UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Developing DNA Vaccines against the Cancer-Related Prostate-Specific Membrane Antigen

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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Vaccines are able to activate many compartments of the immune system in murine settings of infectious diseases and cancer. However, they have thus far had limited success in human trials. The two major problems for cancer are the weak immunogenicity of known target antigens and potential immune tolerance. To overcome these, our laboratory has developed novel DNA fusion vaccines encoding tumour antigens fused to a pathogen-derived sequence from tetanus toxin. The strategy is to activate linked T-cell help from the large anti-microbial repertoire for anti-tumour immunity by using the entire or a partial sequence of Fragment C (FrC) of tetanus toxin.

The aim of this study was to investigate the potential of the DNA fusion vaccine strategy for prostate cancer (PCa). PCa cells express a number of antigens that may serve as targets for vaccine-based therapeutic strategies. The prostate-specific membrane antigen (PSMA) tumour antigen is an attractive immunotherapeutic target due to the several-fold increased expression in the cancer as well as restricted expression in other tissues.

PSMA-derived HLA-A2-binding peptides have been used in clinical trials despite limited knowledge of MHC class I-associated expression by tumour cells, a pre-requisite for successful targeting by cytotoxic T lymphocytes (CTL). To address this, DNA fusion vaccines in which discrete human PSMA-derived HLA-A2-binding epitope sequences (PSMA4, PSMA27, PSMA663 and PSMA711) are genetically fused to a domain (DOM) from FrC were made. The fusion vaccines were tested for their ability to induce epitope-specific $CD8^+$ T cells in HLA-A2 (HHD) transgenic mice. $CD8^+$ T-cell responses against the specific human PSMA epitopes were induced, as assessed by *ex vivo* IFNγ ELISPOT and *in vitro* CTL killing assays. Processing and presentation of the four peptides by tumour cells from an endogenous route, and tumour cell killing by CTL were shown. For PSMA711, only high avidity CTL could kill target cells, suggesting this peptide is presented at lower levels. These pre-clinical data will form the basis for the testing of the vaccines in patients.

Full-length vaccines may also prime $CD8^+$ T cells and would be applicable to patients of all MHC Class I haplotypes. However, we show that only low levels of $CD8^+$ T cell immunity are generated by the full-length human PSMA vaccine. The work presented therefore does not support the full-length vaccine approach and demonstrates the superiority of our novel vaccine design for priming $CD8^+$ T cells.

To address the question of tolerance, a DOM fusion vaccine encoding the mouse PSMA MHC class I-binding peptide, mPSMA636, was created. This vaccine successfully overcame tolerance in mice to prime peptide-specific $CD8^+$ T cells that effectively lysed mouse PSMA-expressing tumour cells *in vitro*.

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Publications

Presentation directly relating to work presented in this thesis:

DNA Vaccination Against Prostate Cancer Tumour Antigen PSMA
G.E. Vittes, K. Harwood, J. Rice, C. Ottensmeier, D. Zhu, and F. Stevenson
Poster Abstract: NCRI Conference 2005

Publication accepted during period of study (not directly related to work presented in this thesis):

DNA Vaccination Induces WT1-Specific T-Cell Responses With Potential Clinical Relevance
Chaise C, Buchan SL, Rice J, Marquet J, Rouard H, Kuentz M, **Vittes GE**, Molinier-Frenkel V, Farcet JP, Stauss HJ, Delfau-Larue MH, Stevenson FK
Blood 2008 (In Press)

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AICD	Activation-induced cell death
APC	Allophycocyanin
APC	Antigen presenting cells
AR	Androgen receptor
B cell	B lymphocyte
BCL	B-cell lymphoma
BCR	B cell receptor
BM	Bone Marrow
BPH	Benign hyperplasia
C-terminus	Carboxyl-terminus
CCR	Chemotactic chemokine receptor
CD	Cluster designation
CDR	Complementarity determining region
CEA	Carcinoembryonic antigen
CLIP	Class II-associated invariant chain
CLP	Common lymphoid progenitor
CpG	Cytidine-phosphate guanosine
CR	Complement receptor
CTL	Cytotoxic T cell
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
DOM1	N-terminal domain of FrC ($TT_{865-1120}$)
DOM2	C-terminal domain of FrC ($TT_{1121-1316}$)
DRiP	Defective ribosomal products
EBV	Epstein Barr Virus
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunospot
EP	Electroporation
ER	Endoplasmic Reticulum
E:T	Effector: Target
FACs	Fluorescence-activated cell sorting
FcR	Fc Receptor
FCS	Foetal calf serum

FITC	Fluorescin isothiocyanate
FOLH1	Folate Hydrolase I
FrC	Fragment C (of TT; $TT_{865-1316}$)
GC	Germinal centre
GCPII	Glutamate Carboxypeptidase II
GFP	Green fluorescence protein
GM-CSF	Granulocyte-monocyte colony stimulating factor
GvL	Graft vs leukaemia
HA	Hemagglutinin
HEV	High endothelial venule
HLA	Human leukocyte antigen
HRPC	Hormone refractory prostate cancer
ICAM	Intracellular adhesion molecule
ICOS	Inducible co-stimulator
Id	Idiotype
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
LCMV	Lymphocytic choriomeningitis virus
LIGHT	Ligand for HVEM
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MPP	Multi-potent progenitor
mTEC	Medullary thymic epithelial cell
N-terminus	Amino terminus
NAAG	Acetylaspartyl glutamate
NAALADaseI	N-acetylated-α-linked acidic peptidase
NK cell	Natural killer cell
NO	Nitric oxide
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCa	Prostate cancer

PCR	Polymerase chain reaction
PCTA-1	Prostate carcinoma tumour antigen
PE	Phycoerythrin
PGE	Promiscuous gene expression
PLC	Peptide loading complex
PRR	Pathogen recognition receptor
PSA	Prostate specific antigen
PSADT	Prostate specific antigen doubling time
PSCA	Prostate stem cell antigen
PSGR	Prostate specific G-protein
PSM'	Prostate specific membrane antigen (cytosolic splice variant)
PSMA	Prostate specific membrane antigen (full-length TM variant)
Rb	Retinoblastoma tumour suppressor
REC	Recycling endosomal compartment
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
scFv	Single chain Fragment variable
SDM	Site-directed mutagenesis
SEM	Standard error of the mean
STEAP	Six transmembrane epithelial antigen
T cell	T Lymphocyte
Tag	SV40 large tumour T-antigen
TAP	Transporter associated with antigen presentation
TCR	T cell receptor
T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells
TfR	Transferrin receptor
Tg	Transgenic
TGF	Transforming growth factor
$T_{H1/2/3}$	$CD4^+$ helper T cell
TILs	Tumour infiltrating lymphocytes
TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor related apoptosis-inducing ligand
TRAMP	Transgenic adenocarcinoma mouse prostate model
Treg	Regulatory T cells
TT	Tetanus toxin
VEGF	Vascular endothelial growth factor

1. Introduction

All cells of the immune system are derived from pluripotent bone marrow-resident $CD34^+$ hematopoietic stem cells (HSCs) by the process of hematopoiesis. Multi-lineage differentiations are tightly regulated through interactions with bone marrow (BM) stromal cells and extracellular matrix. Once committed to differentiation, HSCs progressively lose the capacity to self-renew and give rise to multi-potent progenitors (MPPs). As the name suggests these have multi-lineage differentiation potential and can further differentiate into cells of myeloid or lymphoid lineages ([1] with critical roles in innate and adaptive immunity respectively.

1.1 Innate Immunity

The innate immune response is the first line of defence against pathogens. It produces a rapid but non-specific response that is unaltered with further pathogen encounters. The cells of the innate immune system include granulocytes (eosinophils, basophils, neutrophils and mast cells), monocytes, natural killer (NK) cells and macrophages. Granulocytes are amongst the first cells to migrate to the site of inflammation and actively recruit other immune cells, including those of the adaptive immune response.

In the BM VCAM-1^+MPPs have a high granulocyte/macrophage differentiation potential and thus give rise to common myeloid progenitors (CMP). These differentiate into cells of the granulocyte/monocyte lineage and are released from the BM in a fully matured and functional state. Monocytes can develop into macrophages or act as precursors for dendritic cells (DCs). Upon infection the secretion of cytokines such as GM-CSF, increase the production of these cells leading to elevated numbers in the circulation ready to migrate into the site of inflammation.

Monocytes, macrophages and neutrophils are professional phagocytic cells that can take up and eliminate pathogens within lytic organelles. Activated macrophages and neutrophils can also directly damage pathogens or infected cells via the generation of nitric oxide (NO) or cytokines. Monocytes are able to efficiently remove cells undergoing apoptosis resulting in the removal of potentially inflammatory products. Macrophages secrete cytokines such as TGFβ and TNFα which contribute to the resolution of inflammation, produce extracellular matrix proteins for wound healing [2] as well as enhance cell proliferation and neovascularisation for tissue renewal. Cytokines, chemokines and

hematopoietic growth factors secreted by activated cells of the innate immune system generally serve to amplify defence mechanisms. For example IL-1, IFNα and TNFα recruit and activate phagocytes by increasing chemotaxis.

Initial activation of macrophages is induced by IFNγ produced by cells of the innate immune system such as NK cells. Importantly all phagocytic cells uptake opsonised or apoptotic material and present it via the MHC to generate adaptive immunity. Additionally IL-12 secreted by macrophages drives T-cell differentiation towards a T_{H1} phenotype and induces NK cells to secrete IFNγ. At this point the main source of IFNγ becomes antigen-specific T cells. A second important activation signal for all phagocytic cells is delivered by the recognition of pathogen-associated molecular patterns (PAMPs) on pathogens or damage-associated molecular patterns (DAMPs, also known as alarmins) on dying cells. These are detected via sets of pathogen recognition receptors (PRRs). The PAMP/DAMP interaction results in the phagocytosis of micro organisms or apoptotic cells and secretion of interferons (IFNs), chemokines and hematopoietic growth factors that together function to amplify the response. The widely known PRRs are Toll-like receptors (TLRs), scavenger receptors and lectin receptors. The roles of these receptors in detecting pathogens with respect to DCs have been reviewed by Granucci et al. 2008 [3].

TLRs are transmembrane proteins that recognise conserved structural motifs from a wide range of microbial intruders as well as some self-proteins (i.e. HSPs). To date, 13 TLRs with distinct specificity have been identified in mammals (Table 1, reviewed by [4]. TLRs are located on the plasma membrane or at intracellular sites. TLR signalling is mediated through an intracellular Toll/IL-1R (TIR) domain that associates with adaptor proteins such as myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adaptor inducing IFNβ (TRIF). The signalling cascades lead to the activation of transcription factors NFκB, IRFs and activation protein (AP)-1 that act on interferon stimulatory response elements (ISRE) for production of type I interferons and κB sites for the production of a wide range of pro-inflammatory cytokines (IL1β, IL-6, IL-12, TNF, IFNα and IFNβ) and expression of co-stimulatory molecules. Both are essential for the maturation of immune cells necessary for the induction of adaptive immunity. TLR signalling is tightly regulated as inappropriate activation of TLRs can lead to autoimmune disorders and chronic inflammation, conditions associated with a number of cancers.

TLR	Species	Cellular Location	Ligands
TLR1	Human & Mouse	Surface	Diacyl lipopeptide (bacteria)
TLR2	Human & Mouse	Surface	Triacyl lipopeptides (bacteria), zymosan (fungi)
TLR3	Human & Mouse	Intracellular	dsRNA (virus), Poly-IC, viral RNA (viral)
TLR4	Human & Mouse	Surface	LPS (bacteria), HSP60, HSP70, F protein (from respiratory syncytial virus), vesicular somatitis virus G protein (VSV-G)
TLR5	Human & Mouse	Surface	Flagellin (bacteria)
TLR6	Human & Mouse	Surface	Triacyl lipopeptides (bacteria), zymosan (fungi)
TLR7	Human & Mouse	Intracellular	ssRNA (viral), imidazoquinolines
TLR8	Human & Mouse	Intracellular	ssRNA (viral), imidazoquinolines
TLR9	Human & Mouse	Intracellular	CpG-ODN
TLR10	Human	Surface	Unknown
TLR11	Mouse	Surface	Profilin (protozoa)
TLR12	Mouse	Unknown	Unknown
TLR13	Mouse	Unknown	Unknown

Table 1: Toll-like receptors

The 13 TLRs thus far identified in mammals, their cellular location and known ligands

The C-type lectin receptors are expressed on the surface of phagocytes and function to recognise pathogenic carbohydrate moieties. These include dectin-1 and the macrophage-expressed mannose receptor. The former is involved in fungi recognition (β-glucans) whilst the latter additionally recognises bacterial and fungal glycoproteins.

There are also intracellular PRRs such as the NOD-like receptors (NLRs). These exist in the cytosol in an inactive state but upon PAMP binding change conformation to expose domains that can interact with signalling effector molecules such as adaptor proteins, caspases and kinases, ultimately resulting in phagocytosis and activation of NFκB/MAPK pathways (pro-inflammatory cytokine production). Their location suggests a possible role in detecting microbes escaping detection by extracellular receptors.

Phagocytes also express opsonic receptors. The classic example is the CR1 receptor that mediates endocytosis of antigens opsonised by complement protein C3b. The complement cascade can be triggered in a process where each step is a catalyst for the next. The end stage is the formation of the membrane attack complex (MAC), an insertion of hydrophobic proteins which lyses the target cells by disrupting the osmotic balance. Another example is the mannose binding lectin produced by the liver which circulates and binds mannose on bacteria to facilitate recognition/ingestion by phagocytes or triggers the mannan binding lectin complement pathway. The complement pathways are differentially

triggered but all converge at the C3 stage and result in opsonisation of target and the recruitment of inflammatory cells.

NK cells ($CD3^-TCR^-CD16^+CD56^+$) are a unique set of large granular lymphocytes that represent 5-15% of human peripheral blood lymphocytes. Through the production of immunostimulatory cytokines and direct cytotoxicity they play a major role in rejection of cells infected by viruses. IL-12 induces IFNγ secretion by NK cells whilst chemokines and type I IFNs produced during inflammation drive NK cell migration to peripheral tissues.
NK cells express activating and inhibitory receptors (reviewed by [5]); the killer immunoglobulin-like receptors (KIRs), C-type lectin receptors (NKG2A-E in humans, Ly49 in mice), TLRs and the natural cytotoxicity receptors (NKp30, NKp33 and NKp36). The latter are exclusive to NK cells but their ligands remain unidentified. The C-type lectin receptors are mostly inhibitory and detect expression of HLA-E, HLA-G and non-classical MHC class I homologs (MICA/B). KIRs can be activating or inhibiting in their nature and their predominant ligand is the HLA class-I molecule. The integration of tightly regulated signals from both activating and inhibitory receptors is required for target-cell recognition. Abnormal cells deficient in MHC class I or over-expressing ligands for activating receptors are susceptible to NK-mediated lysis. NK cells also mediate killing of target cells by antibody dependent cellular cytotoxicity (ADCC) through expression of FcγRIII (CD16), triggering apoptosis by the release of perforin and granzymes or by engagement of death receptors (Fas and TRAIL). In contrast to T cells, mature NK cells constitutively express effector molecules and are therefore poised ready for action [6].
NK cells have a role in the killing of tumour cells; those that downregulate MHC class I expression to evade killing by T cells become susceptible to NK-cell lysis. In mice NK cells have been shown to be involved in the rejection of experimentally and spontaneously developing tumours [7]. Human NK cells adoptively transferred into mice can reject human tumours [8]. However, despite the evidence in mouse models there is limited clinical efficacy of activated NK cells adoptively transferred into cancer patients.

1.2 Adaptive Immunity

The adaptive immune response is a slower but highly specific one. This response involves the amplification of lymphocytes that express receptors specific for the antigen (clonal selection). Both B and T cells contribute to the humoral (antibody) and cellular components of adaptive immunity. An important trait of adaptive immunity is the

generation of immunological memory allowing a rapid highly specific secondary response that confers efficient protection against infection. Lymphocytes develop in primary lymphoid tissues and undergo activation to generate immune responses in the optimal microenvironment of secondary lymphoid organs. Healthy individuals have polyclonal populations of both T and B cells whilst clonal populations accumulate with age but are also indicative of disease such as malignancies, infectious diseases and autoimmunity.

1.2.1 B Lymphocytes

B cells constitute ~15% of peripheral blood leukocytes. They express a B cell receptor (BCR) that engages with soluble antigen to trigger activation and subsequent immunoglobulin (Ig) secretion. B cells originate and undergo a differentiation programme in the BM. Early in the hematopoietic process VCAM-1$^-$ MPPs differentiate into common lymphoid progenitors (CLPs). Stromal IL-7 signalling is the major player in B-cell commitment [9] and coordinates with the expression of IL-7Rα by CLPs committed to B-cell differentiation [10]. CLPs further differentiate into various intermediates and finally into a mature naive B cell.

1.2.1.1 B Cell Receptor (BCR) Rearrangement and B Cell Development

The BCR is composed of an Ig heterodimer of two identical heavy (H) and light (L) chains co-expressed and assembled with Igα (CD79a) and Igβ (CD79b) signal transduction molecules. The H and L chains are each composed of a variable (V) region that confers antigen specificity (hypervariable region) as well as constant (C) regions that affect biological function. The H chain variable region is itself created by the somatic joining of three gene segments; variable ($V_{H1\text{-}51}$) diversity ($D_{H1\text{-}27}$) and joining ($J_{H1\text{-}6}$) regions in a process known as V(D)J recombination (Figure 1). The H chain constant region ($C_{H1\text{-}9}$) confers the isotype specificity of the Ig. Only one of the two L chain loci (κ or λ) is used by the Ig. The L chain antigen-binding variable region is composed of a variable (V_L; $V_L\kappa_{1\text{-}40}$ or $V_L\lambda_{1\text{-}31}$) and joining (J_L; $J_L\kappa_{1\text{-}5}$ or $V_L\lambda_{1\text{-}4}$) gene segments. The immense diversity in BCR specificity is created by the possible permutations of the large number of gene segments in each of these regions and for each chain, inaccurate splicing, the addition of random nucleotides during the process of V(D)J recombination and the ability to read D-segments in three reading frames.

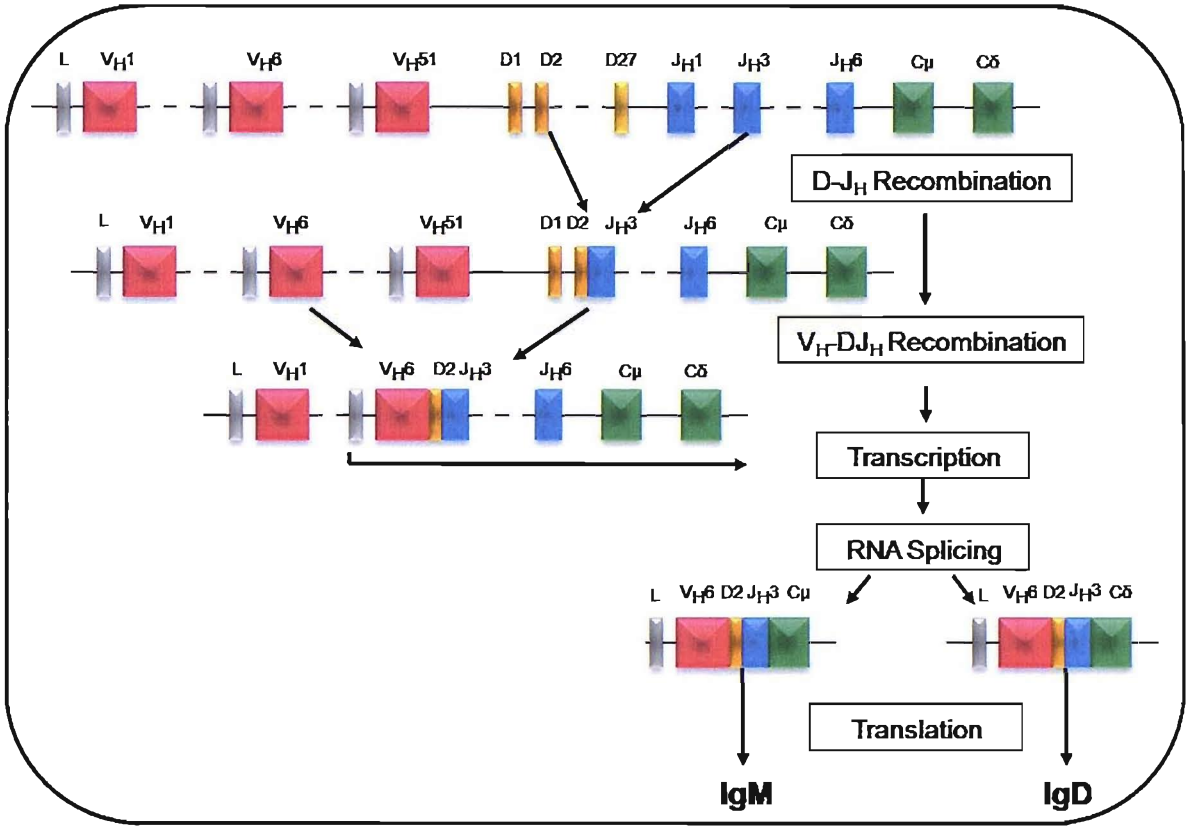


Figure 1: V(D)J Recombination of the heavy (H) chain

The H chain of the BCR Ig is created by the stepwise process of V(D)J recombination that commences in the pro-B cells and is mediated by V(D)J recombinase enzymes. The V (red), D (yellow) and J (blue) segments encode the antigen-recognising variable regions whilst the C (green) segments encode the constant regions of the Ig. Alternative splicing of the mRNA results in the translation of both IgM and IgD molecules.

The process of V(D)J recombination takes place in a step-wise manner mediated by V(D)J recombinase enzymes RAG-1/RAG-2 and terminal deoxynucleotidyl transferase (TdT, see TCR rearrangement section 1.2.2.1.). Pro-B cells are phenotypically defined by the expression of IL-7Rα and D-J_H DNA rearrangement. After an IL-7 signal from BM stromal cells V_H-DJ_H rearrangement is induced, encoding the entire H chain variable region (Figure 1). If the rearrangement is non-productive the process is repeated on the second allele, and if this is unsuccessful the pro-B cell undergoes apoptosis. The productive transcript includes both Cμ and Cδ (IgM and IgD constant regions) but the mRNA undergoes splicing resulting in the expression of only the H chain Cμ in the pre-BCR. At this pre-B cell stage the pre-BCR already assembles with Igα and Igβ signal transduction molecules and a surrogate light chain, signalling for the suppression of further V_H rearrangements (allelic exclusion) and the initiation light chain rearrangement commencing at the Igκ locus. This κ chain transcript incorporates the Cκ gene and is assembled at the surface of what is now an immature B cell with a functional BCR with a membrane Ig. If the Igκ light chain rearrangement fails on both alleles then

rearrangements start on the Igλ locus. A number of markers are associated with each of the B-cell differentiation steps (e.g. $Flt3^-$, $B220^+$, $CD19^+$, c-Kit^+ pro-B cells). The transcription factor Pax5 is able to activate essential components involved in B-cell differentiation whilst simultaneously repressing inappropriate gene expression [11].

Immature B cells become independent of BM stromal cells and are able to exit the BM. They undergo negative selection before leaving the BM to avoid the production of self-reactive B cells. If the BCR binds self-antigen expressed by stromal cells of the BM the developing B cell undergoes apoptosis. However, the BCRs can be edited by further rearrangements of the L chains in an attempt to alter the specificity and avoid programmed cell death. The emigrating B cells are termed functionally immature transitional B cells [12]. There are two stages of transitional B cells (T1 and T2). T2 B cells co-expresses IgM and IgD with the same antigen specificity (same rearranged V_H gene undergoes alternative RNA splicing). T2 B cells can be positively selected by antigen into the long-lived mature pool by virtue of their functional BCR, downstream signalling, and ability to respond to T-cell help [13]. The periphery contains marginal zone B cells or follicular B cells, each subset with distinct anatomic localisation. Naïve B cells die within a few weeks if they fail to encounter antigen in the periphery.

1.2.1.2 B Cell Activation

The BCR assembles with a co-receptor consisting of a CD19, CD21 (CR2) and CD81 (TAPA-1) complex (Figure 2). Activation of peripheral naïve B cells involves BCR cross-linking (Figure 2), the B cell co-receptor and T-cell help. B-cell activation occurs in secondary lymphoid organs that filter antigens out from blood or lymphatic vessels. Cross-linking of multiple BCRs (signal 1) initiates the recruitment and activation of the signalling complex (signalosome). Interaction between immunoreceptor tyrosine based activation motifs (ITAMs) on the Igα/Igβ heterodimer and the src family of tyrosine kinases activate signalling cascades. Phosphorylation of Igα and Igβ creates docking sites for the tyrosine kinases which then phosphorylate the cytoplasmic tail of CD19. This permits CD19 to bind other signalling molecules to amplify the BCR activation signal.
The cross-linking of the BCR leads to increased expression of MHC class II and B7 (CD80 and CD86) co-stimulatory molecules. B cells internalise antigen, process and present it to helper $CD4^+$ T cells (T_H) via MHC class II molecules. The activated T cells provide help (signal 2) by the provision of CD40 ligand (CD40L). Activated B cells also express receptors for cytokines that induce B-cell proliferation.

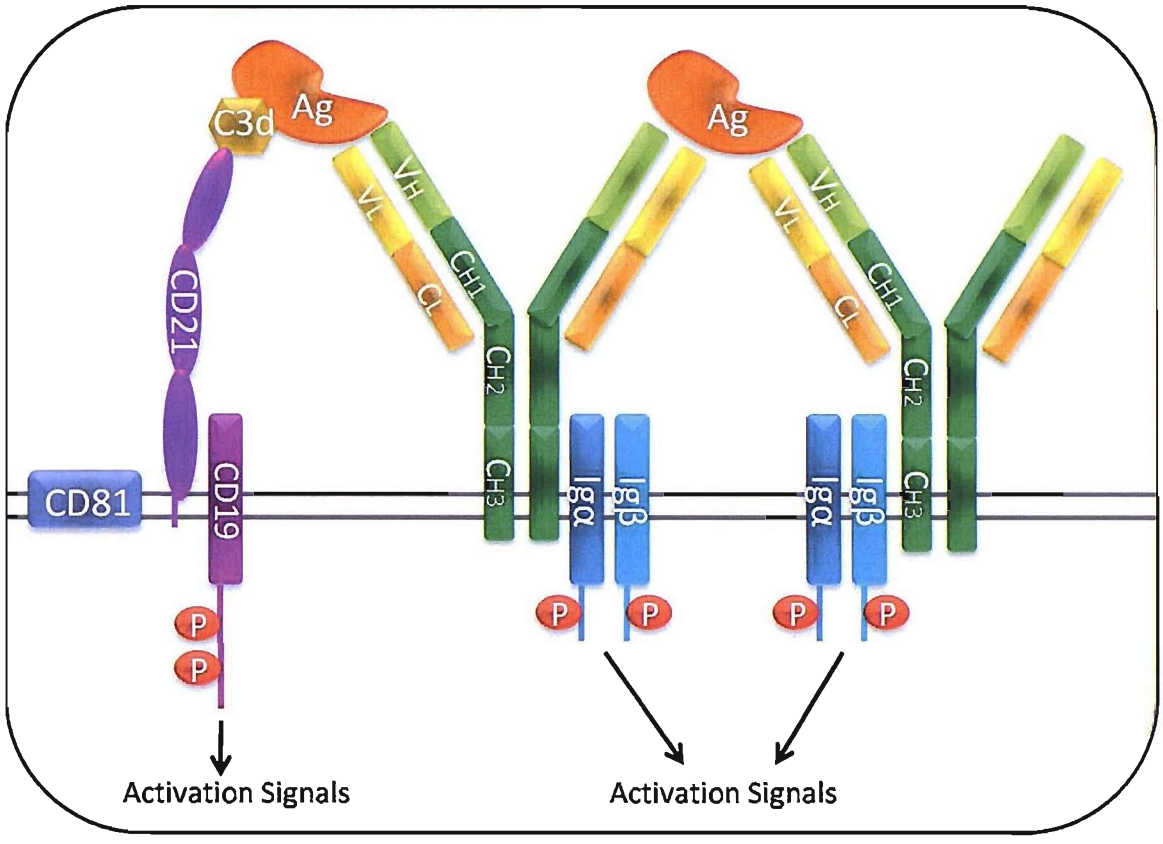


Figure 2: The B-cell receptor (BCR)

Fully re-arranged BCRs (Ig, CD79a and CD79b) assemble with the co-receptor (CD19, CD21 and CD81) on the surface of mature naïve B cells. Cross-linking of BCRs by antigen recognition initiates signalling that is mediated by a series of phosphorylation events. Antigen can be opsonised by C3d, a component of complement that enables recognition by CD21 (complement receptor) to initiate activation signals in the B cell.

Activated B cells and T_H cells migrate to B-cell follicles where they form germinal centres (GCs). Centroblasts are the large, rapidly proliferating B cells within the GC with Ig that undergo somatic hypermutation (SHM) in the V regions. These give rise to smaller non-proliferating centrocytes that require exposure to captured antigens presented by follicular dendritic cells (FDCs) for survival. Due to competition for antigen-presenting DCs only those centrocytes expressing higher affinity Ig survive. Ongoing interactions with T_H cells throughout are necessary for class switch recombination (CSR) where B cells retain Ig specificity but switch isotype. This process leads to memory B-cell and plasma cell (PC) formation. PCs ($CD19^+$ $CD38^+$ $CD138^+$ $CD56^-$) are BM-resident long-lived cells that have re-arranged high affinity B cell receptors and quickly mount a response to secrete antibodies. Factors such as IL-6 and not continued antigen exposure are necessary for the maintenance of PCs and memory B cells.

T cell-independent B-cell activation without GC formation can also occur. Some molecules and micro-organisms can induce B cell activation and proliferation independent

of antigen specificity. This occurs in extrafollicular areas and results in polyclonal B-cell activation that produces short-term PCs secreting low affinity IgM antibodies that are unable to confer complete protection but instead aid early antigen clearance by cells of the innate immune system. Such activation can occur through the secretion of B-cell stimulatory cytokines (e.g. IL-1 and IL-6) by activated cells of the immune system or in an autocrine fashion by B cells activated by LPS or CpG via TLR4 and TLR9 respectively.

1.2.2 T Lymphocytes

T cells express T-cell receptors (TCRs) that engage with antigen-derived peptides presented via MHC molecules. This results in activation and acquisition of effector functions. There are different T cell subsets each fulfilling distinct roles, including $CD4^+$ helper T cells (section 1.2.2.9.2), $CD4^+$ regulatory T cells (Tregs) (section 1.2.2.12) and cytotoxic $CD8^+$ T cells (section 1.2.2.9.1).

Most hematopoietic lineages undergo differentiation and maturation in the BM. However, T-cell precursors migrate from the BM to the thymus where T-cell development takes place. The thymus itself does not contain long term self-renewing progenitor populations and therefore relies on continued importation of BM-derived precursors such as $Flt3^{hi}$ VCAM-1^- MPPs [14]. Alternatively this progenitor population can give rise to CLPs within the BM, which may retain the ability to migrate to the thymus as a T-cell precursor or can remain within the BM and differentiate into B cells.

1.2.2.1 T Cell Receptor (TCR) Rearrangement

The TCR is a heterodimer of two polypeptide chains (αβ or γδ). In peripheral blood the majority of T cells express TCRαβ. Similar to the BCR, TCRs consists of a constant (C) and a variable (V) domain. It is in the V domain where complementarity determining regions (CDRs) are located. The CDRs contact the MHC-peptide complexes and therefore confer the specificity of the TCR. The TCRα and γ chain V regions are assembled from single V ($V_{\alpha 1\text{-}70}$ or $V_{\gamma 1\text{-}14}$) and J ($J_{\alpha 1\text{-}61}$ or $J_{\gamma 1\text{-}5}$) segments whereas TCRβ and δ chains are composed of V ($V_{\beta 1\text{-}67}$ or $V_{\delta 1\text{-}8}$), D ($D_{\beta 1\text{-}2}$ or $D_{\delta 1\text{-}3}$) and J ($J_{\beta 1\text{-}13}$ or $J_{\delta 1\text{-}4}$) segments. The C genes are more restricted as there is only one C segment for TCRα and γ and two for TCRβ and δ (reviewed [15]. TCR recombination commences at the TCRβ locus then proceeds to the TCRα locus. Interestingly, the TCRα locus is structured in such a way that multiple V-J recombinations can occur on the same allele. Recombination terminates once both α and

β chains assemble into a TCR. Thymocytes unable to generate functional rearrangements die by apoptosis. As with BCR rearrangements, allelic exclusion is a hallmark of TCR recombination for the β but not α chain.

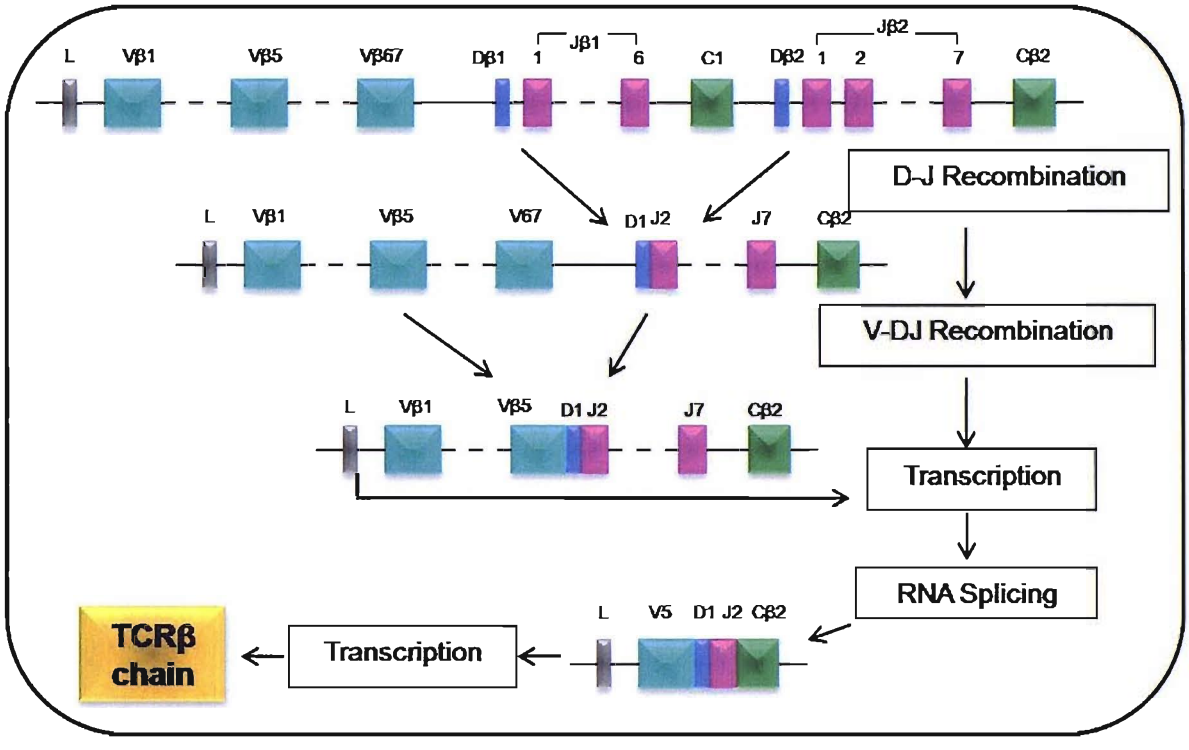


Figure 3: Rearrangement of the TCR β chain

The β chain of the TCR is created by the stepwise process of V(D)J recombination that commences in DN3 developing thymocytes and is mediated by V(D)J recombinase enzymes. The V (turquoise), D (blue) and J (pink) segments encode the CDRs whilst the C (green) segments encode the constant regions that allow the α and β chains to interact.

V(D)J recombinase enzymes (RAG-1 and RAG-2) recognise recombination signal sequences (RSS) that flank the gene segments to initiate the process. The first step in TCR recombination is the D-J followed by V-DJ rearrangements (Figure 3). In those chains without D segments only V-J recombination occurs. Finally the C region is spliced into the transcript to complete the chain. Throughout the process TdT is able to incorporate or delete random nucleotides at the segment junctions in the same manner as for the BCR. The association of two different chains e.g. TCRαβ also contributes towards further diversity.

On the T-cell surface the TCR associates with a cytoplasmic membrane-bound complex of five proteins known as CD3 (Figure 4). The intracellular domains of the CD3 complex are involved in signal transduction upon TCR engagement.

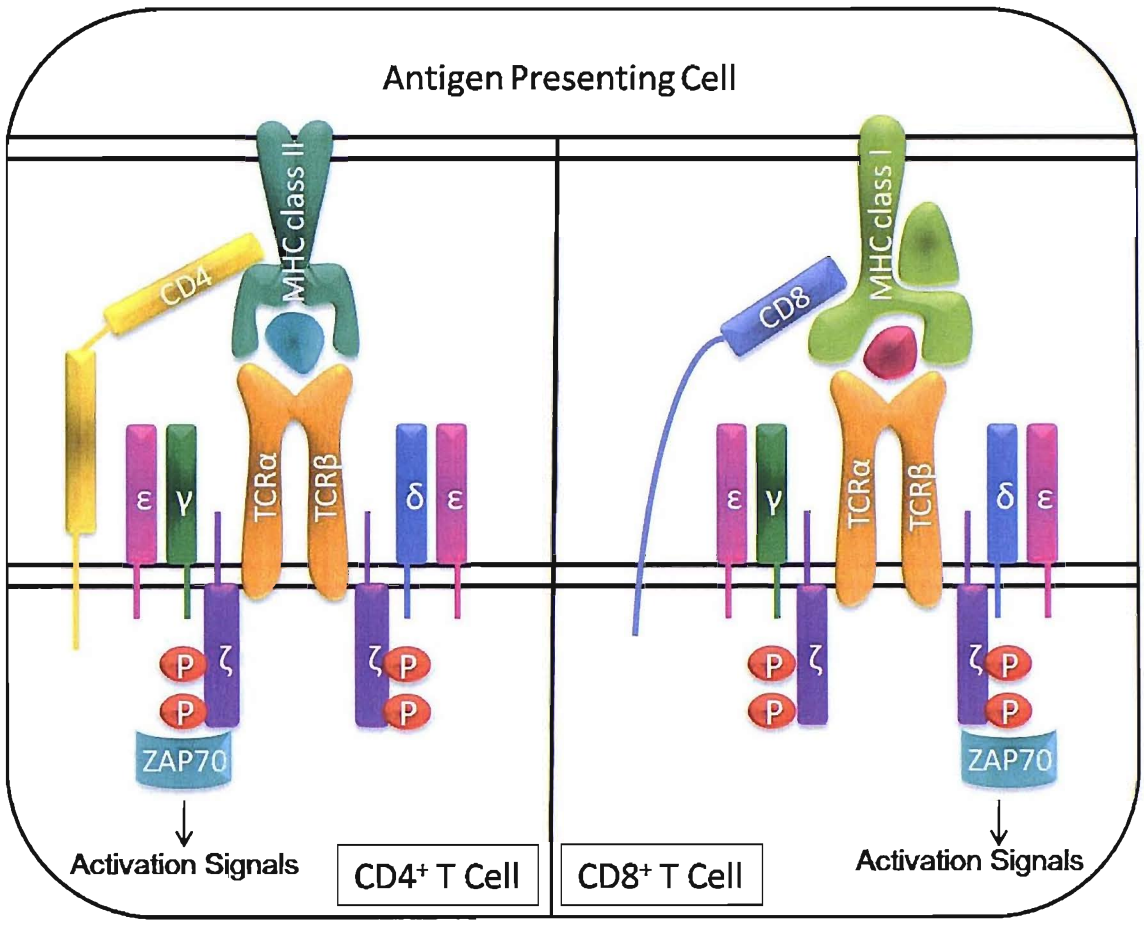


Figure 4: MHC-restricted antigen presentation to the T-cell receptor (TCRαβ)

The mature TCR assembles on the T-cell surface with the CD3 protein complex (1xγ, 1xδ, 2xε and 2xζ). Recognition of antigenic peptide presented by MHC class I or class II molecules on APCs activate $CD8^+$ and $CD4^+$ T cells respectively. This initiates signalling where a series of phosphorylation events (of ITAM motifs) by tyrosine kinases and further recruitment of other signalling molecules occur.

1.2.2.2 γδ T Cells

The majority of $CD3^+$ T cells in humans and primates express TCRαβ (~95%) which will be the focus of the following sections. The other population, γδ T cells, also pose an interesting subset although much less is known about them. In contrast to αβ T cells, γδ T cells are rare in healthy lymph nodes but can be readily found in lymph nodes draining sites of infection and inflammation. This observation is in accordance with their chemokine receptor profile; resting cells express receptors for and can migrate in response to inflammatory cytokines (similar to cells of innate immunity). After TCR triggering their migration profile changes and expression of the lymph node-homing chemokine receptor CCR7 is induced [16]. TCR triggering of γδ T cells requires recognition mostly of small nonpeptide antigen (i.e. CD1-restricted recognition of phospholidpids derived from microbes or necrotic host cells [17] that occurs in an MHC-independent form.

Activated γδ T cells express high levels of co-stimulatory molecules (CD40L, OX40, CD70, CD80, and CD86) and MHC class II molecules. These attributes allow them to provide potent B-cell help and to act as APCs (as efficient as DCs) activating αβ T cells during *in vitro* co-cultures [16, 18].

1.2.2.3 T Cell Development

Precursors of T cells do not express definitive T cell markers when they first migrate to the thymus however there are a range of molecules associated with thymus settling including cytokine receptors, chemokines receptors, ligands for integrins and selectins and proteases (e.g. CD44, PSGL-1 (P-selectin ligand) [19], and CCR9 [20]). The thymic stroma provides numerous signals (e.g. IL-7, stem cell factor and Notch ligands amongst others) which are required for thymocyte survival and proliferation from early stages. The early CD4 and CD8 double negative (DN1) developing thymocytes proliferate in the thymic cortex over the next couple of weeks and acquire lineage-specific markers CD24 (heat stable antigen; HSA) and CD25 at what is now DN2 stage. Notch signalling has an essential role in T-cell lineage induction [21] and is required through the majority of the thymic development process. At the DN1 and DN2 stages Notch signals regulate gene expression in a manner that not only maintains T cell lineage but also represses the development into other cell types that can still arise (NK cells, DCs, B cells). Rearrangement of the TCR (at β, γ and δ loci) commences at the DN3 along with the downregulation of c-kit (tyrosine kinase) and CD44 (adhesion molecule) expression. At this DN3 stage αβ and γδ-T cells diverge. The thymocytes assemble a pre-TCR complex (TCRβ complexed with surrogate α chain and CD3 proteins) and only at this stage have the thymocytes fully committed to T-cell lineage [22].

The checkpoint of β-selection occurs to ensure only DN4 thymocytes with functional TCRβ chains survive. Survival of thymocytes requires signalling through the pre-TCR (through ZAP-70 and activates NFAT and NFκB transcription factors) that induces anti-apoptotic signalling, differentiation, proliferation and allelic exclusion.

1.2.2.3.1 Positive Selection

Importantly, DN4 thymocytes become double positive (DP) cells that express both CD4 and CD8 molecules and the TCRα chain gene rearranges on both alleles. DP cells expressing MHC class I or class II-restricted TCRs undergo positive selection, the rest die by neglect. This occurs by engagement with cortical thymic epithelial cells (cTECs) that act as APCs to present low affinity peptides and thus a protective signal is transmitted

through the TCR. TCR signalling stabilises and selects for the TCRαβ with highest interchain affinity, leading to functional allelic exclusion at the T cell surface. Alongside the repression of RAG gene expression, induction of long-term survival, migration into the thymic medulla and further differentiation occur. The DP cells lose expression of either CD4 or CD8 and retain that which corresponds to the MHC molecule they are able to engage. In this manner only MHC-restricted single positive (SP) thymocytes continue developing.

1.2.2.3.2 Central Tolerance (Negative Selection)

SP cells migrate to the medulla where negative selection occurs. This is the process by which strong stimulation of the TCR by MHC-restricted presentation of the specific peptide results in clonal deletion that eliminates self-reactivity from the T cell repertoire. Autoreactive thymocytes undergo irreversible Bcl-2-regulated apoptosis. Interestingly, the opposite is true for Tregs where high affinity agonistic TCR engagement is required and these cells accordingly display a partially activated phenotype ($CD25^{+}$, section 1.2.2.12). It is thought these cells may not be deleted due to avidity below a threshold or possibly the type of APC presenting the antigen [23]. The APC subsets in the thymus are cTECs, medullary thymic epithelial cells (mTECs), DCs and macrophages. A key feature of central tolerance allowing self-discrimination is the promiscuous gene expression (PGE) of tissue-specific antigens attributed to mTECs [24]. AIRE (autoimmune regulator) is a transcriptional activator that controls the PGE of a subset of tissue-specific antigens, as demonstrated by autoimmune pathology in AIRE-deficient mice [25]. Other thus far unidentified mechanisms controlling PGE are likely to exist. It has been demonstrated that both BM-derived DCs and mTECs can mediate negative selection [26].

Despite the fact that less than 5% of thymocytes survive negative selection and exit the thymus as naïve T cells the process of central tolerance is incomplete. This results in self-reactive lower avidity T cells populating the periphery, as proven by the existence of autoimmune disorders. Some T cells escape due to lack of antigen exposure in the thymus or if they have low avidity for self-antigens. These "escapees" can be controlled by mechanisms of peripheral tolerance (section 1.2.2.4). Immunotherapy aimed at priming T-cell responses against tumour antigens relies on the existence of this self-reactive repertoire.

1.2.2.4 Peripheral Tolerance

Peripheral tolerance has evolved to control self-reactive T cells in the periphery. One mechanism is the inability of lymphocytes to enter immunologically privileged tissues such as testis and the anterior chamber of the eye. This physical protective measure ensures that irreparable damage is not caused by activated lymphocytes.

Another important mechanism concerns DC activation status. Immature DCs capture tumour antigen in the periphery and migrate to the draining lymph nodes where they present it to the remaining repertoire of self-recognising T cells. In the absence of danger signals for DC maturation (section 1.2.2.5) and subsequent lack of co-stimulation, such DCs can tolerise T cells [27]. This leads to brief T-cell proliferation and sub-optimal development of effector function. Exposure to a high dose of antigen results in T-cell anergy (unresponsiveness). However T cells become permanently deleted in the presence of a low antigen dose [28]. The difference is thought to arise from distinct signalling where weak signals may be unable to induce expression of anti-apoptotic signals. This suggests that the strength of the TCR-MHC interaction determines the outcome. The persistence of antigen is another factor that has been shown to be critical in the generation/maintenance of anergy. Persistent antigen suppressed IL-7Rα and Bcl-2 expression and led to T-cell exhaustion [29]. Also, effector $CD8^+$ T cells can become tolerised upon transfer into antigen-expressing hosts [28].

Ligation of T-cell co-stimulatory molecules that inhibit T-cell activation has been implicated in peripheral tolerance. The programmed death-1 ligand-1 (PD-L1) is widely expressed in the periphery and may protect the expressing tissues from self-reactive lymphocytes. This interaction for example is known to promote fetal-maternal tolerance.

A number of features can be associated with anergic T cells. These include the downregulation of the TCR, CD8, TCR-associated signalling molecules (e.g. defect in Src kinase activity and association to the CD3 complex that results in lack of CD3-ζ phosphorylation [30]), and the transcription of anergy-specific genes. A third mechanism of peripheral tolerance is suppression by Tregs (section 1.2.2.12). All the non-deletional mechanisms are potentially reversible. For example the repertoire/activity of Tregs, the activation status of APCs or the expression and presentation levels of antigen could change in response to immunological challenge.

A recent study reported that stromal cells from the cortex of the intestine-draining lymph node were able to present intestinal antigens resulting in the deletional tolerance of naïve $CD8^+$ T cells [31]. They also detected the expression of AIRE and other tissue-specific antigens by lymph node stromal cells. This suggests a peripheral mechanism for deletion

of self-reactive T cells akin to the thymic central tolerance involving mTEC antigen presentation.

1.2.2.5 Dendritic Cell Maturation

Dendritic cells are phagocytic professional antigen presenting cells (APCs) that are part of the innate immune system. The two major DC subsets in humans are $CD11c^+$ myeloid DCs and $CD11c^-$ $CD123^+$ plasmacytoid DCs. Immature myeloid DCs populate peripheral tissues and migrate through afferent lymphatics to lymph nodes where as mature cells they can prime antigen-specific T cells. Plasmacytoid DCs in contrast are thought to migrate from blood into lymph nodes through high endothelial venules (HEVs) (reviewed [32]). They accumulate in inflammed lymph nodes producing type I IFNs that contribute to the innate immune response but can also rapidly expand virus-specific $CD8^+$ T cells.

DCs express a range of receptors, some of which mediate endocytosis, including FcγR, C-type lectin receptors (e.g. DEC-205) and scavenger receptors (e.g. CD91). These receptors along with other markers (CD4 and CD8) can be used to further distinguish DC subsets. Immature DCs are specialised for antigen capture and it is immature myeloid DCs that are found in the epithelia of peripheral tissues. They continuously sample the environment by uptake of antigens through receptor-mediated endocytosis, phagocytosis and macropinocytosis. Upon encounter of antigen and activating danger signals (tissue damage, TLR activation or pro-inflammatory cytokines), DCs maturation takes place. This entails the downregulation of phagocytic activity and simultaneous increased expression of surface MHC class I and class II (due to changes in intracellular transport [33], a range of co-stimulatory molecules (i.e. CD80, CD86, CD70, 4-1BB ligand and OX-40 ligand), adhesion molecules and cytokines (i.e. IL-12 that contributes to a T_{H1} response [34] and type I IFNs). The morphology of DCs also alters with maturation, resulting in extended processes that increase the surface area available for T-cell interaction. These DCs are said to be "licensed" and can effectively initiate and orchestrate adaptive immune responses. In the absence of danger signals DCs remain immature and have toleragenic effects on T cells.

Mature DCs acquire a migratory phenotype by upregulating expression of various chemokines and their receptors, particularly CCR7. This allows migration towards chemotactic gradients generated by the lymph node endothelium and into the T-cell areas of draining lymph nodes. Here, mature DCs present the antigen in the context of MHC molecules (section 1.2.2.6) and provide necessary co-stimulation (section 1.2.2.8) for naïve T cells to differentiate into effector cells. DC subsets differentially express components of

the MHC class I and class II processing pathways resulting in preferential presentation of endocytosed antigen to $CD8^+$ (cross-priming) or $CD4^+$ T cells [35]. Mature DCs also provide cytokines (i.e. IL-12) for the activation of NK cells.

1.2.2.6 Antigen Processing and Presentation

Antigens synthesised within cells or acquired by uptake are processed into peptides and associate with MHC molecules. These complexes are transported to the cell surface where they can be recognised by the T cells via their TCRs. MHC class I and class II processing pathways differ and result in the activation of $CD8^+$ and $CD4^+$ T cells respectively (Figure 4). The CD4 and CD8 co-receptors themselves bind to the MHC protein increasing the binding affinity of the T cell for the antigen-MHC complex and sending activation signals to the T cells.

1.2.2.6.1 MHC Class I Presentation Pathway

The MHC class I molecule is a heterodimer of a membrane-spanning heavy chain (HC) that associates non-covalently with the non-MHC encoded β2-microglobulin. Most nucleated cells express MHC class I molecules on their surface. Peptides bind the groove formed between the α1 and α2 domains whilst the α3 domain is bound by the CD8 molecule on the T cell surface. The peptide-binding cleft is in a closed conformation that can only accommodate peptides of 8-10 amino acids in length.

Cellular proteins are degraded as a means to control the quantity and quality of proteins. Classical MHC class I presentation allows $CD8^+$ T-cell pathogen surveillance that has to work over the background of self-peptide presentation. Yewdell first proposed that rapid viral antigenic peptides were processed from a pool of defective ribosomal products (DRiPs) [36]. The DRiP pool includes viral and defective forms of cellular proteins that are more rapidly degraded than functional cellular gene products. In this manner viral proteins can be detected within an hour of infection rather than the average 1-2 day degradation of gene products.

Poly-ubiquitination of cytosolic proteins targets them to the cytosolic mutlisubunit protease complex known as the proteasome (Figure 5). The proteasome cleaves proteins (reviewed [37]) into long peptides that can be further trimmed by cytosolic proteases [38]. A novel mechanism of peptide splicing carried out by the proteasome has been reported [39]. The covalent fusion of fragments from different parts of the protein generates a "novel" peptide for presentation to $CD8^+$ T cells.

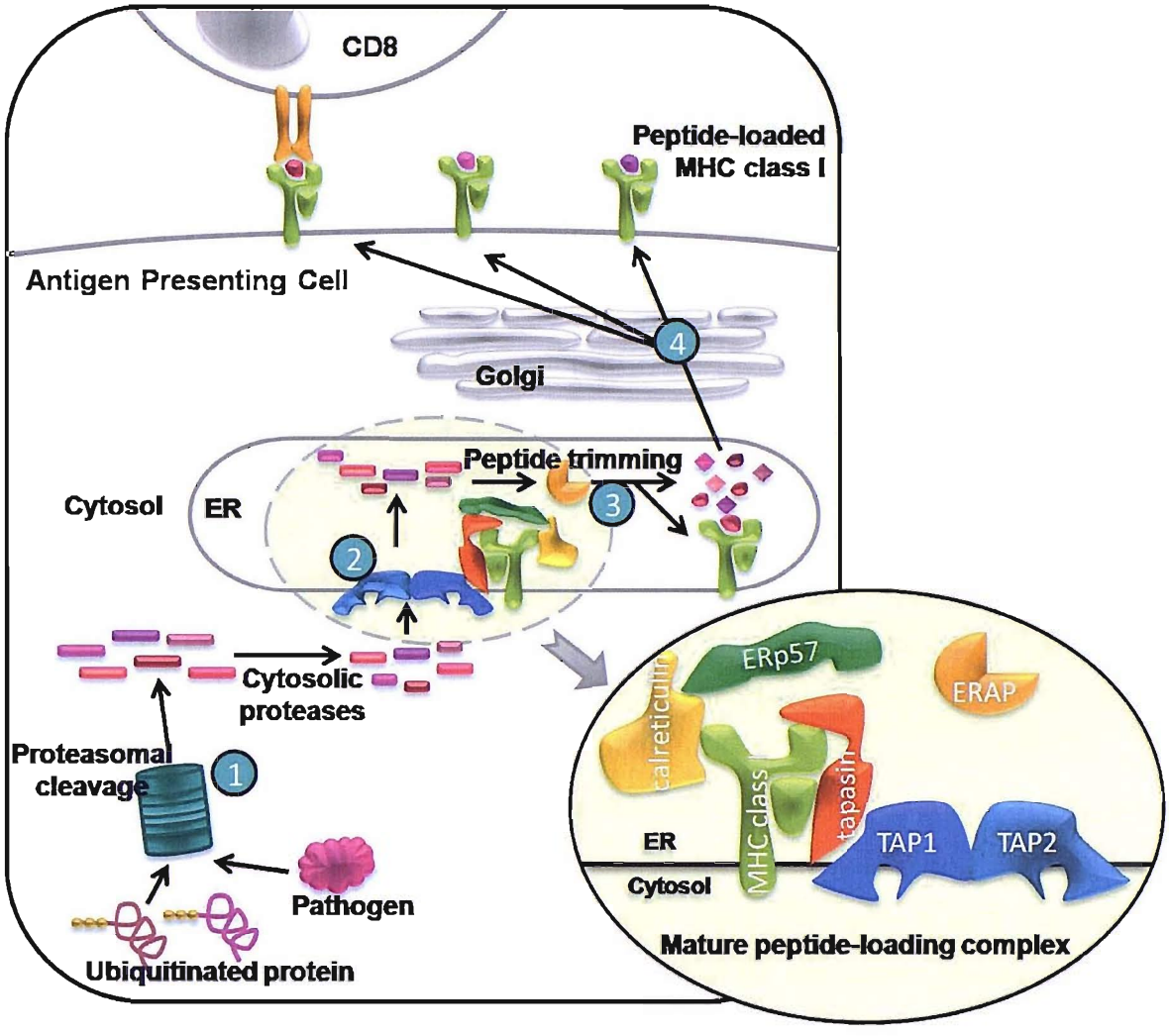


Figure 5: MHC class I antigen presentation pathway

Cellular and viral proteins are presented in the context of MHC class I molecules to activate $CD8^+$ T cells. Ubiquitinated cytosolic proteins are partially degraded by the proteasome (1) and then undergo further degradation by cytosolic proteases. The extended peptides are translocated into the ER by the TAP complex (2) and undergo N-terminal trimming by the ER-resident proteases (ERAAP) (3). The minimal peptides generated are loaded onto the MHC class I molecules with the aid of the peptide-loading complex (PLC) prior to transport to the cell surface via the Golgi.

The proteasome is composed of a 19S regulatory particle responsible for recognition of ubiquitinated target proteins and their translocation into the 20S proteolytic core. The catalytic subunits have different activities; β1 (caspase-like), β2 (trypsin-like), and β5 (chymotrypsin-like). These can be replaced by inducible catalytic subunits (LMP-7, MECL-1 and LMP-2) in the presence of inflammatory mediators (i.e. IFNγ) to create an immunoproteasome that can generate a different peptide repertoire. This may be an important concept to consider for DC-based immunotherapy. Activation/maturation of DCs induces the expression of immunoproteasomes that may present different peptides to those presented by tumours as recently demonstrated in a study. Blocking the expression of immunoproteasome subunits in melanoma patient-derived DCs matured *in vitro* resulted

in greater levels of constitutive proteasomes and consequently much higher lysis of autologous tumour cells by cross-primed $CD8^+$ T cells [40].

The extended peptides generated in the cytosol are translocated to the lumen of the endoplasmic reticulum (ER) in a transporter associated with antigen processing (TAP)-dependent manner. TAP heterodimer (TAP1 and TAP2) is an ATP-binding cassette (ABC) transporter that hydrolyses ATP to form a translocation pore within the ER membrane. The imported peptides undergo further N-terminal trimming by ER amino peptidases associated with antigen processing (ERAAP) within the ER [41] to generate 8-10 amino acid peptides. Long peptides can be shuttled back to the cytosol by the translocating protein Sec61, for further proteasomal degradation. ERAAP has broad substrate specificity and can therefore generate a variety of peptides for presentation. It appears that MHC class I molecules within the ER play an important role in binding and thereby protecting extended peptide precursors from complete unproductive degradation by allowing ERAAP to only trim overhanging amino acids [42].

The MHC class I polypeptides are synthesised and transported into the endoplasmic reticulum (ER). Here, chaperones and accessory molecules ensure their correct folding, association and maintenance in a peptide-receptive state by insertion into the peptide loading complex (PLC). Initially the HC interacts with the chaperones immunoglobulin-binding protein (BiP) and calnexin before dissociating from calnexin and associating with β2-microglobulin, the chaperone calreticulin, ERp57 and a pre-assembled TAP1, TAP2 and tapasin complex (the PLC). The oxido-reductase ERp57 catalyses di-sulphide bond formation to ensure the correct folding and association of the MHC class I molecules [43]. Tapasin is involved in the recruitment of ERp57, stabilisation and connector of TAP and MHC and is essential for normal expression of MHC class I at the cell surface [44]. Tapasin carries out quality control by preventing the premature release of MHC class I molecules loaded with non-optimal peptides. Once loaded, MHC-peptide complexes are released from the PLC and are exported to the cell surface via the Golgi apparatus. Any MHC class I molecules in incorrect conformations are returned to the cytosol, probably through Sec61 channels, to be degraded by the proteasome.

1.2.2.6.2 MHC Class II Presentation Pathway

The MHC class II molecule is a heterodimer of non-covalently associated membrane-spanning α and β-chains. These are expressed predominantly by professional APCs (e.g. DCs and macrophages) and B cells. The antigenic peptides bind the groove formed between the α1 and β1 domains. The peptide-binding cleft is in a more open conformation

than MHC class I so can accommodate longer peptides varying between 9-30 amino acids in length. This pathway functions to present exogenous antigen (acquired by phagocytosis, macropinocytosis, or receptor-mediated endocytosis) to $CD4^+$ T cells.

Newly synthesised MHC class II heterodimers complexed with an invariant chain (Ii) trimer leave the ER and are transported through the Golgi to the surface, early endosomes or late endosomes/lysosomes (Figure 6). The CLIP (Class II associated invariant chain) region of Ii occupies the open-ended peptide-binding groove to prevent undesired premature loading with class I-destined peptides, longer peptides or unfolded proteins. A motif within the Ii directs the MHC class II complexes to a late endocytic compartment so that upon exit from the Golgi network, the majority of MHC class II molecules are diverted from the secretory pathway and targeted directly to late endosomes and lysosomes. The MHC class II molecules can be loaded within these organelles (endocytic pathway) ready for transport to the cell surface when DCs mature [33].

The first step of loading involves the degradation of Ii by endosomal proteases, leaving the CLIP fragment bound at the peptide groove. Antigenic peptides become exchanged for CLIP, a process facilitated by the low pH of the cellular compartment, proteolytic degradation of CLIP and the HLA-DM chaperone. Harsh conditions and degradative proteases within compartments of the endocytic pathway break down endocytosed antigens to generate peptides of a suitable length to bind the MHC class II molecules. The main site of peptide-loading appears to be the late endosome although it has been reported at multiple locations in the endocytic route. HLA-DM stabilises the unloaded MHC class II and supports peptide exchange until a high affinity peptide binds [45]. However, CLIP exchange is not required for cell surface expression of MHC class II molecules that may then be recycled and subsequently loaded with peptide. HLA-DM is also transported to the cell surface with MHC class II molecules and can be internalised to gain access to endocytic compartments. Rocha et al., have reviewed the literature on the transport of loaded and non-loaded MHC class II molecules as well as the location of peptide-loading [46].

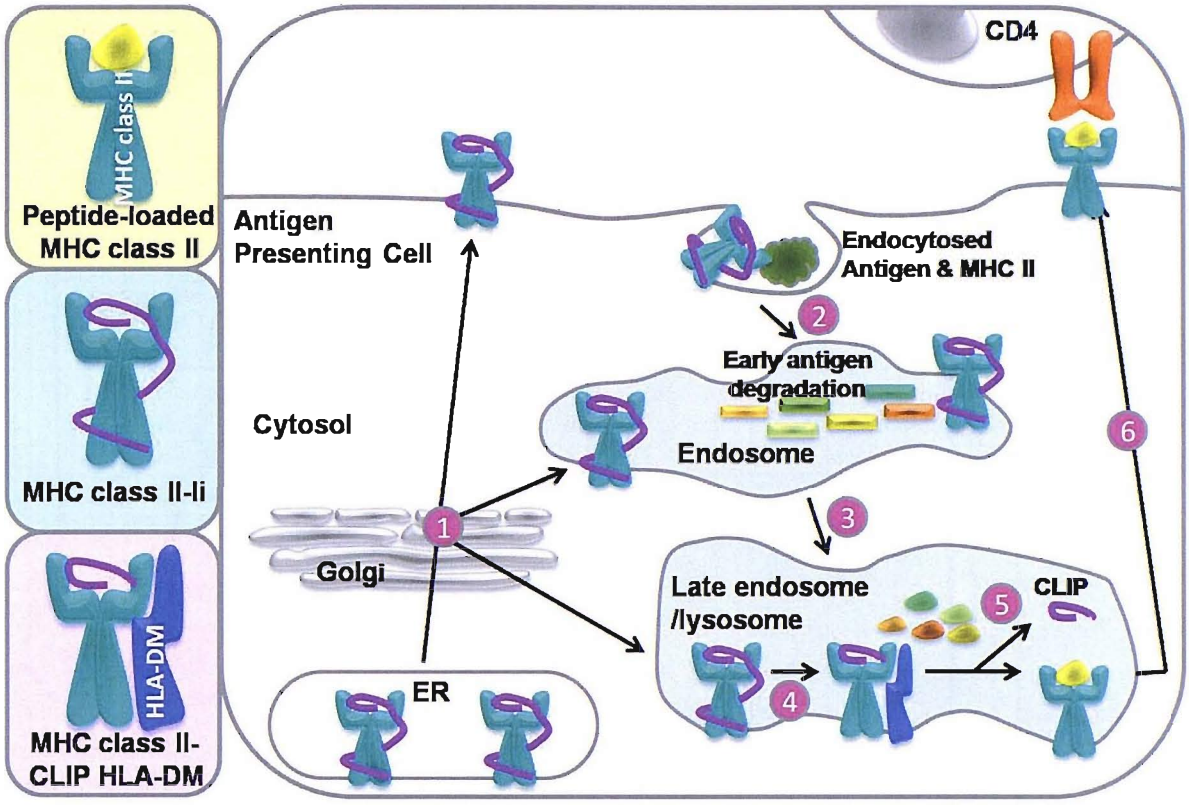


Figure 6: MHC class II antigen presentation pathway

Exogenous proteins are presented in the context of MHC class II molecules to activate $CD4^+$ T cells. Newly synthesised MHC class II proteins are directed to the secretory or endocytic pathways (1). Exogenous antigen and/or MHC class II molecules are endocytosed (2) by the APC and transported through endocytic organelles. Antigen degradation commences in the early endosome where peptide-loading can initially occur. Alternatively the organelle progresses to a late endosome (3), the main compartment for peptide-loading. The Ii is cleaved by endosomal proteases to leave only the CLIP fragment in the peptide-binding groove (4). The HLA-DM chaperone stabilises the MHC class II molecule and catalyses the exchange of CLIP for a suitable peptide (5). The peptide-loaded MHC class II molecules can subsequently be transported to the cell surface (6).

1.2.2.6.3 Cross-Presentation

This pathway of antigen presentation involves the transfer of exogenous antigenic material from donor cells to APCs for cross-presentation to $CD8^+$ T cells via MHC class I molecules. This pathway therefore allows the generation of $CD8^+$ T-cell responses against pathogens that fail to infect DCs as well as cellular pathogens that do not reach the cytosol. DCs likely acquire antigens by internalising infected cell debris into phagosomes which would largely satisfy the preference for intact proteins [47]. The precise mechanisms of cross-presentation remain unclear although it is thought there are three possible pathways in which antigens can be cross-presented by MHC class I molecules (Figure 7). The pathway of choice may be dependent on the type of antigen, APC or the type of receptor mediating phagocytosis.

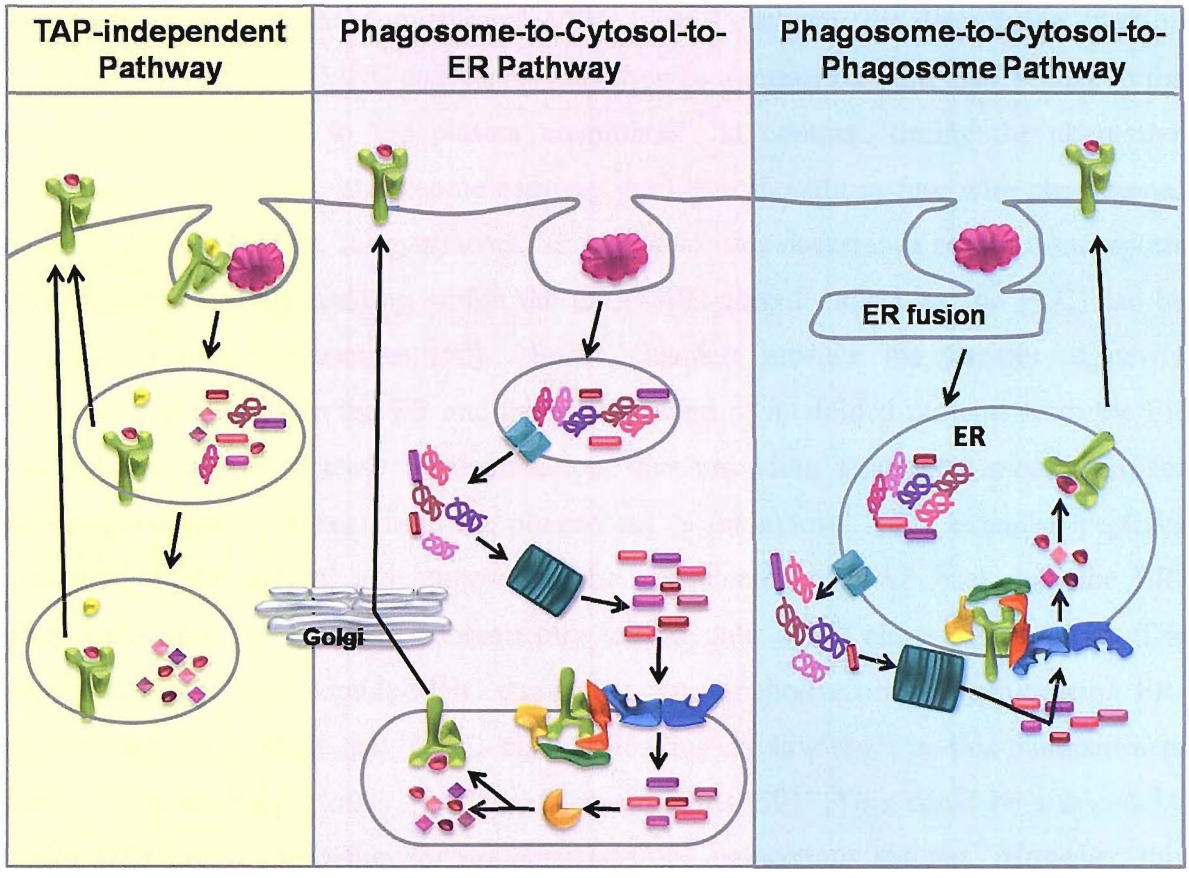


Figure 7: Antigen cross-presentation pathway

There are three routes by which exogenous antigens are presented in the context of MHC class I molecules to activate $CD8^+$ T cells. The TAP-independent pathway loads endocytosed antigen onto MHC class I molecules within the endocytic organelle. The endocytosed antigen can also be transported to the cytosol where it can enter the classical MHC class I presentation pathway (phagosome-to-cytosol-to-ER). Alternatively, the antigen can enter the pahgosome-to-cytosol-to-phagosome pathway where the antigen is transported into the cytosol for degradation by cytosolic antigen processing machinery. The proteasome products are translocated by TAP back into the ER-phagosome for MHC class I-loading and transport back to the surface.

The first and the simplest is proteasome- and TAP-independent cross-presentation. This involves degradation of the internalised antigen within the phagosome by the endosomal proteases [48] and concurrent loading onto co-internalised mature MHC class I molecules. This direct cytosol-independent pathway may play an important role during viral infections that block the cytosolic pathways i.e. TAP inhibition. Similarly, this pathway has also been demonstrated in a *Leishmania major* infection model [49] although in this instance it is suggested by the authors as a possible strategy to evade more efficient TAP-dependent cross-presentation.

It is thought that two TAP-dependent ER-loading pathways occur, both involving antigen egress from endocytic organelles into the cytosol for proteasomal cleavage although there are no well understood mechanisms for this transfer. In the phagosome-to-cytosol-to-ER pathway the extended peptides generated in the cytosol are transported by TAP into the ER

where they can enter the conventional MHC class I pathway for degradation (section 1.2.2.6.1). The peptide-MHC complexes may then be transported from the ER back to the phagosome or directly to the plasma membrane. In contrast, during the alternative phagosome-to-cytosol-to-phagosome pathway the ER is thought to fuse with phagosomes to create ER-phagosome compartments. Evidence for this occurrence comes from reports that proteins normally residing within the ER (MHC class I molecules and PLC) can be identified within phagosomes [50]. Sec61 channels mediate the transfer of newly synthesised proteins into the ER and the translocation of misfolded proteins from the ER into the cytosol for proteasomal degradation, thus providing a potential mechanism for captured antigen to be sent from the phagosome to the cytosol. The extended peptides generated in the cytosol are proposed to be translocated by TAP back into the ER-phagosome (not the ER) and subsequently loaded onto MHC class I molecules. The phagocytosed antigen peptide-MHC complexes are enriched in the antigen-bearing ER-phagosomes suggesting that MHC class I loading is favoured in this compartment compared to the ER or other "irrelevant" phagosomes [50]. This could be a means by which DCs limit competition for presentation from endogenous antigen. However, this donation of ER membrane to phagosomes remains highly controversial and no one has yet been able to demonstrate functional fusion between these two compartments. Additionally the work in this field has relied on the internalisation of latex beads, which may not necessarily precisely mimic the physiological situation.

1.2.2.7 Activation of T Cells

Naïve T cells exported from the thymus circulate between the blood and secondary lymphoid tissues. T cells leave the blood and enter lymph nodes through high endothelial venules (HEVs) on blood vessels. These are lined with endothelial cells expressing the adhesion molecules and chemokines. The interaction between T cell L-selectin (CD62L) and endothelial L-selectin ligand initates "rolling" to slow down the lymphocyte. In response to CCL19 and CCL21 chemotactic gradients detected by the CCR7 chemokine receptor, lymphocytes increase integrin expression for a higher affinity interaction with the endothelium. Lymphocytes can now traverse the endothelium and enter the lymph node.

Foreign antigen can reach the lymph node by passive travel via afferent lymphatics or is picked up in the periphery by DCs that cross-present it in the draining lymph nodes. High cell motility within the lymph node ensures that even rare T cells can be found by APCs

presenting their antigen. It has been estimated that DCs can scan up to 5000 T cells per hour [51].

Two signals are required for full T-cell activation, the first delivered by TCR-MHC interaction which mediates the specificity of the response. This alone results in a state of anergy with limited proliferation and failure to induce cytokine production. For full activation a second signal in the form of co-stimulation is required (section 1.2.2.8) which acts as a regulation step that can serve to lower the threshold of responsiveness for T-cell activation. The expression of adhesion molecules on the surface of T cells and APCs (leukocyte function-associated antigen; LFA-1/ICAM-1 and CD2/LFA-3) enhance T-cell activation by facilitating contact between the cells. Formation of mature immunological synapses requires topological rearrangements that result in central and peripheral supramolecular activation clusters (c- and p-SMAC) containing signalling molecules (TCR, MHC, CD28) or adhesive molecules respectively. The functional significance of immunological synapse formation remains unknown although it is thought to channel effector molecules and cytokines towards target cells.

Engagement of a TCR with an antigen-presenting MHC molecule recruits CD4 or CD8 co-receptors to the site. The Src kinase Lck is activated and recruited to the cytoplasmic domain of the co-receptor. The signal is amplified by simultaneous recognition of MHC by multiple TCRs and co-stimulation. Src family of kinases phosphorylate the TCRζ chain to create a docking site for ZAP-70. Activated (phosphorylated) tyrosine kinase ZAP-70 in turn phosphorylates other substrates including the membrane-associated Linker of Activated T cells (LAT) to create further docking sites for the recruitment of downstream molecules. TCR signalling cascades eventually activate AP-1, NFAT and NFκB transcription factors that initiate transcription of genes that drive proliferation (IL-2) and inhibit apoptosis. Activated T cells are highly dependent on IL-2 for clonal expansion and differentiation into effector cells.

The overall signal strength determines the program of T-cell activation, expansion and differentiation. This is synergistically controlled by various parameters including competition for APCs [52], antigen persistence [53], TCR affinity for the peptide/MHC complex, TCR density, co-stimulation and the duration of T cell-APC contact [54].

1.2.2.8 T Cell Co-stimulation

Co-stimulatory molecules are transmembrane proteins that induce intracellular signalling cascades via their cytoplasmic tails to modify TCR signalling but also induce reverse signalling to simultaneously activate the ligand-providing cells (i.e. APCs). Co-stimulatory signals are regarded as the master switch of T-cell activation as they regulate T-cell responses/activation through the induction or suppression of cytokine production, TCR signalling, survival, apoptosis, expression of other surface molecules and T-cell migration. Co-stimulatory signals can be activating (e.g. CD28, CD27, OX-40, 4-1BB) or negative to attenuate an ongoing response (CTLA-4 and PD-1), and their expression constitutive or induced. The different co-stimulatory molecules and their respective ligands can be seen in Figure 8.

Early in the immune response, CD40 on APCs ligates with CD40L (CD154) provided by $CD4^+$ T cells, to induce APC maturation (section 1.2.2.5) and B-cell activation (GC formation, isotype switch). Importantly, this engagement delivers signals that are also critical for $CD4^+$ T-cell activation as demonstrated by the ability of soluble CD40 to restore the signal delivered through CD40L in $CD40^{-/-}$ mice [55].

The best known co-stimulation molecule is CD28, a member of the CD28/B7 superfamily. Initial T-cell activation critically depends on constitutively expressed CD28 (exclusive to T cells) interacting with CD80/CD86 ligands (APCs) to lower the threshold of activation and drive production of IL-2. CD28 signalling imparts diverse effects, including upregulating CD40L expression on T cells to form a positive feedback loop. It also initiates the expression of inducible co-stimulation molecules (such as ICOS [56] and OX-40 [57]) and regulates T-cell proliferation/survival by means of inducing IL-2 and anti-apoptotic Bcl2 and Bcl-X_L production [58]. CD28 signalling is required for T cells to leave lymphoid tissue and migrate to antigen-bearing sites [59].

CD28 signalling also induces rapid expression of surface T-cell activation attenuator CTLA-4 [60] that competes for binding of the same ligands, providing a means of negative regulation. The importance of both molecules can be appreciated in knockout mice where impaired B and T-cell responses are observed in the absence of CD28 [61] and lethal uninhibited T-cell expansion in $CTLA\text{-}4^{-/-}$ mice [62]. Other negative regulator members of this family are the inducible Programmed Death-1 (PD-1, also expressed by B cells and myeloid cells) and the constitutively expressed B and T lymphocyte attenuator (BTLA,

also expressed by B cells and DCs). Negative co-stimulation plays a role in the maintenance of peripheral tolerance.

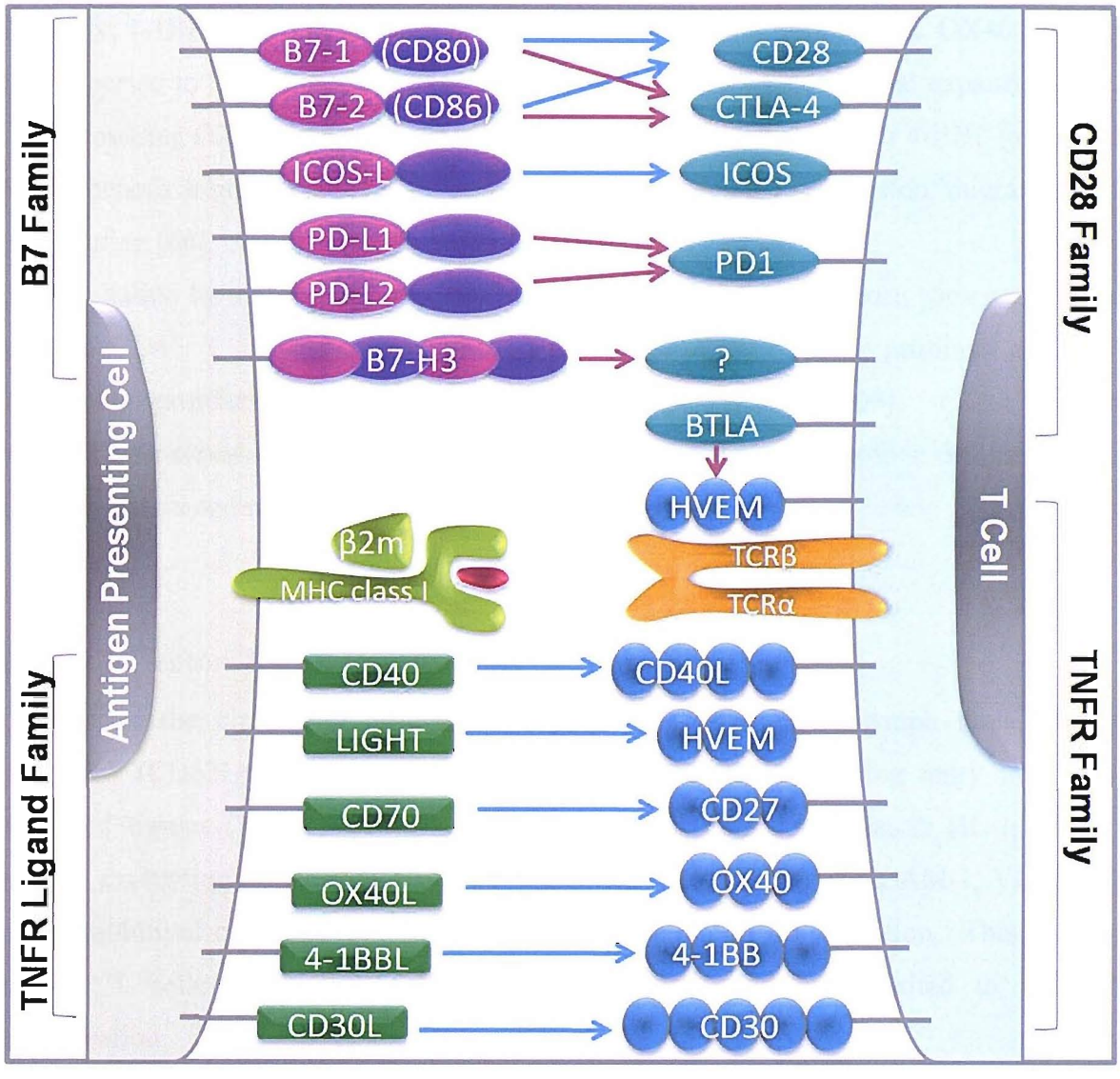


Figure 8: Co-stimulation molecules participating in the activation of T cells

Co-stimulation receptors belong to the CD28/B7 or the TNFR superfamilies. The CD28/B7 family members contain Ig constant-like (pink) or/and Ig variable-like domains (purple/turquoise). The TNFR family members are type I TM proteins containing cysteine-rich motifs (blue) on the extracellular domains whilst the TNF ligands are type II TM glycoproteins (green). Activating and regulatory signalling is demonstrated by the blue and red arrows respectively. Although not depicted the signalling in at least some cases is bi-directional and serves to modify the state of both T cells and APCs. The expression of receptors and ligands is variable and not just restricted to T cells and APCs as depicted. This picture was adapted from a publication by Beier et al., [63].

Co-stimulatory molecules belonging to the TNF superfamily share common signalling pathways through the TNF-related adaptor family. Signalling through these receptors appears to mediate particularly T-cell survival after initial CD28 co-stimulation. Members include the inducible OX40 and 4-1BB, both of which are expressed by activated DCs, B

and T cells. Signalling through these enhance expression of anti-apoptotic Bcl-xL and Bcl-2 [57]. Co-stimulation through OX40 also leads to $CD4^+$ T-cell migration into B-cell follicles, thereby contributing to T_{H2} responses and germinal centre formation [64]. Similarly, 4-1BB signalling induces B-cell proliferation and Ig production. OX40 has also been reported to have a crucial effect on the extent of $CD8^+$ T-cell clonal expansion [65]. Cells providing OX40L (activated B and T cells, DCs and monocytes) or 4-BBL (activated APCs) benefit from reverse signalling effects such as monocyte activation, migration and proliferation [66].

Co-stimulation by the TNF superfamily members CD27-CD70 have been shown to have a role in $CD4^+$ T cell-dependent and -independent $CD8^+$ T-cell priming, critically influencing proliferation, survival and secondary expansion [67-69]. CD27 is also constitutively expressed by B cells for which this signalling plays a role in differentiation, germinal centre and memory cell formation.

1.2.2.9 Effector T Cell Populations

To re-enter the circulation, effector T cells lose expression of lymph node-homing molecules (CD62L) and increase expression of molecules permitting entry into non-lymphoid tissues (LFA-1, VLA-4). Release of inflammatory mediators (IL-1, TNFα) induces expression of selectins (E and P-selectins), integrin ligands (ICAM-1, VCAM-1) and immobilised cytokines by vascular cells near the site of inflammation. This enables effector T cells to penetrate capillary blood vessels and be recruited to sites of inflammation.

1.2.2.9.1 $CD8^+$ Cytotoxic T cells (CTLs)

After only brief engagement of the TCR, primed $CD8^+$ T cells undergo multiple rounds of division and acquire effector function in a program of antigen-independent clonal expansion [54]. The lack of necessity for sustained antigen contact allows these cells to rapidly migrate to sites of infection. Effector $CD8^+$ T cells, also known as cytotoxic T cells (CTLs), use two different apoptosis-inducing mechanisms to kill target cells. The first is by secretion of pore-forming perforin and apoptotic-inducing granzymes, cytotoxic proteins expressed upon priming. These are contained within secretory vesicles to protect the effector cell themselves and are released at the immunological synapse where CTLs are engaged with the target cell for highly specific killing without bystander damage. The second killing mechanism is by exposing death-inducing proteins (FasL and TRAIL) that

engage receptors on the surface of target cells (Fas and TRAILR1/2) to initiate apoptotic signalling [70]. Upon target recognition, activated CTLs also secrete TNFα that can bind TNF receptors to induce apoptosis

CTLs additionally have a role in regulating the immune response in various ways. Through perforin release CTLs can eliminate antigen-presenting DCs to prevent them from reaching lymph nodes and excessively priming T cells [71]. The ability of CTLs to regulate T-cell numbers by fratricide, the killing of other T cells that have acquired MHC class I-antigen complexes, has also been reported. Interestingly, the human T cell lymphotropic virus (HTLV-1) can take advantage of this by naturally infecting $CD8^+$ T cells (*in vivo*) thereby rendering them susceptible to fratricide *ex vivo* [72].
During T cell-APC interactions, T cells are able to acquire molecules from the APCs including MHC-peptide complexes [73]. The biological significance of this remains unknown but could be regulatory to attenuate the immune response through fratricide [74] or the induction of anergy if antigen is presented in a sub-optimal context.

1.2.2.9.2 $CD4^+$ T Cells

Throughout their expansion phase, primed $CD4^+$ T cells retain dependence on antigen for a program of differentiation [53]. Effector $CD4^+$ T cells can differentiate into distinct subsets according to the conditions of priming and subsequent signalling that activates distinct transcription factors. Subsets can be distinguished by cytokine production and effector functions. Regulatory T cells (Treg) suppress immune responses (section 1.2.2.12) whilst T_{H17} produce IL-17 and are implicated in certain infections and in the development of autoimmune disease. The classical subset is known as $CD4^+$ helper T cells as they are critical for both $CD8^+$ T cell and humoral responses and thus play a central role in orchestrating the adaptive immune response. $CD4^+$ T-cell responses can be of T_{H1} (T-bet^{hi} IL-12Rβ2^{hi}) or T_{H2} (Gata-3^{hi} IL-4^{hi}) subtype, the polarisation of the response determined by the amount of antigen, type of APC and most importantly the cytokine mileu. Cytokines secreted by cells of the innate immune system contribute to the shaping of helper T-cell differentiation. In response to infection cells of the innate immune system produce IFNs, IL-12 and IL-18 that drive T_{H1} differentiation and IFNγ secretion by these cells. It is generally regarded that T_{H1} polarisation is preferred for anti-tumour immunity as it activates NK cells and $CD8^+$ T cells. In contrast, T_{H2} $CD4^+$ helper T cells specialise in promoting B-cell activation and differentiation into antibody-secreting plasma cells by production of IL-4, IL-5, IL-6 and IL-13 and play a primary role in infection and allergy.

$CD4^+$ T cells are known as helper T cells as they help prime optimal and functional $CD8^+$ T-cell responses. Help is provided in a number of forms including the induction of APC maturation through the provision of CD40-CD40L co-stimulation. This is demonstrated by the ability to prime $CD8^+$ T cells in the absence of $CD4^+$ T cells when agonistic anti-CD40 MAb is administered [75, 76]. The provision of CD40-CD40L co-stimulation has been implicated in the upregulation of granzyme inhibitors to render DCs insensitive to CTL-mediated killing [77]. However, CD40L signalling alone cannot fully replace the requirement for $CD4^+$ T-cell help as the provision of other co-stimulation (i.e. CD27-CD70, [67-69] and cytokine secretion by activated DCs is also necessary. Cytokines provided by helper T cells such as IFNγ and IL-2 are critical for activation of both APCs and T cells.

Although in some cases $CD4^+$ T-cell help is dispensable for primary responses during infection, it does appear to be an absolute requirement during priming for generating functional memory $CD8^+$ T cells. Memory $CD8^+$ T cells primed and maintained in the absence of $CD4^+$ T-cell help failed to mount a protective secondary response due to the inability to survive, expand or acquire effector function [78-81]. A recent report suggests that help is implicated in epigenetic modifications at the IL-2 and IFNγ loci that allow memory $CD8^+$ T cells to rapidly secrete these cytokines in secondary responses [82]. Besides this finding, the precise mechanisms by which the help "protects" memory $CD8^+$ T cells remain elusive.

$CD4^+$ T cells can additionally mediate tumour rejection in a $CD8^+$ T-cell independent fashion and can kill tumours that are resistant to CTL lysis, despite in some cases the tumours being MHC class II negative [83]. The mechanisms are not clear but are thought to be mediated through cytokines (IFNγ) for the recruitment of effector cells that can mediate killing (i.e. NO-secreting cells such as macrophages, [84] or the provision of death signals (i.e. FasL). Despite the focus on CTL induction as the main effectors in anti-tumour immunity, careful consideration must be taken to provide suitable $CD4^+$ T-cell help as part of any vaccination strategy.

1.2.2.10 T Cell Contraction Phase

At priming the TCR signal determines the programme of T-cell differentiation; clonal expansion, acquisition of effector function and contraction. During the contraction phase when antigen has been eliminated, T cells must switch from being apoptotic-resistant to apoptotic-sensitive to undergo activation-induced cell death (AICD) or intrinsic

programmed cell death. Multiple apoptotic pathways are involved including negative signalling through CTLA-4 and PD-1, death receptors (Fas and TRAILR1/2), cytokine withdrawal, and the expression of pro-apoptotic proteins (Bim). The cytokine IL-2 is essential for clonal expansion but also potentiates AICD through the upregulation of Fas and suppression of cFLIP (inhibitor of Fas signalling) [85]. Activated Fas recruits the adaptor molecule FADD (Fas-associated death domain), caspase-8 and caspase-10 to initiate intracellular apoptotic events [70]. In contrast, intrinsic programmed cell death is determined by the ratio between anti- and pro-apoptotic Bcl-2 family members. Induction of apoptosis is prevented in naïve T cells by the binding of anti-apoptotic Bcl-2 and Bcl-xL to pro-apoptotic Bim [86]. However, during the expansion and contraction phases Bcl-2 levels decrease allowing Bim to initiate apoptosis.

1.2.2.11 Memory T Cells

During the contraction phase <10% of activated T cells survive and differentiate into long-lived CD45RO $CD44^{hi}$ memory T cells. The number of memory T cells generated ultimately depends on the number of effector T cells recruited to the response thereby making it crucial to improve vaccine performance. Memory T cells possess accelerated responsiveness upon re-encounter of antigen to produce vigorous secondary T-cell expansion. The effectiveness of secondary responses is also assisted by the increased frequency of memory compared to naïve cells. Memory cells can persist for years as is the case of immunity against certain pathogens and that elicited by prophylactic vaccination.

Two functionally distinct memory T-cell subsets classified by their migratory and homing properties have been described. The central memory T cells (T_{CM}) retain expression of the lymph node-homing receptor CD62L and CCR7 receptor (similar to naïve cells) so preferentially migrate to secondary lymphoid organs but can also be found at peripheral sites during inflammation [87, 88]. These cells lack effector function but are thought to retain the ability to proliferate and differentiate into effector cells (T_{H1}, T_{H2} or CTL). In contrast, effector memory T cells (T_{EM}) retain effector function but lack the ability to re-enter lymph nodes due to low expression of CD62L and no CCR7, and therefore preferentially home to non-lymphoid tissues where they survey the periphery [87, 88]. T_{EM} cells can secrete cytokines but rely on T_{CM} for back-up effector cells as they cannot undergo expansion.

T cells undergo phenotypic and functional changes to differentiate into memory cells. How these cells are generated is not completely understood. There are two proposed models of differentiation; progressive (linear) development and divergent development.

The progressive development model (appears to have the most support) suggests that a small proportion of fully activated T cells with effector function survive the contraction phase to differentiate into memory T cells. A study reported that at the peak of a $CD8^+$ T-cell response 5-15% of effector cells expressed high levels of IL-7R and these preferentially differentiated into memory cells [89]. This model suggests that T_{EM} convert to T_{CM} as part of the linear differentiation [90]. Equally there is support for divergent development where T cells that do not acquire full effector function differentiate into memory cells capable of mounting potent secondary responses [91].

The mechanisms involved in the maintenance of memory cells and how they work in conjunction are not fully defined. Both cytokine and TCR signalling appear to have roles in memory T-cell maintenance, but it is the former that is critical for homeostatic proliferation and survival [92, 93]. IL-7 and IL-15 have roles in memory $CD8^+$ T-cell maintenance as demonstrated by a reduced number and not absence of these cells in IL-$15^{-/-}$ mice [94]. IL-7 is also important for $CD4^+$ T-cell homeostatic turnover and survival [92, 93]. Signalling through 4-1BB and OX40 has been implicated in the survival of memory $CD8^+$ and $CD4^+$ T cells respectively [95] although the cell type(s) providing the ligands remain undefined. IL-15 treatment induced 4-1BB expression in memory $CD8^+$ $CD44^{hi}$ cells from spleen or BM and the recovery after adoptive transfer of memory $CD8^+$ T cells was 2-3 fold lower in 4-1BB$L^{-/-}$ compared to wild-type mice [96]. This role of TNF family receptor signalling is in accordance with their role in T-cell survival. However, it remains unknown whether this co-stimulation is involved in the selection of activated T cells for the memory pool or only in their maintenance over a lifetime. A different mechanism of $CD4^+$ T-cell maintenance has been described in human subjects receiving a booster injection of tetanus toxoid. There was a bystander expansion of memory populations specific for two other common antigens, with cytokine secretion and kinetics that paralleled the specific response [97]. This observation conceivably ties in with the roles for cytokines and co-stimulation in memory T-cell maintenance.

Vaccination of cancer patients ultimately aims to generate memory responses that through boosting can be expanded to combat tumours. However, the cancer setting may pose a challenge for this aim through persistent and exhaustive antigen exposure that could potentially be detrimental for memory cell differentiation. Although a distinct situation, memory $CD8^+$ T cells from mice with chronic but not acute LCMV infection were unable to undergo homeostatic proliferation and survive long-term [98].

1.2.2.12 Regulatory T Cells

The regulatory T cell (Treg) population accounts for 5-10% of human $CD4^+$ T cells. The transcription factor Foxp3 positively regulates expression of Treg-specific genes and is therefore an exclusive marker. Expression of $Foxp3^+$ is largely restricted to lymphoid tissues (lymph nodes, spleen, BM and thymus) [99]. This cell population can arise in the thymus or differentiate from naïve $CD4^+$ $CD25^-$ $Foxp3^-$ T cells in peripheral lymphoid organs [100]. When naïve $CD4^+$ T cells are exposed to a low antigen dose, IL2 and TGFβ cytokines without co-stimulation, signalling through the TCR differentially activates transcription factors resulting in Foxp3 expression [101]. As a result Tregs express molecules associated with an activated phenotype (CD25, CD62L, CTLA-4), suggesting they are in an antigen-primed state *in vivo*. However, freshly isolated Tregs are in an anergic state as they do not proliferate or secrete cytokines after activation with DCs [102].

Tregs require TCR triggering and IL-2 signalling for activation and mediate their suppressive effects in a contact-dependent but antigen-independent manner. They are able to inhibit the proliferation and function of various cells, including T cells, B cells, NK cells and APCs (through downregulation of CD40, CD80, CD86 and MHC class II, [103]). Tregs release suppressive IL-10, VEGF and TGFβ and express high levels of CTLA-4 to inhibit T-cell activation [104]. The expansion and suppressive function of Tregs themselves are enhanced by TGFβ [105]. Tregs play a critical role in maintaining peripheral tolerance as demonstrated by observed lymphoproliferative disease in humans and mice in their absence [104]. The existence of a regulatory loop has been proposed where tolerogenic DCs induce the generation of Tregs from naïve $CD4^+$ T cells and these in turn programme the production of more tolerogenic DCs. Treg-mediated suppression of anti-tumour immunity is a critical obstacle and is discussed with respect to prostate cancer (PCa) later (section 1.4).

1.3 Cancer Immunotherapy

Prophylactic vaccination against infectious disease mostly aims to generate long-term protective antibody responses that can neutralise the pathogen upon encounter (e.g. influenza A and hepatitis B vaccines). However, CTLs are known to be powerful mediators of immunity against intracellular antigens and can efficiently combat established infections. Similarly, in cancer where the disease is already established the induction of CTLs is highly desired. Cancer immunotherapy can be broadly divided into two categories; passive and active. Some passive immunotherapeutic approaches have led to success in the eradication of tumours. These include the use of monoclonal antibodies (MAb) such as Herceptin® that targets the Her-2/neu tumour antigen expressed in a proportion of breast cancers and the anti-CD20 Rituximab MAb used to treat Non Hodgkin's lymphoma and some other B-cell malignancies. Cellular adoptive transfer therapy is another form of passive therapy, where allogeneic transplantation after chemotherapy is the most successful example. The effector $CD8^+$ T cells within the allograft can effectively suppress the tumour cells in what is known as the graft versus leukaemia effect (GvL). Other adoptive transfer approaches are currently being investigated where autologous immune effector cells from peripheral blood or tumour infiltrating lymphocytes (TILs) are activated and expanded *in vitro* prior to transfer into patients (reviewed by [106]). T cells genetically engineered to express high affinity TCRs, genes that confer resistance to apoptosis or enhance proliferation are more recent developments in this field, reviewed by Rooney, et al. [107]. The success of MAbs and allogeneic transplantation has provided direct evidence that cells of the immune system are able to kill tumour cells *in vivo*.

These observations have encouraged the development of immunotherapeutic strategies designed to elicit the immune attack of cancers *in vivo*. The appeal of active vaccination is the potential induction of tumour-specific immunity against tumour antigens (section 1.3.1) that can mediate tumour rejection and achieve protective long-term immune memory. Many active immunotherapeutic approaches are under exploration with the aim of achieving these goals.

1.3.1 Tumour Antigens

Evidence for the existence of tumour antigens expressed by human tumours came from the observation that TILs from melanoma patients were able to lyse HLA-matched melanoma cell lines. Boon et al., have reviewed the existence of spontaneous T-cell responses against tumour antigens [108]. Immunotherapeutic strategies can specifically target a tumour type

by activating immunity against a particular tumour antigen, or can target a variety of cancers all expressing the same tumour antigen. The latter is the ideal situation where a single vaccine may be used for the treatment of tumours of different origins. The inherent problem with many tumour antigens is their lack of immunogenicity as they are after all 'self' proteins to which tolerance may have been established.

The vast number of tumour antigens identified (reviewed [109, 110]) can be categorised into several broad groupings:

a) The cancer testis antigens are non-mutated antigens expressed in the immune-privileged areas of the testes and by tumours from a variety of origins but not in healthy somatic cells. Examples of these antigens include MAGE, BAGE, RAGE and NY-ESO [111].

b) Lineage-specific tumour antigens are expressed in healthy and neoplastic cells derived from the same tissue. Examples are the melanoma-specific MART-1, gp100, gp75 and tyrosinase proteins expressed by melanocytes and melanoma, as well as the prostate-specific antigen (PSA).

c) The majority of identified tumour antigens are over-expressed in the disease state but also have restricted expression in healthy cells. These include the Her2/neu oncogene, a growth factor receptor that is over-expressed in ~30% of breast cancers and also in ovary, lung, pancreatic and prostate cancers. Other examples are the upregulated expression of Wilm's Tumour 1 (WT1) protein in cancers of lymphoid origin, and the prostate-specific membrane antigen (PSMA). The more restricted the expression in healthy tissue the better the tumour antigen is as an immunotherapeutic target, for example PSMA expression is up to 100-fold higher in prostate cancer (PCa) than in the healthy prostate, which in turn is at least 10-fold higher than that found by other healthy tissues (section 1.5.10).

d) Mutated antigens can give rise to new epitopes that may be immunogenic. Therefore only the mutated areas of these antigens are tumour-specific. These genes include the oncogene ras and the tumour suppressor p53. Mutations can occur at high frequencies, for example p53 mutations are reported in ~50% of cancers. Another example is the idiotype (Id) of B and T cells that give rise to the expansion of a clonal rearrangement unique to the B or T-cell tumour. These tumour-specific antigens are ideal targets to avoid undesired immune attack of healthy cells. However mutations tend to be different in each patient and therefore require the laborious and costly task of making patient-specific vaccines.

e) Chromosomal translocations such as the bcr/abl chromosome rearrangements. The bcr/abl fusion gives rise to a constitutively active tyrosine kinase which leads to enhanced cell proliferation that results in chronic myeloid leukaemia.

f) Viral antigens such as the human papilloma virus proteins (HPV) E6 and E7 are expressed in cervical cancer. These tumour antigens are highly immunogenic proteins targeted for both therapeutic and prophylactic vaccines. Unfortunately only a low proportion of cancers are associated with pathogens.

The design of vaccines particularly those against over-expressed tumour antigens, has to be carefully balanced to avoid potential autoimmunity. The nature of the antigen and its tissue expression profile are important guiding principles for vaccine candidacy. Thus far autoimmunity has not been a problem with the active immunotherapy approaches that have been taken to the clinic; however they have not been particularly effective at rejecting tumours either.

Tumour antigens expressed on the cell surface (i.e. glycoproteins) are accessible to both antibody and T-cell attack. Although T cells are usually the choice of immune mediators, the success of anti-Id antibodies in suppressing lymphoma validates the anti-tumour potential of humoral immunity against surface tumour antigens. Intracellular proteins on the other hand are only susceptible to CTL-mediated attack.

1.3.2 The Role of Spontaneous Immunity: Immunosurveillance and Immunoediting

The concept of cancer immunosurveillance was initially proposed by Thomas and Burnet in the 1950s. More recently, the theory has developed and is now considered the first of the three phases of immunoediting (Figure 9). Abnormal/transformed cells (e.g. damaged cells) that have not undergone apoptosis provide pro-inflammatory signals that recruit and activate cells from the innate and adaptive immune systems. The resulting spontaneous immunity can eliminate the transformed cells in the continuous process of immunesurveillance. Alternatively, an immune equilibrium stage is established where tumour cells colonise sites and continue to divide, but are contained by the immune response. This essentially represents a dormant tumour phase in which the transformed cells are thought to exist for many years. The final immune escape phase favours tumourigenesis resulting in the uncontrollable growth of tumour cells and a clinically detectable cancer. The survival advantage allowing the tumour cells to escape

immunosurveillance becomes a feature of the tumour that establishes. In this manner immune pressure can shape the tumour by driving the selection and outgrowth of variants with phenotypes that evade, suppress and overcome host protection mechanisms.

Smyth et al., and Dunn et al., have reviewed the evidence supporting the process of immunoediting [112, 113]. Strong evidence for roles of both the innate (NK cells and macrophages) and adaptive ($CD4^+$ and $CD8^+$ T cells) immune systems in immunosurveillance comes from numerous mouse knock out or depletion models (i.e. $perforin^{-/-}$, $IFN\gamma^{-/-}$ and $Rag^{-/-}$ [114]) where mice are more susceptible to spontaneous and carcinogen-induced tumour formation. There is also support for the selective survival of tumour cells. In an adoptive CTL immunotherapy mouse model where Fas ligand delivered the death signal, the out-growth of tumour escape variants with Fas resistance was reported [115].

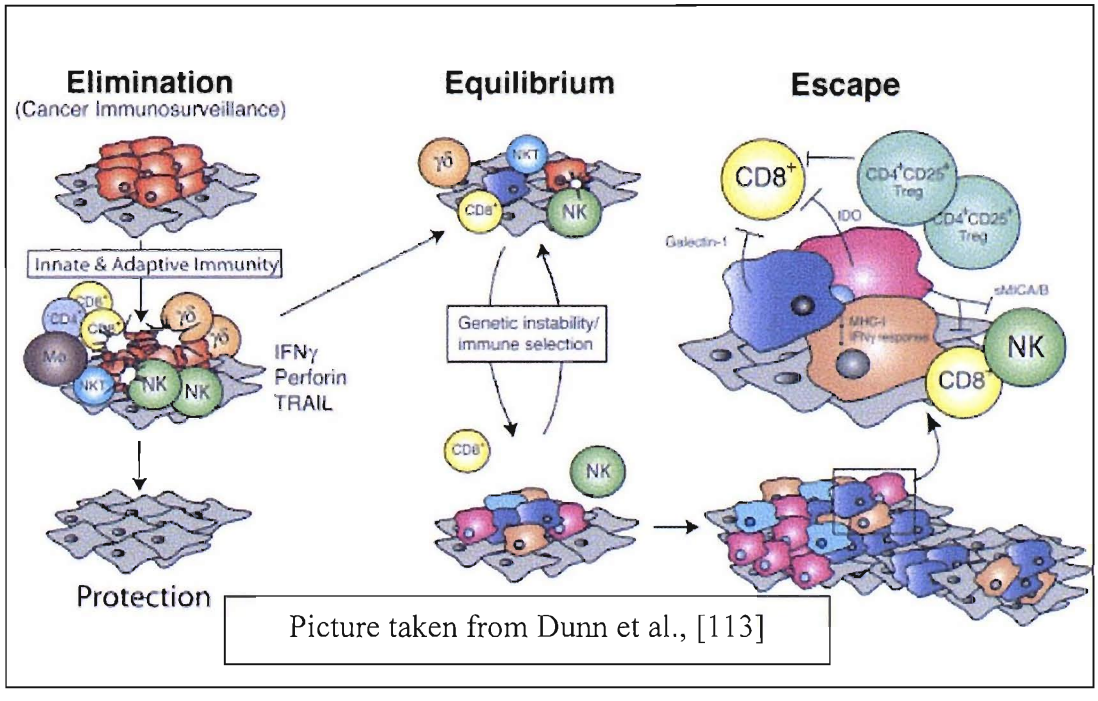


Figure 9: Immunoediting and immunesurveillance

The three stages of tumour immunoediting are depicted. Transformed tumour cells (red) are eliminated by immunosurveillance mechanisms involving a range of cells from the innate and adaptive immune system, to protect from tumour formation. Those that cannot be eradicated enter equilibrium tumour growth where immunoediting eventually gives rise to variants with phenotypes that can escape immune regulation and establish disease. This figure has been taken from Dunn et al., [113].

There is also compelling evidence of immunosurveillance in humans. Firstly, the decline of immune function in the older population makes cancer more prevalent. Secondly, immunosuppressed patients that have received transplants have a higher incidence of particularly virally-associated cancers [116]. Thirdly, cancer patients are known to have

the ability to mount a spontaneous immune response against the tumour as tumour-specific cytotoxic T cells and antibodies have been isolated from patients [117-120]. Fourthly, the presence of TILs is a good prognostic indicator of survival in various cancers including colon and ovarian cancers [121]. Lastly, the association of paraneoplastic neurological degenerative diseases with cancer and anti-tumour immunity has provided the best evidence for immunosurveillance. Some cancer patients can make $CD8^+$ T-cell responses specific for neuronal proteins expressed by tumour cells and which act to suppress tumour growth. In some of these patients the activated T cells enter the CNS and attack neurons causing neurodegeneration. This occurrence is reviewed by Darnell et al., 2004 [122].

1.3.3 The Challenge for Immunotherapy: Evasion, Suppression and Tolerance

The aim of active immunotherapy is to harness and expand the natural ability of the immune system to generate spontaneous anti-tumour immunity to control and ideally eliminate the tumour. However, there are various obstacles to overcome. Firstly, tumour cells develop multiple evasion and suppressive mechanisms (section 1.4.9 and 1.4.10) to go undetected and simultaneously suppress spontaneous immunity. Secondly, immunological tolerance is in place which limits the T-cell repertoire available for immunotherapy to expand. However, peripheral but not central tolerance is potentially reversible. There is evidence suggesting that tolerance may be avoided at least upon adoptive transfer. Tag-specific T cells primed in wild type mice delayed tumour growth when transferred into already tolerised 4-5month old TRAMP mice (spontaneous mouse PCa model expressing Tag as the oncogene, section 1.4.6) and even completely prevented tumour growth in 4/13 mice. The prostatic tissue from the long-term survivors no longer expressed the Tag oncogene suggesting that all the malignant cells were successfully eliminated by the transferred CTLs [123]. Evidence from the TRAMP mouse model also suggests that at a young age when malignant transformation is beginning tolerance to tumour antigens is incomplete and tumour growth can be substantially retarded by the administration of a peptide-loaded DC vaccine [124]. The vaccine was unsuccessful once the tumour was established which unfortunately is the setting for human immunotherapy. These studies indicate that powerful strategies are required for counteracting central and possible tumour-induced peripheral tolerance.

1.3.4 Vaccination Strategies against Tumours

Many active vaccination approaches have been shown to achieve responses against tumours in both pre-clinical mouse models and in the clinic. The intensity of the immune response appears to determine tumour clearance [125] and therefore the focus is on the improvement of vaccine design and delivery.

Vaccination with autologous irradiated tumour cells and tumour lysates or tumour RNA-loaded DCs can target a range of disease-relevant tumour antigens, although the target antigens are of unknown identity making it difficult to monitor the success of vaccination. The other measure of vaccination would be clinical benefit as defined by tumour size or persistence of metastases. Although this is ultimately the most important test, assessment of disease status is difficult to use as a quantitative measure of vaccine efficacy.

The other category of cancer vaccines targets specific tumour antigens. Delivery systems include protein, peptide, loaded DCs, plasmid DNA, and modified viruses. Proteins are taken up by APCs and presented by MHC class II molecules to effectively stimulate $CD4^+$ T-cell and antibody responses, which are only effective against cell surface antigens. Peptides used for vaccination with the aim of priming $CD8^+$ T cells fail to provide the necessary $CD4^+$ T-cell help required for optimal $CD8^+$ T-cell responses. The peptides can also bind to cells other than APCs and may thereby induce tolerance [126]. Peptides, proteins and RNA can also be delivered through the loading of DCs *in vitro* followed by adoptive transfer into patients. DCs must be matured and activated adequately to provide co-stimulation and prevent tolerance induction (reviewed [127]). DNA vaccination offers much promise as it can efficiently prime responses and provides a system that is easy to manipulate to target specific components of the immune system. It is this DNA vaccination approach that was investigated in this project and is discussed below in greater detail.

1.3.5 DNA Vaccines

DNA vaccines comprise a simple system based on an expression plasmid encoding the sequences of interest (i.e. the tumour antigen). They can be injected via a number of routes (intramuscular, intradermal, mucosal, intranodal), where they are taken up by cells at the injection site. The cells transcribe RNA and translate the protein, resulting in the expression of the encoded antigen, which ultimately is presented to the adaptive immune system (Figure 10).

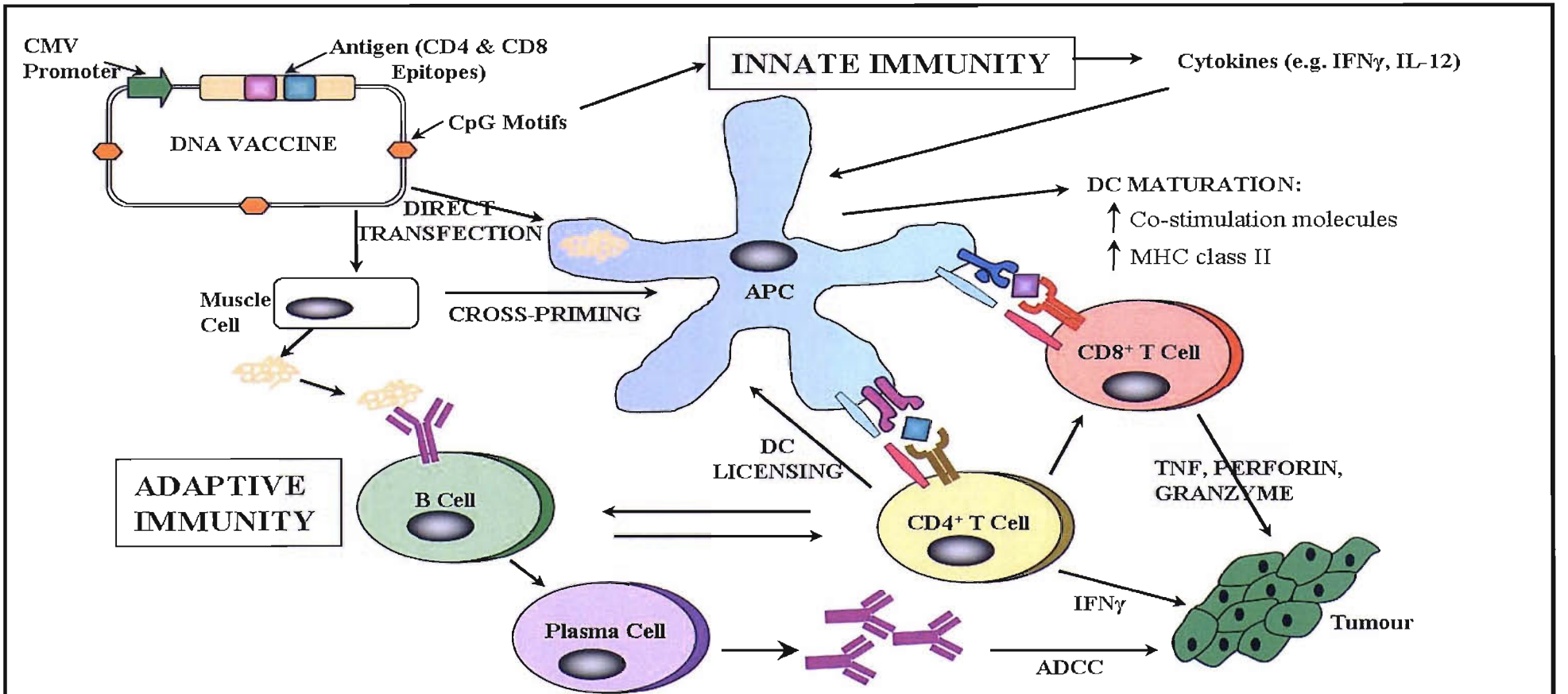


Figure 10:DNA Vaccine Mechanism of Action

Innate and adaptive immunity are activated by DNA vaccines. TLR9 activation by CpG motifs results in secretion of cytokines by the innate immune system that helps support the adaptive immune response. Muscle cells or APCs can be directly transfected with the DNA vaccine upon immunisation. Expression of the encoded protein *in vivo* is driven by a strong promoter. Protein released by these cells can engage with B cell receptors to generate humoral responses. Alternatively APCs can take up protein from apoptotic muscle cells. APCs subsequently process and present peptides to activate $CD4^+$ and $CD8^+$ T cells. Activated $CD4^+$ T cells can license APCs, and provide help to activate B cells and $CD8^+$ T cells, that in turn develop their respective effector mechanisms to kill the tumour.

Plasmids are commercially available and have been engineered for optimal expression in eukaryotic cells. They contain strong promoters active in mammalian cells, such as the viral CMV promoter, to drive high expression levels. The plasmids also have antibiotic resistance genes and an origin of replication for growth in bacteria. Polyadenylation sequences enhance the stability of transcribed mRNA. The plasmid backbone also contains immunostimulatory hypomethylated cytidine-phosphate-guanosine (CpG) motifs that are able to activate innate immunity through TLR9 stimulation (section 1.3.6).

There are advantages in using DNA vaccines over other types of vaccines. The ease of manipulation and production makes them cost-effective in comparison to protein based, live or attenuated vaccines. DNA vaccines can be easily produced with high purity and stability in large scale. Compared to live viral or bacterial vectors, DNA vaccines are safe and can be administered repeatedly without inducing antibody responses against the DNA backbone. Rational design via manipulations such as fusion of immune-enhancing sequences can activate specific pathways of the immune system. Another advantage is that the antigens encoded within the DNA vaccine are expressed endogenously and so the protein is properly folded and undergoes correct post-translational modifications. This allows antibody responses to non-continuous antigen epitopes, which cannot always occur with recombinant protein vaccination, in addition to cytotoxic T-cell activation. It has been demonstrated that the proteins encoded within a DNA vaccine are capable of activating all arms of the immune system.

1.3.6 Innate Immunity Activated by DNA Vaccine

The activation of innate immunity is crucial to provide support for the desired priming of adaptive immunity. DNA vaccination can activate innate immunity through the function of TANK-binding kinase 1 (TBK1), an IκB kinase. Total dependence on TBK1 was demonstrated by the inability of $TBK1^{-/-}$ mice to make humoral or cellular responses to the encoded antigen [128]. TBK1 directly phosphorylates and activates IRF3, leading to its dimerisation and translocation to the nucleus where it binds IRF elements for expression of type-1 IFNs. The dependency on type-1 IFN mediated signalling suggesting that it may be beneficial to target this pathway with a suitable vaccine design.

The other mechanism which has been established for longer is through the stimulation of intracellular TLR9, a receptor present in a number of cells (including B cells, DCs [not human myeloid DCs], NK cells and macrophages) but not expressed by T cells.

Unmethylated CpG motifs within the plasmid backbone are ligands of TLR9 thereby acting as built-in adjuvants to provide danger signals. Unmethylated CpG motifs are several-fold more common in bacterial DNA than mammalian DNA. Evidence first came from a study where similar cytokine production was induced by an empty plasmid and bacterial DNA but also that methylation of the DNA plasmid decreased its immunogenicity [129].

There are various stimulatory effects of TLR9 activation, including APC maturation (section 1.2.2.5), the secretion of pro-inflammatory (IL-1, IL-6, IL-18, IFNβ, IFNα and TNFα) and T_{H1}-biased cytokines (IFNγ and IL-12). T_{H1} cytokines activate NK cells that can kill tolerising immature but not mature DCs by virtue of their low level of MHC class I expression. Therefore TLR stimulation provides an important link between adaptive and innate immunity (reviewed [130, 131]).

Human plasmacytoid but not myeloid DCs express TLR9 resulting in different cytokine and chemokine expression to the mouse immune system in response to TLR9 activation. Initially this was suggested to account for the lower efficacy of DNA vaccines in humans and primates compared to mice. However, equivalent responses to DNA vaccines have been reported in wild type, $TLR9^{-/-}$ and $MyD88^{-/-}$ mice [132, 133]. A separate study found decreased immunity in $TLR9^{-/-}$ mice [134], suggesting that DNA vaccines do not rely on TLR9 stimulation for their activity but CpG motifs are likely to act as adjuvants in settings where there is weak or no T-cell help. TLR9-stimulating capacity of CpGs is further demonstrated by the ability of synthetic CpG oligodeoxynucleotides (ODNs) to act as adjuvants (reviewed by Klinman et al., [135]). These are not analogous to CpG motifs within plasmids/bacterial DNA as they have nuclease-resistant phosphorothioate backbones conferring resistance to degradation and are designed to have a higher concentration of optimal CpG motifs.

1.3.7 Direct and Cross-Presentation of Antigen to the Adaptive Immune System

DNA vaccines are usually administered either via intramuscular or intradermal routes, at mucosal sites or directly into skin cells by gene gun. There is evidence for the participation of BM-derived APCs in priming adaptive immunity following DNA vaccination. Irradiated $H\text{-}2^{bxd}$ chimeric mice were reconstituted with BM of either $H\text{-}2^{b}$ or $H\text{-}2^{d}$ haplotype. Intramuscular immunisation of the recipient mice with a plasmid encoding the influenza A nucleoprotein elicited a CTL response restricted by the MHC

haplotype of the transferred bone marrow only and not the other haplotype expressed by the recipient's myocytes [136]. Other published experiments confirmed this observation [137] and verify that muscle cells at the injection site do not directly present the encoded antigen to T cells.

There are two mechanisms by which the APCs can obtain antigen following intra-muscular DNA vaccination. The plasmid can be directly delivered to APCs that will process antigen via the classical MHC class I pathway. Secondly, APCs can phagocytose apoptotic antigen-expressing (directly transfected) myocytes and proceed to cross-prime $CD8^+$ T cells. Due to cell abundance in the muscle, the majority of cells transfected by intramuscular immunisation are myocytes [138] and only a small proportion of APCs [139]. However, a central role for the involvement of non-APC cells in cross-priming has been demonstrated through the detrimental effect on $CD8^+$ T cell priming and antibody responses by the use of APC-specific promoters [140]. Muscle cells appear to act as antigen reservoirs that maintain the immune response [141]. However, it has also been reported that the removal of muscle 10 minutes after intramuscular injection did not affect the magnitude or longevity of the immune response indicating that APCs can take up the antigen at distal sites and arguing against the necessity for continued antigen expression by the vaccinated muscle [142]. In agreement with this observation, the mRNA transcribed from a DNA vaccine encoding a *Mycobacterium leprae* heat shock protein could be found in a range of tissues 7 days after intra-muscular immunisation, presumably due to migration of directly transfected cells [143].

Gene gun delivers DNA directly into cells in the skin where there are abundant numbers of professional APCs (Langerhans cells) that become directly transfected. Gene gun immunisation with gold particles co-coated with plasmids encoding human CD4 and the *E.coli* LacZ gene resulted in directly transfected cells expressing both membrane bound CD4 and intracellular LacZ. The majority of CD4 and LacZ-expressing cells at the draining lymph nodes were DCs that could present LacZ epitopes to prime $CD8^+$ T cells. Depletion of CD4-expressing cells from the draining lymph node cell population 24 hours post immunisation resulted in the removal of directly transfected DCs and a subsequent 60-70% reduction in T cells with β-gal activity [144]. Keratinocytes in the skin may also be transfected by vaccination and act as a source of antigen for APCs to cross-prime. Therefore antigen can be presented to T cells through both direct and cross-priming mechanisms when DNA vaccines are administered by the intradermal or intramuscular routes of injection.

1.3.8 Antigen Persistence Following DNA Vaccination

The persistence of antigen expression has been reported to range from weeks to months [138, 145]. The immunogenicity of the encoded protein appears to be an important influencing factor. Antigen expression inversely correlated with the onset of antigen-specific CTL expansion, with the CTL population promptly contracting after antigen elimination [146]. This decrease of antigen expression occurs in immune-competent but not immune-incompetent mice [146, 147] suggesting that destruction of antigen-expressing myocytes is likely to be through both CTL and/or antibody-mediated mechanisms.

Antigen persistence appears to have a critical role in determining the efficacy of the immune response. The use of a drug-inducible system revealed that prolonged antigen expression in a primary response limited the magnitude of the $CD8^+$ T-cell effector peak and generated T cells with lower functional avidity [148]. However, during a secondary response those T cells primed under limited antigen expression had a far greater proliferative capacity [148]. This suggests that a vaccine design that limits antigen expression may generate a superior memory T-cell population.

1.3.9 Priming of Helper $CD4^+$ T cells by DNA Vaccines

Following intramuscular DNA vaccination, $CD4^+$ T cells are activated and undergo a differentiation programme usually of a T_{H1} type. However, helper T-cell responses are not necessarily strongly polarised towards one particular subset, as a collaboration of T_{H1} and T_{H2} component responses can be important for the induction of maximal anti-tumour immunity [149]. TLR9 stimulation [150] and the nature of the antigen [151] are both factors that can impact the extent of T_{H1} polarisation.

Primary CTL responses following infection can be independent of $CD4^+$ T-cell help. It is thought that strong stimulation by pathogens (i.e. TLR9 activation) may be responsible for bypassing helper T-cells. However, in the setting of DNA vaccination, helper T cells have been shown to be essential in $CD8^+$ T-cell priming. This has been demonstrated by the seven-fold enhancement in the priming of a $CD8^+$ T-cell response against the ovalbumin (OVA) SIINFEKL peptide when a CD4 epitope was exchanged for CLIP in the Ii sequence and included in a modified vaccine design (resulting in optimal loading of MHC class II) [152]. Further evidence for the requirement of $CD4^+$ T cells comes from the inability of MHC class II-deficient mice devoid of $CD4^+$ T cells to induce a CTL response to a DNA vaccine [153]. $CD4^+$ T cells have also been implicated in the protection of CTLs from activation induced cell death, possibly through specific co-stimulation [154].

Tumour-specific antibodies can also mediate tumour killing. It has been demonstrated that engagement of helper T cells is essential to initiate primary B-cell responses but also to avoid inhibition of memory B cells. Boosting with the non-immunogenic tumour antigen protein alone (that cannot provide T-cell help) resulted in the ablation of the tumour antigen-specific antibody responses primed by DNA vaccination [155]. B cell memory responses therefore rely on the continued provision of T-cell help. A potential problem for antibody-mediated protection is that tumours could release soluble antigens that could cross-link the BCRs resulting in the suppression of B cells. While persistence of plasma cells might maintain antibody levels for some time, memory B cells will be essential for continual differentiation into plasma cells. One way to overcome this could be by repeated vaccination.

1.3.10 Cytotoxic T cell Responses Primed by DNA Vaccination

The kinetics of CTL priming following intramuscular DNA vaccination can vary with different constructs. Generally after a single priming injection $CD8^+$ T cell responses are detectable as early as day 7, tend to peak around day 14 and are detectable for a few weeks [139, 146, 156, 157]. The precise kinetics of the expansion and contraction phases appear to depend on the nature of the antigen encoded within the plasmid and the mechanism by which T cells are primed [157].

In mouse models epitope-specific CTLs have been shown to have a powerful capacity to protect against and kill tumours cells in prophylactic and therapeutic settings [158-160]. Eradication of established tumours by the adoptive transfer of $CD8^+$ T cells in mouse models provide further evidence for the effectiveness of CTL-mediated anti-tumour immunity [161]. Additionally, $CD8^+$ T cells are responsible for the clearance of viral infections. Injection with a DNA vaccine was able to provide long-term $CD8^+$ T cell-mediated protection in a *Leishmania major* model [162]. This demonstrates that DNA vaccines are capable of successfully providing long-term protective $CD8^+$ T cell memory responses. The aim is therefore to induce effector and memory anti-tumour CTL responses.

1.3.11 Humoral Responses Generated by DNA Vaccination

Humoral immune responses play an important role in the control of certain mouse tumour models where antibodies are targeted against cell surface antigens. Antibodies are able to mediate anti-tumour activity directly by ADCP or ADCC.

Knowledge from humoral responses against pathogens is useful to decipher the types and kinetics of responses. In mice, antibody responses elicited by DNA vaccination tend to rise slower and take several weeks to peak in comparison to protein or attenuated viral vaccines. However, DNA vaccines are able to generate persistent antibody responses [139]. Antibody titres are also increased with dose or multiple immunisations [162]. In the therapeutic setting using a Her-2/neu transgenic mouse model, the administration of a Her-2/neu DNA vaccine with electroporation resulted in antibody titres that increased with boosting. The antibodies elicited were primarily of T_{H1} subtype, could mediate ADCC and were indispensable for protection [163]. Another model supporting protective efficacy of antibodies generated anti-idiotype TCR humoral response following DNA vaccination. IgG purified from the sera of immunised mice was transferred into naïve recipients that were subsequently able to reject challenge with T-cell lymphoma [164]. Anti-Id antibodies generated by DNA vaccination have also demonstrated the capacity to protect against mouse B-cell lymphomas [165].

1.3.12 DNA Vaccine Dosage and Volume Administered

The dose of DNA vaccine administered to mice and humans differs greatly on a weight for weight basis. Mice weigh ~20g and are usually administered with up to 50μg of DNA vaccine intramuscularly. The corresponding amount of DNA for humans would be impossible to administer (>100mg). The importance of vaccine dose was shown by a murine study from our laboratory where increasing doses resulted in enhanced $CD8^+$ T-cell responses (reached a plateau at 30μg) [166]. This may be at least in part explained by the strikingly lower levels of plasmid DNA detected in a more restricted number of tissues when mice were immunised with a low dose (20μg) compared to a high (100μg) dose of a DNA vaccine [143].

Increasing the injection volume also significantly improves CTL responses in mice, with doses below 40μl generating significantly reduced immunity [166, 167]. It is thought that the increased hydrostatic pressure created by larger injection volumes may facilitate the uptake of DNA across the plasma membrane. The injection volumes used for mice cannot be proportionally matched for larger animals or humans.

The dose and volume of DNA vaccines may be an important factor explaining why expectations from murine models have not been met in the clinic. However since these cannot be scaled up other methods of enhancing vaccine delivery must be assessed.

1.3.13 Enhancing DNA Vaccine Performance through Modified Delivery Strategies

On the whole weak humoral and cellular anti-tumour responses have been elicited in the clinic by intra-muscular and intra-dermal injections alone. There is a clear need to improve DNA vaccine efficacy for clinical application. One way to improve the efficacy of DNA vaccines is through modified delivery systems.

1.3.13.1 Gene Gun

Intradermal immunisation with a gene gun propels DNA-coated gold particles directly into the epidermis and into the intracellular environment of professional and non-professional APCs. This technology has demonstrated enhanced effectiveness but has also been associated with the induction of a T_{H2}-biased response in some mouse models [151, 168]. Fuller et al., have reviewed the use of gene gun delivery of DNA vaccines in non-human primate models and humans for the induction of immunity against infectious diseases [169].

1.3.13.2 Electroporation

Electroporation represents a promising method for delivering DNA vaccines due to the ease and relatively painless administration. The application of an electric field across the vaccinated muscle makes the cell membrane permeable for passive uptake of the DNA at the injection site. Electroporation increases antigen expression *in vivo* by several fold [117, 170, 171]. Various studies have demonstrated that the increased antigen expression potentially translates into greatly enhanced antibody and CTL responses in the orders of 10-200 fold [166, 171-173]. Electroporation also causes local muscle necrosis [170] that is likely to provide 'danger' signals to recruit cells of the immune system to the site of inflammation and thereby promote the induction of immunity. A single intramuscular DNA injection with electroporation delivered the DNA vaccine with similar efficiency to an adenoviral vector [174]. Suboptimal epitope-specific $CD8^+$ T-cell responses that poorly

protected from tumour when the DNA vaccine was administered in a small volume were efficiently restored with electroporation [166]. These observations suggest that enhancing the uptake of DNA, thereby increasing antigen expression, together with an inflammatory stimulus can improve the immunogenicity of DNA vaccines. Electroporation has been shown to effectively enhance immune responses in non-human primates [172] and is now being tested in the clinic.

1.3.14 Prime-Boost Strategies Enhance Responses to DNA Vaccination

DNA vaccination has proven to be a successful method for priming immune responses. However, further immunisations are required to optimally boost memory responses. One explanation for the high efficacy of prime-boost vaccination is the finding that memory but not effector $CD8^+$ T cells can protect DCs from CTL-mediated killing *in vitro* [175]. Immunisation with naked DNA is poor at boosting immunity so various other boosting strategies are under investigation.

1.3.14.1 Heterologous Boost with Viral Vectors

Gene therapy viral vectors have been adapted for cancer immunotherapy to make them unable to replicate in mammalian hosts (i.e. vaccinia and fowlpox) or modified to make them unable to replicate into infectious virions after infection of a single target cell (i.e. recombinant replication-incompetent adenovirus, retrovirus and lentivirus). These viral vectors lead to efficient expression of the inserted antigen and provide an inflammatory stimulus.

Immunisation with viral vectors weakly primes antigen-specific T cells. The low immunogenicity is likely to be at least in part due to the presence of viral T-cell epitopes that can potentially compete with the less immunogenic tumour antigens and the presence of pre-existing neutralising antibodies specific for the virus. This is demonstrated in a study where canarypox vaccines could elicit CTLs against highly immunogenic CTL epitopes (OVA) but not the less immunogenic CEA antigen. More strikingly was the abrogation of the CTL response primed by a DNA-CEA vaccine when co-injected with the canarypox vector [176].

The use of a naked DNA prime and viral vector boost immunisation protocol allows the amplification of already primed antigen-specific T cells, probably through increased protein expression and a more inflammatory environment provided by the immunogenic viral proteins. In mice, greatly enhanced T-cell frequencies can be generated by a DNA

prime and viral vector boost regimen compared to naked DNA or viral vectors alone [177, 178]. Benefits have also been demonstrated in humans where patients were primed with a DNA vaccine encoding a malaria antigen and boosted with a vaccinia vector. This heterologous prime-boost generated a greater increase in antigen-specific $CD8^+$ T cells (that persisted for several months) compared to the homologous immunisations [178].

Multiple immunisations are likely to be required to generate a strong and on-going immune response in cancer patients but viral vector vaccines would probably only be effective for a single boost as multiple boosts may direct the immune response to the viral components. However, the use of different viral vectors without shared viral epitopes for sequential administration/boosts could evade this limitation.

1.3.14.2 Naked DNA Boost with Electroporation

Naked DNA combined with electroporation at boosting can induce enhanced cellular and humoral recall responses in a prime-boost protocol [160, 166]. Further dissection showed that electroporation at priming and/or boosting can amplify both the CTL (2.5-fold) and antibody (7-fold) responses. However, most effective was electroporation at boosting only as both cellular and humoral responses were ~1.5 fold higher compared to those generated by electroporation at both priming and boosting. Interestingly, electroporation at priming only and boosting with DNA alone generated responses equivalent to those not using electroporation at all [166]. This indicates that the elevated antigen levels provided by electroporation are important only during boosting of $CD8^+$ T-cell and humoral responses.

These findings are mirrored in tumour protection models. Multiple administrations of Her-2/neu DNA vaccines with electroporation to Her-2/neu transgenic mice resulted in significantly enhanced T-cell cytolytic activity and antibody titres that together protected from the spontaneous tumour [163, 179]. In contrast, DNA vaccination without electroporation generated lower levels of cellular and humoral immunity that was only able to offer marginal temporary protection [163]. This clearly demonstrates the powerful ability of DNA vaccines to mobilise both cellular and humoral immunity to provide protection against tumours when the delivery is optimised. This strategy of boosting with electroporation is currently undergoing assessment for the induction of $CD8^+$ T-cell responses in the clinic.

1.3.14.3 Heterologous Prime-Boost with DNA Vaccines Processing Antigen by Alternative Mechanisms

Heterologous immunisation where sequential priming and boosting with vaccines that differentially present antigen (direct or cross-priming) resulted in a ~16-fold increase in the secondary expansion of memory $CD8^+$ T cells compared to the homologous prime-boost regime [157]. The augmentation of the memory response translated into superior *in vivo* killing of adoptively transferred peptide-loaded target cells. This observation was irrespective of the order in which the vaccines were administered. The reason for the effectiveness of this heterologous strategy remains unknown but it was hypothesised that the vaccines may target different APCs at different sites. This could mean that boosting with the reciprocal vaccine may overcome the elimination of antigen-presenting APCs that is mediated by effector T-cell responses.

1.3.15 DNA Fusion Vaccines: Linking Immune-Enhancing Sequences

The administration of a plasmid encoding an autologous antigen alone is generally not sufficient for inducing an effective immune response. The efficacy of DNA vaccines is often enhanced by fusing antigens to various sequences with the aim of targeting the expressed protein to specific processes/pathways/cells. Zhu et al., and Stevenson et al., have reviewed some strategies that are described below [109, 180]. There are many strategies for enhancing antigen processing/presentation (fusion to ubiquitin signals [181, 182]), uptake of antigen by DCs (fusion to Fcγ, [183]), uptake of antigen by B cells (C3d fusion vaccines, although this can also be detrimental [184, 185]), induction of myocyte death (fusion to caspases, [186]) amongst others.

The fusion of xenogeneic/foreign sequences which contain highly immunogenic MHC class II binding peptides that provide strong $CD4^+$ T-cell help is a potent strategy for enhancing immunity to a tumour antigen. Our laboratory was one of the first to successfully demonstrate this concept that is now used by others. Our vaccines encode tumour antigens fused to the bacterial Fragment C (FrC) sequence from tetanus toxin, that result in the dramatic enhancement of tumour-antigen specific immunity through the provision of strong $CD4^+$ T-cell help specific for the FrC component [158-160, 164, 165, 187]. This vaccine design is discussed in detail in section 1.3.16. The potato virus X coat protein (PVXCP) foreign sequence when linked to B-cell tumour Ids can also induce $CD4^+$ T-cell responses that mediate protection against mouse lymphoma and myeloma models [188]. Fusion of the sequences containing the CD4 and CD8 epitopes is likely to result in the uptake of both by the same APC thereby allowing $CD4^+$ T helper cells and $CD8^+$ T

cells to interact with the same APCs, which ultimately lead to optimal $CD8^+$ T-cell responses.

1.3.16 The Vaccine Designs

Our laboratory has created two unique DNA fusion vaccine designs that make use of the FrC sequence. The first is a vaccine encoding the full-length FrC fused to the tumour antigen sequence, which is most effective at inducing humoral responses against tumour antigens. The second vaccine only encodes part of the FrC sequence, DOM, fused to a minimal C-terminal MHC class I-binding epitope from the tumour antigen sequence (Figure 11). This is a more successful design for the induction of epitope-specific $CD8^+$ T cell responses. Both vaccine designs are utilised in this project in order to assess the ability to induce immunity to the PCa tumour antigen PSMA.

1.3.16.1 DNA Vaccine Fused to the Bacterial Tetanus Toxin Sequence, Fragment C

Tetanus toxin is a 150kDa neurotoxin produced by the bacterium *Clostridium tetani*. FrC is an immunogenic but non-toxic 50kDa C-terminal fragment from the microbial tetanus toxin. A DNA vaccine encoding FrC alone elicits protective immunity against *Clostridium tetani* thereby confirming the immunogenic nature of the FrC sequence [189].

Our vaccine design fuses tumour antigens to a codon-optimised FrC sequence (Figure 11) which boosts tumour-specific immunity [164, 165, 187] through the provision of foreign $CD4^+$ T-cell help [155]. Tumour antigens encoded within the vaccines are themselves unlikely to supply significant helper $CD4^+$ T-cell responses due to a limited repertoire and possible tolerance. Following vaccination APCs take up the fusion protein and present MHC class II peptides to prime FrC-specific $CD4^+$ T cells. These in turn provide activation signals to B cells and DCs that are presenting the corresponding FrC MHC class II peptides. Licensed DCs are then able to efficiently cross-prime tumour antigen-specific $CD8^+$ T-cell responses whilst the B cells differentiate into plasma cells and produce antibodies against both components (the tumour antigen and FrC, Figure 11). The advantage of using a foreign sequence to provide T-cell help is clear since the T-cell repertoire for the antigen is large and unlikely to be tolerised. Although the use of foreign sequences will provide $CD4^+$ T-cell help for the induction of antigen-specific CTL responses, they will not take part in the $CD4^+$ T-cell anti-tumour effector phase that is

established. However, they will also not be subject to the deletional or regulatory mechanisms that tumour antigen-specific $CD4^+$ T cells are subjected to.

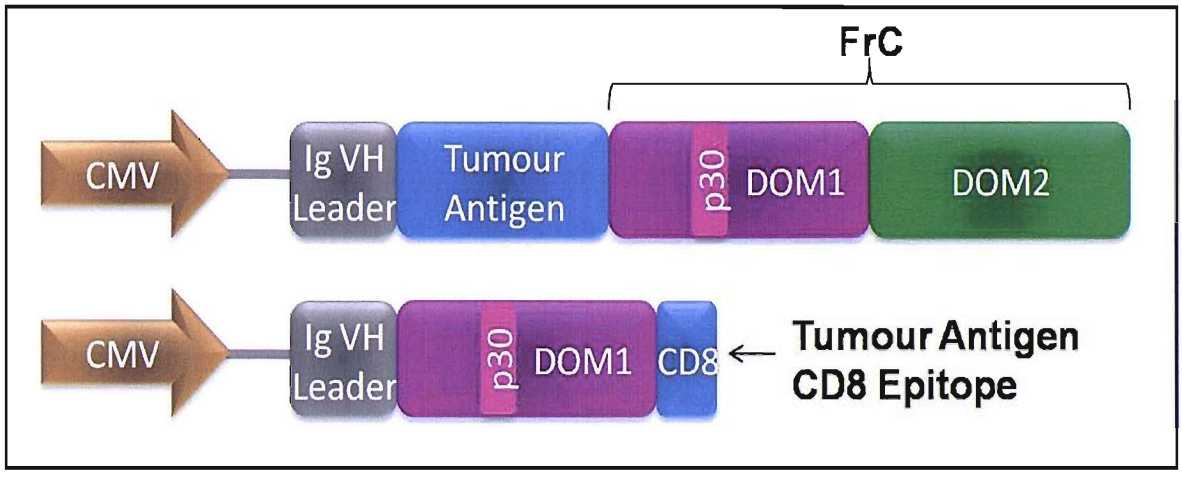


Figure 11: The Vaccine Designs

The bacterial FrC sequence (DOM1 (purple) and DOM2 (green)) is fused to the N-terminal of the tumour antigen (blue). The truncated FrC sequence, DOM1 (purple), is fused to a tumour antigen $CD8^+$ T cell epitope (blue). All vaccines contain the p30 $CD4^+$ T cell epitope (lilac) located within DOM1, the Ig V_H leader sequence (grey) for targeting to the ER and a CMV promoter (brown) to drive expression.

1.3.16.2 DNA Fragment C Fusion Vaccine Effectively Generates Antibody Responses and Protects from Tumour Challenge

In our laboratory DNA vaccines encoding tumour antigen-FrC fusions have been shown to elicit antigen-specific antibody and $CD4^+$ T-cell responses that protected mice from challenge with antigen-expressing tumour cells [165]. The system was first studied using Id determinants from human B-cell malignancies and mouse A31 lymphoma or 5T33 myeloma models. The Id determinants are encoded by variable regions of the tumour immunoglobulin heavy and light chains that assemble as single-chain variable region fragments (scFv). DNA vaccines encoding tumour antigens alone (p.scFvs) were not immunogenic but when fused to FrC (p.scFv-FrC) antibody responses to both FrC and the tumour antigen components were generated [165, 187]. The fusion was an absolute requirement for the induction of tumour antigen-specific antibodies, supporting the concept of provision of cognate T-cell help from the large anti-microbial (FrC) repertoire.

The respective scFv-FrC Id-specific responses were able to protect mice against A31 lymphoma and 5T33 myeloma challenges [165]. The humoral response induced by the p.scFv-FrC vaccine was sufficient for protection as $CD4^+$ T-cell depletion after the induction of the immune response did not abrogate protection against A31 lymphoma

challenge [188]. The precise effector mechanisms that directly protect remain unestablished for both models. Later studies have demonstrated that $CD4^+$ T-cell help is an absolute requirement in the maintenance and expansion of memory B cells. The anti-Id and anti-FrC antibody responses elicited by DNA vaccination are enhanced when boosted (memory B-cell expansion) with Id IgG-FrC protein conjugate in the presence of $CD4^+$ T cells and not when this population is depleted. However, the anti-Id antibody response is completely suppressed when boosted with only the Id IgG protein that is unable to provide $CD4^+$ T-cell help [155]. These experiments provided proof that the FrC vaccine component provides cognate help but also demonstrates the importance of continued $CD4^+$ T-cell help for the ongoing differentiation of memory B cells into Ig-secreting plasma cells.

A similar study with a DNA vaccine encoding the T-cell Id from a mouse T-cell lymphoma model fused to FrC, demonstrated that the fusion made the T-cell Id immunogenic and elicited antibodies against both vaccine components. Purified IgG from vaccinated mice could protect non-immunised mice from T-cell lymphoma challenge [164]. Together these models validate the use of FrC fusions to elicit antigen-specific antibodies and $CD4^+$ helper T cells for anti-tumour immunity.

The FrC fusion vaccines contain an N-terminus leader sequence. The fusion protein expressed from the expression cassette is therefore targeted to the ER and secreted. Studies from our laboratory demonstrated that mice immunised with a DNA vaccine encoding FrC with a 5' leader sequence generated more rapid and ~10-fold higher anti-FrC antibody titres than the leaderless counterpart [190].

1.3.16.3 Immunodominance

The ability of a DNA vaccine to efficiently elicit $CD8^+$ T-cell responses can be hindered when competing CD8 epitopes are encoded within the sequence. This is known as immunodominance, where despite the presence of a large number of potential $CD8^+$ T cell epitopes within the antigen sequence the primed $CD8^+$ T-cell immune response is biased towards the most immunogenic epitope(s), and further narrowed upon boosting [191]. The factors defining epitope immunodominance include the epitope binding affinity for MHC class I molecules, the stability of the peptide-MHC complexes, the frequency of $CD8^+$ T-cell repertoire specific for the epitope, the TCR affinity and the efficiency with which the epitope is processed from the sequence backbone.

The highly immunogenic FrC sequence contains mouse and human MHC class I-binding $CD8^+$ T cell epitopes that compete with weaker epitopes from tumour antigens in the

fusion vaccine [158]. $CD8^+$ T cell epitopes within the sequences of pathogen-derived antigens are often clustered in regions and the FrC sequence is no exception. A minimised FrC vaccine design (section 1.3.16.4) was therefore able to minimise $CD8^+$ T cell epitope competition that is seen at boosting.

1.3.16.4 Optimisation of the DNA Fragment C Fusion Vaccine for the Induction of $CD8^+$ T cell Responses

The FrC sequence is composed of two domains. The N-terminal domain termed DOM1 (amino acids 865-1120 from the tetanus toxin sequence) contains the promiscuous helper T cell epitope, p30 (amino acids 947-967 from tetanus toxin sequence: FNNFTVSFWLRVPKVSASHLE). The p30 $CD4^+$ T cell epitope is a 'universal' MHC class II binder that can be presented by a variety of haplotypes in humans and mice [192]. Within the second DOM2 domain there are several $CD8^+$ T cell epitopes predicted to bind mouse and human MHC class I molecules. Therefore to eliminate competition from potentially immunodominant DOM2-derived $CD8^+$ T cell epitopes, the DNA vaccine was designed to contain only the DOM1 domain of FrC (Figure 11, [158, 159]). This minimised vaccine design (p.DOM-epitope) is therefore able to retain the ability to generate $CD4^+$ T-cell help whilst reducing potential immunodominance.

The second feature of the vaccine is the fusion of only the MHC class I-binding target peptide ($CD8^+$ T cell epitope) from the tumour antigen rather than the entire tumour antigen sequence. The epitope is positioned at the C-terminus and fused to DOM1. The inclusion of only the epitope sequence promotes efficient processing by facilitating peptide liberation. It is necessary to optimise the processing of CTL epitopes as the level of peptide-MHC complexes is a factor regulating the magnitude of CTL responses. With this in mind, mini gene vaccines encoding only a minimal CTL epitope were originally designed to promote efficient epitope processing and they can sometimes successfully prime $CD8^+$ T cells independent of $CD4^+$ T-cell help [193]. However, the T-cell immunity generated in most cases is low compared to DNA vaccines encoding the entire antigen [194]. It is thought a carrier protein protects the epitope from degradation by cytosolic proteases and the presence of a protein rather than only the peptide allows the DOM fusion protein to be taken up and cross-primed by APCs [47, 157, 195]. The removal of most of the DOM sequence so that only the p30 $CD4^+$ T cell epitope sequence is retained and fused directly to a $CD8^+$ T cell epitope results in the poor induction of $CD8^+$ T-cell responses [158]. This is not surprising as less drastic alterations, such as a single nucleotide

substitution, can greatly enhance or be detrimental to peptide processing and subsequent CTL-mediated cytotoxicity [196]. It does however provide evidence for the importance of flanking sequences and the benefit of a protein sequence rather than a short polypeptide. Positioning the MHC class I-binding epitope at the C-terminal rather than N-terminal of a carrier protein has been shown to be optimal for the induction of $CD8^+$ T-cell responses [153]. Peptide liberation from the C-terminal is more efficient as proteasomes generate the correct C-terminal of precursor peptides. These then undergo N-terminal trimming in the ER but no further C-terminal trimming occurs [197, 198].

The third feature of the vaccine design is the inclusion of the BCL_1 IgM V_H tumour leader sequence to target the expressed protein to the ER. Priming of $CD8^+$ T cells via the classical MHC class I processing pathway requires proteasomal degradation of the antigen within the cytosol. After two vaccinations, a DNA vaccine encoding FrC with a 5' leader sequence induced a CTL response with significantly higher cytotoxic ability than those primed by the leaderless vaccine. Upon a third immunisation both constructs $^+$/- leader generated CTL responses with equal cytotoxicity [190]. Therefore the vaccine with a leader sequence was able to elicit a more rapid CTL response. It is thought that the ER-targeted protein can be either trimmed within the ER or taken back into the cytosol by retrograde transport where it can enter the classical MHC class I processing pathway. ER-targeted proteins can be secreted and taken up by DCs that can then cross-prime CTLs.

1.3.16.5 DNA Fusion DOM Vaccine Effectively Elicits Cytotoxic T Cell Responses

The p.DOM-epitope vaccine can prime high levels of tumour epitope-specific CTL responses as shown by a vaccine encoding a C-terminal minimal MHC class I-binding epitope from the carcinoembryonic antigen (CEA) tumour antigen. The p.DOM-CEA vaccine primed CTLs more effectively than the p.FrC-CEA vaccine [158]. Therefore the removal of DOM2 (and competing epitopes) enhanced CEA-specific CTL responses, a result consistent with the phenomenon of immunodominance. The p.DOM vaccine design has been further tested with a colon carcinoma CT26 model where an endogenous retroviral gene product, gp70, contains the H2-L^d-restricted epitope known as AH1. The p.DOM-AH1 vaccine design could efficiently prime CTLs that lysed AH1 peptide-presenting tumour cells *in vitro*. In contrast, the vaccines encoding the full-length gp70 antigen alone or fused to FrC were unable to elicit significant AH1-specific $CD8^+$ T-cell immunity [159]. A similar observation was found with vaccines encoding a mouse MHC

class I-restricted peptide from the human CEA protein. The p.DOM-CEA-peptide vaccine was able to generate CTLs with >2-fold greater lytic capacity than those primed by the p.FrC-CEA-peptide vaccine [158]. The minimised FrC sequence, DOM, is therefore an optimal carrier protein for the induction of CTL responses.

The CTL responses induced by the p.DOM-AH1 vaccine were capable of providing protection against tumour challenge with CT26 cells that was comparable if not superior to the protection mediated by the irradiated tumour cell vaccine [159]. Another experimental model demonstrating vaccine efficacy is the induction of CTL responses specific for $CD8^+$ T cell epitopes encoded within the DOM1 and DOM2 sequences. The CTLs primed by the p.DOM-FrC-epitope vaccines were able to provide superior protection to that generated by the FrC vaccine against EL4-FrC cells, a tumour cell line stably transfected to express FrC [158]. The evidence confirms that epitope-directed vaccination, and more importantly the p.DOM-epitope DNA fusion vaccine design, can effectively prime CTL responses that successfully protect from challenge with antigen-expressing tumour cells.

1.3.16.6 Pre-Existing Immunity to Tetanus Toxoid

These FrC and DOM vaccines have been tested with great success in mice. However unlike mice most humans have pre-existing anti-tetanus toxin antibodies and although unlikely may have some tetanus toxin-specific memory $CD8^+$ T cells. To validate the vaccines for use in humans the effect of pre-existing immunity on the performance of the vaccines has been modelled in mice.

Two studies from our laboratory have immunised mice with tetanus toxin/alum 6 weeks prior to DNA vaccination to generate pre-existing immunity. Humoral immunity generated by a p.scFv-FrC vaccine was reduced at day 21 in the mice pre-vaccinated with tetanus toxin. However, this difference was abolished by day 42 and also with further boosting immunisations [199]. This demonstrates that the pre-existing immunity slows the anti-Id antibody response but does not prevent or diminish it. The second study investigated cellular immunity by vaccinating mice with p.DOM-AH1 and found that equivalent numbers of $CD8^+$ T cells were primed in pre-immune mice. The cytotoxic activity of the primed CTLs was also similar and therefore it appears that pre-existing anti-FrC immunity in mice does not influence the induction of CTLs following immunisation with FrC-encoding vaccines [159]. This observation is in accordance with the finding that only an on-going effector $CD8^+$ T-cell response and not memory $CD8^+$ T cell immunity can hinder priming of epitope-specific $CD8^+$ T cells in response to DNA vaccination [153].

1.4 Prostate Cancer

According to Cancer Research UK (www.cancerresearchuk.org) statistics there were almost 35,000 new PCa cases diagnosed in 2004, which accounted for 24% of all new reported cancers in males. The disease incidence is rising due to a growing elderly population as age remains the strongest pre-determining factor but also as a result of the introduction of serum prostate-specific antigen (PSA) testing that has facilitated earlier diagnosis.

More than 95% of PCas are adenocarcinomas of epithelial cell origin; the remainder are neuroendocrine tumours or sarcomas. The majority of adenocarcinomas arise within the peripheral zone of the prostate [200]. The inflammatory state of prostatic intraepithelial neoplasia is the precursor for PCa and can be found in tissue surrounding the tumour area. Initially the tumour spreads within the prostate gland as the prostatic capsule forms a barrier to protect from invasion of surrounding tissues. Some tumours can remain localised within the prostate for many years making the clinical course of the disease unpredictable. As the locally advanced tumour progresses it extends out of the prostate capsule and spreads to seminal vesicles. Finally the PCa becomes metastatic as it invades vascular and lymphatic tissue with lymph nodes, lungs and bones being the earliest and most common sites.

The current standard treatments for PCa depend on the status of the disease but are only curative in prostate-confined cancer. Radical prostatectomy and local radiotherapy are largely successful for clinically localised PCa giving patients a good prognosis of 90% survival at 10 years [200]. However prognosis is poor for the locally advanced disease as current treatments confer limited benefit. Androgen ablation therapy (i.e. Flutamide that competes with testosterone for binding of androgen receptors) is non-curative but can result in a temporary clinical response in approximately 70-80% of patients [200]. Eventually tumours become hormone-insensitive resulting in the development of androgen-independent (hormone-refractory) disease. Chemotherapy results in temporary responses in more advanced disease or metastatic PCa but unfortunately only delays mortality by a matter of months with ~70% of patients dying within 5 years [200].

1.4.1 Disease Stage Classification: PSA and Gleason Score

PSA is a serine protease also known as kallikrein 3 that is exclusively expressed by the prostate gland and excreted in semen. In PCa PSA is released into systemic circulation likely as a consequence of tissue architecture breakdown. Serum PSA levels aid early diagnosis and the increase in serum PSA levels are used to track tumour progression.

However elevated levels of PSA can be measured in patients with benign prostatic hyperplasia and in chronic inflammation. Therefore serum PSA cannot be used as a definite diagnostic test. The typical cut off for further investigation of 4ng/ml only detects 75% of PCas with a false-positive rate of ~40% [201]. The median PSA levels increase with age and therefore appointing cut off points specific for each age group may improve the use of PSA levels to detect PCa [202]. Despite the highlighted problems, PSA represents the best current clinical marker for PCa.

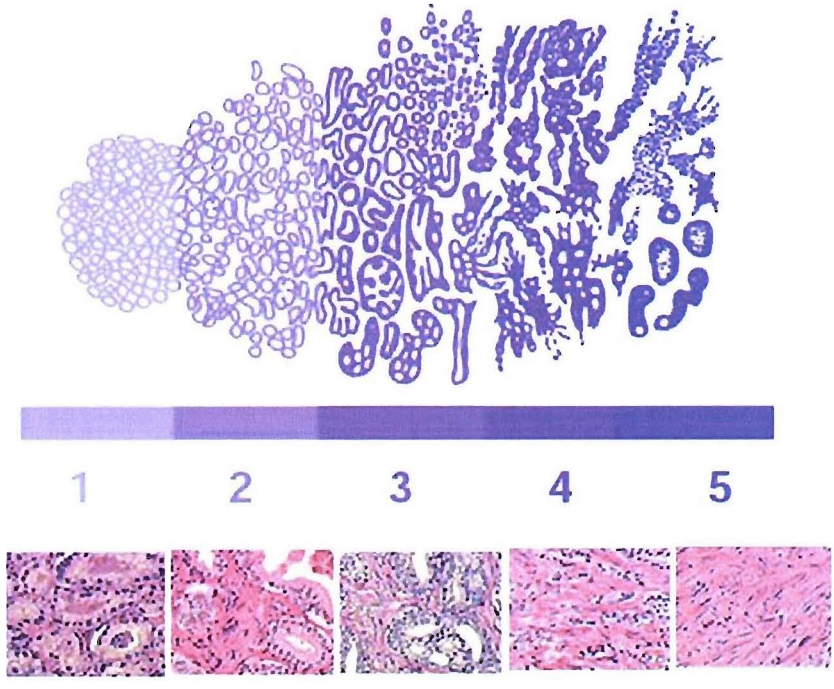


Figure 12: Gleason scoring

Schematic representation of the Gleason grading system (taken from Harnden et al., [203]. The numbers refer to the Gleason grades assigned to the corresponding tissue architecture.

Gleason scoring is a pathologic assessment of PCa disease progression based on the microscopic appearance of the prostate's glandular architecture. Grading is defined by the differentiation of the prostate tumour glands with Grade 1 and 2 being well differentiated, progressing to a Grade 3 moderately, Grade 4 poorly or a Grade 5 undifferentiated tumour (Figure 12). It takes into account the heterogeneity of the disease by giving a grading to the most common and the second commonest pattern of disease and so final scores range from 2-10. The size, shape and spacing of the glands are also taken into consideration. Gleason scores over 8 are associated with aggressive disease and a high risk of systemic disease [203].

1.4.2 Androgen Dependence in Prostate Cancer and Hormone Therapy

In the healthy prostate, androgen signalling is required for prostatic epithelial cell growth, differentiation and survival. PCas generally respond positively to androgen deprivation therapy or castration. However such therapies are ultimately responsible for selectively stimulating the growth of fast dividing prostate cancer cells that do not differentiate in response to androgens but instead respond to increased expression of growth factors and receptors (e.g. EGF and Her-2/neu). The hypothesis is that upon androgen ablation treatment the androgen-dependent cells within the heterogeneous tumour population undergo apoptosis thereby demonstrating a clinical benefit in 80% of patients. Some PCa patients can maintain long-term responses but most eventually relapse to the more aggressive hormone refractory disease as the tumour mass is taken over by the unrivalled growth of the androgen-independent tumour cell population.

This situation has been modelled in the TRAMP PCa transgenic mice (section 1.4.6) where castration led to the growth of androgen-independent tumour cells [204]. Mice implanted with TRAMP-C2 cells (derived from TRAMP model) were castrated to mimic androgen ablation therapy in patients. Castration led to prostate vascular regression but increased tumour cell migration (metastatic potential) [205]. Migration following androgen withdrawal may be in search of non-prostatic tissues where stromal angiogenesis is not androgen-dependent.

1.4.3 The Androgen Receptor in Prostate Cancer

When androgen binds to the androgen receptor (AR), the receptor can positively or negatively modulate downstream gene expression to maintain function and controlled growth of the prostatic epithelium. In this sense the AR can be considered a tumour activator or suppressor. Castrated men or men with a deficiency in 5α-reductase enzyme that converts testosterone to the more potent androgen dihydrotestosterone (DHT) rarely develop PCa and men with PCa have been found to often have single nuclear polymorphisms that encode for higher enzymatic activity [206]. These observations provide support for a role of the AR in the disease process. The AR continues to be expressed in the majority of hormone refractory PCas and is sometimes even amplified. PCa cell lines, human and murine PCas have naturally occurring somatic mutations in the AR gene [207] that lead to aberrant androgen signalling and are associated with hormone therapy failure. Mutations in the ligand-binding domain can broaden ligand specificity resulting in ARs that can be activated by alternative molecules such as oestrogen, steroids and even anti-androgens, thereby conferring a selective advantage in low androgen

environments [208-210]. The pro-inflammatory IL-6 cytokine can also stimulate AR activity in the absence of androgens [211]. Despite the discovery of deregulation in AR signalling, it remains unknown if it mediates or is impartial to progression to androgen-independent disease and growth of the aggressive PCa tumour.

1.4.4 Growth Factor Signalling

PCa is associated with a deregulation of growth factor expression such as insulin-like growth factor-1 (IGF-1), transforming growth factor β (TGFβ) and vascular endothelial growth factor (VEGF) amongst others. This results in altered proliferation, apoptosis and angiogenic signalling. These events lead to apoptotic evasion, uncontrolled proliferation and enhanced invasive potential. The signalling pathways involved have been reviewed [212].

1.4.5 Human Prostate Cancer-Derived Cell Lines

To aid investigations into PCa many human PCa cell lines as well as mouse models have been established. The earliest established human cell lines were the LNCaP, PC-3 and DU145 cells. However, as they do not reflect the full spectrum of heterogeneity observed in the disease other PCa cell lines have been established. A wide range of PCa cell lines have been reviewed in depth by Sobel et al., [213, 214]. The three cell lines originally established remain the best characterised. There have been efforts to evaluate expression of tumour antigens and proteins with relevance to immunotherapy [215, 216].

The PC-3 [217] and DU-145 [218] cell lines are of epithelial origin from a lumbar vertebral (bone) metastasis and a brain tumour mass respectively, and represent androgen-independent disease. MHC class I expression is high for PC-3 cells (HLA-A1 and HLA-24) and moderate for DU-145 (HLA-A3 and HLA-A33). PC-3 and DU-145 express a restricted range of tumour antigens that do not include the AR, PSA or PSMA [215].

The LNCaP cell line was derived from a supraclavicular lymph node metastatic lesion from a 50-year old white man of HLA-A2.1 haplotype [219]. This cell line represents a model of androgen-dependent disease, but has also been used to study progression into androgen independent PCa and bone metastases. LNCaP cells express PSA, PSMA and the AR, as well as many other reported PCa tumour antigens [215]. They express low MHC class I that can only be upregulated by TNFα [201, 215, 220, 221], making the cells poor at antigen presentation. LNCaP cells are unresponsive to treatment by all interferons

(IFNs) due to epigenetic silencing of JAK1 transcription, a Janus family kinase used by receptors of both IFNγ and IFNα/β cytokines [222].

1.4.6 The Transgenic Adenocarcinoma Mouse Prostate (TRAMP) Model

There are various mouse PCa models that mimic the human disease and are used to evaluate molecular events that lead to PCa and to assess therapeutic approaches. Some models have genes implicated in PCa knocked out such as the PTEN tumour suppressor, a gene often lost in PCa, which leads to hyperplasia of the prostate in the mice. Greenberg et al., have reviewed some of the mouse PCa models available [223].

Of particular interest in this study is the transgenic TRAMP mouse model created by Greenberg et al., in the 1990s. It is a spontaneous PCa mouse model where gene expression is perturbed only within the prostate. The rat probasin (rBP) promoter drives expression of the simian virus 40 (SV40) early-region tumour antigens (the oncogene) that have the ability to induce transformation *in vivo*. The SV40 large tumour T antigen (Tag) acts as an oncoprotein by abrogating the function of the tumour-suppressor genes retinoblastoma (Rb) and p53. The small T antigen inactivates protein phosphatase 2A to allow sustained activation of the MAP kinase pathway. The specific targeting of the transgene to the prostate induces prostatic adenocarcinoma in the TRAMP mice [224]. Male TRAMP mice display intraepithelial hyperplasia between 8-12 weeks of age. The onset of metastatic diseases is ~18 weeks and by 28 weeks all have poorly differentiated lymphatic metastases [225]. The TRAMP mice are a good model for human PCa as tumourgenesis is spontaneous and arises in the prostate gland thereby allowing characterisation of the early events occurring in PCa. The loss of Rb and p53 tumour suppressor proteins in the TRAMP mice has been implicated in human PCas whilst malignant histology and progression closely mimic the human disease.

Three epithelial cell lines have been derived from TRAMP mice, TRAMP-C1, C2 and C3. These cell lines were derived from a primary TRAMP prostate tumour of a 32-week old mouse and do not express the Tag antigen *in vitro* or *in vivo*. TRAMP-C1 and TRAMP-C2 cell lines are both tumourigenic syngeneic hosts but TRAMP-C3 only grows *in vitro* [226]. The TRAMP-C1 and C2 cell lines express low levels of MHC class I with <5% of cells expressing H-2K^b or H-2D^b. I*n vivo* passaging is reported to increase levels of MHC class I but quickly declines again after 15 *in vitro* passages [226]. IFNγ treatment restores high levels of MHC class I expression [227]. However, TRAMP-C1 cells are negative for MHC class II [226]. According to the literature TRAMP-C1 and C2 cell lines express a

range of mouse homologues of human prostate-specific genes, including PSCA, PSMA, and PAP [227].

1.4.7 Immunotherapy of Prostate Cancer

The intention is to develop immunotherapy to target PCa patients in remission to prevent the emergence of residual cells (or small tumour mass burden) but also to improve survival of advanced metastatic PCa. The distinct advantage is the retention of the patient's immune capacity and the induction of highly specific anti-tumour immunity. The prostate is an attractive target as it is not an essential organ and can therefore tolerate tissue damage as a result of immunotherapy. Also, many PCa tumour antigens have been described (Figure 13) including prostate specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), prostatic acid phosphatase (PAP), prostein, the six transmembrane epithelial antigen (STEAP), prostate specific G-protein (PSGR), prostate carcinoma tumour antigen (PCTA-1), and the transient receptor potential-p8 (trp-p8, a calcium channel).

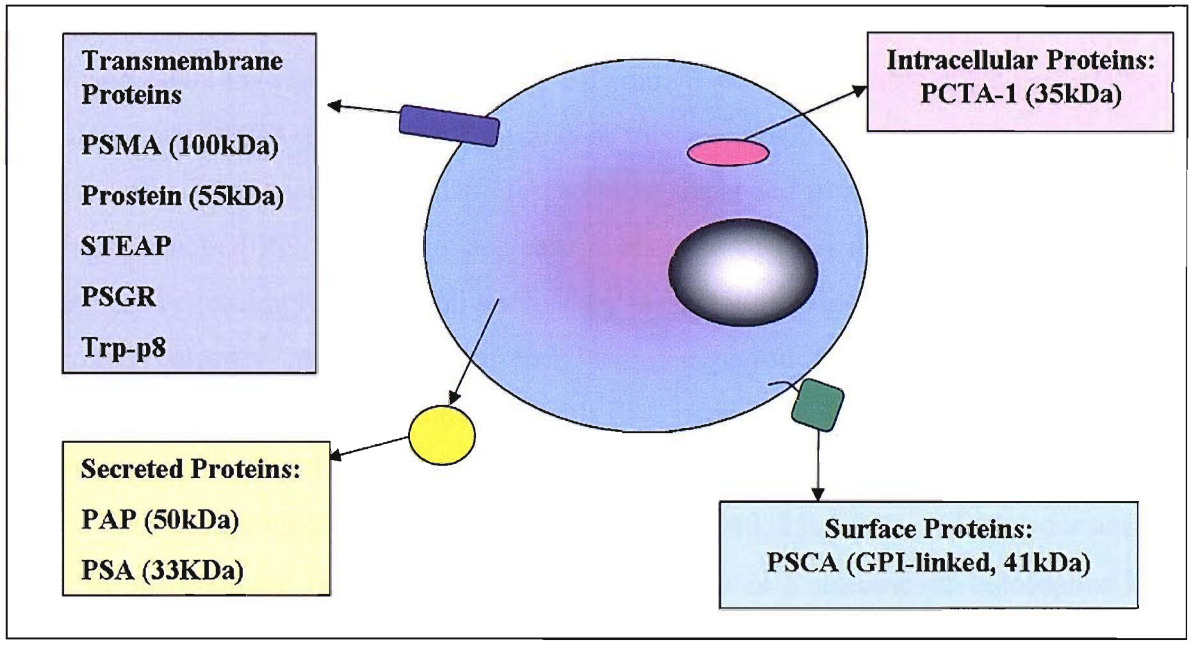


Figure 13: Prostate Cancer Tumour Antigens

Some of the proteins over-expressed by PCa cells that are currently under investigation as potential tumour antigens to target by immunotherapy

Almost 100 PCa molecular cancer markers have been reviewed by Tricoli et al., [228]. These are expressed primarily but not necessarily exclusively in the prostate [229]. Numerous studies have identified human T-cell repertoires in healthy volunteers directed against PCa tumour antigens such as PSMA [230-232], STEAP [233], PSCA [234] and

PSA [235, 236]. More importantly T-cell repertoires against these PCa tumour antigens have also been found in PCa patients, including PSMA [237-239], prostein [240], PSCA [241-243] and PSA [235, 236]. This evidence and the pre-clinical work in mice crucially support the use of immunotherapy strategies to target PCa.

Various types of cancer vaccines have been assessed for PCa therapy and have been subject to reviews [244, 245]. Although thus far human clinical trials have not been as potent as might have been expected from pre-clinical mouse studies, they do document encouraging cellular responses and disease stabilisation in a proportion of patients. There are difficulties with immune monitoring and PSA levels offer the best indication of disease progression. The GVAX® prostate cancer vaccine is comprised of two irradiated PCa cell lines, LNCaP and PC-3 that have been modified to secrete GM-CSF. Improved median survival from the expected 19.5 months were observed with patients receiving low or high doses of this cell-based vaccine (24.0 and 34.9 months respectively) [246]. Two phase II clinical studies enrolling hormone-refractory PCa patients reported post-therapy antibody responses reactive to the tumour cell lysates in 68% and 87% of patients [246]. The GVAX® vaccine has now proceeded to phase III trials to confirm these results. Another vaccine that uses autologous DCs loaded with mRNA from three PCa cell lines (LNCaP, PC-3, and DU-145) was tested in a phase I/II trial. T cells from immunised patients were stimulated with RNA-transfected DCs and in this manner 12/19 patients were found to have specific T-cell responses. $CD8^+$ T cell clones expanded from one patient were able to kill HLA-matched PC-3 cells. A decrease in the rate of PSA increase was found in 13 patients as well as improved overall survival by the immune responders [247].

The other category of vaccines currently being explored for PCa therapy, are those targeting defined antigens. Vaccination with PSA peptides is under investigation and has achieved specific T-cell immunity [248, 249]. Dendritic cell-based vaccines have been loaded with a large range of tumour antigen peptides [240, 250], as well as tumour antigen RNA or recombinant protein [251-253]. Provenge® is a vaccine of autologous DCs loaded with a PAP-GM-CSF recombinant fusion protein. Therapy with this vaccine primed PAP-specific T-cell responses in 38% of HRPC patients and resulted in a >50% decline in serum PSA in 15% of patients [254]. There was an improved median survival and this vaccine is currently on the FDA "fast track" for approval in the USA [244]. The use of recombinant viral vaccines is also being studied. A vaccinia-PSA vaccine, called PROSTVAC, has been tested in a phase I clinical study on patients with locally advanced disease. Stable disease was achieved in 14/33 patients for at least 6 months, and PSA

peptide-specific T-cell responses were observed in 5/7 HLA-$A2^+$ patients (4 of which had stable PSA levels for 6-11 months) [255].

DNA vaccines are also in the pipeline for PCa therapy and results from current trials are eagerly awaited. These include DNA vaccines encoding epitopes, full-length tumour antigens (with viral vector boosting) or xenogeneic tumour antigens. The published studies on DNA vaccination against PCa have thus far only been in pre-clinical models. Clearly the focus must now be on increasing the potency and magnitude of primed immunity.

1.4.8 Tumour Infiltrating Lymphocytes

The presence of TILs in cancers is indicative of an anti-tumour response that has been overcome by the malignant cells. Initially it was wrongly reported that the prostate was an immunologically privileged site without lymphatic vessels. This is incorrect and in fact there are inflammatory responses in the prostate as well as the presence of TILs in as many as 96% of PCa cases [256-258]. Study of TIL populations in PCa is limited by the small amount of tumour material available.

TILs are thought to be primed by APCs that have phagocytosed tumour antigens expressed by engulfed apoptotic cells. Tumour-specific activation is suggested by the higher levels of IFNγ secreted by TILs from PCa compared to benign hyperplasia (BPH) patients [259]. PCa TILs consist of antigen-experienced T cells (effector memory or terminally differentiated) [257, 260]. This is in agreement with a dominant effector memory $CD8^+$ T cell TIL population also found in other cancers [261, 262]. However, reports of perforin expression are contradictory [257, 260]. Recognition of tumour antigen by TILs provides further evidence for tumour-specific activation. Cytotoxic T-cell responses by TILs from PCa metastases against a parathyroid-hormone-related-protein and by prostatic TILs that could kill prostate tumour cells *in vitro* have been reported [263].

Evidence indicates that TILs are suppressed by the tumour microenvironment as they can acquire a cytotoxic phenotype when activated away from the tumour. T cells could be induced to increase T_{H1} cytokine production *in vitro* upon treatment with anti-CD3 antibody to mimic antigen recognition. However, cytokine secretion was decreased in the additional presence of autologous PCa cells [264]. $CD8^+$ T cells from PCa samples but not those from tumour-free prostates have been found to be unresponsive to non-specific stimulus (PHA) [257].

The presence of TILs has been correlated with improved clinical outcome and overall recurrence-free survival in colon [121], ovarian cancer [118], non-small-cell lung carcinoma [265], cervical cancer [266] and urothelial cancer [267]. However, little is known about their actual role in controlling tumour progression. A long-term clinical follow-up of over 300 prostatic adenocarcinoma patients reported a significant correlation between the presence of TILs and good prognosis, whilst absence or weak TILs were associated with a high risk of tumour progression [268]. In contrast, there are other reports where increased inflammatory cell PCa tumour infiltrates are associated with high-risk tumour recurrence [269].

The precise proportion of T-cell subtypes that convey the clinical benefits are not clear. High tumour-infiltration levels of both $CD4^+$ and $CD8^+$ T cells resulted in a better prognosis in non-small-cell lung carcinoma patients [265]. In contrast, a high CD8/CD4 T cell ratio appears to equate to improved survival in a cohort of ovarian cancer patients [118] and is associated with cervical cancers that fail to metastasise [266]. PCa TILs from hormone refractory PCa patients were found to be more frequently $CD8^+$ than $CD4^+$ T cells compared to prostate infiltrate from BPH patients [264, 270]. However, a contradictory report found $CD4^+$ cell dominance [260].

The presence of suppressive $CD4^+CD25^{hi}FOXP3^+$ Tregs has been strongly associated with poor prognosis in different types of solid tumours [118, 271-273]. Suppressive Tregs have been found at higher frequencies within PCa compared to benign prostate tissue from the same patients with early PCa cancer [274]. A recent study reported that 70% of the prostate TILs analysed contained elevated levels of Tregs [275]. A third study found that Tregs were elevated in the peripheral blood of patients with metastatic disease and not localised PCa [276]. The presence of a high $CD4^+$ T cell infiltrate has been reported to be associated with poor PCa survival [277]. However, most PCa studies into TILs have not considered the presence of Tregs despite their identification in tumour tissue, peripheral blood and lymph nodes of patients with other types of cancers. This is likely to have led to incorrect and sometimes contradictory conclusions regarding PCa TILs In a mouse B-cell lymphoma model expressing hemagglutinin (HA) it was shown that naïve $CD4^+$ T cells could differentiate into HA-specific Tregs in tumour-bearing hosts, supporting the importance of phenotyping $CD4^+$ TILs further [278]. In support of human data, mouse Tregs have also been shown to accumulate in tumour draining lymph nodes [105]. The role of Tregs in tumour suppression is discussed in section 1.4.11. There is currently insufficient evidence to conclude on Treg or overall TIL presence in PCa and their respective contribution to disease prognosis.

Immunohistochemical analysis of tumourigenic or benign prostate tissue from the same patients treated with androgen depletion therapy showed that treatment triggered an infiltration of immune cells into the tumour site. The T cells expressed IFNγ (65%), cytotoxicity (TIA-1), activation (CD25) and proliferation markers (Ki67). Other infiltrating cells included macrophages and dendritic cells [279]. The increased antigen source provided through therapy-induced apoptosis of tumour cells together with the increased APC infiltration may have induced the prostate-specific T-cell activation observed. Inflammation that occurs in the prostate during hormone ablation therapy could be due to such a T cell-mediated response. This indicates that androgen manipulation may aid immunotherapy of PCa.

Mouse PCa models have also demonstrated a correlation between tumour infiltration of lymphocytes and a beneficial therapeutic outcome. Interestingly, analysis of cells infiltrating the prostates of 25-week old tumour-bearing TRAMP mice revealed that 95% of prostate-infiltrating $CD8^+$ T cells were antigen-experienced. Overall, $CD4^+$ and $CD8^+$ T cells and Tregs were found to accumulate and the CD4/CD8 ratio was inverted in comparison to wild type mice [280]. However, the authors reported that Tregs were not responsible for the tolerisation of TRAMP mice against the self SV40 large antigen (Tag). Non-tolerised 6-week old TRAMP mice depleted of Tregs until 12 weeks of age and then vaccinated with peptide-loaded DCs were unable to prime responses against this self-antigen that could be primed at 6 weeks of age [280]. This suggests that Tregs are not responsible for tolerance induction in TRAMP mice.

1.4.9 Immune-Suppressive Effects Imposed by Prostate Cancer Tumours

Tumours are known to employ suppressive strategies to prevent induction of immunity and thereby escape immune recognition. The suppressive effects found in PCa have been discovered from patient material, mouse models and PCa cell lines, reviewed by Miller et al., [281].

Tumours can prevent the induction of immunity through secretion of immunosuppressive factors such as IL-10 cytokine, VEGF and TGFβ (reviewed [282]). High levels of these have been found in PCa patients and they have a negative impact on surrounding immune cells [283]. Combined TGFβ overexpression and protective downregulation of the TβRII receptor by tumours are associated with hormone refractory forms of PCa [284, 285]. TGFβ suppression has been modelled in mice where adoptively transferred $CD8^+$ T cells

rendered TGFβ-insensitive are not tolerised *in vivo* by the high TGFβ secreted from TRAMP-C2 tumours [286, 287].

A second suppressive mechanism is the increase of the L-arginine metabolising nitric oxide synthase (NOS) and arginase (ARG) enzymes by tumour cells. These deplete the amino acid availability to T cells. Strong NOS expression is a predictor for poor survival in PCa [288]. Inhibition of these enzymes could activate and restore lytic capacity of PCa $CD8^+$ TILs [257]. A similar situation has been found with tryptophan metabolism where tumours intracellularly express the catabolising enzyme, indoleamine 2,3-dioxygenase (IDO). Tryptophan depletion blocks T-cell proliferation thereby abolishing protection in a mouse immunotherapy model where the tumour cells are transfected to express IDO [289]. A single study reported IDO to be expressed in all 11 prostatic carcinoma patient samples analysed and the majority of these contained >50% IDO-expressing tumour cells [289]. IDO inhibitors are currently being tested in clinical trials after relatively good success in mouse models.

Commonly in cancer, including PCa, suppressive cytokines secreted by tumours disable DC maturation, migration and function. Human and mouse PCa cell lines inhibit the generation and maturation of human DCs *in vitro* [290-292]. PSA derived from PCa cell lines could inhibit human and murine dendropoiesis *in vitro* [293].
Defects in DC maturation not only hamper T-cell priming but also favour T-cell anergy. $CD4^+$ T cell tolerogenicity appears to be an early occurrence in the double transgenic Pro-HA x TRAMP mouse model. Depletion experiments showed that in this model DCs are responsible for presenting the antigen in a tolerogenic setting [294]. Immature myeloid DCs have been found to accumulate in draining lymph nodes of mice during tumour progression. These immature DCs selectively promoted Treg proliferation through secretion of TGFβ [105].

There are negatively signalling co-stimulatory molecules from the B7/CD28 family that have been associated with cancer and blocking these may be a potential immunotherapy strategy. Expression of the ligands by tumours is a candidate mechanism by which anti-tumour T-cell immunity may be inhibited. This has been shown in a mouse model where tumour cells expressing PD-L1 were less susceptible to CTL lysis and as a result more tumourigenic [295]. The PD-1 co-stimulatory molecule is inducibly expressed on activated T cells, B cells, NK-T cells, monocytes and DCs thereby providing a diverse range of potential targets for the PD-L1-expressing tumours to inhibit.

Various types of tumours have been reported to express PD-L1, including melanomas, human lung, ovarian, and colon carcinomas [296]. There are however no reports describing PD-L1 expression levles in prostate carcinomas. Another example of expression of a ligand for a negative co-stimulatiory molecule is the B7x ligand also known as HVEM (ligand for BTLA). Healthy tissue does not express the B7x protein (although mRNA is present) but it is aberrantly expressed in tumours from a variety of origins [297], including 99% of PCas [298]. In a large study of 823 patient samples, the intensity of immunohistochemical staining positively and strongly correlated with the spread and recurrence of PCa [298]. Suppressive tumour-associated macrophages (TAMs) also express B7x, which can be further upregulated by IL-10 and IL6. Through this ligand TAMs can inhibit specific T-cell immunity *in vivo* and abolish protection from tumours in mice [299]. The suppressive effects of multiple cells expressing B7x validates it as a potential immunotherapy target to antagonise.

1.4.10 Tumour Evasion Strategies in Prostate Cancer

Tumour cells acquire tumour evasion mechanisms that allow them to escape from immune recognition. General tumour [109, 300, 301] and PCa-specific [281] evasion strategies have been reviewed. Defective antigen presentation is common in PCa [220, 221, 302]. A high incidence of loss or down-regulated MHC class I expression has been found in prostate carcinoma (34% of primary tumours and 80% of metastases) as a consequence of mutations in the β 2-microglobulin (β2m) or HLA genes, alterations in antigen processing machinery or epigenetic silencing [221, 303, 304]. The resulting lack of tumour recognition and attack by CTLs has been associated with a low degree of $CD8^+$ TIL infiltration in PCa [305]. Low MHC class I expression by LNCaP and TRAMP cells has been widely reported in the literature [220, 227, 230]. Tumours can also develop insensitivity to IFNγ due to defective expression of different IFNγ receptor signalling components, as has been demonstrated for the LNCaP cell line [222]. The resulting inability of IFNγ secreted by activated T cells to upregulate MHC class I on the tumour cells likely affects CTL killing *in vivo*.

Immune escape is also achieved by the down-regulation or loss of tumour antigens. This antigenic drift can be due to selective pressure imposed by immunotherapy [306].

Aggressive behaviour of PCa tumours is associated with increased expression of anti-apoptotic molecules such as cFLIP (inhibitor of caspase-8), Bcl-2 and Bcl-xL (reviewed [211]). Tumours can also down-regulate expression of Fas receptors to avoid being killed.

PCa tumours are thought to be able to induce T-cell death through the secretion of FasL as increased serum FasL levels have been detected in PCa patients [307] as well as the secretion of FasL by three human PCa cell lines [308]. Exosomes purified from LNCaP cell supernatant suppressed T-cell responses through the induction of FasL-mediated apoptosis [309]. However the relevance of FasL expression by tumour cells as a tumour escape mechanism is unclear, since in mice it leads to rejection due to inflammation [310].

There are a large number of other mechanisms of tumour escape applicable to PCa and other cancers, such as the downregulation of co-stimulatory molecules, low tumour ICAM-1 expression [215], and disruption of TCR and CD3ε chains on TILs. Strategies to overcome such evasive and tolerising mechanisms are being explored and evidence from mouse models suggests they can be overcome.

1.4.11 Suppression by Regulatory T cells

Evidence suggests that Tregs can suppress anti-tumour immunity. Tregs isolated from the peripheral blood of PCa patients could suppress T-cell proliferation *in vitro* [274] whilst Tregs isolated from patients with hepatocellular carcinoma could additionally inhibit degranulation and production of granzymes [273]. Although these cells directly impart their suppressive effects, tumours do appear to play a part in the process. The tumour environment allows the generation of tumour antigen–specific Tregs [278] as well as inducing Treg migration and infiltration [271]. PCa tumour samples and PCa cell lines can secrete CCL22 that chemoattracts $CCR4^+$ Tregs *in vitro* [274]. Accordingly, Tregs have been found in intimate contact with and overlying neoplastic epithelium in PCa patient samples [311] and have been shown to proliferate in tumour beds of tumour-bearing mice [105].

Hormones can regulate the immune system, particularly with regards to Tregs and inflammation. Estrogen therapy drove the expansion of $Foxp3^+$ Tregs in mice which correlated with protection from induced autoimmunity [312]. *In vitro* estrogen in combination with TCR stimulation increased Foxp3 expression by $CD4^+CD25^-$ T cells [312]. This finding may be related to the observation that during pregnancy when estrogen levels are high, clinical signs of autoimmunity in humans improve. Similarly, the treatment of male mice with testosterone could inhibit proliferation of T cells mediating autoimmunity, an effect mediated by the AR that could be blocked with the administration of the AR antagonist flutamide [313]. Thus, it is possible that a similar hormone-induced expansion of Tregs may occur in PCa. A recent pilot study of 146 PCa samples found a

correlation between Treg numbers and AR expression [311]. Abrogation of Treg expansion provides a potential mechanism for the effectiveness of anti-androgens in androgen-sensitive PCa.

Depletion of Tregs has been able to enhance vaccine-induced anti-tumour immunity in mouse models, highlighting the detrimental effect they have on the immune response. Interestingly, there are also reports where the adoptive transfer of Tregs has been associated with the regression of inflammation-induced intestinal tumours in mice [314]. Tregs may suppress inflammation and therefore their role at the tumour site may depend on tumour pathogenesis. This could be of interest for inflammation-induced malignancies. The possible role of inflammation in the pathogenesis of PCa is discussed by Goldstraw et al., [315]. Despite the potential beneficial role of Tregs against inflammation-associated tumourigenesis, they are undesired once the tumour is established particularly with the view of developing immunotherapy.

1.5 PSMA (Glutamate Carboxypeptidase II)

1.5.1 Name and Enzymatic Activity

This enzyme is a type II transmembrane protein that hydrolyses α- or γ-linked glutamates. It has various names according to substrate, cleavage position and tissue location. It is known as an intestinal folate hydrolase (FOLH1), a brain N-acetylated-α-linked acidic peptidase (NAALADase I), and a prostate NAALADase (PSMA - prostate specific membrane antigen). These represent functionally distinct expressions of a single gene (FOLH1) that has been designated a single name, glutamate carboxypeptidase II (GCP II), with the nomenclature (EC 3.4.17.21) [316-319].

1.5.2 Function in Non-Prostatic Tissues

The brain GCPII enzyme (NAALADase I) cleaves to inactivate the peptide neurotransmitter N-acetylaspartylglutamate (NAAG) to release N-acetylaspartate (NAA) and glutamate (both products are excitatory neurotransmitters). NAAG is the most prevalent and widely distributed neurotransmitter in the mammalian nervous system and functions to regulate excitatory neurotransmission [320]. There are two NAALADase I knock-out mouse models. One reported early embryonic cell death [321], whilst the other survived without the elimination of NAAG hydrolysis as this function was compensated for by other NAALADases [322].

Intestinal GCPII has a role in folate metabolism and hence regulation of folate absorption [323]. The enzyme is present in the brush border of the small intestine, where it acts to deconjugate poly-γ-glutamated folate into readily absorbable monoglutamate folate that can then be transported across the intestinal mucosa [319, 324, 325]. Deficient deconjugation can lead to lower bioavailability as two thirds of dietary folate is in the polyglutamate form.

In summary, the known roles ascribed to this enzyme include glutamergic neurotransmission, folate absorption, and intestinal folate transport. The function in other tissues remains unknown.

1.5.3 Hypothesised Functions of Prostate Specific Membrane Antigen in the Prostate

PSMA can be found in normal prostate cells, the source from which it was first cloned [326]. Its role in the human prostate remains unknown. It has been suggested that the

enzyme may play a role in the hydrolysis of peptides in prostatic fluid thereby generating glutamate, a component of seminal fluid. Revealing the function of this enzyme in the prostate is of importance to understand its involvement in PCa disease progression.

1.5.3.1 Receptor Internalising a Ligand

The consensus hypothesis and indirect proof suggests that PSMA functions to transport a ligand (possibly folate) into cells. PSMA contains an intracellular MXXXL endocytic motif (MWNLL) that has been shown to mediate internalisation and targeting to endosomes and lysosomes [327, 328]. Endocytic trafficking of TM protein/receptors often serves the purpose of downregulating signalling or to transport nutrients into the cells. Constitutive endocytosis occurs in the LNCaP cell line can be increased by treatment with anti-PSMA antibodies [329].

The extracellular part of human PSMA shares a high degree of sequence (26%) and domain organisation homology with the transferrin receptor (TfR) [330]. TfR is also a type II TM protein expressed as a non-covalent homodimer. It delivers iron to cells after internalisation through the intracellular YXRF motif and subsequent targeting to the REC. The striking similarity between the two proteins supports the hypothesis that PSMA may function as a receptor internalising a ligand.

1.5.3.2 A Multifunctional Protein

Rajasekaran, et al. suggested that the peptidase activity associated with PSMA may be involved in cell migration and proliferation signalling. The prostate gland consists primarily of stromal, epithelial and neuroendocrine cells. Cell balance is achieved through signalling chemokines, growth factors and neuropeptides. The review proposes that PSMA may have similar roles to two other peptidase type II TM proteins; neutral endopeptidase (NEP) and CD26, both of which are involved in the regulation of cell migration. Such a role would account for the increased PSMA expression in PCa and increased enzymatic activity in metastatic PCa [331].

Calveolae are plasma membrane invaginations that provide scaffolds where receptors/signalling molecules can cross-talk and initiate signal transduction. PSMA associates with calveolae in endothelial cells [332] supporting other evidence that PSMA may have signal transduction ability in addition to enzymatic activity and ligand internalisation.

1.5.3.3 A NAALADase

It is unlikely that PSMA functions as a NAALADase since the neurotransmitter substrate would not be present in the prostate. NAALADase activity is independent of PSMA internalisation as LNCaP cells cultured in the presence of phosphate inhibits only the enzymatic function [330].

1.5.4 Human Prostate Specific Membrane Antigen

PSMA is a type II TM glycoprotein. The human PSMA gene is composed of 750 amino acids with a molecular weight of 100kDa [326, 333, 334]. The cytoplasmic (N-terminal), TM and extracellular (C-terminal) domains are 19, 24 and 707 amino acids in length respectively (Figure 14). The protein was originally identified in the membrane preparation of a lymph node prostatic adenocarcinoma cell line, LNCaP, by a MAb [335].

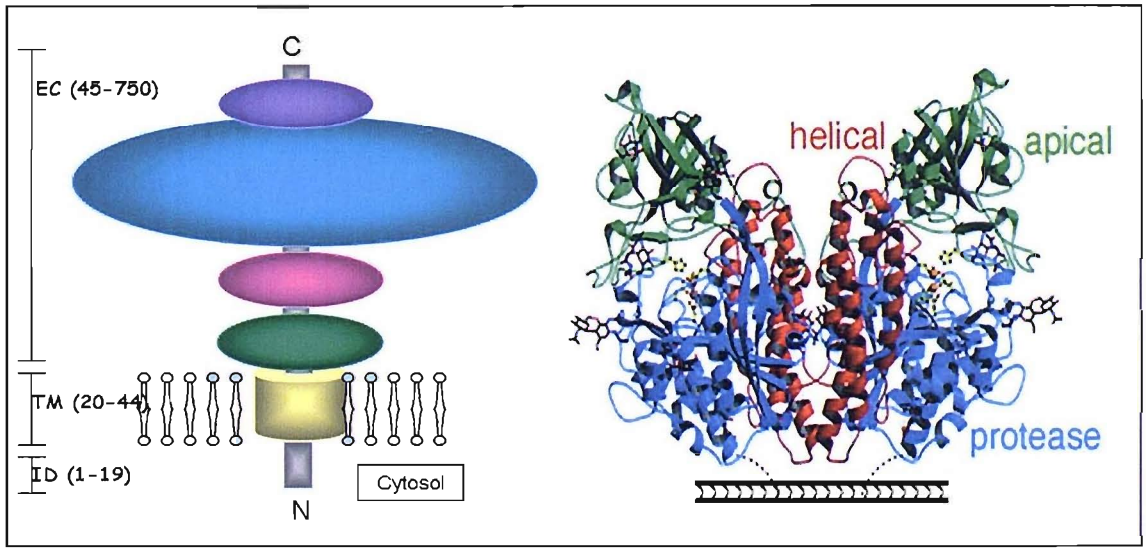


Figure 14: Structure of Prostate Specific Membrane Antigen (PSMA)

Left: a schematic representation of the full-length PSMA protein (adapted from Rajasekaran et al., [330]). The amino acids composing each of the intracellular (IC), transmembrane (TM) and extracellular (EC) domains are shown in brackets. Right: the crystal structure of the dimerised extracellular (amino acids 44-750) portion of the protein created and published by Davis et al., [338].

1.5.5 Mouse Prostate Specific Membrane Antigen (FOLH1)

The mouse homologue is called mouse FOLH1 and is composed of 752 amino acids [336]. The cytoplasmic, TM and extracellular domains for this mouse homologue are 19, 25 and 708 amino acids long respectively. It shares 85% identity and 91% similarity with human

PSMA extracellular domain. Unlike the human gene, it does not appear to be expressed in the mouse prostate [336, 337] so tends to be referred to as mouse FOLH1.

1.5.6 The Structure of Prostate Specific Membrane Antigen

The catalytic activity of the enzyme is in the extracellular domain, the region of very high homology shared between human, mouse, rat and pig GCPII. In contrast the intracellular and TM domains have poor homology.

The native PSMA protein is expressed as a non-covalent homodimer both on LNCaP cells and as recombinant protein. The extracellular domain alone is sufficient for dimerisation [339]. The PSMA crystal structure [338, 340] (Figure 14) has confirmed the symmetric dimer composed of 20 α-helices and 16 β-sheets and the substrate binding cavity at the interface of 3 external domains. These are the protease (residues 57-116 and 352-590), apical (residues 117-351) and helical C-terminal (residues 591-750) domains. The protein contains a Ca^{2+} ion that stabilises a protein loop involved in dimerisation [340], a finding consistent with the observation that chelating agents destabilise the dimer [339]. The dimer interface is very large and therefore it is unlikely that dissociation can occur without some unfolding. Catalytic mechanisms based on the residues in the active site have been proposed [338, 340]

1.5.7 Glycosylation

The extracellular domain of human and mouse PSMA is heavily glycosylated with 10 [326], [336] potential N-glycosylation sites (N-X-S/T motif). The deglycosylated protein has an 84kDa size therefore around 16% of its molecular weight is comprised of sugars [341]. There are 7 conserved predicted glycosylation sites amongst the PSMA homologues of four species.

Glycosylation of native PSMA purified from LNCaP cells is composed of mainly complex type N-glycans but not O-glycans [341]. N-glycosylation is critical for folding and trafficking for targeting to post-Golgi vesicles [342, 343]. The crystal structure has revealed that the glycosylation sites are sufficiently distant from the active site to not directly contact a bound substrate [338]. Therefore the loss of enzymatic activity from disturbed glycosylation must arise from protein misfolding.

1.5.8 Splice Variants

There are multiple PSMA transcription start sites in the prostate (positions –262, -235 and –195 relative to the translation initiation codon) but only for intestinal GCPII (position –138) suggesting tissue specific transcription regulation [326, 334].

There are 5 alternatively spliced variants of prostate PSMA (Figure 15). Variant 1 (GenBank NM_004476) is expressed as the full length 750 amino acid TM protein. Alternative splicing gives rise to the PSM' through the removal of bases 114-379 of GenBank sequence NM_004476 (start ATG for full length PSMA at position 262). Therefore PSM' has a different translation initiation codon resulting in a smaller cytosolic 95kDa protein (amino acids 60-750) that lacks the intracellular and TM domains [344-346]. The mRNA transcript of the PSMA-C variant is 133 nucleotides longer than PSM' but shares the same translation initiation codon so is translated as the same 95kDa cytosolic protein [345]. Splice variant PSMA-D has a different translation start codon located within a novel exon. The translated cytosolic protein contains 24 extra amino acids at the N-terminus followed by the rest of the protein (amino acids 60-750 i.e. 714 total) [345]. Variant PSMA-E encodes a novel exon with a novel translation initiation site that incorporates 11 novel amino acids but is also lacking exon 18 (31 amino acids). Translated PSMA-E is 704 amino acids in length [347].

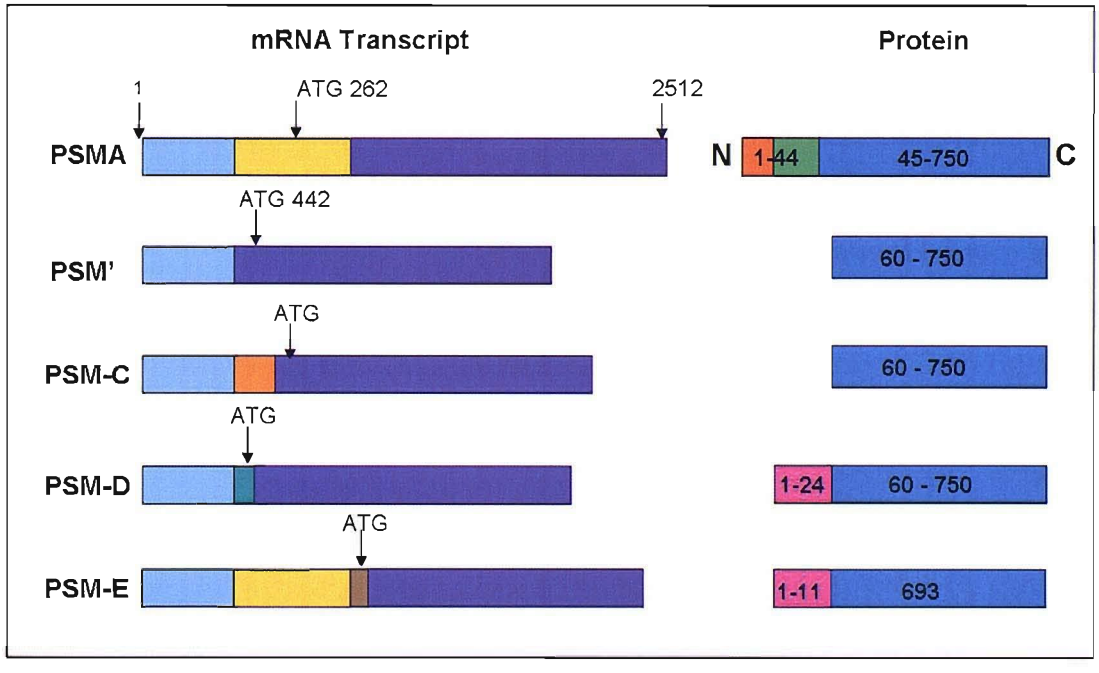


Figure 15: Human PSMA splice variants

The mRNA transcripts, translated protein and the position of translation initiation codons are depicted for each variant. The full-length PSMA is a TM protein (intracellular domain in red and TM in green) and PSM-E is also speculated to be [347]. The novel exons for PSMA-D and PSMA-E are respectively depicted as turquoise and brown in the mRNA transcripts and pink in the protein.

1.5.9 Tissue Expression

In situ hybridization, immunohistochemistry and RT-PCR analysis have shown that human GCPII/PSMA expression can be found in the normal and malignant prostate epithelium (but not in normal endothelial cells), small intestine, brain, kidney, spleen, liver, spinal cord, ovaries and salivary glands. GCPII mRNA has not been detected in the thymus, heart, ovary, colon, leukocytes, placenta, lung, and muscle. There are conflicting reports of the presence and absence of expression in the testis, pancreas, heart and liver [348-352]. The expression of GCPII in various tissues is a concern when targeting therapies towards cells expressing this gene. However, PSMA mRNA levels are higher in the prostate than in the liver (by ~13 fold), the brain and kidney (by ~19-fold), and the spleen (by >100-fold) [229]. All the other 11 tissues tested were negative. PSMA protein levels in the membranes of non-prostatic tissues (brain not tested) have been reported to range between 1.8-51ng/mg of protein (highest levels in ovaries, not a tissue of concern for PCa patients). The PSMA levels in healthy prostate ranged between 519-4,254ng/mg and are therefore several fold higher [353]. The PSMA protein levels in the brain are reported to be 50-300ng/mg of total protein [354], several fold lower than the prostate. The GCPII/PSMA protein is likely to be targeted to the apical membrane in healthy differentiated tissues and therefore unavailable to circulating therapeutics such as MAbs or activated T cells.

Mouse PSMA is predominantly expressed in the hippocampal region of the brain, the kidney, and at lower levels in the ovaries, testis and mandibular gland. There are contradicting reports regarding expression in the prostate. A study reported no expression by Northern blot [336] whilst two studies have found expression in the mouse prostate [345]. There is no expression in the small intestine. All species express some GCPII in the brain and kidney. The universal expression would indicate a central role for this gene in both these tissues with that in the brain already deciphered.

1.5.10 PSMA Expression in Prostate Cancer

Studies using in situ hybridization, immunohistochemical analysis, RT-PCR and RNase protection assays have reported that PSMA is expressed in normal prostate epithelial cells and over-expressed by cells that have undergone malignant transformation into localised and metastatic PCa [355]. PSMA expression inversely correlates with the degree of cancer differentiation. Reports of PSMA positivity range from 56.6% to 100% of PCa cases. A study reported 70.5% of HRPC and 64% of untreated cases to be PSMA positive [351, 356]. PSMA expression levels positively correlate with increasing prostate cancer disease

stage, Gleason score, and time to PSA recurrence after treatment [333, 345, 346, 349, 355, 357-360]. It additionally appears that there is an increase in PSMA expression after androgen ablation therapy [361]. There is also epithelial PSMA staining at metastatic sites, in most lymph node and some bone metastases studied [350, 359].

1.5.11 PSMA Expression in Other Cancers

PSMA is also expressed by endothelial cells of the neovasculature of nearly all types of solid tumours (including bladder, renal, colon, neuroendocrine, pancreatic duct, breast, and lung carcinomas) but not in the tumour or normal endothelial cells [350, 362-365]. A study found 17% of urothelial carcinomas of the bladder (n=346) and 15.5% of non-prostatic adenocarcinomas (n=278) to stain positive for PSMA [351]. Another study reported endothelial PSMA immunohistochemical staining of 47% of renal cell carcinomas, 54% of bladder carcinomas and 16% of colon adenocarcinomas [350]. This will allow immunotherapy developed against PSMA-expressing tumour cells to be applied against these non-prostate cancers.

1.5.12 PSMA Splice Variant Expression in Prostate Cancer

The PSM' variant is predominant in the healthy prostate. However, a switch in mRNA splicing pattern occurs upon malignant transformation that causes the majority of the transcript to be encoded as the membrane bound full-length PSMA [346]. Few studies look specifically for the cancer-related membrane bound form or the other variants, and instead quantify total PSMA levels [345, 346, 357].

The PSMA/PSM' ratio has been reported to increase 10-fold in BPH and 100-fold in PCa [346, 357]. An increased transmembrane PSMA to PSM' ratio has been found to correlate with increasing Gleason score [345, 346, 357]. The results suggest that the PSMA/PSM' ratio may a useful prognostic indicator of the disease.

Single studies have looked at expression of the other variants. The PSM-C variant was found to have similar expression in benign, primary prostate tumour or metastatic tissues. The PSM-D variant expression level was 2-fold higher in lymph node and bone metastases than in primary tumour but similar in primary prostate tumour and benign tissue [345]. The PSM-E mRNA levels were shown to correlate with Gleason score and only in prostatic tumours [347].

1.5.13 PSMA Enzymatic Activity in Prostate Cancer

The increase in PSMA expression levels appears to translate into higher enzymatic activity. Increased NAALADase I activity has been found in PCa samples compared to BPH and normal prostate tissue. There was up to 10-fold greater increase in enzymatic activity of the full-length PSMA protein compared to that of the PSM' variant. As a result the PSMA/PSM' enzymatic activity ratio is also reported to be higher in PCa, following the trend found with mRNA levels. The PSMA enzymatic activity has been found to increase on a per cell basis [331] although the significance of this is unknown.

1.5.14 The Role of PSMA in Prostate Cancer Progression

The precise reason why PSMA is over-expressed in PCa is unclear. Deciphering the role of PSMA in carcinogenesis will allow rational design of therapy to inhibit the function or pathway involved. For the purposes of immunotherapy however it is the expression not function of PSMA that is important.

Augmentation of PSMA expression with PCa severity is likely to provide the cancer cells with more internalised ligand, assuming this postulated function is correct. The extra ligand supply may convey an advantage to the cancer cells. The main ligand candidate, folate, would fit this hypothesis as it is a molecule required for rapid cell division.

It has been speculated that the expression of the cytosolic PSM' in the normal human prostate may place the cells at risk of developing a low intracellular folate environment by converting folate to a non-glutamated from that can readily diffuse out of cells. Folate is required for DNA and RNA synthesis and a deficit can lead to DNA damage, cell cycle arrest, apoptosis and carcinogenesis [366-368].

Angiogenesis is severely impaired in PSMA-null animals at the level of endothelial cell invasion of the extracellular matrix barrier [369]. PSMA inhibition *in vitro* reduced endothelial cell invasion and therefore abrogated angiogenesis [369]. A role in the critical process of angiogenesis would fittingly explain the expression of PSMA in the neovasculature of a wide variety of solid tumours.

1.5.15 PSMA as an Immunotherapy Target

PSMA expression is inhibited by androgens and high expression levels are observed upon spontaneous loss of androgen-dependency in hormone refractory and metastatic PCa

diseases. Tissue sample analysis has shown elevated PSMA levels after physical castration or androgen-deprivation therapy [370]. The high expression levels make PSMA a good immunotherapy target as it may signify a critical role for PSMA in tumour growth/survival.

The expression of the transmembrane PSMA variant in cancer means that the protein is on the cell surface and therefore readily available for MAb targeting. There are various MAbs against human PSMA available and numerous studies use these for selective delivery of toxic compounds to the PSMA expressing cancer cells and for tumour imaging (section 4.1.2.1). An alternative approach investigated in this project is active immunotherapy. The advantage of targeting a membrane protein is that it may be possible to attack via both cytotoxic T cells and antibodies against the PSMA protein.

The prostatic epithelium has highly polarized cells with distinct apical and basolateral surfaces. PSMA is localized to the apical plasma membrane with trafficking occurring directly from the trans-Golgi network. This localization is suggested as a possible obstacle in using therapeutic monoclonal antibodies or active immunotherapy eliciting antibody responses. Unlike the basolateral plasma membrane, the apical membrane has a restricted access imposed by tight junctions that prevent circulating molecules from reaching it. Circulating antibodies may therefore come across epithelial barriers. PCa tumours rapidly lose differentiation and the tissue structure becomes disrupted. This is demonstrated by the fact that PSA, an intracellular protein is secreted at high levels upon prostate carcinogenesis. In poorly differentiated tumour epithelial cells are no longer polarised therefore targeting antibody to PSMA surface-positive tumour cells should not be restricted. In the case of activated T cells, tumour differentiation is irrelevant as T cells recognise PSMA-expressing cells through recognition of peptide-MHC complexes.

2. Materials and Methods

2.1 Cloning

A total of three genes were cloned; human PSMA, mouse PSMA and the chimeric MHC Class I HHD molecule.

2.1.1 RNA extraction

Total RNA was extracted from 30mg C57BL/6 mouse kidney using RNeasy Mini kit (QIAgen, Crawley, UK), and following the manufacturer's protocol. The RNA was eluted in 32µl of RNAse-free water.

Total RNA was extracted from $5x10^6$ RMA-HHD cells by adding 1ml of TRI-Gene Reagent (Sigma-Aldrich, Gillingham, UK) followed by 200µl of chloroform. The cells were shaken vigorously before centrifuging at 12,000 x g for 15 minutes at 4°C. The transparent layer containing RNA was transferred into a fresh RNAse free tube and the RNA precipitated by the addition of 0.5ml isopropanol followed by centrifugation at 12,000 x g for 15 minutes at 4°C. The RNA was washed with 1ml 75% ethanol, then air dried and resuspended in 40µl of water and stored at -80°C.

2.1.2 cDNA synthesis

Human prostate cDNA was generated from human prostate total RNA (BD Biosciences, Oxford, UK) using Superscript First-Strand cDNA Synthesis kit (Invitrogen Ltd, Paisley, UK). 5µg of RNA was reverse transcribed in a total volume of 20µl. In brief, 5µg RNA, 0.5µg oligo(dT_{12-18}), and 0.5mM dNTPs were denatured at 65°C for 5 minutes. The reaction was cooled on ice for 1 minute and then 1X First Strand RT buffer (20mM Tris-HCl, pH8.4; 50M KCl), 5mM $MgCl_2$, 10mM dithiothreitol (DTT) and 40 units of RNaseOUT™ recombinant RNase inhibitor were added. The reaction was incubated at 42°C for 2 minutes prior to the addition of 50 units of Superscript™ II reverse transcriptase enzyme and then incubated at 42°C for a further 50 minutes. The reaction was terminated at 70°C for 15 minutes to inactivate enzymes. The final step was the addition of 1µl of RNaseH and a 20 minute incubation at 37°C to degrade any remaining RNA. The cDNA was stored at -20°C.

C57Bl/6 mouse kidney and RMAHHD cell cDNAs were synthesised in the same manner.

2.1.3 Assessing cDNA Quality

To confirm the cDNA quality, gene specific primers were used to amplify housekeeping mouse and human β-actin genes by PCR.

2.1.4 Polymerase chain reaction (PCR)

All PCRs were carried out with one of the two enzymes listed in Table 2. HotStart Taq® enzyme reactions required an initial 15 minute incubation at 95°C to activate the enzyme. PCRs were carried out with 0.25mM dNTPs and 0.5μM of each primer. The primers used for this project are listed in Appendix I.

Enzyme	Units per 50μl reaction	Buffer Components
HotStart Taq® (QIAgen, Crawley, UK)	2.5	Tris-Cl; KCl; $(NH_4)_2SO_4$; 15mM $MgCl_2$, pH8.7
Pfu DNA Polymerase (Promega, Southampton, UK)	1.25	200mM Tris-HCl pH8.8, 100MKCl, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1mg/ml BSA, 1% Triton® X-100

Table 2: PCR DNA Polymerase enzymes

The number of enzyme units used per PCR reaction and the buffer components.

2.1.4.1 Polymerase Chain Reaction : Human PSMA (hPSMA)

Human PSMA (GenBank accession number NM_004476–2.253KB, 750 amino acids) was cloned with forward and reverse primers designed to anneal to 5' (hPSMAF1) and 3' (hPSMAR1) untranslated regions (UTRs). Human PSMA was amplified from human prostate cDNA in a 25 cycle reaction with HotStart Taq® (QIAgen) enzyme. The PCR cycling conditions were 95°C for 15 minutes followed by 25 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes. A final step at 72°C for 7 minutes was carried out. The PCR product was gel purified (section 2.1.5) and cloned into pGEM-T® Vector System I (Promega, Southampton, UK). This construct was used as the template for sub-cloning into other vectors.

2.1.4.2 Polymerase Chain Reaction: Mouse PSMA (mFolh1 or mPSMA)

Mouse PSMA (GenBank accession number NM_016770, 752 amino acids) was cloned with forward and reverse primers designed to anneal to 5' (mPSMAF1) and 3' (mPSMAR1) UTRs. Mouse PSMA was amplified by PCR from C57Bl/6 mouse kidney cDNA using *Pfu* DNA Polymerase (Promega). The PCR cycling conditions were 95°C for 1 minute 20 seconds followed by 30 cycles of denaturing at 95°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 6 minutes. A final step at 72°C for 7 minutes was carried out. The purified PCR product was purified and cloned into pSTBlue-1 Perfectly Blunt® Vector (Merck Biosciences, Darmstadt, Germany). This construct was used as the template for sub-cloning into other vectors.

2.1.4.3 Polymerase Chain Reaction: HHD MHC Class I Molecule

A forward primer was designed to anneal to the sequence upstream of the start of the coding region (HHDF1) and the reverse primer to the end of the HHD construct (HHDR1). The sequence was obtained from the creators of the RMA-HHD cells (Lemonnier F.A. (Institut Paster, Paris, France)). The HHD gene has a 1.431KB coding region. The PCR was carried out with HotStart Taq® (QIAgen). The PCR cycling conditions were 95°C for 15 minutes followed by 23 cycles of denaturing at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 2 minutes, followed by a final step at 72°C for 7 minutes. The purified PCR product was cloned into pGEM-T® Vector System I (Promega). This construct was used as the template for sub-cloning into other vectors.

2.1.5 PCR Product Purification

All PCR products were separated by 1% agarose gel electrophoresis in 1x TAE buffer (50x TAE stock; 242g Tris, 57.1ml acetic acid and 50mM EDTA made up to 1 litre with dH_2O) and visualised with ethidium bromide under UV light. The 1 KB plus ladder (Invitrogen Ltd) was used to size the PCR products. The PCR products of the correct size were isolated and purified with the QIAquick® Gel Extraction Kit (QIAgen) following the manufacturer's guidelines.

2.1.6 Ligations

pGEM-T® Vector System I (Promega) ligations were set up with 1x ligation buffer (30mM Tris-HCl, pH7.8; 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 10% polyethylene glycol), 50ng pGEM-T® vector, and 3 units of T4 DNA ligase. The ligations were incubated at 4°C overnight.

Ligations into pSTBlue-1 Perfectly Blunt® Vector (Novagen) involved an end conversion blunting reaction with 3µl purified PCR product and 5µl end conversion mix in a total 10µl reaction volume. The reaction was incubated at 22°C for 15 minutes followed by inactivation at 75°C for 5 minutes. The reaction was briefly cooled on ice before 1µl of pSTBlue-1 vector and 3 units of T4 DNA ligase were added and placed at 22°C for a further 15 minutes.

Ligations consisting of digested DNA inserts and plasmids contained 6µl of digested PCR product, 2µl of digested vector, 1x ligation buffer and 1µl of T4 ligase. The ligations were allowed to incubate at 4°C overnight.

2.1.7 Transformations

A 5µl aliquot of the ligation mix was transformed into 50µl of JM109 competent *E. coli* cells (Promega). The cells were incubated on ice for 20 minutes before undergoing heat shock at 42°C for 45 seconds. They were then incubated on ice for a further 2 minutes before the addition of 500µl of Luria Broth (LB). The transformed cells were grown without antibiotic on a shaker at 37°C at 200 RPM for 1 hour prior to plating onto LB-agar plates containing 100µg/ml ampicillin, and grown overnight at 37°C. When cloning into pGEM-T® Vector System I (Promega), 100µl of 0.1M isopropylthiogalactoside (IPTG) and 20µl of 50mg/ml 5-bromo-3-indoyl-b-D-galactopyranoside (X-Gal) (Promega) were spread on the surface of LB-agar plates to allow blue-white colony selection prior to the plating of the transformed cells.

2.1.8 Growing Transformants

Single colonies were used to inoculate 2ml cultures of LB supplemented with 100µg/ml ampicillin. The cultures were grown by shaking at 37°C at 200 RPM for 16-18 hours.

2.1.9 Small Scale Plasmid Purification

Plasmid DNA was purified using QIAprep® Spin Miniprep Kit (QIAgen) following protocol guidelines. In brief, 1.5ml of the cultures were centrifuged at 10,000 x g for 5 minutes and resuspended in 250µl P1 resuspension buffer. The cells were lysed with 250µl of P2 lysis buffer followed by neutralisation with 350µl of N3 buffer. The lysate was centrifuged at 15,000 x g for 10 minutes and the supernatant loaded onto a QIAprep® Spin column for the DNA to be adsorbed to the silica-membrane. The columns were centrifuged and washed in binding PB buffer and PE buffer before DNA elution in 120µl of elution buffer (10mM Tris-Cl pH 8.5).

2.1.10 DNA Sequencing

All clones were confirmed by sequencing using the ABI Prism Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Reactions used 2µl Big Dye Terminator Ready Reaction Mix (labelled A-dye, C-dye, G-dye and T-dye terminators, dNTPs, AmpliTaq DNA polymerase), 1x sequencing buffer (80mM Tris-HCl, pH 9.0; 2mM $MgCl_2$), 5µl mini prep DNA, 1.6 pM primer and were made up to a 10µl volume with water. Cycling conditions; denaturation at 94°C for 10 seconds, primer annealing at 50°C for 50 seconds and extension at 60°C for 4 minutes, for 25 cycles. Sequencing primers were designed in the forward and reverse directions every 300 bases of the target gene. Sequencing was analysed with Lasergene software (DNASTAR Inc, Winsconsin, USA).

2.1.11 Vaccine Assembly

2.1.11.1 The pcDNA3.1 Plasmid

All DNA vaccine expression cassettes were inserted into the expression vector pcDNA3.1 (Invitrogen). The plasmid has been engineered to contain the human V_{H1} leader sequence downstream of the HindIII site and upstream of an added SfiI site for ease of cloning. The downstream NotI site remains so the tumour antigens can be inserted between the SfiI and NotI sites. We also have pcDNA3.1 expression plasmids that additionally have FrC or DOM sequences inserted between the NotI and XbaI sites. The tumour antigens can again be inserted between the SfiI and NotI sites to create the FrC or DOM fusion vaccines that read in the correct open reading frame. A map of the pcDNA3.1 plasmid and the

restriction sites present within the cloning region can be found on (http://www.invitrogen.com/content/sfs/manuals/pcdna3.1_man.pdf).

2.1.11.2 Assembly of p.DOM-epitope Vaccines

The p.DOM-PSMA4, p.DOM-PSMA27 and p.DOM-PSMA711 vaccines were made by Karen Houghton. The p.DOM-PSMA663 and the p.DOM-mPSMA636 vaccines were created by amplifying DOM with a reverse primer (PSMA663R or mPSMA636R respectively, Appendix I) that incorporated the human PSMA 663 or mouse PSMA 636 peptides at the end of the DOM sequence, a stop codon, and a Not I restriction site after the stop codon. The forward primer annealed to the BCL_1 IgM V_H leader sequence that is positioned upstream of DOM and included a Hind III restriction site upstream of the start ATG. The PCR product was inserted into the pcDNA3.1 plasmid (Invitrogen) between the HindIII and NotI restriction sites in the plasmid's polylinker cloning site.

2.1.11.3 Assembly of Human and Mouse PSMA Extracellular (EC) Domain Vaccines

Gene specific primers were designed to amplify the human and mouse PSMA extracellular domain (mPSMAEC: 2.130 KB and amino acids 44-752, hPSMAEC amino acids 44-750). Forward SfiI and reverse NotI restriction sites were incorporated into the primers. Target sequences were amplified with proofreading *Pfu* DNA polymerase (Promega). PCR products were sub-cloned either into pcDNA3.1 expression plasmid alone (to generate mPSMAEC or hPSMAEC) or with 3' FrC or DOM fusion (to generate mPSMAECFrC, hPSMAECFrC, or mPSMAECDOM, hPSMAECDOM) (Figure 47).

2.1.11.4 Assembly of Vaccines Encoding Human PSMA Full-Length or Shortened Versions

The full length human PSMA was altered by PCR to contain an N-terminal BCL_1 leader sequence and ligated into pcDNA3.1 plasmid (HindIII and NotI sites) either alone (p.PSMA) or with a C-terminal DOM fusion (p.PSMA-DOM). These constructs were digested with NheI to remove amino acids 110-586 and leaving 273 amino acids of the protein that encodes part of the extracellular domain and the entire intracellular and TM domains. These were re-ligated to form the human PSMA short versions of the vaccine (p.PSMAshort or p.PSMAshort-DOM).

2.1.12 DNA Digests

PCR products and plasmids were sequentially digested with 10 units of restriction enzymes, with a 15 minute 50°C enzyme inactivation step in between. SfiI and NotI or HindIII and NotI digests were carried out with restriction enzymes (New England BioLabs, Hitchin, UK) in 1x Buffer 2. BamHI and XhoI or EcoRI and XhoI digests were carried out with restriction enzymes from Promega in 1x buffer D.

2.1.13 Assembly of Retroviral Transfection Constructs

2.1.13.1 p.MSCVpuro and p.MSCV-GFP Retroviral Vectors

The murine stem cell virus (MSCV) vectors originated from Clontech (Saint-Germain-en-Laye, France). The p.MSCV-GFP vector was created by Elio F. Vanin (St. Jude Children's Research Hospital, Memphis, Tennessee, USA). The vectors translate the puromycin (puro) antibiotic resistance gene or GFP through an internal ribosome entry site (IRES). The p.MSCV-puro vector cloning site contains BamHI-EcoRI-XhoI restriction sites.

2.1.13.2 Cloning Human and Mouse PSMA into p.MSCV-puro Retroviral Vector

Primers were designed with 5' forward BamHI and 3' reverse XhoI restriction sites. The full Kozak sequence GCCGCCACC was incorporated upstream of the transcription initiation ATG. The PCRs to amplify the full-length human or mouse PSMA sequence were carried out with pfu (Promega). The full-length genes already cloned into either pGEM-T® Vector System I (Promega) and pSTBlue-1 Perfectly Blunt® Vector (Merck Biosciences) were used as templates (sections 2.1.4.1 and 2.1.4.2). A second full-length mouse PSMA sequence was additionally sub-cloned from a plasmid containing a sequence that differed from that in GenBank entry NM_016770 by two amino acids. This construct was given to us by Dr. P. Gregor (Memorial Sloan-Kettering Cancer enter, New York, USA). These genes were inserted into the p.MSCV-puro plasmid (Clontech) between the BamHI and XhoI restriction enzymes. The two coding differences in the construct encoding Dr. Gregor's mouse PSMA sequence were sequentially mutated (G240A and 287N) to match the NM_016770 sequence by site directed mutagenesis (SDM). Briefly, complementary forward and reverse primers with the desired sequence were designed and

used to amplify the entire plasmid by PCR with proofreading *Pfu*. The unmethylated bacterial DNA used as the template was digested with 10 units of DpnI restriction enzyme (Promega). The remaining PCR amplified DNA was purified (eluted in 25µl) and transformed (10µl) into competent JM109 E. coli cells.

2.1.13.3 Cloning HHD MHC Class I into p.MSCV-puro and p.MSCV-GFP Retroviral Vectors

SDM of the single EcoRI restriction site within the HHD sequence was carried out first in order to create a non-coding change that would eliminate this restriction site. This would allow the EcoRI site to be used for sub-cloning. Gene specific primers with forward EcoRI and reverse XhoI restriction sites were used to amplify the HHD sequence. The purified PCR product was digested and ligated into p.MSCV-puro.

2.1.14 Assembly of Protein Expression Constructs Encoding a Secreted form of Mouse PSMA Extracellular Domain

2.1.14.1 Cloning Mouse PSMA Extracellular Domain Fused to a Cλ Purification Tag

The extracellular domain of mouse PSMA with the upstream human immunoglobulin V_{H1} leader sequence was digested from the mPSMAEC vaccine plasmid with HindIII and NotI restriction enzymes. The digested fragment was inserted into a pcDNA3.1 plasmid that already had a C-terminal human immunoglobulin light chain Cλ tag (inserted between NotI and XbaI sites) thereby creating the fusion construct.

2.1.14.2 Cloning Mouse PSMA Extracellular Domain with an N-terminal His(6) Purification Tag

The extracellular domain of mouse PSMA was amplified with a 5' forward primer containing a $His_{(6)}$ tag and inserted into pcDNA3.1 vector using SfiI and NotI sites.

2.1.14.3 Assembly of Human PSMA into p.TNT Vector for *In vitro* RNA Synthesis

The full-length PSMA gene with an upstream BCL_1 leader sequence was amplified by PCR with a forward primer containing an XhoI restriction site and a reverse primer with a NotI restriction site. The PCR product was digested with XhoI and NotI restriction enzymes (Promega) and inserted into the p.TNT™ vector (Promega) which is designed for the convenient *in vitro* expression of cloned genes.

2.1.15 *In vitro* Transcription-Translation (IVTT)

All constructs that contain a T7 promoter were checked by IVTT reactions using *in vitro* TNT® T7 Quick Coupled Transcription/Translation System (Promega) and [^{35}S]methionine. These reactions check that the constructs generate proteins of the correct size. The 25µl total reaction volume includes 0.5µg DNA, 20µl TNT T7 mastermix and approximately 100µCi [^{35}S]methionine. The transcription-translation reactions were incubated at 30°C for 90 minutes. A 10µl aliquot of the reaction was denatured with 1µl β-mercaptoethanol, and 6µl of NuPage loading buffer (Invitrogen, Paisley, UK) at 95°C for 5 minutes and separated on a pre-cast 4-12% gradient polyacrylamide SDS NuPAGE gel (Invitrogen) at 180 volts for 1 hour. The gel was dried on filter paper under a vacuum. Radiographic film was exposed on the radioactive gel in the dark overnight and developed the following morning to visualize the size of the proteins generated by each DNA expression construct.

2.1.16 COS-7 Cell Transfections

COS-7 cells were cultured until confluent then plated at $5x10^5$ per well (6 well plates). The following day they were transfected with 2µg of p.PSMA or p.PSMA-DOM plasmids with 5µl Fugene HD transfection reagent (Roche, following manufacturer's protocol). After 24 hours the cells were harvested and lysates prepared at a $5x10^6$ cells/ml concentration. The lysates were analysed for PSMA expression by Western blot with the 3E2 antibody (section 2.3.2).

2.2 DNA Preparation

2.2.1 DNA Vaccine and Transfection Plasmid Production

Large scale production of DNA vaccines or transfection plasmids were prepared using plasmid purification QIAfilter™ Giga or Mega Kits (QIAgen, Crawley, UK), which yield 10-15mg or up to 2.5mg of DNA respectively. First, glycerol stocks were streaked on LB-agar plates supplemented with 100μg/ml ampicillin, and colonies grown for 16-18 hours at 37°C. Single colonies were used to inoculate 2 ml Luria Broth and cultures were grown by shaking at 200 RPM at 37°C for 6-8 hours. These starter cultures were used to inoculate large cultures of 2 litres or 700ml of LB. Large scale cultures were grown shaking at 200 RPM at 37°C for 16-18 hours. The cultures were centrifuged at 4000 x g for 30 minutes to collect an *E. coli* cell pellet. The DNA is prepared using the QIAfilter™ Giga or Mega Kits (QIAgen, Crawley, UK) according to the manufacturer's guidelines. After elution the DNA was precipitated, washed with 70% ethanol and allowed to dry prior to resuspension in 5 or 2 ml TE buffer (10mM Tris-Cl, pH8.0, 1mM EDTA). Each DNA preparation was checked by sequencing.

2.2.2 DNA Vaccine Storage

The DNA concentration was determined by measuring the absorbance at 260nm wavelength using an eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). The ratio of absorbance at 260nm and 280nm wavelengths (A_{260}/A_{280}) were between 1.8 and 1.9, indicating minimal protein and RNA contamination. Aliquots of 1mg of DNA were precipitated with 0.8 times the volume of isopropanol and 0.1 times the volume of 5M NaCl for storage at -20°C.

2.2.3 DNA Vaccine Preparation for Immunisation

The DNA stored in isopropanol was centrifuged at 14,000 RPM at 4°C for 20 minutes and washed in 70% ethanol. The DNA pellet was allowed to dry before resuspension in sterile 0.9% w/v NaCl (saline) at a 1mg/ml concentration. The DNA was placed on a shaker at a low setting for 30 minutes to allow the DNA to dissolve. The DNA vaccine was made up at 0.5mg/ml in sterile saline for vaccination.

2.3 Protein Expression in HEK293-F Cells

Human embryonic kidney (HEK) 293-F cells (Invitrogen, Paisley, UK) were transfected with the two mouse PSMAEC protein expression constructs (Cλ and $His_{(6)}$ tags). The transfection protocol required 100µl 293fectin™ (Invitrogen, Paisley, UK) and 100µg of DNA each to be diluted in 3mls of Opti-MEM® (Invitrogen). The mixes were allowed to stand at room temperature for 5 minutes before being pooled and allowed to stand at room temperature for a further 30 minutes to allow the DNA complexes to form. The transfection mix was added in a dropwise manner to a 100ml culture of $1x10^6$/ml 293F cells grown in Freestyle™ 293 Expression Medium (Invitrogen). The cells were cultured at 37°C in a rotating incubator. The supernatant was harvested 72 and 144 hours post-transfection. Harvesting involved centrifugation of cells at 300 x g for 5 minutes and the supernatant was replaced with fresh media after the first harvest. The collected supernantant was filtered through Millipore Express™ Plus 0.22µM filters (Millipore UK Ltd, Watford, UK) to ensure exclusion of all cells. The supernantants were then concentrated down to a 10th of the original volume using Vivaspin 10kDa cut off concentrators (Vivascience AG, Hannover, Germany).

2.3.1 $His_{(6)}$ Tag mouse PSMAEC Protein Purification

The concentrated supernatant from the expressed protein was purified using the $His_{(6)}$ tag incorporated at the N-terminal of the protein, and His-Bind columns (Merck Biosciences). The manufacturer's protocol was followed. Briefly, 10ml of 1x Binding Buffer (8x = 4M NaCl, 160mM Tris-HCl, 40mM imidazole, pH7.9) was applied to the His-Bind column. Once it had flowed through, the concentrated supernatant was loaded onto the column. Another 10mls of 1x Binding Buffer was added to the column after loading the supernatant, followed by 10mls of 1x Wash Buffer (8x = 4M NaCl, 480mM imidazole, 160mM Tris-HCl, pH7.9). The final step was to elute the protein from the column in 4mls of 1x Elute Buffer (4x = 4M imidazole, 2M NaCl, 80mM Tris-HCl, pH7.9). The eluted protein was concentrated on a Vivaspin 50kDa cut off concentrator (Vivascience) and dialysed 3 times with 15mls of PBS down to a 1ml volume.

2.3.2 Anti-His and anti-mouse PSMA Western Blot

To confirm the integrity of the purified protein, western blots with antibodies against both the $His_{(6)}$ tag and the denatured mouse and human PSMA protein were carried out. His-tagged purified FrC protein made in-house and LNCaP cell lysate were used as positive

controls for their respective western blots. The LNCaP cell pellet was resuspended in lysis buffer (62.5mM Tris/HCl 1% SDS, pH7.5) at $5x10^6$ cells/ml, and mechanically sheared by plunging through a syringe needle 8-10 times. The lysates were stored at -20°C. Proteins or lysates were denatured with 1µl β-mercaptoethanol and 5µl of 4x SDS-containing loading dye at 95°C for 5 minutes and separated on a pre-cast 4-12% Bis-Tris polyacrylamide NuPAGE® gel (Invitrogen).

The proteins were subsequently transferred onto a nitrocellulose membrane that was briefly equilibrated in 100% methanol then rinsed in 1x NuPAGE® Transfer Buffer (Invitrogen). The gel and membrane were sandwiched between three layers of blotting paper that were first soaked in 1x NuPAGE® Transfer Buffer (Invitrogen). The proteins were transferred at 12 volts for 1-2 hours. The protein gels were stained with Simply Blue™ SafeStain (Invitrogen) to visualize the entire protein sample in order to confirm purity. The membranes were blocked in 5% milk/PBS/Tween-0.1% for 1 hour at room temperature. The membranes were washed then incubated with 1/1000 dilutions of primary antibody (Tetra-His antibody (QIAgen) or anti-mouse/human PSMA antibody clone 3E2 kindly provided by Dr. P. Gregor, Sloan Kettering Institute, section 4.3.2) in PBS-Tween-0.1% at 4°C overnight. Following three 10 minute PBS/Tween-0.1% washes, the membranes were incubated with a 1/1000 dilution of the secondary sheep anti-mouse IgG (gamma chain) HRP-conjugated antibody (The Binding Site, Birmingham, UK) in PBS/Tween-0.1%Tween at room temperature for 1 hour. The HRP conjugate enzyme reaction was visualized by chemilluminescence with an ECL™ Western blotting Detection Reagents (Amersham Biosciences).

2.3.3 Protein Quantification

The concentration of the eluted protein was determined with a BCA™ Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The reactions were placed in a shaker at 37°C for 1 hour and the absorbance of the products measured at 570nm wavelength.

2.4 Cell Lines

Cells were cultured in either RPMI 1640 or DMEM media containing 25mM HEPES and L-glutamine, supplemented with 10% decomplemented fetal calf serum, 1mM sodium pyruvate, 2mM L-glutamine, 0.1mg/ml penicillin, 0.06mg/ml streptomycin, 1% of 100x non-essential amino acids (Invitrogen) and 50µM β-Mercaptoehtanol (complete media)

Adherent cell lines were detached from culture flasks by rinsing the flask in non-supplemented media followed by the addition of sufficient trypsin (500mg/L) EDTA (200mg/L) (Lonza, Wokingham, UK) to form a thin layer at the base of the flask. The flasks were placed at 37°C until cells were detached (typically ~2-5 minutes). The cells were centrifuged at 300 x g for 5 minutes and resuspended in fresh complete media.

A number of cell lines have been used in this project:

- Freestyle™ 293-F cells (Invitrogen, Paisley, UK) are cells in suspension which are derived from the human embryonic kidney cells and allow large-scale transfection/protein expression.
- TRAMP-C1 mouse prostate cancer cell line of H2-K^b and H2-D^b origin were cultured in complete DMEM media with 10nM DHT and 0.005mg/ml bovine insulin (Sigma, Poole, UK) supplements.
- RMA-HHD cells are a mouse lymphoma cell line stably transfected with the transgenic HHD (human β-2 microglobulin fused to HLA-A2 α1, α2 and mouse α3) MHC Class I molecule with the endogenous H-$2D^b$ knocked out. These cells have been kindly provided by Lemonnier F.A. (Institut Pasteur, Paris, France) and were cultured in complete RPMI 1640 media.
- The human LNCaP cell line (CRUK Cell Services) is a lymph node metastasis prostate cancer cell line of HLA-A2 origin and was cultured in complete DMEM media supplemented with 20% fetal calf serum.
- Phoenix Amphotropic and Ecotropic retroviral packaging cell lines were kindly provided by Dr. P. Stevenson (Cambridge University, UK). These were cultured in complete DMEM media.
- NIH3T3 cells are mouse fibroblasts of H-2^d origin were cultured in complete RPMI 1640.
- NIH3T3- mPSMA and the NIH3T3 control cells were created and kindly provided by Dr. G. Gregor (Memorial Sloan-Kettering Cancer Center, New York, USA). These are NIH3T3 cells that have been retrovirally transfected to express mouse PSMA or the vector as a control respectively. These cells were cultured in complete DMEM media.
- YAC-1 are a mouse lymphoma cell line that is sensitive to cytotoxic activity of NK cells

2.5 Retroviral Transfections

2.5.1 Retroviral Transfection Method

The Phoenix packaging retroviral cell lines were plated in 6-well plates at $1.6x10^6$ cells in 6ml of complete media per well on day 1. On day 2, 25μM chloroquine (Sigma-Aldrich) was added to the Phoenix cells prior to calcium phosphate transfection. The 600μl transfection mix consisted of 300μl of 2x HBS (50mM HEPES, pH 7.05; 10mM KCl; 12mM Dextrose; 280mM NaCl; 1.5 mM Na_2HPO_4 buffer), 36μl of 2M $CaCl_2$, 20μg DNA plasmid and the remainder volume made up with sterile water for injection. The mix was briefly vortexed for 15 seconds then added to the cells in a dropwise manner. The media was changed after 9 hours and replaced with 2.5ml fresh complete media. From day 3 (24 hours post-transfection) the transfected Phoenix cells were incubated at 32°C to enhance retrovirus production. The target cells to be transduced with retrovirus were plated in 6 well plates (TRAMP-C1 cells at $1x10^5$ per well and NIH3T3 cells at $3x10^5$ per well). On day 4 (48 hours post-transfection), the retrovirus-containing supernatant from the transfected Phoenix cells was harvested and filtered to remove any cells. This supernatant was added "neat" with 4μg/ml polybrene (Sigma-Aldrich) directly onto the target cells. The transduction plates were centrifuged at 500 x g at 32°C for 90 minutes then cultured for a further 6 hours at 32°C before the retroviral supernatant was replaced with fresh complete media. The transduced cells were cultured at 37°C and analysed for expression of transfected gene on day 6 (48 hours post-transduction). Puromycin antibiotic (Merck Biosciences) was added to cells transduced with the p.MSCVpuro vector two days post-transduction (1μg/ml for TRAMP-C1 and 3μg/ml for NIH3T3 cells).

2.5.2 Analysing Transfected Cells and Selection of a Human PSMA Expressing Clone

Two days post-transduction retrovirally transduced cells (with HHD, or human/mouse PSMA) were washed in PBS and stained with an anti-human HLA-A2 antibody (clone BB7.2, BD Biosciences, San Diego, CA, USA), anti-human PSMA antibody (MBL Ltd, Naka-ku. Nagoya, Japan) or 11C8 anti-PSMA antibody under sterile conditions. Antibody fluorescence was detected on a FACScalibur and analysed with CellQuest software (BD Biosciences, San Jose, CA). The positive population was single-cell sorted into 96 well plates using a BD FACSAria (BD Biosciences). The sorted cells were cultured in complete media supplied with puromycin antibiotic. Once bulked up, clonal populations

were analysed by flow cytometry to re-confirm human PSMA, mouse PSMA or HHD expression.

Cells transduced with human and mouse PSMA were additionally analysed by RT-PCR to confirm the presence of PSMA mRNA transcripts. RNA was extracted and cDNA synthesized (sections 2.1.1 and 2.1.2). Additionally, cell lysates were prepared by exposure of cells to lysis buffer (62.5mM Tris/HCl 1% SDS, pH7.5) at a concentration of $5x10^6$ cells/ml. The lysates were subsequently used for Western blot analysis with the anti-human and mouse PSMA antibody 3E2 (section 2.3.3).

2.6 Flow Cytometry Analysis of MHC Class I Expression

The LNCaP cells were analysed for HLA-A2 expression using the anti-human HLA-A2 antibody (clone BB7.2, BD Biosciences). The cells were analysed before and after TNFα treatment (5, 10, 20ng/ml for 24, 48, 72hrs).

The TRAMP-C1 cells transfected to express mouse PSMA were analysed for H2-D^b expression using two different anti-mouse H2-D^b antibodies. One was conjugated to FITC (clone KH95, BD Biosciences) and the other was biotinylated (clone 28-14-8, Abcam). The latter was used in combination with a streptavidin-APC conjugate (BD Biosciences). The corresponding isotype control antibodies were used for each set of antibody labelling and 1μg of each antibody was used per $1x10^6$ cells labelled.

Ex vivo analysis of H2-D^b expression by TRAMP mPSMA+ cells was also carried out. $1x10^6$ cells were labelled with 10μM CFSE and co-injected with Matrigel™ (BD Biosciences) at a 1:1 ratio in a 150μl volume. The plugs were removed after 5 days and prepared as described in section 2.8.5. The cells were released ($1x10^6$) and were labelled with biotinylated anti-H2-D^b or isotype control antibodies (1μg), followed by a second step with streptavidin-APC. The cells were acquired by flow cytometry and gated on the CFSE+ TRAMP mPSMA+ population for MHC class I analysis.

2.7 Peptides

The four human PSMA peptides PSMA 4 (LLHETDSAV), PSMA27-35 (VLAGGFFLL), PSMA663 (MMNDQLMFL), PSMA711-719 (ALFDIESKV), the mouse PSMA636 (SAVNNFTDV) and the promiscuous MHC class II-binding FrC-derived p30 peptide (FNNFTVSFWLRVPKVSASHLE) were supplied by Peptide Protein Research Ltd (Southampton, UK) and were of > 95% purity as assessed by HPLC. Peptides were

dissolved in 10% DMSO 90%dH_20 to a 1mM concentration, except PSMA663 and mPSMA636 which were dissolved in 100% DMSO at a 10mM concentration. All peptides were aliquoted and stored at -20°C.

2.8 Mouse Experimental Protocols

2.8.1 Mice

HHD transgenic and C57BL/6 male mice aged between 6 and 12 weeks at the beginning of procedures were kept in accordance with Home Office Guidelines. Experiments were performed under Project Licence PPL 70/6401 and Personal Licence PIL 70/18850.

2.8.2 Vaccination

Animals were immunised with DNA vaccines prepared at 0.5mg/ml in sterile saline. A 50µl volume of vaccine was injected into each rear quadricep muscle (total 50µg DNA vaccine per mouse).

2.8.3 Electroporation

To facilitate vaccine delivery in some experiments mice received DNA vaccines delivered by electroporation under anaesthetic. Each mouse received an intra-peritoneal injection of ~10µl/g body mass anaesthetic mix. The anaesthetic mix: 1ml Hypnorm [0.315mg fentanyl citrate, 10mg fluanisone BP] (VetaPharma Ltd, Leeds, UK), 1ml Midazolam [10mg/2ml] (Roche, Welwyn Garden City, UK) was made up to 4mls with water for injection. For vaccination the rear limbs were shaved and 50µl of the 0.5mg/ml vaccine was administered to the rear quadricep. A dab of conductance gel was applied to the muscle and electrodes placed on the skin either side of vaccinated quadricep muscle. A local electric field was applied using a Hear 6p stimulator (Frederick Hear, Bowdoinham, ME) and Elporator 1 software from Dr. I. Mathieson (Inovio AS, Oslo Research Park, Oslo, Norway). The other quadricep muscle was vaccinated and electroporated in the same manner. The electrical field applied to the muscle was composed of 10 trains of 1000 pulses at 1000Hz frequency, each pulse delivered at 1 second intervals and lasting 400µsecs. The electrical field strength applied was approximately 50V. 100µl of 20µg/ml of Naloxone was administered to the mice to aid recovery from the anaesthetic.

2.8.4 Tumour Challenge

In some experiments mice were challenged with a lethal dose of tumour cells to determine vaccine efficacy. Prior to challenge, TRAMP-C1 cells transduced to express mouse PSMA (TRAMPmPSMA+) were expanded in culture for 4 days and for some experiments pre-treated with 20ng/ml IFNγ for 48 hours. The cells were washed in PBS and detached from the culture flask using trypsin (500mg/L)/EDTA (200mg/L) (Lonza). The cells were washed in sterile PBS three times then viable cells resuspended in a single-cell PBS suspension at $7x10^6$ cells/ml. Mice were injected sub-cutaneously with $7x10^5$ TRAMP-C1 cells in 100µl volume. Tumour sizes were monitored and mice euthanised when tumours reached 15mm or became ulcerated.

2.8.5 *In Vivo* Killing of Tumour Cells Co-Injected with Matrigel®

Male HHD mice were vaccinated on day 0 and injected with two different tumour cells in PBS mixed in a 1:1 ratio with Matrigel®. The tumour cells were washed once in PBS and labelled with either 1µM (TRAMP PSMA-HHD+) or 10µM (TRAMP PSMA+HHD+) CFSE at a concentration of $5x10^6$ cells/ml. The reaction was quenched by adding FCS (to an approximate 30% final concentration), then the cells were washed twice in PBS. $1x10^6$ of each cell population were mixed together to give a final volume of 75µl and mixed throroughly with 75µl of Matrigel™. Mice were mildly sedated with 100µl of a 1/100 dilution of Hypnorm [0.315mg fentanyl citrate, 10mg fluanisone BP] (VetaPharma). The mice were then slowly injected subcutaneously with 150µl of the cell/Matrigel™ suspension to allow solid plugs to form.

On day 5, the Matrigel™ plugs were excised and the spleen taken from each mouse. The plugs were individually digested with 1mg/ml collagenase/dispase (Roche) and 0.5mg/ml DNAseI (Sigma) for 2 hours then mashed through a mesh filter and washed in PBS. The cells were resuspended in PBS and analysed by flow cytometry for CFSE fluorescence, acquiring all events possible. The percentages of cells recovered were used to calculate the percentage specific killing using the following formula as previously published [371]:

$$\% \text{ specific killing} = 100 - \left[\frac{(\text{Test mice } \%\text{PSMA+HHD+} / \%\text{PSMA+HHD-})}{(\text{control mice } \%\text{PSMA+HHD+} / \%\text{PSMA+HHD-})} \times 100 \right]$$

2.9 Detection of Vaccination Responses

2.9.1 *Ex Vivo* Enzyme-Linked Immunospot (ELISPOT)

BD™ ELISPOT mouse IFNγ sets (BD Biosciences) were used as recommended by the manufacturer (with some minor modifications) to assess the secretion of IFNγ by T cells. The 96-well plates supplied were coated with 100µl of a 1/200 dilution of the sterile anti-mouse IFNγ antibody (1mg/ml) in PBS overnight at 4°C. Mouse spleens were harvested and mashed through a 70µM nylon mesh. Viable mononuclear splenocytes were isolated with density gradient centrifugation using Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) at 800 x g for 20 minutes at room temperature. The mononuclear cells that form a band at the interface were aspirated with a Pasteur pipette and washed in non-supplemented media. Per well, $2x10^5$ splenocytes were stimulated with varying concentrations of peptide and were incubated at 37°C for 20-22 hours, in a total volume of 200µl.

After the incubation period, the cells on the plate were first lysed by two 5 minute washes with 200µl of dH_20. The plate was then washed three times with PBS-0.05%Tween and the IFNγ cytokine secreted in response to stimulation was revealed by a 2-hour room temperature incubation with 100µl of a 1/250 dilution in PBS-FCS(10%) of the biotinylated anti-mouse IFN-γ antibody (0.5mg/ml) from the ELISPOT kit. A further three washes with PBS-0.05%Tween were carried out prior to a 1-hour room temperature incubation with 100µl of a 1/500 dilution in PBS-FCS(10%) of streptavidin ALP PQ (conjugated to alkaline phosphatase) (MAbtech, Nack a Strand, Sweden). Plates were washed further with four 200µl PBS-0.05%Tween washes and two 200µl PBS washes. The complex was finally developed using the BCIP/NBT substrate kit (Invitrogen) (BCIP is an insoluble substrate for alkaline phosphatase) to reveal a purple precipitate resulting from the enzymatic reaction. The assay was performed in triplicate and responses defined as positive if they were more than twice the "no peptide" background.

2.9.2 Generation of *In vitro* CTL Lines

To establish *in vitro* T-cell lines, spleens from vaccinated mice were mashed through a 70µM nylon mesh and splenocytes placed in *in vitro* cultures at $3x10^6$ cells/ml together with 20units/ml of recombinant human IL-2 (R&D Systems, Abingdon, UK) and 10nM PSMA27 or 0.1µM PSMA711 peptides. To continue CTL expansion beyond one week, the CTLs were re-stimulated with syngeneic peptide-loaded irradiated feeder cells every 7-

10 days. Feeder cells were splenocytes from naïve mice and were first loaded with 10nM PSMA27 or 0.1μM PSMA711 peptides at 37°C for 1 hour. The feeder splenocytes were then washed three times with 50ml of non-supplemented RPMI 1640 media prior to irradiation for 12 minutes at 2500 rad. Aliquots (2mls) of $3x10^5$/ml CTLs and $2.5x10^6$/ml splenocytes together with 20 units/ml of recombinant IL-2 (R&D Systems) were placed per well in 24-well plates.

2.9.3 Detection of Intracellular IFNγ from Stimulated $CD8^+$ T cells

Peptide-specific cells from CTL cultures were assessed for intracellular IFNγ accumulation upon stimulation and simultaneous incubation with Brefeldin A (GolgiPlug™, BD Biosciences), an intracellular protein transport inhibitor.

Viable mononuclear splenocytes were isolated from *in vitro* CTL lines (after 6 days *in vitro* or 6-9 days after the last *in vitro* re-stimulation) as described in section 2.9.1. Splenocytes ($5x10^5$) were incubated with a stimulation source (peptide, $5x10^5$ LNCaP cells or peptide-presenting DCs, or $5x10^5$ peptide-loaded cells) and a 1/200 dilution of Golgiplug™ (BD Biosciences) in 200μl PBS for 4 hours at 37°C. The assay was set up in 96-well plates.

After the incubation period, the surface Fc receptors on the splenocytes were blocked by a 10 minute incubation with 200μl of 2% decomplemented mouse serum and a 1/200 dilution of Golgiplug™ (BD Biosciences) in PBS at 4°C. The CTLs were surface stained in the presence of 1/200 dilution of Golgiplug™ (BD Biosciences) with the APC-conjugated rat anti-mouse CD8a antibody, clone 53-6.7 (BD Biosciences) and the FITC-conjugated anti-mouse MHC class II antibody, clone M5/114.15.2 (Miltenyi Biotec, Surrey, UK) (the latter only when DCs were used in the assay to ensure that $CD8^+$ MHC class II^+ DCs were gated out of the analysis) at 4°C for 20 minutes. The cells were then washed and fixed in 1% formaldehyde/PBS for 10 minutes and washed again twice before being permeabilised with 100μl/well 0.5% saponin-PBS. The intracellular IFNγ staining was then carried out with 0.5μg/well of PE-conjugated rat anti-mouse IFNγ antibody, clone XMG1.2 (BD Biosciences) or the isotype control, in 0.5% saponin-PBS for 20 minutes at 4°C. The cells were washed, resuspended in PBS and antibody fluorescence detected with a FACScalibur (analysed with CellQuest software, BD Biosciences). Up to two hundred thousand events were acquired. Events were gated on CD8 positive (and MHC class II-with the DC experiments) cells and the percentage values represent the percentage of $CD8^+$ cells that are $IFN\gamma^+$.

2.9.4 Chromium Release Cytotoxicity Assay

The ability of the expanded CTLs to kill the target cells was assessed in a chromium (^{51}Cr) release assay. Viable mononuclear cells were separated from the *in vitro* CTL lines using a Lymphoprep™ gradient (section 2.9.1).

When LNCaP or TRAMPmPSMA+ cells were used as targets in chromium assays they were pre-treated wih 5ng/ml TNFα for 24 hours or 20ng/ml IFNγ for 48/72 hours respectively. A large number of each of the target cells were placed into a falcon tube and were loaded with 4 MBq ^{51}Cr sodium chromate (Amersham Biosciences) in a total volume of 300µl. Target cells requiring loading with peptide were additionally incubated in corresponding peptide concentration (1µM or 10µM). Target cells were pulsed for 1 hour at 37°C and were then washed three times with 50ml of non-supplemented RPMI 1640 media to remove any excess peptide and unincorporated radioisotope. Target cells (T) were resuspended to $5x10^4$/ml and 100µl plated with varying numbers of effector (E) CTLs (ideally E:T ratios of 1:1, 1:50, and 1:100) also in a 100µl volume (total 200µl volume per well in a 96-well plate). The wells were plated in triplicate and incubated at 37°C for 5 hours prior to harvesting. The plates were then centrifuged at 300 x g. A 100µl aliquot of the supernatant from each well was transferred into LP3 tubes and assessed for the presence of the ^{51}Cr isotope released by the target cells. The γ-emission by each sample was counted for 5 minutes by an automatic gamma counter.

Spontaneous release (SR) and maximum release (MR) target cell controls were included in the assay, where $5x10^4$ in 100µl target cells were incubated with 100µl fully-supplemented RPMI medium or had 100µl 4% NP40 added at the end of the 5-hour incubation period. These values along with the experimental release (ER) values were used in the calculation of percentage specific lysis for each CTL line:

$$\% \text{ Specific Lysis} = \left(\frac{ER - SR}{MR - SR} \right) X\ 100$$

2.9.5 Anti-FrC and anti-DOM Enzyme-Linked Immunosorbent Assay (ELISA)

To detect vaccine-induced antibody responses, blood samples were taken from vaccinated mice by tail bleeding. Local anaesthetic (Instillagel®, FARCO-PHARMA GmbH, Cologne, Germany) was applied to the tip of the tails and mice were warmed at 37°C for 5

minutes. A 1-2mm section of the tip of the tail was cut with a scalpel and up to 200µl of blood removed per mouse. Whole blood was centrifuged at 300 x g for 5 minutes and the serum was removed, aliquoted and stored at -20°C.

96-well flat-bottomed Nunc Immunos™ ELISA plates (Thermos Fischer, Leicestershire, UK) were coated with 200µl/well of 2µg/ml FrC or DOM protein (expressed in 293F cells) diluted in coating buffer (1.53g Na_2CO_3 anhydrous, 2.92g $NaHCO_3$ in 1 litre dH_2O, pH9.5) at 4°C overnight. The plate was washed three times with 200µl/well PBS-0.1%Tween (also used for all the other washes in this ELISA protocol) prior to blocking in 200µl/well 0.5% BSA in PBS at 37°C for 1 hour. Another three washes were carried out before the addition of 200µl/well of 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400 and 1/12800 dilutions of each serum sample (diluted in PBS-Tween-0.1%) to the plate. In addition FrC or DOM standard samples were used to assign arbitrary units (established in our laboratory). A 1/200 or 1/50 dilution of the FrC or DOM standards equated to 200 units or 500 standard units respectively. Following a 90 minute incubation with the sera, the plate was washed three times before the addition of 200µl/well of a 1/1000 dilution of the secondary sheep anti-mouse IgG (gamma chain) HRP conjugated antibody (The Binding Site) and a further 1-hour incubation. Another three washes were carried out prior to developing the substrate reaction. One o-Phenylenediamine (OPD) tablet (Sigma-Aldrich) and a 1/1000 dilution of H_2O_2 were added to 30ml of substrate buffer (4.68g anhydrous citric acid, 7.3g Na_2HPO_4 in 1 litre dH_20, pH5.5). 200µl of this substrate mix was aliquoted into each well and the reaction was quenched with 80µl/well 2.5M H_2SO_4 when the colour of standard samples had developed sufficiently. The optical densities of each well were measured at 490nm wavelength by a Dynex MRX Platereader (Dynatech Instruments Inc., Santa Monica, USA) and the test sample FrC or DOM antibody levels calculated relative to the standard units.

2.10 Culturing Bone Marrow-Derived Dendritic Cells

Mice were sacrificed and BM was taken from the femur in each leg. Non-supplemented media was plunged through the bone to ensure collection of the entire bone marrow. The bone marrow was mashed through a 70µm nylon and centrifuged at 300 x g. The cells were resuspended in complete RPMI 1640 media at $1x10^6$ cells/ml. The media was supplemented with 20ng/ml recombinant murine GM-CSF (PeproTech, London, UK) and the cells placed in 6 well plates with 3ml aliquots per well. The bone marrow cells were cultured at 37°C and after 48 hours (Day 2) the media was replaced with fresh complete RPMI 1640 supplemented with 20ng/ml recombinant murine GM-CSF. On day 6, the DCs

were directly transferred to non-adherent plates and matured with 1μg/ml LPS (Sigma-Aldrich) for 18-24 hours prior to use for assays on day 7.

2.10.1 *In vitro* Preparation of RNA

The plasmid was linearised with a BamHI digest, a small amount of which was assessed for linearisation on a 1% agarose gel. The remainder of the plasmid was purified, eluted in nuclease-free water and quantified. The RNA was synthesised with the mMESSAGE mMACHINE™ High Yield Capped RNA Transcription kit (Ambion, Huntingdon, UK) following the manufacturer's instructions. The synthesised RNA was precipitated by the addition of 25μl of 7.5M lithium chloride, chilled for 30 minutes at -20°C then centrifuged at 20,000 x g at 4°C for 15 minutes. The pellet was washed with 1ml 70% ethanol and centrifuged at 20,000 x g at 4°C for a further 15 minutes. The RNA pellet was air dried and resuspended in ~20μl of nuclease-free water (depending on the pellet size). The amount of RNA was quantified using an eppendorf BioPhotometer (Eppendorf AG). A 1μg sample of the RNA was loaded on a 1% agarose gel to check the size and quality of the RNA.

2.10.2 Loading Dendrtic Cells with RNA

DCs were loaded with PSMA RNA for use for presentation to the peptide-specific CTLs. DCs were cultured as described in section 2.10. On Day 7 of culture, the DCs were centrifuged at 300 x g for 5 minutes and resuspended at $5x10^7$ DCs/ml in 2x OptiBuffer (Thermo Scientific). The *in vitro* synthesised RNA (40μg) was diluted in 100μl of sterile water and added to 100μl of DCs ($5x10^6$). The DCs/RNA were placed in a Gene Pulser® cuvette (Bio-Rad, Hemel Hempstead) and electroporated with a Bio-Rad Gene Pulser® at 150μFD and 320 volts. The electroporated DCs were immediately transferred into 10mls of pre-warmed complete RPMI 1640 media and allowed to recover at 37°C for 2 hours before used to stimulate CTLs in an IFNγ cytokine secretion assay.

2.10.3 IFNγ Cytokine Secretion Assay

Peptide-specific cells from CTL lines were assessed for IFNγ secretion by co-culturing with PSMA RNA-loaded DCs. Two hours after the DCs were loaded with RNA, they were centrifuged at 300 x g for 5 minutes and resuspended at $5x10^6$/ml. The viable mononuclear cells were separated from the CTL lines by density gradient centrifugation

using Lymphoprep™ (Axis-Shield PoC AS) (section 2.9.1) and were co-cultured at a 1:1 ratio with the RNA-loaded DCs (total of $1x10^6$) for 16 hours at 37°C. The IFNγ-secreting CTLs were identified with the mouse IFNγ secretion assay kit (Miltenyi Biotec) according to the manufacturer's protocol. Briefly, after the 16-hour incubation, the cells were washed twice in PBS-0.5%BSA and resuspended in 90µl of this buffer. A 10µl aliquot of the mouse IFNγ catch reagent was added to the cells and incubated on ice for 5 minutes prior to the addition of 1ml of pre-warmed medium and a further 45-minute incubation at 37°C under a slow continuous rotation. The cells were then washed twice in PBS-0.5%BSA, resuspended in 79µl of the buffer, and 10µl of each the PE-conjugated mouse IFNγ detection antibody from the kit and the FITC-conjugated anti-mouse MHC class II antibody, clone M5/114.15.2 (Miltenyi-Biotec), and 1µl of APC-conjugated rat anti-mouse CD8a antibody, clone 53-6.7 (BD Biosciences). After a 10 minute incubation on ice the cells were washed in PBS-0.5%BSA and antibody fluorescence detected with the FACScalibur (BD Biosciences).

2.10.4 Statistical Analysis

All statistical analysis was carried out with GraphPad Prism® software (GraphPad software, CA, USA). In order to make minimal assumptions about the distribution of the data, the statistical tests used were always non-parametric, non-paired and two-tailed. The Mann-Whitney U test was used for analysis of ELISPOT data and the Logrank Chi-squared test for analysis of *in vivo* protection experiments. Samples are deemed to be statistically different when $p<0.05$. Where population means are quoted the standard error of the mean (SEM) is stated in parenthesis. SEM error bars are also shown in data figures but in some cases are not seen when the SEM is too small.

3. Induction of PSMA-Specific $CD8^+$ T Cell Responses by DNA Vaccines

3.1 Introduction

The aim of this project is to investigate the ability of DNA vaccines to induce immunity against the PSMA tumour antigen. The positive correlation reported between PSMA expression, high Gleason score and poor prognosis [360] supports the development of targeted therapy to directly kill PSMA-overexpressing tumour cells. The apparent role of PSMA in angiogenesis indicates the antigen may also have a critical role in tumour growth and survival. The tumour cells may therefore not be able to shed PSMA expression thereby making PSMA an ideal target.

In this section of the project the expansion of PSMA-specific $CD8^+$ T cells is the focus. Evidence from pre-clinical and clinical studies supporting vaccination aimed at generating PSMA-specific cytotoxic cellular immune responses are described below.

3.1.1 Priming of murine $CD4^+$ and $CD8^+$ T cells Specific for Human PSMA Protect Against Tumour Challenge

In a xenogeneic model, immunisation of mice with a syngeneic mouse DC vaccine transfected to express human PSMA was able to induce cytotoxic T-cell responses that could mediate protection against a human PSMA-expressing tumour [372]. Another study reported that a DNA vaccine encoding a partial human PSMA sequence was able to prime $CD8^+$ T cells that killed a human PSMA-expressing tumour *in vitro* and mediated *in vivo* protection [183]. These studies demonstrate that CTLs are capable of eradicating human PSMA-expressing tumour cells thereby validating the development of such immunisation approaches. Depletion of $CD4^+$ T cells prior to but not after immunisation abrogated CTL activity [183] confirming the expected necessity of $CD4^+$ T cells to assist the generation of CTL responses. However mice and rats do not have tolerised T-cell repertoires specific for the xenogeneic human PSMA antigen. Therefore these studies do not provide direct information on the use of these same vaccines in human patients.

3.1.2 Peptide-Loaded Dendritic Cells as Vaccines in Prostate Cancer Patients

Further evidence supporting the strategy of vaccination to prime cytotoxic cellular responses comes from phase I/II clinical trials. These studies commonly involve only a small number of patients to establish safety. It is difficult to determine effectiveness from small patient cohorts and there is often a problem with the interpretation of outcomes of PCa immunotherapeutic clinical trials. PSA levels and PSA doubling time are used as surrogate markers as a measure of therapeutic success. Difficulties arise due to the heterogeneity of disease between patients making direct comparisons impossible, and the impact that recent treatments such as hormone therapy or radiotherapy may have had. Often these patients are in the process of biochemical (PSA) relapse following remission or have an established metastatic disease. It is important to remember that such patients have a disease that is already beyond current therapies and that creates a challenging setting for immunotherapy.

Encouragingly there have been no reports of immediate or long-term side effects, toxicity, or, most importantly, autoimmunity, in patients receiving PSMA peptide-loaded DC infusions or PSMA DNA vaccination. However the responses observed thus far have not provided much clinical benefit either. It is possible that once vaccines that are able to prime strong immune responses are fine-tuned these could cause undesired autoimmunity.

Most of the clinical studies targeting PSMA have used infusions of autologous DCs loaded with PSMA-derived HLA-A2-binding peptides to activate $CD8^+$ T cells in patients. Details of PSMA HLA-A2-restricted peptides can be found in section 3.1.5 and Table 4. Such studies have used either a single PSMA peptide [250] or a pool of peptides where one or more were PSMA-derived [239, 240, 373]. Positive but limited results have been described from these trials. Murphy et al., carried out a phase I clinical study on 51 HRPC PCa patients (92% with metastatic disease) who received 4 or 5 infusions over 6-8 months. There was an average decrease in PSA levels (31.8ng/ml to 12.1ng/ml) and an increased T-cell lymphoproliferative response to the peptide in patients receiving autologous DCs pulsed with the HLA-A2-binding PSMA711 peptide [250]. 33 of these PCa patients (19 with late stage metastatic disease, D2) proceeded into a phase II study where they received a further 6 infusions of DCs loaded with both PSMA4 and PSMA711 HLA-A2-binding peptides. 9/33 (27%) of patients had >50% decrease in PSA, 4 of which were also responders from the phase I trial and had a total response period of >370 days before biochemical (PSA) relapse. 11/33 (33%) patients exhibited stable disease and 12/19 late stage metastatic disease patients survived >600 days (~20 months), surprisingly longer

than the expected median survival [374]. These clinical outcomes are quite astonishing given the late disease stages treated.

The same group then carried out a phase II trial where HRPC or patients with recurrent disease were administered 6 infusions of DCs pulsed with PSMA4 and PSMA711 peptides at 6-week intervals. 8/25 (32%) HRPC and 11/37 (30%) recurrent disease patients responded with average PSA decreases of 10% and 5% respectively. The non-responders in each group exhibited an increase in PSA of 404% and 100% respectively. 4/8 responders in HRPC and 7/11 responders in the recurrent disease groups were still responding at 291 (~10 months) and 557 (~18 months) days respectively (calculated from the end of the study until disease progressed as defined by a 50% increase in PSA) [373].

Fuessel et al., vaccinated eight HRPC patients with DCs loaded with PSMA4 and another four PCa tumour antigen peptides (from survivin, prostein, and the transient receptor potential p8). It was reported that 1/8 (13%) of the patients had >50% decrease in PSA levels and stable PSA levels were established in 3/8 (38%) patients. Therefore clinical effects were achieved in 50% of the patients. PSA responses lasted between 4-17 weeks but scans revealed that vaccination had not significantly changed the metastatic load. A total of 4 patients (including 3 of the clinical responders) also exhibited immunological responses. Vaccination was able to expand PSMA4-specific cellular responses in two of these responding patients [240].

Waeckerle-Men et al., reported 3/4 HRPC patients immunised with DCs loaded with 4 PCa tumour antigen HLA-A2-binding peptides (from PSMA, PSCA, PAP, and PSA) had evidence of good cellular peptide-specific responses against all the tumour antigen peptides, including PSMA4. These three patients were therefore enrolled for long-term vaccination. After 6 infusions, all 3 patients showed a decrease in PSA doubling time and one patient had a decrease in total PSA levels. The responses lasted up to 32 weeks before the patients exhibited a decline in responsiveness to tumour and control (influenza and tetanus toxoid) antigens. The therapy did not halt clinical progression as metastases developed [239]. As peptides from other tumour antigens were used simultaneously it would be impossible to speculate as to whether the PSMA4-specific cellular responses may have contributed to the decreases in PSA.

Autologous DC peptide loading approaches have been pursued in various other cancers including melanoma [375] and breast cancer [376]. These studies along with the PCa trials described have reported limited effectiveness and inability to halt development of metastases. Repeated DC infusions appear to have an effect on PSA levels and achieve some degree of cellular responses however, they are not durable after cessation of vaccination. Even with long-term vaccination patients were shown to become

unresponsive and disease eventually progressed [239]. The reasons for the limited therapeutic impact of peptide-loaded DCs are likely a combination of non-optimal peptide selection, the relatively low half-life of the peptide-MHC complexes and most importantly the lack of $CD4^+$ T-cell help. Multiple infusions are used to overcome the short peptide-MHC complex half-life restrictions. Improved responses may be observed with a strategy based on stably transfecting DCs with a PSMA-encoding plasmid. Endogenous PSMA expression by the DCs may allow a multi-epitope response although an immunodominant response is most likely. The use of the full-length PSMA would result in the priming of not only PSMA-specific $CD8^+$ but also $CD4^+$ T-cell responses, but potentially also Tregs.

Even though limited, the responses described here are encouraging for vaccination against PSMA. The ability of some patients to generate peptide-specific cellular responses and the presence and correlation of some extent of clinical benefit demonstrate that the induction of peptide-specific immunity against PSMA appears to be feasible. These studies demonstrate both the efficacy and safety of PMSA-targeted vaccination although improvements to enhance effectiveness are required.

3.1.3 Candidate human PSMA $CD4^+$ and $CD8^+$ T Cell Epitopes as Immunotherapeutic Targets

There are algorithms that from a sequence can predict MHC class I (section 3.1.5.1) and class II-binding peptides. $CD4^+$ T-cell epitopes are more difficult to predict than $CD8^+$ T-cell epitopes due to variable pocket binding behaviour. MHC class II-binding peptides can also vary greatly in length as they can be trimmed once bound thereby creating further difficulties with predictions that are usually of set lengths.

In the following sections I will first describe the more limited published data on PSMA $CD4^+$ T-cell epitopes. Although for the moment we are not interested in the study of particular PSMA-specific $CD4^+$ T cells, we have created a full-length PSMA-encoding DNA vaccine that can potentially induce a PSMA-specific $CD4^+$ T-cell response. I will then discuss what is known regarding MHC class I-binding peptides, particularly focusing on the HLA-A2-restricted peptides. It is the PSMA-specific $CD8^+$ T-cell responses that are the focus of the study

A significant effort has been made to identify human PSMA MHC class I and to a lesser extent class II-binding peptides. There are some important criteria that have to be met for

a candidate epitope to be a suitable target for immunotherapy. These are true for the ability to prime and expand successful anti-tumour $CD4^+$ and $CD8^+$ T-cell immunity.

1. Patients must have a repertoire of PSMA epitope-specific $CD4^+$ and $CD8^+$ T cells that have escaped deletion by central tolerance and that can overcome regulatory suppression mediated by peripheral tolerance. Vaccination attempts to expand these existing epitope-specific T-cell repertoires.
2. The epitope-specific $CD4^+$ and $CD8^+$ T cells must demonstrate peptide-specific and MHC-restricted effector activity, and tumours must therefore express MHC class I and/or II.
3. T cells expanded in patients must recognise epitopes that are naturally presented by PSMA-expressing tumour cells in order for the peptide to be biologically relevant. This would allow PSMA-expressing tumour cells to be lysed by CTLs and to stimulate the effector function of the helper T-cells at the tumour site.

3.1.3.1 PSMA-Specific Helper $CD4^+$T cells

Helper $CD4^+$ T-cells are required for the effective priming of $CD8^+$ T cells as well as the generation of memory $CD8^+$ T cells. Helper $CD4^+$ T-cells specific for non-tumour related antigens (such as tetanus toxin) can participate in the CTL induction phase that occurs in the secondary lymphoid organs. Tumour-specific helper $CD4^+$ T cells may additionally aid $CD8^+$ T cells by producing cytokines, growth factors and direct co-stimulation at the tumour site. With this in mind some groups have attempted to define candidate PSMA $CD4^+$ T-cell epitopes that could be incorporated into immunotherapeutic strategies. However, it is currently unclear whether $CD4^+$ T cells are required at the tumour site.

3.1.3.1.1 Expansion of PSMA-Specific Helper $CD4^+$ T cells from Healthy Volunteers and PCa Patients

Investigations have led to the discovery of four human PSMA-specific $CD4^+$ T cell helper epitopes that could be targeted by vaccination. Helper $CD4^+$ T-cell repertoires specific for HLA-DR-restricted PSMA peptides 206, 334, 459, 567, 576, 687 and 730 have been found to naturally exist in PBMCs from healthy volunteers (Table 3) and could be expanded *in vitro* [377, 378]. The T-cell repertoire for PSMA459 is additionally retained and not irreversibly tolerised in PCa patients as *in vitro* T-cell expansion could be carried out from the PBMCs of 3/5 PCa patients in remission [377, 378]. Repertoires specific for the other epitopes in PCa patients' PBMCs were not assessed.

PSMA MHC Class II Binding Peptides	$CD4^+$ T Cells Stimulated from Human PBMCs from Healthy Volunteers	$CD4^+$ T Cells Stimulated from PCa Patient PBMCs (no immunotherapy treatment)	MHC Class II Restriction	Is Peptide Processed and Presented by Autologous DCs Loaded with LNCaP Cell Lysate or Recombinant Protein?	Reference
PSMA206-220 GKVFRGNKVKAQLA	YES	---	HLA-DR04	NO	Schroers 2003 [377]
PSMA334-348 TGNFSTQKVKMHIHS	YES	---	HLA-DR4	YES	Kobayashi 2003 [378]
PSMA 459-473 NYTLRVDCTPLMYSL	YES	YES	HLA-DR04	YES	Schroers 2003 [377]
PSMA567-581 DPMFKYHLTVAQVRG	YES	---	HLA-DR4	NO	Kobayashi 2003 [378]
PSMA576-596 VAQVRGGMVFELANSIVLPF D	YES	---	HLA-DQ	NO	Schroers 2003 [377]
PSMA687-701 YRHVIYAPSSHNKYA	YES	---	HLA-DR04, DR09, DR53	YES	Kobayashi 2003 [378]
PSMA730-744 RQIYVAAFTVQAAAE	YES	---	HLA-DR03, DR04, DR07, and HLA-DQ	NO	Schroers 2003 [377]
	YES	---	HLA-DR53 (not HLA-DR04, DR09)	YES	Kobayashi 2003 [378]

Table 3

PSMA $CD4^+$ T Cell epitopes described within the current literature

Hashed lines (---) indicate that the particular feature was not assessed within the published study.

3.1.3.1.2 Promiscuous $CD4^+$ T Cell Receptors and MHC Class II Molecule Binding Motifs

Interestingly, there appears to be promiscuity in the MHC class II molecule binding of PSMA 687 and 730 peptides but also at the TCR level where peptide can be recognised in the context of more than one MHC class II molecule (Table 3). A $CD4^+$ T cell clone specific for PSMA687 was restricted only by HLA-DR53. However a second T-cell clone from a different volunteer was HLA-DR9 restricted but could also recognise the PSMA687 peptide in the context of HLA-DR53 and DR4 molecules [378].

A study found that several PSMA730-specific T-cell clones from different healthy donors could recognise the peptide presented by both HLA-DR and HLA-DQ as only an antibody that could simultaneously block both HLA-DR and HLA-DQ abrogated recognition [377, 378]. This promiscuity could have favourable implications for vaccination strategies, as immunity against a single helper epitope may induce responses that are biologically relevant in patients of diverse MHC class II haplotypes. This was not a coincidence as both studies selected the MHC class II-binding peptides based on results predicting binding to at least three HLA-DR molecules.

3.1.3.1.3 Processing and Presentation of PSMA Helper $CD4^+$ T Cell Epitopes

The processing of four of these MHC class II-binding peptides was confirmed by the ability of DCs loaded with LNCaP cell lysate or recombinant PSMA protein to present the peptides and stimulate proliferation of the T-cell lines specific for PSMA334, 459, 687, and 730 peptides (Table 3 [377, 378]). The other $CD4^+$ T cell peptides were not presented by the DCs and are therefore not suitable targets for immunotherapy.

3.1.3.2 PSMA-Specific $CD8^+$T cells

Cytotoxic T cells are potent mediators of anti-tumour immunity that are capable of lysing tumour cells and eradicating tumours in many animal models. The clinical evidence discussed in section 3.1.2 suggests that PSMA-specific CTL responses in vaccinated patients may be mediating clinical effects. For these reasons the focus has been on finding MHC class I-binding peptides that can potentially be expanded by immunisation of PCa patients. PSMA $CD8^+$ T-cell epitope investigations have focused on individuals of HLA-

A2, HLA-A3 and HLA-A24 haplotypes as these are the main haplotypes found in the Caucasian and Japanese populations.

3.1.3.2.1 Naturally Existing PSMA-Specific $CD8^+$ T Cell Repertoires in Healthy Volunteers

Cytotoxic T cells specific for a range of peptides have been expanded from PBMCs of healthy individuals of a variety of haplotypes. The HLA-A2-restricted PSMA epitopes reported for which T-cell repertoires have been found in healthy donors are PSMA27, 469, 663 and 711 (Table 4, [230, 231, 238]). These are the peptides of interest in this project and are discussed in greater detail in the following sections (section 3.1.5). T-cell repertoires in healthy volunteers have been found for nine and three HLA-A24- and HLA-A3-restricted PSMA peptides respectively (Table 4, [237, 238, 379, 380]).

3.1.3.2.2 Naturally Existing PSMA-Specific $CD8^+$ T cell Repertoires Retained in PCa Patients

More important is the presence of T-cell repertoires specific for the target epitopes in PCa patients. Peripheral tolerance will have an effect on the availability of and ability to expand $CD8^+$ T cells *in vitro* and *in vivo*. The presence of these $CD8^+$ T cells in patients' PBMCs indicate that despite peripheral tolerance mechanisms, particularly in the presence of several fold higher PSMA expression in PCa, the $CD8^+$ T cells may be expanded. This ability demonstrates that if the suppressive tumour environment in patients can be overcome, these $CD8^+$ T cells are not irreversibly tolerised and may be expanded by suitable immunisation strategies. The majority, but not all, of HLA-A2, HLA-A3 and HLA-A24 epitopes for which T-cell repertoires were found in healthy donors have also been found in PCa patients [237-240, 250, 379, 380], Tables 4 and 5. From the data published on the HLA-A24 and HLA-A3 peptides it appears that the peptide-specific T cells are found in a higher proportion of healthy donors than in PCa patients (Table 5). For example HLA-A24-restricted T cells specific for the PSMA624 peptide were found in 60% of healthy volunteers but only 38% of PCa patients. This trend is apparent for a number of peptides and is not surprising for two reasons. Firstly as described above PSMA-expressing PCa is likely to tolerise PSMA epitope-specific T cells. Secondly it has been reported that PCa patients appear to have an overall degree of non-specific immune suppression as CTLs from PBMCs have an impaired ability to be activated by the positive

control immunogenic peptides derived from EBV and influenza viruses [237]. A clinical trial reported that 76% of HRPC PCa patients (92% of those with metastatic disease) admitted into the study had impaired cellular immunity as judged by the lack of responses to the immunogenic diphtheria, tetanus, streptokinase and mumps antigens in a DTH test [250].

There are also T-cell repertoires for three HLA-A24 peptides that were found in 1/16 (6%) of PCa patients but not in the 5 healthy volunteers [379], Table 5. This would indicate that the number of subjects may need to be larger than currently sampled in order for many of these studies to be representative of a population.

3.1.3.2.3 Processing and Presentation of PSMA $CD8^+$ T cell Epitopes

The processing of 4 (PSMA27, 441. 663, and 711) from 6 of the HLA-A2-binding peptides has been tentatively shown by the ability of the CTLs expanded from human PBMCs to lyse low levels of the HLA-$A2^+$ LNCaP human PCa cell line [230, 231, 238], Table 4. However, the evidence is not conclusive as emphasised by contradictions between different studies.

The processing of 2/3 HLA-A3 and 3/12 HLA-A24-binding peptides have also been demonstrated by killing assays and T-cell IFNγ secretion upon stimulation with cells endogenously expressing PSMA (Table 5, [237, 379, 380]). Again, some of the results can be questioned due to high background but were generally confirmed with CTLs from a number of patients thereby substantiating the findings [237]. The processing of other peptides was not mentioned and may therefore either not be processed or remain untested.

PSMA HLA-A2 Binding Peptides (intracellular IC, transmembrane TM, extracellular EC)	$CD8^+$ T Cells Stimulated from PBMCs of Healthy Volunteers	$CD8^+$ T Cells Stimulated from PCa Patients' PBMCs (+/-peptide loaded DC immunotherapy)	Cytotoxicity Against Peptide Loaded Targets	Cytotoxicity Against PSMA Expressing Tumour Cells (Processing?)	Peptide Specific IgG in Pca Patient's Plasma (# positive patients)	Reference
PSMA4-12 (IC) LLHETDSAV	---	YES (7/10)	---	---	YES (6/10)	Harada 2004 [238]
	---	YES	---	---	---	Fuessel 2006 [240]
	---	YES	---	---	---	Waeckerle-Men 2006 [239]
	---	YES	---	---	---	Murphy 1996 [250]
	NO	---	---	---	---	Lu 2002 [230]
PSMA27-35 (TM) VLAGGFFLL	YES	---	YES	YES	---	Lu 2002 [230]
	---	YES? (epitope PSMA26-34, 2/10)	---	---	NO (0/10)	Harada 2004 [238]
PSMA441-450 (EC) LLQERGVAYI	---	YES (8/10)	YES	YES	YES (5/10)	Harada 2004 [238]
PSMA469-478 (EC) LMYSLVHNL	YES	---	YES	NO	---	Lu 2002 [230]
PSMA663-671 (EC) MMNDQLMFL	YES	---	YES	NO	---	Lu 2002 [230]
	YES	---	YES	YES	---	Mincheff 2003 [231]
	---	---	---	---	YES (4/10)	Harada 2004 [238]
PSMA711-719 (EC) ALFDIESKV	YES	---	YES	NO	---	Lu 2002 [230]
	YES	---	YES	YES	---	Mincheff 2003 [231]
	---	YES (7/10)	---	---	---	Harada 2004 [238]
	---	YES	---	---	YES (4/10)	Murphy 1996 [1250]

Table 4

The PSMA HLA-A2-restricted epitopes described in the current published literature

The four coloured epitopes are those studies within this project. Hashed lines (---) indicate the particular feature was not assessed within the published study.

The information regarding the existence of human T cell repertoires is coloured blue and that on the processing of the peptides is coloured in orange.

PSMA HLA-A24 Binding Peptides	$CD8^+$ T Cells Stimulated from PBMCs of Healthy Volunteers (# of donors)	$CD8^+$ T Cells Stimulated from PCa Patients' PBMCs (# of donors)	Cytotoxicity Against Peptide Loaded Targets	Cytotoxicity Against PSMA Expressing Tumour Cells (Processing?)	Peptide-Specific IgG Detected in PCa Patient Plasma	Reference
PSMA74-83	YES (1/5)	NO	---	---	NO	Kobayashi 2003 [379]
PSMA178-186	NO (0/0)	YES (1/16)	---	---	NO	Kobayashi 2003 [379]
	YES	---	YES	YES	---	Horiguchi 2002 [380]
PSMA227-235	YES	---	YES	YES	---	Horiguchi 2002 [380]
PSMA298-306	YES (1/5)	NO (0/16)	---	---	NO	Kobayashi 2003 [379]
PSMA448-456	YES (1/5)	YES (1/16)	---	---	NO	Kobayashi 2003 [379]
PSMA520-528	NO (0/5)	YES (1/16)	---	---	NO	Kobayashi 2003 [379]
PSMA565-574	NO (0/5)	YES (1/16)	---	---	NO	Kobayashi 2003 [379]
PSMA584-593	NO (0/5)	YES (1/16)	---	---	NO	Kobayashi 2003 [379]
PSMA606-614	YES (1/5)	NO (0/16)	---	---	NO	Kobayashi 2003 [379]
PSMA624-632	YES (3/5)	YES (6/16)	YES	YES	YES (3/30)	Kobayashi 2003 [379]
PSMA624-633	YES (2/5)	YES (1/16)	---	---	NO	Kobayashi 2003 [379]
PSMA699-708	YES (2/5)	YES (1/16)	---	---	NO	Kobayashi 2003 [379]
PSMA HLA-A3 Binding Peptides						
PSMA199-207	YES (4/9)	NO	---	---	NO (0/20)	Matsueda 2005 [237]
PSMA207-215	YES (6/9)	YES (5/15)	---	YES	YES (19/20)	Matsueda 2005 [237]
PSMA431-440	YES (5/9)	YES (3/15)	---	YES	YES (16/20)	Matsueda 2005 [237]

Table 5

PSMA $CD8^+$ T cell epitopes (not restricted by HLA-A2) found in the current published literature

Hashed lines (---) indicate that the particular feature was not assessed within the published study.

3.1.3.2.4 PSMA $CD8^+$ T Cell Epitopes Validated as Immunotherapeutic Targets

There are only a handful of peptides for which the published studies arguably provide evidence for the fulfilment of all the criteria stated in section 3.1.3 and can therefore be considered good immunotherapeutic targets. These are the HLA-A2-binding PSMA27, 441, 663 and 711, the HLA-A3-binding PSMA 207 and 431, and the HLA-A24-binding PSMA178, 227, and 624 peptides. However, as already mentioned, some of the peptide processing data is open to challenge. If validated, and if CTLs specific for the processed peptides can be expanded in patients by immunisation, the CTLs should be able to lyse PSMA-expressing tumour cells *in vivo*.

3.1.4 PSMA MHC Class I-Binding Peptide-Reactive IgG

IgG reactive to various PSMA-specific CTL target peptides has been detected in the plasma of healthy donors and PCa patients. These include the HLA-A2-binding peptides PSMA4, 441, 663, 711, the HLA-A3 peptides PSMA207, 431, and the HLA-A24 peptide PSMA624 (Tables 4 and 5, [237, 238, 379]).

The mechanisms by which the IgG is generated are unknown as is the source of PSMA antigen. The biological role of peptide-specific IgG is not understood and it is a surprising phenomenon given that antibodies often recognise non-continuous epitopes rather than peptide sequences. Harada et al., reported a link between peptide-reactive IgG and the ability to stimulate peptide-specific CTLs from human PBMCs [237]. They speculate that the presence of pre-existing peptide-reactive IgG in PCa patients may therefore be an indication that immunotherapy can elicit cellular epitope-specific responses. If this is true then the presence of PSMA peptide-reactive IgG in PCa patients is an encouraging observation and one that could possibly be used to predict responders to PSMA-targeted immunotherapy. There are also reports that peptide vaccination led to the induction of IgG that could react with CTL-directed peptides and positively correlated with clinical responses and survival in advanced lung and gastric cancer [381-383]. There appears to be some correlation between the presence of IgG reactive to the PSMA HLA-A2-binding peptides and the ability to expand peptide-specific $CD8^+$ T cells from PCa patients. IgG specific for PSMA27 could not be detected in 10 PCa patients and this coincided with the lower number of patients (2/10) with a T-cell repertoire specific for this peptide. At least 4/10 PCa patients had IgG reactive for the other PSMA HLA-A2-restricted peptides and at least 7/10 of these patients also had peptide specific $CD8^+$ T cells (Table 4, [238]). Further investigation to understand the immunology behind this process is clearly required.

3.1.5 Human PSMA HLA-A2-Binding Peptides

The human PSMA peptides restricted by the HLA-A2 MHC class I molecule have been briefly described in the sections above. The three PSMA HLA-A2-binding epitopes that have been most widely investigated are PSMA663 (MMNQLMFL), PSMA4 (LLHETDSAV), and PSMA711 (ALFDIESKV) with the latter two PSMA peptides having already been used in PCa clinical trials where the patients were administered peptide-loaded DC vaccines (discussed in section 3.1.2). Only single studies have reported on PSMA441 (LLQERGVAYI) and PSMA27 (VLAGGFFLL). The published data for the PSMA4, PSMA27, PSMA663 and PSMA711 epitopes (summarised in Table 4) are discussed below.

3.1.5.1 The HLA-A2 Motif and Anchor Residues in Peptides

The anchor MHC class I motifs set the requirement for peptides to be able to bind to their grooves. The length of the peptides is usually determined by the MHC class I allele with the groove on HLA-A2 molecules best accommodating nonamers. The interactions between the peptide and the MHC are mediated through two anchor residues on the peptide sequence. The preferred amino acid specificities at the anchor positions are MHC allele-specific as the side chains of the preferred anchor residues protrude into the pockets of the MHC groove. One of the anchor positions for all MHC alleles is the C-terminal amino acid. The HLA-A2 molecule has a strong preference but not an exclusive requirement for a valine or leucine residue at this position. The second anchor position for the HLA-A2 motif is residue two (P2) with a preference for leucine or methionine amino acids [384]. Algorithms that can predict the ability of a peptide sequence to bind a particular MHC class I molecule have been created. One of the most commonly used predictive algorithms is SYFPEITHI, created by Rammensee et al., [385] which evaluates every amino acid within a given peptide and assigns it a score. The value of a residue at a particular position is dependent on the frequency of the respective amino acid in natural ligands, T-cell epitopes and binding peptides. The overall score corresponds to an estimated binding affinity for the queried MHC class I molecule. The maximum SYFPEITHI score for HLA-A2 peptides is 36. Another predictive database is BIMAS [386], which assigns scores for each residue but in this case calculates a score that corresponds to the estimated half-life of dissociation of the peptide-MHC complexes at 37°C and pH 6.5.

Although the non-anchor residues can be very diverse, there are loose preferences for certain amino acids that have been found in natural ligands such as glutamic acid or proline at P4 for the HLA-A2 motif [384]. This particular preference does hold for PSMA4 and PSMA441 peptides (Table 6).

Position (P)	1	2	3	4	5	6	7	8	9	10	SYFPEITHI Binding Score	BIMAS Estimated Half Time of Dissociation
Amino Acid Preference		L M							V L	V L		
PSMA4	L	L	H	E	T	D	S	A	V		25	484.8
PSMA26	L	V	L	A	G	G	F	F	L		20	375.1
PSMA27	V	L	A	G	G	F	F	L	L		27	400.2
PSMA441	L	L	Q	E	R	G	V	A	Y	I	0.52	919.9
PSMA663	M	M	N	D	Q	L	M	F	L		24	1360.3
PSMA711	A	L	F	D	I	E	S	K	V		26	1055.1

Table 6: PSMA HLA-A2-binding peptides

The peptides conform to the preferred anchor residues at position 2 (P2, orange) and at the C-terminal (P9 or P10, blue). The binding score is predicted by the SYFPEITHI database (http://www.uni-tuebingen.de/uni/kxi/ [385] and half-time dissociation scores predicted by BIMAS (http://www-bimas.cit.nih.gove) [386].

The HLA-A2-binding peptide sequences, except PSMA441, highly conform to the preferred residues at anchor positions resulting in predictions of high HLA-A2-binding scores by SYFPEITHI and high half-time of dissociation scores by BIMAS (Table 6). The selection of these PSMA peptides for study was based on these predictions thereby demonstrating the invaluable contribution the databases have made to this line of research.

The processing of peptides can be assessed by algorithms that predict where the proteasome is likely to cleave a protein. The proteasome possesses catalytic subunits with chymotrypsin-like (cleavage after hydrophobic residues), trypsin-like (cleavage after basic residues) and peptidyl and glutamyl-peptide-hydrolysing (cleavage after acidic residues) specificities [387]. The proteasome cleaves proteins into long peptides that undergo further N-terminal trimming in the cytosol and ER. The C-terminus of peptides leaving the proteasome already constitute the C-terminus of the final MHC-binding peptides. This tool can therefore be valuable to calculate the probability of correct peptide C-terminal

cleavage site and also epitope destroying cleavages. The two algorithms that can be used for this purpose are PAPROC (http://www.paproc.de/) and NetChop (http://www.cbs.dtu.dk/services/NetChop/).

The proteasomal cleavage sites for the human PSMA sequence predicted by these two databases can be seen in Table 7. Net Chop does predict C-terminal cleavage for all six peptides and with high probabilitites for five of the peptides. However PAPROC does not predict cleavage after residues 671 and 719, cleavages that must occur as both PSMA663 and PSMA711 peptides are processed [231], sections 3.2.10 and 3.2.11. Both databases also recognise that there are possible cleavage sites within these peptide sequences. However all predicted cleavages do not occur as proteasomes cannot cleave between the first six or the last four residues of a protein/polypeptide. Therefore if a preferred cleavage occurs the next cleavage will not be within the next six residues.

PSMA HLA-A2-Binding Peptides	PAPROC Predicted C-terminal Cleavage Probability	Net Chop Predicted C-terminal Cleavage Probability
PSMA4	Low	0.88
PSMA26	High	0.55
PSMA27	Intermediate	0.96
PSMA441	High	0.85
PSMA663	No cleavage	0.97
PSMA711	No cleavage	0.97

Table 7: Predicted C-terminal cleavage for HLA-A2-binding peptides

Probabilities for correct C-terminal cleavages for the PMSA HLA-A2-binding peptides as predicted by PAPROC and Net Chop databases.

3.1.5.2 PSMA 4 [LLHETDSAV]

T-cell repertoires specific for PSMA4 were found in PBMCs from 7/10 PCa patients (Table 4, [238]). However the single study attempting to expand PSMA4-specific $CD8^+$ T cells from four healthy donors failed to do so [230].

This peptide has been used in clinical trials vaccinating PCa patients with peptide-loaded DCs. Peptide stimulation of PBMCs *in vitro* verified the presence of PSMA4-specific T cells in the patients prior to vaccination through the secretion of IFNγ and T-cell proliferation assays [238-240, 250]. The existence of a repertoire for this peptide is therefore convincing.

Interestingly these studies were able to demonstrate the increased levels of PSMA4-specific T-cells after vaccination, indicating that the DC vaccines were able to expand PSMA4-specific cellular responses [239, 240, 250]. The clinical responses are discussed in more detail in section 3.1.2. It was found that 6/10 PCa patients had IgG reactive to this peptide in their sera [238].

Despite all this information and the use of the peptide in a number of clinical trials, there does not appear to be any published evidence that the PSMA4 peptide is processed from the full-length protein and presented by cells expressing PSMA. Therefore although there is convincing evidence that the PSMA4-specific T-cell repertoire can be expanded in PCa patients by vaccination, there is no published evidence that cellular immunity against this peptide is of biological significance. There is also no evidence that the PSMA4-specific T-cells in healthy donors or PCa patients have cytotoxic ability, as there are no reported killing assays using peptide-loaded target cells. The first trial to use this peptide was published 12 years ago and so it is clearly desirable to confirm its endogenous processing and presentation by tumour cells.

3.1.5.3 PSMA27 [VLAGGFFLL]

The *in vitro* expansion of PSMA27-specific $CD8^+$ T cells from human PBMCs and thereby the presence of a T-cell repertoire has been demonstrated in at least one from four healthy donors (Table 4, [230]). This repertoire may be retained in PCa patients as a study reported the presence of peptide-specific $CD8^+$ T cells in 2/10 PCa patients although reactivity was in fact against PSMA26-34 [238]. The PSMA26 peptide retains the preferred C-terminal leucine residue and has a P2 valine amino acid, not too different from a preferred leucine (section 3.1.5.1, Table 6). There are no other published studies on PSMA27-specific T cells in healthy donors or PCa patients.
Harada et al., failed to find any PSMA27-reactive IgG in the plasma of 10 PCa patients [238].

The cytotoxicity of the PSMA27-specific CTLs was confirmed by the lysis of peptide-loaded T2 target cells. Additionally, the PSMA27 peptide appears to be processed from the PSMA protein and presented by the LNCaP human PCa cell line. Human PSMA27-specific CTLs were able to lyse up to ~30% of the $PSMA^+$ $HLA\text{-}A2^+$ LNCaP cells after an extended 12-hour chromium release assay, but not after the conventional 4-hour assay.

However the long incubation period in the assay is unsatisfactory and leaves the result open to challenge. The LNCaP cells have very low levels of HLA-A2 expression that can be up-regulated by certain cytokine treatment. The authors in this study reported that they could not up-regulate the HLA-A2 expression with either IFNγ or TNFα and this would account for the poor lysis levels attained. Co-incubation with LNCaP cells for 40 hours induced IFNγ secretion by the PSMA27-specific T cells [230]. Although unlikely, this may have been achieved by APCs cross-presenting the tumour cells. These observations require confirmation in order for this peptide to be applied to human therapy with certainty of its biological relevance.

3.1.5.4 PSMA663 [MMNDQLMFL]

There are data supporting the existence of PSMA663-specific T cells in healthy volunteers that could be expanded *in vitro* by stimulation with peptide or PSMA-expressing DCs (Table 4, [231]). There are however no reports of whether functional T cells specific for this peptide can also be found in PCa patients. There is a report that 4/10 PCa patients did have IgG reactive to PSMA663 [238].

The evidence regarding the ability of PSMA663-specific CTLs to kill PSMA-expressing target cells is contradictory. One study reported that human PSMA663-specific CTLs could lyse LNCaP cells at moderate levels [231] whilst the other study was unable to detect any cytotoxicity against the same LNCaP target cells even after an extended ^{51}Cr release killing assay [230]. The latter was the study that demonstrated LNCaP lysis by PSMA27-specific CTLs and therefore although not ideal they did have a positive control for the assay. Lu et al., used LNCaP cells with low HLA-A2 expression that could not be upregulated [230]. On the other hand Mincheff et al., did not mention the HLA-A2 expression of their LNCaP target cells [231]. This may suggest that the MHC class I status of their cells was not as low as we and others have found.

However the study that demonstrated cytotoxicity also confirmed the processing and presentation of the peptide in an alternative manner. The PSMA663-specific CTLs expanded from human PBMCs using DCs transfected to express the extracellular domain of PSMA could kill T2 cells loaded with PSMA663 peptide [231]. The DCs were therefore able to process and present the PSMA663 peptide in order to expand the CTLs *in vitro*.

3.1.5.5 PSMA711 [ALFDIESKV]

$CD8^+$ T cells specific for the PSMA711 peptide have been found and stimulated from healthy donor PBMCs (Table 4, [230, 231]). This repertoire can be retained in patients and was expanded from the PBMCs of 7/10 PCa patients [238]. It appeared to correlate with the 4/10 PCa patients that had IgG reactive to the PSMA711 peptide [238].

This peptide has been used in a clinical trial vaccinating PCa patients with peptide-loaded DCs. Peptide stimulation of PBMCs *in vitro* verified the presence of PSMA711-specific T cells in the patients prior to vaccination through T cell proliferation assays. Interestingly the clinical study was able to demonstrate increased levels of PSMA711-specific T cells after vaccination, indicating that the DC vaccines were able to expand PSMA711-specific cellular responses in PCa patients [250]. The clinical outcomes of the trial are discussed in section 3.1.2.

These are only two studies that have examined cytotoxicity by the PSMA711-specific CTLs, but they reported opposing results. Both reported the ability to kill peptide-loaded targets thereby confirming the cytotoxicity of PSMA711-specific CTLs. However, one study was unable to lyse LNCaP cells after an extended chromium release assay [230] whilst the other showed very low levels (~15%) of LNCaP lysis after only a 4-hour incubation in a 3H-thymidine cytotoxicity test [231]. Low HLA-A2 expression levels are likely to have contributed to these contradictory results. However one of the studies confirmed the processing through the expansion of PSMA711-specific CTLs using DCs transfected to express the extracellular domain of PSMA. These CTLs were able to kill T2 cells loaded with PSMA711 peptide [231]. The DCs were therefore able to process and present the PSMA711 peptide in order to expand the CTLs.

3.1.5.6 Summary of HLA-A2-restricted PSMA Peptides and Direction of Work Presented in this Study

In summary, T cell repertoires for PSMA4, PSMA27 and PSMA711-specific $CD8^+$ T cells have been found in PBMCs from healthy volunteers and maintained in PCa patients (Table 4). PSMA663-specific T cells have been found in healthy donors but their presence in PCa patients remains unknown.

It is also important to highlight the findings that patients receiving DCs loaded with PSMA4 or PSMA711 peptides were able to expand the peptide-specific T cells as determined by *in vitro* analysis. This validates immunotherapeutic strategies specifically

targeting these epitopes. The challenge now is to expand and activate these T cells to attack PSMA-expressing tumours. Neither PSMA27 nor PSMA663 have been used in any published clinical trial and so the ability to expand the T cells specific for these peptides *in vivo* remains unknown.

The evidence discussed shows that PSMA27, PSMA663 and PSMA711 peptides are processed and presented by tumour cells that express PSMA. However, some of this evidence is open to challenge. The peptide processing evidence has been derived from human systems. Although such human systems are invaluable to assess whether T-cell repertoires exist, any T cells expanded from tolerised humans will be of low affinity. This together with the difficulty in killing LNCaP cells has contributed to the discrepancies. One of the aims in the present study is therefore to use the transgenic HHD mouse model (section 3.1.6), which offers the best option for assessing PSMA peptide processing. The HHD model looks at the ability of vaccines to induce peptide-specific $CD8^+$ T cells. The functional capacity of these T cells in killing tumour cells can then be assessed. None of these 4 PSMA peptides present in the human sequence are present in the homologous mouse sequence. Therefore mice represent a non-tolerised system and are likely to prime high affinity CTLs and provide strong evidence for the ability or inability of the human PSMA peptides to be processed and presented.

PSMA4, PSMA27, PSMA663 and PSMA711 were incorporated into the p.DOM-epitope vaccine design to assess the ability of these to prime $CD8^+$T-cell responses specific for each of the HLA-A2-binding peptides in the HHD mouse model. The reason for testing the various HLA-A2-restricted PSMA peptides is to add to the limited evidence in the literature regarding their processing, especially given that these peptides are in use in various clinical trials. The p.DOM-PSMA27 vaccine is of particular interest as it is currently being tested in a Phase I/II clinical trial coordinated by Professor C. Ottensmeier (University of Southampton, UK).

3.1.6 The HHD Transgenic Mouse Model

Rammensee et al., have provided evidence for highly conserved antigen processing machinery between mice and humans [388]. This results in agreement between peptides processed by HLA-A2-restricted human cells/individuals and that by HLA-A2 transgenic mice [389]. The transgenic mice can therefore process and generate 'human' HLA-specific 8-10mer peptides and provide models for the study of MHC class I-restricted CTL responses. In this manner, the HHD model provides relevant information for patients.

The HLA-A2.1/K^b transgenic mouse model has the homologous mouse HLA-A2 α3 domain substituted for the human sequence, resulting in improved recognition of the HLA-A2 class I molecule by the mouse CD8. However, the retention of endogenous H-2 MHC class I expression leads to competition that begins at the thymic educational stage and results in a reduced size of the HLA-A2-educated repertoire [390].

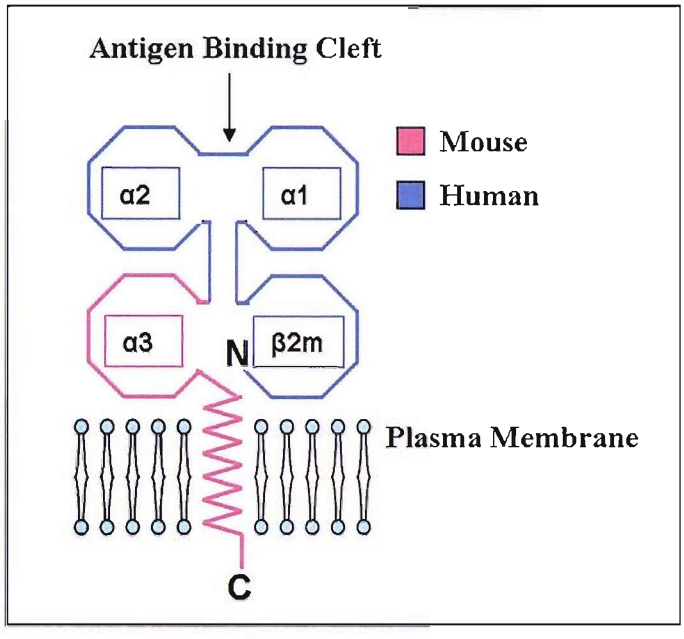


Figure 16: The HHD MHC Class I Molecule

The HHD molecule is a hybrid human HLA-A2 (α1 and α2 domains) and mouse H-2D^b (α3 domain) fused to the human β2-microglobulin (β2m). It therefore retains HLA-A2 peptide specificity but allows mouse CD8 interaction with the mouse α3 domain.

A second-generation HLA transgenic HHD mouse model has overcome this problem. The HHD construct used to generate the animals encodes the HLA-A2.1 α1-α2 and the H-2D^b α3 domains fused to the human β2-m (Figure 16). The HHD transgenic mice have been created on a H-2$D^{b-/-}$ $\beta2m^{-/-}$ double knock out background that is almost devoid of H-2 class I molecules. This supports predominantly positive thymic selection and peripheral activation of HLA-A2.1-restricted mouse CTLs. The creators demonstrated that the HHD mice generated only an HLA-A2-restricted CTL response against influenza A virus [391]. HHD mice have a 5-10-fold reduced number of peripheral $CD8^+$ T cells compared to wild type and the first generation HLA-A2.1/K^b transgenic mice. This does not appear to be as a result of the ~5-fold lower HLA-A2 cell surface expression in HHD mice as a second line of HHD transgenics with 5-fold higher expression of the HHD molecule had the same low peripheral $CD8^+$ T-cell numbers [390]. In spite of this, the HHD mice could generate an influenza virus-specific CTL response that was ~20-30 fold higher than that of the HLA-A2.1/K^b mice [390].

The HHD mouse model generally has an improved capacity to develop HLA-A2.1-restricted CTL responses specific for a range of tumour-derived epitopes compared to the HLA-A2/K^b transgenic mice [392]. These observations highlight the value of the model for testing HLA-A2.1-restricted responses to human antigens.

3.2 Aims

1. To test the efficacy of the p.DOM-epitope vaccine design for the activation of PSMA-specific T cells in the HHD Tg mouse model. The epitopes are the four described HLA-A2-binding peptides PSMA4, PSMA27, PSMA663 and PSMA711.

2. The cytotoxic capacity (functionality) of the CTLs primed by vaccination will be assessed by chromium release assays. This work will additionally reveal the processing and presentation status of the PSMA4 peptide for the first time and convincingly confirm that of the other three PSMA peptides thereby providing substantiating evidence to validate them as immunotherapeutic targets.

3. A third aim is to establish a model to demonstrate *in vivo* killing of human-PSMA expressing tumour cells. This will be used to reveal whether the CTLs primed by the p.DOM-PSMA-epitope vaccines can migrate to the tumour site and display cytotoxic ability *in vivo*.

4. Finally, the ability of full-length PSMA vaccines to prime epitope-specific $CD8^+$ T-cell responses in the HHD Tg mouse model will be explored. These vaccines may potentially prime $CD8^+$ T cells specific for multiple PSMA epitopes simultaneously. If so the full-length vaccines could be a valuable incorporation into the clinic particularly as it would be applicable to patients of all haplotypes.

3.3 Results

3.3.1 DNA Fusion Gene Vaccines Encoding PSMA Epitopes

The first question was the ability of the p.DOM-epitope vaccine design to elicit $CD8^+$ T-cell responses against the PSMA HLA-A2 binding peptides, PSMA4, PSMA27, PSMA663 and PSMA711.

The vaccine constructs incorporate the first domain of FrC, DOM, fused to PSMA4, PSMA27, PSMA663 or PSMA711 epitope sequences to generate the p.DOM-PSMA4, p.DOM-PSMA27, p.DOM-PSMA663 and p.DOM-PSMA711 vaccines respectively (Figure 17). The DOM domain contains p30, a universal $CD4^+$ T cell epitope, encoded within the sequence. The constructs include a leader sequence to ensure targeting of the translated product to the ER where the effectively processed epitopes can be loaded onto respective MHC class I and II molecules.

The same experimental process was used for the evaluation of responses elicited by a single priming immunisation with each of the p.DOM-PSMA-epitope vaccines (p.DOM-PSMA4, p.DOM-PSMA27, p.DOM-PSMA663 and p.DOM-PSMA711). Male HHD mice were vaccinated with one of the p.DOM-PSMA-epitope or the control p.DOM DNA vaccine on day 0 and then sacrificed on Day 14. Spleens were individually processed and analysed *ex vivo* in a peptide-specific IFNγ secretion capture ELISPOT assay. Splenocytes were stimulated for 24 hours with varying concentrations of the respective PSMA peptide or 1μM (previously optimised) p30 peptide. The responses primed by each of these vaccines are shown and described below.

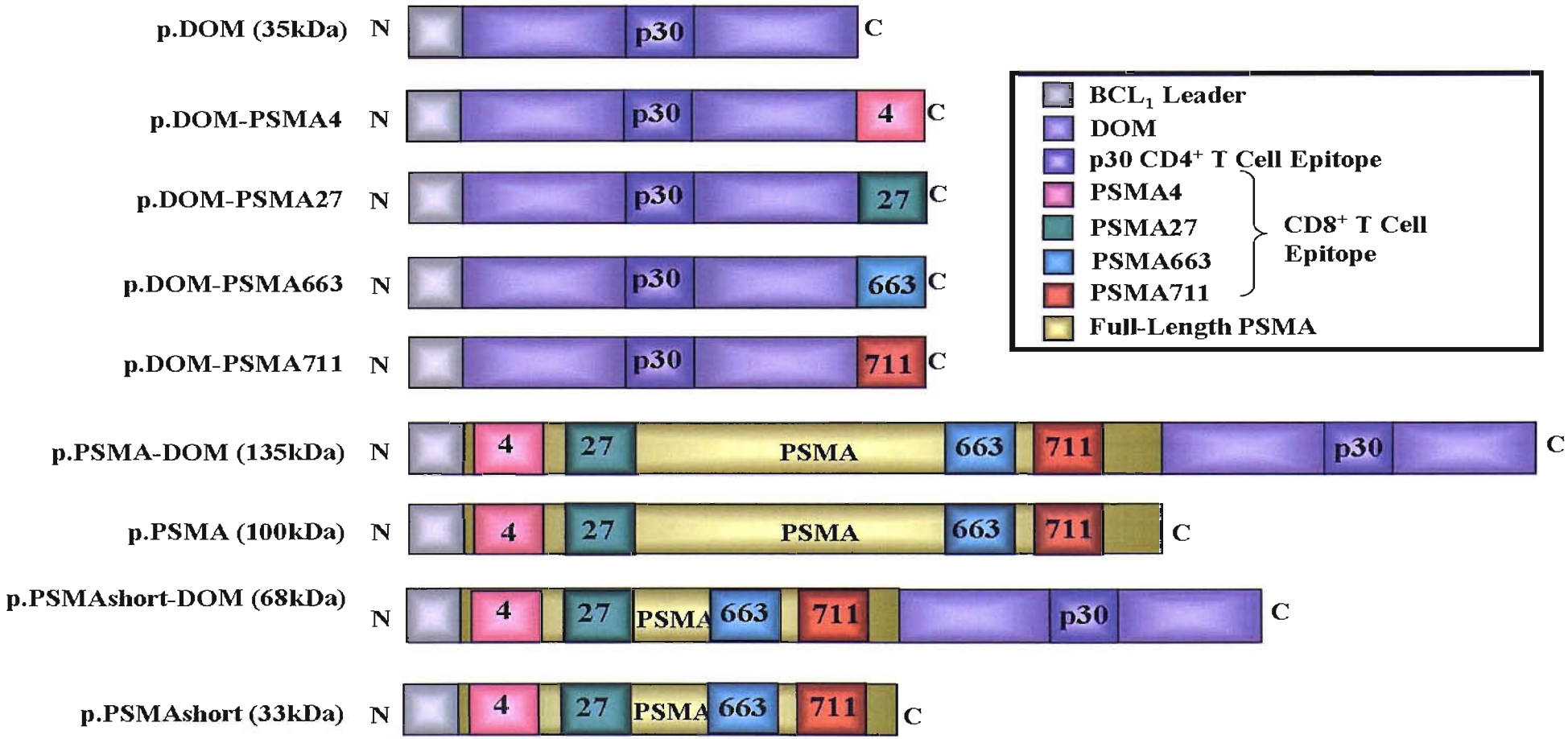


Figure 17: Schematic representation of the human PSMA vaccines investigated in chapter 3

The vaccine open reading frames are represented in this figure. All vaccine constructs contain an N-terminal BCL_1 leader sequence and are inserted into pcDNA3.1 plasmid. Each PSMA HLA-A2-binding peptide and the DOM p30 $CD4^+$ T cell epitopes have been assigned a colour as seen in the key box. The result graphs throughout the relevant sections maintain this colour scheme.

3.3.2 DNA Vaccine Primes Peptide-Specific $CD8^+$ T Cells Against the PSMA27 Epitope

The p.DOM-PSMA27 vaccine was assessed for the ability to elicit epitope-specific $CD8^+$ T-cell responses after a single priming immunisation.

Splenocytes from all but one p.DOM-PSMA27 vaccinated mice responded to the p30 MHC-class II-binding peptide, an indication of vaccine performance. Calculations of population means were carried out only with the mice for which vaccination had been successful. The control p.DOM vaccinated mice secreted IFNγ in response to p30 peptide but not to the PSMA27 peptide thereby demonstrating that it is the PSMA27 epitope encoded within the vaccine that is responsible for the induction of the peptide-specific response. The range of responses reflects individual variability in the results.

The p.DOM-PSMA27 vaccine effectively primed epitope-specific $CD8^+$ T cells in all mice that displayed good vaccine performance (from various separately repeated experiments) as they displayed some degree of response to *ex vivo* PSMA27 peptide stimulation (Figure 18). The degree of stimulation was variable and declined with decreasing peptide concentrations. Responses reached 423 IFNγ spots per million cells when splenocytes were stimulated with 0.1μM PSMA27 peptide (mean 182.4 (± 35.9)) and could be detected with peptide concentrations as low as 10nM (mean response 65.1 (± 14.4)). However, the mean response when stimulated with 1nM PSMA27 peptide (mean 14.6 (±7.2)) was not statistically significant, at least partly due to the few number of mice representing this group. Stimulation with 1μM peptide concentration appears to be approaching the maximum activation capacity of the PSMA27-specific $CD8^+$ T cells as increasing the peptide concentration from 0.1μM (mean 182.4 (± 35.9)) to 1μM (mean 207.4 (± 36.3)) did not greatly enhance responses and in fact 4/14 mice exhibited their maximal response when stimulated with 0.1μM PSMA27 peptide.

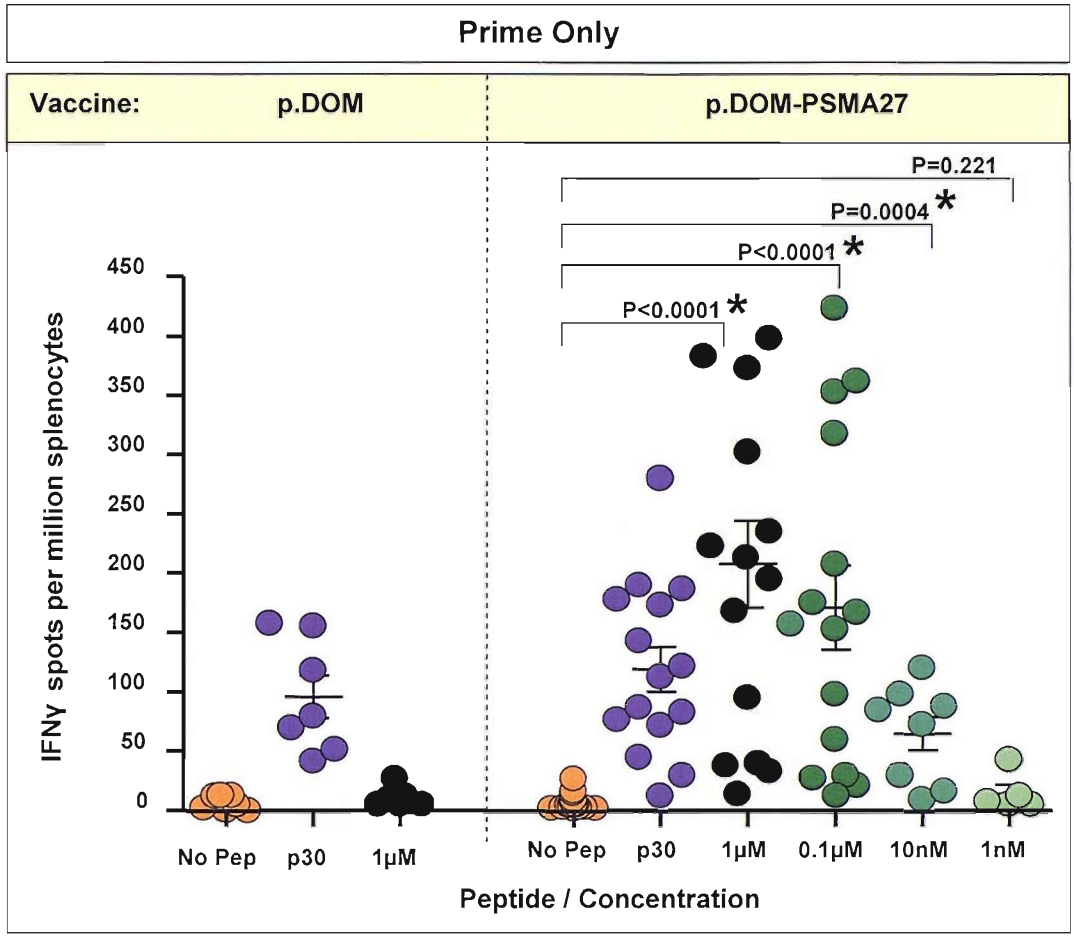


Figure 18: IFNγ responses of *ex vivo* PSMA27-specific CD8$^+$ T cells following peptide stimulation

Male HHD mice were immunised with p.DOM-PSMA27 or p.DOM control vaccines intramuscularly on day 0 (50μg DNA) and sacrificed on day 14. Splenocytes were stimulated in *ex vivo* IFNγ ELISPOT without any peptide to measure background levels (orange), or with p30 (purple) or PSMA27 peptide (green) at different concentrations. Responses from individual spleens are plotted (mean of triplicates) and the mean and SEM for the responses against each peptide/concentration group are shown. The data are pooled from 3 different experiments. (*) indicates were values were statistically significant as assessed by the Mann Whitney test and defined by $p<0.05$. Responses are defined as positive if they are more than twice the "no peptide" background.

3.3.3 DNA Vaccine Primes Peptide-Specific CD8$^+$ T Cells Against the PSMA663 Epitope

The ability of a second vaccine p.DOM-PSMA663, to prime epitope-specific CD8$^+$ T-cell responses was assessed.

The p.DOM-PSMA663 vaccine primed PSMA663-specific CD8$^+$ T-cell responses in all vaccinated mice as they all had some extent of IFNγ response to the PSMA663 peptide (Figure 19). Although the mean of responses were lower than those observed with for the p.DOM-PSMA27 vaccine, the same avidity response pattern was observed. That is the

increase in PSMA663-specific $CD8^+$ T cells secreting IFNγ appeared to reach a plateau when stimulated with 1μM PSMA663 peptide (mean 100 (± 16.8)), an observation supported by 3/8 mice reaching a maximal response when splenocytes where stimulated with 0.1μM PSMA663 peptide (mean 87 (±11.8)). Responses reached 160 spots per million splenocytes when stimulated with 1μM PSMA663 peptide, declined with decreasing peptide concentrations but could be detected with as little as 1nM PSMA663 peptide (mean 17 (±3.7)).

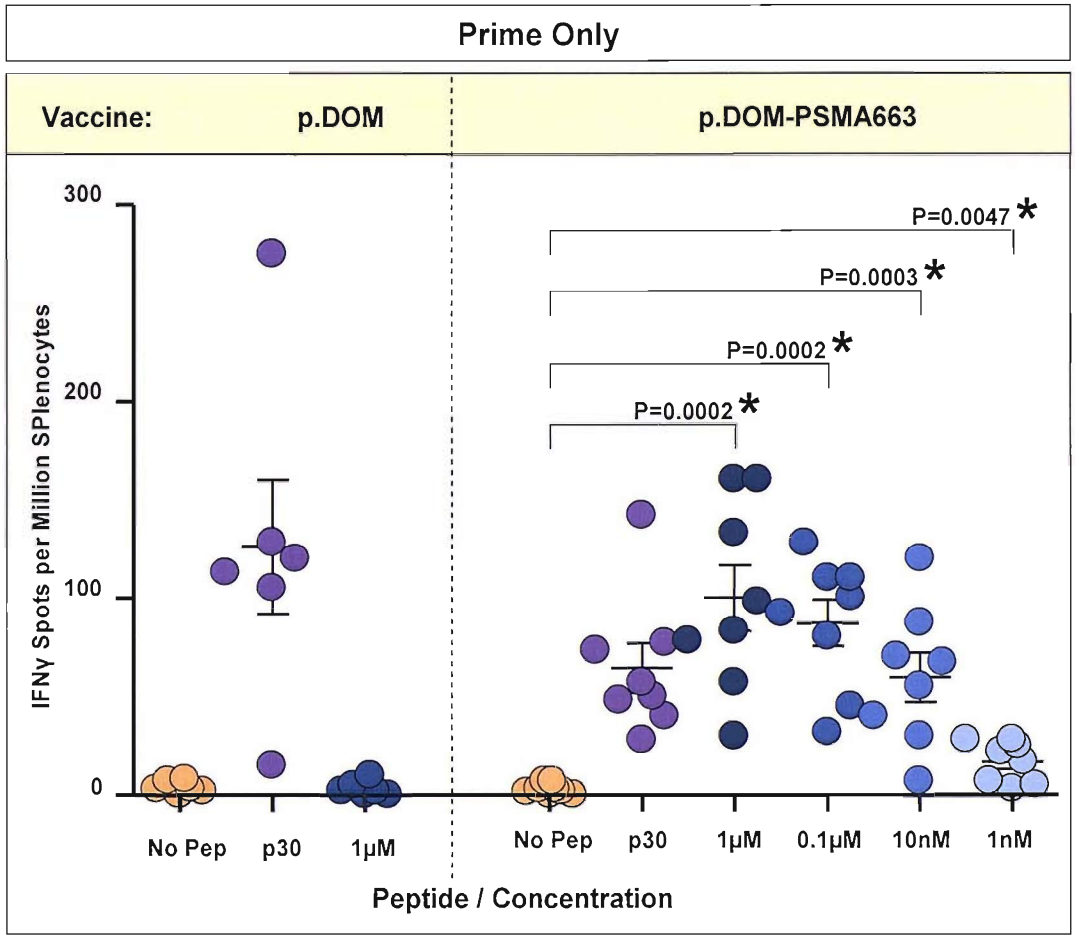


Figure 19: IFNγ responses of *ex vivo* PSMA663-specific $CD8^+$ T cells following peptide stimulation

Male HHD mice were immunised with p.DOM-PSMA663 or p.DOM control vaccines intramuscularly on day 0 (50μg DNA) and sacrificed on day 14. Splenocytes were stimulated in *ex vivo* IFNγ ELISPOT without peptide to measure background (orange), or with p30 (purple) or PSMA663 peptide (blue) at different concentrations. Responses from individual spleens are plotted (mean of triplicates) and the mean and SEM for the responses against each peptide/concentration group are shown. The data are pooled from 2 different experiments. (*) indicates were values were statistically significant as assessed by the Mann Whitney test and defined by $p<0.05$.

Splenocytes from p.DOM-PSMA663 vaccinated mice responded to the p30 MHC class II-binding peptide, which indicates that vaccination was successful. Splenocytes from mice

vaccinated with the control p.DOM vaccine secreted IFNγ in response to p30 peptide but not to the PSMA663 peptide.

3.3.4 DNA Vaccine Primes Peptide-Specific $CD8^+$ T cells Against the PSMA711 Epitope

The p.DOM-PSMA711 vaccine was assessed for ability to elicit epitope-specific $CD8^+$ T-cell responses after a single priming immunisation in the HHD mice. The p.DOM-PSMA711 vaccine effectively primed epitope-specific $CD8^+$ T cells in all vaccinated mice. These could be stimulated with a range of peptide concentrations, even at 1nM PSMA711 peptide (mean 35 (± 15) (Figure 20). 1000 IFNγ spots per million splenocytes was the highest response in a single mouse and this was achieved by stimulation with 1μM PSMA711 peptide (mean 536 (± 91.5)).

The avidity of the PSMA711-specific $CD8^+$ T cells primed was similar to those described for the other two vaccines. The same response pattern was observed where the concentration-dependent increase in the number of PSMA711-specific $CD8^+$ T cells secreting IFNγ reached a plateau when stimulated with 1μM PSMA711 peptide (mean 536 (± 91.5)). That for 0.1μM PSMA711 peptide was not much lower (mean 423 (± 74.5)) with one mouse still yielding a maximal response at this peptide concentration.

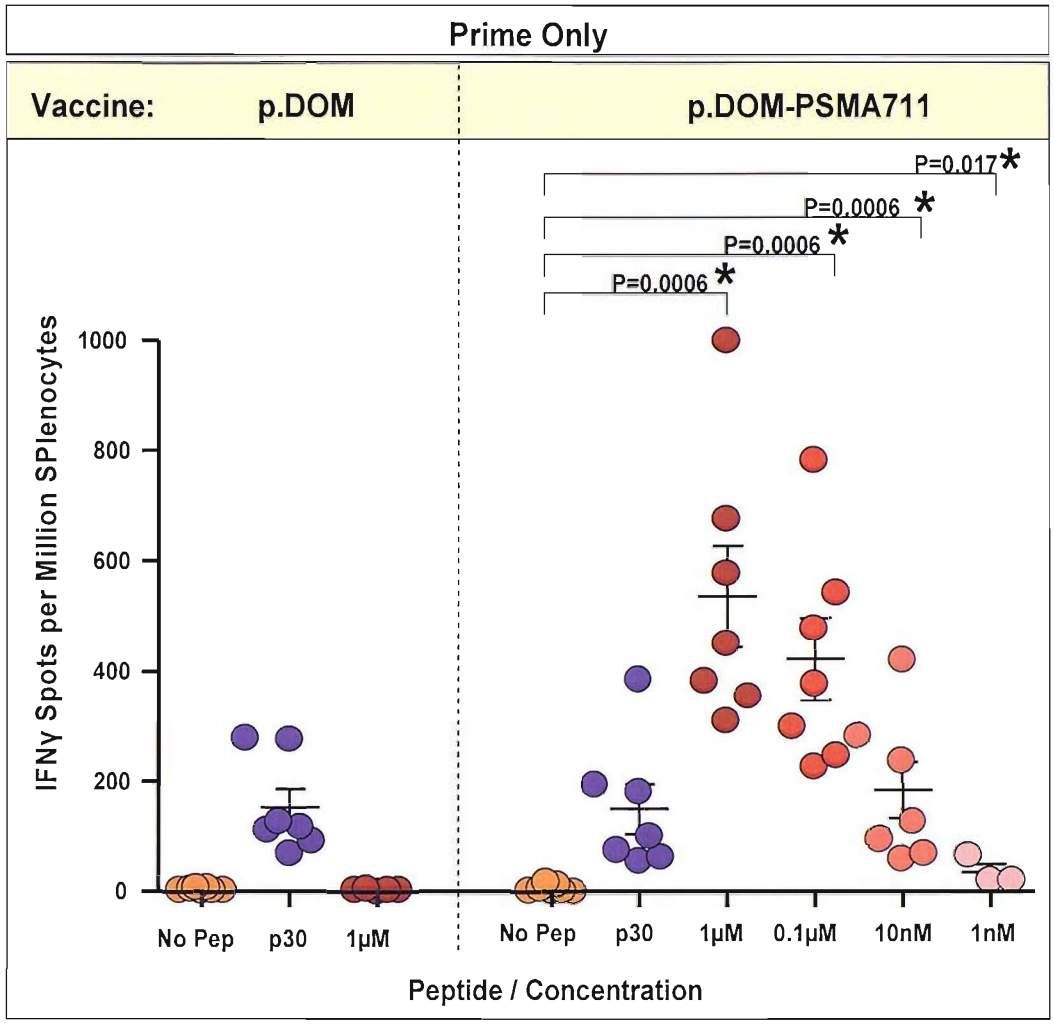


Figure 20: IFNγ responses of *ex vivo* PSMA711-specific CD8$^+$ T cells following peptide stimulation

Male HHD mice were immunised with p.DOM-PSMA711 or p.DOM control vaccines intramuscularly on day 0 (50μg DNA) and sacrificed on day 14. Splenocytes were stimulated in an *ex vivo* IFNγ ELISPOT without any peptide to measure background levels (orange), or with p30 (purple) or PSMA711 peptide (red) at different concentrations. Responses from individual spleens are plotted (mean of triplicates) and the mean and SEM for the responses against each peptide/concentration group are shown. The data are pooled from 2 different experiments. (*) indicates were values were statistically significant as assessed by the Mann Whitney test and defined by $p<0.05$.

All p.DOM-PSMA711 and p.DOM-vaccinated mice responded to the p30 MHC class II-binding DOM peptide, an indication of vaccine performance. The PSMA711 peptide specificity of the response primed by the p.DOM-PSMA711 vaccine is demonstrated by the lack of stimulation (IFNγ secretion) in response to the PSMA711 peptide by splenocytes from p.DOM control vaccinated mice.

3.3.5 DNA Vaccine Primes Peptide-Specific CD8$^+$ T cells Against the PSMA4 Epitope

The epitope-specific CD8$^+$ T-cell response primed by a single p.DOM-PSMA4 immunisation of HHD mice was assessed in a preliminary experiment. The vaccine primed PSMA4-specific CD8$^+$ T cells in 2 from 4 mice (Figure 21), a much lower proportion of responding mice compared to that observed with the other p.DOM-PSMA-epitope vaccines. From this limited data the responses reached 330 IFNγ spots per million splenocytes when stimulated with 1μM PSMA4 peptide. Responsiveness to PSMA4 peptide also declined with decreasing peptide concentrations. It would be incorrect to take these limited data as the average for a population. Nonetheless these results do indicate the ability of the vaccine to successfully prime PSMA4-specific CD8$^+$ T cell responses.

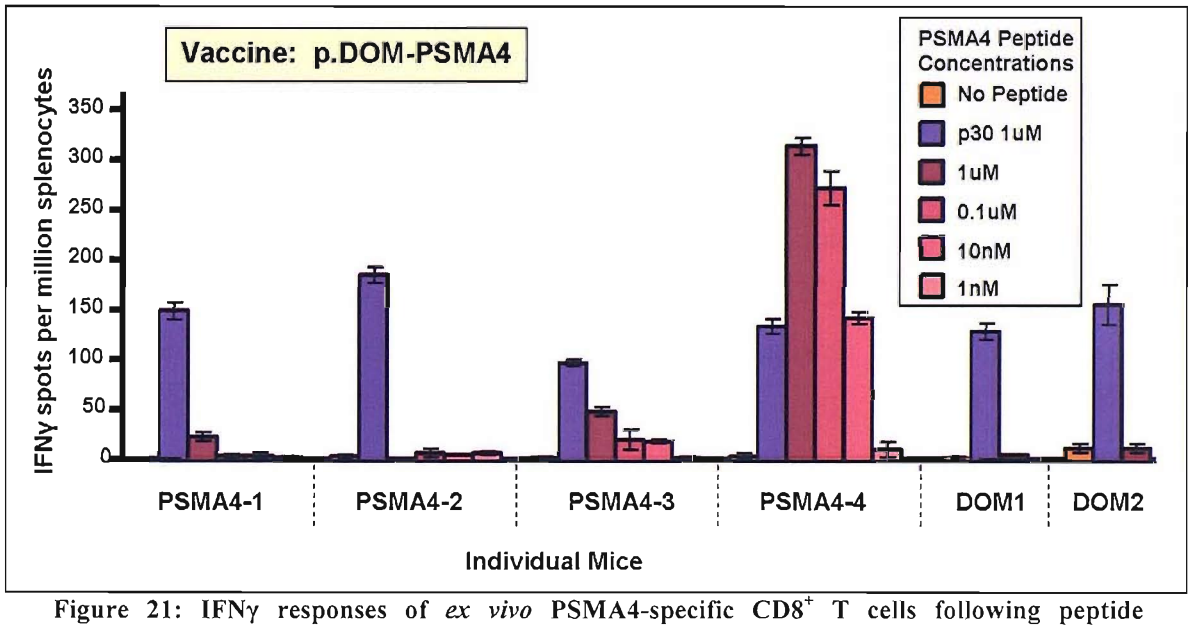


Figure 21: IFNγ responses of *ex vivo* PSMA4-specific CD8$^+$ T cells following peptide stimulation

Male HHD mice were immunised with p.DOM-PSMA4 or p.DOM control vaccines intramuscularly on day 0 (50μg DNA) and sacrificed on day 14. Splenocytes were stimulated in *ex vivo* IFNγ ELISPOT without any peptide to measure background levels (orange), or with p30 (purple) or PSMA4 peptide (pink) at different concentrations. Responses from individual spleens are plotted and the error bars demonstrate the SEM of the triplicates.

3.3.6 The Avidity of Peptide-Specific CD8$^+$ T cells Primed by the p.DOM-PSMA-epitope DNA Vaccines

To summarise the results described thus far (Table 8), all four p.DOM-PSMA-epitope vaccines are able to prime epitope-specific CD8$^+$ T-cell responses. The responses were generally good for p.DOM-PSMA27, p.DOM-PSMA663 and p.DOM-PSMA711 with all

mice for which vaccination had been successful priming $CD8^+$ T cells specific for all three HLA-A2-binding peptides. In contrast, the p.DOM-PSMA4 experiment was only able to prime responses in half of the small number of mice tested.

Vaccine	Responding Mice	Mean Response (1μM Peptide)	Response Range	Mean p30 Response
p.DOM-PSMA4	2/4	179.5	23-313	140.8
P.DOM-PSMA27	13/13	207.4	38-398	127.1
p.DOM-PSMA663	8/8	99.9	30-160	164.7
p.DOM-PSMA711	7/7	536.1	310-1000	150.1

Table 8: Summary of *ex vivo* IFNγ ELISPOT responses for the four p.DOM-epitope human PSMA vaccines

Only those mice for which vaccination was successful as defined by the presence of p30-specific $CD4^+$ T-cell responses, were used for this summary analysis. A positive response was defined as one that was more than twice the "no peptide" background. The mean responses and response range to 1μM of each PSMA4, PSMA27, PSMA663 and PSMA711 peptides are shown.

The second point to analyse is the relative avidity of the responses primed by each of these four vaccines. For each individual mouse in each vaccination group, the highest response for each individual mouse was designated as the 100% response and the responses to the other peptide concentrations expressed as the percentage of the maximal response. The mean for each population at each peptide concentration has been plotted for comparison (Figure 22).

For all four vaccines the avidities of the responding $CD8^+$ T cells were similar in animals receiving only priming injections (Figure 22). The average responsivenss peaked (maximal) when stimulation in the ELISPOTs was carried out with 1μM of the respective peptides. Equally, they appeared to almost lose all sensitivity at 1nM peptide concentration. The 50% of maximal responses for all four vaccine priming-only immunisations is also very similar with approximately half of the epitope-specific $CD8^+$ T cells displaying responsiveness to 10nM peptide concentration. It is important to remember that the T-cell avidity will be influenced by the affinity with which the peptides bind to the MHC. The difference between responses primed by these vaccines appears to only be in magnitude (i.e. quantitative but not qualitative). The p.DOM-PSMA711 vaccine can prime approximately 5-fold more T cells than the p.DOM-PSMA663 and 2.8-fold more T cells than the p.DOM-PSMA27 vaccines.

The data for the prime and EP boost with the p.DOM-PSMA663 vaccines will be discussed in a later section where the ELISPOT data is presented (section 3.3.20, Figure 40).

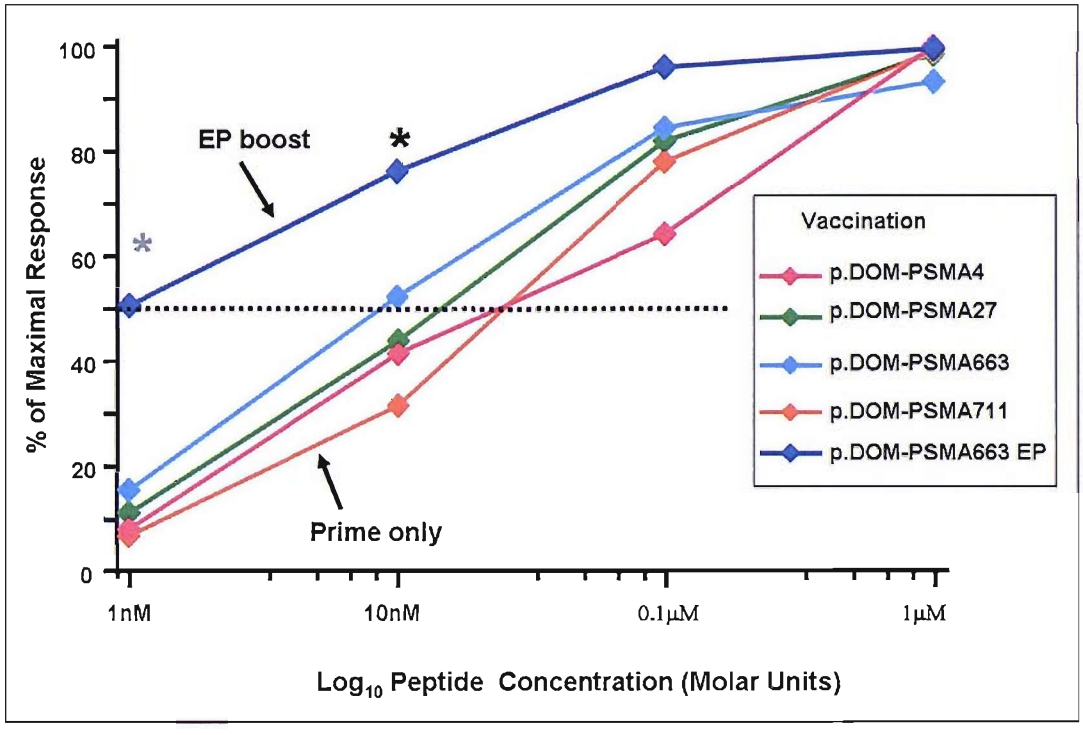


Figure 22: Avidity of CD8$^+$ T cell responses primed by the four p.DOM-PSMA vaccines

Avidity of epitope-specific CD8$^+$ T cell ELISPOT responses elicited by a single priming immunisation with p.DOM-PSMA4, p.DOM-PSMA27, p.DOM-PSMA663 or p.DOM-PSMA711 vaccines. The avidity of T cells generated by a prime + electroporation (EP) boost administration protocol of the p.DOM-PSMA663 vaccine is also shown. The maximal (highest) response for each mouse was assigned 100% and the responses to the other peptide concentrations expressed as a percentage of the maximal response. Average response values for each peptide concentration are plotted. (*) indicates a significant difference between the p.DOM-PSMA663 prime only with prime + EP boost vaccination regimen as assessed by the Mann Whitney test (p=0.01). (*) indicates where there were only two values for p.DOM-PSMA663 EP 1nM (cannot calculate statistics on small sample number) but the difference between prime only and prime + EP boost would be statistically significant (p=0.02) if the average of the two values were representative for three mice (n=3).

3.3.7 DNA Vaccines Encoding Full-Length Human PSMA are Poor at Priming Epitope-Specific CD8$^+$ T Cells

The aim was to explore the ability of DNA vaccines encoding the full-length human PSMA antigen to prime epitope-specific CD8$^+$ T-cell responses and compare them with the level of T cells primed by the four p.DOM-PSMA-epitope vaccines. The main reason for this work was to assess the efficacy of a full-length vaccine which is more applicable in

the clinic for patients of all MHC haplotypes whereas the p.DOM-epitope vaccines thus far described are only therapeutically relevant to the HLA-A2 population.

Two full-length vaccine constructs have been created, p.PSMA and p.PSMA-DOM. As the names suggest, p.PSMA encodes the full-length sequence alone and p.PSMA-DOM additionally has the DOM sequence fused to the C-terminus to provide T-cell help. Schematic representations can be seen in Figure 17.

The p.PSMA and p.PSMA-DOM vaccines were assessed for the ability to prime PSMA4, PSMA27, PSMA663 and PSMA711-specific $CD8^+$ T cells after a single priming immunisation. Male HHD mice were vaccinated with p.PSMA or p.PSMA-DOM DNA vaccines and sacrificed on Day 14. The splenocytes were stimulated for 24 hours in an IFNγ secretion ELISPOT with 10μM or 1μM concentrations of each of the 4 PSMA peptides (data for 1μM not shown) or 1μM p30 peptide. The responses by each mouse were individually assessed (Figure 23).

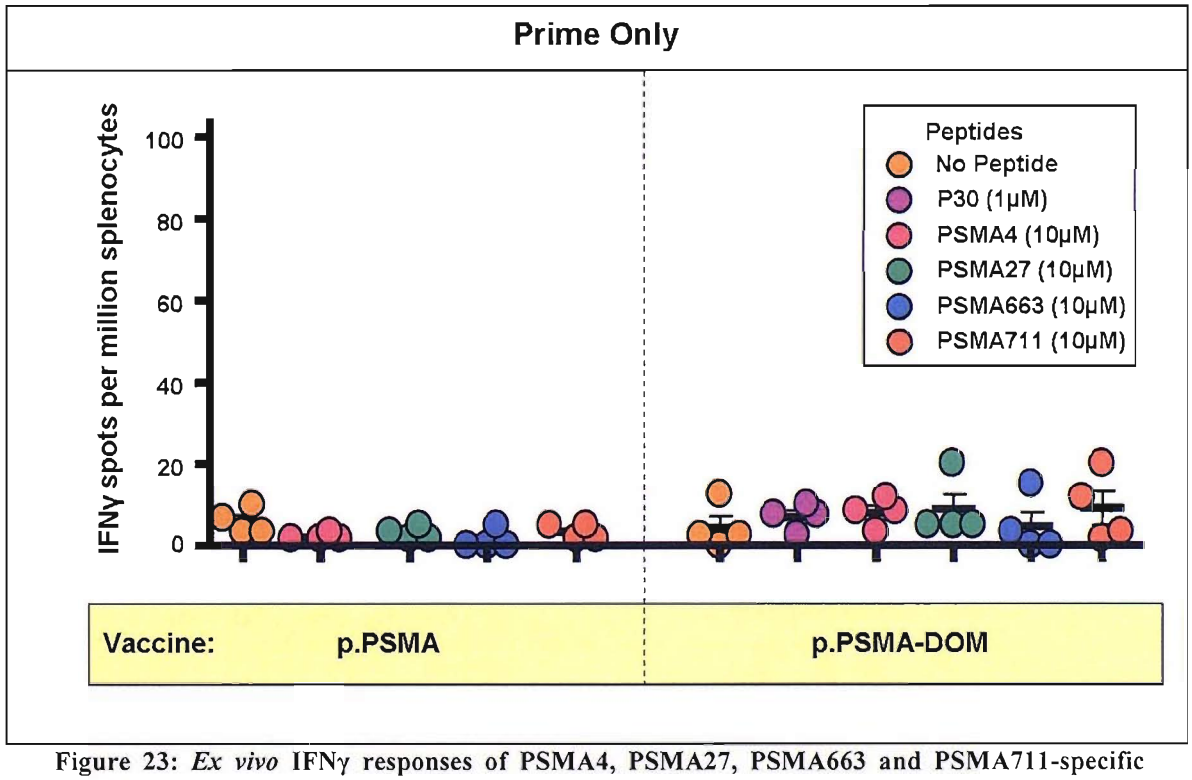


Figure 23: *Ex vivo* IFNγ responses of PSMA4, PSMA27, PSMA663 and PSMA711-specific $CD8^+$ T Cells primed by full-length PSMA vaccines

Male HHD mice were immunised with p.PSMA or p.PSMA-DOM vaccines intramuscularly on day 0 (50μg DNA) and sacrificed on day 14. Splenocytes were stimulated in an *ex vivo* IFNγ ELISPOT without any peptide to measure background levels (orange), or with p30 (purple, at 1μM), PSMA4 (pink), PSMA27 (green), PSMA663 (blue), or PSMA711 (red) peptides at a 10μM concentration. Responses from individual spleens are plotted (mean of triplicates) and the means and SEM for responses against each peptide/concentration group are shown. This data is from one small experiment with only four mice per vaccine group.

The responses primed by both these vaccines to all peptides, including p30, were statistically insignificant. Only four mice were assessed with priming vaccinations as the responses were so low that this immunisation protocol alone was not worth pursuing.

Splenocytes from mice immunised with the p.PSMA-DOM vaccine showed an insignificant response of <5 spots per million splenocytes (after deduction of the no peptide background) when assessed for p30-specific $CD4^+$ T-cell responses. In contrast the p.DOM-epitope vaccines generate p30 T-cell responses that typically range between 40-200 spots per million splenocytes (Figures 18-21). The p30 responses indicate vaccine performance and therefore this marked difference may suggest inefficient *in vivo* expression of the large p.PSMA-DOM construct (~135kDa). To attempt to assess the expression levels of each of these constructs, they were used to transiently transfect HEK 293F cells. The surface levels of the PSMA antigen on the transfected cells were analysed by flow cytometry with anti-human PSMA-FITC conjugated antibody. The p.PSMA construct could readily transfect a moderate proportion of HEK 293-F cells, some which stained with high intensity for the antigen (Figure 24A). Therefore it would be expected that there should be a certain amount of protein available for cross-priming by APCs.

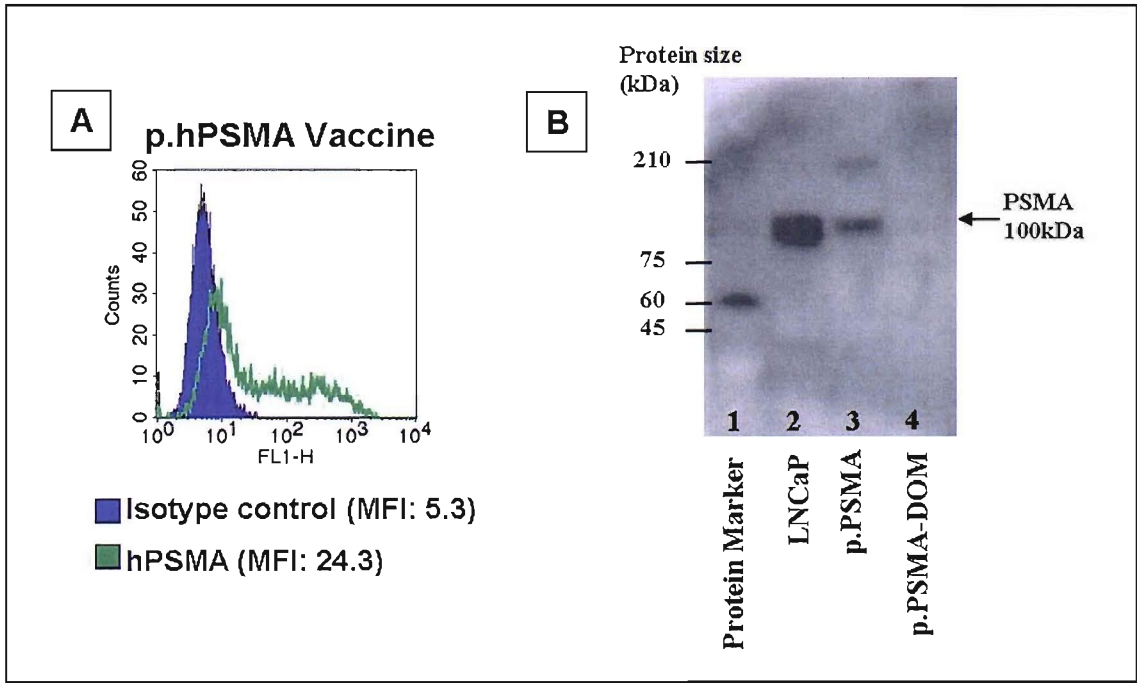


Figure 24: Expression of surface human PSMA by cells transfected with p.PSMA

(A) Transfection of $1x10^7$ HEK293-F cells with 10µl 293fectin™ and 10µg of p.PSMA plasmid, in a 10ml culture volume. The cells were analysed for surface human PSMA surface expression (anti-human PSMA-FITC conjugated antibody or isotype control) by flow cytometry 24 hours post-transfection. (B) COS-7 cells were transfected with 2µg of p.PSMA or p.PSMA-DOM plasmids. Cell lysates were prepared ($5x10^6$ cells/ml) 24 hours post-transfection and a Western blot with the anti-PSMA 3E2 antibody carried out using LNCaP cell line lysate as a positive control.

In contrast, the antibody was unable to stain cells transfected with p.PSMA-DOM, possibly as a result of the fusion interfering with the antibody epitope. COS-7 cells were also transfected with both vaccine constructs and cell lysates were prepared after 24 hours to assess the level of cell-associated PSMA protein. The western blot anti-PSMA 3E2 antibody recognises a denatured epitope so the fusion with the DOM should not interfere with recognition of the PSMA protein. However, the western blot only detected PSMA protein in the lysates of cells transfected with p.PSMA but not with p.PSMA-DOM (Figure 24B). This would suggest that the fusion with the DOM does not allow the expressed protein to remain inside the cell or to localise to the plasma membrane. It is unlikely that the PSMA-DOM protein is secreted due to the inclusion of the PSMA TM domain. Instead it is probably misfolded and therefore retained within the ER and targeted for degradation rather than exportation.

The inability of the p.PSMA and p.PSMA-DOM vaccines to prime $CD8^+$ T-cell responses (specific for PSMA4, PSMA27, PSMA663 or PSMA711) are in sharp contrast to the high peptide-specific $CD8^+$ T-cell responses achieved by a single priming immunisation with the respective p.DOM-PSMA4, p.DOM-PSMA27, p.DOM-PSMA663 and p.DOM-PSMA711 vaccines (Figures 18-21).

3.3.8 Creation of a Target Tumour Cell Endogenously Expressing Human PSMA and HHD

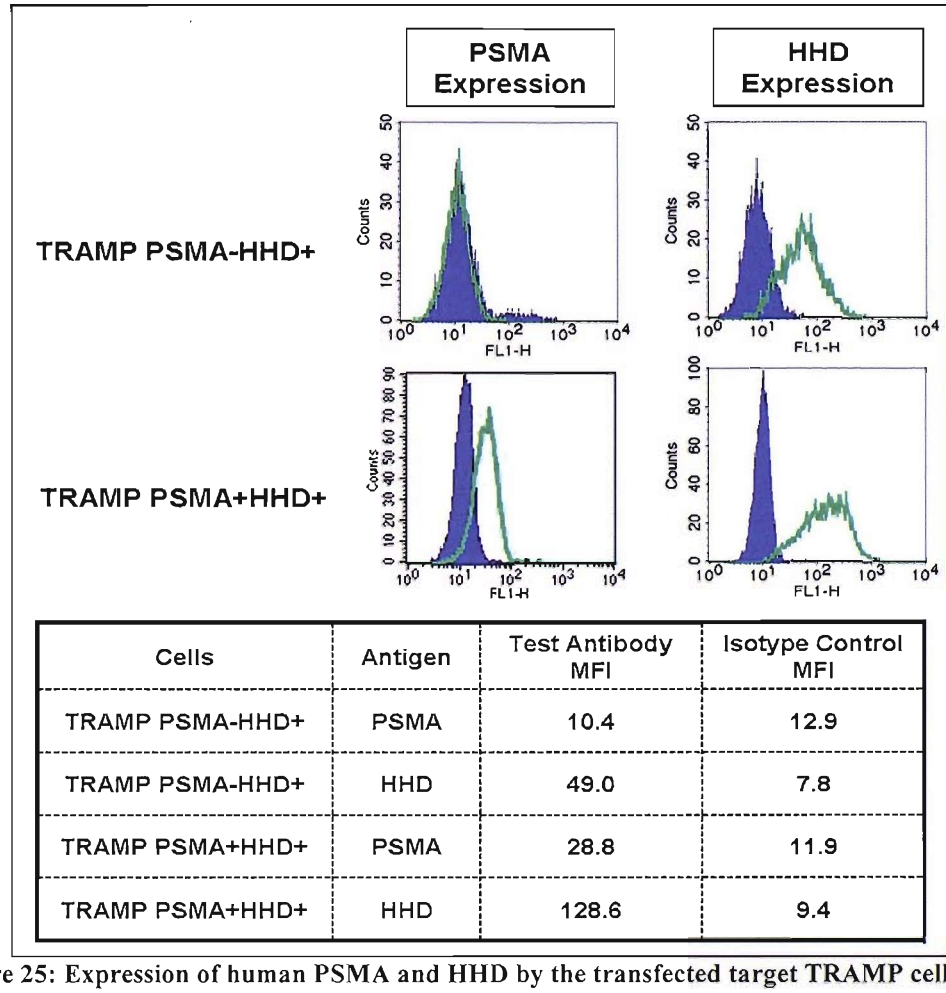
To demonstrate the therapeutic potential of the vaccines, it was necessary to demonstrate that all four of the PSMA peptides (PSMA4, PSMA27, PSMA663 and PSMA711) are processed and presented by cells expressing PSMA endogenously. The goals were therefore firstly to demonstrate the functionality of the peptide-specific CTLs primed by vaccination by assessing if they could display cytotoxic capacity against cells loaded with their respective peptide. Secondly, their ability to specifically lyse target cells expressing endogenous PSMA would reveal the status and efficiency of peptide processing and presentation in association with the chimeric HHD by tumour cells. A cell line expressing both human PSMA and the chimeric HHD MHC class I was therefore required.

The initial plan was to transfect RMA-HHD cells with a human PSMA encoding construct as this would only require a single transfection. Various transfection methods including electroporation, nucleofection system (Amaxa, Cologne, Germany), and lipid-based reagents yielded modest GFP transfection efficiencies but were unsuccessful for the human

PSMA transfections. Ecotropic and amphotropic retroviruses expressing GFP were unable to transduce any RMA-HHD cells to express GFP. RMA cells are thought to be naturally infected with retrovirus thereby requiring the use of amphotropic retrovirus capable of infecting murine and human cells. Despite reports that it is feasible to infect RMA cells with retrovirus, retroviral transduction of the RMA-HHD cells proved fruitless in our hands. However, the TRAMP-C1 mouse prostate cancer cell line can be efficiently transduced by ecotropic retrovirus expressing GFP with >70% efficiency (data not shown). It was therefore decided to endeavour to transduce TRAMP-C1 cells with both human PSMA and HHD-expressing retroviruses.

Two days post-transduction only ~10% of the bulk human PSMA transduced cells (in MSCV-puromycin vector) stained positive for surface PSMA. The surface expression became negligible 7 days post-transduction although the cells continued to grow in puromycin antibiotic selection. RT-PCR analysis indicated the presence of human PSMA mRNA but a western blot on cell lysates confirmed the absence of PSMA protein (data not shown). To overcome this, two days after PSMA transduction, TRAMP-C1 cells were labelled with anti-PSMA-FITC conjugated antibody and single-cell sorted. Clonal cell populations stably expressing PSMA (TRAMP PSMA+HHD-) were grown.

In a second transduction the TRAMP PSMA+ and TRAMP-C1 (non-transfected) cells were infected with an HHD-expressing ecotropic retrovirus. Again, the transduced cells expressing the HHD were labelled with anti-HLA-A2-FITC conjugated antibody and single cell sorted. This process created clones of TRAMP-C1 cells expressing the HHD alone (TRAMP PSMA-HHD+) or human PSMA and HHD (TRAMP PSMA+HHD+). The levels of PSMA and/or HHD expression have been confirmed by flow cytometry and western blot (Figures 25 and 38). As a consequence of order of transduction, both TRAMP PSMA+HHD- and TRAMP PSMA+HHD+ cells express equal levels of PSMA. However, TRAMP PSMA-HHD+ and TRAMP PSMA+HHD+ cell clones express different levels of HHD.



Cells	Antigen	Test Antibody MFI	Isotype Control MFI
TRAMP PSMA-HHD+	PSMA	10.4	12.9
TRAMP PSMA-HHD+	HHD	49.0	7.8
TRAMP PSMA+HHD+	PSMA	28.8	11.9
TRAMP PSMA+HHD+	HHD	128.6	9.4

Figure 25: Expression of human PSMA and HHD by the transfected target TRAMP cells

The TRAMP-C1 cell line was retrovirally transduced once to express human PSMA (TRAMP PSMA+HHD-) or HHD (TRAMP PSMA-HHD+). The cells already expressing human PSMA were transduced a second time to additionally express HHD (TRAMP PSMA+HHD+). The expression levels of these transfectants have been analysed by flow cytometry with an anti-hPSMA-FITC and anti-HLA-A2-FITC conjugated antibodies. All the transfected cell populations are grown from single-cell clones. MFI values for the clonal cell populations are shown in the table.

3.3.9 Ability of PSMA27-Specific CTLs to Kill PSMA and HHD-Expressing Target Cells

The primed PSMA27 epitope-specific $CD8^+$ T cells were tested for cytolytic effector activity against target cells endogenously expressing PSMA and target cells loaded with peptide.

To expand CTL lines, splenocytes from vaccinated mice were cultured *in vitro* in the presence of 10nM PSMA27 peptide and IL-2 in the medium. Previous experiments (data not shown) suggest that expansion with 10nM peptide is optimal. Although a greater proportion of cells respond to higher peptide concentrations in short *ex vivo* assays (Figure 18), it may be detrimental to the survival of the higher avidity $CD8^+$ T cells in culture. Excessive peptide may cause CTL activation induced cell death (AICD) or even fratricide,

both of which would generate sub-optimal lytic responses. Following stimulation *in vitro* for 6 days, standard 5-hour ^{51}Cr release assays were carried out by co-culturing T cells and target cells together at 1:1, 20:1 and 100:1 effector to target (E:T) ratios.

PSMA27-specific CTLs were highly efficient at specifically killing PSMA and HHD-expressing target cells (PSMA+HHD+) with 24%-46% and 52%-79% killing at 20:1 and 100:1 E:T ratios respectively (Figure 26). These three PSMA27 CTL lines (1-3) were derived from mice that responded with 193, 93 and 208 IFNγ secreting cells per million splenocytes respectively in the *ex vivo* ELISPOT (Figure 18). Also, from all the PSMA27 CTL lines thus far tested, any that generated detectable *ex vivo* ELISPOT responses have been able to demonstrate successful specific killing of PSMA+HHD+ targets. Therefore even mice with low *ex vivo* responses can prime PSMA27-specific CTLs with very good cytotoxic capacity. In contrast, the control target cells expressing either PSMA (PSMA+HHD-) or HHD (PSMA-HHD+) alone were not significantly lysed by PSMA27-specific CTLs (0%-26%), indicating the peptide-specificity and the HHD-restriction of the cytotoxicity. The killing of the peptide-loaded target cells (66%-83%) indicates the maximal lysis the CTLs are capable of when the presentation of PSMA27 peptide is not the limiting factor. The lysis of the peptide-loaded targets is only slightly higher than that of the non-peptide-loaded PSMA+HHD+ targets, indicating that the processing of the PSMA27 peptide must be highly efficient and the level of presented PSMA27 peptide very high.

In contrast, the p.DOM CTL lines did not show any cytolytic activity against any of the target cells with background killing of the PSMA and HHD-expressing cells (PSMA+HHD+) ranging only from 6% to 8%. This further confirms that it is the PSMA27 $CD8^+$ T cell epitope component of the p.DOM-PSMA27 vaccine that elicits a PSMA27-specific CTL response.

Overall, the $CD8^+$ T-cell responses elicited in the HHD mice by the p.DOM-PSMA27 DNA vaccine have PSMA27-specific cytolytic activity, a result consistent with their high levels of *ex vivo* IFNγ secretion. This experiment additionally confirms the efficient processing and presentation of the PSMA27 peptide by cells endogenously expressing PSMA.

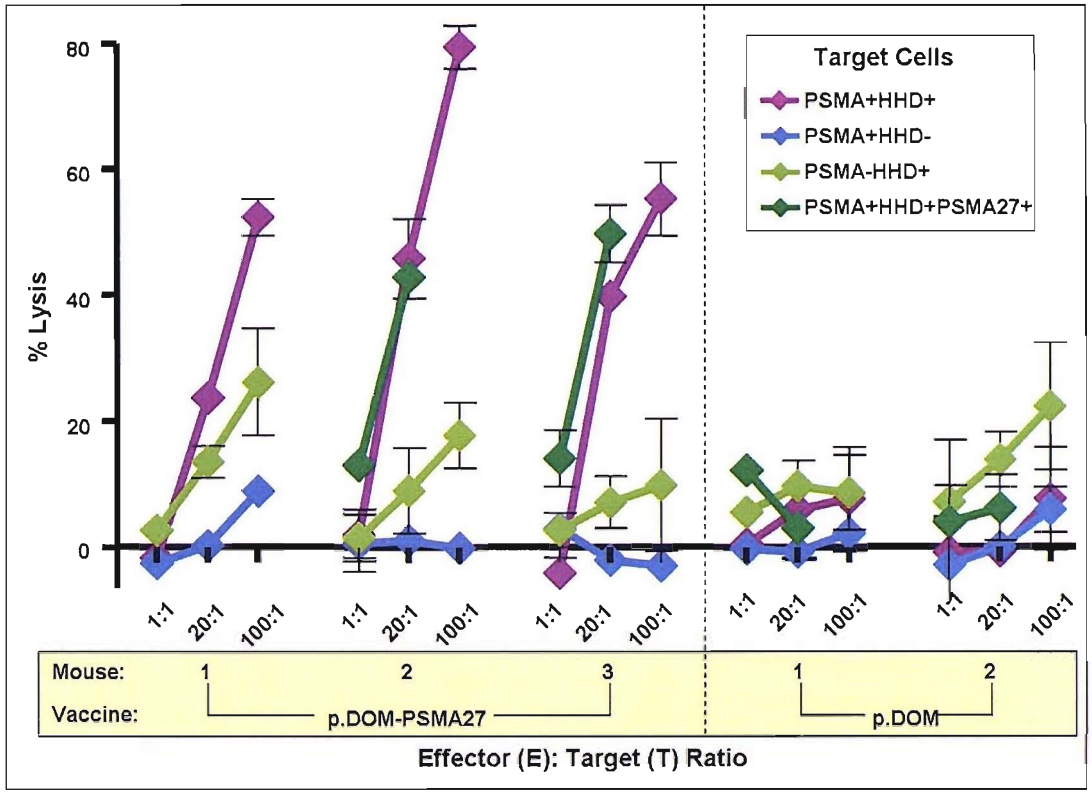


Figure 26: PSMA27-specific CTLs can lyse TRAMP cells expressing endogenous PSMA and HHD MHC class I

Splenocytes from vaccinated male HHD mice (p.DOM or p.DOM-PSMA27 vaccines) were cultured in the presence of 10nM PSMA27 peptide and IL-2. ^{51}Cr release assay was performed after 6 days to assess the ability of the PSMA27-specific CTLs to kill PSMA and HHD expressing target cells (purple), the negative control cells (PSMA+HHD- (blue-grey) and PSMA-HHD+ (light green)), and positive control 1μM PSMA27 peptide-loaded target cells (dark green). Splenocytes from p.DOM-vaccinated control mice were cultured *in vitro* in the presence of PSMA27 peptide as a control. The T cells and target cells were co-cultured at 1:1, 20:1 and 100:1 effector (E): target (T) ratios for 5 hours. The experiment was carried out in triplicates, for which the SEM error bars are shown. The results observed here have been replicated in two other experiments.

3.3.10 Ability of PSMA663-Specific CTLs to Kill PSMA and HHD-Expressing Target Cells

PSMA663-specific CTL lines were expanded from splenocytes of mice immunised with the p.DOM-PSMA663 vaccine, by adding 10nM PSMA663 peptide and IL-2 to the culture. After 6 days of *in vitro* expansion a standard 5 hour ^{51}Cr release assay was performed at 1:1, 20:1 and 100:1 E:T ratios.

The PSMA663-specific CTLs were able to lyse cells expressing PSMA and HHD (PSMA+HHD+) at very high levels ranging from 43%-84% and 77%-94% at 20:1 and 100:1 E:T ratios respectively (Figure 27). This confirms that the PSMA663 peptide must

be processed by these doubly transfected cells and presented by the chimeric HHD MHC class I. The background cytotoxicity of the control cells PSMA+HHD- and PSMA-HHD+ (up to 22% and 26% respectively at 100:1 E:T) indicate that the killing of the PSMA+HHD+ cells is PSMA663 peptide-specific. When the PSMA+HHD+ target cells were loaded with 1μM PSMA663 peptide, their killing by the PSMA663-specific CTLs increased by 2%-20% (to 91-114%). The small increase in cytotoxicity in the presence of excess presented peptide suggests that the PSMA663 peptide is efficiently processed and presented by PSMA-expressing cells.

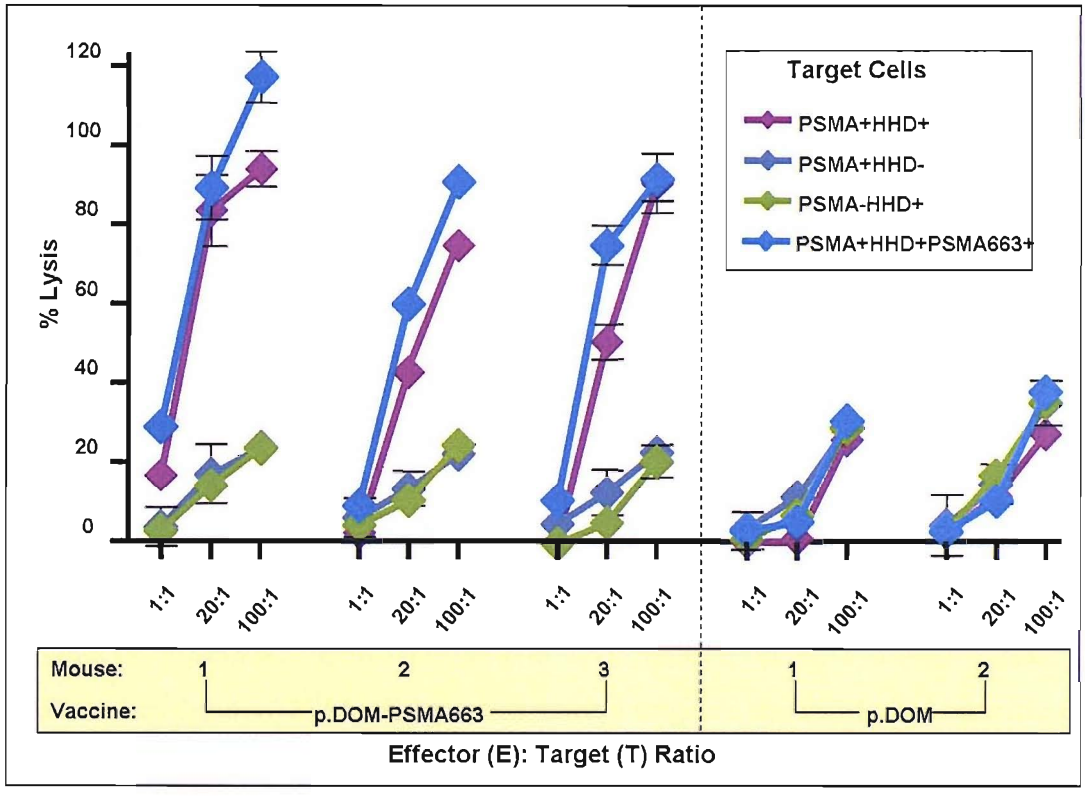


Figure 27: PSMA663-specific CTLs can lyse TRAMP cells expressing endogenous PSMA and HHD MHC class I

Splenocytes from vaccinated male HHD mice (p.DOM or p.DOM-PSMA663 vaccines) were cultured in the presence of 10nM PSMA663 peptide and IL-2. ^{51}Cr release assay was performed after 6 days to assess the ability of the DOM and PSMA663-specific CTLs to kill PSMA and HHD expressing target cells (purple), the negative control cells (PSMA+HHD- (blue-grey) and PSMA-HHD+ (light green)), and positive control 1μM PSMA663 peptide-loaded target cells (light blue). The CTLs and target cells were co-cultured at 1:1, 20:1 and 100:1 E:T ratios for 5 hours. The experiment was carried out in triplicates, for which the SEM error bars are shown. The results observed here have been replicated in one other experiment.

The cultured splenocytes from p.DOM vaccinated mice killed less than 35% of any target cells at all E:T ratios, thereby demonstrating that the PSMA663 $CD8^+$ T cell epitope

encoded within the p.DOM-PSMA663 vaccine is the part of the construct responsible for the induction of PSMA663-specific CTLs.

Overall, the p.DOM-PSMA663 vaccine is able to prime PSMA663-specific CTLs with very high cytotoxic function. More importantly, these CTLs are able to specifically lyse PSMA-expressing target cells thereby confirming that the physiological levels of PSMA663 peptide processed and presented by these cells are sufficient to enable very high levels of CTL-mediated lysis.

3.3.11 Failure of PSMA711-Specific CTLs to Kill PSMA and HHD Expressing Target Cells

Splenocytes from male HHD mice immunised with the p.DOM-PSMA711 vaccine were expanded *in vitro* in the presence of 10nM peptide and IL-2. After 6 days, the CTLs were used in a 5 hour ^{51}Cr release assay at 1:1, 20:1 and 100:1 E:T ratios with the each of the different target cells.

The p.DOM-PSMA711 was able to prime PSMA711-specific CTLs with excellent functional cytotoxic ability as demonstrated by the killing of (84%-96%) PSMA+HHD+ cells loaded with 1μM PSMA711 peptide (Figure 28). The lytic capacity of these PSMA711 $CD8^+$ T cells matched the very high *ex vivo* ELISPOT responses of 450, 1010 and 355 spots per million splenocytes, better than those shown for the other p.DOM-PSMA-epitope vaccines. However, despite their high level of cytolytic capacity (as demonstrated by lysis of PSMA711 peptide-loaded targets), the PSMA711-specific CTLs were unable to kill the non peptide-loaded target cells expressing both PSMA and HHD (PSMA+HHD+, Figure 28). These data confirm that the PSMA711 peptide is not processed from the protein and presented at sufficient levels to enable recognition and lysis by PSMA711-specific CTLs.

As has been shown for the cytotoxicity data presented for the other vaccines, the p.DOM controls were unable to lyse any of the target cells at levels above backround (<35%, only shown for one DOM CTL line), including the peptide-loaded targets. Therefore confirming that the PSMA711 $CD8^+$ T cell epitope encoded at the C-terminus of the vaccine is responsible for the priming of the PSMA711-specific CTLs.

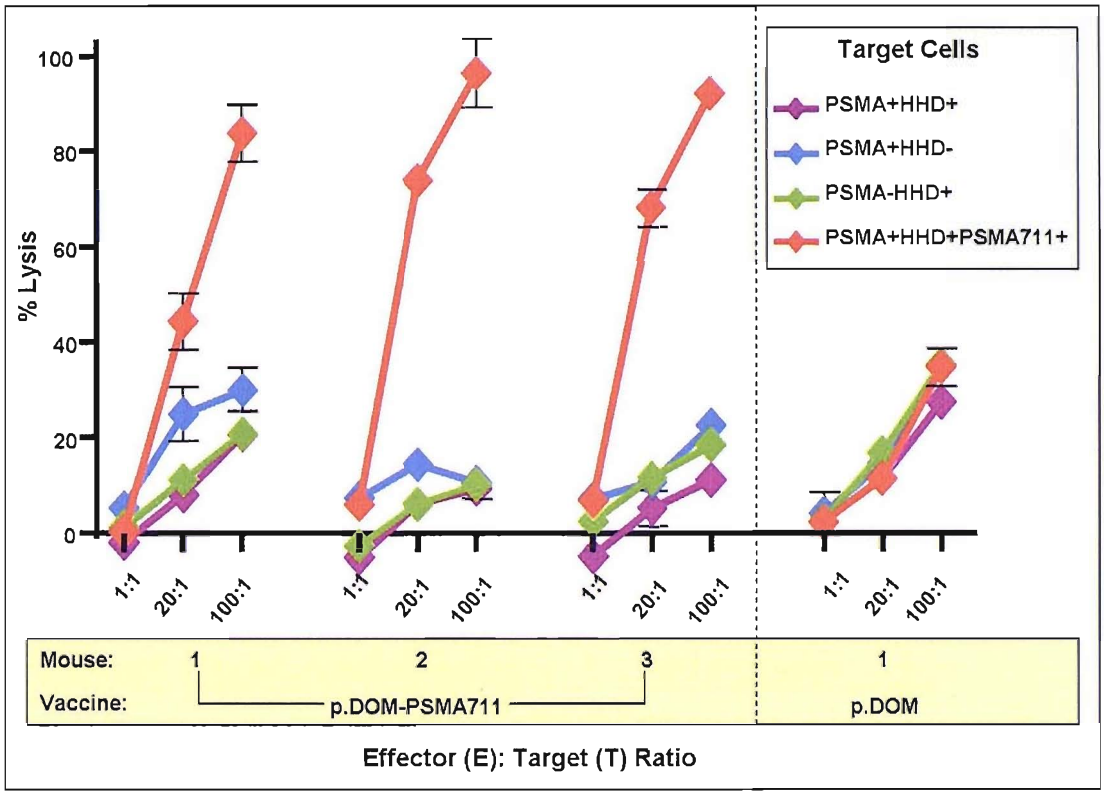


Figure 28: PSMA711-specific CTLs can not kill TRAMP cells expressing endogenous PSMA and HHD MHC class I

Splenocytes from vaccinated male HHD mice (p.DOM or p.DOM-PSMA711 vaccines) were cultured in the presence of 10nM PSMA711 peptide and IL-2. ^{51}Cr release assay was performed after 6 days to assess the ability of the DOM and PSMA711-specific CTLs to kill PSMA and HHD expressing target cells (purple), the negative control cells (PSMA+HHD- (blue-grey) and PSMA-HHD+ (light green)), and positive control 1μM PSMA711 peptide-loaded target cells (red). The CTLs and target cells were co-cultured at 1:1, 20:1 and 100:1 E:T ratios for 5 hours. The experiment was carried out in triplicates, for which the SEM error bars are shown. The results observed here have been replicated in one other experiment.

3.3.12 Ability of PSMA4-Specific CTLs to Kill PSMA and HHD-Expressing Target Cells

The results presented for this vaccine are those obtained from a single preliminary experiment. Only two from four mice for which vaccination was successful, were able to demonstrate a PSMA4-specific response in the *ex vivo* ELISPOT (Figure 21). The splenocytes from p.DOM and p.DOM-PSMA4-vaccinated mice were cultured in the presence of 10nM PSMA4 peptide and IL-2. After 6 days of *in vitro* expansion the PSMA4-specific CTL lines were used in a 5 hour ^{51}Cr release assay at different E:T ratio combinations, depending on the number of CTLs available (1:1, 20:1, 70:1, 100:1, 120:1), against target cells co-expressing PSMA and HHD and the other respective control cells.

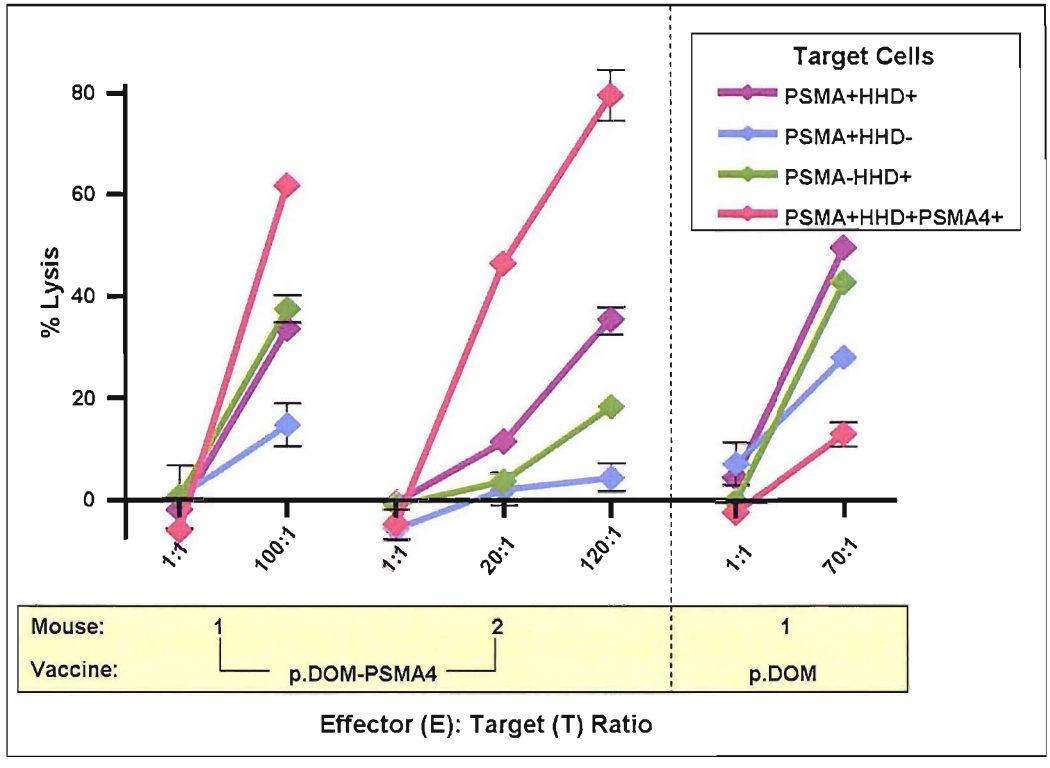


Figure 29: PSMA4-specific CTLs can lyse TRAMP cells expressing endogenous PSMA and HHD MHC class I

Splenocytes from vaccinated male HHD mice (p.DOM or p.DOM-PSMA4 vaccines) were cultured in the presence of 10nM PSMA711 peptide and IL-2. A ^{51}Cr release assay was performed after 6 days to assess the ability of the DOM and PSMA4-specific CTLs to kill PSMA and HHD expressing target cells (purple), the negative control cells (PSMA+HHD- (blue-grey) and PSMA-HHD+ (light green)), and positive control 1μM PSMA4 peptide-loaded target cells (pink). The CTLs and target cells were co-cultured at different E:T ratio combinations (1:1, 20:1,70:1,100:1, 120:1) for 5 hours. The experiment was carried out in triplicates, for which the SEM error bars are shown. These results are of a single preliminary experiment.

The two responding CTL lines can be seen to contain PSMA4-specific CTLs that can kill PSMA4 peptide-loaded target cells with 63% and 84% at 100:1 and 120:1 E:T ratios respectively (Figure 29). Not surprisingly due to very low *ex vivo* ELISPOT responses for PSMA4 CTL number 1, only the higher activity PSMA4-specific CTL line was able to lyse the target cells endogenously expressing PSMA and the chimeric HHD (38% lysis). The control cells PSMA-HHD+ and PSMA+HHD- both underwent background lysis of 19% and 5% respectively, much lower than the 38% specific killing of the PSMA+HHD+ cells. The PSMA4 CTL line 2 displayed rather good ability to lyse peptide-loaded target cells at 84% lysis. However, the killing of the PSMA+HHD+ (non peptide-loaded) target cells was significantly lower at 38% lysis, when compared to that achieved by PSMA27 and PSMA663-specific CTL lines of equal cytotoxic capacity for their respective peptide-loaded targets (Figures 26 and 27). This would suggest that the PSMA4 peptide may

possibly not be as efficiently processed and presented as the PSMA27 and PSMA663 peptides, although more studies would be required for such conclusions.

Overall the results demonstrate that the p.DOM-PSMA4 vaccine can prime epitope-specific $CD8^+$ T cells that acquire epitope-specific cytotoxicity. The killing of PSMA+HHD+ by the single CTL line alone confirms that the PSMA4 peptide is processed and presented at levels that enable PSMA4-specific CTLs to recognise the PSMA-expressing target cells and display cytotoxic activity against them.

3.3.13 Expansion of High Avidity PSMA27 and PSMA711-Specific CTLs

From the four PSMA peptide-specific CTLs assessed for cytotoxicity against cells endogenously expressing PSMA, the PSMA711-specific CTLs were the only ones unable to kill these targets. In order to assess if this was due to low levels rather than the absence of PSMA711 peptide processing and presentation, higher avidity PSMA711-specific CTL lines were expanded. Reports that high but not low avidity $CD8^+$ T cells from melanoma patients were able to lyse tumour targets [393] may indicate that higher avidity PSMA711-specific $CD8^+$ T cells should have a better prospect of killing the PSMA and HHD-expressing target cells.

To test the avidity of the expanded $CD8^+$ T cells, RMA-HHD cells pulsed with a range of peptide concentrations (1μM, 0.1μM, 10nM, 1nM) were used to stimulate the PSMA711-specific $CD8^+$ T cells in an IFNγ intracellular activation assay. Brefeldin A was added to block transport of proteins from the ER, resulting in the intracellular accumulation of IFNγ in activated cells. Flow cytometry was used to analyse the percentage of the $CD8^+$ T cells that were activated ($IFN\gamma^+CD8^+$ T cells).

A PSMA711-specific CTL line was initially expanded with 0.1μM peptide rather than 10nM. Stimulation by RMA-HHD cells loaded with 1μM peptide indicated that 84% of the $CD8^+$ T cells in the CTL line specifically responded to the high concentration of PSMA711 peptide (data not shown). Stimulation by cells loaded with 10 and 100-fold lower peptide concentrations (10nM and 1nM) activated decreasing percentages of PSMA711-specific $CD8^+$ T cells (Figures 30 and 31). Nonetheless a high proportion of $CD8^+$ T cells could still be detectably stimulated by peptide concentrations lower than that used for PSMA711-specific CTL expansion.

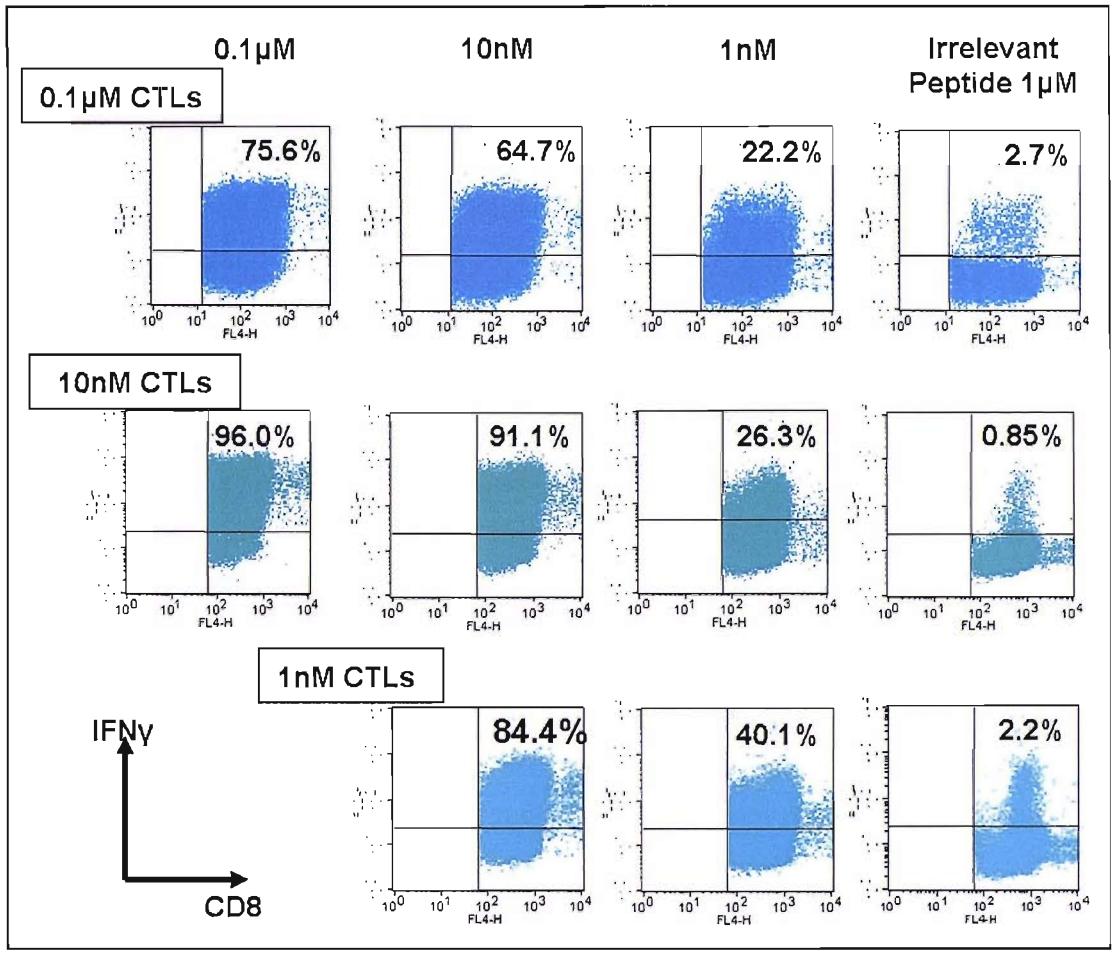


Figure 30: Expansion of high avidity PSMA711-specific CD8$^+$ T cells

Mice were immunised with p.DOM-PSMA711 vaccine on day 0 (50µg DNA) and sacrificed on day 14. Splenocytes were expanded *in vitro* with 0.1µM PSMA711 peptide (top panel) and IL-2. Splenocytes were restimulated with 0.1µM PSMA711 peptide-loaded irradiated splenocytes and IL-2 every 7-10 days for 3 re-stimulations. The peptide concentration used for PSMA711-specific CD8$^+$ T-cell expansion was reduced to 10nM (middle panel) for two re-stimulations and then to 1nM (lower panel) for a further three re-stimulations. 7 days after last re-stimulation, $5x10^5$ lymphocytes were incubated for 4 hours at a 1:1 ratio with RMA-HHD cells loaded with varying PSMA711 peptide concentrations. Intracellular IFNγ was stained as a marker of activation and analysed by flow cytometry. Analysis expressed as percentage of IFNγ positive CD8$^+$ T cells.

The amount of PSMA711 peptide used for CTL expansion was decreased by 10-fold (10nM) for two rounds of re-stimulation and then by a further 10-fold (1nM) for three rounds of re-stimulation. At each stage, the lower avidity T cells expanded with higher PSMA711 peptide concentrations were maintained alongside. This process resulted in the expansion of CD8$^+$ T cells of higher avidity that were responsive to lower peptide concentrations, thereby enriching these CD8$^+$ T-cell populations (Figures 30 and 31). Expansion with the lowest peptide concentration (1nM) generated the highest proportion of CD8$^+$ T cells sensitive to stimulation by all concentrations of peptide-loaded cells. Higher

avidity PSMA711 peptide-specific $CD8^+$ T-cell populations were therefore successfully created in this manner.

High avidity PSMA27-specific $CD8^+$ T cells were also expanded alongside to demonstrate that the same process could be applied to another CTL line with distinct specificity and to assess whether enhanced killing of the PSMA-expressing target could be attained by the higher avidity CTLs. The initial expansion was with 10nM PSMA27 peptide and was then lowered to 1nM and eventually 100pM (Figure 31) in the same way as described above for the PSMA711-specific $CD8^+$ T cells. The same pattern of expansion and enrichment of high avidity PSMA27-specific $CD8^+$ T cells was observed.

The results shown are for single CTL lines at a particular time point during expansion. These CTL lines were tested in the same experimental way a number of times with the same pattern of results observed. However, these results from different time points are not directly comparable so only those for a single experiment are shown. The expansion of higher avidity CTLs was not repeated with other lines as it was laborious, the points of re-stimulation unlikely to be the same and more importantly not required for the objective of the project.

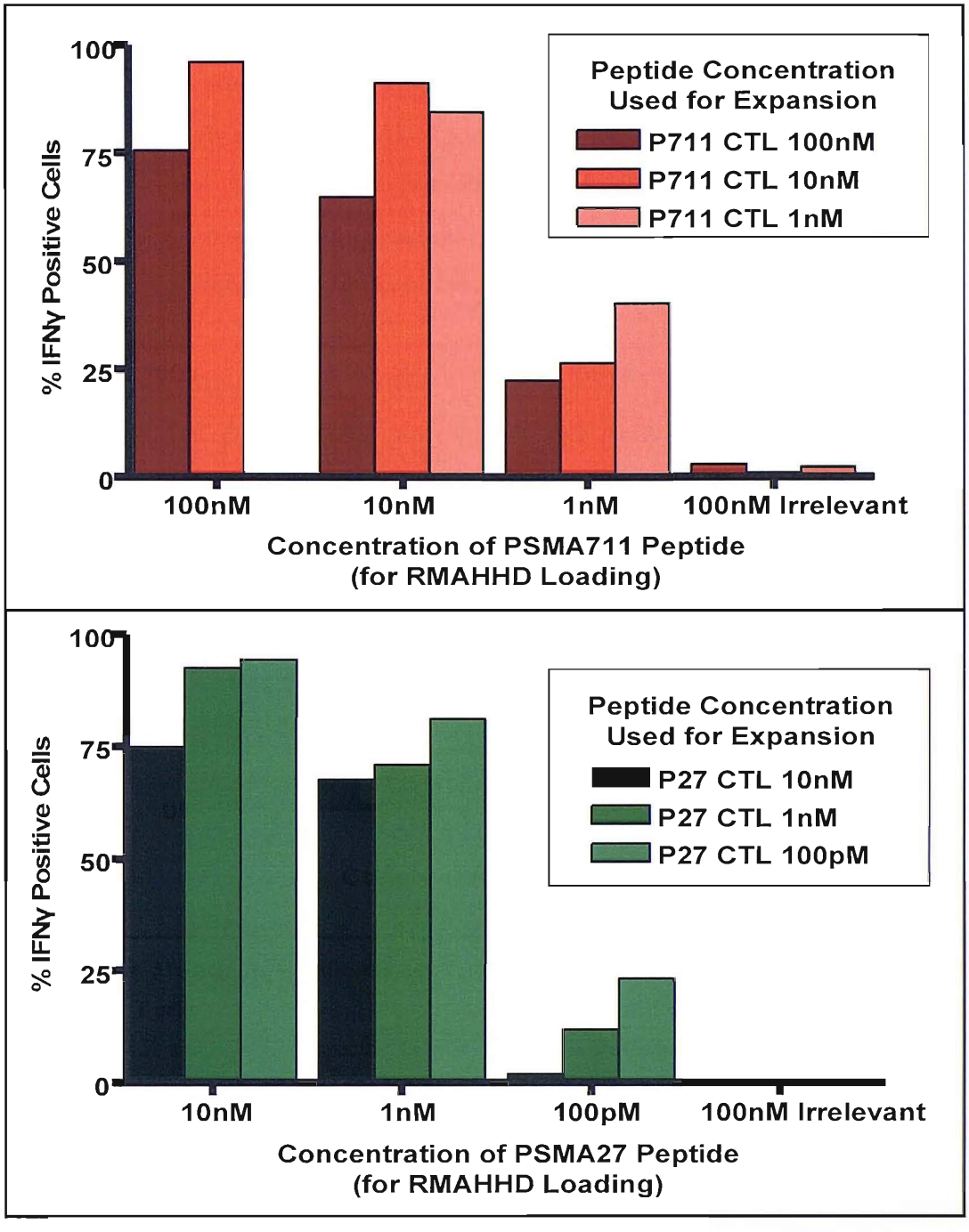


Figure 31: Peptide-specific responsiveness of high avidity PSMA27 and PSMA711-specific CD8$^+$ T cells

PSMA27 and PSMA711-specific CD8$^+$ T cell lines were expanded with IL-2 and 10nM PSMA27 (dark green) or 0.1μM PSMA711(dark red) peptides respectively. After three rounds of expansion a proportion of CD8$^+$ T cells for each line were re-stimulated at 10-fold lower peptide (1nM PSMA27, [middle green] and 10nM PSMA711 [middle red]) for two re-stimulations. The peptide concentration was then lowered by a further 10-fold (100pM PSMA27 [light green] and 1nM PSMA711 [light red]) for another three rounds of expansion. Expansion at the higher peptide concentrations was also maintained alongside. 7 days after the last re-stimulation $5x10^5$ lymphocytes were incubated for 4 hours at a 1:1 ratio with RMA-HHD cells loaded with varying PSMA27 and PSMA711 peptide concentrations. Intracellular IFNγ accumulated due to the added brefeldin A was stained as a marker of activation and analysed by flow cytometry. Analysis expressed as percentage of IFNγ-positive CD8$^+$ T cells. Results shown are from single CTL lines for a particular time point during expansion.

3.3.14 Ability of High Avidity PSMA27 and PSMA711-Specific CTLs to Kill PSMA and HHD-Expressing Target Cells

The ability of the high avidity PSMA27 (pooled 1nM and 100pM) and PSMA711-specific CTLs (pooled 10nM and 1nM) to lyse PSMA and HHD expressing target cells was assessed in a ^{51}Cr release cytotoxic assay. At this point the CTL lines had undergone a number of expansions and had excellent cytolytic activity against the peptide-loaded target cells.

As previously shown with lower avidity CTLs, the PSMA27-specific CTLs were able to lyse the PSMA and HHD double positive TRAMP cells (PSMA+HHD+, Figure 26). However, the difference is in the efficiency of lysis at the various E:T ratios. The high avidity PSMA27-specific CTLs killed PSMA+HHD+ cells at all E:T ratios including at the 1:1 E:T ratio (Figrue 32). In contrast, in the previous experiment, the lower avidity PSMA27-specific CTLs (after brief expansion with 10nM PSMA27 peptide), were unable to kill PSMA+HHD+ target cells at the lower 1:1 E:T ratio although these targets were lysed with good efficiency at 20:1 and 100:1 E:T ratios. At 40:1 E:T ratio, the high avidity PSMA27-specific CTLs were able to lyse 75% of PSMA+HHD+ targets, efficiencies only reached at 100:1 E:T ratio by the lower avidity counterparts.

Background lysis of control PSMA-expressing transduced cells (PSMA+HHD-) was minimal at less than 10%, reconfirming that PSMA27 peptide is processed and presented via the HHD MHC class I.

More striking is the observation that the high avidity PSMA711-specific CTLs lysed PSMA+HHD+ target cells (Figure 32), an ability the lower avidity counterparts did not have (Figure 28). The killing of PSMA+HHD+ targets was 43% and 52% at 40:1 and 80:1 E:T ratios respectively, suggesting relatively high cytotoxic efficiencies against the targets when the T-cell avidity increases by 10-100 fold. The maximal lytic capacity of these PSMA711-specific CTLs appears to be ~80% i.e. that achieved against the peptide-loaded targets. The background lysis of PSMA+HHD- cells is also below 10%, confirming that the PSMA711 peptide is indeed processed and presented via the MHC class I. The levels of PSMA711 peptide presentation appear to be low and therefore only recognised by higher avidity T cells rather than those of heterogenous avidity primed by vaccination.

Overall, these observations confirm the superior cytotoxic capacity of higher avidity CTLs and that it is possible to expand functional high avidity T cells from heterogenous populations *in vitro*. Additionally, processing of PSMA711 peptide is confirmed although the levels presented by PSMA-expressing cells appear to be significantly lower than those of the other PSMA peptides assessed. This may potentially compromise therapies

targeting the PSMA711 peptide and will depend on the avidity of the CTLs that can be primed in patients.

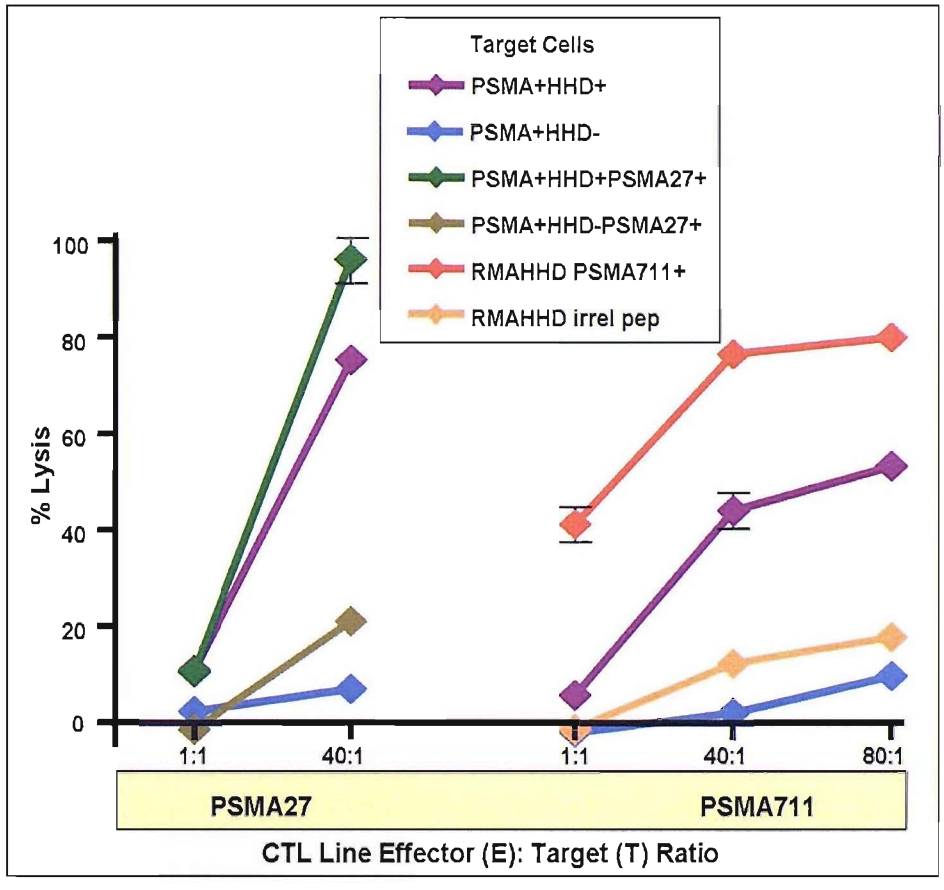


Figure 32: High avidity PSMA27 and PSMA711-specific CTLs can kill cells expressing endogenous PSMA and HHD MHC Class I

High avidity PSMA27 and PSMA711-specific CTLs were used in a standard 5 hour ^{51}Cr release assay against TRAMP cells transduced to express PSMA and the HHD MHC class I molecule. The CTLs had undergone a total of 12 rounds of expansion. The targets used were clonal populations of TRAMP cells stably expressing human PSMA alone (blue) or loaded with PSMA27 peptide (brown). Alternatively these cells had been transduced to additionally express the HHD and were used in this assay unloaded (purple) or loaded with 1μM PSMA27 peptide (green). The PSMA711 CTLs were tested against RMA-HHD cells loaded with 1μM PSMA711 peptide (red) or an irrelevant peptide (orange). The SEM of the triplicate values are shown for each value plotted.

3.3.15 Dendritic Cells Loaded with PSMA RNA Present Peptides to Activate High Avidity PSMA27 and PSMA711-CTLs

An experiment with HHD DCs was designed as an alternative way to assess and confirm the processing of PSMA711 peptide. HHD-derived DCs were used to process and present the antigen from the full-length PSMA sequence. The PSMA27-specific CTLs were

included as a positive control as the processing status of the PSMA27 peptide is clearly established.

Reports where lysate-loaded DCs have been able to cross-prime $CD8^+$ T-cell responses [394] led us to initially load HHD DCs with LNCaP cell lysate. After a 12-hour lysate-pulsing period and maturation with LPS, the DCs were unable to specifically activate the high avidity PSMA27 or PSMA711-specific $CD8^+$ T cells, as assessed by an intracellular IFNγ assay (data not shown).

Rather than refining the tumour lysate loading of DCs, a different PSMA antigen source was used in the form of *in vitro* synthesised PSMA-encoding RNA. This would require the DCs to synthesise the protein and then process it. On day 7 of *in vitro* cultures, immature BM-derived DCs from HHD mice were loaded by electroporation with 40μg PSMA RNA or no RNA as a control. The DCs were allowed to recover for 2 hours before incubation with the high avidity peptide-specific $CD8^+$ T cells for 10 hours. T-cell activation was analysed by IFNγ capture secretion assay and cells were additionally stained for CD8 and MHC class II. Analysis was gated on $CD8^+$ and MHC class II^- cells (to gate out $CD8^+$ DCs) and expressed as the percentage of IFNγ positive $CD8^+$ T cells.

A low proportion of high avidity PSMA27 and PSMA711-specific $CD8^+$ T cells secreted IFNγ in response to incubation with the PSMA RNA-loaded DCs but not upon incubation with the DCs electroporated in the absence of RNA (Figure 33). As positive controls, the $CD8^+$ T cells were incubated with 100pM PSMA27 or 1nM PSMA711 peptide. The RNA-loaded DCs activated 3-fold more PSMA27-specific $CD8^+$ T cells (7.2%) than the positive control 100pM peptide-loaded counterparts (2%). The proportion of activated PSMA711-specific $CD8^+$ T cells was similar when stimulated with RNA loaded DCs (4.7%) or 1nM peptide (6.5%). The results confirm that the PSMA711 peptide is processed from the PSMA protein, presented by HHD derived DCs and can be recognised by high avidity PSMA711-specific $CD8^+$ T cells with similar efficiency to the recognition of cells loaded with 1nM peptide.

With hindsight, this experiment would have ideally used an irrelevant RNA rather than no RNA at all as the negative control and also RNA from the vaccine constructs as positive controls (p.DOM-PSMA27 or p.DOM-PSMA711, as the priming of epitope-specific $CD8^+$ T cells by vaccination demonstrates efficient peptide processing from these constructs). However, due to the much superior efficiency and simplicity of the chromium (^{51}Cr) release assays and the more relevant results generated (direct cytotoxicity), this experiment was not repeated with the improved protocol suggested.

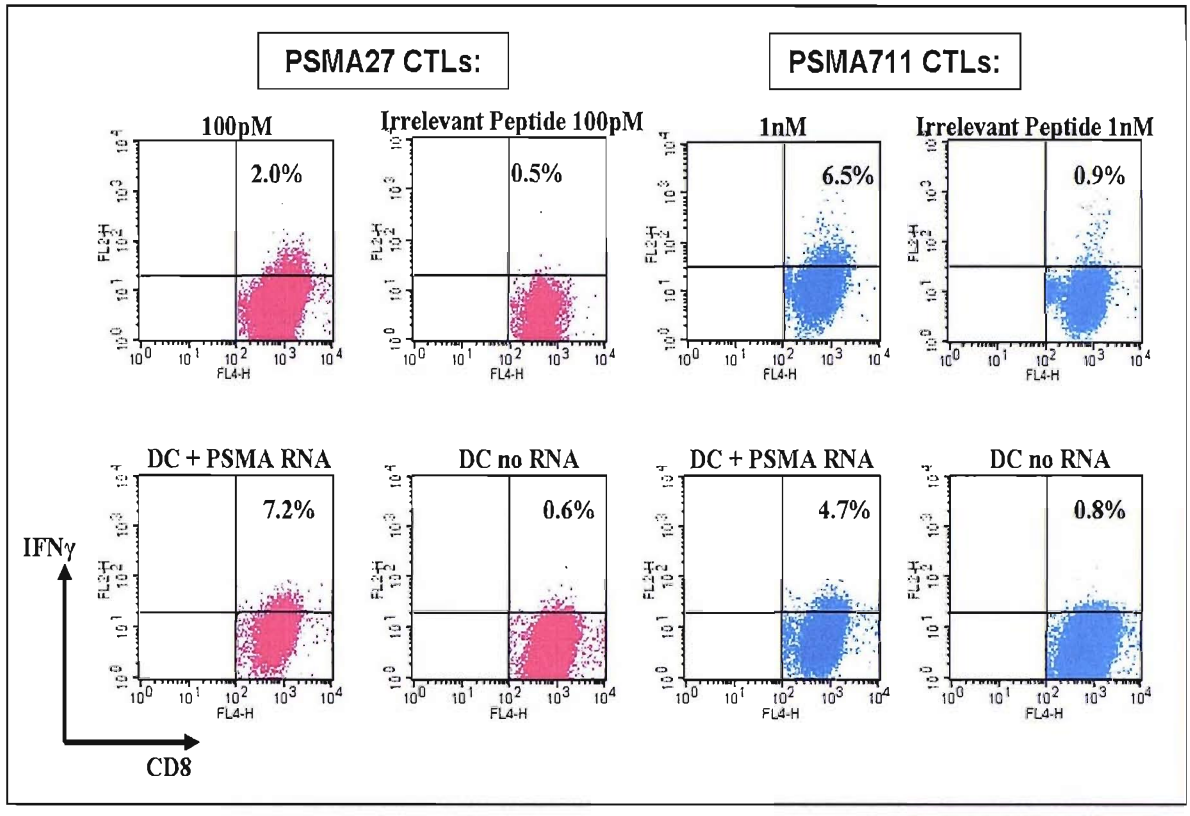


Figure 33: Dendritic cells loaded with PSMA RNA can activate PSMA27 and PSMA711-specific CD8$^+$ T cells

HHD-derived DCs were loaded by electroporation with *in vitro* synthesised RNA or no RNA, and co-cultured for 10 hours with the high avidity 1nM *in vitro* PSMA27 (pink) and 10nM PSMA711 (blue) CTL lines that had undergone 9 re-stimulations. The DCs were also loaded with low peptide concentrations (100pM PSMA27 and 1nM PSMA711) or 1nM irrelevant peptide as positive and negative controls. Secreted IFNγ was captured in a cytokine secretion assay and analysed by flow cytometry. Analysis expressed as percentage of IFNγ positive CD8$^+$ and MHC class II- cells (gates out CD8$^+$ DCs).

3.3.16 Immunity primed by the p.DOM-PSMA663 Vaccine Specifically Eliminates PSMA-Expressing Tumour Cells *in Vivo*

We have thus far demonstrated the ability of our p.DOM-PSMA-epitope vaccines to prime responses in HHD mice (*in vivo*), and the excellent capacity for these to acquire cytotoxic function. It has also been demonstrated that these CTLs can not only lyse peptide-loaded target cells but also cells endogenously expressing PSMA (although in the case of PSMA711 CTLs only high avidity CTLs acquired this ability). The next objective was to demonstrate that these CTLs could kill PSMA-expressing cells *in vivo*, after all this is the ultimate aim of vaccination of patients in a clinical setting.

Not unexpectedly due to MHC class I mismatch, the TRAMP PSMA+HHD+ cells will not form tumours in wild-type C57BL/6 mice, HHD transgenic mice or HLA-A2K^b transgenic mice. They also did not form tumours in immune compromised SCID or RAG$^{-/-}$ mice possibly as a result of NK cell attack. However, inexplicably these cells were not tumourigenic in NK-deficient RAG$^{-/-}$ common γ-chain$^{-/-}$ immune compromised mice either. A novel system of *in vivo* killing was therefore used and adapted for this model. On day 0 male HHD mice were intramuscularly immunised with p.DOM control or p.DOM-PSMA663 vaccines. On day 13 they received by sub-cutaneous injection a mix of two CFSE-labelled tumour cell populations co-injected in a 1:1 ratio with Matrigel™. Matrigel™ is a solubilised membrane preparation of a mouse sarcoma rich in extracellular matrix protein components. Above 4°C it forms a solid "plug" that provides a scaffold for the co-injected cells to grow on. This allows the tumour cells to be retrieved at a later desired time point through the removal of the Matrigel™ plug. The TRAMP PSMA-HHD+ cells were labelled with 1μM CFSE and the TRAMP PSMA+HHD+ with 10μM CFSE, thus allowing differentiation between the two cell populations when mixed. Naïve mice received only a single population of these CFSE-labelled tumour cells ($1x10^6$) whilst the pre-vaccinated mice received a mix of the two cell populations ($1x10^6$ of each, $2x10^6$ total). Five days after the injection of tumour cells/Matrigel™, the plugs were excised. This time point was chosen as Kowalczyk et al., have previously shown detectable CD8$^+$ T-cell infiltrates in Matrigel™ plugs from day 4 (~$1\text{-}2x10^5$) that increased 10-fold ($1\text{-}2x10^6$) by day 6 [395]. Therefore the longer the Matrigel™-embedded tumour cells remained in the mice the higher the CD8$^+$ T-cell infiltrate. Day 5 was the longest period of time that the CFSE-labelled TRAMP cells could be left implanted and still be differentiated from other cells that infiltrate the Matrigel™ plug (background) during analysis (data not shown). The plugs from individual mice were digested with collagenase which allowed the release of the tumour cells for analysis by flow cytometry. The CFSE fluorescence of the two cell populations allowed their quantification and the individual mouse splenocytes were assessed for vaccine responses by IFNγ ELISPOT. The gate for analysis was set according to the cells recovered from the naïve mice that received only single cell populations (Supplementary data II). After the gates were set, cleaner FL1-H/FSC-H plots (orange) were obtained where all the cells within the set areas are counted as belonging to either PSMA+HHD+ 10μM or PSMA-HHD+ 1μM populations (Figure 35). The percentage of cells in each particular population from the total number of both cell populations were calculated and used to determine the percentage specific killing with the following published formula [371]: 100 – ([(%PSMA+HHD+ / %PSMA-HHD+ in immunised test mice) / (%PSMA+HHD+ / %PSMA-HHD+ p.DOM control mice)] x 100).

The values for the p.DOM control mice were taken as the average of the three control mice in the experiment. Therefore, the percentage specific killing by p.DOM-PSMA663-vaccinated mice (Figure 35) is relative to the (average) proportion of cells recovered from p.DOM vaccinated control mice. This confirms that the experiment is valid even though the two cell populations were not recovered at a 1:1 ratio as would be expected.

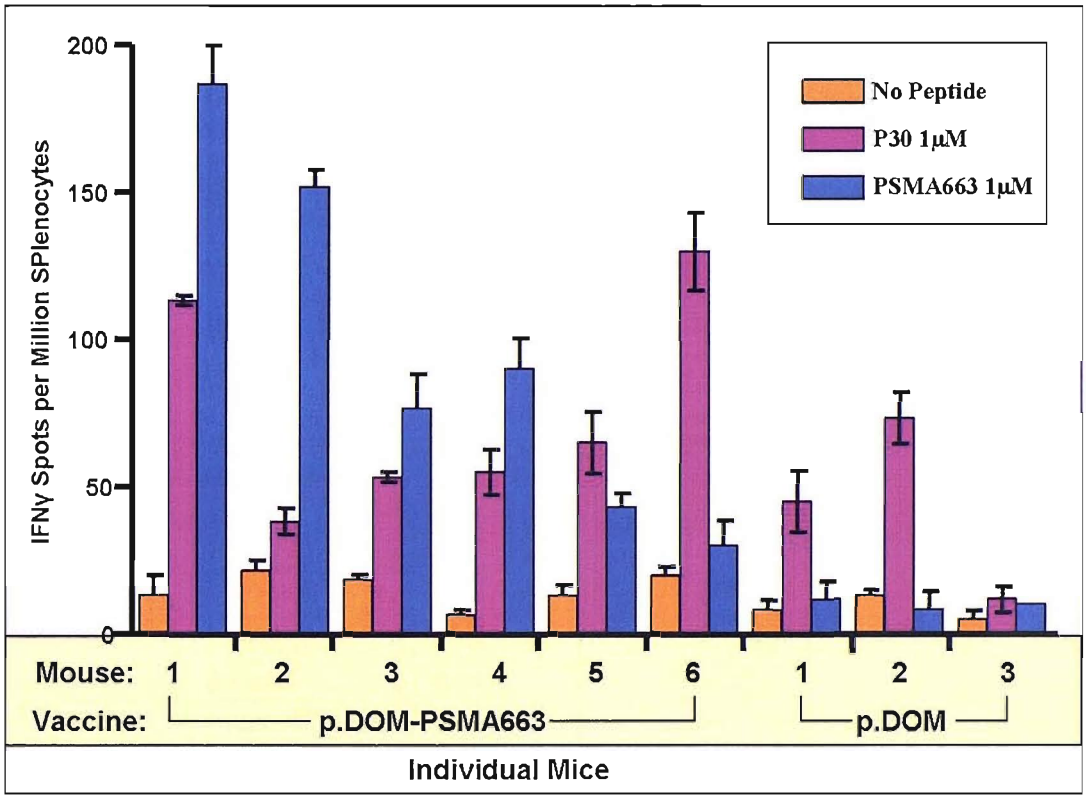


Figure 34: IFNγ responses of *ex vivo* PSMA663-specific CD8$^+$ T cells from mice challenged with PSMA-expressing tumour cells

Male HHD mice were immunised with p.DOM-PSMA663 or p.DOM control DNA vaccines intramuscularly on day 0 (50μg DNA). On day 13 they received a mix of TRAMP PSMA+HHD+ and PSMA-HHD+ tumour cells in Matrigel™. Five days after implantation, the mice were sacrificed and splenocytes stimulated in an *ex vivo* IFNγ ELISPOT without any peptide to measure background levels (orange), or with 1μM p30 (purple) or 1μM PSMA663 peptide (blue). Responses from individual spleens are plotted and the error bars demonstrate the SEM of the triplicates. These responses correspond to the mice in Figure 35 where the percentage specific *in vivo* killing of the TRAMP PSMA+HHD+ cells is calculated from the recovery of the implanted tumour cells.

The ELISPOT data shows that although the responses were variable, all 6 mice immunsed with the p.DOM-PSMA663 DNA vaccine primed PSMA663-specific CD8$^+$ T cells (Figure 34). Three from the six p.DOM-PSMA663-vaccinated mice were able to specifically kill (70.3%, 81.7% and 21.8%) the TRAMP PSMA+HHD+ cells *in vivo* (Figure 35). Similar results were observed in a previous experiment where two from three mice responding to

the p.DOM-PSMA663 vaccine mounted a response that could migrate to the Matrigel™-embedded tumour and specifically eliminate the TRAMP PSMA+HHD+ tumour cells.

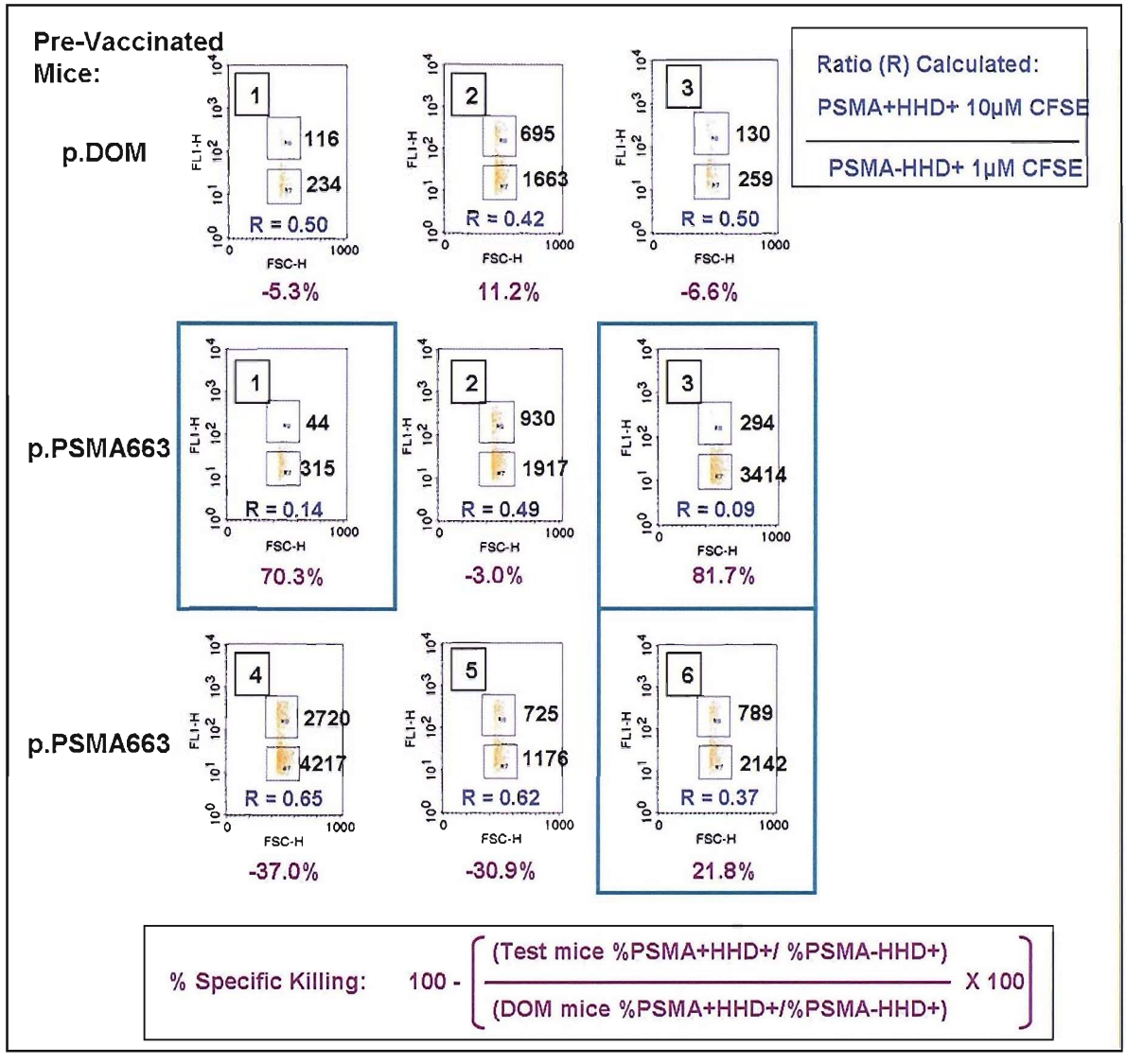


Figure 35: Mice immunised with the p.DOM-PSMA663 can specifically kill implanted PSMA-expressing tumour cells

Male HHD mice were immunised with 50µg of either p.DOM-PSMA663 or p.DOM control vaccines on day 0. TRAMP tumour cells expressing HHD alone or PSMA and HHD were labelled with 1µM or 10µM CFSE respectively, and co-injected subcutaneously in a 1:1 ratio with Matrigel™ on day 13. After 5 days the Matrigel™ plugs were excised and digested with collagenase prior to analysis by flow cytometry for identification of the implanted CFSE labelled TRAMP tumour cells implanted. The total number of cells recovered for each population is shown next to each plot and the ratios of TRAMP PSMA+HHD+ to TRAMP PSMA-HHD+ cells were calculated for each individual mouse (R values in grey). Analysis of specific killing of PSMA+HHD+ cells (pink) is calculated with the previously published formula shown in pink (Kumaraguru 2004). Responding mice are boxed in turquoise. The corresponding *ex vivo* IFNγ ELISPOT responses against PSMA663 peptide stimulation can be seen in Figure 34. These results are representative of two experiments.

There does not appear to be an obvious correlation between PSMA663-specific ELISPOT responses and the ability to kill the PSMA-expressing cells *in vivo*. The three mice that specifically eliminated the TRAMP PSMA+HHD+ cells *in vivo* were the first, fourth and sixth best ELISPOT responders. However, it may be expected that the PSMA663-specific CTLs would migrate away from the spleen and into the tumour site where the PSMA antigen is being presented by the tumour cells.

Two from the three p.DOM-vaccianted control mice have responded to the vaccination and primed p30-specific $CD4^+$ T-cell responses (Figure 34). The CFSE-labelled tumour cells were recovered in the same proportion from the mouse that did not appear to have any *ex vivo* ELISPOT responses as those recovered from the two that did have p30-specific $CD4^+$ T-cell responses. This supports the assumption that the proportion/percentages of cells recovered from the p.DOM-vaccinated mice represent the absence of specific killing of either TRAMP cell population, particularly the PSMA+HHD+ cells despite the divergence from the expected 1:1 ratio. The only difference between the responses elicited by the p.DOM and p.DOM-PSMA663 vaccines is the ability of the latter to prime PSMA663-specific $CD8^+$ T-cell responses. It can therefore only be PSMA663-specific CTLs that are responsible for the specific killing of TRAMP PSMA+HHD+ tumour cells *in vivo*.
A preliminary experiment using this protocol but immunising three mice with the p.DOM-PSMA27 vaccine, revealed a similar result. Only two of the three mice were successfully vaccinated (presence of p30-specific responses), and one of these responding mice was able to specifically kill the TRAMP PSMA+HHD+ cells (56%) in the same manner as described above for the p.DOM-PSMA663 vaccianted mice. This data is not shown as it requires repeating on a larger sample number.

3.3.17 Cytotoxic T-cell Lysis Via Peptides Naturally Presented in Association with HLA-A2 by the LNCaP Human PCa Cell Line

The LNCaP epithelial cell line is derived from a human PCa lymph node metastasis (section 1.2.5). They are HLA-A2 positive cells and expresses high levels of PSMA as analysed by western blot and flow cytometry (Figure 38). It is the only available candidate cell line that to our knowledge meets both requirements of PSMA and HLA-A2 expression and that can therefore be assessed in this setting. As such it is often used as the target cell to assess the cytotoxic ability of human $CD8^+$ T cells *in vitro*. The objective of using them in the present study was to assess whether the HHD CTLs specific for PSMA peptides

could kill a PSMA-expressing target cell in the context of a naturally arisen tumour rather than the transduced TRAMP cell targets created here.

However there are two problems in using this line as a target:

1. Low MHC class I expression.
2. Inability of the human α3 sequence from the MHC class I heavy chain to interact with the mouse CD8 molecule on the HHD-derived T cells.

Although the mouse CD8 will not be able to engage with the human HLA-A2 α3 domain, it is known that when high levels of peptide are presented the mouse HHD CTLs can kill human HLA-A2 cell lines [160]. From the results demonstrated earlier it can be seen that both PSMA27 and PSMA663 peptides appear to be presented at high levels.

3.3.17.1 Upregulation of HLA-A2 by TNFα Treatment of LNCaP Cells

For killing of target cells, they must present a certain amount of MHC class I to enable sufficient peptide presentation to the CTLs. LNCaP cells have low HLA-A2 expression levels that require enhancement for use as targets in a standard ^{51}Cr release assay or for direct presentation. IFNγ is the commonly used cytokine to upregulate MHC class I levels on cells. It has been reported that IFNγ treatment does not upregulate HLA-A2 levels on LNCaP cells [220, 222, 230], an observation we have confirmed (data not shown).
Approximately 10% of the LNCaP cells were HLA-A2 positive before cytokine treatment with a low population MFI of 10.1 (isotype control MFI 5.8, Figure 36A). Treatment with 5ng/ml, 10ng/ml or 20ng/ml TNFα treatment for 72 hours increased the HLA-A2 expression dramatically to MFI levels of 81.9, 101.4 and 97.5 respectively (corresponding to 80.2%, 86.0% and 89.9% of HLA-A2 positive cells, Figure 36B). Also treatment with 5ng/ml TNFα increased HLA-A2 surface expression from MFI levels of 8.9 for untreated cells to 21.3 (76.2% of cells) at 24 hours, 48.2 (79.4% of cells) at 48 hours and 82.2 (85.1% of cells) at 72 hours (Figure 36C). A regimen of 5ng/ml TNFα for 24 hours was chosen as it substantially upregulated HLA-A2 expression without compromising the condition of the cells.

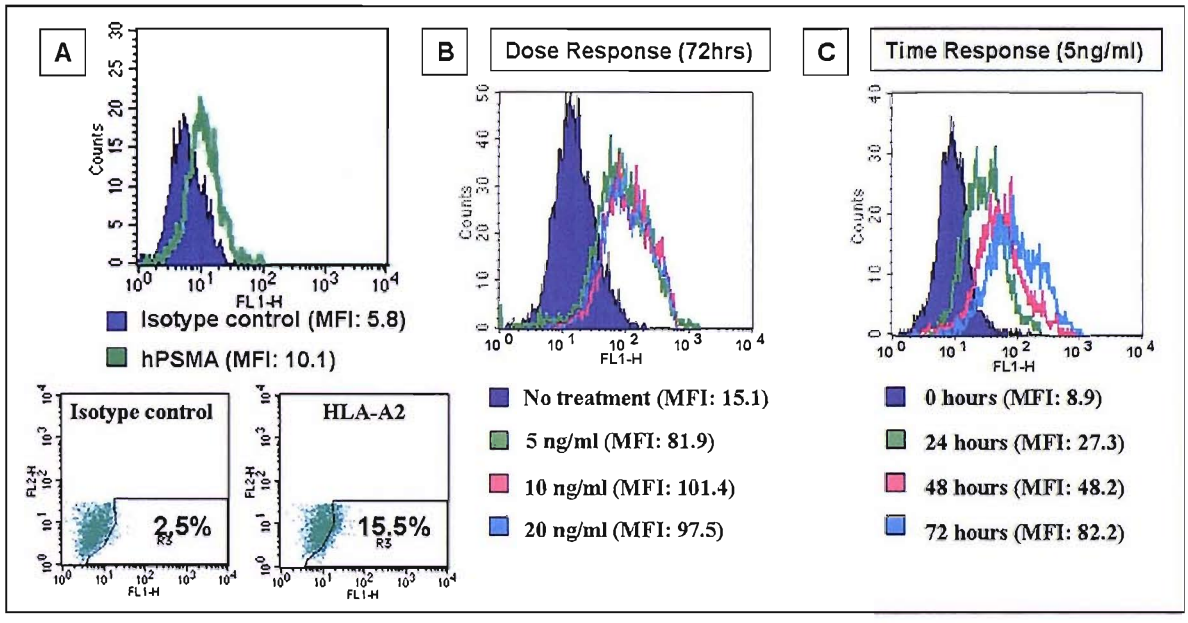


Figure 36: Analysis of HLA-A2 expression by LNCaP cells before and after TNFα treatment

The level of HLA-A2 expression by untreated LNCaP cells was analysed (A). LNCaP cells treated with varying concentrations of TNFα for 72 hours (B) or with 5ng/ml TNFα for 24, 48 or 72 hours (C) were assessed for increased HLA-A2 expression by flow cytometry.

3.3.17.2 Inability of PSMA27-Specific CTLs to Lyse LNCaP Cells

PSMA27-specific CTLs showed no cytotoxicity against untreated LNCaP cells loaded with 1μM PSMA27 peptide (data not shown). Upregulation of HLA-A2 (~70% positive cells) by TNFα treatment and peptide-loading (1μM) results in sufficient peptide-MHC complexes on the surface of LNCaP cells to enable CTL-mediated lysis with up to 60% efficiency (Figure 37). This confirms that a high concentration of peptide-MHC complexes on the cell surface allows CD8-independent recognition and lysis of the target cells. In contrast, the CTLs could kill peptide-loaded RMA-HHD cells with ~90% efficiency (data not shown) thereby suggesting that the MHC expression was still limiting the extent of cytotoxicity. However, the PSMA27-specific CTLs showed no cytotoxicity against TNFα-treated (Figure 37) or untreated (data not shown) LNCaP cells not loaded with peptide. Additionally, the high avidity PSMA27-specific CTLs expanded were also unable to kill TNFα-treated LNCaP cells (data not shown).

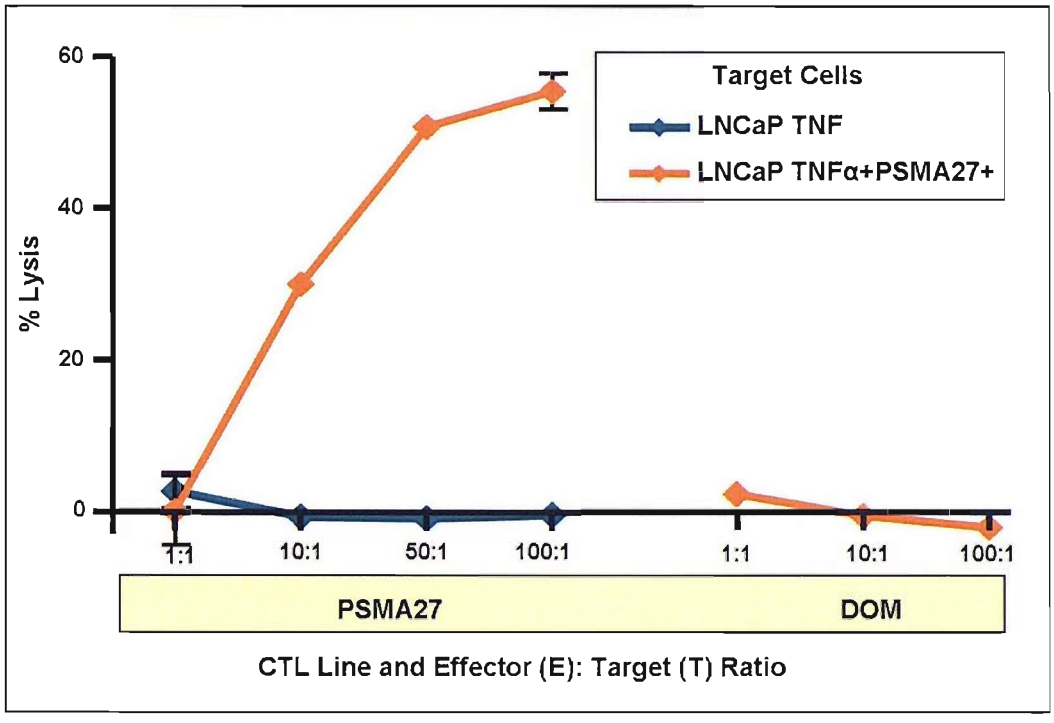


Figure 37: Cytotoxic activity of PSMA27-specific CTLs against TNFα-treated LNCaP cells

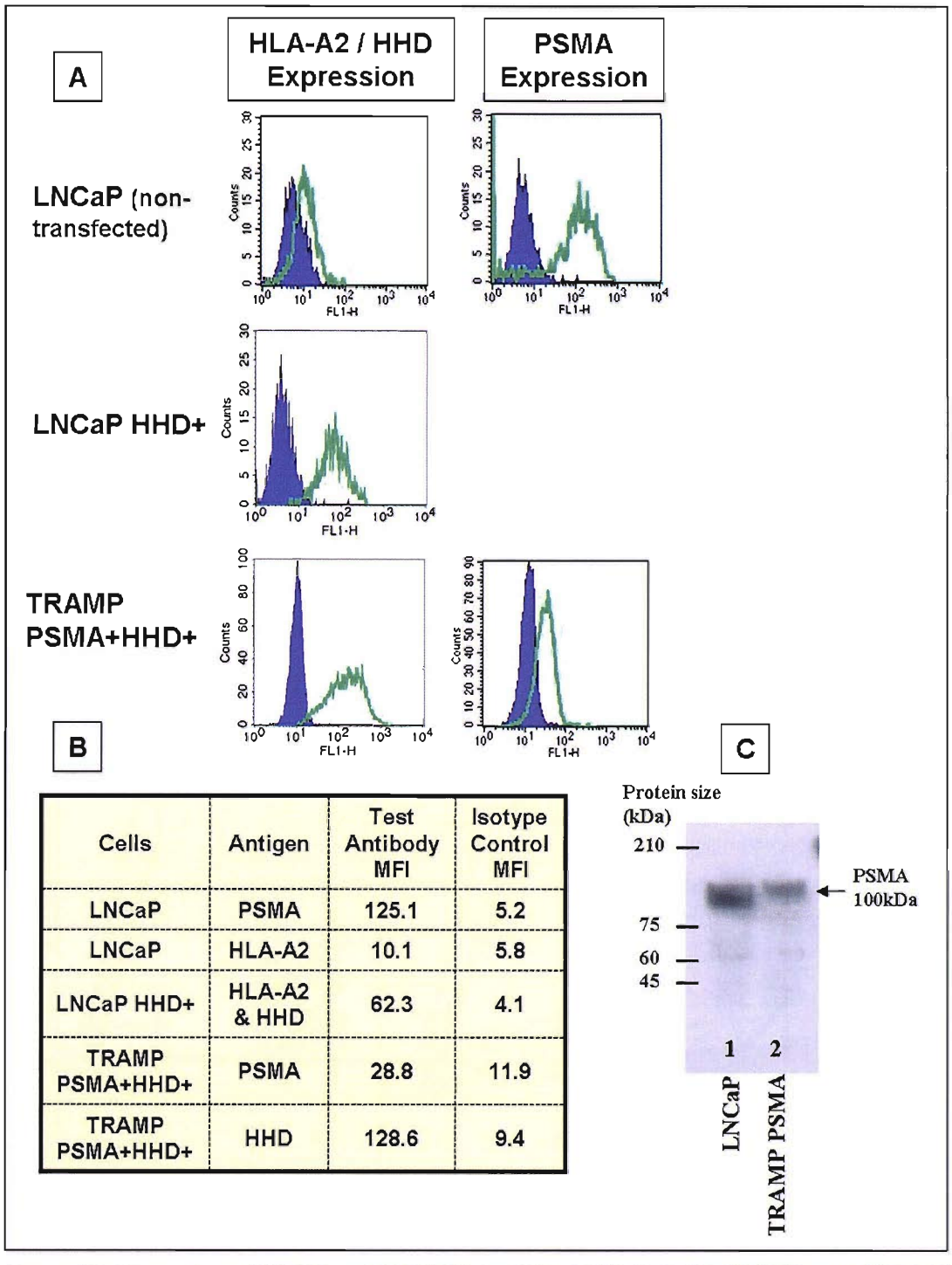
Male HHD mice were immunised with p.DOM-PSMA27 or p.DOM control vaccines via the intramuscular route (50μg DNA) and sacrificed on day 14. CTL lines were generated by *in vitro* culture of splenocytes with 10nM PSMA27 peptide and IL-2. After 6 days of culture the splenocytes were used in a 5 hour ^{51}Cr release assay. The LNCaP target cells were treated with TNFα for 24 hours and were used as either peptide-loaded (orange) or not peptide-loaded (blue) targets.

3.3.18 Generation of LNCaP Target Cells Expressing HHD

The work presented thus far unequivocally demonstrates that the PSMA27 peptide is efficiently processed and presented in context of the HHD MHC class I (Figures 26 and 32). There are only two possible explanations for the inability of the PSMA27-specific CTLs to kill TNFα-treated LNCaP cells. Firstly, low avidity CTLs may require the stabilising CD8-MHC interaction for the recognition of endogenously processed peptides (and not for high concentrations on peptide-loaded targets). It may be that PSMA27-specific CTLs of higher avidity than those expanded in the present study (100pM) are required to overcome lack of CD8-MHC interaction. Secondly the LNCaP cells may potentially have an antigen processing defect developed to evade host immunity that impairs the cells from processing or presenting the PSMA27 peptide.

In order to provide the full engagement of the HHD mouse $CD8^+$ T cells which should result in optimal recognition of the target cells, the LNCaP cells were retrovirally transduced to express the chimeric HHD MHC class I molecule. HHD-expressing amphotropic retrovirus was used to infect the human LNCaP cells. Two days after transduction the cells were labelled with anti-HLA-A2-FITC conjugated antibody and the

positive population sorted. Both HLA-A2 and HHD are labelled by the same antibody (to our knowledge there isn't a discriminating antibody available) so we took advantage of the very low HLA-A2 expression by LNCaP cells and bulk sorted for HHD-transduced LNCaP cells that had very much higher MFI staining than the non-transduced counterparts. In this manner it was guaranteed that the population that was being sorted did express HHD and the higher levels of MHC class I selected for can only potentially enhance cytotoxicity. Single cell sorting was not possible due to a combination of slow growth rates and high sensitivity of the LNCaP cells that resulted in death after sorting in most cases. A comparison of phenotypes (PSMA and HHD/HLA-A2 expression) of LNCaP, HHD-expressing LNCaPs (LNCaP HHD+) and TRAMP PSMA+HHD+ transduced cells can be seen in Figure 38.



Cells	Antigen	Test Antibody MFI	Isotype Control MFI
LNCaP	PSMA	125.1	5.2
LNCaP	HLA-A2	10.1	5.8
LNCaP HHD+	HLA-A2 & HHD	62.3	4.1
TRAMP PSMA+HHD+	PSMA	28.8	11.9
TRAMP PSMA+HHD+	HHD	128.6	9.4

Figure 38: Expression of PSMA and HHD/HLA-A2 by LNCaP, LNCaP HHD+ and TRAMP PSMA+HHD+ cells

The non-transduced LNCaP cell line and the transduced LNCaP HHD+ and TRAMP PSMA+HHD+ cells were analysed for levels of PSMA and HHD/HLA-A2 (cross-reactivity) expression. The flow cytometry plots (A) amd the MFI levels (B) are shown. A western blot with an anti-PSMA antibody demonstrates whole protein levels in cell lysates (prepared at $5x10^6$ cells per ml) of LNCaP and TRAMP PSMA+ cells (C). PSMA expression by LNCaP HHD+ cells is not independently analysed as it is the same for the non-transduced LNCaP cells.

3.3.19 Inability of PSMA27-Specific CTLs to Kill LNCaP Cells Expressing HHD

PSMA27-specific CTLs primed by vaccination were assessed for cytotoxicity of LNCaP cells expressing the chimeric HHD. These targets provide the same MHC class I-CD8 recognition present for the efficient killing of TRAMP PSMA+HHD+ cells (Figure 26). Male HHD mice were vaccinated with p.DOM-PSMA27 or p.DOM control vaccines and sacrificed on day 14. The splenocytes were cultured with 10nM PSMA27 peptide and IL-2 to expand PSMA27-specific CTL lines. After 6 days, a 5 hour ^{51}Cr release assay was performed at different E:T ratios using the various transduced TRAMP cells and LNCaP HHD+ cells as targets (Figure 39).

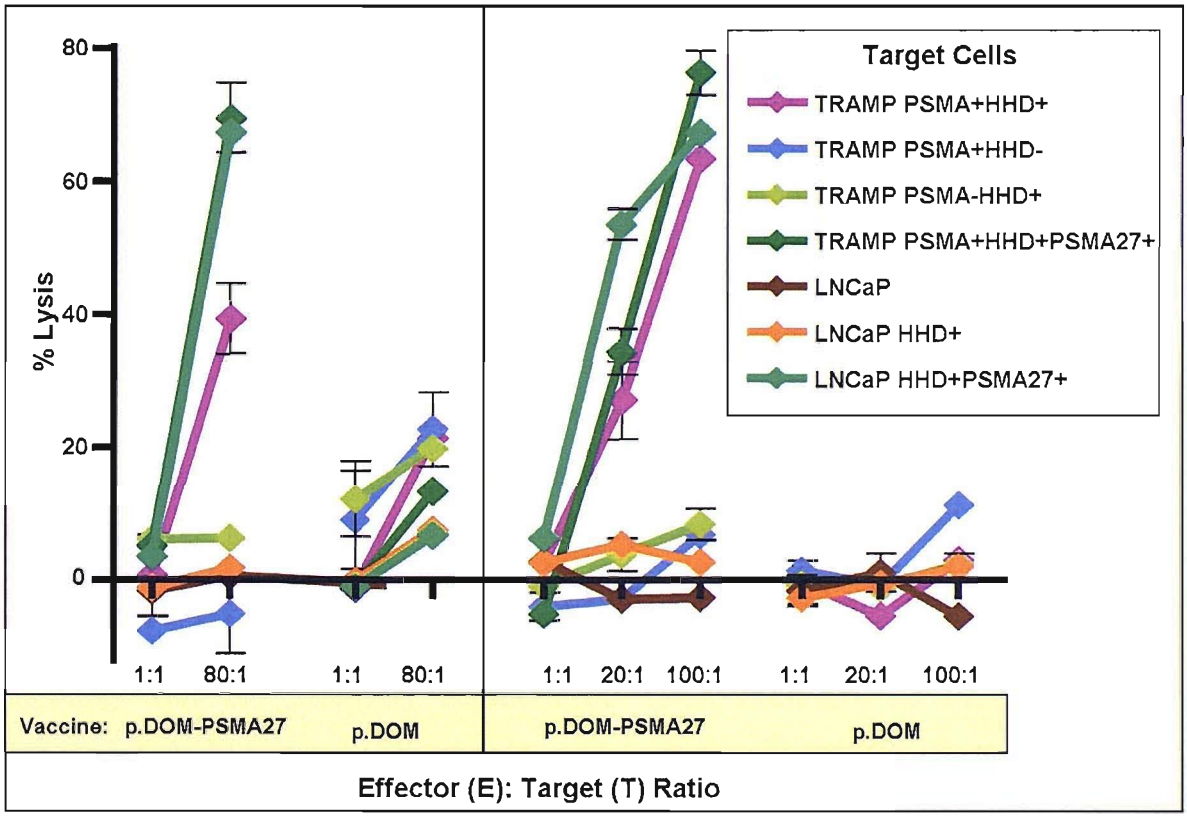


Figure 39: PSMA27-specific CTLs are unable to kill HHD-expressing LNCaP cells

PSMA27-specific CTLs were expanded *in vitro* with 10nM PSMA27 peptide and IL-2 for 6 days. A ^{51}Cr release assay was performed with a range of target cells to assess and compare killing of LNCaP cells transduced to express the HHD (LNCaP HHD+) and TRAMP PSMA+HHD+ cells. Positive control target cells were loaded with 1μM PSMA27 peptide. The error bars represent the SEM of the triplicates. The results shown here are of two separate experiments.

The PSMA27-specific CTLs were able to kill the TRAMP PSMA+HHD+ cells to a high extent (up to 63% lysis) whilst the lysis of the control TRAMP PSMA+HHD- and PSMA-HHD+ target cells remained low at below 10%. In contrast, the same PSMA27-specific CTLs were unable to lyse any LNCaP HHD+ cells at all (Figure 39). The PSMA27-

specific CTLs were however able to kill both TRAMP PSMA+HHD+ and LNCaP HHD+ cells loaded with 1μM PSMA27 peptide to similar extents (78% and 67% respectively). This indicates that the levels of HHD expression were sufficient on the LNCaP HHD+ cells to enable similar recognition by the PSMA27-specific CTLs, despite the lower MFI levels obtained when HHD expression was assessed as compared to that by TRAMP PSMA+HHD+ cells (Figure 38). Our hypothesis to explain the inability to kill these LNCaP HHD+ cells is that there may be some form of antigen processing problem. Investigating this further is not the objective of this project.

3.3.20 Amplifying p.DOM-PSMA663 $CD8^+$ T-Cell Responses with a Prime-Electroporation Boost Vaccination Protocol

Published work from our laboratory has demonstrated that administering a boosting vaccination with electroproation (EP) substantially enhances $CD8^+$ T-cell responses compared to the primed response or after a DNA only boost [166]. This vaccination protocol was applied with the p.DOM-PSMA663, the vaccine that had generated the lowest magnitude $CD8^+$ T-cell responses after a single priming vaccination.

Male HHD mice were intramuscularly immunised with the p.DOM-PSMA663 vaccine (50μg DNA). 28 days later, a boosting vaccination with the same vaccine construct and dose was administered with electroporation immediately after each injection. The mice were sacrificed 8 days later (day 36) and splenocytes assessed for the presence of PSMA663-specific $CD8^+$ T cells responding to varying concentrations of PSMA663 peptide or 1μM p30 peptide in an IFNγ capture ELISPOT.

Boosting with EP effectively enhanced the mean PSMA663-specific $CD8^+$ T-cell responses 6-fold compared to the priming only responses (Figure 40). Responses reached 1070 IFNγ spots per million splenocytes when stimulated with 1μM PSMA663 peptide (mean 623 (± 128.3)), the concentration at which the responses appeared to peak. High responses were also achieved when stimulation was carried out with lower PSMA663 peptide concentrations. Lowering the peptide concentration to 0.1μM only lowered the mean response marginally (mean 601 (± 124.2)), whilst a further 10-fold drop in peptide concentration to 10nM began to have more impact with the mean response at 454 (± 86.8) IFNγ spots per million splenocytes. Stimulation with 1nM PSMA663 peptide still showed a good response with a mean of 277 (± 113.5), however it is statistically insignificant due to the very small sample number (2 mice).

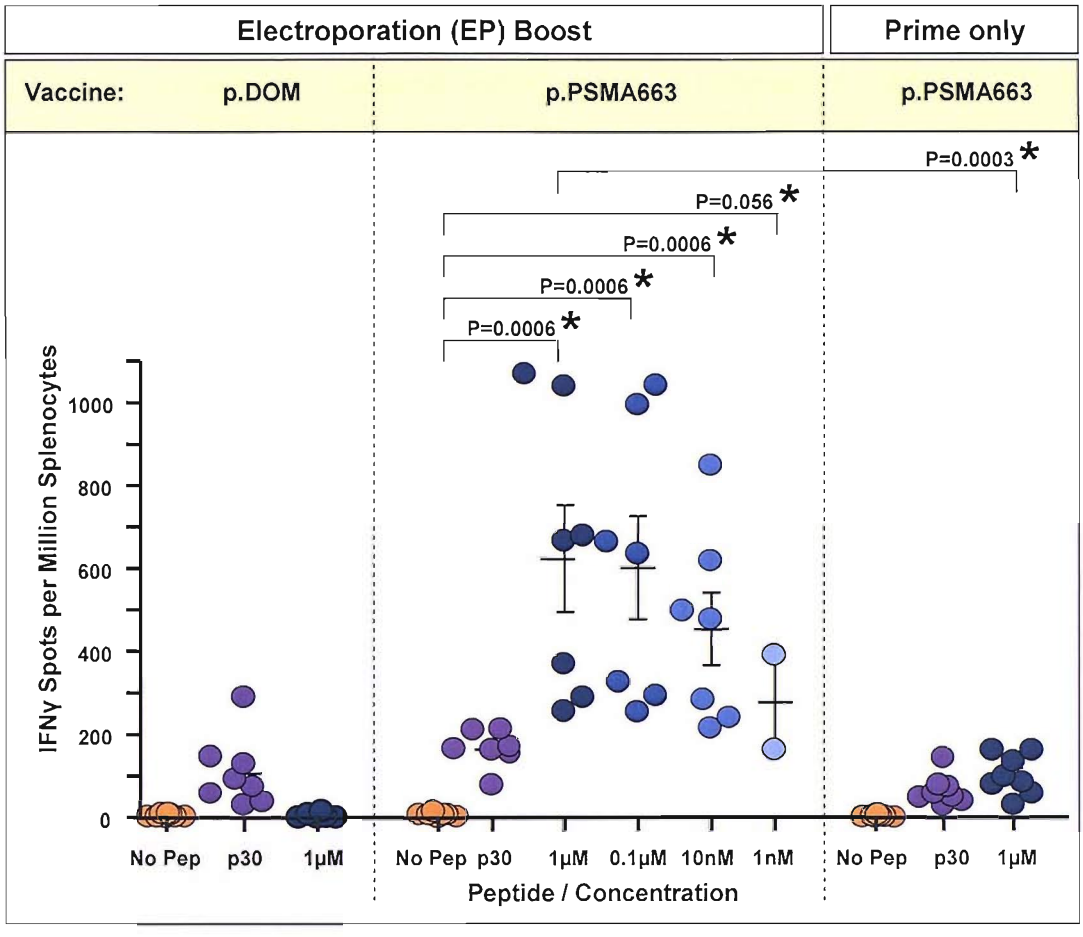


Figure 40: IFNγ responses of peptide-stimulated *ex vivo* PSMA663-specific CD8$^+$ T cells elicited by prime and EP boost

Male HHD mice were primed and boosted on day 28 with the p.DOM-PSMA663 vaccine or p.DOM control vaccines. The boost was accompanied by electroporation (EP) and mice were sacrificed on day 36 (8 days post-boost). PSMA663-specific CD8$^+$ T-cell responses were assessed by peptide stimulation in an IFNγ ELISPOT. The responses of primed only mice day 14 ELISPOT (from Figure 19) were added for comparison. The responses of individual mice were assessed with the average of the triplicates plotted. The mean and SEM error bars for the response against each peptide/concentration are shown. The results are compiled from two seperate experiments.

The avidity of the EP boosted response is higher than that of the primed only response (Figure 19). The maximal response is similar, reached with 1μM PSMA663 peptide. However a clear difference was noted with each 10-fold PSMA663 peptide decrease, particularly at 10nM and 1nM where the differences were statistically significant. Most striking is the difference in peptide concentration at which 50% of the maximal T cell responsiveness was achieved, that is 10nM for the primed only and 1nM for the EP boosted T-cell populations. This indicates that the PSMA663-specific CD8$^+$ T cells expanded by boosting with EP were of 10-fold higher avidity.

All mice responded to p30 peptide stimulation, indicating the presence of p30-specific CD4$^+$ T-cell responses and confirming vaccine performance. The levels of both p30 CD4$^+$

T cell and PSMA663 $CD8^+$ T-cell responses were enhanced by the EP boost when compared to priming alone. As demonstrated earlier for priming only, the p.DOM control mice did not generate any PSMA663-specific responses.

3.3.21 Ability of DNA Vaccines Encoding Full-Length Human PSMA to Generate $CD8^+$ T cells after Prime-EP Single Boost Protocol

Initially, one of the aims of this project was to explore a prime-electroporation boost vaccination protocol using a combination of a DNA vaccine encoding the full-length human PSMA to prime and the p.DOM-PSMA-epitope vaccines for boosting. Priming with a full-length tumour antigen vaccine followed by boosting with p.DOM-epitope vaccines is a vaccination regime that has not been previously studied in our laboratory. We were hoping that the PSMA model would give insight into whether such an approach may be of benefit in patients in terms of being able to prime $CD8^+$ T-cell responses against multiple epitopes at once with a single construct that could then be boosted with the individual p.DOM-PSMA-epitope vaccines. However, a single priming immunisation alone with the full-length PSMA vaccines (p.PSMA and p.PSMA-DOM) in HHD mice was unable to induce a detectable *ex vivo* response (Figure 23). A preliminary experiment in which mice were primed with p.PSMA-DOM or p.DOM control vaccines and then boosted (EP) with a p.DOM-PSMA-epitope vaccine confirmed the absence of a primed response in the p.PSMA-DOM group of mice. The assayed PSMA peptide-specific *ex vivo* responses were the same whether the mice were primed with p.PSMA-DOM or p.DOM vaccines (data not shown). This further confirms the inability of the full-length PSMA vaccines to prime $CD8^+$ T-cell responses.

Given the vast enhancement in generating PSMA663-specific $CD8^+$ T-cell responses after an EP boost with the p.DOM-PSMA663 vaccine compared to priming only (Figure 41), the same protocol was administered with the full-length PSMA vaccines (p.PSMA and p.PSMA-DOM). The aim of this experiment was to assess if these full-length vaccines could generate any CD8 epitope-specific responses.

Male HHD mice were immunised with p.PSMA or p.PSMA-DOM and boosted with the same construct administered with electroporation on day 28. Mice were subsequently sacrificed on day 36 and responses assessed by IFNγ ELISPOT. These prime-boost results are from three pooled experiments with a total of 16 mice per vaccine group, where the same trend was observed in each experiment. The responses were very low and variable as some mice did not respond at all whilst others responded to some peptides. The data has

been analysed in two ways; the statistical significance of mean responses for each group of mice has been calculated but also a threshold of responsiveness has been arbitrarily assigned. In this manner a positive response is defined as any response by a single mouse to a single peptide that is more than twice the "no peptide" background for that particular mouse but also more than the "no peptide" average of the entire group of 16 mice. This was necessary in order to assess the few positive responses to some of the pepides without the large proportion of non-responders obscuring the result through the calculation of population means. This is apparent for example with the responses by p.PSMA-DOM vaccinated mice to PSMA4, PSMA27 and PSMA711 peptides. The response means were identical at 10.4 (±1.9), 10.4 (±2.3) and 10.4 (±2.2) respectively however only the PSMA4 response is calculated as statistically significant by virtue that the variability (SEM) in responses was less than for the other peptides.

The p.PSMA vaccine was able to effectively and significantly prime PSMA663-specific $CD8^+$ T cells (mean 16.4 (±2.8)) in 9/16 mice (of up to 43 IFNγ spots per million splenocytes, Figure 41), confirming a previous report that PSMA663 is the immunodominant peptide and that the processing of the peptide is efficient. As a population (mean responses) there were no statistically significant responses to the other PSMA peptides (PSMA4 mean 8.0 (±1.3), PSMA27 mean 9.8 (±1.9) and PSMA711 mean 9.1 (± 1.4)) for which the values were approximately half of that for the PSMA663 peptide. However, there were two individual mice with responses to PSMA4, three that responded to PSMA27 and three responsive to PSMA711 at levels above the assigned positive threshold. These are only suggestive of minimal responses against the sub-dominant PSMA peptides.

The p.PSMA-DOM vaccine generated $CD4^+$ T-cell responses to the p30 peptide (mean 59.9 (±17.3)) in 15/16 mice upon boosting, suggesting that vaccination was successful in the majority of mice. These were significantly lower than the typical p30 responses achieved after a single administration of p.DOM-epitope vaccines (Figures 18-21). The p.PSMA-DOM fusion vaccine was also able to generate statistically significant $CD8^+$ T-cell responses specific for the PSMA663 peptide (mean 19.9 (±6.8)) in 5/16 mice, that reached up to 115 IFNγ spots per million splenocytes (Figure 41). In addition the responses against PSMA4 (mean 10.4 (±1.9)) were also statistically significant compared to the "no peptide" background (mean 4.7 (±0.8)) but not against PSMA27 (mean 10.4 (±2.3)) and PSMA711 (mean 10.4 (±2.2)) peptides despite the means being the same as explained above. The PSMA663-specific responses were approximately twice as high as

those for the other three PSMA peptides in the p.PSMA-DOM vaccinated mice. Interestingly, there was more variety in the response compared to that seen for the p.PSMA vaccine. There were five, six, and eight individual mice that displayed responses above the threshold against PSMA4, PSMA27 and PSMA711 respectively. These numbers are more than double those observed with the mice vaccinated with p.PSMA, whilst the opposite is the case for PSMA663-specific $CD8^+$ T-cell responses where approximately twice as many p.PSMA-vaccinated mice responded.

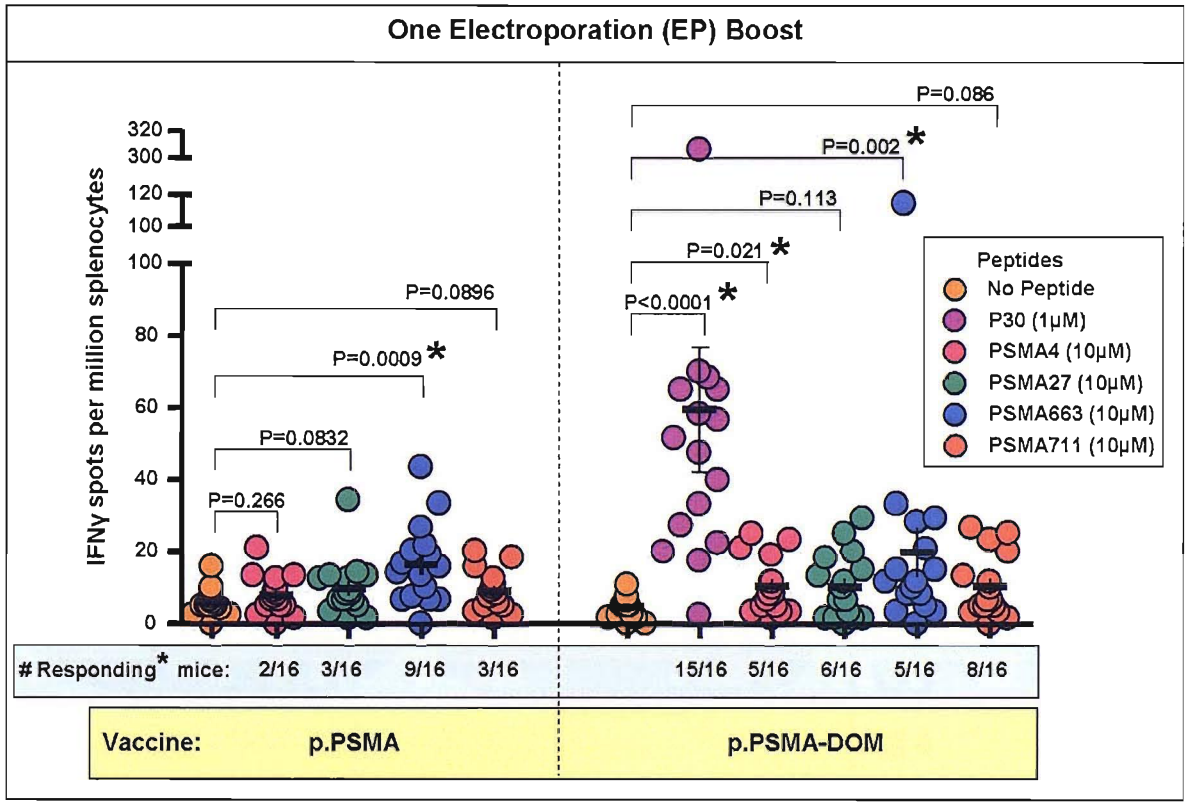


Figure 41: *Ex vivo* IFNγ responses of PSMA4, PSMA27, PSMA663 and PSMA711-specific $CD8^+$ T Cells after prime and one EP boost with full-length PSMA vaccines

Male HHD mice were primed and boosted on day 28 with p.PSMA or p.PSMA-DOM vaccines. The boost was accompanied by electroporation (EP) and mice were sacrificed on day 36 (8 days post-boost). Splenocytes were stimulated in an *ex vivo* IFNγ ELISPOT without any peptide to measure background levels (orange), or with p30 (purple, at 1μM), PSMA4 (pink), PSMA27 (green), PSMA663 (blue), or PSMA711 (red) peptides at a 10μM concentration. The average of triplicates are plotted. The mean and SEM error bars for the response against each peptide/concentration are shown. The results are compiled from three seperate experiments. "Responding* Mice" represents the number of mice that have responded to the particular peptide as defined by a value that is more than twice the "no peptide" background for the particular mouse and more than the "no peptide" population population mean in the vaccine group.

These results further demonstrate the superiority in performance of the p.DOM-PSMA-epitope vaccines with which priming immunisations alone reached epitope-specific $CD8^+$ T-cell responses that are 19-fold, 100-fold and 50-fold higher for PSMA27 (Figure 18),

PSMA663 (Figure 19) and PSMA711 (Figure 20) respectively than those achieved by the full-length p.PSMA-DOM vaccine after a single EP boost. The administration of the p.DOM-PSMA663 vaccine with an EP boost (Figure 40) resulted in ~40-fold higher PSMA663-specific responses than the full-length vaccines administered with the same protocol (Figure 41).

Each individual mouse was also assessed for the number of the four PSMA peptides they responded against. The responding thresholds explained above were used for this analysis. Mice immunised with the p.PSMA vaccine primarily responded to only one PSMA peptide (7/16 [43.75%]) or had no detectable responses against any of the PSMA MHC class I-binding peptides (6/16 [37.5%], Figure 42). The specificity of the responses by those mice that only responded to a single peptide *ex vivo* was always PSMA663. Only 3/16 mice (18.75%) responded to more than one PSMA peptide.

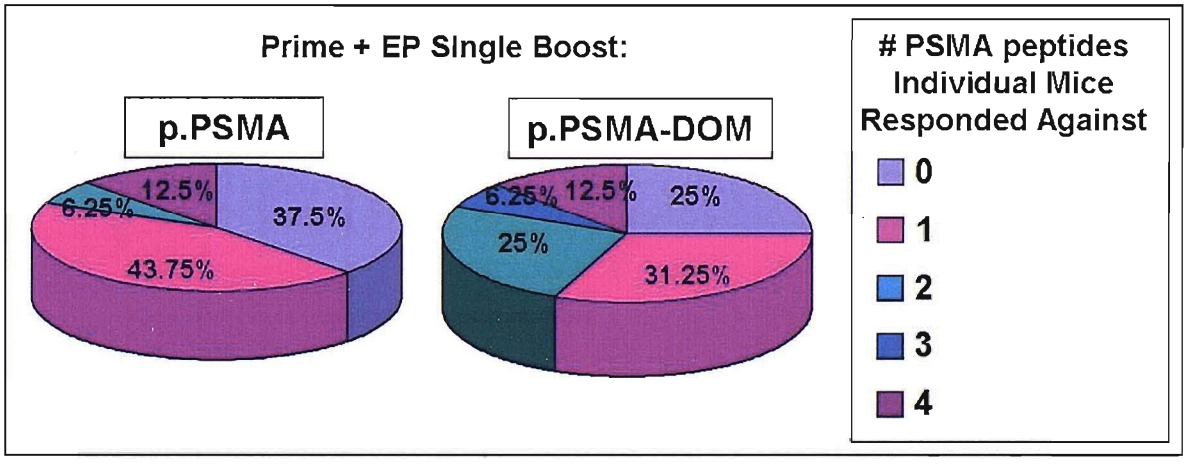


Figure 42: Proportion of individual mice primed and EP boosted once with p.PSMA or p.PSMA-DOM responding to different numbers of PSMA peptides

HHD mice were primed and boosted once with EP on day 28. They were sacrificed on day 36 (8 days post-boost) and splenocytes stimulated in *ex vivo* IFNγ ELISPOT with p30, PSMA4, PSMA27, PSMA663 or PSMA711 peptides. Each individual mouse was assessed for how many PSMA peptides they generated *ex vivo* responses against (according to defined threshold: positive responses were more than the "no peptide" background for the individual mouse and more than the "no peptide" background mean of the population).

In contrast, the splenocytes of 5/16 (31.25%) mice immunised with the p.PSMA-DOM vaccine could respond only to a single peptide, none of which were the immunodominant PSMA663. These single peptide responding mice had specificity for PSMA4 (n=1), PSMA27 (n=2) and PSMA711 (n=2) thereby demonstrating single but heterogenous responses. 7/16 (43.75%) p.PSMA-DOM vaccinated mice generated T-cell responses that *ex vivo* could be stimulated by more than one PSMA peptide (Figure 42). Interestingly, the

mouse with the outlying highest p30-specific response (307 IFNγ spots per million splenocytes) also exhibited higher end responsiveness to all four PSMA peptides (ranging from 21 to 29 IFNγ spots per million splenocytes).

A CTL line was established from the splenocytes from p.PSMA-DOM vaccinated mice by stimulating with irradiated TRAMP PSMA+HHD+ cells and IL-2. After 6 days a ^{51}Cr release assay was performed with these CTLs at a variety of E:T ratios.
The CTL line was able to kill the TRAMP PSMA+HHD+ target cells with relatively good efficiency of 26% at 100:1 E:T ratio compared to the absolute lack of killing of the PSMA+HHD- and PSMA-HHD+ control cells. Despite the low detection of *ex vivo* epitope $CD8^+$ T-cell responses, after only brief *in vitro* expansion they were able to display significant cytotoxicity against the PSMA and HHD-expressing target cells (Figure 43).

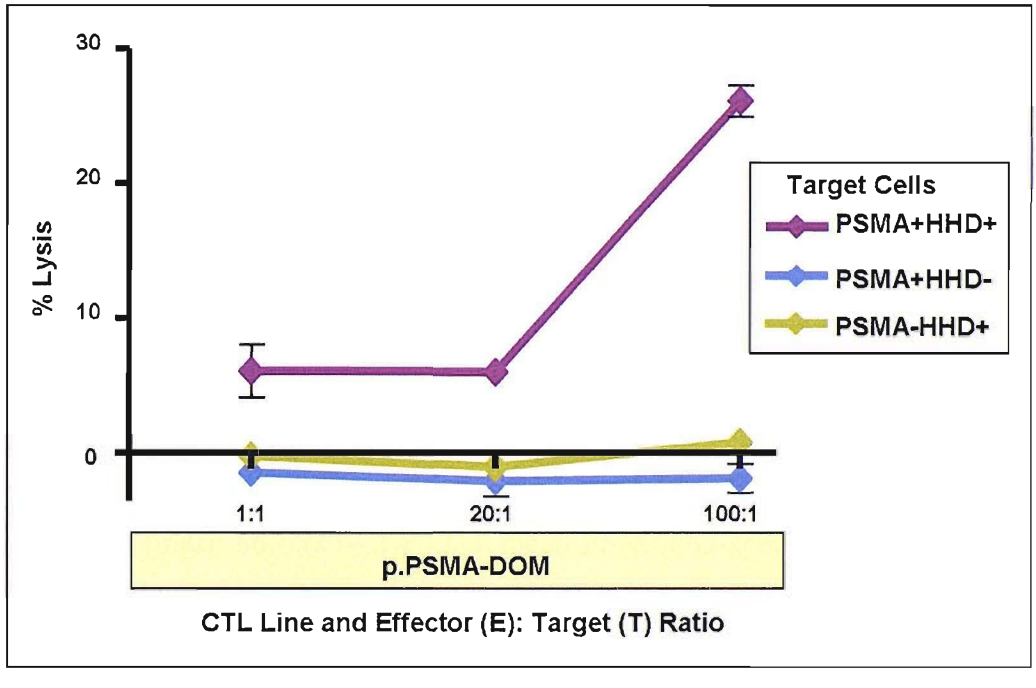


Figure 43: CTLs primed by the p.PSMA-DOM vaccine can kill PSMA-expressing cells
CTLs were expanded *in vitro* with irradiated TRAMP PSMA+HHD+ cells and IL-2 for 6 days. A ^{51}Cr release assay was performed with a range of target cells to assess killing of TRAMP PSMA+HHD+ cells. The error bars represent the SEM of the triplicates. The results shown here are of two separate experiments.

There may be a slight difference in the responses generated by p.PSMA and p.PSMA-DOM vaccines after priming plus a single EP boost immunisation. However, the responses are very low and most of the population means statistically insignificant therefore making these results only suggestive. The differential targeting or possible misfolding of the protein due to the inclusion of the DOM fusion could be responsible for any differences observed. The ability to expand CTLs that kill moderate levels of the

PSMA and HHD-expressing cells demonstrates that the p.PSMA-DOM vaccine must have primed some HHD-restrcited PSMA-specific $CD8^+$ T cells, even if only marginally detectable *ex vivo*.

3.3.22 Ability of DNA Vaccines Encoding Full-Length Human PSMA to Generate $CD8^+$ T cells After a Prime-EP Double Boost Protocol

The epitope-specific responses remained low after a single EP boost to the extent that the immunodominant response could be clearly observed but that to other peptides was more obscure. For this reason a second EP boost step was added to the protocol. Male HHD mice were immunised with p.PSMA or p.PSMA-DOM and boosted with the same construct administered with EP on days 28 and 56. Mice were subsequently sacrificed on day 64 and responses assessed by IFNγ ELISPOT. These prime-double EP boost results are from two pooled experiments with a total of 9 mice per vaccine group. The same trend was observed in each experiment.

The first noticeable difference between the one and two EP boost results is the spread of responses. The "no peptide" backgrounds have substantially increased ~3-fold from 6.0 (±0.9) to 16.1 (±3.0) for p.PSMA and from 4.7 (±0.8) to17.8 (±4.6) for p.PSMA-DOM vaccines (Figures 41 and 44). The only statistically significant $CD8^+$ T-cell responses are to the PSMA663 peptide for p.PSMA (mean 66.6 (±15.6)) and p.PSMA-DOM (mean 70.7 (±32.4)) vaccinated mice. Additionally there were p30-specific helper T-cell responses in the latter group (mean 66.1 (±14.2)). The mean PSMA663-specific responses have been clearly boosted by this two EP protocol 3-4-fold in comparison to the one EP protocol (Figure 41). The proportion of mice responding to the PSMA663 peptide above the defined threshold also increased with the second EP boost from 9/16 (56.3%) to 8//9 (88.9%) for the p.PSMA vaccine and from 5/16 (31.3%) to 5/9 (55.5%) for the p.PSMA-DOM vaccine. In contrast, the proportions of mice responding above the defined threshold for the other three PSMA peptides decreased with the second EP boost. These observations are in accordance to the immunodominance theory where boosting enhances the response to the immunodominant epitope which outcompetes the sub-dominant responses. Surprisingly the mean response to the p30 peptide has remained similar with a one EP boost mean of 59.5 (±17.3) and a two EP boost mean of 66.1 (±14.2).

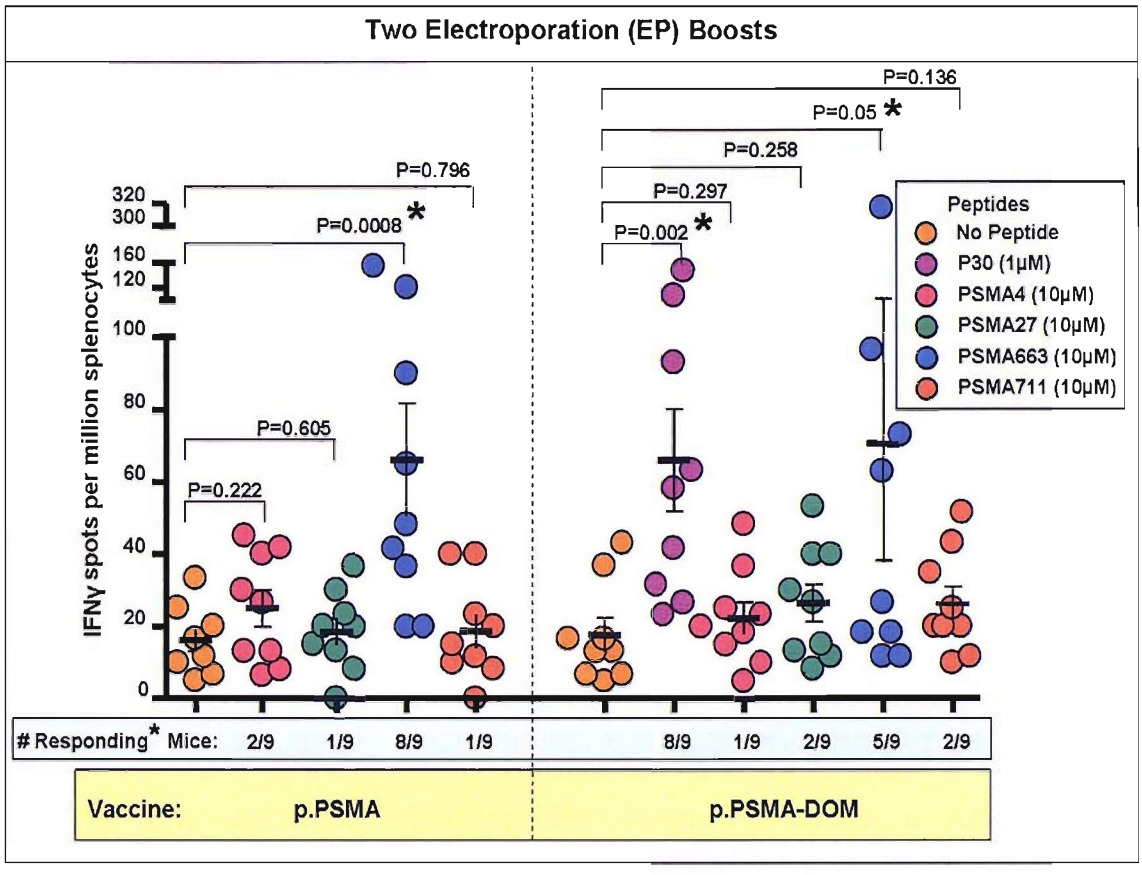


Figure 44: Ex vivo IFNγ responses of PSMA4, PSMA27, PSMA663 and PSMA711-specific CD8+ T Cells after prime and two EP boost with full-length PSMA vaccines

Male HHD mice were primed on day 0 and boosted twice on days 28 and 56 with p.PSMA or p.PSMA-DOM vaccines. Both boosts were accompanied by electroporation (EP) and mice were sacrificed on day 64 (8 days post-boost). Splenocytes were stimulated in an *ex vivo* IFNγ ELISPOT without any peptide to measure background levels (orange), or with p30 (purple, at 1μM), PSMA4 (pink), PSMA27 (green), PSMA663 (blue), or PSMA711 (red) peptides at a 10μM concentration. The average of triplicates for each individual mouse was plotted. The mean and SEM error bars for the response against each peptide/concentration are shown. The results are compiled from two seperate experiments. "Responding* Mice" represents the number of mice that have responded to te particular peptide as defined by a value that is more than twice the "no peptide" background and more than the "no peptide" population mean for the vaccine.

Overall, it appears that DNA vaccines encoding the full-length PSMA sequence can prime some $CD8^+$ T-cell responses specific for the HLA-A2-binding PSMA peptides under investigation in a proportion of mice. This is observed particularly when a single EP boost vaccination protocol is used. However, with two boosting immunisations immunodominance unequivocally takes over. The reason for this could be purely down to numbers where a single immunisation was unable to prime any detectable $CD8^+$ T-cell responses to any peptide and therefore the first EP boost essentially acted as a priming vaccination. The second EP boost would then actually act as a first boost, leading to PSMA663 peptide immunodominance.

3.3.23 Ability of DNA Vaccines Encoding Truncated Human PSMA to Generate $CD8^+$ T cells After Prime-EP Single Boost Protocol

One possible explanation for the low p30 responses in mice primed and boosted once with EP with the p.PSMA-DOM vaccine compared to that achieved by the p.DOM-PSMA-epitope vaccines, could be ineffcicient *in vivo* transfection/expression of the large construct (~135kDa vs ~35kDa). In order to attempt to evaluate this question regarding the molecular size of the construct, shorter PSMA vaccine constructs were created.

Conveniently there are two NheI restriction sites within the human PSMA sequence. When the human PSMA sequence is digested with NheI amino acids 119 to 586 are removed, leaving a 273 amino acid sequence with a conserved open reading frame. This removal creates smaller vaccines p.PSMAshort and p.PSMAshort-DOM, which are ~33kDa and ~68kDa respectively. This is significantly smaller than ~100kDa of p.PSMA and ~135kDa of p.PSMA-DOM.

Male HHD mcie were immunised with p.PSMAshort and p.PSMAshort-DOM vaccines in a prime day 0 and EP boost day 28 protocol. The mice were sacrificed on day 36 (8 days post-boost). Splenocytes were stimulated *ex vivo* with 1μM p30 or 10μM of each of the four PSMA peptides used in this project, for 24 hours. Responses to peptide stimulation are expressed as the number of IFNγ spots per million splenocytes.

Despite the smaller sizes of the p.PSMAshort and p.PSMAshort-DOM vaccines, they were unable to prime any statistically significant $CD8^+$ T-cell responses specific for any of the four PSMA peptides (Figure 45). Mice immunised with the p.PSMAshort-DOM (~68kDa) vaccine generated mean p30 responses of 74.4 (±17.0), not significantly different to that from p.PSMA-DOM (~135kDa)-vaccinated mice. These results would argue that the size of the construct is not affecting peptide processing from the protein. These modified shorter PSMA vaccines are likely to be aberrantly folded and differentially targeted thereby contributing a lot of other variables. These do not represent suitable constructs for the induction of PSMA-specific $CD8^+$ T-cell responses.

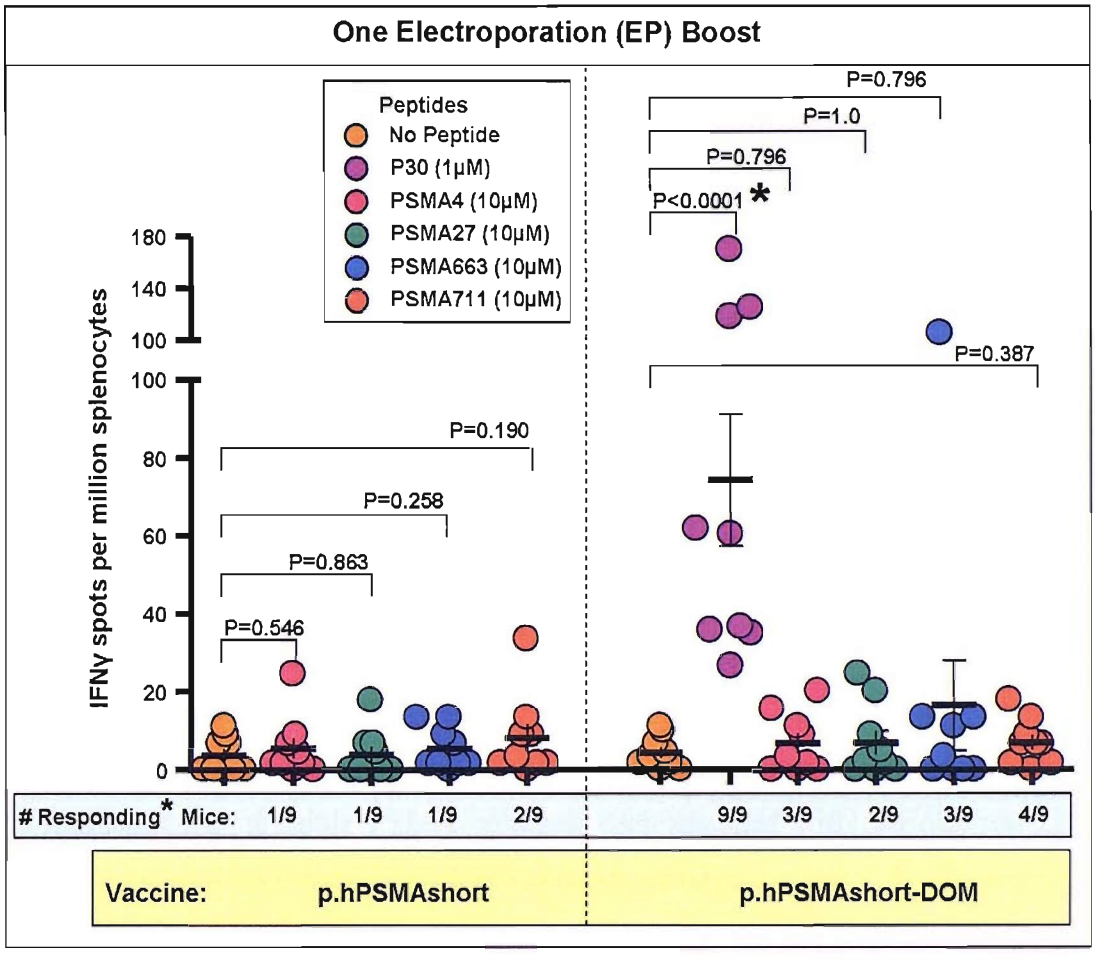


Figure 45: ***Ex vivo*** **IFNγ responses of PSMA4, PSMA27, PSMA663 and PSMA711-specific CD8$^+$ T Cells after prime and one EP boost with vaccines encoding short PSMA proteins**

Male HHD mice were primed and boosted on day 28 with p.PSMAshort or p.PSMAshort-DOM vaccines. The boost was accompanied by electroporation (EP) and mice were sacrificed on day 36 (8 days post-boost). Splenocytes were stimulated in an *ex vivo* IFNγ ELISPOT without any peptide to measure background levels (orange), or with p30 (purple, at 1μM), PSMA4 (pink), PSMA27 (green), PSMA663 (blue), or PSMA711 (red) peptides at a 10μM concentration. The average of triplicates for each individual mouse is plotted. The mean and SEM error bars for the response against each peptide/concentration are shown. The results are compiled from two seperate experiments. "Responding* Mice" represents the number of mice that have responded to te particular peptide as defined by a value that is more than twice the "no peptide" background for the particular mouse and more than the "no peptide" population population mean in the vaccine group.

3.4 Discussion

3.4.1 Induction of $CD8^+$ T-cell Responses by the four p.DOM-PSMA-epitope DNA Vaccines

It has previously been shown that the p.DOM-peptide DNA vaccine format can activate high levels of CTLs specific for H-2-binding epitopes in Balb/c [158, 159, 166] and C57Bl/6 [156, 157] mice, as well as CTLs specific for HLA-A2-binding epitopes in HHD mice [160]. The assessment of the p.DOM-epitope vaccine in the HHD transgenic mouse model enables the validation of candidate HLA-A2-binding epitopes for clinical application within the HLA-A2 positive population. It is estimated that 40-50% of the Caucassian population is of HLA-A2 haplotype thereby making the HLA-A2-binding peptides validated within this mouse model relevant to a large proportion of cancer patients in the western world.

The investigation of multiple PSMA epitopes, each encoded within a distinct p.DOM-epitope vaccine enables their independent validation as target epitopes. It also provides the potential to elicit $CD8^+$ T-cell responses against multiple epitopes by simultaneous administration of the separate vaccines, mixed and injected at a single site or possibly at separate sites [396]. This is yet to be explored. In the present study the p.DOM-epitope vaccine design has been applied to four HLA-A2-binding PSMA epitopes, PSMA4, PSMA27, PSMA663 and PSMA711, all of which have been described in the literature [230, 231, 250] Table 4).

The four p.DOM-PSMA4, p.DOM-PSMA27, p.DOM-PSMA663 and p.DOM-PSMA711 vaccines are all capable of inducing strong $CD8^+$ T-cell responses specific for their respective epitopes. All the epitope-specific $CD8^+$ T cells were capable of generating high levels of IFNγ in response to peptide stimulation *ex vivo* (Figures 18-21). The $CD8^+$ T cells primed by vaccination with specificity to all four peptides appear to be of similar avidity as seen by the requirement of similar peptide concentrations (10nM) for 50% of the maximal response (IFNγ secretion) upon peptide stimulation (Figure 22). The magnitude of the $CD8^+$ T-cell responses elicited by these four vaccines varied, with the lowest responses primed by the p.DOM-PSMA663 vaccine and the highest responses generated by the p.DOM-PSMA711 vaccine, with a difference of ~5-fold. The p.DOM-PSMA27 vaccine, which is of particular interest, also performed well with responses in between those described for the other vaccines. As a result of the effectiveness of the p.DOM-PSMA27 vaccine in the HHD model as well as other supporting data, the vaccine is

currently being assessed in a PCa clinical trial. It is important to highlight that the mouse model is a non-tolerised system and is therefore likely to have T-cell repertoires specific for and probably of higher affinity for these human PSMA epitopes than $CD8^+$ T cells that have escaped central deletion in humans. The HHD model therefore only provides a model for assessment of the vaccines for confirmation that the $CD8^+$ T cell epitope encoded within can be processed and presented to prime immunity. However, the immunity generated is not necessarily indicative of what may occur in patients.

3.4.2 Prime-Boost Vaccination Protocol Enhances $CD8^+$ T Cell Responses

A powerful strategy to enhance primed T-cell responses is to administer a boosting immunisation. In this case we opted to electroporate with the boosting vaccination to optimally boost responses. The data shows that a prime and EP boost vaccination protocol for immunisation with the p.DOM-PSMA663 vaccine enhanced the epitope-specific $CD8^+$ T-cell response ~6-fold compared to a prime only immunisation. Although data is not presented for the other vaccines, this suggests that such boosting would likely be successful with all the p.DOM-PSMA-epitope constructs.

Memory $CD8^+$ T cells have the distinct advantage of rapid and more avid secondary responses making their induction highly desired and essential for vaccine effectiveness. $CD8^+$ T cells possess regulatory functions which can suppress or support immunity. Early after priming they secrete IFNγ to activate DCs to produce IL-12, an important cytokine for T_{H1} differentiation that serves to generate responses of greater magnitude. However, at the other end of the spectrum CTLs can eliminate not only the intended targets but also antigen-presenting DCs [71]. With these opposing roles the exact biology for the efficacy of secondary responses has been unclear. It may be expected that a rapid more avid $CD8^+$ T-cell population would have a detrimental effect on immunity through efficient and quick elimination of antigen-presenting DCs. A recent publication has provided insight into a mechanism whereby human memory $CD8^+$ T cells, similarly to $CD4^+$ T cells [77], can protect DCs from CTL killing *in vitro* [175]. This implies that a quicker responding memory population would protect DCs from early CTL-mediated elimination by the emerging effector cells. The mechanism appears to involve induced expression of a granzyme inhibitor by DCs rendering them resistant to CTL degranulation. Similarly to primed naïve cells, memory $CD8^+$ T cells were also able to enhance IL-12 secretion by DCs [175]. These findings highlight the importance of allowing sufficient time for a memory population to develop prior to boosting immunisations, as is the case in our prime-EP boost regimen, in order to achieve optimal responses. Since $CD4^+$ T cells can provide

similar protection to DCs [77] it may be that the cognate memory helper $CD4^+$ T-cell responses provided by our vaccine design could enhance immunity in a similar fashion.
The helper role of memory T cells may potentially be exploited by the inclusion of tumour-irrelevant CTL epitopes in vaccines, although means of avoiding immunodominance may be necessary. This proof of principle has been demonstrated in a therapy setting where LCMV pre-immune mice were challenged with tumour prior to the administration of a tumour lysate-loaded DC vaccine additionally loaded with the dominant LCMV K^b peptide. These mice exhibited improved tumour protection compared to those receiving the lysate-loaded DC vaccine without the LCMV K^b peptide, but only if pre-immunised 4 weeks but not 1 week earlier [397].

3.4.3 Induction of $CD8^+$ T Cell Responses by DNA Vaccines Encoding Full-Length PSMA

HHD mice receiving a single priming vaccination with DNA vaccines encoding the full-length human PSMA with the DOM sequence fused to the C-terminal (p.PSMA-DOM) or PSMA alone (p.PSMA), were unable to prime PSMA4, PSMA27, PSMA663 or PSMA711-specific $CD8^+$ T cells.
Upon boosting with electroporation the p.PSMA and p.PSMA-DOM vaccines were able to induce statistically significant PSMA663-specific $CD8^+$ T-cell responses. The p.PSMA-DOM vaccine additionally generated a significant PSMA4-specific response. In order to assess the responding mice amongst the many non-responsive ones a threshold for positive IFNγ spots per million splenocytes was set. This is defined as a response to a single peptide that was more than twice the "no peptide" background for the individual mouse but also more than the mean "no peptide" background for the entire group of mice in the vaccine group. The majority of p.PSMA-vaccinated mice exhibited responsiveness to only one peptide, always PSMA663, whilst only a small proportion (3/16) responded to more than one PSMA peptide above the defined threshold. However, most mice immunised with the p.PSMA-DOM vaccine that exhibited responsiveness had $CD8^+$ T cells that could be stimulated by more than one of the PSMA peptides (7/16) but a lower number of mice exhibited PSMA663-specific responses above the threshold. Despite the low responses, this is suggestive of a wider multi-epitope response to the p.PSMA-DOM.

When mice were administered two EP boosting immunisations with the same vaccines (p.PSMA or p.PSMA-DOM) in a longer protocol, it became clear that the immunodominant PSMA663 epitope-specific response dominated for both vaccines. This

is in accordance with the expected narrowing of the immune response towards the immunodominant epitope after boosting. This observation is also in agreement with that reported by Mincheff et al., where multiple rounds of human PBMC stimulation with PSMA (extracellular domain)-transfected DCs generated $CD8^+$ T cells that could kill largely only the target cells loaded with the PSMA663 and not other peptides [231]. Clear immunodominance was not observed until the second boosting vaccination possibly because the absence of detectable $CD8^+$ T cells after priming would have meant there was nothing to boost. Therefore the first boosting vaccination would have essentially acted as another priming immunisation with the capacity to prime multi-epitope specific T cells.

The possible differences observed in immunity primed by the p.PSMA and p.PSMA-DOM vaccines where the latter primed T cells appeared to be specific for a wider range of PSMA epitopes, can potentially be attributed to a number of factors. The most obvious is the fusion to DOM that can provide strong cognate $CD4^+$ T-cell help. As already discussed helper T cells are implicated in protecting DCs from CTL-mediated killing, which in turn may potentially be able to counteract some of the effects of immunodominance. This possibility, though it cannot be discarded, seems unlikely as immunodominance was unequivocally evident with a second boosting vaccination. The answer more likely lies within other differences between the proteins encoded by these two constructs. The PSMA intracellular and TM domains were retained in these constructs as they contained two of the peptides of interest (PSMA4 and PSMA27). The protein translated from p.PSMA is the correctly folded full-length version that is targeted to the plasma membrane of directly transfected cells as confirmed *in vitro*. However, confirmation of the cellular location of the p.PSMA-DOM protein was not obtained. The PSMA component of the fusion protein could not be detected in its native conformation by flow cytometry or denatured by western blot on cell lysates of transfected cells. This suggests that the fusion protein is not localised in an intracellular compartment or on the cell surface despite encoding the PSMA TM domain. Initiation, type and kinetics of an immune response are highly influenced by the form in which the antigen is administered as a result of the impact this has on the access to antigen processing pathways. Some have compared the differential immunity generated by membrane, intacellular and secreted antigen. Boyle et al., showed that DNA vaccines encoding secreted or membrane-bound OVA antigen primed superior CTL responses than that generated by a vaccine encoded intracellular OVA [398]. However, if the vaccines were administered intradermally, a route resulting in high levels of directly transfected APCs, this difference was abrogated and all three forms of antigen primed similar CTL responses [398]. This is in agreement with the finding from our laboratory

that inclusion of a leader sequence that results in secreted antigen is optimal for priming CTL responses [190] (section 1.3.16.4). A partially contradictory finding has been reported where DNA vaccines encoding cell-associated (intracellular or TM) but not secreted OVA were better at priming $CD8^+$ T cells [399]. This suggests that these observations can differ according to the exact construct and the *in vivo* mechanisms of antigen presentation.

DOM or FrC usually confer great stability to the other component in the fusion that in some cases can aid their expression. The PSMA protein is a functional homodimer and its correct folding and association is dependent on correct glycosylation [342]. It could be that the DOM fusion rather than conferring stability in this case may indirectly interfere with stable dimer formation by not allowing correct glycosylation/folding or it may directly interfere through steric hinderance. Assuming this protein is misfolded, it may be quickly targeted for co-translational proteasomal degradation, possibly entering the defective ribosomal protein (DRiP) pool [36]. In contrast, the p.PSMA protein is targeted to the surface potentially resulting in slower processing kinetics. If this occurred however, it would be expected that a greater degree of immunity would be generated by the p.PSMA-DOM vaccines which is not the case.

The T-cell responses primed by the two full-length PSMA constructs were overall equally poor making it impossible to decipher whether any of the situations discussed occurred. This clearly is not the model to pursue such answers regarding fusions to DOM. A smaller monomeric and not membrane-associated protein would be more suitable.

3.4.4 The p.DOM-PSMA-epitope Vaccines Prime Superior $CD8^+$ T Cell Responses in Comparison to the Full-Length PSMA Vaccines

All four p.DOM-PSMA-epitope vaccines can prime far higher levels of the respective epitope-specific $CD8^+$ T cells than the full-length PSMA vaccines. Priming with the p.DOM-epitope vaccines generated PSMA4, PSMA27, PSMA663 and PSMA711-specific $CD8^+$ T-cell responses that on average were ~5-fold (for PSMA663) to ~50-fold (for PSMA711) higher than those to the primed and boosted p.PSMA-DOM vaccination (comparing 10μM to 1μM peptide concentration responses, Figures 18-21, 41).

A similar observation has been previously demonstrated in Balb/c mice with the retroviral antigen product, gp70 and the encoded H2-L^d-restricted AH1 epitope. The AH1-specific responses to the full-length vaccine were inferior to those primed by the p.DOM-AH1 vaccine [159].

As discussed in the vaccine design section of the Introduction (section 1.3.16.4), the enhanced responses against the p.DOM-epitope vaccine sequence are likely due to a combination of factors that optimise peptide liberation. These include the presence of a carrier protein enabling uptake of the protein for efficient cross-presentation, optimal flanking sequences and C-terminal positioning of the peptide. The amount of antigen is thought to be another factor that impacts the efficiency of primed T-cell responses by affecting the amount of peptide presented [399]. However, simply removing part of the PSMA sequence (TM domain retained) did not enhance, and was even detrimental to, the epitope-specific responses (Figure 45). A complex interplay of factors determines the efficiency of processing and rational design of vaccines must be carefully considered. In this case the shorter PSMA protein is unlikely to fold into a structure that resembles the native conformation of the protein and as already discussed the glycosylation pattern and cellular trafficking may be different, potentially resulting in rapid degradation. While it is recognised that a full-length sequence has the advantage of being appropriate for patients of all MHC class I haplotypes, clearly the superior performance of epitope-specific vaccines at priming $CD8^+$ T cells validates clinical testing of this approach.

3.4.5 PSMA4, PSMA27, PSMA663 and PMSA711 Peptides are Processed and Presented by Cells Endogenously Expressing PSMA

Although the four p.DOM-PSMA-epitope vaccines induce high levels of $CD8^+$ T cells, the next question was whether the target epitopes are processed and presented via MHC class I. Although the single study demonstrating PSMA663 processing is convincing the limited current literature regarding PSMA711 and PSMA27 processing is contradictory and weak (section 3.1.5.3). The lack of a suitable target human tumour cell line for use in killing assays has caused difficulty in conclusively determining the processing of these peptides [230, 231]. Surprisingly, despite its use in various clinical trials, there is no published proof of PSMA4 peptide processing.

In the present study the transgenic HHD mouse model has been used to assess the processing and presentation of the four PSMA peptides. A simple procedure for generating HHD-expressing target cells has been developed. The murine PCa tumour cell line, TRAMP-C1, is readily transduced by retrovirus and was infected with both PSMA and HHD-expressing retroviruses to create a suitable target cell to kill for ^{51}Cr release assays. The four p.DOM-PSMA-epitope vaccines (p.DOM-PSMA4, p.DOM-PSMA27, pDOM-PSMA663 and p.DOM-PSMA711) were able to induce epitope-specific CTLs that

could efficiently kill peptide-loaded HHD-expressing target cells. Importantly, the PSMA4, PSMA27 and PSMA663-specific CTLs primed by vaccination could kill the TRAMP-C1 cells transfected to endogenously express PSMA and the HHD MHC class I molecule after only a brief 6-day expansion period *in vitro* (Figures 26, 27, 29). This convincingly demonstrates the ability of PSMA-expressing cells to process and present PSMA4, PSMA27 and PSMA663 peptides. In contrast, only the high avidity PSMA711-specific CTLs that underwent multiple rounds of expansion *in vitro* were able to lyse the PSMA-expressing cells (Figure 32) thus verifying that the PSMA711 peptide is also processed albeit at lower physiological levels than the other PSMA peptides. This is in accordance with a previous report that human PSMA711-specific CTLs were unable to kill the LNCaP target cells whilst the human PSMA27-specific CTLs could [230]. Difficulty with this cell line undergoing CTL-mediated killing is discussed later.

An alternative approach also confirmed the processing of PSMA27 and PSMA711 peptides. Bone marrow-derived HHD DCs loaded with PSMA RNA were able to translate the protein, process it and present both the peptides to the high avidity PSMA27 and PSMA711-specific $CD8^+$ T cells.

From these results it is conclusive that all four PSMA HLA-A2-binding PSMA peptides are indeed processed from the endogenous PSMA protein by the cellular antigen processing machinery, and delivered to the MHC class I presentation pathway for presentation, at what appears to be relatively high levels for PSMA4, PSMA27 and PSMA663 peptides. Highly indicative of peptide processing efficiency is the difference between the killing of peptide-loaded and non peptide-loaded target cells. Loading target cells with PSMA27 and PSMA663 peptides did not greatly increase the extent of target cell lysis. In contrast, loading target cells with PSMA4 peptide did significantly augment the degree of lysis. The results presented suggest that the PSMA27 and PSMA663 peptides are both processed at higher levels than the PSMA4 peptide. These three peptides are therefore suitable targets for immunotherapy in the clinic. However, the lower levels of PSMA711 peptide presentation is critical and brings into question whether such a response in cancer patients would provide any therapeutic benefit.

3.4.6 Epitope-Specific CTLs Primed by p.DOM-PSMA27 and p.DOM-PSMA663 Vaccines Migrate to Tumour Site and Specifically Kill PSMA-Expressing Tumour Cells *In Vivo*

The next objective was to assess whether the epitope-specific CTLs primed by vaccination could mediate protection against the human PSMA and HHD double positive TRAMP-C1 tumour cells *in vivo*. Initially, we sought to demonstrate the principle and validate the method with the immunodominant PSMA663-specific CTLs.

The experiment presented clearly establishes that the PSMA663-specific CTLs primed by the p.DOM-PSMA663 vaccine are able to migrate to the tumour site (Matrigel™-embedded tumour cells) and specifically eliminate the PSMA-expressing cells in half of the mice tested (Figure 35). The same principle has been shown with a single p.DOM-PSMA27-vaccinated mouse although the data is not presented. This observation is important as it demonstrates that PSMA-specific CTL responses primed by p.DOM-PSMA663 vaccination can detect the physiological levels of peptide presented by the PSMA+ tumours and migrate to the site *in vivo*. The LNCaP human PCa cell line expresses far more PSMA protein than the transduced TRAMP cells indicating that the level of PSMA expression by these artificially created PSMA+ TRAMP cells is representative of that which may be found expressed by tumours of PCa patients.

3.4.7 Inability of the Human LNCaP Cell Line to Undergo CTL-Mediated Lysis

The LNCaP cell line is notorious for low MHC class I expression that results in difficulties for CTL-mediated killing. It has been previously reported [220] and confirmed in the present study that TNFα treatment increases the MHC class I levels. Upregulation of HLA-A2 was not sufficient in the work presented here for LNCaP cells to be killed by or to directly present peptide to activate the high avidity mouse HHD PSMA27 or PSMA711-specific $CD8^+$ T cells. Initially we thought this could be attributed to the lack of recognition between the human MHC and the mouse CD8 and possibly due to other evasion mechanisms. The problem of obtaining a suitable target was overcome by using TRAMP-C1 cells transduced to express human PSMA and HHD, thereby providing this mouse MHC class I α3 domain for the interaction with the mouse CD8 to occur (Figure 46).

The killing of human HLA-A2 tumour cells by mouse HHD CTLs has been previously reported [160]. It was thought that the CTLs were able to overcome the lack of recognition between the mouse CD8 and the human MHC for two reasons. Firstly the targets were EBV-transformed tumour cells that expressed high levels of MHC class I. Secondly the CTLs in the killing assay were said to be of high avidity, expanded with 1μM peptide. However, this was a higher peptide concentration than the 10nM/1nM used to expand the high avidity PSMA27-specific CTLs. This would suggest that the PSMA27-specific CTLs in the current study were of 100-1000 fold higher avidity. Despite the extremely high levels of PSMA expressed, the upregulated HLA-A2 and and the already confirmed PSMA27 peptide processing, the high avidity PSMA27-specific CTLs were unable to lyse LNCaP cells. PSMA27-specific CTLs primed by vaccination and only briefly expanded *in vitro* were equally unable to kill LNCaP cells transduced to express the chimeric HHD MHC class I. This suggests that the LNCaP cells may have some other deficiency or evasion mechanism that does not allow them to undergo PSMA27-specific CTL-mediated cytotoxicity.

There appear to be different sub-lines of LNCaP cells which may be the reason for discrepancies regarding MHC class I expression and ability to undergo CTL-mediated killing. The one trait that appears to be conserved is insensitivity to IFNγ treatment. Although most publications report the levels of MHC class I as low, Mincheff, et al. surprisingly do not even mention it and manage to achieve relatively high levels of PSMA663-specific CTL-mediated lysis [231]. A different study assessed two different LNCaP sub-lines, where one was totally HLA-A2 negative whilst the other expressed low levels [400]. Human PAP and STEAP-specific CTLs were able to kill the HLA-$A2^+$ but not HLA-$A2^-$ LNCaP cells. However, the HLA-$A2^+$ LNCaP cells appear to express similar levels of surface MHC class I as those in the work presented here and that by Lu et al., where killing was not achieved after a standard 5-hour chromium release assay [230]. This therefore does not appear to wholly explain the differential observations. Another explanation could be defects in the antigen processing machinery, the status of which could again vary in different LNCaP sub-lines. As a means of assessing antigen processing decoupled from HLA-A2 expression LNCaP cells were transfected with a vaccinia vector to express mouse class I K^d that would present vaccinia peptides. Competent antigen presentation by LNCaP cells was restored by expression of murine K^d as vaccinia-specific CTLs killed the K^d-LNCaP cells [303]. The authors concluded that this demonstrated functional TAP, β2m and other antigen processing machinery and that the defect related specifically to the MHC heavy chain. However, the killing of K^d-LNCaP cells was at relatively low levels of ~25% compared to the 45% lysis of normal prostate

control and DU-145 PCa cells. In agreement with the work presented here, this suggests that LNCaP antigen processing and presentation may be at least partially inhibited. Exploring this observation further was not a direction in which we desired to take this project in the short amount of time remaining. These results confirm that LNCaP cells are poor killing assay targets and explain the difficulties encountered by others to demonstrate LNCaP cell lysis. It also demonstrates how valuable the HHD model and the transduced cells created are.

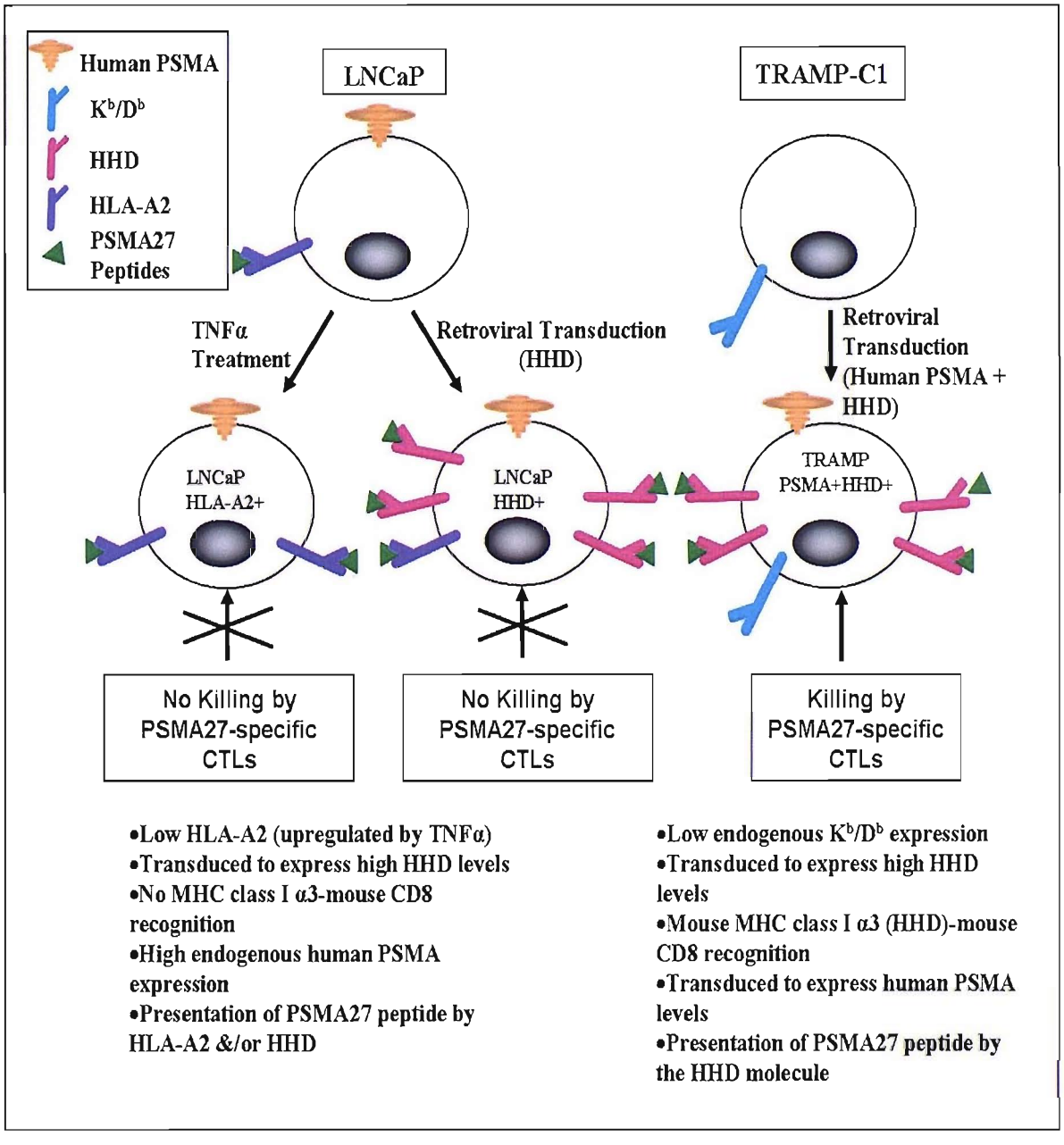


Figure 46: Characteristics of target cells used to assess cytotoxicity by PSMA27-specific CTLs
The TNFα-treated LNCaP cells, LNCaP cells transduced to express HHD and TRAMP-C1 cells transduced to express human PSMA and HHD were used as target cells for chromium release assays. The characteristics (MHC class I and PSMA expression) of each cell line before and after manipulation/transduction are described.

3.4.8 Vaccine Design

There are a number of factors that have to be considered when designing vaccines. One is the antigen that the immune response is targeted against. Ideally the tumour antigen is a protein crucial to tumour survival in order to avoid escape of tumour variants. There is also the form in which the tumour antigen is expressed. Some of these factors that are relevant to the work in the current study are discussed below.

3.4.8.1 Single vs Multi-epitope Immune Attack

In the work presented here a single tumour-derived MHC class I-binding peptide was incorporated into the p.DOM-epitope DNA vaccine design. As a result the $CD8^+$ T cell response elicited is limited to that epitope. It is thought that targeting more than one $CD8^+$ T cell epitope either within the antigen or preferably from different tumour antigens should allow a more diverse attack of the tumour and targeting different antigens should overcome antigen-loss tumour variants. It could also make the vaccine applicable to a wider range of tumours as not all cancers of the same type express the same tumour antigens. However, although polyepitope vaccines can prime CTLs against various epitopes, there usually appears to be a dominant epitope-specific response that becomes narrowed further towards the most immunogenic epitope upon boosting [401]. Yewdell et al., have reviewed the phenomenon of immunodominance extensively [191]. A number of factors are thought to shape immunodominance, including direct competition for the same APC presenting both dominant and subdominant epitopes [402]. This same immunodominant effect is expected to occur and has been demonstrated in the present study when boosting with the full-length PSMA vaccine. Immunodominance may be avoided to achieve diversified CTL responses when the epitopes are administered in separate vaccines [401] and at different injection sites [396]. Vaccination with p.DOM-epitope vaccines encoding for different PSMA CTL epitopes at different injection sites may therefore represent a better strategy for priming CTLs specific for multiple PSMA epitopes. In humans, T-cell repertoires against subdominant epitopes may still be intact and may therefore represent better targets than immunodominant epitopes. If this multi-epitope approach is successful in mice it could be translated into the clinic in an attempt to improve anti-tumour immunity. It would also be possible to target MHC class I-binding peptides from different tumour antigens simultaneously.

3.4.8.2 Tumour-Specific $CD4^+$ T cells

Helper T cells are a requirement for successful priming of $CD8^+$ T cells and establishment of a memory $CD8^+$ T-cell response. They facilitate antigen presentation through secretion of IFNγ, support DC maturation [76], protect DCs from CTL-mediated elimination [77], provide IL-2 to support CTL expansion and rescue CTLs from AICD [154]. These can all be provided by tumour-irrelevant T-cell help as long as the antigen is presented in close proximity to CTL-priming DCs. Foreign CD4 helper T-cell epitopes can be included within the vaccines through the use of foreign sequences such as the FrC/DOM fusion as demonstrated by studies from our laboratory where anti-tumour immunity translated to successful tumour protection. This represents a powerful strategy for mobilising a large repertoire of microbial-specific helper T cells whilst the fusion ensures close proximity of $CD4^+$ and $CD8^+$ T cells during priming (cognate T-cell help).

Another role that may potentially be attributed to helper $CD4^+$ T cells is the aiding of $CD8^+$ T cell tumour infiltration in solid but not "liquid" (haematological) tumours models through modulation of the tumour microenvironment. The presence of $CD4^+$ T cells induces ICAM expression at the tumour site [403], a protein associated with the trafficking of $CD8^+$ T cells to the tumour. Helper T cells at the tumour site secrete cytokines, primarily IFNγ, which enhances tumour MHC class I expression and produce chemokines, which attract immune effector cells (eosinophils and macrophages) to maintain inflammation [403]. Whether tumour-specific helper T cells are a requirement for CTL infiltration of tumours remains a topic of contention. There is some evidence in animal models that $CD4^+$ T cells are required for tumour elimination. An OVA mouse model demonstrated better $CD8^+$ T cell recall responses and protection when OVA-specific rather than irrelevant help was provided [404]. Similarly, the co-transfer of $CD4^+$ and $CD8^+$ influenza HA-specific TCR Tg T cells was necessary for elimination of HA-expressing tumours [403]. However, these are artificial models in non-tolerised systems that do not represent what occurs in cancer patients. This is highlighted in the co-transfer experiment where mice were injected with tumour cells and the T cells transferred on the same day because if the tumour was allowed to establish no tumour regression was observed. Tumours themselves can be inherently inflammatory and this could be enhanced by methods such as irradiation of tumours. Such treatment may be sufficient for the production of chemokines and adhesion molecules to facilitate initial trafficking of immune effector cells to the tumour site. Once anti-tumour immunity is ongoing tumour-specific $CD4^+$ T cells would be primed.

The p.DOM-epitope vaccines prime p30-specific $CD4^+$ helper T-cells in the draining lymph nodes. These will require an inflammatory response signal, probably via a chemokine gradient in order to migrate to the tumour site. We thought the use of the full-length tumour p.PSMA-DOM vaccine should allow the induction of helper $CD4^+$ T cells specific for PSMA and p30 $CD4^+$ T cell epitopes, as well as priming CTL responses. Further boosting with the p.DOM-epitope PSMA vaccines could then boost CTL responses primed by the full-length vaccine. The PSMA-specific $CD4^+$ T cells should migrate to the PSMA-expressing tumour site and support the CTL-mediated attack. Once the tumour begins to be killed by infiltrating CTLs or antibodies (if any are elicited by vaccination), the apoptotic tumour cells can be phagocytosed by the DCs. This would allows the DCs to process and present multiple $CD4^+$ and $CD8^+$ T-cell epitopes from different prostate tumour antigens (epitope spreading) resulting in a positive feedback loop where more tumour-specific T cells are primed and migrate to the tumour.

Although the work presented here has demonstrated that the full-length p.PSMA-DOM full-length vaccine cannot prime any responses, the principle of the approach still stands but the efficacy of the full-length vaccine will be dependent on the tumour antigen under attack. In more recent years there has been increasing literature regarding the discovery of processed and presented MHC class II-binding epitopes, including for PSMA, which in the future may potentially be incorporated into a vaccine design. However, care must be taken as a yet unexplored possibility is that immunotherapy strategies to prime tumour-specific helper T cells can potentially also generate tumour-specific Tregs, particularly at the induction phase when the environment is highly toleragenic.

3.5 Conclusions

The work presented in this chapter has shown that the four p.DOM-PSMA4, pDOM-PSMA27, p.DOM-PSMA663 and p.DOM-PSMA711 vaccines are effective at priming PSMA epitope-specific $CD8^+$ T-cell responses in the HHD mouse model.

CTLs specific for each of the four PSMA peptides investigated can specifically lyse peptide loaded cells and most importantly tumour cells endogenously expressing PSMA. Therefore it has been conclusively demonstrated that PSMA4, PSMA27, PSMA663 and PSMA711 peptides are processed and presented by the MHC class I of cells expressing PSMA endogenously. However the efficiency of processing/presentation of the PSMA711 peptide is significantly inferior to the others, as shown by the fact that only the high avidity CTL line expanded could lyse PSMA-expressing targets. It is therefore unlikely that immunotherapy strategies aimed at inducing immune responses against this peptide may be able to eradicate PSMA-expressing tumours. The other three peptides, PSMA4, PSMA27 and PSMA663, all appear to potentially be good targets.

Even more important is the demonstration that the PSMA663-specific CTLs primed by the p.DOM-PSMA663 vaccine can migrate to the tumour site *in vivo* and specifically kill the PSMA-expressing cells. Unfortunately there has not been sufficient time to carry out all the desired experiments. It is the *in vivo* killing data that must be consolidated for each p.DOM-PSMA-epitope vaccine, particularly with the p.DOM-PSMA27 vaccine to provide proof of principle of what the current p.DOM-PSMA27 clinical trial is attempting to achieve in PCa patients. More recently, we have managed to transduce a cell line of different mouse strain origin to express PSMA and HHD. Fortunately unlike the transduced TRAMP cells, these cells will form tumours in SCID mice thereby allowing us to assess the effects of CTLs in an adoptive transfer model.

The work presented in the study has confirmed the ability to assess the PSMA HLA-A2-binding epitopes in the HHD mouse model. A system where the target TRAMP-C1 cells express the HHD molecule has been created. It should now prove an easier task to transfect these cells with the relevant tumour antigen targeted by vaccination as demonstrated with PSMA here. The use of the HHD model has overcome the problems that have thus far been encountered by the human system that has led to the current contradictory reports in the literature regarding the processing of PSMA epitopes. We have also finally managed to manipulate the HHD system to enable future functional testing of HHD-restricted CTL responses specific to any tumour antigen with relative ease.

4. Assessing Immunity against Autologous PSMA in a Potentially Tolerised Mouse Model

4.1 Introduction

4.1.1 Rationale for Inducing Antibodies against PSMA

Development of active immunotherapeutic approaches using PSMA as a target antigen has generally focused on $CD8^+$ T-cell responses (Chapter 3). PSMA is a cell surface molecule and might therefore be additionally susceptible to antibody attack. PSMA expression correlates with high grade tumours and aggressive tumour behaviour (section 1.5.10) and is therefore an ideal target for antibodies. For this reason I began by trying to establish a mouse model to study the ability of DNA vaccines to generate mouse PSMA-specific antibodies, in addition to $CD8^+$ T-cell responses, and to assess whether these responses were able to convey some protection against a mouse PSMA-expressing tumour. Many PCas, especially with metastatic disease express low levels of MHC class I and are therefore likely to escape T-cell attack. Therefore active immunotherapy capable of eliciting anti-PSMA antibodies may represent an additional approach.

4.1.2 Effects of Antibodies against PSMA

Antibodies can potentially induce anti-tumour effects by modulating signalling pathways activated by the cell surface target to which they bind or through complement-dependent cytotoxicity, ADCP or ADCC. There is evidence supporting the ability of antibodies specific for PSMA to provide anti-tumour activity. However, anti-PSMA antibodies are often endocytosed and this may limit their potential to exert the anti-tumour functions listed above. PSMA-targeted antibody therapy can be divided into two groups. The first is passive immunotherapy using MAbs that specifically recognise the cell surface PSMA protein expressed on tumour cells. The second category is active immunotherapy where vaccines are designed to generate humoral responses against the full-length native transmembrane protein as well as the necessary supporting cellular responses. Studies that have investigated these approaches are described below.

4.1.2.1 Monoclonal Antibody Therapy

Direct cytotoxicity of tumours by PSMA-specific MAbs has been modelled in immune-compromised mice bearing human PSMA-expressing tumours. The MAb 24.4E6 was generated by immunising mice with a PSMA peptide where the sequence (638-657) was predicted to be a B-cell epitope. The MAb was passively administered to SCID mice with established LNCaP tumours and demonstrated anti-tumour activity with a significant retardation in tumour growth [405]. Generally PSMA-specific antibodies alone have exhibited poor performance therapeutically and as mentioned above this may be due to endocytosis by the tumour cell. The conjugation of humanised PSMA-specific MAbs to radionuclides, toxins and drugs that can specifically deliver these toxic compounds to PSMA-expressing cells has proven more successful and efforts have therefore been directed towards this type of therapy. Conjugated MAbs specific for the extracellular portion of PSMA are used in both imaging and therapeutic applications. These initially underwent pre-clinical testing in nude mice bearing LNCaP tumours to ensure the specific delivery of the radioisotopes and internalisation to generate anti-tumour effects [406].

The first PSMA-specific MAb to be used for imaging in the clinic was 7E11-C5 that binds an intracellular PSMA epitope. It therefore only binds dead or necrotic cells where the intracellular PSMA epitope may be accessible for binding. When this MAb is conjugated to the ^{111}Indium isotope it is known as ProstaScint®, a conjugate that despite not directly targeting living tumour cells, can detect apoptotic tumour cells attacked by therapy. This MAb conjugate additionally allows bystander therapy of viable cells in close proximity to dying tumour cells. The humanised MAb J591 conjugated to the ^{90}yttrium radioisotope is also used for imaging. It has very high specificity and sensitivity for metastatic sites [407]. The imaging applicability of the MAbs suggests that they do target the tumour and metastases with high specificity and those targeting extracellular epitopes become internalised so should deliver the chemical conjugates successfully and selectively.

A phase I clinical trial using ^{90}yttrium–labelled MAbJ591 reported anti-tumour activity. Two (7%) responding patients had PSA level decreases >50% that lasted 8-9 months and objective clinical responses. Additionally 6 (21%) partial responders exhibited PSA stabilisation [408]. The J591 MAb conjugated to the ^{111}Indium isotope was recently reported to have specifically targeted at least one metastatic site in 20/27 (74%) of patients with advanced solid tumours. However, stable disease status was only achieved by three of these patients [409]. Other trials with radiolabelled PSMA MAb have demonstrated their specificity and reported similar limited responses [410, 411]. The only serious toxicity observed with radiolabelled MAbs was limited reversible myelosuppression [408].

Other types of conjugates are under investigation. A single chain fragment (scFv) specific for PSMA fused to a truncated Pseudomonas exotoxin lacking its own natural binding site was able to reduce the viability of LNCaP cells by 50% *in vitro* [412]. Drug conjugates are under investigation in mouse models. A humanised MAb conjugated to DM1, a microtubule depolarising agent, was able to effectively penetrate human xenografts in SCID mice and successfully delay tumour growth [413]. This compound is undergoing clinical testing by a commercial company. Therapy with a PSMA antibody conjugated to an inhibitor of tubulin polymerisation improved the median survival and cured 40% of immune compromised mice [414]. Generally the clinical responses yielded by the MAbs taken to clinic are considered to be very limited. Despite the disappointment this field continues to be investigated for therapy of PSMA-expressing tumours.

4.1.2.2 Protection from Human PSMA-Expressing Tumours can be mediated by anti-Human PSMA Humoral Responses in Mice

Balb/c mice vaccinated with NIH3T3 cells expressing the extracellular portion of human PSMA were able to induce a humoral antibody response against the human protein. The antibody-containing sera inhibited LNCaP tumour development when transferred into athymic mice [415]. In spite of the xenogeneic setting, this is the first study to show the anti-tumour activity and therapeutic capability of a PSMA targeted humoral immune response generated by vaccination. Similarly, intramuscular administration of a DNA vaccine encoding the human PSMA transmembrane domain fused to an extracellular PSMA portion generated anti-human PSMA antibody responses against the native protein [416]. No protection experiments were carried out. These studies demonstrate the ability of mice to generate anti-human PSMA humoral responses and the potential of these responses to protect against human PSMA-expressing tumours.

4.1.2.3 Anti-Human PSMA Humoral Responses in Patients

There has been a single clinical trial assessing humoral responses after immunisation of PCa patients with plasmid or adenoviral vectors encoding PSMA. The constructs encoded the extracellular domain of the PSMA protein. Anti-PSMA antibodies were not detected in the patients' sera prior to vaccination or in the sera of healthy individuals. Fluctuating PSMA-specific antibody responses were detected in 86% of patients after vaccination but could not be correlated to efficacy due to patient heterogeneity [417]. The antibody responses were assessed by Western blot for reactivity against a PSMA-expressing tumour

cell lysate. Therefore these humoral responses detected by specificity to denatured protein are not necessarily biologically relevant. Although there may also be antibodies that are able to recognise the native PSMA protein expressed by PCa tumours, this screening does not confirm it. This is the first report of patients' ability to raise anti-human PSMA humoral responses and validates further active immunotherapy approaches aimed at eliciting PSMA-specific antibody responses with therapeutic efficacy.

4.1.3 Xenogeneic Vaccination against PSMA in Mice

Immunisation of mice with DNA vaccines encoding xenogeneic sequences has been used to break tolerance against syngeneic mouse antigens. On the basis of our studies we speculate that xenogeneic sequences are likely to provide $CD4^+$ T-cell help that aids the priming of murine-specific $CD8^+$ T cells and the generation of humoral responses against the mouse antigen, where epitope homology is shared with the human sequence. Single amino acid differences between the xenogeneic and syngeneic sequences can create heteroclitic epitopes that are able to trigger immunity against the autologous protein [418]. Where the aim of vaccination is the priming of $CD8^+$ T cells, multiple immunisations with vaccines encoding the xenogeneic sequence should ideally be avoided in order to prevent eliciting a dominant response specific for the xenogeneic protein.

Humoral responses generated by xenogeneic vaccination targeting PSMA have been studied in a pre-clinical murine model. The study claimed that tolerance to mouse PSMA could be broken in Balb/c and C57Bl/6 mice by immunising with a DNA vaccine encoding the xenogeneic human PSMA sequence and a human PSMA protein boost. The vaccines supposedly elicited anti-human PSMA and anti-mouse PSMA antibodies against the native mouse protein [419]. However, the work presented in this chapter shows that this was not the case as the mouse PSMA protein sequence used was incorrect and the differing amino acids were the same as that found in the human sequence (section 4.2.7). This essentially undermines conclusions of this publication as xenogeneic vaccination in this setting was unable to break tolerance to PSMA.

This principle has been shown *in vitro* by a study that transfected human PBMCs with a fusion of partial sequences from human PSMA, human PSA and mouse PAP. These were used to expand CTLs from human PBMCs that were specific for the relevant tumour antigens including human PSMA. They were additionally able to expand CTLs specific for human PAP thereby demonstrating that the mouse PAP sequence was able to expand

human PAP-specific CTLs [183]. Therefore in some settings xenogeneic sequences can expand syngeneic T cells although the *in vitro* experiment is quite different to the *in vivo* situation.

4.2 Aims

1. The main aim was to establish if the foreign FrC or DOM fusion sequences provided sufficient $CD4^+$ T-cell help to overcome tolerance in the mouse model and induce humoral and cellular responses against mouse PSMA. The initial intention was to assess the ability of a DNA vaccine encoding the mouse PSMA sequence fused to FrC or DOM to generate primarily autologous mouse PSMA antibody responses.

2. The second objective was to investigate mouse PSMA peptides predicted to bind murine MHC class I with the p.DOM-epitope vaccine design. The aim was to prime $CD8^+$ T-cells against the autologous mouse PSMA and to confirm the processing status of the predicted peptide(s).

3. The final question was whether any mouse PSMA-specific antibody and/or CTL responses were capable of mediating anti-tumour immunity to protect mice from a mouse-PSMA expressing tumour.

4.3 Results

4.3.1 DNA Fusion Gene Vaccines Encoding Autologous Mouse PSMA Extracellular Domain

This section of the study investigates the potential of a DNA vaccine design encoding the mouse PSMA tumour antigen fused to the foreign FrC sequence to generate immunity against mouse PSMA. The DNA fusion vaccines incorporate the extracellular domain of the mouse PSMA sequence alone (p.mPSMAEC) or fused to a C-terminal full-length FrC or DOM sequence (p.mPSMAEC-FrC, p.mPSMAEC-DOM). Control vaccines encode FrC (p.FrC) or DOM (p.DOM) alone. The vaccines are schematically represented in Figure 47.

In the experiments described below C57Bl/6 mice were immunised with a priming vaccination on day 0 and boosted with the same vaccine on day 21 with EP. Blood was taken and sera analysed for antibody responses on day 41. In the protection experiments mice were challenged on day 42. This vaccination protocol has been previously used and confirmed to generate superior responses compared to administration of intra-muscular DNA vaccinations without EP [166].

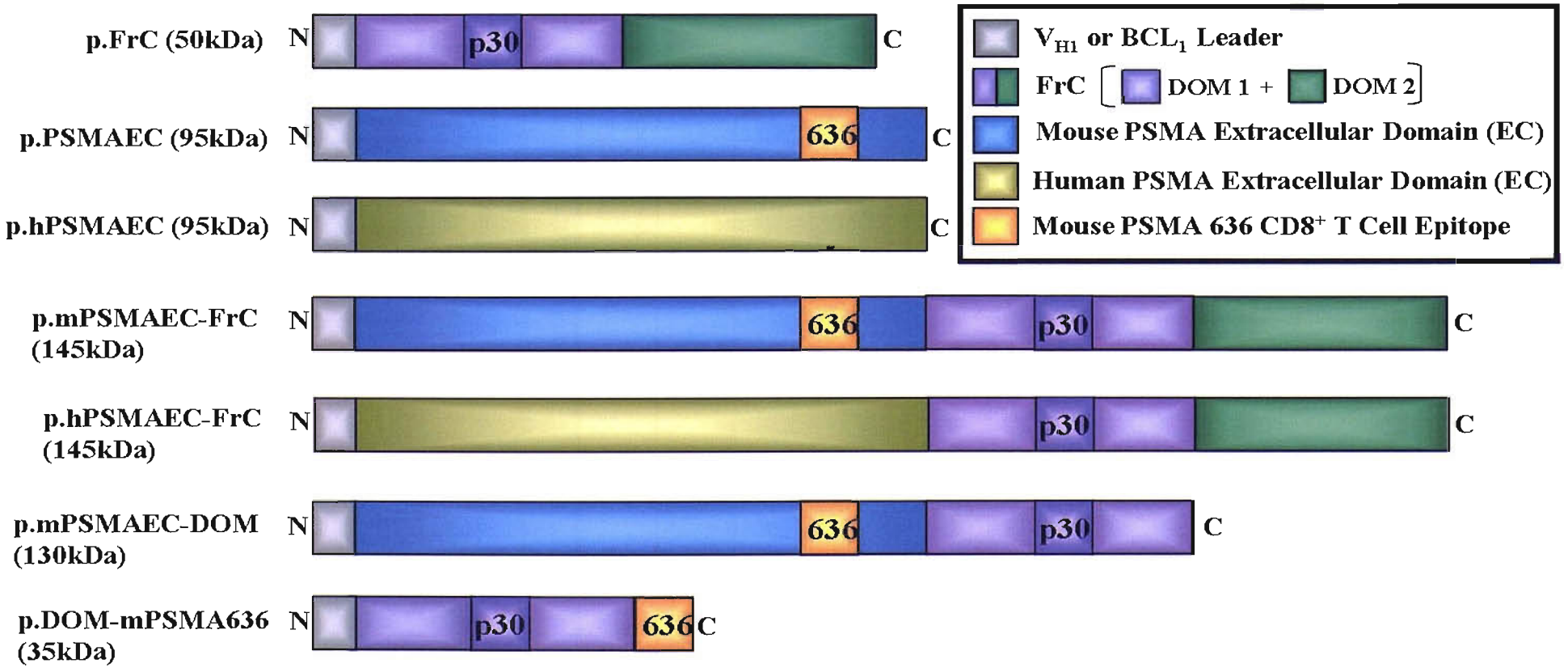


Figure 47: Schematic representation of the mouse PSMA vaccines investigated in chapter 4

The vaccine open reading frames are represented in this figure. All vaccines contain an N-terminal leader sequence (p.DOM-mPSMA636 has BCL_1 leader, the others have a V_{H1} leader) and are inserted into the pcDNA3.1 plasmid. DOM1 and DOM2 are schematically represented as the components of p.FrC. Only the extracellular domain (EC) of mouse and human PSMA are incorporated into the vaccines.

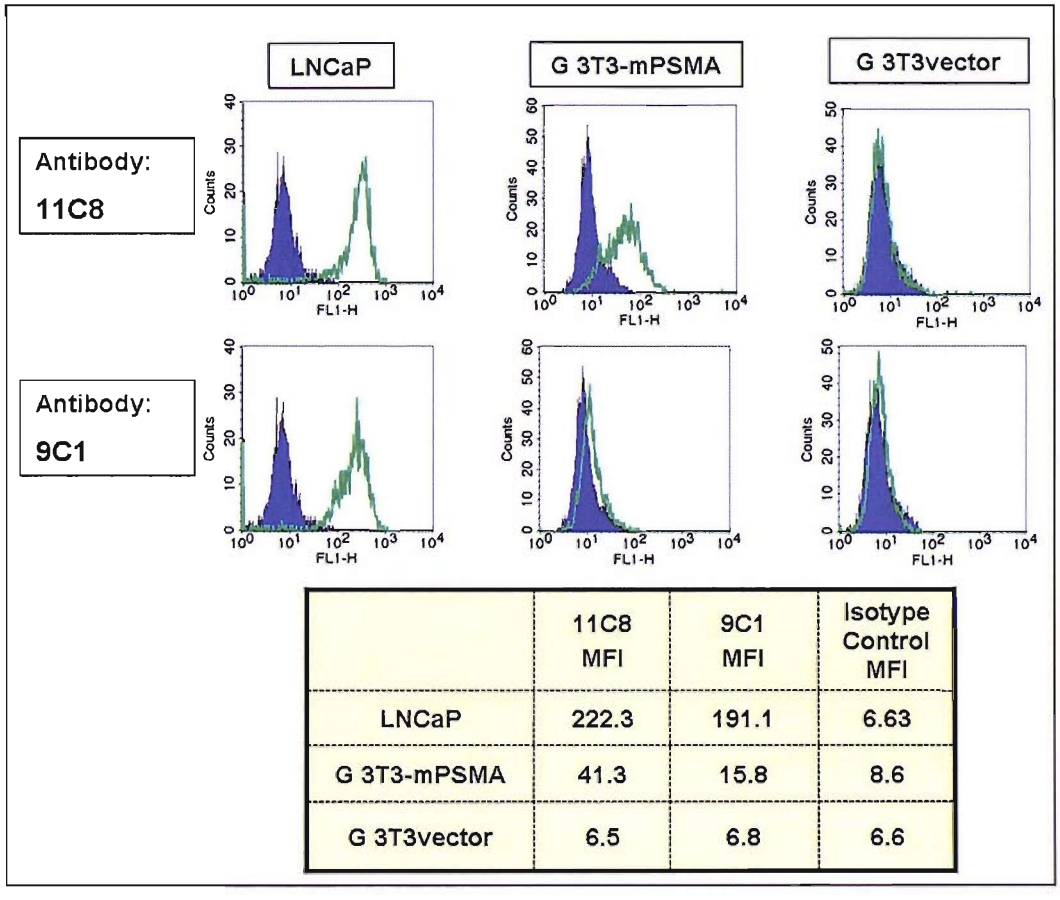
4.3.2 Flow Cytometry and Western Blot Antibodies against Mouse PSMA

Three anti-mouse PSMA antibodies have been kindly gifted by Dr. Gregor (Memorial Sloan-Kettering Cancer Center, New York, USA, Table 9). All three antibodies were raised in C57Bl/6 mice by xenogeneic immunisation with a human PSMA DNA vaccine and human PSMA protein boost [419]. The 3E2 clone recognises a denatured epitope of both human and mouse PSMA. The 9C1 and 11C8 clones (both IgG2b) both cross-react with native human and mouse PSMA although the antibodies stain mouse PSMA-expressing cells with a lower intensity (Figure 48). The precise epitopes recognised by these antibodies are unknown. Table 9 summarises the PSMA antibodies, their reactivity and applications.

Monoclonal PSMA Antibody	Reacts with Human PSMA	Reacts with Mouse PSMA	Recognition of Native or Denatured Epitope (Application)
3E2	YES	YES	Denatured (western blot)
9C1	YES	YES	Native (flow cytometry)
11C8	YES	YES	Native flow Cytometry)
Human PSMA (MBL Ltd)	YES	NO	Native (flow cytometry)

Table 9: Reactivity of PSMA Antibodies

The specificities and applications of the PSMA antibodies 3E2, 9C1 and 11C8 (from Dr. Gregor, Memorial Sloan-Kettering Cancer Center, New York, USA, [419]).



	11C8 MFI	9C1 MFI	Isotype Control MFI
LNCaP	222.3	191.1	6.63
G 3T3-mPSMA	41.3	15.8	8.6
G 3T3vector	6.5	6.8	6.6

Figure 48: Assessing human and mouse PSMA expression with 11C8 and 9C1 monoclonal antibodies

The human and mouse PSMA cross-reactive 11C8 and 9C1[419] antibodies were used to assess surface PSMA expression by human LNCaP cells, cells transfected to express mouse PSMA (G 3T3-mPSMA) or the vector control transfected cells (G 3T3vector). An anti-mouse Ig-FITC conjugated antibody was used for secondary staining. Twice as much 11C8 antibody was used to label the mouse PSMA-expressing cells compared to the human PSMA expressed by LNCaP (3μg vs 1.5μg), and the lower amount did not reach saturation. The same case was found for the 9C1 antibody (5μg vs 2μg).

4.3.3 DNA Vaccine Elicits Antibody Responses against FrC

Sera from mice immunised with p.mPSMAEC-FrC or control p.FrC DNA vaccines were assessed for anti-FrC antibody responses with an in-house established ELISA. Antibody responses against FrC serve as a marker of vaccine performance and confirm the integrity of the product encoded by the vaccine.

Male mice vaccinated with either of these two constructs were able to mount a good humoral response against the foreign FrC vaccine component (Figures 49). The average anti-FrC antibody levels generated by the p.FrC vaccine were approximately 5-fold higher than the p.mPSMAEC-FrC vaccine. This mirrors the p30 responses for the p.DOM-epitope compared to the p.PSMA-DOM human PSMA vaccines, the reason for which

remains unestablished (Chapter 3). This difference can also be appreciated from the anti-FrC levels reached by 50% of the mice in each vaccine group. Half the mice vaccinated with p.mPSMAEC-FrC or p.FrC reached $1x10^4$ or $5x10^4$ FrC standard units respectively.

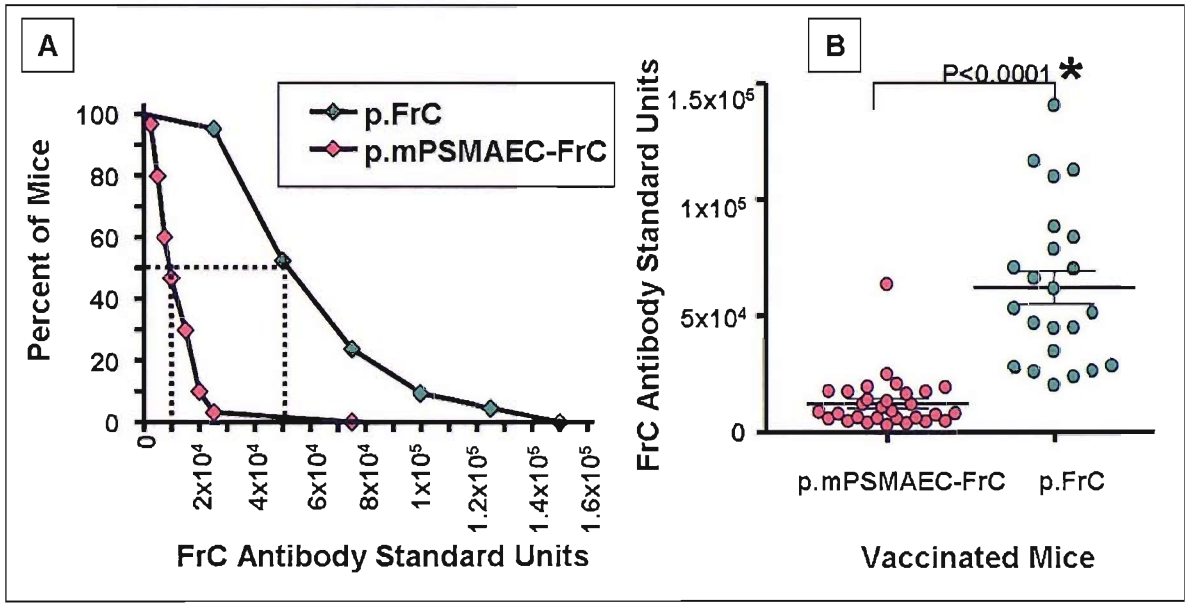


Figure 49: Anti-FrC antibodies raised in individual mice vaccinated with p.FrC or p.mPSMAEC-FrC

Male C57Bl/6 mice were immunised with p.mPSMAEC-FrC (pink) or p.FrC (green) vaccines on day 0 and boosted with EP on day 21. The mice were tail bled or terminally bled for sera on day 41. The level of anti-FrC antibodies (standard units) in individual mice was assessed with an in-house established ELISA. Data are represented as the percentage of mice that achieved particular anti-FrC antibody levels (A) or the result for each individual mouse plotted (B). The dashed lines in (A) designate the 50% response rate and the (*) in (B) signifies a statistically significant difference.

4.3.4 DNA Vaccine Elicits Antibody Responses against DOM

Sera from mice immunised with p.mPSMAEC-DOM or control p.DOM DNA vaccines were assessed for anti-DOM antibody responses with a DOM-specific in-house established ELISA. Antibody responses against DOM serve as a marker of vaccine performance and confirm the integrity of the product encoded by the vaccine.

Male mice vaccinated with these two constructs mounted good humoral responses against the foreign DOM vaccine component (Figures 50). The average anti-DOM antibody levels generated by the p.DOM vaccine were ~4.5-fold higher than those from the p.mPSMAEC-DOM vaccinated mice. This difference can also be appreciated from the anti-DOM levels reached by 50% of the mice in each vaccine group. Half the mice vaccinated with p.mPSMAEC-DOM or p.DOM reached ~$6x10^4$ or ~$2.3x10^5$ standard units respectively.

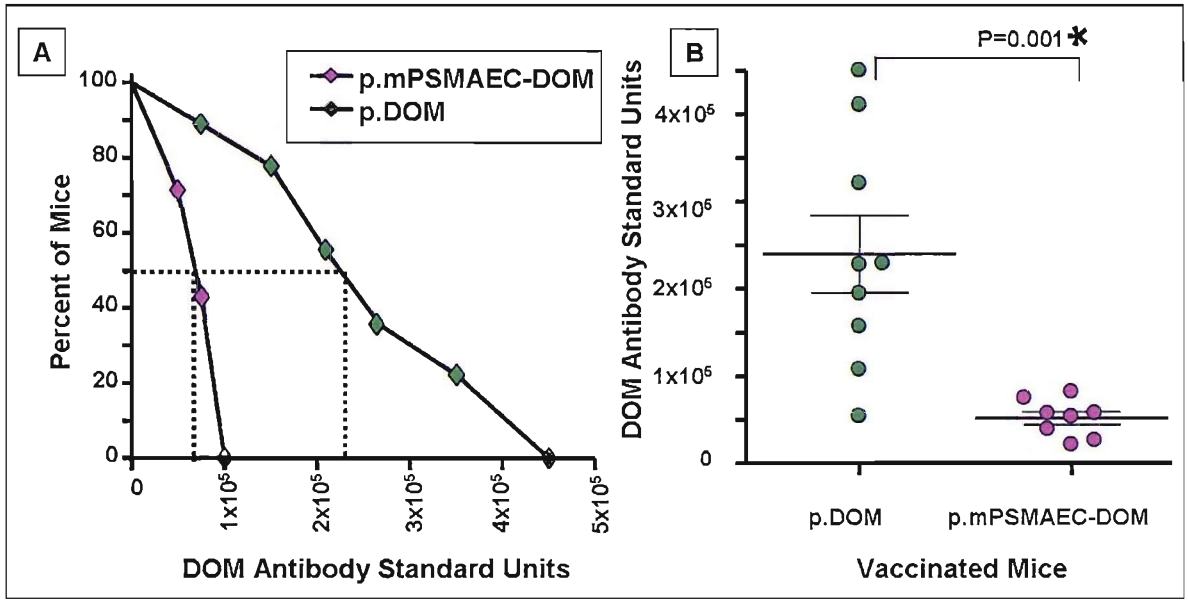


Figure 50: Anti-DOM antibodies raised in individual mice vaccinated with p.DOM or p.mPSMAEC-DOM

Male C57Bl/6 mice were immunised with p.mPSMAEC-DOM (purple) or p.DOM (green) vaccines on day 0 and boosted with EP on day 21. The mice were tail bled or terminally bled for sera on day 41. The level of anti-DOM antibodies (standard units) was assessed with an established in-house DOM ELISA. Data are represented as the percentage of mice that achieved particular anti-DOM antibody levels (A) or the result for each individual mouse plotted (B). The dashed lines in (A) designate the 50% response rate and the (*) in (B) signifies a statistically different result.

4.3.5 Assessment of Antibody Responses against Mouse PSMA Elicited by DNA Vaccination

The detection of anti-mouse PSMA antibodies in the sera of p.mPSMAEC, p.mPSMAEC-FrC and p.mPSMAEC-DOM vaccinated mice has unfortunately not been possible. We initially aimed to develop an anti-mouse PSMA ELISA. We attempted to express the extracellular domain of the mouse PSMA protein with the use of either an N-terminal $His_{(6)}$ or C-terminal human immunoglobulin light chain Cλ purification tags. The human embryonic kidney HEK 293-F expression system was used to ensure correct glycosylation, which is central to protein folding and formation of the functional homodimer. Only a small amount of His-tagged protein could be purified with multiple transfections of HEK 293-F cells as determined by western blot analysis using the 3E2 antibody. However when tested in an ELISA the protein was found to be denatured as it was intensely recognised by the anti-PSMA antibody clone 3E2 (specific for a denatured epitope) but not the native protein-recognising 11C8 antibody. Reactivity with the 9C1 antibody was not assessed.

The Cλ-tagged protein was not expressed at all and so this approach was also abandoned. A third strategy was employed with a construct encoding the mouse PSMA extracellular domain fused to FrC with a linker containing a thrombin cleavage site. The theory behind this approach was that the FrC molecule tends to stabilise the expression of fusion partners. The expressed mPSMAEC-FrC fusion protein was purified using a column made from an anti-FrC polyclonal antibody coupled to sepharose beads. The fusion protein was then to be cleaved by thrombin and the products of the digests separated by the anti-FrC column. Unfortunately only a small amount of protein was purified that would not be recoverable from the last purification step. The problems in expressing the mouse PSMA protein were confirmed by verbal communication with Dr. P. Gregor who had also experienced difficulties when generating the protein for their published study [419].

4.3.5.1 TRAMP Cells Do Not Express Mouse PSMA

Since the protein could not be made we decided to use flow cytometry to assess if anti-mouse PSMA antibodies could be detected in the sera of p.mPSMAEC-FrC or p.mPSMAEC-DOM vaccinated mice. The sera would be used to stain mouse PSMA-expressing cells followed by detection of bound IgG with an anti-mouse Fcγ FITC-conjugated antibody.

TRAMP-C1 cells are reported to express mouse PSMA [227]. However, only traces of mRNA transcripts could be detected by RT-PCR after nested PCR reactions (data not shown). No protein band was seen by Western blot using the PSMA 3E2 antibody on TRAMP-C1 cell lysate samples (data not shown). There is no other mouse cell line that has been reported to naturally express mouse PSMA and therefore it was necessary to create a mouse PSMA transfectant.

4.3.5.2 Differences in Human PSMA Expression by Cells of Different Origins

Dr. Gregor also kindly provided NIH3T3 cells transduced with full-length mouse PSMA (G-NIH3T3-mPSMA) [419]. Expression of mouse PSMA by these cells has been confirmed by flow cytometry analysis using Dr. Gregor's 9C1 and 11C8 antibodies (Figure 48), and western blot using the 3E2 antibody and RT-PCR analysis (data not shown). Sera from p.mPSMAEC-FrC vaccinated mice non-specifically stained the mouse PSMA and vector-transduced control cells with similar intensity, and in some cases at relatively high

levels (data not shown). This significant background would make it difficult to detect any small amount of specific staining.

In light of the background staining it was decided to establish assay conditions using a human PSMA system. The xenogeneic sequence can generate high levels of antibodies in mice that would ease assay optimisation. The TRAMP-C1 and NIH3T3 cells were retrovirally transduced to express human PSMA. Vaccines encoding the human PSMA extracellular domain alone (p.hPSMAEC) or fused to FrC (p.hPSMAEC-FrC) were created (schematically represented in Figure 47).

Sera from C57Bl/6 mice vaccinated with p.hPSMAEC or p.hPSMAEC-FrC vaccines were able to recognise the human PSMA protein naturally expressed by LNCaP cells and that expressed by transduced TRAMP-C1 cells. However the sera could not stain NIH3T3 cells expressing human PSMA. In contrast, the commercial monoclonal anti-human PSMA antibody was able to recognise the protein expressed by all three sets of cells (data not shown). The human LNCaP and the mouse TRAMP-C1 cells are both prostate epithelial cells whilst the NIH3T3 cells are fibroblasts. We therefore speculate that there may be lineage-specific differences in PSMA expression. It appeared that sera from C57Bl/6 mice vaccinated with p.mPSMAEC-FrC were unlikely to recognise the mouse PSMA protein expressed by the G-NIH3T3mPSMA cells.

4.3.5.3 IgG from Vaccinated Mice Non-Specifically Bind Murine Cells

Further analysis using sera from mice vaccinated with p.hPSMAEC, p.hPSMAEC-FrC or p.FrC to stain TRAMP-C1 cells expressing human PSMA was carried out. However, the control and test sera from p.FrC and p.hPSMAEC vaccinated mice stained the TRAMP-C1 cells expressing human PSMA to a similar extent. To assess whether the unexpected recognition was due to the retroviral transduction of the cells, p.FrC sera was used to stain TRAMP-C1 cells before and after retroviral transduction. This assay showed that the sera from p.FrC vaccinated mice non-specifically bound the transduced and non-transduced TRAMP-C1 cells to the same extent (data not shown). This observation was also made using transfected vs non-transfected NIH3T3 and CT26 cells and with sera from p.DOM vaccinated mice (data not shown). It appears that the FrC/DOM components of vaccines generate an element in the sera that can non-specifically bind the surface of murine cell lines. Therefore this approach for analysis of anti-mouse PSMA antibodies was not feasible.

4.3.5.4 Retroviral Transduction of TRAMP Cells to Express Mouse PSMA

We retrovirally transduced TRAMP-C1 cells to express full-length mouse PSMA. A monoclonal antibody is essential as demonstrated by the process of creating the human PSMA transfectants. There was rapid loss of human PSMA-expressing cells despite the antibiotic selection. The same outcome is likely to occur with mouse PSMA transfections due to high sequence similarity.

The mouse PSMA sequence used by Dr. Gregor to create the GNIH3T3-mPSMA cells differs from my sequence by two amino acids. These differences between the sequences was further analysed by site-directed mutagenesis as discussed later (section 4.2.7). Cells transduced with a construct encoding Dr. Gregor's mouse PSMA sequence could be stained by the 11C8 antibody. Although this was not the correct sequence for mouse PSMA in C57Bl/6 mice it was used to enable selection of transfected cells with the monoclonal 11C8 antibody. The positive population was single cell sorted to attain a clonal population of TRAMP-C1 cells expressing mouse PSMA (Figure 51). Similar to the phenotyping of the G NIH3T3-mPSMA cells (Figure 48), a high concentration of antibody was required to saturate the staining reaction.

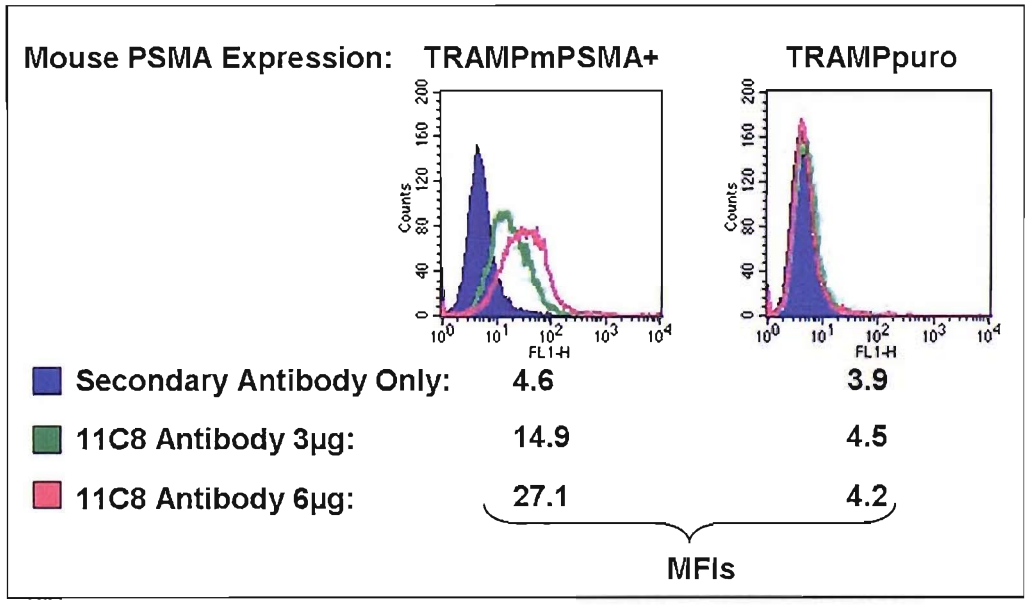


Figure 51: Expression of mouse PSMA by the retrovirally transduced TRAMP-C1 cells

The 11C8 antibody was used to confirm the expression of mouse PSMA in the retrovirally transduced TRAMP-C1 cells or the absence of the protein on the surface of the plasmid (vector) transduced control cells. These cells express Dr. Gregor's mouse PSMA sequence.

4.3.6 Immunisation with p.mPSMAEC-FrC or p.mPSMAEC-DOM DNA Vaccines are Unable to Generate Protective Immunity

Mice vaccinated with p.mPSMAEC-FrC or p.mPSMAEC-DOM vaccines were challenged with a lethal dose of mouse PSMA expressing TRAMP-C1 cells (TRAMP mPSMA+). Neither vaccine was able to generate immunity that could protect mice from tumour growth in either of two experiments for each set of vaccines (Figure 52).

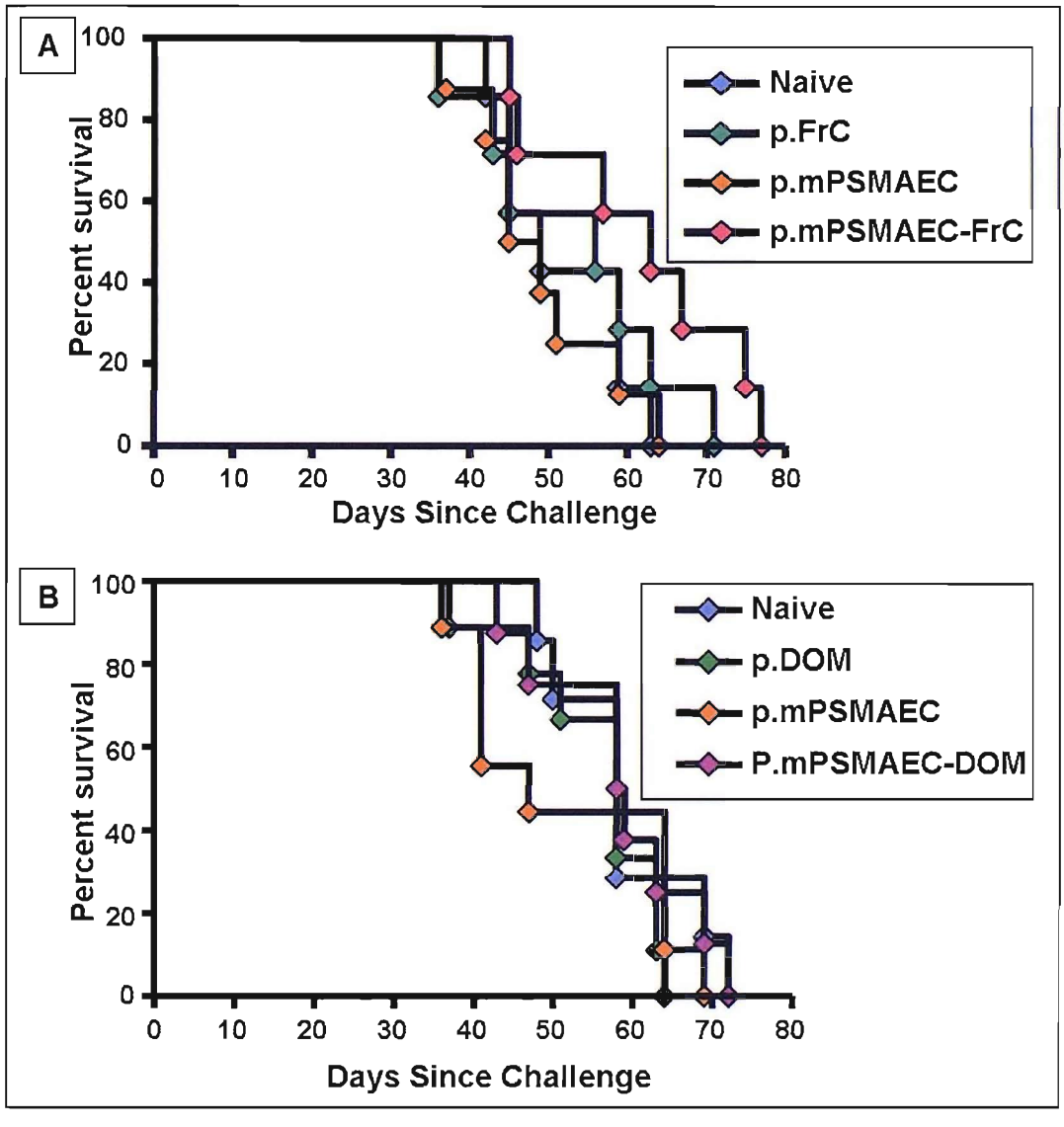


Figure 52: The p.mPSMAEC-FrC and p.mPSMAEC-DOM vaccines failed to protect against mouse PSMA expressing tumour cells

Mice in each vaccine group were immunised on day 0, boosted with EP on day 21 and challenged with a lethal dose of TRAMP mPSMA+ cells subcutaneously on day 42. The graphs show end days from the point when mice were challenged. No protection for either vaccine was also observed in a repeat experiment.

The anti-FrC antibody responses generated by mice challenged in Figure 52A ranged from $2.7x10^3$ to $2.0x10^4$ standard FrC units (average $1.4x10^4$) and so were within the ranges

presented for a larger number of mice in Figure 49. The anti-DOM antibody responses for those mice vaccinated with p.mPSMAEC-DOM and challenged in Figure 52B were those shown on Figure 50 (ranging from $2.1x10^4$ to $8.x10^4$ standard DOM units, average $5.2x10^4$). The FrC and DOM-specific humoral responses indicate that vaccination was successful and therefore the lack of protection was due to either failure of the vaccines to generate immunity specifically against the mouse PSMA antigen or any immunity raised was ineffective and did not provide any significant protection.

4.3.7 Deciphering the PSMA 11C8 and 9C1 Antibody Epitopes

There are two amino acid differences between the mouse PSMA sequence I cloned from a C57Bl/6 kidney and that used by Dr. Gregor [419]. These differences are at amino acid positions 240 and 287 where the sequence cloned in this study has an alanine (A) and asparagine (N) and Dr. Gregor's has a glycine (G) and glutamate (E) respectively (alignment in Appendix III). There are three GenBank sequences (NM_016770, AK002920 and BC119605) that agree with my sequence and one (AF026380) that agrees with Dr. Gregor's with regards to these two amino acids. However the AF026380 sequence was cloned from the Swiss Webster Nude mouse strain whereas the published study used the human PSMA protein to vaccinate C57Bl/6 and Balb/c mice [419]. I confirmed that my sequence was in fact as expressed by both C57Bl/6 and Balb/c mouse strains (confirmed by RT-PCR and sequencing). These two amino acids in question in Dr. Gregor's mouse PSMA agree with those in the human sequence at the corresponding positions when the sequences are aligned (positions 238 and 285 as human PSMA is two amino acids shorter early in the protein sequence, Appendix III).

The ability of both 11C8 and 9C1 antibodies to recognise Dr. Gregor's "incorrect" mouse PSMA sequence but not cells transfected with the correct mouse PSMA construct cloned in the present study suggested that the epitopes for these antibodies must include one or both of the amino acids differing between the sequences. We set out to establish if this hypothesis was indeed the case as this has important ramifications for the findings claimed in the published study. They immunised C57Bl/6 mice with a human PSMA-encoding DNA vaccine and boosted with human PSMA protein. The mice were able to generate anti-human PSMA humoral responses from which the authors made various monoclonal antibodies and a proportion of which cross-reacted with the native mouse PSMA proteins (including 11C8 and 9C1). The study therefore claimed that this xenogeneic immunisation protocol had broken tolerance in the mouse PSMA model [419].

To test this question, the "incorrect" mouse PSMA sequence was cloned into a retroviral vector and site-directed mutagenesis (SDM) was used to change each of the two amino acids back to what they should be (G240A and E287N). These were carried out independently to generate constructs each with only one "incorrect" amino acid but also the E287N construct underwent a second round of SDM to additionally incorporate the G240A change resulting in a construct with both corrective changes (i.e. both amino acids correct for the C57Bl/6 mouse PSMA sequence).

The cells transfected with the construct encoding the "incorrect" protein sequence (p.G-mPSMA) could be recognised by both the 11C8 and 9C1 antibodies (Figure 53). Those cells transfected with the E287N SDM construct (p.G-mPSMA-E287N) were recognised equally well by both antibodies. In contrast, the G240A SDM (p.G-mPSMA-G240A) ablated recognition by both antibodies thereby confirming that the amino acid at position 240 was included in the 9C1 and 11C8 recognition epitopes. There was a chance that the SDM reaction introduced a mistake in a critical part of the vector that did not allow the protein to be expressed at all. The E287N and G240A double SDM construct (p.G-mPSMA-G240A+E287N i.e. the same as my correct sequence) confirms this was not the case as it was created by carrying out the G240A SDM on the existing E287N construct. Therefore two G240A encoding constructs created by independent SDM reactions confirm that this amino acid is critical for 9C1 and 11C8 antibody recognition. Control transductions with the vector plasmid alone (p.MSCVpuro) confirm the level of background staining by each of these two antibodies.

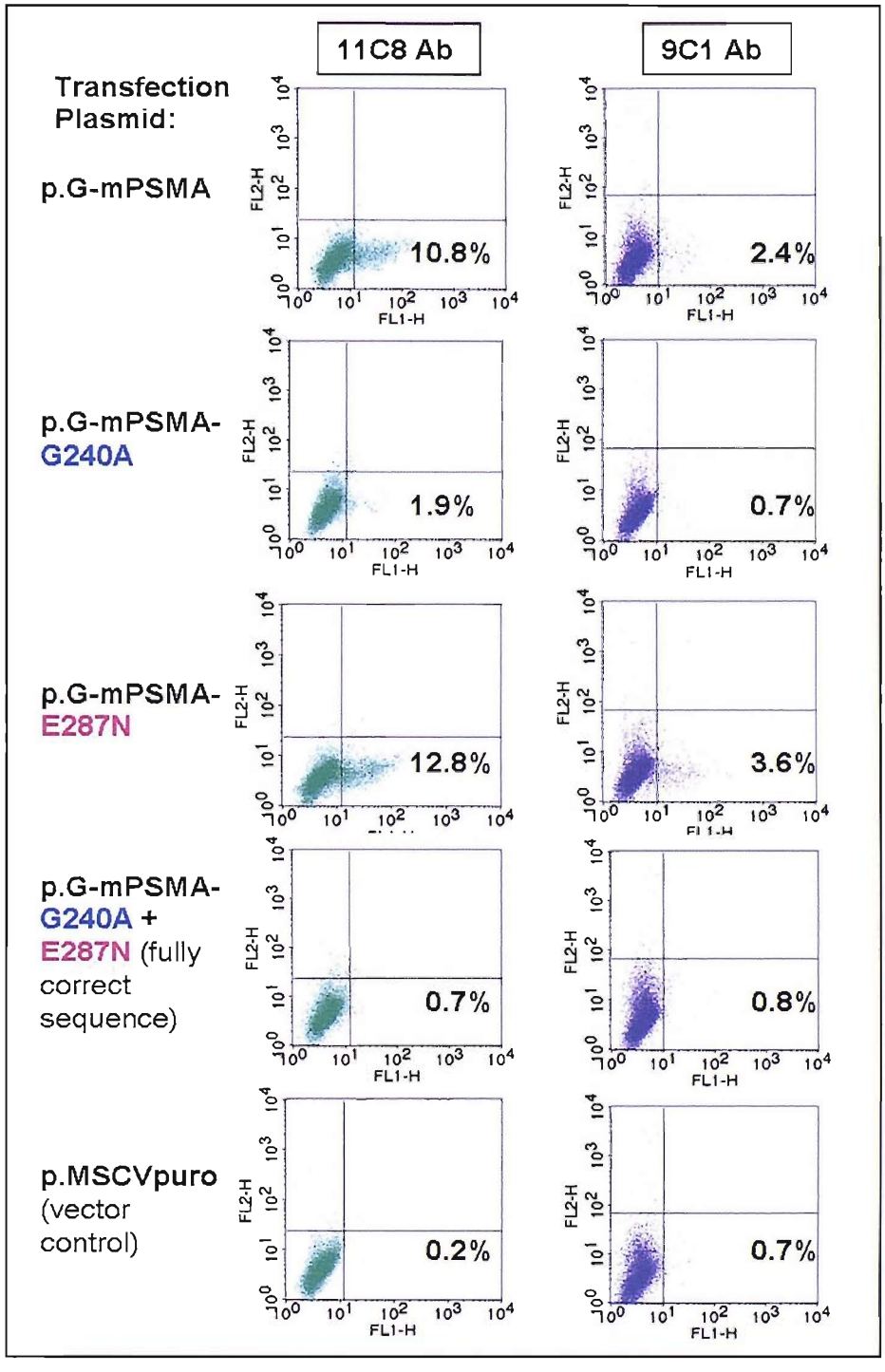


Figure 53: SDM of mouse PSMA sequence reveals 9C1 and 11C8 antibody recognition epitopes

The incorrect sequence from Dr. Gregor underwent sequential SDM to revert it back to the sequence of the C57Bl/6-derived protein (G240A and E287N). TRAMP-C1 cells were retrovirally transduced with the incorrect (p.G-mPSMA), single (p.G-mPSMA-G240A or p.G-mPSMA-E287N) or double (p.G-mPSMA-G240A+E287N) SDM constructs. Mouse PSMA expression was analysed with both 11C8 and 9C1 antibodies. These results were confirmed by two independent experiments.

4.3.8 Predicting K^b or D^b-Binding Mouse PSMA Epitopes

There are no mouse PSMA $CD8^+$ T cell target epitopes described in the literature. The mouse PSMA sequence was analysed for potential MHC class I-binding peptides with the

aim of assessing the ability of PSMA-specific CTLs to attack PSMA-expressing tumours in a tolerised mouse model. The mouse PSMA sequence was put through the SYFPEITHI (http://www.uni-tuebingen.de/uni/kxi/, [385]) and BIMAS (http://www-bimas.cit.nih.gove) algorithms to generate a list of peptides predicted to bind the mouse D^b or K^b mouse MHC class I molecules and the predicted half-time dissociation values. The PAPROC database was additionally used to calculate the probability of correct C-terminal cleavage by the immunoproteasome and to check for epitope-destroying cleavages. Details of these predictive algorithms can be found in section 3.1.5.1.

There were no high scoring K^b-binding peptides predicted so only D^b-binding peptides were considered. The predicted mPSMA86-94 peptide was ranked #1 by BIMAS and SYFPEITHI and has a high probability of C-terminal cleavage. This peptide has been previously investigated with a p.DOM-epitope vaccine but failed to elicit any $CD8^+$T-cell responses in C57Bl/6 mice, possibly as a result of efficient tolerance to this peptide.

The mouse PSMA636-644 (mPSMA636) peptide was chosen due to the high scoring for all the algorithms (second best predicted D^b-binding and estimated dissociation half time) as well as the high probability calculated for the correct proteasomal (C-terminal) cleavage by PAPROC. The high scoring can be attributed to the correct P5 anchor residue and the preferred amino acids at four other positions (Table 10). The mPSMA636 epitope was used to create a p.DOM-epitope vaccine. A schematic representation of this vaccine can be seen in Figure 47.

Position (P)	1	2	3	4	5	6	7	8	9	SYFPEITHI Binding Score	BIMAS Estimated Half Time of Dissociation
Amino Acid Preference for H2-D^b motif	A F S	A G M	I L P V	E V	N	L	D E T	E T Y	M I L		
mPSMA86	A	G	T	Q	N	N	F	E	L	26	302.4
mPSMA636	S	A	V	N	N	F	T	D	V	26	285.1

Table 10: Predicted Mouse PSMA D^b-binding peptides

The peptides conform to the preferred anchor residues at position 5 (P5, purple) but not the C-terminal (P10, blue). This peptide also has the preferred residues at P1, P2, P3, and P7 (pink). The binding score is predicted by the SYFPEITHI database (http://www.uni-tuebingen.de/uni/kxi/, [385] and half-time dissociation scores predicted by BIMAS (http://www-bimas.cit.nih.gove) [386]

4.3.9 DNA Vaccine Primes Peptide Specific $CD8^+$ T cells against the mPSMA636 Epitope

The p.DOM-mPSMA636 vaccine was assessed for the ability to elicit epitope-specific $CD8^+$ T-cell responses in C57Bl/6 mice after a single priming immunisation. Male C57Bl/6 mice were vaccinated with p.DOM-mPSMA636 or the control p.DOM DNA vaccines and sacrificed on day 14. Splenocytes were stimulated with varying amounts of mPSMA636 or 1μM p30 ($CD4^+$ T cell epitope within DOM) peptides for 24 hours in an IFNγ secretion ELISPOT. Positive responses are defined as those that are more than twice the "no peptide" background value.

13/14 (93%) of mice primed with the p.DOM-mPSMA636 vaccine exhibited responses to p30 (ranging 40-255 IFNγ spots per million splenocytes, mean 140.5 (±19.9), data not shown). The control mice immunised with p.DOM all demonstrated responsiveness to the p30 peptide (ranging 185-850 IFNγ spots per million splenocytes, mean 370.5 (±121.6)). These responses demonstrate good vaccine performance. The p.DOM vaccinated mice displayed no response when incubated with the mPSMA636 peptide (Figure 54).

The p.DOM-mPSMA636 vaccine was able to effectively prime epitope-specific $CD8^+$ T cells in 5/14 (36%) vaccinated male mice (1μM group mean 70.4 (±23.6), as revealed by the *ex vivo* analysis (Figure 54). Although the group responses at each peptide concentration were not statistically significant there were two mice that generated high levels of mPSMA636-specific $CD8^+$ T cells (more than 10-fold higher than their background). The responses reached 335 IFNγ spots per million cells when stimulated with 0.1μM mPSMA636 peptide (mean 65.8 (±24.1)). The degree of stimulation was variable and declined with decreasing mPSMA636 peptide concentrations. This confirmed the presence of a mPSMA636-specific T-cell repertoire in a potentially tolerised mouse model.

To attempt to boost responses a prime-EP boost vaccination protocol was used. Mice were primed on day 0, boosted with EP on day 28 and sacrificed 8 days post-boost on day 36. The IFNγ ELISPOT was carried out in the same way as described above.

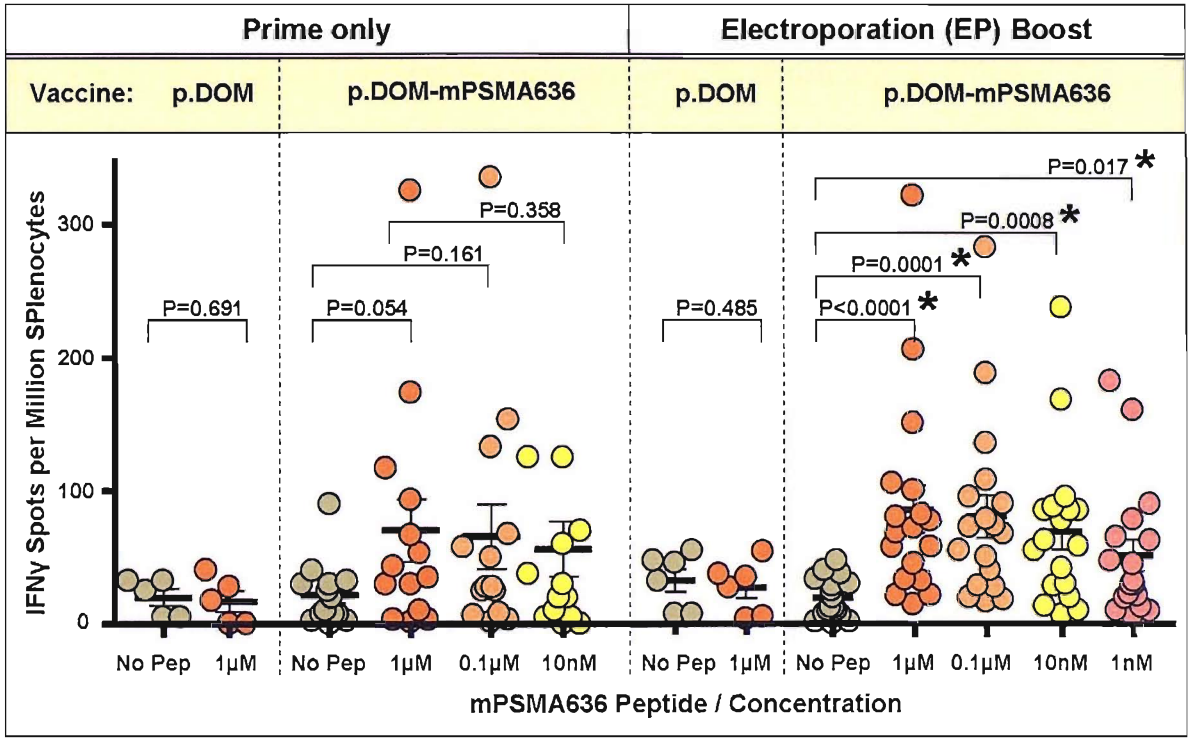


Figure 54: IFNγ responses of ex vivo mPSMA636-specific CD8$^+$ T cells following peptide stimulation

C57Bl/6 mice were immunised with p.DOM-mPSMA636 or p.DOM control vaccines intramuscularly on day 0 and sacrificed on day 14 (Prime only) or boosted with EP on day 28 then sacrificed day 36. Splenocytes were stimulated in *ex vivo* IFNγ ELISPOT without any peptide to measure background levels (grey) or mPSMA636 peptide (orange/yellow) at different concentrations. The p30 responses are not shown as they are on a different scale. Responses from individual spleens are plotted (mean of triplicates) along with the mean and SEM error bars. The data are pooled from two different experiments for each immunisation protocol. (*) indicated were values were statistically significant as assessed by the Mann Whitney test and defined by $p<0.05$.

Good vaccine performance was confirmed by the high p30 responses in 18/18 mice vaccinated with p.DOM-mPSMA636 (ranging 183-1060, mean 471(±58.7), data not shown). These are significantly boosted compared to the primed-only p30 responses. This boost was also observed in the control mice immunised with p.DOM where all (5/5) mice exhibited high levels of responsiveness to the p30 peptide (range 258-1330, mean 699 (±180.9), data not shown).

The mPSMA636-specific CD8$^+$ T cell responses were also enhanced by this boosting protocol although to a much lower extent. 14/18 (78%) mice vaccinated with p.DOM-mPSMA636 exhibited responsiveness to the mPSMA636 peptide, reaching up to 320 IFNγ spots per million cells when stimulated with 1μM mPSMA636 peptide (mean 85.7 (±17.7)). The maximal responses were similar to those achieved by the primed only mice. The extent of stimulation declined with decreasing mPSMA636 peptide concentrations

with response mean 80.2 (±15.8) at 0.1μM, 69.2 (±13.6) at 10nM and 51.5 (±11.7) at 1nM. The group responses at every peptide concentration were statistically significant.

The mPSMA636-specific $CD8^+$ T cell response after prime + boost was of ~10-fold higher avidity than after a prime-only vaccination protocol as seen by the avidity of the 50% response rate (Figure 55). This difference is not significantly different when the *ex vivo* ELISPOT stimulation was carried out with 10nM peptide. Responsiveness to 1nM peptide was unfortunately not assessed for the T cells from primed-only mice. This is the peptide concentration at which a more pronounced difference between the vaccination protocols would be expected, as noted with the p.DOM-PSMA663 (human peptide) vaccine (Chapter 3, Figure 22). These observations are in accordance with reports of T cell affinity maturation where adoptively transferred higher affinity T cells compete more efficiently than lower affinity T cells against an endogenous secondary response [402]. Following engagement, T cells downmodulate antigen presentation by APCs thereby effectively inhibiting stimulation of particularly lower affinity T cells.

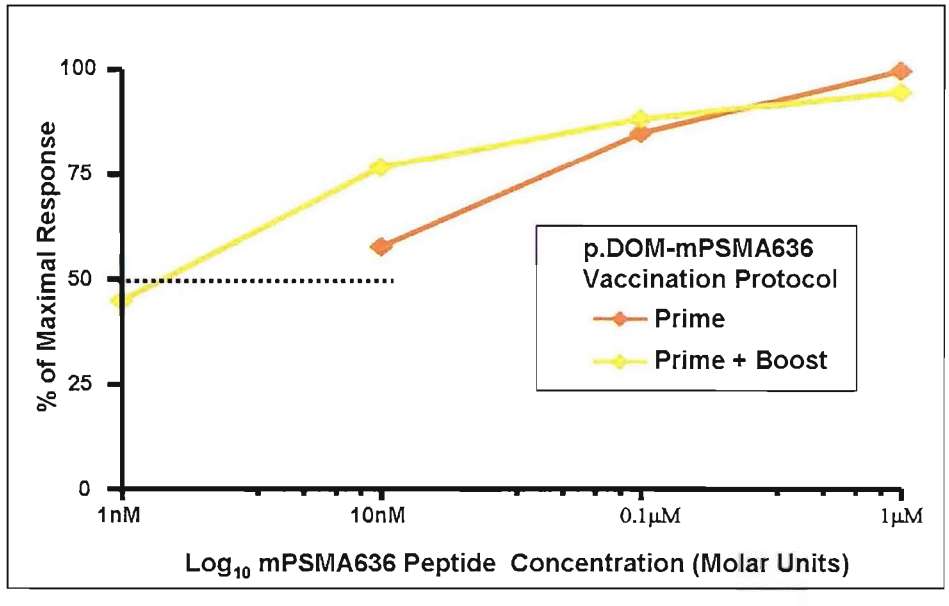


Figure 55: The avidity of CD8+ T cell responses primed and boosted by the p.DOM-mPSMA636 vaccine

The avidity of the p.DOM-mPSMA636 vaccination responses from the prime (orange) or prime + boost (yellow) protocol was analysed. The highest response for each mouse was taken as 100% and the responses to the other peptide concentrations expressed as a percentage of the maximal response. Values for each peptide concentration were averaged and plotted. The differences between the immunisation protocols are not statistically different.

4.3.10 Upregulation of MHC Class I Expression by TRAMP Cells

The TRAMP cells are notorious for low MHC class I expression, a common trait for PCa cell lines. These TRAMP-derived mouse cell lines (C57Bl/6 background) express murine H2-K^b and H2-D^b at low levels [226] that can be reportedly upregulated by IFNγ treatment [227]. To use the TRAMP cells transduced with mouse PSMA as a target cell line for lysis by CTLs specific for the D^b-binding mPSMA636 peptide it would be necessary to increase H2-D^b expression. The baseline MHC class I D^b expression and that after IFNγ treatment were assessed. Pre-treatment H2-D^b levels were confirmed to be very low (Figure 56A) but could be upregulated by a minimum 48-hour IFNγ treatment (Figure 56C). All doses (5, 10 and 20ng/ml) of IFNγ used increased H2-D^b levels to what appeared to be similar levels by eye but the MFI was vastly superior at 20ng/ml (Figure 56B).

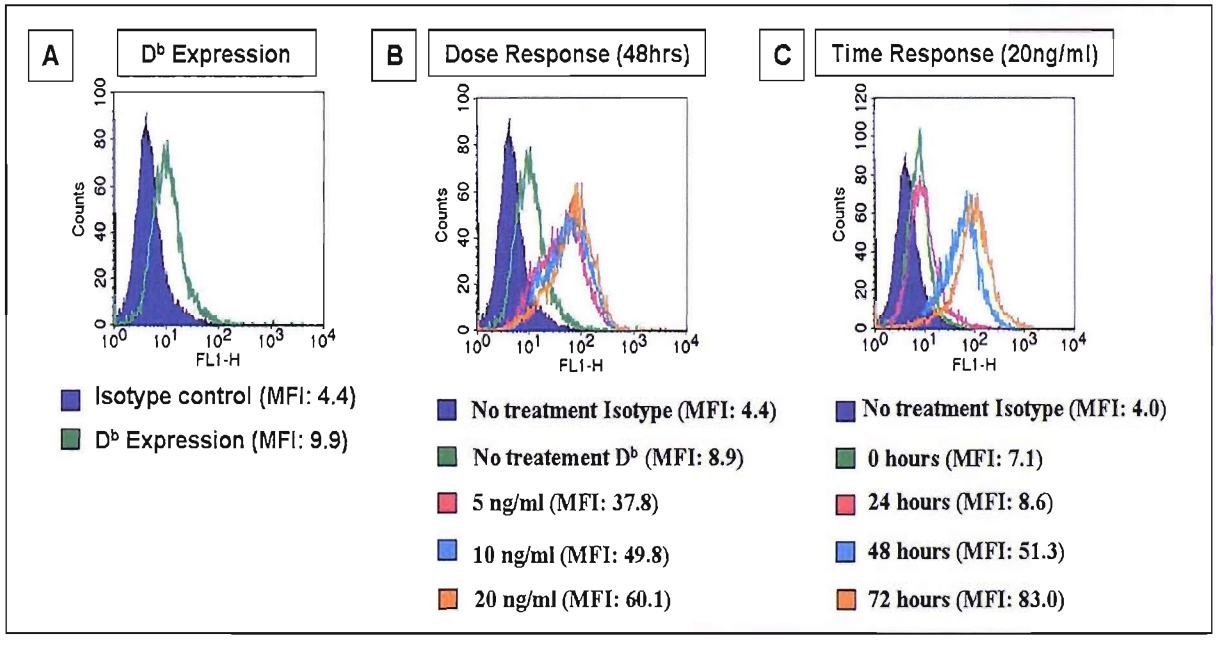


Figure 56: Analysis of H2-D^b expression by TRAMP-C1 cells before and after IFNγ treatment
The level of H2-D^b expression by untreated (A) and IFNγ treated (B and C) TRAMP-C1 cells was analysed by flow cytometry. The cells were treated with varying concentrations of IFNγ for 48 hours (B) or with 20ng/ml IFNγ for 24, 48 or 72 hours (C).

4.3.11 Ability of mPSMA636-Specific CTLs to Kill Mouse PSMA-Expressing Target Cells

The next step was to assess whether the mouse PSMA636 peptide is processed from the full length protein and presented by tumour cells. This confirmation is critical to assess if mice represent a tolerised model for this peptide, thereby making it relevant support for the work with the human PSMA p.DOM-epitope vaccines in patients.

The way that we achieve this aim is to assess the ability of functional mPSMA636-specific CTLs to kill a mouse PSMA-expressing (mPSMA+) cell. As already discussed the TRAMP-C1 cells do not express mouse PSMA (section 4.2.5.1) so a TRAMP mPSMA+ transfectant was created (section 4.2.5.4) by retroviral transduction.

Splenocytes from mice vaccinated with p.DOM-mPSMA636 were expanded *in vitro* with 10nM mPSMA636 peptide and IL-2 (from day 3) for 6 days prior to use in a ^{51}Cr release assay. The target cells for the assay were the transfected TRAMPmPSMA+ or the vector control transfected cells TRAMPcontrol. The TRAMPmPSMA+ cells were used with (TRAMPmPSMA+mPSMA636+) and without peptide-loading. Both sets of target cells were treated with IFNγ for 48 hours prior to the assay.

One line of mPSMA636-specific CTLs was highly efficient at killing the mPSMA636 peptide-loaded target cells and also the cells endogenously expressing mouse PSMA (Figure 57). The *ex vivo* splenocytes used to expand this CTL line had exhibited high levels of stimulation in the ELISPOT assay (325 IFNγ spots per million).

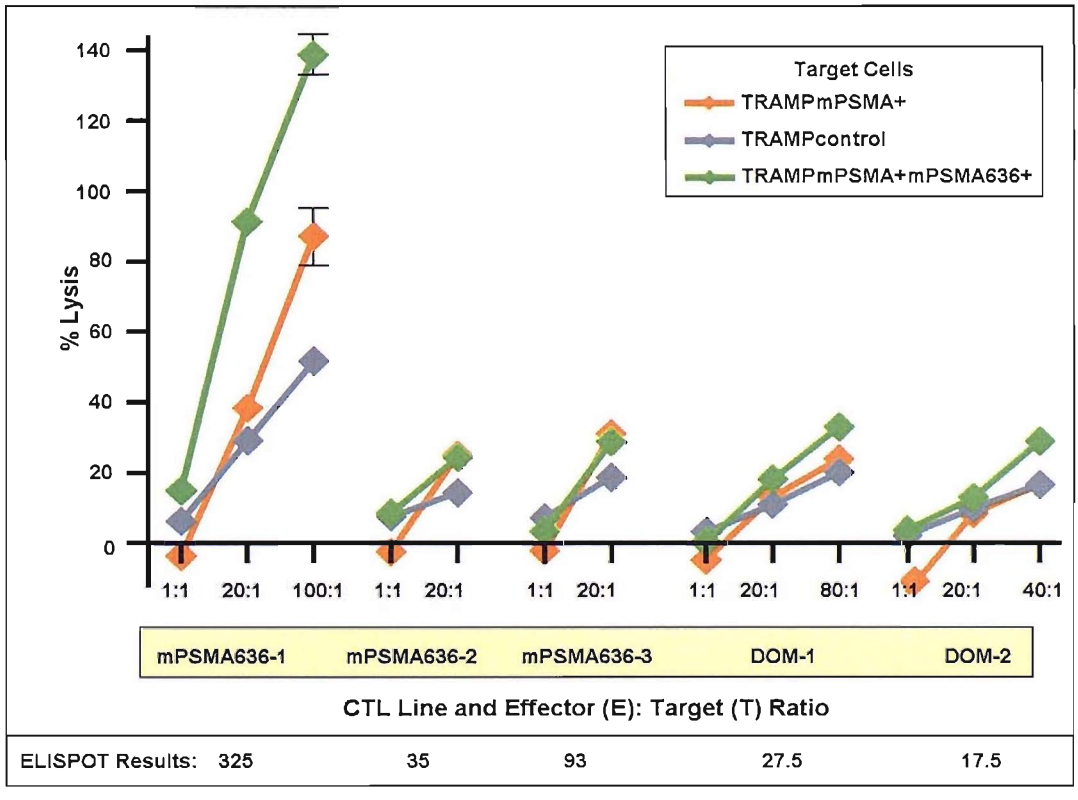


Figure 57: mPSMA636-specific CTLs can lyse cells expressing endogenous mouse PSMA

Splenocytes from vaccinated C57Bl/6 male mice (p.DOM-mPSMA636 or p.DOM) were expanded in the presence of 10nM mPSMA636 peptide and IL-2. After 6 days the ability of mPSMA636-specific CTLs to kill mouse PSMA-expressing cells (TRAMPmPSMA+, orange), the vector transfected control cells (TRAMPcontrol, grey) and the positive control 1μM mPSMA636 peptide-loaded target cells (green) was assessed by ^{51}Cr release assay. Target cells were pre-treated with 20ng/ml IFNγ for 48 hours prior to assay. The experiment was carried out in triplicates for which the SEM error bars are shown.

In contrast, the *in vitro* phase did not expand functional CTLs (only low E:T ratios achieved) of another CTL line despite demonstrating some response in the *ex vivo* ELISPOT (93 IFNγ spots).

This confirms that the mPSMA636 peptide is processed and presented and therefore these experiments in C57Bl/6 mice do represent a tolerised setting. The data also suggests that relatively high levels of *ex vivo* responses are required in order to be able to expand functional CTLs.

The killing of the TRAMP control cells was high at ~50% for the single functional mPSMA636-specific CTL line but still ~35% lower than that of TRAMPmPSMA+ cells. The high background lysis is likely to be due to IFNγ and IL-2 treatment: makes the target cells more susceptible to NK cell killing; the IL-2 used to expand CTL lines would additionally expand NK cells.

To confirm the ability of the mPSMA636-specific CTLs to kill TRAMPmPSMA+ cells further we decided to expand a CTL line with 10nM peptide for 4 weeks (and 3 re-stimulations with freshly isolated irradiated peptide-loaded splenocytes) to ensure high numbers of CTLs and eliminate NK cells. The CTLs were then used in a ^{51}Cr release assay at 1:1 and 100:1 E:T ratios with target cells pre-treated with IFNγ for 48 or 72 hours; TRAMP control cells were the targets loaded with peptide.

The mPSMA636-specific CTLs were able to kill TRAMPmPSMA+ cells treated for 48 or 72 hours equally well (Figure 58). The level of TRAMPcontrol cell killing remained low and the absence of NK cells confirmed by the inability to lyse the NK-cell sensitive YAC-1 cells. The killing of the peptide-loaded target cells was not higher than that of the TRAMPmPSMA+ cells therefore the maximal killing capacity of the CTLs was achieved. These results re-confirm that the mPSMA636 peptide is definitely processed and presented from the mouse PSMA protein backbone.

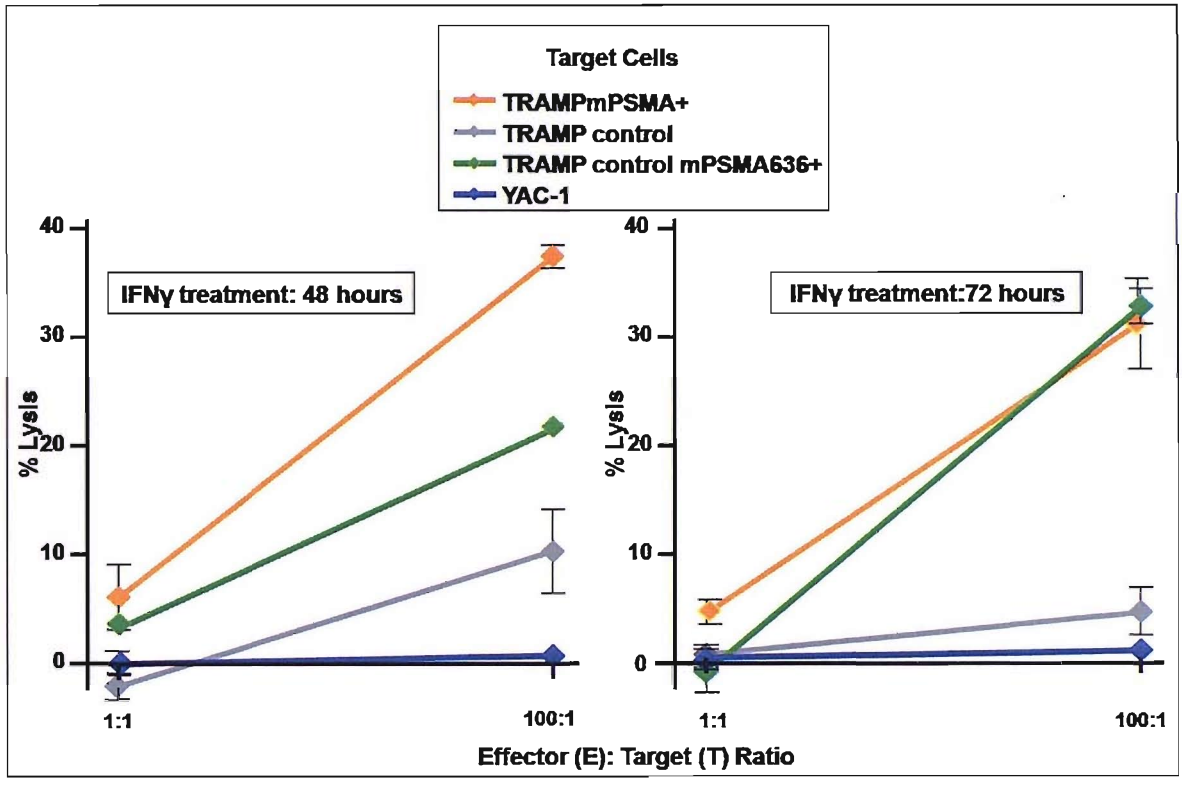


Figure 58: mPSMA636-specific CTLs expanded in vitro for 4 weeks can lyse TRAMP cells expressing endogenous mouse PSMA

Splenocytes from p.DOM-mPSMA636 vaccinated C57Bl/6 male mice were expanded in the presence of 10nM mPSMA636 peptide and IL-2. ^{51}Cr release assay was performed after 4 weeks (3 restimulations) to assess the ability of mPSMA636-specific CTLs to kill mouse PSMA expressing cells (TRAMPmPSMA+, orange), the vector transfected control cells (TRAMP control, grey) and the positive control 1μM mPSMA636 peptide-loaded target cells (TRAMP contol mPSMA636+, green). The target cells were each pre-treated with IFNγ for 48 or 72 hours prior to assay. The T cells and target cells were co-cultured at 1:1 and 100:1 E:T ratios. The experiment was carried out in triplicates for which the SEM error bars are shown.

4.3.12 Immunity Generated by the p.DOM-mPSMA636 Vaccine was Unable to Protect Mice from Mouse PSMA-Expressing Tumours

At this point we had confirmed that the p.DOM-mPSMA636 vaccine could generate epitope-specific T cells, that these secreted IFNγ *ex vivo*, were cytotoxic *in vitro* (functional) and that the tumour cells expressing mouse PSMA presented the mPSMA636 peptide. The next step was to assess if these mPSMA636-specific CTLs could also be functional *in vivo* and prophylactically protect mice from TRAMPmPSMA+ tumour challenge.

Male C57Bl/6 mice received priming and EP boosted immunisations with the p.DOM-PSMA636 vaccine on day 0 and day 28 respectively. These mice were then challenged subcutaneously with a lethal dose of TRAMPmPSMA+ tumour cells on day 36.

Vaccination with p.DOM-mPSMA636 was unable to protect mice from challenge with mouse PSMA-expressing tumour cells (Figure 59).

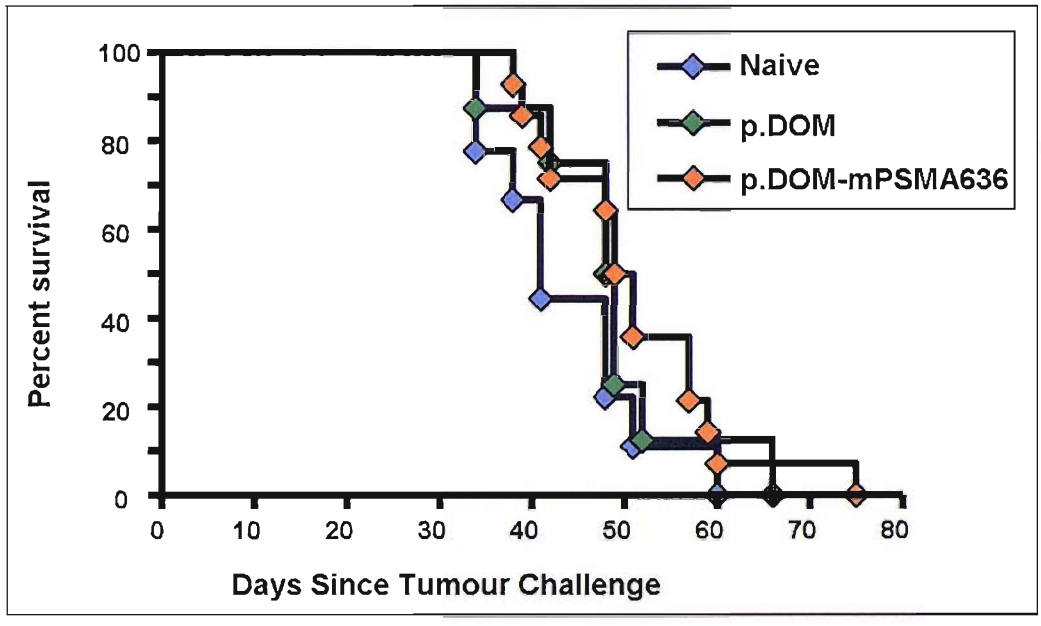


Figure 59: The p.DOM-mPSMA636 vaccine failed to protect against a mouse PSMA-expressing tumour

Mice in each vaccine group were immunised on day 0, boosted with EP on day 28 and challenged with a lethal dose of untreated TRAMP mPSMA+ cells subcutaneously on day 36. The graphs show end days from the point when mice were challenged for naïve (purple) mice, p.DOM (green) or p.DOM-mPSMA636 (orange) vaccinated mice.

We considered whether the low H2-D^b expression of the tumour cells would compromise the ability of these CTLs to kill them. It has been reported that the MHC class I expression of these tumour cells increases *in vivo* [226]. A preliminary experiment co-injecting CFSE-labelled tumour cells with Matrigel™ and excising the plug after 5 days revealed that indeed only a small number of tumour cells recovered at this point expressed H2-D^b and at low levels (Figure 60). It therefore appears to take longer than 5 days for the expression of the murine MHC class I to increase *in vivo* but these 5 days would be critical in allowing the tumour and regulatory mechanisms to establish, leaving the CTLs unable to eliminate the tumour. When the tumour cells were pre-treated with IFNγ and then co-injected with Matrigel™, the majority of tumour cells isolated from the plug after 5 days were still expressing H2-D^b (Figure 60).

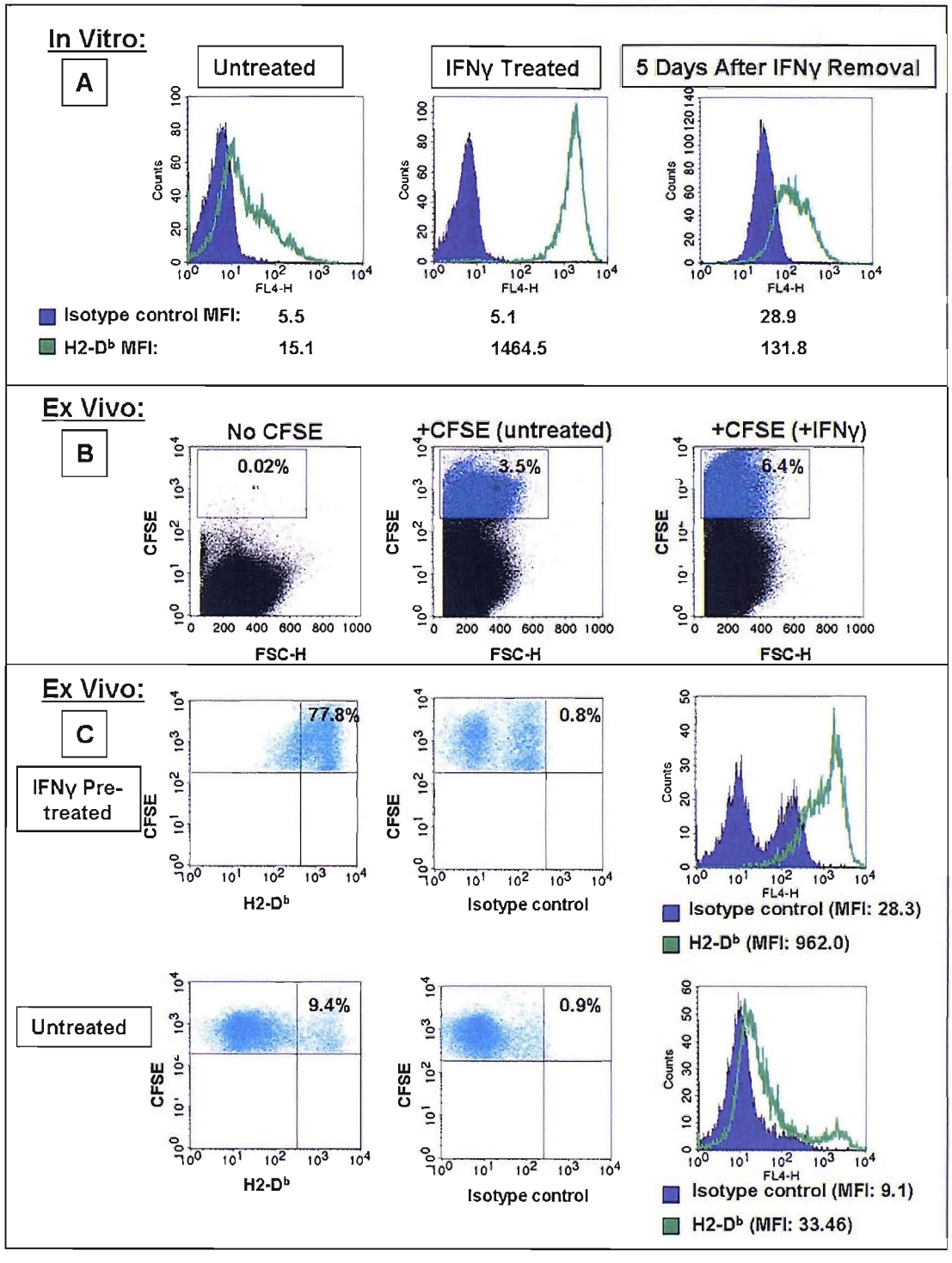


Figure 60: H2-D^b expression of TRAMP mPSMA+ cells *in vitro* and *in vivo*

TRAMP mPSMA+ cells cultured *in vitro* or additionally pre-treated with 20ng/ml IFNγ for 72 hours were analysed for H2-D^b MHC class I expression (A). The IFNγ was then removed and cells cultured *in vitro* for a further 5 days prior to re-analysis of MHC class I (A). Alternatively the TRAMP mPSMA+ cells were CFSE-labelled and co-injected with Matrigel™ into C57Bl/6 mice and excised after 5 days (B). Analysis of CFSE+ TRAMP mPSMA+ cells (C) allowed comparison of H2-D^b expression (after 5 days *in vivo*) in cells pre-treated with IFNγ (C, top panel) or untreated (C, lower panel) prior to *in vivo* passage. Two mice per group were analysed but results shown for a single representative mouse.

We therefore attempted to challenge p.DOM-mPSMA636-vaccinated mice (same prime + boost protocol) with TRAMPmPSMA+ tumour cells that had been pre-treated with IFNγ for 72 hours prior to challenge. However, despite the ensured presence of surface H2-D^b on the tumour cells the mice were not protected from tumour growth in either of two experiments carried out (total of 16 p.DOM-mPSMA636-vaccinated mice, data not shown). The peptide-specific $CD8^+$ T-cell responses of these mice after boosting is unknown as a tetramer would be necessary to gain such information without culling them. However, it is expected to be similar to that shown for other mice in Figure 54. The data demonstrate that the immunity generated by the p.DOM-mPSAM636 vaccine alone with the current immunisation protocol is not sufficiently strong to eliminate mouse PSMA-expressing tumours *in vivo*.

4.4 Discussion

4.4.1 Immunisation with a Mouse PSMA FrC Fusion DNA Vaccine

It has been previously shown that a vaccine encoding the clonal Id of B-cell tumours (scFv) fused to a C-terminal foreign FrC sequence (p.scFv-FrC) can protect mice from challenge with that particular tumour as assessed with the murine A31 lymphoma model [165] (section 1.3.6). The protection was mediated by scFv-specific antibody responses. The $CD4^+$ T-cell help from the FrC fusion component was essential for a sc-Fv-specific humoral response and the fusion was an absolute requirement.

On the basis of this success, the first aim was to elicit a humoral immune response against the self mouse PSMA protein and thereby determine whether the FrC fusion could break potential tolerance to this tumour antigen by means of a DNA vaccine. The vaccines encoding the extracellular domain of mouse PSMA may also potentially prime $CD8^+$ T-cell responses. These were not analysed for two reasons; the inability of HHD mice in chapter 3 to generate human-specific $CD8^+$T cells in response to the full-length human PSMA vaccines (non-tolerised model) as well as the absence of any published mouse PSMA $CD8^+$ T cell epitopes.

Unfortunately it was not possible to determine if the mice vaccinated with p.mPSMAEC-FrC or p.mPSMAEC-DOM vaccines were able to make anti-mouse PSMA antibody responses due to problems developing suitable assays. In any case even if anti-mouse PSMA responses were elicited, they were unable to provide significant protection against the TRAMP-C1 cells transduced to express mouse PSMA. Thus FrC or DOM fusion vaccine designs have therefore proved unsuccessful for targeting mouse PSMA-expressing tumours.

The significant levels of anti-FrC antibodies in the sera of mice vaccinated with p.mPSMAEC-FrC confirm the performance of the DNA vaccine. However, the level of anti-FrC humoral responses may not necessarily correlate with anti-tumour antigen titres. Unpublished data from our laboratory indicates that anti-FrC and anti-Id antibody levels after vaccination with p.scFv-FrC vaccines do not necessarily correlate.

If this project were to be taken further the most important focus would be on making mouse PSMA protein to assess the anti-mouse PSMA humoral response. If none can be identified the problem could potentially be in the folding of the fusion protein. The extracellular domain alone can generate humoral responses that could recognise TRAMP cells transduced to express human PSMA as shown by vaccination of C57Bl/6 mice with

the p.hPSMAEC vaccine. The fusion could potentially introduce misfolding that can severely interfere with the ability to generate anti-PSMA antibodies. The humoral responses elicited by p.hPSMAEC-FrC could not be separately examined for anti-human PSMA antibodies as we had no human PSMA protein either and also the high background staining of control sera during flow cytometry analysis.

If protein folding is an issue the vaccine could be altered to include a longer linker sequence between the PSMA and FrC/DOM components in order to minimise steric hinderance and allow the best opportunity for correct folding. Another factor to contemplate when targeting this protein is that PSMA is found and is functional as a homodimer. It has been reported that the human PSMA extracellular domain protein homodimer but not monomer can elicit PSMA antibodies that can recognise human PSMA-expressing tumour cells [339]. Therefore when targeting PSMA with a DNA vaccine it may be necessary to consider allowing the homodimer to assemble for optimal humoral immunity. The extracellular domain of human PSMA alone can dimerise and this may explain the successful anti-human PSMA antibodies generated by others and in this study with the p.hPSMAEC DNA vaccine.

On the other hand there isn't a strong reason to pursue the generation of PSMA humoral responses further. The MAbs alone have not demonstrated success in the clinic and as a result are now being developed in the context of delivering toxins (section 4.1.2).

4.4.2 Different Molecular Forms of PSMA?

Glycosylation is a post-translational modification that can generate different glycoforms of glycoproteins. In some cases the differential glycosylation can generate proteins with different biochemical and even functional properties. The existence of tissue-specific and tumour-specific differences in the sugar chains on the surface of glycoproteins has been established for some time [420]. Although the polypeptide sequence determines the potential glycosylation sites it is thought that tissue-dictated exposure to the different enzymes involved in distinct glycosylation reactions confers the tissue/tumour-specific glycosylation.

An interesting observation has been the ability of sera from p.hPSMAEC-vaccinated mice to recognise human PSMA expressed by TRAMP-C1 cells but not NIH3T3 despite the MAb being able to recognise both. This suggests that the molecular forms of PSMA expressed by different cell types may not be comparable (section 4.3.5.2). The PSMA protein has 10 potential glycosylation sites and is heavily glycosylated (section 1.5.7) leaving large scope for potential differential glycosylation. It may be that PSMA

glycosylation patterns in prostate cells differ from those of fibroblasts or that those from tumour cells differ from healthy cells. It would be possible to assess if the anti-human PSMA antibodies elicited in C57Bl/6 mice could recognise the human PSMA expressed by TRAMP-C1 cells after treatment with PGNase to remove the surface N-glycosylation. Elimination of recognition would suggest the humoral response focuses on the sugar moieties of the PSMA protein.

Encouragingly, the observation confirms that DNA vaccines injected into and expressed by the muscle induce humoral responses that can recognise the tumour prostate-relevant PSMA form. It may be of interest to investigate whether PSMA expressed by the brain, kidney and small intestine are of the same molecular forms as that expressed by the diseased prostate. Mucin-1 is the most famous example of a tumour antigen defined as such based on its aberrant tumour-specific glycosylation [421]. Targeting any such differences between expressed PSMA, particularly with MAbs, would further relieve concerns of healthy tissue attack.

4.4.3 The p.DOM-mPSMA636 DNA Vaccine Can Prime $CD8^+$ T cells

There are no mouse PSMA $CD8^+$ T cell epitopes described in the literature. A K^b or D^b-binding mouse PSMA epitope is desired for future assessment of protection from a mouse PSMA-expressing tumour by mouse PSMA-specific $CD8^+$ T cells in a tolerised mouse model. The mouse PSMA636 epitope was chosen from those predicted to bind the mouse MHC class I, D^b, by predictive algorithms. The p.DOM-mPSMA636 vaccine was able to generate mPSMA636-specific $CD8^+$ T-cell responses in mice after a prime-only but responses were greatly improved following a prime + boost immunisation protocol. Next, the processing of this peptide was determined by means of a chromium release assay. The mPSMA636 peptide is processed and presented by cells endogenously expressing mouse PSMA thereby allowing CTL-mediated lysis of these target cells. This confirms that the p.DOM-mPSMA636 vaccine was able to break tolerance to prime cytotoxic $CD8^+$ T-cell responses.

The mPSMA636 peptide is likely to be the immunodominant one from the PSMA sequence in C57Bl/6 mice. C57Bl/6 mice immunised thrice with alphavirus particles expressing human PSMA displayed $CD8^+$ T-cell responses that mapped to the PSMA631-645 epitope thereby demonstrating that this peptide includes the immunodominant K^b/D^b-binding peptide within the human PSMA [422]. This longer peptide includes the human SAVKNFTEI sequence that is similar to the corresponding mPSMA636 9mer SAVNNFTGV and the mouse and human PSMA also have identical immediate upstream

and downstream sequences of the 9mer. Therefore the mPSMA636 peptide is also likely to be the immunodominant peptide for the mouse PSMA protein and the ability of the C57Bl/6 mice in this study to retain a T-cell repertoire against this peptide is encouraging.

The response primed by the p.DOM-mPSMA636 vaccine was unable to convey prophylactic protection to the immunised mice. This would suggest that the immunity raised was insufficient and further work with this vaccine must involve developing means of boosting the response. A strategy that could be investigated is boosting with another vaccine encoding a protein that undergoes different pathways of presentation (direct vs cross-presentation) as has been previously demonstrated [157]. Alternatively other help such as Treg depletion could be employed although this is likely to augment undesired retroviral immunity. This vaccine could provide quite a good model as once immunity against the mPSM636 peptide is improved (magnitude and functionality) it could then be assessed for efficacy in the spontaneous TRAMP mouse model.

4.4.4 Xenogeneic Vaccination Cannot Break Tolerance to Mouse PSMA

A published study claimed C57Bl/6 and Balb/c mice primed with a DNA vaccine encoding human PSMA and boosted with the human PSMA protein were able to generate humoral responses against both human and mouse PSMA [419]. It was therefore claimed that B cells recognising mouse PSMA epitopes had not been deleted or anergised and could be activated to break tolerance through the use of xenogeneic-specific $CD4^+$ T-cell help. They found that all the antibodies recognising native mouse PSMA cross-reacted with human PSMA, and 10% of the human PSMA-specific antibodies also recognised the native mouse protein [419]. In contrast there was no response to a DNA vaccine encoding the autologous mouse PSMA.

The authors of this study kindly gifted us the human and mouse PSMA cross-reacting 11C8 and 9C1 antibodies they generated. The inability of these antibodies to recognise cells transduced to express mouse PSMA led us to question their specificity and whether it was actually only for the human PSMA protein. The mouse PSMA sequence used by the authors of the study had two incorrect amino acids that were actually those in the human PSMA sequence. This sequence was confirmed incorrect from that in both C57Bl/6 and Balb/c mice. The SDM experiments in the present study confirmed that the epitopes recognised by both antibodies included one of these two incorrect amino acids. This finding questions those of the published study which in fact did not raise any anti-mouse

PSMA antibodies and therefore did not break tolerance at all. The immunisation protocol they used focused on human PSMA epitopes and this single amino acid dependency explains why the antibodies had much higher affinity for the human PSMA than the incorrect mouse PSMA proteins. This highlights the importance of using 100% correct sequences for immunisation since a single incorrect amino acid can have an immense impact on findings. The published findings have apparently been translated into the clinic where PCa patients are being immunised with either murine or human PSMA DNA followed by crossover of the vaccines for boosting. Although the findings presented here do not necessarily mean that PSMA xenogeneic vaccination is not feasible at all, it is clear that a more thorough investigation and particularly attention to detail is necessary prior to progression to the clinic.

Xenogeneic vaccination of mice with a DNA vaccine encoding the human PSMA sequence primed human PSMA-specific but not any mouse PSMA-specific T cells [423]. It therefore appears that the PSMA model of xenogeneic vaccination cannot overcome T-cell tolerance. Clearly xenogeneic vaccination has not yet proven successful at generating either PSMA humoral or cellular responses. In contrast, the p.DOM-mPSMA636 vaccine constructed in this study was able to break T-cell tolerance in mice (also previously shown in other models [159] thereby demonstrating the effectiveness of our vaccine design and providing further support for its use in the clinic.

4.5 Conclusions

We have shown that C57Bl/6 mice retain a $CD8^+$ T-cell repertoire for the predicted mPSMA636 D^b-binding peptide that the p.DOM-mPSMA636 vaccine was able to prime and expand. The result has confirmed the ability of the p.DOM-epitope vaccine design to elicit a response against PSMA as a self-protein in a fully tolerised setting. The ability of mPSMA636-specific CTLs to kill mouse PSMA-expressing tumour cells *in vitro* confirmed peptide processing and presentation. The future focus would be on improving the magnitude and functionality of the epitope-specific CTL response in order to achieve *in vivo* protection against a mouse PSMA-expressing tumour. This would be essential to establish proof for the role of $CD8^+$T cells in PSMA-specific anti-tumour immunity

5. Appendix I

Table 11: Primers designed for study

Primer Name	Direction	Sequence	Purpose of Primer	Tm
hPSMAF1	Forward	AGATGTGGAATCTCCTTCACG	Cloning human PSMA	63.0
hPSMAR1	Reverse	CCTCTTAGGCTACTTCACTCAAAGTC	Cloning human PSMA	63.4
mPSMAF1	Forward	AGAGATGTGGAACGCACTGC	Cloning mouse PSMA	65.5
mPSMAR1	Reverse	GTGTTTAAGCTACTTCCCTCAGAGTC	Cloning mouse PSMA	63.3
HHDF1	Forward	ATGGCCGTCATGGCGC	Cloning HHD	69.9
HHDR1	Reverse	TCAAGATGTTAACAATCAAGGGTC	Cloning HHD	62.8
mouse Actin F	Forward	GCAATGGAAGAAGAAATCGCCG	cDNA confirmation	73
mouse Actin R	Reverse	ATACAAGGACAGCACCGCCTGAATG	cDNA confirmation	72.4
mPSMAEC SfiI F	Forward	TATGGCCCAGCCGGCCATGGCCATAAAA CCTTCCAATGAAG	Vaccines: p.mPSMAEC	87.3
mPSMA (stop) NotI R	Reverse	TATGCGGCCGCTAAGCTACTTCCCTCAGAG	Vaccines: p.mPSMAEC & protein expression	77.1
mPSMA (no stop) NotI R	Reverse	TATGCGGCCGCAGCTACTTCCCTCAGAG	Vaccines: p.mPSMAEC-fusion	78.8
BCL_1 HindIII F	Forward	TATAAGCTTGCCGCCACCATGGGTTGGAGC	Vaccine: p.PSMAFL	80.8
hPSMAEC SfiI F	Forward	TATGGCCCAGCCGGCCATGGCCAAATCCTCCAATGAAGCTACTAA	Vaccines: p.PSMA EC	87.9
hPSMA HindIII F	Forward	TATAAGCTTACCATGTGGAATCTCCTTCACG	Vaccines: p.PSMAFL	70.9
hPSMA (no stop) NotI R	Reverse	TATGCGGCCGCGGCTACTTCACTCAAAGTCTC	Vaccines: p.PSMA EC/FL	80.7
hPSMA (stop) NotI	Reverse	TATGCGGCCGCTTAGGCTACTTCACTCA	Vaccines: p.PSMA EC/FL	75.8
PSMA663 NotI (stop) R	Reverse	TATGCGGCCGCTTACAGAAACATGAGTTGATCATTCATCAT GTTACCCCAGAAGTCACG	Vaccine: p.DOM-PSMA663	90.3
mPSMA636 NotI (stop) R	Reverse	TATGCGGCCGCTTAAACATCTGTAAAATTATTGACTGCAGAGTTAC	Vaccine: p.DOM-mPSMA636	85.4
mPSMAECHis6F	Forward	TATGGCCCAGCCGGCCATGGCCCATCATCATCATCATC ATATAAAACCTTCCAATGAAGCTAC	mPSMA Protein Expression construct	90.3

Primer Name	Direction	Sequence	Purpose of Primer	Tm
BCL_1 BamHI F	Forward	ATAGGATCCGCCGCCACCATGGGTTGGAG CTGTATCATCTTC	Retroviral Construct: hPSMA	86.2
hPSMA XhoI R	Reverse	TATCTCGAGTTAGTTACCCCAGAAGTCACGCAGG	Retroviral & RNA synthesis Constructs: hPSMA	67.1
V_{H1} Leader BamHI F	Forward	TATGGATCCGCCGCCACCATGGACTGGACCTGGAGGGTCTTC	Retroviral construct: mPSMA	90.2
mPSMA XhoI R	Reverse	TATCTCGAGCTAAGCTACTTCCCTCAGAGTCTC	Retroviral Construct: mPSMA	71.1
HHD SDM EcoRI F	Forward	CTATCTCTTGTACTACACTGAGTTCACCCCCACTGAAAAAGATG	HHD SDM	76.9
HHD SDM EcoRI R	Reverse	CATCTTTTTCAGTGGGGGTGAACTCAGTGTAGTACAAGAGATAG	HHD SDM	76.9
HHD EcoRI F	Forward	TATGAATTCACCATGGCCGTCATGGCGCCCCG	Retroviral Construct: HHD	87.1
HHD XhoI R	Reverse	TATCTCGAGTCACGCTTTACAATCTCGGAG	Retroviral Construct: HHD	70.3
hPSMA NotI F	Forward	TATGCGGCCGCCACCATGGGTTGGAGCTGTATC	RNA synthesis construct hPSMA	85.6
G-mPSMASDMG240A F	Forward	GCTGACTACTTTGTTCCTGCGGTGAAGTCCTATCCAG	G-mPSMASDM: p.G-mPSMA G240A	78.6
G-mPSMASDMG240A R	Reverse	CTGGATAGGACTTCACCGCAGGAACAAAGTAGTCAGC	G-mPSMASDM: p.G-mPSMA G240A	78.6
G-mPSMASDME287N F	Forward	CTTATAGGCATGAGTTGACAAACGCTGTTGGCCTTCCAAG	G-mPSMASDM: p.G-mPSMA E287N	81.0
G-mPSMASDME287N R	Reverse	CTTGGAAGGCCAACAGCGTTTGTCAACTCATGCCTATAAG	G-mPSMASDM: p.G-mPSMA E287N	81.0

5.2 Appendix II: Gates for in Vivo Killing Matrigel™ Experiment

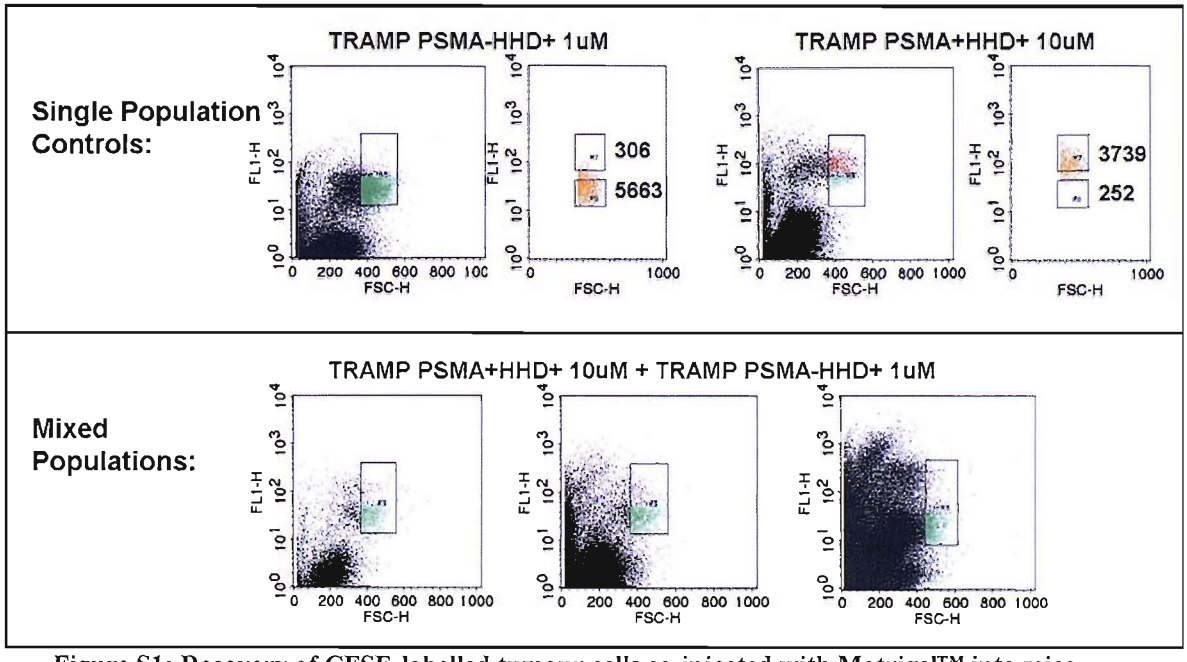
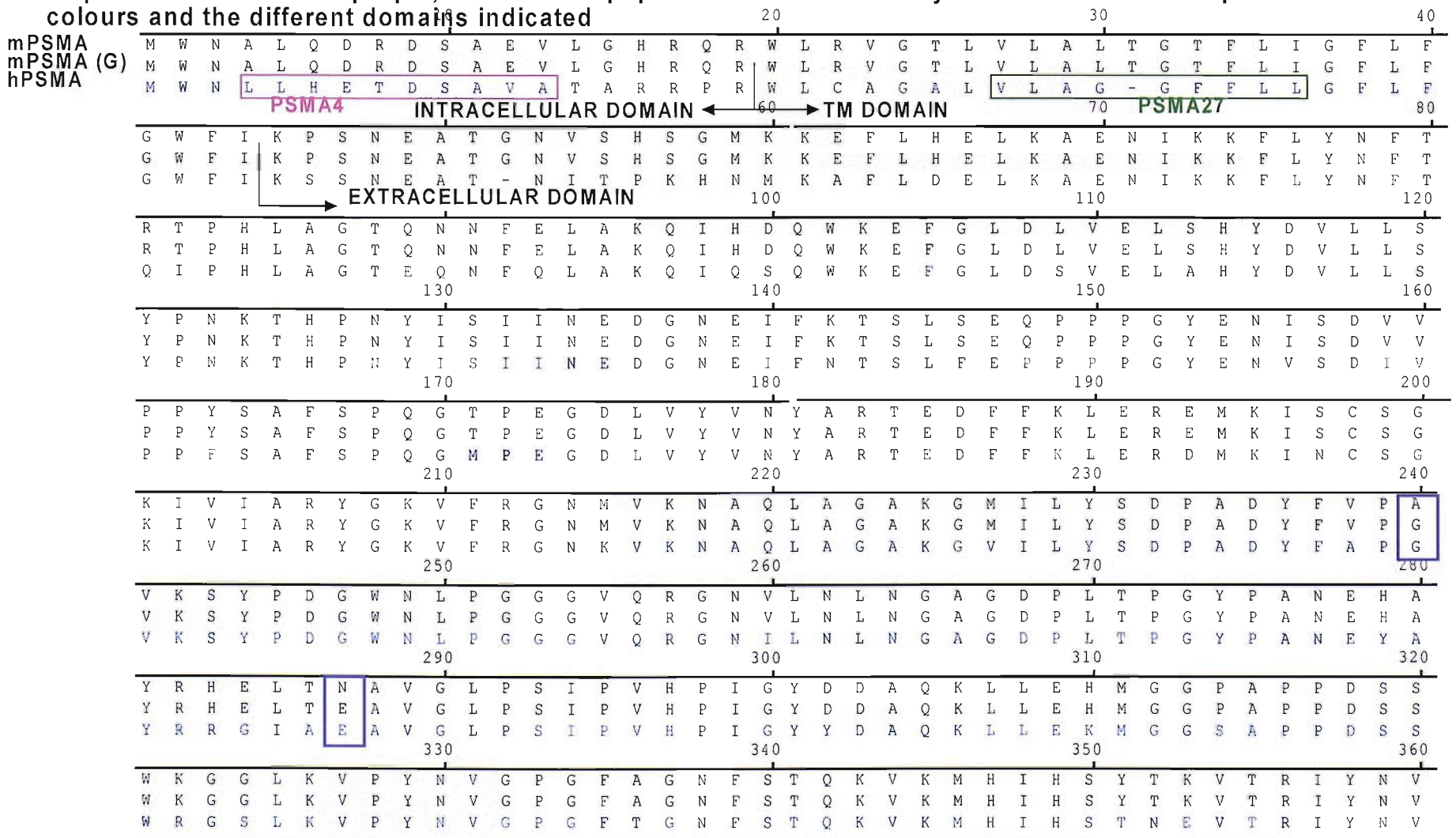
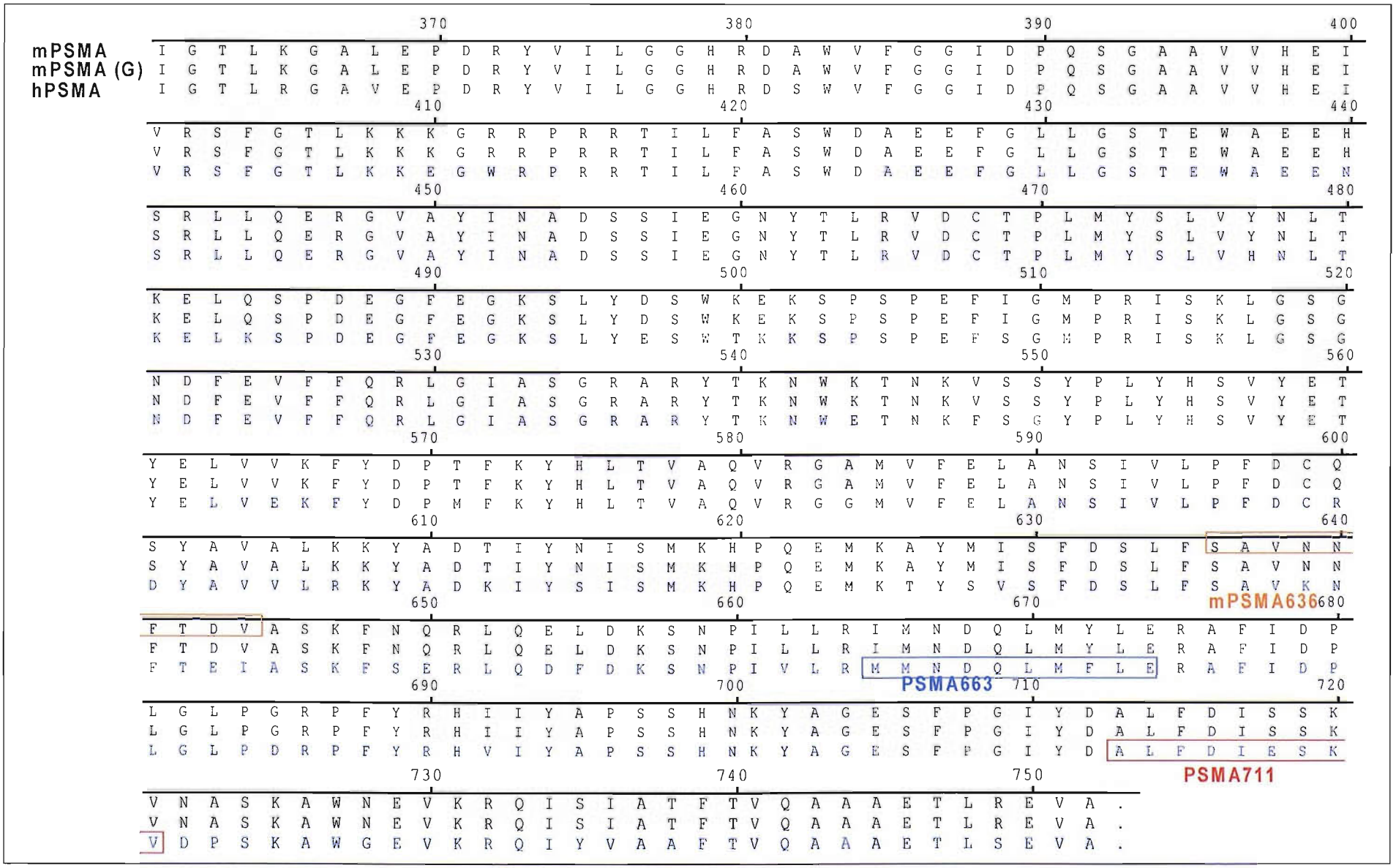


Figure S1: Recovery of CFSE-labelled tumour cells co-injected with Matrigel™ into mice

Naïve HHD mice were co-injected Matrigel™ and transduced TRAMP tumour cells (PSMA+HHD+ and/or PSMA-HHD+) at a 1:1 ratio. The PSMA-HHD+ cells were first labelled with 1μM and PSMA-HHD+ cells with 10μM CFSE. $1x10^6$ of each cell population either kept separate (top panel) or mixed together (lower panel) were mixed with Matrigel™ and subcutaneously injected into mice. After 5 days the Matrigel™ plugs were excised, digested with collagenase and cells analysed by flow cytometry to identify the CFSE-labelled cell populations. The single population controls were used to set the gates for the experimental mice (Figure 35) by ensuring minimal background contamination.

Appendix III: Alignment of mouse PSMA protein sequences (correct sequence: top black, Dr. Gregor's mouse PSMA (G): middle black, human PSMA: blue). The 2 amino acid differences between mPSMA sequences are boxed in purple, the different peptides used in this study are boxed in their respective colours and the different domains indicated




mPSMA
mPSMA (G)
hPSMA
370
380
390
400
I G T L K G A L E P D R Y V I L G G H R D A W V F G G I D P Q S G A A V V H E I
I G T L K G A L E P D R Y V I L G G H R D A W V F G G I D P Q S G A A V V H E I
I G T L R G A V E P D R Y V I L G G H R D S W V F G G I D P Q S G A A V V H E I
410
420
430
440
V R S F G T L K K K G R R P R R T I L F A S W D A E E F G L L G S T E W A E E H
V R S F G T L K K K G R R P R R T I L F A S W D A E E F G L L G S T E W A E E H
V R S F G T L K K E G W R P R R T I L F A S W D A E E F G L L G S T E W A E E N
450
460
470
480
S R L L Q E R G V A Y I N A D S S I E G N Y T L R V D C T P L M Y S L V Y N L T
S R L L Q E R G V A Y I N A D S S I E G N Y T L R V D C T P L M Y S L V Y N L T
S R L L Q E R G V A Y I N A D S S I E G N Y T L R V D C T P L M Y S L V H N L T
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500
510
520
K E L Q S P D E G F E G K S L Y D S W K E K S P S P E F I G M P R I S K L G S G
K E L Q S P D E G F E G K S L Y D S W K E K S P S P E F I G M P R I S K L G S G
K E L K S P D E G F E G K S L Y E S W T K K S P S P E F S G M P R I S K L G S G
530
540
550
560
N D F E V F F Q R L G I A S G R A R Y T K N W K T N K V S S Y P L Y H S V Y E T
N D F E V F F Q R L G I A S G R A R Y T K N W K T N K V S S Y P L Y H S V Y E T
N D F E V F F Q R L G I A S G R A R Y T K N W E T N K F S G Y P L Y H S V Y E T
570
580
590
600
Y E L V V K F Y D P T F K Y H L T V A Q V R G A M V F E L A N S I V L P F D C Q
Y E L V V K F Y D P T F K Y H L T V A Q V R G A M V F E L A N S I V L P F D C Q
Y E L V E K F Y D P M F K Y H L T V A Q V R G G M V F E L A N S I V L P F D C R
610
620
630
640
S Y A V A L K K Y A D T I Y N I S M K H P Q E M K A Y M I S F D S L F S A V N N
S Y A V A L K K Y A D T I Y N I S M K H P Q E M K A Y M I S F D S L F S A V N N
D Y A V V L R K Y A D K I Y S I S M K H P Q E M K T Y S V S F D S L F S A V K N
650
660
670
mPSMA636
680
F T D V A S K F N Q R L Q E L D K S N P I L L R I M N D Q L M Y L E R A F I D P
F T D V A S K F N Q R L Q E L D K S N P I L L R I M N D Q L M Y L E R A F I D P
F T E I A S K F S E R L Q D F D K S N P I V L R M M N D Q L M F L E R A F I D P
690
700
710
720
PSMA663
L G L P G R P F Y R H I I Y A P S S H N K Y A G E S F P G I Y D A L F D I S S K
L G L P G R P F Y R H I I Y A P S S H N K Y A G E S F P G I Y D A L F D I S S K
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730
740
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PSMA711
V N A S K A W N E V K R Q I S I A T F T V Q A A A E T L R E V A .
V N A S K A W N E V K R Q I S I A T F T V Q A A A E T L R E V A .
V D P S K A W G E V K R Q I Y V A A F T V Q A A A E T L S E V A .

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DOI: 10.1182/blood-2008-02-137695 Collection Codes: 13
NEOPLASIA

DNA vaccination induces WT1-specific T-cell responses with potential clinical relevance

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The Wilms' tumor antigen, WT1, is associated with several human cancers, including leukemia. We evaluated WT1 as an immunotherapeutic target using our proven DNA fusion vaccine design, p.DOM-peptide, encoding a minimal tumor-derived major histocompatibility complex (MHC) class I-binding epitope downstream of a foreign sequence of tetanus toxin. Three p.DOM-peptide vaccines, each encoding a different WT1-derived, HLA-A2-restricted epitope, induced cytotoxic T lymphocytes (CTLs) in humanized transgenic mice expressing chimeric HLA-A2, without affecting hematopoietic stem cells. Mouse CTLs killed human leukemia cells in vitro, indicating peptide processing/presentation. Low numbers of T cells specific for these epitopes have been described in cancer patients. Expanded human T cells specific for each of the 3 epitopes were lytic in vitro. Focusing on human $WT1_{37-45}$-specific cells, the most avid of the murine responses, we demonstrated lysis of primary leukemias, underscoring their clinical relevance. Finally, we showed that these human CTL kill target cells transfected with the relevant p.DOM-peptide DNA vaccine, confirming that WT1-derived epitopes are presented to T cells similarly by tumors and following DNA vaccination. Together, these data link mouse and human studies to suggest that rationally designed DNA vaccines encoding WT1-derived epitopes, particularly $WT1_{37-45}$, have the potential to induce/expand functional tumor-specific cytotoxic responses in cancer patients. (Blood. 2008;112:000-000)

Introduction

Despite major advances in chemotherapy and hematopoietic stem cell transplantation, many patients with leukemia will relapse because of reemergence of tumor. However, complete molecular remissions have been achieved by infusion of donor lymphocytes into patients, demonstrating that reactive T cells are able to eradicate leukemic cells in vivo.[1] Recently, various leukemia-associated antigens that are recognized by cytotoxic T lymphocytes (CTLs) in the context of major histocompatibility complex (MHC) class I have been identified.[2] One promising candidate is the Wilms' tumor antigen, WT1, which is expressed by the majority of acute leukemias (both acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML)), blast crisis of chronic myeloid leukemia (CML), and many solid tumors, including those of lung, breast, and colon.[3-6] The finding that blockade of WT1 function in primary leukemic cells significantly slows cell growth in vitro[7] additionally suggests that WT1 is of critical importance to the tumor phenotype and that tumor escape by simple WT1 down-modulation is unlikely to occur. In healthy adults, despite ubiquitous expression during embryogenesis, WT1 expression is limited to renal podocytes, gonadal cells, and a low frequency of hematopoietic precursor $CD34^+$ cells,[4,8-11] where it is expressed at significantly lower levels than those described in tumors (10- to 100-fold).[4] These features indicate that WT1 could be a useful target for therapeutic vaccination.

Antibodies to WT1[12-14] and WT1-peptide specific $CD8^+$ T cells[15-17] have been detected in AML and CML patients, confirming that tolerance is incomplete. Furthermore, the presence of WT1-specific T cells correlates with graft-vs-leukemia (GvL) effects after allogeneic stem cell transplantation in ALL patients,[18] implying that expansion of the WT1-responsive repertoire in leukemic patients may help to mediate tumor clearance.

Facilitating the study of WT1-specific immunotherapeutic approaches, several MHC class I-restricted peptides have been identified, including the HLA-A*0201-restricted WT1.126 ($WT1_{126-134}$; RMFPNAPYL) and the HLA-A*0201 and A*24-restricted WT1.235 ($WT1_{235-243}$; CMTWNQMNL) epitopes.[19-21] T cells with specificity for WT1.126 or WT1.235 have been isolated from the peripheral blood of $HLA\text{-}A2^+$ leukemic patients,[15,22,23] although evaluation of lytic ability has been limited to $HLA\text{-}A2^+$WT1.126-specific cells, which were shown to lyse tumor cell lines.[22] Recently, a novel HLA-A2-binding epitope, WT1.37 ($WT1_{37-45}$; VLDFAPPGA), was identified.[24] High-avidity T cells with WT1.37-specificity were expanded from patients with CML and AML, although the cytotoxic capacity of these cells was untested.[15]

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*C.C. and S.L.B. contributed equally to this work.

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Surprisingly, few groups have investigated vaccine strategies to increase the frequency of clinically relevant WT1-specific T cells in vivo. Nevertheless, clinical trials are underway using the WT1.126 or WT1.235 peptides mixed with adjuvant, and some responses in recipients have been reported.[23,25,26] An early study reported that vaccination using WT1.126 peptide with keyhole limpet hemocyanin plus granulocyte-macrophage colony-stimulating factor induced complete remission in the first tested patient with AML.[23] However, both the functionality of the induced WT1-reactive T cells and the means to achieve significant levels of response in patients require further investigation.

We have previously described a novel DNA fusion vaccine[27,28] that encodes a tumor-derived antigen fused to the fragment C (FrC) of tetanus toxin to provide $CD4^+$ T-cell help, critical for the induction of long-term antitumor immunity.[29-31] Minimizing the FrC sequence to a single domain (DOM) incorporating the MHC class II-restricted peptide, p30,[32,33] reduces the potential for peptide competition. Fusing this single domain to a target MHC class I-restricted epitope then enhances induction of epitope-specific CTL capable of eliminating tumor cells.[34,35] These optimized DNA fusion vaccines, termed p.DOM-peptide, are capable of inducing high levels of epitope-specific CTL from a tolerized repertoire, probably because of provision of high levels of T-cell help via the antimicrobial repertoire.[36]

In the present study, we used humanized HLA-A2 transgenic mice[37] to model a WT1 vaccination strategy based on our optimized p.DOM-peptide design. This model has value for testing the ability of vaccines to induce $CD8^+$ T-cell responses restricted by the human HLA-A2 haplotype and for demonstrating that the target peptide is naturally processed and presented.[38] Murine and human WT1 share a similar expression pattern and 96% identity at the amino acid level,[39] with all 3 HLA-A2-restricted epitopes identical in the 2 species. Thus, WT1-specific immune tolerance is expected to be similar in HLA-A2 transgenic mice and humans, making humanized mice an ideal test-bed for clinical vaccination approaches aimed at breaking tolerance to WT1. We show that T cells with specificity for all 3 WT1-derived epitopes can be expanded by vaccination and can kill human leukemic cells. To make a clinical connection and demonstrate a potential T-cell repertoire, we additionally showed that expanded WT1.37-specific human $HLA\text{-}A2^+$ T cells from healthy donors were able to lyse primary leukemia cells. The ability of our vaccine design to present peptide to these T cells and therefore expand the repertoire was shown by transfecting human cells with the relevant p.DOM-peptide DNA vaccine. Thus, we suggest that vaccinating patients with p.DOM-peptide DNA vaccines to expand functional WT1-specific T cells, including those with specificity for WT1.37, may be an efficient means to induce cancer-specific cytotoxicity in patients with leukemia.

Methods

Construction of DNA vaccines

DNA vaccines are shown schematically in Figure 1. Construction of the p.DOM plasmid containing the gene encoding the first domain of FrC of tetanus toxin (DOM, $TT_{865-1120}$) with a leader sequence derived from the V_H of the IgM of the BCL_1 tumor at the N-terminus has been previously described.[35] Three additional DNA vaccines were constructed encoding $WT1_{37-45}$ (WT1.37; p.DOM-WT1.37), $WT1_{126-134}$ (WT1.126; p.DOM-WT1.126), or $WT1_{235-243}$ (WT1.235; p.DOM-WT1.235) peptides fused directly 3′ to DOM. p.DOM-WT1.126 was constructed by polymerase

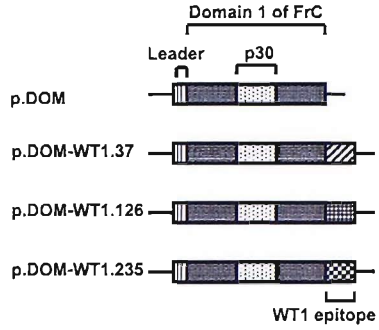

Figure 1. Schematic representation of DNA vaccines. All DNA vaccines encode the leader sequence of the V_H heavy chain gene from the BCL_1 lymphoma upstream of sequence encoding the first domain of fragment C (FrC) of tetanus toxin ($TT_{865-1120}$), which contains a promiscuous MHC II-binding sequence, p30. The control vaccine p.DOM contains no additional sequence, whereas p.DOM-WT1.37 additionally encodes VLDFAPPGA ($WT1_{37-45}$), p.DOM-WT1.126 additionally encodes RMFPNAPYL ($WT1_{126-134}$), and p.DOM-WT1.235 encodes CMTWNQMNL ($WT1_{235-243}$) downstream of the FrC sequence.

chain reaction (PCR) amplification using p.DOM as template with the forward primer 5′-TTTT*AAGCTT*GCCGCCACCATGGGTTGGAGC-3′ and the reverse primer 5′-TTTT*GCGGCCGCTTACAGGTAGGGCG-CATTGGGGAACATCCT*GTTACCCCAGAAGTCACGCAGGAA-3′. The PCR product was gel purified, digested using *Hin*dIII and *Not*I restriction sites, and cloned into the expression vector pcDNA3 (Invitrogen, Paisley, United Kingdom). The vaccines p.DOM-WT1.235 and p.DOM-WT1.37 were constructed similarly using the same forward primer and reverse primers 5′-TTT*GCGGCCGC TTATAAGTTCATCTGATTCCAGGTCATG-CA*GTTACCCCAGAAGTCACGCAGGAA-3′ and 5′-TTT*GCGGCCGC-TTAAGCGCCCGGAGGCGCAAAGTCCAGCAC*GTTACCCCAGAAGTC-ACGCAGGAA-3′, respectively. Restriction sites within primers are italicized and WT1-peptide encoding sequences are underlined. Integrity of the inserted sequence was confirmed by DNA sequencing and translated product size was checked in vitro using the TNT T7 coupled reticulocyte lysate system (Promega, Southampton, United Kingdom).

AQ: 6
AQ: 7

Peptides

The HLA-A*0201-restricted WT1.37 (VLDFAPPGA), WT1.126 (RMFPNAPYL), WT1.235 (CMTWNQMNL) peptides[19-21] and the HLA class II-restricted p30 (FrC-derived: TTFNNFTVSFWLRVPKVSASHLE)[33] and PADRE (KSSAKXVAAWTLKAAA: X = cyclohexylalanine)[40] peptides were synthesized commercially and supplied at more than 95% purity (PPR, Southampton, United Kingdom).

HHD transgenic mice

HHD mice express a transgenic chimeric monochain MHC class I molecule in which the COOH-terminus of human β_2-microglobulin is covalently linked to the NH_2-terminus of chimeric HLA-A2 α1 and α2 domains fused with the murine $H\text{-}2D^b$ α3 domain. These mice lack cell-surface expression of mouse endogenous $H\text{-}2^b$ class I molecules because of targeted disruption of the $H\text{-}2D^b$ and mouse β_2-microglobulin genes.[37]

Vaccination protocol

HHD mice at 6 to 10 weeks of age were injected intramuscularly into both quadriceps with a total of 50 μg DNA in saline solution on day 0. Unless stated otherwise, mice were boosted with the same DNA vaccine delivered with in vivo electroporation on day 28 as previously described.[41] For comparing DNA and peptide vaccination, mice were immunized intramuscularly with p.DOM-WT1.126 or subcutaneously into 2 sites on the flanks with a total of 50 μg WT1.126 and 100 μg PADRE peptides mixed together. Peptides were administered with an equal volume of Incomplete Freunds Adjuvant (IFA). Splenocytes were harvested 10 days later for analysis. Animal experimentation was conducted within local Ethical Committee and

UK Coordinating Committee for Cancer Research (London, United Kingdom) guidelines under Home Office License.

Mouse interferon-γ-ELISpot

Vaccine-specific interferon-γ (IFN-γ) secretion by lymphocytes from individual mice was assessed ex vivo (BD ELISpot Set, BD PharMingen, San Diego, CA) on day 10 or 36, as described previously.[36] Briefly, viable lymphocytes were selected from splenocyte preparations by density centrifugation. Cells ($2–4 \times 10^5$ cells/well) were incubated in complete medium (RPMI 1640, 1 mM of sodium pyruvate, 2 mM of L-glutamine, nonessential amino acids (1% of 100× stock), 50 μM of 2-mercaptoethanol, 100 U/mL of penicillin, 100 μg/mL of streptomycin (all Invitrogen) with 10% heat-inactivated fetal calf serum) with WT1.37, WT1.126, or WT1.235 peptides to assess $CD8^+$ T-cell responses, or with p30 peptide or PADRE peptide to assess $CD4^+$ T cells. Samples were plated in triplicate; control samples were incubated without peptide or with an irrelevant HLA-A2-binding peptide. Data are expressed as the frequency of spot-forming cells (SFCs) per million lymphocytes. For analysis of peptide-specific T-cell sensitivity, splenic lymphocytes from immunized mice were incubated with a range of WT1 peptide concentrations and the frequency of specific cells assessed by ELISpot analysis as described. The number of SFCs/million cells at the peptide concentration inducing the greatest response was assigned a value of 100%. For each peptide concentration tested, the percentage maximal response was then calculated by the formula: (experimental SFCs per million cells/maximal SFCs per million cells) × 100% for each individual animal.

Murine cytotoxic T-cell expansion and detection

For the generation and maintenance of CTL lines, mice were killed at the indicated time points and cell suspensions made from each spleen. Splenocytes were washed and resuspended in 10 to 15 mL complete media per spleen in upright 25-cm^2 flasks together with WT1.37 (10^{-9} M), WT1.126 (10^{-6} M), or WT1.235 (10^{-6} M) peptides. After 7 to 10 days of stimulation in vitro, cytolytic activity of the T-cell cultures was assessed. For further cycles of in vitro restimulation, CTLs were washed, resuspended at 3×10^5/mL with 2.5×10^6/mL syngeneic splenocytes preincubated for 1 hour with the relevant peptide at 10^{-9} M (WT.37) or 10^{-6} M (WT.126 and WT1.235), washed 4 times in unsupplemented RPMI 1640 (Invitrogen), and irradiated at 2500 cGy. Recombinant human interleukin-2 was added to cultures at 20 IU/mL (interleukin-2 (IL-2); Perkin-Elmer, Foster City, CA) and cells were incubated at 2 mL/well of a 24-well plate. Subsequent cycles of in vitro restimulation were carried out similarly every 10 to 12 days. Specific cytotoxic activity was assessed by standard 4- to 5-hour ^{51}Cr release assay as previously described.[42]

Assessment of autoimmune damage

For the detection of autoimmune damage to colony-forming cells in bone marrow, HHD mice were vaccinated as indicated on day 0 and day 28 (+ electroporation). Between days 36 and 42, mice were culled and bone marrow cells collected under aseptic conditions before incubation in Methocult media containing IL-3, SCF, IL-6, and erythropoietin according to the manufacturer's instructions (Stem Cell Technologies, London, United Kingdom). Colonies of hematopoietic cells were enumerated on days 9 to 11 of in vitro incubation.

Patients and healthy donors

Peripheral blood mononuclear cells (PBMCs) from 10 HLA-A*0201-positive healthy subjects were isolated from leukapheresis using Ficoll-Hypaque density-gradient centrifugation and subsequently cryopreserved in complete medium supplemented with 20% heat-inactivated AB serum (SAB) and 10% dimethyl sulfoxide. Tumor cells from 6 patients with $WT1^+$ acute leukemias were isolated from one HLA-A*0201-positive patient with ALL (patient I), 3 from HLA-A*0201-positive patients with AML (patients II, III, and IV), and 2 from HLA-A*0201-negative patients with AML (patients V and VI). WT1 expression was confirmed by WT1-specific RT-PCR (data not shown). Informed consent was obtained from all healthy subjects and patients before blood sampling. Informed consent was provided according to the Declaration of Helsinki.

Cell lines and autologous PHA blasts

Cells used as targets in murine CTL assays were the human leukemia lines C1RA2 ($WT1^-$HLA-A*0201$^+$), KYO-1 ($WT1^+$HLA-A*0201$^-$), or 697 ($WT1^-$HLA-A*0201$^+$) either alone or retrovirally transduced with HHD DNA using standard methods. For human in vitro studies, the pro-B cell line RS 4,11 ($WT1^+$HLA-A*0201$^+$) was purchased from American Type Culture Collection (Manassas, VA), the pre-B leukemia cell line 697 and follicular lymphoma cell line VAL ($WT1^-$HLA-A*0201$^-$) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Phytohemagglutinin-blasts were differentiated from nonadherent PBMCs (1×10^6 cells/mL) by culture in complete medium supplemented with 10% SAB (RPMI 10% SAB), 50 IU/mL of IL-2 (R&D systems, Oxford, United Kingdom), and 2 μg/mL of phytohemagglutinin (Sigma-Aldrich, Saint Quentin Fallavier, France). IL-2 (50 IU/mL) was added every 2 to 3 days. AQ: 14

Human cytotoxic T-cell expansion

Dendritic cells (DCs) were generated from donor monocytes as previously described.[43] Briefly, adherent PBMCs were cultured at 10×10^6 cells/well in 6-well plates with 100 ng/mL of IL-4 and 80 ng/mL of granulocyte-macrophage colony-stimulating factor in AIM V serum free medium (Invitrogen) for 1 day. Maturation was induced by additional 24-hour incubation with 10 ng/mL tumor necrosis factor-α, 20 ng/mL IL-6, 1 μg/mL PGE-2, and 10 ng/mL IL-1β (all R&D Systems, Abingdon, United Kingdom). Mature DCs pulsed for 2 hours at 37°C with 10 μg/mL of WT1-derived peptide in AIM V were then irradiated (50 Gy), washed in RPMI 10% SAB and used as stimulator cells. Autologous $CD8^+$ T cells were purified from healthy HLA-A*0201-positive donor PBMCs by positive selection using CD8 magnetic microbeads (Miltenyi Biotech, Paris, France). The binding fraction was adjusted to 5×10^6 cells/mL, whereas the CD8-negative fraction was irradiated (30 Gy) and adjusted to 5×10^6 cells/mL to be used as helpers. $CD8^+$ T cells were then plated together with stimulator DC at a 10:1 or 5:1 ratio and with helpers at a 2:1 ratio in RPMI 10% SAB. IL-2 (25 IU/mL) and IL-7 (5 ng/mL; R&D Systems) were added every 2 to 3 days from day 3. One million CTLs were restimulated every 10 days in 24-well plates with autologous adherent monocytes from 4×10^6 PBMCs pulsed with 5 μg/mL relevant peptide and 100 ng/mL β_2-microglobulin (Sigma-Aldrich) in serum-free RPMI 1640. IL-2 (25 IU/mL) and IL-7 (5 ng/mL) were added every 2 to 3 days. AQ: 15

Human cytotoxic T-cell detection

$CD8^+$ T cells specific for WT1 peptides were quantified by double-staining using biotinylated HLA-A*0201/WT1.126 or HLA-A*0201/WT1.235 pentamers (Proimmune, Oxford, United Kingdom) and phycoerythrin (PE)-Cy5-coupled anti-CD8 antibody (clone 3B5, Caltag, Burlingame, CA). Briefly, 5×10^5 T cells were incubated for 30 minutes on ice with 0.5 μg of pentamer, washed and incubated for a further 20 minutes at 4°C with 5 μL of anti-CD8 antibody and 200 ng of streptavidin-PE (Invitrogen) concurrently. Fluorescence was determined using a FACS FC500 (Beckman Coulter, Villepinte, France). For determining cytotoxic activity, target cells were either peptide-pulsed (10 μg/mL peptide plus 100 ng/mL β_2-microglobulin 2 hours at 37°C) or unpulsed and labeled with ^{51}Cr sodium chromate for 1 hour at 37°C before plating at 2.5×10^3 cells/well in a 96-well plate. Cytolytic activity at graded effector-to-target ratios was determined in a standard 4-hour ^{51}Cr-release assay. In some experiments, target cells were incubated with the antihuman HLA-class I monoclonal antibody W6.32 (Diaclone, Besançon, France) or with IgG2a isotype control (5 μg/well) for 15 minutes at 4°C before the addition of T cells and for the duration of the assay. Means of triplicate cultures were expressed as (experimental release − spontaneous release)/(total ^{51}Cr incorporated − spontaneous release) × 100%. The average spontaneous release never exceeded 15% of the total incorporated ^{51}Cr. AQ: 16 AQ: 17

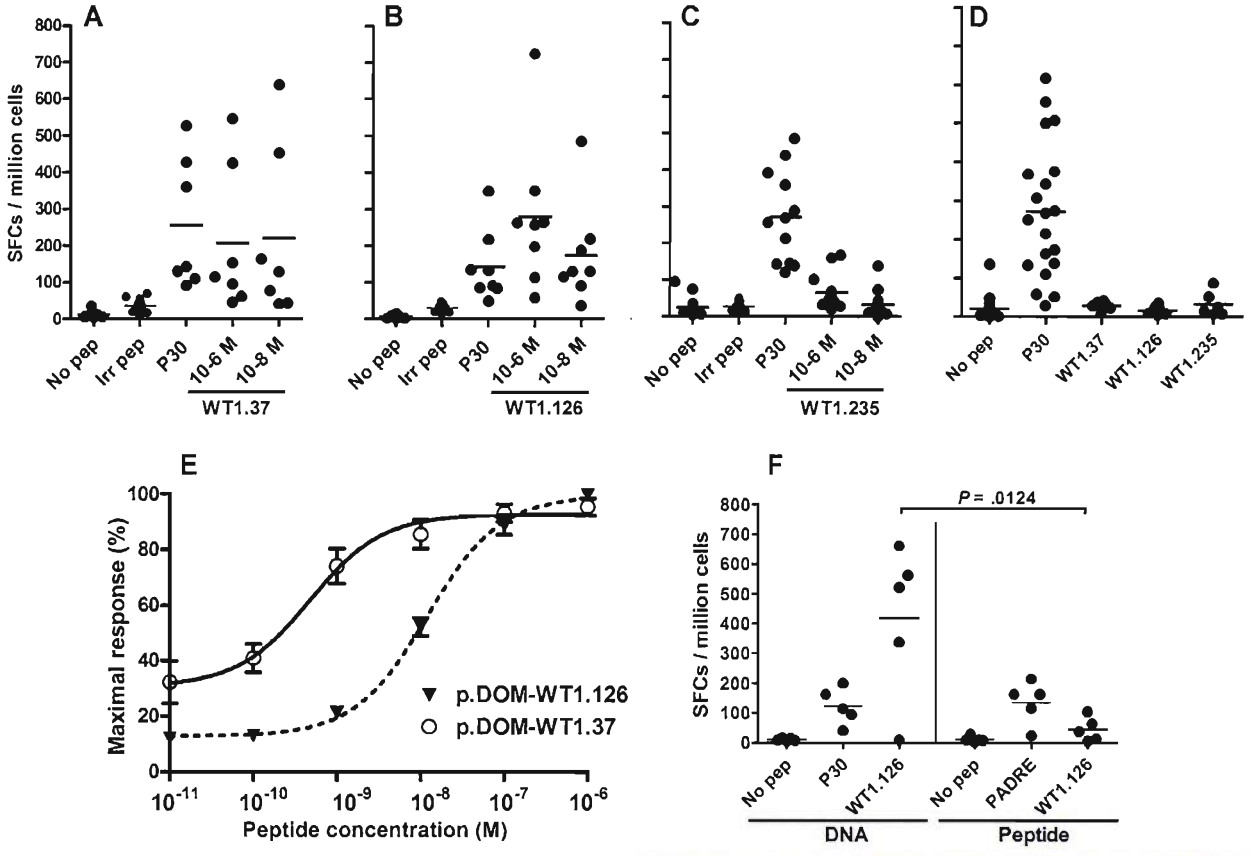

Figure 2. DNA vaccination induces WT1-specific IFN-γ-secreting T cells in HHD mice. HHD mice were vaccinated with (A) p.DOM-WT1.37 (n = 7), (B) p.DOM-WT1.126 (n = 8), (C) p.DOM-WT1.235 (n = 12), or (D) p.DOM (n = 20) and were boosted 28 days later with the same vaccine delivered using in vivo electroporation. On day 36, splenic lymphocytes were harvested by density centrifugation of spleen cells and the frequency of specific T cells assessed by IFN-γ ELISpot following a brief incubation alone (no pep), with an irrelevant peptide (irr pep; 10^{-6} M) with p30 peptide (10^{-6} M) or with the relevant WT1 peptide (10^{-6} M or 10^{-8} M). Data are expressed as the number of spot-forming cells (SFCs) per million lymphocytes and are a combination of 2 of 2 experiments with similar results; group means are represented by a horizontal bar. Responses were considered significant if the frequency of IFN-γ-secreting cells was more than double the frequency detected in wells without peptide. (E) Lymphocytes from mice vaccinated with p.DOM-WT1.37 (n = 4) or p.DOM-WT1.126 (n = 9) were incubated with a range of peptide concentrations and the frequency of specific cells assessed by ELISpot analysis as before. Data are shown as the mean percentage of the maximum obtained for each mouse with the SEM indicated. A nonlinear line of best fit was plotted using GraphPad Prism 4 software. (F) HHD mice were injected with DNA vaccine (p.DOM-WT1.126; DNA) or with peptide vaccine (WT1.126 peptide in IFA mixed with PADRE peptide: peptide). Lymphocytes were harvested on day 10 and the frequency of vaccine-specific T cells assessed by IFN-γ ELISpot following a brief incubation alone (no pep), with the Th peptides p30 or PADRE (10^{-6} M) or with the WT1.126 peptide (10^{-6} M). Group means are represented by a horizontal bar.

VAL cell line transfection

VAL cells (5×10^6 cells) were transfected with 5 μg of plasmid (p.DOM, p.DOM-WT1.37, or p.EGFPmax) using the Amaxa Nucleofector apparatus (Amaxa, Cologne, Germany) according to the manufacturer's protocol for human B cells. Flow cytometry analysis of p.EGFPmax (Amaxa)-transfected cells showed a transfection efficiency of between 60% and 70% at 24 hours. Cells were used as targets in a ^{51}Cr-release assay at this time point.

Results

p.DOM-WT1 vaccines induce WT1-specific T-cell responses in HHD mice

Three DNA vaccines were constructed, each encoding the first domain of FrC of tetanus toxin (DOM) fused to a minimal WT1-derived peptide sequence, and conforming to our previously described vaccine design.[34-36] Specifically, vaccines were p.DOM-WT1.37, p.DOM-WT1.126, and p.DOM-WT1.235, each incorporating sequence encoding the promiscuous helper T-cell epitope, p30, and one of the 3 MHC class I-binding peptides, WT1.37, WT1.126, or WT1.235, respectively (Figure 1). To test vaccine efficacy, humanized HHD mice were immunized on days 0 and 28 with one of the p.DOM-WT1-peptide vaccines or with the control vaccine p.DOM. The booster injection on day 28 was immediately followed by in vivo electroporation at the site of injection, a technique we have previously shown to be of benefit for the activation of T-cell responses, particularly at boosting.[41] On day 36, mice were killed and splenic responses assessed by IFN-γ ELISpot analysis. All vaccines, including the p.DOM control, induced a significant response to the p30 peptide confirming the integrity, in vivo expression, and immunogenicity of the DNA vaccine protein products (Figure 2A-D). Furthermore, 100% of HHD mice immunized with p.DOM-WT1.37 or p.DOM-WT1.126, and 75% of mice immunized with p.DOM-WT1.235, induced significant numbers of WT1-peptide specific IFN-γ-secreting T cells detectable directly ex vivo (Figure 2A-C). $CD8^+$ T-cell responses to WT1.235 were notably lower than responses to either of the other 2 WT1-peptide vaccines, although these were significantly above background levels ($P = .005$, 2-tailed Mann-Whitney U test). As expected, the control p.DOM vaccine failed to induce any significant WT1-specific responses (Figure 2D). Encouragingly, the frequency of T cells specific for the WT1.37 peptide, which has not before been tested in any vaccination strategy, was comparable with that F2

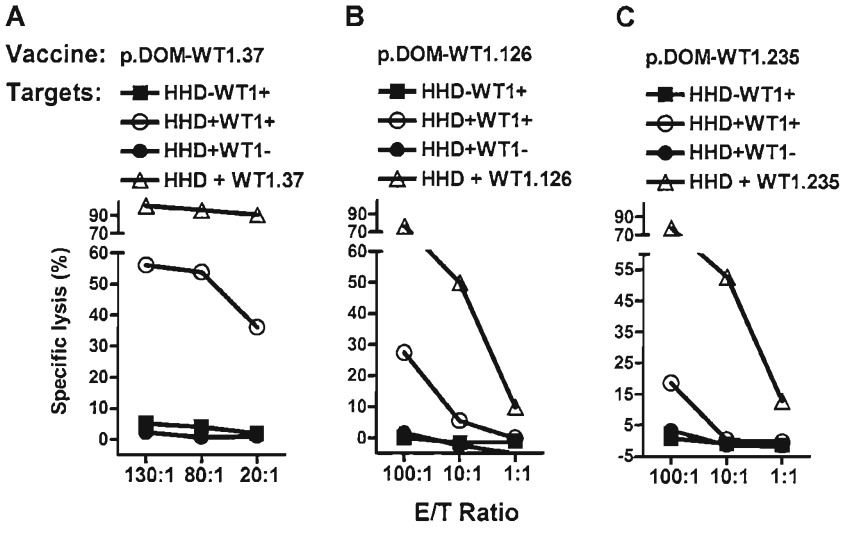

Figure 3. WT1-specific T cells induced by DNA vaccination are peptide specific and kill target cells presenting WT1. Splenocyte cultures from individual mice previously immunized with (A) p.DOM-WT1.37, (B) p.DOM-WT1.126, or (C) p.DOM-WT1.235 were established by stimulation in vitro for 2 weeks before assessing cytotoxicity by ^{51}Cr-release assay. Cytotoxicity was assessed at the effector:target ratios shown, against human tumor cells endogenously expressing WT1 and stably transduced with the chimeric humanized MHC class I molecule, HHD, denoted as HHD^{+}WT1^{+} (specifically, KYO-HHD (A and C) or 697-HHD (B) cells). Cell lines expressing either WT1 (HHD^{-}WT1^{+}) or HHD (HHD^{+}WT1^{-}) were included as negative controls (KYO-1 and C1RA2-HHD, respectively), HHD-transduced cells pulsed with the relevant peptide were used as positive controls.

elicited to the WT1.126 epitope after vaccination (Figure 2A,B). We also noted that T cells responsive to the WT1.37 epitope exhibited no decrease in the frequency of responding cells when incubated with 10 nM rather than 1 μM peptide (Figure 2A). Further analysis of the WT1.37-specific response over a range of peptide concentrations confirmed that these T cells continue to respond to peptide at concentrations approximately 50-fold lower than WT1.126-specific cells (Figure 2E).

One question concerning vaccine design was the comparative performance of the peptide immunogen when delivered via DNA or as an exogenous peptide plus IFA. This was tested using peptide WT1.126, with a source of T-cell help for the exogenous peptide provided by coinjection of the PADRE peptide. Priming of CD8^{+} T-cell responses was clearly superior when using DNA delivery with approximately 9-fold higher levels of IFN-γ-producing T cells detectable at day 10 (P = .012, 2-tailed t test; Figure 2F). Levels of CD4^{+} T-cell help against either p30 (DNA vaccine) or PADRE (peptide vaccine) appeared adequate in both cases (Figure 2F).

p.DOM-WT1 vaccines induce WT1-specific cytotoxic T lymphocytes

To evaluate the cytotoxic activity and peptide specificity of WT1-specific T cells induced by DNA vaccination, splenocytes from immunized mice were restimulated in vitro and their lytic ability tested in a ^{51}Cr-release assay. CTLs from mice immunized with each of the p.DOM-WT1 vaccines demonstrated significant lysis of peptide-pulsed target cells, demonstrating the functionality of WT1-specific T cells induced by pDOM-WT1 vaccination (Figure 3). Lysis was not observed against target cells pulsed with an irrelevant peptide (data not shown).

To ensure that all 3 epitopes are naturally processed and presented by tumor cells, vaccine-induced CD8^{+} T cells were tested for their ability to lyse human leukemia cells endogenously expressing WT1 and transfected with the chimeric HLA-A2 (HHD). T cells specific for each individual peptide lysed human target cells expressing both WT1 and HHD, but not those expressing only HHD or WT1, demonstrating their ability to kill human tumors (Figure 3). In addition, WT1.126-specific T cells induced lysis of target cells expressing HLA-A2 and WT1 in the absence of the HHD molecule (data not shown), indicating lytic activity independent of CD8.

WT1-specific CD8^{+} T cells do not inhibit hematopoietic colony formation

In light of the ability of our DNA vaccine approach to induce WT1-specific T cells, we sought to investigate whether vaccination causes damage to hematopoietic precursor cells known to express WT1.[11] HHD mice were vaccinated twice with one of the 3 p.DOM-WT1 vaccines, with control p.DOM or were left unimmunized before analysis of hematopoietic stem cell frequency in the bone marrow. Ex vivo ELISpot analyses of splenic lymphocytes were performed concurrently and only mice in which a WT1-specific T-cell response was evident were evaluated (data not shown). There was no difference between the frequencies of hematopoietic stem cells (either of the granulocyte-monocyte (CFU-GM; Figure 4A) or erythroid (BFU-E; Figure 4B) lineages) F4 present in mice vaccinated with any of the p.DOM-WT1 DNA vaccines, compared with either p.DOM recipients or unimmunised mice, showing that induction of CD8^{+} T cells against WT1 does not lead to detectable loss of hematopoietic stem cells.

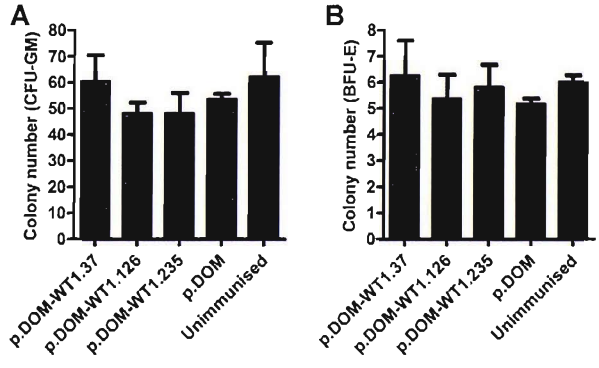

Figure 4. Vaccination with p.DOM-WT1 DNA vaccines does not lead to destruction of hematopoietic stem cells. Groups of mice were injected with the vaccines shown on days 0 and 28 (+ electroporation), or were left unimmunized. Mice were culled between days 36 and 42 and spleen and bone marrow collected. Splenocytes were used to confirm the presence of WT1-specific T cells by ELISpot; nonresponding mice were excluded from analysis (data not shown). Bone marrow cells were assessed for the frequency of (A) colony-forming units granulocyte-macrophage (CFU-GM) and (B) burst-forming units-erythroid (BFU-E). Data are a pool of 3 of 3 experiments with group means and standard deviations shown.

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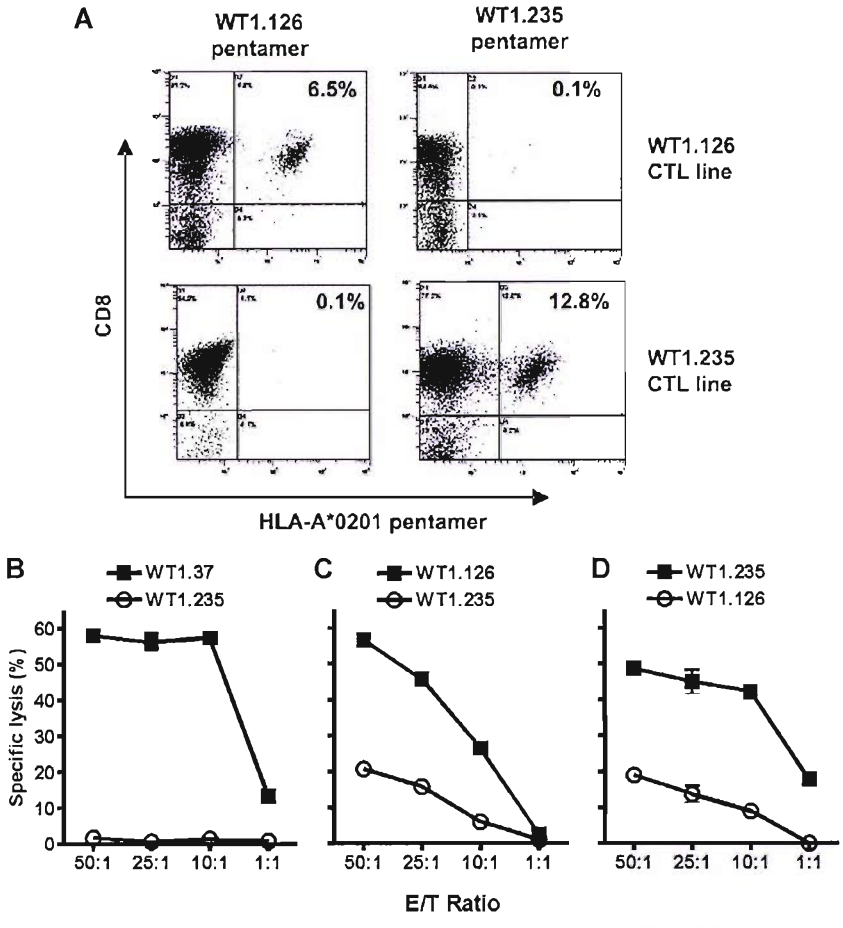

Figure 5. Human WT1-peptide-specific CTLs can be expanded in vitro and specifically kill peptide-pulsed cells. (A) Independently in vitro expanded WT1.126 (upper row) and WT1.235-specific (lower row) $CD8^+$ T cells were identified on day 31 and day 45 of culture, respectively, by double-labeling using specific-HLA-A*0201/peptide-biotin labeled pentamers with streptavidin-PE and anti-CD8-PE-Cy5 labeled antibody. Negative controls were similarly stained with the opposite and irrelevant HLA-A*0201/peptide pentamer. Numbers indicate the percentage of $CD8^-$ cells of whole culture capable of binding pentamer. On day 54 of culture, (B) WT1.37, (C) WT1.126, or (D) WT1.235-peptide-specific $CD8^+$ T cells were tested for their ability to lyse HLA-A*0201 autologous PHA-Blasts (B) or the VAL cell line (C and D) pulsed with the indicated peptides at the effector/target (E/T) ratios shown. For each CTL line generated (3 WT1.37-specific, 1 WT1.126-specific, and 2 WT1.235-specific), the assay was performed twice. Data were similar in each case and representative results are shown.

Human WT1-specific CTL can be expanded from healthy donors

Confirming that a T-cell repertoire specific for these same peptides exists in humans,[15,16,44] WT1-specific $CD8^+$ T cells were successfully expanded from a total of 5 of 10 healthy HLA-$A2^+$ donor PBMCs. Briefly, cells were exposed to autologous DCs pulsed with one of the WT1-derived peptides and were then restimulated at 10-day intervals with autologous monocytes pulsed with the corresponding peptide. Rezvani et al previously reported, using IFN-γ mRNA production in response to peptide stimulation, that T cells specific for WT1.126 or WT1.235 could be identified in 3 of 12 healthy persons.[15] We have used pentamer staining and functional assays to show that 1 of 5, and 2 of 6, healthy donors had a repertoire of T cells specific for WT1.126 and WT1.235, respectively (Figure 5; and data not shown), broadly reflecting the frequency of responders identified previously.[15] WT1.126-expanded cells did not bind to the WT1.235 pentamer and vice versa, confirming the specificity of the multimers used (Figure 5A). CTLs specific for the WT1.37 epitope could not be quantified in this way as HLA-A*0201/WT1.37 pentamers could not be made.

To determine whether WT1.37-specific T cells were expanded and to investigate the functionality of WT1-specific T cells, particularly those specific for WT1.235 and WT1.37 whose lytic ability has not been previously reported, CTL lines were established and used in ^{51}Cr-release assays. T cells of all 3 specificities were able to mediate lysis against peptide-loaded target cells (Figure 5B-D). Peptide-specific cytotoxic responses were detected in 3 of 7 WT1.37 cultures; for WT1.126 and WT1.235, only cultures in which specific cells were detected by pentamer staining exhibited significant levels of lysis.

Human WT1.37-specific CTL kill WT1-expressing tumor cell lines and primary leukemia cells in an HLA-A*0201-restricted manner

Natural processing and presentation of WT1.126 and WT1.235 by HLA-A*0201 and WT1-expressing leukemic cells have been reported.[19,21,22,45] However, it is not known whether this is similarly the case for the WT1.37 epitope, to which particularly avid T cells exist in vaccinated transgenic mice (Figure 2E) and in healthy humans.[15] We therefore chose to focus on human WT1.37-specific T cells and evaluated their ability to kill human tumor cell lines (697 and RS 4,11) and primary tumor cells expressing both HLA-A*0201 and WT1. Our data show that WT1.37-specific CTL efficiently lyse both the 697 and the RS 4,11 cell lines and 4 of 4 primary tumor cell samples from HLA-$A*0201^+$ patients with acute leukemia (either ALL (patient I) or AML (patients II-IV)) (Figure 6A). A significant proportion of the observed lysis against F6 both cell lines and the 2 $WT1^+$HLA-$A*0201^+$ primary AML tumors tested, was HLA-class I-restricted, underscoring the specificity of the effector T cells (Figure 6B). Specificity was further confirmed by the absence of lysis against WT1-expressing, yet HLA-A*0201 negative, primary tumor cells (patients V and VI) (Figure 6C). These data clearly show that the WT1.37 epitope is processed and presented by HLA-A*0201-positive and WT1-expressing human tumor cell lines and primary tumor cells from patients with acute leukemia.

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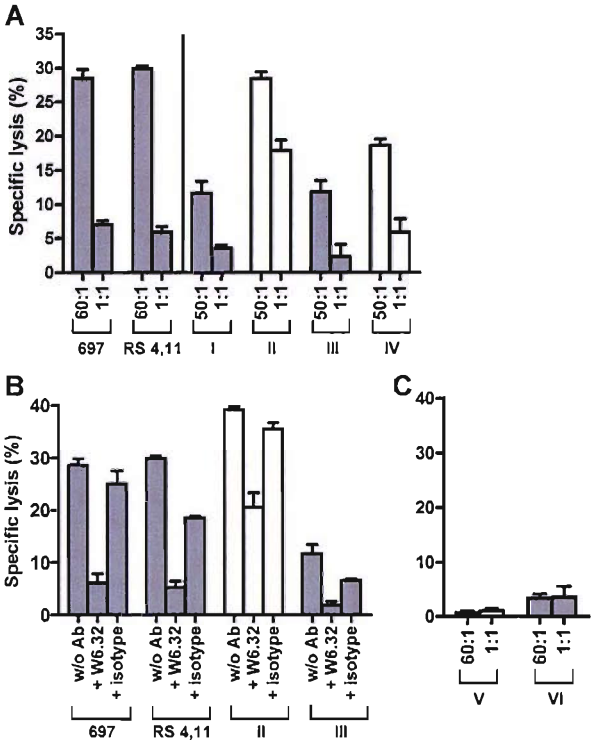

Figure 6. Human WT1.37-specific CTL kill WT1-expressing tumor cell lines and WT1-positive primary leukemia cells in a HLA-class I-dependent manner. WT1.37-specific CTLs were tested in a chromium release assay for lytic activity against (A) 697 and RS 4,11 cell lines (both HLA-A*0201$^+$WT1$^+$) and primary WT1$^-$ leukemia cells from 4 different HLA-A*0201$^-$ patients (I to IV) alone at the effector/target (E/T) ratios indicated or (B) in the presence of an antibody specific for HLA-class I (W6.32), alone (without Ab) or with an isotype control (+ isotype) at an effector/target cell ratio of 60:1 (targets 697, RS 4,11 and sample III) or 100:1 (sample II). (C) WT1.37-specific CTL were tested for lytic activity against target cells from 2 different WT1$^+$ and HLA-A*0201$^-$ patients (V and VI). Data are a combination of 2 experiments with 2 different WT1.37-specific CTL lines, differentiated by white or gray bars.

The WT1.37 peptide is processed and presented similarly by tumors and following DNA vaccination

Finally, human-derived WT1.37-specific CTLs were used to verify that WT1 peptides are processed and presented by human cells expressing the DNA fusion vaccine product. To this end, a human B-cell line, VAL (expressing HLA-A*0201 but not WT1), was transfected either with p.DOM-WT1.37 or with the control plasmids p.DOM or p.EGFP. WT1.37-specific CTL were able to specifically kill these antigen-presenting cells transfected with pDOM-WT1.37, at levels higher than those transfected with control plasmids, confirming that the protein product of pDOM-WT1.37 is expressed, and the WT1.37 epitope presented, by human cells (Figure 7).

Discussion

In this report, we made use of humanized mice to test our DNA vaccination approach against the leukemia-associated antigen WT1. The similar sequence and distribution pattern of WT1 in mice and humans strongly imply that the extent of WT1-specific T-cell tolerance in the 2 species is probably comparable. By using mice transgenic for a chimeric form of HLA-A2, we have been able to model the ability of 3 epitope-specific DNA vaccines to induce HLA-A2-restricted CD8$^+$ T-cell responses against WT1.

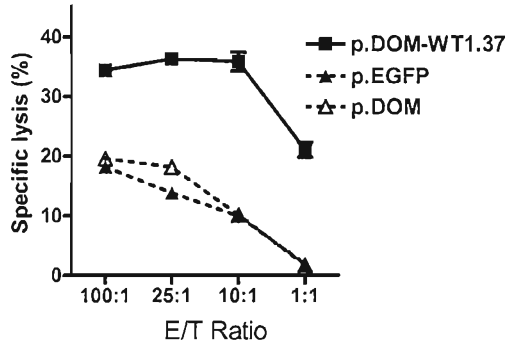

Figure 7. Human B cells transfected with p.DOM-WT1.37 present the WT1.37/HLA complex to, and are lysed by, WT1.37-specific T cells. The VAL follicular lymphoma (HLA-A*0201$^+$WT1$^-$) cell line was transfected with p.DOM-WT1.37 or with control plasmids p.DOM or p.EGFP. Twenty-four hours later, transfectants were tested for their susceptibility to lysis by human WT1.37-specific CTLs. Data shown are representative of 3 experiments performed.

Previous studies have revealed that vaccinating wild-type C57BL/6 mice with peptide-pulsed splenocytes,[46] or peptides in adjuvant,[47,48] can induce T-cell responses against WT1.126 in vivo. In this case, the response was restricted by mouse H-2D^b class I molecules,[46,47] indicating that this peptide can bind to both human and mouse MHC class I molecules but diminishing the relevance to human responses. In contrast, we have made use of HHD transgenic mice expressing only human HLA-A2 MHC class I chimeric molecules. Clearly, DNA vaccination can induce functional T cells to 3 clinically relevant HLA-A*0201-restricted epitopes, WT1.126, WT1.235, and WT1.37, in vivo. The ability of DNA delivery to prime CD8$^+$ T-cell responses against WT1.126 was found to be approximately 9-fold higher than that of exogenous peptide plus adjuvant and a source of T-cell help. Although further comparisons are required, this suggests an advantage for peptide delivery via DNA. Importantly, the CTLs generated also lysed human leukemia cells expressing WT1 from an endogenous source. To link preclinical findings to the clinic, we have studied the natural repertoire of human WT1-specific T cells with similar specificity, to ensure that there is a potential repertoire available for expansion by vaccination.

Using an assay for IFN-γ mRNA, Rezvani et al previously detected CD8$^+$ T cells specific for each of the 3 epitopes, WT1.37, WT1.126, and WT1.235, in the blood of patients with leukemia,[15] showing that a repertoire of WT1-reactive cells persists in cancer patients. Notably, human T cells with specificity for WT1.37 were of approximately 2.5-fold greater avidity than WT1.126-reactive cells,[15] mirroring our findings in p.DOM-WT1.37-vaccinated HHD mice and underscoring the relevance of HHD mice for preclinical modeling. For WT1.37, we have taken this a step further in 2 ways to show that 1) a DNA vaccine can induce high levels of avid CTLs in a setting where there is natural expression of WT1 and therefore the potential for immune tolerance; and 2) WT1.37 is processed and presented naturally by primary tumor cells.

Although CD8$^+$ T cells against all 3 peptides can kill WT1-positive targets, there was no evidence for loss of normal stem cells. Previous studies of WT1 responses in wild-type mice also failed to find any evidence of autoimmune disease,[46,48] and thus far only mild adverse effects have been reported in patients immunized against WT1.126 or WT1.235.[25,26] Our data additionally suggest that this might similarly be the case after clinical vaccination against the WT1.37 epitope.

To date, WT1.126, WT1.235, and a modified version of WT1.235 (CYTWNQMNL), designed to improve binding to HLA-A*2402,[49] are being evaluated in clinical trials for patients

with acute leukemia.[23,25,26,50] In each case, peptide is injected with adjuvant and some success has been reported, with a recent trial showing specific responses after just one injection.[50] However, one problem for peptide vaccines is the failure to provide $CD4^+$ T-cell help, known to be essential for induction of effective $CD8^+$ T-cell memory.[51-53]

Delivery via DCs is an alternative strategy to activate $CD8^+$ T cell responses and could be attractive for AML, especially because AML-derived DC can express leukemia-associated antigens.[54] However, WT1 expression appears to be down-regulated in AML DCs, arguing against the approach for this antigen.[54] We have chosen to use active vaccination with our p.DOM-peptide DNA vaccines specifically designed to provide $CD4^+$ T cell help. Indeed, our previous work has confirmed that $CD4^+$ T-helper cells expanded from the nontolerized tetanus-specific repertoire are critical for effective priming of $CD8^-$ T cells in vivo after p.DOM-peptide immunization.[31] Importantly, we have previously shown in an alternative model that our DNA vaccine design can overcome tolerance in mice.[36] This is probably an issue in patients exposed to high loads of leukemic cells. Other strategies exist to circumvent this toleragenic pressure on the repertoire, including the generation of allogeneic CTLs against mismatched MHC/WT1 peptide complexes. Thus, allogeneic and lytic HLA-A*0201$^-$ CTLs are generated against HLA-A*0201/WT1.126 or HLA-A*0201/WT1.235 complexes in vitro.[19,21] This adoptive therapy approach has to face destruction of the transferred cells; and to avoid this, genes encoding the TCR expressed by these allogeneic CTLs are being transduced into autologous T cells with the intention of subsequent adoptive transfer into patients.[55,56]

Although successful vaccination has the advantage of providing continuous surveillance of emergent tumor, a strong immune response in the clinic is required. It is now evident that performance of DNA vaccines in larger animals, including human subjects, is not optimal. The reason for this appears to be the delivery system which, for intramuscular injection, uses relatively large volumes for mice. Because these cannot be scaled up for human subjects, an alternative strategy to increase transfection rate and inflammation at the site has been sought. One approach is the technique of electroporation, which amplifies responses dramatically, especially at boosting.[41] This exciting technology is now undergoing clinical testing in our institution, and several prostate cancer patients have received p.DOM-peptide DNA vaccines delivered in this way with no serious adverse effects.

One criticism of using epitope-specific vaccines is that tumors may escape immune detection by losing epitope expression. To cover this possibility, DNA vaccines encoding the 3 promising HLA-A*0201-binding epitopes, ideally injected at different sites to avoid competition, could be used to induce a wide range of CTLs. Although encoding a longer sequence from WT1 may achieve this, our previous work has shown that encoding long tumor antigen sequences may compromise epitope presentation.[35] In addition, immunodominance predicts that immune responses will focus on one epitope after subsequent booster injections.[57]

In conclusion, our data reveal that a repertoire of T cells with specificity for the tumor-associated antigen, WT1, exists in humans and humanized transgenic mice, and the preclinical model has revealed a DNA vaccine strategy to induce or expand specific CTLs. This sets the scene for clinical testing of these safe, specific DNA vaccines in cancer patients with HLA-A*0201$^+$ and WT1$^-$ tumor cells, in settings where there is a need to suppress emergence of residual tumor.

Acknowledgments

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Authorship

Contribution: C.C. and S.L.B. conceived, designed, and performed the research, analyzed data, and wrote the report; J.M. assisted with experiments and processed samples; H.R. and M.K. supplied clinical samples; G.E.V. provided essential novel reagents; V.M.-F., J.-P.F., and H.J.S. provided intellectual support; J.R. conceived and supervised the study; M.-H.D.-L. and F.K.S conceived and supervised the study and wrote the report.

Conflict-of-interest disclosure: J.R. and F.K.S. hold a patent relating to the DNA vaccine design described in the article and have declared a financial interest in a company that holds an exclusive license relating to this design. AQ: 19

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References

1. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood. 1995;86:2041-2050.
2. Greiner J, Dohner H, Schmitt M. Cancer vaccines for patients with acute myeloid leukemia: definition of leukemia-associated antigens and current clinical protocols targeting these antigens. Haematologica. 2006;91:1653-1661.
3. Oji Y, Miyoshi S, Maeda H, et al. Overexpression of the Wilms' tumor gene WT1 in de novo lung cancers. Int J Cancer. 2002;100:297-303.
4. Inoue K, Ogawa H, Sonoda Y, et al. Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia. Blood. 1997;89:1405-1412.
5. Koesters R, Linnebacher M, Coy JF, et al. WT1 is a tumor-associated antigen in colon cancer that can be recognized by in vitro stimulated cytotoxic T cells. Int J Cancer. 2004;109:385-392.
6. Oji Y, Yamamoto H, Nomura M, et al. Overexpression of the Wilms' tumor gene WT1 in colorectal adenocarcinoma. Cancer Sci. 2003;94:712-717.
7. Yamagami T, Sugiyama H, Inoue K, et al. Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. Blood. 1996;87:2878-2884.
8. Rauscher FJ III. The WT1 Wilms tumor gene product: a developmentally regulated transcription factor in the kidney that functions as a tumor suppressor. FASEB J. 1993;7:896-903.
9. Lee SB, Haber DA. Wilms tumor and the WT1 gene. Exp Cell Res. 2001;264:74-99.
10. Hosen N, Sonoda Y, Oji Y, et al. Very low frequencies of human normal CD34+ haematopoietic progenitor cells express the Wilms' tumour gene

WT1 at levels similar to those in leukaemia cells. Br J Haematol. 2002;116:409-420.

11. Fraizer GC, Patmasiriwat P, Zhang X, Saunders GF. Expression of the tumor suppressor gene WT1 in both human and mouse bone marrow. Blood. 1995;86:4704-4706.
12. Gaiger A, Carter L, Greinix H, et al. WT1-specific serum antibodies in patients with leukemia. Clin Cancer Res. 2001;7(suppl):761-765.
13. Wu F, Oka Y, Tsuboi A, et al. Th1-biased humoral immune responses against Wilms tumor gene WT1 product in the patients with hematopoietic malignancies. Leukemia. 2005;19:268-274.
14. Elisseeva OA, Oka Y, Tsuboi A, et al. Humoral immune responses against Wilms tumor gene WT1 product in patients with hematopoietic malignancies. Blood. 2002;99:3272-3279.
15. Rezvani K, Brenchley JM, Price DA, et al. T-cell responses directed against multiple HLA-A*0201-restricted epitopes derived from Wilms' tumor 1 protein in patients with leukemia and healthy donors: identification, quantification, and characterization. Clin Cancer Res. 2005;11:8799-8807.
16. Scheibenbogen C, Letsch A, Thiel E, et al. CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. Blood. 2002;100:2132-2137.
17. Gannage M, Abel M, Michallet AS, et al. Ex vivo characterization of multiepitopic tumor-specific CD8 T cells in patients with chronic myeloid leukemia: implications for vaccine development and adoptive cellular immunotherapy. J Immunol. 2005;174:8210-8218.
18. Rezvani K, Yong AS, Savani BN, et al. Graft-versus-leukemia effects associated with detectable Wilms tumor-1 specific T lymphocytes after allogeneic stem-cell transplantation for acute lymphoblastic leukemia. Blood. 2007;110:1924-1932.
19. Gao L, Bellantuono I, Elsasser A, et al. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. Blood. 2000;95:2198-2203.
20. Ohminami H, Yasukawa M, Fujita S. HLA class I-restricted lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte clone specific for WT1 peptide. Blood. 2000;95:286-293.
21. Bellantuono I, Gao L, Parry S, et al. Two distinct HLA-A0201-presented epitopes of the Wilms tumor antigen 1 can function as targets for leukemia-reactive CTL. Blood. 2002;100:3835-3837.
22. Doubrovina ES, Doubrovin MM, Lee S, et al. In vitro stimulation with WT1 peptide-loaded Epstein-Barr virus-positive B cells elicits high frequencies of WT1 peptide-specific T cells with in vitro and in vivo tumoricidal activity. Clin Cancer Res. 2004;10:7207-7219.
23. Mailander V, Scheibenbogen C, Thiel E, et al. Complete remission in a patient with recurrent acute myeloid leukemia induced by vaccination with WT1 peptide in the absence of hematological or renal toxicity. Leukemia. 2004;18:165-166.
24. Smithgall M, Misher L, Spies G, Cheever MA, Gaiger A. Identification of a novel WT1 HLA-A*0201-restricted CTL epitope using whole gene in vitro priming [abstract]. American Society of Hematology Meeting, 2001.
25. Oka Y, Tsuboi A, Taguchi T, et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. Proc Natl Acad Sci U S A. 2004;101:13885-13890.
26. Keilholz U, Menssen HD, Gaiger A, et al. Wilms' tumour gene 1 (WT1) in human neoplasia. Leukemia. 2005;19:1318-1323.
27. Spellerberg MB, Zhu D, Thompsett A, et al. 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. 1997;159:1885-1892.
28. King CA, Spellerberg MB, Zhu D, et al. DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. Nat Med. 1998;4:1281-1286.
29. Stevenson FK, Ottensmeier CH, Johnson P, et al. DNA vaccines to attack cancer. Proc Natl Acad Sci U S A. 2004;101(Suppl 2):14646-14652.
30. Stevenson FK, Rice J, Zhu D. Tumor vaccines. Adv Immunol. 2004;82:49-103.
31. Radcliffe JN, Roddick JS, Friedmann PS, Stevenson FK, Thirdborough SM. Prime-boost with alternating DNA vaccines designed to engage different antigen presentation pathways generates high frequencies of peptide-specific CD8+ T cells. J Immunol. 2006;177:6626-6633.
32. Fairweather NF, Lyness VA. The complete nucleotide sequence of tetanus toxin. Nucleic Acids Res. 1986;14:7809-7812.
33. Demotz S, Matricardi P, Lanzavecchia A, Corradin G. A novel and simple procedure for determining T cell epitopes in protein antigens. J Immunol Methods. 1989;122:67-72.
34. Rice J, Buchan S, Stevenson FK. Critical components of a DNA fusion vaccine able to induce protective cytotoxic T cells against a single epitope of a tumor antigen. J Immunol. 2002;169:3908-3913.
35. Rice J, Elliott T, Buchan S, Stevenson FK. DNA fusion vaccine designed to induce cytotoxic T cell responses against defined peptide motifs: implications for cancer vaccines. J Immunol. 2001; 167:1558-1565.
36. Rice J, Buchan S, Dewchand H, Simpson E, Stevenson FK. DNA fusion vaccines induce targeted epitope-specific CTLs against minor histocompatibility antigens from a normal or tolerized repertoire. J Immunol. 2004;173:4492-4499.
37. Pascolo S, Bervas N, Ure JM, et al. HLA-A2.1-restricted education and cytolytic activity of CD8(−) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. J Exp Med. 1997; 185:2043-2051.
38. Rice J, Ottensmeier CH, Stevenson FK. DNA vaccines: precision tools for activating effective immunity against cancer. Nat Rev Cancer. 2008; 8:108-120.
39. Rauscher FJ III. The WT1 Wilms tumor gene product: a developmentally regulated transcription factor in the kidney that functions as a tumor suppressor. FASEB J. 1993;7:896-903.
40. Alexander J, Sidney J, Southwood S, et al. Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. Immunity. 1994;1:751-761.
41. Buchan S, Gronevik E, Mathiesen I, et al. Electroporation as a "prime/boost" strategy for naked DNA vaccination against a tumor antigen. J Immunol. 2005;174:6292-6298.
42. Rice J, King CA, Spellerberg MB, Fairweather N, Stevenson FK. Manipulation of pathogen-derived genes to influence antigen presentation via DNA vaccines. Vaccine. 1999;17:3030-3038.
43. Ho WY, Nguyen HN, Wolfl M, Kuball J, Greenberg PD. In vitro methods for generating CD8+ T-cell clones for immunotherapy from the naive repertoire. J Immunol Methods. 2006;310:40-52.
44. Rezvani K, Grube M, Brenchley JM, et al. Functional leukemia-associated antigen-specific memory CD8+ T cells exist in healthy individuals and in patients with chronic myelogenous leukemia before and after stem cell transplantation. Blood. 2003;102:2892-2900.
45. Savage P, Gao L, Vento K, et al. Use of B cell-bound HLA-A2 class I monomers to generate high-avidity, allo-restricted CTLs against the leukemia-associated protein Wilms tumor antigen. Blood. 2004;103:4613-4615.
46. Oka Y, Udaka K, Tsuboi A, et al. Cancer immunotherapy targeting Wilms' tumor gene WT1 product. J Immunol. 2000;164:1873-1880.
47. Gaiger A, Reese V, Disis ML, Cheever MA. Immunity to WT1 in the animal model and in patients with acute myeloid leukemia. Blood. 2000;96: 1480-1489.
48. Nakajima H, Kawasaki K, Oka Y, et al. WT1 peptide vaccination combined with BCG-CWS is more efficient for tumor eradication than WT1 peptide vaccination alone. Cancer Immunol Immunother. 2004;53:617-624.
49. Tsuboi A, Oka Y, Udaka K, et al. Enhanced induction of human WT1-specific cytotoxic T lymphocytes with a 9-mer WT1 peptide modified at HLA-A*2402-binding residues. Cancer Immunol Immunother. 2002;51:614-620.
50. Rezvani K, Yong AS, Mielke S, et al. Leukemia-associated antigen specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. Blood. 2007;111:236-242.
51. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. Science. 2003;300:339-342.
52. Janssen EM, Lemmens EE, Wolfe T, et al. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature. 2003; 421:852-856.
53. Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. Science. 2003;300:337-339.
54. Li L, Reinhardt P, Schmitt A, et al. Dendritic cells generated from acute myeloid leukemia (AML) blasts maintain the expression of immunogenic leukemia associated antigens. Cancer Immunol Immunother. 2005;54:685-693.
55. Xue S, Gillmore R, Downs A, et al. Exploiting T cell receptor genes for cancer immunotherapy. Clin Exp Immunol. 2005;139:167-172.
56. Xue SA, Gao L, Hart D, et al. Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. Blood. 2005;106:3062-3067.
57. Palmowski MJ, Choi EM, Hermans IF, et al. Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. J Immunol. 2002;168:4391-4398.

7. References

1. Lai, A.Y., S.M. Lin, and M. Kondo, *Heterogeneity of Flt3-expressing multipotent progenitors in mouse bone marrow.* J Immunol, 2005. **175**(8): p. 5016-23.
2. Huynh, M.L., V.A. Fadok, and P.M. Henson, *Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation.* J Clin Invest, 2002. **109**(1): p. 41-50.
3. Granucci, F., I. Zanoni, and P. Ricciardi-Castagnoli, *Central role of dendritic cells in the regulation and deregulation of immune responses.* Cell Mol Life Sci, 2008.
4. Krishnan, J., et al., *Toll-like receptor signal transduction.* Exp Mol Med, 2007. **39**(4): p. 421-38.
5. Lanier, L.L., *NK cell recognition.* Annu Rev Immunol, 2005. **23**: p. 225-74.
6. Stetson, D.B., et al., *Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function.* J Exp Med, 2003. **198**(7): p. 1069-76.
7. Street, S.E., et al., *Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells.* J Exp Med, 2004. **199**(6): p. 879-84.
8. Guimaraes, F., et al., *Evaluation of ex vivo expanded human NK cells on antileukemia activity in SCID-beige mice.* Leukemia, 2006. **20**(5): p. 833-839.
9. Dias, S., et al., *Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors.* J Exp Med, 2005. **201**(6): p. 971-9.
10. Kondo, M., I.L. Weissman, and K. Akashi, *Identification of clonogenic common lymphoid progenitors in mouse bone marrow.* Cell, 1997. **91**(5): p. 661-72.
11. Pridans, C., et al., *Identification of Pax5 target genes in early B cell differentiation.* J Immunol, 2008. **180**(3): p. 1719-28.
12. Carsetti, R., G. Kohler, and M.C. Lamers, *Transitional B cells are the target of negative selection in the B cell compartment.* J Exp Med, 1995. **181**(6): p. 2129-40.
13. Chung, J.B., et al., *CD23 defines two distinct subsets of immature B cells which differ in their responses to T cell help signals.* Int Immunol, 2002. **14**(2): p. 157-66.
14. Lai, A.Y. and M. Kondo, *Identification of a bone marrow precursor of the earliest thymocytes in adult mouse.* Proc Natl Acad Sci U S A, 2007. **104**(15): p. 6311-6.
15. Hodges, E., et al., *Diagnostic role of tests for T cell receptor (TCR) genes.* J Clin Pathol, 2003. **56**(1): p. 1-11.
16. Brandes, M., et al., *Flexible migration program regulates gamma delta T-cell involvement in humoral immunity.* Blood, 2003. **102**(10): p. 3693-701.
17. Russano, A.M., et al., *CD1-restricted recognition of exogenous and self-lipid antigens by duodenal gammadelta+ T lymphocytes.* J Immunol, 2007. **178**(6): p. 3620-6.
18. Brandes, M., K. Willimann, and B. Moser, *Professional antigen-presentation function by human gammadelta T Cells.* Science, 2005. **309**(5732): p. 264-8.
19. Rossi, F.M., et al., *Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1.* Nat Immunol, 2005. **6**(6): p. 626-34.
20. Schwarz, B.A., et al., *Selective thymus settling regulated by cytokine and chemokine receptors.* J Immunol, 2007. **178**(4): p. 2008-17.
21. Radtke, F., et al., *Deficient T cell fate specification in mice with an induced inactivation of Notch1.* Immunity, 1999. **10**(5): p. 547-58.

22. Moore, T.A. and A. Zlotnik, *T-cell lineage commitment and cytokine responses of thymic progenitors.* Blood, 1995. **86**(5): p. 1850-60.

23. Bensinger, S.J., et al., *Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells.* J Exp Med, 2001. **194**(4): p. 427-38.

24. Derbinski, J., et al., *Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self.* Nat Immunol, 2001. **2**(11): p. 1032-9.

25. Anderson, M.S., et al., *Projection of an immunological self shadow within the thymus by the aire protein.* Science, 2002. **298**(5597): p. 1395-401.

26. Gallegos, A.M. and M.J. Bevan, *Central tolerance: good but imperfect.* Immunol Rev, 2006. **209**: p. 290-6.

27. Jonuleit, H., et al., *Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells.* J Exp Med, 2000. **192**(9): p. 1213-22.

28. Redmond, W.L. and L.A. Sherman, *Peripheral tolerance of CD8 T lymphocytes.* Immunity, 2005. **22**(3): p. 275-84.

29. Lang, K.S., et al., *Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation.* Eur J Immunol, 2005. **35**(3): p. 738-45.

30. Guillaume, S., et al., *Proximal changes in signal transduction that modify CD8+ T cell responsiveness in vivo.* Eur J Immunol, 2003. **33**(9): p. 2551-6.

31. Lee, J.W., et al., *Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self.* Nat Immunol, 2007. **8**(2): p. 181-90.

32. Randolph, G.J., J. Ochando, and S.N.S. Partida, *Migration of Dendritic Cell Subsets and their Precursors.* Annu Rev Immunol, 2007.

33. Pierre, P., et al., *Developmental regulation of MHC class II transport in mouse dendritic cells.* Nature, 1997. **388**(6644): p. 787-92.

34. Stuber, E., W. Strober, and M. Neurath, *Blocking the CD40L-CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion.* J Exp Med, 1996. **183**(2): p. 693-8.

35. Dudziak, D., et al., *Differential antigen processing by dendritic cell subsets in vivo.* Science, 2007. **315**(5808): p. 107-11.

36. Yewdell, J.W., L.C. Anton, and J.R. Bennink, *Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules?* J Immunol, 1996. **157**(5): p. 1823-6.

37. Borissenko, L. and M. Groll, *Diversity of proteasomal missions: fine tuning of the immune response.* Biol Chem, 2007. **388**(9): p. 947-55.

38. Stoltze, L., et al., *Two new proteases in the MHC class I processing pathway.* Nat Immunol, 2000. **1**(5): p. 413-8.

39. Hanada, K., J.W. Yewdell, and J.C. Yang, *Immune recognition of a human renal cancer antigen through post-translational protein splicing.* Nature, 2004. **427**(6971): p. 252-6.

40. Dannull, J., et al., *Immunoproteasome down-modulation enhances the ability of dendritic cells to stimulate antitumor immunity.* Blood, 2007. **110**(13): p. 4341-50.

41. Serwold, T., et al., *ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum.* Nature, 2002. **419**(6906): p. 480-3.

42. Kanaseki, T., et al., *ERAAP synergizes with MHC class I molecules to make the final cut in the antigenic peptide precursors in the endoplasmic reticulum.* Immunity, 2006. **25**(5): p. 795-806.

43. Farmery, M.R., et al., *The role of ERp57 in disulfide bond formation during the assembly of major histocompatibility complex class I in a synchronized semipermeabilized cell translation system.* J Biol Chem, 2000. **275**(20): p. 14933-8.

44. Ortmann, B., et al., *A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes.* Science, 1997. **277**(5330): p. 1306-9.

45. Denzin, L.K., C. Hammond, and P. Cresswell, *HLA-DM interactions with intermediates in HLA-DR maturation and a role for HLA-DM in stabilizing empty HLA-DR molecules.* J Exp Med, 1996. **184**(6): p. 2153-65.

46. Rocha, N. and J. Neefjes, *MHC class II molecules on the move for successful antigen presentation.* EMBO J, 2008. **27**(1): p. 1-5.

47. Norbury, C.C., et al., *CD8+ T cell cross-priming via transfer of proteasome substrates.* Science, 2004. **304**(5675): p. 1318-21.

48. Shen, L., et al., *Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo.* Immunity, 2004. **21**(2): p. 155-65.

49. Bertholet, S., et al., *Leishmania antigens are presented to CD8+ T cells by a transporter associated with antigen processing-independent pathway in vitro and in vivo.* J Immunol, 2006. **177**(6): p. 3525-33.

50. Guermonprez, P., et al., *ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells.* Nature, 2003. **425**(6956): p. 397-402.

51. Miller, M.J., et al., *T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node.* Proceedings of the National Academy of Sciences, 2004. **101**(4): p. 998-1003.

52. Kedl, R.M., et al., *T Cells Compete for Access to Antigen-bearing Antigen-presenting Cells.* J. Exp. Med., 2000. **192**(8): p. 1105-1114.

53. Obst, R., et al., *Antigen persistence is required throughout the expansion phase of a CD4+ T cell response.* J. Exp. Med., 2005. **201**(10): p. 1555-1565.

54. van Stipdonk, M.J.B., E.E. Lemmens, and S.P. Schoenberger, *Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation.* Nat Immunol, 2001. **2**(5): p. 423-429.

55. Essen, D.v., H. Kikutani, and D. Gray, *CD40 ligand-transduced co-stimulation of T cells in the development of helper function.* Nature, 1995. **378**(6557): p. 620-623.

56. Beier, K.C., et al., *Induction, binding specificity and function of human ICOS.* Eur J Immunol, 2000. **30**(12): p. 3707-17.

57. Rogers, P.R., et al., *OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells.* Immunity, 2001. **15**(3): p. 445-55.

58. Parry, R.V., et al., *CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-xL, and IL-2 expression in primary human CD4 T lymphocytes.* J Immunol, 2003. **171**(1): p. 166-74.

59. Mirenda, V., et al., *Physiologic and aberrant regulation of memory T-cell trafficking by the costimulatory molecule CD28.* Blood, 2007. **109**(7): p. 2968-77.

60. Alegre, M.L., et al., *Regulation of surface and intracellular expression of CTLA4 on mouse T cells.* J Immunol, 1996. **157**(11): p. 4762-70.

61. Martins, G.A., et al., *CD28 is required for T cell activation and IFN-gamma production by CD4+ and CD8+ T cells in response to Trypanosoma cruzi infection.* Microbes Infect, 2004. **6**(13): p. 1133-44.

62. Tivol, E.A., et al., *CTLA4Ig prevents lymphoproliferation and fatal multiorgan tissue destruction in CTLA-4-deficient mice.* J Immunol, 1997. **158**(11): p. 5091-4.

63. Beier, K.C., T. Kallinich, and E. Hamelmann, *Master switches of T-cell activation and differentiation.* Eur Respir J, 2007. **29**(4): p. 804-12.

64. Brocker, T., et al., *CD4 T cell traffic control: in vivo evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles.* Eur J Immunol, 1999. **29**(5): p. 1610-6.

65. Bansal-Pakala, P., et al., *Costimulation of CD8 T cell responses by OX40.* J Immunol, 2004. **172**(8): p. 4821-5.

66. Drenkard, D., et al., *CD137 is expressed on blood vessel walls at sites of inflammation and enhances monocyte migratory activity.* FASEB J, 2007. **21**(2): p. 456-63.

67. Taraban, V.Y., T.F. Rowley, and A. Al-Shamkhani, *Cutting edge: a critical role for CD70 in CD8 T cell priming by CD40-licensed APCs.* J Immunol, 2004. **173**(11): p. 6542-6.

68. Taraban, V.Y., et al., *Requirement for CD70 in CD4+ Th cell-dependent and innate receptor-mediated CD8+ T cell priming.* J Immunol, 2006. **177**(5): p. 2969-75.

69. Rowley, T.F. and A. Al-Shamkhani, *Stimulation by soluble CD70 promotes strong primary and secondary CD8+ cytotoxic T cell responses in vivo.* J Immunol, 2004. **172**(10): p. 6039-46.

70. Lavrik, I., A. Golks, and P.H. Krammer, *Death receptor signaling.* J Cell Sci, 2005. **118**(2): p. 265-267.

71. Yang, J., et al., *Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific CD8+ T cells in vivo.* Proceedings of the National Academy of Sciences, 2006. **103**(1): p. 147-152.

72. Hanon, E., et al., *Fratricide among CD8(+) T lymphocytes naturally infected with human T cell lymphotropic virus type I.* Immunity, 2000. **13**(5): p. 657-64.

73. Hwang, I., et al., *T Cells Can Use Either T Cell Receptor or CD28 Receptors to Absorb and Internalize Cell Surface Molecules Derived from Antigen-presenting Cells.* J. Exp. Med., 2000. **191**(7): p. 1137-1148.

74. Cox, J.H., et al., *CTLs target Th cells that acquire bystander MHC class I-peptide complex from APCs.* J Immunol, 2007. **179**(2): p. 830-6.

75. Schoenberger, S.P., et al., *T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions.* Nature, 1998. **393**(6684): p. 480-3.

76. Bennett, S.R., et al., *Help for cytotoxic-T-cell responses is mediated by CD40 signalling.* Nature, 1998. **393**(6684): p. 478-80.

77. Medema, J.P., et al., *Expression of the Serpin Serine Protease Inhibitor 6 Protects Dendritic Cells from Cytotoxic T Lymphocyte-induced Apoptosis: Differential Modulation by T Helper Type 1 and Type 2 Cells.* J. Exp. Med., 2001. **194**(5): p. 657-668.

78. Shedlock, D.J. and H. Shen, *Requirement for CD4 T cell help in generating functional CD8 T cell memory.* Science, 2003. **300**(5617): p. 337-9.

79. Sun, J.C. and M.J. Bevan, *Defective CD8 T cell memory following acute infection without CD4 T cell help.* Science, 2003. **300**(5617): p. 339-42.

80. Sun, J.C., M.A. Williams, and M.J. Bevan, *CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection.* Nat Immunol, 2004. **5**(9): p. 927-33.

81. Janssen, E.M., et al., *CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death.* Nature, 2005. **434**(7029): p. 88-93.

82. Northrop, J.K., et al., *Epigenetic remodeling of the IL-2 and IFN-gamma loci in memory CD8 T cells is influenced by CD4 T cells.* J Immunol, 2006. **177**(2): p. 1062-9.

83. Perez-Diez, A., et al., *CD4 cells can be more efficient at tumor rejection than CD8 cells.* Blood, 2007. **109**(12): p. 5346-54.

84. Corthay, A., et al., *Primary antitumor immune response mediated by CD4+ T cells.* Immunity, 2005. **22**(3): p. 371-83.

85. Refaeli, Y., et al., *Biochemical Mechanisms of IL-2-Regulated Fas-Mediated T Cell Apoptosis.* Immunity, 1998. **8**(5): p. 615-623.

86. Zhu, Y., et al., *Constitutive association of the proapoptotic protein Bim with Bcl-2-related proteins on mitochondria in T cells.* Proc Natl Acad Sci U S A, 2004. **101**(20): p. 7681-6.

87. Weninger, W., et al., *Migratory properties of naive, effector, and memory CD8(+) T cells.* J Exp Med, 2001. **194**(7): p. 953-66.

88. Sallusto, F., et al., *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.

89. Kaech, S.M., et al., *Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells.* Nat Immunol, 2003. **4**(12): p. 1191-1198.

90. Wherry, E.J., et al., *Lineage relationship and protective immunity of memory CD8 T cell subsets.* Nat Immunol, 2003. **4**(3): p. 225-234.

91. Manjunath, N., et al., *Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes.* J Clin Invest, 2001. **108**(6): p. 871-8.

92. Li, J., G. Huston, and S.L. Swain, *IL-7 Promotes the Transition of CD4 Effectors to Persistent Memory Cells.* J. Exp. Med., 2003. **198**(12): p. 1807-1815.

93. Seddon, B., P. Tomlinson, and R. Zamoyska, *Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells.* Nat Immunol, 2003. **4**(7): p. 680-6.

94. Kennedy, M.K., et al., *Reversible Defects in Natural Killer and Memory CD8 T Cell Lineages in Interleukin 15-deficient Mice.* J. Exp. Med., 2000. **191**(5): p. 771-780.

95. Gaspal, F.M.C., et al., *Mice Deficient in OX40 and CD30 Signals Lack Memory Antibody Responses because of Deficient CD4 T Cell Memory.* J Immunol, 2005. **174**(7): p. 3891-3896.

96. Pulle, G., M. Vidric, and T.H. Watts, *IL-15-Dependent Induction of 4-1BB Promotes Antigen-Independent CD8 Memory T Cell Survival.* J Immunol, 2006. **176**(5): p. 2739-2748.

97. Genova, G.D., et al., *Vaccination of human subjects expands both specific and bystander memory T cells but antibody production remains vaccine specific.* Blood, 2006. **107**(7): p. 2806-2813.

98. Wherry, E.J., et al., *Antigen-independent memory CD8 T cells do not develop during chronic viral infection.* Proceedings of the National Academy of Sciences, 2004. **101**(45): p. 16004-16009.

99. Giovanna Roncador, Philip J.B.L.M.S.H.Jorge L.M.-T.K.-L.L.S.P.C.T.Bridget C.F.V.C.F.P.Alison H.B., *Analysis of FOXP3 protein expression in human*

CD4 regulatory T cells at the single-cell level. European Journal of Immunology, 2005. **35**(6): p. 1681-1691.

100. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.

101. Sundrud, M.S. and A. Rao, *New twists of T cell fate: control of T cell activation and tolerance by TGF-beta and NFAT.* Curr Opin Immunol, 2007. **19**(3): p. 287-93.

102. Jonuleit, H., et al., *Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood.* J Exp Med, 2001. **193**(11): p. 1285-94.

103. Cederbom, L., H. Hall, and F. Ivars, *CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells.* Eur J Immunol, 2000. **30**(6): p. 1538-43.

104. Takahashi, T., et al., *Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4.* J Exp Med, 2000. **192**(2): p. 303-10.

105. Ghiringhelli, F., et al., *Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation.* J Exp Med, 2005. **202**(7): p. 919-29.

106. June, C.H., *Adoptive T cell therapy for cancer in the clinic.* J Clin Invest, 2007. **117**(6): p. 1466-76.

107. Leen, A.M., C.M. Rooney, and A.E. Foster, *Improving T cell therapy for cancer.* Annu Rev Immunol, 2007. **25**: p. 243-65.

108. Boon, T., et al., *Human T cell responses against melanoma.* Annu Rev Immunol, 2006. **24**: p. 175-208.

109. Stevenson, F.K., J. Rice, and D. Zhu, *Tumor vaccines.* Adv Immunol, 2004. **82**: p. 49-103.

110. Srinivasan, R. and J.D. Wolchok, *Tumor antigens for cancer immunotherapy: therapeutic potential of xenogeneic DNA vaccines.* J Transl Med, 2004. **2**(1): p. 12.

111. Scanlan, M.J., et al., *Cancer/testis antigens: an expanding family of targets for cancer immunotherapy.* Immunol Rev, 2002. **188**: p. 22-32.

112. Smyth, M.J., G.P. Dunn, and R.D. Schreiber, *Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity.* Adv Immunol, 2006. **90**: p. 1-50.

113. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The immunobiology of cancer immunosurveillance and immunoediting.* Immunity, 2004. **21**(2): p. 137-48.

114. Shankaran, V., et al., *IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity.* Nature, 2001. **410**(6832): p. 1107-11.

115. Liu, K., et al., *CTL adoptive immunotherapy concurrently mediates tumor regression and tumor escape.* J Immunol, 2006. **176**(6): p. 3374-82.

116. Vial, T. and J. Descotes, *Immunosuppressive drugs and cancer.* Toxicology, 2003. **185**(3): p. 229-40.

117. Zhang, L., et al., *Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer.* N Engl J Med, 2003. **348**(3): p. 203-13.

118. Sato, E., et al., *Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18538-43.

119. Naito, Y., et al., *CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer.* Cancer Res, 1998. **58**(16): p. 3491-4.

120. Goodell, V., et al., *Antibody immunity to the p53 oncogenic protein is a prognostic indicator in ovarian cancer.* J Clin Oncol, 2006. **24**(5): p. 762-8.

121. Galon, J., et al., *Type, density, and location of immune cells within human colorectal tumors predict clinical outcome.* Science, 2006. **313**(5795): p. 1960-4.

122. Albert, M.L. and R.B. Darnell, *Paraneoplastic neurological degenerations: keys to tumour immunity.* Nat Rev Cancer, 2004. **4**(1): p. 36-44.

123. Neeley, Y.C., et al., *Partially circumventing peripheral tolerance for oncogene-specific prostate cancer immunotherapy.* Prostate, 2008. **68**(7): p. 715-27.

124. Degl'Innocenti, E., et al., *Peripheral T cell tolerance occurs early during spontaneous prostate cancer development and can be rescued by dendritic cell immunization.* Eur J Immunol, 2005. **35**(1): p. 66-75.

125. Perez-Diez, A., et al., *Intensity of the vaccine-elicited immune response determines tumor clearance.* J Immunol, 2002. **168**(1): p. 338-47.

126. Toes, R.E., et al., *Enhancement of tumor outgrowth through CTL tolerization after peptide vaccination is avoided by peptide presentation on dendritic cells.* J Immunol, 1998. **160**(9): p. 4449-56.

127. Tuyaerts, S., et al., *Generation of large numbers of dendritic cells in a closed system using Cell Factories.* J Immunol Methods, 2002. **264**(1-2): p. 135-51.

128. Ishii, K.J., et al., *TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines.* Nature, 2008. **451**(7179): p. 725-9.

129. Klinman, D.M., G. Yamshchikov, and Y. Ishigatsubo, *Contribution of CpG motifs to the immunogenicity of DNA vaccines.* J Immunol, 1997. **158**(8): p. 3635-9.

130. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses.* Nat Immunol, 2004. **5**(10): p. 987-95.

131. Pasare, C. and R. Medzhitov, *Toll-like receptors: linking innate and adaptive immunity.* Microbes Infect, 2004. **6**(15): p. 1382-7.

132. Spies, B., et al., *Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice.* J Immunol, 2003. **171**(11): p. 5908-12.

133. Babiuk, S., et al., *TLR9-/- and TLR9+/+ mice display similar immune responses to a DNA vaccine.* Immunology, 2004. **113**(1): p. 114-20.

134. Tudor, D., et al., *TLR9 pathway is involved in adjuvant effects of plasmid DNA-based vaccines.* Vaccine, 2005. **23**(10): p. 1258-64.

135. Klinman, D.M., *Adjuvant activity of CpG oligodeoxynucleotides.* Int Rev Immunol, 2006. **25**(3-4): p. 135-54.

136. Corr, M., et al., *Gene vaccination with naked plasmid DNA: mechanism of CTL priming.* J Exp Med, 1996. **184**(4): p. 1555-60.

137. Doe, B., et al., *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, 1996. **93**(16): p. 8578-83.

138. Wolff, J.A., et al., *Direct gene transfer into mouse muscle in vivo.* Science, 1990. **247**(4949 Pt 1): p. 1465-8.

139. Chattergoon, M.A., et al., *Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages/antigen-presenting cells.* J Immunol, 1998. **160**(12): p. 5707-18.

140. Corr, M., et al., *In vivo priming by DNA injection occurs predominantly by antigen transfer.* J Immunol, 1999. **163**(9): p. 4721-7.

141. Ulmer, J.B., et al., *Expression of a viral protein by muscle cells in vivo induces protective cell-mediated immunity.* Vaccine, 1997. **15**(8): p. 839-41.

142. Torres, C.A., et al., *Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations.* J Immunol, 1997. **158**(10): p. 4529-32.

143. Coelho-Castelo, A.A., et al., *Tissue distribution of a plasmid DNA encoding Hsp65 gene is dependent on the dose administered through intramuscular delivery.* Genet Vaccines Ther, 2006. **4**: p. 1.

144. Porgador, A., et al., *Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization.* J Exp Med, 1998. **188**(6): p. 1075-82.

145. Ledwith, B.J., et al., *Plasmid DNA vaccines: investigation of integration into host cellular DNA following intramuscular injection in mice.* Intervirology, 2000. **43**(4-6): p. 258-72.

146. Greenland, J.R., et al., *Plasmid DNA vaccine-elicited cellular immune responses limit in vivo vaccine antigen expression through Fas-mediated apoptosis.* J Immunol, 2007. **178**(9): p. 5652-8.

147. Payette, P.J., et al., *Immune-mediated destruction of transfected myocytes following DNA vaccination occurs via multiple mechanisms.* Gene Ther, 2001. **8**(18): p. 1395-400.

148. Radcliffe, J.N., et al., *Prolonged antigen expression following DNA vaccination impairs effector CD8+ T cell function and memory development.* J Immunol, 2007. **179**(12): p. 8313-21.

149. Hung, K., et al., *The central role of CD4(+) T cells in the antitumor immune response.* J Exp Med, 1998. **188**(12): p. 2357-68.

150. Zimmermann, S., et al., *CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis.* J Immunol, 1998. **160**(8): p. 3627-30.

151. Weiss, R., et al., *Gene gun bombardment with gold particles displays a particular Th2-promoting signal that over-rules the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines.* Vaccine, 2002. **20**(25-26): p. 3148-54.

152. Stevenson, F.K., et al., *DNA fusion gene vaccines against cancer: from the laboratory to the clinic.* Immunol Rev, 2004. **199**: p. 156-80.

153. Wolkers, M.C., et al., *Optimizing the efficacy of epitope-directed DNA vaccination.* J Immunol, 2002. **168**(10): p. 4998-5004.

154. Kennedy, R. and E. Celis, *T helper lymphocytes rescue CTL from activation-induced cell death.* J Immunol, 2006. **177**(5): p. 2862-72.

155. Savelyeva, N., et al., *Inhibition of a vaccine-induced anti-tumor B cell response by soluble protein antigen in the absence of continuing T cell help.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 10987-92.

156. Rice, J., et al., *DNA fusion vaccines induce targeted epitope-specific CTLs against minor histocompatibility antigens from a normal or tolerized repertoire.* J Immunol, 2004. **173**(7): p. 4492-9.

157. Radcliffe, J.N., et al., *Prime-boost with alternating DNA vaccines designed to engage different antigen presentation pathways generates high frequencies of peptide-specific CD8+ T cells.* J Immunol, 2006. **177**(10): p. 6626-33.

158. Rice, J., et al., *DNA fusion vaccine designed to induce cytotoxic T cell responses against defined peptide motifs: implications for cancer vaccines.* J Immunol, 2001. **167**(3): p. 1558-65.

159. Rice, J., S. Buchan, and F.K. Stevenson, *Critical components of a DNA fusion vaccine able to induce protective cytotoxic T cells against a single epitope of a tumor antigen.* J Immunol, 2002. **169**(7): p. 3908-13.

160. Rice, J., et al., *DNA fusion vaccines induce epitope-specific cytotoxic CD8(+) T cells against human leukemia-associated minor histocompatibility antigens.* Cancer Res, 2006. **66**(10): p. 5436-42.

161. Hanson, H.L., et al., *Eradication of established tumors by CD8+ T cell adoptive immunotherapy.* Immunity, 2000. **13**(2): p. 265-76.

162. Gurunathan, S., et al., *Requirements for the maintenance of Th1 immunity in vivo following DNA vaccination: a potential immunoregulatory role for CD8+ T cells.* J Immunol, 2000. **165**(2): p. 915-24.

163. Quaglino, E., et al., *Electroporated DNA vaccine clears away multifocal mammary carcinomas in her-2/neu transgenic mice.* Cancer Res, 2004. **64**(8): p. 2858-64.

164. Thirdborough, S.M., et al., *Vaccination with DNA encoding a single-chain TCR fusion protein induces anticlonotypic immunity and protects against T-cell lymphoma.* Cancer Res, 2002. **62**(6): p. 1757-60.

165. King, C.A., et al., *DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma.* Nat Med, 1998. **4**(11): p. 1281-6.

166. Buchan, S., et al., *Electroporation as a "prime/boost" strategy for naked DNA vaccination against a tumor antigen.* J Immunol, 2005. **174**(10): p. 6292-8.

167. Dupuis, M., et al., *Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice.* J Immunol, 2000. **165**(5): p. 2850-8.

168. Feltquate, D.M., et al., *Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization.* J Immunol, 1997. **158**(5): p. 2278-84.

169. Fuller, D.H., P. Loudon, and C. Schmaljohn, *Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases.* Methods, 2006. **40**(1): p. 86-97.

170. Mathiesen, I., *Electropermeabilization of skeletal muscle enhances gene transfer in vivo.* Gene Ther, 1999. **6**(4): p. 508-14.

171. Widera, G., et al., *Increased DNA vaccine delivery and immunogenicity by electroporation in vivo.* J Immunol, 2000. **164**(9): p. 4635-40.

172. Otten, G., et al., *Enhancement of DNA vaccine potency in rhesus macaques by electroporation.* Vaccine, 2004. **22**(19): p. 2489-93.

173. Luckay, A., et al., *Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques.* J Virol, 2007. **81**(10): p. 5257-69.

174. Lesbordes, J.C., et al., *In vivo electrotransfer of the cardiotrophin-1 gene into skeletal muscle slows down progression of motor neuron degeneration in pmn mice.* Hum Mol Genet, 2002. **11**(14): p. 1615-25.

175. Watchmaker, P.B., et al., *Memory CD8+ T cells protect dendritic cells from CTL killing.* J Immunol, 2008. **180**(6): p. 3857-65.

176. Bos, R., et al., *Characterization of antigen-specific immune responses induced by canarypox virus vaccines.* J Immunol, 2007. **179**(9): p. 6115-22.

177. Hanke, T., et al., *Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime.* Vaccine, 1998. **16**(5): p. 439-45.

178. McConkey, S.J., et al., *Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans.* Nat Med, 2003. **9**(6): p. 729-35.

179. Smorlesi, A., et al., *Evaluation of different plasmid DNA delivery systems for immunization against HER2/neu in a transgenic murine model of mammary carcinoma.* Vaccine, 2006. **24**(11): p. 1766-75.

180. Zhu, D. and F.K. Stevenson, *DNA gene fusion vaccines against cancer.* Curr Opin Mol Ther, 2002. **4**(1): p. 41-8.

181. Rodriguez, F., J. Zhang, and J.L. Whitton, *DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction.* J Virol, 1997. **71**(11): p. 8497-503.

182. Velders, M.P., et al., *Defined flanking spacers and enhanced proteolysis is essential for eradication of established tumors by an epitope string DNA vaccine.* J Immunol, 2001. **166**(9): p. 5366-73.

183. Qin, H., et al., *Enhancement of antitumour immunity by a novel chemotactic antigen DNA vaccine encoding chemokines and multiepitopes of prostate-tumour-associated antigens.* Immunology, 2006. **117**(3): p. 419-30.

184. Ross, T.M., et al., *C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge.* Nat Immunol, 2000. **1**(2): p. 127-31.

185. Bergmann-Leitner, E.S., W.W. Leitner, and G.C. Tsokos, *Complement 3d: from molecular adjuvant to target of immune escape mechanisms.* Clin Immunol, 2006. **121**(2): p. 177-85.

186. Sasaki, S., et al., *Apoptosis-mediated enhancement of DNA-raised immune responses by mutant caspases.* Nat Biotechnol, 2001. **19**(6): p. 543-7.

187. Spellerberg, M.B., et al., *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, 1997. **159**(4): p. 1885-92.

188. Savelyeva, N., et al., *Plant viral genes in DNA idiotypic vaccines activate linked CD4+ T-cell mediated immunity against B-cell malignancies.* Nat Biotechnol, 2001. **19**(8): p. 760-4.

189. Anderson, R., et al., *Immune response in mice following immunization with DNA encoding fragment C of tetanus toxin.* Infect Immun, 1996. **64**(8): p. 3168-73.

190. Rice, J., et al., *Manipulation of pathogen-derived genes to influence antigen presentation via DNA vaccines.* Vaccine, 1999. **17**(23-24): p. 3030-8.

191. Yewdell, J.W. and J.R. Bennink, *Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses.* Annu Rev Immunol, 1999. **17**: p. 51-88.

192. Panina-Bordignon, P., et al., *Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells.* Eur J Immunol, 1989. **19**(12): p. 2237-42.

193. Ciernik, I.F., J.A. Berzofsky, and D.P. Carbone, *Induction of cytotoxic T lymphocytes and antitumor immunity with DNA vaccines expressing single T cell epitopes.* J Immunol, 1996. **156**(7): p. 2369-75.

194. An, L.L., et al., *Quantitative and qualitative analyses of the immune responses induced by a multivalent minigene DNA vaccine.* Vaccine, 2000. **18**(20): p. 2132-41.

195. Wolkers, M.C., et al., *Antigen bias in T cell cross-priming.* Science, 2004. **304**(5675): p. 1314-7.

196. Le Gall, S., P. Stamegna, and B.D. Walker, *Portable flanking sequences modulate CTL epitope processing.* J Clin Invest, 2007. **117**(11): p. 3563-75.

197. Heidi Link Snyder, I.B., ccaron, and J.W.Y.T.W.B.J.R.B. ík, *Promiscuous liberation of MHC-class I-binding peptides from the C termini of membrane and soluble proteins in the secretory pathway.* European Journal of Immunology, 1998. **28**(4): p. 1339-1346.

198. Mo, A.X., et al., *Sequences that flank subdominant and cryptic epitopes influence the proteolytic generation of MHC class I-presented peptides.* J Immunol, 2000. **164**(8): p. 4003-10.

199. Forconi, F., et al., *Insight into the potential for DNA idiotypic fusion vaccines designed for patients by analysing xenogeneic anti-idiotypic antibody responses.* Immunology, 2002. **107**(1): p. 39-45.

200. Swallow, T. and R.S. Kirby, *Cancer of the prostate gland.* Surgery, 2006. **24**(5): p. 173-176.

201. Jain, S., A.G. Bhojwani, and J.K. Mellon, *Improving the utility of prostate specific antigen (PSA) in the diagnosis of prostate cancer: the use of PSA derivatives and novel markers.* Postgrad Med J, 2002. **78**(925): p. 646-50.

202. Loeb, S. and W.J. Catalona, *Prostate-specific antigen in clinical practice.* Cancer Lett, 2007. **249**(1): p. 30-9.

203. Harnden, P., et al., *Should the Gleason grading system for prostate cancer be modified to account for high-grade tertiary components? A systematic review and meta-analysis.* Lancet Oncol, 2007. **8**(5): p. 411-9.

204. Johnson, M.A., et al., *Castration triggers growth of previously static androgen-independent lesions in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model.* Prostate, 2005. **62**(4): p. 322-38.

205. Frost, G.I., et al., *Novel syngeneic pseudo-orthotopic prostate cancer model: vascular, mitotic and apoptotic responses to castration.* Microvasc Res, 2005. **69**(1-2): p. 1-9.

206. Giwercman, Y.L., et al., *The 5alpha-reductase type II A49T and V89L high-activity allelic variants are more common in men with prostate cancer compared with the general population.* Eur Urol, 2005. **48**(4): p. 679-85.

207. Gottlieb, B., et al., *Analysis of exon 1 mutations in the androgen receptor gene.* Hum Mutat, 1999. **14**(6): p. 527-39.

208. Duff, J. and I.J. McEwan, *Mutation of histidine 874 in the androgen receptor ligand-binding domain leads to promiscuous ligand activation and altered p160 coactivator interactions.* Mol Endocrinol, 2005. **19**(12): p. 2943-54.

209. Linja, M.J. and T. Visakorpi, *Alterations of androgen receptor in prostate cancer.* J Steroid Biochem Mol Biol, 2004. **92**(4): p. 255-64.

210. Zhao, X.Y., et al., *Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA PCa 2a.* J Urol, 1999. **162**(6): p. 2192-9.

211. McKenzie, S. and N. Kyprianou, *Apoptosis evasion: the role of survival pathways in prostate cancer progression and therapeutic resistance.* J Cell Biochem, 2006. **97**(1): p. 18-32.

212. Reynolds, A.R. and N. Kyprianou, *Growth factor signalling in prostatic growth: significance in tumour development and therapeutic targeting.* Br J Pharmacol, 2006. **147 Suppl 2**: p. S144-52.

213. Sobel, R.E. and M.D. Sadar, *Cell lines used in prostate cancer research: a compendium of old and new lines--part 1.* J Urol, 2005. **173**(2): p. 342-59.

214. Sobel, R.E. and M.D. Sadar, *Cell lines used in prostate cancer research: a compendium of old and new lines--part 2.* J Urol, 2005. **173**(2): p. 360-72.

215. Carlsson, B., et al., *Characterization of human prostate and breast cancer cell lines for experimental T cell-based immunotherapy.* Prostate, 2007. **67**(4): p. 389-95.

216. Slusher, B.S., et al., *Selective inhibition of NAALADase, which converts NAAG to glutamate, reduces ischemic brain injury.* Nat Med, 1999. **5**(12): p. 1396-402.

217. Kaighn, M.E., et al., *Establishment and characterization of a human prostatic carcinoma cell line (PC-3).* Invest Urol, 1979. **17**(1): p. 16-23.

218. Stone, K.R., et al., *Isolation of a human prostate carcinoma cell line (DU 145).* Int J Cancer, 1978. **21**(3): p. 274-81.

219. Horoszewicz, J.S., et al., *The LNCaP cell line--a new model for studies on human prostatic carcinoma.* Prog Clin Biol Res, 1980. **37**: p. 115-32.

220. Sokoloff, M.H., et al., *In vitro modulation of tumor progression-associated properties of hormone refractory prostate carcinoma cell lines by cytokines.* Cancer, 1996. **77**(9): p. 1862-72.

221. Bander, N.H., et al., *MHC class I and II expression in prostate carcinoma and modulation by interferon-alpha and -gamma.* Prostate, 1997. **33**(4): p. 233-9.

222. Dunn, G.P., et al., *IFN unresponsiveness in LNCaP cells due to the lack of JAK1 gene expression.* Cancer Res, 2005. **65**(8): p. 3447-53.

223. Huss, W.J., L.A. Maddison, and N.M. Greenberg, *Autochthonous mouse models for prostate cancer: past, present and future.* Semin Cancer Biol, 2001. **11**(3): p. 245-60.

224. Greenberg, N.M., et al., *Prostate cancer in a transgenic mouse.* Proc Natl Acad Sci U S A, 1995. **92**(8): p. 3439-43.

225. Gingrich, J.R., et al., *Metastatic prostate cancer in a transgenic mouse.* Cancer Res, 1996. **56**(18): p. 4096-102.

226. Foster, B.A., et al., *Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model.* Cancer Res, 1997. **57**(16): p. 3325-30.

227. Grossmann, M.E., M. Wood, and E. Celis, *Expression, specificity and immunotherapy potential of prostate-associated genes in murine cell lines.* World J Urol, 2001. **19**(5): p. 365-70.

228. Tricoli, J.V., M. Schoenfeldt, and B.A. Conley, *Detection of prostate cancer and predicting progression: current and future diagnostic markers.* Clin Cancer Res, 2004. **10**(12 Pt 1): p. 3943-53.

229. Cunha, A.C., et al., *Tissue-specificity of prostate specific antigens: comparative analysis of transcript levels in prostate and non-prostatic tissues.* Cancer Lett, 2006. **236**(2): p. 229-38.

230. Lu, J. and E. Celis, *Recognition of prostate tumor cells by cytotoxic T lymphocytes specific for prostate-specific membrane antigen.* Cancer Res, 2002. **62**(20): p. 5807-12.

231. Mincheff, M., et al., *Human dendritic cells genetically engineered to express cytosolically retained fragment of prostate-specific membrane antigen prime cytotoxic T-cell responses to multiple epitopes.* Cancer Gene Ther, 2003. **10**(12): p. 907-17.

232. Mincheff, M., S. Zoubak, and Y. Makogonenko, *Immune responses against PSMA after gene-based vaccination for immunotherapy-A: results from immunizations in animals.* Cancer Gene Ther, 2006. **13**(4): p. 436-44.

233. Rodeberg, D.A., et al., *Recognition of six-transmembrane epithelial antigen of the prostate-expressing tumor cells by peptide antigen-induced cytotoxic T lymphocytes.* Clin Cancer Res, 2005. **11**(12): p. 4545-52.

234. Kiessling, A., et al., *Prostate stem cell antigen: Identification of immunogenic peptides and assessment of reactive CD8+ T cells in prostate cancer patients.* Int J Cancer, 2002. **102**(4): p. 390-7.

235. Correale, P., et al., *In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen.* J Natl Cancer Inst, 1997. **89**(4): p. 293-300.

236. Chakraborty, N.G., et al., *Recognition of PSA-derived peptide antigens by T cells from prostate cancer patients without any prior stimulation.* Cancer Immunol Immunother, 2003. **52**(8): p. 497-505.

237. Matsueda, S., et al., *Identification of peptide vaccine candidates for prostate cancer patients with HLA-A3 supertype alleles.* Clin Cancer Res, 2005. **11**(19 Pt 1): p. 6933-43.

238. Harada, M., et al., *Prostate-related antigen-derived new peptides having the capacity of inducing prostate cancer-reactive CTLs in HLA-A2+ prostate cancer patients.* Oncol Rep, 2004. **12**(3): p. 601-7.

239. Waeckerle-Men, Y., et al., *Dendritic cell-based multi-epitope immunotherapy of hormone-refractory prostate carcinoma.* Cancer Immunol Immunother, 2006. **55**(12): p. 1524-33.

240. Fuessel, S., et al., *Vaccination of hormone-refractory prostate cancer patients with peptide cocktail-loaded dendritic cells: results of a phase I clinical trial.* Prostate, 2006. **66**(8): p. 811-21.

241. Dannull, J., et al., *Prostate stem cell antigen is a promising candidate for immunotherapy of advanced prostate cancer.* Cancer Res, 2000. **60**(19): p. 5522-8.

242. Matsueda, S., et al., *Identification of new prostate stem cell antigen-derived peptides immunogenic in HLA-A2(+) patients with hormone-refractory prostate cancer.* Cancer Immunol Immunother, 2004. **53**(6): p. 479-89.

243. Matsueda, S., et al., *A prostate stem cell antigen-derived peptide immunogenic in HLA-A24-prostate cancer patients.* Prostate, 2004. **60**(3): p. 205-13.

244. Vieweg, J., *Immunotherapy for advanced prostate cancer.* Rev Urol, 2007. **9 Suppl 1**: p. S29-38.

245. Slovin, S.F., *Targeting novel antigens for prostate cancer treatment: focus on prostate-specific membrane antigen.* Expert Opin Ther Targets, 2005. **9**(3): p. 561-70.

246. Small, E.J., et al., *Granulocyte macrophage colony-stimulating factor--secreting allogeneic cellular immunotherapy for hormone-refractory prostate cancer.* Clin Cancer Res, 2007. **13**(13): p. 3883-91.

247. Kyte, J.A. and G. Gaudernack, *Immuno-gene therapy of cancer with tumour-mRNA transfected dendritic cells.* Cancer Immunol Immunother, 2006. **55**(11): p. 1432-42.

248. Perambakam, S., et al., *Induction of specific T cell immunity in patients with prostate cancer by vaccination with PSA146-154 peptide.* Cancer Immunol Immunother, 2006. **55**(9): p. 1033-42.

249. Noguchi, M., et al., *Immunological evaluation of neoadjuvant peptide vaccination before radical prostatectomy for patients with localized prostate cancer.* Prostate, 2007. **67**(9): p. 933-42.

250. Murphy, G., et al., *Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen.* Prostate, 1996. **29**(6): p. 371-80.

251. Heiser, A., et al., *Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors.* J Clin Invest, 2002. **109**(3): p. 409-17.

252. Nair, S.K., et al., *Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells.* Nat Med, 2000. **6**(9): p. 1011-7.

253. Barrou, B., et al., *Vaccination of prostatectomized prostate cancer patients in biochemical relapse, with autologous dendritic cells pulsed with recombinant human PSA.* Cancer Immunol Immunother, 2004. **53**(5): p. 453-60.

254. Small, E.J., et al., *Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells.* J Clin Oncol, 2000. **18**(23): p. 3894-903.

255. Eder, J.P., et al., *A phase I trial of a recombinant vaccinia virus expressing prostate-specific antigen in advanced prostate cancer.* Clin Cancer Res, 2000. **6**(5): p. 1632-8.

256. Karja, V., et al., *Tumour-infiltrating lymphocytes: A prognostic factor of PSA-free survival in patients with local prostate carcinoma treated by radical prostatectomy.* Anticancer Res, 2005. **25**(6C): p. 4435-8.

257. Bronte, V., et al., *Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers.* J Exp Med, 2005. **201**(8): p. 1257-68.

258. Frost, P., et al., *Immunosensitization of resistant human tumor cells to cytotoxicity by tumor infiltrating lymphocytes.* Int J Oncol, 2003. **22**(2): p. 431-7.

259. Elsasser-Beile, U., et al., *Comparison of the activation status of tumor infiltrating and peripheral lymphocytes of patients with adenocarcinomas and benign hyperplasia of the prostate.* Prostate, 2000. **45**(1): p. 1-7.

260. Ebelt, K., et al., *Dominance of CD4+ lymphocytic infiltrates with disturbed effector cell characteristics in the tumor microenvironment of prostate carcinoma.* Prostate, 2008. **68**(1): p. 1-10.

261. Pages, F., et al., *Effector memory T cells, early metastasis, and survival in colorectal cancer.* N Engl J Med, 2005. **353**(25): p. 2654-66.

262. Ye, S.W., et al., *Ex-vivo analysis of CD8+ T cells infiltrating colorectal tumors identifies a major effector-memory subset with low perforin content.* J Clin Immunol, 2006. **26**(5): p. 447-56.

263. Correale, P., et al., *A parathyroid-hormone-related-protein (PTH-rP)-specific cytotoxic T cell response induced by in vitro stimulation of tumour-infiltrating lymphocytes derived from prostate cancer metastases, with epitope peptide-loaded autologous dendritic cells and low-dose IL-2.* Br J Cancer, 2001. **85**(11): p. 1722-30.

264. Kramer, G., et al., *Response to sublethal heat treatment of prostatic tumor cells and of prostatic tumor infiltrating T-cells.* Prostate, 2004. **58**(2): p. 109-20.

265. Hiraoka, K., et al., *Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma.* Br J Cancer, 2006. **94**(2): p. 275-80.

266. Piersma, S.J., et al., *High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer.* Cancer Res, 2007. **67**(1): p. 354-61.

267. Sharma, P., et al., *CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma.* Proc Natl Acad Sci U S A, 2007. **104**(10): p. 3967-72.

268. Vesalainen, S., et al., *Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma.* Eur J Cancer, 1994. **30A**(12): p. 1797-803.

269. Irani, J., et al., *High-grade inflammation in prostate cancer as a prognostic factor for biochemical recurrence after radical prostatectomy. Pathologist Multi Center Study Group.* Urology, 1999. **54**(3): p. 467-72.

270. Kramer, G., et al., *Increased expression of lymphocyte-derived cytokines in benign hyperplastic prostate tissue, identification of the producing cell types, and effect of differentially expressed cytokines on stromal cell proliferation.* Prostate, 2002. **52**(1): p. 43-58.

271. Curiel, T.J., et al., *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival.* Nat Med, 2004. **10**(9): p. 942-9.

272. Bates, G.J., et al., *Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse.* J Clin Oncol, 2006. **24**(34): p. 5373-80.

273. Fu, J., et al., *Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients.* Gastroenterology, 2007. **132**(7): p. 2328-39.

274. Miller, A.M., et al., *CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients.* J Immunol, 2006. **177**(10): p. 7398-405.

275. Kiniwa, Y., et al., *CD8+ Foxp3+ regulatory T cells mediate immunosuppression in prostate cancer.* Clin Cancer Res, 2007. **13**(23): p. 6947-58.

276. Yokokawa, J., et al., *Enhanced Functionality of CD4+CD25highFoxP3+ Regulatory T Cells in the Peripheral Blood of Patients with Prostate Cancer.* Clin Cancer Res, 2008. **14**(4): p. 1032-40.

277. McArdle, P.A., et al., *The relationship between T-lymphocyte subset infiltration and survival in patients with prostate cancer.* Br J Cancer, 2004. **91**(3): p. 541-3.

278. Zhou, G. and H.I. Levitsky, *Natural regulatory T cells and de novo-induced regulatory T cells contribute independently to tumor-specific tolerance.* J Immunol, 2007. **178**(4): p. 2155-62.

279. Mercader, M., et al., *T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer.* Proc Natl Acad Sci U S A, 2001. **98**(25): p. 14565-70.

280. Degl'Innocenti, E., et al., *Peripheral T-cell tolerance associated with prostate cancer is independent from CD4+CD25+ regulatory T cells.* Cancer Res, 2008. **68**(1): p. 292-300.

281. Miller, A.M. and P. Pisa, *Tumor escape mechanisms in prostate cancer.* Cancer Immunol Immunother, 2007. **56**(1): p. 81-7.

282. Spaner, D.E., *Amplifying cancer vaccine responses by modifying pathogenic gene programs in tumor cells.* J Leukoc Biol, 2004. **76**(2): p. 338-51.

283. Matthews, E., et al., *Down-regulation of TGF-beta1 production restores immunogenicity in prostate cancer cells.* Br J Cancer, 2000. **83**(4): p. 519-25.

284. Shariat, S.F., et al., *Association of pre- and postoperative plasma levels of transforming growth factor beta(1) and interleukin 6 and its soluble receptor with prostate cancer progression.* Clin Cancer Res, 2004. **10**(6): p. 1992-9.

285. Turley, R.S., et al., *The type III transforming growth factor-beta receptor as a novel tumor suppressor gene in prostate cancer.* Cancer Res, 2007. **67**(3): p. 1090-8.

286. Zhang, Q., et al., *Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive CD8+ T cells: eradication of autologous mouse prostate cancer.* Cancer Res, 2005. **65**(5): p. 1761-9.

287. Zhang, Q., et al., *Blockade of transforming growth factor-{beta} signaling in tumor-reactive CD8(+) T cells activates the antitumor immune response cycle.* Mol Cancer Ther, 2006. **5**(7): p. 1733-43.

288. Aaltoma, S.H., P.K. Lipponen, and V.M. Kosma, *Inducible nitric oxide synthase (iNOS) expression and its prognostic value in prostate cancer.* Anticancer Res, 2001. **21**(4B): p. 3101-6.

289. Uyttenhove, C., et al., *Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase.* Nat Med, 2003. **9**(10): p. 1269-74.

290. Shurin, G.V., et al., *Human prostate cancer blocks the generation of dendritic cells from CD34+ hematopoietic progenitors.* Eur Urol, 2001. **39 Suppl 4**: p. 37-40.

291. Aalamian, M., et al., *Human prostate cancer regulates generation and maturation of monocyte-derived dendritic cells.* Prostate, 2001. **46**(1): p. 68-75.

292. Tourkova, I.L., et al., *Murine prostate cancer inhibits both in vivo and in vitro generation of dendritic cells from bone marrow precursors.* Prostate, 2004. **59**(2): p. 203-13.

293. Aalamian, M., et al., *Inhibition of dendropoiesis by tumor derived and purified prostate specific antigen.* J Urol, 2003. **170**(5): p. 2026-30.

294. Mihalyo, M.A., et al., *Dendritic cells program non-immunogenic prostate-specific T cell responses beginning at early stages of prostate tumorigenesis.* Prostate, 2007. **67**(5): p. 536-46.

295. Iwai, Y., et al., *Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade.* Proc Natl Acad Sci U S A, 2002. **99**(19): p. 12293-7.

296. Dong, H., et al., *Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion.* Nat Med, 2002. **8**(8): p. 793-800.

297. Choi, I.-H., et al., *Genomic Organization and Expression Analysis of B7-H4, an Immune Inhibitory Molecule of the B7 Family.* J Immunol, 2003. **171**(9): p. 4650-4654.

298. Zang, X., et al., *B7-H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome.* Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19458-63.

299. Kryczek, I., et al., *B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma.* J Exp Med, 2006. **203**(4): p. 871-81.

300. Ahmad, M., R.C. Rees, and S.A. Ali, *Escape from immunotherapy: possible mechanisms that influence tumor regression/progression.* Cancer Immunol Immunother, 2004. **53**(10): p. 844-54.

301. Whiteside, T.L., *Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention.* Semin Cancer Biol, 2006. **16**(1): p. 3-15.

302. Restifo, N.P., et al., *Molecular mechanisms used by tumors to escape immune recognition: immunogenetherapy and the cell biology of major histocompatibility complex class I.* J Immunother Emphasis Tumor Immunol, 1993. **14**(3): p. 182-90.

303. Sanda, M.G., et al., *Molecular characterization of defective antigen processing in human prostate cancer.* J Natl Cancer Inst, 1995. **87**(4): p. 280-5.

304. Blades, R.A., et al., *Loss of HLA class I expression in prostate cancer: implications for immunotherapy.* Urology, 1995. **46**(5): p. 681-6; discussion 686-7.

305. Naoe, M., et al., *Correlation between major histocompatibility complex class I molecules and CD8+ T lymphocytes in prostate, and quantification of CD8 and interferon-gamma mRNA in prostate tissue specimens.* BJU Int, 2002. **90**(7): p. 748-53.

306. Bai, X.F., et al., *Antigenic drift as a mechanism for tumor evasion of destruction by cytolytic T lymphocytes.* J Clin Invest, 2003. **111**(10): p. 1487-96.

307. Furuya, Y., H. Fuse, and M. Masai, *Serum soluble Fas level for detection and staging of prostate cancer.* Anticancer Res, 2001. **21**(5): p. 3595-8.

308. Liu, Q.Y., et al., *Fas ligand is constitutively secreted by prostate cancer cells in vitro.* Clin Cancer Res, 1998. **4**(7): p. 1803-11.

309. Abusamra, A.J., et al., *Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis.* Blood Cells Mol Dis, 2005. **35**(2): p. 169-73.

310. Igney, F.H. and P.H. Krammer, *Tumor counterattack: fact or fiction?* Cancer Immunol Immunother, 2005. **54**(11): p. 1127-36.

311. Fox, S.B., et al., *The number of regulatory T cells in prostate cancer is associated with the androgen receptor and hypoxia-inducible factor (HIF)-2alpha but not HIF-1alpha.* Prostate, 2007. **67**(6): p. 623-9.

312. Polanczyk, M.J., et al., *Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment.* J Immunol, 2004. **173**(4): p. 2227-30.

313. Matejuk, A., et al., *Middle-age male mice have increased severity of experimental autoimmune encephalomyelitis and are unresponsive to testosterone therapy.* J Immunol, 2005. **174**(4): p. 2387-95.

314. Erdman, S.E., et al., *CD4+ CD25+ regulatory T lymphocytes inhibit microbially induced colon cancer in Rag2-deficient mice.* Am J Pathol, 2003. **162**(2): p. 691-702.

315. Goldstraw, M.A., J.M. Fitzpatrick, and R.S. Kirby, *What is the role of inflammation in the pathogenesis of prostate cancer?* BJU Int, 2007. **99**(5): p. 966-8.

316. Carter, R.E., A.R. Feldman, and J.T. Coyle, *Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase.* Proc Natl Acad Sci U S A, 1996. **93**(2): p. 749-53.

317. Luthi-Carter, R., et al., *Molecular characterization of human brain N-acetylated alpha-linked acidic dipeptidase (NAALADase).* J Pharmacol Exp Ther, 1998. **286**(2): p. 1020-5.

318. Luthi-Carter, R., et al., *Hydrolysis of the neuropeptide N-acetylaspartylglutamate (NAAG) by cloned human glutamate carboxypeptidase II.* Brain Res, 1998. **795**(1-2): p. 341-8.

319. Pinto, J.T., et al., *Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells.* Clin Cancer Res, 1996. **2**(9): p. 1445-51.

320. Coyle, J.T., *The nagging question of the function of N-acetylaspartylglutamate.* Neurobiol Dis, 1997. **4**(3-4): p. 231-8.

321. Tsai, G., et al., *Early embryonic death of glutamate carboxypeptidase II (NAALADase) homozygous mutants.* Synapse, 2003. **50**(4): p. 285-92.

322. Bacich, D.J., et al., *Deletion of the glutamate carboxypeptidase II gene in mice reveals a second enzyme activity that hydrolyzes N-acetylaspartylglutamate.* J Neurochem, 2002. **83**(1): p. 20-9.

323. Devlin, A.M., et al., *Glutamate carboxypeptidase II: a polymorphism associated with lower levels of serum folate and hyperhomocysteinemia.* Hum Mol Genet, 2000. **9**(19): p. 2837-44.

324. Halsted, C.H., *The intestinal absorption of dietary folates in health and disease.* J Am Coll Nutr, 1989. **8**(6): p. 650-8.

325. Halsted, C.H., et al., *Folylpoly-gamma-glutamate carboxypeptidase from pig jejunum. Molecular characterization and relation to glutamate carboxypeptidase II.* J Biol Chem, 1998. **273**(32): p. 20417-24.

326. Israeli, R.S., et al., *Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen.* Cancer Res, 1993. **53**(2): p. 227-30.

327. Letourneur, F. and R.D. Klausner, *A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains.* Cell, 1992. **69**(7): p. 1143-57.

328. Rajasekaran, S.A., et al., *A novel cytoplasmic tail MXXXL motif mediates the internalization of prostate-specific membrane antigen.* Mol Biol Cell, 2003. **14**(12): p. 4835-45.

329. Liu, H., et al., *Constitutive and antibody-induced internalization of prostate-specific membrane antigen.* Cancer Res, 1998. **58**(18): p. 4055-60.

330. Rajasekaran, A.K., G. Anilkumar, and J.J. Christiansen, *Is prostate-specific membrane antigen a multifunctional protein?* Am J Physiol Cell Physiol, 2005. **288**(5): p. C975-81.

331. Lapidus, R.G., et al., *Prostate-specific membrane antigen (PSMA) enzyme activity is elevated in prostate cancer cells.* Prostate, 2000. **45**(4): p. 350-4.

332. Anilkumar, G., et al., *Association of prostate-specific membrane antigen with caveolin-1 and its caveolae-dependent internalization in microvascular endothelial cells: implications for targeting to tumor vasculature.* Microvasc Res, 2006. **72**(1-2): p. 54-61.

333. Israeli, R.S., et al., *Expression of the prostate-specific membrane antigen.* Cancer Res, 1994. **54**(7): p. 1807-11.

334. O'Keefe, D.S., et al., *Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene.* Biochim Biophys Acta, 1998. **1443**(1-2): p. 113-27.

335. Horoszewicz, J.S., E. Kawinski, and G.P. Murphy, *Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients.* Anticancer Res, 1987. **7**(5B): p. 927-35.

336. Bacich, D.J., et al., *Cloning, expression, genomic localization, and enzymatic activities of the mouse homolog of prostate-specific membrane antigen/NAALADase/folate hydrolase.* Mamm Genome, 2001. **12**(2): p. 117-23.

337. Aggarwal, S., et al., *Comparative study of PSMA expression in the prostate of mouse, dog, monkey, and human.* Prostate, 2006. **66**(9): p. 903-10.

338. Davis, M.I., et al., *Crystal structure of prostate-specific membrane antigen, a tumor marker and peptidase.* Proc Natl Acad Sci U S A, 2005. **102**(17): p. 5981-6.

339. Schulke, N., et al., *The homodimer of prostate-specific membrane antigen is a functional target for cancer therapy.* Proc Natl Acad Sci U S A, 2003. **100**(22): p. 12590-5.

340. Mesters, J.R., et al., *Structure of glutamate carboxypeptidase II, a drug target in neuronal damage and prostate cancer.* EMBO J, 2006. **25**(6): p. 1375-84.

341. Xiao, Z., et al., *Generation of a baculovirus recombinant prostate-specific membrane antigen and its use in the development of a novel protein biochip quantitative immunoassay.* Protein Expr Purif, 2000. **19**(1): p. 12-21.

342. Christiansen, J.J., et al., *N-glycosylation and microtubule integrity are involved in apical targeting of prostate-specific membrane antigen: implications for immunotherapy.* Mol Cancer Ther, 2005. **4**(5): p. 704-14.

343. Barinka, C., et al., *Identification of the N-glycosylation sites on glutamate carboxypeptidase II necessary for proteolytic activity.* Protein Sci, 2004. **13**(6): p. 1627-35.

344. Grauer, L.S., et al., *Identification, purification, and subcellular localization of prostate-specific membrane antigen PSM' protein in the LNCaP prostatic carcinoma cell line.* Cancer Res, 1998. **58**(21): p. 4787-9.

345. Schmittgen, T.D., et al., *Expression of prostate specific membrane antigen and three alternatively spliced variants of PSMA in prostate cancer patients.* Int J Cancer, 2003. **107**(2): p. 323-9.

346. Su, S.L., et al., *Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression.* Cancer Res, 1995. **55**(7): p. 1441-3.

347. Cao, K.Y., et al., *High expression of PSM-E correlated with tumor grade in prostate cancer: a new alternatively spliced variant of prostate-specific membrane antigen.* Prostate, 2007. **67**(16): p. 1791-800.

348. O'Keefe, D.S., D.J. Bacich, and W.D. Heston, *Comparative analysis of prostate-specific membrane antigen (PSMA) versus a prostate-specific membrane antigen-like gene.* Prostate, 2004. **58**(2): p. 200-10.

349. Troyer, J.K., M.L. Beckett, and G.L. Wright, Jr., *Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids.* Int J Cancer, 1995. **62**(5): p. 552-8.

350. Silver, D.A., et al., *Prostate-specific membrane antigen expression in normal and malignant human tissues.* Clin Cancer Res, 1997. **3**(1): p. 81-5.

351. Mhawech-Fauceglia, P., et al., *Prostate-specific membrane antigen (PSMA) protein expression in normal and neoplastic tissues and its sensitivity and specificity in prostate adenocarcinoma: an immunohistochemical study using mutiple tumour tissue microarray technique.* Histopathology, 2007. **50**(4): p. 472-83.

352. Kinoshita, Y., et al., *Expression of prostate-specific membrane antigen in normal and malignant human tissues.* World J Surg, 2006. **30**(4): p. 628-36.

353. Sokoloff, R.L., et al., *A dual-monoclonal sandwich assay for prostate-specific membrane antigen: levels in tissues, seminal fluid and urine.* Prostate, 2000. **43**(2): p. 150-7.

354. Sacha, P., et al., *Expression of glutamate carboxypeptidase II in human brain.* Neuroscience, 2007. **144**(4): p. 1361-72.

355. Bostwick, D.G., et al., *Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 184 cases.* Cancer, 1998. **82**(11): p. 2256-61.

356. Landers, K.A., et al., *Use of multiple biomarkers for a molecular diagnosis of prostate cancer.* Int J Cancer, 2005. **114**(6): p. 950-6.

357. Heston, W.D., *Characterization and glutamyl preferring carboxypeptidase function of prostate specific membrane antigen: a novel folate hydrolase.* Urology, 1997. **49**(3A Suppl): p. 104-12.

358. Kawakami, M. and J. Nakayama, *Enhanced expression of prostate-specific membrane antigen gene in prostate cancer as revealed by in situ hybridization.* Cancer Res, 1997. **57**(12): p. 2321-4.

359. Sweat, S.D., et al., *Prostate-specific membrane antigen expression is greatest in prostate adenocarcinoma and lymph node metastases.* Urology, 1998. **52**(4): p. 637-40.

360. Perner, S., et al., *Prostate-specific membrane antigen expression as a predictor of prostate cancer progression.* Hum Pathol, 2007. **38**(5): p. 696-701.

361. Ghosh, A. and W.D. Heston, *Tumor target prostate specific membrane antigen (PSMA) and its regulation in prostate cancer.* J Cell Biochem, 2004. **91**(3): p. 528-39.

362. Chang, S.S., et al., *Prostate-specific membrane antigen is produced in tumor-associated neovasculature.* Clin Cancer Res, 1999. **5**(10): p. 2674-81.

363. Chang, S.S., et al., *Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature.* Cancer Res, 1999. **59**(13): p. 3192-8.

364. Liu, H., et al., *Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium.* Cancer Res, 1997. **57**(17): p. 3629-34.

365. Baccala, A., et al., *Expression of prostate-specific membrane antigen in tumor-associated neovasculature of renal neoplasms.* Urology, 2007. **70**(2): p. 385-90.

366. Blount, B.C., et al., *Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 3290-5.

367. Jacob, R.A., et al., *Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women.* J Nutr, 1998. **128**(7): p. 1204-12.

368. Courtemanche, C., et al., *Folate deficiency inhibits the proliferation of primary human CD8+ T lymphocytes in vitro.* J Immunol, 2004. **173**(5): p. 3186-92.

369. Conway, R.E., et al., *Prostate-specific membrane antigen regulates angiogenesis by modulating integrin signal transduction.* Mol Cell Biol, 2006. **26**(14): p. 5310-24.

370. Wright, G.L., Jr., et al., *Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy.* Urology, 1996. **48**(2): p. 326-34.

371. Kumaraguru, U., et al., *Concomitant Helper Response Rescues Otherwise Low Avidity CD8+ Memory CTLs to Become Efficient Effectors In Vivo.* J Immunol, 2004. **172**(6): p. 3719-3724.

372. Medin, J.A., et al., *Efficient transfer of PSA and PSMA cDNAs into DCs generates antibody and T cell antitumor responses in vivo.* Cancer Gene Ther, 2005. **12**(6): p. 540-51.

373. Tjoa, B.A., et al., *Follow-up evaluation of a phase II prostate cancer vaccine trial.* Prostate, 1999. **40**(2): p. 125-9.

374. Tjoa, B.A., et al., *Evaluation of phase I/II clinical trials in prostate cancer with dendritic cells and PSMA peptides.* Prostate, 1998. **36**(1): p. 39-44.

375. Tuettenberg, A., et al., *Induction of strong and persistent MelanA/MART-1-specific immune responses by adjuvant dendritic cell-based vaccination of stage II melanoma patients.* Int J Cancer, 2006. **118**(10): p. 2617-27.

376. Brossart, P., et al., *Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells.* Blood, 2000. **96**(9): p. 3102-8.

377. Schroers, R., et al., *Identification of MHC class II-restricted T-cell epitopes in prostate-specific membrane antigen.* Clin Cancer Res, 2003. **9**(9): p. 3260-71.

378. Kobayashi, H., et al., *Identification of naturally processed helper T-cell epitopes from prostate-specific membrane antigen using peptide-based in vitro stimulation.* Clin Cancer Res, 2003. **9**(14): p. 5386-93.

379. Kobayashi, K., et al., *Identification of a prostate-specific membrane antigen-derived peptide capable of eliciting both cellular and humoral immune responses in HLA-A24+ prostate cancer patients.* Cancer Sci, 2003. **94**(7): p. 622-7.

380. Horiguchi, Y., et al., *Screening of HLA-A24-restricted epitope peptides from prostate-specific membrane antigen that induce specific antitumor cytotoxic T lymphocytes.* Clin Cancer Res, 2002. **8**(12): p. 3885-92.

381. Sato, Y., et al., *Immunological evaluation of peptide vaccination for patients with gastric cancer based on pre-existing cellular response to peptide.* Cancer Sci, 2003. **94**(9): p. 802-8.

382. Mine, T., et al., *Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients.* Cancer Sci, 2003. **94**(6): p. 548-56.

383. Mine, T., et al., *Humoral responses to peptides correlate with overall survival in advanced cancer patients vaccinated with peptides based on pre-existing, peptide-specific cellular responses.* Clin Cancer Res, 2004. **10**(3): p. 929-37.

384. Rammensee, H.G., *Chemistry of peptides associated with MHC class I and class II molecules.* Curr Opin Immunol, 1995. **7**(1): p. 85-96.

385. Rammensee, H., et al., *SYFPEITHI: database for MHC ligands and peptide motifs.* Immunogenetics, 1999. **50**(3-4): p. 213-9.

386. Parker, K.C., M.A. Bednarek, and J.E. Coligan, *Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains.* J Immunol, 1994. **152**(1): p. 163-175.

387. Uebel, S. and R. Tampe, *Specificity of the proteasome and the TAP transporter.* Curr Opin Immunol, 1999. **11**(2): p. 203-8.

388. Falk, K., O. Rotzschke, and H.G. Rammensee, *Specificity of antigen processing for MHC class I restricted presentation is conserved between mouse and man.* Eur J Immunol, 1992. **22**(5): p. 1323-6.

389. Shirai, M., et al., *CTL responses of HLA-A2.1-transgenic mice specific for hepatitis C viral peptides predict epitopes for CTL of humans carrying HLA-A2.1.* J Immunol, 1995. **154**(6): p. 2733-42.

390. Firat, H., et al., *Comparative analysis of the CD8(+) T cell repertoires of H-2 class I wild-type/HLA-A2.1 and H-2 class I knockout/HLA-A2.1 transgenic mice.* Int Immunol, 2002. **14**(8): p. 925-34.

391. Pascolo, S., et al., *HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice.* J Exp Med, 1997. **185**(12): p. 2043-51.

392. Firat, H., et al., *H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies.* Eur J Immunol, 1999. **29**(10): p. 3112-21.

393. Yee, C., et al., *Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers.* J Immunol, 1999. **162**(4): p. 2227-34.

394. Bohnenkamp, H.R., et al., *Breast carcinoma cell lysate-pulsed dendritic cells cross-prime MUC1-specific CD8+ T cells identified by peptide-MHC-class-I tetramers.* Cell Immunol, 2004. **231**(1-2): p. 112-25.

395. Kowalczyk, D.W., et al., *A method that allows easy characterization of tumor-infiltrating lymphocytes.* J Immunol Methods, 2001. **253**(1-2): p. 163-75.

396. Liu, J., et al., *Modulation of DNA vaccine-elicited CD8+ T-lymphocyte epitope immunodominance hierarchies.* J Virol, 2006. **80**(24): p. 11991-7.

397. Nakamura, Y., et al., *Helper function of memory CD8+ T cells: heterologous CD8+ T cells support the induction of therapeutic cancer immunity.* Cancer Res, 2007. **67**(20): p. 10012-8.

398. Boyle, J.S., C. Koniaras, and A.M. Lew, *Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when*

antigen is cytoplasmic after intramuscular DNA immunization. Int Immunol, 1997. **9**(12): p. 1897-906.

399. Rush, C., T. Mitchell, and P. Garside, *Efficient priming of CD4+ and CD8+ T cells by DNA vaccination depends on appropriate targeting of sufficient levels of immunologically relevant antigen to appropriate processing pathways.* J Immunol, 2002. **169**(9): p. 4951-60.

400. Machlenkin, A., et al., *Human CTL epitopes prostatic acid phosphatase-3 and six-transmembrane epithelial antigen of prostate-3 as candidates for prostate cancer immunotherapy.* Cancer Res, 2005. **65**(14): p. 6435-42.

401. Palmowski, M.J., et al., *Competition between CTL narrows the immune response induced by prime-boost vaccination protocols.* J Immunol, 2002. **168**(9): p. 4391-8.

402. Kedl, R.M., et al., *T cells down-modulate peptide-MHC complexes on APCs in vivo.* Nat Immunol, 2002. **3**(1): p. 27-32.

403. Marzo, A.L., et al., *Tumor-specific CD4+ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity.* J Immunol, 2000. **165**(11): p. 6047-55.

404. Hwang, M.L., J.R. Lukens, and T.N. Bullock, *Cognate memory CD4+ T cells generated with dendritic cell priming influence the expansion, trafficking, and differentiation of secondary CD8+ T cells and enhance tumor control.* J Immunol, 2007. **179**(9): p. 5829-38.

405. Kinoshita, Y., et al., *Targeting epitopes in prostate-specific membrane antigen for antibody therapy of prostate cancer.* Prostate Cancer Prostatic Dis, 2005. **8**(4): p. 359-63.

406. Smith-Jones, P.M., et al., *Radiolabeled monoclonal antibodies specific to the extracellular domain of prostate-specific membrane antigen: preclinical studies in nude mice bearing LNCaP human prostate tumor.* J Nucl Med, 2003. **44**(4): p. 610-7.

407. Bander, N.H., et al., *Targeting metastatic prostate cancer with radiolabeled monoclonal antibody J591 to the extracellular domain of prostate specific membrane antigen.* J Urol, 2003. **170**(5): p. 1717-21.

408. Milowsky, M.I., et al., *Phase I trial of yttrium-90-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for androgen-independent prostate cancer.* J Clin Oncol, 2004. **22**(13): p. 2522-31.

409. Milowsky, M.I., et al., *Vascular targeted therapy with anti-prostate-specific membrane antigen monoclonal antibody J591 in advanced solid tumors.* J Clin Oncol, 2007. **25**(5): p. 540-7.

410. Bander, N.H., et al., *Phase I trial of 177lutetium-labeled J591, a monoclonal antibody to prostate-specific membrane antigen, in patients with androgen-independent prostate cancer.* J Clin Oncol, 2005. **23**(21): p. 4591-601.

411. Morris, M.J., et al., *Pilot trial of unlabeled and indium-111-labeled anti-prostate-specific membrane antigen antibody J591 for castrate metastatic prostate cancer.* Clin Cancer Res, 2005. **11**(20): p. 7454-61.

412. Wolf, P., et al., *A recombinant PSMA-specific single-chain immunotoxin has potent and selective toxicity against prostate cancer cells.* Cancer Immunol Immunother, 2006. **55**(11): p. 1367-73.

413. Henry, M.D., et al., *A prostate-specific membrane antigen-targeted monoclonal antibody-chemotherapeutic conjugate designed for the treatment of prostate cancer.* Cancer Res, 2004. **64**(21): p. 7995-8001.

414. Ma, D., et al., *Potent antitumor activity of an auristatin-conjugated, fully human monoclonal antibody to prostate-specific membrane antigen.* Clin Cancer Res, 2006. **12**(8): p. 2591-6.

415. Kuratsukuri, K., et al., *Inhibition of prostate-specific membrane antigen (PSMA)-positive tumor growth by vaccination with either full-length or the C-terminal end of PSMA.* Int J Cancer, 2002. **102**(3): p. 244-9.

416. Kuratsukuri, K., et al., *Induction of antibodies against prostate-specific membrane antigen (PSMA) by vaccination with a PSMA DNA vector.* Eur Urol, 2002. **42**(1): p. 67-73.

417. Todorova, K., et al., *Humoral immune response in prostate cancer patients after immunization with gene-based vaccines that encode for a protein that is proteasomally degraded.* Cancer Immun, 2005. **5**: p. 1.

418. Gold, J.S., et al., *A single heteroclitic epitope determines cancer immunity after xenogeneic DNA immunization against a tumor differentiation antigen.* J Immunol, 2003. **170**(10): p. 5188-94.

419. Gregor, P.D., et al., *Induction of autoantibodies to syngeneic prostate-specific membrane antigen by xenogeneic vaccination.* Int J Cancer, 2005. **116**(3): p. 415-21.

420. Parekh, R.B., et al., *Tissue-specific N-glycosylation, site-specific oligosaccharide patterns and lentil lectin recognition of rat Thy-1.* EMBO J, 1987. **6**(5): p. 1233-44.

421. Cloosen, S., et al., *Cancer specific Mucin-1 glycoforms are expressed on multiple myeloma.* British Journal of Haematology, 2006. **135**(4): p. 513-516.

422. Durso, R.J., et al., *A novel alphavirus vaccine encoding prostate-specific membrane antigen elicits potent cellular and humoral immune responses.* Clin Cancer Res, 2007. **13**(13): p. 3999-4008.

423. Gregor, P.D., et al., *CTLA-4 blockade in combination with xenogeneic DNA vaccines enhances T-cell responses, tumor immunity and autoimmunity to self antigens in animal and cellular model systems.* Vaccine, 2004. **22**(13-14): p. 1700-8.