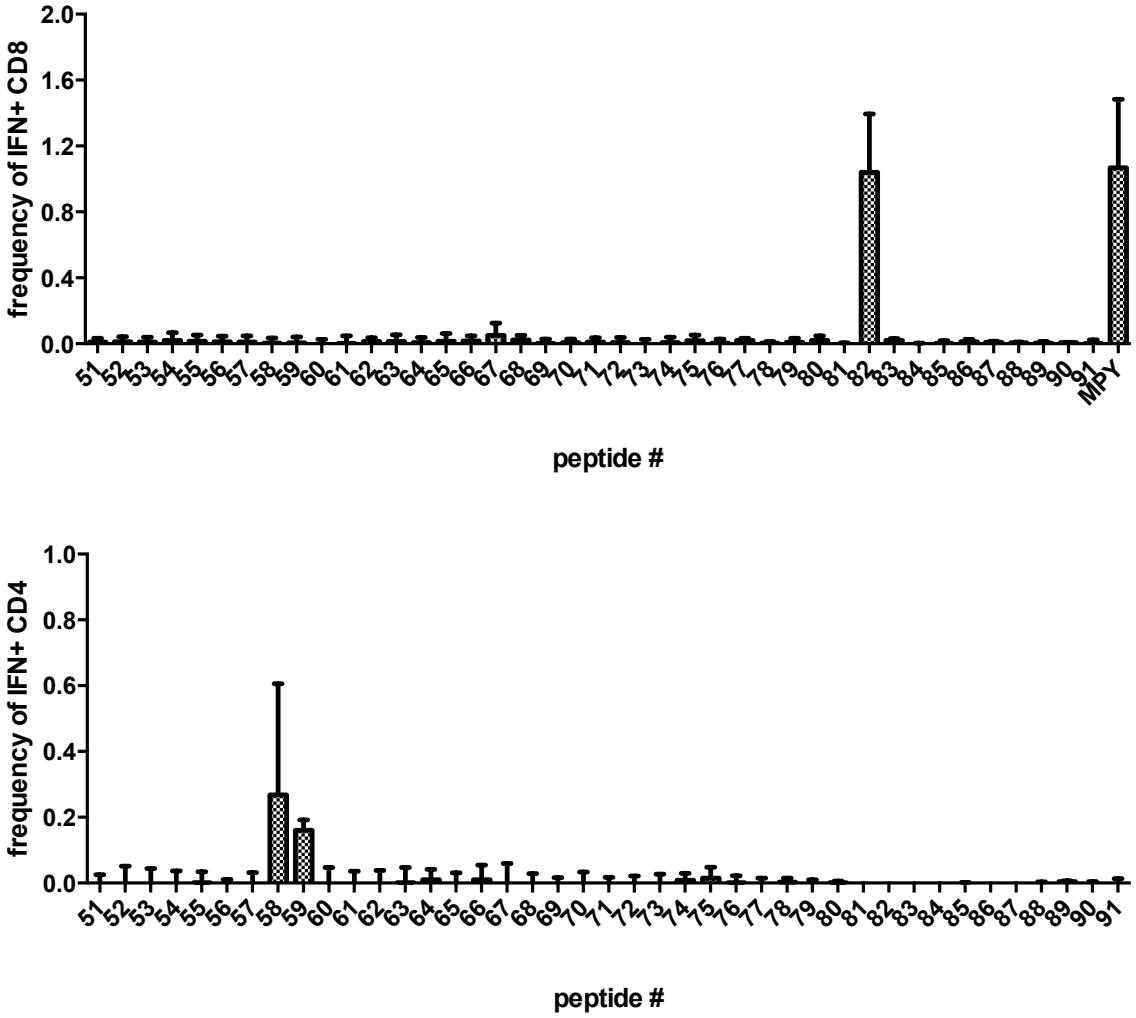

Figure 7. Epitope map of mPlac1 in male BALB/c. Frequency of CD8 T cell (top) and CD4 T cell (bottom) producing IFNγ in response to stimulation with individual peptides from the overlapping mPlac1 peptide library or the MPY peptide.

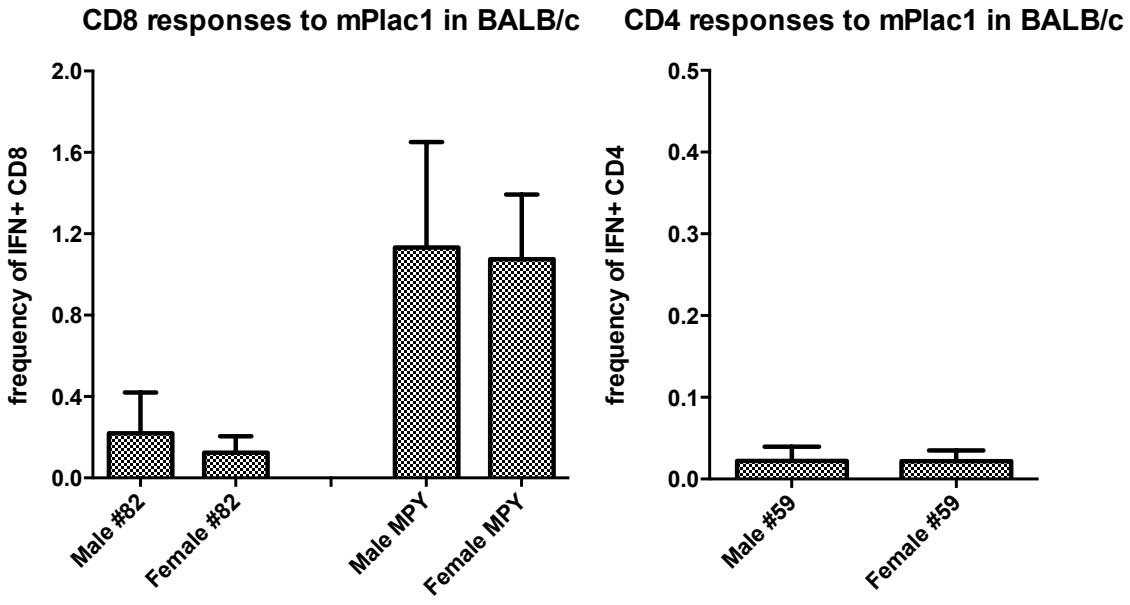

Figure 8. CD8 and CD4 responses to candidate peptides in male BALB/c. Frequency of CD8 T cell (left) and CD4 T cell (right) producing IFNγ in response to stimulation with mPlac1 peptides #82, #59 and MPY.

Can altering the vector order assist in generating an anti mPlac1 immune response?

Another strategy to permit an anti-mPlac1 immune response was to alter the order of the priming and boosting vectors. With another tumour antigen, DCT, VSV has been shown to be an effective priming vaccine vector for subsequent adenoviral boosting[127]. One significant difference between the adenovirus vaccine vector and VSV as a vector is that as a replicating virus, VSV would have more viral proteins present in the presenting cell. We sought to determine if the presence of these more immunogenic peptides provided an environment better suited for breaking whatever immunological tolerance exists with mPlac1. Others have shown that the presence of good CD4 epitopes or even the MHC II invariant chain in a vaccine can increase CD8 responses[128-130]. To test this

strategy, C57BL/6 mice were primed with VSVmPlac1 and boosted with AdmPlac1. CD8 and CD4 T cell responses were analyzed as before by restimulating splenocytes isolated 5 days post Adenovirus boost. No CD4 or CD8 responses to murine Plac1 peptide were detected while the control MHC class I restricted immunodominant VSV epitope peptide RGY was able to stimulate IFNγ production in CD8 T cells (Figure 9). Interestingly, the response to FAL (Ad), the MHC class I immunodoninant epitope of our adenovirus failed to stimulate CD8 T cells. While this could indicate an issue with the adenovirus vaccine boost, this lack of response was attributed to the suboptimal time frame for detecting anti-Adenovirus T cell responses.

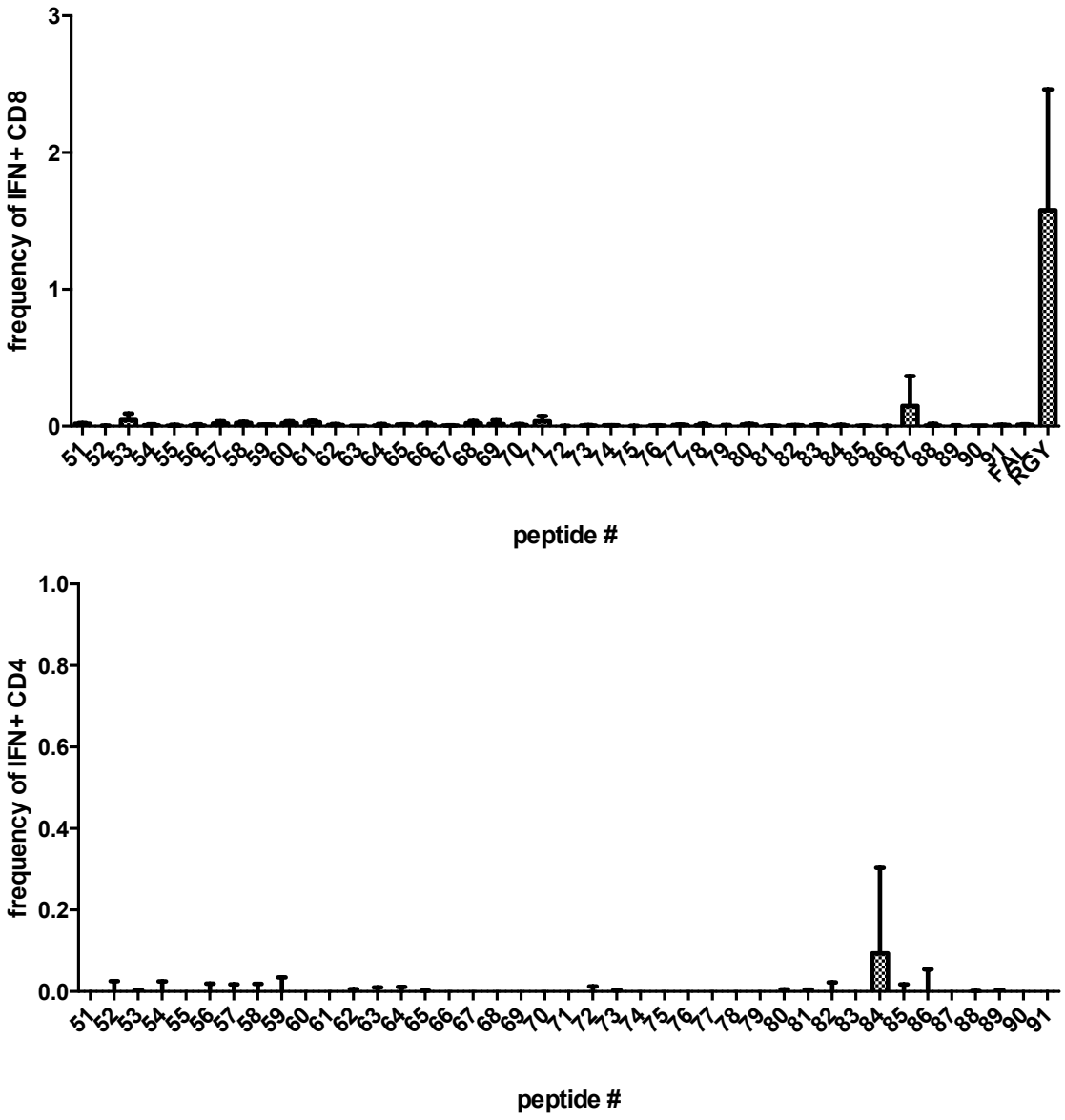

Figure 9. Epitope map of mPlac1 in C57BL/6. Mice were vaccinated with VSVmPlac1 and boosted with AdmPlac1. Frequency of CD8 T cell (top) and CD4 T cell (bottom) producing IFNγ in response to stimulation with individual peptides from the overlapping mPlac1 peptide library or the FAL (Ad) and RGY (VSV) peptides.

Can immune responses to mPlac1 be induced with pharmacological manipulation?

Another strategy used by our group and others to generate immune responses to self antigens is the use of pharmacological agents. Cyclophosphamide (CPA) is a DNA damaging agent that is known to affect various cells of the immune system. On the surface this would seem counterproductive to the goal of generating immune responses. However, low dose treatment with CPA, has been shown to selectively kill regulatory T cells[131]. This is thought to decrease peripheral tolerance and potentiate responses against the tolerized, usually self, antigen. BALB/c mice were treated with CPA, primed with AdmPlac1 and boosted with VSVmPlac1. CD8 and CD4 responses were measured as before by intracellular cytokine staining for IFNγ production in splenocytes. Treatment with CPA failed to generate anti mPlac1 responses above background while CD8 T cell responses to the VSV-N MHC class I restricted epitope were generated (Figure 10).

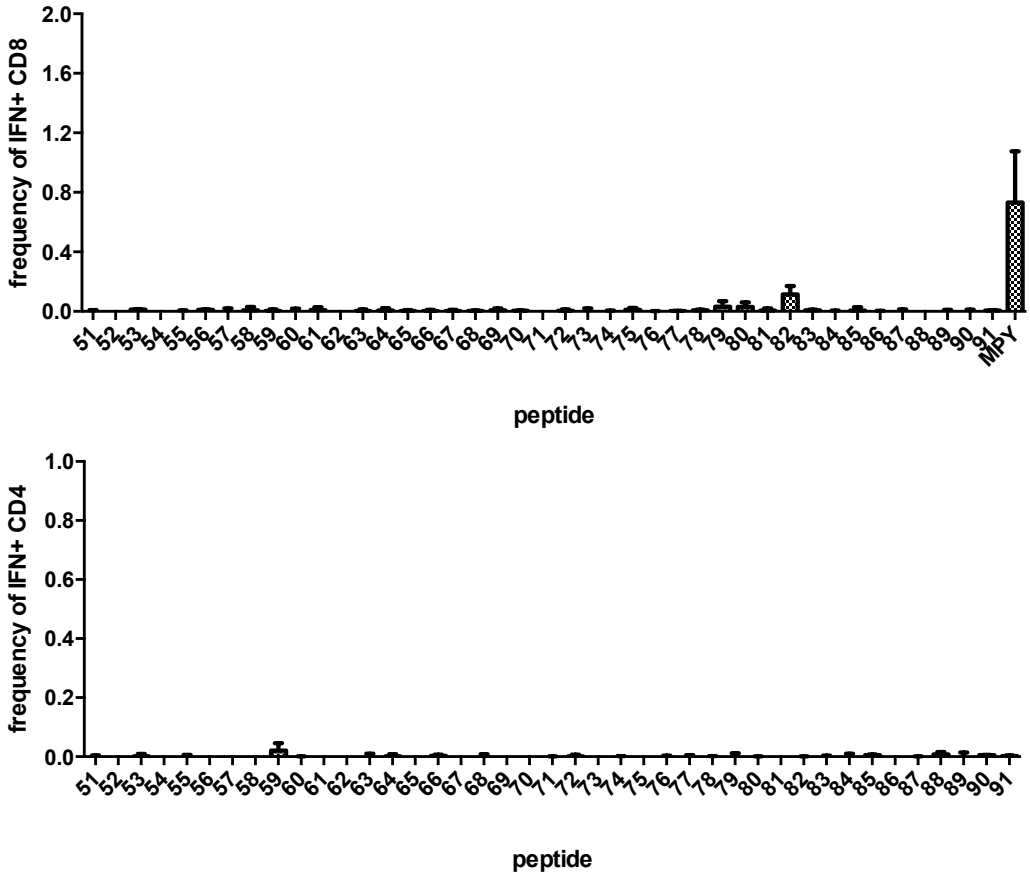

Figure 10. Epitope map of mPlac1 in BALB/c. Mice were treated with cyclophosphamide prior to vaccination with AdmPlac1 and boosting with VSVmPlac1. Frequency of CD8 T cell (top) and CD4 T cell (bottom) producing IFNγ in response to stimulation with individual peptides from the overlapping mPlac1 peptide library or the MPY peptide.

In order to probe the effectiveness of using CPA treatment to generate anti-mPlac1 responses in C57BL/6 mice, the same vaccination protocol that was used in BALB/c above was applied. Instead of using the entire murine Plac1 library to restimulate splenocytes, pools of previously identified weakly positive epitopes were used. While this approach may miss any de novo responses to mPlac1 permitted by the CPA, it would

still allow the detection of enhanced responses to any of the epitopes that were previously classified as weak, potential mPlac1 epitopes. In C57BL/6, CPA failed to enhance the weak CD8 or CD4 responses to the pool of mPlac1 peptides (Figure 11).

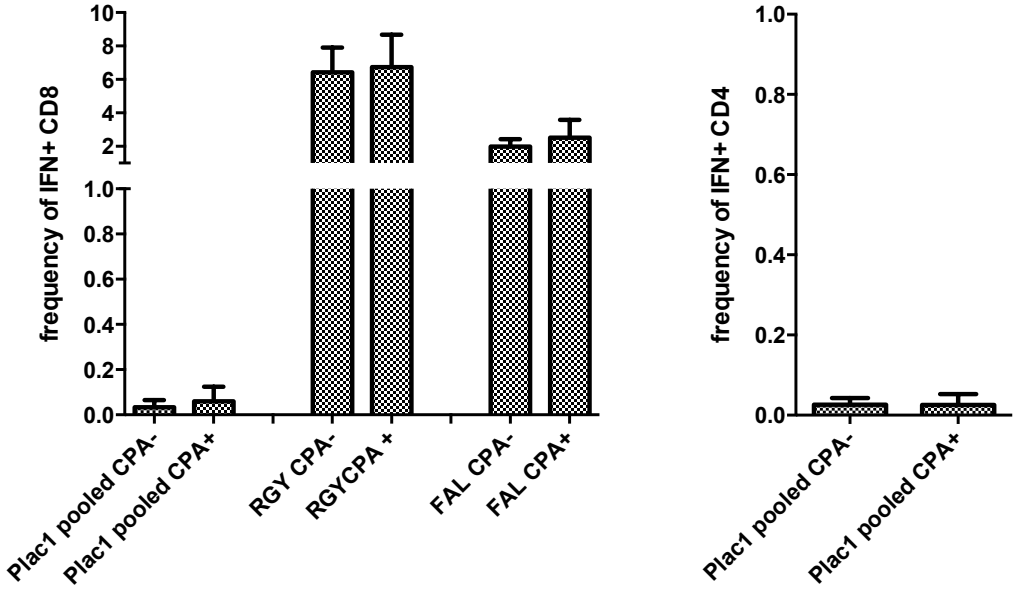

Figure 11. CD8 responses with CPA treatment and mPlac1 vaccination in C57BL/6. Frequency of CD8 T cell (left) and CD4 T cell (right) producing IFNγ in response to stimulation with pooled mPlac1 peptides, RGY (VSV) or FAL (Ad).

2.3 Does the use of xenoantigens facilitate generation of an anti-murine Plac1 immune response in mice?

One commonly used strategy to generate immune responses against autologous antigens is the use of xenoantigens, where vaccination occurs with a homologous protein from another species[132,133]. As previously discussed, depending on the degree of homology, responses may be generated against the homologous antigen. These responses

may then also cross react with the autoantigen, that could not induce a response on its own[134]. Further, the xenoantigen vaccination may involve a process referred to as CD4 licensing where the presence of immunogenic CD4 epitopes facilitate CD8 responses against self antigens. An example of this is observed with the melanoma associated antigen DCT. A simple mutation of murine DCT to include a CD4 epitope of human DCT results in CD8 responses to an epitope shared between murine and human DCT[128]. To apply this strategy to Plac1, we developed two more vaccine vectors, an adenovirus and a VSV each expressing human Plac1 (hPlac1). Expression of the transgene was confirmed by western blot on lysates from cells infected with these viruses (Figure 12).

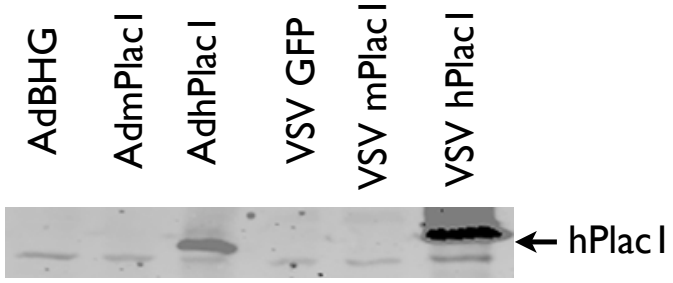

Figure 12. Expression of hPlac1 from vaccine vectors. Western blot analysis of lysates of 293T cells infected with AdBHG, AdmPlac1, AdhPlac1, VSV GFP, VSVmPlac1 amd VSVhPlac1.

Can anti-hPlac1 responses be generated in mice?

As had been done previously with the mPlac1 vaccine vectors, anti hPlac1 immune responses were assessed by performing intracellular cytokine staining on splenocytes isolated 10 days post adenovirus vaccination. In the BALB/c strain of mice, there was only a potentially weak CD8 epitope identified contained within peptide #2 (Figure 13, top). Interestingly, despite the relative lack of a CD8 T cell response, a CD4 epitope was detected within peptides 25-26 (Figure 13, bottom).

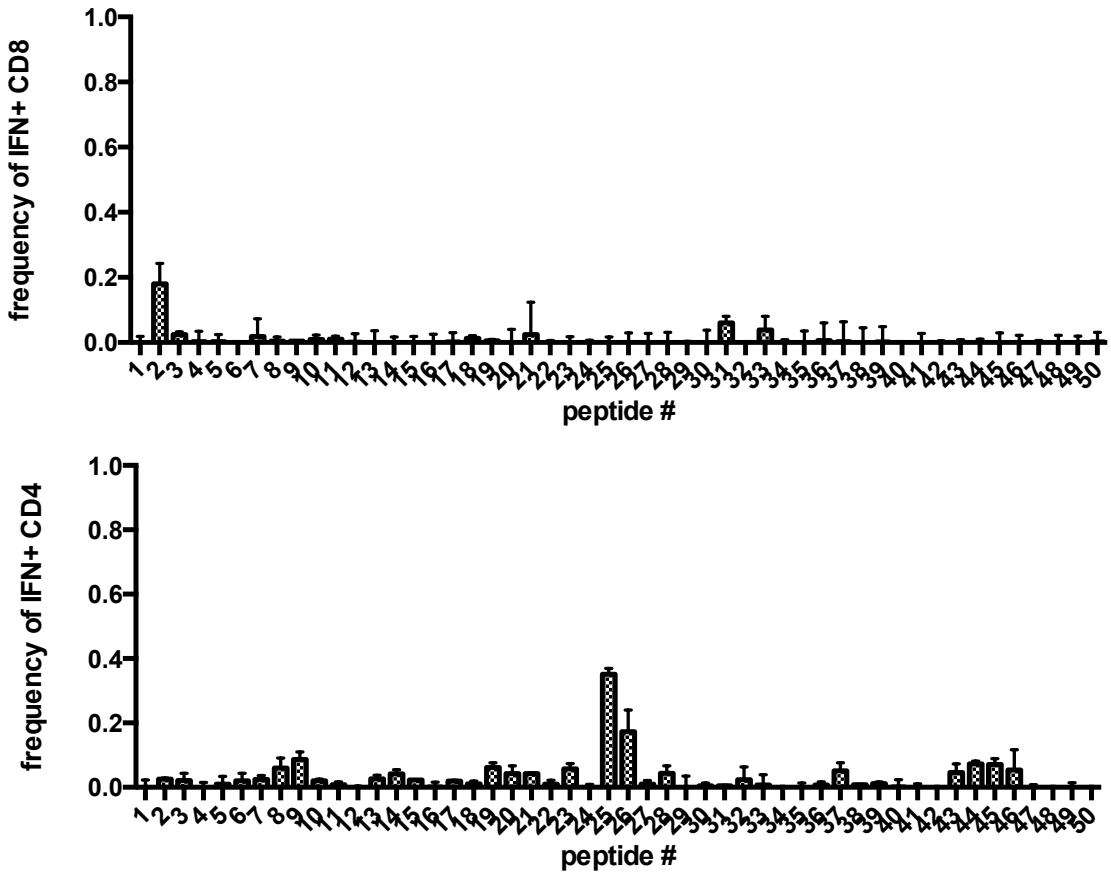

Figure 13. Epitope map of hPlac1 in BALB/c. Mice were vaccinated with AdhPlac1. Frequency of CD8 T cell (top) and CD4 T cell (bottom) producing IFNγ in response to stimulation with individual peptides from the overlapping mPlac1 peptide library.

In contrast to BALB/c, when AdhPlac1 was used to vaccinate C57BL/6 mice, several robust CD8 T cell responses were detected with epitopes within peptides #1 , #11-15 and #26 (Figure 14, top). In addition, several peptides containing CD4 epitopes were identified, most strongly within peptides 44-45 (Figure 14, bottom).

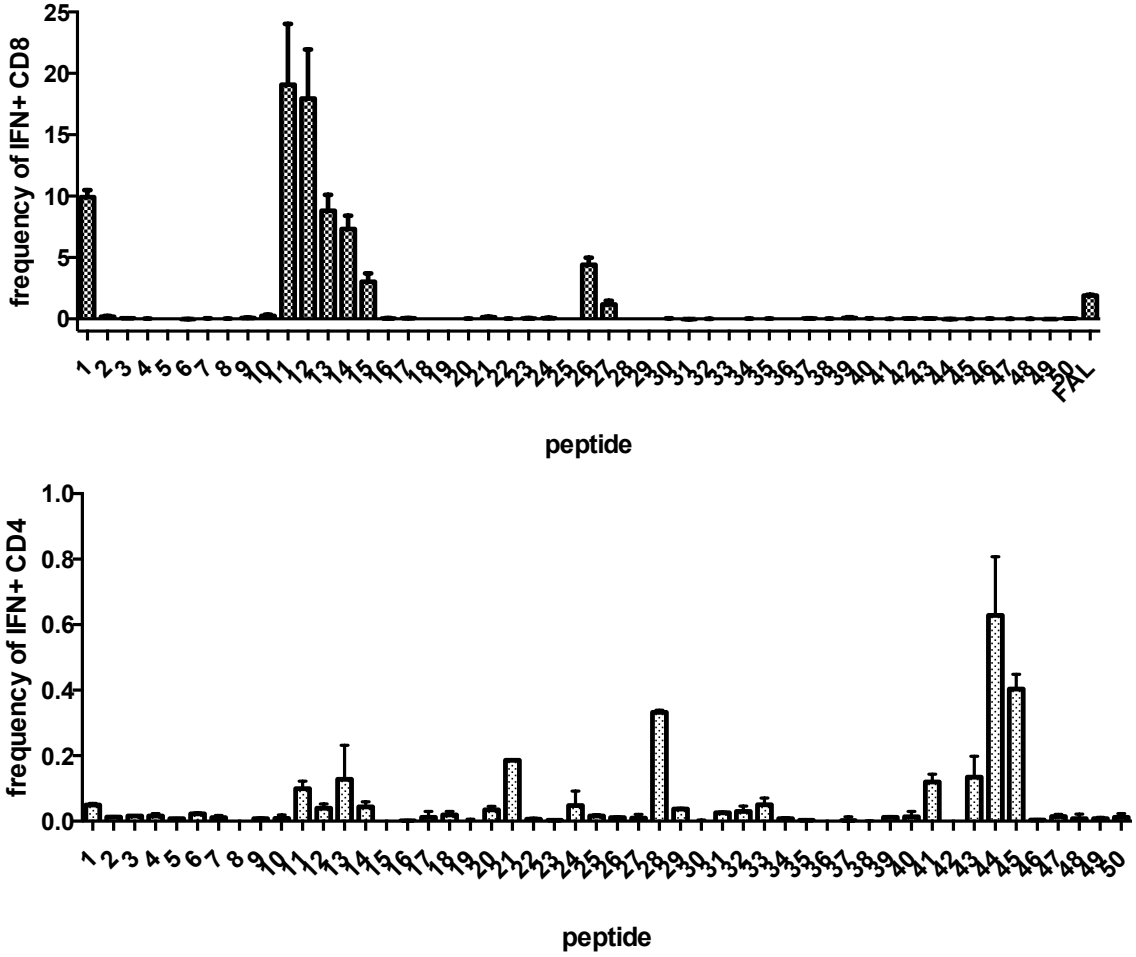

Figure 14. Epitope map of hPlac1 in C57BL/6. Mice were vaccinated with AdhPlac1. Frequency of CD8 T cell (top) and CD4 T cell (bottom) producing IFNγ in response to stimulation with individual peptides from the overlapping mPlac1 peptide library and FAL.

Does the anti-hPlac1 response cross react with mPlac1?

We next sought to determine whether the anti hPlac1 response in C57BL/6 cross reacts with the murine protein. To this end, we primed a response to hPlac1 and attempted to boost this with VSV expressing mPlac1. Mice were vaccinated with AdhPlac1 and boosted with either VSV MT (empty vector), VSVmPlac1 or VSVhPlac1. Splenocytes were analyzed for their ability to be restimulated by the human (Figure 15) and murine (Figure 16) Plac1 library of peptides. Boosting an AdhPlac1 prime with either VSV MT or VSV mPlac1 yielded almost identical anti hPlac1 responses - the VSVmPlac1 was unable to boost any of the preexisting anti hPlac1 responses. Boosting with VSVhPlac1, on the other hand, resulted in increased frequencies of CD8 T cells able to be restimulated by peptides #1, #11, #12. The murine peptide library analysis (Figure 16) revealed than none of the 3 vaccination modalities yielded anti mPlac1 responses. It appears that the anti hPlac1 responses (at the CD8 T cell level) are not useful in generating anti murine Plac1 responses.

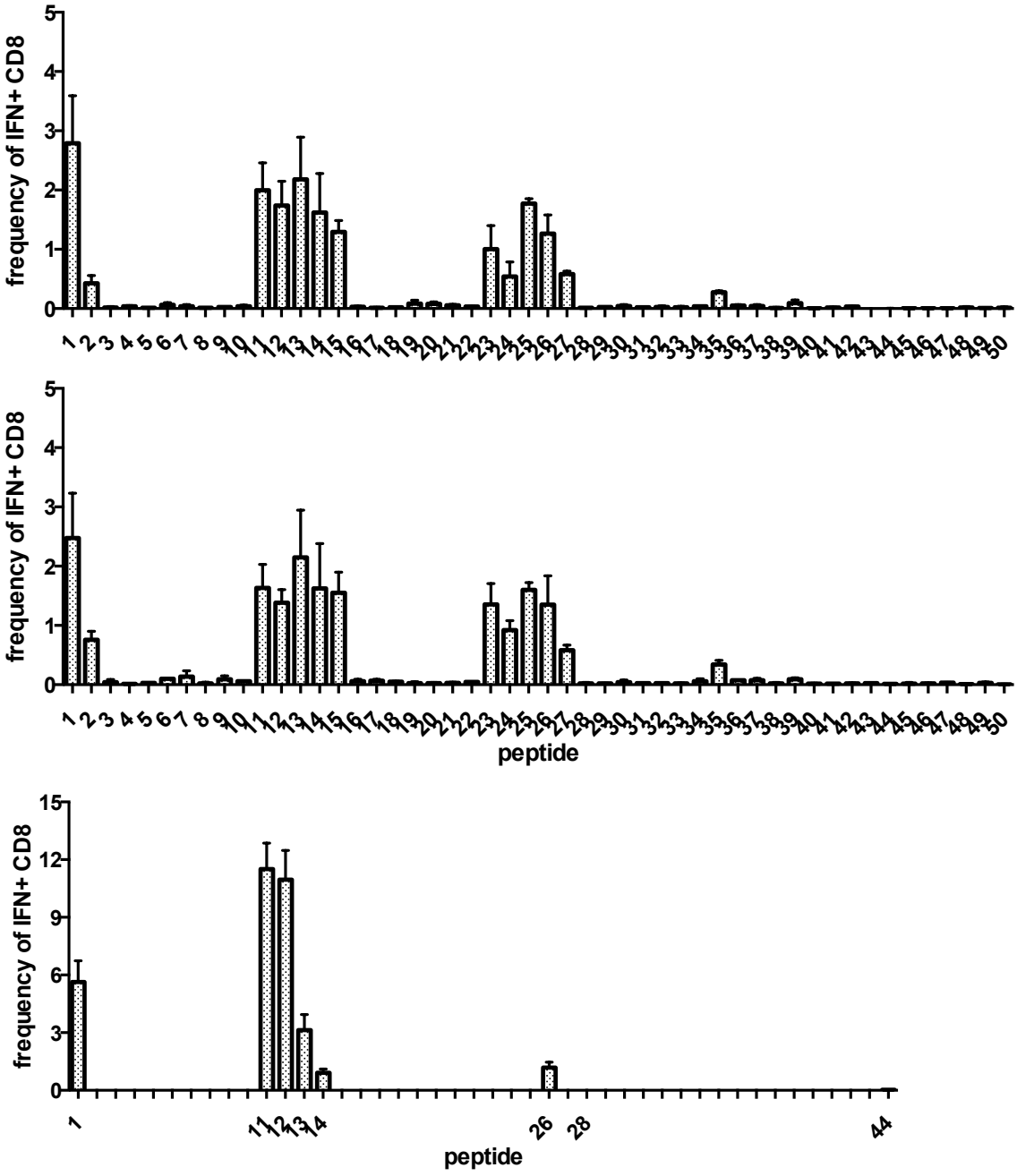

Figure 15. Epitope map of hPlac1 in C57BL/6. Mice were vaccinated with AdhPlac1 and boosted with VSVMT (top), VSVmPlac1 (middle) or VSVhPlac1 (bottom). Frequency of CD8 T cells producing IFNγ in response to stimulation with individual peptides from the overlapping hPlac1 peptide library.

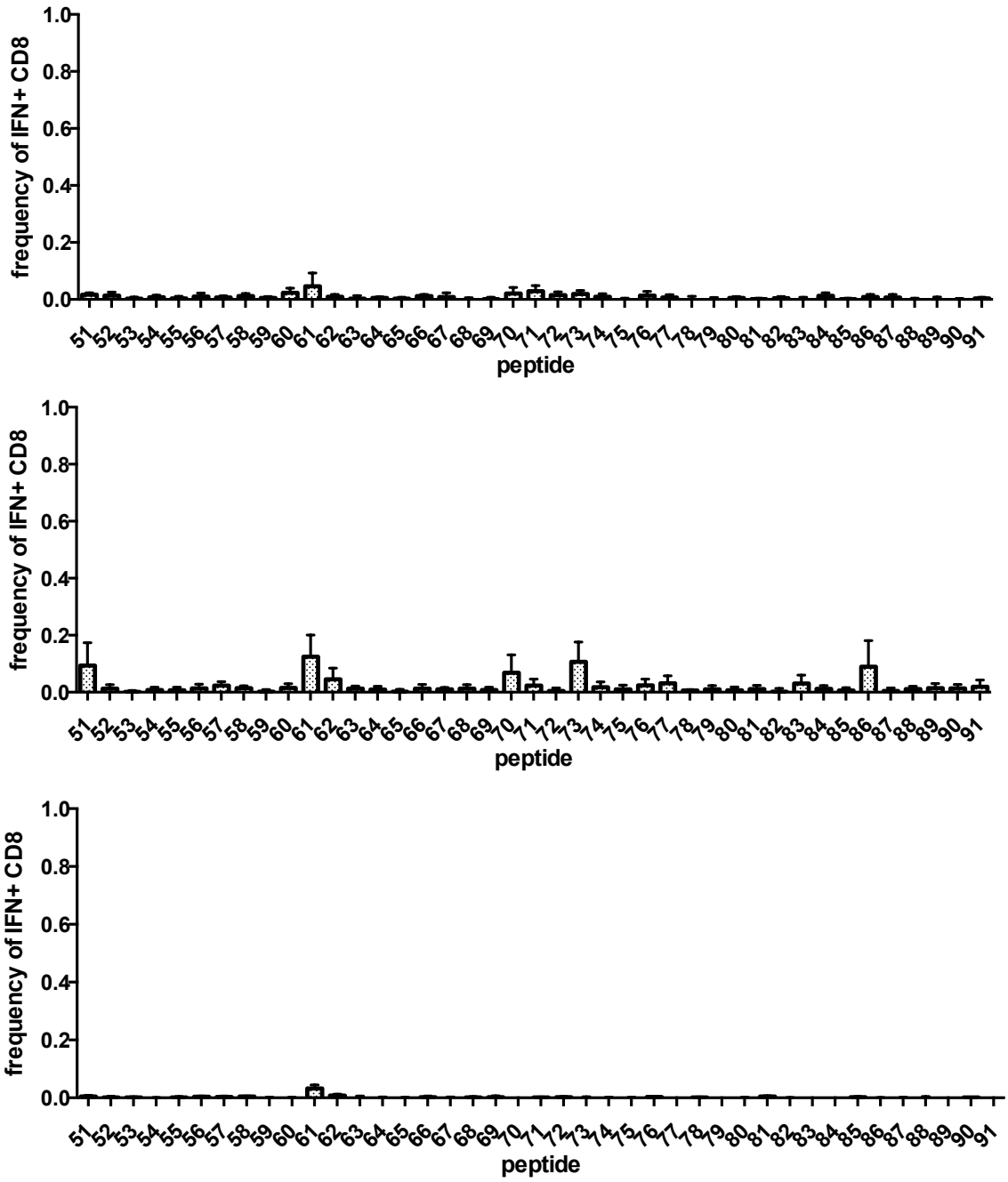

Figure 16. Epitope Map of mPlac1 in C57BL/6. Mice were vaccinated with AdhPlac1 and boosted with VSVMT (top), VSVmPlac1 (middle) or VSVhPlac1 (bottom). Frequency of CD8 T cells producing IFNγ in response to stimulation with individual peptides from the overlapping mPlac1 peptide library.

Are other xenoantigens able to induce anti-mPlac1 immune responses?

The inability of mPlac1 expressing vectors to boost anti hPlac1 CD8 responses is perhaps not surprising given the limited degree of homology between the two antigens. As shown in Figure 17 for the first 171 of 173 or 202 residues of mPlac1 or hPlac1, respectively, there is only 60% identity. In order for a xenoantigen strategy to work, it is thought that the xenoantigen must be sufficiently different from the target such that a response is generated but must also be sufficiently similar such that responses can cross react[134]. With this in mind, vectors containing two new antigens were designed. First, we hypothesized that rat Plac1, given its greater identity to mPlac1 at 90%, would be a superior xenoantigen (Figure 17, lower panel). Second, we designed a fusion protein containing the C-terminal tail of hPlac1 fused to the C-terminus of mPlac1. In mice, this synthetic xenoantigen would contain foreign elements from hPlac1 including the CD4 epitope in C57BL/6 in addition to perfectly matched elements from mPlac1. An additional element to increase the expression and immunogenicity of this synthetic transgene is that it was codon optimized. Codon optimization can increase the expression of a transgene in the presenting cells and has been shown to increase immune responses against the vaccine target[135,136].

```
Mouse  1    MNLRKFLGGTVLVAFMLFSYSEQNQVNVLCSTDWFMVTVHPFLLNNDVYVHFYEVHLGLG  60
            M + KF+G  +L+     + S Q+ + VLCS DWFMVTVHPF+LNNDV VHF+E+HLGLG
Human  1    MKVFKFIGLMILLTSAFSAGSGQSPMTVLCSIDWFMVTVHPFMLNNDVCVHFHELHLGLG  60

Mouse  61   CPPNHVHPHFYQFHYRVTECGIRIKAVSPDVVIYSSEIHYASKGSSTKYVIPVSCAAPRR  120
            CPPNHV PH YQF YRVTECGIR KAVS D+VIYS+EIHY+SKG+ +K+VIPVSCAAP++
Human  61   CPPNHVQPHAYQFTYRVTECGIRAKAVSQDMVIYSTEIHYSSKGTPSKFVIPVSCAAPQK  120

Mouse  121  SPWLTKPYSAKAPSNNMGATPKNDTSYHVFTLPEPSEQPNCSCPPYVYNQK  171
            SPWLTKP S +  S +     K++  Y VF+L + S++PNC CPP V++++
Human  121  SPWLTKPCSMRVASKSRATAQKDEKCYEVFSLSQSSQRPNCDCPPCVFSEE  171
```

```
Mouse  1    MNLRKFLGGTVLVAFMLFSYSEQNQVNVLCSTDWFMVTVHPFLLNNDVYVHFYEVHLGLG  60
            M L KFLGG V    M   YSEQNQVNVLCSTDWFMVTVHPFLLNNDV+VHFYEVHLGLG
Rat    1    MKLIKFLGGVVFFTLMFSGYSEQNQVNVLCSTDWFMVTVHPFLLNNDVFVHFYEVHLGLG  60

Mouse  61   CPPNHVHPHFYQFHYRVTECGIRIKAVSPDVVIYSSEIHYASKGSSTKYVIPVSCAAPRR  120
            CPPNH+HPHFYQF+YRVTECGIRIKAVSPDVVIYSSEIHYASKGSS +YVIPVSCAAPRR
Rat    61   CPPNHIHPHFYQFNYRVTECGIRIKAVSPDVVIYSSEIHYASKGSSARYVIPVSCAAPRR  120

Mouse  121  SPWLTKPYSAKAPSNNMGATPKNDTSYHVFTLPEPSEQPNCSCPPYVYNQKSM  173
            SPWLTKPYSAKAPS+NMGATPKNDTSYHVFTLPEPS+QPNCSCPPYV+NQKSM
Rat    121  SPWLTKPYSAKAPSSNMGATPKNDTSYHVFTLPEPSQQPNCSCPPYVFNQKSM  173
```

Figure 17. mPlac1 Xenoantigen amino acid sequences. Amino acid sequence alignment is shown for murine and human Plac1 (top) and murine and ravine Plac1 (bottom). The alignment was generated using the NCBI BLAST function.

Figure 18 shows the successful expression of the rat Plac1 and optimized mouse/ human fusion Plac1 from the pDC316 adenoviral rescue shuttle vector. Unfortunately, the adenoviruses expressing these transgenes were unable to be rescued by myself or others, including the Fitzhenry Vector GMP lab having extensive expertise in adenovirus rescue. I was however, able to rescue recombinant VSVs expressing these two antigens.

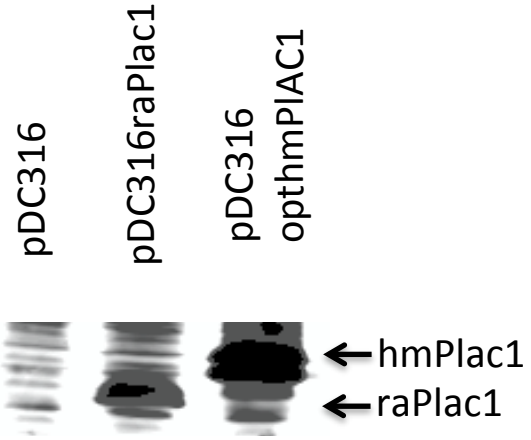

Figure 18. Xenoantigens of mPlac1. Western Blot of lysates from 293T cells transfected with the shuttle vectors for the adenovirus rescue.

Does hPlac1 vaccination of mice generate serum antibodies?

The fact that Plac1 is a membrane protein that would be accessible to antibodies presents the possibility that anti-Plac1 antibodies could be the basis of useful stand alone therapeutics. Given the robust T cell response elicited in C57BL/6 mice to hPlac1 following vaccination, we hypothesized that serum antibodies to hPlac1 may also be generated following this vaccination. To test this, we developed an ELISA-based assay to screen for anti hPlac1 antibodies. We used a modified sandwich ELISA where Flag-tagged hPlac1 was adsorbed to anti Flag antibodies bound to an ELISA plate. Serially diluted sera from naive or hPlac1 vaccinated mice were exposed to these plates and successful binding detected with a secondary anti murine IgG antibody. The vaccinated status of the mice at the time of serum harvest was confirmed using intracellular cytokine staining of splenocytes restimulated with hPlac1 peptide #11 or the immunodominant

VSV peptide, RGY (Figure 19A). There was no Plac1 specific antibody detected above the background in this assay (Figure 19B). The utility of the ELISA plates was confirmed by testing the commercial anti hPlac1 antibody which generated a signal on the hPlac1 lysates above what was seen using the vector control lysate (Figure 19C). The partial detection of signal in the absence of anti Flag capture antibody likely reflects the abundance of hPlac1 in the initial lysate.

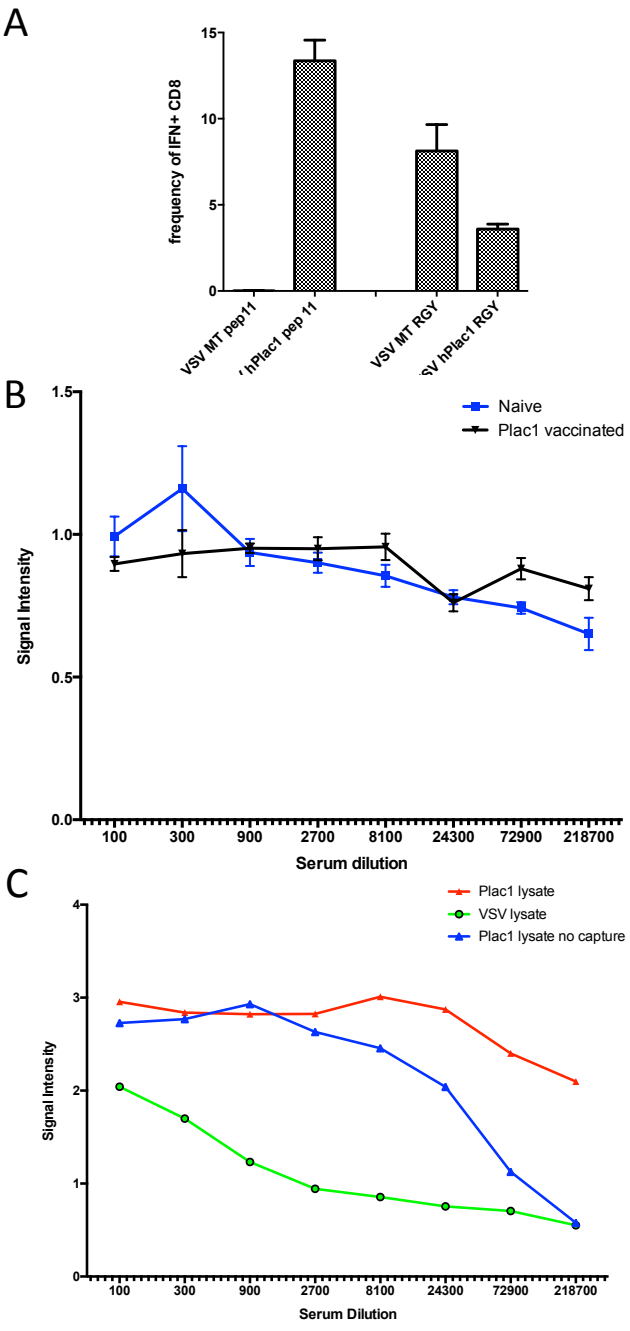

Figure 19. Serum antibodies to hPlac1. **A**. Frequency of IFNγ+ CD8 T cells following restimulation with Plac1 pep#11 and RGY (VSV). **B**. ELISA of serum from Naive or Plac1 vaccinated mice against FLAG captured hPlac1 lysate. **C.** ELISA of Santa Cruz Goat and hPlac1 against a FLAG captured hPlac1 lysate used in B, a Plac1 negative VSV lysate and the hPlac1 lysate in the absence of capture

2.4 Does Plac1 vaccination have any anti-tumour effect on mPlac1 expressing tumours?

Despite the relative inability to generate detectable immune responses against mPlac1 in BALB/c and C57BL/6, we sought whether these vaccine protocols generated any anti tumour activity. With respect to Plac1, the threshold of CD8/4 T cell response required for anti tumour activity is unknown leading to the possibility that in a prophylactic setting, a response below our level of detection may have some anti tumour efficacy. Likewise, the titre of anti Plac1 antibody required to prevent tumour engraftment is unknown. To test this, we employed the subcutaneous BALB/c CT26 tumour model. Mice were vaccinated with adenovirus expressing mPlac1 (or empty vector) and boosted 2 weeks later with VSV expressing mPlac1 (or GFP). At an interval 5 days after the boost, 1 x 10^5 CT26 cells were engrafted subcutaneously on the flank and mice were then monitored for growth of palpable tumours. As shown in Figure 20, the mPlac1 vaccination failed to inhibit CT26 tumour cell engraftment. We next tested the idea that vaccination with human Plac1 as a xenoantigen may be have an effect on CT26 engraftment. As above, mice were vaccinated with AdhPlac1 and boosted with VSVmPlac1 2 weeks later and challenged with CT26 cells 5 days after the boost. Again, this vaccination failed to delay engraftment with CT26, consistent with the inability to generate detectable anti mPlac1 immune responses in BALB/c (Figure 21).

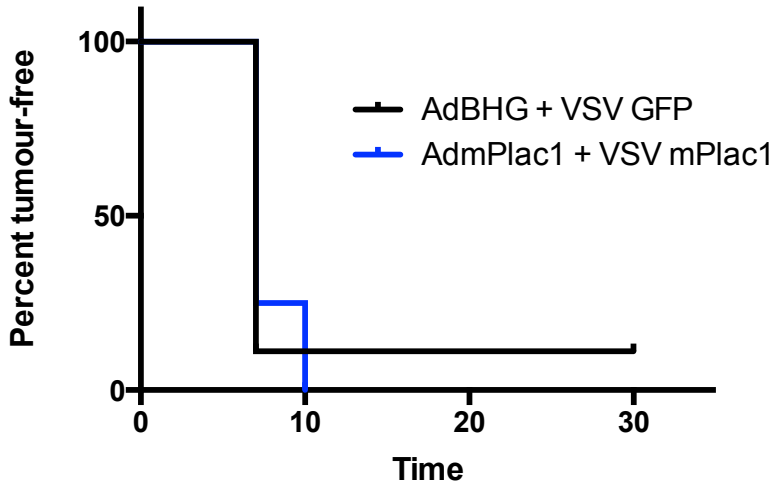

Figure 20. CT26 tumour engraftment in mPlac1 vaccinated mice. Following vaccination using AdmPlac1 and boosting with VSV mPlac1, mice were engrafted with CT26 tumour cells. The tumour free status of these mice is plotted against time in days.

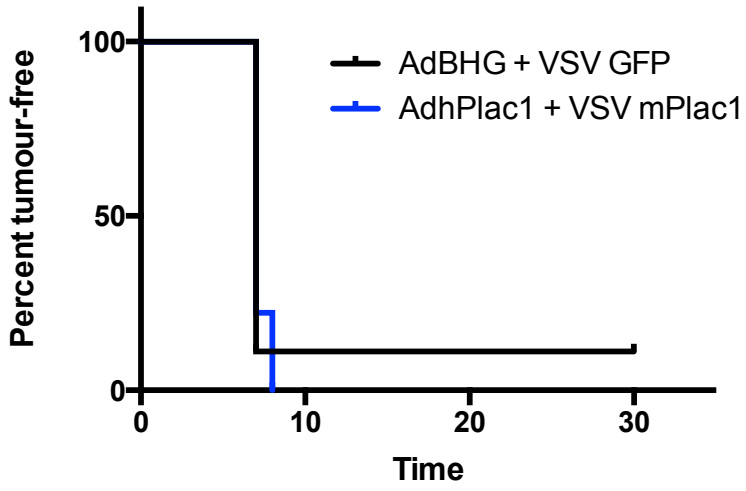

Figure 21. CT26 tumour engraftment in Plac1 vaccinated mice. Following vaccination using AdhPlac1 and boosting with VSV mPlac1, mice were engrafted with CT26 tumour cells. The tumour free status of these mice is plotted against time in days.

We next tested whether we could detect any anti-tumour activity in a different tumour model. The ability to generate anti Plac1 responses, albeit anti human Plac1 responses, in C57BL/6 prompted us to use the lung metastasis model of B16-F10. We injected 2.5 x 10^5 B16-F10 tumour cells intravenously and vaccinated 2 days later with AdhPlac1. At 12 days post vaccination, mice were boosted with either Maraba hPlac1 or VSV expressing the mouse/human fusion transgene described above. Maraba virus is another oncolytic virus used in our group that is as good at boosting adenoviral primed responses as VSV (Pol *et al*, in preparation). To confirm successful vaccination, we measured responses to peptide 12 of the hPlac1 library and the immunodominant VSV epitope, RGY, in the blood. The presence of anti hPlac1 and anti VSV responses confirmed the successful vaccination of the mice. Neither of these vaccination protocols

inhibited tumour growth/engraftment with all mice reaching endpoint in the typical 20-22 days post engraftment (Figure 22).

Despite the suggestion that Plac1 would be immunogenic, none of the strategies employed were able to generate anti murine Plac1 CD8 or CD4 T cell responses. Further, none of these vaccination protocols were able to demonstrate any anti tumour activity in the models tested.

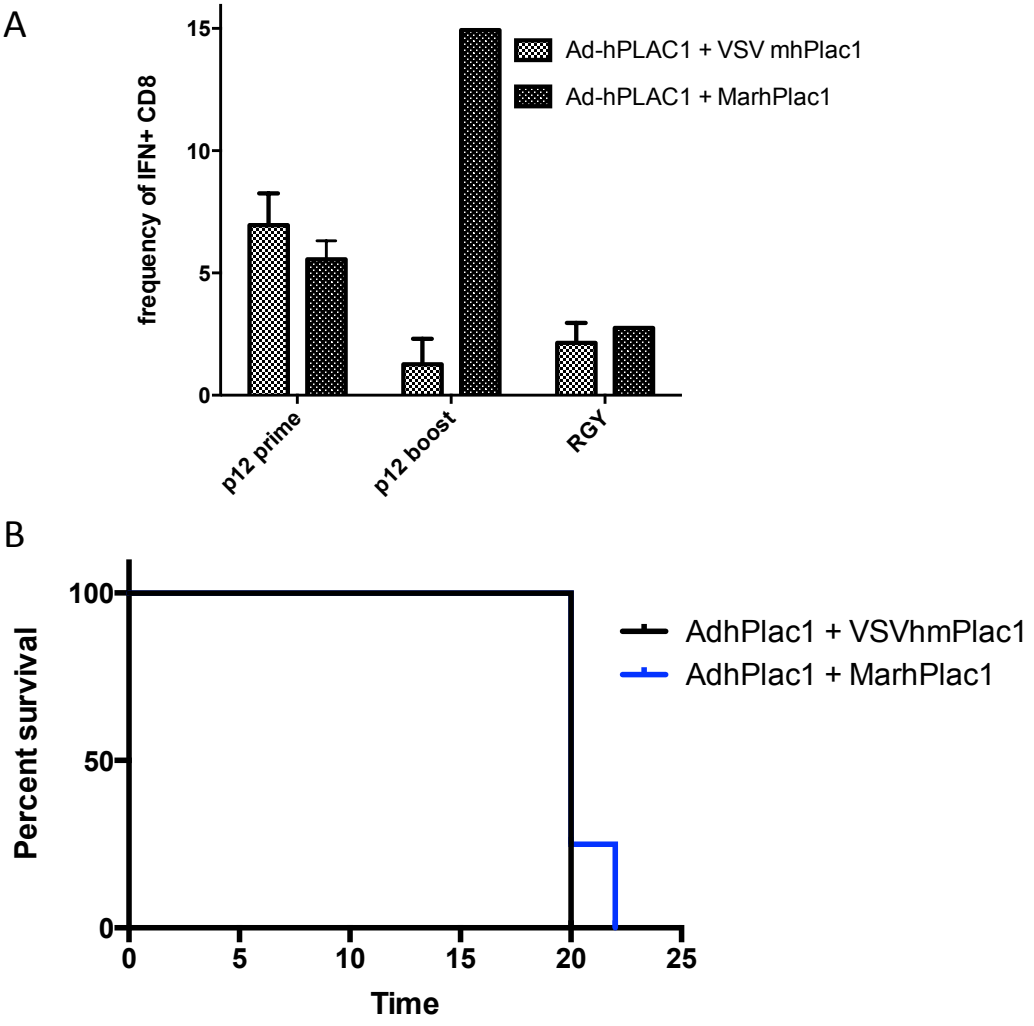

Figure 22. B16-F10 tumour engraftment in Plac1 vaccinated mice. Following vaccination using Ad-hPlac1 and boosting with MarhPlac1 or VSVmhPlac1, mice were engrafted with B16-F10 tumour cells. A. Frequencies of CD8 T cell responses to hPlac1 peptide #12 after prime (left) and boost (middle) and RGY (VSV) peptide after boost are shown. The tumour free status of these mice is plotted against time in days. B. Kaplan-Meier plot showing the survival of engrafted mice in days post engraftment.

- CHAPTER 4 -

Improving Vaccination Strategies Against the Tumour Associated Antigen Gp100

1.0 Introduction

Gp100 is a protein from the group of melanoma associated antigens that has been targeted in a number of clinical trials. The vaccine is most often in the form of gp100 peptide mixed with peptides from other tumour associated antigens or peptide pulsed dendritic cells (DCs)[93,94,137]. One strategy to increase immunogenicity to the self antigen is the modification of the gp100 peptide$_{(209\text{-}217)}$ (HLA-A*0201 restricted) with 210M to improve the stability of the MHC I:peptide complex although the efficacy of this has been disputed[93,95]. The 210M is a substitution of threonine to methionine at position 210 which improves stability of the MHCI:peptide complex by altering the anchor residue[137]. Gp100 peptide vaccination has been successfully used in a small number of patients in combination therapy with anti CTLA-4 antibody (ipilimumab)[96]. Rosenberg *et al* report that the presence of antigen specific T cells doesn't necessarily correspond to anti tumour activity[137]. This is reminiscent of observations made by our group in mice. In the B16-F10 tumour model in C57BL/6 mice, gp100 vaccination fails to match the efficacy of its counterpart melanoma associated antigen, DCT. While vaccination with adenovirus expressing hgp100 induces expansion of CD8 T cells responding to the MHC class I restricted dominant epitope from hgp100$_{(25\text{-}33)}$ and to a lesser extent, the murine homologue mgp100$_{(25\text{-}33)}$, these T cells have limited efficacy against tumours expressing gp100. It is thought that the defect is in the ability of the B16-F10 cells to efficiently process and present the mgp100$_{(25\text{-}33)}$ peptide since overexpression of the rat TAP protein is able to complement this deficiency[98]. Since expression of an exogenous protein isn't a

practical solution in vivo, we sought to study other strategies to efficiently target gp100 in vivo. While its unclear if this inability of generating an anti tumour effect in the B16-F10 model is the result of the same mechanism as what is observed in patients, identifying strategies to address this will most likely be transferable to similar issues in the clinic.

2.0 Results

2.1 Can anti gp100 activity be enhanced by boosting with an oncolytic vector?

The first strategy we chose to investigate was the use of an oncolytic boosting vector. While a defect in B16-F10 has been shown to be at the level of antigen processing, we hypothesized that increasing the number of immune effector cells could mitigate this defect. Indeed, administration of pmel T cells, CD8 T cells responding to gp100, can have anti B16 activity[138]. In addition to increasing the sheer number of anti mgp100 CD8 T cells, boosting may also improve the quality of T cells responsive to gp100. Bridle *et al* (manuscript submitted) have observed that upon boosting, a lower concentration of peptide is required to stimulate the responsive T cells, suggesting that T cells with higher avidity are preferentially boosted. Put together, these observations suggest that boosting the Adhgp100 response is a plausible strategy to induce anti B16-F10 activity in C57BL/6.

We initially tested the hgp100 prime:boost in the B16-F10 subcutaneous model in a prophylactic setting. Mice were vaccinated with an E1/E3 deleted adenovirus expressing human gp100 (Adhgp100). Two weeks following vaccination, mice received

VSVhgp100 as a boosting vaccine followed by subcutaneous engraftment of 1 x 10^5 B16-F10 tumour cells. Immune responses were monitored by evaluating the ability of CD8 T cells from the blood to be restimulated by peptides corresponding to the previously identified immunodominant epitope of hgp100 in C57BL/6 and the corresponding murine peptide. Mice were monitored for signs of palpable tumours at the injection site. Figure 23A shows that the frequency of CD8 T cells able to respond to each of the $hgp100_{(25-33)}$ and the $mgp100_{(25-33)}$ peptides is increased approximately 10 fold from the levels after the prime to 40% of CD8 T cells and 10% of CD8 T cells respectively upon boosting. A frequency of 10% of CD8 T cells is high in comparison to other murine antigens and would be expected to be able to possess anti-tumour activity. The Kaplan-Meier plot for survival is shown in Figure 23B. Priming with Adhgp100 and boosting with VSVhgp100 delayed the onset of subcutaneous tumours but ultimately most of the mice developed tumours.

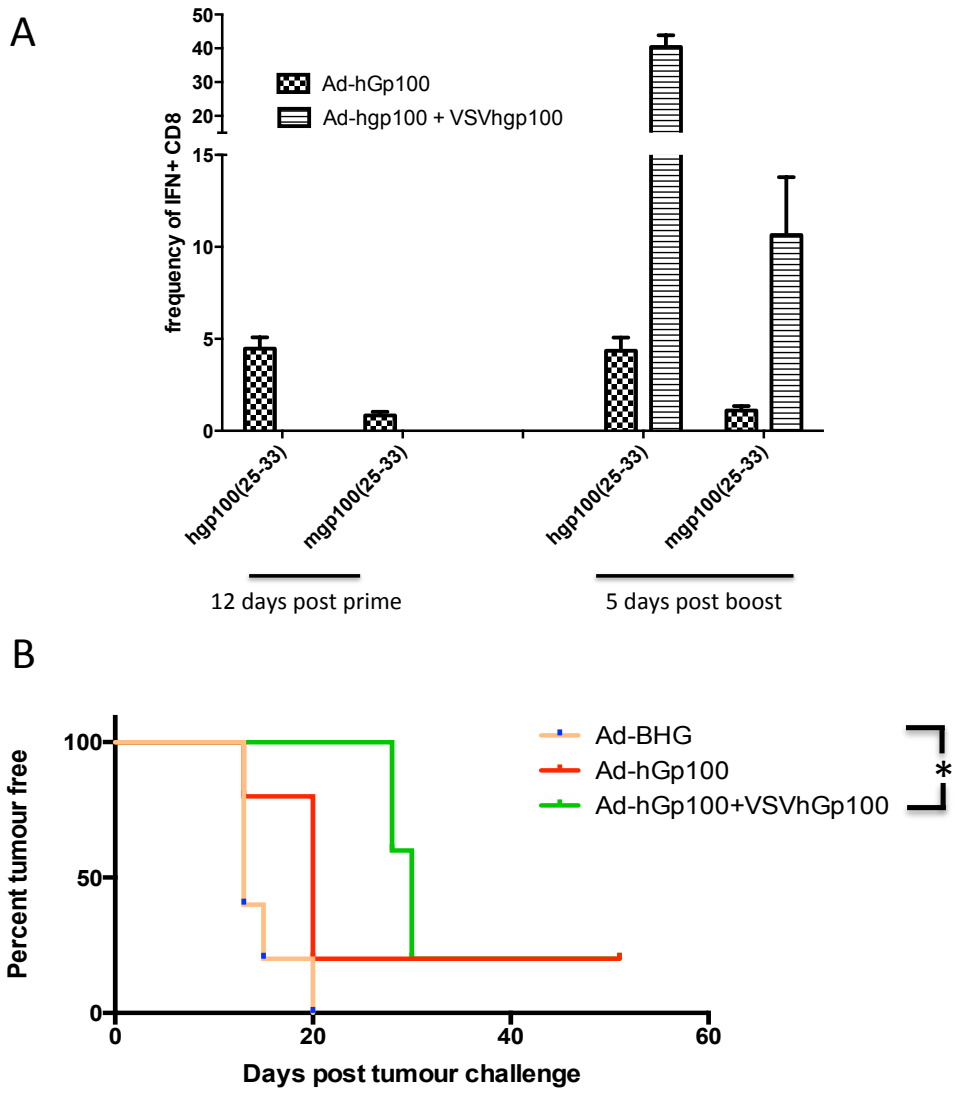

Figure 23. Prophylactic Vaccination with hgp100 prior to C57BL/6 engraftment. **A**: Frequencies of IFNγ+ CD8 T cell from blood taken at 12 days post prime (left) or 5 days post boost (right) following restimulation with $hgp100_{(25-33)}$ or $mgp100_{(25-33)}$ peptides. **B**. Kaplan-Meier plot of survival in days post tumour challenge. (log-rank test; Ad BHG vs Adhgp100+VSVhgp100 = .0015)

After seeing this promising delay in tumour appearance in the prophylactic setting, we set out to test this prime:boost gp100 vaccine in a therapeutic setting, where 5 x 10^4 B16-F10 cells are injected intravenously two days prior to vaccination with Ad hgp100 and boosted with VSV hgp100 12 days after the initial prime, the group receiving the prime and boost had no survival advantage over the sham vaccinated group or the group receiving only the adenovirus vaccine (Figure 24, bottom). This failure is in spite of a robust CD8 response to the MHC class I restricted immunodominant epitope of $hgp100_{(25\text{-}33)}$ as well as the $mgp100_{(25\text{-}33)}$ epitope. It should be noted that the dose of B16-F10 cells was suboptimal leading to variable engraftment, as evidenced by a control mouse not getting a tumour. Subsequent studies used a greater number of B16-F10 cells.

The data from the prophylactic and therapeutic settings suggest that despite increasing the magnitude of the CD8 T cell response with an oncolytic vaccine vector, there was only a marginal improvement in anti tumour activity in the prophylactic scenario and no benefit in the therapeutic setting. This suggests that the defect in mgp100 processing cannot be overcome by simply increasing the number of effector CD8 T cells responding to the $mgp100_{(25\text{-}33)}$ epitope.

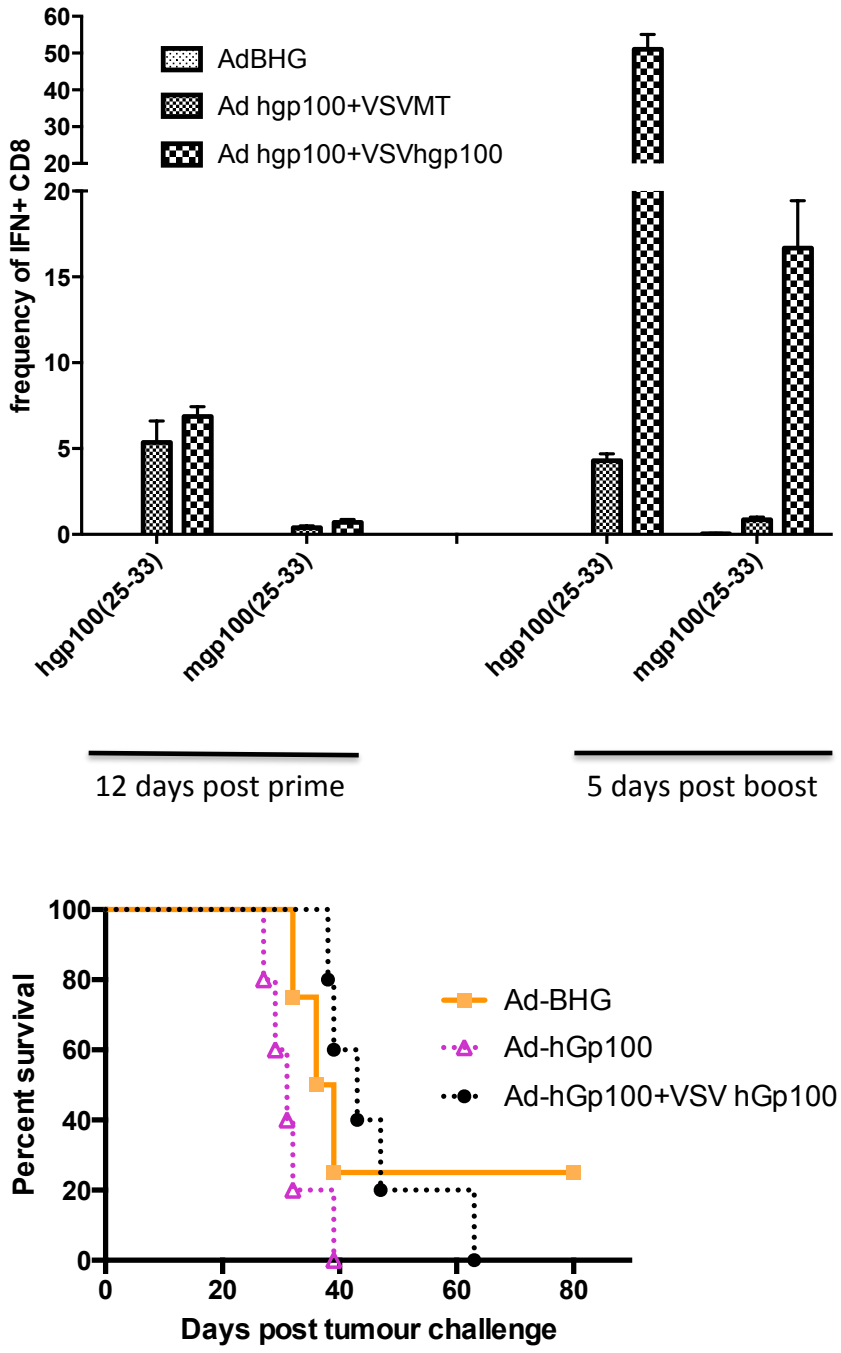

Figure 24. Therapeutic Vaccination with hgp100 after C57BL/6 engraftment. Top: Frequencies of IFNγ+ CD8 T cell from blood taken at 12 days post prime (left) or 5 days post boost (right) following restimulation with $hgp100_{(25-33)}$ or $mgp100_{(25-33)}$ peptides. **Bottom**: Kaplan-Meier plot of survival in days post tumour challenge.

2.2 Can the anti murine gp100 vaccination be improved with pharmacological modification?

We next tested the use of pharmacological agents to modify the immune cell:target interaction with the goal of improving the anti tumour efficacy of the anti mgp100 responses. As with the case of Plac1, we first used low dose treatment with cyclophosphamide (CPA). At this dose, CPA is thought to selectively deplete CD4+CD25+ regulatory T cells (Tregs)[131]. While processing of the $mgp100_{(25-33)}$ epitope appears to be impaired, other mgp100 epitopes may be processed and presented properly. Elimination of Tregs that could be dampening responses to these other epitopes may potentiate anti tumour activity via other CD8 T cell clones as opposed to those responding to $mgp100_{(25-33)}$. C57BL/6 mice were engrafted with 2.5 x 10^5 B16-F10 cells intravenously and treated with cyclophosphamide prior to Adhgp100 vaccination. Twelve days after the adenovirus vaccination, mice were boosted with VSVhgp100 (or empty vector control). Immune responses to $hgp100_{(25-33)}$, $mgp100_{(25-33)}$ and the MHC class I restricted immunodominant epitope of VSV in C57BL/6, RGY, were monitored using intracellular cytokine staining of CD8 T cells from blood at nine days post prime and five days post boost. Figure 25 shows that the CPA treatment didn't alter the frequencies of CD8 T cells responding to the human or mouse $gp100_{(25-33)}$ after the prime or boost or the RGY (VSV) epitope after the boost. This suggests that Tregs are not suppressing the response to the $gp100_{(25-33)}$. While we don't know if responses to other gp100 epitopes are affected by CPA, these other responses still don't have any measurable anti tumour activity as CPA treatment failed to delay onset of lung metastasis in the presence of Ad

hgp100 prime and VSV hgp100 boost (Figure 25D). Both of the hgp100 vaccinated groups showed a modest, but statistically significant extension of survival relative to the control group. This is in contrast to the previous experiment using fewer B16-F10 cells. We attributed this difference to the variability of engraftment using the smaller number of cells.

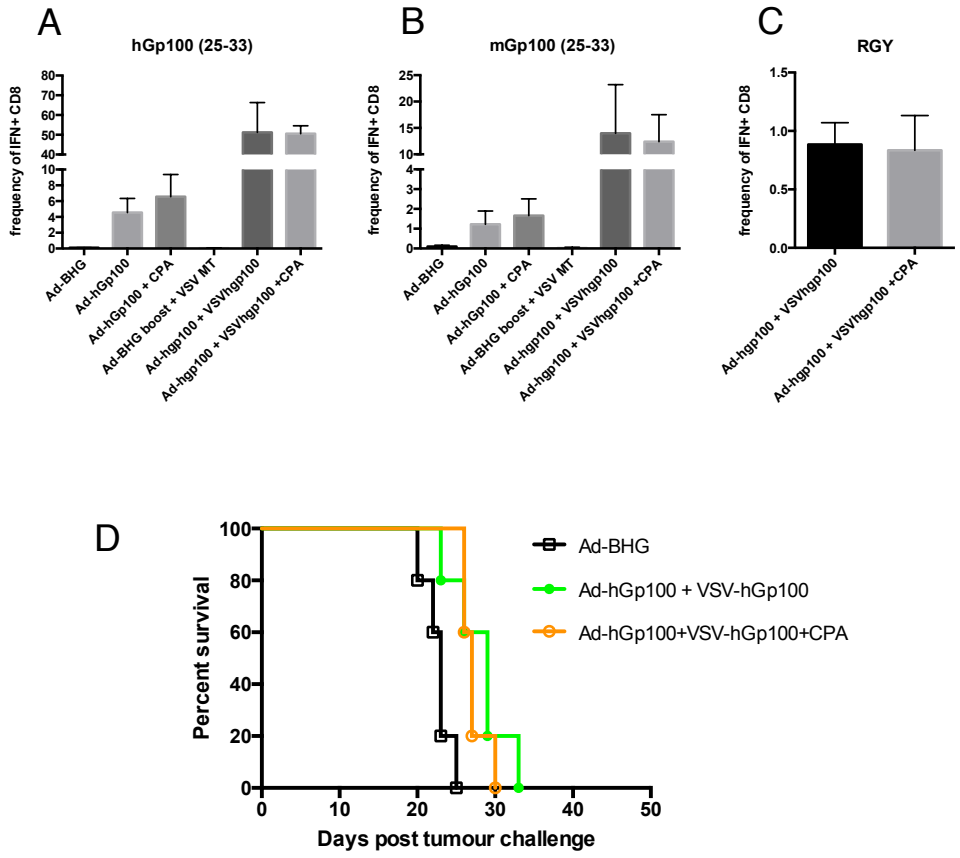

Figure 25. Cyclophosphamide (CPA) treatment and hgp100 vaccination after C57BL/6 engraftment. Frequencies of IFNγ+ CD8 T cell from blood taken at 12 days post prime (left 3 bars) or 5 days post boost (right 3 bars) following restimulation with $hgp100_{(25\text{-}33)}$ (**A**), $mgp100_{(25\text{-}33)}$ (**B**) or RGY(VSV) (**C**) peptides. **D**. Kaplan-Meier plot of survival in days after tumour challenge. (log-rank test; Ad BHG vs Adhgp100+VSVhgp100 = 0.0112; Ad BHG vs Adhgp100+VSVhgp100 + CPA = 0.0021)

Another pharmacological agent that has been used to enhance the anti tumour effect of oncolytic viruses is MS-275. MS-275 is a histone deacetylase inhibitor (HDACi) that enhances viral oncolysis via modification of the interferon response [139]. It has also been shown to increase MHC class I expression in tumour cells, including B16-F10. Finally, MS-275 reduces Treg levels and focuses the immune response to boosting by impairing the primary response to the vector while enhancing the secondary response to the tumour antigen DCT[140]. We hypothesized that these effects could potentially help with the anti B16-F10 activity of the anti hgp100 and mgp100 immune response by increasing the number of targets on the tumour cells and by supporting the anti-tumour response. To test this, C57BL/6 mice were engrafted with 2.5 x 10^5 B16-F10 cells i.v. two days prior to vaccination with 2 x 10^8 pfu of Adhgp100. MS-275 was applied for five consecutive days, beginning on the day of the VSV boost beginning at twelve days post adenovirus vaccination. CD8 T cell responses to $hgp100_{(25-33)}$, $mgp100_{(25-33)}$ and RGY (VSV) were monitored from the blood at 11 days post prime, and 5 days post boost. Figure 26 shows that MS275 treatment did not alter the responses to human or murine $gp100_{(25-33)}$ epitope, as expected and only slightly reduced the response to the VSV epitope RGY. MS-275 treatment failed to improve on the modest extension of survival of mice resulting from the hgp100 vaccination in this aggressive B16-F10 tumour model (Figure 26D).

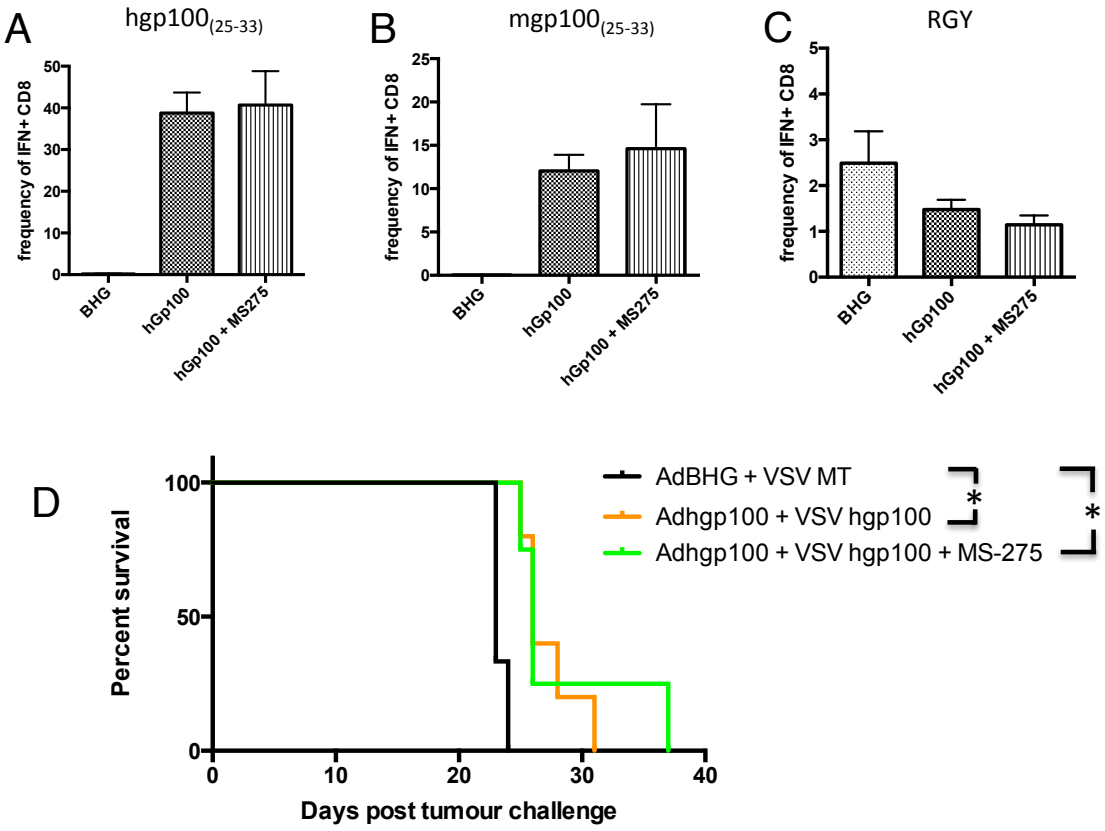

Figure 26. MS-275 treatment and hgp100 vaccination after C57BL/6 engraftment. Frequencies of IFNγ+ CD8 T cell from blood taken at 12 days post prime (left 3 bars) or 5 days post boost (right 3 bars) following restimulation with $hgp100_{(25-33)}$ (**A**), $mgp100_{(25-33)}$ (**B**) or RGY (VSV) (**C**) peptides. **D**. Kaplan-Meier plot of survival in days after tumour challenge. (log-rank test; Ad BHG vs Adhgp100+VSVhgp100 = 0.0046; Ad BHG vs Adhgp100+VSVhgp100 + CPA = 0.0100)

2.3 Does combining gp100 vaccination with DCT vaccination improve anti gp100 mediated anti tumour activity in B16-F10?

A number of groups have had success targeting B16-F10 using another melanoma associated antigen, dopochrome tautomerase (DCT)[141-143]. One of the differences between gp100 and DCT is the ability of Ad DCT to induce MHC I expression in the B16

tumour cells via production of IFNγ by DCT-specific T cells attacking the tumour (Jonathan Bramson, personal communication). This led us to question whether combining vaccination against both DCT and hgp100 may induce not only this increased MHC I expression but other aspects of an immunostimulatory environment in the tumour(s) thereby facilitating a functional anti hgp100 immune response. There is also an observation that pmel T cells can be combined with OT-1 T cells to improve efficacy against B16-OVA[144]. To test this dual vaccination strategy, we combined equal titres of Ad hgp100 and Ad hDCT (or each mixed with the empty vector Ad BHG) for vaccination in the two day old lung metastasis model. Groups of mice were then boosted with a combination of VSV hgp100 and VSV hDCT (or VSV MT). As before, CD8 T cell responses to the $hgp100_{(25-33)}$, $mgp100_{(25-33)}$ and RGY (VSV) were monitored from blood post vaccination. Additionally, responses to the immunodominant MHC class I restricted epitope of hDCT in B16-F10 were monitored using the SVY peptide. Mice were also monitored for development of lung metastasis related pathology. While the group that received both hDCT and hgp100 vaccinations did show enhanced B16-F10 control, it was indistinguishable from the reduced engraftment in the group vaccinated with hDCT expressing vectors (Figure 27). This suggests that the anti B16-F10 tumour activity originates from the hDCT vaccine and that no additional activity can be attributed to the hgp100 vaccination.

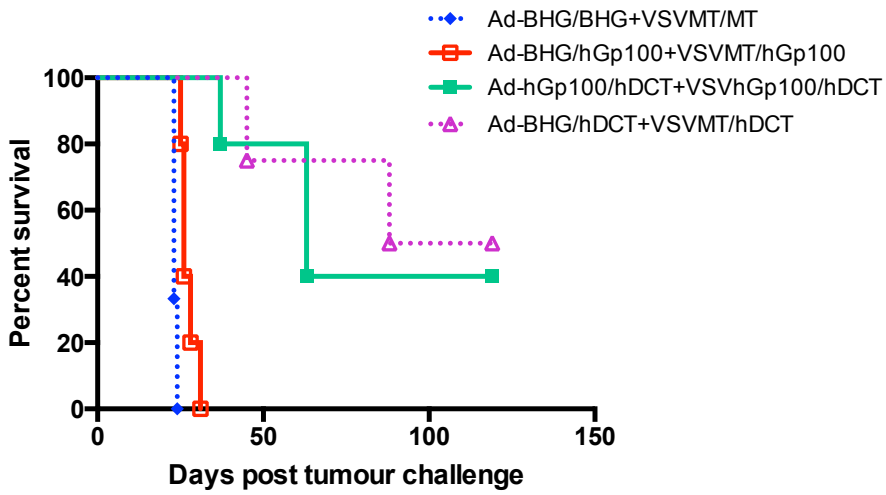

Figure 27. Survival following combination of gp100 and DCT vaccination in B16-F10 tumour model. Mice were vaccinated with the conbination of vectors expressing hgp100 and DCT, or each combined with the empty vector control. The Kaplan-Meier plot of survival post tumour engraftment is shown.

In the immune analysis of this experiment, the normal pattern of boosted $hgp100_{(25\text{-}33)}$ and $mgp100_{(25\text{-}33)}$ responses was observed. Likewise, the anti VSV (RGY) response was present but at a lower level in the DCT or hgp100 vaccinated groups relative to the empty vector group. This is likely a reflection of the immunofocusing phenomenon we have noted previously[113], where immune responses following a boost are focussed on the transgene as opposed to the vector. Finally, a curious observation was made with respect to the SVY (DCT) responses. We noted CD8 T cells that were stimulated to produce IFNγ upon stimulation with the SVY peptide in the group treated with the combination of hgp100 and empty vectors. We also noted that this appeared to be unidirectional in that hDCT vaccination did not result in anti hgp100 responses. After

confirming these results in a repeat experiment, we sought to probe whether this did in fact represent antigen spreading.

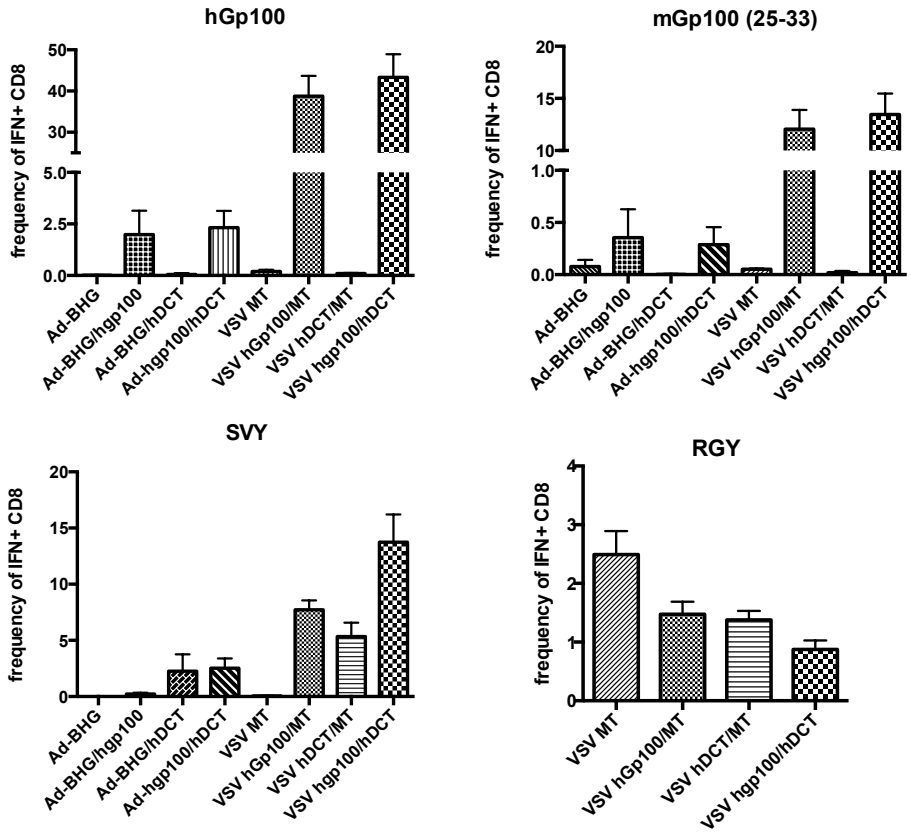

Figure 28. Immune responses using combination of gp100 and DCT vaccination in B16-F10 tumour model. Mice were vaccinated with the combination of vectors expressing hgp100 and DCT, or each combined with the empty vector control. Frequencies of CD8 T cells responding to each the peptides indicated are shown. The left four bars are measured at 12 days after the prime and right four bars measured 5 days after the boost

2.4 Is the anti DCT response in hgp100 vaccinated mice tumour dependent?

Antigen spreading refers to the phenomenon where a vaccine results in immune responses against antigen other than the vaccine target. It has applications clinically in detection of immune escape tumours. The phenomenon has been observed in both mice and in human patients[145-147]. In order to claim that the anti DCT responses observed in hgp100 vaccinated mice were the result of antigen spreading, we needed to show that they occurred only when a tumour is present. To confirm this, we engrafted 2 of 3 groups of mice with B16-F10 2 days before vaccination with Adhgp100. Mice were then boosted 12 days later with VSVhgp100 or the vector control, VSV MT. This last virus was included to assess whether any anti DCT responses observed were the result of the anti hgp100 or were simply due to the oncolysis from the VSV. Immune responses were monitored using intracellular cytokine staining of blood cells at 12 days post prime, 5 days post boost and again at 10 days post boost. In addition to monitoring responses to $hgp100_{(25-33)}$, $mgp100_{(25-33)}$, SVY (DCT) and RGY (VSV), we also used peptide P15E, which corresponds to an endogenous retrovirus in B16-F10[148]. The immune results are shown in Figure 29. At 5 days post boost, frequencies of SVY (DCT) responsive and P15E responsive CD8 T cells of approximately 1% and 3%, respectively were observed in the tumour bearing hgp100 primed and hgp100 boosted group. These responses were not seen in the vaccinated group that did not have a tumour or the group that was boosted with the empty vector, suggesting these were immune mediated spreading responses. We also observed the apparent transient nature of these anti SVY (DCT) and anti P15E

responses in that they were no longer detectable at 10 days post boost whereas the hgp100 responses remained robust at this time-point. Another curious observation with respect to the anti SVY (DCT) and anti P15E responses was the fact these CD8 T cells were IFNγ positive but TNFα negative (Figure 29B).

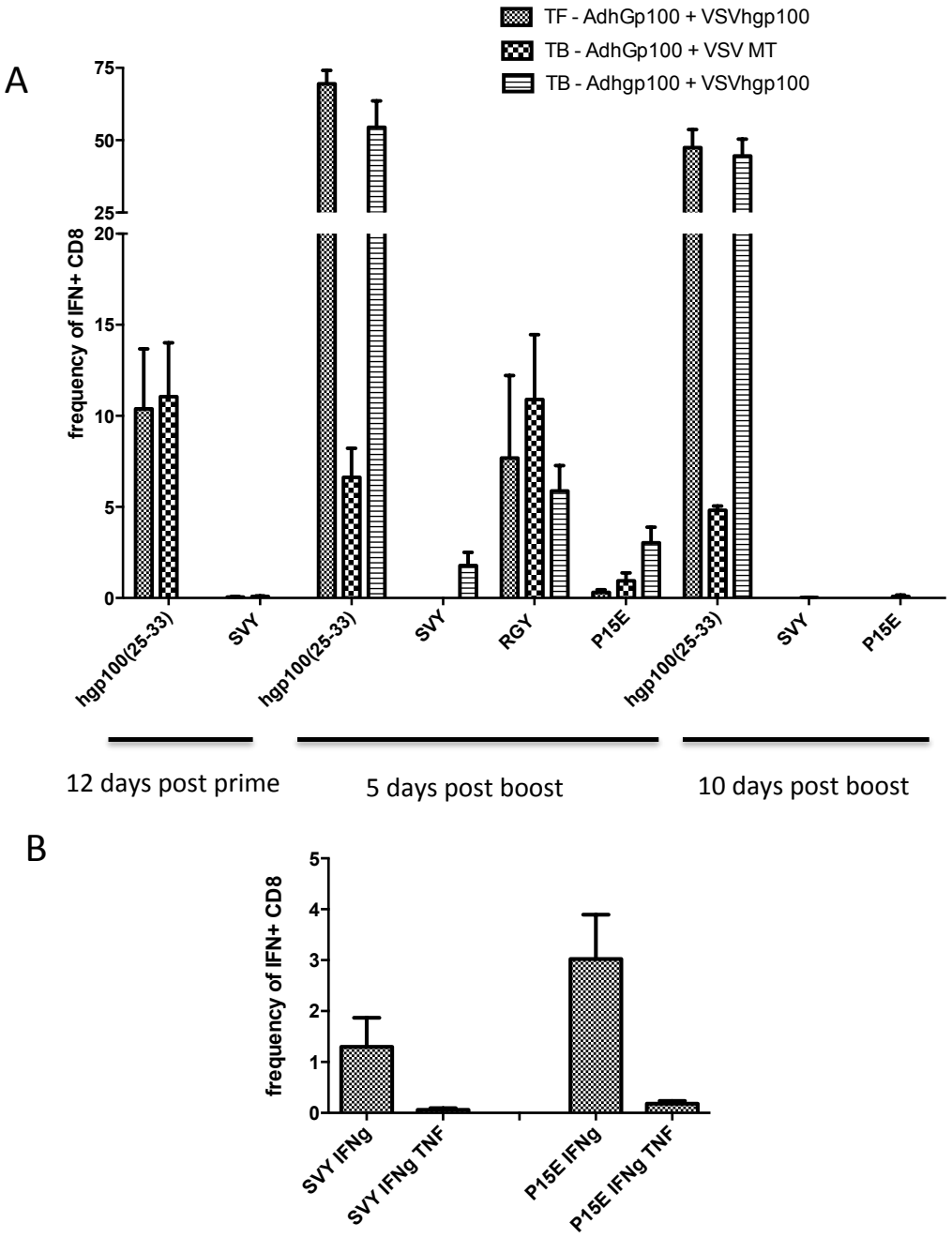

Figure 29. Immune responses after hgp100 vaccination. Tumour bearing (TB) of tumour free (TF) mice were vaccinated with Adhgp100 and VSVhgp100 or Ad hgp100 and VSV MT. **A**. Frequencies of IFNγ positive CD8 T cells following restimulation with the indicated peptides measured at 12 days post prime, 5 days post boost and 10 days post boost. **B.** Frequency of IFNγ and TNFα positive CD8 T cells following restimulation with SVY or P15E

2.5 Is this antigen spreading phenomenon observed in other tumour models?

The next question we sought to answer regarding antigen spreading was if we could detect it in other models. Using the same B16-F10 cells, we repeated the previous experiment in a subcutaneous model. Mice received 1 x 10^5 cells injected subcutaneously on the same day as the adenovirus injections. The interval to the VSV boost was reduced to 9 days. CD8 T cell responses from the blood to the hgp100$_{(25\text{-}33)}$, P15E, SVY (DCT), and RGY (VSV) peptides were monitored at 5 days after the boost. In the subcutaneous model of B16-F10, there were no SVY (DCT) responsive CD8 T cells in the tumour bearing, Ad hgp100 vaccinated, VSV hgp100 boosted group (Figure 30). Responses to P15E were also non existent in this scenario. It is important to note that the hgp100$_{(25\text{-}33)}$ responses and RGY (VSV) responses were as expected. These data suggest that the antigen spreading phenomenon noted in the lung metastasis B16-F10 tumour model is not ubiquitous and is not observed in the subcutaneous version in the time frame studied.

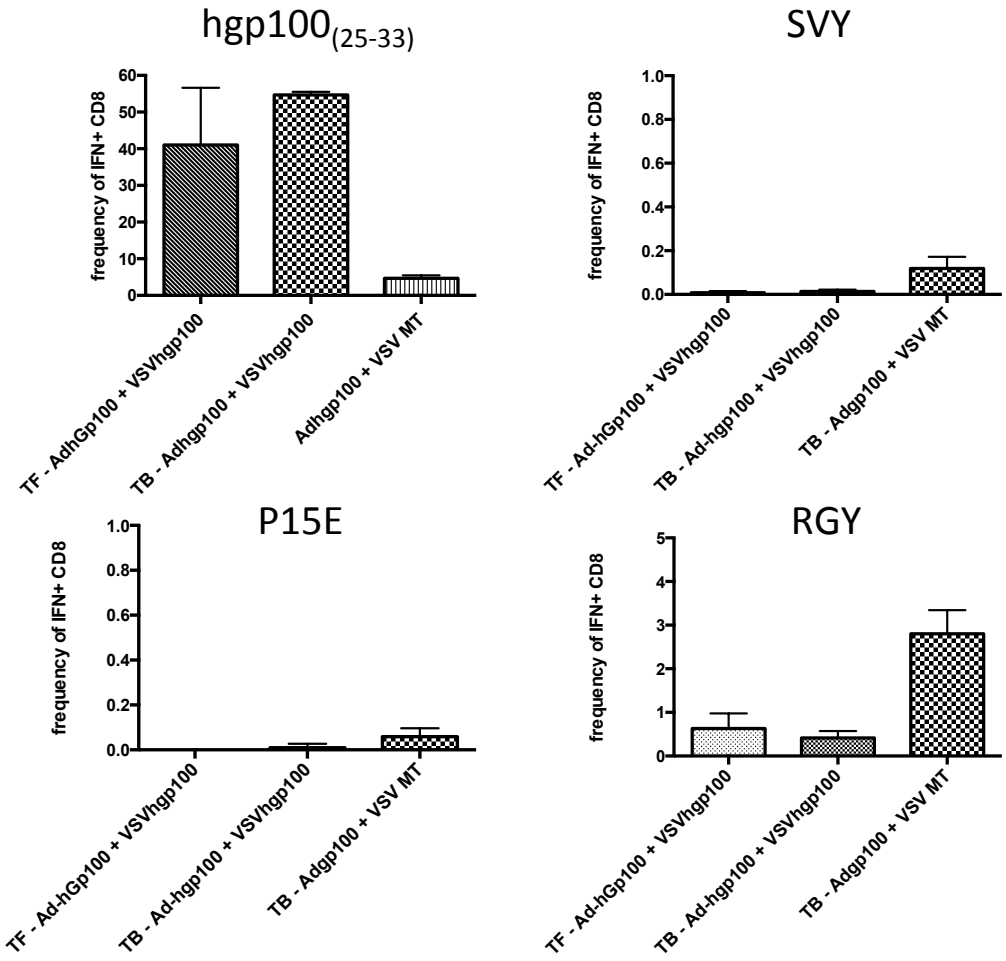

Figure 30. Immune responses after hgp100 vaccination. Subcutaneous B16-F10 tumour bearing (TB) of tumour free (TF) mice were vaccinated with Adhgp100 and VSVhgp100 or Adhgp100 and VSV MT. **A**. Frequencies of IFNγ positive CD8 T cells following restimulation with the indicated peptides measured at 5 days post boost

Of the manipulations to improve the anti tumour activity of the anti gp100 immune response after vaccination, only boosting with VSV was able to provide a small benefit. The use of MS-275 and CPA as pharmacological modifiers of the response also failed to demonstrate any anti tumour activity in the models tested. In addition,

combination of gp100 and DCT vaccination was able to improve on the anti DCT immune response mediated anti B16F10 activity. In order to improve the targeting of B16F10 with gp100 vaccination, alternative interventions need to be explored. The combination of vaccine vectors targeting different antigens do allow for a peculiar observation with respect to anti DCT responses following gp100 vaccination and this warrants more investigation.

- CHAPTER 5 -

Discussion

The work described here focuses on developing a therapeutic strategy to target a newly identified tumour associated antigen (Plac1) and working on strategies to improve targeting of another tumour antigen (Gp100). Targeting these antigens was not as straightforward as simply vaccinating, generating immune responses and watching tumours regress. Instead, the bulk of the experiments involve trying to overcome the barriers to this success. Indeed, the impediments to targeting these two tumour associated antigens appear to occur at different levels. Plac1 targeting in mice is impaired by the inability to generate anti murine Plac1 immune responses. In the case of Gp100, immune responses to murine Gp100 can be generated, but they appear to be ineffectual, implying an impairment at the level of the B16-F10 tumour cells.

1.0 Plac1

The restricted expression of Plac1 made us wonder if autologous Plac1 would be immunogenic without further manipulation. The fact that it is only expressed in testis and placenta[83] suggested the possibility that, especially in females, peripheral tolerance to this antigen would be different than other tumour antigens since it would not be expressed in the tissues and seen by the immune system. Adding to the likelihood of this possibility is the repeated observation that patients with Plac1 expressing tumours often had circulating anti Plac1 antibodies and even Plac1 responsive CD8 T cells[81,87,88]. We were able to confirm expression of hPlac1 mRNA in 5 of 15 tumour samples tested. The differential expression in tumour versus normal tissue was also confirmed as hPlac1 mRNA was not

detected in any of the adjacent tissue samples. The data from Dr. Bramson's group demonstrated the presence of Plac1 responsive CD8 T cells within the tumour of a patient. The data suggested, that in humans, Plac1 was sufficiently immunogenic to induce spontaneous immune responses. In the C57BL/6 and BALB/c strains of mice, this hypothesis was not born out. Simple vaccination with a prime/boost strategy using an adenovirus expressing mPlac1 and a VSV expressing mPlac1 failed to generate meaningful CD8 or CD4 T cell responses against Plac1. We employed several strategies in an attempt generate anti mPlac1 immune responses. The first mouse experiments, like the majority of mouse experiments in general, took place using female mice for sake of convenience. It occurred to us that this may be one antigen in which there could be a difference in tolerance to mPlac1 between the sexes. We considered that central tolerance in males may be less efficient since the gene dose in the thymic epithelial cells would be half in males as it would be in females. We reasoned that Plac1 reactive T cells in males may be less likely to be the subject of negative selection. The only normal expression of Plac1 in male is in testis and at a low level compared to that which is expressed in Placenta. The autoreactive T cells to antigens present in the testis could be controlled through the testis immunoprivileged state[149]. Upon vaccination of male mice with the AdmPlac1 followed by VSV mPlac1 boosting, we initially detected a peptide containing a potential CD8 epitope in the BALB/c strain and only weak responses in C57BL/6. Further investigation revealed that there was no reproducible difference in the ability of male and females to be vaccinated. It appeared that Plac1 is well tolerized in both sexes

of C57BL/6 and BALB/c. To confirm that the lack of responses to Plac1 is the result of tolerance, we would need to study the Plac1 knock out mice. The ability to vaccinate male Plac1 knock out mice (homozygous females appear to be embryonic lethal), would confirm that the inability to vaccinate the wildtype mice is the result of tolerance[84].

The next strategy we used in an attempt to generate anti mPlac1 responses was the inversion of the vaccine vector order. We attempted to prime a response using VSVmPlac1 and boost it with AdmPlac1. This has been shown to work in the context of another tumour associated antigen, hDCT[127]. The rationale for us doing this is that we thought that having more immunogenic proteins in the antigen presenting cell might help provide an immunostimulatory environment that would permit anti mPlac1 CD8 responses. The presence of CD4 epitopes has in fact been shown to result in greater CD8 responses to the same antigen, although there is some debate as to whether the immunogenic CD4 epitope needs to be covalently linked to the potential CD8 eptiope[128]. Our hope was that the replicating VSV would produce more immunogenic epitopes than the replication deficient adenovirus and permit or license responses against the autoantigen mPlac1. An added benefit to this approach was that in the event of successful generation of weak responses, we could more easily manipulate the recombinant VSV to enhance those responses. The recombinant VSV rescue is simpler and quicker than designing and generating the recombinant adenovirus vectors. In the end, this strategy failed to induce any anti mPlac1 CD8 or CD4 responses in the C57BL/6 strain of mice. While reversing the vector order to vaccinate in a more immunostimulatory environment

didn't produce the desired results in this case, the immunogenic nature of the vaccine vector is an important consideration in the design of subsequent vaccine strategies.

Pharmacological manipulation was another tactic we employed to induce anti mPlac1 immune responses. The use of cyclophosphamide as a broad immunosuppressant is well documented, but its use at a low dose can be used to selectively kill regulatory T cells[131,150]. Our rationale was that by eliminating these immunosuppresive cells that we could limit the effects that peripheral tolerance may be having on the ability to generate anti mPlac1 immune responses. Again this strategy was unable, in either C57BL/6 or BALB/c to reveal any meaningful anti mPlac1 CD8 or CD4 responses. Curiously, with BALB/c, there was a very weak response seen with peptide 82, the same peptide to which variable weak responses were noted when vaccinating male and female mice in the absence of cyclophoshamide. We don't know that this apparent weak response is the result of vaccination, as it wasn't deemed significant enough to look for in sham vaccinated animal, or if this peptide just has a higher background activity with MHC I in BALB/c in general. The variability and the weakness of this response suggests that although it may be real, it is almost guaranteed to be not sufficiently robust to possess any anti tumour activity.

From the vaccine manipulation strategies employed here, it became clear that generating anti mPlac1 responses by vaccinating directly with mPlac1 vectors would not be as simple as was originally hypothesized. One of the most commonly used successful

strategies to generate autologous immune responses in the context of therapeutic cancer vaccines has been the use of xenoantigens.

Xenoantigens are the homologous protein from another species. They are seen by the immune system as being foreign, thereby allowing a response. These responses can also cross-react with the autologous antigen. As these statements imply, the degree of homology is an important consideration. An antigen that is too similar with a high degree of homology may not be seen as foreign at all whereas an antigen that is very different may induce a robust response that fails to cross react with the target[134]. The immunogenicity of xenoantigens can be the result of higher avidity T cells specific to the xenoantigen peptides interacting with the autoantigen peptides with lower avidity. Alternatively, xenoantigen immunogenicity may result in the presence of an immunogenic CD4 epitope 'licensing' a weak CD8 response to a less immunogenic shared epitope. This is observed in xenoantigen vaccination against DCT, where mutagenesis of the murine DCT to include the CD4 epitope of the human DCT can result in a CD8 response against the shared CD8 epitope[128]. This is the same principle discussed above with respect to the vector order switching except that in this case the CD4 epitope is, at least initially, covalently linked to the CD8 epitope. In the case of Plac1, we were able to detect an MHC II restricted epitope to human Plac1 in BALB/c, but no CD8 epitopes. In C57BL/6, we detected robust CD8 epitopes to human Plac1 in addition to several potential CD4 epitopes with only a prime vaccination. To determine if this robust anti hPlac1 response could cross react with mPlac1, we asked whether there

were anti mPlac1 responses generated and whether they, or the anti hPlac1 responses could be boosted with VSV expressing the murine or human Plac1. With respect to the anti human CD8 responses, the AdhPlac1 primed, VSV mPlac1 group looked identical to the VSV MT boosted group. This indicated to us that the mPlac1 vaccination cannot lead to further expansion of preexisting CD8 T cells. The VSV hPlac1 group did demonstrate an expansion of the immunodominant epitope responsive CD8 T cells. In contrast, only extremely weak responses were noted upon restimulation with murine peptides in the AdhPlac1 primed, VSV mPlac1 boosted group. While the responses are very weak, they curiously correspond the homologous peptides containing the immunodominant epitopes for hPlac1. This might suggest that this limited activity in vitro is the result of murine peptides being able to weakly stimulate the anti human Plac1 CD8 T cells. These results argue that for T cell responses in C57BL/6, human and murine Plac1 are too divergent for a successful xenoantigen strategy. If human and mouse Plac1 are too divergent, we asked what a useful xenoantigen would look like. Rat Plac1 is much more conserved when compared to murine Plac1. At 90% identity (Figure 17), there is a much greater likelihood of a T cell responding to a rat Plac1 epitope to find a similar or identical eptiope presented from murine Plac1. The risk, of course, is that they are too similar and that rat Plac1 would not be sufficiently immunogenic in mice. Another strategy was the development of a synthetic xenoantigen that consisted of a fusion of mPlac1 with the 39 amino acid tail from human Plac1. In this regard, the immunogenic C-terminal tail would provide the immunogenic CD4 epitope (in C57BL/6) but also contain murine CD8

epitopes perfectly matched to the targets. The plan was to use these transgenes in the adenovirus to prime an anti mPlac1 response that could be boosted by any of the Plac1 boosting VSVs we had in hand. In both these cases, there were technical difficulties with respect to the rescue of these vectors, both on my part and that of the Fitzhenry Vector laboratory, despite the confirmed successful cloning of shuttle vectors used in the rescue process. I was able to rescue VSVs expressing each of these two transgenes but we elected to not look at a complete map of mPlac1 T cell responses induced by them in the absence of the corresponding priming (adenovirus) vector. Studying the success of the rat Plac1 and the synthetic xenoantigens as tools to generate anti mPlac1 responses remains in limbo pending the rescue of these vectors. When they are rescued, there are several possible outcomes. One possibility is that, as designed, either of these transgenes may result in a weak but boostable anti mPlac1 response. It is also possible that the rat xenoantigen is too similar to mPlac1 and doesn't induce any anti mPlac1 (or rat Plac1) T cell responses. It is also possible that the synthetic mouse-human fusion xenoantigen results in responses against the human portion of the transgene but not the desired murine elements. In each of these cases, one possible strategy going forward would be to increase foreign CD4 epitope containing elements from hPlac1, or other known immunogens such as lymphocytic choriomeningitis virus (LCMV) glycoprotein gp33 CD4 epitopes in C57BL/6[151]. This strategy might even be potentiated by including multiple copies of these CD4 epitopes (with flanking sequence to promote correct

cleavage) to maximize the amount of CD4 stimulation in an APC facilitating autologous CD8 responses.

While the focus thus far has been on T cell responses to Plac1, Plac1's expression on the surface of cells makes generating antibody responses a worthwhile endeavor. Given the robust T cell response against hPlac1 in C57BL/6, including the detection of CD4 epitopes, we considered that antibodies to hPlac1 may be produced in vaccinated mice. I developed an ELISA-based assay to test serum from vaccinated mice for anti-hPlac1 antibodies. Sera from Ad hPlac1 primed, VSV hPlac1 boosted or Ad BHG primed, VSV MT boosted mice was tested against FLAG captured hPlac1 in a sandwich ELISA. The sera from the empty vector group were indistinguishable from the sera from the Plac1 vaccinated group with respect to signal from a plate containing hPlac1 lysate. The plate itself was able to produce a signal when exposed to the commercial anti hPlac1 antibody. The vaccine protocol was not designed specifically with the goal of inducing antibody production. A strategy targeted at generating antibodies would more likely involve vaccinating with recombinant protein with adjuvants in order to shift the response to optimize the humoral response[152]. Successful induction of an antibody response could result in the generation of a monoclonal anti hPlac1 antibody with potential clinical applications. The value of such a therapeutic has been realized by others as patents have been applied for with respect to therapeutic applications of anti hPlac1 antibodies[153]. Serum antibodies represent another facet of the xenoantigen vaccination strategy. Another way to target mPlac1 would be through generation of antibodies against a

xenoantigen such as rat Plac1 or the mouse-human Plac1 fusion in the hope the antibodies would then cross react with exposed self antigen on tumour cells.

Despite the inability to detect anti mPlac1 responses in the strategies employed, we investigated whether some of these strategies had any anti-tumour activity in vivo. The frequency of responsive CD8/CD4 T cells or serum antibody titer required to possess anti tumour activity is unknown and it was formally possible that responses below our level of detection would have some effect in vivo. In a prophylactic setting using the subcutaneous CT26 model in BALB/c, we attempted to see if mPlac1 vaccination could delay engraftment. Neither the AdmPlac1 primed, VSVmPlac1 boosted or AdhPlac1 primed VSVmPlac1 boosted groups had any impairment in tumour engraftment as mice developed tumours at the same frequency as the empty vector controls. We also tested the ability of AdhPlac1 vaccination followed by either VSV mhPlac1 (the fusion consisting of mouse Plac1 fused to the human Plac1 C terminal tail) or Maraba hPlac1 to delay B16-F10 engraftment in a lung metastasis model. Both of these groups had CD8 T cells responding to hPlac1 peptide #12 after the prime, but only the Maraba hplac1 boosted group showed a boost in the frequency of the hPlac1 p12 responsive CD8 T cells. Peptide 12 of the hPlac1 library does not correspond to the hPlac1 C-terminal tail which is present in this transgene and thus not surprising that there wasn't a boost of CD8 T cells responding to that epitope. Neither of the groups was able to delay B16-F10 engraftment as all mice reached endpoint in 20-22 days. The inability of the robust anti hPlac1 CD8

T cell response to control mPlac1 expressing tumours is consistent with the observation that the anti hPlac1 T cell responses in C57BL/6 do not crossreact with mPlac1.

Taken together the Plac1 data show that further manipulation is required in order to study the targeting of mPlac1. Ironically, in humans where we really want to target it, Plac1 may in fact be sufficiently immunogenic to target with Plac1 peptides or other vaccine platforms.

2.0 Gp100

The impediments to using gp100 as a tumour antigen target are different than those encountered with Plac1. The previous literature had indicated that the generation of anti mgp100 T cell responses was not the issue but that these T cells failed to control mgp100 expressing B16-F10 tumours[98]. The observations that pmel T cells can impact B16-F10 tumours [138] and that boosting tumour antigen responses can lead to higher quality T cells, suggested that a simple way to overcome the inability of anti mgp100 T cells to affect B16-F10 tumour cells is to generate more, better quality T cells by boosting the adenovirus prime with an oncolytic VSV boost. Boosting with VSV successfully expanded the number of hgp100 and mgp100 CD8 T cells in the blood. In the case of mgp100, this reached a frequency of over 10% of CD8 T cells that could respond to restimulation with $mgp100_{(25\text{-}33)}$. We can't comment on the quality of these T cells, but it is worth considering that boosting with the human antigen, may preferentially result in higher quality hgp100 responsive T cells, which are not necessarily higher quality

mgp100 responsive T cells. In a prophylactic subcutaneous model, this response was sufficient to delay the appearance of B16-F10 tumours but not to prevent their engraftment. We opted to use a different model to study the therapeutic potential of the hgp100 prime:boost regimen. In our experience, the subcutaneous B16-F10 model provides only a narrow window where treatment can occur before tumours ulcerate and mice reach endpoint. Instead we used the lung metastasis model where tumour cells are injected intravenously before forming multiple nodules on the lungs. In this setting, the vaccination led to frequencies of $hgp100_{(25-33)}$ and $mgp100_{(25-33)}$ responsive CD8 T cells similar to those achieved in the prophylactic setting. This implies that the presence of the nascent tumour isn't impacting the ability to generate responses to these antigens. In this initial study, however, there was no difference between the empty vector treated, primed only or primed and boosted mice in terms of the time required to reach tumour dependent endpoint. As noted before, this initial tumour dose was only 1/5 of what we typically use in this model. The elongated and variable tumour take is likely a result of this reduced number of injected tumour cells. In follow up experiments using 2.5×10^5 tumour cells, a modest but significant extension of survival is noted in Adhgp100 primed, VSVhgp100 vaccinated mice. While there is an extension of survival, it is modest and all mice go on to reach endpoint with median survival of 27-29 days as opposed to 23 days with the empty vector vaccination.

One of the strategies to extend survival is the use of pharmacological agents. We attempted to use both cyclophosphamide and MS-275 to generate an environment where

the abundance of mgp100$_{(25\text{-}33)}$ responsive CD8 T cells could have an improved result on the B16-F10 tumours. Cyclophosphamide is an immunosuppressant that is used at low doses to specifically target CD4CD25 regulatory T cells[131]. In the context of hgp100 vaccination, we hope to reduce any immunosuppressive effect of Tregs on the anti mgp100 responses. Interestingly, CPA didn't alter the frequencies of the hgp100$_{(25\text{-}33)}$ or mgp100$_{(25\text{-}33)}$ responsive T cells, unlike its effects on other tumour antigen specific T cells (Pol *et al*, manuscript in preparation). The inability of CPA to alter CD8 T cell responses to alter levels of responses to hgp100$_{(25\text{-}33)}$ or mgp100$_{(25\text{-}33)}$ may be better understood by considering recent data from Dr Jonathon Pol (Figure 31). He showed that CPA was able to affect frequencies of anti SVY (DCT) responses in a tumour dependent manner whereas responses to SIIN (OVA) were unaffected. This suggests that the tumour is suppressing responses to antigens it expresses and presents as opposed to a global suppression of adenovirus induced responses. This is consistent with CPA's inability anti mgp100 responses given that mgp100$_{(25\text{-}33)}$ is poorly presented[98]. In addition to not altering the gp100 $_{(25\text{-}33)}$ responses, CPA treatment failed to extend survival in the B16-F10 lung metastasis model. This suggests that if there are weak T cell responses to other mgp100 epitopes that are being suppressed by Tregs that they cumulatively don't have an meaningful effect on tumour progression.

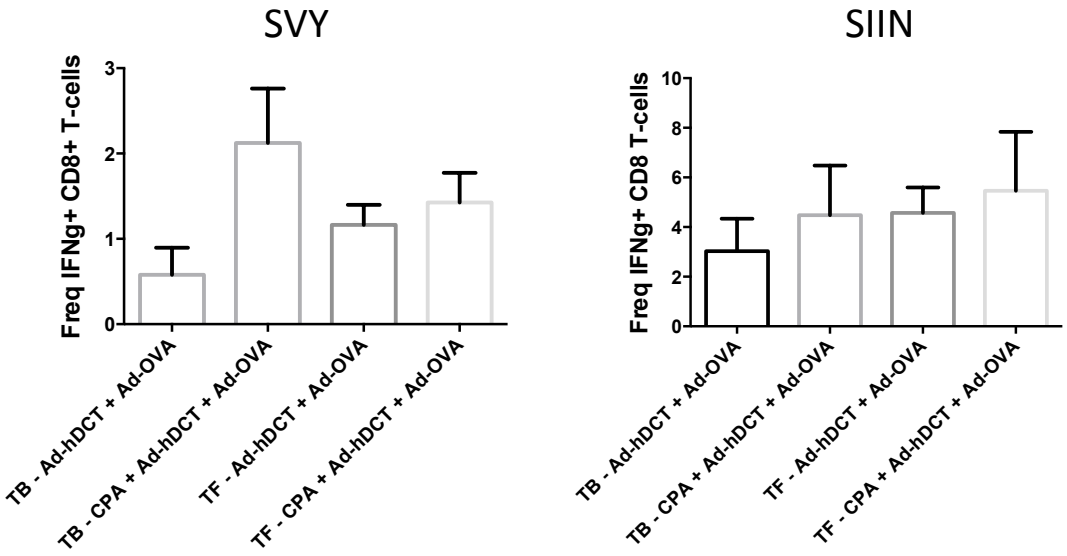

Figure 31. Effect of CPA on DCT and OVA responses. B16-F10 tumour bearing or tumour free C57BL/6 mice were vaccinated with AdhDCT and AdOVA with or without cyclophosphamide (CPA) treatment. Frequencies of IFNγ positive CD8 T splenocytes following restimulation with the indicated peptides measured at 9 days post vaccination. *Data/figure from Dr Jonathon Pol*

The next agent we tested was MS-275. It is a histone deacetylase inhibitor (HDACi) that induces a number of transcriptional changes enhancing viral oncolysis with VSV[139]. Additionally, it can increase MHC I expression in tumour cells, including B16-F10[140]. The increase in MHC I was a potentially useful effect with respect to gp100 vaccination since it has been shown that mgp100$_{(25\text{-}33)}$ processing and presentation are limiting. We hypothesized that the defect in processing may be overcome by increasing the efficiency of presentation by promoting more MHC I expression and perhaps giving the mgp100$_{(25\text{-}33)}$ responsive T cells better targets. Upon vaccination we noted that there was not an MS-275 dependent increase in the frequency of mgp100 $_{(25\text{-}33)}$ or hgp100 $_{(25\text{-}33)}$

responsive CD8 T cells. We also failed to observe a decrease the VSV (RGY) specific CD8 T cells that we would have expected based on previous reports[140]. In addition to its inability to alter hgp100$_{(25-33)}$, mgp100$_{(25-33)}$ or RGY responsive CD8 T cell frequencies, MS-275 failed to build on the modest extension of survival generated by the hgp100 priming and boosting treatment. Further manipulations of the tumour microenvironment may yet yield the ability of the abundant anti mgp100$_{(25-33)}$ CD8 T cells to demonstrate anti tumour activity. Gp100 vaccine enhancements with anti CTLA-4 have been shown in the clinic[154]. One drawback of the anti CTLA-4 treatment is the presence of immune related toxicities, but these can be overcome if treated early enough[97]. Using anti CTLA-4 antibodies, or other immune modifying antibodies such as PD-1 or 41-BB may be of use with respect to gp100 vaccination in that they may derepress T cells responsive to sub dominant epitopes, thereby limiting the reliance on the processing and presentation of mgp100$_{(25-33)}$ on MHC I[143].

The next manipulation we attempted was the vaccine combination including another melanoma associated antigen, DCT. DCT (also known as tyrosinase related protein 2, trp2) vaccination has been used successfully against B16-F10 tumours[141,142]. One of the differences between AdDCT vaccination and Adhgp100 vaccination is the expression of IFN γ by attacking DCT-specific T cells leading to MHC I expression by the tumour ([143], personal communication from Jonathan Bramson). We hypothesized that the presence of a functional anti DCT CD8 T cell response in a tumour may help improve the tumour cells as a target that can be recognized by the robust mgp100$_{(25-33)}$ CD8 T cell

response from Adhgp100/VSVhgp100 vaccination. The combination of pmel T cells, responding to $mgp100_{(25-33)}$, and OT-1 T cells, responding to immunogenic ovalbumin (ova) has been shown to possess increased anti tumour activity in ova-expressing B16 cells[144]. We combined equal doses of the hDCT and hgp100 vaccines (or each mixed with empty vector) in the B16-F10 lung metastasis model. While the group receiving both the hDCT and hgp100 vaccines showed a marked extension of survival, this was no different than for the group that received the hDCT vaccines alone. What we did notice in this experiment was the presence of SVY-responsive CD8 T cells in the absence of hDCT vaccination. This was repeated to ensure there wasn't an issue with reagents or vectors. This result resembles the phenomenon of antigen spreading where dying tumour cells can present multiple epitopes in an immunostimulatory fashion facilitating detection by multiple T cell clones. Antigen spreading has been described in patients[145,146] and in B16 tumours in mice[147]. One key difference between our observation and those reported is that antigen spreading led to dying tumour cells. In our study, mice vaccinated with Adhgp100 and VSVhgp100 reached endpoint quickly with only a marginal extension of survival relative to the empty vector treated group. Its unclear whether that modest extension of survival was the result of a sufficient amount of tumour cell death and T cell recognition to induce antigen spreading to mDCT. To test whether this did in fact represent antigen spreading we looked at responses in tumour free and tumour bearing mice vaccinated with Adhgp100 and VSV hgp100 or Adhgp100 and VSV MT. If the SVY responses were the result of antigen spreading, we would not expect to see them in

mice that didn't have tumours. The VSV MT boosted group is included to separate the effect of oncolysis in the tumour from immune recognition mediated responses. In addition to looking at the SVY responses, we assayed responses to another antigen P15E, an endogenous retrovirus found in B16-F10[148,155]. We were able to detect both SVY responsive and P15E responsive CD8 T cell populations in the tumour bearing Adhgp100 primed; VSVhgp100 boosted group only. These responses were apparent at 5 days post boost but were no longer detectable at 10 days post boost. Investigation revealed that these IFNγ + CD8 T cells were also TNFα negative, further differentiating them from the cells we would expect to see following hDCT vaccination. We next attempted to start to define the determinants of this apparent antigen spreading following Adhgp100 and VSV hgp100 vaccination. We asked if this phenomenon was apparent in the subcutaneous B16-F10 model. In this case we weren't able to detect any SVY or P15E responses despite our ability to generate normal levels of $hgp100_{(25\text{-}33)}$ responsive CD8 T cells. We checked for the SVY and P15E CD8 T cell responses 5 days after the boost. The transient nature of the SVY and P15E responses in the lung metastasis setting indicated that the timing of when to look for responses is important. It is entirely possible that the kinetics in the subcutaneous model are different and that we missed the window where they would have been detectable. That said, we were constrained by the aggressive nature of the subcutaneous model and by the requirement to have sufficient time between the priming vaccination and the boost to generate the magnitude of $mgp100_{(25\text{-}33)}$ response that may be required.

While the specific determinants of antigen spreading induced by hgp100 vaccination in B16-F10 remain elusive, exploiting this phenomenon is an attractive goal. An immunotherapeutic strategy that doesn't rely on one single epitope from a single antigen is presumed to have a better chance at success. Vaccine strategies relying on one antigen are subject to immune escape where selection will occur that promotes the growth of tumour cells that cease to express that particular antigen. Strategies that generate weak responses against many antigens would be more likely to prevent tumours from mutating around the response. The generation of many weak anti tumour responses is the basis behind attempts to use tumour cell lysates or infected cell lysates as vaccines[156]. In the context of the lung metastasis model, efforts should be placed on studying the DCT and P15E responsive CD8 T cells in the hgp100 vaccinated mice with an eye to improving the quality and quantity of these cells. The absence of TNFα production in the IFN γ positive cells indicates that they don't have the normal phenotype expected following DCT vaccination. This begs the question that if the capacity of these cells to produce TNFα is restored by some yet to be determined manipulation, would these cells be able to detect and kill B16-F10 tumours? The impetus for looking at the subcutaneous B16-F10 model for this antigen spreading is that if present, we could more easily study the functionality of these responses by removing the primary tumour and looking at tumour regrowth. I suspect that in the lung metastasis model, the combination of the altered phenotype of the DCT and P15E responsive T cells and the fact there are too few, too late, when the tumour has progressed substantially, is responsible for the lack of anti tumour efficacy.

3.0 Conclusions and Future Directions

In the therapeutic cancer vaccine field targeting specific tumour antigens is likely to encounter situations like that which we found in the case of Plac1. While there are documented strategies to circumvent peripheral tolerance using pharmacological agents such as cyclophosphamide[131], central tolerance is more difficult to overcome. Xenoimmunization is the best strategy currently identified[132]and relies on the hope that weakly autoreactive T cells that escape the thymus can be stimulated by a closely related perhaps slightly more immunogenic antigen. Strategies to best understand how to circumvent tolerance and generate autoantigen responses are likely to be critical in future attempts to develop therapeutic cancer vaccine strategies. Importantly, the ease with which these can be overcome for each prospective tumour antigen is critical to determine when choosing an antigen. In the case of Plac1, responses to the self antigen were not detected and the human xenoantigen did not permit the generation of anti mPlac1 responses. It remains to be seen whether a different xenoantigen or a synthetic xenoantigen such as the fusion protein described herein will be more successful at generating anti mPlac1 responses in mice. This is perhaps a time when studying a potential human disease and treatments in mice is limiting. It possible that in humans, hPlac1 is sufficiently immunogenic and would be a good candidate for future studies.

The case of gp100 demonstrates that the generation of T cells that can respond to the target is not the conclusion of the challenge in terms of generating therapeutic cancer vaccine strategies. Significant responses to mgp100 can be generated without a great

therapeutic benefit. This is another situation that is likely to surface in a clinical setting. Strategies to modify the tumour or tumour microenvironment in order to allow the robust response to have a therapeutic benefit are also critical. While the strategies we employed failed to show a great therapeutic benefit, we described a phenomenon where the gp100 vaccination resulted in a T cell response to other tumour antigens. The exploitation of this phenomenon may provide a way to use these responses to realize the therapeutic potential of vaccines that cannot directly target antigens that are poorly presented.

These studies demonstrate the difficulties encountered in developing therapeutic cancer vaccine strategies and test a number of strategies to overcome the immune system based or tumour cell based limitations on generation successful therapeutic cancer vaccines.

References

1. WHO | Cancer. *WHO*
2. Vogelstein, B. & Kinzler, K. W. The multistep nature of cancer. *Trends Genet.* **9,** 138–141 (1993).
3. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100,** 57–70 (2000).
4. Kelly, G. L. *et al.* Different patterns of Epstein-Barr virus latency in endemic Burkitt lymphoma (BL) lead to distinct variants within the BL-associated gene expression signature. *J. Virol.* **87,** 2882–2894 (2013).
5. Katki, H. A. *et al.* Five-year risks of CIN 3+ and cervical cancer among women who test Pap-negative but are HPV-positive. *J Low Genit Tract Dis* **17,** S56–63 (2013).
6. de-Thé, G. Epstein-Barr virus behavior in different populations and implications for control of Epstein-Barr virus-associated tumors. *Cancer Res.* **36,** 692–695 (1976).
7. Lapointe, N. E. *et al.* Effects of eribulin, vincristine, paclitaxel and ixabepilone on fast axonal transport and kinesin-1 driven microtubule gliding: Implications for chemotherapy-induced peripheral neuropathy. *Neurotoxicology* (2013). doi: 10.1016/j.neuro.2013.05.008
8. O'Brien, S. G. *et al.* Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N. Engl. J. Med.* **348,** 994–1004 (2003).
9. Janeway, C. A. & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20,** 197–216 (2002).
10. Medzhitov, R. & Janeway, C. Innate immunity. *N. Engl. J. Med.* **343,** 338–344 (2000).
11. Beutler, B. Innate immunity: an overview. *Mol. Immunol.* **40,** 845–859 (2004).
12. Li, J. L. & Ng, L. G. Peeking into the secret life of neutrophils. *Immunol. Res.* **53,** 168–181 (2012).
13. Rigby, K. M. & DeLeo, F. R. Neutrophils in innate host defense against Staphylococcus aureus infections. *Semin Immunopathol* **34,** 237–259 (2012).
14. Barker, R. N. *et al.* Antigen presentation by macrophages is enhanced by the uptake of necrotic, but not apoptotic, cells. *Clin. Exp. Immunol.* **127,** 220–225 (2002).
15. Jost, S. & Altfeld, M. Control of human viral infections by natural killer cells. *Annu. Rev. Immunol.* **31,** 163–194 (2013).
16. Zuo, J. *et al.* The Epstein-Barr virus G-protein-coupled receptor contributes to immune evasion by targeting MHC class I molecules for degradation. *PLoS Pathog.* **5,** e1000255 (2009).
17. Picker, L. J. & Butcher, E. C. Physiological and molecular mechanisms of lymphocyte homing. *Annu. Rev. Immunol.* **10,** 561–591 (1992).
18. Masopust, D. & Schenkel, J. M. The integration of T cell migration, differentiation and function. *Nat. Rev. Immunol.* **13,** 309–320 (2013).

19. Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. T cell activation. *Annu. Rev. Immunol.* **27,** 591–619 (2009).
20. Gonzalo, J. A. *et al.* Cutting edge: the related molecules CD28 and inducible costimulator deliver both unique and complementary signals required for optimal T cell activation. *J. Immunol.* **166,** 1–5 (2001).
21. Wang, S. *et al.* Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* **96,** 2808–2813 (2000).
22. Wekerle, T., Blaha, P., Langer, F., Schmid, M. & Muehlbacher, F. Tolerance through bone marrow transplantation with costimulation blockade. *Transpl. Immunol.* **9,** 125–133 (2002).
23. Bossi, G. *et al.* The secretory synapse: the secrets of a serial killer. *Immunol. Rev.* **189,** 152–160 (2002).
24. Woodland, D. L. & Dutton, R. W. Heterogeneity of CD4(+) and CD8(+) T cells. *Curr. Opin. Immunol.* **15,** 336–342 (2003).
25. Prezzi, C. *et al.* Virus-specific CD8(+) T cells with type 1 or type 2 cytokine profile are related to different disease activity in chronic hepatitis C virus infection. *Eur. J. Immunol.* **31,** 894–906 (2001).
26. Glimcher, L. H. & Murphy, K. M. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev.* **14,** 1693–1711 (2000).
27. Santana, M. A. & Rosenstein, Y. What it takes to become an effector T cell: the process, the cells involved, and the mechanisms. *J. Cell. Physiol.* **195,** 392–401 (2003).
28. Liu, Z., Fan, H. & Jiang, S. CD4(+) T-cell subsets in transplantation. *Immunol. Rev.* **252,** 183–191 (2013).
29. Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P. & Yamaguchi, T. Regulatory T cells: how do they suppress immune responses? *Int. Immunol.* **21,** 1105–1111 (2009).
30. Fearon, D. T. & Carroll, M. C. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annu. Rev. Immunol.* **18,** 393–422 (2000).
31. Lanzavecchia, A. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol.* **8,** 773–793 (1990).
32. McHeyzer-Williams, L. J. & McHeyzer-Williams, M. G. Antigen-specific memory B cell development. *Annu. Rev. Immunol.* **23,** 487–513 (2005).
33. Jaiswal, A. I. & Croft, M. CD40 ligand induction on T cell subsets by peptide-presenting B cells: implications for development of the primary T and B cell response. *J. Immunol.* **159,** 2282–2291 (1997).
34. Rus, H., Cudrici, C. & Niculescu, F. The role of the complement system in innate immunity. *Immunol. Res.* **33,** 103–112 (2005).

35. Nash, J. T. *et al.* Immune complex processing in C1q-deficient mice. *Clin. Exp. Immunol.* **123,** 196–202 (2001).
36. Hogquist, K. A., Baldwin, T. A. & Jameson, S. C. Central tolerance: learning self-control in the thymus. *Nat. Rev. Immunol.* **5,** 772–782 (2005).
37. Liu, Y.-J. A unified theory of central tolerance in the thymus. *Trends Immunol.* **27,** 215–221 (2006).
38. Goldrath, A. W. & Bevan, M. J. Selecting and maintaining a diverse T-cell repertoire. *Nature* **402,** 255–262 (1999).
39. Anderson, G. & Takahama, Y. Thymic epithelial cells: working class heroes for T cell development and repertoire selection. *Trends Immunol.* **33,** 256–263 (2012).
40. Alexandropoulos, K. & Danzl, N. M. Thymic epithelial cells: antigen presenting cells that regulate T cell repertoire and tolerance development. *Immunol. Res.* **54,** 177–190 (2012).
41. Koble, C. & Kyewski, B. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J. Exp. Med.* **206,** 1505–1513 (2009).
42. Kyewski, B. & Peterson, P. Aire, master of many trades. *Cell* **140,** 24–26 (2010).
43. Klein, L., Hinterberger, M., Wirnsberger, G. & Kyewski, B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat. Rev. Immunol.* **9,** 833–844 (2009).
44. Sprent, J. & Kishimoto, H. The thymus and central tolerance. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **356,** 609–616 (2001).
45. Sprent, J. & Kishimoto, H. The thymus and negative selection. *Immunol. Rev.* **185,** 126–135 (2002).
46. Nishikawa, H. & Sakaguchi, S. Regulatory T cells in tumor immunity. *Int. J. Cancer* **127,** 759–767 (2010).
47. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting: from immuno- surveillance to tumor escape. *Nat. Immunol.* 1–8 (2002).
48. Dighe, A. S., Richards, E., Old, L. J. & Schreiber, R. D. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity* **1,** 447–456 (1994).
49. Street, S. E., Cretney, E. & Smyth, M. J. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. *Blood* **97,** 192–197 (2001).
50. Smyth, M. J., Crowe, N. Y. & Godfrey, D. I. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int. Immunol.* **13,** 459–463 (2001).
51. Vesely, M. D. & Schreiber, R. D. Cancer immunoediting: antigens, mechanisms, and implications to cancer immunotherapy. *Ann. N. Y. Acad. Sci.* **1284,** 1–5 (2013).

52. Koebel, C. M. *et al.* Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* **450,** 903–907 (2007).
53. Garrido, F. *et al.* Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol. Today* **18,** 89–95 (1997).
54. Nakamura, K., Kitani, A. & Strober, W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J. Exp. Med.* **194,** 629–644 (2001).
55. Teicher, B. A. Transforming growth factor-beta and the immune response to malignant disease. *Clin. Cancer Res.* **13,** 6247–6251 (2007).
56. Muller, A. J. & Prendergast, G. C. Indoleamine 2,3-dioxygenase in immune suppression and cancer. *Curr Cancer Drug Targets* **7,** 31–40 (2007).
57. Uyttenhove, C. *et al.* Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat. Med.* **9,** 1269–1274 (2003).
58. Guleria, I. *et al.* A critical role for the programmed death ligand 1 in fetomaternal tolerance. *J. Exp. Med.* **202,** 231–237 (2005).
59. Dong, H. *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat. Med.* **8,** 793–800 (2002).
60. Iwai, Y. *et al.* Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 12293–12297 (2002).
61. Berger, R. *et al.* Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. *Clin. Cancer Res.* **14,** 3044–3051 (2008).
62. Sznol, M. & Chen, L. Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. *Clin. Cancer Res.* **19,** 1021–1034 (2013).
63. Hou, D.-Y. *et al.* Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. *Cancer Res.* **67,** 792–801 (2007).
64. Pentcheva-Hoang, T., Corse, E. & Allison, J. P. Negative regulators of T-cell activation: potential targets for therapeutic intervention in cancer, autoimmune disease, and persistent infections. *Immunol. Rev.* **229,** 67–87 (2009).
65. Wing, K. *et al.* CTLA-4 control over Foxp3+ regulatory T cell function. *Science* **322,** 271–275 (2008).
66. Scalapino, K. J. & Daikh, D. I. CTLA-4: a key regulatory point in the control of autoimmune disease. *Immunol. Rev.* **223,** 143–155 (2008).
67. Walunas, T. L. *et al.* CTLA-4 can function as a negative regulator of T cell activation. *Immunity* **1,** 405–413 (1994).

68. Ascierto, P. A., Simeone, E., Sznol, M., Fu, Y.-X. & Melero, I. Clinical experiences with anti-CD137 and anti-PD1 therapeutic antibodies. *Semin. Oncol.* **37,** 508–516 (2010).
69. Graziano, D. F. & Finn, O. J. Tumor antigens and tumor antigen discovery. *Cancer Treat. Res.* **123,** 89–111 (2005).
70. Kirkwood, J. M. *et al.* Immunotherapy of cancer in 2012. *CA Cancer J Clin* **62,** 309–335 (2012).
71. Piccart-Gebhart, M. J. *et al.* Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N. Engl. J. Med.* **353,** 1659–1672 (2005).
72. Ferrara, N., Hillan, K. J. & Novotny, W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem. Biophys. Res. Commun.* **333,** 328–335 (2005).
73. Ferrara, N., Hillan, K. J., Gerber, H.-P. & Novotny, W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* **3,** 391–400 (2004).
74. Bogusz, J. *et al.* Mechanisms of action of the anti-VEGF monoclonal antibody bevacizumab on chronic lymphocytic leukemia cells. *Postepy Hig Med Dosw (Online)* **67,** 107–118 (2013).
75. Mendelsohn, J. Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy. *Clin. Cancer Res.* **3,** 2703–2707 (1997).
76. Neal, J. W. *et al.* Cetuximab monotherapy in patients with advanced non-small cell lung cancer after prior epidermal growth factor receptor tyrosine kinase inhibitor therapy. *J Thorac Oncol* **5,** 1855–1858 (2010).
77. Odunsi, K. *et al.* NY-ESO-1 and LAGE-1 cancer-testis antigens are potential targets for immunotherapy in epithelial ovarian cancer. *Cancer Res.* **63,** 6076–6083 (2003).
78. Odunsi, K. *et al.* Efficacy of vaccination with recombinant vaccinia and fowlpox vectors expressing NY-ESO-1 antigen in ovarian cancer and melanoma patients. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 5797–5802 (2012).
79. Jäger, E. *et al.* Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 14453–14458 (2006).
80. Gnjatic, S. *et al.* NY-ESO-1: review of an immunogenic tumor antigen. *Adv. Cancer Res.* **95,** 1–30 (2006).
81. Silva, W. A. *et al.* PLAC1, a trophoblast-specific cell surface protein, is expressed in a range of human tumors and elicits spontaneous antibody responses. *Cancer Immun.* **7,** 18 (2007).
82. Koslowski, M. *et al.* A placenta-specific gene ectopically activated in many human cancers is essentially involved in malignant cell processes. *Cancer Res.* **67,** 9528–9534 (2007).

83. Fant, M. *et al.* PLAC1, a trophoblast-specific gene, is expressed throughout pregnancy in the human placenta and modulated by keratinocyte growth factor. *Mol. Reprod. Dev.* **63,** 430–436 (2002).
84. Jackman, S. M., Kong, X. & Fant, M. E. Plac1 (placenta-specific 1) is essential for normal placental and embryonic development. *Mol. Reprod. Dev.* **79,** 564–572 (2012).
85. Liu, W. *et al.* Identification of a novel HLA-A2-restricted cytotoxic T lymphocyte epitope from cancer-testis antigen PLAC1 in breast cancer. *Amino Acids* **42,** 2257–2265 (2011).
86. Liu, F. *et al.* Identification of two new HLA-A*0201-restricted cytotoxic T lymphocyte epitopes from colorectal carcinoma-associated antigen PLAC1/CP1. *J. Gastroenterol.* (2013). doi:10.1007/s00535-013-0811-4
87. Liu, F.-F. *et al.* The specific immune response to tumor antigen CP1 and its correlation with improved survival in colon cancer patients. *Gastroenterology* **134,** 998–1006 (2008).
88. Dong, X.-Y. *et al.* Plac1 is a tumor-specific antigen capable of eliciting spontaneous antibody responses in human cancer patients. *Int. J. Cancer* **122,** 2038–2043 (2008).
89. Kawakami, Y., Robbins, P. F. & Rosenberg, S. A. Human melanoma antigens recognized by T lymphocytes. *Keio J Med* **45,** 100–108 (1996).
90. Wagner, S. N., Wagner, C., Schultewolter, T. & Goos, M. Analysis of Pmel17/gp100 expression in primary human tissue specimens: implications for melanoma immuno- and gene-therapy. *Cancer Immunol. Immunother.* **44,** 239–247 (1997).
91. Brouwenstijn, N. *et al.* Transcription of the gene encoding melanoma-associated antigen gp100 in tissues and cell lines other than those of the melanocytic lineage. *Br. J. Cancer* **76,** 1562–1566 (1997).
92. Hailemichael, Y. *et al.* Persistent antigen at vaccination sites induces tumor-specific $CD8^+$ T cell sequestration, dysfunction and deletion. *Nat. Med.* **19,** 465–472 (2013).
93. Filipazzi, P. *et al.* Limited induction of tumor cross-reactive T cells without a measurable clinical benefit in early melanoma patients vaccinated with human leukocyte antigen class I-modified peptides. *Clin. Cancer Res.* **18,** 6485–6496 (2012).
94. Oshita, C. *et al.* Dendritic cell-based vaccination in metastatic melanoma patients: phase II clinical trial. *Oncol. Rep.* **28,** 1131–1138 (2012).
95. Lesterhuis, W. J. *et al.* Wild-type and modified gp100 peptide-pulsed dendritic cell vaccination of advanced melanoma patients can lead to long-term clinical responses independent of the peptide used. *Cancer Immunol. Immunother.* **60,** 249–260 (2011).

96. Phan, G. Q. *et al.* Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 8372–8377 (2003).
97. Weber, J. S. *et al.* Patterns of onset and resolution of immune-related adverse events of special interest with ipilimumab: detailed safety analysis from a phase 3 trial in patients with advanced melanoma. *Cancer* **119,** 1675–1682 (2013).
98. Leitch, J. *et al.* CTL-dependent and -independent antitumor immunity is determined by the tumor not the vaccine. *J. Immunol.* **172,** 5200–5205 (2004).
99. Hammill, A. M., Conner, J. & Cripe, T. P. Oncolytic virotherapy reaches adolescence. *Pediatr Blood Cancer* **55,** 1253–1263 (2010).
100. Russell, S. J., Peng, K.-W. & Bell, J. C. Oncolytic virotherapy. *Nat. Biotechnol.* **30,** 658–670 (2012).
101. Msaouel, P., Opyrchal, M., Domingo Musibay, E. & Galanis, E. Oncolytic measles virus strains as novel anticancer agents. *Expert Opin Biol Ther* **13,** 483–502 (2013).
102. Sinkovics, J. G. & Horvath, J. C. Newcastle disease virus (NDV): brief history of its oncolytic strains. *J. Clin. Virol.* **16,** 1–15 (2000).
103. Laurie, S. A. *et al.* A phase 1 clinical study of intravenous administration of PV701, an oncolytic virus, using two-step desensitization. *Clin. Cancer Res.* **12,** 2555–2562 (2006).
104. Jiang, H., Gomez-Manzano, C., Lang, F. F., Alemany, R. & Fueyo, J. Oncolytic adenovirus: preclinical and clinical studies in patients with human malignant gliomas. *Curr Gene Ther* **9,** 422–427 (2009).
105. Dix, B. R., Edwards, S. J. & Braithwaite, A. W. Does the antitumor adenovirus ONYX-015/dl1520 selectively target cells defective in the p53 pathway? *J. Virol.* **75,** 5443–5447 (2001).
106. Ganly, I. *et al.* A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin. Cancer Res.* **6,** 798–806 (2000).
107. Prestwich, R. J. *et al.* Tumor infection by oncolytic reovirus primes adaptive antitumor immunity. *Clin. Cancer Res.* **14,** 7358–7366 (2008).
108. Kaufman, H. L. *et al.* Local and distant immunity induced by intralesional vaccination with an oncolytic herpes virus encoding GM-CSF in patients with stage IIIc and IV melanoma. *Ann. Surg. Oncol.* **17,** 718–730 (2010).
109. Cramer, D. W. *et al.* Mumps and ovarian cancer: modern interpretation of an historic association. *Cancer Causes Control* **21,** 1193–1201 (2010).
110. Diaz, R. M. *et al.* Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. *Cancer Res.* **67,** 2840–2848 (2007).

111. Chuang, C.-M., Monie, A., Wu, A., Pai, S. I. & Hung, C.-F. Combination of viral oncolysis and tumor-specific immunity to control established tumors. *Clin. Cancer Res.* **15,** 4581–4588 (2009).
112. Vigil, A., Martinez, O., Chua, M. A. & García-Sastre, A. Recombinant Newcastle disease virus as a vaccine vector for cancer therapy. *Mol. Ther.* **16,** 1883–1890 (2008).
113. Bridle, B. W. *et al.* Potentiating cancer immunotherapy using an oncolytic virus. *Mol. Ther.* **18,** 1430–1439 (2010).
114. Stojdl, D. F. *et al.* Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat. Med.* **6,** 821–825 (2000).
115. Mire, C. E. *et al.* Recombinant vesicular stomatitis virus vaccine vectors expressing filovirus glycoproteins lack neurovirulence in nonhuman primates. *PLoS Negl Trop Dis* **6,** e1567 (2012).
116. Schwartz, J. A. *et al.* Vesicular stomatitis virus-based H5N1 avian influenza vaccines induce potent cross-clade neutralizing antibodies in rhesus macaques. *J. Virol.* **85,** 4602–4605 (2011).
117. Parks, R. J. Improvements in adenoviral vector technology: overcoming barriers for gene therapy. *Clin. Genet.* **58,** 1–11 (2000).
118. Vemula, S. V. & Mittal, S. K. Production of adenovirus vectors and their use as a delivery system for influenza vaccines. *Expert Opin Biol Ther* **10,** 1469–1487 (2010).
119. Lasaro, M. O. & Ertl, H. C. J. New insights on adenovirus as vaccine vectors. *Mol. Ther.* **17,** 1333–1339 (2009).
120. Bett, A. J., Haddara, W., Prevec, L. & Graham, F. L. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 8802–8806 (1994).
121. Lawson, N. D., Stillman, E. A., Whitt, M. A. & Rose, J. K. Recombinant vesicular stomatitis viruses from DNA. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 4477–4481 (1995).
122. Ng, P. *et al.* A high-efficiency Cre/loxP-based system for construction of adenoviral vectors. *Hum. Gene Ther.* **10,** 2667–2672 (1999).
123. Patel, M. R. & Kratzke, R. A. Oncolytic virus therapy for cancer: the first wave of translational clinical trials. *Transl Res* **161,** 355–364 (2013).
124. Bridle, B. W., Hanson, S. & Lichty, B. D. Combining oncolytic virotherapy and tumour vaccination. *Cytokine Growth Factor Rev.* **21,** 143–148 (2010).
125. Wan, Y., Bramson, J., Carter, R., Graham, F. & Gauldie, J. Dendritic cells transduced with an adenoviral vector encoding a model tumor-associated antigen for tumor vaccination. *Hum. Gene Ther.* **8,** 1355–1363 (1997).
126. Fant, M., Barerra-Saldana, H., Dubinsky, W., Poindexter, B. & Bick, R. The PLAC1 protein localizes to membranous compartments in the apical region of the syncytiotrophoblast. *Mol. Reprod. Dev.* **74,** 922–929 (2007).

127. Bridle, B. W. *et al.* Vesicular stomatitis virus as a novel cancer vaccine vector to prime antitumor immunity amenable to rapid boosting with adenovirus. *Mol. Ther.* **17,** 1814–1821 (2009).
128. Kianizad, K. *et al.* Elevated frequencies of self-reactive CD8+ T cells following immunization with a xenoantigen are due to the presence of a heteroclitic CD4+ T-cell helper epitope. *Cancer Res.* **67,** 6459–6467 (2007).
129. Sorensen, M. R., Holst, P. J., Pircher, H., Christensen, J. P. & Thomsen, A. R. Vaccination with an adenoviral vector encoding the tumor antigen directly linked to invariant chain induces potent CD4(+) T-cell-independent CD8(+) T-cell-mediated tumor control. *Eur. J. Immunol.* **39,** 2725–2736 (2009).
130. Holst, P. J. *et al.* MHC class II-associated invariant chain linkage of antigen dramatically improves cell-mediated immunity induced by adenovirus vaccines. *J. Immunol.* **180,** 3339–3346 (2008).
131. Lutsiak, M. E. C. *et al.* Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood* **105,** 2862–2868 (2005).
132. Sioud, M. & Sørensen, D. Generation of an effective anti-tumor immunity after immunization with xenogeneic antigens. *Eur. J. Immunol.* **33,** 38–45 (2003).
133. Hu, B. *et al.* Human T lymphocyte responses against lung cancer induced by recombinant truncated mouse EGFR. *Cancer Immunol. Immunother.* **55,** 386–393 (2006).
134. Johnson, L. E., Frye, T. P. & McNeel, D. G. Immunization with a prostate cancer xenoantigen elicits a xenoantigen epitope-specific T-cell response. *Oncoimmunology* **1,** 1546–1556 (2012).
135. Frelin, L. *et al.* Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus nonstructural 3/4A gene. *Gene Ther.* **11,** 522–533 (2004).
136. Shin, T. H. *et al.* Induction of robust cellular immunity against HPV6 and HPV11 in mice by DNA vaccine encoding for E6/E7 antigen. *Hum Vaccin Immunother* **8,** 470–478 (2012).
137. Rosenberg, S. A. *et al.* Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. *J. Immunol.* **175,** 6169–6176 (2005).
138. Wongthida, P. *et al.* Activating systemic T-cell immunity against self tumor antigens to support oncolytic virotherapy with vesicular stomatitis virus. *Hum. Gene Ther.* **22,** 1343–1353 (2011).
139. Nguyên, T. L.-A. *et al.* Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 14981–14986 (2008).

140. Bridle, B. W. *et al.* HDAC inhibition suppresses primary immune responses, enhances secondary immune responses, and abrogates autoimmunity during tumor immunotherapy. *Mol. Ther.* **21,** 887–894 (2013).
141. Bronte, V. *et al.* Genetic vaccination with 'self' tyrosinase-related protein 2 causes melanoma eradication but not vitiligo. *Cancer Res.* **60,** 253–258 (2000).
142. Bridle, B. W. *et al.* Immunotherapy can reject intracranial tumor cells without damaging the brain despite sharing the target antigen. *J. Immunol.* **184,** 4269–4275 (2010).
143. McGray, A. J. R. *et al.* Combined vaccination and immunostimulatory antibodies provides durable cure of murine melanoma and induces transcriptional changes associated with positive outcome in human melanoma patients. *Oncoimmunology* **1,** 419–431 (2012).
144. Kaluza, K. M. *et al.* Adoptive transfer of cytotoxic T lymphocytes targeting two different antigens limits antigen loss and tumor escape. *Hum. Gene Ther.* **23,** 1054–1064 (2012).
145. Nesslinger, N. J. *et al.* A viral vaccine encoding prostate-specific antigen induces antigen spreading to a common set of self-proteins in prostate cancer patients. *Clin. Cancer Res.* **16,** 4046–4056 (2010).
146. Corbière, V. *et al.* Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. *Cancer Res.* **71,** 1253–1262 (2011).
147. Nobuoka, D. *et al.* Intratumoral peptide injection enhances tumor cell antigenicity recognized by cytotoxic T lymphocytes: a potential option for improvement in antigen-specific cancer immunotherapy. *Cancer Immunol. Immunother.* **62,** 639–652 (2013).
148. Zeh, H. J., Perry-Lalley, D., Dudley, M. E., Rosenberg, S. A. & Yang, J. C. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *J. Immunol.* **162,** 989–994 (1999).
149. Li, N., Wang, T. & Han, D. Structural, cellular and molecular aspects of immune privilege in the testis. *Front Immunol* **3,** 152 (2012).
150. Luznik, L., O'Donnell, P. V. & Fuchs, E. J. Post-transplantation cyclophosphamide for tolerance induction in HLA-haploidentical bone marrow transplantation. *Semin. Oncol.* **39,** 683–693 (2012).
151. Pavelic, V., Matter, M. S., Mumprecht, S., Breyer, I. & Ochsenbein, A. F. CTL induction by cross-priming is restricted to immunodominant epitopes. *Eur. J. Immunol.* **39,** 704–716 (2009).
152. Coffman, R. L., Sher, A. & Seder, R. A. Vaccine adjuvants: putting innate immunity to work. *Immunity* **33,** 492–503 (2010).
153. Matteo, M. *et al.* Preliminary evidence for high anti-PLAC1 antibody levels in infertile patients with repeated unexplained implantation failure. *Placenta* **34,** 335–339 (2013).

154. Robert, C. *et al.* Efficacy and safety of retreatment with ipilimumab in patients with pretreated advanced melanoma who progressed after initially achieving disease control. *Clin. Cancer Res.* **19,** 2232–2239 (2013).
155. White, H. D., Roeder, D. A. & Green, W. R. An immunodominant Kb-restricted peptide from the p15E transmembrane protein of endogenous ecotropic murine leukemia virus (MuLV) AKR623 that restores susceptibility of a tumor line to anti-AKR/Gross MuLV cytotoxic T lymphocytes. *J. Virol.* **68,** 897–904 (1994).
156. Lemay, C. G. *et al.* Harnessing oncolytic virus-mediated antitumor immunity in an infected cell vaccine. *Mol. Ther.* **20,** 1791–1799 (2012).