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Combination of Immune Stimulatory Strategies to Promote Anti-Tumour Immunity

By

Mohannad Mokhtar Fallatah

ID: 24565482

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ABSTRACT

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$CD8^+$ T cells can kill cancer cells but are tightly regulated by receptors that confer positive or negative signals. Members of the tumour necrosis factor receptor superfamily (TNFRSF) can improve the responses of antigen-specific $CD8^+$ T cells by enhancing their survival, proliferation and differentiation into effector and memory T cells. However, the roles of different members of the TNFR superfamily on $CD8^+$ T cell function has not been fully explored. The aims of this thesis were therefore to investigate the effects of stimulation through different TNFRSF members on augmenting $CD8^+$ T cell responses, controlling tumour growth and $CD8^+$ T cell differentiation into memory cells.

Initially I compared the efficacy of agonist antibodies to TNFRSF members CD27, GITR, 4-1BB and OX40 for their ability to expand adoptively transferred gp100-specific pmel-1 tumour-reactive $CD8^+$ T cells *in vivo*. Anti-CD27 was the most potent agonist and further combined with the TLR ligands PolyI:C and LPS and with inhibitors of the check-point receptor PD-1 to enhance the accumulation of T cells.

For anti-tumour immunity, adoptive T cell transfer and anti-CD27 as a monotherapy was ineffective against a lethal dose of melanoma. However anti-CD27 synergised with PD-1/L1 blockade to confer long term protection dependent on co-transfer of tumour-reactive $CD8^+$ T cells. This combination treatment increased the frequency of adoptively transferred cells, and their expression of effector molecules such as granzyme B, IFN-γ and TNF-α and the transcription factor T-bet, which is associated with T cell effector function. Interestingly in a colon carcinoma model, while anti-CD27 as a monotherapy conferred protection to a minority of mice, this did not synergise with PD-1 blockade. I found that colon carcinoma cells express less PD-L1 compared with melanoma cells suggesting that the efficacy of PD-1 blockade may depend on local concentrations of PD-L1. I then investigated whether the rat anti-mouse CD27 mAb was still effective when converted to a syngeneic isoform to move towards clinical therapy. I found that anti-CD27 of the mouse IgG1 isotype was an effective agonist whereas, when converted to a mouse IgG2a form, CD27 positive cells were depleted.

Finally, across all experiments I noted that adoptively transferred pmel1 $CD8^+$ T cells did not persist after contraction and, in contrast to previous work, IL-2 and the mTORC inhibitor rapamycin did not lead to their increased maintenance. The affinity of pmel-1 cells for gp100 peptide is relatively low. To gain insight into whether the affinity of the TCR/peptide interaction influences memory $CD8^+$ T cell generation, I made use of OT-1 TCR transgenic mice for which a range of altered peptide ligands of different affinities have been described. These data revealed that the magnitude of the primary $CD8^+$ T cell response is dependent on both peptide affinity and density. However OT-1 $CD8^+$ T cells differentiated into memory T cells and expanded equally after secondary stimulation following priming with either the low or high affinity peptide in the presence of anti-CD27. Moreover, the combination of anti-CD27 plus PD-1/PD-L1 delivered with either the low or high affinity peptide synergised for increased OT-1 $CD8^+$ T cell expansion and anti-tumour immunity.

Together my data show that anti-CD27 and PD-1/L1 blockade may be a particularly potent combination for enhancing low affinity $CD8^+$ T cells specific for cancer cells.

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Author's Declaration

I, Mohannad Mokhtar Fallatah, declare that this thesis and the work presented in it are my own and has been generated by me, as the result of my own original research.

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I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

5. I have acknowledged all main sources of help;

6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

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Abbreviations

AICD	activation induced cell death
ATP	adenosine triphosphate
ACT	adoptive T cell transfer
APC	allophycocyanin
APCs	antigen presenting cells
APL	altered peptide ligand
AMPK	aMP-activated protein kinase
ADCC	antibody-dependent cellular cytotoxicity
Ag	antigen
APC	antigen presenting cells
BTLA	B- and T-lymphocyte attenuator
BCL1	B cells leukemic 1
Blimp-1	B lymphocyte-induced maturation protein-1
BCG	bacillus calmette-guérin
BM	bone marrow
BSA	bovine serum albumin
CFSE	Carboxy fluorescein succinimidyl ester
Cat	Cathepsin
CCR7	CC-chemokine receptor 7
CLPs	common lymphoid progenitors
CDR	complementary determining regions
CDC	complement-dependent cytotoxicity
cDCs	conventional or classical DCs
CPM	count per minutes
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T-lymphocyte associated antigen-4
DISC	death inducing signaling complex
DR3	death receptor 3
DR5	Death receptor 5
DCs	dendritic cells
DN	double negative
DP	double positive
dsRNA	double-stranded RNA
DMEM	dulbecco's Modified Eagle Medium
EAE	encephalomyelitis
ER	endoplasmic reticulum
ERAP	endoplasmic reticulum aminopeptidase
ELISA	enzyme-linked immunosorbent assay
Eomes	eomesodermin
EDTA	Ethylenediaminetetraacetic acid
FasL	Fas ligand
FADD	Fas-associated protein with death domain
FAO	fatty acid β-oxidation
FcR	Fc receptors
FCS	Fetal calf serum

FITC	fluorescein isothiocyanate
FDA	food and drug administration
FoxO1	forkhead box protein O1
Fab	fragment of antigen binding
GI	gastrointestinal
GM-CSF	granulocyte macrophage colony-stimulating factor
T_{FH}	helper T cells
Th1	helper T cells 1
HRP	horseradish peroxidase
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HPV16	human Papilloma Virus type 16
HPV	human papillomavirus
iDCs	immature DCs
TIM-3	immunoglobulin mucin-3
IgSF	immunoglobulin superfamily
TRAIL	immunoreceptor tyrosine-based activation motifs (ITAM TNR-related apoptosis-inducing ligand receptor)
ITIM	immunoreceptor tyrosine-based inhibitory motif
ICOS	inducible co-stimulatory
INF-γ	interferon-γ
IL-2	interleukin-2
i.d	intradermally
KLRG-1	killer cell lectin-like receptor G1
KIRs	liller immunoglobulin-like receptors
LPS	Lipopolysaccharide
LAG3	lymphocyte-activation gene 3
LCMV	lymphocytic Choriomeningitis Virus
MHC	major histocompatibility complex
MFI	Mean fluorescent intensity
MDA5	melanoma differentiation-associated protein 5
MDSCs	myeloid derived suppressor cells
NIH	national Institutes of Health
NK	natural killer
NFAT	nuclear factor of activated T cells
ODNs	oligodeoxynucleotides
OVA	ovalbumin
PAMPs	pathogen-associated molecular patterns
PRRs	pattern-recognition receptors
PBLs	peripheral blood lymphocytes
PI3K	phosphatidylinositol 3-kinase
PE	phycoerythrin
pDCs	plasmacytoid DCs
PARP	poly ADP-ribose polymerase
PD-1	programmed cell death-1
PAP	prostatic acid phosphatase
ROS	reactive oxygen species

rmIL-2	recombinant mouse IL-2
rpm	revaluation per minute
RT	room temperature
RPMI	Roswell Park Memorial Institute
SARS	severe acute respiratory syndrome
STAT4	signal transducer and activator of transcription 4
SIV	simian immunodeficiency virus
SP	single positive
s.c.	subcutaneousl
T_{CM}	T central memory
Tfh	T follicular helper
TLRs	the Toll like receptors
TRAFs	TNFR-associated factors
TAP	transporter associated with antigen processing
TAA	tumour associated antigen
TNFRSF	Tumour necrosis factor receptor superfamily
TNF-α	tumour necrosis factor-α
TME	Tumour microenvironment
TDLN	tumour-draining lymph nodes
Type-I INF	type I interferons
WT	wild type
βTMD	β-chain trans membrane domain
γ_c	Common γ chain

Chapter 1. Introduction

In 1900s, Paul Ehrlich was one of the first scientists to hypothesize that the immune system may protect the body from cancer (Schreiber et al., 2011, Teng et al., 2008). However, tools to study and understand the function of the immune system were needed to verify this hypothesis. Years later, Burnet and Thomas built their "cancer immunosurvellance" hypothesis that propose that lymphocytes recognize and eliminate precancerous cells before they develop into clinically visible tumours (Schreiber et al., 2011, Teng et al., 2008). This hypothesis has been expanded into a broader term "cancer immunoediting" after realization that the immune system does not only control tumour quantity, but also tumour quality (Schreiber et al., 2011, Khong and Restifo, 2002). Cancer immunoediting consist of three main phases "the three Es of cancer immunoediting": elimination, equilibrium and escape. The elimination phase corresponds to the immunosurvillavce hypothesis in which new growing tumour cells are recognized and eliminated by innate and adaptive immune cells (Schreiber et al., 2011, Dunn et al., 2004)**.** If tumour cells are not completely eliminated by the immune system during the elimination phase an equilibrium phase develops, that could last for many years. The equilibrium phase is a balanced state between immune cells and tumour cells. The long interaction time between tumour and immune cells can result in dramatic changes in the phenotype of the developing tumours. Finally, the immune system contributes to the selection of tumour variants; these cells are less immunogenic and capable of "escape" from the immune system (Khong and Restifo, 2002, Schreiber et al., 2011)**.**

During the scape phase, different T cell subsets including effector, helper and regulatory T cells as well as myeloid derived suppressor cells (MDSCs) infiltrate into the tumour microenvironment and control T cell fitness and effector function within the tumour. There are also additional factors that may restrict T cell function in the tumour microenvironment, for instance the abundance and expression patterns of the co-inhibitory and co-stimulatory receptors and their ligands (Norde et al., 2012, Pardoll, 2012). Understanding these factors will help in shifting the balance in the equilibrium phase toward the immune system and enable us to find novel therapeutic strategies to eliminate the tumour.

1.1. Overview of the immune system

1.1.1. Innate Immunity

Higher vertebrates rely on innate and adaptive immune cells to fight pathogens. Innate immune cells are relatively antigen nonspecific and act quickly to counter invading microorganisms. Neutrophils, eosinophils, basophils, macrophages and dendritic cells (DCs) mediate the innate immune response (Flannagan et al., 2012, Akira et al., 2006). Cells of the innate immune system are responsible for digesting dying cells, and also for the recognition of invading microorganisms (Akira et al., 2006). The recognition of foreign bodies such as bacteria, parasites, viruses and fungi is mediated by cell surface receptors called pattern-recognition receptors (PRRs), such as the Toll like receptors (TLRs) 1,2 and 5. These receptors can sense microbial components (which are constitutively found in microorganisms) known as pathogen-associated molecular patterns (PAMPs) (Blasius and Beutler, 2010, Kumar et al., 2011). Different PRRs recognise specific PAMPs and can lead to different immune responses (Akira et al., 2006). TLRs can also be expressed intracellularly within the endoplasmic reticulum (ER), endosomes or lysosomes and include TLR3, 7, 8 and 9 (Blasius and Beutler, 2010, Akira et al., 2006). Intracellular TLRs recognise nucleic acids derived from various viruses and bacteria (Akira et al., 2006, Blasius and Beutler, 2010). Innate immune cells play key roles in initiating adaptive immune responses by providing co-stimulatory ligands, secreting proinflammatory cytokines, chemokines, and by introducing fragments of the pathogen to adaptive immune cells (Akira et al., 2006, Kumar et al., 2011).

The complement system is considered to be part of the innate immune system (Dunkelberger and Song, 2010, Sarma and Ward, 2011). Complement proteins are found in soluble or membrane-associated forms (Dunkelberger and Song, 2010). Activation of the complement system contributes in clearing invader pathogens directly by lysing target pathogens, or indirectly by activating immune cells via the secretion of proinflammatory cytokines, or by enhancing phagocytosis by oposinised invader cells (Dunkelberger and Song, 2010).

Natural killer cells (NK) have been classified as lymphocytes based on their origin and morphology (Vivier et al., 2011). NK cells however, are considered as part of the innate

immune system because NK cells lack antigen-specific receptors. NK cells recognise foreign bodies manly via TLRs, and play a key role in killing virus-infected cells, immature DCs (iDCs) as well as tumour cells (Vivier et al., 2011, Della Chiesa et al., 2014). NK cells can be activated by direct recognition of changes in the cell-surface glycoproteins of infected or transformed malignant cell. Activation of NK cells is integrated by two types of surface receptors, activating receptors including killer cell immunoglobulin-like receptors (KIRs) and C-type lectin such as killer lectin-like receptors (KLRs), and inhibitory receptors specific for MHC-I (Murphy et al., 2008). Normal MHC-I expression prevents NK cell-mediated killing, while low levels of MHC-I on the cell surface of the target cell induce NK cell killing (Murphy et al., 2008). NK cells also express receptors for immunoglobulin. The interaction of immunoglobulin receptor with cognate antibody induces antibody-dependent cellular cytotoxicity (ADCC) (Thielens et al., 2012, Campbell and Purdy, 2011). Upon activation, NK cells secrete growth factors like granulocyte macrophage colony-stimulating factor (GM-CSF), and cytokines such as interferon-γ (INF-γ) and tumour necrosis factor-α (TNF-α) to restrict pathogen infection, and also prime of helper T cells 1 (Th1) responses (Vivier et al., 2011, Vivier et al., 2008, Smyth et al., 2005). Perforin and granzyme granule-mediated exocytosis via the immune synapse is the main killing mechanism for NK cells (Smyth et al., 2005). Signalling via TNF death receptors such as the Fas/Fas ligand (FasL) pathway is another mechanism whereby NK cells kill infected as well as transformed cells (Smyth et al., 2005, Zamai et al., 1998).

1.1.2. Adaptive immune system

Adaptive immune cells on the other hand are composed of two major types, B cells (responsible for antibody-mediated humoral immunity) and T cells (responsible for the cellular-mediated immune response). Both cell types possess cell surface receptors that are antigen specific (Smith-Garvin et al., 2009, LeBien and Tedder, 2008). Unlike innate immune cells, B and T cells generate long lasting memory cells. A more detailed description of T cells follows.

1.2. Overview of T cell subsets

T cells express either CD4 or CD8 on their surface. $CD8^+$ and $CD4^+$ T cells recognise different MHC/peptide complexes with $CD8^+$ T cells responding to peptide in association with MHC I while $CD4^+$ T cells recognise MHC II/peptide complexes. Therefore, they recognise antigen from different sources.

1.2.1. $CD4^+$ T cells

$CD4^+$ T cells can be further subdivided into regulatory $CD4^+$ T cells (T_{reg}) which regulate immune responses and effector $CD4^+$ T cells that promote immunity. Effector $CD4^+$ T cells can enhance phagocytosis, antigen presentation by APCs, help B cells to secrete antibodies and orchestrate the immune response against a wide variety of microorganisms and tumour cells(Zhu et al., 2010, Zhu and Paul, 2008). Effector $CD4^+$ T cells enhance DC function by direct cell-cell contact via the ligation of the co-stimulatory receptor CD40 on DCs with the ligand (CD40L) on $CD4^+$ T cells (Cella et al., 1996, Ridge et al., 1998). This interaction induces expression of co-stimulatory molecules on DCs such as B7.1 (CD80) and B7.2 (CD86) as well as cytokine production like IL-12 (Ridge et al., 1998, Taraban et al., 2006, Ma and Clark, 2009). Once activated, effector $CD4^+$ T cells can differentiate into different $CD4^+$ T cell subtypes, that include but are not restricted to Th 1, Th2 and Th17 and T follicular helper (Tfh) cells. Th1 cells play an important role in priming $CD8^+$ responses through DC activation by CD40/CD40L contact. $CD4^+$ Th1 cells can also enhance CTL-mediated antitumor responses by providing interleukin-2 (IL-2) cytokine (Ekkens et al., 2007). Th1 cells also produce cytokines like INF-γ and TNF-α (Romagnani, 1999), whereas Th2 cells drives the humoral immunity, and provide protection against helminths and other extracellular pathogens (Ekkens et al., 2007, Paul and Zhu, 2010). Th2 cells secret IL-4, IL-5, IL-9, IL-13, and TNF-α. Th1-type cytokines are involved in proinflammatory responses that eliminate infected cells (Berger, 2000). Th1 cells induce macrophage activation through INF-γ secretion. INF-γ enhances macrophages to destroying intracellular mycobacteria. Uncontrolled proinflammatory responses can cause autoimmunity (Romagnani, 1999). Th2 cells have been reported to regulate Th1 mediated immune responses and vice versa (Berger, 2000). Th2 cells can also enhance antibody secretion and isotype class switching by B cells to IgE

through IL-4 secretion. The interaction of antigen specific-IgE with FcRI on basophils, eosinophils and mast cells can promotes hypersensitivity reactions (Stone et al., 2010). Th17 cells produce IL-17, IL-21 and IL-22 cytokines (Romagnani, 1999). Th17 cells have been implicated in some autoimmune diseases although their function as helper cells is not fully understood. However, Mitsdoerffer M. *et al,* have shown that Th17 cells can help B cells mature into effector cells and enhance their capacity for antibody secretion and isotype class switching (Mitsdoerffer et al., 2010). IL-17 mediates neutrophil recruitment at the site of infection by promoting local cells to secrete cytokines and chemokines that recruit neutrophils (Crome et al., 2010, Murphy et al., 2008). Th17 cells are involved in multiple inflammatory diseases including asthma, mediated by neutrophil reactivity (Dias and Banerjee, 2013, Cosmi et al., 2011).

1.2.2. Regulatory T cells (T_{reg})

CD4+ T_{reg} cells are identified by their high expression of the IL-2 receptor α chain (CD25), and forkhead box P3 (Foxp3) a transcription factor that is involved in T_{reg} development and function (Miyara and Sakaguchi, 2007). T_{reg} cells inhibit effector T cell functions through inhibitory receptors that include cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and lymphocyte activation gene 3 (LAG3, or CD233). Ligation of these inhibitory receptors with their cognate ligands (CTLA-4 interacts with CD80 (B7-1) or CD86 (B7-2), while LAG3 interacts with (MHC-II receptor) inhibit the priming and differentiation of effector T cells (Hemon et al., 2011, Blank et al., 2006). T_{reg} cells have been reported to form aggregates around DCs, which results in down-regulation of CD80 and CD86 on activated DCs and supresses up-regulation of CD80 and CD86 on immature DCs (Onishi et al., 2008). This process is CTLA-4 dependent (Onishi et al., 2008). In addition to cell to cell contact mediated suppression, T_{reg} cells also suppress activated cells by secreting soluble cytokines such as IL-10 (Miyara and Sakaguchi, 2007). IL-10 has been shown to reduce inflammation particularly in the gut and lung mucosa (Groux and Cottrez, 2003, Iyer and Cheng, 2012, Smallie et al., 2010, Lentsch et al., 1996). Roers *et al.* have reported that IL-10 deficient mice develop colitis (Roers et al., 2004). Furthermore, IL-10 is implicated in reducing the lytic activity of both $CD4^+$ and $CD8^+$ T cells (de la Barrera et al., 2004). In addition to IL-10, T_{reg} cells also secrete TGF-β to supress immune responses by inhibiting the proliferation and cytokine

production of B and T cells, and promoting the generation of T_{reg} cells by inducing the transcription factor Foxp3 (Wan and Flavell, 2008). IL-35 is another inhibitory cytokine that is preferentially expressed by T_{reg} cells. IL-35 seems to be required for T_{reg} maximal suppression activity. However, the mechanism by which IL-35 inhibits effector cells is yet fully understood (Vignali et al., 2008).

1.2.3. $CD8^+$ T cells

$CD8^+$ T cells reduce viral infection and intracellular pathogens by direct killing of infected cells (Prlic et al., 2007, Zhang and Bevan, 2011). $CD8^+$ T cells are considered as potent immune cells that have the ability to suppress and/or eradicate different tumour cells (Prlic et al., 2007). My thesis is concerned mainly with the activation of $CD8^+$ T cells and a more detailed description of $CD8^+$ T cell development and function therefore follows.

1.3. T cell development

T cell maturation from T cell progenitors takes place in the thymus. Because progenitors are not self-renewing, continuous recruitment of hematopoietic precursors from the bone marrow (BM) is crucial to maintain long-term thymopoiesis (Koch and Radtke, 2011). The Weissman group in 1997 showed that common lymphoid progenitors (CLPs) are the main BM population that gives rise to T cells in the thymus (Koch and Radtke, 2011). CLPs enter the thymus through high endothelial venules where they face three fate decisions, death by neglect, death by negative selection or survival by positive selection as described below.

1.3.1 T cell receptor assembly

The journey of thymocyte development starts when progenitor cells enter the cortico-medullary junction. Thymocytes in this anatomical region are $CD4^-CD8^-$ double negative (DN) (Murphy et al., 2008). About 20% of total DN cells contain genes encoding $\gamma{:}\,\delta$ T cell receptor; another 20% of DN cells express genes encoding $\alpha{:}\,\beta$ T cell receptor (Murphy et al., 2008). The DN stage can be divided into four distinct stages according to the adhesion molecules expressed on the cell surface of thymocytes. In the first DN stage (DN1),

thymocytes express CD44 receptor. As thymocytes mature, they upregulate CD25 receptor ($CD4^-$ $CD8^-$ $CD44^+CD25^+$) (DN2), and CD44 receptor starts to downregulate ($CD4^-$ $CD8^-$ $CD25^+CD44^-$) (DN3). (Murphy et al., 2008). In the DN4 stage, thymocytes down regulate CD25 ($CD25^-CD44^-$). The formation of the TCRβ precedes simultaneous expression of both CD4 and CD8 molecules: the double positive (DP) stage ($CD4^+CD8^+$ cells) where the α chain of the TCR CD3 molecules that provide the signalling of the TCR are also assembled (Kreslavsky and von Boehmer, 2010). The final TCR composes two chains (α and β), each of which contains three loops or complementarity determining regions (CDR) that together form the binding region that will contact peptides loaded on MHC-I or II expressed on resident DCs and/or thymic epithelial cells (Goldrath and Bevan, 1999). Loops one and two of the TCR complex are encoded by the V gene segment. The CDR three loop on the other hand is generated by the juxtaposition of the V(D)J segment and provides more diversity (Goldrath and Bevan, 1999).

1.3.2. Negative selection

In the negative selection stage, immature DP thymocytes that have strong binding affinity for self-peptide and either MHC-I or II undergo programmed cell death or apoptosis whereas modest or low binding affinity leads to single positive (SP) cells, i.e. $CD8^+$ or $CD4^+$ single-expressors by down regulation of CD4 or CD8 (Takahama, 2006). In contrast, DP thymocytes that fail to recognise self-antigen/MHC are eliminated by neglect. Those SP thymocytes that survive then migrate to the medulla. Some cells with moderately high affinity to self-peptide/MHC-II escape deletion. Final naïve immature T cells are characterized by the expression of lymph node homing receptors CD62L (or L-selection), CCR7 and low CD44 expression on the cell surface (Takahama, 2006, Sprent and Surh, 2011).

T cell development in the thymus is now complete. Mature $CD4^+$ and $CD8^+$ T cells then migrate to the blood or the lymphatic system via the perivascular space (Takahama, 2006).

1.4. Naïve $CD8^+$ T cell survival in the periphery

In the periphery naïve $CD8^+$ T cells require survival signals for maintenance (Brown et al., 2005, Ferreira et al., 2000). Different groups have shown that the interaction of $CD4^+$ T cells

with MHC-II/self-peptide complexes on DCs is vital for long-term survival, while $CD8^+$ T cells interact with MHC-I/self-peptide for homeostatic proliferation (Brown et al., 2005, Freitas and Rocha, 1999, Gruber and Brocker, 2005). Polic and colleagues ablated the TCR by targeting the constant region after thymic development. They found that T cells decreased with a half-life of about 16 days compared to 162 days without TCR depletion. In another experiment, transgenic $CD8^+$ T cells were transferred into MHC-I deficient or wild-type mice; $CD8^+$ T cells decayed in the hosts that lacked MHC-I in the periphery, indicating the importance of continuous self-peptide MHC-I/II interaction for T cell survival (Brown et al., 2005).

In addition to the TCR/self-peptide MHC interaction, soluble factors such as IL-7, which is produced mainly by stromal cells in the bone marrow and secondary lymphoid organs, has been reported to be essential for $CD8^+$ T cell survival, both during their maturation in the thymus and in the periphery (Brown et al., 2005). Seddon *et al.* targeted the IL-7R *in vivo* using monoclonal antibody (mAb), and showed that the half-life of $CD8^+$ T cells was reduced to about 28 days compared to a control group in which $CD8^+$ T cells did not decline significantly (Brown et al., 2005). Common γ chain cytokines including IL-2, IL-4, IL-15 and have also been shown to be critical for T cell homeostasis (Masse et al., 2007, Boyman et al., 2007). Work performed by Masse and others has shown that common γ chain deficient (γ_c^-) $CD4^+$ T cells displayed decreased survival parameter (detected by low anti-apoptotic Bcl-2 expression) and were smaller in size (Masse et al., 2007).

1.5. Antigen-specific $CD8^+$ T cells

Very few T cells are specific for a given foreign antigen; estimated to be 1 in 100,000 cells within the $CD8^+$ T cell population (Zehn et al., 2009). When the host becomes infected with a microorganism, antigen-specific CD8+ T cells undergo mitosis, differentiate and become cytotoxic T lymphocytes (CTL) (Zehn et al., 2009). Single naïve T cells can undergo up to 15 divisions (Sarkar et al., 2008). After this expansion phase which usually last for 7-8 days in mice and 14 days in human, most of the expanded cells undergo apoptosis; a small

proportion continues dividing to become memory cells (Sarkar et al., 2008, Zhang and Bevan, 2011, Prlic et al., 2007).

1.6. $CD8^+$ T cell priming

In the normal situation, naïve $CD8^+$ T cells circulate throughout the secondary lymphoid organs screening for antigens. These cells have a very low binding affinity to self-peptide MHC-I and the contact duration between the TCR and the MHC-I is very short (Zehn et al., 2009, Gainey et al., 2012, Chieppa et al., 2006). During infection, APCs mainly the DCs, uptake pathogens, migrate to the draining lymph nodes particularly in the T cell zone and present antigen fragments on their cell surface via MHC-I/II. MHC/peptide -specific T cells then bind with greater affinity and for a longer time period, providing the first signal essential for T cell activation (Heath et al., 2004, Steinman, 1991, Chieppa et al., 2006). Activated DCs also provide a stimulatory signal (signal 2) which is important for optimal T cell activation (Heath et al., 2004). These two activation signals along with growth factors, inflammation and $CD4^+$ T cell help drive naïve $CD8^+$ T cells to become CTL and undergo proliferation and differentiation (Guermonprez et al., 2002, Steinman, 1991). $CD8^+$ T cell co-stimulation is the major focus of my PhD. Therefore, co-stimulation will be described in detail in a later section.

1.7. MHC-I restricted antigen presentation and cross-presentation

DCs can prime naïve antigen-specific $CD8^+$ T cells by direct antigen presentation on MHC-I when DCs are the infected cells, as was shown by intracellular imaging in the case of infection with vaccinia virus (Joffre et al., 2012, Chieppa et al., 2006). Direct antigen presentation starts by degrading the protein into oligopeptide fragments by the proteasome (Rock et al., 2010). Oligopeptides undergo further trimming in the cytoplasm by aminopeptidase into smaller peptides and then translocated into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) (Rock et al., 2010). In the ER, some polypeptides can be further trimmed by endoplasmic reticulum aminopeptidase 1

and 2 (ERAP1 and ERAP2 respectively) to generate the optimal peptide size, 7-9 amino acids, for loading onto nascent MHC-I. The newly synthesized peptide-MHC-I complex is then exposed rapidly to the cell surface through the Golgi apparatus (Rock et al., 2010).

DCs can also present antigen indirectly in a process known as “antigen cross-presentation”; where foreign antigen is taken up by uninfected DCs from neighbouring infected cells and presented on MHC-I (Heath et al., 2004). Furthermore, DCs can present pathogen-derived antigen by “cross-dressing”. In this mechanism a pre-formed peptide/MHC-I complex is transferred from the surface of an infected cell to DC without the need for further antigen processing (Leavy, 2011, Wakim and Bevan, 2011).

There are two identified pathways whereby exogenous antigen can be processed and presented to the CTLs by the DCs (i.e. cross-presented), these two pathways are the cytosolic and the vacuolar pathways (Rock and Shen, 2005).

1.7.1. The cytosolic pathway

In the cytosolic cross-presentation pathway exogenous antigen is engulfed and remains temporarily within the phagosome. In the phagosome, DCs raise the pH to avoid peptide degradation by proteolytic enzymes (Rock et al., 2010). Antigen escapes and released in the cytoplasm and degraded into small peptides before they are translocated into in the ER. The optimal peptide length is then loaded onto MHC-I and presented on the cell surface T cell through Golgi apparatus (Rock et al., 2010).

1.7.2. The vacuolar pathway

In contrast to the cytosolic pathway, the vacuolar pathway is independent of the proteasome and TAP, but is inhibited by cysteine protease inhibitors (Rock et al., 2010). Therefore, antigen is most likely processed within the phagosome itself. Cathepsin (Cat) S seems to play a critical role in this pathway as Cat S-deficient mice have a defect in cross-presentation but not with the cytosolic pathway. The importance of Cat is not fully understood, it possibly cleaves antigen into 8 to 9 amino acid fragments (Rock et al., 2010).

1.8. Cross-presentation by DC subsets

DCs can be divided based on their tissue distribution, surface markers and function into two distinct populations, conventional or classical DCs (cDCs) and plasmacytoid DCs (pDCs) (Joffre et al., 2012, Segura and Villadangos, 2009). pDCs accumulate in the blood and lymphoid tissues and enter LNs via blood vessels. In the steady state, pDCs express low levels of CD11c integrin, MHC-II and co-stimulatory molecules (Merad et al., 2013). Plasmacytoid DCs (pDCs) express a narrow range of PRRs, mainly TLR7 and TLR9 (Merad et al., 2013). Once they encounter a foreign antigen, pDCs release high amounts of type I IFN, and acquire the ability to present foreign Ag (Merad et al., 2013). Conventional DCs (cDCs) on the other hand are located in lymphoid and nonlymphoid organs (Merad et al., 2013, Dresch et al., 2012). They can be divided into resident or migratory DCs (Villadangos and Schnorrer, 2007). cDCs have the ability to take up environmental and cell-associated antigen. cDCs are considered superior APCs due to the potency of their Ag processing and presenting (Joffre et al., 2012), their distribution within the body (their localisation within the spleen marginal zone in the steady state increases the chance of encounter with both tissue and blood Ag) and finally their ability to migrate with loaded Ag to the T cell zone in the LNs (Merad et al., 2013, Dresch et al., 2012).

Langerhans cells are another specialized subset of migratory DCs present within the epidermal layers of the skin (Romani et al., 2010, Merad et al., 2008). Langerhans cells take up and process microbial antigens and migrate to secondary lymphoid organs where they meet with naive T cells (Merad et al., 2008, Romani et al., 2010). Finally, inflammatory DCs are another DC subset that are derived from monocytes during inflammation (Romani et al., 2010, Hespel and Moser, 2012).

$CD8^+$DCs have been described for their ability to cross-present Ag. This process is crucial to generate CTL immunity, against pathogens and tumour cells (Heath et al., 2004). Work performed by Matheoud D and colleagues tested cross-presentation of ovalbumin (OVA) to naïve $CD8^+$ T cells in vivo. Bone marrow-derived DCs were first incubated with L-OVA cells (L cells transfected with OVA only expressed in the cytoplasm (Matheoud et al., 2011)) *in vitro.* Then, they were purified and injected into C57BL/6 mice that had previously received Carboxy fluorescein succinimidyl ester (CFSE)-labelled naive OVA-specific (OT-1) $CD8^+$ T cells.

Three days after DC transfer, 50% of OT-1 $CD8^+$ T cells proliferated, 60% were $CD44^+$ (indicating activation) and ~66% produced INF-γ whether antigen donor cells were live or apoptotic (Matheoud et al., 2010). This group also reported that Ag cross-priming by DCs is efficient to eliminate tumour cells *in vivo*. Bone marrow-derived DCs were incubated with live B16 melanoma cells *in vitro,* and then irradiated to avoid transferring live tumour cells. Irradiated DCs were injected twice into recipient mice prior to challenge with live B16 melanoma. Mice immunised with DCs incubated with live B16 tumour cells were protected against tumour growth (Matheoud et al., 2010).

1.9. Cross-presentation and cancer

It is not fully understood which DC subtypes are responsible for the cross-presentation of tumor associated antigen (Joffre et al., 2012). Type I interferons (Type-I INF) have been shown to be important for tumor elimination by enhancing $CD8^+$DC survival and antigen presentation by reducing antigen degradation in the endocytic compartment suggesting that $CD8^+$ DC are a likely candidate (Joffre et al., 2012). Results from a recent study have shown that $CD11b^+CD169^+$ macrophages can also cross-present tumor antigen in the tumor draining LNs and that $CD169^+$ depletion impairs antigen cross-presentation. Currently, it is unclear which of these two APCs play the major role in terms of cross–presenting tumor antigen; of note $CD8^+$DCs also express CD169 (Joffre et al., 2012).

1.10. $CD8^+$ T cell killing mechanisms

After initial activation, CTLs exert effector mechanisms to kill target cells (Harty and Bevan, 1996). Kagi et al. suggested that CTLs and natural killer cells (NKs) kill their target cells in two different ways; the first pathway involves membrane disruption by perforin and cytotoxic granzymes. The second pathway is mediated by the ligation of death receptors on the target cell e.g. FAS ligand (FASL) and by its receptor FAS (CD95) on CTLs or NKs (Russell and Ley, 2002, Shresta et al., 1998, Walsh et al., 1994). FAS/FASL interactions have been reported to be important for eliminating self-reactive lymphocytes and thus, protect the host from autoimmunity (Van Parijs and Abbas, 1996). Both pathways lead to cell death or (apoptosis). CTLs also produce cytokines including TNFα and INF-γ which activate and increase the efficacy of other effector cells such as neutrophils and macrophages (Harty et al., 2000). INF-

γ also clears cytoplasmic viral nucleocapsids and reduces replicative DNA in infected cells in a noncytolytic process (Rodriguez et al., 2003, Lehmann-Grube et al., 1985, Pasquetto et al., 2002, Guidotti et al., 1999). The role of TNFα in clearing viral infection has been shown by Pasquetto V. and colleagues who showed that TNFα synergises with INF-γ to inhibit hepatitis B virus (HBV) replication (Pasquetto et al., 2002).

1.10.1. How does granule exocytosis cause apoptosis?

Several experiments suggest that perforin works as a gateway for cytotoxic granules by forming pores in the plasma membrane of target cells allowing granzymes to enter the cytosol and kill the cell through DNA fragmentation (Russell and Ley, 2002, Trapani and Smyth, 2002). A more recent model suggests that granzymes A, B and H can be internalized in a perforin-independent pathway through receptor-mediated endocytosis via e.g. the mannose 6-phosphate receptor on the target cell (Trapani and Smyth, 2002). However, perforin was important for releasing the granzyme from the endosome. More importantly, perforin forms pores in the membrane of target cells that are crucial for complete cell damage (Lord et al., 2003, Barry and Bleackley, 2002). Both granule components are important in this killing mechanism as the lack of perforin or granzyme impairs CTL killing efficacy and protection against intracellular pathogens (Lord et al., 2003, Walsh et al., 1994, Kagi et al., 1996, Stenger et al., 1998).

Granzymes are serine proteases stored within CTLs and NK cells in granules. Granzymes A, B, C, D, E, F, G, K, L, M and N are found in mice, while granzymes A, B, H, K and M are more restricted to human (Cullen and Martin, 2008). Among all the different granzymes in mice and humans, granzyme A and B are the most abundant and thus, they are the most intensively studied (Chowdhury and Lieberman, 2008).

The process of CTL-mediated killing mechanism starts by forming cytolytic immunological synapse between CTL and the target cell, allowing cytotoxic granules to migrate toward the target cell. Cytolytic immunological synapse also prevent cytotoxic granules from leakage to neighboring cells (Dustin and Long, 2010). Perforin facilitates granzyme entry into the target cells. In the cytosol of the target cell, apoptosis mediated by granzyme B can be initiated in two different pathways, directly through caspase processing and activation, and indirectly through a BID-dependent pathway. The caspase-dependent pathway is initiated mainly by

cleaving caspase-3 which subsequently leads to poly (ADP-ribose) polymerase (PARP) cleavage. PARP cleavage facilitates the apoptotic process in target cells (Cullen and Martin, 2008, Koh et al., 2005, Yu et al., 2002). On the other hand, the BID-dependent mitochondrial pathway starts by activating the pro-apoptotic Bcl-2 family member BID. BID translocates to the mitochondria and activates BAX and BAK (two pro-apoptotic members of BCL2) which leads to the release of the cytochrome *c* and cell death (Cullen and Martin, 2008, Lindsten et al., 2000). Summary of perforin/granzyme killing mechanism is shown in (Fig 1-1).

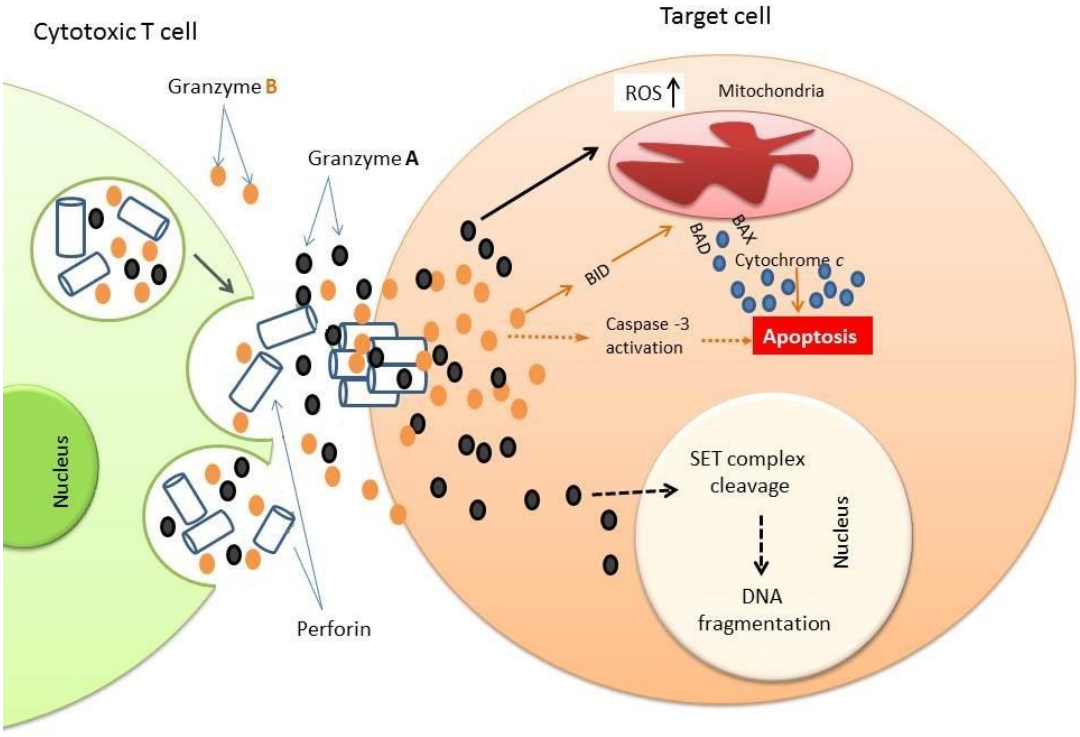


Figure 1-1: Perforin/Granzyme-mediated killing mechanisms. Lytic granules migrate toward the site of contact following target cell recognition by CTL and release perforin and granzyme. Perforin and granzyme. Perforins form pores on the target cell plasma membrane and facilitate granzyme entry into the cytosol. Once in the cytosol, granzyme B (orange colour) induces apoptosis by either activating caspase-3 processing which subsequently activates PARP cleavage and cells death (straight lines), or by activating the pro-apoptotic BID protein. BID translocats into the mitochondria and activates BAK and BAX which increase mitochondrial outer membrane permeabilisation, leading to cytochrome *c* release and apoptosis (dotted orange arrows). Granzyme A (black colour) initiates cell death by increasing ROS release out of the mitochondria (straight black arrows). Granzyme A can also translocate into the nucleus by unknown mechanism and cleave the SET complex within the nucleus resulting in DNA fragmentation and cell death (Black dotted arrows).

Granzyme A can be produced by CTLs and NK cells. In humans, granzyme A has minimal cytotoxic activity even at high concentrations (Lieberman, 2010, Voskoboinik et al., 2015). Granyme A-mediated apoptosis is generally slower when compared to granzyme B-mediated cell death, and apoptosis is induced in a caspase-independent mechanism (Cullen and Martin, 2008). In addition, the outer membrane of the mitochondria remains intact, thus, the cytochrome *c* is not released (Lieberman, 2010). When granzyme A is released in the cytosol of the target cell, the first key act is to disturb inner membrane-associated electron transport complex I, leading to reactive oxygen species (ROS) production and cell death as a consequence (Lieberman, 2010). Granzyme A can also be concentrated in the nucleus by unknown mechanism and initiates nuclear damage by converting the SET complex (consists of base excision repair (BER) endonuclease Ape1, an endonuclease NM23-H1, and a 5'–3' exonuclease Trex1) into a DNA destructive machine. SET cleavage activates the NM23-H1 endonuclease resulting in a single strand DNA damage and apoptosis (Lieberman, 2010). Granzyme A can also open up chromatin by cleaving the linker histone H1 and removing the tail from the core histone making the DNA susceptible to nucleases (Lieberman, 2010). In addition, granzyme A-mediated killing can disrupt the nuclear envelope by cleaving lamins (Lieberman, 2010).

1.11. $CD8^+$ T cells and memory

After initial activation and proliferation of CTLs, 90-95% of effector $CD8^+$ T cells die, leaving 5-10% to become memory cells that live for many years and respond rapidly to subsequent antigen re-encounter (Harty and Badovinac, 2008). $CD8^+$ memory T cells are characterised by their persistent proliferation in the absence of antigen in response to cytokines such as IL-2, IL-7 and IL-15 (Youngblood et al., 2010, Klebanoff et al., 2006, Schluns et al., 2000, Tan et al., 2001). Unlike naive T cells, longevity and turnover of both memory T cells is largely MHC independent (Surh and Sprent, 2008).

1.11.1. Memory CD8$^+$ T cell subsets

CD8$^+$ memory T cells can be classified based on immediate effector function and expression of homing receptors into two distinct populations, central memory (T_{CM}) and effector memory T cells (T_{EM}). T_{CM} express L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7), important for migration to draining lymph nodes and cellular extravasation (Kaech et al., 2002). On the other hand, T_{EM} cells do not express these molecules and therefore they predominantly populate the peripheral tissues and inflammatory sites (Wherry and Ahmed, 2004). Stem cell-like memory T-cells (T_{SCM}) are another subset which have the capacity to differentiate into effector, central and effector memory T cells after antigen reencounter (Gattinoni et al., 2011). These cells have enhanced proliferative and survival capacities compared with central and effector memory T cells (Gattinoni et al., 2011). More recently, studies have identified a new subset of memory T cells called resident memory T cells (T_{RM}); this memory T cell subset is found in peripheral tissues.

Memory CD8$^+$ T cell subsets provide distinct protective immunity. T_{CM} cells produce high amounts of IL-2, and they have the ability to proliferate and differentiate into effector cells in response to Ag stimulation (Sallusto et al., 2004). T_{EM} cells in contrary to T_{CM} cells do not express CD62L, therefore, T_{EM} cell activation can be induced by the interaction with nonprofessional APCs in a milieu that does not require stable cell-cell contact (Sallusto et al., 2004, Schenkel and Masopust, 2014). T_{EM} cells contain high amounts of perforin, and display rapid and immediate effector function. Within few hours following Ag stimulation, T_{EM} cells produce high amounts of IFN-γ, IL-4 and IL-5 (Sallusto et al., 2004, Schenkel and Masopust, 2014). Results from *In vivo* study showed that vaccination route determines memory T cell subset (Wherry et al., 2003). Injecting mice with LCMV systemically induced T_{CM} response, while local injection of Sendai virus generated T_{EM} response (Wherry et al., 2003). T_{RM} cells provide potent recall responses with high proliferative capacity. Because T_{RM} cells are anatomically located in tissues that form a barrier against the outside environment, therefore, they are positioned to provide immediate response against invasive pathogens (Schenkel and Masopust, 2014).

1.12. Co-stimulatory signals (positive regulatory pathway)

Optimal $CD8^+$ T cell priming and differentiation into memory cells requires co-stimulatory signals. This is the focus of my PhD and is described in more detail below.

Most co-stimulatory receptors are members of two large families, the immunoglobulin superfamily (IgSF) or the tumour necrosis factor receptor superfamily (TNFRSF). The IgSF includes the CD28 co-stimulatory receptor, one of the first co-stimulatory receptors to be identified. CD28 is expressed on most resting murine T cells and about 50% of human $CD8^+$ T cells and approximately 80% of $CD4^+$ T cells (Boomer and Green, 2010). This receptor interacts either with B7.1 (CD80) or B7.2 (CD86) on professional APCs like DCs. CD28 interaction with the cognate ligand CD80 or CD86 induces T cell proliferation and cytokine production by increasing TCR sensitivity to Ag-MHC-I/II complex, and prolongs T cell survival by upregulating anti-apoptotic molecules such as Bcl-xl. CD28 signalling also down regulates Fas ligand (CD95L) expression on CTLs, preventing them from activation induced cell death (AICD) and death inducing signalling complex (DISC) (Boomer and Green, 2010). The inducible co-stimulatory (ICOS) is another IgSF member (Peggs and Allison, 2005, Chen and Flies, 2013). ICOS is expressed within 48 hours of T cell activation and binds to ICOS-L (member of B7 family). ICOS/ICOS-L ligation induces both Th1 and Th2 cell proliferation and cytokine production (McAdam et al., 2000). Dong *et al*. showed that T cell activation and proliferation is defective in $ICOS^{-/-}$ deficient T cells (Dong et al., 2001). Furthermore, *in vitro* differentiated $ICOS^{-/-}$ deficient effector Th cells were defective in IL-2 and IL-4 cytokine production (Dong et al., 2001), indicating that ICOS is important for Th cells activation and effector function.

Signal 2 can also be provided by members of the TNFRSF of which there are 30 receptors and 19 ligands (McAdam et al., 2000, Peggs and Allison, 2005). The interactions between individual TNFRSF members with their ligands regulates the frequency of effector and memory $CD8^+$ and $CD4^+$ T cells that are generated from naïve T cells in response to antigen primarily by enhancing cell survival (McAdam et al., 2000, Peggs and Allison, 2005). The interaction can also enhance T cell function by increasing secretion of effector cytokines like interferon-γ (INF-γ). Some members of the TNFRSF include CD40, CD30, CD134 (OX40),

CD137 (4-1BB), CD27 and death receptor 3 (DR3, TNFRSF25) (Wang, 2012, Ramirez-Montagut et al., 2006). Selected interactions between TNFRSF members and their ligands are shown in (Fig 1-2).

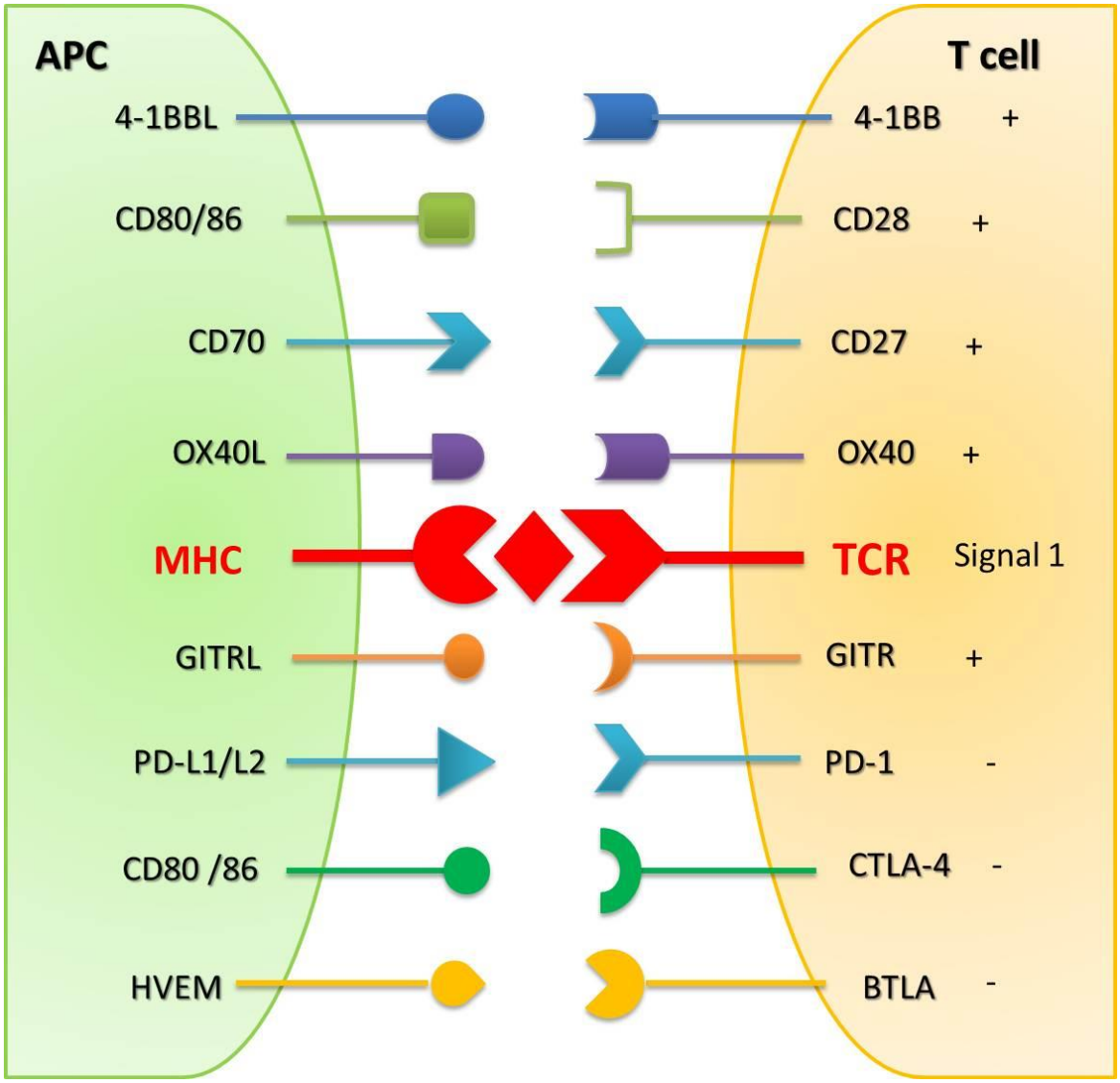


Figure 1-2: co-stimulatory and inhibitory receptors of the immunoglobulin superfamily (IgSF) or the tumour necrosis factor receptor superfamily (TNFRSF). Ligation of costimulatory receptors such as CD28, CD27, 4-1BB, GITR and OX40 with their cognate ligands provides positive signals known as (signal 2) which are essential for full T cell activation, proliferation, survival and effector function. In contrast, co-inhibitory receptors like PD-1, CTLA-4 and BTLA dampen T cell activation by triggering co-inhibitory signals.

1.13. Signalling by members of the TNFRSF

Most TNFRSF receptors signal through TNFR-associated factors (TRAFs) of which there are 6 members; not all TNFRSF members use the same TRAFs (Watts, 2005). TRAFs are adaptors that induce signalling pathways, resulting in activation of the MAP kinase pathways and of classical (canonical) or alternative (non-canonical) NF-kB. NF-kB activation in both pathways is mediated by activation of an IkB kinase (IKK) complex. IKK then mediates phosphorylation-dependent degradation of NF-kB inhibitors in the cytosol thereby releasing the NF-kB transcription factor which translocates into the nucleus and regulates gene expression (81-83). NF-kB activation induces T cell survival and anti-apoptotic molecules such as Bcl2 and Bcl-Xl. How each TRAF adaptor links TNFRSF receptor functions with downstream pathways remains unclear (Chen and Flies, 2013).

In addition, some TNFRSF co-signalling molecules recruit other adaptor molecules. For example, the cytoplasmic tail of CD27 and glucocorticoid inducible tumour necrosis factor receptor (TNFR)-related protein (GITR) binds to the N-terminus of an intracellular mediator of apoptosis (SIVA1), a molecule that is associated with apoptosis. However, the outcome of SIVA1 binding is still under investigation (Nolte et al., 2009). Some of the main TNFRSF members and their ligands are described individually below.

1.13.1. CD27/CD70

CD27 is found on NK cells, memory B cells and naïve T cells (Nolte et al., 2009). CD27 is expressed as a transmembrane homodimer on T cells, and it increases after several cell divisions. CD27 is down regulated on effector T cells, suggesting that CD27 mediates its function in the early stages of the response (Nolte et al., 2009, Wortzman et al., 2013, Denoeud and Moser, 2011) Engagement of CD27 with its only ligand (CD70) enhances $CD8^+$ T cell proliferation and survival resulting in increased numbers of effector and memory T cells (Hendriks et al., 2000, Nolte et al., 2009) CD70 is transiently expressed on activated B cells, DCs and at lower levels on T cells (Wortzman et al., 2013). CD70 can be induced by TLR or CD40 ligation on DCs and B cells and by antigen receptor stimulation on T cells and B cells. In humans, IL-12 and TNF-α induce CD70 expression on peptide stimulated T cells (Lens et

al., 1997). Interaction of CD27 with CD70 enhances antigen-specific T cell responses by promoting T cell proliferation and survival (Rowley and Al-Shamkhani, 2004).

Data from Ramon Arens *et al.* have shown that constitutive stimulation of CD27 on T cells by CD70 improved clearance of influenza virus or the poorly immunogenic murine lymphoma EL4 due to an increase in expansion of antigen-specific $CD8^+$ T cells in the initial phase as well as through increasing effector function per cell (Arens et al., 2004). In our lab, administration of soluble CD70 in the presence of antigen improves T cell proliferation and cytokine production *in vitro.* Furthermore, antigen-specific $CD8^+$ T cells expand massively as a result of both enhanced proliferation and survival *in vivo* (Rowley and Al-Shamkhani, 2004). In contrast, CD27 deficient mice infected with influenza A virus show impaired primary responses to influenza virus by $CD4^+$ and $CD8^+$ T cells (Wortzman et al., 2013). CD27 has been also shown to improve survival of antigen-specific $CD8^+$ T cells important for generation of long-term maintenance of T cells (Hendriks et al., 2000). *In vitro* experiments performed by Perperzak V. *et al.* showed that CD27 improved survival of antigen-specific $CD8^+$ T cells after activation with APC expressing CD80 and CD70. Survival of CTLs was enhanced by autocrine IL-2 production (Peperzak et al., 2010b). Moreover, results obtained by Traban VY *et al*. revealed that blocking CD70 during priming reduced clonal expansion of CD40-induced $CD8^+$ T cells in response to OVA, and resulted in fewer memory T cells. Indicating that CD27/CD70 interaction is crucial for an optimal CD40-mediated priming of $CD8^+$ T cells (Taraban et al., 2006, Taraban et al., 2004). In a similar study conducted by Feau S and colleagues in WT or CD4-depleted mice, results showed that blocking CD70 with mAb for 3 days post immunizing with virus expressing ovalbumin (Vacc-OVA) reduced the magnitude of Ag-specific $CD8^+$ T cells response to levels similar to those generated in CD4-depleted mice (Feau et al., 2012). The number of memory $CD8^+$ T cells was also reduced (Feau et al., 2012).

Targeting CD27 for an effective immunotherapy is currently under investigation. Keler T and others have developed a transgenic mice expressing human CD27 (hCD27) and a fully human IgG1 mAb to hCD27 CDX-1127, to examine the anti-tumour effect of anti-hCD27 Ab in different tumour models. Treating BALB/c hCD27-Tg mice bearing leukemic B cells (BCL1) with multiple injections of 1F5 Ab (days 3, 5, 7, 9, and 11) prolonged mice survival significantly in a dose dependent manner compared to the control group (injected with

hIgG1) (He et al., 2013). Similar results were obtained in CT26 colon carcinoma model. These mice were resistant to CT26 tumour growth after re-challenge with the same tumour, indicating the induction of T cell–mediated antitumor and memory responses (He et al., 2013). The efficacy of antibody was abolished when it was administered at later time points (on days 15, 17, 19, 21, and 23 post tumour inoculation) when tumour size was 0.15–0.2 cm^3 (He et al., 2013). Notably, in the EL4 thymoma model, (which is known for its aggressive growth) tumour was resistant to the treatment, even when the antibody was administered at early time points. The anti-tumour immunity was T cell dependent, as T cell depletion abolished the anti-tumour effect of anti-CD27 antibody (He et al., 2013).

Currently, (CDX-1127) is in its phase I clinical study, in patients with advanced malignancies or solid tumours including metastatic melanoma (Ansell et al., 2013).

1.13.2. OX40/OX40L

In contrast to CD27, OX40 is absent from naive T cells. OX40 is expressed on $CD4^+$ T cells after initial activation with an antigen (Croft, 2010, Watts, 2005, Ishii et al., 2010). OX40 is also found on activated $CD8^+$ T cells, but is more transient compared with $CD4^+$ T cells (Croft, 2010). OX40 expression peaks on $CD4^+$ and $CD8^+$ T cells after two days of activation with antigen *in vitro*, and is down-regulated after 72 hours (al-Shamkhani et al., 1996). OX40L is expressed on activated B cells, T cells, DCs and mast cells (Croft, 2010). Initial studies showed that OX40/OX40L ligation is essential for the clonal expansion of antigen-specific $CD4^+$ T cells, cytokine production and T cell migration (Taraban et al., 2002). The major effects of OX40 seem to be on Th1, Th2 and Th17 cells, and especially on Th2 cells due to the higher expression of OX40 on their cell surface compared with Th1 cells. However, targeting $CD8^+$ T cells by agonistic mAb to OX40 also enhances their expansion *in vivo* (Dawicki et al., 2004, Bansal-Pakala et al., 2004) indicating that OX40 can affect both $CD4^+$ and $CD8^+$ T cells.

Data obtained from several studies suggested that administering anti-OX40 can control infections. In a recent study using Listeria monocytogenes expressing 2W1S peptide as a model antigen, results yielded that anti-OX40 Ab promoted the effector memory T cells (T_{EM})

pool following infection. Notably, anti-OX40 Ab reduced follicular helper T cells (T_{FH}). OX40 deficient mice showed less 2W1S-specific $CD8^+$ T cells (Marriott et al., 2014).

In a another experiment using Lymphocytic Choriomeningitis Virus (LCMV) as a model this time, results also yielded that administering anti-OX40 reduced the frequency of T_{FH} cells and germinal centre cells (Boettler et al., 2013). However, the antibody enhanced virus-specific $CD4^+$ T cells survival and effector function via upregulation of the anti-apoptotic gene Bcl-2 and the transcription factor B lymphocyte-induced maturation protein-1 (Blimp-1) (Boettler et al., 2013).

OX40 has been implicated in enhancing immune responses to infectious disease. Treatment of BALB/c mice with blocking OX40L mAb following influenza A virus infection reduced T cell proliferation and induced T cell apoptosis at infected sites and delayed virus clearance, consistent with the role of OX40L in promoting T cell responses at inflammatory sites (Humphreys et al., 2003b). Hussell T *et al.* administered soluble OX40L and showed increased INF-γ secretion by $CD4^+$ T cells and reduced eosinophilia and *Cryptococcus neoformans* burden confirming the role of OX40 in controlling infection (Humphreys et al., 2003a).

OX40 has also been shown to play a major role in modulating autoimmune diseases. Accumulating data have shown that OX40 OX40L interaction plays a major role in induction of experimental autoimmune encephalomyelitis (EAE) in mice, and blocking OX40/OX40L interaction reduces the severity of the disease. OX40/OX40L blockade also ameliorates different autoimmune diseases such as colitis and collagen-induced arthritis in mice (Croft, 2010). Similar results were obtained by Gaspal MF *et al*. (Gaspal et al., 2011).

The interaction of OX40 with OX40L can enhance tumour regression. Shields and colleagues investigated the role of OX40 co-stimulation during tumour priming with different melanoma tumour models. They showed that targeting OX40 by agonistic antibody or injection with soluble OX40L increased survival in mice depending on the tumour model (Weinberg et al., 2000). The enhancement was due to higher expansion and effector function of antigen-specific $CD4^+$ T cells.

Murine agonistic anti-human OX40 mAb (9B12) has been recently tested in phase I dose-escalation study. 9B12 was designed to induce OX40 signalling of human OX40. Treating patients with one course of the anti-OX40 mAb caused tumour shrinkage in 12 of 30 patients bearing advanced malignancies with acceptable toxicity profile (Curti et al., 2013). 9B12 induced both $CD4^+$ and $CD8^+$ T cell proliferation that lasted for 15-30 days, while $FoxP3^+CD4^+$ T cells remained intact (Curti et al., 2013).

Combination of both anti-OX40 Ab and blocking-CTLA-4 Ab was recently tested in murine prostate tumour cells or MCA-205 murine sarcoma to enhance tumour immunotherapy. Results revealed that combining both agonist anti-OX40 with anti-CTLA-4 caused significant tumour regression and improved overall survival of mice bearing advanced sarcoma cells or prostate cancer compared with mice treated with either Ab alone (Redmond et al., 2014). The combination induced both $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production, but did not change T_{eff}:T_{reg} ratio or T_{reg} effector function within the tumour (Redmond et al., 2014).

Phase II clinical study of anti-OX40 Ab is ongoing (Kovacsovics-Bankowski et al., 2013). These results indicated that administrating anti-OX40 antibody Ab can be useful in inducing durable anti-tumour responses, particularly when combined with checkpoint blockade.

1.13.3. GITR/GITRL

GITR receptor has been found on resting murine and human T cells. Its expression increases rapidly 24-72 hours after initial activation and persists for two days (Kanamaru et al., 2004, Schaer et al., 2012). GITR is predominantly expressed on $CD4^+CD25^+$ T_{reg} (Shimizu et al., 2002). In the mouse, GITR ligand (GITRL) is expressed on B cells, DCs and macrophages upon activation (van Olffen et al., 2009). In humans, GITRL is expressed on endothelial cells, epithelial, pDCs, macrophages and cells of the eye (Placke et al., 2010, Tuyaerts et al., 2007). Co-stimulation through GITR increases proliferation of Th1, Th2, $CD4^+$ T_{reg} cell and $CD8^+$ (Shimizu et al., 2002). GITR/GITRL interaction ameliorates the expression of IL-2Rα and increases IL-4, IL-10, INF-γ and IL-2 secretion by $CD4^+$ T cells (van Olffen et al., 2009, Shimizu et al., 2002). This interaction also induces $CD8^+$ and $CD4^+$ T cell proliferation and IL-2 production (Shimizu et al., 2002, van Olffen et al., 2009). Stimulation through the GITR

receptor also increases the number of Foxp3$^+$ CD4$^+$ T cells, indicating that GITR can act as a co-stimulatory receptor to activate T cells. Expression of GITR on effector T cells makes them more resistant to suppression. In CD25$^+$CD4$^+$ T cells, however, Shimizu et al. showed that *in vitro* stimulating GITR with agonistic mAb abrogated CD25$^+$CD4$^+$ T cell–mediated suppression (Shimizu et al., 2002).

The immune activating effect of anti-GITR antibody (using the rat monoclonal antibody DTA-1) has been tested in different tumour models including B16 melanoma, CT26 colon cancer and A20 lymphoma (Schaer et al., 2012). Moreover, eliminating or modulating GITR on T_{reg} after administration of DTA-1 antibody caused autoimmunity in normal mice; similar to that seen when GITR^{hi} T_{reg} cells were physically eliminated (Shimizu et al., 2002). The major effect of DTA-1 is to decrease T_{reg} stability (Schaer et al., 2012, Ko et al., 2005). Cohen *et al.* showed that treating mice bearing B16 melanoma with DTA-1 modulated intra-tumour T_{reg} stability by reducing Foxp3$^+$ expression. This resulted in a greater T_{eff}:T_{reg} ratio and a greater anti-tumour effect (van Olffen et al., 2009, Cohen et al., 2010). Single administration of DTA-1 mAb to BALB/c mice challenged with Meth A (a BALB/c drived fibroblastoma) tumour cells led to 90% tumour regression without any autoimmunity. Again, DTA-1 abolished intratumoral Foxp3$^+$ CD4$^+$ T_{reg} and increased tumour infiltrating CD4$^+$ and CD8$^+$ T cells (Ko et al., 2005). GITR stimulation can also induce CD8$^+$ T cell response. Data collected by Clouthier *et al*. showed that injecting WT mice with DTA-1 post chronic LCMV infection increased the frequency and total number of LCMV-specific CD8$^+$ T cells (~2 fold increased) compared with the control group, and improved control of LCMV infection without obvious side effect (Clouthier et al., 2014).

1.13.4. 4-1BB/4-1BBL

4-1BB is expressed on activated T cells, NK cells, endothelial cells of some tumours and many other immune and non-immune cells (Moran et al., 2013). Its ligation with 4-1BB ligand on activated DCs, macrophages and B cells induces T cell activation and anti-apoptotic molecules such as Bcl-2 and Bcl-xl. Importantly, it protects antigen-specific T cells from activation-induced cell death (Moran et al., 2013). However, accumulating data suggest that the major effect of 4-1BB is on antigen experienced memory T cells (Snell et al., 2011).

Administration of agonist anti-4-1BB mAb has shown potent anti-tumour affects (Cheuk et al., 2004). A study by Melero I *et al.* revealed that anti-4-1BB mAb reduces tumour growth in mice bearing advanced poorly immunogenic Ag104A sarcoma or the P815 mastocytoma. The anti-tumour effect was mediated by the accumulation of effector T cells and enhancement of both $CD4^+$ and $CD8^+$ T cell activity (Melero et al., 1997). In another study, the combination of anti-4-1BB mAb with IL-12 gene transfer reduced tumour growth and enhanced survival in ~50% of mice injected with the poorly immunogenic tumour B16-F10 melanoma compared to IL-12 or anti-4-1BB alone (Xu et al., 2004). In contrast to Ag104A sarcoma and P815 mastocytoma, the anti-tumour effect in this tumour model was by $CD8^+$ T cells and NK cells, as $CD8^+$ T cell and NK cell but not $CD4^+$ T cell depletion reduced the efficacy of the treatment (Xu et al., 2004). Other studies have shown that *ex vivo* stimulation of cells isolated from tumour-draining lymph nodes (TDLN) with anti-4-1BB mAb prior to transfer into syngeneic mice bearing MCA-205 fibrosarcoma significantly reduced tumour growth and enhanced survival (Li et al., 2003).

1.14. Co-inhibitory receptors (Negative regulatory Pathway)

As well as co-stimulatory signals, co-inhibitory signals can impede ongoing immune responses (Fig 1). Many co-inhibitory molecules belong to the B7 family, including T cell immunoglobulin mucin-3 (TIM-3), B- and T-lymphocyte attenuator (BTLA), lymphocyte-activation gene 3 (LAG3), cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death-1 (PD-1); all have been implicated in tumour immune evasion (Driessens et al., 2009).

1.14.1. PD-1/PD-L1 and PD-L2

PD-1 (CD279) is a type I transmembrane protein that is up-regulated transiently on activated T cells and remains high on chronically stimulated T cells (Keir et al., 2008, Ceeraz et al., 2013). It drives an inhibitory signal after binding with one of its two ligands programmed cell death 1 ligand 1 or 2 (PD-L1 and PD-L2). PD-1 expression is regulated by the transcription factors nuclear factor of activated T cells (NFAT), T-bet, Blimp-1 and Forkhead box protein O1 (FoxO1) (Pauken and Wherry, 2015). NFAT, FoxO1 and Eomesodermin are associated

with high PD-1 expression. While T-bet represses PD-1 expression via binding to the *Pdcd1* enhancer promoter region. Blimp-1 in exhausted T cells is associated with high PD-1 expression (Pauken and Wherry, 2015).

PD-L1 is expressed by numerous immune and non-immune cells, particularly in the presence of inflammatory signals, presumably to avoid collateral damage induced by T cells (Prosser et al., 2012, Keir et al., 2008). In addition to PD-1 receptor, PD-L1 was also reported to bind to CD80, delivering a bidirectional inhibitory signal (Keir et al., 2008).

PD-L2 in contrast is more restricted to DCs and macrophages (Rozali et al., 2012, Yang et al., 2013). Normal fibroblasts, tumour-associated fibroblasts and some other cell types can also express PD-L2 depending on microenvironmental stimuli, resulting in T cell suppression (Latchman et al., 2001, Rozali et al., 2012). Using artificial antigen presenting cells, Parry R *et al.* studied the effect of PD-1 signalling on activated human $CD4^+$ T cells. Magnetic beads coated with anti-CD3, anti-CD28 with or without anti-PD-1 Ab (designated CD3/CD28/PD-1) were used as artificial APCs. Activation of $CD4^+$ T cells with CD3/CD28/control-coated beads induced $CD4^+$ T cell expansion and cytokine production (Parry et al., 2005) whereas CD3/CD28/PD-1-coated beads in contrast, inhibited their activation and reduced IL-2 production (Parry et al., 2005).

While PD-L1 expression protects a host from self-reactive T cells, in the tumour microenvironment malignant cells such as melanoma, lung, renal, ovary and colon carcinomas utilise PD-1/PD-L1 or PD-L2 signalling to escape the immune response by limiting the activity of anti-tumour $CD8^+$ T cells leading to $CD8^+$ T cell exhaustion (Keir et al., 2008, Zhang et al., 2009, Iwai et al., 2002, Harvey, 2014). Animal studies have shown that blocking the PD-1/L1 pathway restores tumour-specific $CD8^+$ T cell function. Tumour cells expressing PD-L1 resisted T cell–mediated lysis and were capable of growing more rapidly than PD-L1 negative cells. Blocking the interaction of PD-1 with PD-L1 pathway using mAb enhanced T cell proliferation, restored their effector function and reduced tumour growth. However, when Ohigahsi and colleagues have investigated the expression of PD-L1 and PD-L2 in human esophageal cancer, their study revealed that there is no correlation between PD-L1 and L2 expression and the frequency of $CD4^+$ and $CD8^+$ tumour infiltrating lymphocytes (TILs). However, they found that there is a strong correlation between

impaired survival and expression of PD-L1, PD-L2 or both ligands (Ohigashi et al., 2005). Overall survival of patients with tumours positive for both PD-1 ligands was significantly worse than that with tumours negative for both. While overall survival of patients positive for either PD-L1 or PD-L2 was slightly better than that with both positive and worse than that with both negative (Ohigashi et al., 2005). Blocking the PD-1 receptor along with other co-inhibitory receptor such as BTLA or TIM-3 improves anti-tumour immunity and increases survival of mice with melanoma (Fourcade et al., 2012). Combining anti-PD-1 with anti-CTLA-4 mAbs also improved survival and dampened tumour growth significantly in mice bearing CT26 colon carcinoma (Binder and Schreiber, 2014).

As well as T cells, PD-1 has been reported to be expressed on naive and activated peripheral B cells with higher levels of PD-1 expression on B cell activation (Agata et al., 1996, Thibult et al., 2013). However, the role of PD-1 on activated B cells is still unclear. Thibult and colleagues have found that PD-1 ligation with PD-L1 or L2 did not affect B cell survival, chemokine synthesis, or isotype class-switching, but clearly reduced proliferation and IL-6 secretion (an important cytokine for B cell growth) (Thibult et al., 2013). Blocking PD-1 co-inhibitory signalling by either anti-PD-L1 and/or anti-PD-L2 restored their proliferation and increased CD80 and CD86 expression on B cells (Thibult et al., 2013) suggesting that PD-1 may impair B c ell, as well as T cell responses.

1.14.2. Mechanisms of PD-1 inhibition

The cytoplasmic domain of PD-1 has a membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) motif, and a C-terminal immunoreceptor tyrosine-based switch motif (ITSM). In vitro experiment by Perry *et al.* revealed that ITSM rather than ITIM is responsible for PD-1 inhibition (Parry et al., 2005). There are multiple pathways that have been recently proposed for how PD-1 supresses T cell activation. The transmission from resting T cells to rapidly dividing effector T cells requires metabolic changes (Jacobs et al., 2008, Macintyre et al., 2014). CD28 signals increase glucose uptake after TCR activation by upregulating surface trafficking of the glucose transporter Glut1 to support the elevated energy and biosynthetic demands of growth, proliferation, and cytokine secretion (Jacobs et al., 2008, Patsoukis et

al., 2015). The effect of CD28 on $CD4^+$ T cells is abolished however when PD-1 co-inhibitory receptor is stimulated (Parry et al., 2005, Patsoukis et al., 2015). In addition to glycolysis inhibition in activated T cells, PD-1 has been recently reported to promote fatty acid β-oxidation (FAO) by increasing the rate-limiting enzyme of FAO, carnitine palmitoyl transferase (CPT1A) inducing lipolysis (Patsoukis et al., 2015). These results indicate that PD-1 is able to reduce and/or inhibit T cell proliferation and cytokine production by reducing glucose uptake and metabolism. PD-1 can also directly supress TCR singling by recruiting SHIP1 and 2 (inhibit effectors of PI3K signalling) (Srivastava et al., 2013, Parry et al., 2005). Moreover, PD-1 signalling reduces T cell proliferation by inhibiting RAS pathway. T cell inhibition can be mediated by expression of BATF, which can repress expression of effector genes. Finally, PD-1 engagement negatively impact T cell motility and stabilisation of TCR/APC interaction (Pauken and Wherry, 2015).

1.14.3. CTLA-4/CTLA-4 Ligand

CTLA-4 is expressed on activated B cells, monocytes, and on activated T cells (McCoy and Le Gros, 1999). It is also highly expressed on T_{regs}. CTLA-4 is homologous to CD28, they both belong to the immunoglobulin (Ig) superfamily, and they bind to the same ligands CD80 (B7-1) and CD86 (B7-2) on APCs (Lenschow et al., 1996). However, CTLA-4 has higher binding affinity to B7-1 and B7-2 than CD28, therefore, CTLA-4 downregulates T cell responses by competing with CD28 for binding to CD80/86 (McCoy and Le Gros, 1999, Greenwald et al., 2005, Rudd et al., 2009). Data from Peggs K *et al.* suggest that blocking CTLA-4 with mAbs both inhibits T_{reg} function and enhances effector T cell function i.e. it has two beneficial roles (Peggs et al., 2009b).

1.14.4. Mechanism of CTLA-4 inhibition

Like PD-1, CTLA-4 has been reported to reduce glucose uptake and inhibit Akt phophorylation. Unlike PD-1, however CTLA-4 blocks Akt in PI3K-independent manner, allowing some level of expression of T cell survival genes such as Bcl-2 and Bcl-xl. CTLA-4 suppresses Akt activity directly by disturbing the type II serine/threonine phosphatase (PSP), this complex is associated with both CD28 and CTLA-4 cytoplasmic tails, and plays a major role in Akt regulation.

CTLA-4 blockade improves immunity to infection. This is supported by a study where simian immunodeficiency virus (SIV) infected monkeys were injected with antiviral therapy and MDX-010 antibody that targets CTLA-4. CTLA-4 blockade increased effector functions for antigen-specific $CD4^+$ and $CD8^+$ T cells, reduced TGF-β production from T_{regs} cells and most importantly reduced the viral level in the draining LNs (Fallarino et al., 2002, Hryniewicz et al., 2006).

It is thought that increasing the ratio of effector T cells to Treg cells within the tumour microenvironment is beneficial (Oleinika et al., 2013, Peggs et al., 2009b, Cohen et al., 2010). This can be achieved by blocking CTLA-4 signalling by mAb (Peggs et al., 2009b). A study by Shrikant *et al.* in mice with solid tumour showed that CTLA-4 blockade increased antigen-specific $CD8^+$ T cell number at the tumour site and mediated tumour regression, but it was not clear whether CTLA-4 blockade was due to a direct effect on effector $CD8^+$ T cells, effector $CD4^+$ T cells or both (Shrikant et al., 1999). Another study by Simpson T *et al.* further investigated the role of blockade of CTLA-4 receptor using mAb. Results showed that the increased number of intratumoral effector T cells was concomitant with T_{reg} depletion through an Fcγ dependent mechanism (Simpson et al., 2013).

1.15. Cancer

1.15.1. Cancer development

Cancer is the second cause of death in the developed world mainly associated with ageing and lifestyle (Urruticoechea et al., 2010). Cancer development (or carcinogenesis) results from an uncontrollable division of cells from tissue (Rakoff-Nahoum, 2006). Carcinogenesis requires the acquisition of six fundamental properties: sustain proliferative signalling, anti-growth suppression, invasion and metastasis, unlimited replicative potential, inducing angiogenesis (maintenance of vascularization) and anti-apoptosis (Rakoff-Nahoum, 2006). The first phase of carcinogenesis is the initiation phase. In this phase, genomic mutation such as point mutation, gene deletion and chromosomal rearrangements occurs in the cancer cell, resulting in irreversible changes. The second phase is characterised by the survival of and maintenance of the mutated cells, this phase is referred to as the promotion phase. Progression phase is when the tumour size increase substantially (Rakoff-Nahoum, 2006). As the tumour grows in size, the cells may undergo further mutations, leading to increasing heterogeneity of the cell population. Some tumour cells lose their adhesive property and detach from the tumour mass, these cells can infect neighbouring tissues,

enter circulating blood and migrate to lymph node, distant tissues and organs from the primary site of grows and develop secondary tumours (Devi).

1.15.2. Conventional cancer treatment

Conventional cancer therapy includes surgery, radiotherapy and chemotherapy. They can be used as monotherapy or in a combination form of treatment, depending on the tumour types and stage of tumour growth (Pajonk et al., 2010, Uramoto and Tanaka, 2014, Bhatia et al., 2009). Early diagnosis and development in conventional therapy has significantly improved survival of cancer patients (Urruticoechea et al., 2010).

Surgery is considered to be the most effective treatment compared to other conventional approaches; almost 100% of excised localized primary tumour is killed, while chemo or radiotherapy kills only a fraction of tumour cells (Urruticoechea et al., 2010).

Radiotherapy is an important strategy for the treatment of cancer patients; about 50% of patients with tumour undergo radiotherapy during their course of illness. Radiotherapy contributes towards 40% of curative treatment for cancer. The main goal of radiotherapy is to reduce tumour growth by damaging cancer cells DNA (Baskar et al., 2012). However, there is accumulation data suggests the presence of DNA repair in different types of tumour including lung cancer (Willers et al., 2013). Thus, radiotherapy alone may not be sufficient in providing full protection again the tumour.

Chemotherapy is another approach that can be applied to treat patients with cancer. Cyclophosphamide (CTX) is a form of chemotherapy which is mainly used to control disseminated tumour (Luqmani, 2005). CTX has a direct cytotoxic activity on tumour cells, but can also reduce total number of $CD4^{+}25^{+}$ regulatory T cells (Motoyoshi et al., 2006). However, the effect of CTX and other chemotherapy drugs is preferential and not exclusive to tumour cells, which means normal cells can also be affected with the treatment, resulting in an unwanted drug side evets like loss of hair, fatigue, nausea, vomiting, blood clotting problems and depressed immune system (Luqmani, 2005). Most of these side effects disappear after the course of treatment. Nevertheless, permanent damage may occur in some organs like kidneys, lungs and heart (Mechanisms of Drug Resistance in Cancer Chemotherapy).

However, conventional treatment does not always provide full protection. For example, cancer relapse is common after resection of visible tumour, about 30% to 55% of patients with non-small cell lung cancer (NSCLC) developed tumour relapse and die despite successful tumour resection. Furthermore, surgery itself can be quite risky, and can increase mortality in some cases. For instant, decrease in lung function has been reported after surgery in most patients (Uramoto and Tanaka, 2014). In addition, some tumour types are relatively resistant to cytotoxic drugs, due to altered transport of the drug, modifications in target molecule, metabolic effects and genetic responses (Luqmani, 2005, Pajonk et al., 2010, Liu, 2009). DNA repair is common in lung cancers, altering the resistance of the affected tumours to many chemotherapeutics as well as radiation (Willers et al., 2013).

1.15.3. Cancer immunotherapy

Although conventional cancer therapies (surgery, chemo and radiotherapy) have shown clinical benefits, some individuals do not response to these kinds of treatments. Furthermore, chemo and radio therapies have always been associated with toxicity (Arens et al., 2013). Therefore, it has proven important to develop alternative strategies to treat cancer with better efficacy and lower toxicity. Data obtained from extensive studies in humans and mice have proven that both innate and adaptive immune cells can play important roles in the recognition and rejection of tumour cells (Schreiber et al., 2011, Hanahan and Weinberg, 2011). Together, these findings are leading to the development of novel biological therapies able to elicit immune-mediated tumour destruction and overcome immune suppression. These immune-modifying treatments are termed 'immunotherapy'. Cancer immunotherapy has been shown to give promising results with metastatic malignancies compared to conventional tumour therapy.

The hallmark of T cell response is specificity and long term memory which can lead to durable responses. In contrast to conventional cancer therapy, the main goal of immunotherapy is to induce strong T cell responses via administration of tumour vaccines like DNA vaccine, peptide and protein vaccine, TLR agonists, ACT, administration of monoclonal antibodies, cytokines and cellular vaccines (Disis, 2014). Combining immunotherapy with T_{reg} cells and/or MDSCs depletion or inhibition of their suppressive

function can further improve the outcome of the treatment (Nishikawa and Sakaguchi, 2014, Draghiciu et al., 2015).

1.15.4. Challenges for cancer immunotherapy

Tumour cells utilise distinct mechanisms to evade rejection by immune system. The tumour microenvironment (TME) consists of multiple cell types such as tumour-associated stroma, fibroblast, MDSCs, T_{reg} cells, and tumour-associated-microphages. Recruitment of these cells can form a physical barrier to T cell entry and suppress tumour-antigen specific T cells proliferation and effector functions (Quail and Joyce, 2013, Gajewski et al., 2013). Tumour cells can also supress effector cells by expressing certain immune checkpoint ligands like PD-L1, the engagement of these ligands with the cognate receptor on effector cells reduce their anti-tumour activity (Lu et al., 2014a). Down regulation of peptide-MHC complex and the secretion of inhibitory cytokines such as vascular endothelial growth factor (VEGF), TGFβ IL-10 and IL-35 can also restrict T cell activity in the TME (Makkouk and Weiner, 2015).

Understanding tumour evasion mechanisms aids to design combinational therapies to overcome immune tolerance and to improve the outcomes of cancer immunotherapy with minimal cytotoxicity (Makkouk and Weiner, 2015). Descriptions for optimising cancer immunotherapy are described below.

1.15.5. Optimizing Cancer Immunotherapy

In the section above I described the development of several novel immunotherapeutics targeting members of the TNFRSF and also PD-1 and CTLA-4. These are all monoclonal antibodies. However, other immunotherapeutic approaches exist including adoptive T cell transfer (ACT), synthetic peptide-based vaccines, cytokine-based vaccines, DC vaccines, whole cells and DNA-based vaccines. These will be described in turn beginning with immunomodulatory monoclonal Abs.

1.15.5.1. Antibody therapeutics in cancer

Antibodies are proteins secreted by differentiated B cells called plasma cells and play an important role in clearing pathogens from a host (Dorner and Radbruch, 2007, Corti and

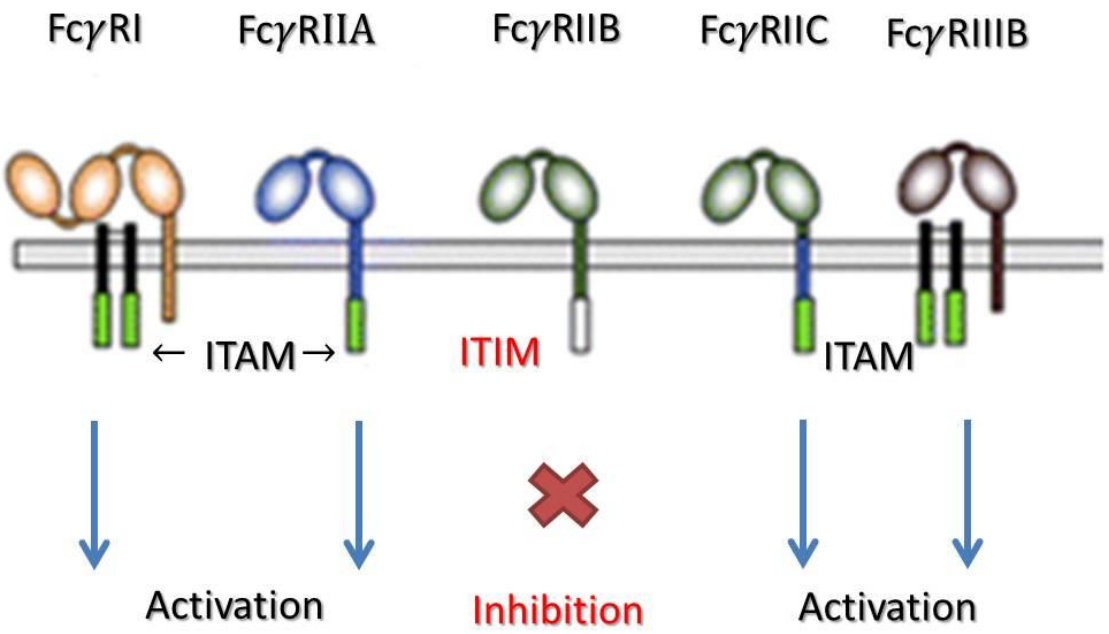
Lanzavecchia, 2013). Antibodies (or immunoglobulin; Ig) can bind to specific antigen and can be divided based on the sequence of the heavy chain into five classes: IgA, IgD, IgE, IgG and IgM. IgG is the most frequent isotype, representing about 75% of all antibodies, and it is also the most frequently used for immunoassay and cancer therapy (Weiner et al., 2010). All antibodies bind to a specific antigen via the fragment of antigen binding (Fab) region which consists of three variable complementary determining regions (CDRs) which together confer antigen specificity. The opposite end of the antibody the crystallising constant fragment (Fc) can bind to Fc receptors (FcR) a family of receptors expressed on different immune cell types (Fig 2) (Weiner et al., 2010). The interaction of the Fc with Fc receptors can lead to different immune reactions such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (Nimmerjahn and Ravetch, 2007, Nimmerjahn and Ravetch, 2008).

Historically, much emphasis was placed on the interaction between the Fab region of an antibody and its antigen. However, in recent years the importance of the Fc/FcR interaction to the efficacy of mAbs is being revealed.

There are different types of Fcγ receptors expressed in immune cells to facilitate their reaction with IgG (Fig 2) (Guilliams et al., 2014). The various FcγRs are functionally divided into activating receptors such as FcγRI, FcγRIIC, FcγRIIIA (CD16a) and inhibitory receptors like FcγRIIB (CD32b)(Guilliams et al., 2014). The high affinity receptor FcγRI is widely expressed on the surface of multiple innate immune cells including natural killer (NK) cells, mononuclear phagocytes, dendritic cells (DCs), mast cells, macrophages and neutrophils. The lower affinity FcγRIIIa on the other hand is more restricted to NK, DCs and macrophages. FcγRIIIa is essential for mediating ADCC (a major mechanism of NK-dependent tumour cell lysate) (Bruhns, 2012, Weiner et al., 2010, Wu and Lanier, 2003, Seidel et al., 2013). The only inhibitory receptor FcγRIIB is found on circulating B cells, on neutrophils, DCs and monocytes. FcγRIIB inhibits FcγRIa and FcγRIIIa-mediated phagocytosis by disrupting ITAM positive signals (Su et al., 2007).

IgG binding to activating FcγRs such as FcγRI and FcγRIIIA in humans, or FcγRI, FcγRIII, and FcγRIV in mice, can initiate phosphorylation of the ITAM in the cytoplasmic domain of the receptor which is mediated by the Src-family tyrosine kinase. The phosphorylation of ITAM

leads to Syk kinase activation (Jonsson et al., 2013), and downstream phosphatidylinositol 3-kinase (PI3K) and nuclear factor (NF)-kB signalling resulting in cell activation, maturation and effector function (Lanier, 2006, Duchemin et al., 1994). FcγRIIb in contrast possesses an ITIM in its cytoplasmic tail which negatively regulates cell function (Fig 1-3) (Weiner et al., 2010, Bruhns, 2012, Daeron et al., 2008, Jonsson et al., 2013). White and colleagues have recently investigated the importance of FcγRs and mAb isotype interaction in determining the activity of the agonist parental antibody rat anti-mouse CD40 antibody (3/23 rat IgG2a). Results revealed that mouse IgG1 variant provided strong humoral and cellular responses against the ovalbumin (White et al., 2011). In contrast, mouse IgG2a isotype did not have any activity. Study in FcR$\gamma^{-/-}$ deficient mice showed that FcγRIIB is crucial for 3/23 activity (White et al., 2011). In another study by by Li and others, results suggested that FcγRIIB is crucial for the antitumor activity of the MD5-1 antibody (an agonistic Armenian hamster IgG2 anti-mouse DR5 antibody). Injecting WT mice bearing stablished MC38 colon carcinoma with MD5-1 antibody inhibited tumour growth significantly compared with mice treated with an isotype control antibody (Li and Ravetch, 2012). The activity of the antibody was abrogated when the antibody was injected into FcγRIIB$^{-/-}$ deficient mice (Li and Ravetch, 2012). There results indicate that FcγRs are important for determining the activity of the antibodies.



Source of image: Blood journal, Volume: 119, Issue: 24

Figure 1-3: FcγRs in human immune cells. Activing FcγRs like FcγI, FcγIIA, FcγIIIB and FcγRIIIC have immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic tail. Activation of ITAMs stimulates cell activation. FcγIIB on the other hand contains an ITIM, which negatively regulates cell function.

Monoclonal Abs can be utilized as powerful tools to eliminate tumour growth via a number of mechanisms (Scott et al., 2012, Melero et al., 2007). As well as targeting co-stimulatory and co-inhibitory receptors, mAbs can exert direct action on the tumour cell (see Table 1-1 for some examples). For instance an anti-Wnt-1 (Wingless-type (Wnt)/β-catenin signalling pathway) antibody induces cell death in different human cancer cell lines, and anti-TNR-related apoptosis-inducing ligand receptor (TRAIL)-R1 and TRAIL-2 antibodies transmit apoptotic signals (He et al., 2004, Ludwig et al., 2003). MAbs can also be conjugated with cytotoxic agents to target tumour cells while avoiding normal cells (Scott et al., 2012, Ludwig et al., 2003). 3- In addition to direct targeting of the tumour cells, mAbs have been used to ablate vascular and stromal cells surrounding the tumour cell. For example an antibody against the fibroblast activation protein, FAP, which is expressed selectively in the majority of human cancer associated fibroblasts, can directly inhibit FAP enzymatic activity.

Anti-FAP can also be conjugated to deliver drugs or toxic reagents to tumour stroma (Zhang and Liu, 2013). In a preclinical study, treatment with FAP5-DM1 (an antibody-toxin conjugate) resulted in long-lasting inhibition and tumour regression in xenograft models of lung, pancreatic, and head and neck cancers with no signs of intolerability (Ostermann et al., 2008).

Table 1-1

Mechanism of action	Tumour type	Example
Induce apoptosis	different human cancers	anti-Wnt-1 Ab, Anti-TRAIL-R1/R2 Abs
Conjugate mAb with cytotoxic agents	Different tumour types	brentuximab vedotin (Adcetris), and ado-trastuzumab emtansine (Kadcyla)
Induction of CDC and ADCC	chronic lymphocytic leukemia	anti-CD20 rituximab, anti-Her2 trastuzumab
Induction of CDC	chronic lymphocytic leukemia	anti-CD20 rituximab
Ablation of tumour vascular and stromal surrounding cells	Colon, breast, lung, head and neck, and pancreas cancer	Anti-FAP Ab Anti-VEGF Ab Anti-EGFR Ab

1.15.5.2 Adoptive T cell transfer (ACT)

Adoptive T cell transfer as a treatment for cancer was first reported in rodents over fifty years ago (24). Understanding of T cell biology, the discovery of the TNFRSF, as well as the identification of soluble growth factors has helped to improve the efficacy of ACT (Maus et al., 2014a, Yee, 2014, Kalos and June, 2013).

1.15.5.2.1. Isolation of tumour infiltrating lymphocytes (TILs) for ACT

The process of adoptive cell transfer involves extracting lymphocytes from the blood or the tumour, selecting the best Ag-specific $CD8^+$ T cells and then co-culturing *ex vivo* with DC-

pulsed with tumour antigen, or with living or dead tumour cells in the presence of interleukin-2 (IL-2) to increase the yield of the tumour antigen-specific $CD8^+$ T cells. Cells are then re-infused back into the patient (Shirasu and Kuroki, 2012, Rosenberg et al., 2008). This strategy increases objective response rates with very little side effects. Although these studies were performed in a small number of patients, these results indicated the efficacy of ACT in treating patients bearing advanced tumour (Galluzzi et al., 2012, Makkouk and Weiner, 2015). Best results were obtained by adoptively transfer into irradiated patients.

Despite these encouraging outcomes using expanded TILs to combat tumours, this approach has some limitations for instance not all tumours can be easily extracted, and in some cases tumour extraction could increase the risk of death due to their distribution within the body e.g. lung, brain and pancreatic cancers (Zhou, 2014, Phan and Rosenberg, 2013). Another limitation for ACT is the difficulty of getting a reasonable percentage of tumour-reactive lymphocytes that can be expanded *ex vivo*. In metastatic melanoma over 80% of the TILs have been reported to be tumour antigen-specific lymphocytes, such a high percentage making it possible to isolate them for ACT. In contrast, less than 3% of expanded TILs were Ag-specific in patients with gastrointestinal (GI) cancers (Zhou, 2014, Phan and Rosenberg, 2013). Therefore, the concept of modifying the T cells to confer the ability to bind to tumour antigen with high affinity was proposed. This can be achieved in two ways by introducing a new TCR or using a CAR; discussed below.

1.15.5.2.2. TCR gene modification

TCR gene modification is a growing field in cancer immunotherapy (Zhou, 2014, Phan and Rosenberg, 2013). Genes encoding TCRs that recognise tumour associated antigen (TAA) are introduced to the patient's T cells using lentivirus or retroviral vectors (Phan and Rosenberg, 2013). Highly avid TCRs can be identified from highly reactive T cells or by immunizing transgenic mice expressing human leucocyte antigen (HLA) associated with human antigen (Phan and Rosenberg, 2013, Sharpe and Mount, 2015). The TCR α and β chains from reactive T cells are isolated and cloned into gene expression viral vectors to transduce lymphocytes that are able to recognise TAA similar to the parental clones (Phan and Rosenberg, 2013, Zhou, 2014). However, because MHC-I downregulation is a common

mechanism of tumour evasion from T cell-mediated immune responses (Bubenik, 2003, Bubenik, 2004), this approach can be insufficient.

1.15.5.2.3. Chimeric antigen receptor (CAR)

More recently, T cells have been genetically engineered, often using virus as a vector, to express chimeric antigen receptors. The first generation of CARs were comprised of a single-chain variable fragment derived from tumour-specific antibody and linked with signalling domain of CD3 zeta (Makkouk and Weiner, 2015, Heslop, 2010, Maus et al., 2014a). Cells expressing these CARs were able to proliferate, secrete cytokines and lyse targeted tumour cells (Makkouk and Weiner, 2015). However, CD3-mediated signalling alone provide only transient cell division, modest cytokine production and limited anti-tumour effects (Brocker and Karjalainen, 1995). Thus, co-stimulatory signals e.g. from CD28 are required in addition to the CD3 zeta signals for full activation. Therefore, second generation CARs were designed to incorporate the intracellular domains of co-stimulatory receptors such as 4-1BB (CD137), OX40 (CD134), CD28 and inducible co-stimulator (ICOS) (Shirasu and Kuroki, 2012, Heslop, 2010). Second generation CARs generate T cells with better activation, proliferation, increased tolerance to regulatory T cells and 20-fold higher cytokine secretion upon stimulation compared with first generation CARs (Heslop, 2010). Third generation CARs include two co-stimulatory domains (Maus et al., 2014b). This is thought to improve CAR-T cell activation and cytokine production (Curran et al., 2012).

1.15.5.3. Peptide-based vaccine and cancer immunotherapy

$CD8^+$ T cells have been defined as potent cells to recognise and kill malignant cells. Based on this principle, MHC-I restricted peptides have been synthesised to stimulate tumour Ag-specific $CD8^+$ T cells as a cancer vaccine. While the first generation of peptide-based vaccines consisted of one or multiple short peptides, the new generation comprised of several long peptides that can bind to both MHC-I and MHC-II on DCs providing not only anti-tumour $CD8^+$ T cell responses but also $CD4^+$ helper T cells (Arens et al., 2013). The length of the peptide used for vaccination and the mobilisation of CD4+ helper T cells are important parameters that could influence the safety and efficacy of the anti-tumour response, even in tumours that do not express MHC-II on the cell surface (Arens et al., 2013).

Furthermore, the use of multiple peptides can overcome tumour antigen-loss variants (Arens et al., 2013). However, peptides when used as monotherapy are poorly immunogenic, and adjuvants are needed to increase their efficacy (Arens et al., 2013). Some adjuvants that are currently used in humans in combination with peptide vaccines include Incomplete Freund's adjuvant (IFA) and Montanide ISA-51. These adjuvants consist of water in oil emulsions. Their main purpose is to control the release of the peptide to protect it from rapid systemic bio-distribution as well as to improve peptide uptake by antigen presenting cells (APCs) (Arens et al., 2013).

1.15.5.4. Synthetic peptide vaccine in combination with mAbs

Using peptide vaccines as monotherapy is often inadequate to provide complete protection from infections and solid tumours (Rosenberg et al., 2004, Ly et al., 2013, Pandey et al., 2013)**.** Therefore, combinations of peptide-based vaccines and immune stimulants have been tested. In a recent *in vivo* experiment conducted in mice with B16 melanoma, vaccination with tyrosinase-related protein-2 peptide ($Trp2_{180\text{-}188}$) (a protein involved in the synthesis of melanin) in combination with synthetic oligodeoxynucleotides (ODNs) (a TLR9 agonist (Bode et al., 2011)) and agonistic anti-4-1BB mAb resulted in complete cure in 5 out of 6 mice. The inclusion of anti-4-1BB mAb in the regimen augmented higher Ag-specific CTL responses ($\sim$1.5 increase compared with peptide alone), and facilitated their migration and infiltration into the tumour site (Sin et al., 2013). These mice also generated memory CD8+ T cells indicating the importance of agonistic mAb. Combining peptide-based vaccine with blocking mAbs also improves their efficacy (Hodi et al., 2010).

1.15.5.5. Cytokines

Cytokines are proteins secreted by different cell types to communicate with one another (Lee and Margolin, 2011). Cytokines have been used in cancer immunotherapy, as they can affect tumour cell growth and survival, either directly such as TNF-α, or indirectly by stimulating the immune response, such as IL-2 and IFN-γ (Disis, 2014, Lee and Margolin, 2011). Some cytokine-based therapies are currently approved by the FDA. For example, IL-2 is now used to treat patients with metastatic melanoma (Schwartzentruber et al., 2011, Vial and Descotes, 1992). However, cytokines can be toxic, especially when they are used at high

concentrations (Vial and Descotes, 1992). Furthermore, in addition to their direct effect on tumour cell growth and survival, some cytokines can be immunosuppressive such as IL-6 (Hegde et al., 2004).

1.15.5.6. DNA-based vaccine

DNA-based vaccine is plasmid DNAs which encode the desired protein for vaccination (Lowe et al., 2007, Fioretti et al., 2010). The expressed protein is then taken up by professional antigen presenting cells such as DCs to be processed and presented to elicit both humoral and cellular-immune mediated responses (Lowe et al., 2007, Bolhassani et al., 2011).

DNA vaccines are easily produced and provide a variety of practical benefits for large scale vaccine production that are not as easily manageable with other forms of vaccines including recombinant protein or whole tumour cells (Fioretti et al., 2010). However, using virus as a vector for gene transfer can induce anti-virus capsule immune responses, affecting the efficacy of the vaccine (Fioretti et al., 2010).

There are now wide variety of cancer-associated antigens that are selected for DNA vaccine such as CDK-4 (tumour specific melanoma antigen), MUC1 (over expressed antigen in breast and ovarian cancer cells) and the Gp100 antigen (melanoma differentiation antigen) (Lowe et al., 2007).

Immunizing mice previously infected with severe acute respiratory syndrome (SARS) coronavirus with DNA vaccine encoding the spike (S) glycoprotein of the virus induced robust humoral and cellular responses, and reduced viral replication significantly in the lung (Yang et al., 2004). Protection was mediated by neutralizing antibody-dependent mechanism (Yang et al., 2004). DNA vaccine has enrolled clinical trials to treat viral and non-viral diseases including human immunodeficiency virus (HIV), human papillomavirus (HPV), hepatitis B virus, melanoma and prostate cancer (Ferraro et al., 2011).

1.15.5.7. Whole-cell tumour vaccination

Whole-cell tumour can also be used to generate anti-tumour responses because they contain all the candidate antigens that may trigger the immune response. In the 1970s Hanna *et al* vaccinated two guinea pigs with irradiated hepatocellular carcinoma cells in

combination with bacillus calmette-guérin (BCG). The vaccination generated protective immunity against a subsequent challenge with syngeneic live tumour cells (Hanna and Peters, 1978). This study indicated the efficacy of vaccinating with live tumour cells.

Tumour cells can be genetically engineered to secrete immunomodulatory cytokines to augment anti-tumour responses and this approach has been validated in several different tumour models including melanoma, colon, renal and lung cancer (Eager and Nemunaitis, 2005). Results collected by Dranoff G and colleagues in mice revealed that injecting irradiated B16 melanoma cells previously transduced to express granulocyte-macrophage colony-stimulating using factor (GM-CSF) induced both $CD4^+$ and $CD8^+$ T cell responses which were responsible for improving overall survival compared with B16 alone (Dranoff et al., 1993).

1.15.5.8. DC-based vaccine in cancer immunotherapy

As mentioned earlier, DCs can take up tumour antigens from living or dying tumour cells and cross-present them to T cells in the adjacent draining lymph nodes to generate cytotoxic $CD8^+$ T cells (Diamond et al., 2011). Therefore, DCs can be utilised as cancer vaccines for effective anti-tumour responses. DCs can be exploited for vaccination against cancer through various means, including nontargeted antigen, vaccines composed of antigens directly coupled to DC antibodies, or ex vivo generated DC pulsed with antigen (Palucka and Banchereau, 2013).

DC-based vaccines have shown encouraging clinical outcomes (Palucka and Banchereau, 2012). An earlier study has shown that a DC-based vaccine is safe and can generate tumour antigen-specific $CD4^+$ and $CD8^+$ T cell responses (Draube et al., 2011). Selection of the best tumour antigen to be loaded on the DC for efficient T cell responses is challenging, because $CD8^+$ T cells with high avidity to non-mutated self-antigen are usually deleted by either central or peripheral tolerance (Palucka and Banchereau, 2012, Palucka et al., 2011). Thus, generating mutated tumour antigens are required to overcome clone deletion.

Sipuleucel-T or Provenge is a novel cancer vaccine that has been approved by the food and drug administration FDA to treat metastatic prostate cancer (Thara et al., 2011). Provenge consists of autologous mononuclear cells which have been activated ex vivo against prostate

cancer antigen prostatic acid phosphatase (PAP) in the presence of GM-CSF. Recent clinical phase III study revealed that Provenge prolonged median survival in patients with advanced prostate cancer (Thara et al., 2011). Advert affects were mostly grade 1 or 2 (Thara et al., 2011).

1.16. Cancer immunotherapy clinical trials

I the section above, I have described different immunotherapy approaches to induce strong anti-tumour immune responses to kill advanced tumour. In this section, I will focus on some immunotherapy strategies which are currently used in clinical trials to treat patients bearing advanced tumour particularly melanoma.

1.16.1. Immune check point blockade and melanoma

The blockade of immune checkpoint is one the most promising approaches to activating therapeutic antitumor in different tumour models including melanoma. Blocking co-inhibitory receptors like CTLA-4 and PD-1 is emerging as a potential approach to produce durable clinical responses in melanoma.

1.16.1.1. Anti-CTLA-4 in the clinic

Ipilimumab is a human anti-CTLA-4 mAb that was approved by the FDA in March 2011 for the treatment of patients with advanced tumours (Graziani et al., 2012, Hodi et al., 2010). The median overall survival of patients with advanced melanoma is only 7-9 months (Middleton et al., 2000). Meta-analysis of a phase II study revealed that only 25% of patients with unrespectable melanoma undergone chemotherapy lived to up to one year (Korn et al., 2008). However, ipilimumab improved the median survival of patients with advanced melanoma. Randomized phase III clinical trial which included 676 patients with stage III or IV melanoma, patients were treated with ipilimumab in combination with gp100 , ipilimumab alone or with gp100 alone. The overall survival in the ipilimumab plus gp100 group, ipilimumab or gp100 alone after one year was 43.6%, 45.6% and 25.3% respectively, and 21.6%, 23.5% and 13.7% after two years respectively, confirming the remarkable efficacy of ipilimumab for melanoma (Weber et al., 2009).

1.16.1.2. Anti-PD-1 mAb

Nivolumab (also known as BMS-936558 or MDX1106) is a human IgG4 anti-PD-1 Ab. In phase 3, randomized study, Nivolumab was given to in patients with metastatic melanoma to determine whether it will improve survival in comparison to dacarbazine (is a commonly used chemotherapy in patients with cancer (Orlandi et al., 1994)). Overall survival in one year in patient treated with Nivolumab was 72.9% compared with 42.1% in individuals initially received dacarbazine (Robert et al., 2015a). The median progress-free survival was 5.1 months in the Nivolumab group and 2.2 in decarbazine. Moreover, the objective response when Nivolumab was administered was significantly higher than the rate in decarbazine group. (40% compared with only 13.9% respectively). The frequency of treatment-related adverse events were low after treatment with Nivolumab, while in the decarbazine group, adverse events were more frequent including gastrointestinal and hematologic toxic events (Robert et al., 2015a).

As well as nivolimab, other PD-1 blockers being tested are Pembrolizumab, MDX-1105 and CT-011. Pembrolizumab (MK-3475) is another humanized anti-PD-1 Ab (IgG4) recently entering phase I clinical trials. In the clinical trial study, one patient with solid melanoma and one patient with Merkel cell carcinoma experienced complete responses of more than 57 and 56 weeks duration respectively, while three patients experienced partial response and fifteen patients bearing different malignancies had stable disease (Patnaik et al., 2015).

CT-011 is a humanized IgG1 monoclonal antibody (Berger et al., 2008). Benson Jr and colleagues have shown that pre-treating human NK cell with CT-011 enhanced NK cell trafficking to multiple myeloma cell, enhanced immune complex formation between NK cells and PD-L1 expressing tumour cells and their cytotoxicity (Benson et al., 2010). Furthermore, results from phase 1 clinical trial conducted in 17 patients with advanced hematologic malignancies revealed that clinical benefit was observed in 33% of the patients with one complete remission. No adverse effect was observed in patient population (Berger et al., 2008).

Anti-CTLA-4 and PD-1 inhibition have been recently combined in the clinic in patients with advanced melanoma. 72 patients were treated with the combination of Ipilimumab (3 mg per kilogram of body weight) and Nivolumab (1 mg per kilogram) every 3 weeks for four doses, while 37 patients received Ipilimumab only (3 mg per kilogram) every two weeks until

the progression of the tumour or unacceptable drug-toxicity. Objective response was 61% in the cohort treated with the combination, with 22% among them with complete responses. In contrary, the objective response was 11% in the group received Ipilimumab only with no complete response (Postow et al., 2015b). Drug-related adverse events of grade 3 or 4 were reported in 54% of the patients who received the combination therapy as compared with 24% of the patients who received ipilimumab alone (Postow et al., 2015b).
These results indicated that dual blockade of co-inhibitory receptors improved response rates compared with monotherapy.

1.16.2. Adoptive cell transfer (ACT)

1.16.2.1. Adoptive T-cell therapy using autologous TILs

ACT with autologous TILs has shown encouraging results in clinical trials, with evidence of durable ongoing complete responses in patients with advanced melanoma. Rosenberg *et al.* showed that TILs can also be isolated from patients with melanoma by resecting the tumour from the patient and divided into multiple tumour fragments or single cell suspension that are individually incubate in the presence of IL-2. Lymphocytes overgrow and destroy tumour cells in 2 to 3 weeks forming pure lymphocytes culture. Individual cultures are then expanded rapidly by incubating with IL-2 and anti-CD3 Ab into the culture for 2 to 3 weeks, generating up to 10^{11} lymphocytes can be obtained for reinfusion into patients (Rosenberg and Restifo, 2015).

1.16.2.2. TCR gene modification

TCRs have now been genetically engineered to recognise various tumour epitopes, for example the gp100 melanoma differentiation antigen, the tyrosinase melanocyte differentiation antigen, NY-ESO-1 cancer/testis antigen, carcinoembryonic antigen (CEA), which is expressed by colorectal and other forms of cancers, and TARP (a nuclear protein expressed in prostate and breast cancer cells) (Wolfgang et al., 2000, Essand and Loskog, 2013). The first successful use of TCR engineered T cells in a clinical trial was in 2006, when a group of patients bearing metastatic melanoma were infused with autologous T cells genetically modified to recognise the melanoma-associated antigen (MART-1). Two out of fifteen patients showed sustained objective clinical responses (Essand and Loskog, 2013).

These results encouraged scientists to increase the efficacy of the treatment. In a second attempt, highly reactive TCR to MART-1 and gp100 were isolated. To overcome central tolerance of T cells expressing a TCR with high affinity for human gp100, highly reactive human $gp100_{145\text{-}162}$ (hgp100)-specific CTLs were generated by immunizing HLA-A*0201 genetically engineered mice with the hgp100. TCRs that had the highest anti-tumour reactivity to hgp100; TCR clone DMF5 (lymphocyte clone DMF5 is more avid to MART-1:27-35 peptide epitope compared with the original clone DMF4 that was targeted in the first attempt) were identified (Johnson et al., 2009). Then, donor peripheral blood lymphocytes (PBLs) were transduced to express α and β chains specific for the $gp100_{(154\text{-}162)}$ or with the human DMF5. Thirty percent of patients that received T cells expressing the DMF5 TCR demonstrated clinical responses as well as loss of skin pigmentation (Johnson et al., 2009), while 19% of donors infused with $gp100_{(154\text{-}162)}$ TCR-transduced cells had complete tumour rejection (Johnson et al., 2009). These results indicate the importance of modifying TCR that is able to bind to TAA with high avidity to eliminate tumour growth.

1.16.2.3. Chimeric antigen receptor (CAR)

CAR-modified T cells have been tested in different tumour models (Shirasu and Kuroki, 2012, Kochenderfer et al., 2010). Early clinical trials revealed that injecting CD19-based CARs into patients infected with $CD19^{+}$ chronic lymphocytic leukaemia gave encouraging outcomes with 82% of individuals being tumour free (Makkouk and Weiner, 2015, Shirasu and Kuroki, 2012). However, and despite promising results observed in earlier clinical trials, using third generation CARs can trigger a lethal cytokine storm (Essand and Loskog, 2013, Heslop, 2010). In a study conducted by the National Institutes of Health (NIH), a 39 year old female with colorectal cancer previously treated with chemotherapy received a high number (10^{10}) of T cells expressing a CAR recognising ERBB2 (HER-2/neu) (member of the epidermal growth factor receptor family) and containing CD28, 4-1BB and CD3 zeta signalling domains after lymphodepletion. Fifteen minutes after injecting the cells she experienced pulmonary toxicity and death on day 4 post cell transfer. This response was due to excessive cytokine release (Morgan et al., 2010). Another potential concern of CARs is that they may reduce the activation threshold of transferred T cells to limits where they could become activated without antigen (Morgan et al., 2010).

1.16.3. Peptide-based vaccine and

Recent studies revealed that synthetic long peptides are highly immunogenic, and can elicit potent $CD4^+$ and $CD8^+$ T cells responses (van Poelgeest et al., 2013, Ly et al., 2013). In a recent clinical phase II study, Poelgeest M *et al.*, showed that vaccinating women bearing Human Papilloma Virus type 16 (HPV16)-induced gynaecological cancer subcutaneously with overlapping synthetic long peptides derived from the virus and dissolved in Montanide resulted in partial tumour regression with minimal side effects (van Poelgeest et al., 2013). Clinical responses were correlated with the enhanced HPV16-specific T-cell responses and cytokine production (van Poelgeest et al., 2013).

1.16.4. Whole-cell tumour vaccination

Patients with stage III melanoma were vaccinated with (DNP)-modified autologous irradiated melanoma cells plus BCG to produce a local inflammation without ulceration. Results showed that overall survival was increased in 44% of the treated patients and delayed-type hypersensitivity (DTH) responses were observed in 47% of the patients. It is worth noting that overall survival in DTH positive patients was doubled (59.3% compared with 29.3% respectively) (Berd et al., 2004).

Tumour cells can be genetically engineered to secrete immunomodulatory cytokines to augment anti-tumour responses. This type of vaccine was also tested in patients with metastatic cancer. For example, five patients with stage IV melanoma were injected with retrovirally transduced autologous tumour cells expressing the GM-CSF gene. One patient had complete tumour regression for more than 36 months after the initial injection (Kusumoto et al., 2001). In another study in Japan, six patients with stage IV renal cell carcinoma received lethally irradiated GM-CSF-transduced autologous renal tumour cell vaccines. The vaccination recruited eosinophils, DCs at the vaccination site as well as enhanced the infiltration of the CD4+ T cells to the tumour site (Tani et al., 2004). The proliferative response of specific T cells was increased in all patients and three of four patients had enhanced cytotoxicity against the tumour (Tani et al., 2004). All patients had skin reaction at the vaccination site.

1.17. Combination cancer immunotherapy

Despite durable antitumor response was observed monotherapy, vaccine treatment alone usually is not efficient to objective regression in advanced tumour. Furthermore, some patients do not fully response to monotherapy. Therefore, combination cancer vaccine might be required to improve the outcome of the treatment.

1.17.1. Synthetic peptide vaccine in combination with mAbs

Peptide vaccine was recently combined with blocking antibody to increase the efficacy of peptide vaccine. In a recent clinical trial, patients with metastatic melanoma were treated with ipilimumab alone (anti-CTLA-4 antibody) gp100 alone (1 mg in the anterior thigh) or a combination of both. Combining ipilimumab with the peptide vaccine improved survival compared to when peptide was injected alone (43.6% and 25.3% respectively at the first 12 month respectively). However, the rate of overall survival in the combination group and ipilimumab alone was similar (43.6% and 45.6% respectively) (Hodi et al., 2010).

1.17.2. Administration of adjuvants after tumour resection

Adjuvant systemic therapy like IFN-α-2b has been injected after surgically resected stage IV melanoma to improve the treatment. Injecting high dose of IFN-α-2b improved relapse-free in randomised clinical trials. However, overall survival is inconsistent across trials (Tarhini and Thalanayar, 2014).

Talimogene laherparepvec (T-VEC) is a herpes simplex virus designed to selectively replicate in tumour cells and produce GM-CSF to induce anti-tumour immune responses. T-VEC was tested and compared with GM-CSF in a phase III clinical trial in patients with solid melanoma stage III to IV. Overall response rate in T-VEC arm compared to GM-CSF was 26.4% and 5.7% respectively. Overall survival with T-VEC was 23.3 months, and 18.9 months with GM-CSF. Side effects resulted from T-VEC treatment were minimal ranged between fatigue, pyrexia and chills. Grade 3 to 4 adverse effect was seen in less than 2% of T-VEC treated patients (Andtbacka et al., 2015).

1.17.3. Combining checkpoint blockade antibodies with cytokines

To improve the anti-tumour immune response of checkpoint blockade antibodies, cytokines are combined with treatment for enhanced immune responses. Administering GM-CSF systemically (sargramostim) plus ipilimumab is in clinical trial phase II currently. A total of 245 patients with advanced melanoma (stage III or IV) were treated with the combination of ipilimumab and sargramostim, or ipilimumab alone. Median overall survival for the group treated with the combination was 17.5 months compared to 12.7 months for ipilimumab alone (Hodi et al., 2014). Grade 3 to5 adverse events were observed In patients treated with both ipilimumab and sargramostim, and 58.3% adverse events occurred in ipilimumab alone (Hodi et al., 2014).

1.18. Aims of the thesis

Many tumour-associated antigens are derived from self-proteins. T cells that recognize these antigens with high affinity are deleted during negative selection in the thymus, whereas those that express low affinity TCRs will be subject to self-tolerance mechanism that operates in peripheral tissues. I set out to investigate the requirement to prime and maintain a $CD8^+$ cytotoxic T cell response directed against gp100, a protein expressed by normal melanocytes as well as the majority of melanomas. Of particular interest is the possibility of inducing an anti-tumour immune response in the absence of overt autoimmunity. A prediction of this hypothesis is that mechanisms of peripheral tolerance to self-tissues and tumours are partially distinct.

To investigate the requirement for priming a gp100-specific $CD8^+$ T cell response, I first established an adoptive transfer approach wherein pmel-1 TCR transgenic T cells are transferred into congenic mice followed by immunisation with gp100 peptides and co-adminsitration of immunomodulatory agents.

Previous studies have demonstrated that enforced co-stimulation through members of the TNFRSF using agonistic mAbs or soluble recombinant ligands is a useful approach to prime $CD8^+$ T cell responses *in vivo*. However, the majority of these studies used model non self-antigens, such as ovalbumin, that elicit high affinity T cells. Thus, detailed examination of how co-stimulation through different members of the TNFRSF affects self-reactive $CD8^+$ T cell responses has not been conducted. Furthermore, engagement of co-stimulatory receptors such as CD27 or 4-1BB has been shown to elicit distinct differentiation programmes in ovalbumin-specific $CD8^+$ T cells which differentially impact effector and memory cell generation. This aspect has not been investigated in the context of self-reactive T cells.

The specific aims of the thesis are:

1. To establish an immunization protocols to expand adoptively transferred pmel-1 TCR transgenic T cells.

2. To compare the effects of signalling by different members of the TNFRSF on pmel-1 TCR transgenic T cell priming and differentiation into effector and memory cells.

3. To assess the impact of co-stimulation via TNFRSF members on anti-tumour immunity/autoimmunity.

4. To investigate the effect of checkpoint blockade in combination with co-stimulation on $CD8^+$ T cell priming and anti-tumour responses.

5. To probe the mechanisms responsible for generation of memory $CD8^+$ T cells following vaccination.

Chapter 2. Material and methods

2.1. Tumour Cell lines

The CT26 colon carcinoma (syngeneic to BALB/c mice), B16-OVA, B16-F10 and B16-BL6 melanoma (syngeneic to C57BL/6 mice) cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, from life technologies) as a base medium. In each case, media was supplemented with 10% fetal calf serum (FCS) to provide essential growth hormones, L-glutamine, penicillin/streptomycin antibiotics and pyruvate (energy source). Cells were maintained at 37°C and 5% CO_2.

2.2. Tumour cell culture and *in vivo* tumour challenge

For CT26 and B16-BL6 tumour challenge, media was removed from flasks; cells were then washed twice with 1x phosphate buffered saline (PBS, from severn biotech) to remove dead cells and any remaining media. Cells were detached by covering the cells with sterile trypsin (0.05%)/ Ethylenediaminetetraacetic acid (EDTA) (1mM) (purchased from Lonza) for 5min at 37°C followed by wash in media to remove trypsin/EDTA. Cells were then washed twice with PBS to remove any media. For CT26 cells, $5x10^5$ cells/mouse were inoculated subcutaneously (s.c.) in PBS, and for B16-BL6 $2x10^4$ were injected intradermally (i.d.).

For all tumour challenge experiments, tumour growth was monitored every 2-3 days by using digital calliper. Individual mice were culled when the mean tumour diameter reached 15mm.

2.3. Mice

Wild-type (WT) C57BL/6, BALB/c and OT-1 transgenic mice on a C57BL/6 background (in which $CD8^+$ T cells are specific for H-2kB and a peptide derived from ovalbumin) were purchased from our Biomedical Facility. Pmel-1 transgenic mice (in which $CD8^+$ T cells are specific for H-2Db and the $gp100_{25–33}$) were purchased from Jackson Laboratories (USA) and were bred in house. In all experiments mice were age and sex-matched.

2.3.1. Generation of the Bcl-2$^+$ pmel-1 mice

To generate pmel-1 mice that carry the Bcl-2 oncogene, I crossed transgenic pmel-1 mice to mice in which the Bcl-2 transgene (VavP-Bcl-2) is over-expressed in all hematopoietic lineages. Generated mice were screened to check for Bcl-2 oncogene expression by flow cytometry. Cells from the blood of these mice were surface stained with Allophycocyanin (APC)-conjugated anti-Thy1.1 and phycoerythrin (PE)-conjugated anti-CD8, prior to fixation and staining with fluorescein isothiocyanate (FITC)-conjugated anti-Bcl-2 (intracellular stain, section 2.7) using an Intracellular Fixation & Permeabilization Buffer Set (eBioscience) according to manufactures instructions.

2.3.2. Experimental mice and cages

To distinguish between individual mice, all mice were ear tagged from 0 to 9 dependent on the number of mice in each box.

2.4 Single cell suspension and sample preparation

Single cell suspension of spleens from 6-8 week old OT-1 or pmel-1 CD8$^+$ transgenic mice was prepared by passing through a 70um filter. Red blood cells (RBC) were removed by adding 5mls RBC lysate buffer (8.2g of Ammonium chloride (NH_4CL) and 1g of potassium hydrogen carbonate ($KHCO_3$) in 1 litter of distilled water) and centrifuged for 5min at 1300 revaluation per minute (rmp). Cells were washed twice with PBS to remove RBC lysate buffer, then resuspended in 5ml of PBS. Cells were counted using a Coulter Counter (particle counter), and cell viability was checked by visualising the frequency of cells excluding trypan blue under the microscope.

2.5 Thymocytes extraction and preparation

To extract the thymus form the mice, Bcl-2$^+$ or WT pmel-1 mice were first anesthetized using mice CO_2 gas chamber. Thymus from each mouse was harvested and transferred immediately into a separate tubes containing PBS. Single cell suspension was performed (see section 2.4) for thymocytes staining (section 2.6.) and analysis.

2.6 Surface stain for flow cytometery

For cell surface staining, $1x10^6$ cells/tube were re-suspended in 100ul of fluorescence activated cell sorting (FACS) buffer consisting of PBS+0.2% bovine serum albumin (BSA). Cells were then incubated with in-house blocking anti-Fc antibody (clone 2.4G2) at a concentration of 10ug/ml in fridge for 15 min. Blocking antibody was then washed twice with FACS buffer by centrifuging at 1300rpm for 5 minutes. Antibodies against target antigens (Table 2-1) were added to the FACS buffer (200ul/tube) at 10ug/ml and incubated in the fridge for ~30 minutes. Next, cells were then washed and re-suspended in 200ul of FACS buffer for analysis. Ki-67 was monitored during the contraction phase after the peak of the response.

Table 2-1: Primary antibodies which were used for a range of applications

Target antigen	clone	Conjugate	Source
CD8	53-6.7	APC/FITC	eBioscience
CD8	YTS169	PE	In house
CD4	GK1.5	APC	eBioscience
Thy1.1 (CD90.1)	HIS51	APC/ eFlour 450/FITC	eBioscience
vβ13	MR12-3	FITC	BD Pharmingen
PD-1	J43	APC	eBioscience
PD-L1 (CD274)	1-111A	APC	eBioscience
PD-L2 (CD273)	122	APC	eBioscience
CD27	LG.7F9	PE	eBioscience
GITR	DTA-1	PE	eBioscience
OX40	OX-86	APC-Cy7-A	eBioscience
4-1BB	17B5	eFlour450	eBioscience
Ki-67	B56	FITC	BD Pharmingen
CD107a	eBio1D4B	eFlour450	eBioscience
Eomes	DAN11MAG	FITC	eBioscience
T-bet	eBio4B10	APC	eBioscience
FoxP3	FJK-16s	FITC	eBioscience
IFN-γ	XMG1.2	APC	eBioscience
TNF-α	MP6-XT22	FITC	eBioscience

IL-2	JES6-5H4	PECy7	eBioscience
Perforin	eBioOMAK-D	FITC	eBioscience
Granzyme B	GB11	PE	eBioscience
Bcl-2	MOPC-21	FITC	BD Pharmingen

2.7 Intracellular staining for flow cytometry

To stain for intracellular proteins, after surface staining (see section 2.6), cells were fixed and pereabilised using the Intracellular Fixation & Permeabilization Buffer Set (eBioscience). Briefly, cells were fixed using fixation buffer for 20mins at 4℃, washed twice in 1x permeabilization buffer to remove any fixation buffer residues by centrifuge at 1300 rpm for 5min. Cells were then left in permeabilisation buffer for 30 mins or 18 hours at 4℃. Cells were then stained with 100ul conjugated antibodies (10ug/ml) against the antigen of interest for 30 mins, then washed once in permeabilisation buffer and resuspended in 100ul of FACS buffer.

For intracellular cytokine staining, blood cells were incubated in a 96 well plate with gentle red blood lysis buffer (ACK Lysing Buffer, Qiagen) for 10 mins at room temperature (RT), then centrifuged and washed with PBS at 3000 rpm for 5 mins. Cells were then resuspended in 200ul of suppelemnted RPMI 1640 media (from life technologies) prior to restimulation with peptide. For restimulation, cells were incubated for 4 hours at 37℃ with 1uM of KVPRNQDWL peptide (in the case of pmel-1 T cells) or with 1nM of SIINFEKL (for OT-1 T cell reactivation) in the presence of 1/1000 dilution of GolgiPlug (BD Pharmingen) to block protein transport and accumulate of cytokines within the cell. After incubation, cells were washed in PBS in the presence of 1/1000 dilution of GolgiPlug (200ul per well). Cells were then fixed with formaldehyde for 15 minutes at RT then were they washed in PBS and left in permeabilisation buffer for 30 minutes. Cells were then washed in permeabilisation buffer and stained for cytokines (10ug/ml) (IL-2, TNF-α and IFN-γ) (Table 2-1) for 30 mins at 4℃, then were washed twice with PBS/0.2%BSA and resuspended in PBS/0.2%BSA (200ul) for flow cytometer.

2.8 In vivo antibody preparation and injection

Antibodies were kept in Tris in -80 for long-term storage. For in vivo injection, the desire antibody was thawed and the dialysis cassette (purchased from ThermoFisher) was hydrated in PBS buffer for 1 minute in RT to give the membrane the flexibility to inject the sample. By using a syringe, antibody was injected into the cassette from the corner of the cassette, and air bobbles were removed to maximize sample contact with the membrane. Float buoy was attached to keep the cassette floating while washing the antibody. PBS buffer was changed every hour for five times in a cold room. The desire antibody was withdrawn using a syringe and stored in -20℃. Mice were injected with the following antibodies (Table 2-2):

Table 2-2: Antibodies for *in vivo* injection, antibodies are generated in house unless otherwise stated

Antibody	Clone	Isotype
anti-CD27	AT12-4	Rat IgG2a
Mouse anti-CD27 (CD16)	3G8	IgG1
Mouse anti-CD27 (CD37)	WR17	IgG2a
4-1BB (CD137)	LOB12.3	IgG1
OX40 (CD134)	OX86	MouseIgG2a
GITR	DTA-1	IgG2b
PD-1 (CD279) Source: BioXcell	RMP1-14	Rat IgG2a
PD-L1 (CD274) Source: BioXcell	10F.9G2	Rat IgG2b
CTLA-4 Source: BioXcell	9D9	Mouse IgG2b
IL-2 (CD25)	JES6.1-1A12	Rat IgG2a
IL-2 (CD122)	S4B6.1	Rat IgG2a
CD40	3/23	Rat IgG2a

For in vivo injection, all antibodies were injected in PBS. All work was performed under a project licence and a personal licence.

2.9 In vivo adoptive cell transfer

2.9.1 Ovalbumin-specific OT-1 cell transfer:

Single cell suspension of spleen and lymph nodes from OT-1 mice were prepared as above (section 2.4.). Next, an aliquot of $1x10^6$ OT-1 T cells was re-suspended in 100ul of FACS buffer with 10ug/ml of 2.4G2 Fc blocking mAb for 10min at 4℃. Cells were washed with FACS buffer then stained with anti-mouse allophycocyanin (APC)-conjugated CD8a (clone: 53-6.7) and in-house phycoerythrin (PE)-labelled H-2Kb tetramers, and then incubated at 4℃ for 30 min. Cells were then washed with FACS buffer and resuspended in 100ul of FACS buffer. The proportion of OT-1 cells was determined using a Canto II flow cytometer gating on $CD8^+$ $tetramer^+$ T cells within a lymphocyte gate. The indicated number of $CD8^+$ $tetramer^+$ T cells were transferred i.v. into sex and aged matched C57BL/6 mice.

2.9.2 Gp100-specific pmel-1 CD8+ T cell transfer:

For pmel-1 CD8+ T cell transfer, splenic single cell suspensions were prepared as above except the proportion of pmel-1 $CD8^+$ T cells was assessed by gating on $Thy1.1^+$ $V\beta13^+$ $CD8^+$ T cells. $1x10^6$ cells were stained in FACS buffer with in-house PE-conjugated anti-$CD8^+$ and anti-mouse/rat CD90.1 (Thy-1.1) APC (clone: HIS51) and fluorescein isothiocynate (FITC)-conjugated mouse anti-mouse v$\beta13^+$ TCR (clone:MR12-3). The indicated number of pmel-1 cells was transferred i.v. into sex and aged matched C57BL/6 recipient mice.

2.9.3 Bcl-2^+pmel-1 T cell transfer:

To determine the proportion of pmel-1 $CD8^+$ T cells in T cells in Bcl-2^+ and Bcl-2^- mice, splenocytes were stained with a commercially available H-2Db-PE-conjugated KVPRNQDWL dextramer (SIGMA-Aldrich) and CD8 as a marker instead of Thy1.1, because the frequency of $Thy1.1^+$ T cells in the Bcl-2^+ pmel-1 mice is lower compared to WT pmel-1 mice. Alternatively, I decided to use anti-v$\alpha1$ antibody but it was not commercially

available. Therefore, I relied on dextramer and CD8 stain to detect pmle-1 T cells. For the transfer, an equal number ($3x10^5$) of WT pmel-1 or Bcl-2^+ pmel-1 T cells was transferred into C57BL/6 recipient mice. Transferred cells were monitored in the blood at different time points by staining with dextramer and CD8**.**

2.10 *In vitro* pmel-1 and OT-1 cell stimulation and proliferation assay

To assess proliferation and cytokine production by pmel-1 or OT-1 $CD8^+$ T cells after in vitro activation, single cell splenic suspensions from pmel-1 or OT-1 mice were prepared as above (section 2.4.) Splenocytes were then re-suspended in complete RPMI 1640 media containing 50uM 2-mercaptoethanol. $1x10^5$ splenocytes were then loaded/well in a 96 well plate with hgp100 (amino acids 25-33) peptide in the case of pmel-1 or with SIINFEKL, SIIQFEKL or SIITFEKL peptides in the case of OT-1 cells at different concentrations. Cells were incubated at 37℃ in 5% CO_2 and 50ul of supernatant was collected at 24hr for the detection of secreted IL-2 and another 50ul after 48hr for detection of INF-γ. At 48 hours [3H]thymidine was added and cells were returned the incubator for a further 18-20 hours.

To measure uptake of [3H]thymidine. [3H]thymidine (1μCi/well) was added ~18 hours to the cell culture prior to cell harvesting. Cells were harvested onto UniFilter glass fiber plates (PerkinElmer,UK) using automated filtermate harvester (PerkinElmer, UK). UniFilter glass fiber plate was left overnight to dry at RT. Incorporation of [3H]thymidine incorporation was measured by adding scintillation fluid then place the plate into the TopCount Microplate Scintillation counter (PerkinElmer). Cell proliferation was measured as count per minutes (CPM).

Cytokines produced in the media were detected by Enzyme-linked immunosorbent assay (ELISA) as follow.

2.11 Enzyme-linked immunosorbent assay

For detection of IL-2 and IFN-γ 96 well Maxisorb (Nunclon) plates were coated with rat anti-mouse IL-2 (BD pharmingen, JES6.1H12) (1ug/ml) or rat anti-mouse INF-γ (HB170) capture Abs (4ug/ml) (100ul/well) and incubated at room temperature (RT) overnight. Plates were then blocked in PBS 1% bovine serum albumin (BSA) and incubated for 1 hour at RT, then

washed (x4) with PBS/0.05% Tween-20. Serial dilution (1/2) of standard recombinant mouse IL-2 (BD Pharmingen) (2ng/ml) top concentration or INF-γ (BD Pharmingen) (4ng/ml) was prepared in RPMI 1640 media and added to the plate (100ul/well) in duplicate. Test sample were similarly added at 100ul/well in duplicate; plates were then incubated for 2hr at 37℃. Plates were then washed (x4) with PBS/0.05%Tween-20 (Sigma-Aldrich) (v/v) and then loaded with biotinylated rat anti-mouse IL-2 (eBioscience,JES65H4) (1ug/ml) or anti-INF-γ Ab (eBioscience) in PBS/1%BSA (w/v) and incubated for 1hr at RT. Plates were washed (x4) with PBS/0.05%Tween-20 and labelled with streptavidin horseradish peroxidase (HRP) (Invetrogen) in PBS/1%BSA (w/v) 100ul/well and incubated for 30min at RT. O-Phenylenediamine (OPD) (Sigma-Aldrich) substrate [1 OPD tablet was dissolved in 25ml citrate buffer (19.2g citric acid/L), 25ml phosphate buffer (28.4g Na_2HPO_4/L), 50ml deionized water dH_2O and 20ul of H_2O_2 (Merk, Feltham, UK)] was added to samples (100ul) for ~30min in darkness for HRP colorimetric detection. To stop the reaction, 50ul/well of 2.5M H_2SO_4 (Merck) was added to stop the reaction. Plates were then loaded into ELISA-reader to read results directly at wavelength of 490nm on a Dynatech MR4000 plate reader.

To drive T cell activation and tumour injection in vivo I tested a number of peptide, Toll-like receptor agonist and antibody combination. The preparation and injection of these is described below.

2.12 Peptide preparation and injection

$OVA_{257-264}$ SII**N**FEKL peptide and its variants SII**Q**FEKL, SII**T**FEKL, and human gp100 (hgp100) amino acids (KVPRNQDWL) peptide were all purchased from Peptide Protein Research Ltd at >95% pure and endotoxin low (Fareham, United Kingdom). Peptides were dissolved in 20% Dimethyl sulfoxide DMSO/H_2O. For *in vivo* use peptides at concentrations of between 0.5-2mgs were injected i.v. on day -1 prior to antibody injection.

2.13 Ovalbumin preparation and injection

Chicken ovalbumin (OVA) (SIGMA-Aldrich.UK) was provided in a powder form and was dissolved in PBS immediately before injection.

2.14 IL-2 complex (IL-2Cx) preparation and administration

For the injection of IL-2 complexes into mice, 1.5ug recombinant mouse IL-2 (rmIL-2); was mixed with 20ug of anti-CD25 (clone JES6.1-1A12; BD Pharmingen) or with 20ug of in-house anti-CD122 (S4B6.1) in PBS and incubated for 15min at RT before injection i.p. (Letourneau et al., 2010).

2.15 Rapamycin preparation and administration

Rapamycin (SIGMA-Aldrich) was dissolved in 100% DMSO. Rapamycin was given at high (1500ug/kg^{-}) or low dose (75ug/kg^{-}) i.p. in a final concentration of 6% DMSO/PBS with anti-CD27mAb. Low dose rapamycin was given daily from day 1 to day 8 post peptide immunization, while the high dose was administered daily from day 4 the peak of the pmel-1 response to day 8.

2.16 Toll-like receptor preparation and injection

Lipopolysaccharide (LPS) preparation and injection

LPS from *Escherichia coli* (from Sigma) was dissolved in distilled water and 10ug/ml was injected either i.p. or i.v. in PBS.

Poly I:C injection and preparation

Poly I:C (from Sigma) was administered i.v. at 50ug/ml in PBS

2.17 In vitro expression of PD-L1/L2 on tumour cells

To assess surface expression of PD-L1 and PD-L2 on tumour cells at rest or after treatment with IFN-γ, CT26 and B16 were incubated with or without 20ng/ml of IFN-γ for 18hrs. Cells were then washed twice with PBS, covered with 1mM EDTA in PBS and left for 10 to 15 min in at 37℃ incubator to detach cells. Cells were then washed twice with PBS and surface Fc receptors were blocked as usual with 2.4G2 (see section 2.4.). In separate tubes, cells were then stained with an APC-conjugated mouse IgG2a isotype control antibody, mouse APC-conjugated anti-PD-L1 or with mouse anti-PD-L2 (APC) then incubated for 30min at 4℃.

Cells were then washed with FACS buffer then re-suspended in 100ul of FACS buffer and analysed on a Canto II flow cytometerStatistical analysis

2.18 Statistical analysis

All data were analysed using the GraphPad Prism software. *t*-test or one way ANOVA was performed using unpaired test. Data was considered significantly different when $P < 0.05$. Data shows Mean ± SEM of group.

Chapter 3. Optimizing anti-tumour CD8$^+$ T cell responses using TNFRSF agonists and checkpoint blockade

3.1. Introduction.

Thymic selection generates a T cell repertoire capable of mounting immune responses against external antigens while largely avoiding self-reactivity (Escors, 2014, Koch and Radtke, 2011). The majority of cancer immunotherapy vaccines are designed to elicit potent CD8$^+$ T cells, due to their specificity and ability to recognise and directly kill malignant cells (Makkouk and Weiner, 2015, Kalos and June, 2013, Topalian et al., 2011, Cho and Celis, 2009, Overwijk et al., 2003). Effective cancer immunotherapy is dependent on generating large numbers of anti-tumour-reactive T cells with appropriate cell trafficking and effector functions (Pandolfi et al., 2011, Abad et al., 2008, Cho et al., 2012). However, many tumour antigens are non-mutated self-derived proteins, resulting in a suboptimal TCR/peptide-MHC activation signal and anergy (Escors, 2014, Sadegh-Nasseri et al., 2010). In addition, tumour cells can downregulate peptide-MHC-I expression, and utilise multiple inhibitory mechanisms within the tumour microenvironment including upregulation of co-inhibitory ligands such as PD-L1/L2, B7-H3 and B7x (B7-H4 or B7S1) to escape tumour reactive CD8$^+$ T cells (Dunn et al., 2002, Schreiber et al., 2011, Kerkar and Restifo, 2012, Zhang et al., 2009, Wang et al., 2014, Abadi et al., 2013). There is now ample evidence that Ag-specific CD8$^+$ T cells become exhausted and dysfunctional upon chronic Ag exposure or cancer, losing their ability to proliferate, release cytokines and kill target cells (Wherry, 2011). Furthermore, exhausted T cells upregulate inhibitory receptors like CTLA-4, PD-1, BTLA, TIM-3 and LAG-3. Ligation of these co-inhibitory receptors with their cognate ligands expressed by APC or tumour cells impedes T cell proliferation and effector function (Nguyen and Ohashi, 2015, Greenwald et al., 2005, Le Mercier et al., 2015, Keir et al., 2008, Binder and Schreiber, 2014, Youngblood et al., 2012, Haymaker et al., 2012). Blucking co-inhibitory signals with mAbs restores effector T cell functions and aids tumour regression *in vivo* (Blank et al., 2006, Bengsch et al., 2014, Ceeraz et al., 2013, Pardoll, 2012).

3.1.1. Synthetic peptides and monoclonal antibodies as a cancer vaccine

Synthetic (or altered) peptide-based vaccine could be used to prime new anti-tumour T cells or expand existing clones (Hoppes et al., 2014, Buhrman and Slansky, 2013, Cho and Celis, 2009, Cho et al., 2012). However, several studies have shown that vaccinating with peptide alone does not elicit strong Ag-specific $CD8^+$ T cell responses (Salem et al., 2005, Ly et al., 2013, Pandey et al., 2013, Arens et al., 2013, Cho and Celis, 2009, Slingluff, 2011). The development of monoclonal antibodies that modulate the function of effector T cells via stimulation of co-stimulatory receptors (CD27, GITR, 4-1BB and OX40) or blocking co-inhibitory receptors have provided a novel mechanism of indirect anti-cancer T cell activity (Croft, 2003, Watts, 2005, Moran et al., 2013, Barber et al., 2006, Ko et al., 2005). Therefore, administering mAbs as adjuvants along with the altered peptide vaccines might be an attractive approach for inducing effective anti-tumour immunity.

3.1.2. Adoptive T cell transfer

In addition to synthetic peptide vaccine, generating TCR that binds to peptide-MHC complex with high avidity is crucial for TAA recognition and to overcome immunologic tolerance (Cho and Celis, 2009, Overwijk et al., 2003, Johnson et al., 2009). Recent studies have shown that human lymphocytes can be modified *ex vivo* and re-infused to mediate tumour regression (Morgan et al., 2006, Johnson et al., 2009, Maus et al., 2014a, Kalos and June, 2013). Thus, I used pmel-1 T cells from pmel-1 transgenic mice to investigate the effect of targeting TNFRSF agonists and checkpoint blockade in controlling solid tumour growth. Pmel-1 mice are on a C57BL/6 background and are genetically engineered to express Vα1 Vβ13 (TCR) on T cells which recognises either the H-$2D^b$-restricted mouse peptide (EGSRNQDWL) or human peptide (KVPRNQDWL) from gp100 (melanoma and normal melanocyte differentiation self Ag) (Zhai et al., 1997). Gp100 is a type I transmembrane glycoprotein predominantly expressed intracellularly. However, gp100 can be processed and presented on both MHC-I or MHC-II molecules to T cells (Bakker et al., 1994).

To generate pmel-1 transgenic mice, RNA was isolated from clone 9 $gp100_{25–33}$-specific H-2Db–restricted $CD8^+$ T cell. α and β variable domains were amplified then co-injected into

fertilized C57BL/6 embryos egg. Generating TCR specific for H-2Db–restricted epitope corresponding to $gp100_{25–33}$ (Overwijk et al., 2003).

An MHC-I stabilization assay performed using RMA/S cells which have low TAP activity and low MHC-I expression on the cell surface (Esquivel et al., 1992, Ljunggren et al., 1990), FACS analysis showed that 50% stabilization of D^b by hgp100 altered peptide at a concentration ~100-fold lower compared with mgp100 peptide (Overwijk et al., 2003) i.e. the binding avidity of the hgp100 peptide to pmel-1 T cells is greater compared with the mgp100 peptide. Because gp100 is also expressed by melanocytes (Le Poole and Luiten, 2008), pmel-1 T cell activation can lead to autoimmune reactions. In support of this, mice with complete tumour rejection after pmel-1 T cell transfer and vaccination with hgp100 with IL-2 or IL-15 exhibited vitiligo (Finkelstein et al., 2004). Vitiligo has also been seen in patients with metastatic melanoma who responded to immunotherapy (Rosenberg and White, 1996).

Pmel-1 T cells are homozygous for the congenic Thy1.1 allele, and the gene is inherited with expected frequency, enabling their easy detection *ex vivo* (Fig 3-1).

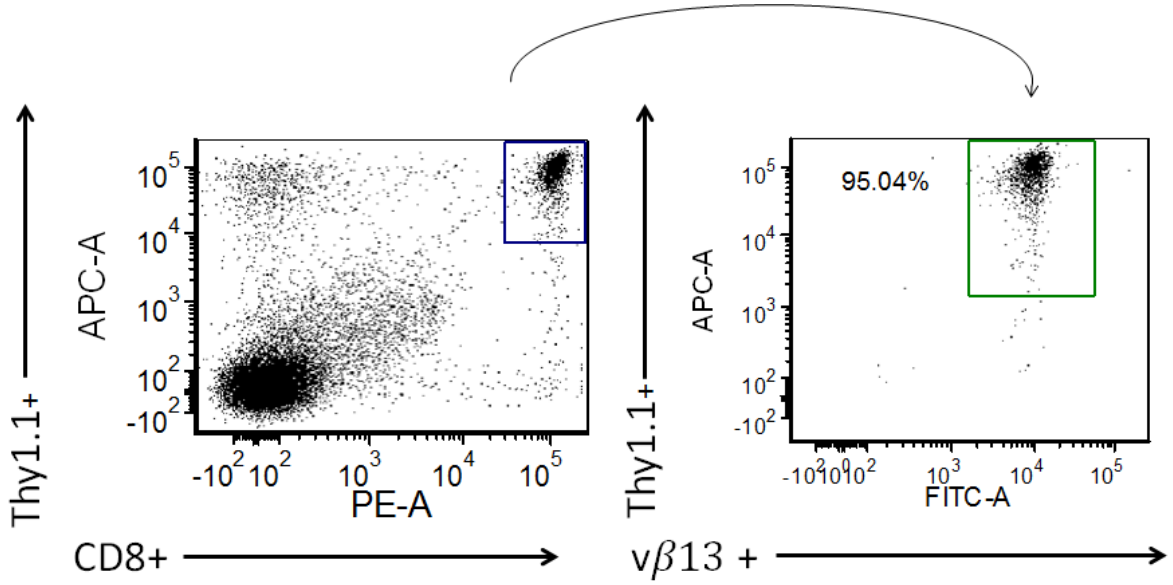


Figure 3-1: Characterization of pmel-1 T cell (gp100-specific CD8+ T cells) from blood. Pmel-1 mice were bled and blood was stained with antibodies against CD8, Thy1.1 and vβ13. Red blood cells were lysed with lysis buffer, then washed with PBS/0.2%BSA and stained for analysis. Cells were gated based on forward and side scatter to approximate lymphocytes. Pmel-1 transgenic mice are $CD8^{+}Thy1.1^{+}$ v$\beta13^{+}$. $Thy1.1^{+}$ gene is inherited with expected frequency and is not defected in reproduction.

I observed distinct patches of white hair growing on some naive pmel-1 mice. Those mice were 4-5 months old; suggesting that $CD8^{+}$ T cells may have encountered antigen and became activated. However, the extent of vitiligo in these mice was not correlated with the percentage of gp100-specific $CD8^{+}$ T cells expressing the activation marker CD44 (Table 3-1).

Table 3-1: Shows the correlation between extent of vitiligo and the percentage of CD44 in mice.

Sex	Age (weeks)	Approximate extent of vitiligo (%)	% of pmel1 cells expressing CD44
F	16-18	60	20
F	16-18	30	9
F	16-18	5	13.5
F	16-18	0	13
F	23	60	ND
F	23	5	13
M	18	5	20
M	18	3	35
M	18	3	28
M	18	3	15
M	18	0	21
F	16	3	62.5
F	16	0	59.7

3.1.3. pmel-1 T cells as a model for ACT in mice bearing tumour cells

Pmel-1 T cell transfer is currently extensively used to treat syngeneic melanoma. Recent study by Cho and others showed that immunizing mice bearing advanced B16 melanoma with TriVax consists of human $gp100_{25}$ peptide (KVPRNQDWL), IL-2 and anti-PD-L1 after pmel-1 cell transfer ($3x10^5$) resulted in a substantial increase in pmel-1 T cell expansion (~90% of total $CD8^+$ T cells) and prolonged their survival for up to day 55 post tumour challenge (Cho et al., 2012). TriVax-treated mice were completely tumour free for up to day ~50 post tumour challenge. Injecting TriVax without pmel-1 cell transfer abrogated the efficacy of the vaccine (Cho et al., 2012), indicating that pmel-1 cells are crucial for controlling tumour growth.

In another study, Klebanoff et al stimulated pmel-1 cells *in vitro* in the presence of IL-15 or IL-2 prior to transfer into WT recipient mice to generate T_{CM} ($CD62L^{hgih}$ and $CCR7^{high}$) and T_{EM} ($CD62L^{low}$ and $CCR7^{low}$) like pmel-1 cells respectively. Results showed that injecting sublethally irradiated WT mice bearing 10-day old B16 melanoma with T_{CM} like pmel-1 cells in combination with fowlpox virus encoding hgp100 and IL-2 reduced tumour growth significantly compared with untreated mice ($P < 0.0001$) and mice injected with T_{EM} cells ($P = 0.0014$) (Klebanoff et al., 2005), and caused complete tumour regression in 100% of the T_{CM}-treated mice for up to day 60 post tumour inoculation compared with T_{EM}- treated mice ($P <$

0.002) (Klebanoff et al., 2005). Similar results were obtained by Gattinoni and others**,** their findings revealed that injecting sublethally irradiated WT mice bearing solid B16 tumour with $CD62L^{High}$ pmel-1 subset in conjunction with hgp100 and IL-2 resulted in a significant delay in the tumour growth compared to untreated mice or $CD62L^{low}$ treated mice (Gattinoni et al., 2005). This was associated with the pronounced expansion of $CD62L^{High}$ subset at the peak of the response (on day 4 post immunization) in blood, spleen and in the tumour (Gattinoni et al., 2005). To further assess the importance of CD62L, WT mice bearing B16 melanoma established for 10 days were injected with $CD62L^{+/+}$ or $CD62L^{-/-}$ deficient pmel-1 cells. $CD62L^{+/+}$-treated mice were more effective in controlling tumour growth compared with $CD62L^{-/-}$-treated mice ($P = 0.014$) (Gattinoni et al., 2005). Therefore, ACT of central memory-like pmel-1 cells are more effective in controlling solid tumour growth compared with effector-like pmel-1 cells.

Adoptive pmel-1 T cell transfer was also combined with immune checkpoint blockade to improve the antitumor responses, recent studies have evaluated the effect of anti-CTLA-4 blocking mAb on pmel-1 T cells in mice with solid tumour, results showed that blocking CTLA-4 receptor or its ligand with mAb increased pmel-1 T cell motility (Ruocco et al., 2012, Pentcheva-Hoang et al., 2014). Similarly, Anti-PD-1 mAb increased pmel-1 T cell accumulation at the tumour site and increased the anti-tumour response compared to mice treated only with pmel-1 T cells (Peng et al., 2012). PD-L1 blockade also resulted in the reduction of 3-day old B16 melanoma following pmel-1 T cell transfer; the anti-tumour response was mediated by enhanced pmel-1 T cell proliferation and cytokine production. Administering gp100-pulsed DC improved the efficacy of the vaccine compared with PD-L1 alone (Pilon-Thomas et al., 2010).

Collectively, vaccinating mice bearing clinically visible tumour with Ag-specific $CD8^+$ T cells alone or with mAbs does not always provide durable protection, other adjuvants might be required for better anti-cancer immunity. Thus, in this section, I tested multiple peptide vaccine formulations with the aim of generating potent tumour-reactive $CD8^+$ T cell responses. These synthetic peptide-based vaccine formulations include adjuvants such as agonist and/or blocking mAbs, TLR agonists (known to be important initiators of innate and adaptive immune responses) (Sin et al., 2013, Bode et al., 2011, Jelinek et al., 2011). I then

assessed the ability of generated CTLs to cause tumour cell regression and improve survival in mice models (see chapter 4).

3.2. Comparison of TNFRSF agonists on CD8$^+$ T cell (pmel-1) activation

To compare the efficacy of TNFRSF agonists in driving T cell activation, C57BL/6 mice were injected with a lethal dose of the aggressive melanoma cell line B16-BL6 i.d. on day -4. On day -1 mice received pmel-1 cells i.v. and were then immunized with hgp100 with or without mAbs (agonists specific for 4-1BB, CD27, OX40 or GITR) on day 0, prior to another injection of mAbs on the following day (day 1). Pmel-1 cells were tracked in blood at different time points by staining for Thy1.1$^+$CD8$^+$ T cells (>95% of these cells in pmel-1 mice are vβ13$^+$) (Fig 3-1). Immunization with hgp100 alone did not have a strong effect on pmel-1 cell accumulation (Fig 3-2). Pmel-1 T cell accumulation with anti-CD27 mAb was at the peak of the response was significantly increased compared with peptide alone. Injecting anti-4-1BB, anti-GITR or anti-OX40 with hgp100 peptide did not induce significant hgp100-specific CD8$^+$ T cell expansion (Fig 3-2).

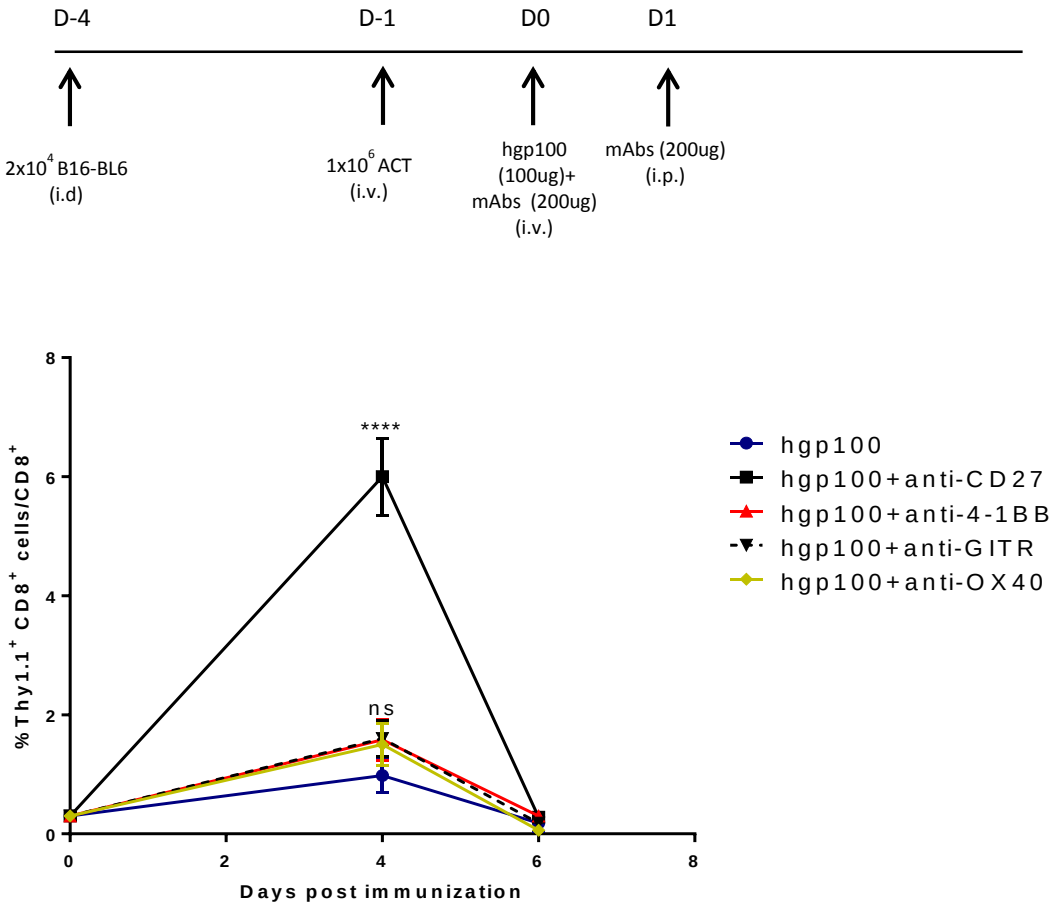


Figure 3-2: Effect of targeting CD27, GITR, 4-1BB and OX40 receptors on pmel-1 T cell expansion. C57/BL6 (n=5) mice were inoculated with $2x10^4$ of B16-BL6 i.d. on day -4. On day -1, mice received $1x10^6$ pmel-1 (i.v.), and were then immunized with hgp100 (100ug) with or without mAb (200ug) i.v. on day 0. Mice received another mAb injection on day 1 (i.p.). Cells were tracked in the blood by staining for Thy1.1 and CD8. Data show the mean %Thy1.1+CD8+ cells out of the CD8+ population +/-SEM. One way ANOVA ****P<0.0001 comparing anti-CD27 with hgp100. Students two-tailed t-test ***P=0.0001. The increase in CD8+Thy1.1+ frequency following immunization with peptide plus agonistic mAbs against 4-1BB, GITR or OX40 was not statistically significant (ns) compared with immunization with peptide alone.

3.3. A high peptide concentration is required for anti-OX40 or anti-GITR to augment pmel-1 expansion

Immunization with 100ug of hgp100 and two injections of anti-4-1BB, GITR or OX40 mAbs (200ug) had little effect on pmel-1 cell expansion (Fig 3-2), possibly because 100ug of the hgp100 peptide is insufficient to trigger maximal activation and expression of anti-4-1BB,

GITR or OX40. Therefore, mice were injected with larger peptide doses (400ug), and pmel-1 responses were tracked within the peripheral blood compartment. Using the same regimen as in (Fig 3-2), increasing peptide dose increased pmel-1 expansion markedly compared such that ~10% of total $CD8^+$ T cells were pmel-1-derived following injection with peptide alone (Fig 3-3) compared with only ~0.8% after injection with 100ug peptide (Fig 3-2). When using the higher peptide dose, anti-CD27 mAb resulted in significant pmel-1 cell expansion (37% of total $CD8^+$ T cells) compared with peptide alone (11% of the total $CD8^+$ T cells). When combined with high dose peptide, anti-OX40 mAb also induced pmel-1 cell proliferation significantly (27% of the $CD8^+$ T cells) compared with mice immunized with peptide alone (Fig 3-3). Targeting the GITR receptor had a small effect on hgp100-specific $CD8^+$ T cell proliferation (17% of total CD8 T cells) (Fig 3-3) while anti-4-1BB did not have any effect on pmel-1 proliferation (Fig 3-3). These data suggest that a high dose of antigen is needed in order to boost pmel-1 cell responses when targeting co-stimulatory receptors GITR and OX40.

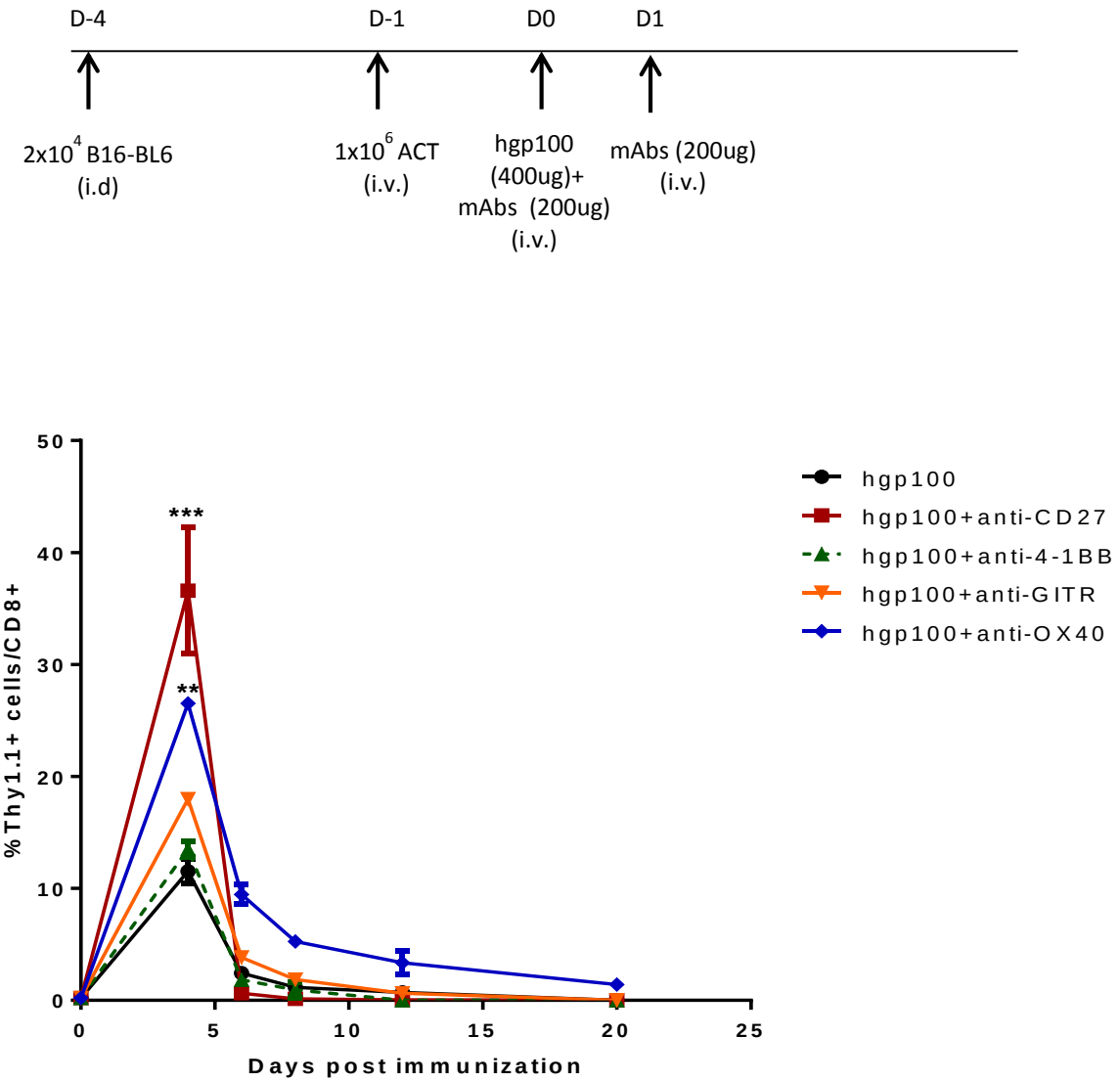


Figure 3-3: Comparing the ability of different TNFRSF agonists to enhance pmel-1 cell accumulation with a high peptide dose (400ug): C57/BL6 mice (n=3), were inoculated with B16-BL6 ($2x10^4$) i.d. on day -4, on day -1 mice received $1x10^6$ CD8+ T cells from pmel-1 mice i.v. and were then immunized with hgp100 (400ug) with or without mAb (200ug) i.v. on day 0. On day 1 mice were left without treatment or were injected with mAb (200ug) i.p. Data points show group means +/-SEM. One way ANOVA ***P=0.005 comparing anti-CD27 with peptide alone, **P=0.0005 OX40 vs peptide alone. * P= 0.012, ***p=0.0002 respectively two tailed Student's t-test.

3.4. TNFSFR expression on pmel-1 T cells *in vivo*

The magnitude of hgp100-specific $CD8^+$ T cells expansion in the previous experiment (Fig 3-2) was low, and increased drastically when high hgp100 peptide dose was given (400ug) (Fig 3-3). Moreover, the size of hgp100-specific $CD8^+$ T cells primary response was remarkably higher when CD27 co-receptor was stimulated with mAb compared with other co-stimulatory receptors (Figs 2-3 and 3-3). One possible reason could be correlated with pattern of expression of these individual receptors. Therefore, I investigated TNFRSF expression on naive or activated pmel-1 T cells *in vivo.* First, $\sim 5x10^6$ pmel-1 $CD8^+$ T cells were transferred into C57BL/6 recipient mice. The following day, mice were either left without treatment or were injected with hgp100 peptide (400ug). Splenocytes were harvested on day 2 (48 hours) or day 3 (73 hours) post peptide injection to assess expression of TNFRSF co-stimulatory receptors on hgp100-specific $CD8^+$ T cells. Expression of CD27 and GITR were detected on naive pmel-1 $CD8^+$ T cells (Fig 3-4). Injecting mice with 400ug of hpg100 peptide further increased their expression on days 2 and 3 post peptide injection (Fig 3-4). OX40 and 4-1BB in contrast were not constitutively expressed on naive cells (Fig 3-4). Peptide injection induced their expression on naive pmel-1 T cells (Fig 3-4), albeit at low levels compared with CD27 and GITR. These results are in line with the literature, and confirm that CD27 and GITR are constitutively expressed on naive $CD8^+$ T cells, while OX40 and 4-1BB co-receptor are upregulated upon stimulation (al-Shamkhani et al., 1996, Watts, 2005, Ronchetti et al., 2004, Croft et al., 2013).

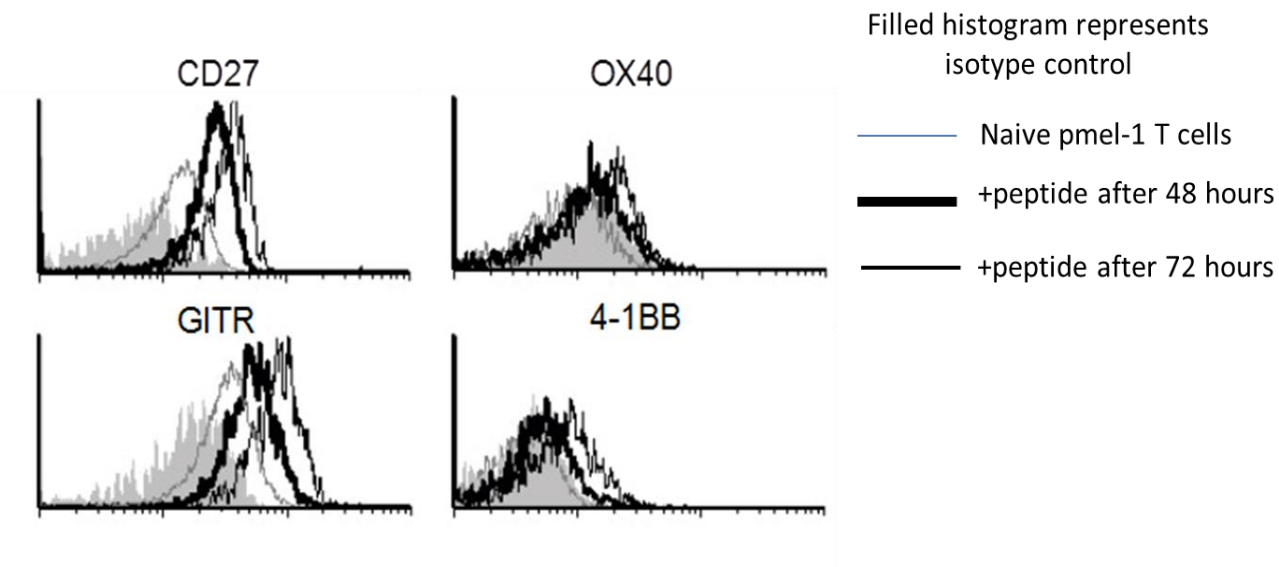


Figure 3-4: Expression of members of the TNF receptor superfamily on pmel-1 CD8+ T cells: C57BL/6 mice (n=3/group) received a high number of pmel-1 CD8+ T cells (~5x10^6) on day -1. On day 0, mice were either left without treatment or were injected with 400ug of the hgp100 peptide. Splenocytes were harvested from both groups on days 2 and 3 post peptide injection and stained for CD8, Thy1.1 and TNFRSF receptors as indicated. Data show the expression of CD27, OX40, 4-1BB and GITR on naive (think grey line), or activated (day 2, thick line, day 3, thick black line) CD8+Thy1.1+ cells; staining with an isotype control is included for comparison (filled light grey). Data are from a representative mouse.

3.5. Vaccination route is critical in influencing the magnitude of the antigen-specific CD8$^+$ T cell response

I investigated another approach that may improve pmel-1 CD8$^+$ T cell responses to control the tumour growth. Evidence suggests that Poly I:C, detected by the melanoma differentiation-associated protein 5 (MDA5) receptor, induces memory T cell formation (Wang et al., 2010). MDA5 is a retinoic acid-inducible gene I (RIG-I) like receptor (RLR), a cytosolic pattern recognition receptor which senses replicating viral RNA in the cytosol (Akira et al., 2006). In the case of viral infection, TLR3 detects extracellular double-stranded RNA (dsRNA) in early endosomes, while the MDA5 receptor senses cytosolic dsRNA resulting in induction of cytokines and type-I INF that are critical for subsequent adaptive immune responses (Akira et al., 2006, Yoneyama et al., 2004, Ishii et al., 2008). Poly I:C is a synthetic

analog of double-stranded RNA which is recognised by TLR3 and MDA5 (Wang et al., 2010, Jelinek et al., 2011, Ngoi et al., 2012). Poly I:C has been used as an adjuvant to improve antigen-specific $CD8^+$ T cell function and enhance memory cell formation through the induction of the anti-apoptotic factors Bcl-3 and Bcl-xl (Wang et al., 2010, Salem et al., 2005). Therefore, I hypothesized that combining poly I:C with peptide vaccination may enhance pmel-1 $CD8^+$ T cell activation. As mentioned above polyI:C has also been included within protocols that successfully induced pmel-1 $CD8^+$ T cells to differentiate to a memory phenotype (Ngoi et al., 2008, Salem et al., 2009, Salem et al., 2005).

Given that, of the TNFRSF agonists tested, anti-CD27 was most effective, I compared poly I:C and anti-CD27 mAb for their ability to induce $CD8^+$ T cell responses. Others have reported success with gp100 peptide vaccination protocols in which peptide was given sub-cutaneously whereas in my previous experiments I have administered gp100 systemically. Therefore to additionally compare the effects of different vaccination routes, groups of mice were injected with pmel-1 $CD8^+$ T cells then immunized either s.c. or i.v. with hgp100 peptide alone, with anti-CD27 mAb, poly I:C or a combination of both aiming to generate potent Ag-specific $CD8^+$ T cell responses. However, unlike other studies (Wang et al., 2010, Salem et al., 2005) , poly I:C did not promote pmel-1 memory cell generation regardless of the vaccination route (Fig 3-5). However, combining poly I:C with 200ug of anti-CD27 mAb enhanced expansion of pmel-1 cells significantly (40%) at the peak compared with anti-CD27 alone (20% of total $CD8^+$ T cells) or with polyI:C alone (5% of the $CD8^+$ T cells) (Figs 3-5A and B). Immunizing mice s.c resulted in an overall lower hgp100-specific $CD8^+$ T cell expansion compared to i.v. injection (Fig 3-5). The combination of poly I:C and anti-CD27 resulted in 20% of total $CD8^+$ T cell being hgp100 specific. Injecting mice with anti-CD27 mAb alone also enhanced pmel-1 $CD8^+$ T cells proliferation significantly compared with peptide alone (13% and 3% of total $CD8^+$ T cells respectively) (Fig 3-5B). Similar to i.v. injection, Poly I:C s.c. alone did not augment strong pmel-1 $CD8^+$ T cells response, the frequency of activated hgp100-specific $CD8^+$ T cells was almost identical to hgp100-induced pmel-1 $CD8^+$ T cells at the peak of the response (Fig 3-5B) (3% of total CD8 T cells).

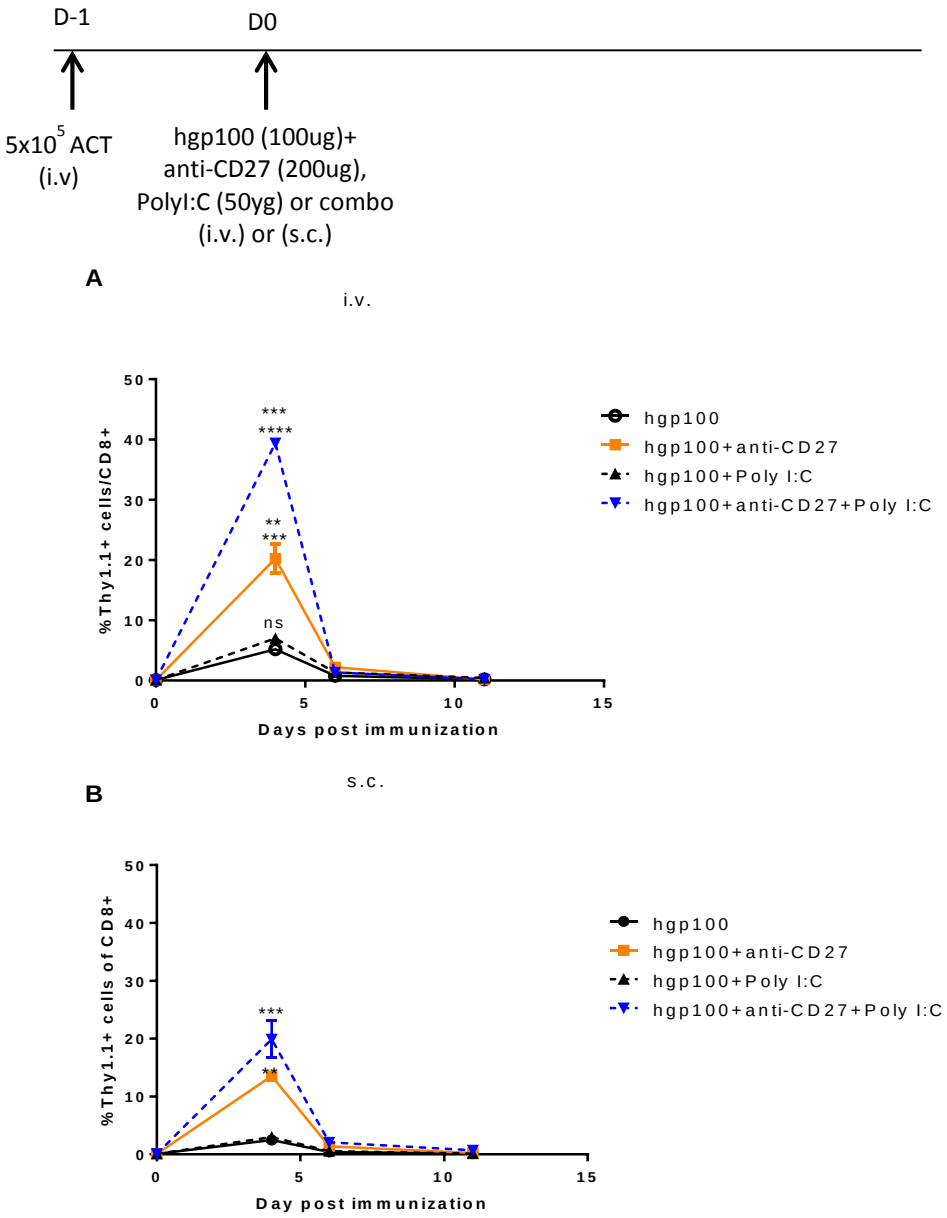


Figure 3-5: Vaccination with hgp100 and poly I:C is inferior to hgp100 and anti-CD27 for the differentiation of pmel-1CD8+ T cells. 5x10^5 pmel-1 cells were transferred into C57BL/6 mice (n=3 per group) on day -1, then immunized i.v. (A) or s.c. (B) with 100ug of hgp100 peptide on day 0 with or without 200ug anti-CD27, poly I:C (50ug) or a combination of both. The frequency of pmel-1 CD8$^+$ T cells was monitored in the blood at different time points by Thy1.1 and CD8 staining. Data show the mean+/-SEM of each group. For (i.v.) arm, one way ANOVA **P < 0.01 between anti-CD27 and Poly IC, ***P<0.001 between the combination group vs anti-CD27 and anti-CD27 and peptide alone, ****P<0001 between the combination vs Poly IC and peptide alone. For (s.c.) arm, **P<0.01 comparing anti-CD27 vs peptide alone and poly IC, ***P<0.001 comparing the combination vs poly IC, and peptide alone. Students two-tailed t-test (A) P=0.003 anti-CD27 vs peptide alone. P=0,008

comparing the combination with anti-CD27, P=0.0001 comparing the combination with poly IC. (B) P=0,0001 comparing anti-CD27 with peptide, P=0.005 comparing the combination with peptide alone, P=0.001 comparing the combination with poly IC.

Thus poly I:C with hgp100 had little influence on the accumulation of $CD8^+$ T cells, whereas the anti-CD27mAb significantly enhanced $CD8^+$ T cell activation. However, the combination of poly I:C and anti-CD27 synergises for $CD8^+$ T cell expansion if they are injected i.v. with the hgp100 short peptide. Potential reasons why poly I:C alone fails to enhance $CD8^+$ T cell activation in this setting will be discussed in a later section (see section 3.10).

3.6. Immunization with lipopolysaccharide (LPS) significantly enhances anti-CD27-induced pmel-1 cell expansion

Given that poly I:C combines well with anti-CD27 to enhance $CD8^+$ T cell accumulation, I additionally evaluated another TLR agonist, LPS, for its adjuvant activity. LPS is a component of the gram-negative bacterial cell wall that causes activation of B cells, macrophages and DCs (Hambleton et al., 1996, Foti et al., 1999, Kearney et al., 1976). It induces the release of T cell stimulatory cytokines such as TNF-α, IL-12 and INF-type I (van Kooten and Banchereau, 2000). Like CD40, LPS up-regulates CD80 and CD86 co-stimulatory receptors (van Kooten and Banchereau, 2000, Pufnock et al., 2011), converting DC into fully activated APC with high cytokine production and expression of co-stimulatory receptors essential for providing co-stimulatory signals for T cell activation and differentiation. To evaluate whether LPS would improve hgp100-specific $CD8^+$ T cell survival, either alone or in combination with anti-CD27, $3x10^5$ pmel-1 cells were transferred into C57BL/6 recipient mice on day -1; on the following day, mice were immunized with hgp100 alone, or in combination with anti-CD27 mAb, LPS, or a combination of both (all delivered i.v.). Immunizing with LPS alone trended towards an increased pmel-1 response (~5% of $CD8^+$ T cells) compared with hgp100 only (~3.5%) at the peak of the response on day 4 post immunization (Fig 3-6). Anti-CD27 proved more effective than LPS and resulted in ~10% of $CD8^+$ T cells being Ag-specific $CD8^+$ T cells (Fig 3-6). Interestingly, the combination of LPS and anti-CD27 mAb resulted in a robust pmel-1 expansion compared with LPS or anti-CD27 mAb alone (~48% of $CD8^+$ T cells) (Fig 3-6). However, the treatment did not prolong their survival and cells returned to baseline after day 8 (Fig 3-6). These results, together with those using poly I:C (Fig 3-5) indicate that

TLR agonists can provide additive/synergistic effects with anti-CD27 mAb in expanding activated antigen-specific $CD8^+$ T cells.

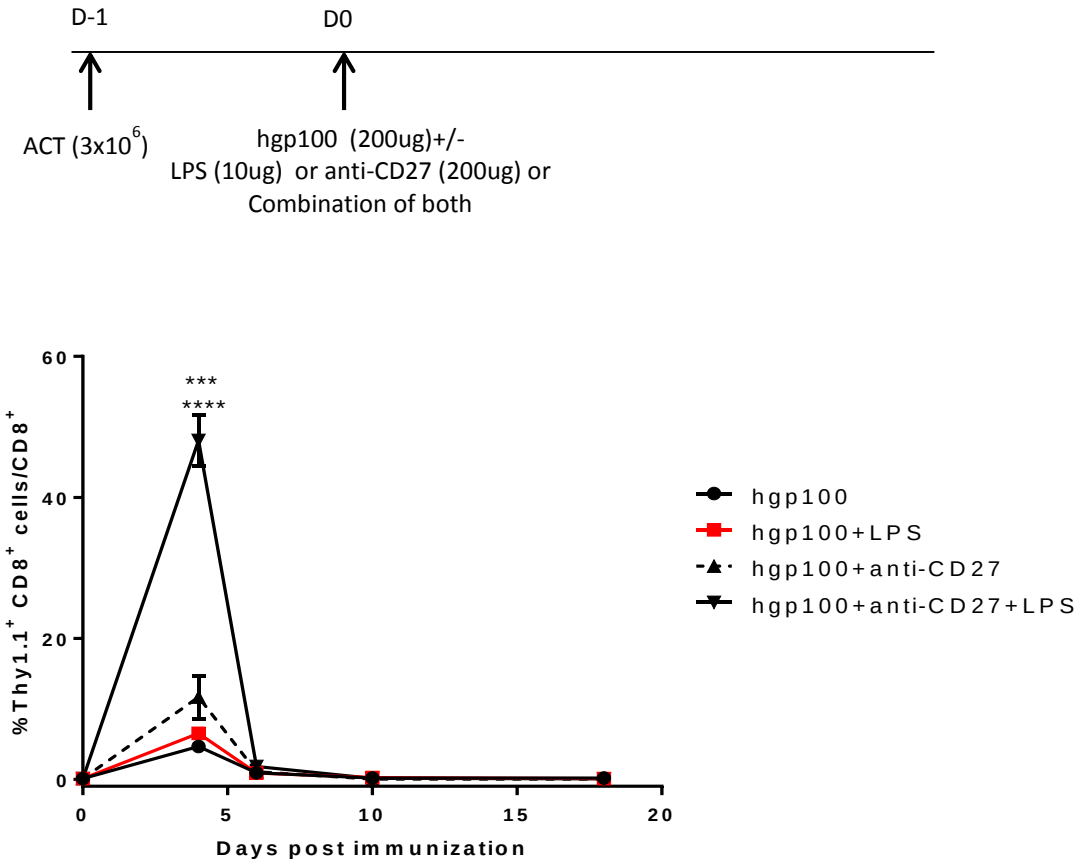


Figure 3-6: Immunization with hgp100 and LPS plus anti-CD27 mAb significantly enhances pmel-1 CD8+ T cell expansion. Pmel-1 CD8+ T cells ($3x10^6$) were transferred into C57BL/6 mice (n=3/group) on day -1. On day 0, mice were immunized i.v. either with hgp100 short peptide (200ug) alone as a control group or with 10ug LPS, 200ug of anti-CD27 mAb or a combination of anti CD27 plus LPS. Pmel-1 cells ($Thy1.1^+CD8^+$) were tracked in the blood at different time points. Data show group means +/-SEM. One way ANOVA ***P<0.001 comparing the combination with anti-CD27, and ****P<0.0001 comparing the combination with hgp100. Students two-tailed t-test **P=0.0016 comparing hgp100+anti-CD27+LPS with hgp100+anti-CD27, ***P=0.0003 comparing hgp100+anti-CD27+LPS with hgp100+LPS.

3.7. Immunization with anti-CD40 mAb with LPS increased pmel-1 T cell expansion and delayed their contraction

Of the TLR agonists tested, LPS in combination with anti-CD27 proved most effective. However, we consistently noted that activated pmel1 $CD8^+$ T cells failed to persist (Fig 3-6). CD40 is a co-stimulatory receptor belonging to the TNFR superfamily that is constitutively expressed on DCs. CD40 ligation with its ligand on $CD4^+$ T cells (CD40L) up regulates co-stimulatory receptors such as CD80 and CD86 and induces cytokine production such as IL-12 (Meunier et al., 2012, Hernandez et al., 2007). Our group has shown previously that CD27 acts downstream of CD40 for T cell activation (Taraban et al., 2004). In addition, data collected by Cho *et al*. revealed that immunizing mice with $Trp2_{180–188}$ peptide (SVYDFFVWL) plus anti-CD40 and poly I:C 3 days after injecting mice i.v. with B16F10 melanoma resulted in a robust primary and secondary Ag-specific $CD8^+$ T cells responses, and increased survival of 70 % of treated mice (Cho and Celis, 2009).

To investigate whether agonist anti-CD40 and LPS are similarly synergistic for naïve pmel-1 $CD8^+$ T cell activation, C57BL/6 mice were injected with $3x10^5$ $CD8^+$ T cells from pmel-1 mice i.v. on day -1, and then immunized with hgp100 peptide (200ug) alone, or with LPS, anti-CD40mAb, or the combination of both. The pmel-1 $CD8^+$ T cell response was then tracked in blood at different time points. Similar to the previous experiment (Fig 3-6), immunizing mice with LPS alone was insufficient to prime strong hgp100-specific $CD8^+$ T cell responses (Fig 3-7). Surprisingly, the hgp100-specific $CD8^+$ T cell primary response generated from vaccinating with anti-CD40 mAb alone was similar to that after vaccination with hgp100 alone. (Fig 3-7). However, combining anti-CD40mAb with LPS increased the pmel-1 T cell primary response significantly (P=0.0057). Importantly the combination of anti-CD40 and LPS (Fig 3-7), but not anti-CD27 and LPS (Fig 3-6), also delayed the contraction of $CD8^+$ T cells (Fig 3-7). The induction of memory $CD8^+$ T cells investigated in more detail in (chapter 5) later on in the thesis.

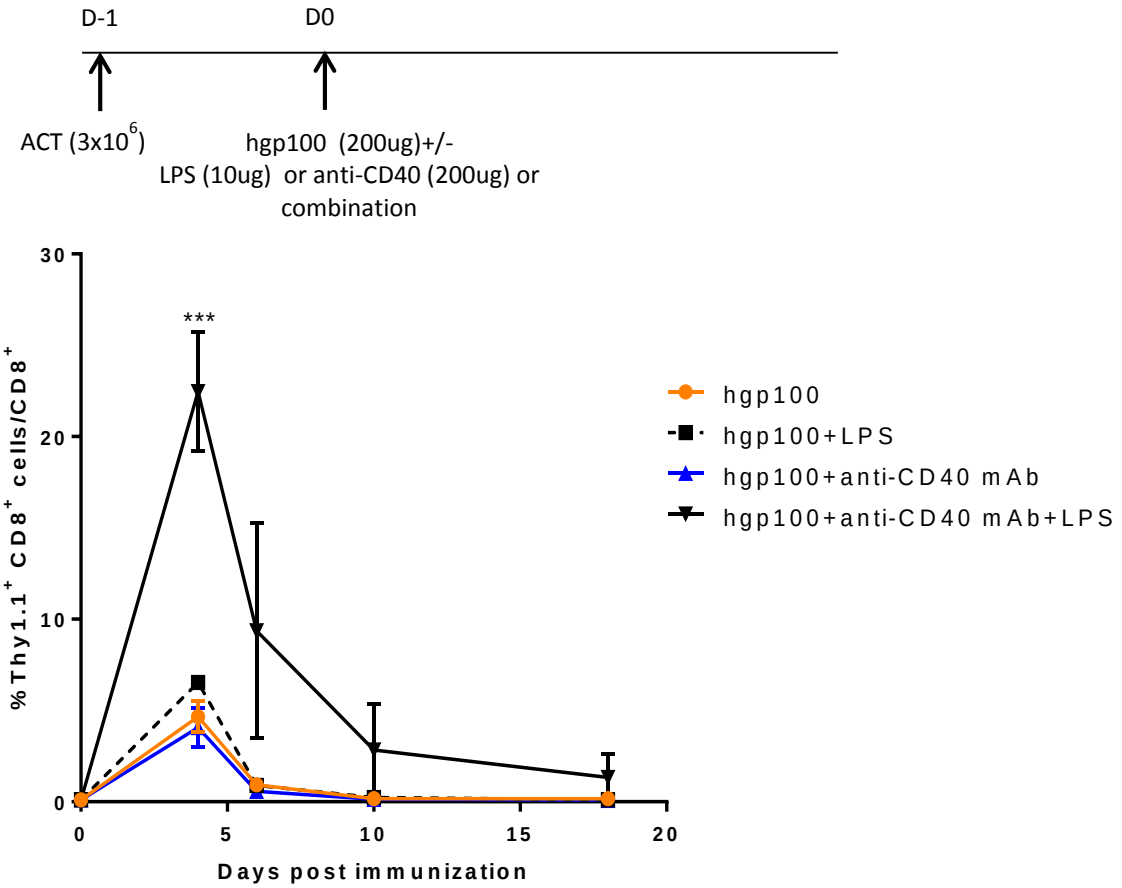


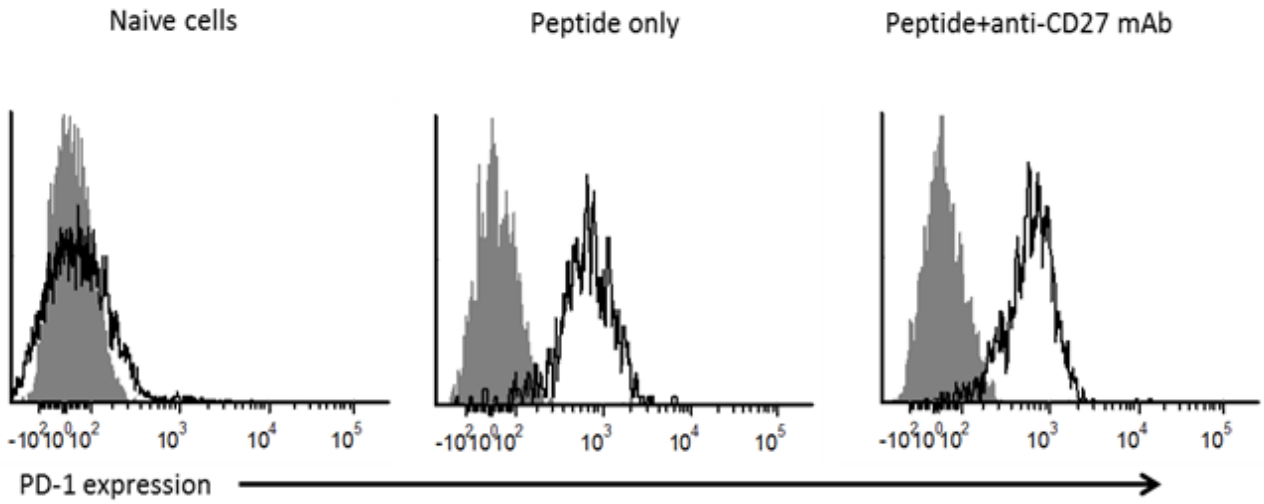
Figure 3-7: Immunization with hgp100 and anti-CD40 plus LPS significantly enhances pmel-1 CD8+ T cell expansion. Pmel-1 cells ($3x10^5$) were transferred into C57BL/6 mice (n=3/group) on day -1. On day 0 mice were immunized i.v. either with hgp100 peptide (200ug) alone as a control group or with 10 ug LPS, 200ug of anti-CD40 mAb or a combination of anti CD40 plus LPS. Pmel-1 cells ($Thy1.1^+CD8^+$) were tracked in the blood at different time points. Data show group means+/-SEM. One way ANOVA ***P<0.001 comparing the combination group with single agent alone. Two tailed student *t* test P=0.008 comparing the combination with LPS, and P=0.005 comparing the combination with anti-CD40.

3.8. PD-1 co-receptor is upregulated on pmel-1 T cell upon activation

As described above, TLR agonists can synergise with anti-CD27 to enhance activation of pmel1 $CD8^+$ T cells. However, in none of my experiments did I notice vitiligo in the mice suggesting that T cell activation is not optimal. For this reason, I chose to focus on inhibitory receptors which might be preventing full T cell activation. PD-1 on activated $CD8^+$ T cells plays a major role in supressing their effector function to avoid collateral tissue damage

Furthermore, PD-1 has been used as a marker for dysfunctional $CD8^+$ T cells, and PD-1/L1 or L2 pathway blockade restored $CD8^+$ T cell responses and effector function (Sakuishi et al., 2010, Binder and Schreiber, 2014, Barber et al., 2006). Therefore, I hypothesised that blocking the PD-1/L1 pathway may increase anti-CD27 induced pmel-1 T cell expansion further. First, I determined whether PD-1 was expressed on activated pmel-1 T cells *in vivo*. To do this, C57BL/6 mice received $CD8^+$ T cells ($3x10^6$) from pmel-1 transgenic mice i.v. on day -1. Mice were then injected with hgp100 peptide alone or with anti-CD27 mAb (200ug) i.v. on day 0. PD-1 expression was assessed on pmel-1 T cells from blood at the peak of the response on day 4. Blood from naive pmel-1 mice was also tested for PD-1 expression as a control. Results showed that activating pmel-1 T cells with hgp100 peptide alone induced PD-1 expression compared with the unstimulated naive T cells (Fig 3-8). Anti-CD27-induced pmel-1 $CD8^+$ T cells were also PD-1 positive (Fig 3-8). These results were in line with previous studies in which PD-1 was shown to be expressed on activated T cells (Parry et al., 2005, Agata et al., 1996, Hong et al., 2013). These findings suggested that blocking the PD-1/L1 co-inhibitory pathway may improve the pmel-1 $CD8^+$ T cell response and improve their effector function and cytokine production.

(A)



(B)

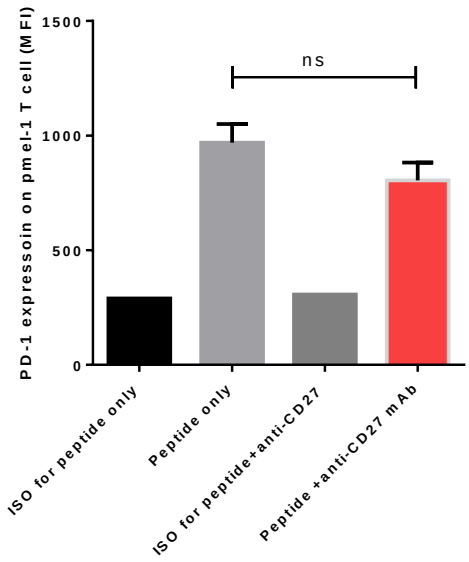


Figure 3-8: PD-1 is upregulated on activated pmel-1 CD8+ T cells. C57BL/6 mice (n=1/group) received pmel-1 T cells ($3x10^6$) i.v. on day -1. Then they were immunized with hgp100 peptide (100ug) alone or with anti-CD27 mAb (200ug) on day 0. Blood samples were collected on day 4 and stained for CD8, Thy1.1 and PD-1. (A) show expression of PD-1 on CD8+Thy1.1+ cells (black line) compared with an isotype control antibody (grey filled histograms) from naive (left), peptide only (middle panel) or peptide+anti-CD27 mAb (right panel) treated mice. (B) MFI of PD-1 on pmel-1 cells.

3.9. Targeting CD27 and PD-1/PD-L1 enhanced antigen-specific $CD8^+$ T cells expansion *in vivo*

Having ascertained that anti-CD27 mAb was the best TNFRSF-targeting mAb tested for pmel-1 T cell expansion (Figs 3-2 and 3-3), I then investigated the potency of targeting CD27 co-receptor in combination with anti-PD-1/anti-PD-L1 immune check-point blockade to further improve pmel-1 T cell responses *in vivo*. $2x10^6$ pmel-1 cells were transferred into C57BL/6 mice; mice were then immunized with the hgp100 peptide alone or in combination with anti-CD27, a blocking anti-PD-L1 mAb or the combination of both mAbs. For these experiments a low dose of peptide was chosen to ensure that any benefit to the combinatorial approach could be readily detected. Delivering hgp100 with agonist anti-CD27 augmented a robust pmel-1 cell expansion with more than 10% of $CD8^+$ T cells being pmel-1^+ at the peak compared with only ~3% with hgp100 alone (Fig 3-9), consistent with previous experiments in which a similar protocol was used (Fig 3-6). Blocking PD-L1 had no

effect on pmel-1 $CD8^+$ T cell expansion; pmel-1 cells comprised only ~1.5% of total $CD8^+$ T cells at the peak of the response (Fig 3-9). However, combining both mAbs (anti-CD27 and anti-PD-L1) improved T cell expansion significantly compared with either anti-CD27 or anti-PD-L1 alone (Fig 3-9).

Despite strong primary responses, at late time points after immunisation pmel-1 $CD8^+$ T cells were undetectable (Fig 3-9). Therefore, to look at whether memory T cells were present but were below the limits of detection, pmel-1 cells were re-stimulated *in vivo* with hgp100 on day 18. Notably, pmel-1 cells did not undergo a strong secondary response irrespective of the priming stimulus (Fig 3-9), indicating that memory cells were very infrequent.

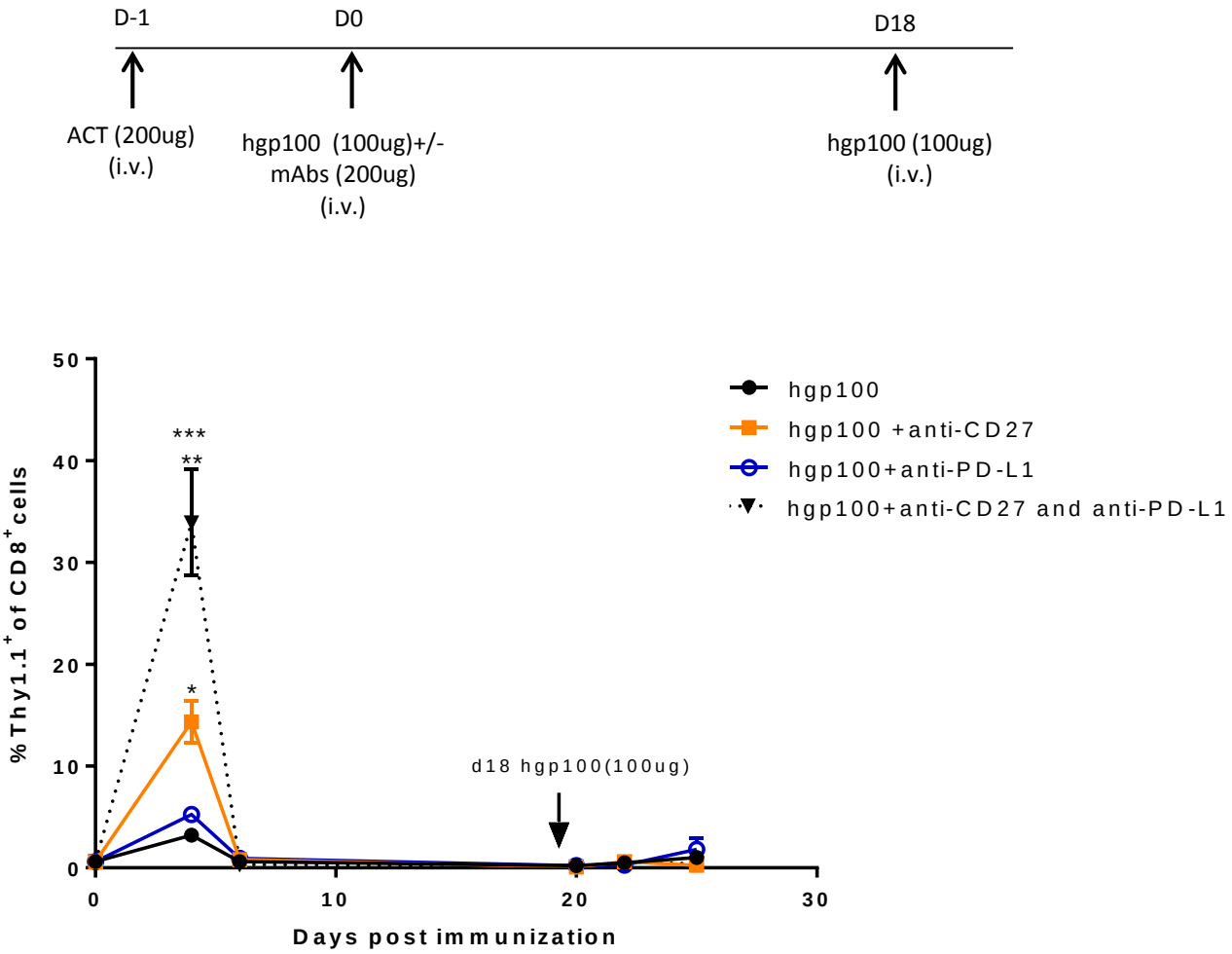


Figure 3-9: Effect of co-stimulation/inhibition with mAbs on pmel-1 CD8+ T cell response. $2x10^6$ Pmel-1 $CD8^+$ T cells were injected i.v. into C57BL/6 mice (3 = group) on day -1, on day 0 mice were immunised with 100ug of hgp100 peptide alone, or with 200ug of anti-CD27, anti-PD-L1 or a combination of both. On day 18 mice were re-stimulated with 100ug of hgp100. Pmel-1 CD8+ T cells were tracked in blood at different time points by staining for Thy1.1 and CD8. One way ANOVA *P=0.05 comparing anti-CD27 with anti-PD-L1, **P<0.01 comparing the combination with anti-CD27, and ***P<0.001 comparing the combination with anti-PD-L1 and peptide alone. Student's *t* test P=0.005 comparing ant-CD27 with peptide alone, P=0.025 comparing the combination with anti-CD27, and P=0.003 comparing the combination with anti-PD-L1. Data show group MEANS +/- SEM.

Because PD-L1 blockade synergised with anti-CD27 *in vivo* in expanding pmel-1 T cells (Fig 3-9), yet failed to induce memory T cells, I investigated whether the combination of anti-PD-1 and anti-PD-L1 would be more potent than anti-PD-L1 alone and might lead to memory T cell differentiation. With this strategy one would ensure that the combination of both mAbs

PD-L1 and PD-L2 are suppressed. Therefore, mice were challenged with the melanoma B16-BL6 on day -6. On day -1, mice were injected with pmel-1 $CD8^+$ T cells, and then immunized with hgp100 peptide alone or with anti-CD27, blocking anti-PD-1 and anti-PD-L1 mixed together (to ensure full signalling blockade) or the combination of all three mAbs on day 0. Mice received another injection of mAbs on day 1. Vaccination with peptide alone had little effect on pmel-1 cell expansion (5% of the $CD8^+$ T cells) as expected (Fig 3-10A). Consistent with (Fig 3-9), immunization with anti-CD27 mAb increased the magnitude of pmel-1 cells on day 4 post immunization drastically compared with peptide alone ($p<0.0001$) (Fig 3-10A). In this experimental set up blocking the PD-1/PD-L1 pathway also enhanced pmel-1 cell proliferation significantly compared with peptide alone ($p=0.0018$) (Fig 3-10A). The combination of both anti-PD-1/L1 and anti-CD27 mAbs augmented a robust pmel-1 T cell expansion significantly compared with anti-CD27mAb alone and anti-PD-1/L1 alone ($p<0.0001$) (Fig 3-10A).

A similar experiment was then performed to investigate CTLA-4 blockade as a means to augment $CD8^+$ T cell expansion. Anti-CTLA-4 alone increased pmel-1 T cell expansion significantly ($p<0.0001$) compared with hgp100 alone (14% compared with 6% of total $CD8^+$ T cells respectively; Fig 3-10B). The combination of anti-CD27 with CTLA-4 blockade further induced pmel-1 T cell expansion compared with anti-CD27 alone or anti-CTLA-4 alone, but was only significantly higher than the anti-CTLA-4 alone group (44% compared with 14% being hgp100-specific $CD8^+$ T cells respectively; $p=0.001$) (Fig 3-10B). These data suggest that anti-CD27 and anti-CTLA-4 can be combined to improve pmel-1 $CD8^+$ T cell responses. Together these results show that blocking CTLA-4 or PD-1 improves the magnitude of responses primed in the presence of anti-CD27. In neither experiment did pmel-1 cells persist. Across all experiments reported in this chapter, vitiligo was never observed in experimental animals.

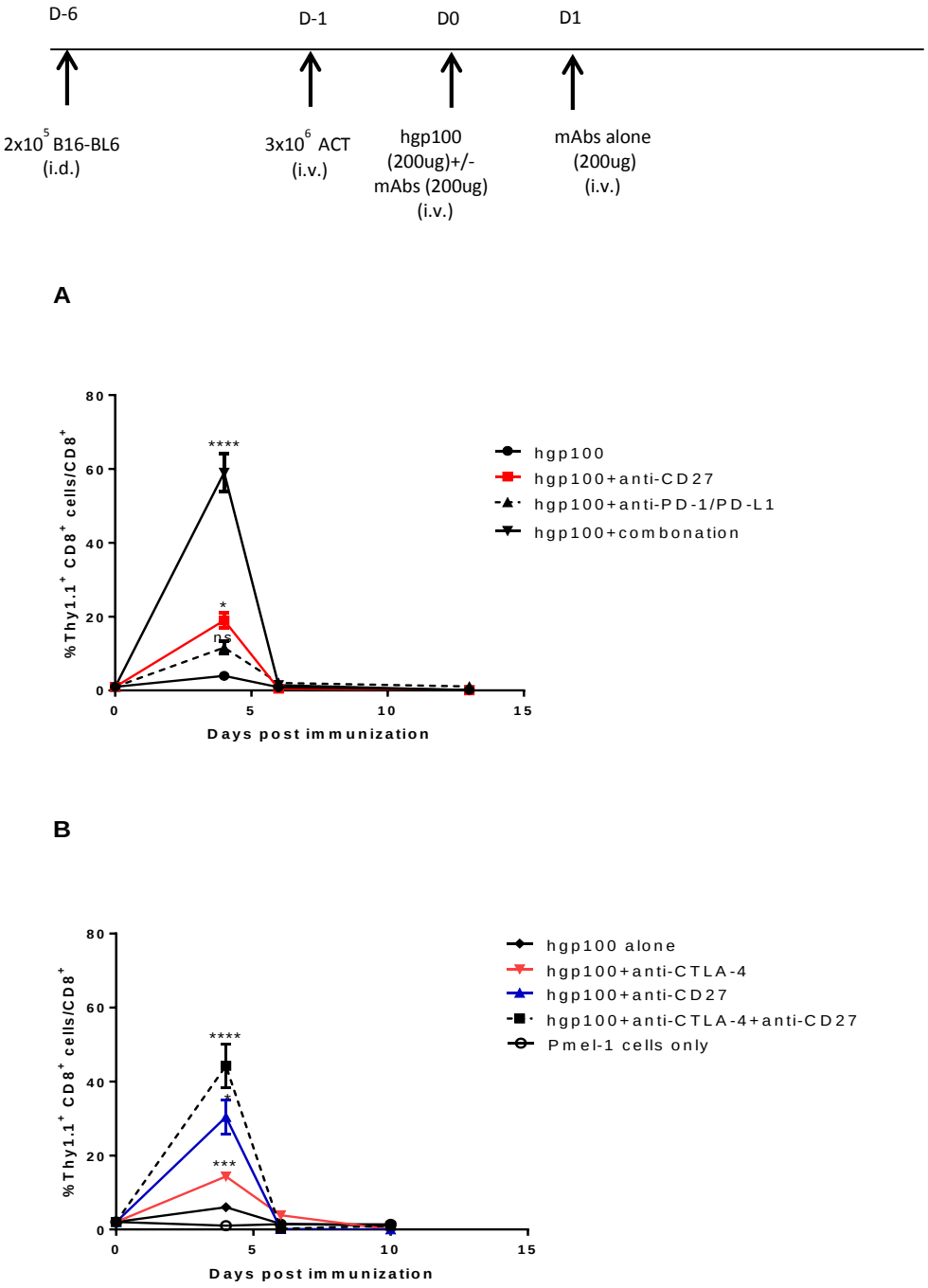


Figure 3-10: Effect of agonist anti-CD27 with or without immune check-point blockade on pmel-1 T cell expansion. C57BL/6 recipient mice (n=5/ group) were inoculated s.c. with $2x10^5$ B16-BL6 on day -6. On day -1, $3x10^6$ $CD8^+$ T cells from pmel-1 mice were transferred i.v. into mice. On day 0 mice were immunized with 200ug hgp100 and left without treatment, or treated with a single injection of 200ug anti-CD27, anti-PD-1, anti-PD-L1 or anti-CTLA-4 alone, or anti-CD27 in combination with either anti-PD-1 and anti-PD-L1 (A), or anti-CTLA-4 blocking mAbs (B). Mice received another injection of mAb on day 1. Pmel-1 $CD8^+$ T cells were identified by Thy1.1 and CD8 at different time points in the peripheral blood. Data show mean+/-SEM of groups. (A) one way ANOVA *p<0.05 comparing anti-CD27 with peptide alone, ****P<0.0001 comparing anti- the combination with peptide, anti-CD27 or anti-PD-1/anti-PD-L1 alone. (B) *p=<0.05 comparing anti-CD27

with anti-CTLA-4 alone, ***P<0.001 comparing the combination with anti-CTLA-4 alone, ****p<0.0005 comparing the combination with peptide alone. Student *t* test (A) p<0.05 comparing hgp100 with anti-PD-1 alone, P<0.0001 comparing anti-CD27 alone with hgp100, and the combination with anti-CD27 or anti-PD/L1 alone. (B) p=<0.005 comparing anti-CTLA-4+anti-CD27 with anti-CTLA-4 alone, p<0.0005 comparing hgp100 with anti-CD27, and p< 0.0001 comparing hgp100 with anti-CTLA-4.

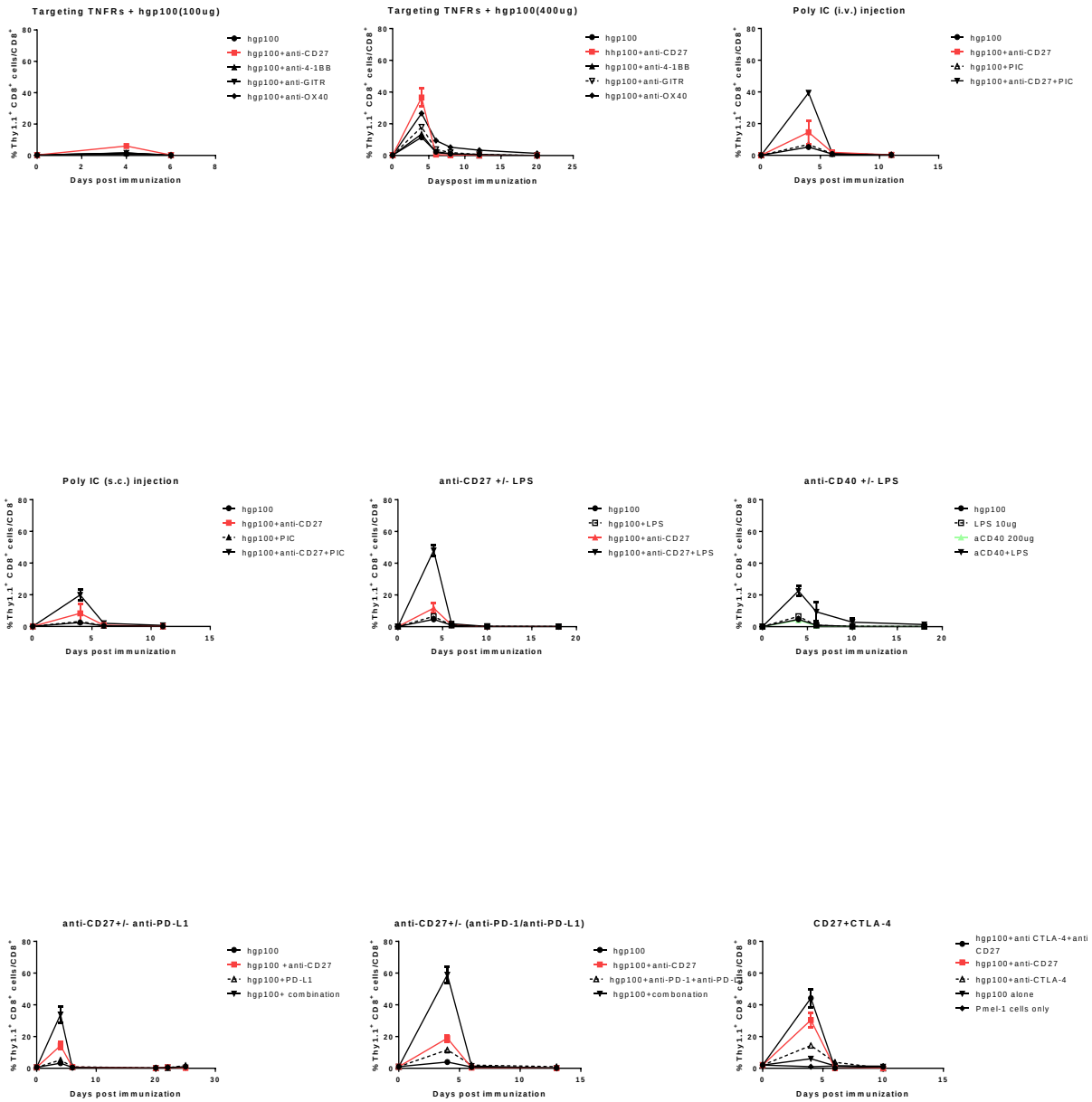


Figure 3-11: Summary of all vaccine strategies in chapter 3 to directly compare the most effective vaccine in generating the most effective immune response.

3.10. Discussion

Recent clinical data have demonstrated the effectiveness of targeting co-inhibitory receptors, namely CTLA-4 and PD-1, as an important therapeutic approach for the treatment of cancer. Long-term therapy however, appears to be limited to a minority of patients (Graziani et al., 2012, Hodi et al., 2010, Hamid et al., 2013). Combining CTLA-4 blockade with PD-1 blockade resulted in significant improvement over administration of single agents as demonstrated in the recent clinical trials (Wolchok et al., 2013). Further improvement may be possible by targeting co-stimulatory receptors with agonistic antibodies in combination with blockade of co-inhibitory receptors. In this result chapter, I have addressed this possibility by examining the effect of combining anti-CD27 with blockade of both PD-1 receptor and PD-L1 in controlling tumour cells growth *in vivo*.

In this chapter, I focused on possible ways to induce strong Ag-specific $CD8^+$ T cells responses by modulating different TNFRs with our without adjuvants such as TLR agonists. To study this, I used $CD8^+$ T cells that are genetically modified to specifically recognise gp100 in the context of $H2\text{-}D^b$. Gp100-specific $CD8^+$ T cells are generated in the transgenic pmel-1 mice. During my project, I noticed white patches (vitiligo) to different extent on naivepmel-1 mice (aged 5 to 6 months old), indicating that at least some pmel-1 T cells have encountered Ag and were activated. Therefore, I measured levels of expression of the activation marker CD44 on pmle-1 T cells in these mice. Notably, the extent of vitiligo was not correlated with the percentage of CD44 (Table 3-1). One possible explanation is that activated pmel-1 T cells started down regulate CD44 receptor by the time point I measured CD44 expression, or, it could be that Ag-experienced pmel-1 T cells have migrated to the skin and could not be detected in blood.

To increase the magnitude of the anti-tumour T cells response, I started by testing different TNFRs including CD27, GITR, OX40 and 4-1BB co-stimulatory receptors to improve T cell proliferation, effector function and survival. Following immunization with hgp100 (100ug) peptide and antibodies, I showed that anti-CD27 significantly increased the primary response of the pmel-1 T cells compared with immunization with peptide alone (Fig 3-2), while anti-OX40, anti-GITR and anti-4-1BB failed to induce durable responses when low peptide dose was administered (Fig 3-2). Increasing peptide dose to 400ug further increased

CD27-induced pmel-1 T cells response (Fig 3-3). Thus, anti-CD27 mAb was used in the rest of the experiments to activate pmel-1 T cell response. Notably, although GITR is constitutively expressed on resting T cells like CD27 co-receptor (Fig 3-4), anti-GITR mAb was insufficient to elicit robust Ag-specific $CD8^+$ T cell response (Fig 3-2), even when higher peptide dose was injected (Fig 3-3). This could possibly be due to the Ab used in the experiments. They might be targeting different region in the receptor, resulting in different activation outcomes. In addition, isotype of DTA-1 mAb rat IgG2b does not engage FcγRIIB efficiently (not strong agonist). Furthermore, stimulating OX40 and 4-1BB co-receptors with mAbs required higher hgp100 peptide doses to induce pmel-1 T cell expansion (Fig 3-2 and 3-3). Unlike CD27 and GITR, these receptors are transiently induced upon activation with TCR. Therefore, persistent Ag (high Ag dosage) might be required to induce the expression of OX40 and 4-1BB on pmel-1 T cells *in vivo* (Figs 3-3). Expression of 4-1BB *in vivo* was induced at low levels after 48 and 72 hours after stimulation with hgp100 peptide alone (Fig 3-4). Higher levels of 4-1BB expression on the activated Ag-specific $CD8^+$ T cells might be required to elicite strong immune stimulatory effect.

To study the efficacy of a particular vaccine on pmel-1 T cell response, low peptide concentration and ACT number ($3x10^3$ to $5x10^5$) were injected into recipient mice, while high transfer of pmel-1 T cells ($1x10^6$-$3x10^6$) were used to generate high frequency of antigen-specific CTLs to kill tumour cells.

Because pmel-1 stimulation did not generate memory cells, I then investigated the efficacy of TLR agonists particularly Poly I:C (TLR3 agonist) and LPS (TLR4 agonist) in enhancing anti-CD27-induced pmel-1 cells expansion and differentiation *in vivo*. First, I tested Poly I:C which has previously been shown to improve Ag-specific $CD8^+$ T cells function and memory T cell cells differentiation (Cui et al., 2014a, Salem et al., 2005). Administering poly I:C together with anti-CD27 was more effective than either treatment alone in expanding pmel-1 T cells (Fig 3-5). However, pmel-1 cells did not persist during the contraction phase. The low pmel-1 T cell response when mice were vaccinated s.c. compared to i.v. could possibly be suboptimal presentation and priming of naive pmel-1 T cells witch do not access extra lymphoid tissues.

Immunizing mice with LPS (TLR4 agonist) and anti-CD27 or anti-CD40 mAbs enhanced pmel-1 cell expansion significantly compared with single agent vaccination (Figs 3-6 and 3-7). The additive effect of LPS was more pronounced in anti-CD27-stimulated pmel-1 cells compared with anti-CD40, but the onset of the contraction phase was sharper and earlier (Figs 3-6 and 3-7).

PD-L1 is expressed by numerous immune and non-immune cells, particularly in the presence of inflammatory signals (Keir et al., 2008, Keir et al., 2007). PD-L2 in contrast is more restricted to DCs and macrophages (Rozali et al., 2012). Normal fibroblasts, tumour-associated fibroblasts and some other cell types can also express PD-L2 depending on microenvironmental stimuli (Rozali et al., 2012). PD-1 inhibits T cell response during the early phase of T cell activation, and reduces proliferation and effector function of CTLs (Keir et al., 2007). I hypothesised that PD-1/PD-L1 blockade in combination with anti-CD27 mAb would improve pmel-1 cells expansion, survival and functional capacity. Result in (Fig 3-8) showed that PD-1 is upregulated on pmel-1 T cell upon activation *in vivo*. Administering anti-CD27 did not further increase PD-1 expression (Fig 3-8).

In the first experiment of PD-1/PD-L1 checkpoint blockade I used anti-PD-L1. Results showed that blocking PD-L1 alone with mAb had little effect on pmel-1 cell priming (Fig 3-9). However, the combination of anti-PD-L1 and anti-CD27 synergised and improved CD27-induced pmel-1 T cells expansion significantly compared with monotherapy (Fig 3-9). In the second attempt, I blocked both PD-1 receptor and PD-L1 to insure full blockade of the inhibitory signal. Injecting mice with anti-PD-1/anti-PD-L1 alone resulted in a remarkable increase in pmel-1 T cell frequency compared with peptide alone (Fig 3-10A). The primary response of pmel-1 cells when anti-PD-1 and anti-PD-L1 were injected was ~2.7 fold higher compared to anti-PD-L1 alone (Fig 3-9 and 3-10A). The combination of both anti-CD27 and anti-PD-1/anti-PD-L1 blockade increased the accumulation of pmel-1 cells drastically compared to monotherapy (Fig 3-10A), resulting in ~34% (when PD-L1 was blocked) and ~60% (when both PD-1 and the ligand were blocked) of total $CD8^+$ being Ag-specific at the peak of the (Figs 3-9 and 3-10A). The synergy between anti-CD27 and anti-PD-1/L1 blockade encouraged me to evaluate another immune checkpoint blockade (CTLA-4), which is a upregulated on effector $CD8^+$ T cells in following TCR activation (McCoy and Le Gros, 1999). Similarly, blocking CTLA-4 receptor alone enhanced gp100-specific $CD8^+$ T cell expansion

compared with the untreated group (Fig 3-10B), and synergised with anti-CD27 toward enhancing the primary response of pmel-1 T cell compared with single agent alone. However, the magnitude of pmel-1 T cell primary response was lower compared to PD-1/L1 blockade (Fig 3-10). Collectively, these results suggested that co-stimulation via CD27 does not overcome inhibitory signals of PD-1 and that combination strategy is needed for optimal responses.

Chapter 4. Effects of augmenting T cell activation on tumour therapy

4.1. Optimising tumour rejection by combining TNFRSF agonists and check-point blockade

The discovery of the ability of the immune system to identify and kill neoplastic cells encouraged cancer immunologist to modulate host immune system to kill cancer cells (Schreiber et al., 2011, Quezada et al., 2011, Dunn et al., 2004). Monoclonal antibody-based cancer therapy is considered to be one of the most successful and promising strategies to eliminate tumour growth both in mice and human (Calemma et al., 2012, Brahmer et al., 2012, Berger et al., 2008, Curti et al., 2013, Hamid et al., 2013, Cohen et al., 2010, Kocak et al., 2006). Monoclonal antibodies can mediate tumour rejection directly by binding to receptors on the cell surface of the tumour cell to trigger cell death, or indirectly by vascular and stromal cell ablation, regulation of effector cells such as T cells, or by the induction of CDC and ADCC (Scott et al., 2012, Calemma et al., 2012, Natsume et al., 2009).

Tumour cells utilise multiple suppressor mechanisms to reduce or inhibit anti-tumour immune responses including up-regulation of immune-check point receptors like PD-L1 and L2 (Postow et al., 2015a, Pardoll, 2012, Peggs et al., 2009a). The interaction of PD-L1 or L2 with the cognate receptor PD-1 on activated T cells reduces their proliferation capacity and supress their effector functions (Peggs et al., 2009a, Rozali et al., 2012, Driessens et al., 2009). Results obtained by Thompson *et al.* revealed that high expression of PD-L1 on renal cell carcinoma (RCC) correlated with poor prognosis (Thompson et al., 2006)**.** The enhanced anti-tumour response of the anti-CTLA-4 blocking mAb encouraged immunologists to test anti-PD-1/L1 neutralizing mAb. Currently, multiple studies have proven that blocking the PD-1/L1 co-inhibitory pathway with mAbs restores tumour Ag-specific $CD8^+$ T cell effector functions and enhanced clinical outcomes in human in different tumour models (Lu et al., 2014a, Berger et al., 2008, Brahmer et al., 2012). In addition, results obtained by Curran and colleagues showed that anti-PD-1 mAb in combination with a cellular vaccine (B16-Flt3-ligand; Fvax) promoted tumour rejection in 25% of mice bearing established B16-BL6 melanoma (Curran et al., 2010, Curran and Allison, 2009). In two independent experiments, Sakuishi *et al*. showed that administering anti-PD-L1 mAb as monotherapy had variable effects on controlling CT26 growth. In the first experiment, anti-PD-L1 showed a trend

toward delayed tumour growth, while in the repeated experiment there was no obvious anti-tumour effect (Sakuishi et al., 2010). This data indicates that PD-1/or L1 blockade alone is insufficient to provide robust protective anti-tumour immune responses.

In the clinic, the blockade of PD-1/PD-L1 signalling pathway has provided persistent clinical benefits of patients with advanced tumour in multiple clinical trials (see section 1.14.2 for more information). Pembrolizumab and Nivolumab are two mAbs (IgG4) directed against PD-1 receptor, both mAbs showed clinical benefits with grade 3 to 4 treatment-related adverse events in 14% of treated patients (Robert et al., 2015b). However, anti-PD-1 drugs are not always beneficial when they are administered as a monotherapy. Some patients may have partial objective response to the treatment; other patients do not responsed to the drug treatment. Thus, current studies are now focusing on testing various combinations of anti-PD-1/L1 in mice including agonist mAbs against co-stimulatory receptors like anti-OX40, anti-4-1BB and anti-GITR to improve the treatment (Lu et al., 2014b, Chen et al., 2015, Guo et al., 2014). Data collected by Guo Z and colleagues revealed that combining anti-PD-1 mAb with anti-OX40 induced tumour regression significantly compared with monotherapy, resulting in 60% of mice bearing 10-days ID8 tumour (a clone of the MOSEC ovarian carcinoma of C57BL/6) with long-term survival. Tumour protection was mediated by $CD4^+$ and $CD8^+$ T cells, as T cells depletion but not NK depletion abolished the anti-tumour immunity conferred by anti-PD-1/OX40 mAb treatment (Guo et al., 2014). Moreover, Lu L *et al.* investigated the synergistic anti-tumour effect of anti-PD-1 and anti-GITR mAbs. There results showed that the combination synergistically inhibited ID8 ovarian cancer growth significantly compared to monotherapy, and improved overall mice survival (resulting in 20% of treated mice with complete tumour regression for up to day 90 post tumour inoculation), while single treatment with either anti-PD-1 or anti-GITR mAb alone exhibited little anti-tumour immune responses (Lu et al., 2014b). The combination increased the frequency of IFN-γ producing $CD4^+$ and $CD8^+$ T cells and decreased T_{reg} cells and MDSC, shifting an immunosuppressive tumour milieu to an immunostimulatory state (Lu et al., 2014b). More recent *in vivo* study showed that combining anti-4-1BB with PD-1 blockade resulted in synergistic antitumor effects, complete B16F10 tumour regression was observed in 7 out of 10 treated mice, injecting PD-1 or 4-1BB alone did not protect mice from tumour growth (Chen et al., 2015). The combination elicited a greater Ag-specific CTL response than either

single-agent alone, and induced Eomes expression (69% of total $CD8^+$ T cells were $Eomes^+$ compared with 64% in 4-1BB and 19% in PD-1-treated mice respectively) (Chen et al., 2015).

Collectively, these results suggest that administering agonist mAb against co-stimulatory receptor like CD27 may synergise with anti-PD-1 mAb toward enhanced anti-tumour immunity.

In this result chapter, I studied the correlation between the size of the Ag-specific $CD8^+$ T cell response (generated in chapter 3) and the anti-tumour response. In the second part of this chapter, I tested the anti-tumour effect of the combination anti-PD-1/ or L1 blocking mAb with anti-CD27 mAb in mice bearing 5-days old CT26 colon carcinoma to improve anti-PD-1/L1-mediated tumour protection.

4.2. Comparison of TNFRSF agonists on $CD8^+$ T cell (pmel-1) activation

The data in chapter 3 show results pertaining to T cell expansion as a result of stimulation with peptide and agonists to TNFRSF members, check-point blockers or TLR agonists. For some of these parameters their effects on an anti-tumour immune response were tracked in parallel and these data follow. In the experiment shown in (Figs 3-2) in which anti-CD27, anti-OX40, anti-GITR and anti-4-1BB were tested for their ability to enhance $CD8^+$ T cell expansion, tumour growth rate was monitored.

C57/BL6 mice were treated with $2x10^4$ of B16-BL6 i.d. on day -4. On day -1, mice received naive $1x10^6$ pmel-1 (i.v.), and were then immunized with hgp100 (100ug) with or without mAb (200ug) i.v. on day 0. Mice received another mAb injection on day 1 (i.p.). Despite increased expansion of the pmel-1 $CD8^+$ T cell population after anti-CD27 treatment (6% of total $CD8^+$ T cells) (Fig 3-2), tumour continued to grow rapidly in all anti-TNFRSF-treated mice similar to the control group (mice treated with hgp100 only) (Fig 4-1). This could be because of the relatively low primary expansion of pmel-1 cells after treatment; only 6% of total $CD8^+$ T cells in mice injected with anti-CD27 mAb and 1.5% in mice treated with 4-1BB, GITR or OX40mAbs were pmel-1 cells (Fig 3-2), which may not have been sufficient to cause tumour regression.

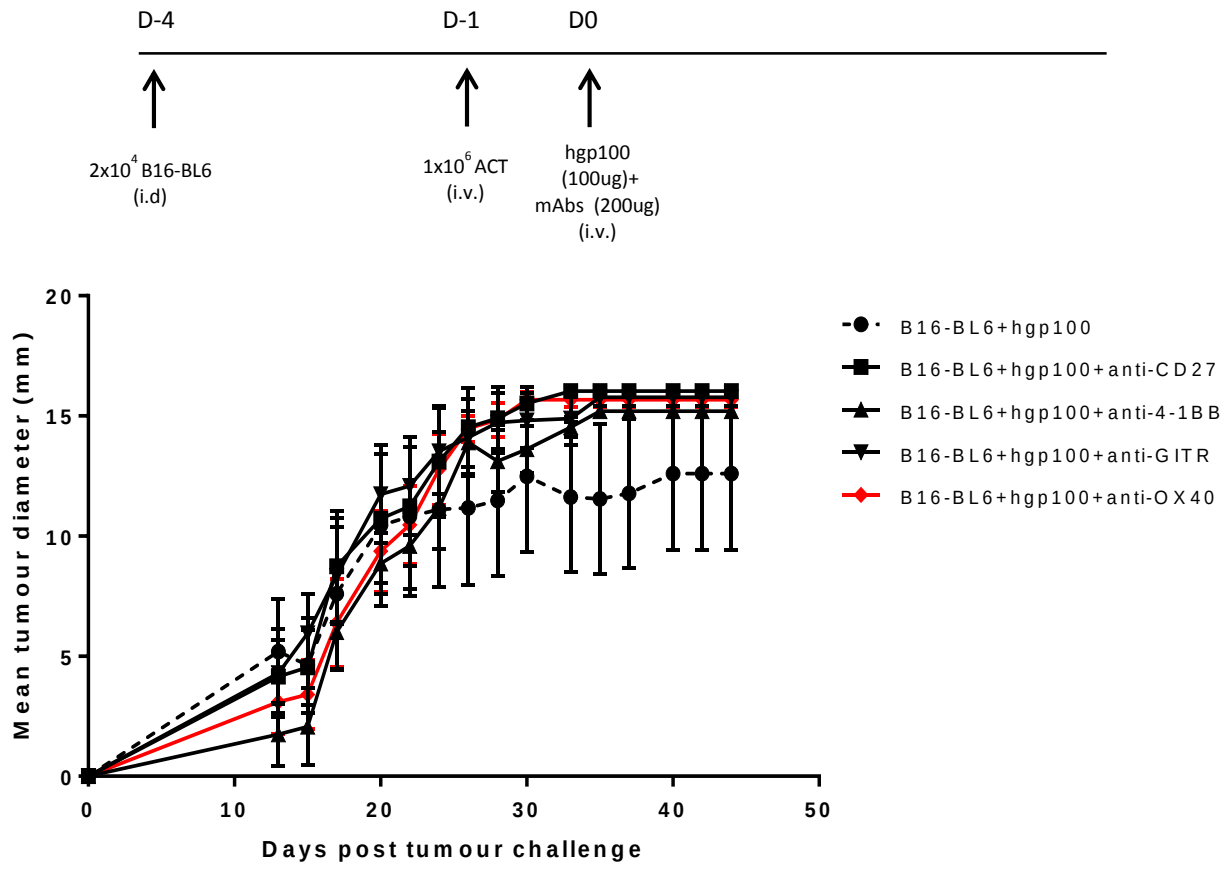


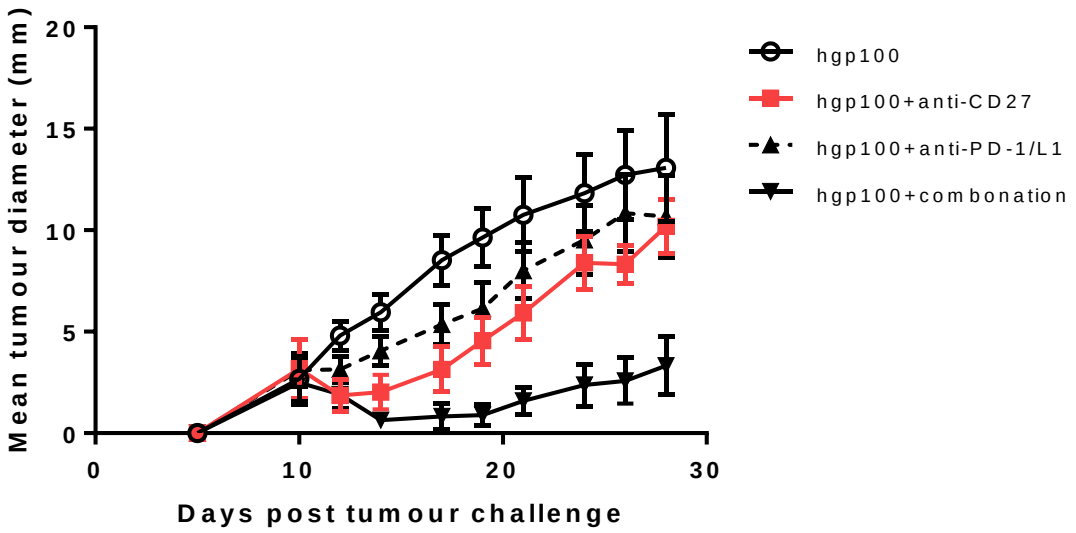
Figure 4-1: Targeting members of the TNFRSF as monotherapy is insufficient to provide anti-tumour therapy. Groups of 5 C57BL/6) mice were inoculated with $2x10^4$ of B16-BL6 i.d. on day -4. On day -1, mice received $1x10^6$ pmel-1 CD8+ T cells (i.v.), and were then immunized with hgp100 (100ug) with or without anti-CD27, anti-4-1BB, anti-GITR or anti-OX40 agonist mAbs (200ug) i.v. on day 0. Mice received another mAb injection on day 1 (i.p.). Mice were culled when the mean tumour diameter reached 15mm. Data represent the mean tumour diameter on the given days following challenge, with points representing the group means +/- SEM. The T cell response for this experiment is represented in (Fig 3-2).

4.3. PD-1 or CTLA-4 blockade with anti-CD27 caused tumour regression and enhanced survival of mice bearing established B16 melanoma

In chapter 3, my data showed that anti-CD27 can be combined with either PD-1 or CTLA-4 blockade to augment $CD8^+$ T cell expansion (Figs 3-10). With regard to the therapeutic effect of anti-PD-1/L1 and/or anti-CD27, hgp100 alone had no effect on controlling tumour

growth (Fig 4-2A). Anti-CD27 mAb had a small anti-tumour therapeutic effect, detected by reduced tumour growth compared with peptide alone (Fig 4-2). Administering anti-PD-1/anti-PD-L1 blocking mAb had little anti-tumour effect as detected by slower tumour growth compared with the control group (Fig 4-2A). Targeting CD27 and PD-1/L1 with mAbs trended towards a more pronounced delay in tumour growth (Fig 4-2A), and resulted in 50% of treated mice with complete tumour regression for more than 100 days post tumour inoculation (Fig 4-2B). The synergistic therapeutic benefit of combining anti-CD27 with blockade of PD-1 was observed in three independent experiments.

A. Mean tumour diameter of each group



B. percentage of mice surviving to the humane end point

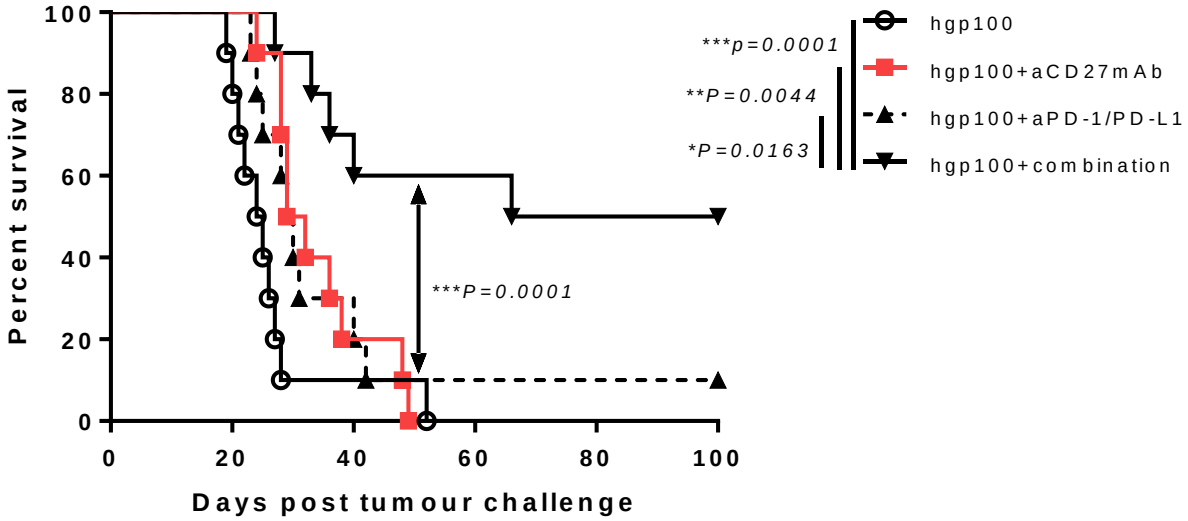
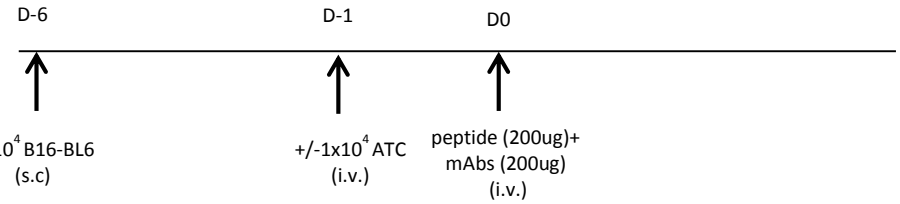
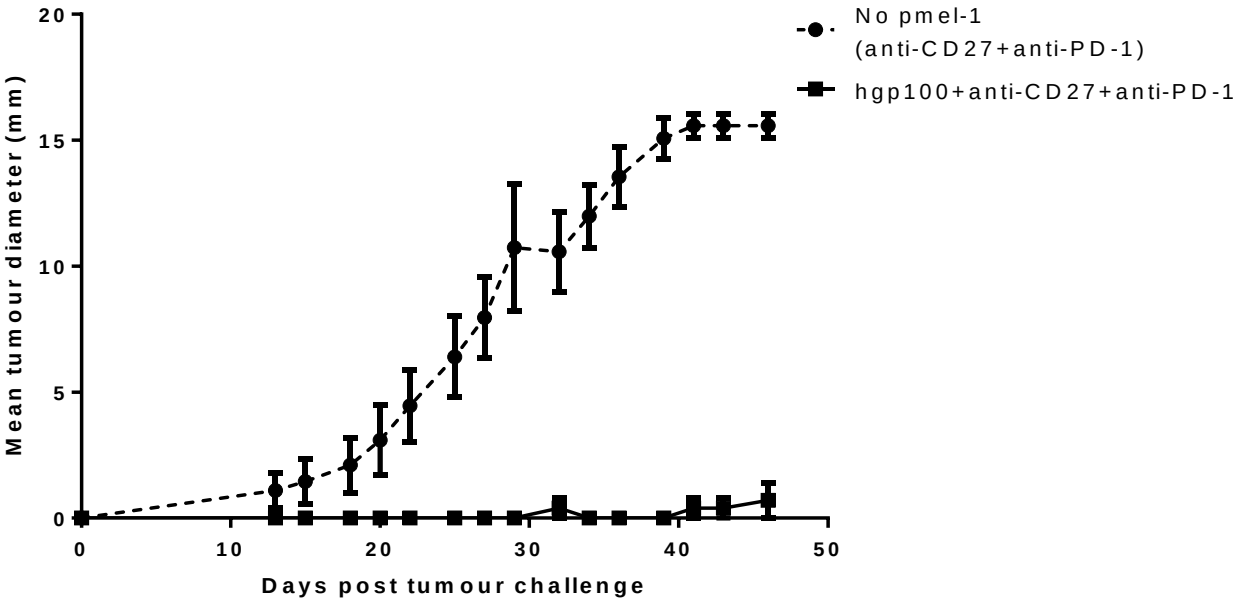


Figure 4-2: Anti-tumour therapeutic effect of adoptively transferred pmel-1 cells activated with anti-CD27 mAb with or without anti-PD-1/L1 blockade in mice bearing established B16-BL6 melanoma. C57BL/6 recipient mice (n=10/ group) were inoculated s.c. with $2x10^5$ B16-BL6 on day-6. $3x10^6$ of pmel-1 T cells were transferred i.v. into mice on day -1. On day 0, mice were immunized with 200ug of hgp100 with an isotype control, or with a single injection of anti-CD27, anti-PD-1/anti-PD-L1 or a combination of both (200ug each mAb) (A). Mean tumour diameter of each group. (B) Shows percentage of mice surviving to the humane end point. Mice were culled when the mean tumour diameter reached 15mm. MEAN+/-SEM on the days shown. *p<0.05,**p<0.005 and ***p<0,0005

An additional group of mice treated with anti-CD27 plus anti-PD-1 blocking mAbs without pmel-1 cell transfer was included to test whether enhanced anti-tumour immunity is pmel-1 T cell-dependent. Interestingly, immunizing mice with mAbs in the absence of pmel-1 cells had no impact on tumour growth and failed to provide long-term protection (Fig 4-3A and B).



A. Shows mean tumour diameter of each groups with or without pmel-1 $CD8^+$ T cell transfer



B. shows percentage of mice surviving with or without pmel-1 CD8$^+$ T cell transfer to the humane end point

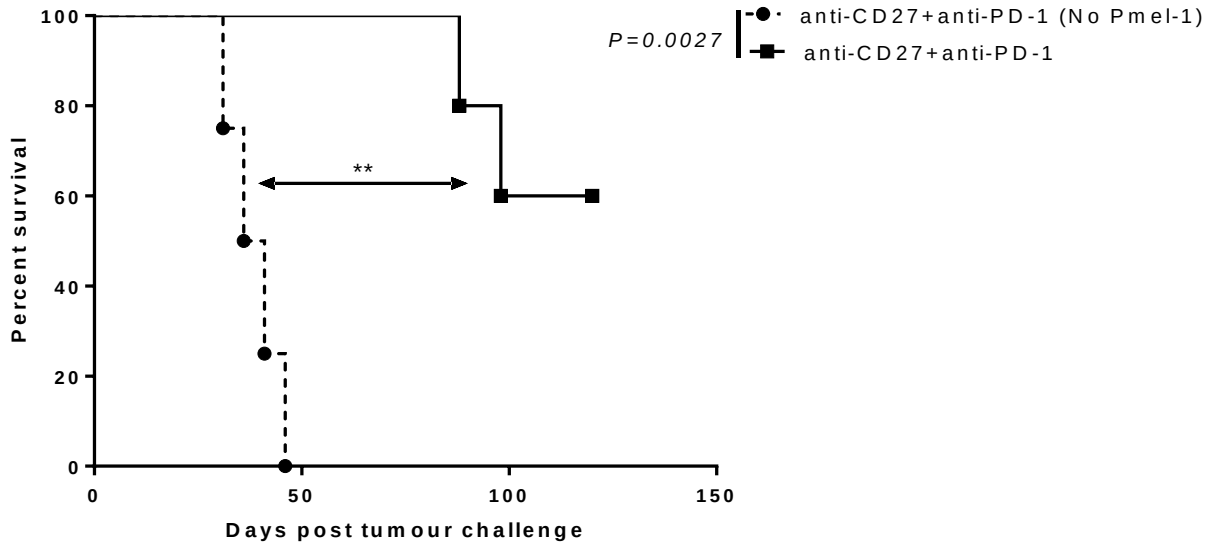
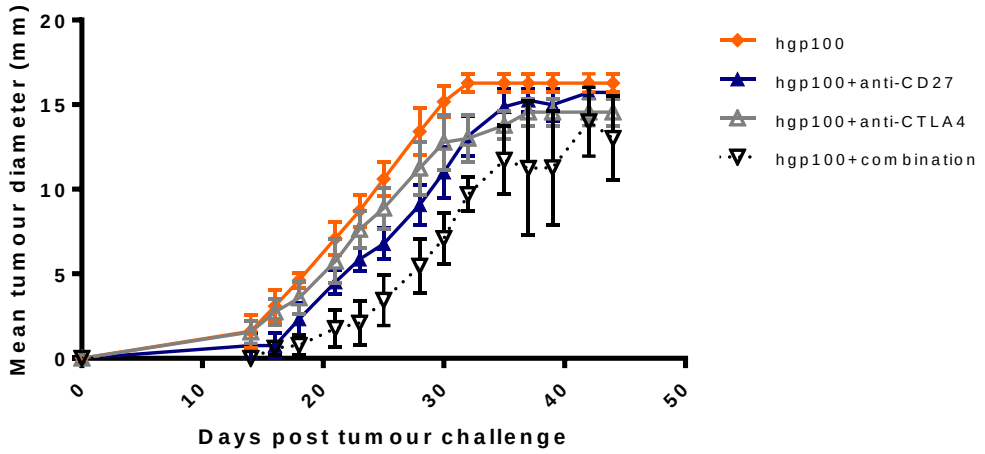


Figure 4-3: The antitumor effect of the combination vaccine is abolished without pmel-1 T cell transfer: To investigate the role of pmel-1 T cell in controlling tumour growth, mice (n=5) were injected with $2x10^4$ B16-BL6 s.c. on day -6, on day -1 one group received $3x10^6$ pmel-1 T cells. Both mice group were immunization with anti-PD-1 mAb with anti-CD27 (200ug for each mAb) on day 0 (A) Mean tumour diameter of each groups with or without pmel-1 CD8+ T cell transfer (B) Percentage of mice surviving with or without pmel-1 CD8+ T cell transfer to the humane end point. Mice were culled when the mean tumour diameter reached 15mm. MEAN+/-SEM on the days shown. *p<0.05,**p<0.005 and ***p<0,0005

Similar experiment in which anti-CTLA-4 was used (accompanying T cell data can be seen in Fig 3-10B) showed that while blocking CTLA-4 alone did not reduce the tumour size (Figs 4-4A and B), the combination of anti-CTLA-4 with anti-CD27 mAb delayed tumour growth, and prolonged survival of mice bearing established B16-BL6 compared with either monotherapy (Figs 4-4A and B) although this was not statistically significant.

A



B

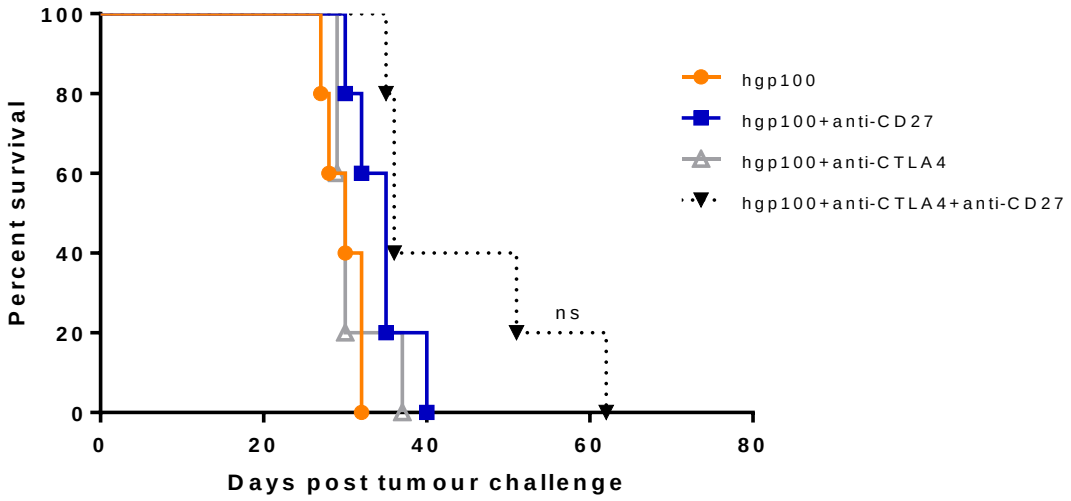


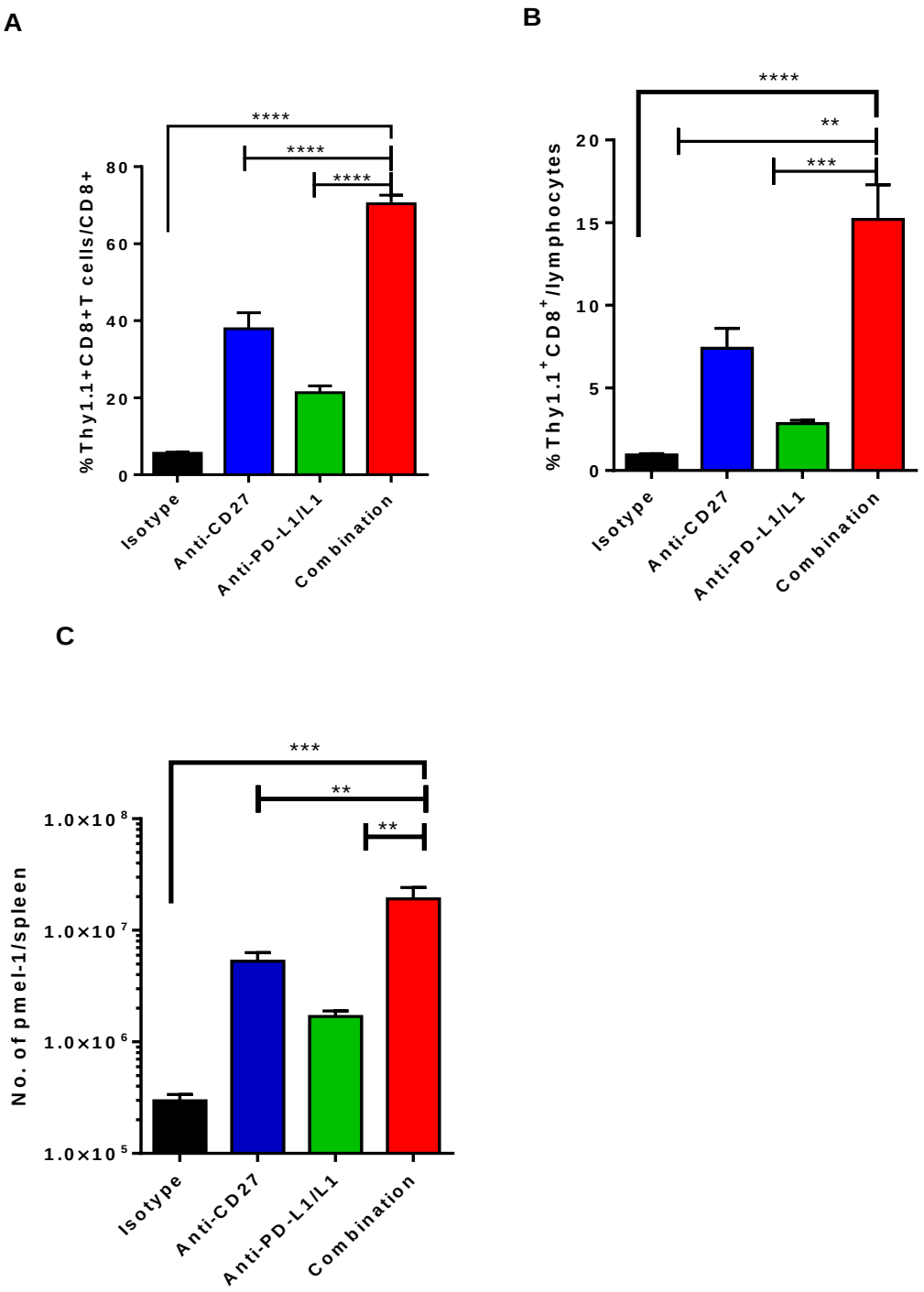
Figure 4-4: Anti-tumour therapeutic effect of pmel-1 cells activated with anti-CD27 mAb with or without anti-CTLA-4 blockade in mice bearing established B16-BL6 melanoma. C57BL/6 recipient mice (n=5/ group) were inoculated s.c. with $2x10^5$ B16-BL6 on day-6. $3x10^6$ of pmel-1 T cells were transferred i.v. into mice on day -1. On day 0, mice were immunized with 200ug of hgp100 alone or with a single injection of anti-CD27, anti-CTLA-4 or a combination of both (200ug each mAb). (A) Mean tumour diameter of each group. (B) Shows percentage of mice surviving to the humane end point. Mice were culled when the mean tumour diameter reached 15mm. MEAN+/-SEM on the days shown.

Collectively, these results show that combining anti-CD27 with anti-CTLA-4 or anti-PD-1 blockade enhances the treatment. Results also suggest that combining anti-CD27 with anti-PD-1/L1 may be more potent compared with the combination of anti-CD27 with anti-CTLA-4. Notably, the anti-tumour effect of pmel-1 $CD8^+$ T cells was correlated with the primary expansion in the blood i.e. the highest frequency of $CD8^+$ T cells was found in the combined anti-CD27/anti-PD-1/L1 group (Fig 14). In addition, pmel-1 $CD8^+$ T cells were crucial to generate sufficient anti-tumour responses (Fig 16C and D).

4.4. The combination of anti-CD27 mAb and anti-PD-1/L1 blockade enhances pmel-1 $CD8^+$ T cell effector functions

The combination of anti-CD27 with PD-1/L1 blockade dampened growth of B16-Bl6 melanoma and resulted in long-term survival in 50% of the treated mice (Fig 4-2). These results also showed that hgp100-specific $CD8^+$ T cells were crucial for effective antitumor immunity (Fig 4-2 and Fig 4-3). Next, I assessed the effector function of pmel-1 T cells in mice treated with the combined therapy and compared it in parallel with the effector function of pmel-1 cells following immunization with either mAb alone. Using the same protocol as in (Fig 4-2), mice were culled at the peak of the response on day 4 post vaccination, and splenocytes were harvested from each group to compare the magnitude of the pmel-1 $CD8^+$ T cell primary response, their ability to produce cytokines as well as expression of perforin and granzyme B. The primary response of the adoptively transferred pmel-1 T cells mirrored pmel-1 T cell expansion in the previous experiments which assessed pmel-1 T cell expansion in blood. Immunization with hgp100 peptide alone had little effect on pmel-1 T cell expansion whereas anti-CD27 mAb increased pmel-1 T cells response remarkably compared with peptide alone ($\sim$40% versus $\sim$7.5% of the total $CD8^+$ T cells respectively) (Fig 4-5A). Approximately 20% of total $CD8^+$ T cells were hgp100-specific when PD-1/PD-L1 signalling pathway was blocked (Fig 4-5A). The combination of both agonist and blocking antibodies improved hgp100-specific $CD8^+$ T cells expansion significantly compared to monotherapy, such that $\sim 68\%$ of total $CD8^+$ T cells ($\sim$16% of the lymphocytes) were hgp100-specific (Fig 4-5A and B). The total number of pmel-1 T cells was dramatically increased in the spleen in response to the combination therapy (1.9×10^7 pmel-1 T cells)

compared with anti-CD27 mAb alone ($5.2x10^6$ cells), or anti-PD-1/L1 alone ($1.68x10^6$ cells)(Fig 4-5C). Figure 4-5D shows the percentage of hgp100-specifiec $CD8^+$ T cells out of the lymphocytes.



(D)

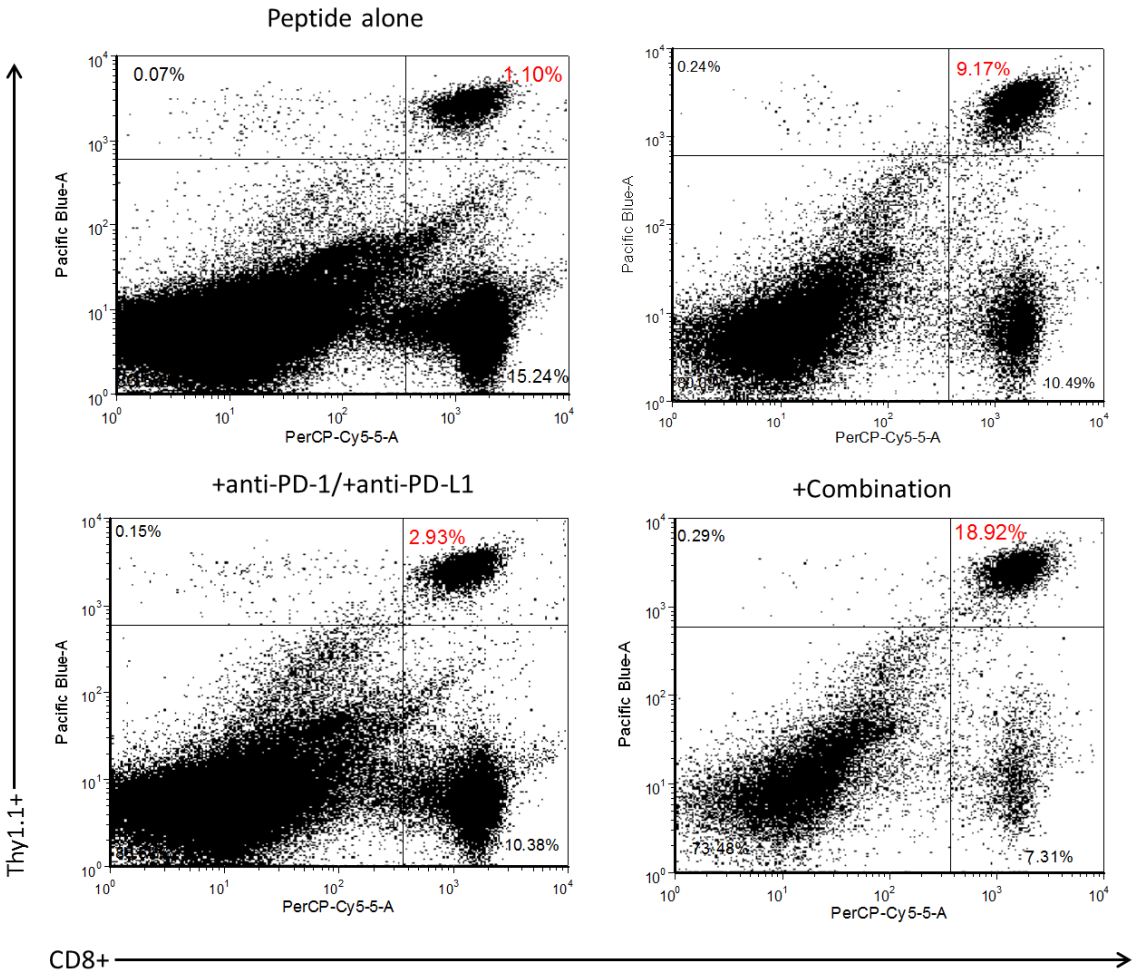
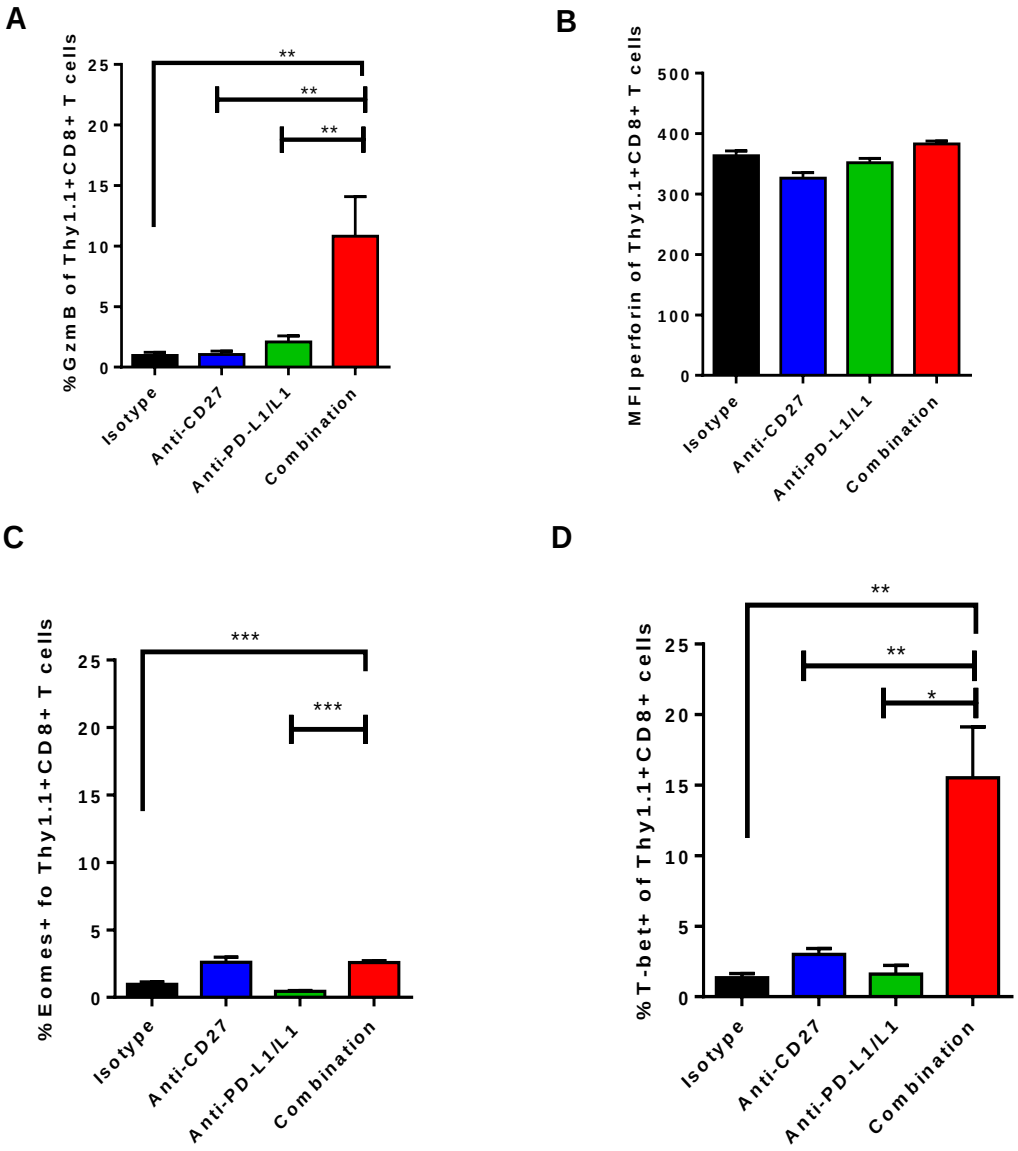


Figure 4-5: Combined anti-CD27 and PD-1/L1 blockade enhances hgp100-specific CD8+ T cells response. C57BL/6 recipient mice (n=5/ group) were inoculated s.c. with $2x10^5$ B16-BL6 on day -6. $3x10^6$ of pmel-1 T cells were transferred i.v. into mice on day -1. On day 0, mice were immunized with 200ug of hgp100 with an isotype control rat IgG2a mAb (clone MC106A5), anti-CD27 (200ug), anti-PD-1 (200ug)/PD-L1 (200ug), or anti-CD27 (200ug) in combination with anti-PD-1 (200ug)/PD-L1 (200ug) blocking mAb. Mice were culled on day 4 post immunization (peak of pmle-1 T cell response) and splenocytes were harvested to analyse pmel-1 T cell response and effector function. (A) Shows the frequency of hgp100-specifiec CD8+ T cells out of total CD8+ T cells and lymphocytes (B). (C) Shows the number of activated pmel-1 CD8+ T cells in the spleen. (D) Flow plots example of the frequency of hgp100-specifiec CD8+ T cells out of lymphocytes. Data show group MEAN+/-SEM. *P<0.05,**P<0.001, ***P<0.001, one way ANOVA.

Perforin/Granzyme B-induced cell death is a major mechanism by which CTLs lyse infected cells as well as transformed cells (Trapani and Smyth, 2002, Lord et al., 2003). The frequency

of granzyme B producing cell out of pmel-1 T cells in mice initially immunized with anti-CD27 mAb plus PD-1/L1 blockade was remarkably higher (11% of hgp100-specific $CD8^+$ T cells were granzyme B positive) compared with anti-CD27 or PD-1/L1 alone (1% and 2% respectively) (Fig 4-6A). I noticed no change in perforin expression between mice groups (Fig 4-6B), reflection of perforin expression is difficult to detect by intracellular flowcytometer compare to isotype. In terms of T-bet and Eomes transcription factor expression, no major difference in Eomes expression was observed between groups. Eomes expression was barely detectable in all groups, less than 4% of pmel-1 T cells were positive for Eomes at the peak of the response in all groups (Fig 4-6C). T-bet expression on contrary was significantly enhanced (15% of pmel-1 T cells were T-bet^+) in response to the combination compared with monotherapy (less than 4% in anti-CD27-induced pmel-1 T cells and 2% in the anti-PD-1/L1- treated group) (Fig 4-6D).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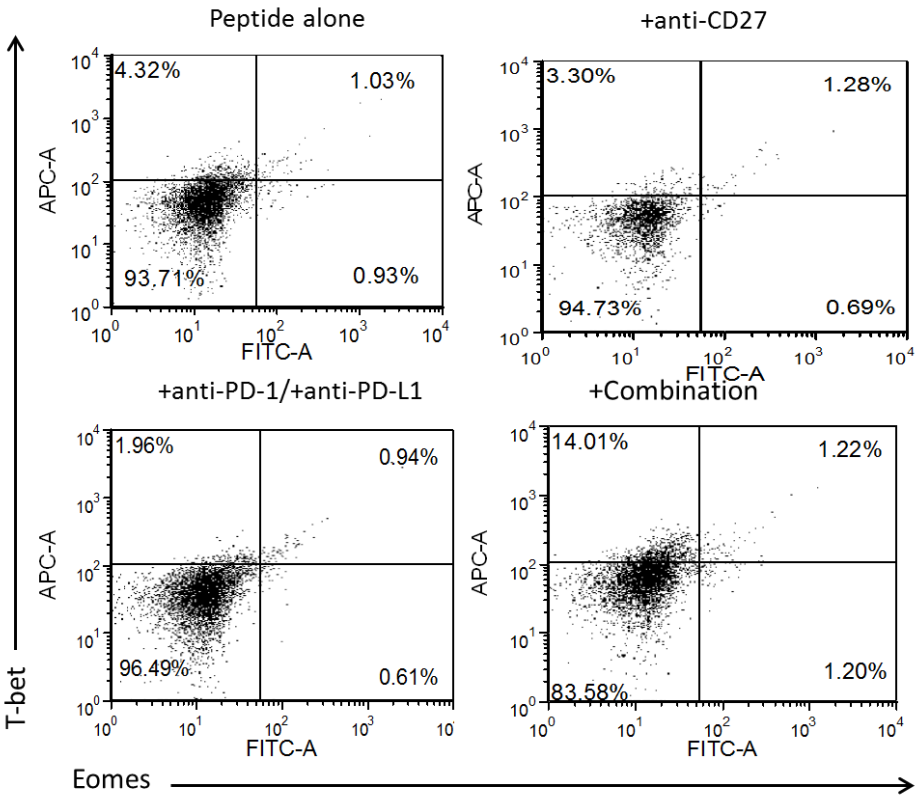
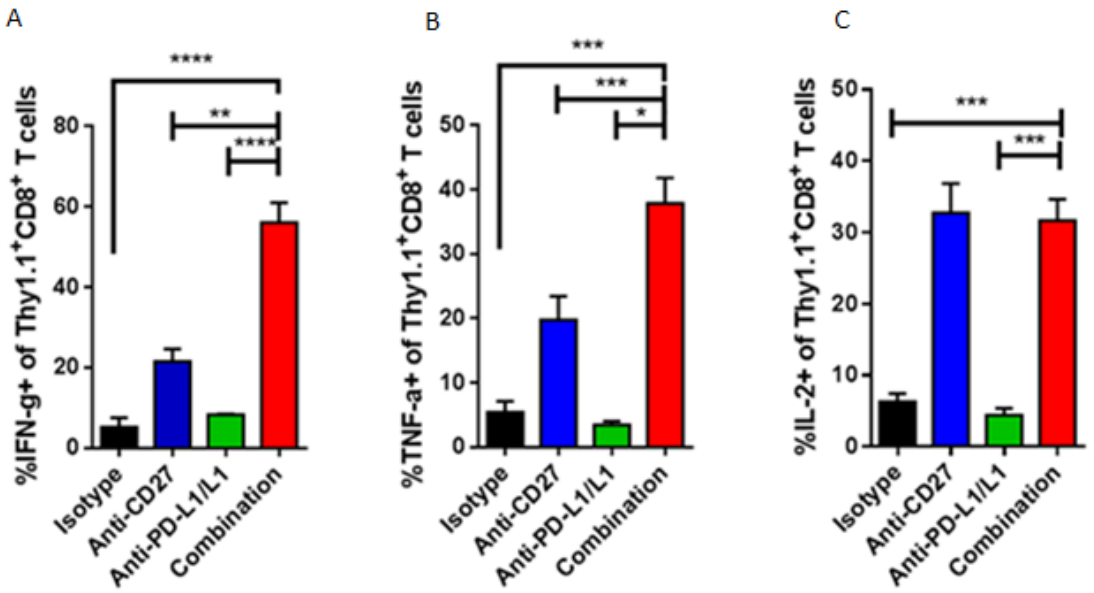


Figure 4-6: Combined anti-CD27 and PD-1/L1 blockade improved hgp100-specific CD8⁺ T cell effector functions. C57BL/6 recipient mice (n=5/ group) were inoculated s.c. with $2x10^5$ B16-BL6 on day -6. $3x10^6$ of pmel-1 T cells were transferred i.v. into mice on day -1. On day 0, mice were immunized with 200ug of hgp100 with an isotype control rat IgG2a mAb (clone MC106A5), anti-CD27 mAb (200ug), anti-PD-1 (200ug)/PD-L1 (200ug), or anti-CD27 (200ug) in combination with anti-PD-1 (200ug)/PD-L1 (200ug) blocking mAb. Mice were culled on day 4 post immunization (peak of the response) and splenocytes were harvested to analyse pmel-1 T cell effector function. (A) Splenic Thy1.1+CD8+ T cell production of granzyme B. (B) Mean fluorescent intensity (MFI) Perforin of Thy1.1+CD8+ T cells (C). Eomesodedrmin expression of hgp100-specific CD8+ T cells (D). T-bet expression of Thy1.1⁺CD8⁺ T cells. (F) Flow plots of Eomes and T-bet expression of Thy1.1⁺CD8⁺ T cells. Data show group means +/-SEM. *P<0.05,**P<0.001, ***P<0.001, one way ANOVA.

With regards to cytokine production, splenocytes were first re-stimulated for 4 hours with hgp100 in the presence of an inhibitor of cytokine secretion prior to intracellular staining. IFN-γ^+ containing pmel-1 T cells were more prevalent in mice immunized with the combination, such that ~60% of hgp100-specific CD8⁺ T cells were IFN-γ^+ compared with 20%

in anti-CD27 mAb-treated mice and ~8% when the anti-PD-1/L1 signalling pathway was blocked (Fig 4-7A). Similar pattern was observed for TNF-α such that immunization with the combination of anti-CD27 and anti-PD-1/L1 increased TNF-α production significantly compared to single agent alone (2-fold higher compared with anti-CD27 mAb alone and 9.5 fold increase compared to anti-PD-1/L1 alone) (Fig 4-7B). However, IL-2 production in the combination group was similar to the frequencies induced by anti-CD27 alone (Fig 4-7C); both vaccines (the combination and anti-CD27 mAb alone) resulted in approximately 30% of antigen-specific $CD8^+$ T cells being $IL\text{-}2^+$ (Fig 4-7C). Very few $IL\text{-}2^+$ pmel-1 T cells were detected following immunization with anti-PD-1/L1 alone (~4%) (Fig 4-7C). Overall, the combination of both mAbs generated more double and triple cytokine producing pmel-1 T cells compared with monotherapy (Fig 4-7D) followed by mice injected with anti-CD27 mAb alone (Fig 4-7D), while anti-PD-1/PD-L1 had no effect in generating these two cell populations as compared with the control group (Fig 4-7D).



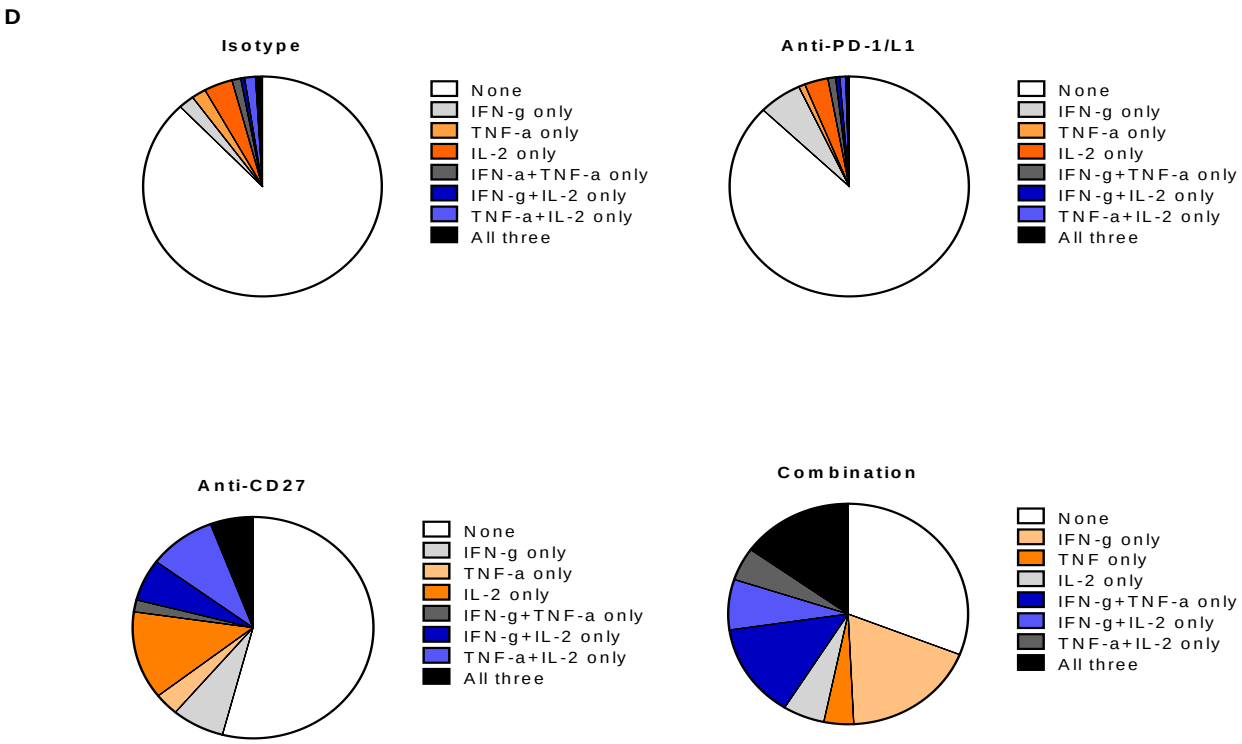


Figure 4-7: The combination of anti-CD27 and PD-1/L1 blockade improved pmel-1 cytokine production. C57BL/6 recipient mice (n=5/ group) were inoculated s.c. with $2x10^5$ B16-BL6 on day -5. $3x10^6$ of pmel-1 T cells were transferred i.v. into mice on day -1. On day 0, mice were immunized with 200ug of hgp100 with an isotype control rat IgG2a mAb (clone MC106A5), anti-CD27 mAb (200ug), anti-PD-1 (200ug)/PD-L1 (200ug), or anti-CD27 (200ug) in combination with anti-PD-1 (200ug)/PD-L1 (200ug) blocking mAb. Mice were culled on day 4 post immunization (peak of the response) and splenocytes were harvested to analyse pmel-1 T cell effector function. (A) Represent IFN-γ production in Thy1.1+CD8+ T cells (B) TNF-α of Thy1.1+CD8+ T cells (C). IL-2 productoin of hgp100-specific CD8+ T cells (D). Pie charts show double and triple cytokine producing cells. Data show group means +/-SEM. *P<0.05, **P<0.005, ***P<0.0005, Students two-tailed t-test.

Surface expression of lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) (surface marker for $CD8^+$ T-cell degranulation after activation (Alter et al., 2004)) was also assessed on the activated pmel-1 cells. Higher frequencies of $CD107a^+$ hgp100-specific $CD8^+$ T cells were observed in response to the combination treatment (~70% of pmel1 $CD8^+$ T cells were $CD107a^+$) compared with anti-CD27 or anti-PD-1/L1 (~60% and ~45% respectively) (Fig 4-8).

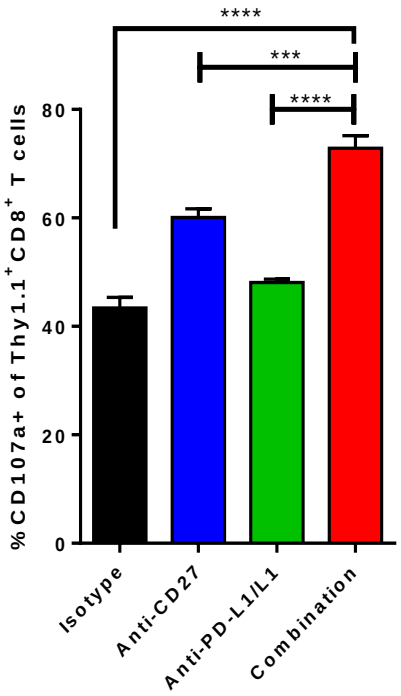
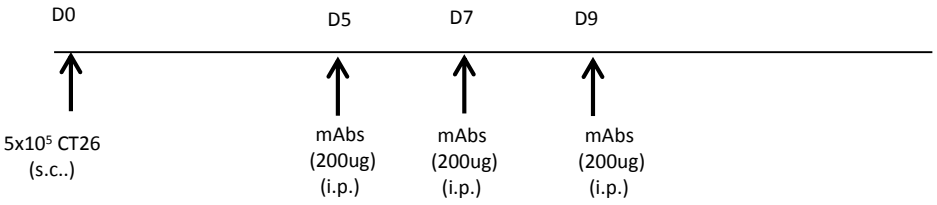


Figure 4-8: Combined anti-CD27 and PD-1/L1 blockade enhances surface expression of CD107a. C57BL/6 recipient mice (n=5/ group) were inoculated s.c. with $2x10^5$ B16-BL6 on day -6. $3x10^6$ of pmel-1 T cells were transferred i.v. into mice on day -1. On day 0, mice were immunized with 200ug of hgp100 with an isotype control rat IgG2a mAb (clone MC106A5), anti-CD27, anti-PD-1 (200ug)/L1 (200ug) or anti-CD27 (200ug) in combination with anti-PD-1 (200ug)/PD-L1(200ug). Mice were culled on day 4 post immunization (peak of the response) and splenocytes were harvested to analyse pmel-1 T cell effector function Cells were restimulated for 4 hours in the presence of Golig-stop, peptide and anti-CD107a and then re-surface stained for CD107a. The percentage of pmel1 CD9+ T cells expressing CD107a is shown. Data show group means +/-SEM. *P<0.05,**P<0.001, ***P<0.001, one way ANOVA.

Together, these results suggested that the combination of anti-CD27 mAb with anti-PD-1/anti-PD-L1 blocking mAb synergised towered generating high frequency of anti-tumour-specific CTLs with potent effector functions.

4.5. Targeting CD27 with PD-L1 has no synergistic effect in reducing CT26 tumour growth

To test the synergistic anti-tumour effect of antiCD27 and anti-PD-L1 mAbs in other setting, BALB/c mice were inoculated s.c. with the syngeneic CT26 colon carcinoma. Mice were then left untreated or injected i.p. with (200ug) mAbs to CD27 or to PD-L1, alone or in combination on days 5, 7 and 9 post tumour inoculation. Untreated mice reached terminal tumour size by day 20 post tumour injection (Fig. 4-9A). Targeting CD27 delayed tumour growth significantly compared with untreated mice (Fig. 4-9A). However, PD-L1 blockade did not have any effect on controlling tumour growth (Fig. 4-9A). More importantly, the combination of anti-CD27 and anti-PD-1 mAbs showed no synergy in dampening tumour size in treated mice (Fig. 4-9A). Stimulating the CD27 co-stimulatory receptor enhanced survival significantly such that 40% of mice exhibited complete tumour regression up to day 100 compared with the untreated group which all died by day ~20 (Fig. 4-9B). In contrast, PD-L1 blockade did not have any therapeutic effect; anti-PD-L1 treated mice were culled day ~20 post tumour challenge similar to the control group (Fig. 4-8B). The combination of both mAbs did not improve survival compared with anti-CD27 used as monotherapy (Fig. 4-9B). Therefore, the anti-tumour immunity observed when both antibodies were combined is most likely due to the effect of targeting CD27. These data contrast with those reported previously in this model (Sakuishi et al., 2010) in which anti-PD-L1 has a modest effect on CT26 tumour growth; anti-CD27 was not evaluated in that report.



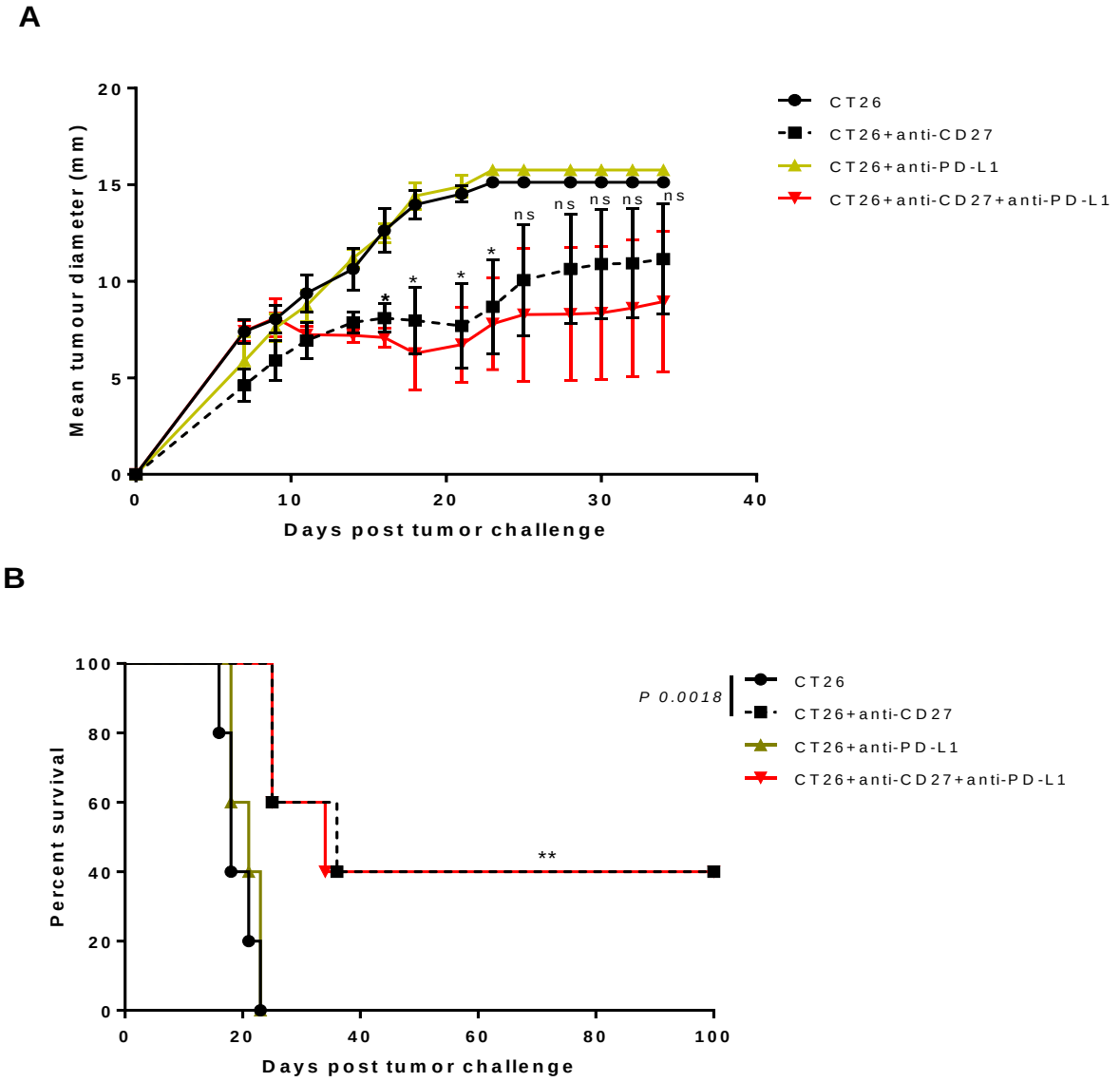


Figure 4-9: Anti-PD-L1 and anti-CD27 mAbs show no synergy in reducing CT26 tumour growth. BALB/c mice (n = 5 per group) were inoculated s.c. with 5x10^5 CT26 tumour cells, then injected i.p. with PBS as a control or with 200ug of anti-CD27, anti-PD-L1 mAbs or a combination of both on days 5, 7 and 9. (A) shows the tumour growth of mice. Each line shows the group MEAN+/-SEM. (B) Percentage of mice surviving to the humane end-point. Mice were culled when the mean tumour diameter reached 15mm. ***P value* ≤ .001 comparing mice treated with anti-CD27mAb with control treated mice, ns=anti-CD27 with the control group, Students two-tailed t-test.

4.6. Blocking PD-1 co-inhibitory receptor does not protect mice with established tumour

PD-L1 blockade does not always result in complete restoration of antigen-specific $CD8^+$ T cell function (Blackburn et al., 2008), and PD-L1 blockade has limited and/or variable effects on CT26 growth (Sakuishi et al., 2010) and (Fig 4-9). To assess whether both PD-L1 and PD-L2 contribute to T cell dysfunction, using the same treatment regimen as in (Fig 4-9), I evaluated anti-PD-1 blockade to test whether blocking PD-1 would improve the treatment. However, while targeting CD27 again had some therapeutic effect, and resulted in complete tumour regression in 20% of the mice (Fig 4-10A and B), PD-1 blockade showed only a trend toward delayed tumour growth in mice bearing established tumour (Fig. 4-10A). Similar to PD-L1 blockade (Fig. 4-9), the combination of anti-CD27 and anti-PD-1 mAbs did not synergise to improve therapy; the treatment outcomes of the treated mice were comparable to those observed when anti-CD27 mAb was injected alone (Fig 4-10A and B).

These results indicate that blocking PD-1 or PD-L1 alone are insufficient to control tumour growth in this tumour model (Figs 4-9 and 4-10). The lack of an anti-tumour effect with PD-1/PD-L1 blockade could be because of negative signals from other co-inhibitory receptors such as TIM-3, CTLA-4 or LAG-3 that can drive effector cells to become dysfunctional after activation (Sakhdari et al., 2012, Woo et al., 2012, Curran et al., 2010). Indeed, a previous report has shown that dual blockade of PD-L1 and TIM-3 increased INF-γ production in TILs compared to single PD-L1 or TIM-3 blockade and resulted in complete tumour regression in 50% of mice bearing CT26 (Sakuishi et al., 2010).

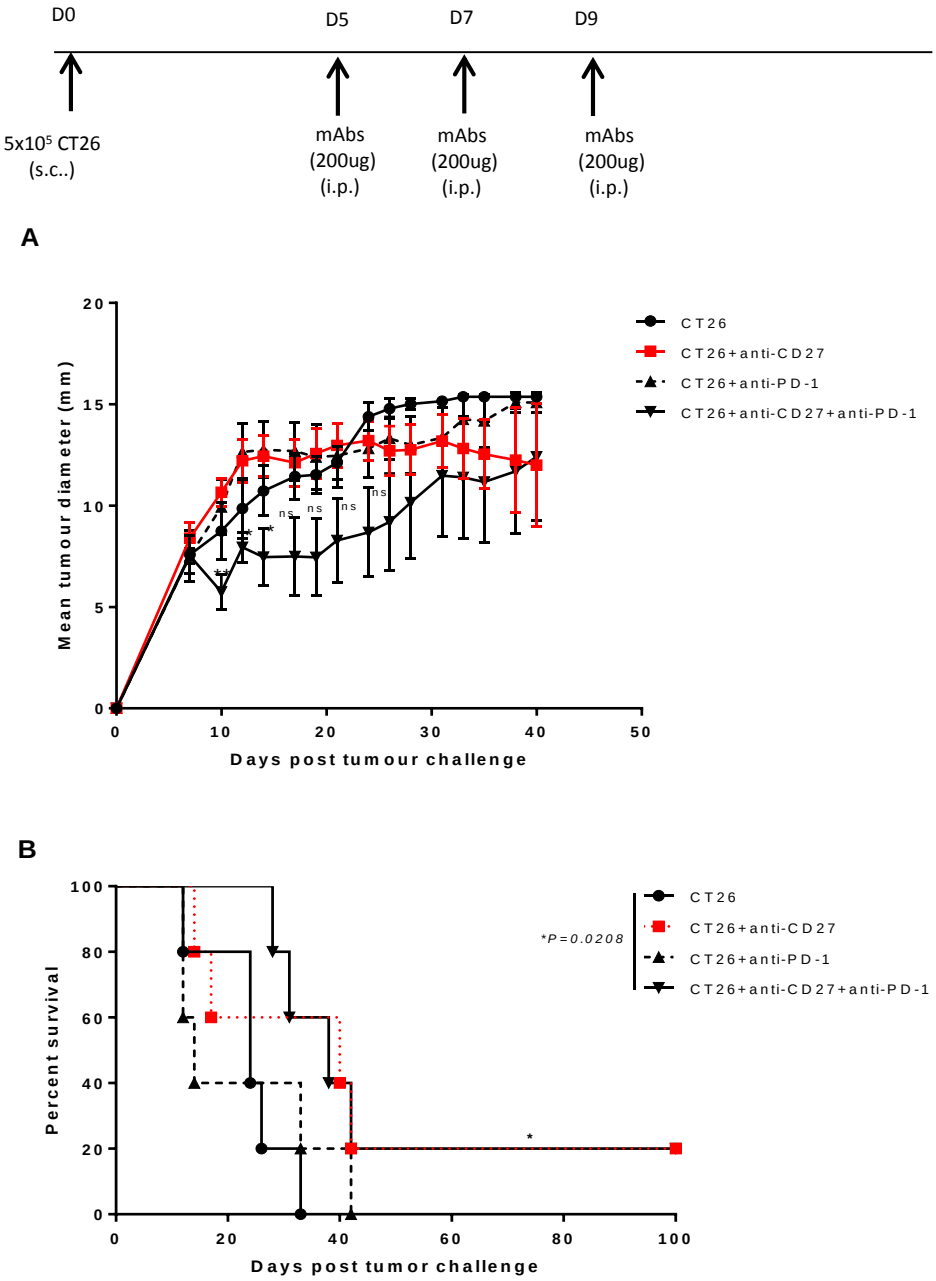
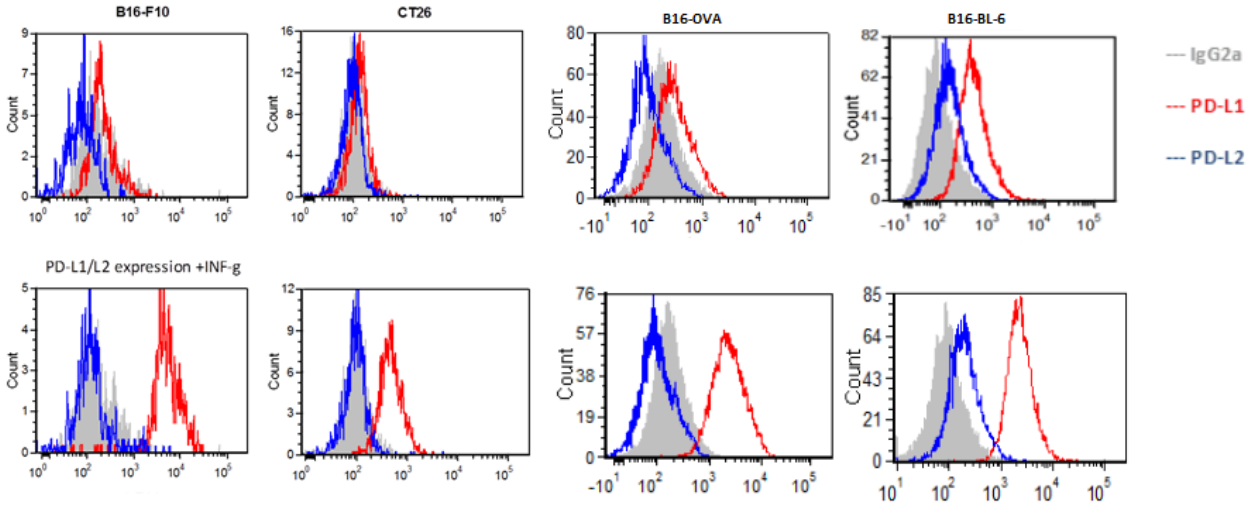


Figure 4-10: Administration of anti-PD-1 and anti-CD27 mAbs into mice bearing established CT26 tumor showed no synergy in reducing tumour growth. BALB/c mice (n = 5 per group) were inoculated s.c. with 5x10^5 CT26 tumour cells , then left without treatment as a control group or injected i.p. with 200ug of mAbs to CD27, PD-1 or a combination of both on days 5, 7 and 9. (A) shows the tumour growth of mice. Each line represents the group MEAN+/-SEM. (B) Percentage of mice surviving to the humane end-point. Mice were culled when the mean tumour diameter reached 15mm. *P<.0.05,**P<0.005 comparing the mean tumour diameter of mice treated with anti-CD27+anti-PD-1 mAbs with untreated mice (A). (B) *P=0.0208 comparing untreated mice with mice injected with anti-CD27+anti-PD-1 mAbs.

4.7. PD-L1/L2 expression on CT26 and B16-F10 in vitro

A study by Lee S. *et al.* suggested that PD-L1 is up-regulated by IFN-γ in multiple tumour cells including lung cancer cells, hepatoma, and colon cancer (Lee et al., 2006). B16 melanoma was also reported to express PD-L1 (Pilon-Thomas et al., 2010, Iwai et al., 2005). Initially, to confirm these results and also to look at PD-L2 expression, cultured CT26 and B16 sub lines (B16-F10, B16-OVA and B16-BL6) were stained with anti-PD-L1 and anti-PD-L2 mAbs. PD-L1 expression was detectable on all B16 sub lines (Fig 4-11). While PD-L1 expression was not observed on CT26 cells (Fig 4-11), IFN-γ induced PD-L1 expression significantly in CT26, but at higher levels on B16 melanoma (Fig 4-10). However, PD-L2 was only detected in B16-BL6 sub lines with or without IFN-γ (Fig 4-11).

(A)



(B)

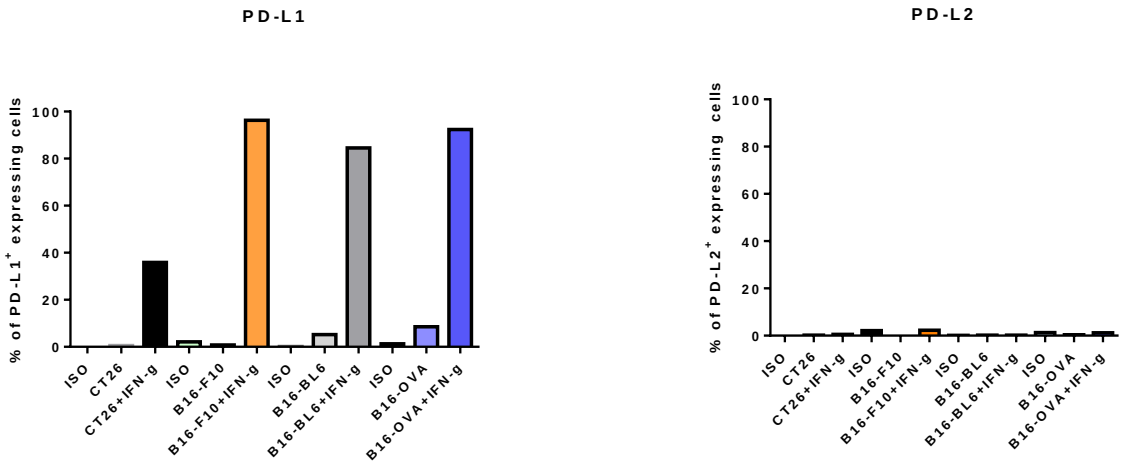


Figure 4-11: PD-L1/L2 expression on CT26 and B16 tumour cells. CT26, B16-F10, B16-OVA or B16-BL6 cells were incubated with or without 20ng of IFN-γ for 18hrs. Cells were then analysed by flow cytometry for PD-L1 or PD-L2 expression. (A) Upper panel shows PD-L1 (red) or PD-L2 (blue) without IFN-γ, while lower panel shows PD-L1 and PD-L2 expression after incubation with IFN-γ. Grey indicates staining with an irrelevant isotype control antibody in each case. (B) Percentage of PD-L1 (left) or (PD-L2) expressing cells.

These results show that PD-L1 is expressed on resting B16 sub lines but not on resting CT26 cells. PD-L1 expression was further induced after 18hrs incubation with INF-γ on all cells. In contrast, PD-L2 was only detected in the highly aggressive B16-BL6 tumour cells

A previous report showed that treating B16.SIY (B16-F10 melanoma cells expressing the SIYRYYGL (SIY) tumour antigen (Spiotto et al., 2002)) with 20ng/ml of INF-γ for 48hrs induced high levels of PD-L1 expression; PD-L2 in contrast was not expressed (Blank et al., 2004). This is consistent with my own data (Fig 24) and also with the observation that PD-L2 is more restricted to APCs (Rozali et al., 2012).

Because PD-L1 is minimally expressed on CT26 cells, this may explain the inability of PD-L1 and PD-1 blocking mAbs to hinder CT26 tumour growth (Figs 4-9 and 4-10).

4.8. Mouse IgG1 isotype is more efficient in controlling tumour growth than the mouse IgG2a isotype

Given that anti-CD27 mAb gave some therapy against CT26 (Figs 4-9 and 4-10) and showed efficacy in combination with PD-1 blockade against B16-BL6, I then investigated the anti-tumour effect of different anti-CD27 mAb isotypes. Previous studies have shown that antibodies of mouse IgG1 isotype bind to the inhibitory receptor FcγRIIB (Kim and Ashkenazi, 2013, Nimmerjahn and Ravetch, 2010, Regnault et al., 1999), whereas IgG2a mAbs interacts with FcγRI, FcγRIII and FcγRIV which trigger cell activation via immunoreceptor tyrosine-based activation motifs (ITAM) (Kim and Ashkenazi, 2013, Furness et al., 2014, Clynes et al., 2000, Kalergis and Ravetch, 2002, Wernersson et al., 1999).

To study this I used a model in which anti-CD27 alone is effective; an immunisation model based on B16-OVA. This was used to compare mouse IgG1 mAb or the mouse IgG2a with the parental rat AT124-1 IgG2a mAb that has been tested previously throughout this thesis. These two murine antibodies are untested in house antibodies.

C57BL/6 mice were injected i.d. with B16-OVA ($2x10^5$) on day -1. Mice were then injected i.v. with ovalbumin (5mg) with isotype control mAbs on days 0 and 1 post tumour inoculation, or in combination with mouse IgG1 (mIgG1) anti-CD27 mAb, mIgG2a anti-CD27, or with the parental rat anti-CD27 (clone AT124-1, rat IgG2a). Mice treated with the isotype control mAb exhibited tumour growth (Fig 4-12A), while mouse IgG1 mAb reduced tumour growth significantly compared with the control group (Fig 4-12A). IgG2a mAb in contrast, did not have any anti-tumour effect (Fig 4-12A). Tumour grew progressively and reached an endpoint (15mm) by day ~22 post tumour challenge. Similar to the IgG1 mAb, the parental AT124-1 mAb showed a remarkable anti-tumour response compared with the control group (Fig 4-12A). With regards to mice survival, mice receiving IgG2a mAb were all culled by day ~23 after tumour inoculation (Fig 4-12B). Anti-CD27 IgG1 isotype to the contrary resulted in 3/5 mice being tumour free for up to day 100 post tumour injection (Fig 4-12B). Similarly, the parental Ab resulted in complete tumour regression in 3/5 treated mice (Fig 4-12B).

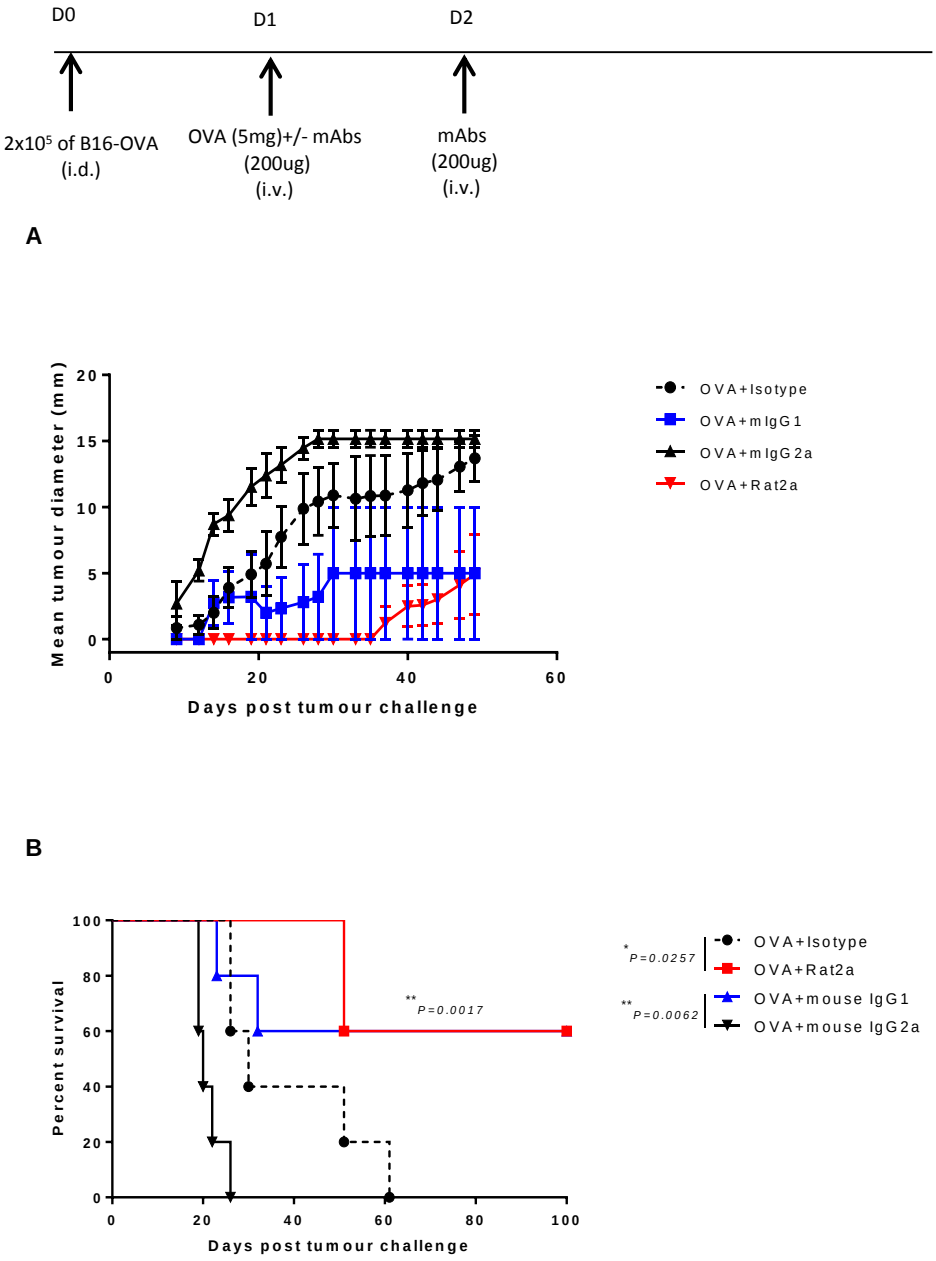


Figure 4-12: Comparison of the anti-tumour effect of the anti-CD27 rat AT124-1 mAb with mouse IgG1 and IgG2a versions of the same mAb on mice bearing established B16-OVA tumour. C57BL/6 mice (n = 5 per group) were inoculated i.d. with 2x10^5 of B16-OVA, then injected i.v. with ovalbumin (5mg) with anti-CD37 (WR17 an isotype control for the mouse IgG2a mAb) mixed 1:1 (100ug each mAb) with anti-CD16 (3G8 isotype mAb for the mouse IgG1 mAb) or with anti-CD27 mAb (rat IgG2a AT124-1, mouse IgG1 or with IgG2a isotypes) on days 1 and 2 post tumour challenge. Mice were culled when the mean tumour diameter reached 15mm of individual mice. Data shows MEAN+/-SEM of group. (A) Shows the mean tumour diameter of mice. (B) Percentage of mice surviving to the humane end-point. Mice were culled when the mean tumour diameter reached 15mm. *P<0.05, **P<0.005, ***P<0.0005.

Data obtained from analysing tumour-reactive CD8⁺ T cells from blood showed that treating mice with the control mAbs did not augment a significant tumour Ag-specific CD8⁺ T cell response (only 1% of total blood CD8⁺ T cells were OVA-specific) as detected by MHC I tetramer labelling (Fig 4-13). Mice treated with the mouse IgG2a mAb had an ineffectual primary OVA-specific CD8⁺ T cell response at the peak of the response (on day 6 post immunization) (~3.5% of total CD8⁺ T cells). Notably, the frequency of OVA-specific CD8⁺ T cell expansion slightly declined by day 8 post immunization and rebounded by day 12 to form ~5% of the CD8⁺ T cells (Fig 4-13). This might be because tumour cells in this group was growing rapidly, and more tumour antigens were exposed to the immune system, resulting in an increase in the number of tumour Ag-specific CD8⁺ T cells. In contrast to mIgG2a mAb, the mouse IgG1 isotype promoted strong expansion of OVA-specific CD8⁺ cells compared with the mouse IgG2a (~13% and ~3.5% respectively of the CD8⁺ T cells) at the peak of the OT-1 T cell response (Fig 4-13). The magnitude of the antigen-specific CD8⁺ T cells response was also significantly increased following administration of the parental rat anti-CD27 mAb, such that ~17% of the CD8⁺ T cells were OVA specific (Fig 4-13). The contraction phase for the rat anti-CD27-activated CD8⁺ T cells was similar to mIgG1-iduced OT-1 T cells (Fig 4-13).

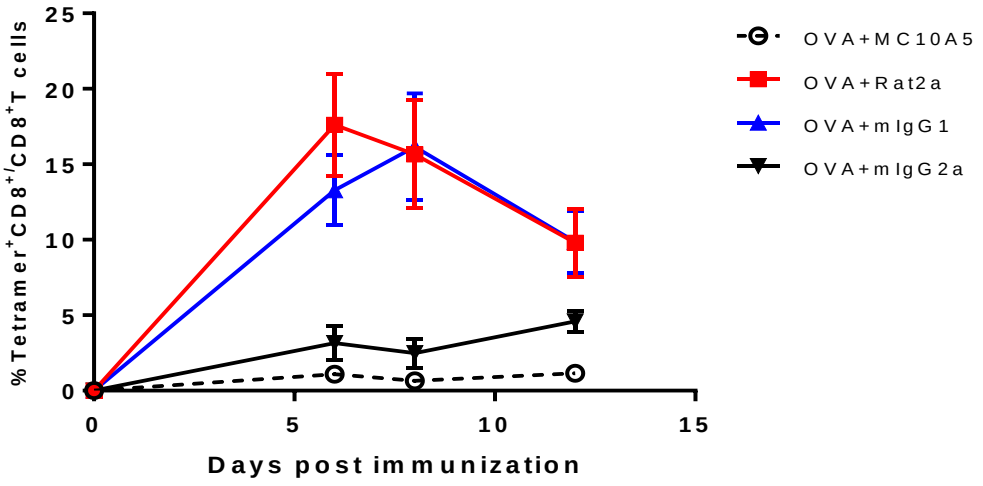


Figure 4-13: OVA-specific CD8⁺ T cell response from blood at different time points as detected by labelled-MHC-1 tetramer and CD8 staining. Data shows MEAN+/-SEM of group. (Full methodology is shown in Figure 4-11).

I also evaluated the effect of each mAb isotype on the frequency of total $CD8^+$, $CD4^+$ and $FoxP3^+CD4^+$ T_{reg} populations from the peripheral blood at the peak of the response on day 6 post priming. The frequency of total $CD8^+$ T cells was significantly reduced when mice were vaccinated with the mouse IgG2a isotype compared with the control group (2% and 9% of the total lymphocytes respectively) (Fig 4-14). Interestingly, no major difference in the frequency of $CD8^+$ T cells was observed in mice initially treated with the mIgG1 or with the control mAb (9% and 8.5% of the total lymphocytes respectively) (Fig 4-14B). Moreover, rat anti-CD27 mAb resulted in a significant increase in the $CD8^+$ T cell population compared with the mIgG1 isotype (Fig 4-14B). The $CD4^+$ T cell population was reduced to only 2% of the lymphocytes in response to IgG2a mAb vaccination (Fig 4-14C). Although both IgG1 and rat anti-CD27 mAbs resulted in a strong OT-1 T cell response (Fig 4-13), both mAbs had no positive effect on the total $CD4^+$ T cell response (Fig 4-14). This might be due to the increased number of antigen-specific and $CD8^+$ T cells response which might affect the proportion of the $CD4^+$ T cells in the blood. Furthermore, there was a small but statistically significant increase in the proportion of $FoxP3^+CD4^+T_{reg}$ cells in blood in mice injected with the mouse IgG1 and mouse IgG2a (Fig 4-14D).

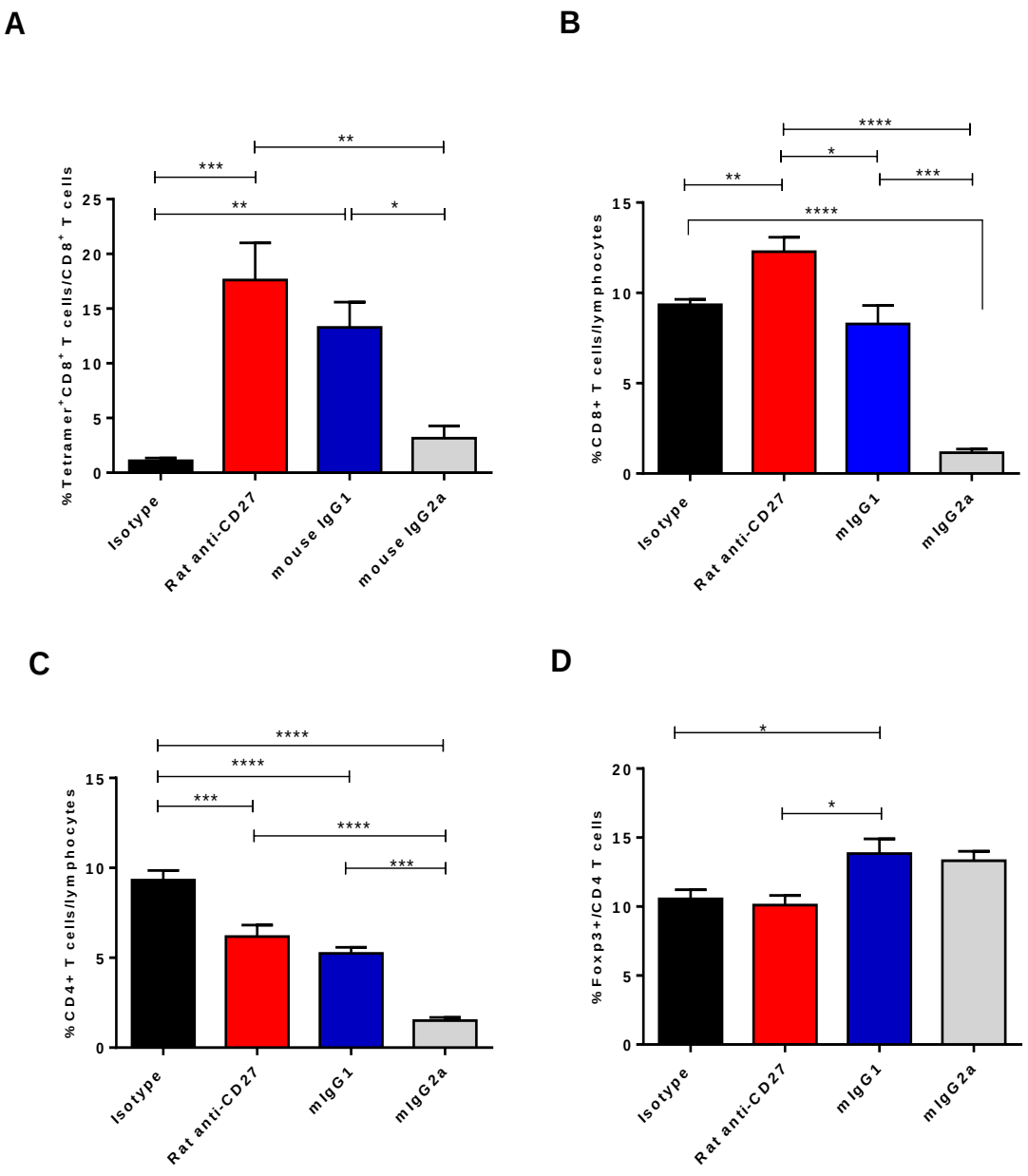


Figure 4-14: Shows the frequencies of (A) OVA-specific $CD8^+$ T cells in the blood at the peak of the response on day 6 post immunization. $CD8^+$ T cells out of the lymphocytes at the peak of the response (B). CD4+ T cells out of the lympohcytes in panel (C). T regulatory cells as detected by the staining of the $FoxP3^+$ and CD4. *P<0.05,**P<0.001, ***P<0.001, ****P<0.0001 one way ANOVA Data shows MEAN+/-SEM of group. (Full methodology is described in Fig 4-11).

These results indicate that the mouse IgG1 isotype is more efficient in controlling tumour growth compared with IgG2a, at least in the B16-OVA model, and that the IgG2a depletes cells which express surface CD27. The enhancement of the antitumor response was correlated with the magnitude of antigen-specific $CD8^+$ T cells expansion at the peak of the response.

4.9. Discussion

In this result chapter, I have investigated the correlation between the primary response of vaccine-induced gp100-specific $CD8^+$ T cells particularly in (Figs 3-2 and 3-10) and the anti-tumour response *in vivo*. In this particular experiment, pmel-1 $CD8^+$ T cells activation following immunization with low peptide dose (100ug) and agonist antibodies against CD27, 4-1BB, OX40 and GITR receptors did not protect mice from the poorly immunogenic B16-BL6 melanoma (Fig 4-1). Tumours continued to grow aggressively in all groups similar to the control group (Fig 4-1), the lack of the anti-tumour response could be possibly because pmel-1 T cells did not expand to an extent that allows a sufficient anti-tumour response.

In the second tumour experiment which involved strong pmel-1 T cell activation, the combination of anti-CD27 and anti-PD-1/anti-PD-L1 mAbs after pmel-1 cell transfer synergised for enhanced tumour rejection compared to each mAb alone, resulting in the long-term survival of 50% of animals that initially received B16 cells (Fig 4-2A).

In vitro experiment showed that resting B16-BL6 cells express both PD-1 ligands (PD-L1 and PD-L2), and incubation with IFN-γ further induced their expression (Fig 4-11), confirming that B16 tumour cells likely provide negative signals via PD-L1 and/or PD-L2. However, anti-PD-1 antibody alone was insufficient to enable tumour rejection. I hypothesise that this may be due to the aggressive and non-immunogenic nature of B16-BL6 and/or the existence of other co-inhibitory receptor/ligand pairings between the B16 tumour and $CD8^+$ T cells. In addition, poor priming of tumour-specific T cells due to the lack of co-stimulatory signals could have prevented optimal expansion and differentiation of T cells. I noticed a marked increase in pmel-1 T cell number in the spleen following immunization with the combined vaccine compared with monotherapy (Fig 4-5).

In addition to the dramatic increase in Ag-specific $CD8^+$ T cells quantity at the peak of the response (Fig 4-5), the combination also improved the quality of the generated CTL as was detected by the measurement of the granzyme B and cytokine production. Pmel-1 T cells produced significantly higher granzyme B and effector cytokines particularly IFN-γ and TNF-α following immunization with anti-CD27 and anti-PD-1/L1 compared with single anti-PD/L1 or anti-CD27 mAb treatment alone (Fig 4-7). Moreover, the combination generated higher

frequencies of double positive and triple positive cytokine producing pmel-1 cells compared with monotherapy (Fig 4-7). I also noticed higher frequencies of peml-1 cells expressing the degranulation marker CD107a when mice where vaccinated with the combination in comparison with mice received single treatment (Fig 4-8). The magnitude of pmel-1 cell expansion and more importantly the superior effector function of the generated CTLs (because the magnitude of Ag-specific $CD8^+$ T cells response is not always a surrogate marker for effective anti-tumour response (Rosenberg et al., 2005)) in mice treated with the combination induced tumour regression of the poorly immunogenic B16-BL6melanoma and improved the overall survival of mice (Figs 4-2A and B). Notably, no vitiligo was observed in the survival mice in response to the combination treatment. Enhancement of CTL effector function following immunization with anti-CD27 and anti-PD-1/L1 mAbs was associated with T-bet expression (control expression of genes required for cytotoxic effector function) (Lazarevic et al., 2013). T-bet expression was significantly higher when mice were injected with the combination compared to monotherapy (Fig 4-6). This is possibly due to the strong TCR signalling resulted from CD27 stimulation and PD-1/PD-L1 blockade. Strong TCR and IL-12 (not detected in this experiment) signalling enhance and sustain the activity of kinase mTOR downstream of STAT4 (signal transducer and activator of transcription 4) leading to T-bet expression (Chi, 2012, Lazarevic et al., 2013).

With regard to CTLA-4 blockade, the anti-B16 response was less potent compared with PD-1 blockade (Figs 4-2 and 4-4), possibly because of inferior quality of the effector function of the pmel-1 cells generated after immunization compared with PD-1 blockade plus anti-CD27. Moreover, anti-PD-1 plus anti-CD27 might recruit more effector Ag-specific $CD8^+$ T cells in the tumour compared with anti-CTLA-4 and anti-CD27. Injecting CTLA-4 locally and anti-PD-1 with anti-CD27 mAbs may provide better therapeutic effect (Binder and Schreiber, 2014, Sandin et al., 2014), although this may lead to activation of self-reactive T cells and autoimmunity. My results were in line with previous observations that combining anti-PD-1mAb with agonist mAb or antagonist mAb against another check point blockade enhances anti-tumour immunity and improve overall survival (Binder and Schreiber, 2014, Guo et al., 2014, Lu et al., 2014b).

Different studies have shown that administering anti-PD-1 or anti-PD-L1 as a monotherapy has a modest impact on tumour growth (Iwai et al., 2002, Binder and Schreiber, 2014, Woo

et al., 2012, Curran et al., 2010), whereas dual immune checkpoint blockade dampens tumour growth significantly (Binder and Schreiber, 2014, Mamalis et al., 2014, Wainwright et al., 2014, Curran et al., 2010). For example, treating mice bearing established Sa1N fibrosarcoma with anti-PD-1mAb alone resulted in only 20% of mice being tumour free. However, dual blockade of PD-1 and LAG-3 co-inhibitory receptor improved survival to 70%. Similar results were observed in MC38 colon adenocarcinoma model (Woo et al., 2012). Consistent with these observations, *Lag3*$^{-/-}$*Pdcd1*$^{-/-}$ mutant mice rejected high dose of B16 melanoma (a tumour that is difficult to cure with immunological intervention (Turk et al., 2004)), and showed increased survival compared with single *Pdcd1*$^{-/-}$ knockout mice (80% and 40% tumour free mice respectively) (Woo et al., 2012).

PD-1/PD-L1 pathway blockade also cooperated with anti-CTLA-4 in reducing tumour growth of different tumour models including CT26, ID8-VEGF ovarian carcinoma and B16-BL6 compared with single treatment (Binder and Schreiber, 2014, Blackburn et al., 2008). The enhancement in tumour protection observed with antibody-mediated immune checkpoint blockade was attributed to restoring exhausted tumour-specific $CD8^+$ T and $CD4^+$ T cell effector functions and expansion within the tumour, thereby shifting the tumour microenvironment from suppressive to inflammatory (Blackburn et al., 2008, Curran et al., 2010).

Next, I tested the anti-tumour effect of the combination anti-CD27 and PD-1 in the CT26 colon carcinoma model. Contrast to the B16-BL6 model, stimulating CD27 alone provided long-term protection against tumour growth in 20-40% of treated mice (Figs 4-9 and 4-10). In contrast, blocking PD-1/PD-L1 pathway alone did not provide any protection against this tumour (Figs 4-9 and 4-10). The combination of both antibodies failed to synergise i.e. long-term survival was not greater than that achieved by the administration of anti-CD27 mAb alone. PD-1/L1 pathway blockade was ineffective in this tumour model, possibly because unlike many tumours including B16-BL6, expression of PD-L1/L2 is low or absent (Fig 4-11). Another possibility for the lack of synergy could be the result of expression of additional inhibitory ligands e.g. LAG-3 and TIM-3.

Consistent with the CT26 tumour data, anti-CD27 demonstrated activity in the B16-OVA model (Fig 4-12). I then tested the anti-tumour therapeutic effect of different isotypes of

the parental anti-CD27mAb (rat AT124 IgG2b). Injecting mice bearing established B16-OVA with mouse AT124 IgG1 inhibited tumour growth significantly (Fig 4-12); similar to the protection induced by the parental rat AT124 IgG2a Ab (Fig 4-12), and eliminated tumour growth in 60% of the treated mice up to day 100 post tumour challenge. In contrast, mouse AT124 IgG2a Ab did not promote any anti-tumour activity (Fig 4-12). The inability of the mouse AT124 IgG2a isotype to confer tumour protection is because the antibody is depleting both $CD4^+$ and $CD8^+$ T cells that are crucial for tumour eradication (Fig 4-14). Suggesting that mouse IgG1 Ab is more potent in generating protective anti-tumour immune response compared with the mouse IgG2a.

Chapter 5. Optimising CD8$^+$ T cell memory

5.1. Introduction

Vaccines play an important role in reducing mortality and morbidity that is caused by infectious diseases. A successful vaccine should elicit strong Ag-specific immune response as well as long-lived memory to protect the host from the disease or reduce its severity (Nabel, 2013). Conventional vaccines were designed to generate neutralizing Abs against the pathogen (Koup and Douek, 2011). However, research in vaccines have shifted toward inducing strong T cell responses due to their potent ability to limit intracellular infections, and because neutralizing Abs are not always sufficient to protect the host from viral infections such as HIV and hepatitis virus (Koup and Douek, 2011). Importantly, T cell responses, particularly those elicited by CD8$^+$ T cells are essential for generating anti-tumour immunity.

The memory T cell compartment consists of Ag-experienced CD4$^+$ and CD8$^+$ T cells that have the ability to proliferate and acquire effector functions rapidly following re-encounter with the same Ag (Nabel, 2013). They can be divided according to surface-receptor expression, effector function, their distribution within the body and trafficking properties into four distinct subsets: T_{EM} that circulate between blood and mucosal sites to survey peripheral tissues, whereas those that are found in the lymph nodes are called T_{CM} cells, tissue-resident memory T cells and stem cell-like memory T cells (Kalia et al., 2006).

5.2. T cell differentiation into long-lived memory cells

The mechanism by which naive T cell differentiate into memory cells is not yet fully understood. The linear model (conventional model) suggest that memory T cells are derived from the Ag-specific CD8$^+$ T cells during the contraction phase (Ahmed and Gray, 1996). *In vivo* experiment showed conducted by Jacob and others showed that marked virus-specific CD8$^+$ T cells (CRE/LOXP recombination system) presented in memory cell precursors, indicating that memory cells are derived from effector cells (Jacob and Baltimore, 1999). Accumulating data by Kaech S and co-workers also supported this notion. In their study, they showed that effector T cells gave rise to long-lived memory T cells that have the

capacity to provide protective immunity (Kaech et al., 2003). The other hypothesis of memory T cell formation suggest that memory T cells are generated through asymmetric cell division that occurs during T cell proliferation or even as early as the first cell division (Kaech and Cui, 2012).

There are several factors that can influence T cell differentiation into memory cells including TCR signal strength, transcription factors, expression of anti-apoptotic genes, metabolic regulation and cytokines. The role of these factors in the generation of memory T cells is described below.

5.2.1. Signal strength and T cell fate

TCR/peptide-MHC signal strength plays a major role in modulating T cell differentiation and effector function. There are three major factors that influence the strength of TCR signalling including Ag density, TCR avidity to peptide-MHC and the duration of TCR/peptide MHC interaction (Corse et al., 2011, Knudson et al., 2013a, Leignadier and Labrecque, 2010). Henrickson SE and colleagues have investigated the correlation between TCR/peptide-MHC interaction and T cell fate decisions *in vivo*. Splenic DCs were pulsed with low (1uM) or high (100uM) $gp_{33–41}$ (KAVYNFAT**C**; an immune dominant of LCMV and activates TCR transgenic $CD8^+$ T cells clone P14) peptide doses. $Gp_{33–41}$-pulsed DC was injected into individual C57BL/6 mice in the foot pad. 18 hours later, mice were injected with P14 $CD8^+$ T cells i.v. and there response to the Ag was followed at different time points from the draining LNs. Results showed that the frequency of activated P14 $CD8^+$ T cells was similar in the first 48 hours. However, the primary response started to decline after 96 hours (peak of the response) in mice injected with 1uM-DC compared with 100uM-DC (Henrickson et al., 2013). More importantly, memory T cell pool was highly affected by the Ag density. The number of memory T cells following re-stimulation with LCMV (30 days post peptide injections) was significantly lower (~5 fold lower) at the peak of the secondary response (on day 5 post re-infection) in 1uM-DC recipient mice compared with the number of memory cells generated in response to 100uM peptide injection (Henrickson et al., 2013). Indicating that memory cell formation is impaired without stable DC-T Cell contact, indicating the important role of peptide density in determining naive T cell fate.

5.2.2. Bcl-2

Bcl-2 (B-cell lymphoma/leukemia-2 gene) is an anti-apoptotic oncogene that was first discovered in B-cell malignancy (Reed, 1994, Chi et al., 2014). Data collected from different studies revealed that Bcl-2 promotes cellular survival and lymphoid homeostasis upon growth factor withdrawal (Boise et al., 1995, Cory et al., 2003, Watts, 2010). Bcl-2 family members share one or more of the constant motifs of the Bcl-2 homology (BH) regions known as (BH1-4) (Lomonosova and Chinnadurai, 2008). The function of each individual protein is determined by the combinations of these constant regions. The anti-apoptotic proteins Bcl-2, Bcl-xl and BcL-w contain the four BH1-4 regions while the pro-apoptotic proteins Bak, Bax and Bok for example have pore-forming BH1-3 regions and a mutated form of the B4 region (Lomonosova and Chinnadurai, 2008). The pro-apoptotic proteins Bim, Bad, Noxa and Nip3 share the BH3 region (Lomonosova and Chinnadurai, 2008). Recent work by Kurtulus S *et al.* showed that both Bim and Bcl-2 proteins were expressed in effector $CD8^+$ T cells have the potential to differentiate into long-lived cells. Bcl-2 expression was required to abrogate the function of the pro-apoptotic protein Bim and maintain memory cells (Kurtulus et al., 2011).

5.2.3. Metabolic regulation and T cell differentiation

Immunometabolism is an emerging field to investigate the correlation between cellular metabolism and T cell differentiation, effector function and longevity (Mathis and Shoelson, 2011). Resting and memory T cells use fatty acid oxidation (FAO) as a major source for the energy (catabolic process). However, during the activation, T cell switches from FAO to aerobic glycolysis and lipid synthesis (anabolic process) to meet the tremendous demand of energy for proliferation and for the production of the effector molecules (Mathis and Shoelson, 2011, Lochner et al., 2015, Kaech and Cui, 2012). The mammalian target of ramapycin (mTOR) signalling pathway plays a key role in regulating naive $CD8^+$ T cells differentiation into CTL by orchestrating nutrient uptake and lipid synthesis. Meanwhile, cellular stress and adenosine triphosphate (ATP) deprivation activate AMP-activated protein kinase (AMPK) (regulate nutrient homeostasis) which therefore inhibits mTOR activity and

induce memory CD8$^+$ T cells formation by switching to FAO (Mathis and Shoelson, 2011, Lochner et al., 2015, Kaech and Cui, 2012).

5.2.4. Common γ chain cytokines

Common γ chain (γ_c) cytokines (IL-2, IL-4, IL-7 and 15) are critical regulators for naive and memory T cells (Ma et al., 2006, Surh and Sprent, 2008). Cytokines of the common γ chain share the same downstream signalling pathway; but they influence T cells at different stages *in vivo*. Cytokines of the common γ_c mediate T cell survival through the activation of the transcription factor STAT5, which in turn migrate to the nucleus and induce expression of Eomes and Bcl-2 (Surh and Sprent, 2008, Belz and Masson, 2010). The important role of common γ_c cytokines in the generation of memory T cells was demonstrated by Decaluwe *H et al.* They showed that despite the potent primary response of the Bcl-2 Tg $\gamma_c^{-/-}$ P14 CD8$^+$ T cells following infection with LCMV (~30% of total CD8$^+$ T cells were Ag specific at the peak of the response), these cells did not survive after the contraction and failed to differentiate into long-lived memory cells. They returned to baseline and become undetectable after day 13 post LCMV infection, (Decaluwe et al., 2010). Indicating γ_c signalling pathway memory T cells differentiation in a Bcl-2-independent manner (Decaluwe et al., 2010).

As mentioned earlier in the introduction of this chapter, successful vaccine should elicit strong CTLs response as well as induce memory T cells formation to eliminate tumour growth and to prevent tumour re-appearance. However, throughout my experiments, I noticed that pmel-1 T cells do not persist during the contraction phase. Therefore, the aim of this result chapter was to test different approaches that have been previously shown to increase Ag-specific CD8$^+$ T cell survival and may promote pmel-1 T cell differentiation into memory cells. These approaches include injecting exogenous IL-2 cytokine, mTOR inhibition using ramapycin drug and incorporation of the anti-apoptotic Bcl-2 protien into pmel-1 cells. I also investigated the role of TCR/peptide-MHC binding affinity in the formation of memory cells. More details about each approach will be described throughout this chapter.

5.3. Low dose of IL-2 complexes (IL-2Cx) do not maintain long-lived CD27-stimulated pmel-1 cells after expansion

IL-2 is a critical cytokine for generating optimal T cell responses to pathogens and secondary responses (Blattman et al., 2003, Kelly et al., 2002, Smith, 1988, Boyman and Sprent, 2012, Matsuoka et al., 2013). During the contraction phase when most effector cells die via programmed cell death, administration of IL-2 increases the memory $CD8^+$ T cell count by rescuing $CD8^+$ T cells from cell death (Boyman et al., 2010, Dooms et al., 2007). However, activating T cells in the presence of IL-2 up-regulates FAS on T cells and increases their susceptibility to FAS-mediated death (Nguyen and Russell, 2001). Consequently, administering IL-2 early can reduce memory $CD8^+$ T cells (Blattman et al., 2003). The biological activity of IL-2 can be maintained *in vivo* by administering recombinant IL-2 with anti-IL-2mAb such as JES6-1 or S4B6 in a complex (abbreviated to IL-2Cx) (Webster et al., 2009, Tomala et al., 2009). IL-2 S4B6mAb binds specifically to the IL-2Rα chain (CD25) which is over expressed on T_{reg} cells. IL-2/S4B6 complexes gives rise to $CD122^{hi}$ cells such as memory $CD8^+$ T cells and NK cells. The antibody JES6-1 interacts with the IL-2 receptor subunit β or the common receptor γ chain. In contrast to IL-2/S4B6-1 complexes, IL-2/JES6-1 gives rise to $CD25^{hi}$ cells such as T_{reg} cells *in vivo*. One limitation of IL-2Cx is its toxicity at high doses (Tomala et al., 2009, Boyman and Sprent, 2012). Injecting multiple doses of IL-2Cx with the TriVax vaccination (hgp100 short peptide, Poly I:C and anti-CD40mAb) increased pmel-1 cell frequencies significantly ($\sim 90\%$ of $CD8^+$ T cells) and generated long-lived $CD8^+$ T cells (Cho et al., 2012). In the TriVax formulation, we hypothesized that IL-2Cx might be promoting memory $CD8^+$ T cell formation. Therefore, to investigate if IL-2Cx would improve anti-CD27-induced memory, WT mice were inoculated i.d. with B16-BL6 tumour and then received pmel-1 $CD8^+$ T cells followed by hgp100 alone, with anti-CD27 mAb or anti-CD27 mAb with two injections of IL-2/JES6-1 or IL-2/S4B6 i.p. Surprisingly, neither IL-2Cx had any effect on pmel-1 $CD8^+$ T cell expansion and a maximum of 10% of total $CD8^+$ T cells was observed with all vaccination modes, comparable to that induced by anti-CD27 (Fig 5-1). In addition, IL-2Cx did not rescue effector $CD8^+$ T cells during the contraction phase from apoptosis (Fig 5-1).

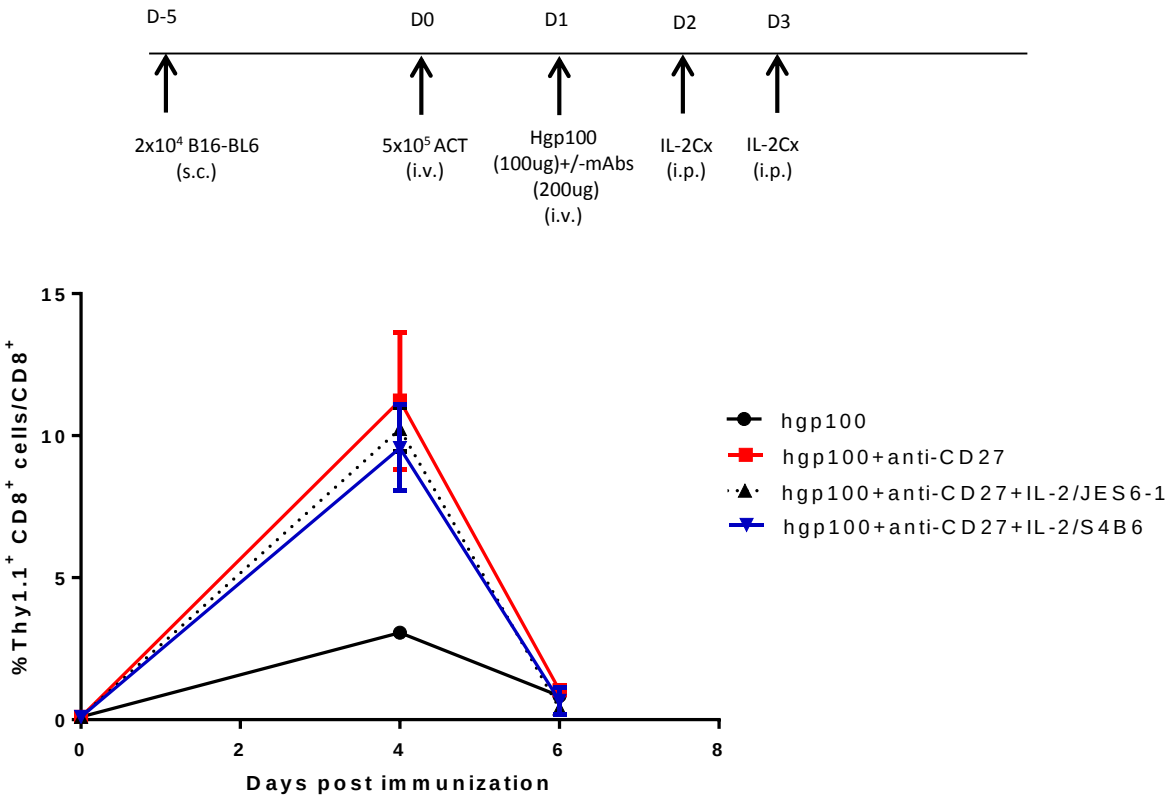


Figure 5-1: Vaccination with IL-2Cx does not increase the frequency of memory CD8⁺ T cells. C57BL/6 (n = 3/ group) were inoculated with $2x10^4$ B16-BL6 tumour cells i.d. on day -5. On day 0 mice received $5x10^5$ pmel-1 cells i.v. and were then immunized on day 1 with hgp100 (100ug) plus 200ug of anti-CD27. On days 2 and 3 some groups were injected i.p. with IL-2Cx directed towards CD25 (1.5ug/ml of mouse recombinant IL-2/JES6.1 20ug/ml), or IL-2Cx against CD122 (1.5ug of recombinant mouse IL-2/S4B6.1 20ug). Pmel-1 CD8⁺ T cells were tracked within peripheral blood at different time points (using antibodies to Thy1.1 and CD8). Data show group means+/-SEM.

5.4. Mammalian target of Rapamycin (mTOR) blockade has little effect on pmel-1 T cell expansion or memory differentiation

Previous studied showed that mTOR inhibition enhances the number and quality of virus-specific memory CD8⁺ T cells (Araki et al., 2009). Therefore, I investigated the role of Rapamycin in promoting memory cell generation in the pmel-1 adoptive T cell transfer model. Following a published protocol (Araki et al., 2009) I injected mice with either low dose ($75ug/kg^{-1}$) from day 0 to day 8 or high dose ($1500ug/kg^{-1}$) Rapamycin from day 4 (the peak of the response) to day 8 post immunization. All mice were immunized with hgp100

(400ug in total) and anti-CD27 mAb (250ug) after pmel-1 cell transfer ($3x10^5$/mouse). The peak of the antigen-specific $CD8^+$ T cell response was similar in all groups (~30% of total $CD8^+$ T cells) (Fig 5-2), and the contraction rate was similar (Fig 5-2).

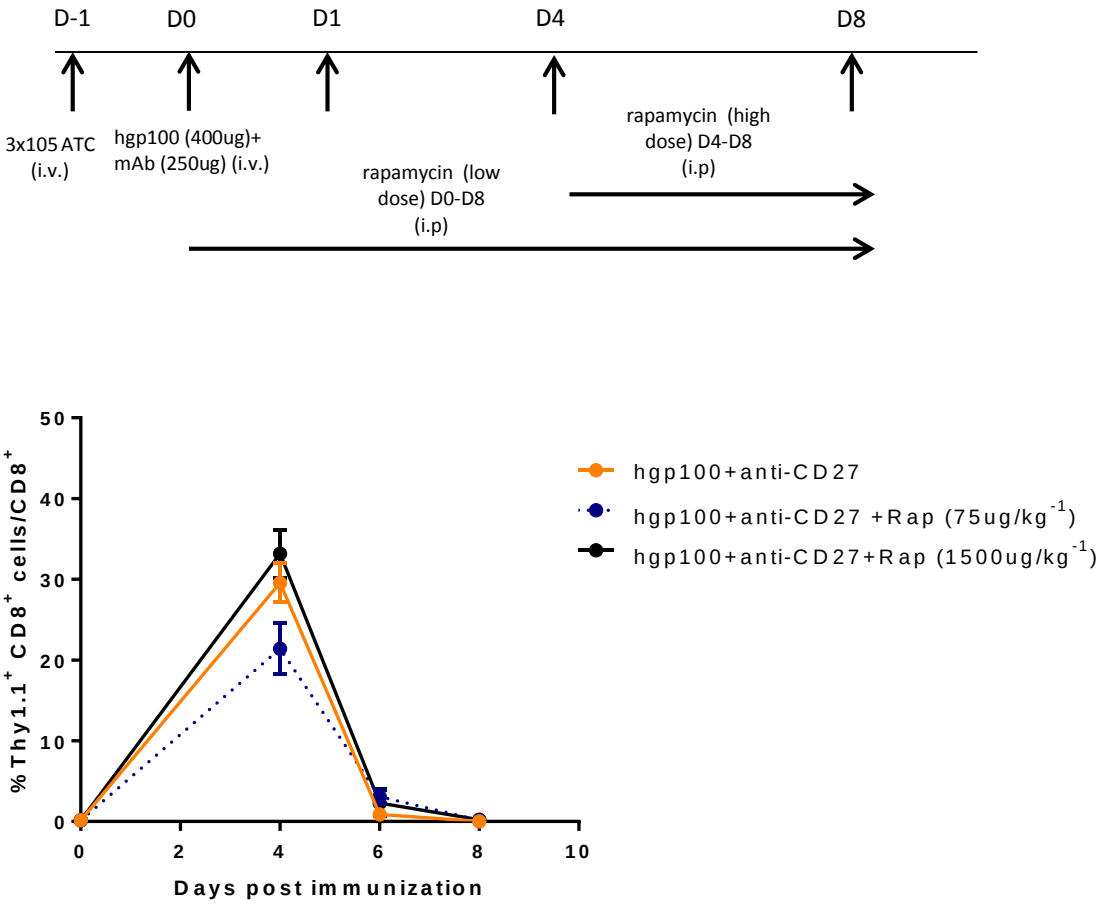


Figure 5-2: Rapamycin does not facilitate memory cell differentiation in the pmel-1 system. C57BL/6 (n = 3/group) mice were injected with pmel-1 cells ($3x10^5$) i.v. on day-1, then immunized with hgp100 short peptide (400ug) and anti-CD27mAb (250ug) on day 0. One group received daily injections of low dose rapamycin (75ug/kg^{-1}) from days 0 to 8. Another group was injected with high dose (1500ug/kg^{-1}) rapamycin from days 4 to 8. Pmel-1 cells were tracked in the blood at different time points by staining for Thy1.1 and CD8. Data show group MEANS+/-SEM.

5.5. Overexpression of the Bcl-2 anti-apoptotic oncogene prolongs hgp100-activated pmel-1 T cell survival

Because Rapamycin and administrant exogenous IL-2 failed to generate memory T cells (Figs 5-1 and 5-2), I then investigated the effect of Bcl-2 over-expression on pmel-1 T cell survival.

To do this, I first generated pmel-1 mice that carry the Bcl-2 protein by breading transgenic pmel-1 mice with mice in which the Bcl-2 transgene (VavP-Bcl-2) is over-expressed in all hematopoietic lineages (Kurtulus et al., 2011). To confirm expression of Bcl-2 in these Bcl-2 x pmel1 mice, I stained the blood sample with antibodies against Thy1.1 CD8 and Bcl-2 (Fig 5-3).

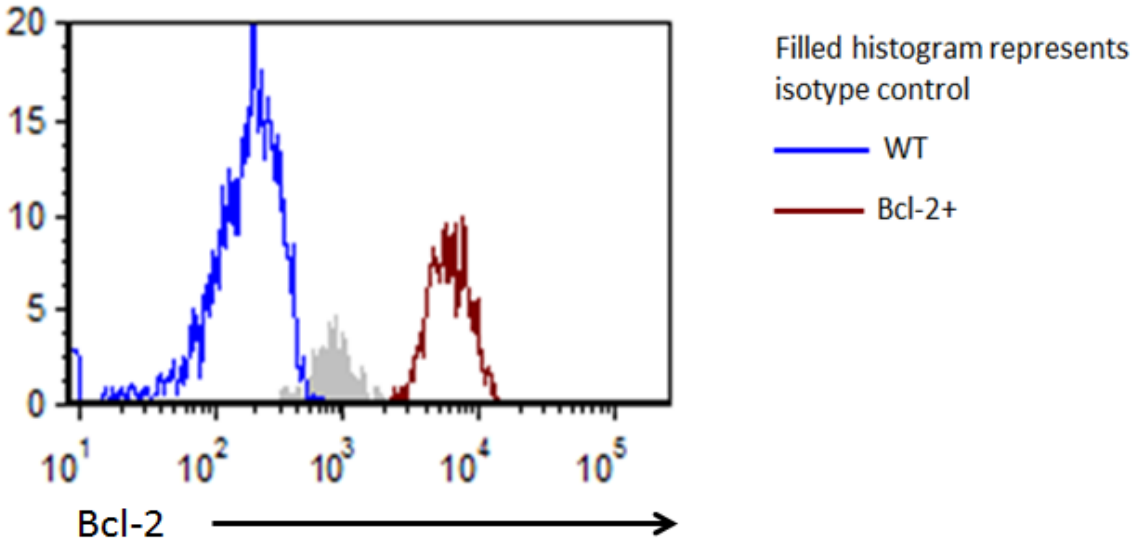


Figure 5-3: Bcl-2 oncogene expression on WT pmel-1 and Bcl-2+ pmel-1 T cells *in vivo*. Mice were bled and stained for Bcl-2 oncogene. Cells were gated on Thy1.1/CD8 T cells. Cells were stained intracellularly with an Isotype control, or with anti-Bcl-2 mAb prior analysing with flow cytometer.

The resulting mice appeared normal. However, the frequency of CD4CD8 double positive (DP) and double negative (DN) cells in the thymus was different (table 5-1) and (Fig 5-4). The increase in the frequency of DN thymocytes in the Bcl-2^+ pmel-1 mouse could be due to the effect of the anti-apoptotic Bcl-2 oncogene, which may prolong their survival in the thymus before they undergo negative selection. Moreover, Bcl-2^+ pmel-1 mice had an increased $CD4^+CD8^-$ and $CD4^-CD8^+$ cell number compared to normal pmel-1 mouse (Fig 5-4), this is probably because these cells in the Bcl-2^+ mouse were more resistant to apoptosis during the positive selection in the DP stage, which led to an increase in the single positive cell population as a consequence.

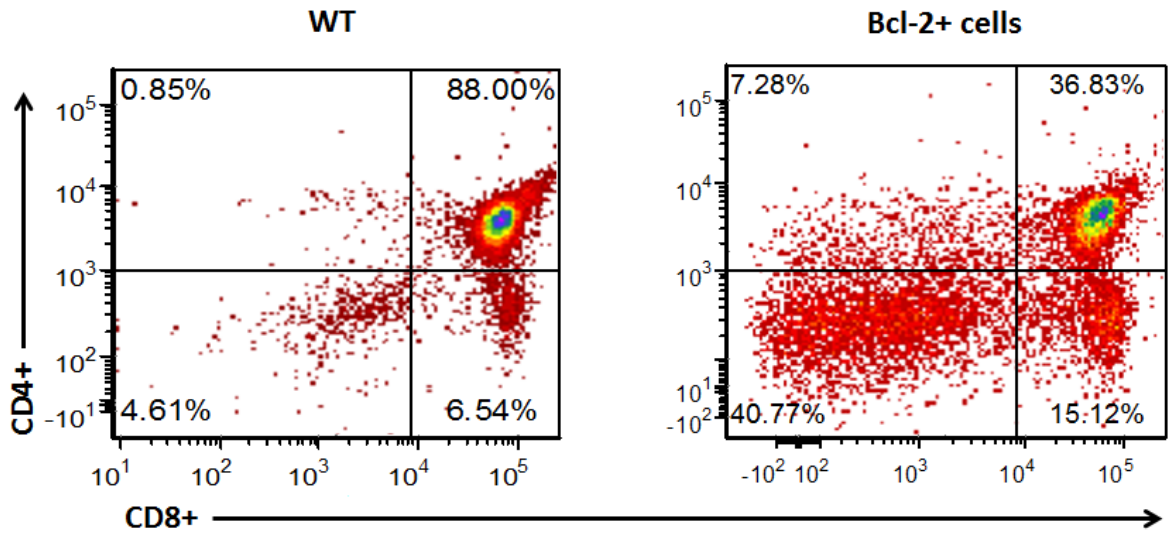


Fig 5-4: Shows the percentage of CD4CD8, double negative (DN) and double positive (DP) thymocytes. Thymocytes from WT or Bcl-2$^+$ pmel-1 mouse were harvested and stained for CD4$^+$ and CD8$^+$. The percentage of each population out of total thymocytes was determined by flow cytometry.

Table 5-1: percentage of the DP and DN cells in the thymus of Bcl-2$^+$ and Bcl-2$^-$ pmel-1 mice.

	% Double positive cells	% Double negative cells
Pmel-1 Bcl-2$^+$ mouse	36.83	40.77
Pmel-1 WT mouse	88	4.61

.

The total blood cell count was significantly higher (p=0.001) in Bcl-2$^+$ mice (7 to 14 mice were used to count number of cells from blood) bearing the pro-apoptotic Bcl-2 gene compared with normal pmel-1 mouse (Fig 5-5C). The total number of thymocytes and splenocytes was also affected by Bcl-2 oncogene. More cells were found in the Bcl-2$^+$ pmel-1 mouse compared with the WT pmel-1 mouse (Figs 5-5B and C). In addition,

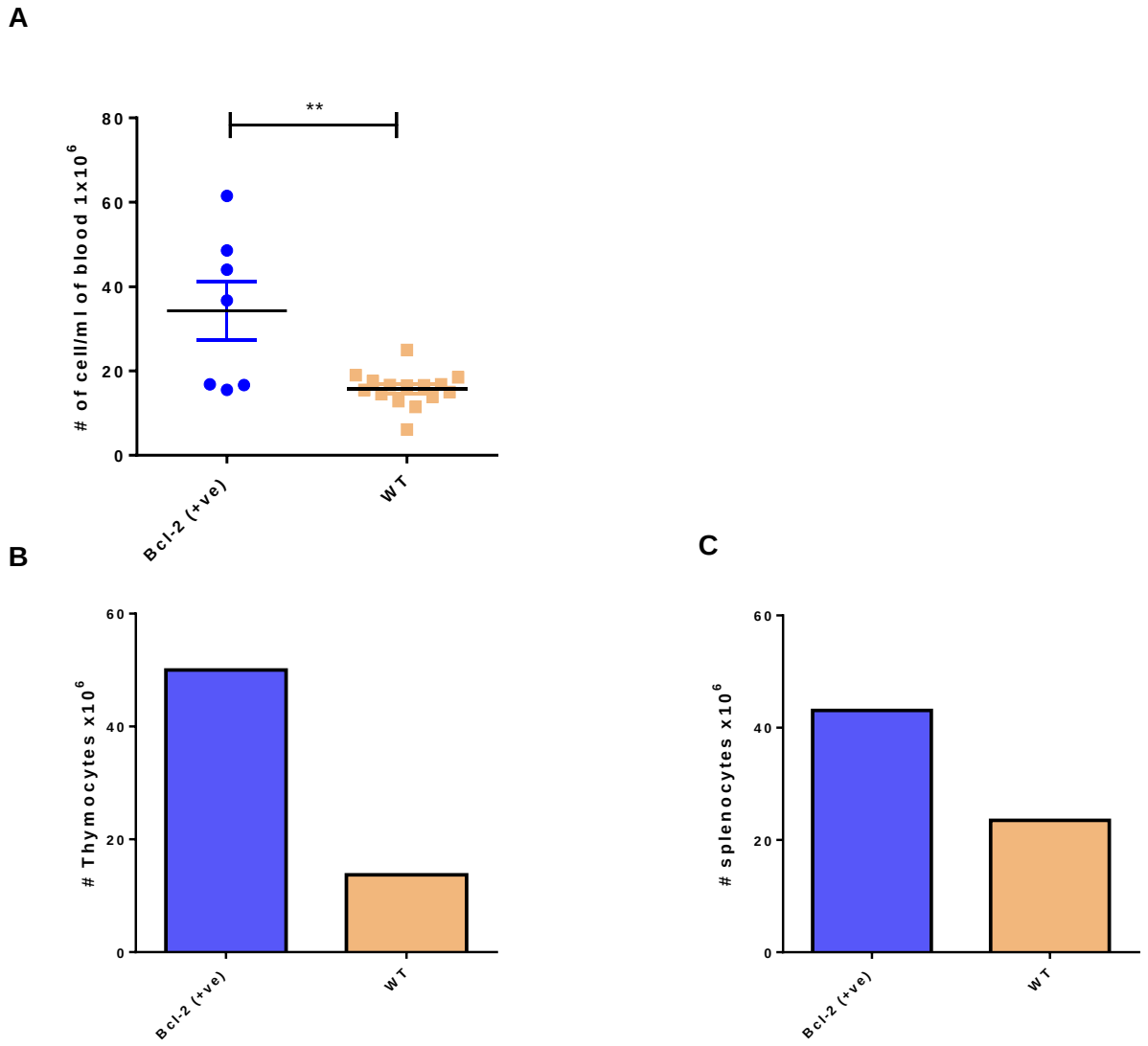


Figure 5-5: Total blood cell count (A), total number of thymocytes (B), and total number of splenocytes (C) in one pmel-1 Bcl-2^{+} and pmel-1 Bcl-2^{-} mice. One mouse was used to count splenocytes and thymocytes.

No difference was noticed in body weight between WT pmel-1 mice and BCL-2^{+} pmel-1 mice (Fig 5-6).

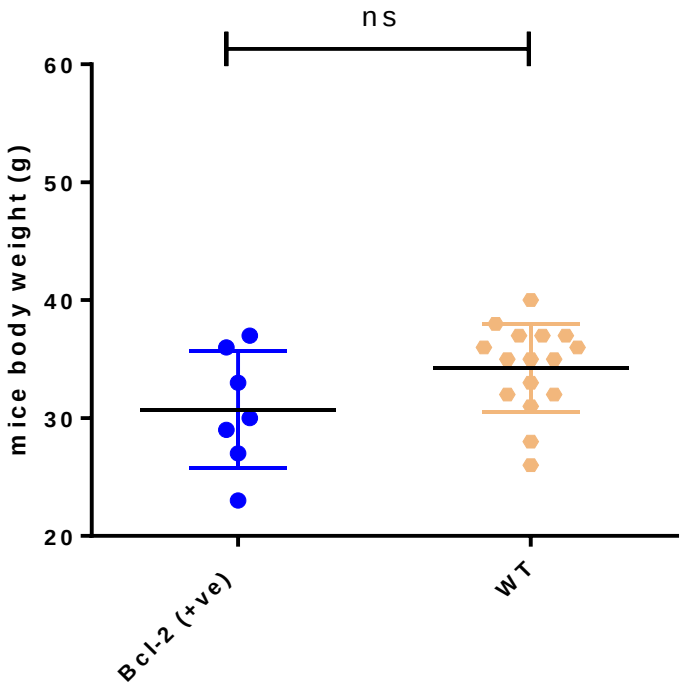


Figure 5-6: Shows mice body weight of pmel-1 Bcl-2^{+} and Bcl-2^{-} pmel-1 mice. Mice were all age and sex matched. Dots represent individual mice and are compared with littermates. Bars represent MEAN+/-SEM.

To investigate the effect of the Bcl-2 oncogene on pmel-1 CD8^{+} T cell survival, an equal number ($3x10^{5}$) of wild-type pmel-1 CD8^{+} T cells or pmel-1 Bcl-2^{+} CD8^{+} T cells was transferred into C57BL/6 recipient mice prior to stimulation with either hgp100 peptide alone or with anti-CD27 mAb. Transferred cells were monitored in the blood at different time points by staining with a commercially available dextramer and CD8.

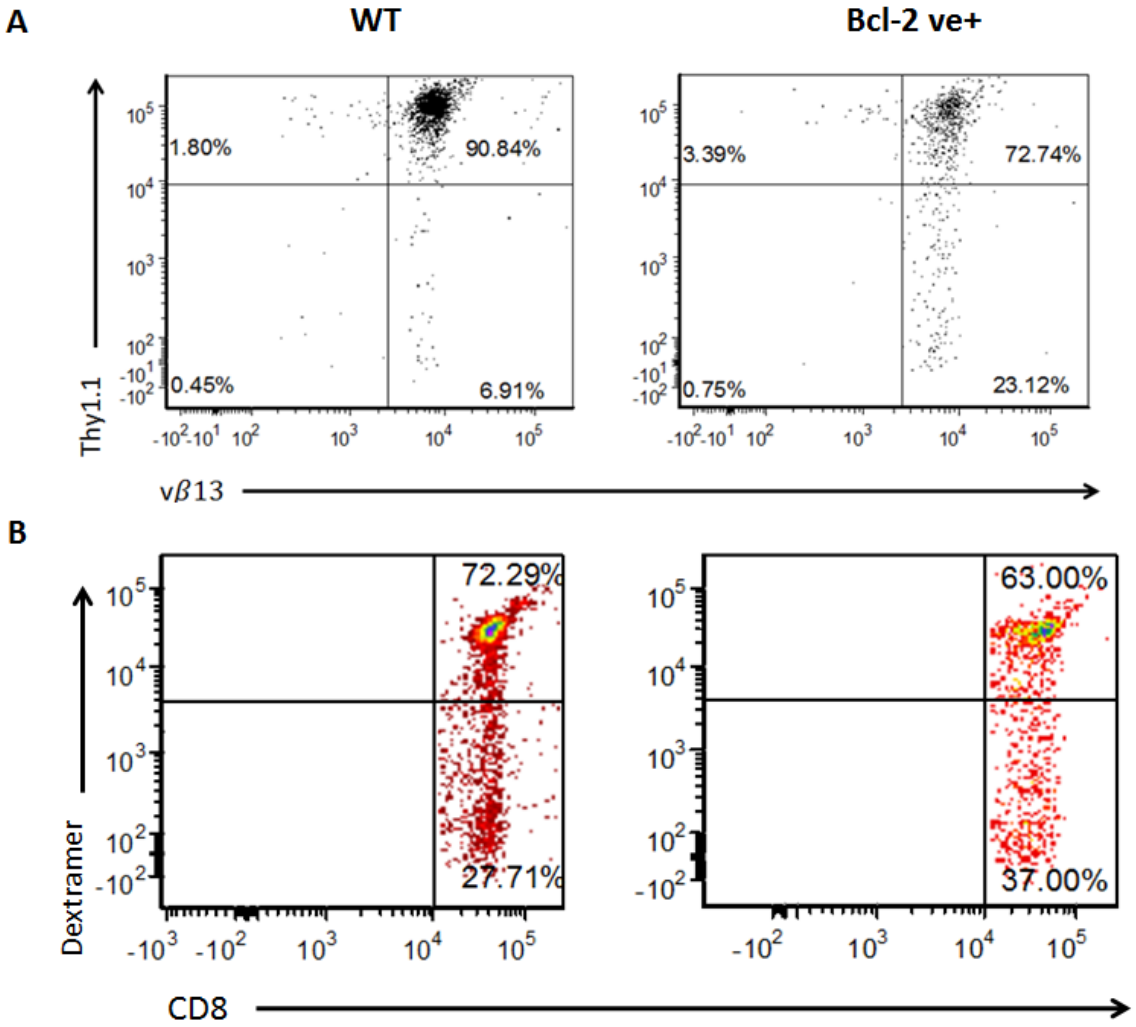


Figure 5-7: Percentage of Thy1.1+vβ+ cells out of total CD8+ T cells from blood (A). (B) shows the percentage of Dextramer+CD8+ (hgp100-specific CD8+ T cells). Pmel-1 mice were bled and blood was stained with antibodies against CD8, Thy1.1 and vβ13 (A) and with dextramer and CD8 in (B). Blood samples were lysed with lysis buffer, then washed with PBS/0.2%BSA and analysed by flow cytometry. Cells were gated based on forward and side scatter to approximate lymphocytes. Dextramer+CD8+ represent hgp100-specific $CD8^+$ T cells.

Immunizing mice with hgp100 peptide alone resulted in a similar primary expansion (~5% of $CD8^+$ T cells) of both WT pmel-1 and $Bcl\text{-}2^+$ pmel-1 $CD8^+$ T cells (Fig 5-8). However, pmel-1 cells that carry the Bcl-2 gene exhibited a slower contraction phase and persisted up to day ~37 post immunization (Fig 5-8). Including anti-CD27mAb within the vaccine increased (~2 fold) the magnitude of activated hgp100-specific $CD8^+$ T cells in both groups compared with peptide only (~17% of total $CD8^+$ T cells) (Fig 5-8). However, interestingly anti-CD27 reduced the persistence of Bcl-2 pmel1 CD8+ T cells after priming.

The similarity in the peak of the response in activated WT and Bcl-2^{+} pmel-1 cells allowed us to directly compare and study the establishment of memory independent of the magnitude of the primary response. To test the secondary response, mice were re-stimulated with hgp100 peptide and anti-CD27 mAb. Notably, adoptively-transferred cells did not significantly expand upon re-stimulating with peptide and anti-CD27 mAb in any group, despite the high frequency of persisting Bcl-2^{+} pmel-1 T cells on day 37 post initial activation (Fig 5-8). These results indicate that Bcl-2 delays contraction of pmel-1 CD8^{+} T cells. It also suggests that anti-CD27 abrogates the prosurvival effect of Bcl-2, and finally Bcl-2 does not fully rescue the defective secondary expansion of pmel-1 T cells *in vivo*.

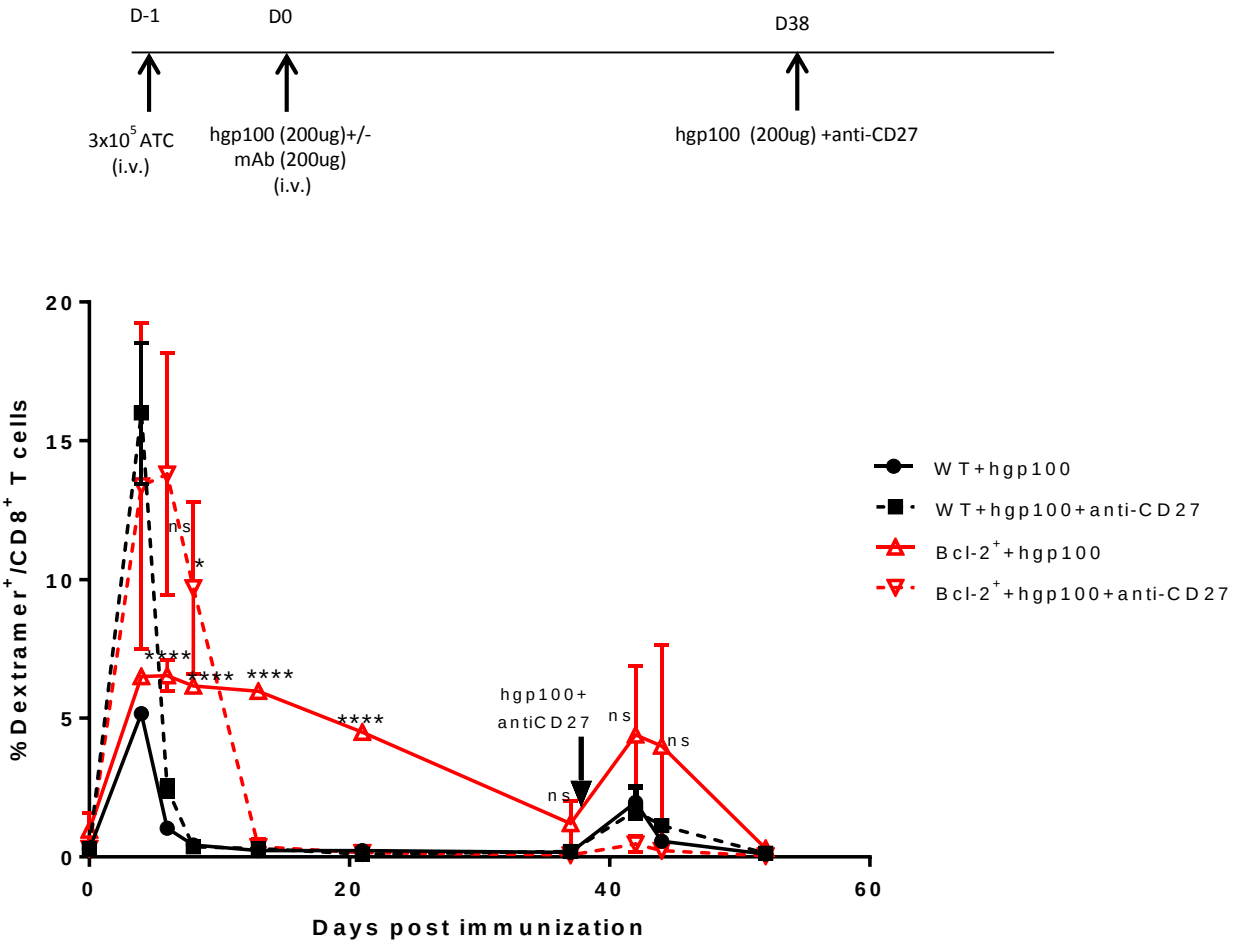


Figure 5-8: The influence of the Bcl-2 anti-apoptotic protein on the survival of pmel-1 CD8⁺ T cells. C57BL/6 mice received equal numbers ($3x10^5$) of wild-type pmel-1 CD8⁺ T cells or pmel-1 Bcl-2⁺ CD8⁺ T cells i.v. on day -1. Mice were then immunized with hgp100 (200ug) alone or with anti-CD27 mAb (200ug) i.v. on day 0. For the secondary response, mice were re-challenged with hgp100 peptide (200ug) plus anti-CD27 mAb on day 38 (indicated by the arrow). Cells were then tracked in the blood by staining for dextramer⁺CD8⁺ T cells. Data show group means +/-SEM. One way ANOVA *P=0.0391 comparing Bcl-2⁺+hgp100+anti-CD27 response with WT+hgp100+anti-CD27, ***P=0.0006 comparing Bcl-2⁺+hgp100 with WT+hgp100 on day 6 post immunization, ****P < 0.0001 comparing Bcl-2⁺+hgp100 with WT+hgp100 on days 8,13 and 21 post immunization. Student's *t* test **P=0.0391* comparing Bcl-2⁺+hgp100+anti-CD27 response with WT+hgp100+anti-CD27, ***P=0.0006 comparing Bcl-2⁺+hgp100 with WT+hgp100 on day 6 post immunization, ****P<0.0001 comparing Bcl-2⁺+hgp100 with WT+hgp100 on days 8, 13 and 21 post immunization. Data show mean+/-SEM of groups.

5.6. OT-1 CD8$^+$ T cells are more sensitive to cognate peptide than pmel-1 CD8$^+$ T cells

OT-1 transgenic mice are on a C57BL/6 background and are designed to recognise ovalbumin residues 257-264 in the context of H-2kB. Previous reports from our laboratory and others have shown that T cells expressing a receptor specific for OVA (OT-1) differentiate into memory cells (Willoughby et al., 2014). Yet, my data suggest that pmel-1 CD8$^+$ T cells seldom do so (Figs 3-2, 3-3, 3-5, 3-6, 3-7, 3-9 and 3-10). TCR binding affinity to peptide can affect the formation of memory T cells (Baumgartner et al., 2012, Corse et al., 2011, Knudson et al., 2013b). Therefore, I performed a proliferation assay *in vitro* to compare the TCR/MHC-peptide binding affinity of OT-1 with pmel-1 cells. 2x10^5 splenocytes from OT-1 or pmel-1 mice were incubated with different peptide concentrations (hgp100 in the case of pmel-1, or SII**N**FEKL peptide for OT-1 T cells) starting from a high peptide concentration of 10uM. Cells were incubated for 72hrs with thymidine added for the last 16 hours. Supernatants were collected after 48hrs to measure cytokine production. Proliferation assays confirmed that OT-1 cells have greater binding affinity compared with pmel-1 cells for their respective peptides (Fig 5-9A). OT-1 CD8$^+$ T cells were ~1000 fold more sensitive to peptide than pmel-1 CD8$^+$ T cells (Fig 5-9A). Kd (peptide concentration that binds to half the receptor sites) for pmel-1 T cells was 4.8x10^{-8}M compared with 2x10^{-11}M for OT-1 cells. Cytokine (IL-2 and IFN-γ) production by OT-1 and pmel-1 T cells after activation with a range of peptide corroborated the findings obtained with the proliferation assay (Figs 5-9B and C). At a peptide concentration of 10^{-8} M, IL-2 production by OT-1 CD8$^+$ T cells was 11170.5pg/ml (which recorded the highest amount of IL-2 produced by OT-1) compared with ~900pg by the pmel-1 CD8$^+$ T cells (Fig 5-9B). INF-γ secreted by OT-1 T cells was also higher compared with pmel-1 T cells (Fig 5-9C). Together, these data indicate that the OT-1 T cells are more sensitive to cognate peptide compared with the pmel-1 T cells.

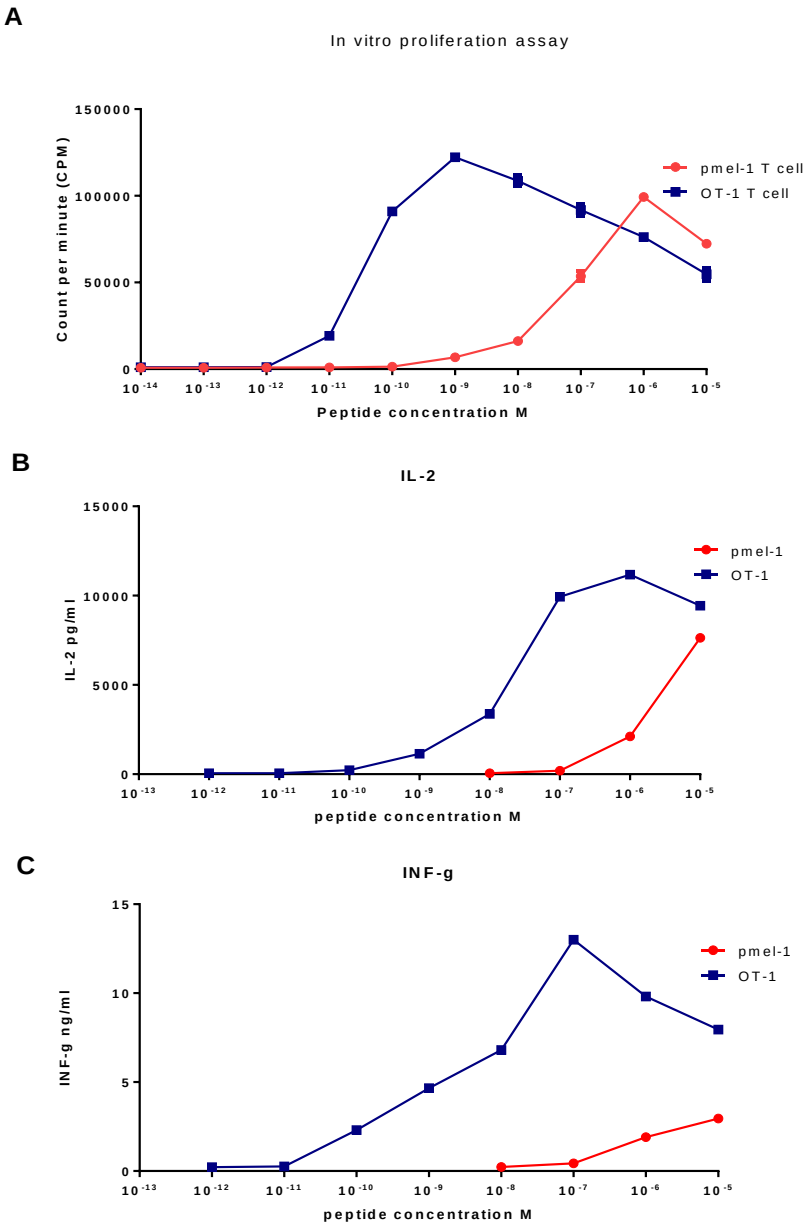


Figure 5-9: Comparison of OT-1 and pmel1 CD8+ T cell peptide affinity in vitro. 2x10^5 splenocytes from OT-1 or pmel-1 mice were incubated with or without peptide (SIINFEKL peptide for OT-1 cells and hgp100 peptide for the pmel-1 cells) at the concentrations indicated in triplicate. For the negative control splenocytes were incubated with the highest concentration (10 uM) of the opposite peptide. Supernatant (50ul) was collected after 48hrs incubation for cytokine analysis, just before adding [3H]thymidine at 1 uCi/well in 100ul/well and incubation for a further 18 hours. (A) Cells were harvested and proliferation was detected by the number of count per minute (CPM). (B) Shows IL-2 production detected by ELISA and compared with a standard curve of known concentration (C) Shows INF-γ secretion by pmel-1 and OT-1 cells, also using a calibrated ELISA. Data show group means+/-SEM. This experiment was performed twice with similar results.

5.7. The extent of Ag-specific CD8+ T cell expansion is associated with the strength of TCR-pMHC signalling.

To directly test if peptide affinity influences the formation of memory T cell precursors, I compared different peptide ligands that only differ in the fourth amino acid of the peptide sequence (SIINFEKL, SIIQFEKL and SIITFEKL) in the OT-1 $CD8^+$ TCR transgenic T cell system. These peptides have equal binding affinity to H-$2K^b$ but differ in their activation of OT-1 cells as measured by their ability to cause release of IFN-γ (Zehn et al., 2009); OT-1 $CD8^+$ T cells are ~18 times less sensitive to SIIQFEKL and ~70 times les sensitive to SIITFEKL compared with SIINFEKL (Zehn et al., 2009). Our data confirm that the OT-1 TCR is more sensitive (~100 fold) to SIINFEKL compared with SIIQFEKL (Fig 5-10). SIITFEKL in contrast showed activation only with the highest peptide concentration (10uM) (Fig 5-10). INF-γ production was higher after 48 hours incubation of OT-1 cells with SIINFEKL compared with SIIQFEKL (~20 higher) (Fig 5-10B) IFN-γ was too low to be detected in the case of SIITFEKL (Fig 5-10). These data confirm that SIINFEKL is a more potent inducer of OT-1 $CD8^+$ T cell proliferation compared to SIIQFEKL or the weakest peptide SIITFEKL.

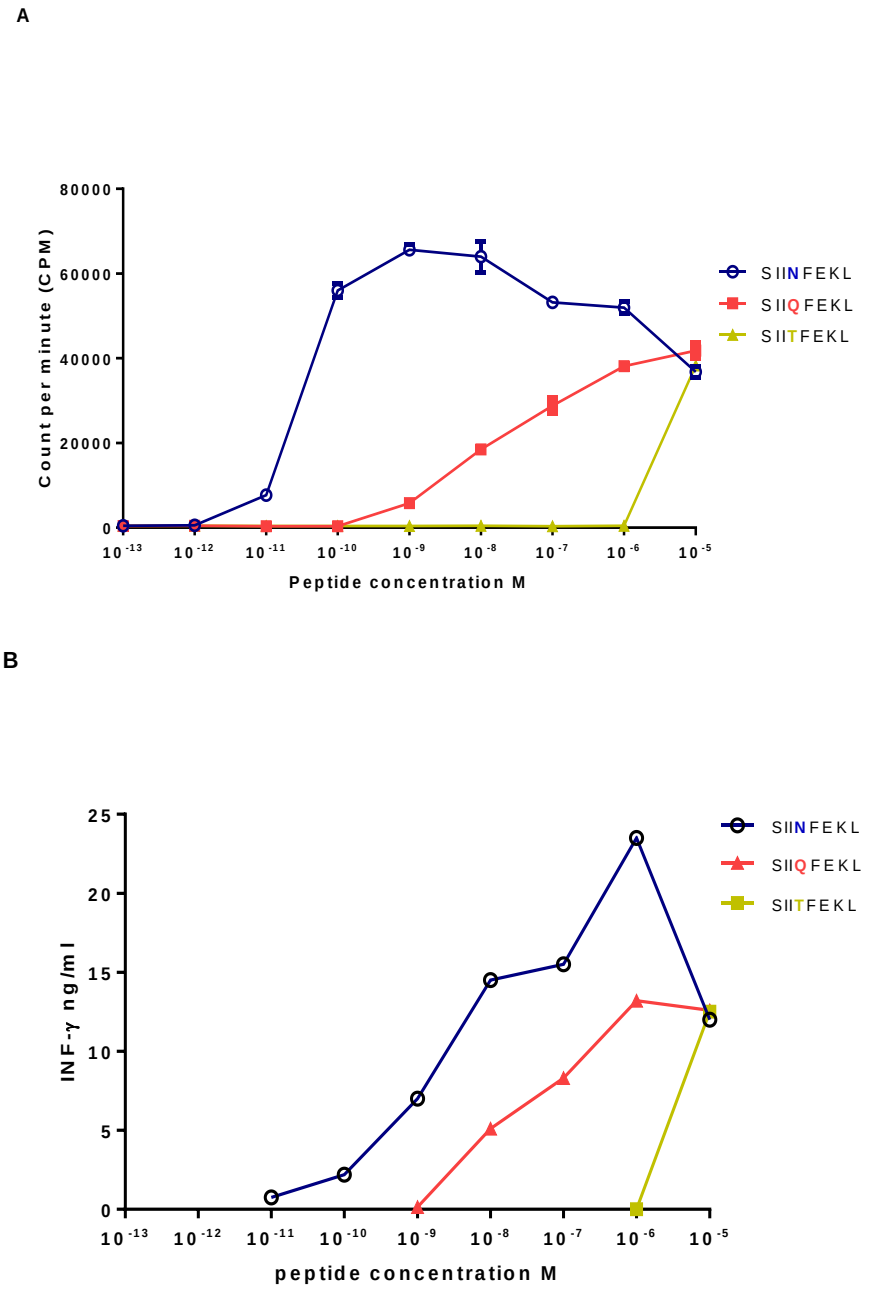


Figure 5-10: Assessment of the stimulatory potency of SIINFEKL variant peptides for OT-1 CD8+ T cell proliferation and cytokine production. 1x10^5 splenocytes from OT-1 TCR transgenic mice were incubated with different concentrations of SIINFEKL, SIIQFEKL or SIITFEKL peptides in triplicate. For the negative control splenocytes were incubated with a high concentration (10 uM) of irrelevant peptide hgp100. Supernatants (50ul) were collected after 48hrs just before adding [3H]thymidine at 1 uCi in 100ul/well and further incubatation for 18 hours. OT-1 cell proliferation was measured by uptake of [3H]thymidine (A). (B) Shows IFN-γ production by splenocytes measured by ELISA. Data show mean+/-SEM of triplicate wells.

5.8. *In vivo* clonal expansion of Ag-specific CD8$^+$ T cell is influenced by Ag affinity.

Several studies have shown that antigen affinity and density can determine CD8$^+$ memory fate decisions (Henrickson et al., 2013, Leignadier and Labrecque, 2010, Zehn et al., 2009). Zehn and collegues have investigated the correlation between Ag binding affinity and CD8$^+$ T cell differentiation into memory T cells. *Listeria monocytogenes* was modified to express different altered peptide ligands (APLs) to ensure that ligands are presented to Ag-specific CD8$^+$ T cells in a physiological context during an infection. Results revealed that differentiation into memory cells was not affected by Ag binding affinity during priming, as was measured by the magnitude of the secondary response after re-stimulation. However, immunization with weaker TCR ligand resulted in earlier T cell contraction compared to higher affinity peptides (Zehn et al., 2009). Injecting mice with *Listeria* can induce inflammatory responses. There is evidence suggests that inflammation such as IFN-γ can influence T cell primary and secondary response (Stoycheva et al., 2015, Zenewicz and Shen, 2007).

In another study, DCs were pulsed *in vitro* with peptides for 3hrs (to generate DC expressing a low density of peptide) or overnight (to express high peptide density) prior to injection into C57BL/6 recipient mice to compare the effect of immunizing with low and high densities of peptides in the formation of memory T cells. The magnitude of the effector CD8$^+$ T cell response was similar after activation with low or high peptide density. However the number of memory CD8$^+$ T cells was significantly reduced in response to low peptide density (Leignadier and Labrecque, 2010).

Thus, to compare the impact of activation with each APL on the Ag-specific CD8$^+$ T cell response. OT-1 cells (1x10^4) were transferred into C57BL/6 recipient mice (I.v.) on day -1. On day 0, mice were immunized with a single dose (100ug) of different peptide ligands (SII**N**FEKL, SII**Q**FEKL or SII**T**FEKL) with or without anti-CD27 mAb. Some mice received another peptide injection on day 1 (200ug) to compare the effect of injecting low and high peptide dose in the generation of memory cells. OT-1 T cell frequency was monitored in blood by staining with tetramer and anti-CD8.

Immunizing mice with 100ug APL alone, including the highest affinity peptide SIINFEKL, was insufficient to detect OT-1 T cell expansion (Fig 5-11A). When combined with anti-CD27 mAb immunization with the weakest peptide stimulus SIITFEKL resulted in only ~0.5% of total $CD8^+$ T cells being antigen-specific $CD8^+$ T cells at the peak of the response (day 4 post immunization) and cells contracted to background levels by day 8 (Fig 5-11A). Injecting mice with SIIQFEKL and anti-CD27 mAb increased OT-1 T cell expansion compared with SIITFEKL and anti-CD27 mAb to around ~8% of total $CD8^+$ T cells before they became undetectable in the blood by day ~15 post immunization (Fig 5-11A). Mice stimulated with SIINFEKL and anti-CD27 mAb further increased OT-1 T cell proliferation (~2.7-fold increase compared with SIIQFEKL and anti-CD27) at the peak of the response (Fig 5-11A). However, OT-1 T cells remained detectable in blood on day 33 post vaccination with SIINFEKL and anti-CD27mAb (Fig 5-11A).

Increasing the antigen dose to 200ug resulted in only limited expansion of OT-1 T cells in the absence of anti-CD27 mAb, (Fig 5-11B). However, OT-1 T cells expanded massively when stimulated with 200ug SIIQFEKL and anti-CD27 mAb compared to when immunized with 100ug resulting in ~2.3 fold increase with the 200ug dose compared to the equivalent 100ug dose (P=0.0301 using one tailed Students t-test) at the peak of the response (day 6 post immunization) (Figs 5-11A and B). As before (Fig 5-10A) the greatest stimulation was seen with SIINFEKL and anti-CD27 mAb although this was not increased compared to when 100ug peptide was used (P= 0.6517) (Figs 5-11A and B).

I did note that OT-1 T cell responses peaked later, at day 8 post immunization, after 200ug immunisation, compared to day 6 when mice were injected with 100ug of the same peptide (compare Figs 5-11A and B). Of note, increasing the SIIQFEKL dose to 200ug induced an OT-1 response showing similar response kinetic and magnitude to that seen after one injection of 100ug SIINFEKL (Fig 5-11C). However, in contrast to the 100ug dose of SIINFEKL, OT-1 T cells did not persist longer than 30 days post immunization after treatment with 200ug SIIQFEKL (Figs 5-11 B and C).

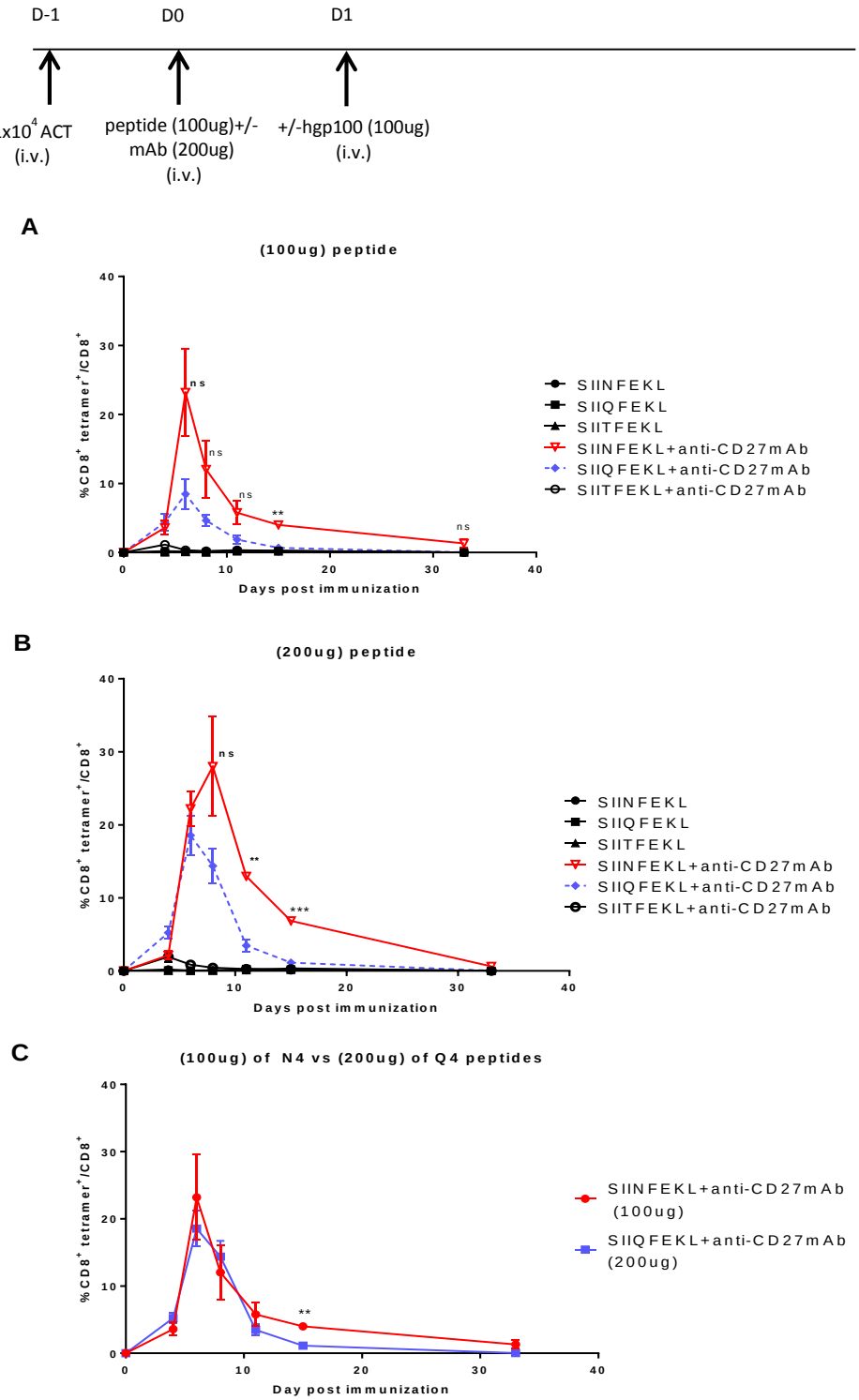


Figure 5-11: Immunization of OT-1 cells with different altered peptide ligands of SIINFEKL alone or with anti-CD27. C57BL/6 mice (n=4) received 1x10^4 OT-1 CD8+ T cells (i.v.) on day -1. On day 0, mice were immunized with SIINFEKL, SIITFEKL or SIIQFEKL either (A) once (100ug),or (B) twice (100ug day 0 and 100ug day 1). In each case mice received peptide alone, or with one injection of anti-CD27mAb 200ug on day 0 (i.v.). (C) Shows data taken from the same experiment to clearly show the kinetics and magnitude of the response after immunisation with 100ug of SIINFEKL and 200ug of SIIQFEKL, delivered with anti-CD27 in each case. OT-1 cells were tracked in the blood by staining for tetramer andCD8. Data show mean +/- SEM for each group. **P= 0.0032 comparing SIINFEKL (200ug)+anti-CD27 with SIIQFEKL (200ug)+anti-CD27 on day 11, ***P=.0005 on day 15 post immunization.

5.9. Ag binding affinity to the TCR and density does not influence the rececall response

In a new experiment, I investigated the ability of APL-stimulated OT-1 $CD8^+$ T cells to induce a secondary response upon Ag re-encounter. Another group was included in this experiment to test the effect of immunizing mice with a very low dose (20ug) of the highly potent SII**N**FEKL peptide. In this experiment all peptides were delivered with anti-CD27 mAb since the previous experiment showed this to be important for OT-1 $CD8^+$ T cell expansion. Injecting mice with 100ug resulted in 23% of the $CD8^+$ T cells being Ag-specific at the peak of the response (Fig 5-12A). While increasing peptide dose to 200ug delayed the peak of the OT-1 T cell primary response to day 8 post immunization and increased the primary response of the transferred OT-1 significantly compared with lower peptide dose (100ug) (39% out total $CD8^+$ T cells compared with 23% respectively) (Fig 5-12A).

Interestingly, decreasing the amount of SII**N**FEKL to 20ug induced maximal OT-1 T cell responses (~18% of the $CD8^+$ T cells) at day 6 post injection, similar to when mice were immunized with 100ug of the same peptide (23% of the CD8+ T cells) (Fig 5-12A).

The contraction phase however was less pronounced when SII**N**FEKL peptide dose was reduced to 20ug compared with higher doses (100ug and 200ug) of the same peptide resulting in 39.3%, 12.26% and 11.82% being antigen-specific $CD8^+$ T cells at a late time point compared with the maximal peak of the response, respectively (Fig 5-12A).

Similar to the first experiment (Fig 5-11A and B), stimulating OT-1 cells with 100ug SII**Q**FEKL peptide plus anti-CD27 mAb increased OT-1 proliferation (12.8% of the $CD8^+$ T cells at the peak of the response) compared with 8% in (Fig 5-12). The magnitude of the primary response of OT-1 cells was further increased after two injections of SII**Q**FEKL compared with a single injection (37.45% of the $CD8^+$ T cells and 12.8% respectively) (Fig 5-12B). The peak of the response in mice injected with two injections of SII**Q**FEKL was significantly higher ($p=0.0418$) in this experiment (37.45%) compared with the response in (Fig 5-12) (18.55%). Nonetheless, the kinetics of the primary response and contraction were similar (Fig 5-11). SII**Q**FEKL-induced OT-1 T cells declined to baseline by day 20 post immunization either with low or high peptide dose (Fig 5-12B).

To examine secondary responses, all mice were re-stimulated with 30ug of SIINFEKL. OT-1 T cells initially primed with 20ug SIINFEKL gradually expanded (forming 27% of total $CD8^+$ T cells) upon re-stimulation before they started to contract by day 11 post re-challenge (Fig 5-12A). 100ug SIINFEKL-induced OT-1 T cells expanded similarly upon re-stimulation, resulting in 24% of the $CD8^+$ T cells being Ag-specific (Fig 5-12A). The magnitude of the secondary response when mice were primed with 200ug of SIINFEKL was also similar (17.2% of total $CD8^+$ T cells (Fig 5-12A). Ag-specific $CD8^+$ T cells in mice initially stimulated with different SIINFEKL concentrations contracted gradually after boosting and formed ~10% of the $CD8^+$ T cells by day 27 post re-stimulation (Fig 5-12A).

With regards to secondary response after priming with 100ug of SIIQFEKL, regardless of the very low frequency of persisted OT-1 $CD8^+$ T cells after the contraction, OT-1 T cells expanded upon Ag re-encounter similar to SIINFEKL-induced OT-1 T cells (to form 15.7% of the $CD8^+$ T cells at the peak of the secondary response) (Fig 5-12B). Likewise, OT-1 T cells in mice initially primed with 200ug of SIIQFEKL, expanded after re-stimulation to form 23.6% of the total $CD8^+$ T cells in the blood (Fig 5-12B).

Comparing the secondary responses after priming with SIINFEKL and SIIQFEKL, despite significant differences in the magnitude of the primary response and in the frequency of memory $CD8^+$ T cells after the contraction, re-stimulation with an Ag resulted in similar levels of peak secondary responses (Fig 5-12C). In addition, immunization with SIINFEKL generated more memory cells after the contraction compared with SIIQFEKL (Fig 5-12C). However, OT-1 T cell expanded similarly after re-stimulation (Fig 5-12C), suggesting that SIINFEKL might promote the generation of T_{EM} cells that do not expand very well following re-challenge.

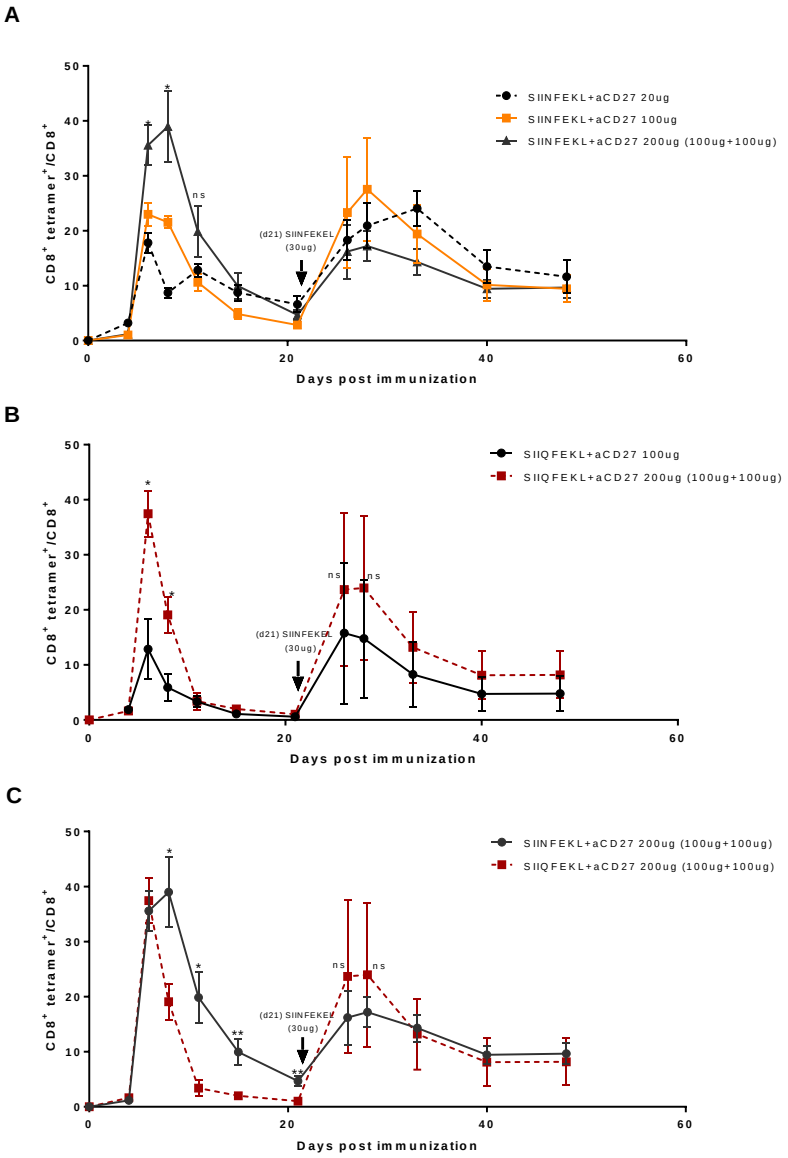


Figure 5-12: Investigating memory CD8+ T cell formation after priming OT-1 CD8+ T cells with different concentrations of SIINFEKL or SIIQFEKL. C57BL/6 mice (n=4) received 1x10^4 OT-1 cells (i.v.) on day -1. On day 0, mice were immunized with SIINFEKL (20ug or 100ug) in combination with anti-CD27 mAb (200ug), or with SIIQFEKL (100ug) plus anti-CD27 (200ug) (i.v.). On day 1, some mice received another injection of peptide (100ug) (i.v.). (A) shows the OT-1 responses in the blood after immunization with SIINFEKL given at different peptide concentrations. (B) shows the OT-1 responses in the blood after immunization with SIIQFEKL (100ug or with 200ug). (C) shows the same data sets as in (A) and (B) but directly compares OT-1 responses immunized anti-CD27 mAb and with high dose SIINFEKL or SIIQFEKL. OT-1 CD8+ T cells were tracked in the blood by staining for tetramer and CD8 T cells. Data show mean +/- SEM of groups. *p<0.05 comparing 100ug with 200ug of SIINFEKL (A), **P<0.005, ns=comparing 200ug of SIINFEKL with 100ug on day 11 post first peptide injection. (B) *p<0.05 comparing OT-1 T cell response on days 6 and 8 post first peptide injection. (C) *p<0.05 and **p<0.005 comparing Ag-specific CD8+ T cell response in the blood at different time points.

5.10. Strength of TCR signalling influences PD-1 expression on OT-1 T cells

In an independent experiment, I then examined PD-1 expression on OT-1 T cells upon stimulation with either SII**N**FEKL or SII**Q**FEKL peptides. My hypothesis was that strong TCR signalling may induces higher PD-1 expression compared to moderate or low TCR signals (Hokey et al., 2008, Greenwald et al., 2005), and that may therefore influence the magnitude of Ag-specific $CD8^+$ T cell response. To test this, OT-1 T cells ($1x10^4$) were adoptively transferred to C57BL/6 recipient mice on day -1. Mice were then immunized with SII**N**FEKL or SII**Q**FEKL alone or with anti-CD27 on day 0. PD-1 expression on OT-1 $CD8^+$ T cells was visualised at the peak of the response on day 6 from blood. Immunization with SII**N**FEKL peptide alone upregulated PD-1 expression on OT-1 $CD8^+$ T cells (Fig 5-13). However, PD-1 expression on SII**N**FEKL-induced OT-1 T cells was higher, but not significantly so, compared to SII**Q**FEKL-induced OT-1 T cell (Fig 5-13). Unlike pmel-1 $CD8^+$ T cells which don't show a further increase in PD-1 expression after anti-CD27 mAb treatment (Fig 3-8), administering anti-CD27 mAb further increased PD-1 expression on OT-1 $CD8^+$ T cells compared with peptide alone (Fig 5-13). Collectively, these results show that the levels of PD-1 expression expressed on the surface of $CD8^+$ T cells vary with the strength of TCR signals.

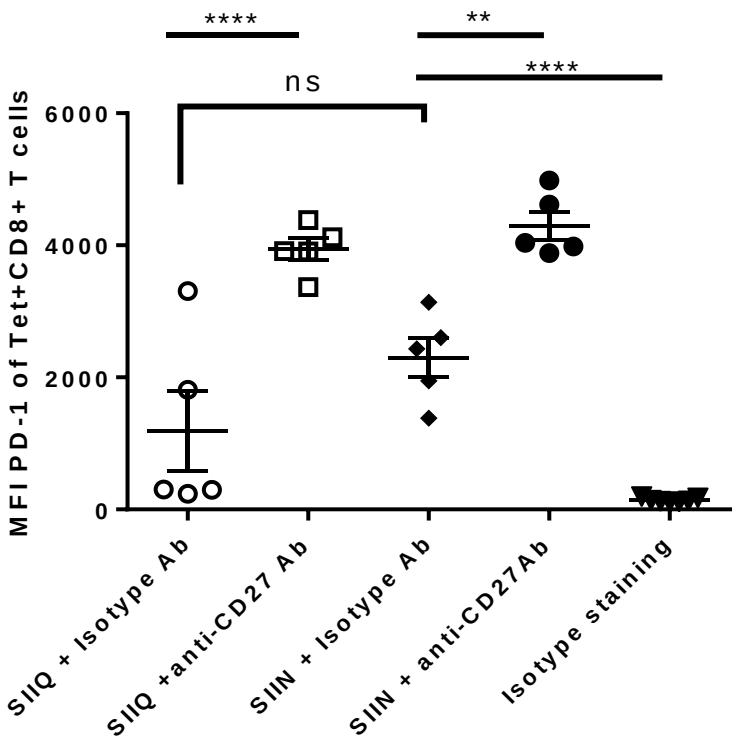


Figure 5-13: Levels of PD-1 co-inhibitory receptor expression on OT-1 CD8+ T cells after activation with SIINFEKL or SIIQFEKL peptides alone or with anti-CD27 mAb. C57BL/6 mice (n=5/ group) were inoculated with B16-BL6 melanoma ($2x10^5$) i.d. on day -6. On day -1, mice received a low number of OT-1 CD8+ T cells ($1x10^4$) i.v. then they were immunized either with 200ug of SIINFEKL or SIIQFEKL peptides in combination with an isotype control mAb, or anti-CD27. PD-1 expression was tested on circulating OT-1 CD8+ T cells 6 days post immunization. Blood cells were stained with anti-PD-1 or with an isotype control antibody (far right column. Data show the mean fluorescence intensity (MFI) after staining with anti-PD-1 or with an irrelevant isotype control antibody (far right group). Each dot represents one individual mouse with groups means represented by the horizontal line; SEM is indicated by the vertical bars. **p<0.001, ***p<0.001, one way ANOVA. Student's two-tailed t-test P<0.005 comparing SIINFEKL alone vs SIINFEKL+anti-CD27, P<0.0005 comparing SIIQFEKL vs SIIQFEKL with SIIQFEKL+anti-CD27.

5.11. The combination of anti-CD27 plus anti-PD-1/L1 mAbs vaccine has different effect on T cell response depending on peptide binding affinity

Since PD-1 expression levels varied between groups after peptide immunisation (Fig 5-13), I next evaluated the influence of combined stimulation through CD27 with anti-PD-1/PD-L1 pathway blockade on antigen-specific $CD8^+$ T cells primed either with SII**N**FEKL or SII**Q**FEKL peptide. A low number of OT-1 T cells ($1x10^4$) were transferred into C57BL/6 recipient mice on day -1. Then they were immunized the following day either with SII**N**FEKL or SII**Q**FEKL plus an isotype control, anti-CD27, a mix of anti-PD-1 and anti-PD-L1 or a combination of anti-CD27/PD-1/PD-L1. Blood samples from mice were harvested at the peak of the response (on day 6 post immunization) to track the OT-1 $CD8^+$ T cell response. The effect of the combination vaccine on the OT-1 $CD8^+$ T cell response was determined by measuring the fold increase in the primary response in the combination group and comparing this with the fold increase over peptide alone and after stimulation with each mAb alone.

OT-1 T cell response was measured at different time points throughout the experiment. Immunization with 100ug of SII**N**FEKL peptide alone did not trigger sufficient OT-1 T cell response (Fig 5-14A). While blocking both PD-1 receptor and the ligand (PD-L1) had a little effect on priming OT-1 T cells response (forming 8.26% Ag-specific CD8+ T cells out of the total $CD8^+$ T cells) (Fig 5-14A). Similar to (Figs 5-11A and 5-12A) targeting CD27 co-stimulatory receptor with mAb further induced OT-1 T cell response at the peak resulting in 43% of total $CD8^+$ T cells being Ag-specific as determined by tetramer staining (Fig 5-14A). The combination of PD-1/PD-L1 blockade with anti-CD27 mAb increased the accumulation of the antigen-specific $CD8^+$ T cells at the peak of the response (66.6% of total $CD8^+$ T cells were Ag-specific). The contraction of the ant-CD27-induced OT-1 T cells was more pronounced during this phase compared to anti-CD27+anti-PD-1/L1-induced OT-1 T cells (Fig 5-14A). To detect the ability of the activated OT-1 T cells to expand following re-encounter with an Ag. All mice were injected with SII**N**FEKL peptide alone on day 28 post first immunization. Results were similar to (Fig 5-12A), mice initially immunized with anti-CD27 mAb or with the combination OT-1 T cells expanded gradually after stimulation (Fig 5-14A). The secondary expansion of OT-1 T cells in mice initially immunized with PD-1/L1 mAb

was not as good as that activated with anti-CD27 or with the combination of anti-CD27 and anti-PD-1/PD-L1 (Fig 5-14).

In the SII**Q**FEKL arm, the kinetic response for SII**Q**FEKL-induced-OT-1 T cells was similar to pmel-1 T cell response (Fig 3-10A). Injecting SII**Q**FEKL peptide alone had no effect on OT-1 expansion (Fig 5-14B). In contrast to immunization with SII**N**FEKL peptide, blocking PD-1/L1 pathway did not induce OT-1 T cell expansion (Fig 5-14B). Injecting mice with anti-CD27 mAb alone drove OT-1 T cells to accumulate, resulting in 21.1% of total $CD8^+$ T cells being Ag-specific at the peak of the primary response (Fig 5-14B). Combing anti-CD27mAb with PD-1/L1 blockade increased the magnitude of OT-1 T cell response significantly at the peak on day 6 compared with anti-CD27 mAb alone (47% and 21.1% respectively) (Fig 5-14B). Like in (Fig 5-12B), SII**Q**FEKL-induced OT-1 T cells in all groups contracted drastically and were barely detectable by day 13 after they peaked (Fig 5-14B). However, apart from PD-1/L1 groups, OT-1 T cells expanded upon re-stimulation with SII**N**FEKL peptide alone (Fig 5-14B).

To detect the effect of immunizing mice with either SII**N**FEKL or SII**Q**FEKL peptides in the generation of memory T cells, I compared the frequency of the activated Ag-specific $CD8^+$ T cells on day 27 (contraction phase) out of the peak of the response on day 6 post immunization. Overall, More Ag-specific $CD8^+$ T cells were detected on day 27 when mice were immunized with SII**N**FEKL peptide compared with SII**Q**FEKL (Fig 5-14C). 13.2% were Ag-specific T cells out of the peak in response to anti-CD27 mAb immunization (Fig 5-14C). Moreover, the frequency of this T cell population was significantly higher at the same time point in the combination-treated mice compared to anti-CD27 alone (24.5% compared with 13.2% respectively) (Fig 5-14C). In contrast to SII**N**FEKL-immunised groups, combing anti-CD27 mAb with SII**Q**FEKL peptide resulted in 3.87% out of the peak of the response being Ag-specific $CD8^+$ T cells (Fig 5-14C). Interestingly, although the primary response of OT-1 T cells was significantly higher when immunizing with the combination compared to anti-CD27-activated OT-1 T cells (Fig 5-14B), the combination resulted in only 1.78% Ag-specific $CD8^+$ T cells out of the peak (Fig 5-14C).

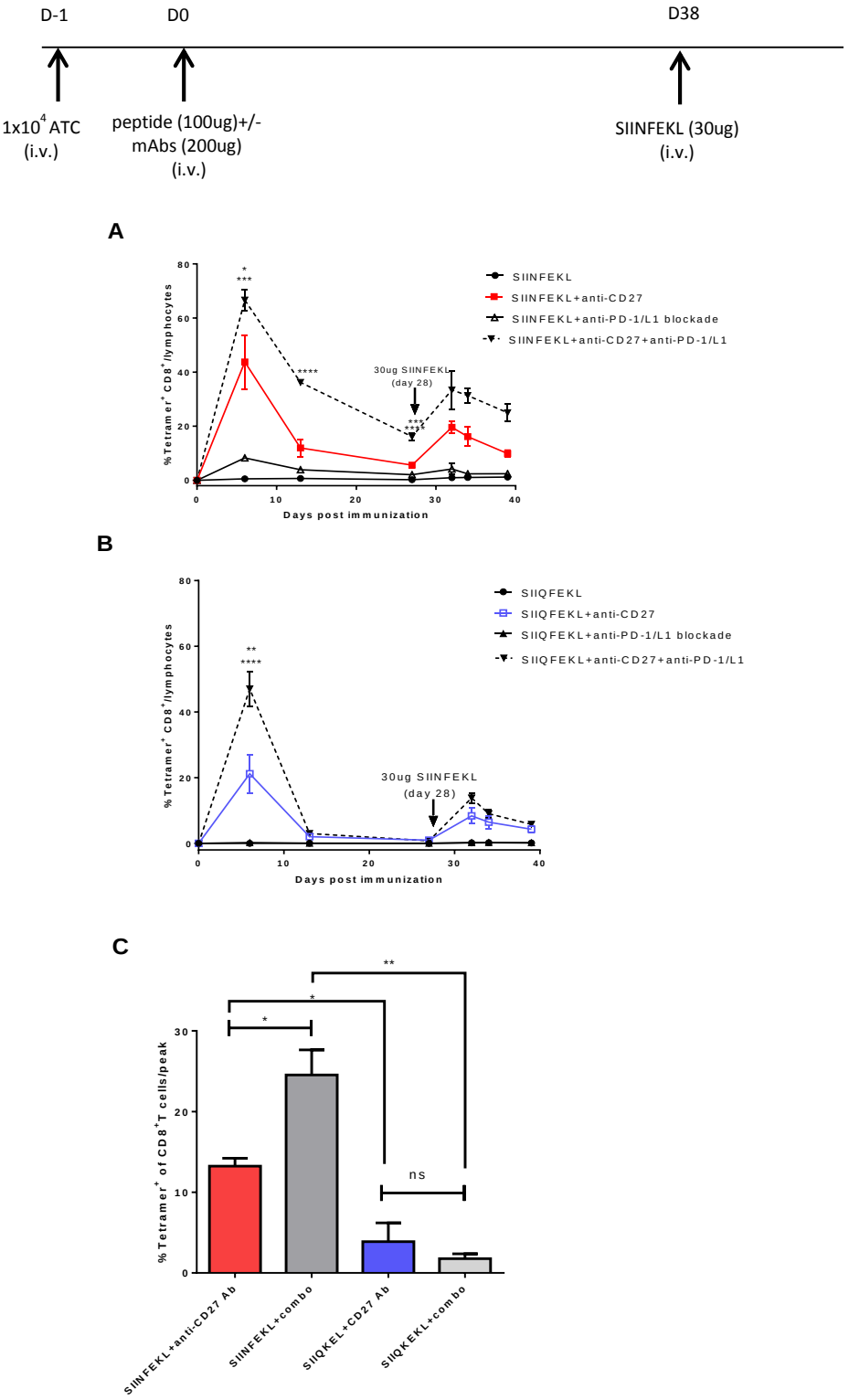


Figure 5-14: The combination of anti-CD27 and PD-1/L1 mAbs has different effect on OT-1 T cell response depending on peptide binding affinity. C57BL/6 mice (n=3) received 1x10^4 OT-1 cells (i.v.) on day -1. On day 0, mice were immunized with SIINFEKL or SIIQFEKL (100ug) in combination with 200ug of control mAbs, anti-CD27 mAb or anti-PD-1/L1 mAb or with the combination (i.v.). All mice were re-stimulated i.v. with 30ug of SIINFEKL on day 28 post priming. (A) shows kinetic response of OT-1 after immunization with SIINFEKL peptide (B) OT-1 T cell response in mice immunized with SIIQFEKL peptide. (C) shows the frequency of OT-1 CD8+ T cells out of the peak. Data show means +/- SEM of groups. (A) One way ANOVA *P=0.01 comparing the

combination with anti-CD27 and ***P<0.001 comparing the combination with anti-PD-1/anti-PD-L1 at the peak. ****P<0.0001 comparing the combination with single treatment on day 13, ***P<0.001 comparing the combination with anti-CD27, and ****P<0.0001 comparing the combination with anti-PD-1/anti-PD-L1. (B)One way ANOVA **P<0.01 comparing the combination with anti-CD27, ****P<0.0001 comparing the combination with either anti-PD-1/anti-PD-L1 alone or with the control. Student's *t* test ns=peak of the OT-1 T cell response comparing the combination with anti-CD27,**P<0.005 comparing the combination with anti-CD27 on days 13 and 27 post priming. (B) *p<0.05 comparing SIIQFEKL+combination with SIIQFEKL+anti-CD27.

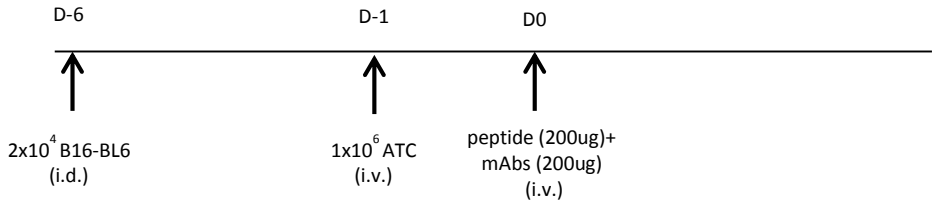
5.12. High and low peptide affinity resulted in a similar anti-tumour immunity

Next, I compared the quality of the anti-tumour response generated following immunization with the combination (anti-CD27 plus anti-PD-1/L1) with either SII**Q**FEKL (moderate affinity) or with SII**N**FEKL (high affinity) in mice bearing B16-OVA melanoma. In two independent experiments, C57BL/6 recipient mice (n=5) were injected with $2x10^5$ B16-OVA tumour cells i.d. (day -6). Five days later (day -1), mice received ($1x10^5$) OT-1 $CD8^+$ T cells i.v. to assess the Ag-specific tumour response and effector function. On day 0, mice were immunized either with SII**N**FEKL or SII**Q**FEKL peptide (200ug) in combination with an isotype control, anti-CD27, anti-PD-1/L1, or a combination of both antibodies (200ug total mAbs). On day 1, mice received another mAb injection (200ug).

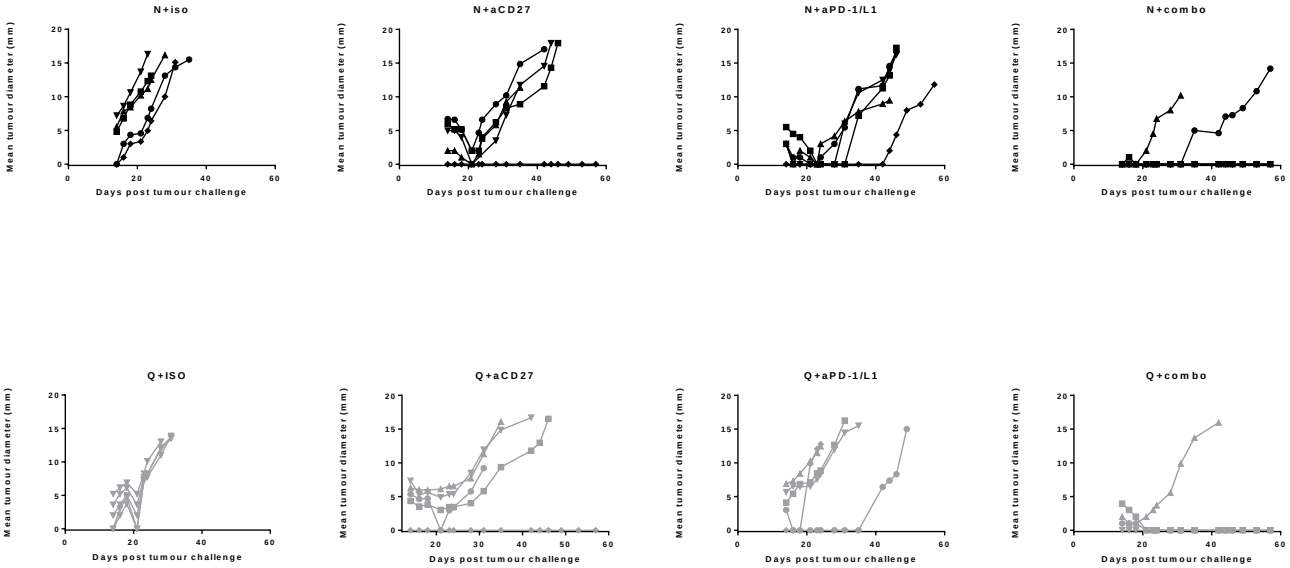
This experiment was performed twice; similar results were obtained in both experiments. Immunization with SII**N**FEKL peptide alone did not have any anti-tumour affect in mice bearing stablished B16-OVA (Fig 5-15A). Combing peptide vaccine with anti-CD27 mAb or with anti-PD-1/L1 blocking mAb had little effect in delaying tumour growth compared with peptide alone (Fig 5-15A), one mouse from anti-CD27-treated group was tumour free until the end of the experiment on day 100 after the tumour injection. Interestingly, combining the two mAbs generated strong anti-tumour immune responses, resulting in 3 out of 5 mice with complete tumour regression (Fig 5-15A).

In regards with mice survival, the control group exceeded end point of tumour growth by day ~37 post tumour challenge (Fig 5-15B). Blocking PD-1/L1 pathway slightly improved mice survival (up to day ~58 compared with ~37 in the control group) (Fig 5-15B), while anti-CD27mAb resulted in 1 out of 5 treated mice with complete tumour regression until the end of the experiment by day 100 post tumour inoculation (Fig 5-15B). Immunizing mice

with the combination enhanced mice survival significantly compared to monotherapy, resulting in 3 out of 5 mice with long-term survival (Fig 5-15B).



Mean tumour diameter (A)



Survival of mice (B)

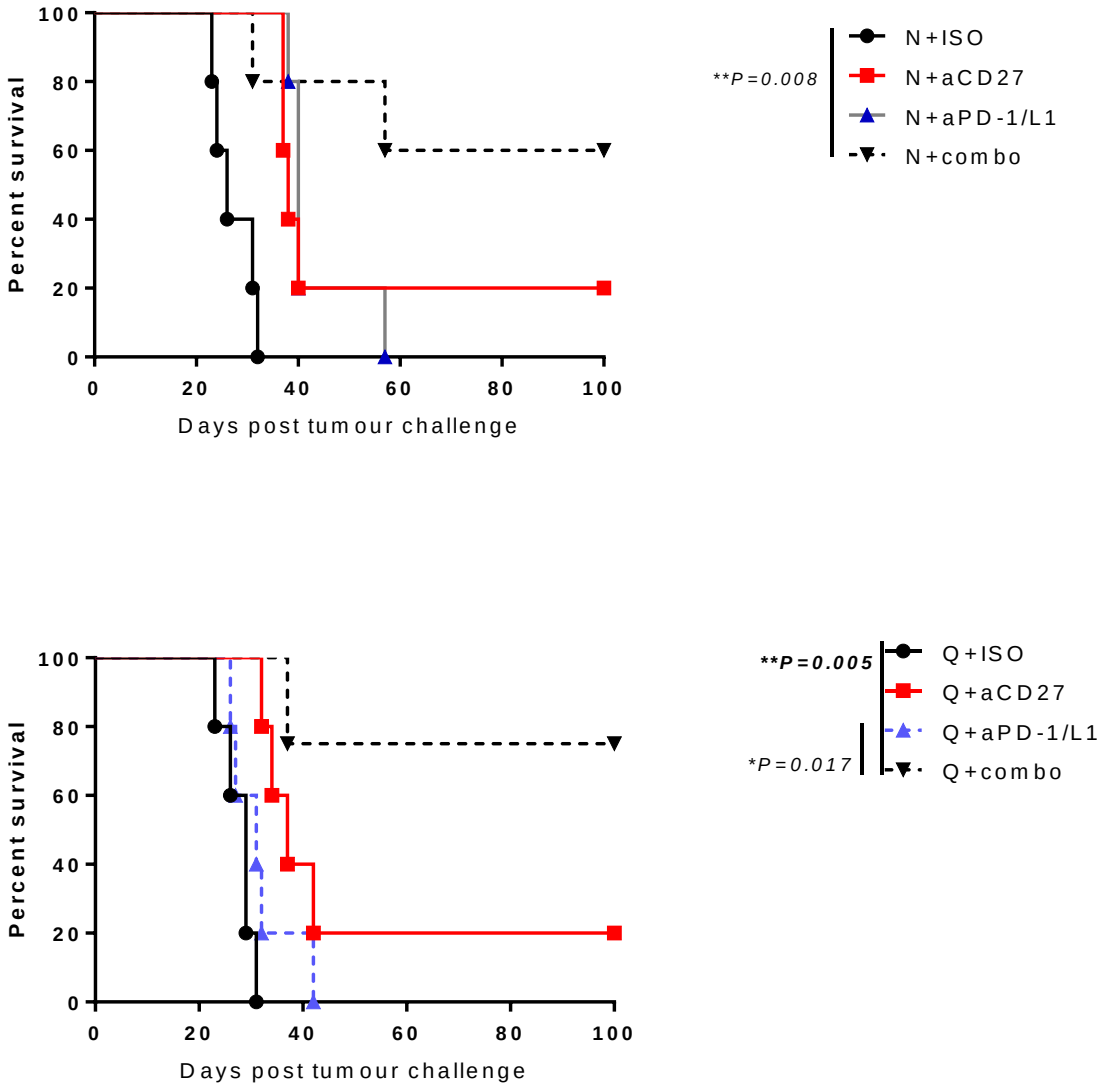


Figure 5-15: Anti-tumour immune response of mice following immunization with SIINFEKL peptide. C57BL/6 recipient mice (n=5/group) were injected with B16-OVA 2x10^5 i.d. on day -6, 1x10^^{5} OT-1 T cells were adoptively transferred i.v. to mice on day -1. Mice were then immunized i.v. by injecting SIINFEKL peptide (200ug) with an Isotype control mAb (clone MC106A5), anti-CD27 mAb (200ug), anti-PD-1 (200ug)/anti-PD-L1 (200ug) or combination of both. On day 1, mice received another mAbs injection (200ug) i.v. Tail bleed was performed to all groups on day 6 post immunization to analyse OT-1 T cell response and effector function. (A) shows tumour growth in mice (B) Survival of mice.

In the other arm of the experiment, mice were initially immunized with SII**Q**FEKL peptide with or without mAbs. Vaccinating mice with peptide alone did not have any effect in

reducing tumour growth, tumour continued to grow significantly in these mice (Fig 5-16A). Administering anti-CD27 mAb with the vaccine reduced tumour growth compared with peptide alone (Fig 5-15A). Similarly, blocking PD-1/L1 pathway delayed tumour growth compared with the untreated mice (Fig 5-15A). Interestingly, immunizing mice with the combination inhibited tumour growth remarkably compared to monotherapy (Fig 5-16A).

In terms of mice survival, in the first experiment, immunizing mice with SII**Q**FEKL peptide alone was insufficient in enhancing mice survival (Fig 5-15B). Administering of anti-CD27 mAb resulted in 1 out 5 mice with long-term survival (Fig 5-15B). Blocking PD-1/PD-L1 interaction had minimal affect in prolonging mice survival (day ~42 post tumour challenge compared with ~36 respectively). Mice immunized with the combination generated robust immunity against the stablished B16-OVA melanoma compared to when mice where immunized with peptide alone or with anti-PD-1/L1, such that 4 out of 5 mice were tumour free for up to day 100 post tumour challenge (Fig 5-15B).

Collectively, these results showed that despite the variety in peptide binding affinity, anti-tumour therapeutic effect following immunization with SII**N**FEKL or SII**Q**FEKL peptide was similar (Fig 5-15). Both peptides induced strong CTLs that can kill tumour cells effectively following immunization with anti-CD27 and anti-PD-1/L1 mAbs, and enhanced survival of mice (Fig 5-15). Immunizing mice with single mAb alone had little anti-tumour effect in both cases (Fig 5-15).

Ag-specific $CD8^+$ T cell response was also detected by conjugated-MHC-I tetramer and CD8 stain. Blood samples were collected from each mice group at different time points. Overall, the kinetic response of OT-1 T cells was similar to the primary response of OT-1 T cells (Figs 5-14). Immunizing mice with SII**N**FEKL generated higher OT-1 T responses and delayed contraction compared with SII**Q**FEKL (Fig 5-16).

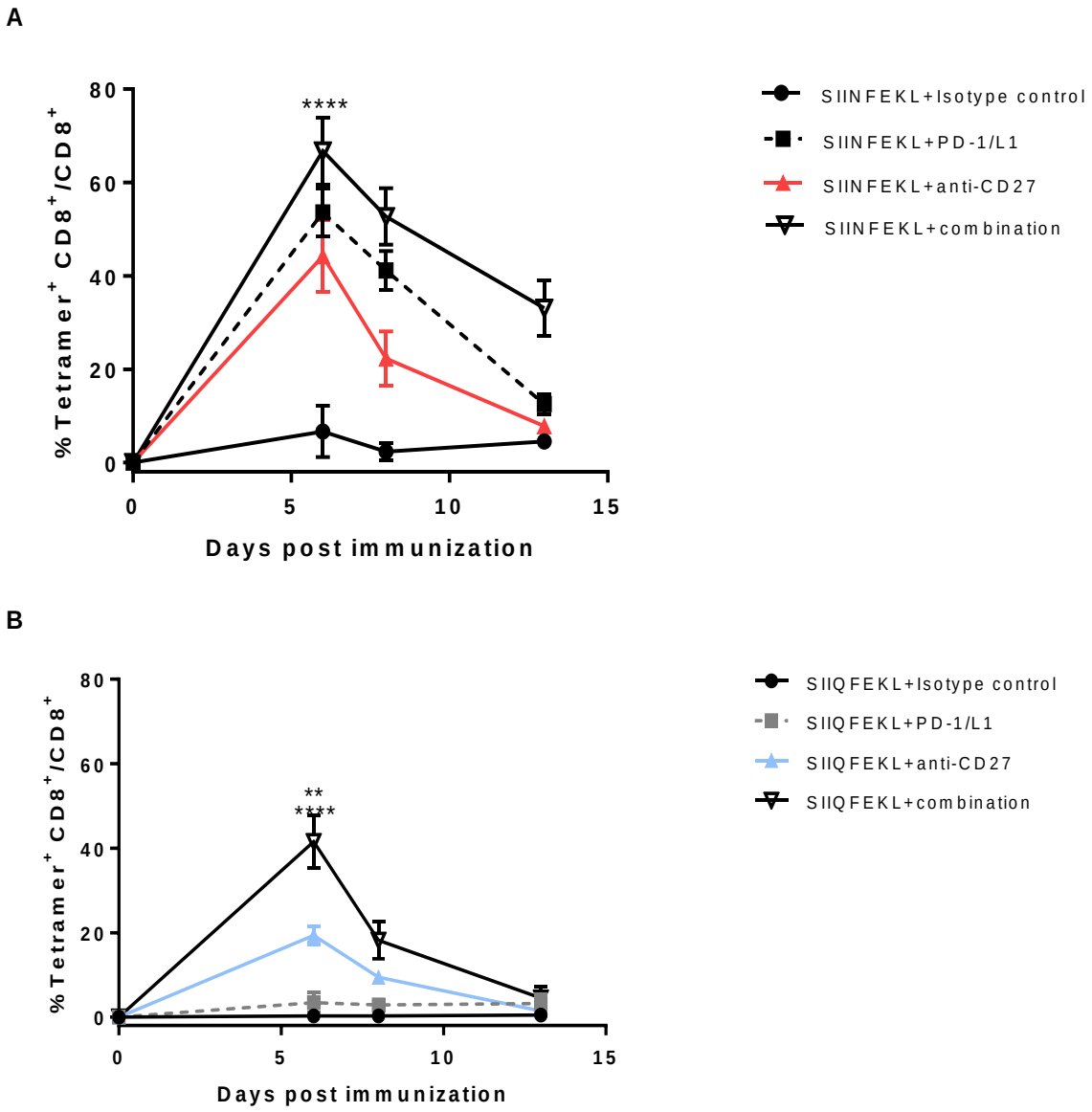


Figure 5-16: OT-1 T cell response from blood in mice bearing solid B16 melanoma following immunisation with different peptide ligands. OT-1 T cell response was detected from blood at different time points upon stimulation with SIINFEKL with or without mAb (A), or with SIIQFEKL peptide (B). Circulating OT-1 T cells were detected by conjugated-MHC-I tetramer and CD8 staining. Data show MEAN+/-SEM of triplicate wells. One way ANOVA for SIINFEKL. ****P<0.0001 comparing the combination with the control. For SIIQFEKL, **P<0.01 comparing the combination with anti-CD27. ****P<0.0001 comparing the combination with anti-PD-1/anti-PD-L1, and the combination with the isotype control.

5.13. Discussion

Successful T cell vaccine should induce high frequencies of CTLs and promote T cells differentiation into long-lived memory T cells to eliminate tumour cells and prevent tumour emergence. However, I noticed that pmel-1 cells did not persist after activation (Figs 3-3, 3-5, 3-6, 3-7 and 3-10), therefore, I tested different approaches like IL-2 injection, mTOR inhibition, and countering apoptosis by Bcl-2 overexpression, to improve pmel-1 T cells survival and promote their differentiation into memory T cells. These approaches will be discussed in detail later in the discussion.

IL-2 cytokine is critical for generating optimal primary and secondary responses respectively (Blattman et al., 2003, Boyman and Sprent, 2012). However, and in contrast to previous reports, IL-2 did not have any effect on priming or on prolonging survival of adoptively transferred pmel-1 T cell (Fig 5-1). In a study by Cho and colleagues using the pmel-1 T cell model, injecting multiple doses of IL-2 on days 2, 3, 5, and 7 after vaccination with TriVax (hgp100_{25} peptide, poly-IC and anti-CD40) sustained activated pmel-1 cells (~90% of total $CD8^+$ T cells) up to day 60 post immunization (Cho et al., 2012). In my study however, mice were given only two injections of IL-2, indicating that higher doses of IL-2 might be required to sustain pmel-1 cell survival. Létourneau S and others have shown that multiple injections are required for strong IL-2Cx activity (Letourneau et al., 2010). One limitation for administering high doses of IL-2 alone or in combination with adjuvants is toxicity. Cancer patients treated with high doses of IL-2 experienced nausea, diarr hoea, vomiting, and malaise (Rosenberg et al., 1989). Furthermore, Krieg C and others have shown that injection high doses of IL-2 reduced cutaneous tumour loads of B16F10 tumour or caused tumour regression in the lung , however, high IL-2 doses-treated mice developed vascular leak syndrome (VLS) (Krieg et al., 2010).

An alternative approach to IL-2 is IL-7. Previous studies have revealed that IL-7 cytokine is critical for naïve and memory T cell homeostasis (Bachmann et al., 2006). In the expansion phase, IL-7 receptor and the anti-apoptotic protein (Bcl-2) are down regulated in effector T cells, but not in memory cell pools (Nanjappa et al., 2008, Schluns et al., 2000). IL-7 administration during the early contraction phase (day 7-14 post infection with LCMV)

enhanced the number of memory $CD8^+$ T cells via the induction of the anti-apoptotic protein Bcl-2 (Kim et al., 2012, Harty and Badovinac, 2008, Youngblood et al., 2010). Hence, injecting IL-7 during the contraction phase in the pmel-1 system might be an alternative cytokine to promote survival of hgp100-specific $CD8^+$ T cells.

It is been shown that blocking mTOR enhances $CD8^+$ T cell survival (Maciolek et al., 2014, Araki et al., 2010). Using a LCMV model, partial mTOR blockade during the activation phase from day 0 to 8, or throughout the experiment (from day 0 to 35 post infection) increased the number of total memory cells significantly, while mTOR blocking during the contraction phase from day 8 to day 35 increased the number of T_{CM} like cells (Araki et al., 2009). However, in the pmel-1 system, injecting multiple doses of rapamycin (an mTOR inhibitor) either at a low dose ($75ug/kg^{-1}$) from day 0 or a high dose ($1500ug/kg^{-1}$) on day 4 post immunization did not improve pmel-1 cell survival (Fig 5-2). The lack of effect of rapamycin here, in contrast to LCMV model, could be related to the inflammation induced by LCMV. There is evidence suggests that inflammation such as INF-γ can influence T cell responses (Stoycheva et al., 2015). In addition, CD27 stimulation could upregulates the prosurvival pim1 gene expression (Peperzak et al., 2010a). Pim1 is a serine/threonine kinase that maintains CTL survival in a manner independent of mTOR (Walpen et al., 2012). Pim1 and mTOR have overlapping function and phosphorylate similar targets, which can partially substitute for each other (Walpen et al., 2012). Thus, pim1 might be overcoming the effect of mTOR inhibition by rapamycin (Fig 5-2). This may also explain the defect of rapamycin in inducing pmel-1 differentiation into memory cell.

Because the Bcl-2 anti-apoptotic gene increases cell survival, we generated pmel-1 cells that carry the Bcl-2 trans gene to address whether Bcl-2 expression would rescue them from apoptosis during the contraction phase. Of note, Bcl-2^+ pmel-1 cells exhibited prolonged survival during the contraction phase upon stimulation with peptide alone compared with WT pmel-1 cells (Fig 5-8). Stimulating CD27 on those cells resulted in a primary expansion level similar to the wild type pmel-1 T cells, but the initiation of the contraction phase was slightly delayed in Bcl-2^+ pmel-1 cells (Fig 5-8). Nonetheless, pmel-1 Bcl-2^+ T cells became undetectable in the blood by day 8 post immunization. Furthermore, they did not respond after re-challenge with peptide plus anti-CD27 (Fig 5-8). One possible reason for the loss of effector pmel-1 cells during the contraction phase could be that pmel-1 Bcl-2^+ cells undergo

apoptosis via the FAS/FASL pathway (Waring and Mullbacher, 1999). Another hypothesis is that activated pmel-1 cells might be expressing high levels of Nor-1, an intracellular transcription factor that has been reported to play a critical role in $CD8^+$ T cells apoptosis and negative selection in the thymus (Thompson and Winoto, 2008, Leignadier and Labrecque, 2010). Strong TCR signals translocate Nor-1 protein toward the mitochondria, where it binds to Bcl-2 exposing the BH3 domain. This converts Bcl-2 into a pro-apoptotic molecule, which then leads to release of the cytochrome *c* from the mitochondria and cell death (Leignadier and Labrecque, 2010, Thompson and Winoto, 2008).

Despite the interventions described above which sought to improve pmel-1 cell survival, rapid contraction was always observed after the peak of the response (Fig 5-1, 5-3 and 5-8). This could possibly be due to the hgp100 binding affinity to TCR. Signals generated from the interaction between the hgp100-MHC complex and TCR might be not sufficient to generate pmel-1 cells that have the potential to become memory cells. Several studies have shown that the strength of T cell receptor signalling determines naïve $CD8^+$ T cell fate (Teixeiro et al., 2009, Knudson et al., 2013b). In an *in vivo* experiment using genetically engineered OT-1 mice expressing a point mutation in the β-chain trans membrane domain (βTMD) which alters TCR signalling, cells expanded similarly to WT OT-1 cells, but completely contracted and failed to generate memory cell pools (Teixeiro et al., 2009, Knudson et al., 2013b). The percentage of mutant T cells polarizing their TCR in the immunological synapse was reduced in βTMD mutant compared with WT cells. Data also revealed that NF-kB activity in βTMD mutant TCR was impaired. Bevan *et al.* have also reported that OT-I TCR with lower binding affinity to cognate peptide had less CD25 (αchain of the IL-2 receptor) expressed on the cell surface compared to OT-1 TCR that bind with higher affinity (Boyman and Sprent, 2012). This may also explain the requirement for higher IL-2 dosage to prolong antigen-specific $CD8^+$ T cell (Akira et al., 2006, Boyman and Sprent, 2012, Boyman et al., 2006). However, Zhen et al. have shown that adoptively transferred OT-1 T cells into WT mice were able to differentiate into memory cells when they were immunized with Listeria encoding either the dominant ovalbumin SIINFEKL peptide or the ~70 fold less sensitive peptide SIITFEKL (Zehn et al., 2009). In contrast, data collected by von Andrian and colleagues revealed that Ag-specific $CD8^+$ T cells that interacted with DCs loaded with a low density of antigen (1µM peptide pulsed DCs) did not form memory cells compared with those primed by high density

antigen (100µM peptide-pulsed DCs) (Henrickson et al., 2013). Therefore, I aimed to study the role of TCR-peptide MHC-I binding affinity in generating memory cells.

In vivo, naïve T cells transiently contact DC (<30min); T cells with stable contacts (>30min) to pMHC move to phase 2 (∼12h) of the activation process, where they undergo full activation accompanied by cytokine production and up-regulation of activation markers such as CD44 and CD69. Phase 3, starts ∼ 1 day later as they leave the LN and is characterized by rapid clonal expansion (Henrickson et al., 2013). There is a vigorous debate about the relationship between effector and memory cells (Restifo and Gattinoni, 2013). The classical theory suggests that Ag-specific $CD8^+$ T cells become highly active, expand and produce high amounts of effector cytokines such TNF-α, INF-γ and GM-CSF (Restifo and Gattinoni, 2013). After they reach the peak of their response, the majority of activated cells die and only 5-10% survive to form the memory cell pool (Wherry et al., 2003). Another model of memory generation suggests that memory cells arise from naïve $CD8^+$ T cells in the early stages of T cell DC interaction. There is cumulative evidence indicating that the strength of TCR-pMHC signal plus pro-inflammatory cytokines such as IL-2 and IL-12 determines naïve $CD8^+$ T cell fate (Smith-Garvin et al., 2010, Corse et al., 2011). Effector and memory $CD8^+$ T cells can arise from the same precursor of naive T cell, via asymmetric division at the early stage after antigen stimulation. Evidence suggests that the proximal daughter cell which is closer to the APC becomes an effector cell, while the distal daughter cell (further from the APC) adopts a memory cell fate (Kaech and Cui, 2012).

To compare the sensitivity of OT-1 T cells with pmel1 T cells I first performed an *in vitro* proliferation assay. Interestingly, the results showed that the binding affinity of the OT-1 TCR to SIINFEKL-MHC-I is higher (∼3 log) than the binding affinity of the pmel-1 TCR for the hgp100-MHC-I complex (Fig 5-9); OT-1 cells are ∼1000 fold more sensitive to cognate peptide than pmel-1 cells (Fig 5-9).

Next, I performed *in vitro* and *in vivo* assays to study the role of Ag affinity in the generation of long-lived $CD8^+$ T cells. To test this, I used the OT-1 T cell model and activated T cells with SIINFEKL, SIIQFEKL and SIITFEKL. These APL bind similarly to H-2Kb, but the avidity to the OT-1 TCR is different (Zehn et al., 2009). The potency of these APLs to activate OT-1 cells was first tested *in vitro* by measuring OT-1 T cell proliferation and cytokine production. The

results confirmed that the OT-1 TCR is much more sensitive to the ovalbumin SIINFEKL peptide, followed by the SIIQFEKL peptide ($\sim$3 log lower), and the weakest peptide SIITFEKL (Fig 5-10A). INF-γ secreted in the media after 48h of activation with SIINFEKL was also higher compared to stimulation with SIIQFEKL and SIITFEKL (Fig 5-10B). *In vivo,* activating OT-1 cells with SIINFEKL, SIIQFEKL or SIITFEKL alone was insufficient to generate strong OT-1 responses; even with high doses of peptide a co-stimulatory signal from CD27 was crucial to trigger their activation (Figs 5-11A and B). These data confirm that signal 1 from TCR-pMHC-1 interaction alone is not enough to induce optimal activation of Ag-specific $CD8^+$ T cells.

In vivo results were consistent with the *in vitro* data. Priming OT-1 T cells with 100ug of SIINFEKL in the presence of anti-CD27 mAb resulted in a strong primary response compared with a moderate response with SIIQFEKL and barely detectable expansion with the weakest SIITFEKL peptide (Fig 5-11A). These results mirrored data observed by Zehn *et al.* In their experiment, they used *Listeria monocytogenes* (which might influence Ag-specific $CD8^+$ T cell response by the induction of inflammatory cytokines like IFN-γ) that expresses different altered SIINFEKL ligands including SIIQFEKL and SIITFEKL and showed that peptide affinity influenced the magnitude of the primary expansion, resulted in $\sim$62%, $\sim$14% and $\sim$8% being Ag-specific $CD8^+$T cells at the peak of the response respectively (Zehn et al., 2009). Doubling the antigen dose to 200ug in my *in vivo* experiment further augmented OT-1 T cell proliferation compared with one injection and delayed the contraction compared with one injection of 100ug (Fig 5-11). In addition, higher amounts of persistent antigen compensated for the lower affinity of the peptide and enhanced SIIQFEKL-induced $CD8^+$ T cell expansion to level comparable with that observed with SIINFEKL-OT-1 stimulated T cells (Figs 5-11B and C). However, increasing dose of SIIQFEKL still resulted in rapid decline (Fig 5-11), suggesting the affinity is important for prolonging the primary response. In contrast, increasing Ag density of the SIITFEKL peptide did not improve OT-1 expansion (Fig 5-11A and B). *In vivo* results also showed that the weaker the affinity of the peptide the earlier OT-1 T cells reached their maximal response and underwent contraction (Figs 5-11A and B). Data collected by Zehn *et al.* suggested that TCRs that bind to weak antigens detach from pMHC/DC and leave secondary lymphoid organs earlier compared to TCRs with a strong binding affinity (Zehn et al., 2009). The reason for this is that weakly stimulated OT-1 cells down-regulate CCR7 (an important receptor for naïve and memory T cells migration (Forster

et al., 2008)), and therefore leave secondary lymphoid organ and appear in the blood earlier than those stimulated with strong antigen (Zehn et al., 2009).

In an independent experiment to study the ability of naïve OT-1 cells to generate memory cell precursors upon stimulating with SIINFEKL or SIIQKEKL, an additional group stimulated with a low concentration of SIINFEKL (20ug) was included to examine whether immunizing with low peptide density will promote naïve $CD8^+$ T cell differentiation into memory cells. Results indicated that OT-1 T cells were able to expand and generate a secondary response upon Ag re-encounter, despite the lower antigen density or peptide binding affinity (Fig 5-12). These observations were similar to Zhen's data in which immunizing with *Listeria monocytogenes* expressing SIINFEKL induced more memory cells compared with the weaker peptide SIIQFEKL (Zehn et al., 2009). Interestingly, although SIIQFEKL-specific $CD8^+$ T cells were barely detectable in blood by day 20 post vaccination, SIIQFEKL-induced $CD8^+$ T cells expanded upon re-challenge with an antigen (Fig 5-12A). Unexpectedly, OT-1 T cells initially induced with a very low SIINFEKL density (20ug) remained in the blood up to day 20 post the first vaccination (Fig 5-12A). However, the level of the secondary response was comparable with 100ug of SIINFEKL. Increasing the SIIQFEKL concentration to 200ug in the initial priming resulted in an increased proportion of Ag-specific memory CD8 T cells, equivalent to SIINFEKL-specific memory OT-1 cells (Fig 5-12B), regardless of the low percentage of persistent OT-1 cells before re-challenge (Fig 5-12B). Collectively, these results indicated that peptide density did not influence the generation of memory $CD8^+$ T cells as judged by secondary expansion, but high affinity peptide resulted in cells that persisted for longer. It also suggested that the number of long-lived $CD8^+$ T cells resulting from SIIQFEKL immunisation might be similar to SIINFEKL-induced memory $CD8^+$ T cells, or they may be fewer but functionally better.

As mentioned earlier, the exact mechanisms of how and what determines the fate of naïve $CD8^+$ T cells differentiation into effector and memory cells is still controversial (Leignadier and Labrecque, 2010, Henrickson et al., 2013, Zehn et al., 2009, Bachmann et al., 2006). For example, my observations were different from another study performed by Leignadier J and colleagues. In their work, they compared the efficacy of T cell responses primed with low and high peptide density, by controlling the antigen load on the surface of DCs. They pulsed DC *in vitro* with the ovalbumin peptide (SIINFEKL) for three hours (low density) or overnight

(strong density), immunized mice with either population and then followed Ag-specific $CD8^+$ T cell responses in LN at different time points. In contrast to our results, the magnitude of the of the Ag-specific $CD8^+$ T cell primary response was similar in both groups (on days 2, 3 and 4 post immunization) (Leignadier and Labrecque, 2010). However, the initiation of the contraction phase in the group primed with DC 3h was earlier compared to those stimulated with high amounts of antigens (Leignadier and Labrecque, 2010); these differences affected the total number of generated memory T cells. In a similar study by Henrickson and colleagues, $CD8^+$ T cell differentiation into long-lived memory cells was correlated with Ag concentration, which strongly influenced the stability of the TCR/pMHC-I interaction and total number of memory cells. Only Ag-specific $CD8^+$ T cells primed with DC expressing large amounts of Ag were able to differentiate into memory cell precursors, whereas T cells induced by low amounts of Ag (short-lived Ag) declined and did not persist (Henrickson et al., 2013). Work by Zehn et al. showed that even OT-1 cells with a very weak clonal expansion at the peak of the response such as OT-1 cells induced by SIIVFEKL peptide which is even weaker than SIITFEKL (Zehn et al., 2009) were able to differentiate into memory cells and respond to a second Ag re-call (Zehn et al., 2009). Thus, these results confirmed that even very weak antigen (<hgp100) can promote naïve T cell to differentiate into memory cell precursors. These findings also suggest that memory cells might be programmed from the initial TCR/pMHC interaction in the early stage of the activation. Of note, initiation and severity of the contraction phase of pmel-1 cells was correlated with the magnitude of primary expansion at the peak of the response. It is possible that these cells undergo apoptosis via the FAS/FASL pathway.

One possible reason for the defect of pmel-1 T cell to differentiate into long-lived memory cells could be correlated with the Ag that pmel-1 TCR recognise. Because gp100 peptide is also expressed in normal cells, generating gp100-specific $CD8^+$ memory T cells might be difficult.

I also showed in (Figs 5-11, 5-12 and 5-14) that the size of Ag-specific $CD8^+$ T cell primary response is correlated with strength of TCR/peptide-MHC signalling. These results were in line with previous studies (Zhong et al., 2013, Tan et al., 2015, Schmid et al., 2010), indicating that high TCR/peptide-MHC binding avidity is crucial for a desirable anti-tumour vaccine. However, tumour Ag-specific $CD8^+$ T cells should not exceed maximum activation.

TCR maximal activation may recruit inositol 5-phosphatase-1 (SHIP-1), which can cause dephosphorylation of proximal signalling targets such as LCK, CD3ζ or ZAP-70 (Hebeisen et al., 2013). Recruitment of SHIP-1 has also been reported to reduce gene expression profile, intracellular signalling and TNFR expression (Hebeisen et al., 2013).

Finally, I compared the anti-tumour response following immunization with high affinity (SIINFEKL) and low affinity (SIIQFEKL) peptide in the B16-OVA melanoma model. Because the combination of anti-CD27 plus anti-PD-1/anti-PD-L1 blocking mAbs elicited strong anti-tumour immune response against the poorly immunogenic B16-BL6 tumour (Fig 4-2A), I used the combination in this study to activate adoptively transferred OT-1 T cells.

Results from two independent experiments showed that both peptide vaccines resulted in a long-term protection for 40-60% of mice bearing established B16-OVA (Fig 5-15). The primary response of the Ag-specific $CD8^+$ T cells was overall lower when mice where initially immunized with SIIQFEKL compared to immunization with SIINFEKL (Fig 5-16). These results were in contrary to previous studies where they show that maximal $CD8^+$ T cells function was correlated with TCR binding affinity to the Ag (Schmid et al., 2010). Tan MP *et al*. In there study, $CD8^+$T cell polyfunctionality was correlated with the strength of TCR–pMHC interaction (Tan et al., 2015). They have shown that activation of human $CD8^+$ T cell clone ILA1 (specific for human telomerase reverse transcriptase peptide $ILAKFLHWL_{540\text{-}548}$) with IL***G***KFLH***T***L (half-life= 14.1 second, kd=4uM) generated better functional profile compared to those generated by weaker ILAKFLH***T***L peptide (half-life=7.3 second, kd=27.6uM) and 4 other lower peptide affinities (Tan et al., 2015). There is evidence suggested that TCR affinity threshold exists, above which T cell function is not improved by further enhancement (Tan et al., 2015). This could be the case in the tumour experiment in (Figs 5-15 and 5-16), where anti-tumour responses generated by immunization with SIIQFEKL and the combination mAbs reached levels where CTL effector function could not be further improved with higher peptide affinity like SIINFEKL in this case.

Immunizing mice with SIINFEKL and the combination of anti-CD27 plus anti-PD-1/PD-L1 delayed the contraction of the Ag-specific $CD8^+$ T cells. While vaccinating with SIIQFEKL and the combination increased the magnitude of the primary response. Therefore, the

antitumour activity induced by the combination could be mediated by slightly different mechanisms.

Chapter 6. General discussion

Conventional cancer therapy such as surgery, chemotherapy or radiotherapy can eliminate tumour cells. However, these therapies are relatively non-specific and normal cells can also be affected by the treatment leading to side effects such as hair loss, fatigue and skin irritation. Furthermore, tumour may relapse after conventional cancer treatment, and some cancers have the ability to resist traditional tumour therapies. Cancer immunotherapy including ACT, mAbs and peptide-based vaccines has emerged as a promising approach for cancer treatment due to its greater specificity and ability to induce strong anti-tumour immune responses.

Tumour cells utilise multiple mechanisms to escape and suppress anti-tumour immune responses, including down regulation of MHC-I, recruitment of suppressor cells and expression of co-inhibitory ligands such as PD-L1 and/or PD-L2 (Bubenik, 2004, Oleinika et al., 2013, Bubenik, 2003, Pardoll, 2012). In addition, many tumour antigens are considered as self, resulting in a weak TCR signal and self-tolerance. Thus, the aim of my PhD project was to establish immunization protocols to expand and maintain adoptively transferred gp100-specific $CD8^+$ T cells by targeting members of the TNFRSF, and to investigate the role of Ag binding affinity in the generation of memory T cells.

First, I targeted different co-stimulatory receptors including CD27, OX40, GITR and 4-1BB by using agonist mAbs in combination with hgp100 vaccine. Anti-CD27 was the most potent in generating a high frequency of pmel-1 T cells at the peak of the response after injection with either a low or high dose of peptide. Therefore, I used anti-CD27 in the rest of my experiments to induce strong pmel-1 T cell responses. Unlike CD27 and GITR, OX40 and 4-1BB are not constitutively expressed on resting $CD8^+$ T cells, they are transiently expressed on activated $CD8^+$ T cells as my data (3-4) and other studies showed (Dawicki et al., 2004, Taraban et al., 2002). This may partially explain the lack of strong T cell stimulation when a low peptide dose was administered. These data suggest that timing the administration of mAbs is important for optimal T cell responses.

To further increase and maintain pmel-1 T cell expansion, I combined anti-CD27 mAb with TLR agonists. Results were similar to those published previously (Salem et al., 2009, Cho et

al., 2012, Cui et al., 2014b), and show that combining LPS (TLR 4 agonist) or poly I:C (TLR3 agonist) with anti-CD27 increased pmel-1 cell expansion significantly compared with injecting single agent alone. Anti-CD27 plus poly I:C was more efficient when it was injected i.v. compared to s.c. injection. This is possibly due to the amount of the Ag presented by the DCs, suggesting that vaccination route can influence the quality of the vaccine. Combining LPS with anti-CD40 also resulted in a significant increase in the pmel-1 T cell primary response compared with monotherapy, and slightly delayed the contraction of the activated pmel-1 cells compared with anti-CD27. I then thought to combine checkpoint blockade such as anti-PD-1/and or PD-L1 with the vaccine to overcome T cell exhaustion and improve pmel-1 T cell persistence. PD-1 expression was detected on activated pmel-1 cells; these results supported previous data in the literature showing that PD-1 is expressed on activated T cells (Agata et al., 1996). Blocking PD-1/PD-L1 in combination with anti-CD27 resulted in an enhanced pmel-1 T cell expansion compared with either agent alone, such that ~60% out of the total $CD8^+$ T cells were Ag-specific at the peak of the response. These results, with previous data (Cho et al., 2012, Barber et al., 2006, Buchan et al., 2015), indicate that stimulating co-stimulatory receptors in combination with checkpoint blockade can enhance T cell expansion. Nonetheless, pmel-1 cells exhibited rapid contraction after the activation and did not persist.

I then tested the ability of the activated pmel-1 T cells to reduce melanoma tumour growth in mice bearing stablished tumours. There was little anti-tumour effect following CD27, OX40, GITR or 4-1BB stimulation when delivered with low dose peptide, possibly because the number of the activated pmel-1 T cells was low after activation. However, immunizing mice with the combination of anti-CD27 and anti-PD-1/PD-L1 after a high transfer of pmel-1 T cells (3×10^6 cells) delivered with hgp100 (200ug) dampened tumour growth significantly resulting in ~50% of the treated mice with complete tumour regression (Fig 4-2). Notably, no vitiligo was observed in the survival mice. The combination also improved the functional profile and cytokine production of pmel-1 T cells remarkably compared to monotherapy. The antitumor therapeutic affect was gp100-specific $CD8^+$ T cell dependent, as injecting the combination in the absence of pmel-1 cell transfer abrogated its efficacy. These results confirmed that high numbers of CTLs with superior effector function are required to

overcome immune tolerance and mediate tumour cell killing (Cho et al., 2012, Sikora et al., 2009).

In my all *in vivo* experiments, I have tested T cell response in the periphery following immunization. However, examining peripheral immune response alone may not be sufficient as a prediction for effective antitumor immune responses. CTLs might be restricted or supressed within the tumour microenvironment by intrinsic and extrinsic factors, some of these factors by which tumour cells supress immune response include down regulation of tumour associated antigen and antigen loss variant, immunosuppressive cytokines and the up-regulation of immunosuppressive enzymes like indolamine-2,3-dioxygenase (IDO) (IDO catalyses tryptophan, supresses T cell proliferation and promote their apoptosis) (Kim et al., 2006, Dai and Dai, 2008), decreased expression of costimulatory molecules on the tumour or APC, alterations in TCR signalling in TILs and cell surface death receptor signalling (Whiteside, 2008), upregulation of inhibitory molecules on the cell surface on the cell surface of cancer cells like PD-L1. The interaction between PD-L1 with PD-1 on activated T cells results in diminished antitumor T-cell responses (Binder and Schreiber, 2014). In addition, suppressive cells such as immature myeloid cells iDC, M2 macrophages, MDSCs, iT_{reg} and nT_{reg} cells are highly enriched in the TME (Lindau et al., 2013, Monjazeb et al., 2013). Immature DC can induce T-cell anergy due to lack of co-stimulatory signalling. MDSCs and M2 macrophages promote T cell dysfunction by different mechanisms including nitric oxide and ROS generation. T_{reg} cells are considered to be significant in limiting antitumor immune responses and promoting tumour growth. T_{reg} cells inhibits CTL function directly by cell-cell contact, indirectly by secretion of inhibitory cytokines such as IL-10, TGF-β and IL-35, or by the competition for growth factors like IL-2 (Sojka et al., 2008). Therefore, examining TILs is important to study the efficacy of a particular vaccine on generating sufficient anti-tumour responses.

Immunizing mice bearing solid colon carcinoma CT26 with anti-CD27 and anti-PD-1/or PD-L1 did not show any synergistic effect against the tumour. Anti-CD27 alone in contrary resulted in 20-40% of mice with long-term survival. Unlike the B16 tumour sublines, CT26 cells only minimally express PD-1 ligands as described *in vitro* in chapter 4. This may partially explain the lack of the synergy in this particular tumour model. This result supports the notion that blocking PD-1/L1 alone has little effect on controlling tumour growth, and additional

checkpoint blockade or adjuvants might be required to increase the efficacy of the vaccine (Binder and Schreiber, 2014, Lussier et al., 2015).

Results also showed that mouse anti-CD27 of the IgG1 isotype is more efficient in controlling tumour growth compared to the IgG2a isotype. This is because mouse IgG1 increased the frequency of T cells, while mouse IgG2a caused T cell depletion. These data are consistent with the literature in which different FcγRs have been targeted and showed that FcγR is critical for the agonist activity of the mAb (White et al., 2011).

Throughout my project I noticed that pmel-1 T cells do not differentiate into memory cells that can expand rapidly after re-stimulation. Therefore, I tested different approaches to rescue pmel-1 cells from apoptosis following the contraction phase. Injecting two doses of IL-2 during the activation phase did not rescue pmel-1 cells form apoptosis. These results were different from other studies, where administering exogenous IL-2 resulted in an enhanced pmel-1 T cell survival (Cho et al., 2012), possibly because they injected multiple doses of IL-2 during the priming and during the contraction phase. My data also showed that pmel1 T cells with enforced expression of Bcl-2^+ exhibited delayed contraction compared with WT pmel-1 cells. Yet, Bcl-2^+pmel-1 T cells did not expand after re-stimulation. Furthermore, administering anti-CD27 to Bcl-2^+ pmel-1 cells did not enhance their survival after activation, probably because anti-CD27 is activating Nor-1 which abrogates Bcl-2 activity. In contrary to Ahmed R, *et al* study, my results also showed that blocking mTOR did not prolong pmel-1 T cell survival, maybe because they used acute LCMV infection as a model in their study, which could trigger an inflammatory response such as IFN-γ and IFN-α and alter the outcome of a T cell response as a consequence.

Accumulating data suggest that TCR/peptide-MHC binding affinity and peptide density play a crucial role in determining the fate of naive T cell differentiation into effector and memory T cells (Corse et al., 2011, Baumgartner et al., 2012). Therefore, I immunized mice with peptides that have variable binding affinity to the TCR. I used OT-1 T cells for this purpose because these cells can differentiate into CTLs as well as into memory cells after activation (Willoughby et al., 2014). First, I showed that the OT-1 TCR is ~1000 fold more sensitive to cognate Ag than pmel-1 T cells. Then I showed that immunization with a low dose (density) of peptide or with a peptide of low binding affinity generated a reduced OT-1 T cell

response compared with high density or high affinity peptides. However, the differentiation into memory cells was not affected by the affinity of the peptide used for activation. These results are in line with Ahmed R data (Zehn et al., 2009), and indicate that peptide affinity determines the magnitude of the primary response but may not have an impact on memory T cell differentiation.

I also showed that immunization with a peptide of low binding affinity i.e. SIIQFEKL can be markedly improved by injecting the combination of anti-CD27 plus anti-PD-1/L1 and that these two mAbs synergised to increase the magnitude of the primary response. Vaccinating with the peptide of high affinity and the same mAb combination prolongs the peak of the OT-1 T cell response. The combination of either high or low affinity peptide with anti-CD27 and anti-PD-1/L1 showed similar significant anti-tumour activity against the established B16 melanoma.

6.1. Clinical relevance

Because many tumour antigens are considered as self, generating strong anti-tumour immune responses remains a challenge. Infusing high numbers of $CD8^+$ T cells that bind to tumour antigen with higher affinity in combination with an altered peptide vaccine has emerged as an alternative immunotherapy approach to overcome immune tolerance (Maus et al., 2014a, Kalos and June, 2013). Furthermore adjuvants like mAbs that can modulate T cell responses have been combined with ACT to increase the efficacy of the treatment. My results suggest that anti-CD27 plus PD-1/PD-L1 blockade can be combined with ACT in the clinic to 1- overcome immune-tolerance, 2- increase the expansion of the adoptively transferred cells to levels that can control tumour growth, and 3- improve effector function and cytokine production of the CTLs. Although vitiligo was not observed in the surviving mice throughout my experiments, patients need to be under observation during the treatment, because infusing $CD8^+$ T cells that can recognise self-antigen might cause autoimmunity (Finkelstein et al., 2004). It is also important to mention that self Ag-specific $CD8^+$ T cells should not be over activated such that they induce adverse effects and collateral damage as a consequence. Therefore, immunization with a peptide that binds to the TCR with moderate affinity might be a better option over a peptide that binds with high affinity. It is worth mentioning that the efficacy of the combined vaccine may vary form one

patient to another, depending on the level of PD-L1 and/or PD-L2 expression in each individual patient (Ohigashi et al., 2005).

6.2. Future work

Because pmel-1 cells contract rapidly after activation, any future work would focuson improving pmel-1 cell survival, and promote their differentiation into long-lived memory T cells that have the capacity to expand rapidly following re-encounter with Ag. A study by Sikora *et al* has shown that administering IFN-α prolonged pmel-1 T cell survival with an effector memory phenotype (Sikora et al., 2009). However, the recall of effector memory cells is weaker than central memory-like T cells (Gattinoni et al., 2005, Klebanoff et al., 2004). Injecting exogenous cytokines like IL-7 or IL-15 might be an alternative approach to prolong pmel-1 T cell survival (Nanjappa et al., 2008, Schluns et al., 2000, Tan et al., 2002). Another line of research would be to compare and study the gene expression patterns of activated OT-1 and pmel-1 cells. This might give insight into understanding the changes at the molecular level to enable manipulation and promotion of pmel-1 T cells into effector and memory T cells. Finally, several studies in mice and humans have shown that dual blockade of PD-1/PD-L1 and CTLA-4 receptors synergized effectively in dampening tumour growth and improving survival compared to monotherapy (Callahan et al., 2014, Binder and Schreiber, 2014). Thus, combining agonist anti-CD27 with a combination of anti-PD-1 and anti-CTLA-4 may further improve the median survival of patients in the clinic. Alternatively, the potential of anti-CD27 to combine with other checkpoint inhibitors like anti-TIM-3 or anti-LAG-3 should be explored to improve anti-tumour immune responses.

7. Conclusion

To summarize the main points of my PhD project, I showed that targeting members of the TNFRSF in combination with hgp100 peptide increases the magnitude of the pmel-1 T cell response. Anti-CD27 was the most potent agonist tested. I also showed that timing and level of expression of co-stimulatory receptors is critical for optimal T cell activation. In addition, the anti-CD27-mediated response can be further enhanced by combining with anti-PD-1/L1 signalling pathway blockade. This combination can be used as a potent vaccine to overcome immune tolerance, eliminate tumour growth and to improve the overall survival of the mice bearing advanced malignancies. The potency of this vaccine was likely through the observed increase in the number of CTLs and their enhanced effector functions. PD-L1 and/or L2 expression is crucial for the efficacy of the treatment, the absence of one or both ligands may abrogate the synergy effect of the combination as was shown in the CT26 tumour model. In addition the potency of the vaccine is highly sensitive to the vaccination route; injecting mice i.v. resulted in a better $CD8^+$ T cell response compared to when the vaccine was injected s.c. Thus, vaccination route should also be considered. Results also revealed that peptide binding affinity determines the magnitude of the primary response, but it does not interfere with memory $CD8^+$ T cell differentiation. Moreover, I showed that the combination of anti-CD27 and anti-PD-1/anti-PD-L1 increases the magnitude of the Ag-specific $CD8^+$ T cell primary response when a peptide of relatively low affinity (SIIQFEKL) was used for vaccination, while the same mAb combination delays the contraction of CTLs when mice were immunized with a high affinity peptide (SIINFEKL). Finally, my results showed that immunizing mice with either SIIQFEKL or SIINFEKL peptides resulted in a similar anti-tumour immune response (~50% of mice survival in the long-term), suggesting that these peptides might have slightly different mechanisms in controlling tumour growth.

8. References

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