# UNIVERSITY OF SOUTHAMPTON

## Inducing Immunity to Haematological Malignancies with DNA Vaccines

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# ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES, CANCER SCIENCES DIVISION

Doctor of Philosophy

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By Jane Katharyn Watkins

Modern treatment regimens are able to bring cancer into remission, but residual disease may persist. One approach to eradicating residual tumour is active vaccination to induce anti-tumour immunity. Vaccination with DNA is an attractive option, as both innate and specific immune responses against defined antigens are activated. Our aim is to develop DNA vaccines against haematological malignancies, with a focus on multiple myeloma (MM), a plasma cell tumour with no known cure.

B cell tumours display an idiotype (Id) derived from immunoglobulin (Ig) variable (V) region. We have shown previously that assembly of V genes as a single chain variable fragment (scFv) format in a DNA vaccine induced weak immune responses. Fusing scFv to a pathogen-derived sequence fragment C (FrC) from tetanus toxin however, promoted Id-specific antibody and T-cell responses and led to protection from a lethal tumour challenge in lymphoma and MM animal models. Validation of this scFv DNA fusion vaccine design has led to current phase I/II clinical trials. Since MM cells are MHC class I positive, there is also an interest in activating cytotoxic $CD8^+$ T cells (CTL). A novel DNA vaccine has emerged in our laboratory, fusing the first domain of FrC (p.DOM) to a MHC class I epitope, which is able to generate potent CTL responses. This design opens up the possibility of attack on intracellular antigens, likely to be presented by MHC class I.

For MM, the cancer testis antigens (CTA) have emerged as tumour-specific intracellular targets. To model DNA fusion vaccine design against CTA, we examined the murine P815 mastocytoma, which expresses the *P1A* gene analogous to human CTA. *P1A* encodes a well-defined MHC class I $H2\text{-}L^d$ motif (AB). A p.DOM-P1A/AB vaccine was constructed and a single vaccination led to detection of activated epitope-specific CTL *ex vivo*, which could be expanded on re-stimulation *in vitro*. These CTL were able to kill P815 tumour cells in an epitope-specific manner. Importantly, in protection experiments approximately 40% of vaccinated mice were protected from tumour challenge. However in some cases there was evidence for immune pressure leading to the growth of antigen loss escape variant tumour cells, highlighting the need to target additional tumour antigen targets.

We also investigated mucin 1 (MUC1), an over-expressed glycoprotein antigen in MM. Early work in our laboratory had shown that vaccination with a DNA vaccine encoding full length human MUC1, either alone or fused to FrC or DOM, could provide low level protection against tumour challenge *in vivo* using wild type mice, with no role for anti-MUC1 antibodies. The protective immunity generated by these vaccines has been re-assessed by lymphocyte depletion, revealing a prominent role for both $CD4^+$ T cells and $CD8^+$ T cells. To improve activation of CTL, two p.DOM.epitope vaccines were constructed incorporating known MUC1 MHC class I epitopes. Reported data suggested that these epitopes could be processed and presented. One p.DOM.epitope vaccine was able to induce potent CTL, compared to vaccines encoding full length MUC1, and these CTL were also able to cross-react and recognise the second epitope. However, there was no killing of $MUC1^+$ tumour cells, revealing limitations in reported epitope presentation.

To evaluate the effect of endogenous expression of human MUC1 on immunity, the MUC1 encoding vaccines were investigated in human MUC1 transgenic (tg) mice. Preliminary data using vaccines encoding full length MUC1 indicated that efficient $CD4^+$ T-cell responses to FrC were generated, indicating that these mice are capable of mounting a normal immune response to foreign antigen. However, these vaccines failed to generate MUC1-specific $CD4^+$ and $CD8^+$ T cells. In contrast, the increased efficiency of the p.DOM.epitope design was able to activate low levels of activated $CD8^+$ T cells in the MUC1 tg model.

These data suggest that both MUC1 and the CTA will be suitable targets for DNA fusion vaccine mediated attack in MM.

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## Authorship declaration

I declare that this thesis represents entirely my work.

This thesis has not been submitted for any other degree.

## Presentations

Parts of this work have been presented at:

- Jane Watkins, Surinder S. Sahota, Jason Rice and Freda K. Stevenson.
  DNA fusion vaccine against a cancer testis antigen provides protection in a murine tumor model: relevance for human multiple myeloma
  Abstract oral presentation (408). *IX*th International Workshop on Mulitple Myeloma, Salamanca, May 2003.

- Freda K. Stevenson, Jason Rice, Jane Watkins, Francesco Forconi, Niklas Zojer, Gianfranco DI Genova, Nigel H. Russell, Christian H. Ottensmeier and Surinder S. Sahota.
  Can we manipulate the immune system to attack myeloma?
  Abstract oral presentation. 4th Annual UK Myeloma Forum Scientific Meeting
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# Abbreviations

| | |
|---|---|
| Ab | antibody |
| ADCC | antibody dependent cellular cytotoxicity |
| AIRE | autoimmune regulator |
| AML | acute myeloid leukaemia |
| APC | antigen presenting cell |
| ATP | adenosine triphosphate |
| β-2M | beta -2 microglobulin |
| BCG | *Bacillus Calmette-Guérin* |
| BCR | B-cell receptor |
| BM | bone marrow |
| CEA | carcino-embryonic antigen |
| CFA | complete Freund's adjuvant |
| CLIP | class-II-associated Ii peptide |
| CLL | chronic lymphocytic leukaemia |
| CML | chronic myeloid leukaemia |
| CMV | cytomegalovirus |
| CR | complete response |
| CTA | cancer testis antigens |
| C-terminal | carboxy terminal |
| CTL | cytotoxic T lymphocyte |
| D | diversity (gene) |
| DAC | 5'-aza 2-deoxycytidine |
| DC | dendritic cell |
| DC-SIGN | DC specific ICAM3 grabbing non integrin |
| DISC | death inducing signalling complex |
| DRiPs | defective ribosomal products |
| DT | diphtheria toxin |
| ER | endoplasmic reticulum |
| ESTs | expressed sequence tags |
| FcR | Fc receptors |
| FL | Follicular lymphoma |
| FrC | Fragment C |
| GC | germinal centre |
| GFP | green fluorescent protein |
| GM-CSF | granulocyte/macrophage colony stimulating factor |
| GvHd | graft versus host disease |
| GvL | graft versus leukaemia |
| HLA | human leukocyte antigen |
| HSPs | heat shock proteins |
| HSV | herpes simplex virus |
| ICAM | intercellular adhesion molecule |
| IFA | incomplete Freunds adjuvant |
| IFN | interferon |
| Ii | invariant chain |
| Ig | immunoglobulin |
| IL | interleukin |
| iNOS | inducible nitric oxide synthase |
| i.p. | intra-peritoneal |
| ISS | immune stimulatory sequences |
| ITAM | tyrosine based activating motifs |

| | |
|---|---|
| ITIM | tyrosine based inhibiting motifs |
| J | joining (gene) |
| KIR | killer immunoglobulin-like receptors |
| KLH | keyhole limpet haemocyanin |
| L | ligand (e.g. CD40L) |
| LCMV | lymphocytic choriomeningitis virus |
| LFA | leukocyte functional antigen |
| LPS | lipopolysaccharide |
| mAb | monoclonal antibody |
| MAGE | melanoma antigen gene |
| MCS | multiple cloning site |
| MECs | medullary epithelial cells |
| MHC | major histocompatibility complex |
| MIP1α | macrophage inflammatory protein-1 alpha |
| MGUS | monoclonal gammopathy of undetermined significance |
| MM | multiple myeloma |
| MR | mannose receptor |
| MUC | mucin |
| MuLV | murine leukaemia virus |
| MVA | modified vaccinia Ankara |
| NK | natural killer cell |
| NO | nitric oxide |
| N-terminal | amino terminal |
| ORF | open reading frame |
| OVA | ovalbumin |
| PAMPs | pathogen associated molecular patterns |
| PBMC | peripheral blood mononuclear cells |
| PLC | peptide loading complex |
| PR | partial response |
| PRR | pattern recognition receptors |
| PVXCP | potato virus X coat protein |
| RAG | recombinase activating gene |
| Rb | retinoblastoma |
| RIP | rat insulin promoter |
| scFv | single chain variable fragment |
| SCID | severe combined immunodeficiency |
| SEREX | serological analysis of cDNA expression |
| SRP | signal recognition particles |
| TAA | tumour associated antigen |
| TAP | transporter associated with antigen processing |
| TCR | T-cell receptor |
| tg | transgenic |
| TGF | transforming growth factor |
| $T_H$ | T helper cell |
| TLR | Toll-like receptor |
| TNF | tumour necrosis factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| TRANCE R | tumour necrosis factor related activation induced cytokine receptor |
| Treg | regulatory T cells |
| TSA | tumour specific antigen |
| V | variable (domain/gene) |
| VNTR | variable number tandem repeats |

# 1 Introduction

There is currently a momentum to find a means of harnessing effective immune responses to attack and eradicate cancer, which has been born from the success of vaccinating against infectious disease. Vaccine design to combat these two areas is not as disparate as it first appears; for example vaccination against infectious organisms has direct relevance in those cancers known to have a viral aetiology, such as hepatitis B in hepatocellular carcinoma[1]. In a similar manner to vaccination against infection, vaccination against cancer requires identification of molecular targets, or tumour antigens which are generally over expressed or unique to tumour cells allowing them to be distinguished by the host immune response. In turn, such tumour antigens need to be delivered to the immune system in a manner that will produce an efficient adaptive immune response and this is indicated in a setting where cancer cell numbers are low, during minimal residual disease. One such delivery vehicle is provided by bacterial plasmid DNA, which when injected into the muscle enters the cell and mimics some aspects of infection. Encoded target proteins are synthesised and presented to cells of both the innate and adaptive immune systems resulting in activation. In practice the opportunity to attack cancer with active vaccination strategies is more difficult in comparison to that for infectious disease with the existence of immunological tolerance and tumour escape mechanisms. Furthermore, preventative vaccination against infectious organisms normally takes place in the absence of the pathogen and in the presence of a healthy immune system. For cancer the circumstances are different and vaccination takes place therapeutically generally in the presence of residual tumour cells and possible immunosuppression. Despite this, strategies to overcome these hurdles are becoming available and the necessary vaccine design optimisations or vaccination protocol modifications require a detailed understanding of how both innate and adaptive immune responses are initiated.

## 1.1 The innate immune system

Infectious micro-organisms can enter the body at various sites although external epithelial layers such as the skin serve as primary barriers to infection. If these barriers are crossed however, infection can occur and disease onset results from the release of toxins and cytolytic effects of the pathogen. This is exacerbated by pathogen replication and spread. Extracellular pathogens spread via the lymphatics and blood, whereas intracellular

pathogens move from one cell to another either by direct transmission or by release and re-infection. One critical role of the innate immune system is to respond immediately to infection and to keep pathogen replication at bay allowing time for the adaptive immune system to become activated.

One of the key early defence mechanisms against extracellular pathogens is the alternative pathway of complement activation, a cascade of enzymatic proteins found in the serum. The complement components (designated by numerals C1-C9 or by letters e.g. Factor D) circulate as proenzymes requiring cleavage and active site exposure before they become functionally active. The alternative complement cascade is initiated by the spontaneous cleavage and conversion of C3 to C3b and C3a. C3b can form covalent bonds with membrane components of host cells or pathogens and can bind to Factor B. Factor B is cleaved by Factor D to yield an active protease, Bb, which remains associated with C3b. Further activation steps are prevented on host cells by complement regulatory proteins. These regulatory proteins are not however present in the membranes of infectious organisms and the C3bBb complex can proceed to convert further C3 proteins, leading not only to amplification of the cascade and opsonisation of C3b on the pathogen surface, but also to cleavage of C5 to C5b and C5a. C5b further binds to C6, C7, C8 and C9 to form the membrane attack complex, a lytic lesion resulting in osmotic lysis of the invading cell. In association with this, C3a and C5a are released and act as inflammatory mediators, recruiting and activating phagocytic cells of the innate immune system, in particular the neutrophils and macrophages.

Monocytes are generated in the BM and leave this site to mature into macrophages in the peripheral tissue where they can be long lived. Neutrophils in contrast, mature in the BM and are released rapidly in response to danger signals. Once they have left the BM they are short lived. Both cell types are recruited to sites of infection by chemotactic agents such as C3a and interleukin (IL)-8. Each cell type expresses a variety of surface receptors, the pattern recognition receptors (PRRs) which allow them to recognise foreign pathogens. Recognition is non-specific; in general, common features that distinguish micro-organisms from mammalian cells are identified (pathogen associated molecular patterns (PAMPs)). These include components of bacterial cell walls, for example lipopolysaccharide (LPS), or intracellular features peculiar, for instance, to bacterial DNA such as hypo-methylated CpG motifs. Examples of PRRs include the Toll-like receptors (TLRs) (Table 1.1), CD14, the mannose receptor (MR) and the macrophage scavenger receptor. Target cell recognition is also efficient when opsonisation has occurred with specific antibody or C3b. Neutrophils

**Table 1.1. TLR and their ligands**

| Receptor | Ligands |
|---|---|
| TLR1 | Tri-acyl lipopeptides |
| TLR2 | Lipoprotein, lipopeptide, petidoglycan, zymosan |
| TLR3 | Double stranded RNA |
| TLR4 | Lipopolysaccharide |
| TLR5 | Flagellin |
| TLR6 | Di-acyl lipopeptides |
| TLR7 | Imidazoquoline (synthetic compound) |
| TLR8 | Unknown |
| TLR9 | DNA CpG motifs |
| TLR10 | Unknown |

Adapted from [2].

and macrophages possess an array of Fc receptors for IgG isotypes as well as a receptor for C3b called CR1. Following recognition, these phagocytes engulf the target cell with membrane, which then buds off inside the cell to form a phagosome. The phagosome fuses with lysosomes and its granules leading to degradation of the ingested material. This is facilitated by the production of cytotoxic oxygen species within the phagolysosome, such as hydrogen peroxide and nitric oxide, as well as the contents of the lysosomal granules such as lysozyme, lactoferrin and defensins. The initial response to infection is then amplified by the release of cytokines such as IL-1, IL-6, IL-8, IL-12 and tumour necrosis factor (TNF) α from the activated phagocytes at the site of infection. Their effects are diverse and include mobilisation of further phagocytes and the initiation of the acute phase response. IL-1, IL-6 and TNFα can induce the synthesis of acute phase proteins from hepatocytes including C-reactive protein, which can bind to LPS of bacterial and fungal cell walls, and mannan binding lectin. Both can lead to opsonisation and activation of complement.

For intracellular pathogens, natural killer (NK) cells serve as an early defence mechanism. NK cells are large granular lymphocytes which express on their surface an array of receptors which recognise phenotypic changes in host cells that are associated with cellular stress. Upon activation, NK cells display spontaneous cytotoxicity via the release of cytotoxic granules, signalling through surface receptors such as Fas ligand (FasL), or through the release of toxic cytokines such as TNFα[3-6]. Their activation is a result of changes in the balance between activating and inhibitory signals received through their surface receptors. The killer immunoglobulin-like receptors (KIRs) recognise human leukocyte antigen (HLA or major histocompatibility complex, MHC) A, B and C resulting in either activating or inhibiting signals depending on the intracellular domain. Those receptors with long intracellular tails contain tyrosine based inhibitory motifs (ITIMs) whereas those with short tails encode tyrosine based activating motifs (ITAMs)[7]. The C-type lectin-like receptors are also classed as either activating or inhibitory. The activating receptors are NKG2C, D and E and the inhibitory receptor is NKG2A. NKG2A, C and E recognise common features of HLA molecules and can therefore survey the changes in their expression. NKG2D recognises the stress induced non-classical HLA molecules MIC-A and MIC-B, commonly expressed on some malignant cells[7, 8]. The natural cytotoxicity receptors (NCRs), NKp30, NKp44 and NKp46 are also activating receptors, but their ligands remain undefined[7].

A final aspect of innate immunity is the promotion of the adaptive immune response through antigen presentation and cytokine production. Of particular importance are

dendritic cells (DC) resident at, or recruited to the site of infection. Their ability to capture antigen and present it in specific ways allows efficient activation of both B and T cells (Section 1.2.3.). DC are the most adept at this process, but the B cells themselves are also capable of this.

## 1.2 The adaptive immune system

Adaptive immunity rests on active interplay between three key players, the B and T lymphocytes for antigen recognition and effector function and the APC which process and present antigen when activated. For B and T cells, antigen recognition is mediated by specific receptors, the B cell receptor (BCR, or immunoglobulin (Ig) or antibody (Ab) when secreted) and the T cell receptor (TCR) respectively. While the BCR can recognise conformational epitopes of antigens, the TCR recognises peptide fragments from the antigen which are presented in association with MHC molecules on the surface of professional APC. Recognition by either antibody or the TCR can lead to cell death and clearance from the body by specific mechanisms. It is now well established that DNA vaccination can induce both effective antibody and T-cell responses in a variety of model system[9].

### 1.2.1 Structural elements: the T cell receptor and thymic selection

The mature T cell pool comprises $CD4^+$ and $CD8^+$ populations, with each cell and its progeny expressing a unique TCR. T cell progenitors leave the bone marrow (BM) at an early stage and migrate to the thymus where they are termed thymocytes. $CD4^+$ and $CD8^+$ T cells have distinct functions, and their lineage, along with their TCR specificity, are determined during their development in the thymus. The thymus comprises a cortex which is densely populated with immature thymocytes and a medulla which contains fewer mature T lymphocytes. Thymocytes migrate through the cortex and undergo TCR rearrangement and education, they then enter the medulla before leaving the thymus to circulate.

The TCR consists of two polypeptide chains, $\alpha$ and $\beta$ (or in some cases $\gamma$ and $\delta$) which dimerise at a 1:1 ratio through disulphide bonds. Each chain forms a variable (V) domain distal to the membrane, which functions in recognising antigen presented as peptide/MHC complexes, atached to a constant domain proximal to the membrane, a transmembrane domain and a cytoplasmic tail (Figure 1.1). The TCR is expressed on mature T cells in association with the CD3 complex and either the CD4 or CD8 co-receptor. Each TCR

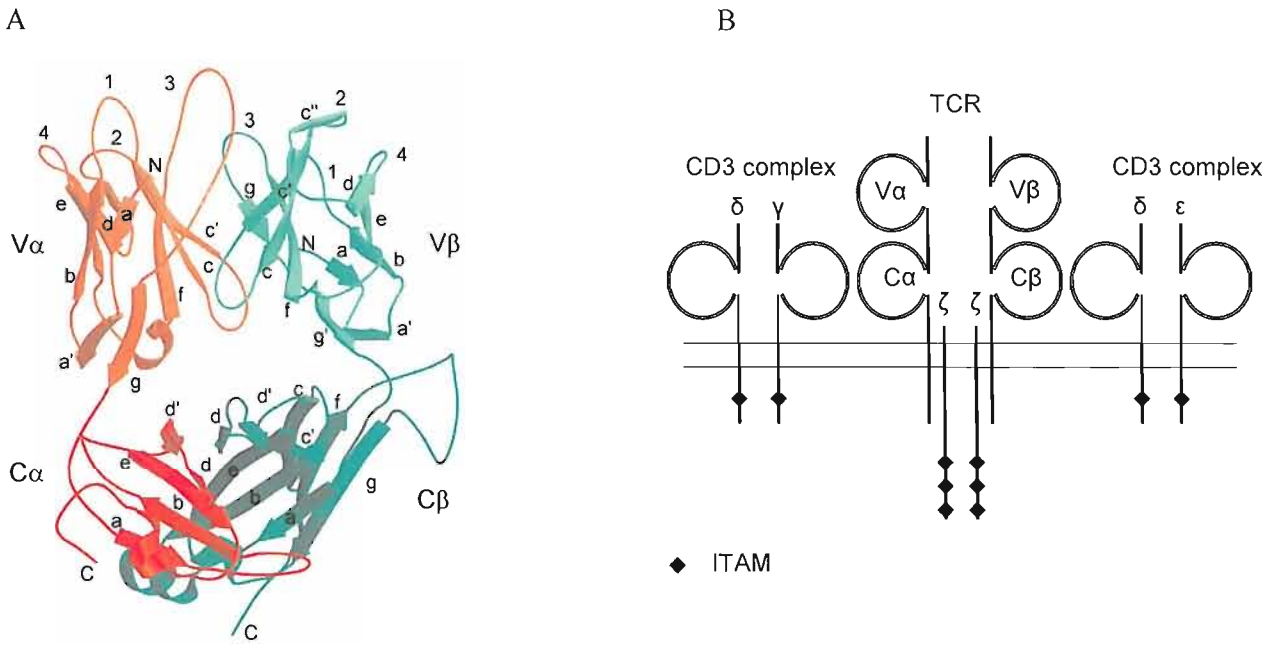



**Figure 1.1. The structure of a human αβ T cell receptor**

A) The ribbon diagram illustrates a human αβ T cell receptor[10]. B) The TCR comprises two polypeptide chains, α and β, or γ and δ. The variable (V) domains of each chain form the antigen recognition site for peptide/MHC complexes. The constant (C) domains are linked to the transmembrane domain and the cytoplasmic tail. The T cell receptor associates with the CD3 complex at the cell surface. Activating signals are delivered to the cell upon TCR recognition of peptide/MHC complex through ITAM motifs found on the cytoplasmic tails of the δ, ε, γ and ζ subunits of the CD3 complex.

polypeptide chain is generated by the rearrangement of a large number of germline gene segments (mapping to chromosome 7 and 14 in humans) by V(D)J recombination. This mechanism confers a vast diversity of TCR molecules since any variable (V), diversity (D) and joining (J) gene segments can be recombined, apparently randomly by the action of the recombinase activating gene (RAG) proteins[11]. The V(D)J functional exon will supply the variable domain of the polypeptide chain, once spliced with the constant domain exon following transcription. Additional diversity is achieved through imprecise joining of the gene segments. In early thymocytes, the β chain is rearranged first, via $D_\beta$ rearranging to $J_\beta$ before $V_\beta$ rearranges to $DJ_\beta$. The newly translated β chain proceeds to assemble at the cell surface with a surrogate α chain and the CD3 complex to form the pre-TCR. The α chain subsequently undergoes multiple rearrangements from available V and J segments (there are no $D_\alpha$ gene segments) of which any can bind the β chain allowing the complete formation of the αβ antigen receptor[12, 13]. At this point both CD4 and CD8 co-receptors are expressed and the thymocytes are termed 'double positive'. The TCR is therefore a highly diverse structure allowing a large number of different peptide/MHC complexes to be recognised. The TCR are required to recognise foreign peptides in association with self MHC and distinguish these from self peptides associated with self MHC. One mechanism by which this is accomplished is through positive and negative selection in the thymus. The thymic stromal epithelium as well as BM derived interdigitating DC and macrophages play a vital role in presenting self peptides in association with self MHC class I and II to developing thymocytes in the cortex[14]. Thymocytes with a non-functional TCR are unable to recognise self peptide/MHC, and unable to receive antigen mediated signals to develop, undergo programmed cell death. In contrast thymocytes with a functional TCR able to recognise self peptide/MHC may have two fates depending on the affinity with which they bind. Binding with high affinity posees a risk of autoimmunity in the periphery and these cells are deleted (negatively selected), whereas those cells whose TCR binds with a low affinity are not deleted and undergo positive selection[15]. Self antigen expressed in thymic medullary epithelial cells (MECs) is not restricted by lineage specificity, allowing central tolerance to be generated to a large number of peripherally expressed self antigens. It has recently been shown that a protein called autoimmune regulator (Aire) is expressed in MECs, and mice with Aire deficiency harbour defects in induction of tolerance[16]. It has been further shown that Aire deficiency does not affect the differentiation or antigen presenting capacity of MECs, but instead regulates gene expression. It is estimated that the number of genes activated to varying levels by Aire is in the range of 200-1200[16], large numbers of which have expression patterns that are otherwise peripherally restricted. Some

of the antigens regulated by Aire are also expressed in tissues targeted by immune attack in AIRE deficient patients[16]. Aire deficiency has also been shown to have direct effects on negative selection of clonal T cells in transgenic mouse models[17]. During negative and positive selection, the decision for co-receptor expression is made with loss of either CD4 or CD8 from the T cell surface. The mechanism by which this occurs is still unclear but most likely depends on the strength of signal received through the TCR and co-receptor[12]. T cells subsequently leave the thymus for recirculation in a mature but naïve state. Before they can perform any effector functions, they need to receive activation signals in the secondary lymphoid organs through interactions with APC.

### 1.2.2 Structural elements: the major histocompatibility complex and recognition by the T cell receptor

There are two classes of MHC molecules, MHC class I which bind and present peptides to $CD8^+$ T cells and MHC class II which bind and present peptides to $CD4^+$ T cells. MHC class I molecules are expressed on most nucleated cells of the body and consist of a large α chain and a smaller non-covalently linked polypeptide beta -2 microglobulin ($\beta_2M$). The α chain forms three domains, α1 and α2 which are distal to the membrane, and α3 which extends through the surface membrane (Figure 1.2). The α1 and α2 domains form the antigen/peptide binding groove which consists of two α helices lying across a platform generated by β sheet. This groove can typically accommodate peptides of 8-10 amino acids. In contrast MHC class II expression is mainly limited to cells of the haematopoietic system such as DC, B cells and macrophages. It consists of 2 polypeptide chains, α and β, each of which forms two domains; α2 and β2 are proximal to the membrane while α1 and β1 form the antigen binding groove distal to the membrane. This groove differs to that seen in MHC class I molecules as the ends of the groove are more flexible, allowing peptides of a longer length to bind, typically in the region of 12-20 amino acids.

MHC molecules present peptides from a plethora of protein antigens and a high degree of genetic polymorphism has evolved to achieve diversity of presentation. The HLA complex on chromosome 6 encodes 3 different types of classical HLA class I molecule, A, B and C as well as 3 different HLA class II molecules DP, DQ and DR. The murine genome displays a similar set of genes where 3 MHC class I molecules exist, H-2 D, K and L together with 2 MHC class II molecules, A and E. Between individuals there exist many different allelic HLA variants, often associated with genetic changes in the regions encoding the antigen binding groove and the surrounding α helices. In many cases, both

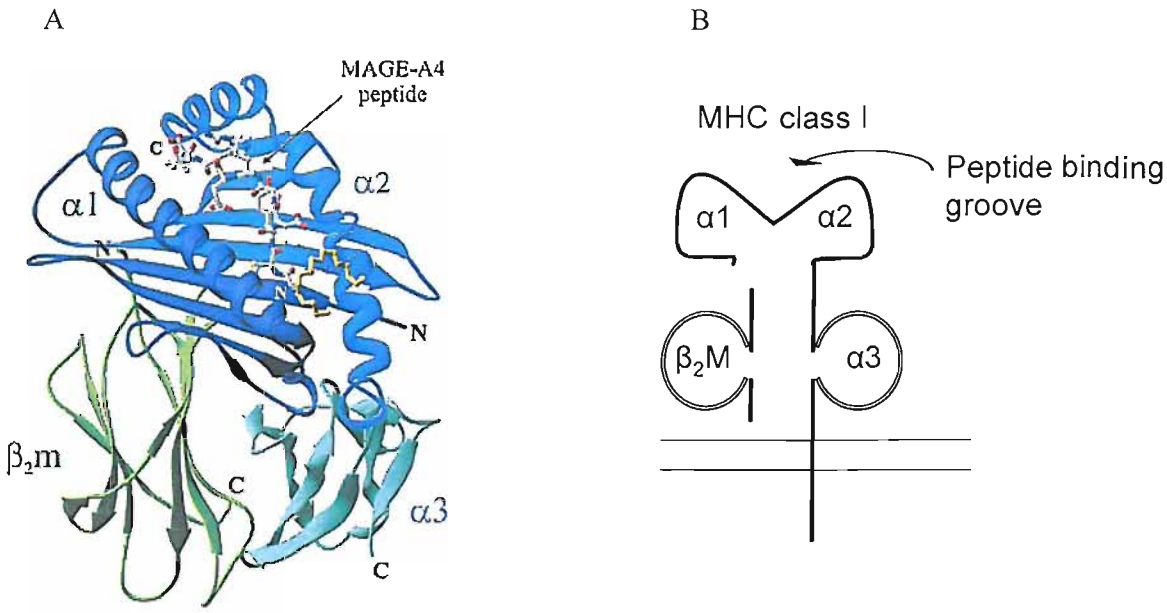



**Figure 1.2. The structure of human MHC class I with MAGE A4 peptide bound**

A) The ribbon diagram illustrates HLA-A2 with a MAGE A4 peptide bound[18]. B) MHC class I comprises one large polypeptide chain which forms three domains, α1, α2 and α3. This chain associates non-covalently with $\beta_2$M. α1 and α2 form the peptide binding groove which can accommodate peptides of approximately 8-10 amino acids.

chromosomes will express a different allelic variant and the individual will be heterozygous, effectively doubling the number of HLA variants expressed on the surface of their cells. In inbred strains of mice however, no allelic variation exists. MHC variation is important because specificity for peptide is partly dictated by the structure of the peptide binding groove. Peptides binding to certain MHC class I variants display similar amino acids at 2-3 sites in the peptide sequence (motif), commonly at or near the N- and C-terminus, and these amino acids bind in pockets along the MHC binding groove. The other amino acids in the sequence can vary to a degree and are generally exposed for recognition by TCR. These sequence motifs allow a certain amount of prediction of which peptides in a given antigen sequence are likely to bind to a given MHC class I allele (Table 1.2). However, there are also important examples of non-motif based peptides presented as post-translationally modified peptides which cannot be predicted[19]. Conversely there are occasions where there is notable inability of peptides with a high predicted value to bind to MHC molecules[20]. Peptides binding to MHC class II molecules are more difficult to predict due to the flexibility in the requirement for N- and C- terminal anchor residues for binding and the ability of this class of MHC to bind longer peptides. Nevertheless there exist web-based *in silico* search engines, such as www.syfpeithi.de, which accumulate reported peptide sequences which have been found to be expressed on both MHC class I and II molecules. These can be used with caution to predict potential peptide sequences in a given protein, as well as provide information on MHC allelic motifs.

### 1.2.3 The antigen presenting cell

DC originate from $CD34^+$ stem cell progenitors in the bone marrow and are the most efficient antigen presenting cells for T-cell activation. DC are highly specialised for monitoring peripheral tissues and play an important role in connecting innate and adaptive immunity. It is now clear that following DNA vaccination at the muscle site, the myocyte becomes directly transfected and can synthesise the encoded antigen endogenously[21, 22]. DC may also be directly transfected to a low level[23] but can also capture antigen secreted from myocytes[24] through phagocytosis of dead or dying cells, fluid phase macro-pinocytosis and receptor mediated endocytosis. Receptor mediated endocytosis is mediated by PRRs that are expressed at high levels on immature DC, including the mannose receptor, DEC-205 and DC specific ICAM3 grabbing non integrin (DC-SIGN)[25]. DC also express Fc receptors (FcR) which can bind to antibody-antigen complexes and mediate their internalisation[26].

**Table 1.2. Examples of peptide motifs**

| MHC allele | Preferred anchor residues | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| HLA-A2 | | L<br>M | | | | *V* | | | V<br>L |
| H-2D[b] | | | | | N | | | | M<br>I<br>L |
| H-2K[b] | | | *Y* | | F<br>Y | | | L<br>M<br>I<br>V | |
| H-2L[d] | | P<br>S | | | | | | | F<br>L<br>M |

The position and favourable amino acid usage for common human and mouse MHC class I alleles.

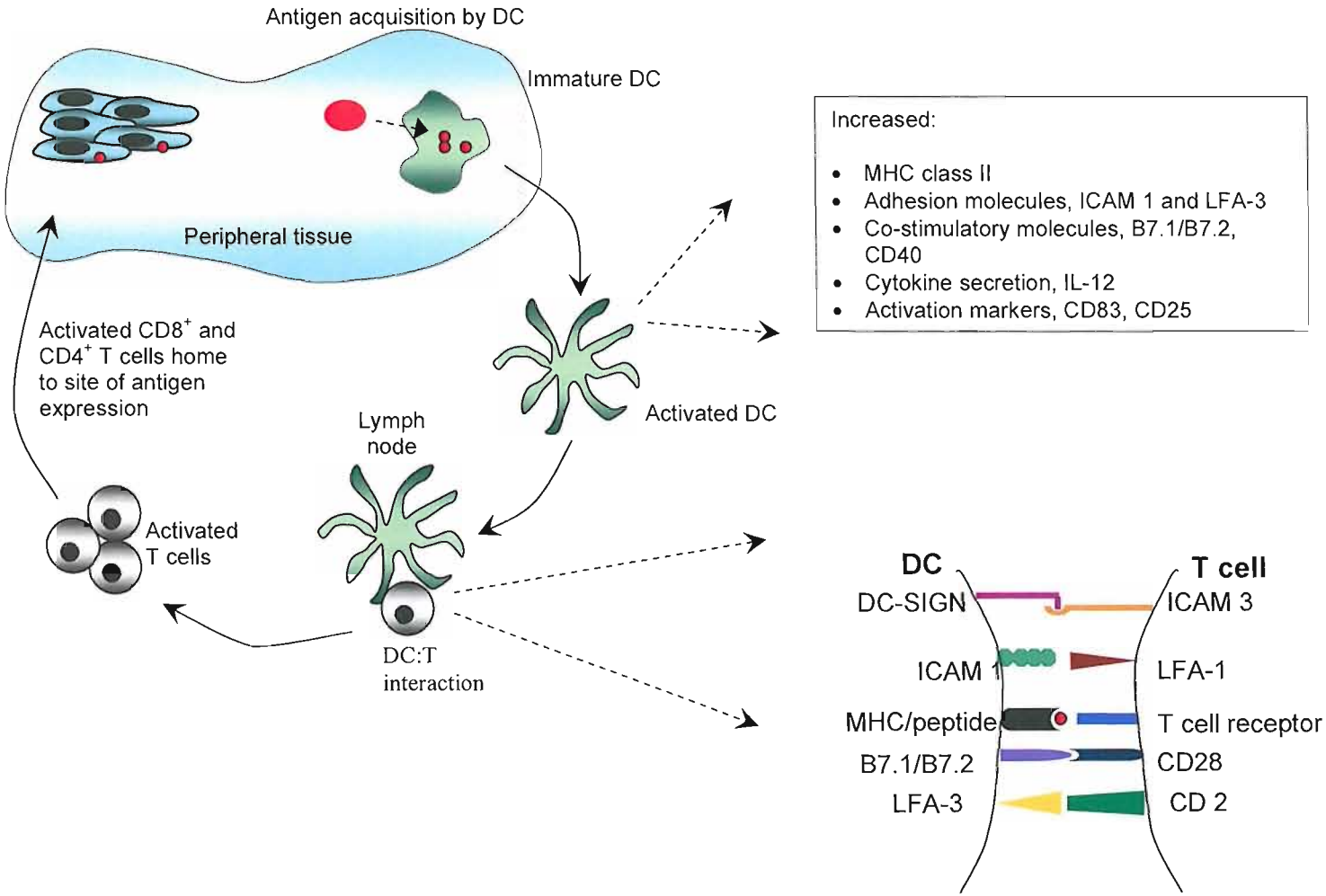
*Underlined italics* indicate auxiliary anchor positions

For a T-cell response to ensue, DC must present at its surface, peptide fragments from endogenous or internalised antigen in complex with MHC class I and II molecules. The DC must also undergo maturation which not only allows the DC to migrate out of the peripheral tissues and home to the draining lymph nodes for T cell interaction but also provides co-stimulatory activation signals to T cells recognising presented peptides (Figure 1.3). DC maturation signals are provided from different sources including infectious micro-organisms[27]. Further activation signals are provided from local release of pro-inflammatory cytokines such as interleukin 1(IL-1), granulocyte/macrophage colony stimulating factor (GM-CSF) or TNFα[27]. DC survival is augmented by receptor-ligand interactions via CD40 and tumour necrosis factor-related activation induced cytokine receptor (TRANCE R)[28], which cascade signals for resistance to apoptosis. Mature DC reveal increased levels of the chemokine receptor CCR7, adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and leukocyte functional antigen-3 (LFA-3) which facilitate DC migration to the lymph node and subsequent adhesion to and egress through high endothelial venules[27, 29]. From here the DC can home to the T cell areas to initiate T cell activation (Section 1.2.4).

### 1.2.3.1 Processing antigen for presentation as MHC class I peptides

Tumour antigen-specific cytotoxic $CD8^+$ T cells (CTL) will be a critical component of an immune response against cancer cells initiated by DNA vaccines. Therefore antigen derived peptide fragments must reach the surface of the APC in complex with an MHC class I molecule. One way this is achieved is following direct transfection of DC with DNA, where the encoded antigen can be synthesised endogenously. A large proportion of MHC class I associated peptides are derived from endogenous proteins targeted for degradation by the ubiquitination-proteasome pathway. These include defective ribosomal products (DRiPs) that arise through mistakes in the translational process[30]. The 26S proteasome is the major source of cytosolic proteolytic degradation and has a 20S catalytic core formed from 2 outer rings, each made up of 7 distinct α subunits, and 2 inner rings each formed from 7 distinct β subunits. The four rings form a cylindrical structure which associates with 19S cap proteins at either end with specificity for ubiquitin[31, 32]. The proteolytic activity is attributed to 3 β subunits, β5 (X), β1 (Y) and β2 (Z) which possess a broad range of substrate specificities. Exposure of cells, in particular APC, to interferon (IFN) γ results in an increased expression of IFNγ inducible proteins which are associated with MHC class I processing, including three proteasome subunits LMP, LMP7 and MECL-1. These subunits replace X, Y and Z

**Figure 1.3. Antigen presenting cells capture antigen and present peptide to T cells**
Figure adapted from[27]



subunits respectively to form the immunoproteasome, and confer distinct proteolytic specificity on the proteasome, notably the increased cleavage downstream of basic and/or hydrophobic residues[32]. This is in line with MHC specificity for carboxy terminal (C-terminal) hydrophobic or basic residues (human) and hydrophobic C-terminals (mouse). The importance of the proteasome and immunoproteasome in generating MHC class I peptides is illustrated by proteasome inhibition studies[33]. The exact nature of the proteasome peptide products are variable, and whilst some peptides produced will fulfil exact binding requirements for MHC class I, it is also known that some peptide products serve as MHC class I precursor peptides which need further trimming. Evidence for both cytosolic and endoplasmic reticulum (ER) amino peptidases exists[34], although their identity is not fully defined. It is apparent that carboxy peptidases do not play a role in trimming peptides for MHC class I presentation within the ER[35]. From these data it has been proposed that proteasome cleavage generates the correct C-terminus of the peptide, but may leave amino terminal (N-terminal) extensions.

Once peptides have been generated they need to access the ER where newly formed MHC class I molecules are being synthesised. Transporter associated with antigen processing (TAP) is an adenosine triphosphate (ATP) dependent pump that is responsible for transporting peptides across the ER membrane. TAP has a preference for transporting peptides typically in the region of 8-16aa, and exhibits some specificity for the C-terminal residue. Like peptides generated by the proteasome (and immunoproteasome) for binding MHC class I molecules, there appears to be a preference for transporting peptides with hydrophobic or basic residues at the C-terminus[36]. Folding of nascent MHC class I heavy chains and association with peptides requires ER resident chaperones (Figure 1.4). The heavy chain is proposed to initially associate with calnexin, and following binding of $\beta_2$m, calnexin is exchanged for calreticulin. The oxido-reductase ERp57 is also required to ensure correct disulphide bond formation within the MHC class I heavy chain[37]. The MHC class I/calreticulin/ERp57 complex is subsequently bound by tapasin and recruited to the TAP heterodimer to form a peptide loading complex (PLC)[37, 38]. Once peptides have traversed the ER membrane they are ideally placed for binding MHC class I molecules in the PLC. Peptide binding by MHC class I promotes dissociation from TAP, allowing the MHC class I/peptide complex to exit the ER and translocate via the Golgi to the cell surface membrane. During MHC class I egress, tapasin plays a role in peptide optimisation through exchange for higher affinity peptides[39] resulting in increased MHC class I stability at the cell surface for TCR recognition.

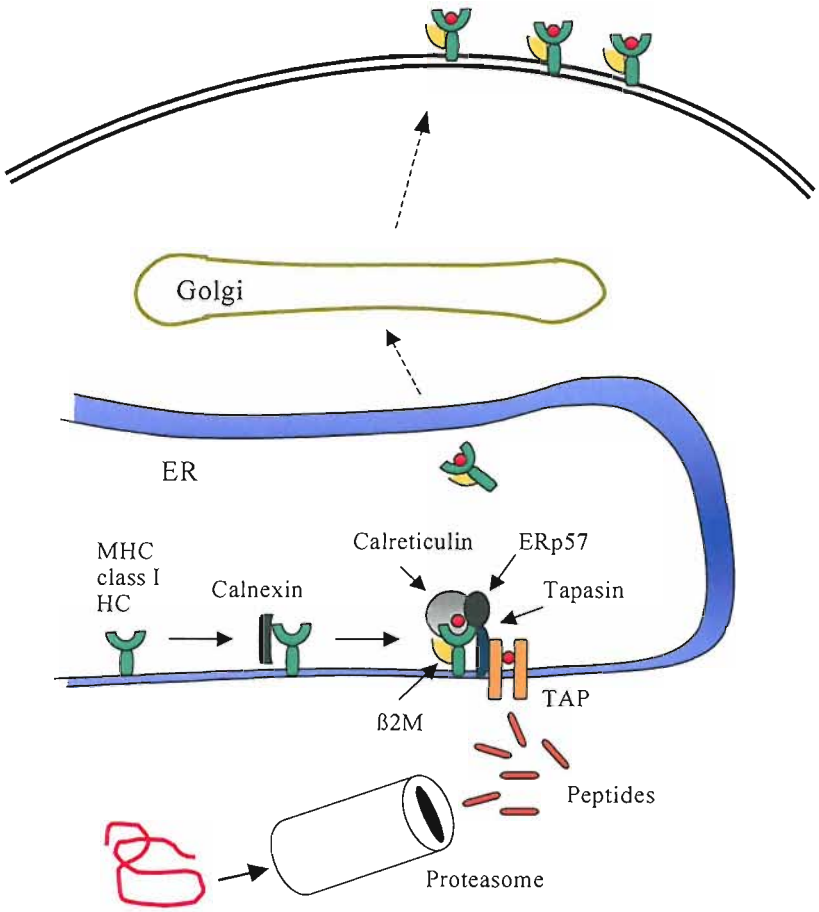



**Figure 1.4. Antigen processing for MHC class I presentation**

Diagrammatic representation of antigen processing for MHC class I presenation[38]. Protein degradation commonly takes place within the cytosol through the action of the proteasome. Peptides generated are transported to the ER via TAP heterodimer in an ATP dependent manner. Once inside the ER peptides associate with newly synthesised MHC class I molecules within the peptide loading complex. Peptide loaded MHC class I molecules are then transported to the cell surface.

An alternative source of peptides is available for binding MHC class I molecules in the ER which bypasses proteasomal cleavage and TAP transport. Here, N-terminal protein signal sequences direct entry of the nascent protein into the ER via signal recognition particles (SRP) and the Sec61p translocon[40]. TAP independent peptides can be generated from the signal sequence which is cleaved prior to protein export. C-terminal sequences can also be efficiently cleaved in the ER[41-43]. It should be noted however that proteins reaching the ER in a misfolded state are likely to undergo retrograde translocation to the cytosol for proteasomal cleavage[44]. Peptides generated this way will be TAP dependent.

### 1.2.3.2 Processing antigen for presentation as MHC class II peptides

In contrast to peptide processing for MHC class I processing, the source of peptides for MHC class II presentation is exogenous. Internalised antigen can be taken up into endosomes or phagosomes, before moving through the endo-lysosomal or phago-lysosomal pathway. Proteins moving through this pathway are exposed to increasingly low pH as well as many acid dependent hydrolases including proteolytic enzymes such as cathepsins, which include both cysteine proteases and aspartic proteases[45]. The end result is the generation of peptide fragments that may be suitable to bind recently synthesised MHC class II molecules arriving from the ER. MHC class II α and β chains are synthesised in the ER where they associate with the trimeric invariant chain (Ii) complex to form a nonameric structure[46, 47]. Ii serves to promote MHC class II assembly and contains within its sequence CLIP (class-II-associated Ii peptide) which binds in the MHC class II groove and prevents ER derived peptides from binding. The Ii also contains a targeting motif in its cytoplasmic domain that directs the transport of MHC class II through the Golgi and trans-golgi network to the endo-lysosomes or phago-lysosomes[47]. The Ii chain is gradually degraded to leave the CLIP fragment bound in the groove which is subsequently replaced by antigenic peptides (Figure 1.5). MHC class II:Ii complexes can be found, if only transiently, in various (early and late) compartments along the endo-lysosomal pathway (termed MIICs) but the identity of the intracellular compartment where Ii degradation and CLIP replacement occurs is still not fully elucidated. There is some evidence that peptide loading takes place in a specialised compartment, CIIV, which are enriched in MHC class II molecules and are distinct from classical endosomes and lysosomes. It appears that at least partial degradation of MHC class II occurs before reaching this compartment[48]. However, there is also evidence to suggest peptide loading occurs in lysosomal MIICs and that non-lysosomal CIIVs are responsible for delivering the peptide:MHC class II complex to the cell surface[49]. In either

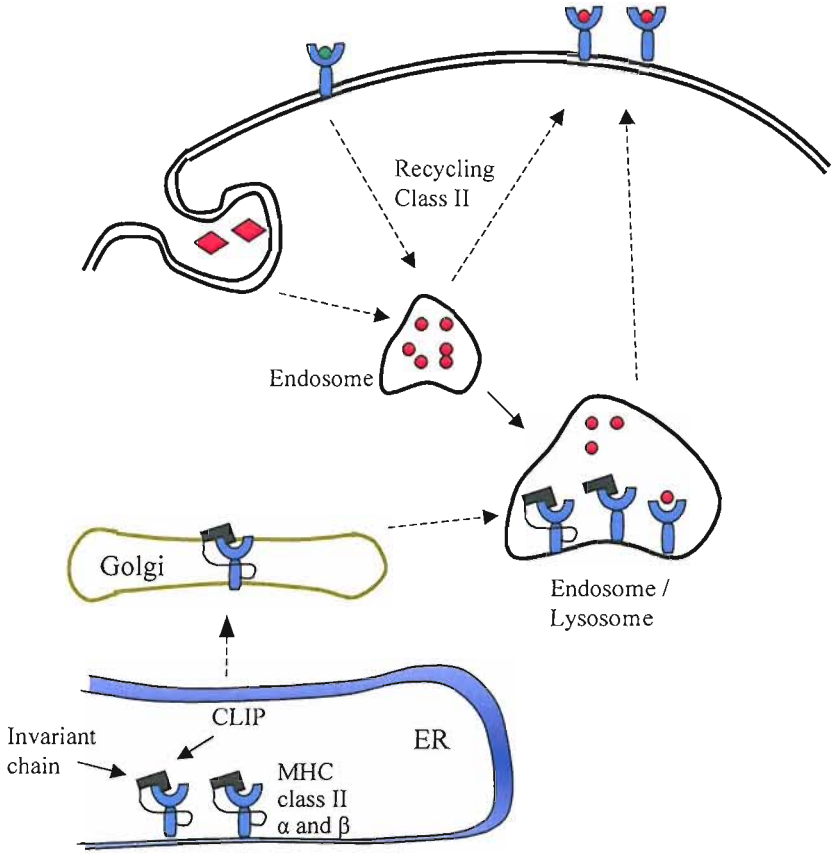



**Figure 1.5. Antigen processing for MHC class II presentation**

Diagrammatic representation of antigen processing for MHC class II presentation[47]. Exogenous antigen is internalised into endosomal compartments where the protein is degraded by an array of proteolytic enzymes in acidic conditions. MHC class II molecules synthesised in the ER associate with the Ii trimer which promotes MHC class II transport through the Golgi to the endosomal compartments. Ii is subsequently degraded and HLA-DM (or H-2M in the mouse) catalyses the exchange of CLIP for antigenic peptides. MHC class II may also recycle from the cell surface for HLA-DM independent peptide exchange in endosomes.

case, CLIP replacement requires HLA-DM (or H2-M in the mouse). HLA-DM is an MHC class II-like molecule that catalyses the replacement of CLIP with peptides derived from exogenous material. HLA-DM can also serve as a peptide editor by promoting exchange of bound peptide for higher affinity replacements[50, 51]. An alternative pathway for peptide binding to MHC class II molecules is achieved by MHC class II 'recycling' from the cell surface during endocytosis or phagocytosis with subsequent peptide exchange occurring in early vesicles both dependently and independently of HLA-DM[47, 52]. The details of this pathway are less well understood and its relative contribution to peptide presentation on MHC class II may vary depending on the antigen or peptide and the degree of protease activity within a given APC type[53, 54].

### 1.2.3.3 Antigen cross presentation

The majority of peptides that associate with MHC class I on APC are sourced endogenously, however it has long been proposed that the immune system must have developed a mechanism whereby APC can present exogenous antigen via the MHC class I pathway. This would allow the immune system to detect tissue tropic micro-organisms that infect non-haematological cells. If such a mechanism exists, it would also provide a route for tumour specific antigens to reach the APC and allow anti-tumour immune responses to be initiated. The first clue that such a mechanism existed came from the Bevan laboratory, where it was demonstrated that injection of H-$2^b$ splenocytes into $F_1$ H-$2^{d/b}$ mice induced CTL activity against minor histocompatibility antigens presented by both H-$2^b$ and H-$2^d$ target cells but were not cross-reactive[55]. This phenomenon has been termed cross-priming whereby an exogenous antigen gains access to the MHC class I processing and presentation pathway and activates $CD8^+$ T cells. This mode of antigen processing and presentation has now been substantiated in several models, but its contribution to the induction of immune responses remains controversial[56].

The RIP mOVA murine model is commonly used to investigate aspects of cross-priming. In these transgenic mice, ovalbumin (OVA) is expressed in the pancreatic islet β cells under the control of the rat insulin promotor. OVA contains a well defined MHC class I epitope (SIINFEKL) for which a specific TCR has been sequenced and cloned and used to generate a TCR transgenic (tg) mouse (OT-1). Using this system, OVA specific OT-1 $CD8^+$ T cells have been shown to become activated following adoptive transfer through cross-priming mechanisms[57]. Irradiation and bone marrow reconstitution experiments have demonstrated the role of host bone marrow derived APC in this process[57, 58]. To validate this, virally

encoded OVA minigene (SIINFEKL) has been fused to green fluorescent protein (GFP). Following vaccination, a direct *ex vivo* observation of OT-1 cells clustering with both GFP positive APC and also with GFP negative APC in the draining lymph nodes was noted[59]. Importantly, the process of cross presentation of exogenous antigen has also been indicated to take place during DNA vaccination[24].

Investigation in this area has also focused on the mechanism of antigen transfer or capture and how antigen reaches the MHC class I pathway. A likely mechanism by which antigen is acquired for cross presentation by professional APC stems from their ability to capture fragments from dead or dying cells during phagocytosis or macropinocytosis during normal monitoring of tissues. Both routes have been demonstrated *in vitro*[60, 61]. DC have also been shown to capture fluorescently labelled plasma membrane with cytosolic proteins from live T cells, B cells and macrophages *in vitro*[62]. Live tumour cells, both murine and human, release 'exosomes' which are membrane vesicles derived from the endosomal compartments containing a discrete set of proteins. Exosomes are immunogenic *in vivo* and their protein components can be cross presented *in vitro*[63]. Another potential mechanism of antigen transfer is through molecular chaperones, of which the heat shock proteins (HSPs) are the best defined. HSPs are released from their intracellular location during cellular stress, e.g. necrosis, and have been shown to associate with cell derived peptides. A receptor for HSP recognition is present on APC (CD91) for endosomal uptake, and exogenous material may gain access to the APC MHC class I pathway in this manner[64]. APC express FcR which bind to antibody constant regions permitting uptake of antibody associated, exogenous antigen as demonstrated *in vitro*[65].

Once antigen has been acquired by the APC, the route taken to reach the MHC class I processing pathway may vary. In some cases there is a dependence for proteasome and TAP suggesting a phagosome/endosome to cytosol route[66]. From here the antigen follows the normal MHC class I pathway of presentation as described earlier (Section 1.2.3.1). However, the details of this pathway remain unresolved and some uncertainty exists regarding overloading of APC with antigen which would result in disruption of the endocytic vesicles. Recently, a plausible mechanism dependent on both TAP and proteasome has been described. Antigen coated beads were shown to be taken up by i*n vitro* cultured DC or macrophages into phagosomal compartments. At the point of phagocytic cup formation, ER membrane components were found associated with the phagosome and it was further demonstrated that ER-phagosome fusion had occurred [67-69]. Several components associated with MHC class I processing were found residing within the

phagosomes within a few hours including TAP, tapasin, MHC class I heavy chain and calreticulin[67, 69]. Additionally, components of the proteasome were also found to associate with the cytosolic side of the phagosome/ER fusion products. Proteasome and TAP inhibiton studies demonstrated that proteins are shunted from the phagosome/ER to the local proteasomes and then back again in a TAP dependent fashion[68]. The peptide loading complex was shown to assemble correctly and peptide/MHC class I complexes were delivered to the cell surface[70]. In conclusion the phagosome/ER vesicle is sufficient for cross presentation. In other cases TAP independent cross presentation has been reported[71]. In support of this, MHC molecules contain an endosomal targeting signal in their cytoplasmic domain, which may allow both recycling from the plasma membrane and interaction with antigen in the endosomal compartment, or alternatively MHC molecules may be directed from the ER to the endo-lysosomal pathway[72].

The subset of APC responsible for cross presentation and priming T cells may also impact on the type of antigen taken up, and the ability of the cell to process and present peptides on MHC class I, and their ability to activate $CD8^+$ T cells. There is evidence to suggest that the $CD8\alpha^+$ DC[73, 74] is the APC subtype with enhanced capability for cross presentation and T-cell activation but this is not discussed further here.

Despite the accumulating evidence to indicate cross-presentation is a viable route for priming an immune response, the physiological input of this process has been called into question. Ochsenbein *et al.*[75] utilised murine tumour cell lines infected with lymphocytic chorimeningitis (LCMV) and demonstrated that when injected as a subcutaneous fragment no detectable CTL were induced to the MHC class I gp33 epitope and no tumour regressions were observed. However, when the transfected tumour cells were injected as single cell suspensions CTL were generated and the tumours were resolved. CTL responses were restricted to the MHC haplotype expressed by the tumour cells, however LCMV was shown to be presented on host APC in context with MHC class II. The conclusions from this report suggest that tumour cells reaching the lymph nodes as single cells can directly prime $CD8^+$ T cells even when they do not express co-stimulatory molecules. $CD4^+$ T cells were critical for direct priming and in this regard the APC may be acting as a bystander cell providing co-stimulation after receiving $CD4^+$ T-cell help[75, 76]. Wolkers *et al.*[77] have also transfected murine tumour cells with known MHC class I epitopes and GFP and demonstrated their ability to reach lymph nodes and interact with epitope-specific $CD8^+$ T cells. Injection of tumours into $TAP^{-/-}$ deficient mice did not affect the efficient induction of $CD8^+$ T-cell responses again suggesting a prominent role for direct priming[77].

### 1.2.4 Activating T cells

#### 1.2.4.1 Early adhesion events

Naïve $CD4^+$ and $CD8^+$ T-cells encounter antigen in secondary lymphoid organs presented by DC through direct and cross-presentation mechanisms. To achieve T-cell activation, a multitude of signals may be required and is initiated through formation of an immunological synapse. Initial interactions between T cells and DC involve adhesion molecules on both cell types resulting in transient connections, allowing the T cell to scan the surface of the DC for MHC/peptide complexes that may be recognised. The primary adhesion event involves DC-SIGN expressed on DC binding to ICAM3 on T cells. The importance of this interaction is indicated by evidence showing that a block in DC-SIGN-ICAM3 interaction can result in up to 60% reduction in DC induced T-cell proliferation[78]. Subsequent interactions involve ICAM1 on DC binding to LFA-1 on the T cell[79]. The increased strength of interaction through adhesion molecules potentiates increased strength of TCR binding to MHC/peptide, which in turn leads to increased avidity of LFA-1 and CD2 expressed on the DC for ICAM1 and LFA-3 on T cells (Figure 1.3)[29]. The function of multiple adhesion events is thought to provide optimal activation signals via intracellular signalling cascades to the T cell, promoting full maturation.

#### 1.2.4.2 Co-stimulation

It has traditionally been thought that T-cells receiving signal 1 alone through the TCR are induced into a state of ignorance or tolerance or undergo programmed cell death. For full activation a second co-stimulatory signal is required and can only be provided by professional APC. However, more recent evidence indicates that this may be more complex and that the requirement for a second signal is more quantitative, and may also be peptide dependent to some degree. Oh, S *et al.*[80] have demonstrated that an increase in signal 1, from increased peptide levels on the DC surface, directly correlates with the number of peptide-specific $CD8^+$ T cells detected by tetramer after vaccination. Notably, this number is enhanced dramatically if co-stimulatory signals are received at the point of $CD8^+$ T-cell priming. The presence of co-stimulation also results, in addition to increased cell numbers, increased functional avidity of $CD8^+$ T cells[80]. There are several types of co-stimulatory signals received by T cells which overlap to a large extent. The first is supplied by the DC as a result of maturation and activation and is exemplified by the B7 family of co-receptors.

The second results from $CD4^+$ T-cell help and is discussed further in Section 1.2.4.3., encompassing co-stimulatory molecules and release of pro-inflammatory cytokines.

The B7 family of co-receptors consist of several members of transmembrane proteins containing at least one immunoglobulin-like domain, and belong to the Ig superfamily. The best characterised of these are B7.1 (CD80) and B7.2 (CD86), expressed on DC which bind to their receptor CD28, constitutively expressed on T cells. This binding occurs at early time points after TCR stimulation and results in IL-2 production and CD25 (IL-2 receptor α chain) expression[81]. IL-2 stimulation drives T-cell entry into the cell cycle to enhance proliferation. CD28 may also impact on the correct molecular organisation of the immunological synapse[82]. Another member of the Ig super family involved in T-cell co-stimulation is ICOS and its associated receptor ICOS-L (or LICOS/B7-H2) (the murine homologues are designated CRP-1 and B7h respectively). T-cell expression of ICOS is dependent on CD28 ligation, suggesting it functions in a synergistic manner to, but at a later time point than CD28[81]. The murine homologue of the human ICOS ligand is expressed on DC but may also be expressed on activated non-haematopoietic cells indicating a possible role in augmenting local T-cell proliferation at peripheral sites[83].

The TNF receptor family provides a further array of activating signals for T cells. DC:T cell interactions involving CD40:CD40 ligand (CD40L), results in two way activation signals for both cell types. CD40:CD40L signals also mediate $CD4^+$ T-cell help, discussed in further detail in Section 1.2.4.3. Other co-stimulatory receptors impact at later time points along the T-cell activation pathway, and include other members of the TNF receptor family, CD27, OX-40 and 4-1BB and their associated TNF receptor ligands, CD70, OX-40L and 4-1BBL respectively. OX-40 is expressed on activated T cells and has been observed as early as 24h post activation and remains expressed for 3-4 days[84]. Likewise, its ligand OX-40L has been shown to be expressed on activated DC with similar kinetics; however, in this instance expression is induced by CD40 ligation[85]. OX-40:OX-40L interaction activates both DC and T cell. Use of anti OX-40 antibodies has been shown to enhance $CD4^+$ T-cell proliferation and survival *in vitro* and *in vivo*[86] by promoting increased expression of the intracellular anti-apoptotic proteins $Bcl\text{-}_{XL}$ and Bcl-2[87]. OX-40 ligation also has comparable effects on $CD8^+$ T cells but this is less well defined[88]. Anti OX-40L antibodies synergise with CD40 ligation to induce DC maturation *in vitro*, with an increase in the co-stimulatory molecules CD80, CD86, CD54, CD40 evident and concomitant release of the pro-inflammatory cytokines, TNFα, IL-12, IL-1 and IL-6[85]. 4-1BB displays similar kinetics and functions to OX-40 expression, i.e. it appears to be

induced on T cells at a later time point than CD28 and generally leads to enhanced proliferation and survival of T cells. There is some indication that expression of 4-1BB is more pronounced on $CD8^+$ T cells than on the $CD4^+$ T-cell subset[89], and 4-1BB has been shown to enhance and broaden the $CD8^+$ T-cell response to influenza type A viral challenge in mice[90]. 4-1BB has also been shown to promote both $CD4^+$ and $CD8^+$ human T-cell proliferation *in vitro*, although $CD8^+$ T-cell proliferation was dependent to some degree on IL-2 production by the $CD4^+$ T-cell population[89]. CD27 expression on T cells increases upon TCR stimulation and its function appears to be parallel to OX-40 and 4-1BB in T-cell survival[91].

Understanding the mechanisms of co-stimulation, which increase both the number of responding T cells as well as promoting their sustained survival will have a large impact on active immunotherapy. The possibility of vaccinating in the presence of co-stimulation enhancers is now being explored[92].

### 1.2.4.3 $CD4^+$ T helper cells: DC licensing and $CD8^+$ T-cell responses

$CD4^+$ T helper cell ($T_H$) subsets have been defined with respect to their cytokine secretion profile, and their ability to promote ensuing immune responses. A $T_H1$ and $T_H2$ spectrum has been described[93, 94], where broadly speaking, $T_H1$-like cells characteristically secrete IFNγ and IL-2 and promote cellular pro-inflammatory immune responses. In contrast, $T_H2$-like cells characteristically secrete IL-4 and IL-5 and promote humoral anti-inflammatory and allergic immune responses. Over the last 20 years the role of $T_H1$-like cells in promoting $CD8^+$ T-cell responses has been extensively researched and this understanding has been pivotal in designing immunotherapeutic strategies for both infectious disease and cancer. Early work described the necessity for 'helper' determinants recognised by $CD4^+$ T cells to be presented on the same APC as the $CD8^+$ T-cell determinants for $CD8^+$ T-cell priming to a model antigen[95]. Much effort has since been focused on the mechanisms whereby $CD4^+$ T cells promote $CD8^+$ T-cell responses and the role of the APC.

Of primary importance is the ability of $CD4^+$ T cells to 'license' DC potentiating their ability to prime other T cells, notably $CD8^+$ T cells. Recognition of MHC class II peptide complexes on presenting APC activates $CD4^+$ T cells, which then promotes a reciprocal activation and maturation of the APC. An important component is the signal generated by CD40 ligation on APC by CD40L on $CD4^+$ T cells. The result is increased expression of co-stimulatory molecules such as ICAM 1, B7.1, B7.2 and MHC class II on APC,

increasing its capacity to present peptides to, and activate other T cells[96]. This effect is both quantitative and qualitative; for example, it has been demonstrated that an increase in the affinity of helper epitopes directly results in an increase in CD40L expression on $CD4^+$ T cells. This in turn promotes the levels of co-stimulatory molecules on APC, thereby leading to enhanced $CD8^+$ T-cell effector function[97]. CD40 ligation further promotes the release of pro-inflammatory cytokines such as IL-1 and IL-12[98], allowing additional T-cell activating signals and in some instances a third co-stimulatory signal required for $CD4^+$ and $CD8^+$ T-cell responses respectively[99, 100]. IL-12 for example, increases expression of CD25 on $CD8^+$ T cells[99], allowing them to respond to a lower concentration of IL-2. DC conditioning in this manner suggests a sequential model of interaction between the three cell types, APC, $CD8^+$ and $CD4^+$ T cells, and precludes the necessity for a three cell cluster[101]. CD40 is also expressed on a small number of $CD8^+$ T cells indicating the possibility of direct activation by $CD4^+$ T cells[102]. The importance of CD40 ligation has been observed *in vivo* using anti-CD40 antibodies, which can by pass (to varying degrees) the need for $CD4^+$ T-cell help[103, 104]. Some viral infections and immune adjuvants, such as complete Freund's adjuvant (CFA), can also negate the need for helper determinants and $CD8^+$ T-cell responses are observed in the absence of $CD4^+$ T-cell activation in this setting. This is attributed to the release of inflammatory signals that can directly activate the APC[105].

The precise role of $CD4^+$ T-cell help in all aspects of $CD8^+$ T-cell responses, including clonal expansion, differentiation and memory formation[106], has now been clarified further. It was originally proposed that priming $CD8^+$ T cells in the absence of help (or co-stimulation) resulted in undetectable immune responses *in vitro* and an inability of $CD8^+$ T cells to respond to a second stimulation *in vivo* despite co-provision of helper determinants. These cells were regarded as tolerised[107]. However it is now known that in some instances effective CD4 independent $CD8^+$ T-cell responses have been described and several factors obviate the need for $CD4^+$ T-cell help, including increased peptide affinity for MHC[108] and increased $CD8^+$ T-cell precursor frequency[109]. Whilst the absence of CD4 help may have no striking effect on the primary response in these cases, the resulting memory T-cell population is likely to be decreased in number, as well as be functionally impaired. In one model for example, female Rag2-/- mice were reconstituted with 90% female bone marrow and 10% male bone marrow[110]. Adoptive transfer of naïve TCR tg $CD8^+$ T cells specific for a male antigen with or without $CD4^+$ T helper cells had no effect on the primary response. However, the memory $CD8^+$ T cell pool formed in the absence of $CD4^+$ T-cell help had a decreased capacity to proliferate or secrete cytokines in response to antigen re-

exposure and were termed 'lethargic'[110]. A similar observation was made when the transferred $CD8^+$ T-cell population were of memory phenotype. Similar findings have been noted in other murine model systems[111, 112]. These data conclude that $CD4^+$ T cells do not simply determine whether or not a $CD8^+$ T cell becomes activated, but appear to supply instructions for differentiation and memory formation, resulting in optimum expansion and effector function on secondary exposure to antigen.

In addition to their ability to promote $CD8^+$ T-cell responses, there is some evidence to suggest that $CD4^+$ T cells can provide help to other $CD4^+$ T cells with different antigenic specificity[113]. The mechanisms by which this occurs have not been fully delineated but there appears to be a requirement for expression of both MHC class II epitopes on the same APC[113]. Recently there has also been evidence to suggest that $CD4^+$ T cells are not alone in their ability to provide help in promoting APC activation of T cells. $CD8^+$ T cells have also been found to activate APC, leading to an increased expression of MHC class II, CD86 and CD40 as well as up-regulated expression of IL-12[114, 115]. Overall a reciprocal arrangement of CD4-CD8 interactions allows regulated help for T-cell activation in both directions, via APC.

It is well established that B cell responses (Section 1.2.8) are also enhanced by $CD4^+$ T cells, involving both contact dependent signals and soluble factors. The B cell receptor can internalise bound antigen and process and present antigenic peptides on MHC class II molecules. Antigen-specific $CD4^+$ T cells subsequently interact with B cells through MHC class II recognition. Signals delivered through MHC class II promote B cell differentiation and proliferation[116]. Once the two cells types are in close proximity, other receptor-ligand interactions can take place. A fundamental signal required for effective B cell responses is CD40L on the T cell binding to CD40 on the B cell. This signal promotes proliferation, antibody production and isotype switching[116]. Other interactions serve to promote the response to CD40-CD40L signalling and include adhesion molecules such as LFA-1 and ICAM-1 and TNF family members OX40 and OX40L. Further signalling through other TNF family members such as CD27 can promote B cell terminal differentiation into plasma cells secreting large quantities of antibody[116]. Soluble cytokines such as IL-4, IL-5 and IL-6 also synergise with CD40 signals and promote proliferation and terminal differentiation of B cells, as well as helping to direct isotype switch events. IL-4 for example, induces switching to IgG1 and IgE, whereas IFNγ induces switching to IgG2a and IgG3 in mice[117].

### 1.2.5 Immunodominance

An important concept for vaccination strategies utilising the APC for processing and presenting antigen is immunodominance. This process was initially described to explain the emerging pattern of CTL responses seen against viral infections, where $CD8^+$ T-cell activity was found to be focused on one or two of the vast array of MHC class I epitopes encoded within viral antigens[118]. As yet, it is not fully clear how this occurs. However, it is likely that factors of antigen processing and presentation, availability of co-stimulatory signals, as well as the TCR repertoire play a part. It is known that for immunodominance to occur both the dominant and sub dominant MHC class I epitopes must be presented on the same APC[119], a situation likely to occur during DNA vaccination. APC processing of a foreign antigen involves degradation, and the rate of degradation may have subsequent effects on the levels of peptide generated and presented[120]. In addition, the flanking residues surrounding a given peptide may influence its ability to be generated[121, 122] with different proteolytic activity found in certain cellular locations having preferences for particular cleavage sites within a given protein. In this respect, the immunoproteasome has been shown to enhance the presentation of some epitopes whilst reducing the presentation or even destroying epitopes in other circumstances[120]. During TAP dependent presentation, TAP specificity will influence the efficiency with which a peptide reaches the ER[123], where the peptides are further subject to amino peptidase N-terminal trimming[34]. Therefore, only a limited spectrum of peptides may reach the cell surface. Once at the surface, peptide affinity for MHC must be strong enough to maintain a stable complex and allow time for $CD8^+$ T-cell recognition and activation. Competition for the APC surface may arise at the T cell level as a result of a combination of factors, including limited APC numbers, TCR affinity for its MHC/peptide complex, the number of MHC/peptide complexes at the cell surface, the ability of the responding T cells to rapidly become activated and acquire effector functions and the level of co-stimulation/cytokines required[119, 124, 125]. Furthermore, effector CTL may remove MHC/peptide complexes from the APC surface[126] or possibly lyse the presenting APC[127], preventing activation of further antigen-specific T cells particularly if the presenting APC are in limiting number. Clonal expansion of one $CD8^+$ T-cell population may also simply overcome other populations which may be influenced by precursor frequency[128]. Finally, for an activated CTL to be effective it must also be able to recognise the same peptide-MHC class I complex on the tumour cell. This will require the tumour cell to have retained the integrity of processing and presentation of the tumour antigen, akin to the APC.

### 1.2.6 $CD8^+$ and $CD4^+$ T-cell effector functions

One goal of active immunotherapy is to induce effector T and B cells that will attack residual cancer cells *in vivo*. For T cells, a program of expansion and differentiation follows initial activation which allows recognition of target MHC-peptide structure through TCR/CD3 complexes and triggers target cell death. The mechanisms by which $CD8^+$ T cells versus $CD4^+$ T cells achieve this differ as most infected host cells or malignant cells are MHC class I positive and MHC class II negative. $CD8^+$ T cells therefore are directly cytotoxic, but there is also evidence to suggest that $CD4^+$ T cells can both play a role in the effector stage of $CD8^+$ T-cell responses as well as mediate killing of MHC class II negative cells by indirect mechanisms.

Cytotoxic T-cells utilise two main pathways for target cell cytolysis following specific peptide recognition via TCR, and these pathways occur simultaneously. The first is the perforin/granzyme pathway. CTL store 'lytic granules' which comprise a family of serine proteases, the granzymes, and perforin, a pore forming protein. Following target cell recognition, CTL secrete the granules in a polarised fashion at the point of CTL:target contact[129]. It is thought that perforin polymerisation allows the formation of a channel in the target cell membrane through which granzymes pass into the intracellular space of the target cell. However, granzyme cell entry has also been shown to occur by receptor mediated endocytosis and a requirement for perforin still remains, suggesting that granzymes are delivered to the target cell within membrane bound compartments and perforin is subsequently required to lyse the membranes for granzyme release once inside the cell[130]. Regardless of the route of entry, once inside the target cell, granzymes can initiate cellular apoptosis through activation of the caspase cascade, resulting in cleavage of signalling proteins, structural proteins and ultimately activation of DNAses[131-134]. The second pathway utilised by CTL to mediate target cell apoptosis is via membrane bound Fas (CD95)-FasL interactions. FasL forms a trimeric complex and its expression is induced on activated T cells by signalling from the TCR/CD3 complex[135]. Fas, expressed on the target cell, contains a death domain which, when trimerised by FasL recruits a large number of proteins, including caspase 8 that associate to form the death inducing signalling complex (DISC). Within the DISC, caspase 8 becomes activated and initiates the caspase cascade and target cell apoptosis[136].

The role of $CD4^+$ T cells in tumour cell killing has been demonstrated conclusively by Bogen and colleagues[137]. Adoptively transferred TCR tg $CD4^+$ T cells can eradicate both

MHC class II positive B cell lymphomas and MHC class II negative myeloma *in vivo* in the absence of B cells and $CD8^+$ T cells

An important mechanism for indirect $CD4^+$ T-cell mediated target cell killing is via the release of cytokines which in turn recruit downstream effector cells. $T_H1$-like $CD4^+$ T cells have been shown to be recruited to the tumour site and release pro-inflammatory cytokines such as IFNγ[138]. IFNγ can slow tumour growth through inhibition of angiogenesis[139] or as yet undefined effects on the tumour stromal cells[140, 141] and use of knockout mice has shown the necessity for both $CD4^+$ T cells and IFNγ in the protection against tumour challenge[138, 140]. Depletion studies have also demonstrated the requirement for $CD4^+$ T-cell IFNγ production for early infiltration of $CD4^+$ and $CD8^+$ T cells into the tumour site[142]. In all cases $CD8^+$ T cells were necessary for optimum tumour eradication suggesting that the early infiltration of tumour specific $CD4^+$ T cells to the tumour site results in modification of the tumour environment to promote $CD8^+$ T-cell responses; for example, IFNγ production can enhance MHC class I and II presentation on the tumour cells as well as alter the proteasome subunit composition[32]. $CD4^+$ T cells have also been shown to be important for recruitment of macrophages into the tumour environment. Macrophages produce inducible nitric oxide synthase (iNOS), the enzyme responsible for nitric oxide (NO) synthesis. NO plays a complex role in the tumour microenvironment, importantly however, NO promotes DNA damage and cell death[143]. Therefore, as expected, the presence of macrophages and increased iNOS helps to promote tumour protection[138]. In addition to $T_H1$ mediated tumour killing, $T_H2$-like $CD4^+$ T cell clones are also responsible for tumour clearance by large scale recruitment of degranulating eosinophils at the tumour site[144].

The key question of whether the $CD4^+$ T-cell response is dictated by peptide specificity has also been raised. Monach *et al.*[145] activated $CD4^+$ T-cells specific for the mL9 antigen expressed by MHC class II negative 6132A tumour cells. In mice challenged with mL9 positive and negative tumour cells at two different sites, only mL9 negative tumours took hold. It was hypothesised that local APC were presenting the peptide allowing peptide-specific recruitment of $CD4^+$ T cells which could mediate indirect mechanisms of killing[141]. In certain circumstances where host cells do express MHC class II, $CD4^+$ T-cell killing may be direct and although this has not been investigated extensively, there is an indication that $CD4^+$ T cells can act in a manner analogous to $CD8^+$ T cells and release cytotoxic mediators, as well as having the ability to directly signal through members of the death domain containing TNF family of receptors, such as Fas, and TNF-related apoptosis-inducing ligand (TRAIL) [146, 147].

In view of these findings, it would clearly be beneficial for immunotherapeutic strategies against cancer to include mechanisms of promoting tumour-specific $CD4^+$ T-cell responses.

### 1.2.7 T-cell clonal expansion and memory

Following activation, the T cell passes through several different stages. Firstly, a large expansion in antigen-specific T cell numbers is observed which peaks typically within 2 weeks following antigen exposure, but ultimately depends on how the antigen is exposed to the immune system and in what quantity. During this time T cells both proliferate and differentiate to the point of effector function commonly measured by CD69 expression, cytokine (IFNγ and IL-2) and perforin/granzyme production. Following the large expansion of antigen-specific T cells, regulatory mechanisms exist to ensure the immune system returns to a steady state level. Hence, a large degree (>95%) of antigen-specific T-cell death is observed during the natural immune response. Finally, following the contraction phase, surviving T cells form a memory pool where an increased antigen-specific T-cell precursor frequency is achieved which results in both a larger and more rapid response upon antigen re-encounter[148, 149]. The factors governing each stage are currently under scrutiny, and several important issues have arisen.

For T-cell expansion, the nature and quantity of antigen will have direct effects on both the number of cells recruited into the response as well as the efficiency of those cells in performing effector functions. When large quantities of antigen are available, a short period of stimulation (2-3 hours) via the TCR and co-stimulatory ligand receptors can be sufficient to provide an autonomous programme of proliferation and differentiation within the T cell, as demonstrated *in vitro* with $CD8^+$ T cells specific for the SIINFEKL peptide[150]. However, the continuing survival and ability of the cells to respond to re-exposure of antigen was not monitored. Others have proposed that an increase in the duration of TCR signalling is required when antigen levels are biologically relevant, allowing both $CD4^+$ and $CD8^+$ T cells to progress through the thresholds of proliferation, differentiation and death/memory formation. This suggestion has stemmed from observations made *in vivo* where a short TCR signal (4 hours) was associated with an arrest in $CD8^+$ T-cell proliferation after 48 hours[151]. 20 hours of TCR stimulation however resulted in an increased capacity for proliferative and effector functions in the $CD8^+$ T-cell compartment. Furthermore both $CD4^+$ and $CD8^+$ T-cell survival is enhanced with increased TCR signal duration[152].

Ultimately the impact of the primary response is seen at the memory cell stage where the size of the memory T-cell pool generated is related to the size of the primary response. In addition the functional properties of memory cells may be influenced by the quality of T cells involved in the primary response which in turn are influenced by the amount of available antigen. Therefore increasing antigen levels to the point of T-cell exhaustion in the primary response is likely to have detrimental effects on the memory cell population[153].

The contraction phase of the T-cell response is marked by a large degree of T-cell death, mediated by several mechanisms which include growth factor withdrawal. As antigen becomes limiting, IL-2 availability decreases and expression of anti-apoptotic members of the Bcl-2 family are down-modulated, the T cells become more susceptible to apoptosis[154]. In addition to mechanisms that cause death by default, active mechanisms also play a role in T-cell death. Perforin for example, has been implicated in limiting the expansion phase for $CD8^+$ T cells and perforin knockout mice display lymphoproliferative disorders[155]. IFNγ is vital for correct contraction of T-cell responses[156]. CTLA-4 expression is also increased on activated T cells and competes with CD28 for binding to B7.1 and B7.2. Its engagement can block co-stimulation through CD28 as well as signal to turn off IL-2 production[157]. Fas mediated T-cell death appears to play a more prominent role in $CD4^+$ T-cell contraction[158]. There are kinetic differences following the contraction phases of $CD8^+$ T cells versus $CD4^+$ T cells in response to viral infection, and this may represent a general phenomenon. $CD8^+$ T-cell contraction occurs earlier than that in $CD4^+$ T cells, and $CD8^+$ memory T cells are stable over time at around 5-10% of the primary response. $CD4^+$ T-cell contraction is delayed and extended in comparison, and memory cell numbers gradually decline over time[159].

The mechanisms controlling the formation of a memory T-cell pool are controversial with suggestions that memory cell formation is a linear progression from effector cells[160] that have received a differentiation signal through CD40[111]. However, the factors determining whether or not an effector T cell undergoes apoptosis or forms a memory cell are under debate. One proposal is that memory cells are derived from cells arriving in the lymph node late in the immune response and therefore receive low levels of stimulation. These cells proliferate and acquire effector function but do not receive sufficient signals to activate the death pathway[161].

Memory T cells are characterised by expression of CD44 and can divide slowly in the absence of antigen or signals through the TCR[162]. Instead, $CD8^+$ T cells require IL-15 for their maintenance[163, 164] and in accordance with this, express high levels of the IL-2Rβ (CD122) component of the IL-15 receptor[164]. The stimulus required for $CD4^+$ memory T-cell homeostasis is not known. However, the functional efficiency of memory cells maintained in the absence of TCR stimulation has been questioned[165, 166]. Two memory $CD8^+$ T-cell subsets have been identified based on CCR7 and CD62L expression, homing preferences and kinetics of acquiring effector function [167, 168]. Central memory cells express high levels of CCR7 and CD62L and home to lymph nodes, whereas the effector memory subset expresses lower levels of both markers and home to peripheral tissues. Both subsets have the potential to produce effector cytokines, such as IFNγ and rapid cytotoxic effector function, but the central memory cells also produce IL-2, proliferate and control viral infection much more effectively than the effector memory subset when adoptively transferred[168]. Similarly, functionally distinct subsets have also been observed in $CD4^+$ T-cell memory cells, but these are less well described[169].

### 1.2.8 B lymphocytes and antibody effector function

The third component of the adaptive immune response is the humoral (antibody) response provided by B lymphocytes. B cells are lineage derived from pluripotent stem cells in the BM, and develop along a complex differentiation pathway from pro-B cells to pre-B and immature B cell stages to acquire a mature B cell status[170], as defined in Figure 1.6. Multiple factors regulate this progression. A pivotal transducing agent is the Ig molecule, a component of the BCR (Figure 1.7). Signalling via the BCR is central to regulating much of B cell differentiation[171]. It functions to recognise antigen, mediated by the V regions of the Ig molecule. The Ig molecule is assembled early in B-cell ontogeny, starting at the pro-B cell stage. Two identical heavy chains and two identical light chains are assembled in a step wise developmental program. The V region domains are functionally rearranged by cutting and splicing together multiple V, D and J gene segments to yield functional $V_HDJ_H$ and $V_LJ_L$ exons. Assembly allows expression of IgM on the surface of mature B cells prior to circulating in the periphery[172]. The BCR is essential to survival in the periphery. Mature B cells encounter antigen in lymphoid tissue, which can lead to the formation of germinal centre (GC) in secondary lymphoid follicles[173]. In the formation of the GC, antigen presented by interdigitating follicular DC, recruits T-cell help leading to B-cell activation, proliferation and initiation of the somatic hypermutation mechanism in order to

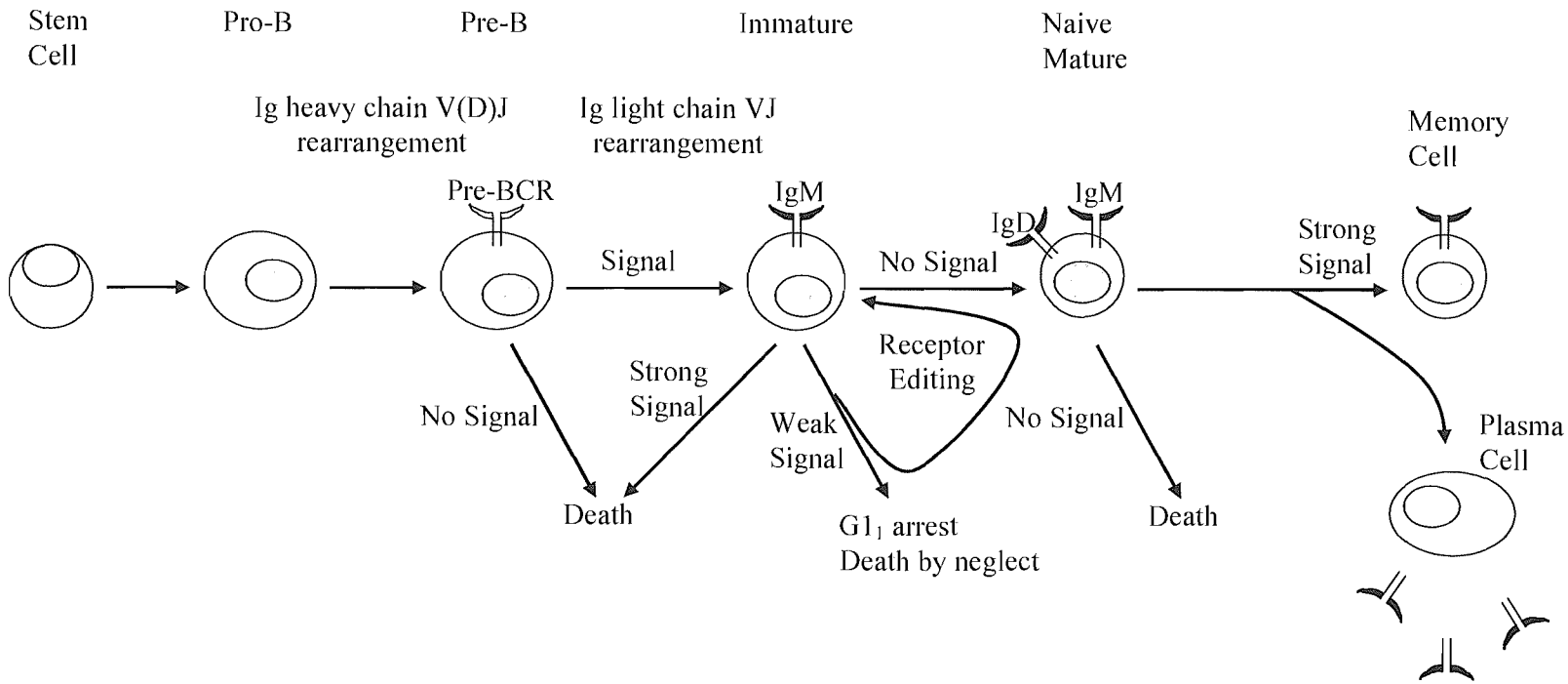



**Figure 1.6. B cell development**

B cells originate in the bone marrow from stem cells and differentiate into pro-B cells. Immunoglobulin heavy chain rearrangement and association with a surrogate light chain allows expression of a pre-BCR at the pre-B cell stage. From the pre-B cell stage it is thought that signals derived from the BCR drive B cell differentiation allowing cells to mature. Immunoglobulin light chain rearrangement occurs and a full BCR is expressed at the immature B cell stage. Figure courtesy of Mr. M. Fox.

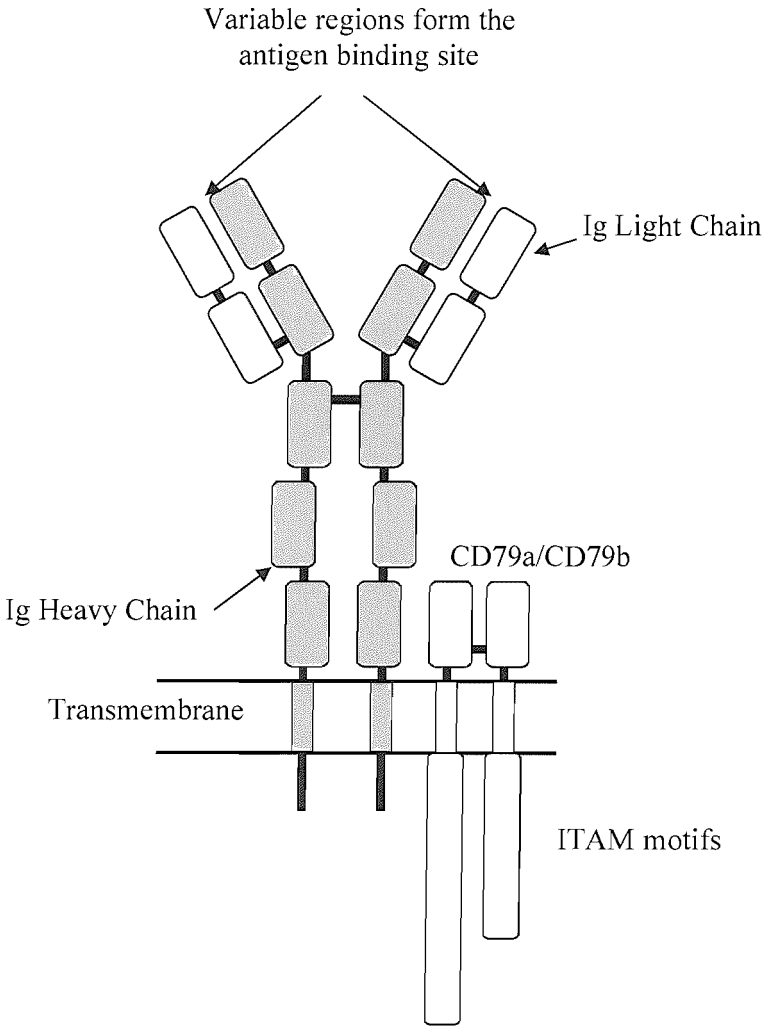



**Figure 1.7. The B cell receptor**

The Ig molecule comprises two heavy and two light chains, whose V regions form the antigen binding site. Association with CD79 heterodimer at the cell surface forms the BCR. CD79 contains within its cytoplasmic domain ITAM motifs which are fundamental to signalling through the BCR both during development and subsequent immune responses. Figure courtesy of Mr. M. Fox.

develop antigen-specific high affinity B-cell clones[174, 175]. Isotype switch events can also follow, and B cells exit the GC to two fates, either differentiation into memory B cells or plasma blasts committed to a plasma cell fate[117, 174]. Antibody secreted by plasma cells mediates humoral responses, able to bind cell surface antigen and interact with effector cells such as macrophages and NK cells by cross linking of FcR, mediating antibody dependant cellular cytotoxicity (ADCC). Additionally, bound antibody can initiate the complement cascade resulting in inflammation and target cell lysis.

### 1.2.9 Origins of B-cell tumours

The interest in our laboratory has been on haematological malignancy, in particular B-cell tumours. A central goal in understanding the origins of B-cell tumours is to define the cell of origin giving rise to disease and its clonal history following malignant transformation. This also defines the most likely normal B-cell counterpart when malignancy occurs and the differentiation status of such a cell. Such analysis has important implications for vaccination strategies. It will be important to target antigens associated with tumour cells which are also expressed by the progenitor cell. Furthermore, defining the stage of neoplastic event can provide insight into critical cellular and molecular mechanisms which may be implicated in the neoplastic process.

B cell tumours can arise at any stage of the normal B-cell differentiation pathway (Figure 1.6), and analysis of Ig V genes can provide pivotal insights into the origin of disease. V(D)J assembly provides a signature CDR3 motif able to define and track any B-cell tumour. V genes can reveal whether the cell of origin has undergone somatic mutation events, generally associated with the GC. In fact, mutational status now enables B-cell tumours to be classified as either having pre-GC origins, where V genes are unmutated, as arising in the GC where V gene mutations display intraclonal heterogeneity or as being post-GC or post-follicular, where tumours reveal no heterogeneity of somatic mutations (Figure 1.8)[176].

Our focus has been on multiple myeloma (MM), a disease characterised by accumulating malignant plasma cells in the bone marrow, typically isotype switched[177]. V gene analysis has revealed a post-GC origin for these tumours[176]. Currently MM remains incurable and disease features include high levels of secreted paraprotein, anaemia and osteolytic lesions[177]. The advent of combination chemotherapy, novel therapeutic drugs and transplantation can now achieve remission in most cases with survival extended to a median

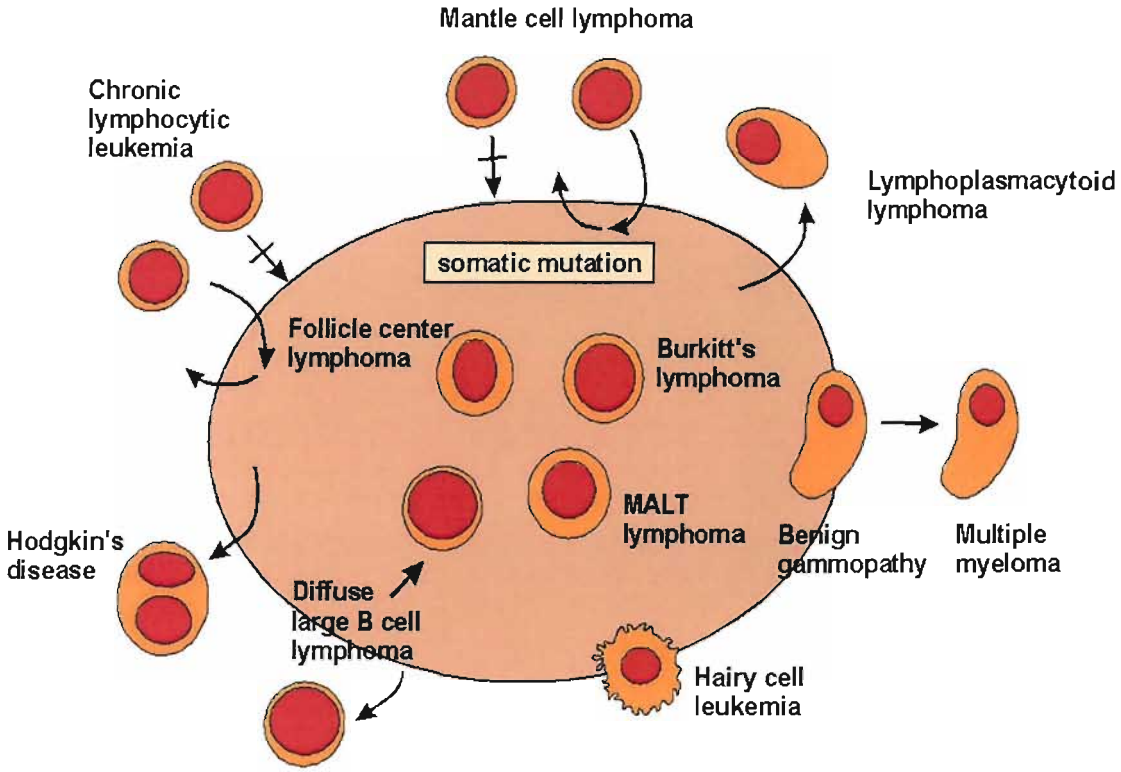



**Figure 1.8. The origins of B cell tumours**[176]

V(D)J recombination provides a signature CDR3 sequence which enables the tumour clone to be defined. The mutational status of V genes can reveal whether the cell of origin has undergone somatic mutation events, generally associated with the GC. Analysis of the mutational status of the V genes allows B-cell tumour classification as either having pre-GC origins, where V genes are unmutated (such as unmutated chronic lymphocytic leukaemia), as arising in the GC where V gene mutations display intraclonal heterogeneity (such as follicular lymphoma) or as being post-GC or post-follicular, where tumours reveal no heterogeneity of somatic mutations (such as multiple myeloma).

of approximately 5 years[177]. This provides a window of opportunity for vaccination against tumour antigens in a setting of minimal residual disease.

## 1.3 Potential barriers to effective T-cell responses against cancer

### 1.3.1 Peripheral tolerance

Whilst central T-cell tolerance is achieved in the thymus (Section 1.2.1), not all self antigens are expressed in this site which allows self reactive T cells to pass into the periphery. This is encouraging for active immunotherapy designed to harness specific T-cell responses against tumour antigens as a large proportion of these antigens are self proteins. However, multiple mechanisms of peripheral tolerance exist to prevent recognition of self (autoreactivity). The issue of peripheral T-cell tolerance is particularly relevant in the setting of malignancy where tumour antigens may persist in the absence of an inflammatory signal. In this situation, DC peptide presentation to T cells can lead to T-cell deletion or anergy. Further modulation of self reactive T-cell activity is provided by the regulatory T cell (Treg, Section 1.3.2.). The normal mechanisms involved in limiting T-cell responses to self antigens will ultimately also play a vital role in tumour immune surveillance and need to be considered when designing immunotherapeutic strategies aimed at activating essentially self reactive lymphocytes.

Our increased understanding of how T-cell responses are initiated has allowed insight into how T-cell responses may fail to become activated. Matzinger and colleagues[107] initially defined tolerance in a model system using the Qa1 antigen which requires $CD4^+$ T-cell help for a CTL response. As expected, vaccination in the absence of helper determinants led to an undetectable CTL response. However, no CTL responses were detected following a boost in the presence of helper determinants[107]. It was concluded that the Qa1 specific T-cell repertoire was rendered tolerant to further activation. The activation state of the DC is now considered to be critical in deciding the fate of the responding T-cell population. Self antigen is generally not recognised by the immune system as it is rarely presented to APC in the presence of activating or 'danger' signals. Recognition of self antigen by T lymphocytes is in the absence of second signals is postulated to lead to a state of either immunological ignorance, a state of functional unresponsiveness or anergy. In circumstances where self reactive T cells are activated, regulatory mechanisms are in place to induce activation induced cell death in responding cells which are not dissimilar to the mechanisms regulating effector T-cell homeostasis via Fas, CTLA-4 and the IL-2 regulatory feedback loop. Murine

models harbouring defects in either of these molecular pathways display an increased propensity for autoimmune pathology[178-180].

Cross-presentation of antigen may also lead to tolerance where activation induced apoptosis leads to deletion as demonstrated by Kurts *et al.*[181]. In this model OT-1 $CD8^+$ T-cells specific for the OVA H-$2K^b$ SIINFEKL peptide proliferate in the lymph nodes draining tissues which express the OVA transgene. However, increased OVA expression led directly to activation induced cell death (deletion) in the OT-1 population[181], whereas low level antigen expression in the pancreas was not cross presented and the $CD8^+$ T cells remained ignorant [182]. It has now been established that for cross-tolerance to occur, the DC must have undergone a threshold level of maturation[183, 184]. As expected, immature antigen expressing DC (receiving no maturation signal) are unable to activate $CD8^+$ T-cell effectors. DC receiving one maturation signal, for example TNFα in the absence of $CD4^+$ T-cell help (and concomitant CD40 ligation) exhibit a 'semi state' of maturation, where an increase in co-stimulatory molecules such as CD86 is observed but these DC are unable to secrete high levels of pro-inflammatory cytokines[183]. If these semi-mature DC are allowed to capture and cross present antigen to $CD8^+$ T cells *in vitro*, $CD8^+$ T-cell proliferation can be detected but effector $CD8^+$ T-cells secreting IFNγ are not generated. These $CD8^+$ T cells are then unable to respond to further stimulation and the majority ultimately undergo programmed cell death. In contrast, DC cross-presenting antigen and matured with anti-CD40 antibodies were able to stimulate $CD8^+$ T-cell effectors *in vitro*. Comparable findings have also been reported for cross tolerance in $CD4^+$ T cells[185].

### 1.3.2 Regulatory $CD4^+$ T cells: a role in peripheral tolerance

Treg cells do not impart permanent functional tolerance in the peripheral T-cell pool but rather modulate T-cell responses to ensure suppression of self reactivity. Therefore it is likely that these cells play a role in suppressing T-cell responses in cancer and/or immunotherapy and will need to be addressed in DNA vaccination strategies.

At least three distinct subclasses of $CD4^+$ Treg have been described in the mouse[186, 187]. Type 1 Treg (Tr1) and Type 3 Treg (Th3) cells characteristically secrete immunosuppressive cytokines, predominantly IL-10 and transforming growth factor (TGF) β respectively. The mechanisms responsible for the initiation of suppression are still unclear, but cytokine release is likely to take effect in an antigen non-specific bystander manner[186]. Both IL-10 and $TGF_\beta$ have multiple and sometimes apparently contradictory

effects on multiple cell types. Notably they have the ability to down modulate professional APC function, antigen expression and co-stimulatory capacity, as well as the ability to impair T-cell function directly by inhibition of IL-2 production[187].

The third subclass of murine Treg cells was originally identified by Sakaguchi *et al.*[188] and were characterised by their expression of the IL-2 receptor α chain (CD25). Sakaguchi demonstrated that transfer of murine BALB/c splenocytes depleted of $CD4^+CD25^+$ T cells into nu/nu mice led to spontaneous autoimmune diseases, which was circumvented by the subsequent transfer of the $CD4^+CD25^+$ T-cell population[188]. Further work by Thornton *et al.*[189] demonstrated that $CD4^+CD25^+$ T cells could inhibit $CD25^-$ T cells when stimulated by anti-CD3 *in vitro*. Since this early work this particular population of lymphocytes have been characterised further. It is now known that they differentiate from immature thymocytes in the thymus into mature, activated cells that are functionally unresponsive to TCR stimulation (are naturally anergic)[190]. However, it seems that for suppression to occur TCR stimulation is required, but subsequent suppressive activity is antigen non-specific[191, 192]. Possible mechanisms of suppressor activity are still controversial. It has been shown that although $CD4^+CD25^+$ T-cells express $TGF_\beta$ and elevated levels of IL-10, a role for soluble suppressor cytokine(s) has not been confirmed. Anti-$TGF_\beta$ blocking antibodies can prevent suppressive activity *in vitro* but transwell systems have indicated that cell contact is necessary[189]. There is also some evidence to suggest $CD4^+CD25^+$ T cells can inhibit IL-2 production in the responder T cells[189]. $CD4^+CD25^+$ T cells have also been shown to constitutively express CTLA-4, and anti-CTLA-4 blocking antibodies can induce autoimmune reactivity suggesting a role for this molecule in suppressor function[193]. However, other work suggests that Treg mediated and CTLA-4 mediated regulation of T-cell activation are functionally separate[194]. Despite the debate surrounding the mechanism of action of $CD4^+CD25^+$ T cells, their functional role *in vivo* has been extended to include the regulation of $CD8^+$ T cells in response to infection[195, 196]. *In vitro* studies suggest $CD4^+CD25^+$ cells can inhibit both proliferation and IFNγ production in the responder $CD8^+$ T cells[192]. Furthermore, a role for Treg cells in suppression of anti-tumour immunity has been demonstrated in murine tumour models[197, 198].

Human $CD4^+CD25^+$ T cells have also been isolated and display an *in vitro* phenotype and suppressor activity similar to their murine counterparts. Human $CD4^+CD25^+$ T cells are functionally unresponsive to TCR stimulation and require cell contact to mediate suppression; with a role for soluble suppressor cytokines thus far ruled out[199-201].

Interestingly, a group of patients with multiorgan autoimmunity display a mutation in the gene *Foxp3* which encodes a transcription factor. Studies in murine cells demonstrated that expression of this gene resided within the $CD4^+CD25^+$ T-cell subset: forced expression in $CD25^-$ T cells converts the phenotype to a suppressive form, with the ability to prevent inflammatory bowel disease in severe combined immunodeficiency (SCID) mice[202].

Although much uncertainty surrounds this cell population, it is becoming increasingly clear that Treg cells reveal a potential to impact on many key areas of immunology, in addition to modulation of self reactive T-cell activation. Their ability to suppress responder $CD4^+$ and $CD8^+$ T-cell populations will have effects on immune responses to pathogens, tumour immunotherapy and vaccination programs.

### 1.3.3 Tumour escape from immunological attack

Peripheral T-cell tolerance and the actions of suppressor T cells are just some of the mechanisms by which tumour cells can 'escape' immune mediated pressure. Other mechanisms include direct effects of secreted or expressed molecules from the tumour cells themselves.

The realisation that various tumour cell types express FasL has led to the suggestion that tumour cells can 'kill' T cells expressing Fas on their surface, allowing tumour cells to 'escape' immune destruction[203]. This possibility however, remains controversial[204, 205]. The expression of FasL on activated T cells following interaction with tumour cells has led to the suggestion that Fas-FasL interactions between T cells themselves may lead to T cell apoptosis or fractricide[206]. Tumour cells have also been shown to down-modulate their MHC class I expression, normally through hypermethylation of HLA loci, mutations in $\beta_2M$ or deficiencies in TAP1/2 expression[207-209], whilst simultaneously increasing their non-classical MHC expression such as HLA-G in response to inflammatory cytokines[210]. These modifications allow increased avoidance of tumour antigen-specific T cells with simultaneous protection from NK cell attack. In a similar manner, tumour antigen expression has also been found to be decreased or absent in response to immune pressure[211]. Tumour cells can also secrete immunosuppressive molecules, for example cytokines, which can inhibit the response of the T cells infiltrating the tumour site[212]. The best characterised immunosuppressive cytokines are IL-10 and $TGF_\beta$. IL-10 can dampen T-cell functions such as proliferation as well as modulate immune responses through effects

on APC. DC exposed to IL-10 have lower levels of co-stimulatory molecules and are more likely to anergise T cells rather than activate them[213].

Some of these difficulties are now being addressed by vaccination regimens such as targeting multiple tumour antigens simultaneously, but others are more likely to require local modulation of the tumour environment[214].

## 1.4 Vaccination against cancer

The goal for immunotherapeutic strategies targeting cancer is to design an optimal vaccine that when delivered to the patient's immune system will raise a relevant, potent and specific immune response to a chosen tumour antigen and to establish immune memory. The strategy will also need to overcome any immunological tolerance or ignorance that may exist, whilst precluding the generation of autoimmune responses. It is likely that the most successful vaccines will target more than one tumour antigen or antigenic epitope at once to circumvent the ability of tumour cells to escape immune pressure through antigen loss. In addition the vaccine would ideally be cost effective, relatively easy to manipulate and have the potential for application to a wide range of patients.

### 1.4.1 Target tumour antigens

An important consideration for immunotherapy is the target tumour antigen. The ideal tumour antigen would be tumour specific and homogeneously expressed within a given tumour clone and at different stages of disease. A tumour antigen that is expressed in a wide variety of tumours of different histological type would also be advantageous, potentially allowing a generic vaccine to be designed. The tumour antigens that have been described to date however only fulfil some of these criteria.

Tumour antigens can be described as tumour associated antigens (TAA) where the antigen is expressed in various tissues but is over expressed within the tumour clone, or tumour specific antigens (TSA) where the antigen expressed is unique to tumour cells arising as a result of re-expression of particular genes, genetic mutations and translocations, or viral transformation. Examples of tumour antigens from these catagories are shown in Table 1.3, and have been reviewed extensively elsewhere[215]. For immunotherapy, the tumour specific antigens are more attractive as no risk of concomitant autoimmunity is posed. For B cell tumours including MM, the B cell idiotype represents a prototypical tumour antigen target

## Table 1.3. Human tumour antigens

| Antigen category | Examples | Tumour specific | Examples of tumours expressing the antigen |
|---|---|---|---|
| Differentiation antigens | Melan-A | No (expression limited to a few tissues) | Melanoma |
| Clonally expressed antigens | Idiotypic determinants of the BCR and TCR | Yes | B cell malignancies<br>T-cell malignancies |
| Onco-viral antigens | Human Papilloma Virus type 16 and 18; E6 and E7 genes | Yes | Cervical cancer |
| Over-expressed antigens | MUC1, carcino-embryonic antigen | No (selective over-expression in tumours) | Epithelial |
| Cancer testis antigens | MAGE, NY-ESO-1 | No (expressed only in immune privileged sites) | Melanoma and various other histological types |
| Genetic mutations | p53, N-ras | Yes | Tumours of various histological type |
| Chromosomal translocations | Bcr-Abl (b3a2 fusion protein) | Yes | Chronic myeloid leukaemia |

Examples of human tumour antigens known to contain immunogenic epitopes recognised by antibody or T cells.

being exquisitely unique to each tumour clone. The use/or generation of tumour specific anti-idiotypic antibody can induce protection upon tumour challenge in animal models of lymphoma and myeloma[216]. For myeloma, which is surface immunoglobulin negative but MHC class I positive, it will also be feasible to harness cellular (T-cell) responses against tumour cells. This has opened up the possibility of targeting other intracellular or secreted antigens associated with MM such as the mucins (e.g. MUC1), and the cancer testis antigens (CTA, e.g. MAGE or NY-ESO-1). The use of additional tumour antigen targets may combat the outgrowth of clonal antigen-loss variants. Our particular interest has been on inducing CTL responses to target CTA and MUC1 and these will be discussed further in Chapters 2 and 3. Importantly in MM, it is known that the $CD8^+$ TCR repertoire to at least some antigenic epitopes within CTA and MUC1 proteins have not been deleted in patients with late stage disease (Tables 2.1 and 3.2). Immunotherapeutic strategies used in this patient group have also demonstrated the ability to activate MUC1 and CTA specific T cells with some notable low level responses to tumour apparent (Tables 2.3 and 3.2).

### 1.4.2 Vaccination strategies against cancer

A number of different vaccination strategies have been described and can be divided into two broad categories, passive immunotherapy and active immunotherapy. Passive immunotherapy describes the administration of immune modulating molecules synthesised *in vitro*. Active immunotherapy, in contrast, activates the immune system to generate an anti-tumour response and to promote immune memory.

#### 1.4.2.1 Passive immunotherapy

Monoclonal antibodies (mAb) specific for tumour antigens or cell surface markers have been a therapeutic option for cancer patients and one success story is anti-CD20 mAb, 'retuximab'. CD20 is expressed on >95% of B cell lymphomas[217], and the administration of retuximab in conjunction with chemotherapy increased response rates in patients with non-Hodgkin lymphoma and mantle cell lymphoma[218]. Radiolabelled anti-CD20 mAb has also been developed in an attempt to increase tumour cell lysis. In phase III trials, the administration of radiolabelled anti-CD20 resulted in increased response rates when compared to retuximab[218]. The main disadvantage of monoclonal antibody therapy is that eventually the antibody will be degraded and further administration will be required.

Allogeneic stem cell transplant using MHC matched donor lymphocytes was originally designed to reconstitute a patients immune system following bone marrow ablation. However it was soon established that even MHC matched donor cells were recognising recipient cells with evident graft versus host disease (GvHd) and that a correlation existed between the severity of the GvHd and the tumour burden. It was in fact soon realised that donor T cells were largely responsible for effective graft versus leukaemia (GvL) effects through recognition of minor histocompatibility antigens expressed on host cells; in some cases these antigens are preferentially expressed on malignant leukaemic cells[219]. Since this time, adoptive cellular therapy has advanced and become an attractive strategy for enhancing immune responses to tumour. Here the aim is to tip the balance in favour of GvL by expanding donor T cells *in vitro* against specific tumour antigens, whilst precluding GvHd. The efficiency of adoptive T-cell therapy has been demonstrated in pre-clinical models and the results of clinical studies look promising[220, 221]. The administration of cytokines for cancer therapy has also been extensively researched as a possible mechanism to activate adaptive immune effector cells non-specifically. Systemic administration of IL-2 and IL-12 have been attempted but with severe toxic side effects[222, 223]. Their use in combination with adoptive T-cell therapy is a more realistic option, and IL-2 has been shown to promote T-cell survival following autologous adoptive transfer into patients with melanoma[220]

### 1.4.2.2 Active immunotherapy

Before the identification of tumour antigens, the methods use to generate immune responses to tumours were non-specific and involved using autologous or allogeneic irradiated tumour cell vaccines or tumour cell lysates. More recently this approach has been rendered more potent with the introduction of cytokine genes into the tumour cells, and the transfection and expression of GM-CSF has had some success in animal models[224]. The main limitations to this approach include accessing sufficient tumour cells followed by the long process of establishing stable transfected clones. The unknown antigen specificity of responses generated also makes clinical trials difficult to monitor and extrapolate.

The identification of tumour antigens has permitted the development of a broad range of immunotherapeutic strategies. The current differences between vaccine design and immunisation protocols utilised by different research groups along with the difficulty in correlating immune responses with clinical responses makes evaluating and comparing the efficiencies of these strategies complicated.

Vaccinating with tumour antigen protein or antigen derived MHC class I and II peptides is a well established approach. The absence of danger signals in these vaccine preparations has led to the combined use of adjuvant, such as incomplete Freunds adjuvant (IFA), stimulatory cytokines such as GM-CSF, or conjugation to xenogeneic sequences such as keyhole limpet haemocyanin (KLH)[214]. Clinical responses have been observed following peptide vaccinations but these are inconsistent[214]. Further improvements to immune responses generated by peptide vaccination have been achieved with the use of peptide analogues, where one or more amino acid changes are introduced to promote binding to the MHC and/or recognition by the TCR[225]. This approach relies on the induction of TCR cross-reactivity with the naturally expressed tumour antigen epitope. One advantage of using peptide vaccines is their ease of synthesis and storage, their major drawback however, is the identification of HLA binding peptides within a given tumour antigen.

A more powerful approach to immunotherapy is the use of antigen loaded autologous DC preparations. Human DC are generated from *in vitro* culture with GM-CSF and IL-4 before being activated through CD40 for example. Various techniques are available to load DC with antigen, including co-culture with MHC class I and II tumour antigen derived peptides, feeding DC with tumour cell lysate or recombinant tumour protein[226, 227]. DC can also be transfected with tumour derived DNA, RNA or viral vectors encoding tumour antigens[226, 228, 229]. Early clinical trials have established that this vaccine modality is safe and is able to activate antigen-specific T-cell responses[230-232]. This strategy is held back not only by the difficulties in isolating human DC but also by the labour intensive DC culture and antigen loading. Autologous DC from cancer patients also commonly harbour defects in activation and cytokine profiles[230].

A further mechanism of activating specific tumour antigen immunity is through genetic immunisation. Bacterial DNA vectors are one such option and are discussed in more detail below (Section 1.5). Viral vectors are also amenable to cancer immunotherapy and include attenuated pox viruses such as modified vaccinia Ankara (MVA) and canarypox, or viral vectors that have been disabled preventing them from replicating in the host such as adenovirus. The DNA sequence of the chosen target tumour antigen can be inserted into the vector and, following vaccination, is transcribed and translated *in vivo*. This approach has resulted in evident T-cell responses in patients[233], but this is associated with neutralising antibodies to the vector preventing a vaccination boost[233, 234].

Several immunotherapeutic approaches mentioned above have been used to target myeloma. In particular, IFNα has been used in conjunction with chemotherapy to try to prolong the maintenance phase of myeloma. However, although relapse free survival is increased, the effect is not dramatic and is associated with severe side effects[235]. Monoclonal antibody therapy has also been used in MM and mAbs designed to target IL-6 in combination with chemotherapy led to favourable responses rates[236]. Vaccination srategies to target the tumour idiotype have also been assessed for efficiency in MM. Patients receiving autologous Id conjugated to KLH following high dose chemotherapy developed anti-Id antibody responses[237], and some patients receiving idiotype peptide pulsed DC developed transient T-cell proliferative responses[238].

Whilst advantages and disadvantages exist for all forms of immunotherapy, we have chosen to focus on DNA vaccination.

## 1.5 DNA vaccines

The idea of DNA vaccination originated from the seminal discovery that any potential antigen encoded by plasmid DNA could be expressed as a protein *in situ* when injected into muscle[239]. Furthermore, vaccines encoding the nucleoprotein from influenza virus were shown to induce both T- and B- cell responses, as well as protect from viral challenge[240]. An application for DNA vaccines in infectious disease and cancer immunotherapy was evident.

DNA vaccines overcome many of the problems highlighted by other active immunotherapeutic strategies mentioned above. Firstly they are simple to construct and store and are cost effective. If necessary, patient specific vaccines can be made with ease. There is no need for additional adjuvants, but it is likely that for successful application to humans, DNA delivery will need to be enhanced, and this is being investigated.

A DNA vaccine is composed of four pivotal features (Figure 1.9). Firstly, a circular plasmid of bacterial DNA provides the backbone within which reside immune stimulatory sequences (ISS) that serve to activate the innate immune system (Section 1.5.1.1). Secondly, within the circular plasmid lies a multiple cloning site (MCS) allowing easy molecular manipulation for target gene insertion. Thirdly, the target gene lies downstream of a strong viral promoter, commonly the cytomegalovirus (CMV) promoter, to enhance gene expression *in vivo*. Finally, the presence of a polyadenylation sequence at the 3' end

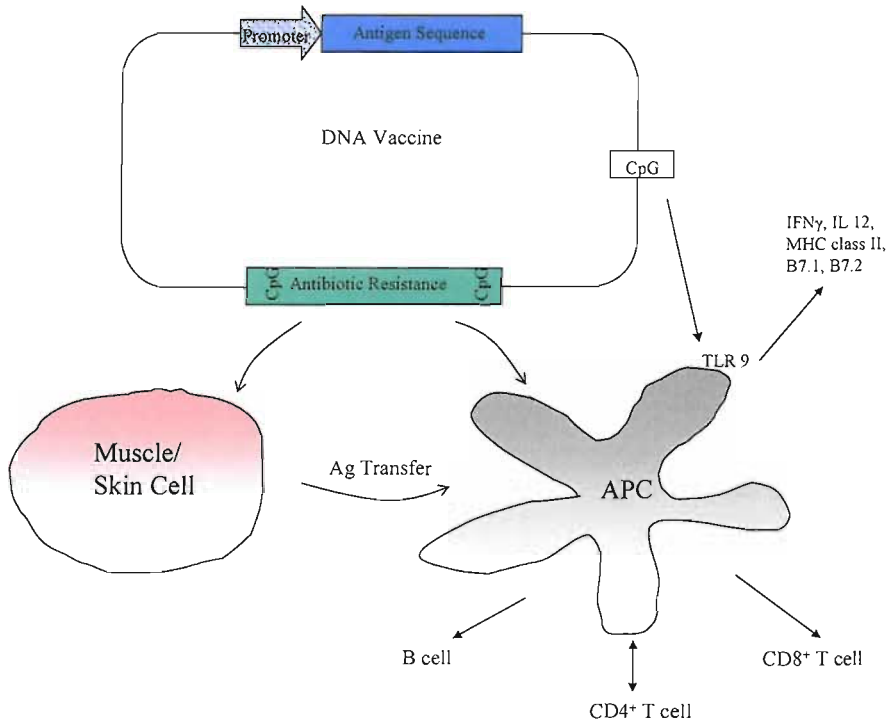



**Figure 1.9. DNA vaccine format**

The DNA vaccine format[241] permits transcription of encoded antigen from a strong viral promoter. Injected naked DNA vaccine may either directly transfect the muscle cell or the APC. The APC may also acquire exogenous antigen for cross-priming. Immunostimulatory sequences in the form of CG dinucleotides (CpG) in the plasmid backbone further activate APC through the Toll-like receptor 9 (TLR-9).

enhances RNA stability. In our laboratory, modifications are also made to enhance target gene expression further. The addition of a Kozak sequence to the 5' end promotes ribosomal recognition[242], we also incorporate a leader sequence which has been shown to enhance antibody responses[243].

### 1.5.1 Pathway of activation of the immune response by DNA vaccination

#### 1.5.1.1 Activation of innate immunity

Bacterial plasmid DNA contains immunostimulatory sequences in the form of hypo-methylated CG dinucleotides or CpG motifs[244]. These sequences, in association with preferential flanking residues, activate cells of the innate immune system in a number of vertebrates[244]. The underlying cause of this immune stimulation is the much reduced frequency of CpG motifs in vertebrate genomes, and where CpG motifs are observed, the flanking residues differ from those in bacterial DNA. Consequently, these fundamental differences allow bacterial DNA to be distinguished within the vertebrate host. DNA containing hypomethylated CpG has recently been shown to activate both human and murine immune cells via TLR-9[245, 246]. TLR-9 is contained intracellularly and has been found to be expressed in B cells, and importantly for DNA vaccination in DC[244, 247]. TLR-9 binding initiates intracellular signalling cascades which lead to cell activation and maturation. For DC, an up-regulation of co-stimulatory molecules such as B7.1, B7.2 and CD40 is seen along with an increase in MHC class II molecules on the cell surface[244] Additional affects include an increase in pro-inflammatory ($T_H1$) cytokine secretion such as IL-12[244]. These changes in phenotype equip the DC with the ability to efficiently prime a T-cell response. It should also be noted however, that only plasmacytoid DC express TLR-9 in humans which, due to their localisation, are unlikely to come into direct contact with plasmid DNA following vaccination. This has raised concerns over the effectiveness of DNA vaccines in humans, however more recently it has been demonstrated that DNA vaccine induced $CD8^+$ T-cell responses are observed in both TLR-9+/+ mice and TLR-9-/- mice suggesting a redundancy in the system[248].

#### 1.5.1.2 Activation of adaptive T-cell immunity

Whilst the plasmid backbone itself can activate the innate arm of the immune system, for adaptive T-cell responses, DNA encoded antigen must be synthesised *in vivo* before being processed and presented on MHC molecules. One fundamental advantage here is that

protein folding and post-translational modifications take place within a mammalian cell. Following vaccine injection in the muscle, there is evidence to suggest that muscle cells are directly transfected with DNA and synthesise encoded antigen[21, 22]. From here endogenous antigen can reach the MHC class I presentation pathway, however myocytes do not express co-stimulatory molecules and therefore no effective T-cell responses can be generated by this route. Instead, as discussed previously, circulating BM derived APC are crucial for the effective induction of an immune response, and are equally as important for generating immune responses following DNA vaccination[249, 250]. Transfected myocytes may act as depots for synthesis of the encoded gene, and from here, secreted antigen can be taken up and processed by local APC[24]. In this situation, antigen can be presented in complex with MHC class II via the exogenous route or alternatively in complex with MHC class I via cross presentation. In addition, APC may also become directly transfected with naked DNA themselves allowing endogenous protein synthesis of antigen[23]. Evidence for both direct transfection of myocytes and of local APC exists and it is likely that both occur to a degree following vaccination. Either way, peptide presentation in association with MHC class I and MHC class II molecules will be achieved permitting T-cell activation.

Importantly, there exist multiple DC subtypes that appear to have different roles in the induction of an immune response, which to some extent will depend on the type and site of exposure to antigen. Plasmacytoid DC primarily reside in the lyphoid organs and the blood, and as mentioned above are unlikely to come into contact with DNA at the injection site; however, they do have a prominent role in producing type 1 interferons which promotes the activation and maturation of other DC as well as T cells in the vicinity[251]. The APC subtypes resident in the tissue are varied, but can be generally described as interstitial DC ($CD11b^{+}CD8\alpha^{-}$). Langerhan cells belong to this group and are well studied examples found in the skin. There is evidence to suggest that interstitial DC can acquire DNA following injection at the muscle and skin, and that these DC can migrate to the local draining lymph nodes[21, 252]. Furthermore, promoting the recruitment of $CD11b^{+}$ DC subset to the site of DNA vaccination with MIP1-α has been shown to augment the T cell response to DNA vaccination[253]. The question of whether these DC are actually responsible for T cell priming has not been evaluated fully with regard to antigen presented via DNA vaccination but this question has been addressed in other model systems. Injection of soluble antigen intravenously led to detectable antigen within $CD8\alpha^{+}CD4^{-}$, $CD8\alpha^{-}CD4^{+}$, and $CD8\alpha^{-}CD4^{-}$ DC subtypes resident in mouse spleen. Whilst both $CD4^{-}$ DC subtypes were capable of inducing $CD4^{+}$ T cell proliferation *in vitro*, only the $CD8\alpha^{+}$

DC subtype was capable of inducing $CD8^+$ T cell proliferation *in vitro*[74]. Since the $CD8\alpha^+$ DC subtype is predominantly found in the secondary lymphoid organs, it has been suggested that this subset acquires antigen in the lymph node, possibly from migrating interstitial DC, for cross presentation to $CD8^+$ T cells. In agreement with this idea, $CD8\alpha^+$ DC were the only DC subtype capable of priming $CD8^+$ T cells following intravenous injection of cellular antigen in $\beta_2M$ deficient cells[73]. Furthermore, radioresistant Langerhan cells were not necessary for CTL priming following administration of viral antigen (HSV) at the skin site, instead the $CD8\alpha^+$ DC subtype were implicated[254]. Injection of DNA plasmid at the skin site using biolistic delivery followed by DC subtype depletion has also demonstrated the importance of $DEC205^+$ DC (incorporating both $CD8\alpha$ positive and negative DC) in T cell responses[255]. From these data, it is clear that further work is needed to define the DC subtypes responsible for T cell priming following DNA vaccination at various sites.

### 1.5.1.3 Requirement for $CD4^+$ T-cell help: the role of Fragment C

As discussed earlier, for optimal T-cell responses $CD4^+$ T-cell help is required in most cases. Following DNA vaccination, there is evidence to show that $T_H1$ $CD4^+$ T-cells play a fundamental role in providing cognate help for both antigen-specific $CD4^+$ and $CD8^+$ T cells[256]. This is achieved through signals to the APC via CD40, increasing its capacity to present both MHC class I and II peptide complexes.

The development of DNA vaccines against B-cell malignancies in our laboratory initially focused on the unique tumour associated idiotype encoded by $V_H$ and $V_L$ genes. DNA vaccination was investigated in murine models of lymphoma (A31) and myeloma (5T33). Injections with plasmid DNA encoding murine A31 lymphoma V genes in a single chain variable fragment (scFv) format failed to induce anti-idiotypic antibody[216, 257]. One possible explanation for this finding was that scFv, being an autologous sequence, was unable to induce a strong inflammatory signal and therefore no $CD4^+$ T helper response was generated. This problem was overcome by fusing scFv to a 'foreign' bacterial derived protein, fragment C (FrC) of tetanus toxin. Vaccination with scFv-FrC yielded high levels of anti-idiotypic antibody affording protection against tumour challenge[216]. Fusion was critical for protection, as presenting scFv and FrC on separate plasmids abrogated the response.

Tetanus toxin belongs to the *Clostridium tetani* family of neurotoxins and comprises two polypeptide chains linked together with disulphide bonds, with a size approximating to 150KDa. This protein can be processed into a 50KDa light (L) chain and a 100KDa heavy (H) chain[258]. The H chain can be cleaved further into an N-terminal portion, $H_N$, and a C-terminal portion, $H_C$ or fragment C. X ray crystallography has demonstrated that FrC contains two domains, an N-terminal jelly roll domain (domain 1) and a C-terminal β-trefoil domain (domain 2) (Figure 1.10)[259]. FrC is non-toxic and has been used successfully in a variety of protein conjugate vaccines[260]. One reason for the success of this strategy is the efficiency with which FrC induces T-cell help. It contains within the first of its two domains a p30 universal helper epitope (FNNFTVSFWLRVPKVSASHLE) which can interact with multiple MHC class II molecules (human and mouse) and activate FrC p30 specific $CD4^+$ T cells[261]. These in turn augment all arms of the adaptive immune response and promote immune memory (Figure 1.11).

### 1.5.1.4 Optimisation of DNA vaccine design

The activation of antigen-specific T cells has also been demonstrated in our laboratory by means of a DNA vaccine encoding scFv-FrC against the surface Ig-negative murine myeloma 5T33. This vaccine mediated protection against tumour challenge in mice[216], and revealed a critical role for idiotype-specific $CD4^+$ T cells (unpublished data) likely to be through indirect mechanisms of tumour cell killing[262]. This result also suggested that intracellular immunoglobulin encoding idiotype was being processed by DC and presented on MHC molecules to T cells. Significantly, these findings open up the potential of an array of other endogenous tumour antigens for targeting by DNA vaccines.

Extending DNA vaccines to target other intracellular tumour antigens, which are presented as peptide fragments on MHC class I molecules prompted modification of DNA vaccine design. Clearly, in relation to myeloma and other tumours which are HLA class I positive, inducing CTL will be a particularly useful line of attack and this was the central aim of this project.

To induce effective anti-tumour CTL responses, a number of factors need to be considered to optimise DNA vaccine design. One aspect concerns APC processing and presentation of DNA vaccine encoded antigen. Here, an important consideration is the phenomenon of immunodominance, which will influence DNA vaccine design targeting single and multiple

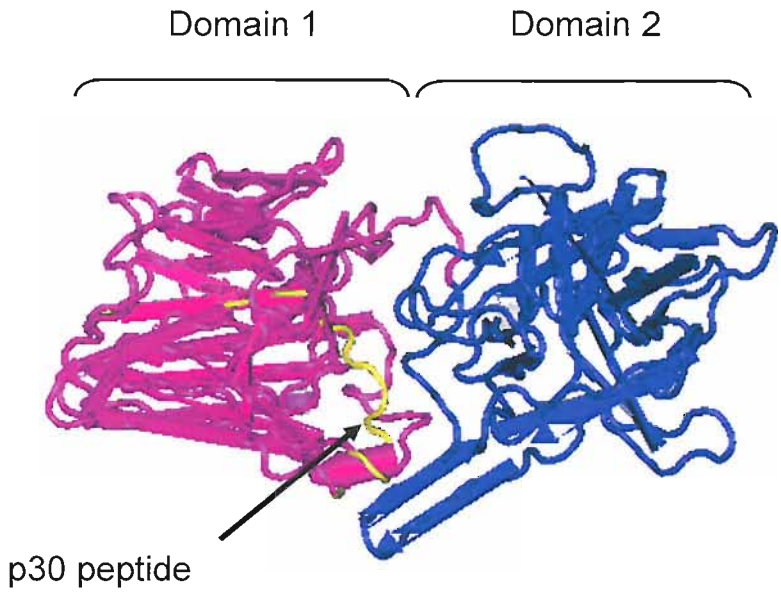



**Figure 1.10. The structure of fragment C of tetanus toxin**

FrC of tetanus toxin contains two domains, an N-terminal jelly roll domain (domain 1) and a C-terminal β-trefoil domain (domain 2)[259]. It contains within domain 1 a p30 universal helper epitope (FNNFTVSFWLRVPKVSASHLE) which can interact with multiple human and mouse MHC class II molecules to activate FrC p30 specific $CD4^+$ T helper cells.

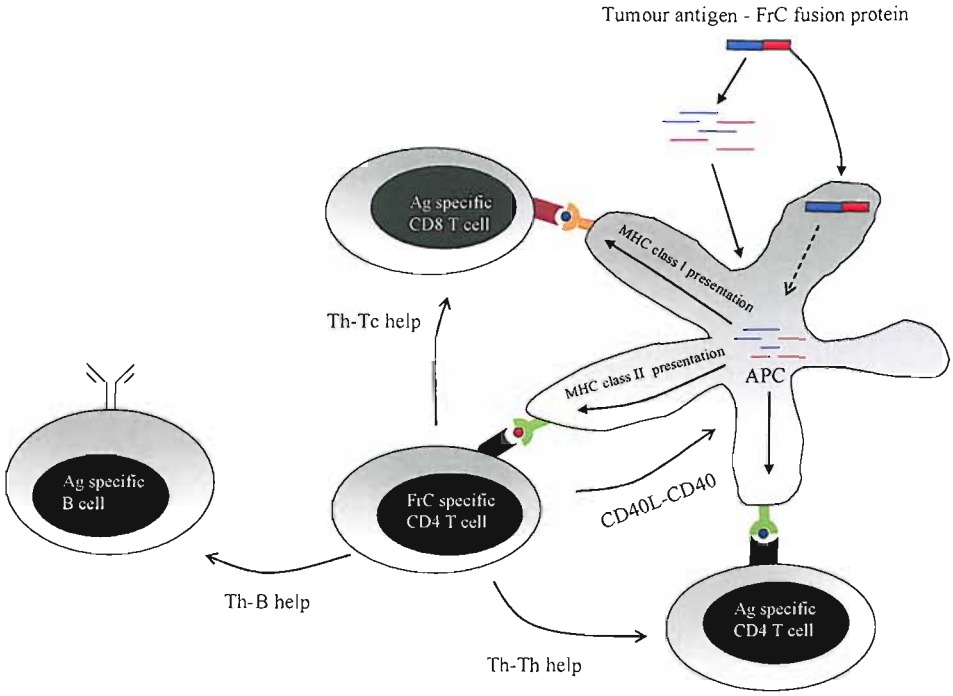



**Figure 1.11. Mechanism of activation of an immune response by FrC fusion vaccines**

DNA vaccine encoded tumour antigen fused to FrC acquired by the APC through direct transfection or cross-priming mechanisms is degraded into peptide fragments for presentation on MHC molecules. $CD8^+$ T cell epitopes encoded within the tumour antigen are presented on MHC class I molecules for recognition by $CD8^+$ T cells. Likewise $CD4^+$ T cell epitopes encoded within the tumour antigen or FrC are presented on MHC class II molecules for recognition by $CD4^+$ T cells. Activation of both $CD8^+$ and $CD4^+$ T cells requires co-stimulatory signals from the APC. Activated FrC or tumour antigen-specific helper $CD4^+$ T cells ($T_H$) augment all arms of the immune response. Firstly, signalling through CD40L-CD40 activates the APC increasing its capacity to provide co-stimulation to effector $CD8^+$ and $CD4^+$ T cells. Secondly, release of soluble factors (cytokines) can also promote activation of $CD8^+$ and $CD4^+$ T cells as well as B cells.

tumour antigen derived epitopes. This has led to the design of a second generation DNA vaccine for optimal CTL induction in our laboratory. The MHC class II 'helper' determinants contained within the first domain of FrC are clearly critical. However, FrC contains potentially immunodominant MHC class I epitopes within its second domain, and it was possible that these would compete with MHC class I epitopes within the fused tumour antigen, and so this domain was removed. Further in design, it was reasoned that the C-end rule would be important in vaccine optimisation to target MHC class I epitopes. Here tethering a known MHC class I restricted antigenic epitope to the C-terminus of encoded protein has been shown to enhance CTL responses[43, 263]. One possible explanation for this observation is that the correct C-terminus of the peptide is already established precluding the necessity for proteasome digestion and any possible concomitant epitope destruction. To test this design, an intracellular MHC class I epitope from carcinoembryonic antigen (CEA) was fused to the C-terminus of full length FrC or the first domain of FrC (DOM). Whilst both vaccines were able to induce CEA peptide-specific CTL as demonstrated by *in vitro* killing assays, fusion of the CEA peptide to the C-terminus of DOM significantly enhanced CTL induction[263]. It was believed that by removing the competing immunodominant murine HLA class I peptides located within the second domain of FrC the CTL response was enhanced. However, the helper T-cell epitopes (p30 and possibly others) within p.DOM remained intact and provided sufficient linked T-cell help for CTL induction.

This design of DNA vaccine has now been validated for a well characterised murine tumour antigen from the CT26 colon carcinoma. CT26 carries an endogenous retrovirus, murine leukaemia virus (MuLV), which encodes the envelope protein gp70. Within the gp70 sequence lies an immunodominant $CD8^+$ T-cell epitope, designated AH1 (SPSYVYHQF). The p.DOM-AH1 vaccine was constructed and shown to induce CTL that can be detected *ex vivo*, and were effective in tumour cell lysis *in vitro*[264]. In protection experiments, only mice vaccinated with p.DOM-AH1 have a survival advantage when compared to vaccines encoding either full length FrC or full length gp70.

Further improvements in vaccine design have been sought using a dual expression vector to deliver a model antigen. Under the control of promoter one (SV40) is the SIINFEKL minigene with leader. Under the control of the second promoter (CMV) is the Ii, however the CLIP region which is normally targeted to MHC class II binding groove, has been replaced with p30 from FrC. This design is proposed to enhance delivery of the 'helper' determinant to the cell surface in complex with MHC class II, and subsequently enhance $T_H$ cell responses. In turn, the increased T-cell help is proposed to promote $CD8^+$ T-cell

responses to SIINFEKL. Comparison of this vaccine design with the p.DOM.epitope design showed the induction of a larger number of IFNγ secreting peptide-specific $CD8^+$ T cells, which had higher avidity and increased ability to lyse cancer cell targets *in vivo* (Thirdborough, S. *et al. in preparation*).

Promoting tumour antigen-specific $CD4^+$ T-cell responses with DNA vaccination has also been addressed in our laboratory. Fusion vaccines encoding scFv from the A31 murine lymphoma model fused to either potato virus X coat protein (PVXCP) or FrC provided protection from tumour challenge[265]. Fusion to FrC promoted Id-specific antibody responses responsible for tumour protection, however, fusion to PVXCP promoted Id-specific $CD4^+$ T cells responsible for tumour protection.

### 1.5.1.5 Optimising DNA vaccine delivery

Improvements to vaccine delivery will be necessary for the successful application of DNA vaccines to humans and are currently being investigated in our laboratory. Experiments designed to assess injection volume and dose (DNA concentration) in mice have shown that both variables have significant effects on T-cell responses induced using the p.DOM.epitope design, confirming other investigations using alternative DNA vaccine designs[21, 266]. Increasing both volume and DNA concentration results in increased numbers of peptide-specific $CD8^+$ T-cells detectable *ex vivo* until a plateau is reached (Buchan, S. *et al. submitted*). These data indicate that in order to induce optimum T-cell responses, a certain level of tissue damage and DNA transfection needs to occur.

Mathieson and colleagues[267] have pioneered plasmid DNA vaccination in combination with electrical stimulus to promote DNA transfection *in vivo*. Enhanced $CD4^+$ and $CD8^+$ T-cells responses as well as elevated antibody levels have been achieved using this method compared to intramuscular injection alone[268]. Our laboratory has also been involved in a collaborative investigation into the use of electroporation in combination with the p.DOM.epitope DNA vaccine design. At suboptimal DNA concentration, both antibody and $CD8^+$ T-cells responses were improved (Buchan, S. *et al. submitted*).

Additional methods to promote DNA vaccine induced responses through enhanced DNA uptake are also currently being analysed in our laboratory. The first is DNA adsorption onto cationic microparticles followed by intramuscular injection. At sizes of less than 5μM, microparticles are efficiently taken up into APC, resulting in enhanced antibody and cellular immune responses in comparison to intramuscular injection of naked plasmid DNA[269].

The second DNA delivery method being analysed involves DNA adsorption onto gold particles followed by gene gun/biolistic delivery. Here, particles are forced under high speed into skin or muscle, with the aim of increasing DNA transfection *in vivo*. This delivery method has been shown by others to amplify both humoral and cellular immune responses to a model antigen in comparison to injection of DNA at the muscle site[270].

### 1.5.1.6 DNA vaccines in the clinic

The validation of the DNA vaccine design in our laboratory against tumour idiotype in the A31 lymphoma and 5T33 myeloma mouse models led to the approval of phase I/II clinical trials designed to evaluate human scFv-FrC vaccines. Three trials are underway, in follicular lymphoma (FL), multiple myeloma and in chronic lymphocytic leukaemia (CLL). In FL and CLL, vaccination is being carried out for patients in remission after chemotherapy. In the case of MM, a unique setting is being explored: vaccination of a normal donor for allo-transfer of educated lymphocytes. Trial patients are being assessed for anti-Id and anti-FrC antibody and T-cell responses. These are being measured by specific ELISA, proliferation and ELISPOT.

DNA vaccines used to immunise against infectious disease such as HIV, hepatitis B and malaria have also reached clinical trial and have demonstrated their safety as well as their ability to promote HIV specific antibody responses in infected patients[271], hepatitis B surface antigen-specific antibody responses[272] and $CD8^+$ T-cell and $CD4^+$ T-cell responses to a malaria parasite circumsporozoite protein in healthy individuals[273]. The combination of DNA priming against the malaria antigen TRAP followed by a boost with a modified vaccinia virus (VV) in healthy individuals also led to detectable T-cell responses that were predominantly $CD4^+$. Levels of T cells were greater after the heterologous prime-boost regimen compared to single vaccination with DNA or VV or homologous DNA-DNA or VV-VV prime boost regimens and resulted in partial protection with delayed parasitemia upon subsequent sporozoite challenge[274]. Optimal responses from DNA vaccination may therefore result from combination with other vaccination strategies.

## 1.6 Aims of this research project

In multiple myeloma, as in other haematological malignancies, conventional therapy can bring disease into remission. This provides an opportunity for vaccination to generate potent immune responses to eradicate residual tumour cells. We have focused on using bacterial plasmid DNA vaccines encoding tumour-associated antigen, which activate both innate and adaptive immunity. Our aim is to develop effective DNA vaccines for potential clinical application in cancer. We have previously formulated use of DNA vaccines to induce protective immunity in murine models of myeloma and lymphoma. Our findings indicate a requirement for co-provision of a pathogen derived sequence, fragment C of tetanus toxin within the DNA vaccine. Fragment C fusion allows the generation of protective anti-Id antibody and T-cell responses, most likely through mechanisms of T-cell help. We have now developed DNA vaccines further to target antigens that are expressed in association with MHC class I molecules. Here, a candidate tumour antigen MHC class I epitope is fused to the C-terminus of the first domain of FrC (DOM), retaining provision of T-cell help.

For myeloma, the CTA and MUC1 tumour antigens have emerged as commonly expressed candidate target antigens, but will require different considerations for active vaccination. This research project is focused on developing DNA fusion vaccines to target these antigens. Specifically, the aims are:

- To develop a tumour model to examine design and efficacy of DNA fusion vaccines targeting CTA.
- To develop and evaluate DNA fusion vaccine designs to target MUC1 in wild type and transgenic models.
- To assess the cellular ($CD4^+$ and $CD8^+$ T cells) immune responses elicited by differing vaccine designs when targeting CTA and MUC1. To analyse the role of $CD4^+$ and $CD8^+$ T cells in providing protection from tumour challenge in these settings.
- To interpret the findings from this thesis to improve the clinical application of DNA vaccines.

# 2 Developing DNA fusion vaccines to target a class of commonly expressed intracellular antigens: the cancer testis antigens

## 2.1 Introduction

The appeal of targeting a universal antigen common to many histologically varied tumours is obvious, as a generic vaccine against such antigens will have widespread application. The usefulness of these antigens is amplified further if their expression is tumour specific. The cancer testis antigens (CTA) fulfil these criteria and appear exemplary. However, their intracellular location will require specific vaccination strategies designed to harness T-cell responses.

### 2.1.1 Human CTA: identification, expression and function

Tumours which naturally regress are associated with immune responses mounted against tumour cells and significantly, offer real hope for vaccine therapies aimed at generating anti-tumour immunity. One of the clearest indications of immune mediated tumour regression has been observed in melanoma associated with vitiligo[275], and provided the basis of the discovery of a new class of TAA, the CTA. This new class of TAA were first identified from CTL clones generated against a patient derived melanoma cell line (MZ2-MEL), which were shown to recognise a short peptide fragment MZ2-E[276]. CTL activity against clones derived from a cosmid library generated from MZ2-MEL led to the identification of the gene encoding MZ2-E, called melanoma antigen gene (MAGE) 1 (subsequently called MAGE A1)[276, 277]. Hybridisation analysis of southern blots of human DNA using a MAGE A1 probe uncovered cross-reacting fragments, which when identified revealed that MAGE A1 was a member of a multigene family[276, 278]. Further refined analysis of genomic and cDNA libraries with this probe yielded the identification of 12 closely related genes, which co-localised on the X chromosome (MAGE A1-12)[278]. Subsequently, two related MAGE gene families were identified, also located on the X chromosome and named MAGE B and C[279-281]. At this time, MAGE A-C were considered to be CTA prototypes as their expression was limited to tumours of various histological types and to normal cells of the testis and placenta, both these sites being immunologically privileged. Now, several MAGE families are known (MAGE A-F)[282], but MAGE D-F by contrast have been shown to be expressed in a restricted panel of normal tissues[283-286].

Similar and additional approaches led to the identification of several other CTA-like genes in humans. T-cell epitope cloning has identified BAGE[287] and 8 members of the GAGE family[288, 289]. Serological analysis of cDNA expression libraries (SEREX), based on natural humoral responses to tumour cells, has delineated the NY-ESO-1[290], CAGE-1[291] and SSX-2 (HOM-MEL-40) genes[292]. Differential gene analysis, which compares mRNA expression in tumour vs. normal or testis tissue, further identified the SAGE[293], HAGE[293] and LAGE[294] genes from human sarcoma and melanoma cell lines. PAGE and XAGE have also been identified using this method[282] as has CTp11 (or SPANX)[295], CT10[296] and MMA1a and 1b[297]. *In silico* comparisons of expressed sequence tags (ESTs) have also yielded identification of a number of CTA homologues, which extends CTA family members further[298]. Clearly a multitude of genes are now known with CTA or CTA-like characteristics. More advanced analysis has revealed that the expression of many of these genes remains restricted to the testis as originally observed, however expression of a growing number of these genes is being detected in non-gametogenic tissues[284-286, 297-299]. Importantly, their expression pattern raises different immunological considerations for intervention. For intracellular antigens, T-cell attack will be necessary and a variety of epitopes predicted to bind to MHC class I and MHC class II molecules have been reported, which are described in Tables 2.1 and 2.2.

Expression of CTA in malignant and normal tissues has been assessed at the RNA level by RT-PCR and to a limited degree by immunohistochemical staining, as only a restricted number of antibodies have been described specific for MAGE A1, A3, A4, C1 and NY-ESO-1[300]. CTA expression has been described in a variety of solid tumours such as bladder cancer and prostate cancer but has been most widely analysed in melanoma, breast cancer and lung cancer[301]. Our focus is on haematological malignancies and in particular multiple myeloma. Expression analysis of CTA in haematological malignancies however, has not extensively documented. Nonetheless CTA expression of a restricted number of genes has been described in acute myeloid leukaemia (AML)[302], chronic myeloid leukaemia (CML)[302], T-cell lymphomas[303], Hodgkins lymphoma[304] and multiple myeloma[300, 305, 306].

Initial findings reported the expression of *MAGE A* and *C*, as well as *BAGE*, *GAGE* and *NY-ESO-1* in multiple myeloma. *MAGE-A1* and *A3* were shown to be expressed in malignant plasma cells by RT-PCR in 62% (13/21) patients[306]. In a separate study, 14/27 stage III myeloma patients were shown to express at least one of the *MAGE A* genes (*A1, 2, 3, 4, 6, 10 or 12*) by RT-PCR. 7/27 from the same patient sample were positive for *BAGE*, 15/27

**Table 2.1. MHC class I epitopes described for human CTA.**

| Gene | HLA binding | Peptide sequence | Processed and presented by tumour cells? | Evidence for T-cell repertoire in humans (‡) | Ref |
|---|---|---|---|---|---|
| MAGE A1 | HLA A1 | EADPTGHSY$_{(161-169)}$ | Nd | Yes | (305) |
| | HLA-A2 | KVLEYVIKV$_{(278-286)}$ | Yes | Nd | (307) |
| | HLA-A3 | SLFRAVITK$_{(96-104)}$ | Yes | Yes | (308) |
| | HLA-A24 | NYKHCFPEI$_{(135-143)}$ | Yes | Yes | (309) |
| | HLA-A28 | EVYDGREHSA$_{(222-231)}$ | Yes | Yes | (308) |
| | HLA-B53 | DPARYEFLW$_{(258-266)}$ | Nd | Yes | (308) |
| | HLA-Cw2 | SAFPTTINF$_{(62-70)}$ | Yes | Yes | (308) |
| | HLA-Cw3/ Cw16 | SAYGEPRKL$_{(230-238)}$ | Nd | Yes | (305, 308) |
| MAGE A2 | HLA-A2 | YLQLVFGIEV$_{(157-166)}$ | Yes | Yes | (310, 311) |
| | HLA-A2 | KMVELVHFL$_{(112-120)}$ | Yes | Yes | (310, 311) |
| MAGE A3 | HLA A1/ B35 | EVDPIGHLY$_{(168-176)}$ | Yes | Yes | (312) |
| | HLA-A2 | FLWGPRALV $_{(271-279)}$ * | Yes † | Yes | (313) |
| | | KVAELVHFL$_{(112-120)}$ | Yes | Yes | (311) |
| | HLA-A24 | IMPKAGLLI$_{(195-203)}$ | Yes | Yes | (314) |
| | | TFPDLESEF$_{(97-105)}$ | Yes | Yes | (314) |
| | HLA-B44/ B35 | MEVDPIGHLY$_{(167-176)}$ | Yes † | Yes | (312) |
| MAGE A4 | HLA-A2 | GVYDGREHTV$_{(230-239)}$ | Yes | Yes | (315) |
| MAGE A10 | HLA-A2 | GLYDGMEHL$_{(254-262)}$ | Yes | Yes | (316) |
| MAGE A12 | HLA-A2 | FLWGPRALV$_{(271-279)}$ * | Yes † | Yes | (313) |
| | HLA-Cw7 | VRIGHLYIL$_{(170-178)}$ | Yes | Yes | (317) |
| MAGE A 1, 2, 3, 6 | HLA-B3701 | REPVTKAEML$_{(127-136)}$ | Yes | Yes | (318) |
| MAGE A1, 2, 3, 4, 6, 12 | HLA-A2 | YLEYRQVPV$_{(240-248)}$ 'heteroclitic' | Yes - native peptides | Nd | (319) |
| BAGE | HLA-Cw16 | AARAVFLAL$_{(2-10)}$ | Nd | Nd | (305) |
| GAGE 1, 2, 8 | HLA-Cw6 | YRPRPRRY$_{(9-16)}$ | Nd | Nd | (305) |

| | | | | | |
|---|---|---|---|---|---|
| GAGE 3, 4, 5, 6, 7 | HLA-A29 | $YYWPRPRRY_{(8-16)}$ | Nd | Yes | (289) |
| LAGE (ORF 2)** | HLA-A2 | $MLMAQEALAFL_{(1-11)}$ | Yes | Yes | (320) |
| NY-ESO-1 (ORF1)** | HLA-A2 | $SLLMWITQC(FL)_{(157-167)}$*** | Yes | Yes | (321-323) |
| | HLA-A31 | $ASGPGGGAPR_{(53-62)}$ | Yes | Yes | (324) |
| NY-ESO-1 (ORF2)** | HLA-A31 | $LAAQERRVPR_{(18-27)}$ | Yes | Yes | (324) |

† - In the presence of IFNγ
‡ - PBMC used were collected from individuals with and/or without cancer
Nd - Not determined
* - Peptide from MAGE A3 and A12 are identical
** - LAGE and NY-ESO-1 both contain two different open reading frames (ORF)
*** - Optimal peptide sequence not fully defined

## Table 2.2. MHC class II epitopes described for human CTA

| Gene | HLA | Peptide sequence | Processed and presented by tumour cells? | Evidence for T-cell repertoire in humans (‡) | Ref |
|---|---|---|---|---|---|
| MAGE A3 | HLA-DP4 | $TQHFVQENYLEY_{(247-258)}$ | Yes | Yes | (325) |
| | HLA-DR11 | $TSYVKVLHHMVKISG_{(281-295)}$ | Yes | Yes | (326) |
| | HLA-DR13 | $AELVHFLLLKYRAR_{(114-127)}$ | No | Yes | (327) |
| | | $LLKYRAREPVTKAE_{(121-134)}$ | Nd | Yes | (327) |
| NY-ESO-1 | HLA-DP4 | $SLLMWITQCFLPVF^{*}_{(157-170)}$ | Yes | Yes | (328) |
| | HLA-DR1 | PGVLLKEFTVSGNILT $RLTAADHR_{(119-143)}$ | Yes | Yes | (329) |
| | HLA-DR4 | 119-143 | Yes | Yes | (329) |
| | HLA-DR7 | 119-143 | Yes | Yes | (329) |
| | HLA-DR11 | 119-143 | Yes | Yes | (329) |
| | HLA-DR53 | 119-143 | Yes | Yes | (329) |

‡ - PBMC used were collected from individuals with and/or without cancer
Nd - Not determined
* - Peptide encodes both MHC class I and class II binding motifs

for *GAGE*, 18/27 for *LAGE-1* and 10/27 for *NY-ESO-1*[305].

Expression analysis carried out in various tumour types has highlighted the degree of heterogeneity of expression between patients[305, 330]. The question of heterogeneity of expression between different tumour cells from the same patient has also been raised, and the limited data described to date illustrate that this too varies between patients. Immunohistochemical data has revealed patient samples with homogenous expression and other patients with intraclonal heterogeneity in myeloma[300, 305, 330]. Interestingly, CTA expression appears to correlate with disease progression, with a much higher percentage of melanoma metastases being positive for at least one CTA compared to their non-metastatic counterparts[331]. Similarly, CTA expression in myeloma also appears to correlate with disease progression, as in the benign condition, monoclonal gammopathy of undetermined significance (MGUS), *LAGE-1* was positive in only 1/6 patient samples, and for myeloma stage I and II samples, only 1/8 samples were positive for *GAGE* by RT-PCR[305]. In contrast 24/27 myeloma stage III patient samples were positive for at least one of the genes tested. This expression pattern has also been observed in myeloma samples using immunohistochemistry[300] and may suggest a functional correlation between CTA expression and malignant clone formation or evolution.

Whilst expression heterogeneity needs to be carefully analysed, it is likely that both the number of different CTA and the number of different T-cell epitopes described for each CTA (Tables 2.1 and 2.2) will allow the incorporation of a high percentage of patients into vaccination trials. Furthermore CTA expression appears to be associated with promoter demethylation[332, 333], and demethylating agents may find a use in combination with targeted vaccination strategies.

Despite the vast array of data describing the identification of CTA, their function remains largely unknown. An approximately 30% homology exists between the conserved C-terminus of the MAGE family of proteins with a necdin consensus sequence[334]. Necdin is a nuclear protein expressed in post-mitotic neurons and is thought to be involved in cell cycle regulation[335, 336]. It can interact with E2F1 in a manner analogous to retinoblastoma (Rb) protein, preventing E2F1 trans-activated genes mediating cell cycle progression. Necdin can also interact with p53 and act as an inhibitor of apoptosis[334]. MAGE D1 (or NRAGE) has also been shown to interact with p75 neurotrophin receptor which can mediate cell apoptosis[336]. The HAGE protein incorporates a DEAD box motif (Asp-Glu-Ala-Asp) that is also found in RNA helicases designed to facilitate correct RNA folding[293, 337].

### 2.1.2 Immunotherapy targeting CTA

CTA specific antibody and CTL can be generated from patients peripheral blood suggesting that in these cases central tolerance is incomplete. The intracellular location of the target proteins however will negate any useful potential of antibody responses either mounted naturally or through vaccination protocols. Antigen-specific $CD8^+$ T cells on the other hand, are likely to be effective at eliminating $CTA^+$ tumour cells and vaccination strategies should focus on generating efficient CTL responses.

Several CTL epitopes have been identified from a number of CTA, using CTL generated from cancer patients, normal volunteers or HLA-A2 tg mice (Table 2.1). The role of $CD4^+$ T cells in immune responses to CTA are also beginning to be explored and MHC class II epitopes have now been described (Table 2.2).

To date, phase I and II clinical trials have assessed the use of peptide and protein vaccination or autologous peptide pulsed DC as well as viral gene delivery. Some encouraging results have been obtained and are summarised in Table 2.3 and selective results are discussed below.

Peptide vaccination protocols are designed to focus the CTL response on one or several predicted MHC class I epitopes within a given antigen; 2 studies are reviewed:
**(a)** 25 metastatic melanoma patients vaccinated with MAGE-3A1 peptide (EVDPIGHLY) resulted in clinical responses in 27% of the cases (3 complete responses (CR) and 4 partial responses (PR)), however there was no evidence of CTL responses[338]. In a follow up study, *in vitro* re-stimulation of peripheral blood mononuclear cells (PBMC) with peptide, $CD8^+$ T-cell cloning and highly sensitive tetramer staining allowed the detection of CTL responses in 4/9 patients with tumour regression and only 1/14 patients with progressive disease suggesting a correlation between peptide-specific CTL induction and clinical response[339].
**(b)** In 12 patients with NY-ESO-1 expressing tumours of various histological types vaccinated with a CTL peptide alone and 50 days later with peptide and GM-CSF, 4/7 patients made a specific CTL response which showed some correlation with stable disease[340].

Whole protein based vaccines are designed to provide the naturally occurring amino acid sequence to the immune system. This allows processing and presentation of encoded MHC

**Table 2.3. Vaccination based clinical trials designed to target CTA**

| Tumour | Vaccine | Adjuvant | No. of Patients | Immune response | Clinical response | Reference |
|---|---|---|---|---|---|---|
| Melanoma | 3 MAGE A1$^+$ melanoma cell lines | None | 57 | 57% demonstrated enhanced IgG levels | Not defined | (341) |
| Melanoma | HLA-A1 epitope from MAGE A3 (EVDPIGHLY) | None | 25 | Not defined | 3 complete response, 4 partial response | (338) |
| Melanoma | HLA-A1 epitope (EVDPIGHLY) from MAGE A3 + MHC class II epitope PADRE | IFA | 18 | 5/14 made specific CTL response | Not defined | (342) |
| Melanoma | HLA-A1 epitope from MAGE A3 (EVDPIGHLY) or canary pox encoding minigene | None | 23 | 4/9 had detectable CTLp | 9 partial response | (339) |
| Melanoma and others | Mix of 3 HLA-A2 NY-ESO-1 peptides | +/- GM-CSF | 12 | 4/7 made specific CD8$^+$ T-cell response | 3 stable disease | (340) |
| Melanoma | MAGE A1, A3, Melan A/MART1, tyrosine and gp100 | None | 22 HLA-A2 | 59% to MHC class I peptides from at least one antigen | Not defined | (343) |
| Melanoma | MAGE A3, MART1, tyrosine and gp100 | None | 131 HLA-A1, A2 | 56% CD8$^+$ T-cell response to at least one antigen | Not defined | (344) |
| Melanoma and others | Recombinant MAGE A3 linked to protein D from H. *Influenzae* | SBAS-2 | 39 (HLA-A1, A2, B44) | Not defined | 2 partial response, 2 mixed response and 1 stable disease | (345) |

a

b

c

**Table 2.3. continued**

| Tumour | Vaccine | Adjuvant | No. of Patients | Immune response | Clinical response | Reference |
|---|---|---|---|---|---|---|
| Melanoma | DC/HLA-A1 MAGE A3 peptide (EVDPIGHLY) + TT or tuberculin | None | 11 | 8/11 made specific CTL response | 6 partial response | (346) |
| Melanoma | DC/peptides from Melan A/MART-1, tyrosinase, MAGE A3 and gp100 | None | 18<br>HLA-A2 | 16/18 made enhanced T-cell response to at least one antigen | 7 partial response | (347) |
| Melanoma | DC/peptides from MART-1, tyrosinase, MAGE A3 and gp100 | None | 2 HLA-A2 | CTL response to several antigens | Progression slowed | (348) |
| Melanoma | DC/peptides from MART-1, tyrosinase, MAGE A3 and gp100 | None | 32 | Not defined | 3 complete response, 5 partial response | (214) |
| Melanoma | DC loaded with KLH and MHC class II (HLA-DP4 and DR13) peptides from MAGE A3 | None | 16 | IFNγ release upon recognition of MAGE A3+, HLA-DP4+ targets | Not defined | (349) |
| Bladder cancer | DC/HLA-A24 MAGE A3 peptide (IMPKAGLLI) | None | 4 | Not defined | 1 complete response, 2 partial response | (350) |
| Gastro-intestinal carcinoma | DC pulsed with HLA-A2 (FLWGPRALV) or HLA-A24 (IMPKAGLLI) epitopes from MAGE A3 | None | 9 | 4/8 made specific CTL response | 3 partial response | (351) |

class II epitopes through the exogenous route. $CD4^+$ T-cells activated by this route may in turn provide T-cell help for $CD8^+$ T-cell responses activated through cross-presentation.

**(c)** In one study of melanoma, 22 HLA-A2 patients were vaccinated with a cocktail of melanoma antigens, including MAGE-A1, MAGE-A3 and the differentiation antigens Melan A/MART 1 and gp100. CTL responses to at least one of the antigens were detected in 59% of the sample[343]. 18% made a specific response to one of the MAGE proteins.

Peptide pulsed DC vaccines provide both peptide-MHC complexes as well as co-stimulation for direct activation of $CD8^+$ T cells and $CD4^+$ T cells.

**(d)** In one study, 11 metastatic melanoma patients were vaccinated with autologous DC pulsed with MAGE-3A1 peptide and recall antigens from tetanus toxin and tuberculin which resulted in 8/11 patients generating a specific $CD8^+$ T-cell response[346]. Regression of skin metastases was evident in 6/11 of these patients.

The data arising from these clinical trials indicates that the CT antigens are weak immunogens. This suggests that effective CTL induction will most likely require $CD4^+$ T helper cell responses. In fact, in a very recent report, vaccine design based on a single peptide with MHC class I and class II specificities was able to generate NY-ESO-1 specific $CD4^+$ and $CD8^+$ T cells *in vitro*[328]. Although it is now clear that immunotherapy can successfully lead to effective immune responses to tumour antigens such as NY-ESO-1, it has also been observed that, as a consequence of immune pressure, antigen and MHC loss tumour variants arise[211]. It may therefore be more advantageous to develop immunotherapeutic protocols aimed at raising multiple T-cell responses against various different intracellular antigens. Another hurdle to overcome with respect to targeting the CTA, and other tumour antigens for that matter, is the possibility of suppression of tumour antigen-specific T cells by $CD4^+$ $CD25^+$ Treg cells *in vivo*. Experiments undertaken *in vitro* with human PBMC have demonstrated that removal of the Treg subset allows the expansion of naturally occurring autoreactive NY-ESO-1 specific $CD4^+$ T cells[352], implying that this may also be the case *in vivo*.

### 2.1.3 Murine CTA

Murine homologues of the human *MAGE* genes have been identified and are represented by the *Mage a1-8*[353] and *Mage b1-4* genes[354, 355]. Like their human counterparts, they are not expressed in normal tissues except germ cells of the testis. As such, they may facilitate the development of a murine tumour model to examine vaccination based immunotherapy for T-

cell attack against CTA. In line with this a Mage a2 H-2K$^d$ CTL epitope has been defined and mice vaccinated with adenovirus encoding the Mage a2 sequence and or peptide sequence have been shown to induce CTL[356]. In addition, the tumour cell line B78H1 was shown to express Mage a2 by RT-PCR, and *in vitro* CTL assays demonstrated endogenous processing of this peptide at low levels. However, limited information regarding expression of the *Mage* family in murine tumour cell lines, as well as the lack of defined MHC class I binding peptides makes this approach more challenging.

In contrast, the P815 mastocytoma, originally induced in DBA/2 (H-2$^d$) mice using methylcholanthrene, is a well studied murine tumour model to evaluate immunotherapeutic approaches to target human CTA. Again, the use of powerful CTL cloning strategies identified a panel of anti-P815 CTL clones, which indicated that the original tumour cell line expressed at least five distinct tumour antigens, P815 -A, -B, -C, -D and -E[277, 357]. CTL epitopes A and B were shown to have a linked expression[358], antigen loss variant P815 cells commonly became resistant to anti-A CTL in the absence of resistance to anti-B CTL (P815A$^-$B+), whereas, resistance to anti-B CTL was always accompanied by resistance to anti-A CTL (P815A$^-$B$^-$). Expression of C, D and E CTL epitopes were shown not to be linked.

The gene encoding tumour antigens A and B has since been cloned, and identified as *P1A*[277]. *P1A* spans approximately 5kb and contains 3 exons, with an open reading frame (ORF) located within exons 1 and 2, encoding a protein of 224aa with a putative molecular weight of 25kDa. The function of the protein remains unknown. However, homology exists to the murine nuclear proteins nucleolin and nucleolar protein[277]. Importantly, analysis of the expression of P1A in normal murine tissues using Northern blots and semi-quantitative RT-PCR revealed that less than 1% of P815 levels could be identified in the spleen, thymus, bone marrow, kidney, liver and lung[277, 359]. In contrast, levels of P1A expression in the testis (spermatogonia) and placenta (labyrinthine trophoblasts) were high, demonstrating a pattern of P1A expression as parallel to human CTA. A precise mapping of CTL epitopes encoded by P1A was determined using tumour derived clones which became resistant to a number of anti-P815 CTL. Resistant clones were found to have point mutations at codons 40 or 42 suggesting the peptide recognised by the CTL was in this region of the gene. Overlapping peptides were synthesised surrounding codons 40 and 42 revealing a nonamer LPYLGWLVF (codon 35-43) which sensitised tumour cell targets to both anti-A and anti-B CTL[358, 360]. Therefore both anti-A and anti-B CTL recognised different regions of the same

epitope, P1A/AB. Transfection of murine MHC class I molecules ($K^d$, $D^d$ and $L^d$) into a fibroblast (H-2K) cell line demonstrated that the P1A/AB epitope was restricted by H-$2L^{d(277)}$.

The gene encoding P815-E (P1E) has also been identified[361]. This gene is virtually identical to the gene encoding the ubiquitously expressed murine methionine sulfoxide reductase (MsrA) from normal somatic cells. However, the P1E gene sequence in P815 tumour cells harbours a single point mutation at position 704 which leads to a Gly→Arg transition at codon 221. There is no evidence that this mutation is oncogenic and a screen of 6 distinct murine mastocytomas and leukaemias were found to be negative. Overlapping peptides spanning this region of the gene were generated and a decamer GYCGLRGTGV (codons 216-225) sufficed to sensitise target cells to lysis by anti-P1E CTL clones. This peptide was found to be expressed in the context of H-$2K^d$, and exhibits the correct binding motifs of tyrosine at position 2 and leucine, isoleucine or valine at position 9 or 10. The genes encoding the P815 antigens C and D have, as yet, not been cloned, and their function remains unknown.

Clearly, the P815 derived P1A/AB epitope presents a useful model antigen in the context of CTL attack on CTA. Several vaccination approaches to target P1A/AB have been investigated as summarised in Table 2.4. The use of live L1210 tumour cells transfected with the P1A gene and B7.1 as a vaccine resulted in 12/15 mice surviving to day 40 as compared with 3/15 control animals when challenged with P815 tumour cells[362]. However, most vaccinated mice were culled due to P815 tumour burden by day 100 indicating a short term effect. A significant proportion of P815 tumour cells collected from animals at later time points (day 40–70) were found to have lost the expression of the AB epitope. Importantly it was also shown with this vaccine that induction of AB specific CTL did not result in an inflammatory response in the testis, nor was fertility affected[359]. Peptide vaccination strategies have used the AB nonamer alone or in conjunction with IL-12 and QS-21 adjuvant (personal communication, Uyttenhove, C.). CTL responses were only detected when IL-12 and QS-21 were co-administered. Several adenoviral vector constructs encoding either the P1A/AB minigene, the first 83 amino acids of P1A, or the first 83 amino acids of P1A fused downstream of the invariant chain sequence were evaluated as vaccines[363]. Vaccination with the minigene vector induced only weak CTL that could be boosted by peptide P1A/AB in QS-21. The remaining two constructs were able to elicit

**Table 2.4. Vaccination approaches designed to target P1A/AB.**

| Vaccine | CTL Response | Protection | Reference |
|---|---|---|---|
| P815 cells | Yes | Yes against P815 | (364) |
| P1A + B7.1 transfected tumour cells | Yes (No inflammatory response in testis) | Yes against P511 60% at day 40 25% at day 25 | (359) (362) |
| P1A/AB peptide<br><br>P1A/AB peptide +IL-12 and QS-21 | No<br><br>Yes | Not tested<br><br>Not tested | Personal communication, Uyttenhove, C. |
| Adenoviral vector encoding AB minigene<br><br>Adenoviral vector encoding 83aa from P1A | Weak (boosted by peptide + adjuvant)<br><br>Yes | Not tested<br><br>Not tested | (363)<br><br>Personal communication, Uyttenhove, C. |
| Chimeric Papillomavirus virus-like particles encoding 90 base pairs of P1A. | Yes | Yes against P815 progressor cell line 60% at day 30 | (365) |
| Semliki Forest viral vector with full length P1A | Yes | Yes against P815 50-60% at day 100 | (366) |
| Phage display particles expressing P1A/AB peptide | Yes | Yes against P815 progressor cell line 70% at day 60 | (367) |
| DNA vaccine encoding full length P1A linked to an epitope tag | Yes | Yes against P815 60% at day 50 | (368) |
| P1A/AB peptide pulsed DC + helper epitopes or IL-12 or extended peptide | Yes but not with peptide P1A/AB alone | Not tested | (369-371) |
| P1A/AB peptide pulsed DC + IL-12 | Yes | Yes against P1.HTR1 100% at day 30 | (372) |
| CD40L matured DC pulsed with P1A/AB peptide | Yes | Yes 89% at day 60 against P815 | (373) |

effective CTL, but protection from tumour challenge was not analysed. Colmereo *et al*[366] alsoused a Semliki Forest viral vector encoding the whole *P1A* gene sequence, where AB-specific CTL were inducible and mediated protection from tumour challenge. However, viral based therapies preclude multiple vaccinations. P1A/AB peptide pulsed DC have also been examined in a vaccination protocol. No CTL activity was detected when vaccinating with the pulsed DC alone, but responses were readily detected by the addition of T helper epitopes from tetanus toxin or the co-administration of IL-12[371] or by increasing P1A peptide length[370]. These studies have demonstrated induction of P1A/AB specific CTL, and confirmed a requirement for $CD4^+$ T-cell help in priming P1A/AB specific $CD8^+$ T cells. Despite the success in generating P1A/AB specific CTL following vaccination and re-stimulation *in vitro*, the actual levels of tumour protection observed *in vivo* are not exceptional. In addition there is little information regarding the actual numbers of activated epitope-specific $CD8^+$ T cells *ex vivo* following different vaccination methods but the indication from this limited data is that no correlation exists between the numbers of peptide-specific $CD8^+$ T cells and the level of tumour cell killing both *in vitro* and *in vivo*. Finally the relationship between the affinity and/or avidity of the P1A/AB specific $CD8^+$ T cells and the levels of tumour protection also remains elusive.

### 2.1.4 Aims of this study

- Evaluate the efficacy with which p.DOM.epitope DNA vaccine design incorporating the P1A/AB motif induces $CD8^+$ T-cell responses.
- Quantify the $CD8^+$ T-cell response following vaccination with p.DOM-P1A/AB.
- Evaluate the ability of activated P1A/AB specific $CD8^+$ T cells to kill tumour cells *in vitro*.
- Assess the ability of p.DOM-P1A/AB vaccination to induce protective immunity.
- Analyse the expression of *P1A* in tumours cells growing in mice following vaccination with p.DOM-P1A/AB.
- Extend the P1A/AB peptide sequence fused to p.DOM to incorporate the P1A encoded putative MHC class II motif. Analyse whether vaccination with p.DOM-ABlong can induce both P1A specific $CD8^+$ T cells and $CD4^+$ T cells.
- Compare the p.DOM-P1A/AB DNA vaccine design with DNA vaccines encoding full length P1A to induce P1A/AB specific $CD8^+$ T cells detectable *ex vivo*. This allows the evaluation of the efficacy with which the putative P1A encoded MHC class II epitope can activate $CD4^+$ T cells and provide T-cell help.

- Compare p.P1A with DNA vaccines encoding full length P1A fused to FrC or DOM to induce P1A/AB specific $CD8^+$ T-cells detectable *ex vivo*. Here additional MHC class II epitopes provided by FrC or DOM may amplify the $CD8^+$ T-cell response.
- Investigate protocols targeting two antigens expressed by the same tumour cell. P815 expresses the P1E epitope as well as the viral CTL epitope AH1.

## 2.2 Results

### 2.2.1 Expression of CTA in a panel of murine tumours

Our initial studies were aimed at identifying a suitable murine tumour model displaying CTA expression. Several murine tumours of diverse histological type were screened using RT-PCR assay for the expression of both murine *Mage a* and *b* family genes, the homologues of the human *MAGE* genes. These tumours included the myeloma cell lines 5T2, 5T13 and 5T33. In 10/10 tumour lines analysed, each was negative for expression of *Mage* genes (Table 2.5). It has been demonstrated that human *MAGE* expression can be induced *in vitro* using a de-methylating agent 5'-aza 2-deoxycytidine (DAC)[374]. We evaluated this agent as an inducer of murine CTA gene expression by incubating the tumour cell lines MOPC-315, EL-4, and P815 for 72 hours with 1μM DAC. For MOPC-315, DAC concentration was titrated further at 1-8μM. RT-PCR analysis of cell lines after treatment revealed that *Mage* expression was not inducible (data not shown). Consequently we opted to utilise the P815 tumour to model our strategy for targeted therapy using DNA vaccines.

### 2.2.2 Expression of *P1A* and *P1E*

We confirmed expression of both *P1A* and *P1E* by RT-PCR. Expression of *P1A* was verified in P815 and a sub-clone P511 and was absent in P1-204, a further P815 sub-clone, and in $BCL_1$ used as a control (Figure 2.1). *P1E* expression was also verified in P815 and its two sub-clones P511 and P1-204 and was absent in $BCL_1$ (Figure 2.1).

### 2.2.3 DNA vaccine constructs

A panel of DNA vaccines were designed and constructed (represented in Figure 2.2). Integrity of each DNA vaccine was confirmed by restriction enzyme digest, DNA sequence analysis and *in vitro* transcription/translation (data not shown). Each of the vaccines expressed a protein of the expected size, calculated approximately by assuming an average molecular mass for each amino acid to be 110 Daltons. The two plasmids, p.AB and p.ABlong encoded proteins too small to be detected using the *in vitro* transcription/translation system.

**Table 2.5. Expression of the *Mage* genes in murine tumours**

| Tumour | Type | *Magea* consensus | *Magea2* | *Magea5* | *Mageb* consensus | *Mageb3* |
|---|---|---|---|---|---|---|
| A31 | B cell (lymphoma) | –* | – | – | – | nd |
| EL4 | T cell (lymphoma) | – | – | – | – | – |
| C6VL | T cell (lymphoma) | – | – | – | – | nd |
| 5T13 | B cell (myeloma) | nd | – | – | –* | nd |
| 5T2 | B cell (myeloma) | nd | – | – | nd | nd |
| 5T33 | B cell (myeloma) | – | – | – | – | – |
| 32Db3a2 | Myeloid cell line | nd | nd | nd | – | nd |
| 32DdR1 | Myeloid cell line | nd | nd | nd | – | nd |
| B16 | Melanoma | – | – | – | – | – |
| CT26 | Colon carcinoma | nd | nd | nd | – | nd |

RT-PCR was performed to evaluate the expression of murine *Mage* genes in various murine tumour cell models. Primers used were either generic (consensus) for all *Magea* or *Mageb* genes or they were specific for the family members *Magea2* and *a5* and *Mageb3*. No tumour cell line screened was positive for *Magea* or *b*.
nd = not determined
* = DNA contamination

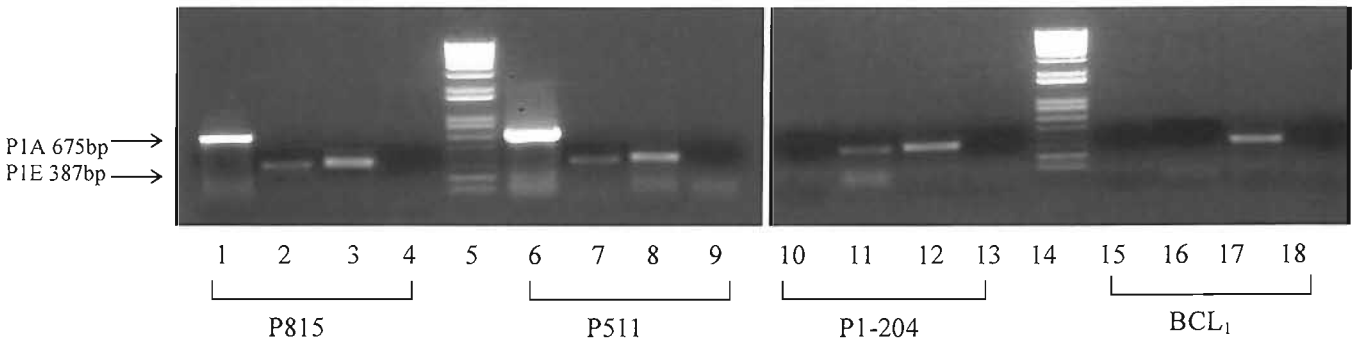



**Figure 2.1. Expression of genes *P1A* and *P1E* in the murine tumour model P815 and its derivatives**

RT-PCR assays were carried out to evaluate the expression of *P1A* and *P1E* and *β actin* in murine tumours. Amplified DNA products from P815 are shown in lanes 1-4, P511 lanes 6-9, P1-204 lanes 10-13 and $BCL_1$ lanes 15-18.
Lanes 1, 6, 10 and 15: P1A
Lanes 2, 7, 11 and 16: P1E
Lanes 3, 8, 12 and 17: β actin control
Lanes 4, 9, 13 and 18: blank
P815 is positive for the expression of *P1A* and *P1E*. P511 is positive for the expression of *P1A* and *P1E*. P1-204 is positive only for the expression of *P1E*. $BCL_1$ does not express *P1A* or *P1E*.

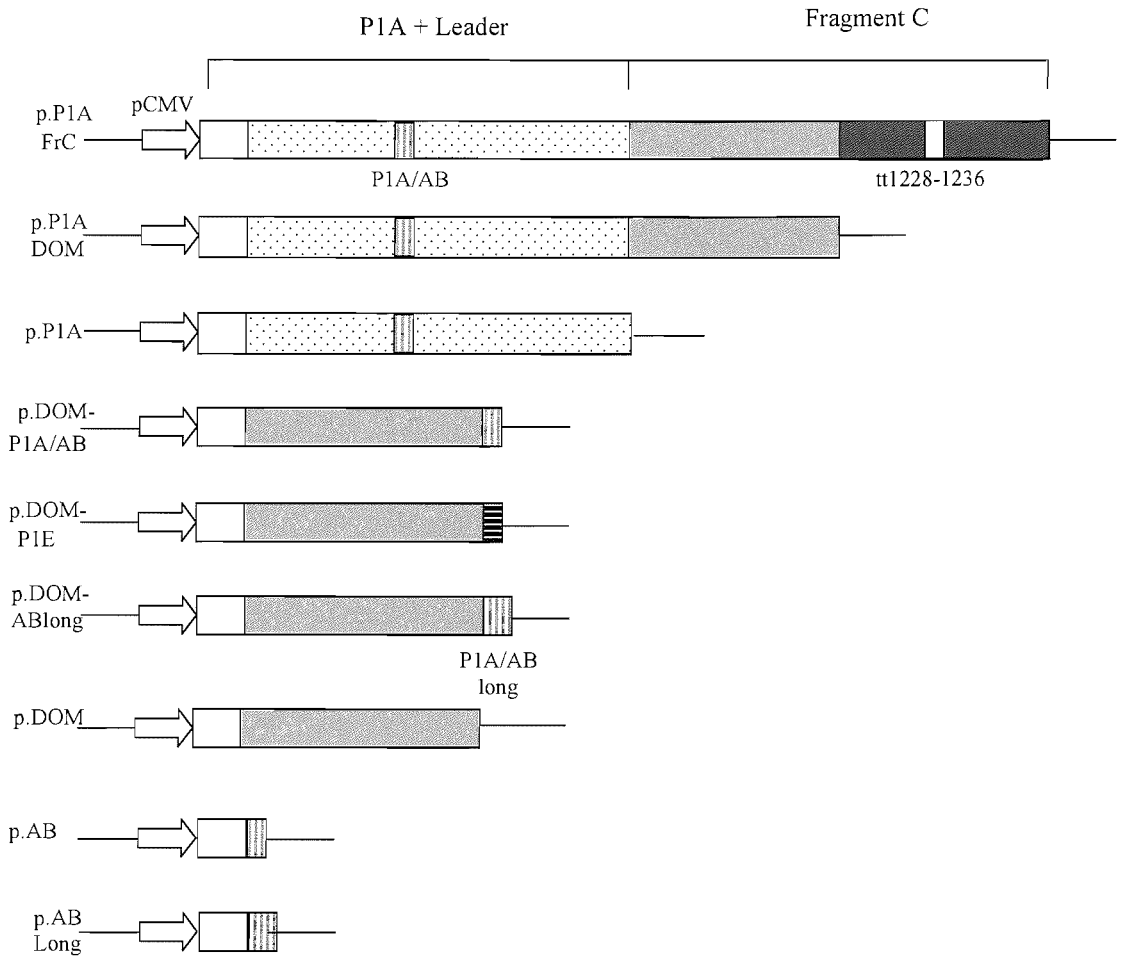



**Figure 2.2. Diagrammatic representation of DNA vaccines encoding P1A derived sequences**

P1A/AB (LPYLGWLVF) maps to codons 35-43 and ABlong (EILPYLGWLVFA) maps to codons 33-44 within the P1A gene. P1E (GYCGLRGTGV) maps to codons 216-222 within the murine methionine sulfoxide reductase (MsrA) gene. tt1228-1236 (GYNAPGIPL) is a H-$2^d$ MHC class I epitope from the second domain of FrC.

### 2.2.4 Monitoring anti-FrC antibody responses following vaccination

Vaccine induced anti-FrC antibody IgG titres were assayed by ELISA to confirm expression and correct folding *in vivo*. Antibody levels were assayed in serum collected from mice at day 42 following 4 vaccinations with DNA constructs (Figure 2.3). Anti-FrC antibody responses were inducible using p.DOM-P1A/AB, p.DOM-ABlong and p.DOM constructs but were not detected following vaccination with p.P1AFrC or p.P1ADOM. This may reflect incorrect folding of P1AFrC and P1ADOM *in vivo*. p.P1A vaccinated mice showed no specific anti-FrC responses as expected.

### 2.2.5 Vaccine p.DOM-P1A/AB can induce epitope-specific $CD8^+$ T-cells detectable *ex vivo*

To investigate the ability of the vaccine p.DOM-P1A/AB to induce epitope-specific $CD8^+$ T cells, groups of 2-3 DBA/2 mice were vaccinated once and spleens taken at day 14 or day 21 for analysis. Pooled splenocytes were stimulated *in vitro* for 4 hours with the AB peptide and then stained with fluorochrome labelled mAbs specific for MHC class II, CD8 and IFNγ and analysed by FACS. MHC class II positive cells were excluded from analysis and the number of $CD8^+$/IFNγ double positive cells was calculated as a percentage of total $CD8^+$ T cells. This vaccination experiment was repeated 4 times: 3 times after one vaccination and spleens taken at day 14 and once after one vaccination and spleens taken at day 21. However, a conclusive positive result was detected only once at day 14 (Figure 2.4). $CD8^+$ T-cells (1.5%) induced with p.DOM-P1A/AB were specifically activated by the AB epitope and produced IFNγ, as compared to 0.07% $CD8^+$ T cells from mice vaccinated with the control vaccine p.DOM and a background staining of 0.25% using isotype control antibodies. The absence of detectable epitope-restricted $CD8^+$ T-cell responses in some of these experiments may be due to the sensitivity of the assay, since only small numbers of cells are activated after one vaccination. It is also possible that by pooling splenocytes from groups of vaccinated mice, individual responses could be masked by animals in which no response occurs. Therefore, subsequent assays were performed on spleens from individual mice. A DNA boost was also given in some experiments to analyse whether this would enhance the numbers of detectable peptide-specific $CD8^+$ T cells. However, again the results of several assays were inconclusive due to a high level of background staining. Meaningful results from a single experiment (day 14, 1 vaccination) and another experiment (day 28, 2 vaccinations) are shown in Figure 2.5. and 2.6. respectively. It was clear from these experiments that not every mouse generated an immune response. At day 14, a

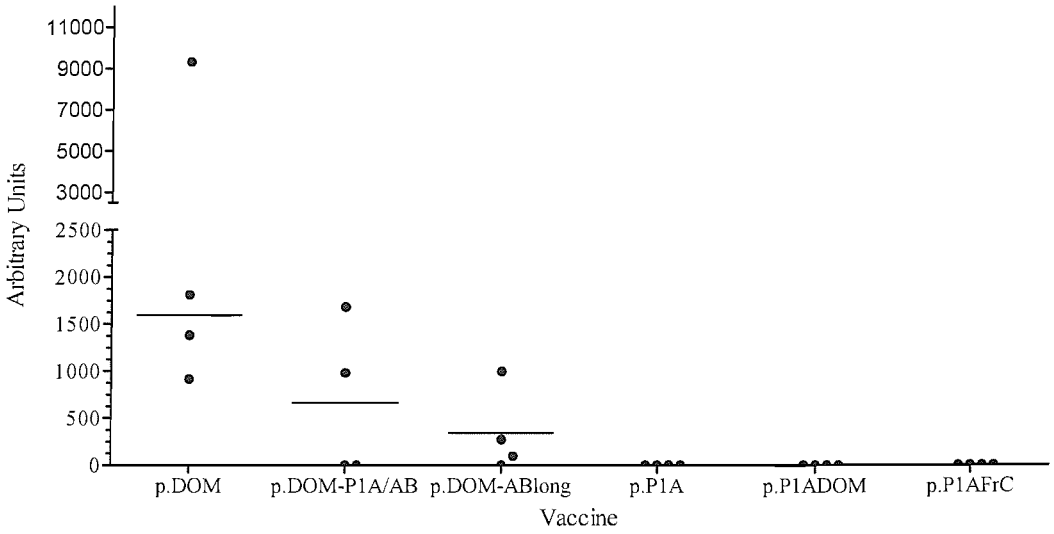



**Figure 2.3. Anti-fragment C antibody response in vaccinated DBA/2 mice**
Groups of DBA/2 mice were vaccinated 4 times with p.DOM-P1A/AB, p.DOM-ABlong, p.P1A, p.P1AFrC, p.P1ADOM or p.DOM (4 mice per group). Serum samples were collected on day 42. Anti-FrC antibody levels were determined by specific ELISA, and are shown as arbitrary units. Black circles (●) denote responses made by individual mice, and black bars (—) the mean response in each group. The induction of anti-FrC antibodies by p.DOM-P1A/AB and p.DOM-ABlong indicate the correct folding of the fusion protein *in vivo*. The absence of a detectable anti-FrC antibody response following vaccination with p.P1AFrC and p.P1ADOM indicate incorrect folding *in vivo*. p.P1A vaccination was used as a negative control as an anti-FrC antibody response to vaccination with this construct was not expected.

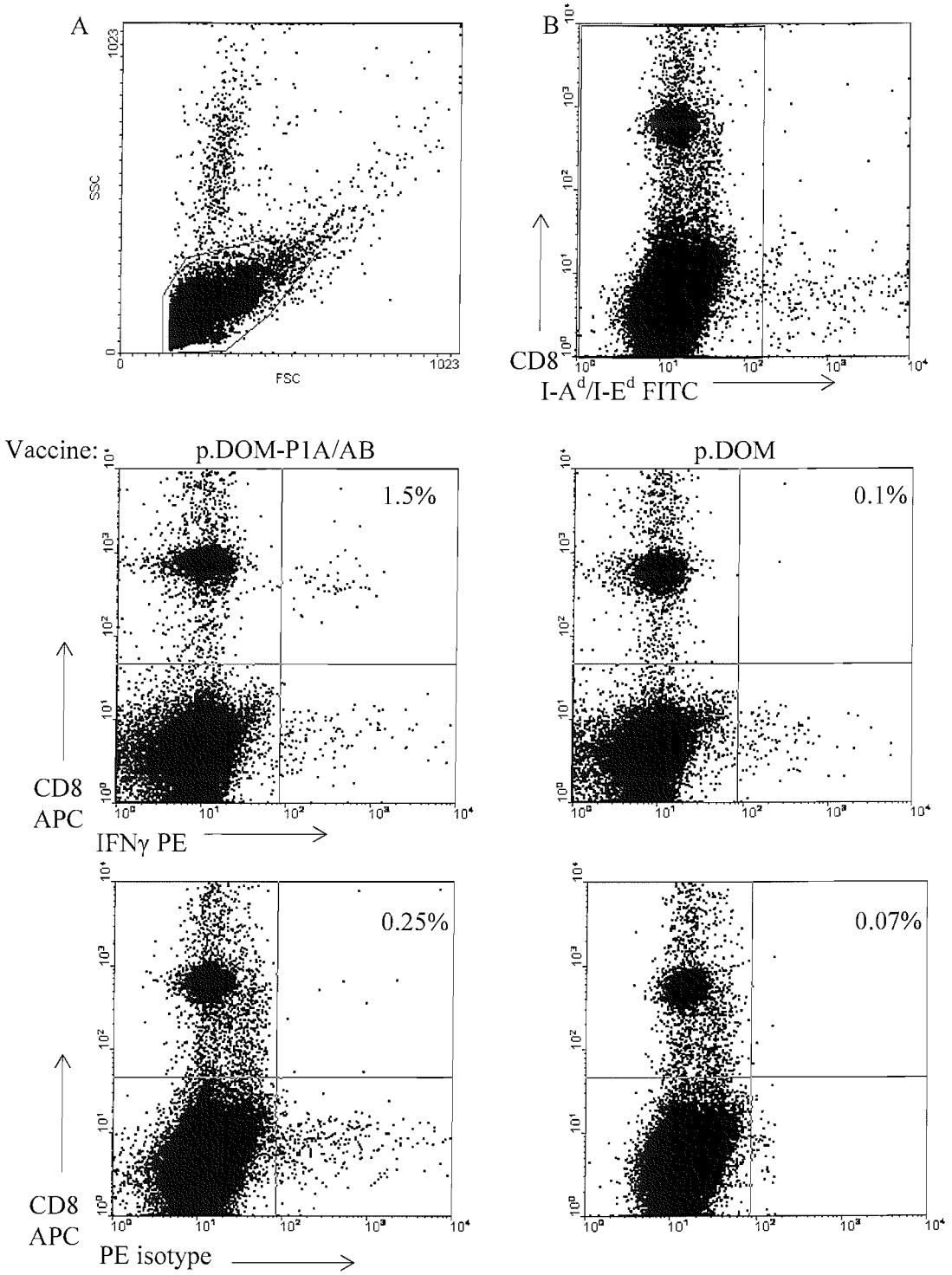



**Figure 2.4. CD8$^+$ T-cell response measured *ex vivo* from mice vaccinated with p.DOM-P1A/AB**

Groups of DBA/2 mice were vaccinated once (50μg) with p.DOM-P1A/AB or p.DOM. Splenocytes were collected and pooled (4 mice) on day 14 and re-stimulated *in vitro* for 4 hours with P1A/AB peptide. Cells were labelled with mAbs specific for MHC class II (I-A$^d$/I-E$^d$) FITC, CD8 APC and IFNγ PE or their isotype controls and FACS analysed. 100,000 events were collected and lymphocytes were gated on according to FSC/SSC properties (A) and MHC class II positive cells were excluded from further analysis (B). Cells positive for both CD8 and IFNγ were calculated as a percent of total CD8$^+$ cells. Vaccination with p.DOM-P1A/AB induced measurable levels of epitope-specific CD8$^+$ T cells (1.5%) compared to background in mice vaccinated with control p.DOM (0.1%) or to cells stained with the isotype control (0.25%).

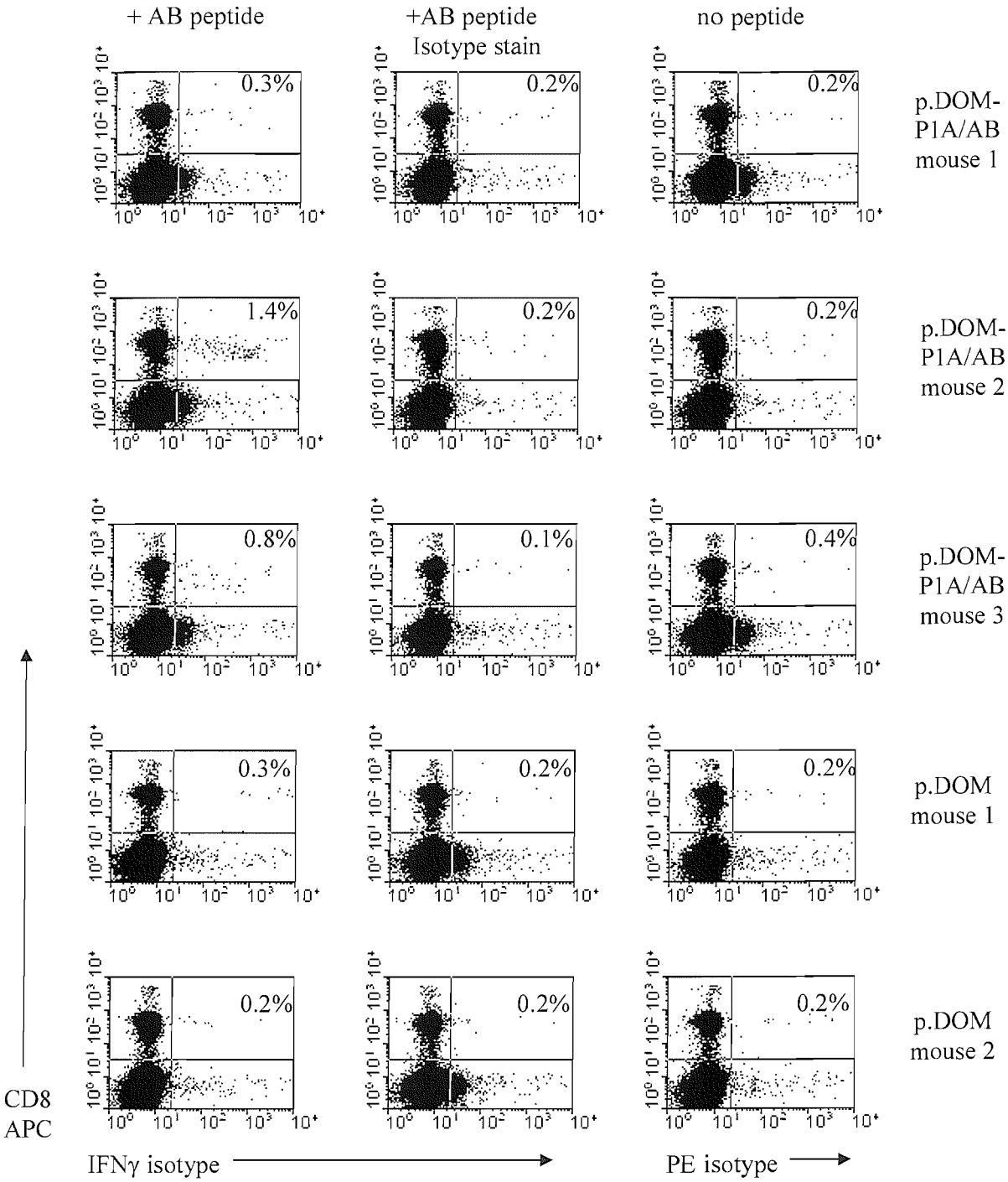



**Figure 2.5. Variability in levels of P1A/AB epitope-specific CD8$^+$ T-cell responses in individual mice following vaccination with p.DOM-P1A/AB**

DBA/2 mice were vaccinated once (50μg) with p.DOM-P1A/AB or p.DOM. Splenocytes were collected individually on day 14 and re-stimulated *in vitro* for 4 hours with or without P1A/AB peptide. Cells were labelled with mAbs specific for MHC class II (I-A$^d$/I-E$^d$) FITC, CD8 APC and IFNγ PE or their isotype controls and FACS analysed. 150,000 events were collected. Lymphocytes were gated on according to FSC/SSC properties and MHC class II positive cells were excluded from further analysis (not shown). Cells positive for both CD8 and IFNγ were calculated as a percent of total CD8$^+$ cells. Vaccination with p.DOM-P1A/AB induced measurable levels of epitope-specific CD8$^+$ T cells in two of four mice (1 experimental mouse not shown) compared to background in mice vaccinated with control p.DOM or to cells stained with the isotype control or stimulated without peptide.

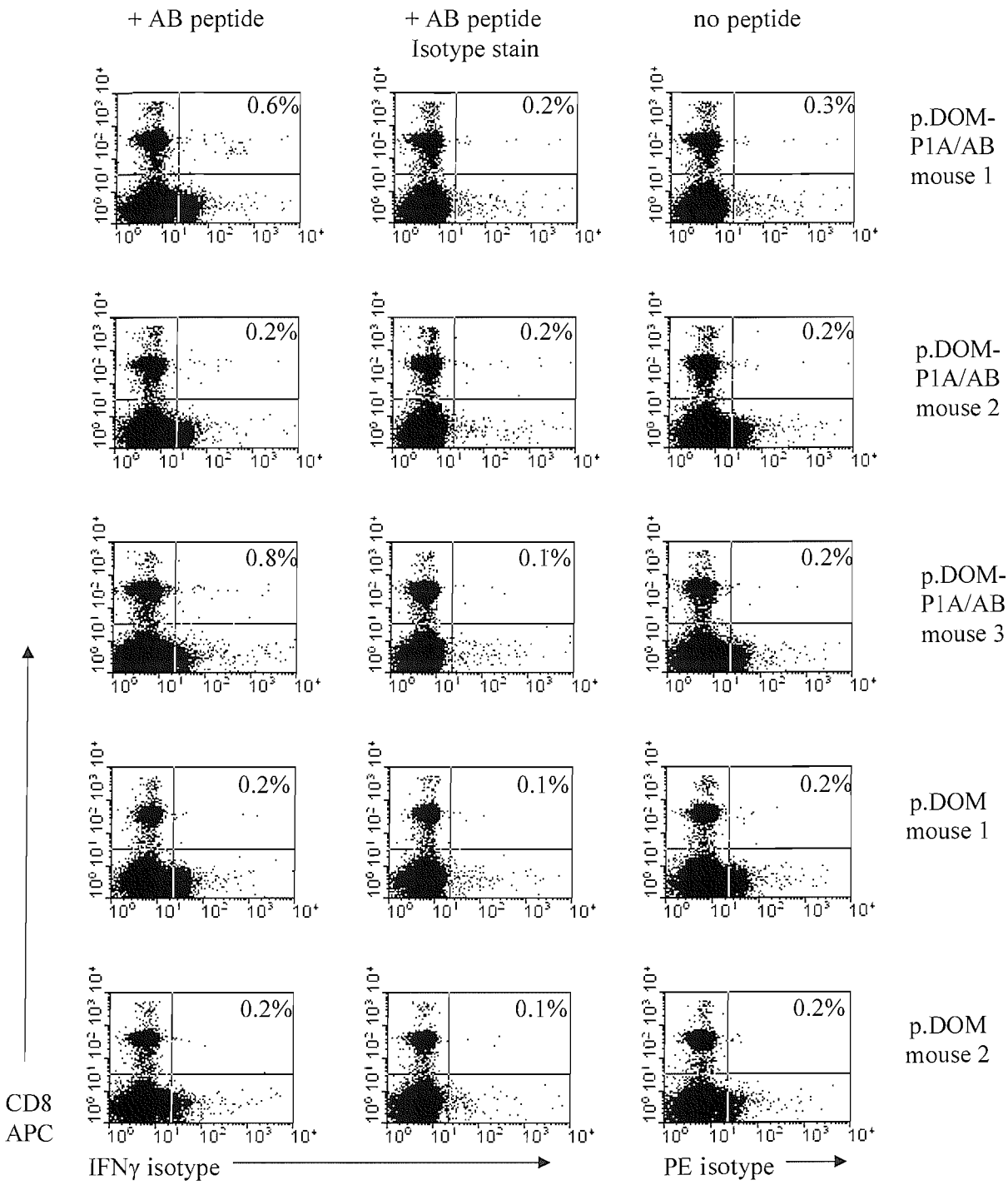



**Figure 2.6. Variability in levels of P1A/AB epitope-specific CD8$^+$ T-cell responses in individual mice following two vaccinations with p.DOM-P1A/AB**

DBA/2 mice were vaccinated twice (2x50μg) with p.DOM-P1A/AB or p.DOM. Splenocytes were collected individually on day 28 and re-stimulated *in vitro* for 4 hours with or without P1A/AB peptide. Cells were labelled with mAbs specific for MHC class II (I-A$^d$/I-E$^d$) FITC, CD8 APC and IFNγ PE or their isotype controls and FACS analysed. 150,000 events were collected. Lymphocytes were gated on according to FSC/SSC properties and MHC class II positive cells were excluded from further analysis (not shown). Cells positive for both CD8 and IFNγ were calculated as a percent of total CD8$^+$ cells. Vaccination with p.DOM-P1A/AB induced measurable levels of epitope-specific CD8$^+$ T cells in two of four mice (1 experimental mouse not shown) compared to background in mice vaccinated with control p.DOM or to cells stained with the isotype control or stimulated without peptide.

peptide-specific $CD8^+$ T-cell response was detected in 2/4 mice, with responses of 1.4% and 0.8% compared to backgrounds of 0.2% and 0.4% respectively in the absence of AB peptide (Figure 2.5., mouse 2 and 3). At day 28, a similar pattern was observed in 2/4 mice revealing a peptide-specific $CD8^+$ T-cell response of 0.6% and 0.8% compared to backgrounds of 0.3% and 0.2% respectively (Figure 2.6, mouse 1 and 3). With these limitations of data it is difficult to assess whether there were any clear quantitative differences at the two sampling time points in terms of numbers of peptide-specific activated $CD8^+$ T-cells measured. In an attempt to overcome the background difficulties encountered with intracellular staining, an ELISPOT assay was employed. The increased sensitivity of this assay allowed detection of peptide-specific T cells *ex vivo* with ease. Responses detected in individual mice after a single vaccination with p.DOM-P1A/AB are shown in Figure 2.7, and are representative of 9 experiments. The cumulative data are shown in Figure 2.8 (A) and indicate the large degree of variation between mice, both in terms of whether a response is induced and the number of responding T cells. A response was considered positive if it was $\geq$ the mean plus 1 standard deviation of the control p.DOM vaccinated mice. Approximately 54% (13/24) of the mice made a response, with the number of IFNγ spots (per $10^6$ lymphocytes) ranging from 28 to 198 (average 56.7) (Figure 2.8A). In 4 experiments, mice were given a boost at day 21 and responses measured at day 28, with the cumulative data shown in Figure 2.8 (B). Variation in mice generating a detectable response (6/11) was again observed suggesting that this is not simply a consequence of vaccination failure. From these data, there is some difficulty in comparing the immune responses at day 14 with those at day 28 due to the large variation among assays. However, it is clear that a DNA vaccine boost does not amplify the $CD8^+$ T-cell response to the AB peptide. Interestingly, a response to the p30 helper epitope was not observed in any mice at any time point (data not shown). The anti-FrC antibody response to vaccination (Figure 2.3) however, indicates that a $CD4^+$ T-cell response is induced by vaccination with p.DOM encoding plasmids in this strain of mice. Additional MHC class II epitopes are likely to exist in the first domain of FrC but this was not analysed further.

### 2.2.6 DNA vaccination with p.DOM-P1A/AB induces cytotoxic T lymphocytes that lyse tumour cells *in vitro*

Although vaccination with p.DOM-P1A/AB can induce IFNγ producing AB epitope-specific $CD8^+$ T cells, it does not necessarily follow that these CTL are capable of lysing

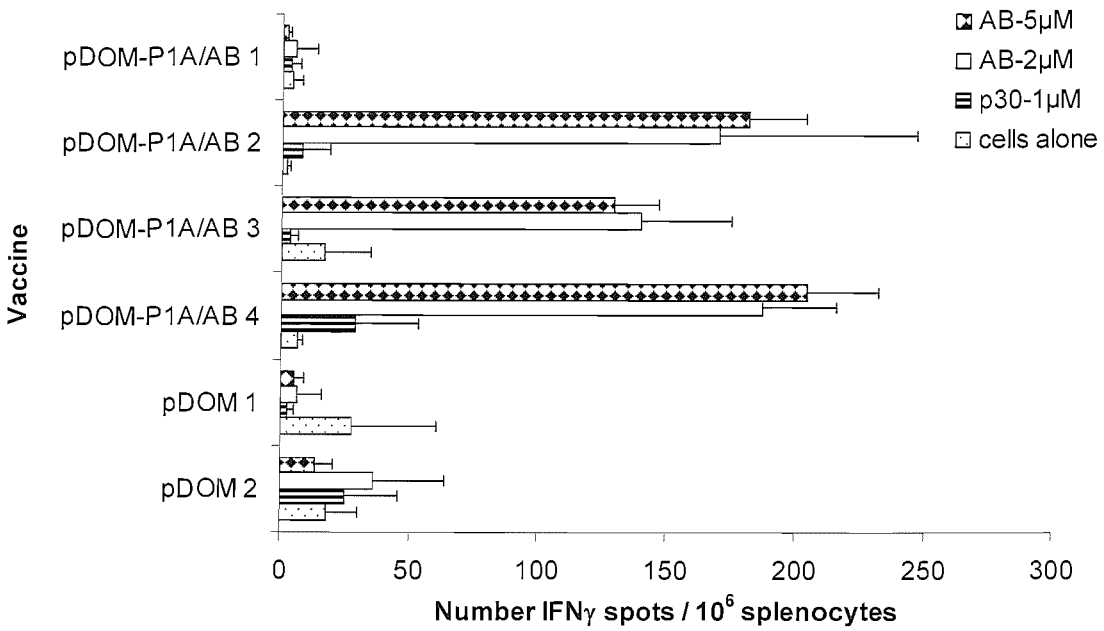



**Figure 2.7. ELISPOT assays allow the detection of epitope-specific IFNγ producing T cells following DNA vaccination with p.DOM-P1A/AB**

Splenocytes from mice vaccinated once with p.DOM-P1A/AB or p.DOM were collected on day 14 and plated in triplicate in a ELISPOT plate coated with purified anti-mouse IFNγ mAb. Cells were re-stimulated *in vitro* for 24 hours with or without the indicated concentrations of P1A/AB peptide or p30 peptide. A secondary biotinylated anti-mouse IFNγ mAb was used to capture IFNγ producing cells, followed by incubation with strepdavidin ALP and development according to the manufacturer's guidelines. Spots were detected using an automated counter. DNA vaccination with p.DOM-P1A/AB induced detectable levels of epitope-specific T cells in three of four mice compared to background numbers observed when cells were incubated in the absence of P1A/AB peptide.

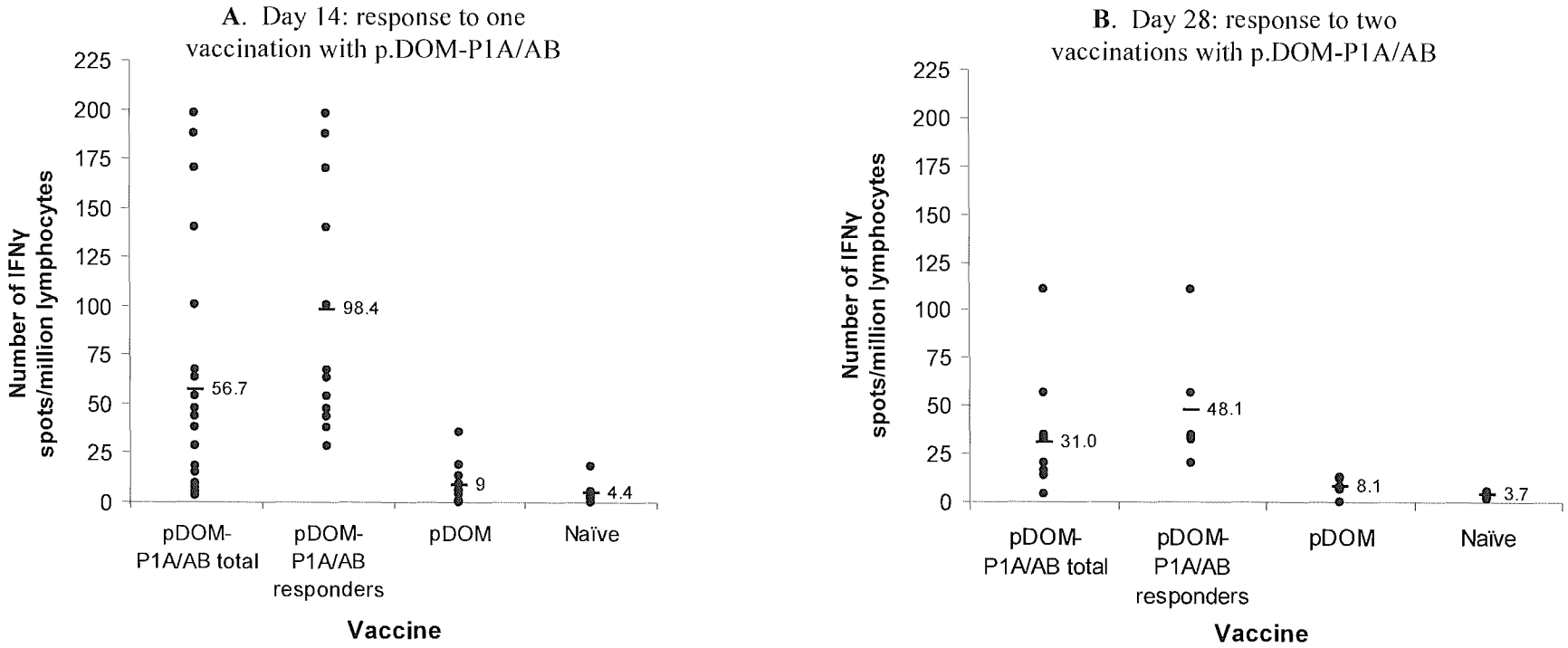



**Figure 2.8. ELISPOT assays were used to detect the range of epitope-specific T-cell responses made in individual mice following one or two DNA vaccinations with p.DOM-P1A/AB**

Splenocytes from mice vaccinated with p.DOM-P1A/AB or p.DOM were collected on day 14 (A) and on day 28 (B) and re-stimulated *in vitro* for 24 hours with 2μM P1A/AB peptide. ELISPOT assays were used to detect epitpe-specific IFNγ producing T cells in individual mice. A: cumulative data for 24 mice vaccinated once with p.DOM-P1A/AB, 14 mice vaccinated once with pDOM and 13 naïve mice. Approximately 54% of mice vaccinated with p.DOM-P1A/AB made a detectable response (≥ the mean plus 1 standard deviation of the control p.DOM vaccinated mice (>18 IFNγ spots per million lymphocytes)), with an average response of 56.7. When the responding mice are plotted alone, an average response of 98.4 IFNγ spots per million lymphocytes is achieved. B: cumulative data for 11 mice vaccinated twice with p.DOM-P1A/AB, 7 mice vaccinated twice with pDOM and 6 naïve mice. Again, approximately 54% of mice vaccinated twice with p.DOM-P1A/AB made a detectable response, with an average response of 31.0. When the responding mice are plotted alone an average response of 48.1 IFNγ spots per million lymphocytes is achieved.

target tumour cells either *in vitro* or *in vivo*. CTL assays were then undertaken following 1 vaccination and splenocytes taken at day 14 before being re-stimulated *in vitro* for 1 week. Antigen-specific $CD8^+$ T cells from pooled splenocytes shown to be activated *ex vivo*, proliferated dramatically from 1.5% of the $CD8^+$ T-cell population to 33% of the $CD8^+$ T-cell population after re-stimulation with 100nM peptide AB (Figure 2.9, 1/1 experiment). Low levels of background IFNγ production is seen in the control vaccinated mice, this may be due to natural low level expression of P1A *in vivo* as has been observed in BALB/c mice[375]. CTL assays performed in parallel demonstrated their ability to lyse tumour targets either pulsed exogenously with the AB peptide ($BCL_1$/AB or P1-204/AB, Figure 2.10 A) or expressing the AB epitope endogenously (P815 or P511, Figure 2.10 B) at effector:target ratios as low as 5:1. The lysis was specific as no killing was observed against un-pulsed control cell lines $BCL_1$ or P1-204 that do not express *P1A*, and only background CTL activity was detected against tumour cell targets when mice were vaccinated with the control construct p.DOM (Figure 2.10, represenatative of 3 experiments). CTL assays were repeated using splenocytes taken at day 21 after one vaccination and confirmed this result (data not shown). To validate the functional link between *ex vivo* peptide-specific IFNγ synthesis and the ability of the T cells to kill target tumour cells, splenocytes from individual mice with a positive ELISPOT response were re-stimulated *in vitro* with peptide AB. Figure 2.11 shows the association between *ex vivo* IFNγ production by $CD8^+$ T cells and the ability of these cells to kill peptide pulsed tumour cells and is representative of 2 experiments.

In a further experiment, pooled splenocytes from mice vaccinated with p.DOM-P1A/AB were re-stimulated with tumour cells transfected with the gene P1A (L1210 $AB^+$). CTL expanded *in vitro* by this route were capable of lysing both AB peptide coated targets and tumour cells expressing the gene endogenously (Figure 2.12). This result confirmed the ability of our DNA vaccine to induce CTL which can recognise the endogenously processed P1A/AB epitope. Lysis levels are generally lower when CTL are re-stimulated using this method than with peptide, probably due to a lower density of available MHC-peptide-AB complexes.

### 2.2.7 Vaccination with p.DOM-P1A/AB mediates protection against tumour challenge *in vivo*

The next aspect of this investigation assessed the ability of the p.DOM-P1A/AB vaccine to induce immunity that would protect from tumour challenge. Vaccinated mice (x2 day 0,

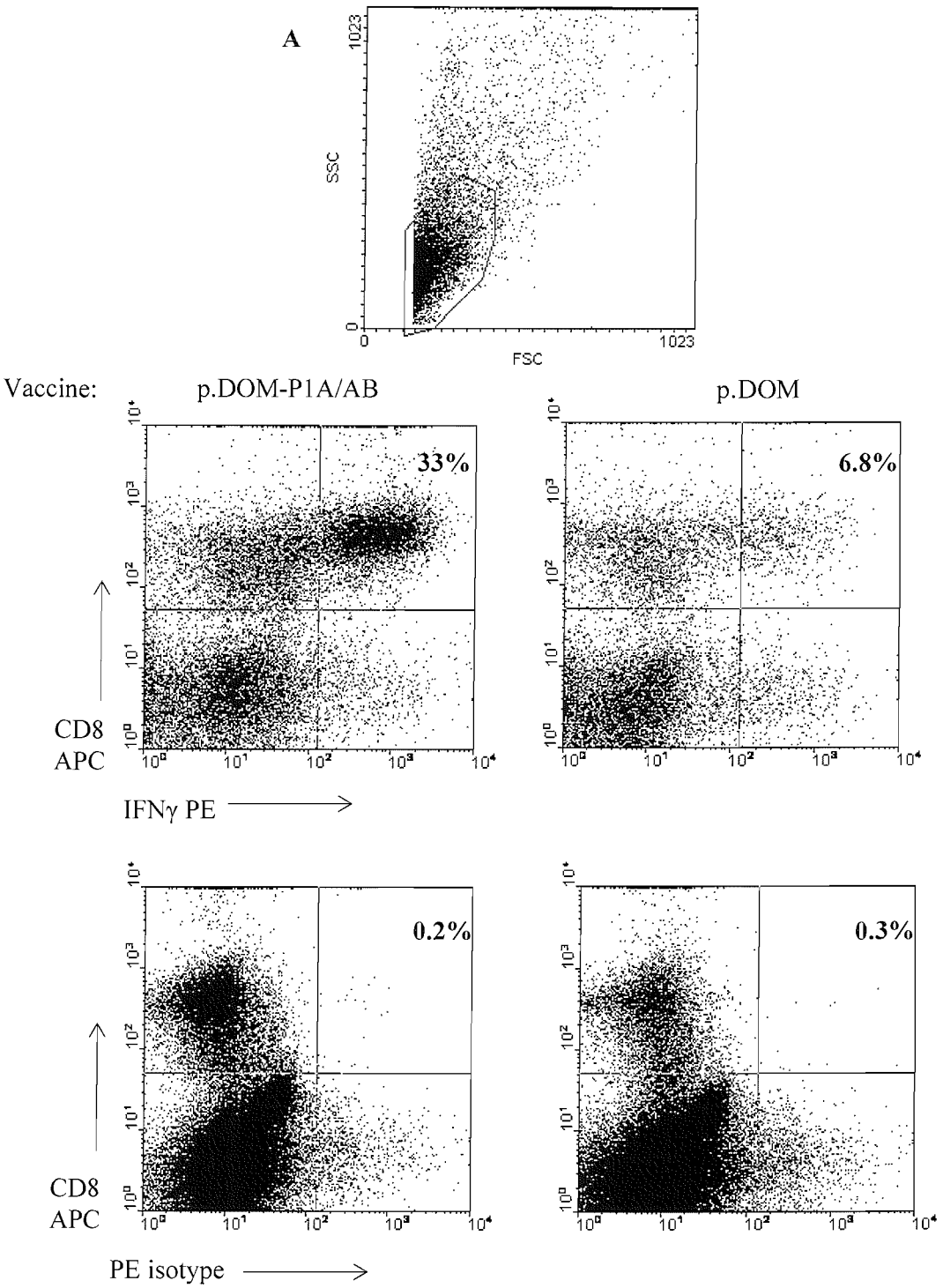



**Figure 2.9. DNA vaccine induced epitope-specific CD8$^+$ T cells expand *in vitro***

Groups of DBA/2 mice were vaccinated once with p.DOM-P1A/AB or p.DOM. Splenocytes were collected and pooled (4 mice) on day 14 and re-stimulated *in vitro* for 6 days with P1A/AB peptide. Cells were labelled with mAbs specific for CD8 APC and IFNγ PE or their isotype controls. Fluorescent staining was analysed by FACS. 100,000 events were collected and lymphocytes were gated on according to FSC/SSC properties (A). Cells positive for CD8 and IFNγ (upper right quadrants) were calculated as a percent of total CD8$^+$ cells. DNA vaccination with p.DOM-P1A/AB induced epitope-specific CD8$^+$ T cells that proliferated *in vitro* (33%) compared to background numbers from mice vaccinated with control p.DOM (6.8%) or to cells stained with the isotype control (0.2%).

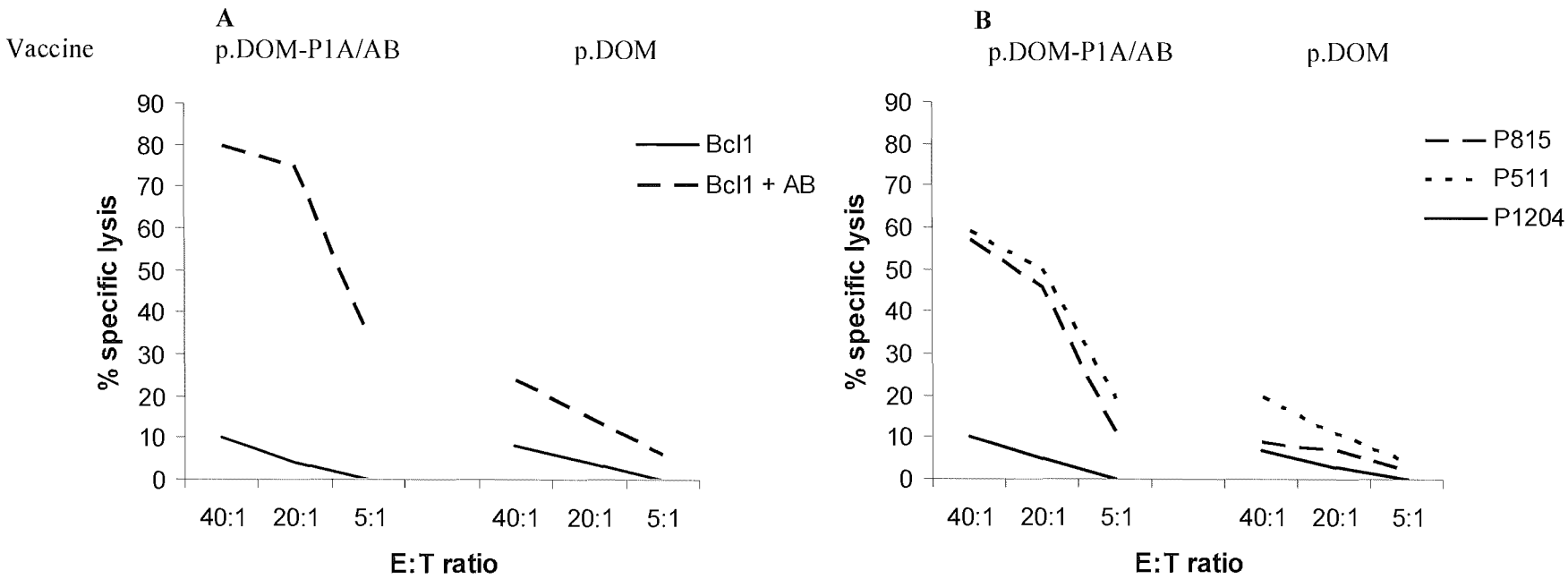
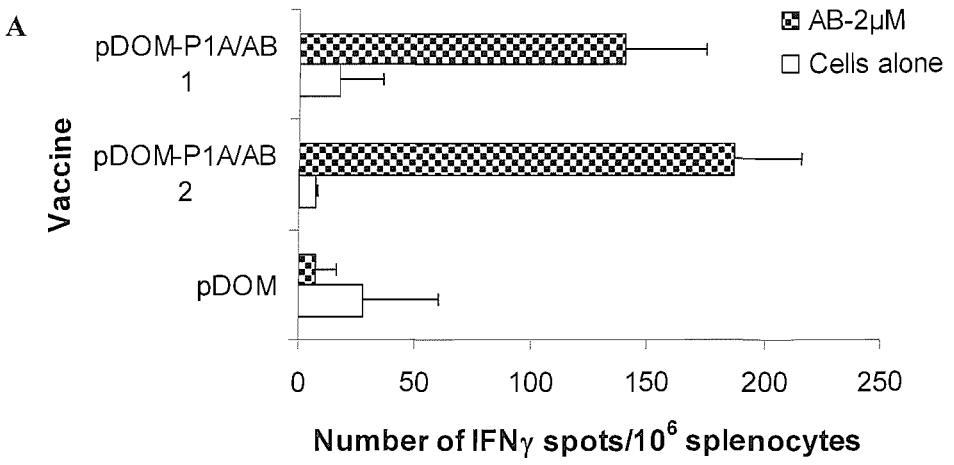



**Figure 2.10. P1A/AB specific $CD8^+$ T cells can lyse both peptide pulsed targets and tumour cells expressing the P1A gene endogenously**

Groups of DBA/2 mice were vaccinated once with p.DOM-P1A/AB or with the control vaccine p.DOM, and spleens were taken on day 14 and pooled (4 mice). Splenocytes were re-stimulated for 6 days *in vitro* with P1A/AB peptide (100nM). A $^{51}Cr$ release assay (5hour) was carried out using tumour target cells. (**A**) No lysis was observed against control P1A negative $BCL_1$ target cells but specific lysis of $BCL_1$ pulsed with P1A/AB was observed. (**B**) Similarly, specific lysis was observed against P815 and P511 tumour cells expressing P1A endogenously, but not against the P815 sub-clone P1-204 which does not express P1A.



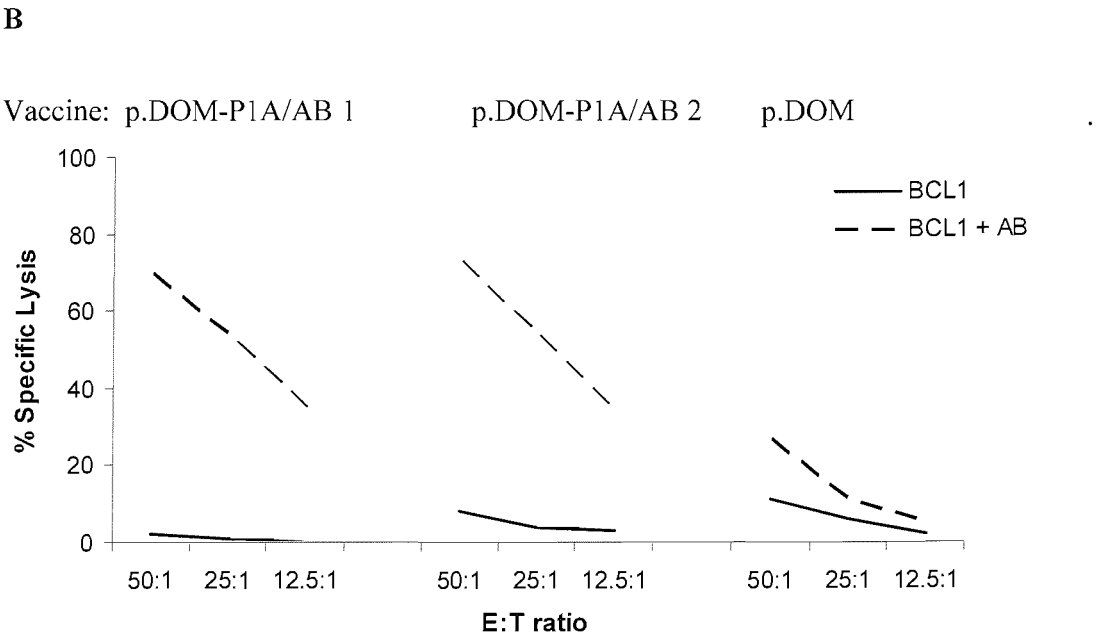




**Figure 2.11. IFNγ release from P1A/AB peptide-specific $CD8^+$ T cells reflects their ability to lyse target tumour cells pulsed with the AB peptide**

**A)** T cells from individual mice vaccinated once with p.DOM-P1A/AB produce a specific IFNγ response to the P1A/AB peptide (measured by ELISPOT assay) compared to mice vaccinated with the control vaccine p.DOM. This effector function associates with their ability to lyse tumour target cells (B). **B)** Remaining splenocytes taken at day 14 from individual mice receiving a single vaccination with p.DOM-P1A/AB or control p.DOM were incubated with peptide (500nM) for 6 days. CTL activity was measured using a $^{51}Cr$ release assay and peptide loaded $BCL_1$ tumour cells as targets. Representative data are shown from similar results obtained in two of two experiments.

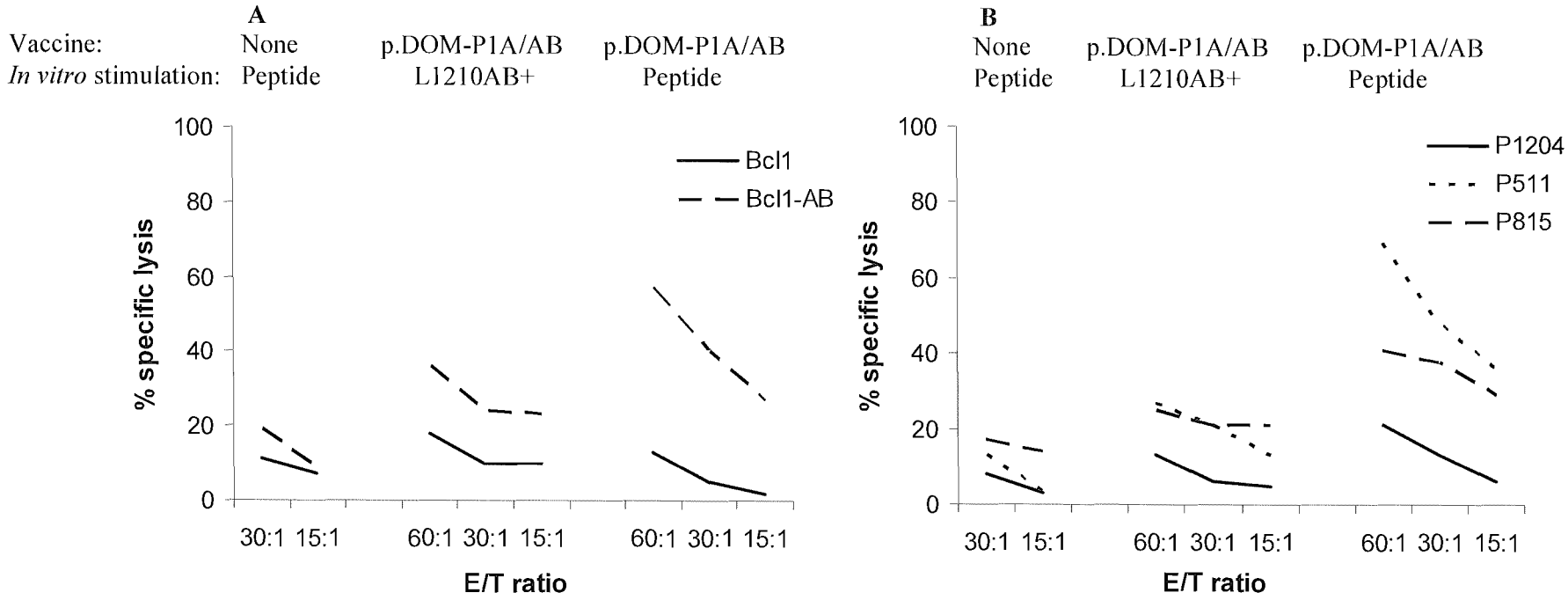



**Figure 2.12. DNA vaccine induced P1A/AB specific T cells can respond to re-stimulation with tumour cells expressing P1A endogenously.**

Groups of DBA/2 mice were vaccinated once with p.DOM-P1A/AB and spleens taken at day 14, and spleens were taken on day 14 and pooled (4 mice). Splenocytes were re-stimulated for 6 days *in vitro* with either 100nM P1A/AB peptide or with Mitomycin C treated L1210AB$^{+}$ tumour cells. A $^{51}$Cr release assay was carried out against tumour target cells. **(A)** No lysis was observed against control P1A negative $BCL_1$ target cells but specific lysis of $BCL_1$ pulsed with P1A/AB was observed. **(B)** Similarly, specific lysis was observed against P815 and P511 tumour cells expressing P1A endogenously, but not against the P815 sub-clone P1-204 which does not express P1A.

day 21) were challenged by intra-peritoneal (i.p.) route with the P815 tumour sub-clone P511. Mice were then monitored for a minimum of 100 days and sacrificed when the ascitic burden reached 20% of the original body weight. Representative data from one experiment (of two) are shown in Figure 2.13. All the mice vaccinated with p.DOM were culled between day 15 and 36 due to burden and 90% of naïve mice were also culled. Significantly 50% of the mice vaccinated with p.DOM-P1A/AB were protected from tumour challenge to day 80 and 40% were protected to day 150 ($p=0.001$, $\chi^2$ Logrank test test, p.DOM-P1A/AB vs. p.DOM). A semi-quantitative RT-PCR analysis of tumour cells arising in the mouse culled at day 81 (Figure 2.13) demonstrated that *P1A* gene expression was lost at this stage (lane 1), relative to a reduced expression of *β-actin* (lane 2) (Figure 2.14). These tumour cells were cultured *in vitro* for 2 weeks to see if levels of gene expression would recover. After this time, *β-actin* expression was comparable to levels seen in the *in vitro* passaged cell line (lane 6, Figure 2.1). *P1A* gene expression however did not recover (Figure 2.14, lane 5) indicating a clonally homogeneous outgrowth of a P1A loss variant. In the second experiment (Appendix A), tumour growth was more aggressive and all naïve mice and 90% of mice vaccinated with p.DOM were culled before day 15. A significant delay in tumour growth was observed in mice vaccinated with p.DOM-P1A/AB ($p=0.0082$, $\chi^2$ Logrank test, p.DOM-P1A/AB vs. p.DOM). However, by day 50 only 10% of these mice were tumour free. Again tumours which emerged were assessed for *P1A* expression at various time points in each of three treatment groups, p.DOM-P1A/AB, p.DOM and naïve. As shown in Figure 2.14, both *P1A* expression and *β-actin* were evident in tumour cells arising in mice in each of the three groups at day 15 (p.DOM-P1A/AB, p.DOM and naïve) (lanes 9, 10, 13, 14, 17 and 18 respectively), at levels comparable with the *in vitro* passaged P511 cell line (lane 6, Figure 2.1). Tumours emerging by day 29 in the p.DOM-P1A/AB vaccinated group had begun to show a level of *P1A* down-regulation (lane 21), which was recoverable when cells were cultured *in vitro* for 2 weeks (lane 25). Tumours arising at day 50 in contrast, had completely lost *P1A* expression (lane 29). These data clearly reveal the immune modulating selection pressure induced by the DNA vaccine which down-regulates tumour antigen expression to allow tumour escape. These observations do however underscore the limitations of vaccines targeting a single MHC class I binding epitope.

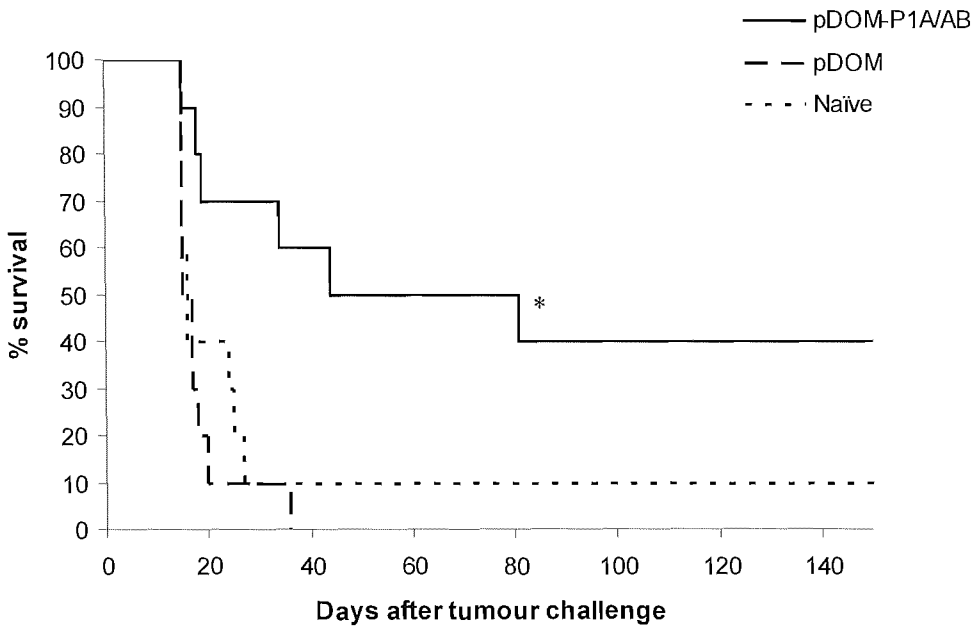



**Figure 2.13. DNA vaccination with p.DOM-P1A/AB generates significant protective immunity against tumour challenge**

10 Mice per group were vaccinated on days 0 and 21 with either p.DOM-P1A/AB or p.DOM or were untreated (naïve). Intra-peritoneal injection of $1x10^4$ P511 tumour cells was performed on day 28 and mice were sacrificed when ascitic burden reached 20% original weight of the mouse. Data from one of two experiments with similar results is shown. Vaccination with p.DOM-P1A/AB induced significant protection compared to p.DOM vaccinated controls ($p=0.001$ using $\chi^2$ Logrank test). (* refer to Figure 2.14).

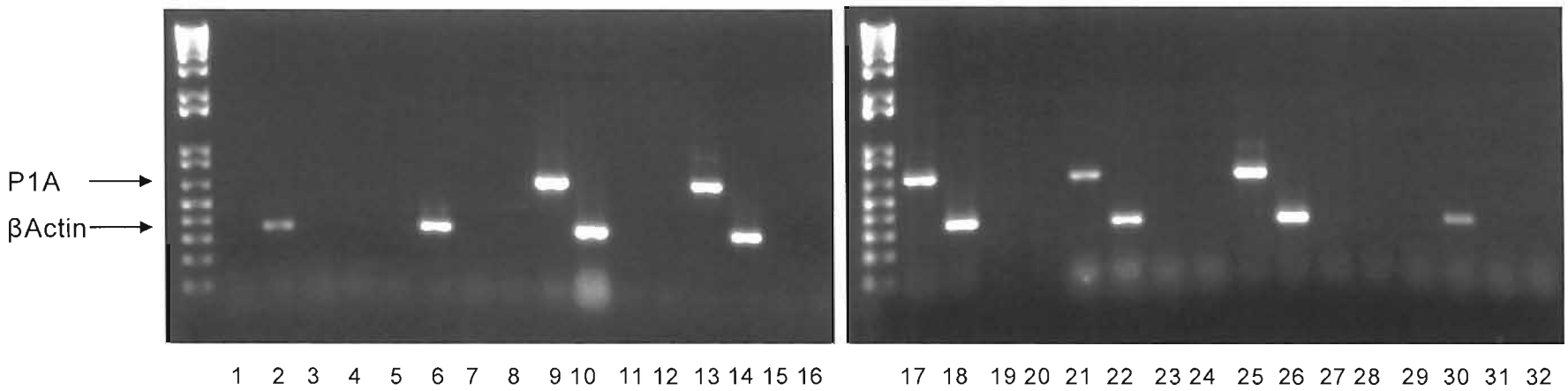



**Figure 2.14. Analysis of *P1A* expression in P511 tumour cells arising in vaccinated mice**

RT-PCR assays were carried out to evaluate the expression of *P1A* and *β actin* in P511 tumour cells arising in vaccinated mice. Amplified DNA products are shown from P511 tumour cells arising on:

Lanes 1-4. Day 81*, mice vaccinated with p.DOM-P1A/AB. 1=*P1A*, 2= *β-actin*, 3+4 = respective controls.

Lanes 5-8. Day 81, mice vaccinated with p.DOM-P1A/AB and cultured *in vitro* for two weeks. 5=*P1A*, 6= *β-actin*, 7+8= respective controls.

Lanes 9-12. Day 15, mice vaccinated with p.DOM-P1A/AB. 9=*P1A*, 10= *β-actin*, 11+12= respective controls.

Lanes 13-16. Day 15, mice vaccinated with p.DOM. 13=*P1A*, 14= *β-actin*, 15+16= respective controls.

Lanes 17-20. Day 15, non vaccinated mice. 17=*P1A*, 18= *β-actin*, 19+20= respective controls.

Lanes 21-24. Day 29, mice vaccinated with p.DOM-P1A/AB. 21=*P1A*, 22= *β-actin*, 23+24= respective controls.

Lanes 25-28. Day 29, mice vaccinated with p.DOM-P1A/AB and cultured *in vitro* for two weeks. 25=*P1A*, 26= *β-actin*, 27+28= respective controls.

Lanes 29-32. Day 50, mice vaccinated with p.DOM-P1A/AB. 29=*P1A*, 30= *β-actin*, 31+32= respective controls.

*P1A* and *β-actin* expression was evident in P511 tumour cells arising on day 15 post-passage in mice from all experimental groups (p.DOM-P1A/AB, p.DOM and naïve) and was comparable to *P1A* expression observed in the *in vitro* cell line (Figure 2.1). P511 tumour cells emerging on day 29 however display a level of *P1A* down-regulation which recovered when the cells were cultured *in vitro* for 2 weeks. P511 tumour cells arising on day 50 and day 81 did not display detectable *P1A* expression which was not recoverable after *in vitro* culture for 2 weeks.

### 2.2.8 A preliminary comparison of DNA vaccines encoding full length P1A

In a previous report using a DNA vaccine encoding the native P1A antigen linked to an epitope tag, it had been shown that peptide-specific CTL could be induced in 80% of mice which protected 40% of mice from a lethal tumour challenge[368]. Consequently it was of interest to compare the efficacy of the p.DOM-peptide design to other DNA vaccines encoding full length P1A. Three vaccines were constructed, p.P1AFrC, p.P1ADOM and p.P1A (illustrated in Figure 2.2) and tested for their ability to induce peptide-specific T cells after one vaccination using ELISPOT as a read-out. As with other antigens, P1A may contain tumour specific MHC class II epitopes, and in fact a putative minimal sequence has been reported in this gene[370]. The aim here was to investigate whether the induction of tumour specific $CD4^+$ T-cell helper/effector cells would complement the AB-specific $CD8^+$ T-cell response for tumour cell attack. Comparison of p.DOM-P1A/AB, p.P1A and p.P1ADOM at day 14 after one vaccination (4 experiments) showed that the expected number of mice (56%, 9/16) vaccinated with p.DOM-P1A/AB made an epitope-specific T-cell response compared with 12% (2/17) of the mice vaccinated with p.P1ADOM and 17% (3/18) of the mice vaccinated with p.P1A (Figure 2.15). p.P1ADOM and p.P1A were unable to induce a mean peptide-specific T-cell responses above control p.DOM values ($p = 0.190$ and $p = 0.419$ respectively using Mann Whitney test and paired Student t test). p.P1AFrC however did induce an AB-specific T-cell response in 55% (6/11) of mice with the mean number of spots being 25 which is favourable in comparison to mice vaccinated with p.DOM-P1A/AB ($p = 0.661$ using paired Student t test). A possible explanation for the absence of a response following vaccination with p.P1A and p.P1ADOM was incorrect folding of the protein *in vivo*, even though each vaccine was sequenced successfully and was efficiently and correctly transcribed and translated *in vitro*. Following 4 vaccinations, serum was collected and anti-FrC IgG titres were analysed by ELISA. Vaccination with p.P1ADOM was unable to induce an anti-FrC IgG response (Figure 2.3) suggesting a flaw in its ability to fold correctly *in vivo* which may also affect transport out of the cell. $CD8^+$ T-cell responses were observed in mice vaccinated with p.P1AFrC however, which also failed to induce anti-FrC antibodies suggesting further investigation is required to explain this data fully.

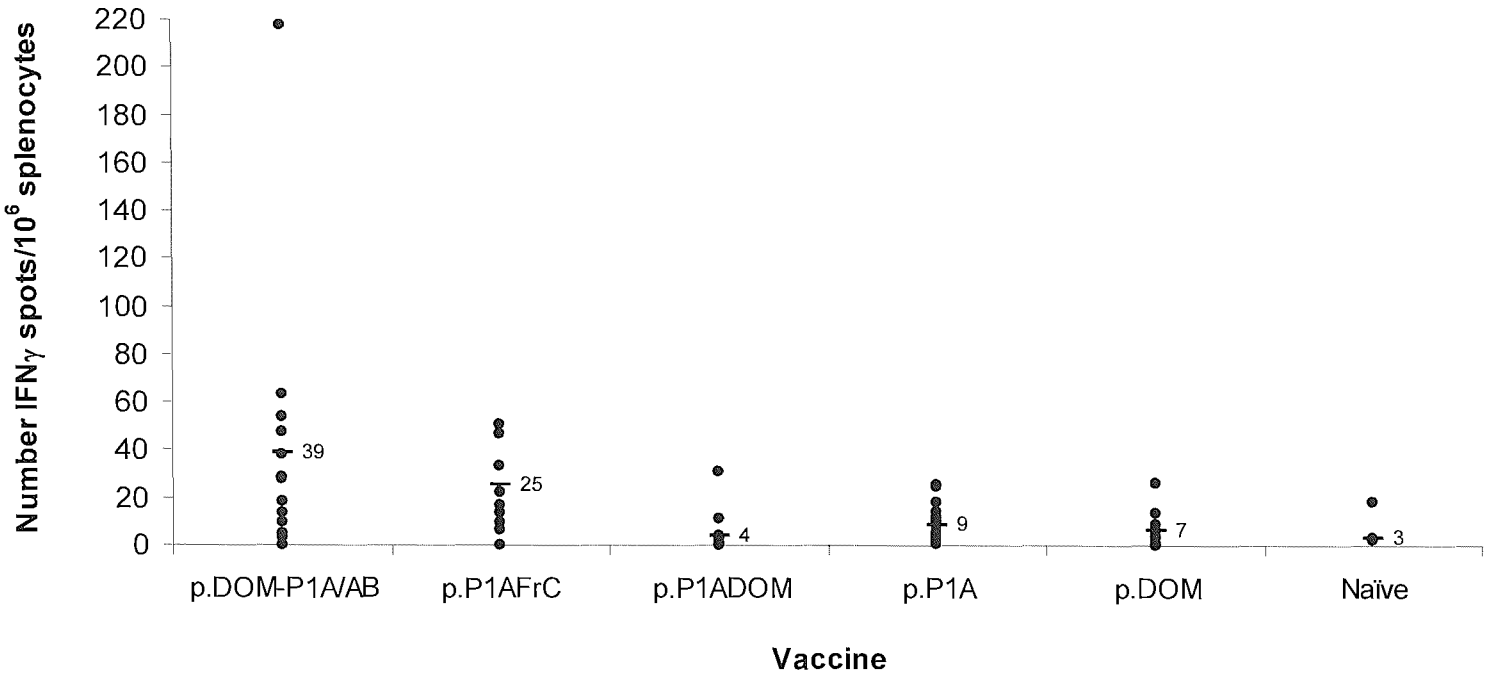



**Figure 2.15. ELISPOT assays were used to compare the number of P1A/AB specific T cells activated following vaccination with DNA vectors encoding full length P1A**

Splenocytes from mice vaccinated with various DNA vaccines encoding P1A were collected on day 14 and re-stimulated *in vitro* for 24 hours with 2μM P1A/AB peptide. ELISPOT assays were used to detect epitope specific IFNγ producing T cells in individual mice. Black circles (●) denote responses made by individual mice, and black cars (– ) the mean response in each group. As shown previously 9/16 mice vaccinated with p.DOM-P1A/AB made a specific T cell response (≥ the mean plus 1 standard deviation of the control p.DOM vaccinated mice (>15 IFNγ spots per million lymphocytes)), with an average response of 39 T cells per $10^6$ splenocytes. Control mice vaccinated with p.DOM (12 mice) or left naïve (6 mice) had background responses of 7 and 3 T cells per $10^6$ splenocytes.

In comparison, only small numbers of mice vaccinated with DNA vaccines encoding the P1A gene (p.P1A (18 mice), p.P1ADOM (17 mice)) made detectable responses, and the mean response was not statistically different from p.DOM vaccinated mice ($p = 0.419$ and $p = 0.191$ using paired Student t test and Mann Whitney test respectively). However, 6/11 mice vaccinated with p.P1AFrC made a detectable T cell response with the average response of 25 per $10^6$ splenocytes, comparable to vaccination with p.DOM-P1A/AB ($p = 0.662$ paired Student t test).

### 2.2.9 A preliminary comparison of DNA vaccines encoding an extended P1A/AB epitope

Further DNA vaccines were constructed to compare p.DOM-P1A/AB with DNA vaccines designed to deliver the AB epitope coupled with the putative P1A MHC class II epitope. Here, the elongated MHC class II epitope incorporating AB nested within it was linked to the C-terminus of p.DOM (p.DOMABlong). Two minigenes were also constructed as controls (p.ABlong and p.AB). As before vaccines were evaluated for their efficacy in inducing AB-specific $CD8^+$ T cells at day 14 following one vaccination using ELISPOT (Figure 2.16). Only small numbers of mice (5 in each group, 2 experiments) have been analysed following vaccination with the minigenes p.AB and p.ABlong and none of these vaccinated animals made a detectable antigen-specific T-cell IFNγ response compared to p.DOM vaccination ($p = 0.6358$ and $p = 0.4172$ using paired Student t test) . In contrast, 5/7 mice vaccinated with p.DOMABlong made a response with the median response being 58 spots per million lymphocytes. This result is very encouraging, however, a larger number of animals need to analysed to allow any statistical analysis. These data may suggest that provision of the extended P1A peptide still allows intracellular processing resulting in presentation of peptides in association with MHC class I. It is of interest to determine if this peptide is also processed and presented in association with MHC class II molecules. To assess the activation of P1A specific $CD4^+$ T cells vs. $CD8^+$ T cells, depletion experiments *in vivo* or *in vitro* need to be undertaken and would constitute a vital part of the future work needed to dissect the mechanism responsible for this result.

### 2.2.10 Evaluation of the vaccine p.DOM-P1E encoding a second P815 tumour antigen epitope

DBA/2 mice have been shown to make a response to an additional P815 tumour antigen P1E, and a H-$2^d$ restricted CTL epitope has been delineated[361]. It was therefore of interest to explore the possibility of 'dual vaccination' by targeting more than one CTL epitope expressed by P815, particularly in a sub-optimal setting where not all mice generate a response to vaccination and tumour cell antigen loss-variants arise when subjected to immune pressure. The p.DOM-P1E vaccine was engineered, and a preliminary evaluation carried out. However, CTL assays performed after one vaccination (at day 14) or two vaccinations (day 28) with a 6 day *in vitro* re-stimulation period yielded no measurable lysis of target cells pulsed with the peptide (data not shown). Modification of design and assessment await further work.

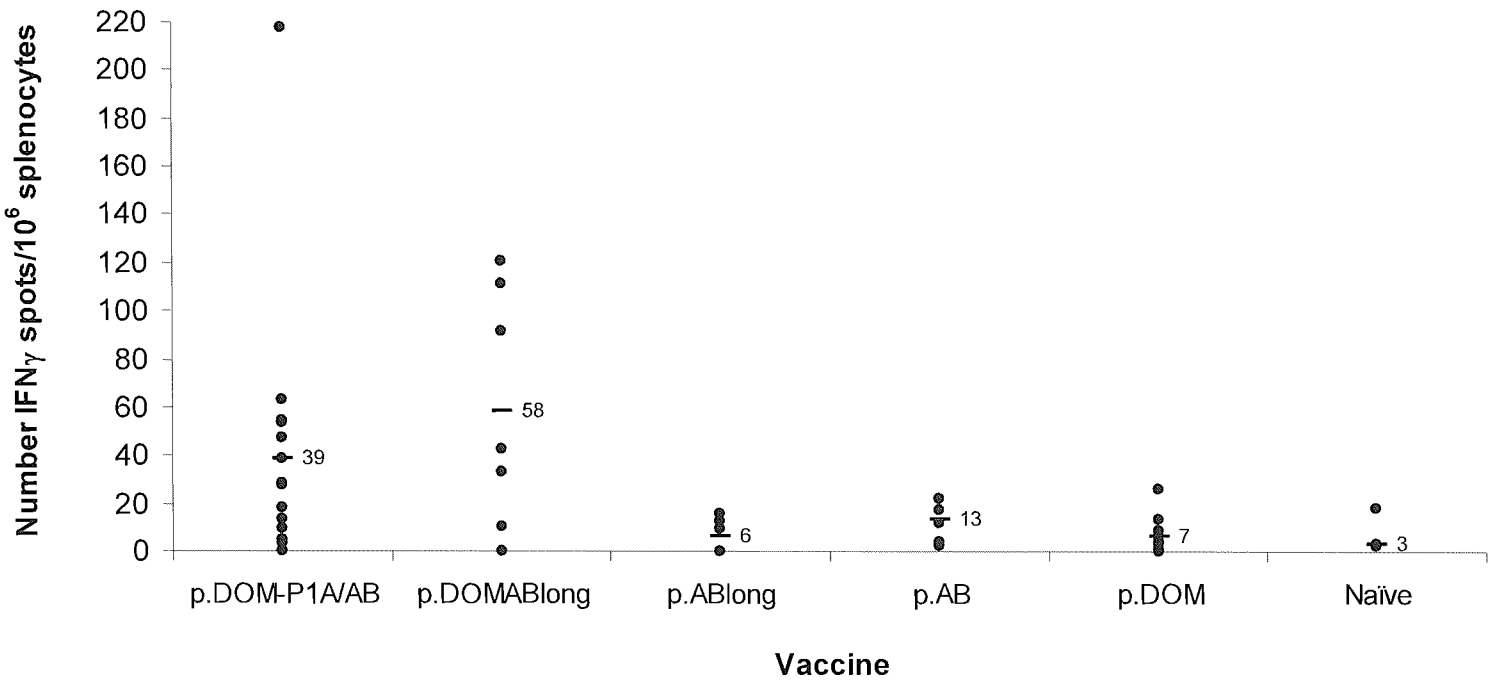



**Figure 2.16. ELISPOT assays were used to compare the number of P1A/AB specific T cells activated after vaccination with DNA vectors encoding two different P1A/AB epitope lengths**

Splenocytes from mice vaccinated with various DNA vaccines encoding P1A were collected on day 14 and re-stimulated *in vitro* for 24 hours with 2μM P1A/AB peptide. ELISPOT assays were used to detect epitope specific IFNγ producing T cells in individual mice. Black circles (●) denote responses made by individual mice, and black bars (—) the mean response in each group. As shown previously 9/16 mice vaccinated with p.DOM-P1A/AB made a specific T cell response (≥ the mean plus 1 standard deviation of the control p.DOM vaccinated mice (>15 IFNγ spots per million lymphocytes)), with an average response of 39 T cells per $10^6$ splenocytes. Control mice vaccinated with p.DOM (12 mice) or left naïve (6 mice) had background responses of 7 and 3 T cells per $10^6$ splenocytes.

In comparison, 5/7 mice vaccinated with DNA vaccines encoding p.DOM fused at its C-terminal to the putative P1A MHC class II peptide (p.DOMABlong) made a detectable epitope specific T cell response, with the average response 58 per $10^6$ splenoctyes. Mice vaccinated with DNA vaccines encoding p.ABlong or p.AB alone in the absence of p.DOM made no detectable T cell response confirming the requirement for fusing to the first domain of FrC ($p = 0.417$ and $p = 0.636$ respectively using paired Student t test in comparison with p.DOM vaccinated mice).

## 2.3 Discussion

This study has focused on targeted immunotherapy to a class of intracellular antigens, the CTA, which in many cases offer a tumour specific target. The intracellular location of target antigens in tumour cells dictates specific strategies for immunotherapeutic intervention, and to be successful will ultimately need MHC class I and possibly MHC class II restricted epitope targeted T-cell attack. Intracellular antigens hold an additional interest in haematological malignancy, in particular MM, since MHC class I molecules are retained by tumour cells allowing presentation and recognition of these intracellular antigens by T cells[376]. Furthermore multiple MHC class I restricted epitopes derived from CTA have been described in human tumours (Tables 2.1 and 2.2) which broadens the spectrum of patients that may benefit and adds the additional possibility of targeting multiple MHC class I epitopes simultaneously.

In order to evaluate the design and use of DNA fusion vaccines to target such intracellular antigens, we identified and selected the P815 murine mastocytoma tumour model for further study, since P815 displays the CTA-like P1A antigen[361]. We first utilised a strategy of inducing responses to a single CTA-derived MHC class I epitope by DNA vaccination in a model setting. Here the C-terminal fusion DNA vaccine design, encoding the P1A/AB nonameric peptide which targets and binds to H-2L$^d$, was employed using an approach developed in our laboratory.

Following DNA vaccination, the efficacy of the p.DOM-P1A/AB design in inducing CTL specific for the P1A/AB epitope was demonstrated. Two assays were used to assess peptide-specific T-cell responses *ex vivo* following a single vaccination. The intracellular staining assay for IFNγ was preferable as it allowed the specific identification of responding $CD8^+$ T cells, whereas the ELISPOT assay cannot differentiate between $CD4^+$ and $CD8^+$ T-cell responses. Although it is assumed that responses to the P1A/AB nonamer *in vitro* would most likely consist of $CD8^+$ T cells, it must be noted that $CD4^+$ T cells have been shown to secrete IFNγ in response to the P1A/AB nonamer[377]. The response of $CD4^+$ vs. $CD8^+$ T cells was not investigated here, but cells expanded *in vitro* with peptide and IL-2 for 6 days and identified with mAbs specific for CD8 and IFNγ clearly demonstrated the expansion of a $CD8^+$ T-cell population. In addition, peptide-specific IFNγ production was shown to associate with the ability of these cells to recognise and lyse tumour target cells *in vitro*, either pulsed exogenously with the peptide or cells processing and presenting the epitope endogenously, using P511 and P815. The CTL lytic activity detected here is

comparable to that obtained using several other vaccine concepts including a Semliki Forest viral vector encoding the full length P1A gene[366], chimeric papillomavirus virus-like particles encoding 90 base pairs of P1A[365], DC pulsed with the P1A/AB nonamer and IL-12[371], P1A/AB peptide with QS-21 and IL-12 (personal communication, Uyttenhove, C.) or phage display particles expressing the AB nonamer[367].

In other model systems the p.DOM-peptide DNA vaccine design has been used to generate responses very effectively in 90-100% of vaccinated mice[264]. Surprisingly, in this setting, only an average of 54% of the mice vaccinated with p.DOM-P1A/AB made a detectable response, which was not increased by a DNA boost. This suggested that the low level response rate was not a consequence of vaccination failure but instead related to differences between individual mice, however the identical genetic makeup of this strain of mice rules out the possibility of MHC differences. Although this issue has not been formally addressed by other research in this area, it is clear from some reports that similar findings have been observed. For example, Warnier G *et al*[363] reported that only 45% of mice vaccinated with an adenovirus encoding the AB epitope made a specific CTL response when the cells were re-stimulated in the absence of cells expressing B7.1. Additionally, vaccination with P1A transfected tumour cells led to a large degree of variation in the lytic activity detected in individual mice[362].

One possible reason for differences between mice is the existence of low level *P1A* expression in normal tissues such as the lung and spleen. This has been reported in BALB/c mice using highly sensitive PCR techniques[375]. As yet, no expression of *P1A* in normal tissues has been reported in DBA/2 mice (with the exception of the testis), but the methods used to detect protein expression may not have been sensitive enough to detect the low levels seen in BALB/c mice. Low level P1A expression may be the reason for detection of P1A/AB-specific CTL in lysis assays. Whilst central T-cell tolerance has been described in P1A transgenic mice, where high levels of P1A expression in the thymus results in deletion of P1A specific T cells[378], this does not occur in wild type DBA/2 mice where P1A expression is likely to be low. Instead, activated T cells in the periphery may be kept in check by peripheral mechanisms of tolerance such as Treg cells, which are not sustained during *in vitro* stimulation where P1A/AB peptide levels are high. Furthermore, in BALB/c mice, low level P1A expression has been shown to result in a restrained effector function of P1A/AB-specific $CD8^+$ T cells[375]. Similar observations have been noted in other model systems[379], where low level antigen expression and exposure leads to a functional impairment in the antigen specific $CD8^+$ T cell population. These data may explain why

mice do not respond uniformly, not only to DNA vaccination but also to other forms of active immunotherapy.

Additional reasons for the discrepancy between individual mice may involve the $CD4^+$ T-cell compartment. Inducing CTL against the P1A/AB epitope has been shown to require $CD4^+$ T-cell help or IL-12. Vaccination with DC pulsed with the P1A/AB nonamer alone has been shown to induce a transient state of functional unresponsiveness in the $CD4^+$ T-cell population[369]. To overcome this, the DC preparation needed to include MHC class II helper epitopes or IL-12[371]. $CD4^+$ T-cell depletion at the time of vaccination was also shown to abrogate the P1A/AB specific $CD8^+$ T-cell response[370]. The theoretical basis for linking p.DOM to the P1A/AB epitope is to act as an immunological alert signal and provide T-cell help. In this setting however, no $CD4^+$ T-cell responses to the encoded 'universal' $CD4^+$ T helper epitope p30 were detected and therefore it may be possible that the minimal threshold level of $CD4^+$ T-cell activation required for optimal P1A/AB specific $CD8^+$ T-cell induction was not being achieved in all vaccinated mice. Other MHC class II epitopes may exist in the first domain of FrC but were not analysed here. The absence of detectable $CD8^+$ T-cell responses in the preliminary testing of the DNA minigene p.AB does however demonstrate the necessity of linking the epitope to p.DOM either because $CD4^+$ T-cell help is initiated or because fusing P1A/AB to DOM enhances the peptides stability or both. Consequently, it suggests that immune stimulatory CpG sequences in the DNA backbone are not sufficient enough to promote a $T_H1$ response that will overcome the need for T-cell help in this setting. There is one caveat however in comparing p.DOM-P1A/AB with the p.AB minigene which is that the ability of the minigene to be transcribed and translated *in vitro* has not been examined and the possibility that the vaccine produces no protein product *in vivo* cannot be ruled out.

Vaccination with p.DOM-P1A/AB was further analysed for its ability to induce immunity in mice that would confer protection from a lethal challenge with the P1A expressing tumour P511. In two of two experiments a statistically significant delay in tumour growth was observed in mice vaccinated with p.DOM-P1A/AB compared with control vaccinated mice. However the number of mice remaining tumour free to the end of each experiment was of concern, with the maximum being 40%. This result however must be correlated firstly with the response rate seen in these mice to vaccination, which was only 54% (discussed above) and secondly to other published work. The first reports of protective immune responses against this tumour were induced by vaccination with P1A expressing tumour cells, P815[364]. Re-challenge with P815 resulted in protection in 90% of mice, but mice were

only monitored over a 30 day period and were not protected against other P1A expressing tumours, suggesting that a multitude of different CTL responses were induced against cells used for vaccination. To prevent this problem, Brandle *et al.*[362] vaccinated mice with tumour cells un-related to P815 which were transfected with the P1A gene and then monitored protection against a subsequent P511 challenge. At day 40, 12/15 animals survived compared to controls where only 3/15 had survived. However, by day 100 only 10-20% of mice survived. Several viral based vectors have also been used to encode P1A or the AB epitope to try and improve protective immune responses, and although some enhanced CTL responses were observed when compared to the cell based therapies, this was not dramatic and did not result in dramatically increased protection from tumour challenge[365, 366].

One reason that may account for the difficulty in generating long term tumour immunity against the P815 tumour cell line is the outgrowth of P1A-loss variants, as detected here after p.DOM-P1A/AB vaccination. This has also been observed in mice vaccinated with tumour cells transfected with P1A, here escaping tumour cells became resistant to CTL clones specific for P1A/AB[380]. In addition, mice given P1A/AB specific TCR tg CTL therapy post-challenge with P1A expressing tumour cells, were unable to control tumour growth. Analysis of P1A expression demonstrated a variety of point mutations within the P1A/AB epitope[381].

Vaccination against P1A/AB and subsequent tumour challenge has also been shown to allow the generation of CTL specific for P1E, an observation indicating epitope spread[372]. These mice were protected from a successive tumour challenge with tumour cells only expressing the P1E antigen. This effect does not appear to have occurred in this setting of DNA vaccination, or if anti-P1E CTL were generated they were ineffective at tumour killing *in vivo*. This compares with a report by Bilsborough J *et al*[361] who demonstrated the induction of P1E specific CTL using tumour cells transfected with the P1E gene and B7-1; however, no protection from tumour challenge was achieved.

P1A/AB specific $CD8^+$ T cells have also been shown to display a restrained effector function *in vivo*[375] and this could be an alternative explanation for the difficulty in generating a good protective immune response against P1A expressing tumours. In this report only 44% of transgenic mice with >50% of $CD8^+$ T-cells expressing clonal TCRs specific for P1A/AB were able to reject P1A expressing tumours (J558) and this was comparable to non-transgenic mice. 100% protection was only achieved when tumours

were transfected with B7-1. Furthermore if transgenic mice were injected with J558-B7-1 on one flank and J558 on the other flank, only the B7-1 positive tumours were rejected. Therefore, even when the $CD8^+$ T-cells specific for P1A were activated *in vivo* they could not reject J558.

The next aspect of this investigation was aimed at comparing our work with that of others using DNA vaccines to target this antigen. Rosato *et al.*[368] encoded the full length P1A gene linked to a tag epitope within a DNA plasmid and demonstrated that 80% (12/15) of vaccinated mice made a CTL response to P1A/AB after three vaccinations and 40% were protected from tumour challenge compared to control vaccinated animals. This result was surprising at first because P1A/AB specific CTL are known to require $CD4^+$ T-cell help. However, a potential $I\text{-}A^d$ MHC class II epitope encoded within P1A/AB has been described[370] and P1A/AB specific $CD4^+$ T-cell responses have been detected in mice using peptide pulsed DC when using extended peptides EILPYLGWLVFA and EILPYLGWLVFAV incorporating both the AB epitope (underlined) and the MHC class II motif. A direct role for P1A/AB specific $CD4^+$ T cells in tumour immunity was however not delineated. It was therefore of interest to compare DNA vaccines encoding full length P1A gene alone or fused to p.DOM or FrC (with both FrC and P1A encoded $CD4^+$ T-cell epitopes) to the p.DOM-P1A/AB design. Increased $CD4^+$ T-cell help may promote $CD8^+$ T-cell responses, but other unknown immunodominant CTL epitopes encoded within P1A may abrogate the P1A/AB specific CTL response. Although only small numbers of mice have been tested, there were no demonstrable responses induced by p.P1A alone or when fused to DOM which contradicts the results obtained by Rosato *et al.*[368] However small differences in the vaccine construct and vaccination protocol may account for this. Rosato linked a 6 amino acid tag sequence to P1A which may allow the formation of MHC class II motifs. They also vaccinated their mice three times with twice as much DNA (100μg as oppose to 50μg) before assessing CTL responses and protection from tumour challenge. It has been demonstrated in our laboratory using the p.DOM-AH1 vaccine that increasing the concentration of DNA (from 5μg to 50μg) or volume it is injected in (from 10μl to 50μl) can improve the CTL response and protection achieved respectively until a plateau is reached (S. Buchan *et al.*, submitted). The plateau may differ for different antigens, and optimal responses to weak antigens such as P1A may require larger concentrations or volumes of DNA. In addition to differences in the vaccine construct and vaccination protocol, further analysis of p.P1ADOM and p.P1AFrC vaccinated mice highlighted the inability of these vaccines to induce an IgG anti-FrC antibody response *in vivo*, suggesting incorrect folding.

The effect of incorrect folding on direct or cross-priming is not known in this setting and remains speculative.

In contrast to p.P1A and p.P1ADOM, p.P1AFrC was able to generate a peptide-specific $CD8^+$ T-cell response in 50% of the mice tested, with an average number of responding cells comparable to those mice vaccinated with p.DOM-P1A/AB. This may reflect the increased ability of full length FrC to promote a $T_H1$ environment and $CD4^+$ T-cell help. The important role of $T_H1$ cytokines in the induction of $CD8^+$ T-cell responses to P1A/AB has been demonstrated by Colmenero *et al.*[382] who found that vaccination with recombinant Semliki Forest virus replicons encoding IL-12 alone into the tumour site, was as efficient at protecting mice from tumour challenge as encoding P1A in the vector. It has also been shown by Grohmann *et al.*[371] that IL-12 can replace helper epitopes when vaccinating with DC preparations pulsed with P1A/AB, an effect which cannot be replaced with IFNγ. IL-12 is proposed to act on the $CD4^+$ T-cell population and provide proliferative and survival signals[383].

Further in this work, we were interested in delivering more precisely tumour antigen-specific MHC class II motifs to promote tumour specific $CD4^+$ T-cell help. As mentioned previously, pulsing DC with extended peptides consisting of both the P1A/AB nonamer and the MHC class II motif was shown to negate the need for IL-12 in vaccinated mice[370]. In light of this, a vaccine incorporating the extended peptide was constructed, p.DOM-ABLong, allowing a comparison of responses when additional antigen-specific $CD4^+$ T helper epitopes were present. Only a small number of animals have however been tested with this vaccine to date, and so far 5/7vaccinated animals have made a detectable P1A/AB specific T-cell response after a single vaccination. The relative contributions of $CD4^+$ T cells and $CD8^+$ T cells have not yet been investigated but it would be of interest to delineate their respective roles and correlate the results with any tumour protection observed. Mice vaccinated with p.ABLong made no detectable P1A/AB specific T-cell responses demonstrating the necessity for p.DOM fusion. However, like p.AB, the possibility that the vaccine is not efficiently transcribed and translated *in vivo* cannot be ruled out.

The final aspect of this investigation was centred on observed tumour antigen loss variants described in human tumours after specific vaccination. It was therefore of interest to investigate targeting more than one antigen simultaneously. P815 is amenable for this investigation as it is known to express a number of other tumour associated antigens, including P1E. It was predicted that concurrent vaccination against P1E may prevent

outgrowth of antigen loss variant clones observed when mice were vaccinated with p.DOM-P1A/AB alone and challenged with P511. However, initial experiments with p.DOM-P1E have been unsuccessful suggesting a modification to strategy to target this epitope maybe necessary. To promote T-cell responses, increased vaccine volume or electroporation *in vivo* could be incorporated into the vaccination protocol. Alternatively a different approach could be taken by targeting a third antigen expressed in P815 such as the MuLV viral envelope protein gp70 encoding the AH1 CTL epitope. In line with this idea, AH1-specific CTL from BALB/c (H-$2^d$) mice vaccinated with p.DOM-AH1 were shown to lyse P815 *in vitro*[264]. It will be of interest to investigate the combined use of p.DOM-P1A/AB and p.DOM-AH1.

The aim of this part of the project was to demonstrate the efficacy of the p.DOM-peptide design in eliciting CTL responses against defined MHC class I motifs in CTA. The P815 model has provided a challenging murine model for human CTA to test vaccine design. For clinical application it will be necessary to demonstrate that the vaccine format can be used to induce CTL responses to human CTA in the HLA-A2 transgenic mouse model and there is interest from our collaborators in Brussels to use such vaccines to target melanoma. Several MHC class I epitopes have been described for MAGE antigens, and future work should involve assessing their ability to be processed and presented. NY-ESO-1 has also been shown to encode a putative nested MHC class I epitope within known MHC class II motifs and it will therefore be of interest to determine whether extended peptides prove to be more effective by enhancing $CD4^+$ T-cell help to poorly immunogenic epitopes.

# 3 Targeting MUC1 with DNA vaccines

## 3.1 Introduction

The mucins (MUC) comprise a family of high molecular weight glycoproteins of which MUC1 was the first to be cloned[384]. MUC1 is the only member of this family to form a type I transmembrane protein and is expressed on ductal epithelium as well as B cells and activated T cells[385, 386]. Expression on epithelial cells is polarised towards the lumen (apical) where the mucins function to lubricate the cell surface and form a protective barrier[387, 388]. MUC1 can also bind to ICAM-1 molecules on endothelium and plays a role in lymphoid cell trafficking[385]. MUC1 is also found in the serum due to an early proteolytic event during synthesis. The two cleavage products remain associated at the cell surface linked non-covalently which allows subsequent shedding of the extracellular domain[389]. MUC1 is also observed as an antigen expressed on the surface of many cancer cell types, including malignant lymphoid cells[390]. Its expression on cancer cells is in an aberrant form, both in relation to its glycosylation state and loss of polar membrane orientation[388]. Despite its aberrant expression in cancer, MUC1 is essentially a self-antigen, suggesting immunological tolerance may exist, although its location may impact on this question. Nevertheless, many investigations in distinct histological cancers have focused on attacking MUC1, either as a target for humoral or for cellular effector arms, with evidence for functional responses[391, 392].

The MUC1 gene yields a protein consisting of an amino terminal signal peptide, which can give rise to two splice variants[393], a degenerate repeat region, a variable number (approximately 20-125) of a 20 amino acid tandem repeat (VNTR, PDTRPAPGST APPAHGVTSA) followed by further degenerate repeats, then a 31 amino acid membrane spanning domain and finally a 69 amino acid cytoplasmic domain (Figure 3.1)[384, 394]. The VNTR varies according to allelic polymorphism. In comparison, the murine muc1 protein shares 34% homology in the tandem repeat region but 87% homology in the transmembrane and cytoplasmic regions. It consists of a fixed number (16) of tandem repeats with potential for O-glycosylation[394, 395].


**Figure 3.1. Human MUC1 shown as an aligned protein sequence (with 16 repeats) with murine muc1**

Sites of homology are marked in red (.). Human MUC1 20 amino acid VNTR (STAPPAHGVTSAPDTRPAP) shares little homology with the murine fixed number 20-21 amino acid degenerate repeat (STSSPVHSGTSSPATSAPXD). High levels of homology exist within the putative signal sequence and C-terminal region. Murine H-2 epitopes (peptide1, 2 and 4) and human HLA-A2 epitopes (L9V, T9A, S9V and A9V) used in the present study are marked in bold.

### 3.1.1 MUC1 is heavily glycosylated

MUC1 is subject to extensive post-translational glycosylation, most likely in the Golgi[396]. Each tandem repeat contains five sites for O-linked glycosylation, and are the serine and threonine residues underlined in the sequence previously, and all 5 residues appear to serve as sites for GalNAc transferase activity in normal cells[397]. Five N-linked glycosylation sites also exist in the extracellular membrane proximal domain between the tandem repeat region and the membrane spanning region[384]. The O-linked carbohydrate can account for more than 50% of the weight of the molecule when expressed. Although the core protein sequence is conserved, the glycosylation pattern can vary depending on tissue type[394], and this may be due to several factors including availability of enzymes and substrates. The carbohydrate elongation steps depend on the different glycosyltransferases available within the cell and in turn, the nature of the residue added affects the addition of subsequent residues. Additional variation is generated in the termination of the carbohydrate chains, utilising Gal, *N-acetylgalactosamine* (GalNAc), fucose or sialic acid[388]. O-linked glycosylation is initiated by transfer of GalNAc to the side chain of a serine or threonine residue, followed by addition of galactose by β1-3 linkage to form a core 1 structure. Two enzymes, $\beta_1$-6N acetylglucosamine transferase ($\beta_1$-6NGlcNAc-T) and α2-3 sialyl transferase (α2-3SA-T) then compete for core 1 substrate (Galβ1-3GalNAc) to either elongate the chain or terminate it respectively. The addition of GlcNAc to core 1 glycans leads to the formation of further core 2 glycans that can be again extended by the addition of polylactosamine side chains (Figure 3.2.A and 3.2.B). Surface MUC1 exists in various stages of molecular maturation and glycosylation and can recycle from the surface into the cell allowing continued carbohydrate modifications, notably addition of large amounts of sialic acid[398].

### 3.1.2 MUC1 expression in malignancy

Malignant transformation of epithelial cells can result in increased levels of MUC1 expression, with loss of polarity (Figure 3.3). This has been shown to occur in carcinomas of the breast, pancreas, prostate, lung and salivary glands amongst others[388]. Increased MUC1 expression has also been described in a variety of haematological malignancies including MM[386, 390, 399, 400], FL[390], B cell lymphoma[401], a proportion of both B and T CML and AML[390]. The functional role for MUC1 expression on malignant cells is not fully delineated, but there is evidence for immune-inhibitory as well immune-stimulatory

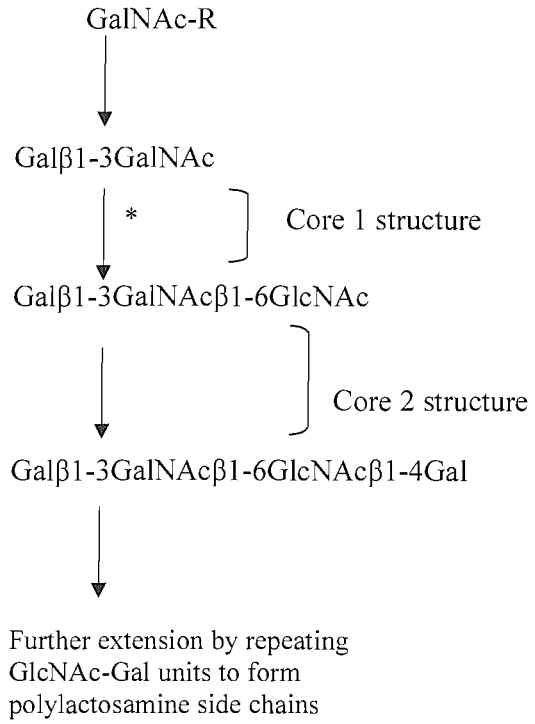
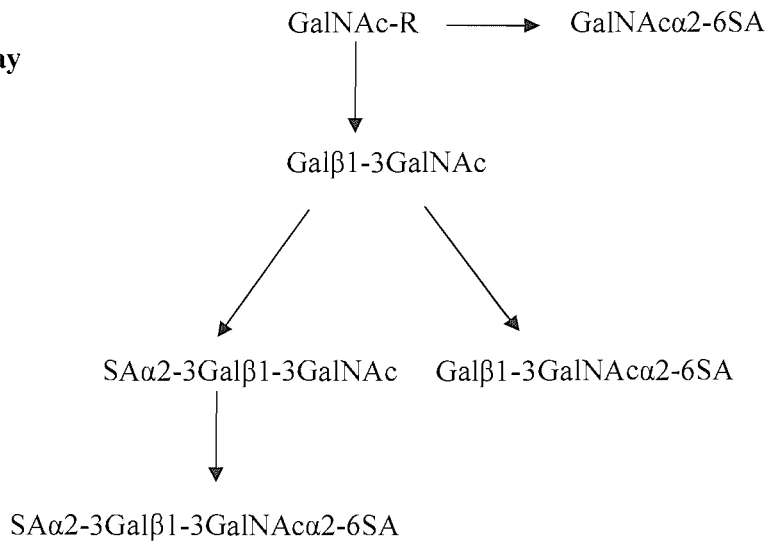



**Figure 3.2. A)The normal O-linked glycosylation pathway**

GalNAc is transferred to the side chain of a Serine or Threonine residue (R), a core 1 structure is formed by the further addition of galactose. * $\beta_1$-6NGlcNAc-T and α2-3SA-T then compete for the same core 1 substrate (Galβ1-3GalNAc) to either elongate the chain or terminate it respectively. The addition of GlcNAc to core 1 glycans leads to the formation of core 2 glycans that can be further extended by the addition of polylactosamine side chains[402].

**Figure 3.2. B) Aberrant O-linked glycosylation pathway found in malignant cells**

Absence of $\beta_1$-6NGlcNAc-T and increased α2-3SA-T results in addition of sialic acid and earlier termination of carbohydrate side chains[402].



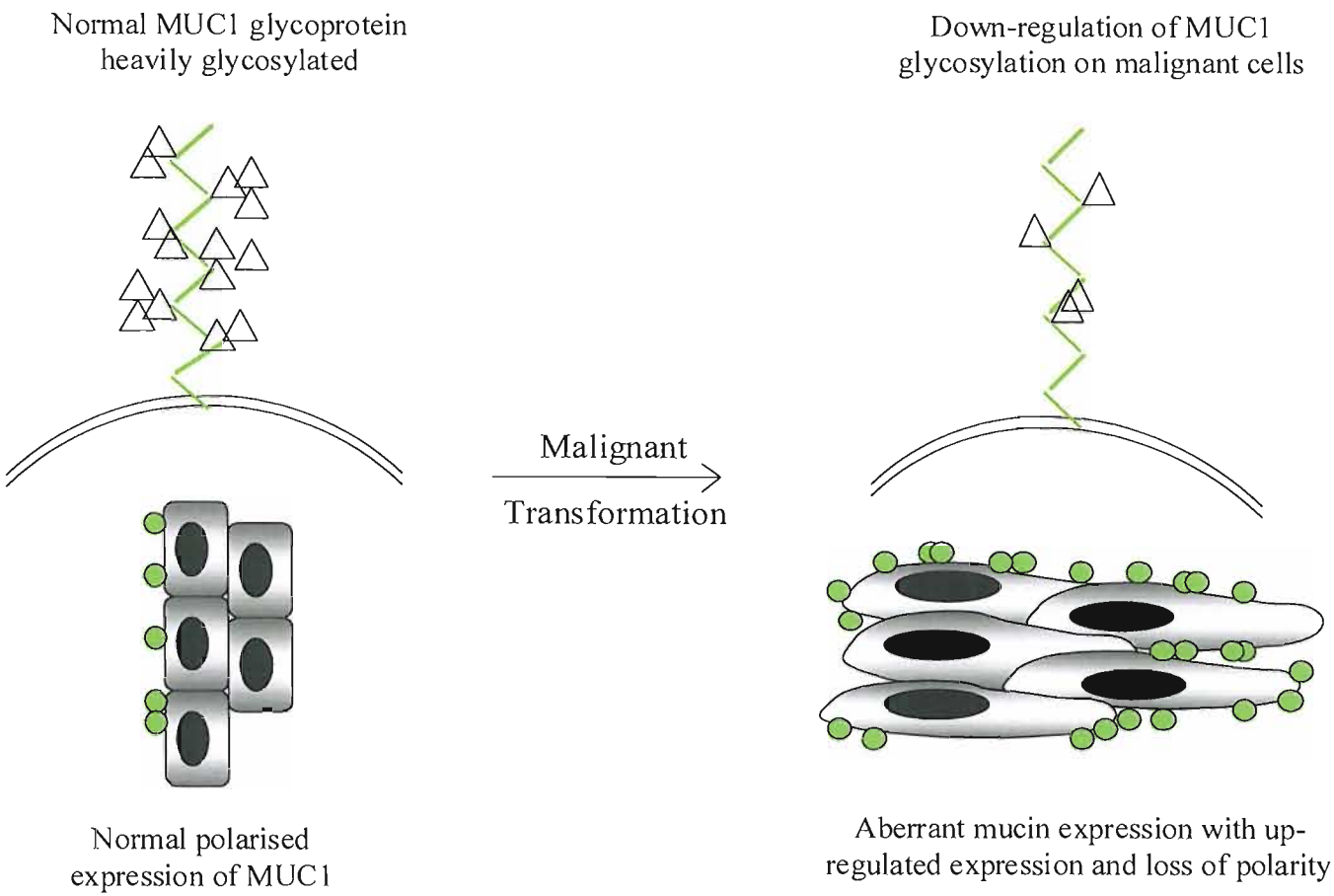



**Figure 3.3. MUC1 expression on normal and malignant cell**

MUC1 (green) is normally expressed as a heavily glycosylated (Δ) surface molecule with polarised expression. During malignant transformation, the carbohydrate side chains are reduced both in number and length. MUC1 expression is additionally increased with loss of polarity.

roles. The presence of MUC1 on tumour cells can enhance survival, with surface expression and shed protein aiding metastasis and suppressing immune responses[403, 404]. Structural studies indicate that the molecule extends more than 1000Å from the cell surface, and the length and sialiation impart a strong negative charge on the molecule which conveys anti-adhesive properties. This may prevent cell-cell contact, help metastatic spread and inhibit CTL interactions[405]. It has also been demonstrated that MUC1 present in supernatant can reversibly inhibit T-cell proliferation and effector function *in vitro*[406]. Contrasted to these findings, MUC1 expression can enhance immunogenicity of malignant cells, stemming from both increased expression and hypo-glycosylation. Aberrant levels of glycosyltransferases have been documented in breast carcinoma cell lines, where $\beta_1$-6NglcNAc-T is absent and α2-3SA-T is increased by 8-10 fold[407]. As a result, the number of carbohydrate chains may be reduced with fewer serine and threonine sites being occupied, and the chains also terminate earlier to result in shorter and less branched carbohydrate structures[408, 409] (Figure 3.2 A and B). Consequently cryptic epitope sequences are revealed from within the protein core sequence and altered carbohydrate side chains which combine to increase immunogenicity of the molecule[410]. Analysis of a number of different breast cancer cell lines revealed that the nature of the sugar residues added to MUC1 within any given malignant cell line is specific[408, 409].

### 3.1.3 Glycosylation and MUC1 processing

An important question which arises is the effect of variable MUC1 glycosylation on processing for MHC class I and II presentation and TCR recognition in tumours. Analysis of fragmentation products by mass spectrometry revealed the main cleavage sites within the VNTR sequence (A**HG**V**T**SAPDTRPAPGSTAPPA) to be between the His-Gly bond and the Thr-Ser bond (bold). However, the presence of even a monosaccharide at Thr (underlined) can inhibit this cleavage event[411]. This demonstrates the effect of carbohydrate on cellular processing and proteolytic events. Furthermore, glycosylated peptides from MUC1, and other glycosylated proteins, have been found in complex with MHC class I and II molecules and were recognised by glycan specific $CD8^+$ and $CD4^+$ T cells[412-415]. Pulsing DC *in vitro* with native MUC1 isolated from tumour cells or a synthetic non-glycosylated peptide conjugated to gold beads has revealed further insights into MUC1 processing. Firstly, glycosylated MUC1 isolated from tumour cells can be captured by APC *in vitro* and taken into the cell through receptor mediated endocytosis but this form of MUC1 is unable to progress through the endocytic pathway. It is eventually re-

cycled to the cell surface, bypassing the MHC class II processing pathway, and is therefore unable to stimulate MUC1 specific $CD4^+$ T-cell hybridomas[416]. These data suggest that for glycosylated MUC1 peptides to reach the cell surface in association with MHC class II, MUC1 must be synthesised endogenously. In contrast non-glycosylated VNTR peptides are taken into the APC by macropinocytosis, efficiently reach the MHC class II processing pathway and lead to $CD4^+$ T-cell stimulation[416]. Interestingly, there appears to be less of an effect of carbohydrate on the presentation of MUC1 peptides on MHC class I molecules. Tumour derived glycosylated forms of MUC1 can be fed to DC and stimulate $CD8^+$ T cells *in vitro* resulting in target cell lysis. However, this is less efficient than using non-glycosylated MUC1 derivatives[417].

### 3.1.4 Naturally occurring immune responses to MUC1 in cancer

Naturally occurring anti-MUC1 antibodies have been detected in breast cancer patients[418] but do not appear to be sufficient to control disease. Likewise, the induction of anti-MUC1 antibodies in cancer patients through vaccination regimens has little effect at the clinical level. High titre anti-MUC1 IgM and IgG antibody responses have been generated in cancer patients when vaccinated with MUC1 peptides coupled to KLH, administered with QS-21 adjuvant[419, 420]. However, the antibodies from the majority of patients were unable to bind to $MUC1^+$ tumour cells. MHC-unrestricted, MUC1 specific, $CD8^+$ T-cell clones have also been identified in lymph node samples from breast, ovarian and MM cancer patients[399, 421-423]. In these cases the $CD8^+$ T cells were thought to bind directly through their TCR to the MUC1 tandem repeat, however additional co-stimulatory signals may have also beeen required[424]. These findings suggest that due to the normal sequestered nature of MUC1, neither immunity nor complete tolerance to this antigen arises. Increased expression, de-polarisation of expression, and hypo-glycosylation seen during malignant transformation therefore leaves this molecule open to immune recognition and attack with a reduced risk of autoimmunity. However, naturally occurring $CD4^+$ T cells have not been detected in patients suggesting a level of tolerance in this T-cell compartment.

### 3.1.5 MUC1 vaccines

Early work targeting MUC1 for immunotherapy in mice utilised human MUC1 encoding vaccines to assess production of anti-MUC1 antibodies, $CD4^+$ and $CD8^+$ T cells, and

protection against tumour challenge. In murine models vaccine formulations to date include:

- peptide/protein alone or conjugated to a carrier protein such as KLH, DT, mannan or *Bacillus Calmette-Guérin* (BCG)[425-427]
- liposomal/MUC1 peptide preparations[428].
- vaccinia vectors (Prof. J. Taylor-Papadimtriou, ICRF, personal communication).
- plasmid DNA vectors[429, 430].
- DC transfected with tumour RNA or adenovirus encoding MUC1[431, 432].

One question firmly addressed in these studies concerned the role of MUC1 reactive antibody in mediating tumour rejection. Early observations indicated that no correlation existed between the induction of anti-MUC1 antibodies and levels of protection achieved[425, 426, 433]. A definitive answer regarding the role of antibody stemmed from the passive transfer of MUC1 reactive antibodies into naïve recipient mice. In this setting, no positive effect on survival following tumour challenge was noted[434].

The role of $CD4^+$ T cells versus $CD8^+$ T cells is still under investigation in response to MUC1 targeted vaccines. Several reports provide direct evidence for a role for $CD8^+$ T cells in anti-MUC1 immunity. In mice, immunisation with a mannan-MUC1 fusion protein led to measurable CTL and tumour protection[435]. The CTL were shown to be specific for MUC1 MHC class I epitopes contained within both the tandem repeat and non-tandem repeat regions[436, 437] and depletion of these $CD8^+$ T cells abrogated tumour protection[435]. Mice immunised with a vaccinia viral vector encoding the human MUC1 cDNA sequence can generate MUC1 specific CTL capable of lysing RMA-MUC1 transfectants *in vitro* and provide protection *in vivo* (Prof. J. Taylor-Papadimitriou, ICRF, London, personal communication). Additionally, mice with spontaneous pancreatic cancer developed MUC1 specific CTL that were able to lyse a B16-MUC1 transfectant *in vitro* and protect from tumour challenge when adoptively transferred[438]. MUC1 transduced DC were also able to induce MUC1 specific $CD8^+$ T cells detected by cytotoxicity assay, and tumour cell killing was demonstrated[431].

With regard to a role for $CD4^+$ T cells in anti-MUC1 immunity, Gerloni M. *et al.*[439] were able to induce $CD4^+$ T-cell proliferative responses to a putative MUC1 $CD4^+$ T-cell epitope when using a transgene vaccination system. Other vaccine formats, such as the 140 amino acid VNTR peptide and DNA plasmids encoding human MUC1, have also been shown to promote $CD4^+$ T-cell responses[426, 440]. More strikingly, Tempero, *et al.*[441] compared

differences between wild type mice able to reject B16-MUC1 spontaneously and MUC1 tolerant mice that were unable to reject the tumour cell line. T-cell subset depletion studies demonstrated a more prominent role for $CD4^+$ T cells than $CD8^+$ T cells in the protection observed, and transfer of this subset to MUC1 tg mice conferred some protection from tumour challenge. The role of $CD4^+$ T cells versus $CD8^+$ T cells may be tumour model and/or vaccination specific; for example, while $CD4^+$ T cells were required for B16-MUC1 rejection, $CD8^+$ T cells were required for Panc02-MUC1 rejection in wild type mice[441, 442].

One drawback of these strategies is the natural immunogenicity of human MUC1 in wild type mice, so that although vaccine efficacy has been demonstrated, its relevance to a human disease setting is minimal since the issue of tolerance cannot be addressed. Consequently, the MUC1 transgenic mouse has been engineered[443], utilising the human MUC1 genomic fragment with its natural promoter. Expression analysis of tissues from MUC1 transgenic mice indicates a similar pattern of expression as in human cells. Overall, comparisons of vaccination protocols between wild type and MUC1 transgenic mice have highlighted a degree of tolerance in the $CD8^+$ T-cell compartments[442], with unknown effects on the MUC1 specific $CD8^+$ TCR repertoire. There also exists a high degree of tolerance in the $CD4^+$ T-cell compartment which consequently limits B cell isotype switching[426, 441]. Immunological responses generated by vaccination of MUC1 tg mice were weaker and more difficult to detect than in wild type mice. Immunotherapeutic strategies often require additional adjuvants such as cytokines or co-stimulatory molecules. For example, MUC1 expressing tumour cells, whilst immunogenic in wild type mice, did not generate MUC1 specific immune responses in their transgenic counter-parts unless modified to express IL-12 or B7.1, or fused with DC[444-446]. Similarly, mannan-MUC1 vaccination was more effective in inducing MUC1 specific CTL precursors if injected with IL-12[447]. The difficulty in generating $CD4^+$ T-cell responses in these mice was evident and parallels the absence of naturally occurring $CD4^+$ T-cells specific for MUC1 in humans. The absence of MUC1-specific $CD4^+$ T-cell help may be one reason for the concurrent difficulty in generating MUC1-specific $CD8^+$ T cells. Importantly, evaluation of tissue samples following generation of MUC1 immune responses in transgenic mice revealed no destructive autoimmune pathology[444, 445] The MUC1 transgenic mouse therefore represents an important and relevant model to assess efficacy of immunotherapeutic strategies targeting MUC1 for application to human clinical trials.

In addition to murine models, the chimpanzee has also been used as a model for vaccination protocols against MUC1. Chimpanzee MUC1 shares a high degree of homology with

human MUC1 protein and the limited research using this animal model has been aimed at inducing $CD4^+$ T-cell responses. Vaccination with a 100 amino acid peptide encoding 5 tandem repeats conjugated to adjuvants led to MUC1 specific T-cell proliferation *in vitro* in all animals tested and to IFNγ production by MUC1 specific $CD4^+$ T cells in 3/4 animals[448]. CTL responses have also been induced in chimps using this vaccine as well as a vaccine incorporating immortilised autologous B cells expressing MUC1[448, 449] These results are encouraging for human application since $CD4^+$ T-cell tolerance in higher primates may not be as complete as that observed in MUC1 transgenic mice.

### 3.1.6 The identification of human HLA-restricted MUC1 epitopes

Early research into the natural cellular immune response to MUC1 led to identification of $CD8^+$ T cells able to recognise hypo-glycosylated MUC1 core peptides in an MHC un-restricted manner[399]. It has since become clear that MUC1 also codes for peptides which can be recognised in a conventional manner, through binding murine MHC, and more recently for human HLA-A2 by specifically using HLA-A2 transgenic mice. Importantly this now allows targeting of specific MUC1 derived epitopes to provide a pre-clinical model in which to assess vaccination strategies. Current data regarding HLA-A2 binding peptides and their immunogenicity are shown in Table 3.1 and is discussed further in relation to our strategy using DNA vaccines in Section 3.1.10.

### 3.1.7 Clinical trials of MUC1 vaccines

An overview of selected phase I and II trials using vaccines to target MUC1 shows the multiplicity of approaches used. (Table 3.2). Current strategies to target MUC1 include:

**Peptide/protein vaccines**

MUC1 peptides (of various length incorporating the VNTR), linked to adjuvant sequences such as KLH and DT, are able to induce anti-MUC1 IgM and IgG antibody responses in breast cancer patients phase I/II trials[419, 420, 450] as well as some proliferative responses[392, 450].

Mannan-MUC1 fusion protein containing 5 tandem repeats of the VNTR has also been shown to induce MUC1 specific antibody responses (69% patients), however there was no reactivity with MUC1 expressing tumour cells. This vaccination protocol was also able to

**Table 3.1. HLA-A2 restricted MUC1 epitopes**

| HLA-A2 peptide sequence | MUC1 region | Predictive algorithm score / HLA-A2 stabilisation / $IC_{50}$ | CTL raised in HLA-A2 mice | CTL repertoire in humans | Presented by tumour cells expressing MUC1 endogenously | Reference |
|---|---|---|---|---|---|---|
| LLLTVLTVV * | Signal sequence | Algorithm score = 412<br>Stabilised HLA-A2 on RMA-S-HHD cells | Yes | Nd | Yes, human tumour cells lysed by specific CTL and recognised by anti-MHC peptide antibodies. | (451, 452) |
| ALASTAPPV | 3' to VNTR | Algorithm score = 69<br>Stabilised HLA-A2 on RMA-S-HHD cells | Yes | Nd | Yes | (452) |
| NLTISDVSV | 3' to VNTR | Algorithm score = 69<br>Stabilised HLA-A2 on RMA-S-HHD cells | Yes | Nd | Yes | (452) |
| **LLLLTVLTV** | Signal sequence | Stabilised HLA-A2 on T2 cells | Yes | Nd | Yes | (453) |
| STAPPVHNV | Degenerate repeat | Stabilised HLA-A2 on T2 cells | Yes | Nd | Yes | (453) |
| FLSFHISNL | 3' to VNTR | Algorithm score = 226<br>Stabilised HLA-A2 on JY cells<br>$IC_{50}$ = 7μM | Yes | Nd | Yes, murine MUC1 transfectants | (454) |

**Table 3.1 Continued**

| HLA-A2 peptide sequence | MUC1 region | Predictive algorithm score / HLA-A2 stabilisation / $IC_{50}$ | CTL raised in HLA-A2 mice | CTL repertoire in humans | Presented by tumour cells expressing MUC1 endogenously | Reference |
|---|---|---|---|---|---|---|
| **ALGSTAPPV** | 3' to VNTR | Algorithm score = 69<br>Stabilised HLA-A2 on JY cells<br>$IC_{50}$ = 10μM | Yes | Nd | Yes, murine MUC1 transfectants | (454) |
| **TLAPATEPA** | 5' to VNTR | Algorithm score = 2<br>Stabilised HLA-A2 on JY cells<br>$IC_{50}$ = 11μM | Yes | Nd | Yes, murine MUC1 transfectants | (454) |
| LLLTVLTVV * | Signal sequence | Algorithm score = 412<br>Stabilised HLA-A2 on JY cells<br>$IC_{50}$ = 6μM | No | Nd | Nd | (454) |
| **STAPPAHGV** | VNTR | Stabilised HLA-A2 weakly | Yes | Nd | Nd | (455) |

Peptide sequences in **bold underlined** indicate those used in this study.

Nd = not determined

* = Same peptide sequence

**Table 3.2. Clinical trials of MUC1 vaccines**

| Tumour | Vaccine | Adjuvant | Patient Number | Immune response | Clinical response | Reference |
|---|---|---|---|---|---|---|
| Breast cancer | MUC1 peptide 1.5 TR conjugated to KLH | QS-21 | 6 | 4/6 IgM reacted with MUC1<br>3/6 IgG reacted with MUC1 | Nd | (419) |
| | | | 9 | 7/9 IgM reacted with MUC1 expressing tumour<br>3/9 IgG reacted with MUC1 expressing tumour | Nd | (420) |
| Breast cancer | MUC1 peptide (106 amino acids) conjugated to KLH | QS-21 | 6 | 6/6 made IgM and IgG reacted with MUC1<br>3/6 T cell IFNγ release to MUC1<br>3/6 made specific proliferative responses | Nd | (392) |
| Breast cancer | MUC1 peptide 20mer linked to diphtheria toxin | None | 13 | 6/12 anti-MUC1 antibody responses | Nd | (450) |

**Table 3.2. continued**

| Tumour | Vaccine | Adjuvant | Patient Number | Immune response | Clinical response | Reference |
|---|---|---|---|---|---|---|
| Breast and pancreatic | MUC1 transfected DC | None | 10 | 4/10 $CD8^+$ T cell IFNγ release to MUC1 nonamers | Nd | (229) |
| Breast and Ovarian | HLA-A2 Peptide pulsed DC | None | 10 | 3/4 $CD8^+$ T cell IFNγ release to MUC1 nonamers | 1PR, 1SD | (456) |
| Various adenocarcinoma | MUC1 5 TR linked to mannan | None | 25 | 13/25 made anti-MUC1 antibody response<br>4/15 made proliferative response<br>2/10 made specific CTL response | Nd | (391) |

CR = Complete response
PR = Partial response
SD = Stable disease
Nd = Not determined

induce proliferative responses (4/15 patients) and CTL responses in 2/10 patients with cancer of various histology[391].

**Dendritic cell vaccines**

MUC1 transfected DC have been shown to elicit MUC1 specific $CD8^+$ T-cell responses in 4/10 breast or pancreatic cancer patients[229]. MUC1 HLA-A2 peptide pulsed DC were also able to elicit MUC1 specific $CD8^+$ T-cell responses in 3/4 breast and ovarian cancer patients[456].

In many of these cases there was no demonstration of reactivity with the native MUC1 or peptide/HLA complexes found on tumour cells. Many of these studies were also phase I/II and hence clinical responses were not formally investigated, but there appeared to be an apparent low level of clinical response against tumour cells in some cases[456]. These observations suggest and warrant the need for further vaccine modifications or protocols. Our strategy is to evaluate DNA vaccines as a means of potentiating effective immune responses against MUC1.

### 3.1.8 MUC1 and multiple myeloma

Increased MUC1 expression has been described in various haematological malignancies, including MM[386, 390, 399]. A range of MUC1 reactive antibodies are available and allow definition of both glycosylated and non-glycosylated forms, and these have been used to determine the levels of MUC1 surface expression on MM cells. The BM-2 anti-MUC1 antibody has been used to show that 92% of MM samples are positive for MUC1 expression[390]. A more detailed analysis using VU-3C6, VU-4H5 and DF3 antibodies revealed that while 5/5 MM patient plasma cells were positive for MUC1, the percentage of cells positive within one clone ranged from 8 to 84%, which depended on the glycosylation state of the MUC1 expressed[386]. MUC1 expression on malignant MM plasma cells was dramatically increased when compared to normal B cells. Work carried out in our laboratory has additionally shown that MUC1 expression in MM patients can be detected as a surface glycoprotein. However, in the absence of surface expression, it could still be detected as mRNA (Dr. F. Forconi, unpublished data). Dexamethasone treatment can also elevate MUC1 expression in MM cells[386] which may augment treatments aimed at targeting this molecule. MUC1 can also be detected in the serum of MM patients, and can therefore be used to monitor disease progression. A notable increase in soluble MUC1 has been observed in MM patients with active disease when compared to minimal disease[400],

signifying the most suitable time for immunotherapy to be whilst the patient is in clinical remission.

### 3.1.9 Preliminary research in our laboratory

MUC1 clearly represents an important, over-expressed tumour antigen with a wide distribution in varyious carcinomas, as well as in haematological malignancies. Although naturally occurring T cell and antibody responses to MUC1 have been detected in cancer patients at a low level, these responses are generally not effective at controlling disease, and a central aim of our study is to utilise DNA vaccines to enhance immunity against MUC1.

This study follows on from preliminary work in our laboratory which evaluated an aspect of DNA vaccines designed to induce anti-MUC1 immunity. The vaccine constructs developed encoded full length human MUC1 sequence, either alone (p.MUC1) or fused to full length FrC (p.MUC1FrC), or to the first domain of FrC, DOM (p.MUC1DOM) (Figure 3.4, Section 3.2). All 3 vaccines were shown to induce anti-MUC1 antibodies in wild type (H-$2^b$) mice and to protect from tumour challenge with RMA-MUC1. However, antibody responses did not correlate with observed protection. Notably tumour cells derived from mice which were not protected by vaccination indicated a decrease in expression of MUC1 from their surface. Antibody-mediated protection was therefore not pursued further. These data implied a role for for cellular immunity and lymphocyte depletion experiments confirmed a role for CTL in mediating protection. This early work also showed that CTL specific for two known MUC1 MHC class I epitopes could be induced by vaccination with either p.MUC1, p.MUC1FrC or p.MUC1DOM in wild type mice. These cells could effectively lyse peptide pulsed target tumour cells *in vitro* (Dr. F. Forconi, unpublished data).

### 3.1.10 Aims of this study

- Assess the ability of DNA vaccines encoding full length MUC1, or MUC1 fused to FrC or DOM to provide protection from a tumour challenge with RMA-MUC1 in wild type mice.
- Delineate the roles of $CD8^+$ T cells and $CD4^+$ T cells in tumour protection.
- Compare the ability of DNA vaccines encoding full length MUC1, or MUC1 fused to FrC or DOM to induce epitope-specific CTL and determine the role of FrC in providing T-cell help.

- Compare the ability of DNA vaccines encoding full length MUC1, or MUC1 fused to FrC or DOM to the p.DOM-peptide design to induce epitope-specific CTL
- Assess the efficacy with which vaccination with the p.DOM-peptide design can induce immunity and provide protection from tumour challenge with RMA-MUC1 in wild type mice.
- Compare the ability of all DNA vaccines encoding MUC1 to induce immunity in the more appropriate MUC1 tg mice.
- Examine the ability of the p.DOM-peptide design to induce MUC1 specific CTL in the human HLA-A2 transgenic mouse for a ready translation into the clinical setting.

## 3.2 Results

### 3.2.1 Vaccine Integrity

The integrity of DNA vaccines encoding full length MUC1 (Figure 3.4), was confirmed by restriction enzyme digests using cloning enzymes. Limited DNA sequencing was performed to confirm that MUC1 was maintained in frame and where applicable, fused to FrC. Due to the guanine/cytosine rich tandem repeat, conventional sequencing of the entire MUC1 nucleotide sequence was difficult to perform. The integrity of all other constructs was confirmed by DNA sequencing and restriction enzyme digest analysis. Protein expression of all vaccines was confirmed by *in vitro* transcription/translation and each of the vaccines expressed a protein of the expected size, calculated approximately by assuming an average molecular mass for each amino acid to be 110 Daltons. Representative results are shown in Figure 3.5.

### 3.2.2 Target tumour cells

RMA-MUC1 is a Rauscher MuLV induced thymoma cell line stably transfected with a plasmid encoding the full-length human MUC1 sequence with 39 repeats (a kind gift from Prof. J. Taylor-Papadimitriou, CRUK London). The transfected tumour cell line was phenotypically characterised by flow cytometry for expression of MUC1, MHC class I (H-$2k^b$ and H-$2D^b$), MHC class II (I-A and I-E), as well as for CD4 and CD8. High levels of MUC1 and both MHC class I alleles were apparent, whereas no MHC class II or CD4 expression was observed. CD8 expression was low (Figure 3.6).

### 3.2.3 Antibody response to vaccination

To determine the ability of the vaccine encoded protein to fold correctly *in vivo*, and to generate an immune response, C57Bl/6 mice were vaccinated with DNA constructs shown in Figure 3.4. Serum was collected on day 28 after two DNA vaccinations on day 0 and day 21. The levels of anti-FrC and anti-MUC1 antibodies of IgG class were determined using a specific ELISA. As expected, vaccination with p.MUC1FrC, p.DOM-MUC1peptide1 and p.DOM-MUC1peptide2 and p.FrC generated detectable levels of anti-FrC antibodies (Figure 3.7). A decreased FrC-specific response was seen when vaccinating with constructs containing DOM probably because of a reduction in the number of epitopes available. It should be noted however, that vaccination with p.MUC1DOM did not generate anti-FrC

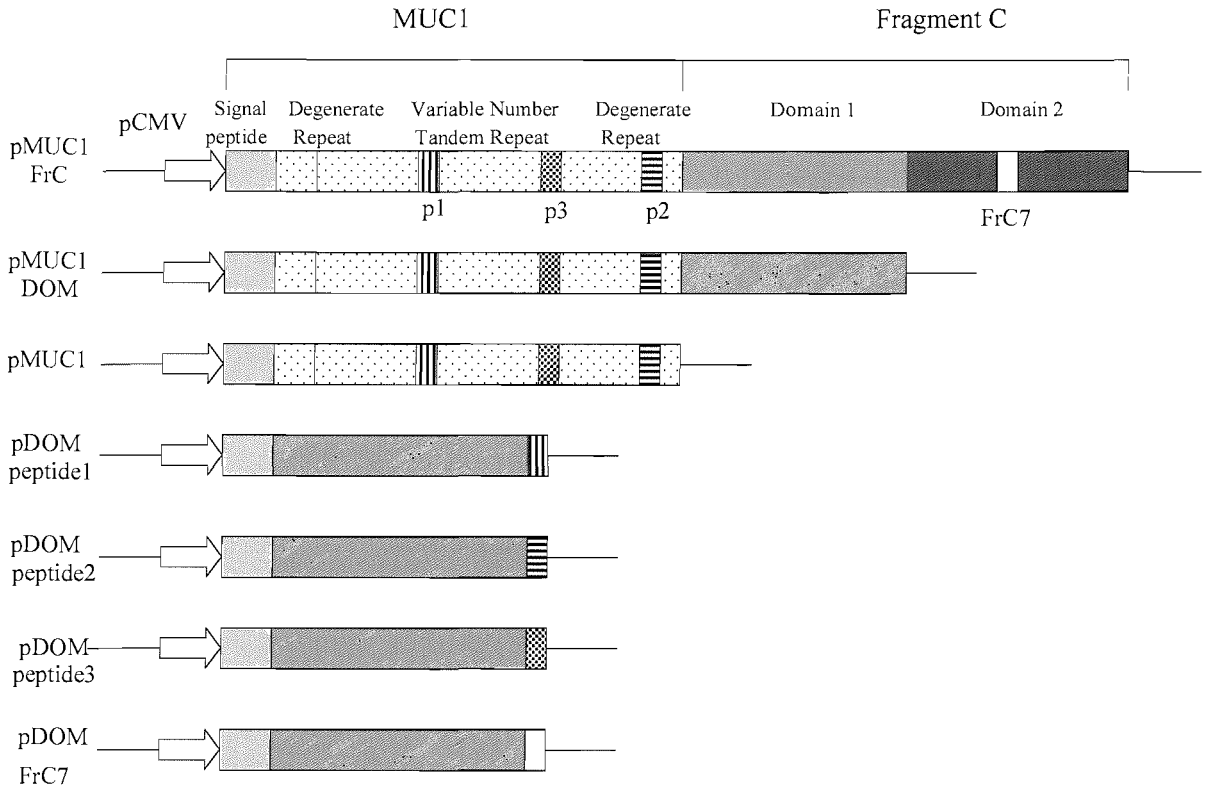



**Figure 3.4. Diagrammatic representation of DNA vaccines containing MUC 1 sequences**

Peptide 1 (SAPDTRPAP) and 3 (STAPPAHGV) are located within the VNTR, peptide 2 (SAPDNRPAL) is located within the degenerate repeat and peptide 4 (TVVTGSGHA) is located within the signal sequence, not shown. FrC7 (SNWYFNHL) is an MHC class I epitope from the second domain of FrC.

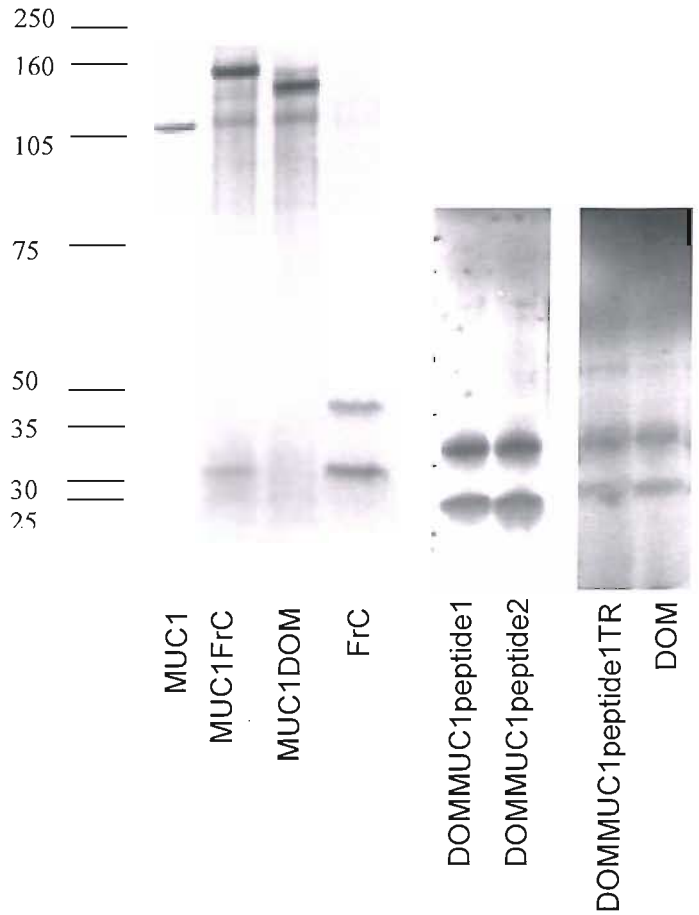



**Figure 3.5. Transcription and translation of DNA vaccines encoding MUC1 *in vitro***

DNA vaccines were transcribed and translated *in vitro* using a reticulocyte expression system. All plasmids produced a protein of expected size and examples are shown. Expected product sizes are as follows: MUC1 - 105KDa, MUC1FrC – 154KDa, MUC1DOM – 133KDa, FrC – 50KDa, DOM-MUC1peptide2 and peptide1TR – 29KDa and DOM– 28KDa.

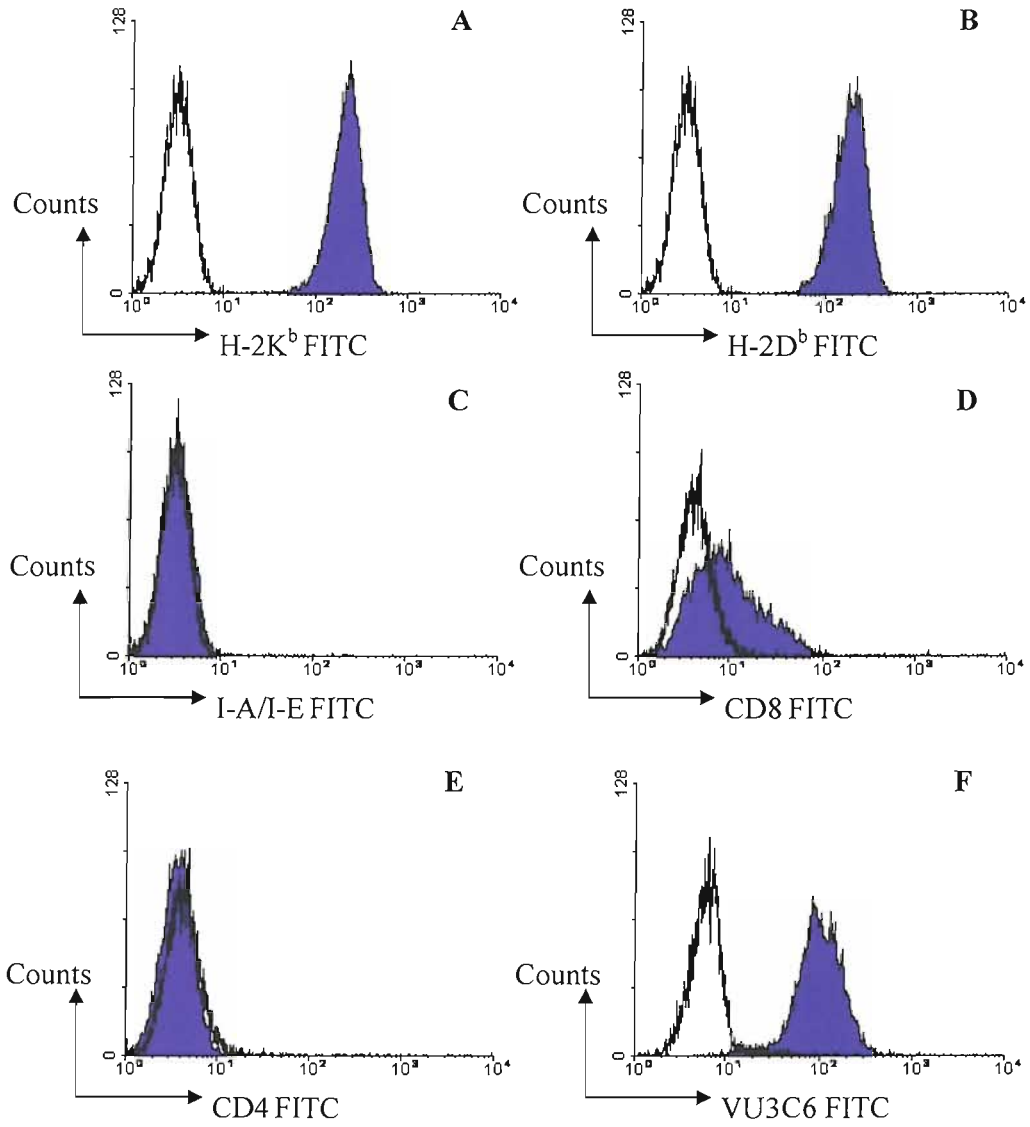



**Figure 3.6. Phenotypic analysis of RMA-MUC1**

RMA-MUC1 cells were incubated with FITC conjugated mAbs or with anti-MUC1 mAb (VU3C6) for 30 minutes, followed by FITC labelled goat anti-mouse Fcγ before FACS analysis. Live cells were gated on and assessed for fluorescent staining of: **A** and **B**, MHC class I (H-2K$^b$ and H-2D$^b$), **C**, MHC class II (I-A and I-E), **D**, CD8, **E**, CD4 and **F**, MUC 1. Cells were positive for expression of both MHC class I isotypes and for MUC1, with low expression of CD8 and no CD4 or I-A/I-E expression. In each case the solid black line represents staining with an isotype control antibody while the purple histogram represents specific staining.

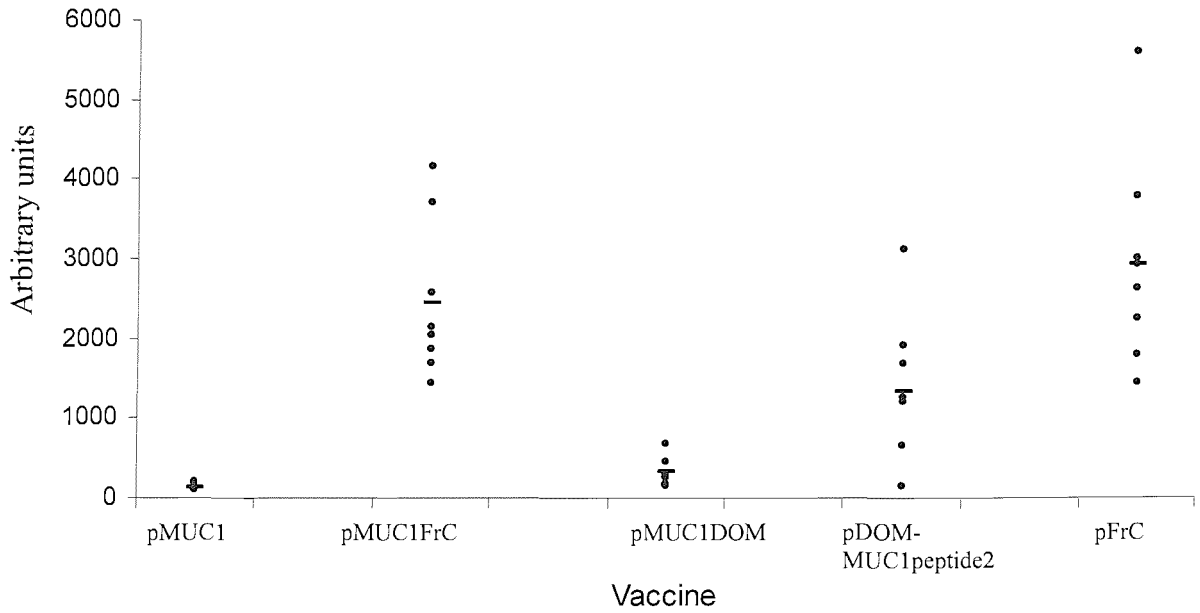



**Figure 3.7. Anti-FrC antibody responses generated in mice vaccinated with MUC1 containing DNA vaccines**

C57Bl/6 mice (8 per group) were vaccinated twice with p.MUC1, p.MUC1FrC, p.MUC1DOM, p.DOM-MUC1peptide2 or p.FrC and serum samples collected on day 28. Anti-FrC antibody was detected using specific ELISA and are given in arbitrary units. Black circles (•) denote responses made by individual mice, and black bars (—) the mean response in each group. Vaccination with p.MUC1FrC, p.DOM-MUC1peptide2 and p.FrC generated detectable levels of anti-FrC antibody ($p = 0.0005$ for p.MUC1 vs p.FrC using paired Student t test). Vaccination with p.MUC1DOM generated an anti-FrC antibody response compared to vaccination with p.MUC1 ($p = 0.0129$ using paired Student t test) , however this was not equivalent to that induced by vaccination with p.DOM-MUC1peptide2 ($p = 0.0124$ using paired Student t test). As expected, higher anti-FrC antibody levels were induced in mice vaccinated with plasmids encoding full length FrC compared with those receiving vaccines encoding only the first domain.

antibodies, despite synthesis of the predicted protein *in vitro* (Figure 3.5). It is possible that the large MUC1 molecule may interfere with correct DOM folding during protein synthesis and inhibit generation of anti-FrC antibodies. The vaccines incorporating p.DOM-MUC1peptide3 and p.DOM-MUC1peptide4 were not assessed for anti-FrC antibodies. The anti-MUC1 antibody response were similarly measured by ELISA using a 24mer MUC1 peptide incorporating 1 full tandem repeat and 4 amino acids from the next tandem repeat to coat ELISA plates. The results are shown in Figure 3.8. As expected, DNA constructs containing full length MUC1 were able to generate detectable antibody to this region of the MUC1 sequence. Fusion of FrC or DOM to MUC1 did not enhance the anti-MUC1 antibody response at this early time point. Fusion of FrC has been shown to enhance the anti-MUC1 antibody response at later time points with an increased number of vaccinations (Dr. F Forconi, unpublished data), consistent with the ability of FrC fusion constructs to provide linked T-cell help.

### 3.2.4 Efficacy of MUC1 DNA vaccines in inducing protective immunity against tumour challenge

DNA vaccines encoding full length MUC1, either alone or fused to FrC or DOM were assessed for their ability to induce immunity that would protect from a tumour challenge with RMA-MUC1. All experiments were performed in C57Bl/6 (H-$2^b$) mice. In a preliminary experiment, groups of 7 mice were vaccinated twice (day 0 and day 21) with p.MUC1, p.MUC1DOM and control plasmid p.DOM. 4 naïve mice were also included. Animals were challenged with $5x10^4$ RMA-MUC1 tumour cells by the i.p. route on day 28 and monitored for 100 days post challenge. Tumour cells were assessed for MUC1 expression on the day of challenge by FACS analysis and were positive (Figure 3.9A). All naïve mice and mice vaccinated with p.DOM were culled between day 15 and 18 due to tumour burden (Figure 3.10), with phenotypic analysis demonstrating no alteration in MUC1 expression on tumour cells from these mice (Figure 3.9B). Vaccination with p.MUC1 and p.MUC1DOM protected approximately 60% and 70% of mice at day 100 respectively (Figure 3.10). This was statistically significant, $p = 0.0003$ and $p = 0.0003$ respectively compared to p.DOM vaccination using $\chi^2$ Logrank test. Assessment of the MUC1 expression on tumour cells which grew progressively in animals previously vaccinated with p.MUC1 and p.MUC1DOM demonstrated a decrease in the level of expression of the cell surface glycoprotein (Figure 3.9C), suggesting immunological pressure to remove MUC1 from the cell surface.

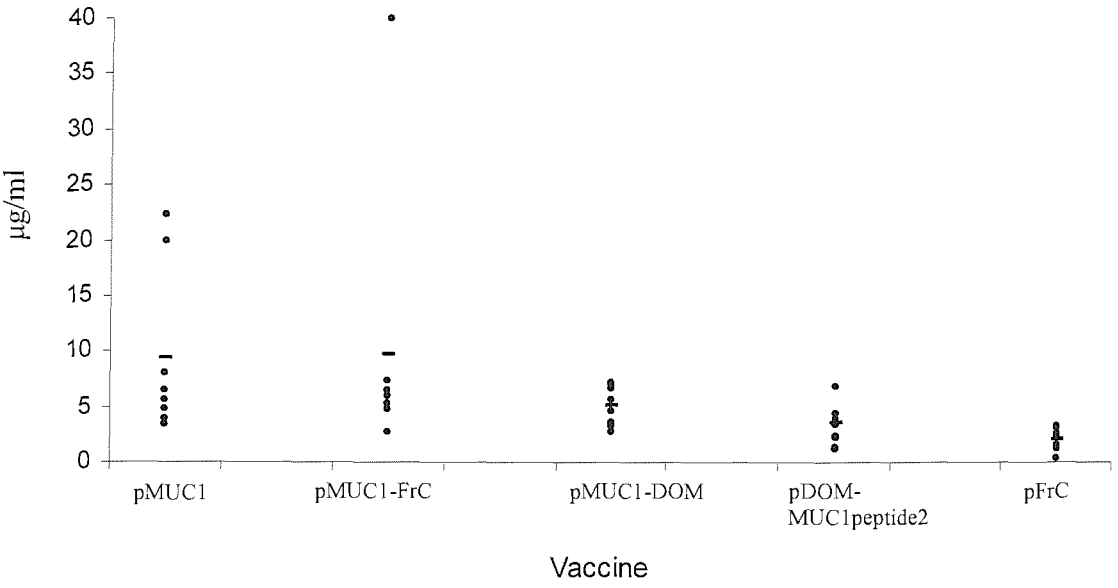



**Figure 3.8. Anti-MUC1 antibody responses generated in mice vaccinated with MUC1 containing DNA vaccines**

C57Bl/6 mice (8 per group) were vaccinated twice with p.MUC1, p.MUC1FrC, p.MUC1DOM, p.DOM-MUC1peptide2 or p.FrC and serum samples collected on day 28. Anti-MUC1 antibody was detected using specific ELISA and were compared to a standard (HMFG1) of known concentration. Black circles (•) denote responses made by individual mice, and black bars (—) the mean response in each group. Vaccination with p.MUC1, p.MUC1FrC, p.MUC1DOM led to significant levels of anti-MUC1 antibody being detected ($p = 0.0094$ for p.MUC1DOM vs. p.DOM-MUC1peptide2 using paired Student t test). Vaccination with pDOM-MUC1peptide2 did not generate significant levels of anti-MUC1 antibody in comparison to p.FrC ($p = 0.058$ using paired student t test).

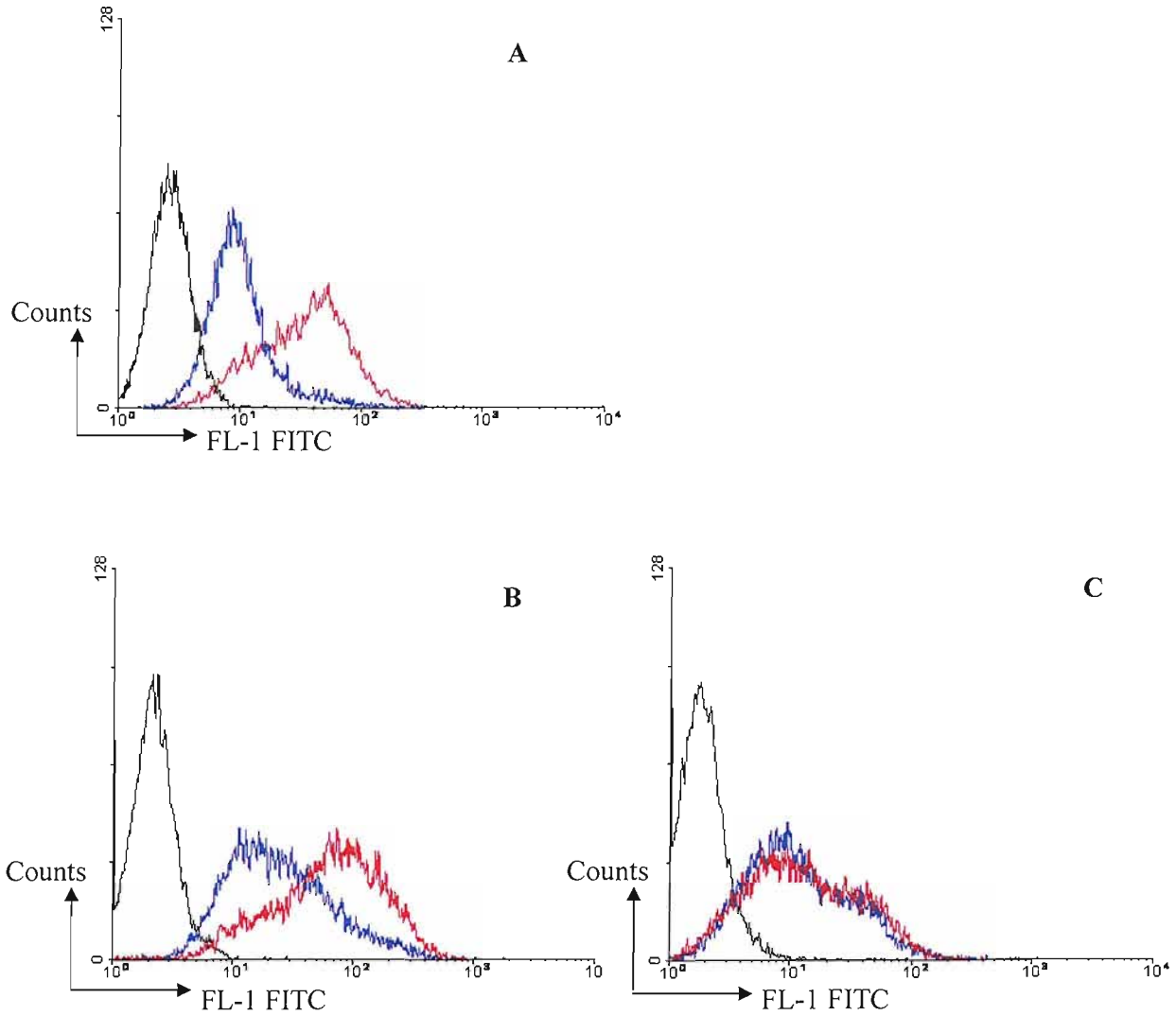



**Figure 3.9. Phenotypic analysis of RMA-MUC1 cells**

RMA-MUC1 tumour cells were assessed for MUC1 expression using HMFG1 anti-MUC1 mAb. Black line illustrates tumour cells alone, blue line illustrates tumour cells incubated with secondary FITC labelled goat anti-mouse IgG alone, and red illustrates tumour cells incubated with HMFG1 followed by FITC labelled goat anti-mouse IgG. **A**, cells were assessed and positive for MUC1 expression prior to challenge. **B**, a representative example of tumour cells that arose in mice vaccinated with control plasmid (p.DOM), these cells remained positive for MUC1 expression demonstrating no down-regulation of the glycoprotein from the cell surface. Tumour cells arising in all other control mice were similarly positive. **C**, a representative example of tumour cells that arose in mice vaccinated with p.MUC1DOM. HMFG1 staining of these cells (red line) was identical to that of secondary FITC staining alone (blue line). This indicates that these cells were negative for MUC1 expression demonstrating the ability of the tumour cells to down-regulate MUC1 from the cell surface. However there was also a possibility that anti-MUC1 antibodies generated *in vivo* remained coated to the RMA-MUC1 cell surface following tumour cell removal from the animal, and may have been able to block HMFG1 staining.

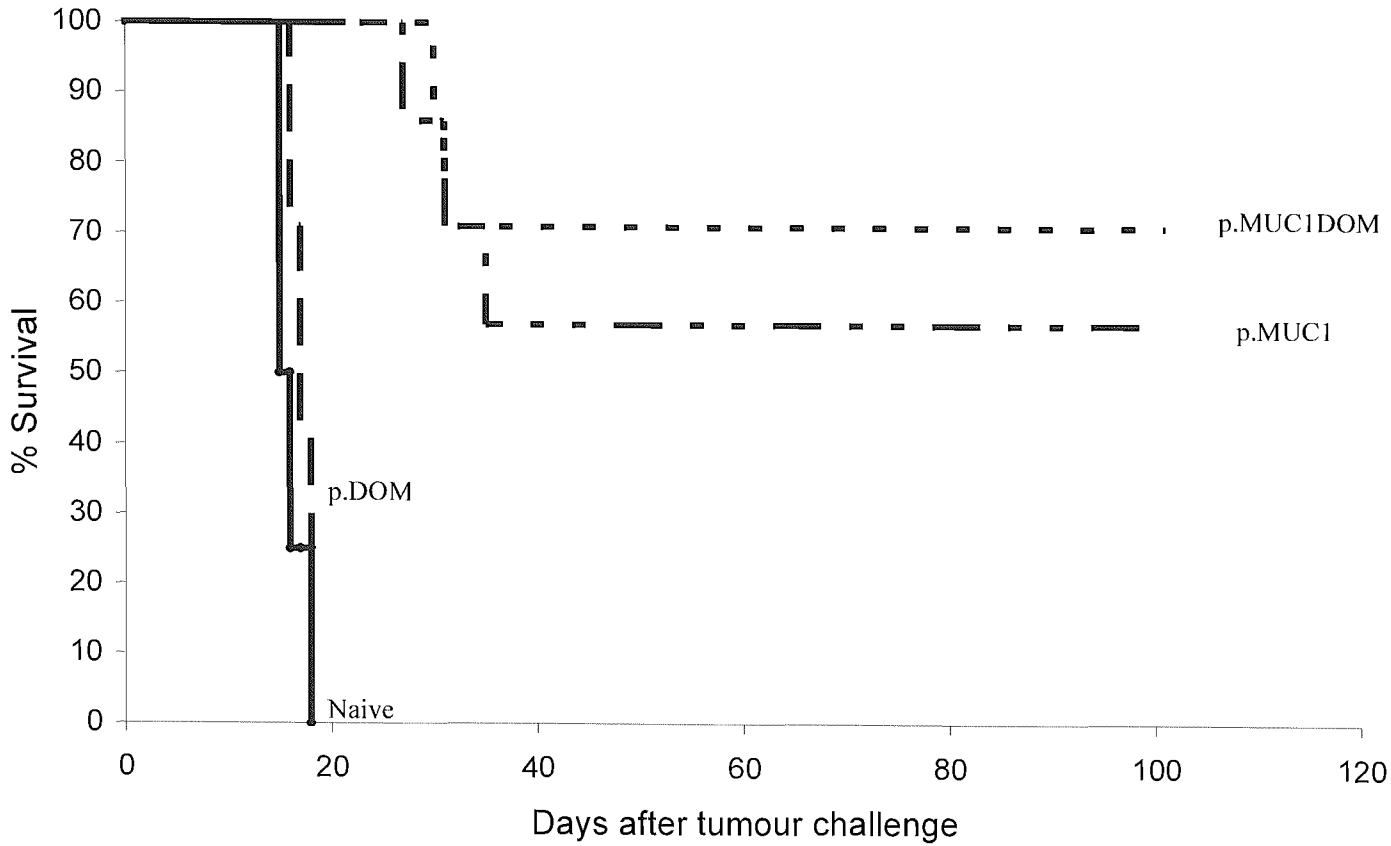



**Figure 3.10. Mice vaccinated with MUC1 containing DNA vaccines are protected from tumour challenge with RMA-MUC1**

C57Bl/6 mice were vaccinated twice with p.MUC1, p.MUC1DOM or control vaccine p.DOM. Naïve mice received no vaccination. Mice were challenged with $5x10^4$ RMA-MUC1 tumour cells i.p. on day 28. Mice were monitored for 100 days and culled when tumour burden (ascites) reached 20% of the original weight of the mouse. Naïve mice and those vaccinated with control plasmid demonstrated no protection from tumour challenge and were culled between day 15-18. Mice vaccinated with p.MUC1 or p.MUC1DOM were protected from tumour challenge, with approximately 60 and 70% of mice surviving at day 100. This was statistically significant compared to p.DOM vaccination ($p = 0.0003$ and $p = 0.0003$ respectively using $\chi^2$ Logrank test)

In a repeat experiment, groups of 8 age and sex matched mice were vaccinated twice (day 0, 21) with p.MUC1, p.MUC1DOM and control plasmid p.DOM. In this experiment, groups of mice vaccinated with p.MUC1FrC and control p.FrC were also included, and naïve mice received no vaccination. Mice were challenged with RMA-MUC1 on day 28 as described above. The results confirmed observations from the first experiment (Figure 3.11). Naïve mice and mice vaccinated with p.DOM had to be culled between days 20 and 30 due to tumour burden. 75% of mice vaccinated with p.FrC were culled before day 20 due to tumour burden. Vaccination with p.MUC1, p.MUC1DOM and p.MUC1FrC significantly protected approximately 75%, 75% and 60% of mice respectively ($p = 0.0341$ for p.MUC1FrC compared with p.FrC using $\chi^2$ Logrank test). The low level of animals surviving tumour challenge following vaccination with p.FrC was not statistically significant compared to p.DOM vaccination using $\chi^2$ Logrank test ($p = 0.169$).

### 3.2.5 Effect of $CD8^+$ and $CD4^+$ T-cell depletion on DNA vaccine mediated protection

To assess the role of $CD8^+$ T cells and $CD4^+$ T cells in the observed DNA vaccine mediated protection, antibodies were used *in vivo* to deplete the relevant subset at the time of tumour challenge. Firstly however, the CD8 and CD4 depleting antibodies were assessed for their depleting efficiency *in vivo* (Figure 3.12). The percentage of $CD8^+$ or $CD4^+$ T cells depleted was assessed by comparison with the same population in non-depleted animals. The CD8 depleting antibody removed at least 95% of the relevant subset and the CD4 depleting antibody removed 89% of the relevant subset. When both antibodies are delivered together, 93% of the $CD8^+$ T-cell population are removed and 76% of the $CD4^+$ T-cell population are removed. Secondly, it is to be noted that the RMA–MUC1 tumour cell line does not express significant levels of CD4 or CD8 (Figure 3.6), which allows use of depleting antibodies without any direct effect on tumour growth. To formally demonstrate this, naïve non-vaccinated mice were depleted of either CD4 or CD8 or given control rat IgG at the time of tumour challenge. Mice were then monitored until culled due to tumour burden. Figure 3.13 shows that neither depleting antibody had any effect on tumour growth. Subsequently, groups of 8 age and sex matched mice were vaccinated twice (day 0, 21) with either p.MUC1DOM or p.DOM. Mice then received 5 doses of either anti-CD4, anti-CD8, or both, normal rat IgG or no treatment at time of tumour challenge. The results, shown in Figure 3.14, indicate cumulative results from 2 experiments: 94% of control mice vaccinated with p.DOM and given rat IgG were culled

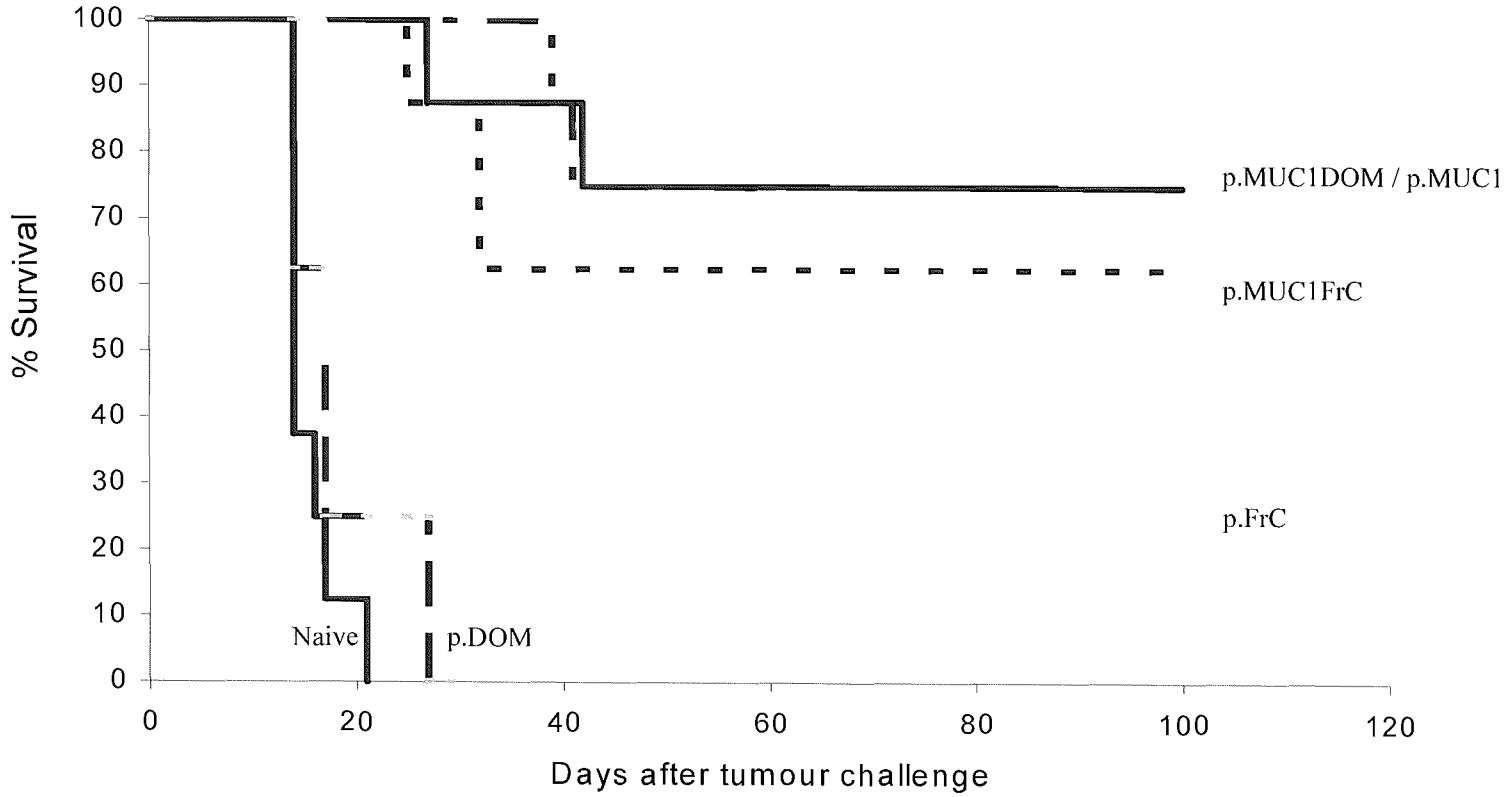



**Figure 3.11. Mice vaccinated with MUC1 containing DNA vaccines are protected from tumour challenge with RMA-MUC1**

In a repeat protection experiment, C57Bl/6 mice were vaccinated twice with p.MUC1, p.MUC1DOM, p.MUC1FrC, or control vaccines p.DOM, or p.FrC. Naïve mice received no vaccination. Mice were challenged with $5x10^4$ RMA-MUC1 tumour cells i.p. on day 28 and monitored for 100 days. Mice were culled when tumour burden (ascites) reached 20% of the original weight of the mouse. Naïve mice and those vaccinated with control plasmid p.DOM demonstrated no protection from tumour challenge and were culled between day 20-30. Mice vaccinated with p.FrC also demonstrated no protection from tumour challenge compared to p.DOM vaccination ($p = 0.169$, $\chi^2$ Logrank test). Mice vaccinated with p.MUC1, p.MUC1DOM or p.MUC1FrC were significantly protected from tumour challenge, with approximately 75%, 75% and 60% of mice surviving at day 100 ($p = 0.0341$ for p.MUC1FrC compared to p.FrC using $\chi^2$ Logrank test).

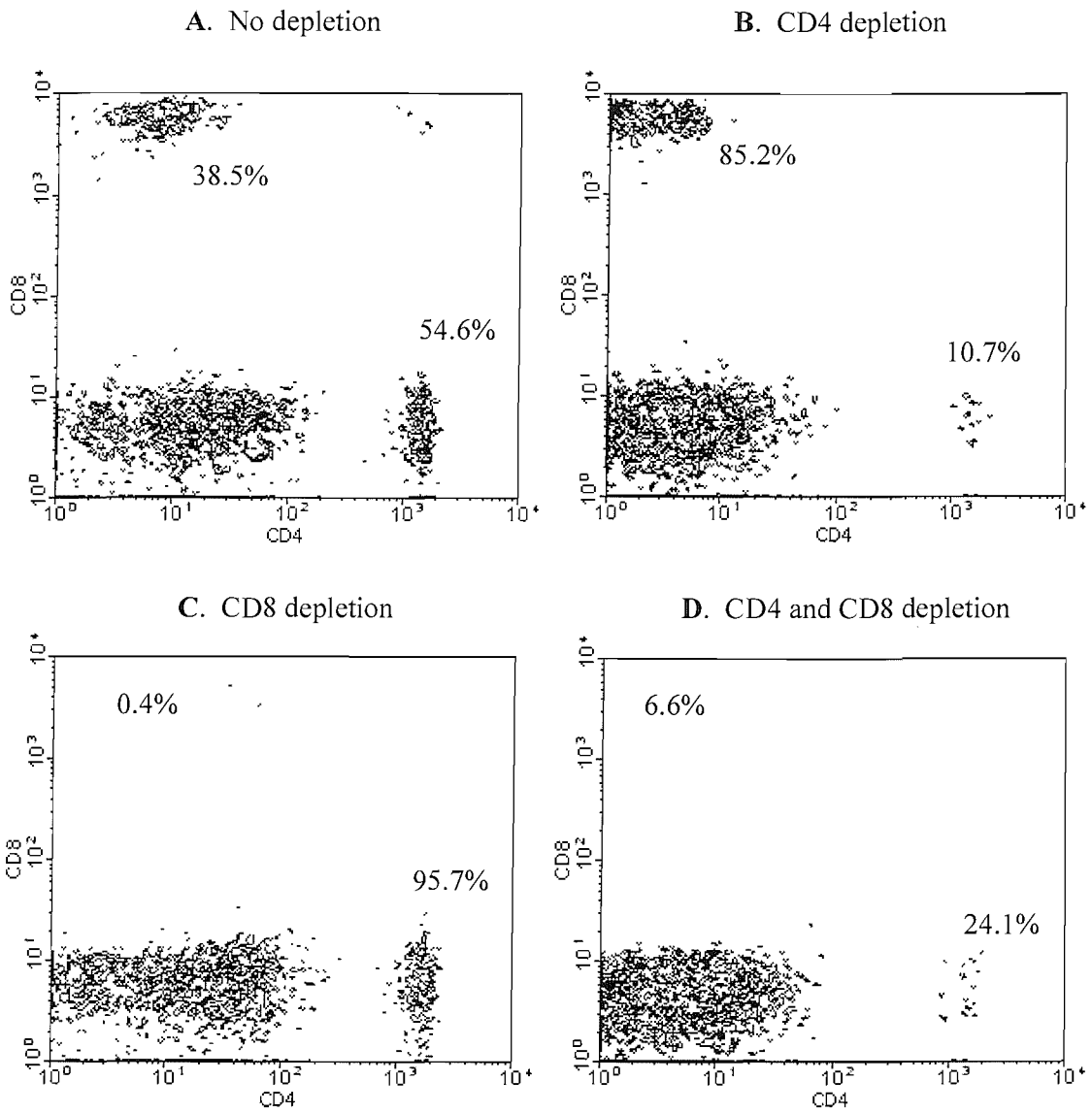



**Figure 3.12. CD8 and CD4 depleting antibodies were assessed for their depletion efficiency *in vivo***

Naïve mice were administered **A**, no antibody, **B**, depleting anti-CD4 antibody, **C**, depleting anti-CD8 antibody or **D**, depleting anti-CD4 and anti-CD8 antibodies. Mice received 5 injections of 100μg of antibody over a period of 10 days. Mice were tail bled and their lymphocytes assessed for populations of $CD8^+$ and $CD4^+$ T cells using fluorescent labelled mAbs specific for CD3, CD8 and CD4 and analysed by FACS. The lymphocytes were gated on based on their FS and SC properties and the plots above generated. The percentages of $CD3^+CD8^+$ or $CD3^+CD4^+$ T cells relative to the same population in mice given no depleting antibodies **(A)** are shown. **B**, CD4 depleting antibody removes 89% of $CD4^+$ T cells. **C**, CD8 depleting antibody removes >95% of $CD8^+$ T cells. **D**, CD4 and CD8 depleting antibodies given simultaneously remove 93% $CD8^+$ T cells and 76% $CD4^+$ T cells.

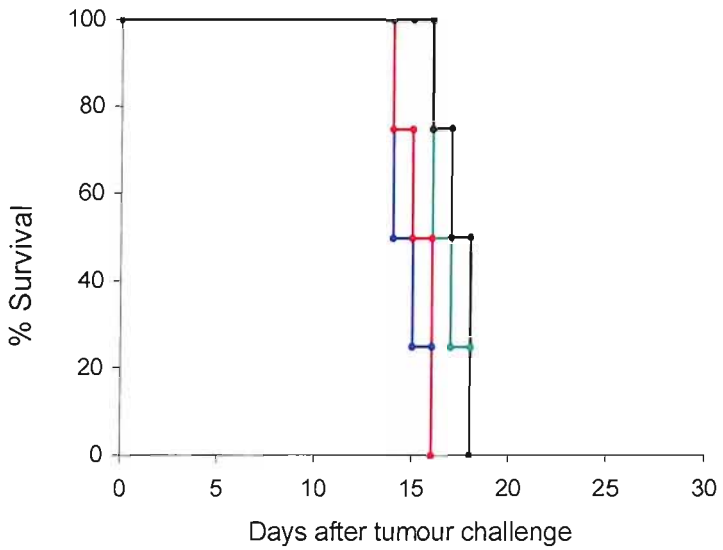



**Figure 3.13. CD4 and CD8 depleting antibodies have no effect on tumour growth in naive animals**

Naïve mice were administered 100μg of depleting anti-CD8 antibody (green line) or depleting anti-CD4 antibody (blue line) or control rat IgG (black line) i.p over a period of 10 days. The red line indicates mice receiving no antibody. Mice were challenged with $5x10^4$ RMA-MUC1 tumour cells and culled when tumour burden reached 20% of the original weight of the mouse. Depleting either the $CD8^+$ or $CD4^+$ T cell populations has no effect on tumour growth.

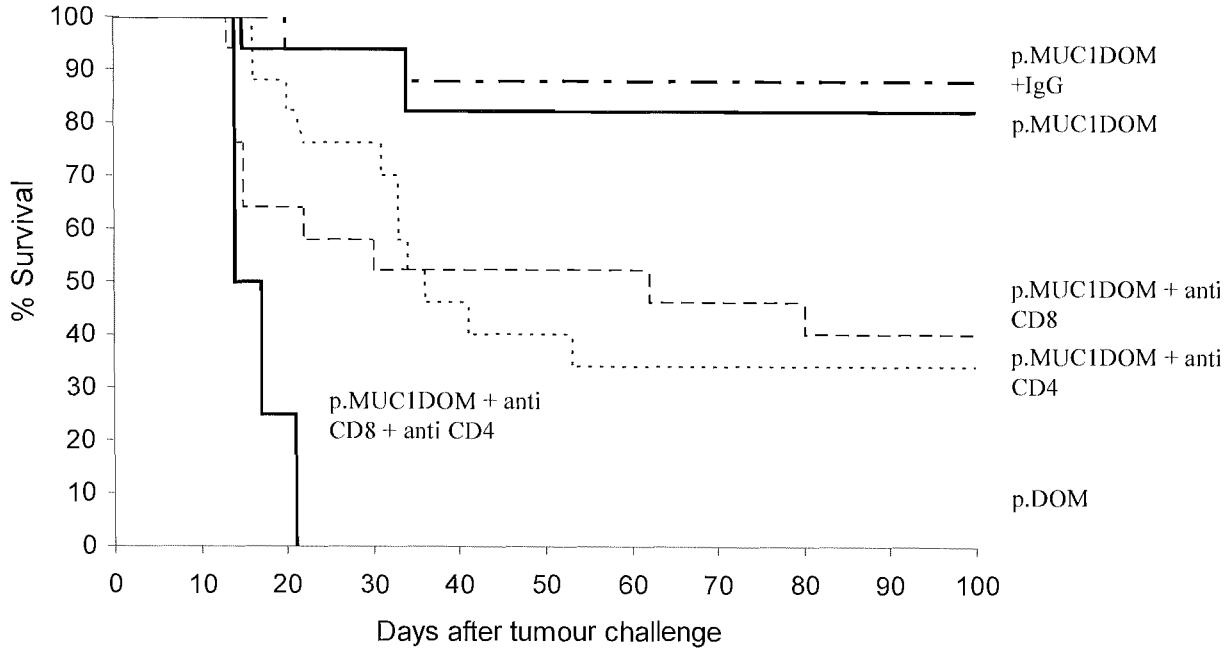



**Figure 3.14. $CD4^+$ and $CD8^+$ T cells play a critical role in the protective immune response to tumour generated by DNA vaccination**

C57Bl/6 mice were vaccinated twice with p.MUC1DOM (day 0 and 21) before being administered 100μg of depleting anti-CD8 antibody, or depleting anti-CD4 antibody, or both, or control rat IgG i.p. at the time of tumour challenge. Further control groups received p.MUC1DOM or p.DOM with no antibody. Mice were challenged on day 28 with $5x10^4$ RMA-MUC1 tumour cells and culled when tumour burden reached 20% of the original weight of the mouse. 94% of mice vaccinated with p.DOM were culled before day 40 due to tumour burden, whereas 88% and 82% of mice vaccinated with p.MUC1DOM and given either rat IgG or no antibody respectively remained tumour free at day 100. However 34% of mice depleted of $CD4^+$ T cells and 40% of mice depleted of $CD8^+$ T cells and 0% of mice depleted of both subsets were tumour free at day 100 indicating a role for both $CD4^+$ T cells and $CD8^+$ T cells in protection. $p = 0.0051$ for CD4 depletion, $p = 0.0034$ for CD8 depletion and $p = <0.0001$ for double depletion compared to p.MUC1DOM using $\chi^2$ Logrank test.

before day 40 after tumour challenge due to tumour burden; 82-88% of mice vaccinated with p.MUC1DOM and given either no treatment or rat IgG were tumour free at day 100. Mice vaccinated with p.MUC1DOM and depleted of $CD8^+$ T cells resulted in 40% of mice being tumour free at day 100, indicating that $CD8^+$ T-cells play an important role in protection mediated by this vaccine. Furthermore, 34% of mice vaccinated with p.MUC1DOM and depleted of $CD4^+$ T cells were tumour free at day 100, also suggesting a critical role for $CD4^+$ T cells in mediating tumour protection induced by vaccination. Depleting both $CD4^+$ and $CD8^+$ T-cell subsets resulted in 0% of mice being tumour free by day 30, highlighting that T cells are responsible for all protective immunity and the synergistic nature with which these two subsets work in this model. This result does correlate well with current literature, where depletion studies have hinted at a combined role for $CD4^+$ T cells and $CD8^+$ T cells in MUC1 targeted immune protection in specific tumour models or modes of vaccination[457].

### 3.2.6 Comparing vaccine design to induce MUC1 peptide-specific CTL

In this part of the investigation, the role of $CD8^+$ T cells in mediating protection against MUC1 expressing tumours was specifically addressed. To confirm preliminary work carried out by Dr. F. Forconi, the ability of vaccines p.MUC1, p.MUC1FrC and p.MUC1DOM to elicit CTL in C57Bl/k mice (against 2 published peptide sequences, SAPDTRPAP and SAPDNRPAL[436, 437]) was assessed. Strong CTL responses against peptide coated EL-4 target cells were observed after two intramuscular injections (day 0, 21) and 1 week *in vitro* stimulation with 20μM of either peptide1 or peptide2 (Figure 3.15). However, the responses seen after vaccination with p.MUC1 and p.MUC1DOM were consistently greater than those seen with p.MUC1FrC in 2 of 2 experiments, indicating possible competition between immunodominant CTL epitopes in FrC and MUC1. An alternative explanation could be due to a decreased protein turnover from the plasmid due to the large size of insert compared to plasmids with smaller coding inserts such as p.MUC1 alone. In addition, the proteasome may not be as efficient in relation to protein turnover with the large and bulky MUC1FrC fusion protein as compared to smaller fusion proteins. To investigate this further, and as specific tetramers were not available, ELISPOT assays were used to assess the quantitative effect of FrC fusion in vaccine design and this is discussed below.

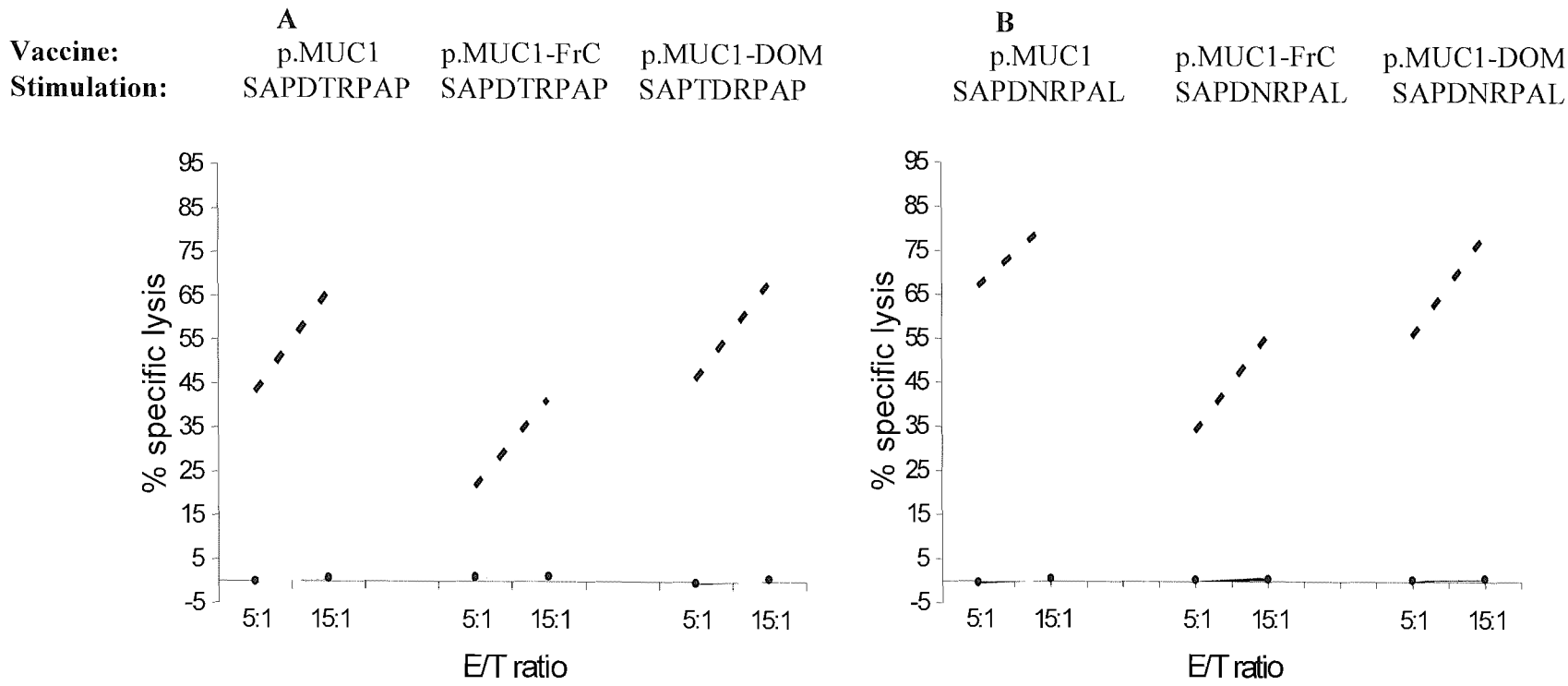



**Figure 3.15. DNA vaccination with MUC1 encoding vaccines efficiently activates MUC1 peptide-specific CTL**

C57Bl/k mice were vaccinated twice with p.MUC1, p.MUC1FrC or p.MUC1DOM and spleens taken on day 35. 1 week *in vitro* re-stimulation was performed with 20μM peptide1 (**A**) or peptide2 (**B**). A standard 5 hour $^{51}Cr$ release assay was performed against peptide coated EL4 targets, EL4 + peptide1 (dotted line, ·····, A), EL4 + peptide2 (dotted line, ·····, B), EL4 + FrC7 (grey line, ) or EL4 alone (black line, **–**). A greater level of lysis was seen against both peptide1 and peptide2 pulsed target cells when immunising with p.MUC1 or p.MUC1DOM as compared to p.MUC1FrC.

To increase the efficacy of DNA vaccines further for CTL induction, the p.DOM.epitope design was employed to target 4 MUC1 epitopes. The p.DOM-MUC1peptide2 vaccine was able to elicit strong CTL responses against peptide2 coated EL-4 targets after a single intramuscular injection (day 14) and one *in vitro* stimulation; this response was maintained and marginally enhanced after two intramuscular injections (day 28) and one *in vitro* stimulation (Figure 3.16, representative of 4 experiments at day 14 and 2 experiments at day 28). The response was specific as no CTL activity was detected against EL-4 alone or EL-4 pulsed with an irrelevant peptide (FrC7), and additionally no CTL activity was detected against any of the targets when mice were vaccinated with the control, p.DOM. IFNγ synthesis is an additional marker for $CD8^+$ T-cell activation and was assessed using intracellular fluorescent antibody staining and FACS analysis. The FACS profile shown in Figure 3.17 mirrors the CTL data, demonstrating peptide-specific $CD8^+$ T-cell expansion. Varying the concentration of peptide used for stimulation (20μM – 0.002μM) or for pulsing target cells (10μM – 0.001μM) had little effect on the response seen, indicating expansion of high affinity CTL (data not shown). In contrast, p.DOM-MUC1peptide1 was unable to induce CTL which could recognise peptide 1 coated EL-4 targets after one or two intramuscular injections (day 14 or 28) with 1 or 2 *in vitro* stimulations with various concentrations of peptide 1 (data not shown). The absence of a response may be a result of poor processing of this peptide, possibly at the level of the proteasome or recognition by TAP. The low affinity of peptide1 for MHC, as suggested by predictive algorithms and the lack of defined anchor residues[458, 459], may also play a role in the inability of this vaccine to induce an observable CTL response. To investigate this further, an MHC class I stability assay was undertaken using a TAP deficient cell line RMA-S. Addition of peptide to these cells in culture can stabilise empty MHC class I molecules on the cell surface. This can be detected using anti-MHC class I antibodies specific for the correctly folded structure. Figure 3.18 shows that peptide 2 is able to stabilise $H\text{-}2D^b$ at concentrations as low as 1μM. Peptide 1 however was unable to stabilise $H\text{-}2K^b$ at any concentration used (0.1-50μM). Stabilisation of $H\text{-}2K^b$ by SIINFEKL was used as positive control.

Following this, a direct comparison of p.MUC1, p.MUC1DOM and p.DOM-MUC1peptide2 vaccines was investigated in parallel after two intramuscular injections (day 0, 21) and 1 *in vitro* stimulation with 0.002μM peptide2. Vaccinating with p.DOM-MUC1peptide2 resulted in a improved CTL response against peptide coated targets when compared to vaccines incorporating the full length MUC1 sequence (Figure 3.19, 1 of 1 experiment). This may be due to immunodominance between MHC class I epitopes within the MUC1 full

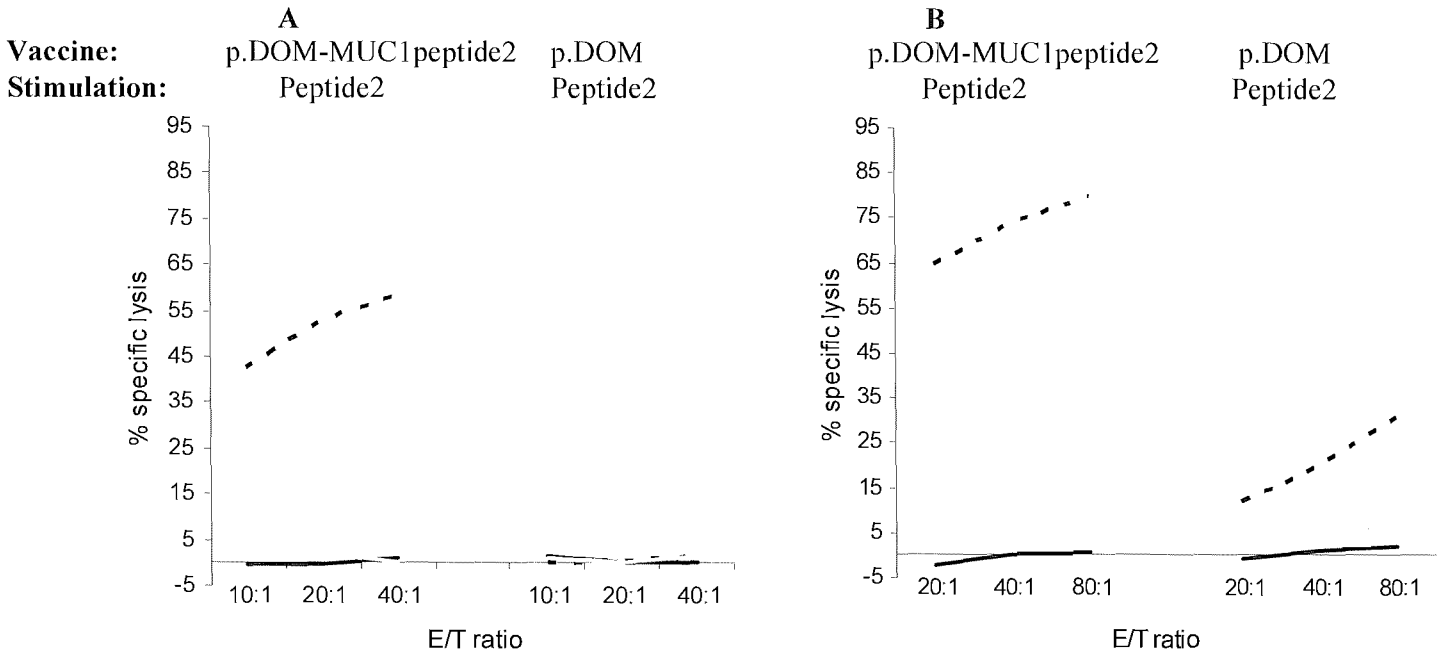



**Figure 3.16. DNA vaccination with p.DOM-MUC1peptide2 efficiently activates peptide-specific CTL**

C57Bl/k mice were vaccinated either once (**A**) or twice (**B**) with p.DOM-MUC1peptide2 and spleens taken at day 14 or 28 respectively. 1 week *in vitro* re-stimulation was performed with 20μM peptide2. A standard 5 hour $^{51}Cr$ release assay was performed against peptide coated EL4 targets: EL4 + peptide2 (dotted line, ·····), EL4 + FrC7 (grey line, ) or EL4 alone (black line, –). Vaccination with p.DOM-MUC1peptide2 activates epitope specific CTL that can lyse peptide pulse tumour cells *in vitro*.

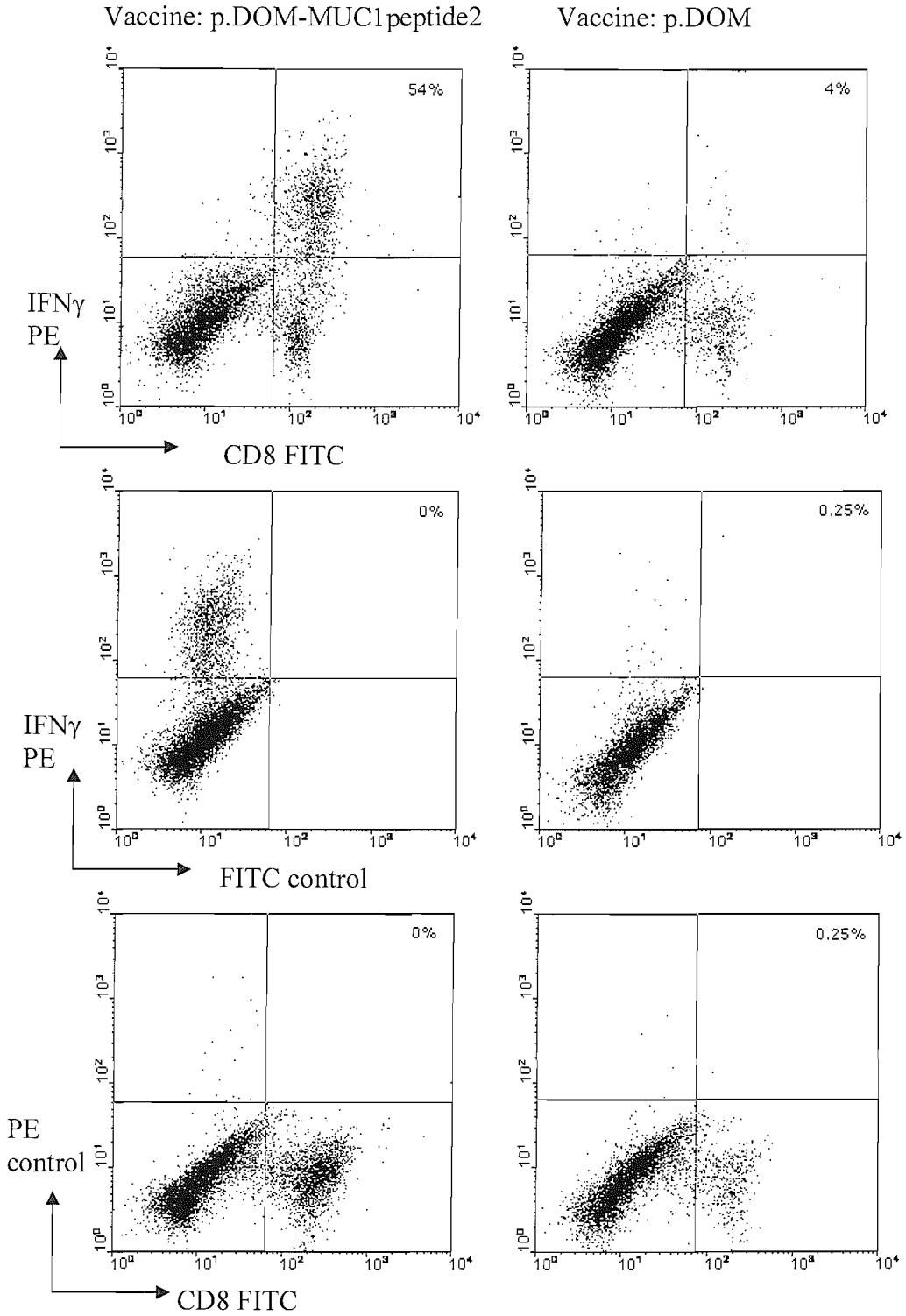



**Figure 3.17. p.DOM-MUC1peptide2 vaccination activates specific CD8$^+$ T cells that expand *in vitro***

C57Bl/k mice were vaccinated once with p.DOM-MUC1peptide2 (left panel) or p.DOM (right panel). Splenocytes were harvested on day 14 and re-stimulated *in vitro* for 1 week with 20μM peptide2. T cells were stained for intracellular IFNγ production after 4 hours incubation with 5μM peptide 2. Percentages of CD8$^+$ T-cells staining positive for IFNγ are given in the top right corner of each plot. Second and third row illustrate specificity of staining by incorporating FITC conjugated isotype controls for CD8 and IFNγ respectively. Vaccination with p.DOM-MUC1peptide2 but not p.DOM generated peptide specific CTL that could be expanded *in vitro*.

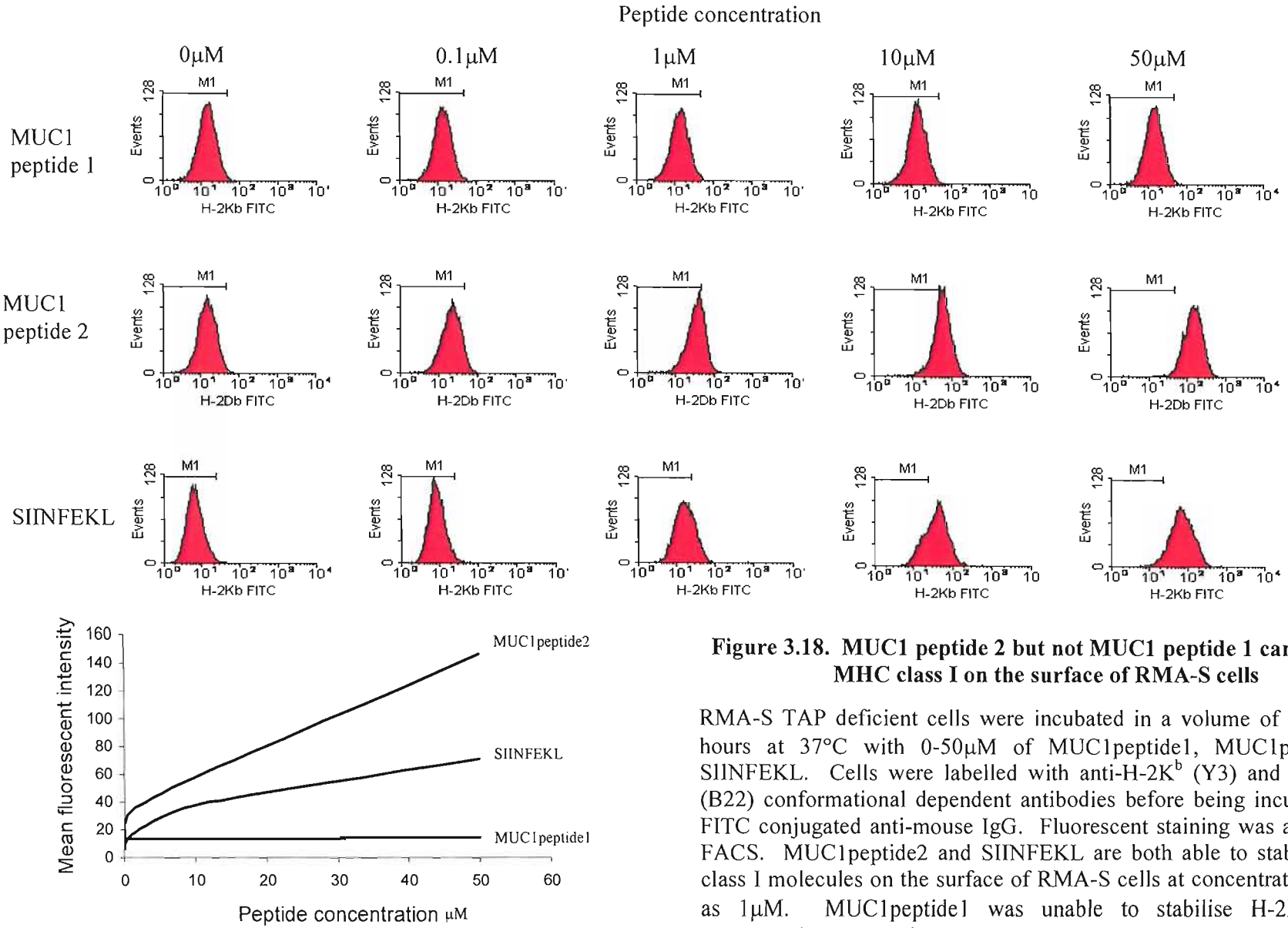



**Figure 3.18. MUC1 peptide 2 but not MUC1 peptide 1 can stabilise MHC class I on the surface of RMA-S cells**

RMA-S TAP deficient cells were incubated in a volume of 1ml for 18 hours at 37°C with 0-50μM of MUC1peptide1, MUC1peptide2 or SIINFEKL. Cells were labelled with anti-H-2K$^b$ (Y3) and anti-H-2D$^b$ (B22) conformational dependent antibodies before being incubated with FITC conjugated anti-mouse IgG. Fluorescent staining was analysed by FACS. MUC1peptide2 and SIINFEKL are both able to stabilise MHC class I molecules on the surface of RMA-S cells at concentrations as low as 1μM. MUC1peptide1 was unable to stabilise H-2K$^b$ at any concentration analysed.

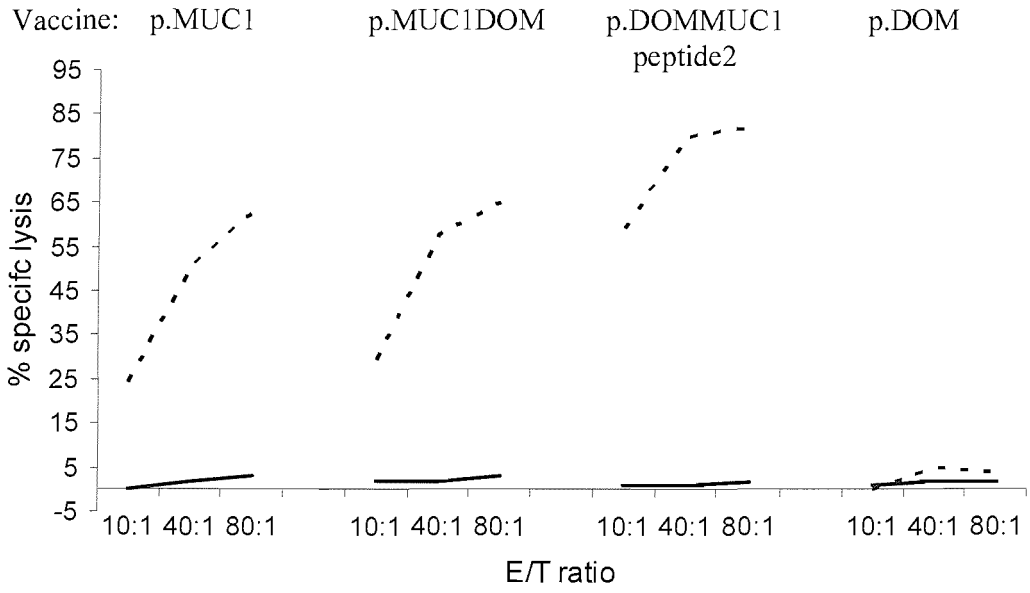



**Figure 3.19. p.DOM-MUC1peptide2 vaccination enhances peptide-specific CTL responses compared to DNA vaccines encoding full length MUC1**

C57Bl/k mice were vaccinated twice with p.MUC1, p.MUC1DOM, p.DOM-MUC1peptide2 or p.DOM and spleens were taken at day 35. 1 week *in vitro* re-stimulation was performed with 0.002μM peptide2. A standard 5 hour $^{51}Cr$ release assay was performed against targets EL4 alone (black line, —) or EL4 + peptide2 (dotted line, ·····). DNA vaccination with p.DOM-MUC1peptide2 design appears to enhance the CTL response to peptide coated targets compared to use of the full length MUC1 sequence.

length sequence as well as enhanced proteolytic cleavage of peptide2 when tethered to the C-terminus of the first domain of FrC, as predicted by the C-end rule[43]. ELISPOT assays were used to compare and quantify the $CD8^+$ T-cell response to peptide2. Mice were injected either once (day 0) or twice (day 0, 21) with p.MUC1, p.MUC1DOM, p.MUC1FrC and p.DOM-MUC1peptide2 and the number of $CD8^+$ T-cell releasing IFNγ specifically was measured directly ex vivo after 24 hour stimulation with 1μM peptide 2. Cumulative results are shown in Figures 3.20 and 3.21, and represent the number of IFNγ producing cells per million splenocytes.

At day 14 following one vaccination there are too few animals in each experimental group to perform statistical analysis to allow a comparison between each vaccine (1 experiment). However, the trend indicates that $CD8^+$ T-cell responses generated to MUC1peptide2 are low with all vaccines. At day 28 following two vaccinations (3 experiments), the p.MUC1 and p.DOM-MUC1peptide2 experimental groups have enough data to perform statistical analysis, and show a significant increase in the number of epitope-specific $CD8^+$ T cells compared to p.DOM control vaccinated mice. The p.MUC1FrC and p.MUC1DOM experimental groups show too much spread for statistical significance over the control vaccinated group but the trend in the response appears to be similar to mice vaccinated with p.MUC1. These data cannot be statistically compared with those at day 14, but indicate an amplification in epitope-specific $CD8^+$ T cells following a DNA vaccine boost. These results complement earlier data and suggest that the provision of additional MHC class II 'helper' epitopes through fusion of FrC or DOM to full length MUC1 does not enhance the $CD8^+$ T-cell response to peptide2. This is due to the natural immunogenicity of human MUC1 in the wild type setting. There is an indication that a similar number of $CD8^+$ T cells are being activated with all DNA fusion vaccines encoding full length MUC1. This suggests the minimum threshold level of peptide-MHC complexes required for $CD8^+$ T-cell activation are reaching the cell surface in all cases. However, it is difficult to relate these findings with the CTL assay results shown in Figure 3.15 because the rate of expansion and avidity of the activated $CD8^+$ T cells from each group has not been analysed. This should form a fundamental part of future work.

Interestingly, vaccination with p.DOM-MUC1peptide2 induces a significantly greater number of epitope-specific $CD8^+$ T cells compared to vaccination with p.MUC1 at day 28. This highlights again the increased efficiency with which this vaccine format can induce epitope-specific $CD8^+$ T cells. It also demonstrates the efficient provision of T-cell help

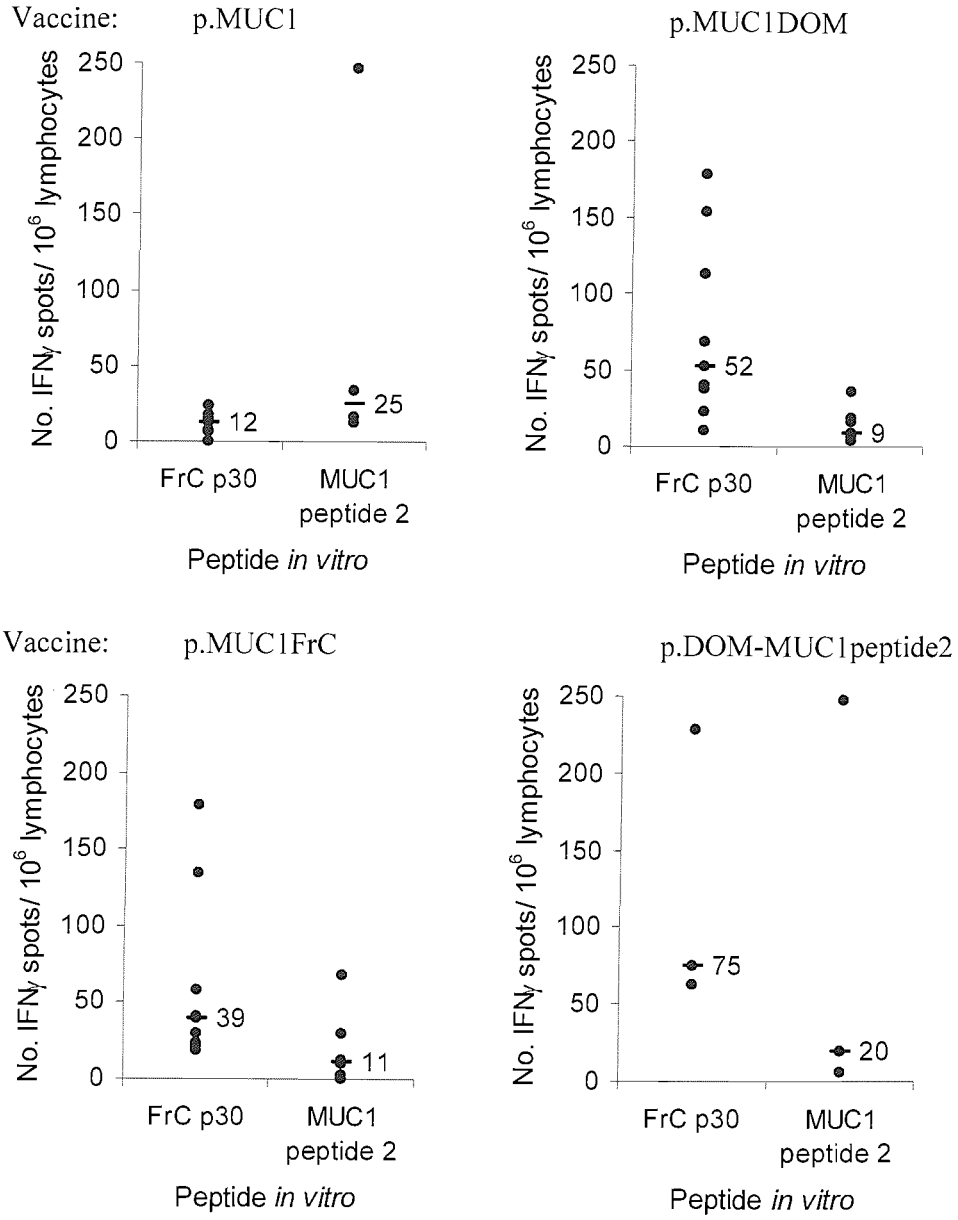



**Figure 3.20. Quantification of CD8$^+$ T-cell activation following one DNA vaccination**

C57/Bl6 mice (a minimum of 3 per group) were vaccinated once with p.MUC1, p.MUC1DOM, p.MUC1FrC, p.DOM-MUC1peptide2 or control vaccine p.DOM. Splenocytes were taken on day 14 and incubated with FrC p30 or MUC1peptide2 for 24 hours. IFNγ producing CD8$^+$ T cells were counted for individual mice (●) and the median is shown (—). Statistical analysis is not possible due to small numbers of animals in each group. The indication is that vaccination with p.MUC1, p.MUC1DOM, p.MUC1FrC and p.DOM-MUC1peptide2 generates a low level of peptide-specific CD8$^+$ T cells.

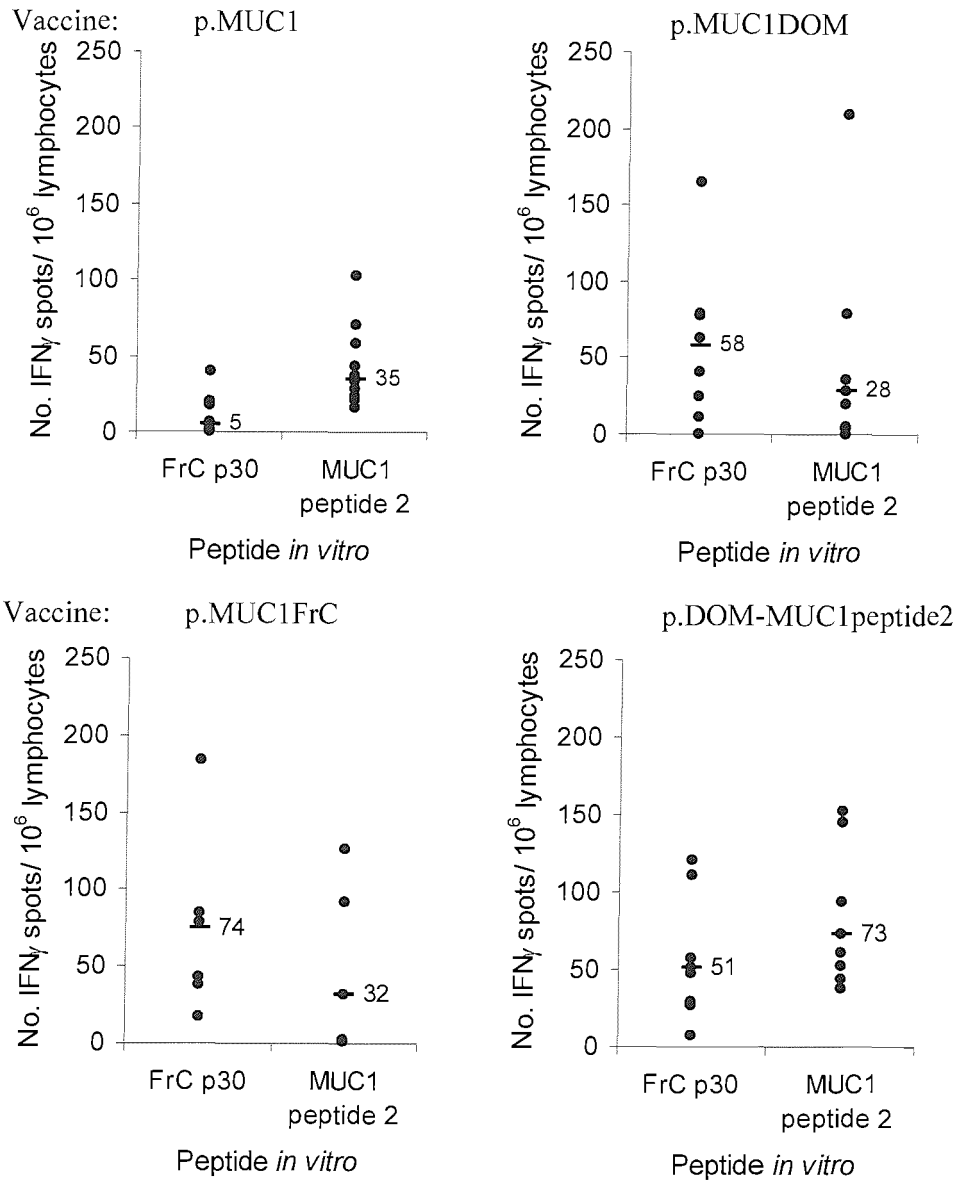



**Figure 3.21. Quantification of CD8$^+$ T-cell activation following two DNA vaccinations**

C57/Bl6 mice (a minimum of 6 per group) were vaccinated twice with p.MUC1, p.MUC1DOM, p.MUC1FrC, p.DOM-MUC1peptide2 or control vaccine p.DOM. Splenocytes were taken on day 14 and incubated with FrC p30 or MUC1peptide2 for 24 hours. IFNγ producing CD8$^+$ T cells were counted for individual mice (•) and the median is shown (–). The trend in the responses indicates that p.MUC1, p.MUC1DOM, p.MUC1FrC and p.DOM-MUC1peptide2 vaccination generates an amplified number of specific CD8$^+$ T cells compared to control vaccination. Due to small numbers of mice in each group and due to data spread, this was only statistically significant for p.MUC1 and p.DOM-MUC1peptide2 vs p.DOM ($p=0.001$ and $p=0.0007$ respectively, Mann Whitney test). For the same reason this data cannot be compared to that at day 14 (Figure 3.20). However, vaccination with pDOM-MUC1peptide2 does generate a significantly greater number of peptide-specific CD8$^+$ T cells compared to p.MUC1 alone ($p = 0.0205$ Mann Whitney test) at day 28.

from p.DOM and/or the enhanced processing of the peptide2 epitope from it's C-terminus. It is also possible that the increased number of activated peptide-specific $CD8^+$ T cells following vaccination with p.DOM-peptide2 accounts for the increased CTL mediated lysis detected against peptide coated targets (Figure 3.19). In all cases the $CD4^+$ T-cell response to FrC p30 peptide was used as a positive control. A $CD8^+$ T-cell response was only detected in conjunction with a $CD4^+$ T-cell response to p30 (except when vaccinating with p.MUC1 alone) however an increased p30 specific $CD4^+$ T-cell response did not necessarily correlate with an increased peptide-specific $CD8^+$ T-cell response following a DNA vaccine boost. This is may indicate that the minimal threshold of T-cell help required for $CD8^+$ T-cell activation to peptide2 was met following vaccination with all MUC1 encoding vaccines.

### 3.2.7 Cross reactivity in the CTL response to MUC1 DNA vaccine

Experimental data described in the previous section show the increased ability of p.DOM.epitope vaccine design to generate elevated numbers of peptide-specific effector CTL in comparison to DNA vaccines encoding the full length MUC1 gene. It was of further interest to find that although the vaccine p.DOM-MUC1peptide1 was unable to elicit any detectable CTL responses, vaccination with the p.DOM-MUC1peptide2 vaccine was able to generate a CTL response that not only recognised EL4 target cells coated with peptide 2 but also when coated with peptide 1 (Figure 3.22, representative of 3 experiments). These two peptides differ in two underlined amino acids, with peptide 1 SAPDTRPAP, and peptide 2 SAPDNRPAL. Peptide 2 contains defined anchor motifs for $D^b$ which are asparagine at position 5 and leucine at position 9, whereas peptide 1 does not contain any defined $K^b$ anchor motifs[458, 459]. This may explain the ability of p.DOM-MUC1peptide2 to elicit vigorous CTL, since these anchor motifs are likely to confer a greater binding affinity of the peptide for MHC class I molecules and increased stability of these peptide/MHC complexes at the cell surface (Figure 3.18). This could effectively lead to higher numbers of MHC class I-peptide complexes at the cell surface for $CD8^+$ T-cell activation. At sufficiently high concentrations, peptide 1 may bind transiently to $D^b$ when loaded externally for *in vitro* assays, and thus allow recognition and target cell lysis by CTL raised against peptide 2. The efficacy of CTL recognition of peptide 2 coated targets is comparably greater than that of peptide 1 coated targets (Figure 3.22) suggesting a heterogeneous population of CTL recognising different determinants from the peptide 2 epitope, only a fraction of which will also be able to recognise peptide 1.

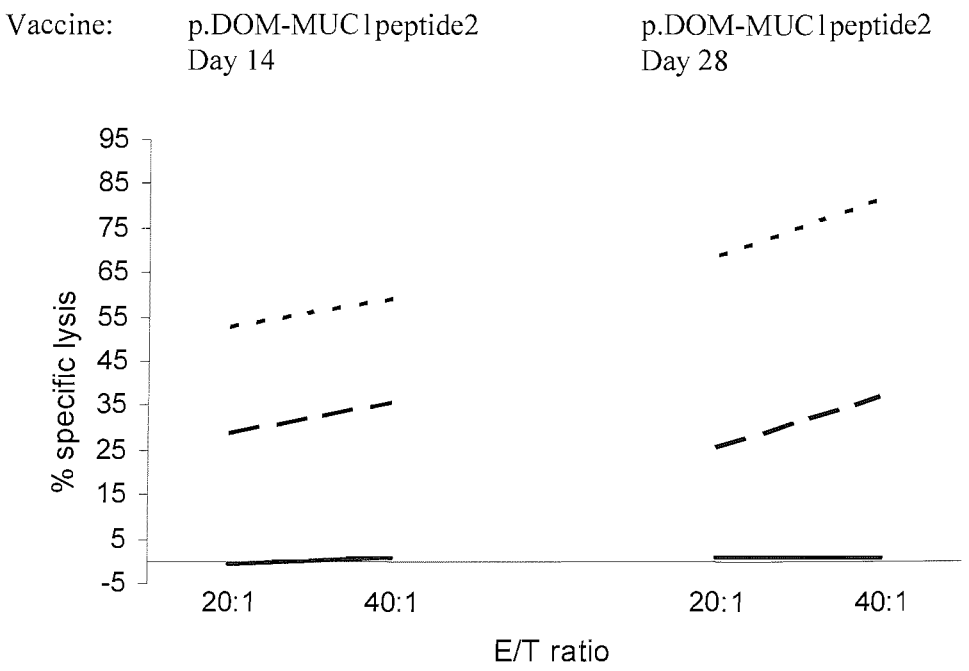



**Figure 3.22. MUC1 peptide 2 specific CTL can also recognise peptide1/MHC complexes**

C57Bl/k mice were vaccinated either once or twice with p.DOM-MUC1peptide2 and spleens taken at day 14 or 28 respectively. 1 week *in vitro* re-stimulation was performed with 0.002μM peptide 2. A standard 5 hour $^{51}Cr$ release assay was performed against EL4 targets, (black line, —), EL4 + peptide2, (dotted line, ·····) and, EL4 + peptide 1, (dashed line, -----). CTL raised against peptide 2 can also recognise and lyse target cells coated with peptide 1.

### 3.2.8 CTL specific for MUC1-derived peptide 2 cannot lyse RMA-MUC1 *in vitro*

The finding that a DNA vaccine format encoding a single MUC1 $K^b$ epitope could elicit effector CTL against both the encoded peptide sequence and an alternative MUC1 epitope sequence was encouraging. However, for this vaccine to be of use *in vivo,* target tumour cells must also process and present either one or both of the epitopes in a recognisable manner. To assess tumour cell processing and presentation of peptide 1 and 2, mice were vaccinated once with p.DOM-MUC1peptide2 and CTL were stimulated *in vitro* for one week with either peptide2 (20μM or 0.002μM) or with Mitomycin C treated tumour cells. CTL were then tested in a $^{51}Cr$ release assay against RMA-MUC1 either coated with or without peptide2. Although CTL stimulated with both peptide or disabled tumour cells could lyse RMA-MUC1 coated with peptide 2, they were unable to kill RMA-MUC1 alone. The inability of peptide 2 specific CTL to kill RMA-MUC1 was confirmed in 4 experiments and one representative experiment is shown in Figure 3.23. In addition, vaccinating C57Bl/6 mice with p.DOM-MUC1peptide2 did not provide protection from tumour challenge (data not shown). These results suggest that neither peptide 1 nor 2 are processed and presented by this tumour cell line. Lack of killing was not due to tumour cell down modulation of MHC class I expression, as confirmed by FACS analysis (Figure 3.6), nor was it due MUC1 surface expression inhibiting T-cell-tumour cell interaction, as peptide-specific CTL were able to recognise peptide loaded MHC class I molecules lyse tumour cells efficiently (Figure 3.23). In addition, the effect of the immunoproteasome on processing and presentation of peptide1 and 2 was assessed. RMA-MUC1 tumour cells were treated with a range of IFNγ concentrations for 24 hours prior to CTL assays, however this did not result in any tumour cell killing *in vitro* (data not shown). There are several possible explanations for these results, including differences in the processing capabilities of MUC1 within the tumour cell and MUC1 encoded by the DNA vaccine and processed within the APC. For instance, glycosylated peptides can be directly presented by MHC class I molecules[19, 460] and there may be differences in the glycosylation pattern of presented peptides by both cell types.

This has highlighted the inadequacy of the methods used to determine MHC class I epitopes in the literature. Peptides 1 and 2 were originally described to be recognised by CTL generated through vaccination with MUC1 protein linked to mannan, indicating that these peptides could be processed and presented from full length MUC1. However, no correlation with the processing and presentation capabilities of the tumour cell was made.

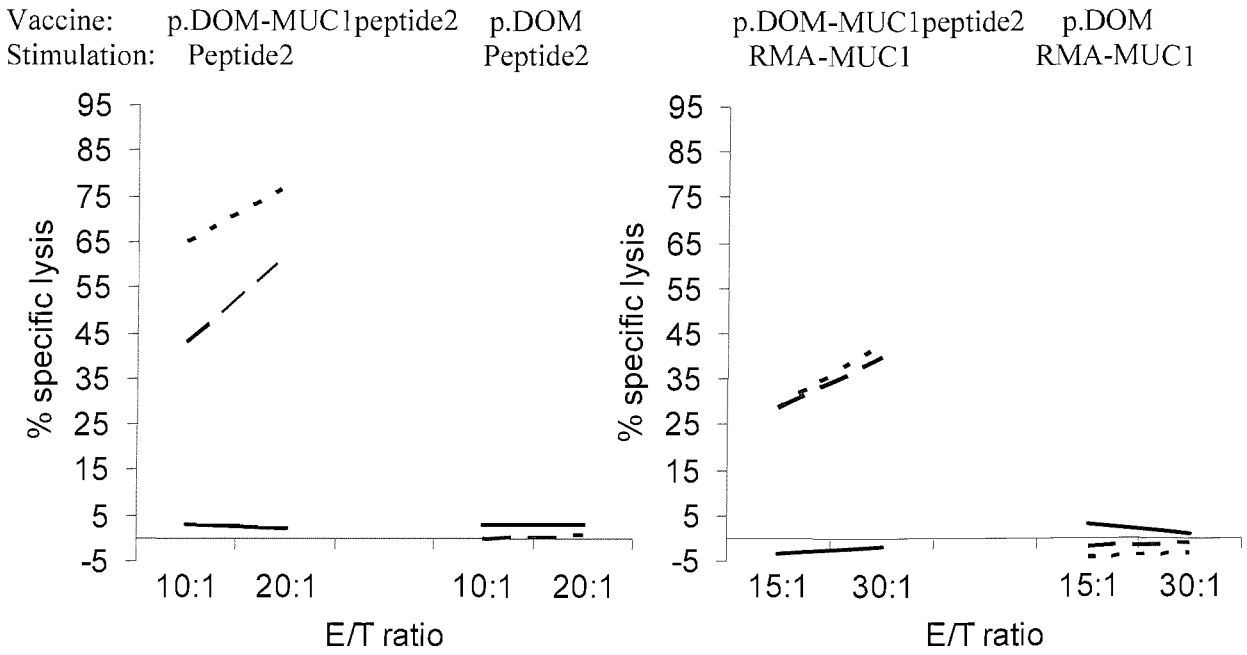



**Figure 3.23. MUC1 peptide 2 specific CTL cannot lyse MUC1$^+$ tumour cells**

C57Bl/k mice were vaccinated once with p.DOM-MUC1peptide2 and spleens taken at day 14. 1 week *in vitro* re-stimulation was performed with either 0.002μM peptide 2 (left panel, representative of 4 experiments) or with Mitomycin C treated RMA-MUC1 cells (right panel, 1 of 1 experiment). A standard 5 hour $^{51}$Cr release assay was performed against RMA-MUC1 targets, (black line, —), RMA-MUC1 + peptide2, (dotted line, ·····) or EL-4 + peptide2, (dashed line, -----). CTL raised against peptide 2 are capable of recognising target cells pulsed with peptide 2 but are unable to lyse targets with endogenous MUC1 expression (RMA-MUC1).

In spite of these problems, the p.DOM.epitope DNA vaccine design was assessed further for generating $CD8^+$ T cells by incorporating other MHC class I epitopes from MUC1. Additional MHC class I epitopes reported to be recognised by MUC1 specific CTL and presented by $MUC1^+$ tumour cells were STAPPAHGV and TVVTGSGHA. DNA vaccines were again constructed for these epitopes and assessed in C57/Bl6 mice using various vaccination and re-stimulation protocols, but no detectable CTL activity was generated (data not shown). In all 4/5 of the p.DOM.epitope vaccines constructed were unsatisfactory and no further attempts have been made this far to define any further MHC class I epitopes that may be of use in generating CTL responses via the p.DOM.epitope DNA vaccine design. The likely reason for the inability of these vaccines to generate a $CD8^+$ T-cell response is the poor processing and presentation of the chosen peptides. STAPPAHGV and TVVTGSGHA are just two of many peptides described as being recognised by CTL following exposure to full length MUC1[438, 461]. However, CTL cross-reactivity was not examined. It is possible that the induced CTL were recognising more than one peptide but only one of these was processed and presented to allow activation.

To investigate further the inability of p.DOM-MUC1peptide1 vaccine to elicit CTL, a additional p.DOM.epitope vaccine was constructed. It has been suggested that H-$2K^b$ can also bind octomeric peptides[462], and that in the case of the MUC1 peptide1 (SAPDTRPAP) a greater binding affinity to H-2 $K^b$ can be achieved through truncating the peptide by removal of the C-terminal proline. However, vaccinating C57/Bl6 mice once or twice with this p.DOM-peptide1TR vaccine and re-stimulating splenocytes at various peptide concentrations did not result in any detectable CTL activity (data not shown). This was not pursued and stabilisation assays were not performed.

### 3.2.9 Investigating the $CD4^+$ T-cell response to MUC1 DNA fusion vaccines

To investigate the role of $CD4^+$ T cells further in mediating protection against MUC1 expressing tumours, as indicated by depletion experiments, a strategy employing ELISPOT assays was utilised. Soares, M. *et al.*[426] used a technique in wild type and transgenic mice to analyse the $CD8^+$ and $CD4^+$ T-cell response to vaccination with the VNTR peptide. They utilised the same 140 amino acid VNTR peptide incorporating 7 tandem repeats in ELSIPOT assays. Lymphocyte depletion *in vitro* demonstrated the induction of $CD4^+$ T-cells specific for this region of MUC1. In a similar manner the response to the VNTR was

assessed following DNA vaccination in our study. Splenocytes from C57/Bl6 mice vaccinated with p.MUC1, p.MUC1FrC and p.MUC1DOM were incubated with a 24mer peptide incorporating one full tandem repeat (20 amino acids) and 4 amino acids from the next repeat for 24 hours, IFNγ producing cells were counted and compared to the control vaccines p.DOM and p.DOM-MUC1peptide2 which were not expected to generate MUC1 specific $CD4^+$ T cells. As shown in Figures 3.24 and 3.25, DNA vaccines encoding full length MUC1 (p.MUC1, p.MUC1FrC and p.MUC1DOM) generate T cells with specificity for the MUC1 tandem repeat, detectable at day 14 (1 experiment) and 28 (3 experiments) after one and two vaccinations respectively. In all cases the number of VNTR specific T-cells activated following vaccination with MUC1 encoding vaccines was significantly greater than control vaccinated mice or mice vaccinated with p.DOM-MUC1peptide2. It is assumed that these represent $CD4^+$ T cells, but depletion experiments undertaken to demonstrate this were unsuccessful due to the presence of high non-specific background values. Analogous to the p30 response discussed above, the MUC1 $CD4^+$ T-cell response was not amplified after boosting. It will be interesting to determine whether MUC1 VNTR-specific $CD4^+$ T cells play a role in the protective immunity generated by DNA vaccination with full length MUC1.

### 3.2.10 Efficacy of MUC1 DNA fusion vaccines in mice transgenic for MUC1

The MUC1 transgenic mouse model provides an important tool for examining vaccination strategies to generate MUC1 specific T-cell responses. In this setting, a level of MUC1-specific tolerance is likely to exist in the $CD4^+$ and $CD8^+$ T cell compartments but, as yet, the peptide specificity of the tolerance remains undefined. To evaluate immunity in this setting, MUC1 DNA fusion vaccines engineered in these studies were evaluated in this tg model. ELISPOTs had been shown to be highly sensitive and specific in wild type mice and were employed to assess T-cell responses here. It was predicted that vaccination with p.MUC1 alone would not generate any detectable immune responses because of the reported MUC1 specific $CD4^+$ T-cell tolerance in the tg model. In the absence of T-cell help it also seemed unlikely that an effective $CD8^+$ T-cell response would be generated. We addressed the question whether fusion of FrC or DOM, in promoting the $CD4^+$ T-cell response to FrC, would in turn provide linked T-cell help for MUC1 specific $CD8^+$ T cells, and possibly the $CD4^+$ T-cell subset. Data shown in Figures 3.26 and 3.27 are the cumulative results from individual mice vaccinated once (day 0) or twice (day 0, 21) with p.MUC1, p.MUC1FrC,

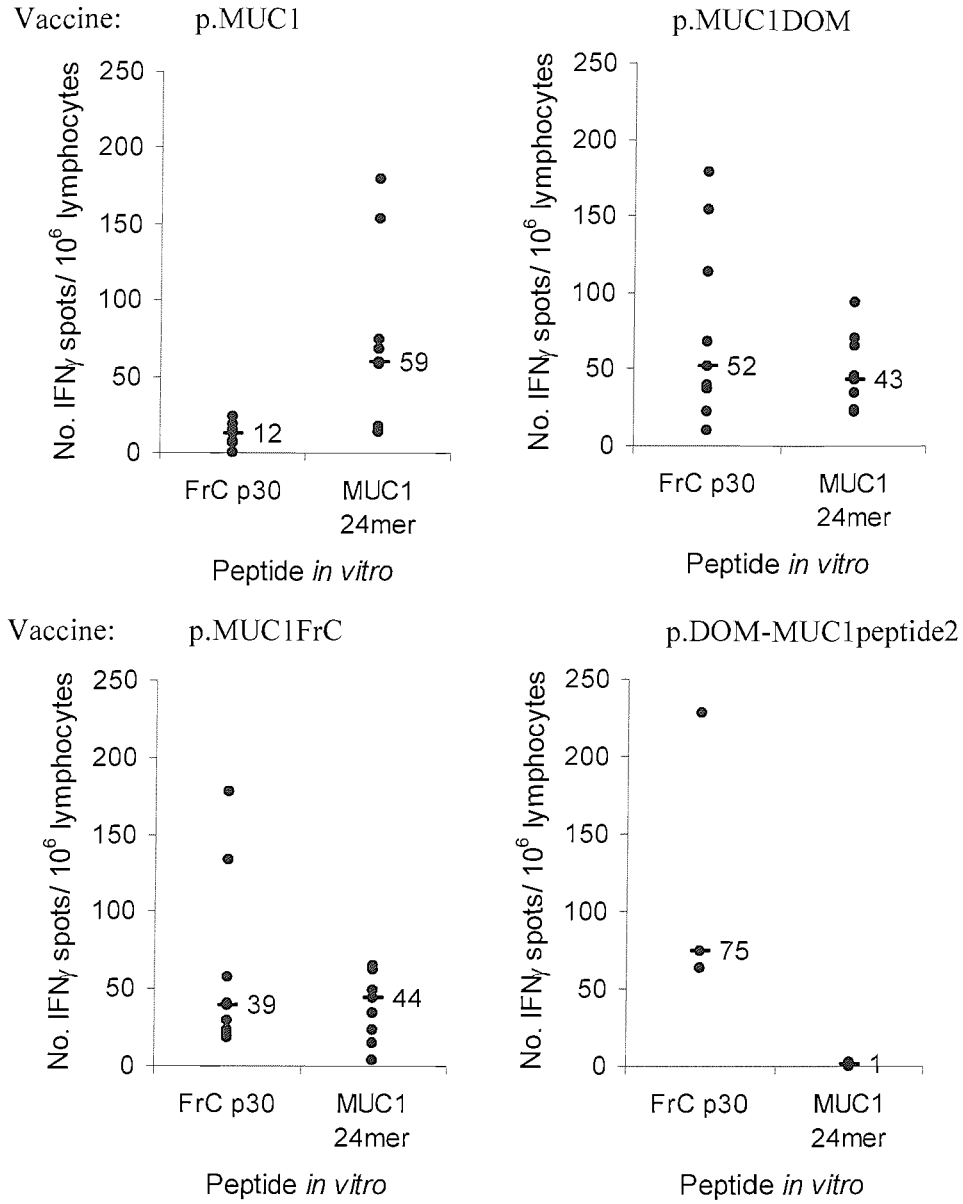



**Figure 3.24. Quantification of the T-cell response to MUC1 VNTR following one DNA vaccination**

C57/Bl6 mice (a minimum of 3 per group) were vaccinated once with p.MUC1, p.MUC1DOM or p.MUC1FrC or control vaccines p.DOM-MUC1peptide2 and p.DOM. Splenocytes were harvested on day 14 and incubated with FrC p30 or MUC1 24mer for 24 hours. IFNγ producing T cells were counted for individual mice (•) and the median is shown (–). Vaccination with p.MUC1, p.MUC1FrC and p.MUC1DOM generated a significant number of VNTR specific T cells compared to p.DOM vaccination ($p = 0.0003$, $p = <0.0001$ and $p = 0.001$ respectively, Mann Whitney test).

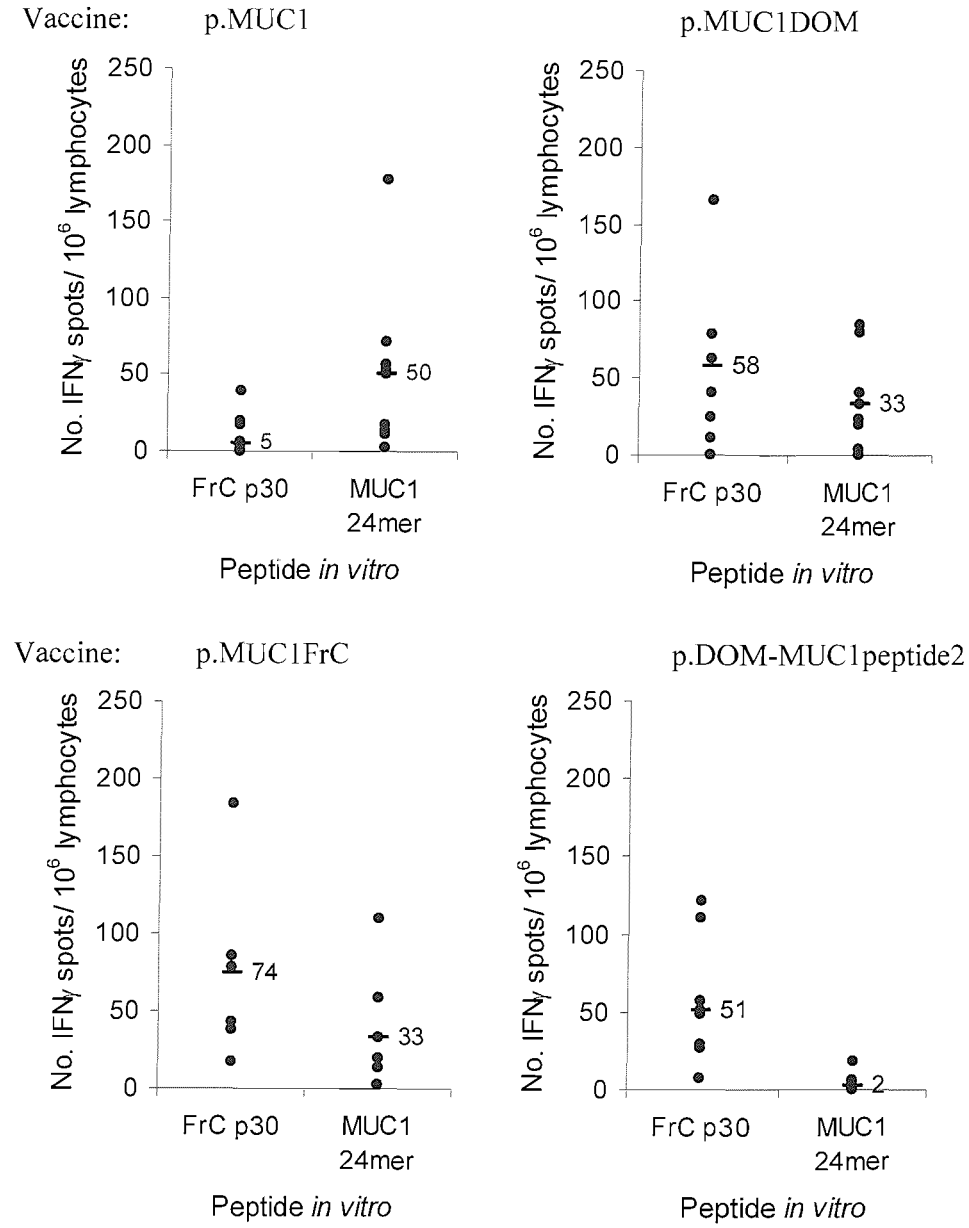



**Figure 3.25. Quantification of the T-cell response to MUC1 VNTR following two DNA vaccinations**

C57/Bl6 mice (a minimum of 6 per group) were vaccinated twice with p.MUC1, p.MUC1DOM or p.MUC1FrC or control vaccines p.DOM-MUC1peptide2 and p.DOM. Splenocytes were taken on day 28 and incubated with FrC p30 or MUC1 24mer for 24 hours. IFNγ producing T cells were counted for individual mice (•) and the median is shown (–). Vaccination with p.MUC1, p.MUC1FrC and p.MUC1DOM generated a significant number of VNTR specific T cells compared to p.DOM vaccination ($p = 0.0017$, $p = 0.0293$ and $p = 0.0152$ respectively, Mann Whitney test). There was no amplification of response following a boost with p.MUC1, p.MUC1FrC or p.MUC1DOM compared to day 14 ($p = 0.182$, $p = 0.607$ and $p = 0.321$ respectively).

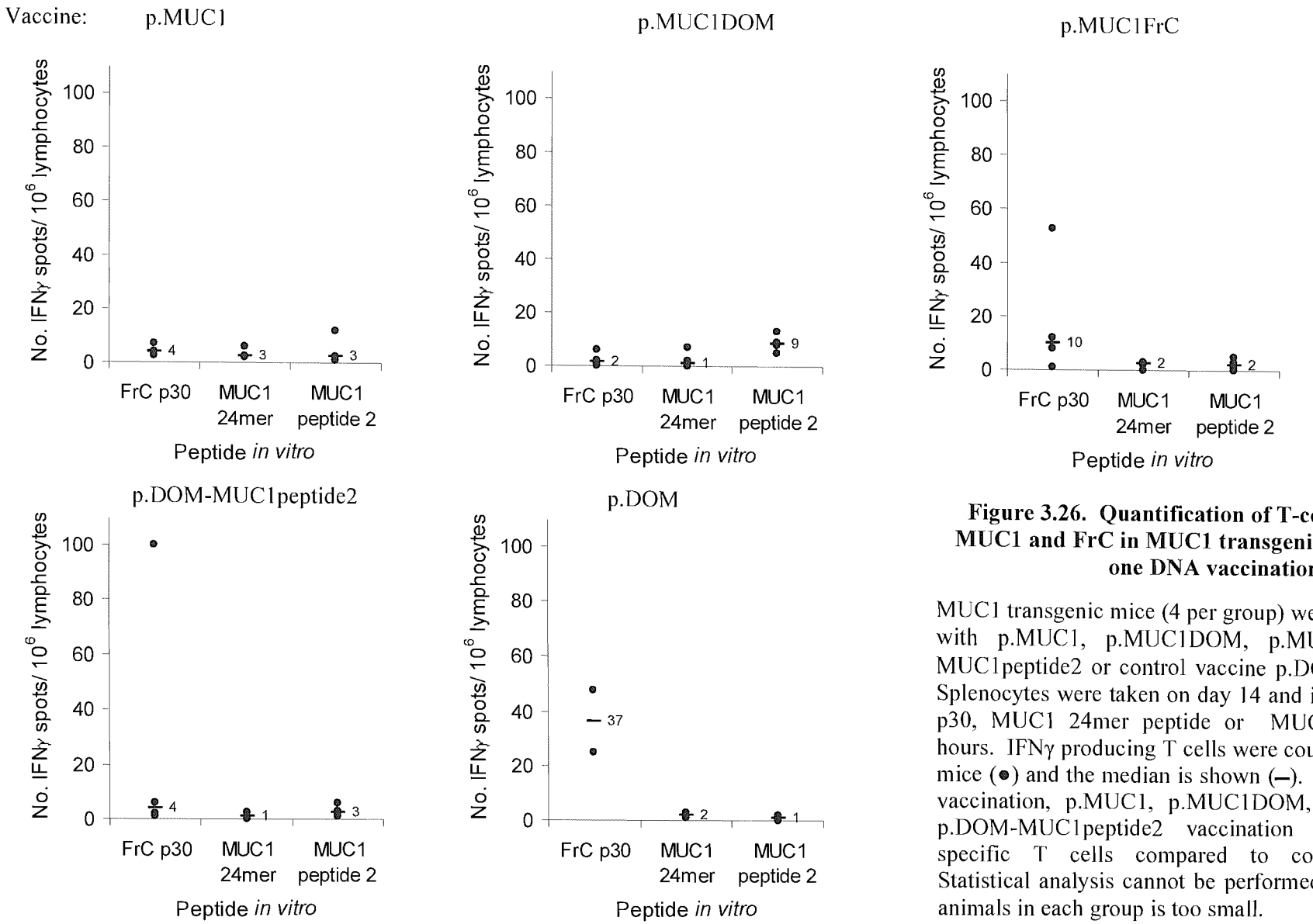



**Figure 3.26. Quantification of T-cell responses to MUC1 and FrC in MUC1 transgenic mice following one DNA vaccination**

MUC1 transgenic mice (4 per group) were vaccinated once with p.MUC1, p.MUC1DOM, p.MUC1FrC, p.DOM-MUC1peptide2 or control vaccine p.DOM (2 per group). Splenocytes were taken on day 14 and incubated with FrC p30, MUC1 24mer peptide or MUC1peptide2 for 24 hours. IFNγ producing T cells were counted for individual mice (●) and the median is shown (–). Following a single vaccination, p.MUC1, p.MUC1DOM, p.MUC1FrC and p.DOM-MUC1peptide2 vaccination do not generate specific T cells compared to control vaccination. Statistical analysis cannot be performed as the number of animals in each group is too small.

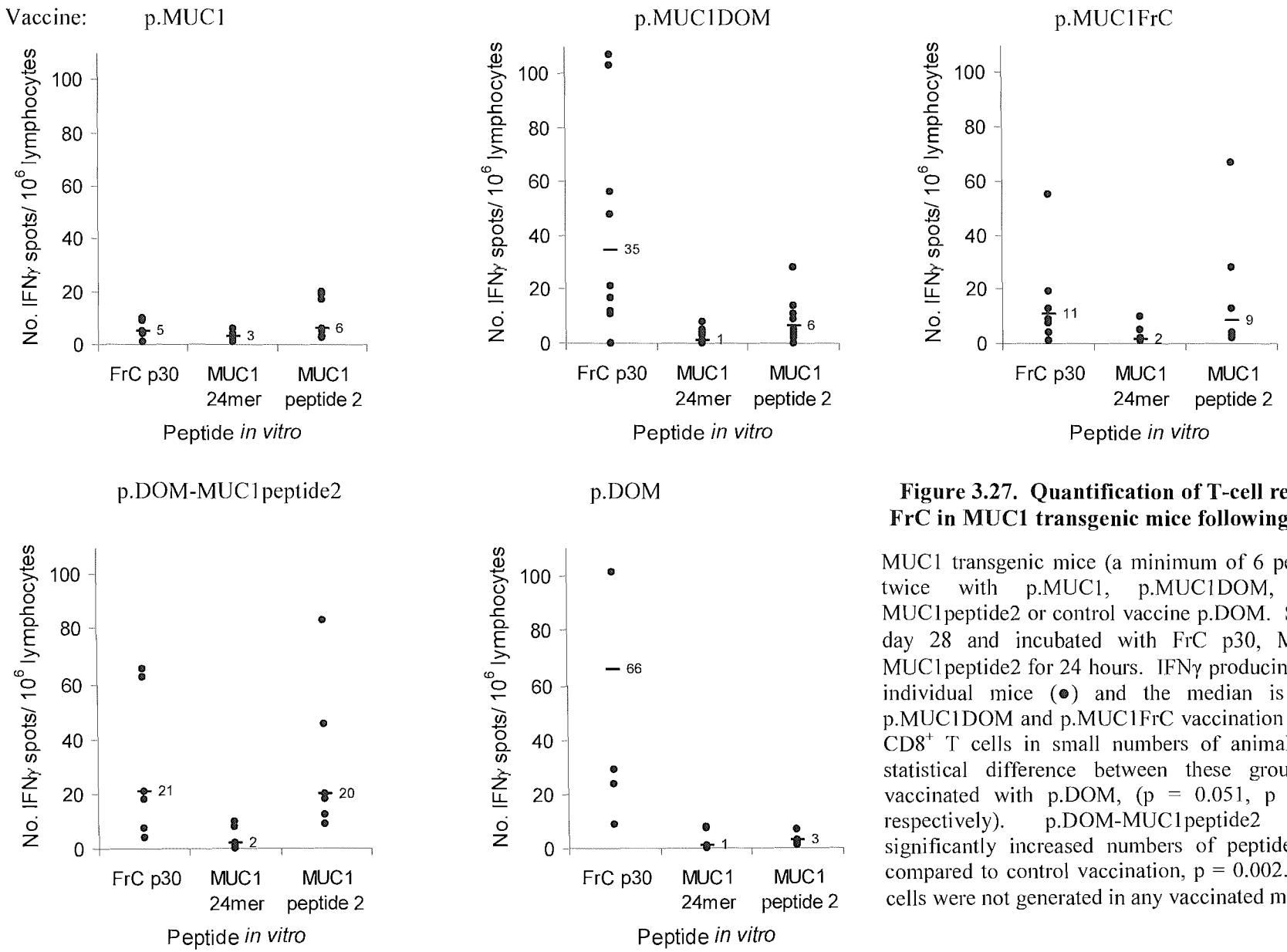



**Figure 3.27. Quantification of T-cell responses to MUC1 and FrC in MUC1 transgenic mice following two DNA vaccinations**

MUC1 transgenic mice (a minimum of 6 per group) were vaccinated twice with p.MUC1, p.MUC1DOM, p.MUC1FrC, p.DOM-MUC1peptide2 or control vaccine p.DOM. Splenocytes were taken on day 28 and incubated with FrC p30, MUC1 24mer peptide or MUC1peptide2 for 24 hours. IFNγ producing T cells were counted for individual mice (●) and the median is shown (–). p.MUC1, p.MUC1DOM and p.MUC1FrC vaccination generate peptide2 specific $CD8^+$ T cells in small numbers of animals. However there is no statistical difference between these groups and control animals vaccinated with p.DOM, ($p = 0.051$, $p = 0.083$ and $p = 0.051$ respectively). p.DOM-MUC1peptide2 vaccination generates significantly increased numbers of peptide2 specific $CD8^+$ T cells compared to control vaccination, $p = 0.002$. MUC1 24mer specific T cells were not generated in any vaccinated mice.

p.MUC1DOM, and p.DOM-MUC1peptide2 using p.DOM as a control. A response was considered positive if it was greater than the mean of the control group plus 1 standard deviation. A single vaccination generated no detectable $CD4^+$ T cell or $CD8^+$ T-cell responses with any of the DNA constructs when compared with the control p.DOM vaccine. However, after a DNA boost, a small number of mice made a $CD8^+$ T-cell response following immunisation with all vaccines, most prominently when using the p.DOM-peptide design. However, no MUC1-VNTR specific $CD4^+$ T-cell responses were evident, suggesting tolerance or regulation to epitopes contained within this MUC1 peptide in this cellular compartment. Further modifications to vaccine design or immunisation protocol may be capable of generating $CD4^+$ T cell responses to the MUC1 VNTR region, such as combining electroporation with DNA vaccination or using pVXCP as a vector. It is difficult to accurately compare the responses seen in the MUC1 tg mice with those in the wild type mice as the number of mice analysed in each group is too low. The trend in the responses indicates however, that whilst both wild type and tg mice respond equally well to fragment C, the numbers of MUC1peptide2-specific $CD8^+$ T cells detected following vaccination of MUC1 tg mice was reduced compared to those seen in the wild type animals. Again this may reflect low level of tolerance in the $CD8^+$ T-cell subset or a smaller TCR repertoire, or even the absence of MUC1 specific helper $CD4^+$ T cells. Treg cells may also be in place in the tg mice but not in the wild type mice, and if present will need to be overcome for a response to be initiated. The addition of FrC or DOM to full length MUC1 was able to generate detectable FrC specific $CD4^+$ T-cell responses, but did not appear to enhance the $CD8^+$ T-cell response to MUC1 as was originally predicted. The comparatively increased ability of p.DOM-MUC1peptide2 vaccine to generate IFNγ producing $CD8^+$ T cells could be due to the linked T-cell help received from DOM specific $CD4^+$ T cells, in addition to the enhanced liberation of the epitope from the C-terminus of p.DOM, which may result in higher numbers of peptide/MHC complexes expressed on the surface of the activating APC. Whether these activated $CD8^+$ T cells prove to be effective at killing remains to be answered and will form a fundamental part of future work in this area. It has been discussed above that CTL responses specific for this epitope are unable to effectively control tumour growth *in vivo*, however no other epitope was available for a read-out of $CD8^+$ T-cell responses at this time. Despite this, it has been demonstrated that the p.DOM.epitope DNA vaccine design is more efficient than DNA vaccines encoding full length MUC1 fused to FrC or DOM in generating $CD8^+$ T-cell responses in a potentially tolerant setting.

### 3.2.11 HLA-A2 restricted CTL are generated by the p.DOM-peptide vaccine design

The preceding data demonstrated the enhanced potency of the p.DOM.epitope DNA fusion vaccine design to generate peptide-specific $CD8^+$ T cells, resulting in greater target cell lysis *in vitro,* as compared to DNA vaccines encoding full length MUC1. This vaccine design was more effective at generating peptide-specific $CD8^+$ T cells in both wild type and MUC1 tg mice. For a potential application to human cancer, this vaccine format was tested in the HLA-A2 transgenic mouse model ($A2/K^b$) mice which express surface human HLA-A2 α1, and α2 in combination with murine α3 and $\beta_2M$ as well as normal murine $H\text{-}2K^{b(463)}$. Therefore during thymic selection the TCR repertoire generated can recognise either MHC molecule. The peptides tested using the p.DOM.epitope design were STAPPAHGV (S9V), ALGSTAPPV (A9V), TLAPATEPA (T9A) and LLLLTVLTV (L9V), all of which contained at least one anchor residue for HLA-A2 and some were known to be processed and presented from endogenous MUC1 within the target tumour cells (Table 3.1). Mice were vaccinated twice with vaccine constructs and splenocytes were re-stimulated *in vitro* with 10μM relevant peptide. This peptide concentration was an estimate and future work should evaluate the optimal re-stimulation concentration. The CTL were assessed for lytic activity using a human cell line T2 pulsed with appropriate peptide on the surface. Prior to pulsing, T2 were assessed for expression of HLA-A2 using the BB7.2 antibody and analysed by FACS (data not shown). Each vaccine was tested a minimum of twice, and the only vaccine capable of generating effective CTL was that encoding ALGSTAPPV (Figure 3.28 representative of 3 experiments). This peptide has a good algorithm score, can stabilise HLA-A2 on JY cells and contains 2 anchor residues for HLA-A2 (L at P2 and V at P9).

A common finding was that only a proportion of the mice, typically 25%, responded to the p.DOM-A9V vaccine. It is likely that this is due to a skewed TCR repertoire, with preference for the endogenous $H\text{-}2K^b$. A mouse model (HHD) has since been obtained to overcome this problem where the endogenous murine MHC molecules have been deleted to yield the singular expression of human HLA-A2 α1, α2 and α3 complex in association with human $\beta_2M^{(464)}$, and would be a more appropriate model to assess HLA-A2 peptide targeted vaccines. However, this was unavailable at this time. Comparison of responses to vaccination using several HLA-A2 restricted epitopes following vaccaination of HLA-$A2/K^b$ or HHD mice has been made in our laboratory (Mr. A. King, unpublished observations) and by other research groups[465]. Variability in the number of mice making $CD8^+$ T-cell responses, as well as low levels of lysis in CTL assays observed following

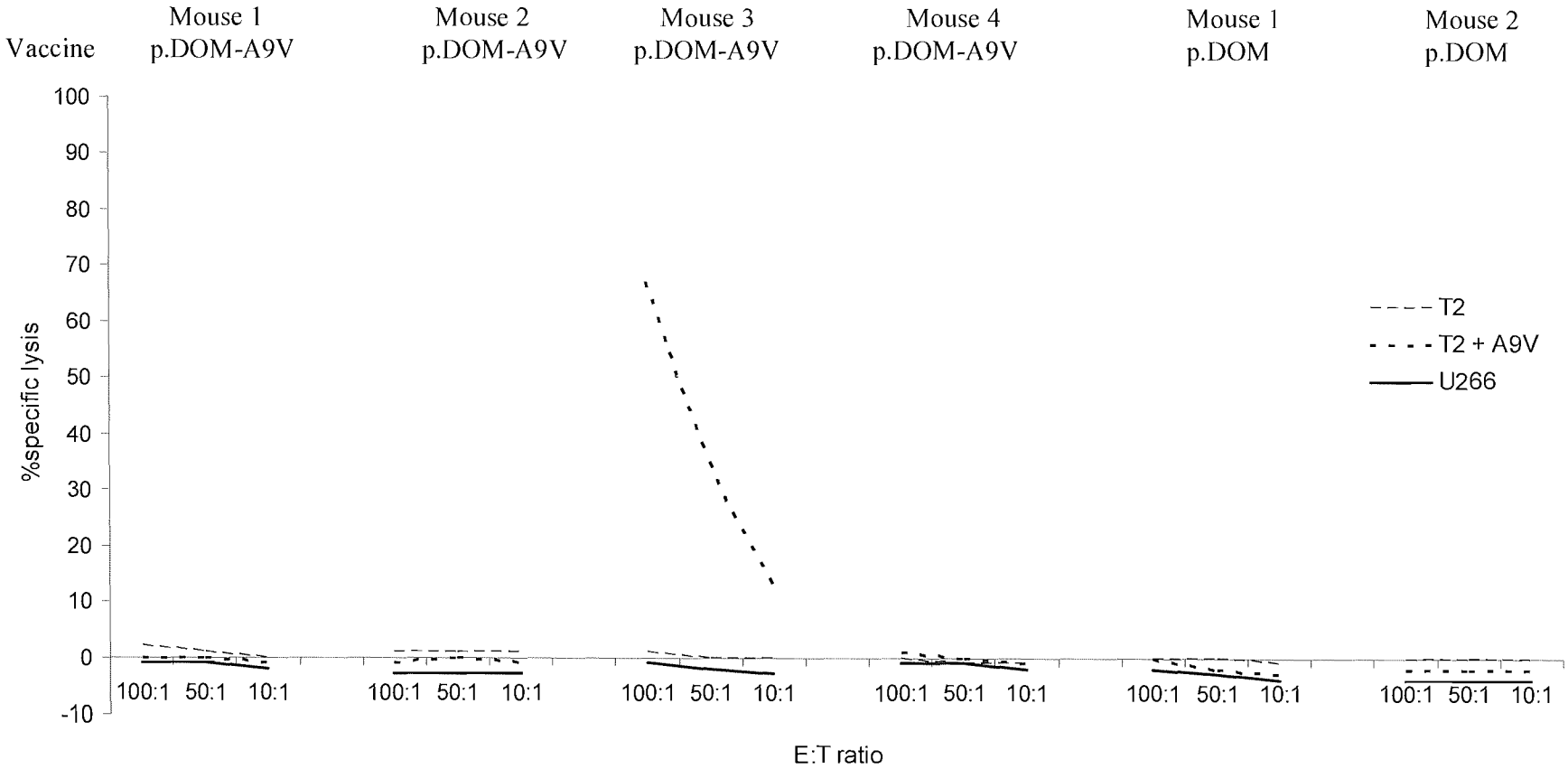



**Figure 3.28. DNA vaccination with p.DOM-A9V activates MUC1 peptide specific CTL in HLA-A2 transgenic mice**

HLA-A2/$K^b$ transgenic mice were vaccinated once with p.DOM-A9V or p.DOM and spleens were taken at day 14. 1 week *in vitro* re-stimulation was performed with 10μM peptide A9V. A standard 5 hour $^{51}Cr$ release assay was performed against HLA-$A2^+$ targets, T2 alone (dashed line, ----), or T2 + peptide A9V (dotted line, ·····) or HLA-$A2^+$ myeloma cell line U266 (black line, —). A peptide specific CTL response was noted in 1/4 vaccinated mice, the absence of a response in other mice is likely due to the absence of the necessary T cell repertoire.

vaccination of HLA-A2/$K^b$ mice is not seen following vaccination of HHD mice. An additional problem with this system is that murine $CD8^+$ T cells cannot interact with human $\alpha 3$ on the T2 targets thereby preventing signal amplification to enhance target cell lysis. Consequently, when CTL were tested for lysis against the human MUC1 positive myeloma cell line U266, it is not surprising that no lytic activity was detected (Figure 3.28). One way to overcome this is through the provision of large numbers of MHC-peptide complexes on the cell surface, i.e. by pulsing cells with peptide. However, to solve this problem, a tumour target cell line will need to be generated which is doubly transfected with the gene encoding the HLA-A2/$K^b$ molecule as well as human MUC1, but this was unavailable at the time of the current studies.

## 3.3 Discussion

The work described here has demonstrated the ability of DNA vaccines encoding human MUC1 to generate specific humoral and cellular immune responses in wild type mice. In this setting, immunity generated by DNA vaccines encoding MUC1 alone or when fused with FrC or DOM mediated protection in mice from tumour challenge with the RMA cell line transfected with human MUC1. Our aim in testing these DNA vaccines in a wild type murine model was to obtain a preliminary read-out of efficacy of vaccine design, since the human MUC1 sequence is naturally immunogenic in these mice. Experimental use of these MUC1 DNA vaccines was then reproduced in MUC1 tg mice, to assess the critical question of whether DNA vaccines can break tolerance.

The cellular compartment responsible for protection in several experimental systems is still unclear in both wild type and MUC1 tg mice. It is unlikely however, that anti-MUC1 antibodies will provide protection. Significant levels of anti-MUC1 antibodies were generated here using a number of DNA vaccine designs, and this response was shown to persist with repeated vaccinations but did not correlate with protection (Dr. F. Forconi, unpublished results). In other murine systems, passive transfer of antibodies from protected wild type mice to MUC1 tg mice again was unable to protect against tumour challenge[434]. In addition, although peptide vaccines based on the tandem repeat linked to a carrier protein induced MUC1 specific antibody in wild type mice, no protection was observed, and notably, T-cell responses were absent[425]. One reason for the inability of anti-MUC1 antibodies to provide protection *in vivo* may be sequestration by serum MUC1 shed by tumour cells, as has been demonstrated in myeloma patients[400], and/or tumour down modulation of MUC1 surface expression as observed here in wild type mice in our experiments.

Substantial evidence from both murine and human studies exists to suggest that specific T-cell responses can be raised against MUC1. In some instances these T-cell responses can provide protection from MUC1 expressing tumour challenge in mice. The role of $CD4^+$ T cells compared with $CD8^+$ T cells is beginning to be addressed. The results obtained by $CD4^+$ and $CD8^+$ T-cell depletion here have demonstrated that both $CD4^+$ T cell and $CD8^+$ T-cell effectors are generated by DNA vaccines encoding full length MUC1 and act synergistically to protect from tumour challenge in wild type mice.

The focus of this work was designed to examine cytotoxic T-cell responses against MUC1 elicited by DNA vaccines. A DNA vaccine encoding full length MUC1 sequence induced

specific CTL to two known MHC class I restricted peptides. The response to one of these peptides (peptide 2) could be enhanced when we utilised vaccine design that promoted $CD8^+$ T-cell responses, with epitope fused to the C-terminus of DOM. This vaccine design generated an increased number of $CD8^+$ T cells as measured by ELISPOT and when compared to DNA vaccines encoding full length MUC1, resulting in increased target cell lysis *in vitro*. The p.DOM-MUC1peptide2 vaccine was also able to induce CTL that not only recognised the delivered peptide but also recognised peptide 1 MHC class I complexes. MUC1-peptide1 lies within the tandem repeat and therefore is present in multiple copies in cells expressing MUC1. In contrast, the p.DOM-MUC1peptide1 vaccine was unable to generate any peptide-specific CTL. The likely reason for this is the lack of defined anchor motifs for H-$2K^b$ in this peptide, as well as its inability to stabilse H-$2K^b$ on the cell surface. In an attempt to increase the binding affinity of this peptide, an additional p.DOM-peptide vaccine was constructed incorporating a truncated peptide 1 sequence (SAPDTRPAP → SAPDTRPA). It has been shown that removal of the proline from the C terminus increases the binding affinity of this epitope to $K^{b(462)}$. This is in accordance with $K^b$ preference for binding octomeric peptide[459]. However this vaccine was still unable to generate detectable $CD8^+$ T-cell responses.

In addition, CTL raised against peptide 2 with the p.DOM-MUC1peptide2 vaccine were unable to lyse RMA–MUC1 *in vitro* and yielded no protective immunity *in vivo*. It was concluded therefore that although peptide 2 is efficiently processed and presented by DC, it is either not processed and presented by the tumour cell, or is being processed in an unrecognisable manner. One possible reason for this is differences in proteasome subunit composition between DC and tumour cell which has been shown to alter the peptide repertoire reaching the cell surface in other systems[32]. Expression of the immunoproteosome can be initiated by increases in the local IFNγ concentration, e.g. from NK cells during the inflammatory response or by T cells during activation. However, treating RMA-MUC1 with IFNγ in this case did not allow any CTL recognition of tumour cells. The exact mechanism responsible for the processing differences was not examined further in these studies. Additionally the glycosylation status of the MUC1 molecule may effect processing and presentation of MHC class I and II peptides within the DC and tumour cell. The extent of glycosylation of MUC1 from endogenous synthesis within the tumour cell, or from DNA vaccine following uptake or endogenous synthesis by DC is not known. Non-glycosylated MUC1 can be efficiently processed and presented as peptides in association with MHC class I and II by DC when taken into the cell *in vitro*[416, 460]. In

contrast, glycosylated MUC1 taken into DC remains in early endosomes with limited degradation[416]. Limited MUC1 degradation may also occur during recycling of MUC1 on tumour cells restricting the MUC1 peptide repertoire and level reaching the cell surface. Consequently, MHC class I and II peptides reaching the cell surface may differ in glycosylation state between DC and tumour cell; CTL activated against a non-glycosylated peptide on DC may not recognise a glycosylated form on the tumour cell.

Further attempts to induce a protective CTL response against defined MUC1 peptides led to the construction of p.DOM.epitope vaccines encoding additional reported MHC class I epitopes derived from MUC1. Two epitopes in particular were considered. Firstly, STAPPAHGV (H-2D$^b$) encoded by the tandem repeat, since CTL against this epitope were found to arise spontaneously in mice with MUC1$^+$ pancreatic cancer[438]. Secondly, TVVTGSGHA (H-2K$^b$) located near the signal peptide, as CTL specific for this peptide were shown to protect against tumour challenge when adoptively transferred in MUC1 tg, mice[461]. Despite these pre-existing reports of efficacy in the literature, the p.DOM.epitope DNA fusion vaccines incorporating these epitopes were not successful in generating peptide-specific CTL.

The ability of DNA vaccines encoding MUC1 to specifically generate CD4$^+$ T cells was investigated. Splenocytes from vaccinated mice were stimulated with an elongated peptide derived from the VNTR, with ELISPOTS as a read out of activated T cells. The results clearly demonstrated the induction of VNTR specific T cells, presumed to be of a CD4 phenotype. Attempts to formally demonstrate the phenotype of the responding T cells by lymphocyte depletion *in vivo* were hampered by a high non-specific background in assays, and future work should attempt to resolve this problem.

Observations arising from investigations in wild type mice were used to directly compare the utility of the constructed DNA vaccines to generate T-cell responses in MUC1 tg mice. It was predicted that p.MUC1 alone would be less immunogenic in MUC1 transgenic mice, and thereby allow a potential role for T-cell help delivered through FrC fusion to be examined. After one vaccination with either p.MUC1, p.MUC1DOM, p.MUC1FrC or p.DOM-MUC1peptide2, no CD4$^+$ or CD8$^+$ T-cell responses were detectable in tg mice. However, following a DNA boost small numbers of mice made a peptide 2 specific CD8$^+$ T cell response as measured by ELISPOT using all vaccines. It is possible that further boosts may increase the number of these CD8$^+$ T cells. The largest numbers of CD8$^+$ T cells generated were detected following two vaccinations with p.DOM-MUC1peptide2,

indicating again the improved ability of this vaccine to generate peptide-specific $CD8^+$ T cells in both the wild type and MUC1 tg settings. In comparison, it has been indicated that generating MUC1 specific $CD8^+$ T-cell responses in MUC1 tg mice is difficult to achieve but can be done by the addition of co-stimulation molecules or cytokines[444, 445, 447], this has not been necessary in this case. However, it has been established previously that MUC1-peptide2 is not presented by RMA-MUC1, and as such it cannot be used to translate observations made in tumour protection studies in wild type mice with MUC1 tg mice. Importantly, this was the only available murine MHC class I epitope which could be used as a read-out at the present time. In agreement with the reported presence of $CD4^+$ T-cell tolerance in the MUC1 tg murine model, it became clear that MUC1-VNTR specific $CD4^+$ T-cell responses were not being generated by DNA vaccination. This was not altered by boosting, or with provision of the foreign FrC protein and its derivatives to provide linked T-cell help. It is likely therefore that the small numbers of MUC1-VNTR specific $CD8^+$ T cells induced, and with absence of MUC1 specific $CD4^+$ T cells, very little protection against tumour will be achieved through vaccination with DNA constructs incorporating the entire MUC1 sequence, although this has not been investigated. The potential for the p.DOM.epitope design to provide protection from tumour challenge cannot be assessed in the absence of a known MHC class I epitope which is processed and presented by target tumour cells expressing MUC1 endogenously.

There is however, a potential to assess this vaccine design using HLA-A2 epitopes and this formed the next phase of the investigation. Here we examined induction of CTL responses by MUC1 specific DNA vaccines in mice transgenic for human HLA-A2 using the p.DOM.epitope design for delivery of specific MUC1 HLA-A2 peptides. Parallel work on CMV epitopes in our laboratory has demonstrated that the HLA-A2 transgenic model ($A2/K^b$) may be a suitable and sufficient screen to test efficacy of DNA vaccines in inducing CTL, allowing for a rapid transfer into the clinical setting (King, A. unpublished data). Several HLA-A2 candidate peptides encoded in MUC1 have been described (Table 3.1), and four vaccines encoding selected epitopes were constructed and evaluated: STAPPAHGV (S9V), ALGSTAPPV (A9V), TLAPATEPA (T9A) and LLLLTVLTV (L9V). Only the DNA vaccine encoding ALGSTAPPV was able to generate detectable CTL in a proportion of mice, and other vaccines were unsuccessful. We believe that these problems are underlined by the HLA-A2 specific TCR repertoire in $A2K^b$ tg mice. In these mice both the HLA-A2 transgene and the endogenous murine $H\text{-}2^b$ molecules are expressed, resulting in a limited TCR repertoire specific for either of the MHC isotypes. Future work

should be aimed at assessing the capacity of p.DOM-A9V vaccine to elicit CTL in the HHD tg mouse model where the TCR repertoire has been generated solely on a background of human HLA-A2, and this may overcome the problem of limited numbers of mice responding. A9V has been shown to be processed and presented by tumour cells expressing MUC1 endogenously, and is recognised by specific CTL resulting in target cell lysis *in vivo*[454], therefore another aspect of future work should aim to establish that CTL generated by p.DOM-A9V can effectively lyse target cells expressing MUC1 endogenously. This will require the generation of a double transfected cell line expressing HLA-A2/$K^b$ and MUC1. On the basis of any observed success, this vaccine could then be assessed in double transgenic mice (HHD crossed with MUC1 tg mice). This would pave the way for use of p.DOM-A9V in a clinical setting to eradicate MUC1 expressing tumours.

# 4 Final discussion

The potential for active immunotherapy as a treatment option for cancer is now viable and strategies are being developed to engage both the humoral and cellular arms of the immune response. It is apparent that with established disease, intervention will most likely be in the setting of minimal residual disease. A prime requirement is knowledge of the nature of tumour associated antigens, as this will determine the strategies used to target specific antigens, and combat tolerance which may exist. It is as yet not clear what the precise requirements for effective immunotherapy are, for instance, whether a combinatorial approach will be required utilising multiple approaches, or delivery of a single agent. To develop an optimal strategy for anti-tumour immunotherapy, the interplay between cells of the immune system, particularly $CD8^+$ T cells and $CD4^+$ T cells, will need to be exploited. In this regard, our strategy has been to use vaccination with bacterial plasmid DNA, with its intrinsic ability to engage all arms of the immune response. We have formulated an approach which links antigen delivery with T-cell help supplied by a bacterial protein, which potentiates responses to encoded antigen. Specific DNA vaccines have been designed able to generate humoral or activated $CD8^+$ T cell responses. Several pre-clinical animal models have now demonstrated the efficacy of this design in activating all arms of the adaptive immune system, promoting protection from tumour challenge. An added advantage is that DNA vaccines are cost effective and simple to construct, and clinical trials initiated in our laboratory have demonstrated that they are well tolerated with few side effects.

The focus of these studies was to develop DNA fusion vaccines for use in the treatment of MM. Several MM-associated antigens have been described. We examined the ability of different DNA fusion vaccine designs to induce responses to two of these antigens, namely the CTAs and MUC1 in pre-clinical animal models.

CTA are particularly suitable intracellular tumour antigen targets for p.DOM.epitope attack. Their expression is essentially tumour specific and a number of CTA derived MHC class I epitopes have been described, presented by different MHC haplotypes, including HLA-A2. Furthermore, successful vaccination harnessing immunity to this group of antigens would find clinical application in a wide variety of patients with tumours of different cellular origin, notably myeloma.

Although murine *Mage* genes have been described, their expression was not identifiable in any of murine tumours models evaluated initially. The P815 mastocytoma was then selected as a suitable tumour to model CTA encoding vaccines in the mouse. P815 expresses the *P1A* gene with an expression pattern which mirrors human CTA. P1A encodes a well defined MHC class I epitope, AB, which was incorporated into p.DOM.epitope design. The aim here was to analyse the ability of p.DOM-P1A/AB design to elicit effective $CD8^+$ T-cell immunity that would protect from a lethal challenge of P815 tumour cells. Notably, a single DNA vaccination was sufficient to activate P1A/AB specific $CD8^+$ T cells that could be identified *ex vivo*. However, it was consistently found that only 55-60% of vaccinated mice made a response following one vaccination. A possible reason for this is that following primary vaccination there is insufficient antigen concentration to allow $CD8^+$ T cells to break through their threshold level of activation in all animals. It is also known that generating P1A/AB specific $CD8^+$ T cell responses *in vivo* requires $CD4^+$ T cell help. However, following vaccination with p.DOM-P1A/AB there was no demonstrable $CD4^+$ T cell response to FrC encoded p30. It is likely that $CD4^+$ T helper cell responses were generated to other MHC class II epitopes within p.DOM, although this was not evaluated. It is also possible that P1A is expressed in several tissues at a low level in DBA/2 mice as has been found in BALB/c mice. In agreement with this, we found that following P1A/AB stimulation of splenocytes from control vaccinated mice, a low level of $CD8^+$ T cells had proliferated, suggesting previous exposure of these T cells to P1A. Depending on the level of expression, this may impart a low level of peripheral T-cell regulation *in vivo* in some, but not all the animals. Treg cells may be playing a part in keeping the $CD8^+$ T cell response to P1A/AB at bay, and their effects may impinge on the variability observed. These issues have not been addressed to date but should form a key question in future research when using DNA fusion vaccines in this tumour model.

P1A/AB specific $CD8^+$ T cells detectable *ex vivo* also proliferated *in vitro* after peptide stimulation. Following expansion, P1A/AB specific CTL were capable of killing tumour cell targets in a peptide specific manner as demonstrated by peptide pulsing $BCL_1$ cells. These CTL were also capable of specifically lysing tumour cells expressing the P1A gene endogenously. The P815 derived tumour target cells were therefore capable of processing and presenting the P1A/AB peptide in complex with MHC class I on the cell surface. The initial tumour challenge experiment led to 40% of the animals surviving at day 150. This was in line with the percentage of animals that made a response to the vaccine. In the repeat experiment however, only 10% of animals survived to day 100, although a significant delay

in tumour growth was seen in p.DOM-P1A/AB vaccinated animals. Analysis of tumour cells arising late in the course of the experiment demonstrated the absence of P1A expression, which was likely to have been caused by immune mediated pressure. This observation highlighted the limitation in using a single MHC class I restricted tumour peptide and quite clearly reveals and important requirement for targeting multiple antigens. Other considerations also arise in a strategy in which p.DOM.epitope DNA vaccines are used to target different antigens. For instance, preliminary experiments have indicated that injecting the DNA vaccines independently at separate sites induces an increased $CD8^+$ T-cell response to both tumour epitopes, compared with vaccines mixed together and injected at the same site (data not shown).

A further aspect of this study was to examine the effect of fusing an elongated AB epitope to p.DOM to incorporate a putative P1A derived MHC class II epitope. It was of interest to evaluate whether we could simultaneously activate P1A specific $CD4^+$ T cells and whether these cells could complement AB-specific $CD8^+$ T cells in tumour eradication. This work is in a preliminary stage and thus far we have shown that this vaccine design can activate AB-specific $CD8^+$ T cells in a comparable manner to p.DOM-P1A/AB. Further work is needed to assess the activation of $CD4^+$ T cells and their role in protective tumour immunity.

The final aspect of this study was to compare the p.DOM-peptide design to DNA vaccines encoding full length P1A. Again this work is at an early stage, but has indicated the AB epitope is not efficiently processed and presented from full length P1A when encoded in a DNA vaccine, either alone or when fused to DOM. Furthermore, only a low level response was seen when FrC was fused to P1A. This may be due to the inability of the vaccines to fold correctly *in vivo* as indicated by their inability to induce anti-FrC antibodies. Furthermore, and in contrast to our findings, others have found that vaccination with DNA encoding full length P1A was able to activate protective tumour immunity. Further work is needed to determine how each of the DNA fusion vaccines are processed and presented *in vivo*.

This tumour model has proved challenging but has allowed the efficacy of the p.DOM.epitope vaccine design in activating peptide specific CTL and their ability to lyse tumour cell targets to be demonstrated in a relevant setting. Various vaccination strategies designed to target P1A have been described including the use of $P1A^+$ tumour cells, peptide vaccination in adjuvant, peptide pulsed DC and viral and DNA vectors encoding P1A as outlined earlier. Only a few of these studies have analysed the tumour protection achieved,

and in all cases this was not remarkable. Comparisons of these strategies with the p.DOM.epitope DNA vaccine design are made with caution, but indicate a similar degree of $CD8^+$ T cell activation and tumour protection. One study has assessed the $CD8^+$ T-cell responses made in individual mice following vaccination with bacterial DNA encoding P1A and the results parallel our data with only a proportion of mice responding. These data indicate an intrinsic limitation with this strain of mouse in their ability to activate AB-specific $CD8^+$ T cell responses, such as low level tolerance or the presence of Treg cells, rather than a fundamental problem with the p.DOM.epitope design. The ability of this vaccine design to activate $CD8^+$ T cells specific for human CTA derived HLA-A2 motifs have also since been demonstrated (data not shown), but in the absence of a suitable tumour target it is difficult to demonstrate the effector function of these CTL.

We next addressed the question of targeting the MUC1 antigen with DNA fusion vaccines. The MUC1 glycoprotein is abnormally expressed in different malignancies such as myeloma. MUC1 is expressed on the surface of ductal epithelial cells by forming a type I transmembrane protein containing a distinctive 20 amino acid variable number tandem repeat. In normal cells MUC1 has a restricted expression pattern on the lumenal side of mucosal epithelium and is extensively glycosylated. It was therefore postulated that the immune system was unlikely to be tolerant to this protein, but this issue has not been resolved. The changes to MUC1 expression pattern and glycosylation state in malignant cells also led to the suggestion that immune responses targeting this antigen on tumours were unlikely to cause simultaneous unwanted autoimmune reactions against normal tissue. For these reasons, MUC1 has long been a favourable tumour antigen target for immune attack and distinct strategies have been documented. These include mannan-MUC1 fusion proteins, MUC1 transfected DC as well as viral or DNA vectors encoding MUC1. The aim of the present study was to assess the use of DNA vaccines encoding MUC1 fused to FrC or its derivatives to elicit effective immunity, initially in wild type mice and subsequently in the MUC1 tg model, where the role of FrC fusion in overcoming potential tolerance could be assessed.

DNA vaccines engineered to express MUC1 with approximately 39 repeats, either alone or fused to FrC or DOM, were shown to be effective at inducing protective immunity in wild type mice. This was expected due to the xenogeneic nature of the antigen. These observations verified the structural integrity of the expressed MUC1 protein. Lymphocyte depletion experiments demonstrated a role for both $CD8^+$ and $CD4^+$ T cells, and earlier work was therefore aimed at defining the specificity of both T cell subsets.

In order to enhance peptide-specific $CD8^+$ T-cell responses, p.DOM.epitope vaccines incorporating documented H-$2K^d$ and H-$2D^b$ peptides were evaluated. The two peptides chosen were SPADTRPAP (peptide1) encoded within the VNTR, and therefore present in multiple copies, and SAPDNRPAL (peptide2) encoded within the degenerate repeat. Both peptides were reported in the literature as being processed and presented from MUC1 encoding vaccines, allowing activation of epitope-specific CTL. A comparison of the p.DOM-MUC1peptide2 design with DNA vaccines encoding full length MUC1 was made. It was demonstrated, using both cytotoxic killing assays against peptide pulsed cells and ELISPOT assays *in vitro*, that an increased MUC1peptide2-specific $CD8^+$ T cell response was generated following vaccination with p.DOM-MUC1peptide2. It is likely that both the removal of immunodominant epitopes from FrC and MUC1, as well as the C-end rule are responsible for this result. However other factors may also play a role, such as reduced protein turnover from DNA vaccines encoding longer sequences. In contrast, p.DOM-MUC1peptide1 was unable to activate peptide specific $CD8^+$ T cells. A possible reason for this was that peptide1 did not contain the correct anchor motifs for H-$2K^b$. Furthermore, peptide1 was not able to stabilise H-$2K^b$ on the cell surface efficiently, and therefore was unlikely to be able to activate $CD8^+$ T cells. It was interesting to find however, that MUC1peptide2 specific CTL were capable of cross-recognition and could lyse MUC1peptide1 coated targets *in vitro*. This was encouraging, in that a single vaccine design was able to induce CTL that could recognise two different peptides encoded within MUC1. It was therefore surprising that MUC1peptide2-specific CTL were unable to lyse RMA-MUC1 *in vitro* or *in vivo*. Treatment with IFNγ did not affect this result, suggesting that it was not due to differences in proteolytic cleavage patterns generated by the two proteasome subtypes. The reason for this was not investigated further but could due to differences in the glycosylation state of the presented peptide by APC from the DNA vaccine and that from the tumour cell. This approach highlighted a potential caveat. Evaluation of reported methods used to determine MUC1 derived MHC class I epitopes do not appear wholly suitable, and has led to the identification of epitopes which are not processed and presented by $MUC1^+$ tumour cells. It is clear however, that the restriction is not due to the p.DOM.epitope fusion DNA vaccine design. With advances in technology able to measure specific peptide-MHC complexes on the cell surface, and the increasing ability to predict peptides likely to be presented, these problems are likely to be overcome.

To analyse vaccine design further, $CD4^+$ T cell responses generated by vaccines encoding full length MUC1 were compared and measured using ELISPOT. Analysing responses

made by both $CD4^+$ T cells and $CD8^+$ T cells in this way made it possible to compare vaccine design in wild type mice vs. MUC1 tg mice. MUC1 specific $CD4^+$ T cell responses have been shown to have some specificity for the VNTR region by others, and this approach was used here. IFNγ production by T cells was measured against a 24mer MUC1 peptide encoding the 20 amino acid repeat with an additional 4 amino acids from the next repeat. MUC1 VNTR specific T cell responses were comparable using p.MUC1, p.MUC1DOM and p.MUC1FrC in wild type mice. Lymphocyte depletion experiments designed to prove the response to VNTR was MHC class II restricted were unsuccessful due to background activity, but should form a fundamental part of future studies.

The most pertinent aspect of this study was to evaluate the efficacy of DNA vaccine design in a setting where MUC1 was expressed endogenously. Mice transgenic for human MUC1 were vaccinated with DNA encoding MUC1 alone or fused to FrC or DOM. p.DOM-MUC1peptide2 was also evaluated in this setting. ELISPOTs were used to measure both the $CD8^+$ T cell response to MUC1peptide2 and the presumed $CD4^+$ T cell response to the VNTR peptide. The response to FrC was also evaluated and indicated that MUC1 tg mice were capable of generating a $CD4^+$ T cell response to a foreign antigen, in a comparable manner to wild type mice.

MUC1 tg mice vaccinated once or twice with p.MUC1 however, were unable to generate MUC1-VNTR specific $CD4^+$ T-cell responses, and the fusion of DOM or FrC did not alter this result. The difficulty in generating MUC1 specific $CD4^+$ T-cell responses in MUC1 tg mice has been demonstrated by several other vaccination strategies. It is possible that peripheral $CD4^+$ T-cell tolerance to MUC1 exists in these mice, and is severely restricting. Further DNA vaccine boosts may lead to activation of small numbers of activated MUC1-VNTR specific $CD4^+$ T cells. It is also possible that these mice display central tolerance to human MUC1 in their $CD4^+$ T-cell compartment, whereby MUC1 specific $CD4^+$ T cells have been deleted from the repertoire.

In contrast to MUC1-VNTR specific $CD4^+$ T cell responses, preliminary data indicate that low levels of activated MUC1peptide2 specific $CD8^+$ T-cell responses were detected in some animals vaccinated twice with p.MUC1FrC, but not with p.MUC1 alone or fused to DOM. These data indicate a difference in the capacity of the MUC1 tg mice compared to wild type mice to respond to DNA vaccines encoding MUC1. However, larger numbers of mice need to be analysed to confirm this. One possible reason for the ability of DNA vaccines encoding MUC1 fused to full length FrC, to generate low level $CD8^+$ T cell

responses, could be the increased numbers of FrC derived MHC class II restricted epitopes. The provision of sufficient FrC linked T-cell help may allow activation of MUC1peptide2 specific $CD8^+$ T cells following 2 vaccinations. To help confirm this idea, the $CD4^+$ T cell response to the whole FrC protein needs to be assessed to give an idea of the level of T-cell help being generated by each vaccine. In comparison to vaccination with DNA vaccines encoding full length MUC1, vaccination with p.DOM-MUC1peptide2 was able to activate the greatest numbers of $CD8^+$ T cells following a DNA boost. This result was statistically significant but again larger numbers of mice are needed to be assessed to be confident with this analysis. These data again indicate the enhanced ability of the p.DOM.epitope design in inducing epitope-specific $CD8^+$ T-cell responses. The ability of these CTL to differentiate to full effector function has not been assessed to date and will form a necessary part of future studies.

The final aspect of this part of the project was to evaluate the p.DOM.peptide DNA vaccine design in activating $CD8^+$ T cells to human HLA-A2 MUC1 epitopes for a potential clinical application in human cancer. HLA-$A2/K^b$ mice were vaccinated with p.DOM fused to 4 described MUC1 HLA-A2 epitopes, STAPPAHGV (derived from the VNTR), ALGSTAPPV, TLAPATEPA and LLLLTVLTV (from the signal sequence). All of these peptides contain at least one anchor residue for HLA-A2 and all had been demonstrated to be presented by tumour cells expressing MUC1 endogenously. The only vaccine capable of activating a $CD8^+$ T-cell response encoded the ALGSTAPPV peptide. The reason for the inability of the other 3 vaccines to induce $CD8^+$ T cell responses may be three-fold. Firstly the mouse model is not ideal and is likely to display a skewed TCR repertoire which is formed from recognition of both human and mouse MHC molecules. Secondly the peptide concentration used *in vitro* for re-stimulation was not optimised and this may have prevented sufficient proliferation of peptide-specific CTL. Thirdly the peptide was not being processed a presented efficiently from the translated vaccine protein product. To answer one of these questions, the vaccines need to be tested in a more appropriate HHD tg model, where the endogenous murine MHC have been removed and the TCR repertoire is HLA-A2 restricted. This model was not available at the time of this study. It will be interesting to see if vaccines that failed to initiate immune responses in the $A2/K^b$ model are capable of activating $CD8^+$ T cells in the HHD model, as has since been found with other p.DOM.epitope vaccines in our laboratory (Mr. A. King, unpublished data). Optimisation of the re-stimulation conditions will also be necessary and will also give an indication of the avidity of the peptide-specific $CD8^+$ T cells. It would also be of interest to cross HHD mice

with MUC1 tg mice and to assess the ability of p.DOM.epitope DNA vaccine design in inducing HLA-A2 restricted $CD8^+$ T cells in this setting. With an appropriate $MUC1^+$/$HLA\text{-}A2^+$ tumour target, the ability of these CTL to lyse relevant target cells could then be assessed.

The CTA and MUC1 tumour antigens offer two very different potential targets in myeloma, most notably in the tolerogenic influence exerted by each. The work undertaken in both wild type and transgenic mice in this study has validated different aspects of vaccine design required to activate therapeutic T cells against these differing antigens. It is expected that this information will have a direct implication in human disease, and has indicated some problems that may arise when vaccinating against these antigens. The tumour antigen specific $CD8^+$ T cell response is vital for protective immunity against CTA expressing tumours but requires T-cell help. In this regard, the p.DOM.epitope DNA vaccine is the most appropriate vaccine design to utilise when targeting these antigens. However, tumour antigen loss is readily achieved, advocating the need to target multiple antigens. CTA though, offer a multiplicity of such targets. In contrast, both antigen-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses are involved in protection against $MUC1^+$ tumours. This suggests that one appropriate DNA vaccine design should incorporate a MHC class II epitope derived from MUC1. The p.DOM.epitope DNA vaccine design was however, very effective at inducing MUC1 specific $CD8^+$ T cells. A combination of vaccines may therefore be needed for optimal T-cell responses targeting MUC1. The CTA and MUC1 will then become available as dual targets in the malignant setting of multiple myeloma.

# 5 Materials and Methods

## 5.1 DNA vaccine design

Vaccine-encoded antigen sequences were inserted into the multiple cloning site of the commercially available vector pcDNA3.1 (Appendix B) (Invitrogen, Paisley, UK) under the transcriptional control of the CMV promoter. Vaccine design is diagrammatically represented in Figure 3.4. for MUC1 encoding vaccines and Figure 2.2 for P1A encoding vaccines.

### 5.1.1 Fragment C encoding vaccines

p.FrC was kindly provided by Dr. D. Zhu, with the Kozak sequence upstream of a leader sequence derived from the IgM $V_H$ of the $BCL_1$ tumour fused to full length Fragment C. The vaccines p.DOM and p.DOM-FrC7 were kindly provided by Dr. J. Rice. p.DOM encodes the amino terminal domain of FrC fused downstream of the Kozak-$BCL_1$ leader sequence. p.DOM-FrC7 is identical to p.DOM with the CTL epitope SNWYFNHL (FrC7, H-2K$^b$) fused to the C-terminus.

### 5.1.2 MUC1 encoding vaccines

The vaccines p.MUC1, p.MUC1FrC and p.MUC1DOM were kindly provided by Dr. F. Forconi. The MUC1 leader (69bp), 5'degenerate sequence (361bp), the VNTR sequence containing 39 identical repeats (2340bp) and the first 81 bp of the 3' degenerate sequence are all encoded downstream of the consensus Kozak sequence (GCC GCC ACC) within the three vaccines. Full length Fragment C or the amino terminal domain of Fragment C are fused to MUC1 at its C-terminus in p.MUC1FrC and p.MUC1DOM respectively. The vaccines p.DOM-MUC1peptide1, p.DOM-MUC1peptide2, p.DOM-MUC1peptide3 and p.DOM- MUC1peptide4 consist of the p.DOM sequence described above fused upstream of the previously published MUC1 nonamer CTL epitopes SAPDTRPAP (H-2K$^b$ or H-2D$^d$), SAPDNRPAL (H-2D$^b$), STAPPAHGV (H-2D$^b$) and TVVTGSGHA (H-2K$^b$) respectively (84, 85). Additionally a truncated version of p.DOM-peptide1 encoding the octomer SAPDTRPA (H-2K$^b$) at the C-terminus was also constructed (p.DOM-peptide1TR). p.DOM S9V, p.DOMA9V, p.DOMT9A and p.DOML9V consist of the p.DOM sequence fused upstream of previously published MUC1 HLA-A2 binding peptides STAPPAHGV,

ALGSTAPPV, TLAPATEPA and LLLLTVLTV respectively.

### 5.1.3 P1A encoding vaccines

The vaccines p.P1A, p.P1AFrC and p.P1ADOM consist of Kozak sequence (GCC GCC ACC) fused upstream of the leader sequence derived from the IgM $V_H$ of the $BCL_1$ tumour and in turn fused to the full length P1A gene. Full length Fragment C or the amino terminal domain of Fragment C are fused to P1A at its C-terminus in p.P1AFrC and p.P1ADOM respectively. p.DOM-P1A/AB and p.DOM-P1E encode the Kozak-$BCL_1$ leader sequence fused upstream of the amino terminal domain of FrC. The previously published immunodominant AB epitope (LPYLGWLVF, H-$2L^d$) or E epitope (GYCGLRGTGV, H-$2D^d$) are fused to the C-terminus respectively. p.DOMABlong consists of p.DOM with an extended AB epitope fused to its C-terminus, EILPYLGWLVFA. p.AB and p.ABlong encode the Kozak-$BCL_1$ leader sequence fused upstream of the immunodominant AB epitope or elongated AB epitope.

## 5.2 DNA vaccine PCR assembly

All assembly primers are given in Table 1, Appendix C.

p.DOM-MUC1peptide1, p.DOM-MUC1peptide1TR, p.DOM-MUC1peptide2, p.DOM-MUC1peptide3, pDOM-MUC1peptide4 p.DOM-P1A/AB, p.DOMABlong and p.DOM-P1E were constructed by a PCR SOEing procedure using p.DOM as template DNA. A T7 forward 5' primer was used with the relevant reverse primer comprising overlapping 3'-region of p.DOM and peptide with stop codon and the *Not I* restriction site.

PCR SOEing was used to assemble the vaccines encoding full length *P1A* gene in a three step procedure. In the first step, the *P1A* sequence was amplified using as template a plasmid encoding full length *P1A* using the forward primer P1AFrC/5p1 (incorporating Kozak-$Bcl_1$ $V_H$ leader) and a downstream primer P1AFrC/3p1 incorporating a CpG linker and 30 base overlap with FrC. In the second step, FrC or p.DOM was amplified from template plasmids encoding either full length FrC or its first domain using the forward primer FrCP1A/5p1 and reverse primers FCAS2 for FrC and DOMP1A for DOM. In the third step, P1A+L was fused to FrC or DOM using the 5' primer P1AFrC/5p1 and the 3' primers FCAS2 and DOMP1A respectively.

The p.AB and p.ABlong minigenes were amplified using $Bcl_1$ $V_H$ leader-MAGEA3 peptide template (Dr. N. Zojer) with the forward primer T7 and reverse primer ABmini+LR and ABLongminiR respectively. All primer sequences are given in Table 1, Appendix C.

The PCR reactions were carried out in a final volume of 50µl with approximately 3µg DNA template, 0.4pmol of each primer, 0.5mM dNTPs, and 1ul of Expand High Fidelity Taq polymerase (Roche, Lewes, UK) in supplied reaction buffer. After an initial step of 95°C for 60 seconds, 5 cycles were performed with a denaturing step of 94°C for 60 seconds, an annealing step of 42°C for 60 seconds and an extension step of 72°C for 3 minutes. This was followed by 30 cycles with a denaturing step of 94°C for 60 seconds, an annealing step of 60°C for 60 seconds and an extension step of 72°C for 3 minutes. A final extension step of 72°C for 5 minutes was carried out. A control reaction containing no template DNA was included to check for contamination. The amplified products were run on an ethidium bromide gel containing 1% agarose, and bands of the expected size were excised and DNA eluted from the gel using the QIAquick Gel Extraction Kit (Qiagen, Crawley, UK) in 35µl of $H_2O$.

## 5.3 DNA vaccine cloning

The DNA inserts were subjected to two sequential restriction enzyme digests for 12 hours at 37°C in a final volume of 40µl with 35µl DNA, 4µl 10x buffer and 1µl *Hind III* or *Not I* (New England Biolabs, Hertfordshire, UK). 8.5µl of double digested DNA was ligated into pcDNA3 linearised with *Hind III* and *Not I* in a final volume of 20µl containing 1µl T4 DNA ligase, 0.5µl vector and 10µl 2x Rapid Ligation Buffer (Promega, T4 DNA ligation kit). The ligation reaction was incubated at room temperature for 1 hour.

JM109 *E. coli* competent cells (Promega) were used for transformation. 10µl ligation product was added to 50µl of JM109 cells and incubated for 30 minutes at 4°C. Cells were heat shocked for 45 seconds at 42°C and replaced at 4°C for 2 minutes. LB medium (0.8ml) was added and the cells were incubated for 60 minutes at 37°C with shaking. Cells were plated on LB/agar plates containing 100µg/ml ampicillin and grown at 37°C for 12-14 hours. Colonies were randomly selected and cultured overnight in 2ml LB medium with 100µg/ml ampicillin at 37°C with shaking. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturers protocol, and eluted in a final volume of 50µl sterile $H_20$. The presence of insert in the vector was confirmed by

migration in comparison to an empty vector plasmid. Following sequence verification (see below), 200µl of culture broth with correct insert were stocked at -80°C with approximately 15% sterile glycerol. For large scale DNA preparation, the glycerol stock was plated on LB agar and grown overnight. Small scale (10ml LB + Amp) cultures were grown for 8 hours at 37°C with shaking. This was used to seed a large volume of LB Amp (1.2L) which was grown overnight at 37°C with shaking. Plasmid DNA was purified using the QIAfilter Plasmid Giga Kit (Qiagen) according to the manufacturers' protocol. DNA yield was quantified by spectrophotometer and stored in aliquots of 1mg as isopropanol precipitates at -20°C until required.

## 5.4 DNA sequence analysis

Clones containing inserts of the correct size were sequenced bi-directionally. 100-250ng plasmid DNA was added to a final reaction volume of 10µl containing 2µl Big Dye (Applied Biosystems, Warrington, UK), 1µl primer (1.6pmol) and 2µl 5 X sequencing buffer. The sequencing primers used are given in Table 2, Appendix C. Vector based primers T7 and Sp6 (MWG, Germany) were used to sequence from upstream and downstream of the plasmid cloning site. pDomseqF1 and pDomseqR1 (Oswel DNA, Southampton, UK) were used as internal forward and reverse primers for p.DOM encoding plasmids. AbseqF1 and ABseqR1 (Oswel DNA) were used to sequence the full length P1A encoding plasmids. The sequencing reaction cycle used was 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes for 30 cycles, with the reaction then held at 4°C until purification. DNA was precipitated using the ethanol/sodium acetate method before resuspension in loading buffer and fractionation on an ABI Prism 377 Automated DNA Sequence Analyser (Applied Biosystems). DNA sequences were evaluated using Macvector 4.5.3 (Oxford Molecular, UK) and Editview 1.0 (Applied Biosystems).

## 5.5 Coupled *in vitro* rabbit reticulocyte transcription/translation

*In vitro* expression of all vaccine constructs was assessed using the TNT® T7 Quick Coupled Transcription/Translation System (Promega) following manufacturers instructions to verify synthesis of encoded antigen. In ribonuclease free conditions, 0.5 - 1µg of purified vaccine plasmid DNA was added to 20µl TNT master mix (containing rabbit reticulocyte lysate), 1µCi $^{35}$S-methionine and $H_2O$ in a final volume of 25µl. The reaction was incubated at 30°C for 90 minutes before being denatured at 95°C for 2 minutes in the

presence of 0.1M DTT and NuPage LDS sample buffer (Invitrogen). Samples were run on a pre-cast 4-12% gradient polyacrilamide gel (Invitrogen) and vacuum dried onto filter paper. A radiographic film was exposed overnight to obtain an autoradiogram.

## 5.6 Cell lines

RMA-MUC1 and RMA-Hygro (both H-$2^b$) were a kind gift from Dr. J. Taylor-Papadimitriou, CRUK London and are transfected with plasmid encoding the full length human MUC1 sequence and empty vector control respectively. Dr. J. Rice kindly provided EL-4 cells.

P815 (H-$2^d$) is a methylcholanthrene induced murine mastocytoma which expresses five tumour antigens A, B (encoded by P1A) C, D and E. P511 is an azaguanine resistant sub-clone of P815 with high expression of P1A/AB. P1-204 is a subclone of P815 that does not express P1A/AB but does express D and E antigens. P511 and P1-204 were a kind gift from Dr. P Coulie, Ludwig Institute for Cancer Research, Brussels. $BCL_1$ is an *in vitro* B cell lymphoma that is negative for antigens A, B and E (C and D have not been tested).

T2 is a human T cell line expressing HLA-A2. U266 is a human myeloma cell line expressing MUC1 and HLA-A2[386].

All cell lines were grown *in vitro* in complete RPMI medium (R10, RPMI supplemented with 10% heat inactivated FCS, 1mM sodium pyruvate, 2mM L-glutamine, non-essential amino acids (1% of 100x stock), 25mM HEPES buffer and 50μM 2-mercaptoethanol [all obtained from Invitrogen]). Hygromycin B was added to RMA-MUC1 at 550μg/ml and to RMA-Hygro cells at 350μg/ml to maintain expression of the transgene. Medium used for growing human cell lines did not contain 2-mercaptoethanol.

### 5.6.1 Phenotypic analysis of MUC1 transfected cell lines

1x$10^6$ RMA-MUC1 cells grown *in vitro* were washed and incubated for 30 minutes at 4°C in the dark with 0.5μg FITC labelled mAbs supplied by BD Pharmingen (Cowley, Oxford). mAbs were specific for murine MHC class I ($K^b$ and $D^b$), MHC class II (I-A/I-E, clone 2G9), CD8 (b.2 clone 53-5.8), CD4 (clone RM4-4) and isotype controls, IgG2a K and IgG2b K. After a final wash the cells were resuspended in PBS and analysed immediately by FACScalibur, using CELLQUEST software (BD Pharmingen). 10,000 events were

collected and live cells were gated on based on their forward scatter/side scatter properties. The FITC staining for each sample was plotted as a histogram and compared directly to an identically gated sample stained with the isotype control.

Purified HMFG1 mAb and HMFG1 supernatants were kindly provided by Dr. J. Taylor-Papadimitriou. HMFG1 is an IgG1 mAb specific for the PDTR epitope within the MUC1 tandem repeat. VU3C6 hybridoma was kindly provided by Dr. S. Mensdorff-poully (Department of Obstetrics and Gynaecology, Academic Hospital Free University, Amsterdam), and purified by Dr. C. King. VU3C6 is also an IgG1 mAb, specific for the RPAP epitope within the MUC1 tandem repeat. MUC1 deglycosylation has no effect on either HMFG1 or VU3C6 binding, however, desialyation increases binding in both cases (Dr. F. Forconi, unpublished results). FITC labelled goat anti-mouse IgG was purchased from Sigma. Anti-saporin IgG1 mAb (SI3) was kindly provided by Dr. R. French (Tenovus) and used as a control.

To assess MUC1 expression, $1x10^6$ RMA-MUC1 cells were incubated with 10μg unlabelled anti-MUC1 mAbs (HMFG1 and VU3C6) for 30 minutes at 4°C in the dark and then washed in PBS, followed by 30 minute incubation with FITC labelled goat anti-mouse IgG (BD Pharmingen). After a final wash the cells were resuspended in PBS and analysed immediately by FACScalibur, using CELLQUEST software. 10,000 events were collected and live cells were gated on using forward scatter/side scatter. The FITC staining for each sample was plotted as a histogram and compared directly with an identically gated sample stained with secondary FITC labelled antibody alone and a sample stained with irrelevant SI3 antibody as controls.

### 5.6.2 Phenotypic analysis of T2 transfected cell lines

Cells were assessed for their continued expression of HLA-A2. $1x10^6$ T2 cells were incubated with 10μg unlabelled anti-HLA-A2 mAb (BB7.2, a kind gift from Professor T Elliott) for 30 minutes at 4°C in the dark and then, as for staining for MUC1 expression, cells were washed, incubated with FITC labelled goat anti-mouse IgG and analysed by FACScalibur, using CELLQUEST software. 10,000 events were collected and live cells were gated on using forward scatter/side scatter. The FITC staining for each sample was plotted as a histogram and compared directly with an identically gated sample stained with secondary FITC labelled antibody alone.

## 5.7 RT-PCR assay for the expression of murine *Mage* genes

Total RNA was extracted from $1x10^6$ diverse tumour cells (32Db3a2, 5T13, 5T2, 5T33, A31, B16, C6BL, CT26 and EL-4) using Tri Reagent (Sigma). Following addition of chloroform, RNA contained within the aqueous phase was removed and precipitated in iso-propanol and stored as an aqueous solution at -80°C until use. Contaminating DNA was removed with 1U DNase I (Stratagene, Amsterdam, Netherlands) treatment, incubated at 37°C for 30-minutes. Enzyme inactivation was achieved by a 20-minute incubation at 65°C followed by phenol chloroform extraction. Reverse transcription was performed using the First Strand cDNA Synthesis Kit (Amersham Biochemicals, Buckinghamshire, UK). 1-5μg total RNA was used in a reaction volume of 15μl containing 1μl oligo d(T), 5μl bulk mix and 1μl DTT and incubated at 37°C for 60 minutes. cDNA was stored at -20°C until use.

PCR primers used for the amplification of murine *Mage a* genes were: Mage a 5p2 and 3p2, these consensus primers amplify all the *Mage a* genes, with a product size of 963 base pairs. Two additional primer sets were designed to distinguish between cDNA and contaminating gDNA and were specific to Mage a2 (primers were MA2/5P1/E3 and MA2/3P1/E4) and Mage a5 (primers were MA5/5P1/E2 and MA5/3P1/E3), giving a product size of 565 base pairs and 440 base pairs respectively. The consensus PCR primer pair used for the amplification of murine *Mage b* genes was B Mage 5' and B mage 3'. The amplified product was 384 base pairs. An additional Mage b3 primer pair was also used, Mage b3/5' and Mage b3/3', yielding a product length of 993 base pairs. All primer sequences are given in Table 3, Appendix C.

In all cases PCR amplifications were carried out in a final volume of 50μl with approximately 3μl cDNA template, 0.4pmol of each primer, 0.5mM dNTPs, and 1μl of Hot Star Taq polymerase (Qiagen) with 5μl of 10x reaction buffer. Control reactions containing no template DNA were included to check for contamination. In all cases the expression of murine β-actin was examined as an internal control, using the primer pair β-actin F and β-actin R (Table 3, Appendix C). The predicted product length was 349 base pairs. Assessment of PCR products was performed on an ethidium bromide-stained agarose gel and the product size was compared to $1Kb^+$ DNA ladder run in parallel.

## 5.8 Isolation of the *P1A* gene

Total RNA was extracted from the tumour cells P815, P511, P1204 and $BCL_1$ and cDNA synthesised. The PCR primer pair used to amplify the full length *P1A* gene product was P1A5P1 and 3P2 (Table 4, Appendix C). The amplified product was 675 base pairs. PCR reaction and cycle conditions are as described for the *Mage* genes. The expression of murine β-actin was examined as an internal control as described. These primers were also used in RT-PCR assays to examine expression of *P1A*. Expression of *P1E* was also assessed by RT-PCR and the primers were P815EF1 and P815ER1 (Table 4, Appendix C), yielding a product length of 387 base pairs.

The amplified PCR product was purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen) and eluted in 35μl of $H_2O$. 8.5μl of eluted DNA was ligated into pGEM-T vector and cloned as described in Section 5.3. Sequence analysis was performed with primers T7, Sp6, AbseqF1 and AbseqR1 (Table 2, Appendix C). Gene sequences were compared to the Genbank database sequence (accession number NMO11635 for P1A and NM026322 for P1E).

## 5.9 Mice

For experiments involving MUC1 encoding vaccines, C57Bl/6 mice (H-$2^b$) were used. These mice were either bred in house under specific pathogen free conditions or purchased from Charles River, UK. Homozygous MUC1tg (SACII) mice were a kind gift from Dr. J. Taylor-Papadimitriou and were re-derived in house before being bred under specific pathogen free conditions. For experimental procedures, the homozygous transgenics were back-crossed onto C57Bl/6. HLA-A2 tg mice (A2-$K^b$) were bred in house under specific pathogen free conditions.

For experiments involving P1A encoding vaccines, DBA/2 (H-$2^d$) were used. These mice were either bred in house under specific pathogen free conditions or obtained from Harlan, UK.

For all experimental procedures, mice were used between 6-12 weeks of age.

## 5.10 Vaccination protocols

For generation of CTL, mice were vaccinated with 25μg DNA in 50μl saline injected into both rear quadricep muscles (total 50μg). The vaccination protocols used are shown below.

| Experiment | Primary vaccination | Vaccination boost | Sacrifice |
|---|---|---|---|
| D14 | Day 0 | None | Day 14 |
| D21 | Day 0 | None | Day 21 |
| D28 | Day 0 | Day 21 | Day 28 |
| D35 | Day 0 | Day 21 | Day 35 |

For protection studies, mice were vaccinated at 6-10 weeks of age with 25μg DNA in saline injected into both rear quadricep muscles on day 0 and day 21. Mice were challenged on day 28 by intra-peritoneal injection of $5x10^4$ RMA-MUC1 or $1x10^4$ P511 tumour cells. Mice were sacrificed when the ascitic tumour burden reached 20% of the original weight of the mouse in accordance with humane endpoint guidelines (UK Co-ordinating Committee for Cancer Research, London). RMA-MUC1 transfectants were tested for MUC1 expression (Section 5.6.1) prior to injection, and, where indicated, ascitic fluid was checked for MUC1 expression on the day of sacrifice. Similarly ascitic fluid from mice challenged with P511 was assessed for P1A expression by PCR as described in Section 5.8.

For $CD4^+$ and $CD8^+$ T-cell depletion, mice received intra-peritoneal injection of 100μg of anti-CD4 antibody clone YTS191.1.2 or anti-CD8 antibody clone YTS169.4.2 or normal rat IgG (Sigma-Aldrich, UK) as a control. Anti-CD8 and anti-CD4 antibodies were purified from hybridoma supernatants in house by Miss S. Buchan using a protein G coupled sepharose column. Injections were carried out on days -3, -1, before tumour challenge (day 0) and days +2, + 5 and +7 subsequent to tumour challenge).

## 5.11 Peptides

SAPDTRPAP* (H-$2K^b$ or H-$2D^d$), SAPDTRPA* (H-$2K^b$), SAPDNRPAL$^\dagger$ (H-$2D^b$), STAPPAHGV* (H-$2D^b$) and TVVTGSGHA (H-$2K^b$) are previously reported peptides and are located in the MUC1 VNTR (*) and 3' degenerate repeat (†)[436, 437]. MUC1 HLA-A2 peptides LLLLTVLTV, STAPPAHGV, ALGSTAPPV and TLAPATEPA are previously described peptides and are outlined in Table 3.1, Section 3.16. FrC7 peptide (SNWYFNHL H-$2K^b$) is located in the C-terminal domain of Fragment C. LPYLGWLVF (P1A/AB) and GYCGLRGTGV (P1E) are previously published peptides[358, 360, 361]. Peptides were

synthesised commercially and supplied at 95% purity (Peptide Protein Research Ltd., Southampton, UK).

## 5.12 MHC stability assay

$1x10^6$ RMA-S TAP deficient cells were incubated with 0.1-50μM MUC1peptide1, MUC1peptide2 and SIINFEKL for 18 hours at 37°C. Cells were incubated with 1μg/ml anti-MHC class I conformational antibody (Y3 for $K^b$ and B22 for $D^b$, kindly provided by Professor T. Elliott, Southampton) for 30 minutes at 4°C. Cells were washed and incubated with secondary FITC conjugated goat anti-mouse IgG (BD Pharmingen) for 30 minutes at 4°C. After a final wash the cells were resuspended in PBS and analysed immediately by FACScalibur, using CELLQUEST software. A minimum of 10,000 events were collected and live cells were gated using forward scatter/side scatter. The FITC staining for each sample was plotted as a histogram and compared directly to samples incubated with no peptide.

## 5.13 Generation of CTL lines and cytotoxicity assays

Splenocytes from vaccinated mice were collected and single cell suspensions prepared in R10. Splenocytes were counted and resuspended in R10 at $3x10^6$ cells/ml with recombinant human IL-2 (rIL-2, 20U/ml, Perkin-Elmer, Foster City, CA) and peptide at indicated concentrations. Splenocytes were then transferred to $80cm^2$ culture flasks and incubated upright at 37°C and 5% $CO_2$. In some cases splenocytes were re-stimulated with Mitomycin C (Sigma) treated RMA-MUC1 or L1210/P1A transfected tumour cells at a ratio of 10:1 (T cells: tumour cells). Mitomycin C is an inhibitor of DNA synthesis and nuclear division and prevents tumour cell out-growing the T cells. Where indicated a second *in vitro* stimulation was performed 7 days later. Here, T-cell cultures were plated in 24 well plates ($10x10^5$/well) with syngeneic feeder splenocytes ($10x10^6$/well) irradiated at 2500 rads for 11.8 minutes. rIL-2 (20U/ml) and peptide were also added. Plates were re-incubated at 37°C and 5% $CO_2$.

T-cell cytolytic activity was examined 6 days after the final *in vitro* stimulation. T cells were selected by density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway), harvested from the interphase, washed and made up to correct dilutions. Target cells were washed and resuspended in a final volume of 300μl containing approximately

200μCi radioactive chromium-51 ($Na_2{}^{51}CrO_4$) and 10μM test or irrelevant peptide, un-pulsed target controls were also prepared. Cells were incubated at 37°C for 1 hour then washed four times before being counted. Targets were plated in 96 U well plates at $5x10^4$ cells/well. Effector T cells were plated at various effector/target ratios in triplicate in a final volume of 200μl. The control plate consisted of targets alone in triplicate at $5x10^4$ cells/well in a final volume of 200μl (to test spontaneous release) and 100μl (to test total release). Plates were incubated at 37°C for 5 hours. 100μl 4% NP40 (detergent lysis) was added to the total release wells. 100μl sample of supernatent from all wells was removed into LMP3 tubes, and counted on a Wallac 1282 compugamma counter for 5 minutes. Percent specific lysis was calculated by the standard formula:

$$\frac{\text{Specific Release} - \text{Spontaneous Release}}{\text{Total Release} - \text{Spontaneous Release}} \times 100$$

## 5.14 Intracellular γ-IFN assay

Spleens from vaccinated mice were collected and single cell suspensions prepared in R10. Viable T cells were selected by density centrifugation and plated at $1x10^6$ cells/well in a 96 U well plate. T cells were incubated for 4 hours at 37°C together with 10U/well rIL2, 1μM/well Golgiplug (BD Pharmingen) and relevant peptide at indicated concentrations. The following wash and staining procedures were all carried out in PBS with 1μM/well Golgiplug. All monoclonal antibodies were supplied by BD Pharmingen.

Cells being stained after an *in vitro* stimulation were washed and blocked with 2% decomplemented mouse serum (15 minutes at 4°C) prior to labelling with 1μg/well FITC anti-mouse CD8b.2 (clone 53-5.8) or an isotype control (IgG2b K) for 20 minutes at 4°C. Cells were washed again, fixed and permeabilised in PBS/0.5% saponin (10 minutes at 4°C) before intracellular labelling with 0.5μg/well PE rat anti-mouse γ-IFN or isotype control (PE rat IgG1) for 20 minutes at 4°C. After a final wash the cells were resuspended in PBS and analysed immediately by FACScalibur, using CELLQUEST software. A minimum of 10,000 events were collected and live cells were gated using forward scatter/side scatter. The FITC/PE staining for each sample was plotted as a dot plot and compared directly to identically gated samples stained with the isotype control antibodies.

Cells being stained directly ex vivo were washed and blocked with 2% decomplemented mouse serum (15 minutes at 4°C) prior to labelling with 1μg/well FITC anti-mouse MHC

class II ($I\text{-}A^d/I\text{-}E^d$ clone 2G9) and 1μg/well CD8 APC (clone Ly-2) or isotype controls (FITC IgG2bK and APC IgG1) for 20 minutes at 4°C. Cells were washed again, fixed and permeabilised in PBS/0.5% saponin (10 minutes at 4°C) before intracellular labelling with 0.5μg/well PE rat anti-mouse γ-IFN or isotype control (PE rat IgG1) for 20 minutes at 4°C. After a final wash the cells were resuspended in PBS and analysed immediately by FACScalibur, using CELLQUEST software. A minimum of 100,000 events were collected and live cells were gated using forward scatter/side scatter. MHC class II positive cells were excluded from further analysis. The APC/PE staining for each sample was plotted as a dot plot and compared directly to identically gated samples stained with the isotype control antibodies.

## 5.15 Mouse γ-IFN ELISPOT assay

Spleens from vaccinated mice were collected and single cell suspensions prepared in R10. ELISPOTs were performed using an available kit: mouse IFNγ ELISPOT set (BD Pharmingen) according to manufactures guidelines. All buffers are given in Appendix D. Briefly, ELISPOT plates were coated with a capture anti-mouse IFNγ antibody overnight before being blocked for 2 hours with R10. Viable T cells were selected by density centrifugation and incubated for 24 hours at $4x10^5$/well and 37°C with indicated concentrations of peptide in triplicate. Cells were removed and plates were washed and incubated with biotinylated anti-mouse IFNγ overnight at 4°C. Plates were washed and incubated with Streptavidin ALP-PQ (MABTECH AB, diluted 1/500) for 1 hour at room temperature. After the final washing steps, plates were incubated with substrate solution and spot development was monitored, the reaction was stopped and after drying the plate was read using the Autoimmune Diagnostika (AID) analyser, software version 3.1 (Autoimmun Diagnostika, Strassberg, Germany).

## 5.16 Measurement of anti-MUC1 and anti FrC antibody response to DNA vaccination

### 5.16.1 Anti-MUC1 ELISA

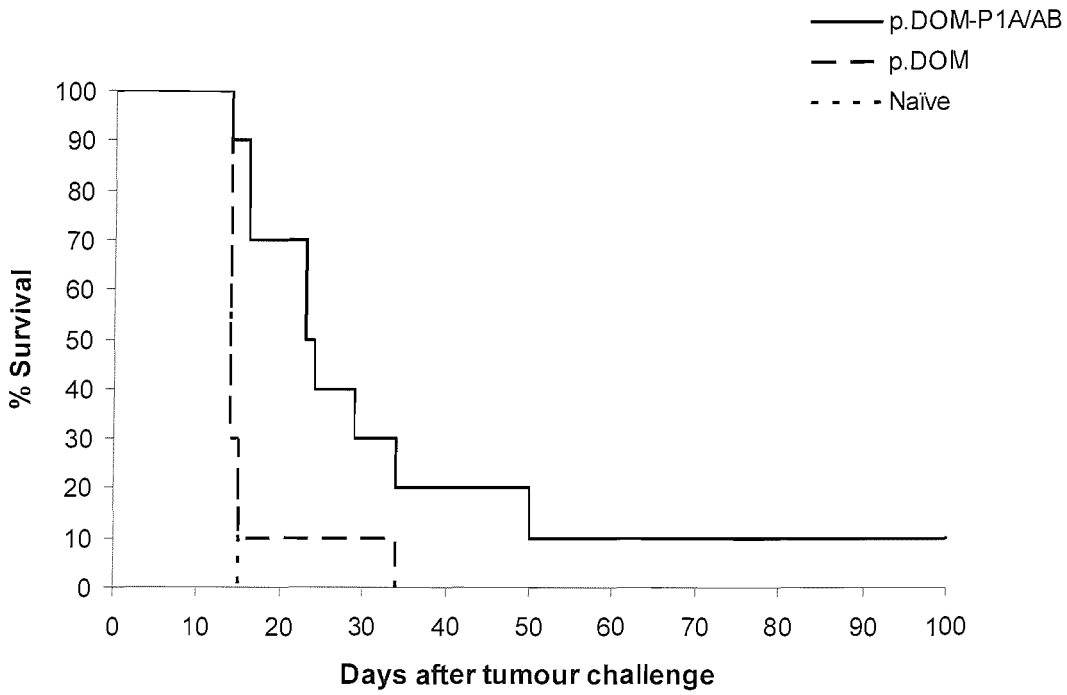
ELISA plates (Nunc immunoplate II, Invitrogen) were coated with streptavidin (Sigma) at 2μg/ml in coating buffer (200μl/well) for 1 hour at 37°C. All buffers are given in Appendix D. Plates were blocked by incubation with PBS/1%BSA (200μl/well) overnight at 4°C.

Plates were washed twice with PBS/1%BSA before addition of biotinylated MUC1 24-mer peptide (TAPPAHGVTSAPDTRPAPGSTAPP, kindly provided by Dr. J. Taylor-Papadimitriou) at a concentration of 1μg/ml in PBS/Tween (200μl/well) and incubated for 1 hour at 37°C. Plates were washed 4 times. Serum samples were collected and serially diluted in PBS/Tween and plated for 90 minutes at 37°C. Plates were washed 4 times before the addition of peroxidase conjugated sheep anti-mouse Fcγ (The Binding Site, Birmingham, UK) in PBS/Tween. Plates were incubated for 1 hour at 37°C and washed 4 times. 200μl/well of fresh substrate buffer was added and the colour reaction allowed to develop. The reaction was stopped by the addition of 2.5M $H_2SO_4$ (80μl/well) and the optical density read on an automatic ELISA reader (Dynatech Instruments Inc., Santa Monica, USA) at 495nm. A HMFG1 standard of known concentration was used as a control.

### 5.16.2 Anti-FrC ELISA

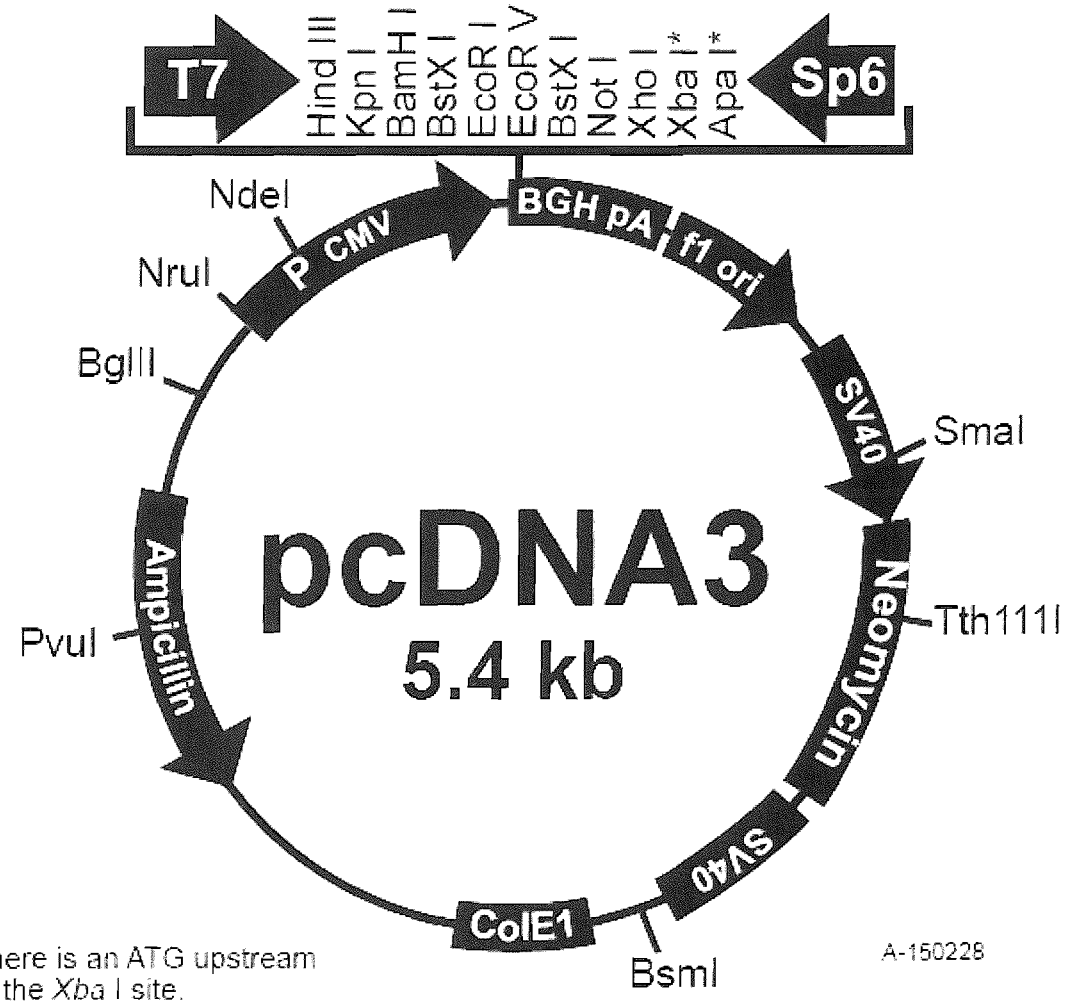
ELISA plates (Nunc immunoplate II, Invitrogen) were coated with a standard concentration (2μg/ml) of FrC protein antigen (Yeast expression and protein purification was performed in house by Dr. R. Snow or Miss K. McCann) in coating buffer (200μl/well) and incubated overnight at 4°C. All buffers are given in Appendix D. Plates were blocked by incubation with PBS/1%BSA (200μl/well) for 1 hour at 37°C and then washed once with PBS/Tween. Serum samples were collected at day 28 and diluted in PBS/Tween and plated for 90 minutes at 37°C. Plates were washed 4 times before the addition of peroxidase conjugated sheep anti-mouse Fcγ (The Binding Site) in PBS/Tween. A further 4 washes were performed before the addition of fresh substrate buffer. The reaction was allowed to develop and stopped and the optical density read as described above. A standardised anti-FrC control was used in every assay.

## 6.1 Appendix A. DNA vaccination with p.DOM-P1A/AB generates significant protective immunity against tumour challenge




10 Mice per group were vaccinated on days 0 and 21 with either p.DOM-P1A/AB or p.DOM or were untreated (naïve). Intra-peritoneal injection of $1x10^4$ P511 tumour cells was performed on day 28 and mice were sacrificed when ascitic burden reached 20% original weight of the mouse. Data from the second of two experiments is shown. Vaccination with p.DOM-P1A/AB induced significant delay in tumour growth compared to p.DOM vaccinated controls (p=0.0082, $\chi^2$ Logrank test, p.DOM-P1A/AB vs. p.DOM).

## 6.2 Appendix B. Vector map of pcDNA3




* There is an ATG upstream of the *Xba* I site.

## 6.3 Appendix C. Primer sequences

Table 1 – Vaccine Assembly Primers

| Vaccine | Forward Primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| pDOM-MUC1peptide1 | **T7**-TAATACGACTCACTATAGGG | TTTTGCGGCCGCTTACGGGGCCGGCCTGGTGTCCGGGGCCGAGTTACCCCAGAAGTCACGCAGGAAG |
| pDOM-peptide1TR | **T7**-TAATACGACTCACTATAGGG | TTTTGCGGCCGCTTAGGCCGGCCTGGTGTCCGGGGCCGAGTTACCCCCAGAAGTCACGCAGGAAG |
| pDOM-MUC1peptide2 | **T7**-TAATACGACTCACTATAGGG | TTTTGCGGCCGCTTACAAGGCGGGCCTGTTGTCCGGGGCCGAGTTACCCCAGAAGTCACGCAGGAAG |
| pDOM-MUC1peptide3 | **T7**-TAATACGACTCACTATAGGG | TTTTGCGGCCGCTTAGACACCGTGGGCTGGGGGGGCGGTGGAGTTACCCCAGAAGTCACGCAGGAAG |
| pDOM-MUC1peptide4 | **T7**-TAATACGACTCACTATAGGG | TTTTGCGGCCGCTTAAGCATGTCCACTTCCGGTAACAACGGTGTTACCCCAGAAGTCACGCAGGAAG |
| pDOM-S9V | **T7**-TAATACGACTCACTATAGGG | TTTTGCGGCCGCTTAGACTCCATGAGCAGGAGGATGGGTGCTGTTACCCCAGAAGTCACGCAGGAAG |
| pDOM-A9V | **T7**-TAATACGACTCACTATAGGG | TTTTGCGGCCGCTTAGACTGGAGGGGCGGTGGAGCCCAAGGCGTTACCCCAGAAGTCACGCAGGAAG |
| pDOM-T9A | **T7**-TAATACGACTCACTATAGGG | TTTTGCGGCCGCTTAAGCTGGTTCCGTGGCCGGGGCCAGAGTGTTACCCCAGAAGTCACGCAGGAAG |
| pDOM-L9V | **T7**-TAATACGACTCACTATAGGG | TTTTGCGGCCGCTTAGACGGTGAGAACGGTAAGAAGAAGAAGGTTACCCCAGAAGTCACGCAGGAAG |
| pDOM-P1A/AB | **T7**-TAATACGACTCACATTAGGG | TTTTGCGGCCGCTTAGAAGACCAGCCACCCTAGATAAGGCAGGTTACCCCAGAAGTCACGCAGGAAG |

Table 1 continued

| | | |
|---|---|---|
| pDOM-P1A/ABlong | **T7**-T AATACGACTCACATTAGGG | TTTTGCGGCCGCTTAAGCGAAGACCAGCCACCCTAGATAAGGCAGAATT TCGTTACCCCAGAAGTCACGCAGGAAG |
| pDOM-P1E | **T7**-TAATACGACTCACATTAGGG | TTTTGCGGCCGCTTAAACTCCAGTACCCCTGAGCCACAGTAGCCGTTAC CCCCAGAAGTCACGCAGGAAG |
| pP1A+L | **P1AFrC/5p1**-TTTTAAGCTTGCCGCCACCATGGGTTGGAGCTGTATCATCT TCTTTCTGGTAGCAACAGCTACAGGTGTGCACTCCATGTCTGATAACAA GAAACCAGACAAAGCC | **P1A+LR**-TTTTGCGGCCGCTTAAGGTGAGAAGCCATCCGGGTTTCC |
| pP1A-FrC/DOM (step 1) | **P1AFrC/5p1**-TTTTAAGCTTGCCGCCACCATGGGTTGGAGCTGTATCATCT TCTTTCTGGTAGCAACAGCTACAGGTGTGCACTCCATGTCTGATAACAA GAAACCAGACAAAGCC | **P1AFrC/3p1**-TTCGTTGTCGACCCAACAATCAAGGTTTTTAGGTCCGGGT CCAGGTGAGAAGCCATCCATCCGGGTTTCCCATTTCCTC |
| pP1A-FrC (step 2) | **FrCP1A/5p1**-GAGGAAATGGGAAACCCGGATGGCTTCTCACCTGGACCCG GACCTAAAAACCTTGATTGTTGGGTCGACAACGAA | **FCAS2**-TTTTGCGGCCGCTTAGTCGTTGGTCCAACCTTCATCGGTCGG |
| pP1A-DOM (step 2) | **FrCP1A/5p1**-GAGGAAATGGGAAACCCGGATGGCTTCTCACCTGGACCCG GACCTAAAAACCTTGATTGTTGGGTCGACAACGAA | **DOM1P1A**-TTTTGCGGCCGCTTAGTTACCCCAGAAGTCACGCAGGAAGG TGAT |
| pP1A-FrC (step 3) | **P1AFrC/5p1**-TTTTAAGCTTGCCGCCACCATGGGTTGGAGCTGTATCATCT TCTTTCTGGTAGCAACAGCTACAGGTGTGCACTCCATGTCTGATAACAA GAAACCAGACAAAGCC | **FCAS2**-TTTTGCGGCCGCTTAGTCGTTGGTCCAACCTTCATCGGTCGG |
| pP1A-DOM (step 3) | **P1AFrC/5p1**-TTTTAAGCTTGCCGCCACCATGGGTTGGAGCTGTATCATCT TCTTTCTGGTAGCAACAGCTACAGGTGTGCACTCCATGTCTGATAACAA GAAACCAGACAAAGCC | **DOM1P1A**-TTTTGCGGCCGCTTAGTTACCCCAGAAGTCACGCAGGAAGG TGAT |
| pAB + L | **T7**-TAATACGACTCACTATAGGG | **Abmini+LR** – TTTTGCGGCCGCTTAGAAGACCAGCCACCCTAGATAAGGC AGGGAGTGCACACCTGTAGC |
| pABlong+L | **T7**-TAATACGACTCACTATAGGG | **ABlongminiR** –TTTTGCGGCCGCTTAAGCGAAGACCAGCCTCCCTAGATA AGGCAGAATTTCGGAGTGCACACCTGTAGC |

Key to table 1 Underlined = *HindII* (GCGGCCGC) or *Not I* (AAGCTT) restriction enzyme recognition site
Red = Stop codon
Blue = Epitope sequence
Green = FrC
Purple = P1A

Table 2 – Sequencing primers

| Primer | |
|---|---|
| T7 | 5'-TAATACGACTCACTATAGGG |
| SP6 | 5'-ATTTAGGTGACACTATAGAA |
| pDomseqF1 | 5'-TAACGAGTACTCCATCA |
| pDomseqR1 | 5'-AAACAGACCAACCAGAGC |
| ABseqF1 | 5'-CAAGCGCATGTCCTCTGTCGATGA |
| ABseqR1 | 5'-TGGCCAGGAACACAGGCACAGTTA |

Table 3 - PCR primers used to amplify murine CTA from total RNA.

| Primer pair | Forward | Reverse |
|---|---|---|
| Mage a 5p2 and 3p2 | 5'-AGTCCTCCCCAGAGTCCTCAGAGA | 5'-SCCATCATAGGTGAKCCCCAGGGC |
| MA2/5P1/E3 and MA2/3P1/E4 | 5'-AAAATCTCCTAAAGCAGAGTTTGAC | 5'-GTCAAGGCCAAAGACCATCTTCA |
| MA5/5P1/E2 and MA5/3P1/E3 | 5'-GCAGAGTTTGACTAGAGTCATCA | 5'-CAGAAAGTCCACCAAGTCATACA |
| Bmage5' and Bmage3' | 5'-GTTCAGCCCACTGCAGAGGAAGCA | 5'-TTCTAGGCGTGCAGAAGTTCTCCT |
| Mage b3/5' and Mage b3/3' | 5'-ATGCCTAGGGGTCAAAAGAGTAAGA | 5'-CTACACATTAGAGGACTTTTGGGATGG |
| β-actin F and β-actin R | 5'-TGGAATCCTGTGGCATCC | 5'-TAAAACGCAGCTCAGTAACA |

Table 4 - PCR primers used to amplify murine P1A and P1E and from total RNA.

| Primer pair | Forward | Reverse |
|---|---|---|
| P1A5P1 and P1A3P2 | 5'-ATGTCTGATAACAAGAAACCA | 5'-CTAAGGTGAGAAGCCAT |
| P815EF1 and P815ER1 | 5'-ATCAGCTTTGAGGAACTGCTCA | 5'-CCGATGGCCATTAAAAAATAA |

## 6.4 Appendix D. Buffers and reagents

**PBS (Phosphate Buffered Saline)**
NaCl - 7.04g/l
$Na_2HPO_4$ - 3.44g/l
$KH_2PO_4$ - 0.79g/l
$dH_2O$ - 1000ml
pH 7.3

**PBS/Tween**
1% - PBS plus 1ml Tween 20 per litre
0.5% - PBS plus 500µl Tween 20 per litre

**PBS/BSA**
PBS plus 1g BSA per litre

**ELISA-coating buffer**
Na2CO3 - 1.58g/l
NaHCO3 - 1.92g/l
dH2O - 1000ml
pH 9.5

**ELISA-substrate buffer**
Citric acid - 4.68g/l
Na2HPO4 - 7.30g/l
dH2O - 1000ml
To 50ml add 10mg orthophenyldiamine (OPD) and 50µl H2O2 immediately before use.

# 7 References


1. Arbuthnot, P., and M. Kew. 2001. Hepatitis B virus and hepatocellular carcinoma. *Int J Exp Pathol 82:77.*

2. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu Rev Immunol 21:335.*

3. Oshimi, Y., S. Oda, Y. Honda, S. Nagata, and S. Miyazaki. 1996. Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. *J Immunol 157:2909.*

4. Mori, S., A. Jewett, K. Murakami-Mori, M. Cavalcanti, and B. Bonavida. 1997. The participation of the Fas-mediated cytotoxic pathway by natural killer cells is tumor-cell-dependent. *Cancer Immunol Immunother 44:282.*

5. Shresta, S., D. M. MacIvor, J. W. Heusel, J. H. Russell, and T. J. Ley. 1995. Natural killer and lymphokine-activated killer cells require granzyme B for the rapid induction of apoptosis in susceptible target cells. *Proc Natl Acad Sci U S A 92:5679.*

6. Baxevanis, C. N., I. F. Voutsas, O. E. Tsitsilonis, M. L. Tsiatas, A. D. Gritzapis, and M. Papamichail. 2000. Compromised anti-tumor responses in tumor necrosis factor-alpha knockout mice. *Eur J Immunol 30:1957.*

7. Papamichail, M., S. A. Perez, A. D. Gritzapis, and C. N. Baxevanis. 2004. Natural killer lymphocytes: biology, development, and function. *Cancer Immunol Immunother 53:176.*

8. Groh, V., R. Rhinehart, H. Secrist, S. Bauer, K. H. Grabstein, and T. Spies. 1999. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci U S A 96:6879.*

9. Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu. 1997. DNA vaccines. *Annu Rev Immunol 15:617.*

10. Kjer-Nielsen, L., C. S. Clements, A. G. Brooks, A. W. Purcell, J. McCluskey, and J. Rossjohn. 2002. The 1.5 A crystal structure of a highly selected antiviral T cell receptor provides evidence for a structural basis of immunodominance. *Structure (Camb) 10:1521.*

11. Bogue, M., and D. B. Roth. 1996. Mechanism of V(D)J recombination. *Curr Opin Immunol 8:175.*

12. Germain, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol 2:309.*

13. Petrie, H. T., F. Livak, D. G. Schatz, A. Strasser, I. N. Crispe, and K. Shortman. 1993. Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes. *J Exp Med 178:615.*

14. Fowlkes, B. J., and E. Schweighoffer. 1995. Positive selection of T cells. *Curr Opin Immunol 7:188.*

15. Goldrath, A. W., and M. J. Bevan. 1999. Selecting and maintaining a diverse T-cell repertoire. *Nature 402:255.*

16. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science 298:1395.*

17. Su, M. A., and M. S. Anderson. 2004. Aire: an update. *Curr Opin Immunol 16:746.*

18. Hillig, R. C., P. G. Coulie, V. Stroobant, W. Saenger, A. Ziegler, and M. Hulsmeyer. 2001. High-resolution structure of HLA-A*0201 in complex with a tumour-specific antigenic peptide encoded by the MAGE-A4 gene. *J Mol Biol 310:1167.*

19. Kastrup, I. B., S. Stevanovic, G. Arsequell, G. Valencia, J. Zeuthen, H. G. Rammensee, T. Elliott, and J. S. Haurum. 2000. Lectin purified human class I MHC-derived peptides: evidence for presentation of glycopeptides in vivo. *Tissue Antigens 56:129.*

20. Andersen, M. H., L. Tan, I. Sondergaard, J. Zeuthen, T. Elliott, and J. S. Haurum. 2000. Poor correspondence between predicted and experimental binding of peptides to class I MHC molecules. *Tissue Antigens 55:519.*

21. Dupuis, M., K. Denis-Mize, C. Woo, C. Goldbeck, M. J. Selby, M. Chen, G. R. Otten, J. B. Ulmer, J. J. Donnelly, G. Ott, and D. M. McDonald. 2000. Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice. *J Immunol 165:2850.*

22. Davis, H. L., C. L. Millan, and S. C. Watkins. 1997. Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA. *Gene Ther 4:181.*

23. Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. Falo, Jr. 1996. DNA-based immunization by in vivo transfection of dendritic cells. *Nat Med 2:1122.*

24. Corr, M., A. von Damm, D. J. Lee, and H. Tighe. 1999. In vivo priming by DNA injection occurs predominantly by antigen transfer. *J Immunol 163:4721.*

25. Engering, A., T. B. Geijtenbeek, S. J. van Vliet, M. Wijers, E. van Liempt, N. Demaurex, A. Lanzavecchia, J. Fransen, C. G. Figdor, V. Piguet, and Y. van Kooyk. 2002. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol 168:2118.*

26. Rafiq, K., A. Bergtold, and R. Clynes. 2002. Immune complex-mediated antigen presentation induces tumor immunity. *J Clin Invest 110:71.*

27. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*

28. Wong, B. R., R. Josien, S. Y. Lee, B. Sauter, H. L. Li, R. M. Steinman, and Y. Choi. 1997. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med 186:2075.*

29. Coombes, B. K., and J. B. Mahony. 2001. Dendritic cell discoveries provide new insight into the cellular immunobiology of DNA vaccines. *Immunol Lett 78:103.*

30. Schubert, U., L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell, and J. R. Bennink. 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature 404:770.*

31. Kessler, B. M., R. Glas, and H. L. Ploegh. 2002. MHC class I antigen processing regulated by cytosolic proteolysis-short cuts that alter peptide generation. *Mol Immunol 39:171.*

32. Morel, S., F. Levy, O. Burlet-Schiltz, F. Brasseur, M. Probst-Kepper, A. L. Peitrequin, B. Monsarrat, R. Van Velthoven, J. C. Cerottini, T. Boon, J. E. Gairin, and B. J. Van den Eynde. 2000. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. *Immunity 12:107.*

33. Schwarz, K., R. de Giuli, G. Schmidtke, S. Kostka, M. van den Broek, K. B. Kim, C. M. Crews, R. Kraft, and M. Groettrup. 2000. The selective proteasome inhibitors lactacystin and epoxomicin can be used to either up- or down-regulate antigen presentation at nontoxic doses. *J Immunol 164:6147.*

34. Saveanu, L., D. Fruci, and P. van Endert. 2002. Beyond the proteasome: trimming, degradation and generation of MHC class I ligands by auxiliary proteases. *Mol Immunol 39:203.*

35. Shastri, N., T. Serwold, and F. Gonzalez. 1995. Presentation of endogenous peptide/MHC class I complexes is profoundly influenced by specific C-terminal flanking residues. *J Immunol 155:4339.*

36. Gromme, M., and J. Neefjes. 2002. Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. *Mol Immunol 39:181.*

37. Antoniou, A. N., S. J. Powis, and T. Elliott. 2003. Assembly and export of MHC class I peptide ligands. *Curr Opin Immunol 15:75.*

38. Purcell, A. W. 2000. The peptide-loading complex and ligand selection during the assembly of HLA class I molecules. *Mol Immunol 37:483.*

39. Williams, A. P., C. A. Peh, A. W. Purcell, J. McCluskey, and T. Elliott. 2002. Optimization of the MHC class I peptide cargo is dependent on tapasin. *Immunity 16:509.*

40. Wilkinson, B. M., M. Regnacq, and C. J. Stirling. 1997. Protein translocation across the membrane of the endoplasmic reticulum. *J Membr Biol 155:189.*

41. Minev, B. R., F. L. Chavez, B. M. Dudouet, and M. S. Mitchell. 2000. Synthetic insertion signal sequences enhance MHC class I presentation of a peptide from the melanoma antigen MART-1. *Eur J Immunol 30:2115.*

42. Norbury, C. C., M. F. Princiotta, I. Bacik, R. R. Brutkiewicz, P. Wood, T. Elliott, J. R. Bennink, and J. W. Yewdell. 2001. Multiple antigen-specific processing pathways for activating naive CD8+ T cells in vivo. *J Immunol 166:4355.*

43. Snyder, H. L., I. Bacik, J. W. Yewdell, T. W. Behrens, and J. R. Bennink. 1998. Promiscuous liberation of MHC-class I-binding peptides from the C termini of membrane and soluble proteins in the secretory pathway. *Eur J Immunol 28:1339.*

44. Pilon, M., R. Schekman, and K. Romisch. 1997. Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *Embo J 16:4540.*

45. Fineschi, B., and J. Miller. 1997. Endosomal proteases and antigen processing. *Trends Biochem Sci 22:377.*

46. Cresswell, P. 1996. Invariant chain structure and MHC class II function. *Cell 84:505.*

47. Hiltbold, E. M., and P. A. Roche. 2002. Trafficking of MHC class II molecules in the late secretory pathway. *Curr Opin Immunol 14:30.*

48. Amigorena, S., P. Webster, J. Drake, J. Newcomb, P. Cresswell, and I. Mellman. 1995. Invariant chain cleavage and peptide loading in major histocompatibility complex class II vesicles. *J Exp Med 181:1729.*

49. Turley, S. J., K. Inaba, W. S. Garrett, M. Ebersold, J. Unternaehrer, R. M. Steinman, and I. Mellman. 2000. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science 288:522.*

50. Katz, J. F., C. Stebbins, E. Appella, and A. J. Sant. 1996. Invariant chain and DM edit self-peptide presentation by major histocompatibility complex (MHC) class II molecules. *J Exp Med 184:1747.*

51. Brocke, P., N. Garbi, F. Momburg, and G. J. Hammerling. 2002. HLA-DM, HLA-DO and tapasin: functional similarities and differences. *Curr Opin Immunol 14:22.*

52. Pathak, S. S., J. D. Lich, and J. S. Blum. 2001. Cutting edge: editing of recycling class II:peptide complexes by HLA-DM. *J Immunol 167:632.*

53. Ramachandra, L., and C. V. Harding. 2000. Phagosomes acquire nascent and recycling class II MHC molecules but primarily use nascent molecules in phagocytic antigen processing. *J Immunol 164:5103.*

54. Pinet, V. M., and E. O. Long. 1998. Peptide loading onto recycling HLA-DR molecules occurs in early endosomes. *Eur J Immunol 28:799.*

55. Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med 143:1283.*

56. Zinkernagel, R. M. 2002. On cross-priming of MHC class I-specific CTL: rule or exception? *Eur J Immunol 32:2385.*

57. Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med 184:923.*

58. Sigal, L. J., S. Crotty, R. Andino, and K. L. Rock. 1999. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature 398:77.*

59. Norbury, C. C., D. Malide, J. S. Gibbs, J. R. Bennink, and J. W. Yewdell. 2002. Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo. *Nat Immunol 3:265.*

60. Brossart, P., and M. J. Bevan. 1997. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood 90:1594.*

61. Inaba, K., M. Inaba, M. Naito, and R. M. Steinman. 1993. Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. *J Exp Med 178:479.*

62. Harshyne, L. A., S. C. Watkins, A. Gambotto, and S. M. Barratt-Boyes. 2001. Dendritic cells acquire antigens from live cells for cross-presentation to CTL. *J Immunol 166:3717.*

63. Wolfers, J., A. Lozier, G. Raposo, A. Regnault, C. Thery, C. Masurier, C. Flament, S. Pouzieux, F. Faure, T. Tursz, E. Angevin, S. Amigorena, and L. Zitvogel. 2001. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med 7:297.*

64. Srivastava, P. 2002. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu Rev Immunol 20:395.*

65. Schuurhuis, D. H., A. Ioan-Facsinay, B. Nagelkerken, J. J. van Schip, C. Sedlik, C. J. Melief, J. S. Verbeek, and F. Ossendorp. 2002. Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses in vivo. *J Immunol 168:2240.*

66. Rodriguez, A., A. Regnault, M. Kleijmeer, P. Ricciardi-Castagnoli, and S. Amigorena. 1999. Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat Cell Biol 1:362.*

67. Guermonprez, P., L. Saveanu, M. Kleijmeer, J. Davoust, P. Van Endert, and S. Amigorena. 2003. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature 425:397.*

68. Houde, M., S. Bertholet, E. Gagnon, S. Brunet, G. Goyette, A. Laplante, M. F. Princiotta, P. Thibault, D. Sacks, and M. Desjardins. 2003. Phagosomes are competent organelles for antigen cross-presentation. *Nature 425:402.*

69. Gagnon, E., S. Duclos, C. Rondeau, E. Chevet, P. H. Cameron, O. Steele-Mortimer, J. Paiement, J. J. Bergeron, and M. Desjardins. 2002. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell 110:119.*

70. Ackerman, A. L., C. Kyritsis, R. Tampe, and P. Cresswell. 2003. Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc Natl Acad Sci U S A 100:12889.*

71. Sigal, L. J., and K. L. Rock. 2000. Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation. *J Exp Med 192:1143.*

72. Lizee, G., G. Basha, J. Tiong, J. P. Julien, M. Tian, K. E. Biron, and W. A. Jefferies. 2003. Control of dendritic cell cross-presentation by the major histocompatibility complex class I cytoplasmic domain. *Nat Immunol 4:1065.*

73. den Haan, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med 192:1685.*

74. Pooley, J. L., W. R. Heath, and K. Shortman. 2001. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. *J Immunol 166:5327.*

75. Ochsenbein, A. F., S. Sierro, B. Odermatt, M. Pericin, U. Karrer, J. Hermans, S. Hemmi, H. Hengartner, and R. M. Zinkernagel. 2001. Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. *Nature 411:1058.*

76. Melief, C. J. 2003. Mini-review: Regulation of cytotoxic T lymphocyte responses by dendritic cells: peaceful coexistence of cross-priming and direct priming? *Eur J Immunol 33:2645.*

77. Wolkers, M. C., G. Stoetter, F. A. Vyth-Dreese, and T. N. Schumacher. 2001. Redundancy of direct priming and cross-priming in tumor-specific CD8+ T cell responses. *J Immunol 167:3577.*

78. Geijtenbeek, T. B., R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, G. J. Adema, Y. van Kooyk, and C. G. Figdor. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell 100:575.*

79. van Kooyk, Y., and T. B. Geijtenbeek. 2002. A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions. *Immunol Rev 186:47.*

80. Oh, S., J. W. Hodge, J. D. Ahlers, D. S. Burke, J. Schlom, and J. A. Berzofsky. 2003. Selective induction of high avidity CTL by altering the balance of signals from APC. *J Immunol 170:2523.*

81. Carreno, B. M., and M. Collins. 2002. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu Rev Immunol 20:29.*

82. Huang, J., P. F. Lo, T. Zal, N. R. Gascoigne, B. A. Smith, S. D. Levin, and H. M. Grey. 2002. CD28 plays a critical role in the segregation of PKC theta within the immunologic synapse. *Proc Natl Acad Sci U S A 99:9369.*

83. Brodie, D., A. V. Collins, A. Iaboni, J. A. Fennelly, L. M. Sparks, X. N. Xu, P. A. van der Merwe, and S. J. Davis. 2000. LICOS, a primordial costimulatory ligand? *Curr Biol 10:333.*

84. Gramaglia, I., A. D. Weinberg, M. Lemon, and M. Croft. 1998. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol 161:6510.*

85. Ohshima, Y., Y. Tanaka, H. Tozawa, Y. Takahashi, C. Maliszewski, and G. Delespesse. 1997. Expression and function of OX40 ligand on human dendritic cells. *J Immunol 159:3838.*

86. Gramaglia, I., A. Jember, S. D. Pippig, A. D. Weinberg, N. Killeen, and M. Croft. 2000. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J Immunol 165:3043.*

87. Rogers, P. R., J. Song, I. Gramaglia, N. Killeen, and M. Croft. 2001. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity 15:445.*

88. Croft, M. 2003. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol 3:609.*

89. Wen, T., J. Bukczynski, and T. H. Watts. 2002. 4-1BB ligand-mediated costimulation of human T cells induces CD4 and CD8 T cell expansion, cytokine production, and the development of cytolytic effector function. *J Immunol 168:4897.*

90. Halstead, E. S., Y. M. Mueller, J. D. Altman, and P. D. Katsikis. 2002. In vivo stimulation of CD137 broadens primary antiviral CD8+ T cell responses. *Nat Immunol 3:536.*

91. Hendriks, J., L. A. Gravestein, K. Tesselaar, R. A. van Lier, T. N. Schumacher, and J. Borst. 2000. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol 1:433.*

92. Zhang, L., Y. Tang, H. Akbulut, D. Zelterman, P. J. Linton, and A. B. Deisseroth. 2003. An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells. *Proc Natl Acad Sci U S A 100:15101.*

93. Swain, S. L., L. M. Bradley, M. Croft, S. Tonkonogy, G. Atkins, A. D. Weinberg, D. D. Duncan, S. M. Hedrick, R. W. Dutton, and G. Huston. 1991. Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development. *Immunol Rev 123:115.*

94. Gor, D. O., N. R. Rose, and N. S. Greenspan. 2003. TH1-TH2: a procrustean paradigm. *Nat Immunol 4:503.*

95. Keene, J. A., and J. Forman. 1982. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *J Exp Med 155:768.*

96. Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. *J Exp Med 180:1263.*

97. Ahlers, J. D., I. M. Belyakov, E. K. Thomas, and J. A. Berzofsky. 2001. High-affinity T helper epitope induces complementary helper and APC polarization, increased CTL, and protection against viral infection. *J Clin Invest 108:1677.*

98. Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med 184:747.*

99. Valenzuela, J., C. Schmidt, and M. Mescher. 2002. The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. *J Immunol 169:6842.*

100. Curtsinger, J. M., C. S. Schmidt, A. Mondino, D. C. Lins, R. M. Kedl, M. K. Jenkins, and M. F. Mescher. 1999. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol 162:3256.*

101. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature 393:474.*

102. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science 297:2060.*

103. Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature 393:480.*

104. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478.*

105. Wu, Y., and Y. Liu. 1994. Viral induction of co-stimulatory activity on antigen-presenting cells bypasses the need for CD4+ T-cell help in CD8+ T-cell responses. *Curr Biol 4:499.*

106. Kaech, S. M., and R. Ahmed. 2003. Immunology. CD8 T cells remember with a little help. *Science 300:263.*

107. Guerder, S., and P. Matzinger. 1992. A fail-safe mechanism for maintaining self-tolerance. *J Exp Med 176:553.*

108. Franco, A., D. A. Tilly, I. Gramaglia, M. Croft, L. Cipolla, M. Meldal, and H. M. Grey. 2000. Epitope affinity for MHC class I determines helper requirement for CTL priming. *Nat Immunol 1:145.*

109. Mintern, J. D., G. M. Davey, G. T. Belz, F. R. Carbone, and W. R. Heath. 2002. Cutting edge: precursor frequency affects the helper dependence of cytotoxic T cells. *J Immunol 168:977.*

110. Bourgeois, C., H. Veiga-Fernandes, A. M. Joret, B. Rocha, and C. Tanchot. 2002. CD8 lethargy in the absence of CD4 help. *Eur J Immunol 32:2199.*

111. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature 421:852.*

112. Johansen, P., P. Stamou, R. E. Tascon, D. B. Lowrie, and B. Stockinger. 2004. CD4 T cells guarantee optimal competitive fitness of CD8 memory T cells. *Eur J Immunol 34:91.*

113. Creusot, R. J., L. L. Thomsen, J. P. Tite, and B. M. Chain. 2003. Local cooperation dominates over competition between CD4+ T cells of different antigen/MHC specificity. *J Immunol 171:240.*

114. Schuler, T., and T. Blankenstein. 2002. Naive CD8(+) but not CD4(+) T cells induce maturation of dendritic cells. *J Mol Med 80:533.*

115. Mailliard, R. B., S. Egawa, Q. Cai, A. Kalinska, S. N. Bykovskaya, M. T. Lotze, M. L. Kapsenberg, W. J. Storkus, and P. Kalinski. 2002. Complementary dendritic cell-activating function of CD8+ and CD4+ T cells: helper role of CD8+ T cells in the development of T helper type 1 responses. *J Exp Med 195:473.*

116. Bishop, G. A., and B. S. Hostager. 2001. B lymphocyte activation by contact-mediated interactions with T lymphocytes. *Curr Opin Immunol 13:278.*

117. Stavnezer, J. 1996. Immunoglobulin class switching. *Curr Opin Immunol 8:199.*

118. Yewdell, J. W., and J. R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu Rev Immunol 17:51.*

119. Grufman, P., E. Z. Wolpert, J. K. Sandberg, and K. Karre. 1999. T cell competition for the antigen-presenting cell as a model for immunodominance in the cytotoxic T lymphocyte response against minor histocompatibility antigens. *Eur J Immunol 29:2197.*

120. Gileadi, U., H. T. Moins-Teisserenc, I. Correa, B. L. Booth, Jr., P. R. Dunbar, A. K. Sewell, J. Trowsdale, R. E. Phillips, and V. Cerundolo. 1999. Generation of an immunodominant CTL epitope is affected by proteasome subunit composition and stability of the antigenic protein. *J Immunol 163:6045.*

121. Mo, A. X., S. F. van Lelyveld, A. Craiu, and K. L. Rock. 2000. Sequences that flank subdominant and cryptic epitopes influence the proteolytic generation of MHC class I-presented peptides. *J Immunol 164:4003.*

122. Serwold, T., and N. Shastri. 1999. Specific proteolytic cleavages limit the diversity of the pool of peptides available to MHC class I molecules in living cells. *J Immunol 162:4712.*

123. Yellen-Shaw, A. J., C. E. Laughlin, R. M. Metrione, and L. C. Eisenlohr. 1997. Murine transporter associated with antigen presentation (TAP) preferences influence class I-restricted T cell responses. *J Exp Med 186:1655.*

124. Pion, S., G. J. Christianson, P. Fontaine, D. C. Roopenian, and C. Perreault. 1999. Shaping the repertoire of cytotoxic T-lymphocyte responses: explanation for the immunodominance effect whereby cytotoxic T lymphocytes specific for immunodominant antigens prevent recognition of nondominant antigens. *Blood 93:952.*

125. Palmowski, M. J., E. M. Choi, I. F. Hermans, S. C. Gilbert, J. L. Chen, U. Gileadi, M. Salio, A. Van Pel, S. Man, E. Bonin, P. Liljestrom, P. R. Dunbar, and V. Cerundolo. 2002. Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. *J Immunol 168:4391.*

126. Kedl, R. M., B. C. Schaefer, J. W. Kappler, and P. Marrack. 2002. T cells down-modulate peptide-MHC complexes on APCs in vivo. *Nat Immunol 3:27.*

127. Roy-Proulx, G., M. C. Meunier, A. M. Lanteigne, S. Brochu, and C. Perreault. 2001. Immunodomination results from functional differences between competing CTL. *Eur J Immunol 31:2284.*

128. Kedl, R. M., J. W. Kappler, and P. Marrack. 2003. Epitope dominance, competition and T cell affinity maturation. *Curr Opin Immunol 15:120.*

129. Griffiths, G. M. 1995. The cell biology of CTL killing. *Curr Opin Immunol 7:343.*

130. Shi, L., S. Mai, S. Israels, K. Browne, J. A. Trapani, and A. H. Greenberg. 1997. Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. *J Exp Med 185:855.*

131. Shresta, S., T. A. Graubert, D. A. Thomas, S. Z. Raptis, and T. J. Ley. 1999. Granzyme A initiates an alternative pathway for granule-mediated apoptosis. *Immunity 10:595.*

132. Beresford, P. J., Z. Xia, A. H. Greenberg, and J. Lieberman. 1999. Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. *Immunity 10:585.*

133. Andrade, F., S. Roy, D. Nicholson, N. Thornberry, A. Rosen, and L. Casciola-Rosen. 1998. Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. *Immunity 8:451.*

134. Talanian, R. V., X. Yang, J. Turbov, P. Seth, T. Ghayur, C. A. Casiano, K. Orth, and C. J. Froelich. 1997. Granule-mediated killing: pathways for granzyme B-initiated apoptosis. *J Exp Med 186:1323.*

135. Vignaux, F., E. Vivier, B. Malissen, V. Depraetere, S. Nagata, and P. Golstein. 1995. TCR/CD3 coupling to Fas-based cytotoxicity. *J Exp Med 181:781.*

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

137. Lundin, K. U., P. O. Hofgaard, H. Omholt, L. A. Munthe, A. Corthay, and B. Bogen. 2003. Therapeutic effect of idiotype-specific CD4+ T cells against B-cell lymphoma in the absence of anti-idiotypic antibodies. *Blood 102:605.*

138. Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky. 1998. The central role of CD4(+) T cells in the antitumor immune response. *J Exp Med 188:2357.*

139. Beatty, G., and Y. Paterson. 2001. IFN-gamma-dependent inhibition of tumor angiogenesis by tumor-infiltrating CD4+ T cells requires tumor responsiveness to IFN-gamma. *J Immunol 166:2276.*

140. Qin, Z., and T. Blankenstein. 2000. CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. *Immunity 12:677.*

141. Mumberg, D., P. A. Monach, S. Wanderling, M. Philip, A. Y. Toledano, R. D. Schreiber, and H. Schreiber. 1999. CD4(+) T cells eliminate MHC class II-negative cancer cells in vivo by indirect effects of IFN-gamma. *Proc Natl Acad Sci U S A 96:8633.*

142. Nakajima, C., Y. Uekusa, M. Iwasaki, N. Yamaguchi, T. Mukai, P. Gao, M. Tomura, S. Ono, T. Tsujimura, H. Fujiwara, and T. Hamaoka. 2001. A role of interferon-gamma (IFN-gamma) in tumor immunity: T cells with the capacity to reject tumor cells are generated but fail to migrate to tumor sites in IFN-gamma-deficient mice. *Cancer Res 61:3399.*

143. MacMicking, J., Q. W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu Rev Immunol 15:323.*

144. Mattes, J., M. Hulett, W. Xie, S. Hogan, M. E. Rothenberg, P. Foster, and C. Parish. 2003. Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. *J Exp Med 197:387.*

145. Monach, P. A., S. C. Meredith, C. T. Siegel, and H. Schreiber. 1995. A unique tumor antigen produced by a single amino acid substitution. *Immunity 2:45.*

146. Thomas, W. D., and P. Hersey. 1998. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. *J Immunol 161:2195.*

147. Vergelli, M., B. Hemmer, P. A. Muraro, L. Tranquill, W. E. Biddison, A. Sarin, H. F. McFarland, and R. Martin. 1997. Human autoreactive CD4+ T cell clones use perforin- or Fas/Fas ligand-mediated pathways for target cell lysis. *J Immunol 158:2756.*

148. Barber, D. L., E. J. Wherry, and R. Ahmed. 2003. Cutting edge: rapid in vivo killing by memory CD8 T cells. *J Immunol 171:27.*

149. Veiga-Fernandes, H., U. Walter, C. Bourgeois, A. McLean, and B. Rocha. 2000. Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nat Immunol 1:47.*

150. van Stipdonk, M. J., E. E. Lemmens, and S. P. Schoenberger. 2001. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol 2:423.*

151. van Stipdonk, M. J., G. Hardenberg, M. S. Bijker, E. E. Lemmens, N. M. Droin, D. R. Green, and S. P. Schoenberger. 2003. Dynamic programming of CD8+ T lymphocyte responses. *Nat Immunol 4:361.*

152. Gett, A. V., F. Sallusto, A. Lanzavecchia, and J. Geginat. 2003. T cell fitness determined by signal strength. *Nat Immunol 4:355.*

153. Wherry, E. J., M. J. McElhaugh, and L. C. Eisenlohr. 2002. Generation of CD8(+) T cell memory in response to low, high, and excessive levels of epitope. *J Immunol 168:4455.*

154. Hildeman, D. A., Y. Zhu, T. C. Mitchell, J. Kappler, and P. Marrack. 2002. Molecular mechanisms of activated T cell death in vivo. *Curr Opin Immunol 14:354.*

155. Kagi, D., B. Odermatt, and T. W. Mak. 1999. Homeostatic regulation of CD8+ T cells by perforin. *Eur J Immunol 29:3262.*

156. Badovinac, V. P., A. R. Tvinnereim, and J. T. Harty. 2000. Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-gamma. *Science 290:1354.*

157. Van Parijs, L., and A. K. Abbas. 1998. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science 280:243.*

158. Tham, E. L., and M. F. Mescher. 2002. The poststimulation program of CD4 versus CD8 T cells (death versus activation-induced nonresponsiveness). *J Immunol 169:1822.*

159. Homann, D., L. Teyton, and M. B. Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat Med 7:913.*

160. Kaech, S. M., S. Hemby, E. Kersh, and R. Ahmed. 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell 111:837.*

161. Sprent, J., and D. F. Tough. 2001. T cell death and memory. *Science 293:245.*

162. Murali-Krishna, K., L. L. Lau, S. Sambhara, F. Lemonnier, J. Altman, and R. Ahmed. 1999. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science 286:1377.*

163. Becker, T. C., E. J. Wherry, D. Boone, K. Murali-Krishna, R. Antia, A. Ma, and R. Ahmed. 2002. Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J Exp Med 195:1541.*

164. Ku, C. C., M. Murakami, A. Sakamoto, J. Kappler, and P. Marrack. 2000. Control of homeostasis of CD8+ memory T cells by opposing cytokines. *Science 288:675.*

165. Kassiotis, G., S. Garcia, E. Simpson, and B. Stockinger. 2002. Impairment of immunological memory in the absence of MHC despite survival of memory T cells. *Nat Immunol 3:244.*

166. Rocha, B. 2002. Requirements for memory maintenance. *Nat Immunol 3:209.*

167. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:708.*

168. Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol 4:225.*

169. Reinhardt, R. L., A. Khoruts, R. Merica, T. Zell, and M. K. Jenkins. 2001. Visualizing the generation of memory CD4 T cells in the whole body. *Nature 410:101.*

170. Meffre, E., R. Casellas, and M. C. Nussenzweig. 2000. Antibody regulation of B cell development. *Nat Immunol 1:379.*

171. Benschop, R. J., and J. C. Cambier. 1999. B cell development: signal transduction by antigen receptors and their surrogates. *Curr Opin Immunol 11:143.*

172. Carsetti, R. 2000. The development of B cells in the bone marrow is controlled by the balance between cell-autonomous mechanisms and signals from the microenvironment. *J Exp Med 191:5.*

173. Kelsoe, G. 1995. The germinal center reaction. *Immunol Today 16:324.*

174. Guzman-Rojas, L., J. C. Sims-Mourtada, R. Rangel, and H. Martinez-Valdez. 2002. Life and death within germinal centres: a double-edged sword. *Immunology 107:167.*

175. Neuberger, M. S., and C. Milstein. 1995. Somatic hypermutation. *Curr Opin Immunol 7:248.*

176. Stevenson, F., S. Sahota, D. Zhu, C. Ottensmeier, C. Chapman, D. Oscier, and T. Hamblin. 1998. Insight into the origin and clonal history of B-cell tumors as revealed by analysis of immunoglobulin variable region genes. *Immunol Rev 162:247.*

177. Kaufmann, H., E. Urbauer, J. Ackermann, H. Huber, and J. Drach. 2001. Advances in the biology and therapeutic management of multiple myeloma. *Ann Hematol 80:445.*

178. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity 3:541.*

179. Watanabe-Fukunaga, R., C. I. Brannan, N. G. Copeland, N. A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature 356:314.*

180. Malek, T. R., and A. L. Bayer. 2004. Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol 4:665.*

181. Kurts, C., H. Kosaka, F. R. Carbone, J. F. Miller, and W. R. Heath. 1997. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *J Exp Med 186:239.*

182. Heath, W. R., C. Kurts, J. F. Miller, and F. R. Carbone. 1998. Cross-tolerance: a pathway for inducing tolerance to peripheral tissue antigens. *J Exp Med 187:1549.*

183. Lutz, M. B., and G. Schuler. 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol 23:445.*

184. Albert, M. L., M. Jegathesan, and R. B. Darnell. 2001. Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. *Nat Immunol 2:1010.*

185. Adler, A. J., D. W. Marsh, G. S. Yochum, J. L. Guzzo, A. Nigam, W. G. Nelson, and D. M. Pardoll. 1998. CD4+ T cell tolerance to parenchymal self-antigens requires presentation by bone marrow-derived antigen-presenting cells. *J Exp Med 187:1555.*

186. McGuirk, P., and K. H. Mills. 2002. Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. *Trends Immunol 23:450.*

187. Roncarolo, M. G., R. Bacchetta, C. Bordignon, S. Narula, and M. K. Levings. 2001. Type 1 T regulatory cells. *Immunol Rev 182:68.*

188. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol 155:1151.*

189. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med 188:287.*

190. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol 162:5317.*

191. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol 164:183.*

192. Piccirillo, C. A., and E. M. Shevach. 2001. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol 167:1137.*

193. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med 192:303.*

194. Sutmuller, R. P., L. M. van Duivenvoorde, A. van Elsas, T. N. Schumacher, M. E. Wildenberg, J. P. Allison, R. E. Toes, R. Offringa, and C. J. Melief. 2001. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med 194:823.*

195. Kursar, M., K. Bonhagen, J. Fensterle, A. Kohler, R. Hurwitz, T. Kamradt, S. H. Kaufmann, and H. W. Mittrucker. 2002. Regulatory CD4+CD25+ T cells restrict memory CD8+ T cell responses. *J Exp Med 196:1585.*

196. Murakami, M., A. Sakamoto, J. Bender, J. Kappler, and P. Marrack. 2002. CD25+CD4+ T cells contribute to the control of memory CD8+ T cells. *Proc Natl Acad Sci U S A 99:8832.*

197. Onizuka, S., I. Tawara, J. Shimizu, S. Sakaguchi, T. Fujita, and E. Nakayama. 1999. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res 59:3128.*

198. Golgher, D., E. Jones, F. Powrie, T. Elliott, and A. Gallimore. 2002. Depletion of CD25+ regulatory cells uncovers immune responses to shared murine tumor rejection antigens. *Eur J Immunol 32:3267.*

199. Levings, M. K., R. Sangregorio, and M. G. Roncarolo. 2001. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med 193:1295.*

200. Dieckmann, D., H. Plottner, S. Berchtold, T. Berger, and G. Schuler. 2001. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med 193:1303.*

201. Woo, E. Y., H. Yeh, C. S. Chu, K. Schlienger, R. G. Carroll, J. L. Riley, L. R. Kaiser, and C. H. June. 2002. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol 168:4272.*

202. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science 299:1057.*

203. Hahne, M., D. Rimoldi, M. Schroter, P. Romero, M. Schreier, L. E. French, P. Schneider, T. Bornand, A. Fontana, D. Lienard, J. Cerottini, and J. Tschopp. 1996. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. *Science 274:1363.*

204. Restifo, N. P. 2000. Not so Fas: Re-evaluating the mechanisms of immune privilege and tumor escape. *Nat Med 6:493.*

205. Chappell, D. B., T. Z. Zaks, S. A. Rosenberg, and N. P. Restifo. 1999. Human melanoma cells do not express Fas (Apo-1/CD95) ligand. *Cancer Res 59:59.*

206. Zaks, T. Z., D. B. Chappell, S. A. Rosenberg, and N. P. Restifo. 1999. Fas-mediated suicide of tumor-reactive T cells following activation by specific tumor: selective rescue by caspase inhibition. *J Immunol 162:3273.*

207. Paschen, A., R. M. Mendez, P. Jimenez, A. Sucker, F. Ruiz-Cabello, M. Song, F. Garrido, and D. Schadendorf. 2003. Complete loss of HLA class I antigen expression on melanoma cells: a result of successive mutational events. *Int J Cancer 103:759.*

208. Seliger, B., D. Atkins, M. Bock, U. Ritz, S. Ferrone, C. Huber, and S. Storkel. 2003. Characterization of human lymphocyte antigen class I antigen-processing machinery defects in renal cell carcinoma lesions with special emphasis on transporter-associated with antigen-processing down-regulation. *Clin Cancer Res 9:1721.*

209. Cabrera, T., M. A. Lopez-Nevot, J. J. Gaforio, F. Ruiz-Cabello, and F. Garrido. 2003. Analysis of HLA expression in human tumor tissues. *Cancer Immunol Immunother 52:1.*

210. Malmberg, K. J., V. Levitsky, H. Norell, C. T. de Matos, M. Carlsten, K. Schedvins, H. Rabbani, A. Moretta, K. Soderstrom, J. Levitskaya, and R. Kiessling. 2002. IFN-

gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. *J Clin Invest 110:1515.*

211. Khong, H. T., Q. J. Wang, and S. A. Rosenberg. 2004. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. *J Immunother 27:184.*

212. Pawelec, G. 2004. Tumour escape: antitumour effectors too much of a good thing? *Cancer Immunol Immunother 53:262.*

213. Steinbrink, K., H. Jonuleit, G. Muller, G. Schuler, J. Knop, and A. H. Enk. 1999. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. *Blood 93:1634.*

214. Parmiani, G., C. Castelli, P. Dalerba, R. Mortarini, L. Rivoltini, F. M. Marincola, and A. Anichini. 2002. Cancer immunotherapy with peptide-based vaccines: what have we achieved? Where are we going? *J Natl Cancer Inst 94:805.*

215. Van den Eynde, B. J., and P. van der Bruggen. 1997. T cell defined tumor antigens. *Curr Opin Immunol 9:684.*

216. King, C. A., M. B. Spellerberg, D. Zhu, J. Rice, S. S. Sahota, A. R. Thompsett, T. J. Hamblin, J. Radl, and F. K. Stevenson. 1998. DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. *Nat Med 4:1281.*

217. Stashenko, P., L. M. Nadler, R. Hardy, and S. F. Schlossman. 1980. Characterization of a human B lymphocyte-specific antigen. *J Immunol 125:1678.*

218. Harris, M. 2004. Monoclonal antibodies as therapeutic agents for cancer. *Lancet Oncol 5:292.*

219. Riddell, S. R., M. Murata, S. Bryant, and E. H. Warren. 2002. Minor histocompatibility antigens--targets of graft versus leukemia responses. *Int J Hematol 76 Suppl 2:155.*

220. Yee, C., J. A. Thompson, D. Byrd, S. R. Riddell, P. Roche, E. Celis, and P. D. Greenberg. 2002. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci U S A 99:16168.*

221. Dudley, M. E., J. R. Wunderlich, P. F. Robbins, J. C. Yang, P. Hwu, D. J. Schwartzentruber, S. L. Topalian, R. Sherry, N. P. Restifo, A. M. Hubicki, M. R. Robinson, M. Raffeld, P. Duray, C. A. Seipp, L. Rogers-Freezer, K. E. Morton, S. A. Mavroukakis, D. E. White, and S. A. Rosenberg. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science 298:850.*

222. Geertsen, P. F., M. E. Gore, S. Negrier, J. M. Tourani, and H. von der Maase. 2004. Safety and efficacy of subcutaneous and continuous intravenous infusion rIL-2 in patients with metastatic renal cell carcinoma. *Br J Cancer 90:1156.*

223. Hurteau, J. A., J. A. Blessing, S. L. DeCesare, and W. T. Creasman. 2001. Evaluation of recombinant human interleukin-12 in patients with recurrent or refractory ovarian cancer: a gynecologic oncology group study. *Gynecol Oncol 82:7.*

224. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R. C. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A 90:3539.*

225. Rosenberg, S. A., J. C. Yang, D. J. Schwartzentruber, P. Hwu, F. M. Marincola, S. L. Topalian, N. P. Restifo, M. E. Dudley, S. L. Schwarz, P. J. Spiess, J. R. Wunderlich, M. R. Parkhurst, Y. Kawakami, C. A. Seipp, J. H. Einhorn, and D. E. White. 1998. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med 4:321.*

226. Nair, S. K., S. Hull, D. Coleman, E. Gilboa, H. K. Lyerly, and M. A. Morse. 1999. Induction of carcinoembryonic antigen (CEA)-specific cytotoxic T-lymphocyte responses in vitro using autologous dendritic cells loaded with CEA peptide or CEA RNA in patients with metastatic malignancies expressing CEA. *Int J Cancer 82:121.*

227. Yu, J. S., G. Liu, H. Ying, W. H. Yong, K. L. Black, and C. J. Wheeler. 2004. Vaccination with tumor lysate-pulsed dendritic cells elicits antigen-specific, cytotoxic T-cells in patients with malignant glioma. *Cancer Res 64:4973.*

228. Wan, Y., P. Emtage, Q. Zhu, R. Foley, A. Pilon, B. Roberts, and J. Gauldie. 1999. Enhanced immune response to the melanoma antigen gp100 using recombinant adenovirus-transduced dendritic cells. *Cell Immunol 198:131.*

229. Pecher, G., A. Haring, L. Kaiser, and E. Thiel. 2002. Mucin gene (MUC1) transfected dendritic cells as vaccine: results of a phase I/II clinical trial. *Cancer Immunol Immunother 51:669.*

230. Buchler, T., J. Michalek, L. Kovarova, R. Musilova, and R. Hajek. 2003. Dendritic cell-based immunotherapy for the treatment of hematological malignancies. *Hematology 8:97.*

231. Figdor, C. G., I. J. de Vries, W. J. Lesterhuis, and C. J. Melief. 2004. Dendritic cell immunotherapy: mapping the way. *Nat Med 10:475.*

232. Raje, N., T. Hideshima, F. E. Davies, D. Chauhan, S. P. Treon, G. Young, Y. T. Tai, D. Avigan, J. Gong, R. L. Schlossman, P. Richardson, D. W. Kufe, and K. C. Anderson. 2004. Tumour cell/dendritic cell fusions as a vaccination strategy for multiple myeloma. *Br J Haematol 125:343.*

233. van der Burg, S. H., A. G. Menon, A. Redeker, M. C. Bonnet, J. W. Drijfhout, R. A. Tollenaar, C. J. van de Velde, P. Moingeon, P. J. Kuppen, R. Offringa, and C. J. Melief. 2002. Induction of p53-specific immune responses in colorectal cancer patients receiving a recombinant ALVAC-p53 candidate vaccine. *Clin Cancer Res 8:1019.*

234. Rosenberg, S. A., Y. Zhai, J. C. Yang, D. J. Schwartzentruber, P. Hwu, F. M. Marincola, S. L. Topalian, N. P. Restifo, C. A. Seipp, J. H. Einhorn, B. Roberts, and D. E. White. 1998. Immunizing patients with metastatic melanoma using

recombinant adenoviruses encoding MART-1 or gp100 melanoma antigens. *J Natl Cancer Inst 90:1894.*

235. Ludwig, H., E. Fritz, G. B. Zulian, and G. P. Browman. 1998. Should-alpha-interferon be included as standard treatment in multiple myeloma? *Eur J Cancer 34:12.*

236. Moreau, P., J. L. Harousseau, J. Wijdenes, N. Morineau, N. Milpied, and R. Bataille. 2000. A combination of anti-interleukin 6 murine monoclonal antibody with dexamethasone and high-dose melphalan induces high complete response rates in advanced multiple myeloma. *Br J Haematol 109:661.*

237. Coscia, M., S. Mariani, S. Battaglio, C. Di Bello, F. Fiore, M. Foglietta, A. Pileri, M. Boccadoro, and M. Massaia. 2004. Long-term follow-up of idiotype vaccination in human myeloma as a maintenance therapy after high-dose chemotherapy. *Leukemia 18:139.*

238. Reichardt, V. L., C. Milazzo, W. Brugger, H. Einsele, L. Kanz, and P. Brossart. 2003. Idiotype vaccination of multiple myeloma patients using monocyte-derived dendritic cells. *Haematologica 88:1139.*

239. Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner. 1990. Direct gene transfer into mouse muscle in vivo. *Science 247:1465.*

240. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, and et al. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science 259:1745.*

241. Stevenson, F. K. 1999. DNA vaccines against cancer: from genes to therapy. *Ann Oncol 10:1413.*

242. Kozak, M. 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J Mol Biol 196:947.*

243. Rice, J., C. A. King, M. B. Spellerberg, N. Fairweather, and F. K. Stevenson. 1999. Manipulation of pathogen-derived genes to influence antigen presentation via DNA vaccines. *Vaccine 17:3030.*

244. Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol 20:709.*

245. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740.*

246. Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A 98:9237.*

247. Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol 5:190.*

248. Spies, B., H. Hochrein, M. Vabulas, K. Huster, D. H. Busch, F. Schmitz, A. Heit, and H. Wagner. 2003. Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *J Immunol 171:5908.*

249. Corr, M., D. J. Lee, D. A. Carson, and H. Tighe. 1996. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J Exp Med 184:1555.*

250. Doe, B., M. Selby, S. Barnett, J. Baenziger, and C. M. Walker. 1996. Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proc Natl Acad Sci U S A 93:8578.*

251. Colonna, M., G. Trinchieri, and Y. J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat Immunol 5:1219.*

252. Garg, S., A. Oran, J. Wajchman, S. Sasaki, C. H. Maris, J. A. Kapp, and J. Jacob. 2003. Genetic tagging shows increased frequency and longevity of antigen-presenting, skin-derived dendritic cells in vivo. *Nat Immunol 4:907.*

253. Sumida, S. M., P. F. McKay, D. M. Truitt, M. G. Kishko, J. C. Arthur, M. S. Seaman, S. S. Jackson, D. A. Gorgone, M. A. Lifton, N. L. Letvin, and D. H. Barouch. 2004. Recruitment and expansion of dendritic cells in vivo potentiate the immunogenicity of plasmid DNA vaccines. *J Clin Invest 114:1334.*

254. Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone, and J. A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev 199:9.*

255. Porgador, A., K. R. Irvine, A. Iwasaki, B. H. Barber, N. P. Restifo, and R. N. Germain. 1998. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J Exp Med 188:1075.*

256. Chan, K., D. J. Lee, A. Schubert, C. M. Tang, B. Crain, S. P. Schoenberger, and M. Corr. 2001. The roles of MHC class II, CD40, and B7 costimulation in CTL induction by plasmid DNA. *J Immunol 166:3061.*

257. Spellerberg, M. B., D. Zhu, A. Thompsett, C. A. King, T. J. Hamblin, and F. K. Stevenson. 1997. DNA vaccines against lymphoma: promotion of anti-idiotypic antibody responses induced by single chain Fv genes by fusion to tetanus toxin fragment C. *J Immunol 159:1885.*

258. Helting, T. B., and O. Zwisler. 1977. Structure of tetanus toxin. I. Breakdown of the toxin molecule and discrimination between polypeptide fragments. *J Biol Chem 252:187.*

259. Emsley, P., C. Fotinou, I. Black, N. F. Fairweather, I. G. Charles, C. Watts, E. Hewitt, and N. W. Isaacs. 2000. The structures of the H(C) fragment of tetanus toxin with carbohydrate subunit complexes provide insight into ganglioside binding. *J Biol Chem 275:8889.*

260. Stevenson, F. K., J. Rice, C. H. Ottensmeier, S. M. Thirdborough, and D. Zhu. 2004. DNA fusion gene vaccines against cancer: from the laboratory to the clinic. *Immunol Rev 199:156.*

261. Panina-Bordignon, P., A. Tan, A. Termijtelen, S. Demotz, G. Corradin, and A. Lanzavecchia. 1989. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur J Immunol 19:2237.*

262. Lauritzsen, G. F., and B. Bogen. 1993. The role of idiotype-specific, CD4+ T cells in tumor resistance against major histocompatibility complex class II molecule negative plasmacytoma cells. *Cell Immunol 148:177.*

263. Rice, J., T. Elliott, S. Buchan, and F. K. Stevenson. 2001. DNA fusion vaccine designed to induce cytotoxic T cell responses against defined peptide motifs: implications for cancer vaccines. *J Immunol 167:1558.*

264. Rice, J., S. Buchan, and F. K. Stevenson. 2002. Critical components of a DNA fusion vaccine able to induce protective cytotoxic T cells against a single epitope of a tumor antigen. *J Immunol 169:3908.*

265. Savelyeva, N., R. Munday, M. B. Spellerberg, G. P. Lomonossoff, and F. K. Stevenson. 2001. Plant viral genes in DNA idiotypic vaccines activate linked CD4+ T-cell mediated immunity against B-cell malignancies. *Nat Biotechnol 19:760.*

266. Widera, G., M. Austin, D. Rabussay, C. Goldbeck, S. W. Barnett, M. Chen, L. Leung, G. R. Otten, K. Thudium, M. J. Selby, and J. B. Ulmer. 2000. Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. *J Immunol 164:4635.*

267. Mathiesen, I. 1999. Electropermeabilization of skeletal muscle enhances gene transfer in vivo. *Gene Ther 6:508.*

268. Tollefsen, S., T. Tjelle, J. Schneider, M. Harboe, H. Wiker, G. Hewinson, K. Huygen, and I. Mathiesen. 2002. Improved cellular and humoral immune responses against Mycobacterium tuberculosis antigens after intramuscular DNA immunisation combined with muscle electroporation. *Vaccine 20:3370.*

269. O'Hagan, D. T., M. Singh, and J. B. Ulmer. 2004. Microparticles for the delivery of DNA vaccines. *Immunol Rev 199:191.*

270. Pertmer, T. M., M. D. Eisenbraun, D. McCabe, S. K. Prayaga, D. H. Fuller, and J. R. Haynes. 1995. Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine 13:1427.*

271. Ugen, K. E., S. B. Nyland, J. D. Boyer, C. Vidal, L. Lera, S. Rasheid, M. Chattergoon, M. L. Bagarazzi, R. Ciccarelli, T. Higgins, Y. Baine, R. Ginsberg, R. R. Macgregor, and D. B. Weiner. 1998. DNA vaccination with HIV-1 expressing constructs elicits immune responses in humans. *Vaccine 16:1818.*

272. Rottinghaus, S. T., G. A. Poland, R. M. Jacobson, L. J. Barr, and M. J. Roy. 2003. Hepatitis B DNA vaccine induces protective antibody responses in human non-responders to conventional vaccination. *Vaccine 21:4604.*

273. Wang, R., J. Epstein, F. M. Baraceros, E. J. Gorak, Y. Charoenvit, D. J. Carucci, R. C. Hedstrom, N. Rahardjo, T. Gay, P. Hobart, R. Stout, T. R. Jones, T. L. Richie, S. E. Parker, D. L. Doolan, J. Norman, and S. L. Hoffman. 2001. Induction of CD4(+)

T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. *Proc Natl Acad Sci U S A 98:10817.*

274. McConkey, S. J., W. H. Reece, V. S. Moorthy, D. Webster, S. Dunachie, G. Butcher, J. M. Vuola, T. J. Blanchard, P. Gothard, K. Watkins, C. M. Hannan, S. Everaere, K. Brown, K. E. Kester, J. Cummings, J. Williams, D. G. Heppner, A. Pathan, K. Flanagan, N. Arulanantham, M. T. Roberts, M. Roy, G. L. Smith, J. Schneider, T. Peto, R. E. Sinden, S. C. Gilbert, and A. V. Hill. 2003. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat Med 9:729.*

275. Yee, C., J. A. Thompson, P. Roche, D. R. Byrd, P. P. Lee, M. Piepkorn, K. Kenyon, M. M. Davis, S. R. Riddell, and P. D. Greenberg. 2000. Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of t cell-mediated vitiligo. *J Exp Med 192:1637.*

276. van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science 254:1643.*

277. Van den Eynde, B., B. Lethe, A. Van Pel, E. De Plaen, and T. Boon. 1991. The gene coding for a major tumor rejection antigen of tumor P815 is identical to the normal gene of syngeneic DBA/2 mice. *J Exp Med 173:1373.*

278. De Plaen, E., K. Arden, C. Traversari, J. J. Gaforio, J. P. Szikora, C. De Smet, F. Brasseur, P. van der Bruggen, B. Lethe, C. Lurquin, and et al. 1994. Structure, chromosomal localization, and expression of 12 genes of the MAGE family. *Immunogenetics 40:360.*

279. Lucas, S., C. De Smet, K. C. Arden, C. S. Viars, B. Lethe, C. Lurquin, and T. Boon. 1998. Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. *Cancer Res 58:743.*

280. Lucas, S., E. De Plaen, and T. Boon. 2000. MAGE-B5, MAGE-B6, MAGE-C2, and MAGE-C3: four new members of the MAGE family with tumor-specific expression. *Int J Cancer 87:55.*

281. Muscatelli, F., A. P. Walker, E. De Plaen, A. N. Stafford, and A. P. Monaco. 1995. Isolation and characterization of a MAGE gene family in the Xp21.3 region. *Proc Natl Acad Sci U S A 92:4987.*

282. Zendman, A. J., D. J. Ruiter, and G. N. Van Muijen. 2003. Cancer/testis-associated genes: identification, expression profile, and putative function. *J Cell Physiol 194:272.*

283. Lucas, S., F. Brasseur, and T. Boon. 1999. A new MAGE gene with ubiquitous expression does not code for known MAGE antigens recognized by T cells. *Cancer Res 59:4100.*

284. Pold, M., J. Zhou, G. L. Chen, J. M. Hall, R. A. Vescio, and J. R. Berenson. 1999. Identification of a new, unorthodox member of the MAGE gene family. *Genomics 59:161.*

285. Stone, B., M. Schummer, P. J. Paley, M. Crawford, M. Ford, N. Urban, and B. H. Nelson. 2001. MAGE-F1, a novel ubiquitously expressed member of the MAGE superfamily. *Gene 267:173.*

286. Kawano, Y., M. Sasaki, K. Nakahira, T. Yoshimine, K. Shimizu, H. Wada, and K. Ikenaka. 2001. Structural characterization and chromosomal localization of the MAGE-E1 gene. *Gene 277:129.*

287. Boel, P., C. Wildmann, M. L. Sensi, R. Brasseur, J. C. Renauld, P. Coulie, T. Boon, and P. van der Bruggen. 1995. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. *Immunity 2:167.*

288. Van den Eynde, B., O. Peeters, O. De Backer, B. Gaugler, S. Lucas, and T. Boon. 1995. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. *J Exp Med 182:689.*

289. De Backer, O., K. C. Arden, M. Boretti, V. Vantomme, C. De Smet, S. Czekay, C. S. Viars, E. De Plaen, F. Brasseur, P. Chomez, B. Van den Eynde, T. Boon, and P. van der Bruggen. 1999. Characterization of the GAGE genes that are expressed in various human cancers and in normal testis. *Cancer Res 59:3157.*

290. Chen, Y. T., M. J. Scanlan, U. Sahin, O. Tureci, A. O. Gure, S. Tsang, B. Williamson, E. Stockert, M. Pfreundschuh, and L. J. Old. 1997. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A 94:1914.*

291. Park, S., Y. Lim, D. Lee, B. Cho, Y. J. Bang, S. Sung, H. Y. Kim, D. K. Kim, Y. S. Lee, Y. Song, and D. I. Jeoung. 2003. Identification and characterization of a novel cancer/testis antigen gene CAGE-1. *Biochim Biophys Acta 1625:173.*

292. Tureci, O., Y. T. Chen, U. Sahin, A. O. Gure, C. Zwick, C. Villena, S. Tsang, G. Seitz, L. J. Old, and M. Pfreundschuh. 1998. Expression of SSX genes in human tumors. *Int J Cancer 77:19.*

293. Martelange, V., C. De Smet, E. De Plaen, C. Lurquin, and T. Boon. 2000. Identification on a human sarcoma of two new genes with tumor-specific expression. *Cancer Res 60:3848.*

294. Lethe, B., S. Lucas, L. Michaux, C. De Smet, D. Godelaine, A. Serrano, E. De Plaen, and T. Boon. 1998. LAGE-1, a new gene with tumor specificity. *Int J Cancer 76:903.*

295. Zendman, A. J., I. M. Cornelissen, U. H. Weidle, D. J. Ruiter, and G. N. van Muijen. 1999. CTp11, a novel member of the family of human cancer/testis antigens. *Cancer Res 59:6223.*

296. Gure, A. O., E. Stockert, K. C. Arden, A. D. Boyer, C. S. Viars, M. J. Scanlan, L. J. Old, and Y. T. Chen. 2000. CT10: a new cancer-testis (CT) antigen homologous to CT7 and the MAGE family, identified by representational-difference analysis. *Int J Cancer 85:726.*

297. de Wit, N. J., U. H. Weidle, D. J. Ruiter, and G. N. van Muijen. 2002. Expression profiling of MMA-1a and splice variant MMA-1b: new cancer/testis antigens identified in human melanoma. *Int J Cancer 98:547.*

298. Scanlan, M. J., C. M. Gordon, B. Williamson, S. Y. Lee, Y. T. Chen, E. Stockert, A. Jungbluth, G. Ritter, D. Jager, E. Jager, A. Knuth, and L. J. Old. 2002. Identification of cancer/testis genes by database mining and mRNA expression analysis. *Int J Cancer 98:485.*

299. Scanlan, M. J., A. O. Gure, A. A. Jungbluth, L. J. Old, and Y. T. Chen. 2002. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol Rev 188:22.*

300. Dhodapkar, M. V., K. Osman, J. Teruya-Feldstein, D. Filippa, C. V. Hedvat, K. Iversen, D. Kolb, M. D. Geller, H. Hassoun, T. Kewalramani, R. L. Comenzo, K. Coplan, Y. T. Chen, and A. A. Jungbluth. 2003. Expression of cancer/testis (CT) antigens MAGE-A1, MAGE-A3, MAGE-A4, CT-7, and NY-ESO-1 in malignant gammopathies is heterogeneous and correlates with site, stage and risk status of disease. *Cancer Immun 3:9.*

301. Scanlan, M. J., A. J. Simpson, and L. J. Old. 2004. The cancer/testis genes: review, standardization, and commentary. *Cancer Immun 4:1.*

302. Adams, S. P., S. S. Sahota, A. Mijovic, B. Czepulkowski, R. A. Padua, G. J. Mufti, and B. A. Guinn. 2002. Frequent expression of HAGE in presentation chronic myeloid leukaemias. *Leukemia 16:2238.*

303. Haffner, A. C., A. Tassis, K. Zepter, M. Storz, O. Tureci, G. Burg, and F. O. Nestle. 2002. Expression of cancer/testis antigens in cutaneous T cell lymphomas. *Int J Cancer 97:668.*

304. Colleoni, G. W., P. Capodieci, S. Tickoo, J. Cossman, D. A. Filippa, and M. Ladanyi. 2002. Expression of SSX genes in the neoplastic cells of Hodgkin's lymphoma. *Hum Pathol 33:496.*

305. van Baren, N., F. Brasseur, D. Godelaine, G. Hames, A. Ferrant, F. Lehmann, M. Andre, C. Ravoet, C. Doyen, G. C. Spagnoli, M. Bakkus, K. Thielemans, and T. Boon. 1999. Genes encoding tumor-specific antigens are expressed in human myeloma cells. *Blood 94:1156.*

306. Pellat-Deceunynck, C., M. P. Mellerin, N. Labarriere, G. Jego, A. Moreau-Aubry, J. L. Harousseau, F. Jotereau, and R. Bataille. 2000. The cancer germ-line genes MAGE-1, MAGE-3 and PRAME are commonly expressed by human myeloma cells. *Eur J Immunol 30:803.*

307. Pascolo, S., M. Schirle, B. Guckel, T. Dumrese, S. Stumm, S. Kayser, A. Moris, D. Wallwiener, H. G. Rammensee, and S. Stevanovic. 2001. A MAGE-A1 HLA-A A*0201 epitope identified by mass spectrometry. *Cancer Res 61:4072.*

308. Chaux, P., R. Luiten, N. Demotte, V. Vantomme, V. Stroobant, C. Traversari, V. Russo, E. Schultz, G. R. Cornelis, T. Boon, and P. van der Bruggen. 1999. Identification of five MAGE-A1 epitopes recognized by cytolytic T lymphocytes obtained by in vitro stimulation with dendritic cells transduced with MAGE-A1. *J Immunol 163:2928.*

309. Fujie, T., K. Tahara, F. Tanaka, M. Mori, K. Takesako, and T. Akiyoshi. 1999. A MAGE-1-encoded HLA-A24-binding synthetic peptide induces specific anti-tumor cytotoxic T lymphocytes. *Int J Cancer 80:169.*

310. Visseren, M. J., S. H. van der Burg, E. I. van der Voort, R. M. Brandt, P. I. Schrier, P. van der Bruggen, T. Boon, C. J. Melief, and W. M. Kast. 1997. Identification of HLA-A*0201-restricted CTL epitopes encoded by the tumor-specific MAGE-2 gene product. *Int J Cancer 73:125.*

311. Kawashima, I., S. J. Hudson, V. Tsai, S. Southwood, K. Takesako, E. Appella, A. Sette, and E. Celis. 1998. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. *Hum Immunol 59:1.*

312. Schultz, E. S., Y. Zhang, R. Knowles, J. Tine, C. Traversari, T. Boon, and P. van der Bruggen. 2001. A MAGE-3 peptide recognized on HLA-B35 and HLA-A1 by cytolytic T lymphocytes. *Tissue Antigens 57:103.*

313. van der Bruggen, P., J. Bastin, T. Gajewski, P. G. Coulie, P. Boel, C. De Smet, C. Traversari, A. Townsend, and T. Boon. 1994. A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. *Eur J Immunol 24:3038.*

314. Katsura, F., M. Eura, K. Chikamatsu, M. Oiso, E. Yumoto, and T. Ishikawa. 2000. Analysis of individual specific cytotoxic T lymphocytes for two MAGE-3-derived epitopes presented by HLA-A24. *Jpn J Clin Oncol 30:117.*

315. Duffour, M. T., P. Chaux, C. Lurquin, G. Cornelis, T. Boon, and P. van der Bruggen. 1999. A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes. *Eur J Immunol 29:3329.*

316. Dutoit, V., V. Rubio-Godoy, P. Y. Dietrich, A. L. Quiqueres, V. Schnuriger, D. Rimoldi, D. Lienard, D. Speiser, P. Guillaume, P. Batard, J. C. Cerottini, P. Romero, and D. Valmori. 2001. Heterogeneous T-cell response to MAGE-A10(254-262): high avidity-specific cytolytic T lymphocytes show superior antitumor activity. *Cancer Res 61:5850.*

317. Heidecker, L., F. Brasseur, M. Probst-Kepper, M. Gueguen, T. Boon, and B. J. Van den Eynde. 2000. Cytolytic T lymphocytes raised against a human bladder carcinoma recognize an antigen encoded by gene MAGE-A12. *J Immunol 164:6041.*

318. Tanzarella, S., V. Russo, I. Lionello, P. Dalerba, D. Rigatti, C. Bordignon, and C. Traversari. 1999. Identification of a promiscuous T-cell epitope encoded by multiple members of the MAGE family. *Cancer Res 59:2668.*

319. Graff-Dubois, S., O. Faure, D. A. Gross, P. Alves, A. Scardino, S. Chouaib, F. A. Lemonnier, and K. Kosmatopoulos. 2002. Generation of CTL recognizing an HLA-A*0201-restricted epitope shared by MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12 tumor antigens: implication in a broad-spectrum tumor immunotherapy. *J Immunol 169:575.*

320. Aarnoudse, C. A., P. B. van den Doel, B. Heemskerk, and P. I. Schrier. 1999. Interleukin-2-induced, melanoma-specific T cells recognize CAMEL, an unexpected translation product of LAGE-1. *Int J Cancer 82:442.*

321. Chen, J. L., P. R. Dunbar, U. Gileadi, E. Jager, S. Gnjatic, Y. Nagata, E. Stockert, D. L. Panicali, Y. T. Chen, A. Knuth, L. J. Old, and V. Cerundolo. 2000. Identification

of NY-ESO-1 peptide analogues capable of improved stimulation of tumor-reactive CTL. *J Immunol 165:948.*

322. Jager, E., Y. T. Chen, J. W. Drijfhout, J. Karbach, M. Ringhoffer, D. Jager, M. Arand, H. Wada, Y. Noguchi, E. Stockert, L. J. Old, and A. Knuth. 1998. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med 187:265.*

323. Bownds, S., P. Tong-On, S. A. Rosenberg, and M. Parkhurst. 2001. Induction of tumor-reactive cytotoxic T-lymphocytes using a peptide from NY-ESO-1 modified at the carboxy-terminus to enhance HLA-A2.1 binding affinity and stability in solution. *J Immunother 24:1.*

324. Wang, R. F., S. L. Johnston, G. Zeng, S. L. Topalian, D. J. Schwartzentruber, and S. A. Rosenberg. 1998. A breast and melanoma-shared tumor antigen: T cell responses to antigenic peptides translated from different open reading frames. *J Immunol 161:3598.*

325. Schultz, E. S., B. Lethe, C. L. Cambiaso, J. Van Snick, P. Chaux, J. Corthals, C. Heirman, K. Thielemans, T. Boon, and P. van der Bruggen. 2000. A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by CD4+ cytolytic T lymphocytes. *Cancer Res 60:6272.*

326. Manici, S., T. Sturniolo, M. A. Imro, J. Hammer, F. Sinigaglia, C. Noppen, G. Spagnoli, B. Mazzi, M. Bellone, P. Dellabona, and M. P. Protti. 1999. Melanoma cells present a MAGE-3 epitope to CD4(+) cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. *J Exp Med 189:871.*

327. Chaux, P., V. Vantomme, V. Stroobant, K. Thielemans, J. Corthals, R. Luiten, A. M. Eggermont, T. Boon, and P. van der Bruggen. 1999. Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4(+) T lymphocytes. *J Exp Med 189:767.*

328. Zeng, G., Y. Li, M. El-Gamil, J. Sidney, A. Sette, R. F. Wang, S. A. Rosenberg, and P. F. Robbins. 2002. Generation of NY-ESO-1-specific CD4+ and CD8+ T cells by a single peptide with dual MHC class I and class II specificities: a new strategy for vaccine design. *Cancer Res 62:3630.*

329. Zarour, H. M., B. Maillere, V. Brusic, K. Coval, E. Williams, S. Pouvelle-Moratille, F. Castelli, S. Land, J. Bennouna, T. Logan, and J. M. Kirkwood. 2002. NY-ESO-1 119-143 is a promiscuous major histocompatibility complex class II T-helper epitope recognized by Th1- and Th2-type tumor-reactive CD4+ T cells. *Cancer Res 62:213.*

330. Dalerba, P., A. Ricci, V. Russo, D. Rigatti, M. R. Nicotra, M. Mottolese, C. Bordignon, P. G. Natali, and C. Traversari. 1998. High homogeneity of MAGE, BAGE, GAGE, tyrosinase and Melan-A/MART-1 gene expression in clusters of multiple simultaneous metastases of human melanoma: implications for protocol design of therapeutic antigen-specific vaccination strategies. *Int J Cancer 77:200.*

331. Brasseur, F., D. Rimoldi, D. Lienard, B. Lethe, S. Carrel, F. Arienti, L. Suter, R. Vanwijck, A. Bourlond, Y. Humblet, and et al. 1995. Expression of MAGE genes in primary and metastatic cutaneous melanoma. *Int J Cancer 63:375.*

332. De Smet, C., O. De Backer, I. Faraoni, C. Lurquin, F. Brasseur, and T. Boon. 1996. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. *Proc Natl Acad Sci U S A 93:7149.*

333. De Smet, C., C. Lurquin, B. Lethe, V. Martelange, and T. Boon. 1999. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Mol Cell Biol 19:7327.*

334. Ohman Forslund, K., and K. Nordqvist. 2001. The melanoma antigen genes--any clues to their functions in normal tissues? *Exp Cell Res 265:185.*

335. Kuwako, K., H. Taniura, and K. Yoshikawa. 2004. Necdin-related MAGE proteins differentially interact with the E2F1 transcription factor and the p75 neurotrophin receptor. *J Biol Chem 279:1703.*

336. Barker, P. A., and A. Salehi. 2002. The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease. *J Neurosci Res 67:705.*

337. Suk, K., S. Kim, Y. H. Kim, S. H. Oh, M. K. Lee, K. W. Kim, H. D. Kim, and Y. S. Seo. 2000. Identification of a novel human member of the DEAD box protein family. *Biochim Biophys Acta 1501:63.*

338. Marchand, M., N. van Baren, P. Weynants, V. Brichard, B. Dreno, M. H. Tessier, E. Rankin, G. Parmiani, F. Arienti, Y. Humblet, A. Bourlond, R. Vanwijck, D. Lienard, M. Beauduin, P. Y. Dietrich, V. Russo, J. Kerger, G. Masucci, E. Jager, J. De Greve, J. Atzpodien, F. Brasseur, P. G. Coulie, P. van der Bruggen, and T. Boon. 1999. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer 80:219.*

339. Coulie, P. G., V. Karanikas, C. Lurquin, D. Colau, T. Connerotte, T. Hanagiri, A. Van Pel, S. Lucas, D. Godelaine, C. Lonchay, M. Marchand, N. Van Baren, and T. Boon. 2002. Cytolytic T-cell responses of cancer patients vaccinated with a MAGE antigen. *Immunol Rev 188:33.*

340. Jager, E., S. Gnjatic, Y. Nagata, E. Stockert, D. Jager, J. Karbach, A. Neumann, J. Rieckenberg, Y. T. Chen, G. Ritter, E. Hoffman, M. Arand, L. J. Old, and A. Knuth. 2000. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. *Proc Natl Acad Sci U S A 97:12198.*

341. Hoon, D. S., D. Yuzuki, M. Hayashida, and D. L. Morton. 1995. Melanoma patients immunized with melanoma cell vaccine induce antibody responses to recombinant MAGE-1 antigen. *J Immunol 154:730.*

342. Weber, J. S., F. L. Hua, L. Spears, V. Marty, C. Kuniyoshi, and E. Celis. 1999. A phase I trial of an HLA-A1 restricted MAGE-3 epitope peptide with incomplete Freund's adjuvant in patients with resected high-risk melanoma. *J Immunother 22:431.*

343. Reynolds, S. R., E. Celis, A. Sette, R. Oratz, R. L. Shapiro, D. Johnston, M. Fotino, and J. C. Bystryn. 1998. HLA-independent heterogeneity of CD8+ T cell responses to MAGE-3, Melan-A/MART-1, gp100, tyrosinase, MC1R, and TRP-2 in vaccine-treated melanoma patients. *J Immunol 161:6970.*

344. Reynolds, S. R., A. Zeleniuch-Jacquotte, R. L. Shapiro, D. F. Roses, M. N. Harris, D. Johnston, and J. C. Bystryn. 2003. Vaccine-induced CD8+ T-cell responses to MAGE-3 correlate with clinical outcome in patients with melanoma. *Clin Cancer Res 9:657.*

345. Marchand, M., C. J. Punt, S. Aamdal, B. Escudier, W. H. Kruit, U. Keilholz, L. Hakansson, N. van Baren, Y. Humblet, P. Mulders, M. F. Avril, A. M. Eggermont, C. Scheibenbogen, J. Uiters, J. Wanders, M. Delire, T. Boon, and G. Stoter. 2003. Immunisation of metastatic cancer patients with MAGE-3 protein combined with adjuvant SBAS-2: a clinical report. *Eur J Cancer 39:70.*

346. Thurner, B., I. Haendle, C. Roder, D. Dieckmann, P. Keikavoussi, H. Jonuleit, A. Bender, C. Maczek, D. Schreiner, P. von den Driesch, E. B. Brocker, R. M. Steinman, A. Enk, E. Kampgen, and G. Schuler. 1999. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med 190:1669.*

347. Banchereau, J., A. K. Palucka, M. Dhodapkar, S. Burkeholder, N. Taquet, A. Rolland, S. Taquet, S. Coquery, K. M. Wittkowski, N. Bhardwaj, L. Pineiro, R. Steinman, and J. Fay. 2001. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res 61:6451.*

348. Andersen, M. H., P. Keikavoussi, E. B. Brocker, B. Schuler-Thurner, M. Jonassen, I. Sondergaard, P. T. Straten, J. C. Becker, and E. Kampgen. 2001. Induction of systemic CTL responses in melanoma patients by dendritic cell vaccination: cessation of CTL responses is associated with disease progression. *Int J Cancer 94:820.*

349. Schuler-Thurner, B., E. S. Schultz, T. G. Berger, G. Weinlich, S. Ebner, P. Woerl, A. Bender, B. Feuerstein, P. O. Fritsch, N. Romani, and G. Schuler. 2002. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J Exp Med 195:1279.*

350. Nishiyama, T., M. Tachibana, Y. Horiguchi, K. Nakamura, Y. Ikeda, K. Takesako, and M. Murai. 2001. Immunotherapy of bladder cancer using autologous dendritic cells pulsed with human lymphocyte antigen-A24-specific MAGE-3 peptide. *Clin Cancer Res 7:23.*

351. Sadanaga, N., H. Nagashima, K. Mashino, K. Tahara, H. Yamaguchi, M. Ohta, T. Fujie, F. Tanaka, H. Inoue, K. Takesako, T. Akiyoshi, and M. Mori. 2001. Dendritic cell vaccination with MAGE peptide is a novel therapeutic approach for gastrointestinal carcinomas. *Clin Cancer Res 7:2277.*

352. Danke, N. A., D. M. Koelle, C. Yee, S. Beheray, and W. W. Kwok. 2004. Autoreactive T cells in healthy individuals. *J Immunol 172:5967.*

353. De Plaen, E., O. De Backer, D. Arnaud, B. Bonjean, P. Chomez, V. Martelange, P. Avner, P. Baldacci, C. Babinet, S. Y. Hwang, B. Knowles, and T. Boon. 1999. A new family of mouse genes homologous to the human MAGE genes. *Genomics 55:176.*

354. De Backer, O., A. M. Verheyden, B. Martin, D. Godelaine, E. De Plaen, R. Brasseur, P. Avner, and T. Boon. 1995. Structure, chromosomal location, and expression pattern of three mouse genes homologous to the human MAGE genes. *Genomics 28:74.*

355. Osterlund, C., V. Tohonen, K. O. Forslund, and K. Nordqvist. 2000. Mage-b4, a novel melanoma antigen (MAGE) gene specifically expressed during germ cell differentiation. *Cancer Res 60:1054.*

356. Van Pel, A., E. De Plaen, M. T. Duffour, G. Warnier, C. Uyttenhove, M. Perricaudet, and T. Boon. 2001. Induction of cytolytic T lymphocytes by immunization of mice with an adenovirus containing a mouse homolog of the human MAGE-A genes. *Cancer Immunol Immunother 49:593.*

357. Brichard, V. G., G. Warnier, A. Van Pel, G. Morlighem, S. Lucas, and T. Boon. 1995. Individual differences in the orientation of the cytolytic T cell response against mouse tumor P815. *Eur J Immunol 25:664.*

358. Lethe, B., B. van den Eynde, A. van Pel, G. Corradin, and T. Boon. 1992. Mouse tumor rejection antigens P815A and P815B: two epitopes carried by a single peptide. *Eur J Immunol 22:2283.*

359. Uyttenhove, C., C. Godfraind, B. Lethe, A. Amar-Costesec, J. C. Renauld, T. F. Gajewski, M. T. Duffour, G. Warnier, T. Boon, and B. J. Van den Eynde. 1997. The expression of mouse gene P1A in testis does not prevent safe induction of cytolytic T cells against a P1A-encoded tumor antigen. *Int J Cancer 70:349.*

360. Van den Eynde, B., H. Mazarguil, B. Lethe, F. Laval, and J. E. Gairin. 1994. Localization of two cytotoxic T lymphocyte epitopes and three anchoring residues on a single nonameric peptide that binds to H-2Ld and is recognized by cytotoxic T lymphocytes against mouse tumor P815. *Eur J Immunol 24:2740.*

361. Bilsborough, J., A. Van Pel, C. Uyttenhove, T. Boon, and B. J. Van den Eynde. 1999. Identification of a second major tumor-specific antigen recognized by CTLs on mouse mastocytoma P815. *J Immunol 162:3534.*

362. Brandle, D., J. Bilsborough, T. Rulicke, C. Uyttenhove, T. Boon, and B. J. Van den Eynde. 1998. The shared tumor-specific antigen encoded by mouse gene P1A is a target not only for cytolytic T lymphocytes but also for tumor rejection. *Eur J Immunol 28:4010.*

363. Warnier, G., M. T. Duffour, C. Uyttenhove, T. F. Gajewski, C. Lurquin, H. Haddada, M. Perricaudet, and T. Boon. 1996. Induction of a cytolytic T-cell response in mice with a recombinant adenovirus coding for tumor antigen P815A. *Int J Cancer 67:303.*

364. Ramarathinam, L., S. Sarma, M. Maric, M. Zhao, G. Yang, L. Chen, and Y. Liu. 1995. Multiple lineages of tumors express a common tumor antigen, P1A, but they are not cross-protected. *J Immunol 155:5323.*

365. Nieland, J. D., D. M. Da Silva, M. P. Velders, K. E. de Visser, J. T. Schiller, M. Muller, and W. M. Kast. 1999. Chimeric papillomavirus virus-like particles induce a murine self-antigen-specific protective and therapeutic antitumor immune response. *J Cell Biochem 73:145.*

366. Colmenero, P., P. Liljestrom, and M. Jondal. 1999. Induction of P815 tumor immunity by recombinant Semliki Forest virus expressing the P1A gene. *Gene Ther 6:1728.*

367. Wu, Y., Y. Wan, J. Bian, J. Zhao, Z. Jia, L. Zhou, W. Zhou, and Y. Tan. 2002. Phage display particles expressing tumor-specific antigens induce preventive and therapeutic anti-tumor immunity in murine p815 model. *Int J Cancer 98:748.*

368. Rosato, A., A. Zambon, G. Milan, V. Ciminale, D. M. D'Agostino, B. Macino, P. Zanovello, and D. Collavo. 1997. CTL response and protection against P815 tumor challenge in mice immunized with DNA expressing the tumor-specific antigen P815A. *Hum Gene Ther 8:1451.*

369. Grohmann, U., R. Bianchi, E. Ayroldi, M. L. Belladonna, D. Surace, M. C. Fioretti, and P. Puccetti. 1997. A tumor-associated and self antigen peptide presented by dendritic cells may induce T cell anergy in vivo, but IL-12 can prevent or revert the anergic state. *J Immunol 158:3593.*

370. Grohmann, U., M. L. Belladonna, R. Bianchi, C. Orabona, S. Silla, G. Squillacioti, M. C. Fioretti, and P. Puccetti. 1999. Immunogenicity of tumor peptides: importance of peptide length and stability of peptide/MHC class II complex. *Cancer Immunol Immunother 48:195.*

371. Grohmann, U., M. C. Fioretti, R. Bianchi, M. L. Belladonna, E. Ayroldi, D. Surace, S. Silla, and P. Puccetti. 1998. Dendritic cells, interleukin 12, and CD4+ lymphocytes in the initiation of class I-restricted reactivity to a tumor/self peptide. *Crit Rev Immunol 18:87.*

372. Markiewicz, M. A., F. Fallarino, A. Ashikari, and T. F. Gajewski. 2001. Epitope spreading upon P815 tumor rejection triggered by vaccination with the single class I MHC-restricted peptide P1A. *Int Immunol 13:625.*

373. Mazouz, N., A. Ooms, V. Moulin, S. Van Meirvenne, C. Uyttenhove, and G. Degiovanni. 2002. CD40 triggering increases the efficiency of dendritic cells for antitumoral immunization. *Cancer Immun 2:2.*

374. Weber, J., M. Salgaller, D. Samid, B. Johnson, M. Herlyn, N. Lassam, J. Treisman, and S. A. Rosenberg. 1994. Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. *Cancer Res 54:1766.*

375. Sarma, S., Y. Guo, Y. Guilloux, C. Lee, X. F. Bai, and Y. Liu. 1999. Cytotoxic T lymphocytes to an unmutated tumor rejection antigen P1A: normal development but restrained effector function in vivo. *J Exp Med 189:811.*

376. Crucian, B. E., L. C. Moscinski, M. Androlewicz, O. F. Ballester, R. H. Widen, and H. Yu. 1997. Assessment of intracellular TAP-1 and TAP-2 in conjunction with surface MHC class I in plasma cells from patients with multiple myeloma. *Br J Haematol 98:426.*

377. Fallarino, F., U. Grohmann, R. Bianchi, C. Vacca, M. C. Fioretti, and P. Puccetti. 2000. Th1 and Th2 cell clones to a poorly immunogenic tumor antigen initiate CD8+ T cell-dependent tumor eradication in vivo. *J Immunol 165:5495.*

378. Sarma, S., X. F. Bai, J. Q. Liu, K. F. May, Jr., P. Zheng, and Y. Liu. 2003. On the role of unmutated antigens in tumor rejection in mice with unperturbed T-cell repertoires. *Cancer Res 63:6051.*

379. Stamou, P., J. de Jersey, D. Carmignac, C. Mamalaki, D. Kioussis, and B. Stockinger. 2003. Chronic exposure to low levels of antigen in the periphery causes reversible functional impairment correlating with changes in CD5 levels in monoclonal CD8 T cells. *J Immunol 171:1278.*

380. Uyttenhove, C., J. Maryanski, and T. Boon. 1983. Escape of mouse mastocytoma P815 after nearly complete rejection is due to antigen-loss variants rather than immunosuppression. *J Exp Med 157:1040.*

381. Bai, X. F., J. Liu, O. Li, P. Zheng, and Y. Liu. 2003. Antigenic drift as a mechanism for tumor evasion of destruction by cytolytic T lymphocytes. *J Clin Invest 111:1487.*

382. Colmenero, P., M. Chen, E. Castanos-Velez, P. Liljestrom, and M. Jondal. 2002. Immunotherapy with recombinant SFV-replicons expressing the P815A tumor antigen or IL-12 induces tumor regression. *Int J Cancer 98:554.*

383. Yoo, J. K., J. H. Cho, S. W. Lee, and Y. C. Sung. 2002. IL-12 provides proliferation and survival signals to murine CD4+ T cells through phosphatidylinositol 3-kinase/Akt signaling pathway. *J Immunol 169:3637.*

384. Lan, M. S., S. K. Batra, W. N. Qi, R. S. Metzgar, and M. A. Hollingsworth. 1990. Cloning and sequencing of a human pancreatic tumor mucin cDNA. *J Biol Chem 265:15294.*

385. Correa, I., T. Plunkett, A. Vlad, A. Mungul, J. Candelora-Kettel, J. M. Burchell, J. Taylor-Papadimitriou, and O. J. Finn. 2003. Form and pattern of MUC1 expression on T cells activated in vivo or in vitro suggests a function in T-cell migration. *Immunology 108:32.*

386. Treon, S. P., J. A. Mollick, M. Urashima, G. Teoh, D. Chauhan, A. Ogata, N. Raje, J. H. Hilgers, L. Nadler, A. R. Belch, L. M. Pilarski, and K. C. Anderson. 1999. Muc-1 core protein is expressed on multiple myeloma cells and is induced by dexamethasone. *Blood 93:1287.*

387. Pemberton, L. F., A. Rughetti, J. Taylor-Papadimitriou, and S. J. Gendler. 1996. The epithelial mucin MUC1 contains at least two discrete signals specifying membrane localization in cells. *J Biol Chem 271:2332.*

388. Finn, O. J., K. R. Jerome, R. A. Henderson, G. Pecher, N. Domenech, J. Magarian-Blander, and S. M. Barratt-Boyes. 1995. MUC-1 epithelial tumor mucin-based immunity and cancer vaccines. *Immunol Rev 145:61.*

389. Parry, S., H. S. Silverman, K. McDermott, A. Willis, M. A. Hollingsworth, and A. Harris. 2001. Identification of MUC1 proteolytic cleavage sites in vivo. *Biochem Biophys Res Commun 283:715.*

390. Brossart, P., A. Schneider, P. Dill, T. Schammann, F. Grunebach, S. Wirths, L. Kanz, H. J. Buhring, and W. Brugger. 2001. The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. *Cancer Res 61:6846.*

391. Karanikas, V., L. A. Hwang, J. Pearson, C. S. Ong, V. Apostolopoulos, H. Vaughan, P. X. Xing, G. Jamieson, G. Pietersz, B. Tait, R. Broadbent, G. Thynne, and I. F. McKenzie. 1997. Antibody and T cell responses of patients with adenocarcinoma immunized with mannan-MUC1 fusion protein. *J Clin Invest 100:2783.*

392. Musselli, C., G. Ragupathi, T. Gilewski, K. S. Panageas, Y. Spinat, and P. O. Livingston. 2002. Reevaluation of the cellular immune response in breast cancer patients vaccinated with MUC1. *Int J Cancer 97:660.*

393. Ligtenberg, M. J., H. L. Vos, A. M. Gennissen, and J. Hilkens. 1990. Episialin, a carcinoma-associated mucin, is generated by a polymorphic gene encoding splice variants with alternative amino termini. *J Biol Chem 265:5573.*

394. Gendler, S. J., and A. P. Spicer. 1995. Epithelial mucin genes. *Annu Rev Physiol 57:607.*

395. Spicer, A. P., G. Parry, S. Patton, and S. J. Gendler. 1991. Molecular cloning and analysis of the mouse homologue of the tumor-associated mucin, MUC1, reveals conservation of potential O-glycosylation sites, transmembrane, and cytoplasmic domains and a loss of minisatellite-like polymorphism. *J Biol Chem 266:15099.*

396. Piller, V., F. Piller, F. G. Klier, and M. Fukuda. 1989. O-glycosylation of leukosialin in K562 cells. Evidence for initiation and elongation in early Golgi compartments. *Eur J Biochem 183:123.*

397. Muller, S., S. Goletz, N. Packer, A. Gooley, A. M. Lawson, and F. G. Hanisch. 1997. Localization of O-glycosylation sites on glycopeptide fragments from lactation-associated MUC1. All putative sites within the tandem repeat are glycosylation targets in vivo. *J Biol Chem 272:24780.*

398. Litvinov, S. V., and J. Hilkens. 1993. The epithelial sialomucin, episialin, is sialylated during recycling. *J Biol Chem 268:21364.*

399. Takahashi, T., Y. Makiguchi, Y. Hinoda, H. Kakiuchi, N. Nakagawa, K. Imai, and A. Yachi. 1994. Expression of MUC1 on myeloma cells and induction of HLA-unrestricted CTL against MUC1 from a multiple myeloma patient. *J Immunol 153:2102.*

400. Treon, S. P., P. Maimonis, D. Bua, G. Young, N. Raje, J. Mollick, D. Chauhan, Y. T. Tai, T. Hideshima, Y. Shima, J. Hilgers, S. von Mensdorff-Pouilly, A. R. Belch, L. M. Pilarski, and K. C. Anderson. 2000. Elevated soluble MUC1 levels and decreased anti-MUC1 antibody levels in patients with multiple myeloma. *Blood 96:3147.*

401. Dyomin, V. G., N. Palanisamy, K. O. Lloyd, K. Dyomina, S. C. Jhanwar, J. Houldsworth, and R. S. Chaganti. 2000. MUC1 is activated in a B-cell lymphoma by the t(1;14)(q21;q32) translocation and is rearranged and amplified in B-cell lymphoma subsets. *Blood 95:2666.*

402. Graham, R. A., J. M. Burchell, and J. Taylor-Papadimitriou. 1996. The polymorphic epithelial mucin: potential as an immunogen for a cancer vaccine. *Cancer Immunol Immunother 42:71.*

403. Zimmermann, G. L., M. J. Krantz, K. P. Kane, and B. M. Longenecker. 2000. A low-level expression of human MUC1 mucin enhances lethality of murine tumor cells. *Cancer Immunol Immunother 49:305.*

404. McDermott, K. M., P. R. Crocker, A. Harris, M. D. Burdick, Y. Hinoda, T. Hayashi, K. Imai, and M. A. Hollingsworth. 2001. Overexpression of MUC1 reconfigures the binding properties of tumor cells. *Int J Cancer 94:783.*

405. van de Wiel-van Kemenade, E., M. J. Ligtenberg, A. J. de Boer, F. Buijs, H. L. Vos, C. J. Melief, J. Hilkens, and C. G. Figdor. 1993. Episialin (MUC1) inhibits cytotoxic lymphocyte-target cell interaction. *J Immunol 151:767.*

406. Chan, A. K., D. C. Lockhart, W. von Bernstorff, R. A. Spanjaard, H. G. Joo, T. J. Eberlein, and P. S. Goedegebuure. 1999. Soluble MUC1 secreted by human epithelial cancer cells mediates immune suppression by blocking T-cell activation. *Int J Cancer 82:721.*

407. Brockhausen, I., J. M. Yang, J. Burchell, C. Whitehouse, and J. Taylor-Papadimitriou. 1995. Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells. *Eur J Biochem 233:607.*

408. Lloyd, K. O., J. Burchell, V. Kudryashov, B. W. Yin, and J. Taylor-Papadimitriou. 1996. Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines. Demonstration of simpler and fewer glycan chains in tumor cells. *J Biol Chem 271:33325.*

409. Muller, S., and F. G. Hanisch. 2002. Recombinant MUC1 probe authentically reflects cell-specific O-glycosylation profiles of endogenous breast cancer mucin. High density and prevalent core 2-based glycosylation. *J Biol Chem 277:26103.*

410. Beum, P. V., J. Singh, M. Burdick, M. A. Hollingsworth, and P. W. Cheng. 1999. Expression of core 2 beta-1,6-N-acetylglucosaminyltransferase in a human pancreatic cancer cell line results in altered expression of MUC1 tumor-associated epitopes. *J Biol Chem 274:24641.*

411. Hanisch, F. G., T. Schwientek, M. S. Von Bergwelt-Baildon, J. L. Schultze, and O. Finn. 2003. O-Linked glycans control glycoprotein processing by antigen-presenting cells: a biochemical approach to the molecular aspects of MUC1 processing by dendritic cells. *Eur J Immunol 33:3242.*

412. Apostolopoulos, V., E. Yuriev, P. A. Ramsland, J. Halton, C. Osinski, W. Li, M. Plebanski, H. Paulsen, and I. F. McKenzie. 2003. A glycopeptide in complex with MHC class I uses the GalNAc residue as an anchor. *Proc Natl Acad Sci U S A 100:15029.*

413. Vlad, A. M., S. Muller, M. Cudic, H. Paulsen, L. Otvos, Jr., F. G. Hanisch, and O. J. Finn. 2002. Complex carbohydrates are not removed during processing of glycoproteins by dendritic cells: processing of tumor antigen MUC1 glycopeptides for presentation to major histocompatibility complex class II-restricted T cells. *J Exp Med 196:1435.*

414. Haurum, J. S., G. Arsequell, A. C. Lellouch, S. Y. Wong, R. A. Dwek, A. J. McMichael, and T. Elliott. 1994. Recognition of carbohydrate by major

histocompatibility complex class I-restricted, glycopeptide-specific cytotoxic T lymphocytes. *J Exp Med 180:739.*

415. Jensen, T., P. Hansen, L. Galli-Stampino, S. Mouritsen, K. Frische, E. Meinjohanns, M. Meldal, and O. Werdelin. 1997. Carbohydrate and peptide specificity of MHC class II-restricted T cell hybridomas raised against an O-glycosylated self peptide. *J Immunol 158:3769.*

416. Hiltbold, E. M., A. M. Vlad, P. Ciborowski, S. C. Watkins, and O. J. Finn. 2000. The mechanism of unresponsiveness to circulating tumor antigen MUC1 is a block in intracellular sorting and processing by dendritic cells. *J Immunol 165:3730.*

417. Hiltbold, E. M., M. D. Alter, P. Ciborowski, and O. J. Finn. 1999. Presentation of MUC1 tumor antigen by class I MHC and CTL function correlate with the glycosylation state of the protein taken Up by dendritic cells. *Cell Immunol 194:143.*

418. von Mensdorff-Pouilly, S., M. M. Gourevitch, P. Kenemans, A. A. Verstraeten, S. V. Litvinov, G. J. van Kamp, S. Meijer, J. Vermorken, and J. Hilgers. 1996. Humoral immune response to polymorphic epithelial mucin (MUC-1) in patients with benign and malignant breast tumours. *Eur J Cancer 32A:1325.*

419. Adluri, S., T. Gilewski, S. Zhang, V. Ramnath, G. Ragupathi, and P. Livingston. 1999. Specificity analysis of sera from breast cancer patients vaccinated with MUC1-KLH plus QS-21. *Br J Cancer 79:1806.*

420. Gilewski, T., S. Adluri, G. Ragupathi, S. Zhang, T. J. Yao, K. Panageas, M. Moynahan, A. Houghton, L. Norton, and P. O. Livingston. 2000. Vaccination of high-risk breast cancer patients with mucin-1 (MUC1) keyhole limpet hemocyanin conjugate plus QS-21. *Clin Cancer Res 6:1693.*

421. Jerome, K. R., N. Domenech, and O. J. Finn. 1993. Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. *J Immunol 151:1654.*

422. Ioannides, C. G., B. Fisk, K. R. Jerome, T. Irimura, J. T. Wharton, and O. J. Finn. 1993. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. *J Immunol 151:3693.*

423. Noto, H., T. Takahashi, Y. Makiguchi, T. Hayashi, Y. Hinoda, and K. Imai. 1997. Cytotoxic T lymphocytes derived from bone marrow mononuclear cells of multiple myeloma patients recognize an underglycosylated form of MUC1 mucin. *Int Immunol 9:791.*

424. Magarian-Blander, J., P. Ciborowski, S. Hsia, S. C. Watkins, and O. J. Finn. 1998. Intercellular and intracellular events following the MHC-unrestricted TCR recognition of a tumor-specific peptide epitope on the epithelial antigen MUC1. *J Immunol 160:3111.*

425. Apostolopoulos, V., G. A. Pietersz, P. X. Xing, C. J. Lees, M. Michael, J. Bishop, and I. F. McKenzie. 1995. The immunogenicity of MUC1 peptides and fusion protein. *Cancer Lett 90:21.*

426. Soares, M. M., V. Mehta, and O. J. Finn. 2001. Three different vaccines based on the 140-amino acid MUC1 peptide with seven tandemly repeated tumor-specific epitopes elicit distinct immune effector mechanisms in wild-type versus MUC1-transgenic mice with different potential for tumor rejection. *J Immunol 166:6555.*

427. Chung, M. A., Y. Luo, M. O'Donnell, C. Rodriguez, W. Heber, S. Sharma, and H. R. Chang. 2003. Development and preclinical evaluation of a Bacillus Calmette-Guerin-MUC1-based novel breast cancer vaccine. *Cancer Res 63:1280.*

428. Samuel, J., W. A. Budzynski, M. A. Reddish, L. Ding, G. L. Zimmermann, M. J. Krantz, R. R. Koganty, and B. M. Longenecker. 1998. Immunogenicity and antitumor activity of a liposomal MUC1 peptide-based vaccine. *Int J Cancer 75:295.*

429. Johnen, H., H. Kulbe, and G. Pecher. 2001. Long-term tumor growth suppression in mice immunized with naked DNA of the human tumor antigen mucin (MUC1). *Cancer Immunol Immunother 50:356.*

430. Graham, R. A., J. M. Burchell, P. Beverley, and J. Taylor-Papadimitriou. 1996. Intramuscular immunisation with MUC1 cDNA can protect C57 mice challenged with MUC1-expressing syngeneic mouse tumour cells. *Int J Cancer 65:664.*

431. Gong, J., L. Chen, D. Chen, M. Kashiwaba, Y. Manome, T. Tanaka, and D. Kufe. 1997. Induction of antigen-specific antitumor immunity with adenovirus-transduced dendritic cells. *Gene Ther 4:1023.*

432. Koido, S., M. Kashiwaba, D. Chen, S. Gendler, D. Kufe, and J. Gong. 2000. Induction of antitumor immunity by vaccination of dendritic cells transfected with MUC1 RNA. *J Immunol 165:5713.*

433. Apostolopoulos, V., G. A. Pietersz, B. E. Loveland, M. S. Sandrin, and I. F. McKenzie. 1995. Oxidative/reductive conjugation of mannan to antigen selects for T1 or T2 immune responses. *Proc Natl Acad Sci U S A 92:10128.*

434. Tempero, R. M., G. J. Rowse, S. J. Gendler, and M. A. Hollingsworth. 1999. Passively transferred anti-MUC1 antibodies cause neither autoimmune disorders nor immunity against transplanted tumors in MUC1 transgenic mice. *Int J Cancer 80:595.*

435. Apostolopoulos, V., G. A. Pietersz, and I. F. McKenzie. 1996. Cell-mediated immune responses to MUC1 fusion protein coupled to mannan. *Vaccine 14:930.*

436. Apostolopoulos, V., J. S. Haurum, and I. F. McKenzie. 1997. MUC1 peptide epitopes associated with five different H-2 class I molecules. *Eur J Immunol 27:2579.*

437. Pietersz, G. A., W. Li, C. Osinski, V. Apostolopoulos, and I. F. McKenzie. 2000. Definition of MHC-restricted CTL epitopes from non-variable number of tandem repeat sequence of MUC1. *Vaccine 18:2059.*

438. Mukherjee, P., A. R. Ginardi, C. S. Madsen, C. J. Sterner, M. C. Adriance, M. J. Tevethia, and S. J. Gendler. 2000. Mice with spontaneous pancreatic cancer naturally develop MUC-1-specific CTLs that eradicate tumors when adoptively transferred. *J Immunol 165:3451.*

439. Gerloni, M., S. Xiong, S. Mukerjee, S. P. Schoenberger, M. Croft, and M. Zanetti. 2000. Functional cooperation between T helper cell determinants. *Proc Natl Acad Sci U S A 97:13269.*

440. Kontani, K., O. Taguchi, Y. Ozaki, J. Hanaoka, N. Tezuka, S. Sawai, S. Inoue, S. Fujino, T. Maeda, Y. Itoh, K. Ogasawara, H. Sato, I. Ohkubo, and T. Kudo. 2002. Novel vaccination protocol consisting of injecting MUC1 DNA and nonprimed dendritic cells at the same region greatly enhanced MUC1-specific antitumor immunity in a murine model. *Cancer Gene Ther 9:330.*

441. Tempero, R. M., M. L. VanLith, K. Morikane, G. J. Rowse, S. J. Gendler, and M. A. Hollingsworth. 1998. CD4+ lymphocytes provide MUC1-specific tumor immunity in vivo that is undetectable in vitro and is absent in MUC1 transgenic mice. *J Immunol 161:5500.*

442. Sivinski, C. L., K. G. Kohlgraf, M. L. VanLith, K. Morikane, R. M. Tempero, and M. A. Hollingsworth. 2002. Molecular requirements for CD8-mediated rejection of a MUC1-expressing pancreatic carcinoma: implications for tumor vaccines. *Cancer Immunol Immunother 51:327.*

443. Rowse, G. J., R. M. Tempero, M. L. VanLith, M. A. Hollingsworth, and S. J. Gendler. 1998. Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. *Cancer Res 58:315.*

444. Smith, M., J. M. Burchell, R. Graham, E. P. Cohen, and J. Taylor-Papadimitriou. 1999. Expression of B7.1 in a MUC1-expressing mouse mammary epithelial tumour cell line inhibits tumorigenicity but does not induce autoimmunity in MUC1 transgenic mice. *Immunology 97:648.*

445. Gong, J., D. Chen, M. Kashiwaba, Y. Li, L. Chen, H. Takeuchi, H. Qu, G. J. Rowse, S. J. Gendler, and D. Kufe. 1998. Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. *Proc Natl Acad Sci U S A 95:6279.*

446. Carr-Brendel, V., D. Markovic, K. Ferrer, M. Smith, J. Taylor-Papadimitriou, and E. P. Cohen. 2000. Immunity to murine breast cancer cells modified to express MUC-1, a human breast cancer antigen, in transgenic mice tolerant to human MUC-1. *Cancer Res 60:2435.*

447. Lees, C. J., V. Apostolopoulos, B. Acres, I. Ramshaw, A. Ramsay, C. S. Ong, and I. F. McKenzie. 2000. Immunotherapy with mannan-MUC1 and IL-12 in MUC1 transgenic mice. *Vaccine 19:158.*

448. Barratt-Boyes, S. M., A. Vlad, and O. J. Finn. 1999. Immunization of chimpanzees with tumor antigen MUC1 mucin tandem repeat peptide elicits both helper and cytotoxic T-cell responses. *Clin Cancer Res 5:1918.*

449. Pecher, G., and O. J. Finn. 1996. Induction of cellular immunity in chimpanzees to human tumor-associated antigen mucin by vaccination with MUC-1 cDNA-transfected Epstein-Barr virus-immortalized autologous B cells. *Proc Natl Acad Sci U S A 93:1699.*

450. Xing, P.-X., M. Michael, V. Apostolopoulos, J. Prenzoska, C. Marshall, J. Bishop, and I. F. C. McKenzie. 1995. Phase I study of synthetic MUC1 peptides in breast cancer. *International Journal of Oncology 6:1283.*

451. Cohen, C. J., N. Hoffmann, M. Farago, H. R. Hoogenboom, L. Eisenbach, and Y. Reiter. 2002. Direct detection and quantitation of a distinct T-cell epitope derived from tumor-specific epithelial cell-associated mucin using human recombinant antibodies endowed with the antigen-specific, major histocompatibility complex-restricted specificity of T cells. *Cancer Res 62:5835.*

452. Carmon, L., K. M. El-Shami, A. Paz, S. Pascolo, E. Tzehoval, B. Tirosh, R. Koren, M. Feldman, M. Fridkin, F. A. Lemonnier, and L. Eisenbach. 2000. Novel breast-tumor-associated MUC1-derived peptides: characterization in Db-/- x beta2 microglobulin (beta2m) null mice transgenic for a chimeric HLA-A2.1/Db-beta2 microglobulin single chain. *Int J Cancer 85:391.*

453. Brossart, P., K. S. Heinrich, G. Stuhler, L. Behnke, V. L. Reichardt, S. Stevanovic, A. Muhm, H. G. Rammensee, L. Kanz, and W. Brugger. 1999. Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. *Blood 93:4309.*

454. Heukamp, L. C., S. H. van der Burg, J. W. Drijfhout, C. J. Melief, J. Taylor-Papadimitriou, and R. Offringa. 2001. Identification of three non-VNTR MUC1-derived HLA-A*0201-restricted T-cell epitopes that induce protective anti-tumor immunity in HLA-A2/K(b)-transgenic mice. *Int J Cancer 91:385.*

455. Apostolopoulos, V., V. Karanikas, J. S. Haurum, and I. F. McKenzie. 1997. Induction of HLA-A2-restricted CTLs to the mucin 1 human breast cancer antigen. *J Immunol 159:5211.*

456. Brossart, P., S. Wirths, G. Stuhler, V. L. Reichardt, L. Kanz, and W. Brugger. 2000. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. *Blood 96:3102.*

457. Plunkett, T., R. Graham, I. Correa, R. Sewell, D. Miles, J. Burchell, and J. Taylor-Papadimitriou. 2004. Protection against MUC1 expressing mouse tumours by intra-muscular injection of MUC1 cDNA requires functional CD8+ and CD4+ T cells but does not require the MUC1 tandem repeat domain. *Int J Cancer 109:691.*

458. Engelhard, V. H. 1994. Structure of peptides associated with MHC class I molecules. *Curr Opin Immunol 6:13.*

459. Rammensee, H. G., T. Friede, and S. Stevanoviic. 1995. MHC ligands and peptide motifs: first listing. *Immunogenetics 41:178.*

460. Hiltbold, E. M., M. D. Alter, P. Ciborowski, and O. J. Finn. 1999. Presentation of MUC1 tumor antigen by class I MHC and CTL function correlate with the glycosylation state of the protein taken Up by dendritic cells. In *Cell Immunol*, Vol. 194, p. 143.

461. Heukamp, L. C., T. van Hall, F. Ossendorp, J. M. Burchell, C. J. Melief, J. Taylor-Papadimitriou, and R. Offringa. 2002. Effective immunotherapy of cancer in MUC1-transgenic mice using clonal cytotoxic T lymphocytes directed against an immunodominant MUC1 epitope. *J Immunother 25:46.*

462. Apostolopoulos, V., I. F. McKenzie, and I. A. Wilson. 2001. Getting into the groove: unusual features of peptide binding to MHC class I molecules and implications in vaccine design. *Front Biosci 6:D1311.*

463. Gotoh, M., H. Takasu, K. Harada, and T. Yamaoka. 2002. Development of HLA-A2402/Kb transgenic mice. *Int J Cancer 100:565.*

464. Firat, H., F. Garcia-Pons, S. Tourdot, S. Pascolo, A. Scardino, Z. Garcia, M. L. Michel, R. W. Jack, G. Jung, K. Kosmatopoulos, L. Mateo, A. Suhrbier, F. A. Lemonnier, and P. Langlade-Demoyen. 1999. H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies. *Eur J Immunol 29:3112.*

465. Ramage, J. M., R. Metheringham, R. Moss, I. Spendlove, R. Rees, and L. G. Durrant. 2004. Comparison of the immune response to a self antigen after DNA immunisation of HLA*A201/H-2Kb and HHD transgenic mice. *Vaccine 22:1728.*