REDIRECTED T CELL ACTIVITY BY HIGH AFFINITY TCR-ANTI-CD3 BISPECIFIC CANDIDATE THERAPEUTICS

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ABSTRACT

T cell antigen receptors (TCRs) on $CD8^+$ T cells recognise endogenously processed peptides bound to major histocompatibility complex (pMHC) antigens presented on the cell surface on almost all types of cells in the body, including tumour cells. The majority of tumour-associated peptide antigens (TAPAs) are derived from non-mutated self-proteins and are therefore subject to immunological tolerance, mainly through negative selection of high avidity T cells in the thymus. In addition, there is low presentation of pMHC on the surface of cancer cells. As a result, T cell responses tend to be weak and ineffective at killing tumour cells. ImmTACs (**I**mmune **m**obilising **m**onoclonal **T** cell receptors **A**gainst **C**ancer) are bispecific soluble biologics comprising a soluble TCR with an enhanced affinity for tumour-associated pMHCI fused to a humanised anti-CD3 single-chain antibody fragment (scFv) which redirect and activate T cells to lyse tumour cells. In this study, the potency, sensitivity, and specificity of ImmTACs was investigated for pMHCI epitopes derived from four tumour associated antigens (TAAs): (1) gp100, (2) MAGE-A3, (3) Melan-A/MART-1, and (4) NY-ESO-1/LAGE-1. A comprehensive range of assays and methodologies have been established to characterise the ImmTAC reagents. Cytokine release assays such as IFN-γ and Granzyme B ELISpot were used to evaluate the specificity and biological activity of ImmTACs. In concentration-response experiments, all four ImmTACs produced EC_{50} values in the range of 100 picomolar or lower demonstrating a high degree of sensitivity despite low epitope numbers. Killing assays, including LDH-release for assessing short-term lysis and IncuCyte technology to visualise long-term killing kinetics in real time, show that redirected T cells potently kill their targets. Furthermore, *in vitro* screening against a panel of antigen negative, primary human cell lines have shown that ImmTACs are highly specific and only activate T cells against target cells presenting their cognate pMHC. The potency of ImmTACs was also investigated using tumour infiltrating lymphocytes (TILs) extracted from tumour specimens and with tumour-derived cancer cells as targets. An HLA-A2, gp100 specific ImmTAC has received phase I clinical trial regulatory approval in the UK and in the US on the basis of this *in vitro* data, which has been used to determine *minimal anticipated biological effect level* (MABEL). The clinical trial is currently in progress.

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ABBREVIATION LIST

General

AE	Adverse event
Apaf-1	Apoptotic protease activating factor 1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
β_2m	β_2-microglobulin
BiTE	Bispecific T cell engager
C-terminus	Carboxyl-terminal end of a protein
CAD	Caspase-activated DNAse
CD (number)	Cluster of differentiation (number)
CDR	Complementarity determining regions
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DMSO	Dimethylsulphoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
EBV	Epstein-Barr virus
EC_{50}	Half maximal effective concentration
EDTA	Ethylenedinitrilotetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
ER	Endoplasmic reticulum
E:T	Effector to target (ratio)
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FDA	Food and drug administration (US)
FIH	First-in-human
Gp100	Glycoprotein 100
HLA	Human leucocyte antigen

ICAD	Inhibitor of CAD
ICAM-1	Intercellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
IL	Interleukin
ImmTAC	Immune mobilising monoclonal T cell receptors against cancer
ITAM	Immunoglobulin receptor family tyrosine based activation motif
K_D	Dissociation constant
kD	Kilo dalton
LAT	Linker for activation of T cells
Lck	Lymphocyte specific protein tyrosine kinase
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
mAb	Monoclonal antibody
MABEL	Minimal anticipated biological effect level
MAGE	Melanoma-associated antigen
MART-1/Melan-A	Melanoma antigen recognised by T cells 1
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MHRA	Medicines and healthcare products regulatory agency
MIL	Marrow infiltrating lymphocyte
mTCR	Monoclonal T cell receptor
MTD	Maximum tolerated dose
N-terminus	Amino-terminal end of a protein
NK	Natural killer (cells)
NOAEL	No observed adverse effect level
NSCLC	Non-small cell lung carcinoma
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohaematogglutinin
pMHC	peptide- Major histocompatibility complex
PTK	Protein kinase C
RLU	Relative light unit

ScFv	Single chain antibody fragment
SEM	Standard error of the mean
SFC	Spot forming cell
SH	Src homology
SMAC	Supramolecular activation clusters
TAA	Tumour-associated antigen
TAP	Transporter associated with antigen processing
TAPA	Tumour-associated peptide antigen
TCR	T cell receptor
Tcm	Central memory T (cells)
Tem	Effector memory T (cells)
Th	Helper T (cells)
Treg	Regulatory T (cells)
TGF-β	Transforming growth factor-beta
TIL	Tumour infiltrating lymphocyte
TLD	Target limiting dose
TNF	Tumour necrosis factor
ZAP-70	Zeta-associated protein of 70 kD

Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine

R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

CHAPTER 1 INTRODUCTION

1.1 The immune system and cancer therapy: General introduction

Cancer remains a largely intractable disease and is a leading cause of death worldwide (GLOBOCAN 2008 http://globocan.iarc.fr/). Until recently, research into the nature of cancer has mainly focused on the cancer cell and on cancer as a genetic disease, as illustrated by the landmark review by Hanahan and Weinburg, published in 2000 (Hanahan & Weinberg, 2000). They proposed six characteristics (hallmarks) of cancer: the capacity to sustain proliferative signalling, to resist cell death, to induce angiogenesis, to enable replicative immortality, to activate invasion and metastasis, and to avoid growth suppressors. However, a decade later it has become evident that cancer is not one disease, but many different diseases. Thus, the focus of attention has shifted from the cancer cell to that of the host, and in particular, the microenvironment in which the cancer grows, a critical component of which is the immune system. A new picture of cancer is now emerging and in 2011, four additional hallmarks were proposed. Two of these hallmarks emphasise the newly recognised dual interaction between cancer and the immune system: firstly, the ability of cancer cells to avoid immune destruction, and secondly, tumour-associated inflammatory responses that promotes tumour growth rather than elimination (Hanahan & Weinberg, 2011).

Standard therapies for cancer include small molecule drugs which are able to target exaggerated functions that allow cancer cells to grow in an uncontrolled manner, such as cell dividing components that are essential for cell cycle, or the evasive growth of cells. Although these drugs have anti-cancer activity, in most cases they have limited efficacy and are associated with severe toxicity because such functions are also features

of normal cells, albeit for most types of cells to a lesser extent. On the other hand, the immune system has the greatest potential for the specific eradication of tumours with no toxicity to normal tissue, as well as the development of immune memory that can prevent cancer recurrence. The immune system can recognise and reject tumours through a process called immunosurveillance. Tumour specificity of the immune response resides in the recognition of tumour antigens by cells of the adaptive immune system, principally, $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells. However, most tumour antigens are 'self-proteins' to which the immune system has limited responsiveness, due to the development of tolerance by clonal deletion or anergy. Furthermore, in many cancers and particularly those forming solid tumours, malignant progression is accompanied by profound immune suppression that hinders an effective anti-tumour response and tumour destruction.

The main premise of cancer immunotherapy is to stimulate or replenish the anti-tumour elements of the patient's immune system. However, two important barriers must be overcome in order for a cancer immunotherapeutic to be effective: namely self-tolerance and tumour subversion of the immune response. The mainstay of immunotherapy has been the use of vaccines but they have had limited success, most likely because they are unable to effectively overcome the obstacles of tolerance and the immune-suppressive tumour microenvironment. Anti-cancer monoclonal antibodies (mAbs), which target specific antigens expressed by cancer cells, are a well-established class of immunotherapeutic agent, apparently with higher therapeutic potential than vaccines. Several Food and Drug Administration (FDA) approved mAbs are now standard treatment for some types of cancer, including trastuzumab for the treatment of breast cancer and rituximab for the treatment of B cell lymphoma. Although transient remissions do occur in patients treated with these drugs, cure rates still remain low.

Some of the most exciting developments in cancer immunotherapy are those approaches that harness the most potent anti-tumour cells of the immune system i.e., $CD8^+$ effector T cells, also known as cytotoxic T lymphocytes (CTLs). Although CTLs have the potential to recognise and destroy malignant cells, there are a number of inhibitory pathways (immune check-points) hard-wired into the immune system that are crucial for the maintenance of self-tolerance in order to prevent autoimmunity. Furthermore, tumours are able to adopt certain immune-checkpoint pathways as a major mechanism of immune resistance, particularly against T cells that are specific for tumour antigens. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is a key immune check-point that attenuates T cell activation. Ipilimumab is a monoclonal antibody to CTLA-4 and is the first agent to demonstrate a survival benefit in patients with advanced melanoma; Ipilimumab was approved by the FDA in March 2011 (Hodi et al., 2010). By blocking CTLA-4, Ipilimumab dampens the inhibitory signals that curtail the full power of CTLs to destroy cancer cells. Blockers of other immune-checkpoint proteins, such as programmed cell death protein-1 (PD-1) are also showing promising anti-tumour effects in early clinical trials.

Another very effective approach for harnessing the anti-tumour potential of T cells is bispecific antibodies. Bispecifics are a novel class of antibodies being developed to recruit pre-existing polyclonal T cell clones for the destruction of tumour cells. Bispecific antibodies are equipped with two non-identical binding arms; one arm engages CD3, a potent signalling protein on the surface of T cells, whilst the other arm binds to an antigen on the tumour cell surface. In B cell lymphoma and leukaemia, the bispecific T cell engager (BiTE) antibodies, developed by MicroMet, have demonstrated impressive clinical results (Nagorsen & Baeuerle, 2011).

Bispecifics as a way of re-directing T cell activity could make a significant contribution in the future to the immunotherapy of cancer. However, the main disadvantage of antibody-based therapies is that they are limited in scope to membrane protein targets and therefore mainly restricted to tissue-specific or lineage-expressed antigens. T cell receptors (TCRs) on the other hand specifically recognise endogenously processed peptides bound to major histocompatibility complex (pMHC) antigens presented on the cell surface. These peptides are derived from proteins in all cellular compartments (Figure 1-1). Thus, this class of antigen provides the most comprehensive range of therapeutic targets, particularly in cancer, as tumour-associated peptide antigens (TAPAs) often provide one of the few distinguishing features on the surface of malignantly transformed cells. A major limitation to TCR-based therapeutic approaches is that TCRs have a natural affinity to pMHC that are several orders of magnitude weaker than mAb binding to protein antigens. However, recent advances have enabled the engineering and production of soluble monoclonal TCRs (mTCRs) that target defined pMHC class I antigens with vastly improved affinities. Furthermore, the improvement in affinity does not appear to have compromised the specificity of the TCR (Liddy et al., 2012). Nevertheless, a significant challenge facing high affinity mTCR-anti-CD3 bispecifics is whether they can work against low numbers of TAPAs per cell which are frequently down-regulated as a mechanism of immune evasion by tumours.

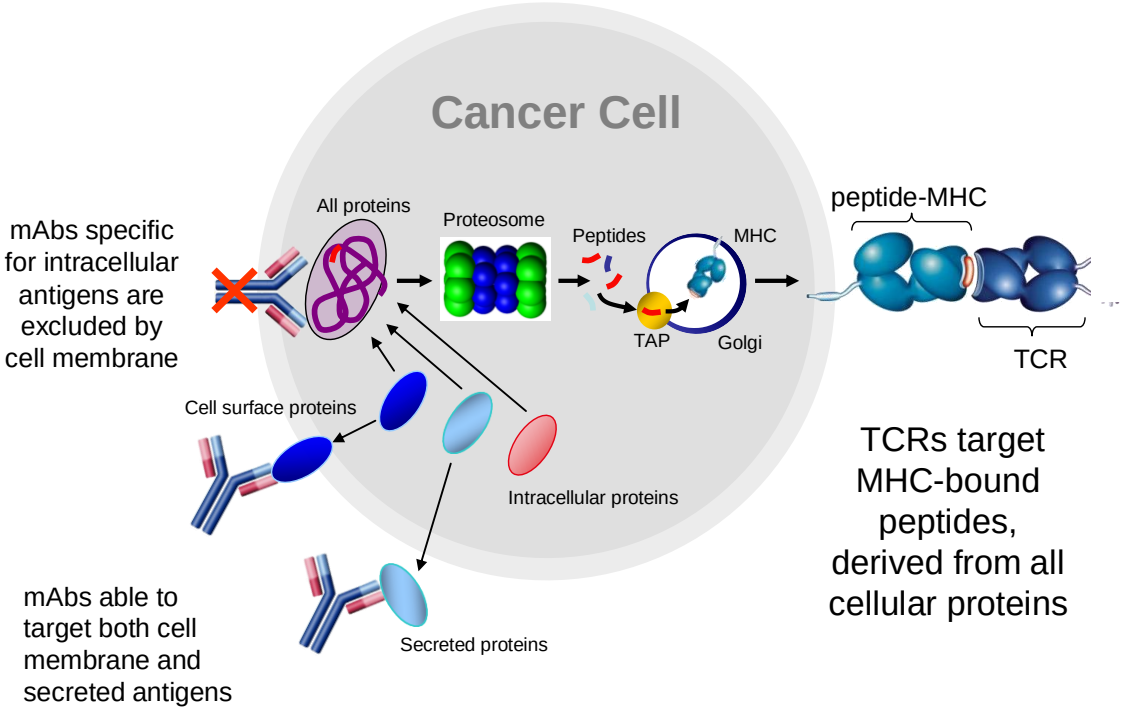


Figure 1-1 T cell receptors (TCRs) as potential targeting molecules.

TCRs bind to antigenic peptides derived from all proteins within a living cell and presented on the cell surface in the context of the major histocompatibility complex (peptide-MHC). In this respect, TCRs have an advantage over antibodies, as the latter are unable to target intracellular proteins.

1.2 Cancer

1.2.1 Cancer and cancer cells

Cancer is a term broadly used to describe a spectrum of diseases in which abnormal cells divide without control and are able to invade other tissues. The development of genomic instability is the most defining feature of cancer cells and lies at the core of neoplastic formation (Knudson, 2001; Maser & DePinho, 2002; Murga & Fernandez-Capetillo, 2007). Genomic destabilisation occurs early on in the evolution of a tumour and is a result of either increased rates of DNA damage, which overwhelm the ability of normal repair mechanisms to restore genomic integrity, or defective repair mechanisms that are unable to repair normal rates of DNA damage (Anderson et al., 2001). Disruption of genomic integrity is manifested as DNA defects, for example, in mitotic checkpoints, impaired non-homologous end joining and imprecise replication (Hoeijmakers, 2001; Lengauer et al., 1998). It is the cumulative effect of all these factors which gives rise to the phenotype of cancer cells. Genomic destabilisation and the high rate of mutations generate genetic diversity in malignant cell populations which drives tumour progression as certain mutant genotypes confer a selective advantage on sub-clones of cells. This enables their outgrowth in the local tissue environment and, eventually, some sub-types acquire the characteristics necessary for invasive growth and for the cancer cells to spread, through the process of metatasis, to other tissues via the blood and lymph system (Hanahan & Weinberg, 2000). Recent evidence indicates that many types of tumours contain a particular sub-population, referred to as cancer stem cells (also called tumour-initiating cells), which, in contrast to the bulk of tumour cells, have the capacity for re-growth and, crucially, formation of new growth from a single cell, i.e., metastasis (reviewed in (Baccelli & Trumpp, 2012)). The heterogeneity of tumours, which not only consist of the original clone and sub-clones, possibly including

cancer stem cells, means that these populations can differ in sensitivity to chemotherapy, radiotherapy, and other treatments, making clinical management challenging (Croce, 2008).

1.2.2 Types of cancer

Cancer includes many types of malignant disease, generally named by the organ or cell type from which the malignancy originates, such as, hepatomas, cancers of liver cells or melanomas, cancers of pigment-producing melanocytes. There are more than one hundred types of cancer which are grouped into broader categories. The five main categories of cancer are: (1) Carcinoma, which begins in the skin or in tissues that line or cover internal organs; (2) Sarcoma, which originates in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue; (3) Leukemia, which starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood; (4) Lymphoma and myeloma, which are cancers that begin in the cells of the immune system; (5) Central nervous system cancers, which originate in the tissues of the brain and spinal cord (for classification of cancers, see National Cancer Institute website: http://www.cancer.gov).

The experiments of this thesis have been particularly focused on malignant melanoma and multiple myeloma.

1.2.2.1 Melanoma

Melanoma is a form of skin cancer that accounts for less than 5% of cases but causes the vast majority of skin cancer deaths which currently affects approximately 120,000 new patients per year in the western world. It is more common in women than men, and

it is particularly common in Caucasians, especially of north-west European extraction living in sunny climates. In 2010, 12,818 people in the UK were diagnosed with melanoma, and in the same year, there were 2,203 deaths from the disease. Incidence rates for melanoma have increased in the last thirty years and, unlike other common cancers, melanoma has a wide age distribution. (GLOBOCAN 2008 http://globocan.iarc.fr/, Cancer Research UK, http://info.cancerresearchuk.org/). Early stage melanoma is treatable with surgical resection, but the prognosis of advanced, metastatic melanoma remains very poor. Advanced melanoma is still associated with an extremely poor median survival, ranging from 2 to 8 months, with only 5% of patients surviving more than five years, and remains one of the most treatment-refractory malignancies. Many agents have been investigated for anti-tumour activity in melanoma but the current treatment options for patients with metastatic disease are limited and do not offer a cure in the vast majority of cases. The chemotherapeutic agent dacarbazine (a DNA alkylating agent; also known as Imidazole) represents the most common option as the standard first-line treatment for metastatic melanoma.

The importance of the immune system in controlling melanoma has been recognised for decades; there have even been case reports of spontaneous tumour regression in patients with metastatic melanoma (Bulkley et al., 1975). This has led to intensive studies of immune-based treatment strategies with biologic response modifiers, i.e. cytokines such as interleukin-2 (IL-2) and interferon-α (IFN-α). However, the outcome of these studies showed that the results with systemically administered immunotherapy are not better than those with chemotherapy and there are associated problems with toxicity. Studies combining immunotherapy and chemotherapy have also shown limited response rates (Mouawad et al., 2010).

Recently, targeted treatment approaches are looking more promising. In March 2011 the FDA approved the use of ipilimumab (Yervoy), a fully human monoclonal antibody against CTLA-4, for the treatment of unresectable, or metastatic, melanoma. Ipilimumab is designed to block the inhibitory molecule CTLA-4, which is expressed on T cells, thereby enhancing T cell activation, leading to increased anti-tumour responses. Significantly, ipilimumab is the first agent that has demonstrated to improve overall survival in patients with metastatic melanoma in randomised Phase III clinical trials (Graziani et al., 2012). This suggests that targeted immunotherapies could be the way forward in modulating anti-tumour immune responses that have the potential to produce durable clinical outcomes.

Other targeted drugs that have shown positive outcomes include small molecule kinase inhibitors. The discovery that melanomas in approximately 50% of patients harbour activating mutations in the serine-threonine BRAF kinase has prompted an intense search for compounds to inhibit BRAF activity. Sarafenib (Nexavar®), a broad-spectrum kinase inhibitor, was the first clinical drug candidate but results were disappointing (Vultur et al., 2010). Greater success has been seen with more selective inhibitors. The treatment of metastatic melanoma harbouring $BRAF^{V600}$ mutation with the drug vemurafenib (Zelboraf) resulted in complete or partial tumour regression in the majority of patients. Vemurafenib has recently been approved as a monotherapy for the treatment of patients with $BRAF^{V600}$ mutation positive unresectable or metastatic melanoma. However, there is a concern with the use of vemurafenib, which will probably apply to all other BRAF inhibitors, and that is the development of resistance by responsive tumours. The heterogeneous nature of tumours means that not all cells within the same tumour may get killed, therefore allowing drug-resistant sub-populations to eventually overtake non-drug resistant populations; alternatively, it could

be that all tumour cells respond to the compound but not to the same extent, allowing some cells to survive while they 'rewire' for growth (Flaherty et al., 2010).

Although ipilimumab and vemurafenib have revolutionised the treatment of malignant melanoma, they have only produced modest improvements in overall survival and, for the majority of patients, neither constitutes a cure for advanced disease. Thus, there remains a clear unmet medical need for new treatment options for malignant melanoma.

1.2.2.2 Multiple myeloma

Multiple myeloma is a cancer of the blood and occurs when plasma cells, which are produced in the bone marrow, become cancerous and proliferate uncontrollably. Healthy plasma cells eventually become crowded out which interferes with the production of other blood cells as well as those involved in regenerating bone. The malignant plasma cells continue to produce antibodies but these are abnormal, monoclonal immunoglobulins and offer no protection against infection (Graham-Rowe, 2011). The bone marrow immune microenvironment is also known to play an important role in the pathobiology of myeloma. Complex interactions between myeloma tumour cells and cells of the bone marrow, such as stromal cells, osteoclasts, osteoblasts, myeloid and lymphoid cells, leads to immune evasion and disease progression (Noonan & Borrello, 2011).

Multiple myeloma is the second most frequent malignancy of the blood (after non-Hodgkins lymphoma), comprising around 1% of all cancers and approximately 10% of haematological malignancies (Rajkumar, 2012). The incidence of multiple myeloma worldwide is 1 to 5 individuals per 100,000 per year, with a higher incidence in the Western world (Parkin et al., 2005). The disease is diagnosed in more men than

women with a prevalence rate in African Americans twice as high as that found in Caucasians (Landgren & Weiss, 2009). The median age at diagnosis is between 63 and 70 years old and the mortality rate is approximately 4 individuals per 100,000 per year (Dimopoulos & Terpos, 2010). In the majority of cases, multiple myeloma is incurable; the best treatments available up until the late 1990s have been chemotherapies, including melphalan (a DNA alkylating agent) and prednisone (a prodrug form of corticosteroid), which gave a life expectancy of three to four years. More recently, the availability of stem cell transplants and the introduction of lenalidomide (an analogue of thalidomide with a complex mode of actions, including direct tumour toxicity, inhibition of the tumour microenvironment, and immuno-modulation) and bortezomib (a proteasome inhibitor) have doubled life expectancy to 7-8 years. However, despite a positive response seen to these new drugs, studies show that once patients relapse, the cancer becomes resistant to the drugs and the long term prognosis of patients remains poor (Graham-Rowe, 2011).

One of the most effective treatments for multiple myeloma is Allogeneic Stem Cell Transplantation (Allo-SCT). Although Allo-SCT is a risky procedure with a high treatment-related mortality, it can induce prolonged periods of disease free survival, and in some incidences, cure patients (Bensinger et al., 1996; Corradini et al., 1999; Gahrton et al., 1991; Gahrton et al., 1995). Furthermore, the success of Allo-SCT suggests that multiple myeloma tumour cells are susceptible to immune recognition (Blade et al., 2010). More recently, autologous stem cell transplants (ASCT) have become the preferred treatment for multiple myeloma because, although slightly less potent against the cancer, it is not associated with the same level of risk for the patient. The efficaciousness of both types of transplantation provides a good rationale for developing

further immunotherapies, possibly with curative potential, for patients diagnosed with multiple myeloma.

1.3 T cell antigens in cancer

The genomic instability of cancer cells gives rise to numerous mutated and aberrant proteins and peptides that deviate from self and therefore can be targets of the immune system. Clinical observations such as the increased rate of tumour formation in immune-compromised individuals (Buell et al., 2005) and the spontaneous, albeit rare, regressions of tumours (Baldo et al., 1992) indicate the presence of anti-tumour activity in the human immune system. Of the different cellular immune effectors involved in anti-tumour immunity, cytotoxic $CD8^+$ T lymphocytes (CTLs) play a key role due to their ability to specifically and effectively kill tumour cells via the recognition of tumour antigens presented on the cell surface of malignant cells. In the early 1990s, ground-breaking studies by Boon and colleagues, and Rosenberg and colleagues, established that T cells found in the peripheral blood of melanoma patients recognised defined antigens expressed by the tumours (Rosenberg, 1997; Van Pel et al., 1995) . MAGE-1 was the first gene reported to encode a human tumour antigen recognised by T cells (van der Bruggen et al., 1991) which subsequently led to the identification of the first $CD8^+$ T cell epitope (Traversari et al., 1992). This discovery heralded the beginning of a new era of antigen-specific T cell immunotherapy. Since then numerous TAAs have been identified utilising both molecular and cellular immunological approaches.

The classical approach employed to identify T cell epitopes involves isolating tumour-reactive T cells from the peripheral blood or tumour tissue of cancer patients which are co-cultured with autologous tumour cells for autologous epitope sensitisation

and induction of proliferation. Subsequently, the stimulated T cells are screened against autologous target cells transfected with genes from a tumour-derived cDNA library. Specific killing of cells transfected with a particular cDNA identifies the protein encoded by this cDNA as a candidate tumour antigen. In order to determine the epitope-containing region, the antigen-specific T cells are co-incubated with target cells expressing only truncated antigen fragments. Then, in order to define the minimal T cell epitope, overlapping synthetic peptides covering the antigen fragment of interest are loaded on to target cells. This approach has rapidly evolved, mainly due to the availability of new technology, and has been integrated with novel strategies such as (1) reverse immunology, (also known as epitope deduction) which predicts epitopes on the basis of known HLA-binding motifs and is performed using dedicated software. Several algorithms are publicly available such as BioInformatics and Molecular Analysis Section (BIMAS) (http://www-bimas.cit.nih.gov/molbio/hla_bind/) and SYFPEITHI (http://www.syfpeithi.de/) (2) biochemical methods which elute and fractionate TAA peptides naturally expressed on tumour cells in the context of MHC molecules by chromatography and mass spectrometry, and (3) DNA microarray technology which allows comparison of gene expression profiles in tumour tissues and normal counterparts (representational difference analysis [RDA], differential display [DD], and serial analysis of gene expression [SAGE]) (Gires & Selinger, 2009; Schultze & Vonderheide, 2001). A useful database of described T cell antigens is maintained by the Cancer Research Institute (http://www.cancerimmunity.org/peptide/) (Van der Bruggen et al., 2013).

1.3.1 Classification of human tumour-associated T cell antigens

Human tumour-associated T cell antigens have been classified into four major groups on the basis of their expression pattern: (1) unique antigens, which are often restricted to a tumour of an individual patient, and shared antigens which are present in many individual tumours and categorised as (2) Tumour-specific antigens (also referred to as cancer testis antigens); (3) differentiation antigens, and (4) overexpressed antigens.

1.3.1.1 Unique Tumour Antigens

These are antigens that are strictly tumour-specific and are not expressed by normal tissue. They are commonly the result of somatic point mutations occurring in many different proteins expressed by tumour cells. However, their expression is often unique to the tumour of an individual patient or restricted to very few patients. The fact that they are not shared by tumours from different patients means that they are not attractive targets for immunotherapy. An example of unique antigens includes abnormal forms of the proto-oncogene, p53.

1.3.1.2 Tumour-specific Antigens (also known as Cancer/Testis (CT) Antigens)

These antigens comprise a family of genes that are present in a wide variety of malignant tumours, but their expression in normal tissues is mostly restricted to MHC-negative testicular germ cells and placental trophoblasts. Epigenetic events, particularly DNA methylation, appear to be the primary mechanism regulating CTA expression in both normal and transformed cells. However, little is known about their specific functions. CT genes fall into two subsets; those encoded on the X-chromosome (CT-X), which are highly expressed in spermatogonia and non-X CT genes, which are encoded on other chromomsomes and tend to be expressed during later stages of

spermatogenesis. Non-X CT genes typically have a less restricted pattern of expression whereas most of the CT-X antigens are not expressed in normal somatic tissues. Examples of CT-X antigens include, NY-ESO and MAGE. An example of a non-X CT antigen is BAGE.

1.3.1.3 Differentiation Antigens

These antigens are expressed by malignant cells but also by their normal counter-parts at a particular differentiation stage. Differentiation antigens were originally identified when T cells from melanoma patients were found to recognise antigens encoded by normal melanocytic differentiation genes, such as those encoded by tyrosinase, Melan-A/MART-1 and gp100/pmel17. Many of these melanocyte lineage-related proteins are involved in the production of melanin. Apart from melanocytes and pigmented retinal cells, these melanocyte differentiation antigens (MDAs) are not expressed on any other normal tissue. In addition to melanoma, T cell differentiation antigens are known to be expressed in other malignancies, for example, carcinoembryonic antigen (CEA) in cases of gut carcinoma, and prostate specific antigen (PSA) in prostate carcinoma. Differentiation antigens are commonly selected as targets for immunotherapy. However, this can often result in autoimmunity towards the corresponding normal tissue. In the case of melanocytes, which to an extent are dispensable, autoimmune toxicity is likely to be tolerable whereas more serious concerns about autoimmune side effects apply to CEA which is expressed in the colon.

1.3.1.4 Overexpressed Antigens

Aberrant over-expression of certain proteins has been found to occur at the mRNA or translational level in malignant cells. The distribution of these antigens in normal

tissues is heterogeneous, with some antigens being expressed in a few normal tissues, while others can be detected ubiquitously. The increased expression of these proteins in transformed cells contributes to their proliferation and survival. Examples include survivin and hTERT (Chaudhuri et al., 2009; Gires & Selinger, 2009).

Table 1-1 Mechanism for generating human tumour associated antigens with examples

Classification of tumour antigen	**Cellular Mechanism**	**Examples**
Unique antigens	Point mutations Frameshift mutations Translocation mutations	CDK4, BRAF, p53 KRAS TGFβII, Bax
Shared tumour-specific antigens (CT antigens)	Reactivation of genes normally expressed in germ cells possibly through DNA demethylation	MAGEs, NY-ESO-1, SSX, CRT-2
Differentiation antigens	Aberrant post-translational modification Deamidation Differential protein splicing Translation of cryptic epitopes Translation from alternative ORF	Tyrosinase Gp100, TRP-2 MART-1, gp100 Gp75
Overexpressed antigens	Gene amplifications	Her2/neu, Akt-1, WT-1, c-Myc

1.3.2 Investigated tumour-associated antigens

In this thesis, epitopes derived from four MHC class I-restricted tumour-associated antigens are investigated: NY-ESO-1 (and LAGE-1), gp100, Melan-A/MART-1 and MAGE-A3.

1.3.2.1 NY-ESO-1 (and LAGE-1)

NY-ESO-1 and LAGE-1 are classed as cancer/testis (CT) antigens and they form part of a subset of CT antigens encoded on the X chromosome (CT-X). They are expressed in different types of cancer, and not in normal tissues except in male germ cells (spermatogonia) (Hofmann et al., 2008). However, little is known about their functions. NY-ESO-1 was originally detected when screening an esophageal carcinoma sample by SEREX (serological analysis of tumour antigens by recombinant cDNA expression cloning), a technique for identifying tumour antigens that elicit an antibody response (Y. T. Chen et al., 1997). The highly homologous antigen, LAGE-1, was subsequently isolated by a subtractive cDNA cloning approach (Lethe et al., 1998). The close homology between NY-ESO-1 and LAGE-1 exists at both the mRNA and protein expression level (de Carvalho et al., 2011). NY-ESO-1 and LAGE-1 are expressed by a wide range of tumours including myeloma (Andrade et al., 2008; van Rhee et al., 2005) and a variety of solid tumours such as ovarian (Odunsi et al., 2003), non-small cell lung cancer (NSCLC) (Gure et al., 2005; Konishi et al., 2004) and melanoma (Barrow et al., 2006; Vaughan et al., 2004). NY-ESO-1 has been an attractive target in clinical trials using a variety of immune-based therapies including vaccines and gene modified T cells (Nicholaou et al., 2006; Robbins et al., 2011). Multiple studies have demonstrated that NY-ESO-1 is immunogenic in human patients (J. L. Chen et al., 2000; Jager et al., 1999), although vaccination approaches alone rarely lead to a clinically significant

response (Bioley et al., 2009; Caballero & Chen, 2009). The epitope of interest in this study is for the immunogenic HLA-A*0201 presented peptide NY-ESO-$1_{157-165}$ (SLLMWITQC) (J. L. Chen et al., 2000; Jager et al., 1998; Liddy et al., 2012; Purbhoo et al., 2006). This same epitope is also present and processed from the LAGE-1 antigen, thus, representing a single therapeutic target (de Carvalho et al., 2011; Lethe et al., 1998).

1.3.2.2 Gp100 and Melan-A/MART-1

Gp100 and Melan-A/MART-1 (Melanoma Antigen Recognised by T-cells-1) are differentiation antigens of the melanocytic lineage which are involved in melanogenesis and cutaneous pigmentation (Gires & Selinger, 2009). Both antigens are highly expressed at over 90% in metastatic melanoma (Boon et al., 2006). Although these antigens represent normal 'self-proteins', T cell responses specific for epitopes derived from these molecules have been reported in melanoma patients (Chi et al., 1997; Hofbauer et al., 2004; Riker et al., 1999). Indeed, immune responses to these antigens have been shown to correlate with clinical response (Engell-Noerregaard et al., 2009; Kawakami et al., 1995; Saleh et al., 2005). However, various vaccination strategies to harness the immune response against these antigens in patients with metastatic melanoma has had limited success to date (reviewed in (Lens, 2008)).

Melan-A/MART-1 was originally isolated by cDNA expression cloning with HLA-A2 restricted melanoma reactive CTL from TIL (tumour-infiltrating lymphocytes) and PBMC (peripheral blood mononuclear cells) (Coulie et al., 1994; Kawakami, Eliyahu, Sakaguchi et al., 1994). It was found to be an immunodominant antigen recognised by the majority of melanoma reactive TIL in HLA-$A2^+$ patients (Kawakami et al., 2000; Kawakami, Eliyahu, Delgado et al., 1994; Kawakami et al., 1995) . One of

the epitopes responsible for this immunodominance is the HLA-A*0201 restricted peptide Melan-A/MART-1_{26-35} (EAAGIGILTV) (Kawakami, Eliyahu, Sakaguchi et al., 1994; Romero et al., 1997; Valmori et al., 1998) and is the epitope targeted in this study.

Gp100 was identified as a melanoma antigen recognised by T cells using three different methods; cDNA expression cloning, direct epitope identification using mass spectrometry and screening candidate molecules using melanoma reactive CTL (Bakker et al., 1994; Cox et al., 1994; Kawakami et al., 2000). The HLA-A*0201 restricted peptide $gp100_{280-288}$ (YLEPGPVTA) was found to be one of three immunodominant epitopes recognised by TIL (Cox et al., 1994; Kawakami et al., 1995) and is the focus of this study.

1.3.2.3 MAGE-A3

The melanoma antigen genes (MAGE) represent the largest gene family among the CT antigens. Like NY-ESO-1 and LAGE-1, the MAGE-A, B, and –C family are localised in clusters on the X-chromosome and are referred to as CT-X-MAGE proteins. MAGE-A3 was identified when analysing CTL responses of a cancer patient to an autologous melanoma cell line (Gaugler et al., 1994). MAGE-A3 is expressed in a range of solid tumours such as melanoma (Brasseur et al., 1995; van der Bruggen et al., 1991), head and neck squamous carcinoma (Atanackovic et al., 2006) and bladder carcinoma (Patard et al., 1995). However, the richest antigen expression of MAGE-A3 is seen in multiple myeloma. Interestingly, although the role of CT antigens in malignancy is poorly understood, MAGE-A3 is known to play an important part in promoting the survival of myeloma cells. Indeed, tumour progression is closely correlated with the level of expression of MAGE-A3 (Atanackovic et al., 2010).

The epitope of interest in this study is for the immunogenic HLA-A*0101 presented peptide MAGE-A3$_{168-176}$ (EVDPIGHLY) (Celis et al., 1994)

1.3.3 Cancer immune escape

A key reason for the limited success of T cell based therapies to date is due to cancer immune escape mechanisms. The inherent genetic instability of malignantly transformed cells results in a heterogeneous population of cells that are able to alter their antigenic profile and avoid immune destruction. Indeed, immunological pressure exerted by immune cells allows for the Darwinian selection of the most fit tumour variants to survive which leads to the outgrowth of a tumour. The cancer immuno-editing theory postulates three phases that describe tumour progression in the context of its interaction with the immune system: elimination, equilibrium, and escape (Schreiber et al., 2011). Elimination refers to the immunosurveillance phase whereby a strongly immunogenic tumour in a highly immunocompetent host induces optimal stimulation of cells from the innate and adaptive immune system (including natural killer (NK), αβ and γδ T cells) and immunoregulatory molecules (including IFN-γ, IL-12, perforin and TRAIL) which act together to detect and destroy the tumour cells. Equilibrium phase occurs when the immune system is not able to completely eliminate the tumour due to low tumour immunogenicity and/or a less immunocompetent host. However, at this stage, the subset of tumour cells, which have not been eliminated are still under immunosurveillance and do not progress or further metastasize. During the equilibrium phase the tumour is not a clinical disease and this phase could be life-long, thus mimicking elimination. On the other hand, continuous pressure by the immune system on genetically unstable cells can lead to the generation of tumour subtypes or variants that are able to avoid immunosurveillance, or changes in the immune system are

induced that weaken its capacity for tumour surveillance. The outcome of either change is tumour escape that leads to the development of clinically apparent tumours in an immunocompetent host (Dunn et al., 2004; Shankaran et al., 2001).

A common mechanism by which tumours avoid immune detection is through down-regulation of MHC molecules and TAAs. Human tumours frequently lose expression of MHC class I antigen (Garrido & Algarra, 2001; Marincola et al., 2000). A study has shown that the frequency of HLA class I antigen loss or down-regulation is as high as 51% in melanomas, with total loss seen in 16% as assayed by immunohistochemical (IHC) techniques (Ferrone & Marincola, 1995). Loss or down-regulation of MHC class I antigen expression abrogates the ability of CTLs to recognise and kill tumour cells (Buell et al., 2005). Complete loss of MHC class I antigen expression is commonly caused by mutation of the β_2 microglobulin (β_2m) gene that results in the functional loss of β_2m expression (Bicknell et al., 1994; Hicklin et al., 1998). β_2m is essential for expression of the HLA class I complex on the cell surface. Loss of MHC class I is irreversible and can be restored only by wild-type β_2m gene transfer (Hicklin et al., 1998; Restifo et al., 1996). However, a number of studies have shown that MHC class I-deficient cells are susceptible to lysis by NK cells (Ljunggren & Karre, 1985; Maio et al., 1991).

Down-regulation of MHC class I can also result from defects in the peptide loading of HLA class I antigens which impairs the assembly and stability of MHC class I molecules. This is caused by abnormalities in the expression and/or function of different component(s) of the MHC class I antigen-processing and –presentation pathway, such as the proteasomal subunits LMP2 and LMP7, the peptide transporters associated with antigen processing (TAP-1 and TAP-2), and the chaperone tapasin

(Seliger et al., 2000). However, in many cases, the expression of these components can be restored by treatment of tumour cells with IFN-γ (Seliger et al., 1997). MHC class I down-regulation can also involve the selective loss of HLA-haplotype, locus or allele (Rivoltini et al., 2002).

In addition, genomic instability within tumour cells may result in the loss or down-regulation of TAA expression creating antigen loss variants that are able to escape T cell recognition. For example, in melanoma, tumour progression is often associated with reduced expression of melanocyte differentiation antigens (MDA) (Cormier et al., 1998; Jager et al., 1997; Maeurer et al., 1996). However, a recent study suggests that CTLs may indirectly eliminate these tumour variants when tumour cells express sufficient antigen to be effectively cross-presented by the tumour stroma (Spiotto et al., 2004).

This section has described some of the cell-autonomous modifications at the level of the tumour cell that directly lead to evasion of immune detection. Section 1.5.8 describes the modifications in immune cells, inflicted on these by tumour cells and which generate an immunosuppressive tumour microenvironment.

1.4 Cell surface antigen presentation

1.4.1 Type of cell surface antigen recognised by antibodies and T cell receptor (TCR)

The adaptive immune system consists of two kinds of lymphocyte; B cells and T cells, which have evolved to survey the host environment for abnormalities. They recognise antigen by two distinct sets of highly variable receptor molecules; the immunoglobulins that function as antigen receptors on B cells and the antigen-specific T cell receptor (TCR) on T cells. Immunoglobulins and TCRs differ in several ways: (1) immunoglobulins are tetramers, comprising four polypeptide chains (two heavy chains, two light chains) and possess two antigen recognition sites, while TCRs are dimers and possess only one binding site, (2) immunoglobulins can be synthesised as either transmembrane receptors or secreted antibodies, whereas TCRs are only expressed naturally as transmembrane receptors, and (3) while antibodies recognise cell surface protein antigens by their overall conformation, T cells recognise peptide (short protein) fragments which have been processed and become bound to MHC class I or II molecules. These MHC-antigen complexes are presented at the cell surface of antigen presenting cells (APCs) (e.g., dendritic cells, B cells, macrophages, and monocytes). This phenomenon is known as MHC-restricted recognition, or MHC restriction (Lefranc & Lefranc, 2001).

1.4.2 MHC class I antigen processing

MHC class I molecules typically bind peptides which have originated from the degradation of the cells' endogenous molecules and are presented to $CD8^+$ cytotoxic T cells. The degradation of peptides destined to be presented by MHC class I molecules begins in the cytosol of the cell in a process involving an organelle called a proteasome,

a multisubunit ATP-dependent protease. Denatured or ubiquitinated (a tag for degradation) proteins are cleaved into peptides by the multicatalytic proteasome subunits. Three subunits, called X (MB1), Y (δ), and Z, mediate its standard catalytic activity and in normal, untransformed cells, the production of MHC class I peptide ligands is an inefficient process. However, in response to the cytokine IFN-γ, this subunit composition is altered, leading to the formation of an immunoproteasome which consists of the catalytic subunits LMP2, LMP7, and LMP10 (MECL-1). The immunoproteasome is more efficient at generating antigenic peptide fragments suitable for binding MHC class I molecules. This process is further enhanced by the proteasome activators, PA28α and β, the expression of which are also modulated by IFN-γ (Pamer & Cresswell, 1998) The antigenic peptides are then transported into the endoplasmic reticulum (ER) by the peptide transporter associated with antigen processing (TAP).

TAP, a member of the ATP-binding cassette (ABC) transporter family, consists of two subunits, TAP1 and TAP2, which form a heterodimer. In the ER, newly synthesised MHC class I–β_2m complexes associate with TAP which binds and translocates peptides of a specific sequence, with a preferential length of 8-16 amino acids (Momburg & Hammerling, 1998). The chaperone molecule, tapasin acts as a bridge between TAP and MHC class I ensuring high peptide-loading efficiency, optimal ligand selection and stabilisation of the MHC class I-loading complex (Barnden et al., 2000). Other co-factors present in the ER, such as the chaperones calreticulin and ERp57, also associate with the TAP-tapasin-MHC class I complex to form a large complex called the peptide loading complex (PLC). Components of the PLC facilitate peptide binding to MHC class I molecules. Peptide occupancy triggers the release of MHC class I- β2m complex from the PLC which is then transported via the Golgi to the cell surface for presentation to $CD8^+$ cytotoxic T cells (Raghavan et al., 2008).

MHC class I peptides are not exclusively generated from endogenous proteins, but can also be derived from endocytosed exogenous antigens, via different intracellular pathways, which are able to access the class I processing and presentation pathway. This phenomenon of cross-presentation (also referred to as cross-priming) is a key mechanistic step in the initiation of $CD8^+$ T cell responses against tumour cells and many pathogens (Shen & Rock, 2006). Cross-presentation was thought to be a specific feature of professional antigen presenting cells (pAPCs), such as dendritic cells. However, a recent study has demonstrated that CTLs from a melanoma patient recognised a cross-presented epitope on autologous tumour cells generated from an exogenous protein after receptor-mediated internalisation (Godefroy et al., 2005). Furthermore, studies have shown that T cells are able to eradicate or arrest growth of large established tumours, including antigen loss variants, by targeting non-malignant stromal cells that have internalised tumour antigens released from dead cancer cells and cross-presented the epitopes on their surface MHC molecules (Spiotto et al., 2004; Spiotto & Schreiber, 2005; B. Zhang et al., 2007). This T cell destruction of stromal cells then leads to bystander killing of cancer cells (Spiotto et al., 2004; Spiotto & Schreiber, 2005; B. Zhang et al., 2008).

1.4.3 MHC class I antigen presentation

Almost, all nucleated cells present MHC class I molecules. The function of MHC class I molecules is to present antigens which have originated inside the cell to cytotoxic $CD8^+$ T cells. There are approximately 10^5 copies of MHC class I molecules expressed on the surface of most nucleated cells and, therefore, many different peptides are simultaneously presented. These include self peptides such as leader signal peptides, enzymes, heat-shock proteins etc, as well as those derived from viruses or intracellular

pathogens which have infected the cell. In contrast, MHC class II molecules are expressed only on B cells, dendritic cells, macrophages and thymic epithelium, and present antigen to $CD4^+$ helper T cells.

MHC class I molecules consist of a heavy polypeptide chain of 44 kDa non-covalently linked to a smaller 12 kDa polypeptide called β_2-microglobulin (β_2m). The class I heavy chain consists of three extracellular domains, designated α1 (N-terminal), α2 and α3, a transmembrane region and a cytoplasmic tail. The heavy chain is encoded by the highly polymorphic major histocompatibility (MHC) gene complex, in humans located on the short arm of chromosome 6. In each individual there are three loci, called human leucocyte antigen (HLA)-A, -B and –C. At each locus there are multiple heavy chain alleles which give rise to the extraordinary polymorphic nature of class I genes. The paternal and maternal derived chromosome 6 combination results in the co-dominant expression of a maximum of six different MHC class I alleles. Although several hundred human heavy chain alleles are known (listed at http://hla.alleles.org/ (Robinson et al., 2013)), specific alleles are preferentially expressed in certain population groups, for example the MHC class I molecule HLA-A*0201 is present in about 50% of Caucasians (New Allele Frequency Database: http://www.allelefrequencies.net/ (Gonzalez-Galarza et al., 2011).

1.4.3.1 Structure and peptide binding of MHC class I molecules

The first X-ray crystallographic structure of a human MHC class I molecule, HLA-A2 (Bjorkman et al., 1987), revealed that both β_2m and the α3 region resemble Ig domains in their folding pattern. The non-polymorphic α3 domain also contains a site which interacts with the coreceptor, CD8, on cytotoxic T cells (Gao et al., 1997). The highly

polymorphic α1 and α2 domains, which are most distal to the membrane, form two extended α-helices above a floor created by strands held together in a β-pleated sheet, forming a peptide binding cleft. The extreme polymorphism concentrated in the α1 and α2 domains allows for the variation in the peptide-binding cleft enabling the binding of the many possible peptides and extends the range of antigens to which the immune system can respond.

The binding cleft or groove is closed at both ends and restricts peptide length to 8-11 residues (averaging 9 residues) and of a linear nature. Antigenic peptides contain specific amino acids at their amino (N)- and carboxyl (C)-termini that bind to invariant sites at each end of all MHC class I molecule peptide binding clefts. Moreover, particular amino acids with defined characteristics form 'anchor residues'. They represent the amino acid side-chains required to fit into allele-specific pockets in the MHC groove. For class I –binding peptides there may be two or three anchor positions, one at the C-terminal end and the other typically at the second residue from the amino terminus of the peptide (termed position P2). The anchor residues are typically hydrophobic residues; by contrast, the remaining amino acids in the sequence can vary almost infinitely (Rammensee et al., 1995). Therefore, a given MHC class I molecule binds a distinct set of peptides sharing a common homologous binding motif, but is still able to present a wide array of peptides to the T cell receptor. Peptide-binding motifs have been defined for numerous MHC class I alleles and this has made it possible to make predictions about the set of peptides with the ability to bind a given MHC class I allele by scanning the gene product sequence of interest with appropriate algorithms. This procedure has become known as reverse immunology and is a potentially useful tool for mining novel tumour antigens from the human genome (Schultze & Vonderheide, 2001).

1.5 T cells and the T cell receptor (TCR)

T cell receptors (TCRs) are the clonotypic antigen-specific receptors presented on the surface of T cells, which recognise peptide antigen in the context of MHC molecules, and are critical to the adaptive immune response. TCRs were first isolated using monoclonal antibodies directed against a clone-specific molecule on T cells (Haskins et al., 1983), and also independently at the same time, by the identification of genes that encode part of the same molecule (Hedrick et al., 1984). The molecule was found to be a heterodimer comprising an α (40-50 kDa) and a β (35-47 kDa) polypeptide chain, linked by a disulfide bond. The αβ heterodimers are very similar in structure to the antigen binding fragment (Fab) of an immunoglobulin (Ig) molecule. They are present on the majority of T cells and recognise peptide antigens presented by MHC class I and II molecules. A small proportion of T cells bear an alternative, although structurally similar, receptor made up of a different pair of polypeptide chains, γ and δ, whose function is less clearly defined at present. The αβ and γδ forms of the TCR, which are structurally unique for each clone of T cells, are additionally both non-covalently associated with CD3, a membrane-bound signalling complex of non-polymorphic polypeptides which includes δ, γ, ε, and ζ chains.

1.5.1 Structure of the T cell receptor

As already eluded to, the αβ TCR framework structure resembles that of an Ig Fab fragment and this had been predicted for some time based on the sequence homology between the molecules (Chothia et al., 1988), before it was conclusively demonstrated in separate X-ray crystallography studies (Bentley et al., 1995; Fields et al., 1995; Garboczi et al., 1996; Garcia et al., 1996). The α and β chains each comprises two extracellular domains, a variable (V) region with homology to the immunoglobulin V

domain, a constant (C) region with homology to the immunoglobulin C domain, and a short stalk segment containing a cysteine residue which forms the interchain disulfide bond. Each chain is anchored into the plasma membrane by a transmembrane domain which has a short cytoplasmic tail. Within the Vα and Vβ domains, there are regions of amino acid hypervariability, known as complementarity determining regions (CDRs) 1, 2 and 3. These are clustered together on the membrane distal part of the TCR to form an MHC-antigen binding site which is analogous to the antigen binding site on antibodies (Garcia, Teyton et al., 1999).

1.5.2 Hypervariability

Similar to B cell immunoglobulin receptors, the T cell receptor (TCR) germline DNA undergoes rearrangement, to produce the diversity of receptors required to recognise the universe of potential pathogens. In αβ T cells, which recognise peptide antigens presented by MHC molecules, most of this receptor diversity is determined by the amino acid sequence encoded in the third complementarity determining region (CDR3) loop of the α and β chain variable domains. The CDR3 regions are formed by rearrangement between variable (Vβ), diversity (Dβ), and joining (Jβ) gene segments in the β chain locus, and between analogous Vα and Jα gene segments in the α chain locus. The existence of multiple such gene segments in the β and α chain loci creates the potential for a large number of distinct CDR3 sequences to be encoded (Figure 1-2). Additional CDR3 sequence diversity is generated by template-independent addition, deletion and substitution of nucleotides at the junction of gene segments during rearrangement. It has been estimated that TCR gene assembly and expression allows for a potential 10^{13}-10^{15} variants of the αβ TCR heterodimer in the mouse (M. M. Davis & Bjorkman, 1988). However, the theoretical number of possible TCRs in humans is

likely to be orders of magnitude larger, as humans possess 54 TCRβ variable genes as compared with the 35 genes in mice, with all other variables being comparable. Thus, the potential number of unique αβ TCRs in human is $> 10^{18}$ (Sewell, 2012).

A key difference between antibodies and TCRs in the generation of diversity is that TCRs do not undergo somatic hypermutation. Somatic hypermutation occurs in response to antigen and involves the introduction of non-templated point mutations in the recombined immunoglobulin variable region V(D)J genes. It is a mechanism for increasing antibody diversity and affinity following antigen recognition (M. M. Davis & Bjorkman, 1988; Tonegawa, 1988). By contrast, the T cell repertoire is shaped by selection processes that operate in the thymus and in the periphery to maintain lower affinities. If hypermutation occurred, those mutations could lead to the emergence of high affinity autoreactive receptors and autoimmunity. Thus, the affinity of TCR-pMHC interaction is 'locked' after gene rearrangement and typically found to be in the low micromolar range (M. M. Davis et al., 1998) compared to antibody-antigen affinities which are in the nanomolar range (Braden et al., 1998).

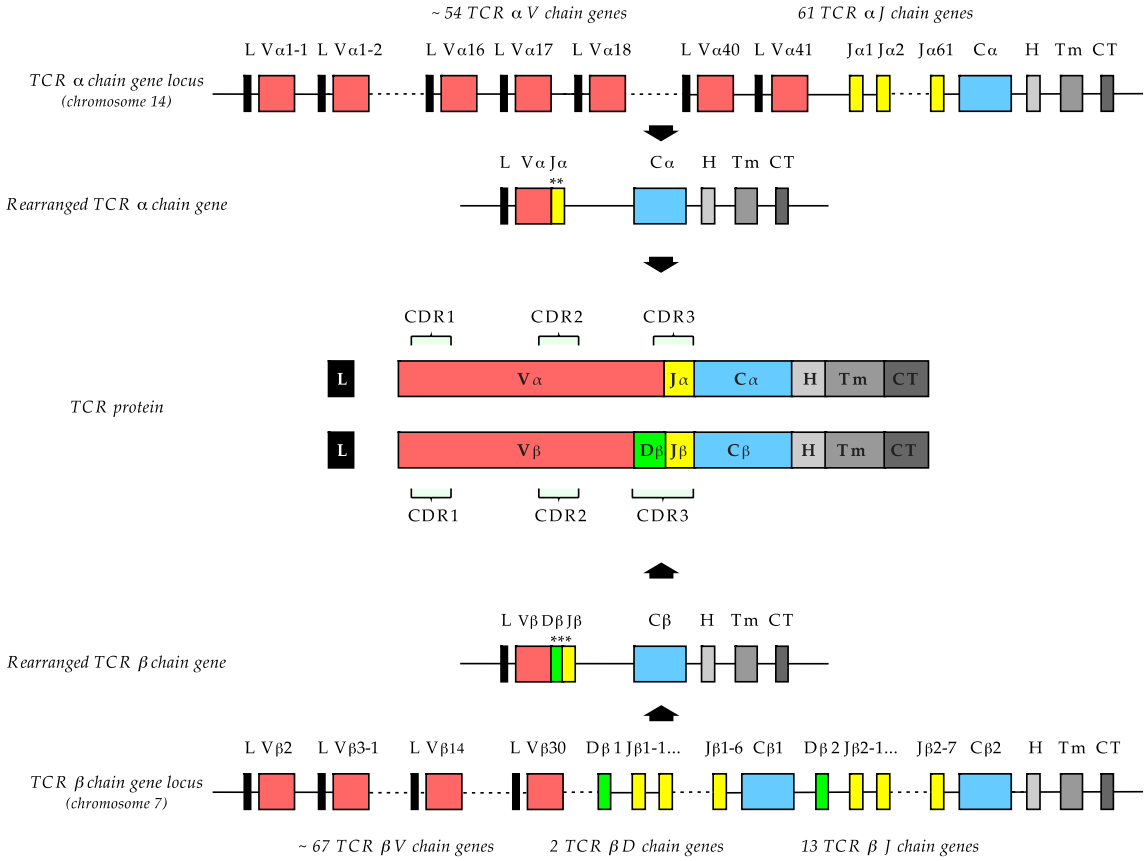


Figure 1-2 T cell receptor (TCR) α and β chain gene rearrangement and expression.

The TCR α and β chain genes are composed of discrete segments that are joined by somatic recombination during T cell development in the thymus. For the α chain (upper part of figure), a Vα gene segment rearranges to a Jα gene segment to create a functional V-region exon. All V segments are preceded by a leader sequence (L). Transcription and splicing of the VJα exon to Cα generates mRNA that is translated to produce the TCR α chain protein. For the TCR β chain (lower part of figure), the variable domain is encoded in three gene segments, Vβ, Dβ and Jβ. Rearrangement of these gene segments generates a functional VDJβ V-region exon that is transcribed and spliced to join to Cβ; the resulting mRNA is translated to produce the TCR β chain. The α and β chains pair soon after synthesis to produce the αβ TCR heterodimer. Diversity is further increased through DNA nuclease activity and random N nucleotide addition at the gene segment junctions, providing almost limitless potential for the TCR repertoire. This method of generating receptor diversity preserves germline-encoded information in the V regions that directly interact with MHC molecules (CDR1 and CDR2) while maximising diversity a the V(D)J junction (region marked by asterisks) that encodes the hypervariable CDR3 that contacts presented peptide antigens. Courtesy of Dr. Bent Jakobsen at Immunocore Ltd.

1.5.3 TCR antigen recognition

The molecular recognition of the pMHC by the TCR is mediated by the three CDRs of each chain of the TCR at the interface with the pMHC complex (Garcia et al., 1996). Co-crystal structures of a number of TCR-pMHC complexes have demonstrated an approximately diagonal orientation of the TCR with respect to MHC, with the Vα domain poised above the N-terminal half of the peptide and the Vβ domain located over the C-terminal portion of the peptide. The hypervariable CDR3 loops of the α chain and β chain are located over the central portion of the peptide (Rudolph et al., 2006). One current model for TCR-pMHC association describe a two step approach, the first step being driven by the conserved, germline-encoded interactions made by the CDR1 and CDR2 loops of the TCR α and TCR β chains, which are largely peptide independent, thereby positioning the highly variable non-germline-encoded CDR3 loops over the peptide. Specific recognition of the presented peptide by the TCR then occurs after the TCR has made the appropriate contacts with the MHC to orient itself correctly on the peptide-MHC complex. T cell activation is triggered only when stable contacts are made with both HLA and peptide (Wu et al., 2002). It is suggested that this paradigm explains how it may be possible for a TCR to efficiently scan the many thousands of peptides presented by MHC on the surface of an antigen presenting cell. However, structural data from TCR-pMHC complexes appear to contradict this model, as the contributions of TCR-MHC and TCR-peptide contacts can vary considerably between different complexes. Also, it has been found that the orientation with which TCRs with the same germline CDRs are positioned over their respective pMHC ligands can vary with up to 60°, indicating that no fixed alignment and, therefore, set of contacts exist between the CDR1 and CDR2s to MHC (Gras et al., 2012). Furthermore, it has been shown that a functional TCR repertoire can be thymically selected even if the CDR1

and CDR2 regions of the TCR β chain were changed to those from a TCR γ chain, which does not recognise MHC class I. Thus, the TCR CDR1 and CDR2s are not obligatory for pMHC recognition and it therefore appears more likely that the TCR adopts a flexible, rather than an ordered, step-wise docking mode when engaging with pMHC (Holland et al., 2012).

Regardless of the various models for the initiation of TCR-pHLA contacts, paired structures of unbound TCRs and TCR-pMHC complexes have revealed considerable flexibility within the CDR loops of the TCR molecule, with stabilisation occurring upon binding to pMHC (Willcox et al., 1999). Hence, a flexible binding mechanism has been proposed for TCR-pMHC docking (Garcia et al., 1998; Garcia, Teyton et al., 1999; Reiser et al., 2003). This TCR 'plasticity' is thought to be an important determinant of the ligand degeneracy that has been reported for both MHC class I- and class II-restricted TCRs (Ishizuka et al., 2008; Wilson et al., 2004).

1.5.4 TCR degeneracy

TCR recognition of peptide-MHC is 'polyspecific', i.e., a particular TCR is not limited to the specific recognition of a single ligand. Indeed, it has been estimated that one TCR can respond to over million different peptides when presented in the context of a single MHC type (Wooldridge et al., 2012). Despite this apparently high level of cross-reactivity, TCRs are still functionally specific because they will recognise only a small fraction (<0.01%) of the peptides that can be presented by a particular MHC molecule (Mason, 1998). T cell cross-reactivity permits effective T cell responses to a vast number of potential peptide sequences bound to MHC molecules with specificity sufficient to distinguish between self and foreign peptides (Sewell, 2012).

TCR-pMHC interactions typically comprise fast kinetics and weak affinity with dissociation constants (K_D), defined at equilibrium as the rate of dissociation divided by the rate of association ($K_D = K_{off}/K_{on}$), ranging from 1-100 μM (Cole et al., 2007; M. M. Davis et al., 1998; Garcia, Degano et al., 1999; van der Merwe & Davis, 2003). These binding properties, shaped during thymic selection, are necessary to enable TCR to effectively trigger the T cell once a rare antigenic complex has been encountered but also to avoid autoreactive responses against self-pMHC (Krogsgaard & Davis, 2005; Rudolph et al., 2006).

1.5.5 TCR thymic selection

The T cell repertoire is generated during thymic development in preparation for the response to pathogenic antigens and also involves strict quality control mechanisms to prevent autoimmunity. Immature thymocytes (thymic T cells) that undergo V(D)J recombination of the TCR genes and express the unselected T cell repertoire are generated in the thymic cortex. The processes of positive and negative selection shape the functional repertoire of $CD8^+$ cytotoxic T cells (Starr et al., 2003).

Positive selection occurs in the thymic cortex and requires that immature, double-positive (DP: $CD4^+CD8^+$) thymocytes be able to recognise self-pMHC molecules on thymic cortical epithelial cells (cTECs). cTECs present a spectrum of peptides that bind weakly to MHC molecules. The presented peptides, which have been generated by a thymoproteasome, are different from the peptides presented in the periphery or in the thymic medulla (Nitta et al., 2010). The low affinity recognition of the cTEC self-pMHC complexes promotes survival of the immature thymocytes; in the absence of recognition, DP thymocytes will undergo apoptotic death through lack of

stimulation. Recognition of the cTEC self-pMHC complexes also determines the commitment of the DP thymocytes to either the CD4 or CD8 lineage.

Negative selection mainly occurs in the medulla, where single-positive thymocytes (SP: $CD4^+CD8^-$ or $CD4^-CD8^+$) encounter thymic epithelial cells (mTECs), and dendritic cells (DCs) that have migrated from the periphery to the thymus. Both mTECs and medullary DCs process peptides through the constitutive proteasome and the more efficient immunoproteasome, and therefore possess the capability to present a vast repertoire of self-pMHC to mirror that presented in the periphery (Groettrup et al., 2010). mTECs also specialise in promiscuous gene expression (pGE), induced by the autoimmune regulator (Aire) (reviewed in (Taniguchi & Anderson, 2011)) which acts as a master switch directing the transcriptional activation of genes for a number of organ-specific antigens. Negative selection results in the clonal deletion of T cells from the repertoire that possess high affinity TCR to self-pMHC thereby conferring immune tolerance to the host and preventing autoimmunity.

Thymic selection generates a TCR repertoire with low affinity for self-pMHC but potentially high affinity for foreign antigens. The result is that, while CTLs can respond vigorously to pathogen-derived antigens, tumour cells appear to evade the CTL response. Most tumour associated peptide antigens (TAPAs) are derived from self and it has been shown that the affinity of TCRs for cancer-associated antigens are of approximately one order of magnitude lower affinity than TCRs recognising viral peptides (Aleksic et al., 2012).

1.5.6 T cell activation in the periphery

1.5.6.1 The immunological synapse

T cell immune responses are initiated by the engagement of the αβ TCR and coreceptor, CD8 or CD4, with its cognate peptide-MHC. The TCR itself has no intrinsic signalling activity and is associated non-covalently on the cell surface with the non-variable CD3 complex (van der Merwe & Davis, 2003). The main role of the CD3 complex is to propagate signals into the interior of the cell (Salmond et al., 2009). T cell activation also involves various antigen independent cell-cell interactions including CD2 (LFA-2), CD40L, LFA-1, and CD28 located on the surface of T cells, which bind CD58 (LFA-3), CD40, ICAM-1, and CD80 (B7.1)/CD86 (B7.2) on the surface of APCs, respectively. Together, these interactions serve to strengthen T cell-APC contact and regulate T cell activation (Bennett et al., 1998; M. K. Jenkins et al., 1991). Costimulatory signals such as the CD28-CD80/CD86 interaction are critical for the activation of naïve T cells that have never encountered antigen (Chai et al., 1999).

Given that the affinity of the interaction between an individual TCR for its specific peptide-MHC complex is relatively low, the successful docking of T cells and targets depends on various pairs of accessory molecules in order for a stable association to be formed. The most important of these is the engagement of the integrin LFA-1 on the T cell with its cognate molecule ICAM-1 on antigen presenting cells (APCs). Antigen-induced up-regulation of LFA-1 binding to ICAM-1 is one of the first critical steps to the formation of a specialised structure at the T cell: APC interface called an immunological synapse or ‘supramolecular activation clusters’ (SMAC) (Grakoui et al., 1999; Monks et al., 1998).

Pioneering fixed-cell imaging studies by Kupfer and colleagues (Monks et al., 1998) revealed that the immunological synapse forms a ‘bull’s eye’ pattern with its

target cell, which may be important for the polarised delivery of cytolytic granules into the immunological synapse space. For example, the bull's eye immunological synapse of cytolytic $CD8^+$ T cells is characterised by a central area known as the cSMAC in which the TCR-CD3 complex, its coreceptors CD8/CD4, and the costimulatory molecule CD28 are positioned for optimal interaction with pMHC, CD80 and CD86 on the APC. This, in turn, is surrounded by a peripheral ring (pSMAC) of molecules such as ICAM-1/LFA-1. The pSMAC may act as a sealing ring, preventing the leakage of cytolytic granule contents to bystander cells (M. R. Jenkins & Griffiths, 2010; Stinchcombe et al., 2001). Furthermore, the ring of integrins is also surrounded by a more distal ring (dSMAC) of membrane proteins like CD43 and CD45 with large ectodomains.

Dynamic studies with planar bilayers have shown that during the formation of the immunological synapse, TCR microclusters initially created in the dSMAC, move centripetally towards the cSMAC region in an actin dependent process (Grakoui et al., 1999). Moreover, there is evidence to suggest that the TCR microclusters are the site where TCR signalling is initiated, and that signalling is sustained by the movement of the TCR microclusters into the cSMAC (Campi et al., 2005; Yokosuka et al., 2005). The TCR microclusters also serve as sites for the initiation of costimulation. The most of important of these costimulatory molecules is CD28 which binds to its ligands CD80 (B7.1) and CD86 (B7.2); these interactions are highly enriched in TCR microclusters. However, T cell activation and immunological synapse formation can also be constrained by the action of inhibitory receptors. For example, the expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) on T cells after activation suppresses T cell signalling by out-competing CD28 for the binding of CD80 and CD86. Programmed-death 1 receptor (PD-1) is another example of an inhibitory molecule

involved in co-signalling in T cells. PD-1 has two ligands, PD-L1 and PD-L2 frequently expressed on tumour cells (Fooksman et al., 2010).

Although studies of the immunological synapse throws light on how T cells function, formation of the mature synapse does not appear to be a pre-requisite for full T cell activation (D. M. Davis & Dustin, 2004; Purbhoo et al., 2004; Thauland & Parker, 2010). Thus, in looser terms, the immunological synapse can be considered to be any structure formed at the interface of functional T cell:APC contacts, whereas the cSMAC and pSMACs are structures restricted to certain conditions (Alarcon et al., 2011). Thus, T cell activation does not always follow a clearly recognisable pattern, probably reflecting that the kinetics and degree of order in the T cell:APC contacts can vary considerably.

T cell activation requires signal transduction by the CD3 complex following engagement of the TCR by its ligand. CD3 contains CD3γ, CD3δ, CD3ε and CD3ζ subunits which have been found to associate with one another to form CD3ζζ homodimers and CD3εδ or CD3εγ heterodimers. Each of the CD3 complex subunits contain immunoreceptor tyrosine-based activation motifs (ITAMS) in their cytoplasmic domains; these are present as single copies in the CD3γ, CD3δ and CD3ε subunits and as three copies in the CD3ζ subunit (Koning et al., 1990; Manolios et al., 1994).

Protein phosphorylation is one of the main forms of chemical modification that controls TCR signalling, and is mediated by a number of protein tyrosine kinases (PTKs). After cognate pMHC molecule binding, TCRs cross-link (cluster) during the formation of an immunological synapse, and the Src family kinase, Lck, is activated leading to the phosphorylation of the CD3 chains and ζ-chain homodimer ITAMS of the TCR complex. These, in turn, provide docking sites for the Syk family kinase, ζ-chain-

associated 70 kD protein called ZAP-70. ZAP-70 becomes activated by Lck-mediated phosphorylation and propagates the TCR signal by phosphorylating downstream signalling molecules, including the adaptor molecules SH2 domain-containing leucocyte protein of 76 kDa (SLP-76) and the linker for activation of T cells (LAT) (Wange & Samelson, 1996; Weiss & Littman, 1994). In addition, the stable recruitment of the coreceptors CD4 or CD8 to the TCR plays a key role in early phosphorylation events during T cell signalling. The cytoplasmic tails of CD4 and CD8 are constitutively associated with Lck which can phosphorylate the ITAMS of the CD3 complex leading to the precipitation of signal transduction within the cell (Smith-Garvin et al., 2009).

1.5.6.2 TCR triggering and considerations for designing bispecific activator molecules

The TCR is able to detect as little as a single agonist peptide MHC on the surface of an antigen-presenting cell (APC). However, it appears that around 3-10 specific pMHC complexes are required for the T cell to embark on the signalling pathways that lead to full activation and deployment of its effector functions (Irvine et al., 2002; Purbhoo et al., 2004). Furthermore, sustained signalling over a period of a few hours has been shown to be essential for cytokine production and proliferation in effector T cells, while naïve T cells require an even longer signalling period (approximately 20 hours) to be activated (Iezzi et al., 1998; Schrum & Turka, 2002; Valitutti, Dessing et al., 1995; Weiss et al., 1987). This raised the question of how relatively low affinity TCRs could remain bound to the same pMHC for long enough to achieve sustained signalling. The serial triggering model addresses this issue by proposing that each cognate pMHC serially engages many TCRs, one after the other. This model is supported by evidence

that, during a 5 hour T cell:APC interaction ~ 100 specific pMHC induced the down-regulation of up to 18,000 TCRs (Valitutti, Muller et al., 1995). The serial triggering model is consistent with the typically short-lived nature of TCR-pMHC interactions and dictates a requirement for an optimal dwell-time range for a given TCR-pMHC complex. Therefore, interactions with half-lives below or exceeding this optimum are predicted to be less effective. This notion is supported by evidence that T cell activation can be impaired by mutations that either decrease or increase the binding half-life of the TCR-pMHC interaction (Kalergis et al., 2001). Of particular relevance here, the serial triggering model could have implications for designing an effective bispecific T cell activator molecule and suggests that the potency of the molecule could be critically dependent on its affinity for the TCR-CD3 complex.

Although it has been known for some time that CD3 transduces signals from the engaged receptor via its ITAMs, exactly how ligation of the TCR is translated into the first signal, in a process referred to as TCR triggering, is still not fully understood. Several models have been proposed to explain how spatio-temporal organisation and interaction of molecules on the cell surface of a T cell enable effective signal transduction within the T cell (reviewed in (van der Merwe & Dushek, 2011)).

One of the mechanisms proposed for TCR triggering is binding-induced segregation or distribution of the TCR-CD3 complex relative to other cell membrane-associated proteins. The kinetic-segregation model proposes that TCR binding to pMHC ligand traps the TCR-CD3 complex in close-contact zones (~15 nm apart), thereby segregating it from the large ectodomain inhibitory tyrosine phosphatases, CD45 and CD148, resulting in the constitutive phosphorylation of TCR-CD3 ITAMs by Lck. The model is supported by a number of key observations; first, inhibitory tyrosine phosphatases are excluded from areas of TCR triggering (J. Lin & Weiss, 2003; Varma

et al., 2006); second, truncation of the large phosphatase ectodomains inhibits TCR triggering (Irles et al., 2003; J. Lin & Weiss, 2003); third, elongation of the pMHC complex inhibits TCR triggering (Choudhuri et al., 2009; Choudhuri et al., 2005); fourth, surface-associated TCR ligands induce TCR triggering more effectively than their soluble counterparts (Geppert & Lipsky, 1987; Ma et al., 2008). Finally, and of particular relevance when considering bispecific T cell activators, is that recognition by engineered TCRs is optimal when the epitope is positioned close to the plasma membrane of the target cell (Bluemel et al., 2010; James et al., 2008). Moreover, the kinetic-segregation model is thought to account for the activating effects of certain antibodies. The best known T cell stimulatory antibodies are anti-CD28 and anti-CD3. Analyses of conventional and superagonist antibodies to CD28, for example, have shown that the triggering of CD28 is sensitive to size-dependent effects. The superagonist antibodies formed a more compact complex which more effectively excluded the large proteins (CD45 and CD148), thus favouring more intense phosphorylation of CD28, leading to more potent signalling (S. J. Davis & van der Merwe, 2006).

1.5.7 T cell effector functions

Prior to the first encounter of cognate antigen, naïve $CD8^+$ T cells are not capable of exerting effector functions such as killing. Naïve $CD8^+$ T cells must be 'primed' or activated in the peripheral lymphoid organs before acquiring cytotoxic effector functions. T cell priming is typically mediated by a specialist type of antigen presenting cells (APC) called dendritic cells (DCs). Naïve $CD8^+$ T cells reach maximal activity after 1-3 days of persistent stimulation of the TCR by its cognate antigen in the context of MHC class I, and a CD28 co-stimulus. This promotes the clonal expansion and

differentiation of the $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs) that kill antigen expressing target cells (through granzymes and perforin), and secrete cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor (TNF). CTLs that exert effector functions following secondary antigen encounter are referred to as effector memory cells (Tem). A distinguishing feature of effector memory $CD8^+$ T cells is their ability to rapidly deploy their arsenal of effector functions. $CD4^+$ T cells recognise peptide presented in the context of MHC class II on the surface of APCs; $CD4^+$ T cells differentiate into T helper (Th1) cells that produce IFN-γ, TNF and interleukin-2 (IL-2). They also possess some cytotoxic activity but one of their key roles is maintenance of antigen-specific $CD8^+$ T cell responses.

1.5.7.1 T cell-mediated killing

CTLs employ two distinct effector pathways to kill infected or malignantly transformed cells: death receptor-mediated and granule-mediated killing. In the death receptor-mediated pathway, CTLs expressing ligands of the tumour necrosis factor (TNF) superfamily on their cell surface can kill target cells expressing the corresponding receptors. Examples of these ligand/receptor pairings include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4, and Apo2L/DR5 (Ashkenazi & Dixit, 1998; Chicheportiche et al., 1997; Peter & Krammer, 1998; Rubio-Moscardo et al., 2005; Suliman et al., 2001). Engagement between a ligand and its receptor initiates a signalling pathway within the target cell that results in the recruitment and activation of caspases. Caspases, a family of cysteine proteases, are the main effectors of apoptotic cell death by cleaving cellular substrates on the carboxy-terminal side of an aspartate residue (Launay et al., 2005).

However, the granule-exocytosis pathway of cytotoxicity is the dominant mechanism by which $CD8^+$ cytotoxic T cells destroy target cells (de Vries et al., 2007; Yasukawa et al., 2000). CTLs contain modified lysosomes filled with cytotoxic proteins, collectively called cytotoxic granules. The granules are synthesized only after the CTL has encountered its specific peptide-MHC and has become activated. The major granule components include the pore-forming protein, perforin, and granzymes. Recognition of a target cell by a CTL triggers the rapid migration of the granules inside the effector cell towards the site of contact i.e. immunological synapse. At this site, the granules fuse with the effector cell plasma membrane and are then secreted into the tight intercellular junction (Cullen & Martin, 2008). Following this 'kiss of death', which can be as short as 20 mins, the CTL can disengage, unharmed, to kill again; thus, CTL can perform serial target cell killing (Cullen & Martin, 2008; Rothstein et al., 1978).

By a mechanism still not fully understood, the cytotoxic granule protein perforin is thought to polymerise within the target cell membrane forming pores that permit passage of other granule constituents, such as granzyme B, into the target cell. An alternative mechanism that has been proposed involves the endocytosis of granzyme B via a specific target cell surface receptor, the mannose-6-phosphate receptor. The internalised granzyme B is retained in an endosome-like vesicle which is then perforated by the perforin, releasing the granzyme B molecules into the cytosol of the target cell (Barry & Bleackley, 2002).

1.5.7.2 The role of granzyme B in T cell-mediated cell death

Granzymes are a subclass of serine proteases displaying the chymotrypsin fold (Smyth et al., 1996). In humans there are a total of five granzymes; A, B, H, K and M.

Granzymes A and B are generally the most abundant, and are the most extensively studied. However, all of the granzymes appear to play a role in the lysis of malignant cells, albeit via different pathways (D. Chowdhury & Lieberman, 2008). Granzyme B is a major constituent of $CD8^+$ CTL granules, promoting target cell death (apoptosis) through proteolysis of a number of cellular substrates. Knockout mouse studies have demonstrated a vital role for granzyme B in target cell DNA fragmentation and apoptosis, with granzyme B-deficient CTLs exhibiting delayed killing, suggesting that this granzyme is required for the rapid and efficient killing of the target cell (Heusel et al., 1994; Pardo et al., 2004).

Once in the target cell cytosol, granzyme B initiates apoptosis primarily through the proteolysis of the BH3-only protein BID (Pinkoski et al., 2001; Waterhouse et al., 2006). Cleavage product truncated BID (tBID) translocates to the mitochondria where it induces the oligomerisation of the BCL-2 family members, BAX and/or BAK in the outer mitochondrial membrane (J. B. Alimonti et al., 2001; Barry et al., 2000; Heibein et al., 2000). This promotes mitochondrial permeabilisation that permits the release of cytochrome *c* in to the cytosol, which leads to the formation of a complex known as the apoptosome, between Apaf-1 and pro-caspase-9 (Jiang & Wang, 2000; P. Li et al., 1997). Assembly of the apoptosome results in caspase-9 activation followed by the activation of down-stream caspases, such as caspase-3 and -7 (Slee et al., 1999). Granzyme B can also bypass the mitochondrial pathway and initiate caspase activation directly and/or cleave caspase substrates such as the inhibitor of caspase-activated deoxyribonuclease (ICAD), thereby allowing CAD to translocate to the nucleus to fragment DNA (Cullen & Martin, 2008) (Figure 1-3).

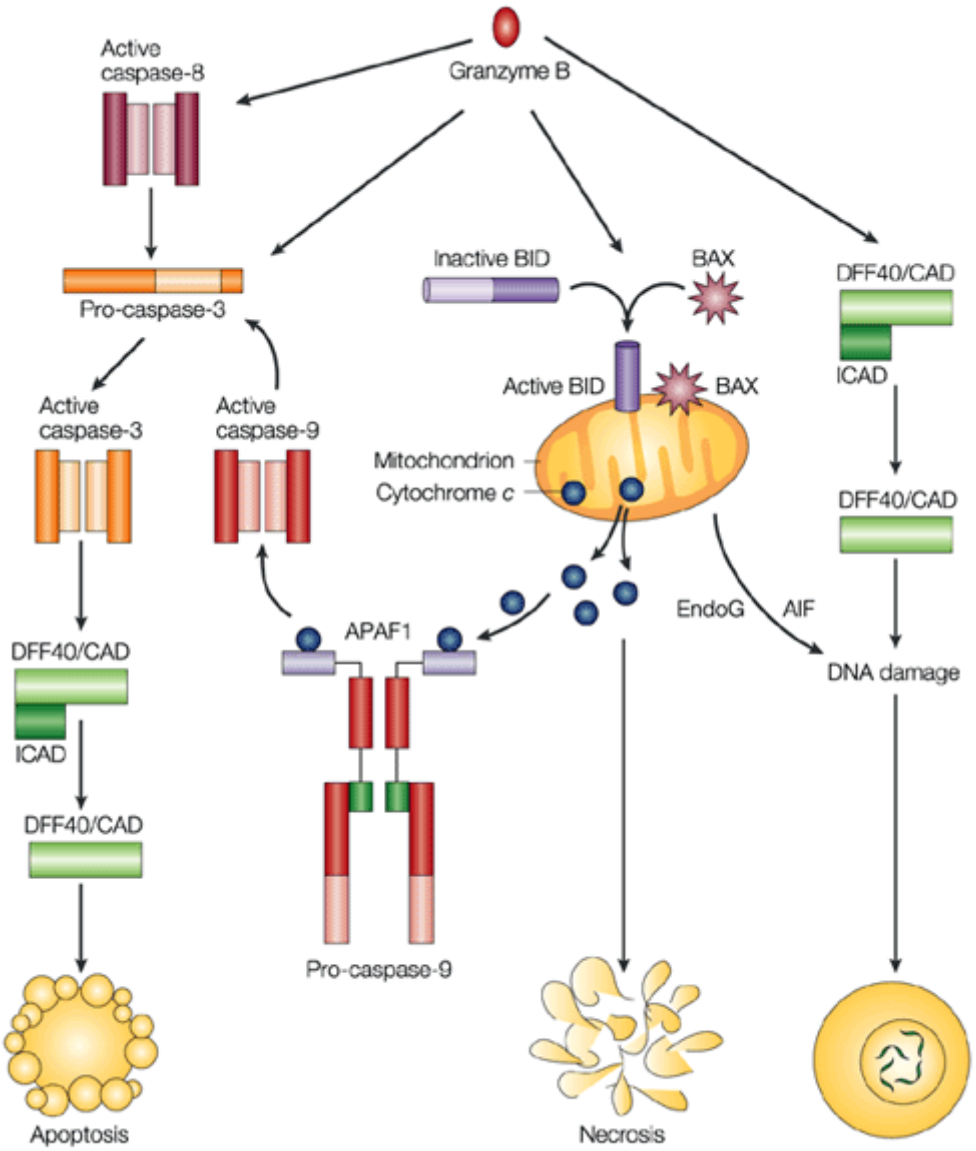


Figure 1-3 Mechanisms of granzyme B mediated cell death.

See section 1.5.7.2 for detailed description (Barry & Bleackley, 2002).

1.5.8 The failure of the immune system against cancer

The main reason for the lack of a robust immune response by T cells against tumour-associated antigens presented on tumour cells is because these antigens are usually derived from 'self' (as described in section 1.3.1). The process of central tolerance i.e., the deletion of high affinity TCRs to self pMHC during thymic development results in low affinity TCRs to self antigens. Direct biophysical measurements show that TCRs specific for tumour antigens bind to their cognate pMHC with affinities that lie approximately one order of magnititude below corresponding affinities of TCRs specific for non-self antigens (Aleksic et al., 2012). The low affinity of anti-cancer TCRs is thought to impair the ability of T cells to mount a full response to tumour cells.

Tolerance to self-antigens impedes an effective immune response against tumours. Even so, it has been shown that T cells do in fact home to tumour sites, at least in some patients. Tumour infiltrating lymphocytes (TILs) have been observed in patients with melanoma (Elder, 1999; Lyle et al., 2000) , colon cancer (Prall et al., 2004) and ovarian cancer (L. Zhang et al., 2003), amongst others, and these studies suggest the presence of TILs correlate with more positive clinical prognosis. However, even when an immune response is mounted, tumours can evolve active mechanisms to avoid detection and destruction by the immune system. In a process described as 'cancer immuno-editing', the immune system can promote the growth of tumour cells by selecting for tumour escape variants with reduced immunogenicity (Dunn et al., 2004). Commonly, immune escape mechanisms involve the loss of tumour-associated antigens or down-regulated MHC antigen expression, described in more detail in section 1.2.3. However, tumours can evade T cell responses by a plethora of complex mechanisms which can lead to an

immunosuppressive tumour microenvironment (reviewed in (Rabinovich et al., 2007)); some of these mechanisms are described below.

Tumours can upregulate suppressive ligands such as PD-LI or PD-L2 that engage PD-1 on the surface of activated T cells, causing T cell anergy or exhaustion (Hamanishi et al., 2007). The release immunosuppressive molecules by tumours such as indoleamine 2,3-dioxygenase (IDO), limits T cell function by depleting the amino acid tryptophan (Mellor & Munn, 2004; Munn & Mellor, 2004). Dendritic cells (DCs) are the most efficient APCs and an effective anti-tumour response depends on the presentation of tumour antigens by DCs; to inhibit this, tumour cells can secrete agents which affect the differentiation and function of DCs. For example, tumour cells can produce large amounts of the angiogenic factor vascular endothelial growth factor (VEGF) which has been shown to suppress the differentiation and maturation of dendritic cells (Gabrilovich et al., 1996). Expression by tumours or non-tumour stromal cells of the immunoregulatory cytokines TGF-β and IL-10 also exert suppressive effects on the immune system (Rabinovich et al., 2007). Transforming growth factor-β (TGF-β) is a pleiotrophic immunosuppressive cytokine that inhibits T cell function (M. O. Li et al., 2006). Indeed, elevated levels of TGF-β levels have been shown to be associated with poor prognosis in a number of different cancers (reviewed in (Drake et al., 2006)). Furthermore, it has been demonstrated that TGF-β acts on CTLs to suppress their cytolytic activity by repressing the expression of perforin, granzyme B, Fas ligand (FasL) and IFN-γ (Thomas & Massague, 2005). IL-10 has been shown to impair DC functionality (Gerlini et al., 2004) and to protect tumour cells from CTL-mediated cytotoxicity by down-regulating TAP1 and TAP2 (Kurte et al., 2004).

Regulatory T cells (Tregs) are a specialised subset of $CD4^+$ T cells that can suppress immune responses. They play an important role in preventing autoimmunity

but can also hamper a robust effector T cell response against tumours through multiple mechanisms, including expression of CTLA-4. Furthermore, the infiltration of Tregs has been shown to correlate with poor prognosis in a number of tumour types (Curiel et al., 2004; Kono et al., 2006). In addition to Tregs, other cell types can promote immune suppression in tumours. Myeloid-derived suppressor cells (MDSCs), a heterogeneous population that includes direct progenitors of DCs, macrophages and granulocytes, can also be recruited into the tumour site and have been shown to inhibit tumour-specific T cell responses by a variety of mechanisms, including nitric oxide, reactive oxidative species and arginase (reviewed in (Gabrilovich et al., 2012)). Finally, tumour stroma cells, which comprise non-malignant host cells and extracellular matrix, exert tumour-promoting and immunosuppressive effects. For example, mesenchymal stem cells block proliferation and function of effector T cells (Aggarwal & Pittenger, 2005).

1.6 Targeted immunotherapy

Recent advances in our understanding of how tolerance, immunity and immunosuppression regulate anti-tumour immune responses have led to the development of more targeted cancer immunotherapeutics that are beginning to show durable therapeutic benefit in the treatment of cancer. To date, the most significant development in cancer immunotherapy was the readout of the ipilimumab phase III trials in late-stage metastatic melanoma in which a clear survival benefit was observed. Furthermore, the survival benefit was still durable after 2.5 years and included a complete response in some patients (Hodi et al., 2010). Ipilimumab, a monoclonal antibody specific to CTLA-4, blocks an important inhibitory signal for activated T cells allowing enhanced T cell stimulation and a more potent anti-tumour response. In March 2011 ipilimumab was approved by the US Food and Drug Administration (FDA)

for the treatment of unresectable or metastatic melanoma. The clinical results with ipilimumab highlight the anti-tumour potential of endogenous T cell responses and therefore support the concept of immune-based therapies as a pathway to achieving durable and long-lasting responses in cancer patients.

1.6.1 Cancer T cell vaccines

There are two kinds of cancer vaccine; prophylactic and therapeutic (Palucka et al., 2010). Prophylactic (preventive) vaccines are used for the prevention of cancers of viral origin, such as human papillomavirus (HPV) which is associated with cervical cancer, and have proved to be very successful (Medeiros et al., 2009) (Munoz et al., 2010). However, in contrast, the development of therapeutic vaccines to treat existing disease has posed a considerable challenge even in patients with minimal residual disease where the goal has been to prevent relapse rather than induce the host to eliminate a solid tumour (Okur & Brenner, 2010).

Therapeutic cancer vaccines developed from the discovery that patients can harbour T cells specific for tumour-associated antigens expressed on their tumours (Boon et al., 2006). The principal objective of cancer vaccination is to amplify the frequency and strength of these pre-existing anti-tumour T cell responses. Early attempts at vaccination met with limited success due to the failure to identify suitable target antigens to mitigate the immunosuppressive tumour microenvironment and generate an effective immune response. However, advances in our understanding of the immune system and in particular that of tumour immunity has led to more sophisticated vaccine strategies which are beginning to show more promise. Various cancer vaccination strategies have been investigated including the use of whole-tumour cells or lysates, dendritic cells, peptide-based approaches, recombinant proteins, and viral and

DNA delivery vectors. The first therapeutic cancer vaccine to be approved by the FDA was Sipuleucel-T (Trade name: Provenge) in April 2010 for the treatment of metastatic hormone-refractory prostate cancer. Sipuleucel-T comprises a complex mixture of autologous peripheral blood mononuclear cells including antigen-presenting cells (APCs) that have been activated in *ex vivo* with a recombinant protein composed of prostatic acid phosphatase (PAP, a tumour-associated differentiation antigen) and the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF). In phase III clinical trials, Sipuleucel-T was found to improve median survival of patients by 4.1 months which was deemed significant even though there was little evidence of actual tumour shrinkage or delay in disease progression (Mellman et al., 2011; Pandolfi et al., 2011).

Despite the huge number of patients that have been treated with cancer vaccines, the overall objective response rate remains low. For example, in 2004, 541 different cancer vaccines had been tested in a total population of 440 patients with metastatic melanoma at the National Cancer Institute (NCI), Bethesda; the overall objective response rate was found to be 2.6% and this is consistent with many other such trials (Rosenberg et al., 2004). Although there have been some promising developments, cancer vaccines are still failing to successfully conquer the barriers of immune tolerance and immune suppression, and more effective strategies are needed.

1.6.2 Adoptive T cell therapy

Adoptive cellular therapy (ACT) presents a promising approach to breaking down the barriers of immune tolerance that has hampered conventional vaccination strategies. ACT involves the administration of autologous or allogeneic T cells which have been manipulated and expanded *ex vivo* to exhibit high specificity for TAA (Rosenberg et al.,

2008). The major break-through for this technology occurred in 1988 with the treatment of patients with metastatic melanoma with autologous tumour-infiltrating lymphocytes (TILs) following host lymphodepletion (Dudley et al., 2005; Rosenberg et al., 1988). However, despite cases of successful treatment of malignant melanoma, the approach did not translate so well for other cancers where TILs could not be easily identified and expanded (R. A. Morgan et al., 2006). In addition, TILs often became tolerant to TAA and are difficult to re-activate into an effective state. Genetically engineering antigen-specifically into T cells offers a powerful way to combat tolerance and can be achieved in a number of ways, as outlined in the following.

1.6.2.1 TCR gene transfer

TCRαβ chains derived from a T cell with a high affinity for a defined MHC-restricted TAA, or a TCR which has been genetically engineered for high affinity for a particular TAA, are transduced into a new T cell using an integrating vector. A T cell is created that possesses both the endogenous (natural) and exogenous (introduced) TAA-specific TCR (Rosenberg et al., 2008). Clinical trials using TCR gene transfer against various tumours expressing distinct TAAs have been carried out and have shown encouraging signs of efficacy. For example, in a recent clinical trial using T cells engrafted with a NY-ESO-1-specific TCR against NY-ESO-1 expressing cancers, metastatic melanoma and synovial cell sarcoma, five of 11 melanoma and four of six synovial cell sarcoma patients showed objective clinical responses. Two of 11 melanoma patients showed complete tumour regression which persisted after one year (Robbins et al., 2011).

1.6.2.2 Chimeric antigen receptors (CARs)

CARs combine the antigen-binding domains from the variable regions of a TAA-specific monoclonal antibody (scFv) linked to the signalling domain of the T cell receptor-ζ (TCR-ζ) and are capable of triggering T cell activation in much the same way as that of the endogenous TCR (Eshhar et al., 1993; Irving & Weiss, 1991). The main advantage CARs have over TCR gene therapy is that they are not MHC-restricted and do not need to be HLA-matched to the patient. This also means that an anti-tumour response can be generated even when MHC molecules have been down-regulated (Okur & Brenner, 2010). First generation CARs, T cells showed limited efficacy in the clinic due to poor *in vivo* persistence (Kershaw et al., 2006; Park et al., 2007). However, successive generations of CARs have incorporated additional signalling domains such as CD28 to harness both TCR activation and costimulation mechanisms (Finney et al., 1998; Friedmann-Morvinski et al., 2005; Maher et al., 2002). To improve persistence, the introduction of 4-1BB (CD137) domains has been shown to enhance CAR-transduced T cell survival and function by inducing production of the anti-apoptotic protein Bcl-x (Zhao et al., 2009). The therapeutic potential of CARs has recently been demonstrated in the clinic when three patients with chronic lymphocytic leukaemia treated with CD19/4-1BB/CD3ζ CAR T cells exhibited strong anti-tumour responses. Two of the three patients went into complete remission and the CAR+ T cells showed encouraging levels of *in vivo* expansion and persistence (Kalos et al., 2011).

1.6.3 Bispecific T cell Activators

Bispecifics are a class of antibodies which are being developed to harness the most potent effector cells of the immune system i.e., cytotoxic T lymphocytes (CTLs), for the elimination of tumour cells. The recruitment of T cells is the major advantage

bispecific antibodies have over conventional monoclonal antibodies which cannot engage T cells. Bispecific antibodies are constructed using cell-fusion or recombinant DNA technology to possess dual non-identical binding arms. One arm binds the monomorphic CD3 complex expressed on the surface of T cells. CD3 is a potent signal transducer associated with the polymorphic TCR. Thus, engagement of CD3 by bispecific antibodies can trigger TCR signalling regardless of the clonotypic antigen specificity of the T cell. The second arm binds to a tumour-associated antigen on the target cell. Bispecific antibodies bind to the tumour thereby decorating it with a matrix of binding sites for T cells. A passing T cell can then engage with the tumour cell via the bispecific antibody and become activated to kill the bound partner. A variety of bispecific antibody formats for recruiting T cells have been developed including diabodies, tandem diabodies, quadroma, F(ab)2 and single-chain antibody (scFv)-based constructs (reviewed in (Carter, 2001; Kufer et al., 2004; von Mehren et al., 2003)). However, most bispecific antibodies are not very efficient at triggering T cell redirected killing against target cells and typically require additional costimulation with anti-CD28 or pre-stimulation of the T cells as well as a high effector to target (E:T) ratio. There have also been issues with Fc-mediated effects and immunogenicity resulting in severe adverse effects such as cytokine release syndrome. To date, the only approved bispecific antibody is catumaxomab (Removab), developed by Fresenius Biotech, which targets CD3 and EpCAM (epithelial cell adhesion molecule), a pan-epithelial differentiation antigen expressed on nearly all carcinomas. Catumaxomab is also termed trispecific because the mode of manufacture ensures that the antibodies retain their natural Fc effector function thus providing a third binding site for cells with an Fc receptor. It is used for the intraperitoneal treatment of malignant ascites (a common manifestation of advanced cancer) in patients with EpCAM-positive carcinomas

(Sebastian et al., 2009). Recently though, one particular format of bispecific antibody has attracted special attention. These molecules are called BiTE (bispecific T-cell engager) antibodies.

BiTE antibodies, developed by Micromet Inc., represent a new class of bispecific T cell activator antibodies that have overcome many of the previous limitations. BiTE antibodies are recombinant protein constructs composed of two binding domains (variable heavy (V_H) and variable light (V_L) chain domains) of two different human IgG antibodies flexibly linked by a short non-immunogenic peptide. The two single-chain Fvs are arranged in tandem on a single non-glycosylated polypeptide chain of approximately 55 kDa molecular weight. One scFv is specific for a tumour-associated surface antigen on target cells and the second scFv is specific for CD3, present on all mature T cells. The small size and short flexible linker of BiTEs facilitate close proximity between T cell and target cell membranes when both cell populations are bound to the respective binding arm. By binding to CD3, BiTE antibodies are able to recruit T cells regardless of their TCR specificity and typically recruit $CD8^+$ cytotoxic T cells of the memory phenotype rather than naïve T cells, resulting in a polyclonal T cell response. The main advantage BiTE antibodies have over other bispecific antibodies is that they do not require a costimulatory signal by CD28 or engagement by CD4 or CD8 coreceptor to induce T cell activation. This feature might be explained by the ability of BiTE antibodies to induce an immunological cytolytic synapse between target cell and cytotoxic T cell which has been shown to bear all the hallmarks of the synapse induced by the interaction of specific TCR and pMHC. The transient nature of the interaction also permits serial killing of many targets by single cytotoxic T cells which results in the lysis of targets at low E:T ratios (Baeuerle et al., 2003).

In vitro, BiTE antibodies have been demonstrated to potently mediate redirected tumour cell killing in culture assays at half maximal concentrations (EC_{50} values) in the low pg/ml range. Furthermore, their potency far exceeds that of some conventional mAb therapies (Dreier et al., 2002). *In vivo*, anti-tumour effects were shown in xenograft and syngeneic mouse models, and in a non-human primate model. There are several BiTE antibodies currently in clinical trials, the furthest developed of these is blinatumomab which targets CD19 on lymphoma and leukemia cells and CD3 on T cells. In a Phase I clinical study in patients with relapsed B cell non-Hodgkin's lymphoma (NHL), treatment with blinatumomab resulted in a 100% response rate with 2 complete and 5 partial responses; furthermore efficacy was seen at very low dose levels (Bargou et al., 2008). In a Phase II trial in patients positive for minimal residual disease of acute B-cell lymphoblastic leukemia (B-ALL) following conventional treatment, blinatumomab induced a complete response in 80% of patients (16 out of 20) (Topp et al.). However, CD19 is not a tumour-specific antigen but is also present on normal B cells. Therefore, adverse events (AEs) of varying severity have been observed with blinatumomab (Nagorsen & Baeuerle, 2011). Furthermore, unexpectedly, in some patients symptoms related to the central nervous system were also observed.

1.6.4 TCR-directed therapeutics

1.6.4.1 The T cell receptor (TCR) as a targeting molecule

The main advantage of using TCRs as a targeting molecule is that they can target intracellular antigens, which are presented on the cell surface as peptides in the context of MHC molecules, whereas mAbs cannot. Notwithstanding the extremely broad range of uses for which they can be applied, mAb are limited to cell-surface TAA, which

represent only a small fraction of the total antigen repertoire. For the treatment of cancer, this is a significant limitation because secreted or cell surface-bound proteins represent a relatively small proportion, probably 10-15%, of malignant state-associated antigens. However, the potential applicability of a TCR-directed therapeutic is governed by the prevalence of both the presenting MHC allele and of the antigen from which the peptide antigen is derived. Although HLA type limits the proportion of patients that could potentially benefit from TCR-mediated cancer therapies, the availability of a large choice of intracellular antigens compared to other cell surface antigens means that pMHC antigens can offer better patient coverage than some antibodies. For example, Her2-neu as an antibody target in breast cancer is present in approximately 22% of the population (Nagai et al., 1993; Slamon et al., 1987), whereas an intracellular antigen such as gp100 is expressed in up to 95% of malignant melanomas (Barrow et al., 2006; Hofbauer et al., 2004; Mocellin et al., 2001; Spagnoli et al., 1995; Wagner et al., 1997). Given that the prevalence of HLA-A2 is approximately 50% in European and North American populations, a TCR specific for gp100 presented by HLA-A2 would therefore give approximately 47% coverage of that patient population.

1.6.4.2 Soluble TCRs

Unlike antibodies, TCRs do not exist naturally in a soluble form. Naturally occurring αβ TCRs are disulphide-linked heterodimers, with each chain comprising the N-terminal variable domain and the constant domain, followed by a transmembrane region and a short C-terminal intracellular signal-transducing tail. In order to engineer a soluble form of monoclonal TCR, a truncated version was designed whereby the native disulphide bond was removed and a new interchain disulphide link was introduced

between TRAC threonine 48 and TRBC serine 57, which were mutated to cysteines, to produce a highly stable TCR framework (Boulter et al., 2003) (Figure 1-4). This resulted in the generation of a highly stable, fully human soluble protein, designated as monoclonal TCR (mTCR).

1.6.4.3 High affinity TCRs

The wild-type affinities (K_Ds) of TCRs for their pMHC are between 1 – 100 μM (M. M. Davis et al., 1998), with binding half-lives of only a few seconds. The low affinity of native TCRs, a result of selection processes in the thymus, means that a monovalent soluble TCR would not be able to bind its target with sufficient affinity to be of therapeutic benefit. However, it has been demonstrated that no inherent genetic or structural limitations exist on TCR affinity (Holler et al., 2000). Utilising phage display technology, native TCR affinity has been improved almost a million-fold to produce TCRs with affinities in the picomolar range, similar to that of antibodies (Y. Li et al., 2005). The process of mTCR affinity maturation using phage display involves PCR mutagenesis to introduce variability into the targeted CDR regions of the α- and β-chains of the TCR. The CDR mutant libraries are then expressed on phage and panned against the specific peptide-MHC. At this stage, the affinity typically increases from micromolar to nanomolar. Creation of second generation phage TCR libraries, and repeated selections against decreasing concentrations of antigen, result in the isolation of TCRs with further incremental improvements in affinity. Optimum affinity mutants are made by combining mutations in several CDRs and making soluble mTCRs through *Escherichia coli* expression and *in vitro* refolding. The affinities of the resultant mTCRs are measured using surface plasmon resonance microchip-based detectors (BIAcore™); by this stage the TCRs are typically in the picomolar affinity range.

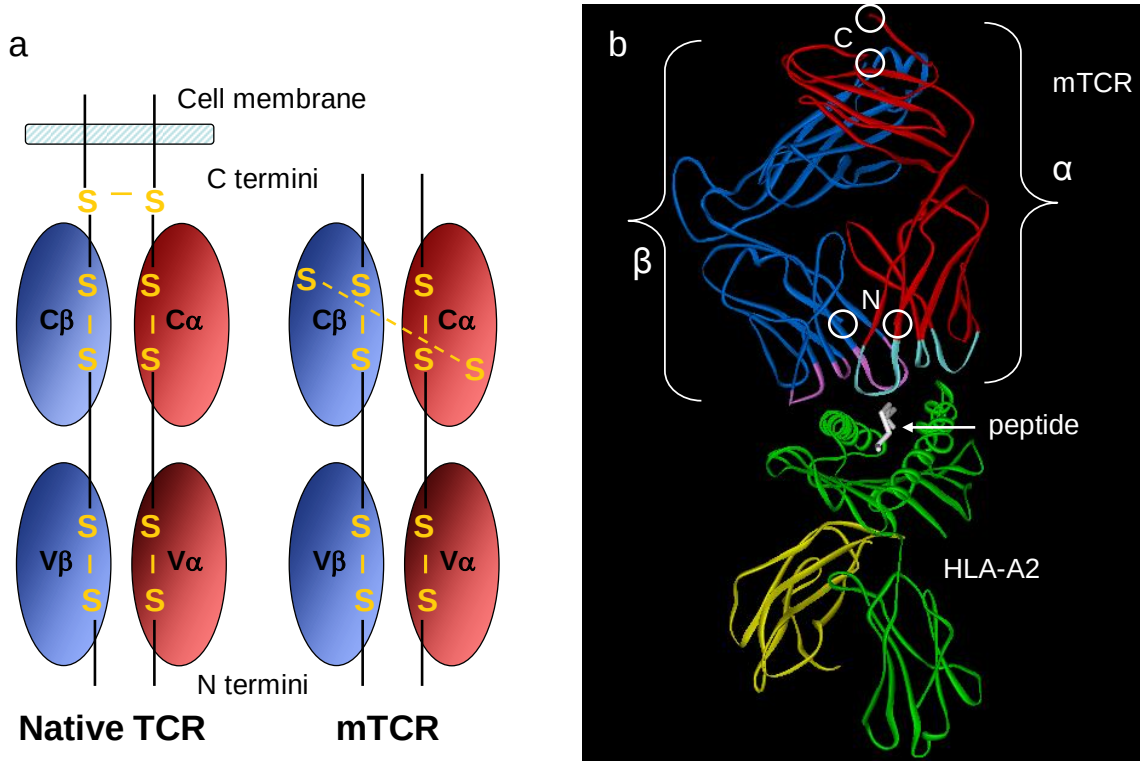


Figure 1-4 The monoclonal TCR (mTCR)

(a) Natural αβ TCRs (left) are disulphide-linked heterodimers, each chain comprising the N-terminal variable domain and the constant domain, followed by a transmembrane region and a short C-terminal intracellular signal-transducing tail. In the mTCR (right), the chains are truncated and the native interchain disulphide bond replaced by an engineered disulphide bond. Based on molecular modelling studies, an optimal site was selected that links the two constant domains producing highly stable and fully human soluble TCR molecules. (b) Crystal structure-based model of 1G4 mTCR bound to NY-ESO peptide-MHC. The ligand consists of MHC class I heavy chain (green) with the 9aa-long antigenic peptide (grey tube) buried within the groove formed between the two helices of the apical α3 domain and the non-covalently associated invariant β2m (yellow). The mTCR, a heterodimer of an α-chain (red) and a β-chain (blue), contacts the antigenic surface through its CDRs (highlighted by light cyan and light magenta colours). The centrally positioned CDR3 loops of both chains predominantly contact the peptide, while the CDR1 and CDR2 loops (appearing furthest from the groove) contribute mostly to the contacts with the relatively conserved HLA α3 domain. The locations of the N- and C-termini of both TCR chains (encircled), can potentially serve as fusion points for the attachment of effector function moieties.

Phage display is highly efficient as it can be used to screen libraries of up to 10^{10} variants and the technology can be applied to any mTCR. Notably, crystal structures of high affinity human TCRs bound to pMHC have shown that the docking mode of enhanced affinity TCRs is identical to that of the parental wild-type TCR, and they exhibit only slight structural alterations in the mutated complementarity determining regions (CDRs) (Sami et al., 2007) (Figure 1-5) .

Fluorescently labelled high affinity mTCRs for TAPAs have permitted the detection of single antigens on tumour cell lines and shown that, as monovalent molecules, they do not cross-react to any detectable extent with self-pMHC (Purbhoo et al., 2006).

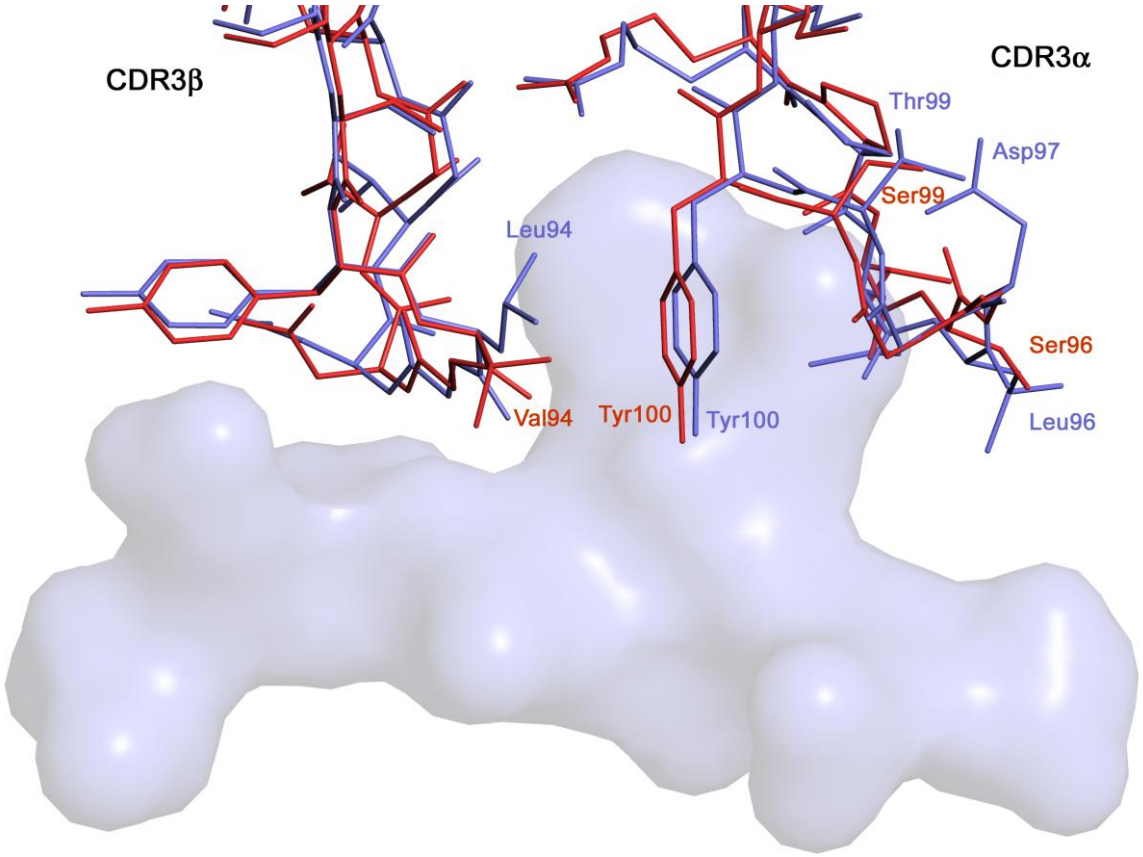


Figure 1-5 Structural alterations in CDR3α and CDR3β loops between wild-type and affinity enhanced mTCR.

CDR3 loops shown as sticks. Wildtype 1G4 TCR specific for NY-ESO-$1_{157-165}$ peptide, binding $T_{1/2}$ = 7 seconds (red), and affinity enhanced 1G4 TCR, binding $T_{1/2}$ = 19 hours (blue), on top of NY-ESO peptide van der Waals surface (light blue) (Sami et al., 2007).

1.6.4.4 Immune-mobilising monoclonal TCRs against cancer (ImmTAC)

Although high affinity mTCRs offer potential as diagnostic tools (Purbhoo et al., 2007; Purbhoo et al., 2006), to be of therapeutic use they require an effector function which can enable the potent killing of target cells. BiTEs have successfully demonstrated the potent cytotoxic potential of the T cell-activating anti-CD3 scFv antibody. Conceptually similar, ImmTACs which are approximately 77 kDa molecular weight, comprise a humanised CD3-specific scFv fused to the high affinity mTCR β chain by a flexible linker; the mTCR part of the fusion binds to an antigenic peptide-MHC on the target cell whilst the other end of the molecule, by binding the ε-chain of the CD3 glycoprotein, is capable of activating any $CD8^+$ CTL, regardless of its intrinsic TCR specificity (Figure 1-6). As is the case for BiTE antibodies, T cell activation induced by ImmTAC molecules does not require a costimulatory signal by CD28 or, CD4 or CD8 coreceptors. Also, the recruited T cells are mostly effector memory or central memory T cells that have encountered antigen previously, rather than naïve T cells. Mechanistically, the main difference between the two reagents is that BiTEs target thousands of antigens per cell whereas ImmTACs will, in most cases, be targeting only a few tens of the cognate HLA-peptide complex. Therefore, ImmTACs have to be highly efficient at initiating optimal contacts for the formation of an immunological synapse.

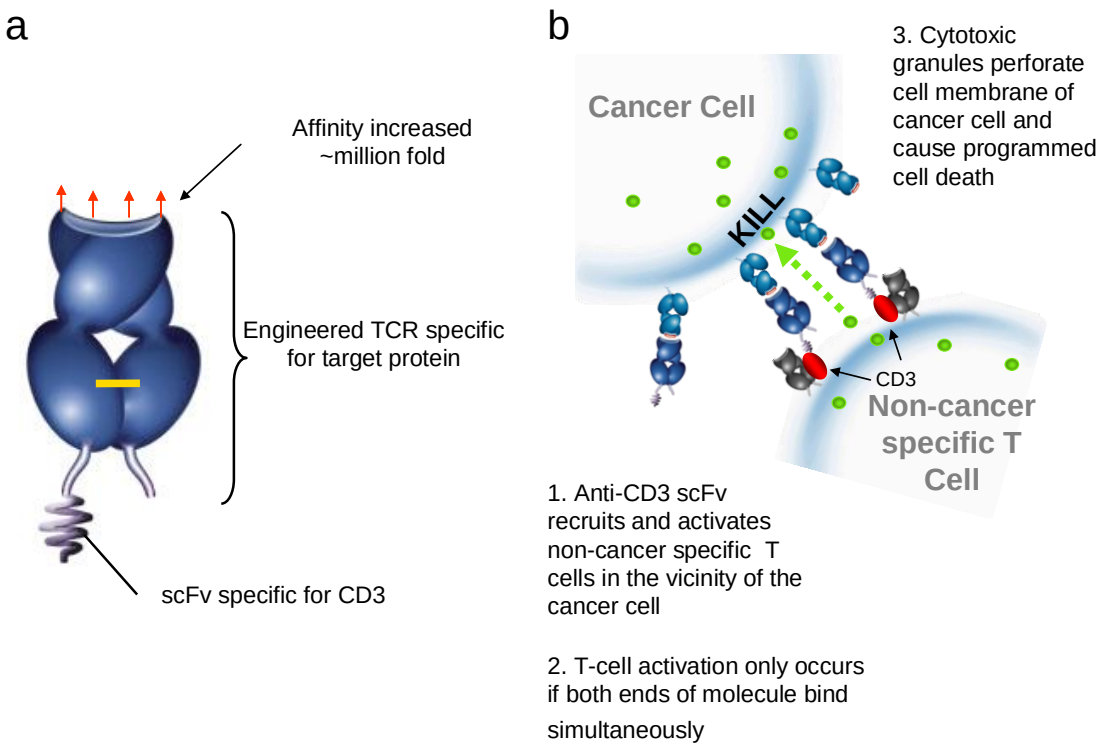


Figure 1-6 Redirection of T cells against cancer with ImmTAC.

(a) ImmTAC molecules comprise a picomolar affinity mTCR, specific for a selected cancer-related peptide-MHC, and an anti-CD3 scFv. **(b)** The mTCR component engages strongly to its ligand on a cancer cell, while the anti-CD3 scFv component activates a proximal T cell, independently of its native specificity. This results in the redirected killing of the bound cancer cell.

1.7 Aims of this thesis

ImmTACs are novel bispecific agents intended for targeted cancer therapy. These molecules have been designed to overcome the limitations of natural TCR-mediated recognition and redirect T cells of the host immune system to kill tumours expressing even very low epitope numbers on their cell surfaces. ImmTACs comprise a soluble TCR with an enhanced affinity for tumour associated pMHC class I fused to a humanised anti-CD3 scFv, thereby combining enhanced epitope targeting with potent T cell activation.

The four ImmTAC reagents investigated in this study comprise a high affinity mTCR specific for the following tumour-associated epitopes: (1) $gp100_{280-288}$, YLEPGPVTA-HLA-A*0201 (Cox et al., 1994); (2) $MAGE\text{-}A3_{168-176}$, EVDPIGHLY-HLA-A*0101 (Celis et al., 1994); (3) Melan-A/$MART\text{-}1_{26-35}$, EAAGIGILTV-HLA-A0201 (Kawakami, Eliyahu, Sakaguchi et al., 1994); (4) $NY\text{-}ESO\text{-}1_{157-165}$, SLLMWITQC-HLA-A*0201 (Jager et al., 1998).

The first objective of this study was to investigate the potency, antigen sensitivity, and specificity of the ImmTAC reagents in *in vitro* cellular assays and assess their suitability for, and potential as, clinical immunotherapies for cancer. Secondly, a further aim was to employ *in vitro* T cell assays to define a safe clinical starting dose, and maximum dose, for the selected first-in-human ImmTAC, which targets the metastatic melanoma epitope $gp100_{280-288}$ presented by HLA-A*0201.

CHAPTER 2 MATERIALS AND METHODS

2.1 TCR-based reagents

TCR isolation and engineering to produce soluble mTCR, mTCR-anti-CD3 scFv and ImmTAC fusions has been described previously (Y. Li et al., 2005; Liddy et al., 2012). Briefly, TCR genes were isolated from T cell clones by PCR, then modified by deletion of the gene segments encoding the transmembrane and cytoplasmic domains and by a single mutation to introduce a cysteine residue in each of the constant domains of the α and β chains. For production of mTCR protein, the modified α and β chain genes were expressed separately in *E.coli* and harvested from the bacteria in the form of inclusion bodies. The protein chains were denatured in buffer containing guanidine, then refolded by dilution into buffer enabling the native conformation. Refolded TCR was purified using anion exchange and size exclusion chromatography. Finally, the TCR reagents were formulated in phosphate buffered saline, typically at a concentration of 1 mg/ml.

High affinity TCR variants were identified from TCR phage display libraries. The TCR β chain was fused to gene III, whereas the α chain was expressed in soluble form; this enabled the formation on the surface of phage particles of TCR proteins corresponding to the extracellular part of the receptor. Libraries with extensive mutations in each CDR region were generated by directed PCR mutagenesis; several individual phage libraries were generated for each CDR. Mutants with increased affinity were isolated by, typically, three rounds of panning with the cognate pHLA complex and the affinity effects of individual and combined sets of mutations were assessed by producing, as described above, soluble mTCR proteins for which the

affinities for pHLA were determined by BIAcore™ surface plasmon resonance binding analysis to immobilised pHLA complexes.

2.2 General Cell Culture

Tissue culture reagents were purchased from Life technologies™ Corporation, and Corning Costar® Tissue culture plasticware was used, except where otherwise indicated.

2.2.1 Human cell lines

A list of cell lines used in this study is presented in Table 2-1. HLA genotype and/or phenotype and expression of various cancer-associated antigens were confirmed by DNA sequencing, RT-PCR, and antibody staining, either by co-workers *in-house* or by external collaborators.

2.2.2 Human primary cells

2.2.2.1 Primary cells grown in culture

A list of the normal human primary cells used in this study is presented in Table 2-2. Primary cells were grown in custom made tissue culture media purchased from the supplier and cultured as per the manufacturer's instructions.

2.2.2.2 Primary tumour tissue

Primary tumour tissue from a lung cancer patient (NSCLC#29) was kindly provided by Dr. Michael Kalos, University of Pennsylvania. The tumour biopsy was supplied as a frozen aliquot which had been processed to obtain single-cell suspension. For use in cellular assays, the vial was thawed and the cells used immediately.

Table 2-1 Cell lines; cell type and source

Cell lines	Cell type	Source
A375	Human melanoma	American Type Culture Collection (ATCC)
Colo205	Human colorectal adenocarcinoma	American Type Culture Collection (ATCC)
EJM	Human multiple myeloma	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)
HCT116	Human colorectal carcinoma	American Type Culture Collection (ATCC)
IM9	Human EBV-transformed B cell line	American Type Culture Collection (ATCC)
Mel526	Human melanoma	Thymed
Mel624	Human melanoma	Thymed
SK-Mel-37	Human melanoma	Ludwig Institute for Cancer Research (LICR)
T2	Human hybrid B and T cell lymphoblast	American Type Culture Collection (ATCC)
U266	Human multiple myeloma	American Type Culture Collection (ATCC)

Table 2-2 Primary cell grown in culture; cell type and source

Primary cell lot number	Cell type	Source
CM12	Cardiac myocyte	ScienCell
HA2	Astrocyte	ScienCell
HEP2	Hepatocyte	ScienCell
NHEM10	Epithelial melanocyte	PromoCell

2.2.2.3 Frozen peripheral blood mononuclear cells (PBMC) derived from multiple myeloma patients

PBMC derived from multiple myeloma patients were purchased from AllCells as frozen aliquots, and were thawed and used on the day of the assay.

2.2.3 Tissue culture media

Cell lines were grown at 37°C with 5% CO_2, in RPMI 1640 (with phenol red) medium supplemented with 10% heat-inactivated foetal bovine serum (HI FBS) (Sera Laboratories International), 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (R10 medium). Cell lines were regularly screened for mycoplasma infection, carried out by Mycoplasma Experience Ltd, UK.

2.2.4 Thawing cell lines

Vials of cryopreserved cells were removed from liquid nitrogen storage and thawed in 37°C water bath while being constantly agitated. Immediately upon thawing cells were transferred into a 15 ml centrifuge tube containing 10 ml pre-warmed R10 medium. Cells were centrifuged at 300 g for 5 mins and the supernatant discarded. The cell pellet was resuspended in 15 ml R10 media and transferred to a T75 flask.

2.2.5 Maintenance and sub-culturing of cell lines

Every 2 to 3 days, cells were split and a portion thereof seeded into fresh media. Care was taken to ensure the cells did not exceed 80-90 % confluency and/or turned the media yellow, indicating a decrease in pH level due to the build up of waste products. For adherent cell lines, the whole content of the tissue culture flask was transferred into a 50 ml centrifuge tube and the flask rinsed with PBS to remove all remains of the

media. Three ml trypsin-0.25% EDTA was added to detach the cells from the inner surface of the flask. The flask was then incubated at 37°C for 2-4 mins and then rinsed with an equal volume of R10 media to inhibit further trypsinisation. The bulk cell suspension was transferred to the 50 ml centrifuge tube and the flask was then rinsed with 10 ml PBS to maximise the number of cells harvested and added to the tube. The tube was then centrifuged at 300 x g for 5 mins and resuspended in R10 medium for counting. Cells were plated into a new flask at the recommended seeding density (between 5,000 and 10,000 cells per cm^2). For suspension cultures, cells were resuspended thoroughly and counted if required. Cells were then split into fresh R10 medium in a new flask.

2.2.6 Counting cells

Cells were thoroughly resuspended, 25 µl of cell suspension was removed and mixed with equal volume of trypan blue 0.4% solution (Sigma-Aldrich) in a single well of a 96-well round bottomed plate. Twenty µl was loaded onto a disposable counting chamber (Nexcelom Bioscience) and inserted into an automated cell counting machine (Cellometer™ Auto T4, Nexcelom Bioscience). The machine automatically calculated the cell concentration and the percentage viability, based on trypan blue exclusion.

2.2.7 Cryopreservation of cells

Cells were centrifuged at 300 x g for 5 mins to remove culture media, and resuspended in freezing mix (90% HI FBS, 10% DMSO (Sigma-Aldrich)). One ml aliquots were frozen down in internal thread cryovials (Nunc) at -70°C using a Mr. Frosty® freezing container (Nalgene). Typically, each aliquot contained 2-5 x 10^6 cells. Once frozen, the cells were stored short- or long-term in liquid nitrogen at -196°C.

2.3 Donor blood samples

2.3.1 Fresh donors

Fresh blood from healthy donors was obtained *in-house* (donor code with prefix FB) and from the National Blood Service (NBS, Bristol) as buffy coats (donor code with prefix Buf). Fresh blood from melanoma patients (donor code with prefix IC) was obtained from Dr. Neil Steven, University of Birmingham. Donors were HLA typed by single strand conformational polymorphism (Tissue Typing Laboratory, Churchill Hospital, Oxford). Peripheral vein blood was collected into 50 ml centrifuge tubes containing 20 µl of 2000 U/ml of the anti-coagulant heparin (Sigma-Aldrich).

2.3.2 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated by Lymphoprep™ (Axis shield PoC, Norway) density gradient centrifugation. Peripheral blood was diluted at a ratio of 1.4:1 with Ca^{2+} and Mg^{2+} free Hanks Buffered Saline Solution (HBSS). To a 50 ml conical leucosep® tube (Greiner Bio-One) containing 15 ml Lymphoprep™, which had been centrifuged at 800 x g for 1 minute to force the Lymphoprep™ below the porous membrane, 35 ml of the diluted blood was carefully poured onto the top of the porous membrane. After checking that the caps of the tubes were properly secured, the tubes were centrifuged at 800 x g for 15 minutes (Sorvall Legend T™) at room temperature in a swinging bucket rotor with the brake off. A 10 ml serological pipette was used to remove as much of the plasma layer as possible without disturbing the cloudy white band of cells at the interface, and discarded. The interface layer, which contained the mononuclear cells, was drawn off using a 5 ml serological pipette and transferred to a fresh 50 ml centrifuge tube for washing. The tube was topped up to 50 ml with HBSS medium and centrifuged at 350 x g for 8 minutes (brake set to maximum). The supernatant was discarded and two

further washes were performed at 200 x g for 10 minutes to remove platelets. After the final wash, PBMC were either frozen down in 1 ml aliquots of between 5 x 10^6 and 2 x 10^7 PBMC following the procedure described in section 2.2.7, or resuspended in R10 medium and kept in the incubator at 37°C/5% CO_2 for further processing.

2.3.3 Isolation of mononuclear cells from bone marrow aspirates of multiple myeloma patients

Fresh bone marrow aspirates from multiple myeloma patients were obtained from Dr. Chris Hatton, Churchill Hospital, Oxford. Typically ~10 ml of bone marrow aspirate diluted in R10 medium was received. The sample was passed through a 30 μm filter (Miltenyi Biotech) to remove bone fragments. The filtered bone marrow aspirate sample was then gently layered onto an equal volume of Lymphoprep™ in a 50 ml centrifuge tube and centrifuged at 445 x g for 35 minutes at room temperature in a swinging bucket rotor without application of the brake. The bone marrow mononuclear cells (BM MNC) at the interface were carefully removed and transferred to a new 50 ml centrifuge tube for washing. The tube was topped up to 40 ml with buffer (Ca^{2+} and Mg^{2+} free PBS, 2 mM EDTA (Sigma-Aldrich)) and centrifuged at 300 x g for 10 minutes at room temperature with the brake set to maximum. The supernatant was then discarded and a final wash was performed at 200 x g for 10 minutes to remove platelets. After the final wash, BM MNC were either frozen down in 1 ml aliquots of between 2 x 10^6 to 2 x 10^7 BM MNC following the procedure described in section 2.2.7 or resuspended in R10 medium and kept in the incubator at 37°C/5% CO_2 for further processing as described in section 2.3.6).

2.3.4 Isolation of $CD8^+$ or $CD4^+$ T cells from PBMC

$CD8^+$ T cells were isolated from PBMC by depletion of non-CD8 T cells using Dynabeads® Untouched™ human CD8 T cell kit as per manufacturer's instructions. Fresh PBMC were centrifuged at 300 x g for 10 minutes and the pellet resuspended in 500 µl Isolation Buffer (Ca^{2+} and Mg^{2+} free PBS, 2% HI FBS, 2 mM EDTA) per 5 x 10^7 PBMC. Added 100 µl heat-inactivated FBS and 100 µl Antibody Mix per 5 x 10^7 PBMC and mixed thoroughly. The Antibody Mix contained biotinylated mouse IgG antibodies for CD4, CD14, CD16 (specific for CD16a and CD16b), CD19, CD36, CD56, CDw123 and CD325 (Glycophorin A). The $CD8^-$ T cells were labelled for 20 mins at 4°C. The cells were washed by adding 10 ml Isolation Buffer per 5 x 10^7 PBMC, inverting the tube several times to mix, and centrifuged at 350 x g for 8 mins. The cell pellet was resuspended in 500 µl Isolation Buffer per 5 x 10^7 PBMC and 500 µl pre-washed Dynabeads®, which are streptavidin coated superparamagnetic polystyrene beads of 1 µm diameter, were added. Gentle tilting and rotation for 15 minutes at room temperature enabled stable binding between the Dynabeads® and the antibody-labelled cells to occur. The bead-bound cells were then vigorously resuspended using a 1 ml pipette tip and 5 ml Isolation Buffer per 5 x 10^7 PBMC was added. The tube was placed in the magnet for 2 minutes where the bead-bound $CD8^-$ T cells were separated to the side of the tube due to magnetic attraction, while the untouched $CD8^+$ T cells remained in the supernatant. The supernatant containing the $CD8^+$ T cells was pipetted off and transferred to a fresh tube. The tube containing the Dynabeads® was removed from the magnet, and the bead-bound $CD8^-$ T cells vigorously resuspended in 5 ml Isolation Buffer, and placed in the magnet again for 2 minutes. This supernatant was also removed and the two supernatants were combined.

The same procedure applied to the isolation of $CD4^+$ T cells from PBMC by depletion of $CD4^-$ T cells using Dynabeads® Untouched™ human CD4 T cell kit. The purity of the enriched $CD8^+$ and $CD4^+$ T cells was typically between 85-90%, as determined by FACS staining.

2.3.5 Generation of $CD8^+$ T cell clones

The HLA-A*0201-restricted MEL187.c5 and EBV176.c4.1 $CD8^+$ T cells, specific for Melan-A/MART-1_{26-35} and EBV $BRLF1_{259-267}$ respectively, were generated as previously described (Adams, 2004).

2.3.5.1 Expansion and culture of CD8+ T cell clones

T cell clones were expanded from cryopreserved stocks. A restimulation mix was prepared as follows: vials of frozen PBMC (2×10^7 cells per vial) from three different donors were thawed and combined in the same tube containing 20 ml R10 medium. The cells were washed by centrifuging at 250 x g for 8 minutes. The supernatant was discarded and the PBMC resuspended in 10 ml R10 medium containing 5 μg/ml Phytohaemagglutinin (Sigma-Aldrich). The cell mixture was incubated at 37°C/5% CO_2 for 1 hour and then subjected to γ-radiation (Gammacell, Nordion) at 33 Gy. For cell culture: a vial of $CD8^+$ T cell clone (approximately 4×10^6 cells) was thawed and washed as above. Cells were resuspended in 4 ml of restimulation mix and seeded into 2 wells of a 24-well tissue culture plate. The plate was incubated at 37°C/5% CO_2 and left until the wells were confluent. Once the wells were confluent (no gaps between cells), cells were split 1:1 into a new well and topped up to 2 ml with R10 culture medium containing 25 μg/ml recombinant human IL-15 (Peprotech). T cell clones could be maintained for up to 10 weeks by replacing half the media with fresh R10

medium containing 25 μg/ml recombinant human IL-15, or cryopreserved according to the method described in section 2.2.7.

2.3.6 Isolation of $CD138^+$ plasma cells and marrow infiltrating lymphocytes (MILs) from BM MNC

MACS beads were used to positively select for $CD138^+$ plasma cells from fresh and frozen BM MNC samples (described in section 2.3.3). Following the manufacturer's instructions, 2 x 10^7 BM MNC were resuspended in 80 μl of chilled MACS buffer (Ca^{2+} and Mg^{2+} free PBS, 0.5% HI FBS, 2 mM EDTA), to which 20 μl anti-CD138 antibody coated microbeads (Miltenyi Biotec) was added. The cells and beads were mixed thoroughly and incubated for 15 minutes at 4°C. The cells were washed by adding 1-2 ml MACS buffer per 2 x 10^7 cells and centrifuged at 300 x g for 10 minutes. The supernatant was completely removed and the cell pellet resuspended in 500 μl MACS buffer and applied to a LS MACS positive selection column (Miltenyi Biotec), which had been equilibrated with 3 ml MACS buffer, held in a QuadroMACS magnetic separator (Miltenyi Biotec). The CD138 negative cells passed through the column and were collected in the flow through. This fraction contained the marrow infiltrating lymphocytes (MILs) and was used in *in vitro* cellular assays the same day. The percentage of $CD4^+/CD8^+$ T cells, within the $CD138^-$ sample, was 12% as determined by FACS staining performed by a co-worker (Dr. Giovanna Bossi). The column was washed with 3 x 3 ml MACS buffer, and the $CD138^+$ cells were eluted by removing the column from the magnetic separator and placing it on a 15 ml centrifuge tube. Then 5 ml of MACS buffer was applied to the column and the plunger was used to flush out the magnetically labelled cells. The $CD138^+$ cells were isolated with 99% purity, as

determined by FACS staining. Following their isolation, the $CD138^+$ cells were used immediately in the *in vitro* cellular assays of this study.

2.4 T cell Activation Assays

2.4.1 Enzyme linked immunosorbent assay (ELISA) for interferon-γ (IFN-γ)

T2 cells were resuspended in R10 medium and peptide-pulsed with Melan-A/MART-1_{26-35} heteroclitic peptide ELAGIGILTV at the stated concentrations for 90 minutes at 37°C/5% CO_2. The cells were washed three times in unsupplemented RPMI 1640 medium by centrifuging at 300 x g for 5 minutes. After the final wash, the peptide-pulsed T2 cells were resuspended in R10 medium at a concentration of 2×10^6 cells per ml. 50 µl of peptide-pulsed T2 cells (1×10^5 cells) were added to each well of a 96-well round bottomed plate (Nunc). $CD8^+$ T cells which had previously been isolated from a buffy coat and stored in liquid N_2, were thawed on the day of the assay, counted, and plated at 1×10^5 cells per well in 50 µl R10 medium. A two times concentrated stock of each mTCR-anti-CD3 scFv test reagent was made up in R10 medium and 100 µl added to the wells to give the final indicated concentration. Each condition was assayed in triplicate in a final well volume of 200 µl. The plate was incubated overnight at 37°C/5% CO_2. The plate was centrifuged at 300 x g for 5 minutes, the supernatants removed, and assayed for IFN-γ using Diaclone ELI-Pair ELISA kit (Gen-Probe). The plate was read on plate reader (VERSAmax, Molecular Devices) using SoftMax Pro 4.6 software. Data was plotted using Prism 4.0 software (GraphPad Software).

2.4.2 Enzyme linked immunospot (ELISpot) assay for interferon-γ (IFN-γ) and granzyme B

The ELISpot assay, derived from the sandwich enzyme-linked immunosorbent assay (ELISA), was originally described by Lalvani *et al* (1997). This was one of the key assays used in this thesis for measuring ImmTAC redirected T cell responses. The main advantage of the ELISpot assay compared to the ELISA is its exquisite sensitivity with which it can measure very low frequencies of analyte-producing cells (Helms et al., 2000). This was particularly important for detecting any potential ImmTAC cross-reactivity against antigen negative target cells. ELISpot kits were used for measuring IFN-γ and granzyme B (BD BioSciences). Each kit included polyvinylidene difluoride (PVDF) membrane-coated 96-well mircowell plates, unlabeled capture antibody, biotinylated detection antibody and enzyme conjugate (streptavidin-HRP). The PVDF-backed plate was pre-coated with 100 µl per well of capture antibody as per the manufacturer's instructions, and the plate incubated overnight at 4°C. The coating antibody was discarded and the wells washed once with 200 µl per well of R10 medium, then blocked with 200 µl per well of R10 medium for 2 hours at room temperature. The blocking medium was discarded, and the required volume of R10 medium was added to the sample wells to result in a final well volume of 200 µl. Target cells were prepared to a cell density of 1 x 10^6 cells per ml in R10 medium and plated at 50 µl (5 x 10^4 cells) per well. A 4 times concentrated stock of ImmTAC test reagent was prepared at the highest test concentration, which was serially diluted 1 in 10 in R10 medium in a separate plate to obtain the other test concentrations. ImmTAC was then added to the ELISpot plate at 50 µl per well to give the final indicated titrated amounts. Effector cells were added at 50 µl per well at a pre-determined cell concentration in R10 medium. Each condition was assayed in triplicate. For untested effector cells it was

necessary to determine the optimal cell concentration in the first ELISpot experiment. This was carried out by testing a range of effector cell concentrations (typically 1 x 10^3-1 x 10^5 cells per well) and incubating with the target cell of interest in the presence of top experimental concentration of the relevant ImmTAC reagent. The plate was then left disturbed overnight at 37°C/5%CO_2. During this period, the immobilised capture antibody specifically captures proteins secreted or released from the effector cells. The following day the cells were discarded and the wells washed three times with PBS containing 0.05% Tween-20 (Sigma) using an automated plate washer (Ultrawash Plus, Dynex Magellan Biosciences). The captured protein was specifically detected by the addition of 100 µl per well biotin-conjugated antibody diluted in PBS containing 10% FBS, and left to incubate for 2 hours at room temperature. After three further washes, horseradish peroxidise (HRP)-conjugated streptavidin diluted in PBS/10% FBS was added at 200 µl per well for 1 hour at room temperature. After this, the sample wells were washed three times with PBS/0.05% Tween-20 followed by two washes with PBS, 3-amino-9-ethyl-carbazole (AEC) substrate reagent set (BD BioSciences) was added for 2-5 minutes for IFN-γ and up to 30 minutes for granzyme B. The resulting appearance of dark spots represented individual IFN-γ or granzyme B producing T cells. The colour reaction was terminated by washing the plate under running tap water. After leaving the plate to dry at room temperature for several hours, the spots were enumerated using an automated ELISpot reader (Immunospot Series 5 Analyser, Cellular Technology Ltd.). Data was analysed using Prism 4.0 software (GraphPad Software).

2.5 Cytotoxicity assays

A number of different cytotoxicity assays were employed to investigate ImmTAC redirected target cell killing, as described in the following.

2.5.1 Analysis of caspase-3/7 activation at an early time point

To assess early manifestations of ImmTAC redirected target cell killing, activity of caspases 3 and 7 was measured using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega). Caspases are proteolytic enzymes that become activated during the process of apoptosis. The Promega Apo-ONE® Homogeneous Caspase-3/7 Assay detects caspase-3/7 activity based on the cleavage of a profluorescent DEVD peptide-Rhodamine 110 substrate [(Z-DEVD)2-R110] to create the fluorescent Rhodamine 110. The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample.

Target cells and effector cells were washed, counted and resuspended in R10 medium without phenol red. Target cells were adjusted to a cell density of 4×10^5 cells per ml and the effector cells at a cell density of 2×10^6 cells per ml. A 2 times concentrated stock of ImmTAC was prepared at 2, 0.2, 0.02, 0.002 nM in R10 without phenol red. In a 96-well round bottomed plate, 25 µl (1×10^4 cells) target cells were mixed with 25 µl effector cells (5×10^4 cells) in each well resulting in an effector to target (E:T) ratio of 5:1, followed by 50 µl of ImmTAC to give the final indicated titrated amounts. All wells were prepared in triplicate in a final volume of 100 µl. The plate was centrifuged (Heraeus Megafuge 1.0R) at 250 x g for 4 minutes and then incubated at 37°C/5%CO_2 for 4 hours. Following the incubation period, the plate was centrifuged at 250 x g for 4 minutes, and 50 µl of supernatant was removed and discarded. As per the manufacturer's instructions, the caspase-3/7 substrate Z-DEVD-

R110 was diluted in the Apo-ONE® buffer, which contains a cell lysis compound and an activity buffer for caspase-3/7, and added 50 µl per well (constituting a 1:1 ratio with sample volume in each well). The samples were then mixed by placing the plate on a shaker at 300-500 rpm for 30 minutes and then placed in the freezer at -20°C overnight. The next day, the plate was thawed and put on the shaker for 2 hours at 300-500 rpm. 80 µl was transferred from each well to the corresponding well on a white 96-well flat bottomed plate and the fluorescence of each well was measured using a spectrophotometer (Wallac VICTOR^{2}™, Perkin Elmer). Data was analysed using Prism 4.0 software (GraphPad Software).

2.5.2 ^{51}Cr-release assay

Mel526 target cells were prepared in R10 medium and labelled with sodium chromate-51 (3.7 MBq (100 µCi) per 1 x 10^6 cells) (Perkin Elmer) and incubated for 1 hour at 37°C/5%CO_2. The cells were washed four times in R10 medium and adjusted to 5 x 10^4 cells per ml. Unstimulated purified $CD8^+$ effector cells were prepared in R10 medium at a cell density of 5 x 10^5 cells per ml and EBV-specific $CD8^+$ T cell clone (EBV176c4.1) at a cell density of 2.5 x 10^5 cells per ml. Two times concentrated stocks of the ImmTAC-gp100 were prepared at 20, 2, 0.2, 0.02, 0.002, 0.0002 nM in R10 medium. In a 96-well round bottomed plate, 50 µl (2,500 cells) target cells was mixed with 50 µl effector cells in each well resulting in an effector to target (E:T) ratio of 10:1 for the unstimulated $CD8^+$ T cells (2.5 x 10^4 cells), and an E:T ratio of 5:1 for the EBV-specific T cell clone (1.25 x 10^4 cells), followed by 50 µl of ImmTAC to give the final indicated titrated amounts. Control wells were set up with target cells alone to calculate spontaneous release; target cells were set up with 1% Triton X 100 (v/v) to calculate maximum release. Each condition was assayed in triplicate in a final volume of 200 µl.

The plate was incubated at 37°C/5%CO_2 for 4 hours. Following incubation, 40 µl were transferred from each well to the corresponding well in a solid scintillant white opaque 96-well microplate LumaPlate (Perkin Elmer). The LumaPlate was sealed with an adhesive cover and read on a TopCount scintillation counter (Perkin Elmer). Percentage cytotoxicity was then calculated using the following equation: Specific lysis = [(experimental lysis – spontaneous lysis)/(maximum lysis – spontaneous lysis)] x 100. Data was analysed using Prism 4.0 software (GraphPad Software).

2.5.3 LDH-release assay

ImmTAC redirected target cell killing was measured in a non-radioactive radioactive cytotoxicity assay using CytoTox96® (Promega). This assay is a colorimetric alternative to the ^{51}Cr-release cytotoxicity assays and quantitatively measures lactate dehydrogenase (LDH); this is an enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a 30 minute coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of cell lysed.

Target cells and effector cells were washed, counted and resuspended in R10 medium without phenol red. It should be noted that primary cell targets (described in section 2.2.2.1) were washed three times to remove culture media which can contain components that are inhibitory to T cells. Target cells were adjusted to a cell density of 2 x 10^5 cells per ml and the effector cells at a cell density of 2 x 10^6 cells per ml. In a 96-well round bottomed plate, 50 µl (1 x 10^4 cells) target cells were mixed with 50 µl effector cells (1 x 10^5 cells) in each well resulting in an effector to target (E:T) ratio of 10:1. When other E:T ratios were used, the target cell number was kept the same, while the number (concentration) of effector cells was adjusted accordingly. A 3 times

concentrated stock of ImmTAC test reagent was prepared at the highest test concentration, which was serially diluted 1 in 10 in R10 medium without phenol red in a separate plate to obtain other test concentrations. ImmTAC was then added to the assay plate at 50 µl per well to give the final indicated titrated amounts. Control wells were set up with effector cells alone to calculate effector spontaneous release, target cells alone to calculate target spontaneous release, and target cells with 80 µg/ml digitonin final to calculate maximum release. Each condition was assayed in triplicate in a final volume of 150 µl. The plate was centrifuged (Heraeus Megafuge 1.0R) at 250 x g for 4 minutes and then incubated at 37°C/5%CO_2 for 4 or 24 hours. Following the incubation period, the plate was centrifuged at 250 x g for 4 minutes and 50 µl transferred from each well to the corresponding well in a 96-well flat bottomed Maxisorb plate (Nunc). The CytoTox96® substrate mix was reconstituted using CytoTox96® assay buffer, as per manufacturer's instructions, and 50 µl added to each well of the plate. The plate was covered with aluminium foil and incubated at room temperature for 30 minutes. Then 50 µl of CytoTox96 stop solution was added to each well and absorbance recorded at 490 nm on a plate reader (VERSAmax, Molecular Devices) using SoftMax Pro 4.6 software. Percentage cytotoxicity was then calculated using the following equation: Specific lysis = [(Experimental – Effector Spontaneous – Target Spontaneous)/(Target Maximum – Target Spontaneous)] x 100. Data was analysed using Prism 4.0 software (GraphPad Software).

2.5.4 IncuCyte and real-time quantification of cell killing

IncuCyte™ FLR technology (Essen Biosciences) enables direct visualisation of caspase-3/7 dependent apoptosis by microscopy at 37°C in real time. This technology is relatively new and has been optimised in our laboratory to monitor the long-term

killing kinetics of ImmTAC redirected T cells *in vitro*. Kinetic measurement of apoptosis is made using CellPlayer 96-well Kinetic Caspase-3/7 reagent (Essen Biosciences) which couples the activated caspase-3/7 recognition motif (DEVD) to Nucview™ 488, a DNA intercalating dye. When added to tissue culture medium, this inert and non-fluorescent substrate crosses the cell membrane where it is cleaved by activated caspase-3/7 resulting in the release of the DNA dye and green fluorescent staining of nuclear DNA. Kinetic activation of caspase-3/7 can be monitored morphologically using live cell imaging, and quantified using the IncuCyte object counting algorithm.

The day before the assay, target cells were prepared in R10 medium without phenol red at a cell density of 2 x 10^5 cells per ml and 100 µl (2 x 10^4 cells) added per well of a 96-well flat bottomed microwell plate. The plate was incubated at 37°C/5%CO_2 overnight to allow the cells to settle/adhere to the bottom of the plate. The following day, 50 µl of the supernatant was carefully removed from each well, leaving the cells undisturbed. A six times stock of ImmTAC reagent was prepared in R10 medium without phenol red and 25 µl was added to the relevant cells to give the final indicated concentration. A 30 µM stock of the CellPlayer 96-well Kinetic Caspase-3/7 reagent was prepared in R10 medium without phenol red and 25 µl added to all wells to give a final concentration of 5 µM. Effector cells were prepared in R10 medium without phenol red at a cell density of 2 x 10^6 cells per ml and 50 µl (1 x 10^5 cells) added per well to give an E:T ratio of 5:1. Each condition was assayed in triplicate in a final volume of 100 µl. Images were taken at intervals of 2 hours and the number of apoptotic cells was quantified using IncuCyte object counting algorithm.

CHAPTER 3 OPTIMISATION AND EVALUATION OF HIGH AFFINITY TCR-ANTI-CD3 FUSIONS FOR TUMOUR CELL RECOGNITION

3.1 Introduction

Epitope counting using fluorochrome-labelled high affinity monoclonal TCRs in single molecule fluorescence microscopy experiments showed that the density of tumour-associated peptide-MHC at the tumour cell surface is as low as 10-50 epitopes per cell on both cell lines and fresh tumour samples (Purbhoo et al., 2006). The low epitope number on tumour cells poses a significant challenge when considering a suitable effector function that could successfully convert the high affinity mTCR from simply a research tool (Purbhoo et al., 2007; Purbhoo et al., 2006) into one that can deliver therapeutic potential.

Unlike antibodies, mTCRs do not possess an Fc fragment or any other effector function. Therefore, targeted cell killing requires conjugation or fusion to a suitable cytotoxic agent or biological modulator. There are two modes of action that can be utilised; first, is a direct mechanism delivering a payload, for example a radioisotope or cytotoxin, to the cancer surface, or second, an indirect mechanism whereby immune activity is redirected against the cancer cell.

3.1.1 Direct mechanisms

Successful use of a radioisotope as a conjugate to kill tumour cells would require the delivery of several thousand molecules per cell. Previous tumour staining experiments (Purbhoo et al., 2007; Purbhoo et al., 2006) indicated that the density of peptide antigen at the tumour cell surface was considerably lower than this level so such an approach was ruled out.

A more promising option was the use of the very potent cell toxin, *Pseudomonas* exotoxin A-derived PE38, where a single molecule is capable of killing a eukaryotic cell. However, although PE38-fused mTCRs were shown to kill peptide-pulsed targets (which can present non-physiologically high numbers of epitope per cell) they were not potent enough to efficiently kill naturally presenting tumour cells (unpublished data). This is probably because the toxin requires internalisation to kill the cell and the use of fluorochrome-labelled high affinity monoclonal TCRs in single molecule fluorescence experiments showed that mTCRs remain bound on the cell surface for over 24 hours with no internalisation detectable (Purbhoo et al., 2007; Purbhoo et al., 2006). This observation pointed strongly to an indirect mechanism of action being the most suitable option for mTCRs.

3.1.2 Indirect mechanisms

Indirect mechanisms for delivering an effector function involve fusing the mTCR to a protein capable of eliciting a potent activation signal to the immune system within the vicinity of the tumour. Such a protein moiety could be a cytokine, a receptor, a bacterial superantigen or an antibody fragment. In order to be effective any such mechanism would need to be capable of delivering an immune system recognisable function to a target cell presenting only 10-50 copies of the relevant peptide target.

The first protein moiety of this kind to be evaluated with the mTCR was the cytokine interleukin-2 (IL-2). A low level of T cell stimulatory effect was demonstrated *in vitro* with high affinity NY-ESO mTCR fused to IL-2 when $CD8^+$ CTLs were stimulated to exhibit activity against NY-ESO peptide-pulsed targets. A small effect was also seen in

an animal model but it was concluded that IL-2 was not a suitable therapeutic candidate based on the toxicity of IL-2 versus the anticipated efficacy that could be achieved. mTCR fused to bacterial superantigen was capable of redirecting T cell activity, producing a low level of target cell killing, and although inadequate, it pointed to a T cell stimulator as the most promising solution.

Cytotoxic T cells (CTL) are considered to be the most potent killer cells of the immune system. They have evolved to eliminate pathogenic cells such as virus-infected and, to a lesser extent, malignant cells. As described in section 1.5.6.2 CTL triggering can be induced with as few as 3 cognate ligands. Therefore, the recruitment of cytotoxic T cells is a highly compelling therapeutic strategy for targeting cancer as these cells offer potent cytotoxicity with exquisite sensitivity. It is long established that T cell triggering can be achieved by monoclonal antibodies specific for the CD3/TCR complex. This has led to the development of a number of bispecific T cell activating antibodies, comprised of a fusion between a monoclonal anti-CD3 antibody and a monoclonal antibody specific for a tumour-associated antigen. Probably the most potent bispecific T cell activator antibodies reported to date are the so-called BiTE (bispecific T-cell engager) antibodies which are a tandem scFv molecule where one scFv has specificity for CD3 whilst the other targets a tumour antigen (Baeuerle & Reinhardt, 2009; Dreier et al., 2002; Topp et al., 2011).

Normally, the activity of T cells is a tightly controlled process involving various cell surface receptors including a specific TCR, the CD3 signalling complex, co-receptors, cell adhesion and costimulatory molecules; ultimately, these interactions can lead to the formation of an immunological synapse with the target cell (Dustin et al., 2010). This high level of control is required to ensure the cytotoxic T cell response is highly specific and that the wrong cells/tissues are not attacked. With bispecific

therapeutics, the anti-CD3 antibody activates T cells by short-circuiting normal T cell activation, bypassing the need for specific TCR, pMHC complex and costimulatory molecules. Therefore, when considering anti-CD3 as an effector function for the mTCR it is vitally important to ensure that specificity is maintained. In particular, it is essential that the anti-CD3 antibody does not activate T cells without the mTCR having bound its target; T cell activation should only be triggered upon concomitant binding to the target cell.

The reagents evaluated in this thesis are called ImmTACs (**I**mmune-**m**obilising **m**onoclonal **T**CRs **a**gainst **c**ancer). These comprise a high affinity mTCR fused to a humanised CD3-specific scFv, resulting in a fusion protein of approximately 76 kDa molecular weight. ImmTACs are designed to redirect and activate both cancer-specific and non cancer-specific T cells to lyse tumour cells. This chapter describes the cellular testing which guided the optimisation of the ImmTAC reagents. I was not involved in the design, engineering or the manufacture of the ImmTAC reagents. My own studies involved *in vitro* testing of ImmTACs in order to assess which reagents might prove to be most potent for therapeutic applications.

3.2 Results

3.2.1 Overview of the testing of different prototype mTCR-anti-CD3 scFv (ImmTACs) for the elimination of tumour cells

As described above, my work involved cellular testing of mTCR-anti-CD3 scFv molecules in order to assess what would be optimal for tumour cell killing with these reagents. At the outset there were a number of questions that I aimed to answer. The first of these was to establish which of the many anti-CD3 antibodies might provide greatest potency. Second, I aimed to examine the optimal way to fuse these bispecifics by examining the potency of both N-terminal and C-terminal fusion molecules and various linkers between the two molecules. Third, I assessed whether addition of a second, tandem anti-CD3 scFv moiety could increase the potency of cellular activation. Access to a range of enhanced TCRs also allowed me to assess the role of TCR binding affinity in the potency of mTCR-anti-CD3 scFv molecules.

3.2.2 Comparison of different anti-CD3 scFv antibodies as fusion partners for mTCR

There are a number of widely used anti-CD3 antibodies available. The humanised monoclonal anti-CD3 antibodies called OKT3 (Xu et al., 2000), 12F6 (B. Li et al., 2005) and UCHT1 (Shalaby et al., 1992) were selected as potential effector function binding partners for the mTCR. These antibodies recognise epitopes on CD3ε found in heterodimers with CD3δ and with CD3γ but not CD3ε expressed in isolation (Salmeron et al., 1991).

The antigen sensitivity mediated by three humanised anti-human CD3 scFv antibodies, OKT3, 12F6 and UCHT1 was evaluated by fusing them to the C-terminus of the TCR beta chain of a high affinity mTCR specific for Melan-A/MART-1_{26-35},

EAAGIGILTV-HLA-A*0201 (ImmTAC-MEL). This mTCR binds to its cognate antigen with a K_D of 39 pM and half-life interaction of 37 hours. The three reagents, ImmTAC-MEL/OKT3, ImmTAC-MEL/12F6 and ImmTAC-MEL/UCHT1 were tested using and interferon-γ (IFN-γ) enzyme-linked immunosorbent assay (ELISA) to measure the activation of unstimulated purified $CD8^+$ T cells in the presence of T2 cells pulsed with varying concentrations of the HLA-A*0201 restricted Melan-A/MART-1_{26-35} heteroclitic peptide ELAGIGILTV (10^{-11}-10^{-5} M) (Figure 3-1a-c).

ImmTAC-MEL/OKT3 shows the least antigen sensitivity of the three anti-CD3 scFv antibodies (Figure 3-1a), activating $CD8^+$ T cells at a peptide concentration of 10^{-7} M. Activation with ImmTAC-MEL/OKT3 did not reach saturation. ImmTAC-MEL/12F6 (Figure 3-1b), is able to activate $CD8^+$ T cells at a 10-fold lower peptide concentration than ImmTAC-MEL/OKT3 with full activation achieved with 1 μM cognate peptide. ImmTAC-MEL/UCHT1 (Figure 3-1c) shows the greatest antigen sensitivity of the three anti-CD3 scFv antibodies; 1 pM ImmTAC-MEL/UCHT1 gave comparable activity to ImmTAC-MEL/12F6 used at a concentration of 10 nM which equates to a 10,000-fold difference in antigen sensitivity (Figure 3-1b,c). In all cases, no activation of $CD8^+$ T cells is seen in the presence of ImmTAC-MEL without targets. This illustrates that the ImmTAC only activates when a multivalent contact is made between the target cell and the T cell.

The huge difference in potency between mTCR fusions with these 3 anti-human CD3 scFv antibodies ensured that UCHT1 was selected as the anti-CD3 scFv antibody effector fusion partner for future ImmTAC reagents and experiments.

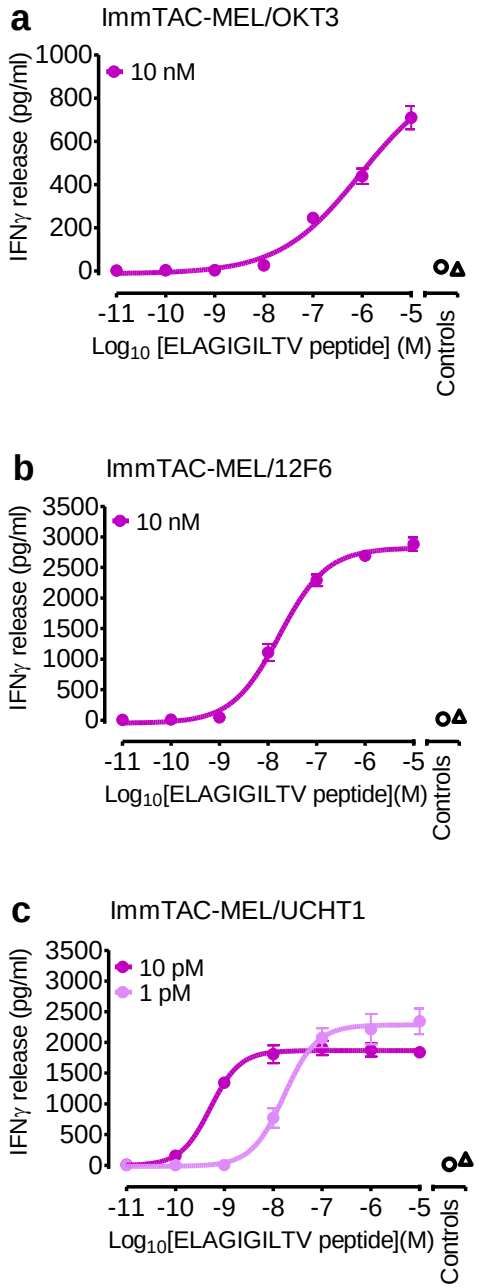


Figure 3-1 The antigen sensitivity of ImmTAC-MEL when fused to different anti-CD3 scFv antibodies.

IFN-γ ELISA assay showing the activation of unstimulated purified $CD8^+$ T cells mediated by ImmTAC-MEL fused at the C-terminus to humanised anti-CD3 scFv antibodies in the presence of T2 cells pulsed with various concentrations of the Melan-A/MART-1_{26-35} heteroclitic peptide ELAGIGILTV (closed circles). Controls are represented by T2 cells pulsed with 10^{-5} M ELAGIGILTV peptide in the presence of $CD8^+$ T cells, without ImmTAC-MEL (open circles) and $CD8^+$ T cells in the presence of ImmTAC-MEL, without targets (open triangles). **(a)** ImmTAC-MEL fused to OKT3, used at a 10 nM. **(b)** ImmTAC-MEL fused to 12F6, used at 10 nM. **(c)** ImmTAC-MEL fused to UCHT1, used at 1 pM (light purple) and 10 pM (dark purple). Data are means ± s.e.m. The data shown are representative of two experiments.

3.2.3 Potency of different anti-CD3 scFv fusion designs

My work described above established that fusions with the UCHT1 scFv were >1000 times more antigen sensitive than those with the other activating anti-CD3 antibodies tested. Next, I aimed to test for the optimal design of ImmTAC-UCHT1 reagents to increase stability, reduce immunogenicity and improve yield and potency so that they would be suitable for manufacture and potential use in the clinic.

3.2.3.1 Fusion of the anti-CD3 scFv to the N-terminus of the TCR beta chain to improve potency

In the first generation ImmTAC constructs the anti-CD3 scFv antibody was fused to the C-terminus of the TCR beta chain via an inter-linker, as molecular modelling based on crystal structures indicated that fusion to the N-terminus of the TCR might interfere with binding to the HLA-peptide complex. However, the presence of anti-CD3 scFv at the C-terminus of the mTCR increases the overall length of the molecule which could impact on its ability to efficiency activate T cells due to the increased distance between the target cell and T cell. To investigate this further, ImmTACs were produced with the anti-CD3 scFv antibody fused to the N-terminus of the TCR beta chain via an inter-linker (Figure 3-3a). This modification makes the ImmTAC reagent more compact and, if functional, could permit closer contact to be made between the target cell and the recruited cytotoxic T cell, potentially resulting in more potent T cell activation. However, there were concerns that the position of the anti-CD3 scFv antibody on the N-terminus could sterically interfere with the binding of the TCR to its target.

I examined the biological activity of an anti-CD3-mTCR N-terminus ImmTAC construct versus the anti-CD3-mTCR C-terminus ImmTAC construct. These experiments were conducted using a different TCR specific for an HLA A*0201-

specific epitope from the cancer/testis antigen NY-ESO-1 (residues 157-165; SLLMWITQC). This TCR binds with a K_D of 18 pM and a half-life interaction of 17 hours.

The NY-ESO-specific ImmTAC (ImmTAC-NYE) with UCHT1 scFv fused either on the C-terminus or N-terminus of the TCR beta chain, were used to activate CTL against T2 target cells pulsed with 10^{-9} M NY-ESO-$1_{157-165}$ heteroclitic peptide SLLMWITQ**V**. The 9V mutaion in the peptide improves binding to HLA A*0201 compared to the wildtype SLLMWITQC sequence (J. L. Chen et al., 2000).

IFN-γ activity measured from redirected unstimulated purified $CD8^+$ T cells in an enzyme-linked immunospot (ELISpot) assay showed that the N-terminus ImmTAC-NYE construct produced approximately a 4-fold improvement in redirected $CD8^+$ T cell activity compared to the C-terminus ImmTAC-NYE (EC_{50} values of 12 pM versus 45 pM) (Figure 3-2).

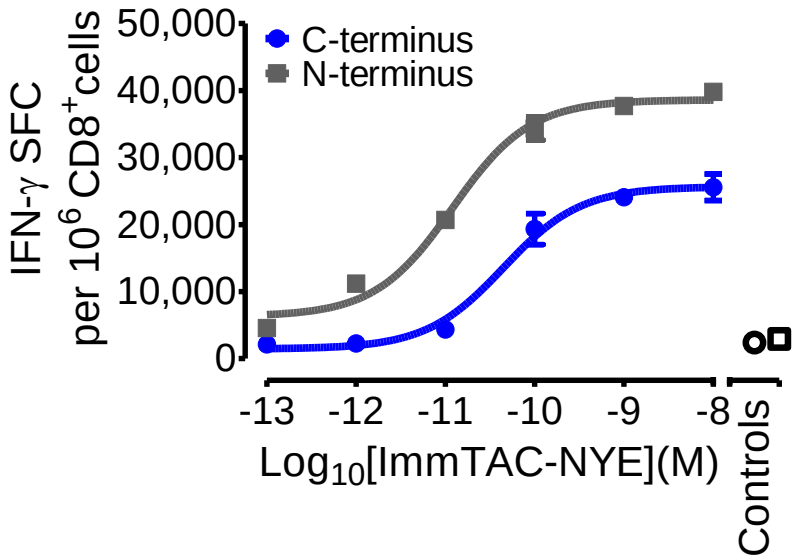


Figure 3-2 The biological activity of N-terminus versus C-terminus ImmTAC-NYE.

IFN-γ ELISpot assay showing the activation of unstimulated purified $CD8^+$ T cells mediated by titrated amounts of N-terminus ImmTAC-NYE (closed grey squares) compared with C-terminus ImmTAC-NYE (closed blue circles) in the presence of T2 cells pulsed with 10^{-9} M NY-$ESO_{157-165}$ heteroclitic peptide SLLMWITQV. Controls (open symbols) represent the relevant ImmTAC-NYE at 1 nM in the presence of T2 cells pulsed with 10^{-9} M irrelevant peptide (P450 $CYP1B1_{190-198}$ FLDPRPLTV) and $CD8^+$ T cells. Data are means ± s.e.m. The data shown are representative of three experiments.

3.2.3.2 Testing for the optimal ImmTAC intra- and inter-linkers

The intra-linker, which links the light chain and the heavy chain variable domains of the anti-CD3 scFv, and the inter-linker, which links the TCR to the anti-CD3 scFv, were both modified to a GS motif (Figure 3-3a), which is often used in fusion proteins in order to ensure flexibility between the fusion partner polypeptides. Further purposes of these modifications were to increase the stability of the ImmTAC and reduce potential immunogenicity. The linker modifications were not expected to affect the biological activity of the ImmTAC.

Experiments examining the role of linker sequences were performed with a TCR specific for and HLA A*0201 epitope in the gp100 protein (YLEPGPVTV). This TCR binds with a K_D of 15 pM and a half-life of 33 hours.

To verify that the modification of the intra- and inter- linkers did not significantly affect the biological activity of the ImmTAC, titrated amounts of the lead therapeutic candidate ImmTAC-gp100, expressed with the first generation linkers or the second generation GS linkers, were compared. The melanoma cell line, Mel526, expressing natural levels of wild-type $gp100_{280-288}$ presented by HLA-A*0201 was used as target. IFN-γ activity was measured from the redirected unstimulated purified $CD8^+$ T cells (Figure 3-3b). There was no significant difference in biological activity between ImmTAC-gp100 with the first generation linkers and ImmTAC-gp100 with the second generation GS motif linkers. However, the second generation linkers are considered more suitable for a therapeutic product and have therefore been used for all subsequent ImmTAC reagents.

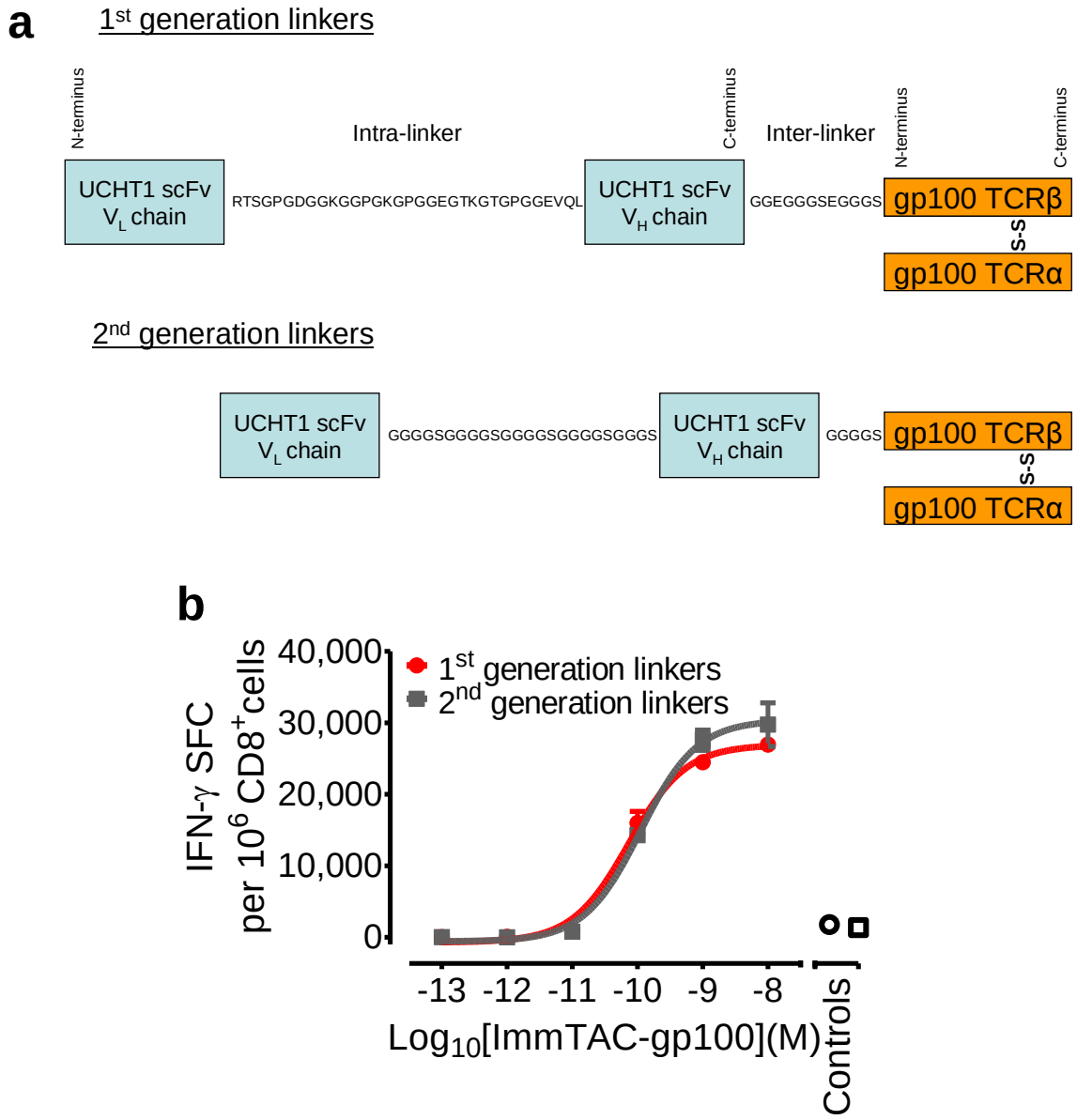


Figure 3-3 The effect of GS motif linkers on the biological activity of ImmTAC-gp100.

(a) A schematic representation of ImmTAC-gp100 showing the difference in the amino acid sequence between the 1st and 2nd generation inter- and intra- linkers. **(b)** IFN-γ ELISpot assay showing the activation of unstimulated purified $CD8^+$ T cells mediated by titrated amounts of ImmTAC-gp100 with the 1st generation intra- and inter-linker (closed red circles) compared to ImmTAC-gp100 with the 2nd generation GS motif intra- and inter-linker (closed grey squares) in the presence of the HLA-$A*0201^+/gp100^+$ melanoma cell line Mel526. Controls (open symbols) represent the corresponding ImmTAC-gp100 at 10 nM in the presence of the HLA-$A*0201^+/gp100^-$ cell line, A375, and $CD8^+$ T cells. Data are means ± s.e.m. The data shown are representative of three experiments.

3.2.3.3 Other modifications

Further modifications were made to the lead therapeutic candidate, ImmTAC-gp100, to make it suitable for clinical testing. These modifications included: (1) truncation of the TCR α-chain as this was found to be chemically unstable, (2) removal of an acetylation site from the N-terminus of the gp100 TCR alpha chain to reduce levels heterogeneity in manufacturing, and (3) removal of a methionine residue from the N-terminus of the gp100 TCR β-chain anti-CD3 fusion to reduce potential immunogenicity.

All of the modified ImmTAC-gp100 reagents were tested in IFN-γ release assays to verify biological activity. None of the modifications significantly affected the potency of ImmTAC-gp100 compared to the non-modified control (Figure 3-4).

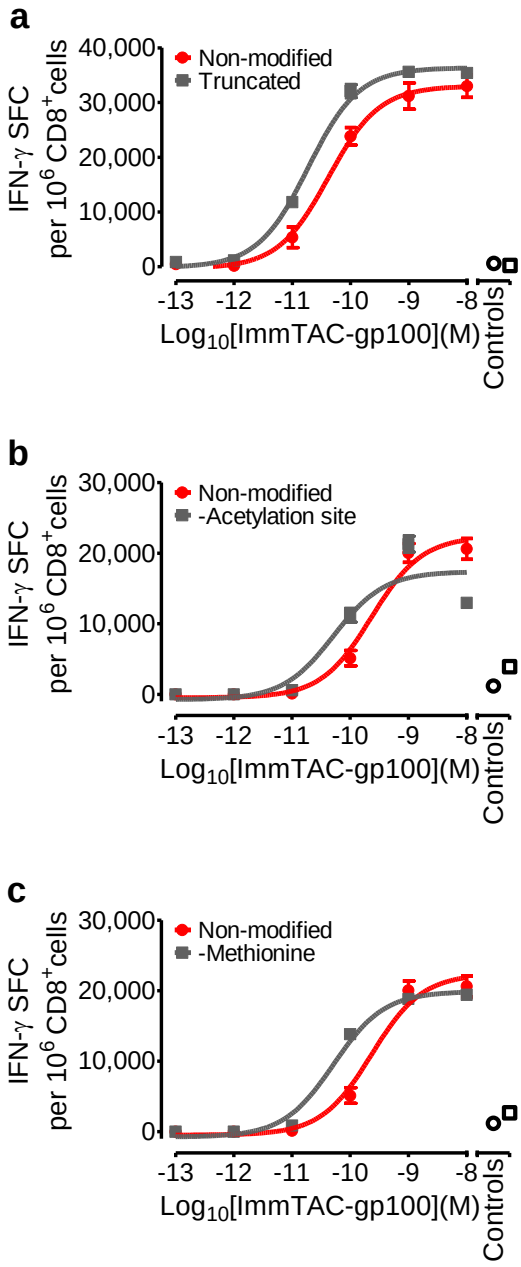


Figure 3-4 The effect of various modifications on the biological activity of the lead therapeutic candidate ImmTAC-gp100.

IFN-γ ELISpot assay showing the activation of unstimulated purified $CD8^+$ T cells mediated by titrated amounts non-modified ImmTAC-gp100 (closed red circles) compared to modified ImmTAC-g100 (closed grey squares) in the presence of the HLA-A*0201$^+$/gp100$^+$ melanoma cell line Mel526. Controls (open symbols) represent the corresponding ImmTAC-gp100 at 10 nM in the presence of the HLA-A*0201$^+$/gp100$^-$ cell line, A375, and $CD8^+$ T cells. **(a)** Truncation of TCR α-chain. **(b)** Removal of acetylation site from N-terminus of TCR α-chain. **(c)** Removal of methionine residue from the N-terminus of the TCR β-chain. Data are means ± s.e.m. The data shown are representative of three experiments.

3.2.4 Testing of mTCR-tandem anti-CD3 scFv fusions

An alternative approach to enhancing effector function potency would be through increasing the payload, i.e., the number of effector function moieties conjugated to each mTCR molecule. An antibody tandem scFv-based lidamycin fusion protein targeting gelatinases, which play important roles in tumour invasion and metastasis, has been shown to enhance therapeutic efficacy in mouse models (Zhong et al., 2010)

To investigate whether a tandem effector function could improve the biological activity of the ImmTAC reagents, a tandem anti-CD3 (UCHT1) scFv version of the ImmTAC was generated and compared with single anti-CD3 (UCHT1) scFv ImmTAC. These experiments were performed with the gp100-specific TCR.

The tandem anti-CD3 scFv ImmTAC-gp100 was constructed with an extra UCHT1 molecule linked via a GS inter-linker to the existing UCHT1 molecule which is fused to the N-terminus of the TCR beta chain via a GS inter-linker (Figure 3-5a). The biophysical properties of the tandem anti-CD3 scFv ImmTAC-gp100 were evaluated by surface plasmon resonance (SPR) technology (BIAcore™) to measure binding to CD3 and HLA-A*0201 presenting $gp100_{280-288}$ heteroclitic peptide YLEPGPVTV (Figure 3-5a-c). To examine whether tandem anti-CD3 scFv ImmTAC-gp100 binds to CD3 with higher affinity than single anti-CD3 scFv ImmTAC-gp100, CD3εγ was immobilised on the chip and single and tandem anti-CD3 scFv ImmTAC-gp100 fusions were injected over the surface. The single anti-CD3 scFv ImmTAC-gp100 dissociated from the bound CD3 with a half-life ($T_{1/2}$) of 16 minutes, whereas tandem anti-CD3 scFv ImmTAC-gp100 had not dissociated substantially even after 20 hours indicating that the avidity of the interaction of the tandem UCHT1 molecules to the surface with immobilised CD3 is very high (Figure 3-5b). To show that the tandem anti-CD3 scFv

ImmTAC-gp100 can bind both CD3 and its cognate pMHC, a bi-functional assay also was performed. HLA-A*0201 presenting $gp100_{280-288}$ heteroclitic peptide YLEPGPVTV was immobilised on the chip followed by tandem anti-CD3 scFv ImmTAC-gp100 or single anti-CD3 scFv ImmTAC-gp100 to saturate the immobilised pHLAs. CD3 was then injected over the sensor surface. Both anti-CD3 scFv-gp100 fusions, bound through their TCR part to the immobilised pHLA, were able to capture soluble CD3 complex, thus demonstrating that both type of subunit of the bispecific molecules are functional (Figure 3-5c).

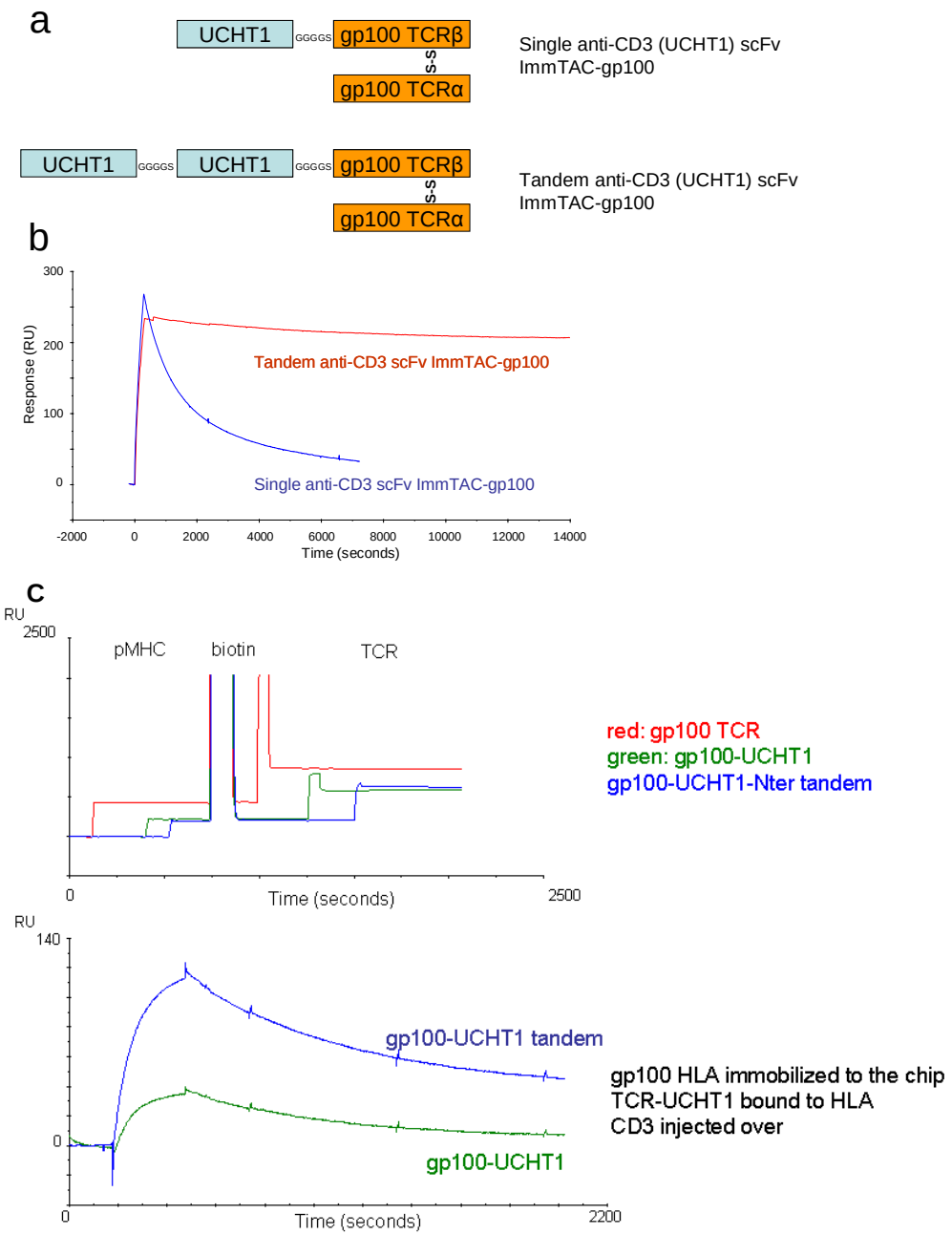


Figure 3-5 The biophysical characteristics of ImmTAC-gp100 with tandem anti-CD3 scFv.

Surface plasmon resonance (SPR) technology was used to characterise the binding of tandem anti-CD3 scFv ImmTAC-gp100 to HLA-A2/gp100 complex and CD3-εγ. **(a)** A schematic representation of the single and tandem anti-CD3 ImmTAC-gp100 molecules. **(b)** CD3 was immobilised to the chip and tandem anti-CD3 scFv ImmTAC-gp100 was injected over the sensor surface. The same procedure was repeated for the single anti-CD3 scFv ImmTAC-gp100. **(c)** Bi-functional assay to show functionality of anti-CD3 scFv and mTCR components of the tandem anti-CD3 scFv ImmTAC-gp100. HLA-A*0201 presenting $gp100_{280-288}$ heteroclitic peptide YLEPGPVTV was immobilised to the chip followed by tandem anti-CD3 scFv ImmTAC-gp100 or single anti-CD3 scFv ImmTAC-gp100 to saturate the immobilised pHLA (upper graph). CD3 was then injected over the sensor surface (lower graph). The data shown are courtesy of Dr. Milos Aleksic at Immunocore Ltd.

The biological activity of the tandem anti-CD3 scFv and the single anti-CD3 scFv ImmTAC-gp100 were compared in T cell activation assays. The melanoma cell line, Mel526, expressing natural levels of wild-type $gp100_{280-288}$ complexed to HLA-A*0201 was used as targets. IFN-γ activity was measured from the redirected unstimulated purified $CD8^+$ T cells (Figure 3-6). The data shows that, at all concentrations from 10^{-13} to 10^{-8} M of the two reagents, the tandem anti-CD3 scFv fusion is inferior to the single anti-CD3 scFv fusion at activating the T cells.

The close proximity of the two anti-CD3 scFv subunits ensures that they would not be able to cross-link two T cells by binding to the cell-surface CD3 on different cells. Indeed, no activation of T cells was seen in the presence of tandem anti-CD3 scFv ImmTAC-gp100 or single anti-CD3 scFv ImmTAC-gp100 without targets.

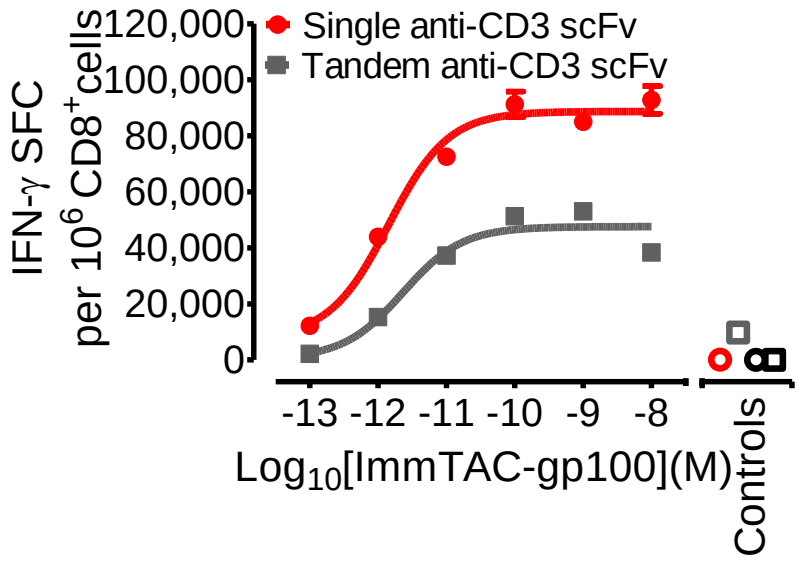


Figure 3-6 The biological activity of tandem anti-CD3 (UCHT1) scFv ImmTAC-gp100 versus single anti-CD3 (UCHT1) scFv ImmTAC-gp100.

IFN-γ ELISpot assay showing the activation of unstimulated purified $CD8^+$ T cells mediated by titrated amounts of tandem anti-CD3 (UCHT1) scFv ImmTAC-gp100 (closed grey squares) compared to single anti-CD3 (UCHT1) scFv ImmTAC-gp100 (closed red circles) in the presence of the HLA-A*0201^+/$gp100^+$ melanoma cell line, Mel526. Controls (open symbols) represent the corresponding ImmTAC-gp100 at 1 nM with $CD8^+$ T cells in the presence of the HLA-A*0201^+/$gp100^-$ target cell line, A375 (coloured open symbols) and in the absence of target cells (black open symbols). Data are means ± s.e.m. The data shown are representative of two experiments.

Next, it was investigated whether the tandem anti-CD3 scFv fusion would be more efficient at recruiting low numbers of $CD8^+$ T cells compared to the single anti-CD3 scFv fusion. The melanoma cell line, Mel526, which naturally presents the wild-type $gp100_{280\text{-}288}$ complexed to HLA-A*0201, was used as a target. IFN-γ activity was measured from the titrating numbers of unstimulated purified $CD8^+$ T cells in the presence of 1 nM tandem or single anti-CD3 scFv fusion (Figure 3-7). The data shows that the tandem anti-CD3 scFv fusion is inferior to the single anti-CD3 scFv fusion at activating the T cells at all the cell concentrations tested (312.5 to 10,000 $CD8^+$ T cells per well). It should be noted that the data shown is representative of one experiment only.

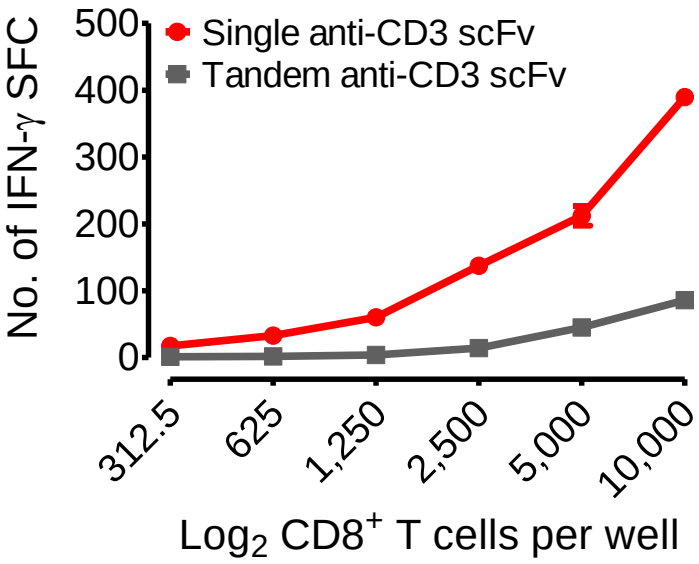


Figure 3-7 Recruitment of $CD8^+$ T cells by tandem anti-CD3 (UCHT1) scFv ImmTAC-gp100 versus single anti-CD3 (UCHT1) scFv ImmTAC-gp100.

IFN-γ ELISpot assay showing the activation of titrating numbers of unstimulated purified $CD8^+$ T cells mediated by 1 nM tandem anti-CD3 (UCHT1) scFv ImmTAC-gp100 (closed grey squares) compared with 1 nM single anti-CD3 (UCHT1) scFv ImmTAC-gp100 (closed red circles) in the presence of the HLA-A*0201$^+$/gp100$^+$ melanoma cell line, Mel526. Data are means ± s.e.m.

3.2.5 The relationship between mTCR affinity and ImmTAC potency

The relationship between mTCR affinity and the biological potency of ImmTAC molecules was investigated next. To examine the relationship between biological activity and ImmTAC-pMHCI affinity, as determined by surface plasmon resonance (SPR) equilibrium measurements, titrating amounts of a panel of seven variants of ImmTAC-gp100 with K_D values spanning over six orders of magnitude (Table 3-1) were investigated. The melanoma cell line, Mel526, presenting natural levels of wild-type $gp100_{280-288}$ complexed to HLA-A*0201 (< 70 epitopes per cell) (Liddy et al., 2012) was used as the target. IFN-γ activity was measured from the redirected unstimulated purified $CD8^+$ T cells (Figure 3-8). All the ImmTAC variants respond in a concentration-dependent manner but are active in different concentration ranges. The two ImmTACs with the highest affinities for pMHCI (K_D values of 0.32 nM and 0.03 nM) are the most potent, activating $CD8^+$ T cells at concentrations as low as 10^{-11} M, with cellular EC_{50} values around 40 pM. Progressive increases in EC_{50} are seen as the affinities of the ImmTAC/pMHCI interactions decrease, with the wildtype and minimally modified ImmTACs showing only limited activation even at the highest test concentration of 10^{-8} M.

To summarise, the data shows the potency of ImmTAC reagents is dependent on the affinity of the interaction with cognate pMHCI antigen. This serves to highlight the significance of the affinity engineering process and the potential of the high affinity mTCR as a targeting reagent.

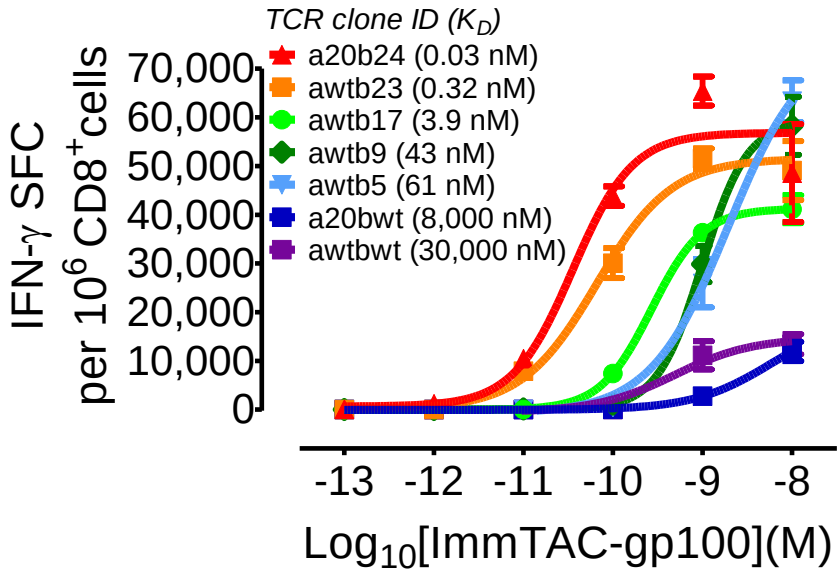


Figure 3-8 The biological activity of a panel of ImmTAC-gp100 affinity variants.

IFN-γ ELISpot assay showing the activation of unstimulated purified $CD8^+$ T cells in the presence of the HLA-A*0201$^+$/gp100$^+$ melanoma cell line Mel526 and titrated amounts of a panel of seven ImmTAC-gp100 affinity variants, colour coded by affinity according to the inset key. Data are means ± s.e.m. The data shown are representative of three experiments.

Table 3-1 Binding kinetics of a panel of seven different ImmTAC-gp100 variants

ImmTAC-gp100	Clone ID	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	T ½ (s)	K_D (nM)
ImmTAC-gp100-(30000nM)	awtbwt	n.d	n.d	n.d	30000
ImmTAC-gp100-(8000nM)	a20bwt	n.d	n.d	n.d	8000
ImmTAC-gp100-(61nM)	awtb5	$1.6x10^{5}$	$9.6x10^{-3}$	72	61
ImmTAC-gp100-(43nM)	awtb9	$1.3x10^{5}$	$5.5x10^{-3}$	126	43
ImmTAC-gp100-(3.9nM)	awtb17	$1.4x10^{5}$	$5.3x10^{-4}$	1000	3.9
ImmTAC-gp100-(0.32nM)	awtb23	$1.7x10^{5}$	$5.6x10^{-5}$	10000	0.32
ImmTAC-gp100	a20b24	$5.3x10^{5}$	$6.7x10^{-6}$	104400	0.03

3.3 Summary

In the experiments described in this Chapter I tested a number of different of mTCR-anti-CD3 scFV fusion molecules to identify the optimal design for their ability to redirect primary $CD8^+$ T cells to recognise tumour cells. I used mTCRs specific for three different HLA A*0201-restricted epitopes: Melan-A/MART-1, NY-ESO-1 and gp100. First, I compared different anti-CD3 scFv molecules and concluded that the UCHT1 antibody was by far the most potent as a mTCR effector fusion partner for activation of $CD8^+$ T cells. All subsequent experiments, therefore, used UCHT1 fusions. I next examined whether fusing the UCHT1 to the C-terminus or the N-terminus of the mTCR produced a more potent T cell activator. From these experiments I concluded that fusing the UCHT1 to the mTCR N-terminal end, which generates a more compact molecule and therefore probably generates closer proximity between the target cell and the T cell, enabled approximately 4-fold more potent T cell engagement. Fusion with two in-line anti-CD3 domains to the mTCR displayed higher affinity for CD3 immobilised on a 2-dimensional surface, but did not produce an improvement in potency; rather this design reduced the potency of the fusion molecule compared to the mTCR fused to a single UCHT1 scFv molecule.

Overall, I conclude that the most potent ImmTACs use a single anti-CD3 scFv domain from the UCHT1 antibody fused to the N-terminus of an mTCR with the highest possible affinity for the pHLA target.

CHAPTER 4 *IN VITRO* CHARACTERISATION OF REDIRECTED T CELL ACTIVATION BY IMMTACS

4.1 Introduction

Studies have shown that $CD8^+$ cytotoxic T cells (CTLs) are the major effector cells involved in the eradication of tumour cells (Celluzzi et al., 1996; Mackensen et al., 2006; Rosenberg et al., 2011). Activation of CTLs is normally initiated through engagement of their TCR with a specific peptide-HLA on the surface of target cells. After triggering through the TCR, CTLs secrete cytokines with cytotoxic and antiviral function, and exocytose lytic granules which deliver cytotoxic molecules directly into the target cell, resulting in apoptosis. Several of these activation markers can be measured in cellular assays to study T cell responses and are therefore also useful for understanding redirected activation of $CD8^+$ T cells with ImmTAC molecules. Many of the T cell activation markers are also of pivotal importance for understanding the therapeutic potential of T cell activation: whilst lytic granule secretion is mainly responsible for direct target cell killing, activated CTLs are also thought to mediate indirect anti-tumour activity by the production of pro-inflammatory cytokines, such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin-2 (IL-2). Indirect stimulation of the immune system through cytokines can have profound anti-tumour effects; for example, a fraction of patients with metastatic melanoma or kidney cancer have responded to treatments with IL-2 and IFN (Dutcher, 2002).

Cytotoxic $CD8^+$ T cells represent a major source of IFN-γ and its secretion is one of the earliest markers of T cell activation. IFN-γ production is induced following antigen or

mitogen stimulation of the T cell receptor. Its release has both immunostimulatory and immunomodulatory effects and has been associated with anti-viral, immunoregulatory, and anti-tumour responses (Schroder et al., 2004). A major anti-tumour effect of IFN-γ is to enhance MHC class I expression on the surface of tumour cells, thus rendering them more susceptible to recognition by tumour-specific $CD8^+$ T cells (J. Alimonti et al., 2000; Shankaran et al., 2001). There have been a number of studies demonstrating a critical role for endogenously produced IFN-γ in promoting host anti-tumour responses (reviewed in (Ikeda et al., 2002)). An example is a study by Schreiber and co-workers (Kaplan et al., 1998) which examined the effects of IFN-γ on the development of both chemically induced and spontaneously arising tumours in mice. Essentially, mice lacking sensitivity to either IFN-γ (i.e., IFN-γ receptor deficient mice) or all IFN family members (i.e., Stat1-deficient mice) developed tumours more rapidly and with greater frequency when challenged with different doses of the chemical carcinogen methylcholanthrene (MCA) compared to wild-type mice. To address the role of IFN-γ in preventing the incidence of spontaneous tumours, the authors monitored the development of tumours in IFN-γ-sensitive and IFN-γ-insensitive (IFN-γ receptor negative) mice that lack the p53 tumour-suppressor gene. They found that IFN-γ-insensitive mice developed tumours more quickly than wild-type mice when bred onto a background deficient in p53. Furthermore, IFN-γ-insensitive $p53^{-/-}$ mice also developed a broader range of tumours compared with mice lacking p53 alone (Kaplan et al., 1998).

In addition to cytokine release, activated $CD8^+$ T cells are capable of inducing lysis of tumour cells via the granule-exocytosis pathway. Cytolytic granules are exocytoxic vesicles stored within CTLs, but they are also found in other cell types such as natural killer (NK) cells. They contain various proteins including the pore-forming protein

perforin and the proteolytic enzyme granzyme B, which upon antigen encounter, release these molecules towards the target cell. Once inside the target cell, granzyme B elicits programmed cell death (apoptosis) via proteolytic activation of pro-caspases and direct cleavage of caspase substrates (D. Chowdhury & Lieberman, 2008; Elgert, 2009). Granzyme B release, therefore, represents an important early marker of T cell activation.

In this chapter, I aimed to assess the potency, antigen sensitivity, and specificity of ImmTAC molecules by measuring the release of two early markers of T cell activation: IFN-γ and granzyme B. A functional T cell assay, the enzyme-linked immunospot (ELISpot) assay, was the tool selected to quantitate these responses. ELISpot, a modified version of the sandwich enzyme-linked immunosorbent assay (ELISA), is a highly sensitive assay and allows detection of cytokine release from T cells at the single cell level (Helms et al., 2000). I have also sought to investigate the ability of ImmTAC reagents to redirect different T cell populations derived from healthy donors as well as T cells derived from cancer patients, including T cells isolated directly from the tumour microenvironment. Finally, the ability of ImmTACs to redirect T cell activity against *ex vivo* primary tumour was investigated as this will be crucial in determining whether ImmTACs could be effective as potential cancer therapeutics.

4.2 Results

4.2.1 The potency and specificity of different ImmTACs to target tumour cell lines and activate $CD8^+$ T cells

In this section, four ImmTAC reagents are investigated for biological activity and specificity. The ImmTACs were engineered on the basis of TCRs isolated from T cell

clones specific for the following tumour-associated epitopes: (1) $gp100_{280-288}$, YLEPGPVTA-HLA-A*0201 (Cox et al., 1994); (2) Melan-A/$MART\text{-}1_{26-35}$, EAAGIGILTV-HLA-A*0201 (Kawakami, Eliyahu, Sakaguchi et al., 1994); (3) NY-ESO-1/$LAGE\text{-}1_{157-165}$, SLLMWITQC-HLA-A*0201 (Jager et al., 1998); and (4) $MAGE\text{-}A3_{168-176}$, EVDPIGHLY-HLA-A*0101 (Celis et al., 1994).

High-affinity mTCRs were generated from the isolated wild-type TCRs using directed molecular evolution and phage display selection (Y. Li et al., 2005). The binding of each ImmTAC to its cognate pMHCI complex has been biophysically characterised by surface plasmon resonance (SPR) equilibrium-binding measurements (Table 4-1). For all four ImmTACs, the antigen binding properties were similar to that of the corresponding unfused mTCR indicating that the integrity of the mTCR binding surface has not been compromised by the process of fusing the anti-CD3 scFv to the mTCR β-chain. The binding of the anti-CD3 scFv component to immobilised CD3εγ protein was similar for all the ImmTAC reagents (Liddy et al., 2012).

To evaluate the biological activity and specificity of the four different ImmTACs, IFN-γ ELISpot assays were conducted to measure the activation of unstimulated purified $CD8^+$ T cells (Figure 4-1a-d). In concentration-response experiments, all four ImmTACs produce EC_{50} values in the range of 100 pM or lower which is consistent with the K_D values for the corresponding ImmTAC/pMHCI interactions. Responses are only seen to the antigen relevant targets.

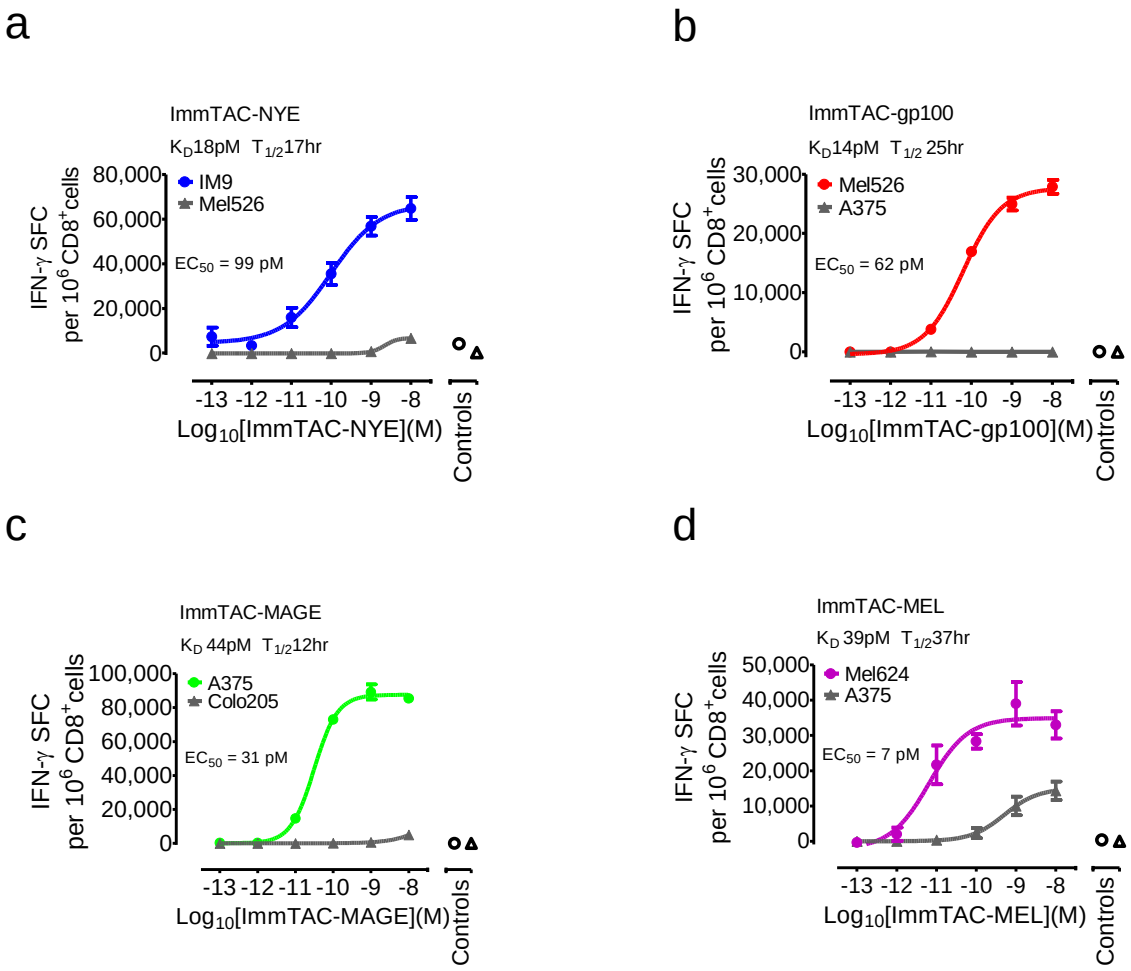


Figure 4-1 The biological activity and specificity of different ImmTAC molecules.

IFN-γ ELISpot assay showing the activation of unstimulated purified $CD8^+$ T cells mediated by increasing concentrations of ImmTAC molecules in the presence of tumour cells expressing endogenous levels of cognate antigen (closed circles). Antigen-negative tumour cells matched for expression of the relevant HLA class I molecules (closed triangles) were used in parallel titrations under identical conditions as specificity controls. The shaped-matched open symbols represent the corresponding ImmTAC-negative controls. Tumour cell line target cells were as follows: **(a)** For ImmTAC-NYE, IM9 EBV-transformed B-lymphoblastoid cells ($NY\text{-}ESO^+$) and Mel526 melanoma cells ($NY\text{-}ESO^-$). **(b)** For ImmTAC-gp100, Mel526 melanoma cells ($gp100^+$) and A375 melanoma cells ($gp100^-$) were used. **(c)** For ImmTAC-MAGE, A375 melanoma cells ($MAGE\text{-}A3^+$) and Colo205 ($MAGE\text{-}A3^-$). **(d)** For ImmTAC-MEL, Mel624 melanoma cells ($Melan\text{-}A/MART\text{-}1^+$) and A375 melanoma cells ($Melan\text{-}A/MART\text{-}1^-$). The equilibrium binding (K_D) and half-life ($T_½$) values determined by SPR are indicated for each ImmTAC-pMHCI interactions (Table 4-1). Data are means ± s.e.m. The data shown are representative of three experiments.

Table 4-1 Binding kinetics of ImmTAC molecules with different specificities

ImmTAC	Target Protein/Peptide	HLA	Peptide used for SPR†	TCR binding to HLA-peptide complex			
				K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	T ½ (h)	K_D (pM)
ImmTAC-NYE	NY-ESO-$1_{157-165}$ SLLMWITQC	HLA*0201	SLLMWITQV	$6.25x10^5$	$1.13x10^{-5}$	17.0	18
ImmTAC-gp100	$gp100_{280-288}$ YLEPGPVTA	HLA*0201	YLEPGPVTV	$5.38x10^5$	$7.61x10^{-6}$	14.1	25
ImmTAC-MAGE	Mage-$A3_{168-176}$ EVDPIGHLY	HLA*0101	EVDPIGHLY	$14.5x10^5$	$1.92x10^{-5}$	12.3	44.5
ImmTAC-MEL	Melan-A/MART-1_{26-35} EAAGIGILTV	HLA*0201	ELAGIGILTV	$1.33x10^5$	$5.20x10^{-6}$	37.0	39

† SPR was carried out using soluble HLA complexed to heteroclitic peptide because these were more stable than the wild-type peptide in these soluble formats.

4.2.2 The relationship between mTCR affinity and the capacity to detect low levels of peptide-MHC class I

Down-regulation of MHC class I antigen expression is a common mechanism by which tumour cells evade immune detection (Cabrera et al., 2003; Seliger et al., 2001). Therefore, in order to be able to eliminate tumours, ImmTACs need to be effective in the presence of low levels of peptide-MHC class I (pMHCI). To investigate further the relationship between the ImmTAC-pMHCI affinity and sensitivity to peptide, a panel of seven variants of ImmTAC-gp100 with K_D values spanning over six orders of magnitude (Table 3-1) were evaluated. T2 cells pulsed with titrating amounts of the heteroclitic $gp100_{280-288}$ YLEPGPVTV peptide (10^{-7}-10^{-12} M) in the context of HLA-A*0201, were used as targets. IFN-γ activity was measured from redirected unstimulated purified $CD8^+$ T cells in the presence of each of the seven ImmTAC-gp100 variants (Figure 4-2). The data shows that there is a clear correlation between the K_D of the ImmTAC reagent and the sensitivity to peptide. For the ImmTAC variants with the higher K_D values, a progressive reduction in peptide sensitivity is observed. This is a strictly hierarchical relationship so that, the higher the K_D, the further the concentration-response curves shift to the right, i.e., higher levels of peptide are required to elicit a T cell response. Indeed, the wild-type ImmTAC-gp100 (K_D = 30 μM) and minimally modified ImmTAC-gp100 (K_D = 8 μM) fail to activate $CD8^+$ T cells at any of the tested peptide concentrations whereas the highest affinity ImmTACs (K_D values of 0.32 nM and 0.03 nM) show the greatest peptide sensitivity and can activate $CD8^+$ T cells at as low as 10^{-10} M peptide which equates to 2-10 pMHCI epitopes per cell. This data highlights the importance of affinity engineering for the capability of mTCR ImmTACs to detect low levels of pMHCI.

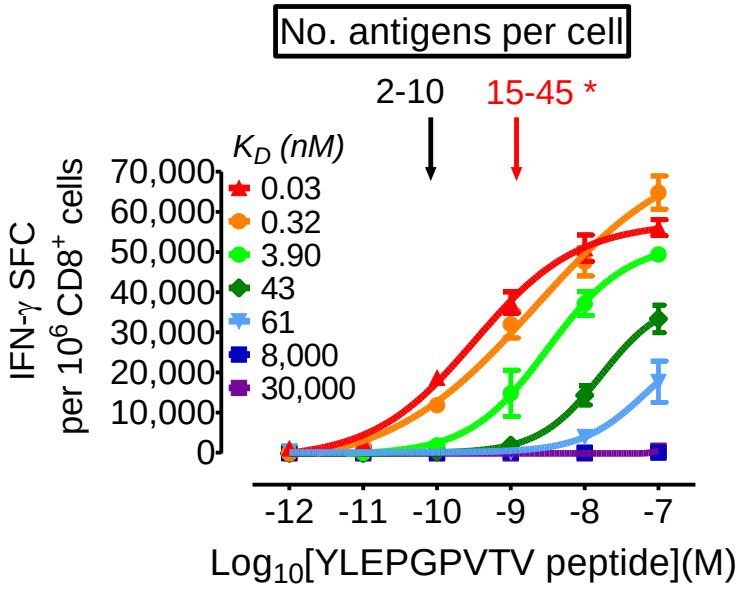


Figure 4-2 The peptide sensitivity of a panel of ImmTAC-gp100 affinity variants.

IFN-γ ELISpot assay showing the activation of unstimulated purified $CD8^+$ T cells in the presence of T2 cells pulsed with titrated amounts of the $gp100_{280-288}$ heteroclitic peptide YLEPGPVTV and different ImmTAC-gp100 variants, colour coded by affinity according to the inset key, each at a concentration of 100 pM. The epitope numbers were determined by single-cell three-dimensional fluorescence microscopy at different concentrations of exogenous peptide and were 0-3 at 10^{-11} M, 2-10 at 10^{-10} M and 15-45 at 10^{-9} M; the latter corresponds to the number of antigens typically detected on tumour cells (indicated with an asterisk). At higher concentrations of peptide, the number presented could not be determined accurately as the large number of fluorescent molecules meant that these could not be individually differentiated under the microscope. Data are means ± s.e.m. The data shown are representative of three experiments.

4.2.3 ImmTAC redirected activation of different T cell populations

The data presented thus far has examined the biological activity of ImmTAC molecules to activate purified unstimulated $CD8^+$ T cells. Next, the capacity of ImmTAC molecules to recruit T cells from the more physiologically relevant mixed population of peripheral blood mononuclear cells (PBMC) was examined. Furthermore, as the activation of T cells by ImmTAC is not dependent on co-receptor binding, ImmTAC molecules should be capable of recruiting $CD4^+$ T cells as well as $CD8^+$ T cells and this was also investigated.

4.2.3.1 Redirection of peripheral blood mononuclear cells (PBMC)

In order to make the *in vitro* assays as physiological as possible, the ability of ImmTAC molecules to activate PBMC, i.e, *ex vivo* unselected effector cells, was assessed. The melanoma cell line, Mel526, expressing natural levels of wild-type $gp100_{280\text{-}288}$ complexed to HLA-A*0201, was used as targets. IFN-γ activity was measured from redirected unstimulated PBMC in the presence of increasing concentrations of the ImmTAC-gp100 (Figure 4-3). The data shows concentration-dependent release of IFN-γ from the redirected PBMC producing an EC_{50} value of 27 pM. The potency seen is comparable to that achieved using purified unstimulated $CD8^+$ T cells confirming that a mixed population of PBMC are equally responsive to ImmTAC stimulation. No activation of PBMC is seen in the presence of ImmTAC without targets. Furthermore, no response is seen against the HLA-A*0201^+, antigen negative control cell line.

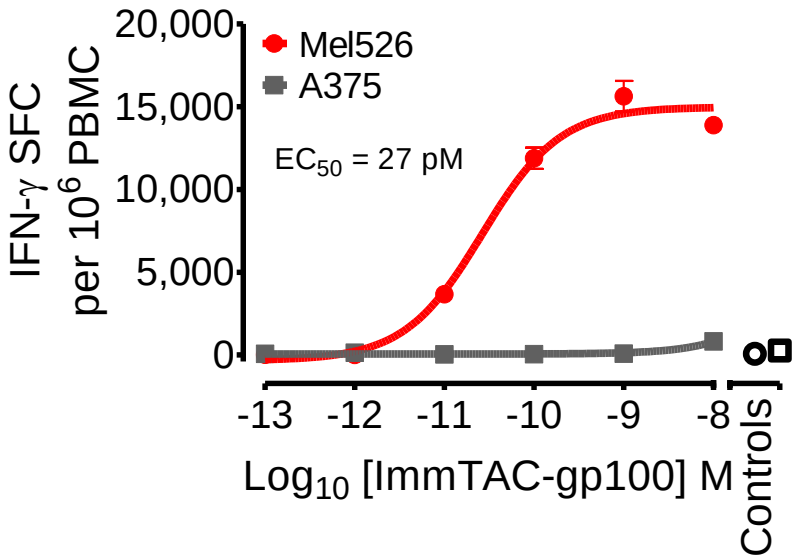


Figure 4-3 ImmTAC-gp100 redirected activity of healthy donor *ex vivo* PBMC.

IFN-γ ELISpot assay showing activation of unstimulated PBMC mediated by increasing concentrations of ImmTAC-gp100 in the presence of the HLA-A*0201^{+}/gp100^{+} melanoma cell line Mel526 (red circles). The HLA-A*0201^{+}/gp100^{-} melanoma cell line A375 (grey squares) was included as a specificity control. The shape-matched open symbols represent the corresponding ImmTAC-gp100-negative controls. Controls also include PBMC in the presence of 10 nM ImmTAC-gp100, without targets (open triangle). Data are means ± s.e.m. The data shown are representative of three experiments.

4.2.3.2 Redirection of $CD4^+$ T cells

PBMC contain a mixture of T cells which includes both $CD8^+$ and $CD4^+$ T cells. $CD4^+$ T cells play a crucial role in anti-tumour immune responses, particularly the $CD4^+$ T helper (Th) lymphocyte subset. $CD4^+$ Th lymphocytes mediate their anti-tumour effects in multiple ways including the release of cytokines such as IFN-γ and TNF-α, and through the recruitment of cells of the innate immune system to the tumour site such as macrophages and NK cells. However, their major contribution for anti-tumour effects is thought to be by providing the required T cell help for generating and augmenting tumour-specific CTL responses, mainly by the secretion of the cytokine interleukin-2 (IL-2). IL-2 is an important growth factor for CTLs and its secretion by $CD4^+$ Th cells can function to recruit and retain CTLs at the tumour site (Kennedy & Celis, 2008). Furthermore, the presence of IL-2 during priming has been shown to be necessary for the secondary expansion of memory $CD8^+$ T cells (Williams et al., 2006). Therefore, the recruitment by ImmTACs of $CD4^+$ T cells, in addition to $CD8^+$ T cells, is highly desirable.

The ability of ImmTACs to activate $CD4^+$ T cells was investigated as follows. The melanoma cell line, Mel526, expressing natural levels of wild-type $gp100_{280\text{-}288}$ complexed to HLA-A*0201, was used as targets. IFN-γ activity was measured from redirected unstimulated purified $CD4^+$ T cells in the presence of increasing concentrations of ImmTAC-gp100 (Figure 4-4). The data shows concentration-dependent release of IFN-γ from the redirected $CD4^+$ T cells. The potency observed with ImmTAC-gp100 redirected $CD4^+$ T cells (EC_{50} value = 58 pM) is similiar to that achieved using purified unstimulated $CD8^+$ T cells (EC_{50} value = 62 pM) (Figure 4-1b) or PBMC (EC_{50} value = 27 pM) (Figure 4-3). The data suggests that the recruitment by

ImmTACs of $CD4^+$ T cells could be beneficial in augmenting the anti-tumour response *in vivo*.

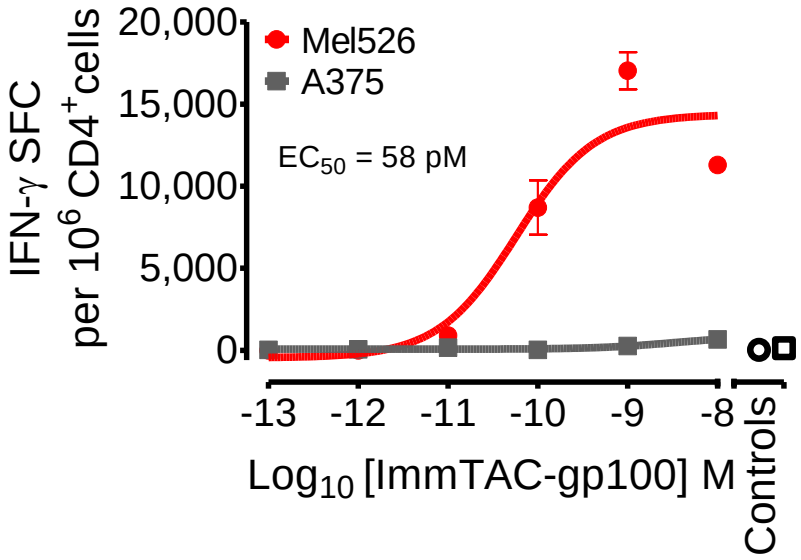


Figure 4-4 ImmTAC-gp100 redirected activity of $CD4^+$ T cells.

IFN-γ ELISpot assay showing activation of unstimulated purified $CD4^+$ T cells mediated by increasing concentrations of ImmTAC-gp100 in the presence of the HLA-$A*0201^+/gp100^+$ melanoma cell line Mel526 (red circles). The HLA-$A*0201^+/gp100^-$ .melanoma cell line A375 (grey squares) was included as a specificity control. The shape-matched open symbols represent the corresponding ImmTAC-gp100-negative controls. Controls also include $CD4^+$ T cells in the presence of 10 nM ImmTAC-gp100, without targets (open triangle). Data are means ± s.e.m. The data shown are representative of two experiments.

4.2.4 The $CD8^+$ T cell subsets and polyfunctional responses activated by ImmTAC redirection

To further determine the effects of ImmTACs under physiological conditions and to examine in more detail the subsets of $CD8^+$ T cells that are activated by ImmTAC, polychromatic flow cytometry experiments were conducted on PBMC directly *ex vivo*. The following flow cytometry data sets are courtesy of Dr. David Price and Dr. Kristin Ladell at Cardiff University (Liddy et al., 2012).

A titration experiment with ImmTAC-gp100 against PBMC pulsed with the $gp100_{280\text{-}288}$ heteroclitic peptide YLEPGPVTV showed that $CD8^+$ T cells are activated in a concentration-dependent manner to elicit multiple effector functions. This includes degranulation, as indicated by the surface mobilisation of lysosomal-associated membrane protein-1 (CD107a), and production of the cytokines IFN-γ, tumour necrosis factor (TNF) and interleukin-2 (IL-2) (Figure 4-5a). At the same time, the use of standard phenotypic parameters revealed which subsets of $CD8^+$ T cells are being activated by ImmTAC-gp100 (Figure 4-5b). The data indicates that the activated $CD8^+$ T cells are mainly distributed in the central memory ($CD45RO^+$, $CD27^+$) and effector memory ($CD45RO^+$, $CD27^-$) compartments. A similar pattern of responses, although weaker, are also elicited when using the melanoma cell line, Mel526, expressing natural levels of antigen as targets (Figure 4-5c). In addition, most of the activated $CD8^+$ T cells showed a terminally differentiated phenotype characterised by the expression of the senescence marker CD57 (Liddy et al., 2012); CD57 identifies $CD8^+$ T cells with maximal lytic capacity (Chattopadhyay et al., 2009). Activation of the $CD4^+$ T cell compartment is also detected and the response is characterised by the production of substantial amounts of TNF and IL-2 (Figure 4-5d-f).

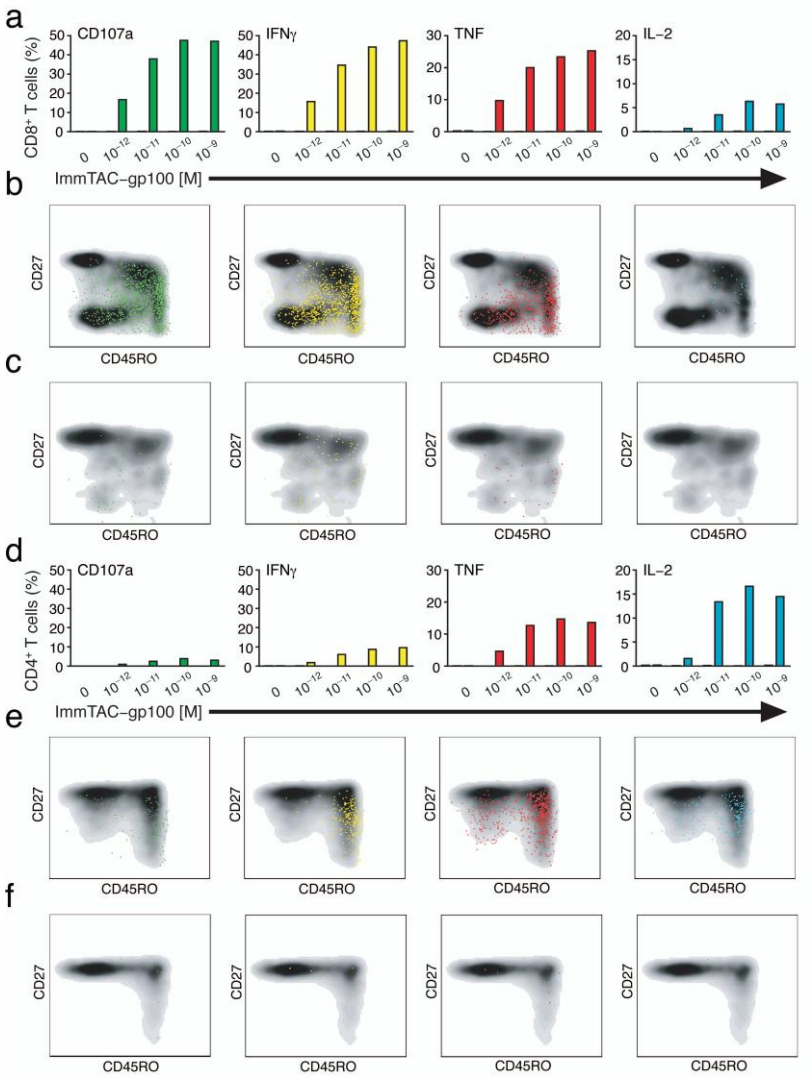


Figure 4-5 Activation of multiple CD8$^+$ T cell effector functions by ImmTAC-gp100.

Polychromatic flow cytometry experiments showing the activation of fresh PBMC mediated by ImmTAC-gp100 at the indicated concentrations in the presence of target PBMC pulsed with the $gp100_{280-288}$ heteroclitic peptide YLEPGPVTV (10^{-6} M) or mock pulsed with medium alone, or target Mel526 melanoma cells expressing endogenous wild-type $gp100_{280-288}$ complexed to HLA-A*0201. Degranulation marker, CD107a (green) and the intracellular production of the cytokines IFN--γ (yellow), TNF (red) and IL-2 (turquoise) are shown. **(a)** The dose-response relationship between the concentration of ImmTAC-gp100 and the percentage of CD8$^+$ T cells activated to express each individual function in the presence of peptide-pulsed PBMC. **(b,c)** The phenotypic profile of CD8$^+$ T cells activated with ImmTAC-gp100 at a concentration of 10^{-11} M in the presence of peptide-pulsed PBMC **(b)** or Mel526 cells **(c)**. The coloured dots depict individual cells that elicited a distinct function, as indicated in **a**, superimposed on cloud plots showing the phenotypic profile of the overall CD8$^+$ T cell population; events shown in **b** correspond to those shown in **a** at a concentration of 10^{-11} M and are colour coded to match. **(d-f)** CD4$^+$ T cell activation data from the same experiments and with the same details described in **a-c**. Courtesy of D. Price and K. Ladell.

4.2.5 Granzyme B release by T cells activated by ImmTAC

Granzyme B is a member of the granzyme family of serine proteases which are located in the cytotoxic granules of CTLs and NK cells. Granzyme B induces the apoptosis of target cells by cleaving and activating members of the caspase family. Conventionally, killing assays such as ^{51}Cr-release assay have been used for the detection of activated CTLs. However, assays that measure the secretion of cytolytic granule contents provide an alternative functional readout of specifically activated and degranulating T cells. The granzyme B ELISpot assay has been validated as a non-radioactive alternative to ^{51}Cr–release assays; results in the ELISpot assay have been shown to correlate with cytolytic responses measured by the classic radioactive ^{51}Cr-release assay (Rininsland et al., 2000) and may even be superior because of its high sensitivity (Malyguine et al., 2007).

Using the ELISpot assay, the ability of ImmTACs to stimulate granzyme B release from unstimulated purified CD8$^+$ T cells was investigated. The Mel526 melanoma cell line expressing natural levels of wild-type gp100$_{280\text{-}288}$ complexed to HLA-A*0201 was used as targets in the presence of increasing concentrations of ImmTAC-gp100 (Figure 4-6). A concentration-dependent release of granzyme B from the redirected CD8$^+$ T cells is observed suggesting that ImmTAC-gp100 is specifically activating CTL mediated apoptosis of the Mel526 melanoma targets via the granule exocytosis pathway. No activity is seen against the HLA-A*0201$^+$, antigen-negative cell line A375, or in the absence of targets.

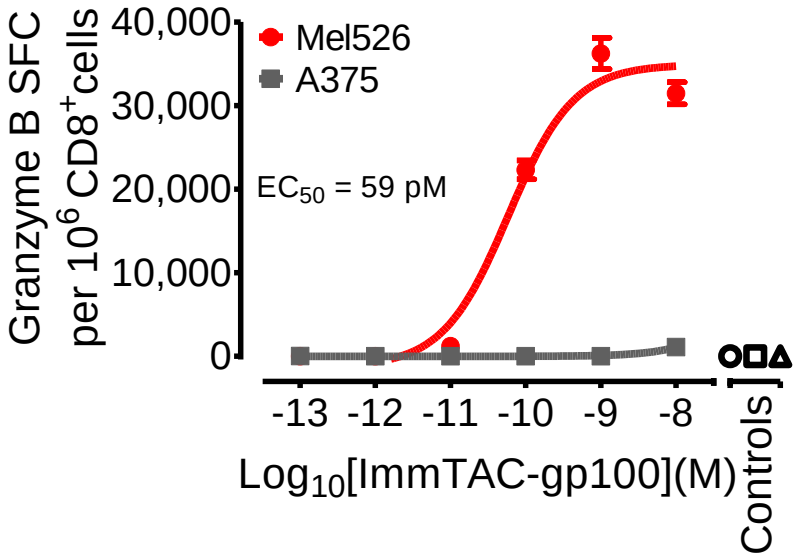


Figure 4-6 Granzyme B release by $CD8^+$ T cells redirected by ImmTAC-gp100.

Granzyme B ELISpot assay showing activation of unstimulated purified $CD8^+$ T cells mediated by increasing concentrations of ImmTAC-gp100 in the presence of the HLA-A*0201$^+$/gp100$^+$ melanoma cell line Mel526 (red circles). The HLA-A*0201$^+$/gp100$^-$ melanoma cell line A375 (grey squares) was included as a specificity control. The shape-matched open symbols represent the corresponding ImmTAC-gp100-negative controls. Controls also include $CD8^+$ T cells in the presence of 10 nM ImmTAC-gp100, without targets (open triangle). Data are means ± s.e.m. The data shown are representative of three experiments.

Next, to test that ImmTACs could stimulate granzyme B release from a more physiologically relevant population of T cells, assays were performed using PBMC as effectors. In this example, EJM, a multiple myeloma cell line expressing natural levels of wild-type MAGE-$A3_{168\text{-}176}$ complexed to HLA-A*0101 was used as targets in the presence of increasing concentrations of the ImmTAC-MAGE (Figure 4-7). As seen for purified $CD8^+$ T cells, concentration-dependent release of granzyme B from the redirected PBMC is also observed producing an EC_{50} value of 30 pM.

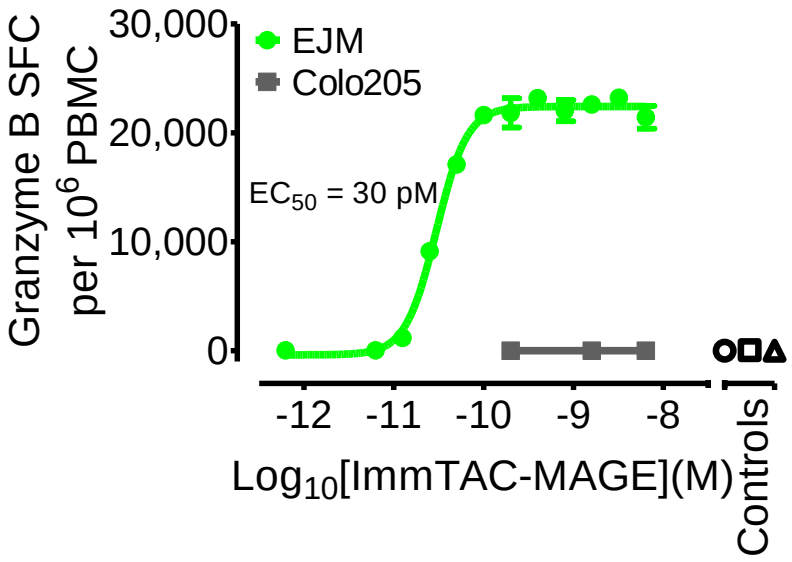


Figure 4-7 Granzyme B release by PBMC redirected by ImmTAC-MAGE.

Granzyme B ELISpot assay showing the activation of unstimulated PBMC mediated by increasing concentrations of ImmTAC-MAGE in the presence of the HLA-A*0101$^+$/MAGE-A3$^+$ multiple myeloma cell line EJM (green circles). The HLA-A*0101$^+$/MAGE-A3$^-$ cell line Colo205 (grey squares) was included as a specificity control. The shape-matched open symbols represent the corresponding ImmTAC-MAGE-negative controls. Controls also include PBMC in the presence of 6.4 nM ImmTAC-MAGE, without targets (open triangle). Data are means ± s.e.m. The data shown are representative of six experiments.

4.2.6 Testing the capacity of cancer patient derived T cells for ImmTAC redirected activation

There is some evidence to suggest that global T cell dysfunction is a common feature in cancer patients (Finke et al., 1993; Nakagomi et al., 1993). It has also been reported that tumour associated antigen (TAA)-specific T cells generated *de novo* in patients with metastatic melanoma are rendered functionally unresponsive, fail to kill melanoma target cells or produce cytokines in response to mitogens (Lee et al., 1999). Furthermore, cancer patients that have received intensive chemotherapy could be immunosuppressed (Mackall, 2000). Therefore, T cells from late stage cancer patients could be less responsive to ImmTAC redirected activation compared to those from healthy donors. To approach this question, the ability of ImmTAC-gp100 to redirect and enhance the biological activity of late stage melanoma patient T cells was investigated. The Mel526 melanoma cell line, expressing natural levels of wild-type $gp100_{280\text{-}288}$ complexed to HLA-A*0201, was used as targets. IFN-γ and granzyme B activity was measured from redirected unstimulated PBMC derived from two different stage IV HLA-A*0201^{+} melanoma patients (Fig 4-8a,b and Fig 4-9a,b). The data shows that these cancer patient T cells are not functionally impaired in the presence of ImmTAC-gp100, and respond in a similar concentration-dependant manner to T cells derived from healthy donors. Interestingly, the PBMC of one patient already appears reactive to melanoma cells without stimulation by ImmTAC-gp100 as evidenced by the presence of raised IFN-γ baseline activity (indicated by the open circle in Figure 4-8a). This data further shows that ImmTAC-gp100 is capable of amplifying an already present anti-tumoural IFN-γ response in patient blood. However, no raised baseline activity is evident for granzyme B release (Figure 4-8b) despite the same patient PBMC being used.

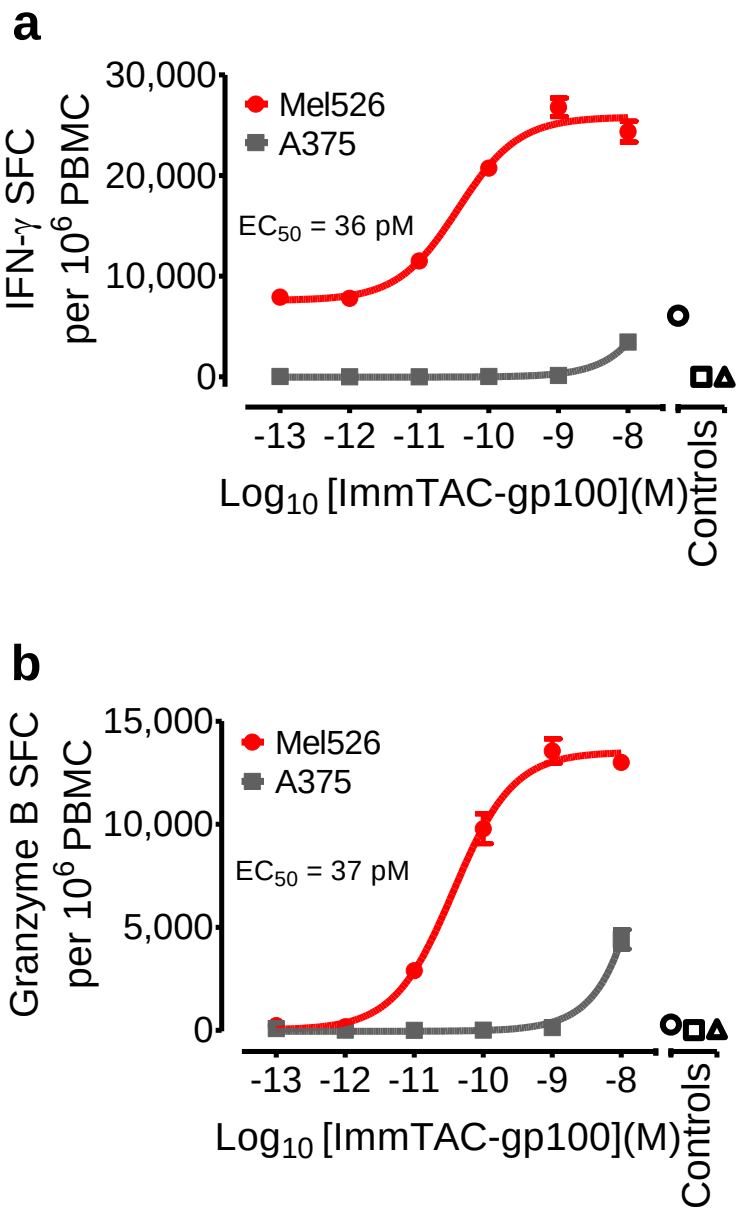


Figure 4-8 ImmTAC-gp100 redirected activity of stage IV melanoma patient PBMC (sample ID: IC/9/18).

ELISpot assays showing the activation of advanced melanoma patient PBMC mediated by increasing concentrations of ImmTAC-gp100 in the presence of the HLA-A*0201^{+}/gp100^{+} melanoma cell line Mel526 (red circles) and the control HLA-A*0201^{+}/gp100^{-} melanoma cell line A375 (grey squares). The shape-matched open symbols represent the corresponding ImmTAC-gp100-negative controls. Controls also include PBMC in the presence of 10 nM ImmTAC-gp100, without targets (open triangles). **(a)** IFN-γ release by redirected melanoma patient PBMC; data shown are representative of three experiments **(b)** Granzyme B release by redirected melanoma patient PBMC; data shown are representative of two experiments. Data are means ± s.e.m.

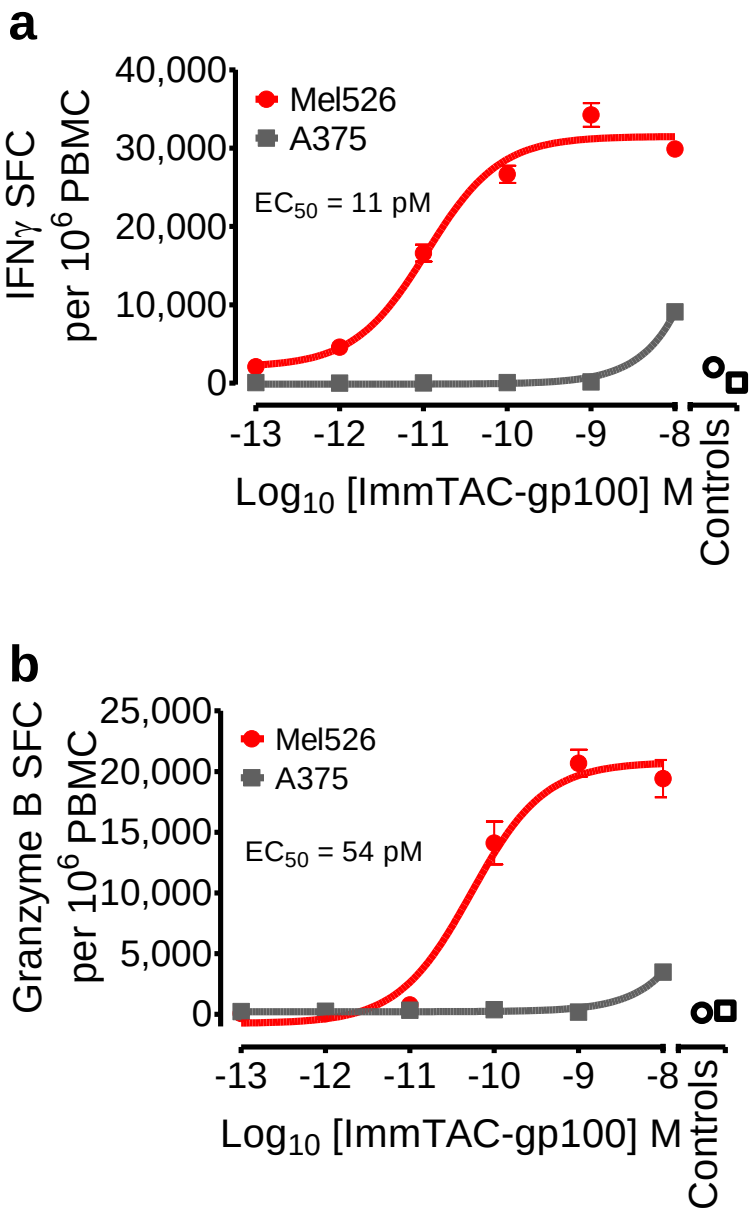


Figure 4-9 ImmTAC-gp100 redirected activity of stage IV melanoma patient PBMC (sample ID: IC/12/20).

ELISpot assays showing the activation of advanced melanoma patient PBMC mediated by increasing concentrations of ImmTAC-gp100 in the presence of the HLA-A*0201^{+}/gp100^{+} melanoma cell line Mel526 (red circles) and the control HLA-A*0201^{+}/gp100^{-} melanoma cell line A375 (grey squares). The shape-matched open symbols represent the corresponding ImmTAC-gp100-negative controls. Controls also include PBMC in the presence of 10 nM ImmTAC-gp100, without targets (open triangles). **(a)** IFN-γ release by redirected melanoma patient PBMC; data shown are representative of three experiments **(b)** Granzyme B release by redirected melanoma patient PBMC; data shown are representative of two experiments. Data are means ± s.e.m.

4.2.7 *Ex vivo* ImmTAC redirected activation of marrow infiltrating lymphocytes (MILs)

Multiple myeloma is a disease that occurs in the bone marrow. It is characterised by the malignant outgrowth of clonal plasma cells that secrete a monoclonal immunoglobulin, and the eventual development of osteolytic bone lesions (Mahindra et al., 2010). The bone marrow tumour microenvironment is known to play an important role in disease progression. Complex interactions of the myeloma tumour cells with various cell types within the bone marrow such as bone marrow stroma, osteoclasts (bone resorbing cells), osteoblasts (bone forming cells), T cells, dendritic cells (DCs) and myeloid-derived suppressor cells (MDSCs) promote cancer cell survival and evasion of immune recognition (Noonan & Borrello, 2011).

A study has shown that activated marrow-infiltrating lymphocytes (MILs) from multiple myeloma patients show significant tumour specificity compared to activated peripheral blood lymphocytes (PBL) from the same patient. Furthermore, activated MILs were shown to target both the terminally differentiated $CD138^+$ plasma cells and their clonogenic precursors (Noonan et al., 2005). However, the immune suppressive microenvironment in myeloma usually prevents the generation of effective anti-tumour responses by MILs. This is supported by evidence that tumour-specific T cells from the myeloma microenvironment are not reactive against autologous tumour cells *ex vivo*, but that this reactivity is restored after stimulation *ex vivo* with DCs that have processed autologous tumour cells (Dhodapkar et al., 2002).

The ability of ImmTAC-MAGE to redirect and enhance the biological of activity of *ex vivo* MILs was investigated. It was not technically feasible to isolate and purify MILs directly from the multiple myeloma patient bone marrow aspirate biopsy samples due to

the low cell numbers. Therefore, the $CD138^-$ population, which contains MILs, was isolated and used in the following *in vitro* cellular assays. The percentage of $CD4^+$ and $CD8^+$ MILs present in the $CD138^-$ fraction was determined by flow cytometry antibody staining experiments in order to calculate the appropriate number of MILs to use. Thus, in the following experiment, IFN-γ release was measured from redirected MILs present in the $CD138^-$ population isolated from bone marrow aspirate samples of two stage III myeloma patients. EJM, a multiple myeloma cell line expressing natural levels of wild-type MAGE-$A3_{168-176}$ complexed to HLA-A*0101, was used as targets (Figure 4-10a,b). The data shows that *ex vivo* MILs from bone marrow aspirate samples of myeloma patients can be activated by ImmTAC-MAGE in a concentration-dependent manner. The potency seen with sample #26 MILs (Figure 4-10b) is similar to that observed with healthy donor T cells using the MAGE-specific ImmTAC (Figure 4-1c) (EC_{50} values of 23 pM and 31 pM, respectively). However, for sample #22, the EC_{50} value is ~ 5-fold higher at 125 pM. It should be noted that due to limited cell numbers that the data are representative of one experiment only for each sample. Further investigations on sample #26 by a colleague, Dr. Giovanna Bossi, demonstrated that redirection by ImmTAC-MAGE resulted in an up-regulation of the activation marker CD25 on both the $CD4^+$ and $CD8^+$ MIL populations and that the MILs were able to lyse EJM target cells in a FACS-based killing assay. As a control, PBMC from a healthy donor were tested in parallel with the MILs, under identical conditions, and no significant differences were observed (data not shown). Therefore, ImmTAC-MAGE can effectively redirect T cells extracted from the myeloma microenvironment.

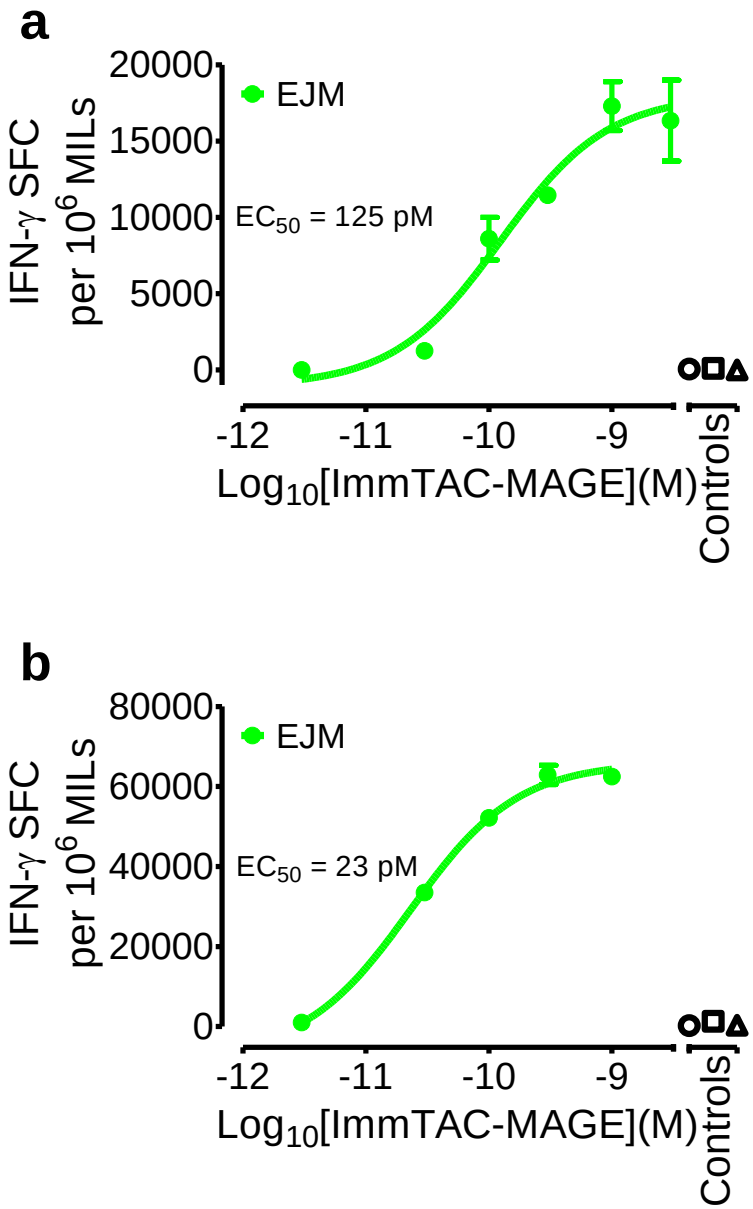


Figure 4-10 ImmTAC-MAGE redirected activity of MILs isolated from the bone aspirate samples of two stage III myeloma patients.

ELISpot assays showing the activation of late stage myeloma patient MILs mediated by titrated amounts of ImmTAC-MAGE in the presence of the HLA-A*0101^{+}/MAGE-A3^{+} myeloma cell line EJM (green). The controls are represented by EJM and MILs, in the absence of ImmTAC-MAGE (open circles), HLA-A*0101^{+}/MAGE-A3^{-} cell line Colo205 with MILs in the presence of 300 pM ImmTAC-MAGE (open squares) and, MILs in the presence of 0.3 nM ImmTAC-MAGE, without targets (open triangles) **(a)** MILs isolated from myeloma bone marrow aspirate sample #22 **(b)** MILs isolated from myeloma bone marrow aspirate sample #26. Data are means ± s.e.m. The data shown are representative of one experiment only.

4.2.8 ImmTAC redirected activation against ex vivo primary tumour cells

Established, continuously growing tumour cell lines have been employed extensively to investigate ImmTAC redirected activity. However, despite representing a convenient and reproducible tool for characterising the ImmTAC reagents, it is possible that the biological properties of tumour cell lines have been altered during the culturing process, and as such, may not completely reflect the characteristics of a clinical tumour (Tveit & Pihl, 1981). Therefore, ImmTACs were also evaluated against fresh tumour samples taken directly from patients.

Working with fresh tumour samples poses three main challenges: first, obtaining fresh tumour sample to begin with; second, the difficultly of processing the samples; and third, the low cell number and poor viability of the tumour cells after processing which involves concerns about their ultimate quality when ready to use in assays. Clinical tumour samples can be obtained from a commercial supplier or in some circumstances directly from the clinic. The limitations that have been experienced with commercial suppliers are that they cannot necessarily supply the tumour of interest. The tumour sample also needs to be of the correct HLA type which reduces the number of suitable samples further. Another issue with commercial tumour samples is the poor quality and/or viability of the samples. Ideally, fresh patient tumour samples are obtained directly from a local oncology clinic so that samples remain fresh during transportation.

For a tumour sample to be suitable for use in cell assays, a single-cell suspension is a prerequisite. Furthermore, tumour tissues often contain many normal cells such as lymphocytes and fibroblasts and therefore need to be purified to obtain an enriched population of tumour cells. For non-solid cancers, such as multiple myeloma, the cancer cells are already in suspension making them relatively easy isolate and purify.

However, for solid tumours such as melanoma, mechanical separation and/or enzymatic treatment of the tumour tissue is performed to make a single-cell suspension but this process can damage the tumour so as to limit the downstream applications. ImmTAC redirected activity is dependent on the targeted epitope being present on the surface of the tumour cells but the use of the enzyme trypsin, for example, which is an effective treatment for the disaggregation of solid tumours, can destroy cell surface molecules such as pMHC. On the other hand, gentler methods are usually not sufficient to produce a single-cell suspension. However, the biggest challenge faced with using fresh tumour samples is the low cell number and poor viability after processing. Although bone marrow aspirate samples from multiple myeloma patients are relatively easy to process, the viability of $CD138^+$ cells, which contain the malignant plasma cells, decreases rapidly, minutes after isolation. To use the $CD138^+$ cells in the cell assays described in section 4.2.8.2 required careful planning and efficient execution of the experiment to ensure the tumour cells were of good quality.

4.2.8.1 ImmTAC-NYE activation of T cells in the presence of freshly isolated lung tumour cells

A clinical lung cancer sample, NSCLC#29, which is known to express high levels of LAGE-1, as determined by quantitative RT-PCR, and is HLA-A*0201 positive (McCormack et al., 2012), was used to test the ability of the NY-ESO-1/LAGE-1-specific ImmTAC, ImmTAC-NYE, to redirect T cell activity against freshly isolated tumour cells. IFN-γ activity was measured from redirected unstimulated PBMC (Fig 4-11). The data shows that IFN-γ is released from the PBMC in the presence of NSCLC#29 and ImmTAC-NYE in a concentration-dependent manner, similar to that observed against tumour cell lines. However, the EC_{50} value at 1.3 nM is considerably higher than that seen against tumour cell lines. The proportion of tumour cells within the sample was not known, and the quality of the material was quite low which may explain the discrepancy in EC_{50} values. Limited tumour cell numbers meant that redirected killing by ImmTAC-NYE could not be evaluated, and it should be noted that the data is representative of one experiment only.

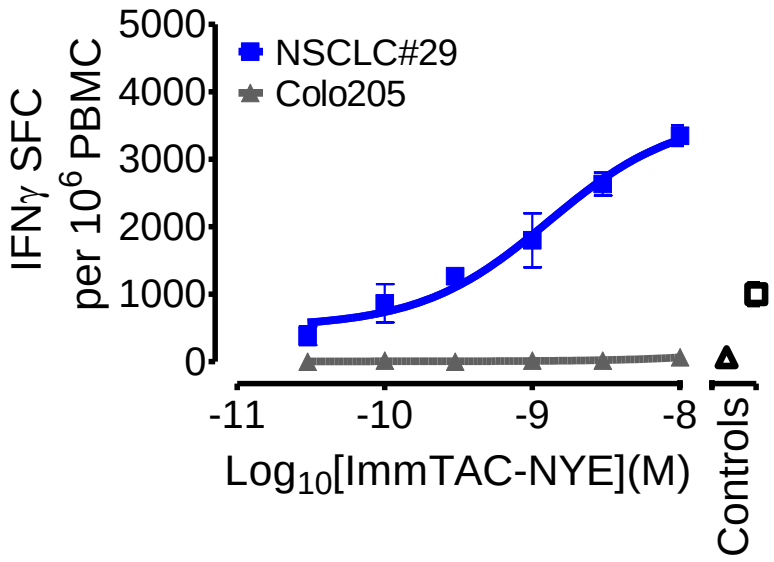


Figure 4-11 ImmTAC-NYE redirected activity against HLA-A*0201^{+}/LAGE-1^{+} freshly isolated lung cancer sample.

IFN-γ ELISpot assay showing activation of unstimulated PBMC mediated by increasing concentrations of ImmTAC-NYE in the presence of freshly isolated HLA-A*0201^{+}/LAGE-1^{+} lung cancer sample, NSCLC#29 (blue squares). The HLA-A*0201^{+}/LAGE-1^{-}/NY-ESO^{-} cell line Colo205 (grey triangles) was used as a specificity control. The shape-matched open symbols represent the corresponding ImmTAC-NYE-negative controls. Data are means ± s.e.m.

4.2.8.2 ImmTAC-MAGE activation of T cells in the presence of myeloma targets derived from fresh primary myeloma tumour sample

CD138 (syndecan-1) is a trans-membrane heparin sulphate proteoglycan that is typically expressed on plasma cells but not on T or B cells. Therefore, CD138 is considered the most specific marker for plasma cells and is expressed on normal and malignant plasma cells (P. Lin et al., 2004).

To investigate the activity of ImmTAC-MAGE against cells from a primary myeloma tumour sample, $CD138^+$ plasma cells isolated from the bone marrow aspirate sample of a stage II multiple myeloma patient (sample #20), which is known to express MAGE-A3, as determined by quantitative RT-PCR, and is HLA-A*0101 positive, were used as targets. IFN-γ activity was measured from redirected unstimulated PBMC isolated from a healthy donor (Fig 4-12). The data shows that IFN-γ is released from the PBMC upon redirection with ImmTAC-MAGE in the presence of $CD138^+$ cells, which contain the malignant plasma cells, whereas activity against the $CD138^-$ cells is minimal. Attempts were made to isolate $CD138^+$ from four other myeloma patient bone marrow aspirate samples but data could not be obtained from these experiments due to the poor viability of the purified $CD138^+$ cells. This serves to highlight the challenges of handling fresh tumour samples.

Furthermore, concomitant FACS-based killing experiments conducted by Dr. Giovanna Bossi, showed that ImmTAC-MAGE used at 0.1 nM redirect T cells to kill 52% of the $CD138^+$ targets. By using an additional phenotypic marker, CD38, which is expressed at higher frequency by myeloma cells (P. Lin et al., 2004), Dr. Bossi found that the

$CD38^+$ sub-population within the $CD138^+$ target cells were preferentially killed (data not shown).

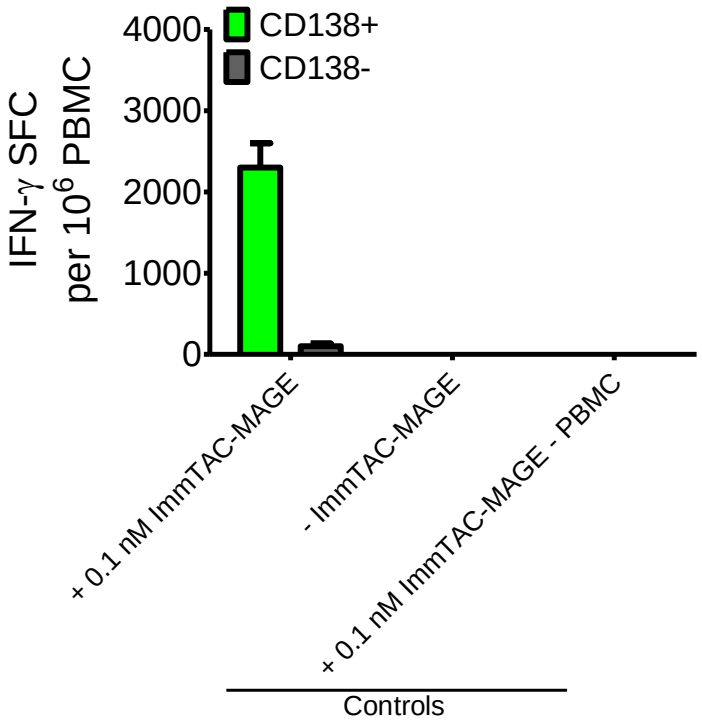


Figure 4-12 ImmTAC-MAGE redirected activity against CD138$^+$ cells isolated from multiple myeloma bone marrow aspirate sample.

IFN-γ ELISpot assay showing activation of unstimulated PBMC mediated by 0.1 nM of ImmTAC-MAGE in the presence of CD138$^+$ cells (green bar) or CD138$^-$ cells (grey bar) isolated from bone marrow aspirate sample of a HLA-A*0101$^+$ stage II multiple myeloma patient (sample #20). Controls are represented by targets and PBMC in the absence of ImmTAC-MAGE, and 0.1 nM ImmTAC-MAGE and PBMC in the absence of targets. Data are means ± s.e.m. The data shown are representative of one experiment only.

4.3 Summary

The experiments described in this chapter show that ImmTAC reagents can potently redirect and activate T cells in the presence of tumour cell lines presenting naturally processed cognate peptide-HLA complex. All 4 ImmTACs elicit cellular EC_{50} values in the range of 100 pM or lower. In peptide-titration experiments I show that the antigen-sensitivity of the ImmTAC reagents is critically dependent on the affinity of the mTCR for its cognate pHLA. The highest affinity mTCR ImmTAC-gp100 was capable of activating $CD8^+$ T cells in the presence of target cells with pHLA densities as low as 2-10 copies per cell. Subsequently, I demonstrated that ImmTACs can redirect T cell activity against *ex vivo* primary tumour in addition to tumour cell lines. From these experiments I conclude that ImmTACs are effective against tumour cells expressing low surface epitope densities, as may be encountered on tumour cells in patients. I also show that ImmTAC redirected responses are specific and limited to cells that express cognate pHLA.

T cell activation assays, measuring IFN-γ release by ELISpot assay and the initiation of the granule-exocytosis pathway of cell cytotoxicity as measured by granzyme B ELISpot, were used for demonstrating ImmTAC redirection of healthy donor unstimulated $CD8^+$ T cells and $CD4^+$ T cells, as well as unselected T cells i.e. PBMC. Furthermore, I was able to show that ImmTACs can potently redirect PBMC from late stage melanoma patients and marrow infiltrating lymphocytes (MILs) from late stage multiple myeloma patients. These results provide the first demonstration that a soluble reagent, based on human T cell receptors, can redirect the activity of non-tumour specific T cells, and enhance the activity of tumour specific T cells, against naturally presenting cancer cells.

Other markers of T cell activation were also investigated such as the degranulation marker CD107a, and cytokines TNF and IL-2, indicating that ImmTACs can induce polyfunctional T cell responses. Furthermore, it was demonstrated that the responding $CD8^+$ T cells are mainly distributed in the central memory ($CD45RO^+$, $CD27^+$) and effector memory ($CD45RO^+$, $CD27^-$) compartments, and the activation of naïve T cells ($CD45RO^-$, $CD27^+$) is minimal.

For the therapeutic ImmTAC candidate, ImmTAC-gp100, the potency data I have presented in this chapter has also been used in the pre-clinical data package, including regulatory documentation for the Food and Drug Administration (FDA) and the Medicine and Healthcare products Regulatory Agency (MHRA), to support its admission into a first-in-human clinical trial. The therapeutic ImmTAC-gp100 candidate is described in more detail in Chapter 6.

CHAPTER 5 TARGET CELL KILLING BY IMMTAC REDIRECTED T CELLS

5.1 Introduction

In the previous chapter, T cell activation assays were performed to assess the early events leading to target cell lysis by ImmTAC redirected T cells. In these assays, the secretion of the cytokine IFN-γ has been analysed as has initiation of the granule-exocytosis pathway of cytotoxicity through the detection of the release of granzyme B. However, for cancer treatment, the most desirable effector function is target cell lysis. Critical to the therapeutic potential of the ImmTAC molecule is that it is capable of redirecting CTLs to lyse tumour cells. In this chapter I have attempted to perform a range of cellular assays to examine target cell fate. As described in section 1.5.7.1, killing by CTL is based, at least in part, on the induction of apoptosis within the target cell. During CTL-induced apoptosis, caspases become activated via the action of death receptors, cytochrome *c*/ Apaf-1, or through direct cleavage by granzyme B. A caspase cascade is triggered which in turn acts upon substrates to begin the final phase of apoptosis whereby the cell is eventually destroyed. Killing assays such as ^{51}Cr-release and other non-radioactive 'release' assays are the main techniques for measuring CTL-induced target cell damage during the final phase of apoptosis. However, in addition to 'release' assays I have investigated other methodologies to examine the short-term manifestations of ImmTAC redirected target cell killing by measuring the activation of caspases. Furthermore, I describe the use of a novel technique to directly visualise ImmTAC redirected target cell killing over longer time intervals. Finally, I attempted to assess the redirected lytic capability of different T cell populations from healthy donors including $CD8^+$ T cell clones, polyclonal $CD8^+$ T cells, and PBMC. As potential cancer

therapeutics, ImmTACs must be able to redirect cancer patient T cells; therefore, the lytic activity of redirected cancer patient PBMC was also assessed.

5.2 Results

5.2.1 Short-term manifestations of ImmTAC redirected target cell killing

In order to assess the earliest manifestations of ImmTAC redirected killing, the activation of caspases in target cells, an event which occurs downstream of granzyme B release, was investigated. Caspases are a family of intracellular cysteine proteases that are responsible for the breakdown of the cytoskeleton and the nuclear membrane, as well as fragmentation of DNA, during apoptotic cell death (Russell & Ley, 2002). Caspases are synthesised in the cytoplasm as inactive, pro-caspase zymogens which comprise several domains including an N-terminal pro-domain, a large subunit and a small subunit. The pro-caspases can be classified into two main groups based on the length of their N-terminal pro-domain. A short pro-domain characterises pro-caspase-3, -6 and -7 which exist as dimers that require proteolysis at internal aspartate residues to generate two large and two small subunits. Fully active enzymes result from heterodimerisation of these subunits to form a caspase tetramer. Caspases 3, 6 and 7 are the main effectors of apoptotic cell death by cleaving cellular substrates, either a down-stream pro-caspase or other cellular proteins, on the C-terminal side of an aspartate residue. Furthermore, activation of these 'so-called' effector or executioner caspases typically results in the irreversible commitment of a cell to apoptosis. Other pro-caspases such as pro-caspase-8, -9 and -10 are characterised by their long pro-domain. These caspases typically exist as monomers and require dimerisation or oligomerisation for activation that can occur in the absence of proteolytic cleavage. Pro-caspase-8, -9 and -10 are known as initiator caspases and can activate effector caspases, such as

caspase-3, thereby triggering a caspase cascade (I. Chowdhury et al., 2008; Launay et al., 2005).

Activation caspase-3 and -7 are reliable markers for the detection of cells undergoing apoptosis, therefore, activation of these particular caspases by ImmTAC redirected T cells was evaluated. The melanoma cell line, Mel526, expressing natural levels of wild-type $gp100_{280-288}$ complexed to HLA-A*0201 was used as targets, and incubated with $CD8^+$ T cells in the presence of increasing concentrations of ImmTAC-gp100 (Figure 5-1). A concentration-dependent increase in the level of caspase-3/7 activity was observed after 4 hours. ImmTAC-gp100 did not stimulate caspase-3/7 activity in the presence of the HLA*0201-positive, antigen-negative cell line, A375.

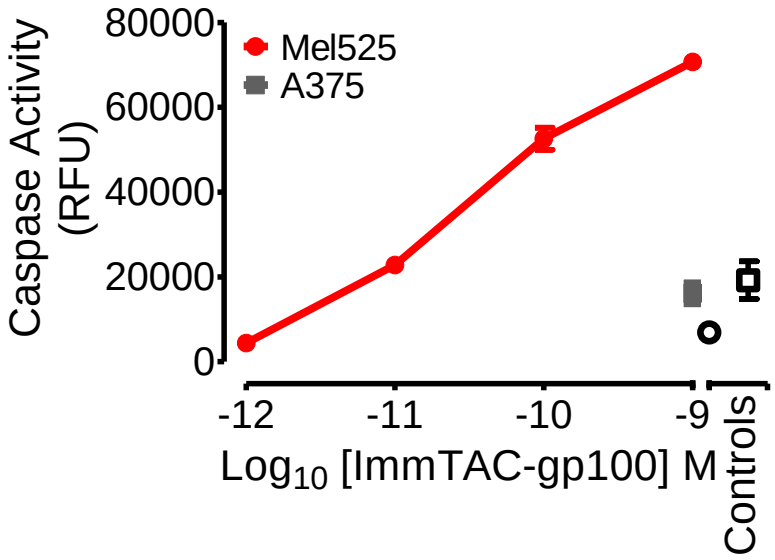


Figure 5-1 Activation of caspases by ImmTAC-gp100 redirected CD8$^+$ T cells.

A 4 hour caspase activation assay (Promega Apo-ONE® Homogeneous Caspase-3/7) showing caspase-3/7 activation in response to unstimulated purified CD8+ T cells mediated by increasing concentrations of ImmTAC-gp100 in the presence of the HLA-A*0201$^+$/gp100$^+$ melanoma cell line Mel526 (red circles) at an E:T ratio of 5:1. The HLA-A*0201$^+$/gp100$^-$.melanoma cell line A375 (grey square) was included as a specificity control at a single concentration of ImmTAC-gp100 (1 nM). The shape-matched open symbols represent the corresponding ImmTAC-gp100-negative controls. Data are means ± s.e.m. The data shown are representative of three experiments.

5.2.2 Short-term target cell killing by ImmTAC redirected T cells

Once the caspase cascade has been activated, the final stage in the process is lysis of the target cell. To measure short-term target cell lysis by ImmTAC redirected T cells, a non-radioactive Lactate dehydrogenase (LDH) release assay (Promega CytoTox96®) was used. LDH is a stable cytosolic enzyme that is released upon cell lysis. The CytoTox96® assay quantitatively measures the amount of LDH released into the cell culture supernatant when a substrate mix is added that converts a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of lysed cells, the absorbance of which can be measured. LDH release killing assays have been well validated for measuring natural cytotoxicity and have been shown to give identical (within experimental error) values as those determined in parallel ^{51}Cr-release assays (Decker & Lohmann-Matthes, 1988; Korzeniewski & Callewaert, 1983).

5.2.2.1 ImmTAC enhancement of cancer-specific T cell potency

The ability of ImmTAC-gp100 to enhance the killing of melanoma cells by a CD8$^+$ T cell clone specific for Melan-A/MART-1 (MEL187.c5) was investigated in a LDH-release killing assay. The melanoma cell line, Mel624, which expresses natural levels of both gp100 and Melan-A/MART-1 antigen in the context of HLA-A*0201 are killed by MEL187.c5 without the presence of ImmTAC-gp100 with levels of specific lysis below 50% after 4 hours at an effector-to-target ratio of 5:1 (Figure 5-2). However, in the presence of ImmTAC-gp100, which binds a different epitope on the same tumour cells, substantially more target cells are lysed by MEL187.c5 under otherwise identical conditions with specific lysis of 80% observed at an E:T ratio of 5:1. Furthermore, the addition of a 100-fold excess of the high affinity gp100-specific mTCR as a competitor

for epitope binding effectively abrogates the enhanced killing effect, reducing the levels of lysis to that seen in the presence of MEL187.c5 alone. The data shows that the enhancement of lytic function mediated by ImmTAC-gp100 is the direct result of binding to the cognate pMHCI epitopes expressed on the cell surface of the target cell and does not occur as a non-specific effect.

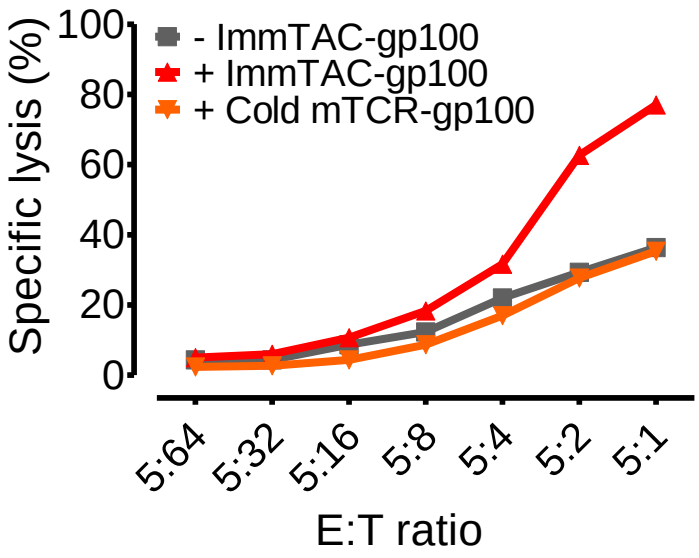


Figure 5-2 Enhanced redirected lysis of tumour cells by cancer-specific T cell clone in the presence of ImmTAC-gp100.

LDH-release killing assay showing lysis of Mel624 melanoma cells over a 4 hour time course by a $CD8^+$ T cell clone (MEL187.c5) specific for Melan-A/MART-1_{26-35} epitope at different E:T ratios (grey squares). Also shown is lysis by redirection in the presence of 1 nM ImmTAC-gp100 (red triangles) and inhibition of redirected lysis mediated by ImmTAC-gp100 with 100 nM cold high affinity mTCR-gp100 (orange inverted triangles). Data are means ± s.e.m. The data shown are representative of one experiment only.

5.2.2.2 Evaluation of ^{51}Cr-release assay and its limitations in measuring ImmTAC redirected killing

Conventionally, target cell lysis by CTL is measured using the four hour ^{51}Cr-release assay, first described by Brunner *et al.* (1968), and is considered to be the gold standard despite the requirement for potentially hazardous and difficult-to-dispose-of radioactive labels. When used as sodium chromate ($Na_2{}^{51}CrO_4$), the ^{51}Cr-isotope is taken up by live cells in the hexavalent form and is released from lysed cells in the trivalent form, which is not re-utilised. The applicability of ^{51}Cr-release to measure redirected lysis of Mel526 melanoma target cells by $CD8^+$ T cell clone (176.c4.1) specific for a BRLF1 epitope derived from Epstein-Barr virus (EBV) and unstimulated purified (polyclonal) $CD8^+$ T cells in the presence of ImmTAC-gp100 was assessed (Figure 5.3a,b). The data shows that a concentration-dependent lysis of the Mel526 melanoma cell targets is detected, albeit at a low level, when redirecting a $CD8^+$ T cell clone with ImmTAC-gp100 (Figure 5.3a), which essentially represents a population of pre-activated T cells. Note that the Mel526 melanoma cells are not killed by this clone, unless redirected by ImmTAC, because they do not present the cognate antigen. In contrast, redirected lysis by unstimulated, polyclonal $CD8^+$ T cells is not detected (Figure 5.3b). Attempts were made to increase the duration of the ^{51}Cr-release assay beyond four hours to allow more time for the unstimulated $CD8^+$ T cells to become activated and kill. However, with extended assay times very high spontaneous release prevented the measurement of specific lysis.

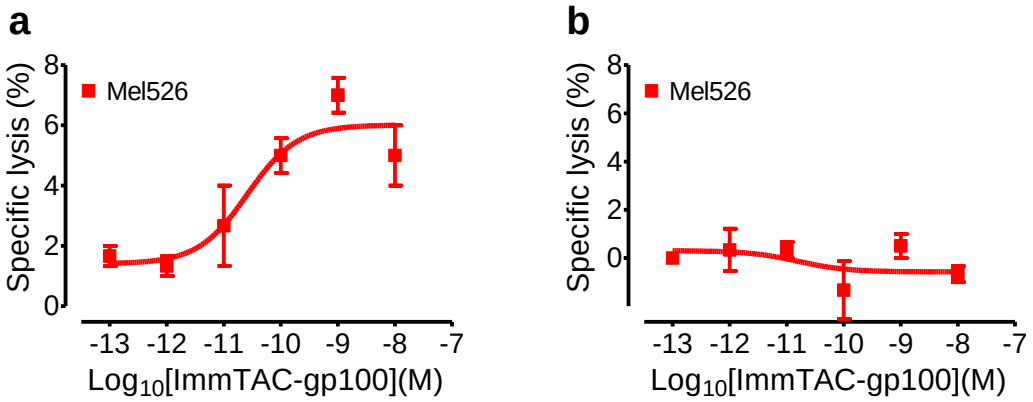


Figure 5-3 Applicability of ^{51}Cr-release to measure redirected lysis of melanoma tumour cells by CD8$^+$ T cell clone and unstimulated purified CD8$^+$ T cells in the presence of ImmTAC-gp100.

^{51}Cr-release killing assay showing redirected lysis of HLA-A*0201$^+$/gp100$^+$ Mel526 melanoma cells by CD8$^+$ T cells in the presence of titrating amounts of ImmTAC-gp100 over 4 hours. **(a)** CD8$^+$ T cell clone (176.c4.1) specific for EBV BRLF1$_{259\text{-}267}$ epitope at an E:T ratio of 5:1. **(b)** Unstimulated purified CD8$^+$ T cells at an E:T ratio of 10:1. Data are means ± s.e.m. The data shown are representative of one experiment only.

5.2.3 An extended time course LDH-assay

The non-radioactive Promega CytoTox96® LDH-release assay has been demonstrated to be a suitable alternative to the more traditional ^{51}Cr-release assay. Figure 5-2 shows an example of redirected target cell lysis as measured by LDH-release after 4 hours when redirecting a CD8$^+$ T clone. However, as was the case for the ^{51}Cr-release assay, redirected target cell lysis was not observed in the same time frame when using unstimulated, polyclonal CD8$^+$ T cells. In contrast to the ^{51}Cr-release assay, the LDH-release assay could be run for a longer time course, up to 24 hours, without the spontaneous release becoming a limiting factor. However, beyond 24 hours the spontaneous LDH release became too high to allow accurate calculation of the percent specific lysis. The potency and specificity of ImmTAC redirected target cell killing was subsequently assessed in 24 hour LDH-release assays, as described below.

5.2.4 ImmTAC redirected killing of tumour cells by polyclonal CD8$^+$ T cells

5.2.4.1 ImmTAC-NYE redirected killing

To extend the LDH-release killing experiments to polyclonal systems, the ability of ImmTAC-NYE to redirect the lytic activity of unstimulated purified CD8$^+$ T cells was evaluated over a 24 hour time period. Various HLA-A*0201 positive tumour cell lines expressing natural levels of NY-ESO-1/LAGE-1$_{157\text{-}165}$ epitope were used as targets (Figure 5-4). With the exception of the SK-Mel-37 cells, after 24 hours concentration-dependent lysis against all the target cell lines is observed although the concentration-response curves and level of killing varied according to the tumour cell lines used. Approximately 100% killing of IM9, A375 and U266 targets is observed whereas only 40% lysis is evident for Mel624 over the time period. No lysis is observed in the absence of ImmTAC-NYE, with the exception of A375 where 30% lysis is evident.

A375 cells appear to be particularly sensitive to lysis and some background lysis of A375 is occasionally observed in the absence of ImmTAC.

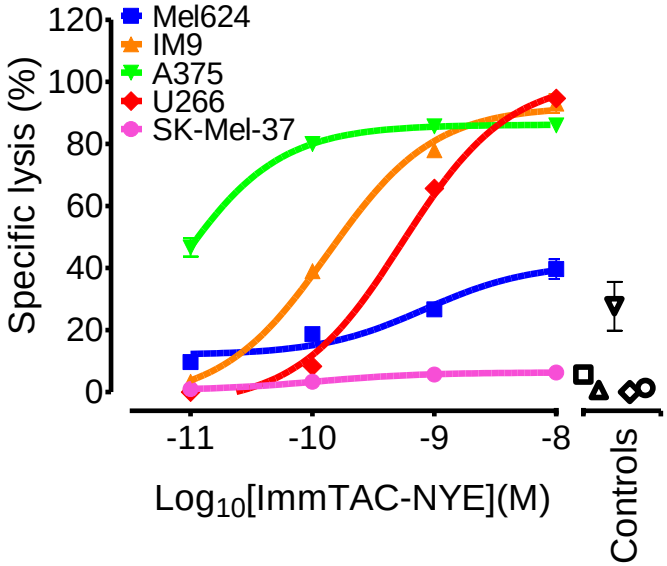


Figure 5-4 Redirected lysis of various tumour cell lines by polyclonal CD8$^+$ T cells in the presence of ImmTAC-NYE.

LDH-release killing assay showing lysis of HLA-A*0201$^+$, NY-ESO-1$^+$/LAGE-1$^+$ tumour cell lines by unstimulated purified CD8$^+$ T cells at an E:T ratio of 10:1 in the presence of increasing concentrations of ImmTAC-NYE over 24 hours. Mel624 melanoma cells (blue squares); IM9 Epstein-Barr virus (EBV)-transformed B-cells (orange triangles); A375 melanoma cells (green inverted triangles); U266 myeloma cells (red diamonds); SK-Mel-37 melanoma cells (pink circles). The shape-matched open symbols represent the corresponding ImmTAC-NYE-negative controls Data are means ± s.e.m. The data shown are representative of two experiments.

5.2.4.2 ImmTAC-gp100 redirected killing

To demonstrate that an ImmTAC of a different specificity could also redirect the lytic activity of polyclonal $CD8^+$ T cells, ImmTAC-gp100 was tested against four melanoma cell lines expressing <70 copies of the HLA-A*0201-restricted $gp100_{280-288}$ epitope per cell (Figure 5-5). After 24 hours concentration-dependent lysis against all the target cell lines is observed although the concentration-response curves and level of killing varied according to the tumour cell lines used. However, the EC_{50} values in the experiments with Mel526 and Mel624 are 30 pM and 51 pM, respectively. The control A375 cells, which express HLA-A*0201 but not gp100, are not lysed.

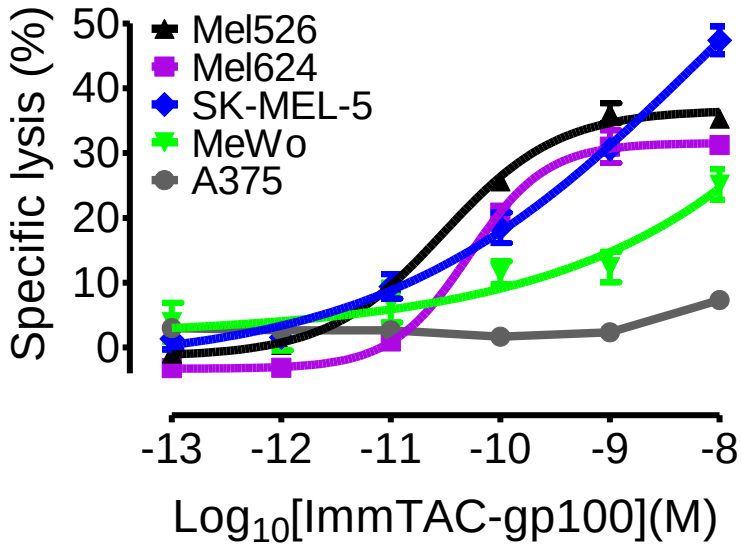


Figure 5-5 Redirected lysis of various melanoma cell lines by polyclonal CD8$^+$ T cells in the presence of ImmTAC-gp100.

LDH-release killing assay showing lysis of four gp100$^+$, HLA-A*0201$^+$ melanoma cell lines by unstimulated purified CD8$^+$ T cells at an E:T ratio of 10:1 in the presence of increasing concentrations of ImmTAC-gp100 over 24 hours. Mel526 cells (black triangles), Mel624 (purple squares), SK-Mel-5 (blue diamonds), and MeWo (green inverted triangles). A375 (gp100$^-$, HLA-A*0201$^+$) melanoma cells (grey circles) were included as a specificity control. Data are means ± s.e.m. The data shown are representative of three experiments.

5.2.5 ImmTAC redirected killing of tumour cells by PBMC

Next, in order to make the *in vitro* killing experiments as physiological as possible, the ability of ImmTAC molecules to redirect PBMC, i.e. unselected effector cells, to lyse tumour cells was assessed. ImmTAC-MAGE was used to redirect the lytic activity of unstimulated PBMC against various HLA-A*0101 positive tumour cell lines expressing natural levels of MAGE-$A3_{168-176}$ epitope (Figure 5-6). After 24 hours concentration-dependent lysis against all the target cell lines is observed. The resulting concentration-response curves varied according to the tumour cell lines used, but potent killing of the tumour cell lines A375 and EJM is seen with EC_{50} values below 50 pM. The control Colo205 cells, which express HLA-A*0101 but are negative for MAGE-A3, are not lysed.

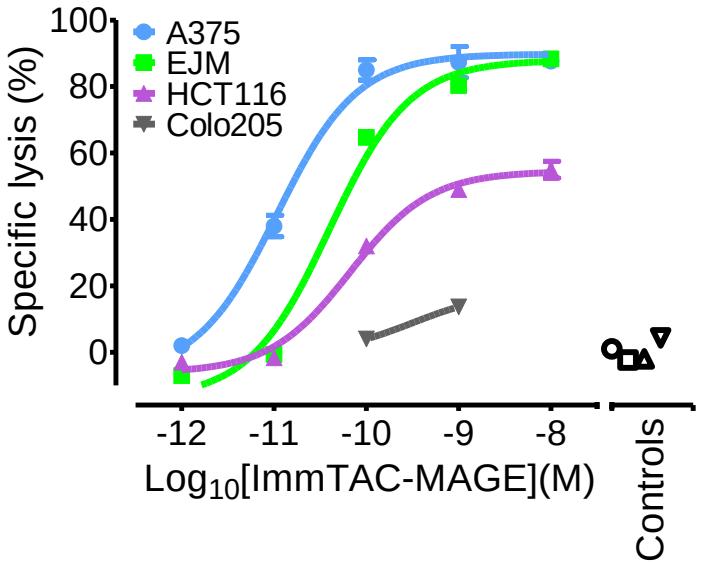


Figure 5-6 Redirected lysis of various tumour cell lines by PBMC in the presence of ImmTAC-MAGE.

LDH-release killing assay showing lysis of HLA-A*0101^{+}, MAGE-A3^{+} tumour cell lines by unstimulated PBMC at an E:T ratio of 50:1 in the presence of increasing concentrations of ImmTAC-MAGE over 24 hours. A375 melanoma cells (blue circles); EJM multiple myeloma cells (green squares); HCT116 colorectal carcinoma cells (purple triangles). The HLA-A*0101^{+}/MAGE-A3^{-} cell line Colo205 (grey inverted triangles) was included as a specificity control. The shape-matched open symbols represent the corresponding ImmTAC-MAGE-negative controls. Data are means ± s.e.m. The data shown are representative of three experiments.

5.2.6 ImmTAC redirected killing of tumour cells by PBMC derived from cancer patients

In the experiments described above, ImmTAC redirected killing has been evaluated using T cells derived from healthy donors. As potential cancer therapeutics, ImmTACs must be able to redirect cancer patient T cells which could be compromised either through the damaging effects of previous treatment regimes, such as chemotherapy, or through progression to a fatigued state caused by the disease.

To investigate whether cancer patient T cells could be redirected by ImmTAC to lyse cancer cell targets, ImmTAC-MAGE was used to redirect *ex vivo* PBMC from a relapse/refractory multiple myeloma patient against EJM targets which are HLA-A*0101 positive and express natural levels of MAGE-$A3_{168-176}$ epitope (Figure 5-7). EJM cells are a myeloma plasma cell line originally established from a patient with terminal refractory myeloma disease. Importantly, EJM's still retain the markers and growth characteristics of the primary tumour (Hamilton et al., 1990) and therefore represent a valuable model to investigate the potential therapeutic utility of ImmTAC-MAGE in myeloma. The data in Figure 5-7 shows concentration-dependent lysis of the EJM target cells after 24 hours. Notably, the potency seen with the cancer patient PBMC is similar to that achieved with healthy donor PBMC (Figure 5-6). This data is representative of a total of six multiple myeloma patient PBMC that were tested. All six donors produced EC_{50} values below 20 pM suggesting that the lytic capability of multiple myeloma patient PBMC is not defective. The control Colo205 cells, which express HLA-A*0101 but are negative for MAGE-A3, are not lysed.

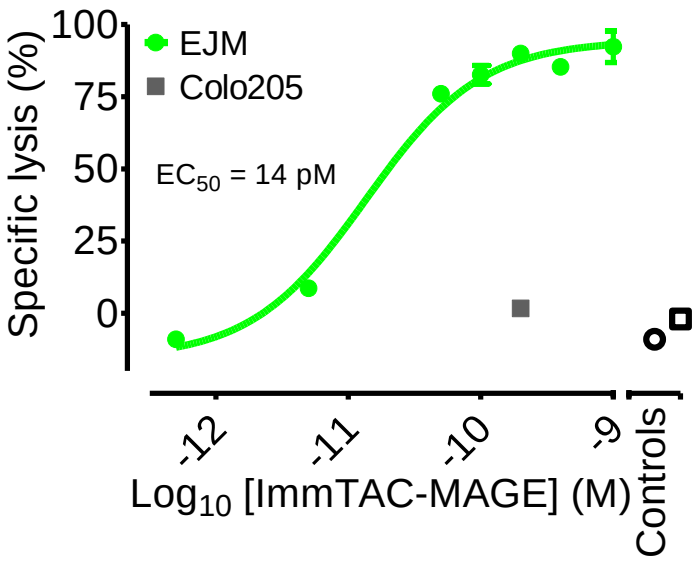


Figure 5-7 ImmTAC-MAGE redirected lysis of multiple myeloma tumour cells by multiple myeloma patient PBMC (sample ID: DV-PB0018).

LDH-release killing assay showing lysis of HLA-A*0101^{+}, MAGE-A3^{+} multiple myeloma cell line EJM by *ex vivo* PBMC from a relapse/refractory multiple myeloma patient at an E:T ratio of 30:1 in the presence of increasing concentrations of ImmTAC-MAGE over 24 hours. The HLA-A*0101^{+}/MAGE-A3^{-} cell line Colo205 (grey square) was included as a specificity control at a single concentration of ImmTAC-MAGE (0.2 nM). The shape-matched open symbols represent the corresponding ImmTAC-MAGE-negative controls. Data are means ± s.e.m. The data shown are representative of six experiments.

5.2.7 Specificity of ImmTAC redirected killing

Thus far, the potency of ImmTAC redirected killing against antigen positive tumour cell lines presenting the relevant HLA molecule has been the main focus of attention. Here, the specificity of ImmTAC-NYE was investigated using HLA-A*0201^{+}, NY-ESO-1/LAGE-1-negative primary human cells derived from normal tissues. The data in Figure 5-8 shows that hepatocytes, astrocytes, cardiac myocytes, and melanocytes are not lysed by redirected ImmTAC-NYE up to a concentration of 1 nM; at 10 nM, a low level of lysis is observed. This slight loss of epitope specificity at high concentrations (10 nM and above) has also been observed with other ImmTAC reagents.

The specificity of the therapeutic ImmTAC candidate, ImmTAC-gp100, is investigated in Chapter 6.

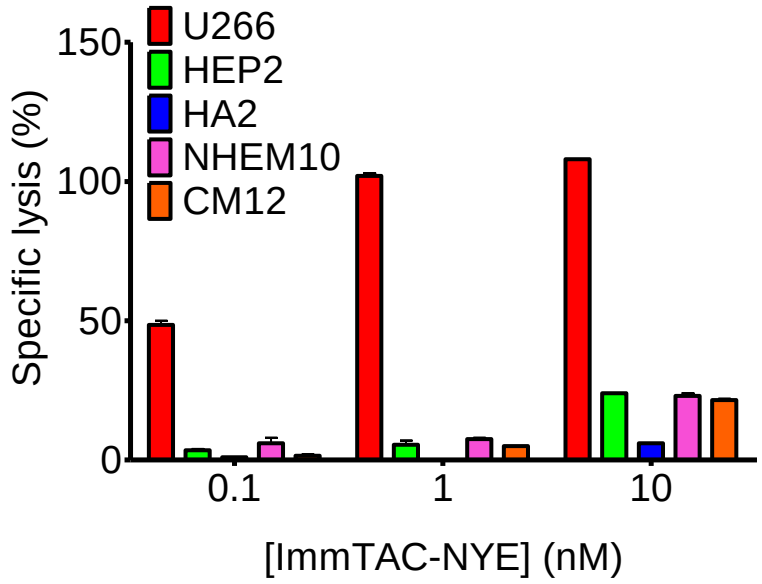


Figure 5-8 Specificity of ImmTAC-NYE redirected killing against a panel of HLA-A*0201^{+}/ NY-ESO-1-/LAGE-1-negative human primary cells

LDH-release killing assay showing lysis of HLA-A*0201^{+},NY-ESO-1^{-}/LAGE-1^{-} hepatocytes (HEP2), astrocytes (HA2), melanocytes (NHEM10), and cardiac myocytes (CM12) co-incubated with purified unstimulated CD8^{+} T cells at an E:T ratio of 10:1 in the presence of 0.1, 1, and 10 nM ImmTAC-NYE over 24 hours. Antigen positive U266 cells were included as a positive control. Data are means ± s.e.m. The data shown are representative of three experiments.

5.2.8 Long-term target cell killing kinetics using IncuCyte FLR technology

5.2.8.1 Target cell killing kinetics beyond 24 hours

In some instances one hundred percent killing of target cells by ImmTAC redirected T cells is not achieved over a period of 24 hours which could be a result of very low epitope number or that the targets are particularly resistant to lysis by $CD8^+$ T cells. The LDH assay is not suitable for assessing target cell killing for longer than 24 hours because the spontaneous release becomes too high thereby decreasing the signal to noise ratio.

IncuCyte™ FLR technology enables direct visualisation of caspase-3/7 dependent apoptosis by microscopy at 37°C in real time. This technology is relatively new and in our laboratory it has been specifically adapted and optimised to monitor the long-term killing kinetics of ImmTAC redirected T cells *in vitro*. In the LDH-release killing assay, the melanoma cell lines SK-Mel-37 and Mel624, which are both HLA-A*0201-positive and NY-ESO-1/LAGE-1-positive, are relatively resistant to ImmTAC-NYE redirected lysis at 24 hours (Figure 5-4). To investigate the long-term killing kinetics of the tumour cell lines SK-Mel-37 and Mel624 by IncuCyte™ FLR, they were co-incubated with unstimulated, purifed $CD8^+$ T cells in the presence or absence of ImmTAC-NYE. The level of caspase-3/7 activation was visualised from images taken at intervals of 10 minutes (Figure 5-9a-h). In agreement with the LDH-release assay, no significant killing of the SK-Mel-37 (Figure 5-9a) or the Mel624 (Figure 5-9e) is observed at 24 hours. However, for Mel624, most of the cells have been killed by 48 hours; the images taken at 48 hours confirm that close to 100% of the cells have been killed at this time point (Figure 5-9h). In the case of SK-Mel-37, significant killing is not evident until 72 hours and is illustrated by the end-point image (Figure 5-9d). Thus,

redirected killing by ImmTACs can be missed in short-term killing assays as such as ^{51}Cr-release and even the extended LDH-release assay. The adaptation and use of the IncuCyte™ technology is therefore of critical importance for assessing ImmTAC potency and specificity under the strictest possible conditions.

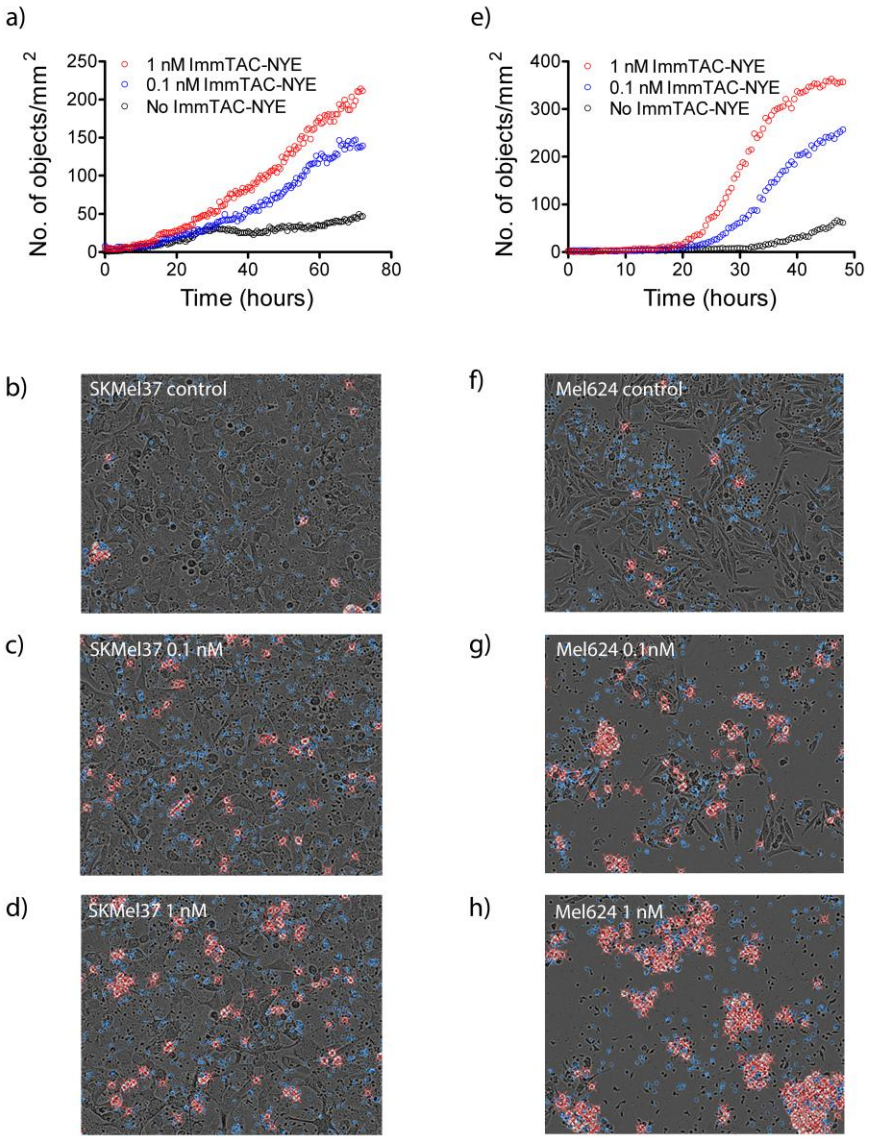


Figure 5-9 Long-term direct visualisation of redirected tumour cell killing mediated by ImmTAC-NYE.

IncuCyte™ FLR technology showing caspase-3/7 dependent apoptosis of target cells in real time. **(a)** SK-Mel-37 or **(e)** Mel624 melanoma cells were incubated with $CD8^+$ T cells (5:1 E:T) in the presence of ImmTAC-NYE at 1 nM (red circles), 0.1 nM (blue circles), or no ImmTAC-NYE (black circles). Images were taken at intervals of 10 minutes. The number of objects/mm^2, a measure of target cells undergoing apoptosis, was determined for each image and plotted against time. Representative endpoint images are shown for SK-Mel-37 at 72 hours **(b,c,d)** and Mel624 at 48 hours **(f,g,h)** in the presence or absence of ImmTAC-NYE at the indicated concentrations. White flashes marked with red-crosses represent cells undergoing apoptosis and are recorded as objects. A size exclusion gate of >100 μm^2 was set to eliminate dead effector cells from the object count and are shown in blue. The data are courtesy of Dr. Namir Hassan at Immunocore Ltd.

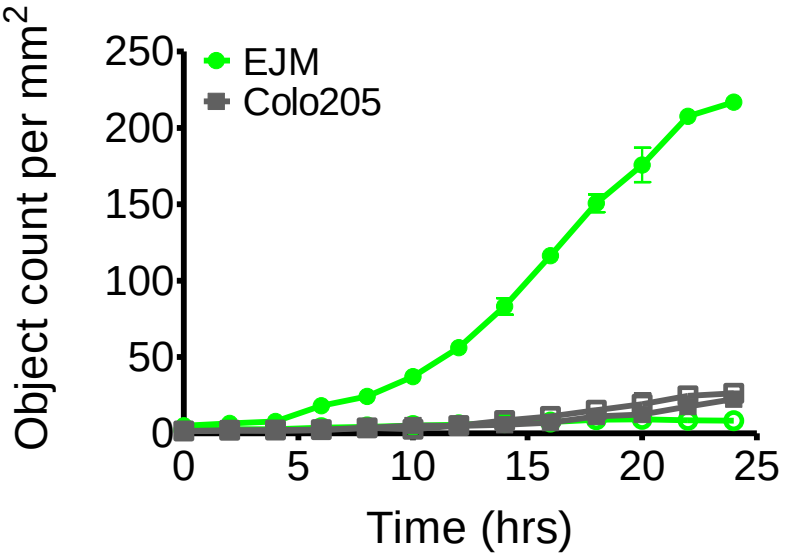
5.2.8.2 The influence of effector cell variability on redirected killing mediated by ImmTAC

In a study by Baeuerle and colleagues (Dreier et al., 2002) investigating the *in vitro* efficacy of bispecific T cell engager (BiTE) molecules, they describe the considerable variation observed in the half maximal concentration values (ED_{50}) and the percent cell lysis between different PBMC donors. Using a 4 hour fluorescence-based cytotoxicity assay, they tested unstimulated $CD8^+$ T cells purified from the PBMC of six healthy donors in the presence of targets and titrated amounts of BiTE reagent. The resulting concentration-response curves produced median half maximal concentrations ranging from 500 pg/ml (9.1×10^{-12} M) to as low a 7 pg/ml (1.3×10^{-13} M) and the percent of cell lysis from 25% to 95%. Furthermore, they showed a correlation between the half maximal concentration values and the level of cell lysis seen; the lower the ED_{50}, the higher the level of cell lysis (Dreier et al., 2002).

The phenomenon of effector T cell variation was also apparent in the experiments conducted for this thesis. To investigate donor T cell variability in more detail $CD8^+$ T cells purified from the PBMC of four healthy donors were co-incubated with the multiple myeloma cell line, EJM, which is HLA-A*0101 positive and express natural levels of MAGE-$A3_{168-176}$ epitope, in the presence of ImmTAC-MAGE. The time to reach maximal killing for each of the donors was assessed by the direct visualisation of caspase-3/7 dependent apoptosis in real time using IncuCyte™ FLR technology. Figure 5-10a-d shows two example data sets to illustrate the variability in redirected killing kinetics between different donor effector T cells where maximal killing (plateau of apoptotic events) is seen at 24 hours for donor FB681 (Figure 5-10a) and at 54 hours for donor FB682 (Figure 5-10c). The respective end-point images, Figure 5-10b for donor

FB681 and Figure 5-10d for donor FB682, show that in both cases nearly 100% of the targets are dead. In 3 out of the 4 donors tested, a 48 hour exposure to ImmTAC-MAGE was sufficient to achieve maximal target cell killing. The control Colo205 cells, which express HLA-A*0101 but are negative for MAGE-A3, are not killed.

a


Object count per mm2
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150
100
50
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EJM
Colo205
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5
10
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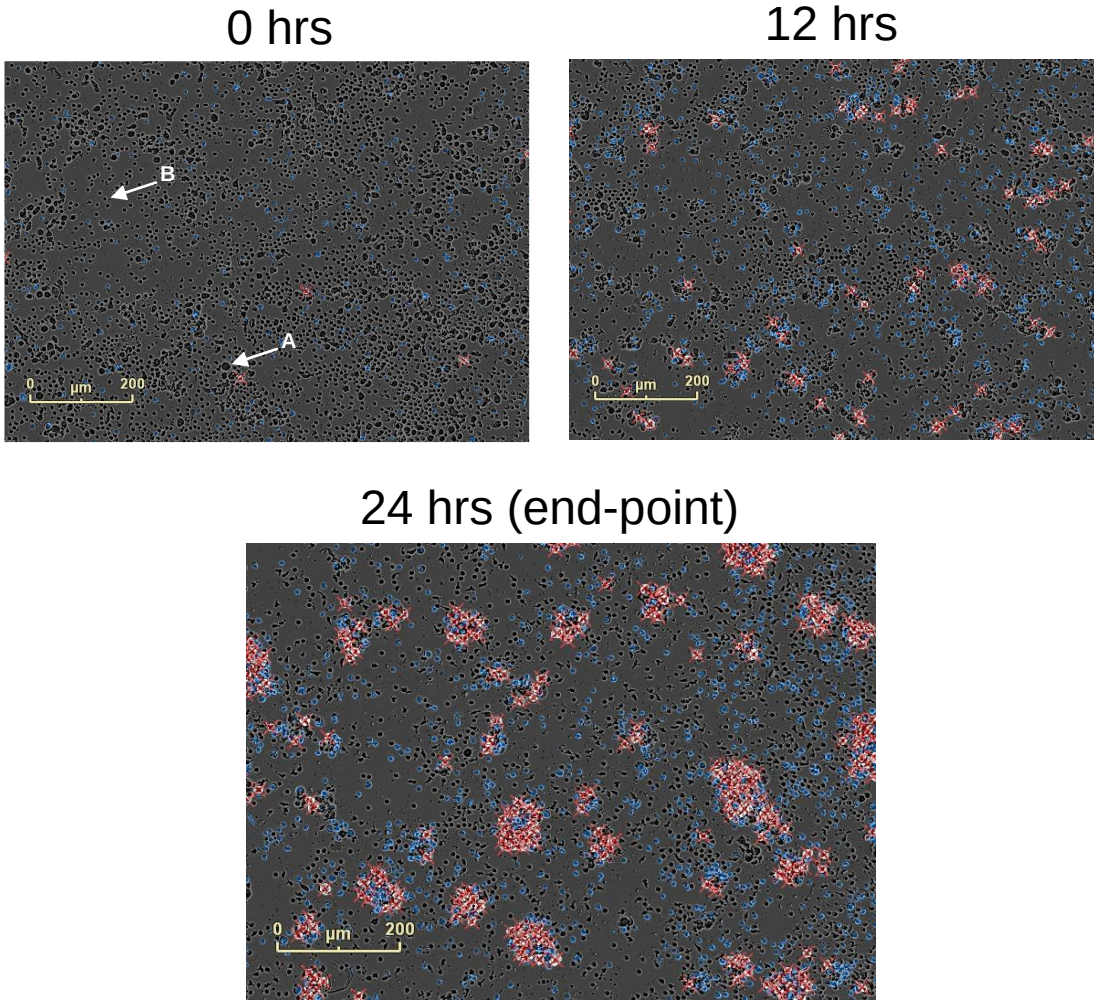
Time (hrs)

b


0 hrs
12 hrs
24 hrs (end-point)
B
A
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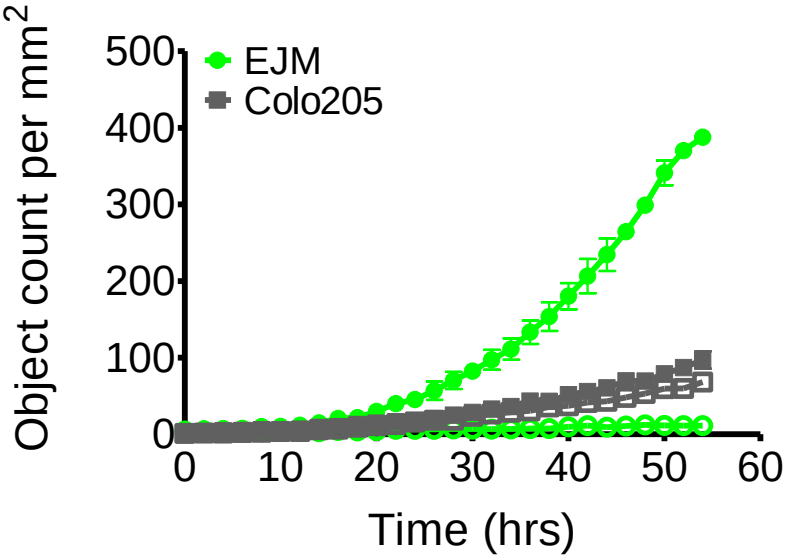
Object count per mm2
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100
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EJM
Colo205
0
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40
50
60
Time (hrs)

d

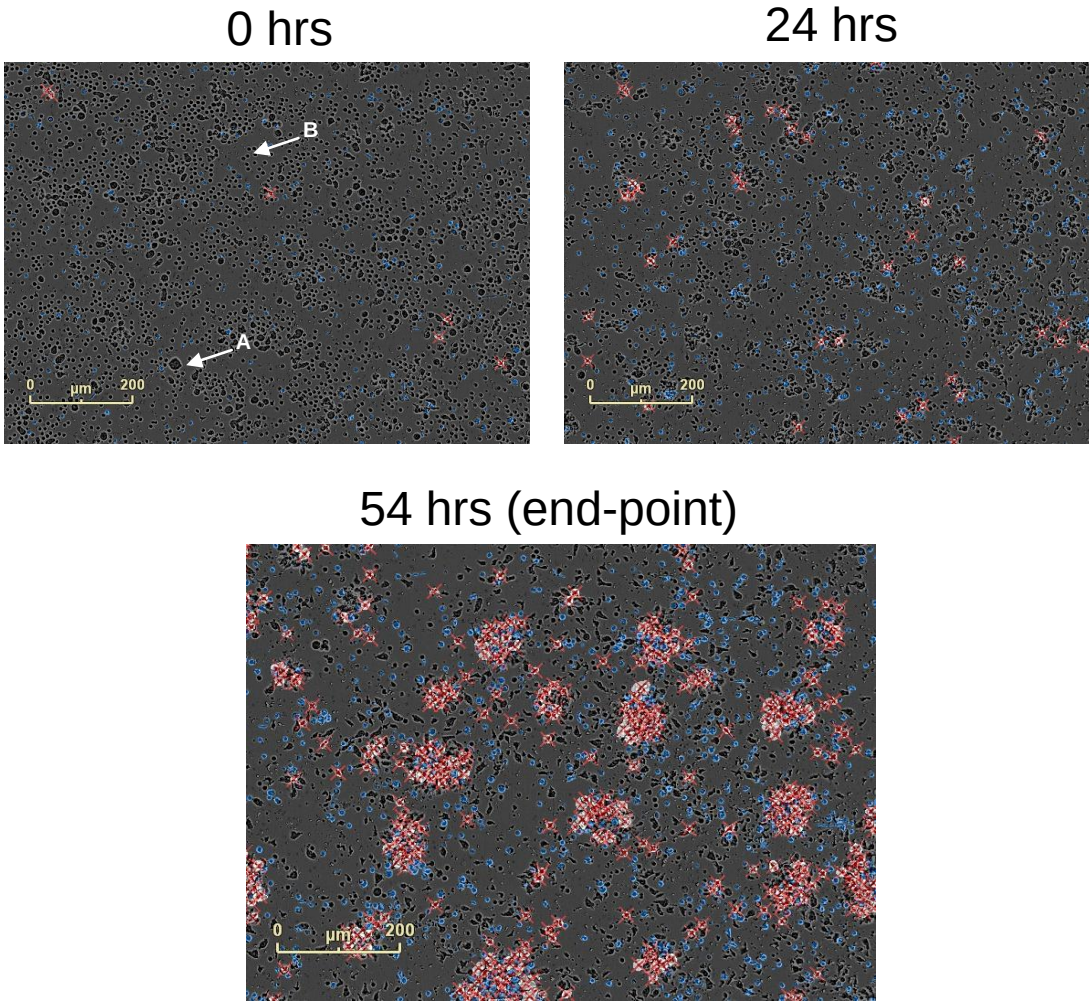
0 hrs
24 hrs
B
A
0
µm
200
54 hrs (end-point)

Figure 5-10 The influence of donor T cell variability on redirected target cell killing.

IncuCyte FLR technology showing caspase-3/7 dependent apoptosis of HLA-A*0101^{+}, MAGE-A3^{+} multiple myeloma cell line EJM (closed green circles) by unstimulated purified CD8^{+} T cells at an E:T ratio of 5:1 in the presence of 0.1 nM ImmTAC-MAGE. The HLA-A*0101^{+}/MAGE-A3^{-} cell line Colo205 (closed grey squares) was included as a specificity control. The shape-matched open symbols represent the corresponding ImmTAC-MAGE-negative controls. Images taken at intervals of 2 hours. The number of objects/mm^{2}, a measure of target cells undergoing apoptosis, was determined for each image and plotted against time. **(a)** Donor FB681 CD8^{+} T cells. **(c)** Donor FB682 CD8^{+} T cells. Images are shown for donor FB681 **(b)** and donor FB682 **(d)** at the indicated time points. White flashes marked with red-crosses represent cells undergoing apoptosis and are recorded as objects. A size exclusion gate of >100 μm^{2} was set to eliminate dead effector cells from the object count and are shown in blue. White arrows showing target cell (A) and T cell (B). Data are means ± s.e.m.

5.3 Summary

In this chapter I have evaluated redirected killing by ImmTAC reagents specific for two different HLA A*0201-restricted epitopes, NY-ESO-1 and gp100, and one HLA-A*0101-restricted epitope, MAGE-A3. ImmTAC mediated activation, after 4 hours, of the 'effector' caspases 3 and 7 in the presence of target cells presenting cognate pMHC, indicates that programmed cell death (apoptosis) has been triggered in the target cell. Despite the early manifestations of target cell killing as evidenced by caspase activation, my experiments show that conventional ^{51}Cr-release assays are not suitable for assessing ImmTAC redirected target cell killing when using unstimulated polyclonal $CD8^+$ T cell populations, although low level redirected killing was observed when using a pre-activated $CD8^+$ T cell clone. Likewise, in a 4 hour LDH release assay redirected killing was only detected when using a $CD8^+$ T cell clone. However, unlike the ^{51}Cr-release assay, the LDH assay could be extended to 24 hours. In the extended LDH-release assays all 3 ImmTACs showed significant lysis of the majority of the relevant tumour cell lines during the first 24 hours. However, the IncuCyte real-time imaging system demonstrated that some cell lines require longer periods for caspase-3/7-dependent apoptosis to occur. It was also evident in the IncuCyte experiments that there is considerable variability in redirected killing kinetics between different donor effector T cells and the time taken to reach maximum target cell lysis. Importantly, I was able to demonstrate that the lytic capability of PBMC derived from cancer patients is not defective; I observed no significant difference in the potency of redirected killing by cancer patient PBMC and that of healthy donor PBMC.

The peptide specificity of ImmTAC redirected target cell killing was also investigated. No redirected killing was observed against cell lines presenting the relevant HLA molecule but negative for the cognate peptide. To investigate specificity

more thoroughly ImmTAC-NYE was tested against a range of HLA-A*0201+/LAGE-1-/NY-ESO-1-negative primary human cells derived from normal tissue. No redirected lysis was observed up to a concentration of 1 nM; at 10 nM, a low level of lysis was observed.

Overall, I conclude that ImmTAC redirected T cells can potently kill tumour cell targets presenting cognate pHLA. Furthermore, target cell killing by ImmTAC redirected T cells is specific and limited to cells that express cognate pHLA.

CHAPTER 6 T CELL ASSAYS TO DETERMINE SAFE STARTING AND MAXIMUM DOSES OF THE THERAPEUTIC IMMTAC CANDIDATE IMCGP100 IN PATIENTS

6.1 Introduction

Of the four ImmTAC reagents evaluated in this thesis, ImmTAC-gp100 was selected as the lead therapeutic ImmTAC candidate to take forward into pre-clinical development, and, subsequently, for testing in a first-in-human clinical trial. In order to distinguish Good Manufacturing Practice (GMP) manufactured, i.e., clinical grade, ImmTAC-gp100 protein from research grade material, the GMP manufactured reagent is referred to as IMCgp100 (the 'new drug' name used for regulatory and clinical trial documentation purposes).

The selection of ImmTAC-gp100 as the first candidate with which to test the ImmTAC concept in humans was based both on potency and specificity data. The results presented in Chapters 4 and 5 demonstrated that ImmTAC-gp100 can induce the full repertoire of CTL activation events in a concentration-dependent manner when combined with target positive melanoma cells *in vitro*. ImmTAC-gp100 is extremely potent and is able to activate unstimulated T cells from unselected populations i.e., PBMC, including those derived from melanoma patients, at concentrations as low as 1 pM, with maximal activity at a concentration of 1 nM. ImmTAC-gp100 is also highly specific for its target and no off-target activity has been observed against target negative cell lines. In addition, gp100 is an attractive target for immunotherapy as up to 95% of all melanoma tissues express the gp100 protein (Barrow et al., 2006; Hofbauer et al., 2004; Mocellin et al., 2001; Spagnoli et al., 1995; Wagner et al., 1997). Furthermore,

the gp100 derived peptide that is the target of ImmTAC-gp100 is presented by the MHC class I molecule variant HLA-A*0201, which is one of the most prevalent HLA types carried by approximately 50% of the North American and Western European populations (New Allele Frequency Database: http://www.allelefrequencies.net/ (Gonzalez-Galarza et al., 2011). Therefore, ImmTAC-g100 could cover approximately 47% of malignant melanoma patients. As described in section 1.2.2.1, there is a clear unmet medical need for new treatment options for malignant melanoma as current treatment options provide only a modest improvement in overall survival and rarely offer a cure. Thus, the intended use of the ImmTAC-gp100 development candidate, IMCgp100, is for the treatment of patients with advanced melanoma at disease Stage IV or unresectable Stage III who are refractory or resistant to standard treatment regimens or for which no standard treatments exist.

The planned Phase I clinical study to assess the safety and tolerability as well as the preliminary efficacy of IMCgp100 in the treatment of patients with malignant melanoma is the first clinical study to use IMCgp100. In order to proceed with clinical testing of IMCgp100, it was necessary to receive approval from the regulatory authorities. To be granted approval, a detailed study plan had to be designed with precise indications of duration of treatment, concentration of IMCgp100, and predictions of possible adverse effects. Therefore, extensive pre-clinical studies have been carried out on IMCgp100 to obtain a full safety and efficacy profile in preparation for the first-in-human Phase I clinical trial. Key to these investigations is establishing a safe starting dose of IMCgp100, with the intention of dose escalating until a *maximum tolerated dose* (MTD), i.e., when unacceptable toxicity becomes an issue, or the *target limiting dose* (TLD), i.e., the set top dose is reached. Finding the starting dose of a new drug is subject to strict rules, as summarised briefly in the following:

For first-in-human (FIH) studies, a safe clinical starting dose needs to be established. Conventionally, the *no observed adverse effect level* (NOAEL) is determined in pre-clinical studies involving one or more relevant animal species. The NOAEL represents the highest dose of a compound that yields no measurable toxicologic effects or adverse events. For calculation of FIH starting doses, the NOAEL is converted to a *human equivalent dose* (HED) which is generally normalised to the body surface area for low molecular weight new chemical entities, or mg per kg bodyweight normalisation for proteins >100 kDa. A safety factor is then applied, generally a dose reduction of at least 10-fold, to give the *maximum recommended starting dose* (MRSD). However, the well-publicised life-threatening cytokine-release syndrome suffered by volunteers in a Phase I clinical trial following administration of the anti-CD28 superagonist monoclonal antibody, TGN1412 (TeGenero AG), in March 2006 (Suntharalingam et al., 2006), highlighted the potential pit-falls in interpreting and extrapolating non-clinical findings to the clinical setting.

After the severe adverse events of the TeGenero TGN1412 case, a broader spectrum of investigations for the calculation of starting dose in man was recommended by the *Expert Scientific Group on Phase One Clinical Trials* for investigational medicinal products (IMP) deemed to be associated with a high risk of toxicity (EMEA/CHMP/SWP/28367/2007). According to the *Expert Scientific Group* recommendations, all relevant pre-clinical *in vivo* and *in vitro* information available is to be considered, resulting in definition of the *minimal anticipated biological effect level* (MABEL). Relevant information includes (1) receptor binding and occupancy data from *in vitro* and *in vivo* studies, (2) concentration response curves determined *in vitro* and *in vivo*, and (3) exposures at pharmacological doses in relevant species. In the

MABEL approach, dose calculations rely on any pharmacologic/biologic effect that typically can be observed at considerably lower concentrations than those required for pathologic responses (i.e., toxicity) as in the NOAEL approach. The starting dose for FIH clinical trials is usually set 10-fold below the MABEL value as safety factors are also applied to this approach for determining a clinical starting dose.

IMCgp100 was considered a high risk IMP because it is an immunomodulator and the mechanism of action is activation of T cells. Furthermore, owing to the entirely human specific nature of IMCgp100, both with respect to the TCR-pHLA and the scFv-CD3 interactions, no relevant species exists in which to test the potential toxicity of IMCgp100. Therefore, *in vitro* cellular assays were used for determining clinical dosage and predicting toxicity. It is anticipated that these studies with IMCgp100 will function as a 'pathfinder' for all future ImmTAC pre-clinical development, and in understanding the predictability of the effects in humans of soluble TCR therapeutics based on *in vitro* assays.

In this chapter I describe the *in vitro* efficacy (potency) experiments that determined the MABEL and hence the starting dose for IMCgp100 in the Phase I clinical trial. The *in vitro* functional cross-reactivity and whole blood assays used to investigate potential sources of IMCgp100 toxicity are also described, as well as the key data sets used to set the top dose, known as the *target limiting dose* (TLD).

6.2 Results

6.2.1 Specificity testing of IMCgp100 redirected T cell activity

Toxicology studies in animals are typically conducted to provide guidance on what may be a safe starting dose for an IMP in humans, what may constitute a toxic dose, and what kind of adverse events (AEs) may be expected in a clinical trial. However, both subunits, and thereby both functionalities, of IMCgp100 are based on completely human specific molecules and as such, no relevant toxicology species exists in which it can be tested. Instead, a series of *in vitro* functional cross-reactivity studies on human primary tissue have been conducted to assess the potential toxicological profile of IMCgp100 by measuring the activation of T cells. Although IMCgp100 has been highly engineered for the specific recognition of its cognate antigen in the context of HLA-A*0201 there is the possibility that it could react unexpectedly to other antigens on normal tissue. In the case of gp100, two kinds of reactivity mechanisms are feasible: on-target reactivity and off-target reactivity (cross-reactivity). For the following experiments, a minimum of three repeats using PBMC from three different healthy donors were conducted.

6.2.1.1 On-target reactivity

Expression of gp100 is not restricted to melanoma; normal tissues that have been shown directly to express gp100 include the retina, melanocytes, the substantia nigra and the thymus (Takase et al., 2005; Wagner et al., 1997). Other tissues are known to contain melanocytes such as the iris, the inner ear and the choroid plexis of the brain but so far no evidence has been reported of gp100 expression in these tissues. Sourcing primary cells from the eye and ear was not possible; therefore, cells cultured from the skin and brain such as melanocytes and astrocytes were some of the most important cell types used to evaluate on-target reactivity.

To investigate on-target reactivity against epidermal skin derived melanocytes, IFN-γ activity was measured from unstimulated PBMC, and granzyme B activity measured from unstimulated purified $CD8^+$ T cells, when co-incubated with the melanocytes in the presence of increasing concentrations of IMCgp100 (Figure 6-1a,b). The data shows IMCgp100 concentration-dependent release of IFN-γ (Figure 6-1a) and granzyme B (Figure 6-1b) from the redirected T cells in response to the melanocytes. The potency measured in the IFN-γ ELISpot shows that the response to the melanocytes is 2.5-fold less than that observed against the HLA-A*0201^+/$gp100^+$ melanoma cell line, Mel526, which was included as a positive control and comparator (EC_{50} values are 51 pM and 20 pM, respectively). No activity is seen against the HLA-A*0201^+/, $gp100^-$ cell line A375, apart from at the highest dose of IMCgp100 tested, 10 nM, where there is some non-specific reactivity occurring. In all cases (in total, melanocyte lots derived from six HLA-A*0201^+ donors were tested), the potency of redirection is weaker against the melanocyte lots than that observed for the melanoma cell line, Mel526. This suggests that there is a therapeutic window between organ specific toxicity and melanoma reactivity.

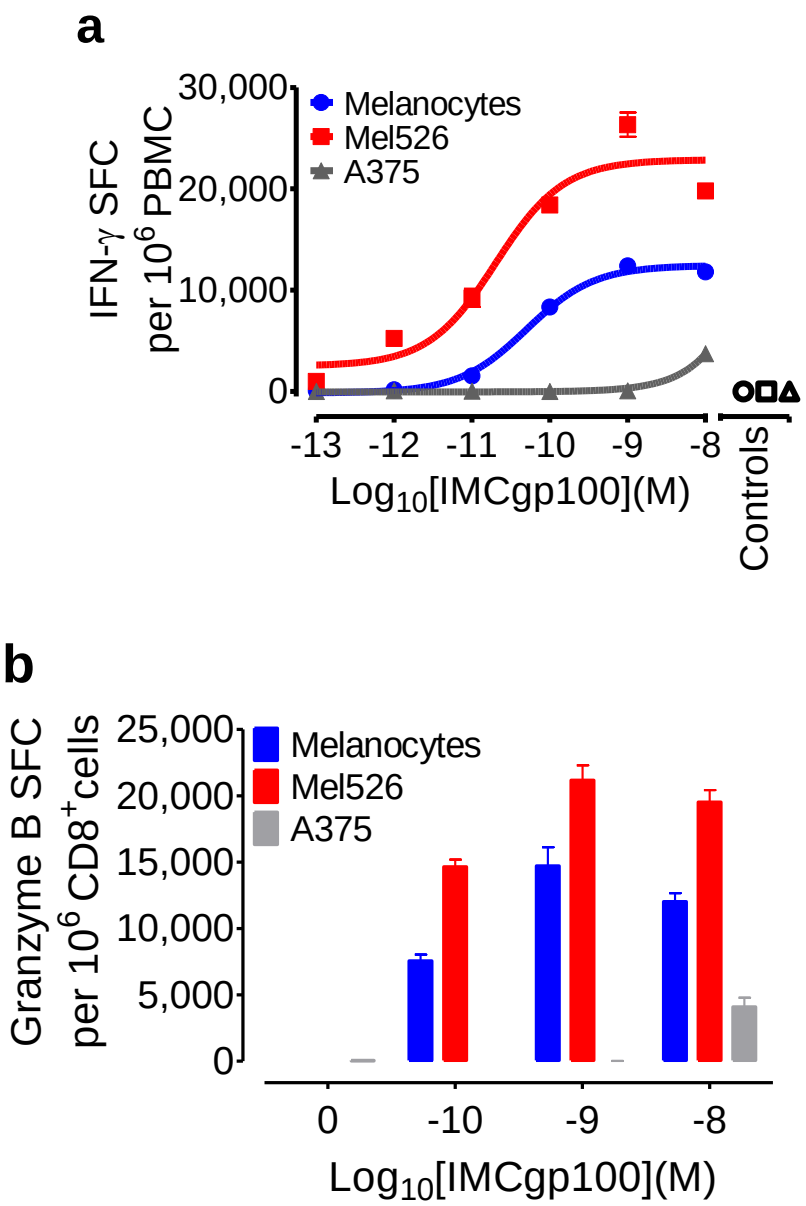

Figure 6-1 IMCgp100 on-target reactivity against epidermal skin derived melanocytes.

ELISpot assays **(a)** IFN-γ activity (line graph) of unstimulated PBMC mediated by increasing concentrations of IMCgp100 in the presence of melanocytes (lot N1, blue circles), positive control HLA-A*0201^{+}/gp100^{+} melanoma cell line Mel526 (red squares) and the negative control HLA-A*0201^{+}/gp100^{-} melanoma cell line A375 (grey triangles). The shape-matched open symbols represent the corresponding IMCgp100-negative controls. **(b)** Granzyme B activity (bar graph) of unstimulated, purified CD8^{+} T cells mediated by increasing concentrations of IMCgp100 in the presence of melanocytes (lot N1, blue bar), Mel526 (red bar), A375 (grey bar). Data are means ± s.e.m. The data is representative of epidermal skin derived melanocytes from a total of six HLA-A*02 positive donors. The data shown are courtesy of Dr. Jane Harper at Immunocore Ltd.

The brain is an immune privileged organ, meaning that there is little or no expression of MHC class I on brain tissue cells (Lampson & Hickey, 1986). In addition, the blood brain barrier (BBB) prevents access of immune cells as well as proteins and the majority of drugs to the brain. However, inflammatory conditions such as mulitiple sclerosis, glioblastoma multiform (GBM) tumours or brain metastases can result in a breakdown of the BBB barrier allowing entry of immune cells. Furthermore, the secretion of cytokines by the immune cells, specifically, IFN-γ, can upregulate MHC class I expression in brain tissue (Deeken & Loscher, 2007; Jarosinski & Massa, 2002; Schneider et al., 2004; Simka, 2009). Astrocytes are a sub-type of glial cells and they are the most abundant cell type in the human brain. Astrocytes can form GBM tumours which have been shown to express gp100 (Saikali et al., 2007). Therefore, the use of astrocytes could represent a valid model to investigate IMCgp100 on-target reactivity against brain tissue.

IFN-γ was measured from unstimulated PBMC, and granzyme B activity measured from unstimulated purified $CD8^+$ T cells, when co-incubated with astrocytes in the presence of increasing concentrations of IMCgp100 (Figure 6-2a,b). Astrocyte samples from a total of five HLA-A*02 positive donors were evaluated. No IMCgp100 reactivity was observed against 4 out of the 5 astrocyte samples. However, the data set presented in Figure 6-2a,b shows that one astrocyte lot (HA2) did induce some IMCgp100 reactivity. The potency of the response observed against this lot of astrocytes is approximately 80-fold less than that observed against the HLA-$A*0201^+/gp100^+$ melanoma cell line, Mel526, which was included as a positive control (EC_{50} values are 2 nM and 24 pM, respectively) (Figure 6-2a). In blocking experiments using cold high affinity gp100-specific mTCR as a competitor for epitope binding, the

response against the astrocytes is effectively abrogated (Figure 6-2b). This suggests that the reactivity seen against lot HA2 astrocytes is gp100 specific and, thus, that this particular sample of astrocytes may have a low level of gp100 presentation.

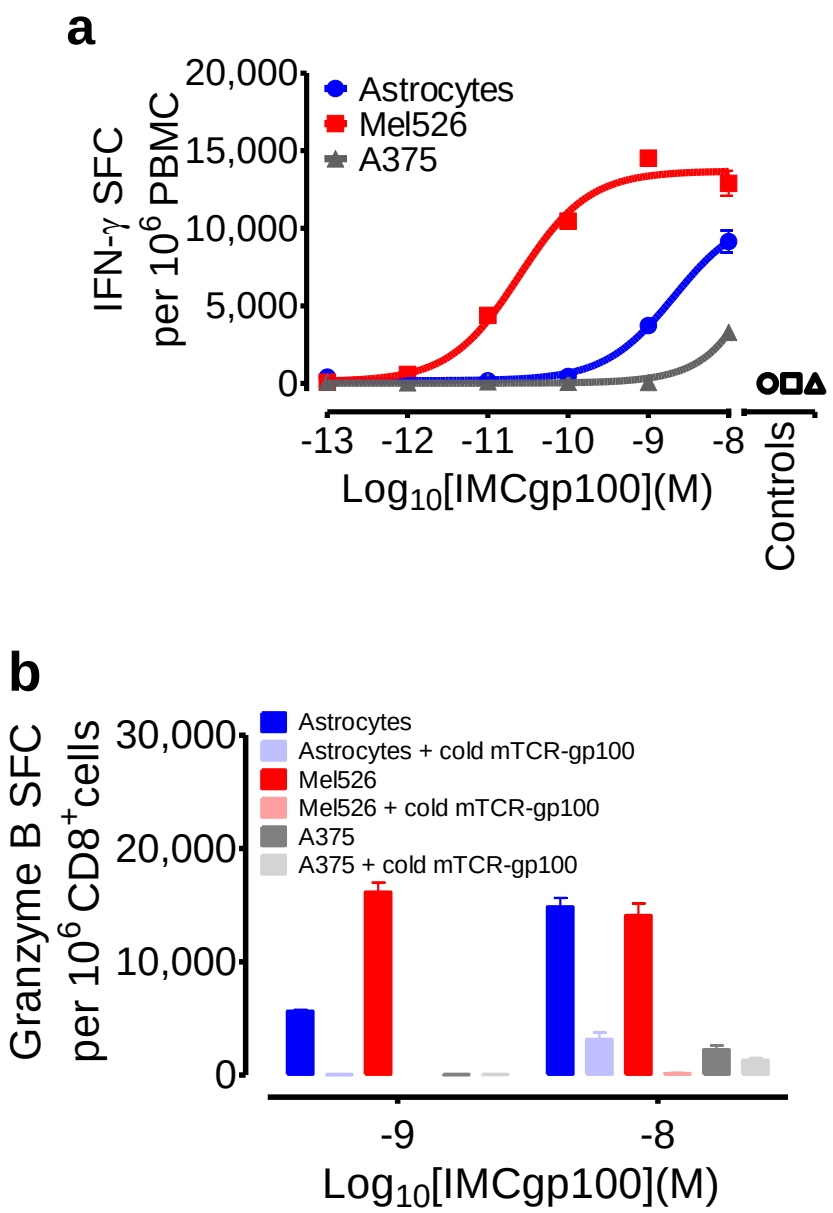

Figure 6-2 IMCgp100 on-target reactivity against astrocytes.

ELISpot assays **(a)** IFN-γ activity (line graph) of unstimulated PBMC mediated by increasing concentrations of IMCgp100 in the presence of astrocytes (lot HA2, blue circles), positive control HLA-A*0201^{+}/gp100^{+} melanoma cell line Mel526 (red squares) and the negative control HLA-A*0201^{+}/gp100^{-} melanoma cell line A375 (grey triangles). The shape-matched open symbols represent the corresponding IMCgp100-negative controls. **(b)** Granzyme B activity (bar graph) of unstimulated, purified CD8^{+} T cells mediated by either 1 nM or 10 nM of IMCgp100 in the presence of astrocytes (lot HA2, dark blue bar), Mel526 (dark red bar), A375 (dark grey bar). The pale coloured bars represent the inhibition of IMCgp100 redirected activity with 1 µM cold high affinity mTCR-gp100. Data are means ± s.e.m. The data shown are courtesy of Dr. Sandrine Buisson and Dr. Jane Harper at Immunocore Ltd, respectively.

6.2.1.2 The use of steroids to control the potential on-target activity of IMCgp100

Some on-target reactivity has been observed for IMCgp100 against primary melanocytes (Figure 6-1). Therefore, adverse events as a result of reactivity of IMCgp100 to tissues expressing both gp100 and HLA-A*0201 cannot be precluded. Studies investigating T cell adoptive therapy for the treatment of patients with melanoma have reported cases of vitiligo (de-pigmentation of sections of skin), uveitis (eye inflammation), and hearing loss caused by the attack of normal melanocytes. In these studies the adverse events were treated and controlled with steroids (Dudley et al., 2005; Palmer et al., 2008; Yeh et al., 2009). As similar toxicities could be exhibited in patients treated with IMCgp100, the effect of the steroid hydrocortisone on IMCgp100 redirected T cell activation was examined (Figure 6-3). The data shows concentration-dependent inhibition by hydrocortisone of IMCgp100 redirected activity. The use of 25 μM hydrocortisone, which is a clinically relevant concentration, almost completely abrogates the activation of T cells even at a high test concentration of IMCgp100 (1 nM) and suggests that this agent could be effective at controlling on-target toxicity.

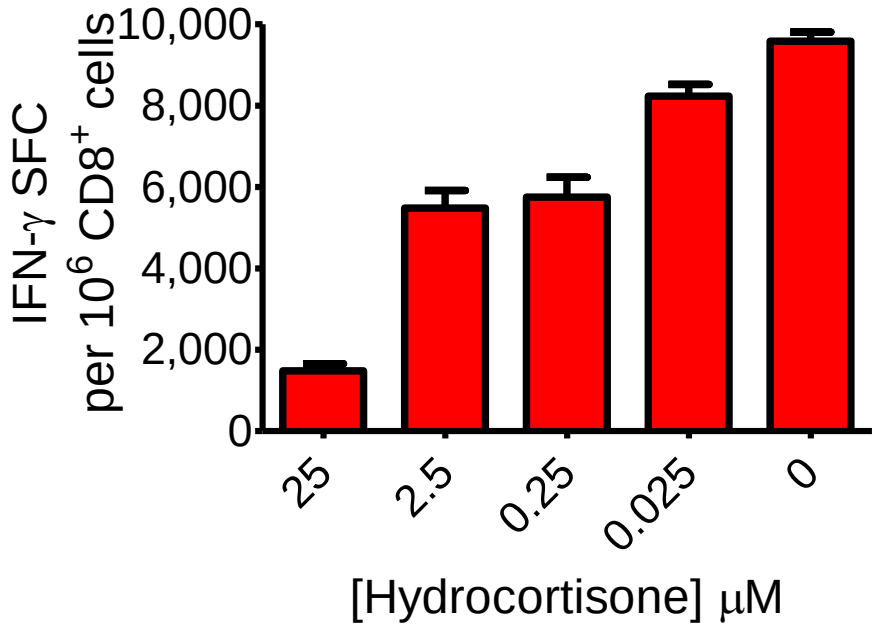

Figure 6-3 The effect of hydrocortisone on IMCgp100 redirected activity.

IFN-γ ELISpot assay showing activation of unstimulated purified $CD8^+$ T cells mediated by 1 nM of IMCgp100 in the presence of HLA-A*0201^+/$gp100^+$ melanoma cell line, Mel526, with titrated amounts hydrocortisone or no hydrocortisone (positive control). Data are means ± s.e.m. The data shown are representative of four experiments.

6.2.1.3 Off-target reactivity (cross-reactivity)

To investigate IMCg100 reactivity to other antigens on normal tissues, a panel of primary cells, representative of a broad range of human organs, were used in functional cross-reactivity studies. The data from these studies are summarised in Table 6-1 and are courtesy of Dr. Jane Harper. Figure 6-4 shows example data sets using cardiac myocytes as targets. No off-target cross-reactivity was observed for any of the tested primary cell types, except at high concentrations (10 nM and above) where a slight loss of specificity is observed against HLA-A*0201^{+} primary cells (as illustrated by the example data set in Figure 6-4a). This non-specific activity is absent in the presence of HLA-A*0201^{-} primary cells (as illustrated by the example data set in Figure 6-4b). These data suggests that at concentrations of approximately 10 nM, IMCgp100 elicits some loss of specificity by recognising and activating T cells in response to HLA-A*0201^{+}, but not to HLA-A*0201^{-}, cells presenting other, unknown peptides than gp100. However, at concentrations up to 1 nM, IMCgp100 is highly specific for its HLA-A*0201^{+}/gp100^{+} target.

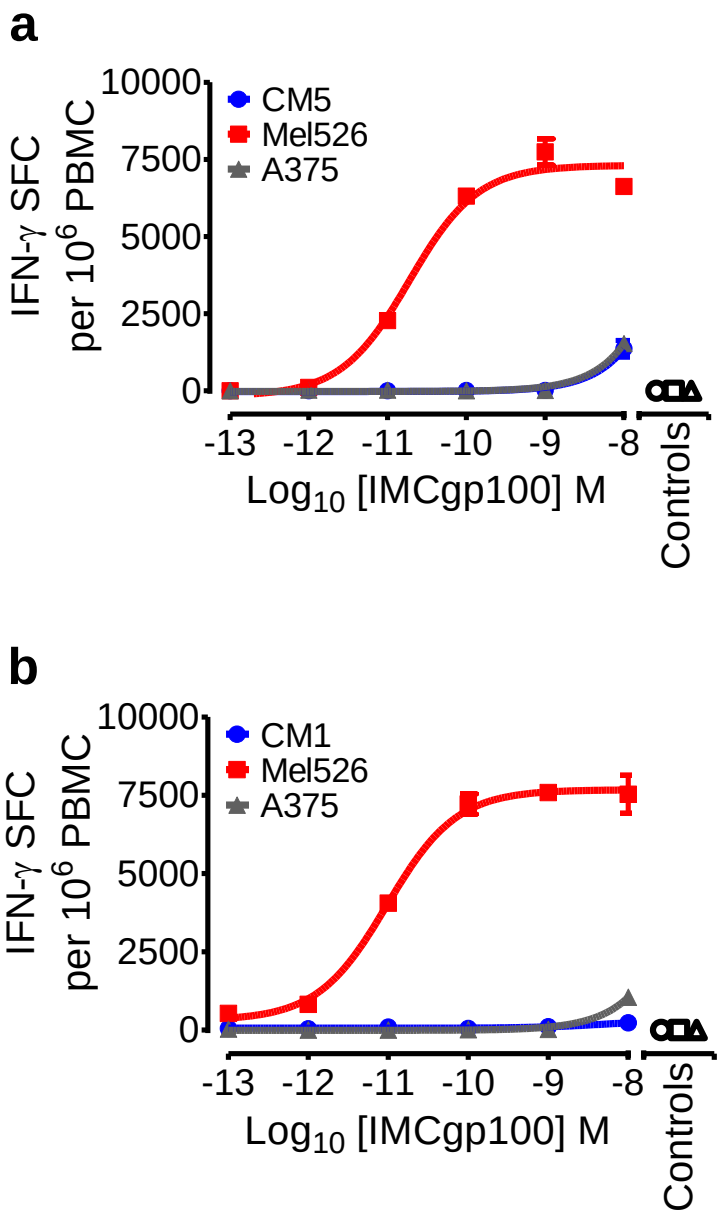

Figure 6-4 IMCgp100 off-target reactivity (cross-reactivity) against cardiac myocytes.

IFN-γ ELISpot assays showing activity of unstimulated PBMC mediated by increasing concentrations of IMCgp100 in the presence of cardiac myocytes (blue circles), positive control HLA-A*0201^{+}/gp100^{+} melanoma cell line Mel526 (red squares) and the negative control HLA-A*0201^{+}/gp100^{-} melanoma cell line A375 (grey triangles). The shape-matched open symbols represent the corresponding IMCgp100-negative controls. **(a)** HLA-A*0201^{+} cardiac myocytes (lot CM5). **(b)** HLA-A*0201^{-} cardiac myocytes (lot CM1). The data shown are courtesy of Dr. Jane Harper at Immunocore Ltd.

Table 6-1 Summary of *in vitro* specificity studies with IMCgp100

Cellular origin	Assay			gp100	Summary
	IFNγ	**GrB**	**N**		
HLA-A*02+ human tissue cells					
Hepatocytes	X	X	2	ND	No reactivity
Colonic smooth muscle cells	X	X	1	NT	No reactivity
Dermal fibroblasts	X	X	3	NT	No reactivity
Pulmonary fibroblasts	X	X	2	NT	No reactivity
Bronchial epithelial cells	X	X	1	ND	No reactivity
Bronchial smooth muscle cells	X	X	1	NT	No reactivity
Renal epithelial cells	X	X	1	ND	No reactivity
Astrocytes	X	X	5	ND	Observed in 1/5 lots
Dermal MVECs	X	X	3	ND	No reactivity
Cardiac myocytes	X	X	1	ND	No reactivity
Skeletal muscle cells	X	X	2	ND	No reactivity
Prostate epithelial cells	X	X	2	NT	No reactivity
HLA-A*02- human tissue cells					
Colonic smooth muscle cells	X	X	1	NT	No reactivity
Pulmonary fibroblasts	X	NT	1	NT	No reactivity
Astrocytes	X	X	1	NT	No reactivity
Cardiac myocytes	X	NT	2	NT	No reactivity

HLA-A*02^{+} and HLA-A*02^{-} primary human cell lines (Sciencell, Lonza and PromoCell) were used as targets in IFNγ and granzyme B (grB) ELISpot assays as indicated. Expression of gp100 was assessed at the transcriptional and translational levels. For RT-PCR, the following gp100-specific primers were used: 5'-TCAGGCGCCAACTGCAGAGC-3' (forward) and 5'-CCCGCCTTGGCAGGACACAG-3' (reverse). For flow cytometry, the anti-gp100 mAb NK1/beteb (HyCult Biotechnology) was used in standard intracellular staining protocols. Each sample was tested in triplicate using unstimulated purified CD8^{+} T cells from 3 separate donors. IMCgp100 was used at a final assay concentration of 1 nM. MVECs, microvascular endothelial cells; N, number of different donors for each tissue tested; ND, not detected; NT, not tested; X, no reactivity (Liddy et al., 2012)

6.2.3 Testing of IMCgp100 in whole blood assays

The superagonist anti-CD28 monoclonal antibody, TGN1412 (TeGenero AG), triggered multiple cytokine release syndrome (CRS, also, in extreme cases such as this, sometimes referred to as 'cytokine storm') in the healthy volunteers enrolled on the phase I trial (Suntharalingam et al., 2006). CRS is an acute immune reaction to the first infusion of a drug, characterised by the systemic release of several inflammatory mediators which trigger a cascade of cytokines, predominantly TNF-α and IFN-γ, followed by IL-6, IL-1β and in some cases IL-2 and IL-8. All therapeutic monoclonal antibodies (mAb) have the potential to mediate CRS, particularly those directed against targets on the surface of lymphocytes (M. R. Walker et al., 2011). As the mode of action of ImmTAC is to activate T cells via anti-CD3 it was important to determine in the pre-clinical studies the potential for ImmTAC mediated CRS.

The majority of immune effector cells are found in systemic circulation including those that express the protein CD3 on their surface such as $CD4^+$ and $CD8^+$ T cells. Bulk activation of these cells in circulation could potentially initiate CRS. Studies on whole blood were carried out to determine the potential for IMCgp100 mediated activation of haematological cells. These investigations were performed *ex vivo* to allow examination of the drug in close to physiological conditions. The blood from healthy HLA*0201^+ donors was incubated with increasing concentrations of IMCgp100. Positive controls for activation included anti-CD28, which activates T cells via the costimulatory pathway, and UCHT1 which is the mAb from which the scFv of ImmTAC is derived. It is important to note that, whilst the UCHT1 mAb is bivalent and can cross link and activate T cells via CD3, the scFv is monovalent and is therefore incapable of cross-linking in the absence of target. The Luminex® assay was used to measure the release of the cytokines IL-1β, TNF-α, IL-6, IFN-γ, and IL-2 (Figure 6-5).

The data shows that IMCgp100 does not induce cytokine release when incubated with *ex vivo* whole blood except at the two highest test concentrations, 10 nM and 100 nM, where some cytokine release is observed for IFN-γ, TNF-α and IL-6. Given that non-specific activation above 1 nM has been observed in the *in vitro* functional assays with control cell lines that do not express gp100, the responses seen in the whole blood assay are not unexpected. Furthermore, the magnitude of the cytokine response in the presence of 10 nM and 100 nM IMCgp100 is considerably less than that observed for the immune stimulatory antibody UCHT1 alone or in combination with anti-CD28 antibody. It should also be noted that these responses did not occur in all the donors tested. In total, four healthy donors were tested.

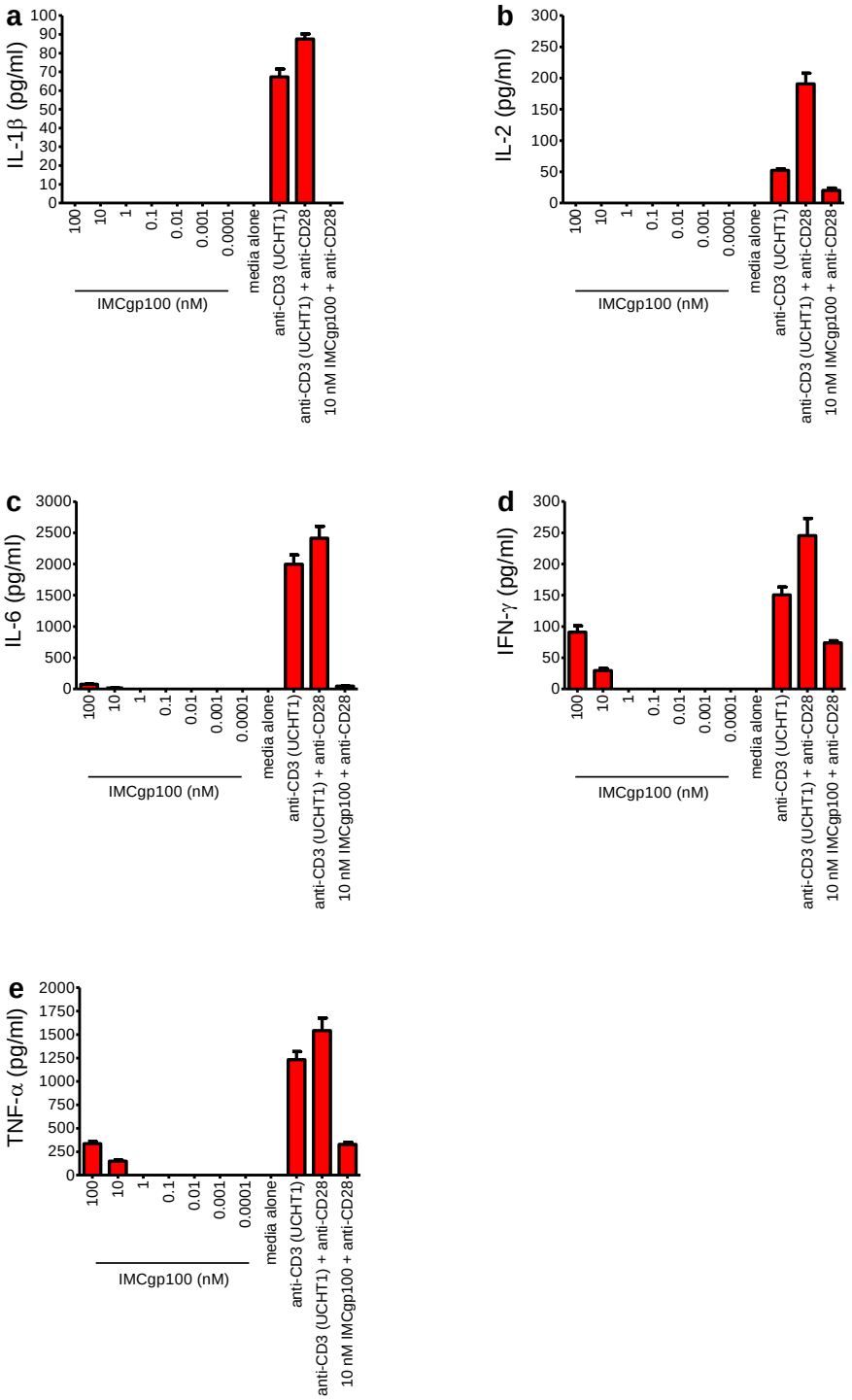

Figure 6-5 Whole blood cross-reactivity assays.

Luminex® assays showing cytokine release mediated by increasing concentrations of IMCgp100 in the presence of whole blood from a healthy donor (FB375) over a period of 4 hours at 37ºC and 5% CO_2. **(a)** IL-1β **(b)** IL-2 **(c)** IL-6 **(d)** IFN-γ **(e)** TNF-α. Controls included anti-CD3 (UCHT1) mAb alone; anti-CD3 (UCHT1) mAb and anti-CD28 mAb; and, IMCgp100 (10nM) and anti-CD28 mAb. Data are means ± s.e.m. The data shown are courtesy of Dr. Sandrine Buisson at Immunocore Ltd.

6.2.4 Applying cellular data for the determination of the MABEL and TLD for IMCgp100

6.2.4.1 Minimal anticipated biological effect level (MABEL) for IMCgp100

In the absence of a relevant toxicology species in which to determine a safe clinical starting dose for IMCgp100 using the conventional *no observed adverse effect level* (NOAEL) method, the *minimal anticipated biological effect level* (MABEL), as derived from the *in vitro* data, was applied. This approach assumes that the *in vitro* data can be used as guidance for what may occur in patients in the clinic. This assumption has been supported by another class of anti-CD3 fusion proteins, the Micromet bispecific T cell engager (BiTE) molecules. BiTE molecules are conceptually similar to IMCgp100 in that they are comprised of a targeting entity (antibody) fused to an anti-CD3 scFv effector function. An anti-CD19/CD3 BiTE molecule was tested in patients with B cell non-Hodgkin's lymphoma as part of a phase I clinical trial; the doses of drug that were efficacious in the *in vitro* assays are the same drug doses that have proven efficacious in plasma. Supporting the predictive value for clinical application of the *in vitro* experimentation, a BiTE dose of 0.06 mg/m^2/24h resulted in 2 complete responses and 5 partial responses in a total of 7 patients (Bargou et al., 2008).

In vitro LDH-release killing assays demonstrating the efficacy of IMCgp100 have been used to determine the MABEL. The MABEL has been defined as the dose of drug to be administered by intravenous (i.v) injection such that the predicted Cmax (peak concentration) in the plasma will be lower than a concentration of drug known to have a minimal biological effect in these assays. The assay has been reproduced with four donor effector T cells and the most sensitive target cell line to IMCgp100 redirected lysis, Mel526, has been used. A high effector-to-target ratio of 10:1 and a long

incubation period of 24 h were used to allow for the most sensitive analysis. Figure 6-6 shows an example data set using Mel526 as targets; the MABEL is calculated as a dose of drug to give a plasma concentration lower than 1 pM or 10^{-12} M (as indicated). The molecular weight of IMCgp100 is 76 kDa, hence the concentration of IMCgp100 in the plasma at which a minimal biological effect is expected is < 76 pg/ml.

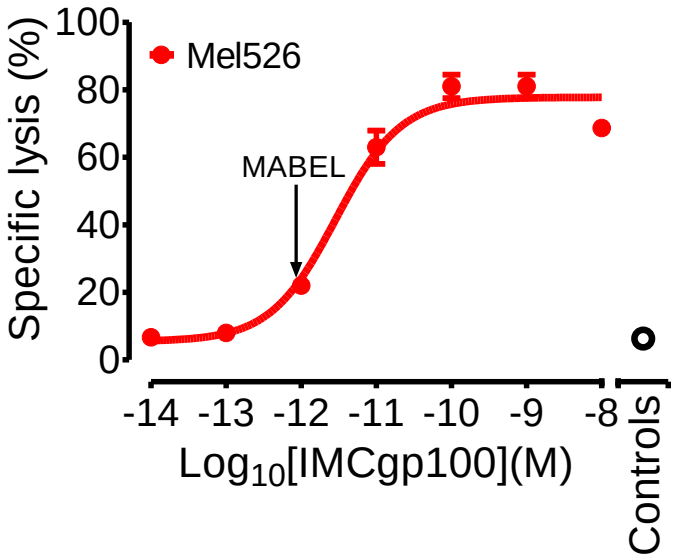

Figure 6-6 *In vitro* efficacy assay used to determine the MABEL.

LDH-release killing assay (Promega CytoTox96®) showing lysis of HLA-A*0201^{+}, gp100^{+} melanoma cell line Mel526 by unstimulated, purified CD8^{+} T cells at an E:T ratio of 10:1 in the presence of titrated doses of IMCgp100 over 24 hours. The open symbol represents the corresponding IMCgp100-negative control. The MABEL is represented by the black arrow. Data are means ± s.e.m. The data shown are representative of four experiments.

6.2.4.2 Setting the target limiting dose (TLD) for IMCgp100

The *target limiting dose* (TLD) is defined as the concentration of drug beyond which a loss of specificity is observed. To determine the TLD of IMCgp100, the HLA-A*0201 positive, gp100 negative melanoma cell line, A375, was co-incubated with unstimulated, purified $CD8^+$ T cells in the presence of increasing concentrations of IMCgp100 (Figure 6-7). The killing data shows that there is no IMCgp100 redirected lysis of the gp100 negative cell line A375 until a concentration of 10^{-8} M (10 nM) is reached. The loss of specificity of IMCgp100 at 10 nM and above has also been observed for a panel of HLA-A*0201 positive primary cells in T cell activation assays (described in section 6.2.1.3). Based on the *in vitro* killing and the functional cross-reactivity assay data, the TLD for IMCgp100 has been set at 1 nM i.e., the maximum plasma concentration will not be allowed to exceed 1 nM until more is known about the safety profile of IMCgp100 in patients.

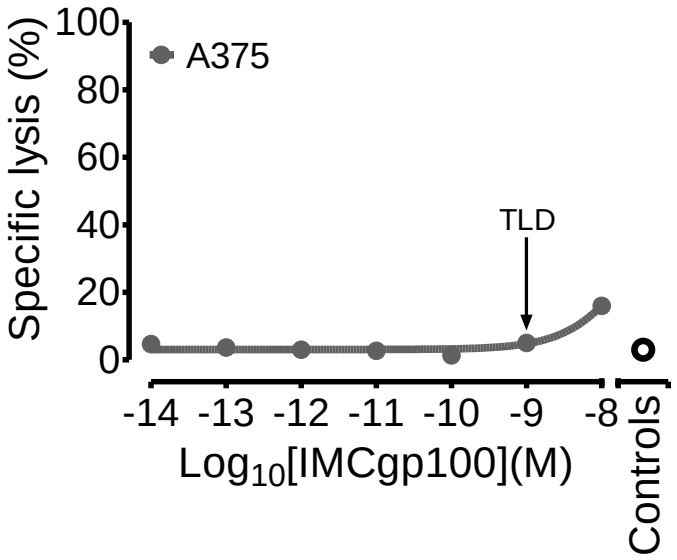

Figure 6-7 *In vitro* killing assay used to determine the TLD.

LDH-release killing assay (Promega CytoTox96®) showing the non-specific lysis of HLA-A*0201^{+}, gp100^{-} melanoma cell line A375 by unstimulated, purified CD8^{+} T cells at an E:T ratio of 10:1 in the presence of titrated doses of IMCgp100 over 24 hours. The open symbol represents the corresponding IMCgp100-negative control. The TLD is represented by the black arrow. Data are means ± s.e.m. The data shown are representative of four experiments.

6.3 Summary

This chapter describes the key data sets used to support the entry of IMCgp100 into a Phase I clinical trial. In the absence of a relevant toxicology species, IMCgp100 was investigated for potential reactivity to tissues other than target melanoma tissue *in vitro*. In functional cross-reactivity assays, that measure T cell activation in the presence of IMCgp100, on-target reactivity is observed against primary tissue that is HLA-A*0201^{+} and known to express gp100, such as epidermal skin derived melanocytes. However, in all cases the reactivity seen against the melanocyte lots was weaker than that observed for the melanoma cell line Mel526, suggesting that there is a therapeutic window between melanoma reactivity and organ specific toxicity. Nevertheless, on-target reactivity of T cells in response to IMCgp100 could possibly result in toxicities in some organs. Therefore, I investigated the use of the steroid, hydrocortisone, as a possible option for controlling on-target toxicity. I was able to demonstrate that hydrocortisone, used at a clinically relevant dose, prevents the activation of T cells even at the highest concentration of IMCgp100 (1 nM) *in vitro*.

No cross-reactivity is observed against HLA-A*0201^{+}, gp100-negative primary cells, suggesting that IMCgp100 is highly specific for its target. However, at high concentrations (10 nM and above), IMCgp100 elicits some loss of specificity by recognising and activating T cells in response to HLA-A*0201^{+}, but not to HLA-A*0201^{-}, gp100-negative cells. The concentration of IMCgp100 beyond which a loss of specificity is observed was used to set the *target limiting dose* (TLD). Based on the *in vitro* killing and the functional cross-reactivity assay data, the TLD for IMCgp100 has been set at 1 nM. *Ex vivo* whole blood assays indicate that IMCgp100 does not cross-react with haematological cells in systemic circulation which could lead to cytokine release syndrome.

Finally, I produced the *in vitro* efficacy data that was used to determine a safe starting dose for the first administration of IMCgp100 in humans, which was set by establishing the *minimal anticipated biological effect level* (MABEL). Based on the *in vitro* data presented in this thesis, IMCgp100 has received phase I clinical trial regulatory approval in the UK.

CHAPTER 7 DISCUSSION

ImmTACs are a novel class of bispecific reagents intended for therapeutic use against cancer. These reagents are comprised of a humanised CD3-specific scFv fused to a high affinity mTCR specific for a tumour-associated pMHCI antigen. In this PhD thesis, the potency, sensitivity, and specificity of ImmTACs is investigated for pMHCI epitopes derived from four tumour-associated antigens: (1) gp100, a melanocyte differentiation antigen, (2) MAGE-A3, a cancer/testis antigen expressed by a wide variety of tumours, (3) Melan-A/MART-1, a lineage-specific antigen expressed by a large proportion of primary and metastatic melanomas, and (4) NY-ESO, a cancer/testis antigen expressed in multiple myeloma, lung cancer, melanoma and several other cancers. The data presented describes the evolution of the ImmTAC molecules, from selecting a suitable effector function providing functional potency, to the modifications investigated to improve the potency, including those which are important for the manufacture of ImmTACs and their potential use in the clinic. A comprehensive range of assays and methodologies have been established to characterise the ImmTAC reagents including functional T activation assays and cytotoxicity assays. However, the main focus of the work was on the developmental pathway of the lead therapeutic candidate ImmTAC molecule from early pre-clinical testing, to the studies required for admission to clinical trials.

$CD8^+$ T cells have evolved to seek out and eliminate abnormal cells in the body. They are guided by T cell receptors (TCRs) which recognise endogenously processed peptides derived from cellular proteins and presented on cell surfaces by MHC

molecules. A highly diverse TCR repertoire is generated to recognise this array of peptides; each one unique to a particular T cell clone. The human TCR repertoire is shaped at an early age in a process called thymic selection; a trade-off between the dangers of failing to recognise abnormal cells and that of potentially devastating auto-reactivity results in a TCR repertoire of approximately 25 million distinct TCRs (Arstila et al., 1999; Robins et al., 2009) which have very low affinities for their relevant pMHC. Given that the majority of tumour-associated peptide antigens (TAPAs) are derived from non-mutated self proteins, T cells often fail to detect and eradicate malignant cells. Even so, the T cell system, through the capacity of TCRs to recognise virtually all pMHC antigens, has a unique, if largely untapped potential for addressing cancer, because MHC presenting TAPAs constitute by far the largest class of therapeutic targets in malignant diseases.

Notwithstanding the failure of the natural T cell repertoire to effectively recognise malignant cells, TCRs do exist that have specificity for TAPAs. However, the affinities of such TCRs are usually too low to instigate a T cell response against tumour cells, and immune escape mechanisms employed by the tumour, such as the down-regulation of MHC and the ability to induce inhibitory signals in T cells, increase the hurdles to an effective immune response. Immunocore Ltd has developed a technology based on the capacity of TCRs to recognise TAPAs to potentially overcome the limitations of the immune system to respond adequately to cancer. To achieve this, TCRs have been engineered in three key ways to turn the natural TCR complex into a potential drug candidate;

(1) A soluble version of the TCR has been generated by deleting the transmembrane regions and introducing a disulphide bond between the Cα and Cβ chains to stabilise the protein

(2) The affinity (K_D) of the TCR for its pMHC has been increased from the micromolar level of naturally selected TCRs to picomolar level

(3) The TCR has been fused to an effector function to replace the natural effector function, the T cell. An anti-CD3 single chain variable fragment (anti-CD3 scFv) has been selected which is capable of activating non-cancer specific T cells when cross-linked to an antigen positive cancer cell via the mTCR

Thus, the ImmTAC functions as a transient linker between the cancer cell and the T cell, forcing an immune response where the natural immune system has failed.

7.1 The testing of TCR-effector function fusions capable of redirecting T cells against cancer cells with an extremely low HLA-peptide antigen target number per cell

Levels of tumour-associated pMHC can be as low as 10-50 epitopes per cell; this has been observed both on cell lines and, much more importantly, on fresh tumour samples (Purbhoo et al., 2006). Therefore, the effector function fused to the mTCR must be highly potent. The use of anti-CD3 monoclonal antibodies to recruit and activate T cells has been employed by a number of bispecific T cell activating antibody therapeutics, most notably, the potent bispecific T cell engager (BiTE) antibodies. The observation that the mTCR is not internalised to any substantial degree when bound to its target cell (Purbhoo et al., 2007; Purbhoo et al., 2006) ruled out the use of toxin payloads that require cellular internalisation. Instead, strategies that worked from the

cell surface were investigated. Anti-CD3 was particularly attractive as it is capable of unleashing the cytotoxic potential of $CD8^+$ CTLs. To find the most applicable anti-CD3 antibody to fuse to the mTCR, three humanised anti-CD3 scFv antibodies were tested; OKT3 (Xu et al., 2000), 12F6 (B. Li et al., 2005) and UCHT1 (Shalaby et al., 1992). As described in Chapter 3, UCHT1 was shown to be at >1000-fold more effective at triggering $CD8^+$ T cell activation than 12F6 and OKT3 when fused to a Melan-A/MART-1-specific mTCR (Figure 3-1). ImmTAC-MEL/UCHT1 when used at 10 pM concentration in cellular assays, was able to activate $CD8^+$ T cells in the presence of target cells loaded with 10^{-9} M peptide, giving a level of peptide presentation close to that found on cancer cells.

One of the main concerns associated with using an anti-CD3 antibody as an effector function is the risk of potentially activating T cells without the simultaneous binding of the mTCR to its target. Whilst the mTCR arm of the fusion binds to an antigenic pMHC on the target cell, the anti-CD3 scFv arm of the molecule, by engaging the invariant ε-chain of the CD3 glycoprotein complex associated with T cell receptors, is capable of activating any $CD8^+$ T cell independently of the intrinsic antigen specificity of its own TCR. The experiments presented with the UCHT1-mTCR fusions, called ImmTACs, show that no activation of CTLs by ImmTAC was observed in the absence of target cells (see, for example, Figure 3-1c). Furthermore, no activation of CTLs by ImmTAC was observed in the presence of antigen-negative target cells containing the correct HLA type (see, for example, Figure 4-1). This illustrates that the biological activity of ImmTAC is highly specific, requiring not only the correct HLA type but also the correct peptide for recognition, and that the ImmTAC only activates when a multivalent contact, i.e. cross-linking, is established between the target

cell and the T cell. This last feature is essential for avoiding systemic toxicity when applied as a therapeutic.

Another important aspect of the anti-CD3 effector function is that it binds CD3 with an affinity that is considerably lower than the mTCR-pMHC interaction. For IMCgp100 (the selected development candidate for ImmTAC-gp100), for example, the TCR affinity for pMHCI is approximately 24 pM whereas the UCHT1 scFv affinity for CD3 has been measured at 38 nM. This difference in affinity should permit the preferential decoration of the tumour target cells with ImmTAC thereby signalling them for destruction by T cells present in the vicinity of the tumour. However, when ImmTACs are administered to patients it is likely that binding to both tumour cells and T cells will occur to a greater or lesser extent with some sequestering of ImmTAC by T cells in the blood before it has bound the tumour cell target. In the Phase I clinical trial with IMCgp100 it has been observed that CD3, CD4 and CD8 staining of T cells transiently reduces after 24 hours following drug dosing, to then gradually reappear after 3 days (Figure 6-1). This could suggest that the CD3, CD4 and CD8 are down-regulated or, perhaps more likely, that T cells migrate from the blood into tissues. Whatever the explanation, which has not been ascertained yet, it appears likely that the changes observed in T cell staining are caused by some kind of contact with the ImmTAC anti-CD3 scFv component. Transient T cell disappearance (lymphopenia) is also consistent with clinical observations made with T cell-engaging BiTE antibody, blinatumomab (Nagorsen & Baeuerle, 2011).

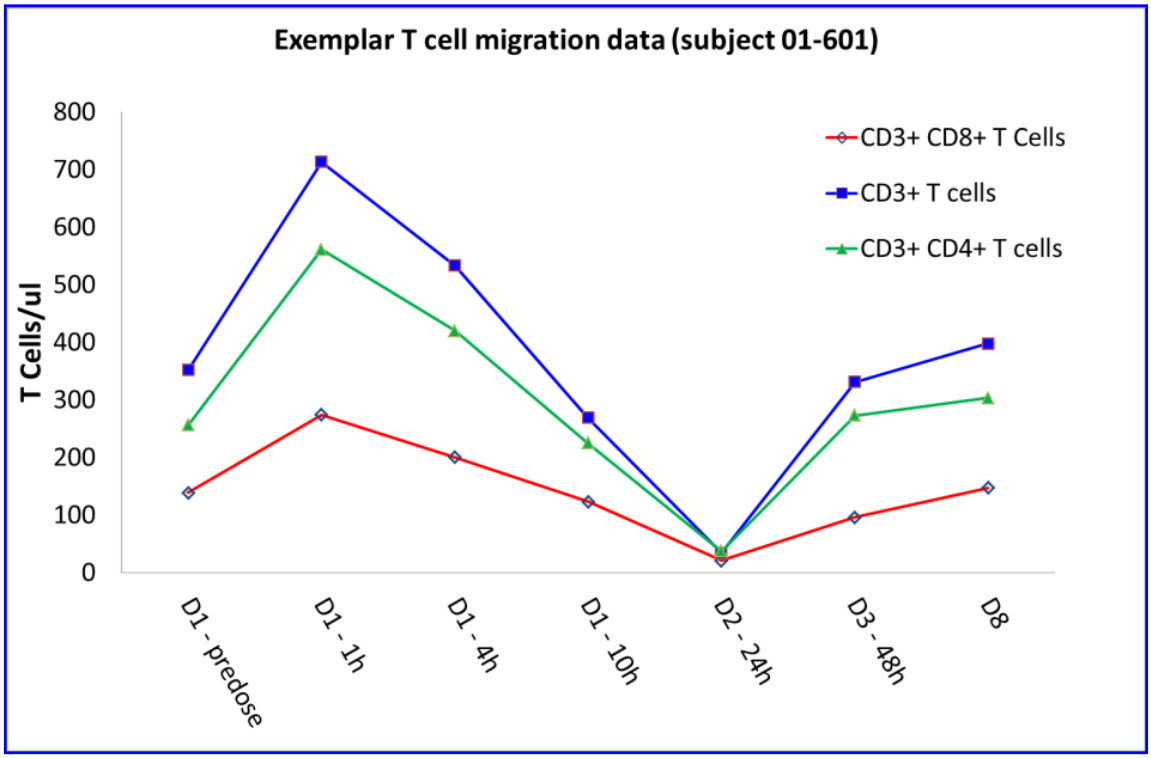

Figure 6-1 Example of T cell staining time course in blood of a melanoma patient treated with a single 4 hour infusion of 405 ng/Kg of IMCgp100.

DX refers to the day after infusion was initiated. Data courtesy of Dr. Stephen Megit at Immunocore Ltd.

The affinity of the binding to CD3 by bispecific T cell activators is considered to be optimal for triggering T cell activation and cytotoxicity (Bortoletto et al., 2002). Studies have also shown that T cells are stimulated best by low affinity ligands of the TCR/CD3 complex (Goldrath & Bevan, 1999). This observation is supported by the experiments described in Chapter 3 comparing single- and tandem anti-CD3 scFv TCR fusions which indicate that the high avidity binding of the tandem anti-CD3 scFv fusion is less efficient at activating T cells than the single anti-CD3 scFv fusion (Figure 3-6 and Figure 3-7). Indeed, the low efficiency of some bispecific antibodies could possibly be attributed to sub-optimal CD3 engagement whereby the affinity of the interaction is too high (Shalaby et al., 1992). This could be because experimental observations indicate that T cell activation requires serial triggering (Valitutti & Lanzavecchia,

1997), which depends on receptor-ligand interactions being of low affinity, and, in particular, having short engagement times (M. M. Davis et al., 1998; Matsui et al., 1991; Weber et al., 1992; Willcox et al., 1999).

While for ImmTACs, the functionally optimal anti-CD3 interaction is not achieved with the highest affinity, the mTCR affinity for the target pMHC appears to be almost directly proportional to potency (Figure 3-8). Here, seven different affinity variants of the gp100-specific ImmTAC (ImmTAC-gp100) were tested with K_D values ranging from 30 pM for the highest affinity variant to the lowest at 30 μM. All the variants were able to activate and redirect unstimulated $CD8^+$ T cells in a concentration-dependent manner when in the presence of HLA-A*0201^+/$gp100^+$ targets. However, the degree of the response varied considerably with the higher affinity variants progressively more potent, activating $CD8^+$ T cells at concentrations as low as 10^{-11} M. This shows that the engineering of high affinity mTCRs is critical for the targeting and $CD8^+$ T cell activating capability of the ImmTAC molecules. Furthermore, the high on-target potency of ImmTACs, which correlates with mTCR-pMHC affinity, means that very small doses should be applicable in the clinic. This is likely to have the significant advantage of minimising any systemic activation of T cells that could occur as a result of scFv-mediated ImmTAC binding to CD3 on T cells.

TCR affinity is also pertinent when considering that down-regulation of MHC expression is a well-documented mechanism by which tumour cells evade immune surveillance (Cabrera et al., 2003; Dunn et al., 2004). Therefore, in order for ImmTACs to be effective immunotherapeutics, they must be able to eradicate tumours expressing particularly low levels of peptide-MHC. The relationship between the peptide-

sensitivity of the ImmTACs and the ImmTAC-pMHCI affinity was investigated using the same set of ImmTAC-gp100 affinity variants described above. As predicted, the highest affinity variants were the most peptide sensitive and were able to activate $CD8^+$ T cells in the presence of T2 cells loaded with 10^{-10} M peptide (Figure 4-2). Epitope counting studies using single-cell three-dimensional fluorescence microscopy have shown that T2 cells pulsed with different concentrations of exogenous the $gp100_{280-288}$ heteroclitic (YLEPGPVTV) peptide produced the following epitope numbers: 0-3 at 10^{-11} M, 2-10 at 10^{-10} M and 15-45 at 10^{-9} M peptide (Liddy et al., 2012). At pulsing concentrations at or above 10^{-8} M peptide it is no longer possible to distinguish the individual epitopes and quantification becomes less accurate. Based on this information, the peptide sensitivity of the highest affinity variants (K_D value = 0.03 and 0.32 nM) equates to 2-10 pMHCI epitopes per cell. Notably, the TCR wild-type affinity ImmTAC (K_D value = 30 μM) and the minimally modified variant (K_D value = 8 μM), were not able to activate $CD8^+$ T cells at any of the tested peptide concentrations, further illustrating the significance of TCR affinity for ImmTAC function.

However, the ultimate test of the capability of ImmTACs at redirecting T cells against targets expressing extremely low levels of peptide-MHCI is to use tumour cells. Studies using high affinity mTCRs to quantify the number of cognate antigens expressed on the surfaces of tumour cells have shown that individual melanoma and myeloma cells present an average of 10-50 copies of the $NY\text{-}ESO_{157-165}$ epitope per cell (Purbhoo et al., 2006). It has also been reported that other tumour associated peptide antigens are similarly presented at extremely low levels on the surfaces of melanoma cells, averaging 20-70 copies per cell for the HLA-A*0201 restricted epitopes $gp100_{280-288}$ and $MAGE\text{-}A3_{168-176}$, and 60-150 copies per cells for $Melan\text{-}A/MART\text{-}1_{26-35}$ (Liddy et al., 2012).

The ability of ImmTACs to potently redirect $CD8^+$ T cell activity against tumour cell lines was demonstrated for all four ImmTAC molecules (see, for example, Figure 4-1). The most valid data sets are those where it was possible to obtain freshly isolated clinical tumour samples to use as targets. The data presented shows that ImmTAC-NYE is able to activate and redirect unstimulated PBMC to freshly isolated HLA-A*0201^+/LAGE-1^+ lung cancer cells (Figure 4-11). However, the potency of the ImmTAC-NYE redirected activity against the lung cancer cells was considerably lower than that seen against tumour cell lines. There are two possible reasons for this; first, the lung tumour sample was composed of a heterogeneous population of cells, probably containing normal lung tissue cells and other normal cells as well as tumour cells; the percentage of tumour cells within the sample could not be established. Second, the viability and quality of the tumour sample cells was poor prior to use in the assay. The data presented also shows that ImmTAC-MAGE is able to activate and redirect unstimulated PBMC to $CD138^+$ cells, which contain the malignant plasma cells, isolated from the bone marrow aspirate sample of a multiple myeloma patient (Figure 4-12).

Taken together the *in vitro* cellular data presented suggests that ImmTACs are able to redirect T cell activity to both tumour cell lines that are known to express low levels of cognate antigen per cell and most significantly, to fresh tumour samples. The down-regulation of MHC by tumours may prevent recognition by naturally occurring tumour specific T cells which require higher levels of epitope to become activated. However, the ability of ImmTAC to detect a low density of pMHC means that ImmTACs have the potential to establish immune recognition of tumours that might normally evade detection.

7.2 ImmTAC redirected T cell responses

The potency of ImmTAC-mediated $CD8^+$ T cell activation is closely correlated to the mTCR-pMHCI affinity. The data presented in Chapter 4 shows that all four ImmTACs activate $CD8^+$ T cells in a concentration-dependent manner, producing cellular EC_{50} values below 100 pM (Figure 4-1); these closely match the K_D values of the corresponding monomeric mTCR-pMHCI interactions as determined by surface plasmon resonance (SPR) equilibrium-binding measurements. However, the potency of the ImmTAC reagents is not only a function of the mTCR binding affinity; the physical length of the ImmTAC molecule also seems to be important.

As described in Chapter 3, in the first generation ImmTAC constructs the anti-CD3 scFv was fused to the C-terminus of the TCR beta chain via an inter-linker. The reasoning for this design was that molecular modelling studies indicated that fusing the effector function to the N-terminus of the TCR beta chain would obstruct the binding of the TCR to its target. However, while the fusion of anti-CD3 scFv to the C-terminus increased the overall length of the construct, its fusion to the N-terminus would produce a more compact molecule which would impose closer proximity between the redirected T cell and the target cell possibly facilitating maximal T cell activation. Surprisingly, the cellular data revealed that the N-terminus ImmTAC construct, i.e. N-UCHT1-TCR-C (Figure 3-3a) is functional and able to redirect $CD8^+$ T cell activity in response to targets presenting the correct HLA with the relevant peptide and does not induce a response in the presence of targets presenting the correct HLA with an irrelevant peptide (Figure 3-2). This suggests that, notwithstanding the molecular modelling predictions, the binding of the mTCR to its pMHCI has not been impeded by the anti-CD3 scFv. Furthermore, the N-terminus ImmTAC redirected activity is 4-fold more potent than that mediated by the C-terminus ImmTAC.

In order for $CD8^+$ T cells to kill their targets, the formation of a cytolytic synapse is critical (Stinchcombe et al., 2001). This structure enables T cells to tightly adhere to targets for the controlled release of granzymes and perforin, the protein mediators of target cell apoptosis. BiTE antibodies have been shown to induce cytolytic synapses that are indistinguishable in structure and composition from naturally formed cytolytic synapses between TCR and pMHC (Offner et al., 2006). Key to this is the length of the inter-cellular bridge formed by the tumour-associated antigen on the target cell, the BiTE antibody, and the CD3ε subunit of the TCR complex. This distance has been estimated to be 12-14 nm for BiTE antibodies (Bluemel et al., 2010), which is very similar to the length of a natural TCR/peptide/MHC complex of ~14 nm (Garboczi et al., 1996; Garcia et al., 1998). At the time of writing this thesis the length of the inter-cellular bridge formed by ImmTACs was not known; indeed the length of the ImmTAC molecule could be difficult to establish by biophysical methods because it is not known how the TCR and scFv portions are organised in relation to each other in the tertiary structure. Nevertheless, the increased potency observed with the N-terminus ImmTAC suggests that synapse formation and function may be more efficient with the more compact N-terminus design as a result of the closer proximity between the target cell and T cell. The potency of target cell lysis by BiTE-redirected cytotoxic T cells has been shown to be optimal when the epitope is positioned close to the plasma membrane of the target cell (Bluemel et al., 2010). Similarly, the antigen sensitivity of an engineered chimeric TCR was found to be modulated by target epitope distance from the cell membrane (James et al., 2008). Indeed, it is a general feature of T cell activation that higher potency is achieved when the T cell and target membranes are in closer proximity to each other, as illustrated by experiments with super-agonistic antibodies to various T cell co-receptors (S. J. Davis & van der Merwe, 2006) The

greater redirected T cell activity observed for the more compact N-terminus ImmTAC fusion lends support to this notion, and indirectly provides evidence in favour of the kinetic-segregation (K-S) model of TCR triggering whereby T cell activation is a result of net phosphorylation. This model proposes that TCR triggering is the result of the exclusion of bulky negative regulators, such as the tyrosine phosphatase CD45, when multiple zones of close contact (~ 15 nm apart) form at the target cell-T cell interface (S. J. Davis & van der Merwe, 2006).

ImmTACs are capable of polyclonal activation of unstimulated $CD8^+$ T cells that includes cytokine production and lytic activity when in the presence of relevant target cells. The cellular assay data sets presented in Chapter 4 shows concentration-dependent release of IFN-γ and granzyme B from ImmTAC-redirected purified $CD8^+$ T cells as well as from unselected T cells present in the more physiologically relevant peripheral blood mononuclear cells (PBMC). Furthermore, ImmTACs have also been shown to elicit concentration-dependent release of the degranulation marker CD107a and the cytokines TNF-α and IL-2 (Figure 4-5). These data demonstrate that multiple effector functions are induced in the ImmTAC redirected $CD8^+$ T cells, comprising both lytic activity and the production of soluble factors. Moreover, the polyfunctional responses induced by ImmTAC redirected activity could help to broaden an immune reaction against tumour cells *in vivo* by recruiting other types of immune cells to the tumour microenvironment.

ImmTACs mainly activate terminally differentiated $CD8^+$ effector memory (Tem) cells (characterised by the standard phenotypic markers, $CD45RO^+$ and $CD27^-$), and central memory (Tcm) cells ($CD45RO^+$ and $CD27^+$) whereas activation of naïve T cells ($CD45RO^-$ and $CD27^+$) is minimal (Liddy et al., 2012). Comprehensive activation

of naïve T cells could potentially be dangerous as became evident in the Phase I clinical trial of the superagonist anti-CD28 monoclonal antibody, TGN1412 (TeGenero AG) (Suntharalingam et al., 2006) (discussed in more detail in section 7.5). The activation of T cells predominantly from the memory compartments, rather than naïve T cells, suggests that ImmTACs do not provide costimulation via CD28. Naïve T cells require CD28 costimulation to become activated whereas memory T cells are less dependent. The apparent lack of T cell costimulation by ImmTAC via CD28 could be important in avoiding differentiation of potentially autoreactive naïve T cell clones. Although ImmTACs do not appear to activate naïve T cells during the relatively short time frame of the assay, it does not rule out the possibility that long-term exposure to ImmTACs *in vivo* could potentially lead to the activation of naïve T cells. However, as naïve T cells are not usually present within the vicinity of tumours, prolonged contact between ImmTAC and naïve T cells that might result their activation, is unlikely.

The primary function of ImmTAC is to recruit $CD8^+$ cytotoxic T cells (CTLs); a subset of T cells recognised as the major effector cell type involved in the potent killing of malignant cells. However, data presented in Chapter 4 shows that ImmTACs can also redirect and activate polyclonal $CD4^+$ T cells (Figure 4-4). Concentration-dependent release of IFN-γ by $CD4^+$ T cells mediated by ImmTAC produced similar cellular EC_{50} values to that observed for $CD8^+$ T cells and PBMC. The recruitment of $CD4^+$ T helper (Th) cells by ImmTACs could have a beneficial role in augmenting anti-tumour immune response *in vivo*. It is well established that $CD4^+$ Th cells play an important part in enhancing and sustaining $CD8^+$ T cell responses (Antony et al., 2005; Pardoll & Topalian, 1998). Moreover, there is increasing evidence that $CD4^+$ Th cells have a more direct role in orchestrating host anti-tumour responses than previously thought. For example, a recent study demonstrated that adoptively transferred tumour-

reactive $CD4^+$ T cells acquire cytotoxic activity and eradicate large established tumours in lymphopenic mouse model hosts (Quezada et al., 2010).

Overall, the data presented in Chapter 4 indicates that the initial redirection event induced by ImmTACs could serve as a catalyst to precipitate the recruitment of additional immune effectors to the tumour site. Furthermore, in addition to ImmTAC-mediated activation of $CD4^+$ T cells, there is evidence that antigen cross-presentation by dendritic cells and epitope spreading may amplify ImmTAC-redirected T cell responses (manuscript in preparation), all of which could contribute to generating a self-sustaining tumour-specific immune response, potentially minimising the necessity for prolonged dosing schedules. Most importantly, it could induce anti-tumour responses that also address antigen-negative cells.

7.3 ImmTAC redirected target cell killing

To be effective *in vivo* ImmTACs must be able to redirect CTLs, irrespective of their native specificity, to lyse tumour cells. CTL induce target cell lysis by two main mechanisms: either by releasing the contents of cytolytic granules in the granule-exocytosis pathway of cytotoxicity, or by engaging cell-surface death receptors, such as members of the tumour-necrosis factor receptor (TNFR) family, which includes Fas (CD95). Both mechanisms trigger the inherent apoptotic response within the target cell. However, the dominant mechanism by which CTLs destroy target cells is through granule-exocytosis pathway. The granules of CTLs contain various proteins; the lytic granules involved in the induction of apoptosis include the pore-forming protein perforin and a family of serine proteases that are known as granzymes. Lysis by CTL involves the formation of a cytolytic synapse with a specifically recognised target cell and the release of the granule contents into the synaptic cleft. The granzymes are then

delivered into the target cell, where they initiate programmed cell death via proteolytic activation of pro-caspases and direct cleavage of caspase substrates.

The mechanism by which ImmTAC-redirected target cell killing is achieved was not specifically investigated but the data presented indicates that granule-exocytosis mediated apoptosis is the primary mode-of-action. Furthermore, the perforin-dependent granule exocytosis pathway has been shown to be important in BiTE antibody-mediated cytotoxicity whereas death receptor-mediated lysis does not appear to play a role (Gruen et al., 2004). One of the earliest markers of granule-exocytosis mediated apoptosis is the release of granzyme B, which is one of the most abundant and best-studied granzymes. In ELISpot assays, concentration-dependent release of granzyme B from ImmTAC-redirected purified $CD8^+$ T cells and PBMC was observed (Figures 4-6 & 4-7). Only recently activated $CD8^+$ T cells are capable of exerting perforin/granzyme B-mediated cytolytic functions (Kaech & Ahmed, 2001). Resting $CD8^+$ T cells (naïve and central memory cells) do not have cytolytic granules containing granzyme B but can express granzyme several days later following antigen encounter – provided they also receive the correct cytokine stimuli (Curtsinger et al., 2005; Trapani & Sutton, 2003). At any one time, up to 20% of all $CD8^+$ T cells in the PBMC of healthy individuals are granzyme B positive due to on-going stimulation with various environmental antigens (Rock et al., 2005). Therefore, the ImmTAC-redirected T cell responses observed in the granzyme B ELISpot are most likely to be those of recently activated $CD8^+$ effector T cells.

Once inside the target cell, granzyme B induces programmed cell death by activating the key executioner (effector) caspases, caspase-3 and caspase-7. ImmTAC-mediated caspase-3/7 activity was detected after 4 hours and the level of activity was concentration-dependent (Figure 5-1). Like the other consequences of ImmTAC

redirected T cell activity, caspase activation was induced in an antigen-specific manner as no activity was observed in the presence of targets expressing the correct HLA molecule but negative for the relevant antigen. Activation of caspase-3/7 by ImmTAC indicates the apoptotic pathway is being triggered in the target cells and that they are doomed to die.

The extent of target cell lysis induced by ImmTAC redirected $CD8^+$ T cells was investigated using the conventional ^{51}Cr-release assay and a non-radioactive alternative, the LDH-release assay, to assess short-term killing kinetics. The levels of ImmTAC-redirected target cell lysis and the time required to reach maximum lysis varied considerably depending on the effector and target cells used. Typically, ^{51}Cr-release and alternative 'release' assays such as the LDH-release assay are conducted over 4 hours. The data presented in Chapter 5 shows that while ImmTAC redirected target cell lysis could be detected within this time frame, it was only possible when redirecting a $CD8^+$ T cell clone but not when using polyclonal unstimulated and purified $CD8^+$ T cells or unselected T cells present in PBMC (Figures 5-2 & 5-3). A T cell clone represents a population of activated $CD8^+$ T cells which are expected to have the same cytotoxic potential, whereas peripheral T cells are a mixture of T cells of varying cytotoxic potential and differentiation state. Therefore, only a fraction of peripheral T cells is likely to have the phenotype of a cytotoxic $CD8^+$ T cell clone. To be effective *in vivo*, it is critical that ImmTACs have the capability to potently redirect unstimulated, peripheral T cells. However, it was clear from the early ImmTAC-redirected target cell killing experiments that the duration of the cytotoxicity assay would need to be extended beyond 4 hours in order to evaluate redirected killing by peripheral T cells. While the ^{51}Cr-release assay was not suited to a longer time course because of prohibitively high spontaneous release, the Promega Cytotox96® LDH-release assay

could be extended to a 24 hour time course without spontaneous release becoming an issue. Thus, the LDH-release assay revealed that despite slow killing kinetics, ImmTACs can potently redirect unstimulated purified $CD8^+$ T cells as well as unselected T cells present in PBMC to kill a range of different tumour cell lines (Figures 5-4, 5-5, 5-6 & 5-7). ImmTAC-redirected killing is concentration-dependent and typically produces cellular EC_{50} values below 100 pM, similar to those observed in the IFN-γ and granzyme B activation assays described in Chapter 4. Furthermore, it has been demonstrated that ImmTAC reagents can induce serial killing, a feature that is likely to contribute to their potency (Liddy et al., 2012).

In contrast to the relatively slow killing kinetics of ImmTAC-mediated target cell lysis, the BiTE antibody, anti-CD19/anti-CD3 single-chain bispecific antibody (bscCD19xCD3), has been shown to potently redirect both T cell clones, and unstimulated polyclonal T cells purified from PBMC, to lyse target cells after 4 hours (Dreier et al., 2002). The key reason for this difference in killing kinetics between BiTE antibodies and ImmTACs is probably due to the levels of target cell antigen being targeted. The Micromet single chain bispecific antibody bscCD19xCD3 targets CD19, a B cell co-receptor expressed on a high percentage of human B-cell malignancies and on normal B cells. The levels of expression of CD19 on chronic B cell leukaemias, and on normal B cells, has been found to number tens of thousands of molecules per cell (Ginaldi et al., 1998). ImmTACs, on the other hand, are potentially targeting less than a hundred epitopes per cell (Liddy et al., 2012). Therefore, it is likely that during ImmTAC-redirected killing there is a greater lag phase prior to synapse formation and target cell lysis. For lysis to occur, a target and/or T cell occupied by ImmTAC must come into close contact. The low epitope numbers on the target cells means that the number of ImmTAC molecules bound on each target cell will also be low. Therefore, it

is speculated that it will take time for T cells to form a close contact zone within which there is a high enough density of bound ImmTAC molecules to generate the binding avidity necessary to trigger potent T cell activation.

While significant lysis of the majority of the tested tumour cell lines occurred during the first 24 hours, the IncuCyte real time imaging system, which measures caspase-3/7 activation, demonstrated that some cell lines require even longer periods for apoptosis to occur (Figure 5-9). This variation in cell lysis could reflect the difference in density of target epitopes on the surface of each cell line. Other factors which may influence the rate of target cell killing include the susceptibility of individual tumour cell lines to lysis and the presence of co-factors, for example, CD80 and CD86 on the tumour cells, which may enhance killing.

The efficacy of ImmTAC redirected target cell killing also varied depending on the PBMC donor used. The use of the IncuCyte real time imaging system to monitor long term redirected killing kinetics revealed that the time taken to reach maximal target cell lysis could vary considerably depending on the donor effector T cells used. For instance, $CD8^+$ T cells purified from the PBMC of two healthy donors were tested against the same target cell line, EJM, in the presence of ImmTAC-MAGE; while the $CD8^+$ T cells from one donor achieved maximal target cell killing by 24 hours, redirected killing by another donor's T cells took over 50 hours before most of the targets were lysed (Figure 5-10). Possible explanations for the variability include various proportions of CD8 memory cells and differences in the cytokine (infectious) state of donors.

Taken together, understanding ImmTAC redirected killing kinetics is hugely important when designing a dosing strategy for the lead therapeutic development

candidate in order to maximise the chances that the drug will be efficacious in the clinic.

7.4 The rationale for the ability of ImmTACs to redirect T cell activation in cancer patients

One of the main obstacles to effective tumour immunotherapy is the ability of tumours to foster a tolerant microenvironment in which they employ a diverse range of immunosuppressive mechanisms that may act in concert to thwart effective immune responses.

Most tumour antigens are derived from 'self' proteins, which are also expressed in normal cells, as opposed to neo-antigens that are uniquely present in cancer cells (Boon et al., 2006; Rosenberg, 1999). The immune system has evolved complex mechanisms for establishing T cell tolerance against self and, by extension, to the majority of tumour antigens. So, ironically, it is the immune system itself that poses the greatest challenge for harnessing immune responses against tumours. 'Tumour-induced anergy' is a well documented phenomenon which was initially demonstrated in two independent studies showing that antigen-specific $CD4^+$ T cells were rendered tolerant during tumour growth *in vivo* (Bogen, 1996; Staveley-O'Carroll et al., 1998). Several studies since have shown that this state of T cell unresponsiveness occurs during the growth of haematologic or solid tumours expressing model or true tumour antigens (Cuenca et al., 2003; D. J. Morgan et al., 1998; Overwijk et al., 2003). T cell anergy is also observed during the progression of spontaneously arising tumours (Willimsky & Blankenstein, 2005) and most significantly, during the progression of human cancers (Lee et al., 1999; Noonan et al., 2005). Therefore, induction of tolerance to tumour antigens, via regulatory mechanisms akin to those that prevent autoimmunity, represents

an important immunosuppressive strategy by which tumour cells might escape T cell mediated anti-tumour responses. Crucially, in order for a cancer immunotherapeutic to be effective against tumours expressing mainly self-antigens, the barrier imposed by immune tolerance must be broken. However, there could be 'a price to be paid' for breaking tolerance to self-antigens in that it involves a potential for inducing tissue-specific autoimmunity. For example, treatment of melanoma patients with anti-CTLA-4 antibodies and peptide vaccination was associated with strong anti-tumour effects and development of vitiligo (de-pigmentation of areas of the skin) as a manifestation of tissue-specific autoimmune responses (Phan et al., 2003).

There have been reports that peripheral blood T cells of patients with certain cancers have decreased expression of TCR-ζ chain, a protein tyrosine kinase that plays a critical role in proximal signalling events leading to T cell activation, implying that systemic T cells are dysfunctional in cancer patients (Gunji et al., 1994; Healy et al., 1998; Matsuda et al., 1995). A marked decrease in the expression of TCR-ζ chain has been observed in the peripheral blood T cells of subsets of patients with malignant melanoma. Cytokine production by T cells from melanoma patients with diminished TCR-ζ chain expression was also shown to be reduced, with lower IL-2 and IFN-γ production compared to levels observed in normal subjects. Furthermore, the overall survival of TCR-ζ chain deficient melanoma patients was significantly shorter than that of patients with normal TCR-ζ.chain expression (Zea et al., 1995). This data is one of several observations indicating that the T cell system can play a pivotal role in controlling malignant melanoma. The corollary to this is that it appears to be generally assumed that many cancer patients, and particularly those affected by late stage disease, have reduced T cell immunity. In this context, the data presented in Chapter 4 showing that fresh *ex vivo*

PBMC derived from late stage cancer patients can act as effector cells for ImmTAC-mediated activity, is encouraging. The lead therapeutic candidate, ImmTAC-gp100, which is intended for the treatment of patients with metastatic melanoma, has been shown to potently redirect PBMC isolated from two stage IV HLA-A*0201^{+} melanoma patients. Notably, redirection of the cancer patients T cells produced similar cellular EC_{50} values to those of healthy donors of less than 40 pM (Figure 4-8 and Figure 4-9). The responses seen with melanoma patient PBMC comprised the production of soluble factors IFN-γ and granzyme B (Figure 4-8 and Figure 4-9), suggesting that the peripheral effector cells of these patients are not functionally defective, at least not to ImmTAC-induced activation, as a consequence of the cancer. Interestingly, the one notable difference between the melanoma patients PBMC and those from healthy donors was the observation of a pre-existing melanoma response. In the *in vitro* cellular assays, it was evident that the T cells from one of the two melanoma patients was already reactive to the Mel526 melanoma cells without stimulation as evidenced by the presence of raised baseline activity in the IFN-γ ELISpot assay (Figure 4-8a). However, there was no raised baseline activity against the HLA-A*0201^{+}, gp100^{-} cell line, A375, suggesting the response was antigen specific. Therefore, ImmTAC-gp100 can potentially serve to significantly augment an already existing anti-tumoural response in patients. Melanoma is unique among solid tumours in being relatively immunogenic, and the existence of tumour associated antigen (TAA)-specific CD8^{+} T cells exhibiting an antigen-experienced phenotype has been demonstrated in patients with malignant melanoma (Pittet et al., 1999; Romero et al., 1998; Valmori et al., 2000). Furthermore, TAA-specific T cells have directly been implicated in spontaneous melanoma regression, occurring more frequently in melanoma than in any other cancer (Mackensen et al., 1994). Functional and phenotypical analysis of circulating tumour-

reactive $CD8^+$ T cells from stage IV metastatic melanoma patients identified a subset of terminally differentiated effector T cells, characterised by the phenotype markers $CD45RA^+CCR7^-$, which produced IFN-γ and exhibited lytic activity *ex vivo* upon stimulation with autologous tumour (Valmori et al., 2002). So despite the many mechanisms by which tumour cells may evade immune recognition such as down-regulation of target antigens and/or HLA expression, absence of costimulatory molecule expression, or secretion of immunosuppressive cytokines, TAA-specific T cell responses can still develop. Nevertheless, the presence of TAA-specific T cells in patients with metastatic melanoma indicate that these responses fail to contain tumour progression. A study investigating Melan-A/MART-1-specific $CD8^+$ T cell responses in patients with late stage melanoma, showed that while primed Melan-A/MART-1-specific T cells that circulate in the peripheral blood display the full arsenal of effector functions, similar cells that reside within the melanoma lesions fail to secrete IFN-γ, an important cytokine in anti-tumour immunity. However, the functional tolerance of the TAA-specific T cells was rapidly reversible *in vitro* upon non-specific PMA/Ionomycin stimulation (Zippelius et al., 2004). The findings of this study suggest that the tumour microenvironment is suppressing T cell effector functions and may explain the failure of tumour-specific responses to effectively counter tumour progression.

In this thesis, it was also found that PBMC from multiple myeloma patients, including those with relapse/refractory disease status, could act as effector cells for ImmTAC-MAGE-induced lysis of target multiple myeloma cells. Notably, there was no significant difference in the potency seen with the patient PBMC compared to those from healthy donors, with cells from all six patient donors producing cellular EC_{50} values below 20 pM (Figure 5-7). The anti-tumour effect of ImmTACs is dependent on

both the expression of tumour antigen and on the activity of $CD3^+$ T cells in and around the tumour. Given that tumour infiltrating lymphocytes (TILs) within the tumour environment may be functionally impaired this could severely hamper the effectiveness of ImmTAC-redirected activity. Multiple myeloma is a malignancy in which it is possible to reliably investigate the effect of the tumour microenvironment on ImmTAC-mediated activity. This is because it is relatively easy to purify both the primary tumour and the immune cells from the tumour bed within the bone marrow. In contrast, solid tumours, such as melanoma, require enzymatic treatment to isolate tumour cells. Alternatively, it maybe necessary to generate cell lines from tumour or TILs which may alter gene- expression profiles and other properties of tumours as well as T cells. Dhodapkar *et al* (Dhodapkar et al., 2002) reported that freshly isolated T cells from the tumour microenvironment of patients with progressive myeloma are unresponsive to autologous tumour *ex vivo*. However, both cytolytic and IFN-γ-producing responses could be restored upon stimulation with DCs that had processed fresh autologous tumour cells. In chapter 4, marrow infiltrating lymphocytes (MILs) from bone marrow aspirates derived from two stage III patients were potently redirected in a concentration-dependent manner by ImmTAC-MAGE against a HLA-A*0101-restricted allogeneic myeloma tumour cell line, EJM (Figure 4-10). The potency exhibited by the redirected MILs in an IFN-γ ELISpot assay was comparable to that achieved using peripheral T cells from healthy donors, producing EC_{50} values in the range of 100 pM or lower. Furthermore, ImmTAC-MAGE redirected MILs did not respond in the presence of targets presenting the correct HLA but negative for the relevant antigen. Therefore, MILs from the myeloma microenvironment contain T cells which appear fully functional and can be effectively redirected by ImmTAC-MAGE. This is a crucial

finding since these T cells are likely to be the initial pool of myeloma target proximal effectors engaged by ImmTAC if used in multiple myeloma patients.

7.5 The relationship between ImmTAC *in vitro* cellular assays and clinical results

IMCgp100 has received Clinical Trials Application (CTA) approval by the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK, and regulatory and ethics approval by the Food and Drug Administration (FDA) in the US.

In the UK, the MHRA has approved a Phase I dose finding study to assess safety and tolerability in HLA-A*0201^{+} patients with advanced malignant melanoma. The primary aim of the trial is to establish the *maximum tolerated dose* (MTD). ImmTACs are entirely human specific, that is both with respect to the TCR-pHLA and the scFv-CD3 interactions, meaning that no suitable animal model exists in which to test the toxicity of IMCgp100. The regulatory authorities endorsed that the clinical trial with IMCgp100 could proceed solely on the support of *in vitro* toxicity data. A comprehensive pre-clinical toxicity data package was produced and was used to set the *target limiting dose* (TLD); the key data sets from these studies are presented in Chapter 6. The starting dose for the Phase I trial was set by establishing the *minimal anticipated biological effect level* (MABEL), predicted from the *in vitro* efficacy (potency) assays, and also described in Chapter 6.

Gp100, the target antigen of IMCgp100, is not only expressed in melanoma tumours but also in some normal tissues. Gp100 expression has been demonstrated in melanocytes, the retina, the substantia nigra (Wagner et al., 1997), and the thymus (Takase et al., 2005) Also, the iris, the inner ear and the choroid plexus of the brain are known to contain melanocytes but currently no evidence has been reported of gp100 expression in these tissues. However, adverse events related to toxicity against normal

tissue have been reported in melanoma trials using other immune-based therapies. For example, melanoma trials using infusions of melanoma/melanocyte-specific $CD8^+$ T cells, *ex vivo* expanded from the patients' own TILs (and of unknown specificity), have reported cases of eye inflammation and hearing loss in some patients (Dudley et al., 2005; Palmer et al., 2008; Yeh et al., 2009), which are most likely caused by the attack of pigmented cells of the retina and melanocytes in the inner eye. Vitiligo is also a common manifestation with many melanoma immunotherapies. Therefore, the expression of gp100 on normal tissue was expected to be a potential source of IMCgp100 toxicity. In the *in vitro* cellular activation assays there is evidence of IMCgp100 redirected activity against epidermal skin derived melanocytes which are HLA-A*0201^+ and known to express gp100 (Figure 6-1). However, in all cases the potency of the response against the melanocytes is weaker than that elicited against the melanoma tumour cell lines. To activate T cells, ImmTACs must bind above a threshold number of cognate antigens. In the case of IMCgp100 the threshold number is thought to be approximately 10 copies per cell (as described in section 7.1). Below this threshold number of cognate antigens, activation of bystander T cells is not detectable. Above this level, T cell activation increases proportionally with the level of antigen presentation on the target cell. Thus, T cell activation via the anti-CD3 scFv arm of the ImmTAC reagent in all likelihood involves an avidity effect. This mechanism of action has been demonstrated for IMCgp100 in experiments (conducted by Dr. Giovanna Bossi) using T2 cells loaded with varying concentrations of gp100 peptide. The level of antigen presentation was then quantified by incubating the loaded T2 cells with fluorescently labelled high affinity gp100 mTCR and counting the number of bound TCRs under the microscope. T cell activation assays using T2 cells, peptide loaded with the same concentrations as used for epitope counting experiments, showed

that the level of IMCgp100 redirected activity was directly proportional to the level of antigen presentation. Therefore, the lower potency observed for IMCgp100 redirected activity against normal melanocytes compared to the potency seen against melanoma cells implies that the number of epitopes on the surface of melanocytes is less than that on the surface of melanoma cells. Importantly, lower expression of gp100 on normal melanocytes means that, all other conditions being equal, a higher concentration of IMCgp100 would be required to trigger T cell activation than would be needed to target and kill melanoma cells. In clinical use, this may translate into a therapeutic window. However, the environment in and around the tumour sites are likely to be more suppressive for T cell activation. Thus, firm predictions about how IMCgp100 redirected T cell reactivity against melanoma and normal melanocytes will compare are not possible purely based on *in vitro* data.

Gp100 is also known to be expressed in certain areas of the brain, most prominently, in the substantia nigra (Wagner et al., 1997). IMCgp100 is too large a molecule to cross the blood brain barrier passively. Therefore, on-target brain toxicity is not expected in the majority of patients where the blood brain barrier has not been compromised. Furthermore, the brain is considered to be an immune privileged organ with low levels or no expression of MHC class I molecules in neurons, glia, oligodendrocytes, microglia or astrocytes (Lampson & Hickey, 1986). Primary cells derived from the substantia nigra or the choroid plexus could not be obtained for experimentation, so astrocytes were selected to investigate potential IMCgp100 on-target activity against brain tissue. Astrocytes are the most abundant cell type in the central nervous system (CNS). They perform a variety of roles including regulating neuronal signalling, maintaining homeostasis at neuronal synapses, and regulating cerebral blood delivery (Figley & Stroman, 2011). Furthermore, astrocytes can form

glioblastoma multiform (GBM) tumours which have been shown to express gp100 (Saikali et al., 2007). No IMCgp100 redirected activity was observed in four of the astrocyte lots tested. However, in one out of the five lots of astrocytes IMCgp100 reactivity was detected (Figure 6-2a). Furthermore, IMCgp100 redirected activity could be abrogated using high affinity gp100-specific mTCR (Figure 6-2b) indicating that some gp100-specific reactivity is occurring. However, as observed for normal melanocytes, reactivity to this particular lot of astrocytes was only seen in the presence of high concentrations of IMCgp100 (1 nM and above), suggesting that the cause of the activity is extremely low presentation of the HLA-A*0201 gp100 epitope. An uncompromised blood brain barrier (BBB) will preclude the entry of IMCgp100 to brain tissue, and therefore the risk of toxicity is considered to be very low. Even so, patients with an overtly compromised BBB, for example, patients that have developed brain metastases, are currently excluded from the trial.

Adverse events due to on-target activity in melanoma trials have largely been tolerable and controllable with the use of steroids (Dudley et al., 2005; Palmer et al., 2008; Yeh et al., 2009). Here, it has been shown that the steroid hydrocortisone inhibits IMCgp100 redirected T cell activity at clinically relevant concentrations (Figure 6-3). The highest test concentration of hydrocortisone (25 μM) used in the *in vitro* assay is equivalent to the maximum plasma concentration (Cmax) that may be expected upon administration of a bolus IV injection of 100 mg of hydrocortisone. Thus, the data indicates that, if toxicities associated with on-target activity are observed with IMCgp100, then they could be managed with the administration of steroids.

Cross-reactivity of IMCgp100 is defined as the binding of the drug to a non-target epitope and the simultaneous triggering of its effector function, i.e. the activation of T cells. There is no toxicologically relevant animal species in which to test IMCgp100 due to a lack of interspecies specificity. Therefore, *in vitro* functional cross-reactivity studies were conducted on human primary tissue to assess the potential toxicological profile of IMCgp100. The panel of human primary cells selected to screen for IMCgp100 cross-reactivity represented all the major organs of the body. In total, eleven HLA-A2^{+}/gp100^{-} and four HLA-A2^{-}/gp100^{-} primary cell lots were evaluated (Table 6-1). No IMCgp100 redirected activity was observed against any of the primary cell lots, except at concentrations of 10 nM and above where a slight HLA-A*0201^{+} dependent loss of peptide specificity is observed. These data suggest that at concentrations of approximately 10 nM, IMCgp100 elicits some loss of specificity by activating T cells in response to HLA-A*0201^{+}cells, but not to HLA-A*0201^{-} cells, presenting other, unknown peptides. Thus, at concentrations up to 1 nM, IMCgp100 is highly specific for its HLA-A*0201^{+}/gp100^{+} target which is 1,000-fold higher than the lowest concentration required for observing anti-tumour cell reactivity. This suggests that organ specific cytotoxicity due to promiscuous cross-reactivity is unlikely to occur when IMCgp100 is administered to patients. However, the study, inevitably, was not exhaustive and was limited by the availability of primary cells.

Another potential source of IMCgp100 cross-reactivity is to cells in systemic circulation. Cytokine release syndrome (CRS) or cytokine storm is an adverse event resulting in the systemic release of cytokines and is sometimes seen upon the first exposure to a therapeutic monoclonal antibody (mAb). CRS can develop within 1-2 hours after infusion and symptoms range from mild to severe, including fatigue, headache, bronchospasm, swelling of the throat, nausea, vomiting, fever, chills,

hypotension, tachycardia and asthenia (Common Terminology Criteria for Adverse Events (CTCAE) version 4.03). CRS is a prominent feature associated with therapeutic mAbs to CD2, CD3, CD20, CD40, CD52, IL-2R, and, by far most dramatically, superagonist (SA) CD28 (M. Walker et al., 2010). The mechanism by which CRS occurs is not fully understood but three principal mechanisms have been proposed. First, the direct binding of mAb to its ligand on the target cell triggers signalling and causes cytokine release by the target cells. Second, binding of the Fc region of the mAb to Fc receptors on non-target cells triggers signalling and causes cytokine release by non-target cells. Third, binding to Fc receptor causes clustering and signalling through the target cell (Bugelski et al., 2009). However, it is likely that some therapeutic mAbs elicit CRS through a combination of these mechanisms. It is thought that anti-CD28 SA mAbs cause CRS primarily through the first mechanism.

While most CRS reactions can be controlled by slowing the infusion rate or by the administration of anti-inflammatory drugs, reactions to an anti-CD28 mAb, TGN1412, constitute a warning of how severe toxicity reactions to immunotherapies can potentially be. In 2006, life-threatening cytokine-release syndrome was triggered in all six healthy volunteers enrolled on a Phase I clinical trial following administration of the CD28 superagonist mAb TGN1412. The clinical events that followed were rapid and severe and can be divided into four phases. The first phase began within 90 minutes of the volunteers receiving an intravenous infusion of the drug, provoking a systemic inflammatory response characterised by a rapid induction of pro-inflammatory cytokines. High cytokine levels in the blood were accompanied by headache, myalgias, nausea, diarrhoea, erthema, vasodilation and hypotension. In the second phase, which occurred within 12 to 16 hours after infusion, the volunteers became critically ill with pulmonary infiltrates and lung injury, renal failure and disseminated intra-vascular

coagulation. The third phase was characterised by a severe depletion of lymphocytes and monocytes and occurred within 24 hours of the infusion. In the fourth phase, the volunteers developed prolonged cardiovascular shock and acute respiratory distress syndrome (Suntharalingam et al., 2006). Fortunately, despite the severity of the events, all six volunteers survived. However, in light of the comprehensive pre-clinical testing which involved human whole blood assays and *in vivo* primate studies, the adverse events of the TGN1412 incident were unexpected. This highlighted the differences that can be encountered in the effects of immunomodulatory agents when tested in *in vitro* and in *in vivo* model systems as opposed to when administered to humans. The European Medicines Agency (EMA) responded immediately by changing the methodology for calculating the starting dose of first-in-human biologicals (discussed in more detail below). However, it took six years to understand why the pre-clinical data, from three test systems (rodents, primates and human cells), used to support the TGN1412 Phase I trial had failed to predict the cytokine storm.

CD28 is a costimulatory molecule present on the cell surface of T cells. Engagement of its ligands CD80 and CD86, on the surface of professional APCs, costimulates T cell responses during antigen recognition. TGN1412 and other anti-CD28 SA mAb trigger particularly strong CD28 signalling, allowing the activation of T cells without simultaneous engagement of the T cell receptor (TCR). Even so, anti-CD28 SA mAbs depend on sub-threshold or 'tonic' TCR signals, which they amplify (Dennehy et al., 2007; Levin et al., 2008). In both rats and mice, anti-CD28 SA mAbs predominantly induce the activation of regulatory T (Treg) cells and have been used to treat experimental autoimmune disease (Beyersdorf et al., 2005; Hunig, 2007). The adverse events, as seen in the human volunteers on the TGN1412 trial, were absent in the rodent model systems. However, a recent study has shown that if mice are first

depleted of Tregs, high levels of circulating pro-inflammatory cytokines are observed upon administration of anti-CD28 SA mAb. Thus, the Treg cell-mediated response appears to be crucial for preventing systemic inflammation in rodents (Gogishvili et al., 2009). So, why did this mechanism fail to protect the human volunteers receiving TGN1412? $CD4^+$ effector memory T (Tem) cells have been identified as the source of the cytokines interferon-γ (IFN-γ), tumour necrosis factor (TNF) and interleukin-2 (IL-2) that mediated the CRS seen in the volunteers (Eastwood et al., 2010; Romer et al., 2011). Multiple exposures to infectious agents during the life of an individual lead to the accumulation of Tem cells. However, this does not occur in laboratory rodents housed under clean conditions. Thus, in the presence of TGN1412, the balance between Treg cell to Tem cell numbers is less favourable in humans compared with mice or rats. Moreover, it is likely that the very high dose of TGN1412 used in the trial exacerbated the problem further as studies in rats have shown that low doses of anti-CD28 SA mAb selectively expand Treg ($CD4^+/CD25^+$) cell populations and do not activate $CD4^+/CD25^-$ ('conventional') T cells (Beyersdorf et al., 2005).

In pre-clinical studies in primates, TGN1412 had been administered to cynomolgus macaques at up to 500 times the dose administered to the human volunteers. Cynomolgus macaques were thought to be an appropriate species for the pre-clinical safety testing of TGN1412 because the amino acid sequences of the extracellular and intracellular domains of cynomolgus macaque CD28 are 100% identical to the human molecule. Furthermore, they both bind TGN1412 with the same affinity (Hanke, 2006). So, why was there no TGN1412-induced CRS in macaques? Unfortunately, what had been overlooked, and has only recently come to light, is that macaque $CD4^+$ T cells lose CD28 expression during their differentiation into Tem cells, whereas human $CD4^+$ T cells do not (Eastwood et al., 2010).

Finally, pre-clinical studies using human peripheral blood mononuclear cells (PBMC) also failed to predict the TGN1412-mediated CRS. When TGN1412 is added in soluble form to PBMC cultures, it does not trigger cytokine or proliferative responses. However, based on a serendipitous discovery, it has since been demonstrated that if T cells are first cultured at high density, TGN1412 can then potently activate these T cells (Romer et al., 2011). It has been proposed that cellular contacts during the high-density pre-culture of PBMC restores the sub-threshold or 'tonic' TCR signal that TGN1412 requires as a 'substrate' for amplification. In contrast, T cells in circulation lose this cell-cell contact dependent sub-threshold signal, and are therefore less responsive. Furthermore, there is evidence to suggest that it was the tissue resident $CD4^+$ Tem T cells that responded immediately to TGN1412 with cytokine release rather than circulating T cells. Pre-culturing PBMC at high density mimics more closely tissue-like conditions and has allowed more detailed *in vitro* investigations of T cell responses to soluble TGN1412 (Romer et al., 2011). An important finding from these studies was that a TGN1412 concentration of 1 μg per ml of blood – a level predicted to be equivalent to the 0.1 mg per kg of body weight dose of TGN1412 administered to the volunteers on the Phase I trial (Duff, 2006) - was not only close to saturation with regard to receptor occupancy but also exceeded the amount required for maximum cytokine release (Romer et al., 2011).

All three pre-clinical test systems failed to provide evidence for the toxic potential of TGN1412 for the distinct and unrelated reasons described above. Notably, it highlighted the limitations of *no observed adverse effect level* (NOAEL)-based calculation, which is the conventional approach for determining first-in-human (FIH) starting doses. NOAEL represents the highest dose of a drug that yields no measurable toxicological effects or adverse events. To calculate FIH starting dose, the NOAEL is

divided by a conversion factor taking into account the body surface area and an additional safety factor (usually ten) is also applied. However, if the animal model failed to respond for some unforeseen reason (in the case of TGN1412, the lack of CD28 expression by macaque $CD4^+$ Tem cells), the requested dose escalation leads to a very high tolerated level (for TGN1412, 50 mg per kg) from which the human starting dose was calculated.

In response to the TGN1412 incident, a guideline was produced by the Committee For Medicinal Products (CHMP) of the European Medicines Agency (EMEA) (EMEA/CHMP/SWP/28367/2007) to identify and decrease the risk with new medicinal products being studied in FIH clinical trials. The guideline highlights the importance of considering *minimal anticipated biological effect level* (MABEL) in deciding the initial dose of a biologic to be used in humans. The MABEL approach selects the starting dose for a FIH study on the basis of the lowest animal dose or concentration required to produce pharmacological activity *in vivo* and/or *in vitro* data in animal/human systems. If TGN1412 had been administered using MABEL-based calculations, the entry dose would have been at least 200-fold lower (Hunig, 2012).

In this thesis, the potential for IMCgp100 to trigger CRS was examined in an *ex vivo* whole blood assay, under physiological conditions. The release of five main cytokines associated with CRS (IL-1-β, TNF-α, IL-6, IFN-γ and IL-2) were measured in the presence of varying concentrations of IMCgp100 (Figure 6-5). No cytokine release was observed up to and including 1 nM IMCgp100. At concentrations above this level (10 and 100 nM) some cytokine release is observed, specifically TNF-α, IL-6 and IFN-γ, although not from all the donors tested. Furthermore, when IMCgp100-mediated cytokine release is seen it is of a much lower magnitude to that observed with the positive control immune stimulatory mAb UCHTI. Although UCHT1 is the

monoclonal antibody from which the anti-CD3 scFv of IMCgp100 is derived, the important distinction between them is that like all antibodies UCHTI is bivalent whereas the scFv component of IMCgp100 is monovalent, and lacks a Fc region. While mAb UCHT1 can cross-link and activate T cells by engaging CD3, the anti-CD3 scFv is incapable of cross-linking in the absence of target. Monovalent binding by the anti-CD3 scFv portion of IMCgp100 and the absence of Fc means that the risk of CRS upon first exposure to IMCgp100 is likely to be minimal. The observation that IMCgp100 used at concentrations above 1 nM can elicit some cytokine release in the whole blood assay is not unexpected. Indeed, non-specific activity at concentrations above 1 nM IMCgp100 has been observed in *in vitro* cellular assays with control cell lines and primary cells that do not express gp100 (as described above). The concentration of IMCgp100 beyond which a loss of specificity is observed defines the *target limiting dose* (TLD) i.e. the maximum dose of IMCgp100 to be administered to patients (Figure 6-7). Based on the *in vitro* killing and the functional cross-reactivity assay data, the TLD for IMCgp100 has been set at 1 nM. As there is no pharmacokinetic data in humans, the Cmax of IMCgp100 in treated patients will be measured throughout the dose escalation and the PK modelled so that the dose of IMCgp100 will not exceed 1 nM until more is known about its safety profile. The primary aim of the Phase I trial is to establish the maximum tolerated dose of IMCgp100, therefore, dose escalation will continue until a *maximum tolerated dose* (MTD) or the TLD is reached.

In the absence of a suitable toxicology species, it was not possible to set a starting dose for IMCgp100 using the conventional NOAEL method. In light of new recommendations by the *Expert Scientific Group on Phase One Clinical Trials* (EMEA/CHMP/SWP/28367/2007), the MABEL was determined solely from *in vitro* cellular assays in order to set a safe starting dose for a first administration of IMCgp100

in humans. This approach relies on the assumption that *in vitro* cellular data can predict what may occur in patients in the clinic. *In vitro* efficacy assays were used to set the MABEL for IMCgp100 using a clinically representative melanoma target cell line. The LDH-release killing assay was deemed to be the most sensitive assay in terms of cellular EC_{50} values compared to activation assays such as IFN-γ ELISpot. The lowest concentration of IMCgp100 that caused measureable redirected lysis of the HLA-A*0201^{+} melanoma target cell line, Mel526, was 1 pM or 76 pg/ml (Figure 6-6).

Based on additional PK data generated in C57Black/6 mice and PK modelling studies, the starting dose of IMCgp100 for FIH clinical trial was proposed to be set at 5 ng/Kg, administered as a 4 hour intravenous infusion, in order to generate a Cmax below the MABEL level of 1 pM. The proposed starting dose and treatment duration was accepted by the regulatory authorities.

Crucially, no adverse events were recorded for patients administered at the first two doses (5 ng/Kg and 15 ng/Kg) for IMCgp100. Therefore, it can be concluded that for a bispecific mTCR-anti-CD3 scFv fusion protein which targets CD3, *in vitro* efficacy assay data can be used to set a safe starting dose for a FIH clinical trial. Furthermore, it appears that the same data sets can predict the dose at which *in vivo* biological responses may occur. Gp100, the target antigen for IMCgp100, is a melanosomal antigen which is also expressed in the skin. Notably, a generalised skin rash has been reported in patients treated with IMCgp100 which is probably the result of the drug targeting the skin.

Concluding remarks

ImmTACs represent a new class of bispecific therapeutics, for the first time enabling the targeting of cancer cells through pMHC, which constitutes a much larger and more

diverse target pool than the cell surface proteins available to antibody targeting. The work described in this thesis has demonstrated the *in vitro* potency of ImmTACs against tumour cells, showing that a full range of T cell responses, including cytokine release and cytotoxicity, can be induced by redirecting non-cancer specific T cells to cancer cells through ImmTAC cross-linking. The work described has also formed the basis for determining a safe starting dose for a first-in-human clinical trial and has therefore contributed to establish a path to the clinic for further ImmTAC molecules in the future. Hopefully, these will become valuable tools in the fight against many forms of cancer, where immunotherapies appear to hold the best promise for more efficacious and safe treatments.

REFERENCES

Adams, K. J. (2004). *The isolation of T cells specific for the melanoma-associated-antigen MART-1 (M.Sc thesis).* University of the West of England, Bristol.

Aggarwal, S., & Pittenger, M. F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood, 105*(4), 1815-1822.

Alarcon, B., Mestre, D., & Martinez-Martin, N. (2011). The immunological synapse: a cause or consequence of T-cell receptor triggering? *Immunology, 133*(4), 420-425.

Aleksic, M., Liddy, N., Molloy, P. E., Pumphrey, N., Vuidepot, A., Chang, K. M., et al. (2012). Different affinity windows for virus and cancer-specific T-cell receptors: Implications for therapeutic strategies. *Eur J Immunol, 42*(12), 3174-3179.

Alimonti, J., Zhang, Q. J., Gabathuler, R., Reid, G., Chen, S. S., & Jefferies, W. A. (2000). TAP expression provides a general method for improving the recognition of malignant cells in vivo. *Nat Biotechnol, 18*(5), 515-520.

Alimonti, J. B., Shi, L., Baijal, P. K., & Greenberg, A. H. (2001). Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. *J Biol Chem, 276*(10), 6974-6982.

Anderson, G. R., Stoler, D. L., & Brenner, B. M. (2001). Cancer: the evolved consequence of a destabilized genome. *Bioessays, 23*(11), 1037-1046.

Andrade, V. C., Vettore, A. L., Felix, R. S., Almeida, M. S., Carvalho, F., Oliveira, J. S., et al. (2008). Prognostic impact of cancer/testis antigen expression in advanced stage multiple myeloma patients. *Cancer Immun, 8*, 2.

Antony, P. A., Piccirillo, C. A., Akpinarli, A., Finkelstein, S. E., Speiss, P. J., Surman, D. R., et al. (2005). CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol, 174*(5), 2591-2601.

Arstila, T. P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., & Kourilsky, P. (1999). A direct estimate of the human alphabeta T cell receptor diversity. *Science, 286*(5441), 958-961.

Ashkenazi, A., & Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science, 281*(5381), 1305-1308.

Atanackovic, D., Blum, I., Cao, Y., Wenzel, S., Bartels, K., Faltz, C., et al. (2006). Expression of cancer-testis antigens as possible targets for antigen-specific immunotherapy in head and neck squamous cell carcinoma. *Cancer Biol Ther, 5*(9), 1218-1225.

Atanackovic, D., Hildebrandt, Y., Jadczak, A., Cao, Y., Luetkens, T., Meyer, S., et al. (2010). Cancer-testis antigens MAGE-C1/CT7 and MAGE-A3 promote the survival of multiple myeloma cells. *Haematologica, 95*(5), 785-793.

Baccelli, I., & Trumpp, A. (2012). The evolving concept of cancer and metastasis stem cells. *J Cell Biol, 198*(3), 281-293.

Baeuerle, P. A., Kufer, P., & Lutterbuse, R. (2003). Bispecific antibodies for polyclonal T-cell engagement. *Curr Opin Mol Ther, 5*(4), 413-419.

Baeuerle, P. A., & Reinhardt, C. (2009). Bispecific T-cell engaging antibodies for cancer therapy. *Cancer Res, 69*(12), 4941-4944.

Bakker, A. B., Schreurs, M. W., de Boer, A. J., Kawakami, Y., Rosenberg, S. A., Adema, G. J., et al. (1994). Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J Exp Med, 179*(3), 1005-1009.

Baldo, M., Schiavon, M., Cicogna, P. A., Boccato, P., & Mazzoleni, F. (1992). Spontaneous regression of subcutaneous metastasis of cutaneous melanoma. *Plast Reconstr Surg, 90*(6), 1073-1076.

Bargou, R., Leo, E., Zugmaier, G., Klinger, M., Goebeler, M., Knop, S., et al. (2008). Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. *Science, 321*(5891), 974-977.

Barnden, M. J., Purcell, A. W., Gorman, J. J., & McCluskey, J. (2000). Tapasin-mediated retention and optimization of peptide ligands during the assembly of class I molecules. *J Immunol, 165*(1), 322-330.

Barrow, C., Browning, J., MacGregor, D., Davis, I. D., Sturrock, S., Jungbluth, A. A., et al. (2006). Tumor antigen expression in melanoma varies according to antigen and stage. *Clin Cancer Res, 12*(3 Pt 1), 764-771.

Barry, M., & Bleackley, R. C. (2002). Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol, 2*(6), 401-409.

Barry, M., Heibein, J. A., Pinkoski, M. J., Lee, S. F., Moyer, R. W., Green, D. R., et al. (2000). Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol Cell Biol, 20*(11), 3781-3794.

Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., & Heath, W. R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature, 393*(6684), 478-480.

Bensinger, W. I., Buckner, C. D., Anasetti, C., Clift, R., Storb, R., Barnett, T., et al. (1996). Allogeneic marrow transplantation for multiple myeloma: an analysis of risk factors on outcome. *Blood, 88*(7), 2787-2793.

Bentley, G. A., Boulot, G., Karjalainen, K., & Mariuzza, R. A. (1995). Crystal structure of the beta chain of a T cell antigen receptor. *Science, 267*(5206), 1984-1987.

Beyersdorf, N., Gaupp, S., Balbach, K., Schmidt, J., Toyka, K. V., Lin, C. H., et al. (2005). Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. *J Exp Med, 202*(3), 445-455.

Bicknell, D. C., Rowan, A., & Bodmer, W. F. (1994). Beta 2-microglobulin gene mutations: a study of established colorectal cell lines and fresh tumors. *Proc Natl Acad Sci U S A, 91*(11), 4751-4755.

Bioley, G., Dousset, C., Yeh, A., Dupont, B., Bhardwaj, N., Mears, G., et al. (2009). Vaccination with recombinant NY-ESO-1 protein elicits immunodominant HLA-DR52b-restricted CD4+ T cell responses with a conserved T cell receptor repertoire. *Clin Cancer Res, 15*(13), 4467-4474.

Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., & Wiley, D. C. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature, 329*(6139), 506-512.

Blade, J., Rosinol, L., Cibeira, M. T., Rovira, M., & Carreras, E. (2010). Hematopoietic stem cell transplantation for multiple myeloma beyond 2010. *Blood, 115*(18), 3655-3663.

Bluemel, C., Hausmann, S., Fluhr, P., Sriskandarajah, M., Stallcup, W. B., Baeuerle, P. A., et al. (2010). Epitope distance to the target cell membrane and antigen size determine the potency of T cell-mediated lysis by BiTE antibodies specific for a large melanoma surface antigen. *Cancer Immunol Immunother, 59*(8), 1197-1209.

Bogen, B. (1996). Peripheral T cell tolerance as a tumor escape mechanism: deletion of CD4+ T cells specific for a monoclonal immunoglobulin idiotype secreted by a plasmacytoma. *Eur J Immunol, 26*(11), 2671-2679.

Boon, T., Coulie, P. G., Van den Eynde, B. J., & van der Bruggen, P. (2006). Human T cell responses against melanoma. *Annu Rev Immunol, 24*, 175-208.

Bortoletto, N., Scotet, E., Myamoto, Y., D'Oro, U., & Lanzavecchia, A. (2002). Optimizing anti-CD3 affinity for effective T cell targeting against tumor cells. *Eur J Immunol, 32*(11), 3102-3107.

Boulter, J. M., Glick, M., Todorov, P. T., Baston, E., Sami, M., Rizkallah, P., et al. (2003). Stable, soluble T-cell receptor molecules for crystallization and therapeutics. *Protein Eng, 16*(9), 707-711.

Braden, B. C., Goldman, E. R., Mariuzza, R. A., & Poljak, R. J. (1998). Anatomy of an antibody molecule: structure, kinetics, thermodynamics and mutational studies of the antilysozyme antibody D1.3. *Immunol Rev, 163*, 45-57.

Brasseur, F., Rimoldi, D., Lienard, D., Lethe, B., Carrel, S., Arienti, F., et al. (1995). Expression of MAGE genes in primary and metastatic cutaneous melanoma. *Int J Cancer, 63*(3), 375-380.

Brunner, K. T., Mauel, J., Cerottini, J. C., & Chapuis, B. (1968). Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology, 14*(2), 181-196.

Buell, J. F., Gross, T. G., & Woodle, E. S. (2005). Malignancy after transplantation. *Transplantation, 80*(2 Suppl), S254-264.

Bugelski, P. J., Achuthanandam, R., Capocasale, R. J., Treacy, G., & Bouman-Thio, E. (2009). Monoclonal antibody-induced cytokine-release syndrome. *Expert Rev Clin Immunol, 5*(5), 499-521.

Bulkley, G. B., Cohen, M. H., Banks, P. M., Char, D. H., & Ketcham, A. S. (1975). Long-term spontaneous regression of malignant melanoma with visceral metastases. Report of a case with immunologic profile. *Cancer, 36*(2), 485-494.

Caballero, O. L., & Chen, Y. T. (2009). Cancer/testis (CT) antigens: potential targets for immunotherapy. *Cancer Sci, 100*(11), 2014-2021.

Cabrera, C. M., Jimenez, P., Cabrera, T., Esparza, C., Ruiz-Cabello, F., & Garrido, F. (2003). Total loss of MHC class I in colorectal tumors can be explained by two molecular pathways: beta2-microglobulin inactivation in MSI-positive tumors and LMP7/TAP2 downregulation in MSI-negative tumors. *Tissue Antigens, 61*(3), 211-219.

Campi, G., Varma, R., & Dustin, M. L. (2005). Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med, 202*(8), 1031-1036.

Carter, P. (2001). Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer, 1*(2), 118-129.

Celis, E., Tsai, V., Crimi, C., DeMars, R., Wentworth, P. A., Chesnut, R. W., et al. (1994). Induction of anti-tumor cytotoxic T lymphocytes in normal humans using

primary cultures and synthetic peptide epitopes. *Proc Natl Acad Sci U S A, 91*(6), 2105-2109.

Celluzzi, C. M., Mayordomo, J. I., Storkus, W. J., Lotze, M. T., & Falo, L. D., Jr. (1996). Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. *J Exp Med, 183*(1), 283-287.

Chai, J. G., Vendetti, S., Bartok, I., Schoendorf, D., Takacs, K., Elliott, J., et al. (1999). Critical role of costimulation in the activation of naive antigen-specific TCR transgenic CD8+ T cells in vitro. *J Immunol, 163*(3), 1298-1305.

Chattopadhyay, P. K., Betts, M. R., Price, D. A., Gostick, E., Horton, H., Roederer, M., et al. (2009). The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. *J Leukoc Biol, 85*(1), 88-97.

Chaudhuri, D., Suriano, R., Mittelman, A., & Tiwari, R. K. (2009). Targeting the immune system in cancer. *Curr Pharm Biotechnol, 10*(2), 166-184.

Chen, J. L., Dunbar, P. R., Gileadi, U., Jager, E., Gnjatic, S., Nagata, Y., et al. (2000). Identification of NY-ESO-1 peptide analogues capable of improved stimulation of tumor-reactive CTL. *J Immunol, 165*(2), 948-955.

Chen, Y. T., Scanlan, M. J., Sahin, U., Tureci, O., Gure, A. O., Tsang, S., et al. (1997). A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A, 94*(5), 1914-1918.

Chi, D. D., Merchant, R. E., Rand, R., Conrad, A. J., Garrison, D., Turner, R., et al. (1997). Molecular detection of tumor-associated antigens shared by human cutaneous melanomas and gliomas. *Am J Pathol, 150*(6), 2143-2152.

Chicheportiche, Y., Bourdon, P. R., Xu, H., Hsu, Y. M., Scott, H., Hession, C., et al. (1997). TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J Biol Chem, 272*(51), 32401-32410.

Chothia, C., Boswell, D. R., & Lesk, A. M. (1988). The outline structure of the T-cell alpha beta receptor. *EMBO J, 7*(12), 3745-3755.

Choudhuri, K., Parker, M., Milicic, A., Cole, D. K., Shaw, M. K., Sewell, A. K., et al. (2009). Peptide-major histocompatibility complex dimensions control proximal kinase-phosphatase balance during T cell activation. *J Biol Chem, 284*(38), 26096-26105.

Choudhuri, K., Wiseman, D., Brown, M. H., Gould, K., & van der Merwe, P. A. (2005). T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature, 436*(7050), 578-582.

Chowdhury, D., & Lieberman, J. (2008). Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol, 26*, 389-420.

Chowdhury, I., Tharakan, B., & Bhat, G. K. (2008). Caspases - an update. *Comp Biochem Physiol B Biochem Mol Biol, 151*(1), 10-27.

Cole, D. K., Pumphrey, N. J., Boulter, J. M., Sami, M., Bell, J. I., Gostick, E., et al. (2007). Human TCR-binding affinity is governed by MHC class restriction. *J Immunol, 178*(9), 5727-5734.

Cormier, J. N., Hijazi, Y. M., Abati, A., Fetsch, P., Bettinotti, M., Steinberg, S. M., et al. (1998). Heterogeneous expression of melanoma-associated antigens and HLA-A2 in metastatic melanoma in vivo. *Int J Cancer, 75*(4), 517-524.

Corradini, P., Voena, C., Tarella, C., Astolfi, M., Ladetto, M., Palumbo, A., et al. (1999). Molecular and clinical remissions in multiple myeloma: role of autologous and allogeneic transplantation of hematopoietic cells. *J Clin Oncol, 17*(1), 208-215.

Coulie, P. G., Brichard, V., Van Pel, A., Wolfel, T., Schneider, J., Traversari, C., et al. (1994). A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med, 180*(1), 35-42.

Cox, A. L., Skipper, J., Chen, Y., Henderson, R. A., Darrow, T. L., Shabanowitz, J., et al. (1994). Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science, 264*(5159), 716-719.

Croce, C. M. (2008). Oncogenes and cancer. *N Engl J Med, 358*(5), 502-511.

Cuenca, A., Cheng, F., Wang, H., Brayer, J., Horna, P., Gu, L., et al. (2003). Extra-lymphatic solid tumor growth is not immunologically ignored and results in early

induction of antigen-specific T-cell anergy: dominant role of cross-tolerance to tumor antigens. *Cancer Res, 63*(24), 9007-9015.

Cullen, S. P., & Martin, S. J. (2008). Mechanisms of granule-dependent killing. *Cell Death Differ, 15*(2), 251-262.

Curiel, T. J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., et al. (2004). Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med, 10*(9), 942-949.

Curtsinger, J. M., Lins, D. C., Johnson, C. M., & Mescher, M. F. (2005). Signal 3 tolerant CD8 T cells degranulate in response to antigen but lack granzyme B to mediate cytolysis. *J Immunol, 175*(7), 4392-4399.

Davis, D. M., & Dustin, M. L. (2004). What is the importance of the immunological synapse? *Trends Immunol, 25*(6), 323-327.

Davis, M. M., & Bjorkman, P. J. (1988). T-cell antigen receptor genes and T-cell recognition. *Nature, 334*(6181), 395-402.

Davis, M. M., Boniface, J. J., Reich, Z., Lyons, D., Hampl, J., Arden, B., et al. (1998). Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol, 16*, 523-544.

Davis, S. J., & van der Merwe, P. A. (2006). The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol, 7*(8), 803-809.

de Carvalho, F., Vettore, A. L., Inaoka, R. J., Karia, B., Andrade, V. C., Gnjatic, S., et al. (2011). Evaluation of LAGE-1 and NY-ESO-1 expression in multiple myeloma patients to explore possible benefits of their homology for immunotherapy. *Cancer Immun, 11*, 1.

de Vries, J. F., von dem Borne, P. A., van Luxemburg-Heijs, S. A., Heemskerk, M. H., Willemze, R., Falkenburg, J. H., et al. (2007). Differential activation of the death receptor pathway in human target cells induced by cytotoxic T lymphocytes showing different kinetics of killing. *Haematologica, 92*(12), 1671-1678.

Decker, T., & Lohmann-Matthes, M. L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods, 115*(1), 61-69.

Deeken, J. F., & Loscher, W. (2007). The blood-brain barrier and cancer: transporters, treatment, and Trojan horses. *Clin Cancer Res, 13*(6), 1663-1674.

Dennehy, K. M., Elias, F., Na, S. Y., Fischer, K. D., Hunig, T., & Luhder, F. (2007). Mitogenic CD28 signals require the exchange factor Vav1 to enhance TCR signaling at the SLP-76-Vav-Itk signalosome. *J Immunol, 178*(3), 1363-1371.

Dhodapkar, M. V., Krasovsky, J., & Olson, K. (2002). T cells from the tumor microenvironment of patients with progressive myeloma can generate strong, tumor-specific cytolytic responses to autologous, tumor-loaded dendritic cells. *Proc Natl Acad Sci U S A, 99*(20), 13009-13013.

Dimopoulos, M. A., & Terpos, E. (2010). Multiple myeloma. *Ann Oncol, 21 Suppl 7*, vii143-150.

Drake, C. G., Jaffee, E., & Pardoll, D. M. (2006). Mechanisms of immune evasion by tumors. *Adv Immunol, 90*, 51-81.

Dreier, T., Lorenczewski, G., Brandl, C., Hoffmann, P., Syring, U., Hanakam, F., et al. (2002). Extremely potent, rapid and costimulation-independent cytotoxic T-cell response against lymphoma cells catalyzed by a single-chain bispecific antibody. *Int J Cancer, 100*(6), 690-697.

Dudley, M. E., Wunderlich, J. R., Yang, J. C., Sherry, R. M., Topalian, S. L., Restifo, N. P., et al. (2005). Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol, 23*(10), 2346-2357.

Duff, G. W. (2006). *Expert Scientific Group on Phase One Clinical Trials: Final Report (Stationary Office, Norwich, UK).*

Dunn, G. P., Old, L. J., & Schreiber, R. D. (2004). The three Es of cancer immunoediting. *Annu Rev Immunol, 22*, 329-360.

Dustin, M. L., Chakraborty, A. K., & Shaw, A. S. (2010). Understanding the structure and function of the immunological synapse. *Cold Spring Harb Perspect Biol, 2*(10), a002311.

Dutcher, J. (2002). Current status of interleukin-2 therapy for metastatic renal cell carcinoma and metastatic melanoma. *Oncology (Williston Park), 16*(11 Suppl 13), 4-10.

Eastwood, D., Findlay, L., Poole, S., Bird, C., Wadhwa, M., Moore, M., et al. (2010). Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4+ effector memory T-cells. *Br J Pharmacol, 161*(3), 512-526.

Elder, D. (1999). Tumor progression, early diagnosis and prognosis of melanoma. *Acta Oncol, 38*(5), 535-547.

Elgert, K. D. (2009). *Immunology : understanding the immune system* (2nd ed.). Hoboken, N.J.: Wiley-Blackwell.

Engell-Noerregaard, L., Hansen, T. H., Andersen, M. H., Thor Straten, P., & Svane, I. M. (2009). Review of clinical studies on dendritic cell-based vaccination of patients with malignant melanoma: assessment of correlation between clinical response and vaccine parameters. *Cancer Immunol Immunother, 58*(1), 1-14.

Eshhar, Z., Waks, T., Gross, G., & Schindler, D. G. (1993). Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A, 90*(2), 720-724.

Ferrone, S., & Marincola, F. M. (1995). Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol Today, 16*(10), 487-494.

Fields, B. A., Ober, B., Malchiodi, E. L., Lebedeva, M. I., Braden, B. C., Ysern, X., et al. (1995). Crystal structure of the V alpha domain of a T cell antigen receptor. *Science, 270*(5243), 1821-1824.

Figley, C. R., & Stroman, P. W. (2011). The role(s) of astrocytes and astrocyte activity in neurometabolism, neurovascular coupling, and the production of functional neuroimaging signals. *Eur J Neurosci, 33*(4), 577-588.

Finke, J. H., Zea, A. H., Stanley, J., Longo, D. L., Mizoguchi, H., Tubbs, R. R., et al. (1993). Loss of T-cell receptor zeta chain and p56lck in T-cells infiltrating human renal cell carcinoma. *Cancer Res, 53*(23), 5613-5616.

Finney, H. M., Lawson, A. D., Bebbington, C. R., & Weir, A. N. (1998). Chimeric receptors providing both primary and costimulatory signaling in T cells from a single gene product. *J Immunol, 161*(6), 2791-2797.

Flaherty, K. T., Puzanov, I., Kim, K. B., Ribas, A., McArthur, G. A., Sosman, J. A., et al. (2010). Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med, 363*(9), 809-819.

Fooksman, D. R., Vardhana, S., Vasiliver-Shamis, G., Liese, J., Blair, D. A., Waite, J., et al. (2010). Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol, 28*, 79-105.

Friedmann-Morvinski, D., Bendavid, A., Waks, T., Schindler, D., & Eshhar, Z. (2005). Redirected primary T cells harboring a chimeric receptor require costimulation for their antigen-specific activation. *Blood, 105*(8), 3087-3093.

Gabrilovich, D. I., Chen, H. L., Girgis, K. R., Cunningham, H. T., Meny, G. M., Nadaf, S., et al. (1996). Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med, 2*(10), 1096-1103.

Gabrilovich, D. I., Ostrand-Rosenberg, S., & Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol, 12*(4), 253-268.

Gahrton, G., Tura, S., Ljungman, P., Belanger, C., Brandt, L., Cavo, M., et al. (1991). Allogeneic bone marrow transplantation in multiple myeloma. European Group for Bone Marrow Transplantation. *N Engl J Med, 325*(18), 1267-1273.

Gahrton, G., Tura, S., Ljungman, P., Blade, J., Brandt, L., Cavo, M., et al. (1995). Prognostic factors in allogeneic bone marrow transplantation for multiple myeloma. *J Clin Oncol, 13*(6), 1312-1322.

Gao, G. F., Tormo, J., Gerth, U. C., Wyer, J. R., McMichael, A. J., Stuart, D. I., et al. (1997). Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2. *Nature, 387*(6633), 630-634.

Garboczi, D. N., Ghosh, P., Utz, U., Fan, Q. R., Biddison, W. E., & Wiley, D. C. (1996). Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature, 384*(6605), 134-141.

Garcia, K. C., Degano, M., Pease, L. R., Huang, M., Peterson, P. A., Teyton, L., et al. (1998). Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science, 279*(5354), 1166-1172.

Garcia, K. C., Degano, M., Speir, J. A., & Wilson, I. A. (1999). Emerging principles for T cell receptor recognition of antigen in cellular immunity. *Rev Immunogenet, 1*(1), 75-90.

Garcia, K. C., Degano, M., Stanfield, R. L., Brunmark, A., Jackson, M. R., Peterson, P. A., et al. (1996). An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. *Science, 274*(5285), 209-219.

Garcia, K. C., Teyton, L., & Wilson, I. A. (1999). Structural basis of T cell recognition. *Annu Rev Immunol, 17*, 369-397.

Garrido, F., & Algarra, I. (2001). MHC antigens and tumor escape from immune surveillance. *Adv Cancer Res, 83*, 117-158.

Gaugler, B., Van den Eynde, B., van der Bruggen, P., Romero, P., Gaforio, J. J., De Plaen, E., et al. (1994). Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J Exp Med, 179*(3), 921-930.

Geppert, T. D., & Lipsky, P. E. (1987). Accessory cell independent proliferation of human T4 cells stimulated by immobilized monoclonal antibodies to CD3. *J Immunol, 138*(6), 1660-1666.

Gerlini, G., Tun-Kyi, A., Dudli, C., Burg, G., Pimpinelli, N., & Nestle, F. O. (2004). Metastatic melanoma secreted IL-10 down-regulates CD1 molecules on dendritic cells in metastatic tumor lesions. *Am J Pathol, 165*(6), 1853-1863.

Ginaldi, L., De Martinis, M., Matutes, E., Farahat, N., Morilla, R., & Catovsky, D. (1998). Levels of expression of CD19 and CD20 in chronic B cell leukaemias. *J Clin Pathol, 51*(5), 364-369.

Gires, O., & Selinger, B. (Eds.). (2009). *Tumor-Associated Antigens*: Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Godefroy, E., Moreau-Aubry, A., Diez, E., Dreno, B., Jotereau, F., & Guilloux, Y. (2005). alpha v beta3-dependent cross-presentation of matrix metalloproteinase-2 by melanoma cells gives rise to a new tumor antigen. *J Exp Med, 202*(1), 61-72.

Gogishvili, T., Langenhorst, D., Luhder, F., Elias, F., Elflein, K., Dennehy, K. M., et al. (2009). Rapid regulatory T-cell response prevents cytokine storm in CD28 superagonist treated mice. *PLoS One, 4*(2), e4643.

Goldrath, A. W., & Bevan, M. J. (1999). Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. *Immunity, 11*(2), 183-190.

Gonzalez-Galarza, F. F., Christmas, S., Middleton, D., & Jones, A. R. (2011). Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. *Nucleic Acids Res, 39*(Database issue), D913-919.

Graham-Rowe, D. (2011). Overview: Multiple lines of attack. *Nature, 480*(7377), S34-35.

Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., et al. (1999). The immunological synapse: a molecular machine controlling T cell activation. *Science, 285*(5425), 221-227.

Gras, S., Burrows, S. R., Turner, S. J., Sewell, A. K., McCluskey, J., & Rossjohn, J. (2012). A structural voyage toward an understanding of the MHC-I-restricted immune response: lessons learned and much to be learned. *Immunol Rev, 250*(1), 61-81.

Graziani, G., Tentori, L., & Navarra, P. (2012). Ipilimumab: a novel immunostimulatory monoclonal antibody for the treatment of cancer. *Pharmacol Res, 65*(1), 9-22.

Groettrup, M., Kirk, C. J., & Basler, M. (2010). Proteasomes in immune cells: more than peptide producers? *Nat Rev Immunol, 10*(1), 73-78.

Gruen, M., Bommert, K., & Bargou, R. C. (2004). T-cell-mediated lysis of B cells induced by a CD19xCD3 bispecific single-chain antibody is perforin dependent and death receptor independent. *Cancer Immunol Immunother, 53*(7), 625-632.

Gunji, Y., Hori, S., Aoe, T., Asano, T., Ochiai, T., Isono, K., et al. (1994). High frequency of cancer patients with abnormal assembly of the T cell receptor-CD3 complex in peripheral blood T lymphocytes. *Jpn J Cancer Res, 85*(12), 1189-1192.

Gure, A. O., Chua, R., Williamson, B., Gonen, M., Ferrera, C. A., Gnjatic, S., et al. (2005). Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. *Clin Cancer Res, 11*(22), 8055-8062.

Hamanishi, J., Mandai, M., Iwasaki, M., Okazaki, T., Tanaka, Y., Yamaguchi, K., et al. (2007). Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci U S A, 104*(9), 3360-3365.

Hamilton, M. S., Ball, J., Bromidge, E., Lowe, J., & Franklin, I. M. (1990). Characterization of new IgG lambda myeloma plasma cell line (EJM): a further tool in the investigation of the biology of multiple myeloma. *Br J Haematol, 75*(3), 378-384.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Hanke, T. (2006). Lessons from TGN1412. *Lancet, 368*(9547), 1569-1570; author reply 1570.

Haskins, K., Kubo, R., White, J., Pigeon, M., Kappler, J., & Marrack, P. (1983). The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J Exp Med, 157*(4), 1149-1169.

Healy, C. G., Simons, J. W., Carducci, M. A., DeWeese, T. L., Bartkowski, M., Tong, K. P., et al. (1998). Impaired expression and function of signal-transducing zeta chains in peripheral T cells and natural killer cells in patients with prostate cancer. *Cytometry, 32*(2), 109-119.

Hedrick, S. M., Cohen, D. I., Nielsen, E. A., & Davis, M. M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature, 308*(5955), 149-153.

Heibein, J. A., Goping, I. S., Barry, M., Pinkoski, M. J., Shore, G. C., Green, D. R., et al. (2000). Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. *J Exp Med, 192*(10), 1391-1402.

Helms, T., Boehm, B. O., Asaad, R. J., Trezza, R. P., Lehmann, P. V., & Tary-Lehmann, M. (2000). Direct visualization of cytokine-producing recall antigen-specific CD4 memory T cells in healthy individuals and HIV patients. *J Immunol, 164*(7), 3723-3732.

Heusel, J. W., Wesselschmidt, R. L., Shresta, S., Russell, J. H., & Ley, T. J. (1994). Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell, 76*(6), 977-987.

Hicklin, D. J., Wang, Z., Arienti, F., Rivoltini, L., Parmiani, G., & Ferrone, S. (1998). beta2-Microglobulin mutations, HLA class I antigen loss, and tumor progression in melanoma. *J Clin Invest, 101*(12), 2720-2729.

Hodi, F. S., O'Day, S. J., McDermott, D. F., Weber, R. W., Sosman, J. A., Haanen, J. B., et al. (2010). Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med, 363*(8), 711-723.

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature, 411*(6835), 366-374.

Hofbauer, G. F., Burkhart, A., Schuler, G., Dummer, R., Burg, G., & Nestle, F. O. (2004). High frequency of melanoma-associated antigen or HLA class I loss does not correlate with survival in primary melanoma. *J Immunother, 27*(1), 73-78.

Hofmann, O., Caballero, O. L., Stevenson, B. J., Chen, Y. T., Cohen, T., Chua, R., et al. (2008). Genome-wide analysis of cancer/testis gene expression. *Proc Natl Acad Sci U S A, 105*(51), 20422-20427.

Holland, S. J., Bartok, I., Attaf, M., Genolet, R., Luescher, I. F., Kotsiou, E., et al. (2012). The T-cell receptor is not hardwired to engage MHC ligands. *Proc Natl Acad Sci U S A, 109*(45), E3111-3118.

Holler, P. D., Holman, P. O., Shusta, E. V., O'Herrin, S., Wittrup, K. D., & Kranz, D. M. (2000). In vitro evolution of a T cell receptor with high affinity for peptide/MHC. *Proc Natl Acad Sci U S A, 97*(10), 5387-5392.

Hunig, T. (2007). Manipulation of regulatory T-cell number and function with CD28-specific monoclonal antibodies. *Adv Immunol, 95*, 111-148.

Hunig, T. (2012). The storm has cleared: lessons from the CD28 superagonist TGN1412 trial. *Nat Rev Immunol, 12*(5), 317-318.

Iezzi, G., Karjalainen, K., & Lanzavecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity, 8*(1), 89-95.

Ikeda, H., Old, L. J., & Schreiber, R. D. (2002). The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev, 13*(2), 95-109.

Irles, C., Symons, A., Michel, F., Bakker, T. R., van der Merwe, P. A., & Acuto, O. (2003). CD45 ectodomain controls interaction with GEMs and Lck activity for optimal TCR signaling. *Nat Immunol, 4*(2), 189-197.

Irvine, D. J., Purbhoo, M. A., Krogsgaard, M., & Davis, M. M. (2002). Direct observation of ligand recognition by T cells. *Nature, 419*(6909), 845-849.

Irving, B. A., & Weiss, A. (1991). The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell, 64*(5), 891-901.

Ishizuka, J., Stewart-Jones, G. B., van der Merwe, A., Bell, J. I., McMichael, A. J., & Jones, E. Y. (2008). The structural dynamics and energetics of an immunodominant T cell receptor are programmed by its Vbeta domain. *Immunity, 28*(2), 171-182.

Jager, E., Chen, Y. T., Drijfhout, J. W., Karbach, J., Ringhoffer, M., Jager, D., et al. (1998). Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med, 187*(2), 265-270.

Jager, E., Jager, D., & Knuth, A. (1999). CTL-defined cancer vaccines: perspectives for active immunotherapeutic interventions in minimal residual disease. *Cancer Metastasis Rev, 18*(1), 143-150.

Jager, E., Ringhoffer, M., Altmannsberger, M., Arand, M., Karbach, J., Jager, D., et al. (1997). Immunoselection in vivo: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma. *Int J Cancer, 71*(2), 142-147.

James, S. E., Greenberg, P. D., Jensen, M. C., Lin, Y., Wang, J., Till, B. G., et al. (2008). Antigen sensitivity of CD22-specific chimeric TCR is modulated by target epitope distance from the cell membrane. *J Immunol, 180*(10), 7028-7038.

Jarosinski, K. W., & Massa, P. T. (2002). Interferon regulatory factor-1 is required for interferon-gamma-induced MHC class I genes in astrocytes. *J Neuroimmunol, 122*(1-2), 74-84.

Jenkins, M. K., Taylor, P. S., Norton, S. D., & Urdahl, K. B. (1991). CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J Immunol, 147*(8), 2461-2466.

Jenkins, M. R., & Griffiths, G. M. (2010). The synapse and cytolytic machinery of cytotoxic T cells. *Curr Opin Immunol, 22*(3), 308-313.

Jiang, X., & Wang, X. (2000). Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J Biol Chem, 275*(40), 31199-31203.

Kaech, S. M., & Ahmed, R. (2001). Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol, 2*(5), 415-422.

Kalergis, A. M., Boucheron, N., Doucey, M. A., Palmieri, E., Goyarts, E. C., Vegh, Z., et al. (2001). Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. *Nat Immunol, 2*(3), 229-234.

Kalos, M., Levine, B. L., Porter, D. L., Katz, S., Grupp, S. A., Bagg, A., et al. (2011). T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med, 3*(95), 95ra73.

Kaplan, D. H., Shankaran, V., Dighe, A. S., Stockert, E., Aguet, M., Old, L. J., et al. (1998). Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A, 95*(13), 7556-7561.

Kawakami, Y., Dang, N., Wang, X., Tupesis, J., Robbins, P. F., Wang, R. F., et al. (2000). Recognition of shared melanoma antigens in association with major HLA-A alleles by tumor infiltrating T lymphocytes from 123 patients with melanoma. *J Immunother, 23*(1), 17-27.

Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Rivoltini, L., Topalian, S. L., et al. (1994). Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc Natl Acad Sci U S A, 91*(9), 3515-3519.

Kawakami, Y., Eliyahu, S., Jennings, C., Sakaguchi, K., Kang, X., Southwood, S., et al. (1995). Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol, 154*(8), 3961-3968.

Kawakami, Y., Eliyahu, S., Sakaguchi, K., Robbins, P. F., Rivoltini, L., Yannelli, J. R., et al. (1994). Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J Exp Med, 180*(1), 347-352.

Kennedy, R., & Celis, E. (2008). Multiple roles for CD4+ T cells in anti-tumor immune responses. *Immunol Rev, 222*, 129-144.

Kershaw, M. H., Westwood, J. A., Parker, L. L., Wang, G., Eshhar, Z., Mavroukakis, S. A., et al. (2006). A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res, 12*(20 Pt 1), 6106-6115.

Knudson, A. G. (2001). Two genetic hits (more or less) to cancer. *Nat Rev Cancer, 1*(2), 157-162.

Koning, F., Maloy, W. L., & Coligan, J. E. (1990). The implications of subunit interactions for the structure of the T cell receptor-CD3 complex. *Eur J Immunol, 20*(2), 299-305.

Konishi, J., Toyooka, S., Aoe, M., Omura, Y., Washio, K., Tsukuda, K., et al. (2004). The relationship between NY-ESO-1 mRNA expression and clinicopathological features in non-small cell lung cancer. *Oncol Rep, 11*(5), 1063-1067.

Kono, K., Kawaida, H., Takahashi, A., Sugai, H., Mimura, K., Miyagawa, N., et al. (2006). CD4(+)CD25high regulatory T cells increase with tumor stage in patients with gastric and esophageal cancers. *Cancer Immunol Immunother, 55*(9), 1064-1071.

Korzeniewski, C., & Callewaert, D. M. (1983). An enzyme-release assay for natural cytotoxicity. *J Immunol Methods, 64*(3), 313-320.

Krogsgaard, M., & Davis, M. M. (2005). How T cells 'see' antigen. *Nat Immunol, 6*(3), 239-245.

Kufer, P., Lutterbuse, R., & Baeuerle, P. A. (2004). A revival of bispecific antibodies. *Trends Biotechnol, 22*(5), 238-244.

Kurte, M., Lopez, M., Aguirre, A., Escobar, A., Aguillon, J. C., Charo, J., et al. (2004). A synthetic peptide homologous to functional domain of human IL-10 down-regulates expression of MHC class I and Transporter associated with Antigen Processing 1/2 in human melanoma cells. *J Immunol, 173*(3), 1731-1737.

Lalvani, A., Brookes, R., Hambleton, S., Britton, W. J., Hill, A. V., & McMichael, A. J. (1997). Rapid effector function in CD8+ memory T cells. *J Exp Med, 186*(6), 859-865.

Lampson, L. A., & Hickey, W. F. (1986). Monoclonal antibody analysis of MHC expression in human brain biopsies: tissue ranging from "histologically normal" to that showing different levels of glial tumor involvement. *J Immunol, 136*(11), 4054-4062.

Landgren, O., & Weiss, B. M. (2009). Patterns of monoclonal gammopathy of undetermined significance and multiple myeloma in various ethnic/racial groups: support for genetic factors in pathogenesis. *Leukemia, 23*(10), 1691-1697.

Launay, S., Hermine, O., Fontenay, M., Kroemer, G., Solary, E., & Garrido, C. (2005). Vital functions for lethal caspases. *Oncogene, 24*(33), 5137-5148.

Lee, P. P., Yee, C., Savage, P. A., Fong, L., Brockstedt, D., Weber, J. S., et al. (1999). Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat Med, 5*(6), 677-685.

Lefranc, M.-P., & Lefranc, G. (2001). *The T-cell receptor factsbook*. San Diego: Academic Press.

Lengauer, C., Kinzler, K. W., & Vogelstein, B. (1998). Genetic instabilities in human cancers. *Nature, 396*(6712), 643-649.

Lens, M. (2008). The role of vaccine therapy in the treatment of melanoma. *Expert Opin Biol Ther, 8*(3), 315-323.

Lethe, B., Lucas, S., Michaux, L., De Smet, C., Godelaine, D., Serrano, A., et al. (1998). LAGE-1, a new gene with tumor specificity. *Int J Cancer, 76*(6), 903-908.

Levin, S. E., Zhang, C., Kadlecek, T. A., Shokat, K. M., & Weiss, A. (2008). Inhibition of ZAP-70 kinase activity via an analog-sensitive allele blocks T cell receptor and CD28 superagonist signaling. *J Biol Chem, 283*(22), 15419-15430.

Li, B., Wang, H., Dai, J., Ji, J., Qian, W., Zhang, D., et al. (2005). Construction and characterization of a humanized anti-human CD3 monoclonal antibody 12F6 with effective immunoregulation functions. *Immunology, 116*(4), 487-498.

Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K., & Flavell, R. A. (2006). Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol, 24*, 99-146.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., et al. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell, 91*(4), 479-489.

Li, Y., Moysey, R., Molloy, P. E., Vuidepot, A. L., Mahon, T., Baston, E., et al. (2005). Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nat Biotechnol, 23*(3), 349-354.

Liddy, N., Bossi, G., Adams, K. J., Lissina, A., Mahon, T. M., Hassan, N. J., et al. (2012). Monoclonal TCR-redirected tumor cell killing. *Nat Med, 18*(6), 980-987.

Lin, J., & Weiss, A. (2003). The tyrosine phosphatase CD148 is excluded from the immunologic synapse and down-regulates prolonged T cell signaling. *J Cell Biol, 162*(4), 673-682.

Lin, P., Owens, R., Tricot, G., & Wilson, C. S. (2004). Flow cytometric immunophenotypic analysis of 306 cases of multiple myeloma. *Am J Clin Pathol, 121*(4), 482-488.

Ljunggren, H. G., & Karre, K. (1985). Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *J Exp Med, 162*(6), 1745-1759.

Lyle, S., Salhany, K. E., & Elder, D. E. (2000). TIA-1 positive tumor-infiltrating lymphocytes in nevi and melanomas. *Mod Pathol, 13*(1), 52-55.

Ma, Z., Sharp, K. A., Janmey, P. A., & Finkel, T. H. (2008). Surface-anchored monomeric agonist pMHCs alone trigger TCR with high sensitivity. *PLoS Biol, 6*(2), e43.

Mackall, C. L. (2000). T-cell immunodeficiency following cytotoxic antineoplastic therapy: a review. *Stem Cells, 18*(1), 10-18.

Mackensen, A., Carcelain, G., Viel, S., Raynal, M. C., Michalaki, H., Triebel, F., et al. (1994). Direct evidence to support the immunosurveillance concept in a human regressive melanoma. *J Clin Invest, 93*(4), 1397-1402.

Mackensen, A., Meidenbauer, N., Vogl, S., Laumer, M., Berger, J., & Andreesen, R. (2006). Phase I study of adoptive T-cell therapy using antigen-specific CD8+ T cells for the treatment of patients with metastatic melanoma. *J Clin Oncol, 24*(31), 5060-5069.

Maeurer, M. J., Gollin, S. M., Martin, D., Swaney, W., Bryant, J., Castelli, C., et al. (1996). Tumor escape from immune recognition: lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen. *J Clin Invest*, *98*(7), 1633-1641.

Maher, J., Brentjens, R. J., Gunset, G., Riviere, I., & Sadelain, M. (2002). Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor. *Nat Biotechnol, 20*(1), 70-75.

Mahindra, A., Hideshima, T., & Anderson, K. C. (2010). Multiple myeloma: biology of the disease. *Blood Rev, 24 Suppl 1*, S5-11.

Maio, M., Altomonte, M., Tatake, R., Zeff, R. A., & Ferrone, S. (1991). Reduction in susceptibility to natural killer cell-mediated lysis of human FO-1 melanoma cells after induction of HLA class I antigen expression by transfection with B2m gene. *J Clin Invest, 88*(1), 282-289.

Malyguine, A., Strobl, S., Zaritskaya, L., Baseler, M., & Shafer-Weaver, K. (2007). New approaches for monitoring CTL activity in clinical trials. *Adv Exp Med Biol, 601*, 273-284.

Manolios, N., Kemp, O., & Li, Z. G. (1994). The T cell antigen receptor alpha and beta chains interact via distinct regions with CD3 chains. *Eur J Immunol, 24*(1), 84-92.

Marincola, F. M., Jaffee, E. M., Hicklin, D. J., & Ferrone, S. (2000). Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol, 74*, 181-273.

Maser, R. S., & DePinho, R. A. (2002). Connecting chromosomes, crisis, and cancer. *Science, 297*(5581), 565-569.

Mason, D. (1998). A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol Today, 19*(9), 395-404.

Matsuda, M., Petersson, M., Lenkei, R., Taupin, J. L., Magnusson, I., Mellstedt, H., et al. (1995). Alterations in the signal-transducing molecules of T cells and NK cells in colorectal tumor-infiltrating, gut mucosal and peripheral lymphocytes: correlation with the stage of the disease. *Int J Cancer, 61*(6), 765-772.

Matsui, K., Boniface, J. J., Reay, P. A., Schild, H., Fazekas de St Groth, B., & Davis, M. M. (1991). Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science, 254*(5039), 1788-1791.

McCormack, E., Adams, K. J., Hassan, N. J., Kotian, A., Lissin, N. M., Sami, M., et al. (2012). Bi-specific TCR-anti CD3 redirected T-cell targeting of NY-ESO-1- and LAGE-1-positive tumors. *Cancer Immunol Immunother*.

Medeiros, L. R., Rosa, D. D., da Rosa, M. I., Bozzetti, M. C., & Zanini, R. R. (2009). Efficacy of human papillomavirus vaccines: a systematic quantitative review. *Int J Gynecol Cancer, 19*(7), 1166-1176.

Mellman, I., Coukos, G., & Dranoff, G. (2011). Cancer immunotherapy comes of age. *Nature, 480*(7378), 480-489.

Mellor, A. L., & Munn, D. H. (2004). IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol, 4*(10), 762-774.

Mocellin, S., Fetsch, P., Abati, A., Phan, G. Q., Wang, E., Provenzano, M., et al. (2001). Laser scanning cytometry evaluation of MART-1, gp100, and HLA-A2 expression in melanoma metastases. *J Immunother, 24*(6), 447-458.

Momburg, F., & Hammerling, G. J. (1998). Generation and TAP-mediated transport of peptides for major histocompatibility complex class I molecules. *Adv Immunol, 68*, 191-256.

Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N., & Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature, 395*(6697), 82-86.

Morgan, D. J., Kreuwel, H. T., Fleck, S., Levitsky, H. I., Pardoll, D. M., & Sherman, L. A. (1998). Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. *J Immunol, 160*(2), 643-651.

Morgan, R. A., Dudley, M. E., Wunderlich, J. R., Hughes, M. S., Yang, J. C., Sherry, R. M., et al. (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science, 314*(5796), 126-129.

Mouawad, R., Sebert, M., Michels, J., Bloch, J., Spano, J. P., & Khayat, D. (2010). Treatment for metastatic malignant melanoma: old drugs and new strategies. *Crit Rev Oncol Hematol, 74*(1), 27-39.

Munn, D. H., & Mellor, A. L. (2004). IDO and tolerance to tumors. *Trends Mol Med, 10*(1), 15-18.

Munoz, N., Kjaer, S. K., Sigurdsson, K., Iversen, O. E., Hernandez-Avila, M., Wheeler, C. M., et al. (2010). Impact of human papillomavirus (HPV)-6/11/16/18 vaccine on all HPV-associated genital diseases in young women. *J Natl Cancer Inst, 102*(5), 325-339.

Murga, M., & Fernandez-Capetillo, O. (2007). Genomic instability: on the birth and death of cancer. *Clin Transl Oncol, 9*(4), 216-220.

Nagai, M. A., Marques, L. A., Torloni, H., & Brentani, M. M. (1993). Genetic alterations in c-erbB-2 protooncogene as prognostic markers in human primary breast tumors. *Oncology, 50*(6), 412-417.

Nagorsen, D., & Baeuerle, P. A. (2011). Immunomodulatory therapy of cancer with T cell-engaging BiTE antibody blinatumomab. *Exp Cell Res, 317*(9), 1255-1260.

Nakagomi, H., Petersson, M., Magnusson, I., Juhlin, C., Matsuda, M., Mellstedt, H., et al. (1993). Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma. *Cancer Res, 53*(23), 5610-5612.

Nicholaou, T., Ebert, L., Davis, I. D., Robson, N., Klein, O., Maraskovsky, E., et al. (2006). Directions in the immune targeting of cancer: lessons learned from the cancer-testis Ag NY-ESO-1. *Immunol Cell Biol, 84*(3), 303-317.

Nitta, T., Murata, S., Sasaki, K., Fujii, H., Ripen, A. M., Ishimaru, N., et al. (2010). Thymoproteasome shapes immunocompetent repertoire of CD8+ T cells. *Immunity, 32*(1), 29-40.

Noonan, K., & Borrello, I. (2011). The immune microenvironment of myeloma. *Cancer Microenviron, 4*(3), 313-323.

Noonan, K., Matsui, W., Serafini, P., Carbley, R., Tan, G., Khalili, J., et al. (2005). Activated marrow-infiltrating lymphocytes effectively target plasma cells and their clonogenic precursors. *Cancer Res, 65*(5), 2026-2034.

Odunsi, K., Jungbluth, A. A., Stockert, E., Qian, F., Gnjatic, S., Tammela, J., et al. (2003). NY-ESO-1 and LAGE-1 cancer-testis antigens are potential targets for immunotherapy in epithelial ovarian cancer. *Cancer Res, 63*(18), 6076-6083.

Offner, S., Hofmeister, R., Romaniuk, A., Kufer, P., & Baeuerle, P. A. (2006). Induction of regular cytolytic T cell synapses by bispecific single-chain antibody constructs on MHC class I-negative tumor cells. *Mol Immunol, 43*(6), 763-771.

Okur, F. V., & Brenner, M. K. (2010). Cellular immunotherapy of cancer. *Methods Mol Biol, 651*, 319-345.

Overwijk, W. W., Theoret, M. R., Finkelstein, S. E., Surman, D. R., de Jong, L. A., Vyth-Dreese, F. A., et al. (2003). Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med, 198*(4), 569-580.

Palmer, D. C., Chan, C. C., Gattinoni, L., Wrzesinski, C., Paulos, C. M., Hinrichs, C. S., et al. (2008). Effective tumor treatment targeting a melanoma/melanocyte-associated antigen triggers severe ocular autoimmunity. *Proc Natl Acad Sci U S A, 105*(23), 8061-8066.

Palucka, K., Banchereau, J., & Mellman, I. (2010). Designing vaccines based on biology of human dendritic cell subsets. *Immunity, 33*(4), 464-478.

Pamer, E., & Cresswell, P. (1998). Mechanisms of MHC class I--restricted antigen processing. *Annu Rev Immunol, 16*, 323-358.

Pandolfi, F., Cianci, R., Pagliari, D., Casciano, F., Bagala, C., Astone, A., et al. (2011). The immune response to tumors as a tool toward immunotherapy. *Clin Dev Immunol, 2011*, 894704.

Pardo, J., Bosque, A., Brehm, R., Wallich, R., Naval, J., Mullbacher, A., et al. (2004). Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. *J Cell Biol, 167*(3), 457-468.

Pardoll, D. M., & Topalian, S. L. (1998). The role of CD4+ T cell responses in antitumor immunity. *Curr Opin Immunol, 10*(5), 588-594.

Park, J. R., Digiusto, D. L., Slovak, M., Wright, C., Naranjo, A., Wagner, J., et al. (2007). Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Mol Ther, 15*(4), 825-833.

Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2005). Global cancer statistics, 2002. *CA Cancer J Clin, 55*(2), 74-108.

Patard, J. J., Brasseur, F., Gil-Diez, S., Radvanyi, F., Marchand, M., Francois, P., et al. (1995). Expression of MAGE genes in transitional-cell carcinomas of the urinary bladder. *Int J Cancer, 64*(1), 60-64.

Peter, M. E., & Krammer, P. H. (1998). Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. *Curr Opin Immunol, 10*(5), 545-551.

Phan, G. Q., Yang, J. C., Sherry, R. M., Hwu, P., Topalian, S. L., Schwartzentruber, D. J., et al. (2003). 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*(14), 8372-8377.

Pinkoski, M. J., Waterhouse, N. J., Heibein, J. A., Wolf, B. B., Kuwana, T., Goldstein, J. C., et al. (2001). Granzyme B-mediated apoptosis proceeds predominantly through a Bcl-2-inhibitable mitochondrial pathway. *J Biol Chem, 276*(15), 12060-12067.

Pittet, M. J., Valmori, D., Dunbar, P. R., Speiser, D. E., Lienard, D., Lejeune, F., et al. (1999). High frequencies of naive Melan-A/MART-1-specific CD8(+) T cells in a large

proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. *J Exp Med, 190*(5), 705-715.

Prall, F., Duhrkop, T., Weirich, V., Ostwald, C., Lenz, P., Nizze, H., et al. (2004). Prognostic role of CD8+ tumor-infiltrating lymphocytes in stage III colorectal cancer with and without microsatellite instability. *Hum Pathol, 35*(7), 808-816.

Purbhoo, M. A., Irvine, D. J., Huppa, J. B., & Davis, M. M. (2004). T cell killing does not require the formation of a stable mature immunological synapse. *Nat Immunol, 5*(5), 524-530.

Purbhoo, M. A., Li, Y., Sutton, D. H., Brewer, J. E., Gostick, E., Bossi, G., et al. (2007). The HLA A*0201-restricted hTERT(540-548) peptide is not detected on tumor cells by a CTL clone or a high-affinity T-cell receptor. *Mol Cancer Ther, 6*(7), 2081-2091.

Purbhoo, M. A., Sutton, D. H., Brewer, J. E., Mullings, R. E., Hill, M. E., Mahon, T. M., et al. (2006). Quantifying and imaging NY-ESO-1/LAGE-1-derived epitopes on tumor cells using high affinity T cell receptors. *J Immunol, 176*(12), 7308-7316.

Quezada, S. A., Simpson, T. R., Peggs, K. S., Merghoub, T., Vider, J., Fan, X., et al. (2010). Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med, 207*(3), 637-650.

Rabinovich, G. A., Gabrilovich, D., & Sotomayor, E. M. (2007). Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol, 25*, 267-296.

Raghavan, M., Del Cid, N., Rizvi, S. M., & Peters, L. R. (2008). MHC class I assembly: out and about. *Trends Immunol, 29*(9), 436-443.

Rajkumar, S. V. (2012). Multiple myeloma: 2012 update on diagnosis, risk-stratification, and management. *Am J Hematol, 87*(1), 78-88.

Rammensee, H. G., Friede, T., & Stevanoviic, S. (1995). MHC ligands and peptide motifs: first listing. *Immunogenetics, 41*(4), 178-228.

Reiser, J. B., Darnault, C., Gregoire, C., Mosser, T., Mazza, G., Kearney, A., et al. (2003). CDR3 loop flexibility contributes to the degeneracy of TCR recognition. *Nat Immunol, 4*(3), 241-247.

Restifo, N. P., Marincola, F. M., Kawakami, Y., Taubenberger, J., Yannelli, J. R., & Rosenberg, S. A. (1996). Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. *J Natl Cancer Inst, 88*(2), 100-108.

Riker, A., Cormier, J., Panelli, M., Kammula, U., Wang, E., Abati, A., et al. (1999). Immune selection after antigen-specific immunotherapy of melanoma. *Surgery, 126*(2), 112-120.

Rininsland, F. H., Helms, T., Asaad, R. J., Boehm, B. O., & Tary-Lehmann, M. (2000). Granzyme B ELISPOT assay for ex vivo measurements of T cell immunity. *J Immunol Methods, 240*(1-2), 143-155.

Rivoltini, L., Carrabba, M., Huber, V., Castelli, C., Novellino, L., Dalerba, P., et al. (2002). Immunity to cancer: attack and escape in T lymphocyte-tumor cell interaction. *Immunol Rev, 188*, 97-113.

Robbins, P. F., Morgan, R. A., Feldman, S. A., Yang, J. C., Sherry, R. M., Dudley, M. E., et al. (2011). Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol, 29*(7), 917-924.

Robins, H. S., Campregher, P. V., Srivastava, S. K., Wacher, A., Turtle, C. J., Kahsai, O., et al. (2009). Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood, 114*(19), 4099-4107.

Robinson, J., Halliwell, J. A., McWilliam, H., Lopez, R., Parham, P., & Marsh, S. G. (2013). The IMGT/HLA database. *Nucleic Acids Res, 41*(D1), D1222-D1227.

Rock, M. T., Yoder, S. M., Wright, P. F., Talbot, T. R., Edwards, K. M., & Crowe, J. E., Jr. (2005). Differential regulation of granzyme and perforin in effector and memory T cells following smallpox immunization. *J Immunol, 174*(6), 3757-3764.

Romer, P. S., Berr, S., Avota, E., Na, S. Y., Battaglia, M., ten Berge, I., et al. (2011). Preculture of PBMCs at high cell density increases sensitivity of T-cell responses, revealing cytokine release by CD28 superagonist TGN1412. *Blood, 118*(26), 6772-6782.

Romero, P., Dunbar, P. R., Valmori, D., Pittet, M., Ogg, G. S., Rimoldi, D., et al. (1998). Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J Exp Med, 188*(9), 1641-1650.

Romero, P., Gervois, N., Schneider, J., Escobar, P., Valmori, D., Pannetier, C., et al. (1997). Cytolytic T lymphocyte recognition of the immunodominant HLA-A*0201-restricted Melan-A/MART-1 antigenic peptide in melanoma. *J Immunol, 159*(5), 2366-2374.

Rosenberg, S. A. (1997). Cancer vaccines based on the identification of genes encoding cancer regression antigens. *Immunol Today, 18*(4), 175-182.

Rosenberg, S. A. (1999). A new era for cancer immunotherapy based on the genes that encode cancer antigens. *Immunity, 10*(3), 281-287.

Rosenberg, S. A., Packard, B. S., Aebersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., et al. (1988). Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N Engl J Med, 319*(25), 1676-1680.

Rosenberg, S. A., Restifo, N. P., Yang, J. C., Morgan, R. A., & Dudley, M. E. (2008). Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer, 8*(4), 299-308.

Rosenberg, S. A., Yang, J. C., & Restifo, N. P. (2004). Cancer immunotherapy: moving beyond current vaccines. *Nat Med, 10*(9), 909-915.

Rosenberg, S. A., Yang, J. C., Sherry, R. M., Kammula, U. S., Hughes, M. S., Phan, G. Q., et al. (2011). Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res, 17*(13), 4550-4557.

Rothstein, T. L., Mage, M., Jones, G., & McHugh, L. L. (1978). Cytotoxic T lymphocyte sequential killing of immobilized allogeneic tumor target cells measured by time-lapse microcinematography. *J Immunol, 121*(5), 1652-1656.

Rubio-Moscardo, F., Blesa, D., Mestre, C., Siebert, R., Balasas, T., Benito, A., et al. (2005). Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. *Blood, 106*(9), 3214-3222.

Rudolph, M. G., Stanfield, R. L., & Wilson, I. A. (2006). How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol, 24*, 419-466.

Russell, J. H., & Ley, T. J. (2002). Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol, 20*, 323-370.

Saikali, S., Avril, T., Collet, B., Hamlat, A., Bansard, J. Y., Drenou, B., et al. (2007). Expression of nine tumour antigens in a series of human glioblastoma multiforme: interest of EGFRvIII, IL-13Ralpha2, gp100 and TRP-2 for immunotherapy. *J Neurooncol, 81*(2), 139-148.

Saleh, F., Renno, W., Klepacek, I., Ibrahim, G., Dashti, H., Asfar, S., et al. (2005). Direct evidence on the immune-mediated spontaneous regression of human cancer: an incentive for pharmaceutical companies to develop a novel anti-cancer vaccine. *Curr Pharm Des, 11*(27), 3531-3543.

Salmeron, A., Sanchez-Madrid, F., Ursa, M. A., Fresno, M., & Alarcon, B. (1991). A conformational epitope expressed upon association of CD3-epsilon with either CD3-delta or CD3-gamma is the main target for recognition by anti-CD3 monoclonal antibodies. *J Immunol, 147*(9), 3047-3052.

Salmond, R. J., Filby, A., Qureshi, I., Caserta, S., & Zamoyska, R. (2009). T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. *Immunol Rev, 228*(1), 9-22.

Sami, M., Rizkallah, P. J., Dunn, S., Molloy, P., Moysey, R., Vuidepot, A., et al. (2007). Crystal structures of high affinity human T-cell receptors bound to peptide

major histocompatibility complex reveal native diagonal binding geometry. *Protein Eng Des Sel, 20*(8), 397-403.

Schneider, S. W., Ludwig, T., Tatenhorst, L., Braune, S., Oberleithner, H., Senner, V., et al. (2004). Glioblastoma cells release factors that disrupt blood-brain barrier features. *Acta Neuropathol, 107*(3), 272-276.

Schreiber, R. D., Old, L. J., & Smyth, M. J. (2011). Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science, 331*(6024), 1565-1570.

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol, 75*(2), 163-189.

Schrum, A. G., & Turka, L. A. (2002). The proliferative capacity of individual naive CD4(+) T cells is amplified by prolonged T cell antigen receptor triggering. *J Exp Med, 196*(6), 793-803.

Schultze, J. L., & Vonderheide, R. H. (2001). From cancer genomics to cancer immunotherapy: toward second-generation tumor antigens. *Trends Immunol, 22*(9), 516-523.

Sebastian, M., Kiewe, P., Schuette, W., Brust, D., Peschel, C., Schneller, F., et al. (2009). Treatment of malignant pleural effusion with the trifunctional antibody catumaxomab (Removab) (anti-EpCAM x Anti-CD3): results of a phase 1/2 study. *J Immunother, 32*(2), 195-202.

Seliger, B., Maeurer, M. J., & Ferrone, S. (1997). TAP off--tumors on. *Immunol Today, 18*(6), 292-299.

Seliger, B., Maeurer, M. J., & Ferrone, S. (2000). Antigen-processing machinery breakdown and tumor growth. *Immunol Today, 21*(9), 455-464.

Seliger, B., Schreiber, K., Delp, K., Meissner, M., Hammers, S., Reichert, T., et al. (2001). Downregulation of the constitutive tapasin expression in human tumor cells of distinct origin and its transcriptional upregulation by cytokines. *Tissue Antigens, 57*(1), 39-45.

Sewell, A. K. (2012). Why must T cells be cross-reactive? *Nat Rev Immunol, 12*(9), 669-677.

Shalaby, M. R., Shepard, H. M., Presta, L., Rodrigues, M. L., Beverley, P. C., Feldmann, M., et al. (1992). Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J Exp Med, 175*(1), 217-225.

Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., et al. (2001). IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature, 410*(6832), 1107-1111.

Shen, L., & Rock, K. L. (2006). Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. *Curr Opin Immunol, 18*(1), 85-91.

Simka, M. (2009). Blood brain barrier compromise with endothelial inflammation may lead to autoimmune loss of myelin during multiple sclerosis. *Curr Neurovasc Res, 6*(2), 132-139.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., & McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science, 235*(4785), 177-182.

Slee, E. A., Adrain, C., & Martin, S. J. (1999). Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ, 6*(11), 1067-1074.

Smith-Garvin, J. E., Koretzky, G. A., & Jordan, M. S. (2009). T cell activation. *Annu Rev Immunol, 27*, 591-619.

Smyth, M. J., O'Connor, M. D., & Trapani, J. A. (1996). Granzymes: a variety of serine protease specificities encoded by genetically distinct subfamilies. *J Leukoc Biol, 60*(5), 555-562.

Spagnoli, G. C., Schaefer, C., Willimann, T. E., Kocher, T., Amoroso, A., Juretic, A., et al. (1995). Peptide-specific CTL in tumor infiltrating lymphocytes from metastatic melanomas expressing MART-1/Melan-A, gp100 and Tyrosinase genes: a study in an unselected group of HLA-A2.1-positive patients. *Int J Cancer, 64*(5), 309-315.

Spiotto, M. T., Rowley, D. A., & Schreiber, H. (2004). Bystander elimination of antigen loss variants in established tumors. *Nat Med, 10*(3), 294-298.

Spiotto, M. T., & Schreiber, H. (2005). Rapid destruction of the tumor microenvironment by CTLs recognizing cancer-specific antigens cross-presented by stromal cells. *Cancer Immun, 5*, 8.

Starr, T. K., Jameson, S. C., & Hogquist, K. A. (2003). Positive and negative selection of T cells. *Annu Rev Immunol, 21*, 139-176.

Staveley-O'Carroll, K., Sotomayor, E., Montgomery, J., Borrello, I., Hwang, L., Fein, S., et al. (1998). Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc Natl Acad Sci U S A, 95*(3), 1178-1183.

Stinchcombe, J. C., Bossi, G., Booth, S., & Griffiths, G. M. (2001). The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity, 15*(5), 751-761.

Suliman, A., Lam, A., Datta, R., & Srivastava, R. K. (2001). Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and -independent pathways. *Oncogene, 20*(17), 2122-2133.

Suntharalingam, G., Perry, M. R., Ward, S., Brett, S. J., Castello-Cortes, A., Brunner, M. D., et al. (2006). Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med, 355*(10), 1018-1028.

Takase, H., Yu, C. R., Mahdi, R. M., Douek, D. C., Dirusso, G. B., Midgley, F. M., et al. (2005). Thymic expression of peripheral tissue antigens in humans: a remarkable variability among individuals. *Int Immunol, 17*(8), 1131-1140.

Taniguchi, R. T., & Anderson, M. S. (2011). The role of Aire in clonal selection. *Immunol Cell Biol, 89*(1), 40-44.

Thauland, T. J., & Parker, D. C. (2010). Diversity in immunological synapse structure. *Immunology, 131*(4), 466-472.

Thomas, D. A., & Massague, J. (2005). TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell, 8*(5), 369-380.

Tonegawa, S. (1988). Somatic generation of immune diversity. *Biosci Rep, 8*(1), 3-26.

Topp, M. S., Kufer, P., Gokbuget, N., Goebeler, M., Klinger, M., Neumann, S., et al. (2011). Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. *J Clin Oncol, 29*(18), 2493-2498.

Trapani, J. A., & Sutton, V. R. (2003). Granzyme B: pro-apoptotic, antiviral and antitumor functions. *Curr Opin Immunol, 15*(5), 533-543.

Traversari, C., van der Bruggen, P., Luescher, I. F., Lurquin, C., Chomez, P., Van Pel, A., et al. (1992). A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J Exp Med, 176*(5), 1453-1457.

Tveit, K. M., & Pihl, A. (1981). Do cell lines in vitro reflect the properties of the tumours of origin? A study of lines derived from human melanoma xenografts. *Br J Cancer, 44*(6), 775-786.

Valitutti, S., Dessing, M., Aktories, K., Gallati, H., & Lanzavecchia, A. (1995). Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J Exp Med, 181*(2), 577-584.

Valitutti, S., & Lanzavecchia, A. (1997). Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. *Immunol Today, 18*(6), 299-304.

Valitutti, S., Muller, S., Cella, M., Padovan, E., & Lanzavecchia, A. (1995). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature, 375*(6527), 148-151.

Valmori, D., Dutoit, V., Lienard, D., Rimoldi, D., Pittet, M. J., Champagne, P., et al. (2000). Naturally occurring human lymphocyte antigen-A2 restricted CD8+ T-cell response to the cancer testis antigen NY-ESO-1 in melanoma patients. *Cancer Res, 60*(16), 4499-4506.

Valmori, D., Gervois, N., Rimoldi, D., Fonteneau, J. F., Bonelo, A., Lienard, D., et al. (1998). Diversity of the fine specificity displayed by HLA-A*0201-restricted CTL specific for the immunodominant Melan-A/MART-1 antigenic peptide. *J Immunol, 161*(12), 6956-6962.

Valmori, D., Scheibenbogen, C., Dutoit, V., Nagorsen, D., Asemissen, A. M., Rubio-Godoy, V., et al. (2002). Circulating Tumor-reactive CD8(+) T cells in melanoma patients contain a CD45RA(+)CCR7(-) effector subset exerting ex vivo tumor-specific cytolytic activity. *Cancer Res, 62*(6), 1743-1750.

Van der Bruggen, P., Stroobant, V., Vigneron, N., & Van den Eynde, B. (2013). Peptide database: T cell-defined tumor antigens, *Cancer Immunity*: Cancer Research Institute.

van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., et al. (1991). A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science, 254*(5038), 1643-1647.

van der Merwe, P. A., & Davis, S. J. (2003). Molecular interactions mediating T cell antigen recognition. *Annu Rev Immunol, 21*, 659-684.

van der Merwe, P. A., & Dushek, O. (2011). Mechanisms for T cell receptor triggering. *Nat Rev Immunol, 11*(1), 47-55.

Van Pel, A., van der Bruggen, P., Coulie, P. G., Brichard, V. G., Lethe, B., van den Eynde, B., et al. (1995). Genes coding for tumor antigens recognized by cytolytic T lymphocytes. *Immunol Rev, 145*, 229-250.

van Rhee, F., Szmania, S. M., Zhan, F., Gupta, S. K., Pomtree, M., Lin, P., et al. (2005). NY-ESO-1 is highly expressed in poor-prognosis multiple myeloma and induces spontaneous humoral and cellular immune responses. *Blood, 105*(10), 3939-3944.

Varma, R., Campi, G., Yokosuka, T., Saito, T., & Dustin, M. L. (2006). T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity, 25*(1), 117-127.

Vaughan, H. A., Svobodova, S., Macgregor, D., Sturrock, S., Jungbluth, A. A., Browning, J., et al. (2004). Immunohistochemical and molecular analysis of human

melanomas for expression of the human cancer-testis antigens NY-ESO-1 and LAGE-1. *Clin Cancer Res, 10*(24), 8396-8404.

von Mehren, M., Adams, G. P., & Weiner, L. M. (2003). Monoclonal antibody therapy for cancer. *Annu Rev Med, 54*, 343-369.

Vultur, A., Villanueva, J., & Herlyn, M. (2010). BRAF inhibitor unveils its potential against advanced melanoma. *Cancer Cell, 18*(4), 301-302.

Wagner, S. N., Wagner, C., Schultewolter, T., & Goos, M. (1997). Analysis of Pmel17/gp100 expression in primary human tissue specimens: implications for melanoma immuno- and gene-therapy. *Cancer Immunol Immunother, 44*(4), 239-247.

Walker, M., Makropoulos, D., Achuthanandam, R., & Bugelski, P. J. (2010). Recent advances in the understanding of drug-mediated infusion reactions and cytokine release syndrome. *Curr Opin Drug Discov Devel, 13*(1), 124-135.

Walker, M. R., Makropoulos, D. A., Achuthanandam, R., Van Arsdell, S., & Bugelski, P. J. (2011). Development of a human whole blood assay for prediction of cytokine release similar to anti-CD28 superagonists using multiplex cytokine and hierarchical cluster analysis. *Int Immunopharmacol, 11*(11), 1697-1705.

Wange, R. L., & Samelson, L. E. (1996). Complex complexes: signaling at the TCR. *Immunity, 5*(3), 197-205.

Waterhouse, N. J., Sedelies, K. A., Sutton, V. R., Pinkoski, M. J., Thia, K. Y., Johnstone, R., et al. (2006). Functional dissociation of DeltaPsim and cytochrome c release defines the contribution of mitochondria upstream of caspase activation during granzyme B-induced apoptosis. *Cell Death Differ, 13*(4), 607-618.

Weber, S., Traunecker, A., Oliveri, F., Gerhard, W., & Karjalainen, K. (1992). Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor. *Nature, 356*(6372), 793-796.

Weiss, A., & Littman, D. R. (1994). Signal transduction by lymphocyte antigen receptors. *Cell, 76*(2), 263-274.

Weiss, A., Shields, R., Newton, M., Manger, B., & Imboden, J. (1987). Ligand-receptor interactions required for commitment to the activation of the interleukin 2 gene. *J Immunol, 138*(7), 2169-2176.

Willcox, B. E., Gao, G. F., Wyer, J. R., Ladbury, J. E., Bell, J. I., Jakobsen, B. K., et al. (1999). TCR binding to peptide-MHC stabilizes a flexible recognition interface. *Immunity, 10*(3), 357-365.

Williams, M. A., Tyznik, A. J., & Bevan, M. J. (2006). Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature, 441*(7095), 890-893.

Willimsky, G., & Blankenstein, T. (2005). Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature, 437*(7055), 141-146.

Wilson, D. B., Wilson, D. H., Schroder, K., Pinilla, C., Blondelle, S., Houghten, R. A., et al. (2004). Specificity and degeneracy of T cells. *Mol Immunol, 40*(14-15), 1047-1055.

Wooldridge, L., Ekeruche-Makinde, J., van den Berg, H. A., Skowera, A., Miles, J. J., Tan, M. P., et al. (2012). A single autoimmune T cell receptor recognizes more than a million different peptides. *J Biol Chem, 287*(2), 1168-1177.

Wu, L. C., Tuot, D. S., Lyons, D. S., Garcia, K. C., & Davis, M. M. (2002). Two-step binding mechanism for T-cell receptor recognition of peptide MHC. *Nature, 418*(6897), 552-556.

Xu, D., Alegre, M. L., Varga, S. S., Rothermel, A. L., Collins, A. M., Pulito, V. L., et al. (2000). In vitro characterization of five humanized OKT3 effector function variant antibodies. *Cell Immunol, 200*(1), 16-26.

Yasukawa, M., Ohminami, H., Arai, J., Kasahara, Y., Ishida, Y., & Fujita, S. (2000). Granule exocytosis, and not the fas/fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4(+) as well as CD8(+) cytotoxic T lymphocytes in humans. *Blood, 95*(7), 2352-2355.

Yeh, S., Karne, N. K., Kerkar, S. P., Heller, C. K., Palmer, D. C., Johnson, L. A., et al. (2009). Ocular and systemic autoimmunity after successful tumor-infiltrating lymphocyte immunotherapy for recurrent, metastatic melanoma. *Ophthalmology, 116*(5), 981-989 e981.

Yokosuka, T., Sakata-Sogawa, K., Kobayashi, W., Hiroshima, M., Hashimoto-Tane, A., Tokunaga, M., et al. (2005). Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol, 6*(12), 1253-1262.

Zea, A. H., Curti, B. D., Longo, D. L., Alvord, W. G., Strobl, S. L., Mizoguchi, H., et al. (1995). Alterations in T cell receptor and signal transduction molecules in melanoma patients. *Clin Cancer Res, 1*(11), 1327-1335.

Zhang, B., Bowerman, N. A., Salama, J. K., Schmidt, H., Spiotto, M. T., Schietinger, A., et al. (2007). Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. *J Exp Med, 204*(1), 49-55.

Zhang, B., Zhang, Y., Bowerman, N. A., Schietinger, A., Fu, Y. X., Kranz, D. M., et al. (2008). Equilibrium between host and cancer caused by effector T cells killing tumor stroma. *Cancer Res, 68*(5), 1563-1571.

Zhang, L., Conejo-Garcia, J. R., Katsaros, D., Gimotty, P. A., Massobrio, M., Regnani, G., et al. (2003). Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med, 348*(3), 203-213.

Zhao, Y., Wang, Q. J., Yang, S., Kochenderfer, J. N., Zheng, Z., Zhong, X., et al. (2009). A herceptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. *J Immunol, 183*(9), 5563-5574.

Zhong, G., Zhang, S., Li, Y., Liu, X., Gao, R., Miao, Q., et al. (2010). A tandem scFv-based fusion protein and its enediyne-energized analogue show intensified therapeutic efficacy against lung carcinoma xenograft in athymic mice. *Cancer Lett, 295*(1), 124-133.

Zippelius, A., Batard, P., Rubio-Godoy, V., Bioley, G., Lienard, D., Lejeune, F., et al. (2004). Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance. *Cancer Res, 64*(8), 2865-2873.

PUBLICATIONS

Liddy, N., Bossi, G., **Adams, K. J.,** Lissina, A., Mahon, T. M., Hassan, N. J., et al. (2012). Monoclonal TCR-redirected tumor cell killing. *Nat Med, 18*(6), 980-987.

McCormack, E., **Adams, K. J.,** Hassan, N. J., Kotian, A., Lissin, N. M., Sami, M., et al. (2012). Bi-specific TCR-anti CD3 redirected T-cell targeting of NY-ESO-1- and LAGE-1-positive tumors. *Cancer Immunol Immunother*.