UNIVERSITY OF SOUTHAMPTON

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School of Medicine

Developing DNA fusion gene vaccines: from pre-clinical models to clinical studies

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ABSTRACT

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DEVELOPING DNA FUSION GENE VACCINES: FROM PRE-CLINICAL MODELS TO CLINICAL STUDIES

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DNA vaccination is emerging as an effective way of generating specific immunity against infectious disease both in mice and humans. By in vivo transfection this approach enables access of antigen to many different processing pathways. Latterly DNA vaccination has been used to induce specific immunity against cancer. Therapeutic vaccination against cancer has several obstacles including the weak immunogenicity of tumour antigens and a potentially tolerized immune system in patients. For the induction of antibody and T cell effector responses, CD4+ T helper responses are critical. Therefore to activate immunity against infectious agents and tumor antigens, we have fused the tumor-derived sequences to genes encoding microbial proteins. We previously showed that a DNA vaccine encoding a tumour antigen linked to Fragment C (FrC) of tetanus toxin induced protective immunity in vaccinated mice, whereas a DNA vaccine lacking the FrC sequence did not.

Many intracellular tumour antigens are expressed as peptides in the groove of the MHC class I molecule. The induction of peptide-specific CD8+ T-cells is therefore important in targeting these antigens. Several changes were made to the design of the vaccine in order to activate CD8+ T cell responses. Firstly the C-terminal domain of FrC was removed and secondly the minimized tumour epitope was placed at the C-terminus of the remaining domain of FrC (DOM). In order to assess whether this design was applicable to a non-viral tumour antigen we chose an H-2 Kb epitope from CEA, referred to as EAQN. The sequence encoding this octamer was fused to the C-terminus of domain 1 of FrC (pDOM.EAQN). This vaccine activated high levels of epitope-specific CTLs whereas vaccines against the whole CEA gene and the CEA gene fused to FrC were unable to induce CTL against EAQN. However the CTL specific for EAQN were unable to lyse tumour cells expressing CEA in vitro suggesting that the tumour cells failed to present the peptide. Several epitopes have been described from CEA that are restricted by the human class I molecule HLA-A2. We placed each of these in turn into our vaccine. All of the vaccines were able to elicit high levels of CD8+ T cells specific for their respective epitopes. A DNA vaccine encoding a recently discovered epitope generated a CTL capable of lysing tumour cells expressing CEA.

In order to move this novel vaccine design towards a clinical trial, an established epitope from a matrix protein (pp65) of cytomegalovirus was studied. The sequence encoding this nonamer was fused to the C-terminus of the first domain of FrC (pDOM.NLV). This vaccine was able to generate high levels of CD8+ T cells specific for this epitope. These CTL were able to lyse cells that were infected with a modified vaccinia Ankara expressing the matrix protein- pp65. We also tested the vaccine format in human cells. We transfected dendritic cells with RNA transcribed from the vaccine. These dendritic cells were potent stimulators of autologous CD8+ T cells and led to 50-fold expansion over 7 days in vitro. A prime-boost strategy was also developed in this model that combined our DNA vaccine with long peptide vaccination.

Finally we studied immune responses in patients who had received this DNA vaccine in the context of allogeneic stem cell transplantation. We monitored the immune responses of donor-recipient pairs after the donor had been vaccinated with pDOM.NLV. Both cellular responses to the FrC component and the CMV epitope have been studied. Increased cellular responses to FrC were detectable post-vaccination. CD8+ T cell specific for the vaccine epitope show a very large expansion post-tranplantation in one of the recipients. However this may be influenced by viral reactivation or by the DNA vaccine.

These data show that relevant pre-clinical models are critical in optimizing the design and application of DNA vaccines. These vaccines can then be taken forward to assess their ability to induce specific anti-viral or anti-tumour immunity in patients.

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Abbreviations

AA	Aplastic anaemia
AEP	Asparaginyl endopeptidase
Ag	Antigen
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
APC	Allophycocyanin
β2-m	β2-microglobulin
BCL	B cell lymphoma
BM	Bone Marrow
BTLA	B and T lymphocyte attenuator
C-terminus	Carboxyl-terminus
CAR	Cancer-associated retinopathy
CCL	Chemokine
CCR	Chemotactic chemokine receptor
CD	Cluster differentiation antigen
CEA	Carcinoembryonic antigen
CLIP	Class-II-associated invariant chain peptide
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CpG	Bacterial unmethylated dinucleotides motifs
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic Cell
DC-SIGN	Dendritic cell specific intracellular adhesion molecule-grabbing nonintegrin
DNA	Deoxyribonucleic acid
DOM	N terminal domain of FrC ($TT_{865-1120}$)
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein Barr Virus
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunospot
ER	Endoplasmic reticulum
ERAAP	Endoplasmic reticulum aminopeptidase associated with antigen processing
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FrC	Fragment C (of tetanus toxin:$TT_{865-1316}$)
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR
GM-CSF	Granulocyte-monocyte colony stimulating factor
GVHD	Graft versus host disease

GVL	Graft versus leukaemia
HEV	High endothelial venule
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HVEM	Herpesvirus entry mediator
ICAM	Intercellular adhesion molecules
ICOS	Inducible T-cell costimulator
Id	Clonal idiotypic protein of B cell malignancies
IDO	Indoleamine 2,3-dioxygenase
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
i.m.	Intra-muscular
IFN	Interferon
ISS	Immuno-stimulatory sequence
i.v.	Intravenous
IVTT	In-vitro transcription and translation
KIR	Killer inhibitory receptor
KLH	Keyhole limpet haemocyanin
LAMP	Lysosome-associated membrane protein
LB	Luria broth
LCMV	Lymphocytic choriomeningitis virus
LFA	Leukocyte functional antigen
LIGHT	Ligand for HVEM
LMP	Low molecular weight protein
LN	Lymph node
LPS	Lipopolysaccaride
miH	Minor histocompatibility antigens
MAC	Membrane attack complex
MAGE	Melanoma antigen-encoding gene
MART	Melanoma antigen recognized by T cells
MCA	Methylcholanthrene
MCM	Monocyte conditioning medium
MDC	Myeloid dendritic cell
MHC	Major Histocompatibility Complex
MIIC	MHC class II compartment
MP	Matrix peptide from influenza
MUC	Mucin glycoprotein
MVA	Modified vaccinia Ankara
NCA	Non-specific cross-reacting antigen
NCR	Natural cytotoxicity receptors
NHL	Non-Hodgkin's Lymphoma

NK	Natural killer cell
N-terminus	Amino-terminus
ODN	Oligonucleotide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCD	Paraneoplastic cerebellar degeneration
PCR	Polymerase chain reaction
PDC	Plasmacytoid dendritic cell
PD-1	Programmed death-1
PE	Phycoerythrin
PLC	Peptide loading complex
pMHC	Peptide-MHC complex
PRR	Pattern recognition receptor
PVR	Poliovirus receptor
RAG	Recombinase activating gene
RNA	Ribonucleic acid
s.c.	Sub-cutaneously
SCID	Severe combined immunodeficiency
SCT	Stem cell transplant
scFv	Single chain Fragment variable
TAA	Tumour-associated antigen
TAP	Transporter associated with antigen presentation
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Tg	Transgenic
T_H	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAIL	Tumour-necrosis factor-related apoptosis-inducing ligand
TRANCE	Tumour necrosis factor-related activation-induced cytokine
Treg	Regulatory T cell
TSA	Tumour-specific antigen
TT	Tetanus toxin
TWEAK	TNF-like weak inducer of apoptosis

1. Introduction

Over the last 20 years evidence has emerged that the immune system has a role in cancer therapy. The first clear indication that immunological manipulation at the molecular level could cause the regression of established human cancers came from the studies of the administration of interleukin-2 (IL-2). This cytokine was administered to patients with metastatic renal cell carcinoma, melanoma and non-Hodgkin's lymphoma and led to the regression of bulky invasive tumours.[1] These initial studies showed that 15-20% of these patients had demonstrable cancer regression. Subsequent studies have confirmed that approximately 10% of patients with these cancers treated in this way achieve a complete response.

Further evidence of the importance of the immune system in the treatment of cancer came from the field of bone marrow transplantation. Sibling bone marrow transplantation has been used in the treatment of various haematological malignancies for many years. Horowitz et al. analysed the clinical results of different sub-groups of patients undergoing this procedure.[2] They found that patients who received non-T-cell depleted transplants and also suffered with graft versus host disease (GVHD) showed decreased cancer relapse when compared with patients who received non-T-cell depleted transplants without GVHD. These data supported an anti-leukaemic effect of GVHD. In addition CML patients who received grafts that were T-cell depleted had higher relapse rates than those who received grafts that were non-T-cell depleted. This suggests that the mediators of this anti-leukaemic effect were the T cells.

A third major advance in the treatment of cancer by the manipulation of the immune system is in the area of monoclonal antibodies. Rituximab the monoclonal antibody against CD20 has been shown to increase patients' survival in patients with Non-Hodgkin's Lymphoma. [3]

The success of these three strategies is encouraging. However these "immunotherapies" are applicable in a limited sub-set of cancer patients and, although the improvements in survival are demonstrable, only a minority of patients benefit from these interventions.

There are many attractions to using active vaccination in the management of both infection and cancer. Active vaccination induces a variety of immune effector pathways and a memory response capable of continually monitoring infection or cancer cell growth. For infection active vaccination can prevent infection and attack resident organisms. In the case of cancer the challenge is to target residual tumour cells after conventional treatment. Whilst the strategy of antibody therapy has been expanding, this therapy is applicable to antigens that are expressed on the tumour surface. Many antigens that are associated with tumours are expressed in the intracellular compartment. Fragments of these proteins will be presented in the context of the Major Histocompatibility Complex molecules (MHC), both class I and class II. The development of a vaccine design that effectively induces CD8+ T cells against intracellular antigens is a central aim. Any vaccine aiming to induce CD8+ T cell immunity will need to activate several different arms of the innate and adaptive immune response. These requirements for the generation of specific CD8+ T cell immunity and a vaccine strategy targeting the activation of these different pathways are discussed below.

1.1. The innate immune response

The innate immune system has evolved to play a central role in the defence against microbial infection and disease. One of the first major mechanisms by which an infection is defended against is the complement system. This is a system of plasma proteins that can be activated directly by the pathogen or indirectly by antibody bound to the pathogen. They then activate a cascade of enzyme reactions that ultimately lead to the recruitment of phagocytes or lead to the assembly of a membrane-attack complex (MAC). Insertion of this lytic pore into the target cell wall can lead to destruction of the pathogen. This cascade can be activated by several different pathogen-derived molecules including lipopolysaccaride (LPS) as well as antibodies that have been secreted by the immune system.

The innate immune response is also mediated through several different cell types such as dendritic cells (DCs), macrophages, monocytes and neutrophils. These cells are able to detect conserved molecular patterns that are essential components of microbial structure or physiology. These molecular patterns are known as pathogen-associated molecular patterns (PAMPS). In reality whilst PAMPs are unique to microbes the organisms containing them are not always pathogenic.[4]

The two most celebrated PAMPs are lipopolysaccharide (LPS) from gram-negative bacteria and peptidoglycan from gram-positive bacteria. PAMPs are recognised by receptors of the innate immune system called pattern recognition receptors (PRRs). It is now clear that Toll-like receptors are key PRRs in the innate recognition of PAMPs and the detection of infection.[4, 5] This family of proteins have extracellular domains that contain leucine-rich repeats and a cytoplasmic Toll/IL-1 receptor (TIR) domain closely resembling that of the interleukin-1 receptor family. Ten of these TLRs have now been identified.

The first TLR that was discovered to be essential for PAMP recognition was TLR-4. This is able to detect the presence of LPS in the host. Lipopolysaccaride is a component of the outer membrane of Gram-negative bacteria. When this is released into the bloodstream it is captured immediately by LPS-binding protein that delivers LPS to CD14 on the surface of mononuclear phagocytes. There are several pieces of indirect evidence that TLR-4 is essential for LPS signalling. Most compellingly, TLR-4 knockout mice have defective LPS-signalling.[6]

TLR-2 recognises several different microbial components including peptidolglycan from Gram-positive bacteria, lipoproteins and lipopeptides from several bacteria, glycophosphatidylinositol anchors from several protozoa and the yeast cell-wall component zymosan. These ligands for TLR-2 are recognised by heterodimers formed between TLR-2 and other TLRs.[7,8] Both TLR-6 and TLR-1 have been implicated in this way.

Flagellin is a monomer obtained from bacterial flagellae. It has been reported that mammalian TLR-5 recognizes bacterial flagellin from both Gram-positive and Gram-negative bacteria, and that activation of the receptor mobilizes the nuclear factor NF-κB and stimulates tumour

necrosis factor-alpha (TNFα) production. In addition deletion of the flagellin genes from Salmonella typhimurium abrogated TLR-5-stimulating activity. [9]

Double-stranded RNA (dsRNA) is a molecular pattern associated with viral infection, because it is produced by most viruses at some point during their replication. It has been shown that mammalian TLR-3 recognizes dsRNA, and that activation of the receptor induces the activation of NF-kappaB. TLR-3-deficient ($TLR\text{-}3^{-/-}$) mice have also showed reduced responses to dsRNA in addition to the viral RNA mimic polyinosine-polycytidylic acid (poly(I:C)).[10] In addition ssRNA has recently been found to be recognized via TLR-7 and 8.[11]

Bacterial DNA has been known to be pro-inflammatory for many centuries. Indeed bacterial crude extracts were used to treat patients with advanced malignancy. Studies to determine the active component within these bacterial extracts revealed that it was the DNA itself.[12] It was then demonstrated in several cell types that within the DNA the immune stimulatory effects resulted from CpG dinucleotides. This effect was related to these hypomethylated dinucleotide motifs in particular contexts.

The cellular response to bacterial DNA is mediated by a Toll-like receptor, TLR-9. TLR-9-deficient ($TLR\text{-}9^{-/-}$) mice did not show any response to bacterial DNA, including proliferation of splenocytes, inflammatory cytokine production from macrophages and maturation of dendritic cells. Also $TLR9^{-/-}$ mice showed resistance to the lethal effect of bacterial DNA without any elevation of serum pro-inflammatory cytokine levels.[13] TLR-9 will be discussed in greater depth in section 1.6.1.

TLRs signal through the adapter molecule MyD88 which recruits other signalling molecules in a pathway that activates NF-κB and the mitogen-activated protein kinases. These induce the transcription of genes encoding inflammatory mediators such as TNFα, IL-1 and IL-6.[14] Stimulation of some TLRs can trigger MyD88-independent signalling pathways.

There is also recent evidence that different TLRs co-operate in DC activation. In human and mouse DCs TLR-3 and TLR-4 can act in synergy with TLR-7, TLR-8 and TLR-9. This

enhanced response increased the production of interleukins 12 and 23 and led to an increase T helper type-1 polarising capacity.[15]

1.1.1. Natural killer cells

Natural killer (NK) cells are another key component of the innate immune system. They were originally defined on a functional basis as they were able to lyse tumour cells without previous stimulation. They are fundamental in defences against certain viruses and are involved in the control of tumour growth in experimental models. Upon activation these cells are able to release chemokines and cytokines that modulate both the innate immune response and the adaptive immune response. They are also able to directly lyse infected cells.

Natural killer cells are able to recognize major histocompatibility complex (MHC) class I molecules via inhibitory receptors that are able to suppress NK function. Tumour cells and virally infected cells that have had these molecules down-regulated are therefore susceptible to lysis.[16] These receptors are termed killer immunoglobulin-like receptors (KIRs). They are able to recognize different allelic groups of HLA-A, HLA-B or HLA-C molecules. Another NK receptor involved in HLA recognition is NKG2A. Each type of KIR is only expressed by a subset of NK cells. The ability of NK cells to kill MHC class I-deficient cells is key as these are cells that cannot be recognized by cytotoxic T cells.

Natural killer cells also express receptors that are able to activate NK-mediated cytotoxic responses. These are collectively known as natural cytotoxicity receptors (NCRs). They include receptors such as NKp46, NKp30 and NKp44. The ligands for these NCRs have still not been identified. However another activating receptor NKG2D has been shown to bind multiple target-cell ligands. These include the major histocompatibility complex class I-related chain A (MICA) and MICB in humans.[17] The equivalent ligands in mice are Rae-1 and H60.[18]

It has also been demonstrated that there is a reciprocal activating interaction between natural killer cells and dendritic cells. The activating influence of NK cells on DCs were cell contact dependent. In addition the secretion of IFN-gamma and TNF also contributed to DC maturation. NK cells were also activated by contact with lipopolysaccharide (LPS)-treated mature DC as indicated by the induced expression of the CD69 antigen, and their cytolytic activity was strongly augmented.[19] This and other data indicate that NK cells have an important role in the development of adaptive immune responses.

1.1.2. Dendritic cells

Dendritic cells (DC) are produced in the bone marrow and are specialised for the uptake, transport, processing and presentation of antigens to T cells.[20] Several differentiation pathways have been described. There are generally three accepted stages of differentiation of DC sub-types: DC precursors, immature DCs and mature DCs.[21] In human blood immature DCs and their precursors are HLA-DR+ mononuclear cells that are traditionally divided into 2 populations. $CD11c^{+}CD123^{lo}$ DCs have a monocytoid appearance and are called "myeloid DCs" (MDCs) whereas CD11c-$CD123^{hi}$ DCs are termed plasmacytoid DCs (PDCs) as their morphological features are similar to plasma cells. Experiments indicate that both DC populations can be derived from flt3-expressing myeloid and lymphoid progenitors.[22] PDCs are involved in innate antiviral immunity and are found in the blood and lymphoid organs and are the principle interferon-α producing cells in the body. MDCs are found in many tissues and are classified into two main subtypes Langerhans cells (which express C-type lectin Langerin) and the interstitial DCs.

MDCs are found within most surface epithelia and solid organs. In these sites they tend to have an "immature phenotype". Immature DCs are very effective at taking up antigen by

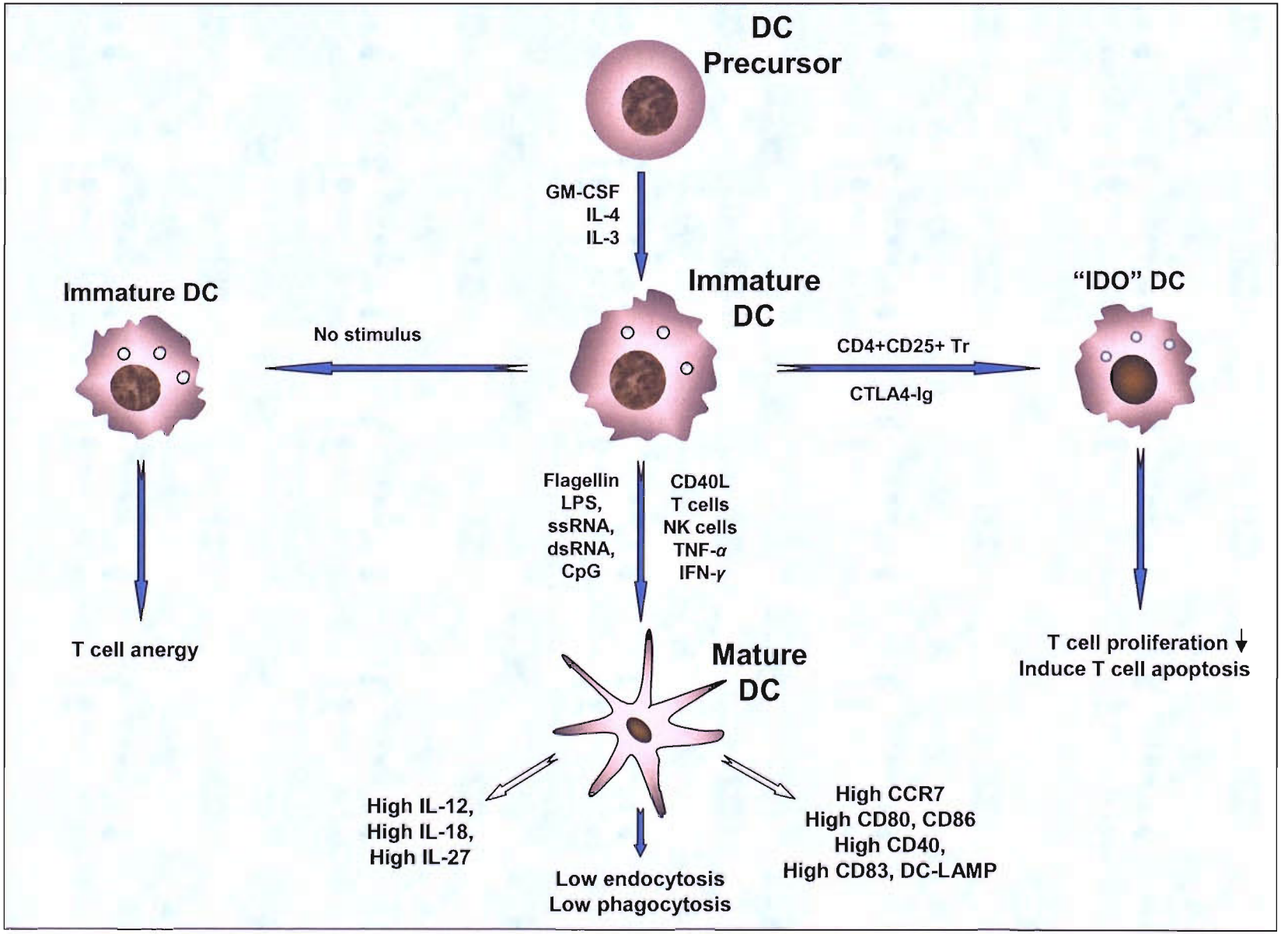


Figure 1. Dendritic cell differentiation and maturation.
Adapted from [20]

several different mechanisms. They are able to capture bacteria, viruses, dead or dying cells through phagocytosis, endocytosis and pinocytosis and have an array of cell surface receptors for antigen uptake.[23] Receptor-mediated uptake of antigen can increase the efficiency of endocytosis by up to 100-fold. Different classes of receptor include the C-type lectin-like receptors DC-SIGN (that includes amongst its ligands several different viruses and also mycobacteria), DEC-205 and the mannose receptor.[24] Other antigen uptake receptors are the FcγRI and FcγRII receptors that are able to bind immune complexes and opsonised cells.[25] In addition integrins can bind apoptotic cells and opsonised antigens. Scavenger receptors can bind and internalise micro-organisms including Gram-positive bacteria (lipoteichoic acid), Gram-negative bacteria (lipopolysaccharide), intracellular bacteria and CpG DNA. [26]

Maturation is a differentiation process whereby DCs are transformed from cells specialized for antigen capture into cells specialized for T cell stimulation. It is characterised by migration to the lymphoid tissues, the development of cytoplasmic extensions and enhanced T-cell activation potential. Mature DCs express high levels of MHC class II, adhesion molecules and high levels of the co-stimulatory molecules CD80 and CD86. They also express several characteristic markers such as CD83 and DC-LAMP. There are a very large number of maturation signals that trigger intracellular signalling via DC receptors. These include all of the TLR agonists that have been described above. MDCs express TLR-1-5 and variably TLR-7 and 8. PDCs express TLR-1, 7 and 9.[27,28,29] Other maturation stimuli include the cytokines IL-1, IL-6, IFNα and the TNF family molecules TNFα, CD40L, FasL and tumour necrosis factor-related activation-induced cytokine (TRANCE) . In addition several cell types can induce this maturation including NK cell, CD8 and CD4 cells.

Maturation results in the increased expression of adhesion and co-stimulatory molecules involved in the formation of the immunological synapse. It also induces the DCs to secrete cytokines that are critical in determining the nature of the immune response. Another important effect of maturation is the induced secretion of chemokines that recruit monocytes and specific subsets of T cells into the local environment.

Finally, maturation also enables DCs to up-regulate an array of chemokine receptors including chemotactic cytokine receptor 7 (CCR7). Ligation of CCR7 enables the recruitment of DCs to the lymph nodes.[30] The ligands of CCR7, CCL19 and CCL21 are secreted by lymphatic endothelium and mature DCs already residing in the T cell areas of lymph nodes to create a chemotactic gradient to which mature DCs migrate.[30]

In this way mature DCs migrate to the lymph nodes where they can present the antigens that have been captured in the peripheral tissues to T cells. There are 3 principal pathways by which antigens are presented by APCs to T cells (described in section 1.3). Before discussing these we will look at the development of the T cell lymphocyte population.

1.2. T lymphocytes

T lymphocytes are a group of cells that are characterized by their expression of a surface receptor known as a T cell receptor (TCR). This is a molecule that has evolved to bind a complex of MHC and antigen-derived peptide. It is a highly variable molecule with the variability concentrated in that part of the molecule that binds to the MHC/peptide complex, the variable region. The MHC molecules exist in 2 forms that are described above. The MHC class I molecule presents peptides generally from an intracellular source (although it may "cross-present" exogenous antigens to this pathway in APCs). The MHC class II molecule is expressed mainly on the surface of APCs and presents exogenous antigen. These two different groups of MHC molecules are recognized by 2 separate lineages of T lymphocyte. The CD8+ lymphocytes bind to MHC class I whereas the CD4+ lymphocytes bind to MHC class II. In this way CD8+ lymphocytes generally respond to intracellular antigens and CD4+ lymphocytes can respond to extracellular antigens.

1.2.1. T cell development

T lymphocyte precursors originate in the bone marrow but migrate to the thymus where all the important events in their development take place. The thymus is a small bi-lobed organ that sits in the superior mediastinum. After migration to the thymus, where they are now termed thymocytes, these cells begin to synthesise the TCR. The TCR is a receptor composed of only two chains which in the largest group of T cells comprises an α and a β chain. These chains are encoded by multiple genes which are rearranged in stages. By recombining multiple genes a very large number of diverse TCR rearrangements are possible.

Each TCR is generated by the rearrangement of a large number of genes from chromosome 7 and 14 in humans by a process known as VDJ recombination. The VDJ exon supplies the variable domain of the TCR which is then spliced to the constant domain after transcription. The β chain rearranges first with the Dβ gene segments rearranging to the Jβ segments and this is followed by Vβ gene segments to DJβ gene rearrangement. If no functional rearrangements can be synthesized from these arrangements then the cell will die.
Once the β-chain gene rearrangement has taken place the β chain is expressed with pTα, a surrogate α chain, and is expressed at the cell surface. This triggers phosphorylation of RAG-2 halting β-chain rearrangement. It also induces proliferation and eventually the expression of the co-receptor proteins CD4 and CD8. The next step is for the α chain to undergo multiple arrangements from its V and J segments. This can proceed through several cycles, as there is a large number of Vα and Jα gene segments, so that productive arrangements are very common. When a functional α chain is produced this pairs with the β chain and the $CD4^+CD8^+$ thymocyte (also known as double positive) is ready to undergo selection.

1.2.2. Positive and negative selection

It is necessary to select thymocytes that, on the one hand, have a functional TCR, and on the other, do not bind self-antigen at too high affinity. T cells that bind MHC:self-peptide complexes with too high affinity could subsequently generate autoimmune disease. Consequently two selection processes take place to ensure this does not happen. Firstly TCRs that have a moderate affinity for MHC:self-peptide complexes are selected (positive selection). Secondly, T cells expressing TCRs that have a high affinity for MHC:self-peptide are deleted (negative selection).

Positive selection is the process that occurs first in vivo . Thymic epithelial cells present self-antigens in the context of either MHC class I or MHC class II molecules to the $CD4^{+}CD8^{+}$ thymocytes. Cells that bind these MHC:self-peptide complexes with a threshold affinity receive a survival signal whereas those that do not die within 3 days.

Negative selection can be mediated by several different cell types within the thymus. The most important of these cell types is the bone marrow-derived dendritic cells and the macrophages. If the mature thymocyte TCR is ligated by a MHC:self-peptide complex from one of these APCs in the thymus then the thymocyte will die by apoptosis. The T cells responding to self-antigens expressed by these cells would be very potent propagators of an autoimmune response and this is presumably why they are eliminated in the thymus. This process of negative selection thereby results in "central tolerance" which is one of the processes preventing auto-reactive T lymphocytes.

However, there are many different tissue-specific proteins that would not normally be expressed outside their organ of secretion such as insulin from pancreatic tissue. But the role of central tolerance induction has recently been revised after the discovery of promiscuous expression of tissue-restricted self-antigens within the cells of the thymus. The autoimmune regulator (Aire) is responsible for inducing a large portion of this gene pool. This is a gene that is expressed in medullary epithelial cells in the thymus. It is thought that Aire activates

200-1200 genes that would otherwise only be expressed in the "peripheral" tissues.[31] Numerous tissue-restricted genes are also up-regulated in mature medullary epithelial cells in the absence of Aire. There are therefore likely to be other mechanisms to allow these cells to express other antigens and maximize the scope of central tolerance.[32]

At the time of this selection process the thymocyte expresses both CD4 and CD8 co-receptor molecules. At the end of the selection process mature thymocytes express only one of these co-receptors. Most of the mature T cells that express CD8 have receptors that recognize peptides bound to the MHC class I molecule and are destined to become cytotoxic effector cells. Almost all of the mature T cells that express CD4 have receptors that recognize peptides bound to the MHC class II molecule and are destined to become cytokine-secreting "helper cells". Once they leave the thymus they will circulate in the blood and lymphoid tissues. Here their conversion from naïve T cell to effector T cell will depend on whether they encounter with an APC presenting a peptide-MHC complex (pMHC) that will bind to their TCR. It will also depend on the context in which this cellular interaction takes place.

1.2.3. T cell interactions with DCs

DCs "prime" or initiate T-cell responses in the secondary lymphoid organs such as the lymph nodes, spleen or mucosal lymphoid tissues. It is estimated that only one naïve T cell in 10^5-10^6 is likely to be specific for a particular antigen.[33] Due to the specificity of the TCR for only a certain number of pMHC complexes it is critical that T cells circulate throughout the body and sample as many pMHC complexes on the surface of APCs as possible. Naïve T cells enter lymphatic tissue by crossing the walls of venules known as high endothelial venules (HEVs). Binding of T cells to HEVs is controlled by a number of adhesion molecules in the surface of the T cells that recognize adhesion molecules on the venules. These adhesion molecules include the integrins, the selectins, members of the immunoglobulin superfamily and some mucin-like molecules. An example is L-selectin that is expressed on leukocytes (also known as CD62L). This binds to mucin-like vascular addressins such as GlyCAM-1 and CD34 on HEVs. This interaction is responsible for the homing of T cells to the lymphoid

tissues. But to enable the T cell to cross the endothelial barrier, the integrins and immunoglobulin-like molecules are necessary. The migration of T cells to the lymphoid tissues is also mediated by the chemokine CCL21 that is expressed in high vascular endothelial cells and binds to the CCR7 chemokine receptor on naïve T cells.

Once in the lymph node the T cell will form serial interactions with DCs. Studies have shown that DCs can scan at least 500 different T cells per hour in the absence of antigen. More than ten T cells can simultaneously engage with these antigen-bearing DCs. In this way DCs are highly efficient in recruiting peptide-specific T cells and will interact with these cells for hours, not minutes.[33] Another study has suggested that T cells are not attracted toward DCs by chemotactic gradients but rather encounter them by chance. In this study they showed T cell/DC contacts were able to occur some distance from the DC soma. In this way the long dendrites, a morphology typical of the mature DC, enabled an individual DC to interact with up to 5000 T cells per hour.[34]

The initial interaction of T cells with APCs is mediated by cell-adhesion molecules. LFA-1, ICAM-3 and CD2 on the surface of the T cells interact with ICAM-1, DC-SIGN and CD48 respectively. This initial transient binding of naïve T cells to APCs gives the cells time to form interactions between the TCR and the pMHC complex. In the rare event when TCRs do recognize a pMHC complex, signalling through the TCR induces a conformational change in LFA-1 which greatly increases its affinity for ICAM-1 and ICAM-2.

Once a specific T cell has interacted with an APC with the required pMHC complex on its surface, a number of factors will determine the response of the T cell. These include the cell type that is presenting antigen, the affinity of the TCR for the corresponding peptide-MHC complex (pMHC), and the level of co-stimulation delivered by the APC. Investigators have also shown that the duration of this process is critical in determining whether T cells will be activated or deleted. In their model, naive T cells required approximately 20 hrs of sustained signalling to be committed to proliferation. Co-stimulation by anti-CD28 facilitates T cell activation by decreasing the time required of this interaction and also protected T cells from

death. These findings explained the essential requirement for professional APCs in T cell priming.[35]

In addition to the requirement for APCs, the state of maturation of these DCs is also critical. T cell stimulation by immature DCs leads to initial T-cell proliferation but only short-term survival. Stimulation by mature DCs results in long-term T cell survival and differentiation into memory and effector T cells. [36]

1.2.4. Co-stimulation

Ligation of the TCR, along with the CD4 or CD8 co-receptor, does not stimulate naïve T cells to proliferate. The naïve T cell requires a second, co-stimulatory signal in order to become activated and divide. This second signal must be delivered by the same APC on which the T cell recognises its antigen. There is a very wide range of molecules on T cells that will provide this signal. The large majority of T-cell co-stimulators belong either to the CD28 or the TNF-receptor families of molecules. Only a few (CD28, CD27 and HVEM) are expressed on T cells in a constitutive fashion. The majority of co-stimulatory molecules are induced only following activation of the cell by antigen recognition through the TCR. All of these constitutively expressed molecules have positive effects on T cell stimulation. But a group of the inducible co-stimulatory molecules can have regulatory or negative effects. These include the cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1) and the B and T lymphocyte attenuator (BTLA)(see table 1).

The best characterised ligands for T cell co-stimulatory molecules are the structurally related glycoproteins B7.1 (CD80) and B7.2 (CD86). The receptor for these molecules is the constitutively-expressed receptor CD28. Ligation of CD28 by the B7 molecules is an absolute requirement for the expansion and activation of naïve T cells. In addition, antibodies to B7 will inhibit T cell responses. Once CD28 has been ligated the expression of other receptors

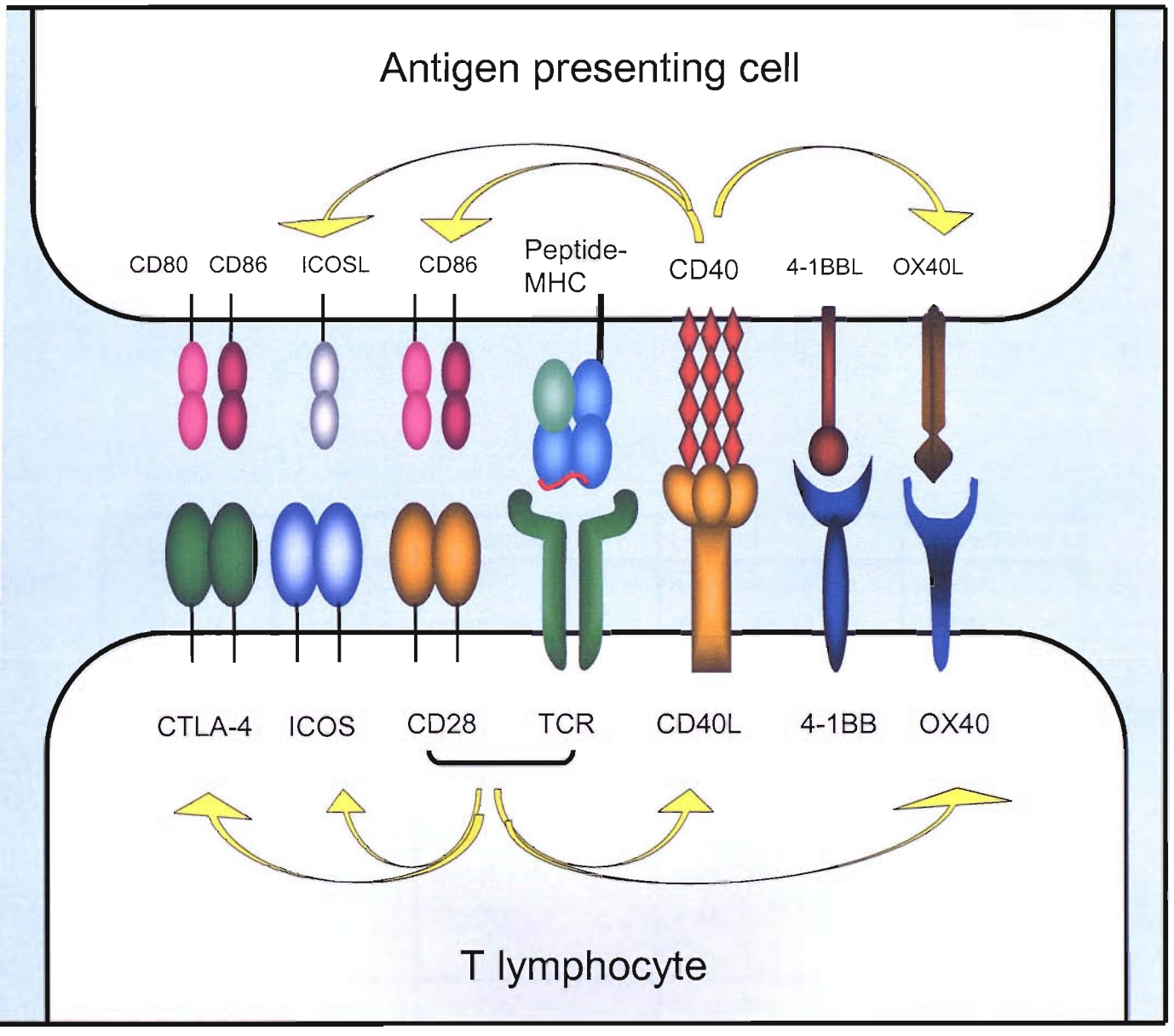


Figure 2. Co-stimulatory receptors.

This diagram illustrates the hierarchy and interdependence of T cell co-stimulation. Upon T cell receptor stimulation, CD28 induces the expression of other co-stimulatory receptors that belong to the immunoglobulin superfamily and tumour-necrosis factor receptor superfamily. The subsequent interaction of CD40L with CD40 up-regulates CD80 and CD86 thus providing a positive feedback loop for the action of CD28. Adapted from [43] and [44].

is induced. This includes the induction of ICOS and OX40.[37,38] Subsequently both CD28 and ICOS are able to strongly up-regulate CD40L on the T cell surface.[39] CD40 is constitutively expressed on all professional APCs and its ligation is a potent signal to up-regulate B7-1 and B7-2. Its ligation also strongly induces ICOS ligand. Both of these therefore supply a positive stimulatory loop for the stimulation of T cells.[40] CD28 ligation also activates the transcription and translation of IL-2 and its receptor IL-2R.[41] It is also able to induce Bcl-xL in the T cell, a molecule that protects against apoptosis.[42]

Expression	Signal	Receptor	Ligand	Superfamily
Constitutive	Positive	CD28	B7-1 (CD80)	CD28/B7
		CD28	B7-2 (CD86)	CD28/B7
		CD27	CD70	TNF/TNFR
		HVEM	LIGHT	TNF/TNFR
Inducible	Positive	ICOS	ICOS-L	CD28/B7
		CD30	CD30L (CD153)	TNF/TNFR
		OX40 (CD134)	OX40L	TNF/TNFR
		4-1BB (CD137)	4-1BBL	TNF/TNFR
	Negative	CTLA-4 (CD152)	B7-1 (CD80)	CD28/B7
		CTLA-4 (CD152)	B7-2 (CD86)	CD28/B7
		PD-1	PD-L1	CD28/B7
		PD-1	PD-L2	CD28/B7
		Unknown	B7-H3	CD28/B7
		BTLA	B7-H4 (BTLA-L)	CD28/B7

Table 1. Expression of T-cell co-stimulatory molecules and their ligands. Adapted from [43] and [44].

It is interesting to note that the different co-stimulatory signals are interdependent and that there is a hierarchy of these signals. The ligation of the B7 molecules with CD28 remains critical in the instigation of T cell responses. Of central importance these B7 molecules are exclusively expressed on professional APCs such as mature DCs, whereas the ligands for the other co-stimulatory molecules are found on other cells including immature DCs.

1.2.5. Peripheral Tolerance

Antigen recognition in the absence of co-stimulation does not lead to T cell expansion but rather to inactivation, inducing anergy. This is where the T cells are unable to produce IL-2 or proliferate. This is one of the mechanisms responsible for "peripheral tolerance" and is critical for maintaining self-tolerance. Central tolerance leads to the deletion of high-affinity T cells that are autoreactive. However this process is incomplete and numerous autoreactive T cells can be detected in the circulation.[45]

Immunoregulation is a method of inducing peripheral tolerance and occurs when one group of cells controls the response of another. This process can be mediated by either "naturally occurring" regulatory T cells (Treg) or "induced" regulatory T cells.

Naturally occurring Tregs are produced in the thymus and express the IL-2 receptor α-chain (CD25). CD4+CD25+ Tregs were found to be critical in the control of autoreactive T cells in vivo.[46] Mice that are thymectomized before day three after birth develop autoimmune diseases affecting numerous organs. Using this model, investigators identified a population of CD4+CD25+ immunoregulatory cells and found that they suppressed the induction of disease post-thymectomy. They also efficiently suppressed disease induced by cloned autoantigen-specific effector cells.[47] In addition depletion of CD4+CD25+ T cells in adult mice also induced autoimmune diseases.[46] These "naturally occurring" regulatory T cells, generated in the thymus, are both anergic and suppressive. They represent 5-10% of all peripheral CD4+ T cells. They are able to suppress CD25- T cells in vitro. These cells markedly suppressed

proliferation by specifically inhibiting the production of IL-2 but the inhibition was not cytokine mediated. It was dependent on cell contact between the regulatory cells and the responders and required activation of the suppressors via the TCR.[48]

The phenotype of the naturally occurring Tregs is characterized by the constitutive expression of several activation markers. These include glucocorticoid-induced TNFR (GITR)[49], OX40[50], L-selectin[51] and CTLA-4[52]. Signals through GITR act to abrogate the immunosuppressive effect of CD25+ Tregs which induces autoimmune disease in one study.[49] Stimulation via CTLA-4 has been more controversial with some investigators finding it reverses the immunosuppressive effect[53] whereas others find it has no effect on the function of these cells.[54]

A recent advance in the study of these CD25+ regulatory cells was discovering the key role played by transcription factor FoxP3.[55] Initially it was discovered that mice lacking this gene developed a lymphoproliferative disease similar to animals that lacked expression of either CTLA-4 or TGF-β. The pathology observed in these mice seemed to result from a lack of T cell regulation.[56] Other studies have shown that Foxp3 is highly expressed by Treg cells and is associated with Treg cell activity and phenotype. The forced expression of FoxP3 in this model also delayed disease in CTLA-4 knock out mice suggesting interlinking of CTLA-4 and FoxP3 pathways.[57]

"Inducible" Tregs were demonstrated by the chronic activation of both human and murine CD4+ T cells in the presence of interleukin (IL)-10. This gave rise to CD4+ T-cell clones with low proliferative capacity, producing high levels of IL-10, low levels of IL-2 and no IL-4. These antigen-specific T-cell clones suppressed the proliferation of CD4+ T cells in response to antigen, and prevented colitis induced in SCID mice.[58] These Tregs are referred to as Tr1 and they exert their influence through IL-10 and are contact independent.

Another set of inducible Tregs were discovered in mice by studying the mechanisms behind tolerance induction in experimental autoimmune encephalomyelitis (EAE) by the oral

administration of myelin basic protein (MBP). T cell clones were isolated from the mesenteric lymph nodes of mice that had been orally tolerized to MBP. These clones were CD4+ and were identical to pathological T helper cell type 1 (TH1) CD4+ clones in T cell receptor usage, major histocompatibility complex restriction, and epitope recognition. However these regulatory cells produced large amounts of TGF-β and lower amounts of other immunosuppressive cytokines.[59] These inducible Tregs are referred to as Th3 and are characterized by the secretion of large amounts of TGF-β and are also contact independent.

DCs may actively be rendered tolerogenic by a number of mechanisms. In humans a subset of monocyte-derived DCs has been described that expresses indoleamine 2,3-dioxygenase (IDO) which inhibits T-cell proliferation and induces T cell death. IDO-positive APCs constituted a discrete subset identified by co-expression of the cell-surface markers CD123 and CCR6.[60] In further studies it was shown that IDO could be up-regulated, selectively and specifically, in splenic DC subsets with particular phenotypes in response to CTLA-4 stimulation. CTLA-4 did not induce IDO expression in macrophages or lymphoid cells. Induction of IDO completely blocked clonal expansion of T cells from TCR transgenic mice following adoptive transfer.[61]

Antigens targeted to immature DCs in vivo can induce tolerance through anergy and abortive proliferation of antigen-specific T cells. In this instance delivery of a DC maturation stimulus induces a full effector T-cell response.[24,62,63] Dhodapkar et al described the immune response to a single injection of immature DCs pulsed with influenza matrix peptide (MP) and keyhole limpet hemocyanin (KLH) in two healthy subjects. Injection of immature DCs in both subjects led to the specific inhibition of MP-specific CD8+ T cell effector function in freshly isolated T cells. This contrasted with previous findings where T cell responses were stimulated using mature DCs. When pre- and post-immunization T cells were boosted in culture, there were greater numbers of MP-specific major histocompatibility complex tetramer-binding cells after immature DC immunization, but these had reduced interferon production, lacked cytolytic activity and even secreted the immunosuppressive cytokine IL-10.[64]

Another mechanism of peripheral tolerance is ignorance. Ochsenbein et al studied a subcutaneous sarcoma model in mice. Tumour cells were capable of inducing a protective cytotoxic T cell response if transferred as a single-cell suspension. However, if they were transplanted as small tumour pieces, the tumour cells did not reach the draining lymph nodes and did not prime cytotoxic T cells. They demonstrated that cytotoxic T cells were not tolerant or deleted because a cytotoxic T cell response was readily induced in lymphoid tissue by immunization with virus or with tumour cells. The inference from this study is that antigens failing to gain access to the lymphoid tissues are ignored.[65]

1.2.6. The influence of CD4 help on CD8 immunity

For some years the central role of CD4+ lymphocyte responses in the immune response has been clear.[66,67] However until recently the mechanisms by which these cells interact with CD8 cells had not been demonstrated. Initially the role of CD4+ cells was thought to be by the production of cytokines, particularly IL-2, that enabled the ongoing proliferation of CD8 cells.[67,68] More recently the interactions between CD4+ lymphocytes and dendritic cells have been studied.[69] CD4+ lymphocytes can be shown to activate DCs so that these cells are subsequently able to prime CD8 cells to become effector CTLs.[70] This effect seems at least in part to be mediated by the ligation of CD40 on dendritic cells. It was also observed that even after CD4+ cell depletion the ligation of CD40 on DCs with an agonistic antibody (Ab) can replace T cell help.[71,72] Another study demonstrated that "licensed" dendritic cells that had been optimally activated could provide a temporal bridge between a CD4+ T cell and a CD8+ T cell.[69,73] The authors of this paper demonstrated a "sequential model" of CD8 priming where the dendritic cell is initially licensed by CD40 ligation by CD4 cells and these DCs can then go on to prime CD8 cells. Further evidence for the importance of CD4+ and CD8+ collaboration comes from studies that look at animals with CD40 and CD40L deficiencies where the CD8 responses are severely impaired.

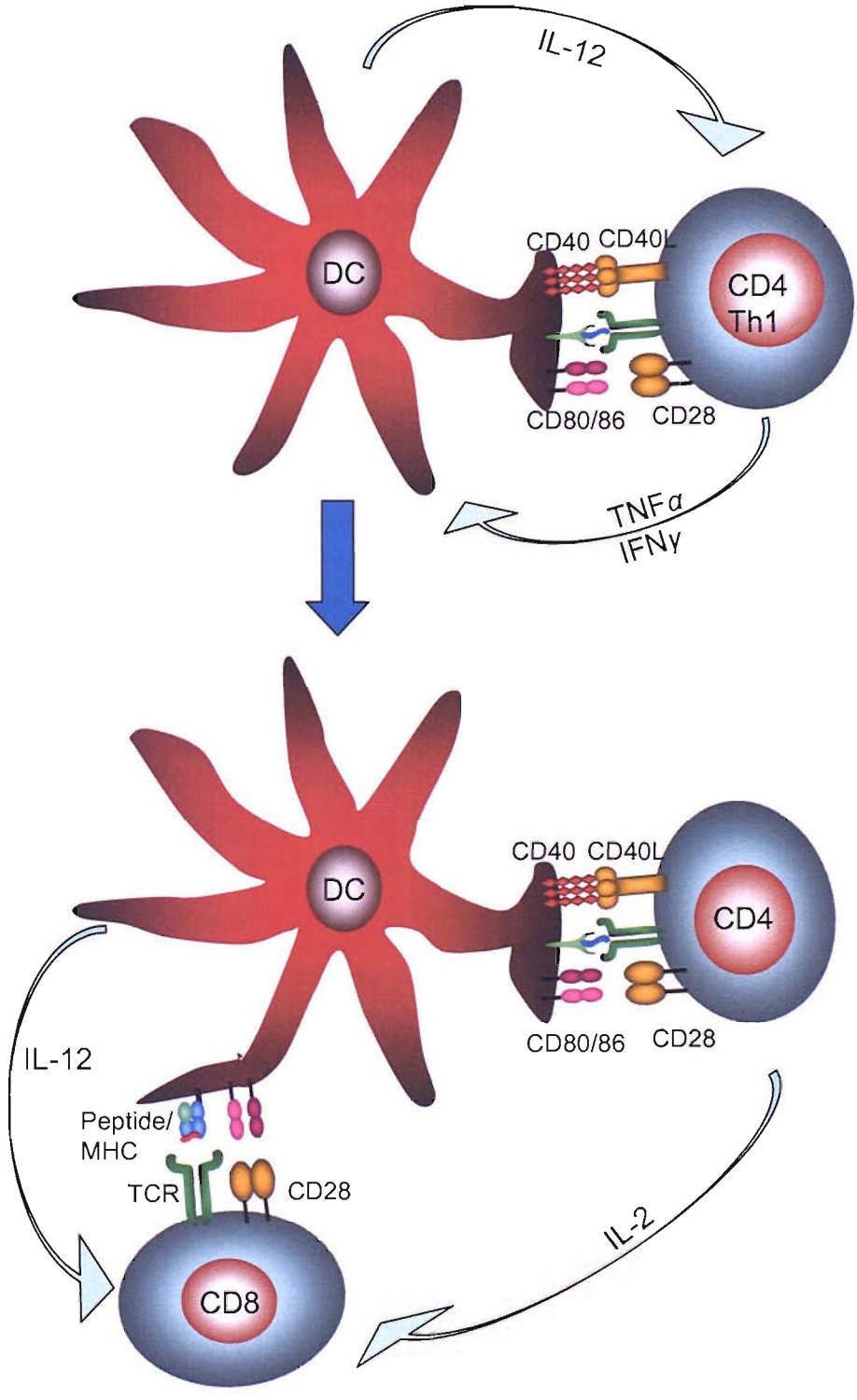


Figure 3. T cell interactions with DCs.

MHC class II/ peptide complexes expressed on the surface of DCs are bound by CD4+ T cells. Subsequent co-stimulatory signals (*e.g.* between CD40 and CD40L) activate the DC presenting the antigen. MHC class II and co-stimulatory molecule expression are up-regulated. In addition cytokines are secreted by the two cells as represented. This fully activated dendritic cell can then go on to prime CD8+ T cells that will in turn result in effective memory CD8+ T cell generation. Adapted from [69].

However CD40-independent T cell help for CD8 T cell responses has also been described. This seems to have two components: firstly the CD40-independent sensitization of DCs by CD4+ T cells and secondly direct cytokine-dependent CD4-CD8 T cell communication.[69,74]

Recently CD4-independent CD8 priming has also been described. This CD4-independent CD8 priming has been demonstrated principally in acute infections with live virus or intracellular bacteria.[75-78] It is likely that the infectious agents provide a plethora of immunostimulatory signals activating the DCs directly and bypassing the need for CD4 help. This effect is also likely to require the provision of maturation signals for the DCs. When a DC matures it up-regulates co-stimulatory molecule expression such as CD80 and CD86 along with MHC class II. Many maturation signals for DCs have been discovered. LPS has been known to activate dendritic cells by binding to Toll-like receptor (TLR) 4. It has also been shown that unmethylated CpG motifs in bacterial DNA can induce DCs to mature by binding to TLR-9.

However several authors have recently demonstrated that whilst CD8 priming is possible without the provision of CD4 help there is a deficit in CD8 T cell memory in this setting. Initially Bourgeois et al demonstrated good CD8 priming but poor CD8 memory responses in the absence of CD4 help in a non-infectious setting; minor histocompatibility antigens.[79] They found that CD40 was critical here in the generation of CD8 T cell memory. They also demonstrated that CD40 is expressed on CD8 cells suggesting a possible direct ligation of this molecule for the mediation of CD4 help. More surprising was the recent demonstration that even in two infectious disease models CD4 T cell help was required for the generation of functional CD8 T cell memory.[80,81] This help was required at the priming stage but was not required during the recall response for secondary expansion. In both of these models the CD8 response was previously thought to be CD4-independent.

1.2.7. Cytotoxic T cells – effector mechanisms

The cytotoxic mechanisms of CD8+ lymphocytes are critical in the elimination of target cells. The CD8+ lymphocytes use one of two main mechanisms to mediate this lysis. The first of these is the perforin/granzyme pathway. CD8+ T cells contain lytic granules that are modified lysosomes. They contain at least 3 different types of cytotoxic effector protein including perforin, granzyme and granulysin. Perforin is a protein which polymerizes in the target's plasma membrane to form a trans-membrane pore.[82] The granzymes comprise at least 3 different serine proteases which can activate apoptosis once in the cytoplasm of the target cell.[83] Granzyme B cleaves and activates caspase-3 which activates a caspase proteolytic cascade which eventually degrades DNA in the target cell. Granulysin is another cytotoxic protein that is able to induce apoptosis in the target cell and has additional anti-microbial activity. Once the cytotoxic T cell has recognized its target cell the lytic granule become polarized to the target cell-T cell interface and the contents are released. The importance of perforin in cytotoxicity is very clear as mice which lack these molecules are defective in their ability to achieve cytotoxicity to many viruses and these mice are also more likely to develop spontaneous tumours.

The second mechanism of target cell cytotoxicity employed by the CD8+ T cells is signalling through the tumour necrosis factor receptor (TNFR) family of receptors. The commonest of these, FasL, is expressed on activated CD8+ T cells. Ligation of Fas on the target cell leads to the activation of the caspases and induces apoptosis in the target cell. Several of the other TNFR family members can also mediate this receptor-mediated target cell apoptosis. These include TRAIL (tumour-necrosis factor-related apoptosis-inducing ligand), TWEAK (TNF-like weak inducer of apoptosis) along with an increasing number of related proteins.

Cytotoxic T cells also release cytokines which contribute to host immunity. These include IFN-γ, TNF-α and TNF-β. These contribute to host immunity in several ways. IFN-γ induces the increase expression of MHC class I and other molecules involved in the peptide loading of

MHC class I molecules. This is also true of some tumour cells that have down-regulated MHC class I. IFN-γ also acts on macrophages and APCs recruiting them to the site of infection and acting as a maturation signal for DCs. TNF-α can act alongside IFN-γ in APC activation and maturation. It can also lead to some target cell killing through its interaction with TNFR. Thus CD8+ lymphocyte effector function is critical for fighting infection but may also have a key role to play in the immunotherapy of cancer.

1.3. Antigen presentation

There are 3 principal pathways by which antigens are presented by APCs to T cells. Firstly endogenous antigen is presented in the context of MHC class I to CD8+ lymphocytes. Secondly exogenous antigen is presented in the context of MHC class II to CD4+ lymphocytes. Lastly exogenous antigen is presented in the context of MHC class I to CD8+ lymphocytes – also known as cross-presentation.

1.3.1. MHC class I antigen processing

Nearly all proteins in mammalian cells are continually being degraded and replaced by new synthesis. In addition, cells eliminate unfolded or damaged proteins whose accumulation may be harmful. Many cellular proteins that are to be degraded in the cytoplasm are first covalently modified with the polypeptide cofactor poly-ubiquitin. This targets the protein to the large cytosolic protease complex termed the proteasome. This is a large barrel-shaped complex of 28 subunits consisting of 4 rings of 7 subunits each. The mammalian proteasome has 6 proteolytic sites 3 in each of the 2 central rings. The hollow core is lined by the active sites of these proteolytic subunits.[84]

In the presence of IFN-gamma these sub-units can be replaced by the proteins b1i and b5i (previously known as low molecular weight protein -7 and -2 respectively). This modified proteasome is referred to as an immunoproteasome.[85] IFNγ is also able to induce a third

subunit known as b2i (previously known as MECL-1) which also displaces a proteasome sub-unit.[84-86] Proteasomes can therefore exist in two forms, the first found constitutively in all cells and the second found in professional APCs and cells that can be stimulated with IFNγ. The IFNγ-inducible components within the immunoproteasome change the specificity of the proteasome, whereby there is increased cleavage of peptides after hydrophobic residues and reduced cleavage after acidic residues.[84,87] Whilst the overlap in repertoire of peptides released from the proteasome and immunoproteasome is considerable, the immunoproteasome is more likely to produce carboxyl-terminal residues that are preferred anchor residues for many HLA class I molecules.

Cytosolic peptides are delivered to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP).[88] Newly synthesized ribosomal associated proteins can also gain access to the ER being normally translocated during their biosynthesis. ER proteins can also be returned to the cytosol via the Sec61 ER-spanning molecule. This mechanism of transport of proteins from the ER to the cytosol is known as retrograde translocation. The proteins may be further degraded in the cytosol by the proteasome.[89]

It is now clear that peptide cleavage can also occur in the ER. This trimming occurs at the amino-terminal of the peptide by an aminopeptidase found in the ER. This enzyme is known as the endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP). This enzyme can be up-regulated by IFNγ.[90]

To be efficiently transported by TAP the peptides must be between 8 and 16 residues long and have an appropriate COOH-terminal residue. Murine TAP prefers a hydrophobic residue whereas human TAP prefers a hydrophobic or positively charged residue. Peptides can be produced in their final form in the cytosol or possess amino terminal extensions of up to 8 residues. In the latter case trimming of these residues would be required.

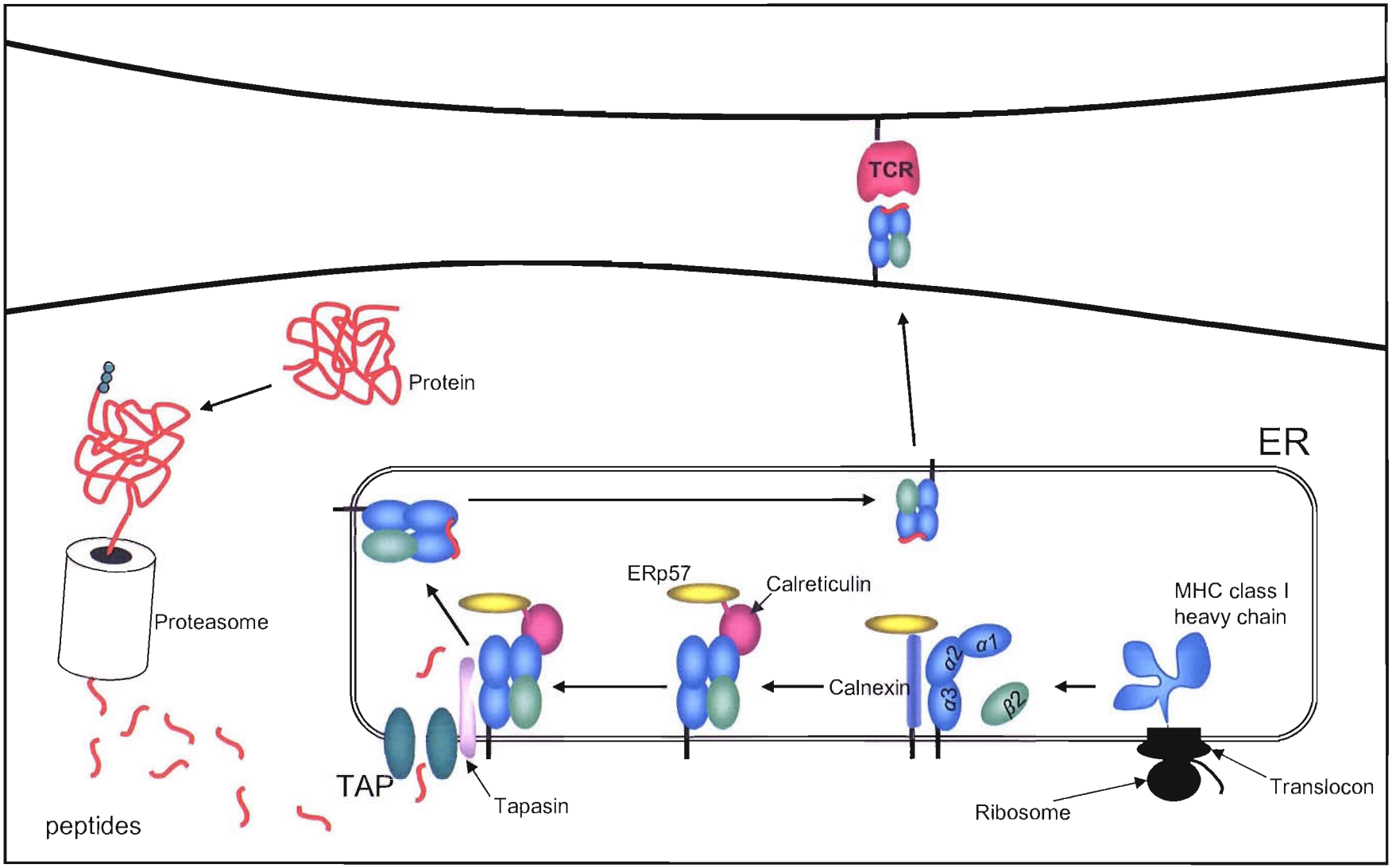


Figure 4. MHC class I antigen processing. Adapted from [84] and [91].

The MHC class I molecule is composed of 2 components: a heavy chain and a non-covalently associated light chain, known as β2-microglobulin (β2-m). Newly synthesized MHC class I heavy chains bind calnexin, a chaperone protein. This retains the MHC class I molecule in the ER in a partly folded state. When β2-m binds to the heavy chain the partly folded heavy chain/β2-m heterodimer dissociates from calnexin and binds to the homologous ER chaperone calreticulin. Once the MHC class I molecule has associated with β2-m and calreticulin the complex of proteins known as the peptide-loading complex (PLC) assembles.[91] This includes tapasin that forms a bridge between the class I molecules and TAP. This close association is presumed to increase the efficiency of peptide loading. Another component of the PLC is ERp57, an oxido-reductase which catalyses disulphide bond formation.[91,92] This may be involved in breaking and reforming disulfide bonds in the MHC class I during peptide loading. Initially the MHC class I may bind low-affinity suboptimal peptides. Subsequently these may be replaced with high-affinity optimal peptides, within the PLC, which stabilise the MHC class I molecule. In this way the PLC is critical for peptide optimisation.[93] Certainly cells defective in tapasin and calreticulin express MHC class I molecules at the cell surface which contain sub-optimal, lower affinity peptides.[94-96] Peptide binding to the MHC class I molecule is followed by dissociation from the PLC and the fully folded MHC-peptide complex is able to leave the ER and begin transport to the cell surface.

The affinity with which MHC class I molecules bind specific peptides will depend on the peptide sequence. This requirement is in addition to specificities required by proteasomal degradation and TAP transportation. Any given MHC class I molecule is only able to bind a small proportion of candidate peptides due to specificities in the peptide-binding cleft. This MHC-peptide complex is stabilised at both ends by interactions between the amino and carboxyl termini of the peptide and sites found at either end of the cleft.[97] In addition, other residues within the peptide serve as anchor residues. These fit into side-pockets located beneath the peptide-binding cleft of the MHC molecule. Peptides that bind to these molecules are usually 8-10 amino acids long. If they are longer than this they can sometimes bind, particularly if they are able to bind at the carboxyl-terminal. Redundant amino acids at the amino terminus can then be cleaved by aminopeptidases in the ER.

MHC molecules are highly polymorphic and therefore display different peptide-binding specificity. There are a large number of different alleles of the MHC class I genes in the human population with each individual only carrying a small selection of them. The differences between the allelic variants are often found at sites within the peptide-binding cleft. This results in different amino acids in critical peptide-interaction sites between the different MHC alleles. Consequently, different MHC variants bind peptides with similar amino acids at "anchor residues" in 2 or 3 particular positions along the peptide sequence. An example of this is in HLA-A2.1 molecule which displays preferences for leucine or methionine at positions 2, and valine or leucine at position 9 in the peptide sequence. [98]

As a result of these preferences investigators have developed predictive algorithms that aim to identify candidate class I-restricted epitopes from protein sequences. These algorithms have identified several epitopes from target antigens. However, limited accuracy may be partly due to secondary requirements for specificity in non-anchor residues. These issues are further discussed in section 1.4.2.

1.3.2. MHC class II antigen presentation

Extracellular antigens are internalised by APCs and enter endocytic vesicles. These antigens initially enter the early endosomes and are rapidly passed to the late endosomes which exhibit a low pH and a high concentration of acid proteases. These proteases are activated at low pH. Amongst their number are the cysteine proteases cathepsins B, D, S and L. These enzymes participate in the processing of internalized antigens into peptides that can be presented to CD4+ lymphocytes in the context of class II. Other proteases also have a role in generating class II specific peptides. The newly discovered asparaginyl endopeptidase (AEP) performs the rate-limiting cleavage in the degradation of Fragment C of tetanus toxin (FrC).[99] However this same enzyme also prevents the presentation of an epitope from another antigen: myelin basic protein. This epitope contains a processing site for AEP and is therefore destroyed by the enzyme. [100]

When they are newly synthesized MHC class II molecules associate with the chaperone invariant chain (Ii) in the ER. This interaction prevents the MHC class II molecule binding the large numbers of unfolded and partly folded polypeptides that exist in the ER. The Ii forms trimers with each subunit associating with the MHC class II molecule α:β heterodimer. Ii binds with part of the protein lying within the peptide-binding cleft of the MHC class II molecule. The molecule is then delivered directly into the endocytic pathway. Within this pathway aspartic and cysteine proteases degrade the Ii leaving the CLIP (class-II-associated invariant chain peptide) fragment of Ii remaining in the peptide-binding cleft of the MHC class II molecule. Whilst associated with CLIP MHC class II molecules cannot bind other peptides. CLIP is displaced from the MHC class II molecule by antigenic peptides in a reaction catalysed by the MHC class II-like molecules HLA-DM (in humans) and H-2M (in mice). In B cells and thymic epithelial cells another atypical MHC class II-like molecule HLA-DO is produced. DO inhibits the function of HLA-DM in all but the most acidic late-endosomal compartments of B cells.

Most newly synthesised MHC class II molecules acquire peptides in late endocytic/lysosomal vesicles. These compartments are referred to as MHC class II compartments (MIIC). If MHC class II molecules do not bind peptide then they aggregate and are rapidly degraded in the acidic pH of the endosome.

MHC class II molecules, like class I molecules, bind to peptides with specific properties. However MHC class II molecules will bind to peptides of far more variable length than class I molecules. Peptides that bind to MHC class II molecule are at least 13 amino acids in length and can be much longer. The peptide lies in an extended conformation along the MHC class II peptide-binding cleft. The binding pockets of MHC class II molecules are more permissive in their accommodation of different amino acid side chains. The distance between anchor residues and the end of the peptide can also vary without apparent loss of binding affinity. Consequently it is much harder to predict which peptides will bind to MHC class II molecule by modelling peptide sequence motifs.

1.3.3. Cross presentation

There is evidence from several models that exogenous antigen can also be presented in the context of the MHC class I molecule. This is known as cross-presentation. When exogenous antigen is cross-presented in this way and subsequently primes a CD8+ response this is termed cross-priming.

Cross-priming was first described by Bevan and colleagues.[101] They used the minor histocompatibility antigens (miH) as targets for cytotoxic T cells. miH are genes with allelic differences of numerous host protein-derived peptides that are presented in MHC class I. In this study they used congenic strains of mice which carry all the same miH information but differ at H-2. If an H-2^b BALB.B mouse was immunised with H-2^b C57BL/10 (B10) splenocytes a CTL response could be demonstrated against minor histocompatibility antigens on B10 targets. The experiment was repeated by immunisation of F1 (BALB/c x BALB.B, H-$2^{d/b}$) mice with cells from H-2^b B10 mice and then re-stimulated with either H-2^b B10 or H-2^d B10.D2 cells in vitro. The CTL generated were able to lyse targets of both the H-2^b and H-2^d haplotype. In this case the H-2^b B10 splenocytes could not have presented the H-2^d-restricted miH antigens directly to the F1 CD8+ T cells. These antigens must have been taken up exogenously by the F1 APCs and presented via H-2^d class I to the responding CD8+ T cells.

Subsequently cross-priming has also been shown in infectious disease. Sigal and colleagues investigated mice that cannot be infected by the poliovirus unless they have the transgene for the poliovirus receptor (PVR).[102] When PVR-transgenic mice were re-constituted with PVR-deficient bone marrow robust CTL responses were elicited against the virus. No CTL responses were seen when the bone marrow used was PVR-deficient and deficient in TAP. Thus the generation of poliovirus-specific CTLs in these experiments required the uptake of

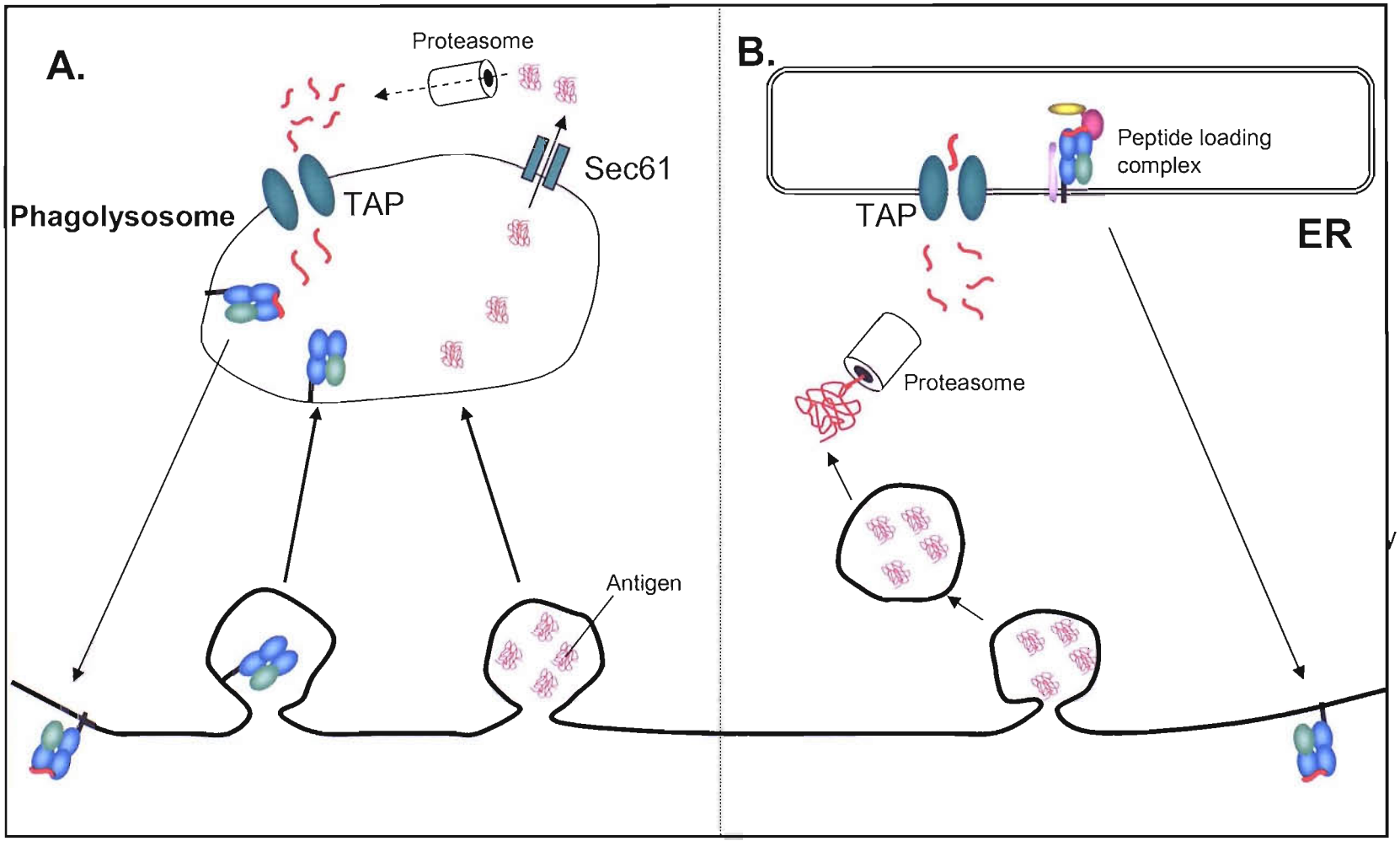


Figure 5. Models of cross-presentation of exogenous antigens to the MHC class I pathway.
A. **Post-Golgi pathway.** Antigens in the phagosome lumen could use SEC61 for translocation to the cytoplasm. After processing by the proteasome, antigens could be translocated to the phagosome lumen through the TAP complex where loading onto MHC class I molecules would occur. Transport to the cell surface from the phagosome lumen could then take place.
B. **Cytosolic pathway.** Antigens are translocated to the cytoplasm by an unknown transporter. There, the antigens are processed by the proteasome to generate the appropriate peptides. These peptides are then translocated to the endoplasmic reticulum (ER) lumen, through the transporter for antigen processing (TAP) complex, where binding to MHC class I molecules occurs.
Adapted from [87].

exogenous antigen by BM derived APCs and the TAP-dependant presentation of epitopes from the virus.

Three recent studies have shown new ways in which MHC class I ligands can be processed and presented. In one study fusion of the endoplasmic reticulum (ER) with the macrophage plasmalemma was described and the ER was also found to be a source of membrane for phagosome formation in macrophages.[103] Since this study other investigators have shown that early phagosomes from human dendritic cells (DCs) contain the peptide-loading complex, incorporating MHC class I, beta2 microglobulin, transporter associated with antigen processing (TAP), calreticulin, tapasin, and ERp57.[104,105] Another study demonstrated that soon after or during formation, phagosomes fuse with the ER.[106] These phagosomes frequently associate with the immunoproteasomes and proteasomes on the cytosolic side and these proteasome/ phagosome complexes are able to cleave peptides and present these within the context of the MHC class I molecule without requiring the usual trafficking of these molecules back to the ER.

Consequently there are now 2 possible routes for class I antigen processing and presentation that have been referred to as the cytosolic and the post-Golgi routes.[87] The cytosolic pathway involves antigen entering in the endosomes and gaining access to the cytosol. From the cytosol peptides are cleaved from the antigen by the proteasome and the transported to the ER by TAP to be loaded onto the MHC class I molecule. The post-Golgi pathway involves proteins being degraded in the phagosomes. Translocation of protein to the cytosol is necessary for proteasomal degradation and a candidate protein for this role is Sec61. After proteasomal degradation the peptides could be transported back to the phagolysosome by TAP and then the peptides can bind to MHC class I molecules [107] (Figure 5).

1.3.4. Immunodominance

There is evidence from several viral infections that, faced with numerous potential epitopes from a large number of proteins, the CD8+ immune response focuses on just a fraction of these. This has been demonstrated in relation to EBV and CMV infection. In CMV infection the CTL response is focussed largely on epitopes from the antigen pp65.[108] Although CTL are found against the immediate early protein (IE1) these are generally of lower frequency.[109] There is also evidence that immunodominance has a role in several other infections including HIV[110], Hepatitis C[111] and influenza.[112]

The molecular and cellular mechanisms underlying immunodominance are now being elucidated. Of principle importance is the ability of peptide to bind to class I molecules. Only a fraction of potential epitopes bind at an affinity above the threshold for the generation of CD8+ immunity.[113] There are also cleavage preferences of the proteasome that govern the probability of a peptide being processed from its parent protein.[114,115] Many potential epitopes are inefficiently processed and are therefore not presented. The number of peptides capable of generating an immune response is further limited by the finite T cell repertoire.

A further possible mechanism for immunodominance is that competition exists between CTL recognising different epitopes, a phenomenon known as immunodomination. Immunodomination in several models is characterised by competition between responding T cell populations rather than by insufficient presentation of subdominant epitopes.[116] Responses to subdominant epitopes in these systems is only obscured when both dominant and sub-dominant epitopes are presented on the same APC.[117] In these models this immunodomination can be abolished by the injection of a surplus of APCs.[118] Kedl and collaborators demonstrated that T cells could compete for response to different antigens presented on the same APC.[119,120] They pulsed DCs with an ova peptide and a second peptide. They then transferred CD8 cells with a transgenic T cell receptor specific for K^b bound to the ova peptide. They demonstrated that these transgenic CD8 cells could compete with CD8 cells specific for the second peptide. It was also shown that the ability of T cells to compete is

affected by their affinity for the specified antigen. They demonstrated that higher affinity T cells competed more efficiently than the lower affinity cells.[120]

Another variable in demonstrations of T cell competition is precursor frequency. It has been observed that competition between responding T cell populations is not usually observed in primary responses. Indeed the vast majority of descriptions of competition have been after multiple immunisations.[121] This may be due to the low precursor frequency during the primary response, any given clone being too small to have an effect on the other responding T cells. Further studies have implicated precursor frequency as an important factor in competition.[122] Prior immunisation against a sub-dominant epitope dramatically reduced the response against a dominant epitope upon subsequent immunisation against both epitopes.

Several mechanisms have been proposed for competition between responding T cell populations. Initially it was thought that the dominant/high affinity T cells killed their APCs thus reducing access to antigen by the lower affinity cells. However competition has now been described in several models in the absence of the destruction of antigen-bearing APCs.[120] Another possible mechanism is the T cell-induced down-modulation of specific antigens from the APC in particular MHC class I. In a variety of experimental model systems T cells have been directly demonstrated to remove and internalise MHC and other proteins from the surface of interacting APCs.[123]

1.4. Immune responses against cancer

1.4.1. Immunosurveillance

As discussed on page 15 the manipulation of the immune response to treat cancer is now established in several different disease settings. These developments took place alongside experiments to determine whether the immune system could naturally recognize and destroy cancer cells without additional therapy. This concept was called immunosurveillance.

One of the first experiments to confirm the importance of the immune system in eliminating cancer was performed by Dighe et al. LPS is able to induce rejection of a fibrosarcoma tumour in syngeneic BALB/c mice. Using a neutralizing monoclonal antibody specific for murine IFN-γ they show that endogenously produced IFN-γ plays an obligate role in mediating this tumour rejection.[124] Other investigators studied mice that were lacking in either the IFN-γ receptor or one of the transcription factors that were responsible for signalling. These mice were much more sensitive to the carcinogen methylcholanthrene (MCA) and developed more tumours and showed shorter tumour latency.[125] Mice from other strains that were IFN-γ knockout showed a higher incidence of spontaneous lymphomas and lung adenocarcinomas.[126] Other evidence has come from studies of mice which were lacking perforin (perforin$^{-/-}$). These mice were again more susceptible to tumours induced by the carcinogen MCA.[127] These mice were also found to have an increased spontaneous incidence of lymphomas.[128] Perforin is a component of the cytolytic granules that are mediators of CD8+ and NK cell cytotoxicity. There is presumably a role for these granules, and the cells from which they come, in the defence of the body against lymphoma.

Major advances in the study of immunosurveillance came with the development of mice that were lacking either of the recombination activating genes (RAG-1 and RAG-2). These enzymes are involved in the repair of double stranded DNA breaks and they are expressed exclusively in the lymphoid department. When the carcinogen methylcholanthrene was administered to these mice they developed many more tumours than their fully

immunocompetent counterparts. In addition many more of these mice developed spontaneous tumours.[129] Consequently there are now considerable data from murine studies that confirm the role of immunosurveillance in controlling the development of spontaneous and induced tumours in mice.

Evidence has also begun to emerge in humans that immunosurveillance also has a role in suppressing cancer development. It has been known for some time that patients who are immunosuppressed and who are immunodeficient are more likely to suffer cancer. However these patients are also more likely to suffer viral infection and this is known to increase the incidence of tumours, such as non-Hodgkin's lymphoma, Kaposi's sarcoma, cervical carcinoma and anal carcinoma.[130] More recently it has also become clear that spontaneous tumours that are non-viral in origin are also more common in immunosuppressed patients.[131] A major review of renal transplant patients in the Nordic countries showed a much higher incidence of cancer for a range of non-virally related cancers (such as colon, lung, bladder, kidney and endocrine) than in the general population.[132]

It has also been discovered in several tumour sites that the presence of lymphocytes in a tumour is correlated with an improved outcome. In a study of cutaneous melanoma the degree of tumour infiltration with lymphocytes was determined. Patients who had the highest degree of infiltration lived up to 3 times longer than patients with no lymphocyte infiltration.[133] In a study of patients with colorectal cancer the degree of tumour infiltration with CD8+ T cells within cancer cell nests was determined. Patients who had tumours that were most significantly infiltrated with these CD8+ cells had a better survival.[134] This has now been repeated in many different tumour types. However the correlation of these immune cells in patients with an improved outcome does not necessarily imply a causative relationship.

Further evidence came from the study by Albert et al. Paraneoplastic cerebellar degeneration (PCD) has been described in patients suffering from carcinoma. It is defined by the presence of antibodies against the tumour and the brain antigen cdr2, found in the patient's spinal fluid and serum. Nevertheless this humoral response has not been shown to be pathogenic. This study looked at 4 such patients and demonstrated that this disease was due to a specific

cellular response. These immune cells were MHC class-I restricted CTL that recognized a fragment of cdr2. This was the first demonstration of tumour-specific CTLs that were able to lyse tumour cells and were detected ex vivo.[135] Of additional interest is the finding that several patients with PND have had tumours that have regressed with the onset of autoimmune disease.[136]

Another paraneoplastic syndrome, cancer-associated retinopathy (CAR), has provided evidence for the role of immunosurveillance in humans. It is characterised by an autoantibody to recoverin which is thought to contribute to the pathogenesis of retinopathy, including apoptosis of retinal cells. It is known that cancer patients who also have the paraneoplastic syndrome, CAR, have a better prognosis. Subsequently Maeda et al demonstrated higher numbers of recoverin-specific CTL in these patients, suggesting that the peripheral activation of recoverin-specific antitumour CTL was likely to contribute to the preferable prognosis of CAR(+) cancer patients.[137]

Half of all patients with small cell lung carcinoma (SCLC) and the paraneoplastic syndrome limbic encephalitis have anti-Hu antibodies that react with central and peripheral nervous system neuronal nuclei. It has been shown that the presence of these antibodies was associated with limited disease stage, complete response to therapy and longer survival.[138] Patients with this condition have been shown to have HLA class I-restricted, CD8-positive cytotoxic T cell activity against five peptides derived from this protein.[139]

All of these studies provide evidence that the immune system may play a role in eliminating cancer cells, even without immunotherapy. But what are the target antigens within these tumour cells that can be recognized by the immune system?

1.4.2. Identifying tumour antigens

The early tumour antigens were discovered by immunohistochemical staining as they were expressed at very high levels on the surface of tumours. Some of them were also secreted at very high levels into the patient's sera. More recently antigens have been discovered by 3 principle methods: genetic methods, reverse immunogenetics and peptide elution.

a. Genetic methods

Different strategies have been employed to discover epitopes from within tumour antigens by using both in vivo and in vitro sensitized T cells. In patients it has been demonstrated that mixed cultures of tumour cells and lymphocytes were able to generate CTL that can lyse autologous tumour.[140,141] In this way Herin et al were able to generate several CTL clones that could lyse an autologous melanoma. Antigen-loss variants of these tumour cells were obtained by selecting cells that were resistant to these same CTL.[142] In this way 6 different antigens capable of generating an immune response were discovered. A cosmid library, encoding fragments of DNA from the tumour cell genome, was used to transfect an antigen loss variant tumour line. The original T cell clones were then used to further characterize which fragments of the genome excited a response from the CTL. In this way the gene MAGE-1 was discovered. Once the gene responsible for producing a CTL response was elucidated, overlapping synthetic peptides covering this truncated antigen fragment were loaded onto target cells to define the minimal T-cell stimulating sequence.

This methodology, using genomic DNA in cosmid transfectants, is both technically demanding and a lengthy process. Latterly cDNA libraries have been prepared from the RNA of tumour cells and co-transfected with HLA genes into simian cos-7 cells. In this way antigens have been expressed from the cells. Again CTL generated from patients were used to discover which antigens had stimulated an immune response in patients. In this way tyrosinase and MART-1 were discovered as antigens that stimulate a CD8+ lymphocyte response. [143,144]

Another approach was based on the analysis of recombinant cDNA expression libraries of human tumours with autologous serum. A large proportion of cancer patients contain antibodies against both surface and intracellular proteins of cancer cells.[145] Therefore the sera of cancer patients can be used to highlight which tumour antigens have elicited a humoral response from these patients. In this way NY-ESO-1 was identified.[146] A repertoire of genes encoding proteins that have elicited an antibody response in cancer patients can be found at http://www.licr.org/SEREX.html.

b. Reverse immunogenetics – epitope prediction

In many different tumours it has been difficult to isolate T cells that have been activated in vivo by tumours and therefore it is not possible to identify tumour antigens and their epitopes using the "genetic methods" above. In this situation an alternative strategy termed "reverse immunology" or "reverse immunogenetics" has been applied. Peptides originating from endogenously expressed antigens of tumour cells are expressed in the context of the class I molecule. However, certain peptides bind more avidly to the class I molecule than others. There are two main factors determining the avidity of this interaction. Firstly, the peptide binding groove of the class I molecule requires peptides of a defined length (between 8 and 11 residues). Secondly, the peptide binding groove of the class I molecule has 2 pockets that display a marked preference for specific amino acids. In the case of the HLA-A2.1 molecule peptide residues two and nine are important in the peptide-class I complex interaction. These are termed anchor residues. Knowing the preferences of different class I molecules for different residues within any given peptide has enabled the prediction of the avidity of this specific peptide-class I interaction. It is now possible to use computer prediction programs that will screen antigenic sequences for motifs that are predicted to bind to a selected HLA molecule (http://www.syfpeithi.de/ and http://bimas.dcrt.nih.gov/molbio/hla_bind/). These predicted peptides can then be synthesized and used for primary in vitro stimulation of naïve T cells with autologous loaded dendritic cells. One of the principal problems with this approach is that whilst it demonstrates the in vitro immunogenicity of any given peptide sequence it does not demonstrate the processing and presentation of that epitope by a target

tumour cell. This can be predicted, to some extent, using a proteasomal cleavage algorithm such as that found at http://www.paproc.de/. But there are many limitations with this predictive algorithm. One of the more important factors is that it does not predict the activity of the immunoproteasome. This catalytic complex of proteins is found in APCs and will destroy some epitopes[147] whilst being necessary for the production of other epitopes.[148] Consequently the ability of the APCs to present any given epitope and the ability of the tumour to present the same epitope has to be independently verified. In vitro-generated CTL lines can be tested for their ability to specifically lyse tumour cells expressing the whole antigen and the relevant MHC class I molecule.

c. Peptide elution

A third way of identifying T cell epitopes is by peptide elution. This approach is based on elution of antigenic peptides from target cells and subsequent determination of peptide sequences with the help of reverse phase HPLC fractionation and Edman degradation. The direct identification of minute amounts of peptides has improved with the advent of sensitive mass spectrometrical methods. Recently, a series of peptides from tumour cell lines[149] and primary tumour tissue[150] were identified with the help of mass spectroscopy.

1.4.3. Tumour antigens

Antigens that are suitable targets for the immune response can be divided into tumour-specific antigens (TSA), which are exclusively expressed by tumour cells, and tumour-associated antigens (TAA), that are expressed on normal tissues but at much lower levels than on the tumour.

There are at least seven categories of tumour antigens that are summarized in table 2. The first group of tumour specific antigens are generated as a result of the mutation of a tumour-suppressor gene or an oncogene. These are ideal antigens as they are expressed exclusively by

tumour cells. Point mutations generate unique epitopes for targeting. However the number of novel epitopes generated in this way is low and restricts the use of these antigens for targeting tumours.

A second group of tumour specific antigens are found on B cell tumours. Each B cell lymphoma expresses a B cell idiotype representing a prototypical tumour antigen target expressed by the tumour clone. The generation of antibody to this idiotypic antibody has been shown to be effective at inducing protection to tumour challenges. [151]

Approximately 15% of tumours world-wide are associated with viral infections. The third group of antigens, of viral origin, are critical in the oncogenic process and are good at evoking a T cell response and therefore make good vaccine targets. These viral antigens are derived from hepatitis B and C, human papilloma virus and Epstein-Barr virus.

The fourth group of antigens are abnormally expressed in tumour cells. An example is HER-2/neu which is a receptor tyrosine kinase homologous to the epidermal growth factor receptor (EGFR). This receptor is over-expressed in many adenocarcinomas. It has been targeted successfully by antibody therapy.

The fifth group of antigens are proteins that are abnormally expressed by tumour cells and are also post-translationally modified. Both MUC-1 and CEA come into this category as they are both under-glycosylated when they are expressed by the tumour.

The sixth group of antigens are the lineage-specific antigens. These are normally expressed only by tissues of a specific lineage. The best examples are those antigens only expressed in the skin in the normal pathways of the production of the pigment melanin. These proteins are also found in melanoma tumours.

The last group of antigens are normally expressed only in male germ cells. These cells do not express MHC class I molecules and consequently do not present peptides from these antigens to T cells. These tumour antigens are therefore effectively tumour specific antigens.

Class of antigen	Antigen	Tumour-specific ?	Tumour types involved
Mutated tumour suppressor genes and oncogenes	Bcr-abl fusion protein	YES	CML
	Mutated p53	YES	Found in 50% of solid tumours
	Cyclin-dependent kinase 4	YES	Melanoma
Idiotype Ig	TCR/ BCR	YES	T-cell and B-cell lymphomas
Viral antigens	EBV	NO	Burkitt's lymphoma
	Human papilloma virus	NO	Cervical Carcinoma
Abnormal gene expression	HER-2/neu	NO	Breast
Post-translationally modified proteins	CEA	NO	Gastro-intestinal tumours
	MUC-1 (Mucin-1)	NO	Breast, gastro-intestinal tumours
Differentiation antigen	Melan-A	NO	Melanoma
	Tyrosinase	NO	Melanoma
Germ cell	MAGE-1	NO	Melanoma and multiple others
	NY-ESO	NO	Melanoma

Table 2. Tumour antigens

Tumours are distinguishable from normal tissues by these numerous tumour antigens. There is also evidence that immunosurveillance happens in vivo. How, therefore, do tumours continue to grow and metastasize in the full view of the immune system?

1.4.4. Tumour evasion of the immune system

Human tumours are often genetically unstable and as a result a population of cells can lose the expression of antigens by mutation. If there is a selection pressure from the immune system this could select out a population of cells that could "escape" the immune response. Certainly it has been well described that some solid tumours can down-regulate MHC class I expression.[152] There are many mechanisms by which this down-regulation can occur. This includes the mutations in the gene for β2-microglobulin. This has also been seen in T cell-based immunotherapy.[153] Down-regulation of class I has also been described with defects in the MHC genes and in the antigen processing and transport pathway. Defects in LMP-2 and LMP-7 have been described as well as in peptide transporters TAP-1 and TAP-2.[154,155] Some of these effects are partly reversible, in some tumours, by the administration of IFN-γ.

Loss of tumour antigen expression is another feature of tumour progression. Decreased expression of gp100, melanoma antigen recognized by T cells-1 (MART-1) and tyrosinase in melanoma tumours is associated with disease progression.[31,156]

A further potential mechanism for immune evasion is defective death receptor signalling. Normally the ligation of Fas by its ligand (FasL) signals through the Fas-associated death domain (FADD) which activates a caspase cascade. The caspase-8 inhibitor, cellular FLICE-inhibitory protein (cFLIP), is expressed in a number of tumours. This expression of cFLIP may allow tumour cells to be resistant to Fas signalling in vivo.[157] In addition the loss of Fas expression in tumours may abrogate this mechanism.[158] Furthermore, numerous tumours have aberrant TRAIL expression by several mechanisms including chromosomal loss and mutation.[159]

Tumour cells also secrete a wide range of cytokines and chemokines that can negatively affect the immune response. Increased concentrations of the cytokine IL-10 are frequently detected in cancer patients. This cytokine can not only inhibit the differentiation of DCs from precursors but also inhibit the maturation from immature DC to the highly activating mature DC. This cytokine also inhibits IL-12 production and the induction of T helper type 1

responses.[160] The inhibitory cytokine TGF-β was originally discovered in supernatants from tumour cells. The immunosuppressive nature of this cytokine and its ability to inhibit immune cells from both the adaptive and innate immune response is well described.[161] In addition tumours also secrete vascular endothelia growth factor that inhibits DC differentiation and maturation through suppression of NF-κB in stem cells.[162] With the secretion of so many inhibitory cytokines the tumour microenvironment is not conducive to T cell activation or indeed to DC activation. Tumour antigen uptake, processing and presentation in these circumstances will not lead to DC activation and maturation. This may lead to T cell anergy and suboptimal activation of NK cells.

A controversial potential tumour escape mechanism is the expression of death receptor ligands by tumour cells. FasL has been shown to induce apoptosis in Fas-expressing target cells. Therefore the description of FasL expression on several tumour cell lines was thought to be a mechanism whereby tumours could evade the immune response. However there were concerns over this work in that several of the antibodies used were non-specific.[163] Other studies have not detected FasL expression on these same melanoma cells.[164] However this second study detected activation-induced cell death of anti-tumour T cells. It was shown that after activation T cells express high amounts of FasL and it was deduced that this induced apoptosis of these tumour-specific T cells and also bystander T cells.[164,165]

Another mechanism of immune evasion is by the creation of immunologically privileged sites. Many solid tumours grow as nodules surrounded by physical barriers such as collagen and fibrin. The cancer cells within these may be invisible to the immune system.[65] In this way tumours create immunological ignorance.

Lastly the immunoregulatory CD4+ CD25+ T cells known as Tregs also seem to be found in higher numbers in certain groups of patients with cancer. Recently Treg in the peripheral blood, amongst the tumour-infiltrating lymphocytes, and in the regional lymph node have been isolated from 65 patients with either pancreas or breast cancer.[166] Another study has shown that lung tumours contain large numbers of these cells and that they have constitutive

high-level expression of CTLA-4. They also demonstrated that the CD4+CD25+T cells mediated potent inhibition of autologous T cell proliferation.[167]

Many of these mechanisms described above could disrupt either the generation of the immune response or the efferent pathways that carry out target cell elimination. The manipulation of the immune response by vaccination may be able to circumvent the mechanisms that interfere with the generation of the immune response. If this is possible then these vaccination strategies will need to be combined with strategies that tackle the mechanisms that disrupt the effector immune response.

1.5. Immunotherapy of cancer

1.5.1. Passive antibody therapy

Several tumour antigens are expressed on the surface of the tumour and these antigens can be targeted with monoclonal antibodies. Two striking successes of this strategy have already been mentioned. A humanized monoclonal antibody known as herceptin targets the growth factor receptor, HER-2/neu. Early analyses of three phase III randomized controlled trials provide solid evidence that herceptin increases progression-free survival, time to first distant recurrence, and overall survival in women with localized invasive breast cancer. The result was highly statistically significant for all women, regardless of age, hormone receptor status, tumour size or the number of positive nodes. Indeed this very encouraging result has meant that two of these trials have been stopped.[168]

Among the mAbs under clinical development for cancer therapy, anti-CD20 mAbs have been most extensively investigated and have shown definitive clinical efficacy. Rituximab is a genetically engineered chimeric anti-CD20 mAb, with mouse variable and human constant regions. Consecutive clinical trials conducted in the US, Europe and Japan[169] have revealed that rituximab is a highly effective agent with acceptable toxicities against indolent and aggressive B cell non-Hodgkin's lymphomas (B-NHLs) as a single agent and in combination

with cytotoxic drugs. A recent French Phase III study in elderly patients with untreated aggressive B-NHL suggested that the addition of rituximab to standard CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) chemotherapy increases the complete response rate and prolongs event-free and overall survival.[170]

In addition antibodies have been combined with toxins to produce immunotoxins. Two commonly used toxins are ricin A chain and Pseudomonas toxin. When a recombinant anti-CD22 antibody was fused to a fragment of Pseudomonas toxin this induced complete remissions in patients with hairy-cell leukemia that was resistant to treatment with purine analogues.[171]

1.5.2. Adoptive T-cell therapy

One approach to generating cellular immunity against tumours is to generate T-cell clones specific for tumour antigens ex vivo.[172] There are many problems with this approach including the huge investment in cost and time in generating these T cell populations. It is also possible that these clones having been handled ex vivo for so long may not have normal homing and survival.[173] There is some evidence of antitumour responses to EBV-induced T-cell clones in patients with EBV-induced posttransplantation lymphoproliferative diseases.[174] However the main concern with these tumour-specific T cell clones has been survival of the cells when they are returned to the patient. This may be partly circumvented with the co-administration of IL-2.[175] This is further discussed in section 4.1.4.1.

Sibling bone marrow transplantation (BMT) has been used in the treatment of various haematological malignancies for many years. Horowitz et al. analysed the clinical results of different sub-groups of patients undergoing this procedure.[2] They found that patients who received non-T-cell depleted transplants who also suffered with graft versus host disease (GVHD) showed decreased cancer relapse when compared with patients who received non-T-cell depleted transplants without GVHD. These data supported an anti-leukaemic effect of GVHD. In addition CML patients who received grafts that were T-cell depleted had higher

relapse rates that those that received grafts that were non-T-cell depleted. This suggests that the mediators of this anti-leukaemic effect were the T cells.

The clinical efficacy of transferring lymphocytes in haematological malignancy has been further emphasized by the results of the administration of donor lymphocyte infusions (DLI) to patients with relapsing disease after allogeneic BMT. Durable remissions have been achieved with this treatment modality particularly in patients with chronic myeloid leukaemia.[176,177] Clinical studies have demonstrated that the DLI-mediated graft-versus-leukaemia effect correlated with a significant rise in alloreactive T cells.[178] Although graft-versus-host disease is a complication of DLI, T-cell dose-escalation allows tumour responses to be achieved without this life-threatening side-effect.[179]

1.5.3. Active vaccination

There have been a plethora of vaccination strategies to stimulate immune responses to cancer. Initially, without the precise definition of tumour antigens autologous tumour cells were used in vaccines. At first these tumour cells were simply irradiated. More recently tumour-cell vaccination, using autologous tumour cells transfected with granulocyte-macrophage colony-stimulating factor (GM-CSF), led to promising results.[180] This approach has gone onto phase I studies in patients. Another approach is to transfect into these autologous tumour cells co-stimulatory molecules. Tumour cells transfected with B7 using this approach have generated significantly improved immune responses against the tumour.[181] In addition malignant B cell tumours have been stimulated by ligation of CD40 and these cells up-regulate co-stimulatory molecules and stimulate T cell immunity.[182] This approach has also gone forward to a phase I trial.[183]

Once tumour antigens became more clearly defined the antigens themselves, or fragments of these antigens, were used to vaccinate patients. For instance the clonal idiotypic protein derived from the murine B cell lymphoma, BCL1, was used to protect mice from tumour challenge.[184] The idiotypic protein from B cell lymphoma was also conjugated to KLH

(keyhole limpet haemocyanin) and this conjugate protein was administered with soluble GM-CSF. Vaccination was associated with clearance of residual tumour cells from the blood of patients and long-term disease-free survival.[185] In an effort to target T cells, peptide vaccination has also been used. This has generated T cell responses and the anti-tumour effects of these responses have been demonstrated in vitro. In addition peptide vaccination is easy and has little toxicity associated with its administration. But disparities exist between in vitro and clinical responses.[186]

Using dendritic cells to vaccinate against specific antigens is attractive as these are known to be the most potent APC in the immune system. DCs are loaded with antigen in many different ways: using either peptide[187], protein[188], cellular debris, exosomes[189], apoptotic tumour cells, viruses[190], DNA and RNA[191]. They are clearly very potent stimulators of immunity. However to produce clinical grade DC vaccines is extremely expensive, both in terms of time and finance.

Viruses have also been used extensively to induce immunity. Viral vaccines can be injected directly into patients without the need for DCs. Vaccinia has been the most widely used viral vector for experimentation on viral vaccines against cancer. It has a short life cycle and rapid spread, strong lytic ability, a large cloning capacity and well-defined molecular biology. More recently modified vaccinia Ankara (MVA) has been used because of its limited replication potential. Adenovirus has also been extensively used to deliver tumour antigens for vaccination. Nevertheless one recent report highlights difficulties in using viral vectors. Investigators have shown functional exhaustion and deletion of CTL following immunization with a recombinant adenovirus. This seemed to be related to a large antigen load in the peripheral tissues.[192] Alphavirus has also been used and has shown much promise as a vaccine vector.[193] Viruses are often used in prime-boost protocols that are discussed later. This is partly because they produce large amounts of antigen and have been shown to be very effectively used in conjunction with DNA vaccination.

1.6. DNA vaccination

Wolff and collaborators demonstrated that the injection of plasmid DNA into murine muscle led to the expression of gene-encoded proteins from within this DNA. They demonstrated that luciferase and B-galactosidase activity could be detected from this murine tissue, demonstrating that these genes were successfully transcribed and translated.[194] Subsequently a DNA plasmid encoding a nucleoprotein from influenza virus induced antibody, T cell responses and protected animals against viral challenge.[195] DNA vaccines comprise a circular plasmid backbone and a gene insert that encodes the antigen of interest. This gene is placed under the influence of a strong viral promoter. It is immediately preceded with a short Kozak sequence which enables ribosomal recognition.[196] These vaccines also incorporate a number of other genes including antibiotic resistance genes and a polyadenylation sequence that serves to enhance the stability of the mRNA. DNA vaccine can influence both innate immunity and the adaptive immune response.

1.6.1. DNA vaccines and innate immunity

The plasmid backbone consisting of bacterial DNA contains several immunostimulatory sequences. These are unmethylated CG dinucleotides or CpG motifs with specific flanking residues. These motifs are found in bacterial genomes at a 20-fold greater frequency than in mammalian DNA.[197]

Recently Toll-like receptor 9 (TLR-9) has been identified as the "pattern recognition receptor" for bacterial DNA in both mice and humans.[13] TLR-9 is an intracellular receptor expressed by DCs, NK cells, B cells and macrophages. As this receptor is intracellular the CpGs require cell uptake for activation to occur. Interaction between CpGs and TLR-9 activates the Toll/IL-1 signalling pathway and triggers an inflammatory response. CpGs have multiple effects including the secretion of multiple cytokines including TNF-alpha, IL-6, IL-12 and IFN-gamma.[198] Krieg et al showed that optimal B-cell activation requires a DNA motif in which an unmethylated CpG dinucleotide is flanked by two 5' purines and two 3'

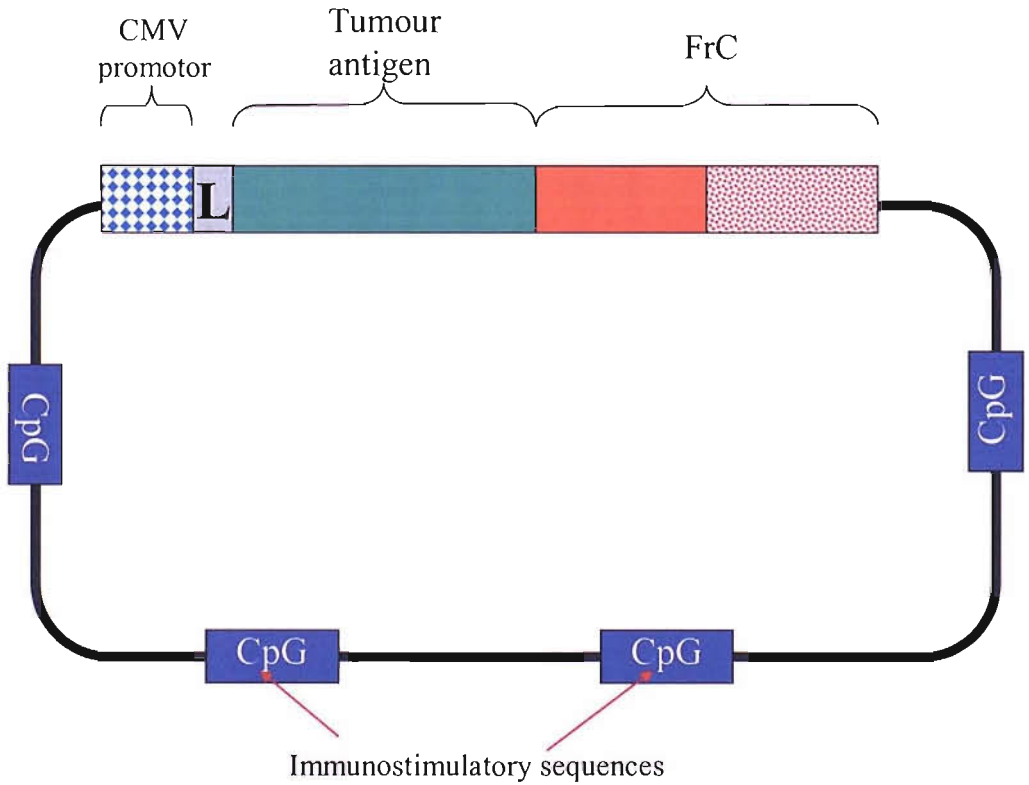


Figure 6 - Diagram of DNA fusion vaccine for eliciting immunity against a tumour antigen

pyrimidines.[199] Oligodeoxynucleotides containing this CpG motif induce more than 95% of all spleen B cells to enter the cell cycle. He hypothesized and later demonstrated that there are specific dinucleotide motifs that appear less commonly in vertebral DNA than in bacterial DNA.[199]

The cellular response to bacterial DNA is mediated by a Toll-like receptor, TLR-9. TLR-9-deficient (TLR-$9^{-/-}$) mice did not show any response to bacterial DNA, including proliferation of splenocytes, inflammatory cytokine production from macrophages and maturation of dendritic cells. Also TLR-$9^{-/-}$ mice showed resistance to the lethal effect of bacterial DNA without any elevation of serum pro-inflammatory cytokine levels.[13]

It has also been shown that human TLR-9 expression in human immune cells correlates with responsiveness to bacterial DNA (deoxycytidylate-phosphate-deoxyguanylate –DNA also known as CpG). The ability to respond to immunostimulatory CpG-DNA can be achieved by expressing TLR-9 in human cells that do not respond to CpGs under normal circumstances. Transfection of either human or murine TLR-9 conferred responsiveness yet required species-specific CpG-DNA motifs for initiation of the Toll/IL-1R signal pathway via MyD88. The optimal CpG motif for human TLR-9 was GTCGTT, whereas the optimal murine sequence was GACGTT.[200]

However it is clear that CpGs are diverse and that different CpGs activate different sub-types of cells. Using synthetic oligodeoxynucleotides (ODNs) groups have shown certain CpGs activate murine B cells, macrophages and DCs but not T cells or NK cells.[198] In mice this correlates with the cell types that have been shown to express TLR-9.

CpGs stimulate B cells to proliferate and up-regulate co-stimulatory molecules. Whilst CpGs do not interact with T cells directly the CD4 response becomes skewed by the cytokine milieu to a T_H1 response. CpGs also act on APCs to up-regulate co-stimulatory molecules CD80 and CD86, up-regulate MHC class II leaving them with a mature phenotype capable of priming a CD8 immune response.

A recent study has looked at TLR-9 expression on human NK cell. Flow cytometric analysis revealed that a third of human NK cells expressed basal levels of TLR-9. This expression of TLR-9 in human NK cells was confirmed by immunoblot analysis. Human NK cells cultured in the presence of CpG-ssODN plus immobilized IgG or antibody-coated tumour cells secreted large amounts of IFN-γ, whereas cells stimulated with Ab alone or CpG-ssODN alone did not.[201]

Most of the studies investigating the properties of CpGs have been performed on oligonucleotides with comparatively few studies looking at whole plasmid DNA. However, more recently, plasmid DNA has been shown to induce a TLR-9-dependent DC activation.[202] In this study plasmid DNA was found to be less efficient than ODNs containing CpGs.

1.6.2. DNA vaccines and the adaptive immune response

After DNA plasmid injection there is in vivo transcription and translation of the target gene. As described above this was first described in muscle cells. Tang et al showed that the delivery of plasmid DNA–coated particles directly into the skin of mice using a biolostic device could elicit a specific immune response against the encoded protein.[203] When DNA vaccines are delivered by this intradermal route the skin-derived DCs (Langerhans cells) are directly transfected and were thought to play a key role in antigen presentation to T cells. Subsequently Cho and colleagues used tissue-specific promoter-directed gene expression and were able to demonstrate that, even in cutaneous delivery of DNA vaccine by gene gun, the predominant mechanism of CD8+ T cell responses was by cross-priming.[204]

There is evidence from the intramuscular injection of fluorescent labelled plasmid DNA that there is uptake of DNA by the muscle cells. However it is not certain whether the immune response is generated by cross-priming of these cells or by direct transfection of APCs. Bone marrow derived APCs are certainly required for an immune response. Doe et al studied severe combined immunodeficiency mutation (SCID) mice that expressed either H-2d or H-2b.

These were re-constituted with spleen cells from immunocompetent F1 mice (H-2b/d) and then vaccinated with a DNA plasmid encoding either an H-2d or an H-2b restricted class I epitope. CTL responses were only generated when the recipient SCID haplotype matched the haplotype of the DNA vaccine epitope. They then re-constituted SCID mice with spleen cells from immunocompetent F1 mice with or without bone marrow from these mice. When chimeric bone marrow was also transferred then H-2b SCID mice were able to mount a CTL response against an H-2d epitope. However without the bone marrow transfer these mice had no CTL response to the MHC mismatched epitope plasmid. This study suggests that bone marrow derived cells, presumably DCs, are involved in the class I MHC presentation of viral antigens after i.m. immunization with plasmid DNA.[205] Other experiments using bone marrow chimeras performed by Corr and colleagues have confirmed the central role of bone marrow derived APCs in DNA vaccination.[206] However there is evidence for direct transfection of APCs leading to MHC class I processing and presentation.[207]

1.6.3. DNA vaccines for inducing immunity against cancer

The initial focus in this laboratory was using DNA vaccines to induce immunity against the idiotypic determinants (Id) of the immunoglobulin expressed by B cell tumours.[151,208] This clonally expressed target represents a tumour-specific antigen. When mice were immunised with DNA encoding Id V genes in a single chain variable fragment (scFv) format there was no induction of anti-Id antibody. But by fusing the gene encoding the Id to Fragment C (FrC) of tetanus toxin the performance of the vaccine was dramatically enhanced. Antibody responses were seen in the animals against the Id protein from the tumour and this response protected against a lethal challenge of tumour cells. Importantly fusion of the gene encoding Id to the gene encoding FrC was required since the delivery of the vaccine via two separate plasmids, one encoding the Id and one encoding FrC, was unable to induce a response.[151]

The mechanism behind the success of this fusion DNA vaccine can be explained by current knowledge of how T-cell help is provided to B cells (Figure 7). FrC is a highly immunogenic derived sequence that contains several MHC class II epitopes. One of these is a universal

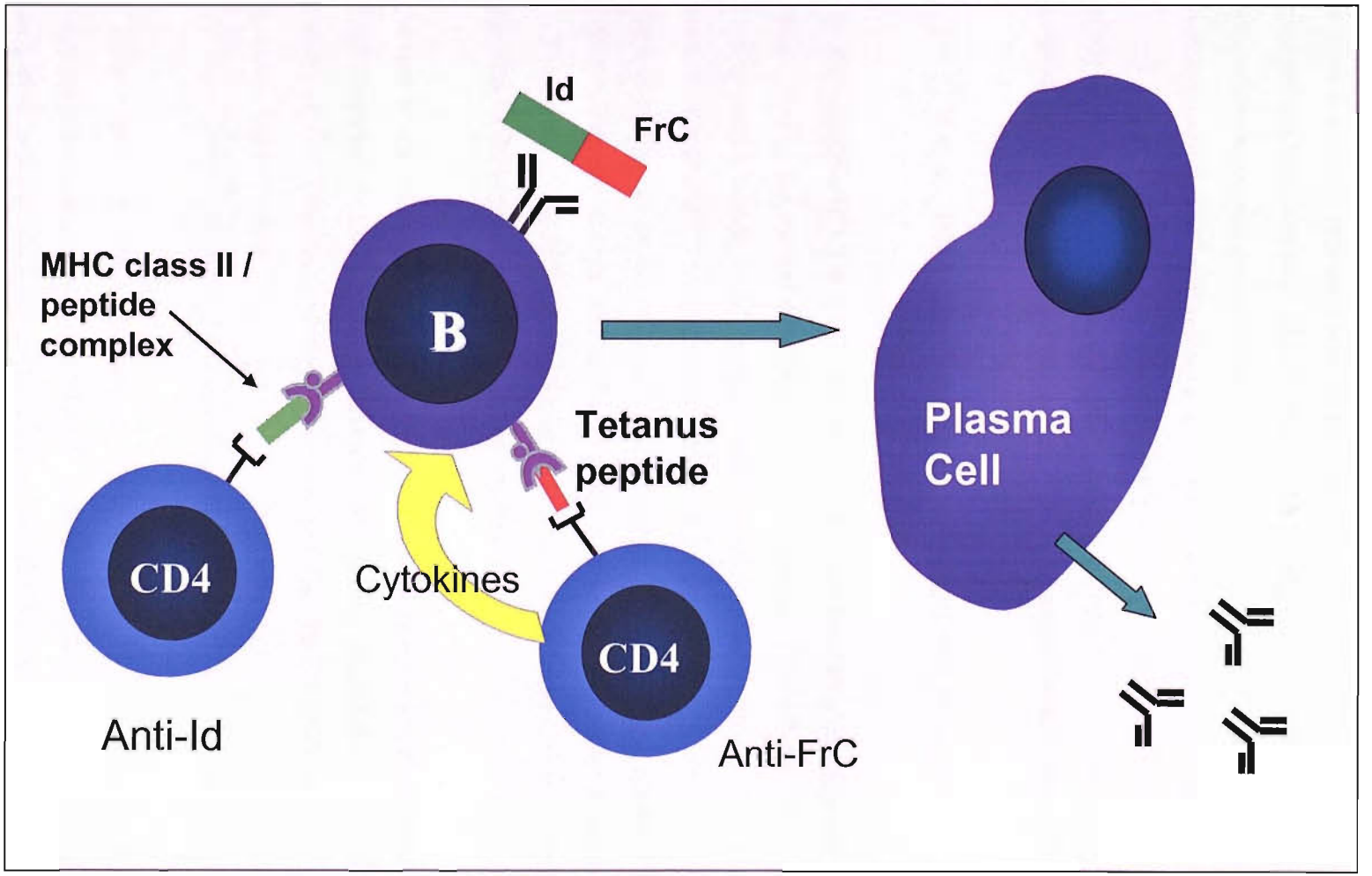


Figure 7. Proposed mechanism of action of FrC-Idiotypic protein vaccine

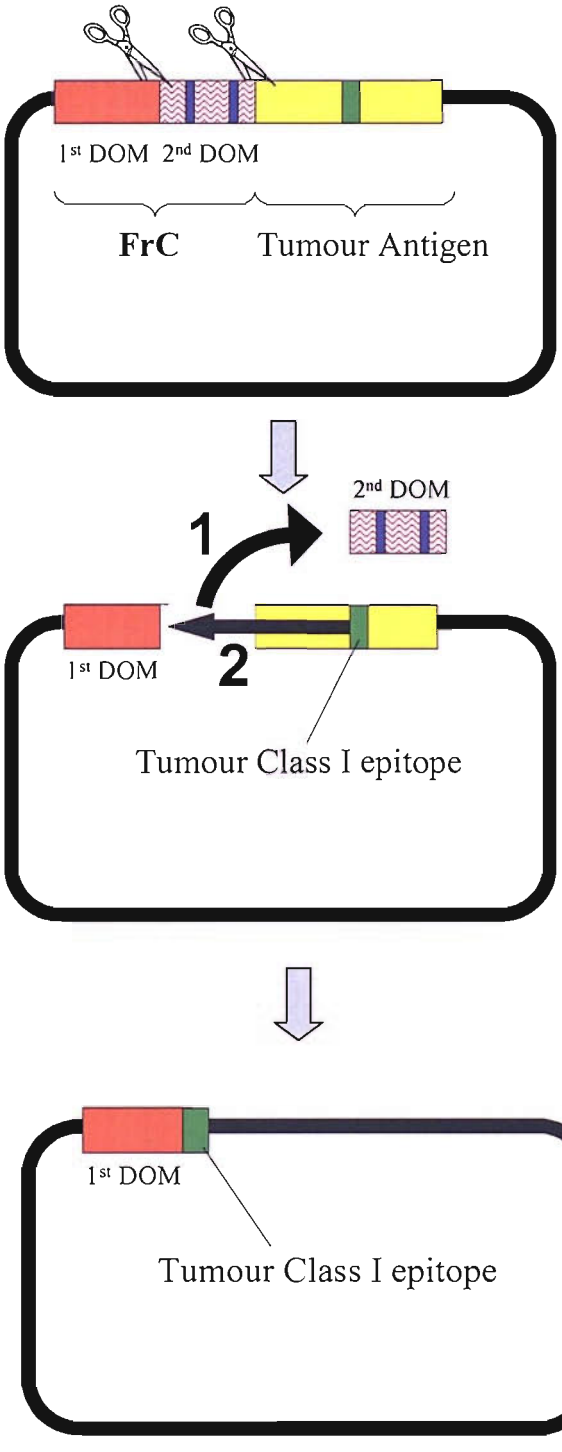
MHC class II epitope within the first domain known as p30.[209] This epitope has been shown to bind to a range of mouse and human class II haplotypes.[210, 211] The proposed mechanism involves the fusion protein Id-FrC binding to the sIgM on antigen-specific B cells. These B cells then process and present MHC class II peptides from both Id and FrC to CD4+ T cells. The repertoire for FrC will be large, as several class II epitopes have been described.[99] CD4+ T cells recognising these FrC epitopes will provide co-stimulatory molecules and cytokines which allow differentiation of the anti-Id specific B cells. Whilst CD4+ T cells specific for Id are produced they appear inadequate in promoting an antibody response.

1.6.4. DNA vaccines to induce CD8 responses

For the induction of CD8 T cell responses there have been two changes made to the vaccine design. Firstly, the second domain of FrC was removed. This domain includes several class I binding motifs which could compete with tumour-derived epitopes. The first domain was retained as it included the leader sequence and the promiscuous MHC class II epitope (p30). The second change to the vaccine was to remove the target tumour epitope from its position within the tumour antigen and to fuse it to the C terminus of the first domain of FrC (Figure 8). The combination of the first domain of FrC with the tumour target epitope fused to the C terminus led to high levels of CTL in two models.[212]

The first model using class I epitopes from the second domain of FrC itself showed that CD8 T cell responses to these could be enhanced if they were placed at the C terminus of the first domain of FrC and the second domain was omitted. Poor CD8 responses were seen to a candidate peptide when it was fused to the whole FrC molecule. However when this epitope was fused to the C terminus of the first domain a high level of CD8 immunity was induced.[212]

A follow-up study looked at immune responses to an H2-Ld-restricted epitope from the retroviral gene product gp-70, called AH1. A DNA vaccine encoding the whole gp-70 antigen was a poor inducer of CTL and this was not significantly improved by the fusion of the full length FrC molecule. Again, high levels of CTL were induced with the minimised FrC construct described above; with the AH1 epitope fused to the C-terminus of the first domain



1. Removal of the second domain of FrC with its potentially competing epitopes

2. Taking the tumour class I epitope out of its embedded site and placing it at the C terminus of the first domain of FrC

Fusion vaccine consisting of the first domain of FrC fused to the tumour class I epitope

Figure 8. Schematic representation of the modifications to the FrC-tumour antigen DNA fusion vaccine that were necessary for the effective induction of CD8+ T cell immunity.

of FrC. The vaccine was able to provide protection against tumour cells that expressed this antigen and to kill these tumour cells in vitro.[213]

The mechanism for the enhanced success of this vaccine design is again thought to be due to the provision of T cell help (Figure 9). Either the DNA vaccine or the protein translated from the vaccine is taken up by the APCs. This leads to the presentation of the class II epitope from FrC on the APC surface. Responding CD4 cells then provide licensing signals to the DC to mature and thereby increase its capability of priming T cell responses. The class I epitope encoded within the vaccine is presented on the same licensed APC and a CD8+ T cell response is primed.

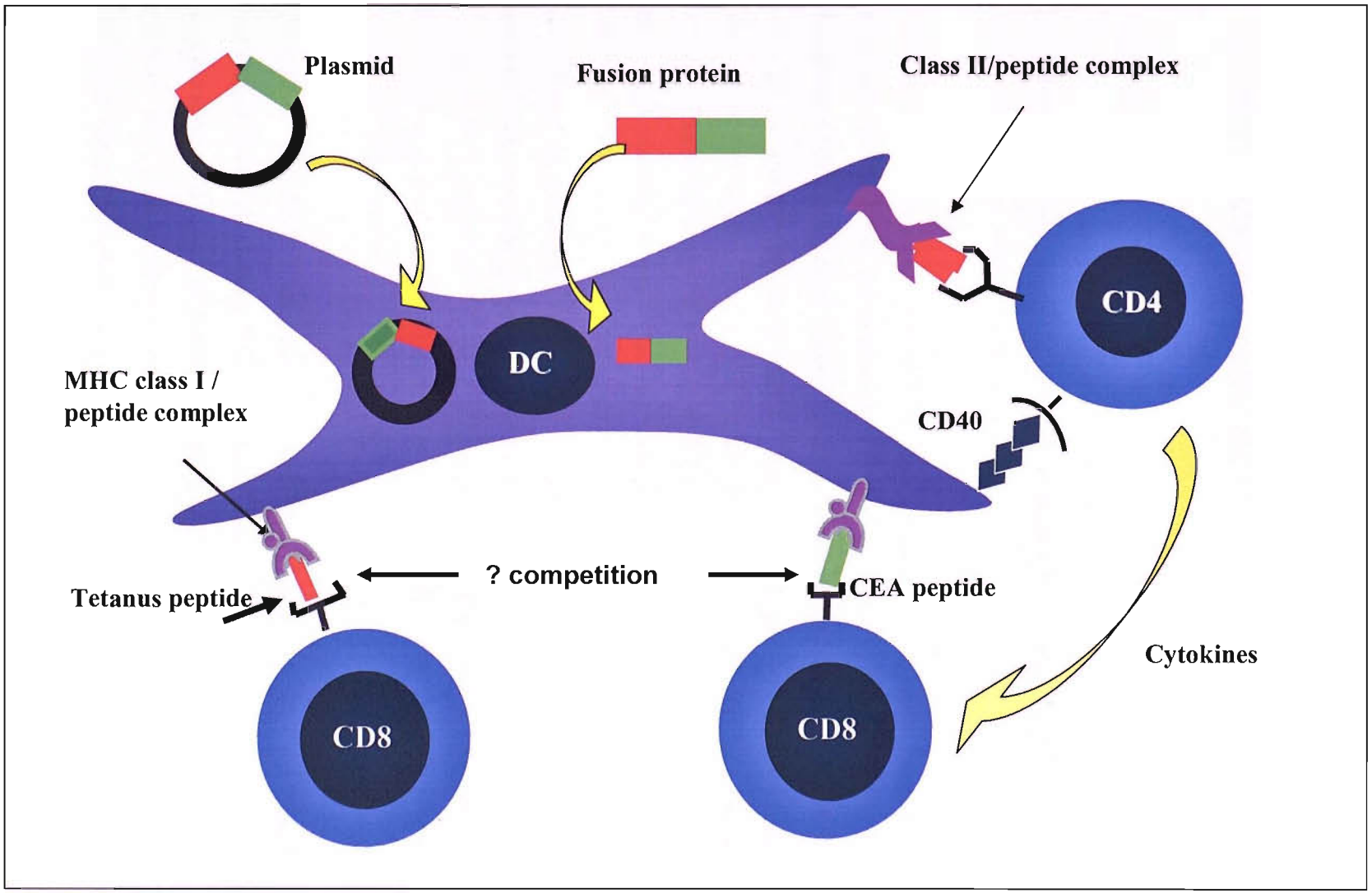


Figure 9. Potential mechanism for DNA vaccines capable of inducing CD8+ responses

2 Materials and methods for chapters 3 and 4

2.1 Mice

C57Bl/k aged between 6 and 12 weeks were used for the experimental procedures and were bred in house under pathogen free conditions.

CEA.Tg mice were kindly provided by Dr M. Ilyas (CRUK, South Mimms, Herts. UK). A complete description of the generation of the CEA.Tg mice has been published previously.[214] Lines were established from founder animals by continuous backcrossing with C57Bl/6 mice. Only half of the progeny carry the CEA transgene and therefore PCR analysis was used to identify the CEA-positive mice. Mice were bled at 6 weeks and the DNA extracted using the DNeasy Tissue Extraction Kit (Qiagen, UK). Approximately 200ng of DNA were amplified in a 25μl reaction volume. The following CEA-specific primers were used: forward 5'-GGACTTTTTAACACAGAATTGGG-3'; reverse 5' CCTTGTGCCCATGGAACACAGAC-3'. 100mM of these primers were used together with the DNA, 0.2mM deoxynucleotide triphosphates, Amplitaq polymerase and the recommended reaction buffer. The amplification consisted of 35 cycles of 94°C for 1 minute, 55°C annealing for 1 minute and 72°C extension for 1 minute. A final step for 10 minutes at 72°C was performed. The PCR product was run on a 1% agarose gel and CEA-positive DNA yielded a 380-bp fragment.

The A2-K^b and HHD transgenic mice were provided by Dr V. Cerundolo (Institiute of Molecular Medicine, Oxford, UK). The A2-K^b transgenic mouse model is frequently used as an immunological model of human immunity and vaccine development. The mice express a chimeric HLA class I molecule HLA-A2.1/D^b composed of the α1 and α2 domains of HLA-A2.1 and the α3 transmembrane and cytoplasmic domains of H-$2D^b$ on a predominantly C57BL background. A2-K^b mice are capable of making CTL responses restricted by both HLA- A2.1 and H-2^b class I molecules. [215]

HHD mice express a transgenic chimeric monochain class I molecule in which the C terminus of the human β2-microglobulin is covalently linked to the N terminus of chimeric HLA-A2.1 (HLA-A2.1 α1 and α2 domains fused with the D^b α3 domain). H-$2D^b$ and mouse β2-m genes were disrupted by homologous recombination, resulting in complete lack of serologically detectable cell surface expression of mouse endogenous H-2^b class I molecules. On cell surfaces, both strains express similar amounts of chimeric HLA-A2.1/D^b molecules.[216]

2.2 Cells

The CEA-expressing MC38 cells (MC38-CEA) were a gift from Dr. J. Primus (La Jolla, CA, US). They had been produced by transducing the murine colon adenocarcinoma cell line MC38 with human CEA cDNA using the retroviral expression vector pBNC.[217] The cell line was subsequently cloned and routinely examined for stable CEA expression as measured by the cell surface reactivity using anti-CEA mAb COL-1. The parent MC38 line and the MC38-CEA-2 line were grown in DMEM containing high glucose and 10% heat-inactivated foetal bovine serum.
EL-4 cells were provided by Dr. J. Rice and T2-A2 cells were provided by Prof. T. Elliott. These cell lines were grown in 1640 RPMI supplemented with 10% heat-inactivated FCS and 25mM HEPES buffer (all from Invitrogen).

The EL-4 tumour was also transfected with the human CEA gene with the transmembrane domain. $5x10^6$ cells were electroporated in the presence of 25μg of plasmid DNA. The cells were pulsed at 0.3 kV and 975μF using a Gene Pulser II (Bio Rad) and cultured for 48 hours in the absence of selection antibiotic. After this time 2mg/ml of geneticin was added to the cells. These cells were then sub-cloned. The presence of CEA expressing tumours was confirmed by cell surface reactivity using the monoclonal anti-CEA mAb COL-1 as described below. This cell line showed stable expression of the molecule over a 4 month period.

Jurkat cells that had been transfected with the chimeric molecule HLA-A2.1/D^b (α1α2 domains from HLA-A2.1 and α3 domain from the murine D^b) were a kind gift from Prof.

C.A. Sherman, Scripps Institute, California. These were cultured in 0.25 mg/ml geneticin in CRPMI/10% FCS.

L929 cells were originally derived from murine connective tissue tumour. These cells had been retrovirally infected with the fully human HLA-A2.1 molecule and were a kind gift from Dr Philip Stevenson (University of Cambridge). These cells were grown in Minimum essential medium (Invitrogen) with 2 mM glutamine and Earle's BSS and contained 1.5g/L sodium bicarbonate (Invitrogen), 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate supplemented with 10% heat-inactivated FCS.

2.3 Phenotypic analysis of CEA expressing lines

The phenotype of the MC38 and the MC38-CEA lines were determined by FACS analysis. $1x10^6$ cells of interest were incubated with 0.5 μg of the following antibodies K^b (FITC, BD Pharmingen), ICAM (FITC, BD Pharmingen), IgG2a isotype control (FITC, BD Pharmingen) and the monoclonal anti-CEA: COL-1 (Zymed). The COL-1 antibody samples were washed twice and further stained with a secondary sheep-anti-mouse FITC labelled antibody. Cells were analysed using a FACScalibur, using CELLQUEST software from BD Pharmingen. Cells were gated on their forward scatter/side scatter properties.

2.4 Plasmid DNA

The expression vector pcDNA3 (Invitrogen, Paisley, UK) was used for the construction of all of the DNA vaccines used in these studies. This vector consists of a multiple cloning site that is under the transcriptional control of the promoter from the immediate early gene of human cytomegalovirus (CMV). It also contains transcription termination and a polyadenylation sequence from the bovine growth hormone gene to enhance RNA stability. There is also a neomycin resistance gene in a separate expression cassette that is under the control of the SV40 promoter. This promoter is immediately preceded by the SV40 origin of replication. There is also an ampicillin resistance gene for selection of the plasmid in bacterial cultures.

The vaccines pCEA and pCEA.FrC were kindly provided by Dr. D. Zhu. These vaccines consist of the CEA leader sequence with the 7 domains of CEA without the membrane-spanning sequence. In the case of the second vaccine this is fused to the sequence encoding both domains of FrC from tetanus toxin at its C-terminus. All vaccines in these studies were placed in the multiple cloning site with a Kozac sequence (GCC GCC ACC) placed at the beginning of the sequence.

pmembCEA consisted of the CEA leader sequence, the 7 extracellular domains of CEA and the membrane spanning sequence.

pFrC and pDOM were kindly provided by Dr D. Zhu and Dr J. Rice respectively. Both encode the Kozac sequence upstream of the leader sequence derived from the IgM VH of the BCL1 tumour fused to the beginning of the FrC sequence. In the case of pFrC all of the FrC sequence is encoded. With pDOM only the amino terminal domain of FrC is encoded.

pDOM.EAQN, pDOM.CAP-1 and pDOM.CAP-1/6D were all constructed using the amino terminal domain of FrC (pDOM) fused to sequences encoding class I epitopes from CEA. EAQN is an octamer: EAQNTTYL (H-2K^b). CAP-1 is a nonamer: YLSGANLNL (HLA-A2.1) and CAP-1/6D also a nonamer: YLSGADLNL (HLA- A2.1). p(L-)DOM.EAQN was constructed from the terminal domain of FrC (pDOM) fused to a sequence encoding EAQNTTYL but the leader sequence was omitted.

pDOM.NLV was also constructed using the amino terminal domain of FrC fused to a sequence encoding a class I epitope from the pp65 protein of HCMV: NLVPMVATV (HLA-A2.1).

2.5 Plasmid DNA assembly

pDOM.EAQN, p(L-)DOM.EAQN, pDOM.EAQD, pDOM.CAP-1, pDOM.CAP-1/6D, pDOM.CAP-1/8D and pDOM.NLV were constructed using pDOM as a template for the reaction. A standard PCR SOEing procedure was used with assembly primers used as stated

in Table 3 and Table 4. The forward 5' primer used was complementary to a sequence in the T7 sequence. The reverse 3' primer in each case encoded an overlapping 18 nucleotides from the 3' terminus of pDOM, the relevant sequence from the class I epitope, a stop codon and the Not I restriction site. A PCR reaction was carried out in a volume of 50 μl with 500ng of pDOM plasmid as template. 20pmol of each primer were used along with 0.5mM DNTPs and 0.6 μl of Expand High Fidelity Taq polymerase (Roche Biochemicals, UK) in the supplied reaction buffer. After an initial denaturing step of 94°C for 5 minutes, 30 cycles were performed with a denaturing step of 94°C for 30 seconds, an annealing step of 42°C for 30 seconds and an extension step of 72°C for 3 minutes. A final extension step for 5 minutes at 72°C was performed. The products of the PCR reaction were run on a 1% agarose gel and bands of approximately 880 base pairs were excised. DNA was extracted from the gel into 45μl using QIAquick Gel Extraction Kit (Qiagen, UK).

The PCR products were to a restriction enzyme digest using Hind III and Not I. The reaction was in a volume of 50μl with 43μl of DNA, 1μl each of Hind III and Not I (New England Biolabs, UK) and 5μl of the reaction buffer. The digestion was performed over 2½ hours. An empty pcDNA3 vector was linearised with Hind III and Not I and ligated to

Vaccine	Reverse Primer (5'-3')
pDOM.EAQN	ATATGCGGCCGCTTACAGGTAGGTTGTGTTCTGAGCCTCGTTACCCCAGAAGTCACG
pDOM.EAQD	ATATGCGGCCGCTTACAGGTAGGTTGTGTCCTGAGCCTCGTTACCCCAGAAGTCACG
pDOM.CAP-1	ATATGCGGCCGCTTAGAGGTTGAGGTTCGCTCCCGAAAGGTAGTTACCCCAGAAGTCACG
pDOM.CAP-1/6D	ATATGCGGCCGCTTAGAGGTTGAGGTCCGCTCCCGAAAGGTAGTTACCCCAGAAGTCACG
pDOM.CAP-1/8D	ATATGCGGCCGCTTAGAGGTCGAGGTTCGCTCCCGAAAGGTAGTTACCCCAGAAGTCACG
pDOM.IMI	ATATGCGGCCGCTTAAACCCCAACCAGCACTCCAATCATGATGTTACCCCAGAAGTCA
pDOM.NLV	ATATGCGGCCGCTTAAACCGTAGCCACCATGGGCACCAGGTTGTTACCCCAGAAGTCACG

Table 3. Reverse primers for the construction of DNA vaccines

Name of primer	Sequence (5'-3')	Vaccines used to construct
Leader +	TAATACGACTCACTATAGGGAGAGCCACCATGGGTTGGAGCTGTATCATC	pDOM.EAQN, pDOM.EAQD, pDOM.CAP-1, pDOM.CAP-1/6D, pDOM.CAP-1/8D, pDOM.IMI pDOM.NLV
Leader -	TAATACGACTCACTATAGGGAGAGCCACCATGAAAAACCTTGATTGTTGG	p(L-)DOM.EAQN

Table 4. Forward primers for the construction of DNA vaccines

Name	Sequence (5'-3')	Used to transcribe	Direction
T7-FrC	TAATACGACTCACTATAGGGAGAGCCACCATGGGTTGGAGCTGTATCATC	pDOM.NLV, pDOM.CAP-1	Forward
T7-GFP	TAATACGACTCACTATAGGGAGAGCCACCATGGTGAGCAAGGGCGAGG	GFP	Forward
polyA-GFP	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACTTGTACAGCTCGTC	GFP	Reverse
polyA-CAP-1	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACAGGTAGGTTGTGTTCTG	pDOM.CAP-1	Reverse
polyA-NLV	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAACCGTAGCCACCATGG	pDOM.NLV	Reverse

Table 5. Primers for transcription of RNA

the digested PCR products in a final volume of 10 μl containing 1μl of T4 DNA ligase, 1μl of linearised pcDNA3, 1μl of 10x Ligation Buffer (Promega, T4 DNA ligation kit) and 7μl of the PCR product digest. The ligation reaction was performed at room temperature for 2 hours.

2.6 Transformation of plasmid DNA into competent bacteria

All DNA vaccines were transformed into XL1 blue competent bacteria (Promega, Southampton, UK). 10μl of ligation was incubated with to 50μl of the competent E.coli and this was incubated on ice for 20 minutes. Bacteria were subsequently heat shocked at 42°C for 30 seconds and then returned to ice for 2 minutes. 950μl of Luria Broth (LB) was added and the bacteria were incubated on a shaking platform for 1 hour at 37°C to enable. The bacteria were then plated out onto LB agar that contained 100μg/ml ampicillin for 12-18 hours at 37°C for the selection of transformed bacteria.

Colonies that had grown over this time-period were selected and cultured overnight in LB medium containing 100μg/ml ampicillin. The cells were then lysed and the DNA purified using the QIAprep Spin Miniprep Kit (Qiagen). The DNA was eluted into a final volume of 100 μl of sterile H_2O. DNA constructs were initially assessed by restriction digestion to confirm the presence of a cloned insert of the correct size and to assess the quality of the DNA. 6 μl of the DNA eluted was incubated with 1 unit of Hind III and Not I (New England Biolabs, UK) in the recommended reaction buffer for 45 minutes at 37 °C. Any clone that was chosen contained one insert at the expected size. Any clone that was found to have the correct sequence was stored at -80 °C with approximately 15% sterile glycerol.

2.7 Amplification of plasmid DNA

For large scale amplification of the DNA plasmids the glycerol were plated on LB agar containing 100 μg/ml ampicillin overnight. Individual colonies were picked from this plate and grown for 8 hours in LB/ampicillin at 37 °C. This culture was then used to inoculate three 400ml flasks of LB/ampicillin which were then grown at 37 °C for 18 hours on a rotating platform. DNA was then extracted from these cultures using an ion-exchange resin (QIAfilter

Giga Kit, Qiagen, UK) using the manufacturer's instructions. A 1 in 40 dilution of the final DNA in H_2O was quantitated using a Biophotometer (Eppendorf AG, Germany). They were stored at -20 °C until required for quality control checks or vaccination.

2.8 In vitro transcription/ translation reaction

An in vitro transcription and translation reaction was used to check the ability of each vaccine to deliver a protein product of the correct size. This was performed using the TNT T7 Coupled Reticulocyte Lysate System (Promega). In this system 500ng of plasmid DNA is incubated with rabbit reticulocyte lysate (20 μl of TNT T7 mastermix) and 100 μCi of ^{35}S-labelled methionine. This allows transcription and translation to proceed with the incorporation of the radioactive methionine for detection of the final protein product. This reaction is incubated for 90 minutes at 37 °C. The final product is denatured in the presence of 2-mercaptoethanol and NuPage loading buffer (Invitrogen) for 5 minutes at 95 °C. Samples were then run on a pre-cast 4-12% gradient polyacrylamide gel (Invitrogen) ar 150 volts for 1 hour. The gel was then dried onto filter paper for 45 minutes and the exposed to radiographic film overnight. Rainbow markers (Amersham) were run alongside the samples to confirm the presence of protein bands of the expected size.

2.9 Sequence analysis of DNA plasmids

Every DNA plasmid used was sequenced to ascertain the sequence of the cloned insert. This was performed using the Big Dye reaction kit (Applied Biosystems, UK). 5 μl (100 μg/ml) of plasmid DNA was added to 1 μl of primer (1.6pmol), 2 μl of sequencing buffer and 2 μl of Big Dye reaction mixture. This mixture contains DNA polymerase and ddNTPs that are labelled with different fluorochromes. The primers used enabled sequencing of the insert from both directions and are listed in table 2. The sequencing reaction was performed at the optimal Big Dye conditions: denaturing at 96°C for 10 seconds, annealing at 50°C for 5 seconds and then extending at 60°C for 3 minutes. These 3 steps were repeated for 30 cycles on a GeneAmp PCR System 9700 (Applied Biosystems). Samples were precipitated with 2μl of

Name	Sequence (5'-3')	Position of binding	Direction
pCMVp1	CTATATAAGCAGAGCTCT	From b.p. 803 within CMV promoter	Forward
pJR5p1	GGCACAGTCGAGGCTGATCA	From b.p. 1029 within pcDNA3	Reverse
Fcf1	GTTAGCTTCTGGCTGCGCGTTC	From b.p. 220 of DOM1 of FrC	Forward
Fcf2	TCCTACACTAACGGTAAACT	From b.p. 832 of DOM2 of FrC	Forward
Fcr1	CGGGTAACCAAACGATGTGTTCGT	From b.p. 980 of DOM2 of FrC	Reverse
CEA1320f	TGCACAGTATTCTTGGCTG	From b.p. 1320 of CEA	Forward
CEA361f	GCCTTCACCTGTGAACCT	From b.p. 361 of CEA	Forward
CEA1930r	GCGTGATTTTGGCGATAAAGA	From b.p. 1930 of CEA	Reverse
CEA1460r	TGACTGTAGTCCTGCTG	From b.p. 1460 of CEA	Reverse
CEA1023r	CAGGTTAAGGCTACAGC	From b.p. 1023 of CEA	Reverse

Table 6. Sequencing primers

3M sodium acetate and 25μl of ethanol on ice for 10 minutes. This precipitated was then centrifuged at 16000rcf for 30 minutes and washed once in 160μl of 75% ethanol. DNA was then resuspended in 1.2μl of loading buffer and formamide. It was then sequenced on an ABI Prism 377 DNA sequencer (Applied Biosystems). Sequences were analysed using MacVector 4.5.3 (Oxford Molecular, UK) and Editview 1.0 (Applied Biosystems) software.

2.10 Vaccination

Mice were vaccinated with 50μl of DNA plasmid in saline at a concentration of 0.5mg/ml into both rear quadriceps muscles. Mice were subsequently sacrificed on day 14 and their spleens harvested. Single cell suspensions were prepared by dispersing splenocytes through a 70μm nylon mesh. Mononuclear cells were then isolated over a lymphoprep (AS Pharma, U.K.) density gradient at 800rcf for 20 minutes. These cells from each mouse were then either pooled or kept separately for experimentation.

2.11 Peptide vaccination

A long peptide was synthesized that contained the PADRE sequence: AKX*VAAWTLKAAA and the sequence NLVPMVATV from pp65 of CMV (Peptide Protein Research Ltd, UK). The whole sequence includes an adaptor sequence and is KSS-AKX*VAAWTLKAAA-NLVPMVATV (referred to as PADRE-NLV). The previously described immunostimulatory synthetic oligonucleotides "1826" (5' TCCATGACGTTCCTGACGTT 3') containing 2 CpG motifs was synthesized with a nuclease-resistant phosphorothioate backbone (MWG, Germany). Vaccinations were given subcutaneously at the base of the tail. 100nmol of peptide was used with or without 25μg of the oligonucleotides 1826 (referred to as CpG-ssODN).

A long peptide was also synthesized combining the PADRE epitope with the MHC class I epitope from CEA: CAP-1. The sequence of this long peptide was KSS-AKX*VAAWTLKAAA-YLSGANLNL. Vaccinations were carried out in an identical protocol.

2.12 Detection of intracellular interferon-gamma by FACS

Splenocytes were plated out in a 96-well plate at $1x10^6$ per well. Cells were then either re-stimulated with 1 μM of peptide or no peptide for 6 hours in the presence of 10 units/ml of recombinant human IL-2 and 1 μl per well of Brefeldin A (GolgiPlug, BD Pharmingen, CA) Cells were pelleted and washed once in PBS containing Brefeldin. Surface Fc receptors were blocked by the addition of 2% normal mouse serum in PBS/Brefeldin for 10 minutes at 4°C. Splenocytes were then labelled with APC conjugated anti-mouse CD8b.2 (clone 53-5.8, BD Pharmingen) and FITC conjugated anti-mouse Mouse MHC class II (2G9; BD Pharmingen) at 1μg/well for 20 minutes at 4°C. Cells were then fixed in 1 % formaldehyde-PBS for 10 minutes at 4°C. The fixing agent was removed by an additional wash and cells permeabilised in 0.5% saponin (Sigma) for 5 minutes. Then the cells were incubated for 20 minutes in the presence of 0.5μg/well PE-conjugated anti-mouse IFN-γ (clone XMG1.2) or an isotype control (both BD Pharmingen), diluted in 0.5% saponin-PBS. Cells were then washed in PBS and analysed on a FACScalibur. One hundred thousand events were collected from a live lymphocyte gate. Subsequently the MHC class II positive cells were excluded and the proportion of CD8 cells that were also IFN-γ positive was determined.

2.13 Generation of CTL lines

Splenocytes were harvested from mice 14 days after vaccination as described above. For some experiments the splenocytes from several animals were pooled and in others the splenocytes were cultured separately for each mouse. Splenocytes were cultured in 1640 RPMI with 10% FCS at $5x10^6$/ml in the presence of 20U/ml of IL-2 and peptide for 6 days at 37°C with 5%CO2.

2.14 Cytotoxicity assays

After 6 days of in vitro stimulation viable cells from the CTL lines generated above were isolated by density gradient centrifugation as described above. Target cells were washed and resuspended in a final volume of 300 μl containing 200 μCi of chromium-51. Then either with or without the addition of peptide the cells were incubated at 37°C for 1 hour. The targets were then washed well and then plated out in a 96 U-bottomed plated at $5x10^3$/well. Effector cells from the CTL lines were then plated out at a range of ratios (30:1 to 120:1). Control wells contained target cells alone in order to assess spontaneous release of isotope. The effectors and targets were incubated for between 4 and 6 hours at 37°C. After this time 2% Nonidet P40 was added to half of the target alone wells to assess maximum lysis.

After centrifuging at 400 rcf for 5 minutes, 100 μl of supernatant was transferred from each well to LMP3 tubes. Gamma activity was assessed in a Wallac 1282 compugamma counter for 5 minutes for each tube. Specific lysis was calculated by the standard formula:

$$\left\{ \frac{\text{Release by CTL - Release by targets alone}}{\text{Release by 2\% Nonidet P-40 – release by targets alone}} \right\} \text{X } 100\%$$

2.15 Viral infections to provide relevant targets for cytotoxicity assay

MVA that expressed the antigen pp65 from CMV and a control MVA were a kind gift from Dr Naeem Khan (Cancer Sciences, Birmingham). These were used to transfect Jurkat cells that had been previously transfected with the chimeric class I molecule HLA-A2.1/D^b. These cells were suspended at 2.5 x 10^6/ml in PBS/BSA and virus was added at a multiplicity of infection between 3 and 10. Cells were then allowed to infect on a rotator for 1 hour at 37°C. After 1 hour complete medium was added to the cells. Then the cells were placed on rotator

for a further 3 hours. After this time the cells were spun down and the media replaced with 100μCi of 51-chromium/10^6 cells in R10. After 1 hour of labelling the assay continued as above.

X31 influenza A virus (H3N2) was a kind gift from Philip Stevenson (University of Cambridge). 100μl of infected allantoic fluid was added to 10^6 target cells in serum free conditions. The 2 target lines were the Jurkat cells transfected with the chimeric class I HLA-A2.1/D^b and L929 cells that had been transfected with fully human HLA-A2.1. After incubation for 1 hour chromium was added to the cells and virus for a second hour. After this time the cells were washed 4 times in serum containing medium. These cells were then placed in a cytotoxicity assay as described above.

2.16 ELISPOT assay for IFN-γ release from single Ag-specific murine T cells

Ninety-six well plates (Millititer, Millipore, Bedford, MA) were coated overnight at 4°C with 5 μg/ml of the primary anti- IFN-γ mAb (Mabtech, Stockholm, Sweden). The Ab-coated plates were washed four times with PBS and blocked with RPMI 1640 containing 10%FCS for 2 hours at room temperature. 2-4x10^5 splenocytes were added to each well in RPMI/10%FCS and incubated in the presence of peptide or with no peptide for 14-18 hours at 37°C. Peptides were added at concentrations of between 0.1-25μM directly to the wells. After the overnight incubation the wells were washed four times with PBS containing 0.1% Tween-20 (Sigma) followed by 2-hour incubation with 1 μg/ml of the secondary Ab (biotin-conjugated anti-IFN-γ mAb; Mabtech, Sweden). Plates were then washed four times in PBS with 0.1% Tween-20. Avidin-bound biotinylated ALP (Mabtech) was added to the wells for 1 hour at room temperature. The plates were washed four times in PBS with 0.1% Tween-20 followed by an 8 minute incubation with the substrate NBT/BCIP (Zymed, CA). Tap water was added to stop the reaction. Spots were counted using an ELISPOT plate reader (AID, Germany) and analysed using ELISPOT reader software (AID, version 2.6).

2.17 Enumeration of epitope-specific CD8 T cells using HLA class I tetramer complexes

HLA class I tetramer complexes conjugated to PE that incorporated the human HLA-A2.1 epitope from CMV (NLVPMVATV) were purchased from Proimmune, Oxford UK. HLA class I tetramer complexes that were chimeric (composed of the α1 and α2 domains of HLA-A2.1 and the α3 transmembrane and cytoplasmic domains of H-2D^b) were also purchased from Proimmune.

Splenocytes were taken from vaccinated mice on day 14 post vaccination. They were then stained with 0.4 μg of tetramer per 10^6 cells. Splenocytes were then labelled with APC conjugated anti-mouse CD8b.2 (clone 53-5.8, BD Pharmingen) and FITC conjugated anti-mouse Mouse MHC class II (2G9; BD Pharmingen) at 1μg/well for 20 minutes at room temperature. Cells were then washed in PBS and analysed on a FACScalibur. One hundred thousand events were collected from a live lymphocyte gate. Subsequently the MHC class II positive cells were excluded and the proportion of CD8 cells that were also tetramer positive was determined.

2.18 Dendritic Cells

Dendritic cells (DCs) were generated by plating PBMCs in a 6-well tissue culture plate (Nunc, UK). The cells were allowed to adhere for 2 hours at 37°C. After this time the non-adherent fraction was removed and the adherent cells were cultured in 5% FCS with 1000U/ml GM-CSF (Schering-Plough) and 500U/ml IL-4 (R&D). Cytokines were added to the cultures on days 0, 2, 4 and 6. Dendritic cells were transfected with RNA on day 6 and then re-plated in monocyte-conditioned medium (MCM) at 50%(v/v) in order to induce maturation. Mature DCs were collected on day 7.

MCM was prepared by coating bacteriological plates with human immunoglobulin for 24 hours at 4°C. Then $7x10^7$ PBMCs were layered onto the plates for 1 hour at 37°C. Non-adherent cells were removed and the adherent cells were incubated in medium containing 1% serum at 37°C for 24 hours. After this time the medium was collected as MCM.

2.19 Production of in vitro-transcribed mRNA

The insert from the plasmids pDOM.NLV, pDOM CAP-1 and pcDNA3.GFP were amplified by PCR using the primers in Table 5. Primers for transcription of RNAThe reverse primers incorporate a poly A tail into the PCR product that allows for subsequent stabilisation of the RNA. These PCR products were then run on a 1% agarose gel and then purified with a QIAquick gel purification kit. Transcription was carried out in a 100μl reaction at 37°C for 2 hours using a T7 MessageMachine kit (Ambion) generate 5' m^7GpppG-capped in vitro-transcribed mRNA. Purification of the IVT mRNA was performed by DNase I digestion followed by LiCl precipitation and 70% ethanol wash according to the manufacturer's instructions. RNA concentration was assayed by spectrophotometrical analysis at OD260.

2.20 DC transfections

DCs were washed twice in serum free medium and resuspended to a final concentration of $2x10^6$ cells/ml in Opti-MEM. Subsequently 0.5 ml of the cell suspension was mixed with 20μg of IVT mRNA and electroporated in a 0.4-cm cuvette using an Easyject Plus device (EquiBio, Kent, United Kingdom). A voltage of 300V was used combined with a capacitance of 150μF in a total cuvette volume of 200μl. The cells were checked for GFP expression 24 hours after by flow cytometric analysis. $5x10^5$ cells were washed in PBS containing 1%BSA. Cells were then resuspended and analysed on a FACScalibur. Gating was performed on cells exhibiting a large forward scatter and side scatter profile (DCs) so as to exclude contaminating lymphocytes. Gated DCs were then evaluated for EGFP expression.

2.21 Immunophenotyping of DCs

DCs were studied both prior to maturation and subsequent to maturation to ascertain their immunophenotype. The following antibodies were used: anti-CD1a-FITC, anti-CD14-FITC, anti-HLA-DR-PE, anti-CD83-FITC, anti-CD80-FITC and anti-CD86-FITC. Non-reactive isotype-matched antibodies were used as controls (all antibodies were from BD Pharmingen). Cells were analysed on a FACScalibur.

2.22 DC culture with CD8 cells

CD8 cells were isolated using a CD8 isolation kit (Miltenyi Biotec, UK) using the manufacturers instructions. Briefly the following hapten-conjugated antibodies were added to PBMCs: CD14, CD4, CD19 and CD56. Cells were then washed and anti-hapten beads are incubated with the cells. The cells were then run through a magnetic column and cells the CD14-,CD4-, CD19-, CD56- population that did not bind to the column were collected. These CD8 cells were then placed in a 96-well culture plate at 5x104 cells per well. 5x103 DCs that were transfected with the pDOM.NLV were then placed in each well. Cells were co-incubated in the presence of interleukin-7 at a concentration of 5ng/ml. After 3 days IL-2 was added at 20 IU/ml. Cells were taken on day 7 for tetramer analysis and a ^{51}Cr release cytotoxicity assay.

3 DNA Vaccination against carcinoembryonic antigen

3.1. Introduction

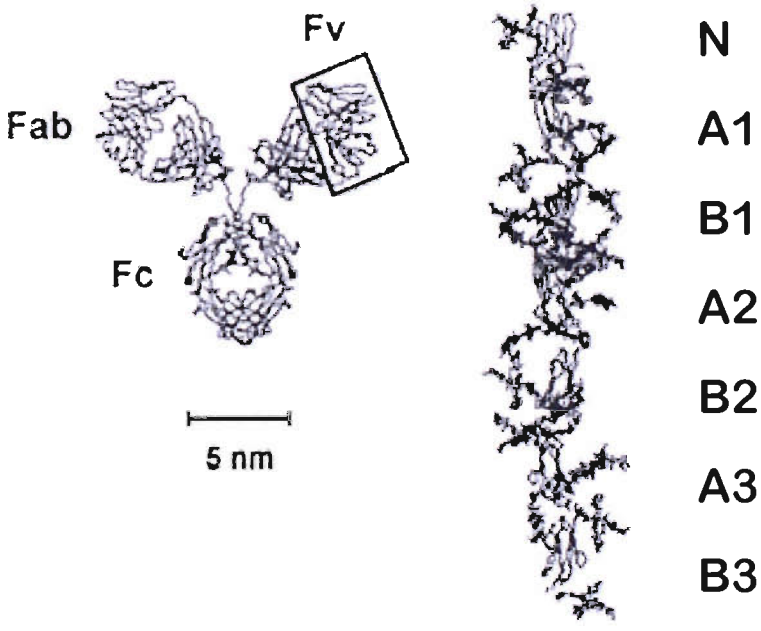
It is 40 years since carcinoembryonic antigen (CEA) was described as one of the first tumour associated antigens. It was initially isolated from human colon cancer extracts and then from the serum of patients with colorectal cancer and other carcinomas.[218] Sera from healthy individuals were noted to have very low levels of CEA. Consequently the measurement of serum CEA levels attracted much research into how this may be applied to diagnosis, staging and monitoring of these diseases. Serum CEA levels now have a central role in the monitoring of colorectal cancer post-surgery. There are also many exciting applications for CEA as a target antigen for radioimmunolocalisation of colorectal tumours. In the last 15 years CEA has been actively pursued as a possible target for the immunotherapy of cancer.

3.1.1. Structure and function

CEA is a 180 kDa glycoprotein that consists of approximately 50% carbohydrate.[219] It is a member of the Ig superfamily and consists of two types of immunoglobulin domain.[220] An N-terminal domain of 108 amino acids shows homology to the Ig variable domain and this is combined with six domains homologous to the Ig constant domain. In CEA there are three Ig constant-like domains of type A containing 93 amino acids and three of type B containing 85 amino acids (Figures 10 and 11).

Recently the structure at low resolution of the seven domains of CEA was determined by X-ray and neutron scattering.[221] CEA was found to be a monomer with dimensions of 20 x 8 nm implying extended carbohydrate structures. CEA can therefore be seen as a "bottle-brush" with the immunoglobulin-like domains tilted at a 160° angle against each other.

Figure 10. The structure of CEA



The structure of CEA as determined by x-ray and neutron solution scattering. The carbohydrate chains are represented in full. The domains of CEA are given. The immunoglobulin G model is given as comparison.

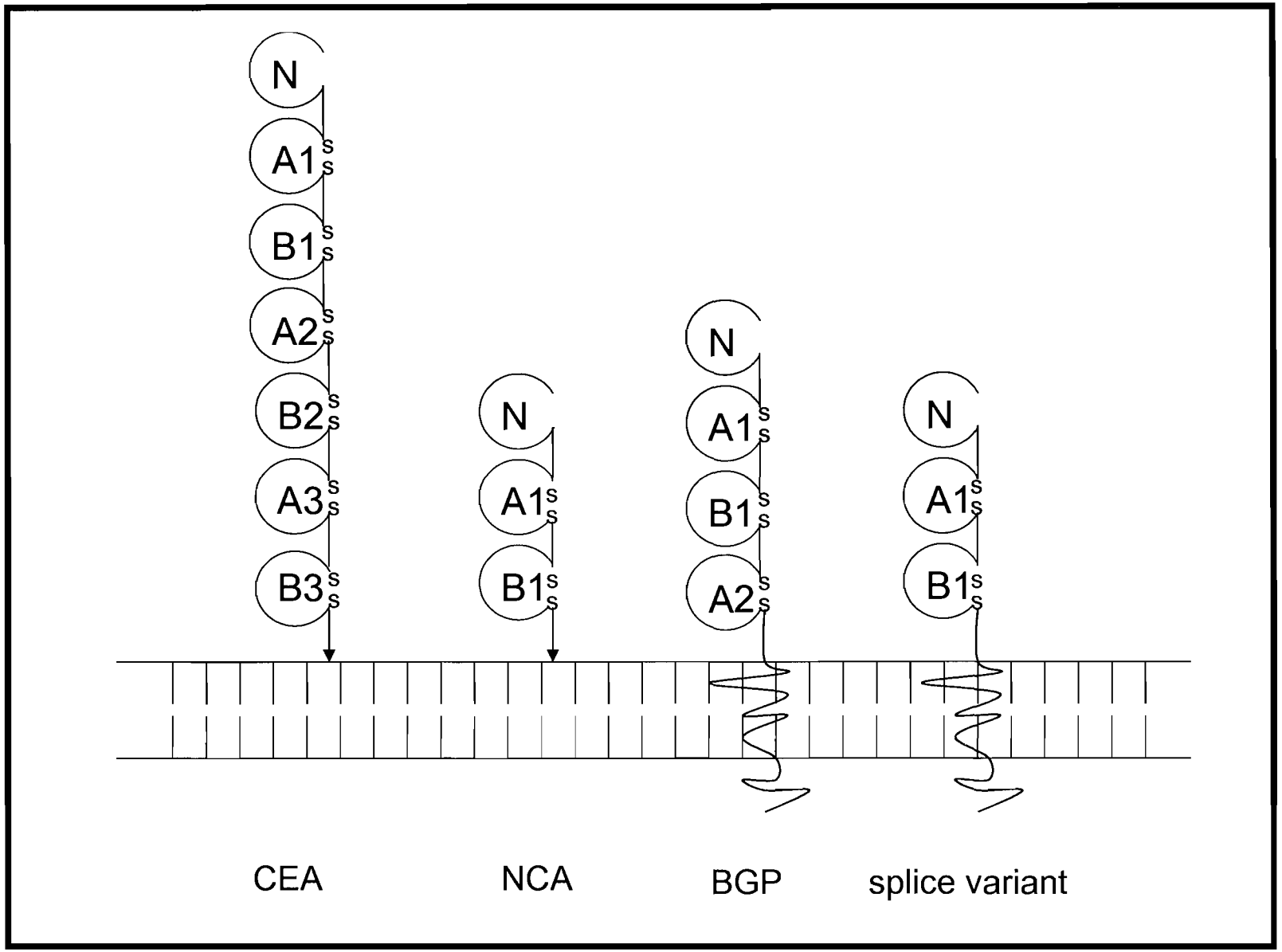


Figure 11. The structure of CEA, NCA and BGP

CEA is attached to the cell membrane via a glycosyl phosphatidyl inositol (GPI) moiety. The significance of this GPI-linkage is uncertain. The presence of the anchor appears to impose conformational restraints, and its removal may alter the structure of a GPI-anchored protein. Release of GPI-anchored proteins from the cell surface by specific phospholipases may play a key role in regulation of their surface expression and functional properties.[222,223]

CEA is a member of a family of related proteins, the so-called CEA family. Altogether 29 different genes have been identified in the human CEA gene family. The proteins are similar in structure all consisting of the N terminal domain which is homologous to the immunoglobulin variable domain and having additional Ig constant domain-like regions. The next family member described was non-specific cross-reacting antigen (NCA).[224] The other important member of this family is biliary glycoprotein (BGP).[225] These molecules are relevant to a vaccine designed against CEA as they have a high degree of homology for CEA and are more widely expressed.

3.1.2. Expression in normal tissues

CEA is expressed in columnar epithelium of the colon and in the stomach, oesophagus, cervix and prostate.[226-228] BGP is expressed much more widely (see table below).[226,229] NCA also has a fairly broad expression in epithelial cells of different organs and in granulocytes and monocytes.[230] Studies of immunoelectron microscopy using specific antibodies for CEA, NCA and BGP demonstrate that all four molecules are specifically localised to the apical surface of mature enterocytes. No staining is seen at the basolateral aspect of these epithelia.[231] In addition to this, CEA in normal colon is released via CEA-coated vesicles.

Antigen	Structure	Tissue Expression	Membrane linkage
CEA	N-A1-B1-A2-B2-A3-B3	Colon, stomach, oesophagus, cervix and prostate	GPI-linked
NCA	N-A1-B1	Epithelial cells of many different organs, granulocytes and monocytes	GPI-linked
BGP	N-A1-B1-A2	Entire gastro-intestinal tract, throughout the hepato-biliary tract, the genitor-urinary system, granulocytes and lymphocytes	Hydrophobic transmembrane domain

Table 7. Members of the CEA family

3.1.3. Expression in tumour cells

Carcinoembryonic antigen is expressed in a number of tumour types. It is over-expressed in nearly all colorectal cancers, 70% of lung cancers and in 50% of breast cancers. In addition it is over-expressed in most of the other common gastro-intestinal malignancies.[232] Contrary to the strict apical localisation of CEA in normal colon epithelial cells, CEA is expressed over the entire surface of colonic adenocarcinomas.

Another difference between CEA from tumours and CEA from normal epithelium is the degree of glycosylation. As mentioned above CEA in normal epithelium is heavily N-glycosylated and is expressed as a large 200 kD protein. In colon cancer cells it was noted that the protein is only 170 kD. Biosythesis in the presence of tunicamycin led to the expression of an even smaller molecular weight form whether the source of tissue was normal or cancerous. The authors inferred that the colonic carcinomas expressed an under-glycosylated form of CEA.[233] Follow-up studies revealed that specific galactosyltransferase (B1,3Gal-T) activity

was found to be lower in adenocarcinomas than in normal mucosa. Using RT-PCR it was also found that the level of a B1,3Gal-T mRNA in adenocarcinoma tissue was on average 30-fold lower than in normal colonic mucosa.[234]

3.1.4. Function

Several studies with tumour cell lines have demonstrated that CEA can act as homotypic intercellular cell adhesion molecules when expressed on the tumour cell surface.[235] These CEA-CEA interactions have been studied using site directed deletions and point mutations. Using these and other techniques it has been demonstrated that 3 sub-domains within the N-domain are directly involved in this cell adhesion phenomenon.[236,237] In vitro studies have also demonstrated that overexpression of CEA has an instrumental role in tumorigenesis through the inhibition of cell differentiation. Overexpression of CEA in several cell lines has been shown to block cellular polarization, disrupt tissue architecture and block the differentiation of colon carcinoma cell lines.[238] It is also possible that CEA and NCA play a role in the innate immune defense protecting the colon. The N-domain of CEA is recognized by the virulence associated Opa proteins in Neisseria gonorrhoeae and Neisseria meningitides. Mutation studies reveal that these Opa proteins bind to a 15 amino acid sequence in this domain.[239]

3.1.5. CEA in vaccines against cancer

Early strategies using CEA in vaccination aimed at generating antibody and CD4 responses to CEA by using recombinant protein vaccines. In one of the first trials to target CEA, Samanci et al treated 18 patients with subcutaneous injections of CEA protein with or without granulocyte-macrophage colony-stimulating factor (GM-CSF).[240] Patients had minimal residual disease at the time of treatment. IgG antibody responses were documented in 9/9 patients receiving the CEA/GM-CSF vaccine compared with only 3/9 patients treated with CEA alone. In addition T-cell proliferative responses were seen together with T cells that

secreted IFN-γ in response to CEA as detected by ELISPOT. No clinical responses were seen in this trial.

In another approach Foon et al used an "anti-idiotype antibody vaccine" approach. They took a monoclonal antibody designated "8019" which recognizes a CEA epitope. This monoclonal antibody was injected into BALB/c mice which subsequently generated an antibody response against this "idiotype antibody". This "anti-idiotype" antibody (called CEAVAC) generated by the BALB/c mice mimics a specific epitope on CEA and was subsequently used to immunize patients with CEA-expressing tumours. In the phase I trial CEAVAC was able to induce an antibody response against CEA. In a phase II study all 32 patients generated an IgG response to CEA. In addition all 32 patients generated T-cell proliferative responses to CEA. No survival benefit was demonstrated in this patient cohort.[241]

It is not clear how effective CEA antibodies will be in the treatment of CEA-expressing malignancies. Several studies have documented an anti-CEA antibody response in un-treated patients who have colorectal cancer. This antibody response is not effective at inducing tumour regression in these patients. The reasons for this are not clear. Certainly many of the patients have high levels of serum CEA which may block antibody activity.

Several other investigators have sought to generate CD8+ cellular responses to CEA. One of the first pre-clinical studies seeking to generate cellular immunity against CEA was using a recombinant CEA-vaccinia vaccine. The extracellular domain of human CEA was expressed via a viral expression cassette. This vaccine was able to generate humoral immunity against CEA that protected against CEA-expressing tumour.

Transgenic mice have been developed that express human CEA with a tissue distribution similar to that of humans. In addition these transgenic mice had measurable serum CEA levels. They are therefore an invaluable model in the assessment of vaccines against CEA. Peripheral T-cell tolerance to CEA in these mice has been demonstrated by the absence of any immune response to CEA when vaccinated with whole protein in adjuvant.[214]

In contrast the CEA-vaccinia vaccine was able to generate robust IgG antibody responses and CD4 responses to CEA. CTL responses to a MHC class I restricted epitope (EAQNTTYL) were demonstrable after 2 weeks in vitro stimulation. Vaccination was effective in providing protection from a CEA-expressing tumour in these mice.[242]

Tsang et al used this same vaccinia vaccine expressing the extracellular domain of CEA in three cohorts of patients.[243] In this phase I trial there was no toxicity observed in any of the cohorts or any objective clinical response. However this group went on to expand the low numbers of CTL that had been stimulated by this vaccination. Algorithms were used to predict peptides that would bind to MHC class I molecules and in particular HLA- A2.1. These peptides were then used to stimulate PBMCs taken from patients vaccinated with the vaccinia-CEA construct and unvaccinated patients. The peptide that generated the largest expansion of CEA specific CTL was designated CAP-1. This peptide was not able to generate an immune response from patients who were not vaccinated with vaccinia-CEA. Although the vaccine could induce a CD8+ T-cell response in vaccinated patients, the levels of CTL were low and took several weeks to expand in vitro.

There are several problems with using vaccinia as a vaccine. Some patients will have pre-existing antibody responses to vaccinia that may diminish the response to vaccination. Even in patients who do not have pre-existing antibodies it has also been demonstrated that when vaccinated they generate high levels of circulating anti-vaccinia antibodies following the initial immunization. It is thought that these antibodies diminish the host response to the CEA vaccine during subsequent vaccinations.[244]

Recombinant avian pox viruses (avipox) such as the canarypox (ALVAC) are potential candidates for immunization protocols in that they can infect mammalian cells and express the inserted transgene, but do not replicate in mammalian cells. In addition humans do not have pre-existing antibody responses to this virus. Consequently a new canarypox vaccine incorporating CEA was designed (ALVAC-CEA). This virus encoded the extracellular domain of CEA and was also effective at generating antibody, CD4 and CD8+ responses in

mice transgenic for CEA. The CTL responses were again demonstrated after several rounds of in vitro stimulation.[245]

This ALVAC-CEA vector was subsequently used to vaccinate colorectal cancer patients. Marshall et al vaccinated 20 patients with ALVAC-CEA. An increase in the T-cell precursor frequency, as documented by limiting dilution cytolytic assays was found in 7/9 evaluated patients. These CTL responses were seen after only 2 in vitro stimulations and this represented an improved CTL response over the vaccinia vaccine. No objective clinical responses were seen.[246]

It has been noted in many clinical settings that giving sequential injections of different vectors encoding the same recombinant antigen can be very effective at boosting the immune response to that antigen. This so-called "heterologous prime-boost" protocol was applied to CEA using a combination of two different viral vectors: vaccinia-CEA and ALVAC-CEA. Patients were either vaccinated with the ALVAC-CEA vaccine followed by the vaccinia-CEA vaccine or vice versa.[247] In 6/6 patients receiving vaccinia and then ALVAC vaccines there was an increase in CAP-1 specific T cell numbers as measured by ELISPOT. ELISPOT is a sensitive technique which examines the ability of a specific peptide to elicit IFN-gamma secretion from PBMCs. It enables ex vivo CTL responses to CEA to be examined for the first time. These ex vivo CTL responses to CEA represent further advances in vaccination strategy. No objective clinical responses were seen.

Two further studies have attempted to generate epitope-specific CD8+ T cell immunity against CEA. This study used the CEA-vaccinia vaccine to stimulate immunity in H-2^b restricted mice transgenic for human CEA.[248] A CTL specific for the MHC class I-restricted epitope from CEA (EAQNTTYL) was generated from the splenocytes of these vaccinated animals using peptide stimulation in vitro. After 5 weeks of in vitro stimulation this CTL was not able to lyse CEA-expressing tumour. However pre-treatment of the tumour with IFN-gamma resulted in lysis of the CEA-expressing tumour by the CTL. Subsequent studies have also suggested a role for this epitope in the CD8+ response to CEA in H-2^b restricted

mice.[242,249,250] Given these encouraging results we chose this epitope for generating CD8+ responses against CEA-expressing tumours.

More recently epitope specific vaccines have been used to target CEA in HLA- A2.1 transgenic mice.[251] A minigene plasmid construct was made of a novel epitope from CEA: IMIGVLVGV. This vaccination vector was transfected into doubly attenuated *Salmonella typhimurium* by electroporation. Not only was this vaccine strategy able to generate an immune response specific for this epitope but these CTL were able to lyse tumour in vitro. In addition some mice vaccinated in this way were protected against a tumour challenge with CEA-expressing tumour.

3.1.6. Targeting epitope-specific immunity with analogue peptides

Tumour antigens are often poor stimulators of CD8+ T cell immunity. In order to improve the immunogenicity of tumour peptides some investigators have systematically altered residues in these peptides and looked at the efficacy of these "analogue peptides" at generating CD8+ immunity. The immunogenicity of tumour-derived peptides depends on two key interactions. Firstly the ability of the peptide to bind to the MHC class I molecule, and secondly the affinity of this peptide-MHC complex for the TCR. Investigators have targeted each of these interactions in turn to improve CD8+ T cell responses against tumour epitopes.

The interaction between peptide and MHC class I has two major requirements. Firstly the peptide binding groove of the class I molecule requires peptides of a defined length (between 8 and 11 residues). Secondly the peptide binding groove of the class I molecule has 2 pockets that display a marked preference for specific amino acids. In the case of the HLA- A2.1 molecule peptide residues two and nine are important in the peptide-class I complex interaction. These are termed anchor residues. The first analogue peptides that were developed altered the anchor residues in order to increase the binding affinity of the peptide for the MHC class I molecule and consequently to increase immunogenicity of the peptide. Examples of this include an analogue of gp100 $_{209}$ peptide that is 100-fold more potent in activating naïve T cells than the wild type peptide. The dissociation rate of this modified

peptide from the HLA- A2.1 molecule is more than 100-fold lower than the wild type peptide.[252]

Further analogue peptides such as CAP-1/6D discussed below have alterations in non-anchor residues within the peptide: the TCR binding region of the peptide. These peptides have a similar affinity for the MHC class I molecule as the wild-type epitope but demonstrate an enhanced ability for generating CD8+ responses. These are termed "heteroclitic epitopes".

Zaremba et al systematically modified the amino acid residues of CAP-1 (YLSGANLNL), an epitope from CEA, in an attempt to enhance the immunogenicity of this epitope.[253] They discovered an analogue peptide where the asparagine at position 6 was substituted by an aspartic acid that they designated CAP-1/6D. This epitope was studied to see if a CTL from a patient vaccinated with vaccinia-CEA that had been stimulated with CAP-1 peptide could recognize this epitope. Targets loaded with CAP-1/6D were lysed at concentrations 100-fold less than those targets loaded with CAP-1. It was therefore deduced that this analogue peptide represented an "enhancer agonist" peptide that may be capable of stimulating CD8+ immunity more effectively.

At the same time it was recognized that to be an effective analogue peptide, T cells generated by it would have to be able to maintain their ability to recognize the native configuration of the peptide-MHC configuration on the tumour cell surface. To demonstrate this CAP-1/6D was used to generate CTL in vitro from unimmunised healthy HLA- A2.1 donors. On two occasions the CAP-1/6D peptide was capable of priming a CD8+ T cell response whereas CAP-1 was not. These lines that were generated by CAP-1/6D were tested for their cross-reactivity i.e. their ability to recognize CAP-1 loaded targets. The line from one volunteer showed cross-reactivity against CAP-1 loaded targets and was also able to lyse human tumour cells that expressed CEA. However the CTL generated from the other volunteer demonstrated reduced cross-reactivity and there was only appreciable lysis of the target cells when loaded with high concentrations of CAP-1 (10μM). No data on tumour lysis were available from this second line. This analogue peptide CAP-1/6D has therefore been shown to be very good at

generating high levels of CTL. What is less certain is how predictable cross-reactivity to CAP-1 will be.

However following this study Fong et al used dendritic cells pulsed with CAP-1/6D to treat patients with CEA-expressing tumours.[187] They used Flt3 ligand to expand their numbers of DCs greatly and then matured them in vitro with keyhole limpet hemocyanin. These mature DCs were then loaded with peptide and administered to patients as a cellular vaccine. They used tetrameric MHC/peptide complexes to follow the epitope-specific CD8+ responses. An expansion of epitope-specific CD8+ cells was demonstrated in 7/12 patients. They did not, however, demonstrate the ability of these CD8+ cells to kill CEA-expressing tumour. They also did not demonstrate the ability of these CTL to recognize CAP-1 pulsed targets. They did document tumour regression in 2 out of 12 patients both of whom had had a sizeable increase in the numbers of CAP-1/6D specific CTL.

One concern in the production of analogue peptides with alterations in the TCR-binding regions such as CAP-1/6D is that they may generate CTL whose TCRs may have a reduced affinity for the wild-type peptide. If difficulties in generating a response to these weaker epitopes can be overcome by more effective delivery of the antigen through optimised vaccine designs then there will be no need for the use of analogue peptides. Consequently there will be fewer concerns over analogue peptides generating CD8+ T cell responses that have a reduced specificity for the target peptide.

A further consideration when looking at cellular immune responses to a molecule such as CEA is that it is heavily N-glycosylated. Peptides carrying N-linked GlcNAc residues are probably not presented by MHC class I.[254] Previous studies looking at the presentation of another N-glycosylated tumour-associated antigen tyrosinase have noted that such an epitope is presented with an amino acid substitution. YMNGTMSQV that includes the glycosylation site at position 3 is presented to MHC I as the peptide, YMDGTMSQV.[255] The Asn at position 3 is converted to Asp by the peptide N-glycanase which also cleaves the glycan from Asn at the N-glycosidic linkage.

Both of the candidate class I epitopes that have been most widely used in the wild type mouse and in the human HLA- A2.1 setting both have N-glycosylation motifs. The epitopes EAQNTTYL and YLSGANLNL both have the motif "NXT" at positions 4 and 8 respectively (YLSGANLNL is followed by a serine in its embedded site). We therefore sought to test two other epitopes derived from these that could be generated by post-translational modification of the above epitopes: EAQDTTYL (EAQD) and YLSGANLDL (CAP-1/8D) respectively.

MHC allele	Epitope	Name	Comment
H-2K^b	E A Q N* **T** T Y **L**	EAQN	Wild type peptide
H-2K^b	E A Q D **T** T Y **L**	EAQD	Substitution at position 4
HLA-A2	Y **L** S G A N L N* **L** (S)	CAP-1	Wild type peptide
HLA-A2	Y **L** S G A D L N **L**	CAP-1/6D	Substitution at position 6
HLA-A2	Y **L** S G A N L D **L**	CAP-1/8D	Substitution at position 8
HLA-A2	I **M** I G V L V G **V**	IMI	Wild type peptide

Table 8. CEA epitope sequences used in chapter 3

Anchor residues are denoted in bold type. Potential N-glycosylation sites are marked with an asterisk. The eighth residue of CAP-1 is a potential N-glycosylation site by virtue of a serine that is next amino acid in the CEA sequence thereby fulfilling the motif: NXS/T.

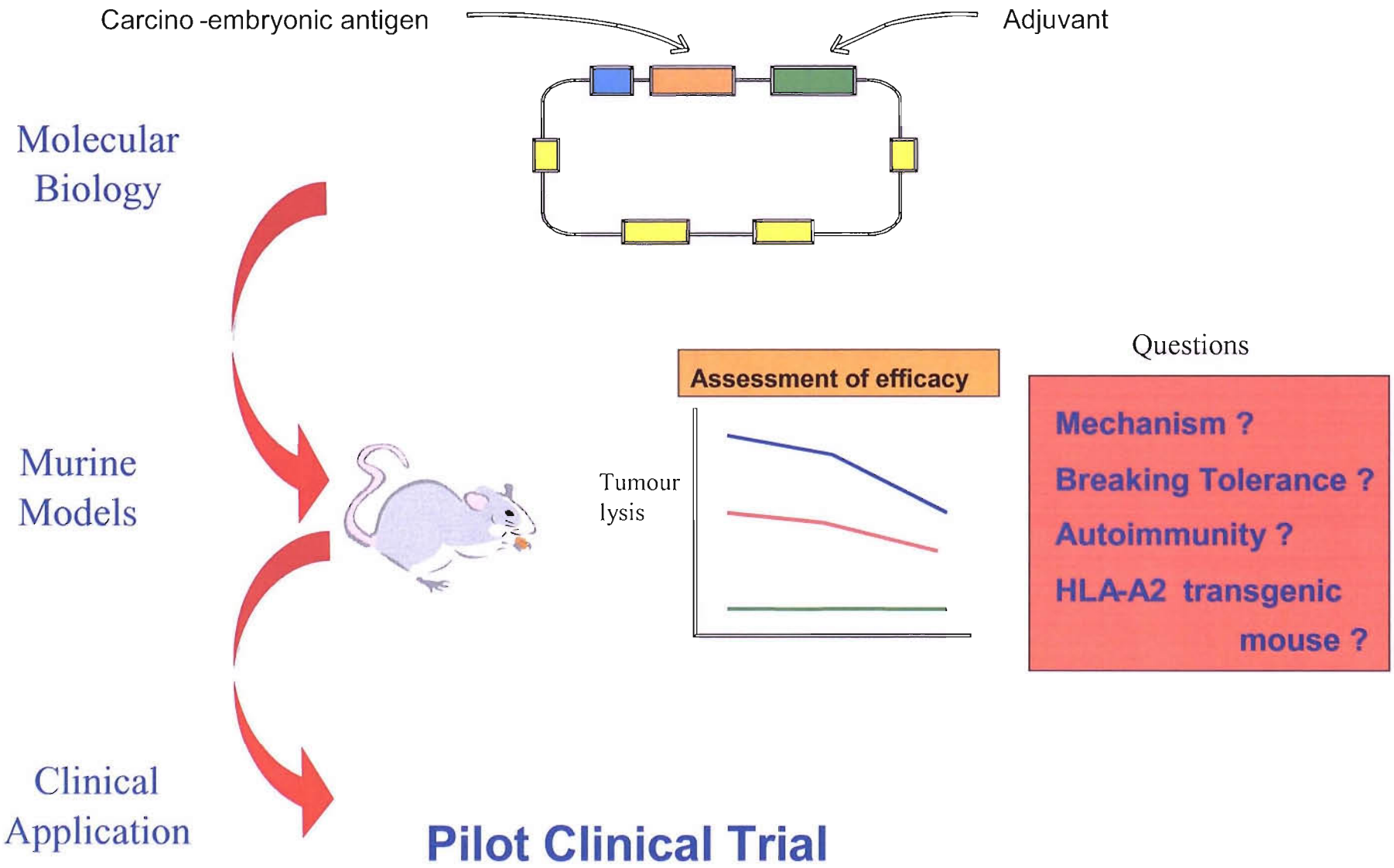


Figure 12 - Study aims

3.1.7. Study Aims

1. In order to determine a strategy that is effective at inducing a CD8+ response to CEA a novel vaccine design against a CEA epitope was examined in wild type mice. In this setting an epitope from CEA previously identified by several other investigators as a good target epitope was used (EAQNTTYL).

2. In order to determine if this vaccination approach was likely to be effective at breaking tolerance to CEA in patients we examined vaccination in mice transgenic for human CEA.

3. To take this strategy towards a clinical trial it was necessary to determine if this vaccination approach is effective at stimulating immunity to CEA in mice transgenic for the common human MHC class I molecule, HLA- A2.1. Again the candidate epitopes most widely used both in pre-clinical models and in clinical trials were used (YLSGANLNL).

We also studied the widely used analogue epitope, CAP-1/6D (YLSGADLNL), to examine if this epitope was more effective at stimulating immunity to CEA than CAP-1 in these HLA-A2.1 transgenic mice. We examined two further analogue peptides in an attempt to explore the possibility of post-translational modification of the epitopes above: EAQDTTYL (EAQD) and YLSGANLDL (CAP-1/8D).

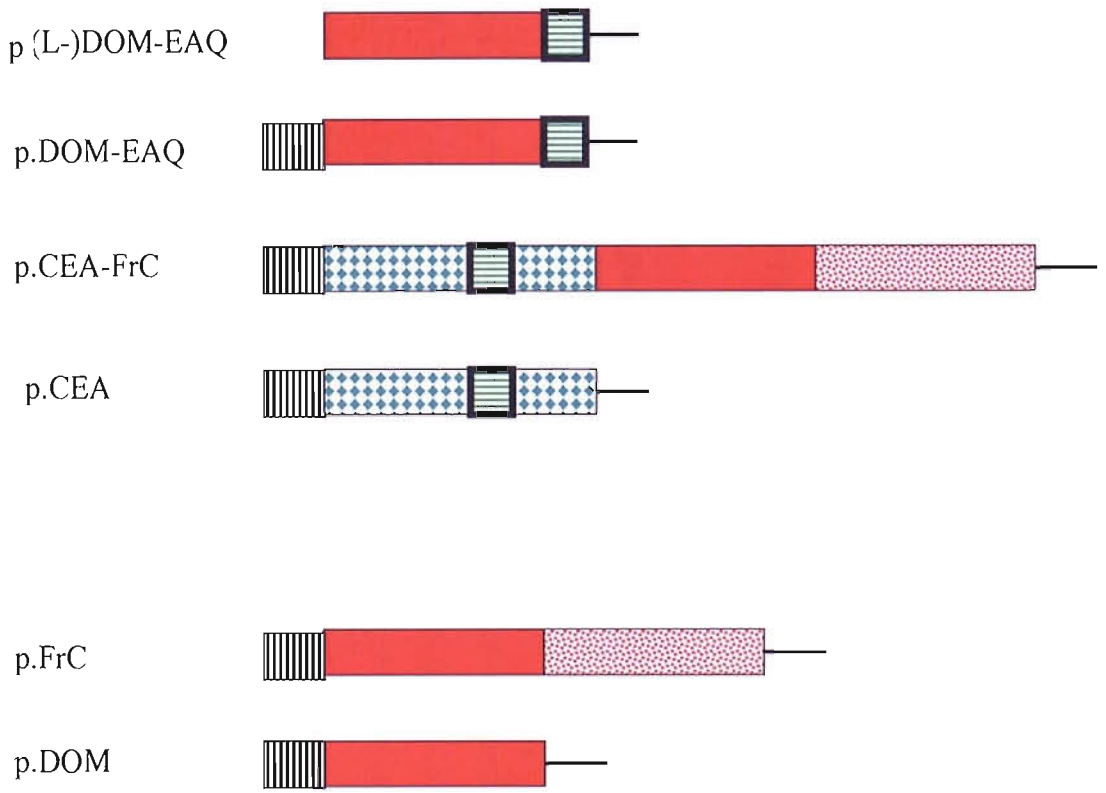


Figure 13 - Schematic diagram indicating DNA vaccine design.

Vaccines were assembled and inserted into pcDNA3 using Hind III and Not I restriction enzyme sites. As indicated DNA sequences included those encoding the amino-terminal ( ) and the carboxy –terminal ( ) domains of full-length FrC. The EAQ epitope ( ) from CEA is either embedded in the CEA sequence ( ) or at the carboxyl terminus of pDOM. Most constructs encode the BCL1 leader sequence at the amino terminus ( ).

3.2. Results

DNA vaccination against CEA in H-2K^b mice

3.2.1. Validation of plasmid sequence.

Confirmation of integrity for all the DNA vaccines used in this study was by DNA sequencing, restriction digestion and in vitro transcription and translation (IVTT). This included the pDOM, pDOM.EAQN, pDOM.EAQD, pCEA, pCEA.FrC, pDOM.CAP-1, pDOM.CAP-1/6D, pDOM.CAP-1/8D and pDOM.IMI vaccines. All sequences were correct and the digests and IVTT assays yielded products of the expected sizes.

3.2.2. Phenotypic analysis of the MC38, MC38-CEA tumour and transfected EL-4 tumour.

These tumours were analysed as described in the Methods and Materials section. The wild type tumour had low class I expression as measured by FACS analysis. The transfected line MC38-CEA had higher levels of class I expression but it was still low. Both lines had up-regulation of the class I when treated with interferon-gamma. This finding was not true for murine ICAM on either tumour. The MC38 tumour did not express CEA whereas the transfected line expressed high levels of the transgene (Figure 14).

The EL-4 cell line that had been transfected with the full length human CEA showed surface expression of this antigen over a 4 month period in vitro culture when measured by FACS analysis (Figure 15). The double transfectant MC38-CEA-A2D^b that had been transfected with both the human molecule CEA and the chimeric MHC class I molecule HLA-A2.1/D^b was also analysed. It was found to express high levels of both molecules (Figure 15).

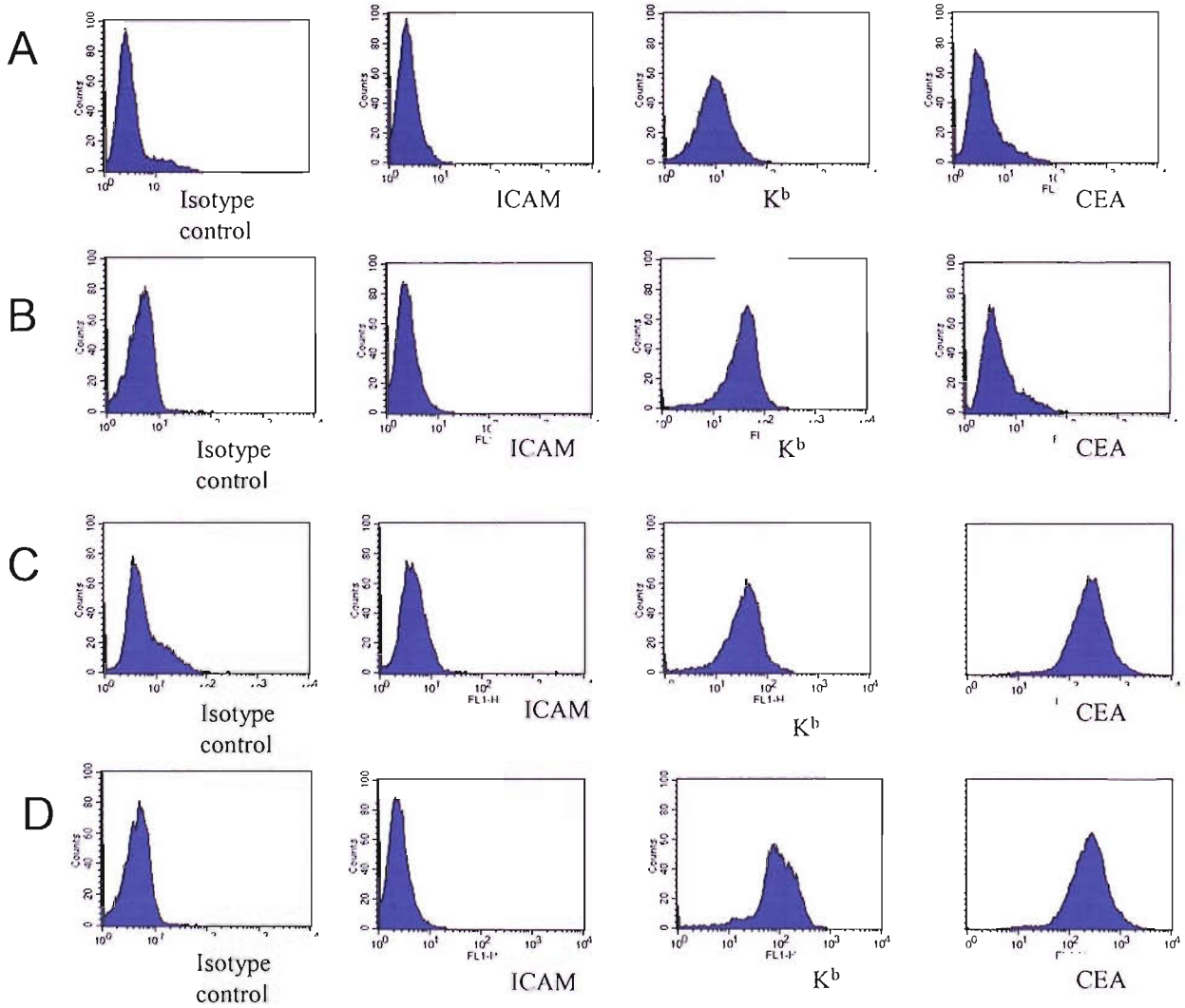


Figure 14. The phenotype of MC-38 and MC-38-CEA with and without interferon-γ.

The phenotype of the MC38 (panel A) was determined by FACS analysis. $1x10^6$ cells were incubated with 0.5 μg of the following FITC labelled antibodies against Kb, ICAM, IgG2a isotype control and the unlabelled monoclonal anti-CEA: COL-1 (Zymed). The COL-1 antibody samples were washed twice and further stained with a secondary sheep-anti-mouse FITC labelled antibody. Cells were analysed using a FACScalibur using CELLQUEST software from BD Pharmingen. Cells were gated on their forward scatter/side scatter properties. This was then repeated for MC38 cells that had been pre-treated with 100iU/ml of murine IFN-gamma for 48 hours (panel B). MC-38-CEA cells were also analysed in a similar way (panel C) and then after pre-treatment with IFN-gamma (panel D). This result was repeated on one other occasion.

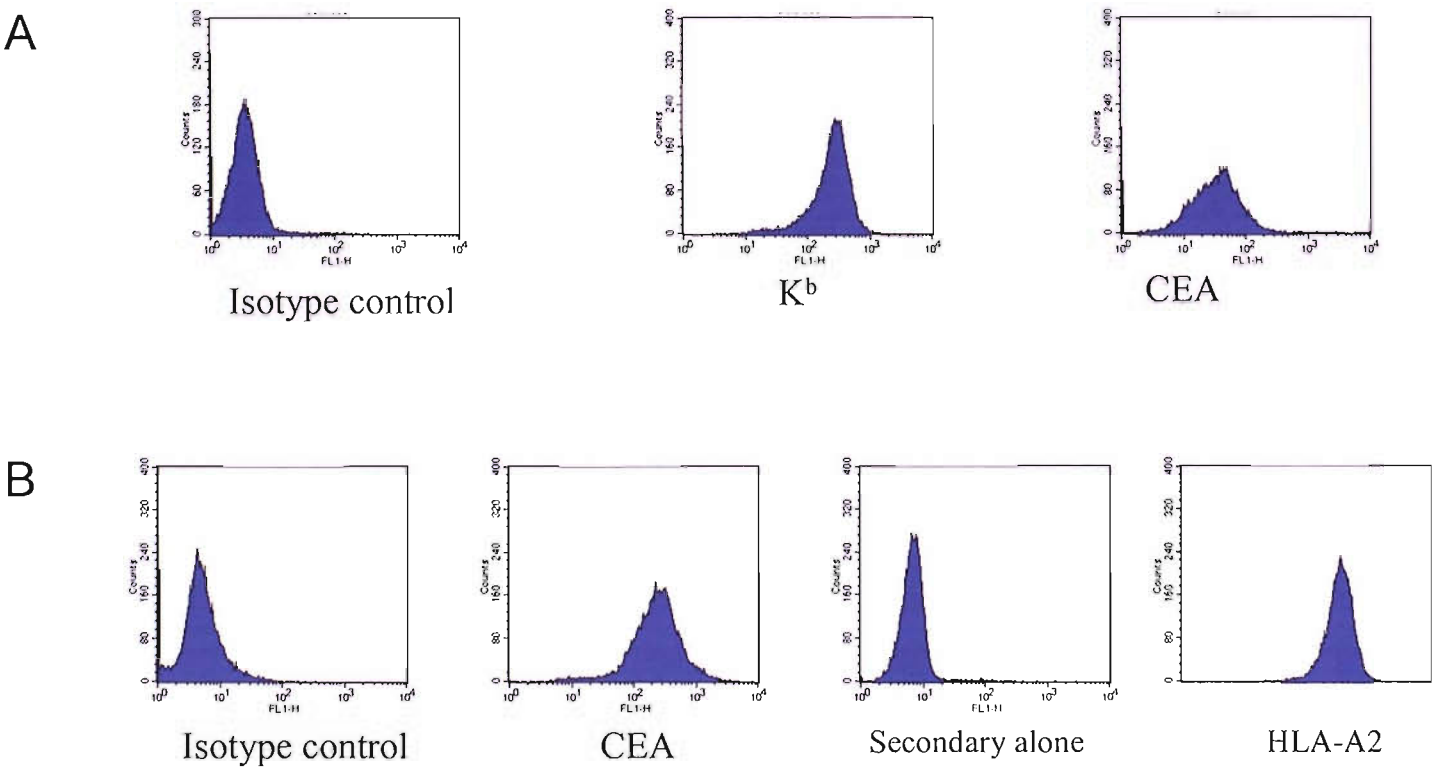


Figure 15. The phenotype of EL-4-CEA and MC38-CEA-A2Kb.

The phenotype of the EL-4-CEA transfected cell line (panel A) was determined by FACS analysis. $1x10^6$ cells were incubated with 0.5 μg of the following FITC labelled antibodies against Kb, IgG2a isotype control and the unlabelled monoclonal anti-CEA: COL-1 (Zymed). The COL-1 antibody samples were washed twice and further stained with a secondary sheep-anti-mouse FITC labelled antibody. Cells were analysed using a FACScalibur using CELLQUEST software from BD Pharmingen. Cells were gated on their forward scatter/side scatter properties. This was then repeated with MC38-CEA-A2Kb (panel B) with the addition of also staining with the bb7.2 antibody against HLA-A2 followed by secondary sheep-anti-mouse FITC labelled antibody. This experiment was repeated on one other occasion producing a similar result.

3.2.3. The DNA vaccine pDOM.EAQN primes epitope-specific CD8 IFN-gamma secreting cells

Groups of mice were vaccinated with the DNA vaccines as described above. 14 days later they were sacrificed and splenocytes pooled. FACS analysis was performed to detect peptide-specific IFN-gamma secretion directly ex vivo (figure 8). Priming of tumour-specific CD8 T cells was observed following one injection of pDOM.EAQN, with 0.7% of CD8 T cells staining with IFN gamma in response to 18 hours of peptide stimulation in vitro. pCEA did not induce a peptide-specific CTL response that was detectable ex vivo (Figure 16).

3.2.4. The DNA vaccine pDOM.EAQN activates specific cytotoxicity

The ability of these IFN-gamma-secreting CD8 T lymphocytes to mediate specific cytotoxicity was assessed by a standard ^{51}Cr release assay. After vaccination the splenocytes were pooled from 4 animals and were stimulated with EAQN peptide and IL-2 for 6 days. pDOM.EAQN vaccination induced cytotoxic CD8 cells that were able to lyse EL-4 cells that were loaded with peptide (figure 9). There was no lysis of an EL-4 target that was not loaded with peptide. The control vaccine pDOM did not induce cytotoxicity above the background level. The vaccines pCEA and pCEA.FrC did not induce cytotoxicity that was detectable in this assay. This was consistent with their inability to induce specific IFN gamma production (Figure 17).

3.2.5. pDOM.EAQN is able to induce specific cytotoxicity in mice transgenic for CEA

Groups of mice that were transgenic for human CEA were vaccinated as above. These mice express human CEA with a tissue distribution similar to that of humans. In addition these transgenic mice have significant levels of serum CEA in their blood. They should therefore be tolerant to human CEA.

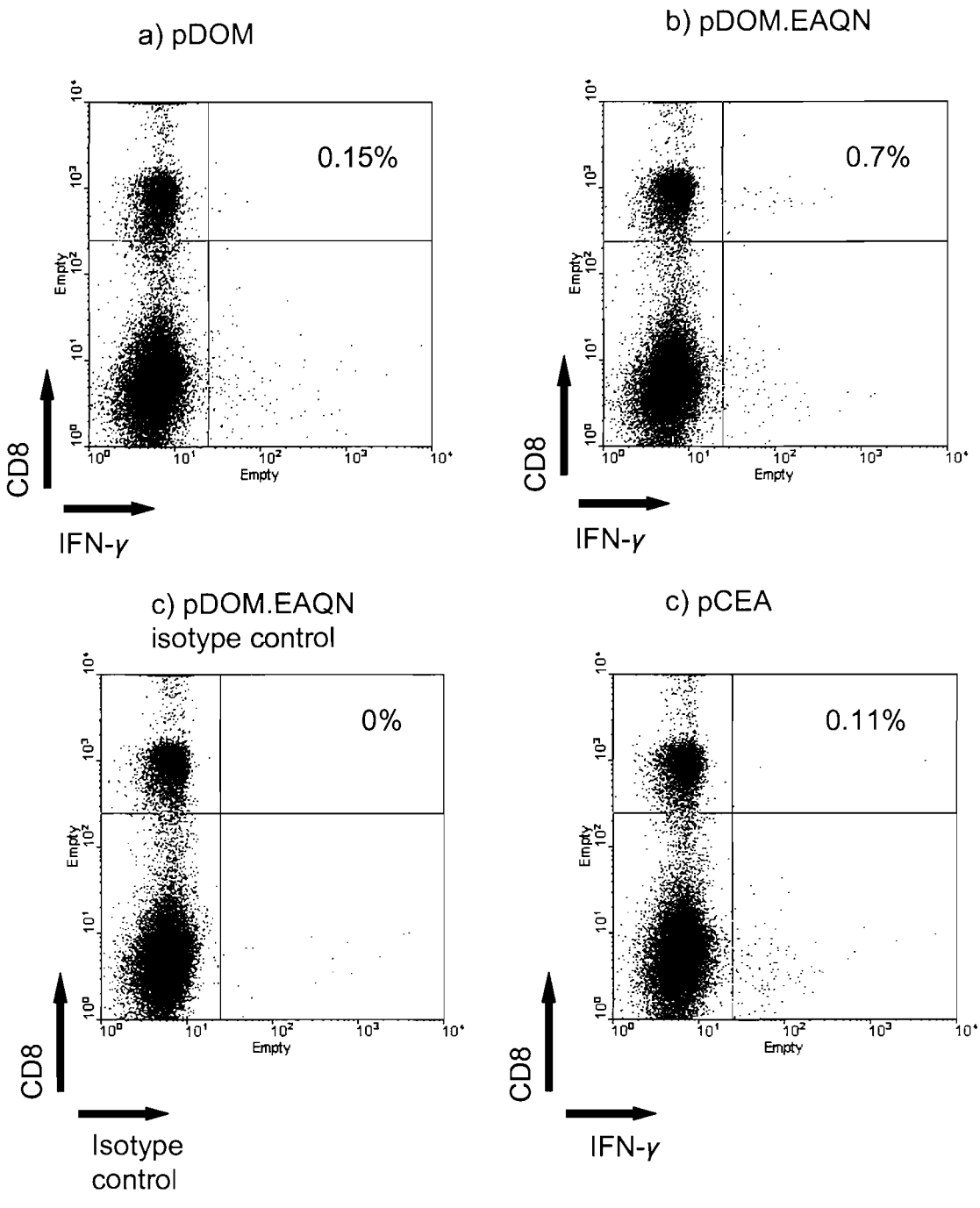


Figure 16. The DNA vaccine pDOM.EAQN primes epitope-specific CD8 IFN-gamma secreting cells

Groups of 4 wild type C57Bl/k mice were vaccinated with either pDOM, pDOM.EAQN or pCEA. Mice were culled at day 14. Splenocytes were pooled and stimulated with 10μM EAQN peptide for 6 hours in the presence of Brefeldin and 10iU/ml of IL-2. They were then permeabilised (see section 2.12) and ex vivo FACS analysis (see section 2.12) was used to measure the percentages of CD8+ T cells that stained with intracellular IFN-γ antibody. Splenocytes from mice that had been vaccinated with pDOM.EAQN were permeabilised and also stained with an isotype control as demonstrated above. Similar results were obtained in 3 of 3 experiments.

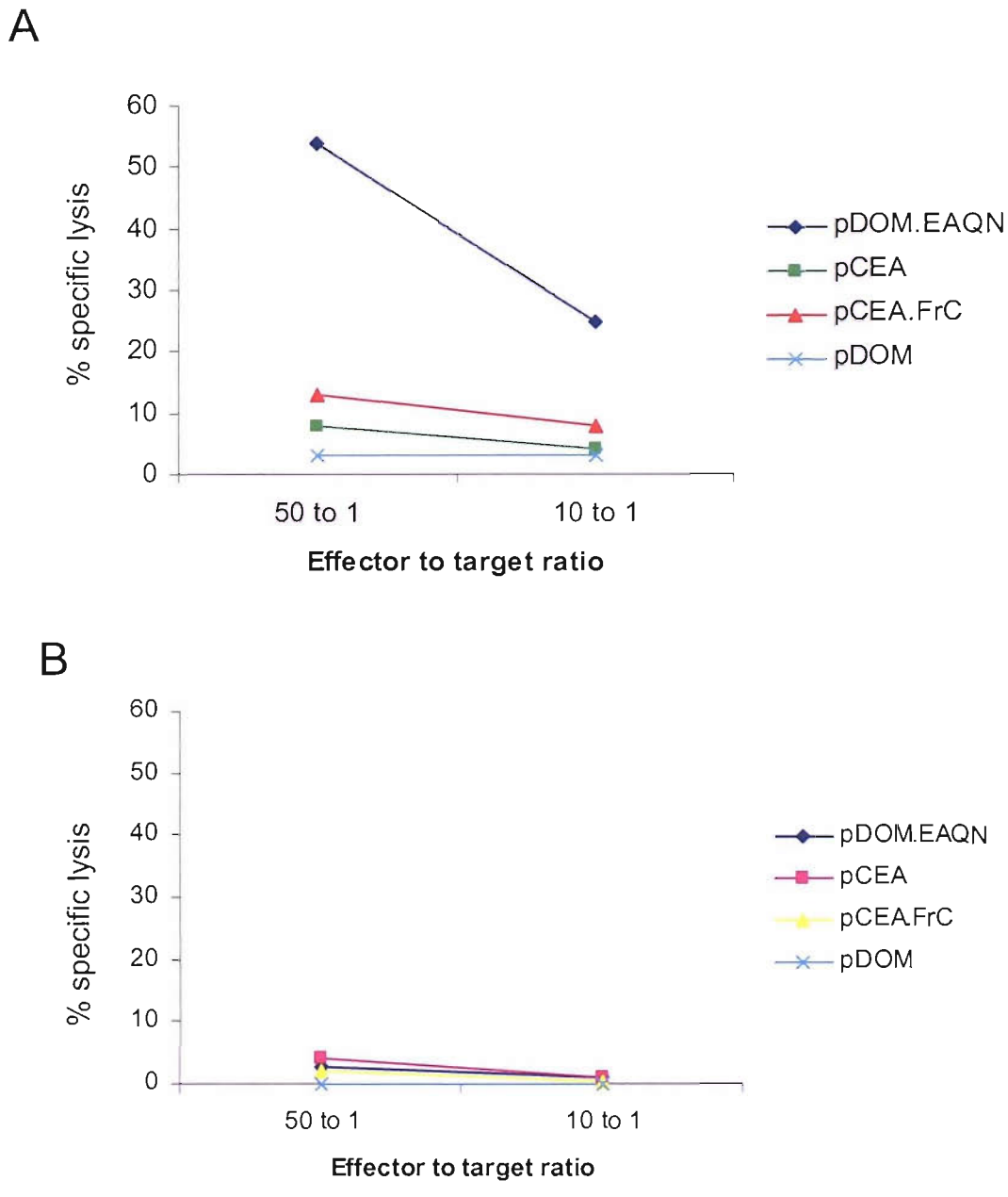


Figure 17. The DNA vaccine pDOM.EAQN activates specific cytotoxicity.

Groups of 4 mice were vaccinated against either pDOM.EAQN, pCEA, pCEA-FrC or pDOM. On day 14 splenocytes were pooled and stimulated in the presence of IL-2 and 10μM EAQN peptide. On day 6 of in vitro stimulation they were tested in a standard chromium release killing assay (section 2.14) against MC38-CEA cells pulsed with 10μM EAQN peptide (panel A) and against MC38-CEA with no peptide (panel B). This experiment was repeated with a similar result.

Mice were sacrificed on day 14 and pooled splenocytes were pulsed with peptide for 6 days in the presence of IL-2. Their cytotoxicity was assessed in a standard ^{51}Cr release assay. CTL from the mice vaccinated with pDOM.EAQN but not pDOM were able to induce lysis in peptide-pulsed targets (Figure 18).

3.2.6. CTL induced by pDOM.EAQN cannot lyse CEA-expressing tumours in vitro

The vaccine, pDOM.EAQN, is capable of eliciting high levels of CTL that are specific for the CEA-derived epitope. It is also able to induce specific CD8 cytotoxicity when injected into mice transgenic for CEA. However these CD8 cells will only be relevant to the treatment of cancer if they can lyse tumour that expresses CEA. In order to demonstrate this, mice were again vaccinated with pDOM.EAQN and CTL lines were generated with peptide and IL-2 as described as above. However whilst these lines could specifically lyse tumour cells pulsed with peptide they could not lyse unpulsed tumour cells (Figure 19).

One possibility was that the MHC class I surface expression was too low and consequently the cells were treated with IFN gamma to up-regulate class I expression. However the CTL still showed no specific lysis of these targets. To assess whether the inability to kill CEA expressing tumours was specific to this cell line the cytotoxicity of the CTL was assessed against EL-4 that had been transfected with CEA. However there was still no specific lysis of this target.

3.2.7. A DNA vaccine without leader, p(L-)DOM.EAQN, showed equivalent ability in stimulating cytotoxic T cell responses.

In order to assess whether this vaccine format could be processed from a cytosolic route a leaderless construct p(L-)DOM.EAQN was made. Mice were vaccinated with both pDOM.EAQN and p(L-)DOM.EAQN to see if these vaccines would generate responses that

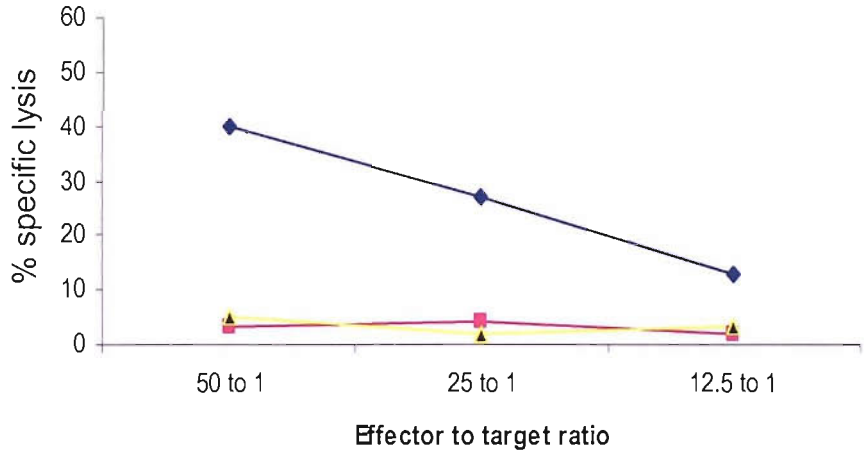


Figure 18. The pDOM.EAQN is able to stimulate an immune response from mice that are transgenic for human CEA.

Groups of 4 mice, transgenic for human CEA, were vaccinated with pDOM.EAQN. On day 14 mice were sacrificed and splenocytes cultured in the presence of EAQN peptide and IL-2 for 6 days. CTL activity was then measured by a ^{51}Cr release assay (see section 2.14) using MC-38-CEA cells as targets either with (◆) or without (▲) 10μM EAQN peptide. Splenocytes from mice vaccinated with pDOM were also tested against MC-38-CEA that were loaded with EAQN peptide (■). This experiment was repeated twice with similar results.

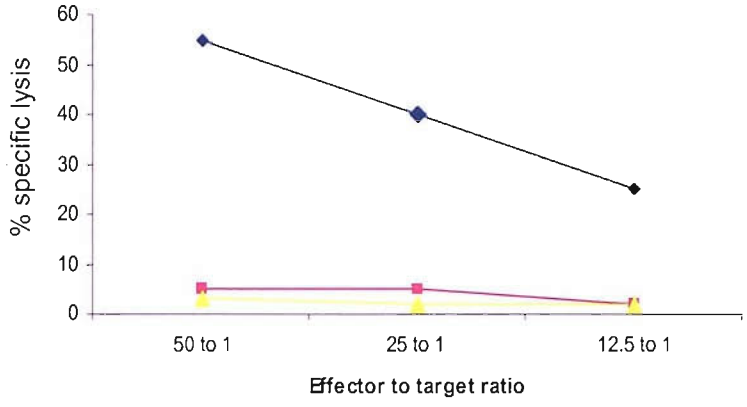


Figure 19. The CTL generated by pDOM.EAQN are not able to kill EL-4-CEA cells that express CEA.

Groups of 4 wild type C57Bl/k mice were vaccinated with pDOM.EAQN. On day 14 mice were sacrificed and splenocytes cultured in the presence of EAQN peptide and IL-2 for 6 days. CTL activity was then measured by a ^{51}Cr release assay (section 2.14) using EL-4-CEA cells as targets either with (♦) or without (▲) peptide. A CTL generated by pDOM was also tested against EL-4 that were loaded with EAQ peptide (■). This experiment was repeated with a similar result.

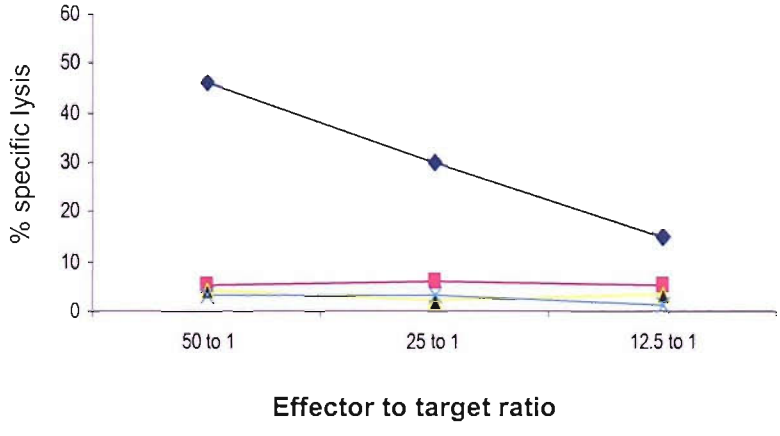


Figure 20. The CTL generated by pDOM.EAQN are not able to kill MC-38-CEA cells that express CEA.

Groups of 4 wild type C57Bl/k mice were vaccinated with pDOM.EAQN. On day 14 mice were sacrificed and splenocytes cultured in the presence of EAQN peptide and IL-2 for 6 days. CTL activity was then measured by a ^{51}Cr release assay (section 2.14) using MC-38-CEA cells as targets either with (♦) or without (▲) peptide. This CTL was also tested against MC-38-CEA that had been pre-treated with IFN-gamma (X).A CTL generated by pDOM was also tested against MC-38-CEA that were loaded with EAQ peptide (■). This experiment was repeated 3 times with similar results.

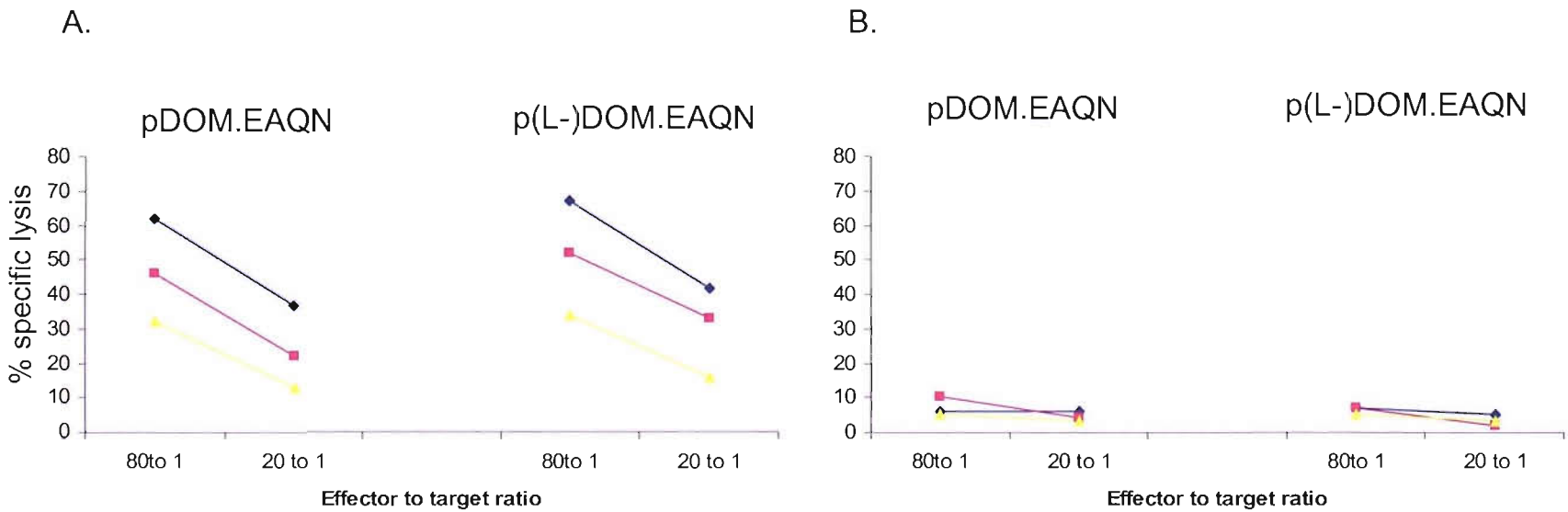


Figure 21. CTL generated by pDOM.EAQN and p(L-)DOM.EAQN show equivalent ability in generating cytotoxicity against EAQNTTYL.

Groups of 4 wild type C57Bl/k mice were vaccinated with either pDOM.EAQN or p(L-)DOM.EAQN. On day 14 splenocytes were pooled and cultured for 6 days in the presence of IL-2 and EAQN peptide (5μM - ◆ ; 1μM - □ ; and 0.2 μM - △). Panel A: they were then tested in a standard 51Cr release cytotoxicity assay (section 2.14) against peptide pulsed EL-4 cells. Panel B: they were also tested against MC-38-CEA tumour. This experiment was repeated twice with similar results.

were significantly different. In both cases spleens were taken from vaccinated animals on day 14 and cultured in the presence of peptide over 6 days in the presence of IL-2. Both vaccines generated CTL that were equivalent in their ability to lyse peptide pulsed targets after a number of different stimulation conditions (Figure 21). However neither vaccine was capable of stimulating a CTL response that could lyse the unpulsed tumour. It must be noted that this comparison was performed after a period of in vitro culture and therefore a quantitative comparison is less reliable than with an ex vivo analysis.

3.2.8. The DNA vaccine pDOM.EAQD did not elicit high levels of CTL specific for this analogue epitope

Groups of C57Bl/k mice were vaccinated with the pDOM.EAQN and pDOM.EAQD vaccines as described. They were then sacrificed on day 14 and the splenocytes pooled.

The two different cell lines were stimulated with EAQN or EAQD peptides respectively at 10 μM and IL-2 for 6 days. pDOM.EAQN vaccination induced cytotoxic CD8 cells that were able to lyse MC38 cells that were loaded with peptide (Figure 22). Again there was no background lysis against unloaded MC38 cells. There was 20% lysis of the MC38 cell line pulsed with EAQD peptide. pDOM.EAQD vaccination did not induce a CTL that could lyse MC38 cells pulsed with either the EAQD or the EAQN peptide. There was no lysis of an un-pulsed MC38-CEA tumour with CTL induced by either the pDOM.EAQN or the pDOM.EAQD vaccine. The control vaccine pDOM did not induce cytotoxicity above 10% lysis targets loaded with either peptide. Therefore pDOM.EAQD did not elicit a CD8+ lymphocyte response specific for the epitope EAQDTTYL.

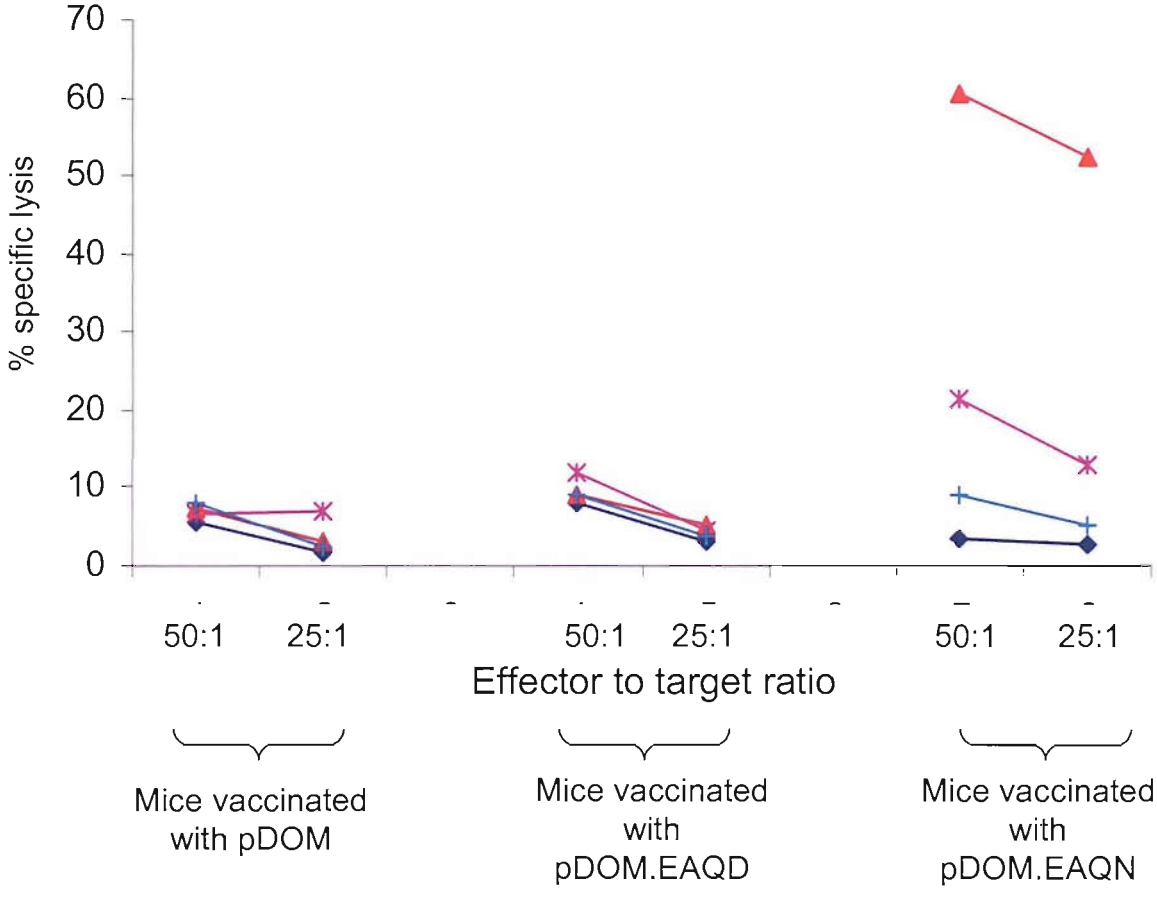


Figure 22. The DNA vaccine pDOM.EAQD did not elicit high levels of CTL specific for this analogue epitope and did not lyse the MC38-CEA tumour in vitro

Groups of 4 wild type C57Bl/k mice were vaccinated with pDOM.EAQN, pDOM.EAQD or pDOM. On day 14 mice were sacrificed and splenocytes cultured in the presence of EAQN or EAQD peptide and IL-2 for 6 days. CTL activity was then measured by a 51Cr release assay (see section 2.14) using MC-38 cells as targets either pulsed with with EAQN peptide (▲) or EAQD peptide (X) or no peptide (+). The tumour lines were also tested against MC-38-CEA cells with no peptide (♦)

DNA vaccination against CEA in mice transgenic for HLA-A2.1

3.2.9. The DNA vaccines pDOM.CAP-1 and pDOM.CAP-1/6D prime epitope-specific CD8+ IFN-gamma secreting cells in mice transgenic for HLA-A2.1

HHD mice express chimeric HLA-A2.1 (with $\alpha 1\alpha 2$ domains of human HLA-A2 and $\alpha 3$ cytoplasmic domain of D^b) but do not express murine $H\text{-}2^b$ class I molecules. Groups of HHD transgenic mice were vaccinated with the DNA vaccines: pDOM.CAP-1, pDOM.CAP-1/6D and pDOM. 14 days later they were sacrificed and splenocytes pooled. These cells were placed in an ELISPOT assay to detect IFN-gamma secretion directly ex vivo (Figure 23). Effective priming of high levels of tumour-specific CD8 T cells was observed following one injection of pDOM.CAP-1 or pDOM.CAP-1/6D. ELISPOT results reveal that there were high numbers of CTL that could be detected ex vivo by stimulation with relevant peptide post-vaccination with both of these vaccines. The vaccine pDOM.CAP-1/6D generated very high numbers of CTL that were specific for that epitope consistently (in 6/6 mice). pDOM.CAP-1 was less consistent in the generation of CTL and these CTL were lower in number than with the analogue vaccine.

3.2.10. CTL that were generated by vaccination with pDOM.CAP.1/6D recognised CAP-1 peptide poorly ex vivo.

After vaccination with pDOM.CAP-1/6D spleens were taken on day 14 and analysed for IFN-gamma secretion with the ELISPOT assay. This demonstrated that large numbers of cells to secreted IFN gamma in response to CAP-1/6D. Between 180 and 230 spots per million splenocytes were detected ex vivo at the lowest concentration of peptide. These cells did not secrete IFN-gamma in response to CAP-1. In every mouse tested less than 25 spots per million splenocytes were detected. This demonstrates a lack of cross-reactivity in these CTL (Figure 24 and Figure 25). Conversely splenocytes were isolated from mice vaccinated with

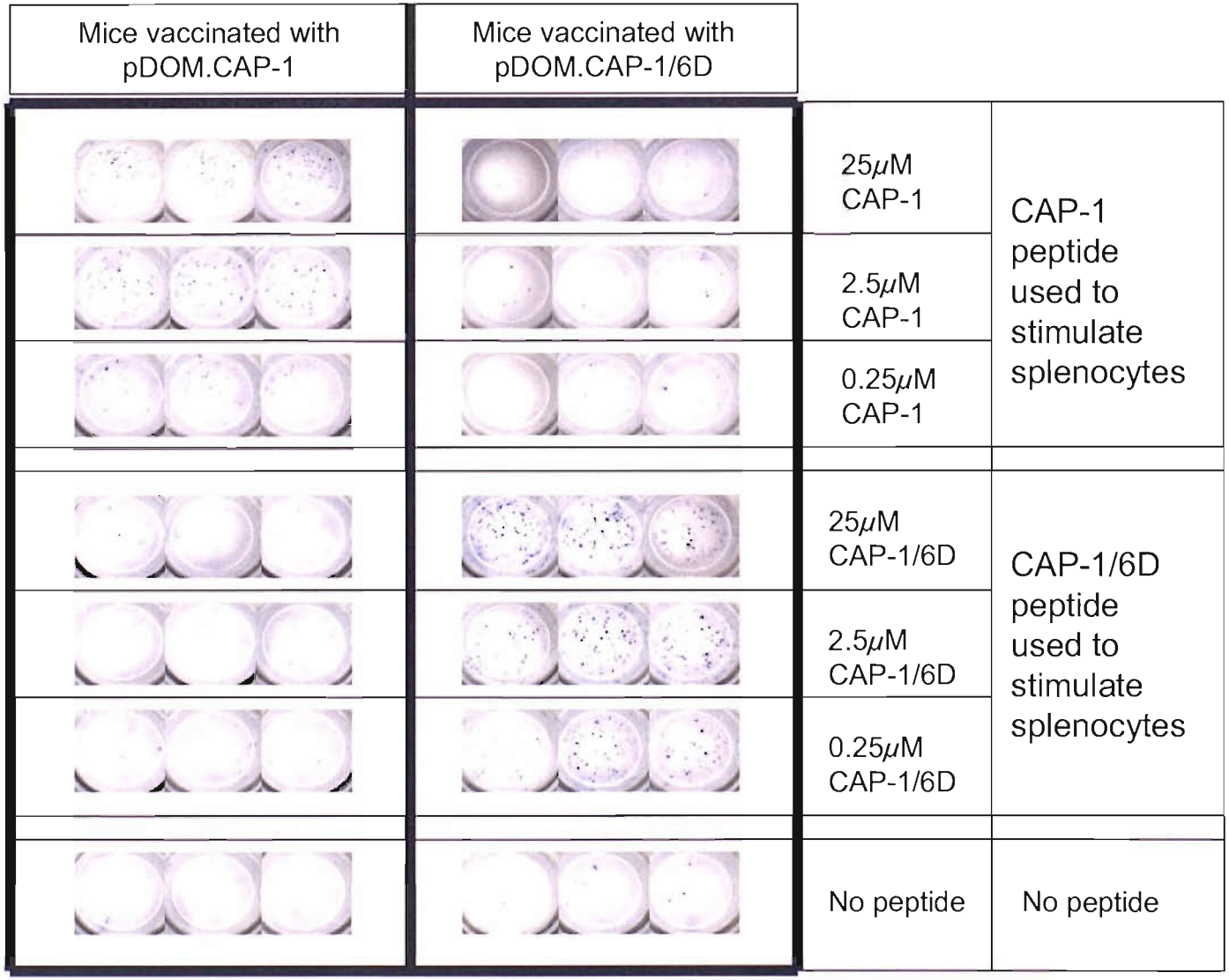


Figure 23. The DNA vaccines pDOM.CAP-1 and pDOM.CAP-1/6D prime epitope-specific CD8+ IFN-gamma secreting cells in HHD mice. HHD mice were vaccinated with either pDOM.CAP-1 or pDOM.CAP-1/6D. Spleens were taken on day 14 and placed in the ELISPOT plate wells in the presence of 25μM, 2.5μM or 0.25μM CAP-1 peptide or CAP-1/6D peptide (see methods section 2.16). Cells were incubated for 18 hours and then plates developed in the standard fashion (see section 2.16). This experiment was repeated with a similar result.

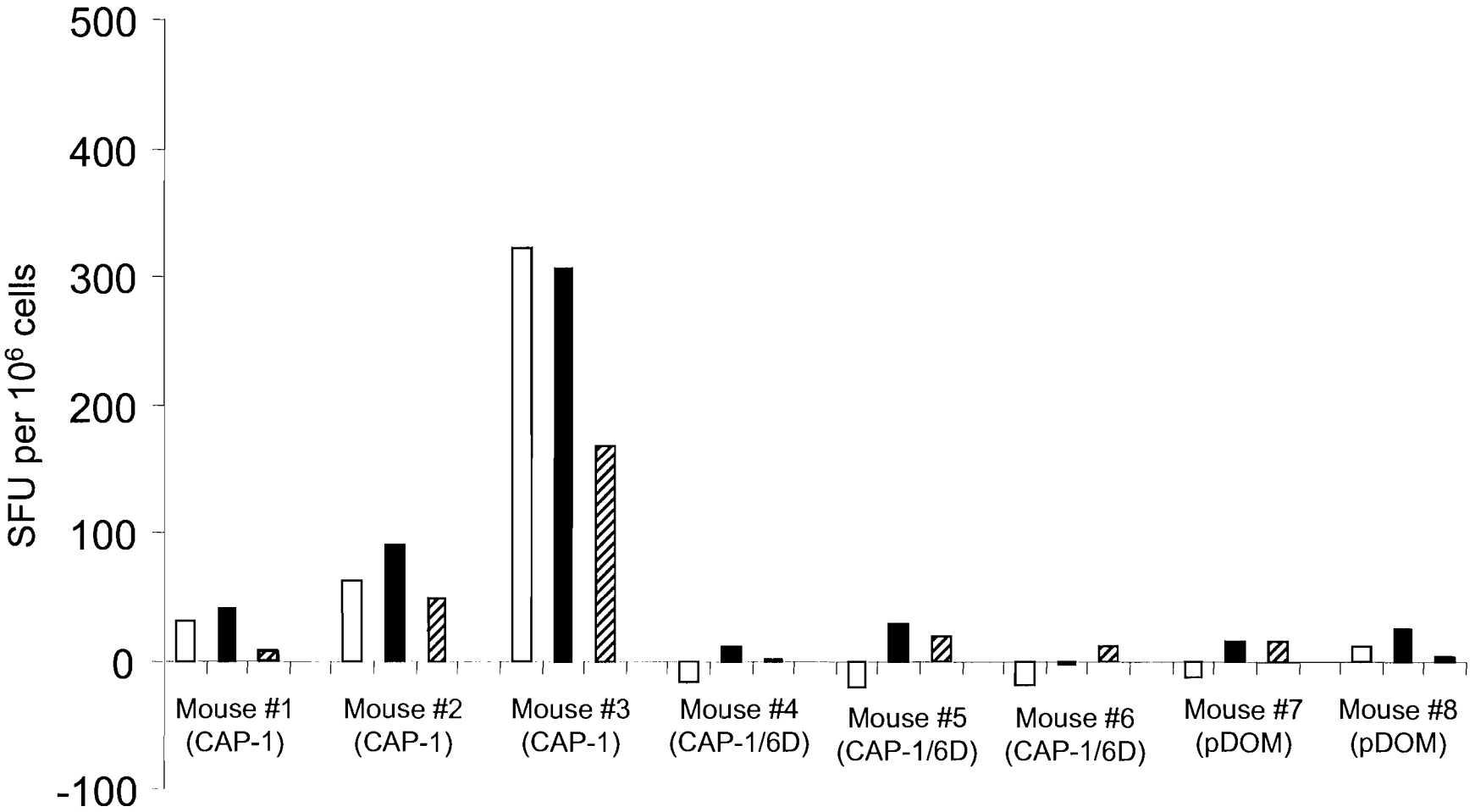


Figure 24. CTL that were generated by vaccination with pDOM.CAP.1/6D recognised CAP-1 peptide poorly ex vivo. HHD mice were vaccinated with pDOM, pDOM.CAP-1 or pDOM.CAP-1/6D. Spleens were taken on day 14 and placed in the ELISPOT plate wells in the presence of 25μM (open bars), 2.5μM (shaded bars) or 0.25μM (striped bars) CAP-1 peptide (see methods section 2.16). Cells were incubated for 18 hours and then plates developed in the standard fashion (see section 2.16). This experiment was repeated with a similar result.

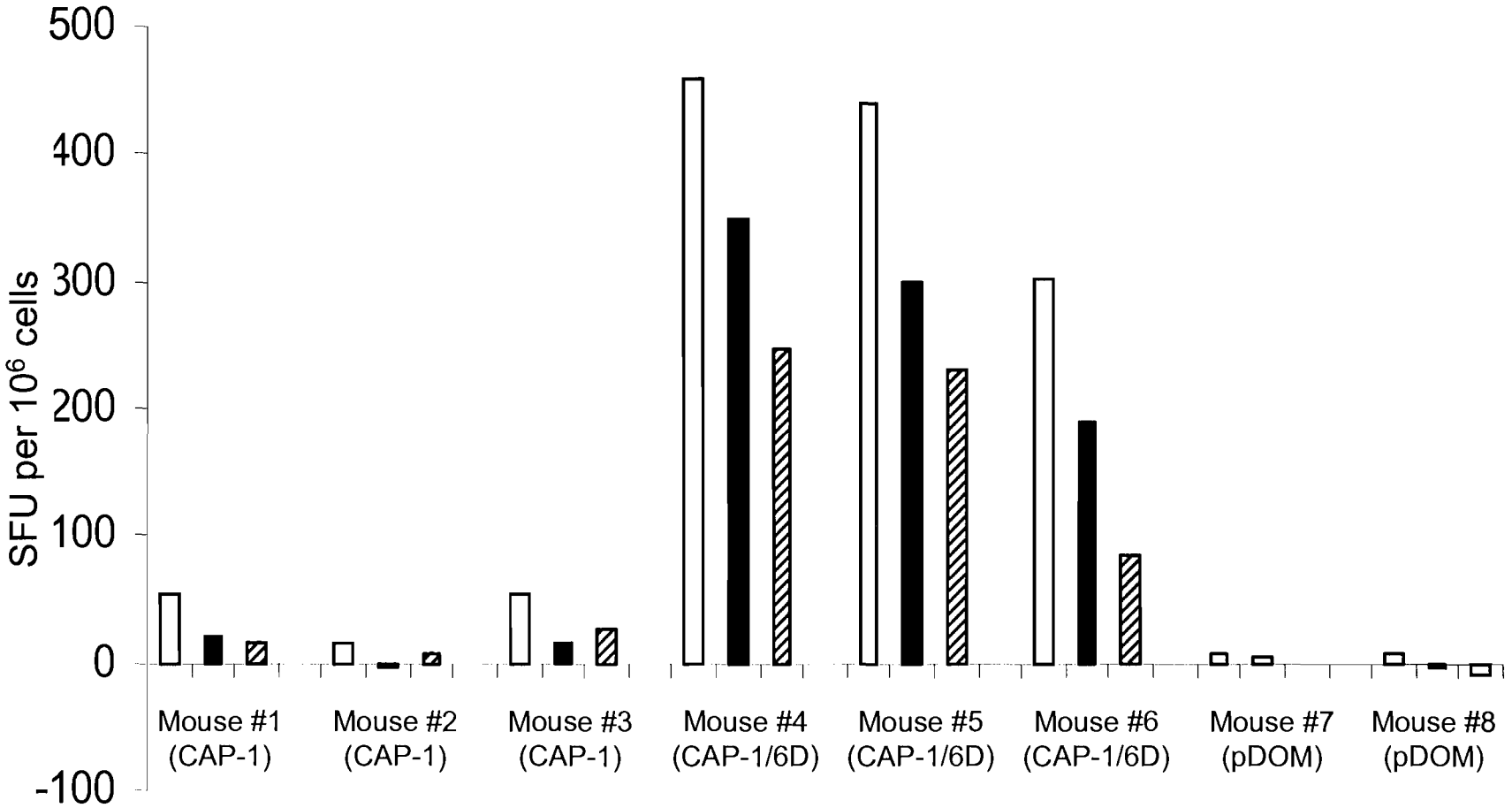


Figure 25. CTL that were generated by vaccination with pDOM.CAP.1 recognized CAP-1/6D peptide poorly ex vivo. HHD mice were vaccinated with pDOM, pDOM.CAP-1 or pDOM.CAP-1/6D. Spleens were taken on day 14 and placed in the ELISPOT plate wells in the presence of 25μM (open bars), 2.5μM (closed bars) or 0.25μM (striped bars) CAP-1/6D peptide(see methods section 2.16). Cells were incubated for 18 hours and then plates developed in the standard fashion (see section 2.16). This experiment was repeated with a similar result.

pDOM.CAP-1 and tested against the two peptides. Large numbers of spots were detected by IFN-gamma ELISPOT in response to the CAP-1 peptide however there were very few spots detected when these cells were tested with the CAP-1/6D peptide. Splenocytes isolated from mice vaccinated with the control vaccine pDOM did not respond to either CAP-1 of CAP-1/6D.

3.2.11. The DNA vaccines pDOM.CAP-1 and pDOM.CAP-1/6D activate specific cytotoxicity

Mice that had been vaccinated with pDOM, pDOM.CAP-1 and pDOM.CAP-1/6D were sacrificed on day 14. Splenocytes were isolated as above and stimulated for 6 days in the presence of either CAP-1 or CAP-1/6D peptide and IL-2. The ability of the IFN gamma secreting CD8+ T lymphocytes to lyse targets was assessed by a standard ^{51}Cr release assay. pDOM.CAP-1 was able to induce CTL that were able to lyse T2 cells that had been loaded with CAP-1 peptide (Figure 26). In a parallel assay CTL generated by pDOM.CAP-1/6D were able to lyse target cells loaded with CAP-1/6D (Figure 27). Interestingly the CTL generated by pDOM.CAP-1/6D were able to lyse targets that had been loaded with CAP-1 peptide after 6 days in vitro stimulation (Figure 28). This level of lysis was not as high as mice that had been vaccinated with CAP-1. This experiment was repeated on another occasion with similar results.

3.2.12. CTL induced by the vaccines pDOM.CAP-1, pDOM.CAP-1/6D and pDOM.CAP-1/8D were not able to lyse human tumour cells SW480

HHD mice were vaccinated with pDOM.CAP-1, pDOM.CAP-1/6D and pDOM.CAP-1/8D. On day 14 splenocytes were generated with peptide and IL-2 as described as above. However whilst these lines could specifically lyse SW480 tumour cells that express CEA pulsed with peptide they did not lyse unpulsed tumour cells (Figure 29). It remained a possibility that this was due to low class I surface expression and consequently the cells were pre-treated with IFN gamma to up-regulate class I expression. However in a chromium

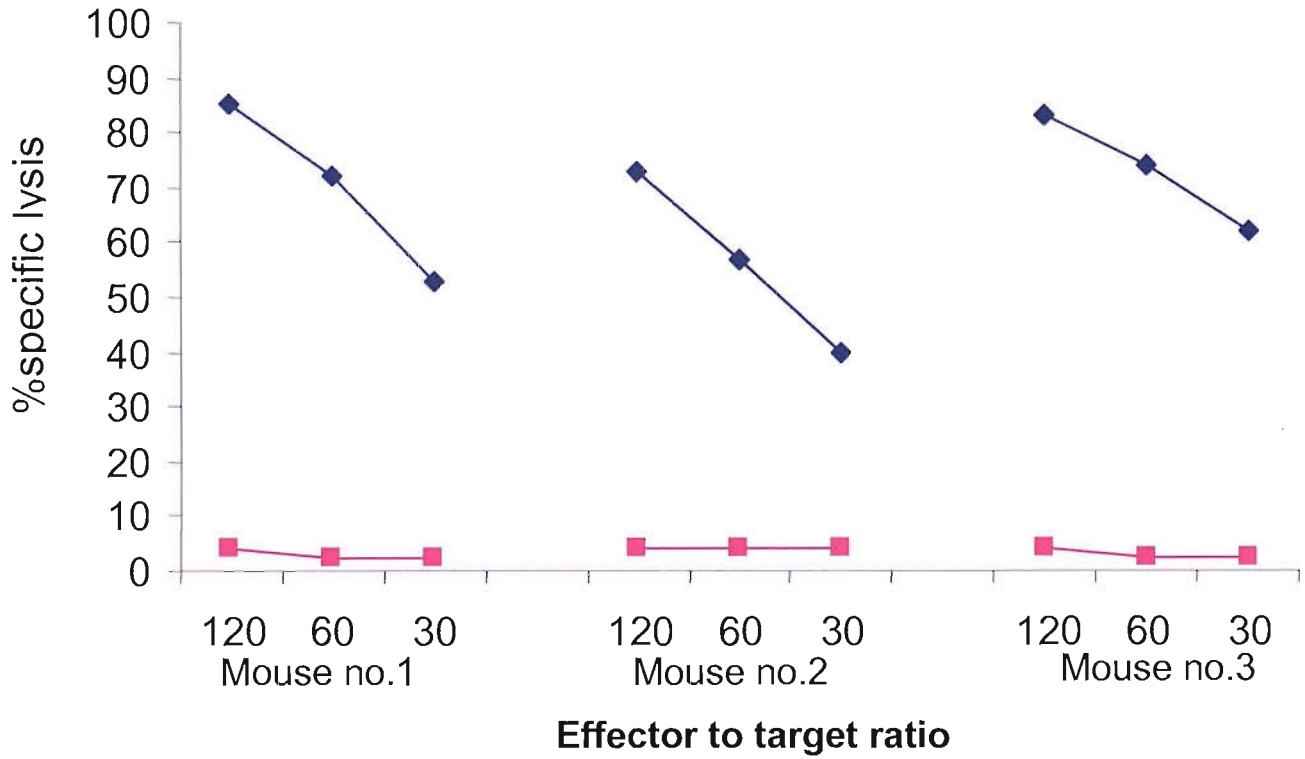


Figure 26. Cytotoxic activity of CTL from mice vaccinated with pDOM.CAP-1 against T2 cells loaded with CAP-1

HHD mice were vaccinated with pDOM.CAP-1. At day 14 splenocytes were re-stimulated with 10 μM CAP-1 peptide for 6 days in vitro in the presence of 10iU/ml IL-2. CTL activity was then measured by a ^{51}Cr release assay (see section 2.12) using T2 cells loaded with 10 μM CAP-1 peptide (♦) or no peptide (■) as target cells. The result was repeated on two occasions with similar results.

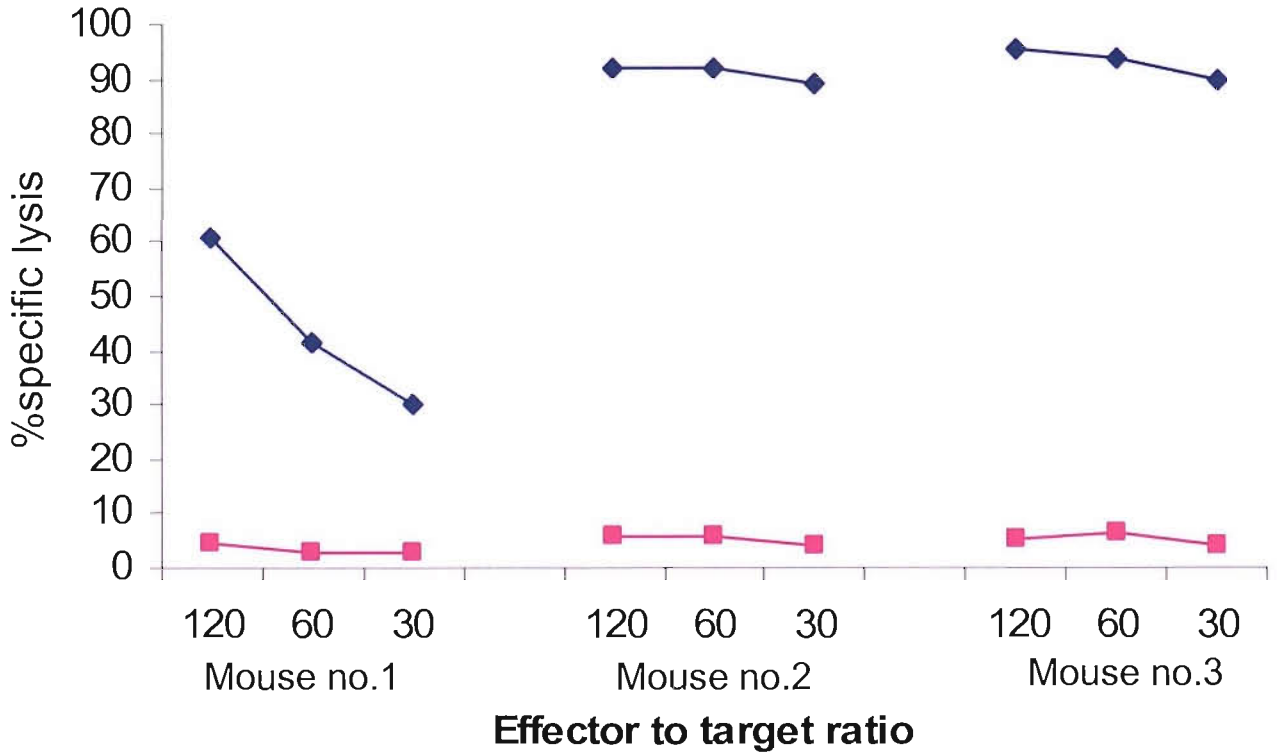


Figure 27. Cytotoxic activity of CTL from mice vaccinated with pDOM.CAP-1/6D against T2 cells loaded with CAP-1/6D

HHD mice were vaccinated with pDOM.CAP-1/6D. At day 14 splenocytes were re-stimulated with 10 μM CAP-1/6D peptide for 6 days in the presence of 10iU/ml IL-2 in vitro. CTL activity was then measured by a ^{51}Cr release assay (see section 2.12) using T2 cells loaded with 10 μM CAP-1/6D peptide (♦) or no peptide (▪) as target cells. The result was repeated on two occasions with similar results.

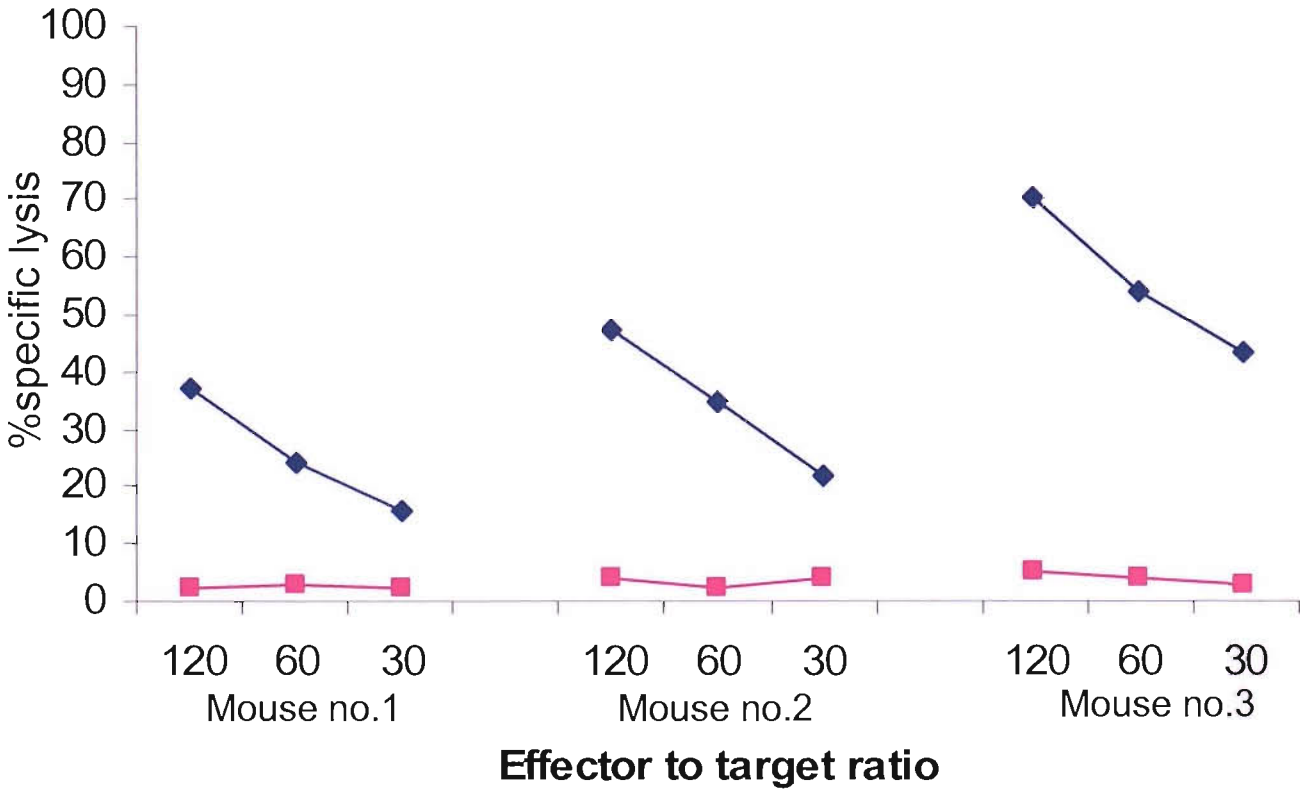


Figure 28. Cytotoxic activity of CTL from mice vaccinated with pDOM.CAP-1/6D against T2 cells loaded with CAP-1

HHD mice were vaccinated with pDOM.CAP-1/6D. At day 14 splenocytes were re-stimulated with 10 μM CAP-1/6D peptide for 6 days in vitro in the presence of 10iU/ml IL-2. CTL activity was then measured by a ^{51}Cr release assay (see section 2.12) using T2 cells loaded with 10 μM CAP-1 peptide (♦) or no peptide (■) as target cells. Representative data are shown and the result was repeated on two occasions.

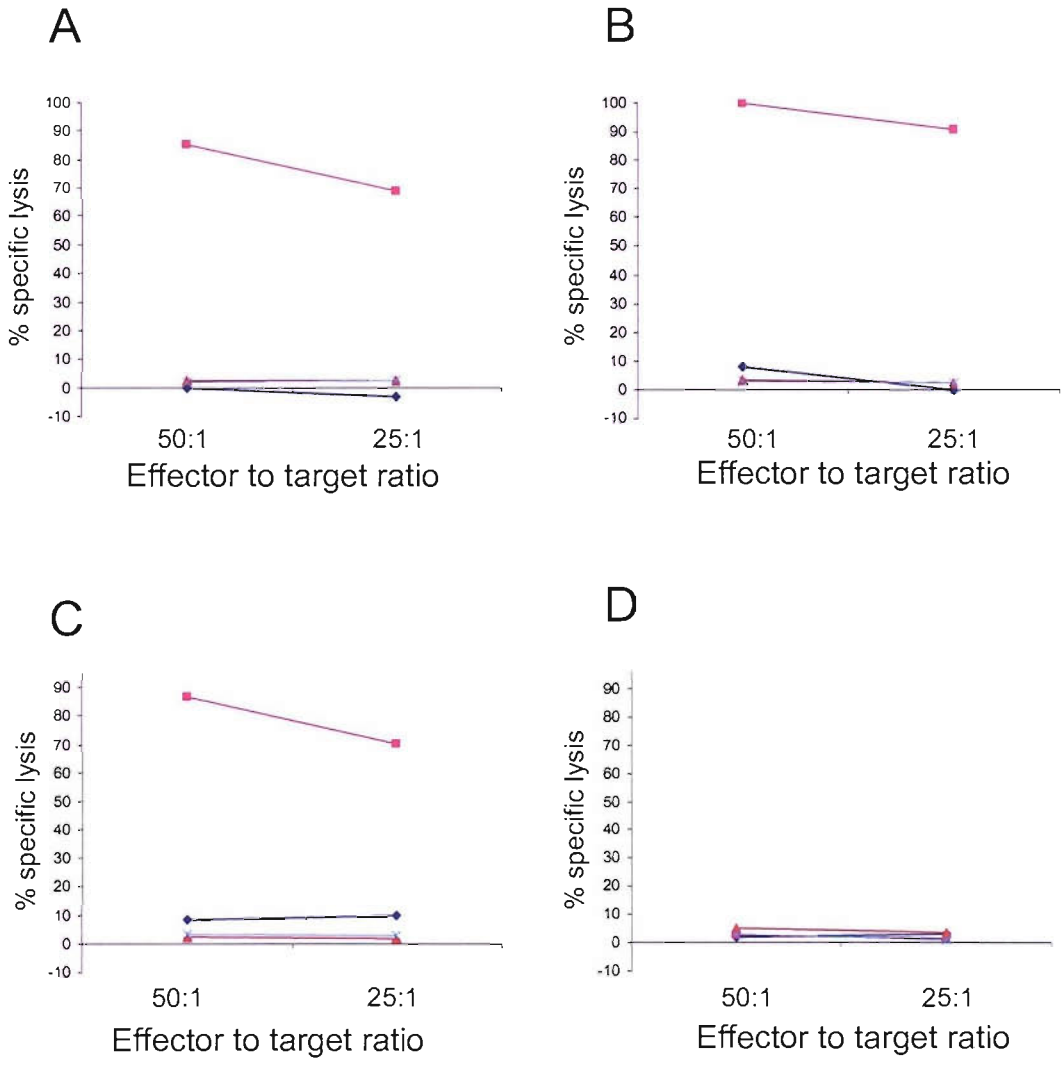


Figure 29. CTL induced by the vaccines pDOM.CAP-1, pDOM.CAP-1/6D and pDOM.CAP-1/8D were not able to lyse human tumour cells SW480.

Groups of 4 HHD mice were vaccinated with either pDOM.CAP-1 (A), pDOM.CAP-1/6D (B), pDOM.CAP-1/8D (C) or pDOM (D). After 14 days splenocytes were isolated and pooled and stimulated in vitro in the presence of 10 μM of the respective peptides and 10iU/ml IL-2. The resultant cells were tested in a standard 51Cr release assay (see section 2.12) against peptide-pulsed T2 cells (■), T2 cells alone (♦), SW480 cells (▲) or SW480 cells treated with IFN-γ (x). This experiment was repeated with a similar result.

release assay these cells were still unable to lyse SW480 human tumour cells expressing CEA. The murine CD8 T cells were only able to lyse tumour cells that were loaded with peptide. It is known that CD8 T cells are dependant on the interaction between CD8 and the $\alpha 3$ domain of MHC class I to function effectively. In this experiment murine CD8 will not bind to the human $\alpha 3$ domain of the fully human HLA-A2.1 molecule. Therefore it is perhaps not surprising that the murine CTL was unable to lyse the human tumour SW480.

3.2.13. CTL against a model epitope from influenza could lyse cells infected with influenza that also expressed chimeric HLA-A2.1/D^b but not fully human HLA-A2.1.

To address this issue the vaccine design was used to elicit CTL against a model epitope from the matrix protein of influenza. This is a well known nonamer that binds with high affinity to the MHC class I molecule, HLA-A2.1: GILGFVFTL. The DNA encoding this peptide was fused to the amino terminus of the gene encoding the first domain of FrC to give the vaccine pDOM.GIL.

CTL were generated in the usual way with groups of 4 mice being vaccinated against pDOM.GIL and the control vaccine pDOM. After 14 days the splenocytes were harvested and the splenocytes pooled. The CTL were then stimulated in vitro in the presence of 10 μM peptide and IL-2. After 6 days the cells were tested in a cytotoxicity assay against cells infected with influenza or pulsed with peptide. Target cells included L929s that had been transfected with the fully human HLA- A2.1 molecule and Jurkat cells that had been transfected with the chimeric HLA-A2.1/ D^b molecule.

The CTL that had been induce by the vaccine pDOM.GIL were able to lyse chimeric Jurkat cells that were both infected and peptide pulsed. However these CTL were only able to lyse L929 cells that were peptide pulsed. They were not able to lyse infected L929s. This shows the dependence of the CD8+ T cells on the interaction between murine CD8 and the $\alpha 3$ domain of MHC class I to lyse infected cells. However they are able to function independently of this interaction when they are lysing targets pulsed with high levels of peptide (Figure 30).

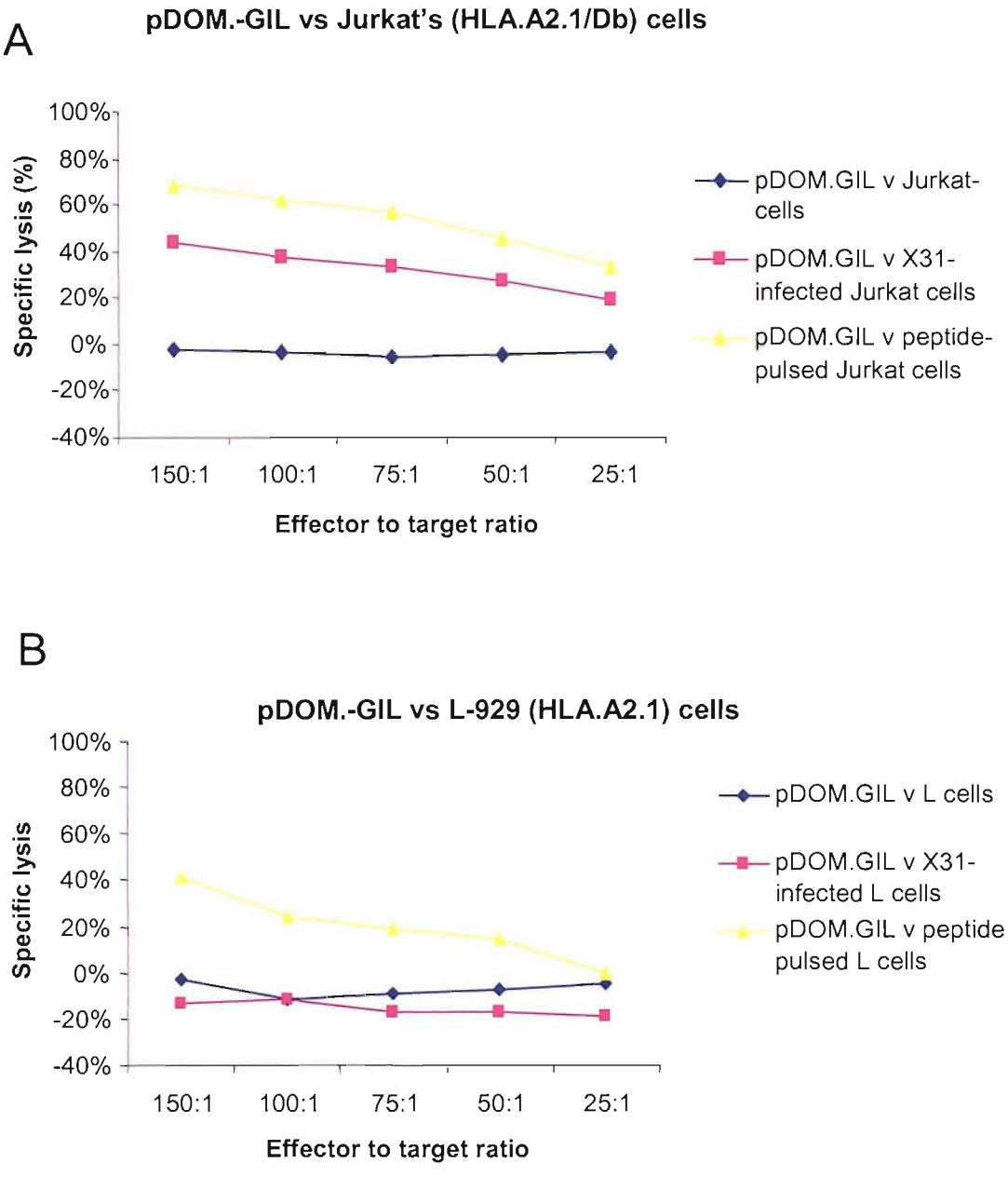


Figure 30. CTL induced by the vaccine pDOM.GIL is able to lyse a chimeric HLA-A2.1/Db-expressing target but not a fully human HLA-A2.1 target.

Groups of 4 HHD transgenic mice were vaccinated with pDOM-GIL. On day 14 splenocytes were pooled and stimulated in the presence of 10 μM GIL peptide and 10 iU/ml IL-2. Cytolytic activity was then tested in a standard 51Cr- release assay (see section 2.12) against peptide-pulsed or influenza-infected Jurkat-HLA-A2.1/D^b target cells (panel A). CTLs from mice vaccinated with pDOM-GIL were also tested against peptide-pulsed and influenza-infected L929-HLA-A2.1 cells (panel B).

3.2.14. CTL induced by the vaccine pDOM.IMI induced low level lysis of the tumour line MC38-CEA-A2K^b

In order to assess CTL activity in the presence of CD8-MHC class I interaction we used a target cell line that expressed the chimeric HLA-A2.1 molecule. A murine colorectal cell line, MC38, had been previously transfected with CEA and the chimeric HLA-A2.1 molecule (referred to as MC38-CEA-A2K^b).

Having the DNA vaccine design optimized makes it easy to substitute epitopes that are demonstrated to be relevant targets from within tumour antigens. Recently another potential HLA-A2.1 epitope from CEA has been identified by Zhou et al as a good target for CD8+ responses: IMIGVLVGV.[251] They generated a CD8-mediated immune response against this epitope and it was effective at lysing tumour cells expressing CEA. HHD mice were vaccinated with pDOM.CAP-1, pDOM.CAP-1/6D, pDOM.CAP-1/8D and pDOM.IMI and pDOM. CTL were generated using the method described above. CTL were tested after 6 days of in vitro culture against the transfectant MC38-CEA-A2K^b. This cell line expresses high levels of the chimeric class I molecule HLA.A2.1/K^b together with CEA. All the CTL lines were able to lyse peptide-pulsed cells apart from the control vaccine pDOM. The CTL generated by pDOM.IMI induced 20% lysis in unpulsed MC38-CEA-A2K^b cells. This level was significantly higher than the level of lysis induced by the cells taken from mice vaccinated with the control vaccine (8%). pDOM.CAP-1/6D induced a CTL that produced 14.7% lysis from the un-pulsed MC38-CEA-A2K^b cells. Neither pDOM.CAP-1 nor pDOM.CAP-1/8D generated a CTL that was able to lyse tumour alone (Figure 31).

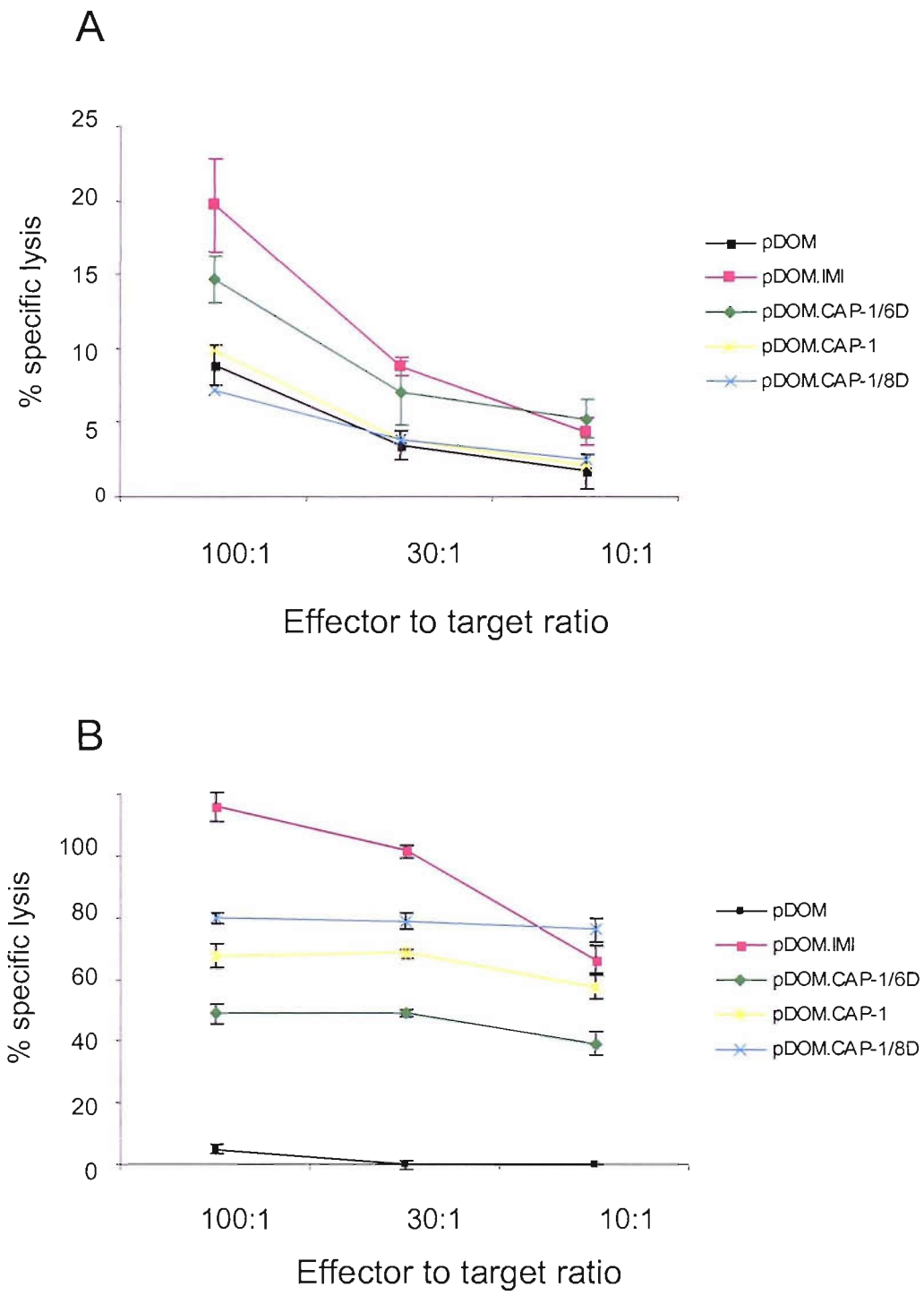


Figure 31. CTL induced by the vaccine pDOM.IMI induced lysis of the tumour line MC38-CEA-A2Kb.

Groups of 4 HHD mice were vaccinated with the following vaccines: pDOM, pDOM.IMI, pDOM.CAP-1, pDOM.CAP-1/6D and pDOM.CAP-1/8D. On day 14 the spleens were pooled and stimulated in vitro in the presence of 10 μM of each respective peptide and 10iU/ml of IL-2 for 6 days. They were then tested against MC-38-CEA-A2Kb without peptide (panel A) or with the relevant peptide (panel B).

3.3. Discussion

CEA is a tumour associated antigen found at very high levels on >95% of colorectal carcinomas. Consequently it has attracted much attention as a target for immunotherapy. There is no consensus of opinion as to what vaccine strategy should be used against this antigen. Vaccines that have effectively induced antibody against CEA have not yielded any clinical benefit for the patient. This study sought to build on previous work and generate a DNA vaccine that elicited CD8+ T cell-mediated immunity against CEA. . By studying this vaccine in pre-clinical models we aimed to move towards a clinical trial in colorectal cancer patients. Our strategy therefore was three-fold:

1. Firstly to demonstrate that we could induce CD8+ immunity to this antigen in wild-type mice.
2. Secondly to study this vaccine in mice transgenic for human CEA. This would enable us to study the ability of the vaccine to "break tolerance" and induce immunity against the self-antigen. It would also enable us to study any potential auto-immunity that followed vaccination.
3. Thirdly to study this vaccine in mice transgenic for the human MHC class I molecule. In this way we would be able to assess the induction of CTL against epitopes known to bind to human HLA-A2.1.

The DNA vaccine design in H-2K^b mice

Previous work had developed a DNA fusion vaccine that combined a pathogen-derived sequence from tetanus toxin (FrC) fused to full length tumour sequences. However whilst this vaccine was successful in eliciting antibody and CD4+ lymphocyte responses was not optimized to elicit strong CD8+ T-cell responses.[151,212]

The vaccine design, containing elements of the FrC gene fused to tumour genes, was modified to increase specific CD8+ T-cell immunity against candidate antigens. One potential problem was that two H-2K^b-restricted peptides from the second domain of FrC were previously found to be capable of stimulating a CD8 response. Because of the potential competition from these epitopes with the tumour-derived epitope the second domain of FrC was removed. It was then demonstrated that the immune response to the tumour epitope could be enhanced when the second domain of FrC was omitted and the tumour epitopes were fused to the C terminus of only the first domain of FrC. In fact, it was also shown in this study that if candidate peptides were placed at the C-terminus of the whole of FrC they were not as effective at generating a CD8 immune response as when the peptides were fused directly to the C-terminus of the first domain of FrC.[212]

A follow-up study looked at immune responses to an H2-Ld-restricted epitope from the retroviral gene product gp-70, AH1. A DNA vaccine encoding the whole gp-70 antigen was a poor inducer of CTL and this was not significantly improved by the fusion of the full length FrC molecule. Again the efficacy of the minimised FrC construct described above with the AH1 epitope fused to the C-terminus of the first domain of FrC was demonstrated with high levels of CTL being induced. The vaccine was able to provide protection against tumour cells that expressed this antigen, to kill these tumour cells in vitro and provide protection in vivo.[213]

This study sought to use this modified DNA vaccine design to target the human antigen, CEA, not only in wild type mice but also in mice transgenic for this human molecule. However there is no consensus of opinion as to which vaccine strategy should be used against this antigen. Consequently we sought to develop a vaccine that would induce high levels of cytotoxic T cells. Several studies have suggested a suitable target epitope presented by CEA-expressing cells is the H-2K^b -restricted peptide EAQNTTYL. By vaccinating mice with vaccinia virus expressing CEA, CTLs to the EAQNTTYL epitope were generated that were then able to lyse CEA-expressing tumour pre-treated with IFNγ.[248] It was uncertain from this study whether the IFN-γ mediated its effects by up-regulating the expression of MHC class I or whether it had effects on the processing of the epitope. Three other studies have vaccinated mice with either vaccinia or fowlpox expressing CEA and shown protection against CEA-

expressing tumours which was, at least partly, attributed to CTLs that they demonstrated against EAQNTTYL.[242,249,250] Therefore we chose this epitope from CEA to examine improved vaccine delivery for the generation of CD8+ immunity and its efficacy against tumours expressing this antigen.

The H-2K^b -restricted epitope EAQNTTYL (EAQN) is derived from the 6th domain of CEA. We have also tested vaccines that include the whole CEA sequence and the whole of the CEA sequence fused to the FrC sequence. As anticipated from the modified design it has been demonstrated here that the pDOM.EAQN design can elicit high levels of CTL that are detectable immediately ex vivo. It was also not possible to detect any IFN-γ secretion from the CD8 lymphocytes from mice vaccinated with the pCEA vaccine ex vivo.
Splenocytes from mice vaccinated with pDOM.EAQN were able to lyse peptide-pulsed targets after 6 days of in vitro stimulation. The construct pCEA and pCEA.FrC were unable to elicit a detectable cytotoxic response after an identical period of in vitro stimulation.

The pDOM.EAQN vaccine was also capable of generating peptide-specific CTL when injected into transgenic mice. These mice express CEA in cells of their gastro-intestinal tract in a pattern that is very similar to the native expression in human gut, and all have measurable serum CEA levels.[214] They are therefore a useful model when trying to evaluate the impact of immune tolerance to CEA as well as the effectiveness of vaccine strategies in overcoming tolerance. It was therefore interesting that the pDOM.EAQN vaccine was able to generate CD8+ responses in this challenging tolerogenic model. It is uncertain whether the generation of these CTL represents "breaking tolerance" in these mice. Given that all of the evidence from this study points to this epitope not being expressed by the tumours it is it is also possible that this epitope is not expressed by the epithelial cells of the gastro-intestinal tract in these animals. Their immune system may have never seen this epitope in the context of the MHC class I molecule before and therefore it would be effectively a foreign antigen. This would mean that we were unable to study tolerance and auto-immunity in this model.

The vaccine format that incorporates candidate epitopes fused to the C-terminus of the first domain of FrC seems to perform consistently better than constructs that only contain the whole antigen, such as CEA or gp70. It is probable that part of this reason is the provision of CD4 help for the CD8 immune response from the universal helper epitope p30 from the first domain of FrC. Maecker et al have demonstrated that CTL responses to DNA vaccination with the CTL epitope alone in the form of a minigene are dependant entirely on the presence of CD4 help.[256] Whilst some groups have demonstrated that responses to minigenes are possible in the absence of CD4 help this may be dependant on the CD8 epitope.[257] Certainly the presence of CD4 help is emerging as a major influence particularly on the development of an effective CD8 response. Recent studies demonstrate that T-cell help is 'programmed' into CD8+ T cells during priming, enabling these CD8+ cells to further expand on re-encounter with antigen in the development of effective memory responses.[258]

These studies also demonstrate that vaccines incorporating epitopes fused to the C-terminus of the first domain of FrC are consistently better at inducing CD8 responses than constructs that are linked to the whole of FrC. Initially this was thought to be due to competition between the class I epitopes that reside in the second domain of FrC and the candidate epitopes from the antigen that is being vaccinated against. Certainly there are two epitopes in the second domain of FrC that are extremely effective at stimulating a CD8 response. It is possible that these epitopes lead to the suppression of CEA-specific CTL activity. This would be expected to be most important at boosting vaccinations.[121] However the fact that these two second-domain FrC epitopes themselves produce a better CTL response when they are placed at the C-terminus of the first domain of FrC than when they are in their embedded site suggests that there must be other relevant mechanisms here.[212]

A second element in this vaccine design that may enhance presentation of MHC class I epitopes such as EAQN and AH1 is that they are processed more efficiently from the C-terminus of the first domain of FrC than from their embedded site in the whole CEA molecule and gp70 respectively. It is well described now that the the context of any potential epitope is critical to the probability that that epitope will be processed and presented. One example of this is the so-called "C-end rule". This was first described in the analysis of TAP-independent

routes of antigen processing.[90] It was discovered that peptides are efficiently liberated from the C-terminus of membrane proteins in the ER and that this was an extremely effective method of peptide delivery to the MHC class I complex.

There is also a rapidly expanding body of data on the influence of flanking residues on the processing and presentation of MHC class I peptides. More is being learnt about the cleavage motifs of the proteasome, the preferred sequences around cleavage sites. In this way it is possible to assess the likelihood of cleavage of an epitope from its site via this cytosolic protease pathway. It may be that many of these epitopes that have been assessed are more easily cleaved from the end of the first domain of FrC than they are from their embedded site in the native antigen. Certainly EAQNTTYL, the epitope from CEA is preceded by a proline and this makes it less likely that it is cleaved from its embedded site within CEA. In addition to proteasomal enzymes in the cytosol, aminopeptidases in the ER are also involved in epitope processing.

It is also likely that the size of the DNA vaccine is critical in the ability of the gene product to be transcribed and translated. Quinn et al examined in vivo protein expression by DNA vaccines in myocytes. They found an inverse correlation between gene size and protein expression.[259] In addition it has been described that there is an increase in peptide-class I complex formation obtained with cytosolic minigene products relative to full-length proteins. Anton et al compared the efficiency of generating antigenic peptides from various polypeptide contexts expressed by recombinant vaccinia viruses. They looked in particular at the processing and presentation of an immunodominant peptide from nucleoprotein.[260] They found that a minigene construct generated 55,000 complexes per cell, whereas the full-length gene generated only 10^5 copies per cell. Small increases in cell surface presentation of these complexes was associated with improved CTL generation.[261] The fact that the construct pCEA.FrC is much larger than the pDOM.EAQN construct will lead to lower levels of gene expression and consequently in the generation of peptide-class I complexes. The pCEA construct is also much larger than pDOM.EAQN but also lacks the CD4+ T cell help afforded by the first domain of FrC. It is likely that both of these factors are important.

Although constructs encoding smaller genes lead to increased protein expression this is complicated by the inability of minigene constructs to be cross-presented.[262] There is evidence that DNA vaccines stimulate CD8+ responses largely through cross-priming. Therefore the presence of the first domain of FrC may protect the tumour peptide for presentation through this route.

A leader sequence was included as there was evidence that specifically targeting antigens to the ER led to higher levels of CTLs in a previous model.[263] Surprisingly there was no difference in this model with or without the leader sequence. Both of these constructs stimulated CD8+ immunity.

The CTL elicited by the vaccine do not lyse H-2K^b tumour cells that express CEA.

The vaccine pDOM.EAQN was able to induce high levels of specific CTL in both wild-type and transgenic mice. These CTL generated by the vaccine were not able to lyse the tumour MC38-CEA or the transfectant EL4-CEA. There are several possible explanations of this finding. Firstly it may be that the tumours do not express EAQNTTYL-class I complexes. It may be that it is not possible to cleave this peptide from its native site. This epitope was chosen because of evidence from Schlom and colleagues using vaccinia viruses encoding the whole of CEA to vaccinate mice.[248] They found that an in vitro cell line generated from these mice with the EAQN peptide was able to lyse the tumour MC38-CEA in vitro. On closer examination this CTL showed minimal tumour lysis in a standard 6-hour ^{51}Cr release assay but marginally higher levels in a 24-hour indium-release assay. This, together with the very high concentration of peptide required to propagate the CTL, implies that the TCRs of the CTL were of very low affinity for the EAQNTTYL peptide-class I complexes. In addition when this EAQNTTYL-specific CTL were adoptively transferred to mice bearing CEA-expressing tumour this CTL had no effect on overall survival. Interestingly lysis of tumour was inhibited by D^b antibodies but not by K^b antibodies raising further doubts that this octamer was the relevant epitope expressed by the tumour.

Another possibility explaining the inability to kill the tumour is that the epitope is differentially processed by the proteasome and the immunoproteasome. Schlom et al demonstrated that CTL generated above only lysed tumour after pre-treatment with IFN-gamma. This may be due to up-regulation of class I on the tumour surface that has been demonstrated here. It may also be due to the induction of the immuno-proteasome in these tumour cells. However in this study CTL induced by pDOM.EAQN could still not induce lysis of the MC38-CEA line even after it had been pre-treated with IFN-gamma.

The tumour line may also glycosylate the EAQNTTYL epitope that has a potential N-glycosylation site at position four. It may be that this epitope is normally glycosylated as CEA is heavily N-glycosylated in vivo. Before entry into the proteasome, sugars can be removed from glycosylated polypeptides by cytosolic peptide *N*-glycoamidase.[264] This *N*-glycanase removes N-linked glycans by cleaving the glycan from Asn and converts the Asn residue to Asp.[265] However this conversion would yield the peptide EAQDTTYL which I have studied. This epitope was unable to generate an immune response.

Another possibility was that this epitope is differentially glycosylated in the cells that are transfected during vaccination (probably myocytes) and the tumour. Several tumours have been shown to aberrantly glycosylated surface antigens such as CEA. In this circumstance it may be that the tumour presents an unglycosylated EAQNTTYL. However in our study the CTL generated by the vaccine pDOM.EAQN are able to respond to the unglycosylated peptide so this explanation seems unlikely. Therefore it is likely that this epitope is either not expressed on the surface of the tumour cells or is expressed at such a low level that it was not possible to lyse these target cells.

Testing of the candidate peptide sequences reported to be restricted by the human HLA- A2.1 molecule

All 4 vaccines (pDOM.CAP-1, pDOM.CAP-1/6D, pDOM.CAP-1/8D and pDOM.IMI) were able to generate high levels of peptide-specific CTL in mice transgenic for the human MHC molecule: HLA- A2.1. The HHD transgenic mouse is a demanding test-bed for any vaccine and this model is discussed more fully in the next chapter.

What was particularly of interest was the success of the pDOM.CAP-1 vaccine in generating an immune response. Many groups have had difficulties in generating immunity to the CAP-1 epitope and this was the reason for resorting to the analogue peptide CAP-1/6D. Whilst this peptide does not have a higher affinity for the class I molecule it does generate CTL more effectively than its wild-type counterpart. Therefore this peptide is being used in a larger clinical trial loaded onto dendritic cells and infused into cancer patients. In a similar result the pDOM.CAP-1/6D was more effective at consistently inducing CTL in mice transgenic for HLA- A2.1 than pDOM.CAP-1. What was noticeable from our study was that these CTL when analysed ex vivo were very poor at recognising the wild-type CAP-1 peptide. After 6 days stimulation in vitro these CTL did show cytotoxicity against CAP-1 loaded targets but this did not match the cytotoxicity generated by pDOM.CAP-1. It is difficult to extrapolate a result from a transgenic mouse into a human. However it does parallel a result found in another study. Zaremba and colleagues found that they were unable to generate in vitro CTL using CAP-1 peptide but could do so using the CAP-1/6D analogue in two healthy volunteers. Only one of these CTL showed cross-reactivity to the CAP-1 peptide and was able to lyse a CEA-bearing tumour in vitro.[253] This highlights the dangers of using analogue peptides in clinical trials. It may be that some analogue peptides are more effective at generating CTL but that these do not have cross-reactivity for the wild-type peptide. This may particularly be a feature of analogue peptides where the altered amino acid is not in an anchor residue but is in the TCR-interacting region of the peptide. Optimisation of vaccine design, incorporating CD4 help and pathogen-derived sequences, enables the stimulation of CD8 immunity to the weaker wild-type peptide. This may be a lower risk strategy when attempting to generate CD8 immunity against tumours.

Interestingly the CTL generated by pDOM.CAP-1/6D were able to lyse targets that had been loaded with CAP-1 peptide after 6 days in vitro stimulation. One possible explanation of this is the expansion of low affinity CTL by stimulation with high concentrations of peptide in vitro. These lower affinity CTL may have a greater cross-reactivity to the wild type peptide.

In the HLA- A2.1 transgenic mouse it was again very difficult to generate CTL that were able to lyse tumour that was not pulsed with peptide. Initially the CTL were tested against the human tumour SW480. This is a human tumour expressing the human class I molecule HLA-A2.1 and CEA. None of the transgenic murine CTL were able to lyse this cell. The most likely factor here is the inability of the mouse CD8 molecule to bind human MHC class I. In a different model CTL generated against influenza were not able to lyse target cells that expressed the fully human HLA-A2.1. However these CTL were able to lyse infected cells that were expressing the chimeric molecule HLA-A2.1/D^b. Since this experiment has been performed Purbhoo et al. have confirmed that the CD8 molecule is critical for cytotoxic T cell function. They showed mutations in the alpha-3 domain of MHC class I that fully abrogate binding to CD8 did not affect interaction with the TCR. Despite this, cells expressing mutant MHC class I did not activate CTL. They showed that this is dependent on the ability of the coreceptor to effect signal transduction and does not derive from CD8-mediated stabilization of TCR-MHC complexes. There are data that indicate that CD8 can contribute in a very limited degree to the stabilization of some cell surface human TCR/pMHC interactions. This contribution to stability appears to be greater in murine systems which may be due to higher binding affinity of the TCR/pMHC interaction. This is presumably less true for murine cells transgenic for the human class I molecule HLA-A2.1/D^b. In addition it was interesting that in the above paper Purbhoo et al were unable to detect binding of murine CD8 to human MHC.

Consequently I sought a target cell that would have an alpha-3 region that could bind to the murine CD8 on the CTL that I had generated. MC38-CEA-A2K^b is such a cell line with the chimeric class I molecule. When tested against this line the CTL induce by the vaccine pDOM.IMI showed specific lysis of the target cell. The vaccine pDOM.CAP-1/6D showed lower levels of lysis against this tumour. This is only a preliminary result and needs to be tested against the tumour in vivo as well as in vitro.

Further Work

Future work will involve further analysis of the CTL generated by the pDOM.CAP-1/6D and pDOM.IMI vaccines. Preliminary results suggest that the pDOM.IMI vaccine is able to induce CTL that lyse tumour cells and possibly also pDOM.CAP-1/6D.

A double transgenic mouse that expressed both human CEA and the human class I molecule HLA-A2.1 will be obtained to test these vaccines further. The above vaccines will be studied in vivo to see if they are able to protect against a tumour challenge in these mice.

This model, that expresses the commonest human MHC class I molecule along with CEA throughout their gastro-intestinal tract, in a manner that exactly reflects human expression, provides an excellent model for generating CD8+ lymphocytes against CEA in patients. In this way tolerance and auto-immunity can be studied.

Rammensee has recently eluted a peptide from 2 colorectal tumours that is derived from the CEA sequence. This direct approach for discovering relevant tumour epitopes has been reliable in identifying epitopes that are both processed and presented by tumour cells. This would make a sensible further target epitope with which to study the CEA transgenic mouse.

4. DNA Vaccination against cytomegalovirus

4.1. Introduction

Cytomegalovirus (CMV) was first isolated from the salivary gland and kidney of two dying infants with cytomegalic inclusion bodies in 1956. Even today congenital infection is very common. In the US alone there 40,000 congenital infections per year, 10% of which leave the infant with a severe neurological deficit.

The other important clinical setting for CMV disease is in patients who are immunocompromised. In transplantation CMV is the commonest opportunistic infection and causes significant mortality and morbidity. There are 4,000 transplant-associated CMV infections per year in the US; 1,600 of these develop severe CMV-related disease each year and 10% of these will go on to die.[266,267] CMV infection is also an increasing problem in patients who have AIDS.

Although CMV is often neglected it is apparent from the above that it is an extremely destructive infection. Treatments are available including the antiviral drug ganciclovir. However this drug has many side-effects including marrow toxicity. The development of effective vaccines against this virus is therefore of paramount importance.

4.1.1. Cytomegalovirus

Human cytomegalovirus (CMV) is also known as Human herpesvirus 5 (HHV5) and is a member of the subfamily β-Herpesvirinae of the family Herpesviridae. These are enveloped viruses with an icosahedral capsid that encloses a double-stranded, linear DNA genome. It is one of the largest viruses known to infect humans having a genome of 230,000 kDa that encodes approximately 230 proteins. The DNA is wrapped around a nuclear core protein and

this is surrounded by a matrix protein (pp65 or UL83). The virus enters the host by fusion of the virus envelope with the cell membrane or by phagocytosis and infectious particles are detectable 1 day after inoculation.

CMV infection produces immediate-early (IE), early and late CMV antigens. IE antigens appear in the nucleus of CMV-infected cells 1 to 3 hours after infection and remain present throughout latent infection. Many of the IE antigen gene products direct transcription of both viral and cellular genes. Early antigens appear in the cytoplasm 3-24 hours after infection and they direct viral DNA synthesis. Late antigens appear in the nucleus and cytoplasm greater than 24 hours after infection and they direct the production of structural nucleocapsid proteins.

CMV antigens have a number of other effects. The IE gene product upregulates transcription and expression of IL-2 and the IL-2 receptor.[268] The IE and early gene products also up-regulate adhesion molecules such as intracellular adhesion molecule-1 and lymphocyte functioning antigen-3.[269] Ultraviolet irradiation can block this up-regulation which destroys viral infectivity.

4.1.2. Evasion of the immune system

Following primary infection CMV persists for the life-time of the infected carrier. The principle site of latency in the peripheral blood is the monocyte. However the virus only causes disease in patients who are immunocompromised or in neonates whose immune system is not yet developed. The immune surveillance is therefore effective at controlling this virus without ever eliminating it. The reason for this is the multiple immune evasion mechanisms that the virus employs.

Most of the immune evasion mechanisms that are involved in CMV infection interrupt the presentation of immunogenic peptides in the context of the major histocompatibility complex (MHC) class I molecule. Infection of a cell with CMV leads to the down-regulation of MHC class I. Expression of the viral early gene products gpUS2 and gpUS11 lead to the retrograde

translocation of the alpha chains of the MHC class I from the endoplasmic reticulum(ER) to the cytosol.[270,271] These class I molecules then undergo proteasomal degradation. Another mechanism is the retention of MHC class I complexes in the ER by the product of US3.[270] Finally another glycoprotein product of US6 is able to block TAP-mediated translocation of peptides into the ER. All of these mechanisms inhibit the expression of MHC class I and therefore the CD8-mediated response to CMV.[272]

This low expression of class I should render CMV-infected cells more susceptible to attack by natural killer cells. However CMV uses at least two mechanisms to evade NK immunity. Firstly CMV-infected cells express CMV pgUL18 which is a class I homologue.[273] Secondly another CMV antigen, gpUL40 has been shown to up-regulate the non-classical class I molecule HLA-E. This inhibits NK-mediated lysis by binding to a C-type lectin receptor on NK cells. [274]

4.1.3. The immune response to CMV

Despite these multiple immune evasion mechanisms the human immune system is able to effectively control infection in the absence of immunosuppression. Consequently much work has focused on the existing immune response to CMV in the immunocompetent seropositive individual and what aspects of this immune response are essential for disease control.

CTL have been raised from asymptomatic seropositive donors by stimulating their peripheral blood lymphocytes in vitro using autologous fibroblasts infected with CMV laboratory strains, or by using EBV-transformed B cell lines infected with recombinant viruses or loaded with peptides.[275,276] Initial investigations focused on the immediate early protein 1 (IE1) as a possible target for cellular immune response. However, limiting dilution assays (LDA) of PBMCs from seropositive volunteers revealed a high frequency against the matrix protein pp65 and to a lesser degree against the other proteins.[108]

It is now known that pp65 is present in the cell before the expression of immune evasion genes.[277] It is a main component of the enveloped sub-viral particle known as dense bodies

that are delivered into the cytosol through the entry process. Therefore pp65 gains access to the antigen processing machinery of the cell before viral gene expression.
Since the finding that the cellular immune response is focussed on this protein it has been extensively studied. In patients that are HLA-A2-positive there is an immunodominant peptide that induces a response from the vast majority of these patients (NLVPMVATV).[108]

The development of fluorochrome-conjugated, peptide loaded MHC class I tetrameric complexes (tetramers) has enabled the T cells that recognise this immunodominant epitope to be studied much more easily. It has been confirmed that this epitope is very important in the immune response to CMV. A study looking at immunocompromised patients at risk of CMV showed that patients who recovered high numbers of T cells that are specific for this epitope were not at risk of infection. However patients who did not, had a high rate of CMV disease.[278]

4.1.4. Immunotherapy for CMV

4.1.4.1. Adoptive transfer

Adoptive transfer of cells has been used in the treatment of CMV infection. Riddell et al derived CD8+ CMV-specific clones as treatment following allogeneic transplantation for haematological malignancy.[279,280] These clones were derived from the donor prior to transplant and then isolated and expanded in vitro before being re-delivered to the patient 30 to 40 days after transplantation during the period of immunosuppression and vulnerability to infection. Three escalating doses were given and immune responses were examined before, during and after these infusions. Before the infusions only 3 of 14 had CMV-specific CTL responses. After infusion all of the patients had CTL responses equivalent to the donor. By examining the Vβ T cell rearrangements it was possible to show that all of these CTL had identical T cell receptor Vβ sequence to the infused clones.

In the recipients of the CMV-specific CTL, cytolytic responses were demonstrated that were equivalent to those in the donor. However after the last infusion these responses declined over the ensuing weeks. This finding was similar to findings in class II knockout mice where the virus-specific CTL fail to persist. Therefore the absence of CD4 help was proposed as a reason for this failure of persistence.

Subsequent phase II studies included virus-specific CD4 cells. The CTL persistence in this study was augmented. However it is not certain whether this result was due to fewer of these patients receiving prednisolone and cyclosporine.

In a recent study Cobbold et al have also demonstrated that epitope-specific CD8+ lymphocytes can be isolated from the blood of stem cell transplant donors using staining with HLA-peptide tetramers followed by selection with magnetic beads. CMV-specific CD8+ cells were then infused directly into 9 transplant recipients. CMV viremia was reduced in every case and eight patients cleared the infection, including one patient who had a prolonged history of CMV infection that was refractory to antiviral therapy.[281] 48% of the patients who received adoptive transfer required ganciclovir. It is difficult to know what proportions of CMV-specific CD8+ cells were transferred and what proportion were transplanted initially. This study was particularly important as there were no CD4+ lymphocytes that were transferred with this technique. CD4+ responses were followed in some of these patients but showed no correlation with the degree of CMV-specific CD8+ T cell expansion.

Concerns over the lack of CD4 help for adoptively transferred CTL has led to the development of another technique. PBMCs containing both CD4 and CD8 cells have been isolated from stem cell donors. These are then expanded in vitro using whole CMV antigen pulsed onto monocyte-derived dendritic cells. This led to the expansion of CD4 and CD8 cells. These cultured cells can then be transferred back into the patient. There are many advantages of this system including the absence of live virus and the presence of CD4 positive cells. However the need for 3 weeks of in vitro culture clearly has an impact on the feasibility of this treatment for large numbers of patients.[282]

4.1.4.2. Anti-CMV vaccine development

In the development of an effective vaccine against a persistent virus such as CMV the aim of the vaccine strategy has to be clearly stated. If it is to provide a prophylactic vaccine then the induction of antibody by the vaccine would be desirable. In this way the host could prevent the virus accessing the principle sites of infection and establishing latency within those cells. If however the aim is to treat someone who already has persistent infection to prevent reactivation-related diseases then a vaccine that induces cellular responses is desirable.

Initially efforts focused on the development of a prophylactic vaccine that would induce neutralising antibodies. Plotkin and colleagues developed an attenuated CMV referred to as the Towne strain in the 1970s.[283] This was tested both in renal transplant recipients and in women of child-bearing years.[284] Although this vaccine was shown to be immunogenic the use of a live virus in the transplant population presents a potential risk in immunocompromised patients.

To circumvent this problem, attenuated poxviruses were used as an alternative. These viruses have a limited potential for replication in humans. These have therefore been used as vehicles for the expression of recombinant proteins derived from the CMV virus, principally gB a major target of the neutralising antibody response to CMV. A vaccine using a canarypox encoding gB was able to produce antibody and CTL responses to inserted genes in animal models. Disappointingly they only induced minimal levels of antibody in human trials. However they did prime the antibody response for a Towne strain-booster stimulus.[284]

Recombinant proteins have also been used to generate antibody responses against CMV. A recombinant form of CMV gB protein was evaluated in combination with MF59 a powerful adjuvant. Results of a trial in 46 seronegative volunteers showed the stimulation of antibody for at least 12 months. Whether antibody against CMV gB protein will be sufficient to prevent recurrence of primary disease is still debated.[285]

Recently several vaccination strategies have sought to induce a CD8+ T cell response to CMV. Peptides have been used to stimulate CD8+ responses to CMV. The two most successful approaches have involved the use of lipopeptides and the use of peptides incorporating T cell helper epitopes. Lipidated peptides have been shown to be immunogenic against several viruses including influenza, HIV and hepatitis B. Lipopeptide vaccines have also been made using the pp65 epitope: NLVPMVATV.[275] These vaccines also incorporate T cell help in the form of class II epitopes from tetanus and also "PADRE" epitopes. The PADRE epitopes are synthetic non-natural epitopes that have been designed to bind to a wide range of HLA-DR class II molecules.

In a similar study it has also been shown that in an unlipidated peptide vaccine the NLVPMVATV epitope can induce epitope specific immunity when combined with an avid class II epitope. The peptide sequence KSS-AKX*VAAWTLKAAA-NLVPMVATV (where X* indicates cyclohexyl-alanine) combines the universal helper epitope PADRE with the NLV epitope from pp65. This long peptide vaccine was injected into mice that are transgenic for HLA- A2.1 and was able to elicit a CD8+ T cell response from these mice that was detectable after in vitro peptide stimulation. The vaccine was weakly immunogenic when administered in saline alone. But when administered with CpG-containing single stranded DNA (CpG-ssODN) there was an enhanced CD8+ T cell response to NLVPMVATV.[286]

4.1.5. Study Aims

The study aimed to develop a vaccine against the immunodominant epitope of pp65 (NLVPMVATV) thereby activating CD8+ T cell immunity against this antigen. In order to test this vaccine we immunised mice that were transgenic for the HLA-A2.1 MHC class I molecule. We then examined CD8+ responses to the vaccine from these animals.

In order to take this vaccine further towards clinical trial we tested it in a human in vitro assay to see if we could stimulate specific human CD8+ T cells to generate cytotoxicity against this epitope.

In addition we compared the performance of the pDOM.NLV to the long peptide vaccine mentioned above. We also sought to develop a novel strategy for a prime-boost vaccination protocol to see if the response to the DNA vaccine could be enhanced.

4.2. Results

4.2.1. The DNA vaccine pDOM.NLV primes epitope-specific CD8 IFN-gamma secreting cells

Two strains of mice were used that expressed the human molecule HLA- A2.1. The first strain was the A2-K^b mouse that expresses the chimeric HLA- A2.1 molecule (with $\alpha 1$ and $\alpha 2$ from HLA- A2.1 and $\alpha 3$ from H-$2D^b$). This mouse still expresses the endogenous murine MHC class I molecules H-$2K^b$ and H-$2D^b$. The second strain of mouse was the HHD mice. These express the chimeric HLA- A2.1 molecule but the H-$2K^b$ and the H-$2D^b$ have been knocked out.

Groups of A2-K^b mice that were transgenic for the chimeric class I molecule HLA-A2.1/D^b were vaccinated with the DNA vaccines as described above. Fourteen days later they were sacrificed and splenocytes pooled from 4 mice and used in an intracellular cytokine assay. FACS analysis was performed to detect peptide-specific IFN-gamma secretion directly ex vivo (Figure 32). Effective priming of high levels of epitope-specific CD8+ T cells was observed following one injection of pDOM.NLV, with 0.85% of CD8 T cells staining with IFN gamma in response to peptide stimulation in vitro.

The pDOM.NLV plasmid was also used to vaccinate HHD mice that only express the chimeric HLA-A2.1/D^b molecule. They do not express the H-2^b murine class I molecules. After 14 days splenocytes were used in an interferon-gamma ELISPOT assay. Large numbers (120-245) of spots were elicited at the highest concentration of NLV peptide in every mouse. In addition there were large numbers of spots produced by lower levels of peptide. The cells from animals vaccinated with the control pDOM vaccine produced small numbers of spots at each concentration (between 3 and 18 spots) (Figure 33).

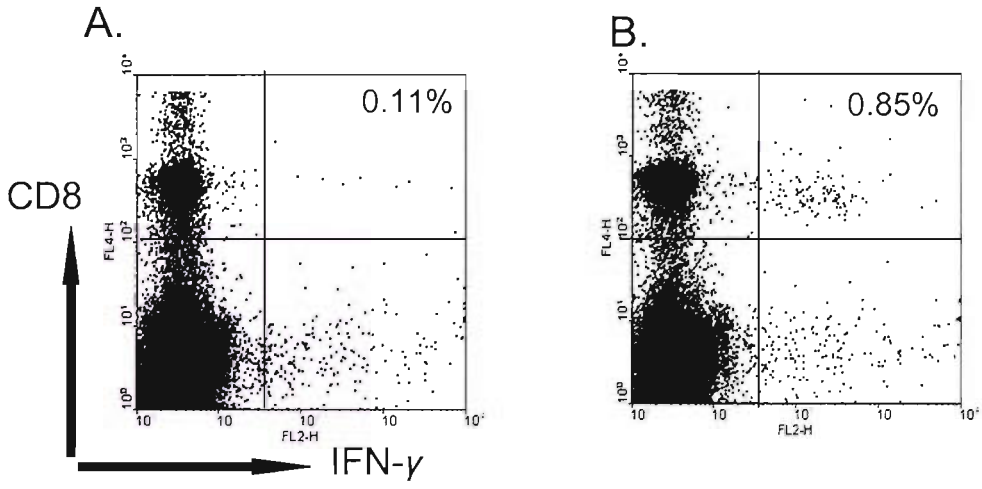


Figure 32. The DNA vaccine pDOM.NLV primes epitope-specific CD8 IFN-gamma secreting cells in A2-K^b mice.

Groups of 4 HHD mice were vaccinated with either pDOM or pDOM.NLV. Mice were culled at day 14 and splenocytes harvested. These cells were stimulated with 10 μM NLV peptide (panel B) or with no peptide (panel A) in the presence of Brefeldin and 10iU/ml of IL-2. They were then permeabilised (see section 2.12) and ex vivo FACS analysis (see section 2.12) was used to measure the percentages of CD8+ T cells that stained with intracellular IFN-γ. Similar results were obtained in 2 experiments

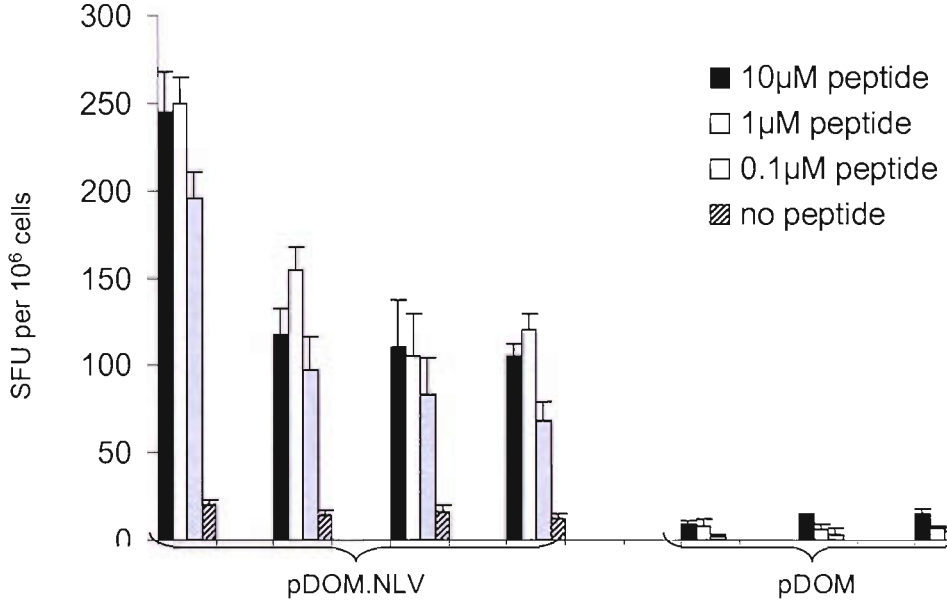
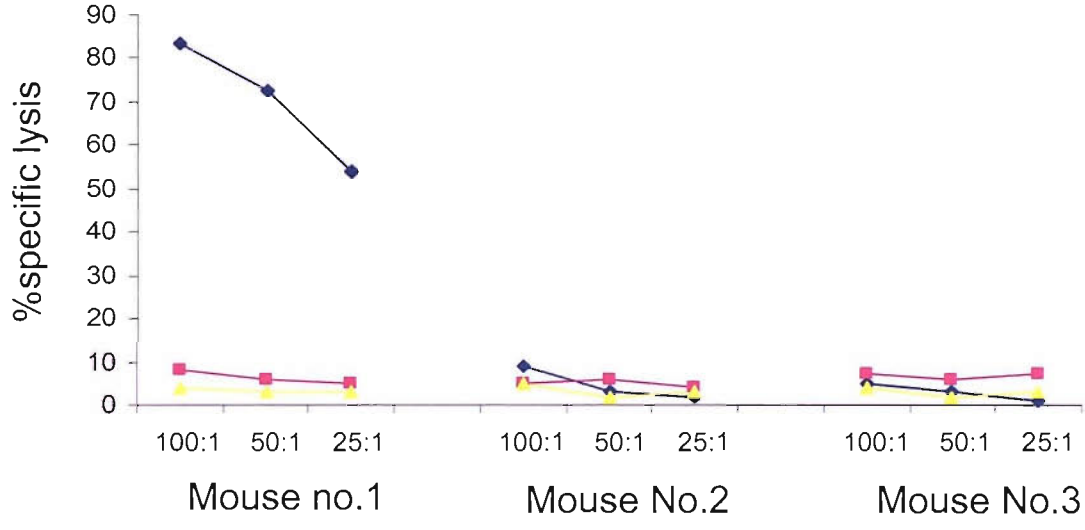


Figure 33. The DNA vaccine pDOM.NLV primes epitope-specific CD8 IFN-gamma secreting cells in HHD mice.

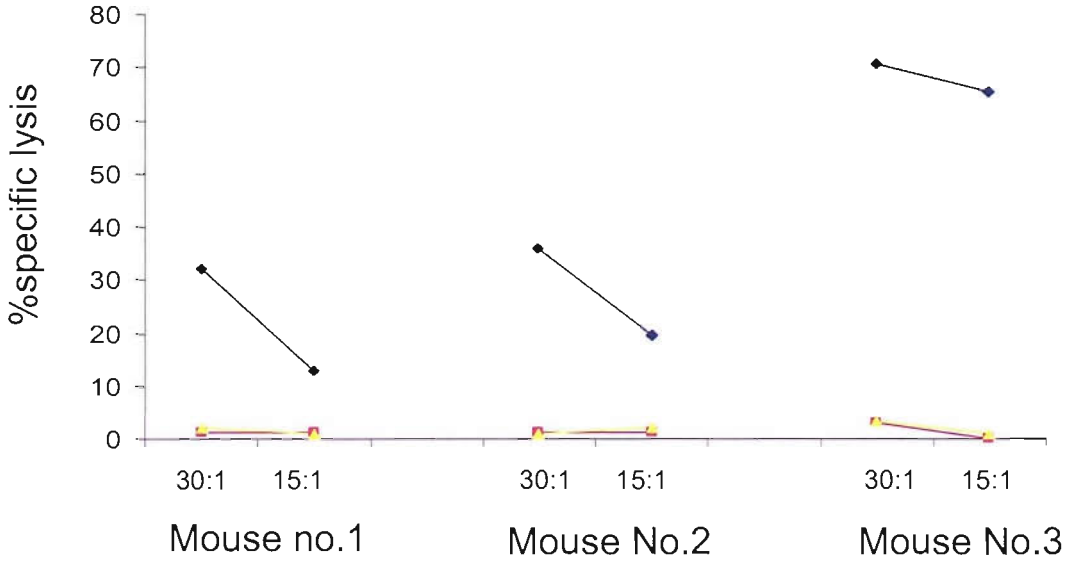
HHD mice were vaccinated with pDOM.NLV or pDOM. On day 14 the mice were culled and splenocytes were placed in an ex vivo ELISPOT assay. Cells were incubated for 18 hours in the presence of varying concentrations of peptide: filled bars, 10μM; open bars, 1μM; dotted bars, 0.1 μM; striped bars, no peptide. The plates were then developed in the standard fashion (see section 2.16). Each bar represents the number of spot forming units per million splenocytes. The experiment was repeated with similar results.

Figure 34. The DNA vaccine pDOM.NLV activates specific cytotoxicity in A2-Kb mice



CTL generated by the vaccination of A2-Kb mice with pDOM.NLV were detectable by cytolytic activity. Mice were vaccinated with pDOM (■) or pDOM.NLV(♦). At day 14 splenocytes were re-stimulated with NLV peptide for 6 days in vitro in the presence of 10iU/ml IL-2. CTL activity was then measured by a ^{51}Cr release assay (see section 2.12) using T2 cells loaded with NLV peptide as target cells. CTL from mice vaccinated with pDOM.NLV were also tested against T2 cells with no peptide (▲). Representative data are shown and the result was repeated on three occasions.

Figure 35. The DNA vaccine pDOM.NLV activates specific cytotoxicity in HHD mice



CTL generated by the vaccination of HHD mice with pDOM.NLV were detectable by cytolytic activity. Mice were vaccinated with pDOM (■) or pDOM.NLV(♦). At day 14 splenocytes were re-stimulated with NLV peptide for 6 days in vitro in the presence of 10iU/ml IL-2. CTL activity was then measured by a ^{51}Cr release assay using T2 cells loaded with NLV peptide as target cells. CTL from mice vaccinated with pDOM.NLV were also tested against T2 cells with no peptide (▲). Representative data are shown and the result was repeated on three occasions.

4.2.2. The DNA vaccine pDOM.NLV activates specific cytotoxicity

The ability of these IFN-gamma-secreting CD8 T lymphocytes to mediate specific cytotoxicity was assessed by a standard ^{51}Cr release assay. Fourteen days after vaccination of the A2-K^b mice splenocytes were stimulated with NLVPMVATV peptide and IL-2 for 6 days. pDOM.NLV vaccination induced cytotoxic CD8+ cells in some mice that were able to lyse T2 cells that were loaded with peptide. There was no lysis of an un-pulsed T2 target cell. The control vaccine pDOM did not induce cytotoxicity above the background level. This experiment was repeated twice with similar results. Across these experiments 4 out of 13 mice responded to the pDOM.NLV vaccine generating high levels of cytotoxicity (up to 85% lysis). The remaining mice had responses that were equivalent to the pDOM responses (<8% lysis)(Figure 34).

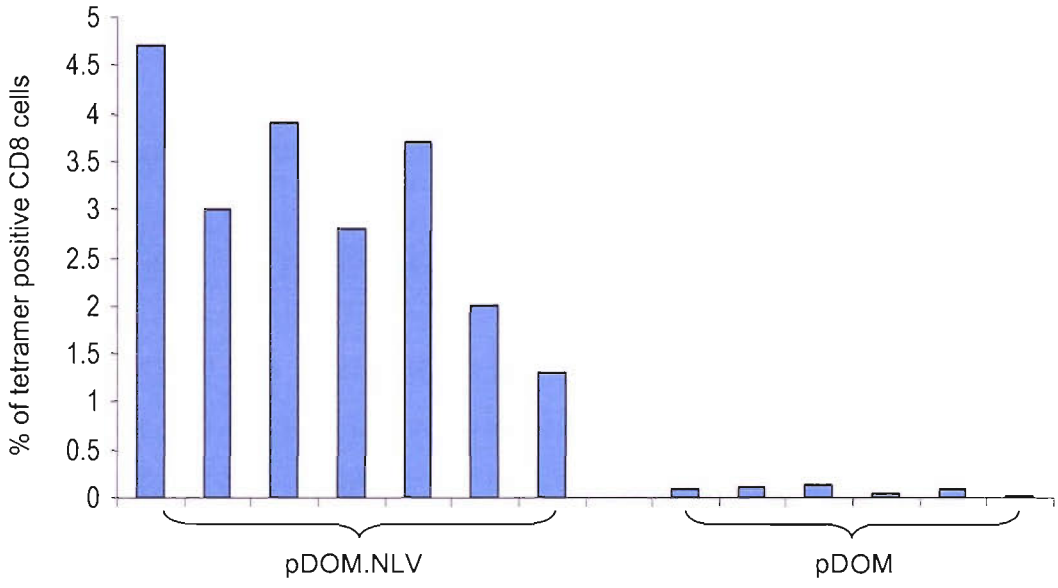
The HHD mice were also vaccinated with the pDOM.NLV vaccine. In these mice the vast majority of mice (>95%) produced CTL that were specific for the NLVPMVATV epitope (Figure 35).

4.2.3. The vaccine pDOM.NLV elicits high numbers of CTL that bind a tetramer specific for the NLVPMVATV epitope

HHD mice were vaccinated with either pDOM or pDOM.NLV. After 14 days they were sacrificed and the splenocytes harvested. These cells were then dual stained with anti-CD8 antibodies and also a chimeric tetramer. This tetramer had chimeric HLA-A2.1/D^b class I molecules covalently bound to the NLVPMVATV peptide.

The CD8+ lymphocytes derived from mice vaccinated with the pDOM.NLV vaccine consistently had a high proportion of cells that stained with the chimeric tetramer. Between 1.3 and 4.7% of the CD8 in these individual mice are positive for the tetramer (Figure 36). CD8+ lymphocytes from the mice vaccinated with the control pDOM vaccine had very low levels of tetramer positive staining (<0.2%).

Figure 36. The vaccine pDOM.NLV elicits high numbers of CTL that bind a tetramer specific for the NLVPMVATV epitope



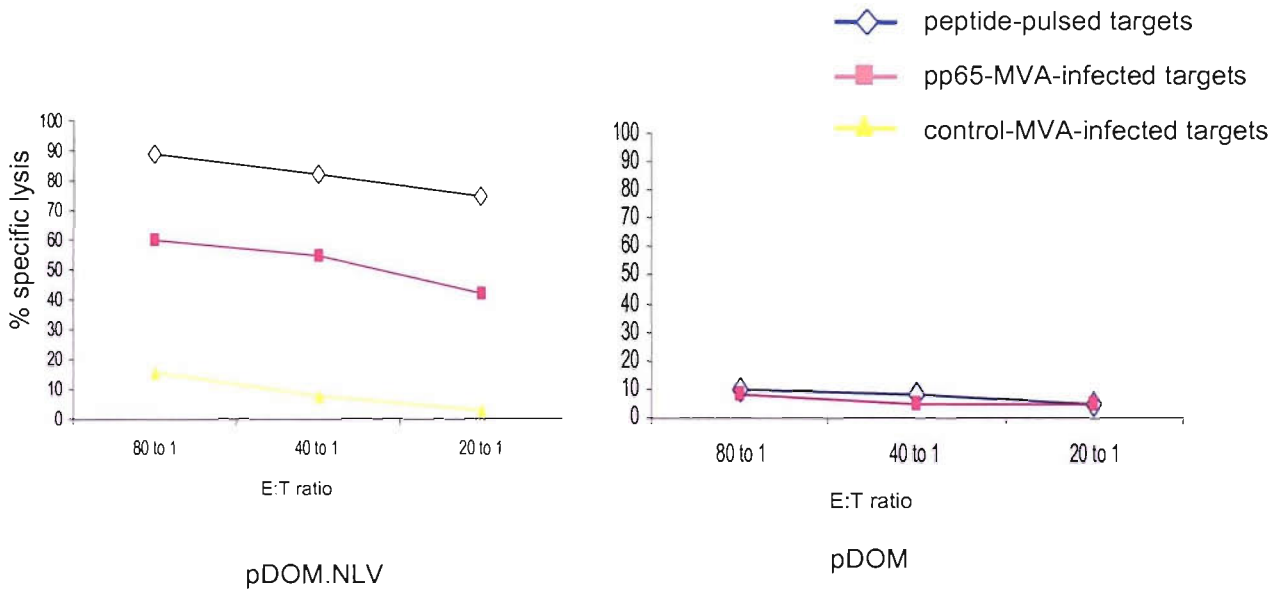
HHD mice were vaccinated with either pDOM.NLV or pDOM. Splenocytes were taken from vaccinated mice on day 14 post vaccination. They were then stained with 0.4 μg of NLVPMVATV chimeric (HLA-A2/Db) tetramer per 10^6 cells. Splenocytes were then labelled with APC conjugated anti-mouse CD8b.2 (clone 53-5.8, BD Pharmingen) and FITC conjugated anti-mouse Mouse MHC class II (2G9; BD Pharmingen) at 1μg/well for 20 minutes at room temperature. Cells were then washed in PBS and analysed on a FACScalibur. One hundred thousand events were collected from a live lymphocyte gate. Subsequently the MHC class II positive cells were excluded and the proportion of CD8 cells that were also tetramer positive was determined. This experiment was repeated 2 further times with similar results.

4.2.4. CTL induced by the pDOM.NLV vaccine are able to lyse targets that have been infected with MVA expressing pp65

HHD mice were vaccinated with either pDOM or pDOM.NLV. On day 14 splenocytes were pooled and cultured in the presence 10μM peptide and IL-2. After 6 days of in vitro culture the cells were tested in a chromium release killing assay.

The target cells used were Jurkat cells that had been transfected with the chimeric human class I molecule HLA-A2.1/D^b. These cells were infected with either MVA that expressed pp65 from CMV or a control MVA. Another group of target cells were also pulsed with peptide. The CTL induced by the vaccine pDOM.NLV demonstrated high levels of specific lysis of both the peptide pulsed and the pp65-expressing MVA. The peptide pulsed targets showed 89% lysis when the effector to target ratio was 80:1 and the targets infected with MVA showed 60% lysis at the same ratio. The cells from the mice vaccinated with pDOM when tested against these two targets only induced 10.1% lysis and 8.7% lysis respectively in these two lines. This pDOM.NLV CTL induced low levels of lysis (14% at effector to target ratio 80:1) from the target cells infected with the control MVA. This vaccine is therefore able to induce cytotoxic T cells that are able to kill target cells that present peptide from an endogenous route.

Figure 37. CTL induced by the pDOM.NLV vaccine are able to lyse targets that have been infected with MVA expressing pp65



Mice were vaccinated with either pDOM.NLV or pDOM. Fourteen days after vaccination splenocytes were cultured for 6 days in the presence of 10μM peptide and in the presence of 10iU/ml IL-2. They were then placed in a chromium release assay against Jurkat cells that expressed the chimeric MHC class I molecule HLA-A2.1/D^b. These target cells were either peptide pulsed (◊), infected with pp65-expressing MVA (■) or infected with control MVA (▲). This experiment was repeated with a similar result.

Results from testing the vaccine in an in vitro human model

4.2.5. The phenotype of human dendritic cells

Dendritic cells were generated from adherent PBMCs from healthy volunteers in the presence of GM-CSF and IL-4. They had a phenotype that was consistent with immature dendritic cells. They had high levels of expression of CD1a and low levels of expression of CD14. They had intermediate expression of HLA-DR and low levels of expression of CD83 a marker for mature dendritic cells. In addition the co-stimulatory molecules CD80 and CD86 were low (Figure 38).

After incubation with monocyte-conditioned medium these dendritic cells displayed a fully mature phenotype. They lost their expression of CD1a and gained CD83 surface expression. In addition they had high levels of expression of CD80, CD86 and HLA-DR.

4.2.6. The assessment of RNA transfection of the dendritic cells

DCs were prepared as described in section 2.18 and were then transfected with RNA transcribed from a GFP expressing plasmid. Flow cytometric analysis 24 hours after transfection revealed that more than 45% of immature DCs expressed the protein GFP demonstrating successful transfection. If the cells were matured prior to transfection this resulted in a lower transfection rate (16.2%). Consequently for the following experiments DCs were transfected and then the maturation stimulus was delivered on the day of transfection. They were then used 24 hours after transfection when they had assumed a mature phenotype (Figure 39).

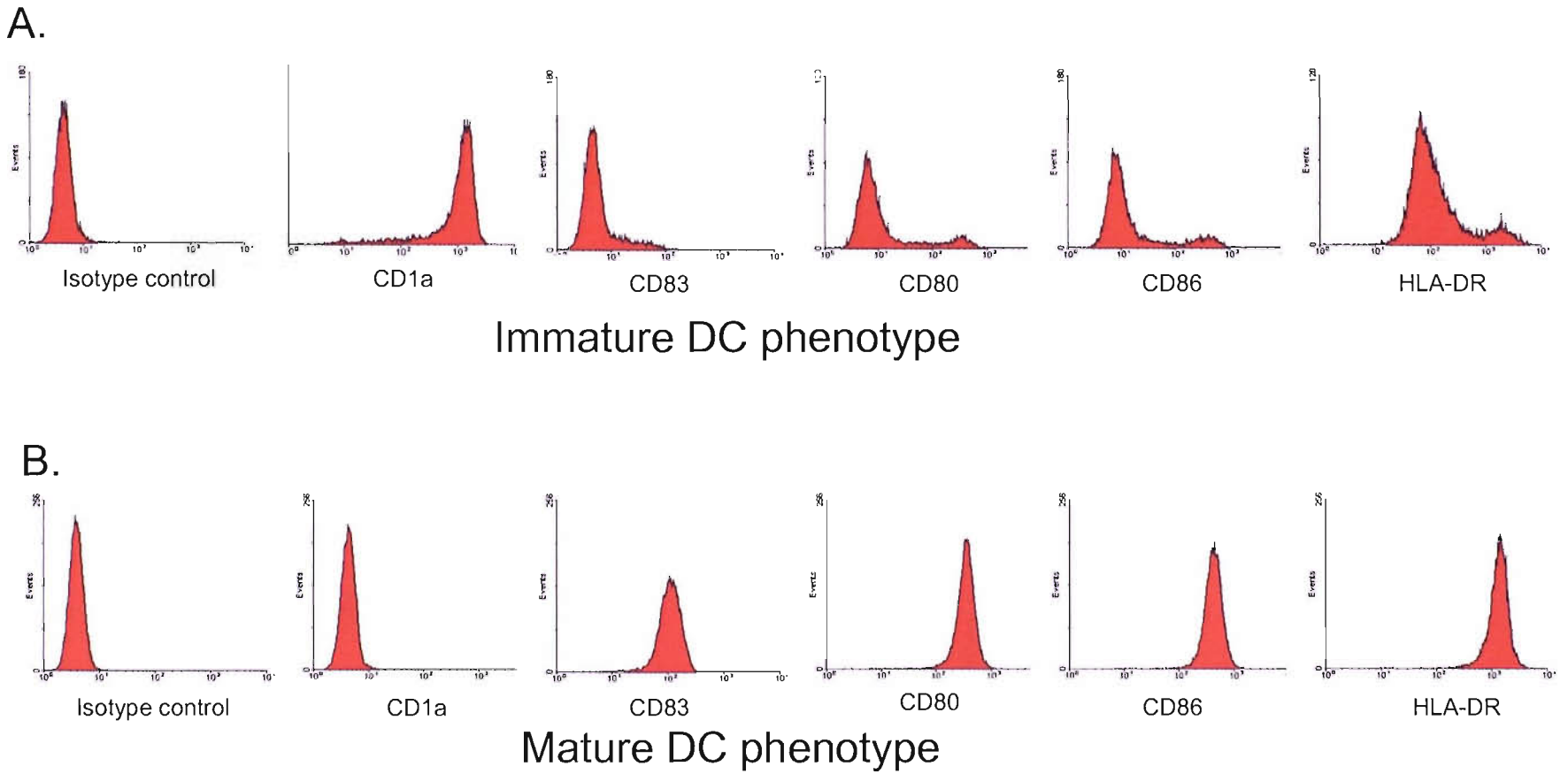


Figure 38. The phenotype of human dendritic cells - dendritic cells phenotype changes with and without monocyte-conditioned medium.

Dendritic cells that had either been cultured in the presence (panel B) or absence (panel A) of monocyte conditioned medium were stained for the expression of the above surface markers (see Materials and Methods - section 2.21 and section 4.2.5). This experiment was repeated on 3 occasions with similar results.

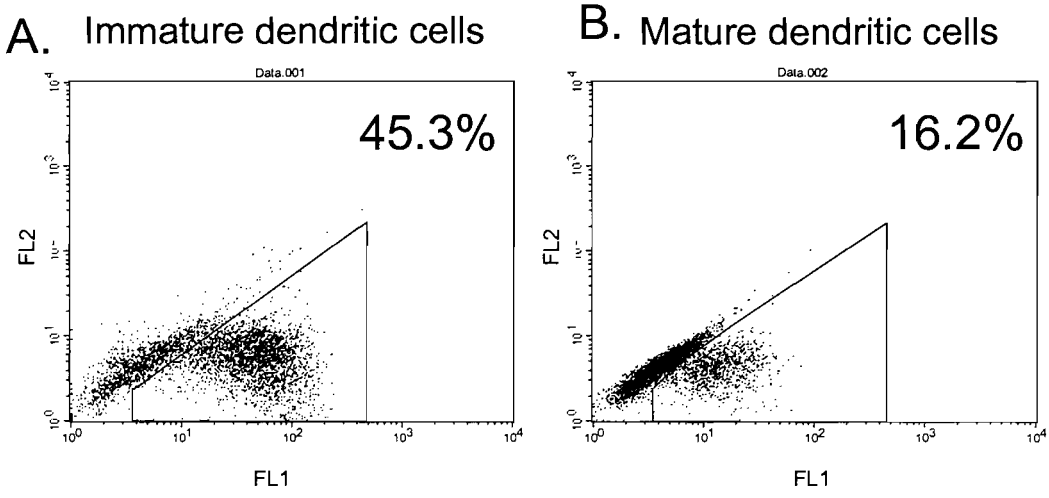


Figure 39. The assessment of RNA transfection of the dendritic cells - immature dendritic cells are more easily transfected with RNA electroporation.

Dendritic cells were prepared from monocytes as described in section 2.18. They were transfected on day 8 of differentiation. The "immature DCs" received only GM-CSF and IL-4 over this time (panel A), whereas the "mature DCs" had received monocyte-conditioned medium for 24hrs in addition to these cytokines. In vitro transcribed RNA was produced from plasmid DNA as described in section 2.19. Cells were electroporated in the presence of RNA transcribed from a GFP-expressing plasmid. 24 hours later they were analysed using FACS to determine the quantity of GFP protein that they were expressing. This experiment was repeated with a similar result.

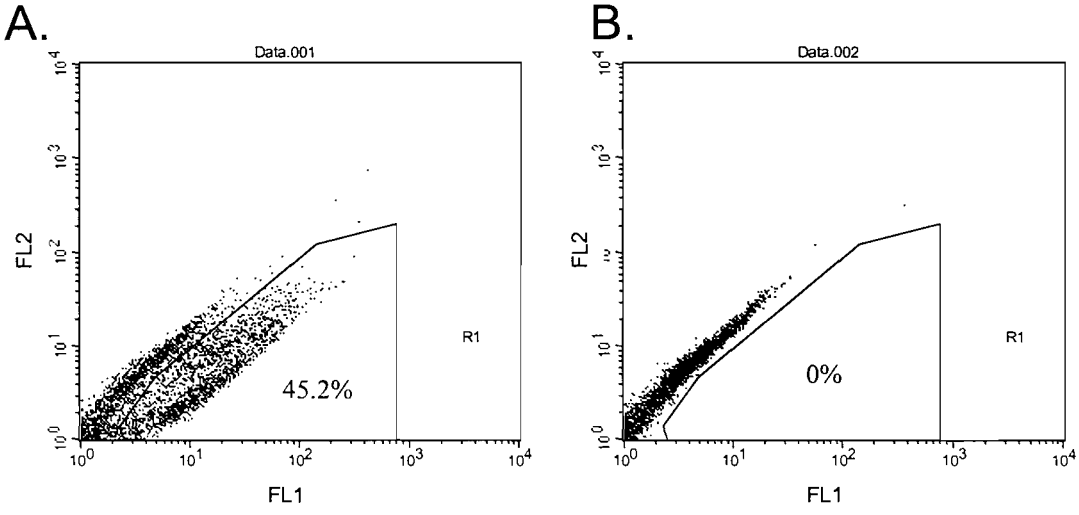


Figure 40. Transfection of immature dendritic cells with the GFP plasmid reveals high levels of transfection.

Dendritic cells were prepared from monocytes as described in section 2.18. In vitro transcribed RNA was produced from plasmid DNA (pDOM.NLV and GFP) as described in section 2.19. Immature dendritic cells were electroporated in the presence of RNA transcribed from either a GFP-expressing plasmid (panel A) or with the experimental plasmid pDOM.NLV (panel B). The cells were analysed using FACS analysis to determine the proportion of cells that were expressing GFP. Cells from panel B were used in the CD8+ lymphocyte stimulation shown overleaf.

4.2.7. Transfection of the DCs with RNA from the vaccine pDOM.NLV leads to an expansion of tetramer-positive CD8+ cells.

CD8+ T cells were isolated from a healthy volunteer, who was CMV seropositive (see methods section 2.22). Autologous DCs were prepared as described in section 2.18. RNA was transcribed from the plasmids pDOM.NLV or a control construct (pDOM.CAP-1) as described in section 2.19 The DCs were then electroporated in the presence of RNA transcribed from each vaccine. The CD8+ lymphocytes were then co-incubated with DCs that had been transfected with RNA for 7 days in the presence of IL-7 and then IL-2.

After seven days of stimulation there was a very large expansion of CD8 cells that were detected by the epitope-specific tetramer. Before stimulation 0.07% of CD8 cells were detected by the NLVPMVATV tetramer. After 7 days of stimulation with the DCs that had been transfected with RNA transcribed from pDOM.NLV, 3.6% of the CD8 cells were specific for this epitope. There was therefore a 50-fold expansion in the numbers of these cells in an in vitro stimulation assay (Figure 41).

The CD8+ T cells that had been stimulated by the DCs transfected with RNA from the control vaccine were also examined for tetramer binding by FACS analysis. After stimulation there was no expansion of the number of CD8 cells that interacted with the NLVPMVATV tetramer (0.07% of total CD8+ cells). It was noted in this tetramer analysis that there were two distinct populations of tetramer positive cells with different fluorescence intensities. This has been described in other studies and seems to be related to clones within the tetramer-positive population that have different avidities. Of note is that both populations are present in the donor at baseline and both expand in response to transfected DC.[287,288]

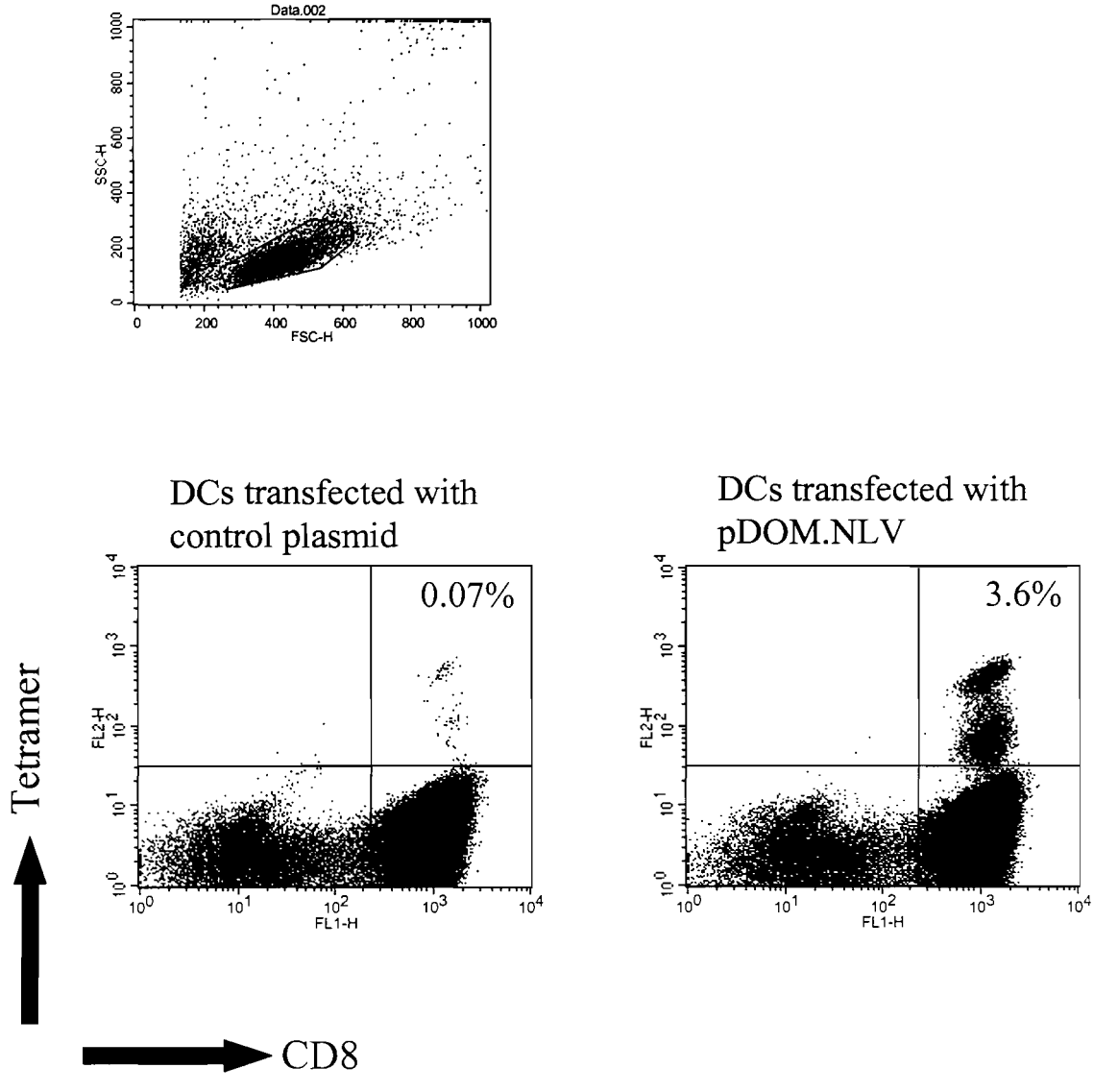


Figure 41. Transfection of the DCs with RNA from the vaccine pDOM.NLV can lead to an expansion of the number of tetramer-positive CD8+ cells.

Dendritic cells were prepared from monocytes as described in section 2.18. In vitro transcribed RNA was produced from plasmid DNA (pDOM.NLV vaccine or a control vaccine) as described in section 2.19. DCs were transfected with RNA derived from either the pDOM.NLV vaccine or a control vaccine. The DCs were then matured for 24hours in the presence of monocyte-conditioned medium. They were then co-cultured with CD8+ T cells for 6 days. They were stained with anti-CD8 antibody and NLVPMVATV-specific tetramer as described in the Materials and Methods section 2.17. Cells were gated on a live lymphocyte gate. The results above are expressed as the percentage of CD8+ lymphocytes that are positive for the NLVPMVATV tetramer. This result was repeated on one other occasion with a similar result.

4.2.8. The pDOM.NLV vaccine sequence elicits peptide-specific cytotoxicity from human CD8+ lymphocytes in vitro.

These CD8+ lymphocytes that had been stimulated by co-incubation with RNA-transfected DCs were also tested in a standard ^{51}Cr release cytotoxicity assay. The first group of CD8+ T cells had been co-cultured with DCs that had been transfected with RNA from pDOM.NLV. This CTL was able to lyse T2 cells that had been pulsed with the peptide NLVPMVATV. They induced 90% lysis of peptide-pulsed targets at an effector to target ratio of 40:1 (Figure 42). They had only low background levels of lysis against unpulsed targets. The CD8 cells that had been stimulated with DCs transfected with RNA from the control vaccine were only able to induce 22% lysis at an effector to target ratio of 40:1.

4.2.9. The pDOM.NLV vaccine elicits a higher number of tetramer positive CD8+ lymphocytes than the long peptide vaccine

Long peptide vaccines that combine avid class II epitopes with target class I epitopes have been shown to enhance T cells responses to the class I epitopes. One group of class II peptides commonly used are the PADRE epitopes (see section 4.1.4.2). One such peptide sequence KSS-AKX*VAAWTLKAAA-NLVPMVATV(where X* indicates cyclohexyl-alanine) combines the universal helper epitope PADRE with the NLV epitope from pp65 (PADRE-NLV).

HHD mice were vaccinated with either the pDOM.NLV vaccine, the control plasmid (pDOM) or the long peptide (PADRE-NLV used with CpG-ssODN) (see section 2.11). After 14 days individual spleens were harvested and stained ex vivo with antibodies against CD8 and tetramer specific for the epitope NLVPMVATV. This tetramer is a chimeric tetramer (with $\alpha 1\alpha 2$ from HLA-A2.1 and $\alpha 3$ from D^b). Mice that had been vaccinated with pDOM.NLV revealed a very high proportion of CD8+ lymphocytes (2.82-4.85%) that were also tetramer positive. Mice vaccinated with the long peptide also revealed a high level of

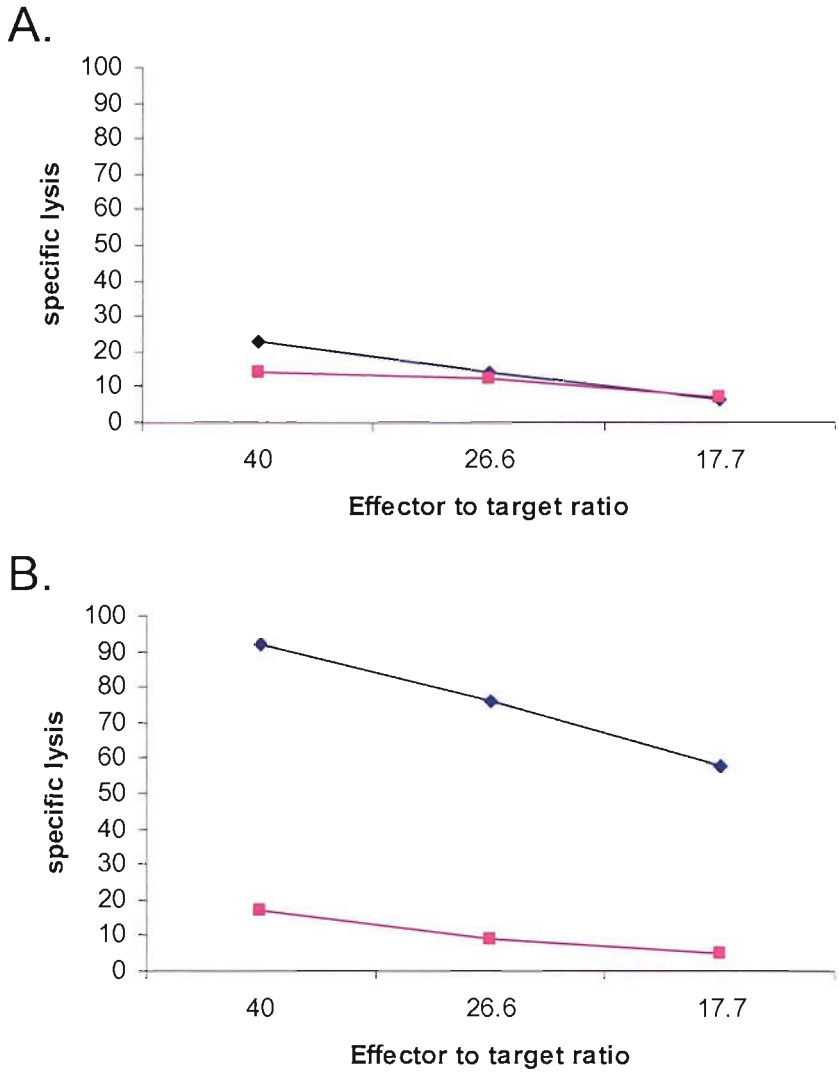


Figure 42. The pDOM.NLV vaccine elicits peptide specific cytotoxicity from human CD8+ lymphocytes stimulated in vitro.

Dendritic cells were prepared from monocytes as described in section 2.18. In vitro transcribed RNA was produced from plasmid DNA (pDOM.NLV vaccine or a control vaccine) as described in section 2.19. Half of the DCs were transfected with RNA transcribed from a control plasmid (panel A) and half transfected with RNA from the plasmid pDOM.NLV (panel B) . The DCs were then matured for 24hours in the presence of monocyte-conditioned medium. These DCs were used to stimulate autologous CD8+ T cells in the presence of 10iU/ml IL-2 . After 7 days in vitro culture they were tested in a standard ^{51}Cr release cytotoxicity assay against peptide pulsed T2 cells (♦) and unpulsed target cells (■).

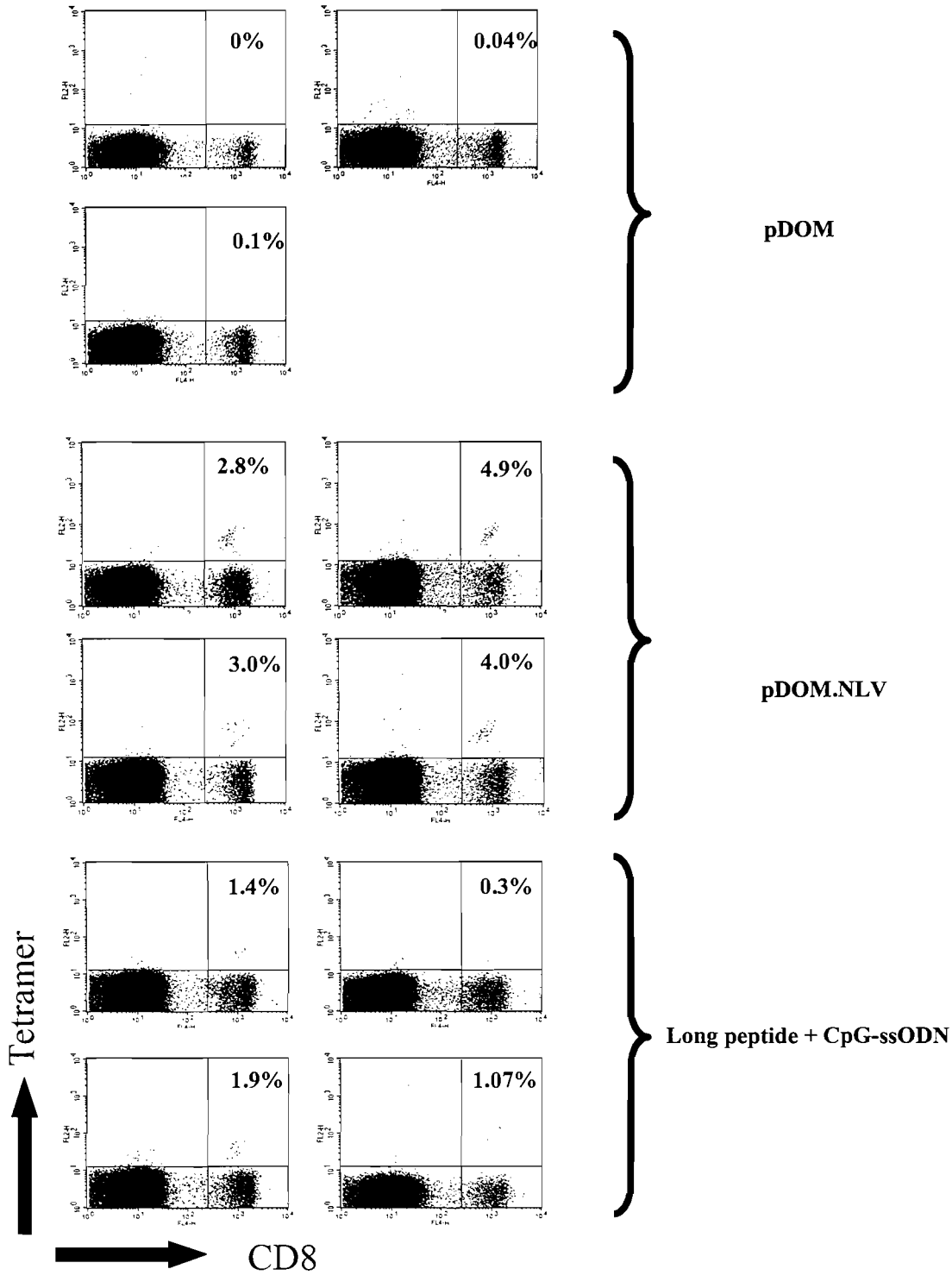


Figure 43a. The pDOM.NLV vaccine elicits a higher number of tetramer positive CD8+ lymphocytes than the long peptide vaccine. Four HHD mice in each group were vaccinated with either pDOM, pDOM.NLV or the long peptide with CpG-ssODN. On day 14 the mice were culled and splenocytes isolated. They were then stained with 0.4 μg of NLVPMVATV chimeric (HLA-A2/Db) tetramer per 106 cells. Splenocytes were then labeled with APC conjugated anti-mouse CD8b.2 (clone 53-5.8, BD Pharmingen). Cells were then washed in PBS and analysed on a FACScalibur. One hundred thousand events were collected from a live lymphocyte gate. Subsequently the MHC class II positive cells were excluded and the proportion of CD8 cells that were also tetramer positive was determined. This result was repeated with similar results.

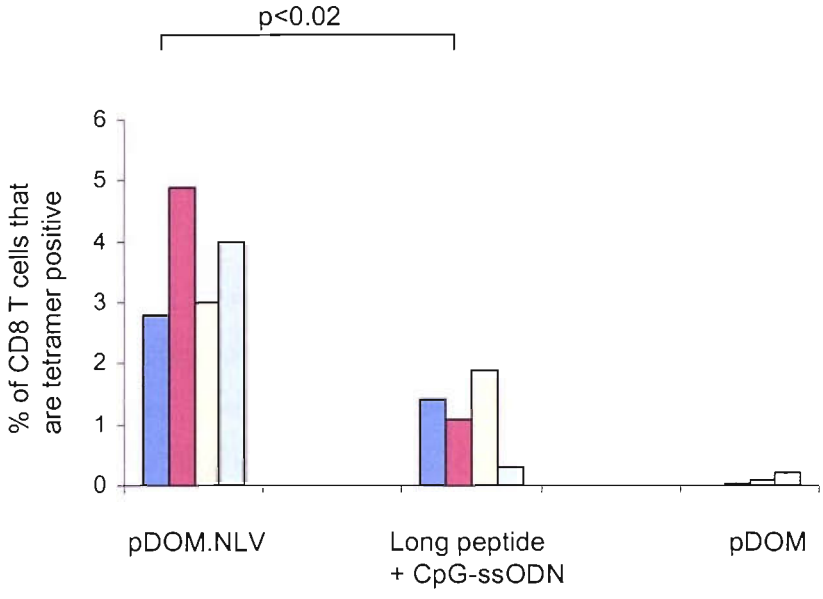


Figure 43b. The pDOM.NLV vaccine elicits a higher number of tetramer positive CD8+ lymphocytes than the long peptide vaccine.

The pDOM.NLV vaccine elicits a higher number of tetramer positive CD8+ lymphocytes than the long peptide vaccine. Four HHD mice in each group were vaccinated with either pDOM, pDOM.NLV or the long peptide with CpG-ssODN. On day 14 the mice were culled and splenocytes isolated. They were then stained with 0.4 μg of NLVPMVATV chimeric (HLA-A2/Db) tetramer per 106 cells. Splenocytes were then labeled with APC conjugated anti-mouse CD8b.2 (clone 53-5.8, BD Pharmingen). Cells were then washed in PBS and analysed on a FACScalibur. One hundred thousand events were collected from a live lymphocyte gate. Subsequently the MHC class II positive cells were excluded and the proportion of CD8 cells that were also tetramer positive was determined. This result was repeated with similar results. On the previous page the original FACS plots are shown

tetramer positive cells (0.3-1.9%) but this was significantly lower than the proportion elicited by the pDOM.NLV vaccine ($p<0.02$) (Figure 43). The control vaccine pDOM elicited a very low level of tetramer-positive CD8+ lymphocytes (0.2%). This experiment was repeated on one other occasion with similar results.

4.2.10. The pDOM.NLV vaccine in a heterologous prime-boost combination with the long peptide elicits a very high proportion of tetramer positive CD8+ lymphocytes

A group of 4 HHD mice were vaccinated as above with "priming" vaccination of pDOM.NLV. Twenty-one days later they received a "boost" vaccination with the same vaccine; a so-called homologous prime-boost protocol. A second group of mice was vaccinated with PADRE-NLV peptide and CpG-ssODN and also had a homologous boost on day 21 with the same vaccine. A third group of mice were vaccinated with a heterologous prime-boost protocol receiving a DNA vaccination on day 0 (pDOM.NLV) and then a long peptide with CpG-ssODN boost on day 21. The last group of mice received this heterologous DNA-long peptide vaccination protocol but without the CpG-ssODN. The spleens were all taken on day 31 and analysed using antibody against CD8 and two different tetramers specific for NLVPMVATV. One tetramer was the chimeric tetramer (with $\alpha1\alpha2$ from HLA-A2.1 and $\alpha3$ from D^b) with the other being fully human ($\alpha1\alpha2\alpha3$ all from HLA-A2.1).

The homologous prime-boost protocol pDOM.NLV followed by pDOM.NLV induced a high level of tetramer-positive CD8+lymphocytes (0.4-8.4%). However this level was not significantly higher than with a single DNA vaccination alone ($p=0.55$). Again the homologous prime-boost protocol with repeated vaccination with long peptide and CpG induced a high level of tetramer-positive CD8+lymphocytes (1.0-7.7%) however this again was not significantly higher than a single vaccination ($p=0.24$). But the heterologous prime-boost protocol DNA followed by long peptide and CpG-ssODN yielded very high levels of tetramer positive CD8+ cells (15-59%) (Figure 44). This was significantly higher than the

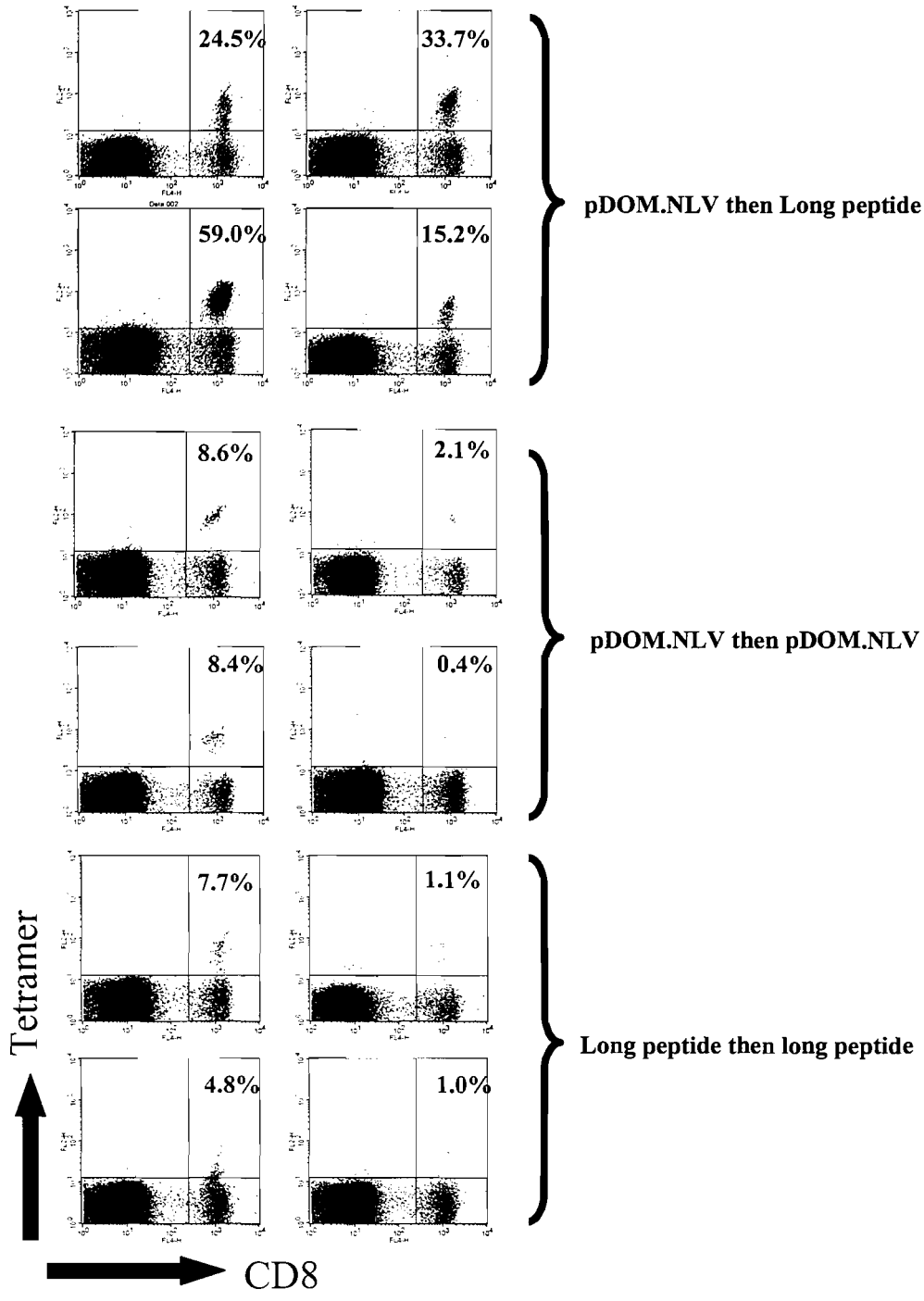


Figure 44a. The pDOM.NLV vaccine when used in a heterologous prime-boost combination with the long peptide elicits a very high proportion of tetramer positive CD8+ lymphocytes.

Four mice in each group were vaccinated with pDOM.NLV or long peptide on day 0 and boosted with pDOM.NLV or long peptide with or without CpG-ssODN on day 21. Ex vivo tetramer analysis was performed on day 31. The splenocytes were then stained with 0.4 μg of NLVPMVATV chimeric (HLA-A2/Db) tetramer per 10^6 cells. Splenocytes were then labeled with APC conjugated anti-mouse CD8b.2 (clone 53-5.8, BD Pharmingen). Cells were then washed in PBS and analysed on a FACScalibur. One hundred thousand events were collected from a live lymphocyte gate. Subsequently the proportion of CD8 cells that were also tetramer positive was determined. This result was repeated with similar results. This result was repeated yielding similar results

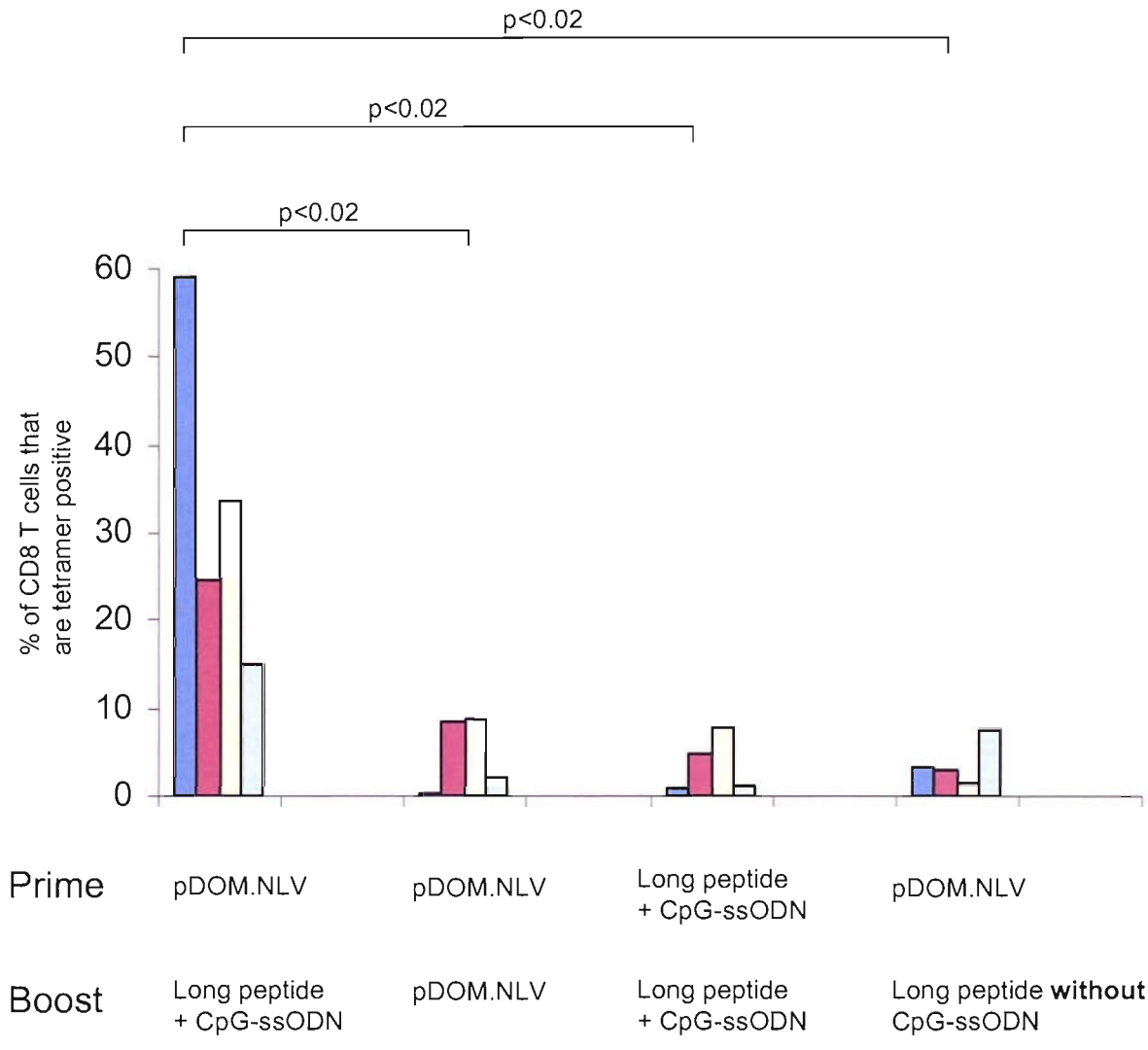


Figure 44b. The pDOM.NLV vaccine when used in a heterologous prime-boost combination with the long peptide elicits a very high proportion of tetramer positive CD8+ lymphocytes.

Four mice in each group were vaccinated with pDOM.NLV or long peptide on day 0 and boosted with pDOM.NLV or long peptide with or without CpG-ssODN on day 21. Ex vivo tetramer analysis was performed on day 31. The splenocytes were then stained with 0.4 μg of NLVPMVATV chimeric (HLA-A2/Db) tetramer per 10^6 cells. Splenocytes were then labeled with APC conjugated anti-mouse CD8b.2 (clone 53-5.8, BD Pharmingen). Cells were then washed in PBS and analysed on a FACScalibur. One hundred thousand events were collected from a live lymphocyte gate. Subsequently the proportion of CD8 cells that were also tetramer positive was determined. This result was repeated yielding similar results. Comparisons within the groups was performed using Mann Whitney U test. This result was repeated yielding similar results.

other prime-boost protocols ($p<0.02$). If CpG-ssODN were not included on the second vaccination of this prime-boost then there was no increase in the level of tetramer-positive population (1.5-7.4%, $p=0.88$). This experiment was repeated on one other occasion in the NLVPMVATV model.

This finding was repeated in another model HHD mice received homologous prime-boost vaccination with either pDOM.CAP-1 or a long peptide incorporating PADRE with CAP-1 peptide KSS-AKX*VAAWTLKAAA-YLSGANLNL. Another group of mice received a homologous prime-boost vaccination with pDOM.CAP-1 followed by long peptide with CpG-ssODN. This revealed a similar result to the above with the heterologous prime boost being significantly higher than the homologous DNA-DNA or peptide-peptide vaccinations ($p=0.05$ and $p=0.03$ respectively) (Figure 45).

4.2.11. The heterologous prime-boost vaccination protocol elicits epitope-specific CTL of lower avidity than from vaccination with pDOM.NLV alone.

HHD mice were used from the above experiment which had received either a homologous prime-boost vaccination with pDOM.NLV or heterologous vaccination with pDOM.NLV followed by long peptide. The splenocytes were isolated ex vivo on day 31. They were then stained with anti-CD8 antibody and the fully human tetramer ($\alpha1\alpha2\alpha3$ HLA-A2.1) specific for NLVPMVATV. Mice that had been vaccinated against a homologous DNA prime-boost vaccination showed a population of CD8+ lymphocytes that were also positive for the fully human tetramer (4.2% and 4.7%). The mean fluorescence intensity of this tetramer staining was lower than with the chimeric tetramer. However mice that had received the heterologous prime-boost vaccination (pDOM.NLV followed by long peptide and CpG) had very low levels of tetramer positive CD8+ lymphocytes (1.1% and 1.0%) even though these cells had shown much higher levels with the chimeric tetramer (24.5% and 15.2% respectively). This demonstrates that these cells are of lower avidity. This staining of the effector cells with the fully human tetramer is a preliminary result and has not yet been repeated (Figure 46).

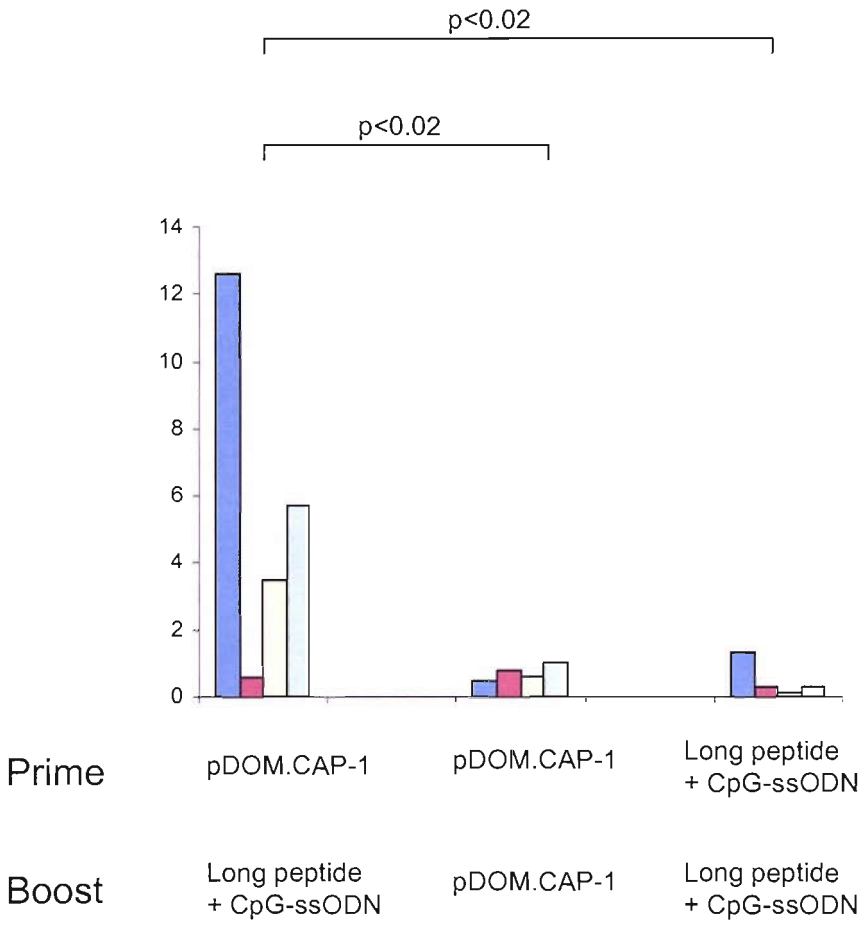
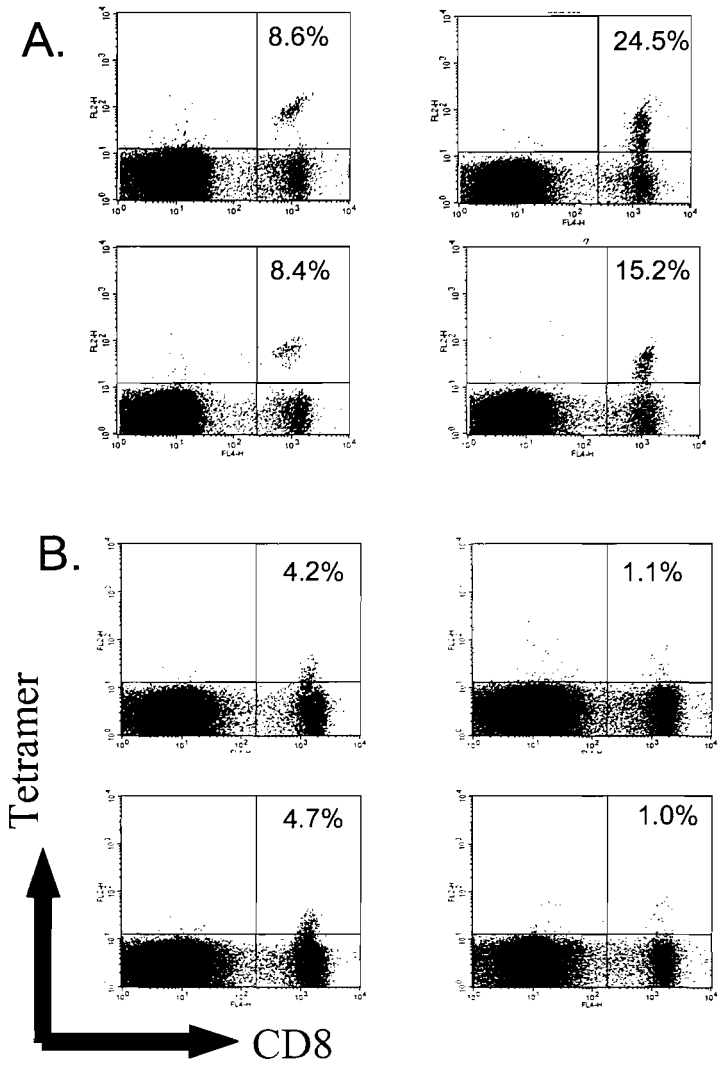


Figure 45. The heterologous vaccination protocol with DNA followed by peptide is also valid for another epitope..

Four mice in each group were vaccinated with either pDOM.CAP-1 or long peptide containing PADRE and CAP-1 with CpG-ssODN. Twenty-one days later they received a "boost" vaccination with either the pDOM.CAP-1 DNA vaccine or the long peptide vaccine. The splenocytes were assessed ex-vivo 12 days after the second vaccination. The splenocytes were then stained with 0.4 μg of YLSGANLNL chimeric (HLA-A2/Db) tetramer per 10^6 cells. Splenocytes were then labeled with APC conjugated anti-mouse CD8b.2 (clone 53-5.8, BD Pharmingen). Cells were then washed in PBS and analysed on a FACScalibur. One hundred thousand events were collected from a live lymphocyte gate. Subsequently the proportion of CD8 cells that were also tetramer positive was determined.



Prime (day 0)	pDOM.NLV	pDOM.NLV
Boost (day 21)	pDOM.NLV	Long peptide + CpG-ssODN

Figure 46. The heterologous prime-boost vaccination protocol elicits epitope-specific CTL that are lower in avidity than the CTL from vaccination with pDOM.NLV alone.

Mice were vaccinated on days 0 and 21 with the vaccines pDOM.NLV and long peptide with CpG-ssODN. The splenocytes were harvested on day 31. The cells stained with 0.4 μg of the chimeric NLVPMVATV A2/Db tetramer (panel A) and the fully human HLA-A2 tetramer (panel B) per 10^6 cells. Splenocytes were then labeled with APC conjugated anti-mouse CD8b.2 (clone 53-5.8, BD Pharmingen). Cells were then washed in PBS and analysed on a FACScalibur. One hundred thousand events were collected from a live lymphocyte gate. The percentages indicate the proportion of CD8+ lymphocytes that were also NLVPMVATV tetramer positive. This experiment is a preliminary result and has not yet been repeated.

4.3. Discussion

This study uses the immunodominant HLA- A2.1 epitope from CMV, NLVPMVATV, fused to the C-terminus of the first domain of FrC (pDOM.NLV). This vaccine is highly effective at inducing peptide-specific CD8 immunity in mice transgenic for the human HLA- A2.1 molecule. Following one injection, primed epitope-specific CD8+ T cells were detected in individual mice by tetramer binding. Substantial, reproducible levels of specific T cells were induced in all mice, compared to the control pDOM vaccine.

These data demonstrate that the p.DOM-NLV vaccine design offers a potent and reproducible method of inducing peptide-specific T cells with effector function. It confirms the efficacy of the single epitope design, which focuses the CTL response onto the target peptide[289] and mimics the natural focusing on this immunodominant peptide.[290] Although the power of the epitope-specific CTL to kill infected cells appears assured, it could be argued that attack on multiple epitopes would be more appealing. Multiple presentation from a single vaccine has the problem of epitope competition, especially at the point of boosting.[291] However, there appears to be less competition when vaccines are delivered separately.

An attenuated canarypox vaccine expressing full length pp65 containing multiple epitopes has been tested recently in transgenic mice.[292] It is difficult to compare efficacy, but viral vectors add the problem of immune blocking[293] and the dangers of live virus, especially for immunosuppressed patients. Neither applies to naked DNA. One criticism leveled at DNA vaccines is their limited performance in large animals, largely due to dose/volume restrictions. Physical methods including electroporation may solve this problem.[294]

The A2-K^b transgenic mouse model is frequently used as an immunological model of human immunity and vaccine development. The mice express a chimeric HLA class I molecule HLA-A2.1/D^b composed of the α1 and α2 domains of HLA-A2 and the α3 transmembrane and cytoplasmic domains of H-2 D^b on a predominantly C57BL background. A2-K^b mice are capable of making CTL responses restricted by both HLA- A2.1 and H-2^b class I molecules.[215]

Using these mice in vaccination studies assumes conserved antigen processing and presentation to generate the appropriate 8-10-mer CTL epitopes for the HLA- A2.1 molecule. It also assumes physiological peptide selection by human class I molecules in murine APCs and an appropriate CD8 T-cell repertoire containing TCRs capable of positive selection on the epitope-HLA- A2.1 complex. There are reports that not all components of the antigen presentation machinery function normally across species barriers. These mice nonetheless have been used to identify CTL epitopes that have subsequently been applied to human studies.

There are several limitations to this transgenic mouse as a model. Firstly they have a limited TCR repertoire. A comparison of these A2-K^b mice with the related HHD transgenic mouse, where the H-2 class Ia gene had been invalidated, was made. The A2-K^b mice showed a greatly restricted ability to respond to HLA- A2.1 epitopes when compared to the HHD H-2 class I gene knockouts.[216] Our study shows that 35% of mice respond to the vaccination in the A2-K^b mice demonstrating the ability of this transgenic model to generate CD8 T-cell responses. The remaining 65% of mice generated only background levels of CTL. In contrast greater than 95% of the HHD mice responded to the pDOM.NLV vaccine. These two findings taken together suggest that the reduced response to pDOM.NLV in the A2-K^b mice is governed by a restricted TCR repertoire.

The HHD double knockout mice have a larger "HLA-A2.1-educated" CD8 T cell repertoire than the classical A2-K^b transgenics. The simultaneous expression of HLA-A2.1 and H-2 class I molecules in the A2-K^b mice results in reduced HLA-A2.1 education of mouse CD8 T cells. Subsequent studies in peptide vaccination have confirmed this restricted TCR repertoire.[295] Interestingly 100% of A2-K^b mice vaccinated with pDOM.CAP-1 and related constructs produced high levels of CTL suggesting that the TCR repertoire for this construct is present in a larger proportion of these transgenic mice.

The chimeric nature of the HLA- A2.1 molecules makes the assessment of the efficacy of the CTL against relevant virally infected cells difficult. Mouse cells cannot be infected by human

CMV. It is also difficult to assess murine CTL against human targets because of the key role of CD8 in cytotoxicity. Whilst murine CD8 can interact with the murine H-2^b α3 domain of the chimeric HLA-A2.1/D^b molecule it cannot do so with the wild type human α3 domain. CD8 acts as not just a co-receptor that helps to establish the TCR-class I complex, but also crucially in the provision of src-family kinase, Lck. Lck seems to play several unique roles in CD8 lymphocyte signalling.

Consequently we tested this vaccine in a Jurkat tumour line that had been transfected with the chimeric molecule HLA-A2.1/D^b and then infected with a vaccinia virus that expressed pp65 from CMV. The CTL induced by the vaccine pDOM.NLV was able to lyse both peptide-pulsed and virally infected cells. This shows that the CTL generated by this vaccine are of a sufficient avidity to lyse target cells that are presenting the peptide presented via an endogenous route.

We sought to further validate the DNA vaccine construct using a human in vitro assay. The pDOM.NLV vaccine was used to generate RNA that was transfected into DCs from an HLA-A2.1 donor. These transfected DCs were then used to stimulate autologous CD8 cells. Using tetramers it was possible to see a 60-fold expansion in the number of CD8+ T cells that recognized the NLV epitope. It was also demonstrated that these CTL were far more effective at lysing peptide pulsed targets. These data show that the vaccine is able to be processed by human APCs and presented to autologous CD8 cells successfully. They provide further evidence that justify the progression to a clinical trial.

We also compared the DNA vaccine to a long peptide vaccine that has been successfully used in the clinical setting. In the HHD mice the vaccine pDOM.NLV induced a higher level of tetramer-positive CTL than that from the long peptide. It was noted again that a homologous prime-boost vaccination protocol was not successful in increasing the response to either DNA or long peptide vaccination. As in many other settings a heterologous prime-boost vaccination protocol was very successful at eliciting a large increase in the number of CD8+ cells specific for this epitope NLVPMVATV. However it has been achieved here without the use of viral

vectors. Not only does this avoid immune responses to these vectors but also avoids the infection risk of live virus in immunocompromised patients.

The reasons as to why homologous prime-boost vaccination strategies do not increase specific CD8+ responses over responses generated by a single vaccination are not clear. Certainly some vaccine delivery systems elicit an immune response to not just the delivered antigen but also the viral vector that is used to deliver the antigen.[244] Although DNA has been shown to prime an immune response in several different studies it is not effective at boosting an immune response.[296,297] This may be due to the relative lack of antigen generated by these vectors. In this situation a low number of antigen presenting cells presenting the target epitope may be eliminated very early by the recently generated effector T cells specific for the antigen. A boosting vaccination is more effective with vectors that generate a larger amount of antigen such as viral vectors and electroporation. Long peptide vaccination seems to deliver an adequate amount of antigen in this heterologous prime-boost protocol. This would be consistent with the higher level of antigen in this system.

The dependence of this boost on the presence of CpG was less expected. In the original paper looking at this long peptide, the presence of CpG was found to increase the response to the peptide but there was still some response to peptide without CpG. Therefore to find the boost response completely abrogated in the absence of CpG was surprising. In addition some studies have suggested that the need for adjuvant in the "boost" vaccination is less critical. However this may be due to the fact that this study looks at an antibody prime-boost and not a cytotoxic T cell prime-boost phenomenon.

Previous studies looking at prime-boost vaccination have suggested that not only does the quantity of antigen-specific T cells increase with prime-boost vaccination but the avidity of T cells also increases.[298] However other studies have suggested that in fact prime-boost actually leads to the expansion of lower avidity T cells.[121] Results from our study seem to concur with this second study. Whilst the T cells activated by the heterologous prime-boost strategy are of a higher number they seem to be of a lower affinity than the T cells vaccinated by

pDOM.NLV. This may be due to higher levels of antigen delivered in the peptide boost vaccination leading to an expansion of lower avidity CD8+ lymphocytes.

Priming with naked DNA has been shown to be particularly effective, however boosting may require an alternative delivery system. In this study an effective boost would be with specific peptides that target the same T cell epitope. However recent investigations have revealed that naked DNA itself can also be used in a prime/boost strategy, with boosting relying on the physical change in delivery obtained by electroporation.[296] Testing in human subjects is required before decisions on the optimal CMV vaccine can be reached, but gene-based vaccines have a clear potential to suppress this difficult virus.

Further Work

The vaccine design of pDOM.NLV is highly effective at priming an immune response. However the limited performance of DNA vaccination in large animals, largely due to dose/volume restrictions is acknowledged. Physical methods including electroporation with this construct will be studied.

In addition prime-boost strategies will be explored. Initially the long peptide boost will be further studied in particular looking at the mechanisms behind the efficacy of this strategy. This will include studying the CpG dependence of the "boost" vaccination which will be studied in TLR-9 knockout mice. We will also look at the contribution CD4-help makes to this prime-boost strategy. In particular we will determine whether it is of benefit to have the same CD4-helper epitope in the "boost" vaccination as in the priming vaccination.

Also we will continue to look at the affect of prime-boost strategies on the avidity of the resulting CTL. We will seek to confirm the preliminary result that heterologous boosting with peptide leads to a lower avidity of CTL. We will also see whether the amount of peptide used in the boost vaccine influences the avidity of the CTL generated.

This study also provides further evidence that justifies the progression to a Phase I clinical trial. The early stages of this clinical trial are described in the next chapter.

5. Vaccination of patients against cytomegalovirus

5.1. Introduction

5.1.1. Clinical setting

As discussed in chapter 4, cytomegalovirus infection often causes severe disease in immunocompromised patients. In particular, it has been identified as a major cause of morbidity and mortality in patients following allogeneic stem cell transplantation. In the early days of transplantation CMV disease often developed within 100 days of transplantation with a severe pneumonitis. This complication is the most serious manifestation of infection in this clinical setting with a mortality of 60-80% without ganciclovir and up to 50% even when treated with antiviral pharmaceutical agents. With the advent of ganciclovir and a pre-emptive treatment regimen the incidence of early CMV disease in transplant recipients fell from approximately 25% to 5%. The pp65 antigen from CMV can be detected in a patient's blood by quantitative PCR with very high levels of sensitivity, long before CMV disease becomes apparent. In this way very low viral loads can be treated with ganciclovir at the earliest available time-point and the risk of disease is minimised.

However this dramatic reduction in early disease has been accompanied by an increase in late CMV disease (>100 days). In a recent study, CMV-seropositive patients were studied prospectively for late infection. Late CMV disease developed in 26 of 146 (17.8%) patients with a median of 169 days after transplantation and the mortality rate was 46%. Thirty-eight percent of patients surviving late disease went on to have a further episode. The incidence of CMV disease after 100 days is increased with chronic GVHD and the prior use of antiviral therapy.[299] The recommendation from this paper and others is that surveillance should be performed weekly and that treatment should begin as soon as infection is detected. Unfortunately disease may occur in patients in whom pp65 PCR detection techniques and

antigen detection techniques are negative.[299] In addition CMV may also become resistant to ganciclovir.[300]

There are many different risk factors for CMV disease post-transplantation. These include the CMV serostatus of both the donor and the recipient. The patients who suffer the highest incidence of disease are the recipients who are seropositive and therefore susceptible to viral reactivation once immunosuppressed.[301] These patients are particularly at risk if the donor is CMV-seronegative.[302] Recipients who are seronegative are at lower risk of disease even if the donor is seropositive.[303] The patients with the lowest risk of all are seronegative recipients from a seropositive donor. For these patients the main risk is from the administration of contaminated blood products.

Another factor that influences the incidence of CMV disease is the conditioning regimen that seeks to destroy existing cells to allow for replacement with graft cells. For instance a high incidence of infection has been observed when high doses of alemtuzumab-1H (immunoglobulin G1 humanized monoclonal antiCD52) is employed as a "reduced toxicity" conditioning regimen. These patients show a high incidence of CMV infection with very early reactivation of the virus.[304] This monoclonal antibody causes T cell depletion and thereby allows the virus to reactivate.

CD34 selection also leads to a notable increase in CMV disease and associated mortality.[305] In contrast the addition unmodified peripheral blood stem cell transplant recipients had a lower incidence of CMV infection when compared to bone marrow transplant recipients. They also displayed a trend towards a lower incidence of CMV disease in a recent randomized trial.[306] The number of T cells transferred with each transplantation technique is clearly critical in preventing CMV reactivation and disease.

5.1.2. CMV-specific CD8+ lymphocytes in recipients

Poor T-cell proliferative responses to CMV following allogeneic stem cell transplantation are associated with CMV disease.[307,308] Certainly many of the factors listed above which lead to increased risk of disease also suppress T cell responses. Investigators have found that before day 40 post-transplantation the majority of recipients do not have functional T-cell proliferative responses. Between day 40 and 90 the responses depend on the use of ganciclovir. The majority of patients who did not receive ganciclovir had persisting T cell responses whereas only a minority of patients who have received ganciclovir have preserved T cell responses.[309] There are thought to be two possible mechanisms for this effect. Firstly ganciclovir can inhibit antigen-induced T-cell proliferation because of effects on cellular DNA synthesis. Secondly prolonged therapy with ganciclovir may also cause sufficient suppression of CMV replication to preclude in vivo expansion of virus-specific T-cells.

In recent years the CMV-specific CD8+ T cell reconstitution in the recipient has been studied using peptide-MHC class I tetramers. In sibling donor-recipient pairs who were both seropositive the recovery of CMV-specific CD8+ T cells is rapid (median 37 days). This is not the case for recipients of matched unrelated donors who receive T cell depleting alemtuzumab-1H as standard treatment and also receive prophylactic ganciclovir. In this study high levels of CMV-specific CD8+ T cells were also associated with protection against CMV disease. Recovery of CMV-specific CTL to levels greater than $10x10^6$/L was associated with protection from disease.[310]

5.1.3. Vaccination against CMV

Chapter 4 provided pre-clinical data that supported the testing of a DNA fusion vaccine in a clinical trial. This vaccine incorporates a single domain of FrC of tetanus toxin fused to a single epitope of pp65 of CMV.

The CMV pp65-derived peptide is recognised by CTL only in the context of HLA A*0201, consequently only HLA-identical sibling donors expressing this HLA type were asked to participate in the study. Neither the disease entity nor the conditioning regime were used as patient selection criteria, in keeping with other trials in the transplant population. We felt that it was the allograft and subsequent immunosuppression that would dictate the immune reconstitution in the patients rather than the underlying haematological malignancy. Ideally we would have vaccinated only CMV seronegative donors, but the lack of donors led us to vaccinate independently of the serostatus of the donor/recipient. In addition it was unclear whether pre-existing CMV-specific responses would interfere with the activation of an anti-CMV T-cell response.

The dose of vaccine administered was based on a regimen used in the previous four DNA vaccine trials conducted from our laboratory. Anti-FrC responses were seen at this dose in these trials.

5.1.4. Monitoring T cell responses

The primary endpoints of the trial were:

1. To determine whether the DNA vaccine could generate or possibly augment a CD8+ T-cell response to pp65 from CMV.
2. To determine whether it is possible to transfer an anti-CMV immune response from a stem cell donor to an allogeneic stem cell transplant recipient.

To elucidate the answer to either of these questions it was necessary to monitor the CMV-specific CD8+ T cell responses. Several assays have been used to assess levels of antigen-specific CD8+ T cells in the past. In the past the most established assay for measuring cytolytic activity has been the chromium-release cytotoxicity assay. This assay has many advantages including the measurement of the most important effector function of the CD8+ T cells. However for the monitoring of human responses 3 or 4 rounds of stimulation have been

required to obtain sufficient levels of CTL to be detectable in chromium-release assays. Quantative comparisons after several rounds of in vitro expansion are very difficult. Assays that can detect immune responses immediately ex vivo are more reliable. In addition chromium-release cytotoxicity assays also require a very large number of cells and it would not be feasible to follow a patient's ongoing T-cell response in this way.

Quantitative frequency estimations by the chromium-release assay were obtained using the limited dilution assay (LDA). However this assay is not applicable to serial monitoring of patient responses due to its complexity and labour-intensity.

Two assays have recently emerged as the techniques most suited to following T-cell responses from patients. Both ELISPOT and tetramer analysis allow antigen-specific T cells to be detected ex vivo without the need for in vitro expansion.[311] The ELISPOT assay has been compared to the LDA by several groups.[312-315] They have found that ELISPOT correlates well with LDA and has approximately the same sensitivity. Most groups have found that ELISPOT can detect cells of a frequency of at least 1/100,000. Any frequencies lower than this would require prior ex vivo expansion of cell numbers.[312] In all of these assays the interassay variability is always proportionately higher when the numbers of responding cells are low.[316]

One of the central problems in monitoring immune responses to a vaccine, in patients who are receiving immunosuppression, is that cell numbers available for performing assays are very low. In addition when cells are frozen and then retrieved, cell losses can occur. One group used an external quality assurance panel to assess 11 laboratories for their ability to retrieve viable cells from cryopreservation. They found that a median of only 35% of cells were retrieved and only 86% of these cells were viable.[317] In addition any viable T cells that are retrieved post-freezing seemed to yield lower responses to antigen stimulation. Mwau et al found responses 2 to 3- fold higher when using fresh cells as opposed to cryopreserved cells.[318]

In view of this we planned to look for T-cell responses using fresh cells. In this way the number of cells available for use in assays was maximised. However this decision has two potential drawbacks. Firstly there are concerns over reproducibility as each time-point has to be analysed separately with no ideal internal control. Maecker and colleagues have recently shown more consistent results when using cryopreserved PBMCs.[316] In addition the amount of work that the trial generates is far higher when using fresh cells as each time-point generates a separate assay.

5.2. Methods

5.2.1. Ethical and Regulatory Approval

Ethical approval was obtained from the Local and Regional Ethical Committee in Southampton University Hospitals Trust. In addition approval was sought from the national Gene Therapy Advisory Committee for the use of this plasmid for clinical trial.

5.2.2. Preparation of vaccine for clinical trial

The DNA vaccine pDOM.NLV was prepared for clinical trial by the National Blood Service in Bristol. The bulk preparation and sterile fill was performed in accordance with Good Manufacturing Practice guidelines at the MCA approved laboratory at the National Blood Service in Bristol. Vaccine batches were assessed for sterility, purity and endotoxin level prior to use. They were also tested by restriction enzyme digest and nucleotide sequencing of the whole plasmid.

5.2.3. Donor and Patient Selection

Eligibility of Donors

1. Donor for allogeneic transplantation of an HLA identical sibling.
2. Likely time to transplant $\geq$8 weeks
3. Donor is HLA A*0201 positive.
4. WHO performance score 0
5. Donor must be greater than 18 years of age.
6. No medical condition, requiring immunosuppressive/steroid treatment in the 2 months prior to vaccination. Inhaled steroids will be allowed.
7. Known donor CMV status; CMV sero-positive and CMV sero-negative donors will be allowed

8. Donor must be able and willing to travel to the centre for vaccination, blood tests and follow up.
9. The donor and patient must give informed consent to vaccination and GTAC monitoring of their health records.
10. If of child bearing age the donor must be willing and able to take contraceptive measures for a period of 6 months.

Eligibility of Patients

1. HLA A*0201 positive recipient of allograft (stem cells or bone marrow).
2. Known CMV status; CMV sero-positive and CMV sero-negative – recipients will be allowed
3. Patient must be greater than 18 years of age.
4. Informed consent to study and GTAC monitoring of health records.
5. No uncontrolled sepsis or clotting disorder
6. Platelet count >50.000

Exclusion criteria

Patients and Donors

1. Presence of clinically significant levels of anti-DNA antibodies, anti-muscle antibodies or rheumatoid factors or who have active autoimmune disease.
2. Presence of antibodies to HIV.
3. Presence of severe medical condition other than the cancer requiring transplant and transplant related problems in the patient.
4. Pregnancy, lactation, or not using contraceptive measures (see Page 52).

5.2.4. Vaccination schedule

HLA-A2.1 stem cell transplant donors were vaccinated intramuscularly on weeks 0, 1, 2, 4, 8 and 12 with 1mg of the DNA vaccine pDOM.NLV (as per protocol - Figure 47). The vaccine was supplied as 1mg/ml in standard PBS.

5.2.5. Preparation of peripheral blood mononuclear cells

PBMC were isolated from heparinised blood by centrifugation over Lymphoprep. Cells were washed twice with RPMI (Invitrogen) and counted. Following a further wash a proportion of cells were then analysed in an Interferon-gamma ELISPOT assay or labelled with MHC class I tetramer complexes and then analysed by flow cytometry. The remaining PBMCs were frozen at 0.5-1x10^7/ml in 50% decomplemented human AB serum (Sigma-Aldrich), 40% RPMI (Invitrogen) and 10% DMSO (Sigma-Aldrich) and stored in liquid nitrogen for future use.

5.2.6. IFN-γ ELISPOT assay to detect antigen specific CD8+ T-lymphocytes

96-well ELISPOT plates (Millipore, Bedford, USA) were precoated overnight at 4^0C with 10μg/ml anti-IFNγ antibody (mAb1-DIK, Mabtech, Nach, Sweden) in sterile filtered PBS. 1 x 10^5 and/or 4 x 10^5 PBMC in 200μl of RPMI/10% pooled human AB serum (Sigma-Aldrich) were incubated in triplicate with either NLVPMVATV or GILGFVFTL peptide (Peptide Protein Research Ltd. Eastleigh UK) at 1μg/ml or PHA 5μg/ml (Sigma-Aldrich) or medium alone. After incubation for 16 hours at 37°C in 5% $C0_2$, the cells were discarded. Wells were washed 6 times with PBS/0.1% Tween-20 (Sigma-Aldrich). Biotinylated anti-IFNγ antibody (7-B6-1, Mabtech) at 1 μg/ml was added and the plates were then incubated at 37°C in 5% $C0_2$ for 90 minutes. Wells were again washed 6 times with PBS/0.1% Tween-20 and then streptavidin-conjugated alkaline phosphatase (Mabtech) was diluted 1:1000 in PBS/1% BSA and added at 100μl per well. The plates were incubated at 37°C in 5% CO2 for 60 minutes. An alkaline phosphatase-conjugate substrate kit was used to detect the IFNγ-producing cells (Zymed Laboratories Inc. San Fransisco CA). After spot development was complete, the reaction was stopped with tap water. The spots were counted by a computer assisted video image analyser (Autoimmun Diagnotika GmbH, Strassberg, Germany) using ELISPOT reader software (AID, version 3.1). Background spots were subtracted. Results are reported as the number of spot forming units / 10^6 PBMC.

Figure 47. Flow chart of vaccinations and investigations.

WEEK	-2	0	1	2	3	4	8	12	16+
Vaccination		*	*	*		*	*	*	
Physical Examination	*	*	*	*	*	*	*	*	*
Chest X-Ray	*								*
Toxicity Assessment	*	*	*	*	*	*	*	*	*
Weight	*	*	*	*	*	*	*	*	*
Vital signs	*	*	*	*		*	*	*	
Blood Count, ESR Clotting	* 15 ml		*	*	*	*	*	*	*
U+E, LFT, ‡CPK	* 10 ml			*		*	*	*	*
Autoimmune profile including Anti-muscle antibodies Anti-nuclear antibodies Anti-DNA antibodies Rheumatoid factors Serum electrophoresis	* 10 ml					*	*	*	*
PCR on PBL for CMV viral load		* 5ml		*		*	*	*	*
anti-tetanus antibodies		* 10 ml		*		*	*	*	*
CMV reactive CTL by Tetramer staining and intracellular cytokines		* 30 ml	*	*	*	*	*	*	*
CMV-reactive CTLs (Cytotoxic T cell assay)		* 100				*	*	*	
Tetanus-reactive helper T cells (ELISPOT)		* 20		*	*	*	*	*	
Total volumes (1000 ml)		195	40	85	60	195	195	195	95

5.2.7. Enumeration of epitope-specific human CD8 T cells using HLA class I tetramer complexes

HLA class I tetramer complexes conjugated to PE that incorporated the human HLA- A2.1 epitope from CMV (NLVPMVATV) were purchased from Proimmune, Oxford UK. In human volunteers PBMCs were taken from HLA- A2.1 positive donors and stained with 0.4 μg of tetramer per 10^6 cells. One μg of anti-CD8 mAb conjugated with FITC (BD Pharmingen) and anti-CD3 conjugated with PerCP (BD Pharmingen) were also added to the cells. They were then incubated at room temperature for 30 minutes and washed in PBS before analysis on FACScalibur using CELLQUEST software (Becton Dickinson). Cells were gated on a live lymphocyte gate and then a CD3+ gate. The proportion of CD8+ cells that were labelled with the tetramer was then calculated.

The proportion of CD8 T cells specific for the influenza matrix peptide GILGFVFTL was also determined with tetrameric complexes. HLA class I tetramer complexes conjugated to PE that incorporated the human HLA-A2.1 epitope from Influenza matrix peptide (GILGFVFTL) were purchased from Proimmune, Oxford UK. In human volunteers PBMCs were taken from HLA-A2.1-positive donors and stained with 1 μg of tetramer per 10^6 cells. Again 1μg of anti-CD8 mAb conjugated with FITC (BD Pharmingen) and anti-CD3 conjugated with PerCP (BD Pharmingen) were also added to the cells. They were incubated at 37°C for 20 minutes and washed in FACS buffer before analysis on FACScalibur using CELLQUEST software (Becton Dickinson).

5.2.8. Anti-FrC ELISAs

Anti-FrC IgG antibodies were measured using WHO human antitoxin standards (NIBSC Herts, UK). Briefly, ELISA plates were coated with E coli expressed FrC (0.5ug/ml diluted 1/2000 in coating buffer) 200 µl per well and incubated at 4^0C overnight. They were washed with PBS/Tween and blocked with blocking buffer for 1 hour at 37°C. The TT anti-sera standard (IgG) at 10iU/ml was diluted in PBS starting at 1/100 dilution. Patient samples and

an internal control serum were also diluted in PBS. Plates were washed with PBS/Tween and then doubling dilutions of standard and test samples added, 200 μl per well. Plates were then incubated for 1.5 hours at 37°C. They were then washed x4 in PBS/Tween before the addition of anti-Fc γ-HRP (Sigma) at a 1/2000 dilution and incubated for a further hour at 37°C. After 4 washes, 200 μl of fresh substrate buffer was added to each well. The colour was allowed to develop and the reaction was then stopped by the addition of 80μl per well of 2.5M H_2SO_4. The optical density was read on an automatic ELISA reader.

5.2.9. Interferon-γ ELISPOT assay for T-cell responses to FrC

96-well ELISPOT plates were pre-coated overnight at 4°C with 10 μg/ml anti-IFNγ antibody (mAb 1-DIK, Mabtech, Nacka, Sweden). 2 x 10^5 PBMC in 200μl of C-RPMI /10% decomplemented pooled human AB serum (Sigma-Aldrich) were incubated in triplicate with either FrC (at 20μg/ml, 2μg/ml or 0.2μg/ml), P30 (1μg/ml or 0.1μg/ml), medium alone (negative control) or 5μg/ml PHA (Sigma-Aldrich). After incubation for 48 hours at 37°C in 5% CO_2, the cells were discarded and the wells were washed 6 times with 150μl of filtered PBS/0.1% Tween-20 (Sigma-Aldrich). Biotinylated anti-IFNγ antibody at 1μg/ml (7-B6-1, Mabtech) in filtered PBS/1%BSA (Sigma-Aldrich) was added (100μl per well) and the plates were incubated for 90 minutes at 37°C in 5% CO_2. The wells were then washed another 6 times with filtered PBS/ Tween. Strepavidin conjugated alkaline phosphatase (Mabtech) diluted 1:1000 in filtered PBS/1%BSA was then added to each well (100μL) and the plates were again incubated for 1 hour at 37°C in 5% CO_2. After 6 further washes with filtered PBS/Tween, IFNγ spots were detected using an alkaline phosphatase-conjugate substrate kit (Zymed Laboratories Inc. San Francisco CA). Once spot development had finished the reaction was stopped by washing the plates with tap water. Once the plates had dried, a computer assisted video image analyser (Autoimmun Diagnostika GmbH, Strassberg, Germany) was used to count the spots. Background spots were subtracted.

5.2.10. Selection of NLVPMVATV tetramer positive $CD8^+$ T cells

PBMCs were re-suspended in FACS buffer at $1x10^7$ cells/ml and stained with PE-labeled HLA-A*0201/NLVPMVATV labeled tetramer complexes (5µl per $1x10^7$ cells). Cells were also stained with anti-CD8 mAb conjugated with FITC (BD Pharmingen) (10µl per 10^7 cells). They were incubated in the dark at 20°C for 30 minutes and washed in FACS buffer. Cells were sorted on a FACSvantage by gating on a live lymphocyte gate and then gating on $CD8^+$/tetramer positive or $CD8^+$/ tetramer negative cells. These samples were sent to the Wessex regional genetics laboratory where chimerism studies were performed to determine if the cells were of donor or recipient origin.

5.2.11. Dendritic Cells

Dendritic cells (DCs) were generated by plating PBMCs in a 6-well tissue culture plate (Nunc, UK). The cells were allowed to adhere for 2 hours at 37°C. After this time the non-adherent fraction was removed and the adherent cells were cultured in 5% FCS with 1000U/ml GM-CSF (Schering-Plough) and 500U/ml IL-4 (R&D). Cytokines were added to the cultures on days 0, 2, 4 and 6. Dendritic cells were transfected with RNA on day 6 and then re-plated in monocyte-conditioned medium (MCM) at 50%(v/v) in order to induce maturation. Mature DCs were collected on day 7.

MCM was prepared by coating bacteriological plates with human immunoglobulin for 24 hours at 4°C. Then $7x10^7$ PBMCs were layered onto the plates for 1 hour at 37°C. Non-adherent cells were removed and the adherent cells were incubated in medium containing 1% serum at 37°C for 24 hours. After this time the medium was collected as MCM.

5.2.12. DC culture with CD8 cells

CD8 cells were isolated using a CD8 isolation kit (Miltenyi Biotec, UK) using the manufacturers instructions. Briefly the following hapten-conjugated antibodies were added to PBMCs: CD14, CD4, CD19 and CD56. Cells were then washed and anti-hapten beads are incubated with the cells. The cells were then run through a magnetic column and cells the CD14-, CD4-, CD19-, CD56- population that did not bind to the column were collected. These CD8 cells were then placed in a 96-well culture plate at $5x10^4$ cells per well. $5x10^3$ DCs that were transfected with the pDOM.NLV were then placed in each well. Cells were co-incubated in the presence of interleukin-7 at a concentration of 5ng/ml. After 3 days IL-2 was added at 20 IU/ml. Cells were taken on day 7 for tetramer analysis and a ^{51}Cr release cytotoxicity assay.

5.3. Results

5.3.1. Responses in normal volunteers

5.3.1.1. Ex vivo analysis of the proportion of CD8+ lymphocytes that also bind NLVPMVATV tetramer in normal volunteers.

PBMCs were collected from 9 CMV sero-positive, HLA-A2.1-positive donors. These volunteers were all immunocompetent and had no history of CMV disease. Tetramer analysis was used to assess the proportion of CD8+ cells that were capable of recognizing the NLVPMVATV epitope. A wide range of values was found with most volunteers having very low levels of tetramer positive cells (Figure 48). Eight out of nine volunteers had between 0.07% and 0.15% of their CD8+ specific for the NLVPMVATV epitope. One volunteer had 1.2% of her peripheral CD8+ cells that were tetramer positive. A control group of volunteers who were CMV sero-negative but HLA-A2.1-positive were also examined. The background level of tetramer staining in this group was always less than 0.02% of the CD8+ population.

5.3.1.2. Ex vivo analysis of IFN-gamma production by CD8+ cells in response to CMV peptide

PBMCs were taken from 7 volunteers and examined for IFN-gamma production by ELISPOT assay. $4x10^5$ PBMCs were placed in each well of 96-well ELISPOT plate that had been coated with anti-IFN-gamma antibody. The cells were stimulated with NLVPMVATV peptide or were incubated without peptide. The plate was developed with the method described above. Again a wide range of results were demonstrated reflecting the tetramer data. The volunteer who had 1.2% tetramer positive CD8+ cells had the highest number of spot forming units (SFU) in the IFN-gamma assay (810 spots / 10^6 PBMCs). All the other volunteers had much lower numbers of SFUs in the ELISPOT assay (less than 120 spots / 10^6 PBMCs). The background level of staining determined by PBMCs with no peptide added was always <10% of the positive wells. A representative set of results is shown in Figure 49.

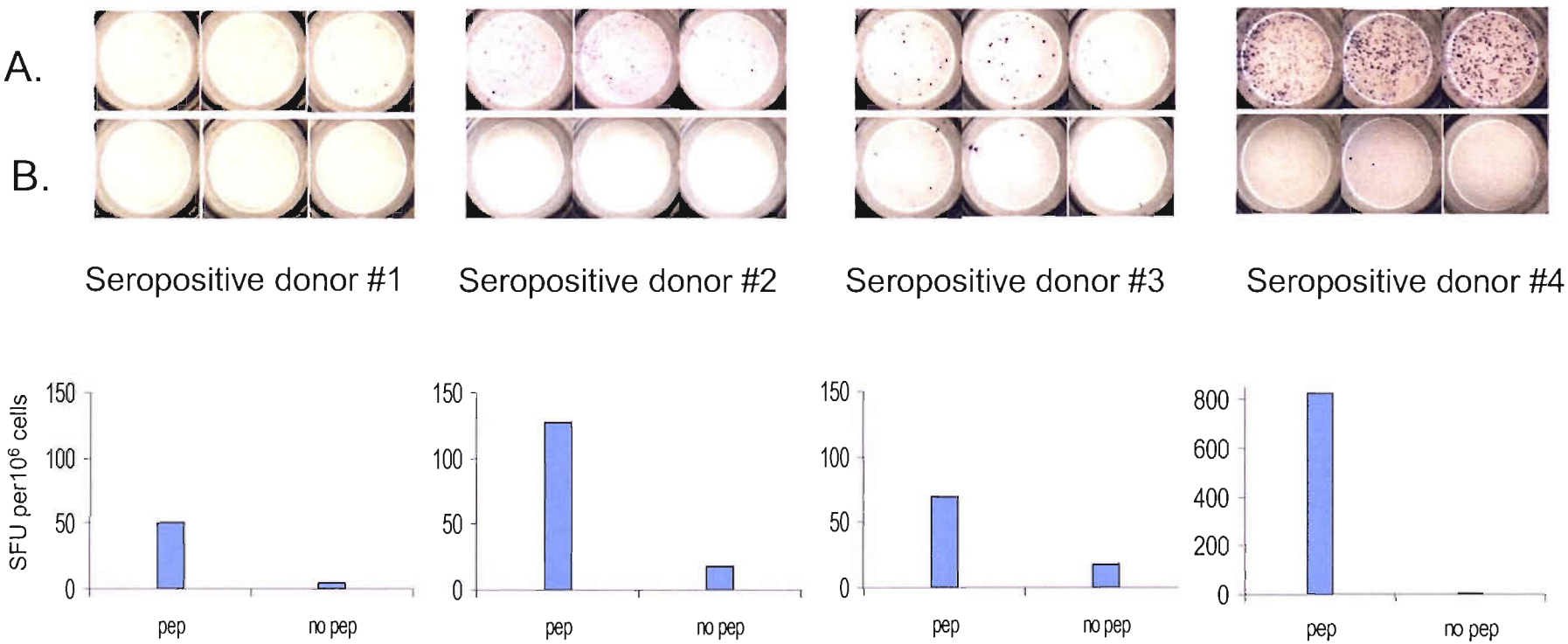
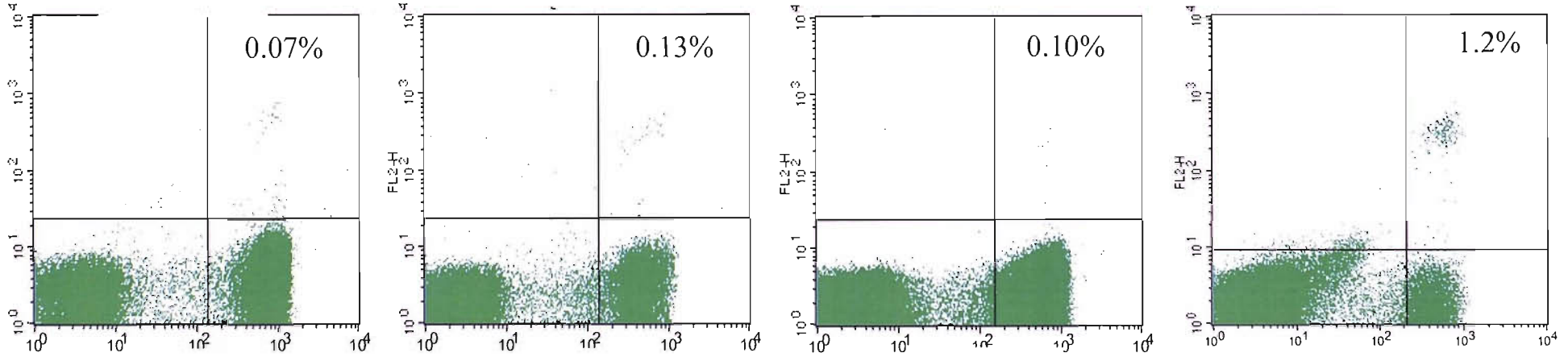


Figure 48. Healthy volunteers who are seropositive for CMV display a wide range of CTL responses to CMV.

$4x10^5$ PBMCs from volunteers were isolated and plated in the ELISPOT plate in the presence of 10microM NLVPMVATV peptide (panel A) or in the absence of the peptide (panel B) for 16 hours. An interferon-gamma ELISPOT was then performed as described in the Materials and Methods (section 5.2.6). Results are expressed as the number of spot forming units (SFU) per million cells.



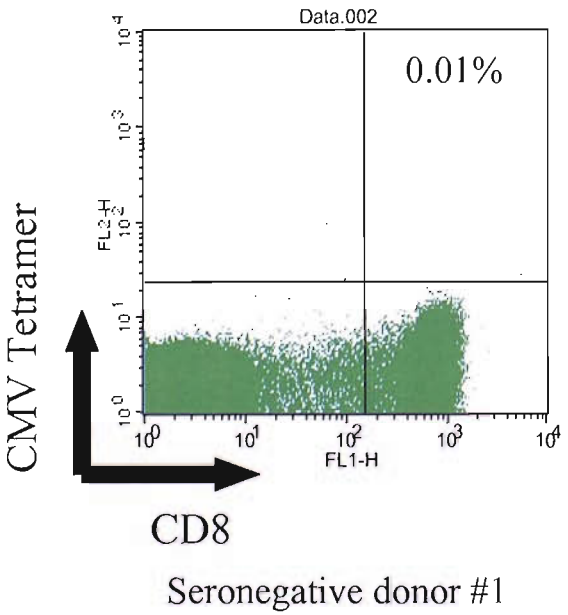


Figure 49. Ex vivo analysis of the proportion of CD8+ lymphocytes that also bind NLVPMVATV tetramer in normal volunteers.

Ex vivo PBMCs were assessed with tetramer staining (see section 5.2.7). These cells were stained with 0.4 μg of NLVPMVATV.HLA-A2.1 tetramer per 10^6 cells. One microgram of anti-CD8 mAb conjugated with FITC (BD Pharmingen) and anti-CD3 conjugated with PerCP (BD Pharmingen) were also added to the cells. They were then incubated at room temperature for 30 minutes and washed in PBS before analysis on FACScalibur using CELLQUEST software (Becton Dickinson). Cells were gated on a live lymphocyte gate and then a CD3+ gate. The proportion of CD8+ cells that were labelled with the tetramer was then calculated.

Figure 50. Allogeneic SCT recipient clinical characteristics

Recipient	Age	Sex	Diagnosis	Conditioning intensity	Stem cell source	Stem cell dose
1	23	F	ALL	Standard	BM	7.71 $x10^6$/kg
2	57	M	CLL	Reduced	PBSCs	5.38 $x10^6$ / kg
3	20	F	AA	Standard	BM	7.44$x10^6$/kg

Figure 51. Donor/ Recipient HLA class I and class II type and CMV serostatus

Donor/ Recipient 1	
HLA-A	0201
HLA-B	44, 57
HLA-C	04, 06
HLA-DR	DRB1*07
HLA-DQ	DQB1*02, 0303
HLA-DP	DPA1*0103/05, DPB1*0401, 1301

	CMV Serostatus
Donor 1	Seronegative
Recipient 1	Seronegative

Donor/ Recipient 2	
HLA-A	0201
HLA-B	27, 35
HLA-C	01, 04
HLA-DR	DRB1*04, 11
HLA-DQ	DQB1*0301/04, 0302/07

	CMV Serostatus
Donor 2	Seropositive
Recipient 2	Seropositive

Donor/ Recipient 3	
HLA-A	01, 0201
HLA-B	08, 51
HLA-C	0701, 01
HLA-DR	DRB1*03011/0306, 04
HLA-DQ	DQB1*02,0301/0304

	CMV Serostatus
Donor 3	Seropositive
Recipient 3	Seropositive

5.3.2. NLVPMVATV-specific responses in vaccinated patients

5.3.2.1. Donor 1

Donor 1 was CMV seronegative and heterozygous for the MHC class I molecule HLA.A2.1. Her peripheral blood revealed very low levels of NLVPMVATV-specific CD8+ lymphocytes when labelled with a NLVPMVATV-specific tetramer analysis prior to vaccination (<0.1% of peripheral CD8+ lymphoctes). In addition, on stimulation with the NLVPMVATV peptide in the ELISPOT assay, the number of spots was not higher than background (Figure 53). The donor was monitored until week 46 post vaccination. At no point did the level of tetramer staining increase to above 0.05%. Similarly at no stage during this time did stimulation with the NLVPMVATV peptide elicit any number of spots over background.

Using a tetramer specific for the influenza matrix peptide: GILGFVFTL donor 1 was found to have low levels of GILGFVFTL-specific CD8+ lymphocytes prior to vaccination (0.25% of CD8+ lymphocytes). This showed only minor fluctuations throughout the study period. ELISPOT responses to the GILGFVFTL peptide varied between 20 and 66 spots per million PBMCs. (Figure 52)

5.3.2.2. Recipient 1

Recipient 1 was also seronegative for CMV. This patient was neutropenic and lymphopenic prior to transplantation and therefore tetramer analysis was performed on a small number of cells. A NLVPMVATV-specific population was not detected prior to transplantation nor was a convincing population detected after transplantation. IFN-γ ELISPOT also revealed no spots above background from either NLVPMVATV or GILGFVFTL stimulation at any time point. Both of these analyses were continued up to 32 weeks post-transplantation (Figure 54).

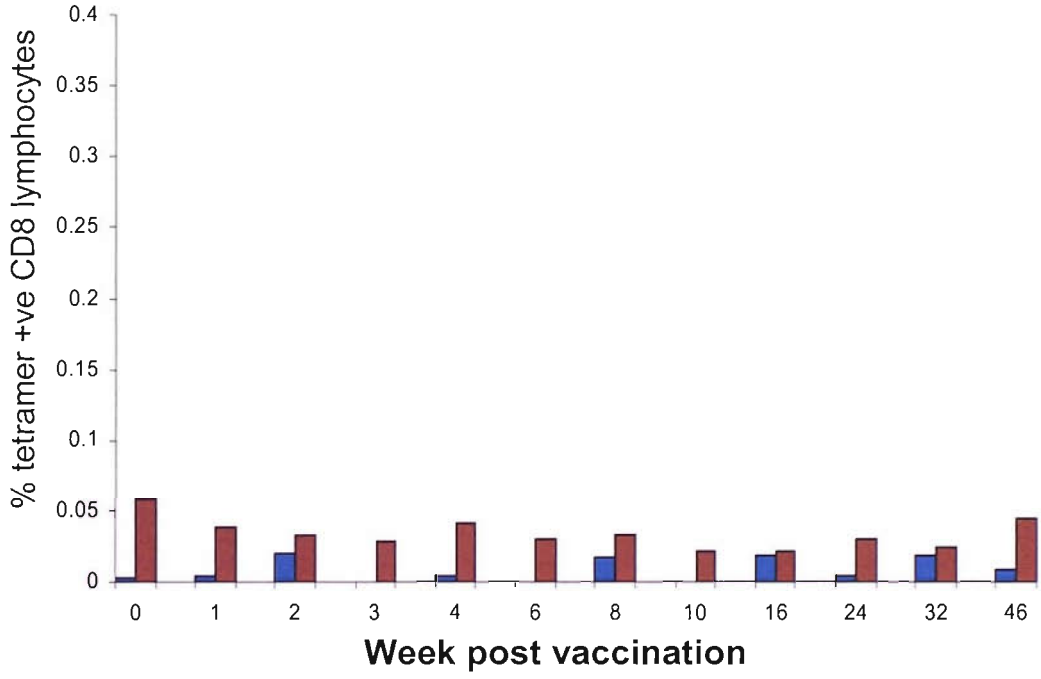


Figure 52. The proportion of CD8+ lymphocytes that bind NLVPMVATV-specific tetramer from Donor 1.

PBMCs were isolated from Donor 1 at the above time-points. They were then stained with NLVPMVATV(■) or GILGFVFTL (■) tetramer (see materials and methods section 5.2.7). Results are expressed as the percentage of CD8+ lymphocytes that are staining positively for the respective tetramers.

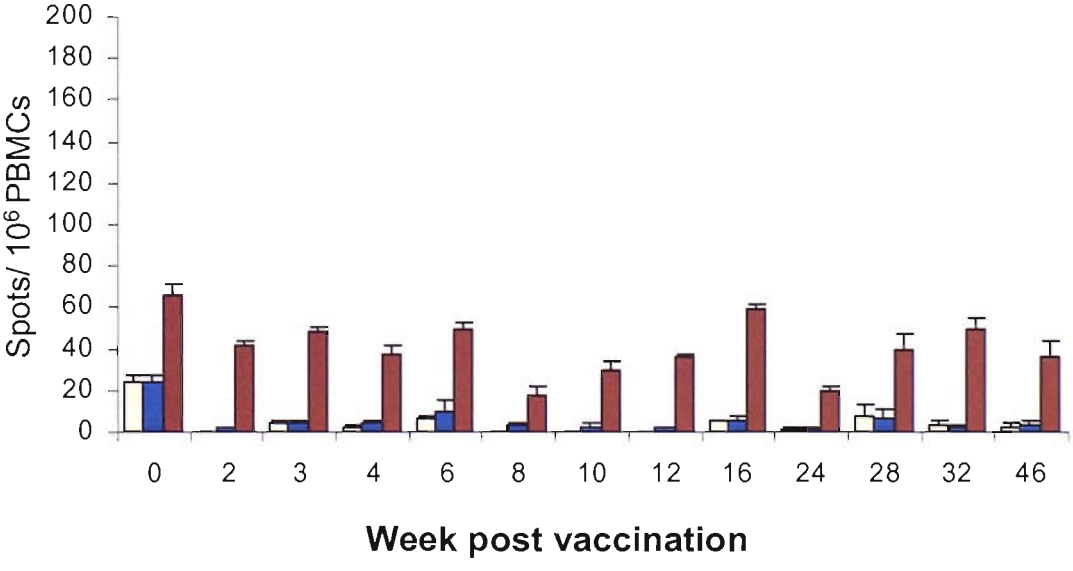


Figure 53. IFN-γ ELISPOT responses to NLVPMVATV from Donor 1.

PBMCs were isolated from Donor 1 at the above time-points. They were then incubated on ELISPOT plates for 16 hours with no peptide (□), 10μM NLVPMVATV (■) or 10μM GILGFVFTL (■). The cells were washed from the ELISPOT plate and a standard IFN-γ ELISPOT was performed (see section 5.2.6). Results are expressed as the number of spots per 10^6 PBMCs.

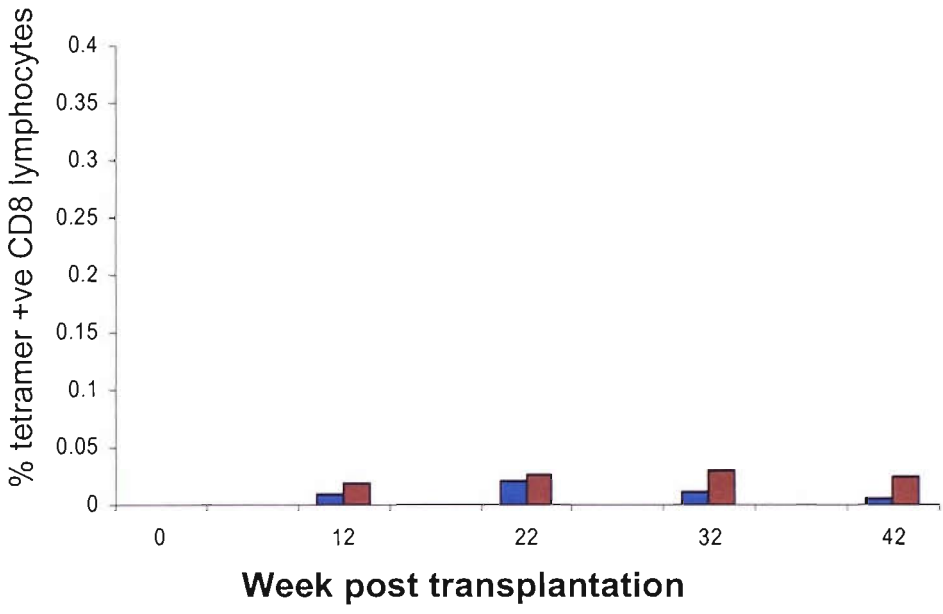


Figure 54. The proportion of CD8+ lymphocytes that bind NLVPMVATV-specific tetramer from Recipient 1

PBMCs were isolated from Recipient 1 at the above time-points. They were then stained with NLVPMVATV(■) or GILGFVFTL (■) tetramer (see materials and methods section 5.2.7). Results are expressed as the percentage of CD8+ lymphocytes that are staining positively for the respective tetramers.

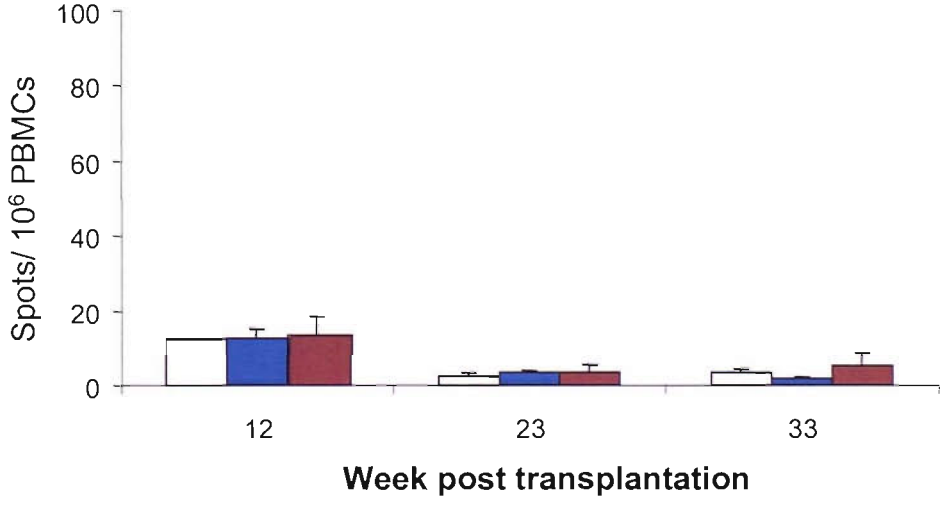


Figure 55. IFN-γ ELISPOT responses to NLVPMVATV from Recipient 1

PBMCs were isolated from Recipient 1 at the above time-points. They were then incubated on ELISPOT plates for 16 hours with no peptide (□), 10μM NLVPMVATV (■) or 10μM GILGFVFTL (■). The cells were washed from the ELISPOT plate and a standard IFN-γ ELISPOT was performed (see section 5.2.6). Results are expressed as the number of spots per million PBMCs.

In conclusion both Donor 1 and Recipient 1 displayed very low numbers of CD8+ T cells specific for either the NLVPMVATV epitope or the GILGFVFTL epitope throughout the study period.

5.3.2.3. Donor 2

Donor 2 was CMV seropositive and was homozygous for HLA-A*0201. Before vaccination, 2.9% of his peripheral CD8+ lymphocytes were stained with the NLVPMVATV-specific tetramer. In contrast only 0.1% of his CD8+ lymphocytes were specific for the influenza epitope GILGFVFTL. Between weeks 2 and 3 post-vaccination the proportion of CD8+ lymphocytes staining with the NLVPMVATV-specific tetramer fell to 1.9% before rising to 2.7% by week 8. This was followed by another fall at week 12 before rising to 4.1% at week 24. Although results from the ELISPOT do not exactly mirror those from the tetramer analysis, a similar pattern can be discerned. Interferon-γ producing cells were at their lowest number in week 2 before rising and reaching their maximum at week 32 post vaccination. Responses increased again to reach a maximum of 1550 spots per million PBMCs (Figure 56).

The population of CD8+ lymphocytes that were specific for GILGFVFTL was very low and was always less than 0.07% . Interferon-γ ELISPOT for this peptide was always fewer than 20 spots per million PBMCs throughout the study period.

It is uncertain whether this decrease in NLVPMVATV-specific CD8+ T cells and then subsequent increase simply represents assay variability or whether it is a true representation of T cell migration and expansion. A seropositive donor was followed for a similar period of time and had a mean of 0.55 ± 0.15% of CD8+ T cells. This shows that the assay is reproducible but not without variation.

5.3.2.4. Recipient 2

Recipient 2 was also CMV seropositive. The first tetramer analysis performed on week 4 post-transplantation revealed that 4.6% of his CD8+ lymphocytes were specific for NLVPMVATV. By week 6 this percentage had risen to 18.3 %. PCR for the pp65 antigen from CMV was positive at week 4 but returned to undetectable levels without requiring therapy at this stage. However the PCR became positive again on days 45 and 48 post-transplantation. Ganciclovir therapy was therefore started on day 52 post transplantation. The pp65 PCR was negative again on day 59 and therefore ganciclovir therapy was discontinued on day 66. Following this ganciclovir therapy the proportion of CD8+ lymphocytes that were NLVPMVATV-specific detected by tetramer fell to 4% before rising again to 18.6% (Figure 58).

The IFN-γ ELISPOT assay revealed that prior to vaccination there were 200 spots per 10^5 PBMCs prior to vaccination. This climbed steeply to 550 spots per 10^5 PBMCs at week 12 before it dropped to a much lower level between weeks 16 and 20 and then rose again after week 30.

Similar to donor 2 very few GILGFVFTL-specific CD8+ lymphocytes were detected by tetramer throughout the study period (<0.02% throughout). In addition very few spots were produced in the IFNγ ELISPOT assay when stimulated by GILGFVFTL peptide (<15 spots per million PBMCs throughout the study).

In summary then recipient 2 revealed dramatic rises in the proportion of NLVPMVATV-specific CD8+ T cells after transplantation. These levels fluctuated with the administration of ganciclovir. It is not certain whether this is a response to reactivation of the virus or whether DNA vaccination of the donor had a role in the rapid expansion of these cells.

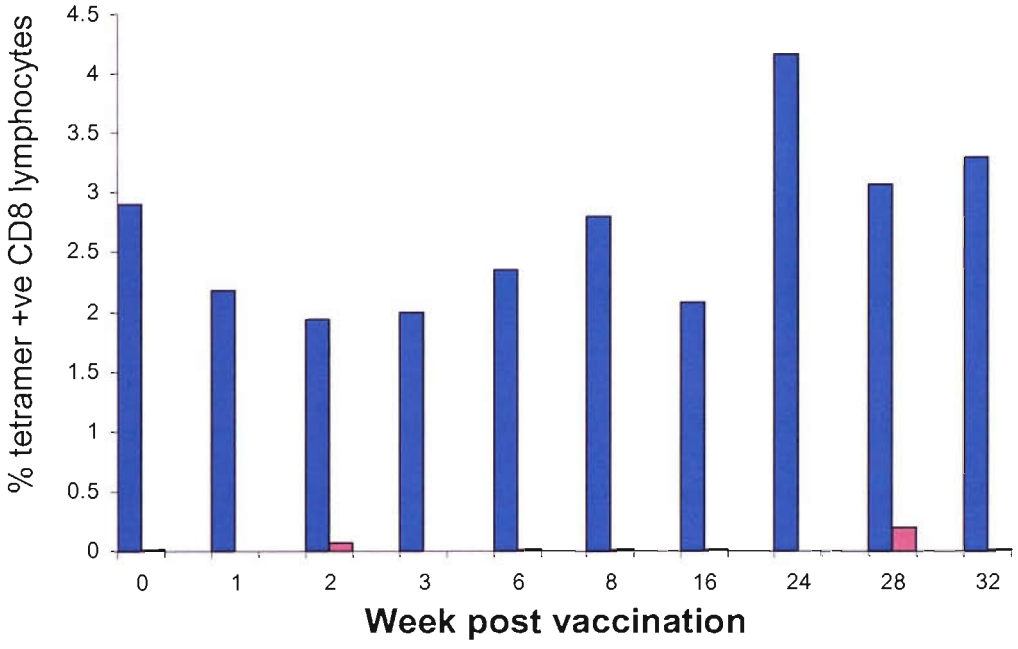


Figure 56. The proportion of CD8+ lymphocytes that bind NLVPMVATV-specific tetramer from Donor 2

PBMCs were isolated from Donor 2 at the above time-points. They were then stained with NLVPMVATV(■) or GILGFVFTL (■) tetramer (see materials and methods section 5.2.7). Results are expressed as the percentage of CD8+ lymphocytes that are staining positively for the respective tetramers.

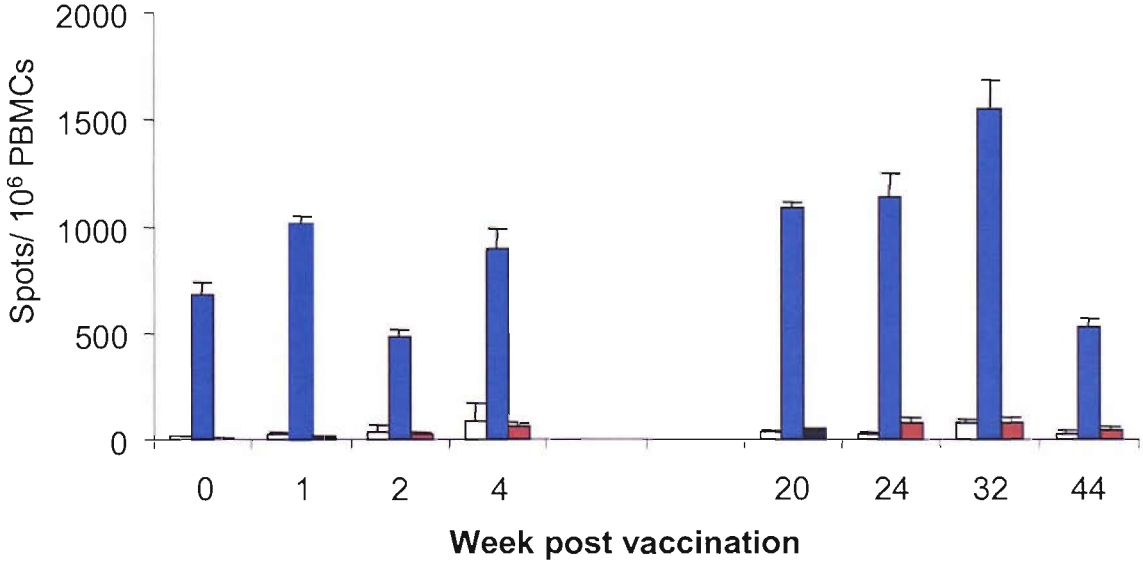


Figure 57. IFN-γ ELISPOT responses to NLVPMVATV from Donor 2

PBMCs were isolated from Donor 2 at the above time-points. They were then incubated on ELISPOT plates for 16 hours with no peptide (□), 10μM NLVPMVATV (■) or 10μM GILGFVFTL (■). The cells were washed from the ELISPOT plate and a standard IFN-γ ELISPOT was performed (see section 5.2.6). Results are expressed as the number of spots per million PBMCs.

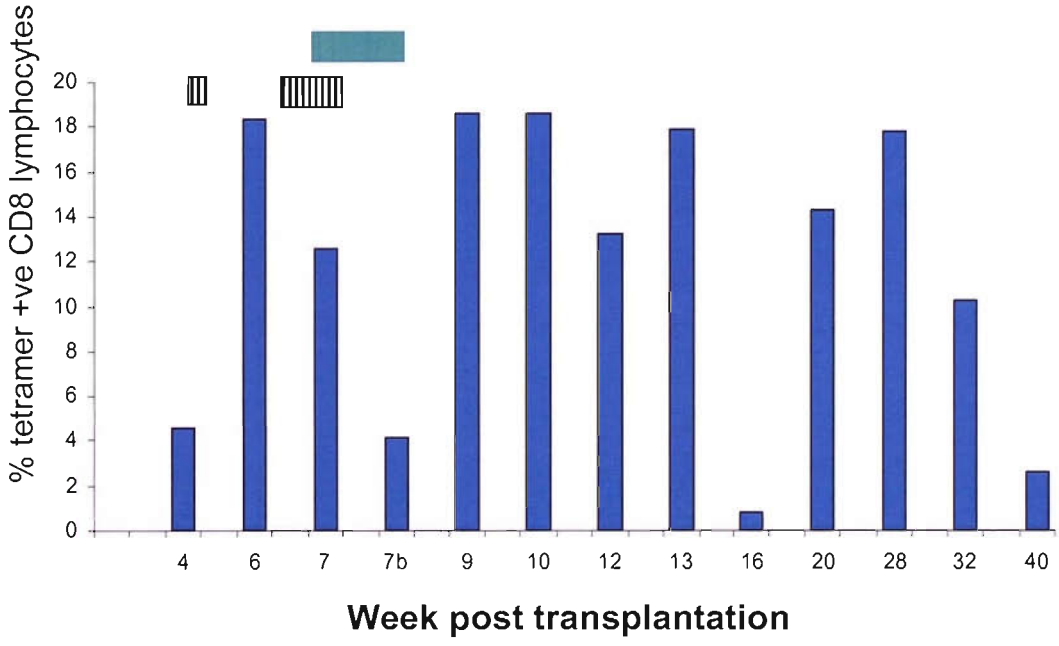


Figure 58. The proportion of CD8+ lymphocytes that bind NLVPMVATV-specific tetramer from Recipient 2

PBMCs were isolated from Recipient 2 at the above time-points. They were then stained with NLVPMVATV(■) tetramer (see materials and methods section 5.2.7). Results are expressed as the percentage of CD8+ lymphocytes that are staining positively for the respective tetramers. CMV reactivation is indicated by the striped bar. Ganciclovir therapy is indicated by the green bar.

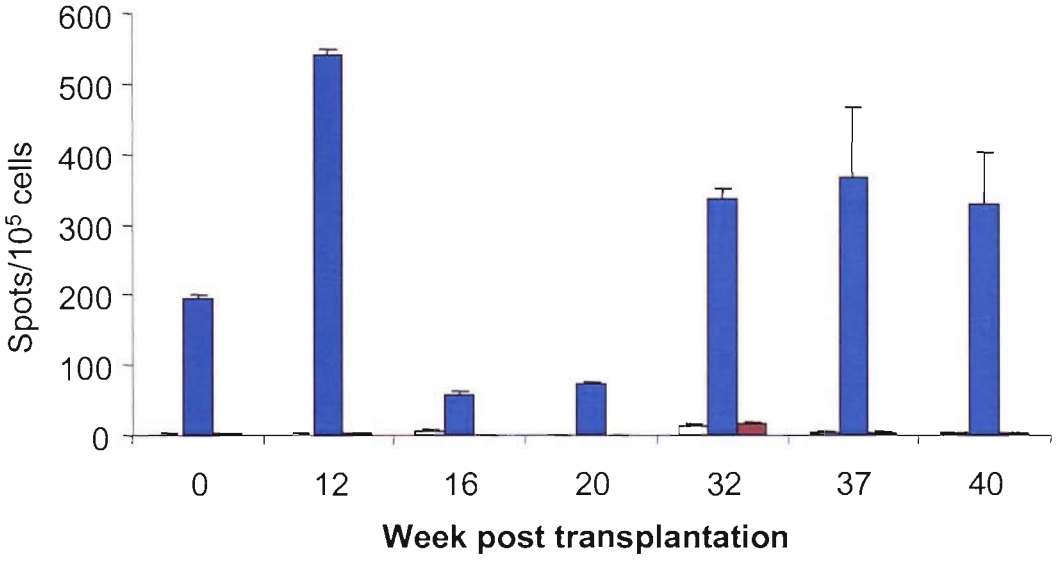


Figure 59. IFN-γ ELISPOT responses to NLVPMVATV from Recipient 2

PBMCs were isolated from Recipient 2 at the above time-points. They were then incubated on ELISPOT plates for 16 hours with no peptide (□), 10μM NLVPMVATV (■) or 10μM GILGFVFTL (■). The cells were washed from the ELISPOT plate and a standard IFN-γ ELISPOT was performed (see section 5.2.6). Results are expressed as the number of spots per million PBMCs.

5.3.2.5. Donor 3

Donor 3 was CMV seropositive and heterozygous for HLA-A*0201. Tetramer analysis revealed that 0.1% of her peripheral CD8+ lymphocytes were NLVPMVATV-specific prior to vaccination. After vaccination this proportion of CD8+ T cells rose to 0.27% at week 3. This proportion then fluctuated between 0.03 and 0.3%. Towards the end of the study period there was a late increase in NLVPMVATV-specific CD8+ T cells that reached 0.62% at week 44. The ELISPOT results mirrored these results showing low levels of cells responding to the NLVPMVATV peptide throughout the study period. Before vaccination there were only 280 spots per million PBMCs. After vaccination the number decreased to 93 on week 3 and then varied between 210 and 583 spots per million PBMCs for the rest of the study period (Figure 60).

Very low levels of GILGFVFTL-specific CD8+ lymphocytes were detected throughout the study period with tetramers (0.05-0.29%). Again very low numbers of IFN-γ spots were detected by the ELISPOT assay when stimulated with the GILGFVFTL peptide throughout the study period.

In summary donor 2 has an expansion of NLV tetramer-binding CD8+ T cells over the study period. The number of cells that secrete IFN-γ in response to the peptide over the study shows a more modest rise.

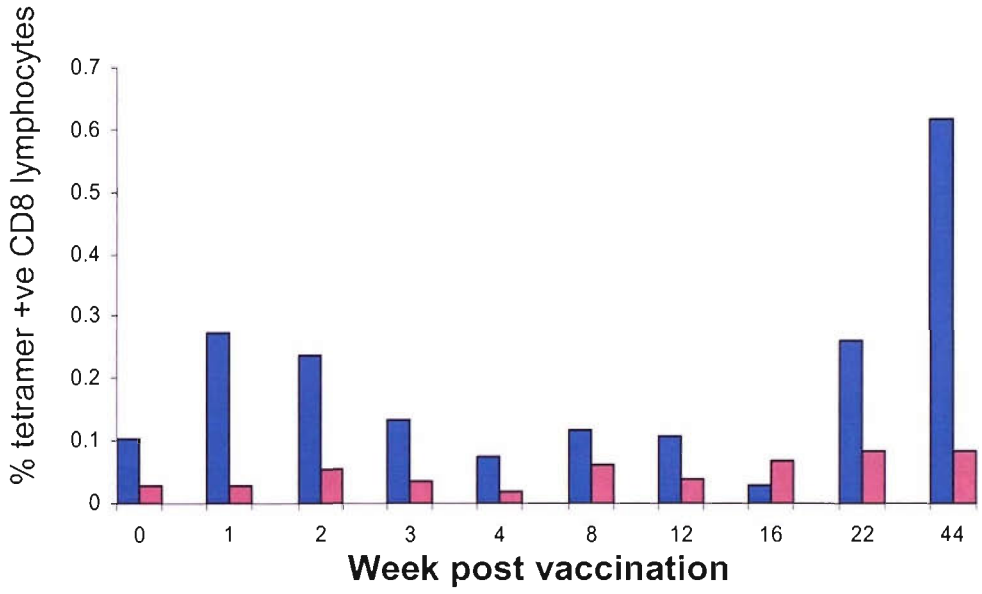


Figure 60. The proportion of CD8+ lymphocytes that bind NLVPMVATV-specific tetramer from Donor 3

PBMCs were isolated from Donor 3 at the above time-points. They were then stained with NLVPMVATV(■) or GILGFVFTL (■) tetramer (see materials and methods section 5.2.7). Results are expressed as the percentage of CD8+ lymphocytes that are staining positively for the respective tetramers.

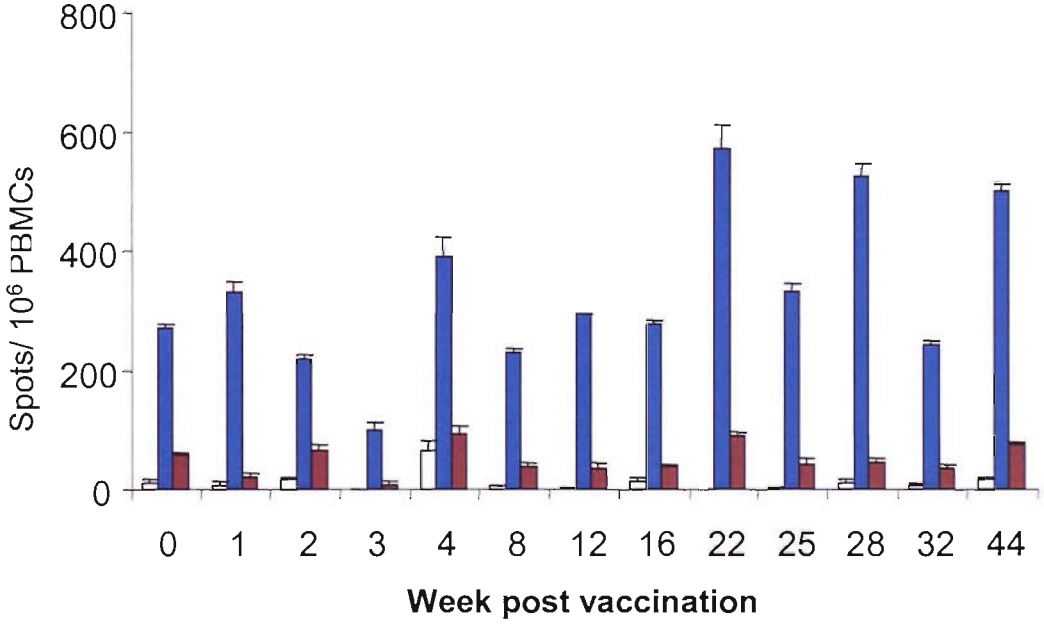


Figure 61. IFN-γ ELISPOT responses to NLVPMVATV from Donor 3

PBMCs were isolated from Donor 3 at the above time-points. They were then incubated on ELISPOT plates for 16 hours with no peptide (□), 10μM NLVPMVATV (■) or 10μM GILGFVFTL (■). The cells were washed from the ELISPOT plate and a standard IFN-γ ELISPOT was performed (see section 5.2.6). Results are expressed as the number of spots per million PBMCs.

5.3.2.6. Recipient 3

Recipient 3 was CMV seronegative when tested several months prior to transplantation. However a baseline tetramer analysis performed 3 weeks prior to transplantation revealed that 2.5% of her CD8+ lymphocytes were specific for NLVPMVATV. This is a very high level for a HLA-A*0201 heterozygote. Serology repeated at this time was postive for CMV IgG and weakly positive for CMV IgM indicating a recent primary CMV infection. This level of NLVPMVATV-specific CD8+ T cells fell and was 1.27% one week pre-transplant. Reactivation of CMV occurred very early in the post-transplant period. PCR was positive for pp65 on day 14 and ganciclovir was commenced on day 25. Tetramer analysis could not be performed until week 10 after transplantation due to haemolysis and low peripheral lymphocyte counts. On week 10 post-transplantation the proportion of CD8+lymphocytes that stained with the NLVPMVATV tetramer was 1.36% (Figure 62).

Before transplantation 890 IFN-γ spots per million PBMCs were detected on stimulation with the NLVPMVATV peptide. This level had fallen to 120 spots per million PBMCs by week 10. Low levels of GILGFVFTL-specific cells were detected by tetramer and ELISPOT throughout the study period. Less than 0.1 % of CD8+ lymphocytes were detected by tetramer analysis with the GILGFVFTL tetramer throughout the study period. Fewer than 60 spots were elicited by the GILGFVFTL peptide in the IFN-γ ELISPOT assay at both time points studied.

In summary there is little evidence of an expansion of NLVPMVATV-specific CD8+ T cells in recipient 2.

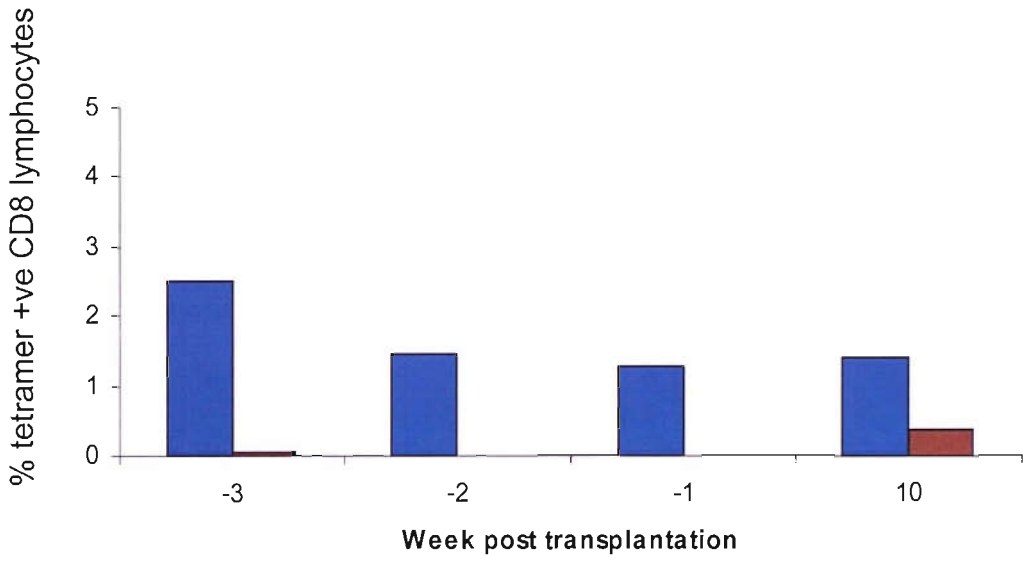


Figure 62. The proportion of CD8+ lymphocytes that bind NLVPMVATV-specific tetramer from Recipient 3

PBMCs were isolated from Recipient 3 at the above time-points. They were then stained with NLVPMVATV(■) or GILGFVFTL (■) tetramer (see materials and methods section 5.2.7). Results are expressed as the percentage of CD8+ lymphocytes that are staining positively for the respective tetramers.

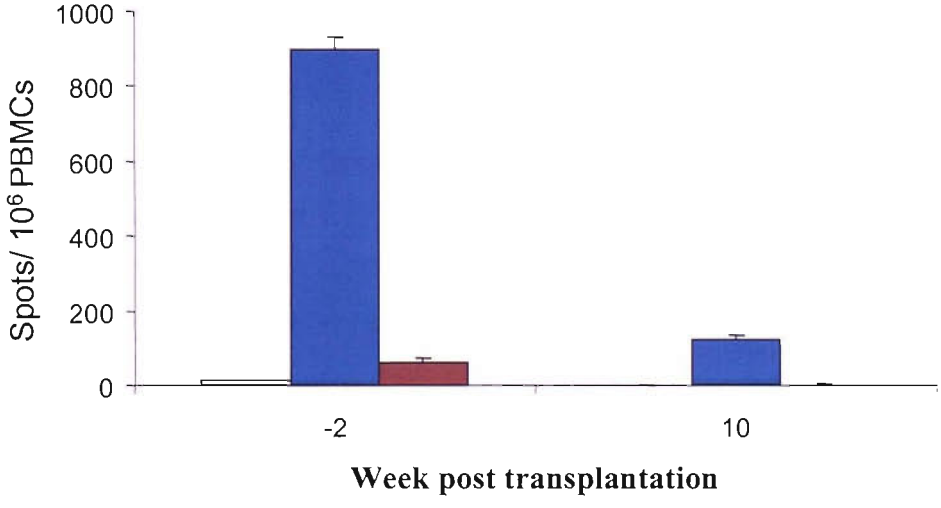


Figure 63. IFN-γ ELISPOT responses to NLVPMVATV from Recipient 3

PBMCs were isolated from Recipient 3 at the above time-points. They were then incubated on ELISPOT plates for 16 hours with no peptide (□), 10μM NLVPMVATV (■) or 10μM GILGFVFTL (■). The cells were washed from the ELISPOT plate and a standard IFN-γ ELISPOT was performed (see section 5.2.6). Results are expressed as the number of spots per million PBMCs.

5.3.3. Chimerism studies

Given that the CD8+ T-cells specific for NLVPMVATV became so high in recipient 2 it was important to determine whether these were of donor origin. It is routine practice in stem cell transplantation to identify polymorphic differences between the stem cell donor and recipient prior to transplantation. These differences are then used to assess chimerism post-tranplantation. Consequently markers which distinguished between donor 2 and recipient 2 had already been determined. PBMCs from recipient 2 were stained with anti-CD8 antibody and the NLV-specific tetramer in the usual manner. Tetramer-positive, CD8-positive cells were subsequently sorted from the PBMCs using a FACSvantage. After selection over 90% of the CD8+ T-cells were specific for NLVPMVATV (Figure 64). The tetramer-negative, CD8-positive population of cells were also sorted. Both sets of cells were sent to the Wessex regional genetics laboratory for chimerism studies. This identified that 100% of the tetramer positive CD8+ positive cells were of donor origin. This compared to only 40% of the PBMC that were of donor origin.

5.3.4. In vitro stimulation of CD8+ lymphocytes

The ex vivo assays described thus far sought to detect epitope-specific CD8+ T cells. The most sensitive of these assays is the ELISPOT. In this setting it will only detect cells that are able to secrete interferon-γ in response to peptide stimulation ex vivo. This includes effector CD8+ T cells and effector memory CD8+ T cells. Central memory cells will not be detected by this assay. In attempt to detect this subtype of CD8+ lymphocyte and to expand any epitope-specific CD8+ T cells we stimulated these cells in vitro for 7 days before tetramer analysis (Figure 65).

Monocytes were obtained from the PBMCs of donor 1 and differentiated into DCs using the protocol described in section 2.18. These dendritic cells were matured using LPS and IFN-γ. CD8+ lymphocytes were then isolated from donor 1 PBMCs collected at 0, 4, 8 and 16 weeks post-vaccination.

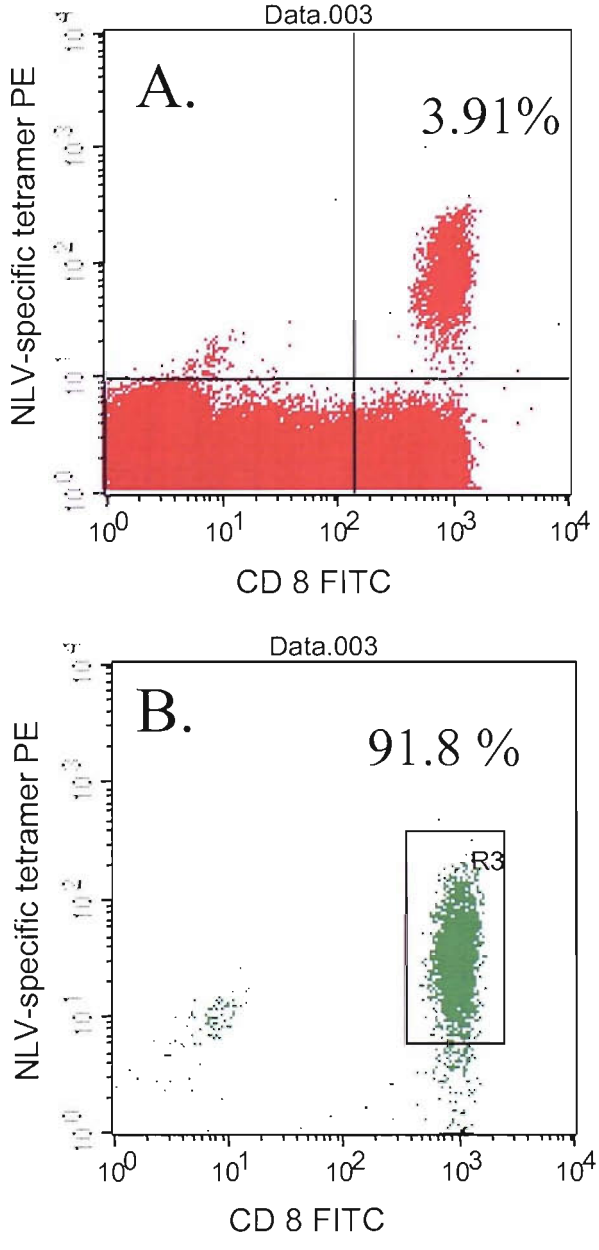


Figure 64. Chimerism studies in receipient 2

PBMCs were isolated from recipient 2 on week 6. PBMCs were re-suspended in FACS buffer at 1x10^7 cells/ml and stained with PE-labeled HLA-A*0201/NLVPMVATV labeled tetramer complexes (5µl per 1x10^7 cells). Cells were also stained with anti-CD8 mAb conjugated with FITC (BD Pharmingen) (10μl per 10^7 cells). They were incubated in the dark at 20°C for 30 minutes and washed in FACS buffer. Cells were sorted on a FACSvantage by gating on a live lymphocyte gate and then gating on CD8+/tetramer positive or CD8+/ tetramer negative cells. Chimerism studies were performed to determine if the cells were of donor or recipient origin. Analysis of peripheral blood of recipient 2 before and after PE-labelled NLVPMVATV tetramer FACS selection. A). Prior to selection 3.91% of the $CD8^+$ T-cell population of recipient 2 were specific for NLVPMVATV. B). Post selection over 90% of the $CD8^+$ T-cell population were NLVPMVATV specific. FL2-H = PE labelled NLVPMVATV tetramer.

The cells that had been selected in this way were found to be entirely of donor origin whereas only 40% of the mononuclear cells in the recipients blood were of donor origin.

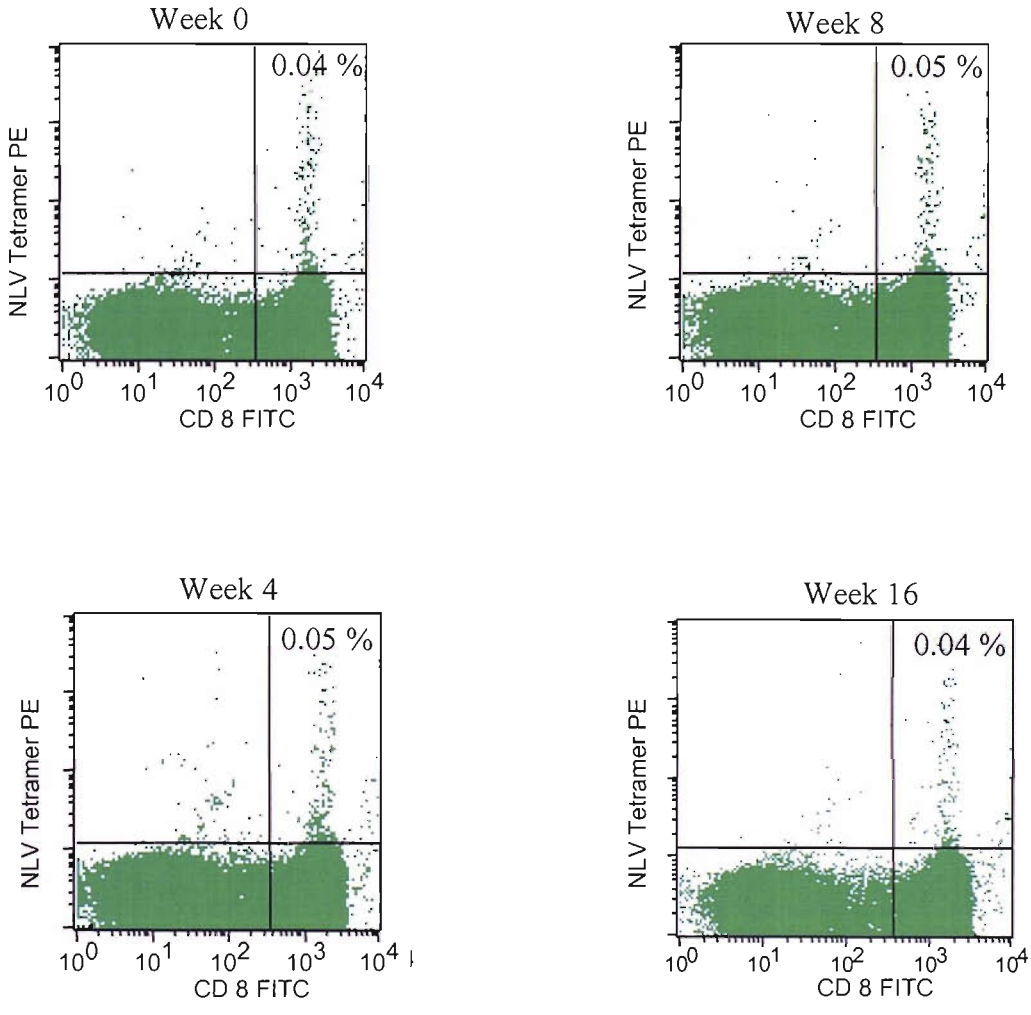


Figure 65. NLVPMVATV-specific responses from Donor 1 after in vitro stimulation

CTLs isolated from Donor 1 at weeks 0, 4, 8 and 16 were incubated with NLVPMVATV-pulsed mature autologous dendritic cells (see sections 5.2.11 and 5.2.12). Cells were co-incubated in the presence of interleukin-7 at a concentration of 5ng/ml. After 3 days IL-2 was added at 20 IU/ml. After 7 days the CTLs were analysed by FACS to asses the number of NLVPMVATV-specific CTLs (see section 5.2.7). Results are expressed as the percentage of CD8+ lymphocytes that were positive for the NLVPMVATV tetramer. Vaccination with pDOM.NLV had not induced a specific CTL population which could be expanded in vitro.

After 7 days of incubation the cells were stained with anti-CD8 antibodies and NLVPMVATV-specific tetramer. They were then analysed by FACS analysis to determine the proportion of CD8 cells that were tetramer-specific. The results were very similar for each time point. The proportion of CD8 cells that were tetramer-positive varied between 0.04 and 0.05%. This is very low and there was no expansion post-vaccination. CD8+ cells that were specific for matrix peptide of influenza were expanded by peptide-pulsing mature dendritic cells in identical protocol. These CD8+ T-cells were expanded 50-fold.

5.3.5. Cellular responses to FrC in vaccinated patients

The DNA vaccine pDOM.NLV was designed to elicit CD8+ T-cell immunity against the NLV epitope. Nonetheless it does contain the universal T-helper epitope p30 along with a single domain of FrC. Therefore it may elicit CD4+ T-cell responses and antibody responses to these elements. ELISPOT assays were used to assess the former and ELISA assays to assess the latter.

5.3.5.1. Donor 1

Interferon-γ ELISPOT assays were performed with cells being stimulated with FrC and p30 before and after vaccination. A specific response to incubation to FrC or p30 was not detected either prior to vaccination or during follow-up to week 20 (Figure 66).

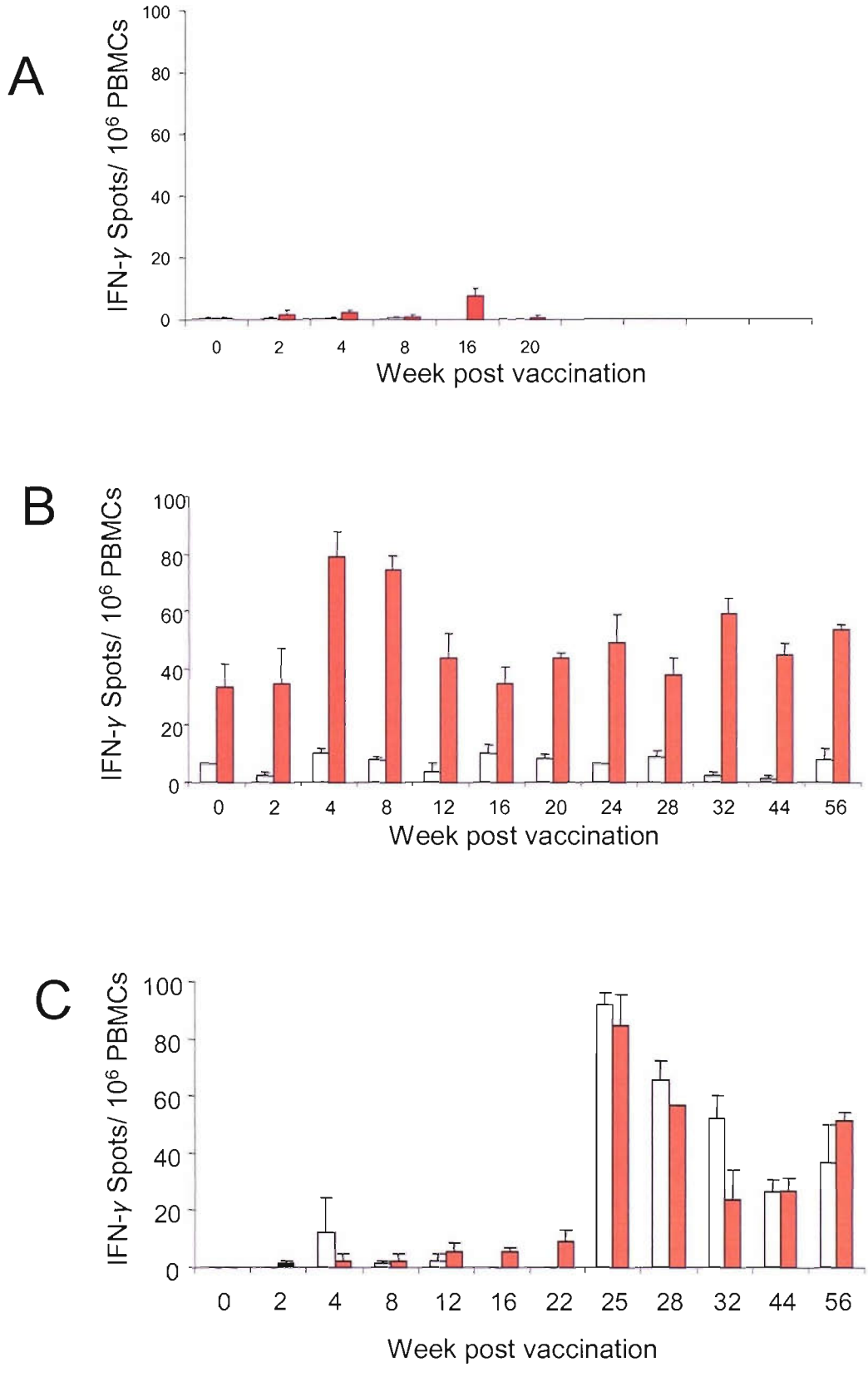


Figure 66. Cellular responses to FrC in Donors 1, 2 and 3

PBMCs were isolated from Donor 1 (panel A), Donor 2 (panel B) or Donor 3 (panel C) at the above time-points. PBMCs were then incubated with no protein (□) or FrC (■) for 48 hours on an ELISPOT plate pre-coated with anti-interferon-gamma antibody (see materials and methods section 5.2.9). The cells were washed from the plate using PBS/0.1% Tween-20 and then the plate was developed in the standard fashion using biotinylated anti-IFNγ antibody, streptavidin-conjugated alkaline phosphatase and alkaline phosphatase-conjugate substrate as described in the materials and methods section. Results are expressed as spot forming units per million PBMCs.

5.3.5.2. Donor 2

Interferon-γ ELISPOT for donor 2 revealed low level cellular responses to FrC pre-vaccination with 34 spots per million PBMCs. After vaccination this increased to 78 spots per million PBMCs on week 4 post-vaccination. The number of spots elicited by FrC returned to pre-vaccination levels by week 16 at 35 spots per million PBMCs (Figure 66).

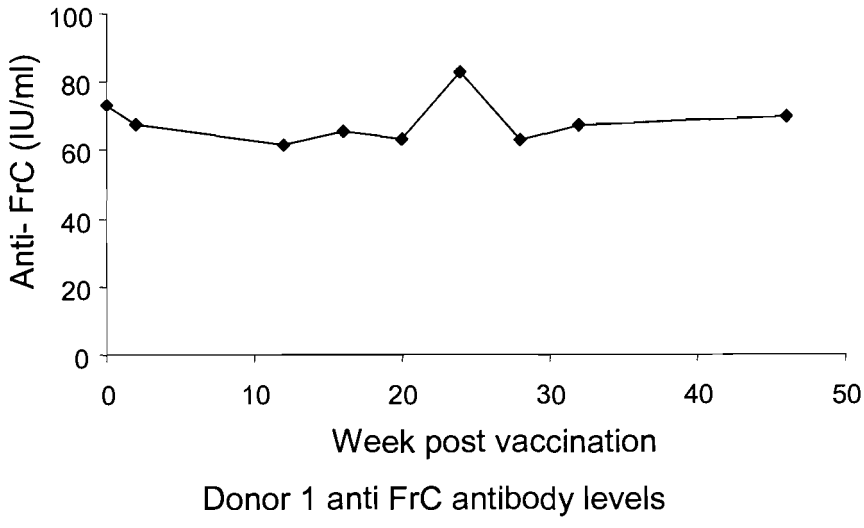
5.3.5.3. Donor 3

Interferon-γ ELISPOT for donor 3 revealed no cellular responses to FrC pre-vaccination. Over the course of the trial there were no specific cellular responses to FrC although the background responses became higher towards the end of the study period (Figure 66).

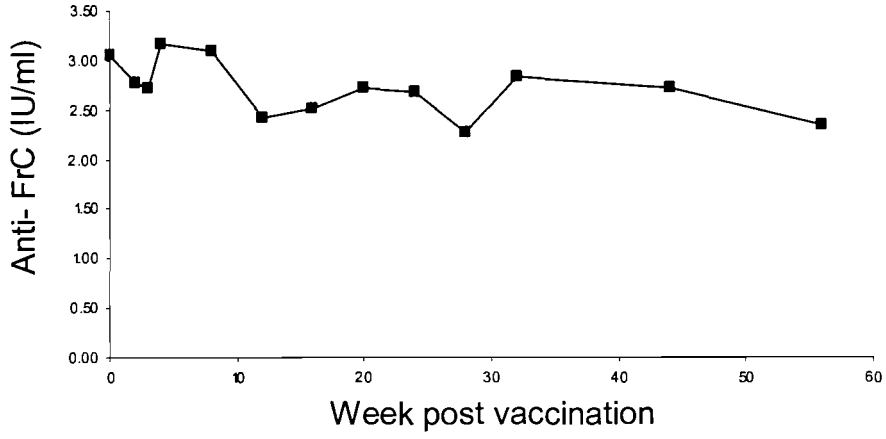
5.3.6. Antibody responses to FrC

The DNA vaccine only contained the first domain of FrC. Initially antibody responses were assessed against the whole of FrC with an ELISA.

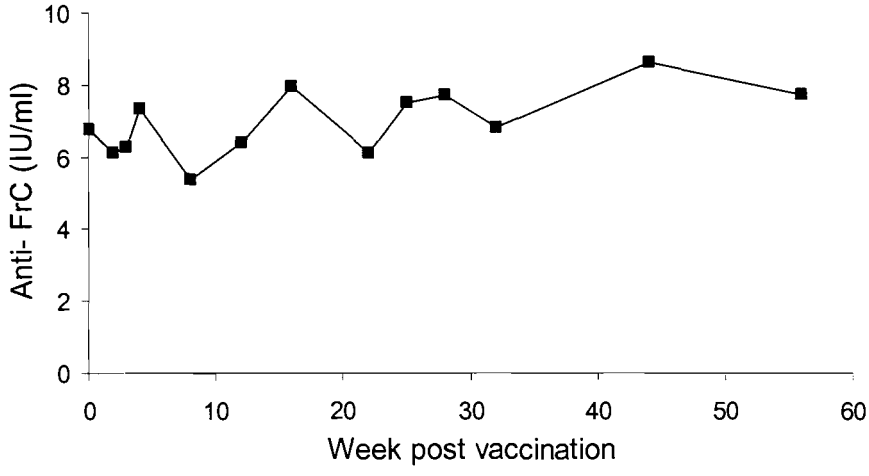
Donor 1 had a low level of antibody against FrC pre-vaccination. After vaccination there was no significant increase in the anti-FrC antibody (Figure 67). Donors 2 and 3 also had low levels of anti-FrC antibody prior to vaccination. Again there was no increase in the level of antibody after vaccination in either donor. The ELISA was performed with the whole of FrC. The recipients 1 and 2 had low levels of anti-FrC antibody soon after transplantation. Over the course of the study period the levels of antibody declined steadily until 50 weeks. Recipient 3 had low levels of antibody immediately after transplantation that rose until week 10 but declined thereafter (Figure 68).



Donor 1 anti FrC antibody levels



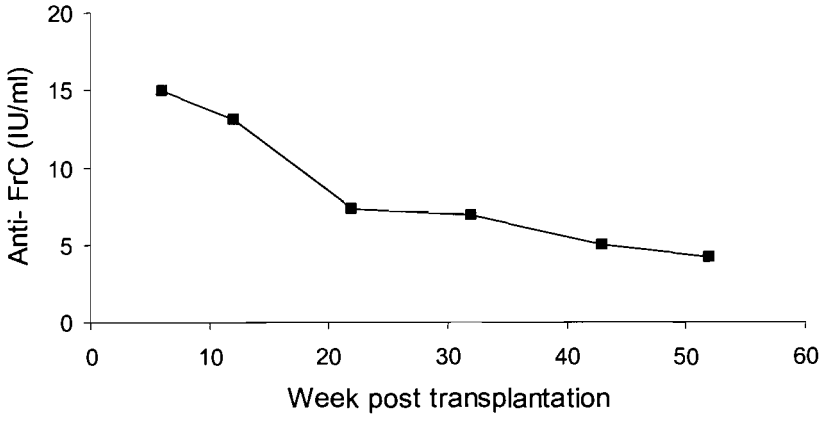
Donor 2 anti FrC antibody levels



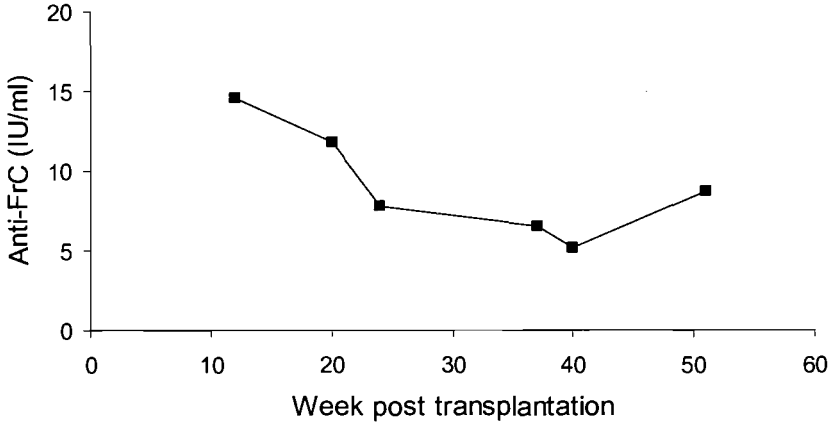
Donor 3 anti FrC antibody levels

Figure 67. Antibody responses to FrC in the transplant donors

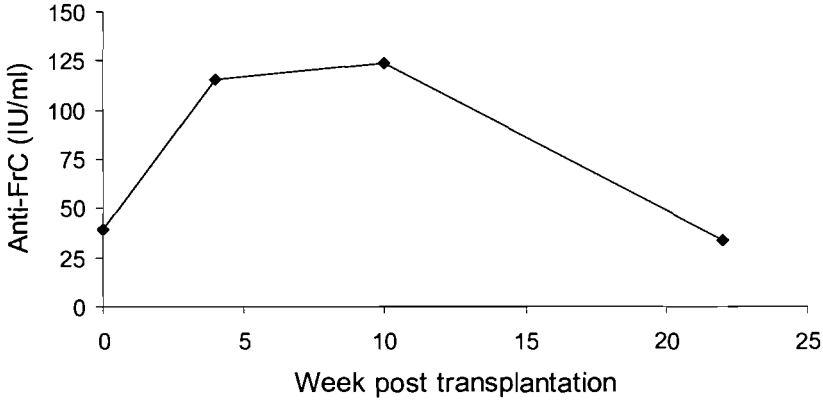
Serum was collected from the donors at the above time-points. ELISAs were then performed to look for anti-FrC antibody as described in the materials and methods section 5.2.8.



Recipient 1 anti-FrC antibody level



Recipient 2 anti FrC antibody level



Recipient 3 Anti-FrC antibody level

Figure 68. Antibody responses to FrC in the transplant recipients

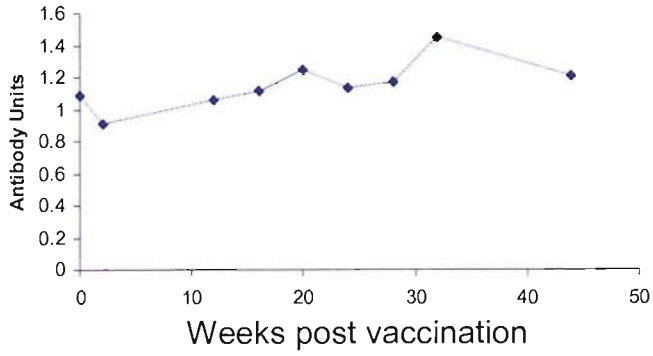
Serum was collected from the recipients at the above time-points. ELISAs were then performed to look for anti-FrC antibody as described in the materials and methods section 5.2.8.

5.3.7. Antibody responses to pDOM

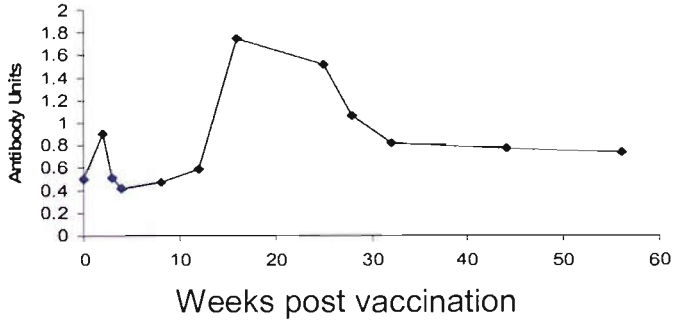
Donor 1 had anti-pDOM antibody levels that rose slightly over the 44 week period. There was no obvious peak response (Figure 69).

Donor 2, 3 and 4 all had increases in anti-pDOM antibody levels that peaked at approximately 16 weeks. The antibody levels increased between two and four-fold for these 3 donors over this time.

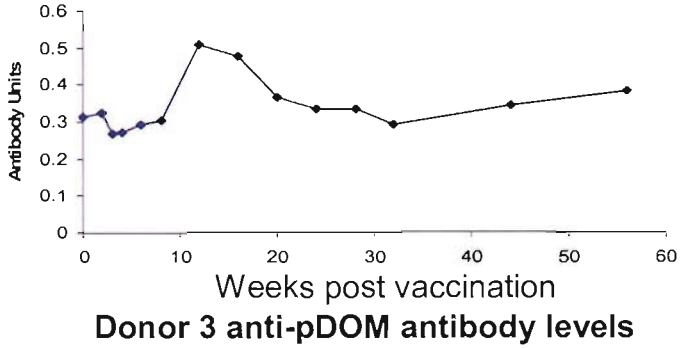
In summary whilst none of the donors had a detectable antibody response to FrC at least 3 of them had an antibody response to pDOM. The kinetics of this response was similar in these 3 donors.



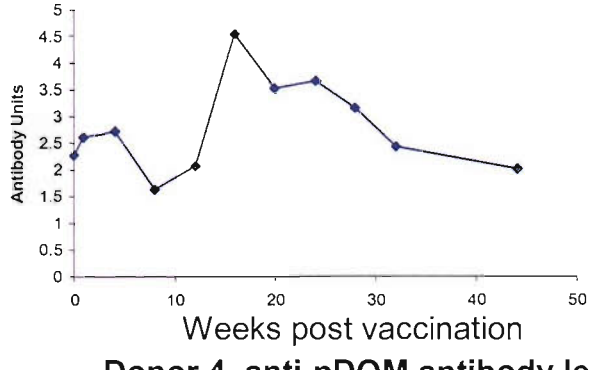
Donor 1 anti-pDOM antibody levels



Donor 2 anti-pDOM antibody levels



Donor 3 anti-pDOM antibody levels



Donor 4 anti-pDOM antibody levels

Figure 69. Antibody responses to pDOM in the transplant donors

Serum was collected from the recipients at the above time-points. ELISAs were then performed to look for anti-FrC antibody as described in the materials and methods section 5.2.8.

5.4. Discussion

The aim of this trial was to use the DNA vaccine pDOM.NLV to induce or augment a specific CD8+ T-cell response to NLVPMVATV. The clinical setting chosen to test this construct was the vaccination of normal individuals who were donating stem cells to patients suffering from haematological malignancy. Any specific immunity generated by these donors could then be transferred to the recipients and may be of clinical benefit to them.

In these subjects we obtained periodic samples from the peripheral blood unlike the situation in mice where responses in lymphoid tissues, particularly spleen, are easy to assess. The monitoring of responses in only the peripheral blood has several limitations. Upon vaccination antigen-specific T cells will migrate to the regional lymph node where they will encounter antigen presenting cells. Monitoring the peripheral blood for these antigen-specific T cells may see no change or even a drop in the level of antigen-specific T cells. Secondly there are dynamic temporal considerations. After vaccination in mice there is a rapid T cell expansion phase and then a rapid contraction phase where the majority (>90%) of responding T cells die.[319] If this situation is mirrored in human CD8+ T cell responses to vaccination this will present several challenges in detecting the induction of specific immune responses. If the expansion and contraction phases in the human are similarly short-lived then we are more likely to detect memory responses. Effector memory cells should be detectable ex vivo. However central memory cells will need to be stimulated in vitro before they will be detectable by their ability to secrete interferon-γ.[320]

The responses of both the donors and recipients were monitored using immunological assays looking at responses to both aspects of the vaccine: responses to the specific epitope NLVPMVATV and also to FrC. Ex vivo NLV-tetramer and ELISPOT assays were able to quantify the number of NLV-specific CD8+ cells. In addition in one patient we were able to stimulate CD8+ cells in vitro with peptide-pulsed mature DCs. This technique is particularly good at expanding the number of peptide-specific CD8+ lymphocytes but is also able to detect central memory T cells discussed above.

In this study we vaccinated both CMV seropositive and seronegative volunteers. However the serostatus of the volunteer will have a large effect on the response of the immune system to vaccination. Consequently these are two very different clinical scenarios in which to test the vaccine. Volunteers who are seronegative require the vaccine to prime an immune response. Any specific T cell that is targeted will be naïve. To be activated these naive T cells require higher concentrations of antigen than memory T cells. This presents a considerable challenge for any DNA vaccine as these vaccines are known to produce very low levels of antigen when injected into humans. Our murine vaccination protocols deliver a far higher dose of vaccine per unit mass which may explain higher responses to DNA vaccination when compared to humans.

The vaccination of seropositive individuals presents very different challenges since these volunteers already have high levels of T cells that have already encountered CMV antigens. In the case of the epitope NLVPMVATV of pp65 individuals can have very high levels of effector memory cells in their peripheral blood. These cells should be much more sensitive to the lower levels of antigen produced by DNA vaccination. Vaccination in this setting seeks to boost pre-existing immunity.

Vaccination of CMV-seronegative patient

Donor 1 was the only CMV seronegative donor recruited to this clinical trial. There were no specific NLVPMVATV responses prior to vaccination that could be detected by ELISPOT or tetramer analysis. There was also no specific CD8+ T cell response to this epitope detected by these assays after vaccination. Consequently we stimulated CD8+ lymphocytes from weeks 0, 4, 8 and 16 after vaccination with peptide-pulsed mature DCs. There was no expansion of tetramer-positive CD8+ cells detectable at any time after vaccination.

There are two possible interpretations of this result. Firstly the vaccination has not been successful at eliciting a CD8+ T cell response to NLVPMVATV. Secondly that it has induced

a response but that it was at such a low level that we have not detected it with our assays. We will examine these possibilities in turn.

DNA vaccines are known to be less effective in humans than in mice. This may partly be dose related phenomenon. Both the mass of DNA delivered and the volume it is delivered in are known to be important in vaccination. Mice tend to receive approximately 50-fold greater quantities of DNA in approximately 50-fold higher volumes than our human volunteers. This almost certainly contributes to their lower efficacy in humans. Nonetheless DNA vaccines have been successfully used to induce low level specific immunity in several clinical trials.[297,321-323] However it has generally been observed that higher levels of response have been noted when higher levels of DNA vaccine are administered. In some of the most robust data from clinical trials in DNA vaccination McConkey et al noted that they only achieved responses with higher levels of DNA.[297] Wang et al also vaccinated patients with a range of DNA concentrations. They had evidence of immune responses to only 500μg of DNA but noted more consistent results when higher levels of vaccine were administered.[323] We had decided on a level of 1mg of DNA from our previous vaccine trials in lymphoma. In these trials we found responses to FrC could be detected after vaccination with 500μg of DNA and above. However this was a memory response to a previously encountered antigen and would therefore have required lower levels of antigen expression. Priming an immune response may require higher levels of DNA. An escalating dose of DNA will therefore be considered for continuation of this trial.

Another factor that might have contributed to a lack of NLVPMVATV-specific CD8+ T cell response was the absence of a cellular response to FrC in donor 1. This volunteer had received a full course of tetanus toxoid vaccination as a child and yet cellular responses were not detected prior to DNA vaccination. Nor were there responses after DNA vaccination. Although pDOM.NLV is designed to induce CD8+ responses it contains the first domain of FrC that contains the promiscuous helper epitope p30. This helper epitope is known to be restricted by certain MHC class II alleles. These alleles are expressed by 57.2% of the caucasian population.[210] She does not express any of these alleles. In consequence there were no cellular or antibody responses to the FrC component of the DNA vaccine. It is possible

that this is further evidence that donor 1 has not responded to the FrC component of the DNA vaccine. In the absence of CD4+ help it is likely that the vaccine will be less successful at inducing an antibody response.

The second possibility is that there has been an immune response to the vaccine but that it has been at such a low level that it has not been possible to detect it with the assays used here. As discussed in the introduction (section 5.1.4) the ELISPOT and tetramer assays have been well validated by a number of groups as both specific and sensitive for the detection of low level patient responses. Several groups have shown that this assay is as sensitive as a limited dilution assay.[312-315] However other commentators have suggested that in vitro expansion may be necessary to reveal very low level responses.[320] We have performed in vitro expansion in this one patient but this has not been successful in revealing a small population of responding cells.

Vaccination of CMV-seropositive patients

The responses in the seropositive donor-recipient pairs are more complex. It has been noted that whilst DNA is effective at priming an immune response it is not effective when used as a boosting vaccination in a heterologous prime-boost vaccination strategy.[296,297,324] This is thought to be related to the high level of pre-existing CTL that are able to kill the APC loaded with low amounts of antigen.[324] Only high levels of antigen during this second vaccination can overcome the lack of antigen-loaded presenting cells. This clinical setting could be considered a "virus prime" (the initial CMV infection) followed by a DNA boosting vaccination. DNA may not be the correct strategy in augmenting T cell immunity in this scenario.

An additional problem is that it is not clear whether responses by effector memory CD8+ T cells could be monitored from sampling peripheral blood. T cells specific for the antigen may be recruited to the site of vaccination. This could result in no change in the level of these cells in the peripheral blood or even a drop in their frequency.

Donor 2 was CMV seropositive and was also homozygous for HLA-A2.1. Therefore the levels of CD8+ lymphocytes in his peripheral blood specific for NLVPMVATV were very high. For the first 3 weeks after vaccination the proportion of CD8+ cells that were tetramer positive fell before rising again at week 6. Then the level fluctuated throughout the follow-up period. This drop and rise in level of epitope-specific CD8+ cells may represent normal variation in the number of NLV-specific cells present in the peripheral blood or it may represent migration of specific CTLs from the blood to the tissues in the early period after vaccination. Certainly we have seen similar patterns in response to tetanus toxoid vaccination in some normal volunteers (personal communication Dr Di Genova). These individuals have decreasing numbers of antigen-specific T cells in the peripheral blood in the early weeks after vaccination.

Donor 2, unlike donor 1, had a cellular response to FrC as determined by the ELISPOT assay. This peaks at between weeks 4 and 8 after vaccination. Donor 2 had no detectable antibody response to FrC. However the DNA vaccine encodes only the first domain of FrC (DOM1). The antibody response to this first domain alone increases 3.5-fold between weeks 0 and 16 after vaccination. There are two possible explanations as to why antibody responses to DOM1 increase after vaccination whereas antibody responses to FrC do not. Firstly it is possible that altered folding of the first domain uncovers antigenic determinants. The antibody response may be against these. Alternatively it may be that it is the quantity of antibody response that cannot be detected by an ELISA against the whole of FrC. Here low level responses against determinants in the first domain might be masked by high pre-existing responses against the whole molecule.

The responses from recipient 2 were also of interest. There was a 2 to 3-fold increase in the number of NLVPMVATV-specific CD8+ cells after vaccination as assessed by tetramer analysis and ELISPOT. By day 20 after transplantation NLV-specific cells comprised 11.6% of the total CD8+ population which rose to 19% by week 6. It has been described before that patients who are seropositive for CMV and receive a transplant from a seropositive donor can have large increases in the numbers of NLVPMVATV specific cells. Despite this a reactivation of CMV was detected from the fifth post-transplant week and required

ganciclovir therapy. We cannot discount the possibility that vaccination of the stem cell donor with pDOM.NLV aided the expansion of NLVPMVATV-specific T cells but equally we cannot at this stage provide strong evidence that such a link exists.

Donor 3 was also seropositive for CMV but was heterozygous for HLA-A2.1 and consequently has a much lower level of CD8+ lymphocytes specific for NLVPMVATV. The frequency of these cells slowly rose after vaccination before falling at week 4. After this levels fluctuated. The level of cells responding to NLVPMVATV peptide stimulation in the ELISPOT assay rose over the 44 week follow-up period. Again levels were fluctuant. There seem to be no cellular responses to FrC as determined by ELISPOT assay. This was made difficult to assess because of high levels of background labeling towards the end of the study period. We were only able to follow recipient 3's responses for a few weeks before I left the trial. Therefore it is hard to assess the level of CD8+ T cell responses in their peripheral blood.

In summary it is important to note the different clinical settings in which vaccination has been performed. Following responses in patients who have been previously infected with CMV and those who have not, presents separate challenges. So far only one CMV-seronegative donor has been recruited in the trial and immune responses to the vaccine have not been detected in this individual or the recipient of her transplant. If this lack of response is confirmed in other seronegative donors then we will have to examine ways of improving vaccine delivery to these patients. Increasing the dose of vaccine delivered is one possibility discussed above. Secondly electroporation of the vaccinated muscle is a technique that is undergoing assessment in several groups. This has been effective at priming immune responses and in boosting DNA vaccine responses.[296, 325] The increased transfection of muscle and local inflammation may be able to counterbalance the low levels of antigen expression induced by DNA vaccination alone. Our group has found that mice which receive an initial DNA vaccination followed several weeks later by electroporation have greatly enhanced immune responses.[296] The prime/boost strategy was also effective if electroporation was used at both priming and boosting. It may well be that boosting immune responses with other methods

may be effective. Boosting vaccinations with long peptides and CpG that has been discussed in chapter 4 may be one such strategy.

The pDOM.NLV vaccine may have induced an immune response in the seropositive donors in this current trial. A low level dynamic response may have occurred in the blood of donors 2 and 3 in the early weeks after vaccination. Vaccination may have also contributed to the high frequency of NLV-specific CD8+ cells observed in the blood of recipients 2 and 3 after transplantation.

The ongoing slow recruitment to this trial is a concern. It may well be that this trial may have to be repeated in normal volunteers. This would present the opportunity of optimizing the vaccine delivery using one or several of the methods described above.

6. Concluding remarks and further clinical applications

In order to develop a successful DNA vaccine for use in patients it is valuable to have demonstrated efficacy in pre-clinical models. In addition testing immunological principles in pre-clinical models can inform vaccine design for clinical testing.

Our laboratory had previously demonstrated that when a plasmid encoding a weakly immunogenic tumour specific antigen was injected intramuscularly a poor humoral response was seen. However when a gene encoding a pathogenic protein (FrC) was fused to this tumour sequence high levels of antibody and protective immunity against a tumour challenge were induced.

We then demonstrated that the CD8+ T cell response from animals vaccinated with plasmids encoding whole tumour antigens was ineffective even when these genes were fused to the FrC gene. Three modifications were made to this vaccine design to maximise the CD8+ response. This included reducing the size of the pathogen sequence to include only the first domain of FrC, minimising the tumour antigen sequence to only encode the MHC class I epitope of interest and fusing this epitope sequence to the C terminus of the first domain of FrC. These 3 modifications greatly enhanced the CD8+ T cell response.

In order to generate a vaccine to induce a CD8+ T cell response specific to CEA we turned to the literature for candidate epitopes. Based on murine experiments we chose an H-2K^b epitope from CEA that has been described in several publications: EAQNTTYL. The vaccine design was effective at inducing large numbers of epitope-specific CTL. Similar to our previous findings these CTL were at far higher levels than when the vaccine encoded the whole of CEA, even when this sequence was fused to the whole of FrC.

Surprisingly the CTL specific for EAQNTTYL were not able to lyse tumours expressing CEA, in spite of several reports that cite this peptide as a focus for CD8+ T cell immunity to this antigen.[242,248-250] As discussed in chaper 3 there are several limitations to this previous research on EAQNTTYL. This included long periods of in vitro stimulation without characterisation of the final population of cells. In addition the CTL were not effective in a standard 6-hour chromium release assay and low level lysis over 24 hours was not K^b-restricted. Finally adoptive transfer of this CTL was not effective at protecting against tumour. Therefore it is unlikely that this epitope is presented by these tumour cells in the context of the class I molecule. To determine whether glycosylation was playing a role in post-translational modification of this epitope we tested a vaccine where the fourth position was converted from asparagine to an aspartic acid. However this epitope, EAQDTTYL, was not able to induce CD8+ T cell immunity.

For the second area of study this vaccine design was also able to induce these CTL in mice transgenic for human CEA. Given that all of the evidence from this study points to this epitope not being expressed by the tumours it is difficult to know the significance of this finding. If this epitope is also not expressed by the epithelial cells of the gastro-intestinal tract in these animals then this does not represent breaking tolerance. Their immune system will have never seen this epitope in the context of the MHC class I molecule before and therefore it is effectively a foreign antigen. This meant that we were unable to study tolerance and auto-immunity in this model.

The third part of the study sought to demonstrate efficacy of the vaccine in mice transgenic for the human MHC class I molecule: HLA-A2.1. Using individual epitope-specific DNA vaccines we were able to generate high levels of CD8+ T cells against 4 epitopes studied. However none of these CTL were able to lyse a human tumour line expressing CEA. Two of these CTL were able to induce lysis of a murine tumour line that had been doubly transfected with CEA and the chimeric HLA-A2.1 molecules.

We also highlighted some of the dangers in using analogue peptides to enhance CD8+ T cell responses. In our model mice vaccinated with a vaccine encoding this analogue peptide

demonstrated very little response ex vivo to the wild type peptide. Caution must be used when utilising analogue peptides. A better strategy is to optimise vaccine design to induce higher levels of immunity to weakly immunogenic tumour epitopes.

These findings also illustrate the dangers of using reverse immunogenetics to find potential target epitopes from tumour antigens. In all of the papers identifying the two most popular epitopes from CEA, the H-2K^b epitope EAQNTTYL, and the HLA-A2.1 epitope CAP-1, there are several common features. Firstly both peptides were predicted to bind with high affinity to their respective MHC class I molecules. Secondly subjects were vaccinated against the whole antigen and then splenocytes or PBMCs were stimulated with peptide in vitro. This in vitro stimulation was in the presence of IL-2 and continued for long periods of time (4 to 8 weeks in both cases). The resulting cells were then able to induce low level cytotoxicity of interferon-treated, CEA-expressing tumours. There are several concerns over these studies including the stimulation for very long periods of time in the presence of IL-2 and the numbers of NK cells in the cultures. But in retrospect neither group of studies convincingly demonstrate the presentation of these epitopes by CEA-expressing tumours.

Newer techniques have led to more reliable identification of tumour MHC class I restricted epitopes.[150] The most direct of these is peptide elution directly from the tumour surface. Peptide sequences can then be determined with the help of reverse phase HPLC fractionation and Edman degradation. In this way an HLA-A2.1-restricted epitope of CEA has recently been described. We could study whether this epitope is presented by the tumour expressing the chimeric HLA-A2.1 molecule and the gastro-intestinal cells in the transgenic mice. If it is, then this would enable us to study tolerance and auto-immunity in mice transgenic for both CEA and HLA-A2.1.

In Chapter 4 we explored the pDOM-epitope design in a pre-clinical model using an epitope with established clinical relevance and with none of the issues of tolerance and autoimmunity that would be encountered with a tumour-associated antigen. We chose an epitope known to be presented by HLA-A2.1 cells infected with CMV: NLVPMVATV. This vaccine had the additional advantage that it could be tested in donors of stem cells for allogeneic

transplantation. These individuals have normal immune systems that are not subject to any of the immunosuppressive effects of a tumour load. The vaccine was again able to induce high levels of epitope-specific CD8+ T cells which in this case were able to lyse a cell line that had been infected with a MVA expressing pp65 from CMV. There are clear advantages of this model, where the peptide is known to be processed and presented by target cells, and our vaccine design can be used to induce effective epitope-specific immunity.

To move closer to the clinic we wanted to assess performance of this vaccine in a human in vitro model. To do this we transfected DCs with RNA transcribed from the vaccine. These DCs were able to stimulate autologous CD8+ lymphocytes to expand 50-fold in seven days. In addition this CTL was able to lyse peptide-pulsed targets.

In chapter 5 we demonstrate how we have started a clinical trial of this vaccine in patients. We administered the vaccine to donors of stem cells for transplantation. The immune response to both aspects of the vaccine were studied in these individuals. After transplantation the immune responses were also studied in the sibling transplant recipients. This study presented very different clinical settings in which vaccination has been performed. Following responses in patients who have been previously infected with CMV and those who have not, presents different challenges. In addition these patients receive their transplantation as soon as is clinically possible and this makes recruitment and preparation for entry to the trial very difficult. This may, in part, explain the recruitment problems that we have faced.

Unfortunately we have recruited only one donor who had no pre-existing immunity to CMV where priming of the immune response could be tested. The CMV-seronegative donor 1 and recipient 1 do not seem to have any immune response to either part of the vaccine: the FrC domain or the CMV-epitope. We were not able to expand any population of epitope-specific CD8 T cells with in vitro stimulation. The CMV-seropositive donor 2 had equivocal responses to the NLV epitope but more robust responses from the ELISPOT assay in response to FrC. Recipient 2 had dramatic changes in the number of CD8 T cells that were specific for the NLV-epitope. Using chimeric studies these cells were found to be of donor origin at a time when only 35% of peripheral blood cells were of donor origin. It was uncertain whether

this was solely related to a reactivation of virus or whether this was influenced by vaccination. The third donor has a rise in the number of NLV-specific CD8 T cells over the course of the study as measured by tetramer and ELISPOT. There were no detectable specific antibody responses to FrC in this individual. The second and third donors showed antibody responses to the first domain of FrC

At this early stage in the trial we have started to delineate some of the issues that are central for testing this vaccine in patients. Clearly it needs to be tested in many more individuals before firm conclusions are drawn. To obtain more reliable recruitment, we will start a trial in normal volunteers outside of a transplant setting. This will enable us to recruit larger numbers of volunteers and administer an escalating dose of vaccine.

In addition we would be able to try and enhance the vaccine delivery. We know that the immune response to DNA vaccination is highly dependant on the dose of vaccine and the volume in which it is delivered. We also know that the dose of vaccine relative to body mass is far lower in this study than in the murine studies. This may be compounded by the ratio of injection volume to muscle mass being far lower in humans than in mice.[326] A recent study from our laboratory demonstrated that a suboptimal dose and volume of DNA vaccine was rendered effective by electroporation. This primed higher levels of epitope-specific CD8+ T cells that were able to protect mice from tumour growth.[296]

Further enhancement of the immune response to this epitope could also be induced using a prime-boost vaccination strategy as that described in chapter 4. We combined the vaccine with a long peptide that was effective at inducing an immune response to this epitope. When these vaccines were used in a heterologous prime-boost protocol they were able to induce far higher levels of specific CD8+ T cells than with homologous prime-boost vaccination. In this way low level immune responses could be greatly enhanced.

One of the concerns with this vaccine design is that it only targets a single MHC class I epitope from a virus or tumour. In murine models there are several examples of immunity

against single epitopes that are able protect against tumour or virus.[212,327] However it is less certain whether this principal can be applied to human tumours and viruses that may display higher levels of genetic instability. Some viruses and tumours develop mutations within the epitope sequences and subsequently "escape" elimination by the immune response. In these circumstances CD8+ T cell responses to subdominant epitopes develop, as the immunodominant CTL no longer kill the APCs presenting epitopes from the antigen. If mutation occurs it would be possible to administer vaccines of an identical design against different epitopes to target escape mutants.

A phenomenon that may potentiate the use of vaccines against a single epitope is the phenomenon of epitope spreading. This is seen in both infectious disease and vaccination against cancer. Mice vaccinated against a single epitope from LCMV developed CD8+ responses against a number of different epitopes even in the absence of viral evasion by mutation.[327] In addition patients vaccinated with peptides from a HER-2/neu, a tumour antigen, revealed evidence of epitope spreading. Cellular responses to peptides from HER-2/neu other than those used to vaccinate the patient were seen in 84% of individuals.[328] Therefore targeting a single epitope may generate immunity against a number of epitopes.

Another concern in the cancer setting is that this vaccine design targets only the CD8+ T cell arm of the immune response. As discussed in section 1.4.4 some tumours are able to down-modulate expression of MHC class I molecule which may enable them to avoid detection by CD8+ T cells. The plan will be to administer the vaccine in patients who have had surgery or chemotherapy prior to vaccination and therefore have minimal residual disease. This setting will minimise the possibility of tumour escape via this mechanism. However escape via the down-regulation of MHC class I may be circumvented by other treatment strategies that could activate natural killer cells.

In summary results presented in this thesis confirm that pDOM.epitope vaccination is effective at inducing specific CD8+ T cell immunity in a number of different models. We have highlighted difficulties in the use of reverse immunogenetics in tumour epitope

discovery as CEA-expressing tumours were found not to present several epitopes. As these epitopes may also not be presented by gut cells in CEA-transgenic mice the significance of the generation of CTL to these "CEA-derived epitopes" is uncertain. We have also highlighted potential difficulties in the use of analogue peptides. A pDOM.NLV vaccine against CMV was effective at inducing high levels of CTL specific for this epitope and first steps have been taken to test this vaccine in patients with some provocative results. This trial needs to be expanded to optimise vaccine delivery to humans and reproduce the impressive results from vaccinated mice.

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