Combined Adjuvant For Stimulation of Cellular Immunity

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Declaration

I hereby declare that I alone composed this thesis and the work presented here in my own except stated otherwise.

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Abstract

Vaccination has important clinical potential in the immunotherapy of both infectious disease and cancer. The central aim of the studies reported in this thesis has been the development of vaccination strategies that will be effective for therapeutic applications in cancer. Using ovalbumin antigen in a mouse model, we have examined a combination of recently developed adjuvants referred to as CASAC (combined adjuvants for synergistic stimulation of cellular immunity) to optimise the efficacy of vaccination induced T cell mediated immunity. These studies have examined the effect of repeated rounds of vaccination with one, or alternating cycles of two different helper peptides. The hypothesis was that repeated stimulation of the same clonal population of $CD4^+$ T helper cells with a single MHC class II peptide could induce anergy, exhaustion, clonal deletion and/or stimulation of regulatory T-cells, resulting in reduced and shorter lived immunity. These studies have also examined the effect of inclusion of other immune regulatory components, and in particular a cocktail of cytokines generated by phytohemagglutinin stimulation of human peripheral blood mononuclear cells (referred to as IRX-2). Another important issue for vaccination mediated immune therapy that was addressed is the age-associated loss of immunological competence (immunesenescence).

A comparative analysis is reported of the magnitude and duration of responses to vaccination with a single MHC class-I presented peptide in combination with the same or alternating MHC class-II presented peptides. In addition, we have quantified the number of antigen specific CD8 T cells, their effector and memory subsets and *in vivo* antigen specific cytolytic activity to examine the effect of CASAC vaccination alone or in combination with IRX-2. The induction of immunological responses in young and aged immune backgrounds was also examined.

Vaccinations with ovalbumin peptides in the CASAC adjuvant have shown higher percentages and numbers of antigen specific CD8 T cells with improved cytolytic activity when helper functions are stimulated by two different class-II peptides used in alternating cycles of vaccination, rather than repeated stimulation by the same class-II peptide. This conclusion can be confirmed with a repeated experiment. Analysis of T cells with a regulatory (Treg) phenotype ($CD4^+CD25^+Foxp3^+$) showed that their expansion was reduced with the alternating T-helper peptide vaccination regimen. The addition of IRX-2 to the CASAC vaccination regimen was found to enhance the *in vivo* antigen specific cytolytic activity. This was particularly significant in the aged mice which were found to have increased levels of Tregs.

Table of Content

AML	Acute myeloid leukaemia
AC	Alpha casein
ADCC	Antibody- dependant cell-mediated cytotoxicity
APC	Allophycocyanin
APC	Antigen presenting cells
BCG	Bacillus Calmette-Guérin
BCR	B-cell receptor
CASAC	Combined Adjuvant for Synergistic Activation of Cellular immunity
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
CHOP	Cyclophosphamide, Hydroxydaunorubicin Oncovin (vincristine), Prednisone
CLL	Chronic lymphocytic leukaemia
CNX	Calnexin
CRT	Calreticulin
CTL	Cyototoxic lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic cells
DLI	Donor lymphocyte infusion
DOM	Domain of tetanus toxin
DPBS	Dulbecco's phosphate buffered saline
DTH	Delayed Type Hypersensitivity

ED	Extra domain
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FAP	Familial adenomatous polyposis
FBS	Foetal bovine serum
FDA	Food and Drug Association
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good Manufacturing Practice
HBV	Hepatitis B virus
HBV-Tg	Hepatitis B virus transgenic mouse
HC	Heavy chain
HNSCC	Neck squamous cell carcinoma
HPV	Human papilloma virus
IBD	Inflammatory bowel disease
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IFN-γ	Interferon-gamma
IL	Interleukin
ISCOMs	Immune stimulating complexes

KIR	Killer cell immunoglobulin-like receptor
KLH	Keyhole limpet hemocyanin
KLRG-1	Killer lectin like receptor G1
LAG-3	Lymphocyte Activation Gene-3
LCMV	Lymphocytic choriomeningitis virus
MAGE	Melanoma-associated antigen 3
MALT	Mucosal-associated lymphoid tissue
MC-38-CEA	murine colon adenocarcinoma cells expressing human carcinoembryonic antigen
MHC	Major histocompatibility complex
MMP	Mitochondrial membrane potential
MTX	Methotrexate
MVA	Modified vaccinia Ankara
NF-κB	nuclear factor kappa B
NHL	Non-Hodgkin's lymphoma
NK	Natural killer cells
NSAID	Nonsteroidal anti-inflammatory drug
NSCLC	Non-small cell lung cancer
OVA	Ovalbumin
PADRE	Pan DR epitope
PAMPs	Pathogen Associated Molecular Patterns
PBlue	Pacific Blue
PBMC	Peripheral blood mononuclear cells
PD-1	Programme death 1

PD-L1	Programme death 1 ligand
PEG	Polymer poly-(ethylene glycol)
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
RCC	Renal cell carcinoma
R-PE	R-Phycoerythrin
RPMI	Roswell Park Memorial Institute
SCID	Severe combined immunodeficient
ssRNA	Single stranded RNA
TAA	Tumour associated antigens
TAP	Transporter associated with Antigen Processing
TCR	T-cell receptors
TH	T-helper
TIRAP	toll-interleukin 1 receptor adaptor protein
TMV	Tumour micro vesicles
TNF α	Tumornecrosis factor-alpha
TNF-α	Tumornecrosis factor-alpha
Tpsn	Tapasin
TR	Tregs
TRAM	TRIF-related Adaptor Molecule
TRICOM	Triad of co-stimulatory molecules
Tregs	Regulatory T-cells
TRIF	TIR-domain-containing adapter-inducing interferon-β

VEE	Venezuelan equine encephalitis virus
VIN	Vulvar intraepithelial neoplasia
WT-1	Wilms Tumour Protein

CHAPTER 1

Introduction

This thesis describes studies aimed at enhancing the generation of CD8 T-cell responses by peptide vaccination, using a number of novel vaccination regimens. Peptide vaccination has the potential to be of therapeutic value for the treatment of cancer and chronic infectious diseases but would require the right combination of adjuvants, timing, induction of suitable helper functions, etc., without the stimulation of immune suppressive functions. In this chapter, previously published studies that have provided the basis for studies that are described in later chapters will be discussed.

1.1 Vaccination

Vaccination is the administration of antigenic material to produce immunity against certain diseases. There are also emerging vaccination strategies for the induction of antigen specific tolerance, but they are not the subject of this review. Antigens used in vaccinations range from live viruses or bacteria to peptides of proteins associated with the disease target. Edward Jenner marked the beginning of vaccination by using cowpox to give protection against smallpox in humans in 1796. Injecting the harmless form of a disease organism, Jenner utilized the specificity and memory of the acquired immune response to lay the foundation to modern vaccination strategies[1]. The two main types of cancer vaccination are prophylactic and therapeutic cancer vaccines. The latter are substantially more challenging, as the disease would have co-existed for a long time with the immune system and have finally escaped immune surveillance. At this stage, almost by definition, tumour tolerance would have been induced[2, 3].

A previously published study by Chan *et al.* (2006), showed an example of tumour escape by acute myeloid leukaemia (AML) cells by downregulation of the B7.1. Downregulation of B7.1 molecule, a co-stimulatory molecule required for activation of CD8 T-cells, enabled the tumour to escape the immunesurveillance[4]. The modern history of cancer vaccination started in the 1970s when Hanna *et al.*, showed tumour regression in vaccinated guinea pigs, using irradiated hepatocellular carcinoma cells and bacillus Calmette-Guérin (BCG) as an adjuvant[5, 6]. BCG, an attenuated *Mycobacterium bovis* was a vaccine first used in humans in 1921 to confer immunity towards tuberculosis[7]. BCG contains pathogen-associated molecular patterns that

stimulate the innate immune system and thus provide Th1 responses that lead to improved generation of cytotoxic T-cells, providing the protective immunological responses in the vaccinated guinea pigs. BCG is currently studied for its efficacy as an adjuvant used in vaccinations against autologous tumour cells in a number of clinical studies. For instance, a Phase III study of the benefits of BCG as an adjuvant (Patented as OncoVax®) in 2005 showed statistically significant 5-year survival and recurrence free survival in Stage II colon cancer patients[8]. In various forms of tumour cell vaccination, tumour cells maybe rendered replication incompetent (e.g. by ionizing irradiation) and administered alone or in combination with different adjuvants (e.g. BCG). Alternatively, the tumour cells may be genetically modified to express immune stimulatory cytokines such GM-CSF, IL-2, IL-12, etc., or co-stimulatory ligands such as B7.1 (CD80), B7.2 (CD86) and 4-1BBL (CD137)[9-12]. All such modifications enhance the immunogenicity of the tumours and achieve better immune stimulation, resulting in improved tumour rejection by the immune system.

Peptide vaccination and peptide loaded dendritic cell-based immune therapy of cancer has also been attempted[13] and Bakker *et al.*, in 1995 generated anti-melanoma cytotoxic T-cells by presentation of melanoma tumour associated antigens[14] on dendritic cells (DC). Immature monocyte-derived DC were loaded with Tumour associated antigen [15] peptides and then used in an autologous transplant to induce antigen-specific T-cell responses[16]. Immature DC were also injected into adjuvant pre-treated sites in mice and were found to be more effective than ex-vivo matured DC. This strategy showed that the injected DC acquired a lymph node migratory capacity comparable to ex-vivo matured DC[17]. TAA such as MAGE-A3 and NY-ESO-1 have been utilised in peptide vaccinations in combination with a range of different adjuvants for several clinical trials in melanoma, non-small cell lung cancer (NSCLC) and ovarian carcinoma[18-20]. This subset of TAA, which are normally expressed in the testis and tumour cells, are also known as cancer testis antigens and expressed by a range of tumours. MAGE-A3 for example is found in lung cancer (35-50%), bladder cancer (35%), head and neck cancer (49%) and melanoma (74%), while it is absent in normal healthy cells[18]. Cancer vaccination strategies also include DNA vaccines where vectors (usually plasmids) encoding TAA are delivered to muscles as a strategy for the expression of TAA at high levels in sites away from the tumour (i.e. less immune suppressed environment) in combination with adjuvants,

e.g. super antigens expressed by pathogens (e.g. fragments of tetanus toxoid). In an example of this strategy, Chaise *et al.*, demonstrated in 2008 that vaccination with a plasmid containing the gene encoding Wilms tumour antigen (WT1), fused to the first domain of tetanus toxin (DOM), could induce in a transgenic mouse expressing human HLA-A2 the activation of cytotoxic T-cells that were able to kill HLA-$A2^+$ human leukemic blasts *in-vitro*[21]. Genes for TAA were also introduced into viral vectors such as modified vaccinia Ankara (MVA) virus in combination with co-stimulatory factors as a DNA vaccine that generated tumour-specific immune responses and antitumor activity[22]. This approach was shown by Hodge *et al.* to induce the rejection of murine colon adenocarcinoma cells expressing human carcinoembryonic antigen (MC-38-CEA) following vaccination with MVA expressing CEA and a Triad of co-stimulatory molecules (TRICOM which includes B7.1, ICAM-1 and LFA-3)[22]. Cancer vaccination strategies are still largely in clinical trials, but there has been a variety of approved immunotherapy methods (see Table 1.1). The presence of a variety of approved immune therapies and vaccination for cancer indicates its potential therapeutic value[2].

Other notable immunotherapy products are Ipilimumab and Provenge. Ipilimumab, a monoclonal antibody against CTLA-4 (an inhibitory receptor for T-cell generation) has been extensively reviewed[23] and shown to have therapeutic benefits in metastatic melanoma[24]. Sipuleucel-T (Provenge) on the other hand, is an autologous cellular immunotherapy approved by the American Food and Drug Association (FDA) for the treatment of metastatic castration-resistant prostate cancer[25]. It's efficacy against metastatic prostate cancer has been proven in a clinical trial where there was a reduction of 22% in the risk of death, representing a 4.1 months improvement in the median survival[26].

Established therapies	Indication
Monoclonal antibodies	
Rituximab	NHL, CLL
Ibritumomab tiuxetan	NHL
Tositumomab	NHL
Alemtuzumab	CLL
Gemtuzumab	AML
Trastuzumab	Breast cancer
Cetuximab	Colorectal cancer
Panitumumab	Colorectal cancer
Bevacizumab	Colorectal, lung
Immune adjuvants	
BCG	Superficial bladder cancer
Imiquimod	Basal cell carcinoma, VIN, actinic keratosis
Cytokines	
IFN-α	melanoma, RCC
IL-2	melanoma, RCC
TNF-α	Soft tissue sarcoma, melanoma
Supportive therapy	
G(M)-CSF	Myelosuppressive chemotherapy
Leucovorin	MTX rescue
Prophylactic immune therapy	
HBV vaccine	Hepatocellular carcinoma
HPV vaccine	Cervical cancer
Antibiotics (*H. pylori*)	Gastric cancer, MALT lymphoma
NSAIDs (FAP, ulcerative colitis)	Colorectal cancer
Bone marrow transplantation	
Allogeneic	Hematologic malignancies
DLI	Hematologic malignancies

Table 1.1: Approved Immune therapies for cancer: Abbreviations: AML, acute myelogenous leukaemia; BCG, bacillus Calmette-Guérin; CLL, chronic lymphocytic leukaemia; DLI, donor lymphocyte infusion; FAP, familial adenomatous polyposis; HBV, hepatitis B virus; HPV, human papilloma virus; MALT, mucosal-associated lymphoid tissue; MTX, methotrexate; NHL, non-Hodgkin's lymphoma; NSAID, nonsteroidal anti-inflammatory drug; RCC, renal cell carcinoma; VIN, vulvar intraepithelial neoplasia. (Dougan et.al. 2009)[2]

1.2 Immunity

The immune system can be divided into the innate immune system which encompass a system that does not require a specifically acquired immunity against a certain antigen, and the adaptive immune system which encompasses specifically acquired immunity against antigens or pathogens. The innate immune system is the frontline of the body's defence system against bacteria and virus infections. It works by toll-like receptors recognising certain pathogen-associated molecular patterns[27]. This recognition allows up-regulation of transcription factors such as NF-κB, IRF3, IRF5, etc, which eventually lead to the production of inflammatory cytokines and other effector molecules.

After the onset of innate immunity, the secondary line of defence, adaptive immunity, takes over in the form of specifically acquired immunity. This is composed of B-cell mediated humoral immunity, involving the production of antibodies and the antigen specific cellular immunity which involves the activation of cytotoxic T-cells. B-cells produces about 10^8-10^9 B-cell receptor (BCR) on their cell surface and each B-cell when matured and terminally differentiated will only produce a single type of antibody which in turn will only recognize one specific antigen[28]. When a pathogen enters the body, it will be presented to a very large array of B-cells expressing about 10^5 different antibody molecules making a very diverse variety of antibody molecules. The recognition of a pathogen by B-cell receptor molecule triggers the expansion of the B-cell clone to produce a high enough concentration of a given antibody to ensure effective eradication of infection. The complex of antibody and antigen activates phagocytosis when the antibody is detected by the antibody receptors on phagocytes. This complex would also lead to NK cell antibody-dependant cell-mediated cytotoxicity (ADCC). Single antibody detection is not sufficient to elicit a response and it would require multiple antibodies to bind and trigger phagocytosis by cross-linking the surface receptors for antibodies. However, in immunotherapy especially with monoclonal antibodies, the efficacy of these antibodies are higher as they have been specifically selected for increased binding to pathogens. This eventually leads to the eradication of the pathogen[1].

Antibody mediated responses have played a crucial part in development of immunotherapy and now represent well established treatments for different types of

diseases including cancer. For instance Rituximab, a widely used monoclonal antibody, targets CD20 which is expressed on the surface of B cells, including non-Hodgkin's lymphoma (NHL) cells. When used in combination with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP), Rituximab therapy induces 76% complete remissions contrasted to 63% for CHOP treatment alone. However, antibody mediated rejection of solid tumours are less efficient, unless the antibody is engineered to induce more efficient ADCC which involves NK and macrophage mediated lysis of the antibody decorated target cells. One such example is cetuximab which targets colorectal cancer expressing high epidermal growth factor receptor (EGFR)[29]. Therefore based on our basic understanding of the immune system, we and many others predict that a much more effective tumour rejection response could be induced by the activation of cell mediated immunity. Cell mediated immunity could be induced by activating the host's immune system with different vaccination regimens.

Cell mediated, antigen specific, immunity is manifested by the activation of T-cells which are cytotoxic cells that are able to recognize and eradicate cells that are infected by intracellular organisms such as viruses. T-cells differentiate in the thymus and are capable of recognizing cells by the presence of foreign antigen derived peptides in the context of their own MHC molecule[1]. T-cell immunity differs from humoral immunity as they are capable of screening cells with their T-cell receptors (TCR) for the recognition of antigen in the context of major histocompatibility complex (MHC)[1]. The two major groups of MHC molecules are the MHC Class I which is recognized by $CD8^+$ effector T-cells and the MHC Class II molecule which is recognized by $CD4^+$ helper T-cells[1]. Antigens are taken up by the professional antigen presenting cells (e.g. dendritic cells) and are processed to small fragments which are then presented by the MHC molecules. This will be further elaborated in section 1.3. In addition to presentation of antigen on MHC molecules, other co-stimulatory factors are also required for activation of cytotoxic T-cells. Two main factors which are involved in the activation of T-cells are the B7.1 and B7.2 co-stimulatory molecules which bind to the activatory receptor CD28 and inhibitory receptor CTLA-4 on T-cells. B7 family members of co-stimulators were first characterized to activate T-cell proliferation and to up-regulate the expression of IL-2 mRNA[30]. B7-CD28 interaction stimulates the proliferation and the activation of

cytotoxic resting T-cells[31]. However, it wasn't until 1993 when the B7 family was shown to have distinct members, B7.l and B7.2[32]. About the same time, it was also shown that B7.1 and B7.2 can bind not only to the activatory receptor CD28, but also to Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), the activation of which induces T-cell apoptosis[33] (Figure 1.1).

T-cell receptors are different from B-cell immunoglobulins (antibodies) in terms of recognition of foreign antigens. Antibodies are capable of recognizing pathogens and their immunogenic products within the body. Antibodies are secreted as soluble molecules which patrol the extracellular spaces of the body. T-cells receptors however recognizes composite ligands made up of short sequences of peptide bound to a self MHC complex. These interactions enable killing of cells infected with intracellular pathogens because they carry foreign antigenic peptides on their surface[34].

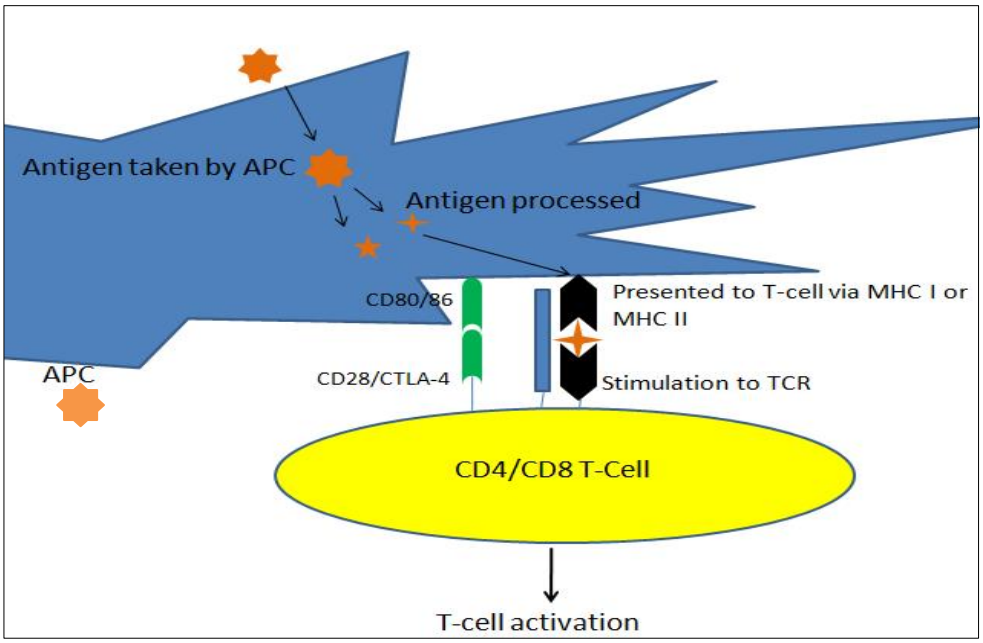


Figure 1.1 Antigen presentation: Presentation of antigen and the requirement of co-stimulatory factors to activate T-cells. Abbreviations: APC, Antigen presenting cells; TCR, T-cell receptor; MHC, Major Histocompatible Complex.

In the human body, there are also two types of T-cells subdivided by their TCR due to the presence of 4 loci of TCR which are α, β, γ and δ. The majority of T-cells expresses a variable alpha chain, a variable beta chain, a constant alpha chain and a constant beta chain termed αβ T-cells because of the αβ heterodimers found on their cell surface (Figure 1.2 a and b). The other T-cell type, named γδ T-cells, constitute a minority group of T-cells (about 10% of total T-cells). The γδ T-cells are predominant until birth when the αβ T-cells takes over. In our studies, we will be focusing only on αβ T-cells. However, it is noted that a number of studies have shown efficacy for γδ T-cells activated with zoledronate to treat metastatic solid tumours in combination with other therapies in the clinic[35].

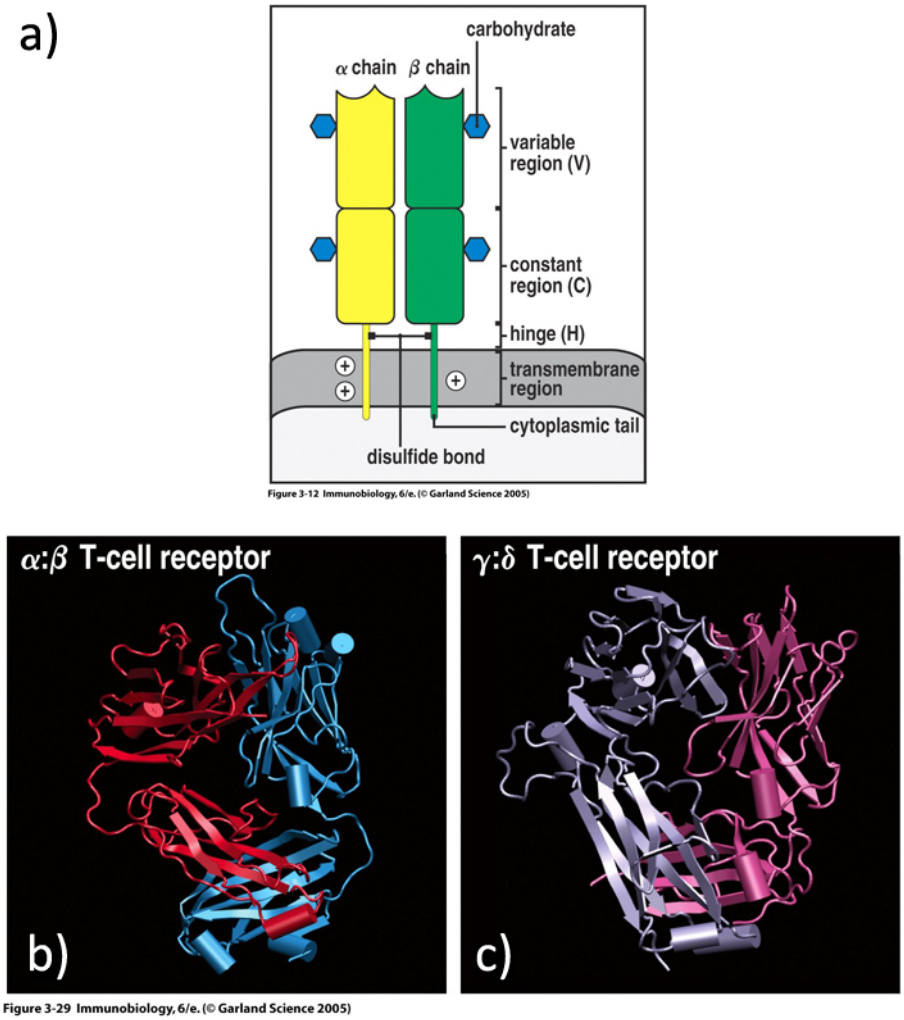


Figure 1.2 T-cell receptors: a) A schematic representation of αβ. Protein crystallographs of αβ T-cell receptor (b) which shows similarity to δγ T-cell receptor (c) (Adapted from Figure 3-12 and 3-29, Immunobiology, 6/e Garland Science 2005)[28]

1.3 MHC Class I and Class II antigen presentation for activation of $CD4^+$ and $CD8^+$ T-cells

Histocompatibility genes H-1, H-2 and H-3 were first characterized in transplantation studies when numerous genes which control the acceptance and rejection of transplants were discovered on antigen presenting cells in mouse models[36]. The product of these genes were found to fall into the MHC Class I and Class II glycoproteins which are involved in antigen presentation to T-cells. Figure 1.3 shows a schematic representation of MHC Class I and Class II genes[37]. MHC Class I molecules are made up of a heavy 44kDa protein non-covalently linked to a smaller 12kDa polypeptide called a β_2-microglobulin. As seen in Figure 1.3, the larger 44kDa polypeptide is organized into three globular domains namely α1, α2 and α3. The whole MHC Class I molecule protrudes from the cell membrane. MHC Class II molecule, which is also a transmembrane glycoprotein, consists of an α and β polypeptide chain which is subdivided into 2 globular domains, α1 and α2; β1 and β2. Both MHC Class I and MHC Class II molecules present antigens but the peptides that are presented by them are different. MHC Class I molecules are only capable of presenting polypeptides of 8-9 amino acid residues. This is due to the fact that the binding groove of Class I molecule is closed at both ends and the binding takes place at peptide positions 2, 3, 5 and 7. Class II molecule on the other hand has an open groove and therefore able to present peptides ranging from 8 to 30 amino acids. The major anchorage pockets of the Class II are peptide positions 1, 4, 6, 7 and 9 with position 1 being the most important[38].

Antigens that are taken up by antigen presenting cells (APC) such as dendritic cells (DC), by phagocytosis or pinocytosis, are generally presented on MHC Class I molecules and presented to $CD8^+$ T-cells for priming an immune response (This is called cross presentation). However, some antigens are digested by the immune proteasomes to form smaller peptide fragments and transported to the lumen of the endoplasmic reticulum via a peptide loading complex composed of calreticulin (CRT), Tapasin, Transporter associated with Antigen Processing (TAP) dimer and thiol oxidoreductase ERp57. ER aminopeptidase[39] might be involved in trimming the peptide to an 8-10 amino acid sequence. The MHC Class I heavy chain associates with Calnexin chaperone to initiate folding and disulfide bond formation before binding to the peptide loading complex and beta-2-microglobulin (β2m). Once

peptide is loaded onto the MHC Class I, it will dissociate from the other complexes to form an MHC Class I molecule with β2m and the loaded peptide[34]. This is schematically presented in Figure 1.4.

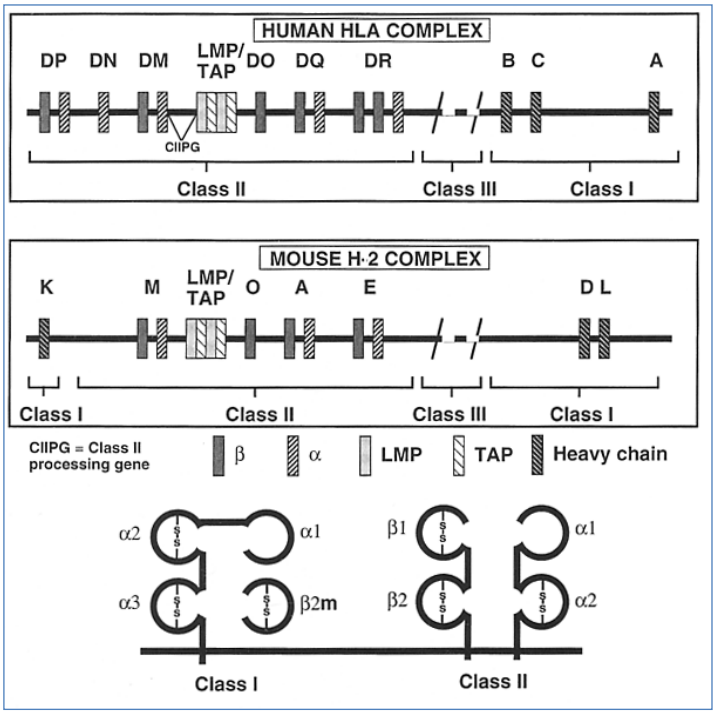


Figure 1.3 Schematic Representations of the Gene Organization of the Human HLA and Mouse H-2 Major Histocompatibility Complexes. Also presented is the organization of Class I and Class II molecules (from Germain R.N, 1994)[37]

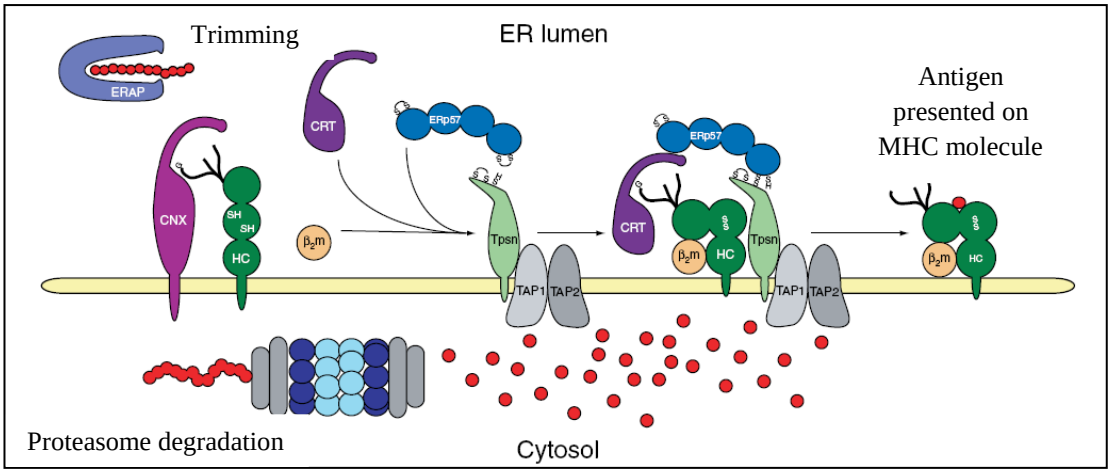


Figure 1.4 Antigen processing and MHC Class I peptide presentation: Schematic presentation of antigen processing and MHC class I presentation (adapted from Cresswell et. al, 2005). Abbreviations ERAP, ER aminopeptidase; ER, endoplasmic reticulum; CNX, Calnexin; CRT, calreticulin; HC, Heavy chain; TAP, Transporter associated with Antigen Processing; Tpsn, Tapasin.

Some infectious agents such mycobacteria replicate in the membrane bound vesicles in macrophages and are thus inaccessible to cytosolic proteasomes for degradation which will only occur after phagocytosis. However, during macrophage activation, proteases in these vesicles will degrade the protein into peptide fragments that bind to MHC Class II molecules and then presented to $CD4^+$ T-cells. MHC Class II complexes are formed in the ER lumen together with an Invariant Chain (li) which blocks unspecific binding of peptides to the MHC class II molecules[34]. The complex is then transported to acidified vesicles where the li is cleaved by proteases leaving a short peptide fragment called CLIP on the MHC Class II complex. CLIP, which blocks peptide binding to MHC Class II, is released when HLA-DM binds to the MHC Class II in the presences of antigen peptide fragments. Once CLIP is released, peptides of 15-24 amino acid length bind to the MHC Class II molecule, which is then transported to the membrane for presentation[34]. This process is represented in Figure 1.5.

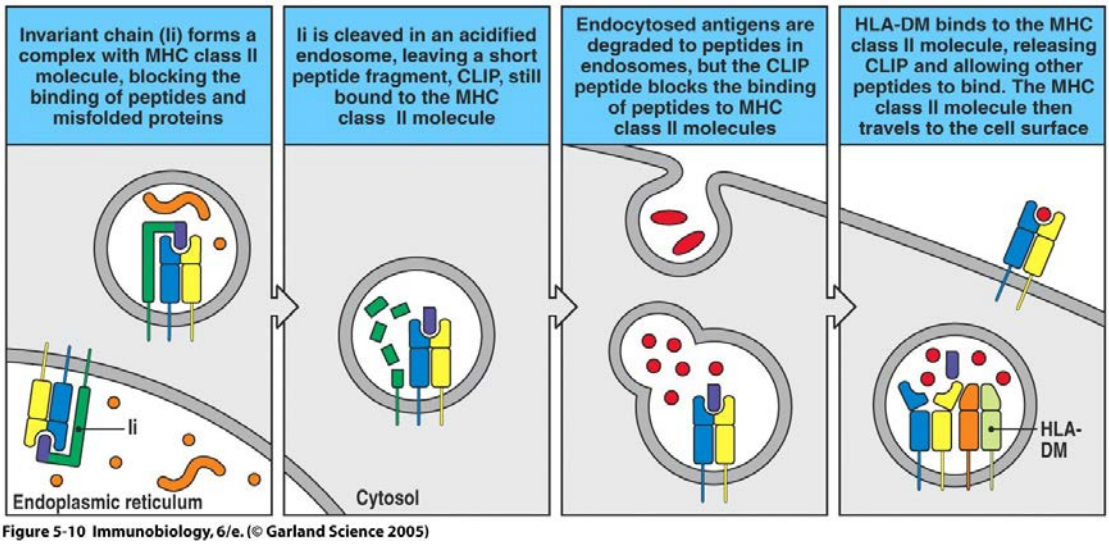


Figure 1.5: MHC Class II presentation: (From Figure 1.5-10, Immunobiolgy, 6/e Garland Science 2005) [34]

Upon presentation of antigen on MHC molecules, T-cell priming can take place. The MHC Class I-antigen complex is recognized by the T-cell receptor (TCR) and the CD8 molecule. MHC Class II presented antigen on the other hand is recognised by TCR and CD4 molecule. This activates the T-cells via several signalling pathways and triggers clonal expansion as well as effector functions including the up-regulation of cytokines such as IFN-γ, IL-2 and other inflammatory cytokines. T-cell activation will then take 4-5 days from its resting phase to a full blown activated state and trafficking of the activated T-cells to the site of infection[34]. The need for co-stimulatory factors is also noted and was explained in the previous section (Section 1.2). Generally, activation of $CD8^+$ T-cells leads to effector functions while CD4+ T-cells provide help.

1.4 Peptide vaccination and $CD4^+$ T helper cells

Work on cancer vaccination, as well as other peptide vaccinations has been centred on the activation of $CD8^+$ T-cells, whereby MHC Class I restricted peptides are utilised either in combination with adjuvants and administered directly as a vaccine or by loading MHC class I restricted peptides onto dendritic cells ex-vivo before administration. However in a published review by Janssen *et al.*, a consistent deficiency in memory $CD8^+$ T-cells was shown when there was absence of $CD4^+$ T-cells or T-helper cells (T_H)[40]. Depletion of $CD4^+$ cells prior to vaccination was shown by Schoenberger *et al.*, to block cytolytic capacity of peptide-specific T-cells in an *in vitro* stimulation assay after 14 days of vaccination[41] showing the importance of $CD4^+$ T-cell help. In the absence of help, secondary exposure to the antigen shows no secondary expansion of the $CD8^+$ effector cells *in vivo*[40]. This clearly shows the importance of $CD4^+$ T_H cells in the generation of an effective immune response in terms of continued protective immunity provided by the antigen specific $CD8^+$ effector T-cell[40]. A study by Sun *et al.* showed that mice that lack $CD4^+$ T-cells were able to mount a primary response to *Listeria monocytogenes* with similar capacity to wild-type mice for eradication of infection. However, the protective capacity then decreases in the CD4 deficient mice, suggesting that $CD8^+$ memory T-cells generated without $CD4^+$ T_H cells are unable to mount a response against a secondary infection[42].

Rocha *et al.* suggest that generation of $CD8^+$ effector T-cells and $CD8^+$ memory T-cells are two separate entities and the generation of a strong effector response does not end with the generation of $CD8^+$ memory T-cells. This implies that there is a separate process of $CD8^+$ memory T-cell differentiation that might mimic B-cell memory generation, which depends on the help of antigen-specific $CD4^+$ T cells. This process is irreversible and occurs progressively during the late expansion phase of the primary immune response with a similar mechanism; that is, the triggering of CD40 expression on B or $CD8^+$ T-cells.[43]. $CD4^+$ T-cell activation as an effect of MHC Class II peptide presentation produces cytokines that help the activation and expansion of $CD8^+$ T-cells, eosinophils and macrophages, leading to more effective immune mediated protection including protective immunity against cancer and for therapeutic vaccinations against cancer[44].

Although it is clear that $CD4^+$ T-cells are required to provide help for the proliferation of $CD8^+$ T-cells and the differentiation of memory $CD8^+$ T-cells, the method by which it is achieved is not entirely clear. An earlier model of help was introduced by Mitchison in 1987 whereby for the $CD4^+$ T-cells to provide help in the form of cytokines, a cluster of 3 cells were required. This model requires $CD4^+$ and $CD8^+$ T-cells to be bind to the same APC, so that the local paracrine action of cytokines secreted by $CD4^+$ T-cells binding the MHC class II molecules on the APC, would support the clonal expansion of the adjacent $CD8^+$ T-cells which are bound to the MHC class I molecules of the same APC[45]. However, the probability of this happening is very low and a new model was introduced by Schoenberger *et al.* in 1998, whereby the $CD4^+$ cells activate DC via CD40L-CD40 interaction resulting in a "licensed" DC to become fully competent to activate naive $CD8^+$ T-cells. This enables activated $CD4^+$ T-cells to amplify their helper function as they could license the DC to support $CD8^+$ T-cell responses[41]. Ridge *et al.* also showed clear evidence that when DC are pre-exposed to activated $CD4^+$ T-cells, they become able to support $CD8^+$ T-cell responses *in vitro*[46].

Toll-like receptors (TLR) were shown to trigger a variety of inflammatory responses and to mediate the activation of DC when bound to Pathogen Associated Molecular Patterns (PAMPs). The recognition of PAMPs by TLRs suggests the possibility of direct activation of DC and therefore bypassing the need for $CD4^+$ T-cell help as TLR mediates the release of cytokines that lead to an inflammatory environment[47, 48]. However, the presence of an inflammatory environment does not render $CD4^+$ T_H help redundant as observed in the chronic infections of lymphocytic choriomeningitis virus (LCMV). In the absence of $CD4^+$ T-cell, LCMV infection causes impaired cytotoxic lymphocyte (CTL) responses[49]. Specifically, the absence of $CD4^+$ T-cells causes an inability to control viral replication and the functional inactivation of CTL responses in mice[49]. CD40L deficient mice which lack the ability to mount $CD4^+$ T-cell responses were also shown to allow progression of LCMV[50]. Therefore, the vaccine mediated induction of effective immunological responses against cancer and even many infectious agents (e.g. HBV)[51] appeared to require adequate activation of not only $CD8^+$ T-cells but also the corresponding $CD4^+$ T-cells. Wells *et al.* also showed that in a peptide vaccination study with combinations of TLR agonists and the presence of helper peptides or activating

antibody against CD40 are necessary to generate maximum antigen specific CD8 T-cells responses[52].

Memory T-cells play an important role in increasing the survival against a previous exposure to a pathogen in a general population[53]. After pathogen clearance, excess number of pathogen specific T-cell clones occupy the lymphoid compartment and dominates the T-cell repertoire. In order to restore the immune homeostasis, 90% of effector T-cells undergo apoptosis or programmed cell death[54]. The 10% of the remaining population differentiates into small population of memory cells which will persist and protect a subsequent infection[55].. The benefit of memory T-cells (especially central memory T-cells), lies in its capability of self renewal through stimulation with normal levels of IL-7 instead of requiring an antigen presenting cell to stimulate its proliferation leading to rapid response against a pathogen compared to naïve T-cells[56]. In a model suggested by Wherry *et al.*, (2003), generation of effector memory or central memory T-cells are part of a continuum in a linear differentiation of naive T-cells to effector T-cells to effector memory T-cells to central memory T-cells. This was demonstrated in a LCMV infection C57BL/6 mouse model whereby clearance of pathogen leads to decrease in effector memory T-cells characterized by antigen specific $CD62L^{lo}CCR7^{-}CD27^{lo/int}$ CD8 T-cells and an increase in central memory T-cells characterized by antigen specific $CD62L^{hi}CCR7^{+}CD27^{hi}$ CD8 T-cells[57]. The central memory T-cells generated were also shown have significantly improved antigen driven proliferation rate both *in vivo* (adoptive transfer of T-cells) and *in vitro*. Therefore, in order to generate an immunological response which would be able to protect against relapse in cancer or provide an improved secondary infection, generation of central memory T-cells will play a crucial role in the development of a vaccine.

1.5 Peptide vaccination and Tregs

The use of peptides for stimulation of T-helper function is important for the induction of effective immunity but in cases where repeated vaccination is required to generate optimal CD8 T-cell responses, there will be a risk of generating antigen specific regulatory T-cells (Tregs: $CD4^+CD25^+FoxP3^+$). In 1959, where clonal selection was first described, discrimination between self and non-self by the immune system was first established[58]. This led to the discovery of T and B cell clonal deletion in their respective immature stages of development where these clones are exposed to self antigens. The deletion of the potential self reactive clones were found to be incomplete, therefore leading to the notion that a regulatory system would be needed to avert immune mediated responses against self antigens[58, 59]. This was first shown in detail in 1969 where neonatal thymectomy of normal mice which were 2-4 days old were found to lead to developmental arrest of the ovaries in female mice. Thymectomy after 7 days of birth did not cause a similar problem with the development of the ovaries and thymus grafting enabled restoration of ovarian development showing that the developmental arrest was of an autoimmune nature[59, 60].

Another concept leading to the notion of possible presence of these regulatory T-cells surfaced following the observation of autoimmune responses in the thymetomised mice. An important observation was made by Penhale *et al.* in 1973, showing that thymectomy of adult rats, followed by sub-lethal irradiation, caused development of autoimmune thyroiditis in rats. Transfer of T-cells, and specifically $CD4^+$ T-cells, was shown to prevent the disease. This pointed towards the presence of cells within the $CD4^+$ population that confer inhibitory immune regulation, then averting the autoimmune thyroiditis[61]. It was only in 1985 that experimental proof showed direct correlation between $CD4^+$ T-cells which are $CD25^+$ and immune regulation. A critical experiment was as illustrated in Figure 1.6 A. Whole splenic $CD4^+$ T-cells, or $CD25^-$ $CD4^+$ T-cells, were transferred into immune deficient nude or severe combined immunodeficient (SCID) mice. Mice transfused with whole $CD4^+$ T-cells developed normally as the transfusion involved both effector and regulatory T-cells. However, when the spleen cells were depleted of $CD25^+$ T-cells,

(i.e. the regulatory cells), mice develops autoimmune disease, inflammatory bowel disease (IBD) and hyper reactivity to non-self antigens[62].

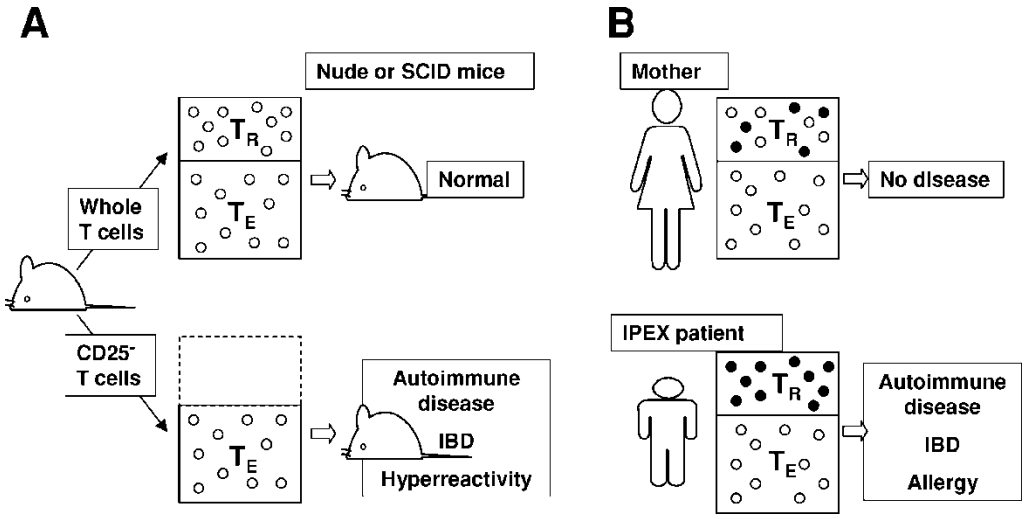


Figure 1.6 Tregs discovery: (A) Transfer of T cell suspensions depleted of $CD25^+CD4^+$ Tregs (TR) cells induces autoimmune disease and IBD, demonstrating heightened immune responses to nonself-antigens in athymic nude or SCID mice. (B) In male children afflicted with IPEX, their mothers with hemizygous defects of the FOXP3 gene bear defective and normal Tregs (TR) cells as a mosaic because of random inactivation of the X chromosome in each Treg cell. The mothers are completely normal because normal Tregs (TR) cells dominantly control the activation and expansion of effector T (TE) cells that mediate autoimmune disease, IBD, and allergy. However, in the affected children there are only defective Tregs, hence autoimmunity. Open circles represent intact TR or TE cells; closed circles indicate defective TR cells (Sakaguchi, 2004)[59].

IPEX is an X-linked immunodeficiency syndrome associated with autoimmune disease in multiple endocrine organs, IBD, atopic dermatitis and fatal infections in humans. Initial identification of Tregs and its correlation with the FoxP3 gene came with the identification of the Scrufy mice. These mice exhibit an X-linked recessive mutant with lethality in hemizygous males within a month of birth, exhibiting hyper-activation of $CD4^+$ T cells and overproduction of pro-inflammatory cytokines, a similar phenotype to IPEX in humans. The murine FoxP3 (forkhead box P3) gene that encodes for Scrufin, was identified and found to be mutated in the Scrufy mice in 2001. That enabled the identification of the human orthologue of the murine FoxP3 which was also found to be mutated in the IPEX patients in the same year[63, 64].

From that point onwards, the phenotyping of Tregs and the role of FoxP3 as the master controller of regulatory T-cells took centre stage in the study of immune regulation[59, 65]. Tregs in mice are phenotyped as $CD4^{+}CD25^{+}FoxP3^{+}$ T-cells, while Tregs in human are much more complex and phenotyped as $CD4^{+}CD25^{high}CD127^{-}FoxP3^{+}$ T-cells[59, 66].

Tregs either in human or mice are divided into two main types, naturally occurring Tregs (nTregs) and induced Tregs (iTregs). Naturally occurring Tregs are produced in the thymus after exposure to self antigens. These Tregs are the core player in averting autoimmune diseases in humans as discussed earlier. Induced Tregs however are regulatory T-cells that obtain a regulatory function after exposure to antigen. The iTregs produce IL-10, TGF-β and are converted from non-FoxP3 $CD4^{+}$ T-cells[67]. The iTregs are antigen specific Tregs and have been shown to be generated when there is continuous antigen stimulation in the absence of a co-stimulatory function. One of the earliest discoveries of this condition was shown by Apostolou *et al.* in 2004 when minute doses of antigen were delivered subcutaneously via an osmotic pump, leading to generation of antigen specific Tregs[68]. This discovery was important to denote the fact that continuous stimulation of $CD4^{+}$ T-cells could lead to the generation of iTregs. These are antigen specific regulatory cells that are able to cause major limitations in attempts to induce better protective immunity against cancer or chronic infections by repeated vaccinations. However, the coralory has been repeated vaccinations with class II presented peptides to induce protective tolerance against autoimmune diseases such as autoimmune diabetes[69].

Therefore in this study (see Chapter 3), the focus was on manipulating the T-helper peptide stimulation in order to generate an improved immunological response, by avoiding the stimulation of Tregs.

1.6 Peptide vaccination and adjuvants

As previously discussed in section 1.4, vaccination with a single class I peptide would result in a suboptimal immunological response. However, with the inclusion of adjuvants such as TLR agonists, a strong CD8 T-cell response could be generated without T-helper peptides despite not generating recall responses[47, 48]. History of using an adjuvant (from the word adiuvare which means to aid in Latin) to improve vaccination outcome started in 1926 when the diphtheria toxin in addition with a potassium alum precipitate generated high antigenic responses for veterinary vaccinations against diptheria[70, 71]. This is because classical vaccination in the absence of adjuvants is insufficient for generation strong immunological responses for poorly immunogenic pathogens (Tuberculosis, malaria, HIV, etc). This is particularly relevant for patients that suffer from immune dysfunction, the elderly or in conditions of chronic infection. To further understand how adjuvants are able to enhance the immune response generated in response to vaccination, understanding of the parts of the immune system that can be exploited is required (Figure 1.7).

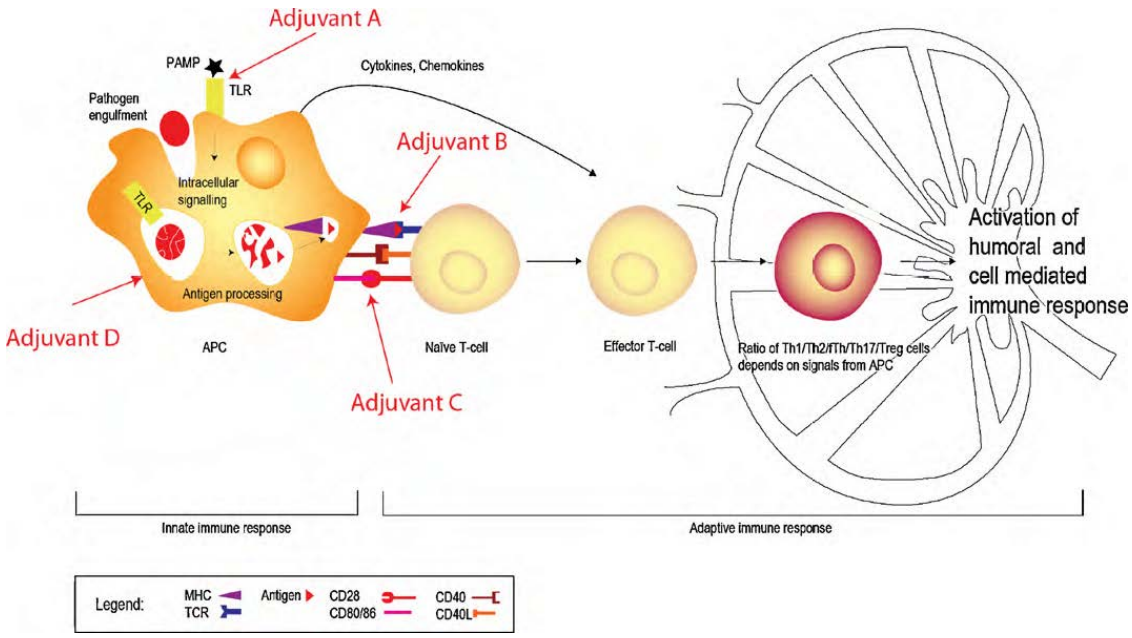


Figure 1.7 Exploiting the immune system for discoveries of new adjuvant: The activation of T-helper cells (Th1 and Th2) requires specific signals from an antigen presenting cell (APC). There are several targets for adjuvants to interact with this process and to modulate the adaptive immune response. Adjuvants can impact at different levels such as: (1) Recognition of a pathogen associated molecular pattern [27] by a toll-like receptor (TLR). (2) Presentation of antigens by the major histocompatibility complex (MHC) to the T-cell receptor (TCR). (3) Recognition of co-stimulatory signals like CD28 or CD40L on T-cell by CD80/86 or CD40 ligands. (4) Intracellular signalling processes in the APC (adapted from Geert Leroux-Roels, 2010)[71].

As described in section 1.2, the immune responses can be divided into innate immunity and acquired immunity. Adjuvants A and D (Figure 1.7), represent PAMPs which are recognised by TLRs on APC. These danger signals in the form of microbial cell wall, virus DNA, etc., provide the initial stimulation that primes the immune system to start producing pro-inflammatory cytokines such as tumour necrosis factor (TNF), type 1 interferon, IL-1, etc, by NF-κB mediated stimulation of gene expression. The use of TLR agonists will be further elaborated in the following section on novel adjuvants. Some adjuvant such as adjuvant B shown in Figure 1.7 represent emulsions or alum precipitates that allow for improved antigen presentation by MHC molecules. The depot effect created by emulsion also enables the slow release of antigen as well as increased recruitment of APCs to the site of

vaccination. The proper presentation of the antigen then allows for an improved stimulation of T-cells, leading to improved immunological responses. An example of such an adjuvant is MF59, a squalene in water emulsion that improves the response against influenza vaccine in older individuals (Podda *et al.*)[72]. This again will be further reviewed in the discussion of classical adjuvants in the following section. Adjuvant C (Figure 1.6) represents a co-stimulatory molecule needed for the efficient stimulation of T- and B-cells. This co-stimulatory signal is usually provided by "licensed" APC in the form of B7 family members (B7.1, B7.2, etc). One example of using such an adjuvant was presented in a study by Chan *et al.* in 2005 where primary AML cells were genetically modified by infection with a lentivirus that encodes for B7.1, plus IL-2 as an additional stimulating factor allowing the expansion of $CD8^+$ T-cells when co-cultured with autologous peripheral blood mononuclear cells (PBMC)[9]. This has also led to a clinical trial in AML patients (unpublished data). A list of adjuvants that exploit the innate immune system can be seen in Table 1.2. The types of adjuvants used today can be divided into classical adjuvants, novel adjuvants and adjuvant systems[71].

1.6.1 Classical adjuvants

Classical adjuvants are those that enable the enhancement of antigen presentation by APCs utilising the depot effects. A good example of a classical adjuvant is alum salts which have been used extensively for vaccination against hepatitis A (Havrix™, Vaqta™ and Avaxin™), hepatitis B (Engerix-B™ and Recombivax™ HB), human papillomavirus (Gardasil™), etc[71]. In its early days of use, aluminium salts were shown to be working by providing a depot of the antigen at the vaccination site, thus improving the attraction of APC to the site, and enhances the uptake of antigen by APC for presentation[73]. However, in 2004, Morefield *et al.* used alpha casein (AC) which was labelled with a green fluorescent dye to determine its uptake by APCs when the AC was adsorbed onto alum salt. This showed that alum absorbed AC was better taken up by the APC compared to non-absorbed AC. The multivalent presentation of AC in combination with alum salt increased the efficacy of its uptake by APC[71, 74].

Adjuvant	Major Immunostimulatory Component(s)	Innate Receptors or Pathway Activated	Principal Immune Responses Stimulated
Licensed Adjuvants			
Alum	aluminum salts	NLRP3 inflammasome (?)	Ab, Th2 (+ Th1 in humans)
MF59 and AS03	squalene-in-water emulsions	tissue inflammation (no receptors defined)	Ab, Th1 + Th2
AS04	MPL plus alum	TLR4 and inflammasome (?)	Ab, Th1
Adjuvants in Widespread Experimental Use or in Late Stage Clinical Development			
Poly-IC (also Poly-ICLC)	synthetic derivatives of dsRNA	TLR3, MDA5	Ab, Th1, $CD8^+$ T cells
MPL and formulations (AS01, AS02)	MPL and QS-21	TLR4 (MPL), ? (QS21)	Ab, Th1
Flagellin, flagellin-Ag fusion proteins	Flagellin from *S. typhimurium*	TLR5	Ab, Th1 + Th2
Imiquimods	imidazoquinoline derivatives	TLR7, TLR8 or both	Ab, Th1, $CD8^+$ T cells (when conjugated)
CpG oligodeoxynuceotides and formulations (IC31, QB10)	synthetic phophorothioate-linked DNA oligonucleotides with optimized CpG motifs	TLR9	Ab, Th1, $CD8^+$ T cells (when conjugated)
CAF01	trehalose dimycolate (cord factor)	Mincle	Ab, Th1, Th17
ISCOMS and ISCOMATRIX	saponins	mechanism undefined	Ab, Th1+ Th2, $CD8^+$ T cells
IFA (and Montanide formulations)	mineral or paraffin oil + surfactant	mechanism undefined	Ab, Th1 + Th2
CFA	IFA + peptidoglycan, trehalose dimycolate	NLR, inflammasome, Mincle, TLR?	Ab, Th1, Th17

Table 1.2 Types of adjuvants currently in use: Table list the type of adjuvants in use that exploit the innate immunity to provide help for better induction of immunological responses to vaccines. The pathway that is activated for the adjuvant and the principal immune responses stimulated are indicated. (Coffman *et al.*, 2010(Coffman, 2010 #5820))

Emulsions are also widely used as classical adjuvants. Emulsions are normally made of oil and water combinations. One typical example of an emulsion is complete Freund's adjuvant (CFA), used in many animals studies, created by Jules Freund especially for the stimulation of B-cells to produce antibodies. CFA has been used extensively in generation of hybridomas for the production of monoclonal antibodies[75]. CFA contains heat-inactivated *Mycobacterium tuberculosis*, paraffin oil and surfactant, which was used in a priming stage together with an antigen followed by boosting with an incomplete Freund's adjuvant (IFA) which does not contain the inactivated mycobacteria. However, due to its reactogenic properties in the human body, CFA has been banned for human use[71, 76]. This has paved the way for other more refined emulsion adjuvants such as MF59 which has been described earlier in this section. Another key emulsion that is widely used at the present time is montanide. Montanide which is a squalene based adjuvant has been widely used for

several types of cancer vaccination studies. Montanide was used instead of IFA in cancer vaccination targeting the extra domain (ED-b) of fibronectin[77]. There has also been phase II studies on a commercial montanide vaccine for non-small-cell lung cancer called CimaVax EGF targeting epidermal growth factor showing that 73% of vaccinated patients generated antibodies against EGF[78].

Other classical adjuvants include liposomes and virosomes. They are synthetically made nanospheres that encapsulates antigen, serving as antigen delivery vehicles. Modulation of lipid composition, size and charge of the liposomes have been studied and the inclusion of the synthetic polymer poly-(ethylene glycol) (PEG) in liposome compositions has extended the liposome's blood-circulation time while reducing mononuclear phagocyte system uptake[79]. However, liposomes do not generally have immune activation properties and only act as a delivery system[71]. A variety of approved liposomes used in cancer treatment and other diseases has also been approved[79]. However, the use of liposomes is not restricted to vaccination, but also a variety of other applications including drug delivery (see Figure 1.8)

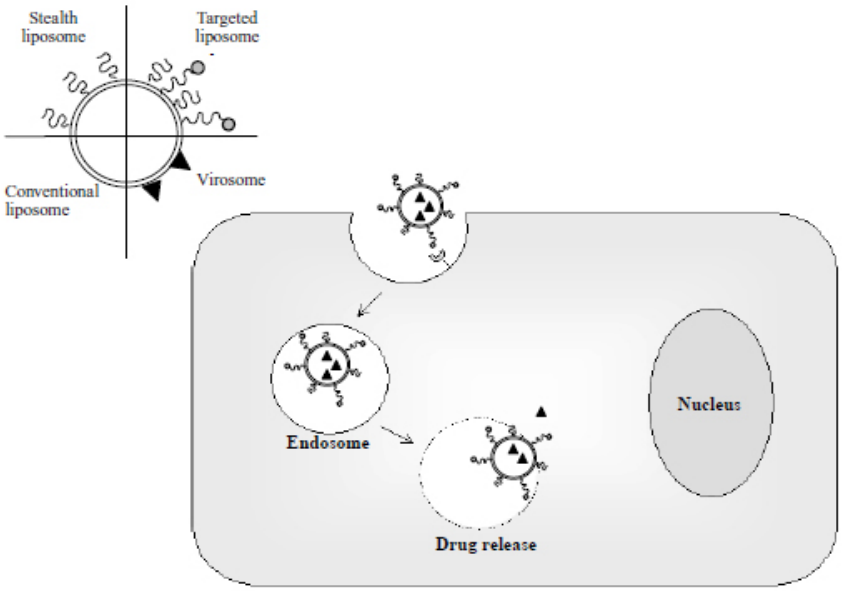


Figure 1.8 Types of liposomes: The different types of liposomes - conventional, stealth and targeted, as well as virosomes and their uptake by target cells including APC (Adapted from Immordino *et al.*, 2006)[79].

1.6.2 Novel Adjuvants

Novel adjuvants are more refined products made by understanding the nature of the immune system, in order to generate an improved vaccination response. Amongst the key novel adjuvants are the Toll-like receptor (TLR) agonists. TLRs are a family of pathogen associated molecular pattern [27] immune-recognition receptors that are utilised by the innate immune cells (macrophages, dendritic cells and monocytes) to recognise certain molecular patterns presented by microbial pathogens[80]. Recognition of these danger signals induce the innate immunity directly and generate a pro-inflammatory environment which leads to enhancement of acquired immunity (activation of B- and T-cell responses). TLR agonists have also been shown to be able to skew the T helper responses to either the Th1, Th2 or Th17 types[71, 76]. TLR agonists will be discussed in more depth later. A list of TLR agonists are listed in Table 1.2 and Figure 1.9.

One of the many TLR agonists in relatively common use is MPL, monophosphoryl lipid A (Corixa) which is a detoxified version of lipopolysaccharide[81] from *Salmonella minnesota*. MPL, like LPS binds to TLR-4 on the surface of APCs (monocyte and dendritic cells)[76], leading to activation of NF-κB and production of inflammatory cytokines such as TNF-α, promoting the generation of acquired immune responses. LPS activates NF-κB through the MyD88 and TRIF pathways but MPL activates NF-κB only through the TRIF signalling pathway as established by Mata-Haro, *et al.* (2007). This study also showed that MPL signalling activity leads to a Th1 type of response instead of the mixed Th1 and Th2 responses showed by LPS[76, 82]. LPS mediated stimulation was shown to be a more rapid inducer of NF-κB activation via MyD88, while MPL activation is considered to be a slower inducer of NF-κB activation by the TRIF signalling pathway. MPL which is considered as a safe adjuvant for use in human applications has been approved for hepatitis B and human papillomavirus vaccination (Ceravix)[83, 84].

Double stranded RNA, generated in a subtype of viral infections was discovered by Alexopoulou *et al.* (2001) to activate TLR-3 in mammalian dendritic cells. Viral infection of mammalian cells resulted in activation of an innate immune response mediated by type I IFNs, IFN-α and IFN-β as well as interleukin (IL-6 and IL-12). Expression of TLR-3 is found intracellularly in APCs compared to TLR-4 which is

found on the extracellular matrix. Alexopoulou *et al.* used a 293T cell line which was genetically modified to produce TLR-3, TLR-6 and TLR-9 with a reporter gene that is dependent on NF-κB activation. This allowed the identification of NF-κB activation by TLR-3 stimulation, using Polyinosinic:polycytidylic acid (Poly I:C) which is a synthetic double stranded RNA[80]. Longhi *et al.* in 2009 showed that Poly I:C was an effective adjuvant in a dendritic cell based HIV-Gag protein vaccine in mice. Poly I:C was shown to be able to generate a predominantly Th1 response[85]. Poly I:C was shown to be able not only to stimulate predominantly Th1 responses, but also the activation of NK cells[86].

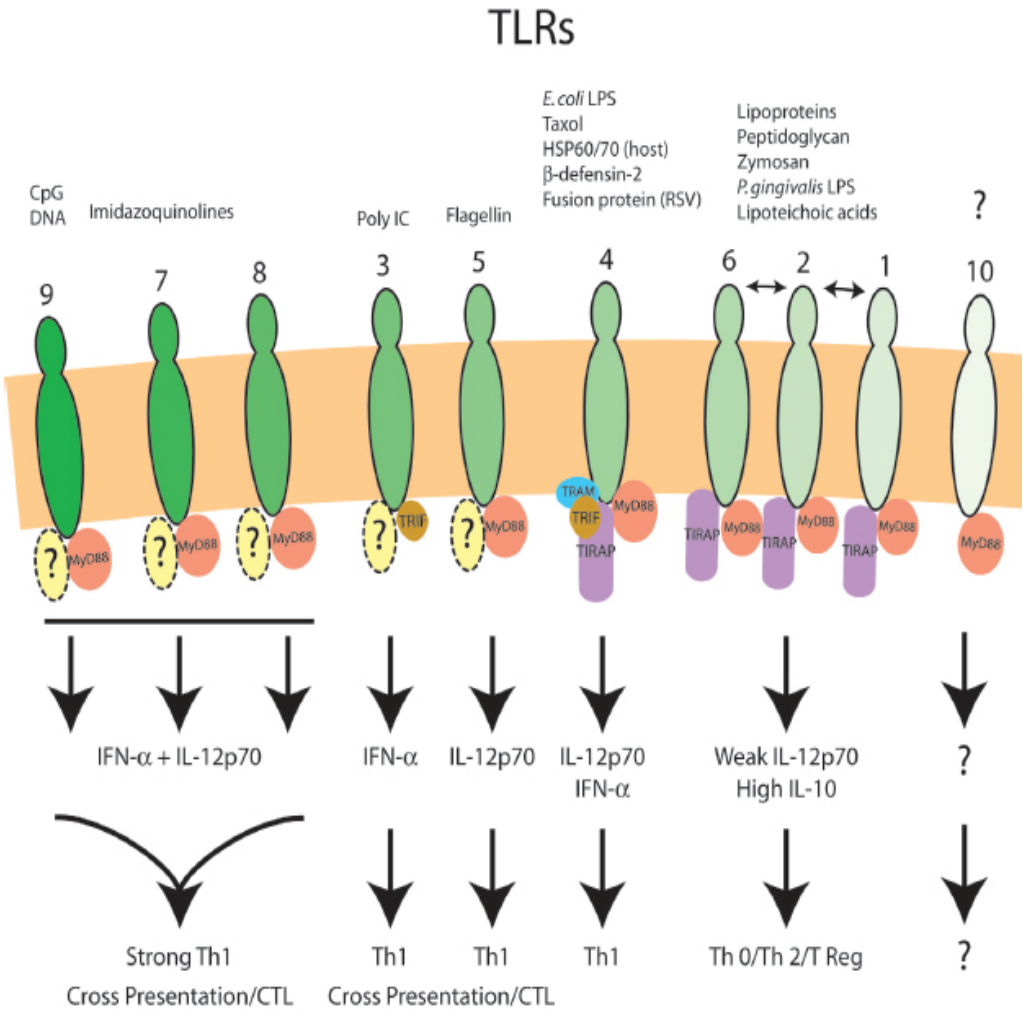


Figure 1.9 TLR agonists and the TLR receptors: The binding of TLR agonist to its receptor stimulates the indicated activation pathways (TRIF, TIRAP, TRAM or MyD88) in the activation of NF-κB in APCs, leading to the production of different types of cytokines. Depending on the type of cytokines involved Th1, Th2 or Th17 (not shown here but referred to in Table 1.2) responses are activated (Pulendran B., 2004)[87].

Poly I:C has shown efficacy when co-administered with PR8 from H1N1 influenza virus administered by an intranasal route, resulting in antibody responses as indicated by increased levels of IgA and IgG in Balb/C mice[88]. This has made Poly I:C a key adjuvant in human influenza protective vaccinations[89]. Although TLR-3 was shown to be expressed at a very low level in mice and upregulated only in response to LPS activation, TLR-3 is widely expressed in human dendritic cells without the need of LPS activation, thus making TLR-3 agonists potent adjuvants in the human setting[90].

Single stranded RNA (ssRNA) from influenza virus was first found to induce generation of IFN-α in dendritic cells by TLR-7 recognition of ssRNA and stimulation of MyD88 signalling pathway, by Diebold et.al. and Lund *et al.* in 2004[91, 92]. The use of Loxorobine, a TLR-7 agonist, led to the discovery of TLR-8 activation with ssRNA[93]. ssRNA rich in guanosine and uracil are good activators of TLR-7 and TLR-8 found in the endosome of monocytes and dendritic cells. ssRNA can be easily degraded in the intracellular matrix by RNAase and therefore requires modification to enable optimal use in an adjuvant setting. These studies have led to the discovery of small synthetic compounds of the imidazoquinolines such as imiquimod (TLR-7 agonist) and resiquimod (TLR-7 and TLR-8 agonist) as potent TLR-7 and TLR-8 agonist that can be used as adjuvants. Other guanosine and uracil analogues were also used to avoid the breakdown by RNAase[76]. Imiquimod is currently use in clinic studies to treat melanoma and recent studies show that imiquimod used topically on the skin of mice post vaccination with genetically modified *Listeria monocytogenes* (modified to express Tyrosinase related protein-2, $TRP2_{180\text{-}188}$ which is an antigen up-regulated in melanoma) increases survival rate in B16 melanoma challenged mice[94].

TLR-9 is the only intracellular TLR that binds to DNA sequences from bacteria and viruses. CpG nucleotide motifs are recognized by TLR-9, initiating the NF-κB signalling pathway through MyD88, leading to the production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IFN-γ and IFN-α[71]. In vaccinations, synthetic CpG optimized motifs of 18-25 bases, are widely used as adjuvants[76].

Another novel adjuvant is immune stimulating complexes (ISCOMs) which are solely used as delivery vehicles[71]. ISCOMs are cage-like nanoparticle saponins that

are purified from the bark of a South American tree called *Quillaja saponaria*. These saponins are made up of phospholipids and cholesterol. ISCOMs are used to encapsulate antigen for delivery in vaccination studies. Solubilisation of antigens and antigen-free ISCOM allowed the formation of ISCOMATRIX. These matrixes are not recognised by any of the pattern recognition receptors but enhance antigen uptake by APCs and prolong the retention of the vaccine in the draining lymph nodes. Maraskovsky *et al.* (2009) showed that the use of ISCOMATRIX adjuvant induces a broad range of specificity to the vaccine antigen which includes robust antibody, $CD4^+$ and $CD8^+$ T-cell responses[95].

1.6.3 Adjuvant systems - Combination of classical and novel adjuvants: The challenge of therapeutic vaccination especially in chronic diseases such as cancer, and chronic infections established by HBV or HIV, as well as vaccination in immune suppressed individuals, such as the elderly, has marked the need for improved vaccine adjuvants. This has led to the use of a combination of two or more type of adjuvants that will allow the generation of an enhanced immune response. The use of combinations of adjuvants could allow for synergistic enhancement of responses to vaccinations. Pharmaceutical companies such as GSK have developed a number of adjuvant systems that are used in clinical application (Table 1.3).

Adjuvant system	Components	Current applications
AS01	MPL, QS21, liposome-based	Malaria, tuberculosis
AS03	α-Tocopherol oil-in-water emulsion-based	H5N1 influenza, seasonal influenza, H1N1v influenza (*Arepanrix*™, *Pandemrix*™
AS04	MPL absorbed on aluminium salt	Hepatitis B (*FENDrix*™), HPV (*Cervarix*™), *Herpes simplex* virus (HSV) vaccine,
AS15	MPL, QS21, CpG, liposome-based	cancer immunotherapy, Non-Small Cell Lung Cancer (NSCLC), melanoma

Table 1.3 GSK biological adjuvant systems, its components and current application in the clinic[76].

There is also a number of studies in which combinations of adjuvants are used (for example see Wells *et al.,* 2008). The combination of adjuvants used by Wells *et al.*, consist of an emulsion made of squalene and Tween 80, 2 TLR agonists (combination of a MyD88 and a non-MyD88 signalling pathway TLR agonist), recombinant IFN-γ plus an MHC Class I presented peptide with an MHC Class II presented peptide or anti-CD40 administered intradermally. They have called this combination of adjuvant Combined Adjuvant for Synergistic Activation of Cellular immunity (CASAC)[52]. In order to determine the best combination of adjuvants to generate $CD8^+$ T-cell responses, Wells *et al.,* utilised ovalbumin as the target antigen. [52]

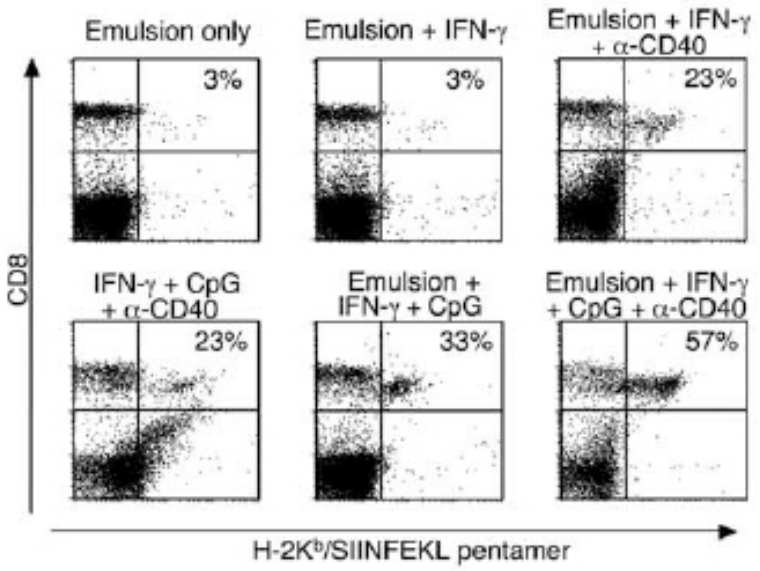


Figure 1.10 Generation of antigen specific CD8 T-cells by different combinations of adjuvant: Percentage of SIINFEKL specific $CD8^+$ T-cells in different combination of adjuvants (Emulsion used is MPL plus TDM). In the emulsion only and emulsion plus IFN-γ vaccination with SIINFEKL, low levels of SIINFEKL specific CD8+ T-cells were generated. However with the inclusion of an anti-CD40, which acts to substitute for a helper peptide to license APC, the percentage of SIINFEKL specific $CD8^+$ T-cells were seen to have increased. By removing the anti-CD40 and introducing a second TLR agonist (CpG ODN 1826), the percentage of SIINFEKL specific CD8+ T-cells were found to have increased again compared to the single TLR plus anti-CD40 combination. The best combination however was described when an emulsion, two TLR agonist, IFN-γ and anti-CD40 was included in the SIINFEKL vaccination generating the highest percentage of SIINFEKL specific CD8 T-cells. (adapted from Wells. *et al.*, 2008)[52]

Initially a series of adjuvants were tested and the immune responses were quantified with a pentamer (ProImmune) which is made of five murine H-2Kb/SIINFEKL complex bound together in a coiled-coiled matrix tagged with a fluorescent dye. Pentamers are very specific and bind only to TCR that are antigen specific. Combining pentamers and fluorescently labelled anti-CD8 allows the quantification of the percentage of SIINFEKL specific $CD8^+$ T-cells. The combination of adjuvants used and the generated immune responses could be seen in Figure 1.10. The best combination was determined after two rounds of vaccination, which were given 10 days apart, followed by quantification of immunological responses 10 days later. The vaccination not only produced high levels of SIINFEKL specific $CD8^+$ T-cells but also about 99% lysis of antigen positive target cell in an *in vivo* cyototoxic lymphocyte (CTL) assay, using SIINFEKL pulsed splenocytes as antigen positive target cells (Figure 1.11 a). These mice were injected with the peptide pulsed splenocytes that were labelled with a green fluorescent dye, Carboxyfluorescein diacetate, succinimidyl ester (CFSE), intravenously. The mice were left overnight (16-18 hours) before they were culled to harvest the spleens. Splenocytes were obtained and analysed by Fluorescence activated cell sorting (FACS). Recall responses were also observed after the vaccinated mice were rested for about 3 months before rechallenging with SIINFEKL peptides showing good memory CD8 T-cell generation (Figure 1.11 b). The rested mice were also examined for *in vivo* CTL activity and were found to be still able to produce good clearance of target cells (Figure 1.11 c), showing that the generated memory T-cells within the population were long lived and able to mount a recall response.

The potential showed by CASAC was immense and as discussed in the previous section, the use of combination of adjuvants was shown to generate the best response. Therefore, a series of combinations of different emulsion, TLR agonists and helper peptides were tested (Figure 1.12 a, b and c). This led the authors to conclude that the combination of CASAC is important in order to induce efficient immunological responses.

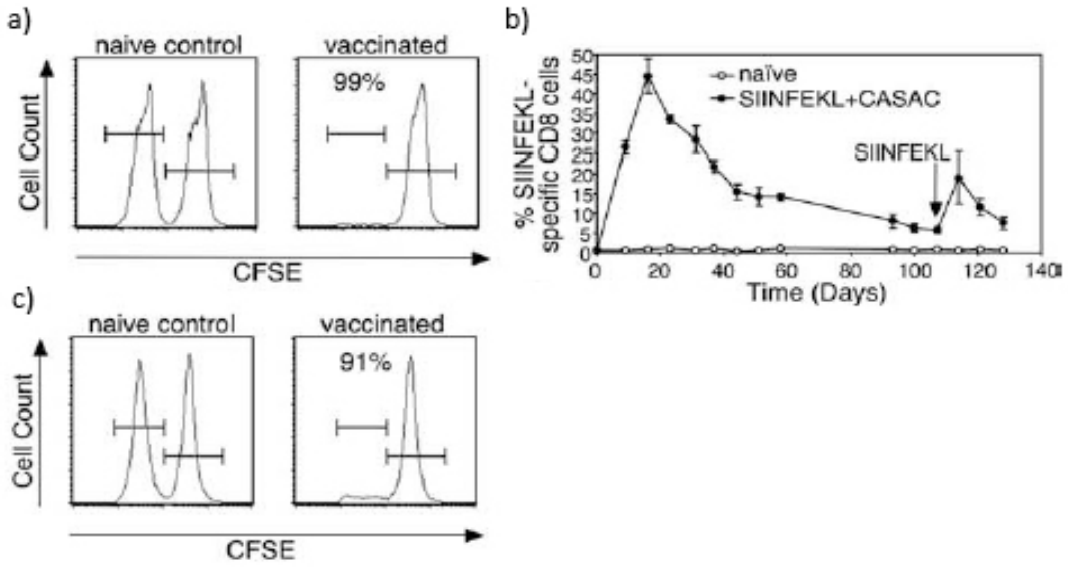


Figure 1.11 Generation of functional CD8 T-cell responses by CASAC: a) In the vaccinated mice challenged with peptide pulse splenocytes, 99% of antigen positive target cells which were labelled with low levels of CFSE dye were cleared 10 days after second round of vaccination compared to no clearance in the naive mice. Non-target (antigen negative) cells labelled with high levels of CFSE were not affected and were used as a reference to calculate the percentage of target cells lysis. b) Mice were then rested for about 3 months and a rechallenge with SIINFEKL showing the presence of a recall response. c) The generated memory CD8 T-cells were also shown to have cytolytic function as when the rested mice were challenged with antigen positive cells, they were able to mount an effective *in vivo* CTL response as indicated by the clearance of 91% of target cells, but no cell lysis in the unvaccinated mice. (adapted from Wells *et al.*, 2008)[52]

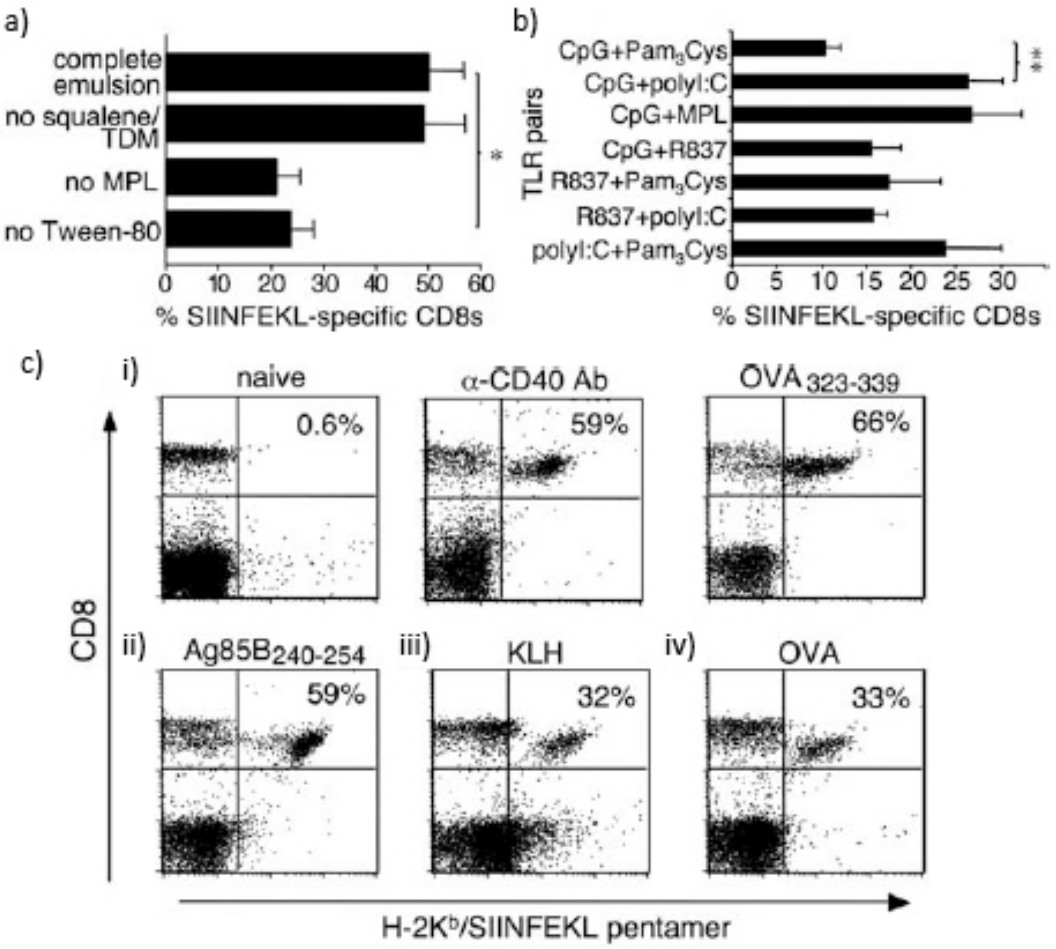


Figure 1.12 Comparison of different combinations of CASAC: a) CASAC in combination with complete emulsion (squalene and Tween80) or TDM emulsion generated the highest percentage of SIINFEKL specific $CD8^+$ T-cells making the emulsion a crucial part of the vaccination as in absence of the non-emulsion vaccine did not generate as good a response. b) Combination of TLR agonist that provided the best percentages of SIINFEKL specific $CD8^+$ T-cells were combinations of a MyD88 and non-MyD88 signalling pathway activation. c) anti-CD40 antibody or an ovalbumin T-helper peptide was shown to be required for generation of highest percentages of SIINFEKL specific $CD8^+$ T-cells. In these studies, the use of anti-CD40, a class II presented (OVA or unrelated), or whole protein (OVA or unrelated) was required. (Adapted from Wells *et al.*, 2008)[52]

In order to test the efficacy of the adjuvant combination, Wells *et al.* substituted OVA with TRP-2 to vaccinate against a self antigen which has been shown to be up-regulated in melanoma cells. Vaccinating the mice with TRP-$2_{180-188}$ peptide in combination with CASAC, increased levels of IFN-γ expressing cells. Higher percentages of IFN-γ expressing cells were seen in mice vaccinated with increasing quantities of peptide (Figure 1.13 a). The improvements in IFN-γ production were also translated into improved clearance of target cells (splenocytes) that were pulsed with the TRP-$2_{180-188}$ peptide (Figure 1.13 b). Intravenous injection of B16 melanoma cells in the vaccinated mice, resulted in the recovery of 2-3 $\log_{10}$ lower levels of B16, 3 days later, in the lungs of the TRP-2 peptide vaccinated mice (Figure 1.13 c)

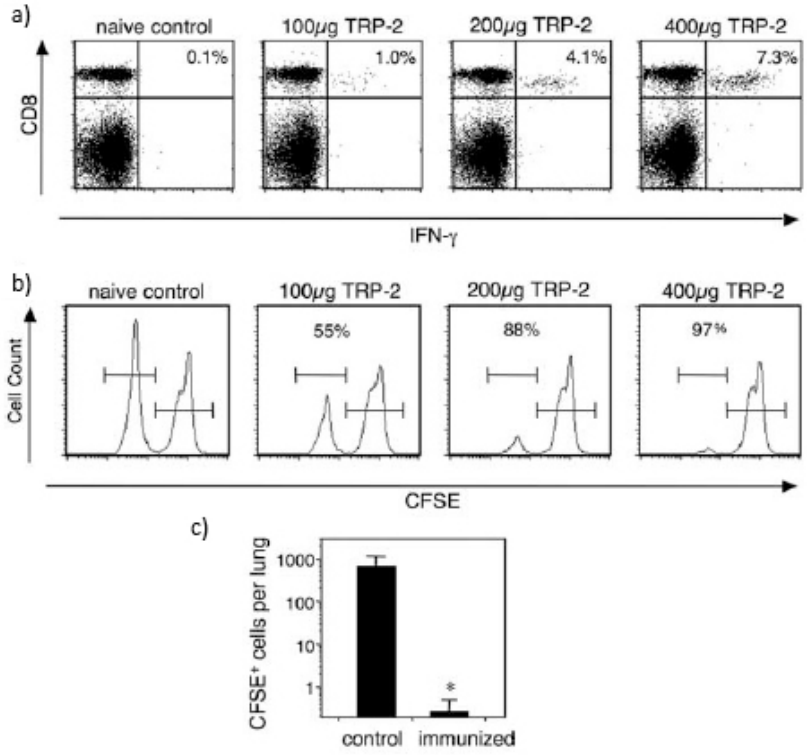


Figure 1.13 Vaccinating against a self antigen with CASAC: a) Mice were vaccinated with CASAC plus TRP-$2_{180-188}$ peptide ranging from 100 - 400 µg. The increasing quantity of peptide resulted in increased detection of antigen specific IFN-γ producing cells. b) Challenging the mice with an *in vitro* CTL assay using peptide pulsed splenocytes also showed the improved lysis of antigen positive target cells. c) In the vaccinated mice, there was substantially reduced recovery of B16 melanoma cells found in the lungs after 3 days of intravenous inoculation of B16. (adapted from Wells *et al.*, 2008)[52]

In a therapeutic setting, CASAC was also shown to be effective in the B16 melanoma tumour model. B16 cells were injected subcutaneously (10^5 cells per mouse) and vaccination with CASAC and TRP-$2_{180\text{-}188}$ were conducted on days 3, 11 and 19. Vaccination without CASAC showed only partial improvement in tumour diameter and survival compared to the unvaccinated mice. In the mice vaccinated with TRP-2 in combination with CASAC, there was little increase in the tumour size of the mice and 80% rejected the previously inoculated tumour and survived for the duration of the study (Figure 1.14 a and b). CASAC/TRP-2 vaccination followed by CD8 antibody mediated depletion of CD8 T-cells substantially abrogated the therapeutic effect of vaccination, demonstrating the involvement of CD8 T-cells in the CASAC mediated immune protection.

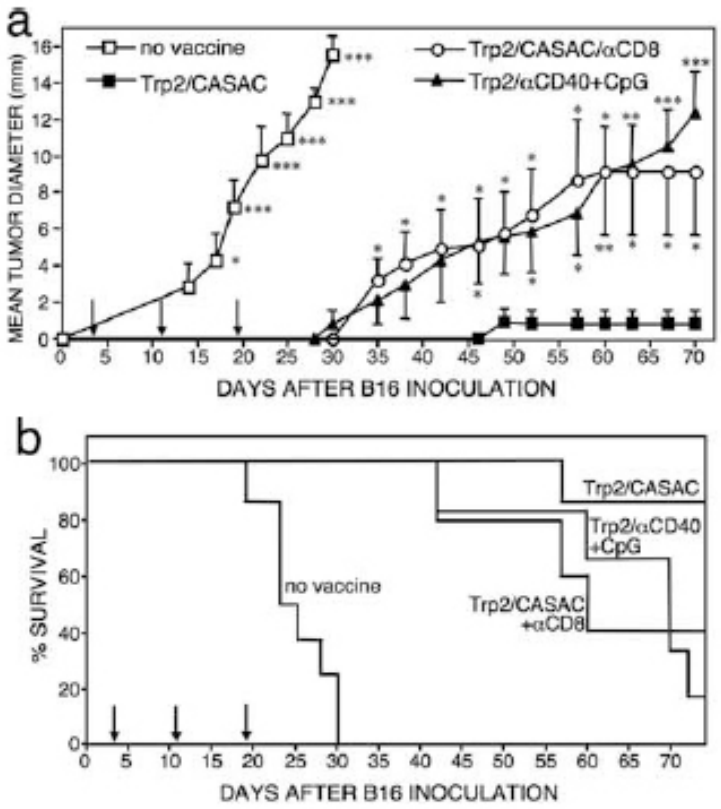


Figure 1.14 Tumour rejection by CASAC vaccination: a) Mean tumour diameter recorded in the B16 innoculated mice and the effect of TRP-$2_{180\text{-}188}$ vaccination with CASAC, CASAC plus a depleting anti-CD8 or CpG plus anti-CD40. The best tumour rejection was obtained when CASAC is use as an adjuvant for the TRP-2 vaccination. b) Similar results were seen in the percentage of mice that survived the tumour inoculation. Vaccination resulted in death of all mice while 80% of mice vaccinated with CASAC survived till day 70 post the inoculation. (Adapted from Wells *et al.*, 2008)[52]

In summary, CASAC vaccination improved the percentages of antigen specific T-cells generated by peptide vaccination against a foreign antigen (OVA) as well as a self antigen (TRP-2). CASAC was also shown to be an excellent activator of dendritic cells and stimulated the production of IL-12 which promotes strong Th1 responses and cross presentation of antigen[52]. CASAC vaccination has been extensively employed in studies described in the experimental sections of this thesis.

1.7 Peptide vaccination and IRX-2

IRX-2 is a combination of biological agents containing physiological quantities of IL-1β, IL-2, IL-6, IL-8, GM-CSF, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) generated by phytohemagglutinin (PHA) activation of human PBMC under Good Manufacturing Practice (GMP) conditions by IRX Therapeutics[96]. IRX-2 has immunomodulatory properties that restore immune responsiveness, induce tumour infiltration in head and neck squamous cell carcinoma (HNSCC), increasing the mean 5 year survival of patients in a phase II clinical trial[97]. IRX-2 was also found to have adjuvant properties when it was administered as 4 or 9 daily doses in a vaccination against a mouse immuno-dominant peptide derived from human prostate-specific membrane antigen (NFT). Increased T-cell responses were observed when IRX-2 was used in combination with incomplete Freund's adjuvant or NFT peptide conjugated with keyhole limpet hemocyanin (KLH) or with irradiated cells expressing NFT. This was shown in a delayed type hypersensitivity (DTH) response where there was the most swelling found in the footpad of mice challenged with the target peptide after vaccinating the mice in combination with IRX-2 (Figure 1.15)[98].

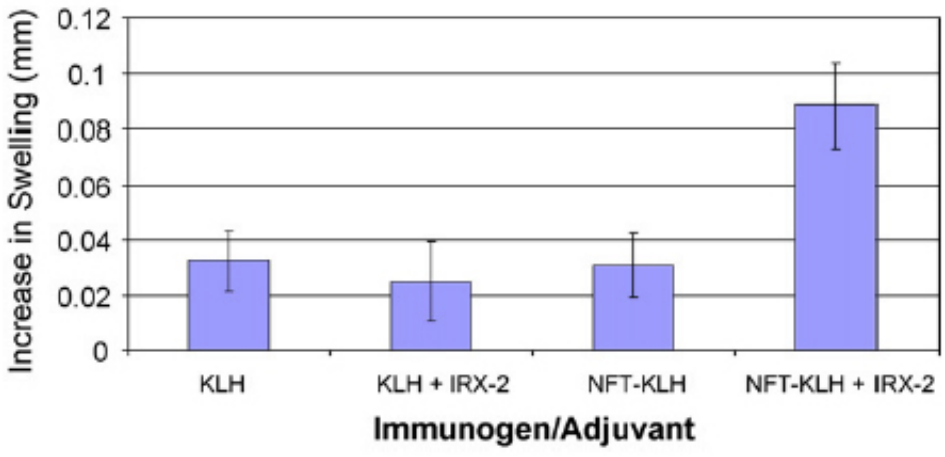


Figure 1.15 DTH effects by IRX-2: Increased swelling was measured (0.01mm sensitivity caliper) at the area of the mouse footpad where the challenge with antigen (NFT) was conducted. A prime and boost vaccination was conducted with IRX-2 was given 9 daily doses subcutaneously (from Naylor *et al.*, 2010)[98].

Splenocytes or cells from lymph nodes of the vaccinated, as well as the unvaccinated mice were investigated for their ability to produce IFN-γ when challenged *in vitro* with the target antigen to determine T-cell responses. Naylor *et al.*, reported the improved production of IFN-γ in both enzyme-linked immunosorbent assay [99] and enzyme-linked immunosorbent spot (ELIspot) assays in mice which were vaccinated with NFT plus IFA and IRX-2 showing data that show increased T-cell responses in IRX-2 vaccinated mice compared to IFA vaccination in the absence of IRX-2 (Figure 1.16). This has prompted our interest in incorporating IRX-2 into our peptide vaccination studies to investigate its potential for synergy with CASAC.

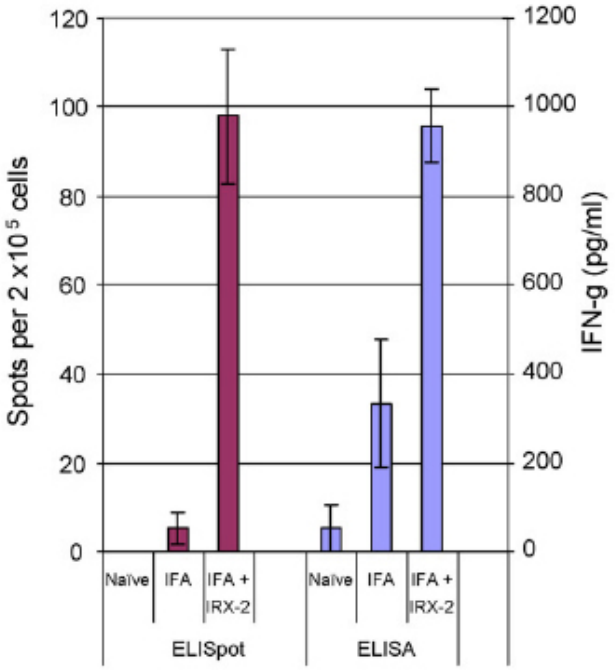
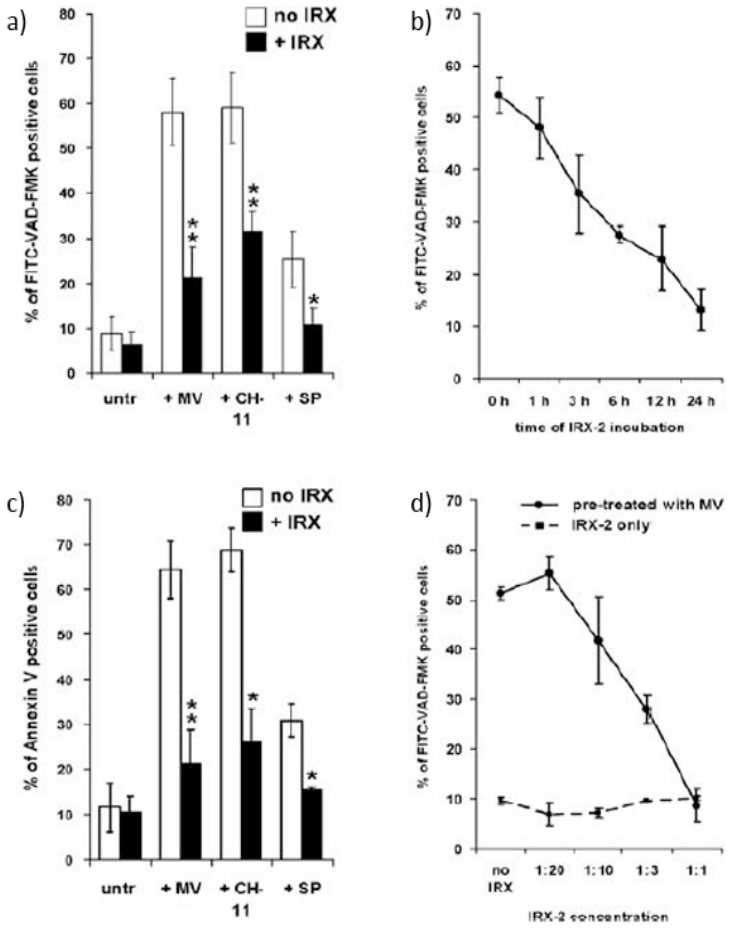


Figure 1.16 ELIspot and ELISA for IFN-γ in IRX-2 vaccinations: Data obtained from both ELIspot and ELISA assay show a significant increase in IFN-γ production when comparing IFA versus IFA plus IRX-2 vaccination with NFT peptides. The increase in the production of IFN-γ shows the potential of IRX-2 in generating T-cell responses in mice (Naylor *et al.*, 2010)[98]

Another key feature of IRX-2 that has prompted our interest in its investigation is its anti-apoptotic properties reported by Czystowska *et al.* (2009) and the protective action of IRX-2 on T-cells that were exposed to tumour cells[100, 101]. Many types of

cancer such as head and neck squamous-cell carcinoma (HNSCC) and melanoma expresses Fas ligand (FasL), while some cancers are capable of secreting micro vesicles [102] with membrane bound FasL. This leads to elimination of activated Fas+ effector T-cells by the FasL/Fas pathway, thus leading to a poor immunological response against the tumour[100]. MV isolated from sera of oral carcinoma patients were also shown to have the capacity to induce caspase-3 cleavage, DNA fragmentation, cytochrome c release, loss of mitochondrial membrane potential (MMP) and TCRζ-chain down-regulation in activated T lymphocytes, which leads to T-cell apoptosis, shown to be blocked by the presence IRX-2[100, 103].

Pre-incubation of Jurkat cells (which is a $CD8^+$ human T-cell line) with IRX-2, protects from MV, CH-11 and staurosporine induced caspase activation and apoptosis[100]. Protection of Jurkat cells was seen to be directly correlated with the duration of IRX-2 incubation time. Similar effects were also seen in primary $CD8^+$ and $CD4^+$ T-cells making IRX-2 a potent agent for protecting against T-cell apoptosis (Figure 1.17)[100]. The mechanism of protection was later elucidated by Cysztowska *et al.*, in 2011 when IRX-2 was shown to prevent over expression of Fas in the activated T-cells which were induced by MV, rather than by blocking the FasL/Fas pathway that leads to apoptosis. They were also able to discover the up-regulation of cFLIP, a protease-deficient caspase homologue widely regarded as an apoptosis inhibitor, making the activated T-cells more resistant to MV induced apoptosis[101, 104]. This is important in terms of vaccination because in 2006, Wang *et al.*, showed that vaccinating mice against an autologous inactivated auto-reactive T-cells, in a model to eliminate auto-reactive T-cells, showed increased Fas^+ T-cells in the vaccinated mice[105]. Therefore, IRX-2 could play a part in stimulating better responses to vaccination, especially in the context of chronic disease or vaccination in the elderly population.



e)

	$CD8^+$ cells		$CD4^+$ cells	
	FITC-VAD-FMK+ cells	*P*-value[b]	FITC-VAD-FMK+ cells	*P*-value[b]
Untreated cells	14.8 ± 4.8	—	12.6 ± 2.3	—
No IRX-2+CH-11 Ab	52.8 ± 4.9	—	41.2 ± 9.8	—
+IRX-+CH-11 Ab	15.0 ± 3.5	0.0010	15.4 ± 6.1	0.0510
No IRX-2+MV	71.0 ± 8.0	—	45.7 ± 1.6	—
+IRX-2+MV	26.5 ± 10.3	0.0006	13.9 ± 6.4	0.0211

Figure 1.17 IRX-2 protects Jurkat cells and primary T-cells from MV induced apoptosis: Jurkat cells (human $CD8^+$ T-cell line) were pre-incubated with or without IRX-2 24 hours prior to treatment with MV, CH-11 antibody (a Fas activating antibody) or staurosporine (SP - a drug that induces apoptosis) for 3 hours. FITC-VAD-FMK staining indicates caspase activation while annexin V staining indicates cell apoptosis. a) and b) show that IRX-2 was capable of protecting Jurkat cells from caspase activation caused by MV, CH-11 antibody or SP treatment in the presence or absence of IRX-2. Increasing IRX-2 incubation time leads to better protection especially from MV. c) and d) shows protection of IRX-2 from cell apoptosis. In Table 1.e), primary T-cells were treated in the same manner as Jurkat cells, again showing IRX-2 mediated protection against apoptosis. (Cysztowska *et al.*, 2009)[100]

In 2011, Shiling *et al.*, also showed that IRX-2 favours the expansion of effector T-cells rather than Tregs in an *in vitro* human model[106]. $CD3^{+}CD4^{+}CD25^{-}$ naive human T-cells were co-cultured with immature dendritic cells and irradiated PCL-13 (a head and neck cancer cell line) in the presence or absence of IRX-2 for 10 days. The cultured cells were then identified for effector T-cells (Teff: $CD25^{+}CD122^{-}CD132^{-}CD152^{-}FoxP3^{-}$) and Tregs (Tr1: $CD25^{+}CD122^{+}CD132^{+}CD152^{+}FoxP3^{+}$). Based on phenotypic marker analysis, IRX-2 was shown to have favoured the expansion of Teff instead of Tr1 (Figure 1.18 a)[106]. The expression of Tr1 associated markers as well as IL-10 and TGF-β, were seen to be increased in the co-culture of PCL-13 and T-cells in the absence of IRX-2, thus supporting the data showing Teff cell expansion. In the presences of IRX-2, Treg markers were significantly reduced compared to the non-IRX-2 treatment. IFN-γ, which supports Teff cell expansion, was also seen to have increased (Figure 1.18 b and c)

The evidence provided by Schilling *et al.*, suggests the importance of blocking the expansion of Tregs in response to vaccination as Tregs could impede immune responses generated by the vaccine, thus reduce efficacy. The expansion of effector T-cells on the other hand would lead to an improved generation of immunological responses that could increase the efficacy of vaccination. The evidence summarised suggests that IRX-2 could play an important role in enhancing protective immunity in response to vaccinations against tumour associated antigens.

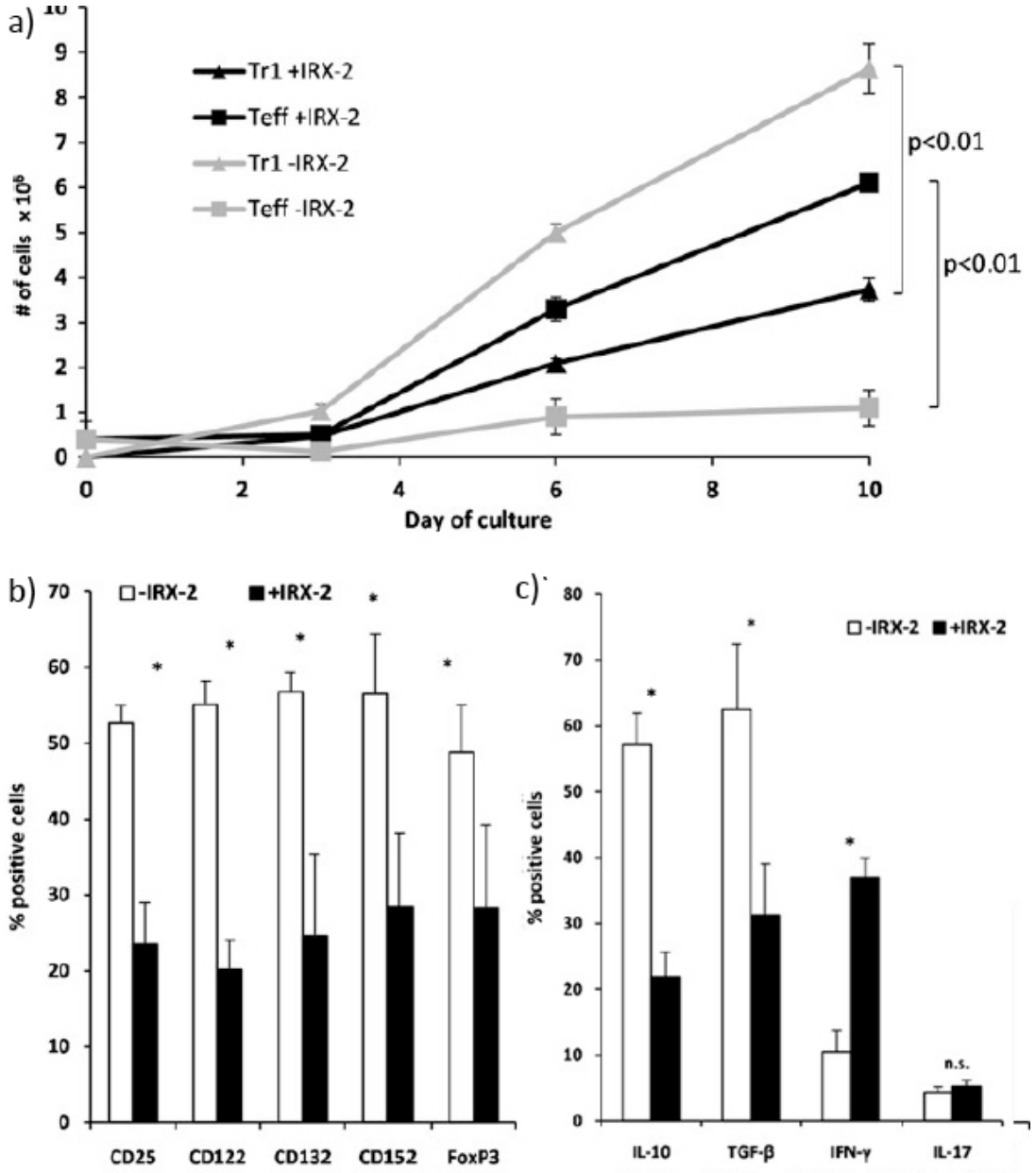


Figure 1.18 Generation of Teff with use of IRX-2: a) Numbers of effector T-cells (Teff) and regulatory T-cells (Tr1) were analysed post *in vitro* co-culture of T-cells with immature dendritic cells and PCL-13 tumour cell line with IRX-2, showing increased Teff and decrease Tr1 cells compared to the absence of a non-IRX-2 in the co-culture. b) IRX-2 incubation leads to reduced the expression of Treg phenotypic markers compared to non-IRX-2 co-culture. c) IRX-2 reduces the expression of Tregs related cytokines (IL-10 and TGF-β) while increasing IFN-γ expression compared to a non-IRX-2 co-culture system. (Schilling *et al.*, 2011)[106]

1.8 Vaccination in the exhausted, anergized and senescent immune states

The potential for vaccination as an immunotherapy in the clinic is usually investigated in young mice which have potent immunological responses. These vaccination studies have proven to indicate efficacy of a range of therapeutic vaccination strategies. However, the vast majority of these vaccinations have failed to produce a therapeutic response in clinical studies of human malignancy. Patients treated in the clinic had either chronic exposure to their malignant cells and are usually at a much older age. Therefore two major differences between murine models of cancer immune therapy and clinical human studies in immunotherapy of cancer are:

1. Chronic exposure to cancer and the interaction between the tumour and the immune system.

2. The aged, possibly exhausted, and immune senescent status of the majority of cancer patients.

Cancer patients would have lived with the disease for years before symptoms start to show. Therefore, the immune system would have seen the disease and been exposed to the tumour mediated immune suppression, including anergy and clonal exhaustion of specific effector T-cells. Long term exposure to tumours, and aging of the thymus can also cause patients to lose the ability to generate naive T-cell or maintain a repertoire with the required diversity to respond to vaccination[107, 108]. Similarly, a chronic infection like LCMV could lead to similar immune perturbations seen in cancer patients (Figure 1.19).

Three main pathways have been identified that causes a negative regulatory effect in the immune system[109]. These are:

1) Tregs (refer to section 1.5)

2) Cell surface inhibitory markers such as PD-1, KLRG-1, etc.

3) Cytokines (IL-10, TGF-β, etc).

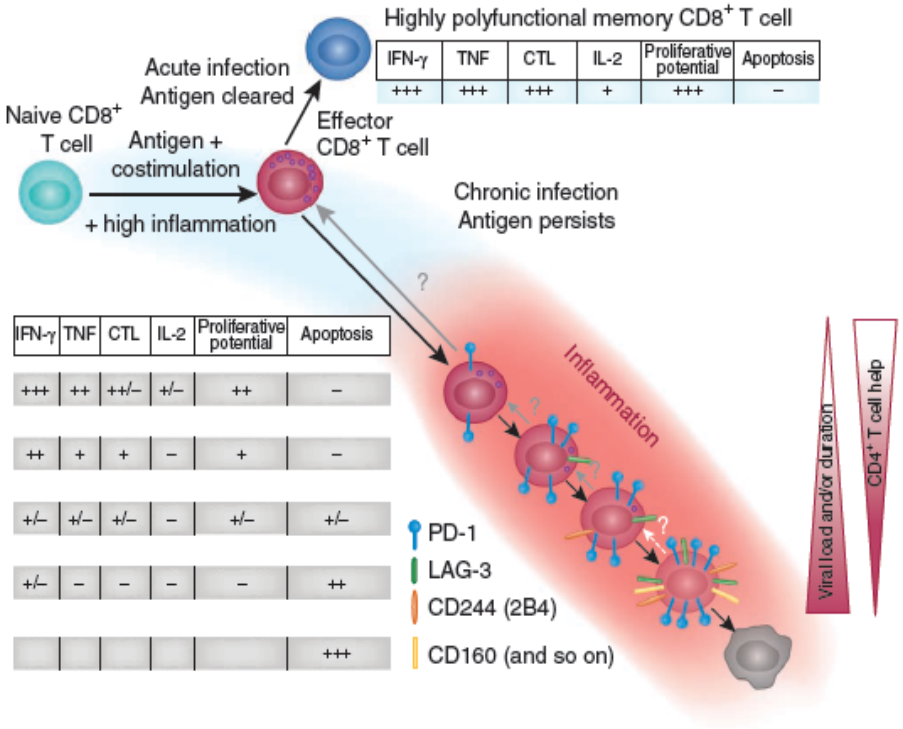


Figure 1.19 Model of exhaustion: Exhausted antigen specific $CD8^+$ T-cells were first identified in chronic LCMV infection. Exhaustion occurs in a hierarchical manner with CD8 T-cells losing their properties one by one, IL-2 production, proliferative capacity and ex-vivo killing are lost first followed by loss of TNF production. Severe exhaustion follows via the loss of IFN-γ production or beta-chemokines production or the ability to degranulate. The altered expression of markers associated with exhaustion and immunesenescence markers are also indicated. (Wherry, 2011)[109].

One of the best known inhibitory receptors is programme death 1 (PD-1). The PD-1/PD-L1 pathway regulate both the induction and the maintenance of peripheral tolerance. PD-L1 protein on tolerogenic DCs can induce T-cell tolerance. PD-L1 is constitutively expressed on APCs and T cells and is further up-regulated by pro-inflammatory cytokines. PD-1 has a negative effect on the survival and proliferation of exhausted and senescent T-cells leading to cell death and apoptosis[110]. The mechanism of action of PD-1 can be seen in Figure 1.20. The activity of PD-1 in the thymus is required to ensure that T-cells specific for self antigens are eliminated in the course of their thymic maturation. However in diseased patients where the T-cells have been exhausted and expresses high levels PD-1, the blockade of PD-1 may help to reinvigorate the T-cells. A model was suggested by Sharpe *et al.*, in an extensive review in 2007 and Figure 1.21 shows a schematic diagram of the proposed use of a PD-1/PD-L1 blocker[110].

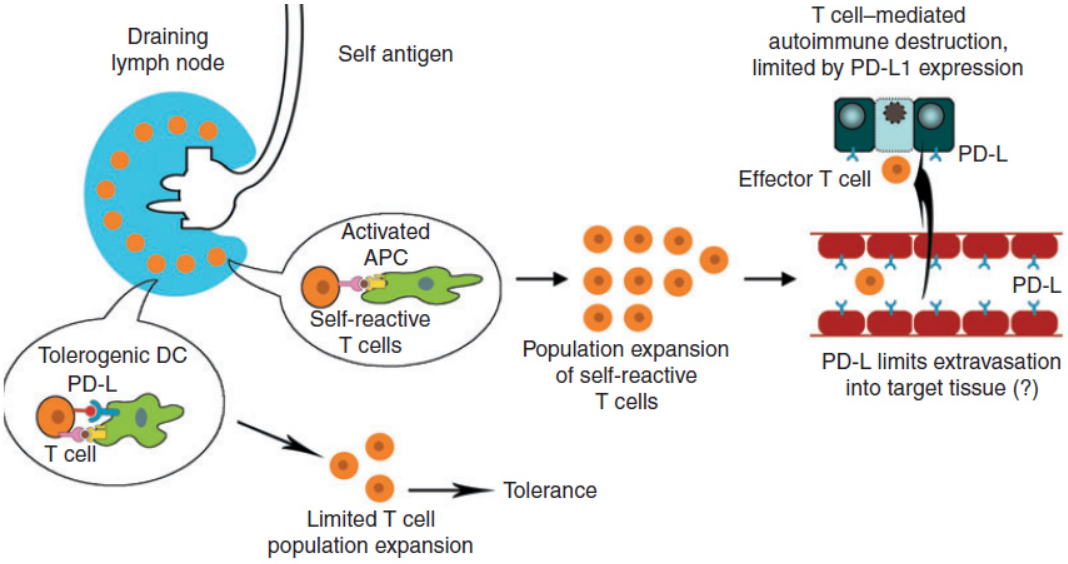


Figure 1.20 PD-1/PD-L1 natural pathway: Normal expression of PD-1 and PD-L1 (PD-L) in the thymus where PD-1/PD-L1 pathway causes depletion of self-reactive T-cells, avoiding the occurrence of autoimmune diseases (from Sharpe *et al.*, 2007)[110].

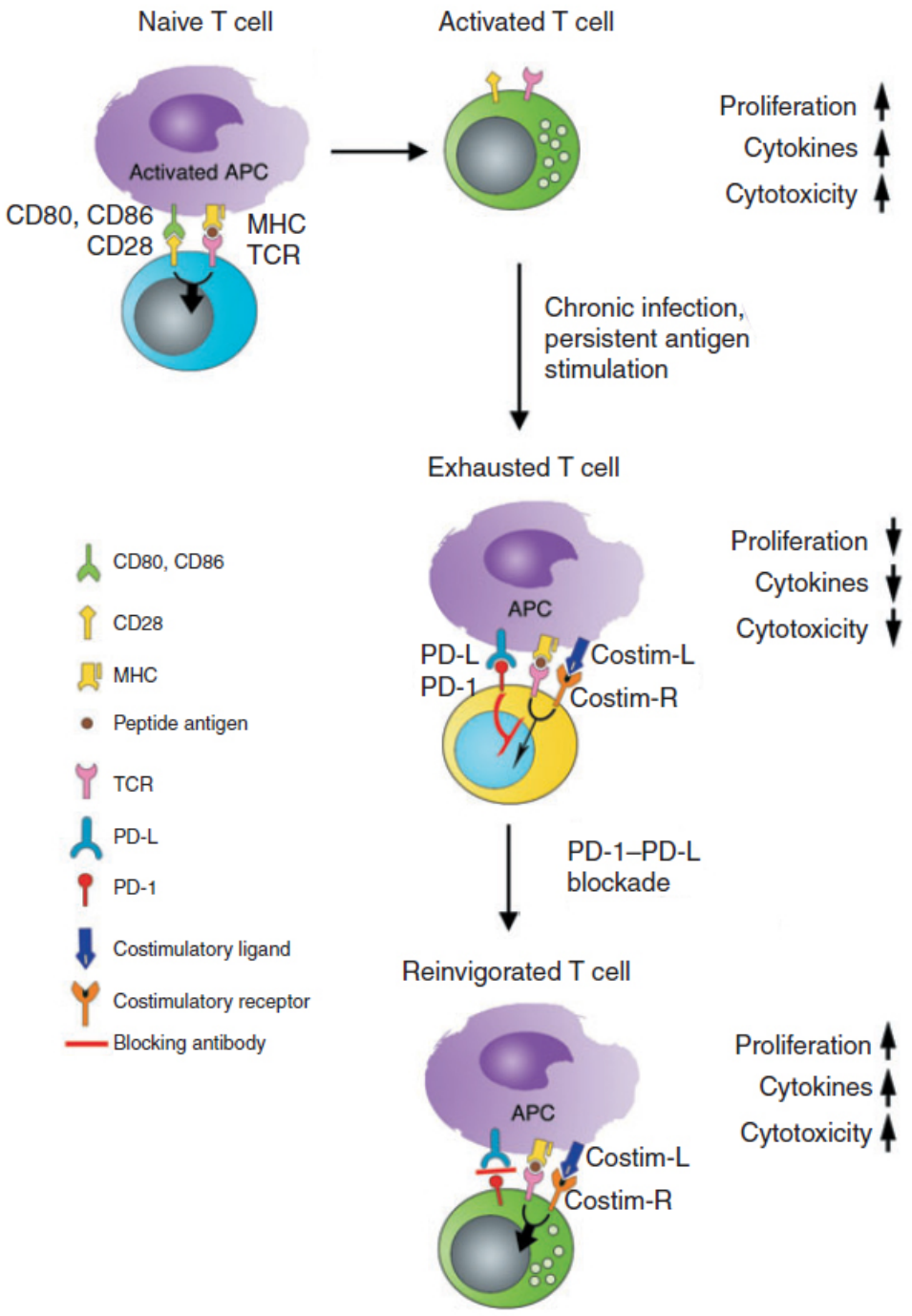


Figure 1.21 Possibility of reinvigorating exhausted T-cells: In an activated T-cell state where exhausted cells are expressing high levels of PD-1, the interaction between PD-1 and PD-L1 causes the depletion of these T-cells and the elimination of the exhausted clone of antigen specific T-cells. Blocking antibodies have then been introduced to reinvigorate the exhausted T-cells (Adapted from Sharpe *et al.*, 2007)[110].

Killer lectin like receptor G1 (KLRG-1) has also been shown to have been up-regulated in highly activated T-cells and NK cells, especially in antigen experienced T-cells[111]. However, in senescent T-cells, KLRG-1 was shown to stop proliferation resulting in terminal differentiation. This has been reviewed extensively by Henson *et al.*, in 2009 and its proposed mechanism of action is shown in Figure 1.21[112]. KLRG-1 plays an important role in maintaining the ability of a T-cell population to proliferate thus affecting the generation of antigen specific T-cells especially when vaccinating the aged mice. In mice, KLRG-1 has been shown to be up-regulated in mice aged more than 18 months old. In humans however, CD57 is the primary marker of immunesenescence in individuals aged above 65 years[113-116].

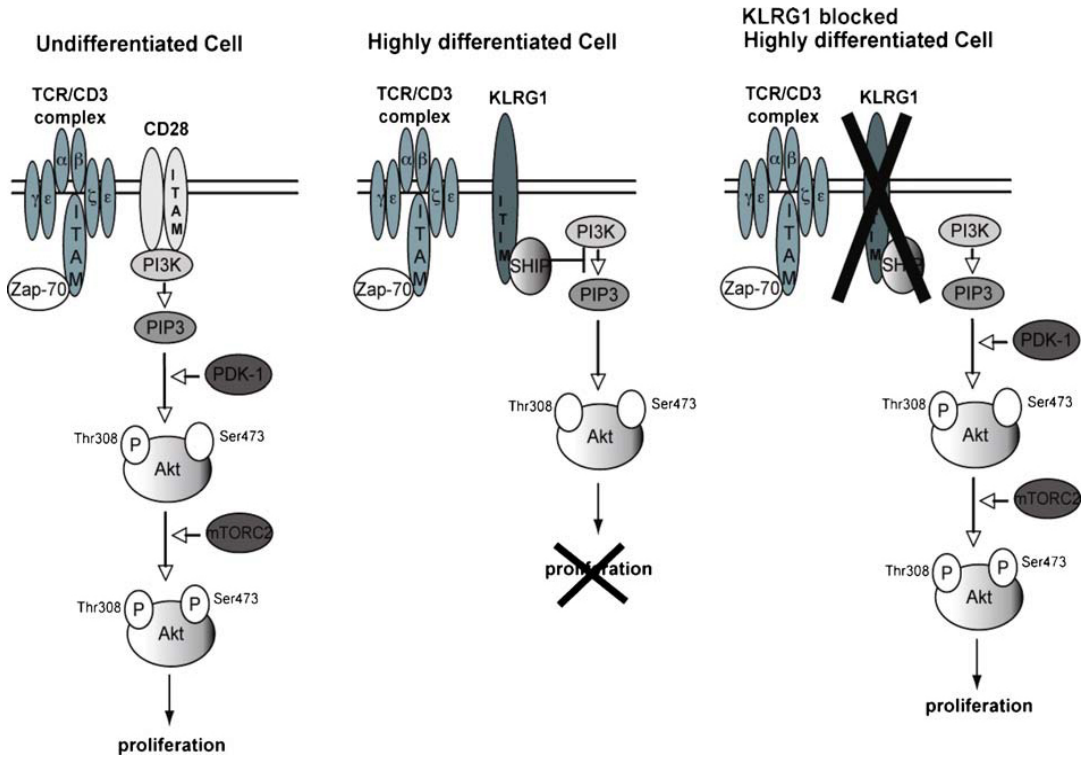


Figure 1.22 Blockade of T-cell differentiation by KLRG-1: In naive/undifferentiated T-cells, no KLRG-1 was found on the cell surface of the T-cells. This enables the T-cells to undergo proliferation by stimulation of CD28 pathway, leading to PI3K/Akt signalling thus proliferation can take place. However, in differentiated cells, the KLRG-1 marker replaces CD28 and causes inhibition of the PI3K/Akt pathway by blocking the conversion of PI3K to PIP3 required for Akt activation. This leads to terminal differentiation of the cells. However, the blockade of KLRG-1 would again restore the proliferative function of the T-cell, leading to continued proliferation. (Adapted from Henson *et al.*, 2009)[112]

Other cell surface markers that play roles in immune dysfunctions are, LAG-3 which affects cell cycle progression, and the inhibitory receptor CTLA-4 which competes with CD28 for co-stimulatory ligands[109].

There are also important inhibitory immune regulatory cytokines, including IL-10 and TGF-β. IL-10 is a key cytokine that leads to up-regulation of Tregs when combined with TGF-β. Evidence has shown that IL-10 is up-regulated in chronic infections and is linked to T cell dysfunction during persistent viral infection[117]. Blocking of IL-10 was shown to enhance viral control and to improve T-cell responses for clearance of virally infected cells *in vivo* as previously shown by Brooks *et al.*, in 2006[117]. There was also correlation between IL-10 production by monocytes in HIV infected humans due to increased ligation of PD-1, thus causing impairment of $CD4^+$ T-cells[118]. TGF-β has been linked to T cell exhaustion through the phosphorylation of Smad2, especially in chronic LCMV infection as shown by Tinoco *et al.*, in 2009[119]. However, there are cytokines such as IL-7, IL-21 and IL-2 which are positive immune regulatory factors in chronic infection. They are important in fostering more functional T-cell responses[109].

Aged mice which are >18 months old, are considered immunosenescent. An extensive review by Maue *et al.* (2009)[107], has highlighted several important observations in immunosenescent mice (Table 1.4). The factors indicated in Table 1.4 would hamper the generation of immunological responses against infections and diseases such as cancer. These markers of immune dysfunction in the aged mice are also indicative of immune senescence in human aging. Vu *et al.* (2002), showed the presence of a correlation between increased influenza induced mortality and age of patients. Patients aged >65 years old have a higher risk of death compared to younger patients[108] . Vaccination in the aged population was also found to be a challenge as age associated thymic involution leads to poorer thymic output of naive T-cell, thus diminishing the repertoire available for generation of new immunological responses in humans as stated by Aspinall *et al.*[120].

$CD4^+$ T cells	$CD8^+$ T cells
Reduced T-cell receptor signaling intensity	Decline in responsiveness to newly encountered antigens
Defects in activation, differentiation and expansion after stimulation	Age-related declines in $CD8^+$ T-cell repertoire diversity
Reduced production of interleukin-2	Nonmalignant expansion of individual $CD8^+$ T-cell clones
Impaired ability to provide cognate help to B cells after immunization	Impaired generation of $CD8^+$ T-cell memory

Table 1.4 Impairment of CD4 and CD8 T-cells in aged mice. (Maue *et al.*, 2009)[107]

1.9 Objectives of this study:

The focus of this project is the study of T-cell responses to peptide vaccination in combination with adjuvant combination referred to as CASAC and immune regulation by IRX-2. Specifically, the following chapters will focus on the primary objectives as below:

1. The use of alternating class II presented T-helper peptides for vaccination induced stimulations of CD8 T-cell responses

2. To study the effects of IRX-2 as a potential immunomodulatory agent to enhance potency of vaccination induced generation of antigen specific CD8 T-cells.

3. Development of a peptide vaccination strategy for generation of functional antigen specific $CD8^+$ T-cells in aged mice.

CHAPTER 2

General Materials and Methods

2.1 Materials

2.1.1 Reagents, chemicals and buffers

AKFVAAWTLKAAA, 95% pure (PADRE); *Peptide Protein Research Ltd.*

Ammonium chloride (NH_4Cl); *SIGMA*

Cell Trace™ Carboxyfluorescein diacetate, succinimidyl ester (CFSE); Invitrogen
Cell Trace™ Far red DDAO-SE (DDAOSE); *Invitrogen*

Chicken Ovalbumin, ≥98% Ovalbumin (OVA); *SIGMA*

CpG ODN 1826 (5'-tccatgacgttcctgacgtt-3'), Phosphothiorate modified (S-Oligos); *SIGMA-Genosys*

Detoxified Monophosphoryl Lipid A from S. minnesota (MPL); *Invivogen*

Dimethyl sulfoxide - Hybri-Max™, sterile-filtered, BioReagent, suitable for hybridoma, ≥99.7% (DMSO); *SIGMA*

Dulbecco's phosphate buffered saline (DPBS); *SIGMA*

Ethylenediaminetetraacetic acid (EDTA); *SIGMA*

Fetal bovine serum (FBS); *Gibco*

Flow-count™ Counting Beads; *Beckman Coulter*

Foxp3 / Transcription Factor Staining Buffer Set; *eBioscience*

Freund's Adjuvant, Complete (CFA); *SIGMA*

Ionomycin calcium salt from Streptomyces conglobatus, ≥98% (Ionomycin); *SIGMA*

IRX-2; *IRX Therapeutics*

Isoflo® Isofluorane; *Abbot's*

ISQAVHAAHAEINEAGR, >95% pure (ISQ, $OVA_{323-339}$); *Peptide Protein Research Ltd.*

Monensin sodium salt, 90-95%; *SIGMA*

Nuclease-free water; *Promega.*

Penicillin/Streptomycin; *SIGMA*

Phorbol 12-myristate 13-acetate, (PMA); *SIGMA*

Polyinosinic–polycytidylic acid sodium salt (Poly I:C); *SIGMA*

Potassium bicarbonate ($KHCO_3$); *SIGMA*

Recombinant mouse Interferon Gamma (mIFN-γ); *Peprotech*

RMFPNAPYL, >95% pure (WT-$1_{126–134}$); *Peptide Protein Research Ltd.*

Roswell Park Memorial Institute media (RPMI-1640); *SIGMA*

SIINFEKL peptide, >95% pure (SIINFEKL, $OVA_{257-264}$); *Peptide Protein Research Ltd.*

Sodium citrate pH 3; 0.05M – Polyethylene glycol 3350 12% (v/v) solution; *SIGMA*

Sodium hydroxide 1.0N solution; *SIGMA*

Squalene, ≥ 98%; *SIGMA*

TEWTSSNVMEERKIKV, >95% pure (TEWT, $OVA_{266-281}$); *Peptide Protein Research Ltd.*

Tween® 80 - Cell culture tested; *SIGMA*

X-vivo 15 media; *Lonza*

2.1.2 Monoclonal antibodies and pentamers

All antibodies used were purchased from Ebioscience unless otherwise stated:

R-Phycoerythrin (R-PE) pentamer (H-2K^b specific for SIINFEKL - ProImmune); Pacific Blue (PBlue) conjugated anti-mouse CD3 (17A2); Peridinin-chlorophyl-Cy5.5 (PE-Cy5.5) or Allophycocyanin (APC) anti-mouse CD8a (53-6.7); APC anti-mouse CD62L (MEL-14); Fluorescein isothiocyanate (FITC) anti-mouse CD44 (IM7); FITC or PerCp-Cy5.5 anti-mouse CD4 (RM4-5); APC anti-mouse CD25 (PC61.5); Phycoerythrin (PE) or PerCP-Cy5.5 anti-mouse FoxP3 (FJK-16s); PE Rat IgG2a kappa isotype control (eBR2a); PE or un-conjugated anti-mouse CD28 (37.51); PE Anti-mouse IFN-γ (XMG 1.2 - BDBioscience); FITC Anti-mouse Interleukin-17 (eBio17B7); PE-Cy7 Anti-mouse Interleukin-4 (BVD6-24G2); Pblue Anti-mouse Tumour Necrosis Factor-α (MP6-XT22); PE anti-mouse GATA-3 (TWAJ); eFluor® 660 Anti-Human/Mouse T-bet (eBio4B10); PE Anti-Human/Mouse RORγt (AFKJS-9); PE Anti-Mouse $OVA_{257-264}$ (SIINFEKL) peptide bound to H-2Kb (25-D1.16); APC anti-mouse CD152 (CTLA-4) (UC10-4B9); PE anti-mouse CD274 (PD-L1) (MIH5); FITC anti-mouse CD279 (PD-1) (RMP1-30); PE anti-mouse CD223 (Lag-3) (eBioC9B7W (C9B7W)), Rat IgG1 K Isotype Control PE and PE-Cy7 anti-mouse KLRG1 (2F1)

All volumes or concentrations of antibodies used were as suggested by the manufacturers.

2.1.3 Preparation of RBC lysis solution

8.26 g ammonium chloride (NH_4Cl), 1 g potassium bicarbonate ($KHCO_3$) and 0.037 g EDTA were added to 1 litre of distilled water. The solution was filtered through a 0.2 µm filter (Milipore) and stored in room temperature.

2.2 Peptide Vaccination

2.2.1 Animal Model

C57BL/6 mice were selected as the animal model. The mice were bought from HARLAN and were housed according to Home Office regulations. Female C57BL/6 (H2 Haplotype = b) mice aged 6-8 weeks were purchased and were left to adapt to the environment for at least 1 week at the Biological Services Unit before starting the experiments. Aged female C57BL/6 mice were also purchased from the same source aged 18 months for experiments involving immunesenescence. At the end of each study, mice were humanely killed by a Schedule 1 method.

2.2.2 Preparation of vaccine components

All vaccine components were prepared under sterile conditions using sterile solutions and plastics in a Class II microbiological safety cabinet (Howorth airtech Ltd.)

2.2.2.1 Protein and peptides

Chicken Albumin (OVA) and synthetic sequences of its peptides were used in all vaccination studies as a model antigen. SIINFEKL (MHC Class I), ISQAVHAAHAEINEAGR (MHC Class II) and TEWTSSNVMEERKIKV(MHC Class II) were in vials of 5 mg and diluted to 5 mg/ml by adding 100 µl of DMSO and 900 µl of DPBS and vortexed (Labinco L46 vortex) for 10 seconds. In cases where peptides did not dissolve well, 1 µl of 1N sodium hydroxide solution was added and vortexed. This was repeated until peptides had completely dissolved. The peptides were then divided into 4 aliquots of 250µl each in 1.5 ml tubes (Eppendorf) and stored in at -20^{o}C (Leibherr -20^{o}C Freezer). To avoid degradation of peptides, each tube of peptides would only undergo a maximum of 3 freeze thaw cycles.

40 mg of Ovalbumin was weighed with a scale (Denver Instruments, APX-153, max = 150 g, sensitivity = 0.001 g) and diluted with 0.5 ml of DMSO and 4.5 ml of DPBS to obtain an 8 mg/ml concentration in a 15 ml Falcon tube (Beckton Dickinson). Ovalbumin was prepared fresh each time it was required.

2.2.2.2 TLR agonists and mIFN-γ

MPL: MPL was prepared by diluting the stock vial (1 mg) with 200 µl of 100% ethanol to make a stock solution of 5 mg/ml. MPL was made fresh each time it was required.

CpG: 10 mg of CpG was diluted in 2 ml of nuclease-free water to make a stock solution of 5 mg/ml. The stock solution was then divided into 200 µl aliquots in 0.5 ml tubes (Eppendorf) and stored at -20^{o}C.

Poly I:C: 25 mg of Poly I:C was diluted in 5 ml of nuclease-free water to make a stock solution of 5 mg/ml. The stock solution was then divided into 200 µl aliquots in 0.5 ml tubes and stored -20^{o}C.

mIFN-γ: 100 µg of recombinant mouse IFN-γ was diluted in 1 ml of DPBS to make a 100 µg/ml stock solution. The stock solution was then divided into 50 µl aliquots in 0.5 ml tubes and stored at -20^{o}C.

2.2.2.3 Emulsion

A standard 5 ml emulsion (0.4% Tween 80 and 8% Squalene) for CASAC vaccination was prepared by adding 20 µl of Tween 80 into a 15ml Falcon tube followed by 400 µl of Squalene and 4520 µl of DPBS. The mixture was sonicated (Sonics Vibra cell sonicator) on ice at 100% amplitude for 5 seconds and allowed 30 seconds for cooling. A total of 4 rounds of sonication were conducted. The solution was clear prior to sonication (Figure 2.1 a) and after 4 rounds of sonication (Figure 2.1 b), the emulsion mixture was formed as a milky white mixture (Figure 2.1 c).

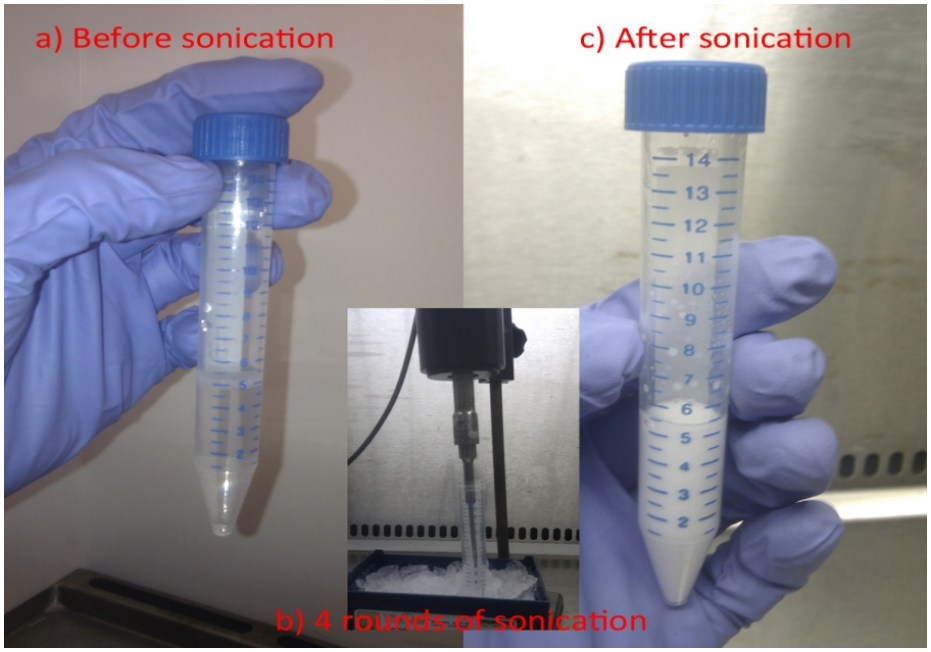


Figure 2.1 Preparation of emulsion: a) The emulsion mix prepared was clear after mixing squalene, Tween 80 and DPBS. b) 4 rounds of sonication at 100% amplitude for 5 seconds with 30 seconds cooling intervals. c) Final emulsion mix, milky white in colour.

2.2.3 Vaccine preparation

2.2.3.1 The alternating T-helper peptide vaccination regimen involving emulsion plus 2 TLR agonists as the adjuvant

The vaccine mixture comprises of an emulsion (emulsion prepared as described in section 2.2.2.3), 2 TLR agonist (Poly I:C - 10 µl from 5 mg/ml stock to make 50 µg/mouse and CpG - 5 µl from 5 mg/ml to make 25 µg/mouse), DPBS to make up the volume to 100 µl of the non-emulsion part and a combination of peptides as required by the vaccination regimen. For each mouse, 20 µl of each indicated peptide was added into the vaccine mixture from a stock solution of 5 mg/ml to make 100 µg/mouse (Table 2.1).

	µg per mouse	µl per mouse	Volume typical for 5 mice
Vaccine component:			
Poly I:C	50	10	50
CpG	25	5	25
SIINFEKL	100	20	100
ISQ	100	20	100
TEWT	100	20	100
DPBS	-	25	125
	FINAL VOLUME	100	500

Table 2.1: A typical vaccine preparation for vaccination of 5 mice in a group.

2.2.3.2 Alternating T-helper peptide vaccination regimen with a single TLR agonist and reduced peptide concentrations

In a separate vaccination regimen for the alternating T-helper peptide regimen involving reduced peptide concentration and a single TLR agonist (Poly I:C), the mixture was prepared without the presence of emulsion. 10 µl of Poly I:C (50 µg/mouse, from a stock solution of 5mg/ml) was mixed with the combinations of peptides as indicated in the example in Table 2.1. Here the peptide concentration was reduced to 25 µg/mouse where only 5 µl of each peptide (from a stock solution of 5 mg/ml) was added as indicated in the vaccination groups.

2.2.3.3 Priming with OVA protein in CFA for the alternating T-helper peptide vaccination

5ml stock solution of Ovalbumin (8 mg/ml) was mixed with 5 ml of CFA to make a final concentration of 4 mg/ml Ovalbumin. The mixture was then emulsified by either sonication on ice at 100% amplitude for 4 x 5 seconds, allowing 30 seconds for cooling between pulses, or by continuously passing the mixture between two 5 ml glass syringes (Scientific Laboratory Supplies) attached together with a three way valve (DJB Microtech ltd.) until a stable emulsion was formed. To check for stable formation of emulsion, the emulsion was dropped into a beaker of water. The emulsion was regarded as stable if the drop formed on the surface of the water does not disperse (Figure 2.2 a and b). After the priming dose, the boosting dose was prepared as in section 2.2.3.2.

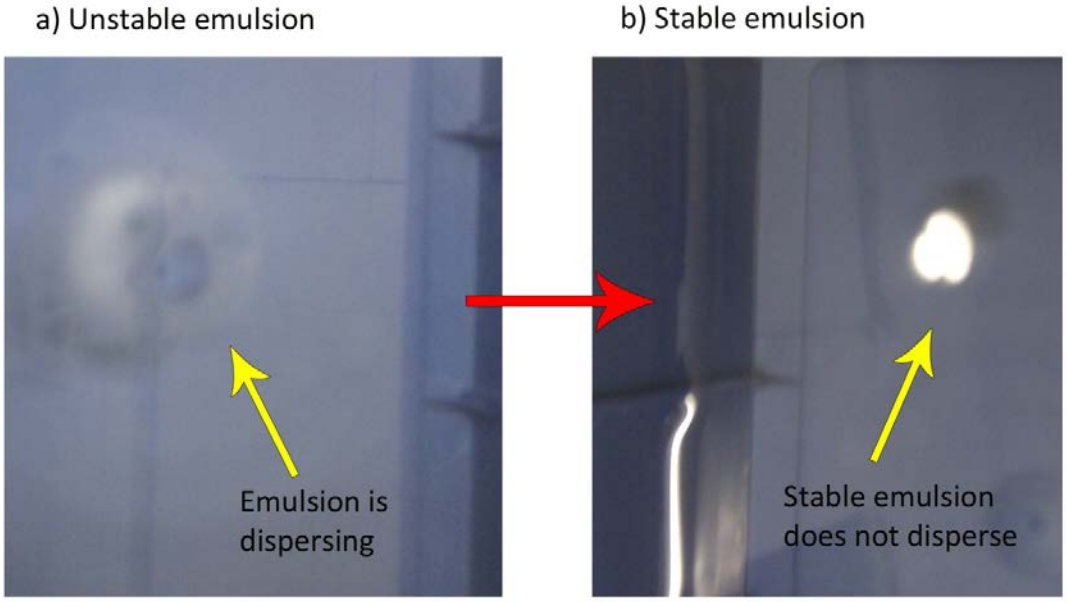


Figure 2.2 Emulsion formation: Emulsion prepared with pushing the mixture of CFA and OVA through 2 glass syringes. a) Emulsion formed after 10 rounds of pushing between syringes was not stable and starts to disperse in water. b) Stable emulsion is formed after approximately 100 rounds pushing the mixture through the glass syringes. Emulsion formed did not disperse after dropping into water.

2.2.3.4 Preparation of the combined adjuvant for synergistic activation of cellular immunity (CASAC)

There were 2 versions of CASAC used in our studies. Preparations were as described below:

Poly I:C version - The vaccine comprises of 2 separate parts. The first part comprises of an emulsion which was made as described in section 2.2.2.3 and left in a 37^{o}C water bath (Clifton unstirred waterbath) to be kept warm for administration. The second part was made by combining 20 µl of an MHC class I presented peptide (100 µg/mouse, 5mg/ml stock solution), 20 µl of an MHC class II presented peptide (100 µg/mouse, 5mg/ml stock solution), 10 µl Poly I:C (50 µg/mouse, 5 mg/ml stock solution), 5µl CpG (25 µg/mouse, 5 mg/ml stock solution), 1µl recombinant mIFN-γ (100 ng/mouse, 100 µg/ml stock solution) and 44 µl DPBS to make a final volume of 100 µl. 100 µl of emulsion and 100 µl of the adjuvant were then mixed and vortexed for 1 minute. The vaccine prepared was administered within a 2 hours period.

MPL version - The preparation of the MPL version of CASAC was similar to that of the Poly I:C version. In this version, 10 µl of MPL (25 µg/mouse, 5 mg/ml stock solution) replaced Poly I:C and the vaccine was prepared as described above.

2.2.3.5 Preparation of CASAC with IRX-2 in the vaccine mixture

IRX-2 is a combination of biologic agents containing physiological quantities of IL-1β, IL-2, IL-6, IL-8, GM-CSF, interferon-gamma (IFN-γ) and tumornecrosis factor-alpha (TNF-α) generated by phytohemagglutinin (PHA) activation of human PBMC under GMP conditions by IRX Therapeutics.

IRX-2 was obtained in aliquots of 10ml. In our studies, the lot number of IRX-2 used was **#042908** which comprised of:
IL-2 (6.3 ng/ml); IL-1β (0.71 ng/ml); IFN-γ (2.48 ng/ml); TNF-α (2.1 ng/ml); IL-8 (34.4 ng/ml); IL-6 (1.01 ng/ml) and GM-CSF (0.52 ng/ml) as quantified by IRX Therapeutics.

The preparation of CASAC was as stated in section 2.2.3.4 but instead of inclusion of 44 µl of DPBS, 100 µl of IRX-2 was added into the vaccine mixture to make the final volume 156 µl of the first part of CASAC. 156 µl (equal volume) of emulsion was then added to the vaccine mixture and was vortexed for 1 minute. The prepared vaccine was then administered within a 2 hours period.

In situations where IRX-2 is required to be given as a daily dose, IRX-2 is then thawed to room temperature and injection of 100 µl of IRX-2 was conducted near the site of vaccination (i.d.)

2.2.3.6 Preparation of rechallenge dose

In vaccinations where a rechallenge with class I peptides were required, 20µl of the SIINFEKL peptides (100 µg/mouse, from a stock solution of 5 mg/ml) was mixed with 80 µl of DPBS to make a final volume of 100 µl. Rechallenge was conducted with an i.d. injection (Section 2.2.4.1)

2.2.4 Vaccine administration

2.2.4.1 Intradermal injection (i.d.)

For i.d. injections, mice were anesthetized with isofluorane using an anaesthetic system (VetTech Solutions Ltd - Figure 2.3). 5 mice were placed in the anaesthetic chamber and the system was turned on (2 litre/minute of oxygen and a flow rate of 4 for isofluorane) for the initiation of anaesthesia. Once the mice were anaesthetized, one mouse was removed from the chamber and placed on the anaesthetic nozzle to ensure the mouse continued to receive isofluorane. At this stage, the flow rate for isofluorane was brought down to flow rate of 2. The anaesthetized mouse was then shaved (Wella contour clipper) on both the right and left flank. Area of injection was sterilized with an antiseptic, normally Chlorohexidine (Vetasept). Vaccine was then injected with a 0.3 ml insulin syringe with a 30 gauge needle (Beckton Dickinson) intradermally by pinching the skin layer and administering the vaccine into the pinched skin Half of the vaccine dose was administered with into each flank of the mouse. A bulge would appear if the intradermal vaccination was successful (Figure 2.4). The mice were monitored until they were fully awake before returning them to their holding rooms.

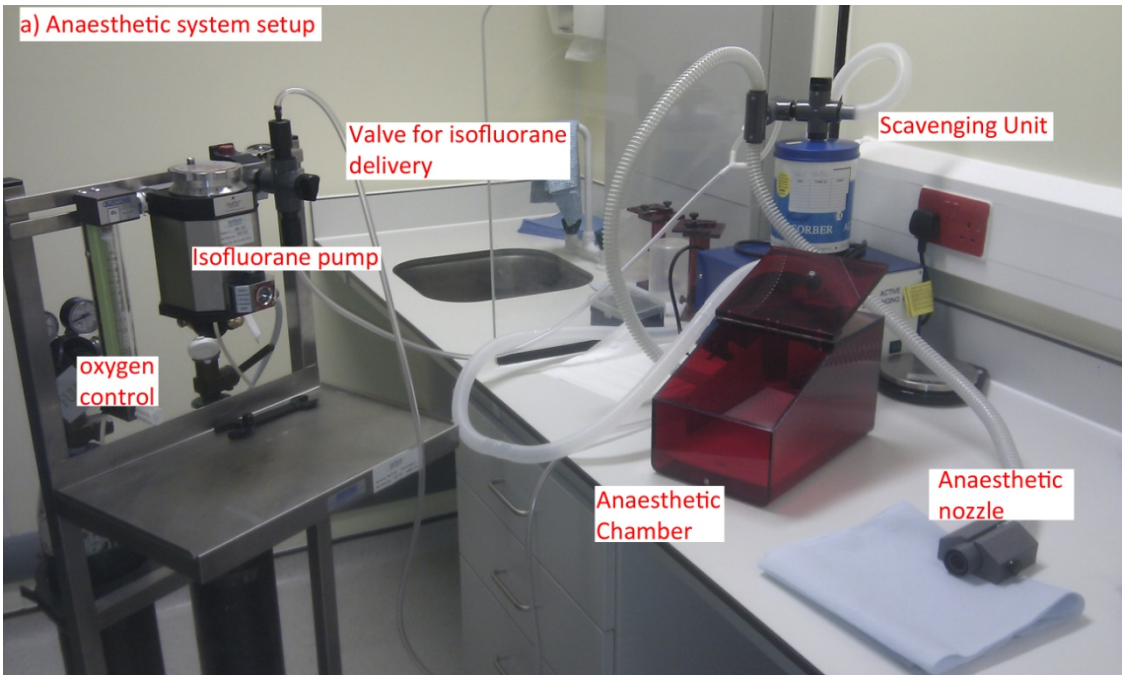


Figure 2.3 VetTech Solutions Ltd. anaesthetic system: Components of the system were as labelled.

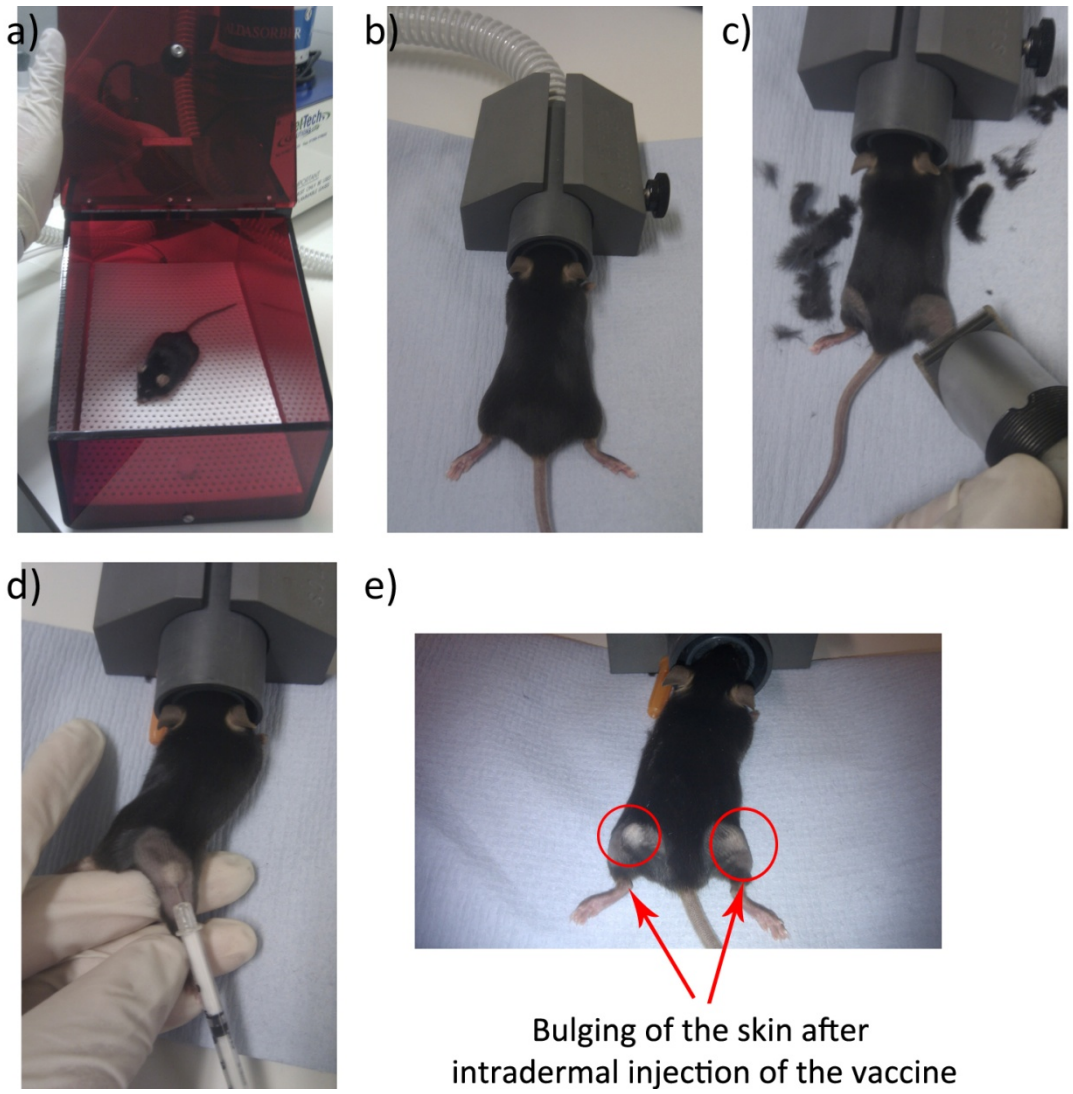


Figure 2.4 Intradermal injection: a) Initiation of anaesthesia in the anaesthetic chamber. b) Mouse transferred to anaesthesia nozzle for continued administration of anaesthetics. c) Mouse was shaved on both flanks. d) Vaccine was administered intradermally with an insulin syringe. e) After the i.d. vaccination on both flanks, bulging of the skin at the vaccination site could be observed indicating the intradermal delivery of the vaccine solution.

2.2.4.2. Intraperitoneal injections (i.p.)

Intraperitoneal injections were conducted for the priming dose of vaccine. Mouse was scruffed by the back of the neck and turned over to expose the abdomen after it had been restrained. A 0.3 ml insulin syringe with 30 gauge needle was used for the i.p. injection. The needle was inserted to the middle of the abdomen and then the priming dose was delivered.

2.3 Harvesting of blood and tissue

2.3.1 Bleeding by tail vein

Mice were bled through their tails. A sterile sharp scissors (Scientific Laboratory Supplies) or a No. 10 disposable blade (Swann Morton) was used to clip 1mm of the tip of the tail. 1.5 ml tube (pre-filled with 20 µl of sodium citrate as anticoagulant) was used to collect 100-150 µl of blood from a mouse. In cases where absolute cell counting was conducted, a 1.5 ml tube containing dried sodium citrate was used. These tubes were prepared by adding 20 µl of sodium citrate and left open to dry in a biological safety hood for 1-2 days.

2.3.2 Harvesting the spleen

In analyses where splenocytes was required, mice were first humanely killed with a schedule 1 procedure. Here we used a carbon dioxide (CO_2) chamber (VetTech Solutions Ltd.) with rising levels of CO_2 at a rate of 4 litre/minute. A maximum of 10 mice could be humanely killed at once. Mice were left in the chamber till rigor mortis had set in. A mouse was then removed from the chamber and placed into a biological safety cabinet. It was sprayed with chlorohexidine for sterilization purposes. An incision was made on the right abdominal flank, as the mouse was placed on its back, with a sterile pair of scissors. A pair of tweezers was then used to slowly remove the spleen. Spleen was then placed into a 50 ml Falcon tube (Beckton Dickinson) filled with 10 ml of RPMI complete media (RPMI-1640, 10% FBS and 1% penicillin/streptomycin).

2.4 Fluorescent Assisted Cell Sorting (FACS) analysis of immunological responses

2.4.1 FACS machine and setup

In all of the FACS analyses, a 3-laser FACS Canto II (Beckton Dickinson) was used. In a multi-colour FACS analysis, compensation for the fluorochrome involved was conducted using single colour stained samples and an unstained sample for control. Compensation was calculated by the BD FACS Diva software (Beckton Dickinson) and manually readjusted with FlowJo software (Tree Star) where required. In the FACS analysis, 2 staining protocols were used. Surface staining was conducted for markers on the cell surface (i.e. CD3, CD8 and CD4) while intracellular staining was conducted for intracellular markers (i.e. FoxP3 and IFN-γ).

2.4.2 Analysis of Antigen specific T-cells

2 µl of R-PE H-2K^{b} SIINFEKL pentamer was added to 40 µl of whole blood in wells of a 96 well plate (Greiner Bio One). Pentamers were titrated with antigen specific OT-1 splenocytes to saturation point and were tested from batch to batch for variability. Incubation was conducted in the dark for 15 minutes followed by addition of 2 µl of PBlue anti-mouse CD3, 1 µl PE-Cy5.5 anti-mouse CD8a, 1 µl APC anti-mouse CD62L and 1 µl FITC anti-mouse CD44. After 15 minutes of incubation, red blood cells were lysed with 200 µl of RBC lysis solution for 7 minutes or until the mixture turned clear. Plates were centrifuged (Rotanta 460R centrifuge) for 5 minutes at 400 xg. Supernatant was discarded and cells were resuspended with 200 µl of DPBS. This was repeated twice and finally resuspended with 200 µl of DPBS. Cells were analysed on the FACS machine and the data obtained was analysed with FlowJo (TreeStar).

To gate for the SIINFEKL specific CD8 T-cells, the gating strategy was as outlined in Figure 2.5. The population of lymphocytes was gated by size (forward scatter - FSC) and granularity (side scatter - SSC) of the cells (Figure 2.5 a). The lymphocyte population was then gated for CD3 and CD8 positive cells (Figure 2.5 b). $CD3^{+}CD8^{+}$ T-cells were then gated for $CD8^{+}SIINFEKL^{+}$ T-cells. The data is presented as a dot plot of CD8 vs. SIINFEKL in which the percentage of $CD3^{+}CD8^{+}SIINFEKL^{+}$ T-cells are determined (Figure 2.5 c). In order to set the gates on the SIINFEKL positive cells, negative gating was confirmed with

unvaccinated mouse PBMC stained with SIINFEKL pentamer (Figure 2.5 c-I) or a vaccinated mouse PBMC stained with an irrelevant LCMV pentamer (Figure 2.5 c-II). SIINFEKL pentamer staining of OT-1 splenocytes which are almost 100% transgenic with a T cell receptor recognizing SIINFEKL, served as a positive control (Figure 2.5 c-III). This gating strategy was then applied for the stained vaccinated samples to determine the percentage of SIINFEKL specific $CD8^+$ T-cells (Figure 2.5 c-IV).

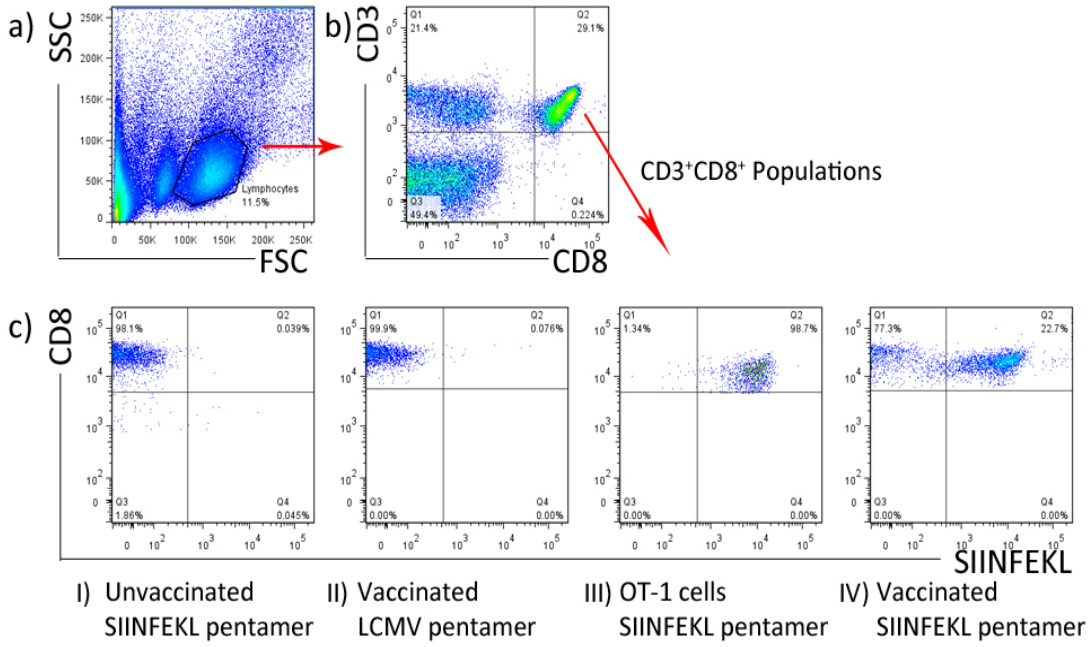


Figure 2.5 Gating strategy for SIINFEKL specific $CD8^+$ T-cells: a) FSC vs. SSC gating of cells to determine the lymphocyte population according to size and granularity based on light scattering. b) Identification of the $CD3^+CD8^+$ T-cells. c) A negative control can be obtained by I) staining PBMC from unvaccinated mice with SIINFEKL pentamer or II) staining the PBMC from vaccinated mice with LCMV pentamer. III) Positive control for SIINFEKL pentamer by staining of OT-1 splenocytes with the SIINFEKL pentamer. IV) An example of SIINFEKL pentamer stained PBMC from a CASAC/SIINFEKL vaccinated mouse.

2.4.3 Analysis of FoxP3$^+$ cells

Cell surface marker staining was conducted with 1µl FITC anti-mouse CD4 and 1µl APC anti-mouse CD25 on 40 µl of whole blood aliquots in a 96 wells plate. Incubation was for 30 minutes in the dark at room temperature. RBC lysis conducted as stated in 2.4.2 and 2 washing steps with DPBS was carried out before reconstituting with 200 µl of Fix/Permeabilization buffer (Ebioscience). Cells were fixed and permeabilized for 30 minutes in the fridge (dark and cold). Cells can be left in this condition up to 18 hours with no significant difference in data obtained from cells incubated for 30 minutes. Cells were then washed with 200 µl of ice-cold Permeabilization Buffer for 3 times. The cells were finally resuspended in 100 µl of ice-cold Permeabilization buffer. 2 µl PE anti-mouse FoxP3 was used to stain the cells. 2 µl of PE Rat IgG2a kappa (isotype control) was used as an isotype control for FoxP3 staining. Staining was conducted in the fridge for 30 minutes. Cells were then washed twice with Permeabilization buffer and once with DPBS before being resuspended in 200 µl of DPBS for FACS analysis. Data were analysed with FlowJo and the gating strategy for PBMC stained for Tregs is shown in Figure 2.6. A population of lymphocytes was gated based on the FSC and SSC (Figure 2.6 a). $CD4^+$ population was then gated on the lymphocytes (Figure 2.6 b) followed by $CD4^+CD25^+$ T-cells (Figure 2.6 c). The $CD4^+CD25^+$ were then determined for $FoxP3^+$ staining by using an isotype control (Figure 2.6 d). The gating was then applied to a stained sample to determine the percentage of FoxP3 stained cells (Figure 2.6 e).

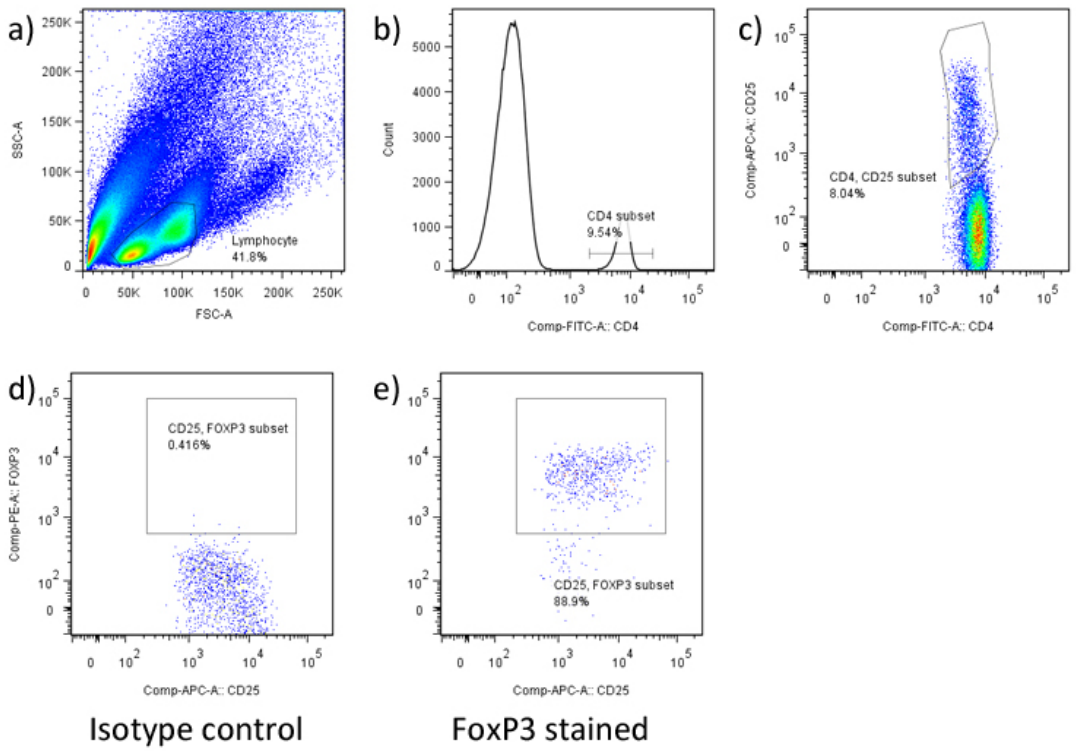


Figure 2.6 Gating for Tregs: a) Lymphocytes were gated based on their FSC and SSC. b) Lymphocytes were then gated for $CD4^+$ and c) for $CD4^+CD25^+$ cells. d) The $CD4^+CD25^+$ were stained with an isotype control to determine the gating for $FoxP3^+$ cells and e) the gating applied to a stained sample, identifying the $CD4^+CD25^+FoxP3^+$ cells (Tregs).

2.4.4 Intracellular Cytokine analysis

Spleen from mice were made into single cell suspensions by mashing the cut up tissue with a syringe before filtering it through a 40 µm cell strainer (Beckton Dickinson) in sterile DPBS. After one round of washing, 1 ml of RBC lysis buffer was added to the pellet and resuspended gently. After 7 minutes the cells were washed twice with DPBS and counted in a haemocytometer to make a final concentration of 1 X 10^6 cells/200 µl in complete RPMI medium (10% Foetal calf serum (Dulbecco), 1% Penicillin/Streptomycin (Sigma) and Roswell Park Memorial Institute (RPMI) media (Sigma). 2 µl of 10mM Monensin (final concentration of 3 µM - SIGMA), 0.2µl of 1mg/ml anti-mouse CD28 (final concentration of 1 µg/ml) and 1 µl of 5mg/ml peptide (for each peptide and final concentration of 25 µg/ml)

were added to the cell suspension and incubated for 5 hours in 37^{o}C, 5% CO2 incubator (Thermo Scientific) to produce cytokines. PMA (50 ng/ml)/Ionomycin (1 µg/ml) stimulation was used to provide positive controls for staining. After the incubation period, cells were washed twice with 200 µl of DPBS before resuspending in a final volume of 100 µl of DPBS. Depending on subset of T-cells and type of cytokines to be analysed, the list of antibodies used is as below:

CD4 subsets: 1.0 µl Percp-Cy5.5 Anti-mouse CD4
1.0 µl PE Anti-mouse IFN-γ
0.5 µl FITC Anti-mouse IL-17
2.5 µl PE-Cy7 Anti-mouse IL-4
1.0 µl Pblue Anti-mouse TNF-α

CD8 subsets: 1.0 µl PercpCy5.5 Anti-mouse CD8
1.0 µl PE Anti-mouse IFN-γ

Surface markers such as CD4 and CD8 were first stained for 30 minutes in the dark. 2 washing steps followed by resuspensing the cells in 100 µl of DPBS. Cells were transferred to BD Falcon™ 5 ml round-bottom tubes (Beckton Dickinson). Cells in tubes were vortexed and 100 µl of IC fixation buffer (Ebioscience) was added drop-wise. Cells were then left to be fixed for 20 minutes in the dark. 1ml of ice-cold 1 X Permeabilization buffer was added and centrifuged at 400 x g for 5 minutes. This step was repeated twice and cells were resuspended with 100 µl of 1 X Permeabilization buffer and transferred to a 96 well plate for subsequent staining with cytokine specific antibodies as shown above. Staining was conducted for 30 minutes in the fridge, followed by 2 rounds of washing with 1 X permeabilization buffer and then DPBS before resuspending to a final volume of 200 µl. FACS analysis followed.

2.4.5 Absolute cell count

20 µl of Flow-Count™ counting beads (1000 beads/µl) was added to 20 µl of whole blood from samples collected in tubes with dried anticoagulant. Cells were mixed gently by pipetting and lysed with 200 µl of RBC lysis buffer for 7 minutes. Cells were then centrifuged for 5 minutes at 400 x g and the supernatant was discarded. Finally, these cells were resuspended in 200µl of DPBS ready for FACS analysis.

2.4.6. Surface staining of senescence, exhaustion and activatory markers

Surface staining for these markers was conducted in a similar fashion to that described in section 2.4.2. The antibodies and their concentrations were as follow: 2 µl APC anti-mouse CD152 (CTLA-4); 2 µl PE anti-mouse CD274 (PD-L1); 2 µl FITC anti-mouse CD279 (PD-1); 1 µl PE anti-mouse CD223 (Lag-3); 1 µl PE-Cy7 anti-mouse KLRG1 and 2 µl PE anti-mouse CD28. Cells were also stained with CD3, CD8 and CD4 antibodies as previously described. Gating strategy for CD28/CTLA-4 is shown in Figure 2.7. Monocytes and lymphocytes were selected based on size (FSC) and granularity (SSC). Monocytes do not express CD28 or CTLA-4 and were used to determine the negative gating.

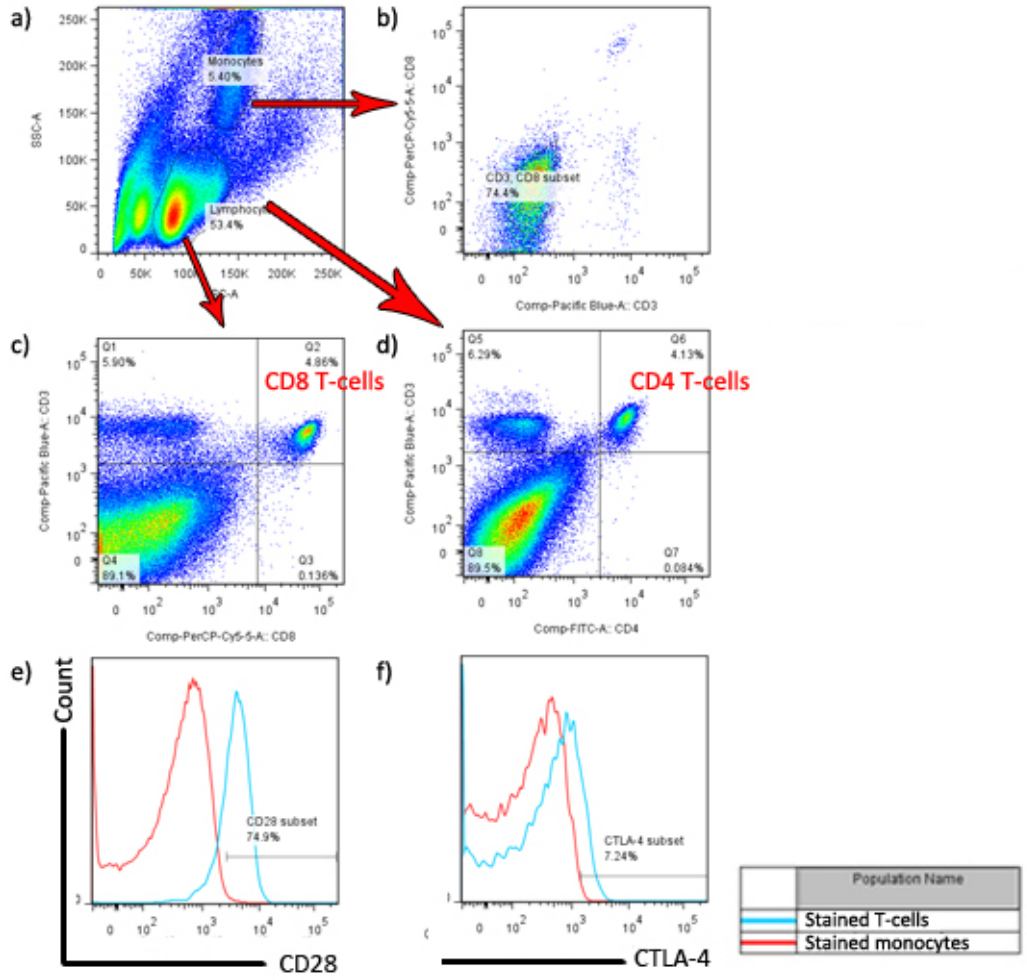


Figure 2.7 Gating strategy for CD28 and CTLA-4: a) Lymphocytes and monocytes were gated according to FSC and SSC. b) The monocytes were then gated for $CD3^{-}CD8^{-}$ cells and used as a negative control for stained T-cells shown in e and f. CD4 and CD8 T-cells gating is as shown in c and d. Percentage of CD28 and CTLA-4 in these cells were determined with the negative gating of monocytes (e and f).

CpG has been shown to upregulate expression of PD-L1 in mouse monocytes[121]. Therefore, CASAC vaccinated mice and unvaccinated mice could be utilised to determine the gating for PD-L1 (Figure 2.8).

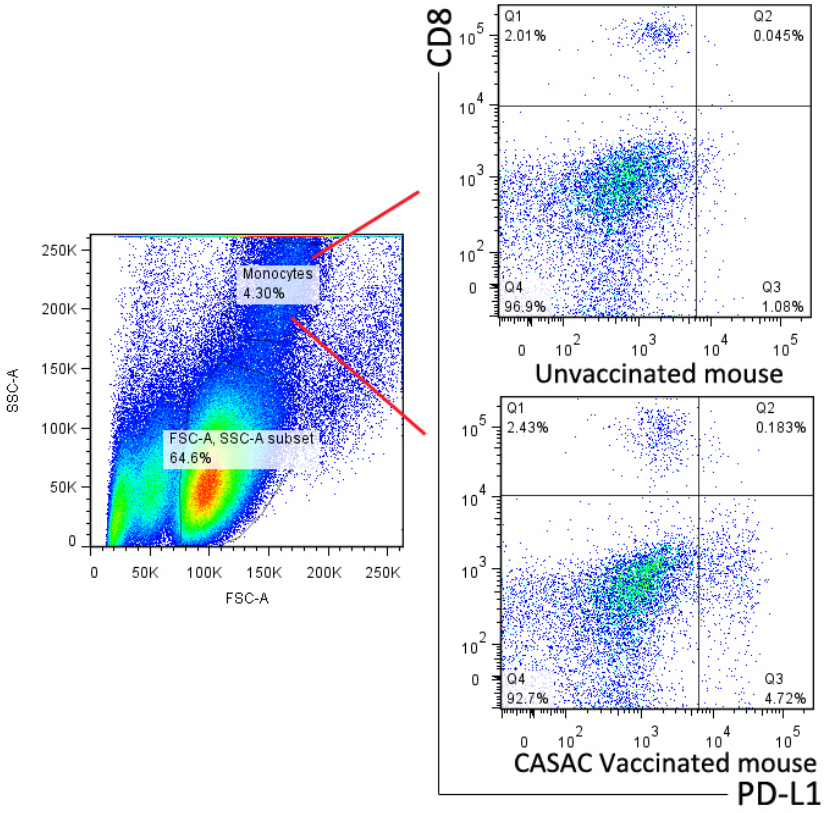


Figure 2.8 Gating strategy for PD-L1: Gating strategy for PD-L1 utilising monocytes from unvaccinated and vaccinated mouse. In the CASAC vaccinated mice, the monocytes express PD-L1, which is absent in unvaccinated mouse. Therefore the gate set here for CD8 vs. PD-L1 was utilised to determine PD-L1 expression in T-cells.

Gating strategy for PD-1 and KLRG-1 was based on the expression of PD-1 and KLRG-1 positive cells in activated T-cells (antigen specific CD8 T-cells). In young mice which are not antigen experienced, $CD3^{+}CD8^{+}$ T-cells showed almost no PD-1 and KLRG-1 expression[111]. This enables the gating strategy for PD-1 (Figure 2.9 a and b) and KLRG-1 (Figure 2.9 c and d) to be conducted based on stained CD8 T-cells from unvaccinated young mice as a negative control while stained SIINFEKL specific $CD8^{+}$ T-cells were used as positive controls. An isotype control was used in determining the gate for LAG-3 (Figure 2.9 d and e)

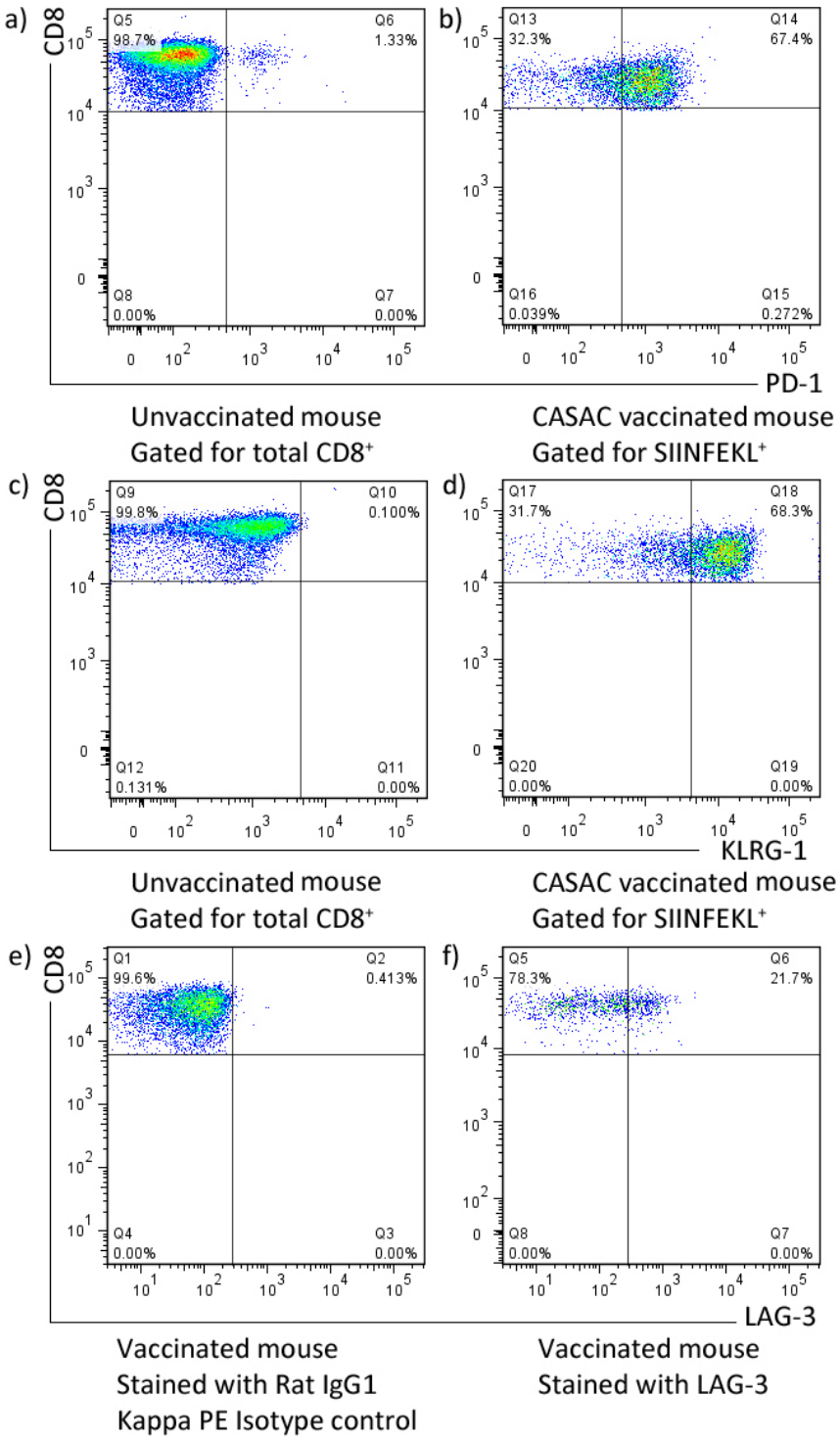


Figure 2.9 Gating strategy for PD-1, KLRG-1 and LAG-3: Gating for PD-1 conducted with unvaccinated mouse PBMC for $CD3^{+}CD8^{+}$ T-cells as negative control (a) and SIINFEKL specific CD8 T-cells for positive control (b). Gating for KLRG-1 conducted with unvaccinated mouse PBMC for $CD3^{+}CD8^{+}$ T-cells as negative control (c) and SIINFEKL specific CD8 T-cells for positive control (d). Gating for LAG-3 was by staining PBMC from vaccinated mice with an isotype control (e) and the gate was applied to a stained sample from the vaccinated mice (f).

2.5 Functional analysis of T-cells responses

2.5.1 In-vivo Cytolytic T-Lymphocyte (CTL) assay

Spleens were collected from healthy C57Bl/6 female mice. One healthy spleen was required to provide sufficient cells for 5 sets of *in vivo* CTL assays. Spleens from mice were made into single cell suspensions by cutting with a scalpel and then mashing with a syringe before filtering it through a 40 µm Cell strainer in sterile DPBS. After one round of washing, 1 ml of RBC lysis buffer (1 ml per spleen) was added to the pellet and resuspended gently. After 7 minutes of lysis, the cells were washed twice with DPBS, resuspended in DPBS and were counted with a haemocytometer. Splenocytes were then made to a final density of 1 X 10^7 cells/ml in serum free X-vivo 15 media (Lonza).

Cells were separated into two 50 ml Falcon tubes (Beckton Dickinson) of equal volume. Antigen positive target cells for the *in vivo* CTL assay were made using 1 X 10^7 splenocytes/ml pulsed with 10µg/ml SIINFEKL peptide. After 1 hour incubation, the peptide pulsed splenocytes were washed twice with DPBS. Cells were resuspended in DPBS at 2-5 X 10^7 cells/ml. These cells were then tested for presences of SIINFEKL in MHC-I $H2K^b$ by staining a small aliquot of the cells with 2 µl of PE Anti-Mouse $OVA_{257-264}$ SIINFEKL peptide bound to H-2Kb. Almost 100% of splenocytes were shown to be loaded with SIINFEKL peptides (Figure 2.10 a). The target cells were then labelled with 0.3 µM of DDAOSE or CFSE (DDAOSE/CFSE low). DDAOSE is a succinimidyl ester similar to CFSE but has excitation at 640nm and emission of 663-687. Antigen negative cells were not pulsed with peptide and were labelled with 3 µM of DDAOSE (DDAOSE high). After 7 minutes of incubation in the dark, cells were washed with RPMI complete medium at 10 fold of the staining volume, repeated twice. Cells were finally resuspended in DPBS at a concentration of 1 X 10^8 cells/ml. A ratio of 1:1 (antigen positive target cells : antigen negative cells) was obtained (Figure 2.10 b). The cell mixture was then injected intravenously (i.v.) through the tail vein in a volume of 100 µl containing 10^7 of the antigen positive and antigen negative cells. An aliquot of the cell mixture was incubated in a 37^{o}C, 5% CO_2 incubator.

Sixteen to eighteen hours after i.v. injections of cells, mice were humanely killed by schedule 1 procedure and spleens were harvested. Spleens were made into single cell

suspension as mentioned above in DPBS and analysed with FACS (Figure 2.10 c). Cell mixture incubated with the same period of time was also used as a negative control to ensure no changes occur to the percentage of antigen positive and antigen negative cells (Figure 2.10 d). Percentage of antigen specific cell lysis was calculated based on the formula below :

$$\%\ \text{of cell lysis} = \left(1 - \frac{\dfrac{\text{Antigen positive cells in test sample}}{\text{Antigen negative cells in test sample}}}{\dfrac{\text{Antigen positive cells in control sample}}{\text{Antigen negative cells in control sample}}} \right) \times 100\%$$

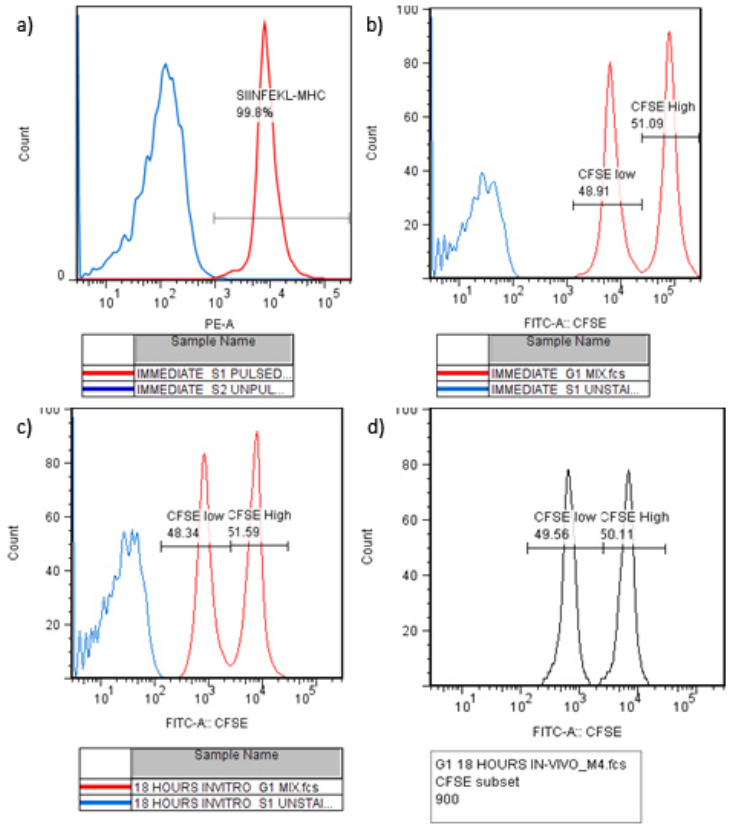


Figure 2.10 In-vivo CTL assay gatings: a) Histogram shows the peptide pulsed (red line) and unpulsed (blue line) splenocyte populations after staining with anti-mouse OVA bound to H-2K^b PE antibody. b) CFSE low and CFSE high splenocytes were at an approximately 1:1 ratio (antigen positive : antigen negative). c) *In vitro* mixed population of target and non-target cells which were left for the same period as the *in vivo* CTL assay confirmed the presence of the two cell populations at a ratio of approximately 1:1. d) A representation of the recovered splenocytes from an emulsion only vaccinated mouse, where the percentages of cells obtained confirmed the presence of the two cell populations at a ratio of approximately 1:1.

2.5.2 In-vitro proliferation of antigen specific Tregs

Splenocytes from vaccinated mice were made into single cell suspension and stained with 3 µM CFSE as described in 2.5.1. Cells were then resuspended at a density of 1 X 10^6 cells/ml in complete RPMI medium and aliquoted into 48 well plates (Greiner Bio One). 25 µg of each peptide (ISQ and TEWT or PADRE) was added to the culture. On day 4, cells were analysed for proliferation as indicated by lower intensity of CFSE. 250 µl of cells were collected from each well into a 96 well plate. Cells were washed twice with DPBS and resuspended in 100 µl DPBS. Cells were stained with LIVE⁄DEAD® Fixable Far Red Dead Cell Stain (0.2 µl/well from stock solution (Invitrogen) for 15 minutes in the dark at room temperature. Cells were washed twice with RPMI medium and 1 X DPBS before resuspension in 100µl DPBS. Cells are then stained with anti-mouse CD4 and FoxP3. Staining was conducted as described in section 2.4.3 and after completion of staining and washing, cells were resuspended in 200 µl of DPBS and subjected to FACS analysis.

2.6 Statistical analysis

Two-tailed Student's t test was conducted on data with a normal distribution to determine significance. However, in instances where a normal distribution of data was not seen, Mann Whitney test was conducted to determine statistical significance. All statistically analysis were conducted using GraphPad Prism (GraphPad Software, Inc)

Standard error of mean (SEM) were used in all the analysis conducted. SEM is a hypothetical quantity of error from the mean value of samples from a population. In all my analysis, I assumed that the vaccinated or unvaccinated mice were samples from a population (by definition the entire mice population). However, this is inaccurate as the vaccinated and unvaccinated mice are the population that I have been studying. Therefore the use of standard deviation (SD) which is defined as the average deviation of the samples values from the mean in a population would be a more accurate statistical analysis.

Multiple comparisons should also be considered when comparing three or more groups. In this case an analysis of variance (ANOVA) could be conducted. Further analysis can be conducted with the Bonferroni method to determine the differences in between each groups[122].

CHAPTER 3

Alternating T-helper peptide vaccination to enhance $CD8^+$ T-cell responses.

3.1 Introduction and Study objectives

3.1.1 Introduction

Peptide vaccination, particularly when used in combination with appropriate adjuvants can generate the stimulation and expansion of antigen specific T-cells. If the antigenic peptide used is an MHC I (Class I) restricted peptide, the stimulated T-cells will be of the $CD8^+$ subset. In the absence of a T-helper peptide which is usually an MHC II (Class II) restricted peptide, antigen specific CD8 T-cells can still be generated but with less expansion of the memory CD8 T-cells[40]. A strong CD8 response without help does not necessarily generate memory T-cells[43]. However, repeated stimulation of the same population of $CD4^+$ T-cells with a CD4 restricted peptide (T-helper peptide) could generate antigen specific Tregs as shown by Apostolou *et al.,* (2004) when minute doses of the antigen were delivered by an osmotic pump[68]. Belkaid *et al.* (2009) stated that Tregs are important in controlling collateral damage in inflammatory situations[123] and therefore in vaccination, the repeated stimulation of the same population of $CD4^+$ T-cells could suppress the generation and expansion of $CD8^+$ T-cell responses.

In the light of this background information, T-helper peptides are on the one hand necessary for induction of effective and long term immunological responses. On the other hand, repeated stimulation of $CD4^+$ T-cells by multiple rounds of vaccination could prove counterproductive, resulting in greater stimulation of Tregs and reduced or shorter lived duration of $CD8^+$ mediated responses (e.g. by exhaustion and/or eventual depletion of a specific clone of $CD4^+$ T-helper cells). We hypothesised that by alternating the T-helper peptides in consecutive rounds of vaccinations, it would be possible to obtain robust and long term $CD8^+$ T-cell responses required for disease clearance (e.g. as in cancer therapy), whilst avoiding the generation of Tregs. Chicken ovalbumin (OVA) was utilised as a model antigen as it is well studied. In a standard peptide vaccination protocol, an MHC class I presented OVA peptide, i.e., SIINFEKL, and an MHC class II presented OVA peptide (as T-helper peptide, ISQAVHAAHAEINEAGR - ISQ) were used repeatedly to vaccinate mice (Figure 3.1 a). In an alternating T-helper peptide vaccination protocol, SIINFEKL was used together with two alternating T-helper peptides ISQ and another OVA derived

peptide restricted by class II, TEWTSSNVMEERKIKVY (TEWT) in consecutive rounds of vaccination (Figure 3.1 b)

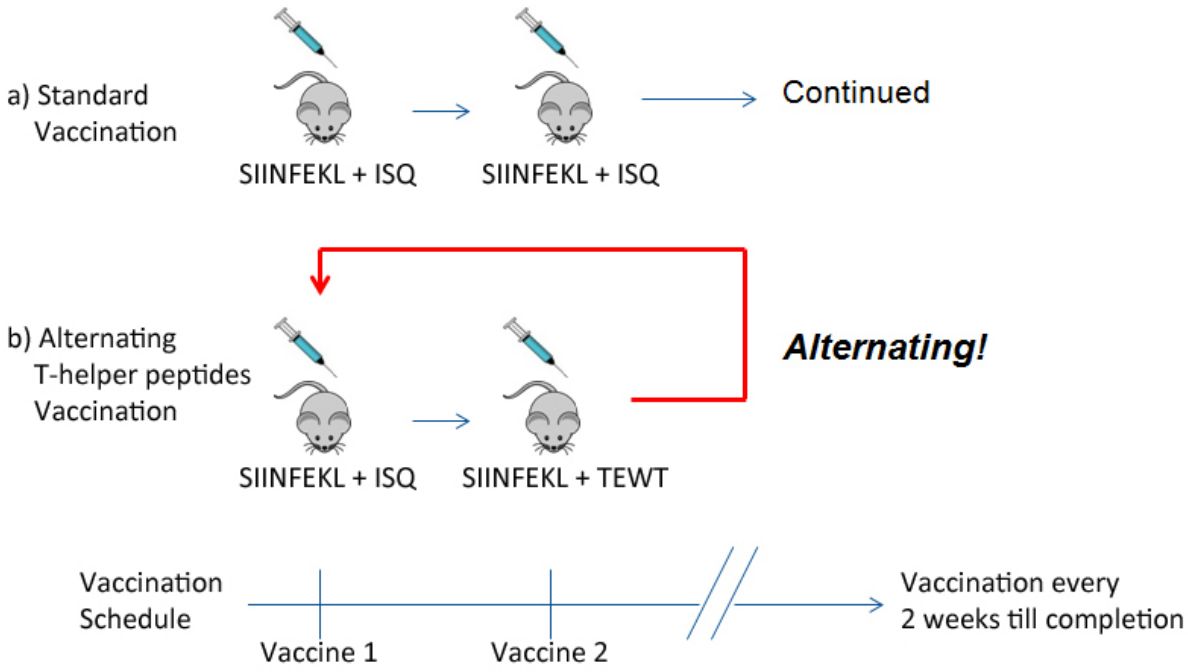


Figure 3.1 Concept of alternating T-helper peptides: a) A standard vaccination protocol where the same set of class I and class II peptides were administered at every vaccination time point. b) An alternating T-helper peptide vaccination protocol where the same class I peptide was used together with alternating T-helper peptides at consecutive vaccination rounds.

3.1.2 Study Objectives

a) Investigate the optimum vaccination regimen and adjuvants required to generate enhanced antigen specific CD8 T-cell responses through alternating T-helper peptide vaccinations.

b) Assess if the magnitude of Treg expansion differs in the two vaccination protocols.

c) Functional analysis of antigen specific $CD8^+$ T-cell responses generated by the two vaccination protocols.

d) Investigate the type of memory CD8 T-cells generated by the two vaccination protocols.

3.2 Alternating T-helper peptide vaccination to promote SIINFEKL specific $CD8^+$ T-cells.

3.2.1 Vaccination design

Effective vaccination requires one or more adjuvant to provide sufficient innate stimulation. Adjuvants from CASAC[52], which is a combination of a class I peptide, a class II peptide, two TLR agonist (which activates independent NF-κB pathway), mIFN-γ and emulsion (Tween-80 and squalene) were adapted to investigate the use of alternating T-helper peptides. The adjuvant chosen for this study was a combination of TLR-3 agonist, Poly I:C (50µg); TLR-9 agonist, CpG ODN 1826 (25µg) and emulsion (4% Squalene and 0.2% Tween 80). 100µg of each peptide were used to vaccinate each mouse. The groups of mice vaccinated are shown in Figure 3.2 a). The vaccination schedule was as described in Figure 3.2 b).

a)

Groups	Adjuvant	SIINFEKL	ISQ	TEWT
Unimmunized	-	-	-	-
G1	+	+	-	-
G2	+	+	+	-
G3	+	+	-	+
G4	+	+	+	+
G5 (Vaccine 1,3,5,..)	+	+	+	-
G5 (Vaccine 2,4,6,..)	+	+	-	+

b)

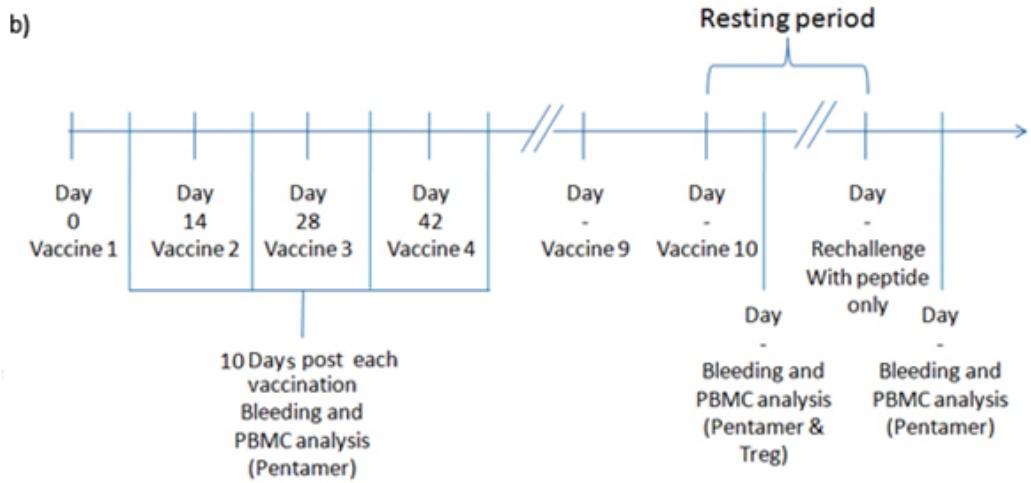


Figure 3.2 Vaccination and analysis time points: a) Vaccination group and components of vaccine. Adjuvant used in this design consists of Poly I:C (50µg/mouse), CpG (25µg/mouse), 0.2% Tween 80, 4% Squalene and PBS. Each peptide included in the vaccine is 100µg/mouse. b) Vaccination schedule with vaccination and analysis time points. Intra dermal vaccination was conducted while collection of PBMC for analyses were conducted by tail vein clipping. Analyses as described in section 2.4.2.

3.2.2 Vaccination with Poly I:C and CpG produces high percentages of SIINFEKL specific $CD8^+$ T-cell

In this vaccination protocol, high percentages of SIINFEKL specific $CD8^+$ T-cells were obtained with no significant differences between groups vaccinated with a single class I peptide alone or in combination with a T-helper peptide. SIINFEKL specific $CD8^+$ T-cells were analysed 10 days post each vaccination on PBMC collected by tail vein using an H-2Kb/SIINFEKL pentamer. FACS gating was as presented in Figure 3.3. $CD3^+CD8^+$ T-cells in the unvaccinated mice stained with SIINFEKL pentamer were used as a negative control to set the background gate according to Figure 3.3 and the same gating strategy was then applied to other samples which were pentamer stained. A representative example of a stained sample is shown in Figure 3.3. Positive control staining was also conducted with the use of OT-1 splenocytes which produced almost 85% SIINFEKL specific $CD8^+$ T-cells (Figure 3.4).

The use of Poly I:C and CpG in the vaccine generated 40-75% SIINFEKL specific $CD8^+$ T-cells after 7 rounds of vaccination. From the second vaccine onwards, the percentages of SIINFEKL specific $CD8^+$ T-cells increased consistently across all groups. No statistically significant differences were seen by comparison of the alternating T-helper peptides with the repeated use of the same one or two helper peptides. Even in the absence of a class II T-helper peptide, the group vaccinated with SIINFEKL alone generated similar responses to groups vaccinated with T-helper peptides. The data was not significantly different using a Student's t test analysis (Figure 3.5).

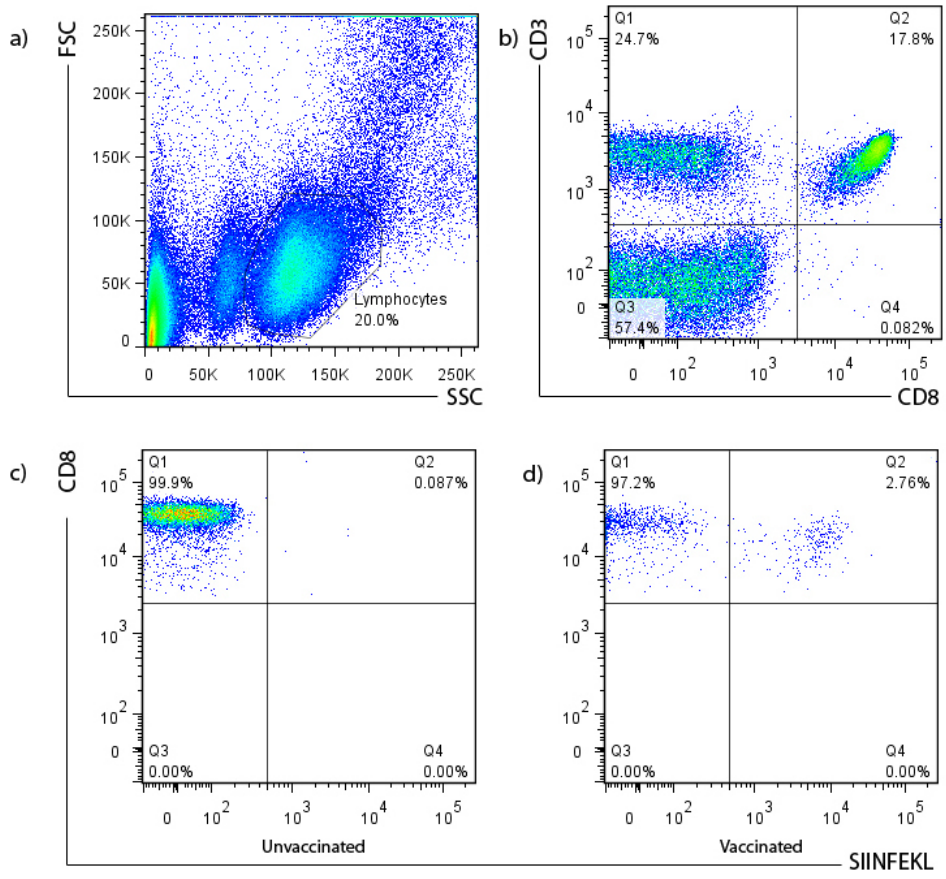


Figure 3.3 Gating strategy for SIINFEKL specific CD8+ T-cells: a) The lymphocyte population was identified and then b) gated for $CD3^{+}CD8^{+}$ cells. The $CD3^{+}CD8^{+}$ population was gated for $CD8^{+}$ and $SIINFEKL^{+}$ pentamer staining. c) Scatter plot shows staining with SIINFEKL pentamer on a PBMC sample from an unvaccinated mouse while d) shows staining on a PBMC sample from a vaccinated mouse 10 days after the first vaccination.

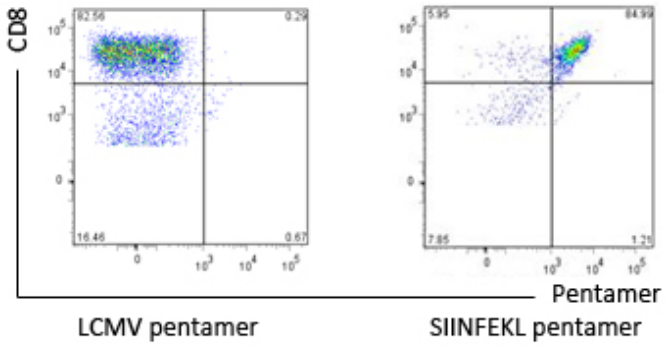


Figure 3.4 Pentamer staining of OT-1 splenocytes: Splenocytes obtained from an OT-1 transgenic mouse in which all T-cells express has SIINFEKL specific T-cell receptor. The left panel shows LCMV specific pentamer staining and the right panel shows SIINFEKL specific pentamer staining in the OT-1 splenocytes.

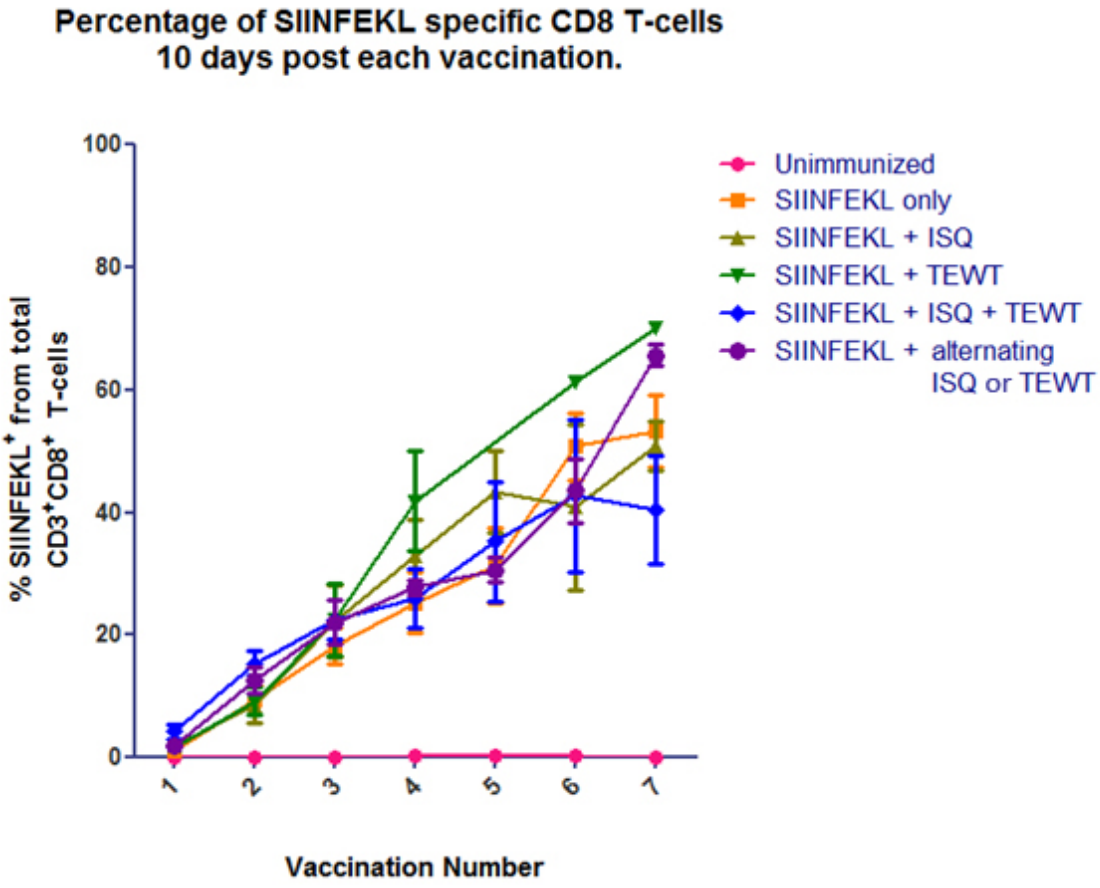


Figure 3.5 Percentage of SIINFEKL specific CD8$^+$ T-cells detected 10 days post each vaccination: The data shown was not significantly different when comparing between the groups at each time point with a Student's t test analysis. Mean ± SEM percentage is shown. (n=5)

3.2.3 SIINFEKL specific $CD8^+$ T-cells generated have a predominantly effector memory phenotype

Having found that the presence of T-helper peptides did not enhance the generation of SIINFEKL specific $CD8^+$ T-cells (see section 3.2.2); memory subsets were investigated in the SIINFEKL specific $CD8^+$ T-cells to determine if the use of T-helper peptides had enhanced the generation of the memory $CD8^+$ T-cells. In this study we found that SIINFEKL specific $CD8^+$ T-cells generated were found to be predominantly of the effector memory phenotype. Memory subsets were identified with CD44 and CD62L markers[124] (see Figure 3.6 a). Unvaccinated mice were analysed for memory subsets within the $CD3^+CD8^+$ T-cells as no SIINFEKL specific $CD8^+$ T-cells were detectable prior to vaccination (Figure 3.5). Percentages of memory subsets on $CD3^+CD8^+$ T-cells of unvaccinated mice are shown in Figure 3.6 b.

In the population of SIINFEKL specific $CD8^+$ T-cells about 95% of all SIINFEKL specific $CD8^+$ T-cells were found to have the effector memory phenotype in all of the vaccinated groups (Figure 3.6 c). Therefore, presence of T-helper peptides did not impact on memory CD8 T-cell type. There were few central memory cells within the SIINFEKL specific $CD8^+$ T-cells. This is probably due to the repeated stimulation with the vaccine which emulates a persisting need of effector memory SIINFEKL specific $CD8^+$ T-cells as in an infection where the antigen has not been eliminated[57]. The high percentage of effector memory T-cells within this population was also previously seen in CASAC vaccinations, producing high percentages of antigen specific T-cells[52].

The percentage of naïve T-cells in the unvaccinated mice is significantly higher compared to the vaccinated mice (Figure 3.6 d and 6 e). Almost no naive T-cells were found within the SIINFEKL specific $CD8^+$ T-cells. This is consistent with other studies and to be expected from the continuum linear differentiation of cells from naive → effector → effector memory → central memory cells[57].

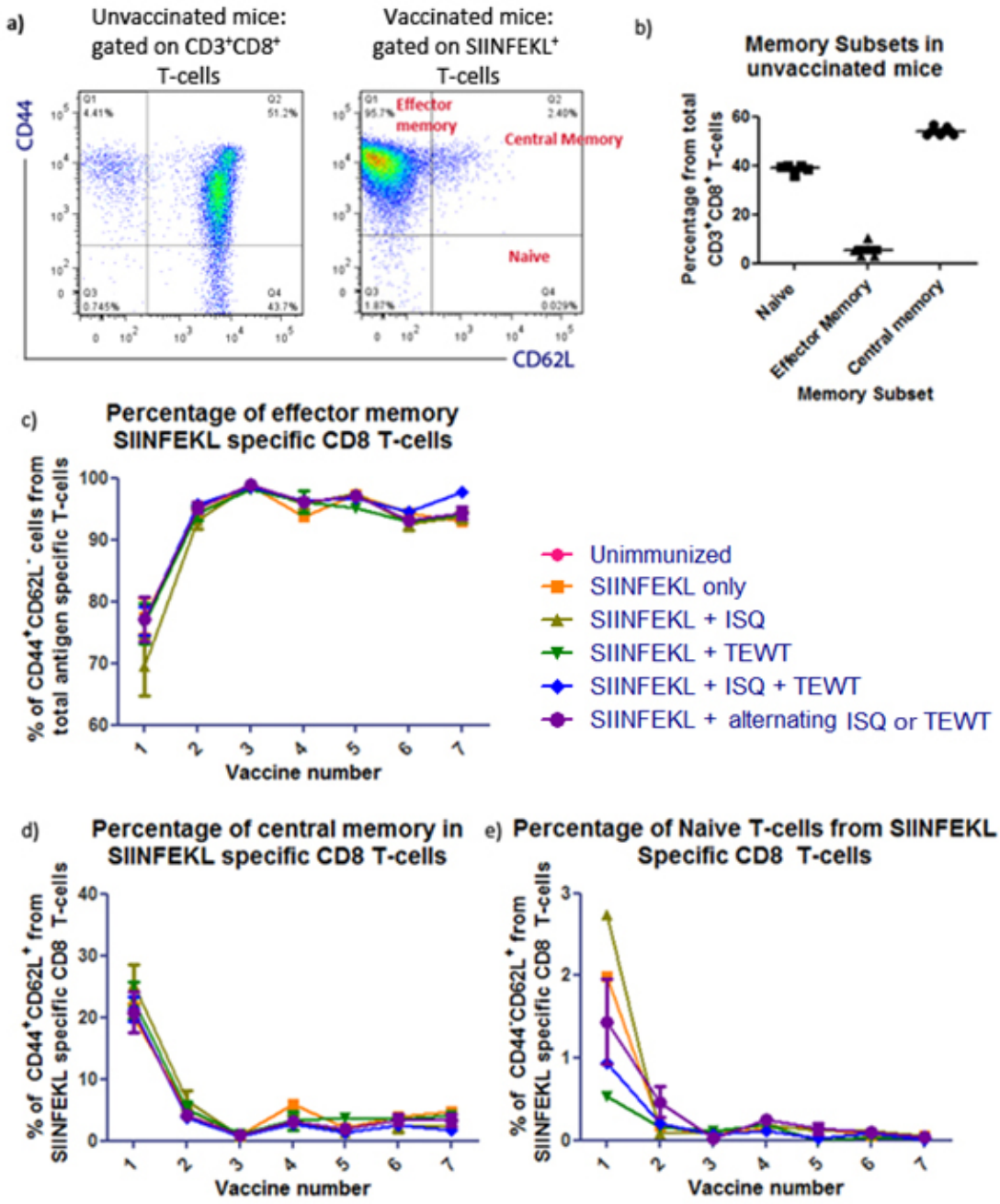


Figure 3.6 Memory phenotype analysis: a) Gating strategy for memory T-cells subsets. b) In unvaccinated mice, memory subsets were determined from total $CD3^+CD8^+$ T-cells in order to understand the percentages in normal resting mice. Percentage of c) effector memory, d) central memory and e) naïve T-cells within the SIINFEKL specific $CD8^+$ T-cells. Mean ± SEM is shown. (n=5 mice per group)

3.2.4 Summary

In all the vaccinated mice, either in the presence or absence of T-helper peptides, no significant differences were evident in the generation of SIINFEKL specific $CD8^+$ T-cells. The percentage of SIINFEKL specific $CD8^+$ T-cells increased consistently throughout the 7 cycles of vaccination with an effector memory phenotype for the majority of T-cells. Low percentages of central memory T-cells were also found within the SIINFEKL specific $CD8^+$ T-cells but no significant differences were seen between the different vaccinated groups. The continuous expansion and proliferation of SIINFEKL specific $CD8^+$ T-cells was probably due to the effect of the strong adjuvant used. Poly I:C and CpG ODN1826 are capable of causing a synergistic effect on NF-κB activation by two different signalling pathways[52, 125]. High quantity of peptides (100µg) might also be a cause for the continuous expansion of SIINFEKL specific $CD8^+$ T-cells. Therefore any effects by T-helper peptides appeared to be masked by this vaccination strategy.

The original plan for these studies was to proceed to ten rounds of vaccination. However, following the eighth vaccination some of the mice were unwell and humanely killed. Therefore, the study was terminated at this point. Spleen, lymph nodes, liver, lungs and heart tissue were sent for pathological examinations to establish possible illness due to the vaccination regimen resulting to lymphocytes infiltrating organs. However, pathology results showed very little invasion of lymphocytes into these organs. Therefore, we were unable to conclude the primary cause of the illness suffered by the vaccinated mice.

To study the effects of T-helper peptides, an experiment which induces less CD8 T-cells responses was designed. The vaccine adjuvant consisted of Poly I:C only. Poly I:C is a weaker TLR agonist compared to CpG in mice because APC in mice express lower level of TLR-3 which requires stimulation by the TLR-4 agonist by LPS[126, 127]. The high numbers of vaccinations conducted also eliminates the need for an emulsion which provides the depository effect. Peptide quantity was also reduced from 100µg to 25µg to reduce the percentage of SIINFEKL specific $CD8^+$ T-cell generated. Resting mice for a period of approximately 50 days also allowed investigation of a recall response[52].

3.3 The use of a single TLR agonist to analyse the effect of T-helper peptides on the generation of SIINFEKL specific $CD8^+$ T-cells.

3.3.1 Vaccination design

The groups remained the same as shown in Figure 3.2 a. The vaccine components and the vaccination schedule are shown in Figure 3.7 a and b, respectively.

a)

Groups	Adjuvant	SIINFEKL	ISQ	TEWT
Unimmunized	-	-	-	-
G1	+	+	-	-
G2	+	+	+	-
G3	+	+	-	+
G4	+	+	+	+
G5 (Vaccine 1,3,5,..)	+	+	+	-
G5 (Vaccine 2,4,6,..)	+	+	-	+

b)

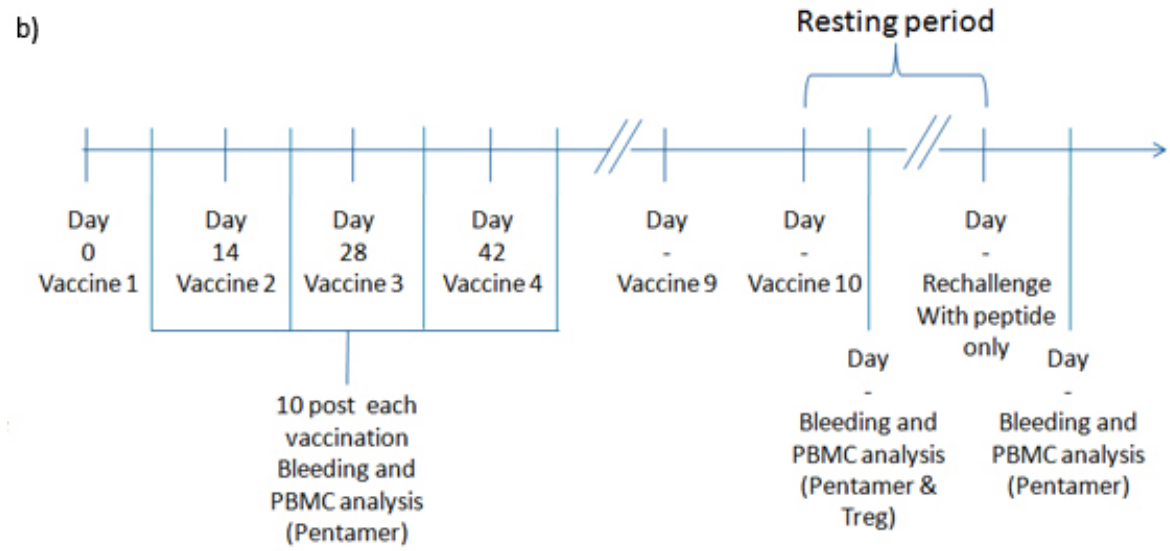


Figure 3.7 Vaccination schedule and analysis time points: a) Vaccination groups and components of vaccine. Adjuvant used in this design was Poly I:C (50µg/mouse) in PBS. Each peptide included in the vaccine is 25µg/mouse. b) Vaccination schedule and analysis time points. Intra dermal vaccination was conducted while collection of PBMC for analysis was conducted by a tail vein clipping. Analysis parameters were as described in section 2.4.2.

3.3.2 Peptide vaccination with a single TLR agonist produces low percentage of SIINFEKL specific $CD8^+$ T-cells

Vaccination with Poly I:C and use of a reduced quantity of peptides (25µg/mouse) produced lower percentages of SIINFEKL specific $CD8^+$ T-cells compared to the previous vaccination regimen. However, vaccination with SIINFEKL alone and vaccination with SIINFEKL together with alternating T-helper peptides produced lower percentages of SIINFEKL specific $CD8^+$ T-cells compared to vaccinations with the same one or two T-helper peptides. This was consistent following each of the 8 rounds of vaccination with SIINFEKL and SIINFEKL plus alternating T-helper peptides. Resting the mice for 50 days followed by a challenge with SIINFEKL peptides in PBS did not produce a significant increase in the percentage of SIINFEKL specific $CD8^+$ T-cells, therefore no evidence of a recall response, except in the SIINFEKL + ISQ group. Even then the data was not significantly different with a Student's t test analysis when compared to SIINFEKL plus TEWT and SIINFEKL plus ISQ and TEWT.

Groups vaccinated with one or two peptides produced higher percentages of SIINFEKL specific $CD8^+$ T-cells after the 8th round of vaccination. The three groups vaccinated with ISQ, TEWT and ISQ plus TEWT as T-helper peptides generated higher percentages of SIINFEKL specific $CD8^+$ T-cells from vaccine 4 onwards. (Figure 3.8)

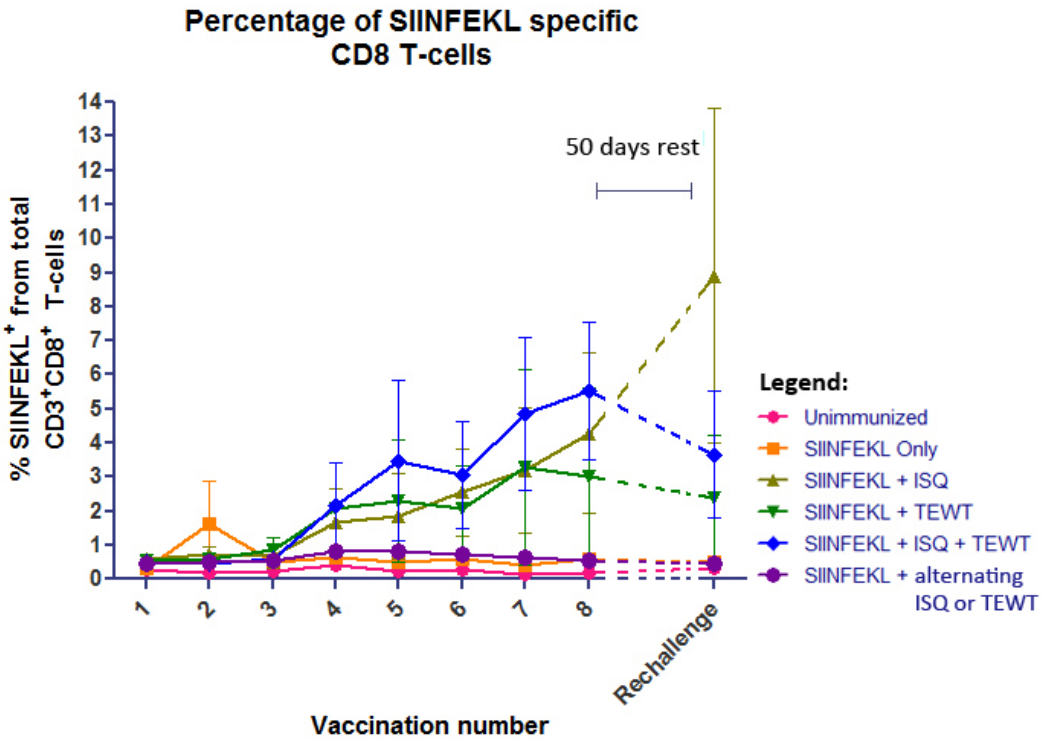


Figure 3.8 Percentage of antigen specific T-cells generated 10 days after each round of vaccination: Group vaccinated with SIINFEKL + alternating ISQ or TEWT T-helper peptide did not produce comparable percentage of SIINFEKL specific CD8+ T-cells when compared to groups vaccinated with SIINFEKL + ISQ, SIINFEKL + TEWT and SIINFEKL + ISQ + TEWT. A challenge with SIINFEKL plus PBS took place 50 days after resting the mice post the 8th vaccine. Mean ± SEM percentage. (n=5)

3.3.3 SIINFEKL specific CD8$^+$ T-cells have a predominant effector memory T-cells phenotype

Referring to Figure 3.8, levels of SIINFEKL specific CD8$^+$ T-cells in groups vaccinated with alternating T-helper peptide vaccination or SIINFEKL only vaccination were at background (similar to unvaccinated group). Therefore, the data for memory subsets of these groups was not meaningful. After rechallenge with SIINFEKL, vaccination with one or two T-helper peptides generated a predominant effector memory T-cell phenotype within the SIINFEKL specific CD8$^+$ T-cells and were not significantly different when compared between these groups (Figure 3.9 a). The percentage of central memory cells and naive cells detected were low and was

not significantly different between those groups (Figure 3.9 b and c) after the challenge with SIINFEKL.

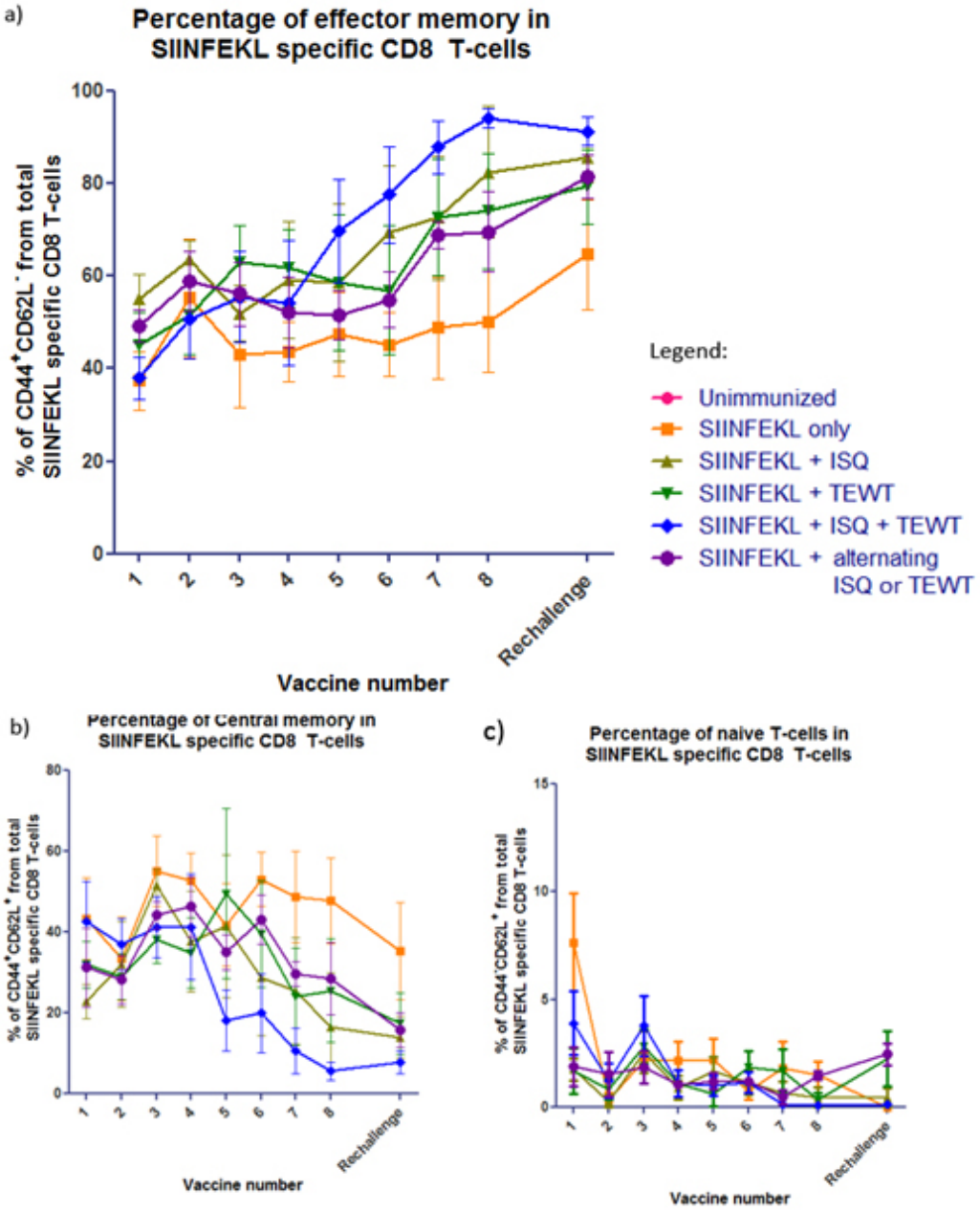


Figure 3.9 Memory subsets of SIINFEKL specific CD8$^+$ T-cells: a) Percentage of effector memory, b) central memory and c) naive memory cells in total SIINFEKL specific CD8$^+$ T-cells present 10 days after each round of vaccination and rechallenge. No significant differences were found when comparing the groups vaccinated with one or two T-helper peptides after the rechallenge. Mean ± SEM percentage is shown. (Student's t test analysis, n=5)

3.3.4 Vaccinations with a single TLR agonist and T-helper peptides do not expand Tregs

The stimulation of $CD4^+$ T-cells with a T-helper peptide might increase the percentage of Tregs and therefore the Treg population was analysed after eight rounds of vaccination. The percentage of Tregs from total $CD4^+$ T-cells did not show differences between the different vaccination groups (Figure 3.10). The mean percentage of Tregs in each group was between 2.5% to 4% of the total $CD4^+$ T-cells.

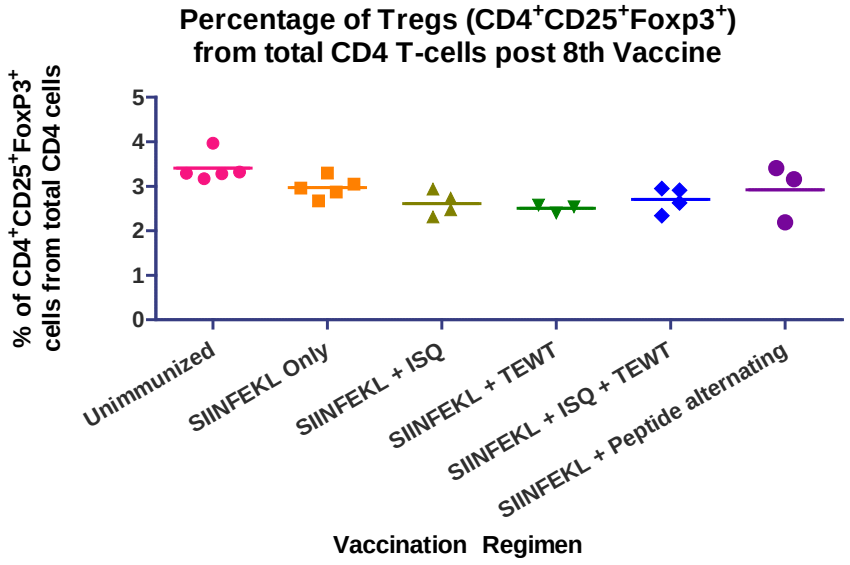


Figure 3.10 Percentages of Tregs from total $CD4^+$ T-cells after the 8th vaccination: No significant differences were seen using Student's t test analysis between the groups. Mean percentages are shown.

3.3.5. Summary

Vaccination with a single TLR agonist (Poly I:C - 50µg per mouse) and a lower level of peptide (25µg instead of 100µg) generated lower percentages of SIINFEKL specific $CD8^+$ T-cells compared to when two TLR agonist (Poly I:C 50µg and CpG 25µg per mouse) were used in combination. This lowered the level of responses and allowed the benefits of adding T-helper peptides to be seen. However, the use of alternating T-helper peptides, failed to generate significantly improved SIINFEKL specific $CD8^+$ T-cell responses. In fact the lowest levels of SIINFEKL specific $CD8^+$ T-cells were obtained when the helper peptides were completely excluded or when the two helper peptides (ISQ and TEWT) were used in cycles of alternating vaccination. In groups which produced SIINFEKL specific $CD8^+$ T-cells (with inclusion of one or two helper peptide), the predominant cells had an effector memory phenotype. In order to further investigate the effect of using alternating T-helper peptides, a priming step with OVA protein prior to the start of the vaccination regimen was introduced. In this approach, mice were inoculated with the OVA protein in complete Freund's adjuvant (CFA) 14 days prior to the vaccination with 50µg of poly I:C and 25µg of each of one or more peptides as described in the next section. (3.4). Priming with complete Freund's adjuvant (CFA) provides an initial exposure of OVA antigens to the mice and therefore potentially generates a more robust $CD8^+$ T-cell response[128].

3.4. Peptide vaccination following initial priming step with whole OVA protein.

3.4.1. Vaccination design

Mice were primed with an intraperitoneal (i.p.) administration of 400µg of OVA protein in CFA and then vaccinated according to the groups (n=5) shown in Figure 3.11 a. The adjuvant used in this study was similar to that described in section 3.3.1 where 50µg of Poly I:C and 25µg of each peptide were mixed in PBS and administered intradermally. The vaccination schedule and the analysis time points were as indicated (Figure 3.11 b). In this study, PBMCs were analysed for the presence of SIINFEKL specific $CD8^+$ T-cells 3 and 10 days after each round of vaccination, in order to determine whether day 10 analysis may miss responses that could be detectable on day 3.

a)

	Priming	Vaccine			
Groups	CFA + OVA	Adjuvant	SIINFEKL	ISQ	TEWT
G1	-	-	-	-	-
G2	+	+	-	-	-
G3	+	+	+	-	-
G4	+	+	+	+	-
G5	+	+	+	-	+
G6	+	+	+	+	+
G7 (Vaccine 1,3,5,..)	+	+	+	+	-
G7 (Vaccine 2,4,6,...)	+	+	+	-	+

b)

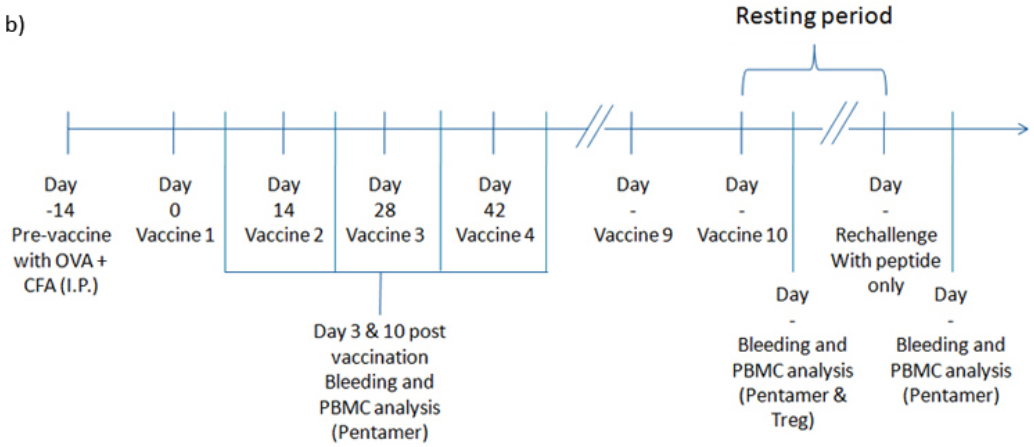


Figure 3.11 Vaccination design: a) Vaccination groups and vaccine components. Mice were primed intraperitoneally with 400µg of OVA protein in CFA. Adjuvant used in this design was Poly I:C (50µg/mouse) in PBS. Each peptide included in the vaccine was 25µg/mouse. b) Vaccination schedule and analysis time points. Intradermal vaccination was conducted while collection of PBMC for analysis was by a tail vein clipping. Analysis parameters were as described in section 2.4.2.

3.4.2 Analysis of PBMC 10 days after each round of vaccination shows a higher percentage of SIINFEKL specific $CD8^+$ T-cells percentage compared to 3 days after vaccination

PBMC were harvested and analysed at three days and ten days after each round of vaccination (Figure 3.12). An initial priming vaccination with whole OVA protein in CFA enhanced the generation of a subsequent response to SIINFEKL vaccination with alternating T-helper peptides. Mice in groups 1 to 6 (either without a helper peptide or with the same helper peptide/s without alternating between the helper peptides) showed hints of lower percentages of SIINFEKL specific $CD8^+$ T-cells than mice in group 7 where alternating T-helper peptides was used although it is not statistically significant. The responses over the first 5 doses of the vaccine were highly variable and the reasons for this were not clear. This study also showed a trend that PBMC analyzed 3 days after vaccination had lower percentage of SIINFEKL specific $CD8^+$ T-cells compared to samples analyzed 10 days after vaccination, although the data was not significantly different. In subsequent studies, responses to vaccination were examined only ten days after each round of vaccination.

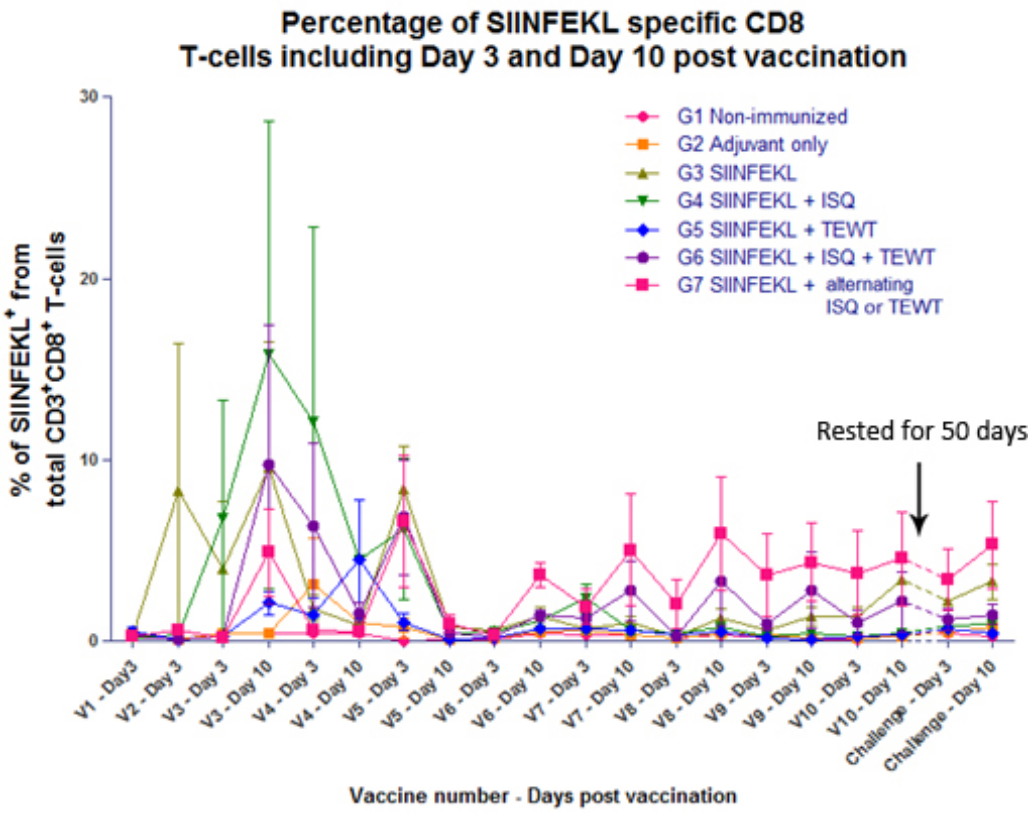


Figure 3.12 Percentage of SIINFEKL specific CD8$^+$ T-cells 3 and 10 days after each round of vaccination: Student's t test conducted comparing 3 and 10 days showed no statistically significant variation. Mean ± SEM percentage was shown. (n=5)

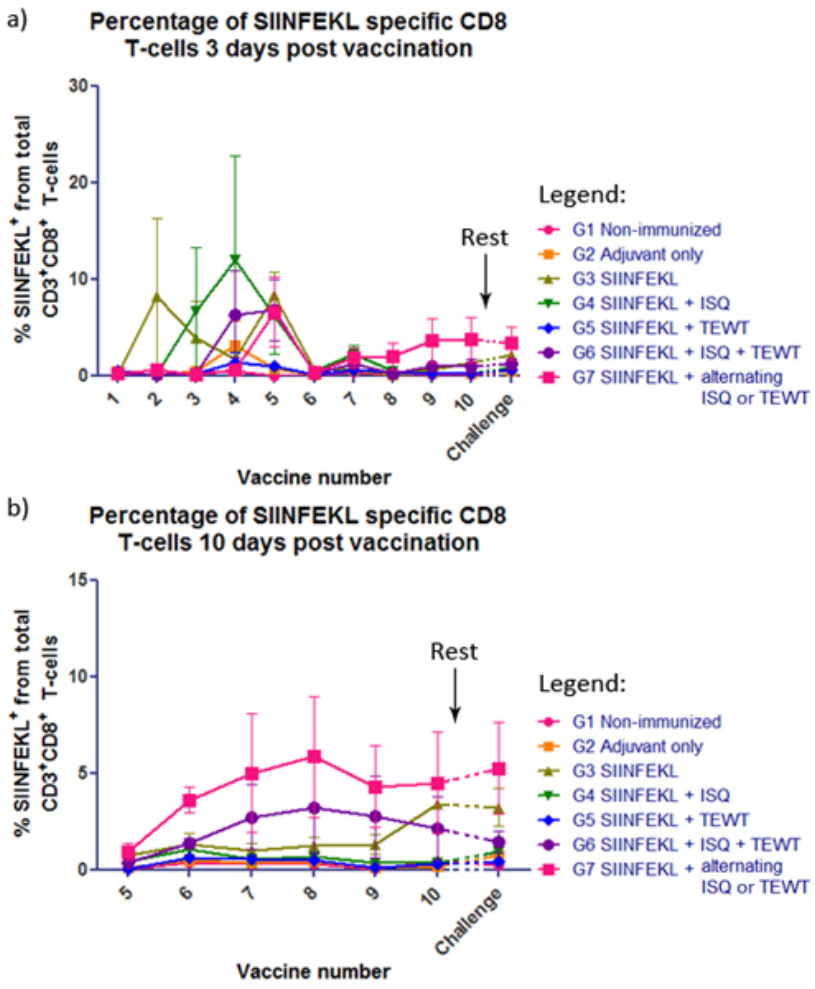


Figure 3.13 Alternative presentation of data: a) Percentage of SIINFEKL specific $CD8^+$ T-cells obtained 3 days after each vaccination. b) Percentage of SIINFEKL specific $CD8^+$ T-cells obtained 10 days after each vaccination. Means ± SEM (n=5)

An alternative presentation of the data presented in Figure 3.12 is shown in Figure 3.13 with data for analysis at 3 and 10 days after vaccination shown separately. All data points at 3 or 10 days after vaccination showed vaccine numbers 1 to 4 to have highly variable percentages of SIINFEKL specific $CD8^+$ T-cells. For the presentation of data 10 days after each vaccination (Figure 3.13 b), data for vaccine rounds 1 to 4 were omitted as the data were inconsistent and not significantly different. The highest percentage of SIINFEKL specific $CD8^+$ T-cells were detected in mice vaccinated with SIINFEKL in combination with alternating T-helper peptide (ISQ or TEWT) but the detected increase was not significantly different when compared to each of the other vaccinated groups.

3.4.3 Reduced percentage of Tregs in mice vaccinated with alternating T-helper peptides

To investigate if the basis for the increased percentage of SIINFEKL specific $CD8^+$ T-cells in mice vaccinated with the alternating T-helper peptides might be due to reduced suppression, the frequency of Treg was examined after the tenth round of vaccination (Figure 3.14). Cells with a Treg phenotype ($CD4^+CD25^+FoxP3^+$) comprised between 2 and 7.5%. The lowest mean percentage of Tregs was found in mice that were vaccinated with SIINFEKL in combination with the alternating T-helper peptides, suggestive that there may be reduced Tregs mediated suppression in this vaccination regimen. Vaccination with the adjuvant in the absence of peptides did not significantly alter the percentage of CD4 T-cells with a Treg phenotype. The reduced level of Tregs in mice vaccinated with the alternating T-helper peptides and SIINFEKL was statistically significant when compared with SIINFEKL alone ($p=0.015$) and SIINFEKL plus ISQ ($p=0.034$). The use of increased number and quantity of T-helper peptides was expected to increase the percentage of Tregs but in the SIINFEKL plus ISQ plus TEWT group, this increase was not observed. The basis for this observation is not clear and we are yet to be able to explain this data.

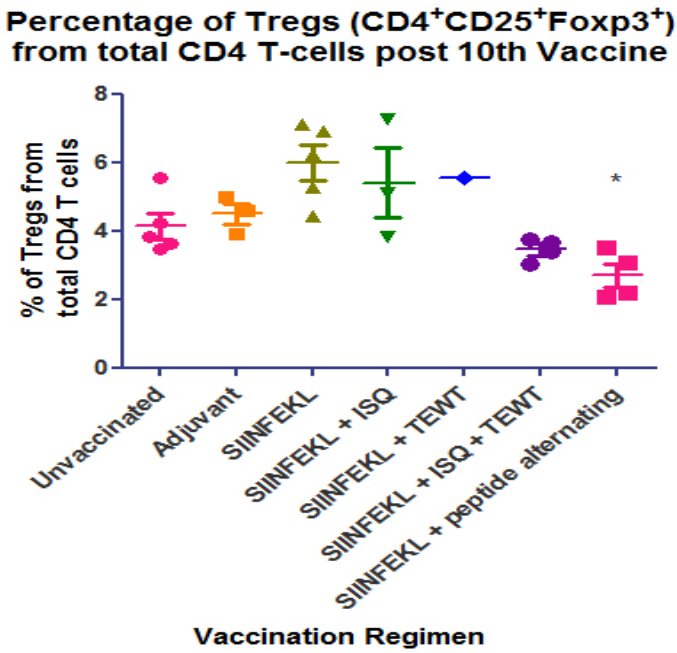


Figure 3.14 Percentage of Tregs in the total $CD4^+$ T-cells after 10 rounds of vaccination: Mean percentage shown. (n=1-5, *$p<0.05$ when compared to SIINFEKL or SIINFEKL plus ISQ, Student's t test analysis)

3.4.4 Summary

The inclusion of a priming step with CFA and the whole OVA protein in this experiment has enabled the enhancing effect of alternating T-helper peptides vaccination on generation of SIINFEKL specific $CD8^+$ T-cells to be revealed. Compared to previous experiments where only 0.4% SIINFEKL specific $CD8^+$ T-cells were generated with this peptide vaccination regimen (refer to Figure 3.8), the data now showed the generation of an average of 4.5% SIINFEKL specific $CD8^+$ T-cells. This higher level of response maybe due to the lower levels of Tregs in mice vaccinated with the alternating T-helper peptides and priming with OVA protein.

With the combination of adjuvant, peptide quantity and priming step for the vaccination established, further investigation was undertaken to study the underlying mechanism of improved CD8 T-cell response generated by vaccination with the alternating T-helper peptides.

3.5 Vaccination with the aid of alternating T-helper peptides generates enhanced SIINFEKL specific $CD8^+$ T-cell responses

3.5.1 Vaccination design: Two similar experiments were designed. In the first, mice were vaccinated every 7 days (weekly). In the second, the standard 14 days vaccination cycle was used (biweekly). The aim was to assess whether shortening the interval between vaccinations would enhance SIINFEKL specific $CD8^+$ T-cell responses. Analysis were conducted 10 days after the indicated cycles of vaccination (Post vaccination 2, 4, 6, 8 and 10). The vaccination protocol for both the weekly and bi-weekly cycles are shown in Figures 3.15 and 3.16, respectively.

a)

	Priming	Vaccine			
Groups	CFA + OVA	Adjuvant	SIINFEKL	ISQ	TEWT
G1	-	-	-	-	-
G2	+	+	-	-	-
G3	+	+	+	-	-
G4	+	+	+	+	-
G5	+	+	+	-	+
G6	+	+	+	+	+
G7 (Vaccine 1,3,5,..)	+	+	+	+	-
G7 (Vaccine 2,4,6,...)	+	+	+	-	+

b)

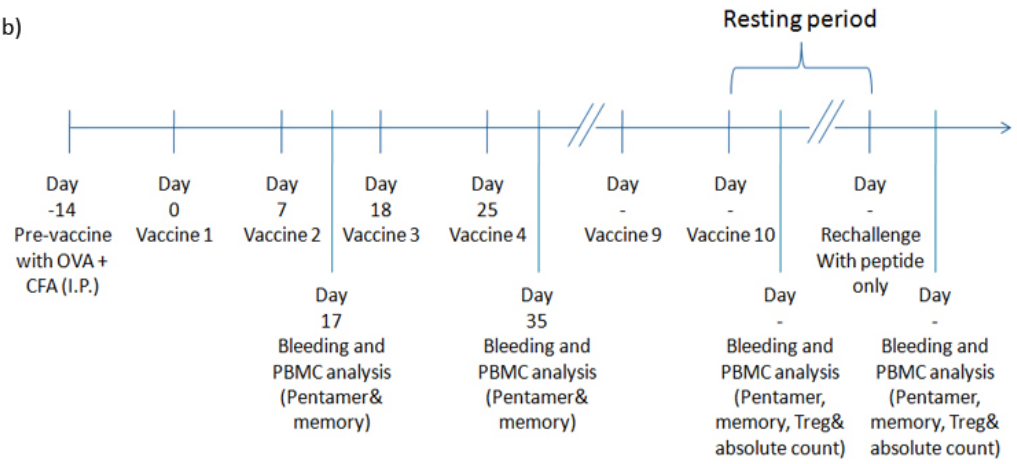


Figure 3.15 Weekly vaccination protocol: a) Vaccination groups and vaccine components. Mice were primed intraperitoneally with 400µg of OVA protein in CFA. Adjuvant used in this was Poly I:C (50µg/mouse) in PBS. Each peptide included in the vaccine was 25µg/mouse. b) Vaccination schedule and analysis time points. Intradermal vaccination was conducted while collection of PBMC for analysis was by tail vein clipping. Analysis parameters were as described in section 2.4.2.

a)

	Priming	Vaccine			
Groups	CFA + OVA	Adjuvant	SIINFEKL	ISQ	TEWT
G1	-	-	-	-	-
G2	+	+	-	-	-
G3	+	+	+	-	-
G4	+	+	+	+	-
G5	+	+	+	-	+
G6	+	+	+	+	+
G7 (Vaccine 1,3,5,..)	+	+	+	+	-
G7 (Vaccine 2,4,6,...)	+	+	+	-	+

b)

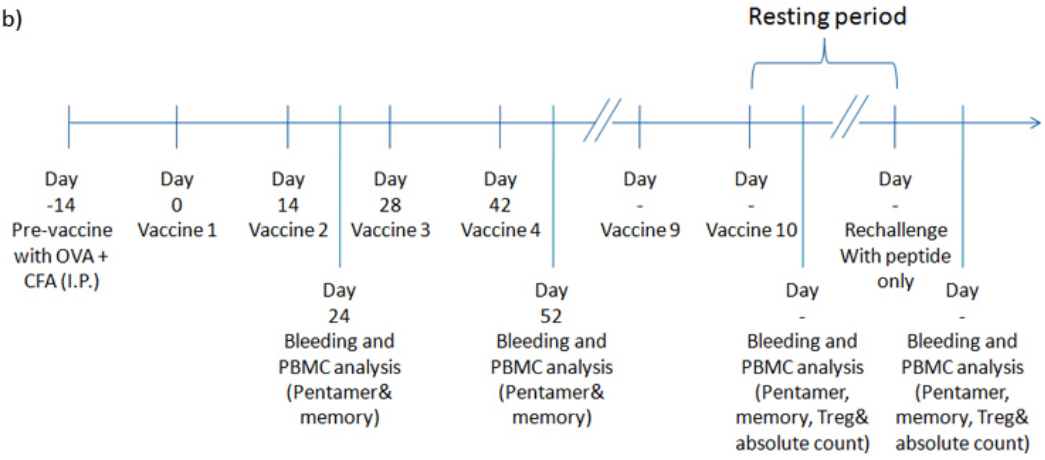


Figure 3.16 Biweekly vaccination protocol: a) Vaccination groups and vaccine components. Mice were primed intraperitoneally with 400µg of OVA protein in CFA. Adjuvant used in this was Poly I:C (50µg/mouse) in PBS. Each peptide included in the vaccine was 25µg/mouse. b) Vaccination schedule and analysis time points. Intradermal vaccination was conducted while collection of PBMC for analysis was by tail vein clipping. Analysis parameters were as described in section 2.4.2.

3.5.2 Vaccination on a weekly basis generates SIINFEKL specific $CD8^+$ T-cell but was not significantly enhanced by alternating the T-helper peptides

Vaccination 7 days apart resulted in generation of SIINFEKL specific $CD8^+$ T-cells for all vaccinated groups. However there was no evidence of significant enhancement by the presence of one or two helper peptides, or the use of the alternating helper peptides. A recall response was also observed in all the vaccinated groups (Figure 3.17 a). There was a detectable trend to improved SIINFEKL specific CD8+ T-cell cell numbers (Figure 3.17 b). However, there was no statistically significant differences amongst the different vaccination groups.

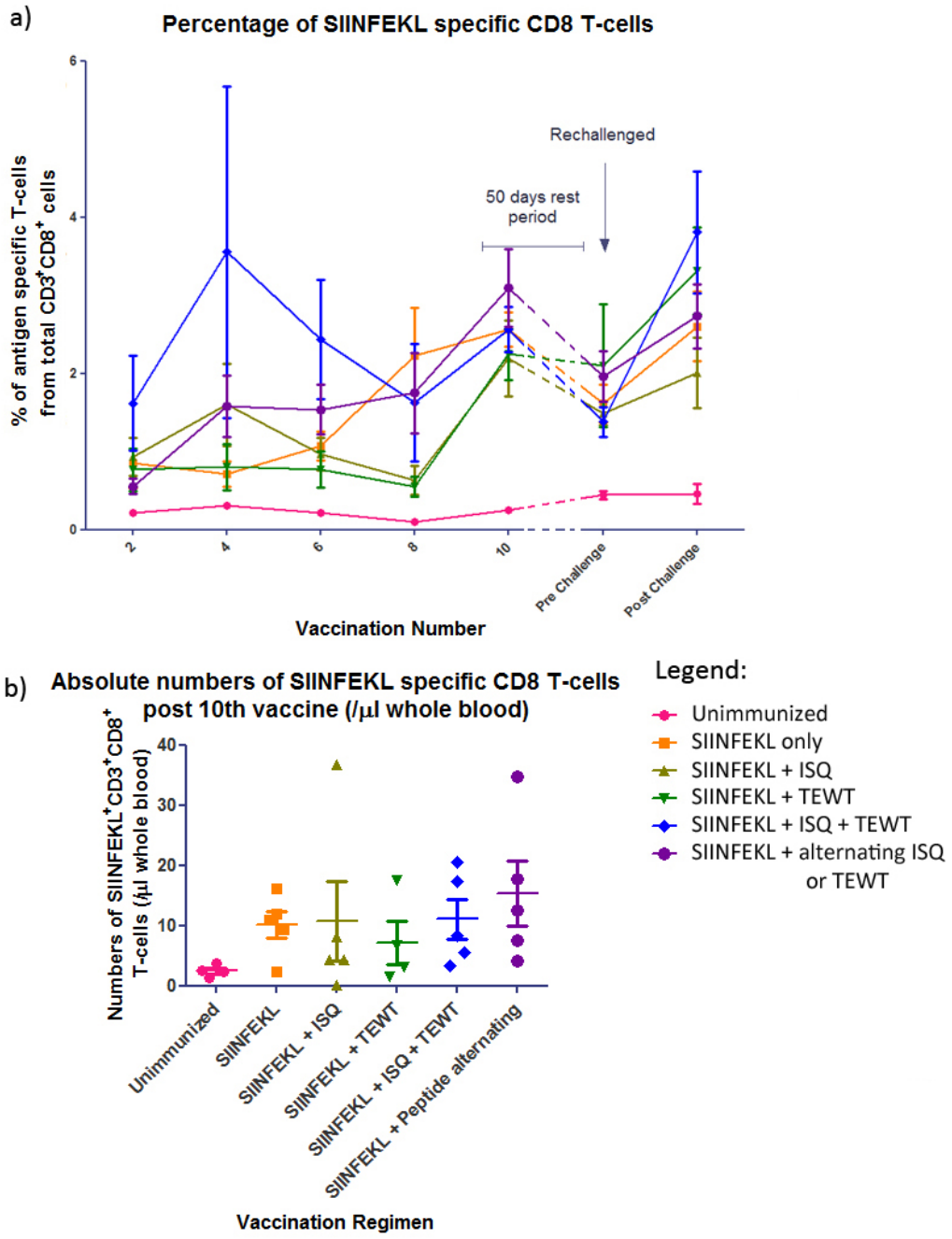


Figure 3.17 SIINFEKL specific CD8 T-cells in weekly vaccination: a) Percentage of SIINFEKL specific $CD8^+$ T-cells generated in the weekly vaccination regimen. After 10 rounds of vaccination, mice were left to rest for 50 days before a rechallenge with SIINFEKL peptide (100µg/mouse) in PBS. Mean ± SEM percentage shown. (n=5). b) Absolute numbers of SIINFEKL specific $CD8^+$ T-cells generated after 10 rounds of vaccination were obtained using counting beads (SIINFEKL specific $CD8^+$ T-cells/µl of blood). Mean percentage shown. (Student's t test was carried out between the groups but no significant differences were observed when comparing between the vaccinated groups.)

3.5.3 Vaccination generates long lasting central memory phenotype within the SIINFEKL specific CD8$^+$ T-cells

In section 3.5.2, a recall response was observed in Figure 3.17 a. To investigate the effect of the different vaccination regimens on the effector and central memory T-cells, the memory phenotype of the SIINFEKL specific CD8$^+$ T-cells were examined.

After resting the mice for 50 days, the SIINFEKL specific CD8$^+$ T-cells were found to be "quiescent" presenting with a central memory phenotype (designated as Pre challenge in Figure 3.18) with less effector memory phenotype compared to previous analysis time points. This phenomenon of increased central memory phenotype is desirable in vaccinations as they provide long term protection. Central memory T-cells generated, which have self renewing properties, remained within the SIINFEKL specific CD8$^+$ T-cells but effector memory T-cells were depleted as these cells lacked CD127 expression which is an IL-7 receptor crucial for self renewal. The change in the percentages of central and effector memory cells has been shown by other studies[57, 129] to be caused by the conversion of effector memory cells to central memory cells.

By challenging the mice with 100µg of SIINFEKL peptide in PBS (i.d.), a recall response was observed as an increase in the percentage of SIINFEKL specific CD8$^+$ T-cells (Figure 3.18 a). Reduced levels of central memory T-cells (Figure 3.18 b) and the increased levels of effector memory T-cells were observed within the SIINFEKL specific CD8$^+$ T-cells cells, providing the basis for the robust recall responses presented. This was likely to be driven by the conversion of central memory T-cells to effector memory T-cells. However, there were no significant differences when comparing the different regimens. Therefore the use of alternating T-helper peptides was not shown to be superior or inferior in generating effector or central memory T-cells in the weekly vaccination regimen.

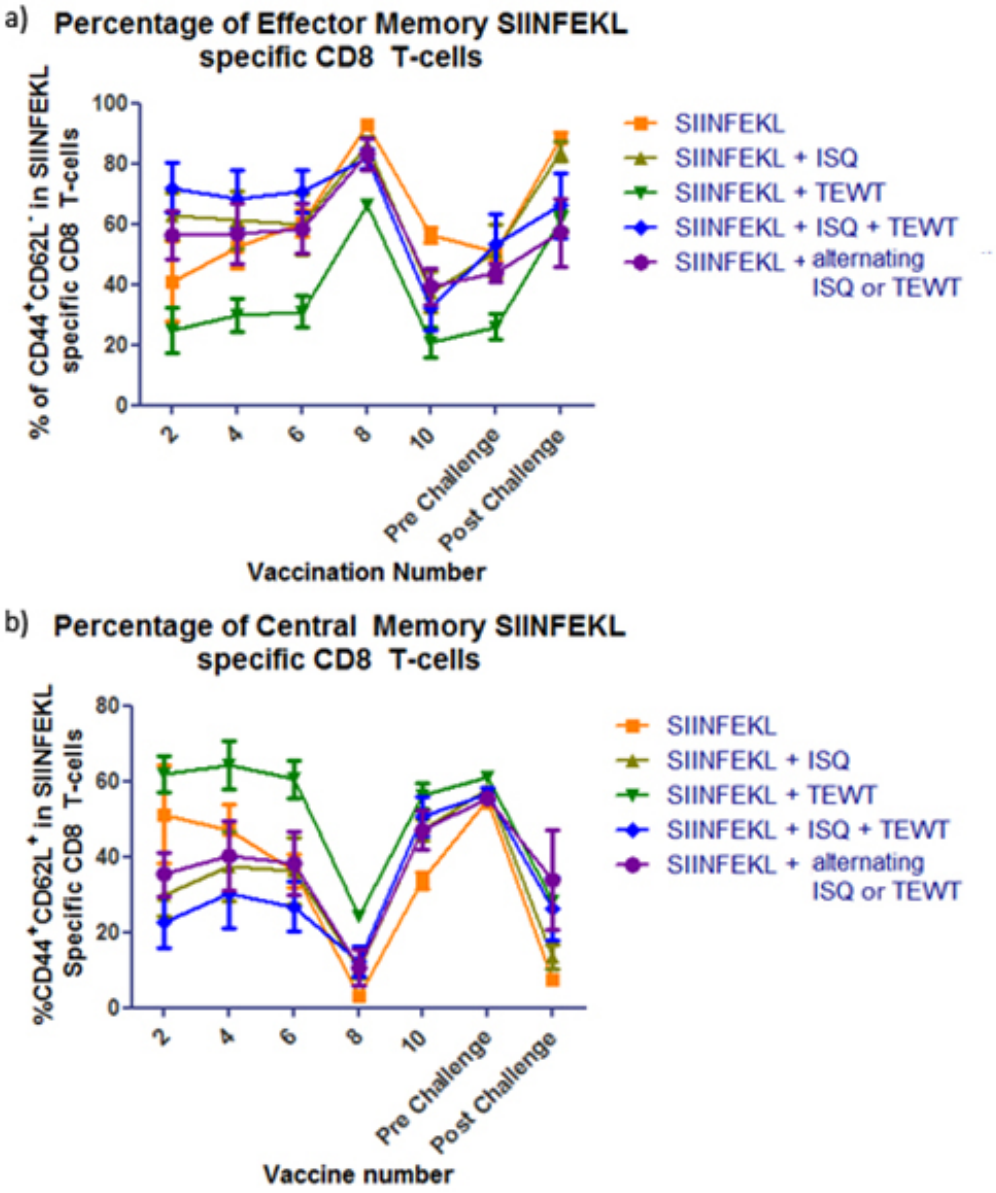


Figure 3.18 Subsets of memory T-cells: Percentage of a) effector memory ($CD44^+CD62L^-$) and b) central memory ($CD44^+CD62L^+$) in the SIINFEKL specific $CD8^+$ T-cells obtained 10 days after the indicated vaccine rounds for the weekly vaccination regimen. Mean ± SEM percentage is shown. (n=5)

3.5.4 Weekly vaccination with alternating T-helper peptides does not significantly affect the numbers or percentage of Tregs

No significant changes were seen in the percentage of Tregs generated in each of the various vaccination groups (Figure 3.19 a). Vaccination with alternating T-helper peptides did not change the percentage of Tregs. However, SIINFEKL plus ISQ, SIINFEKL plus TEWT, and SIINFEKL plus ISQ and TEWT resulted in significantly increased numbers of Tregs when compared to unvaccinated mice or mice vaccinated with SIINFEKL plus alternating T-helper peptides (Figure 3.19 b). In section 3.4.3 (Figure 3.14), SIINFEKL alone and SIINFEKL plus ISQ vaccination resulted in higher percentage of Tregs compared to vaccination with alternating T-helper peptides. Here the absolute cell numbers of Tregs showed a statistically significant increase in groups vaccinated with SIINFEKL plus ISQ (111 ± 8.7 Tregs/µl blood), SIINFEKL plus TEWT (107 ± 11 Tregs/µl blood) and SIINFEKL plus ISQ and TEWT (113 ± 6 Tregs/µl blood) when compared to unvaccinated mice (62 ± 11 Tregs/µl blood) as well as SIINFEKL plus alternating T-helper peptide vaccinated mice (72 ± 8 Tregs/µl blood).

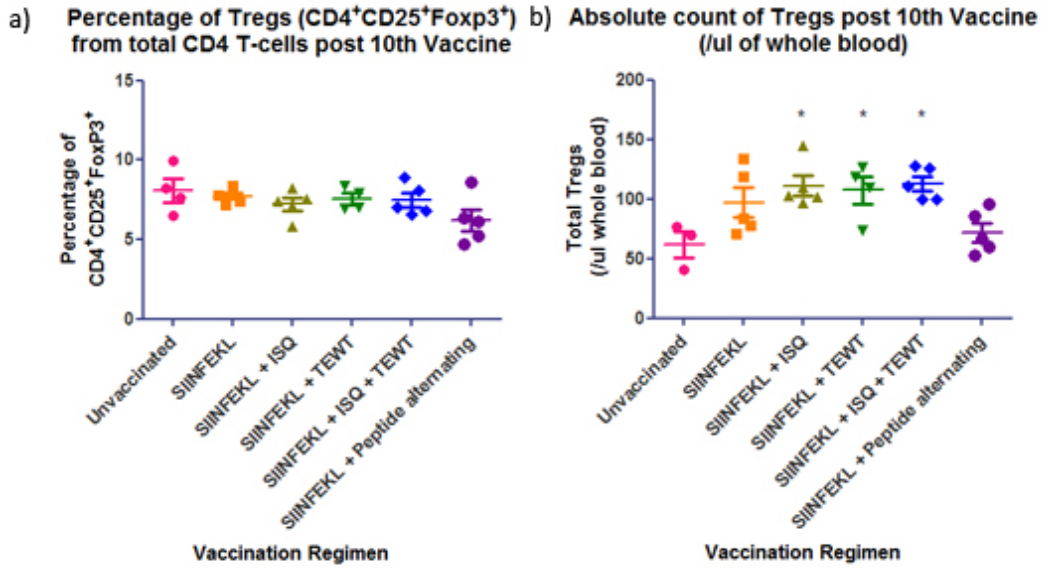


Figure 3.19 Tregs analysis on weekly vaccination: a) Percentage of Tregs in the total CD4 T-cells after the 10th vaccination round. b) Absolute cell count of Tregs post the tenth vaccine using counting beads. Data obtain 10 days post the tenth vaccination. Mean ± SEM percentage shown. (*p>0.05 compared to group which was unvaccinated or group vaccinated with SIINFEKL plus alternating T-helper peptide, Student's t test analysis)

3.5.5 Priming step and biweekly vaccination is required for generation of robust SIINFEKL specific $CD8^+$ T-cell in alternating T-helper peptide vaccination

Investigation into vaccination conducted every 14 days showed the improved generation of SIINFEKL specific $CD8^+$ T-cells with the alternating T-helper peptides vaccination as shown in Figure 3.19 a. After the 8th round of vaccination, the percentage of SIINFEKL specific $CD8^+$ T-cells generated were higher than the rest of the vaccinated groups at 11.6 ± 4.6% compared with the highest percentage of 3.6 ± 0.9% either with one or two helper peptides without alternating these peptides (Figure 3.20 a). The data obtained for the groups vaccinated with the alternating T-helper peptides was shown to be significantly higher than each of the unimmunized, SIINFEKL only and SIINFEKL plus TEWT vaccinated groups ($p<0.05$, Student's t test). After the tenth, vaccination the mean percentage of SIINFEKL specific $CD8^+$ T-cells was 29.9 ± 15.8% compared to maximum of 8.3 ± 2.7% in the other groups. The data was significant when compared to the unimmunized and SIINFEKL plus TEWT vaccination ($p<0.05$, Student's t test).

SIINFEKL specific $CD8^+$ T-cells number were determined after the tenth vaccination (Figure 3.20 b). Vaccination with alternating T-helper peptides generated the highest number of SIINFEKL specific $CD8^+$ T-cells compared to all the other groups at 477 cells/µl of blood. This data is statistically significant when compared to unimmunized (3 cells/µl of whole blood), SIINFEKL plus TEWT (29 cells/µl of whole blood) and SIINFEKL plus ISQ and TEWT (37 cells/µl of whole blood), ($p=0.05$. Comparison with other groups was not statistically significant. This data did not show normal distribution and thus Mann Whitney Test was employed)

After the tenth round of vaccination, the mice were rested for 50 days and a prechallenge analysis of the percentage of SIINFEKL specific $CD8^+$ T-cells was conducted. This showed a decrease in the percentages of SIINFEKL specific $CD8^+$ T-cells in all groups. The challenge with 100µg of SIINFEKL (in PBS) then showed a recall response where the percentage of SIINFEKL specific $CD8^+$ T-cells were seen to have increased in each of the vaccinated groups. Cell numbers were also quantified (Figure 3.20 d). Despite the higher absolute number of SIINFEKL specific $CD8^+$ T-cells in the group with alternating T-helper peptides after challenge

with SIINFEKL peptides (837 cells/µl of whole blood), the data was not statistically significant when compared to other vaccinated groups.

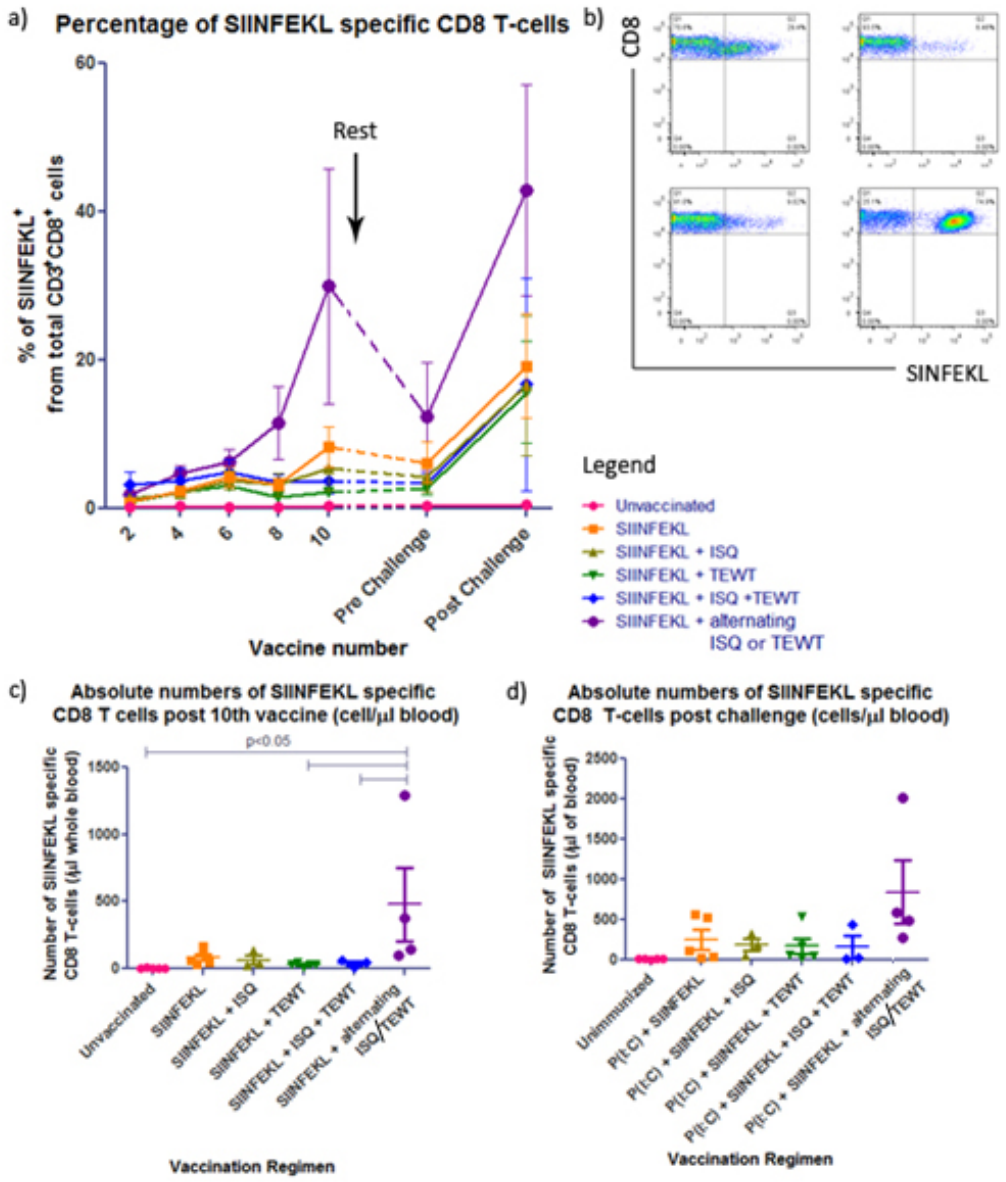


Figure 3.20 Percentage and absolute numbers of SIINFEKL specific CD8 T-cells: a) Percentage of SIINFEKL specific $CD8^+$ T-cells generated 10 days post vaccination number 2, 4, 6, 8 and 10; including the pre- and post-challenge percentages. The resting period is shown in the dotted lines for 50 days prior to a rechallenge. b) Representative plots of SIINFEKL specific CD8 T-cells gating in vaccinated mice. c) Absolute cell numbers of SIINFEKL specific $CD8^+$ T-cells post the tenth vaccination. Peptide alternating vaccination was only found to be significantly different when compared to SIINFEKL + TEWT and SIINFEKL + ISQ + TEWT. d) Absolute cell numbers of antigen specific T-cells generated after the rechallenge with SIINFEKL only. No significant differences were observed. (Student's t test employed in Figure 3.20a, Mann Whitney Test employed in Figure 3.20 b and c)

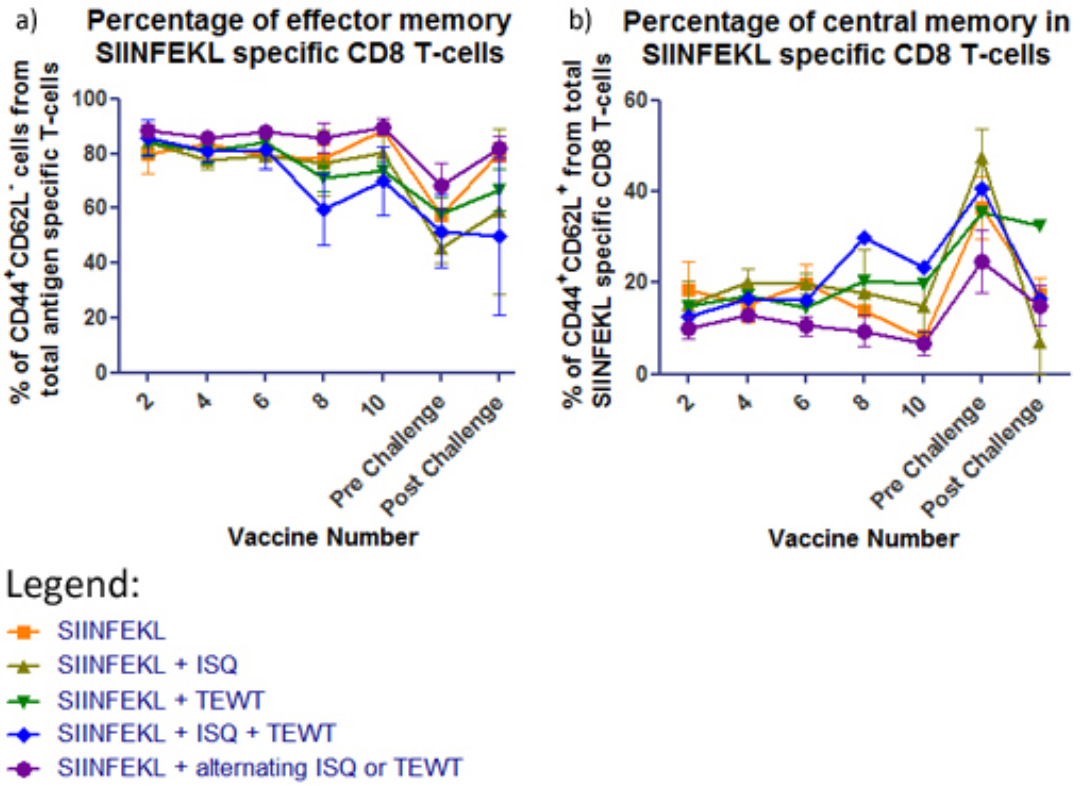


Figure 3.21 Memory subsets: Percentage of a) effector memory ($CD44^+CD62L^-$) and b) central memory ($CD44^+CD62L^+$) in the SIINFEKL specific $CD8^+$ T-cells obtained 10 days after the indicated vaccine rounds for the biweekly vaccination regimen. Mean ± SEM percentage is shown (n=5).

The percentages of central and effector memory T-cells in the biweekly vaccination were similar to the previous experiment in section 3.5.3 (Figure 3.17), where the dominant population was effector memory cells (Figure 3.21 a). During the rest period, there was loss of effector memory cells but increased percentage of the central memory population (Figure 3.21 b). The percentage of effector memory cells then increased again after the challenge with SIINFEKL as described previously in section 3.5.3.

The increase in the percentage and the absolute cell number of SIINFEKL specific $CD8^+$ T-cells in the group vaccinated with the alternating T-helper peptides showed the need for a priming step. Timing of the vaccination was also crucial as the weekly cycles of vaccination did not produce an improved response by the alternating helper peptides.

3.5.6 Vaccination with the alternating T-helper peptides does not result in expansion of Tregs

With weekly cycles of vaccination, the alternating T-helper peptides regimen did not result in an expanded Tregs population. Similar results were obtained with the biweekly cycles of vaccination. There was no significant differences in percentages and absolute cell numbers in the group vaccinated with alternating T-helper peptides compared to the unvaccinated group. This might be one of the main reasons for the increased numbers of SIINFEKL specific $CD8^+$ T-cells obtained by this vaccination regimen (Figure 3.22 a). Groups vaccinated with SIINFEKL and SIINFEKL plus ISQ showed significantly higher percentage of Tregs when compared to alternating T-helper peptide vaccination (Figure 3.22 a). This shows that in cases where T-helper peptide was administered repeatedly over a long period of time, there is bound to be upregulation of Tregs and alternating T-helper peptides restricts the expansion of Tregs.

A very important observation when comparing the numbers of Tregs generated in the weekly to the biweekly vaccination was the increased numbers of Tregs in the weekly vaccination (Comparing Figure 3.19 b to Figure 3.22 b). The unvaccinated mice showed about 50 Tregs/µl of blood for both sets of experiments showing that the data obtained in the two vaccinations are comparable. However, the weekly vaccination showed a significantly higher number of Tregs especially in the group vaccinated with SIINFEKL plus ISQ, SIINFEKL plus TEWT and SIINFEKL plus ISQ and TEWT compared to the biweekly vaccination. There was no significant differences when comparing the SIINFEKL and SIINFEKL plus alternating T-helper peptide vaccinations in the weekly and biweekly schedule. This shows that Treg expansion was seen after the repeated rounds of vaccination at short time intervals but this was avoided when using alternating T-helper peptides.

Analysis of antigen specific Tregs was also conducted in this batch of mice. After the *in vivo* CTL assay was conducted, splenocytes were harvested from the mice and made into a single cells suspension. 1×10^6 splenocytes from each mouse were labelled with 3µM of CFSE and subjected to T-helper peptides (25µg of ISQ and 25 µg of TEWT) stimulation for 4 days. The cells were CFSE-labelled in order to observe antigen specific Treg proliferation. Proliferation of cells was measured by

CFSE dilution and detection by FACS analysis. Four days after the *in vitro* stimulation, cells were stained for FoxP3 and evaluated for intensity of CFSE staining. The $FoxP3^{+}$ cells that proliferated represent the antigen specific Tregs (Figure 3.22 c). The data obtained showed a significant reduction of Tregs in groups vaccinated with SIINFEKL plus TEWT, SIINFEKL plus ISQ and TEWT and SIINFEKL plus alternating T-helper peptides compared to SIINFEKL vaccinated mice. However, results of Treg proliferation were puzzling because the unvaccinated and SIINFEKL vaccinated splenocytes showed significantly increased percentage of proliferated antigen specific Tregs when compared to groups which were vaccinated with 1 or 2 T-helper peptides where generation of induced-Tregs was anticipated. Based on Figure 3.22 d, a possible explanation might be that the number of cells recovered post the stimulation was insufficient in the groups vaccinated with T-helper peptides despite inclusion of a cell death exclusion dye and the selection of only singlet cells by FACS analysis.

This experiment should be repeated in future studies to investigate the generation of antigen specific Tregs in repeated vaccination with one or two T-helper peptides either in an alternating or non-alternating fashion

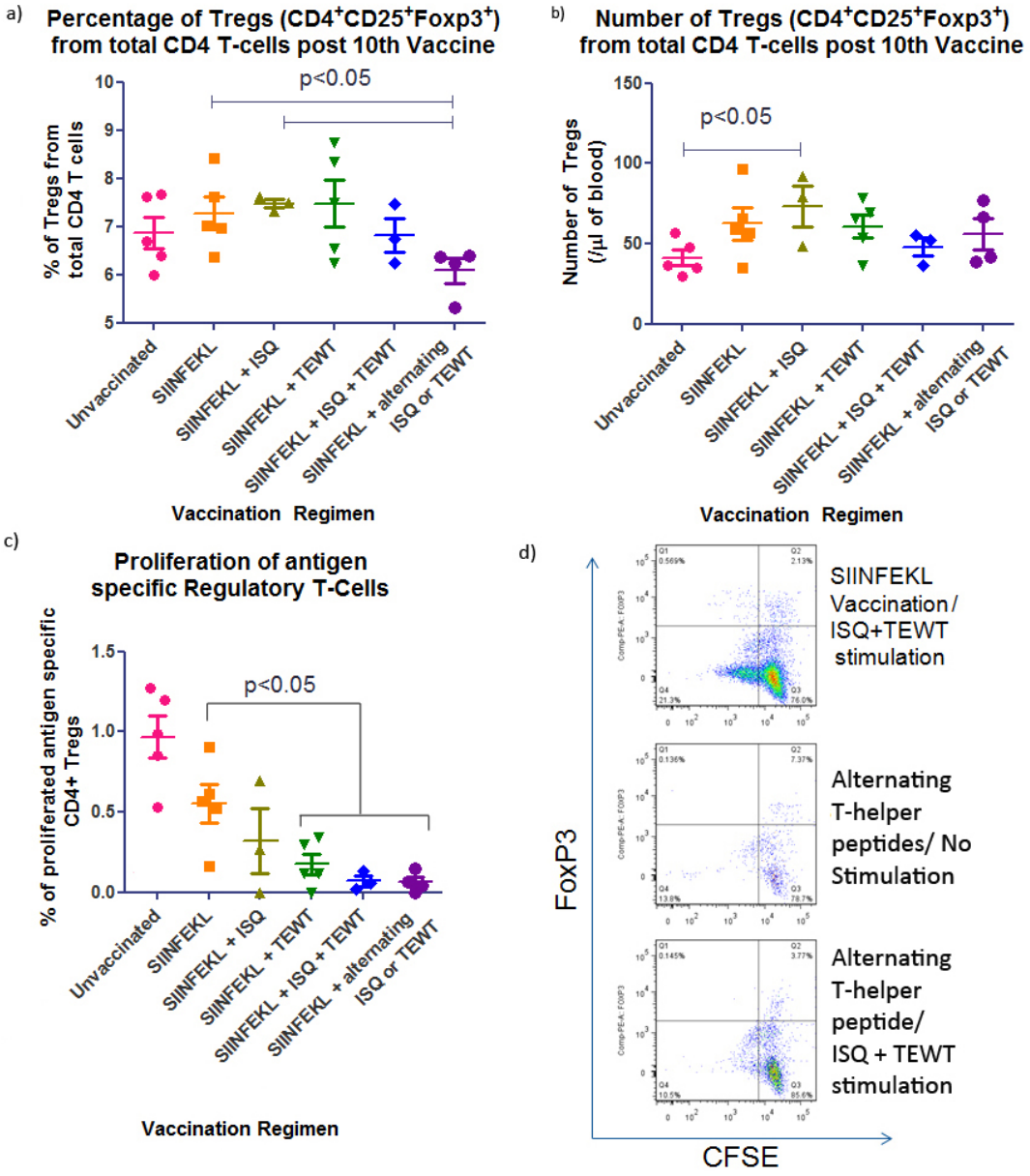


Figure 3.22 Tregs analysis: a) Percentage of Tregs from total $CD4^+$ T-cells quantified after 10 rounds of vaccination. b) Absolute numbers of Tregs quantified. c) Percentage of proliferated antigen specific Tregs ($CD4^+FoxP3^+$) after four days of *in vitro* stimulation with ISQ and TEWT. Mean ± SEM was shown. (Student's t test for analysis of statistical significance) d) Representative example of gating strategy of the proliferation of antigen specific Tregs.

3.5.7 Vaccination with alternating T-helper peptides generates SIINFEKL specific $CD8^+$ T-cells with improved in-vivo cytolytic activity

The SIINFEKL specific $CD8^+$ T-cells were found to be increased in the mice vaccinated with SIINFEKL plus alternating T-helper peptides. In order to investigate the functionality of the SIINFEKL specific $CD8^+$ T-cells, *in vivo* CTL killing of antigen positive target cells was examined. Target cells for the *in vivo* CTL assay were made using 10^7 cells/ml splenocytes which were peptide pulsed with 10µg/ml SIINFEKL. The target cells were then labelled with 0.3µM of DDAOSE (DDAOSE low). Non-target cells were not pulsed with peptide and labelled with 3µM of DDAOSE (DDAOSE high). The 1:1 mixture of antigen positive (DDAOSE Low): antigen negative cells (DDAOSE high) was then injected intravenously (100µl containing 1 X 10^8 cells) into the vaccinated mice. The following day splenocytes were harvested and analysed by flow cytometry. Figure 3.23 a, shows representative histogram of the recovered antigen positive and antigen negative cells in each group of mice.

The *in vivo* CTL killing assay showed a significant increase in target cell lysis only in the mice vaccinated with the alternating T-helper peptides compared to the unvaccinated mice. There was clear evidence of *in vivo* antigen specific lysis within the other groups, but the increased cytolytic activity was not significant because of the low numbers of mice and the variation in the magnitude of responses in individual mice. Similarly the higher level of *in vivo* CTL activity in mice vaccinated with the alternating helper peptides was not statistically significant when compared to other vaccination groups (Figure 3.23 b).

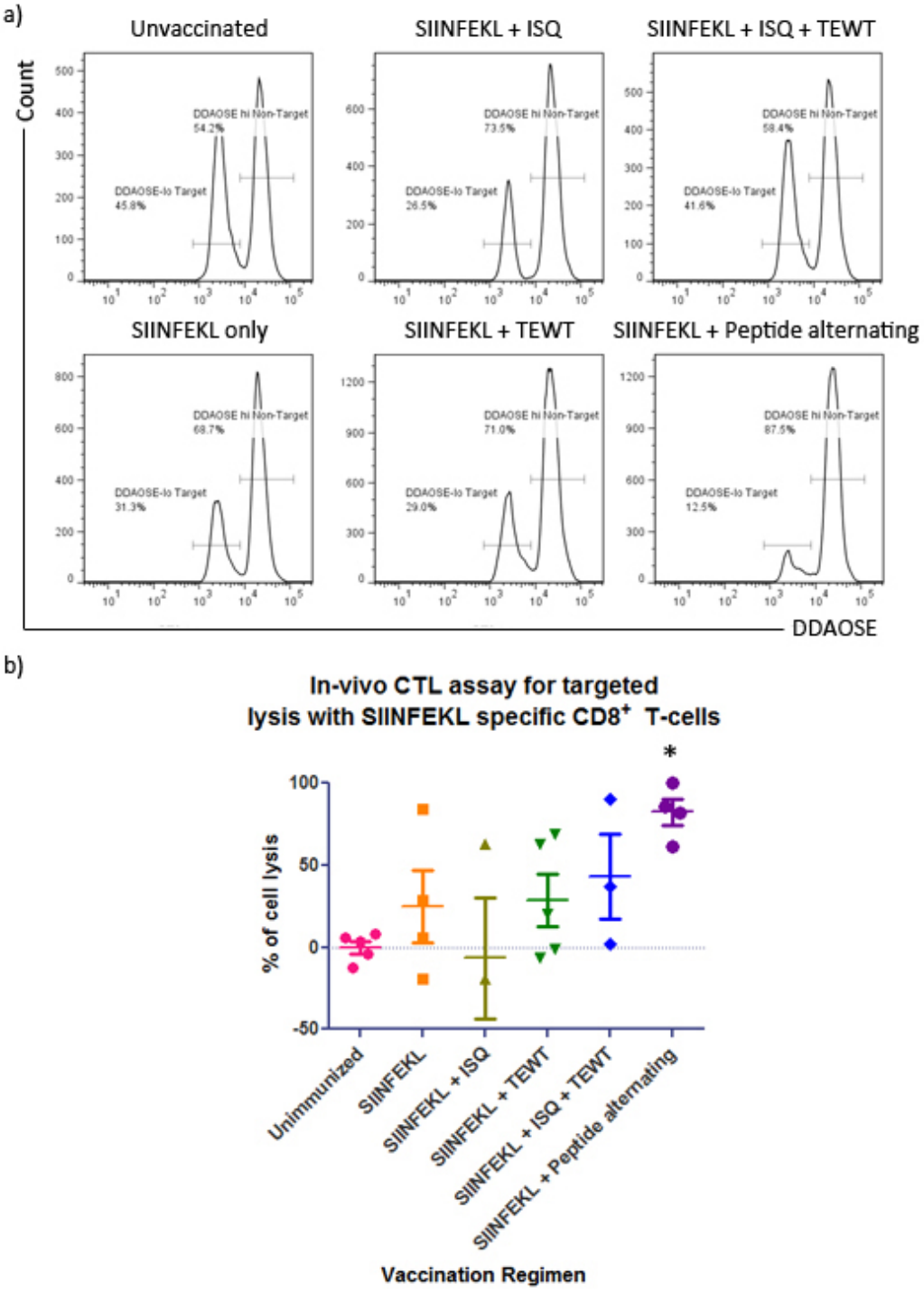


Figure 3.23 In-vivo CTL assay: a) Representative histogram showing the levels of target and non-target cells for each group in the *in vivo* CTL killing assay of DDAOSE labelled splenocytes. DDAOSE low cells are target cells whereas DDAOSE high cells are non-target cells. b) Percentage of target cell lysis generated in each group post flow cytometry analysis. The percentage of cell lysis was calculated based on the calculation in section 2.3.1. Mean ± SEM percentage shown. ($p>0.05$, Student's t test analysis was conducted, n = 3-5)

Percentage of IFN-γ producing $CD8^+$ T-cells were examined with an intracellular cytokine analysis assay. Splenocytes obtained from the mice were stimulated with SIINFEKL peptide, WT-1 peptide or subjected to PMA/Ionomycin stimulation (Refer to section 2.4.4 for intracellular cytokine analysis). Figure 3.22 a, shows representative plots of IFN-γ producing $CD8^+$ T-cells post stimulation. All vaccinated groups were found to have IFN-γ producing $CD8^+$ T-cells after 5 hours of *in vitro* stimulation with SIINFEKL peptide (25 µg/ml final concentration) (Figure 3.22 b).

Vaccination with alternating T-helper peptides produced significantly higher levels of $CD8^+IFN\text{-}\gamma^+$ cells when compared to splenocytes stimulated with an irrelevant peptide (WT-1). There were however no significant differences in the percentage of $CD8^+IFN\text{-}\gamma^+$ responses to SIINFEKL peptide between the vaccinated groups. Furthermore, no significant differences were detected in the amount of IFN-γ produced (as measured by geometric MFI) by the different groups (Figure 3.22 c)

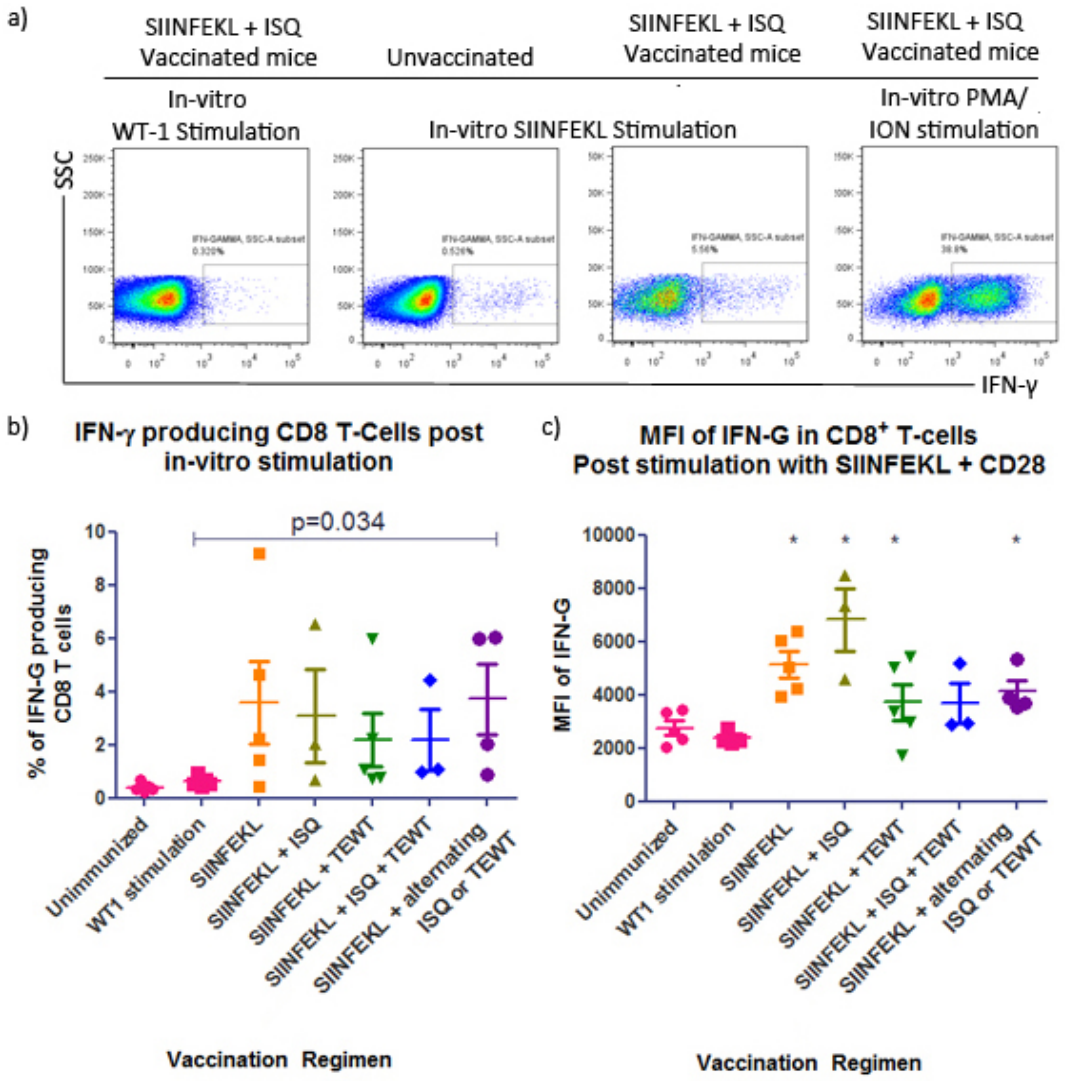


Figure 3.24 IFN-γ analysis: a) Representative plot for gating of IFN-γ^+ CD8 T-cells. Splenocytes from SIINFEKL plus ISQ vaccinated mice were stimulated with an irrelevant peptide (here WT-1 was used) to provide the negative gating for IFN-γ producing cells. Splenocytes from unvaccinated mice were stimulated with SIINFEKL peptide to provide a negative gating as well. In order to obtain a positive gating for IFN-γ, splenocytes from SIINFEKL plus ISQ vaccinated mice were stimulated with PMA/Ionomycin. b) Percentage and b) Geometric MFI of IFN-γ producing CD8 T-cells after a 5 hours *in vitro* stimulation and *in vitro* cytokine staining (post challenge with SIINFEKL)

3.6. Discussion

Peptide vaccination is a cheap and simple method of augmenting cellular immunity. Improved antigen specific T-cell responses can be obtained if the right vaccination regimen is implemented. The experiments reported in this chapter addressed a number of specific questions:

a) Optimum vaccination regimen and adjuvants required to generate enhanced antigen specific CD8 T-cell responses through alternating T-helper peptide vaccinations.

Selection of an adjuvant is important as not all antigens used are strongly immunogenic[28]. Combinations of vaccine components such as different TLR agonist which activate the NF-κB[130] by different signalling cascades is important for production of inflammatory cytokines that support the proliferation, differentiation, and survival of lymphocytes[131]. The CASAC[52] vaccine adjuvant was modified to include a single TLR agonist (Poly I:C) in order to be able to address the requirement for helper functions. In addition, peptide quantities in the vaccine were reduced to a level where the benefits of T-helper peptide addition could be evaluated. Repeated vaccination with single or two T-helper peptides generated high percentage of SIINFEKL specific $CD8^+$ T-cells as determined by pentamer staining. However the group vaccinated with alternating T-helper peptides failed to produce any responses. The introduction of a priming step which involved the use of CFA, an adjuvant derived from heat inactivated mycobacterium in an paraffin oil and surfactant mixture[132], enhanced the percentage and number of SIINFEKL specific $CD8^+$ T-cells. The priming step was found to be essential for improved CD8 T-cell responses to vaccination with alternating T-helper peptides[128]. The frequency of vaccination was also established to be best when conducted every 14 days compared to every 7 days.

b) Differences in the magnitude of Treg expansion caused by the two vaccination protocols.

In all the different vaccination protocols, continuous stimulation of a single $CD4^+$ T-cell epitope with a T-helper peptide lead to the generation of Tregs. This was most clearly evident when the vaccination was conducted every 7 days (Figure 3.19). By alternating T-helper peptide in the vaccination, there was no significant increase in either the percentages or numbers of Tregs, even after 10 rounds of vaccination. Banerjee *et al.* (2006)[133] have reported that vaccination with cytokine matured dendritic cells expanded FoxP3 Tregs in melanoma patients[133]. The importance of generating antigen specific CD8 T-cell responses while avoiding expansion of Tregs was also stressed by Zou *et al.*, (2006)[134]

c) Functional analysis of antigen specific $CD8^+$ T-cell responses generated by the two vaccination protocols.

All vaccinations regimens showed increases in the percentage of IFN-γ producing CD8 T-cells following *in vitro* peptide stimulation with SIINFEKL. Comparison between regimens did not show any significant differences, possibly due to the variation in the magnitude of responses. The *in vivo* CTL killing assay however, did show that the use of alternating T-helper peptide induced significantly better killing of target cells compared to the other vaccination regimens

d) Analysis of the type of memory CD8 T-cells generated by the two vaccination protocols.

All vaccination regimens stimulated SIINFEKL specific $CD8^+$ T-cells, predominantly comprising of the effector memory phenotype ($CD44^+CD62L^-$) when analysed 10 days after vaccination. After 50 days of rest, SIINFEKL specific CD8+ T-cells were predominantly of the central memory phenotype ($CD44^+CD62L^+$), which is indicative of conversion from effector to central memory cells. Central memory provides better long-term protective immunity and this was shown to be the case when the recall response was examined after 50 days of rest and rechallenged

with SIINFEKL peptide in the absence of adjuvant. This pattern of conversion from effector memory T-cells to central memory T-cells and recalled again from central memory T-cells to effector memory T-cells has been reported by Wherry *et al.*[57] and Seder *et al.*[129].

3.7 Conclusion and future studies

The data obtained from the experiments described in this chapter suggest that vaccination with alternating T-helper peptides generates enhanced cytotoxic antigen specific CD8 T-cells with reduced expansion of Tregs. However, more studies are required to further establish the benefits of the alternating T-helper peptides in vaccinations. Investigation into the generation of antigen specific $CD4^+$ T helper cells as well as characterization of their function could provide a more detailed understanding. Different models of diseases should also be selected to study the effects of alternating T-helper peptide vaccination to break tolerance, especially when vaccinating against self antigens that are over expressed in cancer. For instance, WT-1 in leukaemia would be an excellent choice as it has been shown to be upregulated in leukemic cells and already used extensively in vaccination against cancer associated antigens[135].

Further analysis of the phenotype of the T-cells generated will also be crucial to determine the nature of the immunological responses generated. Markers such as PD-1, KLRG-1 and LAG-3 can be used to determine whether alternating T-helper peptide vaccination could reduce the risk of vaccination induced exhaustion of effector CD8 T-cells[109].

The ultimate test of a vaccination regimen will always be its protective or therapeutic efficacy. By pitting the vaccine against a well established tumour or a chronic infectious disease, it will be possible to assess whether vaccination with alternating T-helper peptides vaccination has the ability to enhance the immune mediated rejection of a chronic infectious disease or cancer.

CHAPTER 4

IRX-2 as a potential immunomodulatory agent to enhance the efficiency of vaccinating against a target antigen

4.1 Introduction and Study objectives

4.1.1 Introduction: IRX-2 is a combination of biologic agents containing physiological quantities of IL-1β, IL-2, IL-6, IL-8, GM-CSF, interferon-gamma (IFN-γ) and tumornecrosis factor-alpha (TNF-α) generated by phytohemagglutinin (PHA) activation of human PBMC under GMP conditions by IRX Therapeutics[96]. It has immunomodulatory properties that restore immune responsiveness, induces tumour infiltration in head and neck squamous cell carcinoma, increasing patient's survival outcome at 5 years[97]. IRX-2 was also found to have adjuvant properties when administered to mice as 4 or 9 daily subcutaneous injections of 100µl with an immuno-dominant peptide derived from human prostate-specific membrane antigen (designated as NFT) which is found to be antigenic in Balb/c mice. Increased T cell responses were observed when IRX-2 was used in combination with incomplete Freund's adjuvant, NFT peptide conjugated with KLH or with irradiated cells expressing NFT[98]. These observations prompted the question of whether IRX-2 could enhance the efficacy of generating of cyototoxic T-cells by CASAC vaccination[52].

We hypothesized that by including IRX-2 into CASAC vaccination, we would be able to generate more potent CD8 T-cell responses than is possible with the use of CASAC or IRX-2 individually.

4.1.2 Study Objectives

a) Investigation of different combinations of TLR agonists for assessment of synergy with IRX-2

b) Investigation of the effects of IRX-2 on the memory and regulatory populations of T-cells

c) Investigation of the potency of antigen specific CD8 T-cell responses generated by the combined adjuvant vaccination.

4.2. Optimizing the use of IRX-2 with CASAC vaccination.

4.2.1 Vaccination design

As shown in Chapter 3 and by Wells *et al.* (2008), different combination of vaccine components affect the level of antigen specific T-cells as well as their functional competence (e.g. cytotoxic activity). Thus comparison of the two best combinations of CASAC with IRX-2 was conducted as shown in G3 and G5 (Table 4.1 a). G3 includes the use of MPL (a TLR-4 agonist) while G5 includes Poly I:C (a TLR-3 agonist). Despite stimulating different NF-κB activation pathways, Poly I:C and MPL induced signalling are independent of the MyD88 pathway which is stimulated by CpG in order to enhance vaccination efficacy[52, 125, 130]. ISQ peptide, an MHC class II presented T-helper peptide was used to provide help as part of the CASAC vaccination.

a)

GROUP	Vaccine components
G1 Emulsion without peptide	Emulsion
G2 CASAC (MPL)	Emulsion + SIINFEKL + ISQ + CpG + MPL + IFN-γ
G3 CASAC (MPL) + IRX-2	Emulsion + SIINFEKL + ISQ + CpG + MPL + IFN-γ + IRX-2
G4 Emulsion + IRX-2	Emulsion + SIINFEKL + ISQ + MPL + IRX-2
G5 CASAC (Poly I:C) + IRX-2	Emulsion + SIINFEKL + ISQ + CpG + Poly I:C + IFN-γ + IRX-2

b)

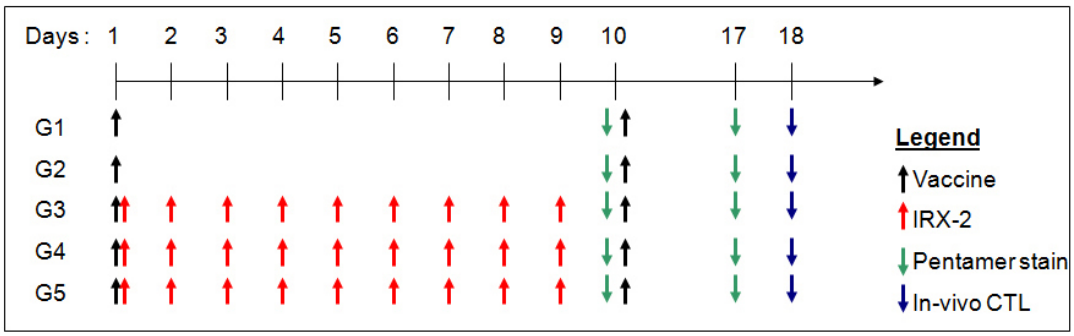


Table 4.1 Vaccination design: a) Outline description of vaccination groups using CASAC and IRX-2. b) Outline of vaccination schedule. Refer to section 2.2.3.4 and 2.2.3.5 for the concentration of CASAC and IRX-2 used.

IRX-2 has been recommended by Naylor *et al.* and IRX Therapeutics to be administered in 9 consecutive injections of 100µl/mouse (equivalent to 0.6ng of IL-2) at or near the site of vaccination[98]. The use of IRX-2 is as indicated in Table 4.1. Two rounds of vaccination with CASAC ~~was~~were conducted according to the schedule suggested by Wells *et al.* (2008)[52]. The percentages of SIINFEKL specific $CD8^+$ T-cells were quantified on days 10 and 17 after the first vaccination. An *in vivo* CTL assay was conducted on day 18 to investigate the cytolytic activity generated by the different vaccination regimens.

4.2.2 IRX-2 does not significantly enhance the generation of SIINFEKL specific $CD8^+$ T-cells responses in combination with CASAC

In this study, two versions of CASAC adjuvant were employed. Group 2 and 3 utilised the MPL version while MPL were substituted with Poly I:C in Group 5. An adjuvant comprising emulsion and just one TLR agonist (MPL) was also tested (Group 4). Results presented in Figure 4.1 a show that all vaccination groups with CASAC generated SIINFEKL specific $CD8^+$ T-cells at levels significantly above background (Group 1 - emulsion only) 17 days after the first round of vaccination. Some enhancement was seen in the percentage of SIINFEKL specific $CD8^+$ T-cells present at day 17 in mice vaccinated with CASAC + IRX-2 compared to CASAC alone. However, differences were not statistically significant. Group 3 and Group 5 generated 11.2 ± 3.8% and 8.3 ± 1.8% SIINFEKL specific $CD8^+$ T-cells respectively compared to 4.7 ± 1.5% in Group 2 ($p=0.15$) (Figure 4.1 b). A single TLR agonist (MPL) + IRX-2 vaccination (Group 4) did not stimulate SIINFEKL specific $CD8^+$ T-cells above the level in Group 1 -emulsion only control group.

Although the data was not significantly different, IRX-2 did appear to be providing some help towards generation of increased levels of SIINFEKL specific $CD8^+$ T-cells. The data also shows that in the presence of IRX-2 either in the Poly I:C or MPL version of CASAC, the generation of SIINFEKL specific $CD8^+$ T-cells were not impaired.

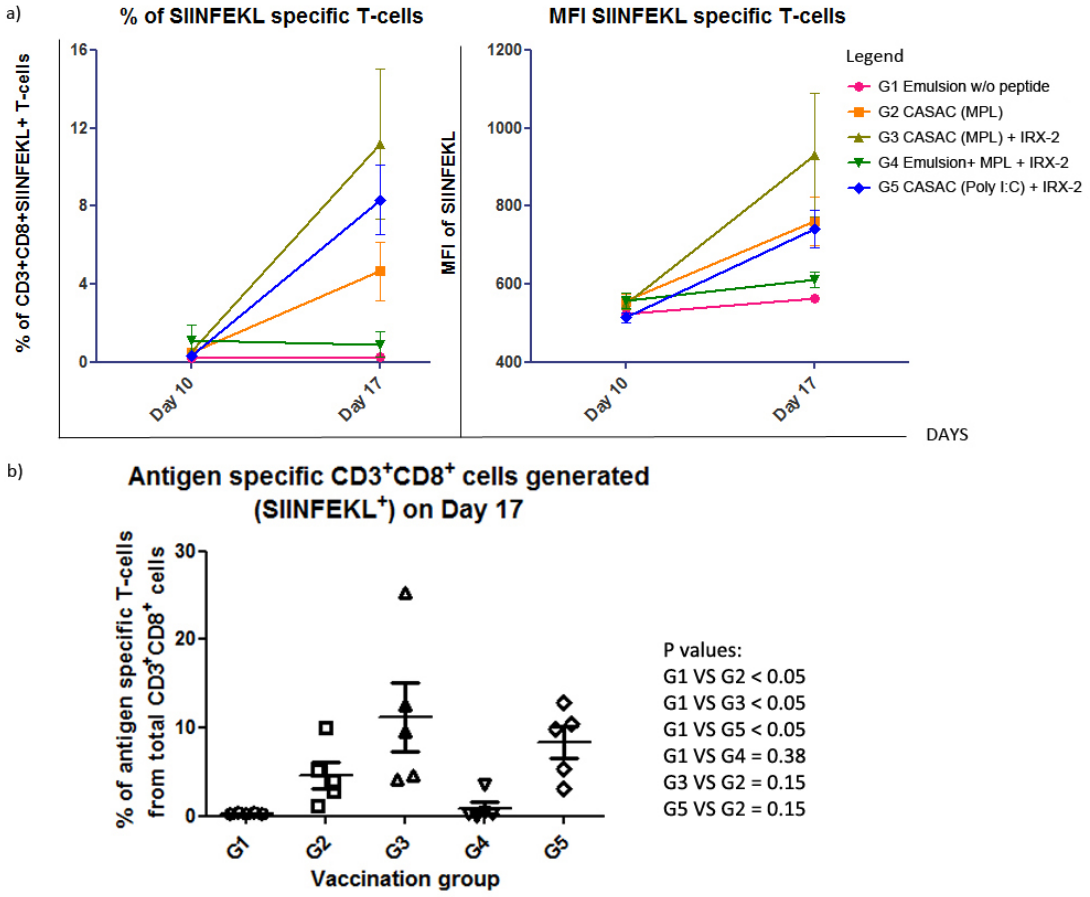


Figure 4.1 Analysis of SIINFEKL specific CD8 T-cells in CASAC plus IRX-2 vaccinations: Vaccine components were as describe in Table 4.1. Concentration of vaccine components were as described in section 2.2.3.4 and 2.2.3.5. a) The changes in percentage of SIINFEKL specific $CD8^+$ T-cells and geometric MFI generated on day 10 and day 17 after the first vaccination. b) Comparison of percentages of SIINFEKL specific $CD8^+$ T-cells in each groups on day 17 based on SIINFEKL pentamer staining (P values were calculated by Student's t test analysis)

4.2.3 CASAC vaccination, either in the presence or absence of IRX-2, generates maximal target cell killing

To determine if there was any improvement or impairment to the functionality of the SIINFEKL specific $CD8^+$ T-cells generated by vaccination in the combined presence of IRX-2 with CASAC, an *in vivo* CTL killing assay was conducted. The method was as described in section 2.5.1. Target cells were splenocytes pulsed with SIINFEKL peptide (10µg/ml). The SIINFEKL loaded, target cells were stained with CFSE dye at a concentration of 0.3µM (CFSE low cells) while the non-target cells which were not loaded with SIINFEKL, were stained with a log higher concentration of CFSE at 3.0µM (CFSE high cells). Equal numbers of target (CFSE low cells) and non-target cells (CFSE high cells) were mixed together making the cell concentration 1 X 10^8 cells/ml (in PBS). 100µl of the cell mixture were injected intravenously by tail vein injection of the vaccinated C57BL/6 mice and left overnight (16-18 hours).

The splenocytes recovered from the vaccinated mice showed lower levels of the CFSE low (target) compared to the CFSE high (non-target) cells (Figure 4.2 a). *In vivo* CTL killing showed all groups vaccinated with CASAC in the presence or absence of IRX-2 to produced similar percentages of target cell ~~lysis~~lysis. CASAC vaccinated mice in Group 2 lysed 89 ± 3% of target cells (Figure 4.2 b). The inclusion of IRX-2 in CASAC vaccination showed similar levels of *in vivo* target cell ~~lysis~~lysis compared to CASAC only vaccination with 94 ± 0.6% and 92 ± 1.4% in G3 and G5 with no significant differences in the levels of ~~lysis~~lysis (Figure 4.2 b). The data presented showed that the use of either Poly I:C or MPL version of CASAC in combination with IRX-2 produced no significant differences compared to CASAC by itself.

Despite no convincing expansion of SIINFEKL specific CD8+ T-cells detected by pentamer staining of mice vaccinated with emulsion + MPL + IRX-2 (Group 4), antigen specific cytolytic activity was detected, although at reduced levels (mean of 40 ± 9%) compared to the other vaccination groups. (Figure 4.2 b).

Further studies were needed to assess the possible benefits of IRX-2 to the outcome of vaccination.

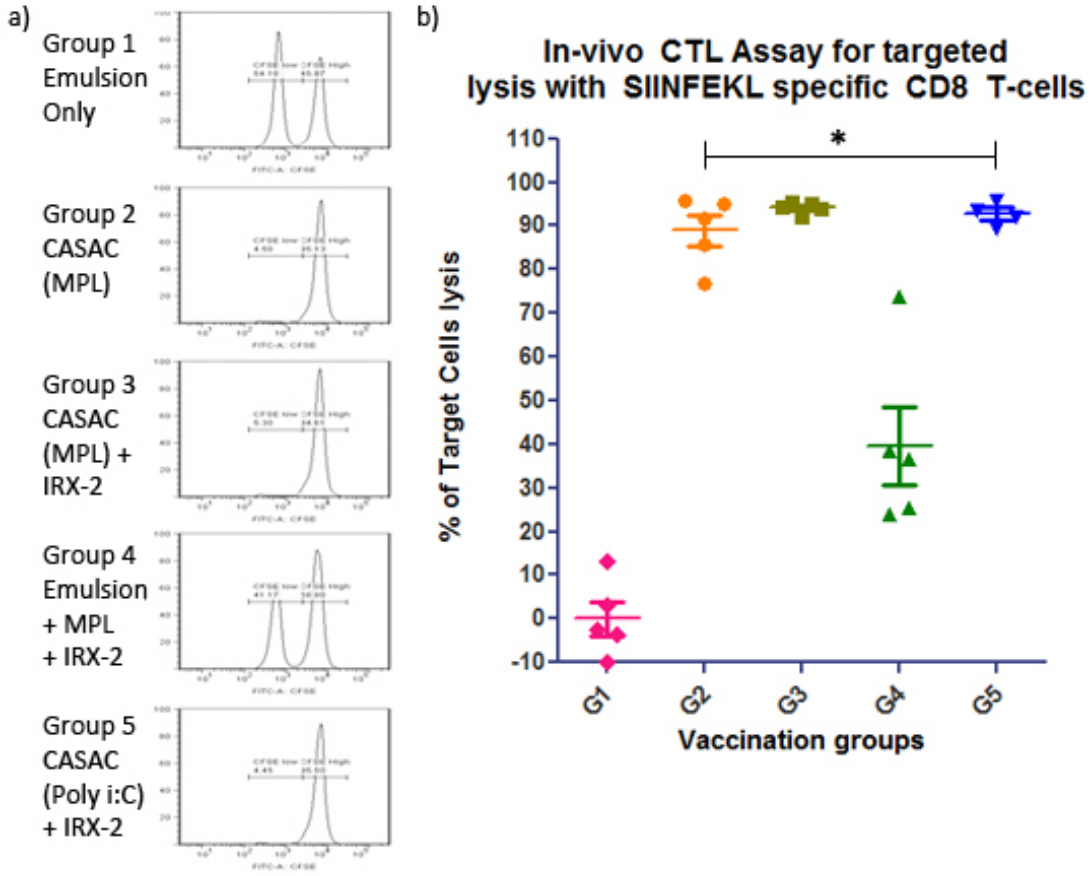


Figure 4.2 In-vivo CTL assay: a) Representative FACS plot of *in vivo* CTL activity in each vaccination group 18 hours after the tail vein injection of the cell mixture. Target cells which were loaded with SIINFEKL were CFSE low while non-target cells were CFSE high. b) Analysis of target cell lysis by the SIINFEKL specific $CD8^+$ T-cells in each group. All vaccination groups showed statistically significant higher levels of lysis of antigen pulsed splenocytes (CFSE low, target cells) than the emulsion only control group (Group 1) (*$p<0.005$, Student's t test analysis conducted, n=5)

4.2.4 Summary

IRX-2 was shown by Naylor *et al.* (2010) to have the ability of increasing T-cell specific immune responses (DTH) against human prostate-specific membrane antigen (NFT) in mice, when combined with IFA or other adjuvants as described in section 4.1.1[98]. The inclusion of IRX-2 in CASAC (either MPL or Poly I:C version) vaccination, however, did not generate significant differences in the generation of SIINFEKL specific $CD8^+$ T-cells or target cell lysis when compared to CASAC only vaccination (MPL version). Vaccination using emulsion + MPL + IRX-2 was conducted to investigate the effect of inclusion of just a single TLR agonist, but no significant increase in SIINFEKL specific $CD8^+$ T-cells was observed when compared to an emulsion only vaccination (without peptides). However, there was a significant increase in target cell lysis within the group vaccinated with emulsion + MPL + IRX-2 when compared to the emulsion only vaccination. Unfortunately, this data could not be used to assess the possible benefits of addition of IRX-2 because a comparator group containing emulsion + MPL (without IRX-2) was not examined. In this experiment, IRX-2 was administered separately to the vaccine as an immunomodulator. In the next set of studies (section 4.2.1), IRX-2 was incorporated into the vaccine to act as a component of the adjuvant.

In one arm of the vaccination regimen, IRX-2 was included directly into the vaccine (mixed with emulsion, adjuvant and peptides) and in the other arm IRX-2 was administered as in its standard protocol (administered in 5 daily doses or 9 daily doses after the first vaccination near the site of vaccination). IRX-2 has previously been shown to provide protection against apoptosis by downregulating FasL on primary T-cells which were co-cultured with tumour micro vesicles (TMV)[100]. Therefore, the inclusion of the IRX-2 into the adjuvant mix and its administration with each round of vaccination as an adjuvant might protect the activated T-cells from FasL/Fas induced apoptosis because activated T-cells in mice up-regulate FasL[105]. IRX-2 also promotes translocation of NF-κB to the nucleus thus stimulating the production of pro-inflammatory cytokines which sustain T-cell responses[101]. On this basis, it was speculated that $CD8^+$ T-cell responses could be improved if IRX-2 was mixed into the vaccine and administered as an adjuvant rather than a separately administered immunomodulator.

Investigation into generation of Tregs and subsets of memory T-cells was also performed to provide additional information on the efficacy and mechanism of IRX-2 synergy with CASAC as IRX-2 has been shown to favour generation of effector T-cells over regulatory T-cells in an *in vitro* setting[106].

The two different versions of CASAC (MPL and Poly I:C version) in the presence of IRX-2 showed no differences in the percentage of SIINFEKL specific $CD8^+$ T-cells or the level of target cell lysis. Therefore only the Poly I:C version was used for subsequent studies because it is more readily available and less expensive. Poly I:C is a TLR-3 agonist that activates the TRIF pathway for the stimulation of NF-κB mediated responses while CpG is a TLR-9 agonist that activates the MyD88 pathway. This synergy between a MyD88-dependant and a MyD88-independent[136] activation of NF-κB[137] improves the stimulation of T-cells and has shown efficacy in CASAC vaccination[52].

4.3 Potential efficacy of IRX-2 as an immune adjuvant for peptide vaccination.

4.3.1 Vaccination design

The ability of IRX-2 to act as an immune adjuvant for inclusion in peptide vaccination or as a separately administered immunomodulator for the enhancement of efficacy of peptide vaccination was further examined. In these studies, IRX-2 was included in the vaccine preparation (with or without TLR agonists as indicated in Table 2). Alternatively, IRX-2 was given as a potential immunomodulator by intradermal injections for 5 consecutive days starting on day 2 after the first vaccination. In the study conducted by Naylor *et al.*, 4 doses and 9 doses of IRX-2 administered 1 day after the first vaccination, was shown to produce similar responses[98]. The vaccine combinations and groups were as indicated in Table 2. For the outline of the vaccination schedule, see Figure 4.3.

Analysis of the immunological responses in the vaccinated mice included pentamer staining for SIINFEKL specific T-cells, and the quantification of Tregs and memory T-cells subsets. *In vivo* CTL killing assay was also carried out to determine the cytolytic activity of the SIINFEKL specific $CD8^+$ T-cells generated.

Group	Vaccination combination
G1	No vaccination
G2	E + OVA
G3	E + OVA + IRX-2 (5D)
G4	E + OVA + IRX-2 (IV)
G5	E + OVA + IRX-2 (IV + 5D)
G6	E + OVA + P + C + I
G7	E + OVA + P + C + I + IRX-2 (5D)
G8	E + OVA + P + C + I + IRX-2 (IV)

Legend :

E = emulsion
C = CpG ODN1826
I = IFN-Gamma
IRX-2 (5d) = IRX-2 given in 5 doses
OVA = Ova peptides (SIINFEKL + ISQ)
P = Poly I:C
IRX-2 (IV) = IRX-2 included in the vaccine

Table 4.2 Vaccination groups: Eight groups of mice were vaccinated as described above. The combination of "E + OVA + P + C + I" represents CASAC vaccination (See G6). IRX-2 as an immunomodulator is given in 5 consecutive doses after the first vaccination. The final concentration of emulsion was 0.2% Tween80 and 4% squalene (Refer to section 2.2.3.4 and 2.2.3.5 for CASAC and CASAC + IRX-2 preparation).

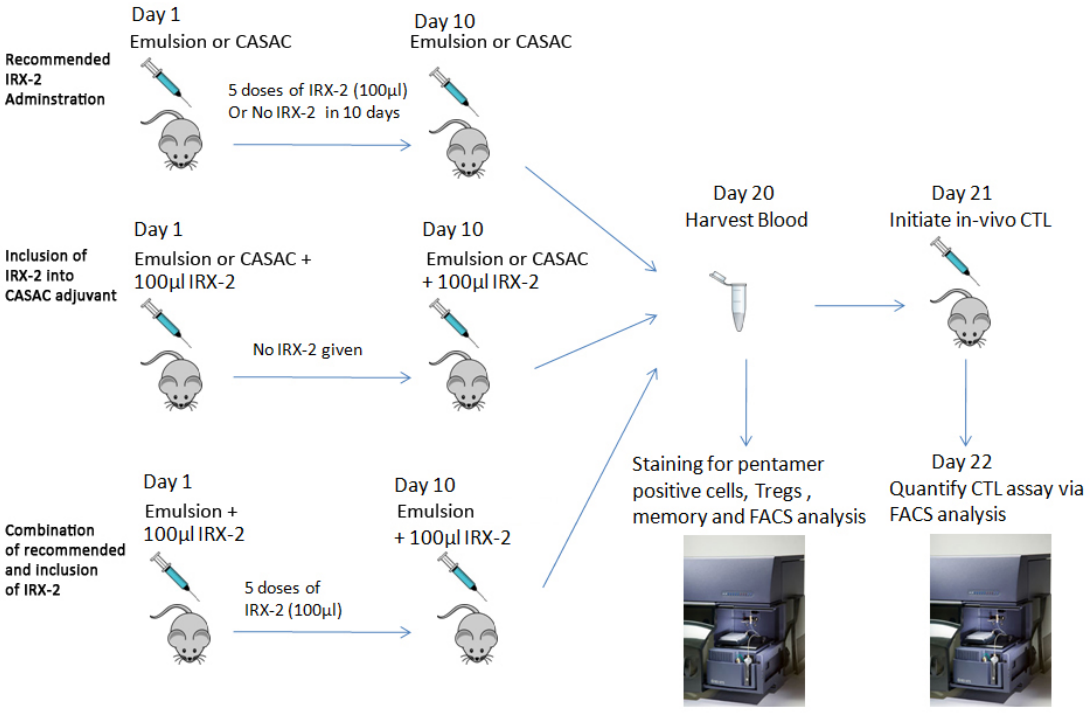


Figure 4.3 Vaccination schedule of all the groups including the analysis time points

4.3.2 Different IRX-2 vaccination regimens generate similar levels of SIINFEKL specific CD8$^+$ T-cells

The inclusion of 100µl of IRX-2 in the CASAC vaccine preparation, instead of administering it as 5 daily doses of 100µl after the first vaccination, generates the same percentages of SIINFEKL specific CD8$^+$ T-cells (approximately 20%). The percentage of SIINFEKL specific CD8$^+$ T-cells generated by CASAC alone was about 5% lower than the groups vaccinated with CASAC plus IRX-2 (Figure 4.4 a). The data is also corroborated by the geometric MFI generated (Figure 4.4 b). Despite the increase in the percentages of SIINFEKL specific CD8$^+$ T-cells in the groups vaccinated with both CASAC and IRX-2, compared to the CASAC only group, the detected increase was not statistically significant.

IRX-2 in combination with emulsion (0.2% Tween80 and 4% squalene) did not stimulate the generation of SIINFEKL specific CD8$^+$ T-cells (Figure 4.4 a). It was shown by others that IRX-2 requires the presence of other adjuvants such as IFA or conjugation of peptide to KLH to induce the expansion/activation of antigen specific

$CD8^+$ T-cells responses[98]. Therefore, IRX-2 by itself is not a effective immune adjuvant for peptide vaccination, but it may be able to act synergistically with other adjuvants such as Poly I:C or CpG, to enhance the response to vaccination.

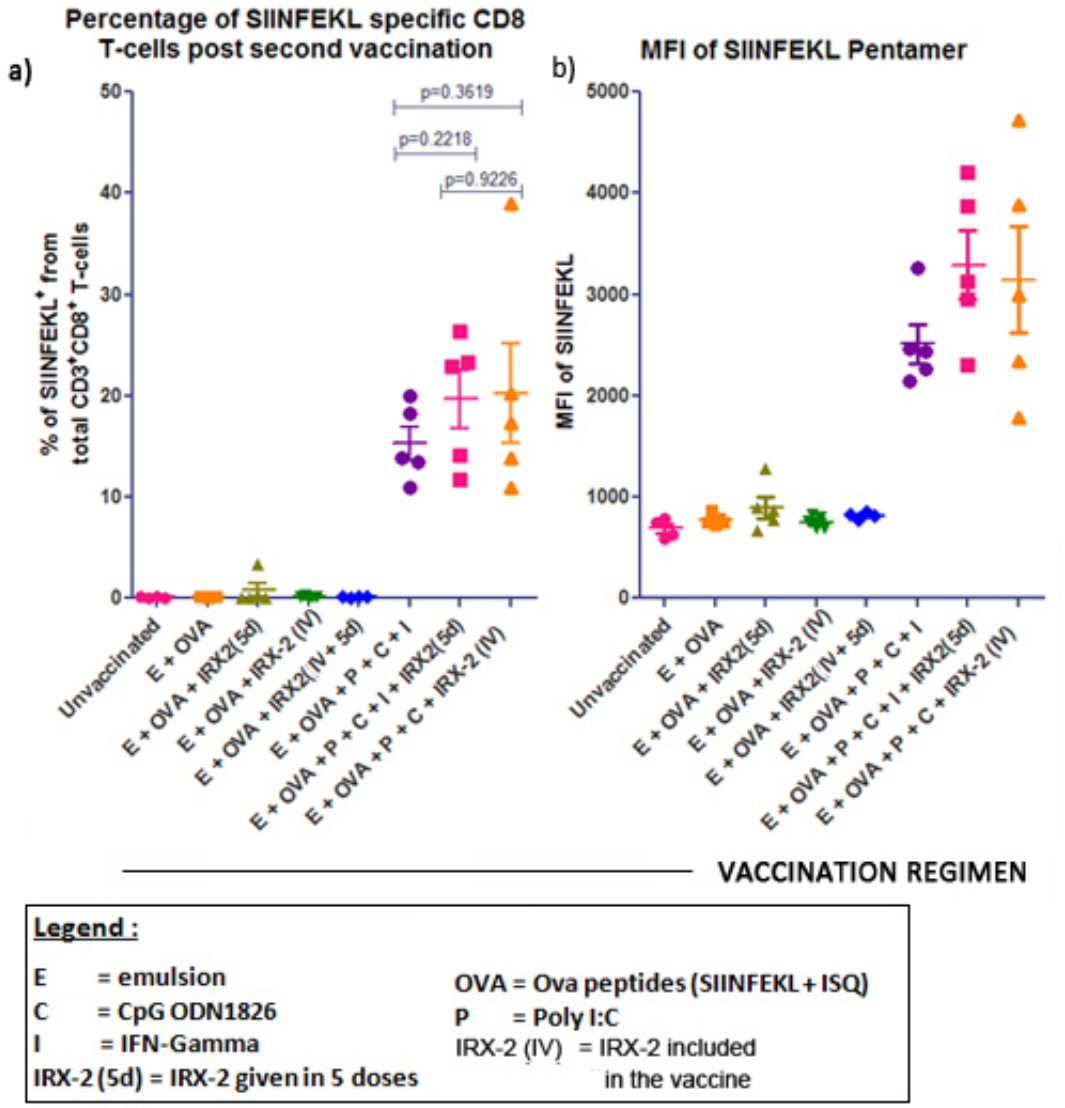


Figure 4.4 Analysis of SIINFEKL pentamer staining: a) Percentage of SIINFEKL specific $CD8^+$ T-cells generated on day 20 in the emulsion and emulsion with IRX-2 (in the vaccine or as 5 daily doses) and the CASAC vaccinated mice (with or without IRX-2). b) Geometric MFI of SIINFEKL pentamer positive CD8 T-cells corroborated with the percentage of SIINFEKL specific $CD8^+$ T-cells. Mean ± SEM percentage and geometric MFI shown (Student's t test analysis, n=5).

4.3.3 CASAC plus IRX-2 vaccination generates effector memory SIINFEKL specific CD8 T-cells without increasing Tregs

CASAC has been shown to generate predominantly effector memory SIINFEKL specific CD8 T-cells[52]. In this experiment, over 90% of the SIINFEKL specific $CD8^+$ T-cells were found to have an effector memory phenotype ($CD44^+CD62L^-$) in all the CASAC vaccinated mice (Figure 4.5 a). This was observed either with or without IRX-2. The inclusion of IRX-2 in CASAC showed a statistically significant increase of 4% in the effector memory cells compared to CASAC only vaccination (Figure 4.5 a). There was no significant differences in the percentage of cells with a central memory phenotype ($CD44^+CD62L^+$) when comparing the two groups (Figure 4.5 b). No naive cell phenotype was seen in groups vaccinated with CASAC (with or without IRX-2). In the unvaccinated mice, analysis of memory T-cell subsets were conducted on the $CD3^+CD8^+$ cells (data not shown but similar to that in section 3.2.3).

Repeated stimulation of $CD4^+$ T-cells may lead to generation of Tregs which could suppress the activation and proliferation of antigen specific $CD8^+$ T-cells. However, none of the vaccinated groups showed significant differences in the percentages of Tregs ($CD4^+CD25^+FoxP3^+$) after two rounds of vaccination which included a T-helper peptide [138] (Figure 4.5 d).

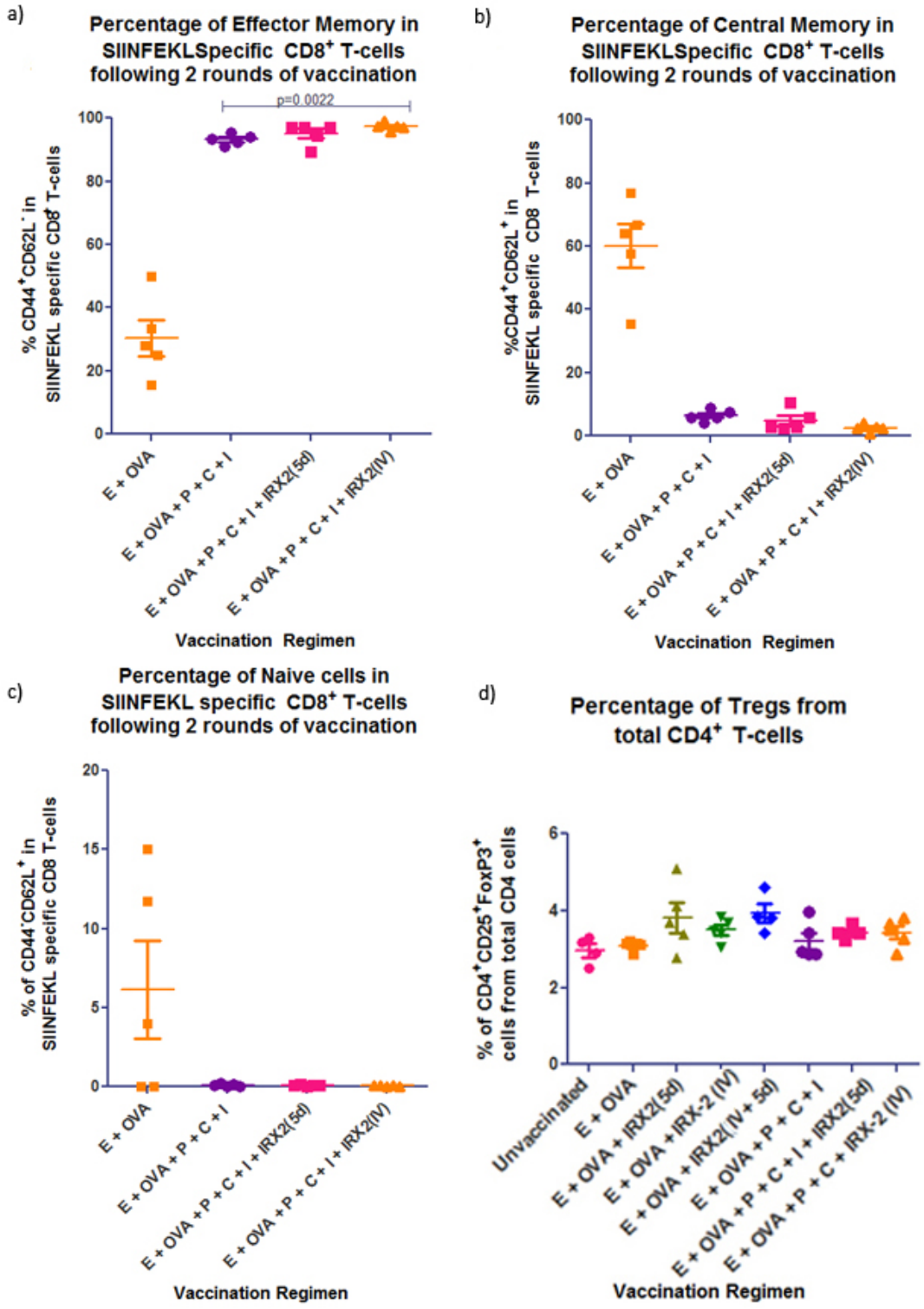


Figure 4.5 Analysis of Memory subsets and Tregs: a) The percentages of effector memory SIINFEKL specific CD8 T-cells generated by CASAC vaccination at 10 days after the second vaccination. b) The percentages of central memory SIINFEKL specific CD8 T-cells 10 days post the second vaccination. c) The percentages of naive CD8 T-cells 10 days post the second vaccination. d) The percentage of Tregs from total $CD4^+$ T-cells 10 days post the second vaccination. (Student's t test analysis)

4.3.4 CASAC vaccination generates maximal cell killing in an in-vivo CTL killing assay

In section 4.2.3, the *in vivo* CTL killing assay showed no significant differences in the potency of CASAC compared to CASAC plus IRX-2 vaccination. Similar results were obtained in this experiment where over 95% of target cell (peptide loaded, CFSE low) lysis was detected (Figure 4.6). The inclusion of IRX-2, either in the vaccine or given as 5 daily intradermal injections after the first vaccination with emulsion and OVA peptides showed small, consistent, but not statistically significant increases (about 5%) in the *in vivo* CTL activity (Figure 4.6). Given that CASAC by itself could induce the *in vivo* lysis of over 95% of the target cells, the experimental design used here appears to be not suitable for assessment of any additional effects of IRX-2 in the stimulation of *in vivo* CTL activities.

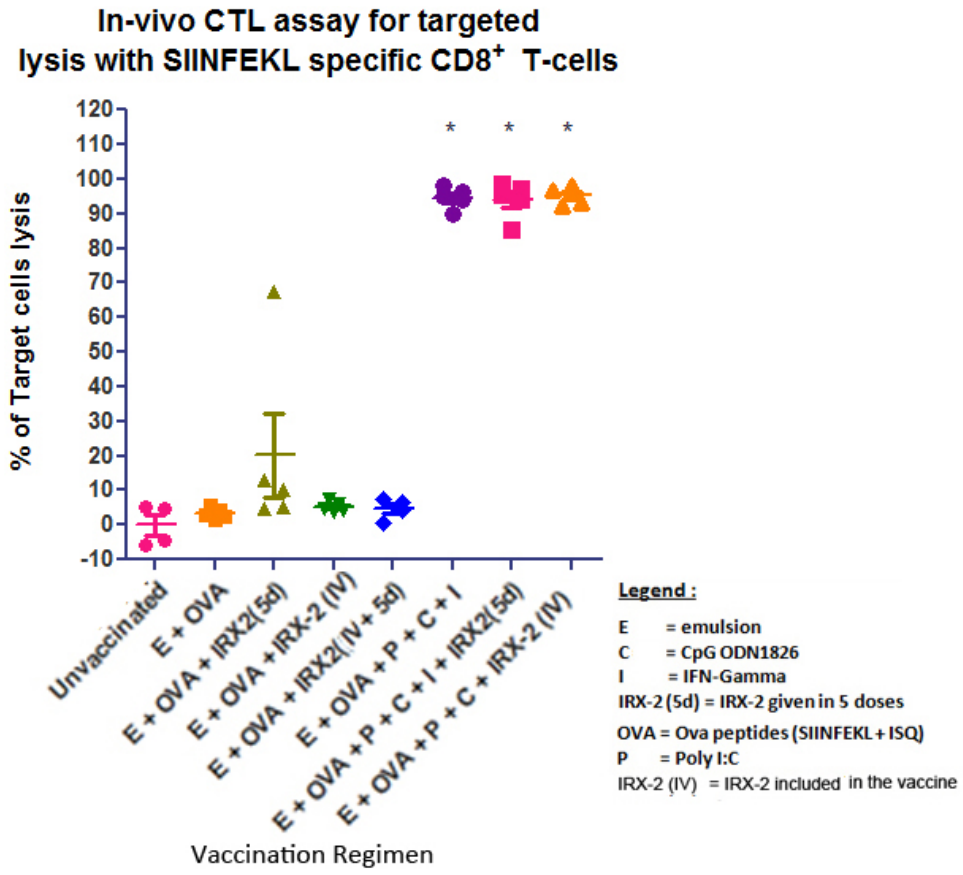


Figure 4.6 In-vivo CTL assays for the different vaccination regimens conducted on day 22 post the second vaccination. (*p<0.001 compared to all groups vaccinated with emulsion and OVA peptide without the TLR agonists (Poly I:C and CpG) or the unvaccinated group; Student's t test analysis, n=5)

4.3.5 Summary

OVA peptides (SIINFEKL + ISQ) vaccination with emulsion and IRX-2 did not promote generation of SIINFEKL specific $CD8^+$ T-cells beyond the background levels. This was the case whether IRX-2 was included in the vaccine or administered as daily intradermal injections after the first round of vaccination. In contrast, OVA peptides (SIINFEKL + ISQ) vaccination with CASAC generates high percentages of SIINFEKL specific $CD8^+$ T-cells. The combination of CASAC with IRX-2 administered either on a standard 5 daily doses or included in the vaccine preparation, did not enhance levels above the very high percentages of SIINFEKL specific $CD8^+$ T-cells obtained with CASAC alone. These studies were therefore unable to assess the potential immune modulatory or immune adjuvant activity of IRX-2, as evaluated by either percentage of SIINFEKL specific $CD8^+$ T-cells or by *in vivo* CTL activity.

The memory subset of T-cells generated by CASAC vaccination corroborated with the data generated by Wells *et al.* (2008), where the SIINFEKL specific $CD8^+$ T-cells were found to be predominantly of the effector memory phenotype. Schilling et.al., (2011) also showed the ability of IRX-2 to expand effector memory subset instead of Tregs. In the present studies, IRX-2 combined with CASAC showed a significant 4% increase in the effector memory T-cell subset. CD4 T-cells in the vaccinated mice (CASAC ± IRX-2) showed no alteration in the percentage with regulatory phenotype ($CD4^+CD25^+FoxP3^+$)

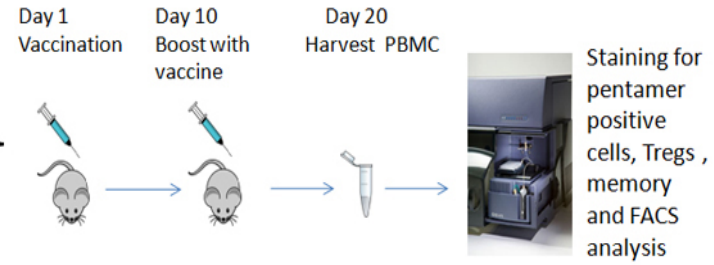
Based on this data and also results from others[98, 106], it would appear that IRX-2 by itself does not generate antigen specific CD8 T-cell responses. However, when IRX-2 was combined with other adjuvants, it was shown to have immunomodulatory activities. Our data shows that CASAC alone is a very potent adjuvant and that inclusion of IRX-2 does not promote any further stimulation of the very high levels of T-cell activation induced by CASAC. Dilution of CASAC in order to stimulate suboptimal CD8 responses was therefore undertaken in order to be able to study the effects of IRX-2 in combination with CASAC.

4.4. Analysis of IRX-2 in combination with reduced concentration of CASAC.

4.4.1 Vaccination design: CASAC was diluted from its standard concentration in order to weaken the CD8 T-cell responses, thus facilitating the investigation of the effects of IRX-2. Here, CASAC was diluted to a level where it would induce no significant SIINFEKL specific CD8 T-cells response by itself. The quantity of peptide for the vaccination remained as used previously (100µg of peptide per mouse). Dilution of the other components of the vaccine is shown in Figure 4.7.

a)

Group	Vaccination
G1	Unvaccinated
G2	Emulsion + OVA
G3	Standard CASAC dose
G4	5X Diluted CASAC
G5	25X Diluted CASAC
G6	100X Diluted CASAC
G7	500X Diluted CASAC
G8	5000X Diluted CASAC



b)

Content of CASAC with dilution injected per mice depending on dilution factor	
SIINFEKL (Class I peptide)	= 100ug (Undiluted)
ISQ (Class II peptide)	= 100ug (Undiluted)
Tween 80	= 0.2% of final volume (Undiluted)
Squalene	= 4% of final volume (Undiluted)
Recombinant mIFN-γ	= 100ng (diluted according to dilution factor)
CpG	= 25ug (diluted according to dilution factor)
Poly I:C	= 50ug (diluted according to dilution factor)

Figure 4.7 Vaccination design for CASAC dilution: a) Groups and dilution of CASAC, ranging from undiluted to 5000X dilution in 5 different groups and the vaccination schedule. b) Peptides and emulsion concentrations were unchanged while recombinant mIFN-γ, CpG and Poly I:C were diluted as indicated by the dilution factor. As an example in the 5X dilution, 100 µg of SIINFEKL, 100 µg of ISQ, 0.2% of Tween80, 4% of Squalene, 20 ng of recombinant mIFN-γ, 5 µg of CpG and 10µg of Poly I:C were combined to make up a 100 µl vaccine in PBS.

4.4.2 5000X diluted CASAC vaccination generates background levels of SIINFEKL specific $CD8^+$ T-cells

The percentage of SIINFEKL specific $CD8^+$ T-cells generated declined with increasing dilutions of CASAC (Figure 4.8 a). In the group vaccinated with emulsion and OVA peptides (SIINFEKL and ISQ), background levels of 0.4% SIINFEKL specific $CD8^+$ T-cells were detected. The undiluted CASAC vaccination generated an average of 27 ± 4.6% SIINFEKL specific $CD8^+$ T-cells. The percentages of SIINFEKL specific $CD8^+$ T-cells declined with increasing CASAC dilutions. At 5X dilution of CASAC, an average of 19 ± 2.5% SIINFEKL specific $CD8^+$ T-cells were generated. At 25X, 100X, 500X and 5000X dilution, the percentage of SIINFEKL specific $CD8^+$ T-cells generated were 8 ± 1.8%, 3.5 ± 1%, 0.8 ± 0.2% and 0.4 ± 0.04% respectively. The data generated for undiluted CASAC and all dilutions till 500X were significantly higher than obtained by vaccination with the OVA peptides plus emulsion. At 5000X dilution of CASAC, the percentage of SIINFEKL specific $CD8^+$ T-cells generated showed no significant difference compared to emulsion plus OVA peptide vaccination.

The percentage of effector memory in SIINFEKL specific $CD8^+$ T-cells declined with the reducing concentrations of CASAC (Figure 4.8 b), but was still significantly higher for the group vaccinated with CASAC diluted up to 25-fold. The percentages were similar for the higher dilutions when compared to the emulsion and OVA peptide vaccination.

Treg phenotyping showed significantly lower levels in the standard CASAC and up to 100-fold dilution of CASAC groups compared to the emulsion and OVA peptides vaccination. However the, emulsion and OVA peptides, 500- or 5000-fold diluted CASAC showed a significant increase in Treg levels when compared to the unimmunized mice (Figure 4.8 c).

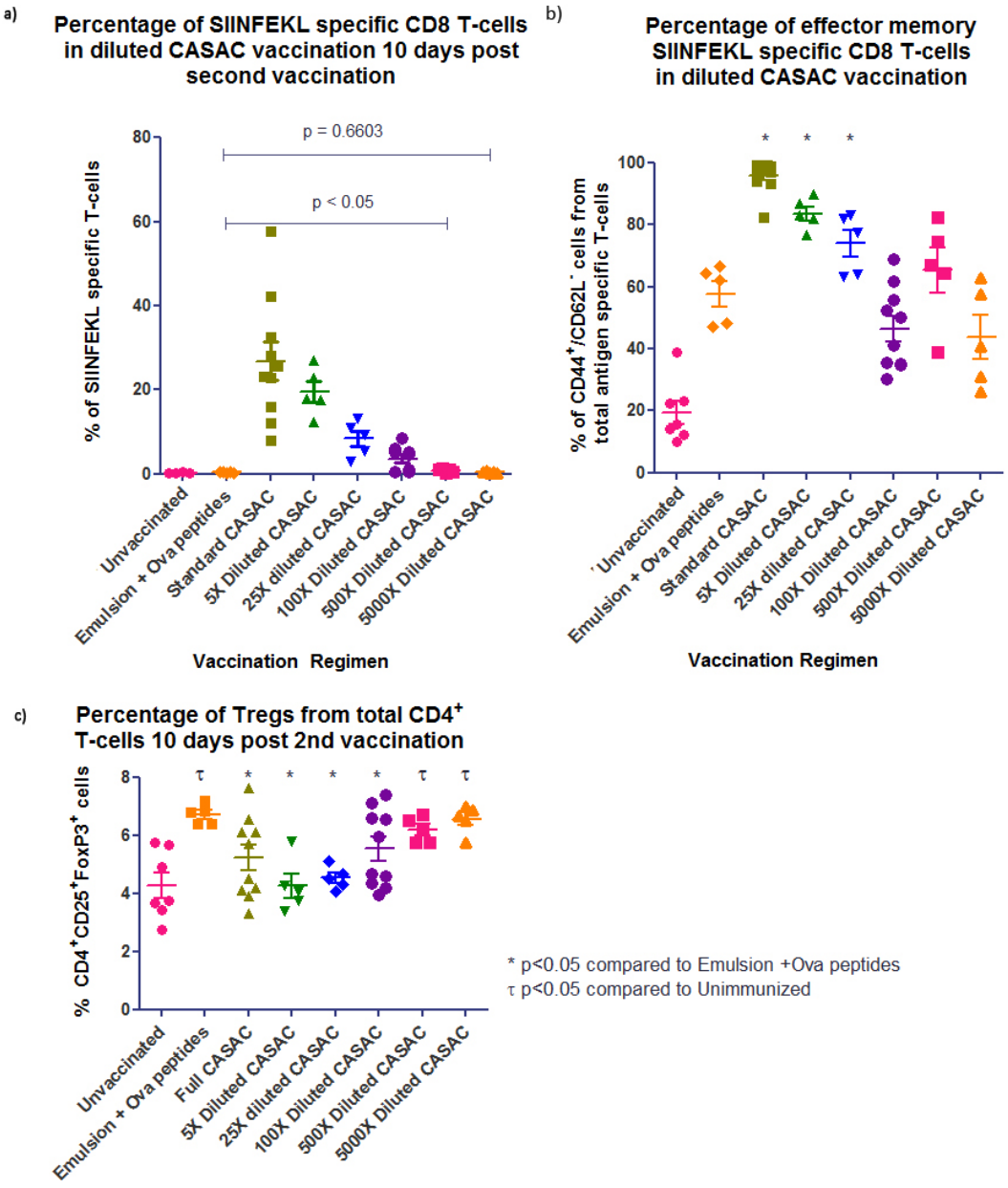


Figure 4.8 Analysis of immunological effects of dilution of CASAC: a) Percentage of SIINFEKL specific $CD8^+$ T-cells generated following vaccination with diluted CASAC preparations. b) Percentage of effector memory phenotype ($CD44^+CD62L^-$) in the total antigen specific T-cells. c) Percentage of Tregs ($CD4^+CD25^+FoxP3^+$) in the total $CD4^+$ T-cells generated after the second round of vaccination. (p values calculated by Student's t test analysis)

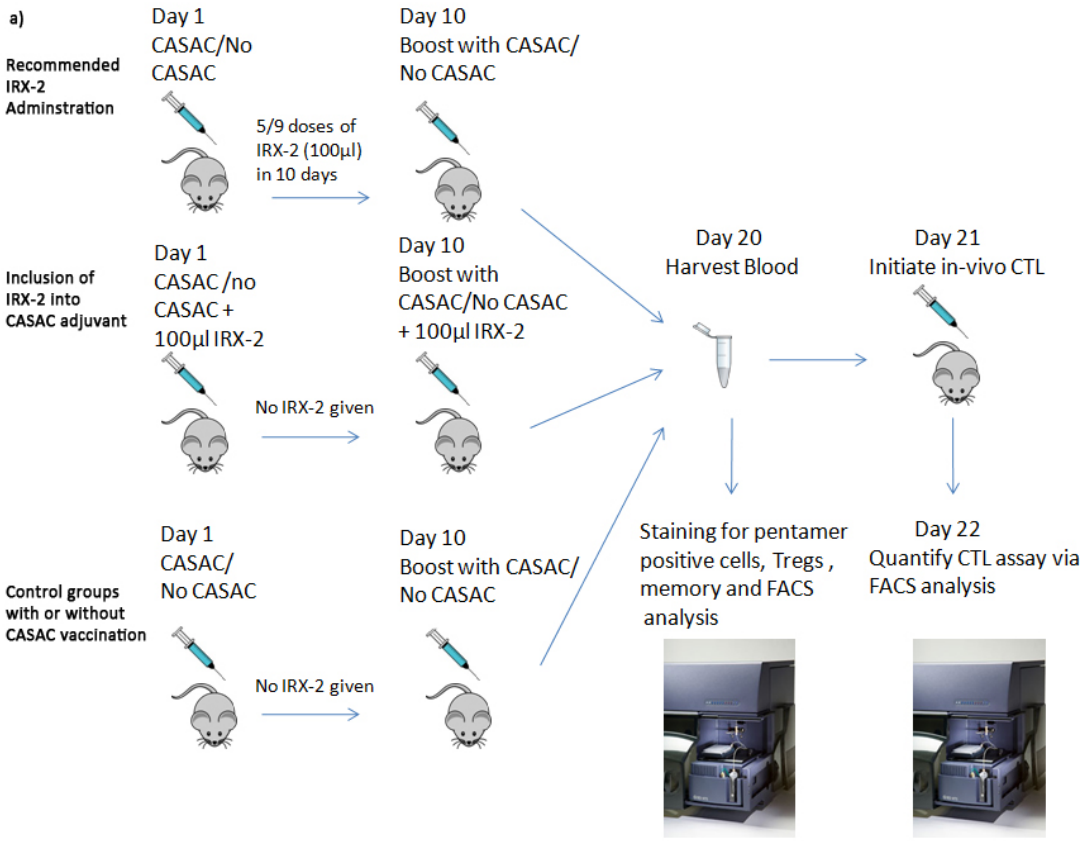
4.4.3 Selection of CASAC dilution for IRX-2 study

To study the potency of IRX-2, 100-fold dilution of CASAC was chosen. This dilution of CASAC generates a significantly higher percentage of SIINFEKL specific $CD8^+$ T-cells compared to the emulsion only vaccination against OVA peptides. Percentage of Tregs generated in the 100X diluted CASAC was between the levels of Tregs generated in the unimmunized group and emulsion only vaccination against OVA peptides.

4.4.4 Vaccination design for 100X diluted CASAC and IRX-2

100X diluted CASAC, chosen as previously described, was combined with standard IRX-2 administration of 5 and 9 daily doses starting after the first vaccine. IRX-2 was also included in the CASAC vaccine mix (Figure 4.9)

a)



b)

Groups	Vaccination Regimen
G1	Unimmunized
G2	Emulsion + OVA
G3	Emulsion + OVA + IRX-2 (9 Doses)
G4	Emulsion + OVA + IRX-2 (5 Doses)
G5	Emulsion + OVA + IRX-2 (in the vaccine)
G6	100X diluted CASAC
G7	100X diluted CASAC + IRX-2 (9 Doses)
G8	100X diluted CASAC + IRX-2 (5 Doses)
G9	100X diluted CASAC + IRX-2 (in the vaccine)

Content of 100X diluted CASAC injected per mice	
SIINFEKL (Class I peptide)	= 100ug
ISQ (Class II peptide)	= 100ug
Tween 80	= 0.2% of final volume
Squalene	= 4% of final volume
Recombinant mIFN-γ	= 1ng
CpG	= 0.25ug
Poly I:C	= 0.50ug

Figure 4.9 Vaccination design with 100-fold diluted CASAC in the presence or absence of IRX-2: a) Vaccination schedule for the inclusion of IRX-2 into 100X diluted CASAC. b) Vaccination groups and the components of the 100X diluted CASAC.

4.4.5 Inclusion of IRX-2 in the vaccine significantly increased percentage of SIINFEKL specific $CD8^+$ T-cells generated by vaccination with a 100-fold diluted CASAC

In OVA plus IRX-2 vaccination, only background levels of SIINFEKL specific $CD8^+$ T-cells were detected with no significant difference compared to the unvaccinated mice. All CASAC vaccinated mice showed significantly higher levels of SIINFEKL specific $CD8^+$ T-cells compared to the non-CASAC vaccinated mice. Inclusion of IRX-2 in the 100-fold diluted CASAC vaccination showed a significant increase in percentage of SIINFEKL specific $CD8^+$ T-cells compared to the 100-fold diluted CASAC vaccination (1.3 ± 0.3% compared to 0.6 ± 0.1%, p=0.047). The 5 and 9 daily doses of IRX-2 did not enhance the generation of SIINFEKL specific $CD8^+$ T-cells with 100-fold diluted CASAC (0.5 ± 0.06% and 0.9 ± 0.2% compared 0.6 ± 0.1%) (Figure 4.10)

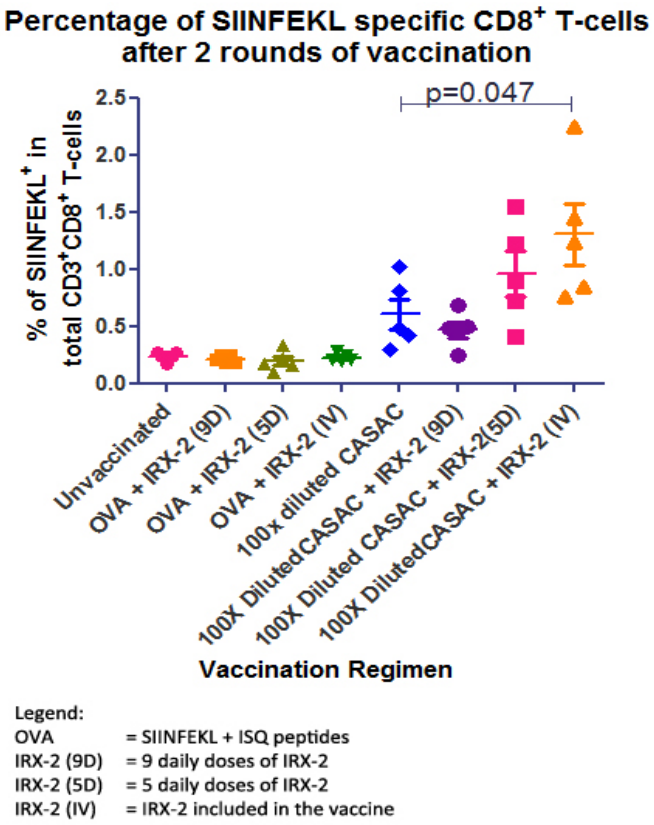


Figure 4.10: Percentage of SIINFEKL specific $CD8^+$ T-cells generated 10 days after the second vaccination with 100-fold diluted CASAC (Student's t test analysis, n=5)

4.4.6 100-fold diluted CASAC ± IRX-2 vaccination generates predominantly effector memory SIINFEKL specific $CD8^+$ T-cells without expanding the Treg population

SIINFEKL specific $CD8^+$ T-cells were phenotyped for memory subset to investigate the effects of IRX-2 on the CASAC vaccination (100-fold dilution). In all the CASAC vaccinated groups (CASAC, CASAC + 5 doses IRX-2, CASAC plus 9 doses IRX-2 and CASAC + IRX-2 in the vaccine), the SIINFEKL specific $CD8^+$ T-cells had a predominantly effector memory phenotype ($CD44^+CD62L^-$, see Figure 4.11 a). Within the CASAC vaccinated groups, CASAC + IRX-2 in the vaccine generated the highest percentage of effector memory SIINFEKL specific CD8 T-cells (84 ± 2.5%). This increase was significant when compared to CASAC only vaccination in which 69 ± 3% of the cells were of the effector memory phenotype ($p=0.042$). The other combination of 100-fold diluted CASAC and IRX-2 did not produce any significant changes in the effector memory cells.

All CASAC vaccination regimens produced similar percentages of central memory SIINFEKL specific CD8 T-cells ($CD44^+CD62L^+$) (Figure 4.11 b). All vaccinated groups showed less than 5% naive T-cells (CD44-CD62L+). This shows that the SIINFEKL specific CD8 T-cells were all antigen experienced (Figure 4.11 c).

In all non-CASAC vaccinated groups, there was no change in the percentage of Tregs compared to the unvaccinated mice (Figure 4.11 d). CASAC only vaccination produced no significant difference when compared to the unvaccinated mice. There was a significant increase in the CASAC + IRX-2 (IV) vaccination, 6.6 ± 0.4% Tregs of total $CD4^+$ T-cells compared to 4.2 ± 0.2% unvaccinated mice ($p=0.0078$). However, there was no significant differences when comparing CASAC + IRX-2 with the CASAC only vaccinated group.

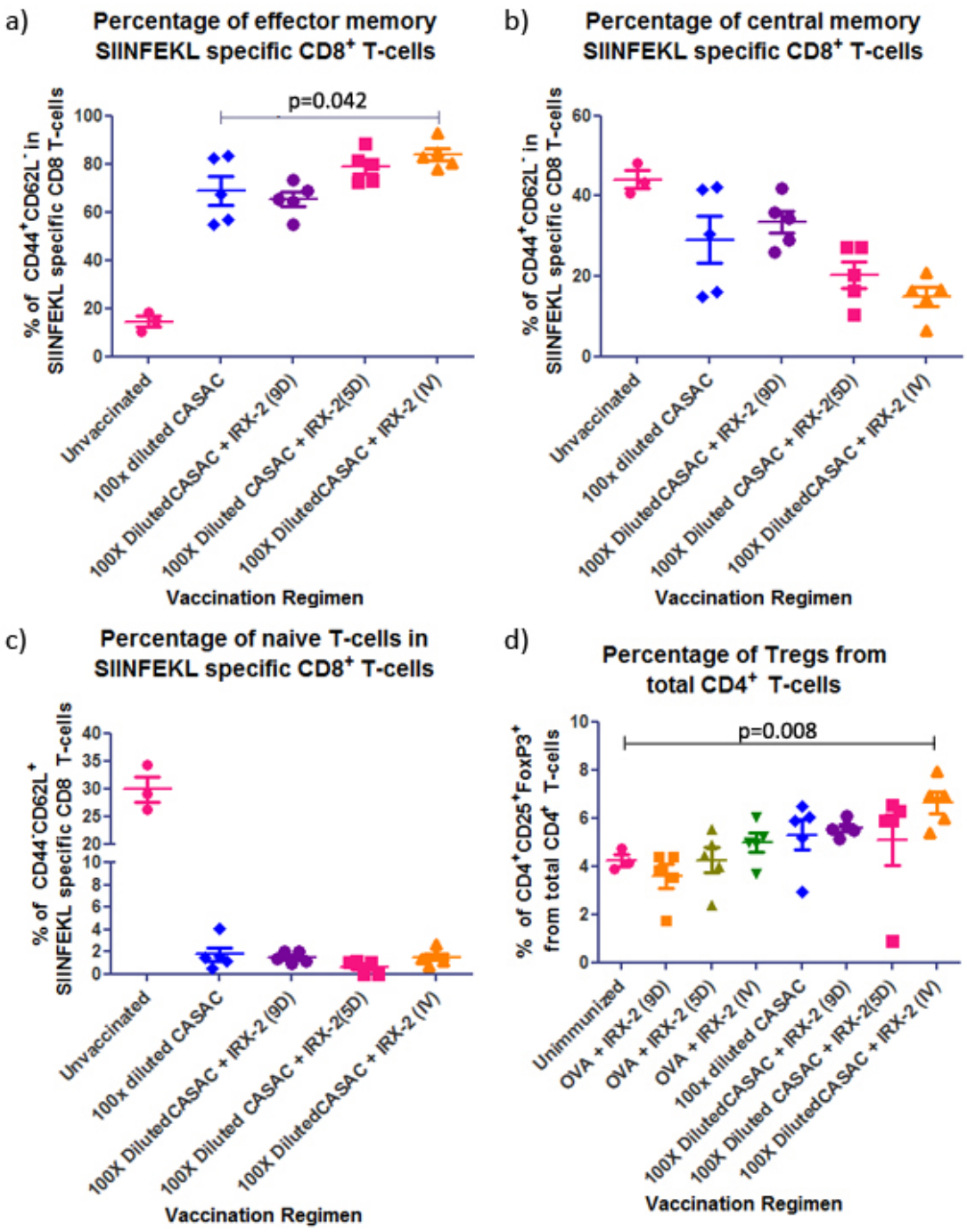


Figure 4.11 Analysis of memory subsets and Tregs: Percentage of effector memory (a), central memory (b) and naive T-cells (c) in the SIINFEKL specific $CD8^+$ T-cells after 2 rounds of vaccination with the indicated combinations of adjuvant. d) Percentage of Tregs in the total $CD4^+$ T-cells. (Student's t test analysis)

4.4.7 IRX-2 enhances the in-vivo CTL activity when used in combination with 100-fold diluted CASAC vaccination

Effects of IRX-2 on the *in vivo* CTL mediated lysis of antigen positive target cells were masked in the earlier studies reported in this chapter (see section 4.2.3 and 4.3.4). This was interpreted to be due to induction of maximal SIINFEKL specific $CD8^+$ T-cells responses by vaccination with the full strength CASAC. The dilution of CASAC to 100-fold of its original concentration of TLR agonists and mIFN-γ reduced the killing of target cells to 4.2 ± 1.2% (Figure 4.12 a). This reduction in the *in vivo* lysis of antigen positive target cells enabled the investigation of the effects of IRX-2 in CASAC vaccination. Vaccination with the OVA peptides plus IRX-2 did not produce any significant increases in the *in vivo* CTL activity.

The combination of 100-fold diluted CASAC and IRX-2 given as 9 daily doses increased the *in vivo* lysis of target cells to 11.5 ± 3.8%, although the increase was just below the significance level (p=0.071). In the 5 daily dose administration of IRX-2 + 100-fold diluted CASAC vaccination, 12.2 + 2.1% of target cells were lysed and this was significantly higher compared to the 100-fold diluted CASAC alone (p=0.012). The combination of IRX-2 in 100X diluted CASAC given as two doses mixed in the vaccine generated significantly higher percentage of *in vivo* CTL activity (11.7 ± 2.7%) when compared to 4.2 ± 1.2% lysis in the group vaccinated with 100-fold CASAC alone (p=0.036). There was a similar increase in the *in vivo* CTL activity of about 2 fold when 100-fold diluted CASAC was used in combination with IRX-2, given as 5 daily doses or mixed in the vaccine.

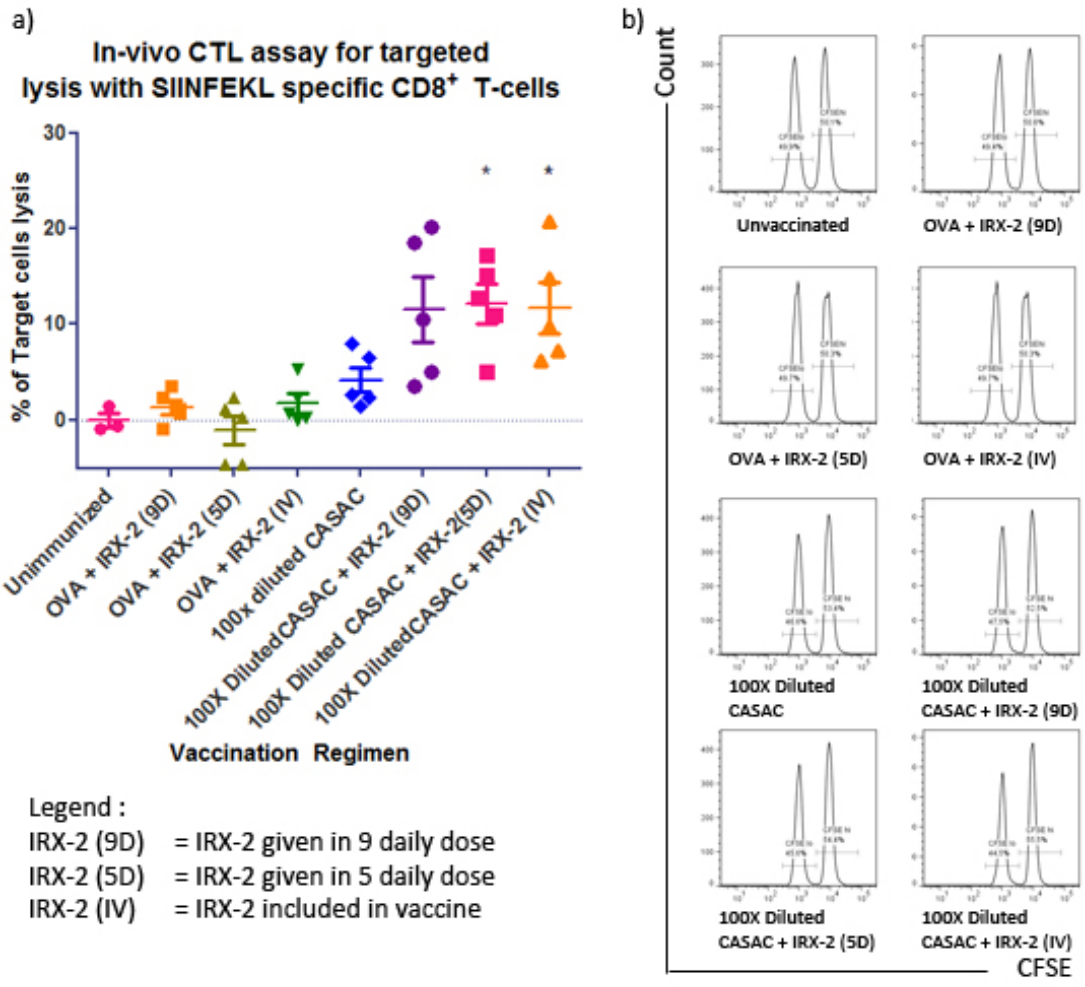


Figure 4.12 *In vivo* CTL assay: a) Percentage of target cell lysis based on *in vivo* CTL killing assay by SIINFEKL specific CD8 T-cells 11 days after the second round of vaccination. b) Representative plot for the *in vivo* CTL assay (*$p<0.05$ when compared to 100X diluted CASAC, Student's t test analysis, n=5).

4.4.8 Summary

The mixture of adjuvants referred to as CASAC can be diluted to produce lower levels of SIINFEKL specific $CD8^+$ T-cells. This involved reducing the concentration of the two TLR agonists and mIFN-γ, but maintaining the concentration and composition of the emulsion and OVA peptides. The decrease in the efficacy of vaccination with the diluted CASAC enabled the investigation of the effects of IRX-2, as an immune adjuvant or immune modulator, for enhancing cellular immunity in response to peptide vaccination.

IRX-2 alone was a poor adjuvant for peptide vaccination. Even in the presence of tween/squalene emulsion, IRX-2 did not provide sufficient stimulation to generate potent antigen specific CD8 T-cell responses. However, the combination of IRX-2 with a 100-fold diluted CASAC showed that IRX-2 can increase the potency of CD8 responses generated in vaccination with a combination of two TLR agonists. In this setting, IRX-2 was also able to increase the percentage of effector memory SIINFEKL specific $CD8^+$ T-cells, without causing a significant increases in Tregs. This may provide the basis for the detected increase in the potency of the SIINFEKL specific $CD8^+$ T-cells in the *in vivo* lysis of antigen positive target cells. Therefore, IRX-2 may be a suitable immunomodulator to enhance the potency of a weak vaccine as modelled here with the use of a diluted CASAC adjuvant. In addition, IRX-2 may provide a means to achieving better immunological responses when vaccination, even with a strong adjuvant such as CASAC, is not able to provide sufficient immunity.

4.5. Discussion

The study reported in this chapter aimed to combine a potent adjuvant, CASAC, with IRX-2 which has been shown to have immunomodulatory activities. The objective was to increase the efficacy of generating potent CD8 T-cells responses, particularly against poorly immunogenic targets such as tumour associated antigens or antigens associated with chronic infections

a) Investigation of different combinations of TLR agonists for assessment of synergy with IRX-2

As stated in section 1.6.2, multiple potential combinations of TLR agonists in CASAC are able to produce similarly high levels of T-cell activation. Here it was shown that the Poly I:C and MPL version of CASAC in combination with IRX-2 does not a significant increase in the very high level of response that can be achieved even without IRX-2. The addition of IRX-2 did show increase of a few percentage in the level of SIINFEKL specific $CD8^+$ T-cells, but this was not statistically significant. The absence of detectable improvements could be because CASAC is a very potent combination of adjuvants. Therefore, to investigate the potential synergy between IRX-2 and other adjuvants, lower concentrations of CASAC were used. A CASAC formulation with a 100-fold lower concentration of Poly I:C and CpG was tested in combination with 3 different regimens of administering IRX-2. Vaccination with this 100-fold diluted CASAC yielded much lower percentages of SIINFEKL specific $CD8^+$ T-cells, but still significantly above background. Although the addition of IRX-2 did not significantly increase the fraction of SIINFEKL specific $CD8^+$ T-cells, the combination did significantly increase the lysis of the antigen positive target cells in the *in vivo* CTL assay. This is consistent with previously published data showing improved T-cell responses when IRX-2 was used for vaccination in combination with other weak adjuvants such as IFA[98]. The increase in potency may also be attributed to the demonstrated ability of IRX-2 in translocating NF-κB to the nucleus, leading to improved production of pro-inflammatory cytokines that enhance Th1 responses. Protection from apoptosis may also play a key role as most activated CD8 T-cells would go into apoptosis after the clearance of antigen[100, 101].

b) Effects of IRX-2 on the memory population and Tregs

Tregs were shown to be slightly increased in IRX-2 plus CASAC vaccination compared to unvaccinated mice, although previous studies have reported no expansion of Tregs when IRX-2 was co-culture with primary T-cells in the presence of immature DCs and irradiated tumour cells. The expansion of effector memory T-cells seen in our vaccinated mice (100X diluted CASAC + IRX-2) was consistent with the previous findings that IRX-2 favours expansion of effector T-cells[106]. However, the increase seen in percentage of Tregs did not block the generation of higher levels of SIINFEKL specific $CD8^+$ T-cells, or the *in vivo* cytolytic activity induced by the combined use of 100-fold diluted CASAC and IRX-2. Instead, the increase seen in the *in vivo* cytolytic activity potentially rendered the increase in Tregs insignificant to the vaccination. The most effective means of delivery of IRX-2 appeared to be as a component of the vaccine mixture rather than the intradermal injections for 5 or 9 consecutive days after the first round of vaccination.

c) The potency of antigen specific CD8 T-cell responses generated

IRX-2 was shown to be a potential modulator/enhancer of cellular immunity, improving the potency of *in vivo* CTL activity. There is a possibility that the increase in the cytolytic activity is caused by improved survival of the T-cells generated. As mentioned in section 1.7, IRX-2 is able to block T-cell apoptosis, thus improving the survival of the activated T-cells. Such improved survival would lead to improved efficacy of the T-cells generated.

4.6 Conclusion and future Studies

In summary, IRX-2 by itself is not an effective adjuvant for vaccine mediated induction of peptide specific CD8 T-cell responses. However, when combined with other adjuvants, IRX-2 can enhance the efficiency of vaccination mediated induction of antigen specific $CD8^+$ T-cell responses. Therefore, IRX-2 maybe a useful component in vaccination against diseases where immunological responses are difficult to achieve. Examples include vaccination against self antigens (e.g. WT-1 for myeloid leukaemias and solid malignancies) or therapeutic vaccination against chronic infections (~~eg~~e.g. chronic HBV infection)

Another possible use of IRX-2 could be to reverse age associated immunological deficiencies referred to as immune senescence or exhaustion. The aged population is more difficult to efficiently vaccinate as there is a much more restricted repertoire of T-cells available[107]. This is at least in part a product of age associated thymic atrophy hence a restricted ability to produce new naive T-cells[139]. In addition, the presence of a higher percentage of regulatory T-cells, hampers the ability to generate antigen specific CD8 T-cells in the aged population[140]. Therefore, IRX-2 is a potential candidate for improving immunological responses to vaccination in the aged population[109].

CHAPTER 5

Peptide vaccination for generating functional SIINFEKL specific $CD8^+$ T-cells in aged mice

5.1 Introduction and study objective

5.1.1 Introduction

Vaccination for cancer and infectious disease can alter potentially effective means of immune therapy. However, such vaccination studies are usually carried out in young animals which have potent immunological responses, in part due to a proficient thymic output of naive T-cells, thus providing a sufficiently diverse population of immune competent T-cell. However, this is not necessarily true for the older individuals with a substantially atrophied thymus, and an immune system that is much less functional in multiple other ways[120]. In particular, patients requiring therapeutic vaccinations are generally older, especially true of cancer sufferers. Long term exposure to tumours, environmental factors and aging of the thymus causes patients to lose the ability to generate a complex enough repertoire of T-cells with the required diversity and functional competence for generating therapeutically effective immunological responses to vaccination[107, 108, 139].

To better emulate the situation in humans, mice that have aged more than 18 months could be used[114-116]. Aging in mice is associated with weak $CD4^+$ and $CD8^+$ T-cells responses due to deficiency in $CD4^+$ naïve T-cells and a reduced repertoire of $CD8^+$ T-cells, leading to less efficient generation of $CD8^+$ T-cell responses, including memory T-cells[107]. Here we hypothesize that the use of CASAC in combination with IRX-2 could lead to the generation of enhanced CD8 T-cell cytolytic activity in the aged mice. Responses in both the aged and the young C57BL/6 mice were compared to the effects of vaccination with CFA as a standard adjuvant.

Aged mice show increased expression of Killer cell lectin-like receptor G1 (KLRG-1) an inhibitory receptor expressed by NK and T-cells is the primary marker used to identify senescence in mice. In humans however, CD57 is an equally useful marker of senescence[113]. Increased expression of exhaustion markers such as PD-1 and LAG-3 has also been correlated with age and are known to be up-regulated with continuous exposure to antigen[109]. The increased expression of PD-1 and LAG-3 have been associated with tolerance to both self and tumour associated antigens[141]. Increased presence of Tregs and expression of CTLA-4 on the surface of T-cells has

also been seen as part of the exhaustion mechanism. Therefore, the mechanisms involved in generating $CD8^+$ T-cell responses could be investigated in terms of these phenotypic changes pre and post vaccination.

5.1.2 Study Objectives:

a) To investigate differences in senescence and exhaustion markers in T-cell population subsets between aged and young mice pre-vaccination

b) To investigate the ability of aged and young mice to generate functional antigen specific $CD8^+$ T-cells responses following vaccination with CASAC in combination with IRX-2 compared to a standard vaccination with CFA.

c) To investigate changes in expression of exhaustion, senescence, activation markers and to study Treg population changes after vaccination.

5.2 Pre-vaccination differences between the young and aged mice.

5.2.1 Lower numbers of lymphocytes in aged mice

Blood was collected from aged (>18 months old) and young mice (6-8 weeks old) from two different batches of C57BL/6 mice pre-vaccination. Cell count was conducted using counting beads of equal volume to anti-coagulated blood obtained by tail vein clipping. The initial lymphocyte cell numbers recorded for young mice were 6495 ± 673 cells/µl blood (batch one) and 4630 ± 512 cells/µl blood (batch two), values which were consistent with previous reports (Doeing *et al.*, 2003), 8500 cells/µl[142]. Lymphocyte count for aged mice however were lower at 3385 ± 358 cells/µl blood (batch one) and 2254 ± 259 cells/µl blood (batch two) ($p<0.001$). This could be explained by thymic involution in the aged mice causing less T-cells to be produced and also the limited lifespan of naïve T-cells in the aged mice leading to loss of cell numbers[120]. The cell numbers and percentages of $CD8^+$ and $CD4^+$ T-cells were also found to be decreased significantly in the aged compared to the young mice as shown in Figure 5.1.

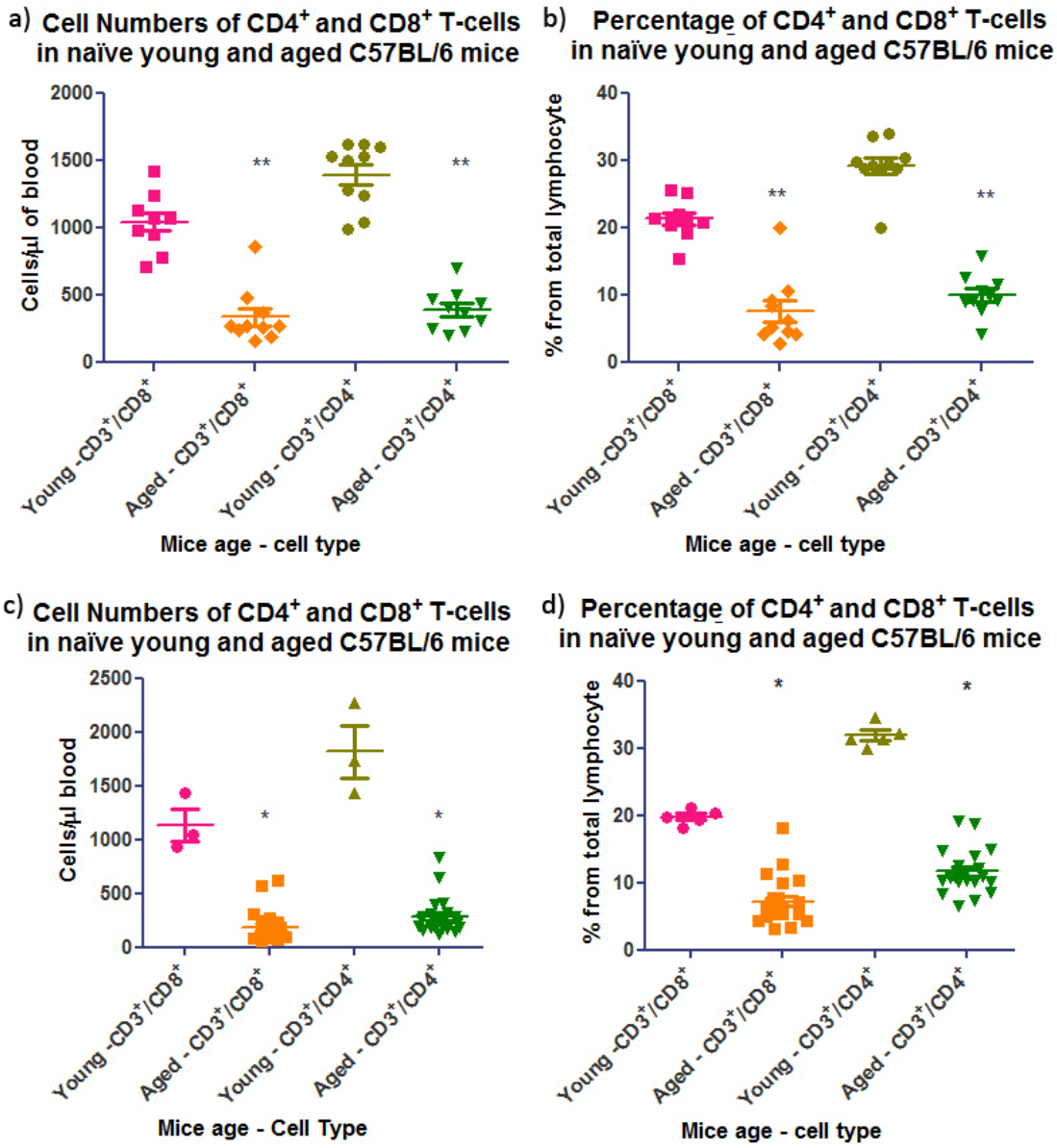


Figure 5.1 Quantification of CD8 and CD4 in aged and young mice: Quantification of $CD3^+CD8^+$ and $CD3^+CD4^+$ T-cells of total lymphocytes. a) & b) represents the cell numbers and percentages in the first batch of mice while c) & d) represents the cell numbers and percentages in the second batch of mice prior to vaccination. Mean ± SEM percentages is shown. (* $p<0.001$ comparing aged and young mice, Student T-test, n=3-20)

5.2.2 Increased expression of senescence and exhaustion markers by T-cells in aged compared to young mice

Percentages of PD-1 and KLRG-1 positive cells were analysed on the $CD3^+CD8^+$ T-cell population. The percentage of cells expressing KLRG-1, a marker for senescence was significantly increased from 2.3% to 22.6% of the $CD3^+CD8^+$ T-cells in the aged population compared to the young ($p=0.0051$). PD-1, an important marker for exhaustion is also expressed on more $CD3^+CD8^+$ T-cells in the aged C57BL/6 compared to the young. In the young, 1.2% of $CD3^+CD8^+$ T-cells was PD-1 positive and increased to 5.7% in the aged ($p=0.0184$). Up-regulation of KLRG-1 is consistent with previously published studies of aged mice (>18 months old)[109, 111, 113]. Representative plots were shown in Figure 5.2 a and results were shown in Figure 5.2 b.

Differences in expression of KLRG-1 and PD-1 in young and aged CD8 T-cells were confirmed in another cohort of young and aged C57BL/6 mice (Figure 5.3). In this cohort, the expression of LAG-3, a terminal differentiation marker associated with exhaustion and PD-L1, which is the ligand for PD-1, were also investigated. Aged mice showed significant up-regulation in the percentage of LAG-3 in $CD8^+$ T-cells compared to young mice. LAG-3 was expressed by 16.9% of aged $CD8^+$ T-cells compared to 9.8% of young $CD8^+$ T-cells ($p=0.0272$). No expression of PD-L1 was found in both aged and young mice. Analysis of PD-1, KLRG-1, LAG-3 and PD-L1 by CD4 T-cells was performed. Unfortunately, data was of poor quality, but suggestive of increased expression of these markers by CD4 T-cells from aged mice (data not shown)

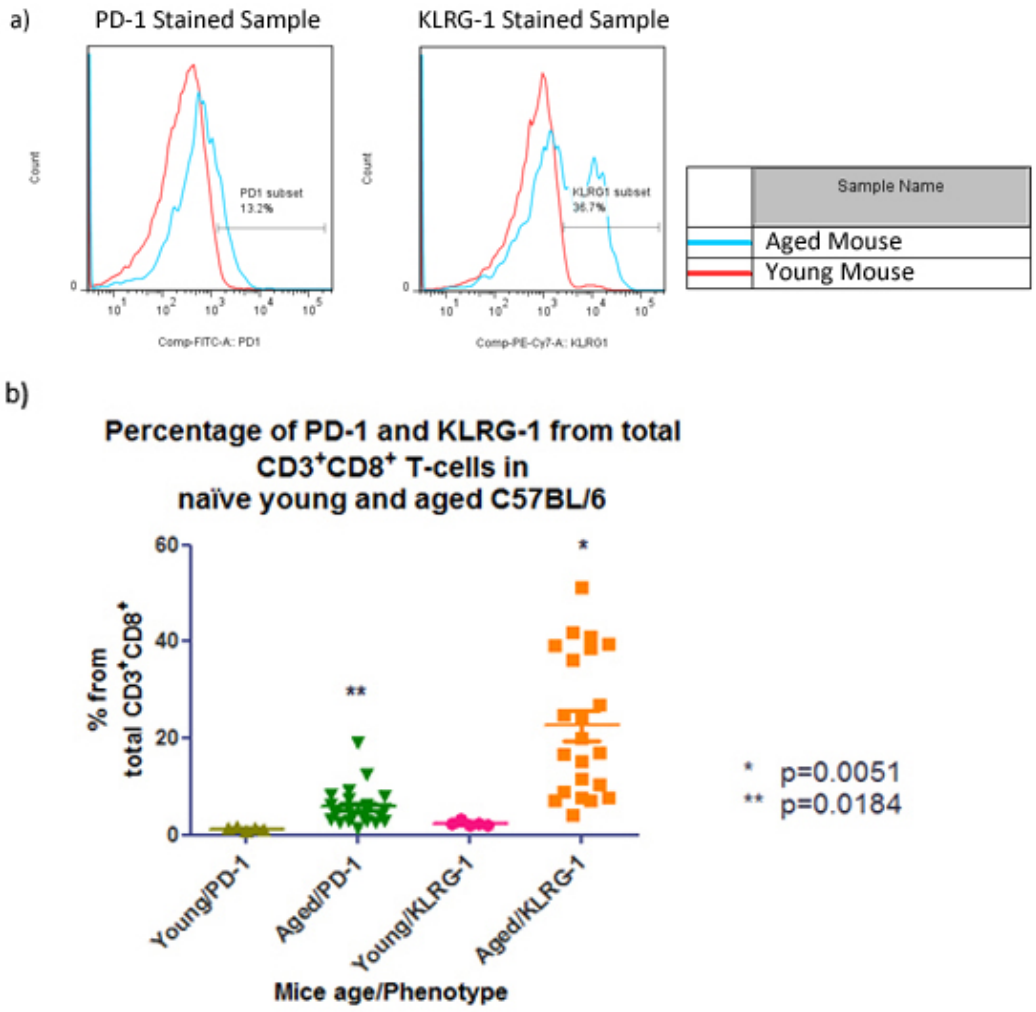


Figure 5.2 Expression of PD-1 and KLRG-1 in young and aged mice: a) A representative plot of PD-1 and KLRG-1 in young and aged mouse (Gating strategy for PD-1 and KLRG-1 was shown in Section 2.5.6) b) Percentages of PD-1 and KLRG-1 positive cells of total $CD3^+CD8^+$ T-cells in the aged (>18 months old) and young (6-8 weeks old) C57BL/6 mice. Mean ± SEM percentages shown. (n=5-20, Student T-test analysis)

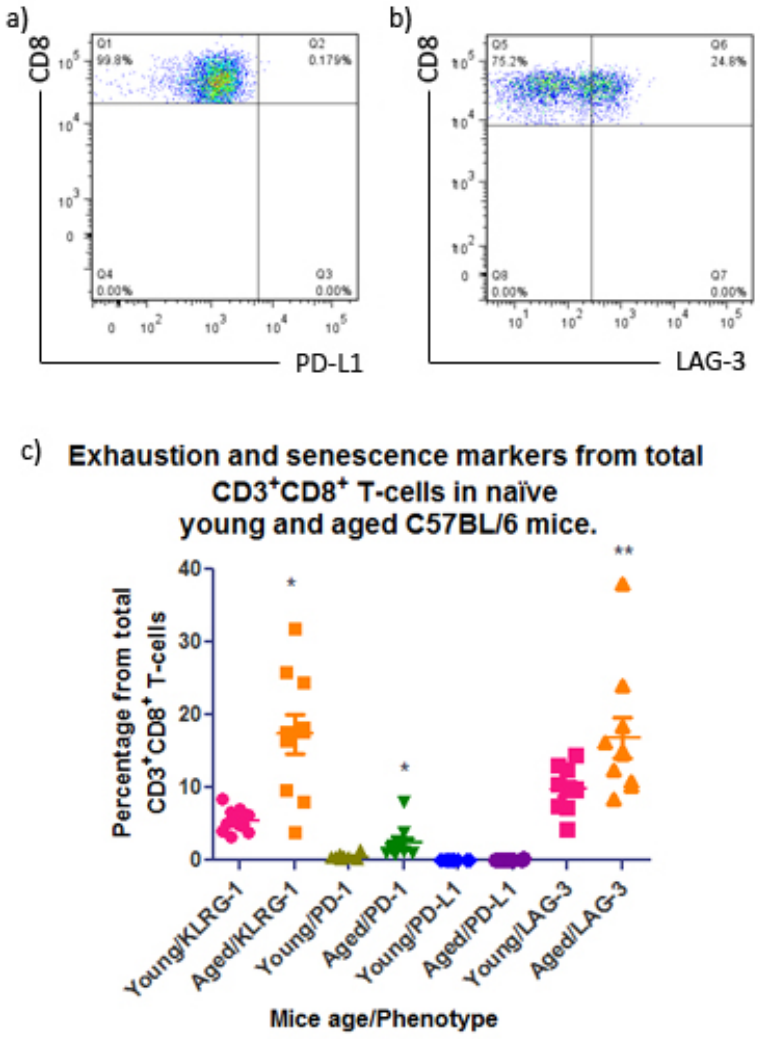


Figure 5.3 Expression of senescence and exhaustion markers in young and aged mice: A representative plot for a) PD-L1 and b) LAG-3 in aged C57Bl/6 mice (Gating strategy for both PD-L1 and LAG-3 was shown in materials and method section 2.5.6). c) Percentage of KLRG-1, PD-1, PD-L1 and LAG-3 in CD8+ T-cells of aged and young mice prior to vaccination. Mean ± SEM percentages is shown. (n=10, * $p<0.05$, ** $p<0.005$, comparing aged to young mice. Student T-test analysis)

5.2.3 No differences in expression of co-stimulatory molecules by T-cells in young or aged mice

T-cell receptor (TCR) activation together with co-stimulation via CD80 or CD86 generates antigen specific $CD8^+$ T-cell responses[32]. CD28 and CTLA-4 competes for CD80 and CD86 co-stimulation. CD28 is constitutively expressed by T-cells and activated by engagement with CD80 or CD86. CTLA-4 is an inhibitory receptor that is only expressed at the surface when a T-cell is activated. CTLA-4 upregulation typically last for about 48 hours although it has been reported that CTLA-4 is constitutively upregulated in mice with chronic viral infection suggesting association with an exhausted phenotype[109].

In this study, CD28 was found to be expressed by the majority of CD4 and CD8 T-cells with no significant differences in young or aged mice (Figure 5.4). CTLA-4 was expressed by a very small percentage of CD4 and CD8 T-cells with no indication of increased expansion in aged mice (Figure 5.4).

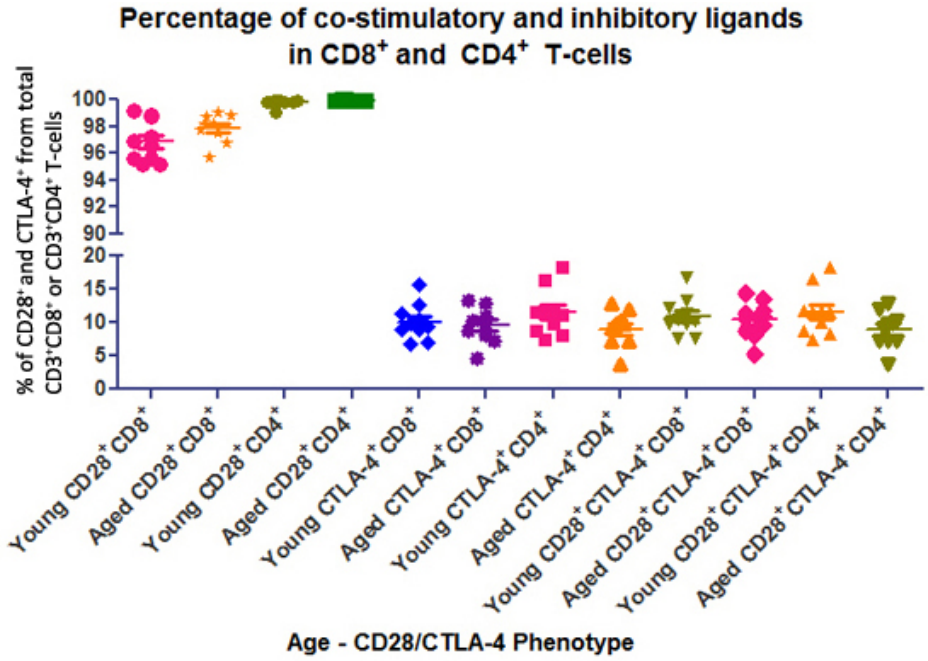
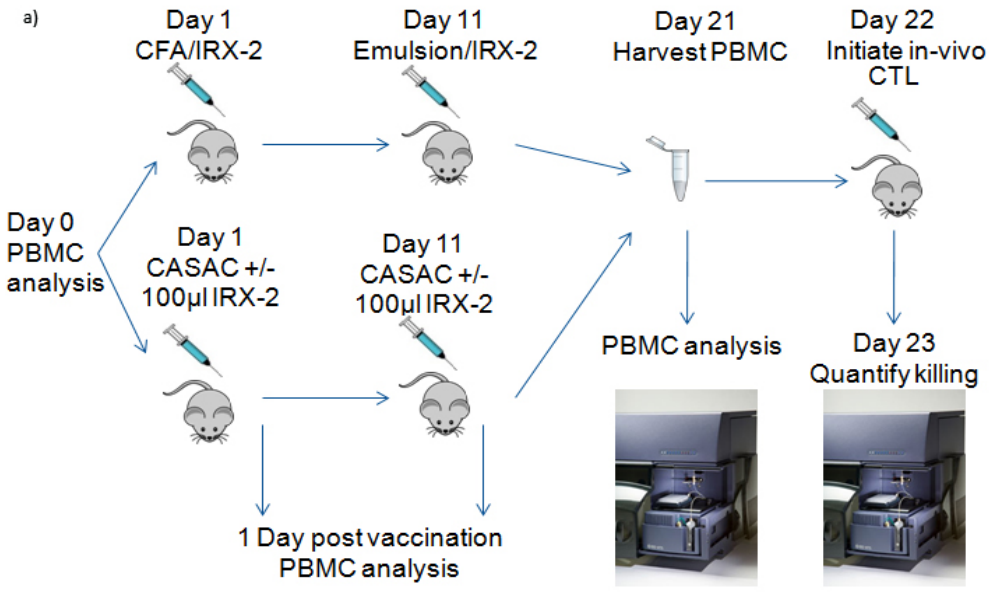


Figure 5.4 Expression of CD28 and CTLA-4 in young and aged mice: Percentages of CD28, CTLA-4 and double positive T-cells in young and aged mice prior to vaccination. Gating strategy for CD28 and CTLA-4 was shown in materials and methods section 2.5.6. Mean ± SEM percentages shown. (n=10)

5.3 Vaccination design

Young (6-8 weeks old) and aged (>18 months old) C57BL/6 mice were vaccinated on Days 1 and 11 followed by analysis and quantification of SIINFEKL specific $CD8^+$ T-cells and the study of various T-cell subsets, on day 21.

On day 22, an *in vivo* cytotoxic T-cell killing assay was initiated, with the analysis of SIINFEKL specific cytolytic activity on day 23 (Refer section 2.6.1 for *in vivo* CTL assay). Splenocytes obtained from the vaccinated mice were used to determine levels of cytokines produced by $CD4^+$ T-cells by an *in vitro* peptide stimulation assay. Vaccination protocol is displayed in Figure 5.5.



b)

	Group	CASAC	CFA	IRX-2
YOUNG	G1	-	-	-
	G2	-	+	-
	G3	-	-	+
	G4	+	-	-
	G5	+	-	+
AGED	G6	-	-	-
	G7	-	+	-
	G8	-	-	+
	G9	+	-	-
	G10	+	-	+

c)

Vaccine components:
CASAC :
Poly I:C and CpG (TLR agonist)
OVA peptides(SIINFEKL and ISQ)
mIFN-γ
Emulsion (Tween 80 and Squalene)
IRX-2 included where required
CFA: CFA + OVA peptides (1st)
Emulsion + OVA peptides (2nd)

Figure 5.5 Vaccination design for young and aged mice: a) Vaccination protocol and experimental flow to analyse the effects of CASAC with/without IRX-2 in aged C57BL/6 mice compared to the young. b) Vaccination groups. c) Vaccination components. The concentration of the components are: 50µg/mouse of Poly I:C, 25µg/mouse of CpG, 100µg/mouse of SIINFEKL and ISQ, 0.2% Tween 80, 4% Squalene, 100, 100µl/mouse of IRX-2, 100ng of mIFN-γ and 1:1 ratio of CFA to vaccine. IRX-2 was included into the mixture of the vaccine in the indicated groups.

5.4 Vaccination with CASAC generates functional SIINFEKL specific CD8 T-cells in aged mice

5.4.1 CASAC vaccination generates improved target cell killing in aged mice compared to CFA

Previous published studies have shown that vaccination of older humans induces poorer responses[120, 143-146]. A good example of this phenomenon in a mouse model was vaccination of aged (>12 months old) and young (6-8 weeks old) Balb/C mice against attenuated Venezuelan equine encephalitis virus (VEE) replicon particle which showed protection in the young mice but not in the aged[147]. Comparison of *in vivo* CTL activity induced by vaccination of aged or young mice using standard CFA showed that although responses were significantly above background (unvaccinated mice) for all mice, responses were significantly weaker in aged mice (16% compared to 54% respectively, p=0.0051) (Figure 5.6 b).

The use of CASAC in the presence or absence of IRX-2 resulted in substantially higher levels of *in vivo* CTL activity as indicated by 95-98% lysis of antigen positive target cells both in the young and aged. This was a significant increase compared to unvaccinated, CFA or IRX-2 alone vaccinations ($p<0.005$).

IRX-2 alone did not induce *in vivo* CTL activity (Figure 5.6). In the young mice, IRX-2 alone generated 7% target cell lysis but was not significantly different compared to unvaccinated young mice (p=0.27). In the aged mice, IRX-2 vaccination generated 33% target cell lysis but again was not statistically significant when compared to the unvaccinated aged mice (p=0.15). This was in agreement with the data shown in section 4.2.4 (Figure 4.7).

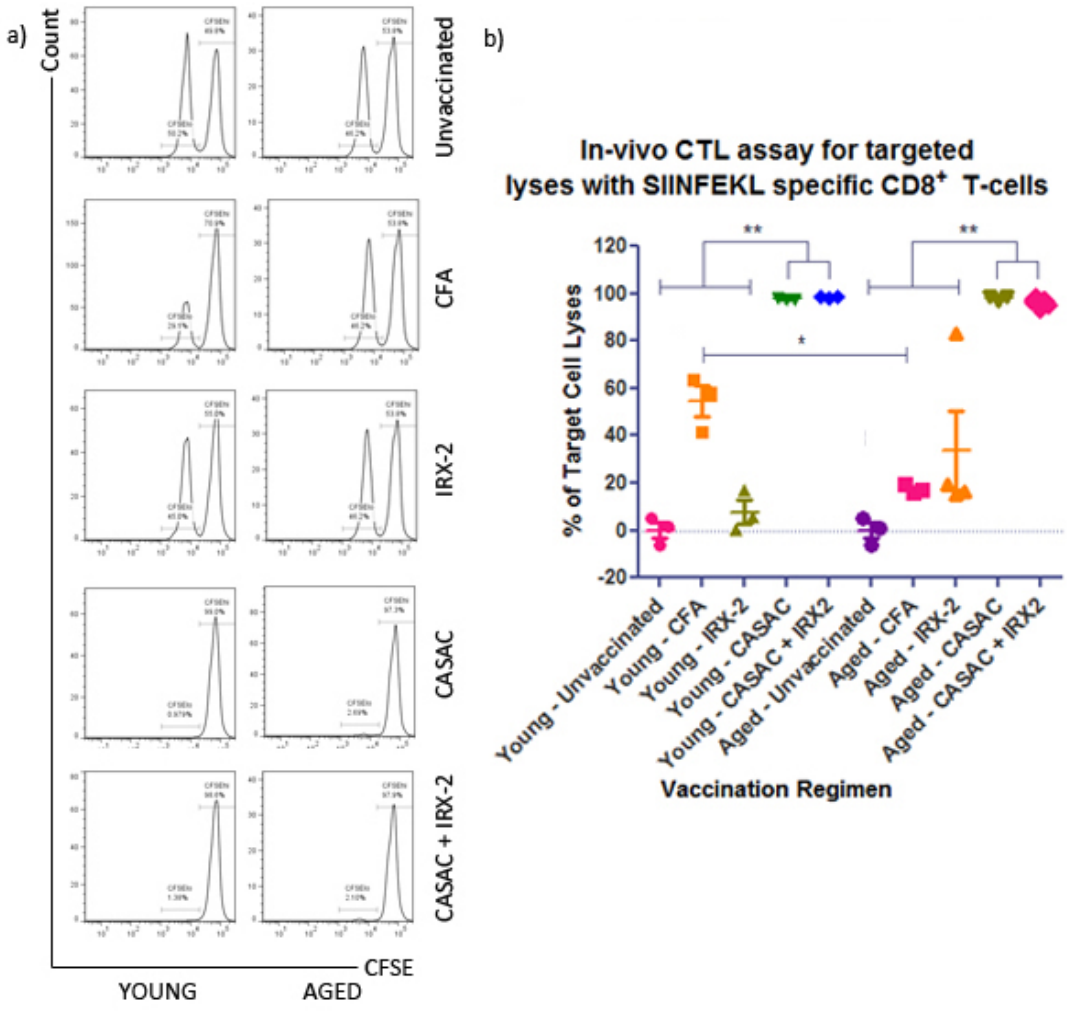


Figure 5.6 In-vivo CTL activity was quantified on day 23 after 2 rounds of vaccination: Antigen positive target cells, loaded with SIINFEKL peptide were labelled with low concentration of CFSE (0.3µM) and antigen negative non-target cells were labelled with high concentration of CFSE (3.0µM). The mixture of target and non-target cells (1:1 ratio) were injected intravenously into the groups of mice and recovered after 18 hours. a) Representative plots for target cell lysed in each group. b) Percentage of target cells lysed in each group. Mean ± SEM percentage shown. (n=3-7) (*$p<0.05$, **$p<0.005$, Student T-test analysis)

5.4.2 IRX-2 can enhance response to vaccination with CASAC in the aged mice

In chapter 4.1.3, where young mice were used, it was shown that CASAC promotes maximal cytolytic activity of SIINFEKL specific $CD8^+$ T-cells. The enhancing effect of adding IRX-2 was only revealed when CASAC was diluted to 100 fold.

In experiments to compare immune responses in young and aged mice, one experiment using undiluted CASAC induced maximal cytolytic activity in all mice and therefore the impact of IRX-2 could not be seen (Figure 5.6). However in a second experiment (Figure 5.7), antigen specific cytolytic activity after vaccination of aged mice with CASAC was lower (75 ± 7%), and in this setting the significant enhancing effect of IRX-2 was observed (92 ± 3%) (p=0.0258). This was only seen in the aged mice. In the young mice, CASAC vaccination resulted in the *in vivo* lysis of 98 ± 0.7% of the antigen positive target cells both in the presence and absence of IRX-2 treatment.

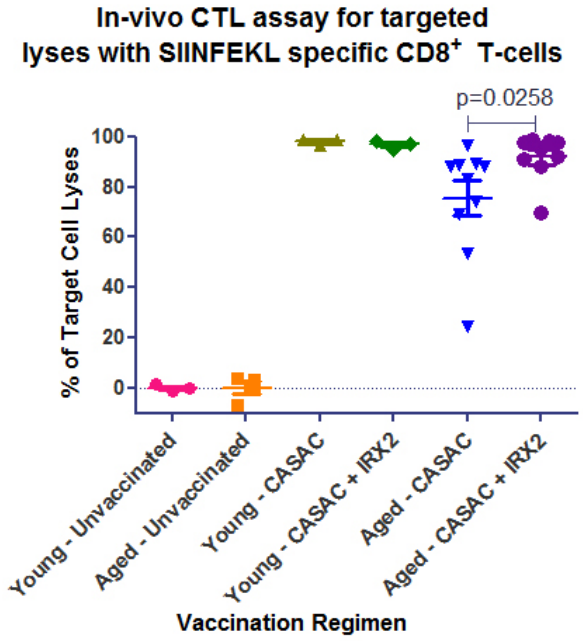


Figure 5.7 In-vivo CTL activity in vaccinated young and aged mice: The percentage of antigen positive target cell lysis in young and aged mice. *In vivo* CTL activity was quantified on day 23 after 2 rounds of vaccination. Antigen positive target cells, loaded with SIINFEKL peptide were labelled with low concentration of CFSE (0.3µM) and antigen negative non-target cells were labelled with high concentration of CFSE (3.0µM). The mixture of target and non-target cells (1:1 ratio) were injected intravenously into the groups of mice and recovered after 18 hours. Mean percentage ± SEM were shown. (n=3-10) (Student T-test analysis)

5.5 Quantification of SIINFEKL specific $CD8^+$ T-cells.

5.5.1 Numbers of SIINFEKL specific CD8 T-cells generated are higher in the young compared to the aged mice despite similar percentages

The *in vivo* CTL data shown in section 5.4.1, indicate the improved cytolytic activity of SIINFEKL specific $CD8^+$ T-cells in the aged mice vaccinated with CASAC compared to CFA. Therefore, we analysed the quantitative generation of SIINFEKL specific $CD8^+$ T-cell responses. When comparing CASAC in the presence or absence of IRX-2 to CFA, there was much lower percentage of SIINFEKL specific $CD8^+$ T-cells in the CFA vaccinated mice, consistent with the induction of a lower level of *in vivo* CTL activity (Figure 5.8 a). Despite the similar percentage of SIINFEKL specific $CD8^+$ T-cells generated in the young and the aged mice that were CFA vaccinated, the absolute numbers of these cells were lower in the aged (12 ± 5 cells/µl blood) compared to in the young (27 ± 7 cells/µl blood), although this differences was not statistically significant ($p=0.16$) (Figure 5.8 b).

No differences were found in the percentage of SIINFEKL specific $CD8^+$ T-cells when comparing CASAC vaccination in the presence or absence of IRX-2 between the young and the aged (Figure 5.8 a). However, SIINFEKL specific $CD8^+$ T-cells numbers were lower in the aged compared to the young (Figure 5.8 b). In the aged mice, CASAC in the presence or absence of IRX-2 generated between 140-200 SIINFEKL specific $CD8^+$ T-cells/µl blood. This was significantly lower when compared to in the young mice (between 620-790 SIINFEKL specific $CD8^+$ T-cells/µl blood, $p<0.05$)

The generation of lower absolute numbers of SIINFEKL specific $CD8^+$ T-cells in the aged mice could be attributed to the lower numbers of T-cells (or total lymphocytes) compared to the young mice.

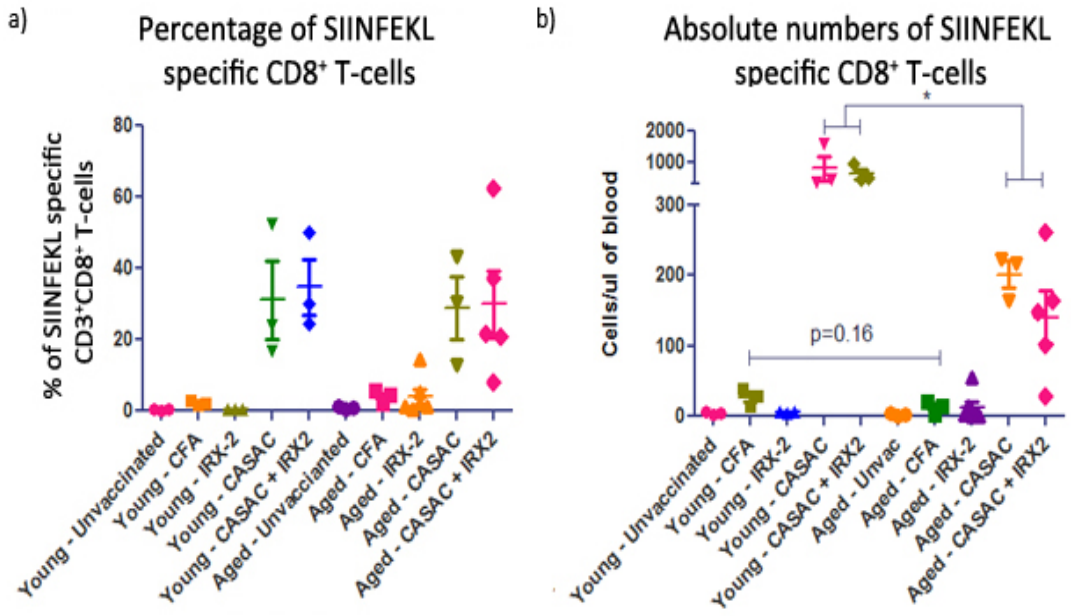


Figure 5.8 SIINFEKL specific CD8$^+$ T-cell in young and aged mice: After 2 rounds of vaccination, whole blood was collected on day 21 and percentage (a) and absolute numbers (b) of SIINFEKL specific CD8$^+$ T-cells were quantified. Mean ± SEM percentage and numbers were shown. (n=3-7, *p<0.05, Student T-test analysis was conducted)

5.5.2 IRX-2 enhanced the cytolytic activity of SIINFEKL specific CD8$^+$ T-cells in CASAC vaccination, but without detectable changes in the percentages or absolute numbers of antigen specific T-cells

In one experiment, vaccination of aged mice using CASAC combined with IRX-2 was shown to significantly increase the *in vivo* lysis of antigen positive target cells (Figure 5.7). SIINFEKL specific CD8$^+$ T-cells in these mice were quantified to assess any differences in the population generated in response to vaccination with CASAC or CASAC + IRX-2.

No significant differences in the percentage or absolute numbers of SIINFEKL specific CD8$^+$ T-cells generated by vaccination with CASAC and CASAC + IRX-2 were found between the young and aged mice (Figure 5.9). The significant increase was *in vivo* lysis of antigen positive target cells that was seen by addition of IRX-2 was therefore not due to quantitative differences in SIINFEKL specific CD8 T-cell populations. This is reflective of the results obtained in section 4.3.5 and 4.3.7 where IRX-2 in combination with 100 fold diluted CASAC did not enhance the generation of SIINFEKL specific CD8$^+$ T-cells but did increased the *in vivo* cytolytic activity of CD8$^+$ T-cells generated by a suboptimal formulation of CASAC (100 fold dilution).

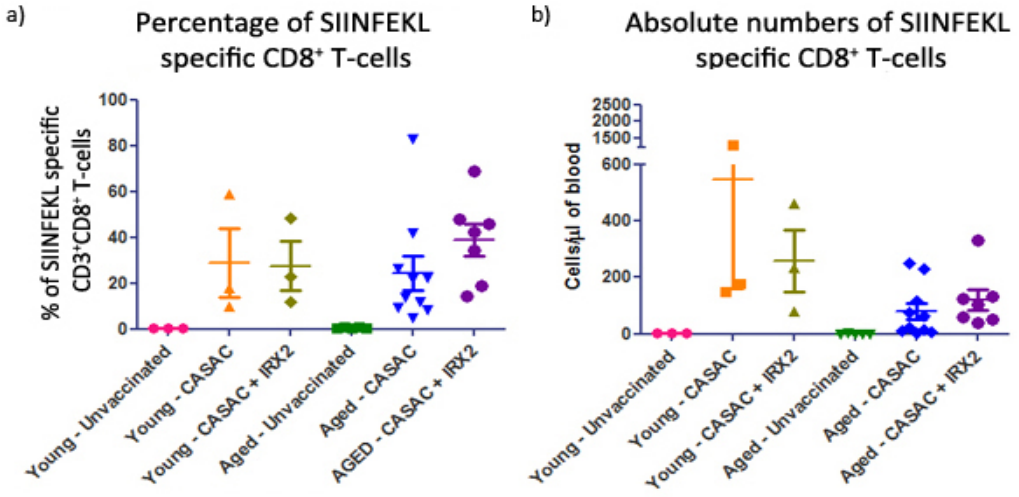


Figure 5.9 Quantification of SIINFEKL specific CD8 T-cells in mice where IRX-2 enhances cytolytic activity: After 2 rounds of vaccination, whole blood was collected on day 21. Percentage (a) and absolute numbers (b) of SIINFEKL specific CD8$^+$ T-cells were quantified. Mean ± SEM percentage and cell numbers shown. (n=3-10, Student T-test analysis)

5.5.3 Summary

CFA vaccination generated SIINFEKL specific $CD8^+$ T-cells in aged mice. These CD8 T-were less cytolytic in the aged compared to the young CFA vaccinated mice. This data suggests that aging of mice causes decline in the ability of the vaccination induced cytolytic activity of SIINFEKL specific $CD8^+$ T-cells. The cytolytic activity of CASAC stimulated SIINFEKL specific $CD8^+$ T-cells in the aged mice was higher than after CFA vaccination and similar to that generated in young mice. Therefore CASAC is a superior adjuvant for the induction of cellular immune responses in aged mice compared to CFA. When CASAC alone did not produce maximal cytolytic activity, addition of IRX-2 to the vaccination regimen generated SIINFEKL specific $CD8^+$ T-cells with greater cytolytic activity. However this enhancement was not due to an increase in the absolute number or percentage of the SIINFEKL specific $CD8^+$ T-cells. This led us to investigate the differences caused by CASAC and CASAC plus IRX-2 vaccination in the aged mice. We speculated that there might be differences in senescence and exhaustion markers by the SIINFEKL specific $CD8^+$ T-cells generated by the different vaccination strategies in the two age groups. Young and aged mice after vaccination were also assessed for differences in T-cell activation levels, Tregs percentage and type of CD4 T-helper cell activity induced by CASAC alone or CASAC plus IRX-2

5.6 Analyses of T-cell subsets in the aged and young mice following vaccination.

5.6.1 Analyses of senescence and exhaustion markers in SIINFEKL specific CD8 T-cells

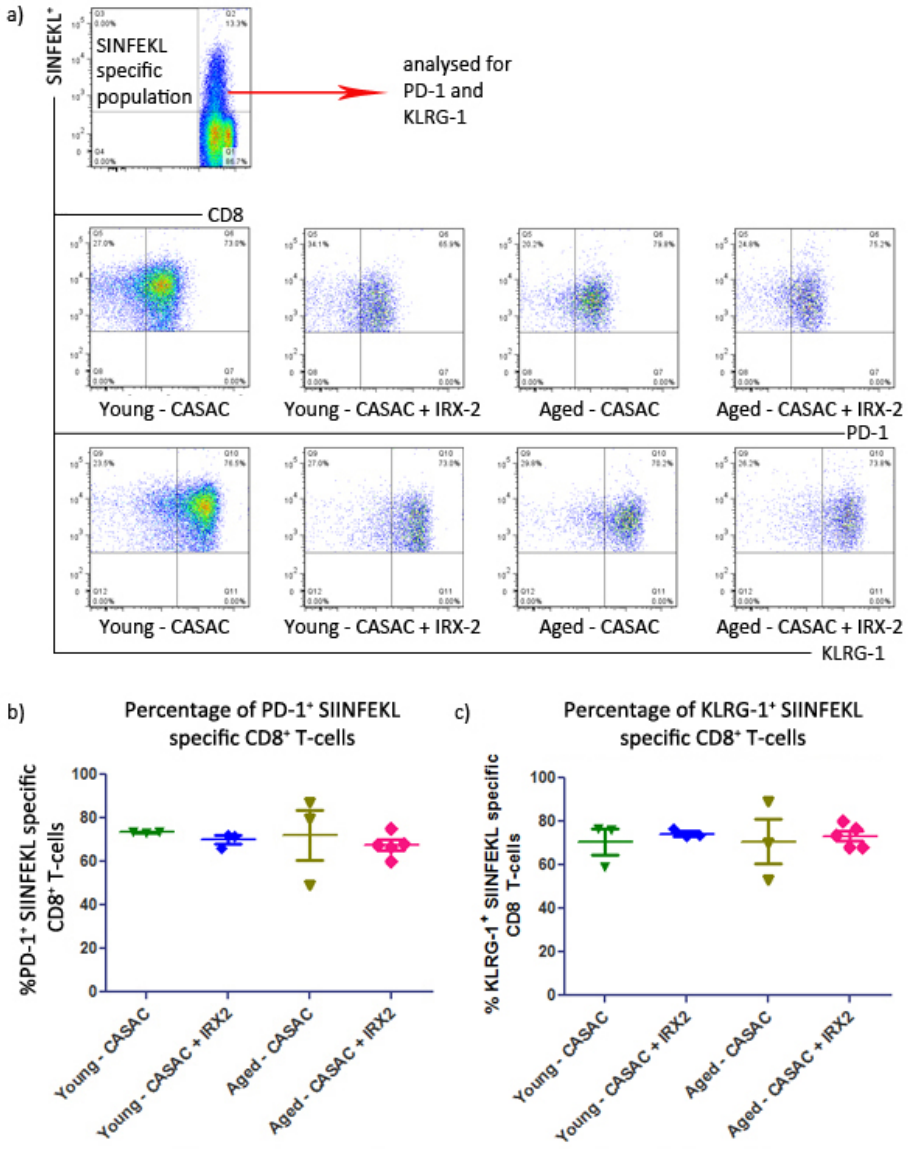


Figure 5.10 Analysis of subset of SIINFEKL specific CD8 T-cells: After 2 rounds of vaccination, SIINFEKL specific $CD8^+$ T-cells were analysed for senescence and exhaustion markers. a) Representative plots of SIINFEKL specific $CD8^+$ T-cells for KLRG-1 and PD-1 from each group. b) Percentage of $PD\text{-}1^+$ in SIINFEKL specific $CD8^+$ T-cells. c) Percentage of $KLRG\text{-}1^+$ in SIINFEKL specific $CD8^+$ T-cells. Mean ± SEM percentages shown. (n=3-5, Student T-test analysis showed no statistically significant data)

To determine if the SIINFEKL specific $CD8^+$ T-cells generated show differences in the expression of senescence and exhaustion markers, the percentages of KLRG-1^+ and PD-1^+ cells following CASAC vaccination with or without IRX-2 treatment was assessed. Analysis at day 10 after the second round of vaccination showed the majority of SIINFEKL specific $CD8^+$ T-cells expressed PD-1 and KLRG-1 in both young and aged mice and for all vaccine regimens. No significant differences were found. This result was similar to the results shown by Kao *et al.*, where an acute LCMV infection generated antigen specific CD8 T-cells expresses high percentage PD-1 and KLRG-1. After an infection has been cleared, KLRG-1 and PD-1 act to regulate the T-cell responses generated[111]. Therefore high expression of PD-1 and KLRG-1 in SIINFEKL specific $CD8^+$ T-cells does not necessarily indicate exhaustion or senescence because these studies were carried out 10 days after the second round of vaccination. If antigen specific CD8 T-cell responses had been tested after a resting period, more informative data might have been provided. Higher level of PD-1^+ and KLRG-1^+ SIINFEKL specific $CD8^+$ T-cells in the aged compared to the young mice would be anticipated[111].

5.6.2 Analysis of memory T-cells subsets generated in vaccinated mice

In chapter 4, effector memory SIINFEKL specific $CD8^+$ T-cells were shown to be predominantly generated with CASAC in the presence or absence of IRX-2. Effector memory $CD8^+$ T-cells can have potent cytolytic activity while central memory $CD8^+$ T-cells have the ability to renew and convert to effector memory T-cells[57, 129]. Percentage of effector and central memory CD8 T-cells in the SIINFEKL specific $CD8^+$ T-cell population in young and aged mice were analysed (Figure 5.11). No significant differences were seen between both CASAC and CASAC plus IRX-2 vaccination either in the young or the aged. The majority of SIINFEKL specific CD8 T-cells were of the effector memory phenotype. This data is similar to results in chapter 4, where effector memory T-cells were predominantly generated in the CASAC or CASAC plus IRX-2 vaccination.

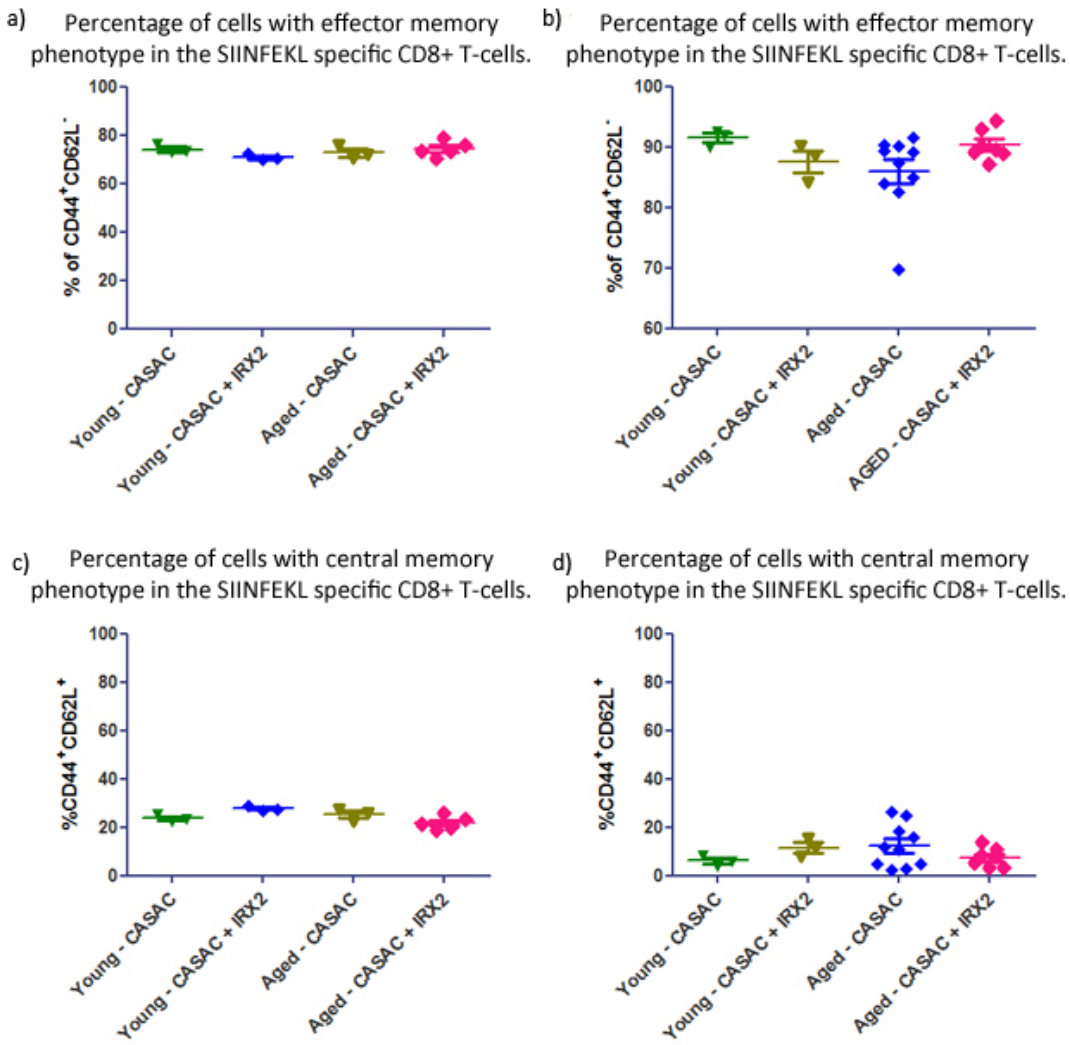


Figure 5.11 Analysis of memory subsets: Percentage of effector memory in SIINFEKL specific $CD8^+$ T-cells generated in a) cohort 1 and b) cohort 2 of the young and aged C57Bl/6 mice after 2 rounds of vaccinations. Percentage of central memory in SIINFEKL specific $CD8^+$ T-cells generated in c) cohort 1 and d) cohort 2 of the young and aged C57Bl/6 mice after 2 rounds of vaccination. Mean ± SEM of percentages are shown. (n=3-10, Student T-test analysis found no statistically significant)

5.6.3 Increased percentage of Tregs in aged mice correlated with lower $CD8^+$ T-cell in-vivo lytic activity induced by CASAC vaccination but was overcome by inclusion of IRX-2

In one of the two experiments, vaccination of aged mice using CASAC plus IRX-2 produced significantly increased *in vivo* lysis of antigen positive targets compared to CASAC alone (Figure 5.7). This effect was not due to qualitative differences in SIINFEKL specific $CD8^+$ T-cells generated by CASAC alone compared to CASAC with IRX-2 (Figure 5.9). The possible involvement of Tregs was therefore examined.

In the experiment where CASAC alone induced maximum lytic activity and hence no benefit from addition of IRX-2 was seen, no significant differences in percentage of Tregs present in unvaccinated young or aged mice and vaccination did not induce any significant changes (Figure 5.12 a). However, in the experiment where CASAC alone did not stimulate maximum SIINFEKL specific lysis in aged mice, the percentage of Tregs in the unvaccinated aged mice was significantly higher than the unvaccinated young mice (12.9 ± 0.8 % and 7.7 ± 0.3% respectively; p=0.0048) (Figure 5.12 b). The unvaccinated group is indicative of the percentages of Tregs present at the start of the vaccination regimen for the other groups. Higher frequencies in the aged mice at the beginning of this experiment may explain why maximum stimulation of lytic activity by CASAC vaccination could not be achieved. In this setting, inclusion of IRX-2 improved the killing potency significantly in the CASAC plus IRX-2 vaccinated aged mice.

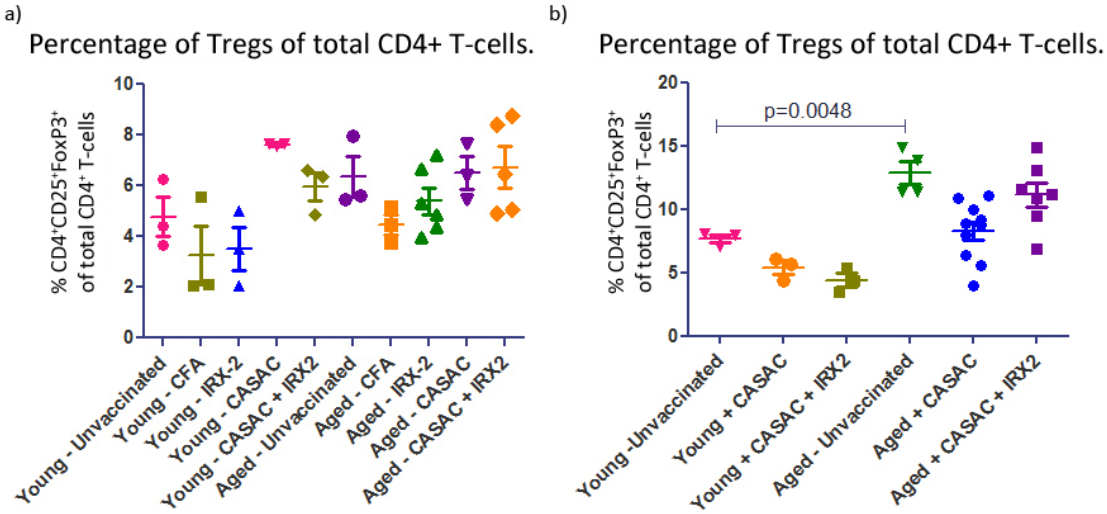


Figure 5.12 Percentages of Tregs after vaccination: Percentages of Tregs after the second vaccination in two independent experiments (a and b). Percentages of Tregs were analysed in the blood on day 21, after 2 rounds of vaccination. Mean ± SEM percentages shown. (n=3-7, Student T-test analysis conducted)

5.6.4 Percentages of co-stimulatory receptors on T-cells showed no significant changes 24 hours post vaccination

The percentages co-stimulatory receptor CD28 and the inhibitory receptor CTLA-4, 24 hours after each rounds of vaccination was investigated. A previous study by Krummel *et al.*, has established that CD28 is constitutively expressed on T-cells but CTLA-4 expression is induced on T-cells between 0 and 48 hours after stimulation of CD28 but down-regulated at 72 hours[148]. The data showed no significant differences either in the CD8 or CD4 T-cell expression of CD28 and CTLA-4 (Figure 5.13).

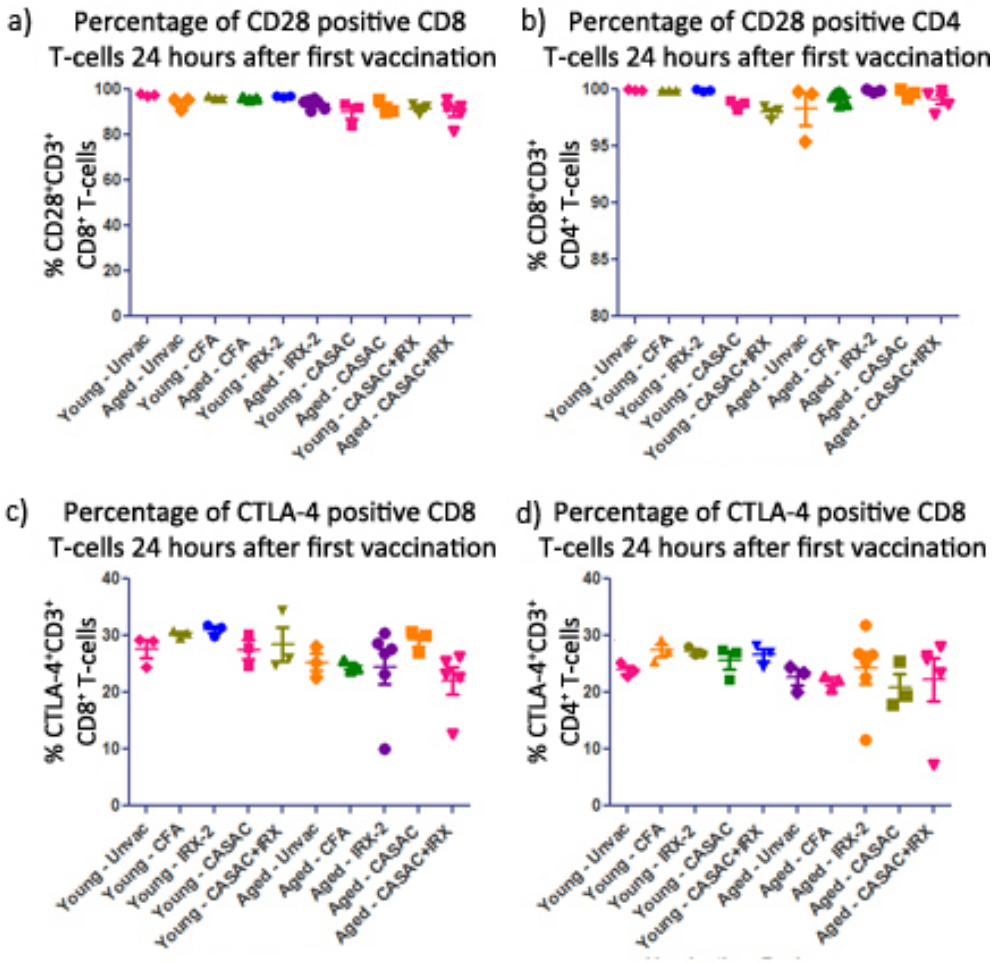


Figure 5.13 Analysis of CD28 and CTLA-4: Percentage of $CD28^+$ on CD8 T-cells (a) and CD4 T-cells (b). Percentage of CTLA-4^+ in CD8 T-cells (c) and CD4 T-cells (b) 24 hours after the second vaccination. 24 hours after the first round of vaccination were not shown as results were similar. Mean ± SEM percentages and geometric MFI shown. (n=3-5)

5.6.5 Predominant Th1 cytokine production by the CD4 T-cells after CASAC vaccination in both aged and young mice

In the CASAC vaccination regimen, the class II presented ISQ peptide was used to stimulate CD4 T-cells that provide help to CD8 T-cells. In a model suggested by Wherry, (2011), inclusion of T-helper peptides provides the necessary help in order to reduce exhaustion of $CD8^+$ T-cell responses[109]. Therefore, the type of help that was provided by the use of the T-helper peptide was investigated to further elucidate the nature of responses generated by CASAC vaccination and to see if addition of IRX-2 altered help for generation of improved SIINFEKL specific $CD8^+$ T-cell responses. Splenocytes harvested for the *in vivo* CTL assay performed on day 23 after 2 rounds of vaccination were used for evaluation of cytokine production by CD4 T-cells. Splenocytes were stimulated with PMA/Ionomycin (positive control), TEWT (negative control - an irrelevant T-helper peptide) or ISQ (T-helper peptide used for vaccination). $CD4^+$ T-cells were then analysed for TNF-α, IL-4, IFN-γ and IL-17. The gating strategy for the cytokines was as presented in Figure 5.14.

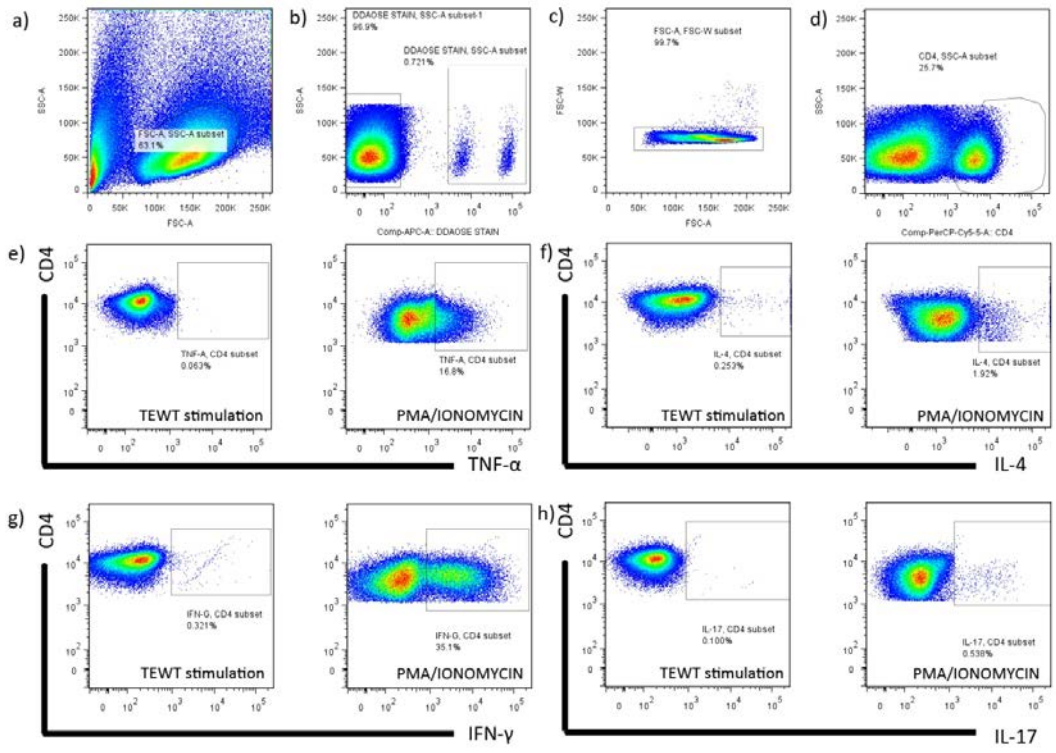


Figure 5.14 Gating strategy for cytokines analysis: a) Lymphocytes were gated and b) separated those cells which are non-DDAOSE (DDAOSE is a dye used for *in vivo* CTL assay) labelled. c) These cells were then gated for singlet cells before d) gating for CD4+ T-cells. CD4+ gated cells were then gated for e) TNF-α, f) IL-4, g) IFN-γ and h) IL-17 using irrelevant peptide stimulated cells to form the negative gate and confirmation of positively stained cells with PMA/Ionomycin stimulated cells.

Analysis of IFN-γ producing $CD4^+$ T-cells showed a significant increase in CASAC vaccinated mice both in the aged (1.2%) and the young (0.7%) cohorts compared to the unvaccinated aged (0.8%) and young mice (0.3%). CASAC plus IRX-2 vaccination did not induce responses above those seen with CASAC alone (Figure 5.15).

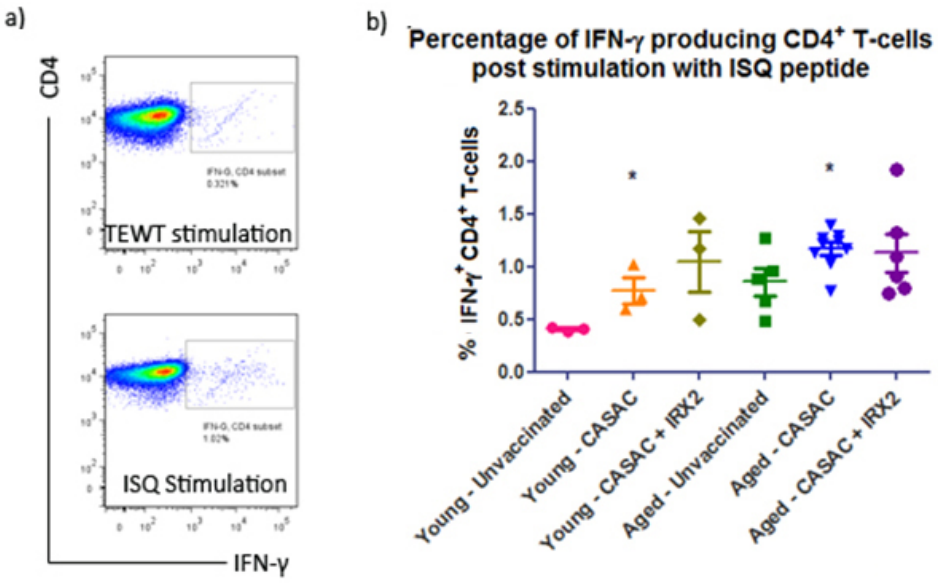


Figure 5.15 Analysis of IFN-γ producing CD4 T-cells: a) Dot plots representative of IFN-γ produced by TEWT stimulated splenocytes (irrelevant peptide) and ISQ stimulated splenocytes (helper peptide used in vaccination) b) Percentage of IFN-γ producing $CD4^+$ T-cells between different vaccination regimen after stimulating splenocytes for 4 hours with 25µg/ml of ISQ peptide. Mean ± SEM percentages shown (n=3-7, *p<0.05 compared to unvaccinated groups)

Analysis of Th2 cytokines showed no differences in the production of IL-4 between vaccinated and unvaccinated mice. When comparing the aged and the young mice, the background production of IL-4 (Figure 5.16) in the unvaccinated mice was found to be significantly increased in the aged population. Likewise, TNF-α producing $CD4^+$ T-cells (Figure 5.16) were shown to be significantly increased in the unvaccinated aged mice compared to young mice. There were also significantly high percentages TNF-α producing $CD4^+$ T-cells in the CASAC vaccinated young mice (0.4%) compared to unvaccinated mice (0.2%) ($p<0.001$). A similar trend was found in the aged population for CASAC (0.7%) and CASAC plus IRX-2 (0.61%)vaccination when compared to the unvaccinated mice (0.1%) ($p<0.05$).

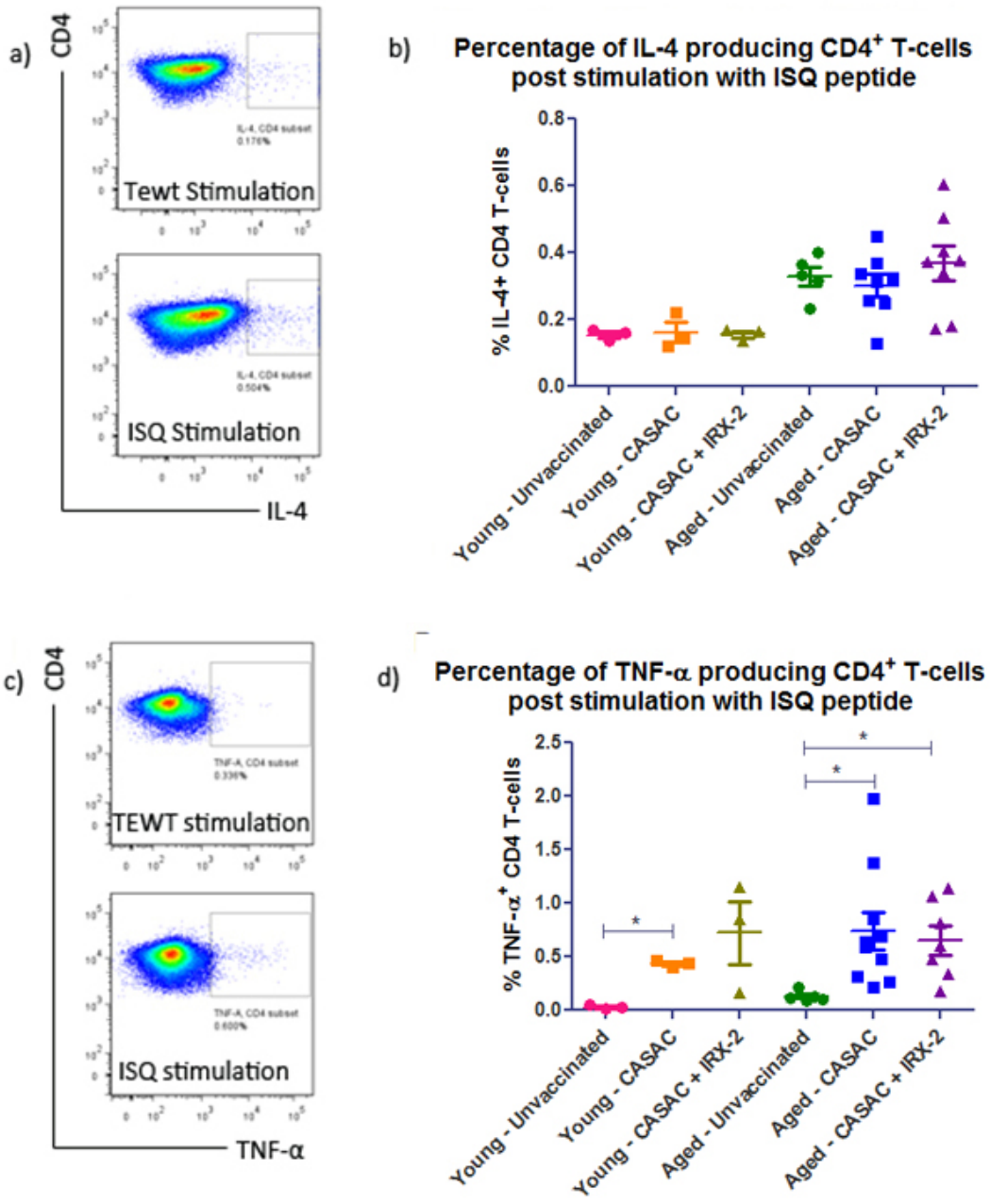


Figure 5.16 Analysis of IL-4 and TNF-α producing CD4 T-cells: a) Dot plots representative of IL-4 production by TEWT stimulated splenocytes (irrelevant peptide) and ISQ stimulated splenocytes (helper peptide used in vaccination). b) Percentage of IL-4 producing $CD4^+$ T-cells between different vaccination regimens post 4 hours stimulation with ISQ peptide. c) Dot plots representative of TNF-α produced by TEWT stimulated splenocytes (irrelevant peptide) and ISQ stimulated splenocytes (helper peptide used in vaccination). d) Percentage of TNF-α producing $CD4^+$ T-cells between different vaccination regimens after stimulating splenocytes for 4 hours with 25µg/ml of ISQ peptide. Mean ± SEM percentages shown. (n=3-7, *$p<0.05$, Student T-test analysis)

No production of IL-17 by CD4 T-cells from any vaccination group was above the background levels seen in unvaccinated mice. This shows that CASAC vaccination does not stimulate help via IL-17 (Figure 5.17).

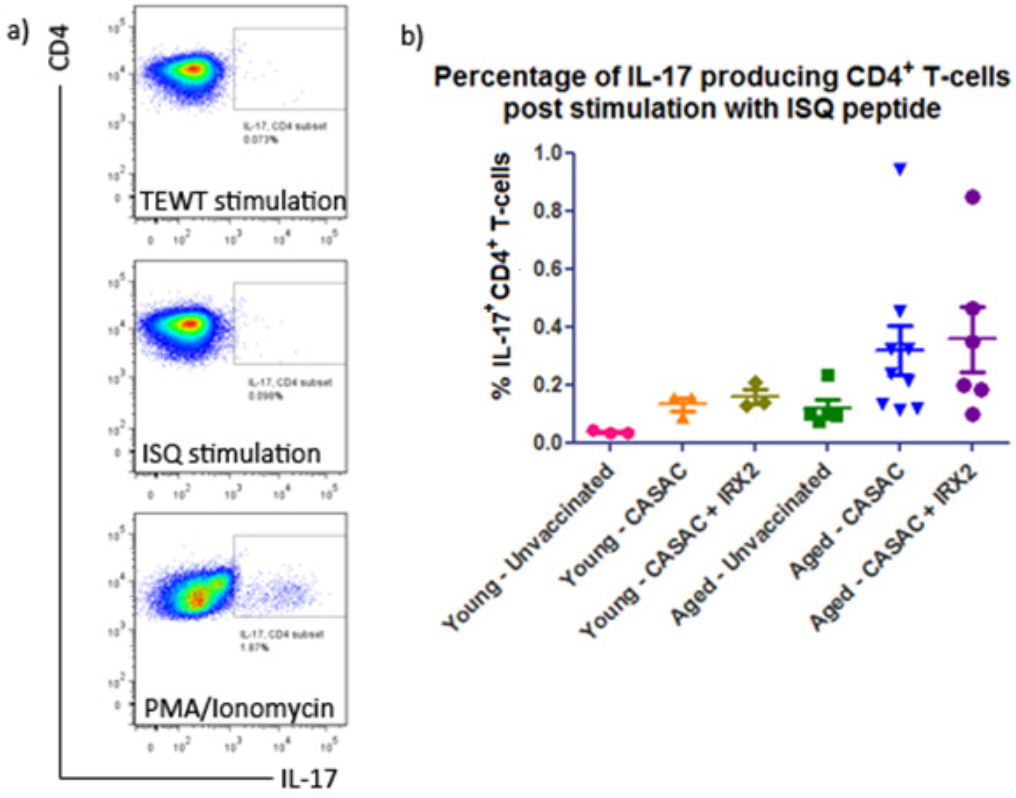


Figure 5.17 Analysis of IL-17 producing CD4 T-cells: a) Dot plots representative of IL-17 produced by TEWT stimulated splenocytes (irrelevant peptide), ISQ stimulated splenocytes (helper peptide used in vaccination) with PMA/Ionomycin stimulated splenocytes induced as positive control to show the CD4 T-cell population contains IL-17 producing cells. b) Percentage of IL-17 producing $CD4^+$ T-cells between different vaccination regimens after 4 hours stimulation with 25µg/ml ISQ peptide. Mean ± SEM percentages shown. (n=3-7, Student T-test analysis conducted showed no significant differences)

5.6.6 Summary

In one of the two experiments, vaccination of aged mice by CASAC plus IRX-2 was found to produce superior SIINFEKL specific $CD8^+$ T-cell lytic activity compared to CASAC alone. The enhancing effect of IRX-2 in the one experiment was found to correlate with higher percentage of Tregs in this group of aged mice at the start of the experiment. Comparison of expression of senescence and exhaustion markers and the central/effector memory composition of SIINFEKL specific $CD8^+$ T-cells generated by CASAC alone or CASAC plus IRX-2 in young or aged mice did not show any differences. Expression of co-stimulatory molecules by CD4 and CD8 T-cells after vaccination was similar for all regimens and in both young and aged mice. Analysis of the type of CD4 T-cell help induced by CASAC showed a predominant Th1 phenotype (IFN-γ and TNF-α) with no differences detected by addition of IRX-2 or between the young and aged mice.

5.7 Discussion

In this chapter, we investigated the potency of CASAC in the presence or absence of IRX-2 in generating CD8 T-cell responses in the aged mice.

a) Expression of senescence and exhaustion markers in T-cell subsets of young and aged mice.

In the data obtained, we demonstrated increased expression of senescence/exhaustion marker (KLRG-1, PD-1, PD-L1 and LAG-3) in the $CD8^+$ T-cells isolated from the aged C57Bl/6 mice (>18 months old). Although previous studies have indicated the presences of a higher percentage of CTLA-4 expressing T-cells in the aged mice[149], the data obtained in our study here showed no changes in the percentage of CTLA-4 or CD28 expressing cells. A major difference in the aged mice was reduced number of lymphocytes, almost one half of the numbers in young mice. Previous studies have demonstrated the reduced ability of aged mice to respond to a range of vaccination strategies[120, 143-145, 147]. This was clearly reflected in the CFA vaccinations in the present study where cytolytic activity of the SIINFEKL specific CD8 T-cells generated was much reduced in the aged population.

b) The ability of young and aged mice to generate functional antigen specific T-cell responses following vaccination with CASAC.

CASAC was much more efficient than CFA in the vaccination mediated stimulation of antigen specific cytolytic activity in the aged mice. Use of CASAC as an adjuvant for vaccination mediated generation of SIINFEKL specific $CD8^+$ T-cells improves the cytolytic activity of CD8 T-cells in the aged mice to a similar level to that achievable by CASAC vaccination in the young mice. However, when vaccination was conducted in aged mice with increased Tregs percentages, the *in vivo* cytolytic activity was reduced. In this setting, inclusion of IRX-2 enhanced the responses to CASAC vaccination resulting in increased cytolytic activity of the SIINFEKL specific $CD8^+$ T-cells generated. This could be attributed to the ability of IRX-2 protecting the stimulated T-cells from apoptosis as well as improving the production

of inflammatory cytokines which are crucial for generation of good CD8 T-cell responses[100, 101].

c) Changes in expression of exhaustion, senescence and activation markers by T-cells and changes in Treg percentages after vaccination.

Understanding of responses to CASAC and IRX-2 mediated peptide vaccination was improved by the analysis of expression of senescence and exhaustion markers by antigen specific T-cells, changes in Treg frequencies, expression of co-stimulatory receptors by T-cells and T helper subtypes. In the present study, we did not to see differences in senescence and exhaustion markers on the SIINFEKL specific CD8+ T-cells. This however, could possibly have been more informatively investigated if the aged mice were left to rest for a period of time after vaccination as shown by other groups examining responses to chronic LCMV infection[150]. There were also no differences seen in the co-stimulatory receptors, CD28 and CTLA-4. Although no significant differences were found between the vaccination regimen in terms of T helper cytokine production, we were able to elicit CASAC (in the presence or absence of IRX-2 promotes skewing towards Th1 as there were IFN-γ and TNF-α positive CD4 T-cells after splenocytes from vaccinated mice were stimulated with T-helper peptide, ISQ. Wells. *et al.*, (2008), showed similar findings where CASAC skewed a Th1 response[52].

5.8 Conclusion

In conclusion, the data presented here shows that CASAC is a very potent adjuvant for peptide vaccinations to generate antigen specific CD8 T-cells with cytolytic activity in both aged and young mice compared to CFA. In cases where a weaker immunological response is generated with CASAC alone, the combination of CASAC and IRX-2 can increase the potency of the CD8 T-cell responses.

5.9 Future studies

In the present studies, generation of antigen specific CD8 T-cells in aged mice was evaluated very close to the vaccination when the response is likely at its peak. It will be important to assess whether the potent T-cell responses generated by CASAC in aged mice are maintained. This would require re-challenge after a prolonged period of time after vaccination. Furthermore, the OVA peptide used in the present studies is a very immunogenic foreign antigen. It will be important to test efficacy of CASAC in vaccinations to self antigens such as WT-1 to see if tolerance to self antigens can be broken in aged mice. This could have major therapeutic implications for development of therapeutic vaccinations in the treatment of cancer which predominantly affects older people.

CHAPTER 6

Discussion

Peptide vaccination has the potential to play an important role in immune therapies especially in cancer and chronic infectious diseases. The use of specific peptide sequences from TAA found in cancer, and antigens derived from viruses, enables the immune system to be activated in a targeted manner, thus leading to generation of specific responses. These short sequences were also safer compared to full length antigen, as cross reactivity against off-target antigens could be reduced. However, the use of short sequences of peptides reduces the immunogenicity of the antigen. Additional help in the form of adjuvants and T-helper peptides to boost the targeted responses is required for improved efficacy of peptide vaccination.

The initial aim of this study was to evaluate and improve CASAC vaccination. In a previously published study by our colleagues (Wells *et al.*, 2008), CASAC has been shown to generate strong CD8 T-cell responses against both foreign (OVA) and self-antigens (e.g. TRP-2) after just 2 doses of vaccination. CASAC vaccination induced high percentages of antigen specific CD8 T-cells, produced high levels of IFN-γ producing CD8 T-cells and achieved greater than 90% *in vivo* lysis of antigen positive target cells. CASAC vaccination against TRP-2 also induced rejection of B16 tumours which were inoculated 3 days prior to vaccination[52].

Despite the significantly improved CD8 T-cell response in CASAC vaccinated mice, there were several other issues that need to be addressed prior to introducing it into the clinic. In the studies conducted, only 2 doses of vaccine were administered. However, peptide vaccination has often been shown to require more than 2 doses of vaccine. Solares *et al.* has shown that 4 rounds of HPV16 (E7 epitope) peptide vaccination was required to obtain clearance of human papillomavirus in 3 out of 7 patients[151]. In another study conducted by Schwartzentruber *et al.*, a complete cycle of 12 doses of gp100 peptide vaccination, in combination with IL-2, was administered to melanoma patients, for up to 3 cycles[152]. However, one of the implications of multiple rounds of vaccination is the activation of regulatory T-cells (Tregs). Tregs were previously shown to be induced with antigen persistence[68], causing a variety of suppressive reactions against the cytolytic T-cells by inducing the production of factors such as IL-10 and TGF-β, as well as increased levels of CTLA-4 expression[153]. Therefore, in this study we investigated the effect of vaccination regimens involving the use of alternating T-helper peptides. These studies have shown to suggest that the alternating T-helper peptide regimen was able

to generate significantly improved percentage and numbers of antigen specific CD8 T-cells with improved *in vivo* cytolytic activity. Ten rounds of vaccinations with the alternating T-helper peptides resulted in no expansion of Tregs. The level of Tregs at the end of this vaccination regimen was lower than the level in the standard vaccination with either one or two T-helper peptides administered repeatedly (Figure 3.18 and Figure 3.20 a). However, an additional experiment repeating the same vaccination protocol would be required to provide conclusive evidence of observations. Although an improved cytolytic activity was seen in our studies, further studies should be conducted in order to verify that the data obtained is reproducible. CASAC vaccination involving the use of T-helper peptides could benefit from these findings especially when vaccinating against tumour-associated antigens, which are in fact self-antigens. Other colleagues in the laboratory have found that CD8 T-cell responses mediated by CASAC vaccination against WT-1 can be improved with 4 doses of vaccine, compared to 2, as quantified by *in vivo* cytolytic activity (data yet to be published. Wilms Tumour 1 (WT1) is a TAA that is up-regulated in acute myeloid leukaemia[135] and a range of solid tumours, currently receiving attention for therapeutic vaccinations in leukaemia and solid cancers[21, 154, 155].

Cancer immunoediting is an important issue to consider when designing vaccination regimens. Patients in the clinic would have lived with the cancer for a long period of time before the development of symptoms. Tumours evolve in the face of a proficient immune system in a number of steps, including immune surveillance-mediated “elimination" through a phase of "equilibrium" in which immune surveillance succeeds in keeping the tumour growth in check, to "escape” from immune surveillance. In the first 2 phases, symptoms of the disease would have been unnoticed but upon reaching the "escape" phase, patients present with tumours that are poorly immunogenic, possibly immunoevasive[3, 156]. Schreiber *et al.* suggest that effective immunotherapies will require to increase both the quality and the quantity of immune effector cells and to break tumour induced immune tolerance. Therefore the use of CASAC which has already been shown to increase CD8 T-cell responses could be an effective strategy for therapeutic vaccination against cancer. In order to further enhance the efficacy of CASAC, we then studied the combination of CASAC and IRX-2.

IRX-2 is a combination of biological agents generated by PHA activation of human PBMC, under GMP conditions, by IRX Therapeutics[96]. Previous studies by Naylor *et al.*, have shown that IRX-2 is capable of inducing T-cell responses against target antigens by the administration of IRX-2 in combination with other adjuvants such as IFA or KLH conjugated peptides[98]. Pre-treatment of activated T-cells with IRX-2 was shown in another study to prevent apoptosis induced by tumour microvesicles. The activation of Fas/FasL pathway leads to apoptosis of the activated T-cells when tumour microvesicles (FasL +ve) were co-cultured with activated T-cells (Fas +ve) in the absence of IRX-2[100]. The protection conferred by IRX-2 has been shown to act directly by down-regulation of Fas in the activated T-cells[101]. This is crucial especially for vaccinations against cancer cells, which have evolved to escape immune surveillance as previously discussed. Melanoma and HNSCC cells have been shown to upregulate FasL[102] and IRX-2 is currently being studied to see if this "escape" mechanism could be overcome by IRX-2 therapy[96, 157, 158].

In terms of vaccination, we hypothesized that the inclusion of IRX-2 in CASAC vaccination could improve the CD8 T-cell responses. For instance on the activated T-cells Fas could be down-regulated, leading to longer lasting CD8 responses. Previous studies by Schilling *et al.* have shown that IRX-2 can promote the expansion of effector T-cells in favour of Tregs, thus leading to an improved potency of CD8 responses. In our studies, the combination of CASAC and IRX-2 vaccination in young C57Bl/6 did show a marginal improvement in the CD8 T-cell responses. The *in vivo* cytolytic activity generated was near maximal in the CASAC vaccination alone, thus masking an increase in the cytolytic potency. In order to further investigate the benefits of IRX-2, CD8 responses of a 100 fold diluted CASAC in the presence or absence of IRX-2 were compared. The data obtained suggests that despite a similar level of antigen specific CD8 T-cells, IRX-2 inclusion significantly increased the *in vivo* cytolytic activity of antigen specific CD8 T-cells.

Immune senescence and exhaustion play important roles in determining the efficacy of peptide vaccination. The aging process causes involution of the thymus, thus decreasing lymphocyte output[139], leading to a decline in T-cell repertoire, reduced responsiveness towards newly encountered antigen and impaired generation of memory T-cells[107]. Constant exposure to an antigen (TAA or antigen from chronic infectious diseases) causes exhaustion, loss of cytolytic activity, proliferation

potential and finally apoptosis of the antigen specific T cell clones[109]. These conditions mirror the immune status of most patients in the clinic who are suffering from cancer and chronic infectious diseases (e.g. chronic HBV or HIV infection). Even with respect to sporadic infections such as influenza, immunosenescence has been closely related to the increased infection associated deaths in the older individuals compared to young[159].

With tumour and age associated immune senescence in mind we were interested in investigating the effects of vaccination in aged (>18 months old) compared to young C57BL/6 mice (6-8 weeks old). Preliminary investigation showed that there were significantly increased percentages of KLRG-1 and PD-1 expressing T cells in the aged mice, indicating the increased presence of immune senescent and exhausted T cells in these compared to young mice. We also found reduced numbers of lymphocytes in the aged mice compared to young mice. This too has been shown to be the case by other investigators[139]. We then investigated the efficacy of OVA vaccination with the CFA adjuvant. The results indicated poorer *in vivo* cytolytic activity in the aged mice compared to the young mice. Vaccination against OVA with CASAC ± IRX-2 generated high levels of *in vivo* cytolytic activity (>90% lysis of antigen positive cells) in both the young and the aged mice, suggesting that CASAC is a superior adjuvant compared to CFA for these applications (Figure 5.6).

However, in another batch of aged mice, CASAC vaccination did not produce the previously detected maximum *in vivo* cytolytic activity. The inclusion of IRX-2 in this second CASAC vaccination of the aged mice produced a significant increase in the *in vivo* cytolytic activity, suggesting an enhancement by IRX-2 (Figure 5.7). Upon investigation, we found that the percentage of Tregs in this batch of aged mice was significantly increased compared to the young mice (Figure 5.12 b). In the batch of aged mice that responded equally well to CASAC and CASAC plus IRX-2, the percentage of Tregs in the aged mice were not significantly different from the percentages found in the young mice (Figure 5.12 a). Therefore, we conclude that the increase in Tregs reduces the efficiency of *in vivo* cytolytic activity mediated by CASAC vaccination. This however is rescued by the inclusion of IRX-2 in the vaccination. IRX-2 has the potential to enhance *in vivo* cytolytic responses in peptide vaccination without significantly changing the number or ratio of antigen specific T-

cells. This suggests that IRX-2 has the potential of enhancing the cytolytic activity of CD8 T-cells generated in response to vaccination.

CHAPTER 7

Summary and Future Studies

In summary the data presented in this thesis has shown that peptide vaccination could be enhanced by use of alternating T-helper peptides in repeated rounds of vaccination, in order to avoid vaccination induced expansion of regulatory T cells. This however would require a repeat of the experiment with similar conditions. Groups of unvaccinated, SIINFEKL only, SIINFEKL plus ISQ, SIINFEKL plus ISQ plus TEWT and SIINFEKL plus alternating T-helper peptide vaccination should be included in the vaccination. Based on the data obtained in previous experiments for the *in vivo* CTL lysis assay (this assay showed the clearest outcome to the efficacy of alternating T-helper peptide vaccination), a power calculation to obtain sample size was conducted using the mean value and standard deviation that has been recalculated for the alternating T-helper peptide vaccination group (83 ± 8%). Power and Sample size calculator (http://www.statisticalsolutions.net/pss_calc.php), a free sample size calculator generated a required sample size of 8 mice per group (with a power of 0.8 and a $p<0.05$). Previous studies and our results show that CASAC vaccination generates strong CD8 T-cell responses. However, in conditions where CASAC was unable to induce maximal responses, the inclusion of IRX-2 could enhance these effects.

Based on the studies conducted, one could suggest additional studies to enable better understanding of vaccination induced responses to chronically experienced antigens. Such studies would include:

1. Breaking of tolerance caused by cancer and chronic infectious diseases: Cancer cells have been shown to be able to induce tumour tolerance by what Schreiber and others have called “cancer immunoediting”. It would be interesting to investigate the possibility of vaccinating against self-antigens such as WT-1. Such studies would seek to determine whether inclusion of IRX-2 in CASAC based vaccination can promote easier breakdown of tolerance to tumour-associated self-antigens. Vaccinating against antigens from infectious agents such as hepatitis B virus could also be conducted, for instance in transgenic animals that constitutively express viral antigens, or possibly even are chronic carriers of the infection. Such studies could have great implications for peptide vaccination. Based on the CD8 T-cell responses generated in the present study, it would be expected that CASAC, either in the presence or absence of IRX-2, would be able to break tolerance to such chronically experienced antigens. Vaccination against tumour stem cells such as leukemic stem

cells would also be an area very much worth pursuing. This is because tumour stem cells are much more resistant to traditional therapies, such as chemotherapy, and thus immune therapies like vaccination could possibly play an important role in tumour clearance[160, 161].

2. Vaccination against models of spontaneous disease formation: In experimental models of tumour or infections in mice, there is usually insufficient interactions with the immune system to allow an adequate representation of the conditions prevailing in the normal pathogenesis of cancer or the establishment of chronic infections[162]. However, the use of animal models of spontaneous disease formation, for instance aflatoxin induced development of hepatocellular carcinoma in a hepatitis B virus transgenic mouse model (HBV-Tg)[163] would allow for the disease to progress along a more natural course of development, including prolonged interactions between the tumour and the immune system, prior to vaccination.

These “more natural” models of disease development and progression, allowing the establishment of chronic interactions between the immune system and the disease may provide better representation of the normal pathogenesis of the disease. These models are therefore likely to be better informative of the potential clinical efficacy of vaccination mediated immune therapy of cancer and infectious disease.

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