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DNA FUSION VACCINES AGAINST HPV16 E7 ANTIGEN-ASSOCIATED CANCERS

By

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ABSTRACT

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To date, the success of cancer vaccines in human clinical trials has been limited. One of the reasons for this is the immunological tolerance to tumour antigens found in cancer patients. A novel DNA fusion vaccine design which links a pathogen-derived domain (DOM) of fragment C from tetanus toxin to a peptide epitope from a tumour antigen has been developed in our laboratory. The microbial sequence is able to activate a non-tolerised pool of helper T cells, providing T-cell help for immune induction against the linked tumour-specific sequence.

The main aim of this project was to produce a therapeutic DNA vaccine against human papillomavirus (HPV)-associated cancers. A number of DNA fusion vaccines against the E7 antigen from HPV16 were constructed, including pDOM.$E7_{49\text{-}57,}$ which encodes a well described H-$2D^b$-binding epitope from E7 fused to the DOM sequence. $CD8^+$ T-cell responses to the vaccines were demonstrated using flow cytometry and functional assays. Importantly, these responses were stronger than those induced by a published synthetic long peptide strategy. *In vivo* tumour challenge experiments showed that DNA vaccines had a protective and therapeutic effect. The vaccines were then tested in transgenic mice which develop spontaneous E7-expressing tumours in a setting of tolerance. DNA vaccine-mediated E7-specific $CD8^+$ T-cell responses were successfully induced in these mice, together with a reduction in the mass of spontaneous tumours. This is the first demonstration of pDOM-epitope DNA vaccine-mediated therapy for spontaneous tumours and bodes well for translation into the clinic.

One limiting factor for DNA vaccination in humans may be the delivery system. Electroporation (EP) is one approach which may overcome this. Therefore, a secondary aim of this project was to investigate the impact of EP on immune responses to DNA vaccination in more detail. EP proved essential for generating T-cell and antibody responses to the pDOM.$E7_{49\text{-}57}$ vaccine in sub-optimal conditions. This information will be crucial for the planning of therapeutic vaccination protocols in patients.

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Declaration of Authorship

I, ……., declare that the thesis entitled:

'DNA Fusion Vaccines Against HPV16 E7 Antigen-associated Cancers'

and the work in the thesis are my own, and have been generated by me as result of my own original research.

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

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Abbreviations

AID	Activation-induced cytidine deaminase
AIDS	Acquired immunodeficiency syndrome
AIM	Absent in myeloma
APC	Antigen-presenting cell
APC	Allophycocyanin
ASC	Apoptosis-related speck
B cell	B lymphocyte
BCR	B-cell receptor
BM	Bone marrow
bp	Base pair(s)
C	Constant (region of Ig loci)
CAM	Cell adhesion molecule
CAT	chloramphenicol acetyl transferase
CD	Cluster designation
CIN	Cervical intraepithelial neoplasia
CLIP	Class II-associated invariant peptide
CO_2	Carbon dioxide
CpG	Cytosine-phosphate-guanine
CSR	Class-switch recombination
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic lymphocyte antigen
d	Day
D	Diversity (region of Ig loci)
DAI	DNA-dependent activator of interferon-regulatory factors
DAMP	Damage-associated molecular patterns
DAPI	4,6-diamidino-2-phenolindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DOM1	N-terminal domain of tetanus toxin
DOM2	C-terminal domain of tetanus toxin
ds	Double-stranded
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot

EP	Electroporation
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FOXP3	Forkhead box P3
FrC	Fragment C of tetanus toxin
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
H & E	Haematoxylin and eosin
HEK	Human embryonic kidney
HER-2	Human epidermal growth factor receptor 2
HEV	High endothelial venuole
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMGB	High-mobility group box
HPV	Human papilloma virus
hr	Hour(s)
HSIL	High-grade squamous intraepithelial lesion
i.m.	Intramuscular
i.p.	Intraperitoneal
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
IRF	Interferon regulatory factor
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
IVTT	*In vitro* transcription and translation
J	Joining (region of Ig loci)
KIR	Killer cell immunoglobulin-like receptor
LB	Luria broth
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LSIL	Low-grade squamous intraepithelial lesion

MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minutes
MVA	Modified vaccinia virus Ankara
n/s	Not statistically significant
NF-κB	Nuclear Factor-κB
NHS	National Health Service
NK	Natural killer cell
NLR	NOD-like or nucleotide-binding leucine-rich repeat containing receptor
OD	Optical density
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PRR	Pattern recognition receptor
RAG	Recombinase-activating genes
Rb	Retinoblastoma protein
RIG	Retinoic-inducible gene
RIP	Rat insulin promoter
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RSS	Recombination signal sequences
s.c	Sub-cutaneous
SDS	sodium dodecyl sulphate
SEREX	Serological identification of antigens by recombinant expression cloning
SFC	Spot-forming cells
SHM	Somatic hypermutation
SIV	Simian immunodeficiency virus
SOE	Splicing by overlapping extension
STING	Stimulator of interferon genes
T cell	T lymphocyte
T reg	Regulatory T cell
TAP	Transporter associated with antigen presentation

TCR	T-cell receptor
TEC	Thymic epithelial cell
Tg	Transgenic
TGF	Transforming growth factor
TIFF	Tagged image file format
TIL	Tumour-infiltrating lymphocyte
TIR	Toll/IL-1R homology domains
TLR	Toll-like receptor
TRA	Tissue-restricted antigens
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing IFNβ
TT	Tetanus toxin
UK	United Kingdom
US	United States
V	Variable (region of Ig loci)
WT	Wild-type

1. Introduction

1.1. The Immune System

The vertebrate immune system has evolved to protect the host against invading pathogens. It consists of multiple mechanisms which work in concert, and can be divided into two arms: innate and adaptive immunity. Innate immunity provides a rapid response based on the recognition of stereotyped pathogen-derived molecules. This initial response is essential as the induction of adaptive immunity is slower (5-10 days). However, the adaptive immune system is able to respond to a wider variety of antigens in a more specific manner. Furthermore, the induction of adaptive immunity also leads to the formation of immunological memory which provides more rapid responses upon secondary exposure. The principal components of each arm of the immune system are described below.

1.1.1. Innate Immunity

Innate immunity constitutes the first line of defence in the immune response, providing a rapid but relatively non-specific response against pathogens. The first component is the physical barrier provided by epithelium on all body surfaces. When this is breached, soluble factors such as complement and effector cells, for example natural killer cells, are induced to eliminate invading microorganisms. The first step in the induction of immunity is the recognition of danger. The innate immune system recognises pathogen-derived molecular components via a limited range of receptors. The recognition of these signals and how they induce innate immunity is described below.

1.1.1.1. Danger-sensing Mechanisms

The innate receptors that sense invading pathogens are collectively known as pattern recognition receptors (PRRs). Their ligands are specific pathogen-associated molecular patterns (PAMPs). These are conserved molecular structures such as lipopolysaccharide (LPS), which are present only in microorganisms or nucleic acids found in unusual cellular locations due to infection or cell damage. Additional damage-associated molecular patterns (DAMPs) provide further signals which alert the innate immune system to cellular stress or damage, ensuring that an appropriate response is mounted. For example, changes in the phospholipids of the cell membrane or glycoproteins on the cell surface of a dying cell can induce its phagocytosis by monocytes [1].

One group of important PRRs are the Toll-like receptors (TLRs). These constitute a group of highly conserved membrane-bound glycoproteins, containing leucine-rich repeats (LRRs) in their extracellular domains and a Toll/IL-1R homology (TIR) cytoplasmic signalling domain [2]. There are 12 TLRs found in mammals, expressed primarily in immune cells such as dendritic cells (DCs), macrophages and B cells, but also in fibroblasts and epithelial cells. TLRs 1, 2 and 6 recognise lipids and are expressed on the cell surface, as is TLR-4 which recognises LPS amongst other things. In contrast TLRs 3, 7, 8 and 9, which recognise nucleic acids, are expressed intracellularly on the membranes of the endosomal compartments. The ways in which nucleic acids are sensed by these and other receptors will be discussed in detail in the context of DNA vaccines in section 1.2.4.2.

After ligand binding, TLRs signal via adaptor molecules which bind the TIR domain and then initiate downstream signalling cascades, ultimately inducing changes in gene expression. MyD88 is a crucial adaptor molecule which directs a signalling pathway that leads to the activation of transcription factors such as nuclear factor (NF)-κB, resulting in the production of proinflammatory cytokines. Signalling through some TLRs, namely 3, 4, 7 and 9, also results in production of type I interferon (IFN). Furthermore, TLR-3 and -4 can direct type I IFN production through a MyD88 independent mechanism via TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) [2].

NOD-like receptors (NLRs; also known as nucleotide-binding leucine-rich repeat containing receptors) are soluble intracellular proteins expressed by macrophages and DCs which sense microorganisms and danger signals. Structurally, NLRs are composed of three domains; (i) a c-terminal ligand-sensing domain containing LRRs, (ii) an oligomerisation domain and (iii) an effector domain at the N-terminus. The central oligomerisation domain is responsible for the formation of NOD signalosomes which are created when NLRs such as NOD1 and NOD2 dimerise and recruit the kinase RIP2, leading to downstream signalling and activation of NF-κB. Another molecular complex formed by multiple NLRs is the inflammasome [3]. The inflammasome is so-called because of its ability to activate inflammatory caspases. *In vitro* studies have shown that inflammasomes assemble in response to disruption of cellular integrity, leading to caspase-1 induction which in turn cleaves the proinflammatory cytokine interleukin (IL)-1β into its active form [4].

1.1.1.2. Cellular Mechanisms

Once PAMPs and DAMPs have been sensed by the cells of the innate immune system via the mechanisms described above, suitable responses are induced. As already mentioned, the first step in this process is a change in gene expression which leads to the production of inflammatory cytokines. These polypeptides are crucial to the regulation of the immune response. Chemokines are an important group of cytokines which control the migration of immune cells into sites of infection. Once activated and in the inflammatory site, the cells of the innate immune system can begin to instigate their effector functions, detailed below.

Neutrophils are the most abundant of all immune cells circulating in the blood. They produce granules containing enzymes such as lysozyme and collagenase, and microbicidal molecules such as defensins and cathelicidins. After recruitment to a site of inflammation, neutrophils attack invading pathogens for just a few hours before they undergo apoptosis.

Mononuclear phagocytes circulate in the blood as monocytes and on recruitment to a site of inflammation, mature into macrophages and continue to divide. These cells have a phagocytic function which allows them to ingest and destroy pathogens.

DCs are related to mononuclear phagocytes and like them, are also phagocytic. DCs express PRRs which detect pathogens and produce cytokines in response to them. There are many subsets of DCs, divided according to location, surface molecule expression and function. The major divide in both mouse and human is between plasmacytoid and myeloid (conventional) DCs. Plasmacytoid DCs are major producers of type I IFN [5]. Conventional DCs can be separated into several subsets, responsible for further cytokine production but also the priming of T cells. Therefore, DCs are not only crucial in innate immune response, but also provide a link to the adaptive immune system by activating T cells (discussed in detail in section 1.1.2).

Natural Killer (NK) cells are able to kill infected or stressed cells without the need for prior activation, unlike the cytotoxic T lymphocytes (CTLs) of the adaptive immune system. This phenomenon is tightly controlled to prevent autoimmunity by signalling via the activating and inhibitory receptors that they express. There are many activating receptors expressed by each NK cell, including natural cytotoxicity receptors, C-type lectin-like receptors and killer cell immunoglobulin-like receptors (KIRs). One key receptor is NKG2D, a C-type lectin-like receptor which recognises major histocompatibilty (MHC) class I-like stress-induced self ligands such as MICA and MICB in humans [6] and H-60 and Rae-Iβ in mice [7]. However, just as important are the inhibitory receptors expressed by these cells. Again, receptors of KIR and C-type lectin-like receptor families

predominate. MHC class I is a key ligand recognised by several of these receptors. As almost all healthy cells express MHC I on the cell surface, its presence provides a very useful indication that NK activation is not required. In contrast, cells which have been infected with virus or bacteria often down-regulate or lose MHC I expression. This is of particular importance in tumour immunology as many solid tumours reduce surface MHC I expression in order to escape CTL attack [8], potentially rendering them susceptible to NK cytotoxity, as shown *in vitro* [9] and in experiments in mice [10].

1.1.1.3. Soluble Factors

As well as the cells of the innate immune system, soluble factors also contribute to the first line of defence against pathogens. These include the plasma proteins of the complement system which recognise immunoglobin (Ig) bound to the surface of pathogens via the classical pathway, microbial surface structures via the alternative pathway or mannose residues on microbial glycoproteins via the lectin pathway. Once identified, pathogens can then be phagocytosed.

1.1.2. Adaptive Immunity

Adaptive immunity is mediated by B and T lymphocytes. Although the effector functions of these two lineages are quite distinct, there are similarities. Both cell types express hypervariable antigen receptors which allow them to respond to an almost infinite number of pathogens, ensuring that the body is well protected. B cells produce antibodies which bind to the surface proteins on microorganisms, targeting them for destruction. T cells recognise short peptides which result from intracellular processing in antigen-presenting cells (APCs). These peptides are then displayed on MHC molecules on the surface of the APC. Once activated, T cells secrete cytokines and gain cytotoxic function. Importantly, T cells are also able to help the antibody response. B and T cells are discussed in more detail below.

1.1.2.1. B-cell Development

The development of B cells from stem cells in the bone marrow (BM) into mature B cells capable of responding to antigen and producing Ig is a multi-step process. The first phase of this is antigen independent and takes place in the BM. The second phase involves

antigen encounter in the secondary lymphoid organs. The general scheme of B-cell development is shown in Figure 1.1.

B cells recognise antigen via the B-cell receptor (BCR), which consists of surface-expressed Ig and CD79 (Ig-α and Ig-β). The structure of Ig is shown in Figure 1.2. The germline Ig gene loci consist of many variable (V), diversity (D), joining (J) or constant (C) gene segments. In order to produce the mature Ig heavy chain polypeptides one of each of the V and D gene must be spliced together with a J gene located upstream of the Cμ gene. The light chain contains only V, J and C regions therefore recombination involves V to J only. An example of the joining of these segments during V(D)J recombination is depicted in Figure 1.3. Recombination signal sequences (RSSs) flanking each gene segment are cleaved by recombinase-activating genes (RAG-1 and -2), cutting one DNA strand, resulting in a hairpin of coding DNA and a double-stranded break at the RSS. At this point, additional diversity is produced by the loss and addition of extra nucleotides (N and P additions). The cut gene segments are joined by double-stranded break DNA repair enzymes and VDJ segments are then transcribed along with all constant regions, resulting in the mature gene product. Translation ceases at the stop codon after the Cμ but this segment can be removed from the transcript by RNA splicing to allow IgD production in naïve B cells. Mature naïve B cells expressing rearranged BCRs then exit the BM, enter the blood and migrate to the peripheral lymph nodes.

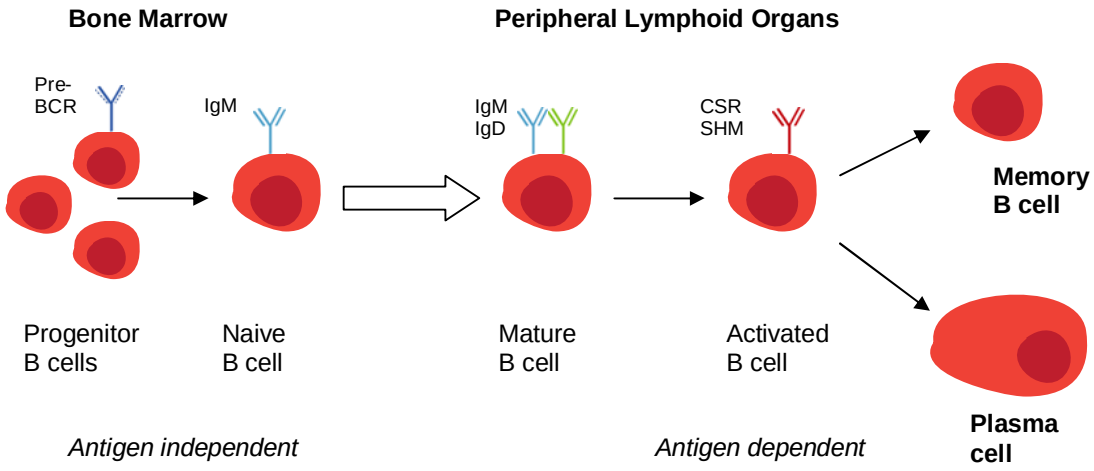


Figure 1.1. B-cell development

Naïve B cells originate in the bone marrow. Here, genetic rearrangement of the immunoglobulin (Ig) genes takes place and the surrogate light chain (dashed line) found in the pre-BCR is replaced with the mature light chain (solid line) to make IgM and IgD (shown in blue and green). Naïve B-cells become activated after antigen encounter in the periphery. Activated B cells migrate to the germinal centre where they undergo class-switch recombination (CSR) and somatic hypermutation (SHM). They then mature further into plasma cells which secrete Ig or memory cells which are able to mount a rapid response upon antigen re-encounter.

Ligation and cross-linking (when antigen binds to multiple Ig) of the BCR initiates a series of intracellular signalling cascades via immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD79 co-receptor, activating the B cell. This causes clonal expansion which produces a clone of identical daughter cells expressing the same BCR. During proliferation of a B-cell clone, affinity maturation also takes place, whereby the B cells which produce Ig with the highest affinity for the antigen are selected. This is achieved by somatic hypermutation (SHM) of the variable Ig genes which are responsible for antigen binding. One of the mediators of SHM is activation-induced cytidine deaminase (AID). These processes usually take place in germinal centres, specialised tissues found in the lymph nodes during antigen exposure. To allow production of IgG, E and A, class-switch recombination (CSR) occurs. To achieve this, the upstream C gene segments are removed at the DNA level. Activated B cells then differentiate further in the germinal centres, either into antibody-secreting plasma cells or memory B cells.

T-cell help is of critical importance to most antibody responses. So-called thymus-dependent antigens, which are proteins, only fully activate a B cell in the presence of costimulation by $CD4^+$ T cells. Helper T cells also secrete cytokines which induce CSR and aid affinity maturation by supporting germinal centre formation. In contrast, thymus-independent antigens can induce an antibody response in the absence of T-cell help. Antigens recognised by this pathway are multivalent repetitive molecules able to cross-link multiple BCRs such as bacterially derived LPS. In this way, the immune system has developed a means of mounting a rapid response against pathogens which can start prior to the induction of cognate T-cell help.

1.1.2.2. B-cell Effector Function

The five different classes of Ig have different functions; for example, IgG is the major Ig responsible for clearance of target cells by opsonisation and antibody-dependent cell-mediated toxicity. IgA is found in mucosal secretions and so constitutes an antibody ‘first line of defence’. IgE is important for clearing parasitic infections. Some B cells produce natural antibodies, which are not class-switched or mutated and use only certain V genes. These antibodies are cross-reactive and thus are able to respond to a variety of antigens very rapidly. An important aspect of B-cell effector function is their ability to differentiate into memory B cells which will proliferate rapidly upon antigen re-encounter and produce class-switched, high affinity antibody much sooner than a primary response would.

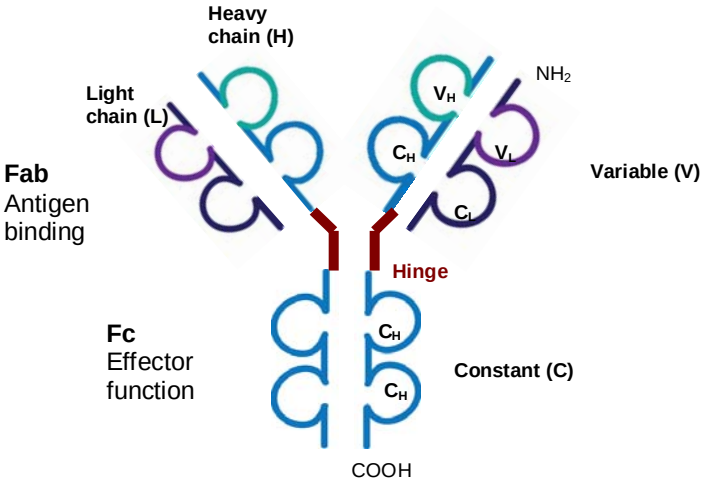


Figure 1.2. Immunoglobin structure

Schematic representation of IgG (analogous to IgA and IgD). Heavy (H) and light (L) polypeptide chains joined by disulphide bonds. The Fc portion consists of two constant (C) regions donated by each heavy chain (in contrast to IgM and IgE which have three). The Fab region is associated with a light chain and it is here that the antigen-binding variable (V) regions are found.

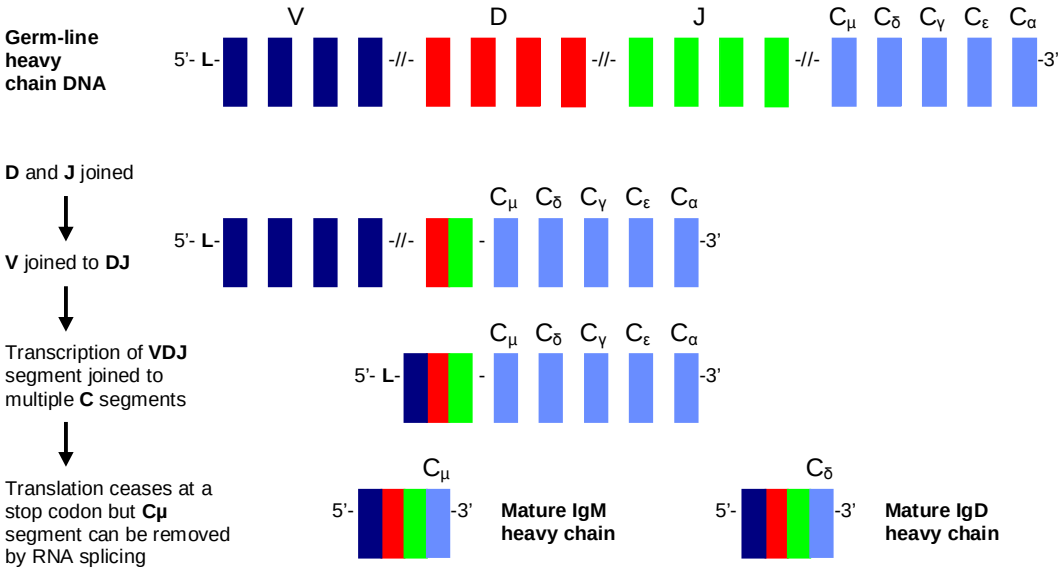


Figure 1.3. Ig heavy Chain V(D)J recombination

The Ig heavy chain is encoded by four gene segments: variable (V), diversity (D) joining (J) and constant (C). There are many different copies of each gene segment available for use (though only 4-5 shown for each here), as shown in the representation of heavy chain germ-line DNA. To make the mature template, first one D and one J segment are joined. Then, a V gene is added, including a leader sequence (L). The DNA is transcribed until the stop codon found at the end of each C gene segment. In naïve B cells, RNA splicing between recombination signal sequences (RSSs) can remove the Cμ gene to allow expression of Cδ which results in the production of IgD. After antigen encounter, class switch recombination (CSR) takes place which removes upstream C genes at the DNA level to allow expression of IgG, E and A.

1.1.2.3. B-cell Tolerance

Immunological tolerance is the mechanism whereby immune cells distinguish between self and non-self, preventing inappropriate immune responses which can damage normal tissue (autoimmunity). Autoreactive B cells undergo apoptosis but can also be rescued from this fate by receptor editing. During this process, B cells with a specificity for self antigen are able to reactivate RAG genes and express a different Ig light chain, changing their specificity. As well as these central tolerance mechanisms, peripheral tolerance ensures that any B cells which react to self antigens in the periphery do not cause autoimmunity. T-cell help is crucial in this process as it provides a second checkpoint prior to activation; so if a B-cell recognises a self antigen in the absence of T-cell help, it will become anergised and undergo apoptosis. One mechanism which controls this is the expression of chemokine receptors. Reduced expression of these surface molecules prevents B-cell migration to the follicles, indirectly causing them to die. However, autoreactive B cells can also be killed directly when Fas ligand (FasL) on T cells interacts with Fas on the B cells.

1.1.2.4. T-cell Development

T lymphocytes originate in the foetal liver, relocating to the thymus during gestation. In the adult, stem cells from the BM continue to seed the thymus. It is here that the immature lymphocytes (thymocytes) develop into mature T cells. There are several intermediate developmental stages, shown in Figure 1.4. The epithelial cells, DCs and BM-derived macrophages within the thymus provide the necessary cytokine environment and stimuli for T cell maturation. As the immature T cells pass through the cortex of the thymus, they begin to express RAG-1 and RAG-2; the genes which control rearrangement of the T-cell receptor (TCR) genes. The process of producing a mature TCR from the V, D, and J gene segments and constant region which compose the TCR β-chain is similar to that which produces a BCR, described in section 1.1.2.1. When an in-frame gene is produced, the β-chain protein will associate with a CD3 molecule and an invariant protein called pre-Tα to form the pre-TCR. This marks the transition from pro-T cells to pre-T cells. The function of the pre-TCR is to perceive survival signals which direct high levels of proliferation and drive further development. At the next stage, the genes of TCR α-chain (V and J only) are also rearranged, when combined with the constant region, this results in production of the mature TCR. There is also a T-cell subset which utilises different TCR genes, the γ and δ chains; these cells develop in a similar way and are described in more detail in 1.1.2.12.

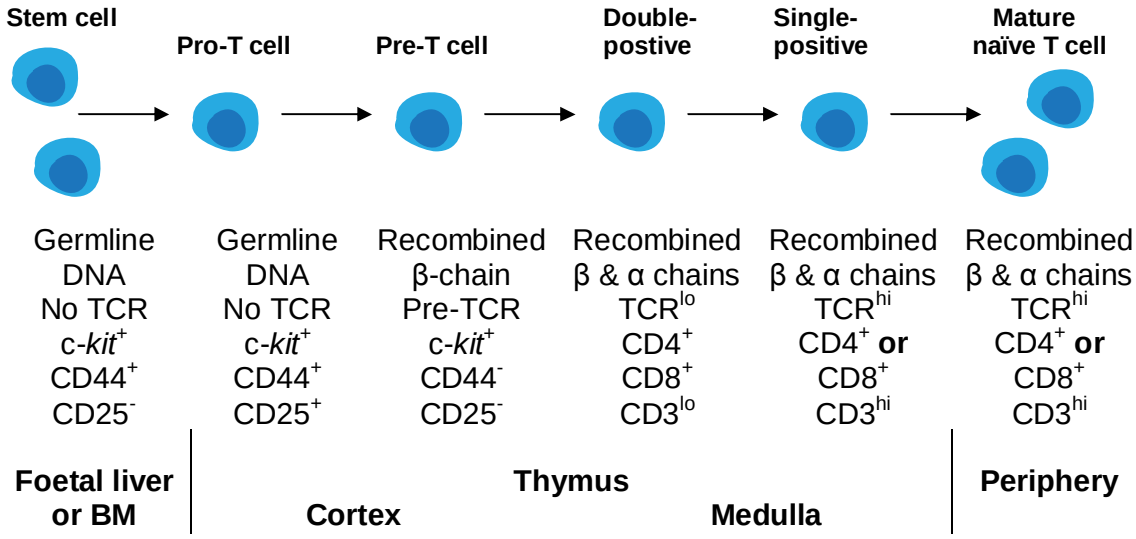


Figure 1.4. T-cell development

T cells begin their journey in the foetal liver, or bone marrow (BM) in the adult, as pluripotent stem cells. After migration into the thymus, the cells begin to differentiate. During the early stages of development, Pro- and Pre-T cells, the T-cell receptor (TCR) α- and β-chain genes have not yet been rearranged and no TCR is expressed on the cell surface. After recombination of the TCR genes, T cells migrate to the thymic medulla. Here CD4 or CD8 expression is lost, producing single-positive cells which undergo positive and negative selection based on their affinity for antigen. Mature naïve T cells home to peripheral lymphoid sites where they may encounter antigen.

After TCR expression, the cells are 'double positive' as they express both CD4 and CD8 surface molecules. They also express the chemokine receptor CCR7, which causes them to migrate into the medulla of the thymus. The final stage of development involves selection of useful T cells which are able to recognise peptides in the context of MHC, at the expense of cells that have a high affinity for self peptides, which could lead to autoimmunity. Thymocytes which have an intermediate affinity for self MHC-peptide complexes presented by epithelial cells in the thymus are positively selected. After this, MHC I^+ cells will lose CD4 expression and MHC II^+ cells will lose CD8 expression to produce 'single positive' cells. Negative selection removes cells which do not recognise any MHC-peptide complexes by neglect, whereas those which recognise self peptides with a high avidity are deleted by apoptosis. The presentation of peptide to T cells will be discussed in detail in section 1.1.2.6, but the mechanisms involved in the presentation of antigen to thymocytes may be slightly different to this [11]. Cortical thymic epithelial cells (TEC) exhibit an unusually high level of macroautophagy [12], allowing them to sample more self peptides. This is a process thought to have evolved as a starvation response whereby whole portions of cytoplasm, including organelles are engulfed in a

macroautophagosome; the fusion of these vesicles with endosomes and lysosomes allows loading of peptides onto MHC class II molecules.

Negative selection is of paramount importance as it forms the basis of central tolerance (discussed further in section 1.1.2.5). However, the fact that some low avidity T cells specific for self peptides escape central tolerance mechanisms and develop into mature T cells has allowed the advent of immunotherapy against the self antigens expressed by cancer cells.

1.1.2.5. T-cell Tolerance

There are two types of T-cell tolerance, central and peripheral. Central tolerance is mediated during the early stages of T-cell development, in contrast to peripheral tolerance which is a means of controlling mature T cells in the tissues.

1.1.2.5.1. Central Tolerance

The basis for central tolerance is negative selection in the thymus whereby T cells that express a TCR which binds a self peptide at a high avidity undergo apoptosis. This relies on the ability of cells in the thymus to present all possible self peptides to the developing T cells. In order to achieve this, tissue-restricted antigens (TRA) must be expressed in the thymus. A subset of 'mature' medullary TECs which have high levels of MHC II and costimulatory molecules on their cell surface are largely responsible for this 'promiscuous gene expression', evidenced by single-cell analysis of TRA expression in $CD80^{hi}$ medullary TEC [13]. This phenomenon is known to be controlled by the transcription factor autoimmune regulator (AIRE). Humans with a defective copy of the *AIRE* gene develop autoimmune polyendocrine syndrome type 1, a rare and severe autoimmune condition [14]. Transgenic mice which lack this protein exhibit a dramatic reduction in the expression of TRA in the thymus and therefore develop multi-organ autoimmunity [15]. However, when it comes to the severity and nature of this autoimmunity, there do seem to be familial differences in humans and strain-specific differences in mice; suggesting that other genetic and environmental factors play a role [16].

After a T cell with an autoreactive TCR is detected by a medullary TEC, it will be deleted (recessive tolerance) or directed to the regulatory T-cell (T reg) lineage (dominant tolerance) which controls peripheral tolerance (discussed below). Evidence from transgenic mice which express the ovalbumin (OVA) protein under the control of the rat insulin

promoter (RIP) in a membrane-bound form (RIP-mOVA mice), suggests that the majority of OVA-specific, autoreactive $CD8^+$ T cells are deleted after direct presentation of antigen by medullary TECs; in contrast, $CD4^+$ T cells are deleted by BM-derived DCs which cross-present antigen acquired from medullary TECs [17]. Another transgenic mouse model in which the haemagglutinin antigen is expressed in $AIRE^+$ medullary TECs demonstrates that they can direct autoreactive thymocytes to the T reg lineage independently of thymic DCs [18].

1.1.2.5.2. Peripheral Tolerance

Peripheral tolerance is maintained by a discrete $CD4^+$ T cell population known as regulatory T cells (T regs). This is necessary because, despite central tolerance mechanisms, healthy individuals still harbour self-reactive T cells in the periphery and under the right circumstances, they can become activated [19]. Studies in athymic nude mice show that transfer of thymocytes depleted of the $CD25^+$ $CD4^+$ $CD8^-$ subset leads to autoimmunity and demonstrable suppressive effects *in vitro* [20]. More recently, the transcription factor forkhead box P3 (FOXP3) has been identified as a marker for T regs, and furthermore its transfection into naïve T cells causes them to develop a T reg phenotype, providing evidence that FOXP3 is an instrumental regulator of T reg development [21]. When autoreactive T cells are detected as such in the thymus, they are subsequently rendered suppressive. These $CD25^+$ $CD4^+$ $FOXP3^+$ T regs develop in the thymus alongside their effector counterparts. This can be demonstrated in an experimental setting using TCR transgenic mice that also express the cognate antigen, which leads to the development of regulatory T cells against the antigen [22]. Data from non-obese diabetic mice supports the physiological relevance of this mechanism as antigen-specific T regs are more potent suppressors of diabetes than polyclonal T regs [23]. The development of human T regs remains less well understood. Although it is thought to be similar to that of mice, some key differences include the presence of the unique microenvironment of Hassall's corpuscles and the fact that human T regs are already apparent *in utero* (human T regs have been reviewed recently in [24]).

The suppressive function of T regs is mediated by the production of inhibitory cytokines such as IL-10, IL-35 and transforming growth factor (TGF)β; metabolic suppression including the consumption of IL-2 which is required by activated T cells and cytolysis via perforin and granzyme B [25], reviewed in [26]. As well as these effects on activated T cells, T regs can also suppress DCs, demonstrated by reduced CD80 and CD86 surface expression after incubation with T regs *in vitro*, a phenomenon which seems to be

controlled by cytotoxic lymphocyte antigen 4 (CTLA-4) expression [27]; and T regs can also reduce $CD4^+$ T cell-DC contact time [28].

As well as the thymically derived 'natural' T regs described above, a suppressive phenotype may be induced in naïve T cells. These 'induced' T regs seem to be particularly relevant in mucosal sites, such as the gut, where they have been implicated in oral tolerance [29]. Two subsets of induced T regs have been identified: type 1 T reg cells which produce IL-10 and TGFβ but do not express FOXP3 [30] and T_H3 cells which secrete TGFβ and do express FOXP3 [31].

Tumour-specific T regs have been associated with poor prognosis, for example in patients with cervical intraepithelial neoplasia, a precursor to cervical cancer [32]. This underlines the importance of the impact T regs can have on tumorigenesis and their potential to hinder cancer immunotherapy.

1.1.2.6. Antigen Presentation to T cells

In contrast to B cells, which recognise whole antigens, T cells recognise short peptides. These peptides require processing via intracellular machinery which trims proteins and longer peptides to size. These peptides are then transported to the cell surface where they are displayed on MHC molecules. MHC class I molecules are found on almost all somatic cells and usually bind endogenous peptides. MHC class II molecules are found on specialised APCs such as DCs which are able to acquire and process exogenous antigen. Importantly, T cells only recognise antigen bound to an MHC molecule which they express themselves, a phenomenon called MHC-restriction.

1.1.2.6.1. MHC class II Antigen Presentation

Activation of $CD4^+$ T cells is mediated via peptide presentation on MHC II molecules which are primarily expressed in professional APCs, such as DCs. The MHC II molecule, depicted in Figure 1.5, consists of two non-covalently linked polypeptide chains. The molecule has two Ig-like domains; α2 and β2, and a peptide-binding cleft which is formed by the polymorphic α1 and β1 domains. MHC II molecules are found in the endoplasmic reticulum (ER) of DCs with a portion of a chaperone protein called the invariant chain (Ii) bound to the peptide-binding groove. This serves to stabilise the molecule and provides an endosomal targeting sequence [33]. These complexes are then transported to endosomal compartments (the MHC II compartments), where Ii is cleaved into small fragments. One of the resulting fragments is Class II-associated invariant peptide (CLIP) which remains

bound to the peptide-binding groove [33]. CLIP continues to stabilise the MHC II molecules and prevents endogenous peptides binding to them.

Meanwhile, secreted protein, apoptotic vesicles and pathogens are endocytosed by the DCs. This potentially antigenic protein is sequestered in the phagolysosome compartments, where lysosomal enzymes break it down into peptides [34]. At this point, the antigenic peptides can replace CLIP in the peptide-binding groove of MHC II. The rate of CLIP dissociation is accelerated by the catalyst-chaperone molecule human leukocyte antigen (HLA)-DM (or H-2M in mice), which may also have a role in peptide selection by catabolising multiple peptide exchanges until a stable complex is formed [33], [35]. The complexes are then transported to the cell surface, probably through tubular structures [34].

These surface-bound MHC II-peptide complexes can then be recognised by $CD4^+$ T cells and generate a response as described in section 1.1.2.11.1.

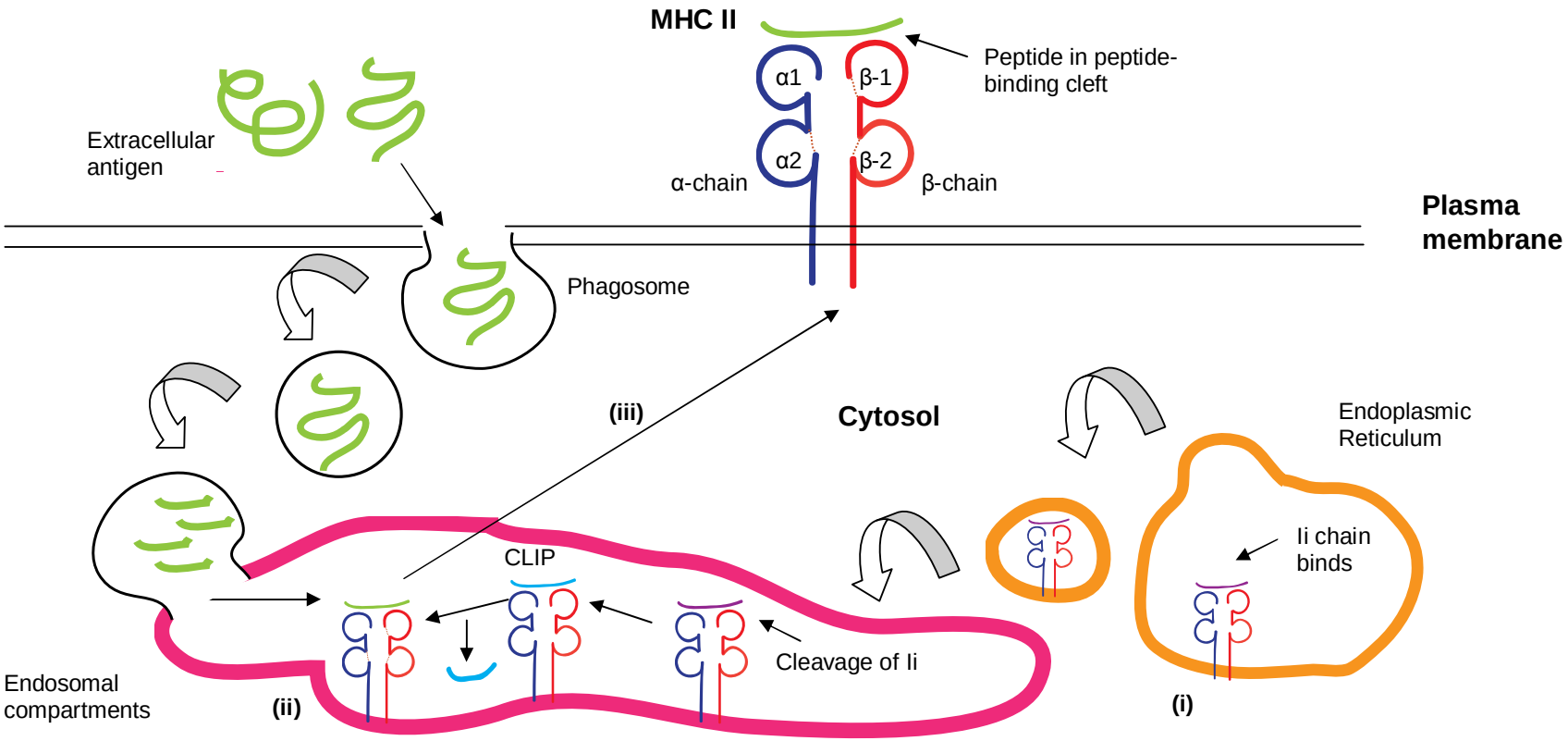


Figure 1.5. The exogenous antigen presentation pathway for MHC II

The mature MHC II molecule consists of two non-covalently linked polypeptide chains; the α-chain (shown in blue) and the β-chain (shown in red). The structure of the molecule is maintained by disulphide bonds (indicated by a dashed orange line). **(i)** The MHC I molecule is assembled in the endoplasmic reticulum with the invariant chain (Ii) bound to the peptide-binding cleft (shown in purple). **(ii)** After transport to the endosomes, Ii is cleaved leaving Class II-associated invariant peptide (CLIP) bound in its place (shown in light blue). **(iii)** Finally, CLIP is removed, allowing phagocytosed peptides (shown in green) delivered to the endosomes to replace it before the MHC II-peptide molecule is transported to the cell surface.

1.1.2.6.2. MHC I Presentation of Endogenous Antigen

$CD8^+$ T cells are activated by MHC I-peptide complexes. Unlike MHC II, MHC I is expressed on almost all nucleated cells and usually presents endogenous antigen from within the cell. This pathway, shown in Figure 1.6, is crucial in identifying and eliminating cells infected with virus.

In the first step of the MHC I presentation pathway, proteins are degraded by proteases within the multi-subunit complex called the proteasome and then peptidases in the cytosol, creating shorter peptides. These peptides are transported from the cytosol to the ER by transporter associated with antigen presentation (TAP), where they can bind nascent MHC I molecules. MHC I molecules consist of a transmembrane glycoprotein α-chain (the heavy chain) and the β_2-microglobin (depicted in Figure 1.6). Disulphide bonds are created between the loops of the α-chain and β_2-microglobin, resulting in two Ig-like domains and a peptide-binding cleft (see Figure 1.6). The association between the α-chain and the β_2-microglobin is a non-covalent one and requires peptide binding to stabilise the interaction. Peptides are loaded onto the MHC I molecule by the peptide loading complex which consists of several proteins: TAP, tapasin, calreticulin and ERp57 [36]. These stable MHC I-peptide complexes travel to the cell surface via the Golgi [34].

1.1.2.6.3. MHC I Presentation of Exogenous Antigen

Although MHC I molecules usually present endogenously derived antigen, specialised cells such as DCs and macrophages can direct exogenous antigen to the MHC I-presentation pathway, via a process called cross-presentation.

For cross-presentation to occur, exogenous peptides must be processed and transported to the ER where they can bind MHC class I molecules. The pathways that control this are still incompletely understood, and several models have been proposed (reviewed in [37], [36], [33] and [34]). The proposed pathways, shown in Figure 1.7, rely on endocytosis of exogenous antigen by the DC and then either (i) the generation of peptides within the endocytic compartments, which could later be delivered to the ER; or (ii) transport of antigen out of the endocytic compartments into the cytosol where it can enter the endogenous pathway. The major proposed routes are depicted in Figure 1.7. One theory is that phagocytosed antigen may be delivered to the ER as a result of the fusion of the two compartments, creating an novel cellular compartment containing machinery capable of processing and loading peptides onto MHC I molecules [38]. Although the ability of DCs

to transport antigen out of the phagosome to the cytosol for proteasomal degradation has been demonstrated [39], the exact details of how this occurs are still unknown. One proposed mechanism by which antigen may travel from the phagosome to the cytosol is via the ER dislocon complex which usually removes misfolded proteins from the ER. Proteins involved with this process have previously been found in phagosomes [40] and would clearly be available when the phagosome-ER fusion pathway is implicated. It is also possible that membrane 'leaking' could occur as with viral peptides such as Tat from HIV-1 which can spontaneously transverse the lipid bilayer. Finally, if phagosomes containing antigen were to rupture this would allow antigen to escape into the cytosol. However, the toxic contents of the phagosome would also escape, which would carry a risk of cell death. During the course of a *Crytococcus neoformans* infection, the fungus can escape the phagosome and leave the cell viable [41], [42]; providing at least some evidence that this possibility should not be ruled out.

Antigen may also access this pathway by mechanisms other than phagocytosis. One suggestion is that antigen may be passed from cell to cell via gap junctions, as has been demonstrated *in vitro* [43] though in practice, this would have to be a highly selective process to prevent a reduction in the sensitivity of CTL-mediated lysis.

Once MHC I-peptide complexes are displayed on the surface of an activated DC, they are able to induce a $CD8^+$ T-cell response as described in section 1.1.2.11.2.

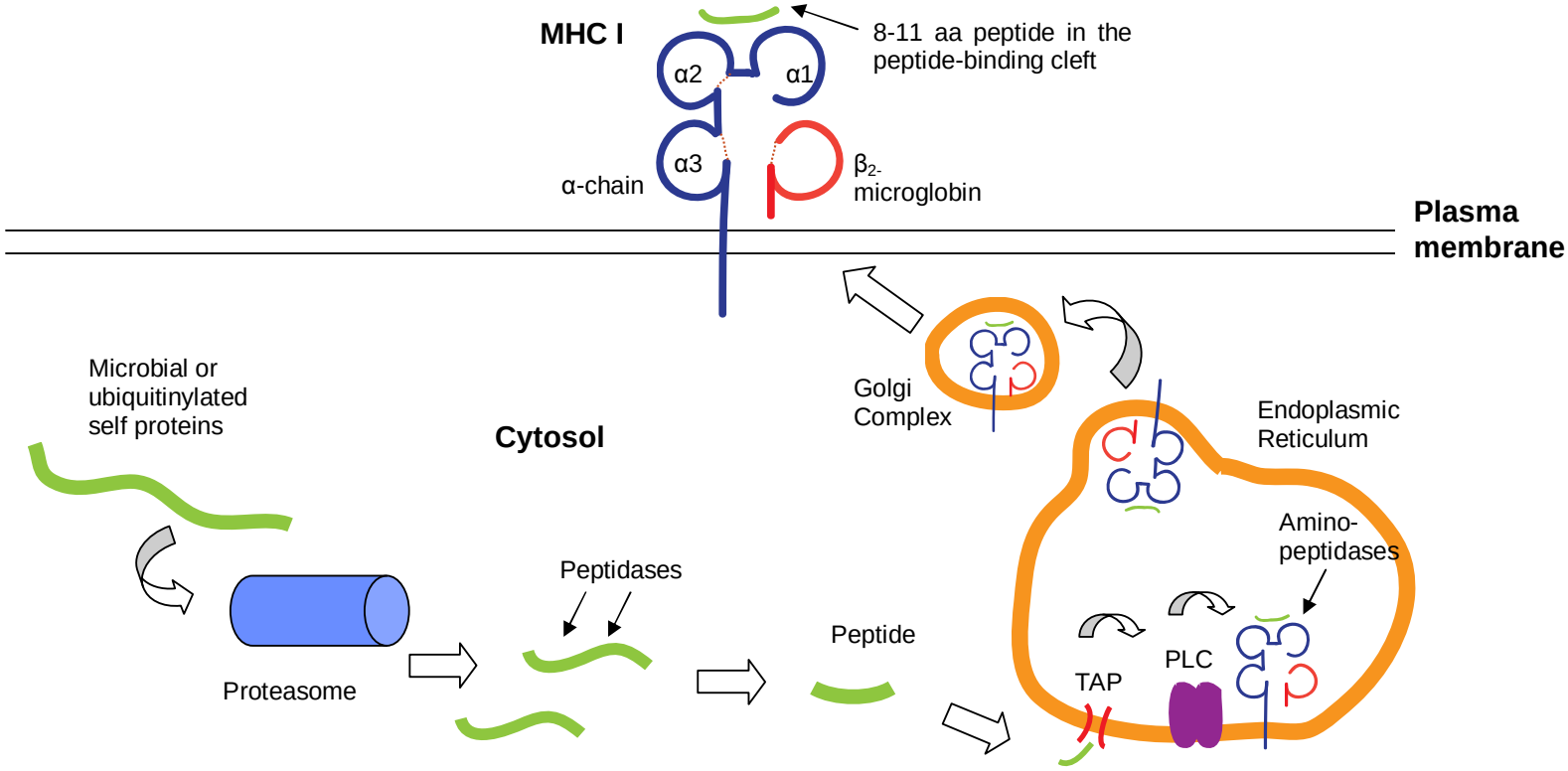


Figure 1.6. The endogenous antigen presentation pathway for MHC I

The mature MHC I molecule consists of two non-covalently linked polypeptide chains: the transmembrane α-chain (shown in blue) and the β_2-microglobin (shown in red); its structure is maintained by disulphide bonds (indicated by a dashed orange line). In order to produce a stable MHC I molecule that will be expressed on the surface it must first bind peptides of 8-11 amino acids (aa) in length which are generated by proteolysis of ubiquitinylated or viral proteins as shown. Short peptides are transported into the endoplasmic reticulum (ER; shown in orange) by transporter associated with antigen processing (TAP) and then loaded onto nascent MHC I molecules there by the peptide loading complex (PLC; shown in purple). Further trimming by amino-peptidase action may occur after peptide loading. This creates the mature MHC I-peptide molecule which is finally trafficked to the cell surface via the Golgi complex.

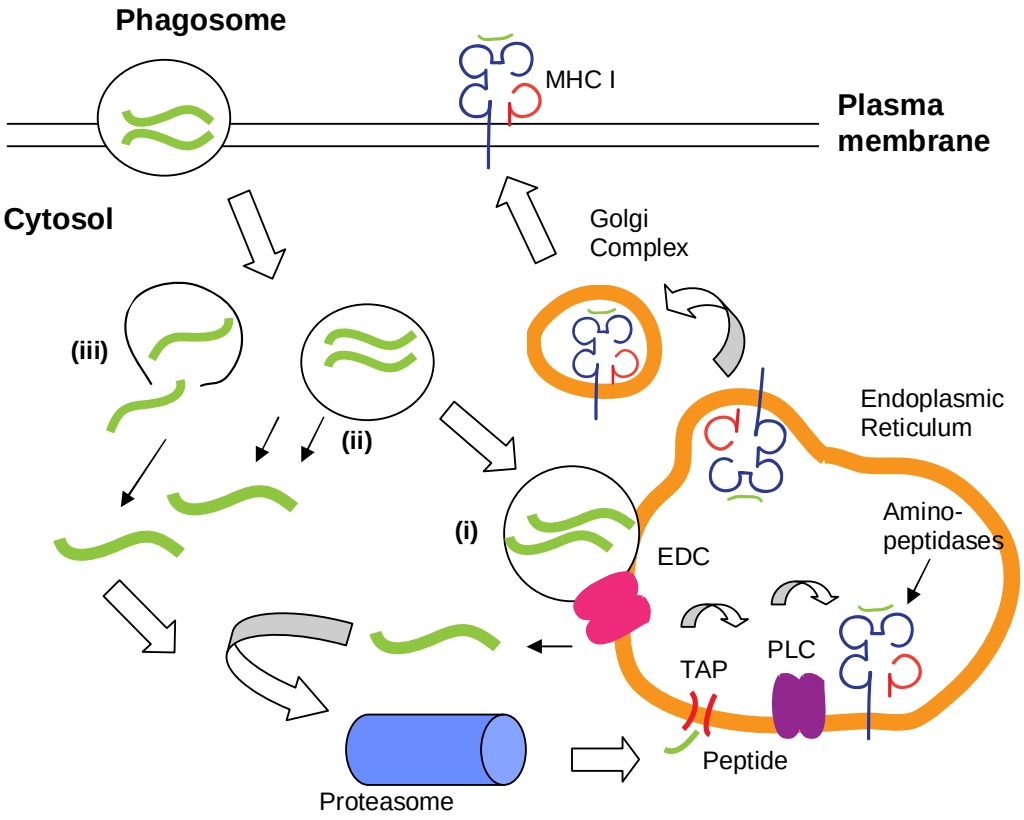


Figure 1.7. Proposed mechanisms for cross-presentation

Exogenous antigen (shown in green) is ingested by DCs via the process of phagocytosis. In order to be presented to $CD8^+$ T cells, antigens must be degraded into short peptides by the proteasome in the cytosol and then loaded onto MHC I molecules in the endoplasmic reticulum (ER). Proposed models include **(i)** fusion of the phagosome with the ER, providing the ER dislocon complex (EDC) as a mode of transport into the cytoplasm; **(ii)** leakage or spontaneous exit of antigen from the phagosome and **(iii)** rupture of the phagosome.

1.1.2.7. Co-stimulation

As described above, when a naïve $CD8^+$ T cell encounters a mature DC displaying a MHC I-peptide complex recognised by its TCR, a second signal is required to induce T-cell activation. Signal 2 is provided by co-stimulatory molecules on the surface of the APC which interact with their ligands on T cells. This leads to T-cell activation and acquisition of effector functions; without this signalling, T cells become tolerised and unresponsive to the peptide. The best described co-stimulatory mechanism is the interaction of the CD28 receptor expressed by T cells and its ligands CD80 and CD86 which are expressed by DCs; however there are many more including other members of the CD28 family, as well as the

tumour necrosis factor (TNF) family and the T-cell Ig and mucin (TIM) family (reviewed in [44]). CD28 ligation causes an intracellular signalling cascade which ultimately results in IL-2 production, prevention of anergy and increased cell survival [45]. Interestingly, it has become clear that co-stimulation can also provide inhibitory signals. CTLA-4 is a well described inhibitory co-stimulatory molecule which mediates its effects in several ways; for example, CTLA-4 signalling reverses the reduction in cell motility which occurs during TCR ligation, reducing T-cell/APC contact time [46]; and CTLA-4 has been shown to be crucial for suppressive function of T regs by reducing CD80 and CD86 expression on DCs [27].

1.1.2.8. T-cell Activation

T-cell activation is a multi-step process. 'Signal 1' is received when the TCR binds a MHC-peptide complex (the generation of these molecules is described in sections 1.1.2.6.1 and 1.1.2.6.2). However, further signals are required. 'Signal 2' is mediated by co-stimulation with other molecules on the T-cell surface interacting with the APC (described in section 1.1.2.7). This should ensure activation of the T cell, however it has been proposed that a third signal may exist in the form of inflammatory cytokines. IL-1 has been shown to enhance $CD4^+$ T-cell responses and IL-12 for $CD8^+$ T-cell responses; in both cases via direct action on the T cells [47].

T-cell activation leads to the formation of the immunological synapse. This is a complex of molecules composed of the TCR, CD3 and CD4 or CD8 expressed on the T-cell which cluster at the site of the interaction. Ligation of these molecules results in intracellular signalling pathways mediated by the immunoreceptor ITAMs found in the TCR/CD3 complex. The ensuing signals lead to changes in gene expression in the T cell which change its phenotype from naïve to activated (reviewed in detail in [48]). At this point, T cells are ready to instigate their effector functions, described in section 1.1.2.11.

1.1.2.9. DC Licensing

As professional APCs, DCs are crucial to the generation of T-cell responses; especially in the case of cross-presented antigens. However, in their resting state, DCs express low levels of MHC and co-stimulatory molecules on the cell surface. As a result, they are inefficient at T-cell priming. The process of DC maturation involves stimulation by PRRs, inflammatory cytokines or $CD4^+$ T cells, or a combination of these. $CD4^+$ T cells are able to 'licence' the DC via interactions between CD40 molecules on the surface of the DC and

CD40L (CD154), which is expressed by T helper cells [49]. This renders a DC 'mature' and thus able to activate $CD8^+$ T-cell responses [50]. Whilst primary responses, particularly to pathogens which directly infect DCs or those that provoke a strong inflammatory response [51], [52] may be induced in the absence of T-cell help, secondary responses are not [53]. In addition, mature DCs secrete cytokines themselves, providing positive feedback for T cells [54]. Therefore, this phenomenon is crucial to the development of primary $CD8^+$ T-cell responses to cross-presented antigens which develop in a non-inflammatory environment.

1.1.2.10. T-cell Migration

Appropriate T-cell migration is crucial to the T-cell response as T cells must be able to circulate in order to encounter antigen. Responses must subsequently be mounted in the correct location, requiring further migration. Naïve T cells circulate in the blood and lymphatic system until they encounter antigen in the lymph nodes. Lymphocyte homing to the lymph nodes is controlled by cell adhesion molecules (CAM) which mediate interactions between them and the high endothelial venuoles (HEV) which separate the bloodstream from the lymph node, as shown in Figure 1.8. Initial, weak interactions are mediated by L-selectin and its ligand on the HEV, before stronger interactions between integrins on the two cell types are made. Meanwhile, in a site of infection or vaccination, antigens are phagocytosed by APCs such as DCs which are able to process and present them as described in sections 1.1.2.6.1 and 1.1.2.6.2. Antigen-experienced DCs then enter the draining lymph node of the tissues and present the antigen to naïve T cells which they encounter there. Naïve T cells which do not encounter an antigen specific for the TCR that they express leave the lymph nodes and continue to the thoracic duct where they rejoin the blood to recirculate. T cells which do encounter their cognate antigen and therefore become activated exit the lymph nodes as effector cells. As a consequence of activation, certain surface molecules are up-regulated to ensure that the T cells migrate to the appropriate peripheral site. For example, T cells which home to the gut express α4β7-integrin which binds to the mucosal vascular addressin cell adhesion molecule 1 (MADCAM-1) expressed in the gut [55]. This enables the immune system to direct activated T cells to where they are needed.

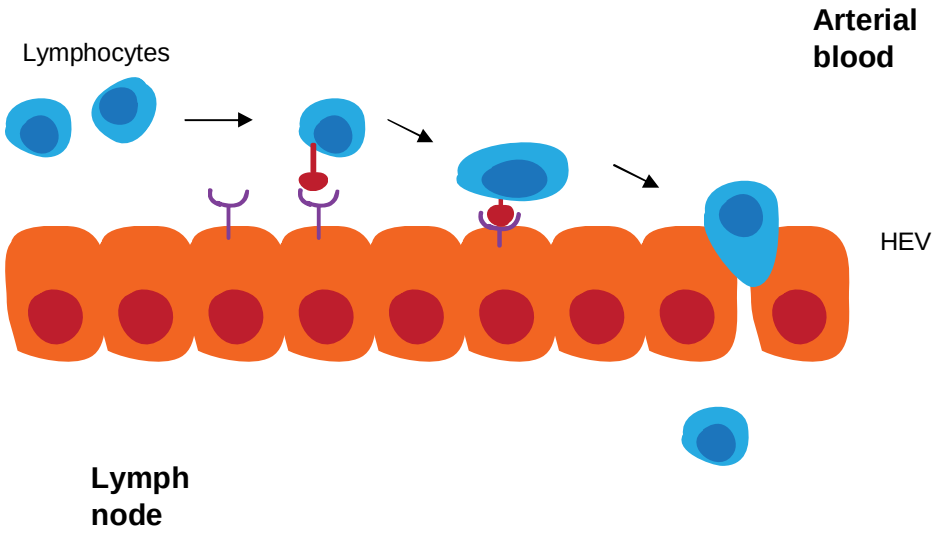


Figure 1.8. Migration of naïve lymphocytes into the lymph nodes

Naïve T cells in the blood home to the lymph nodes due to interactions between cell adhesion molecules (CAM). L-selectin expressed on the T and B cells binds to its ligand (both shown in red) on the high endothelial venuole (HEV) with a low affinity, causing rapid detachment until a stronger interaction is formed between integrins on the surfaces of the two cells (shown in purple). Finally the lymphocytes migrate through the HEV barrier towards chemotactic factors in the lymph nodes.

Another factor which influences T cell migration is the presence of chemokines. These molecules are produced constitutively in the lymphoid organs and in response to inflammatory signals in other sites. Chemokines bind to heparin sulphate proteoglycans on endothelial cells and are thus displayed to lymphocytes circulating in the bloodstream which have bound to the endothelium via L-selectin. Ligation of the chemokine receptor causes affinity of lymphocyte integrins to increase, aiding migration through the blood vessel wall. Migration itself is also controlled by chemokines as they induce remodelling of cytoskeletal elements within the cell [56]. The molecular pathways which control this are starting to be elucidated [57]. There are many types of chemokines and each act on different immune cell subsets. For example, CCL-2 recruits monocytes, whereas CXCL-9 recruits effector T cells.

1.1.2.11. αβ T-cell Effector Function

T cells can be divided into two major subsets based on their expression of either CD4 or CD8 surface molecules. $CD4^+$ T cells constitute a functionally diverse group of cell subsets which have far-reaching roles in supporting and controlling many immune functions. Here

$CD4^+$ T cells with helper functions will be discussed. Another subset of $CD4^+$ T cells is the regulatory T cells which are involved in peripheral tolerance, which have already been discussed in detail in section 1.1.2.5. $CD8^+$ cells are specialised killer cells which target infected or abnormal cells.

1.1.2.11.1. Helper T cells

$CD4^+$ helper T cells (T_H) are critical in supporting the immune response. So far, four separate subsets have been described: T_H1, T_H2, T_H17 and T_{FH} (follicular helper cells). Each of these subsets is derived from $CD4^+$ naïve T cells which have undergone distinct differentiation programmes according to environmental cues such as cytokines. This process is controlled by specific transcription factors; for example, T-bet is induced in T_H1 cells [58] and GATA-3 in T_H2 cells [59]. T_H1 cells are induced in response to intracellular pathogens and are characterised by their secretion of IFNγ. The IFNγ that they produce activates macrophages, increasing phagocytosis and microbe killing. It also acts on B cells to stimulate class switching to specific IgG subclasses, increases opsinisation and enhances cytolysis mediated by $CD8^+$ T cells. T_H2 cells are induced in response to helminth infections and allergens and are characterised by the production of IL-4. IL-4 is of critical importance for antibody class switching to IgE, which has a role in autoimmune diseases such as asthma. T_{FH} cells are a more recently described subset [60], [61] that also secrete cytokines which influence antibody production such as IL-21 and IL-4, making them similar to the T_H2 subset. However, the defining feature of these cells is their expression of the chemokine receptor CXCR5, which allows them to home to the B-cell follicles where they can influence B-cell development (reviewed in [62]). T_H17 cells are so-called because they produce the cytokine IL-17. The role of these cells seems to be to clear certain pathogens, for example IL-17 is required during a *Candida albicans* infection due to its stimulatory effect on neutrophils [63]. However, these cells have also been linked to autoimmunity such as psoriasis. A recent clinical trial showed that a TNF inhibitor successfully treated this disease due to a reduction in local inflammation; specifically cytokines associated with T_H17 cells [64]. The physiological relevance of this subset has been reviewed in detail in [65].

1.1.2.11.2. Cytotoxic T cells

$CD8^+$ T cells kill target cells in a contact-dependent fashion facilitated by the interactions between the T cell and the target. These interactions are mediated by MHC I-peptide

complexes and the accessory molecules which mediate co-stimulation. Upon ligation of the TCR of an activated $CD8^+$ T cell with a corresponding MHC-peptide molecule on a target cell, cytolysis is initiated by one of two distinct pathways: (i) cytotoxic granule release or (ii) Fas binding.

The primary mode of CTL-mediated lysis of virally infected and cancerous cells is the cytotoxic granule exocytosis pathway. Changes in gene expression in activated CTLs cause accumulation of cytotoxic granules. One recently identified transcription factor which is crucial to the development of terminally differentiated effector T cells is B lymphocyte-induced maturation protein 1 (Blimp-1); mice lacking this protein have dramatically reduced intracellular levels of the toxin granzyme B [66]. In order to deliver the cytotoxic molecules to the target cell, another constituent of the vesicles is required. Perforin creates a pore in the membrane of the target cell, through which granzymes enter. These serine proteases then cause membrane and DNA damage, leading to apoptosis [67], [68].

The Fas receptor (CD95) is expressed on the surface of a broad range of lymphoid, myeloid and non-haemopoetic cell types [69]. When Fas is bound by its ligand, produced by CTL, an apoptosis pathway is triggered within the target cell. Fas-mediated apoptosis is involved in eliminating autoreactive T cells, evidenced by the fact that individuals lacking Fas or Fas ligand develop autoimmunity [70]. Although CTLs have been shown to kill tumour cells in this way in a mouse model [71], the physiological relevance of this is unknown.

As well as their lytic function, $CD8^+$ T cells also produce the cytokines IFNγ and IL-2. IL-2 is particularly important for the $CD8^+$ T-cell response as it acts in an autocrine manner as a survival factor.

1.1.2.11.3. T-cell Immunodominance

Although the diversity of the TCR means that T cells can potentially recognise thousands of peptide epitopes, in practice the T-cell response focuses on just a few for any given antigen. These peptides are classed as dominant, whereas peptides that produce a very weak or infrequent response are classed as subdominant. Immunodominance is controlled by three major factors: (i) the quantity of MHC-peptide complexes on APCs, (ii) the presence of suitable TCRs within the T-cell repertoire, and (iii) the ability of immunodominant responses to suppress subdominant responses [72]. Proteasomal digestion profiles correlate with immunodominance hierarchies for HIV antigens [73] and studies of lymphocytic choriomeningitis virus (LCMV) infection show that the immunodominant peptide binds MHC with higher affinity than sub-dominant ones [74].

These data demonstrate that presentation of a particular MHC-peptide complex relies on efficient processing of the peptide and high affinity binding of the peptide to the MHC. However, so-called 'holes' in the T-cell repertoire are particularly relevant for T-cell responses directed against self peptides as central tolerance mechanisms delete T cells with a high affinity for self antigens (as described in section 1.1.2.5.1). T-cell competition can take the form of T cells of the same specificity competing for the same APC, or so-called cross-competition, which involves T cells of different specificities competing for the same APC; however this phenomenon appears to be more important for secondary immune responses [75]. For $CD4^+$ T cells, another factor is HLA-DM, the catalyst-chaperone protein involved in MHC II-peptide presentation (see section 1.1.2.6.1 for more information) which favours immunodominant peptides [76].

1.1.2.11.4. T-cell Memory

A crucial function of T cells is to ensure that a memory population remains after the initial effector phase. Just like secondary antibody responses, memory T cells are able to produce a rapid response upon re-exposure to an antigen [77]; therefore inducing them is a crucial target of cancer immunotherapy.

The factors which control $CD8^+$ T-cell memory development are unclear. There are two possibilities (i) cell fate could be pre-determined such that some CTL are destined to become memory cells, while some are destined to die; (ii) environmental factors, for example signal strength and cytokine environment could influence cell fate. The former theory of pre-determined cell fate is supported by the fact that two populations can be identified during the effector phase; those which express low levels of IL-7R are terminally differentiated effector cells which do not persist beyond the effector stage, whereas those which express high levels of IL-7R are the memory cell precursors which provide long-term protection [78]. The latter theory relies on the principle that T cells compete for resources such as cytokines, and those which lose out will die during the contraction phase. In fact, exogenous IL-2, -4, -7 and -15 do increase T cell survival *in vitro* [79]. However, more refined *in vivo* experiments using apoptosis-defective T cells ($bim^{-/-}$) as competitors show that wild-type cells are not affected by the persistence of large numbers of cells which presumably consume large amounts of cytokines [80]. It has been demonstrated recently that naïve T cells activated in the presence of strong IL-2 signals acquire characteristics of terminally differentiated effector cells, in contrast to those which are activated in the presence of weak IL-2 signals, which become long-lived memory cells [81]. Furthermore, prolonged IL-2 signalling drives terminal effector differentiation [82];

indicating that the quality of the cytokine signalling may be more in important for T-cell memory development than the presence of cytokines *per se*.

Therefore, perhaps both pathways exist, with T-cell fate being somewhat pre-determined but still requiring certain additional signals.

A small proportion of $CD4^+$ T cells also persist beyond the effector stage of the response to become memory $CD4^+$ T cells. The factors in control of this process are not completely understood; IL-7 signals may have a role [83], so may TCR signal strength [84] and possibly asymmetric cell division, as suggested in the case of $CD8^+$ memory cells [85]. More importantly, the physiological role of these cells is also unclear because, whilst primary CTL and antibody responses rely on cognate T-cell help, the subsequent memory responses may not.

1.1.2.12. γδ T cells

γδ T cells are a separate lineage of T cells which express an alternative TCR consisting of a γ-chain and a δ-chain in place of the more common α- and β-chains. Their development is analogous to αβ T cells but they have limited diversity and function.

Historically, it was thought that their main purpose was to provide protection against a limited number of common microbes at epithelial surfaces; however recent data from mice is uncovering various other important functions, dependent on subset (reviewed in [86]). Their actions can be both protective, as in the case of their role in neonatal immunity [87]; and pathogenic demonstrated by their exacerbation of allergic airway hyperresponsiveness [88].

1.1.2.13. NK-T cells

NK-T cells are a subset of αβ T cells which are not MHC-restricted. Instead they recognise microbial and self glycolipid antigens bound to the MHC-like molecule called CD1-d, and produce IFNγ and IL-4 in response [89]. The unusual fate of these cells may be due to selection by interactions with thymocytes presenting lipid antigens in the context of CD1-d. NK-T cells do not subsequently express CCR7, and thus do not migrate to the medulla but exit the thymus at this stage; often becoming resident in the liver. NK-T cells do not express CD4 or CD8 but express surface markers usually found on NK cells. Many NK-T cells exhibit a stereotyped TCR consisting of a specific α-chain, leading to the term invariant NK-T cells [89].

1.2. Cancer Immunotherapy

Cancer immunotherapy uses the immune system as a weapon against the disease. The field of cancer immunotherapy is vast but falls into two categories: passive and active immunotherapy. Passive immunotherapy is mediated by transferring exogenous immune agents into patients. Examples include monoclonal antibodies such as anti-CD20 which is used to target B-cell malignancies with great success [90]; and allogeneic transplant can have a potent graft-versus-leukaemia effect, though this must be balanced with the likelihood of graft-versus-host disease [91]. Active immunotherapy is mediated by immunisation of patients to induce immunological memory, and this is the focus of this section. Tumour cells themselves have been used as vaccines, usually with an adjuvant that renders them more immunogenic [92]. DCs that have been pulsed with protein, peptide, DNA or RNA derived from tumours can also be used as a vaccine. This strategy has proven effective in a mouse model of myeloma [93] and in a clinical trial targeting prostate cancer, in which all patients developed an anti-tumour immune response [94]. The alternative to cellular vaccines is the use of protein, peptide [95], DNA [96], or viral vectors [97] derived or including material from tumour as a vaccine. The portions of tumour used to stimulate the immune system in this way are called tumour antigens, and these are described below.

1.2.1. Tumour Antigens

Tumour antigens are molecules expressed by a tumour against which a patient can potentially produce an immune response. They may be proteins/glycoproteins, carbohydrates or glycolipids. *In vitro* assays using T cells from patients have been used to identify tumour antigens from tumour-derived expression libraries [98]. More recently, there have also been advances in the field of T-cell epitope prediction with the emergence of algorithms which model protease cleavage sites and peptide binding to MHC molecules, leading to the creation of databases such as SYFPEITHI [99]. Another important approach is the serological identification of antigens by recombinant expression cloning (SEREX) technology. This has been developed to identify spontaneously induced IgG serum antibodies against potential tumour antigens [100]. The ‘reverse immunology’ approach of epitope prediction together with SEREX has lead to the identification of numerous tumour antigens (tumour antigens are reviewed in [101]).

In cancers that are associated with viruses, such as human papillomavirus (HPV) which causes cervical cancer [102], the virus can provide a source of tumour antigen. However, most tumour antigens are self molecules. They can be lineage specific, such as prostate-

specific membrane antigen, which is expressed in normal and cancerous prostate tissue [103]. Tumour antigens may be different to the naturally occurring protein in a variety of ways. They may be altered by splicing, mutation or carbohydrate modification, such as mucins which may have reduced glycosylation levels in adenocarcinoma [104]. Some tumour antigens are aberrantly expressed, such as the cancer/testis antigens, a large group of proteins which are only expressed in germline cells in healthy individuals, but often found in tumours [105]. Others are over-expressed in tumours, such as the human epidermal growth factor receptor (HER)-2, which is over-expressed in 25% of all breast cancers [106].

1.2.2. Spontaneous Immunity

The identification of tumour antigens has paved the way for cancer immunotherapy; however, evidence that immune responses can be raised against them is equally important. Immune surveillance of tumours was first proposed in the 1950s by Burnet and Thomas and is a widely accepted phenomenon today. Data collected in immunodeficient nude mice in the 1970s indicated that the immune surveillance theory was incorrect as these mice did not appear to be more susceptible to spontaneous or chemically induced tumours. However, these data were misleading for several reasons: the short-term nature of the experiments, increased sensitivity of the strain to the chemical used to induce tumours and the fact that nude mice are not completely immunocomprimised since they do possess some T cells as well as NK cells [107]. More refined modern data from other transgenic mouse strains do provide evidence for the importance of natural immunity to tumour. For example, sarcomas induced by the chemical methylcolanthrene are increased in incidence in mice lacking T cells, B cells and NK T cells compared to wild-type mice with intact immune systems [108].

The theory of immunoediting describes carcinogenesis as three stages: (i) elimination, when tumour cells are removed by immunosurveillence; (ii) equilibrium, when tumour cells maintain a steady state; and (iii) escape, when tumour growth is uncontrolled [109].

Immune responses have also been documented in cancer patients. Tumour-specific T cells are found in malignant melanoma, carcinomas, haematological malignancies and virally-induced cancers (reviewed in [110]). Importantly, there is growing evidence that spontaneous immune responses can control cancer growth. A recent study in colorectal cancer patients found that those with more T cells at the tumour site had prolonged

survival rates [111]. Antibodies against tumour antigens certainly exist in many patients, leading to their use as identifiers of these antigens by the SEREX approach [100].

This evidence suggests that immune responses against tumour antigens may be able to control tumour growth, and therefore that active immunotherapy could be used as a potential cancer treatment. However, there are several challenges for immunotherapy to overcome before an immune response can be induced, as described below.

1.2.3. The Challenges for Immunotherapy

Although immune responses to tumour antigens have been documented, there are several reasons why they may not be successful in removing tumours. Firstly, cancerous cells often exhibit mechanisms of immune evasion, reviewed in [112] and [113]. Tumour cells can 'hide' from the immune system by reducing the amount of antigen that they present to lymphocytes. This can be mediated by the down-regulation of molecules involved in antigen processing such as tapasin and decreased expression of MHC class I molecules on the cell surface [114]. Some cancerous cells acquire mechanisms that inhibit CTL-mediated killing. For example, *in vitro* studies have found that serpin, a protease which inactivates granzyme B, can be expressed by tumour cells, inhibiting the ability of CTLs to kill them [115]. The tumour microenvironment can also be immunosuppressive due to the local cytokine and chemokine milieu, which can inhibit immune responses and lymphocyte entry to the tumour [116]. Altered amino acid metabolism in the tumour microenvironment can also aid tumour cell escape. L-arginine is one amino acid whose metabolism can influence the immune response in many ways. For example, L-arginine is depletion can lead to functional impairment and reduced proliferation of T cells [117].

Aside from potential immune suppression, many tumour antigens are normal self proteins and as such, the immune system is often tolerised to them. This is due to clonal deletion during central tolerance induction and by the suppressive actions of T regs which constitute peripheral tolerance (immunological tolerance is described in detail in section 1.1.2.5). There is another subset of immune cells with a regulatory function called myeloid-derived suppressor cells, a heterogeneous population of myeloid progenitor cells and immature myeloid cells. These cells accumulate in cancer patients and mediate immune suppression by producing reactive oxygen species (ROS), peroxynitrite and enzymes which catabolise L-arginine [118].

1.2.4. DNA Vaccines

A DNA vaccine is a simple means by which antigen can be delivered to the host immune system. The vaccines consist of a bacterial plasmid into which antigenic sequences are cloned. After vaccination, cells at the injection site can take up the DNA vaccine. Within a transfected cell, the DNA will be transcribed and translated to produce the protein that it encodes. The protein can then be presented to the host immune system, which can lead to an immune response. All arms of the immune system can be selectively targeted by DNA vaccines; however, the effectiveness of this targeting is dictated by the vaccine design. For example, additional immunostimulatory sequences can also be incorporated into the vaccine design. Sequences which induce a helper T-cell response are particularly useful as these cells enhance both B-cell and CTL responses. In fact, the ease of DNA plasmid manipulation is one of the major advantages to this mode of vaccination. The design of DNA vaccines is described in more detail in sections 1.2.4.7 and 1.2.4.8.

1.2.4.1. Production of DNA vaccine-encoded Proteins *in vivo*

DNA vaccines are most commonly injected directly into a muscle site through the skin, making muscle cells (myocytes) the main antigen depot. Experiments using DNA encoding green fluorescent protein (GFP) have demonstrated transfection of muscle fibres as well as mononuclear cells at the site after intramuscular injection [119]. When the protein encoded by DNA vaccine is fused to a secretory signal sequence, as ours are, secreted protein is available to stimulate B-cell responses (as described in section 1.1.2.2).

In contrast to antibody production, the induction of T-cell mediated immunity requires processing of mature proteins into smaller peptides for presentation on MHC molecules. Although most somatic cells express MHC class I molecules and are therefore able to present peptides to $CD8^+$ T cells, in practice this process is more often carried out by professional APCs such as DCs (as described in section 1.1.2.6). Myocytes directly transfected with DNA are able to present antigen on MHC I molecules; however, this means that they will become a target for immune cells. In fact there is evidence for both T-cell and antibody-mediated destruction of DNA-transfected myocytes [120]. After DNA transfection, muscle cells can exhibit increased expression of MHC I and co-stimulatory molecules, increased expression of genes involved in antigen processing and presentation, and increased production of cytokines and chemokines [121]; which could enhance antigen presentation. However, it is perhaps more pertinent to discuss how professional APCs such as DCs obtain DNA vaccine-encoded antigen. Non-muscle cells found to be transfected at the site of a DNA injection were identified as adipocytes, endothelium, fibroblasts and

connective tissue [119]. This suggests that the DCs are not usually directly transfected at the injection site, and instead obtain the DNA exogenously via a process known as cross-presentation, described in section 1.1.2.6.3. However, it is possible that some professional APCs may be directly transfected at the injection site. It has been demonstrated in mice that DCs can obtain DNA vaccine-derived antigen and subsequently migrate to the draining lymph nodes where they can prime both $CD4^+$ and $CD8^+$ T cells [122].

1.2.4.2. Innate Immune Responses Stimulated by DNA Vaccines

As the backbone of a DNA vaccine is a bacterially derived plasmid, it contains PAMPs which are recognised by the PRRs of the innate immune system (described in section 1.1.1). There are several pathways which control this process, as shown in Figure 1.9. Hypomethylated DNA containing CpG repeats is found in the bacterial plasmids which form the backbone of DNA vaccines, and this is a PAMP which is recognised by the PRR TLR-9 [123]. TLR-9 is almost exclusively expressed in the endosomal compartments of cells such as DCs, where it can recognise endocytosed CpG [2]. Activation of TLR-9 leads to the production of IL-12, IFNα, IFNβ and TNFα inflammatory cytokines which enhance T_H1-polarised responses of the adaptive immune system and activate NK cells. B cells and DCs that receive TLR-9 activation will also up-regulate the expression of co-stimulatory molecules, improving T-cell activation capabilities [124]. This adds a crucial adjuvant effect to DNA vaccines; however, transgenic mice which do not express TLR-9 still exhibit robust $CD8^+$ T-cell responses after DNA vaccination [125]. This is probably due to more recently identified pathways by which cytosolic double-stranded DNA (dsDNA) can be recognised as a danger signal. This relies on the fact that DNA is not found outside the nucleus in normal cells. When DNA appears in the cytoplasm as a result of infection or tissue damage, it will be recognised by receptors in the cytoplasm which will in turn initiate downstream signalling cascades [126]. One of the major regulators of this process is the prominent transcription factor NF-κB which controls changes in gene expression leading to production of proinflammatory cytokines such as type I IFN [127].

One cytosolic DNA sensor is DNA-dependent activator of IFN-regulatory factors (DAI), which can recognise DNA from a variety of sources and initiate a signalling cascade leading to the expression of type I IFN [128]. However, studies in transgenic mice which lack this molecule show that immune responses to DNA remain intact [129], indicating that redundancy exists in the pathways involved in sensing cytosolic DNA. Recently, another cytosolic sensor was identified as IFI16 (p204 in mice); detection of dsDNA by this molecule leads to IFNβ production in both macrophages and mouse embryonic

fibroblasts [130]. Interestingly, it is clear that sequence of the dsDNA does not play a role in its recognition by IFI16, in contrast to TLR-9. Another sensor of cytosolic DNA is the absent in myeloma (AIM) 2 inflammasome. This is a complex formed of AIM 2 and apoptosis-related speck-like (ASC) proteins and its formation leads to activation of the transcription factor NF-κB [131]. The creation of this complex also leads to the activation of caspase-1, which cleaves IL-1β into its active form and causes cell death by pyroptosis [132]. These intracellular mechanisms for sensing dsDNA share some common regulators: stimulator of interferon genes (STING) [133] and high-mobility group box (HMGB) proteins [134].

Another pathway which has been identified in intracellular dsDNA recognition involves the RNA-sensing molecule retinoic acid-inducible gene (RIG)-1. It has been demonstrated that AT-rich DNA is converted into RNA which then induces type I interferon production in both mouse and human DCs [135].

Although these pathways have evolved to sense damaged or infected cells, DNA vaccination can take advantage of them. DNA sensed by these pathways will also lead to the activation of innate immunity. This will aid the adaptive immune response by releasing pro-inflammatory cytokines and activating the professional APCs required for cross-presentation of DNA vaccine-encoded antigen.

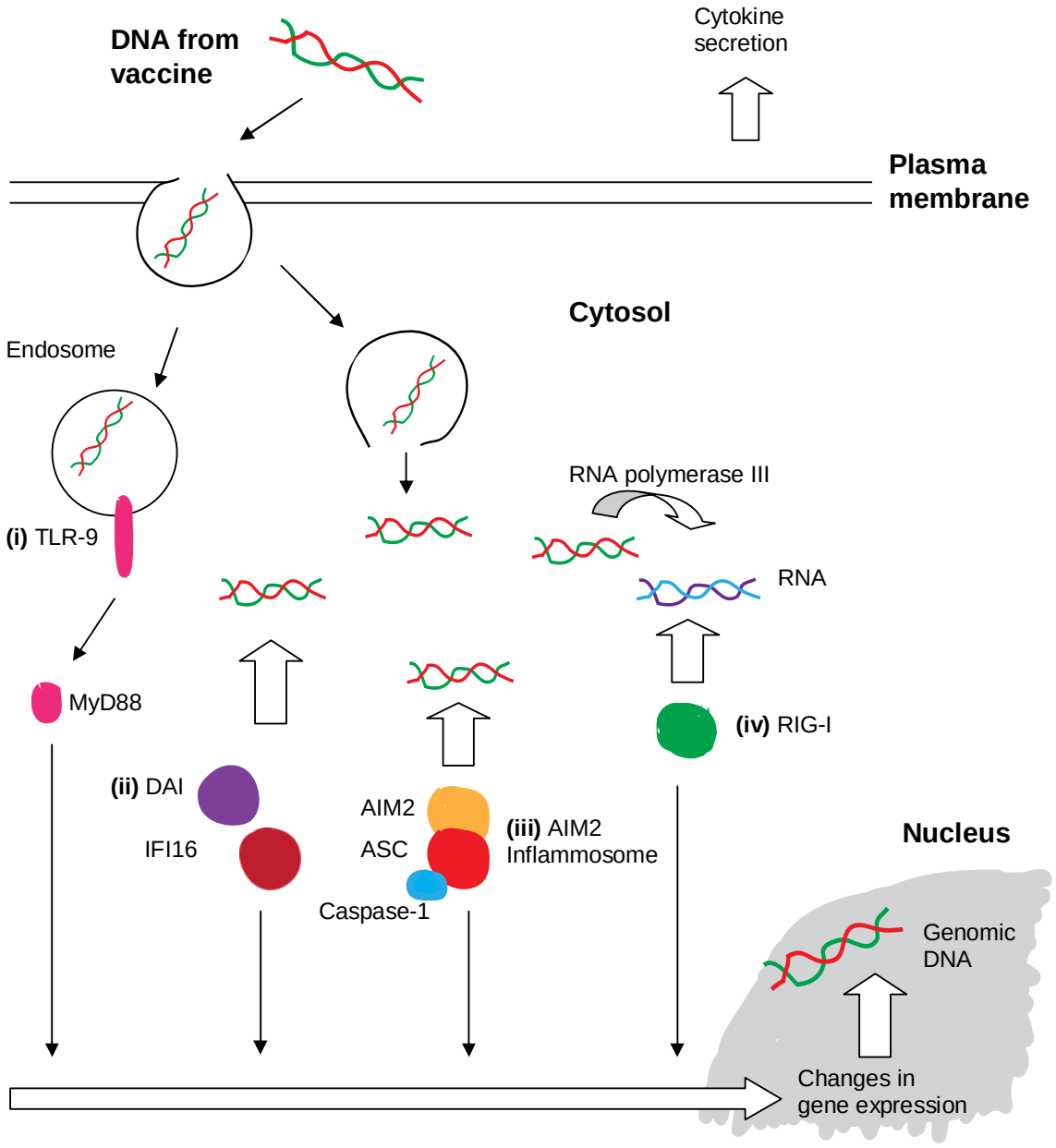


Figure 1.9. Potential DNA vaccine-sensing mechanisms of the innate immune system

Several different mechanisms ensure redundancy in the sensing of DNA by the innate immune system. Under normal physiological circumstances, these mechanisms ensure that infected or damaged cells are recognised. However, when a DNA vaccine enters a cell much as a myocyte or DC, the same pathways will be activated. **(i)** Toll-like receptor (TLR)-9 recognises DNA in the endosomal compartments of DCs and recruits the adaptor molecule MyD88. **(ii)** DNA-dependent activator of interferon-inducible factors (DAI) and IFI16 are cytosolic sensors that recognise DNA in this unusual location. **(iii)** The absent in myeloma (AIM) 2 inflammasome is another of the cytosolic sensors which is a complex of proteins formed when AIM2 binds the adapter molecule apoptosis-related speck-like (ASC) protein. The protease caspase-1 is also recruited, so this pathway also has the potential to lead to cell death. **(iv)** Retinoic acid-inducible gene I (RIG-I) recognises double-stranded RNA which can be generated from a DNA transcript in the cytoplasm by RNA polymerase III-mediated conversion. All of these pathways lead to activation of transcription factors such as nuclear factor (NF)-κB which modulate the expression of cytokines.

1.2.4.3. Adaptive Immune Responses Stimulated by DNA Vaccines

The main goal of DNA vaccination is to stimulate adaptive immunity against the encoded target antigen (adaptive immunity is described in detail in section 1.1.2). A schematic representation of the induction of adaptive immunity by DNA vaccines is shown in Figure 1.10. Naïve B cells with the appropriate BCR recognise membrane-bound or secreted antigen produced by DNA vaccine-transfected muscle cells. In mouse models of lymphoma and myeloma it has been demonstrated that IgG production and protective immunity can be induced by DNA vaccines containing antigenic determinants from the tumour linked to a helper sequence [136]. Mature DCs cross-present DNA vaccine-derived antigen as MHC I-peptide complexes which can be recognised by naïve $CD8^+$ T cells with the appropriate TCR. Activated $CD8^+$ T cells are cytotoxic and are able to kill cells expressing the same MHC I-peptide complex on their surfaces. Both effector and memory $CD8^+$ T cells can be induced by DNA vaccines in mice [137]. DCs displaying DNA vaccine-derived MHC II-peptide complexes stimulate T-cell help which aid both CTL and antibody responses [96].

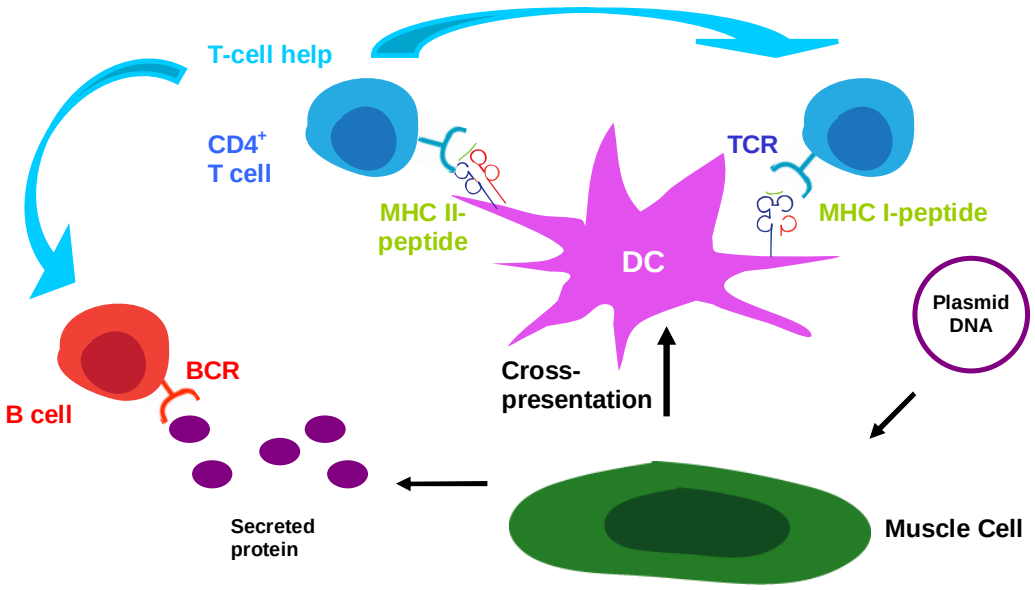


Figure 1.10. Activation of adaptive immunity by DNA vaccines

B cells recognise membrane-bound or secreted protein derived from DNA vaccines via the B-cell receptor (BCR) and produce antibody. Dendritic cells (DCs) are either directly transfected with or obtain DNA vaccine-derived antigen from the extracellular environment, process it into short peptides and then present it to $CD8^+$ and $CD4^+$ T cells via major histocompatibility (MHC) class I or II molecules respectively. For $CD8^+$ cells, this occurs via cross presentation. T cells recognise MHC-peptide complexes via the T-cell receptor (TCR) and gain effector functions: $CD4^+$ T cells become helper cells and $CD8^+$ T cells become cytotoxic.

1.2.4.4. DNA Vaccine Dose and Volume

Several studies have shown that DNA vaccines can certainly induce immune responses in mice [138], [137], [136]. However, ultimately they must succeed in generating responses in rather larger human subjects. Early DNA vaccine clinical trial data proved disappointing, possibly due to the issues with scaling up the dose and volume of DNA vaccines given.

The optimal DNA dose in humans is unknown, but in mice $CD8^+$ T-cell responses do not appear to be greatly affected by DNA dose [139]. Perhaps more important is the question of injection volume. The typical 50μL intramuscular injection volume in mice would equate to approximately 175mL in humans; making it unfeasible to scale-up. Studies in mice have revealed that reducing the volume of a specified dose of a DNA vaccine can result in dramatically reduced vaccine-specific $CD8^+$ T-cell responses [139]. Larger injection volumes will cause increased hydrostatic pressure on the muscle, leading to greater inflammation at the site and improved myocyte transfection which will be crucial for inducing an immune response. The reduction in inflammation associated with smaller injection volumes may prevent strong responses to similar DNA vaccines in patients. For this reason, it is necessary to improve the efficacy of DNA vaccines by utilising some of the strategies detailed below.

1.2.4.5. Delivery of DNA Vaccines

Despite the success of DNA vaccines in small animal models, it is clear that further refinements must be made in order to translate them into effective immunotherapy for patients. The process of delivering the DNA to cells is one area where improvement is sought; with several different methods being actively investigated.

One strategy used to protect the DNA and to facilitate cell transfection is the physical linkage of a DNA vaccine to an inert carrier. These carriers may consist of microparticles, nanoparticles or polymer (reviewed in [140]).

Gene gun vaccination is a needleless method of vaccination which employs gold particles coated with DNA vaccine for administration into the epidermis. This has proved successful in raising immunity to low doses of DNA in both pre-clinical models and clinical trials for infectious diseases [141]; possibly because the DNA is directly delivered to DCs in the skin [142]. DNA vaccines can also be enclosed in cationic liposomes to protect against degradation by nucleases and increase transfection efficiency. There is evidence that this does induce greater immune responses in mice compared to naked DNA vaccine alone when low doses are used [143].

DNA vaccines can also be applied directly to the skin and then 'tattooed' into the dermal and epidermal layers of the skin. This results in reduced antigen expression compared to a standard intramuscular injection but, perhaps surprisingly, more rapid immune induction, with protective immunity to influenza established within two weeks [144].

An important strategy to improve transfection of cells at the injection site after a standard intramuscular injection with naked DNA is electroporation (EP). This method is used routinely to transfect cells with DNA or RNA *in vitro*, and has now been adapted to permit *in vivo* transfection. The technique usually involves intramuscular injection of a DNA vaccine, followed by several pulses of electricity given across the injection site. The electric fields create a large membrane potential, giving rise to transient pores in the cell membrane through which the DNA can enter [145]. Myocyte transfection has been visualised using DNA plasmids encoding reporter proteins such as β-galactosidase or luciferase. Results show that the level of β-galactosidase expression is proportional to the number of pulses given directly after injection, but if the duration of the pulses exceeds a critical amount, reporter gene expression decreases. The reason for this is probably the muscle damage that results from EP, demonstrated by the presence of necrosis in the muscle fibres three days after injection, which also increases with longer pulse durations [145]. Interestingly, evidence suggests that this muscle damage may be related to the DNA itself. When mice were given EP after injection of saline solution alone, no muscle damage was present 7 days later, in marked contrast to those given plasmid DNA [146]. Although there is clearly a need to balance the level of muscle damage with vaccine efficacy, it is also clear that some damage is beneficial to the immune response. This is due to the local inflammation it causes [147] and the release of apoptotic vesicles containing the protein encoded by the DNA vaccine, which could be engulfed by DCs for cross-presentation [148]. It is thought that the increased rate of cell transfection with vaccine and local inflammation caused by EP result in the improved immune responses seen in mouse models. Importantly, it has been demonstrated that EP can 'rescue' reduced $CD8^+$ T-cell responses induced with a sub-optimal vaccine volume in a mouse model [139]; which is very promising for translation to the clinic.

In fact, EP is already being used in the clinic. In Southampton, our DNA vaccine design has been tested in a phase I/II study being carried out in patients with recurrent prostate cancer. There have been promising results after vaccination with DNA encoding a prostate cancer antigen linked to a microbial sequence from tetanus toxin with or without EP. EP was well tolerated, immune responses were induced and data indicate that patients who received EP had increased vaccine-specific antibody responses [149]. However, the effects

of EP on $CD8^+$ T-cell responses are less clear at this stage (L Low, personal communication).

1.2.4.6. Prime-boost Strategies for DNA Vaccination

Immunisations are typically given in a prime-boost setting in order to maximise immune responses, and DNA vaccines against cancer are no exception. However, instead of a simple prime-boost with multiple injections of DNA, heterologous boosting with a different modality derived from the same antigen has emerged as an attractive way of boosting immune responses to DNA vaccines. Some of the strategies currently under investigation are described below.

Modified vaccinia virus Ankara (MVA) is an attenuated form of vaccinia virus commonly used as a vehicle to deliver antigen, which can also be used to boost immune responses to a naked DNA vaccine. One informative study using antigens from the parasite *Plasmodium falciparum* clearly demonstrated that a primary vaccination with DNA followed by a booster vaccination with MVA encoding the same antigen induced T-cell responses and protected mice against challenge. In contrast, a second DNA vaccination failed to boost T-cell responses or confer protection after a primary vaccination of either DNA or vaccinia virus [150]. This shows the potential of viral vectors as a boost for DNA vaccines; however, it is likely that the success of viral vectors will be limited by any pre-existing immunity, or immunity that develops against the virus itself after multiple vaccinations, as the production of neutralising antibodies against viral vectors is well documented [151]. Thus, viral vectors may be less appropriate as cancer vaccines since it is likely that repeated vaccinations will be necessary to maintain an ongoing response against the tumour. CTL epitopes within a viral vector may also be immunodominant, resulting in immune responses against the virus instead of the tumour antigen, particularly if the tumour antigen is only weakly immunogenic (more information about immunodominance can be found in section 1.1.2.11.3). As a primary vaccination of MVA followed by either a homologous or DNA boost results in far lower T-cell responses than a DNA/MVA regimen [150], this suggests that immune responses to the virus dominate the primary response. Immundominance has also been reported in a melanoma clinical trial in which $CD8^+$ T-cell responses to the MVA vector out-competed those to the target antigen [152]. In contrast, a DNA vaccine prime, which directs the immune response to the antigen of interest, followed by the highly immunogenic MVA boost succeeds in inducing protective anti-malarial T-cell responses in human subjects [97].

There is some evidence from vaccines against infectious diseases that boosting DNA vaccine-induced responses with the same antigen in the form of a protein can prove very effective at stimulating antibody production [153], [154], [155]. Similarly, virus-like particles containing a tumour antigen can boost responses induced by a DNA vaccine encoding the same antigen, resulting in modest improvements in protection from subsequent tumour challenge [156].

A primary DNA vaccination can also be boosted more successfully by giving EP (see section 1.2.4.5 for more information on EP) with DNA at the time of boosting [139], particularly in sub-optimal conditions; presumably due to increased inflammation and cell transfection EP causes [145].

1.2.4.7. Adjuvants for DNA Vaccines

Adjuvants are additives which can be given with vaccines to enhance their immunogenicity. Aluminium-based adjuvants were first discovered in the early 20th century and are still the most commonly used in clinical practice today. Aluminium salts are utilised primarily for their ability to help stimulate robust antibody responses but they appear to have effects on both innate and adaptive immunity, though the exact mechanisms which mediate this are still not fully described [157].

More recently, many cytokines and co-stimulatory molecules have been used to enhance immune responses to DNA vaccines [158], either in the form of recombinant protein or as part of the genetic information in the DNA vaccine itself. One commonly used cytokine is granulocyte-macrophage colony-stimulating factor (GM-CSF) which enhances both cellular and humoral immunity. When GM-CSF was co-expressed with a tumour antigen in a mouse model it protected against subsequent tumour challenge, but interestingly this was not the case when the two components were given in separate plasmids [159]. As DNA vaccines are more likely to transfect somatic cells at the injection site which do not express the relevant co-stimulatory molecules for T-cell activation, it is possible that augmenting this signal may increase T-cell responses. In fact, DNA vaccination with a plasmid encoding the co-stimulatory molecule CD86 at the same time as a DNA vaccine encoding viral antigens dramatically increased both $CD4^+$ and $CD8^+$ T-cell responses in mice and chimpanzees [160].

Modifications to DNA vaccines which alter their intracellular targeting can also be thought of as a form of adjuvant as they can affect immunogenicity. The cellular location of antigen is important because it must be processed in specific compartments before it can

be presented to the immune system (see section 1.1.4.2). To this end, DNA vaccines have been targeted to the cytosol, ER or lysosomal and endosomal compartments (reviewed in [161]).

DNA can be targeted to the ER with a signal sequence taken from a secreted protein. In the ER, protein folding and initial glycosylation occur and secreted proteins traffic through to the Golgi, so this route gives rise to mature proteins which can be recognised by B cells. Indeed targeting to the ER does produce robust antibody responses [136], but $CD8^+$ T-cell responses are also effectively induced [162]. Even so, many attempts have been made to improve these responses. One example is a DNA vaccine encoding a minimal $CD8^+$ T-cell epitope fused to an ER-targeting signal peptide, along with a modified version of the invariant chain in which a helper T-cell epitope replaces CLIP to direct it to MHC II molecules [163]. This vaccine results in a slower development of T-cell responses yet a larger peak when compared to a simple vaccine encoding the minimal epitope fused to a helper T-cell sequence. Interestingly, a heterologous prime-boost strategy using these two vaccines in combination is more effective than either vaccine alone in a homologous setting. These results demonstrate how easy it is to manipulate DNA vaccines for intracellular targeting purposes and how complex the consequences can be.

Another way to provide an adjuvant for a DNA vaccine is to physically fuse the antigenic DNA to immunostimulatory sequences. The E7 gene from HPV16 has been fused to the *Escherichia coli* β-glucuronidase gene which appeared to improve its immunogenicity and efficacy in tumour therapy experiments in mice [164]. Similar results have been found after fusion of E7 to the *Mycobacterium tuberculosis* heat shock protein 70 gene [165]. Several tumour antigens have been fused to tetanus toxin DNA in the past as a way of inducing helper T cells from a non-tolerised pool [96]. This leads to augmented antibody [136] and $CD8^+$ T-cell responses, even in a tolerised repertoire [137], [166]. This is the strategy employed in our vaccines, which are described below.

1.2.4.8. Our DNA Fusion Vaccines

In order to overcome some of the issues described above, we have developed novel DNA fusion vaccines. Within our DNA fusion vaccines, pathogen-derived DNA is linked to specific sequences from tumour antigens. The microbial sequence is able to activate a non-tolerised pool of $CD4^+$ T cells, providing T-cell help for immune induction against the linked tumour antigen sequence. This is particularly important in cancer where there is often tolerance to overcome, as most tumour antigens are self proteins. A schematic

representation of the basic design which encodes only a minimal $CD8^+$ T-cell epitope is shown in Figure 1.11, but larger portions of antigen may also be used. The microbial sequence used in our vaccines is derived from tetanus toxin (TT), which is produced by the *Clostridium tetani* bacterium. The structure of TT is shown in Figure 1.12. The protein consists of the N-terminal ~50kDa light chain and C-terminal ~100kDa heavy chain linked by a disulphide bridge [167]. The heavy chain is responsible for binding of and internalisation into neurones; the light chain is an intracellular metalloprotease that cleaves synaptic proteins at the neural-muscular junction, resulting in spastic paralysis [168]. The C-terminal end of the heavy chain contains a ~50kDa domain known as Fragment C (FrC), which is not toxic and thus safe for *in vivo* use [169]. It is this portion of TT that is used in our vaccines [96]. Within FrC there are two domains, DOM 1 and DOM 2. DOM 1 is composed of four helices and sixteen β-strands, some of which form a jelly-roll motif similar to motifs found in plant lectins; DOM 2 exhibits a β-trefoil motif, which are often involved in recognition and binding [170].

Crucial to the success of this strategy is the presence of a 'promiscuous' helper T-cell epitope within FrC. This peptide, known as p30, which consists of residues 947-1315 of TT was identified after it was found to be recognised by a $CD4^+$ T cell clone from a human donor [171]. It has been subsequently demonstrated that p30 is recognised by donors with many different MHC II haplotypes [172], and mice [166]; leading to the term 'promiscuous'. This means that vaccines containing FrC could potentially induce p30-specific $CD4^+$ T cells in many patients.

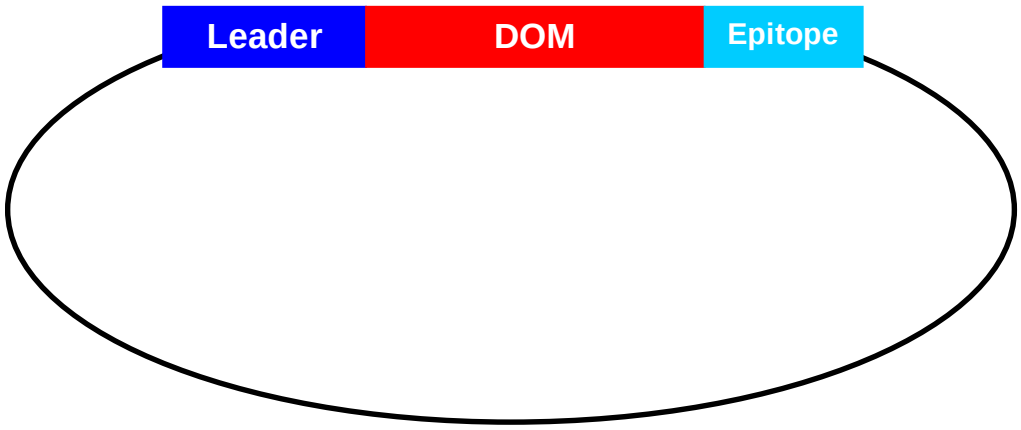


Figure 1.11. DNA vaccine design used in this study

The vaccines are based on a commercial DNA plasmid (pCI, Promega) that has been modified to produce fusion genes under the control of the immediate/early enhancer and promoter region from cytomegalovirus by cloning into the multiple cloning region. The vector backbone contains an ampicillin resistance gene to facilitate cloning and, due to its bacterial origin, contains sequences which are recognised by the innate immune system. The fusion gene consists of a leader sequence which ensures that the protein produced is targeted to the ER; domain 1 from FrC of tetanus toxin (DOM) which stimulates helper T cells; and a peptide epitope recognised by $CD8^+$ T cells.

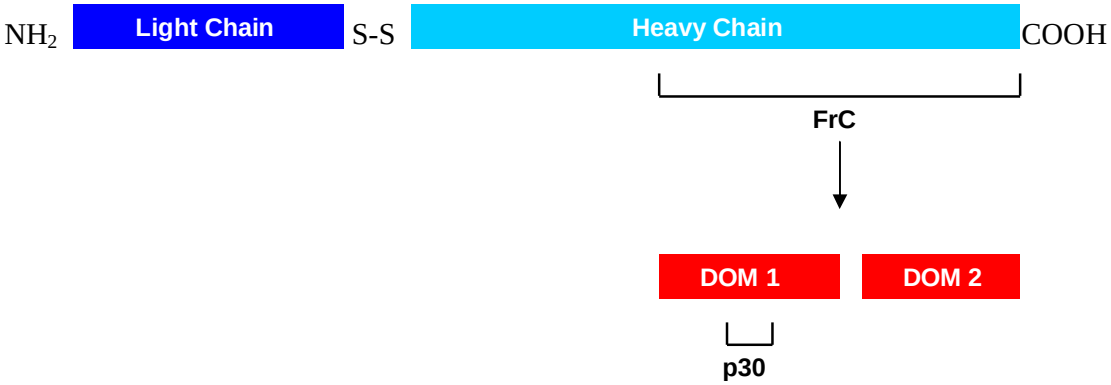


Figure 1.12. The structure of tetanus toxin (TT)

TT protein is 1315 amino acids in length, consisting of a 50kDa light chain and a 100kDa heavy chain linked by a disulphide bond. The heavy chain controls binding and internalisation into neurons; the light chain is responsible for the cleavage of synaptic proteins. The COOH-terminal fragment within the heavy chain known as fragment C (FrC) contains two domains, domain 1 (DOM 1; $TT_{865-1120}$) and domain 2 (DOM 2; $TT_{1121-1315}$). DOM 1 contains a promiscuous helper T cell epitope called p30 ($TT_{947-967}$).

While helper T cells induced by microbial sequences such as DOM within a DNA vaccine can enhance a response, this must be balanced against any DOM-specific $CD8^+$ T cells that are induced concurrently. This is important due to the phenomenon of immunodominance (described in section 1.1.2.11.3) which focuses the immune response against so-called 'dominant' peptide epitopes. When the full length FrC is fused to an antigenic peptide epitope in a DNA fusion vaccine, the $CD8^+$ T-cell response is diverted to dominant peptides derived from DOM 2 instead of the target CTL epitope [173]. Therefore, only the DOM 1 portion of FrC is used here, in order to minimise this phenomenon.

Our vaccines also include a leader sequence, derived from the V_H gene from the IgM of the BCL_1 tumour, which targets the encoded protein to the ER and was found to improve vaccine-specific antibody and CTL responses [174]. As described in section 1.2.4.1, DNA vaccines are thought to prime T cells via cross-presentation and indirect evidence from MHC-mismatched tumour challenge experiments supports this in the case of the pDOM-epitope design. In these experiments, $CD8^+$ T cells proliferated *in vivo* in response to challenge with pDOM-epitope transfected tumour cells of either matched or mismatched MHC [163]. This shows that pDOM-epitope DNA can induce immune responses, even if an APC is not directly transfected; presumably via cross-presentation. Furthermore, experiments using several OVA-specific DNA vaccines which targeted the antigen to different cellular locations, it was clear that secreted OVA induced better CTL responses than cytoplasmic OVA; providing more evidence that cross-presentation is important [175].

Using the pDOM-epitope strategy, we have demonstrated that a pathogen-derived domain (DOM) of fragment C from tetanus toxin combined with an MHC class I-binding peptide of interest can activate high levels of $CD8^+$ T lymphocytes against the peptide epitope and induce protective or therapeutic immunity against tumour [96].

1.3. The Human Papillomavirus

The human papillomavirus (HPV) family is a large group of double-stranded DNA viruses which can infect humans and cause disease. The classification of the viruses is shown in Table 1.1. Each HPV sub-type is classified as high-risk, low-risk or cutaneous depending on the frequency at which they cause cancer and their prevalence in the population [176]. Low-risk HPV types cause benign warts at cutaneous or mucosal sites. However, high-risk HPV types are associated with cancer.

Evolutionary Group	Sub-types	Category
Alpha	60+	Low-risk, high-risk & Cutaneous
Beta, Gamma, Mu & Nu	40+	Cutaneous

Table 1.1. The classification of human papillomaviruses

There are 5 evolutionary groups which comprise of over 100 sub-types. Each sub-type is further classified as cutaneous, high-risk or low risk. Information taken from [176].

1.3.1. Epidemiology

A recent meta-analysis of published data found that the overall world-wide prevalence of HPV infection was 10.4% in women with normal cytology, and that the most commonly found type was HPV16 at 2.5%, followed by HPV18 at 0.9% [177]. Worryingly, these are both high-risk types, with HPV16 being responsible for 60-70% of cervical carcinomas and >80% of vulval and penile carcinomas caused by HPV [178]. There are also reports of HPV DNA being found in other cancers, the most compelling example being head and neck cancer [179]; however its association with cervical cancer is the strongest. Cervical cancer is the second most common cancer in the female population worldwide and HPV DNA is found in almost all cases. According to the World Health Organisation, there are around half a million new cases of cervical cancer every year and over a quarter of a million deaths (figures from 2010). Coupled with the fact that the incidence of this disease peaks in young women in their thirties as well as the elderly, the impact of HPV is not trivial.

1.3.2. Virus Structure

HPV is a double-stranded circular DNA virus housed within an icosahedral capsid. The small (~8kb) papillomavirus genome, shown in Figure 1.13, is highly conserved among the different types. It consists of just eight genes which are split into two groups according to the timing of their expression and their function. Early-expressed genes E1, E2, E4, E5, E6 and E7 are involved in non-structural functions; and late-expressed genes L1 and L2 have structural functions [178].

1.3.3. Virus Life Cycle

The life cycle of HPV, shown Figure 1.14, takes advantage of the infection site it targets, the mucosal epithelium. The high cell turnover and constant cell shedding which are found in this tissue facilitate viral replication and transmission to the next host.

On entry to the host, the virus infects a basal keratinocyte, probably a stem cell, at a low copy-number [102]. This occurs after a microabrasion of the overlying epithelium, revealing the underlying basal stem cells. If low copy-number virus merely infects a suprabasal non-stem cell, the predicted outcome is that the infection will be quickly cleared as the cells shed [180]. *In vitro* evidence suggests that virus entry to the cell is initiated by binding heparin sulphate proteoglycans in the cell membrane [181].

In the basal cells, E1 and E2 are thought to play a role in the formation of a stable episome in which the virus can replicate. E2 is a DNA-binding protein that recognises a motif in the non-coding region of the genome; after binding occurs E1 can be recruited to the viral origin to carry out its helicase function and replication can begin. Replication occurs concurrently with the host cell during S-phase in the cell nucleus. Copy number increases by 5 to 10-fold in the basal layer, followed by a period of little replication until the host cell enters the proliferative squamous layer when viral DNA is dramatically increased. At this time, as the epithelial cells are differentiating, the viral genes E6, E7, L1 and L2 are highly expressed and the mature virus is assembled (HPV biology is reviewed in [176] and [102]).

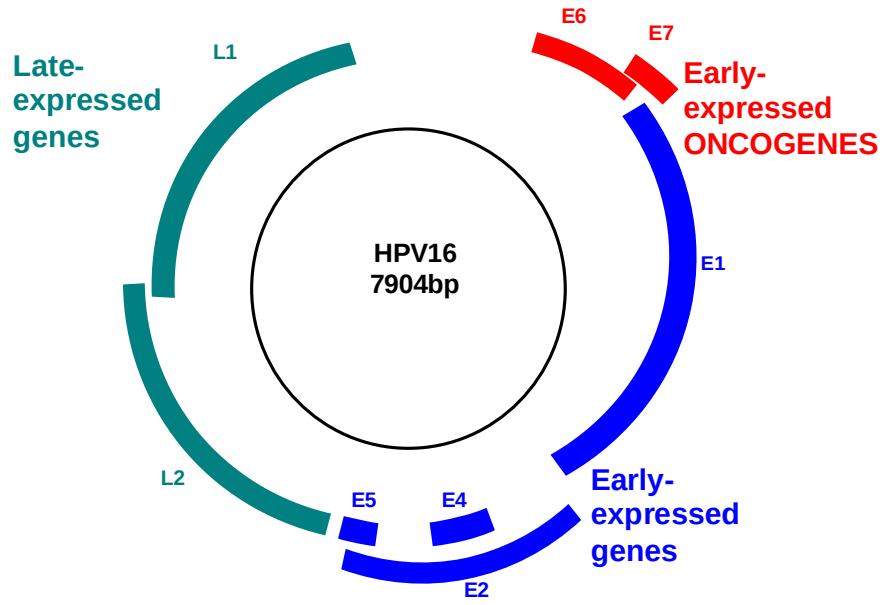


Figure 1.13. The HPV genome

The double-stranded DNA virus is composed of eight coding regions which consist of the six early-expressed genes (E1-2 & 4-7) which function in viral replication and pathogenicity; and the two late-expressed genes (L1 & 2) which form the coat proteins (adapted from [176])

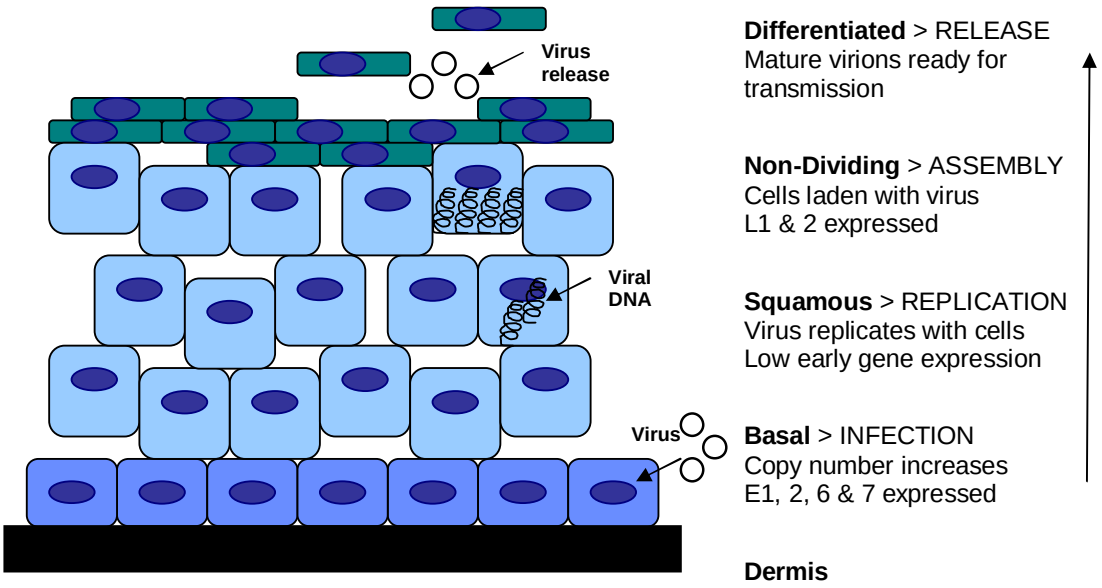


Figure 1.14. HPV infection of mucosal epithelium

The virus life cycle follows the progression of the keratinocytes through the cell cycle. Viral genes are expressed at different levels in each of the stratified layers, depending on function. The process takes around three weeks, which is also the length of the epithelial differentiation programme.

1.3.4. Immune Evasion

The major way in which HPV evades the host immune system is closely linked to its life cycle and the site that it targets. As discussed in section 1.3.3, the virus replicates within the nucleus of epithelial cells which later die of natural causes, not necrosis. This results in very little inflammation which would normally alert the immune system to a viral infection. Furthermore, the virus does not infect other cell types or lead to viremia so there is little antigen for the host immune system to react to. Even in the keratinocytes, viral gene expression is tightly regulated [102].

However, viral antigen will inevitably be exposed to the host immune system, so further attempts to avoid immune activation are made. Many viruses have evolved mechanisms to increase cell proliferation and reduce cell apoptosis, in order to maximise production of virus. These include mechanisms which interfere with various aspects of host adaptive immunity, and HPV is certainly no exception. Methods employed by HPV include inhibition of cytokine production or skewing of cytokine profiles, modulation of adherence molecules, modulation of intracellular signalling and impairing antigen presentation (reviewed in [182]). Molecular mimicry is another strategy employed by pathogens whereby their proteins resemble those of the host; taking advantage of the host's tolerance to self antigens. There is some evidence that HPV16 E7 has sequence similarities to human proteins which may be indicative of this [183].

1.3.5. Immunity to HPV

Despite the array of immune evasion strategies, both high- and low-risk HPV infections resolve spontaneously in the overwhelming majority of cases, as demonstrated by many population studies [184], [185], [186]; providing evidence for the presence of an immune response.

Although it is difficult to separate genetic and environmental factors, there is also evidence that there may be some heritable component to HPV susceptibility. Studies of Swedish population data have shown a convincing familial association with cervical cancer risk [187], [188] though a criticism of studies such as these is the fact that they are unable to control for HPV exposure. Little is known about the nature of this genetic association but genes involved with the immune response have been implicated. Polymorphisms in the interferon regulatory factor 3 (*IRF3*) gene are found to be associated with HPV persistence and a variant of the *TLR-2* gene is associated with progression to cervical intraepithelial

neoplasia stage 3 (CIN3) or cervical cancer [189]. Many studies have also investigated the relationship between particular HLA genes and cervical cancer (reviewed in [190]). Interestingly, there is evidence to suggest a protective role for the HLA class II genes *DQB1*13* and *DQB1*0603* [190].

Serological data shows that most women who are positive for HPV16 DNA produce IgG antibodies against the L1 protein [191]. In addition, studies in healthy donors provide evidence that there is a $CD4^+$ T-cell response against E6, E7 [192] and E2 proteins [193]. Furthermore, both $CD4^+$ and $CD8^+$ T cells been found in greater numbers in regressing HPV-positive genital warts versus non-regressing cases [186].

This evidence demonstrates the importance of the immune response in clearing HPV infections and preventing cervical cancer. It also indicates that immunotherapy may be useful in patients who do progress to cervical cancer.

1.3.6. Progression to Cervical Cancer

Although there is clearly a successful immune response in most HPV-infected individuals, as discussed above, a minority of patients do progress to cancer. Understanding not only the success of the immune response but also, and perhaps more importantly, its failure will be important in developing effective therapy for the disease. In order to clarify the stages of CIN that can lead to cervical cancer, Table 1.2 describes the clinical terminology.

In many cancers immune tolerance prohibits an effective response. However, cervical cancer is caused by a virus and as such, will provide non-self antigen, unlike many other cancers. Even so, HPV-specific regulatory T cells may impair the immune response against HPV. In patients with CIN, although specific helper T cells are present, specific regulatory T cells are also associated with HPV16 persistence [32]. Regulatory T cells from LN and tumour biopsies do inhibit effector function of their T helper cell counterparts [194], and $CD25^+$-cell depleted cultures from CIN patients reveal increased responses to E6 and E7 peptide pools [195] in *in vitro* studies. Therefore, regulatory T cells are likely to modulate anti-cancer immunity *in vivo*; thus increasing the chance of progression to cervical cancer.

Disease	Grade	Description
LSIL	CIN1	Viral cycle still regulated
HSIL	CIN2	Production of virions restricted to epithelial surface, viral DNA becomes more widely expressed, deregulation of oncogenes
	CIN3	
Cervical Cancer		Later stages of viral life cycle are lost, usually no episomes, viral DNA integrates host genome

LSIL, low-grade squamous intraepithelial neoplasia lesions; HSIL, high-grade squamous intraepithelial neoplasia lesions; CIN, cervical intraepithelial neoplasia

Table 1.2. The stages of cervical intraepithelial neoplasia (CIN) which can lead to cervical cancer

The first signs of disease are low-grade squamous intraepithelial neoplasia lesions (LSIL) and it is thought that the majority of these lesions do not progress. Those that do go on to form high-grade squamous intraepithelial lesions, at which point the normal life cycle of HPV breaks down. The expression of viral proteins is altered as E7 is more widely expressed throughout the epithelium but E1, E2, E4, L1 and L2 expression becomes restricted and is eventually lost in cervical cancer. Summarised from [176].

One reason why some HPV-infected individuals progress to cervical cancer is immunosuppression. There is a peak of cervical cancer prevalence in the elderly, explained by age-related immunosuppression which has been demonstrated in women aged over 65 years with a persistent HPV infection [196]. This also fits with data from individuals with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and transplant recipients, who have an increased risk of HPV-related cancers [197]. This also confirms the importance of the immune response in preventing HPV-related cancers.

Another important factor to consider is viral integration. High-risk HPV integration is seen even in early-stage CIN and by the time the disease progresses to cervical cancer, most tumours exhibit the phenomenon [198]. This does not occur as part of the natural HPV life cycle (which is shown in Figure 1.14), but as a result of viral gene deregulation [199].

High levels of viral gene expression are facilitated by integration, and in high-risk subtypes, this includes the oncogenes E6 and E7 which have transforming potential [200] and thus cancer can ensue. As integration of the viral DNA into the host DNA probably occurs after the accumulation of mutations in both genomes over a period of time, viral persistence increases the risk of cervical cancer development. This provides a link between failure of the immune response and progression to cancer.

1.3.7. Current treatments for Cervical Cancer

The treatment of cervical cancer is dependent on the stage of the disease at diagnosis but consists of the standard course of action for tumours: surgery, radiotherapy and chemotherapy. Due to their invasive nature and the obvious impacts on fertility, these treatment options are certainly not desirable.

Early diagnosis is crucial for preserving fertility and curing the disease, so in many countries there is a screening programme in place. In the UK, women aged 25-64 are invited to undergo a smear test every 3-5 years to identify any cellular abnormalities in the cervix. Around 80% of women in the target age group are tested, which should ensure a 95% reduction in deaths (figures from the NHS Website*). However, a major proportion of the disease burden is in less economically developed countries where funding and administering such a programme would be considerably more difficult.

With all infectious diseases, the mantra 'prevention is better than cure' holds true and thus vaccines are the ultimate goal. HPV is no exception and, in fact, recently two prophylactic vaccines against high-risk HPV have been licensed. These vaccines are based on virus-like particles first produced more than 15 years ago [201], [202] which consist of the L1 and L2 proteins from HPV. Gardasil (produced by Merck Chemicals Ltd) includes L1 and L2 from HPV6, 11, 16 and 18 [203] and Cervarix (produced by GlaxoSmithKline) includes L1 and L2 from HPV16 and 18 [204]. These vaccines induce protective antibody responses that prevent infection with these particular strains of HPV [203], [204]. However, women who are already infected with HPV16 or 18 at the time of vaccination with Cervarix do not clear the virus more frequently or quickly than untreated controls [205]. Unfortunately, this means that for the many thousands of women already infected with high-risk HPV, these vaccines will not reduce their chances of progressing to cancer.

* http://www.cancerscreening.nhs.uk/cervical/about-cervical-screening.html

1.3.8. E7 Oncoprotein

1.3.8.1. Gene structure and function

The E7 gene, shown in Figure 1.15, is translated into a 98 amino acid protein with several functions. It is thought that E7 is involved in viral replication but, certainly for high-risk HPV types, E7 is an oncoprotein which interferes with several host pathways (reviewed in [206]). *In vitro* studies have shown that the E7 protein from high-risk HPV16 is sufficient for the immortalisation of human epithelial cells, in contrast to E7 protein from low-risk HPV6. It seems that whilst the other viral oncoprotein, E6, is not sufficient for transformation of human cells, it acts in synergy with E7 [200].

A major oncogenic function of the E7 gene is its ability to bind retinoblastoma susceptibility gene product, Rb. The *Rb* gene is a tumour-suppressor gene which regulates progression from G1 to S phase of the cell cycle [207]. E7 has been shown to cause degradation of Rb protein *in vitro* [208], thus removing its inhibition of the transcription factor E2F which usually halts the cell cycle, leading to aberrant proliferation. In conjunction with the actions of E6; namely that which causes the degradation of another tumour-suppressor gene, *p53* [209], this unchecked proliferation may allow mutations that permit viral integration to develop.

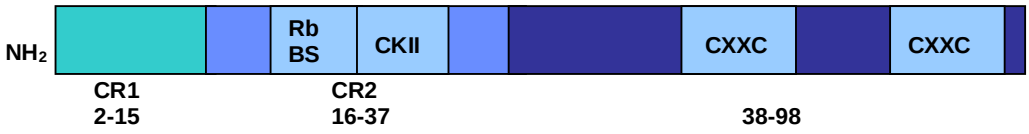


Figure 1.15. The E7 oncoprotein

Within the E7 oncoprotein there are two conserved regions (CR) which share homology with E1A from adenovirus and some sequences from SV40. In CR2, there is an Rb binding site (BS) and a casein kinase II phosphorylation site (CKII). The COOH terminus contains a zinc-binding domain consisting of two CXXC motifs. Schematic adapted from [210].

E7 also aids the virus to avoid immune surveillance by host $CD8^+$ T cells which lyse virally infected cells in an MHC class I-dependent manner. Viral interference with this process is well documented [211], and HPV is no exception. *In vitro* chloramphenicol acetyl transferase (CAT) assays show that MHC class I heavy chain promoter-driven expression is reduced by co-transfection with plasmids containing E7 from high-risk HPV16 but not low-risk HPV6 [212]. *In vivo* challenges with HPV16 E6 and E7-expressing TC-1 tumour cells sometimes give rise to escape variants in vaccinated mice. Sub-lines derived from these tumours which express fewer MHC I molecules on the cell surface are more oncogenic in subsequent challenges [213]. Human cervical carcinomas also exhibit the phenomenon of reduced or lost MHC expression [214], [215], [216], so this is an important caveat to bear in mind when developing immunotherapy to induce $CD8^+$ T cell mediated killing, which relies on the presence of target MHC class I-peptide complexes on the tumour surface.

1.3.8.2. Immune Responses to E7

There is some evidence that patients with CIN or cervical cancer do produce an immune response to HPV16 E7, as both $CD4^+$ [217] and $CD8^+$ T cells [218] can be detected in *ex vivo* assays, though these responses are certainly not found in all patients. Tumour-infiltrating lymphocytes (TILs) have also been reported and seem to correlate with a reduced likelihood of metastasis [219]. Importantly, this cell-mediated immune response seems to be observed more frequently in the later stages of cervical lesions, which may explain why it may ultimately be ineffective [220].

The natural immune responses described above expose the potential for generating cell-mediated immunity against cervical cancer. However, these responses alone fail to control the tumour. So the question is this: Can we improve them with immunotherapy?

2. Materials and Methods

2.1. Vaccine Design and Construction

2.1.1. Polymerase Chain Reaction (PCR)

Vaccines were constructed by PCR. Forward and reverse oligonucleotide primers were used to amplify DNA fragments from a DNA template. The resulting fragments encoded the target sequence flanked by specific endonuclease restriction sites at either end to allow for subsequent ligation into a vector. Forward primers encoded an *Nhe*I site followed by the start of the fusion gene and reverse primers encoded the end of the fusion gene followed by a *Not*I site. Details of the primer sequences can be found in Table 2.1 and the PCR conditions used are given in Table 2.2. A typical PCR mix was as below:

0.1µg template DNA
5µL 10x PCR buffer (containing 15mM $MgCl_2$)
5µL 2.5mM dNTPs
50pMol forward primer
50pMol reverse primer
1µL 5 units/µL HotStar Taq polymerase
<50µL H_2O

All PCR reagents were purchased from Qiagen, Crawley, UK except dNTPs which were purchased from Promega, Southampton, UK. Primer details are given in Table 2.1.

Some of the vaccines included the domain 1 (DOM) from fragment C of tetanus toxin. DOM had been fused to the IgM signal sequence from the BCL_1 tumour previously [173]. In constructs where DOM was absent, the signal sequence was directly fused to the E7 sequence.

All vaccines were constructed using the pCI vector (Promega, Eastleigh, UK); a map of the plasmid including the restriction sites present can be found on the manufacturer's website*. After fusion genes described below were constructed, they were then ligated into the pCI vector as described in section 2.1.2.

* http://www.promega.com/figures/popup.asp?partno=e1731&product=pci+mammalian+expression+vector&fn=0685va

To make pDOM, the forward primer NheI-BCL_1F1 and reverse primer pJR5p1 were used in a PCR with pcDNA3.DOM as a template. This resulted in a DNA fragment consisting of DOM with an upstream *Nhe*I restriction site and a downstream *Not*I restriction site from the multiple cloning region of the pcDNA3 backbone.

The validated pDOM construct was used as a template to make the pDOM.$E7_{49-57}$ construct. The NheI-BCL_1F1 forward primer and HPVconsR1 reverse primer were used in a PCR to make the fusion gene encoding the BCL_1 leader fused to DOM followed by the H-$2D^b$-resistricted $E7_{49-57}$ epitope from HPV16 E7 (RAHYNIVTF), flanked by upstream *Nhe*I and downstream *Not*I restriction sites.

Two initial PCRs were carried out to make the p$E7_{43-77}$ construct. In the first, the 5' end of the $E7_{43-77}$ sequence was fused to the BCL_1 leader sequence using the NheI-BCL_1 F1 forward primer and the HPVconsR3 reverse primer with pDOM as a template. In the second, the $E7_{43-77}$ sequence was amplified using TC-1 cell cDNA as a template with the forward primer HPVconsF1 and reverse primer HPVconsR5. These two fragments of DNA were then fused in a splicing by overlapping extension (SOE) PCR using the primers NheI-BCL_1 and HPVconsR5. This resulted in a fusion gene encoding the BCL_1 leader fused to the $E7_{43-77}$ sequence, flanked by upstream *Nhe*I and downstream *Not*I restriction sites.

Two initial PCRs were also carried out to make the pDOM.$E7_{43-77}$ construct. In the first, the NheI-BCL_1 F1 forward primer and HPVconsR4 reverse primer were used in a PCR with pDOM as a template in order to fuse the 5' end of the $E7_{43-77}$ sequence to DOM. This resulted in one DNA fragment consisting of DOM fused to the 5' end of the $E7_{43-77}$ with an upstream *Nhe*I site. In the second reaction, the E7 sequence was amplified using the forward primer HPVconsF2 and the reverse primer HPVconsR5 in a PCR using the finished and validated p$E7_{43-77}$ construct as a template. This yielded a DNA fragment consisting of the 3' end of the $E7_{43-77}$ followed by a downstream *Not*I site. The two DNA fragments were then fused in a SOE PCR using the primers NheI-BCL_1 and HPVconsR5. This resulted in a fusion gene encoding DOM (including the upstream BCL_1 leader sequence) fused to the $E7_{43-77}$ sequence, flanked by upstream *Nhe*I and downstream *Not*I restriction sites.

The pE7GGG construct was made from two initial PCRs. The E7GGG modified version of the E7 oncogene itself has been described before [221]. In the first PCR, the forward primer NheI-BCL_1 F1 and reverse primer slbHPVR1 were used with the pDOM construct as a template. This resulted in a DNA fragment encoding the BCL_1 leader fused to the beginning of E7GGG sequence. In the second PCR, the forward primer slbHPVF1 and reverse primer slbHPVR2 were used amplify the E7GGG sequence in a PCR using pBSC/E7GGG plasmid as a template. The two fragments were then combined in a SOE

PCR using NheI-BCL_1 F1 and slbHPVR2 primers. This resulted in a gene encoding the BCL_1 leader sequence fused to E7GGG sequence, flanked by upstream *Nhe*I and downstream *Not*I restriction sites.

The pDOM.E7GGG construct was also made from two initial PCRs. In the first, the forward NheI-BCL_1 F1 primer and reverse slbHPVR3 primer were used to make a DNA fragment encoding DOM fused to the 5' end of the E7GGG sequence using pDOM as template. In the second PCR, the slbHPVF2 forward primer and slbHPVR2 reverse primer were used to amplify the E7GGG sequence from the validated pE7GGG construct as a template. These two fragments were subsequently fused in a SOE PCR using NheI-BCL_1 F1 and slbHPVR2 primers. This resulted in a gene encoding DOM (including the BCL_1 leader sequence) to the E7GGG sequence, flanked by upstream *Nhe*I and downstream *Not*I restriction sites.

The pDOM.$E7_{11-20}$ and pDOM.$E7_{86-93}$ vaccines were made in a PCR using the pDOM construct as a template and the NheI-BCL_1 forward primer and the reverse primers HPVconsR6 and R7 respectively. This resulted in fusion genes encoding the BCL_1 leader fused to DOM followed by the A*0201-restricted $E7_{11-20}$ (YMLDLQPETT) and $E7_{86-93}$ (TLGIVCPI) epitopes from HPV16 E7 flanked by upstream *Nhe*I and downstream *Not*I restriction sites.

2.1.2. DNA Purification and Ligation

Fusion gene PCR products were visualised on a 1-1.5% agarose gel under UV light next to a lane of DNA standards from 200-10,000bp (Hyperladder I; Bioline, London, UK) or 100-2,072bp (100bp ladder; Invitrogen, Carlsbad, US). DNA bands of the correct size excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol.

Purified PCR products were subsequently digested with *Nhe*I (Promega) and *Not*I (NEB, Ipswich, US) restriction endonuclease enzymes along with the pCI vector during 1-2hr incubation with the supplied buffers at 37ºC.

Digested PCR products were separated on an agarose gel and purified as described above. The fusion gene inserts were ligated into the pCI backbone at a ratio of approximately 5:1 using 1µL 3 units/µL T4 DNA ligase and 2x ligation buffer (both Promega) in a final volume of 10-15µL for 3-4hr at room temperature or overnight at 4ºC.

Primer	Direction	Sequence 5'-3'
NheI-BCL$_1$F1	Forward	AAA *GCT AGC* CGC CAC CAT GGG TTG GAG C
pJR5p1	Reverse	GGC ACA GTC GAG GCT GAT CA
HPVconsF1	Forward	TTT GCA ACA GCT ACA GGT GTG CAC TCC GGA CAA GCA GAA CCG GAC AGA GCC
HPVconsF2	Forward	TTC CTG CGT GAC TTC TGG GGT AAC GGA CAA GCA GAA CCG GAC AGA GCC
HPVconsR1	Reverse	TTT T*GC GGC CGC* TTA AAA GGT TAC AAT ATT GTA ATG GGC TCT GTT ACC CCA GAA GTC ACG CAG GAA
HPVconsR3	Reverse	GGC TCT GTC CGG TTC TGC TTG TCC GGA GTG CAC ACC TGT AGC TGT TGC
HPVconsR4	Reverse	TTT GGC TCT GTC CGG TTC TGC TTG TCC GTT ACC CCA GAA GTC ACG CAG GAA
HPVconsR5	Reverse	TTT T*GC GGC CGC* TTA ACG AAT GTC TAC GTG TGT GCT TTG
slbHPVF1	Forward	TAC AGG TGT GCA CTC CAT GCA TGG AGA TAC ACC T
slb HPVF2	Forward	TGA CTT CTG GGG TAA CAT GCA TGG AGA TAC ACC
slbHPVR1	Reverse	AGG TGT ATC TCC ATG CAT GGA GTG CAC ACC TGT A
slbHPVR2	Reverse	TTT T*GC GGC CGC* TTA TGG TTT CTG AGA ACA
HPVconsR6	Reverse	TTT T*GC GGC CGC* TTA AGT TGT CTC TGG TTG CAA ATC TAA CAT ATA GTT ACC CCA GAA GTC ACG CAG G
HPVconsR7	Reverse	TTT T*GC GGC CGC* TTA GAT GGG GCA CAC AAT TCC TAG TGT GTT ACC CCA GAA GTC ACG CAG G

Table 2.1. Primers used in vaccine construction

Restriction sites are shown in italics and stop codons are underlined. Primers were designed by SLB except NheI-BCL$_1$F1 which was designed by SND and pJR5p1 which was designed by JR. Primers were synthesised by MWG (Ebersberg, Germany), except slbHPVF1, F2, R1 and R2 and HPvconsR6 and R7 which were synthesised by Sigma-Aldrich.

Construct	Primers	Conditions
pDOM	NheI-BCL_1 F1, JR5p1	5min 94ºC, 5 cycles of 1min 94ºC, 2min 54ºC, 1min 72ºC, then 25 cycles of 1min 94ºC, 2min 50ºC, 1min 72ºC, then 5min 72ºC
pDOM.$E7_{49-57}$	NheI-BCL_1F1, HPVconsR1	15min 94ºC, 5 cycles of 1min 94ºC, 1min 55ºC, 2min 72ºC then 20 cycles of 1min 94ºC, 2min 60ºC, 2min 72ºC
$pE7_{43-77}$	(i) NheI-BCL_1F1, HPVconsR3 (ii) HPVconsF1, HPV consR5 (iii) NheI-BCL_1F1, HPV consR5	(i), (ii) & (iii) 15min 94ºC, 5 cycles of 1min 94ºC, 1min 55ºC, 2min 72ºC then 25 cycles of 1min 94ºC, 1min 62ºC, 2min 72ºC then 10min 72ºC
pDOM.$E7_{43-77}$	(i) NheI-BCL_1F1, HPVconsR4 (ii) HPVconsF2, HPV consR5 (iii) NheI-BCL_1F1, HPV consR5	(i), (ii) & (iii) 15min 94ºC, 5 cycles of 1min 94ºC, 1min 55ºC, 2min 72ºC then 25 cycles of 1min 94ºC, 1min 62ºC, 2min 72ºC then 10min 72ºC
pE7GGG	(i) NheI-BCL_1F1, slbHPVR1 (ii) slbHPVF1, slbHPVR2 (iii) NheI-BCL_1F1, slbHPVR2	(i) 2min 94ºC, 5 cycles of 30s 94ºC, 30s 54ºC, 1min 72ºC then 25 cycles of 30s 94ºC, 30s 60ºC, 1min 72ºC then 5min 72ºC (ii) & (iii) 25 cycles of 30s 94ºC, 30s 54ºC, 1min 72ºC then 5min 72ºC
pDOM.E7GGG	(i) NheI-BCL_1F1, slbHPVR1 (ii) slbHPVF1, slbHPVR2 (iii) NheI-BCL_1F1, slbHPVR2	(i) & (ii) 2min 94ºC, 5 cycles of 30s 94ºC, 30s 54ºC, 2min 72ºC then 25 cycles of 30s 94ºC, 30s 60ºC, 2min 72ºC then 5min 72ºC (iii) 2min 94ºC, 5 cycles of 30s 94ºC, 30s 54ºC, 3min 72ºC then 25 cycles of 30s 94ºC, 30s 60ºC, 3min 72ºC then 5min 72ºC
pDOM.$E7_{11-20}$	NheI-BCL_1 F1, HPVconsR6	5min 94ºC, 5 cycles of 1min 94ºC, 1min 55ºC, 2min 72ºC, then 25 cycles of 1min 94ºC, 1min 60ºC, 2min 72ºC, then 15min 72ºC
pDOM.$E7_{86-93}$	NheI-BCL_1 F1, HPVconsR7	5min 94ºC, 5 cycles of 1min 94ºC, 1min 55ºC, 2min 72ºC, then 25 cycles of 1min 94ºC, 1min 60ºC, 2min 72ºC, then 15min 72ºC

Table 2.2. PCR conditions used in vaccine construction

Numbers in parentheses indicate (i) reaction 1, (ii) reaction 2 and (iii) SOE reaction where applicable.

2.1.3. Vaccine Plasmid Transformation into Competent Bacteria and Larger Scale Production

Ligated constructs were transformed into 50-100µL competent *E. coli* (JM109, Promega) by incubation on ice for 10-20 minutes prior to a 45-second heat-shock at 42ºC. After returning to ice for a further 2min, Luria broth (LB; Sigma-Aldrich, St. Louis, US) was added to a total volume of 1mL and the cells were incubated for 1hr at 37ºC with shaking at 200RPM. After this incubation, the cells were pelleted by centrifugation at 7000 x *g* for 5 minutes, resuspended in 100µL LB and spread onto a Luria agar plate containing 100µg/mL ampicillin (Calbiochem, Merck, Darmstadt, Germany). Plates were inverted and incubated at 37ºC overnight.

Single colonies were used to inoculate 5mL LB containing 100µg/mL ampicillin in 30mL tubes, and these clones were incubated overnight at 37ºC with shaking at 200RPM.

Cells were pelleted by centrifugation at 1500 x *g* for 5 minutes and plasmid DNA was recovered using the Miniprep Kit (Qiagen) according to the manufacturer's protocol. The integrity of the DNA vaccines was verified by restriction digestion and subsequent visualisation by agarose gel electrophoresis to validate the size of the inserted gene as described in section 2.1.2. DNA sequencing analysis (see section 2.2.1) was also carried out to confirm the DNA sequence of the constructs.

Larger scale production of validated DNA vaccines was carried out in a similar way using the Gigaprep Kit (Qiagen) according to the manufacturer's protocol. The resulting DNA was digested and sequenced again as before and quantified using a photometer (Eppendorf, Cambridge, UK). All vaccines had an A_{260}: A_{280} ratio of 1.8-2.0, indicating DNA purity and minimal protein contamination.

DNA vaccines were stored in 1mg aliquots containing 10% 3M sodium acetate and 80% isopropanol at -20ºC.

2.2. Vaccine Validation

2.2.1. DNA Sequence Analysis

DNA sequencing was carried out using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, US). Each sequencing reaction consisted of 500ng of DNA, 3.2pMol of primer, 2µL of BigDye® and 2µL of 5x buffer in a total volume of 10µL. The BigDye® Terminator ready reaction mix consists of A-dye, C-dye, G-dye and T-dye terminators labelled with dichloro R6G, ROX, R110 and TAMRA respectively; dNTPs (dATP, dCTP, dGTP and dTTP) and AmpliTaq DNA polymerase. Primer

sequences are shown in Table 2.3. Sequencing reactions consisted of a 1 minute denaturation step at 96ºC followed by 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes.

The completed sequencing reactions were precipitated with 70µL/tube of a master mix consisting of 550µL 100% ethanol and 40µL 3M sodium acetate on ice for 10-20 minutes before centrifugation at 15,000 x *g* for 30 minutes. Supernatants were aspirated and pellets were washed in 150µL 70% ethanol during a further centrifugation at 15,000 x *g* for 10 minutes. Supernatants were aspirated and pellets were dried at 37ºC before resuspension in 10µL formamide.

Sequences were resolved with a 3130*xl* Genetic Analyzer (Applied Biosystems) and analysed using Lasergene software (DNA Star, Madison, UK).

2.2.2. *In vitro* Transcription and Translation (IVTT) Test

To ensure that the cloned genes in the DNA vaccines were transcribed and translated into protein of the expected size, an *in vitro* transcription and translation (IVTT) test was carried out using the TnT Quick Coupled Transcription/Translation Kit (Promega). The kit contains rabbit reticulocyte lysate, RNA polymerase, salts, nucleotides and ribonuclease inhibitor in a master mix. It uses the T7 RNA promoter which is located upstream of the multiple cloning site in pCI. A typical reaction mix consisted of 20µL master mix, ~0.5µg DNA and approximately 100µCi ^{35}S methionine (Amersham Biosciences, GE Healthcare, Little Chalfont, UK); reactions were incubated for 1.5hr at 30ºC in a total volume of 25µL. A 10µL aliquot of the completed reaction was denatured with 1µL β-mercaptoethanol and 6µL NuPage loading buffer (Invitrogen) in a total volume of 24µL for 5 minutes at 95ºC. Denatured reactions were run on a pre-cast 4-12% sodium dodecyl sulphate (SDS) polyacrylamide NuPage gel (Invitrogen) alongside 12µL denatured Rainbow marker (Amersham Biosciences) for ~1hr at 180V. Gels were dried on filter paper under a vacuum for 1-2hr. The dried gel was exposed to radioactive film overnight and developed to reveal the radioactive bands. The size of the bands was compared to the markers to ascertain if a product of the expected size was seen.

Primer	Direction	Sequence 5'-3'	Position of Binding
FrC-F1	Forward	GTT AGC TTC TGG CTG CGC GTT C	From 220bp into DOM1 of FrC
pCI-intA	Forward	ACT CTT GCG TTT CTG ATA GGC	From 925bp into pCI
pCI-polyA	Reverse	CAC TGC ATT CTA GTT GTG G	From 1147bp into pCI

Table 2.3. Primers used in sequencing reactions

FrC-F1, designed by JR, synthesised by MWG; pCI.intA and pCI.polyA as advised by Promega, synthesised by Sigma-Aldrich.

2.3. DOM-His Protein Production

DOM-His Protein was made in human embryonic kidney (HEK) 293F cells (Invitrogen). The cells were cultured in Freestyle Expression medium (Invitrogen) in Erlenmeyer flasks with no antibiotic. After thawing, cells were cultured for 1-2 weeks prior to transfection. 100mL cell cultures were transfected with 100µg of a pcDNA3.DOM-His construct (kindly supplied by KJ McCann) with 100µL 293-Fectin reagent (Invitrogen), each in a total volume of 3mL Opti-MEM (Invitrogen). The two reagents were mixed after 5 minutes at room temperature, pooled and then incubated together for a further 30 minutes before the mixture was added drop-wise to the culture. Cells were replaced in an incubator at 37ºC, 8% CO_2, shaking at 125RPM.

After 3 days supernatants were harvested by centrifugation at 500 x *g*. Cell pellets were resuspended in fresh media and replaced in the incubator. After another 3 days the process was repeated to maximise protein yield. Cell supernatants were filtered through a 0.22µm filter (Millipore, Billerica, US) prior to concentration to exclude all cell debris.

Filtered supernatants were concentrated using Vivaspin 20 concentrators (Sartorius Stedim Biotech, Aubagne, France) by centrifugation at 1028 x *g* at 4ºC. Every 1-2hrs flow-through was removed and more supernatant was added until a total final volume of ~15mL/culture was achieved.

Concentrated protein was purified using a His-Bind column (Novagen, Merck, Darmstadt, Germany) in accordance with the manufacturer's protocol. The pH of the concentrate was adjusted to pH 8.5 prior to application to ensure adequate binding to the column. The His tag binds to divalent cations in the column, causing it to remain in the column whilst contaminants are washed through with 10mL of wash buffer (4M NaCl, 480mM imidazole, 160mM Tris-HCl, pH7.9) diluted 1/8. The protein was subsequently liberated using 4mL elution buffer (4M imidazole, 2M NaCl, 80mM Tris-HCl, pH7.9) diluted 1/4. The eluted protein was then dialysed 3 times with 15mL PBS to a total volume of 2mL.

2.4. DOM-His Protein Validation

The protein size was verified by electrophoresis. A 10µL aliquot of the protein was denatured with 1µL β-mercaptoethanol and 6µL NuPage loading buffer (Invitrogen) in a total volume of 24µL for 5 minutes at 95ºC. Denatured products were run on a pre-cast 4-12% SDS polyacrylamide NuPage gel (Invitrogen) alongside 12µL denatured Rainbow marker (Amersham Biosciences) for ~1hr at 180V. The gel was subsequently stained with

Simply Blue Safe Stain (Invitrogen); after which a band of the expected size (35kDa) was visible.

Further validation was also performed by western blot using an anti-His tag antibody. The SDS gel was transferred to a nitrocellulose membrane which had been equilibrated with methanol and rinsed in NuPAGE transfer buffer (Invitrogen) over ~2hr at 12V. The membrane was blocked with 5% milk/0.1% Tween-20 PBS for 1hr at room temperature before incubation with anti-tetra-His antibody (Qiagen) diluted 1/1000 in 0.1% Tween-20 PBS overnight at 4ºC. The membranes were then washed for 5 minutes in 0.1% Tween-20 PBS three times before the addition of the secondary sheep-anti-mouse IgG-HRP (The Binding Site) antibody, diluted 1/1000 in 0.1% Tween-20 PBS for 1hr at room temperature. The HRP conjugate enzyme was visualised by chemiluminescence with ECL Western blotting detection reagents (Amersham Biosciences).

The protein was quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Scientific, Rockford, US) according to the manufacturer's protocol (carried out by KJ McCann).

2.5. Cell Lines

TC-1 cells were a kind gift from CJM Melief (first batch) and TC Wu (second batch). TC-1 cells are derived from C57BL/6 mouse lung tissue which was immortalised by retroviral transduction with a HPV16 E6/E7-expressing vector and further transduction with a vector containing the activated human *ras* oncogene [222]. EL-4 cells are derived from a C57BL/6 mouse T-cell lymphoma. The YAC-1 cell line is an NK-sensitive cell line derived from an A/Sn mouse lymphoma. Ecotropic phoenix cells are used in retroviral transfections as a packaging cell line which infects rodent cells.

Phoenix cells were maintained in DMEM (Lonza, Basel, Switzerland) plus 10% FCS (Gibco, Invitrogen, Carlsbad, US), 50μM 2-β-mercaptoethanol, 100U/mL penicillin and 100μg/mL streptomycin, supplemented with 1mM pyruvate (Gibco), 2mM glutamate (Gibco) and 1x non-essential amino acids (Gibco). All other cell lines were maintained in RPMI 1640 (Gibco) plus 10% FCS (Gibco), 50μM 2-β-mercaptoethanol, 100U/mL penicillin and 100μg/mL streptomycin, supplemented with 1mM pyruvate (Gibco), 2mM glutamate (Gibco) and 1x non-essential amino acids (Gibco), referred to as complete media. The selective antibiotic Geneticin (Gibco) was added to all TC-1 cultures at 400μL/mL to maintain transgene expression. TC-1 cells were passaged using Trypsin Versene Solution (Lonza, Basel, Switzerland) to remove the adherent live cells from the flask. Cells were then washed in complete media to remove the trypsin and obtain a single-

cell suspension. Some washes were carried out with RPMI 1640 with no additions, referred to as incomplete media. Cells were centrifuged for 5 minutes at 400 x *g* during wash steps.

2.6. Retroviral Transfection of TC-1 cells with H-2D^b

TC-1 cells were transfected with the H-2D^b heavy chain following the protocol developed in Dr G Nolan's laboratory (Stanford University, US, protocol available online[*]). The H-2D^b coding sequence (NCBI accession no. NM_010380) was cloned into the pmscv vector (Clontech, Saint-Germain-en-Laye, France) containing a puromycin resistance gene. To achieve this, cDNA from a C57/BL6 mouse (kindly provided by S Thirdborough) was amplified in a PCR using the Db-F1 and Db-R1 primers shown in Table 2.4. Restriction sites were added in a second PCR using the Db-F2 and Db-R2 primers the H-2D^b gene was then inserted into the pmscv-puro plasmid between the *Eco*RI and *Bam*HI restriction sites as described in section 2.1.2. Ecotropic phoenix cells were seeded in 6-well plates at a density of 1.4-1.6x10^6 cells/well in a volume of 5mL DMEM media 18-24hrs prior to transfection. Immediately prior to transfection, choloroquinine was added in 1mL of media to a final concentration to 25µM. A transfection cocktail of 20µg DNA in TE buffer, 36µL 2M $CaCl_2$ and 200µL HBS buffer in a volume of 300µL sterile H_2O was then added in a drop-wise manner. The media was changed 9hrs after transfection to remove the choloroquinine, and again after 24hrs. At this point, cells were placed at 32ºC for 24hrs. After this incubation, supernatant was harvested, 4µg/mL polybrene was added to it and this was used to replace media in one well of a 6-well plate containing TC-1 cells at confluency. Viral supernatant was removed after 8hrs and replaced with complete media. Transfected TC-1 cells were subsequently grown for a prolonged period in 3µg/mL puromycin to select for cells carrying the transgene (this concentration is lethal to untransfected cells). H-2D^b expression was confirmed by FACS as described in section 2.7.

2.7. TC-1 Tumour Phenotyping by Flow Cytometry

TC-1 cell surface expression of H-2D^b was analysed by FACS following incubation with anti-mouse H-2D^b-FITC antibody (clone KH95) or isotype control, (1µg/10^6 cells; both Becton-Dickenson and Company (BD), Franklin Lakes, US) for 25 minutes at 0-4ºC. After washing in PBS (VWR International, Lutterworth, UK), cells were analysed immediately

[*] http://www.stanford.edu/group/nolan/retroviral_systems/phx.html

or fixed in 150µL 1% formaldehyde. Results were collected on a FACS Calibur (BD) and analysed using CellQuest Pro software (BD).

Primer	Direction	Sequence 5'-3'	PCR Conditions Used
Db-F1	Forward	ATG GGG GCG ATG GCT CCG	5min 94ºC, 7 cycles of 1min 94ºC, 1min *x*ºC, 2min at 72ºC where *x* starts at 62ºC and decreases
Db-R1	Reverse	CGA GAT TGT AAA GCG TGA	by 1ºC each cycle, until 55ºC followed by 18 further cycles at this temperature, then 15min 72ºC
Db-F2	Forward	AAA *GAA TTC* GCC GCC ACC ATG GGG GCG ATG GCT CCG CGC	15min 94ºC, 20 cycles of 1min 94ºC, 1min 62ºC, 2min 72ºC, then 7min 72ºC
Db-R2	Reverse	AAA *GGA TCC* TCA CGC TTT ACA ATC TCG GAG	
			Sequencing primer binding at pos 637 of D^b gene
Db-F3	Forward	GTG ACC CAT CAC CCC AGA TC	*Sequencing primer binding at pos 827 of D^b gene*
Db-R3	Reverse	GT GTA ATT CTG CTC CTT CCC	

Table 2.4. Primers used in the construction of retroviral plasmid containing the H-2D^b gene

Restriction sites are shown in italics and stop codons are underlined. Primers were synthesised by Sigma-Aldrich.

2.8. E6/E7 Spontaneous Tumour Measurement and Phenotyping by Flow Cytometry

Thyroids were excised and the trachea and oesophagus removed prior to determining their mass or conducting flow cytometric and immunhistochemical analysis in all experiments. For flow cytometry, thyroid tissue was incubated in PBS plus 1mg/mL collagenase (Roche, Basel, Switzerland) and 0.5mg/mL DNase (Sigma) for 2hrs at 37ºC. The cells were subsequently ground through a 70µ mesh filter (BD) and washed in PBS. Anti-mouse H-2D^b-FITC antibody (clone KH95) surface and anti-mouse CD8a-PE surface antibodies (clone 53-6.7; both BD) were at 1µg/10^6 cells added and cells were incubated on ice for 30 minutes. After washing in PBS, cells were fixed in 150µL 1% formaldehyde (Sigma) in PBS for 10 minutes on ice and subsequently washed in PBS twice more. Cells were then permeabilised 100µL 0.5% saponin in PBS containing 1µg/10^6 cells anti-HPV16 E7 antibody (clone TVG 10Y; Santa Cruz, Santa Cruz, US) and incubated on ice for 30 minutes. After a wash in 0.5% saponin followed by another in PBS, anti-mouse IgG2a-APC secondary antibody (Southern Biotech, Birmingham, US) was then added and cells were incubated for a further 15 minutes on ice. A final wash in PBS was carried out before cells were analysed using a FACS Canto and FACS Diva software (both BD). For each sample 100,000 events were collected.

2.9. Haematoxylin and Eosin Staining and Immunofluorescence Staining on Frozen Tumour Sections

Fresh tumour samples were embedded in OCT (RA Lamb, Thermo Scientific, Basingstoke, UK) and frozen in a bath of isopentane on dry ice. Sections of 10µm thickness were cut using a Cryo-Star HM 560 (Microm, Walldorf, Germany) and mounted onto microscope slides before air drying for at least 30 min. Slides were then stained with haematoxylin and eosin dyes.

For immunofluoresence, mounted sections were fixed with 100% acetone for 10 min, air dried and then marked around sections with barrier pen. Residual OCT was washed away with PBS and then sections were blocked with 10% goat serum (as host of the secondary antibody is goat) for 30 min. The rat anti-CD8α antibody (clone 53-6.7; BD) was added in 100µL PBS + 0.05% Tween20 and incubated for 2 hours at RT. After incubation, 3 PBS washes were carried out before adding a goat anti-rat secondary antibody conjugated to Alexa Fluor® 488 (Molecular Probes, Invitrogen, Paisley, UK) in 100µl PBS + 0.05% Tween20. After a 45min incubation in covered box, sections were washed 3 times in PBS and counterstained with 4,6-diamidino-2-phenolindole (DAPI; Invitrogen) for 20min at

RT. A final wash in PBS was carried out before mounting in Vectashield (Vector Laboratories, Peterborough, UK). Staining was visualised using an Olympus CKX41 inverted microscope equipped with a reflected fluorescence system running under Cell^B software (Soft Imaging Solutions, Olympus, Southend-on-Sea, UK). Fluorescence images were collected as separate tagged image file format (TIFF) files (DAPI and Alexa Fluor® 488), merged using Adobe Photoshop CS4 software (Adobe Systems Inc, San Jose, US) and signal levels adjusted to fill the whole grey scale.

2.10. Experimental Protocols *in vivo*

2.10.1. Mice

Male C57BL/6 mice aged 8-12 weeks were obtained from the University of Southampton Biomedical Research Facility; E6/E7 transgenic mice were obtained from Y Paterson (University of Pennsylvania; [223]); HHD mice were obtained from V Cerundolo (Human Immunology Unit, Oxford; [224]), both strains were then bred in the University of Southampton Biomedical Research Facility. All mice were handled in accordance with UK Home Office legislation. Experiments were carried out under project licence no. 70/6407 (JR) using personal licence no. 70/19802 (ELR) and no. 30/5256 (SLB).

2.10.2. DNA vaccination

For immunisation, DNA vaccines were given by intramuscular (i.m.) injection at 0.5 mg/mL in 0.9% w/v saline in a volume of 50µL in each hind leg tibialis anterior muscle. All injections given with electroporation (see below) were given in the quadriceps muscle. Where indicated, vaccine concentration and injection volumes were adjusted such that dose remained constant at 25µg/leg.

2.10.3. Electroporation (EP)

In vivo EP was carried out on anaesthetized mice. Usually, mice were anaesthetised by inhalation of isofluorane and oxygen gases administered by a Penlon Sigma-Delta anaesthetic vaporiser (InterMed, South Portland, US). Some mice were anaesthetised with 1:1:2 midazolam (5 mg/mL), hypnorm (0.315 mg/mL fentanyl citrate and 10 mg/mL fuanisone) and water respectively by intraperitoneal injection. Hind legs were shaved and DNA vaccines were given in the quadriceps muscle. Immediately following immunization, conductance gel was applied to the site and the electrodes were placed onto the skin. 10

trains of 1000 square wave pulses were delivered at a frequency of 1000 Hz at 1 second intervals using a custom-made pulse generator (Inovio Biomedical Corporation, Bluebell, US) as previously described [139], [145].

2.10.4. Peptide Vaccination

Mice were vaccinated with synthetic 'long peptide' (residues 43-77 of HPV16 oncoprotein) in 200µL saline by sub-cutaneous (s.c.) injection in the dorsal flank in accordance with a published method [225]. This relatively large injection volume leads to the dispersal of the fluid over a large area under the loose skin found in this site. Peptide dose was 150µg/40nMoles per mouse; always given mixed with 50µg CpG ODN 1826 on a phosphorothiate backbone (5'-TCC ATG ACG TTC CTG ACG TT-3'; Sigma).

2.10.5. Tumour Challenge

Mice were challenged with $5x10^4$ TC-1 tumour cells in 100µL PBS by s.c. injection in the dorsal flank. Resultant tumours were palpated every 2-3 days. Mice were culled when the humane endpoint of a diameter of 15mm or more was reached in accordance with UK Home Office regulations.

2.10.6. Blood Sampling

Blood samples were usually collected from vaccinated mice by tail bleeding. Instillagel™ local anaesthetic (Farco-Pharma, Cologne, Germany) was applied to the tail and mice were warmed to 37ºC for 5 minutes. A 1-2mm section of the tail was cut with a scalpel and 100-200µL blood was collected from each mouse (100µL for FACS analysis; 200µL for serum). At the end of some experiments the final blood sample was taken by cardiac puncture under general anaesthesia (induced as described in section 2.10.3). If blood samples were to be used for FACS analysis, ~20µL/100µL heparin sodium (1000 IU/mL; Wockhardt, Wrexham, UK) was added.

2.10.7. *In vivo* Cell Depletion

C57BL/6 mice were subjected to $CD4^+$ T cell depletion using a rat anti-mouse CD4 antibody (clone YTS 191.1.2; Cancer Research UK Monoclonal Antibody Service, London, UK) or rat IgG (Sigma-Aldrich) as a control. Mice were given 100µg of anti-CD4 antibody or control IgG in 200µL PBS by intraperitoneal (i.p.) injection. The antibody was

given 3 days prior to tumour challenge and then every two days until 5 days after challenge. After this time, mice were given 1-2 injections/week until day 27. A 50µL blood sample was taken from mice in both the treated and control groups to monitor the level of $CD4^+$ T-cell depletion by FACS analysis. The blood samples were incubated with Red Blood Cell Lysis Solution (Qiagen) and washed twice in PBS. The cells were then incubated with 2.5µg anti-CD3-PE (clone 17A2), 1µg anti-CD4-APC (clone RM4.5) and 2.5µg anti-CD8a-FITC (clone 53-6.7) antibodies (all BD) for 25 minutes on ice. After two further washes in PBS, cells were fixed in 150µL 1% formaldehyde. Wash steps were carried out with centrifugation at 400 x *g*. Results were collected using a FACS Calibur and analysed using Cell Quest Pro software.

2.11. Analysis of Immune Responses *ex vivo*

2.11.1. Enzyme-Linked Immunosorbent Spot (ELISpot) Assays

ELISpot assays were carried out on splenocytes directly *ex vivo* to assess T cell responses using the BD ELISpot Kit. In order to detect IFNγ production, 96-well plates were coated with purified anti-mouse IFNγ antibody (BD) diluted 1/200 in sterile PBS overnight at 4ºC. Whole spleens were aseptically excised from immunised mice. From this point, the assay was carried out in a laminar flow hood under sterile conditions. Spleens were ground through a 70µ mesh filter (BD) and then washed in incomplete media and pelleted by centrifugation at 400 x *g* for 5 minutes. Mononuclear cells were separated by density centrifugation using Lymphoprep™ (Axis-Shield, Dundee, UK) which separates monocytes and lymphocytes from the bulk cell population during a 20 minute centrifugation at 937 x *g*. After washing as before, cells were centrifuged at 544 x *g* and resuspended in a small volume. Cells were then counted and $2x10^5$ cells were incubated with or without peptides (Peptide Protein Research, Wickham, UK) at varying concentrations in a total volume of 200µL/well, in triplicate. The cells were incubated at 37ºC, 5% CO_2 and 99% humidity for 22hr.

Following incubation, cells were lysed with MilliQ water (2 washes, 5 minutes each) and washed three times with 200µL/well PBS containing 0.05% Tween-20 (Sigma-Aldrich). The biotinylated anti-mouse IFNγ antibody (BD) was diluted 1/500 in PBS containing 10% FCS and 100µL/well was incubated in the plates for 2hrs at room temperature or overnight at 4ºC. After this incubation, plates were washed as above. Streptavidin-alkaline phosphatase secondary antibody (Mabtech, Nacka Strand, Sweden) was diluted 1/500 in PBS containing 10% FCS and 100µL/well was then added to the plates. After a final

washing step which included two further washes with PBS alone, 100µL/well of the developer (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium; Zymed Laboratories, Invitrogen, Carlsbad, US) was added according to the manufacturer's protocol. Spots appeared within approximately 5 minutes, at which point the reaction was quenched with distilled water. When dry, plates were read and the number of spots was evaluated using an automated plate reader (Autoimmun Diagnostika, Strasberg, Germany).

2.11.2. Tetramer Staining

Peripheral blood was lysed using Red Blood Cell Lysis Solution (Qiagen), washed in PBS, blocked with 2% normal mouse serum and washed in PBS prior to staining. Cells were incubated with H-2D^b-E7$_{49-57}$-PE tetramer in 100µL PBS for 10min at room temperature. After washing in PBS, cells were stained with 0.6µg anti-mouse CD8a-APC (clone 53-6.7) and 1µg anti-mouse MHC II-FITC (clone 2G9; both BD). Cells were then washed in PBS and either fixed in 1% formaldehyde or analysed immediately in PBS. Wash steps were carried out by centrifugation at 400 x *g*. Results were analysed using a FACS Calibur (BD). Typically, 150,000-250,000 events were collected for each sample.

2.11.3. Intracellular Cytokine Staining

For intracellular cytokine staining, viable lymphocytes were separated by density centrifugation as described in section 2.11.1. Cells (1.5-2x10^6) were re-stimulated *in vitro* in U-bottomed, 96-well plates for 4hrs at 37ºC, 5% CO_2 and 99% humidity with 1µM peptide, 10U/mL IL-2 and 0.5% Golgi Plug (BD) containing brefeldin A to prevent cytokine secretion. After blocking in 2% normal mouse serum, cells were stained with 1µg/10^6 cells anti-mouse CD8a-APC (clone 53-6.7) and 1µg/10^6 cells anti-mouse MHC II-FITC (clone 2G9) or an isotype control (all BD) and then fixed in 1% formaldehyde overnight. Cells were subsequently permeablised with 0.5% saponin and stained with 1µg/10^6 cells anti-mouse IFNγ-PE antibody (clone XMG1.2) or isotype control (both BD). Wash steps were carried out by centrifugation at 400 x *g* for 5 minutes. Results were analysed using a FACS Calibur (BD). Typically, 50,000-200,000 events were collected for each sample.

2.11.4. Chromium Release Assays

CTL lines were derived from the spleens of immunised mice. Each was ground through a 70µ mesh filter (BD) and washed in incomplete media. Usually, 60% of the cells were used to seed a line in 15mL complete media; if not, the volume was scaled accordingly. CTL lines were then incubated in *vitro* with 1nM $E7_{49-57}$ peptide and 20U/mL IL-2 for 6-7 days prior to the assay. Target cells were labelled with 3-5MBq ^{51}Cr for 1hr at 37ºC and then washed to remove unincorporated Cr^{51}. Some targets were also incubated with peptide during this time; either with the $E7_{49-57}$ peptide as a positive control or an irrelevant peptide as a negative control (CMTWNQNML; residues 235-243 of Wilms tumour antigen). The target cells used are described in detail in section 2.5. CTL lines were separated by density centrifugation as described in section 2.11.1 and then incubated with $3\text{-}5x10^3$ target cells at given ratios in a total volume of 200µL in triplicate. The cell mixtures were then incubated in 96-well plates at 37ºC, 5% CO_2 and 99% humidity for 5hr. Cells were then pelleted by centrifugation at 450 x *g* and supernatants were harvested. The amount of ^{51}Cr in the supernatants was then measured using a Wizard 1470 γ counter (Perkin-Elmer). Specific ^{51}Cr release was calculated according to the following equation:

$$\% \text{ release} = \frac{(\text{test release} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} \times 100$$

Test release is the amount of ^{51}Cr released when target cells were incubated with effector cells. Spontaneous release is the amount of ^{51}Cr released when target cells were incubated with media alone. Total release is the amount of ^{51}Cr released when target cells were incubated without effectors but lysed with 4% Nonidet P-40 (Roche, Basel, Switzerland) prior to harvest.

2.11.5. Enzyme-Linked Immunosorbent Assays (ELISAs)

For ELISAs, 96-well plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) were coated with 2µg/mL DOM protein (see section 2.3) in 200µL/well coating buffer (1.6g/L Na_2CO_3 and 2.9g/L $NaHCO_3$ in dH_2O) overnight at 4ºC. Plates were washed with PBS containing 0.1% Tween-20 three times and then blocked with PBS containing 1% BSA for 2hr at 37ºC. Plates were washed as before and then serum samples were plated out at several different dilutions, along with the standard serum, initially diluted 1/50 and then doubling dilutions down the plate, in duplicate. After washing as before, goat anti-mouse Ig-HRP (The Binding Site, Birmingham, UK) was added at 1/1000 dilution in 0.1%

Tween-20 PBS and incubated for 1hr at 37ºC. Plates were washed for a final time and then the o-phenylenediamine dihydrochloride (OPD) developer (Sigma-Aldrich) was added. Reactions were stopped with the addition of 2.5M sulphuric acid when colour change was visible. Results were measured using a plate reader (Dynex Technologies, Chantilly, US) to analyse the optical density (OD) at 490nm wavelength. The OD values of the standard serum at different dilutions, the first being 1/50 dilution (equivalent to 500 arbitrary units/mL), followed by 1/2 dilutions thereafter, were plotted on a graph as a standard curve. OD values from the test sera which did not fall on the straight part of the standard curve were omitted from analysis. At least two readings in U/mL were averaged to obtain a final value for each sample. Results were detectable from ~10U/mL.

2.12. Statistical Tests

During the data analysis various statistical tests were used. Mann-Whitney U tests were conducted on non-parametric data such as ELISpot results and t tests on parametric data such as FACS results. Survival in tumour challenge experiments was assessed using Chi square log rank tests. Correlations between two variables were analysed by linear regression. Results where $P<0.05$ were considered statistically significant. Data were sometimes combined from multiple experiments conducted in the same way and showing the same trend in order to increase statistical power.

3. Induction of HPV16 E7-specific Immune Responses by DNA Vaccines

3.1. Introduction

The aim of this project was to produce a therapeutic DNA vaccine against HPV16-associated cancers. A number of vaccines have been compared in this study with the goal of producing the most effective immunotherapy possible, by harnessing both $CD8^+$ and $CD4^+$ T lymphocytes and the innate immune system. Successful immunotherapy of this kind would provide a potential treatment for the thousands of women who are diagnosed with cervical cancer every year.

3.1.1. Vaccines Used in this Study

Several vaccines (shown in Figure 3.1A-F) were constructed in order to investigate the ability of DNA vaccines to elicit responses against HPV16 E7 oncoprotein, which contains a well-described H-$2D^b$-restricted $CD8^+$ T-cell epitope (residues 49-57 of E7) [226]. The vaccines encode sequences from E7 either alone or fused to DOM (domain 1 of FrC) in the pCI vector. The presence of DOM provides T-cell help for $CD8^+$ T cells and for antibody responses partly due to the presence of p30, a promiscuous $CD4^+$ T-cell epitope (see section 1.2.4.8 for further information). Adequate provision of $CD4^+$ T-cell help will be crucial to the success of therapeutic anti-cancer vaccines in the clinic where immune tolerance is likely to restrict immune responses.

In order to make the E7 oncogene safe to use in its entirety, three amino acids within the Rb-binding site have been mutated to glycine (G) [221]. The resulting full-length gene vaccine is called pE7GGG, shown in Figure 3.1A. The E7GGG gene was also fused to the 3' terminus of DOM to make the pDOM.E7GGG vaccine (Figure 3.1B). Although the increased size of this vaccine may actually be detrimental to immune responses in wild-type mice, the inclusion of DOM may be necessary for therapeutic efficacy in a tolerant setting.

'Long peptide' DNA vaccines which encode amino acids 43-77 of the E7 gene were also constructed. The long peptide vaccine ($pE7_{43-77}$) is shown in Figure 3.1C; a fusion gene in which the long peptide is fused to the 3' terminus of DOM ($pDOM.E7_{43-77}$) was also made, shown in Figure 3.1D. These vaccines were designed on the basis of the previous success of vaccination with synthetic $E7_{43-77}$ long peptide, which was reported to induce both $CD8^+$ and $CD4^+$ T cells due to the presence of the $E7_{49-57}$ CTL epitope and the $E7_{48-57}$ helper T-

cell epitope [225]. This approach of using a longer peptide is thought to have many benefits over short peptides containing only minimal CTL epitopes. One of most important is the potential presence of helper T-cell epitopes, another is that antigen presentation of longer peptides is increased in the draining lymph node; where they are only taken up and processed by professional antigen presenting cells such as DCs [95]. Vaccination with $E7_{43-77}$ synthetic long peptide induced tumour rejection in mice, though this effect was most potent when CpG was given as an adjuvant [225]. The efficacy of this synthetic $E7_{43-77}$ long peptide vaccine with CpG will be compared to our DNA vaccines. In the case of pDOM.$E7_{43-77}$, this will allow us to directly compare the efficacy of DNA versus peptide vaccination for delivering the same antigen. The E7 component of the two vaccines will be the same; the only difference will be that the synthetic peptide will be delivered with CpG as an adjuvant to stimulate innate immunity via TLR-9 and the DNA, which will also stimulate innate immunity by multiple mechanisms, will include DOM to induce extra $CD4^+$ T-cell help.

The final test vaccine is based on the pDOM-epitope design described in section 1.2.4.8. This vaccine, shown in Figure 3.1E, encodes only the $E7_{49-57}$ minimal $CD8^+$ T-cell epitope fused to the 3' terminus of the DOM sequence (pDOM.$E7_{49-57}$). For clinical relevance, two other pDOM-epitope vaccines have been designed which each encode an HLA-A*0201-restricted epitope (residues 11-20 and 86-95 of E7). A DNA vaccine which encodes DOM alone, shown in Figure 3.1F, is used as a control in all experiments.

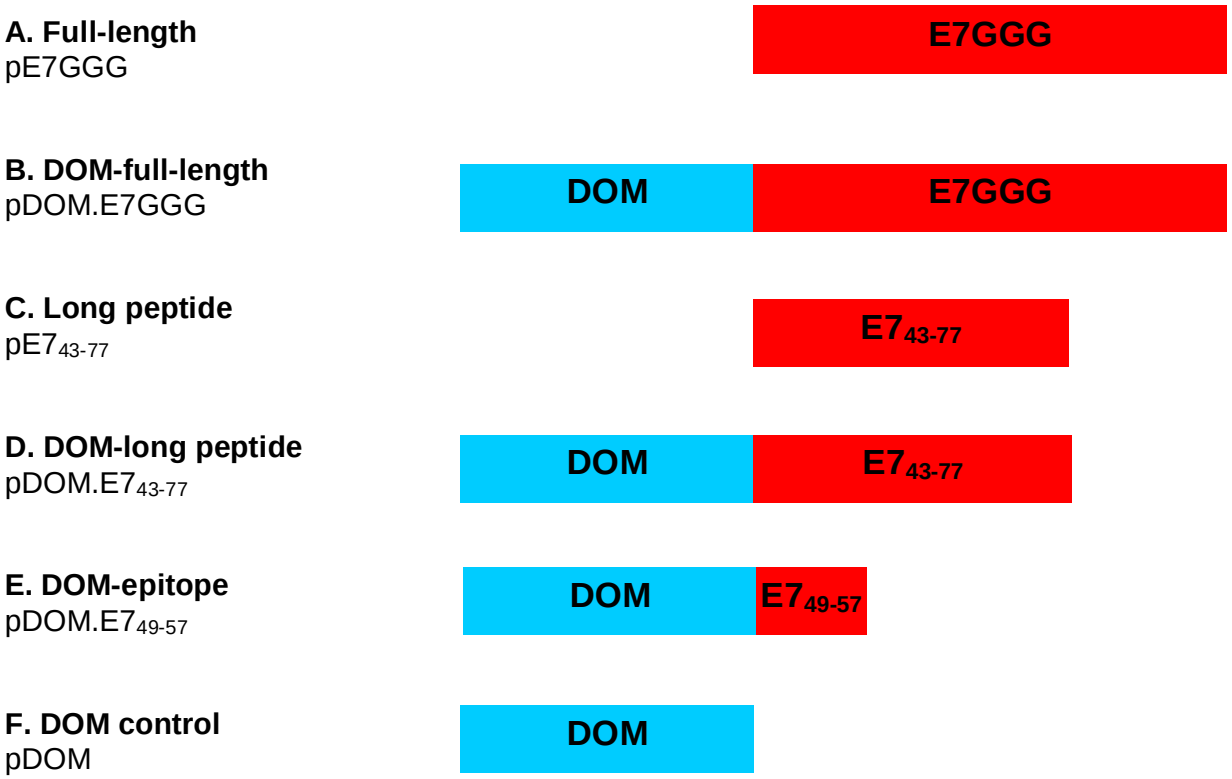


Figure 3.1. A schematic representation of the fusion vaccines used in this study

The protein product of each vaccine is shown.

3.1.2. The TC-1 Tumour Model

In order to investigate the potential of our DNA vaccines as immunotherapeutic agents against E7-expressing cancers, the TC-1 tumour model was used. TC-1 is a cell line derived from C57/BL6 mouse lung epithelium which has been transduced with E6 and E7 oncoproteins from HPV16 as well as the activated human c-Ha-*ras* gene [222]. This resulted in a tumorigenic cell line; tumours appear in 100% of congenic mice within 10-20 days of sub-cutaneous injection with $1x10^5$ cells/mouse [222].

A major issue which arises with this model is the reduced expression of MHC class I molecules on the tumour cell surface. It is well documented that tumours cells can down-regulate MHC I molecules in order to avoid CTL-mediated killing which requires peptides to be presented in the context of MHC I. In this case, the problem is compounded by the expression of the E7 oncoprotein. It has been demonstrated the E7 is able to reduce MHC I heavy chain promoter activity *in vitro* [212] and that interruption of the E7 gene in cell lines results in increased MHC I expression [227]. Therefore, TC-1 cells clearly have the potential to exhibit MHC I down-regulation. However, it has been reported that the parental line does express MHC I and only becomes MHC I negative in escape variants resulting from *in vivo* challenges in immunised mice [213]. Nevertheless, this does demonstrate that the TC-1 cells have the capacity to change their phenotype due to selective immune pressure.

3.1.3. Aims

The aim of this project was to produce a therapeutic DNA vaccine against HPV. The DNA vaccines described above were tested for their ability to induce E7-specific T cells and to protect against challenge with E7-expressing tumours *in vivo* in a prophylactic or therapeutic setting. This chapter will concentrate on investigating the description of the immune responses induced by the vaccines.

3.2. Results

3.2.1. $E7_{49-57}$-specific $CD8^+$ T cell Induction by the DNA Vaccines

In order to investigate the induction of $CD8^+$ T cells by all the vaccines described in section 3.1.1, lymphocytes from vaccinated mice were analysed by intracellular cytokine staining. This allows us to confirm the phenotype of the induced cells as either $CD8^+$ or $CD4^+$ T cells, as well as measuring their ability to produce IFNγ. This is a particularly important point as there is a putative $CD4^+$ T-cell epitope within E7 (amino acids 48-54) [228] which overlaps with the $E7_{49-57}$ $CD8^+$ T-cell epitope; and it has been suggested that the two may cross-react (CJ Melief et al., personal communication to S Buchan).

Mice were immunised with the DNA vaccines shown in Figure 3.1 or synthetic $E7_{43-77}$ long peptide with CpG for comparison. Lymphocytes were isolated 14 days after vaccination, restimulated with $E7_{49-57}$ peptide for 4hr *in vitro* and then stained with anti-CD8-APC and anti-MHC II-FITC antibodies. The cells were then fixed and permeabilised before staining with anti-IFNγ-PE antibody. Cells were gated on forward and side scatter and MHC II negativity to reduce background staining. The results, shown in Figure 3.2, are expressed as the percentage of IFNγ positive cells within the CD8 positive cell population.

After this primary vaccination, all the vaccines induced IFNγ-producing $CD8^+$ T cells at a level that was statistically significant when compared to the background level observed in mice given the pDOM control vaccine ($P<0.05$ in t test). The full-length E7 gene vaccines (pE7GGG and pDOM.E7GGG) proved to be the most effective. After pE7GGG vaccination, the mean percentage of $CD8^+$ IFNγ-producing cells was ~8%. The incorporation of DOM resulted in ~5% $CD8^+$ T cells producing IFNγ. This reduction may be due to the increased size of the gene product, which will reduce the rate of its translation into protein. However, there was not a statistically significant difference between the two groups when they were compared in a t test, due at least in part to the range of the data, highlighting the variation in the individual immune responses to these vaccines. The pDOM-epitope vaccine ($pDOM.E7_{49-57}$) was also effective, resulting in a mean of ~2.5% of $CD8^+$ T cells producing IFNγ. The pDOM-long peptide DNA vaccine ($pDOM.E7_{43-77}$) induced a very similar number of $CD8^+$ T cells (a mean of ~2%), indicating that the long peptide is of no additional benefit in this format. However, without DOM ($pE7_{43-77}$) the mean percentage of $CD8^+$ IFNγ-producing T cells was reduced (~1%). Synthetic long peptide vaccination with CpG also induced $CD8^+$ IFNγ-producing cells, albeit at a low level, with a mean of 0.5% IFNγ-positive $CD8^+$ T cells induced.

The level of background staining was <0.5% when cells from test mice were stained with the relevant isotype control or when cells from control mice vaccinated with pDOM were stained with anti-IFNγ.

Although all the vaccines induced an E7-specific $CD8^+$ T-cell response, there was a difference between the levels of response induced by some of the vaccines. The best responses were induced by the full-length vaccines; perhaps because the E7 protein is a small enough that its translation is efficient and because it is foreign protein in these wild-type mice. The pDOM-epitope vaccine also induced a strong $CD8^+$ T-cell response, and only responses to the pE7GGG vaccine showed a statistically significant improvement on this (P=0.0042 in a t test). Interestingly, $CD8^+$ T-cell responses to the long peptide vaccine were increased by the addition of DOM (P=0.0115). Crucially, all the DNA vaccines induced a stronger E7-specific $CD8^+$ T-cell response than the soluble long peptide did (P<0.05 in a t test).

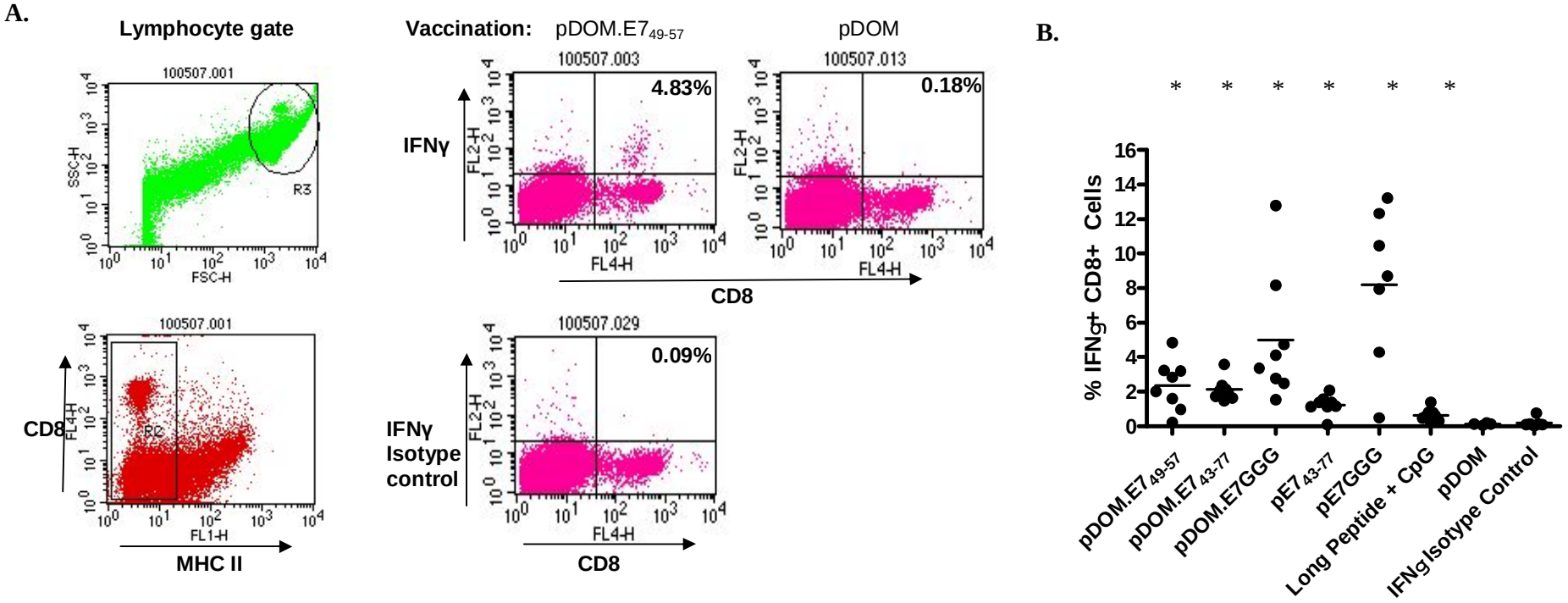


Figure 3.2. Intracellular IFNγ staining in CD8$^+$ T cells after a primary DNA vaccination

Groups of 4 mice were vaccinated with one of the vaccines shown as described in the Materials and Methods. Splenocytes were harvested 14 days after vaccination. Lymphocytes were isolated, restimulated with $E7_{49-57}$ peptide and stained with anti-CD8-APC and anti-MHC II-FITC antibodies. After permeabilisation, cells were also stained with anti-IFNγ-PE antibody. **A**. Representative set of FACS plots. **B**. Results expressed as the percentage of IFNγ and CD8 double positive cells. * indicates a statistically significant difference (P<0.05 in a t test) between the response induced by a test vaccine when compared to that induced by the pDOM control vaccine. Data were collected on a FACS Calibur and analysed using Cell Quest software. Data are combined from two experiments showing the same trend carried out jointly by ELR and SLB.

3.2.2. $CD8^+$ T-cell Responses against Putative E7 epitopes

The strong $E7_{49-57}$-specific responses seen after vaccination with full-length E7 gene constructs seen in Figure 3.2 suggest that this peptide is immunodominant. To confirm this, the existence of other $CD8^+$ T-cell epitopes exist in the E7 protein was investigated. Mice were vaccinated with the full-length pE7GGG vaccine and 14 days later lymphocytes were isolated and used in an IFNγ ELISpot. In this assay, lymphocytes were stimulated overnight with 1μM each of several different E7 peptides or $E7_{49-57}$ for comparison.

Epitopes were chosen due to their predicted binding of $H\text{-}2D^b$ by the SYFPEITHI [99] and BIMAS [229] algorithms and by proteasomal cleavage sites available for epitope liberation predicted by the NetChop 3.1 Server [230]. Several $H\text{-}2D^b$ binders were predicted, as shown in the SYFPEITHI results depicted in Figure 3.3A. However, although both predicted the $E7_{49-57}$ as dominant, the scores of the other predicted epitopes differed between the two algorithms; highlighting their limitations. Previous reports had indicated that residues 4-12, 66-74 and 71-79 may be recognised [226] so these were tested along with residues 85-93.

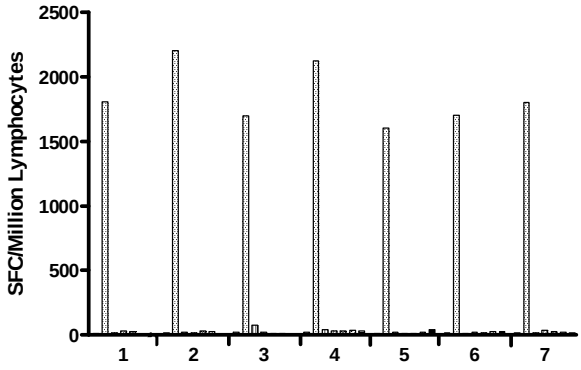
As shown in Figure 3.3B, despite the presence of strong $E7_{49-57}$-specific responses, no responses to any of the other E7 peptides were seen. Background responses in mice vaccinated with pDOM were low, despite the presence of a strong response to the DOM-derived p30 peptide (Figure 3.3C).

These results indicate that the $E7_{49-57}$ peptide is immunodominant, supported by its high predicted binding score. Based on this data, no $CD8^+$ T-cell epitopes which could add to or compete with $E7_{49-57}$-specific responses induced by the pE7GGG vaccine seem to exist.

A.

Pos		Score
49	R A H Y **N** I V T **F**	23
4	D T P T **L** H E Y **M**	18
30	D S S E **E** E D E **I**	18
74	V D I R **T** L E D **L**	18
57	F C C K **C** D S T **L**	16
71	S T H V **D** I R T **L**	16
20	T D L Y **C** Y E Q **L**	15
7	T L H E **Y** M L D **L**	13
14	D L Q P **E** T T D **L**	12
25	Y E Q L **N** D S S **E**	12
59	C K C D **S** T L R **L**	12
75	D I R T **L** E D L **L**	12
79	L E D L **L** M G T **L**	12
81	D L L M **G** T L G **I**	12
85	G T L G **I** V C P **I**	12
68	C V Q S **T** H V D **I**	11
46	E P D R **A** H Y N **I**	10
66	R L C V **Q** S T H **V**	10
76	I R T L **E** D L L **M**	10

B.



C.

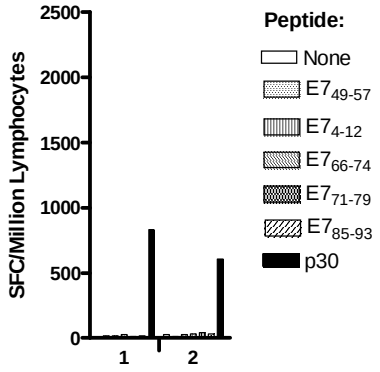


Figure 3.3. CD8$^+$ T-cell responses against several putative epitopes within E7

A. H-2D^b-binding scores of potential E7 nonomer epitopes predicted by the SYFPEITHI algorithm (Pos, position). Mice were vaccinated with **B.** pE7GGG or **C.** pDOM control vaccine; 14 days later lymphocytes were isolated and IFNγ responses to various E7 peptides or the DOM-derived p30 peptide were measured by ELISpot. Bars represent responses to each peptide in individual mice (numbered). SFC, spot-forming cells. Data are representative of two experiments.

3.2.3. E7-specific $CD4^+$ T-cell Responses Induced by the DNA Vaccines

To ascertain whether our vaccines induced an E7-specific $CD4^+$ T cell-response, lymphocytes from the mice discussed in 3.2.1 were also analysed in a second intracellular FACS assay. IFNγ production was assessed as described in section 3.2.1 after a 4hr restimulation with $E7_{48-57}$, a short peptide encoding the previously described $CD4^+$ T-cell epitope $E7_{48-54}$ [228], or the $E7_{43-77}$ long peptide, which also encompasses this but could contain additional epitopes. After restimulation, the lymphocytes were stained with anti-CD4 surface antibody instead of anti-CD8.

The results, shown in Figure 3.4, revealed that this primary vaccination with the vaccines did not induce a strong $CD4^+$ T-cell response after a short restimulation with these peptides.

After restimulation with the $E7_{48-57}$ short peptide (Figure 3.4A), the only vaccines which induced $CD4^+$ T cells at a statistically significant level when compared to the pDOM control vaccine were pE7GGG, pDOM.$E7_{43-77}$ and synthetic long peptide with CpG ($P<0.05$ in a t test). After a restimulation with the $E7_{43-77}$ long peptide, the results showed the same trend but none of the differences reached statistical significance (t test; Figure 3.4B).

The experiment was repeated with an overnight peptide restimulation to ensure ample time for peptide processing from the $E7_{43-77}$ long peptide. However, no E7-specific $CD4^+$ T cell responses were revealed as shown in Figure 3.5. As a control, some of the lymphocytes were incubated with DOM-derived p30 peptide which is known to stimulate strong $CD4^+$ T-cell responses (see Figure 3.3C). As shown in Figure 3.5C, p30-specific responses could be detected in lymphocytes from mice immunised with one of the vaccines containing DOM. However, these p30-specific responses are relatively weak. Therefore it is still possible that the assay is not fully optimised.

Several other experiments using both ELISpot and intracellular FACS analysis also showed very little evidence for an E7-specific $CD4^+$ T-cell response after vaccination with any of the vaccines. This indicates that if there is a $CD4^+$ T-cell response to the $E7_{48-54}$ epitope, it is certainly not a strong one. Furthermore, it appears that no extra $CD4^+$ T-cell epitopes exist within the 43-77 portion of the E7 gene. However, it is possible; perhaps likely that other $CD4^+$ T-cell epitopes do exist within the E7 gene.

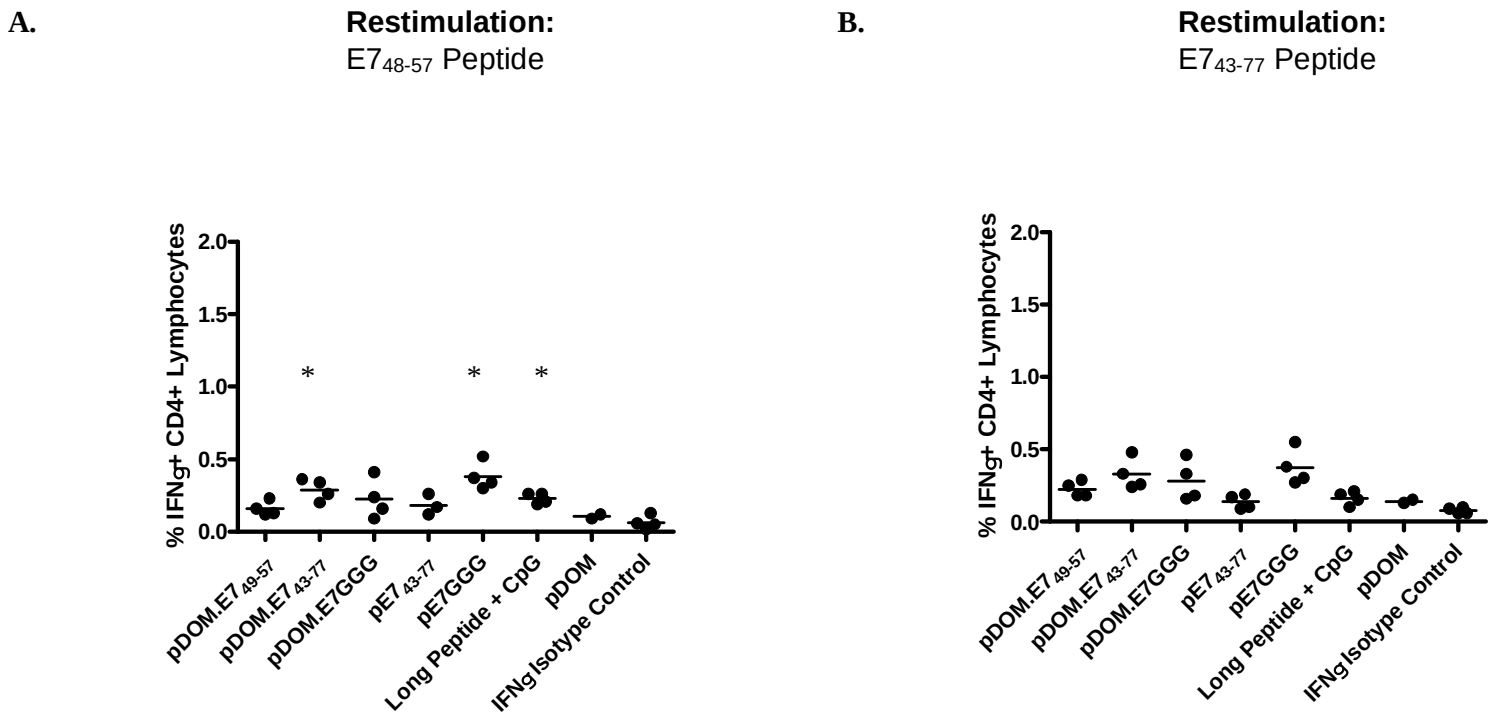


Figure 3.4. Intracellular IFNγ staining in CD4$^+$ T cells after a short restimulation

Groups of 4 mice were vaccinated with one of the vaccines indicated above and splenocytes were harvested 14 days later. Lymphocytes were isolated, restimulated with peptide and stained with anti-CD4-APC and anti-MHC II-FITC antibodies. After permeabilisation, cells were also stained with anti-IFNγ-PE antibody or isotype control. **A**. IFNγ production after restimulation with $E7_{48-57}$ peptide. **B**. IFNγ production after a 4hr restimulation with $E7_{43-77}$ peptide. Results are expressed as the percentage of CD4 and IFNγ double positive cells. * indicates a statistically significant difference (P<0.05 in a t test) between the response induced by a test vaccine when compared to that induced by the pDOM control vaccine. Data were collected on a FACS Calibur and analysed using Cell Quest software. Experiment was carried out jointly by ELR and SLB.

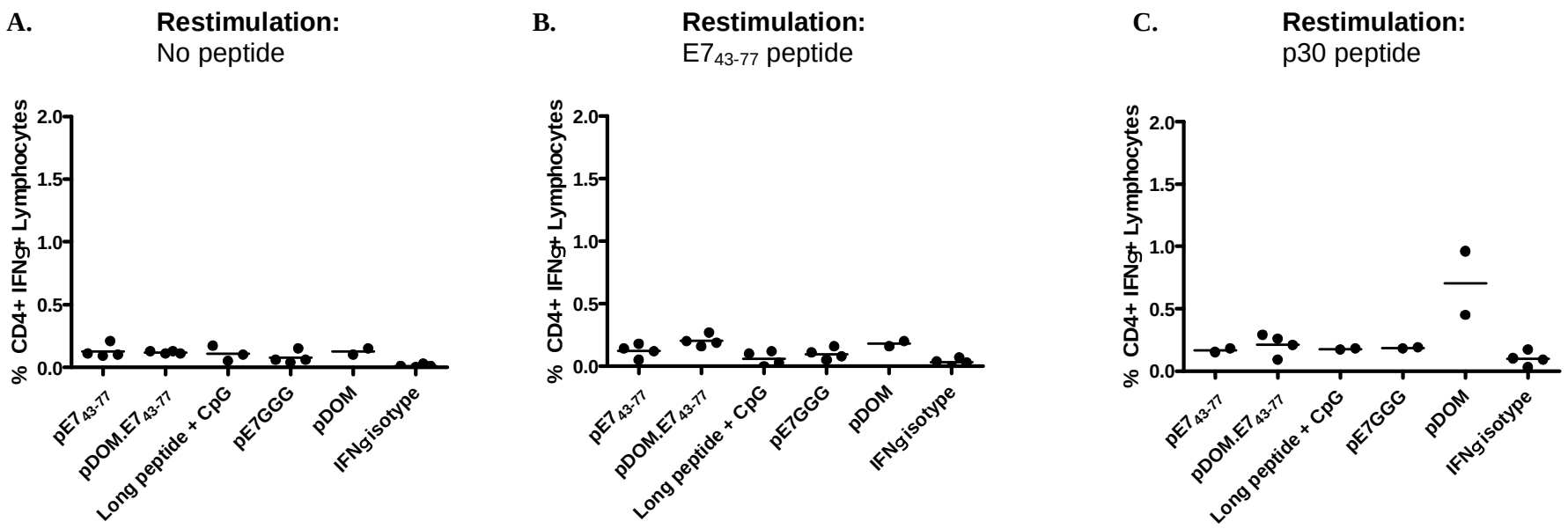


Figure 3.5. Intracellular IFNγ staining in CD4$^+$ T cells after overnight restimulation

Groups of 4 mice were vaccinated with one of the vaccines indicated above and splenocytes were harvested 14 days later. Lymphocytes were isolated, restimulated with peptide overnight before addition of a protein transport inhibitor and a further 4 hr incubation prior to staining with anti-CD4-APC and anti-MHC II-FITC antibodies. After permeabilisation, cells were also stained with anti-IFNγ-PE antibody or isotype control. IFNγ production after restimulation with **A**. no peptide; **B**. $E7_{43-77}$ peptide; or **C.** DOM-derived p30 peptide as a positive control. Results are expressed as the percentage of CD4 and IFNγ double positive cells. Data were collected on a FACS Calibur and analysed using Cell Quest software.

3.2.4. Primary Kinetics of the $CD8^+$ T-cell Response to DNA and Peptide Vaccination

As it was possible that the kinetics of the primary $CD8^+$ T-cell response induced by DNA and soluble peptide could vary, this was investigated using an H-$2D^b$-$E7_{49-57}$-specific tetramer to allow serial sampling. Blood samples were collected 8, 10 and 14 days after vaccination, and immune responses were analysed by flow cytometry. As well as tetramer, cells were also stained with anti-MHC class II antibody in order to gate out APCs which could bind the tetramer non-specifically; and with anti-CD8 antibody so that the tetramer-positive cells could be expressed as a percentage of the total number of $CD8^+$ T cells. Representative FACS plots are shown in Figure 3.6A.

By 8 days after vaccination with the pDOM-epitope (pDOM.$E7_{49-57}$), or long peptide (pDOM.$E7_{43-77}$) DNA vaccines, or with synthetic long peptide with CpG, low-level E7-specific $CD8^+$ T-cell responses were detectable. Interestingly, responses appeared to peak at d10 in the blood after both DNA and peptide immunisation (Figure 3.6B and C), indicating no difference in kinetics. At d14 post vaccination responses had not fallen much in most cases. In fact, responses to the pDOM.$E7_{43-77}$ vaccine were higher in some mice, though the difference was not statistically significant (using a t test); probably due to the variation between individuals, as shown by the error bars.

At the peak of the response on d10, tetramer-positive cells were found at a considerably higher frequency after pDOM.$E7_{49-57}$ vaccination compared to synthetic long peptide with CpG (P=0.0076; Figure 3.6B), confirming the findings shown in Figure 3.2. As shown in Figure 3.6C, when responses to the pDOM.$E7_{43-77}$ vaccine were compared to those to long peptide with CpG at d10 post vaccination, more tetramer-positive CD8-positive cells were found in the DNA vaccinated group. However, responses in some of the mice in the DNA vaccine group did not peak until d14, when the difference between the two groups was greater. However, these differences were not statistically significant at any time point or when the peak response from each group was compared (using a t test).

Using tetramer as a read-out, the p$E7_{43-77}$ vaccine seemed to fail to induce responses in almost all mice and was significantly worse than both pDOM.$E7_{43-77}$ and synthetic long peptide with CpG (P<0.04) at d10 (Figure 3.6C). This supports the data shown in Figure 3.2 where it induced only weak IFNγ responses in $CD8^+$ T-cells.

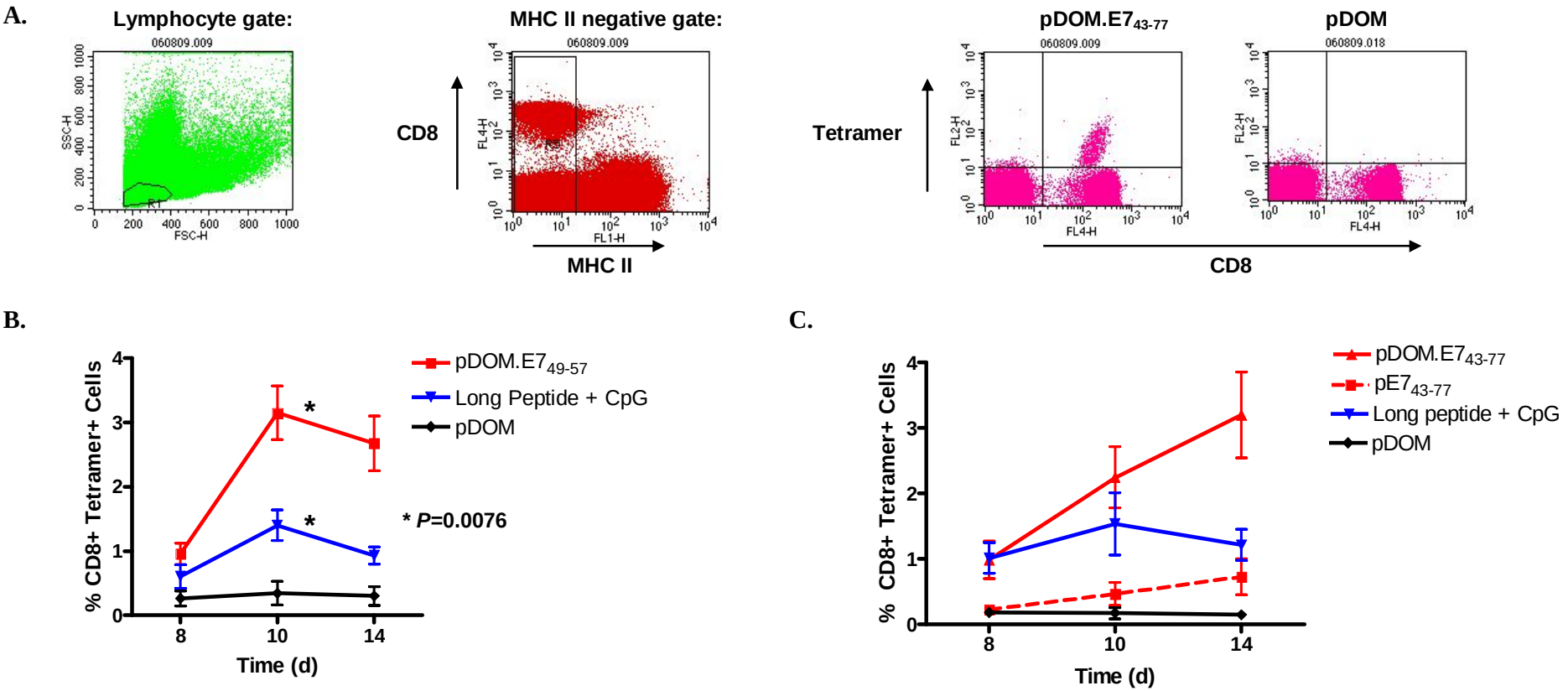


Figure 3.6. Primary kinetics of the CD8$^+$ T-cell responses stimulated by DNA vaccines compared to soluble long peptide with CpG

Groups of 6 mice were vaccinated with the indicated DNA vaccines or synthetic $E7_{43-77}$ long peptide with CpG and blood samples were taken at various time points. After red blood cell lysis, cells were stained with an $E7_{49-57}$-specific tetramer and anti-MHC II-FITC and anti-CD8a-APC antibodies. **A.** Representative FACS plots. The kinetics of the primary CD8$^+$ T-cell response to **B.** pDOM.$E7_{49-57}$ and **C.** pDOM.$E7_{43-77}$ or p$E7_{43-77}$ DNA vaccines compared to synthetic long peptide with CpG or pDOM control vaccine. Data are combined from two experiments showing the same trend for each plot. *P* values were generated in a t test.

3.2.5. Memory and Secondary $CD8^+$ T-cell Responses to DNA and Peptide Vaccination

To discover whether the same trends held true upon secondary vaccination, mice discussed in Figure 3.6 were boosted and $CD8^+$ T-cell responses were also assessed 10 days later. E7-specific $CD8^+$ T-cell responses were monitored immediately before and after boosting using $E7_{49-57}$-specific tetramer as before. As shown in Figure 3.7, 51 days after vaccination, E7-specific $CD8^+$ T cells were still detectable in the blood, with around 1-1.5% tetramer and CD8 double-positive cells in many of the vaccinated animals compared to <0.5% in controls vaccinated with pDOM. As the effector phase of the response is over by this point, these cells constitute the memory pool available for boosting with vaccine. Vaccination with pDOM.$E7_{49-57}$ gave rise to a larger T-cell memory pool than synthetic $E7_{43-77}$ long peptide with CpG (P<0.03 in a t test; Figure 3.7A), as did the pDOM.$E7_{43-77}$ vaccine (P=0.0001; Figure 3.7B). Unsurprisingly given the poor primary responses induced by it, memory responses to the p$E7_{43-77}$ vaccine were not detectable above background.

Mice vaccinated with pDOM.$E7_{49-57}$ at prime were subsequently boosted with either pDOM.$E7_{49-57}$ with electroporation (EP; to improve immune responses, see Materials and Methods for further information) or synthetic long peptide with CpG. The DNA/DNA+EP strategy was more successful than DNA/peptide or peptide/peptide at inducing robust $CD8^+$ T cells (P<0.0001). Interestingly, even though primary vaccination with DNA gave rise to a larger memory pool than long peptide vaccination (P=0.0289), when these groups were boosted with peptide, there was no difference in the secondary responses, as shown in Figure 3.7.

When responses generated by pDOM.$E7_{43-77}$ vaccination were compared to those induced by synthetic long peptide with CpG, the same trends were observed as for the pDOM.$E7_{49-57}$ vaccine; perhaps unsurprising given their similar structure and the previous observation that no $CD4^+$ T-cell responses are stimulated by this portion of the E7 sequence (Figure 3.4 and Figure 3.5). Again, the DNA/DNA+EP strategy was more successful than peptide/peptide (P=0.0001). Due to the finding that a peptide boost stimulated a similar frequency of E7-specific $CD8^+$ T cells following a primary vaccination of either DNA or peptide for the pDOM.$E7_{49-57}$ vaccine, the DNA/peptide strategy was not tested for the pDOM.$E7_{43-77}$ vaccine. As at priming, secondary responses to the p$E7_{43-77}$ were very weak, confirming that this vaccine format is ineffective at inducing $CD8^+$ T cells.

These results confirm the validity of a DNA vaccine approach for inducing E7-specific $CD8^+$ T cells in these mice. The successful generation of a pool of memory $CD8^+$ T cells

and the ability to expand these cells will be crucial in cancer patients, where multiple vaccinations are expected to be required.

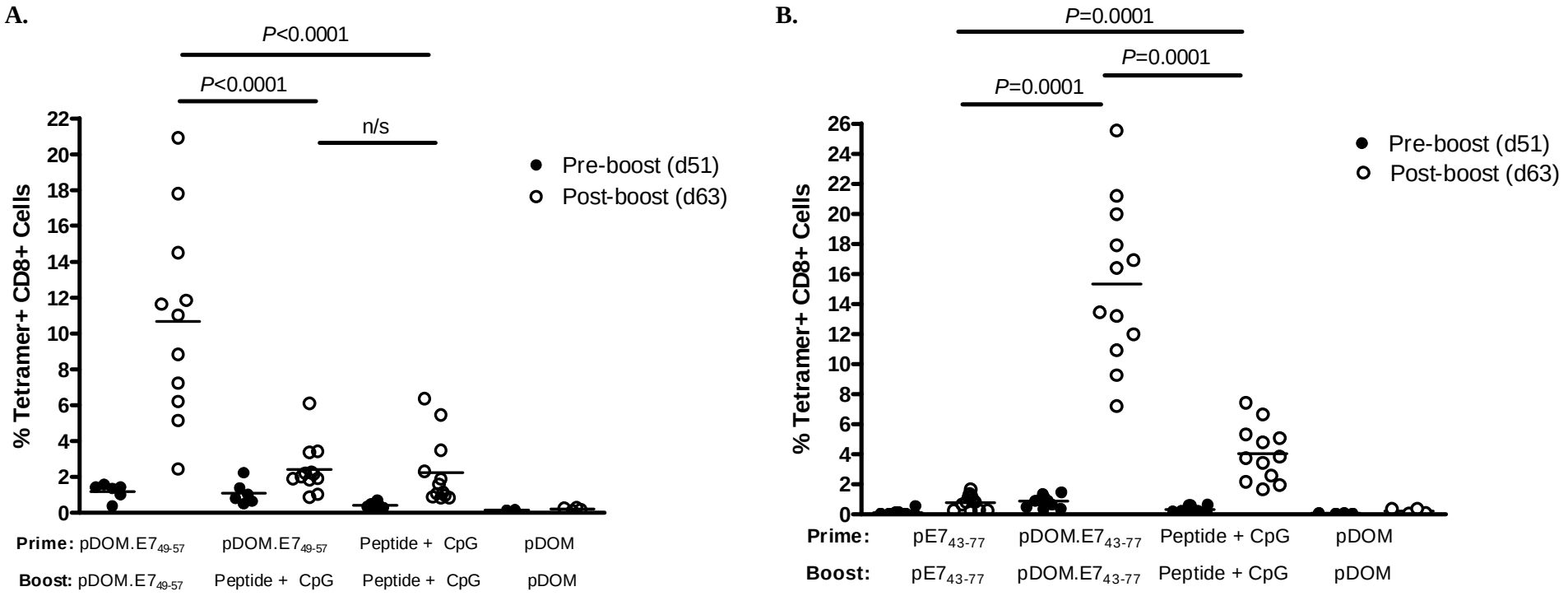


Figure 3.7. Secondary CD8$^+$ T-cell responses stimulated by DNA vaccines compared to soluble long peptide with CpG

Groups of 6 mice were vaccinated with the indicated DNA vaccines or synthetic $E7_{43-77}$ long peptide with CpG and blood samples were taken at various time points. Cells were stained with an $E7_{49-57}$-specific tetramer and anti-MHC II-FITC and anti-CD8a-APC antibodies as before. **A.** Responses to pDOM.$E7_{49-57}$ and **B.** pDOM.$E7_{43-77}$ or $pE7_{43-77}$ DNA vaccines compared to synthetic long peptide with CpG. Booster DNA vaccinations were given with electroporation 53 days after primary vaccination; synthetic long peptide with CpG was given as before. Responses were assessed 10 days after boost; pre-boost memory responses measured on d51 are also shown for comparison. Data are combined from two experiments showing the same trend for each panel. *P* values were generated in a t test.

3.2.6. CTL-mediated Killing of Target Cells *in vitro*

As the vaccines clearly induced IFNγ-producing $CD8^+$ T cells, the next question was whether these cells were cytotoxic and could lyse E7-expressing target cells *in vitro*. In order to investigate this, a chromium release assay was carried out. In this assay, target cells are labelled with radioactive ^{51}Cr and then incubated with different numbers of effector cells. After 5hr, the cell supernatants are harvested and the amount of ^{51}Cr that they contain is quantified, giving a measure of target cell lysis by the effector cells. The target cells used were TC-1 cells which express the HPV16 E7 antigen endogenously [222] and EL-4 cells, which do not. Some of the cells were also incubated with either the $E7_{49-57}$ peptide or an irrelevant peptide (from WT-1, see Materials and Methods for more information) for 1hr prior to the assay as positive and negative controls respectively.

Mice were immunised with the pDOM.$E7_{49-57}$ vaccine as before. After 14 days, splenocytes from 4 mice were harvested and pooled. The cells were restimulated *in vitro* for 7 days in the presence of 1nM $E7_{49-57}$ peptide and 20U/mL IL-2. After the restimulation, lymphocytes were isolated and processed as described in the Materials and Methods, and subjected to a 5hr-chromium release assay.

At a ratio of 80 effector cells to 1 target, ~100% of the $E7_{49-57}$ peptide-pulsed EL-4 cells were killed, compared to ~5% of EL-4 cells alone or pulsed with irrelevant peptide, as shown in Figure 3.8A, indicating peptide-specific lytic activity. TC-1 cells were also lysed at a ratio of 80:1, however only at the modest level of ~10%. Even labelling TC-1 cells with $E7_{49-57}$ peptide only led to a slight increase in lysis to ~30%. As $CD8^+$ T-cell mediated killing is reliant on MHC class I expression, this was thought to be a limiting factor.

In order to investigate whether reduced MHC I expression could explain the low level of CTL lysis, expression of $H\text{-}2D^b$, the restriction element for the $E7_{49-57}$ peptide; was analysed by flow cytometry. The results, shown in Figure 3.8B, revealed that the cells did not express high levels of $H\text{-}2D^b$. It had previously been reported that the level of $H\text{-}2D^b$ expression in these cells could be increased by incubating them with IFNγ [231]; and this is confirmed in Figure 3.8B and C.

The chromium release assay was then repeated using CTL lines from individual mice vaccinated with pDOM.$E7_{49-57}$ or pDOM 14 days previously and expanded in the same way. However, in this assay the targets used were TC-1 cells that had been incubated with 200U/mL IFNγ for the previous 48hr, in order to increase their surface MHC I expression. The results, shown in Figure 3.9, confirmed that these TC-1 cells were more effectively

lysed by the CTL effector cells. Even at a relatively modest ratio of 10 effector cells to each target, ~70% of the TC-1 cells were lysed, whether pulsed with $E7_{49-57}$ peptide or not; indicating that the peptide is processed efficiently. This level of lysis was higher than that of EL-4 cells pulsed with $E7_{49-57}$ peptide, ~35% of which were lysed at the 10:1 ratio in this experiment.

In this assay the lysis of the YAC-1 cell line which is known to be sensitive to NK cell mediated killing was also analysed. No lysis of YAC-1 cells was observed, indicating that the lysis of the other target cells was indeed peptide specific, and not due to NK-like activity. There was also little background killing of the target cells using effector cells derived from mice vaccinated with the control vaccine, pDOM (Figure 3.9B), which demonstrates that the lysis is not an *in vitro* artefact caused by IL-2 and peptide stimulation; rather that vaccination has induced the peptide-specific response.

Similar results were obtained with CTL lines harvested from mice immunised with each of the vaccines ($pE7_{43-77}$, $pDOM.E7_{43-77}$, pE7GGG, and pDOM.E7GGG). The majority of the mice tested (2-4 for each vaccine) exhibited a comparable level of specific lysis of the target cells as seen in Figure 3.9 (data not shown).

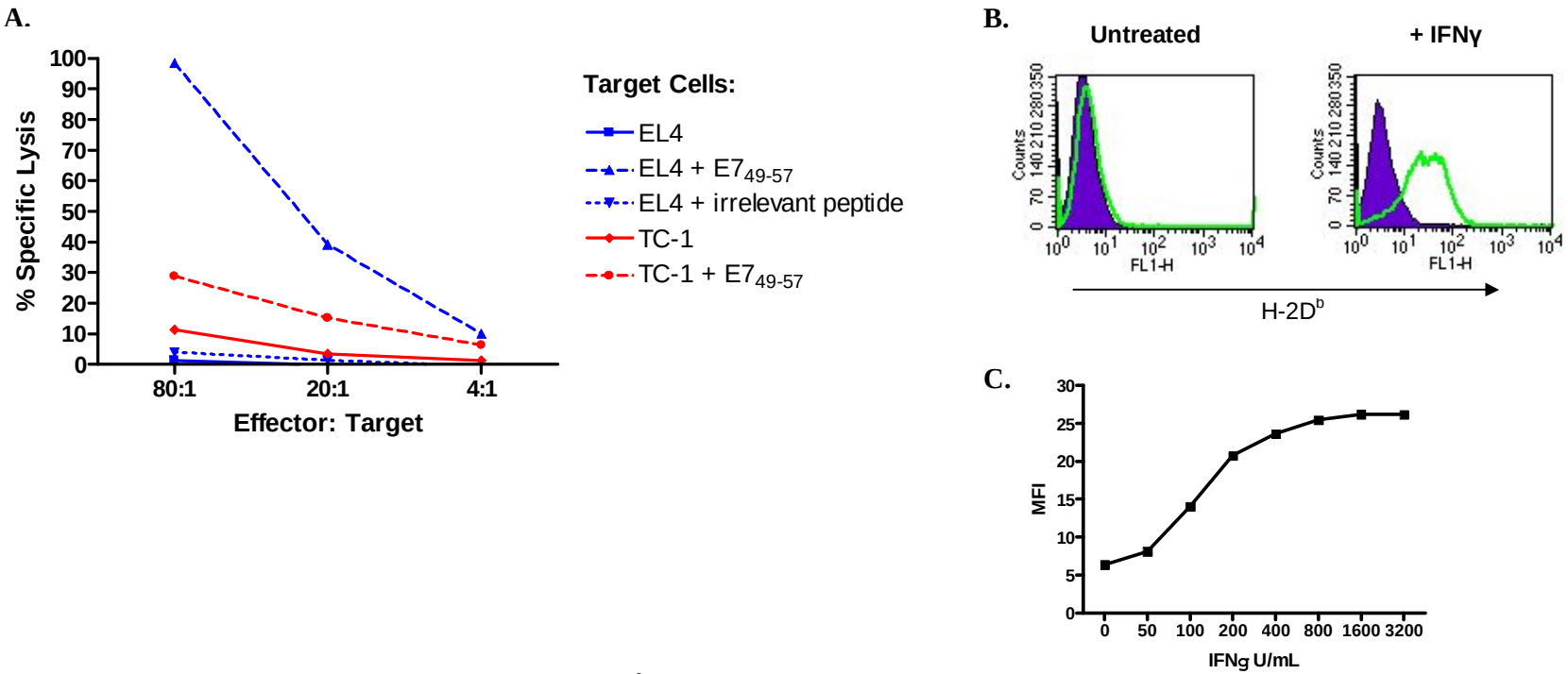


Figure 3.8. TC-1 Cell killing *in vitro* and *in vitro* H-2D^b expression

A chromium release assay was carried out using short-term CTL lines pooled from 4 mice vaccinated with pDOM.E7$_{49-57}$. CTL effector cells were expanded in vitro for 7 days in the presence of 1nM E7$_{49-57}$ peptide and 20U/mL IL-2. Chromium release was measured after a 5hr incubation of effector and target cells. **A**. Specific killing of TC-1 or EL-4 target cells alone or pulsed with E7$_{49-57}$ or an irrelevant peptide. Experiment carried out jointly by ELR and SLB. **B**. TC-1 cell expression of H-2D^b were analysed by FACS with or with prior incubation with 400U/mL IFNγ. **C.** A dose-response of TC-1 cell treatment with IFNγ against H-2D^b. MFI, mean fluorescence intensity.

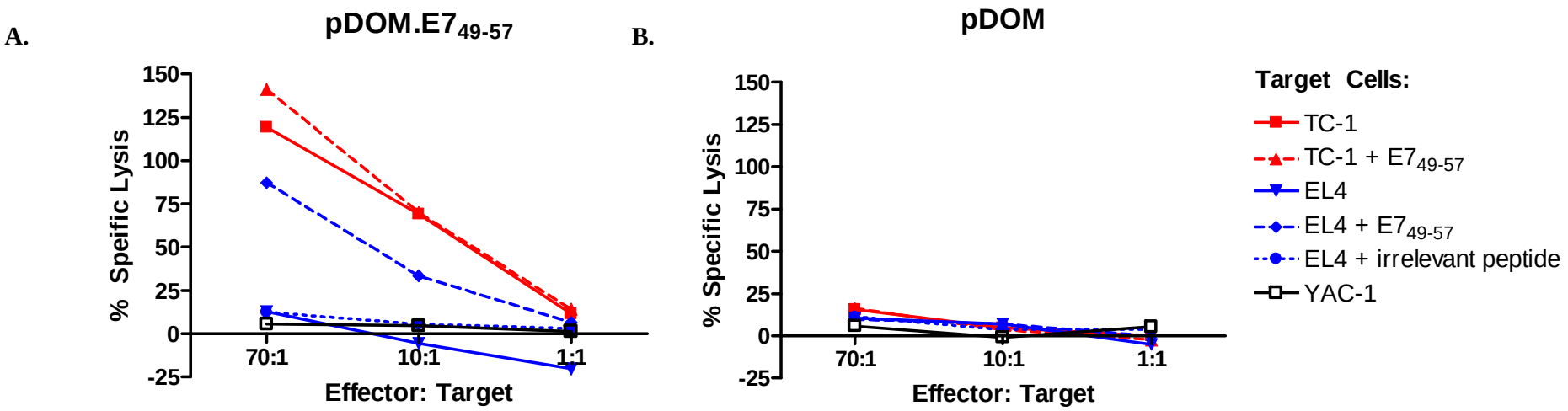


Figure 3.9. CTL-mediated lysis of IFNγ treated TC-1 cells *in vitro*

A chromium release assay was carried out using individual short-term CTL lines from mice immunised with the indicated DNA vaccines 14 days previously. Lines were expanded *in vitro* for 7 days in the presence of 1nM $E7_{49-57}$ peptide and 20U/mL IL-2. Chromium release was measured after a 5hr incubation of effector and target cells. TC-1 cells were incubated with 200U/mL IFNγ for 48hr prior to the assay. Killing of TC-1, EL-4 or NK-sensitive YAC-1 target cells alone or pulsed with $E7_{49-57}$ or an irrelevant peptide by CTL lines derived from **A.** pDOM.$E7_{49-57}$-vaccinated mice or **B.** pDOM-vaccinated control mice. Plots are representative of 2/3 pDOM.$E7_{49-57}$-vaccinated mice and 2/2 pDOM-vaccinated control mice.

3.2.7. Variant TC-1 Cells

TC-1 cells are used frequently in tumour immunotherapy studies with some success [232], [233] and have been reported to express significant levels of MHC I molecules (TC Wu and M Smahel, personal communications). To ensure the reduced MHC class I expression observed in Figure 3.8B was not particular to the batch of cells that we had acquired, we obtained a new batch from their original source (TC Wu, Johns Hopkins University, US). These cells will be referred to as 'variant TC-1 cells' throughout the subsequent sections.

The variant TC-1 cells were tested for H-2D^b expression by FACS analysis. As shown in Figure 3.10, the cells did express H-2D^b; however it was still at a relatively low level, and could still be dramatically increased by incubation with IFNγ. In other experiments (not shown), the level of H-2D^b expression varied. These results suggest that the TC-1 cells have a highly plastic expression of MHC class I molecules, which may be influenced by environmental factors both *in vitro* and *in vivo*.

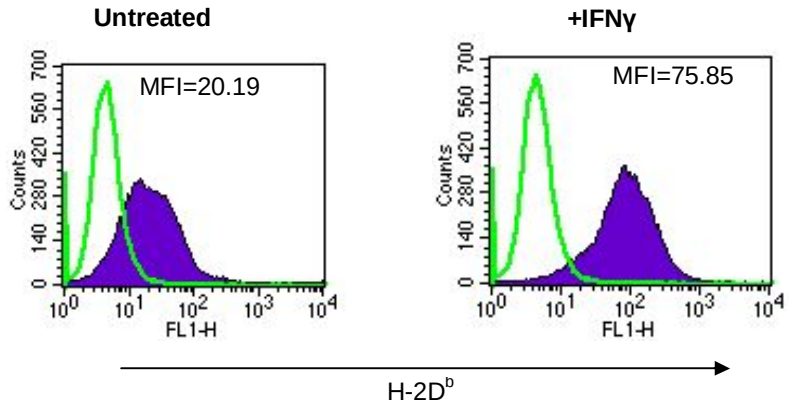


Figure 3.10. Variant TC-1 cell expression of H-2D^b *in vitro*

TC-1 cells were analysed by FACS for *in vitro* H-2D^b surface staining with or without prior treatment with 400U/mL IFNγ for 48hr. Open histograms show staining with isotype control; filled histograms show staining with anti-H-2D^b-FITC antibody. H-2D^b mean fluorescence intensity (MFI) is indicated.

3.2.8. CTL-mediated Killing of Variant TC-1 Cells *in vitro*

To confirm whether the increased expression of H-2D^b on the variant TC-1 cells increased their susceptibility to CTL-mediated killing *in vitro*, the ^{51}chromium release assay was repeated using these cells as targets.

Splenocytes from mice vaccinated with the pDOM.E7$_{49-57}$ or pDOM control vaccine were incubated with 1nM E7$_{49-57}$ peptide in the presence of 20U/mL IL-2 *in vitro* for 7 days. These short-term CTL lines were then used in a 5hr chromium release assay as before.

The results, shown in Figure 3.11, confirmed that the variant TC-1 cells were efficiently lysed by pDOM.E7$_{49-57}$-specific CTLs, unlike the original TC-1 cells (see Figure 3.8A). Approximately 50% of the TC-1 cells were lysed at a ratio of 50 effectors to 1 target, and this did not increase much at a ratio of 100:1. However, there was increased lysis of TC-1 cells which had been pre-incubated with IFNγ, as before. At a ratio of 50:1, 70% of TC-1 target cells were lysed, indicating that the additional increase in MHC class I expression further enhances susceptibility to CTL-mediated lysis. The lysis of TC-1 cells was greater than lysis of EL-4 cells that had been pulsed with the E7$_{49-57}$ peptide, which was ~30% at a ratio of 50:1. There was no lysis of EL-4 cells that had been pulsed with an irrelevant peptide, demonstrating that the lysis seen was peptide specific. Lines from mice vaccinated with the pDOM control vaccine exhibited increased background killing against the IFNγ-treated TC-1 cells, however this was still at a much lower level than test mice.

Short-term CTL lines isolated from mice immunised with the other vaccines also effectively lysed the variant TC-1 cells (data not shown).

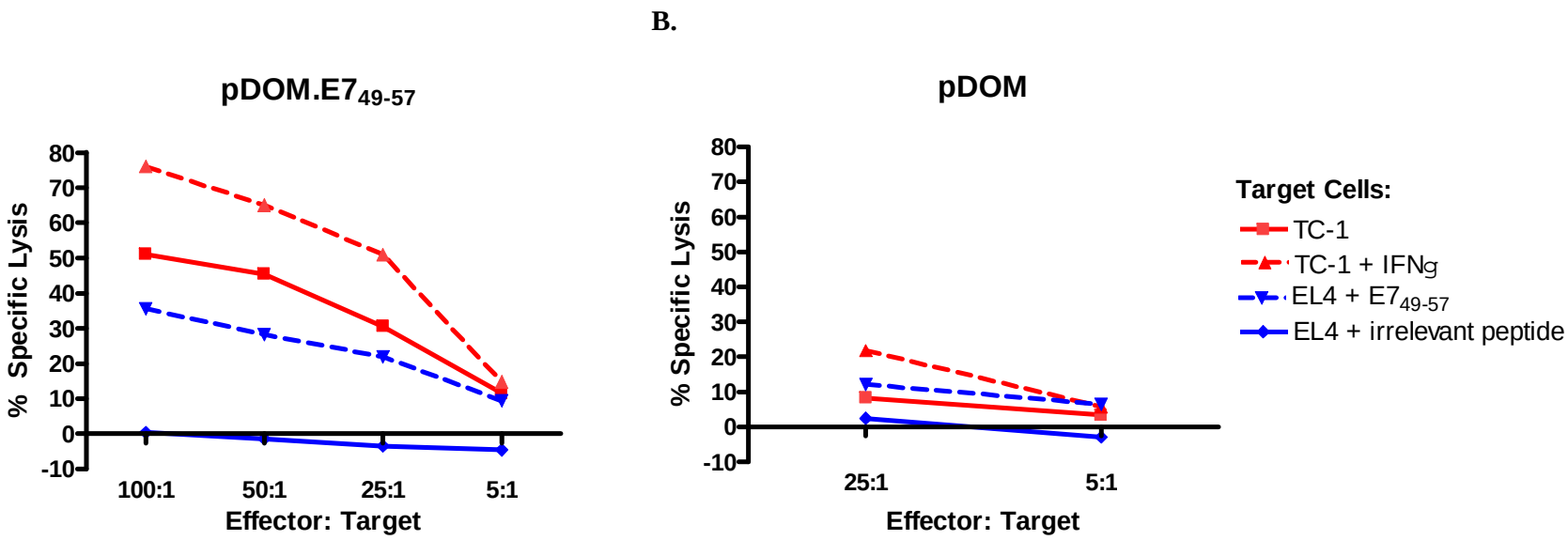


Figure 3.11. CTL-mediated lysis of variant TC-1 cells *in vitro*

A chromium release assay was carried out using individual short-term CTL lines from mice vaccinated with **A.** pDOM.E7$_{49-57}$ or **B.** pDOM control vaccine on d0 and boosted with EP 14 days later. Splenocytes were harvested 8 days after boost and CTLs were expanded *in vitro* for 7 days in the presence of 1nM E7$_{49-57}$ peptide and 20U/mL IL-2. The target cells used were EL-4 cells or variant TC-1 cells either alone or pulsed with E7$_{49-57}$ or an irrelevant peptide. Where applicable, variant TC-1 cells were incubated with 400U/mL IFNγ for 48hr prior to the assay. Chromium release was measured after a 5hr incubation of effector and target cells. Plots are representative of two mice tested with each vaccine.

3.2.9. HLA-A*0201-restricted E7-specific $CD8^+$ T-cell Responses Induced by DNA

So far, all of the E7-specific responses investigated have been H-$2D^b$-restricted responses. In order to demonstrate that the work discussed here could be applicable for humans, it was necessary to induce CTLs against an HLA-A*0201-resistricted human T-cell epitope. To facilitate this, HHD transgenic mice were used. These mice express a chimeric MHC I molecule partially derived from HLA-A*0201 and partially derived from endogenous H-2 but no H-2 in its wild-type form [224]; see Materials and Methods for further information. To analyse HLA-A*0201-resistriced responses, two pDOM-epitope fusion vaccines encoding two previously identified epitopes, residues 11-20 and 86-93 of E7 [234] were constructed.

Mice were vaccinated with pDOM.$E7_{11-20}$, pDOM.$E7_{86-93}$ or pDOM control vaccine and an IFNγ ELISpot was carried out 14 days later. Lymphocytes were isolated and incubated overnight with the corresponding E7-derived peptide or the DOM-derived p30 peptide at a concentration of 1μM. As shown in Figure 3.12A, a strong $E7_{11-20}$-specific T-cell response could be detected in 7/8 mice vaccinated with pDOM.$E7_{11-20.}$ However, no $E7_{86-93}$-specific responses were observed after pDOM.$E7_{86-93}$ vaccination, despite the presence of detectable p30-specific responses in the same mice, an indicator of successful immunisation (Figure 3.12B). Background responses found in the absence of peptide were low, as were those found against both E7 peptides in lymphocytes from pDOM-vaccinated mice (Figure 3.12C).

These data confirm that the pDOM-epitope design has the potential to induce E7-specific responses in cancer patients.

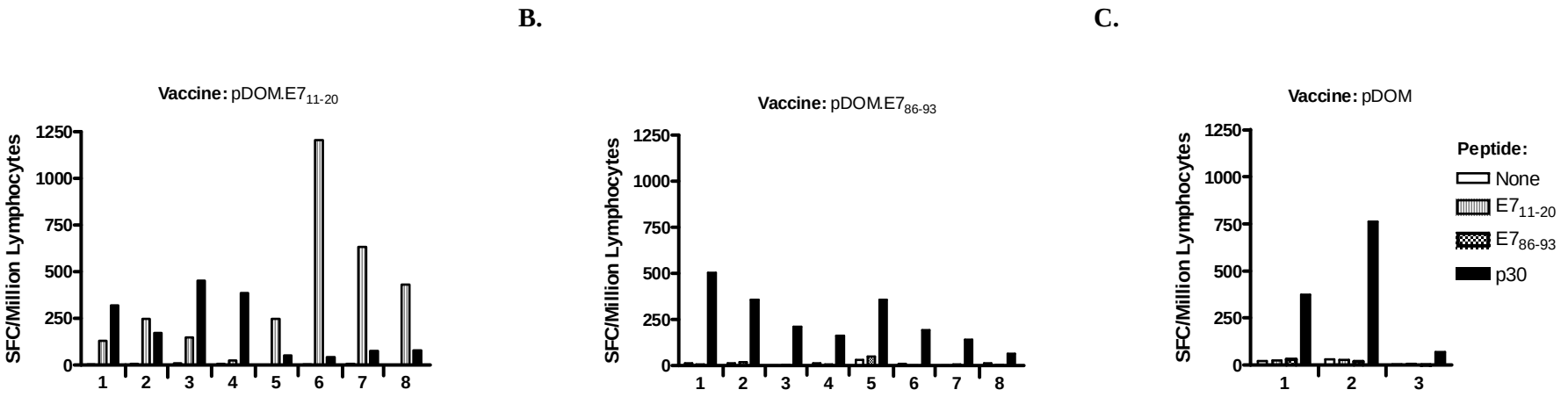


Figure 3.12. HLA-A*0201-restricted E7-specific CD8$^+$ T-cell responses

Mice were vaccinated with **A.** pDOM.E7$_{11\text{-}20,}$ **B.** pDOM.E7$_{86\text{-}93}$ or **C.** pDOM control vaccine. IFNγ responses were assessed 14 days later in an *ex vivo* ELISpot assay after overnight restumulation with E7-derived (residues 11-20 or 86-93) or DOM-derived (p30) peptides as indicated. SFC, spot-forming cells. Each set of bars represents an individual mouse (numbered 1-8). Data are combined from two experiments showing the same trend.

3.3. Discussion

3.3.1. DNA Vaccines Induce Robust E7-specific $CD8^+$ T-cell Responses

All DNA vaccines tested here induced an E7-specific $CD8^+$ T-cell response detectable in *ex vivo* assays (Figure 3.2). The best responses were induced by the vaccines encoding the whole E7 gene (pE7GGG and pDOM.E7GGG); however the pDOM-epitope and pDOM-long peptide vaccines were also very effective. In contrast, previous work in our lab has shown that full-length gene vaccines are often not this successful (unpublished observations) due to the phenomena of immunodominance, immunological tolerance and peptide production.

In the case of the E7 protein, although not all possible epitopes were tested here, no competing CTL epitopes appear to exist (Figure 3.3), a finding which indicates that the $E7_{49-57}$ epitope is immunodominant. Therefore, removing the rest of the antigen is not necessary to reveal a response, as it is in other models where the target epitope is subdominant [173]. The viral origin of E7 probably means it is a highly immunogenic protein in these wild-type mice. As a result, the provision of DOM-specific T-cell help is not required to induce a $CD8^+$ T-cell response, as it is in a setting of tolerance [166]. Another reason why the pE7GGG vaccine may be so immunogenic is the length of the E7 gene. At only ~300bp in length it is relatively small so will be efficiently transcribed and translated, unlike many other tumour antigens which could be 1000+bp long. Furthermore, although it has been demonstrated that responses are induced by several other peptides when positioned at the C-terminus of the DOM sequence [173]; suggesting that peptide processing from this position is effective, it appears that the processing of the $E7_{49-57}$ peptide epitope may be superior from the full-length E7 protein. Certainly, peptide processing is not improved by positioning the epitope at the 5' end of DOM. Therefore, it is likely that $E7_{49-57}$ peptide production from the E7 gene is not impaired, both in terms of protein translation and subsequent peptide processing. Another factor could be the persistence of the protein product of the pE7GGG vaccine. If the E7GGG protein did have a longer half-life than that of the DOM-epitope protein it could potentially be recognised by more DCs leading to greater T-cell responses. However, there are data which suggest that the persistence of antigen may actually have an inhibitory effect on $CD8^+$ T-cell induction ([235]) so the true effect of this is unknown.

Other sequence-specific factors may also account for the performance of the pE7GGG vaccine. $CD8^+$ T-cell responses to the full-length gene could potentially be augmented by the presence of sequences within the E7 gene which induce cognate $CD4^+$ T-cell help;

though we have found little evidence of this in response to the $E7_{48-57}$ and $E7_{43-77}$ peptides in *ex vivo* assays (Figure 3.4 and Figure 3.5), there may be other epitopes outside this region. Previous reports have described a $CD4^+$ T-cell response to the $E7_{43-77}$ synthetic long peptide only after *in vitro* restimulation for 7 days [225], suggesting that there is only a weak response, however it may still be physiologically relevant. Another possibility is that the E7 gene sequence could stimulate innate immunity which may be crucial for DC activation at the site of injection in order to facilitate cross-presentation. However, the pCI plasmid backbone itself provides 16 CpG motifs for this purpose [236]. In addition, there is growing evidence that several non-sequence specific cytosolic DNA-sensing mechanisms exist in several cell types which also lead to the production of type I IFN [128], [130]. Finally, as the pE7GGG construct encodes the E7 protein in its native form, protein folding should be optimal. It is thought that the pDOM-epitope construct should also fold into a mature protein, based on the previous success of similar vaccines ([96]); however this potential advantage cannot be ruled out.

Despite the apparent superiority of the full-length E7 gene vaccines, the pDOM-epitope format is still very effective and may be a better strategy in a clinical setting due to the potential for the development of tolerance to persistent HPV infection. Certainly, other tumour antigens to which this approach may be applied may require the benefits of T-cell help, lack of T-cell competition and efficient transcription and translation which the pDOM-epitope design provides.

3.3.2. DNA Vaccines Induce Superior $CD8^+$ T-cell Responses Compared to Synthetic Long Peptide with CpG

To put the $CD8^+$ T-cell responses induced by our vaccines into context, we compared them to those stimulated by a synthetic 'long peptide' vaccine given with CpG and previously reported as a successful strategy [225]. However, in our hands, after a single vaccination all of the DNA vaccines induced superior IFNγ production in $CD8^+$ T cells (Figure 3.2) and this was not due to differences in kinetics (Figure 3.6). Furthermore, DNA vaccines also out-performed the synthetic long peptide in a prime-boost setting (Figure 3.7). $CD8^+$ and $CD4^+$ T-cell responses to synthetic long peptide vaccination were previously analysed after one week-restimulations [225] which would expand the low-level responses. As the data described here were collected immediately *ex vivo* this could explain why the approach proved less successful in this study.

The synthetic long peptide approach has now been taken into the clinic in the form of multiple E7-derived overlapping peptides 25-30 amino acids in length. Half of patients with HPV16-positive vulvar intraepithelial neoplasia treated with the E7 long peptides displayed complete regression [237]; and in patients with advanced cervical carcinoma E6 and E7 synthetic long peptide vaccination induced T-cell responses, and a reduction in tumour burden was seen in 6/35 cases [238]. Interestingly, when pools of E6 and E7 peptides were given in the same site, E6-specific T-cell responses dominated; however E7-specific responses were induced if the two pools were given at different sites [238]. A more detailed phase II study in cervical cancer patients revealed that the T-cells induced by the synthetic long peptide vaccines were $CD8^+$ and $CD4^+$ effector cells but also $CD4^+CD25^+FOXP3^+$ regulatory cells [239], which could limit vaccine efficacy. Given the superior immune induction by DNA vaccines in mice, this confirms the validity of investigating the DNA vaccine approach as a potential immunotherapy against HPV16-associated cancers.

3.3.3. Lysis of TC-1 Tumour Cells *in vitro*

The next question was whether the $CD8^+$ T cells induced by the DNA vaccines were able to lyse target cells *in vitro*. In order to analyse this, the TC-1 tumour model was used. As shown in Figure 3.8, these cells expressed a low level of H-$2D^b$ MHC I molecules, which prevented their lysis by pDOM.$E7_{49\text{-}57}$-specific CTLs unless they were pre-treated with IFNγ to up-regulate its expression (Figure 3.9). The role of IFNγ signals in TC-1 cell biology has been investigated previously and found to be of critical importance. TC-1 cells transfected with a non-functional IFNγ receptor escape immunotherapy due to their resistance to MHC class I up-regulation, and treatment with depleting anti-IFNγ antibody reduces the number of T cells found in both mutant and parental TC-1 tumours in immunised mice [240]. Down-regulation of surface MHC class I expression is a method of immune evasion often employed by tumours [241] and viruses [211], in fact the E7 gene is itself responsible for this effect [212]. However, it is important to note that the result of this can be two-fold; firstly it can enable transformed or infected cells to resist CTL-mediated lysis, but secondly, it could also render them susceptible to NK attack [242].

Despite the finding here that TC-1 cells express low levels of surface MHC I molecules, previous reports had stated that the cells did express significant amounts, and only escape variants which emerged after passage in immunised mice had lower or no expression [213]. Therefore, another batch of the TC-1 cells was acquired. These 'variant' TC-1 cells did seem to express slightly higher levels of H-$2D^b$; however it could still be increased by

incubation with IFNγ (Figure 3.10), and always remained plastic. The level of MHC I expression in different sub-lines of TC-1 cells *in vitro* was followed for several weeks but the results were not clear, however there was a weak trend towards increased MHC I expression after longer periods of culture (data not shown), perhaps due the lack of selective pressure. The variant TC-1 cells do appear to be slightly more susceptible to lysis *in vitro* (Figure 3.11). These data highlight the complications of the TC-1 tumour cell model. The results also confirm that the $CD8^+$ T cells induced by our vaccines are functional in that they are able to lyse tumour cells *in vitro*; this provides a basis for *in vivo* experiments.

3.3.4. Induction of HLA-A*0201-restricted E7-specific $CD8^+$ T-cell Responses

In order to confirm that this approach would be applicable to E7-associated tumours in patients, the presence of the human $CD8^+$ T-cell epitopes had to be confirmed. One of the pDOM-epitope designs tested did indeed induce $E7_{11-20}$-specific $CD8^+$ T cells in HHD transgenic mice (Figure 3.12A). However, this epitope would require further validation to ensure that it is processed and presented by E7-expressing tumours in patients. Recent data suggest that the related epitope $E7_{11-19}$ is recognised by human CTLs and is expressed on HPV16-expressing tumour cell lines [243]; indicating that it may be a better target. Despite previous reports that the $E7_{86-93}$ peptide binds to HLA-A*0201 [234] and that a weak $E7_{86-93}$-specific T cell response can be detected in HPV^+ individuals [244], no responses to the pDOM.$E7_{86-93}$ vaccine were seen in HHD transgenic mice (Figure 3.12B). This can be explained by the finding that the epitope is not processed by the mouse antigen processing and presentation machinery in HLA-A*0201 transgenic mice [245].

A T-cell epitope such as $E7_{11-20}$ that binds to a member of the HLA-A2-like superfamily of related HLA molecules, including HLA-A*0201, should be recognised by 30-50% of the human population, depending on ethnicity [246]. In order to cover >90% of the world's population, epitopes which bind to each of the four HLA supertypes (A2-, A3-, B7- and B44-like) would need to be targeted [246]. By producing a vaccine appropriate for each haplotype, a universal immunotherapy against HPV16-related cancers could be provided.

3.3.5. Conclusions

Using a murine model, it has been demonstrated that our DNA vaccines induce $CD8^+$ T-cell responses to the H-$2D^b$-restricted $E7_{49-57}$ epitope and that these T cells are functional;

they produce IFNγ and acquire lytic ability. Furthermore, they also develop a memory pool allowing effective subsequent boosting. Importantly, these responses are significantly stronger than those induced by synthetic $E7_{43-77}$ long peptide with CpG, validating our approach. In addition, there is a potential HLA-A*0201-restricted epitope within the E7 gene which confirms the potential for translation into human therapy. The assessment of therapeutic vaccination with these constructs will be the subject of the next chapter.

3.3.6. Future Work

Further investigation into the mechanisms which control the efficacy of the various vaccines could be carried out. For example, it is not known whether the processing of the $E7_{49-57}$ epitope is more efficient from the pDOM-epitope or pE7GGG construct. This could be addressed by transfecting cells with each construct *in vitro* and then using them in a killing assay as a proxy for peptide expression. In order to control for the transfection efficiency, the constructs first must be cloned into bicistronic plasmids which co-express the green fluorescent protein (GFP) marker. These modified constructs could then be used to transfect target cells *in vitro* prior to transfer into pDOM-$E7_{49-57}$ vaccinated mice. The clearance of these cells would correlate with the expression of $E7_{49-57}$ on the cell surface, revealing any differences in the processing and presentation of the $E7_{49-57}$ peptide from two vaccines.

4. The Production of an E7-specific Therapeutic Vaccine

4.1. Introduction

In the previous chapter it was demonstrated that DNA vaccines can induce E7-specific $CD8^+$ T-cell responses; and that these cells produce IFNγ and lyse target cells. This sets the scene for the continuation of the investigation *in vivo* to determine if the cells induced by the vaccines provide effective immunotherapy. This was approached in two ways: firstly, using the TC-1 tumour cells described in the previous chapter as an *in vivo* challenge; and secondly by using transgenic mice which develop tumours spontaneously.

4.1.1. Therapeutic Vaccines against E7-expressing Tumours in Mice

Therapeutic vaccination against HPV16 E7 aims to induce E7-specific $CD8^+$ T cells which can kill tumour cells *in vivo*. Several studies in mice have successfully induced immune responses against the HPV16 E7 oncoprotein using a variety of approaches, reviewed in [247]. However, producing an impressive therapeutic benefit, where immunotherapy is able to control an existing E7-expressing tumour, has proven difficult. In one study, a DNA plasmid encoding the E7 gene fused to a lysosomal target signal was given in conjunction with a plasmid aimed at improving helper T-cell responses and another plasmid aimed at reducing DC apoptosis [248]. It was necessary to include all three components to achieve a therapeutic effect against an established E7-expressing tumour, illustrating the complex nature of immunotherapy. Another strategy under investigation uses an intracellular pathogen to deliver antigen. Recombinant *Listeria Monocytogenes* expressing E7 fused to listeriolysin O (LLO) has been used in a therapeutic setting to treat established E7-expressing tumours [249]; this was only successful with the fusion protein, and not with E7 alone. Ordinarily, *Listeria* directs the E7 antigen to the MHC class II processing pathway but the actions of LLO direct antigen to the MHC class I processing pathway, so its requirement indicates that $CD8^+$ T cells account for the therapeutic effect. Interestingly, these *Listeria*-based vaccines also have some effect in a tolerised mouse model [223] used in this study (see next section).

Another popular method for delivering antigen in a therapeutic setting is peptide vaccination. The major limitation of peptide vaccination with minimal $CD8^+$ T-cell epitopes is the lack of T-cell help. One approach to overcome this is to use a 'long peptide' strategy which can stimulate both $CD8^+$ and $CD4^+$ T-cell responses. A portion of the E7 protein (residues 43-77) has been used in this way with some success. $E7_{43\text{-}77}$ peptide

vaccination in conjunction with CpG greatly improved survival of mice with established E7-expressing tumours [225]. Based on this result, overlapping long peptides from HPV16 E6 and E7 emulsified in the adjuvant Montanide are now being used in clinical trials. Phase I and II data in cervical cancer patients show that the vaccines are safe and induce both E6- and E7-specific $CD8^+$ and $CD4^+$ T-cell responses [238], [239]. However, when this approach was compared to DNA vaccination in the previous chapter, the $CD8^+$ T-cell response induced was lower than those induced by DNA vaccines (see Figure 3.2).

4.1.2. E6/E7 Transgenic Mice

E6/E7 transgenic mice express the E6 and E7 oncoproteins from HPV16 under the control of the thyroglobulin promoter, however they are also expressed in the neonatal thymus [223]. The effect of this is two-fold: firstly, the mice are tolerised to the HPV16 proteins, and secondly, they develop tumours in their thyroid glands. This is important as in many cancers the tumour antigens available to target with immunotherapy are self antigens to which the immune system is tolerised (see section 1.1.2.5 for information on immunological tolerance). Those self-reactive T cells which escape central tolerance mechanisms may be of lower avidity and such responses may also be suppressed in the periphery. Therefore, these mice which express the E7 oncogene as a self protein allow us to test our vaccines in an experimental setting which more closely resembles the clinical one. The benefits of using spontaneous tumour models have been shown before for other cancer antigens [250], [251].

Previous data collected in E6/E7 mice revealed that the E7-specific T-cells induced were of lower frequency and avidity [223]; but that *Listeria*-based E7 vaccines could reduce the mass of spontaneous tumours [252].

4.1.3. Aims

The aim of this part of the project was to assess the potential of the DNA vaccines described in Chapter 3 as immunotherapy for HPV16-associated cancers. This chapter focuses on their ability to protect against challenge with E7-expressing tumours *in vivo*. Firstly, transplantable E7-expressing tumours were used as a challenge in both a prophylactic and therapeutic setting. Secondly, E6/E7 mice were used to test the efficacy of the DNA vaccines against spontaneously developing tumours.

4.2. Results

4.2.1. DNA Vaccine-induced Protection against *in vivo* TC-1 Tumour Challenge

The low expression of MHC I that we found on the original batch of TC-1 cells could enable them to escape CTL-mediated destruction *in vivo*. However, local inflammation and cytokine production *in vivo* may be able to increase MHC class I expression on the surface of tumour cells. In the previous chapter it was demonstrated that this is the case with *in vitro* IFNγ treatment, and others have shown that genetically modified TC-1 cells which express a non-functional IFNγ receptor can escape immune attack *in vitro*; in contrast to the parental TC-1 cells [240]. This suggests that local IFNγ production is crucial for rejection of TC-1 tumours *in vivo*. In order to investigate whether our vaccines could induce tumour rejection *in vivo*, a tumour challenge experiment was carried out.

Firstly, a dose-response experiment was conducted to reveal the optimal tumour dose for the subsequent experiments. TC-1 cells were implanted by sub-cutaneous injection, causing a discrete tumour nodule to form under the skin. These tumours were easily measured by palpation every 2-3 days and mice were culled when the tumour reached 15mm diameter. By 30 days after tumour implantation, all mice had succumbed to tumour at doses of $5x10^4$ and $1x10^5$ TC-1 cells/mouse, as shown in Figure 4.1A. A dose of $5x10^4$ cells/mouse was used for all subsequent experiments; this dose has been used in the past by others [225], [253].

Tumour challenge experiments were then undertaken in which immunised mice were challenged with TC-1 cells 14 days after vaccination. As shown in Figure 4.1B, the best protection from the TC-1 challenge was afforded by the pE7GGG vaccine, which protected 100% of mice. The addition of DOM in the pDOM.E7GGG vaccine resulted in ~60% protection, possibly due to the increased size of the gene product reducing the level of its transcription and translation, however the difference between the groups vaccinated with pDOM.E7GGG and pE7GGG was not statistically significant. Similarly, ~60% of the mice given either the pDOM.$E7_{43-77}$ or the p$E7_{43-77}$ vaccine also survived. When compared to the survival of the group given the control vaccine (pDOM), immunisation with all these vaccines resulted in a statistically significant improvement in protection ($P<0.005$ in a Chi square log rank test). For the p$E7_{43-77}$ vaccine, this is surprising given the low level of $CD8^+$ T cells found to be induced by the construct in Chapter 3. Surprisingly, only ~20% of those in the group which received the pDOM.$E7_{49-57}$ vaccine survived the tumour challenge, despite the fact that this vaccine is effective at inducing $E7_{49-57}$-specific $CD8^+$ T

cells (see Chapter 3). Vaccination with long peptide and CpG also failed to protect the mice, in contrast to previous reports [254]. Given the low numbers of mice (n=8-9) in this experiment and reduced MHC I expression found on the original TC-1 cells, these data may not be reliable. In all subsequent experiments the 'variant' TC-1 cells were used.

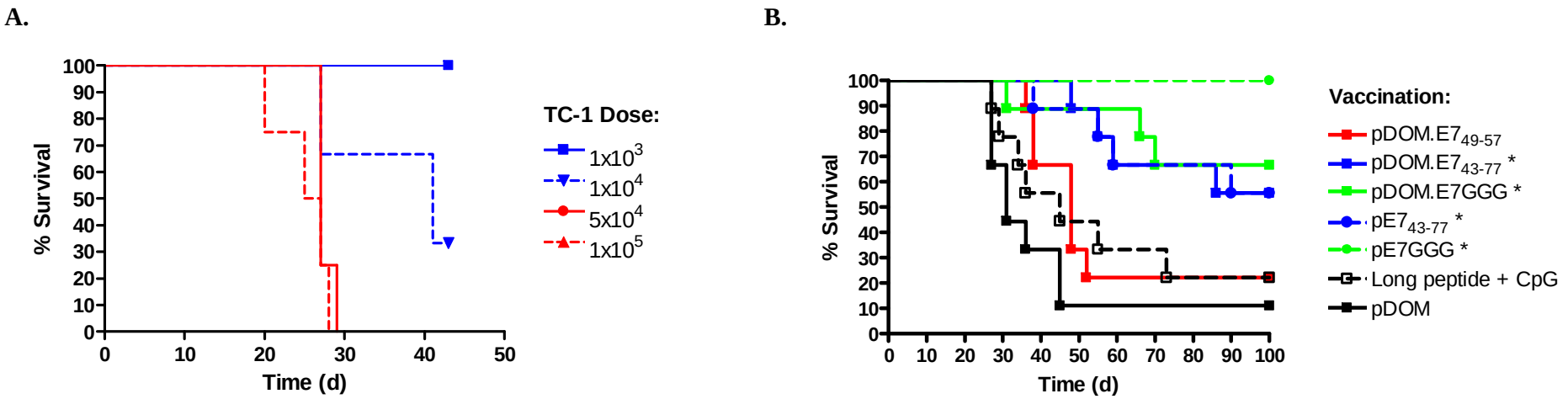


Figure 4.1. Tumour challenge experiments *in vivo* using original TC-1 cells

Mice were injected s.c. with TC-1 cells in 100µL PBS and tumours were palpated every 2-3 days. **A.** A dose-response experiment was carried out in groups of 3-4 naïve mice. Each group was given the indicated number of TC-1 cells/mouse. Experiment carried out by SLB. **B.** Groups of 8-9 mice were vaccinated with the indicated DNA vaccines or long peptide ($E7_{43-77}$) and CpG 14 days before challenge (day 0) with $5x10^4$ TC-1 cells/mouse. Vaccines marked with an asterisk (*) caused a statistically significant improvement in survival when compared to the pDOM control vaccine in a Chi square log rank test ($P<0.005$). Experiment shown in **A**. carried out by SLB.

4.2.2. DNA Vaccine-induced Protection against *in vivo* Challenge with Variant TC-1 Cells

To ascertain whether the increased *in vitro* killing of the 'variant' TC-1 cells correlated with *in vivo* protection, DNA-vaccinated mice were challenged with these TC-1 cells. First a dose-response experiment was carried out to determine the appropriate dose of TC-1 cells to use in challenge experiments. As shown in Figure 4.2A, $1x10^4$ cells failed to implant in naïve mice so the higher dose of $5x10^4$ cells/mouse was used in all subsequent experiments.

Groups of 8-9 mice were vaccinated with pDOM.$E7_{49-57}$, pE7GGG or pDOM control vaccine and then boosted with the same vaccine 28 days later. Mice also received electroporation (EP) with the boost (see Materials and Methods for further information on EP). Mice were then challenged with $5x10^4$ TC-l cells by sub-cutaneous injection 8 days after boost. The results are shown in Figure 4.2B. Both test vaccines induced statistically significant improvements in survival when compared to the pDOM-vaccinated control group (P=0.0001 in a Chi square log rank test). Vaccination with the pE7GGG vaccine protected 75% of the mice and vaccination with the pDOM.$E7_{49-57}$ vaccine protected 50% of the mice. This level of survival is approximately twice that which was achieved with the original TC-1 cells (Figure 4.1B); though it must be noted that the vaccination schedule is different as these mice have received a booster injection.

Based on these findings, we decided to use variant TC-1 cells for all further experiments in this project. They will henceforth be referred to simply as 'TC-1' cells.

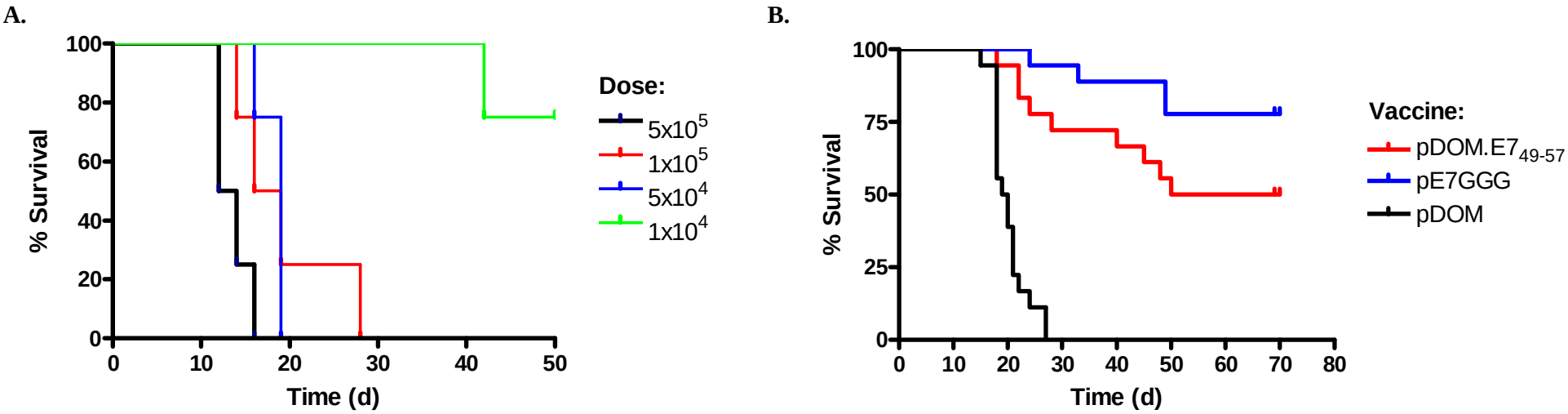


Figure 4.2. DNA vaccine protection against *in vivo* challenge with 'variant' TC-1 cells

A. Dose-response to the indicated numbers of variant TC-1 cells in groups of 4 naïve mice. **B.** Groups of 8-9 mice were vaccinated with pDOM.E7$_{49\text{-}57}$, pE7GGG or the pDOM control vaccine and boosted 28 days later with EP. Eight days after boost, mice were challenged with $5x10^4$ TC-1 cells/mouse by s.c. injection and resultant tumours were palpated every 2-3 days. Both test vaccines caused a statistically significant improvement in survival when compared to the pDOM control vaccine in a Chi square log rank test (P=0.0001). In **B**, data are combined from two experiments showing the same trend.

4.2.3. DNA Vaccines as Immunotherapy for TC-1 Tumours *in vivo*

As the ultimate goal for DNA vaccines against cancer is efficacy in a therapeutic setting, we wanted to test what effect ours had on established E7-expressing TC-1 tumours. In this experiment, mice were challenged with $5x10^4$ TC-1 cells by subcutaneous injection and given DNA vaccine as therapy three days later. Tumours were palpated every 2-3 days and their growth monitored. Tumour sizes in the different treatment groups were compared 19 days after tumour challenge. The results, shown in Figure 4.3, demonstrate the potential of DNA vaccines to work in this setting. When compared to mice given the pDOM control vaccine, pDOM.$E7_{49-57}$-vaccinated animals had significantly smaller tumours (P=0.0392), with 2/18 being tumour-free at this point. The reduction in tumour size was even greater in the group which received the pE7GGG vaccine (P<0.0001) and 3/17 were tumour-free at d19. In comparison, all of the mice given pDOM had developed tumours by this point and they were on average almost twice the size of those found in the pE7GGG group. However, despite the differences seen at d19, all mice eventually developed tumours and ultimately succumbed to tumour, demonstrating how aggressive the TC-1 cells are *in vivo*.

These data support the results of the tumour protection experiment shown in Figure 4.2 which showed that the full-length E7 vaccine is more effective than the pDOM-epitope vaccine in a prophylactic setting. There are several potential mechanisms which could explain this discrepancy, one of which is the possibility that the E7GGG sequence could contain $CD4^+$ T-cell epitopes to provide cognate help for the $CD8^+$ T-cell response.

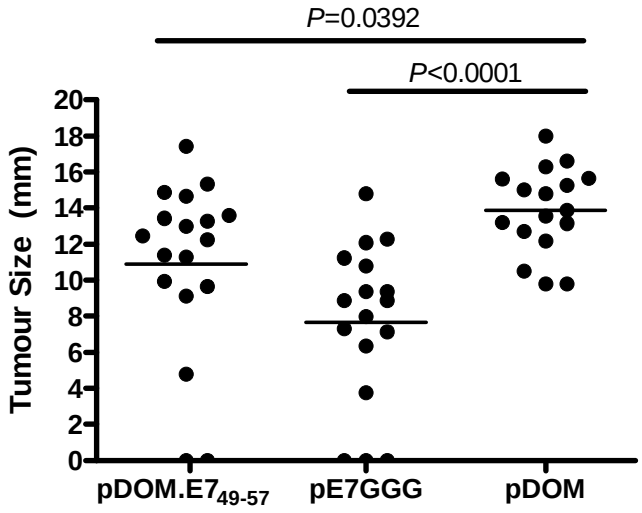
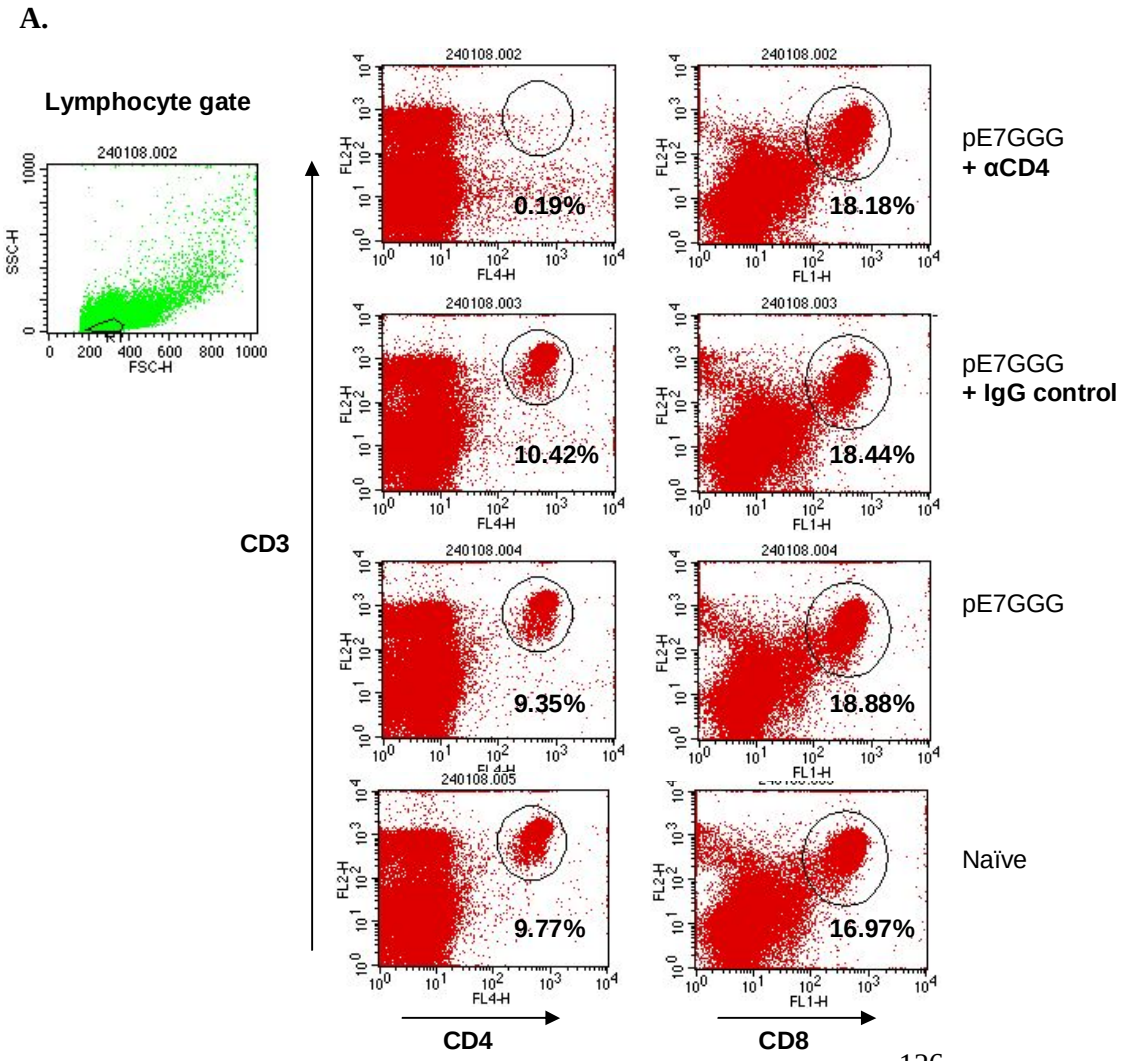


Figure 4.3. DNA vaccine-mediated reduction in tumour size

Groups of 8-9 mice were challenged with $5x10^4$ TC-1 cells by subcutaneous injection. After 3 days, each group was vaccinated with either pDOM.$E7_{49-57}$, pE7GGG or pDOM control vaccine and tumours were monitored every 2-3 days. The size of the respective tumours 19 days after challenge is shown. Lines represent group means. Data are combined from two experiments showing the same trend. *P* values were generated in a t test.

4.2.4. E7GGG-mediated Protection against TC-1 Challenge in $CD4^+$ T cell-depleted Mice

One explanation for the observation that pDOM.$E7_{49-57}$ vaccine is less successful than the pE7GGG vaccine at conferring protection against TC-1 tumour challenge *in vivo* (see Figure 4.1 and Figure 4.2) could be the presence of cognate $CD4^+$ T-cell epitopes in the E7 sequence. Even though no evidence of $CD4^+$ T-cell epitopes within the $E7_{43-77}$ long peptide sequence was found in Chapter 3, it is likely this viral protein does contain some in other regions of the protein. Therefore we conducted a tumour protection experiment in which $CD4^+$ T cells were depleted during the effector phase to see if $CD4^+$ T cells were implicated in pE7GGG-mediated protection against TC-1 tumour challenge. Groups of 7-9 male mice were vaccinated with pE7GGG 14 days before they were challenged with $5x10^4$ TC-1 cells/mouse. After immune induction, mice were treated with rat anti-mouse CD4 antibody in order to deplete $CD4^+$ T cells or rat IgG as a control. Depletion at this time point should enable us to investigate tumour protection in the absence of $CD4^+$ T-cell help. To ensure that the depletion had been successful, blood samples were taken approximately 2hr prior to challenge on d0 and stained with anti-CD4, anti-CD8 and anti-CD3 antibodies. Flow cytometric analysis, confirmed that the group given anti-CD4 did lack $CD4^+$ cells but not $CD8^+$ cells, as shown in Figure 4.4A. CD3 and CD4 double-positive cells constituted 0.19% of the lymphocyte population in samples from depleted mice, compared to an average of 9.85% in the other groups. This corresponds to 98% depletion. Depletion continued throughout the experiment (injections of depleting or control antibodies were given every 2-4 days until d27) and sample mice were re-tested on d13 when depletion was 89% and d20 when depletion was 90%. The survival of the mice is shown Figure 4.4B. As expected, naïve mice succumbed by d55 (all but one by d35) however, all the other groups did not. Surprisingly, even the CD4-depleted mice were able to resist the challenge, indicating that at the time of challenge, $CD4^+$ T cells are not required for TC-1 tumour rejection.



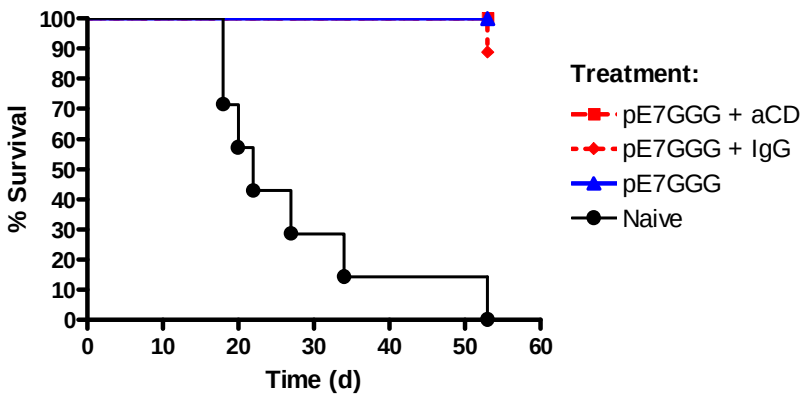


Figure 4.4. pE7GGG-mediated *in vivo* protection in mice depleted of CD4$^+$ cells

Mice were vaccinated with pE7GGG 14 days prior to challenge with 5x10^4 TC-1 cells on d0. 9 injections of anti-CD4 antibody or control IgG were given from d-3 to d27. Depletion was confirmed by FACS staining with anti-CD3, CD4 and CD8 for comparison **A.** Representative FACS plots from individual mice from each treatment group on d0. **B.** Survival. Data from test groups were compared to data from the naïve controls using a Chi square log rank test (*P*=0.0001 in all groups).

4.2.5. Comparison of DNA Vaccine and Soluble Long Peptide-mediated Immunotherapy of TC-1 tumours

Although it was demonstrated that DNA vaccines could have a beneficial effect in a therapeutic setting (Figure 4.3), all mice eventually succumbed to tumour. Therefore, a comparison with synthetic $E7_{43-77}$ long peptide was carried out. This strategy of vaccination with 'long' peptides had been previously reported as an effective therapeutic vaccine in this model [225]; therefore it could serve as a positive control. The authors of the published data indicated that $CD8^+$ and $CD4^+$ T cells were responsible for the therapeutic effect of the peptide vaccine. However, in Chapter 3, synthetic long peptide with CpG was shown to induce fewer $CD8^+$ T cells than the DNA vaccines and no cognate $CD4^+$ T-cell responses could be detected at all. Therefore, how the synthetic $E7_{43-77}$ long peptide might function in this model was unclear.

As before, mice were challenged with $5x10^4$ TC-1 cells and vaccinated with DNA or peptide with CpG three days later; the results are shown in Figure 4.5. After treatment with the pE7GGG vaccine a significant reduction in tumour size was seen at d19 (P=0.0002) and 4/18 mice were tumour-free at this point, similar to the previous experiment. Treatment with the pDOM.$E7_{43-77}$ long peptide DNA vaccine also resulted in reduced tumour size (P=0.0062) to a similar degree as the pDOM.$E7_{49-57}$ vaccine; as would be predicted given their only very slight difference in structure and analogous immune induction shown in Chapter 3. As predicted given its poor performance in inducing immunity demonstrated in Chapter 3, the p$E7_{43-77}$ vaccine was not of any therapeutic benefit.

Suprisingly, synthetic long peptide vaccination caused a dramatic reduction in tumour size at d19 ($P<0.0001$), with 8/18 mice tumour-free at this point and furthermore, 7/18 (39%) of the mice survived the experiment, a statistically significant difference ($P<0.0001$). Treatment with the pE7GGG vaccine also led to prolonged survival (P=0.0003), but this was not as dramatic with only 1/18 (5.5%) surviving the experiment (Figure 4.5B).

These results do not confirm the findings shown in Figure 4.1B. As previous investigators (TC Wu, M Smahel and CJ Melief, personal communications) had reported that their clones of TC-1 cells displayed a phenotype more like that of the variant TC-1 cells, it was decided that the original TC-1 cells should not be used any further in this project.

Interestingly, in the control group given CpG alone, there was a trend towards mice having reduced tumour sizes at d19 as well. Though this did not reach statistical significance, associated prolonged survival did when compared to that for the mice treated

with pDOM control vaccine (P=0.0401). Based on this result it was hypothesised that the CpG, which is given with the peptide vaccine, might be having an effect on the tumour separate to $CD8^+$ T-cell mediated attack. However, these data were quite weak due to the low numbers of mice given CpG alone (n=9, one experiment); therefore, further experiments were undertaken to investigate this possibility.

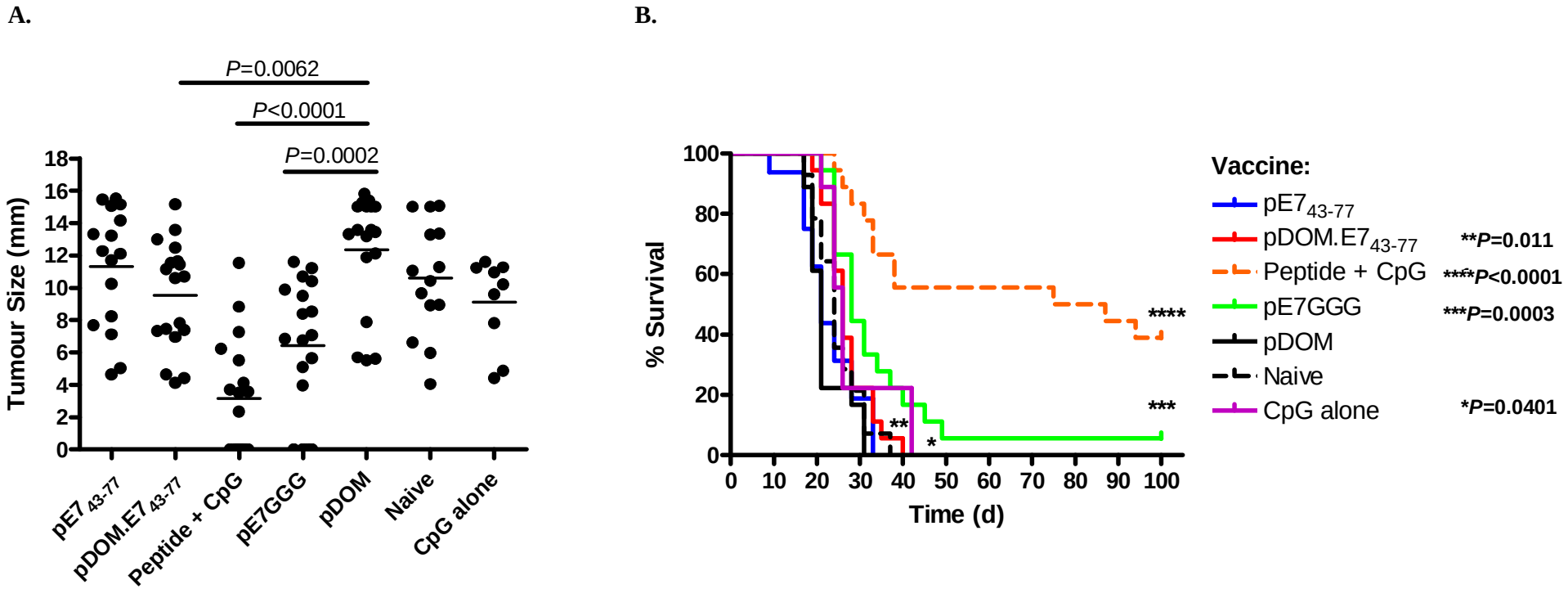


Figure 4.5. DNA vaccine and peptide-mediated reduction in tumour size *in vivo*

Groups of 6-9 mice were challenged with $5x10^4$ TC-1 cells by subcutaneous injection. After 3 days, each group was vaccinated with DNA vaccines shown above, pDOM control vaccine, soluble long peptide with CpG, CpG alone or left untreated (naïve). Tumours were palpated and measured every 2-3 days. *P* values were generated in a t test. **A.** The size of the respective tumours 19 days after challenge. **B**. Overall survival in the same mice. Lines represent group means. Data are combined from two experiments showing the same trend. *P* values were generated in a Chi square log rank test when compared to pDOM-vaccinated animals.

4.2.6. Stimulation of Innate Immunity after TC-1 Challenge in Naïve Mice

As $CD4^+$ T-cell help did not appear to be important during the effector phase of in vivo protection against the TC-1 tumour (Figure 4.4), another explanation for the superior therapeutic effect of the $E7_{43-77}$ long peptide vaccine was sought. Innate immunity could potentially alter the cytokine milieu *in vivo* which could affect tumour susceptibility to CTL-mediated lysis: for example, local IFNγ could increase MHC class I expression on TC-1 cells which have been shown to express variable levels of MHC I *in vitro* (Figure 3.8, Figure 3.9 and Figure 3.10). Furthermore, activation of innate immunity could lead to NK cell-mediated killing of MHC I negative TC-1 target cells.

To investigate this, mice were injected with CpG. CpG is known to stimulate innate immunity through TLR-9 which is expressed on specific immune cell subsets such as DCs, causing activation and cytokine release [124]. Two groups of 9 naïve mice were challenged with $5x10^4$ TC-1 cells/mouse as before and 5 days later, one group of mice was given CpG by sub-cutaneous injection close to the site of tumour inoculation in a protocol analogous to synthetic peptide vaccination.

Surprisingly, just one dose of CpG did significantly improve the median survival of the mice from 17 days to 21 days (P=0.0003 in Chi square log rank test), as shown in Figure 4.6. These data suggest that innate immunity could have a significant role in the rejection of TC-1 tumours. This may be especially important in a therapeutic setting where immune responses must be raised rapidly.

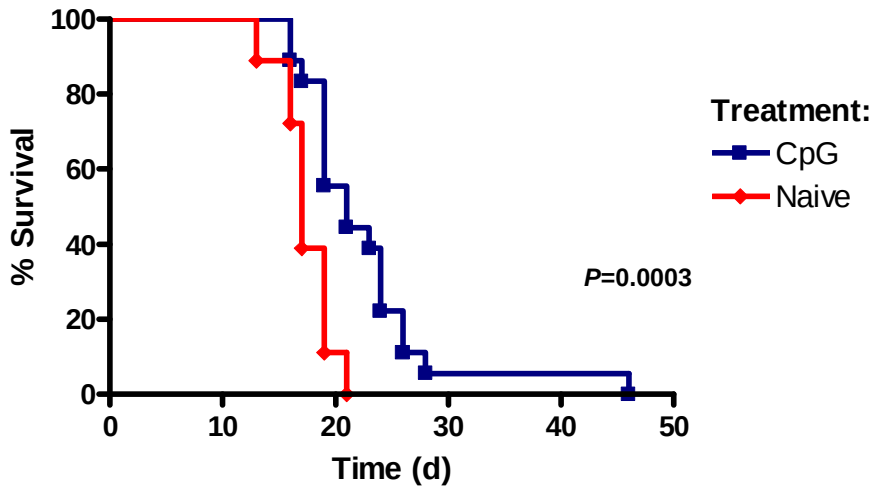


Figure 4.6. Survival in naïve mice challenged with TC-1 and given CpG therapy

Groups of 9 naïve mice were challenged with $5x10^4$ TC-1 cells/mouse by subcutaneous injection. 5 days later, one group was given a subcutaneous injection of CpG. Data are combined from two experiments showing the same trend. *P* value was generated in a Chi square log rank test.

4.2.7. Transfection of TC-1 cells with H-2D^b Heavy Chain

In order to remove the complication of variable MHC I expression found in TC-1 cells, they were retrovirally transfected with a plasmid containing the D^b heavy chain (for details see Materials and Methods). This is important because if the TC-1 cells down-regulate H-2D^b expression *in vivo* they may escape CD8$^+$ T-cell attack and could also become susceptible to NK cells. NK susceptibility could explain the discrepancy between induction of CD8$^+$ T cells and the level of protection afforded by DNA versus synthetic long peptide vaccine.

After transfection with pmscvD^b but not empty vector, it was clear that these 'TC-1-D^b' cells expressed more MHC I, as shown in Figure 4.7A. To assess whether this rendered the TC-1-D^b cells more susceptible to CTL-mediated attack, a 5hr chromium release assay was carried out. Mice were vaccinated with the pDOM.E7$_{49-57}$ or pDOM control vaccine and 14 days later short-term CTL lines were isolated and restimulated *in vitro* with E7$_{49-57}$ peptide for 6 days. As shown in Figure 4.7B, the parental TC-1 cells were lysed efficiently, but not as highly as EL-4 cells pulsed with the E7$_{49-57}$ peptide, as found before (see Chapter 3). Lysis of the TC-1-D^b cells was 10-20% higher than that of the parental TC-1 cells at both 80:1 and 40:1 effector: target cell ratios (3/4 pDOM.E7$_{49-57}$-vaccinated mice); indicating

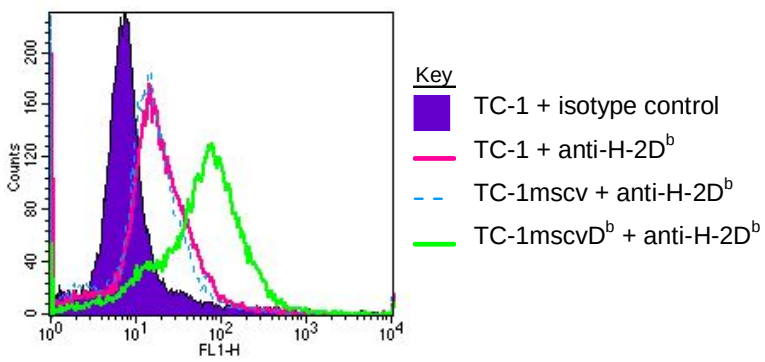
that the TC-1-D^b cells are more susceptible to CTL-mediated lysis. Lysis of TC-1-mscv cells which were transfected with empty plasmid was not notably increased. Background killing in lines from pDOM-vaccinated mice was low, as was lysis of the NK susceptible YAC-1 cell line, indicating that there was little NK-like activity in the cultures.

4.2.8. DNA Vaccine-mediated Immunotherapy against TC-1-D^b Tumour Challenge

Prior to using the TC-1-D^b cells for *in vivo* tumour challenge experiments, naïve mice were injected with the cells to confirm that they would passage. The standard dose of $5x10^4$ cells/mouse used for the parental TC-1 cells failed to implant in 20-40% of naïve mice, as shown in Figure 4.8. Therefore, the dose was increased to $1x10^5$ cells/mouse in subsequent experiments.

To assess whether the increased level of MHC I expression on TC-1-D^b cells may render them more susceptible to CTL-mediated immune attack *in vivo*, mice were vaccinated with DNA and then challenged with $1x10^5$ TC-1-D^b cells 14 days later. Interestingly, the level of protection afforded by both the pDOM.$E7_{49-57}$ and pE7GGG vaccines was 100%, as shown in Figure 4.9; compared to the 50 and 80% protection respectively which was previously observed against the parental TC-1 cells (Figure 4.2B). This confirms that these cells do behave differently in a challenge setting, suggesting that the increased MHC I expression observed *in vitro* may be maintained *in vivo*, as expected. However, technical issues complicate confirming this.

A.



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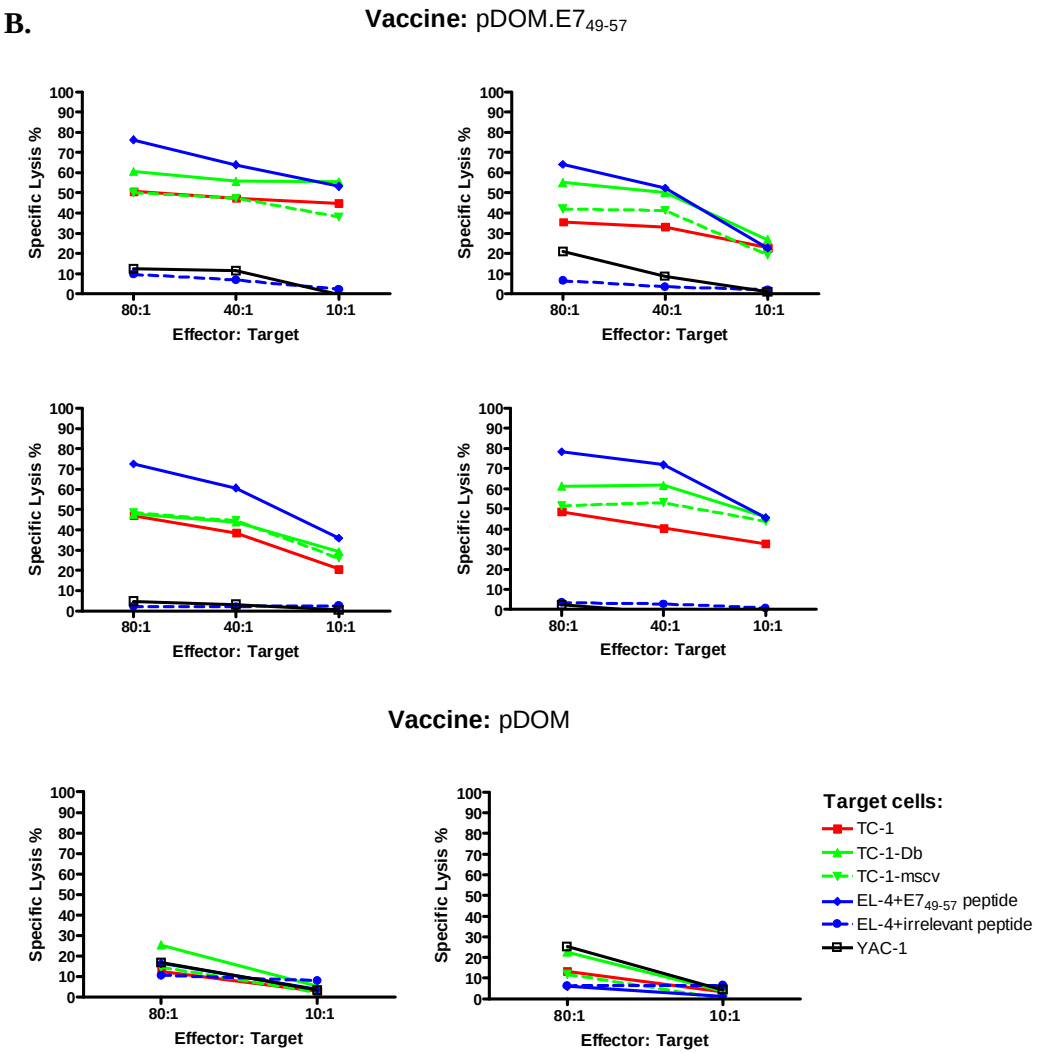


Figure 4.7. H-2D^b expression and CTL lysis of TC-1 cells transfected with the D^b heavy chain

A. FACS plot showing D^b expression before and after retroviral transfection with pmscv-D^b or empty pmscv plasmid. Cells were stained with anti-H-2D^b-FITC antibody or isotyoe control. **B.** Target cell lysis by short-term CTL lines isolated from mice vaccinated with pDOM.E7$_{49-57}$ or pDOM control and restimulated *in vitro* for 6 days in the presence of 20U/mL IL-2 and 1nM E7$_{49-57}$ peptide EL-4 cells were pulsed with E7$_{49-57}$ peptide or irrelevant peptide as positive and negative controls respectively. Each plot represents an individual mouse.

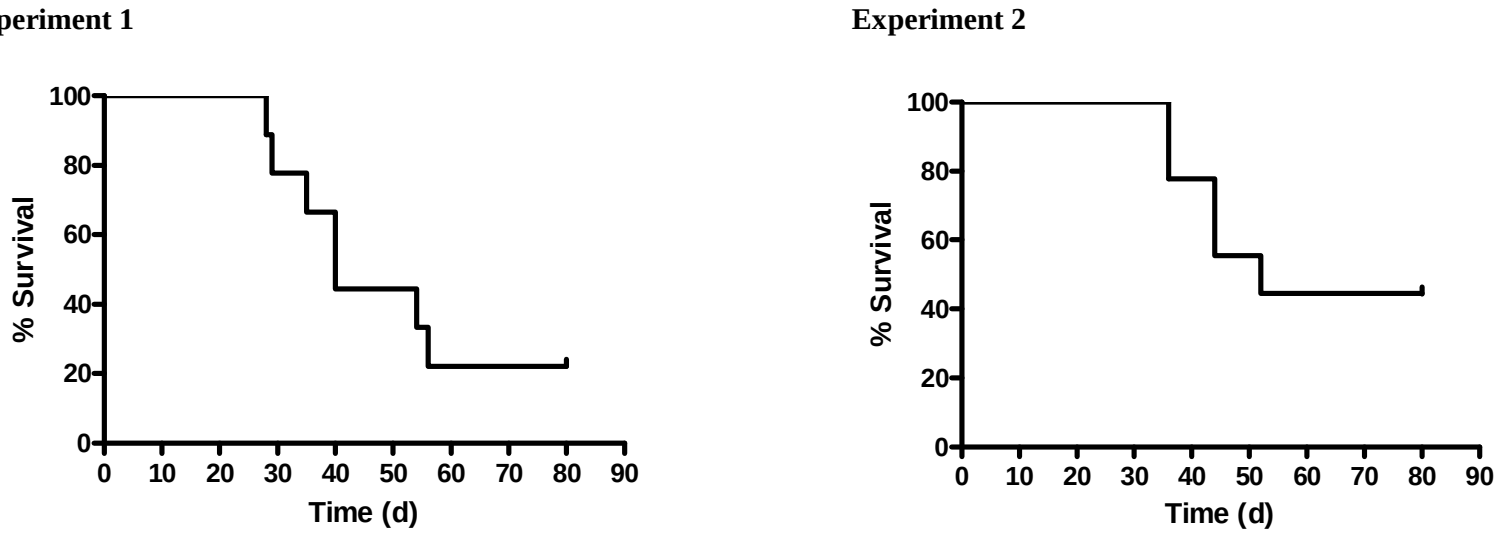


Figure 4.8. *In vivo* passage of TC-1-D^b cells

Groups of 9 naïve mice were challenged with 5x10^4 TC-1-D^b cells by subcutaneous injection. Tumour growth was monitored every 2-3 days and mice were sacrificed when they reached the endpoint of 15mm in size.

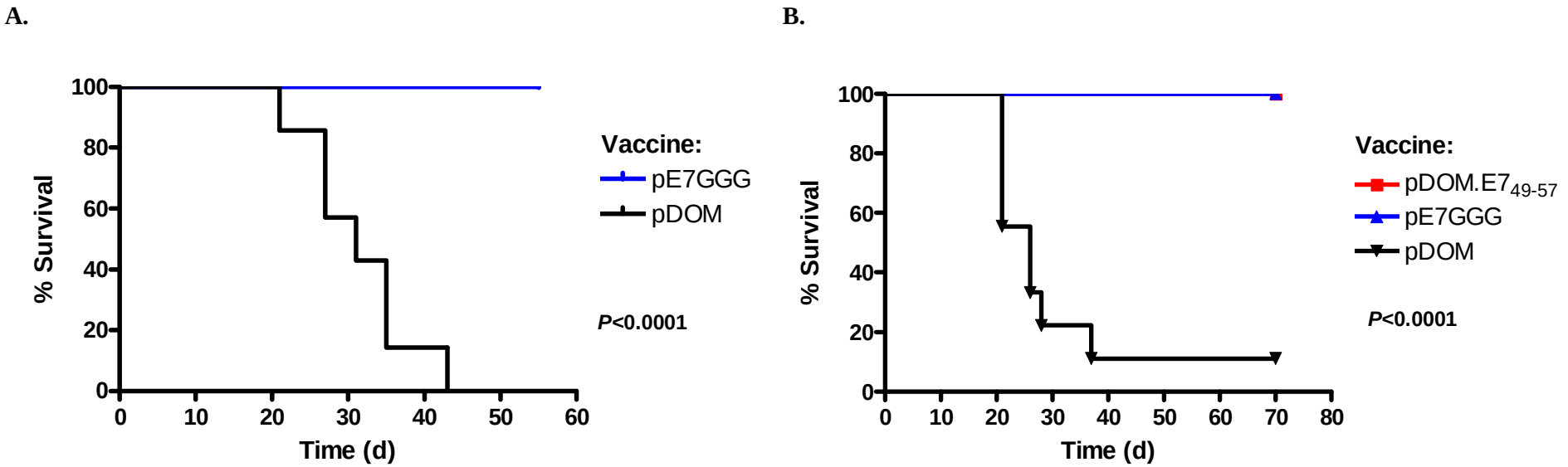


Figure 4.9. DNA vaccine-mediated protection against TC-1-D^{b} tumour challenge *in vivo*

Groups of 7-9 mice were vaccinated with **A.** pE7GGG or pDOM control vaccine or **B.** pDOM.E7$_{49-57}$, pE7GGG or pDOM control vaccine, and challenged with 1x10^{5} TC-1-D^{b} cells by subcutaneous injection 14 days later. Tumour size was monitored every 2-3 days and mice were sacrificed when tumours reached 15mm. *P* values were generated in a Chi Square Log rank test.

As the level of protection was increased against these cells, a therapy experiment was carried out. In this situation, TC-1-D^b cells were implanted prior to DNA vaccination, the lag between challenge and therapy could provide a window for the cells to lose MHC I expression as selective pressure acts *in vivo*. Mice were challenged with $1x10^5$ TC-1-D^b cells and then immunised with pDOM.$E7_{49-57}$, pE7GGG or pDOM control vaccine three days later, as described previously. However to improve the efficacy of the therapy to this high dose of tumour cells, the mice were boosted with DNA and electroporation (EP) 14 days after the primary vaccination. As shown in Figure 4.10, vaccination with both pDOM.$E7_{49-57}$ and pE7GGG significantly reduced the tumour size on d17 post challenge ($P<0.05$); however this did not lead to a statistically significant increase in survival (data not shown). This may be due to the fact that, even at this high dose, not all control animals developed tumours, which could obscure any subtle effects. Also, the tumour growth is slowed by vaccination but not prevented. This course of events mirrors that seen with the parental TC-1 tumour, and could indicate that the tumour cells down-regulate MHC I expression *in vivo*, despite the transfection with H-$2D^b$.

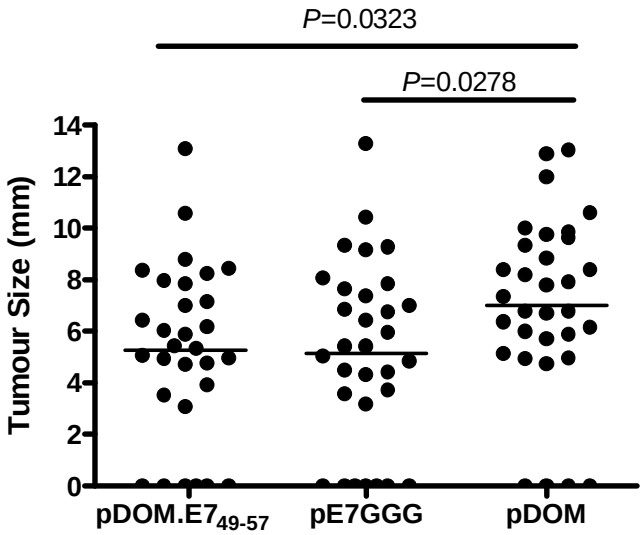


Figure 4.10. DNA vaccine-mediated therapy for TC-1-D^b tumours

Groups of 8-24 mice were challenged with TC-1-D^b cells by subcutaneous injection. Mice were then vaccinated 3 days later with pDOM.$E7_{49-57}$, pE7GGG or pDOM control vaccine and subsequently boosted 14 days after prime with the same DNA vaccine plus *in vivo* electroporation (EP). Tumours were monitored every 2-3 days. Data indicate the size of individual tumours in each group on d17 post challenge. *P* values were generated in a t test. Data are combined from two experiments showing the same trend.

4.2.9. DNA Vaccine-mediated Immunotherapy in Spontaneous Tumour Model

The data demonstrating immune induction and immunotherapeutic potential presented so far have been collected in wild-type mice, in which E7 is a foreign antigen. However, in many cancers, the tumour antigens available to target with immunotherapy are self antigens to which some degree of immune tolerance may exist (see section 1.1.2.5 for information on immunological tolerance). Those self-reactive T cells which escape central tolerance mechanisms may be of lower avidity and subject to other regulatory mechanisms in the periphery. Therefore, it is important to test our vaccines in an experimental setting which more closely resembles the clinical one. In the following experiments, E6/E7 transgenic mice have been used to this end. These mice express the E6 and E7 oncoproteins from HPV16 under the control of the thyroglobulin promoter [223]. As a result, the mice are tolerised to the HPV16 proteins, and furthermore, they develop thyroid hyperplasia. While this proliferation in the thyroid may not be true neoplasia (G Thomas, personal communication), hyperplastic thyroids are referred to as tumours here, as they have been elsewhere [223], [252]. This model allows us to explore the ability of our DNA vaccines to induce $CD8^+$ T cells in a setting of tolerance and to investigate whether they can prevent or treat these spontaneous tumours.

4.2.9.1. $CD8^+$ T-cell Induction in E6/E7 Transgenic Mice

Firstly, the induction of immunity by our DNA vaccines was tested in the E6/E7 transgenic mice, and compared to responses seen in wild-type mice. To this end, mice were vaccinated with the pDOM.$E7_{49-57}$ vaccine and 14 days later, the number of responding E7-specific T cells was assessed in an IFNγ ELISpot assay. Isolated lymphocytes were incubated overnight in the presence of varying concentrations of $E7_{49-57}$ peptide and subsequently the number of IFNγ SFCs per million lymphocytes was assessed.

As shown in Figure 4.11, $E7_{49-57}$-specific responses could be induced in the transgenic mice, though at an approximately two-fold lower level than in wild-type mice (P=0.0071). This indicates that tolerance to the E7 antigen does exist in these mice. However, responses to the DOM-specific p30 peptide were similar in both the transgenic and wild-type mice; indicating that the transgenic mice do not have generalised immunosuppression and that the level of DOM-specific T-cell help afforded by the DNA vaccine was equivalent. Interestingly, when the avidity of the T cells was measured by $E7_{49-57}$ peptide dilution, there appeared to be no significant difference between the two mouse strains, as shown in Figure 4.11B, with responses typically peaking at 5 or 50nM peptide concentration. This

was found in several independent experiments, with a small reduction in avidity observed in only one of five experiments carried out (data not shown).

To ascertain whether DOM-specific $CD4^+$ T cells were required to generate a $CD8^+$ T-cell response in these mice, the experiment was repeated using the pE7GGG vaccine. This is important because any T cells which are stimulated by the E7 sequence are potentially tolerised, unlike those induced by DOM. As shown in Figure 4.12A, there was a trend towards a lower frequency of E7-specific T cells being induced in the transgenic mice compared to wild-type mice but this did not reach statistical significance due to the range of the data. Interestingly, when the avidity of the T cells induced by the pE7GGG vaccine in transgenic mice was compared to that of those induced in wild-type mice, in 2/4 experiments it appeared that they were of lower avidity; one example of which is shown in Figure 4.12B. Responses to the $E7_{49-57}$ peptide were typically <50 SFCs/million in mice vaccinated with the pDOM control vaccine (Figure 4.11B). In previous experiments, no difference was seen in the level of background in wild-type and E6/E7 transgenic mice (data not shown).

These results demonstrate that the DNA vaccines used in this study are able to induce immune responses in these mice, where tolerance exists. Of the approximately 100 mice tested, only around 10% failed to respond.

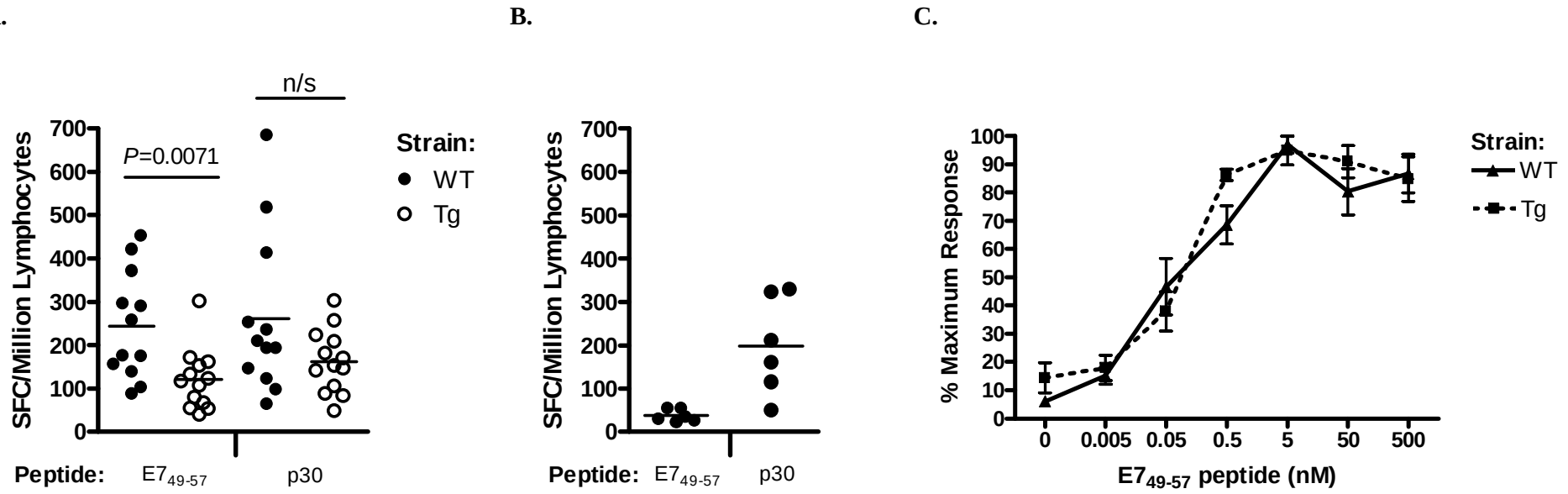


Figure 4.11. Induction of E7-specific immunity by the pDOM.E7$_{49\text{-}57}$ epitope vaccine in E6/E7 transgenic mice

Groups of 4-5 wild-type (WT) or E6/E7 transgenic (Tg) mice were vaccinated with the pDOM.E7$_{49\text{-}57}$ or pDOM control vaccine. After 14 days, splenocytes were isolated and an IFNγ ELISpot was carried out. **A.** IFNγ production after overnight restimulation with 50nM E7$_{49\text{-}57}$ or 1μM DOM-specific p30 peptide in splenocytes from pDOM.E7$_{49\text{-}57}$-vaccinated mice. **B.** Comparative data from pDOM-vaccinated WT mice. Data are combined from three experiments showing the same trend. **C.** IFNγ response to varying concentrations of E7$_{49\text{-}57}$ peptide shown as a percentage of the maximal response. SFC, spot-forming cells. Data are representative of several experiments. *P* value was generated in a Mann-Whitney U Test.

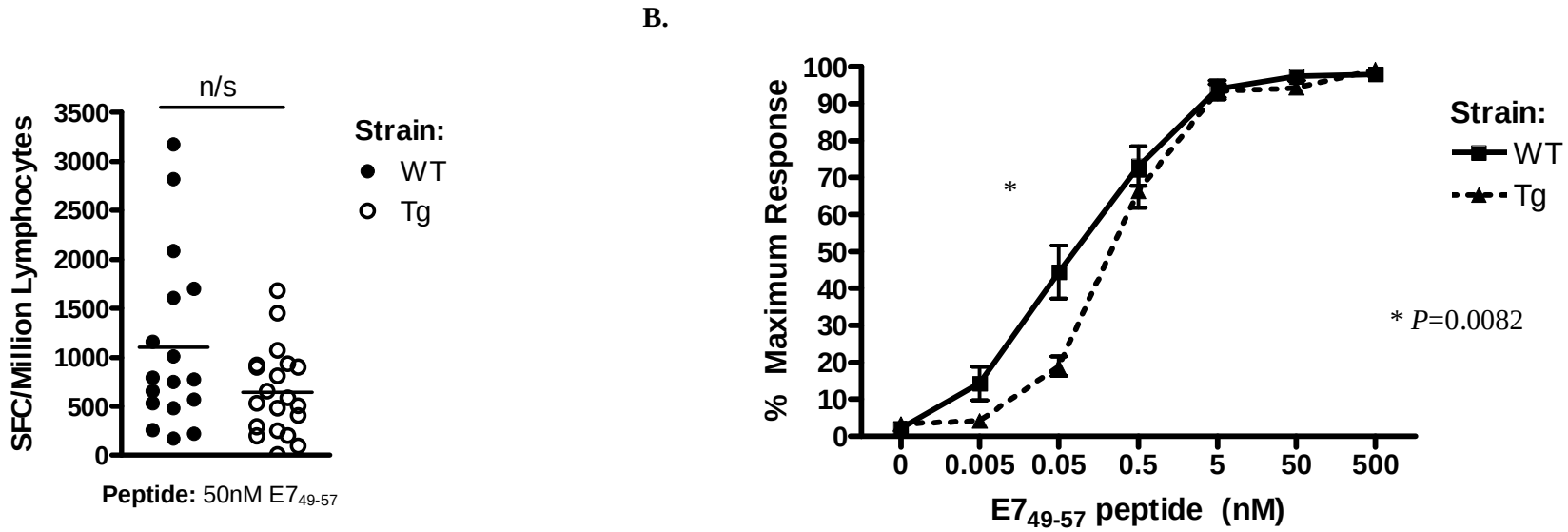


Figure 4.12. Induction of E7-specific immunity by the pE7GGG full-length gene vaccine in E6/E7 transgenic mice

Groups of 4-6 wild-type (WT) or E6/E7 transgenic (Tg) mice were vaccinated with the pE7GGG vaccine. After 14 days, splenocytes were isolated and an IFN$_\gamma$ ELISpot was carried out. **A.** IFNγ production after overnight restimulation with 50nM E7$_{49\text{-}57}$ peptide. Data are combined from four experiments showing the same trend. **B.** IFNγ response to varying concentrations of E7$_{49\text{-}57}$ peptide shown as a percentage of the maximal response. SFC, spot-forming cells. Data are representative of several experiments. *P* value was generated in a t test.

4.2.9.2. The Development of Spontaneous Tumours in Naïve E6/E7 Transgenic Mice

One of the major questions which the E6/E7 transgenic mouse model allows us to answer is whether our DNA vaccines can offer protection against the development of spontaneous tumours. Before investigating this, the development of the spontaneous tumours was first tracked in naïve mice. The graph depicted in Figure 4.14A shows the size of tumours (as a mass) in mice which were sacrificed due to external signs of illness; namely breathing problems and weight loss. As expected, larger tumours are found in older mice (P=0.0004, R^2=0.2752 in a linear regression). Younger mice, which showed no signs of ill health, were also sacrificed for comparison. As shown in Figure 4.14, at 6 weeks of age, the thyroid did not appear to be dramatically enlarged; however by 17 weeks of age hyperplasia had begun. Based on these data, a time point of 30 weeks of age was chosen as the endpoint for the therapy experiments as monitoring tumour growth based on external signs of illness proved too difficult to judge accurately. This should allow the progression of tumours to a size large enough to measure in naïve mice, without allowing them to get so large that the mice would have to be culled for humane reasons before experimental completion.

The nature of the developing thyroid tumours was assessed by haematoxylin and eosin (H & E) staining. Example slides are shown in Figure 4.13. Staining was also carried out on a section from a normal wild-type thyroid (kindly provided by S James) for comparison and the structural differences are clear, with the E6/E7 thyroid lacking cellular organisation. An experienced pathologist (G Thomas) confirmed that the thyroid proliferation is likely to be hyperplastic, not neoplastic. However, for simplicity, hyperplastic thyroids are referred to as tumours here, as they have been elsewhere [223], [252]. To confirm continued expression of the E7 antigen *in vivo*, a hyperplastic thyroid from a 49-week old naïve E6/E7 mouse was excised and stained with anti-E7 antibody for analysis by flow cytometry. The results, shown in Figure 4.15, confirm that E7 is expressed in the E6/E7 thyroid glands, with a three-fold increase in the MFI compared to staining with an isotype control antibody. This is as expected due to the enlarged thyroids found in naïve E6/E7 mice shown in Figure 4.14.

The actions of the E7 oncoprotein can reduce MHC I expression experimentally [212] and probably also affect its expression in the E7-expressing tumour cell line TC-1 (see Chapter 3). Therefore, H-2D^b expression in the E6/E7 thyroid was also analysed. As shown in Figure 4.15C, approximately 3% of the E7-positive cells were also positive for H-2D^b in this naïve mouse. This demonstrates the potential for CD8$^+$ T-cell attack but local inflammation may be required to up-regulate MHC I expression. This thyroid tissue was

also stained with anti-CD8 antibody but no CD8-positive cells were seen (data not shown). These results confirm that the thyroid tumours which develop in these mice may be susceptible to CTL attack but that no endogenous E7-specific immune response seems to exist, as expected.

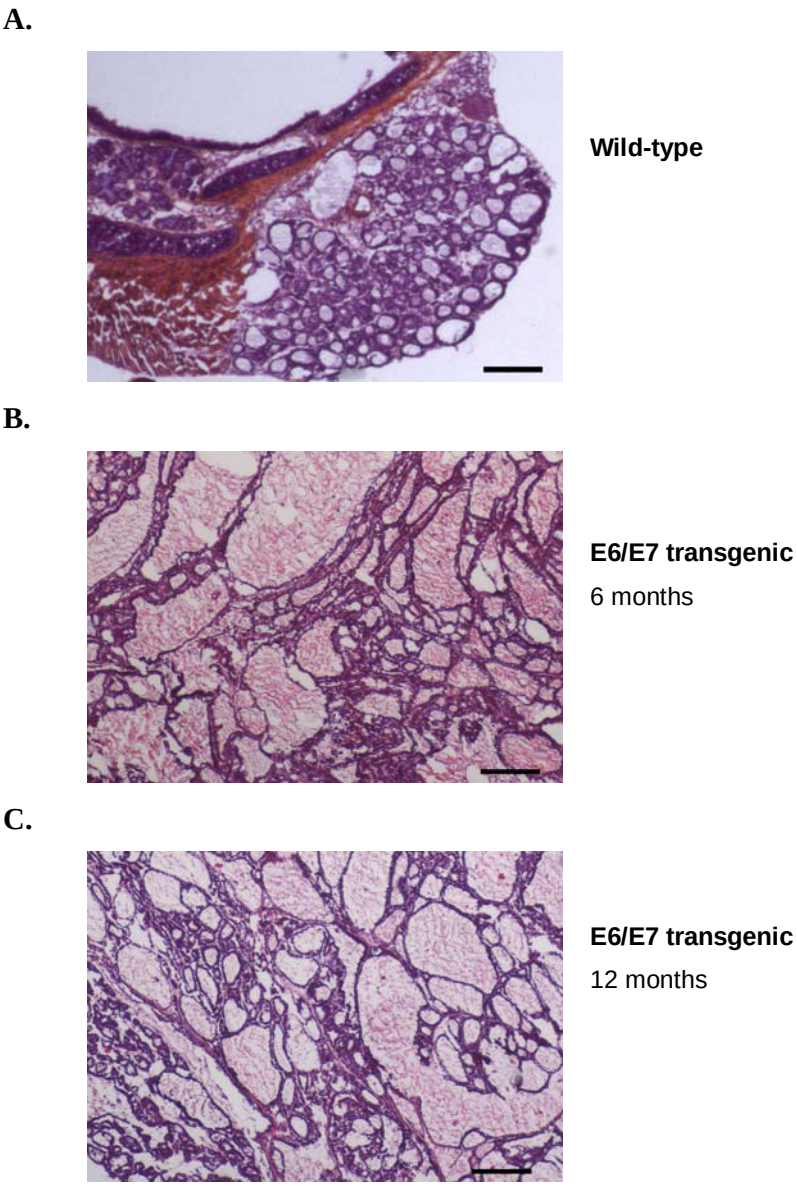
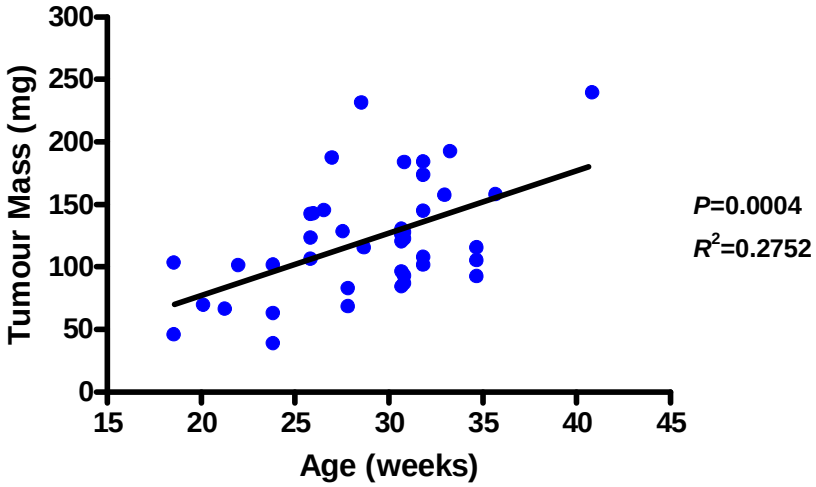


Figure 4.13. Haematoxylin and eosin (H & E) staining of thyroid sections

Thyroids were frozen in OCT, cut into 10µm sections and mounted on microscope slides. Sections were then stained with haematoxylin (a nuclear stain, blue) & eosin (a structural stain, red). Thyroid sections from **A.** wild-type or E6/E7 transgenic mice at **B.** 6 months and **C.** 12 months of age. Scale bar represents 200µm. Sections were cut and stained by S James. Pathology was judged by G Thomas.

A.



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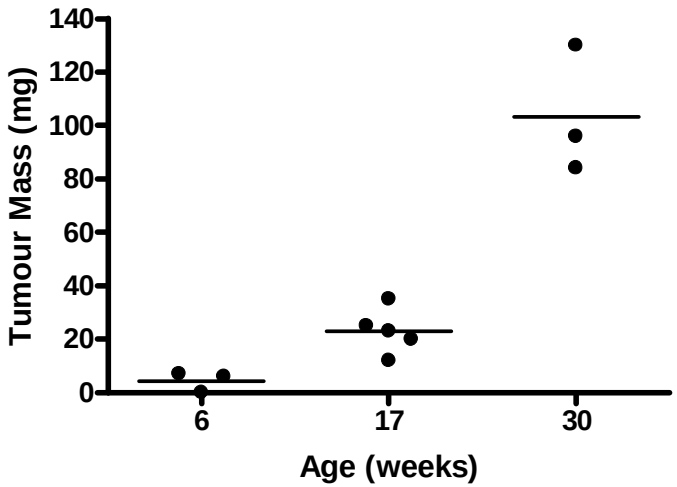


Figure 4.14. Development of spontaneous tumours in naïve E6/E7 transgenic mice

The mass of individual tumours was monitored in naïve E6/E7 transgenic mice **A.** which exhibited signs of breathing difficulties or weight loss assumed to be caused by the presence of enlarged thyroid glands and **B.** which did not appear to be sick, at the given time points of 6, 17 and 30 weeks of age. *P* value was generated by linear regression.

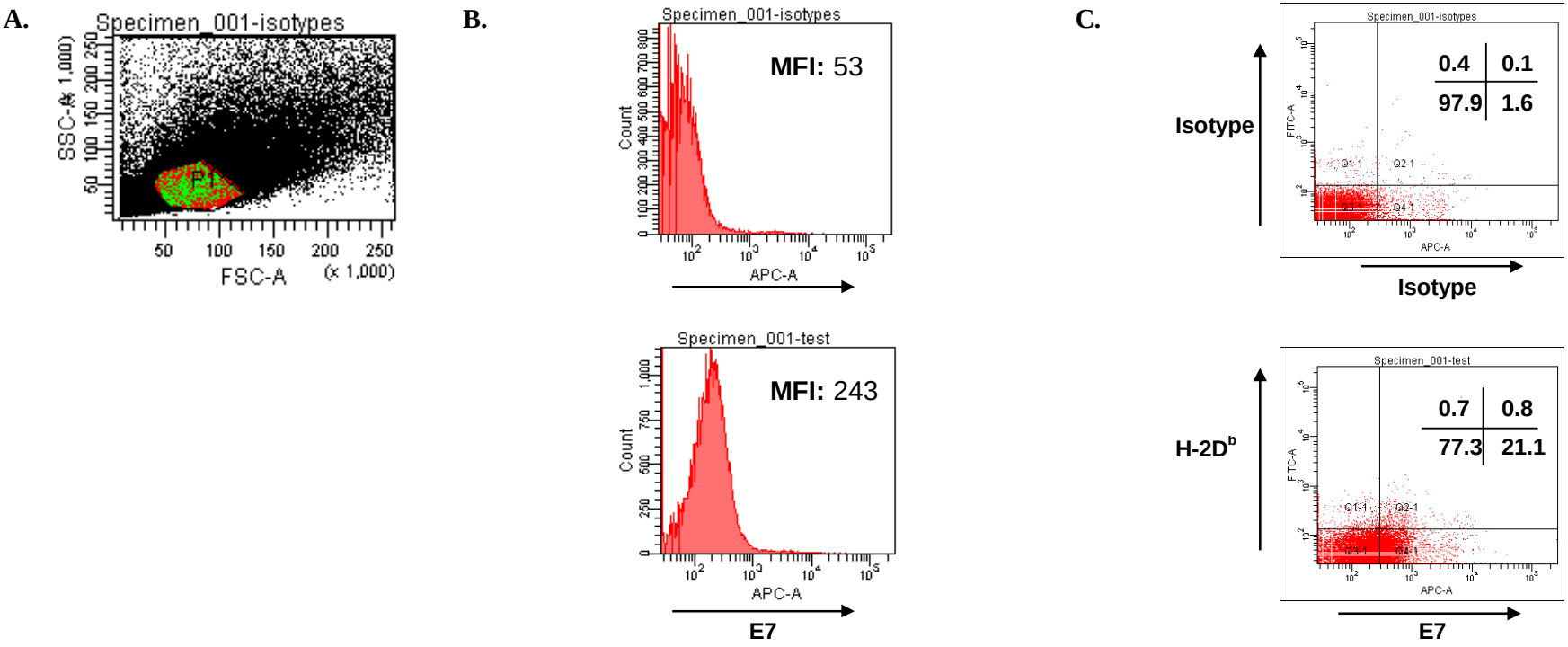


Figure 4.15. Phenotypic analysis of thyroid tissue from an E6/E7 transgenic mouse

A thyroid gland was excised and processed to obtain a single-cell suspension before staining with anti-E7 and anti-H-2D^b antibodies for flow cytometry. **A.** Forward/side scatter plot. **B.** Histograms show intracellular staining of thyroid tissue with anti-E7 antibody or isotype control. MFI, mean fluorescence intensity. **C.** Plots show staining with anti-H-2D^b and anti-E7 antibodies. Figures indicate percentages of cells in each quadrant.

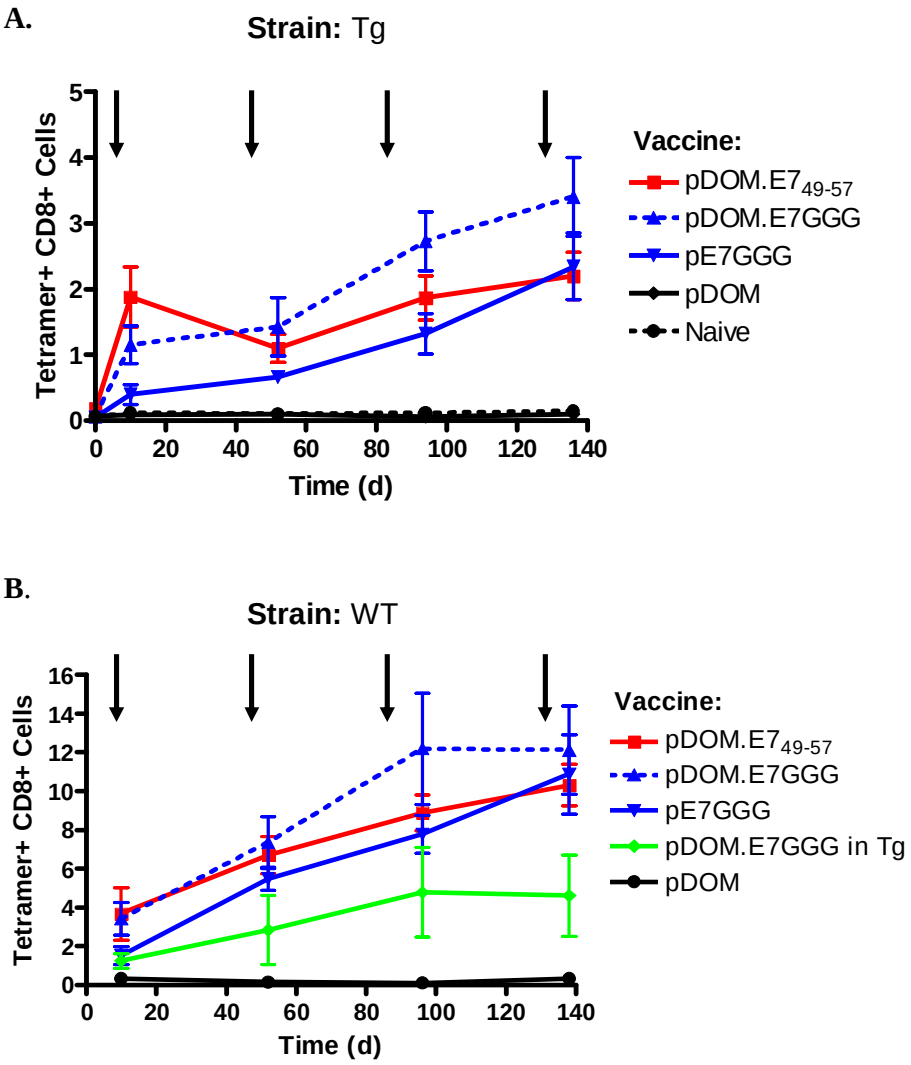
4.2.9.3. DNA-vaccine Mediated Therapy of Spontaneous Tumours in E6/E7 Transgenic Mice

To assess the performance of E7-specific DNA vaccines as therapy for these spontaneous tumours, groups of 6-9 E6/E7 transgenic mice were vaccinated from 6-7 weeks of age, and given three further injections at 6-week intervals. The E7-specific $CD8^+$ T-cell response was monitored at baseline and 10 days after each injection using the H-$2D^b$-$E7_{49-57}$ tetramer. Prior to vaccination, no E7-specific $CD8^+$ T cells could be detected, as expected; shown in Figure 4.16A. After each injection of DNA vaccine, responses to pDOM.$E7_{49-57}$, pDOM.E7GGG and pE7GGG generally increased slightly. However, one exception was that responses to the first pDOM.$E7_{49-57}$ vaccination were not matched by the second vaccination. Responses to the pDOM.E7GGG vaccine appeared to be greater than those to pE7GGG, indicating that DOM may be of benefit to the $CD8^+$ T-cell response in this setting of tolerance.

As a control, an identical experiment was carried out in wild-type mice. Responses followed the same course but at an increased level, as expected, with 2- to 3-fold more CD8-positive cells being tetramer positive; shown in Figure 4.16B. The pDOM.E7GGG vaccine appears to have generated a stronger $CD8^+$ T-cell response than pE7GGG after the third vaccination. However, this is probably due only to the presence of one particularly high responder skewing the data for this time point with approximately 26% of CD8-positive cells also being tetramer-positive, compared to a mean of approximately 9% in the rest of the group; as indicated by the large error bars.

To assess any impact on thyroid hyperplasia, four weeks after the last vaccination, at approximately 30 weeks of age; mice were sacrificed and tumours were weighed. The results, shown in Figure 4.16C, revealed that tumour mass was significantly reduced in mice vaccinated with pDOM.$E7_{49-57}$, pDOM.E7GGG and pE7GGG when compared to untreated controls (P<0.04). In all groups, the mean thyroid mass was approximately 25% lower than that found in naïve or pDOM-vaccinated controls. However in some individual mice the reduction was even more dramatic.

For the first time, this indicates that the $CD8^+$ T cells which are induced by our DNA vaccines are able to attack spontaneously occurring tumours in a setting of tolerance and significantly affect their behaviour *in vivo*, leading to significantly reduced tumour growth.



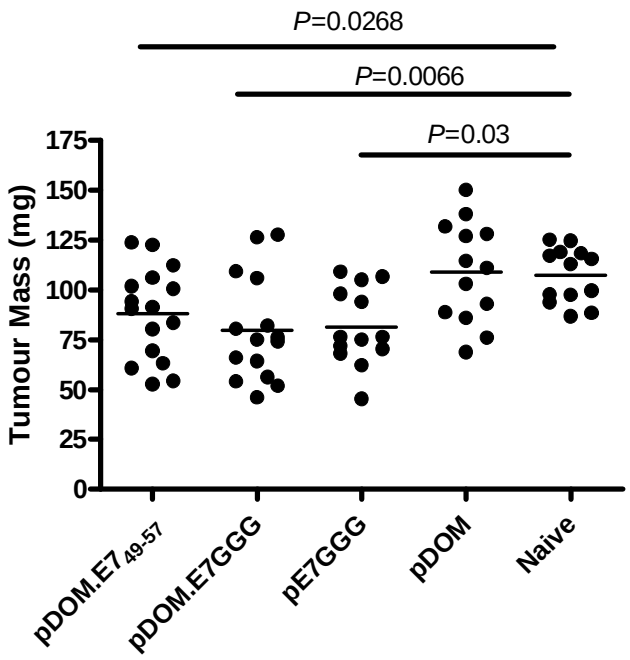


Figure 4.16. DNA vaccine-mediated therapy of spontaneous tumours

Groups of 6-9 mice were vaccinated with DNA as indicated 4 times from 6-7 weeks of age at 6 week intervals. $E7_{49-57}$-specific $CD8^+$ T-cell responses in the blood were assessed using an $E7_{49-57}$-specific tetramer 10 days after each vaccination. Arrows indicate vaccination time points **A.** $CD8^+$ T cells responses in E6/E7 transgenic (Tg) or **B.** wild-type mice (WT). **C.** Mass of spontaneous tumours found in the thyroid 4 weeks after the last vaccination (40 weeks of age) of the mice in **A.** Data shown in **A** and **C** are combined from two experiments showing the same trend. *P* values were generated in a t test.

4.2.9.4. Assessment of Tumour-infiltrating Lymphocytes in Spontaneous Tumours of E6/E7 Transgenic Mice

The data shown in the previous section indicate that DNA vaccines do have an effect on spontaneous tumour growth in E6/E7 transgenic mice. The presumed mechanism is via $CD8^+$ T-cell mediated destruction of the hyperplasic thyroid tissue. Although $CD8^+$ T-cell induction had been demonstrated in association with regression of spontaneous tumours in these mice (Figure 4.16), it is important to know whether these cells, which were detected in the blood and spleen, do migrate to the tumour site.

To investigate this, some of the tumours shown in Figure 4.16 were snap-frozen in OCT on dry ice and later cut into sections for immunohistochemical analysis. These sections were stained with anti-CD8 antibody and with DAPI nuclear stain. The results are shown in Figure 4.17, with CD8 staining in green and nuclei shown in blue. Although these data are not quantitative, it is clear that $CD8^+$ T cells are present in the thyroids of these mice, and is suggestive that vaccination may augment this. However, these findings must be confirmed by quantitative methods before drawing any conclusions on the comparisons between each treatment group.

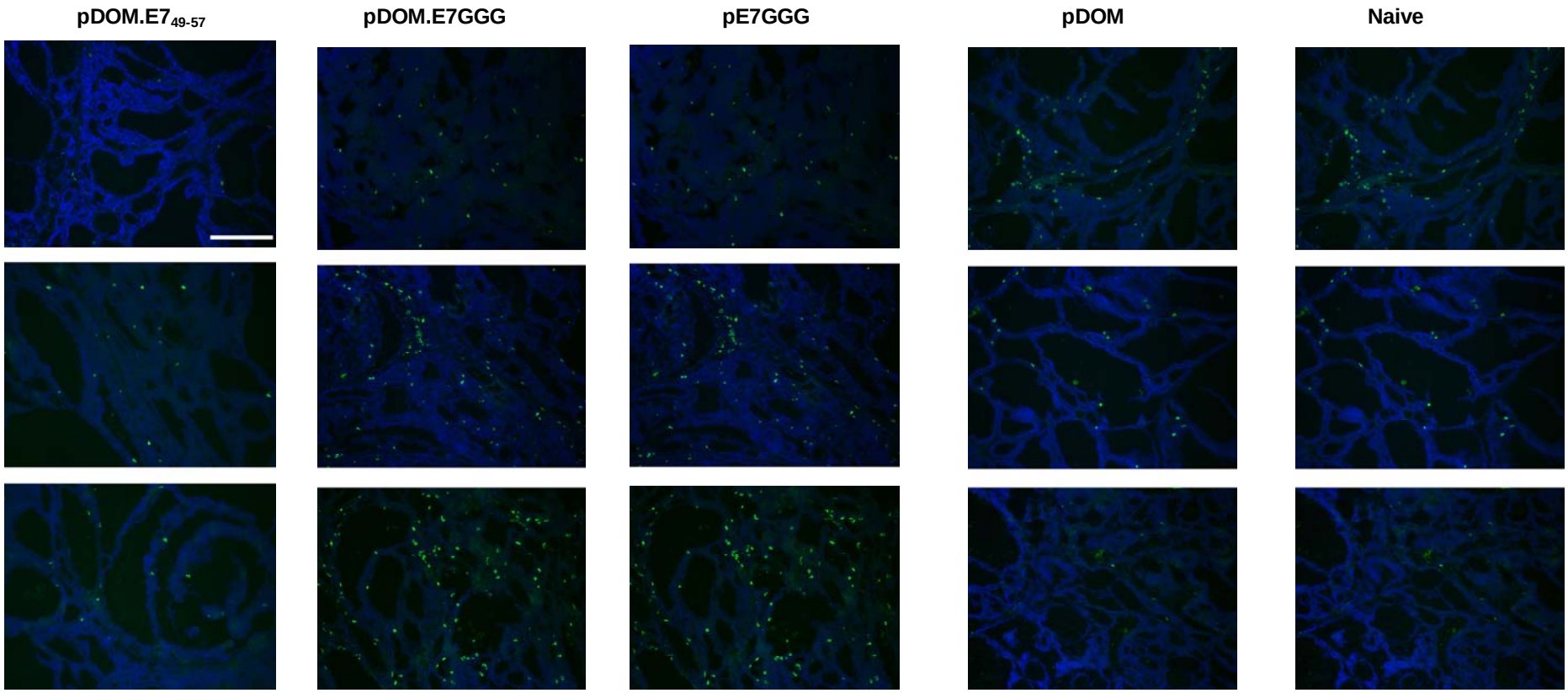


Figure 4.17. Immunofluoresence on thyroid sections from E6/E7 transgenic mice

Some of the tumours from Figure 4.14C were frozen in OCT and subsequently 10µm sections were cut and mounted on microscope slides for staining. Nuclear staining is visualised with DAPI (blue) and $CD8^+$ cells with anti-CD8α antibody/Alexa Fluor 488 secondary antibody (green). Each panel is from an individual representative mouse for each group (several sections were stained for each of 3-4 mice per group). White scale bar in the first panel represents 200µM. Sections cut and staining carried out by S James.

4.2.9.5. Induction of E7-specific Immunity in Aged E6/E7 Transgenic Mice

The results shown in Figure 4.16 are encouraging; however DNA vaccination commenced in young mice with little discernable tumour. In a clinical situation tumour development and immune tolerance may be more advanced. Therefore, DNA vaccination was carried out in older E6/E7 transgenic mice to see if it was still possible to induce immunity under these circumstances.

In this experiment, E6/E7 transgenic mice were vaccinated at 26-30 weeks of age. By this point, thyroid hyperplasia has been shown to be dramatically increased compared to mice of 6 weeks of age (Figure 4.14B). Age-matched wild-type controls were included for comparison in order to take into account any normal age-related changes in the immune response.

The results of an IFNγ ELISpot carried out 14 days after injection with pE7GGG, pDOM.E7GGG or pDOM control vaccine are shown in Figure 4.18A. Both the pE7GGG and pDOM.E7GGG vaccines induce an IFNγ response upon stimulation with the $E7_{49-57}$ peptide in these mice. Interestingly, these data indicated that the presence of DOM in the vaccine does not seem to be necessary for induction of $CD8^+$ T cells in transgenic mice of this age, when there may be greater immune tolerance.

The thyroids of these mice were excised and weighed as before. As expected, all mice had tumours of at least 50mg. Interestingly, when tumour mass was compared to IFNγ responses in each individual mouse, there was a correlation, as shown in Figure 4.18B. In the pDOM.E7GGG-vaccinated group the mice with the greatest IFNγ responses had the smallest tumours (P=0.0277, R^2=0.3985 in a linear regression analysis). This could indicate that these mice have lower levels of immune suppression. The same trend was observed in mice vaccinated with pE7GGG but the presence of an anomaly prevented this from being a statistically significant difference.

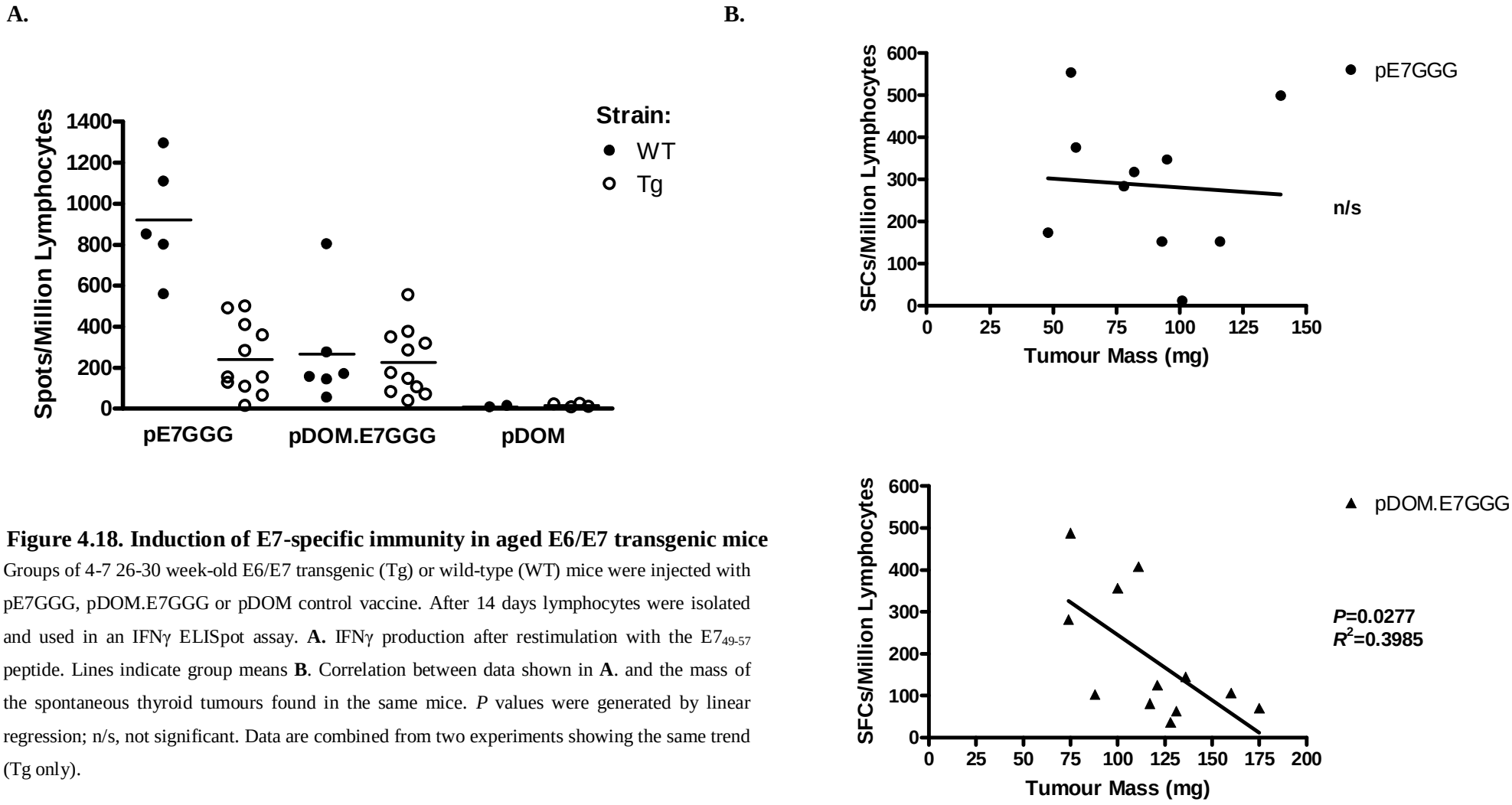


Figure 4.18. Induction of E7-specific immunity in aged E6/E7 transgenic mice

Groups of 4-7 26-30 week-old E6/E7 transgenic (Tg) or wild-type (WT) mice were injected with pE7GGG, pDOM.E7GGG or pDOM control vaccine. After 14 days lymphocytes were isolated and used in an IFNγ ELISpot assay. **A.** IFNγ production after restimulation with the $E7_{49-57}$ peptide. Lines indicate group means **B**. Correlation between data shown in **A**. and the mass of the spontaneous thyroid tumours found in the same mice. *P* values were generated by linear regression; n/s, not significant. Data are combined from two experiments showing the same trend (Tg only).

4.3. Discussion

4.3.1. DNA Vaccine-mediated Protection Against TC-1 Tumour Challenge *in vivo*

Despite the finding in the previous chapter that TC-1 cells expressed low levels of MHC I *in vitro*, the full-length E7 and long peptide DNA vaccines afforded 50-100% protection against an *in vivo* challenge (Figure 4.1). The ability of DNA vaccines to protect against challenge with a tumour which expresses only low levels of MHC I may be surprising, however it is possible that local IFNγ production will up-regulate its expression. Local inflammation and innate immune activation at the tumour site will lead to local IFNγ production, which could explain how these MHC I-negative tumours are rejected. However, as the orginal TC-1 tumour cells did not have the chacteristics observed in previous publications [255], [240] they were not used in future *in vivo* experiments.

The 'variant' TC-1 cells which were shown to express slightly higher levels of H-2D^b *in vitro* in the previous chapter were also rejected by mice vaccinated with pDOM-epitope (pDOM.E7$_{49-57}$) or full-length E7 (pE7GGG) DNA vaccines (Figure 4.2). In fact, the level of protection rose from 20% to 50% in pDOM-epitope-vaccinated mice, indicating that these tumours are more susceptible to CTL-mediated attack. In a therapeutic setting, the pDOM-epitope and full-length E7 vaccines significantly reduced the growth of these tumours (Figure 4.3). However, neither conferred complete protection.

Interestingly, the synthetic E7$_{43-77}$ long peptide vaccine given with CpG out-performed all the DNA vaccines as a therapeutic (Figure 4.5); despite the comparatively low numbers of CD8$^+$ T cells shown to be induced by this strategy in Chapter 3. There are two possibilities that could explain this: (i) the CD8$^+$ T cells induced by the long peptide are not genuinely of lower frequency but are simply not found in the blood or spleen; or (ii) the therapeutic benefit of the long peptide is not controlled solely by CD8$^+$ T cells. Efforts were made to visualise TILs in TC-1 tumours (data not shown) with a view to revealing any differences in infiltrating cells or MHC class I expression but technical issues make this difficult. Firstly, by definition, tumours which escape the immune response are unlikely to have a large CD8$^+$ T-cell infiltrate; whereas those which do regress have thus disappeared and are therefore not available for analysis. Secondly, even if one attempts to catch the tumours during the short temporal window between implantation and rejection, analysis by standard flow cytometry or immunohistochemistry methods is hampered by the non-specific antibody binding to the large amounts of necrotic tissue found in a tumour site. The

possibility that tumour rejection may be controlled by effector mechanisms other than $CD8^+$ T cells is discussed in the following section.

4.3.2. Mechanism of TC-1 Tumour Rejection *in vivo*

In Chapter 3 it was demonstrated that the E7-specific $CD8^+$ T-cell response induced by the full-length vaccines was superior to that induced by the pDOM-epitope construct and synthetic long peptide induced even weaker responses. However, it was unclear whether these differences would explain the varying levels of protection. There is also the possibility that there may be other $CD8^+$ T-cell epitopes within the E7 gene that could stimulate more $CD8^+$ T cells of different specificities. There have been other epitopes suggested by H-$2D^b$-peptide binding studies [226] and predicted by algorithms (SYFPEITHI). However, when we used several of these peptides in an *ex vivo* ELISpot assay on lymphocytes from pE7GGG-vaccinated mice, we found that they induced no significant IFNγ production (Figure 3.3). The results obtained with the synthetic long peptide certainly indicated that mechanisms other than $CD8^+$ T cell attack could be involved.

One explanation for the superior protection afforded by the full-length E7 gene vaccine compared to the pDOM-epitope vaccine (Figure 4.2) could be that the full-length vaccine might induce an E7-specific $CD4^+$ T-cell response. However, the *ex vivo* data described in Chapter 3 suggested that whilst the $CD8^+$ T-cell response after vaccination with full-length E7 vaccines was strong, only weak $CD4^+$ T-cell responses were induced. However, as only the $E7_{43-77}$ long peptide was used in *ex vivo* assays carried out here, rather than the full-length E7 protein, the possibility of $CD4^+$ T-cell epitopes outside this region could not be excluded. Therefore, the importance of a cognate $CD4^+$ T-cell response was investigated by depleting $CD4^+$ cells immediately prior to tumour challenge. This did not reduce the efficacy of the pE7GGG vaccine (Figure 4.4), providing more evidence that $CD4^+$ T cells are not essential during the effector phase for protection; although they are likely to be necessary for CTL priming. T-cell help may be of less importance during the effector phase of the $CD8^+$ T-cell response in these wild-type mice in which the E7 molecule is highly immunogenic and no immunological tolerance exists. Indeed, high levels of $CD8^+$ T cells are induced by this vaccine. The situation may be different in a tolerant setting where T-cell help has previously been shown to be required for tumour rejection [166].

Stimulation of the innate immune response in TC-1 tumour-bearing mice was also investigated. This is particularly relevant for TC-1 tumour cells as they expresses low levels of MHC class I and could therefore be susceptible to NK attack. Interestingly, just one dose of CpG did retard tumour growth (Figure 4.6), corroborating previous findings of others [256] and suggesting that stimulating innate immunity could be very important for tumour rejection in this model. Our DNA vaccines will be sensed in the cytosol by recently identified mechanisms [130], [128] and in addition the backbone of the bacterial plasmid itself contains CpG sequences [236] which will stimulate TLR-9 [124]. Recent evidence has shown that synthetic CpG, which is based on a phosphorothioate backbone instead of the natural phosphodiester backbone of DNA, has a higher affinity for TLR-9 [257]. This opens up the possibility that the innate immunity stimulated by synthetic CpG versus plasmid CpG motifs may be different, both qualitatively and quantitatively.

However, the site of injection is perhaps more important. Synthetic long peptide with CpG was injected sub-cutaneously in the flank of the mouse, very close to the site of the tumour, in order to follow the published protocol [225]. In contrast, DNA vaccines are injected in a distant site by intramuscular injection in the hind legs. Therefore, while the stimulation of innate immune cells such as DCs in the muscle site may be important and effective for cross-presentation; this may not have any effect on the tumour. For TC-1, as for many solid tumours, this could prove crucial due to the need to up-regulate surface expression of MHC class I molecules. Therefore, in this very artificial system of transplantable mouse tumours, the synthetic long peptide plus CpG strategy could have an advantage. However, in a clinical setting, where it may not be feasible to inject an immunotherapeutic agent so close to the tumour site, the results may be different.

Another aspect of the innate immune system which may be relevant in this model is NK cell attack. These innate immune cells lyse foreign cells on the basis of the absence of self MHC molecules. As discussed above, the TC-1 tumour cells used in this study down-regulate surface MHC I expression, as many solid tumours do. This could render them more susceptible to NK-mediated killing than CTL-mediated killing. Therefore, the TC-1 cells were retrovirally transfected with the H-2D^b heavy chain, resulting in increased D^b expression and an increased sensitivity to CTL-mediated killing *in vitro* (Figure 4.7). It was hoped that these tumour cells could be used as model for CD8$^+$ T-cell mediated immunotherapy. Both the pDOM.E7$_{49\text{-}57}$ and pE7GGG vaccines mediated efficient protection against challenge with these TC-1-D^b cells (Figure 4.9). However, the increased MHC I expression and CTL sensitivity *in vitro* did not result in improved DNA-vaccine mediated therapy of these tumours (Figure 4.10). In order to escape the CD8$^+$ T-cell response, the TC-1-D^b cells must either proliferate at such a rate as to win the race against

the immune response; or somehow lose H-2D^b expression to evade detection. The retroviral transduction of these cells clearly puts them at a disadvantage in naïve mice, where 5x10^4 cells fail to implant (Figure 4.8), in contrast to the parental cell line (Figure 4.2A). The reasons for this are unknown but it is likely that selective pressure *in vivo* could drive the cells to lose the transduced H-2D^b gene. On the other hand, the speed of tumour growth may simply be to fast for the immune response to beat. The primary CD8$^+$ T-cell response peaks at d10-14 post injection and perhaps this is just not fast enough to eradicate aggressive transplantable tumours such as TC-1.

This loss of MHC I expression seen in TC-1 as well as many other solid tumour tumours represents a major hurdle for immunotherapy to overcome if CTL induction is the goal. However potential ways to deal with this issue are under active investigation. Given their proven ability to up-regulate MHC I expression on tumour cells *in vitro,* cytokines have obvious potential. Intratumoural injection of constructs expressing GM-CSF improves rejection of MHC I negative tumours in mice [258]. Another way of tackling this may be to use HDAC inhibitors and methyltransferases which modulate epigenetic changes in gene expression and have been shown to increase MHC I expression on TC-1 cells *in vitro* [255].

4.3.3. Induction of Protective Immunity Against Spontaneous Tumours in Transgenic Mice Tolerised to the E7 Antigen

It has been demonstrated that our DNA vaccines can induce E7-specific CD8$^+$ T cell responses in E6/E7 mice (Figure 4.11 and Figure 4.12), which have been shown to exhibit tolerance to the E7 antigen [223]. Interestingly, these CD8$^+$ T cells did not seem to be of reduced avidity, in contrast to previous data obtained by vaccinating the mice with a *Listeria*-based E7 vaccine [223]. However, the previous reports used limiting E7$_{49\text{-}57}$-specific tetramer dilutions as a measure of avidity and the results of this may be clouded by the lower frequency of E7$_{49\text{-}57}$-specific T cells in these mice. Here avidity was determined using varying concentrations of peptide in an IFNγ ELISpot. However, in these mice, the number of SFC/million lymphocytes does not increase dramatically even when high peptide concentrations are used. This makes the uncovering of statistically significant differences very difficult. Therefore, based on this data it is not possible to say whether or not E7$_{49\text{-}57}$-specific T cells are of reduced avidity in E6/E7 transgenic mice. Chromium release assays using target cells pulsed with varying concentrations of peptide are another potential method to determine avidity which have been used in the past [259], and could be tried in this model.

In naïve E6/E7 transgenic mice thyroid hyperplasia is apparent by 17 weeks of age and thyroids increase in size continuously until mice are sacrificed (Figure 4.14). This enlargement of the thyroid glands appears to be hyperplastic, not neoplastic when visualised by immunohistochemistry (as judged by an experienced pathologist, Dr G Thomas). However, these 'tumours' still provide a useful model system in which to test E7-specific immunotherapy.

E7 expression can be demonstrated by flow cytometry but interestingly, in the one sample tested, only approximately 3% of the E7-positive thyroid cells express H-2D^b (Figure 4.15). This could potentially allow the spontaneous tumours to escape the CD8$^+$ T-cell response; however this result should be substantiated with multiple replicates. In Chapter 3, it was shown that TC-1 tumour cells which express E7 can have very low levels of MHC I molecules on their surface. This is to be expected as it is known that the E7 oncoprotein interferes with MHC I expression both experimentally [212] and in HPV16/18-positive cervical cancer patients [215]. Therefore, the presence of this apparent MHC I down-regulation in this model mirrors the clinical situation. However, this phenomenon will present a challenge for CD8$^+$ T-cell mediated immunotherapy. Local cytokine production could potentially induce increased MHC I expression, as seen *in vitro* for the TC-1 cells in Chapter 3 and discussed previously. Therefore, MHC class I expression may be higher in immunised mice.

Vaccination of E6/E7 transgenic mice with DNA from 6 weeks of age with regular boosts resulted in the maintenance of CD8$^+$ responses over time; albeit at a lower level than in wild-type mice, and this correlated with a reduction in the mass of the spontaneous tumours (Figure 4.16). Despite a trend towards the pDOM.E7GGG vaccine inducing more CD8$^+$ T cells than pE7GGG, the protection afforded by the different vaccines was very similar. This is surprising given the previous findings from our lab which demonstrated the importance of DOM-specific T-cell help in a setting of tolerance [166]. However, it is worth noting that these are merely the responses in the blood; and if the immune cells are attacking the tumour, it is possible that they will migrate to the site where it is growing, thus disappearing from the blood. Therefore, it is possible that there are differences not revealed using these techniques. The presence of a therapeutic effect indicates that the CD8$^+$ T cells induced by the DNA vaccines do migrate to the thyroid and are able to attack the E7-positive cells there. Preliminary immunofluoresence data supports this, with positive staining seen using anti-CD8 antibody (Figure 4.17). Importantly, the fact that the DNA vaccines have an effect on the thyroid tumours indicates that MHC I is expressed by

these cells. However, as the level of MHC I expression has not been confirmed at this time, it could be a limiting factor.

Encouragingly, even in mice which were over 25 weeks old, when tumours are known to be much larger (Figure 4.14), IFNγ production was still seen in response to the pE7GGG and pDOM.E7GGG vaccines (Figure 4.18), paving the way for a later therapy experiment. It is possible that at a later time point, tolerance may be more severe and therefore the presence of DOM in the vaccines may be more of an advantage. Although the E7 gene sequence may itself induce $CD4^+$ T cells, it has been shown previously that tumour-specific $CD4^+$ T cells induced by vaccination in tumour bearing mice can develop a regulatory phenotype [260] which could impair CTL-mediated tumour cell lysis. Therefore, the fact that the pDOM-epitope vaccine is equally effective as the highly immunogenic full-length gene vaccine may be critical. This is because the use of a pDOM-epitope construct means that it will be possible to circumvent the threat of potentially tolerising tumour-specific $CD4^+$ T cells whilst retaining T-cell help, due to the presence of DOM.

4.3.4. Conclusions

We have demonstrated the ability of our vaccines to induce $CD8^+$ T-cell responses and confer some level of protection and therapy against TC-1 tumour challenge. We have shown that the superior results found with the full length gene pE7GGG vaccine compared to the pDOM.$E7_{49-57}$ epitope vaccine are not due to cognate $CD4^+$ T-cell help but that innate immunity may instead be the key to rejection of TC-1 tumours. We have also succeeded in inducing an E7-specific $CD8^+$ T-cell response in a tolerised mouse model of cancer. Furthermore, these responses were accompanied by a reduction in the mass of spontaneously developing tumours. This is the first time that this DNA vaccine design has been proven successful in a spontaneous tumour model. These data confirm that the DNA vaccine approach is a valid strategy for treating cancers *in vivo* and that they have the potential to provide an effective immunotherapy in humans.

4.3.5. Future Work

There are still some questions to answer regarding the mechanism of tumour protection in the TC-1 model. As the tumour is known for its ability to down-regulate MHC class I molecules, it is important to confirm whether or not NK cells are implicated in rejection of

TC-1 tumours. This could be achieved by depleting NK cells *in vivo* using anti-NK1.1 antibody. As it is possible that the $E7_{43-77}$ synthetic long peptide with CpG may mediate its effects by engaging NK cells, another way of answering this question may be to use this approach as therapy for TC-1-D^b cells. Due to their high expression of MHC class I molecules, these tumours should be resistant to NK-mediated lysis; though it is possible that these might be able to down-regulate H-$2D^b$ expression *in vivo*. This could also tested *in vitro* using TC-1-D^b cells as targets for effector cells derived from mice vaccinated with the synthetic peptide. Another possible explanation for the superior therapeutic effect of the synthetic peptide with CpG could be the route of delivery. Therefore, this should be investigated by injecting the peptide at a site which is distant to the tumour.

The E6/E7 transgenic mouse model could be used to investigate any number of different vaccines or vaccine regimens in the setting of tolerance. As MHC I expression may be a limiting factor for CTL killing in this model, as it presumably is in cervical cancer patients, it may be of interest to test therapeutic agents which may increase it. As it is known that IFNγ can increase MHC I expression in these cells [240], agents aimed at increasing cytokine production may be beneficial. For example TLR-7 agonists such as short hairpin RNA have been shown to induce type I interferon production in DCs [261] and other TLR-7 agonists are known to increase IFNγ production in human $CD4^+$ T cells, especially those in the effector memory subset [262]. Given its success against transplantable tumours in the wild-type mice, it would be interesting to test the synthetic long peptide with CpG as a therapeutic for E6/E7 thyroid tumours. Perhaps in a setting of tolerance and one where the tumour is found in a site which is distant to the site of vaccination, this strategy may not prove so successful.

Although there are some data which indicate that $CD8^+$ T cells do migrate to the thyroid tumours after vaccination, this should be confirmed using quantitative methods. This could also be extended to include $CD4^+$ cells, both helper and regulatory subsets which could reveal the level of peripheral tolerance found in these mice.

Finally, since we have shown that it is possible to induce immunity in older E6/E7 transgenic mice, it would be interesting to repeat the therapy experiment with DNA vaccination starting at a later time. This would test them in an even more stringent environment as the tumours would be larger and the level of tolerance would potentially be increased to due to prolonged exposure to the antigen, which could result in increased peripheral tolerance.

5. The Effect of Electroporation on DNA Vaccination in a Prime-boost Setting

5.1. Introduction

This part of the project is focused on the investigation of how electroporation (EP) affects immune responses to DNA vaccines. EP is a process in which an electrical field is passed across a cell membrane, resulting in a large membrane potential. This causes transient pores to appear in the membrane, enabling nucleic acid to enter the cell through them. EP also causes tissue damage at the site, leading to cell death and inflammation.

EP can be applied to the site of a DNA vaccination with a view to improving immune responses; however, the finer details of how the two aspects of increased cell transfection and tissue damage impact on the immune response are still unknown. Some of the previous work that has informed this project is discussed below.

5.1.1. Electroporation Increases the Transfection of Cells by DNA Vaccines

When EP is given at the site of vaccination, the increased membrane permeability it causes allows the DNA to enter the cell passively, bypassing the need for endocytosis and thus increasing the rate of transfection, resulting in increased protein production [145]. Although this technique was originally developed for the purpose of gene therapy [263], it was also hoped that increased antigen delivery to the cell would increase immune responses to a DNA vaccine. However, it is not clear how important the dose of a DNA vaccine is, with dose-response experiments in mice seeming to plateau after a 'critical' dose was reached [139]. This suggests that improved transfection may not be of benefit to primary $CD8^+$ T-cell responses.

5.1.2. Electroporation Causes Muscle Damage

The other element of the adjuvant properties of EP is the increased muscle damage at the injection site. This can be measured by determining the levels of circulating creatine phosphokinase, performing histological analysis and detecting the presence of inflammatory cells in the muscle [146]. After EP is administered, the ensuing inflammation recruits cells that express CD11c (DCs), MHC class II (professional APCs) and F4/80 (macrophages) to the site [264], [119]. It has been shown that apoptotic vesicles are taken

by DCs and cross-presented to T cells [148], therefore this inflammation is likely to enhance the immune responses by increasing antigen presentation.

Previous studies have shown that the muscle damage caused by EP begins to resolve by 14 days after treatment [147] and the technique has proven safe and well tolerated in clinical trials [149].

5.1.3. Electroporation and the Immune Response

It is clear that EP can increase the transfection of muscle cells at a vaccination site, and at the same time cause damage to the muscle. What is less clear is how these two effects influence the immune response to the DNA vaccine. Early studies using a DNA vaccine containing the gag gene from HIV-1 showed that giving EP at the time of vaccination increased antibody titres against the gag protein. However, the effect of EP on the T-cell response was less clear. Mice which received EP did have an increased number of gag-specific $CD8^+$ T cells, but this effect was most striking with DNA doses of 0.2µg and 2µg, becoming much less so when a 20µg dose was used [265].

More recent data from our laboratory supported this in part, with increased primary antibody responses seen in mice given EP at the time of primary DNA vaccination. When EP was given in a prime-boost setting, the antibody response was reduced compared to mice which were only given EP with the booster vaccination. EP proved crucial for the $CD8^+$ T-cell response when the injection volume was sub-optimal. However, when injection volumes were optimal, there was a trend towards a reduction in $CD8^+$ T-cell induction with EP; and in a prime-boost setting the strongest $CD8^+$ T-cell response was induced by only giving EP with the secondary vaccination [139].

These data suggest that giving EP with a DNA vaccination can improve immune responses. EP may be particularly useful for inducing T-cell responses when sub-optimal injection volume or doses are used, which could be the case in the clinic. This evidence is certainly encouraging, but the conditions of DNA vaccination in conjunction with EP must be optimised in order to induce the best possible immune responses.

5.1.4. Vaccination Site

An added complication to this study is the vaccination site. Our DNA vaccines are usually administered in the mouse tibialis anterior muscle; however the electroporator device used in this study has been optimised to deliver DNA to the mouse quadriceps muscle. This is

an important consideration as there may be differences between the two muscles which affect the immune responses generated in them. Studies carried out some time ago indicated that when a DNA plasmid encoding the hepatitis B surface antigen (HBs) was injected into the mouse tibialis anterior, it induced a superior antibody response compared to the same plasmid administered in the quadriceps, though the difference was less clear for the CTL response [266]. This highlights how different muscle sites may potentially induce different immune responses, making comparisons all the more complex.

5.1.5. Aims

The aim of this study was to fully investigate immune responses induced by the pDOM.$E7_{49-57}$ DNA vaccine. T-cell and antibody responses were assessed after injection of DNA alone into the mouse tibialis anterior or quadriceps muscles, or injection into the quadriceps in conjunction with EP. $CD8^+$ T-cell responses to the $E7_{49-57}$ peptide were measured by flow cytometry using an H-$2D^b$-$E7_{49-57}$ tetramer. Functionality was confirmed by ELISpot assay, in which $CD4^+$ T-cell responses to the p30 peptide from DOM were also measured. Antibody responses against DOM were measured by ELISA. These immune responses were studied after both primary and booster vaccinations. The results should help to build a clearer picture of the way the immune response is shaped by the addition of EP.

5.2. Results

5.2.1. Primary $CD8^+$ T-Cell Responses Induced by pDOM.$E7_{49-57}$ Vaccination with or without Electroporation in the Quadriceps Muscle

To investigate the effect of muscle electroporation (EP) after DNA vaccination on primary $CD8^+$ T-cell responses, mice were vaccinated with the pDOM.$E7_{49-57}$ vaccine into the quadriceps muscle. The vaccine dose remained constant at 25µg/leg (i.e. 50µg/mouse) but the injection volume used was either the standard 50µL or a sub-optimal 10µL in each leg. This is an important comparison as in a clinical setting injection volumes may be sub-optimal.

Blood samples were collected 10 days after vaccination, and immune responses were analysed by flow cytometry using an H-$2D^b$-$E7_{49-57}$ tetramer. Cells were also stained with anti-MHC class II antibody in order to gate out APCs which could bind the tetramer non-specifically; and with anti-CD8 antibody so that the tetramer-positive cells could be expressed as a percentage of the total number of $CD8^+$ T cells. A representative example of the FACS plots from a mouse vaccinated 10 days previously with pDOM.$E7_{49-57}$ alone is shown in Figure 5.1A, together with a FACS plot from a mouse given pDOM control vaccine with EP.

As shown in Figure 5.1B, a 50µL injection of DNA induced a $CD8^+$ T-cell response, with an average of approximately 1% of cells being both CD8 and tetramer positive 10 days after vaccination. When EP was given with such an injection, there was a trend towards an improved response, with an average of approximately 2.5% of cells being double-positive; however this difference was not statistically significant. When an injection volume of 10µL was used, tetramer staining was not above background (<0.5% in pDOM vaccinated mice). However, if EP was given with this sub-optimal volume, significantly more $CD8^+$ T cells were tetramer-positive (P=0.0006 in a t test), demonstrating the potential of using this approach to improve immune responses to the p$E7_{49-57}$ DNA vaccine.

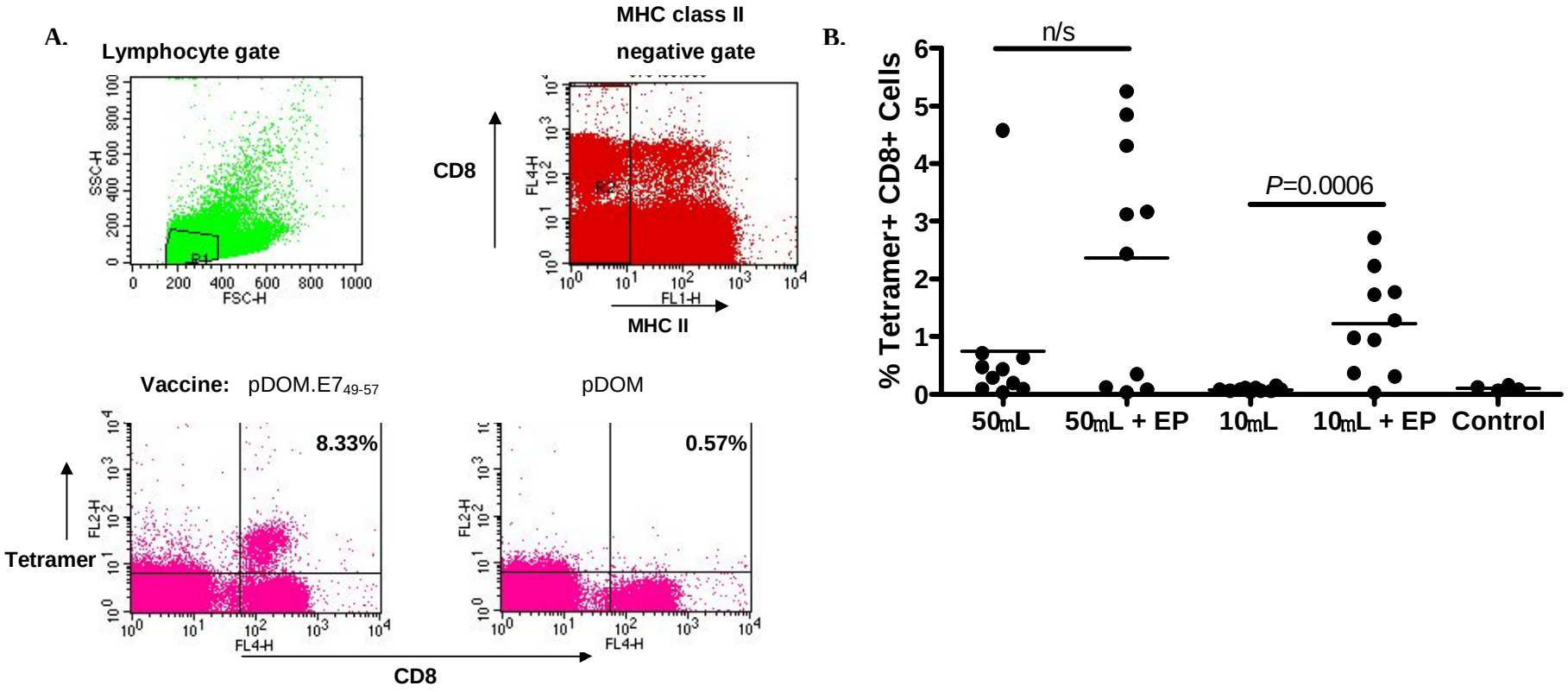


Figure 5.1. Electroporation (EP) increases T-cell responses to the pDOM.E7$_{49-57}$ DNA Vaccine injected into the quadriceps muscle

Groups of 5 mice were injected with 10µL or 50µL of pDOM.E7$_{49-57}$ DNA in the quadriceps muscle, either with or without EP. Control mice received 50µL of pDOM with EP. After 10 days, blood samples were taken and cells were stained with E7$_{49-57}$ tetramer and anti-MHC II-FITC and anti-CD8-APC antibodies as described. **A**. Representative FACS plots showing gating strategy and example results. **B**. percentage of tetramer-positive, CD8-positive cells after 10 days. Lines indicate means. Data are combined from two experiments showing the same trend. *P* values were generated using a t test.

5.2.2. Primary $CD8^+$ T-Cell Responses Induced by pDOM.$E7_{49-57}$ Vaccination in the Tibialis Anterior Muscle

The standard vaccination protocol used in our laboratory is to inject DNA into the tibialis anterior muscle. Therefore, it was important to compare immune responses generated via that method with those induced by the EP protocol, where DNA is injected into the quadriceps. The reason for using this different route is that the electroporator used in this study was designed specifically for use in the mouse quadriceps muscle. Due to the distance between the electrodes, it is not suitable for use in the tibialis anterior.

Groups of 4-6 mice were vaccinated with the pDOM.$E7_{49-57}$ DNA vaccine alone in the tibialis anterior or with EP in the quadriceps and the kinetics of the $CD8^+$ T-cell response were tracked, as shown in Figure 5.2A. Eight days after injection, some tetramer-positive $CD8^+$ T cells were already present in the blood of the vaccinated mice. In mice vaccinated with DNA alone in the tibialis anterior, the response peaked 10 days after injection. Surprisingly, at this point fewer tetramer-positive $CD8^+$ T cells were found in mice given DNA with EP into the quadriceps compared to those which were given DNA alone in the tibialis anterior (P=0.0125 in a t test), suggesting that vaccination into the tibialis anterior is more effective than vaccination into the quadriceps. However, by 14 days post-injection the mice from both groups had comparable levels of tetramer-positive $CD8^+$ T cells. The background level of staining in blood samples from mice given the pDOM control vaccine with EP was always <1%.

In order to ensure that giving DNA with EP into the quadriceps did not cause a delayed response which peaked after day 14, the experiment was repeated over a longer period of time. Blood samples were taken 7-17 days after pDOM.$E7_{49-57}$ vaccination with EP in the quadriceps. The results, shown in Figure 5.2B, demonstrate that the response peaks 14 days after injection and regresses after that.

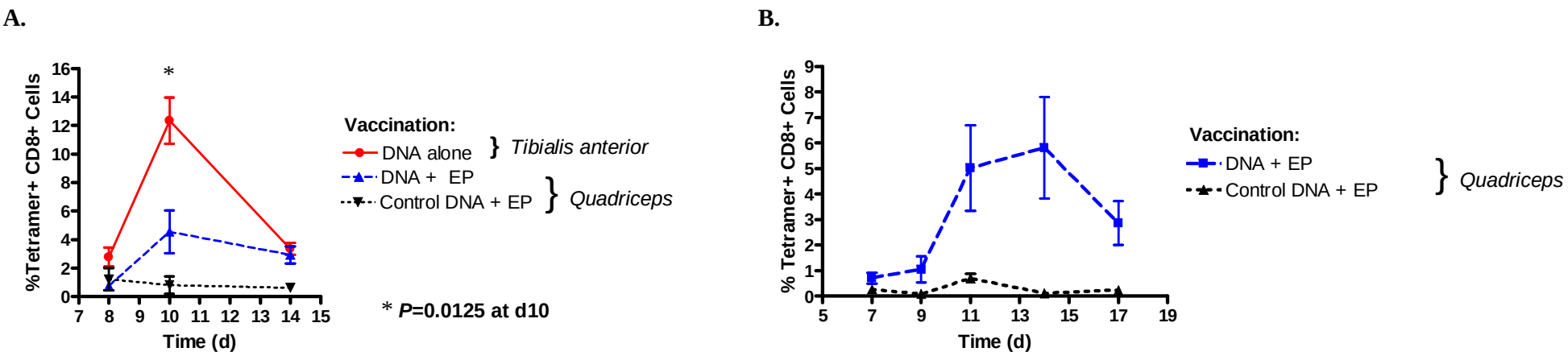


Figure 5.2. H-2D^{b}-E7$_{49\text{-}57}$ tetramer staining after pDOM.E7$_{49\text{-}57}$ vaccination into the tibialis muscle or into the quadriceps muscle with EP

Groups of 4 mice were immunised with pDOM.E7$_{49\text{-}57}$ and 8, 10 and 14 days post-vaccination, blood samples were taken and analysed by flow cytometry. Cells were gated on forward and side scatter and MHC II negativity as before. CD8 and tetramer double positive cells were calculated as a percentage of total CD8 positive cells. **A**. The changing percentage of tetramer and CD8 double positive cells over time. Data are combined from two experiments showing the same trend. *P* values were generated in a t test. **B**. An extended experiment to monitor the kinetics of responses to DNA with EP over a longer period of time.

5.2.3. $CD8^+$ T-Cell Responses Induced by pDOM.$E7_{49-57}$ Vaccination with or without Electroporation in a Prime-boost Setting

Choosing the right time at which to boost will be crucial to raising robust immune responses. If a booster vaccination is given too early, the T-cell response may still be dominated by effector cells, rather than the memory cells which booster vaccinations are designed to expand. When using EP, the muscle damage caused must be taken into consideration. Especially when multiple vaccinations are given as this may affect immune responses. Here, two different time points have been compared for boosting; day 28 and day 53 post injection. The vaccination schedule is shown in Figure 5.3.

5.2.3.1. Early Boost

Previous work investigated the consequences of boosting a DNA vaccination with DNA and EP 28 days later. The results showed that $CD8^+$ T-cell responses to a DNA vaccination were boosted more effectively when the secondary vaccination was given with EP rather than another injection of DNA alone [139]. We wanted to see if this was also the case using the pDOM.$E7_{49-57}$ vaccine.

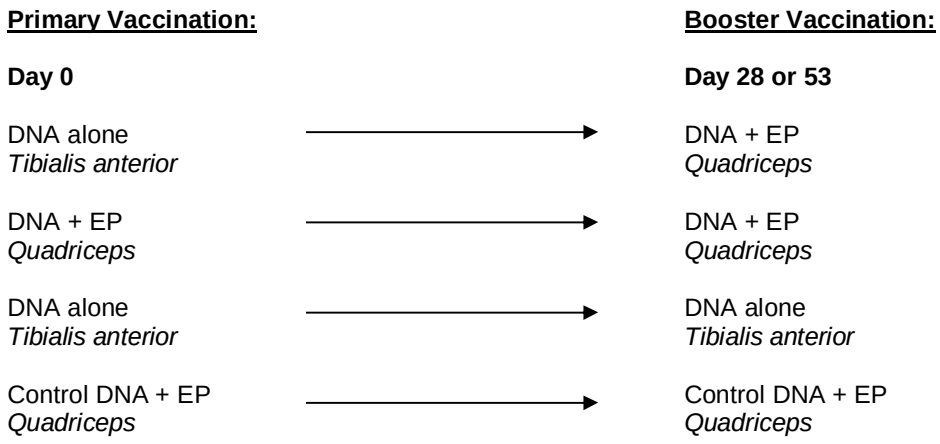


Figure 5.3. Prime-boost DNA vaccination schedule

Mice were vaccinated with DNA (pDOM.$E7_{49-57}$) with or without electroporation (EP) as primary and booster vaccinations. Mice which received DNA alone were vaccinated into the tibialis muscle as per the standard protocol. Mice given EP with the vaccine were injected into the quadriceps as per the EP protocol. Some mice were given an 'early' boost after 28 days, and some a 'late' boost after 53 days. Control animals were vaccinated with the pDOM vaccine (control DNA) with EP at both time points.

In order to investigate this point, groups of 6 mice were immunised with the pDOM.$E7_{49-57}$ vaccine and then boosted 28 days later with or without EP at both time points, as shown in Figure 5.3. In these experiments, injections of DNA alone were given into the tibialis muscle, where they induce robust $CD8^+$ T-cell responses (see Figure 5.2) whereas DNA injections given with EP were given into the quadriceps muscle. We were unable to do a direct comparison due to the fact that the electrodes have been specifically designed for use on the mouse quadriceps muscle and not the tibialis anterior.

The H-$2D^b$-$E7_{49-57}$ tetramer was used as before to measure E7-specific $CD8^+$ T cells in the blood 10 days after prime and then again 10 days after boost. The results, shown in Figure 5.4A, confirmed the primary kinetics data shown in Figure 5.2A, with fewer tetramer-positive $CD8^+$ T cells found in the blood 10 days after DNA vaccination with EP in the quadriceps compared to DNA alone in the tibialis anterior ($P<0.05$ in a t test compared to both the other test groups given DNA alone).

As shown in Figure 5.4A, the prime-boost strategy which induced the most $CD8^+$ T cells was a primary vaccination with DNA in the tibialis anterior followed by a DNA boost with EP in the quadriceps (DNA/EP). This expanded approximately twice as many tetramer-positive $CD8^+$ T cells compared to the group which received EP at both time points (EP/EP), and this difference was statistically significant ($P=0.0087$ in a t test). Interestingly, even though a vaccination of DNA alone induced significantly more tetramer-positive $CD8^+$ T cells than a vaccination of DNA with EP at priming, after a homologous boost, there was no longer a statistically significant difference between the two groups (EP/EP and DNA/DNA) when compared in a t test. A vaccination of DNA alone at both time points also induced approximately half as many tetramer-positive $CD8^+$ cells compared to the heterologous strategy ($P=0.0163$ in a t test). This suggests that a vaccination of DNA alone in the tibialis anterior does not boost effectively at this time point and also that a vaccination of DNA with EP in the quadriceps can boost a weak primary response. In fact, the responses after a DNA boost with EP were approximately three-fold higher after either a primary vaccination of DNA alone or DNA with EP. Therefore, whatever the reason for the limitation of responses after a primary vaccination of DNA with EP in the quadriceps; it does not occur at boosting.

In order to confirm the number of effector T cells still present in the blood at the time of boosting, the experiment was repeated and after 28 days, the number of tetramer positive $CD8^+$ T cells in the blood was assessed. The results, shown in Figure 5.4B, indicate that responses have waned by this point, though they are still readily detectable. Interestingly, there is a trend towards fewer $CD8^+$ T cells being tetramer positive at this time point after an injection of DNA with EP into the quadriceps muscle compared to an injection of DNA

alone in the tibialis anterior. The number of E7-specifc T cells present as this point may affect the effectiveness of the boost as it is possible that these effector cells may lyse APCs bearing the $E7_{49-57}$ peptide. Therefore, we reasoned that boosting later, when the effector response may have contracted further, may improve secondary responses.

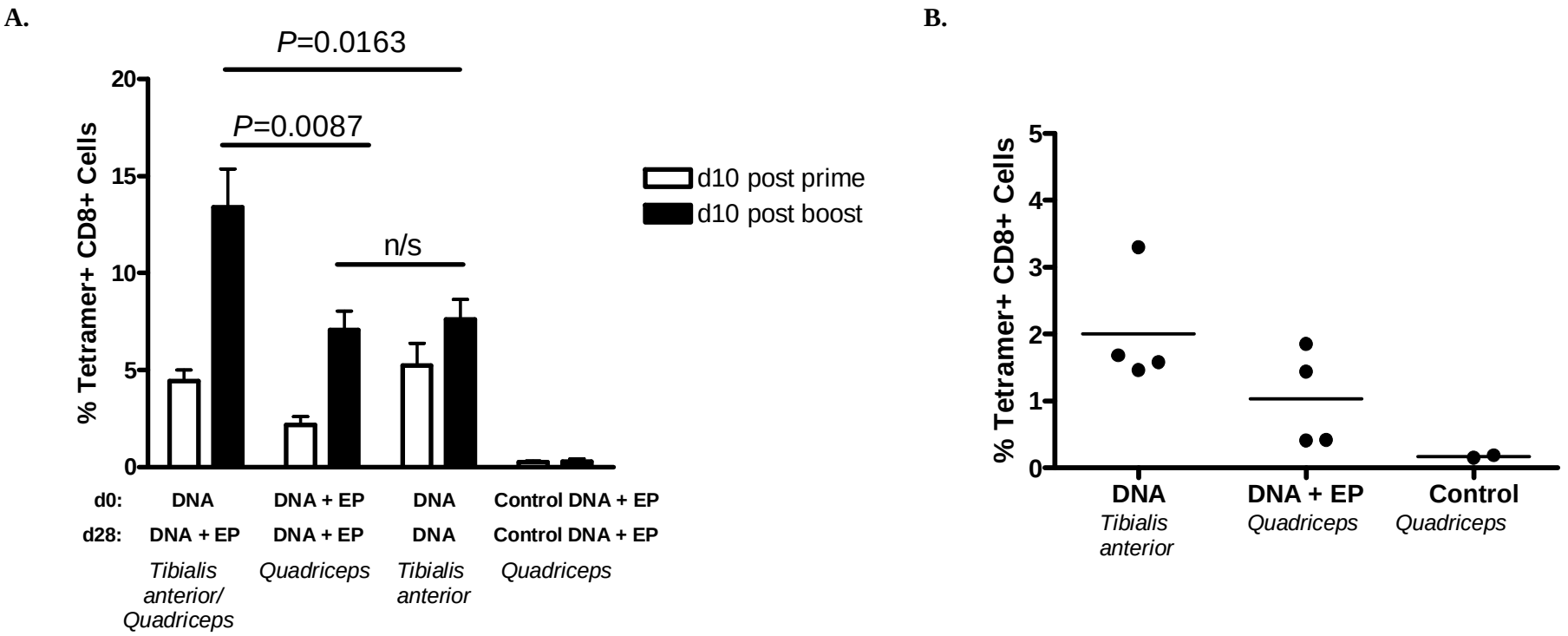


Figure 5.4. CD8$^+$ T-cell responses induced by pDOM.E7$_{49\text{-}57}$ after an early boost with or without electroporation (EP)

A. Groups of 4-6 mice were vaccinated with the pDOM.E7$_{49\text{-}57}$ (DNA) or pDOM (control DNA) vaccines with EP in the quadriceps or without EP in the tibialis anterior and then boosted 28 days later. Blood samples were taken and stained with an E7$_{49\text{-}57}$-D^b tetramer 10 days after prime (open bars) and 10 days after boost (filled bars). Error bars represent variation between individual mice. Data are combined from two experiments showing the same trend. *P* values were generated using a t test. **B.** Groups of 2-4 mice were vaccinated as before and after 28 days, blood samples were taken and stained with an E7$_{49\text{-}57}$-D^b tetramer. Data are representative of two experiments. Bars represent group means.

5.2.3.2. Late Boost

As the data in Figure 5.4B showed that there were still E7-specific $CD8^+$ T cells circulating in the blood 28 days after a primary immunisation, boosting at a 'late' time point may be advantageous. Researchers often wait 50 days from the time of priming before giving a booster vaccination [225], [163]. Data from viral infections indicate that by this point the effector T-cell population should have contracted [267]; leaving only memory T cells, and no effector cells which might impair responses. In order to see if a later boost improves immune responses, a second prime-boost experiment was carried out in which mice were not boosted until 53 days after the primary immunisation.

Groups of 6 mice were vaccinated with pDOM.$E7_{49-57}$ or pDOM control vaccines either with EP in the quadriceps or without EP in the tibialis anterior. Tetramer staining was carried out on blood samples as before. The primary response in these mice, depicted in Figure 5.5A, showed the same trend and kinetic as in Figure 5.2 and Figure 5.4. After an injection into the quadriceps with EP approximately three-fold fewer $E7_{49-57}$-specific $CD8^+$ T cells were found in the blood 10 days later (P=0.001 in a t test).

A further blood sample was taken 51 days after the DNA prime to assess the level of memory T cells remaining in the blood. The results, shown in Figure 5.5B, confirmed that a small population of tetramer positive $CD8^+$ T cells was detectable at this time point. In blood samples from some mice, the level of staining was too low to distinguish from background (usually <0.5% of $CD8^+$ T cells), but in most animals 0.5-1.5% of $CD8^+$ T cells were also tetramer-positive. This low level of tetramer staining indicates that the effector phase of the $CD8^+$ T-cell response is over, and that only a small pool of memory cells remains in the blood. As expected, the number of tetramer-positive cells present at this time point seems to be lower than at day 28 (see Figure 5.4B), indicating either that the effector phase may not have fully regressed at the time of early boost, which may effect its efficacy; or that the memory pool is diminishing over time.

Interestingly, there was a trend towards more tetramer-positive $CD8^+$ T cells being present after an injection of DNA alone in the tibialis anterior compared to those which received DNA with EP in the quadriceps muscle, though this was not a statistically significant difference. This could suggest that despite the reduced peak response in mice which received EP, the size of $CD8^+$ T-cell memory population is not affected. However, it is possible that there is a real difference between the groups, but that the tetramer may not be sensitive enough to detect it due to the low frequency of the memory T cells.

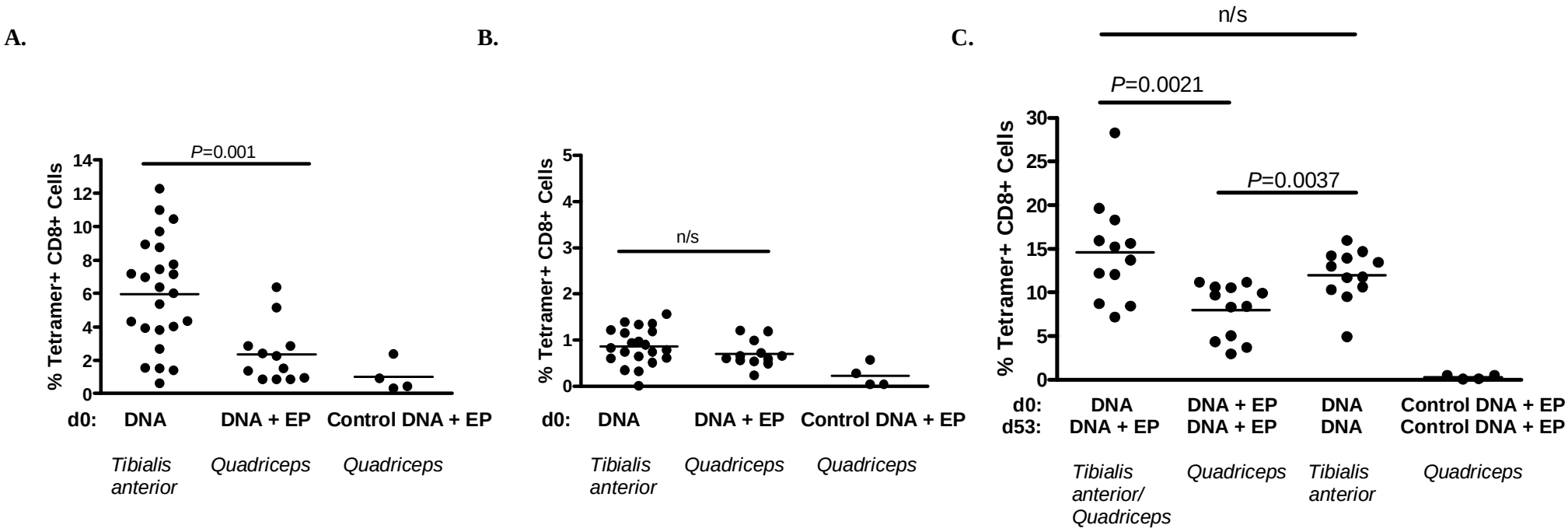


Figure 5.5. CD8$^+$ T-cell responses induced by pDOM.E7$_{49\text{-}57}$ vaccination after a late boost with or without electroporation (EP)

Groups of 6 mice were vaccinated with the pDOM.E7$_{49\text{-}57}$ (DNA) or pDOM (control DNA) vaccines with EP in the quadriceps or alone in the tibialis anterior. Blood samples were taken at various time points and CD8$^+$ T-cell responses were analysed using the H-2D^b-E7$_{49\text{-}57}$ tetramer as before. **A.** E7$_{49\text{-}57}$-specific CD8$^+$ T cell responses 10 days after primary vaccination. **B.** E7$_{49\text{-}57}$-specific CD8$^+$ T cells remaining in the blood 51 days after vaccination. **C.** E7$_{49\text{-}57}$-specific CD8$^+$ T cell responses d10 days after boosting. *P* values were generated using a t test; n/s, not significant; d, day. Data are combined from two experiments showing the same trend.

These mice were then boosted with the pDOM.$E7_{49-57}$ DNA vaccine, either with DNA alone in the tibialis anterior muscle or with EP in the quadriceps muscle 53 days after the primary vaccination, as shown in Figure 5.3. Tetramer analysis was then carried out 10 days later. In all the groups, the mean percentage of CD8 and tetramer double positive cells at this time point was approximately two-fold higher than it was at the peak of the primary response (Figure 5.5A and C).

As with the early boost, the most successful strategy was giving a primary vaccination of DNA alone followed by a DNA boost with EP, which induced a mean of ~15% tetramer-positive $CD8^+$ T cells, as shown in Figure 5.5C. However, a homologous prime-boost with DNA alone in the tibialis anterior induced a similar number of tetramer-positive $CD8^+$ T cells, with a mean of ~13%. This indicates that at this time point, an injection of DNA alone in the tibialis anterior is able to boost primary responses just as well as DNA with EP in the quadriceps, contrary to the findings after an early boost (see Figure 5.4). Vaccination into the quadriceps with EP at both prime and boost induced a mean of ~6% of tetramer-positive $CD8^+$ cells. This reduction in $CD8^+$ T-cell responses seen in mice given EP at both time points was statistically significant when compared to the groups which received DNA/EP or DNA/DNA (P=0.0021 and P=0.0037 respectively; results were generated using a t test). The likely explanation for this could be the reduced pool of memory $CD8^+$ T cells available for boosting seen in Figure 5.5B. As with the early boost, the fold-increase from primary to secondary responses was very similar in each group; again indicating that DNA with EP in the quadriceps is more efficient as booster vaccination than a primary vaccination.

In order to confirm these data using a functional assay, an IFNγ secretion capture ELISpot assay was carried out on the mice discussed above as described in the Materials and Methods. Lymphocytes were isolated and incubated overnight in the presence of varying concentrations of the $E7_{49-57}$ peptide, 1μM p30 peptide from DOM or without peptide to give the background level of IFNγ-producing cells. In all groups, the number of IFNγ-positive spot-forming cells (SFC) was increased after incubation with higher concentrations of the $E7_{49-57}$ peptide, with half the maximum response reached at 0.5nM and the maximum response reached at 50nM (Figure 5.6). This suggests that there is no difference in the avidity of the $CD8^+$ T cells induced by each vaccine regimen. In order to conduct a statistical analysis, responses to 50nM peptide were compared. The results, shown in Figure 5.7A, reveal that a primary vaccination with DNA alone in the tibialis anterior followed by a DNA boost with EP in the quadriceps induces twice as many IFNγ-positive SFC compared to a vaccination of DNA with EP in the quadriceps at both time

points (*P*=0.0009 in a Mann-Whitney test). Vaccination with DNA alone at both time points induced double the number of IFNγ-positive SFC compared to a vaccination of DNA with EP in the quadriceps at both time points (*P*=0.0027 in a Mann-Whitney Test). Again, there was no statistical difference between the two groups given a primary vaccination of DNA alone and then boosted with DNA with or without EP.

The ELISpot assay also allowed assessment of $CD4^+$ T-cell responses. The presence of DOM in the vaccine gives us the opportunity to investigate how EP affects the level of T-cell help induced. To do this, cells were incubated with the well described p30 peptide from DOM. The data, shown in Figure 5.7B, followed the same trend as the $CD8^+$ T-cell response. DNA vaccination with EP in the quadriceps at both prime and boost induced fewer IFNγ-producing SFC than vaccination with DNA alone at prime followed by a boost of DNA with EP or vaccination with DNA alone at both time points (*P*=0.0008 and *P*=0.0086 respectively; using a Mann-Whitney test). There was also no statistically significant difference between the number of p30-specific IFNγ-producing SFC induced by a primary vaccination of DNA alone when followed by either a boost of DNA alone or a boost of DNA with EP.

These data show that vaccination with DNA alone in the tibialis anterior muscle can provide an effective boost for both $CD8^+$ and $CD4^+$ T cells 53 days after prime, in contrast to the results generated after a boost at day 28, which required the addition of EP to improve responses, possibly due to the persistence $CD8^+$ effector T cells. This may have clinical relevance as in humans, the kinetics of the $CD8^+$ T cell response are unclear; making the use of EP advantageous.

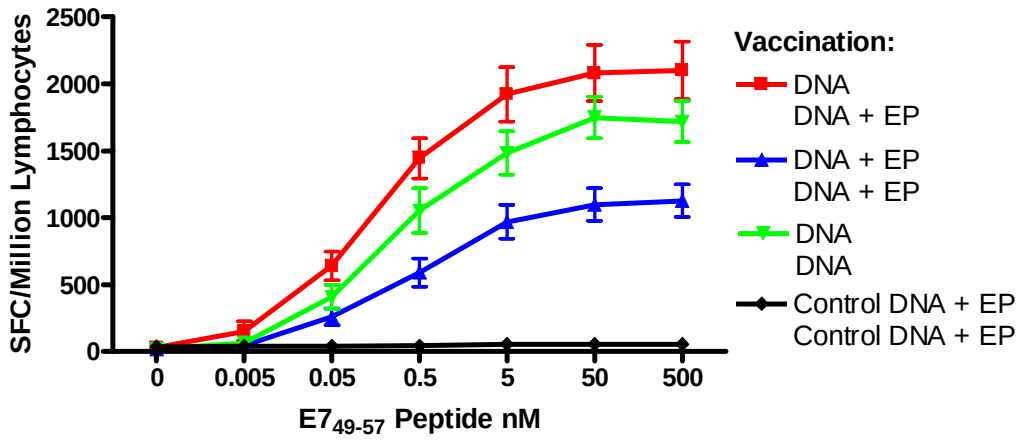


Figure 5.6. *Ex vivo* IFNγ production in response to varying concentrations of $E7_{49-57}$ peptide after a late boost with pDOM.$E7_{49-57}$ with or without electroporation (EP)

Mice were primed and boosted 53 days later with pDOM.$E7_{49-57}$ with or without EP. Lymphocytes were harvested and used in an ELISpot assay 10 days after boosting. The number of IFN_{γ} positive spot-forming cells (SFC) per million lymphocytes after incubation with different concentrations of the $E7_{49-57}$ peptide is shown. Error bars represent variation between individual mice. Data are combined from two experiments showing the same trend.

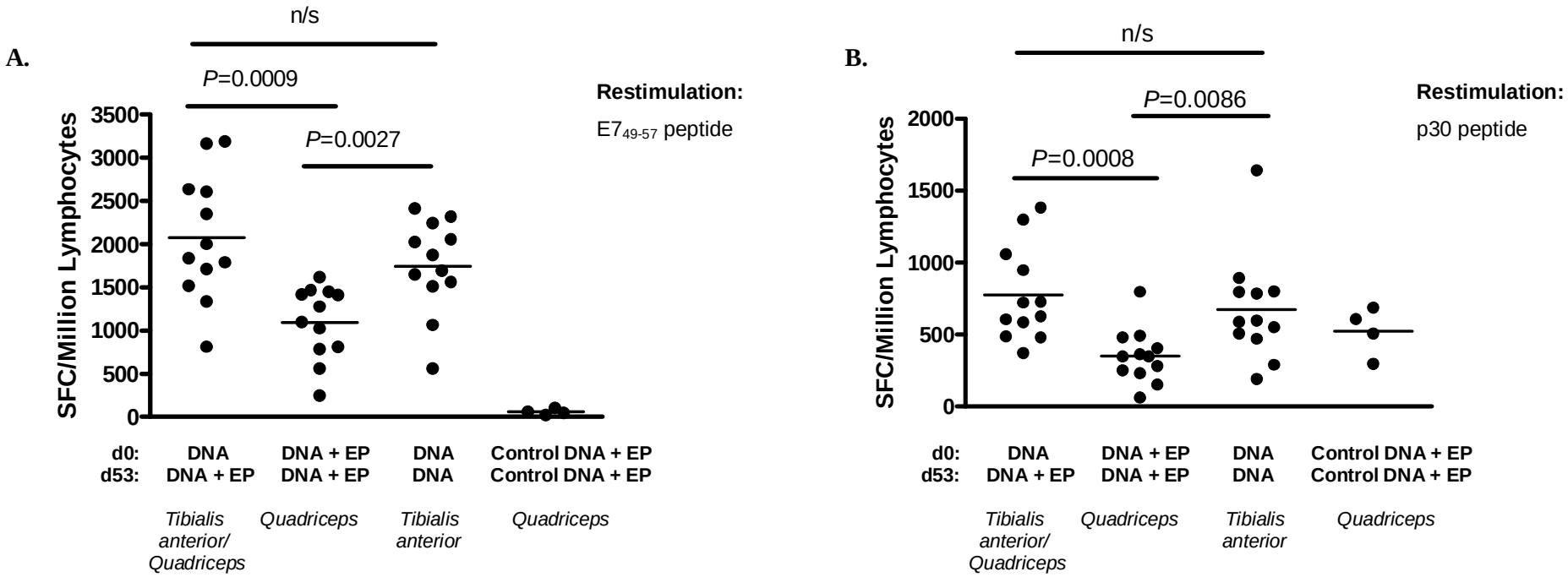


Figure 5.7. *Ex vivo* IFNγ production induced by pDOM.E7$_{49-57}$ vaccination after a late boost with or without electroporation (EP)

Mice were vaccinated with pDOM.E7$_{49-57}$ and boosted 53 days later with or without EP. Lymphocytes were harvested and used in an IFNγ ELISpot assay 10 days after boosting. The number of IFN$_\gamma$ SFC per million cells after incubation with **A.** 50nM E7$_{49-57}$ peptide or **B.** 1µM DOM-specific p30 peptide. Lines indicate means. The statistical analysis was conducted using a Mann-Whitney test; SFC, spot-forming cells; n/s, not statistically significant; d, day. Data are combined from two experiments showing the same trend.

5.2.4. DOM-specific IgG Induced by pDOM.E7$_{49-57}$ Vaccination with Electroporation

The presence of DOM in the pDOM.E7$_{49-57}$ vaccine enables the assessment of antibody responses against the DOM protein. Therefore, DOM-specific IgG was also monitored to see what effects EP might have on antibody titres in this model.

Groups of 4-6 mice were vaccinated into the quadriceps muscle with either 10 or 50µL of the pDOM.E7$_{49-57}$ vaccine with or without EP and serum samples were collected 42 days later. An ELISA was carried out on DOM-His protein-coated 96-well plates to determine titres of DOM-specific IgG in the serum. As shown in Figure 5.8, after a 50µL injection DOM-specific IgG titres were the same regardless of whether EP had been given with the injection or not. However, this was in marked contrast to responses to the suboptimal injection volume of 10µL. In these mice, responses were not detectable (<10U/mL) unless EP was given. Interestingly, when EP was given the responses were comparable to those of the mice given an optimal dose, indicating that EP can rescue antibody responses following a sub-optimal injection volume. DOM-specific IgG was not detectable in serum from naïve mice (<10U/mL).

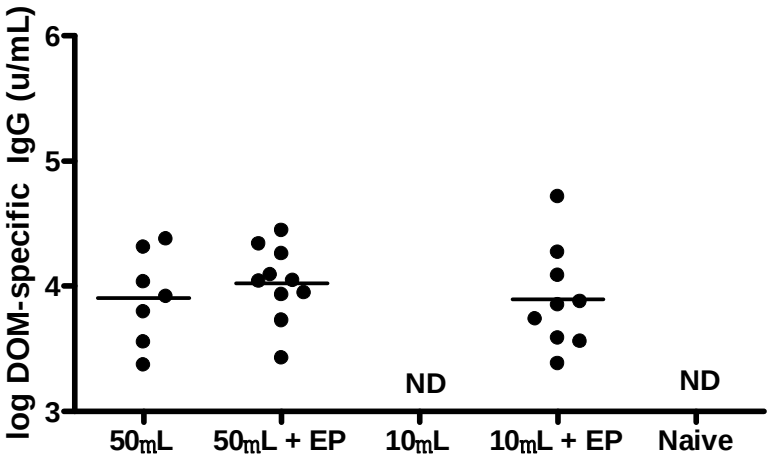


Figure 5.8. DOM-specific IgG induced by the pDOM.E7$_{49-57}$ vaccine after injection into the quadriceps with or without electroporation (EP)

Groups of 4-6 mice were vaccinated with pDOM.E7$_{49-57}$ in the quadriceps either alone or with EP. Blood samples were collected 42 days later and an ELISA for DOM-specific IgG was carried out on the sera. Data are combined from two experiments with similar results and log-normalised. Lines indicate the mean log DOM-specific IgG titre in units/mL (U/mL). ND signifies that a response was not detectable (<10U/mL).

The IgG response generated by the injection condition which had proved optimal for T-cell responses, that is a 50µL injection volume given in the tibialis anterior was also investigated. This was compared to a 50µL injection given with EP into quadriceps. The results, shown in Figure 5.9A, reveal that 35 days after injection, DOM-specific IgG titres were significantly higher in the group which received DNA alone (P=0.0013). This effect only reached statistical significance when data from several experiments were combined due to the spread of the data. As shown in Figure 5.9B, the same pattern was observed after a booster injection; a homologous prime-boost with DNA alone into the tibialis anterior induced significantly higher levels of IgG compared to a homologous prime-boost with EP into the quadriceps (P=0.0134). There was also a trend towards a primary DNA injection into the tibialis anterior followed by a booster vaccination with EP into the quadriceps being more effective than a homologous prime-boost with EP into the quadriceps, but this did not reach statistical significance.

Mice which were not vaccinated or which were vaccinated with an irrelevant antigen (synthetic $E7_{43-77}$ long peptide with CpG) did not produce detectable levels of DOM-specific IgG when tested in the same way (<10U/mL; data not shown).

These results do not corroborate previous data from our lab which showed that a DNA vaccine induced stronger antibody responses when given with EP, even though the optimal injection volume was used [139]. This difference could be due the strains of mice used; in Buchan *et al*, BALB/c mice were used, a strain dominated by Th2 responses, in contrast to the C57BL/6 mice used in this study which are Th1-polarised [268], [269].

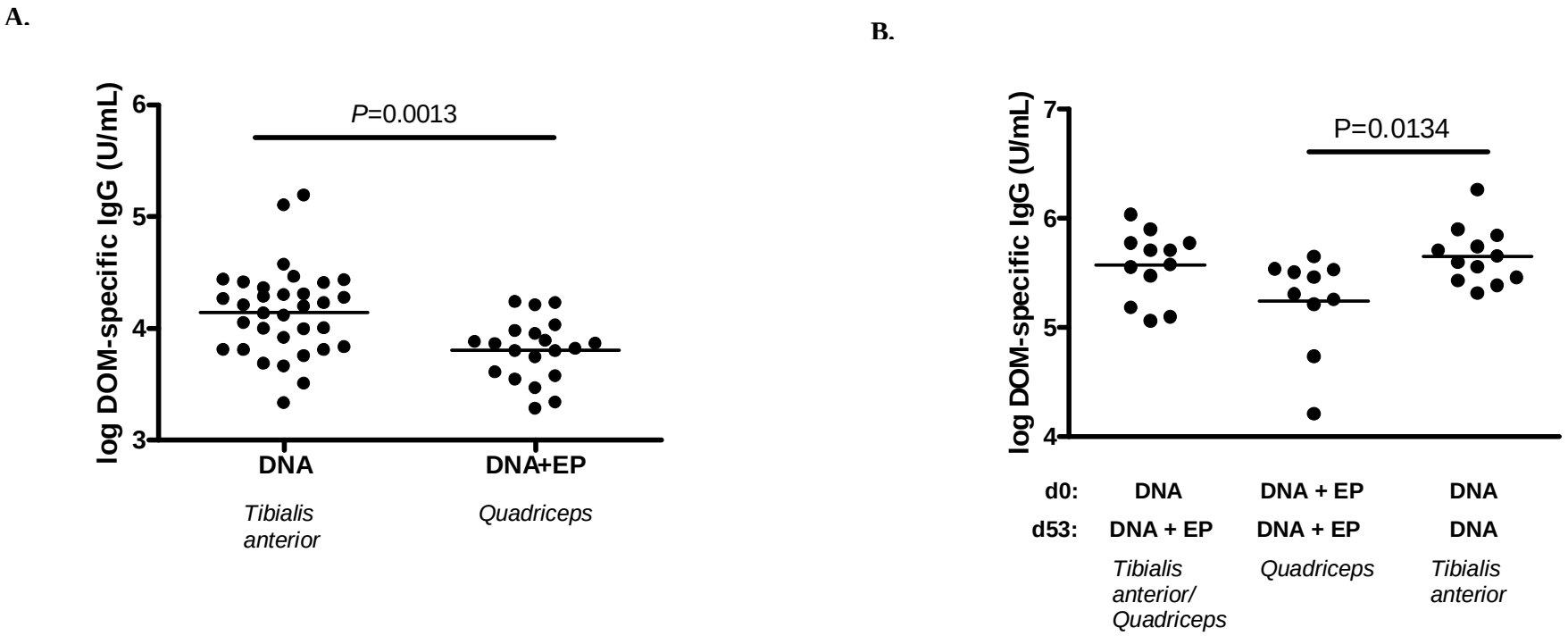


Figure 5.9. DOM-specific IgG induced by the pDOM.E7$_{49\text{-}57}$ vaccine after injection into the tibialis muscle alone or into the quadriceps muscle with electroporation (EP)

Groups of 4-6 mice were vaccinated with pDOM.E7$_{49\text{-}57}$ (DNA) either alone into the anterior tibialis or with EP into the quadriceps (DNA+EP). **A.** DOM-specific IgG in the sera of mice vaccinated 35 days earlier. **B.** DOM-specific IgG in the sera of mice primed and then boosted as shown 53 days later. Samples collected 10 days post boost. Lines indicate the mean log DOM-specific IgG titre in units/mL (U/mL). Data are combined from four (A) or two (B) experiments with similar results and log-normalised to allow statistical analysis. *P* values were generated using a t test.

5.3. Discussion

5.3.1. Electroporation can Rescue a Sub-optimal Injection Volume in the Quadriceps Muscle

The ability of EP to rescue vaccine-specific T-cell responses to a sub-optimal injection volume in the mouse quadriceps muscle has been confirmed using the pDOM.E7$_{49-57}$ vaccine, as has been shown previously in other models [139], [270]. In contrast, the vaccine-specific CD8$^+$ T-cell responses after an injection of the standard 50µL volume in the quadriceps did not appear to be significantly improved by the addition of EP (Figure 5.1). It is known that EP (i) increases transfection efficiency and (ii) causes muscle damage. Both these two effects could potentially contribute to the improved immune responses to a sub-optimal injection volume. There is evidence that the dose of DNA is less important than the volume in which it is given [139]; so it is likely that the increased transfection may not be as important as the muscle damage. Injection volume determines the hydrostatic pressure on the muscle, and thus the amount of damage and inflammation caused. EP is known to cause inflammation, so it seems likely that this replaces the inflammation which is missing from a sub-optimal injection volume. In a clinical setting, injection volumes are likely to be sub-optimal so other ways of generating local inflammation are required. The additional inflammation provided by EP [146], [119], [264], [147] could be crucial for recruiting APCs to the site, where they can acquire antigen for cross-presentation. These data support the importance of EP for improving DNA vaccine responses in patients. In fact, a recent clinical trial carried out here in Southampton has demonstrated that EP does indeed increase DOM-specific antibody levels after DNA vaccination in prostate cancer patients [149], though the effect on T-cell responses are less clear at this point (L Low, personal communication).

5.3.2. DNA Injection into the Tibialis Anterior Muscle Induces a Superior CD8$^+$ T-cell response Compared to Injection in the Quadriceps even with Electroporation

Having confirmed the importance of EP in improving responses in the quadriceps muscle, it was demonstrated that an injection of DNA alone into the smaller tibialis anterior muscle was actually superior to an injection of DNA with EP given in the quadriceps muscle (Figure 5.2). Although few comparisons appear to have been made to date, one other study which did directly compare the two injection sites did support this. When a DNA vaccine was administered in the tibialis anterior, it induced a superior antibody response compared

to the same plasmid administered in the quadriceps. Though the difference was less clear for the CTL response, a higher dose of DNA was required in the quadriceps to achieve a CTL response similar to that generated by injection into the tibialis anterior [266]. This surprising result may also hinge on inflammation. As the tibialis anterior is a smaller muscle, it could incur more damage than the quadriceps after an identical injection. Using microscopy to visualise the result of a 50µL injection, it was shown that this volume is sufficient to exceed the fluid capacity of the mouse tibialis anterior muscle [270]. Therefore, inflammation may not be a limiting factor in this site. There could also be other differences between the two muscle sites, perhaps their distance from draining lymph nodes or how well used or developed they are. Unfortunately, a direct comparison between the two injection sites was not possible during this study as the available electroporator is designed for use with injections in the mouse quadriceps only. However, others have tested the effects of EP in the tibialis anterior. One recent study found that EP did increase immunogenicity of a DNA vaccine given in the tibialis anterior; however it is worth noting that the injection volume used was 20µL which may be sub-optimal [271].

5.3.3. Electroporation can Rescue an 'Early' Boost but is not Required for a successful 'Late' Boost

Although giving a primary pDOM.E7$_{49\text{-}57}$ DNA vaccination with EP into the quadriceps appears to induce fewer E7-specific CD8$^+$ T-cells than an injection of DNA alone into the tibialis anterior, as a mode of boosting EP is very successful (Figure 5.4 and Figure 5.5). This is particularly true when the boost is given at the 'early' time point of 28 days after the primary vaccination, as shown in Figure 5.4. In contrast, an injection of DNA alone into the tibialis anterior fails to boost CD8$^+$ T-cell responses to a high level; confirming previous findings from our laboratory [139]. However, when DNA alone is given as a boost 53 days after priming, it can induce CD8$^+$ T-cell responses at the same level as a DNA boost given in combination with EP (Figure 5.5). Therefore, it seems that a longer rest period between prime and boost improves T-cell responses to the pDOM.E7$_{49\text{-}57}$ DNA vaccine after boosting. The importance of the interval between vaccinations has been documented before, with a greater rest period between DNA vaccinations increasing antibody responses in *Rhesus macaques* [272] and longer intervals between *Listeria monocytogenes* infections also increasing CD8$^+$ T cell-responses in mice [273].

One possible explanation is that an ongoing CTL effector response could limit boosting by attacking APCs bearing the relevant peptide. It has been demonstrated that when peptide-bearing DCs are infused into mice which have been previously immunised against the

same peptide, the DCs disappear from the draining lymph node. Importantly, this effect is more rapid when the DCs are injected during the effector rather than the memory phase of the $CD8^+$ T-cell response; suggesting that the $CD8^+$ T cells do eliminate antigen-presenting DCs [274].

5.3.4. The Low $CD8^+$ T-cell Primary Response to DNA vaccination with EP in the Quadriceps Restricts Secondary Responses

After injection with an optimal volume of pDOM.$E7_{49-57}$, it was clear that stronger $CD8^+$ T-cell responses were induced by vaccination in the tibialis anterior compared to the quadriceps, even if EP was also given. There was also trend towards the development of a larger $CD8^+$ T-cell memory population if the vaccine was given in the tibialis anterior rather than in the quadriceps with EP (Figure 5.5B). However, this difference was not statistically significant. It may be that the tetramer used was not sensitive enough to detect a difference, or that any difference was not quantitative, but qualitative. It is also important to note that memory T-cell responses were analysed in the blood, where effector memory cells will be found; not in the lymph nodes, where central memory T cells reside [267].

By investigating the responses to a booster vaccination, it was possible to expand and thus further assess the memory cells induced by the different protocols. After an 'early' or 'late' boost with EP, far more $CD8^+$ T cells were expanded when the primary vaccination had been given in the tibialis anterior without EP (Figure 5.4 and Figure 5.5 respectively). This indicates that the lower frequency of effector T cells induced by a primary DNA vaccination in the quadriceps with EP during the primary response does limit secondary responses. In fact, despite the low frequency of tetramer $CD8^+$ T cells in terms of absolute numbers; the fold-increase from the primary to the secondary $CD8^+$ T-cell response is no different in mice given DNA alone or DNA with EP at priming followed by DNA with EP at boosting. It is known that the secondary T-cell response develops from the pool of memory cells left after the contraction of the effector phase. As the frequency of memory cells is proportional to the effector cell frequency during viral infections [275], this finding is not unexpected. Even so, the reduced response seen in mice which received both injections in the quadriceps with EP could be exacerbated by the application of EP twice in the same muscle. However, increasing the time interval between immunisations from 28 days (Figure 5.4) to 53 days (Figure 5.5C) does not improve $CD8^+$ T-cell responses in mice which were primed with EP, suggesting that prevailing muscle damage is not a concern. Furthermore, this is supported by the fact that other studies have found that EP-related muscle damage begins to resolve within 14 days [147], [145].

5.3.5. Electroporation and Antibody Production

There was no detectable DOM-specific IgG antibody production after a 10µL pDOM.E7$_{49\text{-}57}$ DNA injection into the quadriceps unless EP was given with the injection (Figure 5.8). This mirrors the T-cell response, again probably due to the low level of inflammation caused by a small injection volume being increased by the addition of EP. A recent clinical trial carried out in prostate cancer patients here in Southampton has shown that EP is safe and well tolerated. Furthermore, after vaccination with a pDOM-epitope vaccine specific for a relevant tumour antigen, patients which also received EP have increased DOM-specific IgG compared to those who received DNA alone initially or throughout [149;276].

What is perhaps more surprising is that in this model, EP does not appear to be of benefit when the pDOM.E7$_{49\text{-}57}$ vaccine was injected into the quadriceps in an optimal 50µL volume; which also follows the same trend as the T-cell response. This does not agree with previous data generated by our lab which showed that EP did increase IgG titres generated by DNA vaccination [139]. One key difference between these two studies is that in the previous study BALB/c mice were used; but in the present study, C57BL/6 mice were used. These two strains are known for their differences in helper T-cell polarisation, evidenced by their responses to the pathogen *Leishmania major*. As a strong T_H1 response is required to clear this intracellular parasite, T_H1-polarised C57/BL6 mice are protected; however BALB/c mice do not produce IFNγ but instead T_H2 cytokines such as IL-4 and -5, rendering them susceptible [277]. As another hallmark of the T_H2 response is antibody production, perhaps in BALB/c mice there is a greater capacity to boost humoral responses but in the C57/BL6 mice used here, the immune response is dominated by cell-mediated immunity.

When using optimal injection volumes, primary DOM-specific IgG titres were greater after vaccination with DNA alone in the tibialis anterior compared to a DNA injection into the quadriceps with EP (Figure 5.9A). This follows the same trend as the $CD8^+$ T-cell response which was stronger after DNA vaccination in the tibialis anterior; indicating that this site is also optimal for the induction of antibody responses. When a primary DNA vaccination given in the tibialis anterior was boosted by another injection into the tibialis anterior or the quadriceps with EP, IgG titres were analogous. However, if both primary and booster vaccinations are given in the quadriceps with EP, DOM-specific IgG was reduced (Figure 5.9B). As T-cell help is crucial to antibody induction by DNA vaccines [136], the reduced frequency of p30-specific T cells after DNA vaccination into the

quadriceps with EP seen in Figure 5.7B may be responsible for the concomitant reduction in DOM-specific IgG.

5.3.6. Conclusions

This study has confirmed that, in the context of a sub-optimal injection volume, EP is crucial in generating both $CD8^+$ T-cell and antibody responses to DNA vaccination in the mouse quadriceps muscle. This is probably due to the additional inflammation it provides. In addition, the data demonstrate that there is an intrinsic difference between the immune responses induced after DNA vaccination in the mouse tibialis anterior versus quadriceps muscles. This can also be explained by the effects of local inflammation at the vaccination site. In the smaller tibialis anterior muscle, more damage is caused by a given injection volume, removing the need for EP. However, when boosting even with the injection being given into the tibialis anterior, DNA alone fails to boost $CD8^+$ T-cell responses as successfully as DNA with EP in the quadriceps. Interestingly, a boost of DNA alone is successful at a later time point, underscoring the importance of the timing of booster vaccinations. Therefore, the recommendations for future DNA vaccine regimens in mice would be to prime with DNA alone in the tibialis anterior and boost with EP; particularly for earlier time points. However, in clinical setting where injection volumes are probably sub-optimal for DNA vaccination, EP should be given with every injection. Early clinical trial data support this, with a protocol of DNA vaccination with EP inducing greater DOM-specific IgG titres than DNA alone, even when given with multiple injections [149].

5.3.7. Future Work

In order to investigate the mechanism which limits T-cell responses to an early pDOM.$E7_{49\text{-}57}$ boost, the phenotype of the T cells present at various time points after the DNA prime could be studied. This would reveal whether there are effector cells present at the time of the early boost, and whether these cells disappear by the time of the late boost. If the hypothesis is correct, this would provide evidence for the effector T cells being the limiting factor for an early DNA boost. During the current study, attempts were made to achieve this but technical issues meant no conclusive evidence was found. Perhaps future refinement of phenotypic markers for memory T cell will facilitate this. To provide more information for translation to the clinic, it may be of benefit to investigate repeated immunisations with sub-optimal injection volumes to understand how boosting works under these conditions. As immunological tolerance exists against many cancer antigens,

vaccination strategies should also be investigated under these circumstances where regulatory T cells may dampen down inflammation.

The discrepancy between vaccine efficacies in the mouse tibialis versus quadriceps muscles demonstrates that the physiology of different muscle sites may vary. These differences are likely to be accounted for at least in part by the overall size, but there may be other factors involved. For example, proximity to the draining lymph node, muscle development and frequency of usage. The differences between mouse and human muscles is likely greater still so it is probably of little value to extend the investigations into the detailed physiology of mouse muscles. Instead, it may be useful to compare DNA vaccinations in different muscle sites in patients. Many vaccinations are given in the deltoid muscle in adult humans, and this was the case in early DNA vaccine clinical trials conducted by our laboratory (A Mander, personal communication; unpublished results). However, more recently EP has been added to the protocol and vaccination with or without EP is now carried out in the quadriceps [149]. It would be interesting to compare the efficacy of DNA vaccination in various muscle sites in humans in order to maximise responses to tumour antigens which are often only weakly immunogenic.

6. Concluding Remarks

Cervical cancer is the second most common cancer in the female population worldwide and HPV DNA is found in almost all cases. According to the World Health Organisation, there are around half a million new cases of cervical cancer every year and over a quarter of a million deaths. HPV is also associated with other anogenital cancers and HPV DNA has been found in other cancers, such as head and neck cancer [179]. The high-risk subtype HPV16 is responsible for 60-70% of cervical carcinomas and >80% of vulval and penile carcinomas caused by HPV [178]. Prophylactic vaccines [203], [204] licensed in the recent past hold great promise for the future but have already been discounted as therapeutic vaccines [205]. Therefore there is a real need for the development of novel therapies. This is especially important as most cervical cancer cases occur in less economically developed countries, where screening programmes and preventative vaccination may not be carried out on a large enough proportion of the population to be effective.

The aim of this study was to develop a therapeutic DNA vaccine against HPV16 E7 antigen. Using a murine model, it has been demonstrated that our DNA vaccines induce $CD8^+$ T-cell responses to the H-$2D^b$-restricted $E7_{49-57}$ epitope and that these T cells are functional as they produce IFNγ and acquire lytic ability. Furthermore, they also develop a memory pool allowing effective boosting. In this model, the full-length gene vaccine is very effective due to an absence of immunological tolerance, T-cell competition and its short length. However shorter versions of the E7 gene including a pDOM-epitope design, which may have certain advantages in a clinical situation, are also able to stimulate a strong $CD8^+$ T-cell response. Importantly, the $CD8^+$ T-cell responses induced by the DNA vaccines described here are significantly stronger than those induced by a published synthetic $E7_{43-77}$ long peptide with CpG, validating our approach. Both pDOM-epitope and full-length E7 gene DNA vaccines confer some level of protection and therapy against challenge with the transplantable E7-expressing tumour TC-1. Surprisingly, $E7_{43-77}$ long peptide with CpG proved to be an extremely effective immunotherapeutic agent against these tumours, despite the low $CD4^+$ and $CD8^+$ T-cell responses induced by it. This indicates that $CD8^+$ T cells are not solely responsible for tumour rejection in this model. In support of this, the rate of tumour growth can be reduced by treatment with CpG alone in naïve mice, suggesting that innate immunity could be involved. This may be linked to the low MHC class I expression of TC-1 which has also been observed here and could promote NK-cell mediated lysis. However another explanation could be that the site of

peptide vaccination is physically much closer to the tumour than the DNA injection site in the muscle. It is obviously important to be aware of which factors operating in mouse models are relevant for the clinical setting and more investigation is required of these variables.

In order to increase the clinical relevance of the findings in wild-type mice, the DNA vaccines were also tested in a tolerised mouse model of cancer. It was demonstrated that E7-specific $CD8^+$ T-cell responses could be generated in this setting. This is encouraging for translation into patients as the persistent HPV infections which are associated with malignant disease may result in immunological tolerance. The E6/E6 transgenic mice used here also develop thyroid hyperplasia leading to spontaneous tumours. For the first time, it was possible to assess the performance of our DNA vaccines as immunotherapy against spontaneous tumour development and the results showed that they did indeed have an effect. Preliminary data provide evidence that $CD8^+$ T cells migrate to the tumour site and therefore are the likely mediators the effect; perhaps in contrast to therapy of TC-1 tumours.

This study has also confirmed that EP is crucial in generating $CD8^+$ T-cell responses to DNA vaccination in sub-optimal conditions. This was demonstrated in the context of smaller injection volumes and premature boosting. The findings also revealed a fundamental difference in vaccination efficacy in two different muscle sites. EP is known to increase inflammatory and immune cell infiltration at the site of injection. Given the requirement for cross-presentation, recruitment of DCs to the site of DNA vaccination is crucial. Therefore, the adjuvant effects of EP in certain conditions are likely to be due to inflammation. In the clinical setting, injection volumes are thought to be sub-optimal, boosting strategies may need to be swift and muscle physiology could be unclear. Therefore, EP should be given with DNA vaccination in order to augment immune responses.

These data add to the growing evidence that therapeutic DNA vaccination is a valid strategy for treating cancers in humans. A recent clinical trial in prostate cancer patients here in Southampton has shown that DNA vaccines are safe and well tolerated, even with the addition of EP. Results showed that DOM-specific IgG was generated and that this was increased in patients which also received EP [149]. Preliminary data on showed that approximately 60% of patients developed tumour-specific $CD8^+$ T-cell responses and that these responses may also have been improved by EP though numbers were small [278].

Based on these findings a new clinical trial in chronic myeloid leukaemia is beginning, with the hope of demonstrating a clinical benefit. As the HLA-A*0201-restricted epitope targeted here using our pDOM-epitope strategy in HHD transgenic mice proved successful, an E7-specific pDOM-epitope vaccine could also potentially be of value in the clinic.

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