NOVEL ADENOVIRUS VACCINE VECTORS

Thesis submitted for the degree of Doctor of Philosophy

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Developing Novel Adenovirus Vaccine Vectors

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Abstract

Replication defective adenoviruses have emerged as promising vectors for delivery of vaccine antigens. The development of new vaccine vectors has recently focused on serotypes to which the human population is less exposed in order to circumvent pre-existing anti vector immunity. This thesis describes the construction and optimisation of ChAdOX1, a new vector based on chimpanzee adenovirus Y25, which has recently been manufactured to clinical grade for a Phase I human trial.

Comparative immunogenicity studies between vectors of different serotype were performed in mice, with careful consideration of the infectious titer of vector preparations, since this parameter can confound studies based solely on viral particle estimation. After intramuscular administration, HAdV-5 (*Human adenovirus C*) based vectors elicited superior transgene product specific T cell and antibody responses compared to a selection of chimpanzee adenovirus vectors (from *Human adenovirus E*) including ChAdOX1.

The construction of ChAdOX1 in a bacterial artificial chromosome (BAC), enabled precise and flexible modification of the genome by recombination mediated genetic engineering (recombineering). Reverse genetics was performed to identify vector determinants of immunogenicity. Chimeric ChAdOX1 vectors were created by replacement of native virus associated RNA (VA-RNA) and fiber sequences with the corresponding sequences from HAdV-5. Using these chimeric vectors, the importance of innate immunity and vector transduction in determining vector immunogenicity was investigated. Though the mechanisms responsible ultimately remain unclear, superior transgene product specific immune responses with HAdV-5 correlated with higher levels of transgene expression *in vivo* after vector administration. The current study has conclusively demonstrated that neither VA-RNA sequences, nor the fiber protein, are responsible for differences in immunogenicity between vectors, contrary to hypotheses based on previous studies.

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Abbreviations

Ab	Antibody
ACK	Ammonium chloride potassium
ADCC	Antibody-dependent cellular cytotoxicity
HAdV	Human adenovirus
APC	Antigen presenting cells
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
CD	Cluster of differentiation
ChAd	Chimpanzee adenovirus
CXCR	Chemokine receptor
DC	Dendritic cell
DLN	Draining lymph node
DMSO	Dimethyl Sulfoxide
ds	Double stranded
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ELISpot	Enzyme-linked immunosorbent spot assay
FcR	Fc Receptor
FP9	Fowlpox strain virus FP9
g	The acceleration due to gravity
GC	Germinal Centre

GFP	Green fluorescent protein
GSK	Glaxo Smith Kline
GST	Glutathione S-transferase
h	Hour
HIV	Human immunodeficiency virus
i.m.	Intra-muscular
i.p.	Intra-peritoneal
i.v.	Intra-venous
IC	Immune complex
ICS	Intracellular cytokine staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ifu	Infectious units
kb	Kilobase
kDa	Kilodalton
KO	Knock-out
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
mBC	Memory B cell
ME	Multi epitope string
MEM	Minimum essentials medium

MHC	Major histocompatibility complex
MVA	Modified vaccinia virus Ankara
NLR	NOD-like receptors
NYVAC	New York vaccinia virus
OD	Optical density
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS/T	0.05% Tween 20 in phosphate buffered saline
PCR	Polymerase chain reaction
P:I ratio	Viral particles : infectious units ratio
pfu	Plaque forming units
Poly (I:C)	Polyinosinic–polycytidylic acid
PRR	Pattern recognition receptors
RLR	RIG-1-like receptor
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Room temperature
SAdV	Simian adenovirus
s.c.	Subcutaneous
sec	Second
SCS	Subcapsular sinus
SFC	Spot forming cell
ss	Single-stranded

TB	Tuberculosis
TCR	T-cell Receptor
T_{FH}	T-follicular helper cell
T_H	T helper cell
TIP	Synthetic polyepitope string
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAP	Thrombospondin-related adhesion protein
wt	Wild type
BM-DC	Bone marrow dendritic cell
GM-CSF	Granulocyte macrophage colony stimulating factor
Y25	Chimpanzee adenovirus Y25
T_{EFF}	Effector T cell
T_{EM}	Effector memory T cell
T_{CM}	Central memory T cell
M1	Influenza A Matrix Protein 1
NP	Influenza A Nucleoprotein
ANOVA	**AN**alysis **O**f **VA**riance
EC_{50}	Half maximal effective concentration

Papers Published from this Thesis

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1 Introduction

1.1 Aims of this study

The principal aim of this study was to generate a novel replication defective adenovirus vaccine vector, with low human seroprevalence, for clinical application. The laboratory had recently obtained a sample of the chimpanzee adenovirus isolate, Y25, from Prof. Göran Wadell at Umeå University in Sweden. The initial challenge was to sequence the virus and generate a genetically stable replication defective vector, which could be amplified to high titer for clinical manufacture.

Once constructed, the potential of the new vector to elicit transgene product specific immune responses was assessed in mice and compared to existing vectors in current clinical use. It has been established that adenovirus vectors of different serotype may differ considerably in potency, though the mechanisms that govern these differences remain poorly understood. The current pre-clinical study attempts to address this important question, with a focus on immune responses after intramuscular injection since this is the primary route of administration for clinical studies conducted by the laboratory. Using a reverse genetics approach, the molecular determinants of adenovirus vector immunogenicity are explored.

1.2 The Global Importance of Vaccines

Since its birth in the late eighteenth century, vaccination has arguably become the most effective tool for prevention of infectious disease. Vaccines have saved the lives of millions during the past two centuries; significantly reducing morbidity and mortality from infectious disease, particularly in the developed world.

Edward Jenner first coined the term 'vaccination' in the late 1790's when he showed that protection against smallpox could be conferred by subcutaneous inoculation of naïve individuals with pus from an infection with the related but less virulent cow-pox virus [1]. Indeed, the term 'vaccination' is derived from the Latin name for cow-pox, *Variola vaccinia*. Vaccination was a significant advance from the prior practice of variolation, which first became widespread in Europe during the eighteenth century and involved the subcutaneous transfer of live smallpox from infected to uninfected persons. Though this procedure had a lower fatality rate compared to natural infection, 3% of variolated individuals died from the disease, became the source of another epidemic, or suffered from diseases such as tuberculosis and syphilis transmitted by the procedure itself. Edward Jenner's safer 'vaccination' approach culminated in the global eradication of smallpox by 1980; among the greatest public health achievements to date [2].

The principle of vaccination has since been used to target a multitude of other infectious pathogens. The global eradication of a second pathogen, rinderpest, an ancient viral disease of cattle that has preceded famines in much of Africa, Asia and Europe, was confirmed last year [3]. In addition, the global disease burden of many other human pathogens has been significantly reduced.

Global incidence of poliomyelitis has reduced by over 99% since the World Health Organization (WHO) launched the Global Polio Eradication Initiative in 1988. Diseases including diphtheria, measles, *Haemophilus influenza* type b (Hib), mumps, rubella, yellow fever, and hepatitis A and B have all been controlled, at least in some regions, by effective vaccination programs [4].

However, despite these successes, major infectious diseases including malaria and human immunodeficiency virus / acquired immunodeficiency syndrome (HIV/AIDS) have been refractory to traditional vaccination approaches. In addition, many existing vaccines, such as the Bacillus Calmette-Guérin (BCG) vaccine against *Mycobacterium tuberculosis*, have variable global efficacy [5]. The vast majority of currently licensed vaccines are based on closely related, live attenuated or inactivated whole pathogens. However, in the case of malaria, the multistage lifecycle of the parasite, inter-strain polymorphism, and the challenge of *in vitro* parasite culture have made this approach difficult [6]. The development of novel vaccine strategies and platforms is therefore the focus of current research in the fight against infectious disease.

1.3 Mechanisms of Vaccine Immunity

The vast majority of vaccines in use today have been developed empirically; using a similar experimental approach as the one used by Jenner over two centuries ago. Recently however, vaccine development has shifted to a more rational approach based on an increasingly detailed understanding of microbial pathogenicity and the host response to infection. Advances in molecular microbiology have enabled selection of specific antigenic targets within a pathogen that are most susceptible to host immunity. This, together

with the birth of recombinant DNA technology, has lead to the development of subunit vaccines focused on specific microbial components rather than the entire organism. The immunological mechanisms responsible for the protection afforded by our current vaccines are beginning to be revealed. Most current vaccines confer protection through humoral antibody mediated immunity [7]. However, for some intracellular vaccine targets including liver stage malaria, HIV, and tuberculosis, a robust cytotoxic CD8+ T cell response is likely to be important for protection [8]. Novel vaccine platforms, including recombinant adenovirus vectors, have therefore been designed to target both cellular and humoral arms of the adaptive immune response. In addition, an appreciation of the importance of innate immunity in tailoring the adaptive response has enabled protective cellular and humoral responses to be enhanced through the use of rationally designed adjuvants [9]. An overview of our current understanding of the host immune response is described in this section.

1.3.1 Innate Immunity

The innate immune system is the first line of defense against invading pathogens. Innate immune mechanisms act immediately and have broad specificity, enabling resistance against a wide range of pathogens. Pattern recognition receptors (PRRs) on innate immune cells recognise pathogen associated molecular patterns (PAMPs) associated with infectious agents, enabling discrimination of pathogens from self antigens. These receptors are germline encoded, and innate immune cells do not therefore require clonal expansion to respond to infection, unlike the canonical T and B lymphocytes of adaptive immunity. Innate mechanisms can therefore respond to an infection

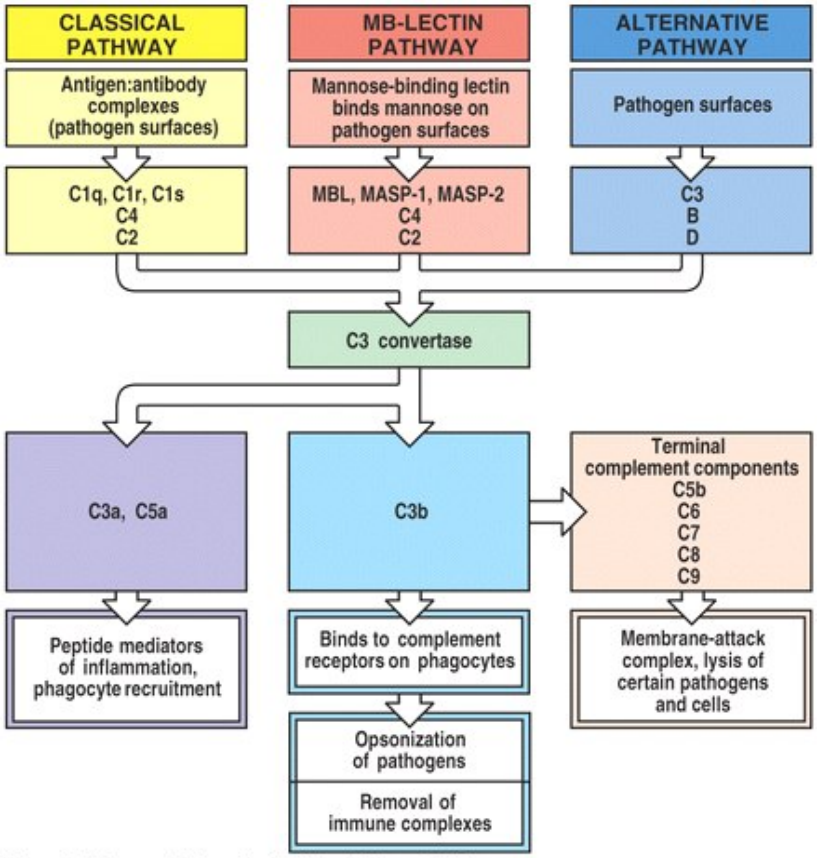
within hours, in contrast to the 3-4 days required for the initiation of adaptive responses. However, innate immune responses lack the fine specificity and immunological memory afforded by adaptive immunity. Vaccination therefore requires engagement of adaptive immunity, but stimulation of the innate response is critical in initiating and tailoring the adaptive response to effectively respond to a specific pathogen. The interplay between innate and adaptive immunity in the context of recombinant adenovirus vector administration is an important topic in this study, and the specific focus of Chapter 6.

1.3.1.1 The Complement System

The complement system comprises a range of plasma proteins which, when activated on a pathogen surface, react with one another eventually culminating in opsonisation of the pathogen and initiation of an inflammatory response. The system is activated through a triggered proteolytic cascade. Many complement proteins are zymogens; proteases which are themselves activated by proteolytic cleavage. There are three main pathways of complement activation, each leading to the activation of the C3 convertase, which initiates the effector functions of the cascade [10,11] (Figure 1.1). The 'classical' pathway is initiated by the accumulation of complement protein C1q on the surface of the pathogen. C1q may bind directly to polyanionic components of bacterial cell walls, via the C-reactive protein to phosphocholine residues on bacterial polysaccharides, or become activated upon interaction with antibody-antigen complexes. The mannose binding lectin (MBL) pathway is activated upon binding of the MBL serum protein to mannose residues on the surface of microorganisms. The alternative pathway is initiated through binding of spontaneously activated C3 protein to the surface of a pathogen. Activation of the

complement cascade can initiate a number of effector functions to combat invading pathogens. The activated C3 convertase, cleaves the central complement protein C3 into C3a and C3b. C3a is a peptide mediator of inflammation, leading to phagocyte recruitment to the site of infection (see Section 1.3.1.2). C3b binds to complement receptors on phagocytes, triggering the opsonisation of pathogens. Complement receptors are also important in the trafficking and presentation of antigen to B cells (Section 1.3.2.2). Complement receptor CD21, expressed on B cells, acts a co-stimulatory molecule and triggers B cell receptor clustering and activation. C3b can also initiate the assembly of a 'membrane attack complex' composed of complement proteins C6-C9 on the surface of selected pathogens such as the bacterium *Neisseria*, the causative agent of gonorrhea [12]. The complex forms a channel spanning the bacterial membrane, leading to cell lysis.

Figure 1.1: Overview of the complement system. From Janeway *et al*, Immunobiology 6th Edition, 2005 [11]



1.3.1.2 Cellular Innate Immunity

Macrophages, neutrophils and dendritic cells are the phagocytic cells of the innate immune system. They recognise pathogens by virtue of cell surface receptors and internalize them by phagocytosis into phagosomes. These endocytic vacuoles become acidified, and may fuse with lysosomes containing anti microbial peptides, proteases and reactive oxygen species, which destroy the pathogen. Upon phagocytosis, macrophages and neutrophils may also convert molecular oxygen to the superoxide ion O_2- via NADPH oxidase, which is subsequently converted to hydrogen peroxide and other toxic by-products in a process termed the respiratory burst [13].

Tissue resident macrophages are typically the first leukocytes to encounter pathogens that have crossed the physical barrier of the surface epithelia. In addition to pathogen clearance, macrophages become activated upon pathogen recognition by pattern recognition receptors (PRRs) (see Section 1.3.1.3) and secrete pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, and IL-12. TNF-α secretion leads to vasodilation and increased vascular permeability, increasing the entry of IgG antibodies, complement proteins, and leukocytes to the site of infection. Fluid drainage to lymph nodes is also increased, enhancing the efficiency of antigen presentation to lymphocytes for initiation of adaptive responses. TNF-α and IL-1β also induce activation of the vascular endothelium to up-regulate surface molecules E-selectin and ICAM-1, which in combination with the chemo-attractant cytokine CXCL-8 promote rapid recruitment of neutrophils, and later lymphocytes, to the site of infection [14]. Neutrophils are recruited in large numbers, and are short lived. Their primary function is in endocytosis and

pathogen clearance. Dendritic cells are specifically adapted for antigen presentation to lymphocytes and will be discussed in section 1.3.1.3.

Other leukocytes of the innate immune system are non-phagocytic. Eosinophils and basophils degranulate rapidly in response to immunoglobulin E (IgE) immune complexes on the surface of parasites, releasing lytic mediators to destroy the pathogen. Basophils are the least abundant of the circulating granulocytes and are phenotypically similar to tissue resident mast cells. They also contribute to the inflammatory response by promoting vasodilation through the secretion of histamine.

Natural killer (NK) cells are derived from the common lymphoid progenitor and perform a role analogous to that of cytotoxic T cells in the adaptive immune response; initiating perforin and granzyme mediated lysis of virally infected cells [15]. However, unlike T and B lymphocytes they do not require antigen presentation and clonal expansion, and can therefore act rapidly to control viral infection prior to the onset of adaptive immunity. NK cells are believed to recognise cells infected by viruses primarily by the reduced expression of MHC class I molecules on the cell surface; viruses commonly downregulate MHC class I expression on host cells as an immune evasion strategy to avoid recognition by cytotoxic T lymphocytes [16]. The cytotoxic activity of NK cells is stimulated by macrophage derived cytokines such as IL-12 and the secretion of antiviral type I interferon (IFN-α or IFN-β) by infected cells. NK cell mediated cytotoxicity can also be activated by engagement of FcγRIII (CD16) with IgG immune complexes on the surface of infected cells; this mechanism is termed antibody-dependent cellular cytotoxicity (ADCC). In addition, NK cells produce large amounts of IFN-γ in response to

proinflammatory cytokines IL-12 and TNF-α. Secretion of IFN-γ by NK cells prior to the onset of adaptive immunity is important for the control of certain infections such as *Listeria monocytogenes* [17].

Several minor lymphocyte subpopulations display semi-invariant receptor diversity, limited gene rearrangement, and do not undergo clonal expansion in response to infection. These lymphocyte subsets, which include γ:δ T cells, natural killer T cells (NKT) cells, and B1 cells therefore act essentially as innate cells, and are termed innate-like lymphocytes. B1 lymphocytes can rapidly and independently generate functional but low affinity IgM antibodies, particularly to bacterial capsular polysaccharide antigens, prior to the onset of adaptive immunity. [18]. NKT cell receptors are comprised of an invariant α chain and one of three variable β chains, and recognise glycolipid antigens presented by CD1d molecules [19]. Activated NKT cells secrete both pro- and anti-inflammatory cytokines including IL-4, IL-10 and IFN-γ. The γ:δ T cell subset displays an alternative T cell receptor composed of a γ and δ chain as opposed to the α and β chains of conventional T lymphocytes. The biological function and receptor diversity of γ:δ T cells appears to vary with tissue distribution, and is not fully understood [20]. They have been implicated in the recognition of non-peptide phospho-antigens, particularly those present on mycobacteria [21,22]. Upon activation, γ:δ T cells secrete proinflammatory cytokines, and may also play a role in antigen presentation to T lymphocytes [23].

1.3.1.3 Dendritic cells and the initiation of adaptive immunity

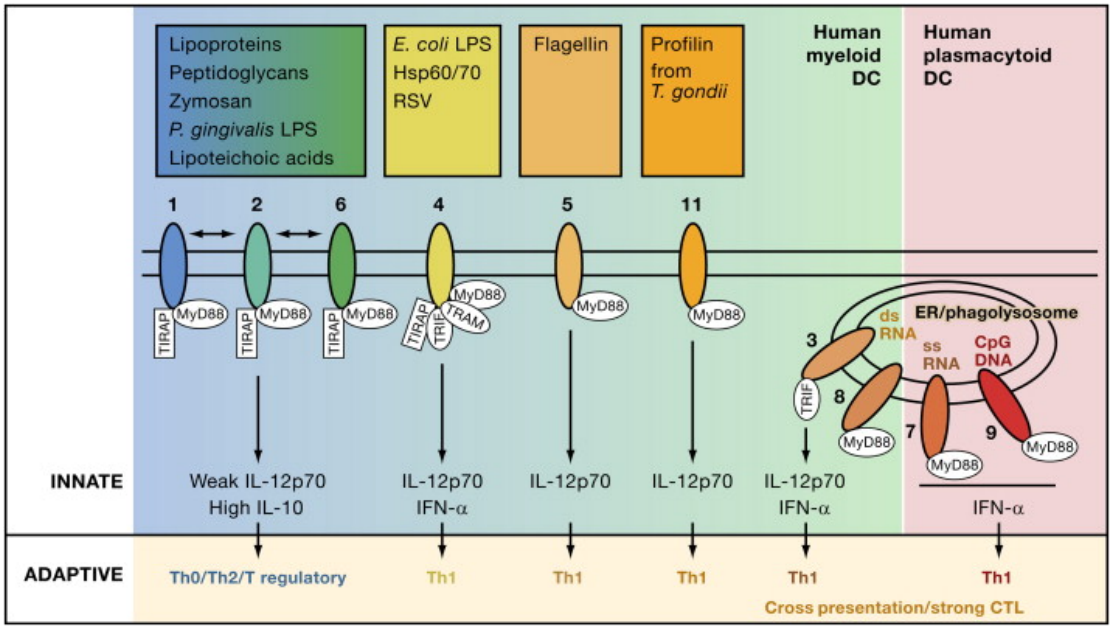
Dendritic cells (DCs) are professional antigen presenting cells; their primary function is to present antigen to T lymphocytes, initiating the onset of adaptive immunity. Understanding the mechanisms through which DCs capture

and present antigen after adenovirus vector administration will be essential for the development of more efficacious vaccine vectors. DCs arise from two distinct haematopoetic lineages; myeloid and plasmacytoid. Myeloid (or conventional) DCs respond to a wide range of microbial stimuli and have been further divided into additional phenotypic subsets including skin resident Langerhans cells, and BDCA-3+ DCs (similar to the CD8α+ subset in mice) which are particularly efficient at pro-inflammatory cytokine secretion and cross presentation of exogenous antigen to CD8+ T cells (see Section 1.3.2) [24]. Plasmacytoid DCs are important mediators of antiviral immunity, secreting large amounts of type I interferon upon activation [25]. Both myeloid and plasmacytoid DCs are proficient at sampling the environment for non-self antigen. Dendritic cells are highly phagocytic and also ingest extracellular antigen non-specifically by macropinocytosis.

DCs, like macrophages, recognise pathogen associated molecular patterns (PAMPS) through a wide range of pattern recognition receptors (PRRs). Perhaps the best characterized of the non phagocytic PRRs are the Toll-like-receptor (TLR) family [26]. These type I membrane glycoprotein receptors are differentially expressed on a variety of innate immune cells, including macrophages, dendritic cells, and B cells, and even on some non-immune fibroblast and epithelial cells. The TLRs recognise a remarkably broad range of structurally unrelated ligands (Fig. 1.2). TLRs may be expressed on the cell surface (TLR 1,2,4,5,6) for detection of extracellular microbial components, or intracellularly (TLR 3,7,8,9) for detection of pathogens within endosomal compartments. The functional differences between myeloid and plasmacytoid DCs are reflected in their differential expression of TLR molecules, with

plasmacytoid DCs expressing TLR7 and TLR9 which recognise viral nucleic acid (Figure 1.2). In addition to the TLRs, two cytosolic PRR families the NOD-like receptors (NLRs) and the RIG-1 like receptors (RLRs) have recently been discovered [27] [28]. These PRRs are particularly important in the recognition of intracellular bacteria and viruses, including adenovirus (Section 1.4.2).

Figure 1.2 – Differential DC expression and subcellular location of the Toll-like receptors. Stimulation of specific TLRs leads to induction of distinct DC cytokine profiles and subsequent polarisation of the adaptive immune response. (From Pulendran and Ahmed, 2006 [29])



Signaling through PRRs stimulates dendritic cell maturation via activation of the transcription factor NF-κB. Mature dendritic cells upregulate expression of MHC and co-stimulatory molecules (CD80/CD86) for efficient antigen presentation and activation of naïve T lymphocytes. Migration of DCs from the periphery to secondary lymphoid organs, the site of naïve T-cell priming, is facilitated by upregulation of chemokine receptor 7 (CCR7). In addition, proinflammatory (IL-12, IFN-α) or antinflammatory (IL-4, IL-10) cytokines are secreted; the nature of these cytokines is dependent upon the PRR engaged and hence the nature of the pathogen stimulus (Figure 1.2). These cytokines act to polarize the T helper adaptive response towards a T_H1 or T_H2 bias (see Section 1.3.3.3). Adaptive immunity is therefore tailored by the nature of the innate stimulus in order for an appropriate response to be mounted.

1.3.2 Antigen processing and presentation

1.3.2.1 Presentation to T lymphocytes

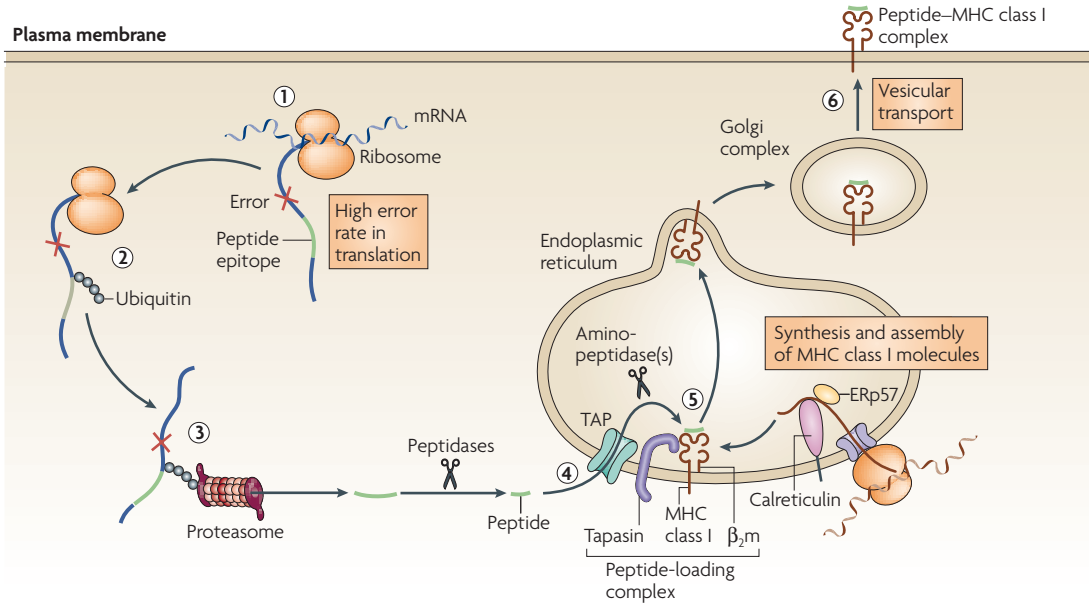
Conventional T lymphocytes recognise peptide antigen displayed on the surface of antigen presenting cells on major histocompatibility complex (MHC) molecules. There are two classes of MHC molecules that display peptides derived from two topologically distinct intracellular compartments. MHC class I molecules, expressed on all nucleated cell but most abundant on haematopoietic cells, display endogenous peptides derived from the cytosol and nucleoplasm. Antigens presented on MHC class I are recognized by cytotoxic CD8+ T lymphocytes which become activated to destroy the infected cell. MHC class II molecules are expressed only on professional antigen presenting cells including

dendritic cells, macrophages and B cells. They present antigen within vesicular compartments (such as endosomes and lysosomes) to CD4+ T lymphocytes. Antigen presented on MHC class II molecules may enter vesicles through endocytosis, phagocytosis or macropinocytosis from the extracellular environment.

The classical MHC class I processing pathway is shown in Figure 1.3 [30,31]. Cytosolic peptides (often defective ribosome products from rapid viral transcription [32]) are targeted by ubiquitination for proteasome degradation. T lymphocytes and NK cells activated during infection secrete IFN-γ, which together with production of type I IFN from virally infected cells, leads to a change in conformation of the proteasome. This new conformation, termed the immunoproteosome, has different cleavage specificity and generates antigenic peptides of optimum size (8-10 amino acids) and nature for high affinity interaction with MHC class I molecules. These peptides are transported into the lumen of the endoplasmic reticulum (ER) by the TAP transporter for MHC class I assembly. In the ER lumen, further trimming of peptides may occur by aminopeptidases such as endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP), also upregulated by IFN-γ [33]. MHC class I molecules are dimers, comprised of a variable α chain and a smaller invariant β2-microglobulin chain. Newly synthesized MHC class I α chains assemble in the ER in association with a chaperone protein, calnexin. Upon binding to the β2-microglobulin subunit, calnexin dissociates and the partially folded MHC molecule binds to the TAP associated chaperone protein tapasin. Further chaperone proteins including calreticulin and ERp57 form a peptide loading complex which stabilizes the peptide:MHC complex. High affinity peptide:MHC

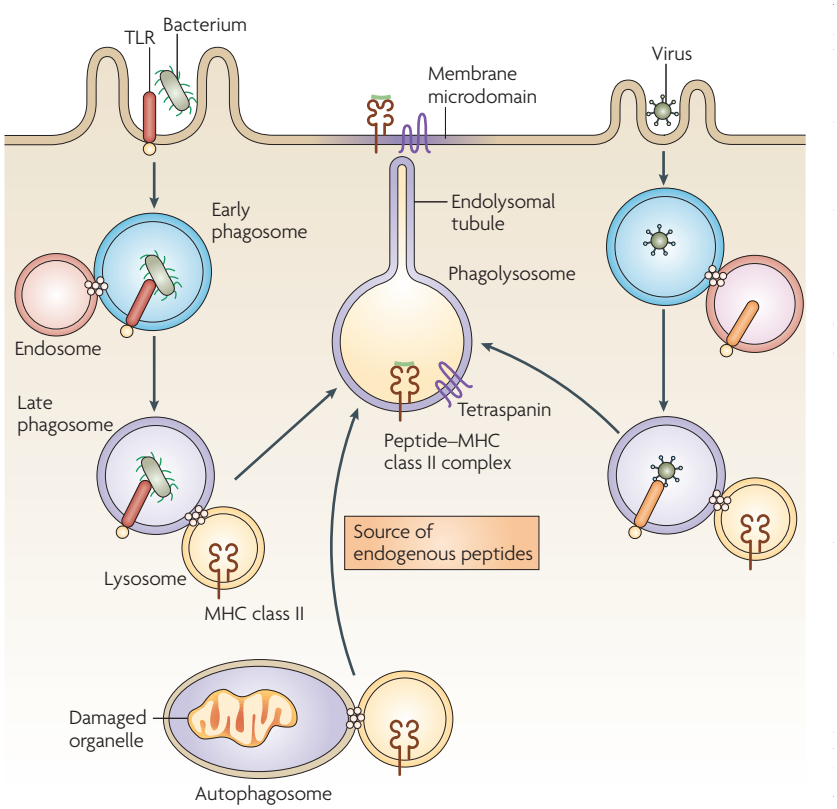
complexes are transported via the Golgi apparatus to the cell surface for surveillance by CD8+ T cells .

Figure 1.3: Processing and presentation of endogenous peptide on MHC class I molecules. From Vyas *et al* 2008 [31]



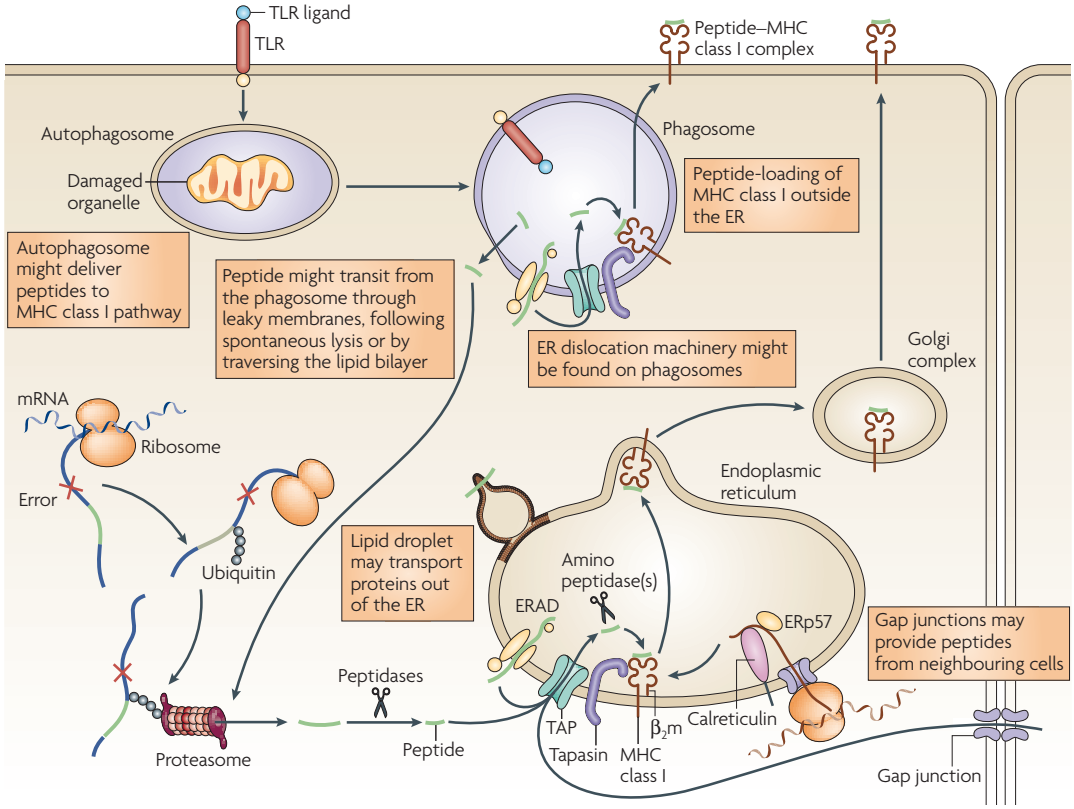
The classical MHC class II pathway is shown in Figure 1.4 [30,31]. In the endocytic pathway, peptide antigens are degraded by acid proteases of the cathepsin family within endosomes. Intramolecular disulphide bonds are reduced by the gamma inducible lysosomal thiol reductase (GILT). Peptide antigens are introduced to MHC class II molecules by fusion of late phagosomes with MHC containing vesicles. Newly synthesized MHC class II heterodimers are prevented from binding endogenous misfolded polypeptides in the ER lumen by association with an invariant chain peptide (Ii). Upon fusion with phagolysosomes, cathepsin S degrades the invariant chain leaving a small fragment (termed CLIP) remaining in the MHC peptide-binding groove. An accessory protein, HLA-DM, similar in nature to MHC class II, catalyses the release of the CLIP fragment in exchange for exogenous peptide antigens. Peptide:MHC class II complexes of sufficient affinity are translocated to the cell surface for presentation to CD4+ T cells.

Figure 1.4: Processing and presentation of exogenous peptide on MHC class II molecules. From Vyas *et al* 2008 [31]



There is a degree of plasticity in this system. Mechanisms have evolved to enable presentation of antigens from vesicular compartments on MHC class I molecules; a process known as cross presentation. Cross presentation, a unique property of dendritic cells, enables the induction of CD8+ T cell responses against viruses and other intracellular pathogens that have not directly infected an antigen presenting cell. However, the mechanisms through which cross presentation occur remain controversial, and are discussed in Figure 1.5 [31,34]. In addition, autophagy has been implicated in the reverse process; presentation of endogenous antigens on MHC class II molecules (Figure 1.4).

Figure 1.5: Potential mechanisms of cross presentation. From Vyas *et al* 2008 [31]



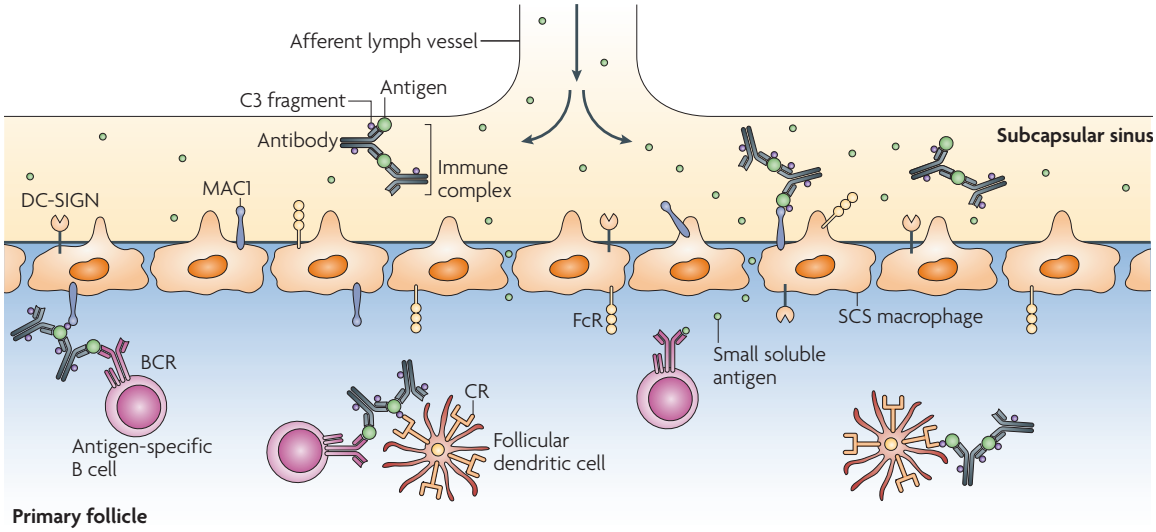
1.3.2.2 Presentation to B lymphocytes

B cell receptors (BCRs) recognise antigen in its native form. BCR engagement and subsequent activation of naïve B cells occurs in secondary lymphoid organs such as the lymph nodes and spleen. Small soluble antigens may initiate B cell responses through direct diffusion into lymphoid tissue, passing through pores in the sub capsular sinus to the B cell follicles [35]. However, membrane associated antigen presentation via macrophages, dendritic cells and follicular dendritic cells is believed to be the primary mechanism of B cell priming [36] (Figure 1.6). An immunological synapse is formed, as in T cell priming, between the antigen-presenting cell (APC) and the B lymphocyte [37]. Formation of the synapse promotes receptor clustering and activation, and is also believed to stimulate antigen internalization for presentation on MHC class II to enable CD4+ T cell help [38].

Macrophages in the sub-capsular sinus are phenotypically distinct; they display reduced phagocytic activity and are thus able to retain antigen in native conformation on the cell surface. Complement receptors such as MAC1 have been implicated in surface retention of antigen [39]. These sub capsular sinus macrophages have recently been implicated in the direct presentation of large particulate antigens and immune complexes to follicular B cells [39,40]. Recently migrated dendritic cells in high endothelial venules (HEVs) of secondary lymphoid organs have also been implicated in presentation of antigen to B cells as they enter the lymph nodes [41]. Dendritic cells in HEVs express receptors FcγRIIB and DC-SIGN, which mediate the internalisation of IgG immune complexes and carbohydrate antigens respectively into non degradative

endosomes for recycling to the cell surface [41]. Classically, follicular dendritic cells (FDCs) have been considered the most important presenters of antigen to B cells. These cells are non-haematopoetic, originating from mesenchymal cells, and thus represent a population entirely distinct from classical DCs. They are non migratory, residing in B cell follicles of secondary lymphoid organs. FDCs express high levels of complement receptor 1 and 2 for retention of complement coated antigen. They also express Fc receptors for presentation and accumulation of immune complexes [42]. Indeed, FDCs are believed to act as 'antigen-depots' within germinal centres, aiding affinity maturation of B cell clones [43]. Marginal zone (MZ) B cells, which display characteristics of both between innate and adaptive immunity, are believed to be responsible for transporting complement coated antigen across the sub capsular sinus to FDCs.

Figure 1.6: Antigen presentation to B cells. From Batista *et al*, 2009 [44].



1.3.3 Adaptive Immunity

Conventional T and B lymphocytes represent the cellular and humoral arms of the adaptive immune response respectively. In contrast to cells of the innate response, which respond to a wide range of stimuli, T cell receptors (TCRs) and B cell receptors (BCRs) respond to antigens of a single unique specificity. During lymphocyte development, these receptor specificities are generated by somatic DNA recombination events between a series of gene segments; a process termed V(D)J recombination. This process generates staggering combinatorial diversity; estimates predict there could be as many as 10^8 different TCR specificities in every human individual [45]. Upon engagement of the TCR or BCR with antigen of the required specificity, lymphocyte activation stimulates clonal expansion and differentiation into effector cells. Importantly, a significant subset of these antigen experienced lymphocytes persist after antigen has been eliminated. These 'memory' lymphocytes ensure a more rapid and effective response upon secondary encounter with the pathogen, and form the basis of immunological memory.

1.3.3.1 T lymphocytes

Naïve T lymphocytes are primed in secondary lymphoid organs in response to the presentation of specific peptides on MHC molecules (Section 1.3.2) by antigen presenting cells. However, engagement of the TCR with peptide:MHC complexes must be accompanied by a second co-stimulatory signal from the same APC before activation of naïve T cells may occur. APCs stimulated by pathogen recognition upregulate co-stimulatory glycoproteins CD80 and CD86 which engage the CD28 receptor on T cells to provide this second

activation signal. Binding of peptide:MHC complexes to the TCRs of naïve T cells in the absence of co-stimulation induces T cell anergy; a mechanism to prevent autoimmunity. This highlights the importance of innate stimulus for adaptive immunity; an essential consideration in vaccine design. Activated T cells upregulate additional molecules that sustain or modify co-stimulatory signals with the APC including CD40 ligand, and 4-1BB. IL-2 secretion provides an autocrine signal to drive T cell clonal expansion and differentiation into effector cells (Sections 1.3.3.2 and 1.3.3.3). After pathogen clearance, most effector T cells are eliminated by apoptosis; termed the contraction phase of the response. However, a population of antigen experienced memory T cells remains, expressing the IL-7 receptor to promote survival. These cells can be further classified into two distinct subsets; effector memory T cells (T_{EM}) and central memory T cells (T_{CM}). T_{CM} cells express lymphoid homing markers (CCR7 in humans, CD62L in mice), and rapidly proliferate in response to receptor crosslinking. T_{EM} cells can perform more immediate effector functions than T_{CM} cells but lack the proliferative capacity of T_{CM}. An effective T cell response will require a different balance of T_{EFF}, T_{EM} and T_{CM} induction for different pathogens, an important consideration for vaccine development (Section 4.2.3).

1.3.3.2 CD8+ T lymphocytes

Presentation of antigen on the surface of infected cells by MHC class I activates cytotoxic CD8+ T lymphocytes to destroy the infected cell. Effector CD8+ T cells mediate cell death through the release of lytic granules containing perforin and granzyme proteases. Perforin monomers polymerise within the membrane of target cells to create a pore in the lipid bilayer, promoting cell lysis.

CD8+ T cells also promote apoptosis of target cells through upregulation of Fas ligand. Activated CD8+ T cells also secrete cytokines including IFN-γ and TNF-α. TNF-α activates macrophages and promotes inflammation as described in Section 1.3.1. IFN-γ is a principal cytokine of the immune response, performing a vast and diverse range of functions [46]. These include i) upregulation of multiple components of the MHC class I and II antigen presentation pathways; ii) induction of antiviral components including upregulation of PKR and ADAR (Section 6.1); iii) maintenance of the inflammatory response and recruitment of macrophages and leukocytes to sites of infection; iv) activation of antimicrobial mechanisms within phagocytes including nitric oxide synthesis and production of reactive oxygen species; v) upregulation of Fc receptors and complement pathways vi) upregulation of co stimulatory molecules including CD86; vii) induction of T_H1 immunity (Section 1.3.3.3) [46]. Due to the central importance of IFN-γ for CD8+ T cell effector function, enumeration of vaccine antigen specific IFN-γ secreting T cells has become a universal readout for T cell responses in vaccine studies.

1.3.3.3 CD4+ T lymphocytes

CD4+ T lymphocytes act primarily by activating and augmenting the functions of other cell types; for this reason they have been termed 'helper' T cells. Indeed, CD4+ T cell involvement can be required in naïve CD8+ T cell priming if the antigen presenting cell displays a low abundance of co-stimulatory molecules [47]. Upon activation, CD4+ T cells can differentiate into a variety of subsets with different phenotypic and functional characteristics. Classes of CD4+ T helper cell include T_H1, T_H2, T_H17, regulatory T cells (T_{reg}) and T follicular

helper (T_{FH}) cells. Class selection is dependent upon the local environment and cytokine milieu during clonal expansion, and hence influenced by components of both innate and adaptive immunity (Figure 1.2).

Differentiation to T_H1 is induced by pro-inflammatory cytokines including IL-12, IFN-γ, and type I IFN, through activation of the transcriptional regulator T-bet [48]. T_H1 cells activate microbicidal effector mechanisms in macrophages by secretion of IFN-γ, TNF-α and co-stimulation via CD40 ligand. They recruit macrophages and NK cells to sites of infection and also stimulate phagocyte differentiation in the bone marrow by secretion of IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). T_H1 cells also secrete IL-2, inducing T lymphocyte proliferation and hence increase the effector T cell population. T_H1 responses involving secretion of IFN-γ induce B cells to express powerfully opsonising and complement fixing IgG antibody subtypes.

Differentiation to T_H2 is induced in the presence of IL-4, through activation of transcriptional activator GATA-3 [49]. T_H2 cells are important during parasitic helminth infections and secrete IL-4, IL-5, and IL-13, which induce IgE production and eosinophil mediated pathogen clearance [50].

Regulatory T cells (T_{regs}) suppress excessive or chronic immune responses and hence prevent immune pathology. They secrete immunosuppressive cytokines IL-10 and TGFβ. The mechanism of T_{reg} development is not presently clear, but it appears to involve activation of the transcription factor Forkhead box protein 3 (Foxp-3)[51].

Differentiation to T_H17 is induced by cytokines IL-1β and IL-6, through activation of the transcription factor ROR-γ-t. T_H17 cells secrete the highly pro-inflammatory cytokines IL-17 and IL-22. T_H17 cells have been shown to be

important for the clearance of certain pathogen such as *Candidia albicans*, but the broad receptor distribution of IL-17 and IL-22 can lead to extensive tissue inflammation and autoimmune responses upon T_H17 activation [52].

T follicular helper (T_{FH}) cells reside in the B cell follicles of secondary lymphoid organs and act as specialized providers of B cell help (Section 1.3.3.4). Differentiation is induced by activation of transcriptional regulator Bcl6, and identification of T_{FH} is primarily by high expression of chemokine receptor CXCR5. T_{FH} are important in establishing germinal centre formation and class switching of B cells and can assume characteristics of other T helper subsets, though the precise mechanisms that underpin these processes remain unclear [53].

1.3.3.4 B lymphocytes and antibodies

Conventional B cell lymphocytes (also termed B-2 cells, or follicular B cells) represent the humoral arm of the adaptive immune response, initiating the production of antibodies (secreted forms of the BCR) in response to infection. Presentation of antigen to B cells by sub capsular sinus macrophages and follicular DCs simulates internalization of antigen by BCRs for presentation on MHC class II molecules (Section 1.3.2.2 and Figure 1.5). Activated B cells migrate from follicles to the border between B and T cell zones of secondary lymphoid organs. Here, CD4+ T cells with receptor specificity for the same antigen engage with displayed peptide:MHC complexes and deliver activating signals to the B cell to promote proliferation and differentiation into effector cells. These signals include interaction between CD40, CD30, 41BB and ICOS (inducible co-stimulator) and their cognate ligands.

The requirement for linked T and B cell recognition helps to ensure self tolerance, and has important consequences for vaccine design. It enables humoral responses to poorly immunogenic or non-peptide antigens to be augmented by T cell responses to a different antigen by physically combining the two. This approach has been used in the development of the *Haemophilus influenza* type b (Hib) conjugate vaccine, where the capsular polysaccharide of Hib has been chemically liked to the tetanus toxoid, a protein against which children are routinely vaccinated and elicit robust T cell responses [54,55].

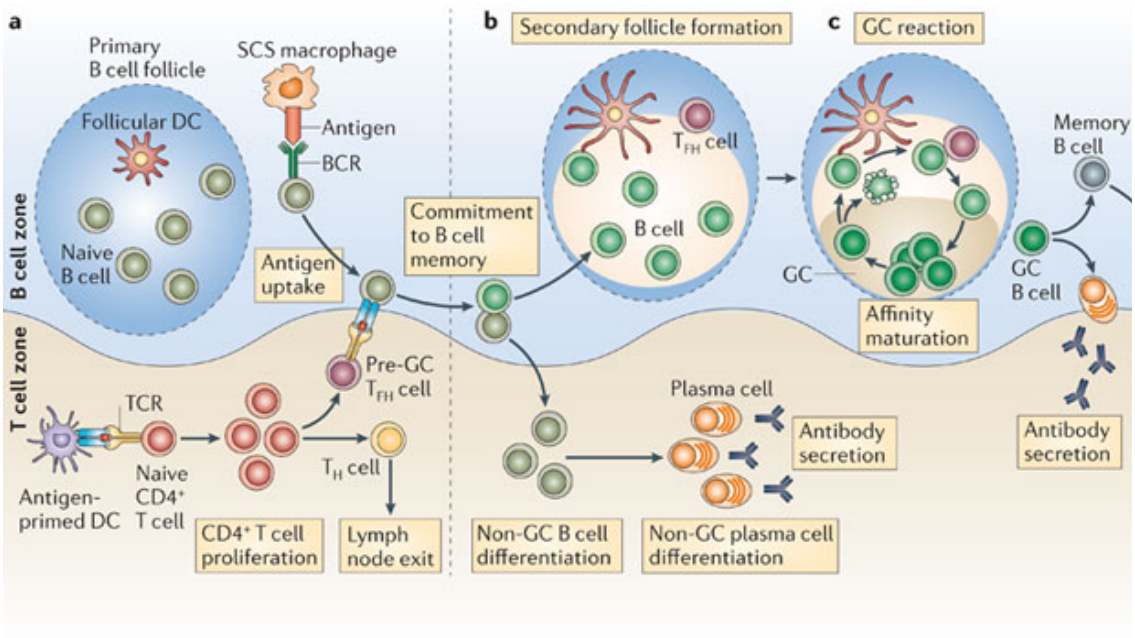
Proliferating B cells may differentiate immediately into short lived plasma cells which secrete antibodies, or continue to proliferate in secondary B cell follicles eventually leading to the formation of a germinal centre (Figure 1.7). Within germinal centres, the processes of somatic hypermutation and affinity maturation occur to generate high affinity antibodies to pathogen antigens. T_{FH} cells are vital in this process, promoting survival of activated B cells with high affinity for antigen [53]. T_{FH} cells also promote class switching of immunoglobulin. Naïve B cells express cell surface IgM and IgD only, though the most abundant circulating immunoglobulin is the opsonising isotype IgG. Cytokines, secreted either by the cognate T_{FH} cell, or that are present in the environmental milieu, influence selection of isotypes IgA, IgG or IgE (Section 1.3.3.3). The different isotypes perform a wide array of effector functions. High affinity IgG and IgA, (the latter particularly prevalent at mucosal surfaces) can neutralize bacterial toxins and viruses. These isotypes can prevent the adhesion of viruses and bacteria to cell surfaces, inhibiting pathogen entry. IgG subtypes promote Fcγ receptor mediated opsonisation of pathogens by phagoctytosis, and together with IgM, can also promote pathogen clearance by activation of the

complement cascade. The IgE isotype is important in the response to parasite infection, mediating eosinophil dependent pathogen clearance.

Affinity matured B cells from germinal centres may differentiate into long lived plasma cells, or memory B cells. Memory B cells provide immunological memory in response to antigen restimulation, with rapid clonal expansion, plasma cell generation, and the induction of a secondary germinal centre reaction [56].

Mature B cells can occasionally be reactivated in response to highly repetitive non-peptide structures without CD4+ T cell engagement. These antigens are termed 'thymus independent', and often include capsular polysaccharides of bacteria that induce clustering of B cell receptors. Thymus independent activation is important in the recall response to the conjugate Hib vaccine, but cannot initiate an effective primary response in children, since naïve B cell activation and establishment of immunological memory requires linked T and B cell recognition.

Figure 1.7: The B cell differentiation pathway. Adapted from McHeyzer-Williams 2012 [56].



1.3.4 Correlates of protective immunity and novel vaccine platforms

Despite their success, the vast majority of vaccines in current use have been developed empirically, with little or no understanding of the mechanisms by which they generate protective immunity. However, for many infectious agents including the global pandemic agent human immunodeficiency virus (HIV) the empirical approach to vaccine development has failed, despite decades of research. This has prompted a shift from empirical to rational vaccine design, and highlighted the need to understand the immunological mechanisms by which vaccines confer protection.

Most currently licensed vaccines are live attenuated; comprising intact but much less virulent pathogens. Such vaccines including those against smallpox, yellow fever, measles, mumps, and rubella mimic the exposure of an individual to a live infection, and often confer protection for decades after vaccination. Though these vaccines engage multiple arms of the immune system we now appreciate that the primary mechanism of protection is through antibody mediated immunity [7]. Pathogen specific serum IgG is the main correlate of protection for most of these vaccines, neutralizing pathogens to prevent cell entry and facilitating opsonisation by macrophages. Mucosal IgA is an important correlate for some live attenuated vaccines including oral polio and rotavirus [57]. However, cellular immunity certainly contributes to the protective efficacy of many live attenuated vaccines, particularly in the case of the tuberculosis vaccine Bacillus Calmette-Guérin (BCG) (an attenuated strain of the related *Mycobacterium bovis*) where IFN-γ secreting CD4+ T cells are believed to be critical in stimulating macrophage activation and control of *M. tuberculosis* infection [58].

However, the attenuated whole organism approach is challenging for some pathogens such as malaria, since infectious sporozoites are difficult to prepare in sufficient quantity *in vitro* (Section 1.2). Furthermore, in the case of malaria natural infection does not provide complete protection against reinfection, so an alternative mechanism of immunity must be sought. Some pathogens including HIV have been considered too dangerous for development of a live attenuated vaccine, due to the potential of mutation to cause reversion to virulence [59]. In addition, pathogens that mutate rapidly (such as HIV), those that are highly polymorphic (such as the *Plasmodium* parasite, the causative agent of malaria) or exist as multiple serotypes (such as dengue virus) and those that cause persistent or latent infection (such as HIV and hepatitis C virus) are particularly challenging targets for vaccination [60].

Research into the molecular mechanisms of pathogenesis has led to the recent development of subunit vaccines, which target the immune response to specific components of the pathogen. Licensed subunit vaccines include the inactivated toxoids of tetanus and diphtheria, polysaccharide conjugate vaccines (Section 1.3.3.4) and the hepatitis B surface antigen which forms particles of recombinant protein to enhance B cell stimulation and augment antibody responses targeted against the viral envelope. Recently, two licensed Human Papilloma Virus (HPV) vaccines Cervarix and Gardisil have been developed based on so called 'virus-like-particles' formed by the HPV major capsid protein L1. HPV L1, expressed as a recombinant protein in *Saccharomyces cerevisiae*, forms particles closely resembling the live HPV virus but which lack the viral DNA preventing virulence [61,62]. Recombinant protein subunit vaccines act primarily via induction of antigen specific neutralizing or opsonizing antibody

subtypes. However, these vaccines are often poorly immunogenic alone, requiring the use of 'adjuvants' to provide the innate stimulus required for a robust adaptive response. Advances in understanding of pattern recognition and innate immunity (Section 1.3.1) has enabled rational design of vaccine adjuvants with many recently developed adjuvants containing TLR agonists [9]. For example, the licensed adjuvant AS04, a component of the Cervarix vaccine, contains the TLR4 agonist monophosphoryl lipid A [63].

Though recombinant protein vaccines induce high antibody titers when used in combination with an appropriate adjuvant, induction of cellular immunity is often poor. There is strong evidence that T cell immunity may be critically important for protection against many pathogens for which an effective vaccine remains elusive, including pre-erythrocytic malaria [64,65], HIV [66,67,68], and tuberculosis [58]. The focus of much research has therefore turned to the development of gene-based vaccines [69]. In this system, DNA encoding specific pathogen antigens is delivered to host cells enabling *in vivo* expression of antigen and presentation on MHC class I molecules for efficient induction of CD8+ T cell responses. Since antigens are expressed *in vivo* the gene based system circumvents difficulties in recombinant protein expression and purification, affording simplicity and greater flexibility in subunit design. In some cases, gene based vaccines have been developed against antigens that have been challenging to express *in vitro* as recombinant proteins, such as the malarial merozoite surface protein reticulocyte-binding protein homologue 5 (RH5) [70,71]. Since T cell epitopes are linear, gene based vaccines designed specifically to elicit T cell responses often consist of a series of T cell epitopes rather than the full length protein [72]. Indeed there is evidence that direct presentation of

peptides on MHC class I molecules is more efficient in the context of so called 'mini-gene' cassettes than within the native protein [73]. To circumvent the challenges of polymorphism of pathogens such as HIV, some gene based vaccines are being designed containing 'mosaic' antigens designed to increase the breadth of the T cell response. Mosaic constructs are assembled *in silico* by from natural antigen sequences and are designed to provide maximal coverage of potential T cell epitopes across multiple alleles of a particular antigen [74].

The most simple gene based vaccines, developed two decades ago, are DNA plasmid vectors [75]. These plasmids were first administered into mice via a biolistic system based on gold microprojectiles, but recent strategies involve the use of biodegradable nanoparticles, liposomes, and electroporation [76]. However, despite early promise in preclinical rodent models [77], efficacy and immunogenicity of DNA vaccines in human clinical trials has been comparatively poor [78,79]. This may be due to inefficient DNA delivery or insufficient innate stimulation; TLR9 expression is less abundant in humans than rodents, and it is likely that TLR9 stimulation by unmethylated plasmid CpG sequences contributes significantly to the potency of DNA vaccines in mice [69]. Attention has therefore turned to the use of recombinant viral vectors for gene based vaccine delivery. In this system the viral vector provides both the delivery system for the encoded antigen and the innate immune stimulus required for the generation of robust T cell responses. Indeed recombinant viral vector vaccines remain the most potent inducers of T cell immunity of all vaccine platforms [80]. The first viral vector was developed by the laboratory of Bernard Moss in the mid 1980's; a live replication-competent recombinant vaccinia virus (VACV) expressing hepatitis B virus surface antigen which demonstrated protective

efficacy in chimpanzees against hepatitis B infection [81]. Most viral vectors in use today have been rendered replication defective; either empirically by continuous passage *in vitro* as in the case of modified vaccinia virus Ankara (MVA) [82], or by targeted gene deletion. Replication defective vectors are considered to be safer for use in humans, and often display immunogenic potential similar to their wild type derivatives, as is demonstrated by the comparative immunogenicity of VACV and MVA based vectors [83]. Vectors based on a wide range of viruses have been developed including VACV, fowlpox virus, attenuated canarypox virus, lentivirus, flavivirus, measles virus, sendai virus, and adenovirus. Many studies have combined different viral vector platforms in heterologous prime boost regimens, and this approach has proved successful in recent clinical trials with an adenovirus prime and MVA boost regimen eliciting unprecedented frequencies of IFN-γ T cell responses in humans [84]. Heterologous regimens are required to maximize vaccine insert specific responses because, in contrary to DNA vaccines, immune responses against proteins of the vector itself limit vaccine efficacy when the same vector is used for repeat immunizations [85].

There are as yet no DNA or viral vector vaccines licensed for use in humans, due in part to the long, arduous path to human licensure [86]. A chimeric viral vaccine for Japanese encephalitis virus (JEV), IMOJEV®, was recently licensed in Australia and Thailand based on the attenuated yellow fever vaccine strain YFV-17D [87]. The vector was generated by replacement of two structural genes prM and E from YFV-17D with capsid proteins from an attenuated strain of JEV, but since the capsid is chimeric the pathogen specific antigen is not purely gene based. Many gene based vaccines have however been

licensed for veterinary use. Licensed DNA vaccines include those against West Nile virus in horses [88], haematopoetic necrosis virus in schooled salmon [89], and melanoma in dogs [90]. The first licensed viral vector vaccine was a live VACV recombinant expressing rabies glycoprotein G [91], administered in baits for oral uptake by foxes in Europe and by raccoons in the United States. Since then, there have been many other licensed veterinary vaccines mainly based on poxvirus vectors including fowlpox and attenuated canarypox virus (ALVAC) vectors [80]. However, a replication defective recombinant adenovirus (HAdV-5) expressing foot and mouth disease (FMDV) capsid and protease antigens is set for licensure in the United States for protection of livestock. Protective immunity has been demonstrated in both cattle and swine seven days after a single immunization with the recombinant vaccine [92,93].

1.4 The Adenovirus as a Vaccine Vector

1.4.1 Adenovirus

Adenoviruses were first isolated from adenoid tissue in 1954 by two independent groups attempting to identify the causative agents of acute respiratory illness [94,95]. However, it has since become clear that adenoviruses are far from the major aetiological agents of respiratory disease; though infection is common much is subclinical and adenoviruses are responsible for only a small percentage of clinical respiratory illness. However, particular serotypes such as HAdV-4 and HAdV-7 have been associated with outbreaks of acute respiratory disease (ARD) among military recruits. In the 1970's an oral enteric vaccine containing these two serotypes was implemented by the US military and successfully controlled the disease; cessation of vaccine administration in 1996 has led to a return of adenovirus associated respiratory illness [96].

A total of 52 human adenovirus (HAdV) serotypes have been identified to date, and these have been classified into seven viral species (A-G) by serum neutralization, heamaglutinin inhibition, and genome sequence analysis (Table 1)[97,98]. Adenoviruses isolated from great apes (chimpanzees, bonobos and gorillas) have been found to be cluster phylogenetically into human adenovirus species B, C, and E [99]. The current study compares the use of four different viruses as vaccine vectors: one human adenovirus (HAdV-5, *Human adenovirus C*) and three adenoviruses isolated from chimpanzees (ChAd63, AdC68 and ChAdY25, all *Human adenovirus E*).

Human adenoviruses belong to the genus *Mastadenovirus*, along with other adenovirus species isolated from mammals including monkeys, mice,

horses, pigs, dogs, cattle and sheep. Other adenovirus genera include *Siadenovirus* (reptiles and birds), *Atadenovirus* (reptiles, birds, marsupials, mammals), *Aviadenovirus* (birds), and *Ictadenovirus* (sturgeon)[98].

Table 1.1: **Classification of Human Adenoviruses. Adapted from Hall *et al* [97].**

* Number of fiber shaft repeat sequences shown for individual serotypes within each species, not necessarily representative of all serotypes within a particular species.

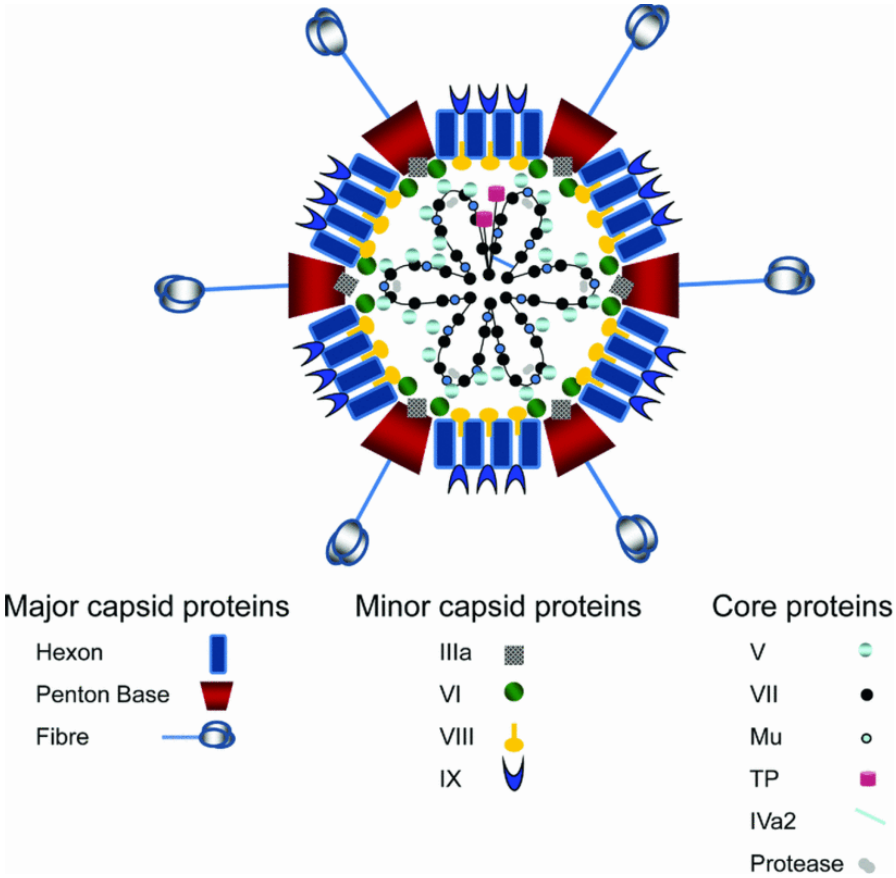
Species	Serotypes	Associated disease	Primary attachment molecule	GC content of genome (%)	No. repeats in fiber shaft*
A	12, 18, 31	Gastroenteritis	CAR	48-49	23
B	3, 7, 16, 21, 50 (B:1) 11, 14, 34, 35, (B:2)	Respiratory (B:1) or urinary tract disease (B:2)	CD46/ CD80/CD86	50-52	6
C	1, 2, 5, 6	Respiratory disease	CAR	57-59	22
D	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	Keratoconjunctivitis	CAR (Sialic acid for 8, 19, 37. CD46 for 37, 26?)	57-61	8
E	4	Respiratory disease / conjunctivitis	CAR?	57-59	12
F	40, 41	Gastroenteritis	CAR (long fibers)	Unknown	22
G	52	Gastroenteritis	Unknown	Unknown	Unknown

Human adenoviruses have not only been implicated in respiratory disease. Species HAdV-A, HAdV-F and HAdV-G have been associated with gastroenteritis, HAdV-D with keratoconjunctivitis and HAdV-B2 with urinary tract disease (Table 1). Oncogenic potential has been demonstrated for some serotypes in rodent models (mainly attributed to E1 and E4 gene products [100]), but transformation of human cells lines has proved far less efficient and adenoviruses have yet to be associated with tumorigenesis in humans [101,102].

Adenoviruses are non enveloped viruses with a linear double stranded DNA (dsDNA) genome of between 30-38kb, encoding 30-40 viral genes. The viral particle is 90nm in diameter and icosahedral in shape with fibers projecting from each of the twelve vertices [103]. The vast majority of the capsid consists of

trimeric hexon (polypeptide II) capsomeres (240 in total), with pentamers of penton base (polypeptide III) protein at the vertices, anchoring the trimeric fibers (polypeptide IV) to the capsid (Figure 1.8). Minor capsid proteins pVIII, and pIX stabilize the virion structure, pVI promotes release of the virus from endosomes after cell entry, and pIIIa is important for assembly of new virions. The virion core contains at least six core proteins and the DNA genome. Polypeptides pV, pVII and Mu are small basic DNA binding proteins which act to maintain the viral DNA in a highly condensed state within the virion. Protein IVa2 is present in much lower abundance within the virion, also in complex with the viral DNA, but plays an important structural role in encapsidation of the viral genome. IVa2 is also critical in the regulation of late gene expression during viral replication, initiating activation of the viral major late promoter (MLP) that drives expression of the structural capsid proteins [104]. The terminal protein (TP) is covalently attached to the 5' ends of the viral DNA, facilitating circularization of the genome and priming of early DNA replication upon nuclear entry. The cysteine protease cleaves several viral precursor proteins during multiple stages of the viral infection and replication cycle.

Figure 1.8: Schematic diagram of the adenovirus virion. From Hall *et al*, [97].



The adenovirus infection cycle is initiated by binding of the virus to the cell membrane and subsequent internalization into endosomes. However, despite decades of research, the precise mechanisms of viral entry *in vivo*, in the contexts of both natural infection and vector administration, are still unclear. The classical pathway of human adenovirus entry (except for members of species HAdV-B, and some members of HAdV-D) is via attachment to the coxsackie and adenovirus receptor (CAR), a 46kDa transmembrane protein that also functions as a high affinity receptor for coxsackie B viruses [103]. Interaction between the C-terminal knob domain of the adenovirus fiber protein and CAR facilitates initial attachment of the virus to cells. A second, lower affinity interaction between the viral penton base and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins on the cell surface (specifically via an arginine-glycine-aspartate, or RGD motif within the penton base) initiates clathrin dependent endocytosis through phosphoinositide-3-kinase and Rho GTPase signaling [105]. However, the initial discovery of CAR as a receptor for adenoviruses arose from *in vitro* infection studies [106]. The *in vivo* relevance of the CAR receptor is less clear, since expression of the receptor on surface epithelia is restricted to tight junctions between cells and the basolateral membrane where it is believed to function as a cell adhesion molecule [107]. Since adenovirus infects from the apical surface, access to CAR *in vivo* may be limited. Indeed, it has been proposed that the high affinity fiber-CAR interaction may be more important during viral escape, since excess fiber proteins produced during the replication cycle may disrupt CAR-CAR homodimer interactions disrupting the integrity of the epithelial surface [108]. Utilisation of CAR for viral entry may be restricted to pre-existing lesions within the epithelium [109]. Alternatively, a rare spliceform of CAR containing an additional

eighth exon (CAR Ex^8) has been identified on the apical surface of polarized epithelia and may be responsible for mediating cell attachment [110].

CAR is not the only cell surface molecule that has been implicated in fiber mediated virus attachment. Members of *Human adenovirus C* have been also been shown to mediate interactions with heparin sulphate glycosoaminoglycans via a basic KKTK motif within the fiber shaft [111]. Members of *Human adenovirus D* that cause keratoconjunctivitis (HAdV-8, HAdV-19, HAdV-37) have been shown to use sialic acid as a cell attachment receptor [112]. Members of species *Human adenovirus B* (with the possible exception of HAdV-3 and HAdV-7) have been found to interact with CD46, via their short fiber proteins [113,114]. CD46 is expressed on almost all nucleated cells in humans and acts as an inhibitory complement receptor. The molecule is utilized by a number of other pathogens to gain cell entry, including measles virus, human herpesvirus-6, and *Streptococcus* species [115]. Studies using *Human adenovirus B* viruses in mice are, however, confounded by the fact that expression of the murine homolog of CD46 is restricted to the testis [116]. Co-receptors CD80 and CD86 have also been implicated in attachment of *Human adenovirus B* viruses, though the relevance of this interaction for cell entry remains controversial [117].

Use of the adenovirus in the gene therapy field has revealed the potential of blood coagulation factors to promote non-fiber mediated cell attachment. In 1999, a high titer arterial injection of an HAdV-5 based gene therapy vector caused severe liver damage and the subsequent death of patient Jesse Gelsinger [118]. It has subsequently been discovered that the strong hepatic tropism of HAdV-5 vectors is CAR-independent, instead mediated by blood coagulation factors including Factor VII, IX, and X and complement component C4-binding

protein (C4-BP)[119]. Factor X (FX) was found to directly bridge heparin sulphate proteoglycans on hepatocytes and hexon trimers on the HAdV-5 capsid [120]. However, it has also been argued that the main relevance of the FX interaction *in vivo* is protection of the virus from antibody and complement mediated clearance (Andrew Byrnes, personal communication).

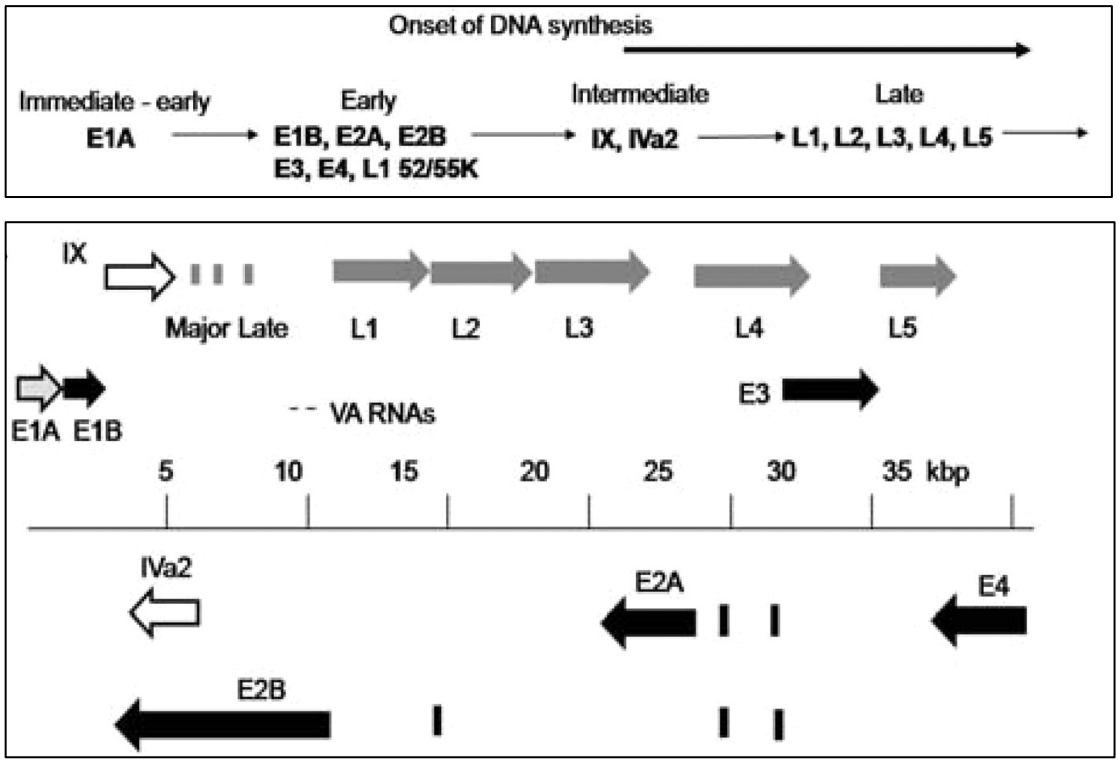
Furthermore, not all adenovirus entry is via clathrin dependent endocytosis. Interaction of HAdV-2 with α_v integrins on the surface of epithelial cells has been shown to stimulate clathrin independent macropinocytosis concomitantly with clathrin mediated uptake [121]. HAdV-3 has also been shown to stimulate membrane ruffling and non selective fluid phase uptake via low affinity interactions with α_v integrins and CD46. This mechanism seems to be of particular importance in HAdV-3 infection since inhibitors of macropinocytosis have been shown to prevent HAdV-3 entry [122].

Following internalization, rapid acidification of endosomes leads to stepwise capsid disassembly, and subsequent release of the disassembled virus from the endosome into the cytosol. The process is rapid for most adenoviruses but is dependent on serotype; members of HAdV-C typically require just 15 minutes for endosomal escape while members of HAdV-B have been found associated with endosome components for over an hour post internalization [123,124]. Once in the cytosol, partially disassembled virions translocate towards the nucleus along microtubules, driven by the motor protein dynein [125]. Upon arrival at the microtubule organizing centre (MTOC), the virion is transferred from the microtubule network to the nuclear pore complex (NPC) for entry of viral DNA into the nucleus. The mechanism of nuclear entry has been studies in detail for members of *Human adenovirus C* [126]. Docking at the

nuclear pore complex is mediated by interactions between hexon proteins and the NPC protein CAN/Nup214. Soluble histone H1 proteins then interact with a conserved acidic motif on the hexon, triggering binding of further import factors and the final stages of capsid disassembly. The viral DNA is released from interaction with the viral capsid and transported through the NPC. Once in the nucleoplasm, the nuclear DNA binding protein template activating factor-I (TAF-I) associates with pVII, remodeling the viral chromatin to facilitate transcription of the viral genome.

The adenovirus genome contains five early transcriptional units (E1A, E1B, E2, E3, and E4), two delayed early units (IX, IVa2) and one late transcriptional unit that is processed to generate five families of late mRNA (L1-L5), all of which are transcribed by nuclear RNA polymerase II and exported to the cytoplasm for translation (Figure 1.9). Most of these units are alternatively spliced to generate a multitude of mRNAs from a single primary transcript; indeed the phenomenon of alternative splicing was first discovered in adenovirus replication and represents perhaps the greatest contribution of adenovirus research to the field of cellular biology [127].

Figure 1.9: Transcription map of human adenovirus. From Morris *et al* [128]. Early transcriptional units are shown in black, intermediate early units in white, and late units in dark grey.



Deletion of the E1 transcriptional unit renders recombinant vectors, including those used in this study, replication defective. However, interactions involving E1 proteins are still relevant to vector development, since they profoundly effect viral growth and yield during vector production in E1 complementing cell lines. The immediate early gene, *E1A*, is the first adenovirus gene to be transcribed upon entry to the nucleus. The two spliceforms of E1A, 12S and 13S, initiate a transcriptional cascade of early viral gene expression. Expression from the adenovirus early gene regions (E1, E2, E3 and E4) is required to prepare the cellular environment for optimum viral replication. In addition to their role as transactivator proteins, E1A 13S and 12S act to accelerate progression of the cell cycle into S phase. E1A proteins bind to, and sequester, retinoblastoma protein (pRb), a principal regulator of cell cycle progression [129]. In quiescent cells, pRb sequesters the nuclear E2F transcription factor responsible for activating transcription of many S phase genes; hence pRb blocks cell cycle progression. By sequestration of pRb, E1A releases the cell from cell cycle arrest, and furthermore promotes transcription of viral E2 genes, which are also activated by E2F [129]. E1A proteins may also inhibit or alter the activity of chromatin modifying enzymes such as the acetyltransferase PCAF (p300/CBP associated factor), which control gene expression by regulating the acetylation state of nucleosomes, and hence the accessibility of the DNA. These properties have enabled E1A proteins of some adenoviruses (particularly HAdV-12) to transform murine cells *in vitro*.

The second E1 transcriptional unit, *E1B*, also generates two polypeptides; E1B-19K and E1B-55K. E1B-19K is a potent inhibitor of apoptosis by blocking the action of the death-inducing Bax protein [130]. E1B-55K functions in concert

with a product of the E4 region, E4Orf6, to form an E3-ubiquitin ligase complex which is essential for efficient viral replication. The E1B-55K/E4Orf6 complex targets the key tumor suppressor p53 for proteasome mediated degradation, and also targets components of the DNA damage MRN complex, since the double stranded viral genome can activate cellular DNA damage responses (genome termini are recognized as a double strand break)[131]. E1B-55K/E4Orf6 is also required later in the replication cycle for the selective export of late viral mRNAs to the cytoplasm for translation, and may also be involved in splice site recognition during processing of late transcripts [132].

E4Orf6 is the only locus within the E4 region in which mutations have any significant affect on viral growth rate [133]. However, functions for other members of E4, generated from alternative splicing of a common precursor mRNA, have since been revealed. The highly conserved E4Orf3 has been shown to perform an antagonistic function with E4Orf6 in the regulation of viral mRNA splicing [134], and has also been shown to physically interact with E1-55K [135]. In addition, E4Orf3 has been shown to disrupt nuclear bodies; punctate multiprotein structures that form the sites of important cellular and antiviral processes including cell cycle control, DNA damage repair, apoptosis and antiviral responses to type I IFN [136]. E4Orf4 functions as part of an auto-regulatory feedback loop to control the activity of E1A by binding to protein phosphatase 2A (PP-2A) and promoting the phosphorylation of E1A [132]. The interaction between E1 and E4 gene products is an important consideration in vector design, and is discussed in detail in Chapter 3.

Products of the E3 region serve primarily to combat cellular antiviral defenses. Though these proteins perform important functions in the context of

adenovirus infection they are not required for *in vitro* virus culture and are deleted from many recombinant vectors including those used in this study. The E3-19K glycoprotein targets MHC class I molecules for retention in the endoplasmic reticulum, preventing the display of viral antigens to CD8+ T cells [137]. Integral membrane proteins E3-10.4K and E3-14.5K form a complex termed the receptor internalization and degradation (RID) complex, which, as the name suggests, down-regulates cell surface expression of the epidermal growth factor (EGF) receptor, TNF-related apoptosis-inducing ligand (TRAIL), and the apoptotic Fas receptor. E3-14.7K also inhibits TNF induced apoptosis and inflammation. The gene encoding the adenovirus death protein (ADP), though located within the E3 region, is expressed only at low levels from the E3 promoter. ADP functions primarily during the late stages of viral replication, when, expressed at high levels from the major late promoter, it triggers cell death and subsequent release of new viral progeny.

The E2 region encodes the molecular machinery required for viral replication, and is separated into two transcriptional units, E2A (encoding the DNA binding protein, DBP), and E2B (encoding the pre-terminal protein and the viral DNA polymerase).

Human adenoviruses also contain short RNA polymerase III transcripts, termed virus associated RNAs (VA-RNAs). HAdV-A, HAdV-B:1 and HAdV-F members express a single VA-RNA transcript, all other human adenoviruses express two, termed VA-RNA I and VA-RNA II. Expressed early in the replication cycle, VA-RNA accumulates to very high levels, up to 10^8 copies per infected cell [138]. VA-RNA has been shown to saturate and block the activity of the anti-viral serine/threonine double-stranded RNA activated protein kinase (PKR) [139].

PKR functions to phosphorylate and inactivate the eukaryotic translation initiation factor eIF2 in response to type I IFN, leading to a blockade in protein synthesis upon viral infection. In addition to removing this cellular blockade of protein synthesis, VA-RNA molecules can also interact with components of the RNA interference machinery, including DICER, since they possess a similar structure to pre-miRNAs. VA-RNA molecules can block DICER, preventing anti-viral gene silencing, and can even generate viral miRNAs (termed svaRNAs or mivaRNAs) which target cellular genes such as the pro apoptotic regulatory protein TIA-1 [140]. The consequences of VA-RNA expression for the function of replication defective vectors are less clear, though recent *in vitro* studies have implicated VA-RNA transcripts in induction of type I interferon in the absence of E1 [141] (Section 1.4.2). The significance of adenovirus VA-RNA in this context is investigated further in Chapter 6 of this study.

In HeLa cells, adenovirus DNA replication begins 5-6 hours post infection. The inverted terminal repeat (ITR) sequences at both ends of the linear genome, facilitate circularization and act as an origin of replication. The adenovirus polymerase is recruited to the replication origin by association with the pre-terminal protein (which is covalently bound to each end of the genome), and forms a pre-initiation complex with the adenovirus DNA binding protein and cellular transcription factors nuclear factor I (NF-I) and Oct-I. Chain elongation requires the polymerase, DNA binding protein, and the cellular topoisomerase-I. These components are sufficient to reconstitute complete adenovirus DNA replication *in vitro*; indeed adenovirus DNA was the first eukaryotic template to be replicated *in vitro* [142].

The onset of DNA replication leads to enhanced expression of late viral genes from the major late promoter (MLP). Intermediate gene products IVa2 and pIX are also expressed during this period, and are required for maximal late gene expression [104,143], in addition to the late gene products L4-22K and L4-33K [144]. During the early phase of the viral replication cycle, activity of the MLP is low and transcription is focused on the L1-52/55K reading frame, the product of which is involved in viral DNA encapsidation [145]. But after the initiation of viral DNA replication, activity is considerably enhanced and transcription is allowed to proceed through the entirety of the major late transcriptional unit (L1-L5). Late mRNAs, approximately twenty in total, are generated from a single major late primary transcript through alternative splicing and differential poly(A) site utilization. All late transcripts contain the 5' tripartite leader sequence, important for stabilization, nuclear export, and translation of late mRNAs. Products of the late L1-L5 units encode structural proteins and other proteins required for assembly of new viral progeny. Translation of these products at ribosomes is facilitated by a number of viral factors including VA-RNA I, and the L4-100K protein, which promotes selective translation of viral mRNAs by preventing formation of the 5'-m^7GpppN (methyl-G) cap structure required for translation of cellular mRNA [146]. Late viral mRNAs continue to be translated since the tripartite leader abrogates the requirement for the methyl-G cap by initiating translation through an alternative mechanism termed RNA shunting [147]. L4-100K also performs an additional function as a chaperone protein in the formation of hexon trimers during capsid assembly [148].

Assembly of new virions occurs in the nucleus, so structural proteins require nuclear import after synthesis in the cytoplasm. Protein VI has been

implicated in nuclear import of hexon monomers, prior to its role in capsid stabilization within the virion [149]. Packaging and encapsidation of viral DNA into new virions requires viral proteins L1-52/55K, L4-22K, and IVa2, the latter has been proposed to function as a packaging motor, stuffing viral DNA into the capsid [150]. These proteins form a complex at a specific packaging domain located at the left end of the viral genome which is also essential for formation of correctly packaged virions [151].

Finally, the L3 encoded 23 kDa viral protease is required for cleavage of the precursors of proteins VI, VII, VIII, TP and μ to complete assembly of mature infectious virions. The viral protease requires viral DNA and a C-terminal fragment of pVI (pIVc) as cofactors for catalytic activity; recent evidence suggests that pVIc functions as a molecular 'sled' enabling the protease to move along the DNA and interact with its substrates (Walter Mangel, personal communication).

Once protease activity completes the maturation process, new infectious viral progeny are released from the cell (approximately 10^4 virions per cell). Release of viral progeny is facilitated by the adenovirus death protein (ADP, E3-11.6K), which promotes death of infected cells late in the viral life cycle [152]. The complete life cycle of adenovirus infection is summarized in Figure 1.10.

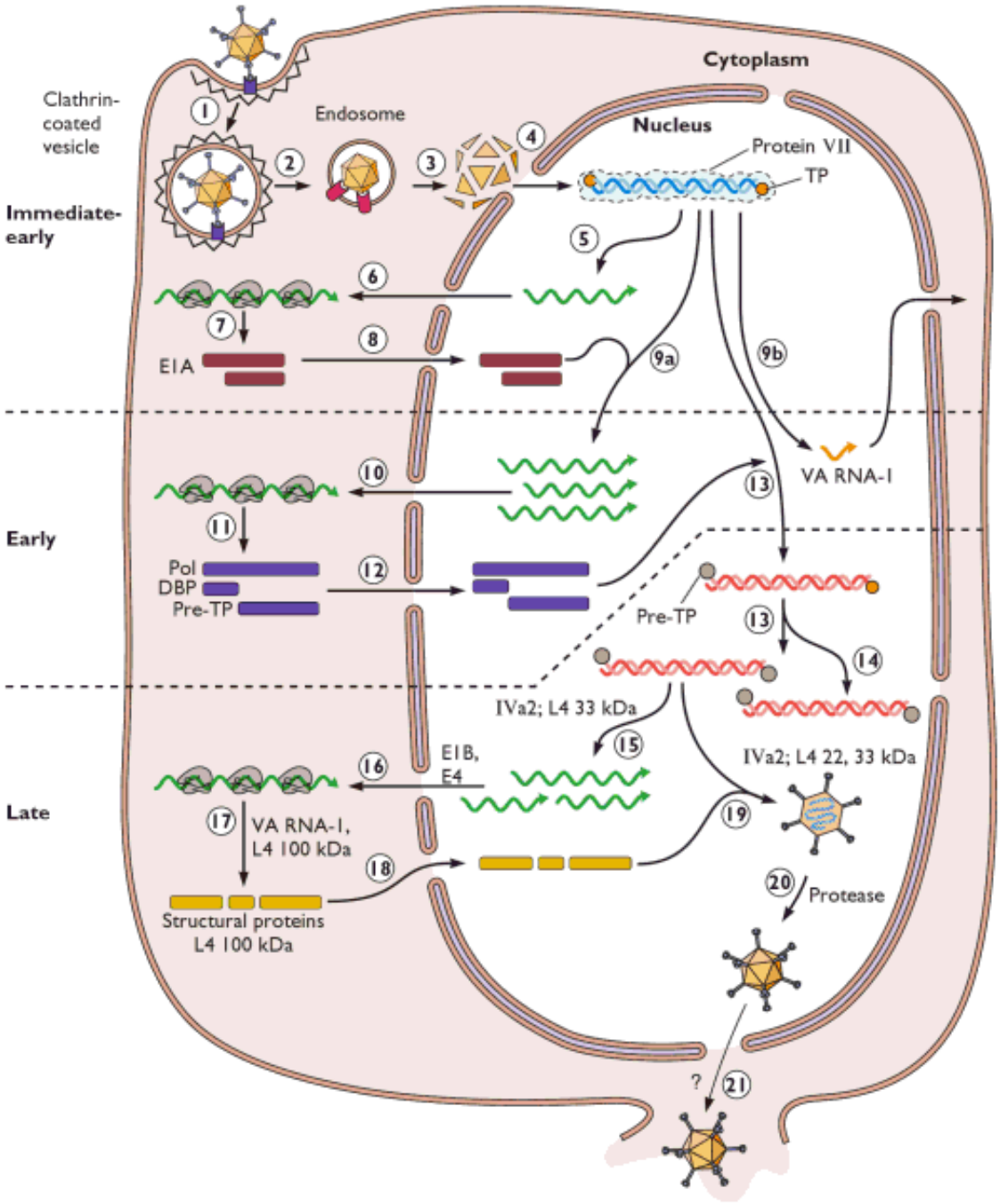


Figure 1.10: Stepwise summary of the human adenovirus replication cycle. Adapted from Principles of Virology 3rd Edition, Flint *et al* [153]. Adenoviruses enter cells by endocytosis, and after escape from the endosome translocate to the nucleus (1-4). Upon entry of the viral DNA in complex with pVII and TP, the immediate early gene

E1A is transcribed by RNA polymerase II, and the protein synthesised at ribosomes in the cytoplasm after prior mRNA splicing and export (5-7). E1A is imported into the nucleus and initiates activation of other early viral genes including those required for DNA replication, and cellular genes involved in cell cycle progression (8-9a). Transcription of VA-RNA genes by RNA polymerase III also occurs during the early phase of transcription (9b). Early viral pre-mRNAs are processed, exported and translated in the cytoplasm (10-11). These include products of the E2A and E2B regions involved in the initiation and propagation of viral DNA synthesis, including pTP, DBP and polymerase (12-13). Newly synthesized copies of the viral genome may serve as templates for further rounds of DNA replication (14), or transcription of late viral genes (15). Maximal efficiency of transcription from the major late promoter (MLP) requires pIVa2 and L4-33K. Late mRNAs are selectively exported from the nucleus through the action of the E1B-55K/E4Orf6 complex. Efficient translation in the cytoplasm is facilitated by VA-RNA I which prevents PKR mediated cellular blockade of protein synthesis, and 100K promotes selective translation of viral mRNAs by inhibiting formation of the 5' cap required for translation of cellular mRNAs (16-17). Late mRNAs include structural capsid proteins, which are imported into the nucleus for assembly of new virions within the nucleus (18-19). Capsid assembly requires a DNA packaging signal at the left end of the genome, and encapsidation of the viral genome also requires IVa2 and L4-22K proteins. 100K protein, in addition to its role in translation, also acts as a chaperone protein to promote assembly of hexon trimmers during construction of the viral capsid. Non-infectious, immature virions contain precursors of several viral proteins which are cleaved to their mature forms by the viral L3 protease, which enters the virion core (20). Mature virions are released from the host cell, assisted by the adenoviral death protein (ADP) which triggers apoptosis (21). Approximately 10^4 progeny virions are released per infected cell.

1.4.2 Replication defective adenoviruses as vaccine vectors

The development of the adenovirus E1 complementing human embryonic kidney-293 (HEK-293) cell line, by Frank Graham in the late 1970's [154], facilitated the development of replication defective E1 deleted adenovirus vectors (also termed 'first generation' adenovirus vectors). First generation vectors were initially developed for gene therapy applications, with a particular focus on cystic fibrosis due to the strong tropism of many serotypes for the upper respiratory tract. However, expression of the encoded *CTFR* transgene was transient in clinical trials, declining considerably after two weeks [155]. Administration of the vector was associated with anti-vector and anti-transgene product immune responses. It was later established that induction of CD8+ T cells after vector administration leads to a decline in target gene expression by destruction of vector transduced cells, while neutralizing antibodies against the viral vector reduce the effectiveness of repeat administrations [156].

However, the strong and sustained transgene-specific immune responses elicited by these delivery agents, together with their broad tissue tropism, prompted the use of adenoviruses as vaccine vectors. The first adenoviruses developed for vaccine applications were replication competent, with recombinant transgenes inserted in place of the E3 region (which is not required for viral replication) [157]. However, the potent immunogenicity and efficacy of human adenovirus based vectors in non-permissive or semi permissive species (human adenoviruses cannot replicate productively in many rodent species including mice) suggested that sufficient antigen was expressed in the absence of viral replication [158]. The use of replication defective first generation vectors has reduced the potential for side effects in clinical application, and lowered the

risk of vaccine dissemination, which had previously been demonstrated for the live enteric HAdV-4 vaccine [159].

Replication defective adenoviruses have become a popular viral vectored vaccine platform. The adenovirus genome is well characterised and easy to manipulate, simplifying the construction of recombinant vectors. The viral genome remains episomal upon infection, significantly reducing the risk of insertional mutagenesis. Adenoviruses can be made replication defective by deletion of a single transcriptional unit, E1, and cultured to high titer in E1 complementing cell lines such as HEK-293 and PER.C6 [160]. Deletion of a second unit, E3, increases the insert capacity to 8kb, allowing greater flexibility in antigen design without affecting viral replication. The insert capacity of vectors is limited by the size of the genome that can be physically packaged into viral capsids; the recombinant vector genome must not exceed 105% of the size of the parental virus [161]. Further deletions have been made to increase insert capacity, including deletion of the entire adenoviral coding sequence to generate 'gutless' vectors, but these modifications require complementation of deleted genes to enable viral replication [160]. Robust expression of the recombinant transgene can be achieved using strong constitutive heterologous promoters such as the major immediate early promoter of human cytomegalovirus (CMV IE) [162].

Since replication defective adenovirus vector vaccines were first developed in the late 1980's [163], their use has been widespread in both preclinical and clinical vaccine programs targeting a wide range of pathogens [164,165]. Recently, an adenovirus vector vaccine against foot and mouth disease virus (FMDV) in cattle was conditionally licensed for use in the US. However, in

humans, the first generation of vaccine vectors based on the widely studied serotype HAdV-5, showed poor efficacy in a recent HIV-1 clinical trial (Merck's Phase IIb STEP trial) [166] despite encouraging pre-clinical data [167]. A large proportion of human adults possess significant titers of neutralising antibodies to common human serotypes such as HAdV-2 and HAdV-5 and these have the potential to reduce the potency of viral vector vaccines by inhibiting vector mediated delivery of the encoded transgene. Furthermore, the STEP trial was terminated prematurely due to the emergence of a trend towards increased susceptibility to HIV in vaccinated individuals with high titer pre-existing neutralizing antibodies against the HAdV-5 vector [168], though this relationship has since been shown to wane with time [169] and is of marginal statistical significance. It was hypothesized that the increased rate of infection could be due to induction of vector specific CD4+ T cells creating a pool of target cells for HIV infection, though subsequent studies have found this hypothesis to be incorrect [170,171]. Nevertheless, the issue of anti-vector immunity has discouraged the use of common human serotypes for clinical use. Pre-existing anti vector immunity has since been addressed through the development of new vectors based on serotypes to which the human population is less exposed, including those of chimpanzee origin [172]. Chimpanzee adenovirus vectors have been shown to be highly immunogenic in animal models and now recently in clinical malaria vaccine trials [84,173,174]. Use of rare human serotypes such as HAdV-26 and HAdV-35 [85,175], and non-human adenoviruses such as canine adenovirus [176] and porcine adenovirus [177] have also been explored.

Adenovirus based vaccine vectors are arguably the most potent inducers of protective CD8+ T cells. Replication defective adenoviruses have consistently

been shown to elicit superior CD8+ T cell responses in comparison to other leading vector technologies including MVA, Fowlpox (FP9), and DNA based vectors, and give enhanced protective efficacy in pre-clinical malaria and HIV challenge models [167,178,179]. The mechanisms that underlie the potency of adenovirus vectors are not fully understood, and will be investigated further in this study.

Adenoviruses and their vectors are potent inducers of type I interferon, and pro-inflammatory cytokines including TNFα, IL-6, IL-12, IFN-γ and chemokines MIP-1α, MIP-1β, RANTES and IP-10 shortly after infection [180,181]. Activation of innate immunity is required to adjuvant transgene product specific adaptive responses (Section 1.3.2); hence adenovirus vectors can induce robust cellular and humoral immunity in the absence of additional adjuvants. Innate immunity and induction of type I interferon has been shown to be critical for adaptive responses to adenovirus vectors; mice lacking the interferon α and β receptors (IFNαβR -/-) display significantly reduced adaptive responses [182]. Induction of innate immunity is independent of viral transcription; UV/psoralen inactivated vectors can still induce robust pro-inflammatory cytokine responses [183]. The innate immune response to adenovirus appears to be mediated by both TLR dependent and TLR independent pathways. Plasmacytoid DCs (pDCs) produce particularly high levels of type I interferon in response to viral infection, and are believed to sense adenovirus DNA through a TLR9 dependent, MyD88 dependent pathway [182]. The same study showed, however, that in non pDCs including conventional DCs and macrophages, sensing of adenovirus is TLR independent and mediated through cytosolic sensing of viral DNA. However, there appears to be

considerable functional redundancy within signaling pathways that contribute to innate recognition of adenovirus vectors. A study from the Barouch laboratory recently demonstrated that within the TLR signaling pathway, only the absence of MyD88 had a significant impact on the ability of replication defective adenovirus vaccine vectors of multiple serotype to elicit transgene specific CD8+ T cell responses [184]. Responses were not significantly diminished in mice lacking TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR9, IL-1R or IL-18R [184]. A recent study has also implicated the adenovirus VA-RNA in induction of type I interferon (but not pro-inflammatory cytokines) by replication defective vectors in both murine fibroblasts and bone marrow derived DCs [141]. Cytosolic recognition of VA-RNA transcripts occurs via the pattern recognition receptor retinoic acid-inducible gene I (RIG-I) [185]. Recent studies have shown that vectors derived from different adenovirus serotypes induce different levels of type I interferon, with potential implications for vector potency [186]. The contribution of type I interferon production to vector immunogenicity is a highly complex phenomenon, and will be discussed further in Chapter 6. Several studies have implicated high levels of interferon with lower transgene product specific responses [186,187], the latter correlating interferon production with lower transgene expression [187]. However, other studies have questioned the ability of the interferon response after vector administration to affect expression of the recombinant transgene [188].

In contrast to many viral vector vaccine platforms, including MVA and other poxvirus based platforms, transgene expression from replication defective adenoviruses is persistent after *in vivo* administration [189]. Indeed, low levels of transcriptionally active vector genome, detected up to a year post

administration, have been shown to maintain functional CD8+ effector T cell populations [190]. The CD8+ T cell kinetic after adenovirus vector administration typically displays a protracted contraction phase and sustained memory population, characteristic of persistent antigen expression and more commonly associated with low-level persistent infections such as CMV. Consequently, the CD8+ effector response typically peaks between two and three weeks post administration, while poxvirus vectors that give rise to acute, rapidly cleared infections, peak much earlier and at a lower magnitude [179]. Consistent with the persistence of antigen expression, the CD8+ T cell memory population after adenovirus vector administration is composed mainly of T_{EM} cells, with little evidence of T_{CM} accumulation. Since T_{CM} cells have the maximum proliferative potential it has classically been thought that these are the most important subset for vaccine applications. However, recent studies have shown that in fact T_{EM} cells, with more immediate effector functions, are the strongest correlates of protection in many disease states including HIV infection (using a highly pathogenic SIV model in rhesus macaques)[68] and pre-erythrocytic malaria [65].

It is believed that a combination of direct priming and cross priming of dendritic cells contribute to initiation of the potent CD8+ T cell responses. Certainly adenoviruses are capable of infecting both murine and human DCs *in vitro* [191,192,193], and there is also evidence of direct infection of murine DCs *in vivo* [194]. However, adenoviruses have been shown to infect a wide range of tissue resident cells at sites of administration, and by expressing recombinant transgenes using tissue specific promoters, studies have shown that comparable CD8+ T cell responses can be achieved without direct priming of DCs,

presumably through cross presentation [195]. The ability to efficiently cross prime CD8+ T cells implies that a significant amount of antigen is available for exogenous uptake by DCs and subsequent presentation on MHC class II molecules, though paradoxically, adenoviruses are weaker inducers of CD4+ T cell responses, eliciting magnitudes comparable to naked DNA vaccines [196]. The laboratory of Jonathan Bramson has recently proposed a model for CD8+ T cell induction by adenovirus vectors after intramuscular injection, whereby professional APCs in the draining lymph node are required for initial priming but not maintenance of the memory T cell population [197]. Bramson and colleagues showed directly that doxycycline mediated termination of transgene expression before the peak of the CD8+ effector T cell response attenuated expansion, and termination up to day 30 post immunization yielded a reduced memory population [198]. The same laboratory later showed that CD8+ T cells are primed in the draining lymph nodes and these sites are critical for maximal T cell expansion [199]. However, by removal of draining lymph nodes, the authors showed that antigen presentation by non-hematopoetic APCs outside the draining lymph nodes was required to sustain the memory CD8+ T cell population [199]. The identity of the non-hematopoetic APCs has not been confirmed, but muscle cells surrounding the site of injection and endothelial cells peripheral to the lymph nodes have been proposed as likely candidates [197].

The potential of an adenovirus vector to elicit transgene product specific immune responses is largely dependent upon the serotype of virus upon which the vector is based [200,201]. It is however currently unclear how differences in the tropism of different vector serotypes contribute to differences in immunogenicity. The comparatively low immunogenicity of *Human adenovirus B*

derived vectors in both mice and macaques [201] may relate to their alternative receptor usage. Mice do not express the CD46 receptor utilized by these viruses [116], and while macaques widely express a homologue of CD46, recent evidence suggests that engagement of the CD46 receptor by HAdV-35 vectors may inhibit CD4+ T cell activation [202].

Despite the impressive potency of adenoviruses as vaccine vectors, the magnitude of cellular and humoral responses obtained after a single immunization are unlikely to afford sufficient protective efficacy for all clinical applications. Adenovirus vectors have therefore been used extensively in heterologous prime boost regimens in both pre-clinical and clinical applications. Since pre-existing anti vector immunity precludes short-term use of the same vector for repeat administrations, adenoviruses of different serotype have been used sequentially to induce elevated CD8+ T cell frequencies both pre-clinically [203] and clinically [204]. However, perhaps the most potent heterologous prime boost regimen involves priming with a recombinant adenovirus, and boosting with an MVA based vector [205]. This immunization regimen, using chimpanzee adenovirus based vector ChAd63 as a priming agent, has translated into unprecedented transgene specific T cell potency in a recent clinical trial [84]. The mechanisms underpinning the potency of MVA as a boosting vector, considering the comparatively weak priming responses elicited by this vector, are at present unclear. However, a recent study using the related vaccinia virus, identified the efficiency of direct antigen presentation to dendritic cells as the critical factor in the ability of this vector to expand the memory population in the face of cognate T cell self-regulation [206].

Adenovirus vectors used in the context of heterologous prime boost regimens have also been shown to elicit potent humoral responses. Adenovirus-MVA regimens have been shown to induce high-titer and protective antibody responses against blood stage malaria parasites in mouse models [207,208], and these pre-clinical studies have translated into robust humoral immunity in recent clinical trials [173,174]. Indeed, the use of adenoviruses as priming agents has been shown to abrogate the requirement for potent adjuvants to enhance antibody titers elicited by recombinant protein vaccines [209]. It is believed that efficient priming in this context is mediated by superior germinal centre formation after adenovirus vector administration, perhaps as a consequence of persistent transgene expression (de Cassan *et al*, manuscript in preparation).

1.5 Outline of thesis

- **Chapter 2** provides a description of the materials and methods used during the course of this study
- **Chapter 3** describes the construction and optimization of a new replication defective vector, ChAdOX1, based on chimpanzee adenovirus Y25
- **Chapter 4** provides a comparative assessment of the immunogenicity of new vector ChAdY25/ChAdOX1 with existing vectors HAdV-5, AdC68 and ChAd63 in the mouse model.
- **Chapter 5** investigates mechanisms responsible for differences in immunogenicity between adenovirus vectors of different serotype with a focus on infectivity and transgene expression.
- **Chapter 6** investigates the role of innate immunity on the immunogenicity of adenovirus vectors.
- **Chapter 7** provides a discussion and draws conclusions from the results presented in the current study.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Material	Supplier	Catalogue
0.2µm syringe filter	Sartorius	16534
1.1X Thermo-Start ReddyMix PCR Master Mix	Thermo Scientific	AB-1960
10mM dNTPs	Sigma Aldrich, UK	DNTP100
15mL centrifuge tubes	Fisher, Inc.	FB55950
2-Deoxy D Galactose	Sigma Aldrich, UK	D4407
2-β Mercaptoethanol [50µm]	Gibco	31350-010
50mL centrifuge tubes	Greiner	227261
6-well tissue culture plates	Fisher, Inc.	TKT-520-
70µM cell strainers	BD Biosciences	352350
Adenovirus QuickTiter™ Immunoassay kit	Cell Biolabs	VPK-109
Agar	Sigma Aldrich, UK	A6686
Agarose	Sigma Aldrich, UK	A9539
Ampicillin	Sigma Aldrich, UK	A5354
Anti Influenza A M1 mouse monoclonal	AbCam	ab22396
Anti V5 tag (SV5-Pk1) mouse monoclonal	AbCam	Ab27671
Anti-Influenza A nucleoprotein mouse	AbCam	Ab20343
Anti-mouse CD11c APC	eBioscience	17-0114
Anti-mouse CD127 eFluor 450	eBioscience	48-1271
Anti-mouse CD16/32 (Fc block)	eBioscience	14-0161
Anti-mouse CD4 APC-A780	eBioscience	47-0042

Anti-mouse CD62L PECy7	eBioscience	25-0621
Anti-mouse CD8α PerCP-Cy5.5	eBioscience	45-0081
Anti-mouse IFN-γ APC	eBioscience	17-7311
Anti-mouse IgG1biotin	BD Biosciences	553441
Anti-mouse IgG2a biotin	BD Biosciences	553388
Anti-mouse interleukin-2 (IL-2) PE	eBioscience	12-7021
Anti-mouse TNFα FITC	eBioscience	11-7321
Anti-rabbit IgG alkaline phosphatase conjugate	Sigma Aldrich, UK	A8025
Benzonase ® Nuclease	Novagen	70664-3
Black 96 well assay plate	Costar	3915
Bovine Serum Albumin	PAA: Cell culture	K41-001
BrightGlow Luciferase Reagent	Promega	E2620
Chloramphenicol	Sigma Aldrich, UK	C7795
Cytofix/Cytoperm™ fixation/permeabilization solution kit	Becton Dickinson	555028
D Biotin >99%	Sigma Aldrich, UK	B4501
D-Galactose	Sigma Aldrich, UK	G0750
DH5α sub-cloning efficiency cells	Life Technologies	18265-017
Diethanolamine buffer	Thermo Scientific	34064
Difco MacConkey Agar Base	Becton Dickinson	281810
Dimethyl suloxide (DMSO)	Sigma Aldrich, UK	D2650
D-Luciferin Potassium Salt	Caliper Life Sciences	122796
Dulbecco's PBS	Sigma Aldrich, UK	D8537
Dulbecco's Modified Eagle's Medium	Sigma Aldrich, UK	D6546
ELIspot colour development kit	BioRad	170-6432
Endotoxin Free water	Sigma Aldrich, UK	W3500

Ethanol	Ethanol	32221
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, UK	E7889
Expand PCR High Fidelity Kit	Roche	11 732 641 001
ExtrAvidin Alkaline Phosphatase	Sigma Aldrich, UK	E2636
Fœtal Calf Serum (FCS)	Sigma Aldrich, UK	F-2442
Gateway® LR Clonase® II Enzyme Mix	Life Technologies	11791-020
Gene pulsar cuvette 0.1 cm	BioRad	165-2089
GenElute Mamalian Genomic DNA	Sigma Aldrich, UK	G1N350
Glycerol, for molecular biology	Sigma Aldrich, UK	G5516
Goat anti-mouse whole IgG alkaline phosphatase conjugate	Sigma Aldrich, UK	A-3562
Golgi Plug	Becton Dickinson	555028
ImmPACT DAB	VectorLabs	SK-4105
Isoflo	Oxford University	n/a
Kanamycin	Sigma Aldrich, UK	K0254
L-Leucine	Sigma Aldrich, UK	L8000
LB Agar	Sigma Aldrich, UK	L7025
LB Broth	Sigma Aldrich, UK	L7275
L-Glutamine 200mM	Sigma Aldrich, UK	G7513
Lipofectamine™	Life Technologies	11668-019
LIVE/DEAD® Aqua	Life Technologies	L34957
M9 minimal salts	Sigma Aldrich, UK	M6030
MAIP ELIspot Plates	Millipore	MAIPS4510
Max efficiency DH5α competent *E. Coli*	Life Technologies (Invitrogen)	18258-012
MEM α-modification	Sigma Aldrich, UK	M-4526

Methanol	Sigma Aldrich, UK	32213
Micro-fine Insulin Syringe with needle 1mL 29gx12.7mm	Nu-care products	M4891
Microlance needle 26g x 5/8 inch	Nu-care products	M4300
Microvette tubes (CB300K2E)	Sarstedt	16.444
MinElute gel extraction kit	QIAGEN	28606
Mouse IFN-γ ELISPOT kit (ALP)	Mabtech	3321-2A
Mouse IgG1 K Isotype Control Purified	eBioscience	14-4714-85
Mouse IgG2a K Isotype Control Purified	eBioscience	14-4724-85
Murine Granulocyte/Macrophage Colony	Peprotech	315-03
NP40 Tergitol	Sigma Aldrich, UK	NP40S
NUNC Maxisorp 96-well plates	Fisher, Inc.	DIS-971-030J
Omniscript Reverse Transcriptase Kit	QIAGEN	205113
One Shot ccdB Survival 2 T1R competent *E. Coli*	Life Technologies (Invitrogen)	A10460
OptiMEM™ Reduced Serum Medium	Life Technologies	31985
PBS/Tween Sachets	Sigma Aldrich, UK	P3563
Pen/strep [100U penicillin 100μg strep]	Sigma Aldrich, UK	P0781
Phire Hot Start DNA Polymerase (F-120)	New England Biolabs	F-120S
Phospha-light ™, Reporter Assay for Secreted Alkaline Phosphatase (SEAP)	Applied biosystems	T1016
Phosphate Buffered Saline (PBS)	Sigma Aldrich, UK	D-8537
Photinus luciferase protein	Sigma	L9506
Phusion High Fidelity PCR Master Mix	New England Biolabs	M0531
Phusion Hot Start High Fidelity DNA	New England Biolabs	F-540S
Plastipak syringe 1mL Luer slip	Nu-care products	M0013
pNPP (20mg tablets)	Sigma Aldrich, UK	N-2765
Poly (I:C)	Sigma Aldrich, UK	P1530

Polyclonal rabbit anti-mouse IFN-β	Chemicon	AB2215
Proteinase K	QIAGEN	1017738
QIAGEN plasmid midi kit	QIAGEN	12143
QIAprep spin miniprep kit	QIAGEN	27106
Quantitect SYBR Green PCR Kit	QIAGEN	204143
Rat anti-mouse IFN-β monoclonal (7FD3)	AbCam	ab24324
Recombinant human Factor X	Cambridge Bioscience	HCX-0050
Recombinant Luciferase protein	Sigma Aldrich, UK	A7641
Recombinant mouse IFN-β	PBL Interferon Source	12400-1
Reporter Lysis Buffer (5X)	Promega	E3971
Restriction endonucleases	New England Biolabs	Various
RNeasy Mini Kit	QIAGEN	74104
RPMI-1640	Sigma Aldrich, UK	R0883
S.O.C. medium	Life Technologies	15544-034
Smart Ladder DNA marker	EurogenTec	MW-1700-02
Sodium azide	Sigma-Aldrich, UK	S2002
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, UK	L4390
SYBR Safe in DMSO	Life Technologies	S33102
T150 culture flasks	BD Falcon	353025
T4 DNA Ligase	New England Biolabs	M0202
Tris-acetate-EDTA	Fisher, Inc.	BP1332-1
Trizma® HCL buffer solution (pH7.8)	Sigma Aldrich, UK	T2569
U bottom plates, with lid, 96-well	Jencons	734-0027
V bottom plates, 96-well	Fisher, Inc.	DIS-210-150L
Zero Blunt TOPO PCR Cloning Kit	Life Technologies	45-0245

Sucrose	Fluka	84097
1M $MgCl_2$	Sigma Aldrich, UK	63069-100ML
Caesium Chloride	Sigma Aldrich, UK	203025
Ultraclear centrifuge tubes 14.0 ml	Beckman Coulter	344060
Slide-A-Lyser Dialysis Cassettes, 10K	Thermo Scientific	66455
TrypLE™ Express	Life Technologies	12605010
Blasticidin (10mg/ml)	Melford Labs	B1105
Trypan Blue	Sigma Aldrich, UK	T8154
T25 Cell Culture Flask	Fisher, Inc.	TKV123011R
24 well tissue culture plate	Fisher, Inc.	TKT190010Y
HYPERFlask	Fisher, Inc.	TKV128020J

2.1.2 Solutions

- **ACK Lysis Buffer**: 8.29g NH_4Cl (0.15M), 1g $KHCO_3$ (1mM), 37.2mg Na_2EDTA in 800mL dH_2O. pH adjusted to 7.2-7.4 with HCl (1M) before making a final solution up to 1L with dH_2O.
- **ELISA / ELISpot Coating Buffer**: 15mM sodium carbonate and 35mM sodium bicarbonate capsules were dissolved in dH_2O and autoclaved.
- **Complete αMEM Medium**: Minimal Essential Media (MEM) α-modification was supplemented with 5mL L-glutamine (2mM), 5mL pen/strep (100U penicillin, 100μg streptomycin), 500μl 2-Mercaptoethanol (50μm) and 50mL of heat inactivated foetal calf serum (FCS) (10%).

- **Complete DMEM Medium**: Dulbecco's Modified Eagle Medium was supplemented with 5mL L-glutamine (2mM), 5mL pen/strep (100U penicillin, 100μg streptomycin), and 50mL of foetal calf serum (FCS) (10%).
- **Diethanolamine Buffer**: A 5x concentrate was purchased (Thermo Scientific) and diluted with dH_2O before use.
- **FACS buffer:** 2.5g (0.5%) Bovine Serum Albumin (BSA) was added to 500mL PBS.
- **DNA Loading Dye:** 0.4% orange G, 15% Ficoll® 400, 10mM Tris-HCl (pH 8.0), 50mM EDTA (pH 8.0) in dH_2O.
- **Phosphate buffered saline (PBS)** 0.01M: NaCl 0.138M, KCl 0.0027M, pH 7.4: made by dissolving tablets in dH_2O.
- **PBS/Tween (PBS/T)** 0.01M: NaCl, 0.138M, KCl 0.0027M, pH 7.4 Tween-20 0.05%. Sachets dissolved in dH_2O.
- **Tris-acetate-EDTA** (TAE) buffer: Made from 50x concentrate with dH_2O.
- **M63 Minimal Agar Plates:** 5X M63 solution was prepared by dissolving 10g $(NH_4)_2SO_4$, 68g KH_2PO_4, 2.5mg $FeSO_4 \cdot 7H_2O$ in 1L dH_20 and adjusted to pH 7 with addition of KOH. To prepare the agar, 7.5g agar was dissolved in 400ml dH_20, prior to addition of 100ml 5X M63 solution. After equilibration to 50°C, 0.5ml 1M $MgSO_4 \cdot 7H_2O$, 0.5mg d-biotin, 25mg L-leucine and a carbon source at a final concentration of 0.2% (either galactose or 2-deoxy-galactose and glycerol). The antibiotic chloramphenicol was added at a concentration of 12.5 mg/ml. 500ml agar was used to pour 12-20 plates.

- **M9 Salt Solution:** 6g Na_2HPO_4, 3g KH_2PO_4, 1g NH_4Cl, 0.5 g NaCl was dissolved in 1L dH_20.
- **NP-40 lysis buffer:** 50mM Tris HCL pH8.0. 150mM NaCl, 5mM MgCl2, 0.6% (v/v) NP-40 Tergitol.
- **Cesium Chloride solutions:** CsCl solutions of density 1.25g/ml or 1.35g/ml were prepared in 10mM Tris pH7.8 buffer with filter sterilized deionised H_20.
- **Lysis buffer for vector preparation:** 10 mM Tris, 1 mM $MgCl_2$. pH 7.8, filter sterilized.
- **Vector storage buffer:** 10 mM Tris, 7.5% w/v sucrose. pH 7.8, filter sterilized.
- **LB Agar / LB Broth:** Tablets (Sigma) dissolved in dH20 (1 tablet per 50ml). Antibiotics added at the following working concentrations: Ampicillin 100µg/ml, Chloramphenicol 15µg/ml, Kanamycin 25µg/ml.

2.1.3 Cell lines

HEK293 and 293TREX cell lines (Invitrogen) were maintained by the Viral Vector Core Facility at the Jenner Institute, University of Oxford. Human lung carcinoma cell line A549 was obtained from the European Collection of Cell Cultures (ECACC) (Cat. 86012804). Human ovarian carcinoma cell line SKOV-3 was received from Clemens Thoma, Department of Clinical Pharmacology, University of Oxford. All cell lines were maintained in complete DMEM, but 293TREX cells were supplemented with 5 µg/ml blasticidin.

2.2 Methods: General Molecular Biology Techniques

2.2.1 Genome sequencing and phylogenetic analysis

Sequencing of the Y25 viral genome was performed by Eurofins MWG Operon AG using GS SLX Titanium Series technology. Previously reported sequences were obtained from Genbank, accession numbers as follows:

ChAd3 CS479276; **ChAd63** CS479277; **HAdV-5** AC_000008; **SAdV-22** AY530876; **SAdV-25** AF394196, **HAdV-1** AF534906; **HAdV-2** J01917; **HAdV-4** AY458656; **HAdV-6** FJ349096; **HAdV-12** X73487; **HAdV-16** AY601636; **HAdV-30** DQ149628; **HAdV-10** AB369368, AB330091; **HAdV-17** HQ910407; **HAdV-9** AJ854486; **HAdV-37** AB448776; **HAdV-8** AB448767; **HAdV-7** AB243119, AB243118; **HAdV-11** AC_000015; **HAdV-21** AY601633; **HAdV-34** AY737797; **HAdV-35** AC_000019; **HAdV-40** NC_001454; **HAdV-41** DQ315364; **SAdV-21** AC_000010; **SAdV-23** AY530877; **SAdV-24** AY530878; **HAdV-3** DQ086466; **HAdV-18** GU191019; **HAdV-31** AM749299; **HAdV-19** AB448774; **SAdV-25.2** FJ025918; **SAdV-30** FJ025920; **SAdV-26** FJ025923; **SAdV-38** FJ025922; **SAdV-39** FJ025924; **SAdV-36** FJ025917; **SAdV-37.1** FJ025921; **HAdV-14** AY803294; **SAdV27.1** FJ025909; **SAdV-28.1** FJ025914; **SAdV33** FJ025908; **SAdV35.1** FJ025912; **SAdV31.1** FJ025906; **SAdV34** FJ025905; **SAdV40.1** FJ025907; **SAdV3** NC_006144; **Y25** JN254802.

Sequence alignment was performed with DNAStar Megalign software using the ClustalW algorithm [210]. Phylogenetic and bootstrap analyses were performed

utilising the neighbour joining method with 100 bootstrap reconstructions for each gene. Trees were modified in FigTree v1.3.1

2.2.2 Agarose Gels

DNA samples were analysed by 1% agarose gel electrophoresis (100V, 50min) in 1x Tris-acetate-EDTA (TAE) buffer stained using SYBR Safe. DNA was visualised on a UVP transilluminator, and the length of DNA fragments estimated by comparison to the molecular weight marker SmartLadder (Eurogentec). Fragments for cloning or sequencing applications were purified from agarose gel using the QIAGEN MinElute DNA purification kit according to manufacturer's protocol.

2.2.3 DNA restriction digest

Restriction endonucleases were obtained from New England Biolabs (NEB), UK. Reactions were performed in one of four NEB reaction buffers, the identity of which was dependent upon compatibility with the specific restriction endonuclease. For basic cloning, a standard 20µl digest was performed:

NEB Restriction Endonuclease	-	1µl
NEB Buffer (1-4) 10X	-	10µl
dH_2O	-	To total volume 20µl
DNA	-	0.5-1µg

2.2.4 DNA Ligation

T4 DNA ligase (NEB, UK) was used in cloning schemes to join molecules of double stranded DNA together. Specifically, the enzyme catalyzes the formation of a phosphodiester bond between the 3' hydroxyl end of one nucleotide with the 5' phosphate end of another. For a typical 10µl µl ligation:

T4 DNA Ligase (20U) - 1µl
NEB Ligase Buffer (10X) - 1µl
Restriction digested insert DNA
Restriction digested vector DNA } Total volume 8µl
dH_2O

Note: In this instance, enzyme units (U) are Cohesive End Units as described by NEB. For restriction digested DNA molecules with compatible cohesive ('sticky') ends, the molar ratio of insert to vector DNA was 3:1.

2.2.5 High fidelity PCR

Phusion High Fidelity PCR Master Mix (Finnzymes, distributed by NEB, UK) was used to amplify gene fragments for cloning or sequencing applications where nucleotide sequence of the amplified transcript was important. PCR was performed according to the following protocol (50µl reaction):

Reagent:	Volume:	Final Concentration:
Phusion High Fidelity Master Mix (2X)	25µl	1X
Forward Primer (10µM)	2.5µl	0.5µM
Reverse Primer (10µM)	2.5µl	0.5µM
dH_20	15µl	
Template DNA (5ng/ul)	5µl	25ng total

PCR Programme:

98°C	2 min	Initial denaturation	
98°C	15s	Denaturation	x 35 cycles
55-65°C	30s	Annealing	
72°C	2 min	Elongation	
72°C	10 min		
4°C	Hold		

Note: NEB recommend allowing 15-30s/kb so elongation times were adjusted to suit the length of amplicon.

2.2.6 Colony PCR Screening

This technique enabled the screening of recombinant DNA clones direct from the bacterial colony without the need for prior DNA purification. Bacterial colonies were picked using a 10µl pipette tip and transferred to a PCR tube containing 5µl dH_20. The tip was then used to streak a reference agar plate before discard. The PCR tube was then sealed and incubated at 95°C for 10min to lyse the bacterial cells and release the DNA. After briefly cooling, a PCR mix based on the Phire Hot Start PCR reagent (Finnzymes, distributed by NEB, UK) was added to the tube:

Reagent:	Volume:	Final Concentration:
Phire Hot Start Polymerase	0.4µl	1U
Phire Reaction Buffer (5X)	4µl	1X
dNTP (10mM)	0.4µl	200µM
Forward Primer (10µM)	1µl	0.5µM
Reverse Primer (10µM)	1µl	0.5µM
dH_20	8.2µl	

PCR was then performed (total reaction volume 20µl) according to the following programme:

98°C	30s	Initial denaturation	
98°C	5s	Denaturation	x 35 cycles
55-65°C	5s	Annealing	
72°C	20s	Elongation	
72°C	1 min		
4°C	Hold		

NEB recommend allowing 10-15s/kb so elongation times were adjusted to suit the length of amplicon.

2.2.7 Restriction cloning of recombinant antigens

Antigen DNA sequences were either obtained from existing constructs (TIPeGFP, eGFP, *Photinus* Luciferase) or synthesized by GeneArt® (Invitrogen) (Ag85A, NP+M1). In the case of the Secreted Alkaline Phosphatase (SEAP) antigen, viral DNA from an existing adenoviral vector (ChAd63-SEAP) received from Okairos AG (Rome, Italy) was amplified by high fidelity PCR (Section 2.2.5) with primers designed to include convenient restriction endonuclease sites for sub cloning. Recombinant antigens were cloned into Invitrogen Gateway™ 'Entry' vectors for efficient generation of recombinant adenovirus molecular clones (Section 2.2.8). Transgene expression cassettes within 'Entry' vectors contained the human cytomegalovirus IE promoter (CMV IE) with intron A to drive constitutive high level expression of recombinant antigen *in vivo* [211]. A multiple cloning site immediately after CMV IE allowed insertion of antigen sequences prior to the bovine growth hormone polyadenylation signal (*bgh*-

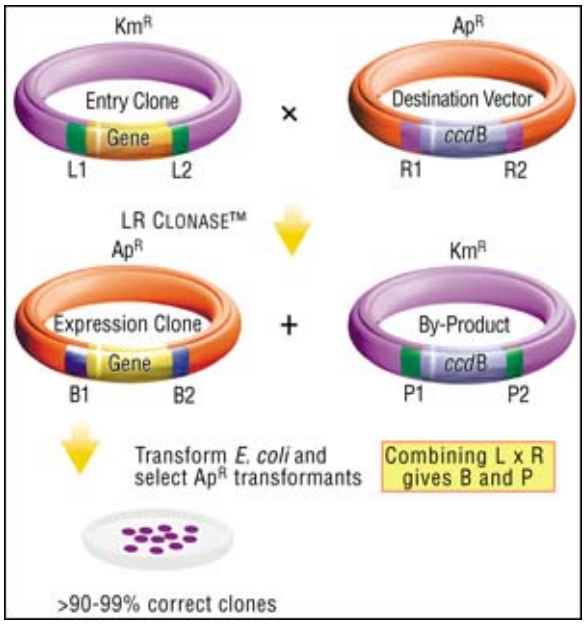
PolyA), a specialized termination sequence for protein expression in eukaryotic cells. Constructs for expression of Ag85A, NP+M1 and pLuciferase also contained a *tet* operator sequence within CMV IE for repression of transgene expression during virus production in 293TREX cells which constitutively express the Tet repressor protein.

To generate new 'Entry' clones, antigen sequences were excised from existing plasmids by restriction digest and ligated into 'Entry' vectors treated with the same restriction enzymes (Sections 2.2.3 and 2.2.4). Ligation products were transformed into 50µl chemically competent DH5α *E. Coli* by heat shock at 42^{o}C according to manufacturers protocol. After recovery of bacteria in SOC medium (Invitrogen) for 1 hour at 37^{o}C, cells were plated onto LB agar containing an appropriate antibiotic for selection and incubated overnight at 37^{o}C. Selected colonies were picked from plates and used to inoculate 2ml cultures which were incubated for 12-16 hours at 37^{o}C in a shaking incubator. DNA was extracted from bacterial culture by QIAGEN Miniprep, according to the manufacturer's protocol. Clones were then screened for the correct nucleotide sequence by diagnostic restriction digest of miniprep DNA. Alternatively, colonies were screened for positive clones by colony PCR (Section 2.2.6). Correct clones were selected and used to inoculate 150ml cultures, providing sufficient material for a QIAGEN Midiprep which typically yields up to 100µg transfection grade DNA. After a final restriction screen, 'Entry' vector DNA was used to generate a recombinant adenovirus molecular clone using Gateway™ technology (Section 2.2.8).

2.2.8 Generating recombinant adenovirus vectors using Gateway™ technology

Gateway™ is a universal cloning technology that takes advantage of the site specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to transfer a DNA sequence of interest into multiple vector systems. Gateway™ technology [212] was utilized to generate recombinant adenovirus genomic clones, prior to transfection of the genomic DNA into mammalian cells to generate recombinant virus. Vaccine antigens were directionally inserted into the adenoviral genome at the E1 locus by *in vitro* Gateway™ recombination. 'Destination' vectors containing the E1 E3 deleted adenoviral genome were constructed by inserting a cassette consisting of the *CcdB* lethal gene from the *E. Coli* F plasmid, flanked by specific phage lambda *attR* sites at the E1 locus. Note that these vectors were propagated in CcdB Survival (Invitrogen) *E. Coli* cells which are resistant to the action of CcdB. Vaccine antigen constructs, including the CMV promoter and BGH poly A region, were cloned into 'Entry' vectors, between specific phage lambda *attL* sites. The LR Clonase™ enzyme was used to perform a site specific recombination between *attL* sites in the 'Entry' vector and *attR* sites in the 'Destination' vector to directionally insert vaccine antigen constructs into the 'Destination' vector according to the manufacturers protocol (Figure 2.1). *In vitro* recombination products were transformed into standard DH5α *E. Coli* (or DH10B for BAC vectors), and negative selection against the presence of *CcdB* ensured that over 90% of resulting colonies were positive for the recombinant 'Expression' clone.

Figure 2.1: Overview of the Gateway™ system. From bioresearchonline.com.



2.2.9 BAC *GalK* Recombineering

2.2.9.1 Principal

Recombineering (recombination-mediated genetic engineering) is a technique enabling the precise modification of BAC DNA by homologous recombination. For insertion of specific DNA sequences, a PCR amplified cassette is generated containing the target sequence flanked by homology arms to facilitate homologous recombination with the BAC vector. *GalK* recombineering, performed using a specifically modified strain of *E Coli*, is particularly advantageous since both positive and negative selection can be conferred using the *GalK* gene. This enables convenient removal of the marker gene, allowing seamless modification of BAC DNA.

The modified *E. Coli* strain, SW102, developed by Soren Warming (NCI-Frederick, MD) [213], was derived from the DY380 strain [214]. DY380 contains a stably integrated defective λ prophage containing λ-Red encoded genes, *exo*, *bet* and *gam*, which are essential for homologous recombination. These genes are expressed under the strong phage promoter pL, and under stringent control of the temperature sensitive *c1857* repressor. This repressor system prevents expression of these genes at 32^{o}C, but once the temperature is raised to 42^{o}C expression is permitted enabling efficient homologous recombination to occur. This inducible system is essential to ensure that recombination occurs only at the desired locus.

SW102 cells have been modified to include the galactose operon with the galactokinase (*galK*) gene deleted. Galactokinase, is essential for galactose metabolism, catalysing the first step in the galactose degradation pathway; the

phosphorylation of galactose to galactose-1-phosphate. *GalK* funtion can be restored *in trans*, enabling the bacteria to utilize galactose as the sole carbon source. Hence, the inclusion of *galK* in recombineering cassettes enables positive selection of recombinants on minimal media containing galactose. Conveniently, galactokinase also efficiently catalyzes the phosphorylation of a galactose analog, 2-deoxy-galactose (DOG). The product of this reaction cannot be further metabolized, leading to a toxic accumulation of 2-deoxy-galactose-1-phosphate. Hence, the inclusion of DOG in place of galactose in selection media enables negative selection of *GalK* recombinants.

2.2.9.2 Amplification of homology cassette

Primers were designed to include a 50bp homology region to the target BAC to facilitate homologous recombination. Oligonucleotides for PCR amplification of the targeting cassette were therefore typically 70-80 nucleotides in length. These were synthesized by Eurofins MWG Operon with HYPUR purification, a technique based on gel electrophoresis which guarantees >90% purity of product. PCR was performed using the Phusion Hot Start high fidelity PCR reagent, according to the following protocol (50ul reaction):

Reagent:	Volume:	Final Concentration:
Phusion HF buffer (5X)	10µL	1X
Phusion Hot Start Polymerase	0.5µL	1U
dNTPs (8mM)	1.25µL	200µM each
Primer F (1µM)	2.5µL	0.5 µM
Primer R (1µM)	2.5µL	0.5 µM
Template DNA (4ng/µL)	2.5µL	10ng total
DMSO	1.5µL	3%
Water	29.25µL	

PCR Programme:

98°C	30s	Initial denaturation	
98°C	10s	Denaturation	x 35 cycles
68°C	10s	Annealing	
72°C	40s	Elongation	
72°C	5 minutes		
4°C	Hold		

NEB recommend allowing 15-30s/kb so elongation times were adjusted to suit the length of amplicon. After PCR, 2ul DpnI restriction endonuclease was added and the reaction mix incubated at 37°C for 1 hour. DpnI cleaves methylated DNA, and is required to eliminate residual template DNA. The PCR product was then separated on a 1% agarose gel by electrophoresis and purified from the gel using a QIAGEN MinElute Gel Extraction kit. DNA was eluted in 10µl with a typical concentration of 20-100ng/µl.

2.2.9.3 Method

SW102 cells containing the target BAC vector were cultured overnight at 32°C with chloramphenicol selection (12.5 mg/ml). 1 ml of the culture was diluted in 50 ml Luria–Bertani (LB) medium containing chloramphenicol and incubated in a shaking waterbath at 32°C to an OD_{600} of 0.6. Then, 25ml of the culture was transferred to a separate conical flask and incubated at 42°C for exactly 15 min in a shaking waterbath to induce λ-Red recombination genes. The remaining culture was left at 32°C as an uninduced control. After 15 min, both induced and uninduced cultures were briefly cooled on ice and transferred to

two 50ml Falcon tubes (BD Biosciences). Cells were pelleted at 5000g at 0°C for 5 min. The supernatant was decanted, and the pellet resuspended in 10 ml ice-cold dH2O. Cells were pelleted once again, and this wash step repeated a further time. After the second wash and centrifugation, the supernatant was removed and the kept on ice prior to transformation with the PCR amplified target cassette. Transformation of SW102 cells was achieved by electroporation in a 0.1 cm cuvette (BioRad) at 1.8 kV, 25 μF, and 200Ω using a BioRad GenePulser. At least 100ng of purified PCR product was used for transformation. After electroporation, bacteria were recovered in 1ml LB for 1 hour in a 32°C shaking waterbath. After recovery, the SW102 cells were washed twice in 1X M9 salts to remove the rich LB medium. After the second wash, the pellet was resuspended in 1 ml of 1X M9 salts before plating on M63 minimal media plates containing either galactose (for positive selection) or 2-deoxygalactose and glycerol (for negative selection). Plates were incubated for 3-4 days at 32°C prior to the emergence of recombinant colonies.

2.2.10 Gap repair using BJ5183 *E. Coli*

Bacterial artificial chromosome and plasmid based constructs for gap repair were linearised by restriction digest at the insertion locus, the restriction endonuclease heat inactivated prior to transformation. DNA for 'repair' containing homology to linear ends of the BAC/plasmid was purified, either by QIAGEN MinElute (if a PCR product) or phenol purification (if linear virus genomic DNA). A fresh culture of BJ5183 *E. Coli* (Stratagene) was grown to an OD_{600nm} of 0.6, and washed twice in ice cold distilled H_20 to generate

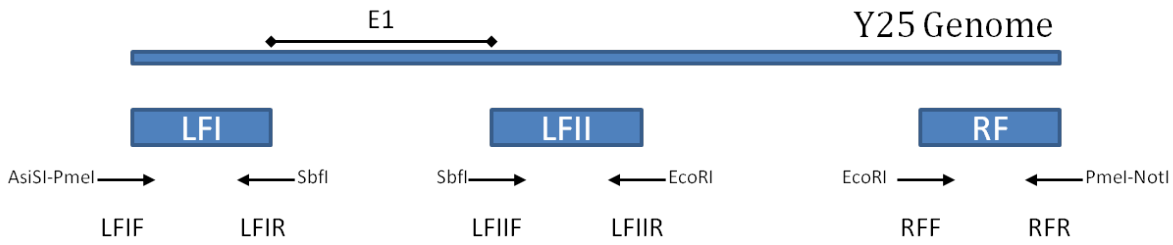
electrocompetent cells. Simultaneous transformation of competent cells with both BAC/plasmid and 'repair' DNA was performed using a BioRad GenePulsar with 0.1cm cuvette at 1.8 kV, 25μF, and 200Ω. A molar excess of 'repair' DNA to vector DNA (ratio of approx 1:25) was used to promote 'gap repair' homologous recombination. Cells were incubated for an hour after electroporation at 37°C in a shaking waterbath before plating onto agar containing an appropriate antibiotic. Since proteins required for recombination are expressed constitutively in BJ5183 cells, plates and cultures were incubated for the minimum time possible to prevent erroneous recombination.

2.3 Methods: Generating BAC-ChAdY25

2.3.1 Construction of Y25 BAC rescue vector

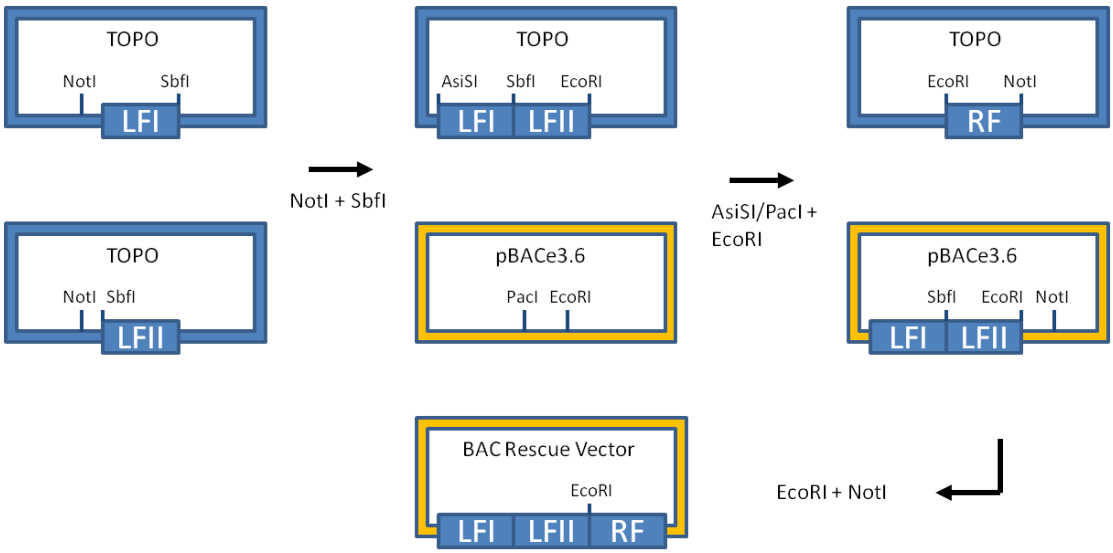
A molecular clone of the Y25 virus was generated by gap repair homologous recombination using a BAC vector containing regions of homology to both ends of the viral genome (Section 3.2). The first step in the construction of the BAC 'rescue' vector was amplification of homology arms from Y25 viral DNA using Phusion™ high fidelity PCR (Figure 2.2).

Figure 2.2: Amplification of homology arms for Y25 BAC 'rescue' vector. Primer sequences are given in Table 2.1. LFI – Left Flank I, LFII – Left Flank II, RF – Right Flank.



The next step was to clone the amplified fragments into Invitrogen TOPO® vectors using the PCR Blunt II TOPO kit (Invitrogen), following manufacturer's instructions. The steps involved in assembly of the BAC rescue vector by restriction cloning are outlined below in Figure 3.3:

Figure 2.3: Cloning scheme to construct Y25 BAC 'rescue' vector



The LFI fragment was cloned into the LFII-TOPO vector using NotI and SbfI. The LFI/LFII cassette was then cloned into the pBACe3.6 vector, taking advantage of the complementary 'sticky ends' left by enzymes PacI and AsiSI, such that ligation resulted in removal of the PacI site in the pBAC vector. This enabled PacI to be used later as a unique site at the E3 locus of the adenoviral genome. The final RF homology region was cloned into pBAC-LFI/II using EcoRI and NotI to generate the final rescue vector. The genome of the Y25 virus was incorporated into the BAC by BJ5183 gap repair as described in Section 2.3.2, using EcoRI to linearise the vector prior to recombination.

2.3.2 Generating the E1 E3 deleted ChAdY25 vector

For gap repair insertion of the Y25 genome into the BAC vector, BJ5183 electrocompetent *E. coli* cells (Stratagene) were co-transformed with 20ng BAC vector and 500ng Y25 genomic DNA and selected using the chloramphenicol resistance marker on pBACe3.6. Resulting colonies were screened by colony PCR

for insertion of the genome at both flanks, and for deletion of the E1 region; a consequence of homologous recombination between BAC and genome at LFII instead of LFI (Section 3.2.2). E1 deleted clones were selected, and genome integrity was checked by restriction digest.

Galactokinase (*GalK*) based recombineering was performed as described in Section 2.2.9 to delete the Y25 E3 region, leaving in its place a unique *PacI* site. *GalK* replacement of the E3 region was performed using primers E3delLH and E3delRH (Table 2.1). The PCR amplified *GalK* gene was flanked by *PacI* sites, enabling subsequent *GalK* deletion by restriction digestion and re-circularization rather than by counterselection to leave a unique *PacI* site at E3. A unique *AsiSI* site was introduced at the E1 locus in place of *SbfI*, again using *GalK* recombineering. The *GalK* gene was PCR amplified with flanking *AsiSI* sites using primers E1AsiSIinsLH and E1AsiSIinsRH (Table 2.1). The *AsiSI* site at the E1 locus was subsequently used to insert a modified *attR1 attR2* Gateway® cassette (Invitrogen) for site specific directional insertion of recombinant antigens at the E1 locus (Section 2.2.8). The *attR1 attR2* destination cassette was PCR amplified from the pAd-PL-DEST vector (Invitrogen) and inserted into a PCR Blunt II TOPO vector for modification (Invitrogen). A chloramphenicol antibiotic resistance marker between *attR* sites was replaced by the kanamycin resistance *AphAI* gene since pBACe3.6 based vectors already confer resistance to chloramphenicol. The modified *attR* destination cassette was inserted into the E1 E3 deleted BAC-ChAdY25 vector by restriction cloning with *AsiSI*.

2.3.3 Generating E4 modified ChAdY25 vectors including ChAdOx1

The E4 region was modified to optimise growth rate and yield in human cell lines. Two *GalK* insertions were made, to delete either the entire native E4 region excluding the E4 promoter (using primers E4delLH/E4wholedelRH), or only open reading frames (Orfs) 4, 6 and 7 (using primers E4delLH / E4Orf4delRH). Subsequent counter-selection replacements of *GalK* with regions of the HAdV-5 E4 genome were performed using PCR products amplified from an HAdV-5 genomic clone pAd-PL DEST (Invitrogen). Negative selection against *GalK* using 2-deoxygalactose was performed as described in Warming *et al [213]* and Section 2.2.9.

2.3.4 Generating chimeric ChAdOX1 vectors ChAdOX1-Ad5VA and ChAdOX1-Ad5Fiber

Chimeric ChAdOX1 based vectors were constructed through BAC *GalK* recombineering, using the *GalK* gene for both positive and negative selection as described previously (Sections 2.3.2 and 2.3.3). Primers used in the construction of ChAdOX1-Ad5VA and ChAdOX1-Ad5fiber are described in Table 2.2. In the construction of ChAdOX1-Ad5VA, native VA-RNA sequences were deleted by *GalK* replacement (using primers VADelGalKF and VADelGalKR), with recombinants selected on M63 minimal plates containing galactose. *GalK* replacement was performed (using primers Ad5insVAF and Ad5insVAR, with plasmid pAD-PL-DEST as template). Selection against the presence of GalK was performed on plates containing 2-deoxygalactose (DOG). Figure 2.4 describes the construction of ChAdOX1-Ad5VA. The ChAdOX1-Ad5fiber chimeric was constructed via a similar process. Primers GalKFiberinsF and GalKFiberinsR

were used for *GalK* insertion at the fiber locus, leaving the N-terminal fiber tail region intact but deleting the fiber knob and shaft domains. Primers Ad5FiberinsF and Ad5FiberinsR were used to insert the corresponding regions of the HAdV-5 fiber in place of *GalK*.

Figure 2.4: Replacement native ChAdOX1 VA-RNA with HAdV-5 VA-RNA sequences through BAC *GalK* recombineering

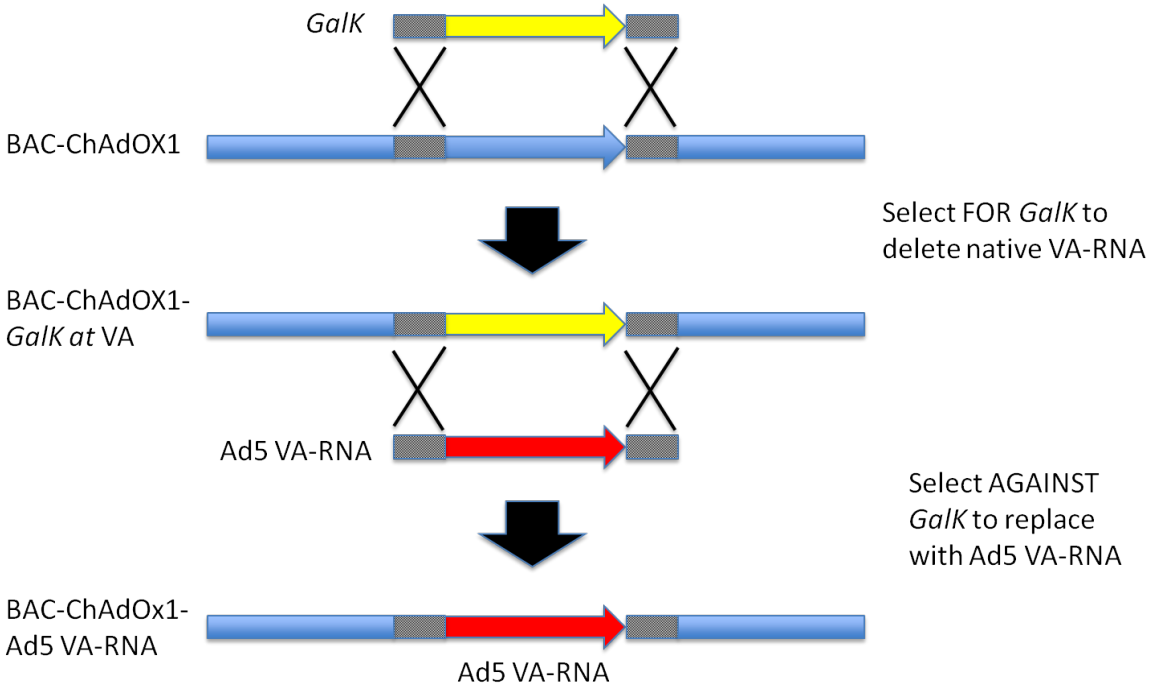


Table 2.1: Primers used in the construction of ChAdY25/ChAdOX1. Restriction sites **bold**; 50 bp homology regions for recombineering in *italic*

Purpose	Primer Name	Template	Sequence 5'- 3'
Amplification of LFI homology arm of rescue	LFI Fwd	Y25 gDNA	GCGATCGCGTTTAAACCCATCATCAATAATATACCTCAA
	LFI Rev		CCTGCAGGCTTTCAAAGTGTAGATCTGACTCG
Amplification of LFII homology arm of rescue	LFII Fwd	Y25 gDNA	CCTGCAGGAGTGAGTAGTGTTCTGGGGCGGGGGAGGAC
	LFII Rev		GAATTCCGGTAACATCGCCAATCTCAAG
Amplification of RF homology arm of rescue	RF Fwd	Y25 gDNA	GAATTCGCAATTTTTAAGAAAATCAACA
	RF Rev		GCGGCCGCGTTTAAACCATCATTCAAATATATACCTCAAAC
Replacement of Y25 E3 region with *GalK*	E3del LH	*GalK* cassette	*TCATCCCGAACTTTGACGCCATCAGCGAGTCGGTGGACGGCTACGATTGA***TTAATTAA**CCTGTTGACAATTAATCATCGGCA
	E3del RH		*CATTTTTAATCTTTATTTTTACTGGAGGGTAAAGGGGTAGGGGGTTAGTTGA***TTAATTAA**TCAGCACTGTCCTGCTCCTT
Replacement of *SbfI* site at E1 with *GalK*	E1 AsiSIins LH	*GalK* cassette	*CGAGAAGAGTTTTCTCCTCCGCGCCGCGAGTCAGATCTACACTTTGAAAG***GCGATCGC**CCTGTTGACAATTAATCATCGGCA
	E1 AsiSIins RH		*GTCATTCTGGCCCTCATGCAGGTCCTCCCCCGCCCCAGAACACTACTCACT***GCGATCGC**TCAGCACTGTCCTGCTCCTT
Replacement of native Y25 E4 region with *GalK*	E4delLH	*GalK* cassette	*CTGAAGCAGAAAAAAATAAAGTTCAAGTGTTTTATTGATTCAACAGTTTTC***CGGACCG**CCTGTTGACAATTAATCATCGGCA
	E4 wholedel RH		*TAGAGCTCAGCCTTTTCTCTGACTCTTGCGCCTGCCGTGCTCGGTAAGCT***CGGACCG**TCAGCACTGTCCTGCTCCTT
	E4Orf4del RH		*CGGGACCTGCATTTTGAAGTGCTCCGAGACCGTTTGGAATAAAGTTAATC***CGGACCG**TCAGCACTGTCCTGCTCCTT
Replacement of *GalK* with variants of HAdV-5 E4 region	Ad5E4Orf6insLH	pAdPL-DEST	*CTGAAGCAGAAAAAAATAAAGTTCAAGTGTTTTATTGATTCAACAGTTTTC*CCTACATGGGGGTAGAGTCATAATCG
	Ad5E4Orf6/7insLH	pAdPL-DEST	*CTGAAGCAGAAAAAAATAAAGTTCAAGTGTTTTATTGATTCAACAGTTTTC*TCACAGAACCCTAGTATTCAACCTG
	Ad5insE4Orf4RH	pAdPL-DEST	*CGGGACCTGCATTTTGAAGTGCTCCGAGACCGTTTGGAATAAAGTTAATC*ATGACTACGTCCGGCGTTCCATTTGG
	Ad5insE4wholedelRH	pAdPL-DEST	*TAGAGCTCAGCCTTTTCTCTGACTCTTGCGCCTGCCGTGCTCGGTAAGCT*ATGACTACGTCCGGCGTTCCATTTGG
	Ad5Orf4insOrf4delRH	pAdPL-DEST	*CGGGACCTGCATTTTGAAGTGCTCCGAGACCGTTTGGAATAAAGTTAATC*ATGGTTCTTCCAGCTCTTCCCGCTC

Table 2.2: Primers used in the construction of ChAdOX1-Ad5VA and ChAdOX1-Ad5Fiber chimeric vectors. 50 base pair homology regions for

Purpose	Primer Name	Template	Sequence 5'- 3'
Replacement of native VA-RNA sequences with *GalK*	VADelGalKF	ChAdOX1	*GCGCGCGCAGTCGTGGATGCTCTATACGGGCAAAAACGAAAGCGGTCAGC*CCTGTTGACAATTAATCATCGGCA
	VADelGalKR		*TGTTGCGGTGGAGGGTGGTGGGGGCGCATCTGCCGCAGTACGGGATGCAT*TCAGCACTGTCCTGCTCCTT
Replacement of *GalK* with HAdV-5 VA	Ad5insVAF	ChAdOX1 GalK at VA	*GCGCGCGCAGTCGTGGATGCTCTATACGGGCAAAAACGAAAGCGGTCAGC*GGGCACTCTTCCGTGGTCTG
	Ad5insVAR		*TGTTGCGGTGGAGGGTGGTGGGGGCGCATCTGCCGCAGTACGGGATGCAT*CTGGGAAAAGCAAAAAAGGGGCTCG
Replacement of native fiber with *GalK*	GalKFiberinsF	ChAdOX1	*TCTTCAGATGGATTCCAAGAAAAGCCCCTGGGGGTGTTGTCCCTGCGACTG*CCTGTTGACAATTAATCATCGGCA
	GalKFiberinsR		*GCTTCAGAGTTTTCCATAGTGGTGGGAACAATGGGGTTGGCATGCAGGGT*TCAGCACTGTCCTGCTCCTT
Replacement of *GalK* with HAdV-5 fiber.	Ad5FiberinsF	ChAdOX1 GalK at Fiber	*TCTTCAGATGGATTCCAAGAAAAGCCCCTGGGGGTGTTGTCCCTGCGACTG*TCCGAACCTCTAGTTACCTCC
	Ad5FiberinsR		*GCTTCAGAGTTTTCCATAGTGGTGGGAACAATGGGGTTGGCATGCAGGGT*TTATTCTTGGGCAATGTATGA

2.4 Methods: General Virology

2.4.1 Transfection of adenovector DNA

Adenovirus vector genomes were linearised at specific PmeI (Y25/ChAdOx1, ChAd63) or PacI (HAdv-5, AdC68) sites to remove non viral plasmid sequences (including BAC sequence, bacterial replication origins and antibiotic resistance markers). Linearisation of the viral genome also releases the inverted terminal repeat (ITR) regions at the end of the genome, allowing replication of the viral DNA in mammalian cells. After heat inactivation of the restriction endonuclease, 6μg linearised viral DNA was used to transfect a T25 tissue culture flask (Corning) of adherent HEK293 cells or 293TREX cells (Invitrogen) at 80-90% confluency. DNA was digested in a total volume of 100μl for 2hr, and after heat inactivation of the restriction endonuclease at 65^{o}C for 30min, 15μl was run on a 1% agarose gel to check the efficiency of linearisation. The remaining 85μl was added to 215μl OptiMEM media without serum (Invitrogen). This DNA-OptiMEM mix was then added to the same volume of Lipofectamine 2000 reagent (Invitrogen) diluted 1:10 in OptiMEM. The solution was incubated at room temperature for 30min to allow the formation of DNA-Lipofectamine complexes before adding dropwise onto the cell monolayer. A further 1.4ml OptiMEM media was added to the T25 flask before incubating the cells at 37^{o}C, 5% CO_2 for 4 hrs. After 4hrs, the media was replaced with 5ml complete DMEM to limit toxicity.

It is important for optimum production of complementing E1 proteins and viral replication that cells are actively dividing. After 3-4 days, confluent cell

monolayers were passaged to maintain cell cycle progression. Media was transferred into a T75 tissue culture flask and 2ml trypsin solution (TrypLE Express, Invitrogen) was added to the monolayer. After 2min at 37^{O}C, cells were detached and complete DMEM was added before transfer of the cell suspension into the T75 flask. After a further 2-3 days, cells were examined for typical adenovirus cytopathic effect (CPE) as a sign of productive viral replication.

2.4.2 Passaging recombinant adenoviruses

Infected cells from the initial transfection were removed from the flask using a cell scraper, and the harvested material was transferred to a 50ml falcon tube. Virus was then released from the cells into the supernatant by three cycles of freeze-thaw; tubes were frozen on dry ice, and thawed briefly in a 37^{o}C waterbath. After freeze-thaw, the cell debris was pelleted by centrifugation at 1500g for 5 min and the supernatant used to infect new HEK293 or 293TREX cells in a 6-well plate to generate a pre-master virus stock. Wells were infected with 1ml, 500µl, 250µl, 125µl, 60µl, or 30µl of supernatant and incubated at 37^{o}C and 5% CO_2 for 48 hours. After this period, wells beginning to show clear signs of CPE were harvested as described previously. Significant cytotoxicity before this time would generally indicate cell death due to excessive multiplicity of infection rather than the emergence of new virus progeny. Pre-master material was used to infect a HYPERFlask^{TM} (equivalent of 10X T150 flasks, Corning) to generate sufficient quantities of recombinant virus for purification. Pre-master material was titered, and HYPERFlasks^{TM} were infected at a multiplicity of infection of five infectious virions per cell (MOI=5). Once characteristic CPE was displayed, cells

were harvested and pelleted by centrifugation in 250ml polypropylene tubes. This time the supernatant was discarded and the cells suspended in 10ml Lysis Buffer (10 mM Tris, 1 mM $MgCl_2$. pH 7.8) for virus purification.

2.4.3 Purification using Cesium Chloride Ultracentrifugation

Recombinant adenovirus was purified by sequential discontinuous and isopycnic cesium chloride (CsCl) ultracentrifugation. Prior to centrifugation, virus lysate was subjected to three cycles of freeze-thaw. After the first cycle, 250U Benzonase Nuclease (Sigma Aldrich, UK) per ml of lysate was added and the material incubated at room temperature for 1hr. After freeze thaw, cell debris was pelleted by centrifugation and the supernatant carefully added onto a discontinuous cesium chloride density gradient consisting of 1.35g/ml CsCl layered below 1.25g/ml CsCl in Beckman Ultraclear centrifuge tubes. Tubes were centrifuged for 2hrs at 110,000g in an Optima L-80 XP Ultracentrifuge (Beckman Coulter) to isolate adenovirus particles by rate zonal separation. Banded adenovirus was extracted by 19G needle and syringe before being added to a new ultracentrifuge tube containing 1.35g/ml CsCl. Adenovirus particles were separated from residual contaminants by isopycnic centrifugation at 160,000g for 16-18 hrs (at 4^{o}C). After the second centrifugation, virus was extracted and dialysed in virus storage buffer (10 mM Tris, 7.5% w/v sucrose. pH 7.8) using Slide-a-Lyser dialysis cassettes (Thermo Scientific, UK). Recombinant virus in storage buffer was dispensed into single use aliquots and stored at -80^{o}C.

2.4.4 Infectivity titration methods

Infectious titer of vector preparations was assessed by single cell infectivity on HEK293 or 293TREX cells. For vectors expressing fluorescent transgenes (TIPeGFP, eGFP, mCherry) infected HEK293 cells were visualized and enumerated directly by fluorescent microscopy. For vectors without a fluorescent marker, infectivity of HEK293 or 293TREX cells was assessed by immunostaining for expression of the Hexon capsid protein.

Tenfold serial dilution of virus in complete DMEM was performed in sterile eppendorf tubes. Two serial dilutions were performed per virus, and 50µl of each dilution (10^3 to 10^10) was added per well of a 96 well plate containing HEK293 cells or 293TREX cells at 80-90% confluency. For immunostaining, 96 well plates should be pre coated with poly-L-Lysine (Sigma, 100µg/ml) prior to seeding to improve cell adhesion to the plate. Plates were incubated for 48 hours at 37^{o}C, 5% CO_2, with additional media (50µl) added after 24 hours to improve the condition of cells. After 48 hours, single infected cells expressing a fluorescent marker were enumerated, and an infectious titer calculated in infectious units (ifu) per ml. For immunostaining, media was aspirated from the cell monolayer and cells were fixed with ice cold methanol for at least 30 min. Plates were then washed three times in PBS before blocking for an hour with 1% BSA/PBS. Detecting mouse polyclonal anti-Hexon antibody (Cell Biolabs Inc., US) was added at 1:1000 dilution and incubated for an hour at room temperature. Alternatively, the detecting antibody may be targeted against the transgene. Mouse monoclonal antibodies AA5H (AbCam, Ab20343), and GA2B (AbCam Ab22396) were used to detect influenza A Nucleoprotein and Matrix 1 protein

respectively. Mouse monoclonal antibody SV5-Pk1 (AbCam, Ab27671) recognizes the PK(V5) tag present in the Ag85A construct, so could be used to detect transgene expression from this vector. After incubation of the primary antibody, cells were washed a further three times prior to addition of a secondary anti-mouse HRP-conjugated antibody (Cell Biolabs Inc, US) again at 1:1000 dilution. After another hour incubation, plates were washed five times in PBS prior to development. To develop, 50 μl of freshly prepared DAB solution (ImmPACT DAB, Vector Labs) was added to each well and plates incubated at room temperature until brown foci appear, representing single infected cells.

2.4.5 Quantification of Virus Particles

The number of adenovirus particles in a purified preparation may be estimated by measuring viral DNA content by spectrophotometric absorption at 260nm as described by Maizel *et al* [215] in 1968. Briefly, samples were diluted 1:10 in virus storage buffer containing 1% sodium dodecyl sulphate (SDS) to release viral DNA from capsids. After incubating at room temperature for 10min, absorbance at 260nm was measured using a Beckman Coulter DU 730 Spectrophotometer. An absorbance of 1.00 (AU, 1 cm pathlength) at 260nm corresponds to 1.1×10^{12} viral particles/ml.

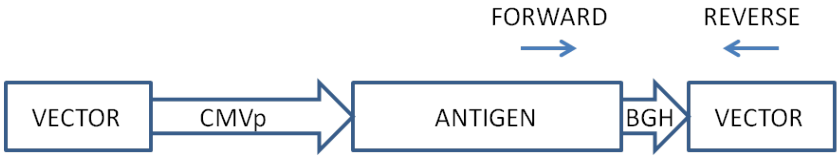
2.4.6 Vector Quality Control

DNA was extracted from CsCl purified virus using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma). Identity and purity of the virus preparation was confirmed by PCR, using the ABgene ReddyMix™ PCR Master

Mix (Thermo Scientific) according to manufacturer's protocol. Specific PCR assays are described below. Each primer is designated a Jenner (J) oligo number.

2.4.6.1 Identity PCR

Identify the presence of the correct antigen insert:



Forward Primers:

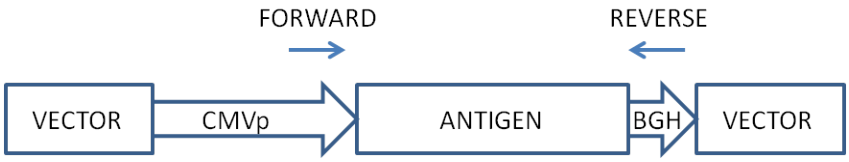
GFP-VC-F (J492)	5'-TCGCCGACCACTACCAGCAGAACACCC-3'
Luciferase (J556)	5'-AAGGGCCCAGCGCCATTCTA-3'
85AU2 (J322)	5'-GCGGACCAGCAACATCAAGT-3'
NP+M1r2 (J225)	5'-GCAGATGCAGCGGTTCAAG-3'
SEAPF (J834)	5'-ATGCTGCTGCTGCTGCTGCTG-3'

Reverse Primers:

AdL (Ad5) (J31)	5'-CTTAAGCCACGCCCACACATT-3'
AdC63L (J311)	5'-CTCGTTCAGGCTGGCGGGATTA-3'
AdC68L (J601)	5'-CTTCCGCTCATGCTGCTGCAACACAC-3'
Y25E1delR (J544)	5'-GCTCATGATGCTGCAACACAC-3'

2.4.6.2 Flank-Flank PCR

Confirm that the vector transgene is the correct size (no deletions):



Forward Primer:

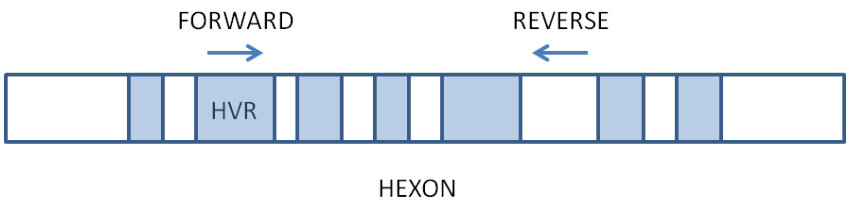
CMV(LP)idF (J711) 5'-TTTCCATGGGTCTTTTCTGC-3'

Reverse Primer:

BGHidR (J690) 5'-GCGATGCAATTTCCTCATTT-3'

2.4.6.3 Serotype PCR

Confirm vector serotype. Forward primers are located in 'hyper variable regions (HVR)' with a sequence specific to a particular serotype. The reverse primer is located in a conserved region, with a sequence common to multiple serotypes.



Forward Primers:

Ad5hexF (J626) 5’-GAAATAAACCTAGAAGAAGAGGACGATG-3’

AdY25hexF (J627) 5’-CACTATGGAAACACACACATTTGGTG-3’

AdC68hexF (J628) 5’-GCACAGATGACAGCAGCTCTTCTA-3’

AdC63hexF (J629) 5’-GAAGGATTCCGACAGCAAAATG-3’

Reverse Primer:

UniversalhexR (J630) 5’-CGTTGAAGGACTGGTCGTTGGTGTCG-3’.

2.5 Methods: *In vitro* Vector Characterisation

2.5.1 *In vitro* infectivity of cell lines by flow cytometry

Cells were seeded in 24 well plates at a density of $2.5x10^5$ cells / well and incubated at 37°C, 5% CO_2 overnight. For studies in the A549 cell line, cells were infected with recombinant adenoviral vectors expressing GFP at different multiplicities of infection, and incubated for a further 24 hours. For Factor X studies, SKOV-3 cells were infected with vectors in serum free media with or without human recombinant Factor X (Cambridge Bioscience) at a concentration of 8ug/ml. After an incubation period of 90 min to enable virus entry, cells were washed and the media replaced with complete DMEM for a further 24 hour incubation. After overnight infection of both A549 and SKOV-3, cells were washed in PBS, detached by brief treatment with trypsin solution (TrypLE Express, Invitrogen) and pelleted by centrifugation at 1500g for 5 min in 96 well cluster tubes (Fisher Scientific). Cells were resuspended in 200µl FACS buffer and samples run on a Cyan ADP flow cytometer (Beckman Coulter). The percentage of cells expressing GFP, and the mean fluorescent intensity of fluorescent cells were calculated. Further analysis was performed using FloJo (TreeStar Inc.).

2.5.2 Quantitative RT-PCR for RNA expression

A549 cells in a 6-well plate were infected with viral vectors and incubated for 24 hours at 37°C, 5% CO_2. Cells were washed in PBS, detached by brief treatment with trypsin solution (TrypLE Express, Invitrogen), and pelleted in a

microfuge. RNA was extracted using the QIAGEN RNA Miniprep kit and eluted in RNAse-free dH_20. cDNA was generated from adenoviral VA-RNA I transcripts using the Omniscript Reverse Transcriptase kit (QIAGEN) according to manufacturers protocol and using primers RTVAF (5'-GTGGTCTGGTGGATAAATTCGCAAG-3') and RTVAR (5'-GTCGCACACCTGGGTTCGACA-3'). Samples without the addition of the reverse transcriptase enzyme were included as a control. cDNA samples were diluted 10 fold, prior to quantitative PCR analysis. Quantitative PCR (QPCR) was performed using the Quantitect SYBR Green I PCR kit in a 20µl reaction:

Quantitect SYBR Green Master Mix - 10µl

Forward Primer (10µM) - 1µl

Reverse Primer (10µM) - 1µl

dH20 - 3µl

Template DNA - 5µl

Note: The same primers were used in both QPCR and CDNA synthesis.

QPCR was performed on a LightCycler 480 Real-Time PCR System (Roche Applied Science) and analysis performed using the integrated software.

2.6 Methods: Animal Procedures

2.6.1 Animals

All mouse procedures were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act Project Licence (PPL 30/2414) and were approved by the University of Oxford Animal Care and Ethical Review Committee. Mice were housed primarily in the Wellcome Trust Centre for Human Genetics animal facility (Functional Genetics Facility (FGF)) under Specific Pathogen Free (SPF) conditions. Animals were transferred to the Radiobiology Research Institute, Churchil Hospital, Oxford for imaging studies (Section 2.6.4). Female BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were 6-10 weeks old at the start of experiments and obtained either from Harlan Laboratories, Oxfordshire, UK or Charles River Laboratories, UK.

2.6.2 Immunisation

Mice were anaesthetised before each immunisation using inhalation anaesthetic comprised of a mixture of isofluorane (2-3.5%) and oxygen (1-2l/min). Vaccinations were administered intramuscularly (i.m.) into the *musculus tibialis* using a 26G needle. A total volume of 50µL was administered, with viral vector vaccines prepared in sterile, endotoxin free PBS (Sigma).

2.6.3 Tail Vein bleed

Mice were pre-warmed on a heat box (37^{o}C) before being transferred to a restrainer for blood collection. Tails were nicked prior to collection of blood in serum tubes.

2.6.4 *In vivo* luminescence imaging

Imaging studies were performed at the Radiobiology Research Institute, Churchill Hospital, Oxford under the supervision of Dr Sean Smart, and with assistance from Karolis Bauza. Before delivery to the institute, all animals were injected with viral vectors expressing *Photinus* luciferase at the Functional Genetics Facility. Prior to imaging, animals were anaesthetised as described previously before subcutaneous injection of 0.75mg D-Luciferin (Caliper Life Sciences, UK) into the scruff of the neck in a volume of 50µL using a 26G needle. Animals were kept under anaesthetic for a further 8 min to allow dissemination of the luciferin substrate before image capture on an IVIS200 imaging system (Caliper Life Sciences, UK). Exposure times were adjusted to avoid oversaturation of pixels and flux measurements converted to photons per second for comparative assessment of luminescence at different timepoints. Luminescence image data was analysed using Living Image 4.0 software.

2.7 Methods: Murine *Ex Vivo* Characterisation and Immunology

2.7.1 Splenocyte preparation

Mice were sacrificed by cervical dislocation and spleens removed by dissection. Spleens were crushed in PBS, passed through a sterile 70μm cell strainer and centrifuged (500g, 5min). Supernatants were discarded and pellets resuspended in ACK lysis buffer for 5 min before addition of 25mL PBS. The mixture was immediately centrifuged again (500g, 5min) and resuspended in 5mL complete αMEM medium. Splenocytes were counted using a CASY counter (Schärfe Systems, Germany) and resuspended at appropriate concentrations in complete αMEM medium.

2.7.2 Peripheral Blood Mononuclear Cell (PBMC) preparation

8-10 drops of blood were collected from the tail vein of mice into 200μL 10mM EDTA. 1mL of ACK lysis buffer was added and cells were centrifuged for 4 min (4000rpm). Supernatants were discarded and pellets were again resuspended in 1mL ACK lysis buffer and centrifuged as before. Supernatants were removed and the cell pellet was re-suspended in complete αMEM medium.

2.7.3 Bone marrow dendritic cell isolation, culture, and transduction studies

Naïve mice were sacrificed and the hind limbs removed for extraction of bone marrow dendritic cells. The lumen of the hind leg bone was flushed with complete media using a 26G needle, and the resulting suspension filtered

through a 70μm cell strainer. ACK lysis was performed before cells were washed in PBS and resuspended in RPMI media supplemented with 10% FCS, 2μM L-glutamine and 20ng/ml murine recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF). Cells were cultured (37^{o}C, 5% CO_2) in 6-well plates at a concentration of 2x10^6 cells per well for seven days prior to use. Transduction studies were performed in 24-well plates, at a cell density of 3x10^5 cells per well. Vectors expressing TIPeGFP were incubated with cells for 24 hours, before the cells were harvested and resuspended in FACS buffer. Cells were surface stained with an allophycocyanin (APC) conjugated anti-mouse CD11c monoclonal antibody (eBioscience, clone N418). The LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life technologies) was used to assess cell viability. CD11c+GFP+ cells were enumerated on a Cyan ADP flow cytometer (Beckman Coulter).

2.7.4 Draining Lymph Node preparation

Mice were sacrificed and the draining popliteal lymph nodes (LNs) were excised. Lymph nodes were collected into 2mL PBS and subsequently crushed in a 6-well plate. Cells were recovered by passage through a 70μm strainer (Corning) and centrifuging at 1350 rpm for 5 minutes. Supernatants were discarded and pellets resuspended in NP-40 lysis buffer to release transgene product for *ex vivo* detection.

2.7.5 Skeletal Muscle Tissue Preparation

Tibialis anterior muscles were removed, and macerated in NP-40 lysis buffer with glass beads using a Mini Beadbeater (Biospec Products) at maximum

speed (4800 oscillations / min) for 3 x 1 min. Homogenised material was centrifuged at 4000rpm for 4 min, and supernatant collected.

2.7.6 *Ex vivo* Luciferase quantification

Supernatants from lymph nodes and muscle extracts were assayed for *Photinus* luciferase activity. 50µl of sample was transferred to a 96 well plate, and diluted with an equal volume of 1X reporter lysis buffer (Promega - diluted from a 5X concentrate in H_20). Samples were then subjected to a single freeze-thaw cycle at -80^oC to lyse any intact cells. 50µl of sample in lysis buffer was transferred to a black 96-well assay plate (Costar) and an equal volume of reconstituted BrightGlow™ Luciferase Substrate (Promega) added. After a 5 min incubation period, luminescence was measured using a Varioskan Flash luminometer (Thermo Scientific).

2.7.7 Interferon Gamma ELISpot assay

MAIP ELISpot plates (Millipore, UK) were coated with anti-mouse-IFN-γ mAb (Mabtech, UK) at 5µg/mL in carbonate-bicarbonate coating buffer. Plates were blocked with complete αMEM medium for at least two hours. Splenocytes were re-suspended at a concentration of 1x10^{7}cells/mL. 50µL of cells were plated per well in duplicate and diluted two fold down the plate. 50µL peptide diluted in complete medium was added to test wells and complete medium alone was added to control wells. Peptides were added at a final concentration of 2µg/mL. Plates were incubated at 37°C, 5% CO_2 in a humidified incubator for 18-20 hours. Plates were then washed 6 times with PBS and incubated for 2 hours with biotinylated anti-mouse-IFN-γ mAb (Mabtech, UK) diluted to 1µg/mL in

PBS, followed by an incubation with a streptavidin alkaline phosphatase polymer diluted to 1µg/mL in PBS. Spots were developed by addition of Colour Development Buffer (BioRad, UK) and counted using ELISpot AID software (Autoimmun Diagnostika, Germany). Responses were expressed as 'spot forming cells' (SFC) per million splenocytes. Background responses in media-only wells were subtracted from those measured in peptide-stimulated wells.

Table 2.3: Epitopes used in vector immunogenicity studies

EPITOPE	ANTIGEN CONSTRUCT	CD8+ / CD4+	SEQUENCE
*Pb*9	TIPeGFP	CD8+	SYIPSAEKI
$EGFP_{200-208}$	TIPeGFP	CD8+	HYLSTQSAL
p15	TIPeGFP/ Ag85A	CD4+	MTFLTSELPGWLQANRHVKPT
SIV-gag A11	TIPeGFP	CD8+	AAVKNVVMTQTL
p11	Ag85A	CD8+	WYDQSGLSV
$NP_{147-155}$	Nucleoprotein, Influenza A NP-M1	CD8+	TYQRTRALV
Luc	*Photinus* luciferase	CD8+	GFQSMYTFV

2.7.8 Intra-cellular cytokine staining (ICS)

To analyse the percentage of cytokine producing peripheral blood and spleen $CD4^+$ and $CD8^+$ T lymphocytes, cells were collected from spleen and blood samples as previously described. Cells were restimulated for 5 hours at 37°C in the presence of Brefeldin A and peptides at a final concentration of 1µg/mL per peptide. Following stimulation, plates were stored at 4°C overnight. Cells were transferred to V-bottom plates and centrifuged (500g, 5 min). Supernatants were discarded and cells resuspended in anti-mouse CD16/CD32 (Fc block) for 15

minutes on ice and in the dark. Cells were washed in FACS buffer, and a surface stain applied which included fluorophore conjugated anti-CD8α PerCP-Cy5.5, anti-CD4 APC-A780, anti-CD127 Pacific Blue and anti-CD62L PE-Cy7 (BD Biosciences). After a 30 min incubation, cells were permeabilised using Cytofix/Cytoperm (BD Biosciences), and washed in FACS buffer. An intracellular stain including anti-IFN-γ APC, anti-TNF-α FITC and anti-IL-2 PE (BD Biosciences) was applied for 30 minutes in the dark. Samples were resuspended in 1% PBS Formalin and data acquired using a Cyan ADP flow cytometer (Beckman Coulter). Analysis was performed using FloJo (TreeStar Inc.). Background responses in unstimulated cells were subtracted from the stimulated responses prior to analysis. To assess polyfunctionality the Boolean gate platform in FlowJo was used with individual function gates to create all possible combinations of response patterns. Pestle version 1.3 (Mario Roederer, National Institutes of Health) was used to prepare data for the subsequent analysis of response profiles. The data analysis program, simplified presentation of incredibly complex evaluations (SPICE) (Mario Roederer, National Institute of Health) was used to analyse data and to generate graphical representations of functional T cell responses using background-deducted data.

2.7.9 Whole IgG ELISA

Blood was collected from tail veins into Microvette tubes, or by cardiac puncture into eppendorf tubes following cervical dislocation. It was stored at 4°C for at least 12 hours to allow clotting, before centrifugation at 13000rpm for 4min. Serum was removed, transferred into clean eppendorf tubes and stored at -20°C prior to use. Nunc-Immuno Maxisorp Plates (Thermo Scientific, UK) were

coated with recombinant protein at a concentration of 1µg/mL in coating buffer overnight at room temperature. Plates were washed with PBS/T and blocked with 1% BSA in PBS for at least 1 hour. Sera were diluted 3-fold down the plate, at an initial dilution of 1:100, and incubated for 2 hours at room temperature. After a further wash with PBS/T, Goat anti-mouse total IgG conjugated to alkaline phosphatase (Sigma Aldrich, UK), was added for 1 hour at room temperature. Following a final wash, *p*-nitrophenylphosphate (pNPP) diluted in diethanolamine buffer to a concentration of 1mg/mL was used as a developing substrate (Pierce, UK). Optical density (OD) was read at 405nm using a Model 550 Microplate Reader (Bio-Rad, UK). Serum antibody endpoint titres were taken as the x-axis intercept of the dilution curve at an absorbance value three standard deviations greater than the OD_{405} for serum from a naïve mouse. A standard positive serum sample and naïve serum sample were added as controls for each assay. Naïve mouse serum was negative for antigen-specific responses to all antigens. GFP specific antibodies, following immunisation of mice with TIPeGFP and eGFP expressing vectors, were measured using recombinant GFP protein (Millipore, UK). *Photinus* luciferase specific antibodies were measured using recombinant *Photinus* luciferase protein (Sigma, UK)

2.7.10 IgG Isotype ELISA

To detect antigen-specific IgG1 and IgG2a responses, Nunc-Immuno Maxisorp Plates (Thermo Scientific, UK) were coated with recombinant protein as described previously. After blocking and addition of serum, plates were washed and either biotin anti-mouse IgG1 or IgG2a (BD biosciences) were added at a 1:5000 dilution for 1 hour at room temperature. Plates were washed again

and Extravidin Alkaline Phosphatase (Sigma Aldrich, UK) was added for 30 minutes. Development was performed as described for the total IgG ELISA.

2.7.11 Type I IFN ELISA

Nunc-Immuno Maxisorb Plates (Thermo Scientific, UK) were coated with 5μg/ml monoclonal rat anti-mouse IFNβ antibody [7F-D3] (Abcam, Cambridge, UK, Ab24324). After an overnight incubation at room temperature, plates were blocked with 3% BSA/PBS for 1 hour at room temperature. Serum samples were added and serially diluted 2-fold down the plate. Recombinant mouse IFNβ (PBL Interferon Source, NJ, US, 12400-1) was diluted in 2% BSA/PBS to generate a standard curve. Samples and standards were incubated for 2 hours at room temperature. After washing in PBS/T, plates were incubated with 400 Units/ml polyclonal rabbit anti-mouse IFNβ antibody (Chemicon, CA, US, AB2215) for 2 hours at room temperature. After a further wash, plates were incubated with goat anti-rabbit IgG alkaline phosphatase conjugated antibody (Sigma A8025) diluted 1:5000 and incubated for 1 hour at room temperature. Plates were developed with *p*-nitrophenylphosphate (pNPP) diluted in diethanolamine buffer as described previously.

2.8 Serum neutralisation assays

Human serum samples (pre-vaccination) were collected from trial volunteers; UK samples (adults) were obtained from trials MAL34 (NCT00890760) and VAC33 (NCT00890019). Gambian samples (adults and infants 2-6 years old) were obtained from trial VAC41 (NCT01373879). HEK293

cells were infected with ChAdOX1 or ChAd63 vectors expressing secreted alkaline phosphatase (SEAP). Recombinant adenoviruses were incubated with five serial dilutions of human serum in FBS–DMEM prior to infection. The final serum dilutions were 1:18, 1:72, 1:288, 1:1152, 1:4608; each serum sample was tested in duplicate. Supernatants were collected and assayed for SEAP concentration using the Phospha-light™ chemiluminescent reporter assay for secreted alkaline phosphatase (Applied Biosystems) according to the manufacturer's instructions. Luminescence intensity was measured using a Varioskan Flash luminometer (Thermo Scientific). Neutralisation titers were defined as the serum dilution required to reduce SEAP concentration by 50% compared to wells infected with virus alone. Neutralization titer was calculated by linear interpolation of adjacent values. Assays were performed by Nick Edwards at the Jenner Institute, University of Oxford.

3 Generation of novel chimpanzee adenovirus vector ChAdY25 / ChAdOx1

3.1 Introduction

Adenovirus vectored vaccines have been widely used in early stage clinical trials targeting a range of diseases including malaria, HIV, influenza, hepatitis C, and cancer [165]. However, despite the large number of clinical trials, to date only a handful of serotypes (HAdV-5, HAdV-6, HAdV-35, HAdV-26 and two chimpanzee adenovirus serotypes ChAd63 and ChAd3) have been assessed as vaccine vectors in humans [80,84,166,204]. There is a need for development of new vectors for clinical application, both to assess the utility of different serotypes and to enable deployment of multiple vaccines within a single population, since anti-vector immunity has been shown to limit the efficacy of a second immunization with the same vector [211]. New vectors will need to be based on viruses that have low seroprevalence in humans, and are able to elicit robust transgene product specific immune responses.

This chapter describes the development of a new adenoviral vector, based on a chimpanzee adenovirus isolate, Y25. Some of the data in this chapter has recently been published [216]. The Y25 isolate was first described by Hillis *et al* in the late 1960's [217], but since then studies on this isolate have been limited and the genomic sequence was reported for the first time by our laboratory [216]. The sequence data facilitated construction of an E1 E3 deleted replication defective vector from the wild type Y25 genomic DNA, for *in vivo* expression of recombinant vaccine antigens.

The first recombinant adenovirus vectors were generated by homologous recombination in mammalian cells between purified wild type viral DNA and a shuttle vector containing the recombinant transgene. However, this process is not efficient. A more desirable method has since been developed involving generation of a molecular clone of the viral genome in bacterial vectors that can be propagated and genetically manipulated in *E. Coli* prior to transfection into mammalian cell lines for virus production. A recent protocol by Zhou *et al* [218], describes a direct cloning approach involving piecemeal assembly of adenovirus genomic DNA fragments into plasmid based vectors by restriction cloning. However, the direct cloning approach is technically challenging and viral genomes can be unstable in high copy plasmid based vectors. Moreover, this method relies upon convenient location of restriction endonuclease sites within the adenovirus genome. A cloning scheme of this nature must be redesigned for every new vector, and the flexibility of modifications to the genome is limited by restriction site availability.

In this study, the preferred approach was to generate a molecular clone of the virus in a bacterial artificial chromosome (BAC). Unlike plasmid vectors, BACs are present in single copy within *E. Coli* conferring increased genetic stability [219]. The entire chimpanzee adenovirus genome was incorporated into the BAC vector in a single step by homologous recombination, similar to the method described by Chartier *et al* [220], rather than by piecemeal assembly. Precise modification of the viral genome was then achieved through BAC recombineering (recombination mediated genetic engineering) [213]. This technique, again based on homologous recombination, is highly flexible and independent of sequence specific elements within the genome, enabling precise

and seamless modification of the ChAdY25 vector, such that the vector genome contains no truncated genes or unnecessary sequence. The construction and optimization of the new ChAdY25 vector to increase yield and productivity in E1 complementing cell lines is described here. The optimized version, renamed ChAdOX1, has recently been manufactured to clinical grade expressing an influenza antigen construct and currently in Phase I trials at the University of Oxford (Gilbert *et al*, manuscript in preparation).

3.2 Results

3.2.1 Y25 clusters phylogenetically with human and chimpanzee adenoviruses belonging to the species *Human adenovirus E*.

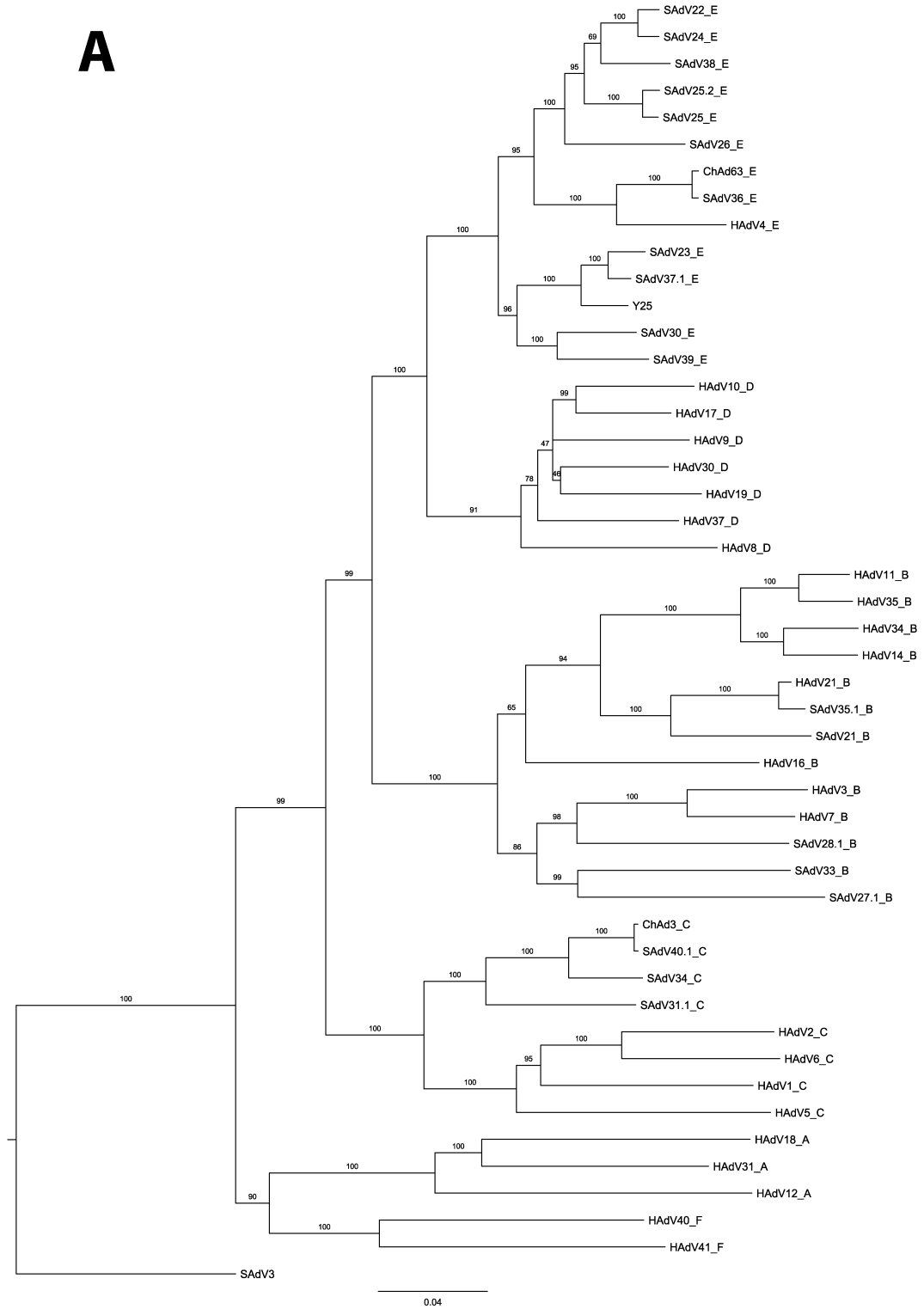
The chimpanzee adenovirus isolate Y25, was first described by Hillis *et al* [217]. Upon receipt from Prof. Goran Wadell, Umea University, Sweden, the wild type virus was propagated and the viral genome sequenced (GenBank accession no. JN254802). Previous literature on this isolate is scarce, but the genome sequence data has confirmed early serological indications that this adenovirus is related to Human adenovirus 4 (HAdV-4) [221]. Simian adenoviruses (SAdV) isolated from great apes are not phylogenetically distinct from human adenoviruses (HAdV); chimpanzee adenoviruses isolated to date have been found to group phylogenetically within the species *Human adenovirus B, Human adenovirus C* and *Human adenovirus E* [99]. *Human adenovirus E* has only one member isolated from humans (HAdV-4) but includes many chimpanzee adenoviruses, most notably the vaccine vector candidates ChAd63, AdC68 (SAdV-25), AdC7 (SAdV-24) and AdC6 (SAdV-23) [222,223,224,225].

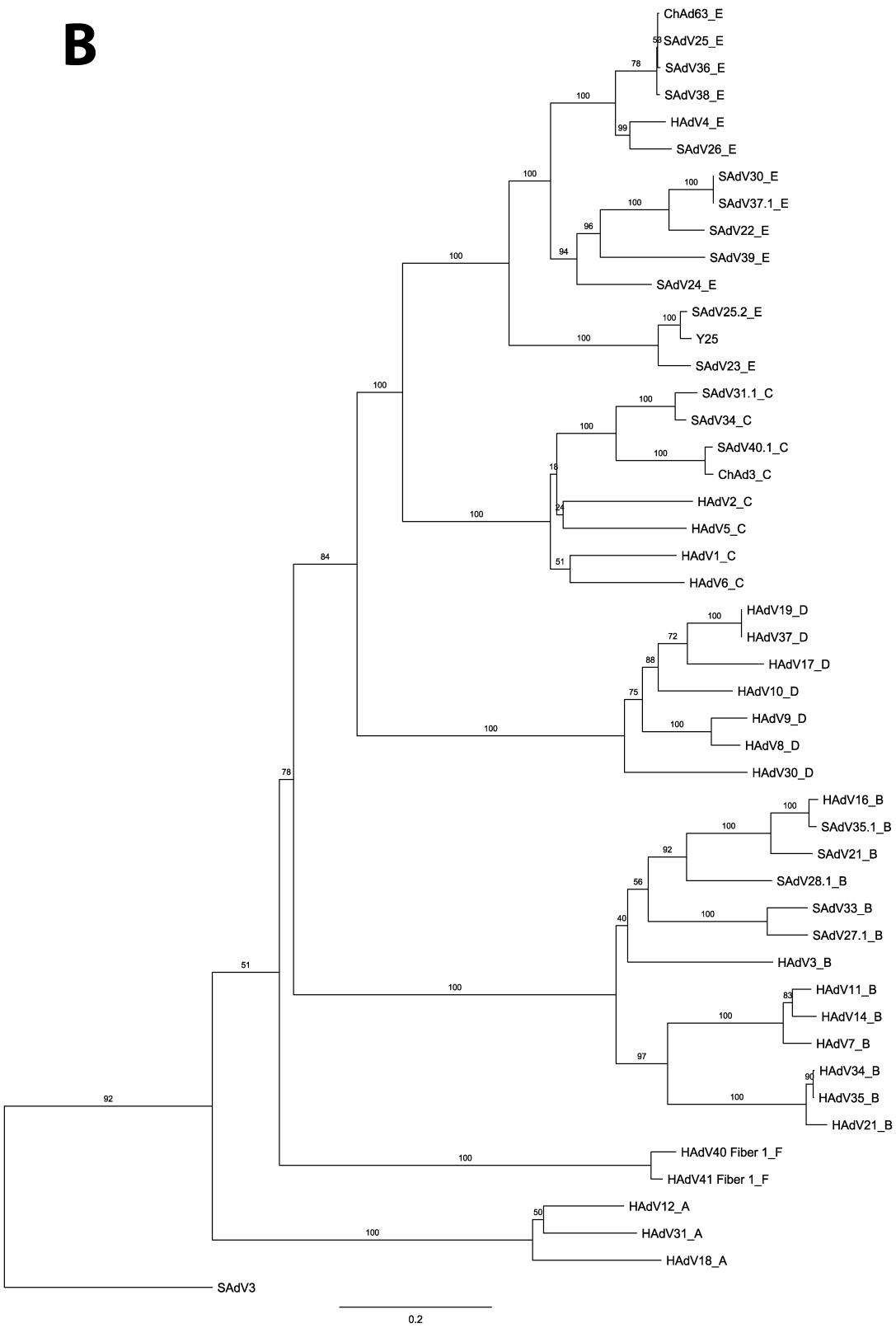
Phylogenetic trees were constructed based on the DNA sequences of hexon and fiber proteins since these are the major surface-exposed capsid components and are believed to be the primary determinants of vector tropism and serum neutralization (Figure 3.1). The phylogenetic analysis indicates that chimpanzee adenovirus Y25 also groups with *Human adenovirus E* viruses. A SAdV isolated from a rhesus macaque (SAdV-3) is a member of *Simian adenovirus A* and was included as an out-group, since, unlike the chimpanzee (and other great ape) derived adenoviruses it does not group within the human

adenoviral species. Trees based upon the viral DNA polymerase (commonly used to classify adenovirus species), and complete genome sequences also group Y25 in *Human adenovirus E* (data not shown). Interestingly, both hexon and fiber DNA sequences from Y25 and SAdV-23 appear to cluster separately from other *Human adenovirus E* members, in agreement with early serological data suggesting that Y25 and SAdV-23 (CV-32) are more closely related to each other than to their other species E counterparts [221].

In order to assess cross-neutralization of Y25 by another chimpanzee adenovirus belonging to *Human adenovirus E*, serum was obtained from a recent Phase I clinical trial in which human volunteers vaccinated with a recombinant ChAd63 malaria vaccine developed very high neutralization titers against ChAd63 (>1:1,000) [84]. Serum from these subjects did not neutralise Y25 (data not shown), supporting the phylogenetic evidence that Y25 is a distinct serotype from ChAd63, despite being a member of the same species. Neutralisation by sera raised against other chimpanzee adenoviruses has not been assessed.

Figure 3.1: Phylogenetic analysis of adenovirus sequences. Phylogenetic trees based on sequence alignment of amino acid sequences of (A) the hexon protein and (B) the fiber protein of different human (HAdV-) and chimpanzee (SAdV-) adenovirus serotypes including Y25. Clustering of sequences into each of the six human adenovirus species A-F is indicated below. The rhesus macaque derived adenovirus SAdV-3 does not group phylogenetically within the human adenovirus species, and is thus included as an out-group. Published in Dicks *et al* [216].




B
ChAd63_E
SAdV25_E
SAdV36_E
SAdV38_E
HAdV4_E
SAdV26_E
SAdV30_E
SAdV37.1_E
SAdV22_E
SAdV39_E
SAdV24_E
SAdV25.2_E
Y25
SAdV23_E
SAdV31.1_C
SAdV34_C
SAdV40.1_C
ChAd3_C
HAdV2_C
HAdV5_C
HAdV1_C
HAdV6_C
HAdV19_D
HAdV37_D
HAdV17_D
HAdV10_D
HAdV9_D
HAdV8_D
HAdV30_D
HAdV16_B
SAdV35.1_B
SAdV21_B
SAdV28.1_B
SAdV33_B
SAdV27.1_B
HAdV3_B
HAdV11_B
HAdV14_B
HAdV7_B
HAdV34_B
HAdV35_B
HAdV21_B
HAdV40 Fiber 1_F
HAdV41 Fiber 1_F
HAdV12_A
HAdV31_A
HAdV18_A
SAdV3
0.2

3.2.2 Rescue of molecular clones of ChAdY25

To generate a molecular clone of the Y25 genome, a BAC gap repair vector was constructed containing PCR-amplified regions of homology to the left and right flanks of the viral genome, similar to the method described in Chartier *et al* [220] (Section 2.3.1). In this study, an extra homology flank downstream of the adenovirus E1 region (Left Flank II, LFII) was included to enable deletion of E1 concomitant with genome insertion into the BAC (Figure 3.2). The E1 region is essential for viral replication, hence the ability to delete E1 at this stage would render the new vector immediately replication incompetent. Prior to gap repair insertion, the quality of the Y25 genomic DNA preparation was assessed by analytical restriction digest (Figure 3.3A).

Replication incompetent (E1-deleted) BAC clones were successfully identified by restriction digest and PCR screening (Figure 3.3C-D). Transfection into E1 complementing HEK293 cells [226] confirmed the ability of all candidate clones to generate infectious virions, which had the expected adenovirus morphology by electron microscopy (Figure 3.3B).

Further genetic modifications were performed through BAC recombineering. The galactokinase (*GalK*) recombineering system in SW102 *E. coli* was employed for both positive and negative selection of recombinant clones [213](Section 2.2.9). Using this technique the entire adenovirus E3 region, not essential for replication, was deleted, increasing insert capacity of the new vector by approximately 5kb (Section 2.3.2). The new E1/E3 deleted vector was termed ChAdY25. Bacteriophage lambda site specific recombination sequences *attL* and *attR* were introduced at the E1 locus to enable efficient and directional insertion of recombinant antigens using Gateway® technology (Invitrogen) (Section 2.2.8).

Figure 3.2: Generation of a molecular clone of Y25 by gap repair insertion of Y25 genomic DNA into the pBAC 'rescue vector'. The 'rescue vector' pBACe3.6 contains regions of homology to both left and right flanks of the Y25 genome. Recombination between BAC vector and the left side of the viral genome can either occur at Left Flank I (LFI) such that the E1 region is included in the resulting BAC clone, or more desirably at Left Flank II (LFII) such that E1 is deleted. Published in Dicks *et al* [216].

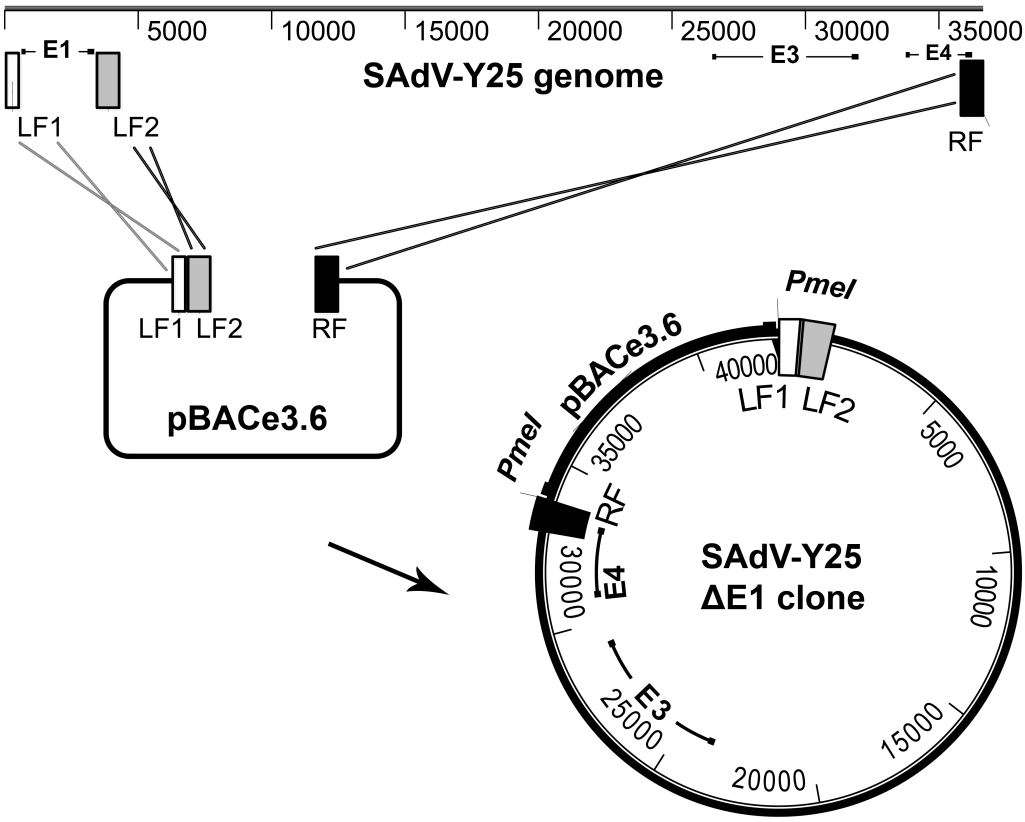
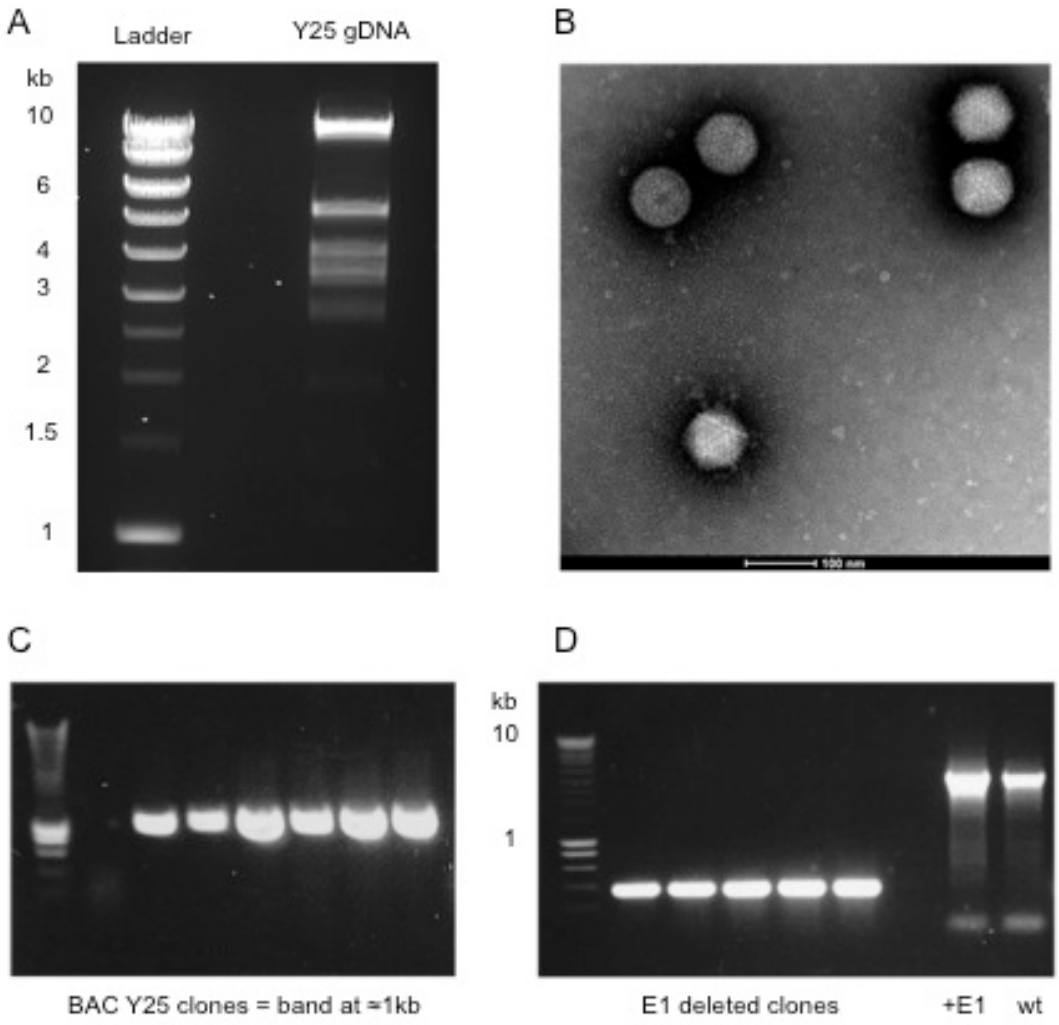


Figure 3.3: Confirmation of successful genome rescue and E1/E3 deletion of BAC-ChAdY25 (A) *Pme*I restriction digest of wild type Y25 gDNA, used for gap repair insertion into the BAC **(B)** Rescue of recombinant ChAdY25 vectors in HEK293 cells generates infectious virions displaying the typical features of adenoviruses. Transmission electron micrograph of ChAdY25 adenovirus particles (virions shown are from the E4 modified ChAdY25-E version, see Figure 3.4A) Image courtesy of Tom Walter and Jonathan Grimes, Division of Structural Biology (STRUBI), University of Oxford. **(C)** PCR screen for genome insertion into the BAC. **(D)** PCR screen for E1 deletion of BAC Y25 clones (+E1 = E1 positive BAC Y25 clone. wt = wild type Y25 gDNA). DNA Smartladder MW1700-10 (Eurogentec) was used to estimate sizes of bands in A, C and D.



3.2.3 Optimisation of simian adenoviral vector growth and yield

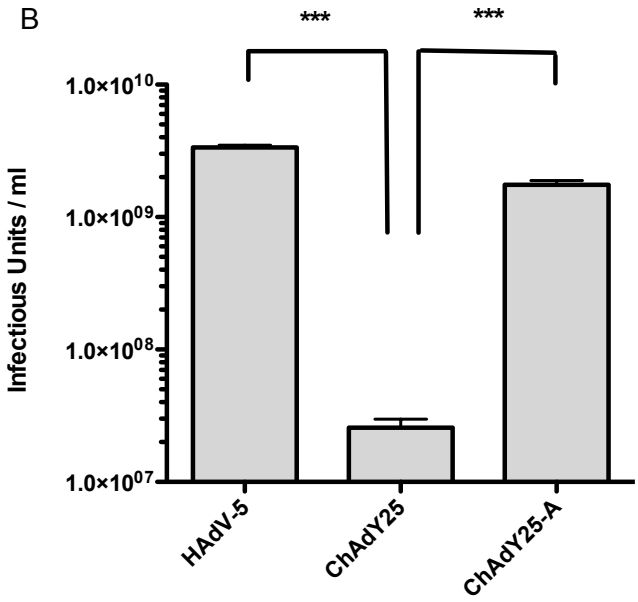
The first generation E1/E3 deleted ChAdY25 vectors grew inefficiently in HEK293 cells, and viral yield was approximately two logs lower than for HAdV-5 based vectors (Figure 3.4, B). It has previously been shown that modification of the adenovirus E4 region is necessary for the efficient propagation of some non-HAdV-5 based vectors in HEK293 cells [201,227]. Although HEK293 cells do support propagation of adenoviruses from other serotypes, yield is often lower and it has been speculated that this is due to a suboptimal interaction between the HAdV-5 E1 proteins expressed by the cell line and vector-encoded E4 gene products required for replication. Of particular importance is the product of *E4Orf6*, a multifunctional protein implicated in late viral mRNA splicing, selective export of viral mRNA, viral DNA synthesis, and inhibition of apoptosis. The function of E4Orf6 is dependent on an interaction with the E1B-55K protein.

A series of five ChAdY25 vectors with variant E4 loci were generated, incorporating different regions of the E4 locus from HAdV-5, referred to as ChAdY25-A to -E (Figure 3.4, A). Details of the construction of these E4 modified vectors are provided in Section 2.3.3. To our knowledge, previously reported E4 modifications to non HAdV-5 recombinant vectors have concerned the introduction solely of *E4Orf6* from HAdV-5. In the case of chimpanzee adenovirus vector ChAd63, the entire native E4 locus was deleted prior to insertion of the HAdV-5 *E4Orf6* gene [201]. As expected, a similarly modified Y25 based vector, ChAdY25-A, significantly improved virus yield in HEK293 cells (Figure 3.4, B). This improvement in yield enabled production of enough virus to perform comparative immunogenicity studies using ChAdY25-A. Little further improvement in yield was given with vectors ChAdY25-B to E (data not shown).

Figure 3.4 Modification of the ChAdY25 E4 locus to improve vector yield in HEK293 cells. (A) E4 Modified ChAdY25 vectors labeled ChAdY25-A to –E. **(B)** Virus yield of ChAdY25 based vectors expressing GFP before and after *E4Orf6* replacement. HEK293 cells were infected at a multiplicity of infection of 10 ifu/cell and incubated at 37°C for 48hours before harvesting. Infectious titer of the harvested material was measured by quantifying GFP positive foci 48hrs post infection. Bars show mean and SEM (n=6). *** p<0.001. Published in Dicks *et al* [216].

A

	Y25 E4 modified construct	
	Ad5 E4 ORFs	Y25 E4 ORFs
ChAdY25	Nil	ALL
ChAdY25-A	E4Orf6 only	Nil
ChAdY25-B	E4Orf6 only	E4Orf1,2,3
ChAdY25-C	E4Orf6/7	Nil
ChAdY25-D	E4Orf6/7	E4Orf1,2,3
ChAdY25-E	E4Orf4,6/7	E4Orf1,2,3



3.2.4 Optimal E4 modification is required to allow accurate infectious titration in the absence of a marker gene.

A principal aim of this study is to compare the immunogenicity of adenovirus vectors of different serotype in mice. The vast majority of immunogenicity studies of adenoviral vectors to date have based immunization doses on viral particle number (a spectrophotometric measurement of absorbance at 260nm or real-time PCR to quantify viral DNA, see Section 2.4.5) [179,228,229,230]. While rapid and straightforward, this measurement is not only susceptible to nucleic acid contamination, but provides no information on the ability of viral particles in the vector preparation to infect cells – which is required for synthesis of the recombinant antigen protein and hence for induction of an immune response. It has previously been shown that the ratio of viral particles to infectious units (often termed P:I ratio) can vary over several orders of magnitude between virus preparations, and significantly affects immunogenicity [172]. Work from our laboratory has subsequently shown that titration based on infectious units is required for unbiased comparisons of the immunogenicity of adenoviral vectors [216].

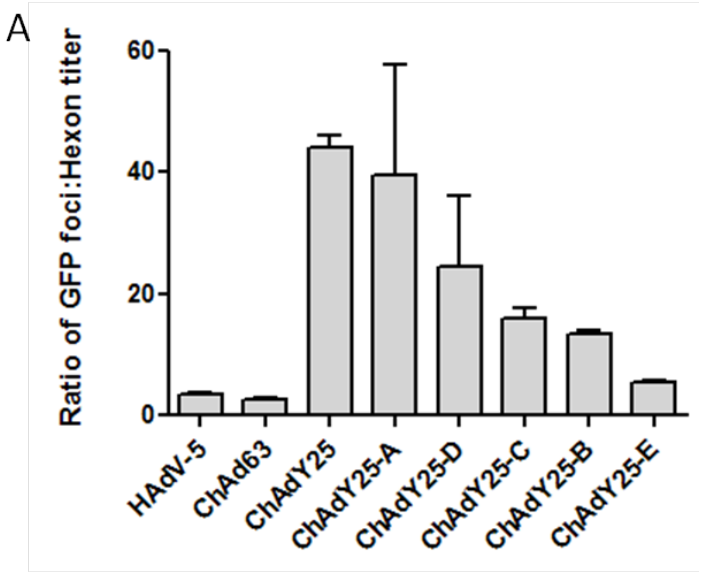
For the first comparative immunogenicity studies using ChAdY25-A (described in Chapter 4), a GFP-tagged model epitope string was employed to facilitate titration by infectious units, allowing direct enumeration of single infected HEK293 cells expressing the fluorescent transgene (Section 2.4.4). However, no vaccine antigen constructs for clinical use, and indeed few pre-clinical constructs, contain a marker gene. An assay using a commercial anti-hexon antibody to identify single adenovirus-infected cells was therefore

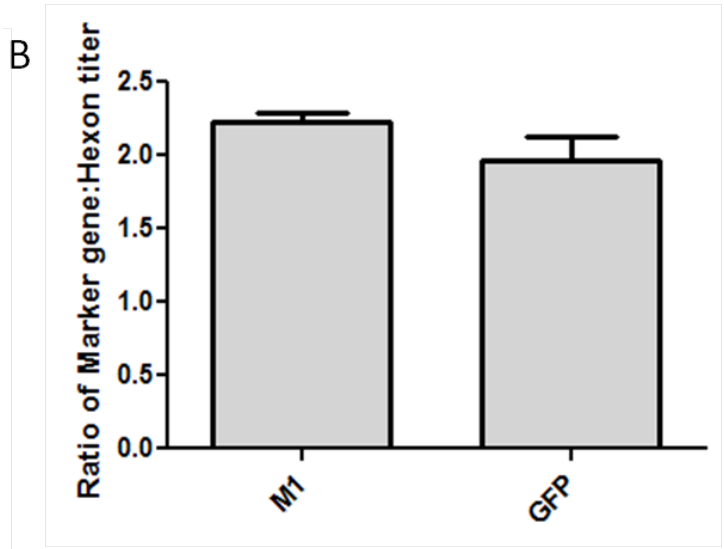
adopted [231]. This mixture of two monoclonal antibodies performs well for species E chimpanzee adenovirus vectors and vectors based on species C virus HAdV-5. However, the assay relies on the assumption that the rate of hexon accumulation relative to transgene expression is consistent between vectors. Hexon accumulation is likely to be a function of the rate of replication in the E1 complementing cell line and hence a similar assumption is made when comparing vectors using any infectivity assay including traditional plaque assays. Consequently there is potential for bias in the titration if the growth rates are unequal, which could lead to inaccurate determination of doses for immunogenicity. Infectious titers of GFP expressing recombinant adenovirus vectors obtained by enumerating GFP foci were therefore compared to titers obtained using anti-hexon based assays (Figure 3.5).

For HAdV-5 and ChAd63 vectors, GFP based titers were approximately twofold higher than the anti-hexon titers. However, for Y25-based vectors, the sensitivity of the anti-hexon assay varied remarkably with genetic modification of the adenovirus E4 region. No further improvement in the GFP:hexon infectious unit ratio was observed by extending the incubation period beyond 48 hours (data not shown). For the original ChAdY25 vector which had an unmodified E4 region and exhibited poor productivity, anti-hexon titers were over 40 fold lower than GFP titers, suggesting that the rate of hexon production is considerably slower than for HAdV-5 and ChAd63 vectors. This was to be expected, given the poor yield of E4 unmodified ChAdY25 described previously. Surprisingly however, the titer of the modified ChAdY25-A vector expressing Ad5 *E4Orf6* only (the same modification as has been made to ChAd63) was still 30 fold lower by anti-hexon staining, despite a marked improvement in yield.

However, the other E4 modified vectors performed better and the output of the anti-hexon immunoassay for ChAdY25-E was comparable to HAdV-5 and ChAd63 based vectors (Figure 3.5). ChAdY25-E, the optimal E4 modified vector, contains the *E4Orf4*, *Orf6* and *Orf6/7* coding regions from Ad5, and the *E4Orf1*, *2* and *3* coding regions from Y25 (Figure 3.4, A). A Y25 based vector expressing the entire Ad5 E4 cassette showed inferior hexon accumulation to ChAdY25-E (data not shown). To determine whether the output of the anti-hexon assay was transgene dependent, a ChAdY25-E vector expressing matrix protein 1 of influenza A virus (M1) was generated. To detect M1 transgene expression, a primary M1 specific monoclonal antibody (see Section 2.4.4) was used in place of the commercial anti-hexon in an immunostaining assay. Figure 3.5 shows that the ratio of transgene gene titer to anti-hexon titer is similar for both GFP and M1 antigens. **ChAdY25-E** was selected for clinical application, and subsequently renamed **ChAdOX1**.

Figure 3.5 Modification of the ChAdY25 E4 region to improve hexon expression for infectivity titration on HEK293 cells. **(A)** Ratio of GFP titer to anti-hexon titer for different Y25 E4 modified vectors expressing the TIPeGFP antigen. Titers assessed 48hrs post infection. **(B)** Ratio of transgene: hexon expression for ChAdY25-E based vectors expressing TIPeGFP and Influenza Matrix protein 1 (M1) recombinant transgenes. Data is representative of at least two independent experiments. Error bars show mean and SEM of two independent serial dilutions. Published in Dicks *et al* [216].

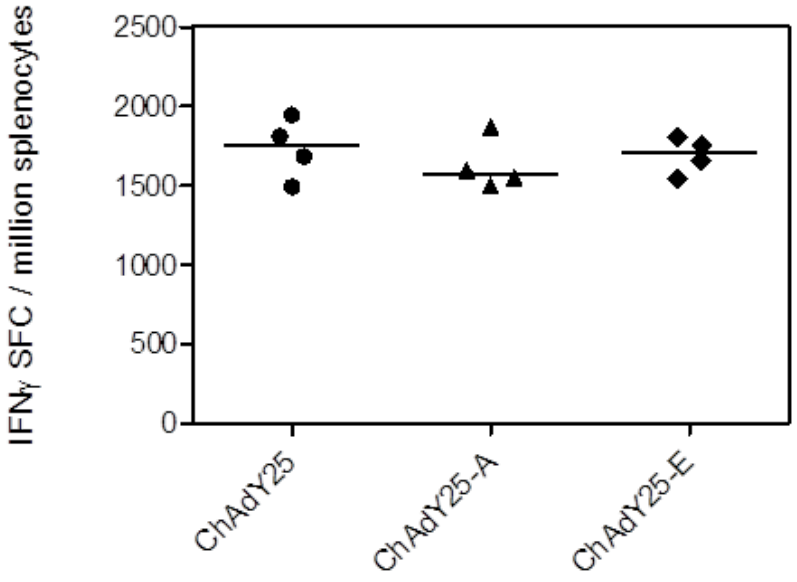




3.2.5 E4 modification has no effect on vector immunogenicity

The effect of E4 modification on adenovirus vector immunogenicity was investigated. The cellular immunogenicity of three different E4 modified Y25 based vectors were tested in parallel; the E4 wild type vector ChAdY25, the ChAdY25-A vector containing only Ad5 *E4Orf6*, and the final E4 modified ChAdY25-E vector (ChAdOX1). All three vectors expressed a model antigen TIPeGFP, consisting of an epitope string fused to the N-terminus of enhanced GFP [232]. The TIP epitope string contains several murine T cell epitopes including *Pb*9, a dominant H2-K^d restricted $CD8^+$ T cell epitope from the circumsporozoite protein of *Plasmodium berghei*. The vectors were titered on GFP infectious units to avoid the differences in the readout of the hexon assay, and thus ensure accuracy of the titer. The data indicate that E4 modification has no effect on vector immunogenicity (Figure 3.6). This is not surprising, since deletion of the E1 region severely reduces *in vivo* expression of adenoviral proteins by replication incompetent vectors. It does however highlight the ability to compare vectors with markedly different ratios of viral particles to infectious units (P:I ratios) if immunisation is based on infectious titer. If the same comparison had been performed with immunisation based on viral particles, the lower proportion of infectious virions in the ChAdY25 native E4 preparation would likely have manifested in lower immunogenicity, giving a misleading result [216].

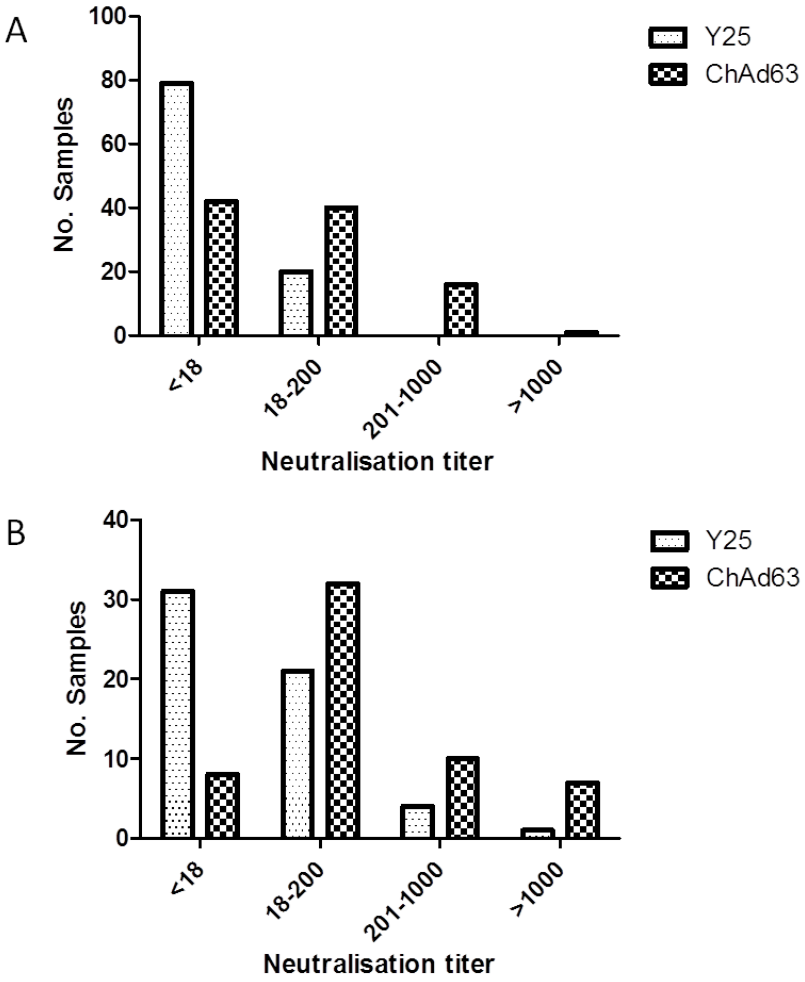
Figure 3.6. E4 modification has no effect on vector immunogenicity. Balb/c mice were immunised intramuscularly with 10^8 infectious units of Y25 vectors expressing model antigen TIPeGFP with either a native E4 locus (ChAdY25), Ad5E4Orf6 expressed at E4 (ChAdY25-A) or the clinical ChAdY25-E vector (ChAdOX1). Responses to $CD8^+$ epitope *Pb9* were measured two weeks post immunisation by IFN-γ spleen ELISpot. Ratios of total viral particles to infectious units (P:I ratios) of vector preparations were as follows; ChAdY25 131, ChAdY25-A 17.4, ChAdY25-E 13.4. Published in Dicks *et al* [216].



3.2.6 Seroprevalence of vector neutralising antibodies in humans

Virus neutralising antibodies acquired through natural exposure may have been responsible for limiting the potency of vectors based on common human serotypes such as HAdV-5 in recent clinical trials [166]. Adenoviruses of chimpanzee origin, including ChAd63, have previously been shown to circumvent this issue to a large extent since neutralisation titers in human sera against these viruses are generally much lower [233,234]. It is, however, not uncommon for human sera to display some degree of neutralisation against vectors of chimpanzee origin and it was therefore important to establish the human seroprevalence of vector neutralising antibodies against Y25. Two previously uncharacterised panels of human sera from British and Gambian adults were analysed using virus neutralisation assays against ChAd63 and Y25 (Section 2.8). Neutralising antibody titers against Y25 were in general lower than against leading chimpanzee adenovirus vector ChAd63, in both UK and Gambian populations. In the UK cohort of 100 individuals, not a single individual possessed a 50% neutralisation titer of > 200, which is considered the threshold for a titer of clinical relevance during pre-vaccination screening (Figure 3.7). In the Gambian cohort, only five individuals possessed a neutralisation titer > 200 against Y25, verses seventeen against ChAd63 (Figure 3.7). The low seroprevalence of Y25 in two human populations suggests that Y25 based vectors could be efficacious in a clinical setting.

Figure 3.7 Anti-vector neutralising antibody titers. Neutralising antibody titers were measured in human sera from **(A)** UK (n=100) and **(B)** The Gambia (n=57) against Y25 and ChAd63 vectors. Y25 data published in Dicks *et al*, [216].



3.3 Discussion

This chapter describes construction of a novel chimpanzee adenovirus vaccine vector from the viral isolate, Y25, which from phylogenetic analysis, seems to belong to the species *Human adenovirus E*. Many promising vector candidates are members of this viral species, including ChAd63, the first chimpanzee adenovirus vector to enter clinical trials in man [84,173]. A molecular clone of the Y25 virus was constructed to enable modification of the viral genome prior to generation of recombinant virus. Most adenovirus molecular clones for vector development have been derived using multi-copy plasmid based systems [218], but in this study the molecular clone was constructed in a bacterial artificial chromosome. This has not previously been attempted for a chimpanzee adenovirus vector, though BAC derived vectors have been described for human adenoviruses including HAdV-5 [235]. The entire chimpanzee adenovirus genome was inserted into the BAC in a single step by homologous recombination, similar to the method described by Chartier *et al.* [220], but in a manner that allowed simultaneous E1 deletion (Figure 3.2). Advantages of the BAC system include improved genetic stability compared to multi-copy plasmids[236]; and the capacity for precise modification of the viral genome through BAC recombineering. BAC recombineering enabled Y25 based vectors with E3 deletions and variant E4 loci to be created seamlessly, such that the vector genome contained no truncated genes or unnecessary sequences. It should be noted that recombineering has been attempted with multi-copy plasmid based systems but typically leads to mixed populations of modified and parental plasmids. A protracted process involving DNA extraction, dilution and

retransformation is then required to obtain a clonal population, and there are also issues with plasmid multimerisation [237,238].

The first E1 E3 deleted ChAdY25 vectors grew poorly in E1 complementing HEK293 cells and exhibited a log reduction in yield compared to similar HAdV-5 based vectors. In agreement with previous literature [227], modification of the viral E4 locus to replace the native *E4Orf6* gene with the HAdV-5 version (ChAdY25-A) enabled Y25 based vectors to achieve similar yields to HAdV-5 vectors (Figure 3.4).

A chief aim of the study is to reliably compare immunogenicity of ChAdY25 with existing vectors and our laboratory has demonstrated that a careful consideration of the infectious titer of vector preparations is essential to achieve consistent and reliable data [216]. The infectious titer of vectors can be assessed easily and reliably, by enumerating single transgene positive infected cells using a fluorescent marker such as GFP. However, where marker genes cannot be employed, an alternative unbiased infectivity assay must be sought. An immunostaining assay was employed to identify single infected cells by detecting accumulation of hexon, the most abundant protein in the adenovirus capsid. Hexon immunostaining offers several advantages over the plaque assay, traditionally the assay of choice. Plaque assays require long incubation periods and titers are often inconsistent. Furthermore, the plaque assay is inherently insensitive for the detection of transgene-expressing virions since not all virions induce plaque formation.

However, it is vital that the readout of any infectivity assay accurately reflects the transduction frequency of recombinant vectors. When infectious titers of GFP expressing recombinant adenovirus vectors obtained by

enumerating GFP foci were compared to titers obtained using anti-hexon based assays, it was found that the output of the hexon assay was over twenty fold lower for E1/E3 deleted ChAdY25 vectors than for HAdV-5 and ChAd63 (Figure 3C). Furthermore, though modification of the native Y25 E4 region to incorporate the *E4Orf6* gene from HAdV-5 was essential to increase yield of the new vector in HEK293 cells (Figure 3.4), it was not sufficient to increase the output of the infectivity assay to a level comparable to HAdV-5 (Figure 3.5).

We therefore created a series of vectors expressing variant E4 loci and found that the frequency of hexon positive infected 293 cells compared to transgene expressing cells was dependent on the precise nature of the E4 modification. Vectors ChAd63 and ChAdY25, despite the high degree of sequence identity between them, required different genetic modifications for optimal hexon immunopositivity (equivalent to HAdV-5). While ChAd63 required only the presence of Ad5 *E4Orf6* at E4, ChAdY25 required a chimeric E4 locus containing all six E4 open reading frames, three from HAdV-5 and three from Y25 (ChAdY25-E). Interestingly, the inclusion of Ad5 *E4Orf4* in this vector increased the frequency of hexon immunopositivity by over fourfold compared to the ChAdY25-D vector, containing the other five ORFs only (Figure 3.5). The E4Orf4 protein, in complex with protein phosphatase 2A (PP-2A), has been shown to down regulate transcription from E2 and E4 adenoviral promoters and is therefore believed to form part of a negative feedback loop contributing to the temporal regulation of early viral gene expression [239]. In addition, E4Orf4-PP2A has also been shown to regulate alternative splicing of late viral transcripts which may explain the enhanced accumulation of late hexon protein [240]. While the addition of HAdV-5 E4Orf4 and E4Orf6/7 increased the rate of hexon

production for ChAdY25-E, the relationship between this parameter and viral yield during propagation is unclear, since the addition of these proteins did not increase viral productivity in HEK293 cells (data not shown) under the conditions tested.

This work has demonstrated that vectors which exhibit sub-optimal growth or infectious titer readout without E4Orf6 replacement should not be prematurely dismissed as vaccine candidates. In many high throughput studies, chimpanzee derived adenoviruses are discarded after failing to grow in E1 complementing mammalian cell lines, often without any modification to the vector backbone at all [241]. This could ultimately limit the repertoire of serotypes tested, and cause potentially efficacious vectors to be overlooked.

It has previously been demonstrated that chimpanzee adenovirus vectors, including ChAd63, are less susceptible to neutralising antibodies in humans than vectors based on common human adenovirus serotypes such as HAdV-5 [233,234,242]. In a recent clinical trial assessing efficacy of a HAdV-5 vector expressing HIV-1 antigens, T cell immunogenicity was significantly lower in individuals with a pre existing anti-HAdV-5 neutralisation titer of >200 [166]. In a previous study by Dudareva *et al*, the percentage of serum samples exhibiting 50% ChAd63 neutralizing titres >200 was just 4% in a Kenyan population [233] – somewhat lower than the values reported in this study for the UK (~17%) and Gambian (~30%) sera tested. Nevertheless, it is clear from the same paper that ChAd63 is significantly less seroprevalent than HAdV-5 (4% ChAd63, 23% HAdV-5), in common with other chimpanzee adenoviruses [233,234,242]. In this study, neutralisation titers against ChAdY25 were lower and less prevalent than those against ChAd63 in both the UK and Gambian sera tested (Figure 3.7).

Whether differences in seroprevalence of this magnitude affect the clinical utility of the vectors remains to be determined. In this regard, it is worth noting that our laboratory have observed T cell immunogenicity of ChAd63-based vaccines comparable to that achieved in UK-based clinical trials in ongoing clinical studies in both Kenya and the Gambia (A. V. S. Hill *et al.,* unpublished data).

4 A comparative assessment of the immunogenicity of adenovirus vectors of different serotype in mice

4.1 Introduction

The low seroprevalence of neutralising antibodies against ChAdY25 in two human populations has indicated that Y25 / ChAdOX1 based vectors could be particularly efficacious in the clinic. However, it was important to ascertain whether differences in the nature of capsid proteins responsible for the reduction in seroprevalence have any effect on the potency of ChAdOX1 as a vaccine vector. Encouraging preclinical immunogenicity data in mice with the ChAd63 vector has translated into unprecedented antigen specific T cell frequencies in human clinical trials [84,173,174]. Therefore, it was important to compare the immunogenicity of ChAdOX1 to existing chimpanzee vectors, including ChAd63, in mice. The comparative studies included two additional adenovirus vectors, AdC68 and HAdV-5. AdC68 was the first chimpanzee vector to be described, based on *Human adenovirus E* virus SAdV-25, and has been extensively used in pre clinical studies [179,223,228]. HAdV-5 is the most studied adenovirus serotype, and the serotype upon which the first replication defective adenovirus vaccine vectors were based [163]. Though the issue of anti vector immunity has reduced the use of HAdV-5 for clinical application in recent years, it still remains a popular vector for preclinical studies. HAdV-5 is a member of a different viral species (*Human adenovirus C*) to the three chimpanzee vectors (all *Human adenovirus E*) in this study.

There have been a number of previous studies comparing HAdV-5 based vectors with *Human adenovirus E* chimpanzee adenovirus vectors in mice; some demonstrate comparable or superior immunogenicity with HAdV-5 [201,242], while others indicate superior immunogenicity with chimpanzee vectors [179,211,228]. However, the vast majority of these studies have been performed basing immunization doses on viral particle count, rather than infectious titer. As discussed in Chapter 3, viral particle estimation is based on quantification of viral DNA and hence provides no information regarding the infectivity of the vector preparation; critical for efficient delivery of the encoded transgene. Our laboratory [216], and others [172] have shown that the ratio of estimated viral particles to infectious units (P:I ratio) can exhibit significant natural variability and can confound comparative immunogenicity studies when immunization doses are based on viral particles. In this study, care was taken to accurately determine the infectious titer of vector preparations. Immunization doses were based on infectious units, since this approach has been shown to correct for differences in P:I ratio between preparations [216]. Nevertheless, it is always desirable to minimize as far as possible the variables that might bias or introduce artifacts into comparative studies. As described in Chapter 2, during the vector production a fixed multiplicity of infection, based on an infectious unit titration, was used for the final stage of viral amplification prior to purification. This allowed preparation of stocks of different viruses with very similar total yields and P:I ratios (Section 4.2.1). This approach has enabled a reliable comparative assessment of vector immunogenicity to be conducted, the results of which are described in the following chapter.

4.2 Results

4.2.1 HAdV-5 vectors elicit superior antigen specific T cell and antibody responses compared to ChAd63, AdC68, and ChAdY25 based vectors.

Comparative immunogenicity studies were performed using E1 E3 deleted adenovirus vectors expressing the model antigen TIPeGFP [232](Section 3.2.5). All three chimpanzee adenovirus vectors had a similar E4 modification (deletion of the native E4 and replacement with HAdV-5 *E4Orf6*, Figure 3.4A construct ChAdY25-A) though studies presented in the previous chapter have shown that differences in the nature of the E4 locus have no effect on immunogenicity (Section 3.2.5). No modification of the E4 locus of HAdV-5 TIPeGFP was required. Immunization dose was based on infectious titer as determined by enumeration of single GFP^+ 293 cells (Section 2.4.4), since anti-hexon staining would be insufficiently sensitive for ChAdY25-A (Section 3.2.4) and similarly for AdC68 (data not shown). Antigen specific T cell responses were assessed across a range of doses, from 10^3 to 10^8 infectious units two weeks post immunization, the peak of the effector $CD8^+$ T cell response [211].

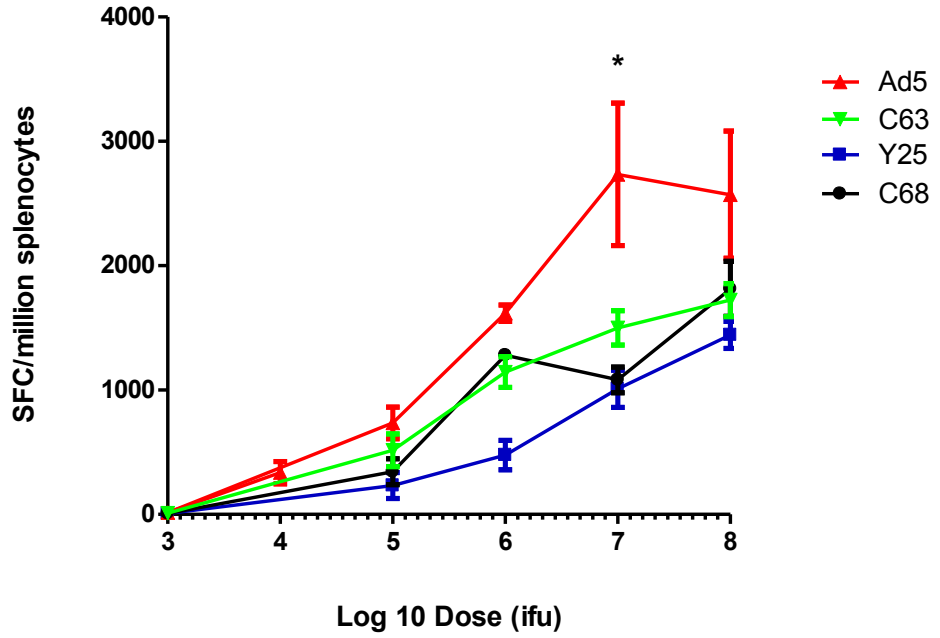
Responses to Balb/c epitopes *Pb*9 (CD8+) and p15 (CD4+) in the N-terminal TIP epitope string, and $EGFP_{200\text{-}208}$ (CD8+) in the GFP antigen, were determined by IFN-γ ELIspot (Section 2.7.7). In the case of all epitopes, the HAdV-5 vector elicited a higher frequency of transgene product specific T cell responses compared to the chimpanzee adenovirus vectors, particularly at doses greater than 10^5 ifu (Figure 4.1A-C). The three chimpanzee adenovirus vectors

induced comparable T cell responses at each dose throughout the five log range. Responses appeared to reach a plateau at 10^8 ifu. Dose response experiments were performed with each vector separately, with a common group immunised with 10^6 ifu HAdV-5 TIPeGFP (approximately the half maximal effective concentration (EC_{50}) dose) included to assess variability across experiments. There were no statistically significant differences in responses in this control group across the individual experiments (Figure 4D).

In addition to the comparative analysis of T cell responses in the spleen, serum was harvested for assessment of transgene product specific antibody induction. Immunoglobulin G (IgG) is the major effector immunoglobulin isotype in blood and tissue fluid, accounting for over 75% of circulating immunoglobulin. Anti-GFP IgG responses after immunization with 10^8 ifu of recombinant vector were measured by ELISA. Titers after immunization with HAdV-5 were a log higher than those after ChAd63, and almost two logs higher than those after AdC68 and ChAdY25 (Figure 4.2).

Figure 4.1: Cellular immunogenicity of HAdV-5 is superior to chimpanzee adenovirus vectors ChAd63, AdC68 and ChAdY25. Balb/c mice (4 /group) were immunised intramuscularly with 10^3 -10^8 infectious units of HAdV-5-TIPeGFP, AdC68-TIPeGFP, ChAd63-TIPeGFP or ChAd-Y25-A TIPeGFP. Two weeks post immunisation, spleens were harvested and cellular responses against $CD8^+$ epitopes **(A)** Pb9, and **(B)** EGFP, and **(C)** $CD4^+$ epitope P15, were assessed by IFN-γ ELISpot. **(D)** There was no significant difference in magnitude of the EGFP CD8+ response after 10^6 ifu HAdV-5 TIPeGFP across the individual dose response (DR) experiments. P:I ratios of vector preparations were as follows; HAdV-5 15.8, ChAd63 24.9, ChAdY25-A 17.41, AdC68 31.6. Graphs show mean response and SEM. Statistical analysis performed by one-way ANOVA, with bonferroni posttest. Asterisk (*) indicates HAdV-5 response is significantly higher ($p<0.05$) than all three ChAd vectors.

A



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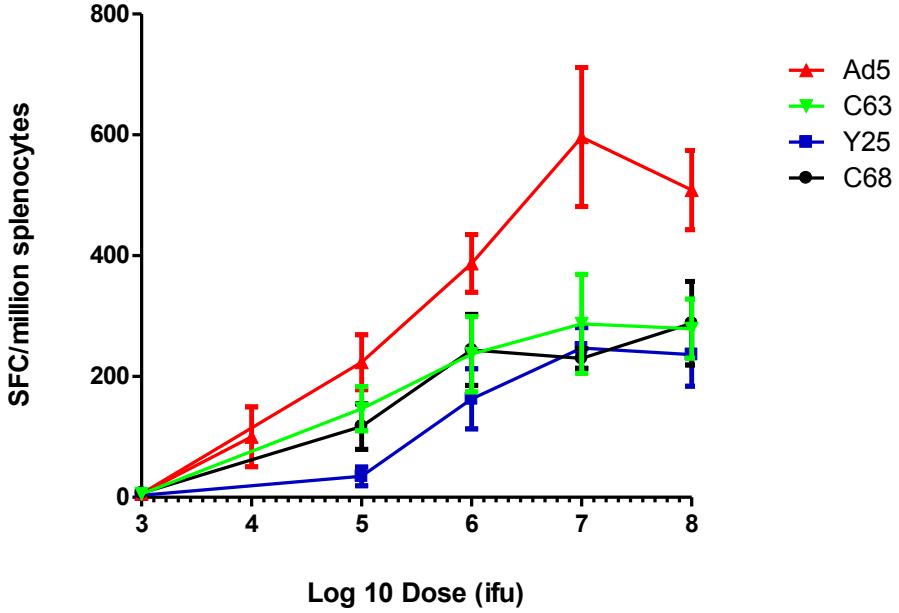
800
600
400
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SFC/million splenocytes
3
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Log 10 Dose (ifu)
Ad5
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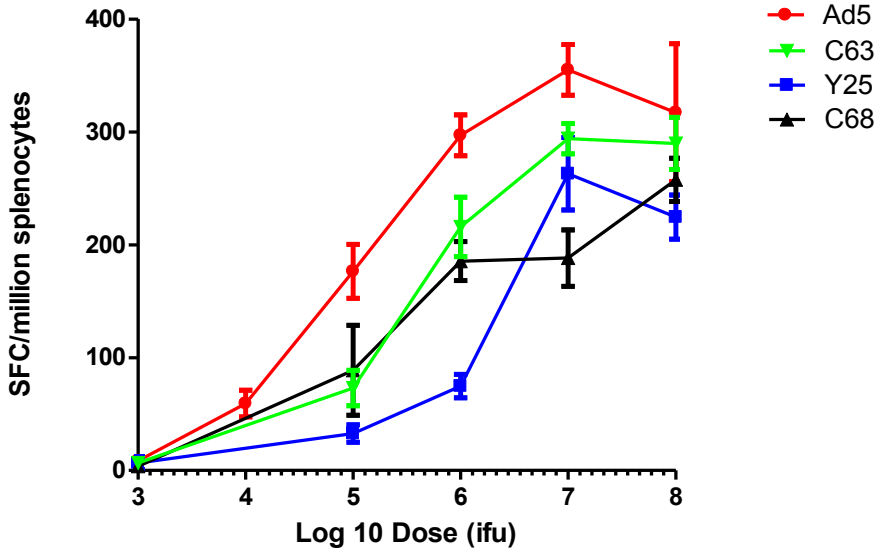
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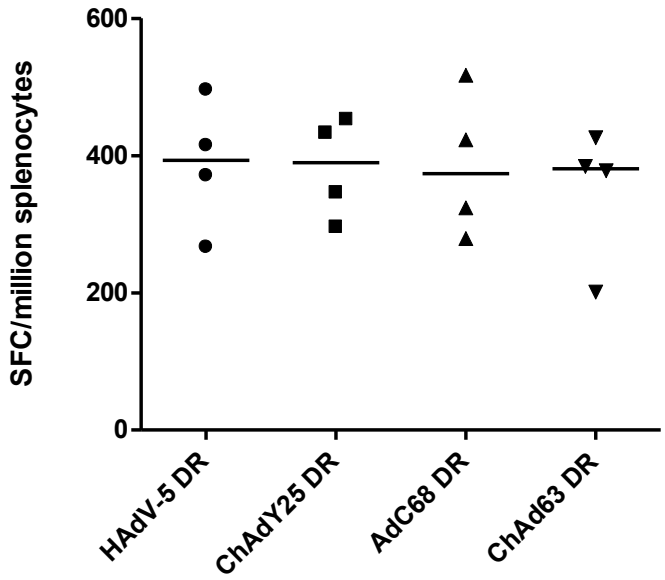
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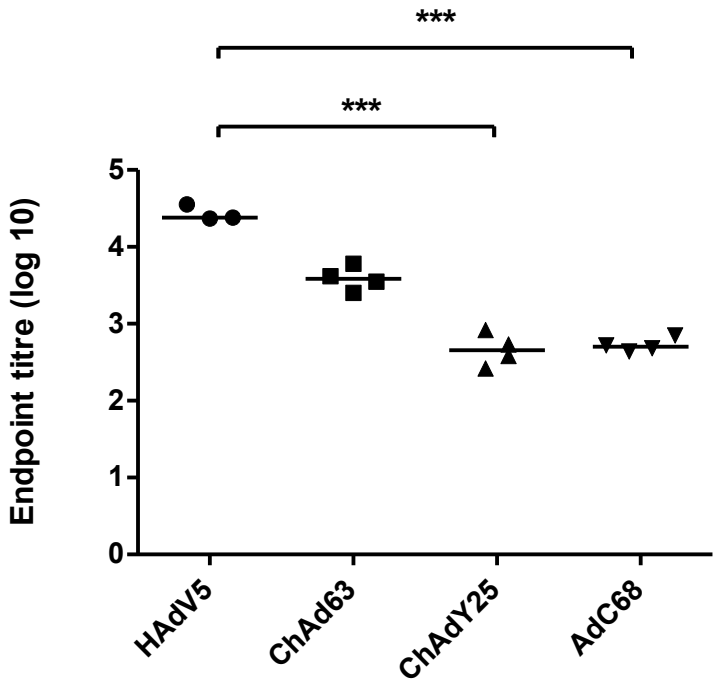
SFC/million splenocytes
HAdV-5 DR
ChAdY25 DR
AdC68 DR
ChAd63 DR

Figure 4.2: HAdV-5 vectors elicit superior transgene product specific antibody titers. Serum was collected two weeks post immunization from Balb/c mice from the previous experiment (Figure 4.1) immunised with 10^8 ifu. Anti-GFP IgG endpoint titers were measured by ELISA against recombinant GFP protein. Graph shows mean response. Statistical analysis by one-way ANOVA with Bonferroni post test. HAdV-5 titer is significantly greater than both AdC68 and ChAdY25, $p<0.001$ (***).



4.2.2 The superior immunogenicity of HAdV-5 based vectors in mice is independent of transgene antigen and mouse strain.

The striking differences in immunogenicity between HAdV-5 and the three chimpanzee adenovirus vectors from *Human adenovirus E* demonstrated previously could potentially have been a specific feature of using the TIPeGFP model antigen, or the inbred Balb/c strain of mice. It was therefore important to show that this result could be repeated using other vaccine antigens, particularly those destined for clinical application.

Antigen 85A is a leading candidate antigen for subunit vaccines against *Mycobacterium Tuberculosis*. MVA85A [243], a recombinant modified Vaccinia virus Ankara (MVA) based vector expressing antigen 85A is currently in Phase III clinical efficacy trials in South Africa, and is therefore the most advanced MTB vaccine candidate in clinical development since the deployment of Bacillus Calmette-Guérin, (BCG). HAdV-5 and ChAdOX1 (ChAdY25-E) based recombinant vectors expressing antigen 85A were constructed for comparative immunogenicity studies. The Pk (V5) Epitope Tag (GKPIPNPLLGLDST) was fused to the C-terminus of 85A, enabling transgene expression *in vitro* to be detected using a V5 monoclonal antibody. This enabled confirmation that titration of both vectors was equally sensitive by anti-hexon and anti-V5 immunostaining (data not shown). Once again, the HAdV-5 85A vector delivered higher frequency T cell responses two weeks post immunisation, both against the CD8+ (p11) epitope, and CD4+ (p15) epitope (Figure 4.3).

The ChAdOX1 vector has been shown to elicit comparable magnitudes of transgene product specific T cell responses to existing chimpanzee adenovirus vectors in mice (Figure 4.1), and has low human seroprevalence (Figure 3.7). It is also free from commercial ownership, and has therefore become an attractive vector for clinical application at the University of Oxford. Influenza vaccine ChAdOX1 NP+M1, in which the recombinant vector encodes the nucleoprotein and Matrix-1 proteins from Influenza A, recently became the first ChAdOX1 based vector to enter trials in humans (Gilbert *et al*, manuscript in preparation). Prior to the clinical study, immunogenicity of the ChAdOX1 NP+M1 vector was compared to a similar HAdV-5 based vector in mice (Figure 4.4). Vectors were titered using the hexon immunostaining assay, and Figure3.5-B from the previous chapter, indicates that the assay sensitivity is comparable between NP+M1 and GFP expressing vectors ChAdOX1 vectors, and hence between ChAdOX1 (ChAdY25-E) and HAdV-5 (Figure3.5-A). Spleen IFN-γ ELISpot responses against dominant CD8+ T cell epitope TYQRTRALV were comparable between the two vectors at the higher doses; the dose response curve appeared to plateau at 10^7 infectious units. However, at a log lower dose of 10^6 infectious units, responses induced by the HAdV-5 vector were significantly superior to the ChAdOX1 vector (Figure 4.4).

Finally, a study comparing immunogenicity of HAdV-5, ChAd63 and ChAdOX1 (ChAdY25-E) vectors expressing the TIPeGFP model antigen was performed in an alternative inbred mouse strain, C57BL/6. SIV-gag, a dominant CD8+ epitope in C57BL/6 present within the N-terminal TIP epitope string was used to assess CD8+ T cell responses (Section 2.7.7). At the lower dose of 10^6 ifu, HAdV-5 once again elicited a superior mean IFN-γ ELISpot response in

comparison to ChAd63 and ChAdOX1 vectors, though the difference was not statistically significant (Figure 4.5A). More strikingly, only vector HAdV-5 TIPeGFP generated a detectable transgene product specific IgG antibody response (Figure 4.5B). While HAdV-5 TIPeGFP generated a robust anti-GFP antibody response, mice immunised with ChAd63 and ChAdOX1 vectors failed to seroconvert.

In conclusion, the superior ability of HAdV-5 based vectors to generate transgene product specific effector T cell and antibody in comparison to chimpanzee adenovirus vectors from species *Human adenovirus E* is independent of the epitope, transgene, and inbred mouse strain studied.

Figure 4.3: HAdV-5 vectors expressing antigen 85A elicit superior antigen specific CD8+ and CD4+ T cell responses compared to ChAdOX1. Balb/c mice were immunised intramuscularly with HAdV-5 85A or ChAdOX1 85A vectors at doses ranging from 10^6 to 10^8 ifu (infectivity titer by hexon immunostaining). Spleens were harvested two weeks post immunization, and T cell responses against **(A)** CD8+ epitope *P*11 and **(B)** CD4+ epitope P15 were measured by IFN-γ ELISpot. Graphs show mean response and SEM. Statistical analysis performed by one way ANOVA with Bonferroni post test. **p<0.01, ***p<0.001

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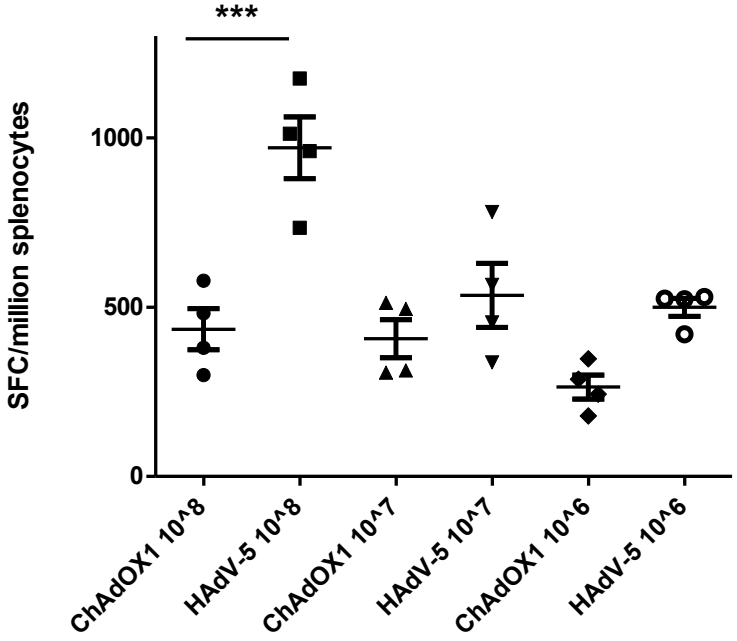

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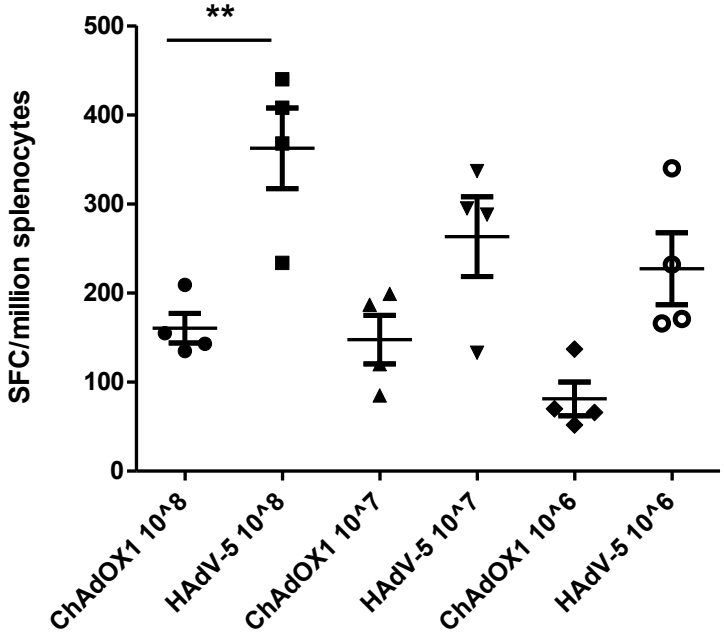


Figure 4.4: HAdV-5 vectors expressing Influenza A antigens nucleoprotein (NP) and Matrix 1 (M1) elicit higher frequencies of antigen specific CD8+ T cells compared to ChAdOX1 at intermediate dose. Balb/c mice were immunised intramuscularly with HAdV-5 NP+M1 or ChAdOX1 NP+M1 vectors at doses ranging from 10^8 – 10^6 infectious units (hexon immunostaining). Spleens were harvested two weeks post immunization, and T cell responses against dominant CD8+ T cell epitope TYQRTRALV (NP residues 147-155) were measured by IFN-γ ELISpot. Graph shows mean response and SEM. Statistical analysis performed by one-way ANOVA with Bonferroni post test. ***p<0.001

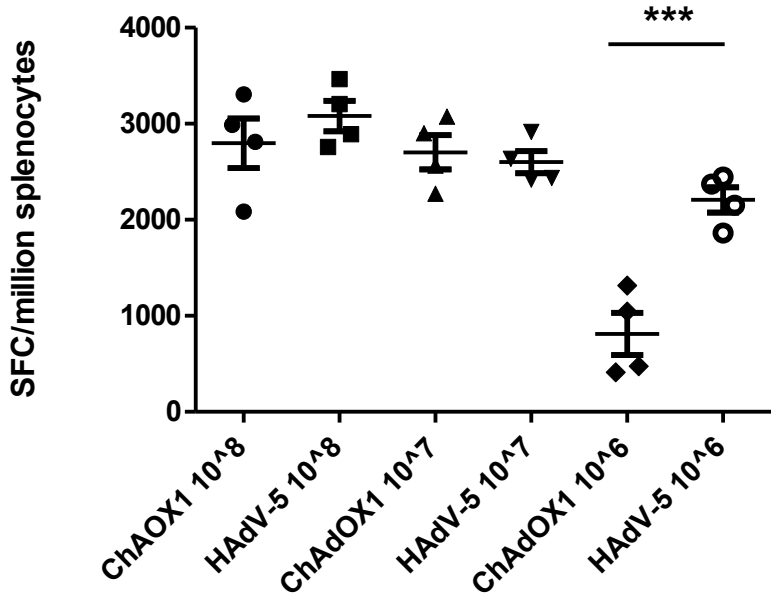
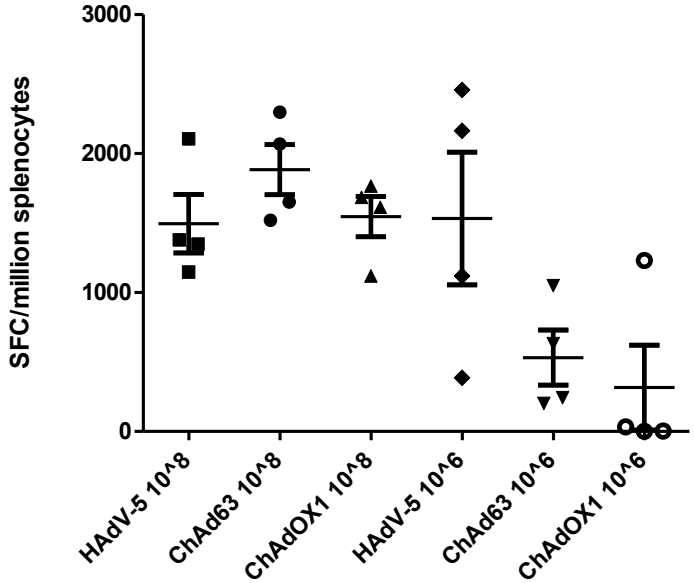


Figure 4.5: HAdV-5 vectors deliver higher frequencies of transgene product specific responses in C57BL/6 mice. C57BL/6 mice were immunised intramuscularly with 10^8 or 10^6 ifu of HAdV-5, ChAd63 or ChAdOX1 vectors expressing TIPeGFP. Infectivity titers were measured by GFP focus assay. **(A)** Spleens were harvested two weeks post immunization, and T cell responses to dominant CD8+ epitope SIV-gag were measured by IFN-γ ELISpot. No statistically significant differences between groups by one-way ANOVA. **(B)** Anti-GFP IgG antibodies were measured in sera of mice immunised with 10^8 ifu of recombinant vector two weeks post immunization by GFP ELISA. Dashed line indicates limit of detection. Graphs show mean response and SEM.

A



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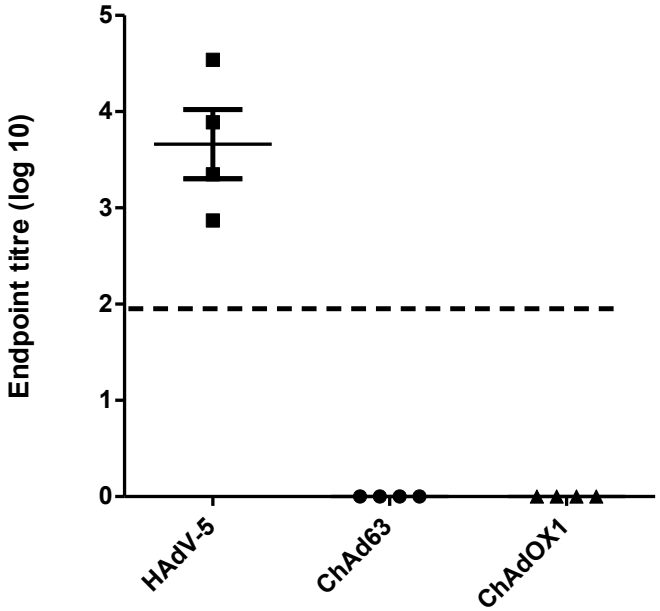


4.2.3 The magnitude of the effector CD8+ T cell response elicited by HAdV-5 based vectors peaks later and is more durable compared to species E chimpanzee adenovirus vectors.

The previous immunogenicity studies had all been performed two weeks post immunization, generally considered to be at or near the peak of the CD8+ effector T cell response after adenovirus vector administration [199,211]. However, it was important to test the hypothesis that the observed superiority of HAdV-5 based vectors could simply be due to differences in kinetic of the immune response. The kinetic of the CD8+ effector T cell response for each vector was monitored by blood sampling from the same individuals at time points up to eight weeks post vaccination. Intracellular cytokine staining (ICS) and flow cytometry was performed on blood samples to assess antigen specific effector responses. Previous studies, including those from our own laboratory, have indicated that conclusions drawn from assessment of T cell responses in the blood and spleen are generally consistent [211,244]. Figure 4.6 indicates that at the high dose of 10^8 ifu the HAdV-5 based vector maintains superior transgene product specific CD8+ T cell responses throughout the eight week period post vaccination. In addition, the peak of the effector response after HAdV-5 administration is later than after ChAd vector immunization, and indeed the difference in the magnitudes of responses is greater three weeks post immunisation compared to two weeks (the interval for the previous studies). At the lower dose of 10^6 ifu, CD8+ effector responses elicited by all four vectors

follow a similar kinetic with the HAdV-5 based vector trending to superiority without statistical significance (in agreement with Figure 4.1).

Antigen experienced CD8+ T cells exhibit substantial heterogeneity *in vivo*; differing in cytotoxic potential, cytokine secreting ability, longevity, and anatomical localization [245,246]. The phenotype and functionality of CD8+ T cells, primed in secondary lymphoid organs, can be influenced by a number of factors including the nature of the antigen presenting cell, local cytokine milieu, and the polarity of CD4+ T helper (T_H) response. However, of particular importance in the generation of a lasting memory response is the strength and duration of antigen encounter. Bachmann *et al* [245] have catagorized antigen experienced CD8+ T cells into three main subsets according to expression of the lymph node homing receptor CD62L and the IL-7 receptor CD127. These two markers have been used to broadly define central memory T cells (CD62L+CD127+), effector memory T cells (CD62L-CD127+), and effector T cells (CD62L-CD127-). Effector CD8+ T cells (T_{EFF}) have superior cytolytic potential and cytokine secreting capacity but have a short half life and limited proliferative capacity. Effector memory T cells (T_{EM}) have a longer half life and enhanced proliferative potential compared to T_{EFF} cells but may have reduced cytolytic potential. Central memory T cells (T_{CM}) are located mainly in secondary lymphoid organs due to upregulation of CD62L, and have superior proliferative capacity but reduced cytolytic and cytokine secreting potential compared to the T_{EFF} and T_{EM} subsets. Reduction in antigen load has been associated with a contraction of the T_{EFF} CD8+ response to memory T_{EM} and T_{CM} cells, whereas antigen persistence leads to maintenance of the T_{EFF} population.

Due to the marked differences in CD8+ effector T cell kinetic between different adenovirus vectors at the high 10^8 ifu dose, the memory phenotype of induced CD8+ T cells from the previous kinetic study was investigated. Figure 4.7 indicates that, while IFN-γ+ CD8+ T cells are phenotypically similar between groups, HAdV-5 does maintain a slightly elevated population of T_{EFF} cells up to five weeks post immunisation compared to ChAd63 and ChAdOX1 vectors. The significance of this result will become clear in the following chapter (Chapter 5). By eight weeks post immunisation, the relative proportion of T_{EFF} and T_{EM} cells is similar between all three vectors. The proportion of T_{CM} cells observed was very low, but this was to be expected since sampling was performed in blood and the majority of CD8+ T_{CM} cells reside in secondary lymphoid organs [246].

Figure 4.6: CD8+ T cell kinetic differs with vector serotype. Balb/c mice (6/group) were immunised intramuscularly with either **(A)** 10^8 ifu or **(B)** 10^6 ifu of HAdV-5, ChAd63, AdC68 or ChAdOX1 vectors expressing the model antigen TIPeGFP. Blood was collected on days 12, 21, 36, and 56 post vaccination and the percentage of *Pb9* specific CD8+ IFN-γ+ T cells was determined by intracellular cytokine staining (ICS). Graphs display mean and SEM. Statistical analysis was performed by one-way ANOVA with Bonferroni post test. Statistical significance indicated where HAdV-5 responses are significantly greater than all three ChAd vectors. **p<0.01, ***p<0.001

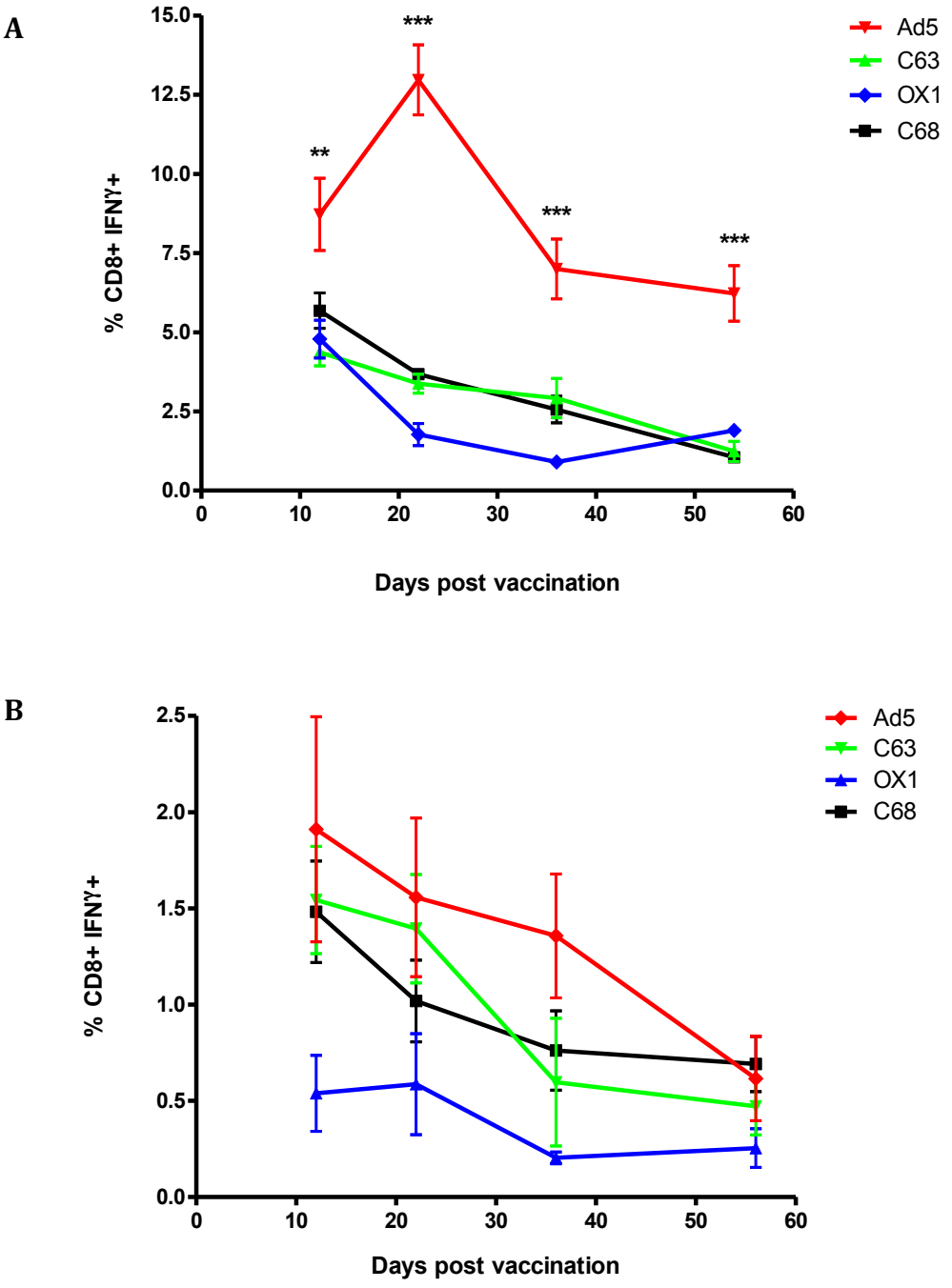
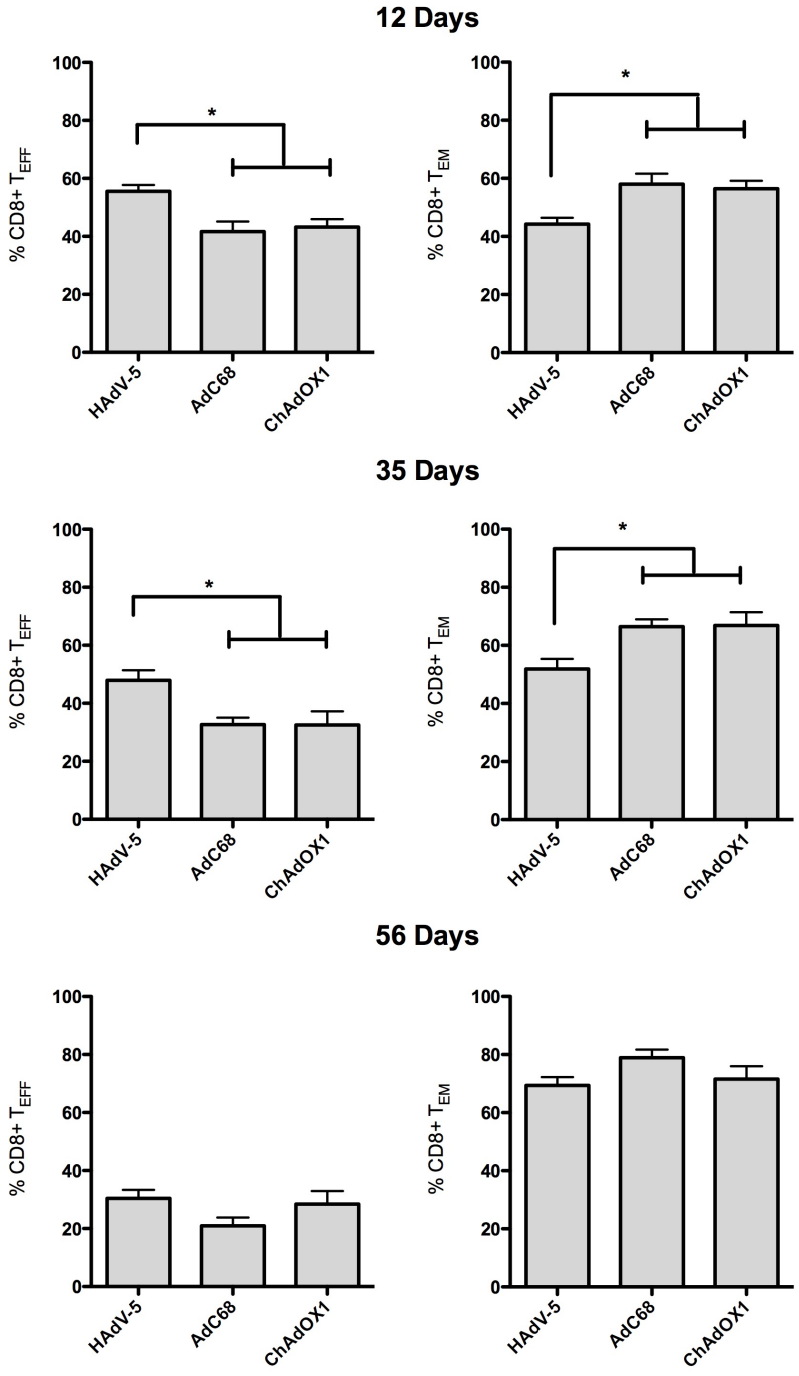


Figure 4.7: The CD8+ T cell memory phenotype differs after immunisation with HAdV-5 and ChAd based vectors. *Pb*9 specific IFN-γ+ CD8+ T cells shown in Figure 4.6 were stained for expression of surface markers CD127 and CD62L to indicate memory phenotype. Relative percentages of *Pb*9 specific IFN-γ+ CD8+ T_{EFF} and T_{EM} cells on days 12, 35 and 56 post immunization are shown below. Less than 1% of *Pb*9 specific CD8+ T cells displayed a T_{CM} phenotype (data not shown). Bars show mean and SEM. Statistical analysis performed by one-way ANOVA with Bonferroni post test. *p<0.05

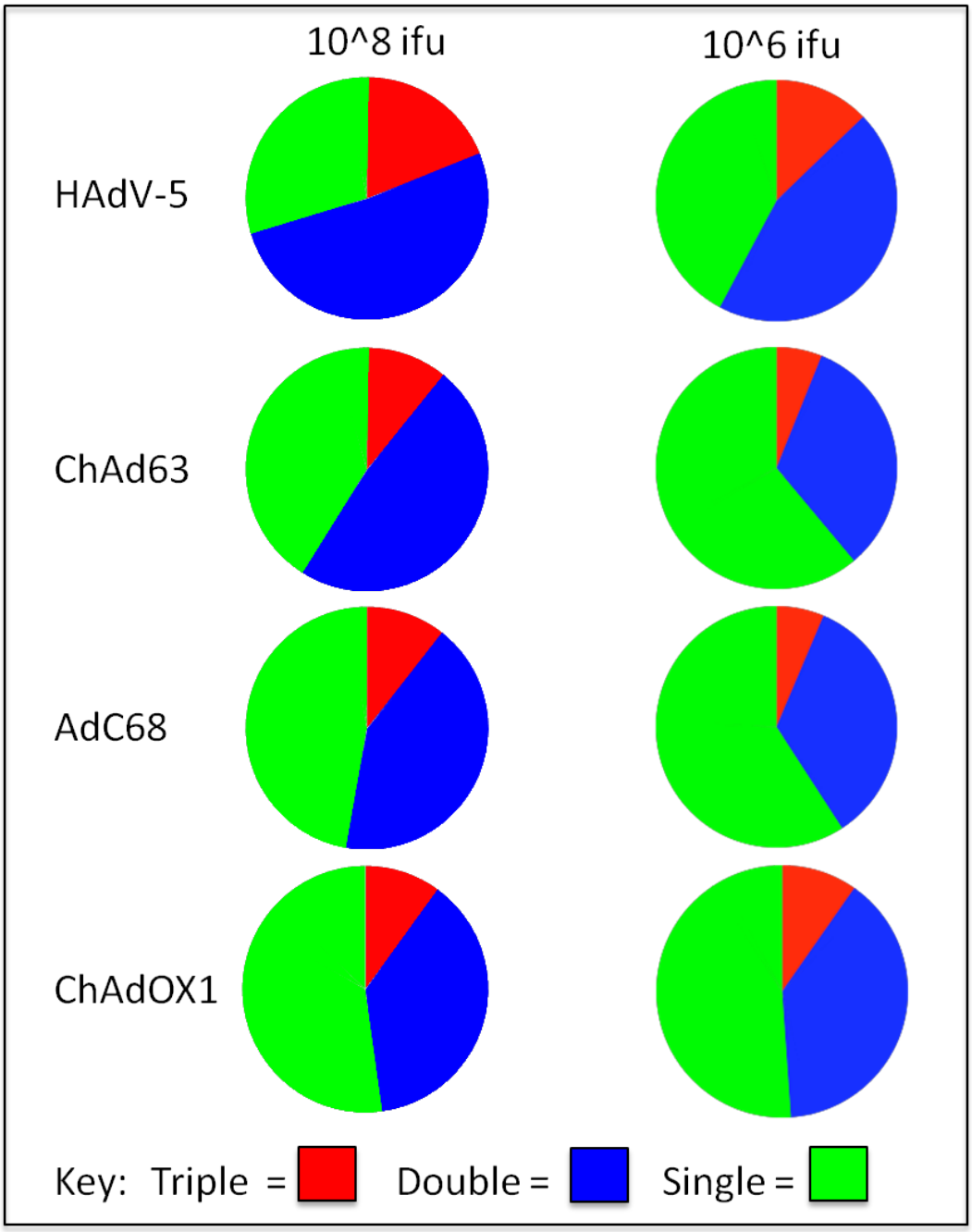


4.2.4 CD8+ T cells induced by HAdV-5 and ChAd based vectors are similar in functionality of cytokine secretion.

Thus far, the ability of transgene product specific CD8+ T cells to secrete the pro-inflammatory cytokine IFN-γ has been the only parameter used to assess the effector function of vaccine induced CD8+ T cells. Despite the importance of this cytokine in the development of an effective T cell response (Section 1.3.3.2) it is not the only cytokine secreted by antigen experienced T cells. Indeed, recent studies have suggested that the ability of primed CD8+ and CD4+ T cells to secrete multiple cytokines is an important requirement for establishing a protective response [247]. Interest in T cell 'multi-functionality' increased after a study in mice by Darrah *et al* in 2007, which prospectively correlated the proportion of CD4+ T cells simultaneously secreting cytokines IFN-γ, IL-2 and TNFα with increased protection from *Leishmania major* infection [248]. Other studies have suggested the importance of CD8+ T cell 'multi-functionality', including a study by Betts *et al* that associated multi-functionality of human CD8+ T cells with control of viremia in HIV-infected non-progressers [248]. Though the significance of CD8+ T cell 'multi-functionality' has been disputed for other diseases [224,249], it was important to establish whether there are differences in the 'multi-functionality' of the CD8+ T cell response between adenovirus vectors of different serotype, in addition to the difference in magnitude and persistence. Figure 4.8 shows that the multi-functionality of the CD8+ T cell response between vectors is comparable. HAdV-5 vectors induced a slightly higher proportion of double and triple positive CD8+ T cells compared to the ChAd vectors, though evidence from our own laboratory suggests that this is

likely a function of the higher overall magnitude of the CD8+ T cell response after HAdV-5 administration (Spencer *et al*, manuscript in preparation). Data for CD4+ responses are not shown since the magnitude of the response to the P15 CD4+ epitope was not sufficient to reliably quantify multifunctional responses.

Figure 4.8: Multi-functionality of CD8+ T cell responses after vaccination with HAdV-5, ChAd63, AdC68 or ChAdOX1 based vectors. Multifunctional CD8+ T cell responses were studied using blood collected at the day 12 time point from the kinetic experiment shown in Figure 4.6. Pb9 specific CD8+ T cells were identified expressing one or more of the cytokines IFN-γ, TNF-α and IL-2 by flow cytometry. The proportion of CD8+ T cells secreting one cytokine, two cytokines, or all three cytokines in response to Pb9 re-stimulation are show below.



4.2.5 Transgene product specific IgG antibodies share a similar T_H1 subclass bias for both HAdV-5 and ChAd based vectors.

There are four subclasses of Immunoglobulin G (IgG) in mice, IgG1, IgG2A, IgG2B and IgG3. Induction of IgG2A, IgG2B and IgG3 subclasses is stimulated by IL-12 and indicative of a T_H1 response, while IgG1 induction is indicative of a T_H2 response [250]. IgG2A and IgG2B subclasses bind to Fc receptors on phagocytes with the highest affinity[251], and together with IgG3 fix complement more efficiently than IgG1[252]. The balance between T_H1 and T_H2 subclasses can be investigated by comparing titers of subclasses IgG2A and IgG1 [209,253,254]. De Cassan *et al* have previously shown that intramuscular administration of adenovirus vaccine vectors based on HAdV-5 skew humoral responses to a cytophilic T_H1 subclass [209]. Furthermore, Xiang *et al* have shown that vectors based on HAdV-5 and AdC68 induce a similar T_H1 bias in transgene product specific IgG responses after subcutaneous and intranasal administration[254]. The data in Figure 4.9 agree with these previous studies and indicate that this T_H1 bias is also true for ChAd63 and ChAdOX1 based vectors. There is little difference between the ratio of IgG2A and IgG1 between HAdV-5 and ChAd based vectors.

Figure 4.9: IgG antibody responses are biased towards a T_H1 subclass after vaccination with HAdV-5 and ChAd based vectors. Balb/c mice (6/group) were vaccinated intramuscularly with 10^9 ifu of HAdV-5, ChAd63, AdC68 and ChAdOX1 vectors expressing TIPeGFP. Five months post vaccination serum was collected and anti-GFP IgG antibodies of subclass IgG2A (T_H1) or IgG1 (T_H2) were measured by ELISA. **A** Endpoint ELISA titers for the IgG2A and IgG1 subclasses**. B** Ratio between IgG2A and IgG1 titers shown in A. No significant differences were observed between vector groups by one-way ANOVA (Bonferroni post test). Bars represent mean and SEM.

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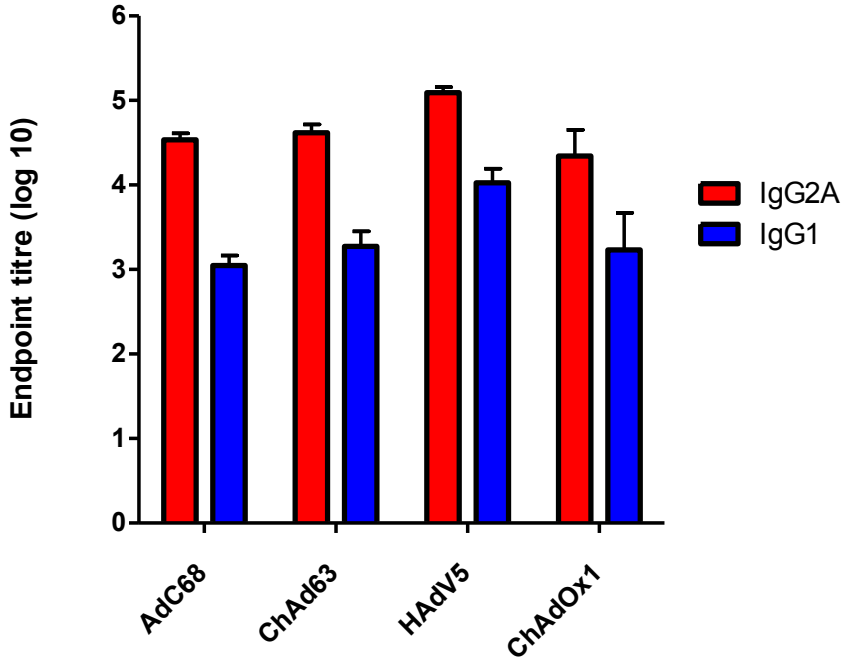

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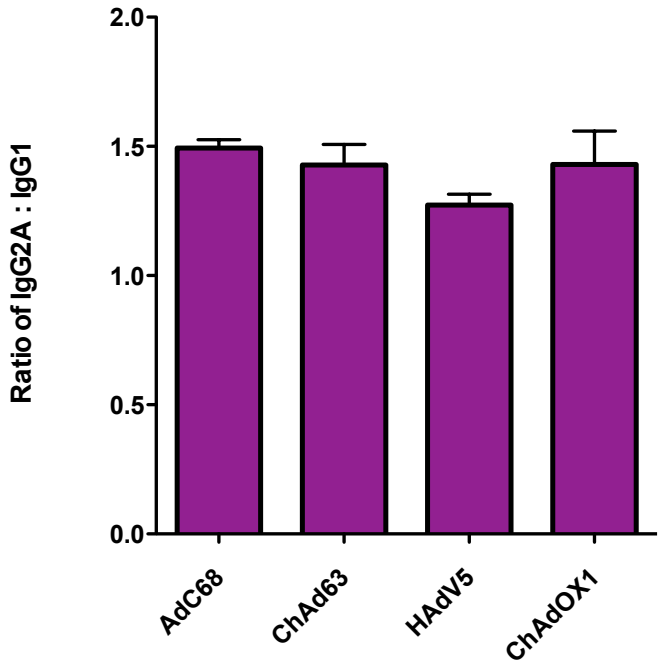

4.2.6 ChAd vectors vary in their ability to act as effective priming agents for transgene specific antibody responses in the context of a heterologous prime boost with modified vaccinia virus Ankara.

In both pre-clinical and clinical vaccine settings, adenovirus vectors have been shown to act as highly effective priming agents in the context of heterologous prime boost immunisation regimens [255,256,257]. Indeed, for many clinical applications, a single adenovirus vector immunisation may be insufficient for protective immunity in humans. Modified vaccinia virus Ankara (MVA) vectors in particular have shown a remarkable capacity to boost adeno-vector induced responses both in pre clinical models, and in human clinical trials [165,224,258]. The ability of vectors based on HAdV-5, ChAd63, AdC68 and ChAdOX1 to prime a response boosted with an MVA vector expressing the same recombinant antigen was assessed. Mice were primed with a 10^8 ifu dose of adenovirus vector expressing TIPeGFP followed eight weeks later with a boosting immunization of MVA TIPeGFP. Despite the HAdV-5 vector generating a higher magnitude of CD8+ T cells immediately prior to the boost (in agreement with Figure 4.6), responses were comparable one week after the MVA boost, with all four vectors priming a robust response post boost (Figure 4.10A). Responses post boost were also comparable when a lower priming dose of 10^6 ifu of each adenovirus vector was used (data not shown). However, anti-GFP antibody responses varied significantly between the different serotypes both before and after the boosting immunization (Figure 4.10B). HAdV-5 and ChAd63 vectors primed significantly higher boost responses than the ChAd68 vector.

Responses post boost after priming with ChAdOX1 were highly variable; in two out of six individuals there was no increase in the response after MVA, while the other four individuals achieved endpoint titers comparable to the ChAd63 and HAdV-5 vaccinated groups.

In conclusion, transgene product specific CD8+ T cell responses after boosting with recombinant MVA are largely independent of the adenovirus serotype used to prime the response, in mice. However, there are significant differences between post-boost transgene product specific IgG responses after priming with adenovirus vectors of different serotype. Differences in immunogenicity between adenovirus vectors are therefore relevant to both single dose and prime boost vaccine regimens.

Figure 4.10: Transgene product specific CD8+ T cell responses are comparable after adenovirus-MVA prime boost regimens with adenovirus vectors of different serotype, but antibody responses vary significantly. Balb/c mice were vaccinated intramuscularly with HAdV-5, ChAd63, AdC68 or ChAdOX1 vectors expressing TIPeGFP at a dose of 10^8 ifu. After eight weeks, the same mice were boosted with 10^6 plaque forming units (pfu) of MVA-TIPeGFP administered into the opposite leg as the adenovirus injection. **(A)** Pb9 specific CD8+IFN-γ+ responses were measured by flow cytometry, both immediately before ('Pre') and one week after ('Post') the MVA boost vaccination. HAdV-5 responses were significantly higher pre-boost (P<0.001) but there was no significant difference in magnitude after the boost **(B)** At the same time points, anti-GFP IgG responses were measured in serum by endpoint ELISA. Dashed line represents limit of detection. All statistical analyses performed by one-way ANOVA with Bonferroni post test.

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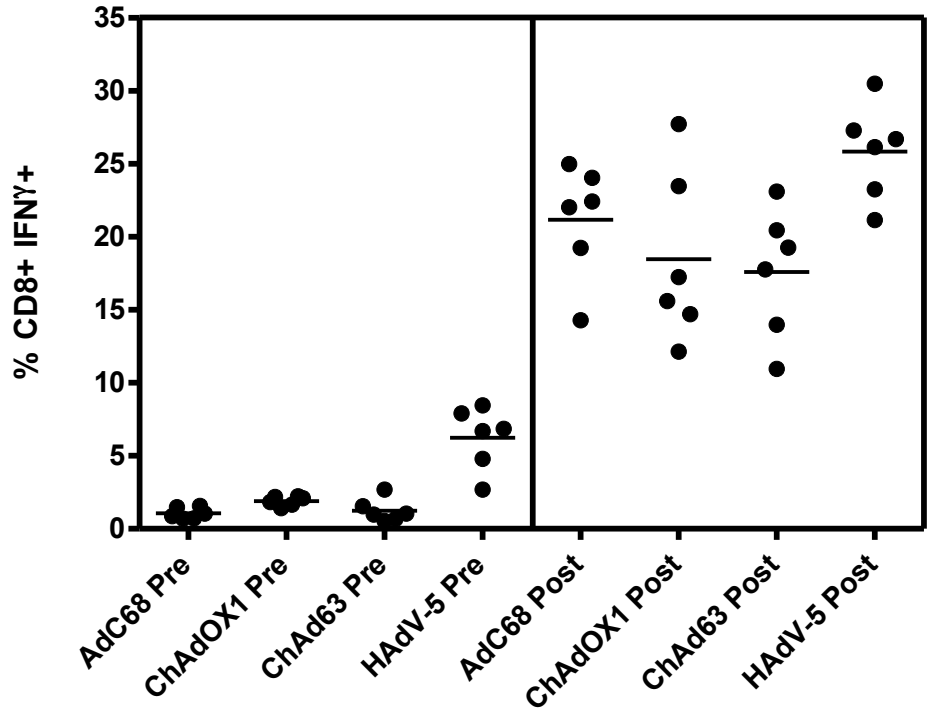

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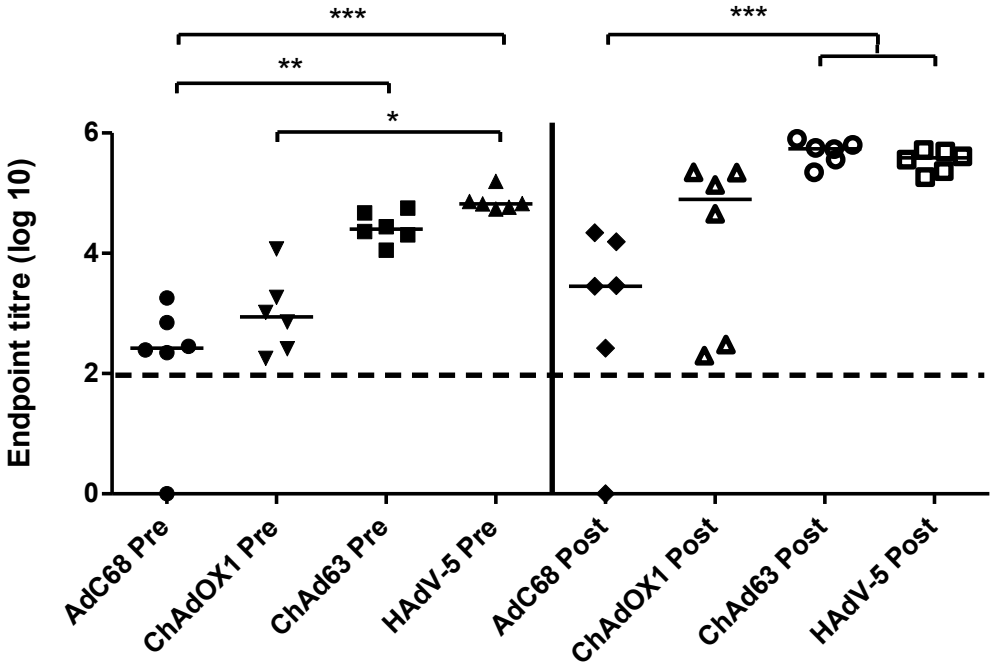
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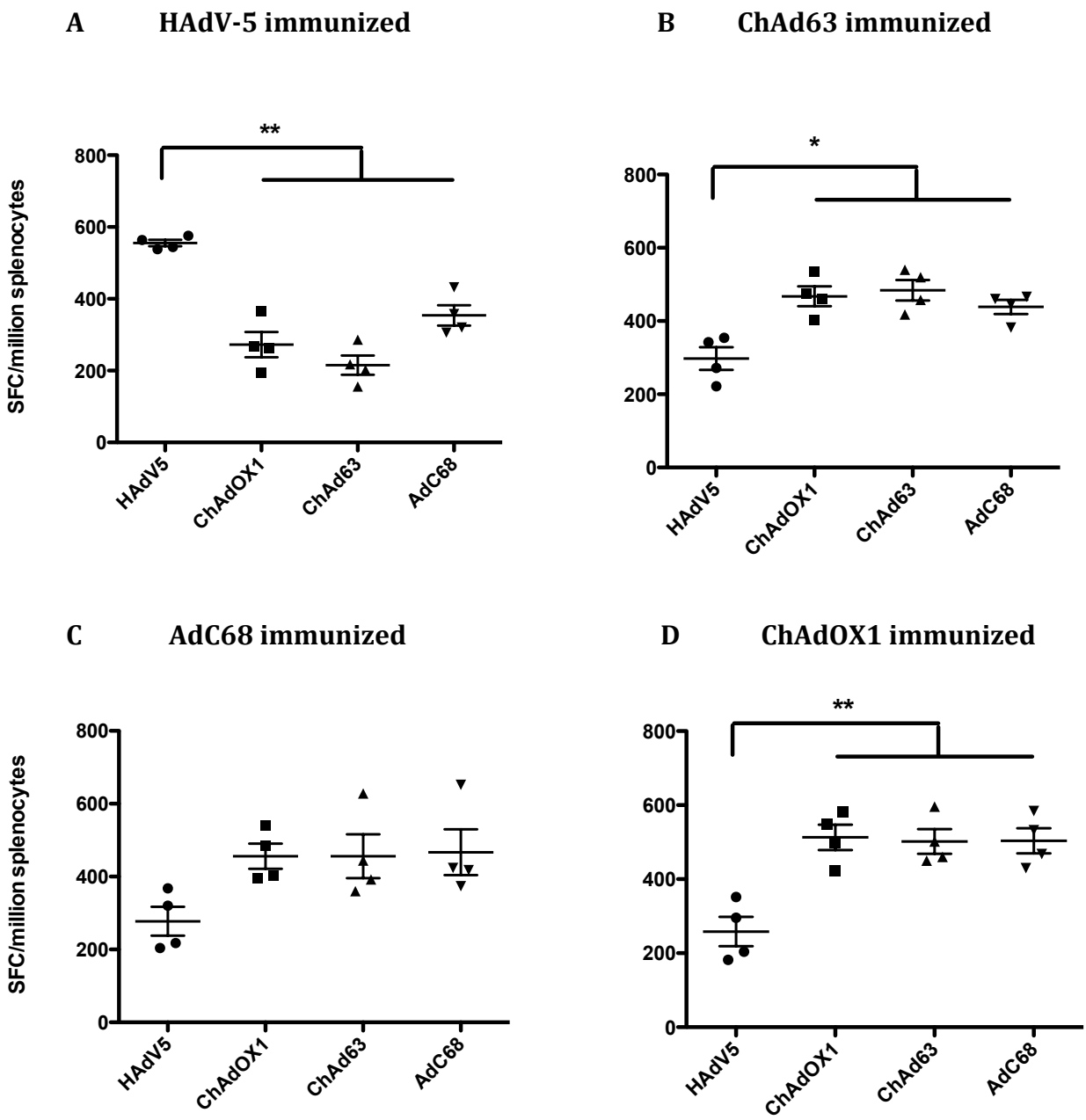
4.2.7 Anti vector T cell responses are highly cross reactive between members of *Human adenovirus E*, but less so with HAdV-5.

Replication defective recombinant adenovirus vectors were first developed as transgene delivery agents for gene therapy applications. However, in most applications, transgene expression was not sustained beyond a few weeks post administration [259]. The level of transgene expression has also been shown to be important in vaccine applications, and is investigated further in Chapter 5. A primary cause of the decline in transgene expression is the induction of CD8+ T cell responses against both transgene product and viral vector, leading to cytotoxic killing of vector transduced cells [156,260]. Induction of innate immunity is also believed to play an important role in silencing of vector delivered transgene expression [261], and is the focus for Chapter 6. Induction of CD8+ T cells against immunodominant components of the viral vector has also been shown to limit the breadth of the transgene product specific response by reducing the magnitude of the CD8+ responses to subdominant epitopes within the encoded transgene [262].

Anti-vector T cell responses against HAdV-5, ChAd63, AdC68 and ChAdOX1 were assessed in mice by stimulating splenocytes from vaccinated individuals with heat inactivated preparations of each CsCl purified virus. Figure 4.11 shows that anti-vector T cell IFN-γ ELISpot responses are highly cross reactive between the ChAd derived members of *Human adenovirus E*, perhaps reflecting the high degree of sequence homology between them (over 90% identity across the entire E1 E3 deleted genome). Responses against the HAdV-5 vector were less cross reactive with members of *Human adenovirus E*, although

the magnitude of the HAdV-5 response against the HAdV-5 vector protein was comparable to the magnitude of the ChAd vector response against each of the ChAd vector preparations.

Figure 4.11: Cross reactivity of anti-vector responses against HAdV-5, ChAd63, AdC68 and ChAdOX1 vectors. Balb/c mice (4/group) were immunized with 10^9 ifu of **(A)** HAdV-5 **(B)** ChAd63 **(C)** AdC68 or **(D)** ChAdOX1 vectors expressing TIPeGFP. After two weeks, spleen T cell responses against heat inactivated CsCl purified material from each vector preparation were assessed by IFN-γ ELISpot. Tests for statistical significance were performed by one-way ANOVA with bonferroni post test. *p<0.05, **p<0.01



4.3 Discussion

Adenovirus vaccine vectors based on HAdV-5 elicit superior transgene product specific cellular and humoral immune responses compared to vectors based on ChAd63, AdC68 and ChAdOX1 after intramuscular immunization in mice (Figure 4.1 and 4.2). This observation was not transgene dependent or mouse strain dependent (Figure 4.3-4.5) and in the case of the effector CD8+ T cell response, was maintained up to eight weeks post immunization (Figure 4.6). However, it is important to note that these differences in immunogenicity do not necessarily predict that *Human adenovirus E* ChAds are likely to be non-efficacious vaccine vectors in murine challenge models. Indeed, work from the laboratory of John Harty has shown that *Pb9* specific CD8+ T cell frequencies greater than 1% are sufficient to induce sterile immunity upon *Plasmodium berghei* sporozoite challenge [263]. This immunogenicity threshold was reached after administration of each of the four vectors at the 10^8 ifu dose (Figure 4.6).

The superior immunogenicity of *Human adenovirus C* HAdV-5 based vectors over *Human adenovirus E* based vectors observed here is in agreement with some prior publications [201,242] but not others [179,211,228]. This lack of consensus within the field has highlighted the importance of carefully considering the infectivity of vaccine preparations, since the majority of previous studies have failed to do so. Colloca *et al* have recently compared a large number of vector serotypes, and have also concluded that *Human adenovirus C* based vectors were, as a group, more effective at inducing transgene specific IFN-γ ELISpot responses in mice than *Human adenovirus E* based vectors [201]. This has been the most comprehensive study of adenovirus vaccine vector

immunogenicity to date, with 26 serotypes tested, although vector infectivity was not built into the readout of the study.

It is also important to note that the use of high vaccine doses may confound comparative studies, since a plateau in the magnitude of CD8+ T cell frequencies may be reached. Such a phenomenon was observed in the dose response study (Figure 4.1), and also confounded the comparison between HAdV-5 and ChAdOX1 vectors expressing influenza NP and M1 antigens at the 10^8 and 10^7 ifu doses (Figure 4.4). Previous studies have typically administered doses over 10^10 viral particles in mouse experiments [179,211], which is the comparable to the maximum doses currently used in human clinical trials [173]. Administration of such high doses may explain why some previous studies have failed to demonstrate differences in immunogenicity between vectors of different serotype.

The current study has also highlighted a difference in kinetic of the CD8+ effector response after immunisation with HAdV-5 and ChAd vectors from *Human adenovirus E*. At the high 10^8 ifu dose, the frequency of the *Pb9* specific CD8+IFN-γ+ response elicited by HAdV-5 based vectors peaked a week later and was more durable than for ChAd63, AdC68 and ChAdOX1 based vectors (Figure 4.6). Analysis of the memory phenotype of these cells hinted that there may be a delay in contraction to memory after HAdV-5, with a slightly higher proportion of effector CD8+ T cells (T_{EFF}) compared to the ChAd vectors for at least five weeks post immunisation (Figure 4.7). By eight weeks post immunisation, the proportion of T_{EFF} to effector memory CD8+ T cells (T_{EM}) was similar for all four vectors, with the majority adopting an effector memory phenotype, in agreement with previous studies [179,264]. A delay in contraction to a memory phenotype

is often associated with persistence of antigen presentation [245]. This would suggest that either the initial delivery of antigen by HAdV-5 is more efficient and hence clearance requires longer, or that *de novo* antigen expression persists for longer after vaccination. The efficiency of antigen expression from HAdV-5 compared to the ChAd vectors will be addressed in the following chapter (Chapter 5).

Functionality of the effector CD8+ T cells induced (defined as expression of cytokines IFN-γ, IL-2 and TNF-α), and IgG isotype of transgene specific antibodies were very similar between all four adenoviral serotypes studied (Figure 4.8 and 4.9). HAdV-5 elicited a CD8+ T cell population with a slightly higher proportion of triple and double cytokine expressing cells, though recent work has shown that this may be directly related to the higher magnitude of the effector response (Spencer *et al*, manuscript in preparation). Immune responses elicited between vectors of different serotype therefore differ mainly in terms of magnitude and kinetic, and these two parameters may be related. It is however worth noting that there are other factors that affect T cell functionality other than secretion of IFN-γ, IL-2 and TNFα. Upregulation of CD107a (LAMP-1) by CD8+ T cells is commonly used as a marker of degranulation and could indicate the cytotoxic potential of activated CD8+ T cells [265]. In addition, the ability of activated CD8+ T cells to proliferation upon re-stimulation can be measured by labeling with carboxyfluorescein succinimidyl ester (CFSE)[266]. However, based on the data acquired, subsequent chapters will assume that differences in the nature of the immune response between vectors are primarily related to magnitude and not functionality.

This study has demonstrated that the differences in immunogenicity between vectors of different serotype may have consequences for prime boost regimens in addition to single dose approaches, particularly regarding IgG antibody induction (Figure 4.10). Mice were given a priming dose of one of the four recombinant adenovirus vectors, followed eight weeks later with a boosting dose of an MVA vector encoding the same recombinant antigen. This heterologous prime boost strategy, with an eight-week interval between vectors, is currently the regimen of choice for clinical studies in our laboratory [84,173,174]. In this study, the magnitude of transgene product specific IgG responses differed markedly between the groups, both before and after the MVA boost. In contrast, CD8+ T cell responses were comparable one week after the boosting immunization, although the response may a reached a plateau. However, the CD8+ T cell kinetic study (Figure 4.7) demonstrated that the proportion of CD8+ T cells displaying a memory phenotype differs between HAdV-5 and the ChAd vectors up to five weeks post immunization but is comparable by eight weeks (the time of the boost). Therefore, variation in the timing of the boosting immunization may have lead to differences in post boost responses that were not detected here.

Though the ultimate aim of viral vector vaccines is to induce CD8+ T cell activation, T cell responses against vector components can both reduce the breadth of the transgene product specific response [262], and reduce transgene expression through T cell mediated elimination of vector-transduced cells [260]. Studies using heat inactivated material from purified vector preparations demonstrated that anti-vector T cell responses were highly cross reactive between members of *Human adenovirus E*, but less so with HAdV-5 (Figure 5.12).

Importantly, the magnitudes of anti-vector responses against the cognate material were comparable between all four vectors. However, the use of heat inactivated vector material is not ideal for the study of anti-vector T cell responses. Magnitudes of responses are low, perhaps reflecting the fact that heat inactivated protein is not as efficiently processed and presented as the peptides more commonly used in an ELISpot assay. Furthermore, the protein sequences from different vectors almost certainly contain different T cell epitopes, some likely to be more immunodominant than others. Attempts were made to assess anti vector responses against a protein sequence common to all four vectors, HAdV-5 E4Orf6. However, differences in the level of expression of this protein between vectors confounded the comparative assessment (data not shown).

This study has clearly demonstrated that HAdV-5 vectors elicit superior magnitudes of transgene product specific CD8+ T cell and antibody responses in mice. However, though selection of adenovirus vectors for clinical application is still based largely on comparative assessment in mice [201], the hierarchy of vector efficacy in mice may not necessarily match that in humans. Indeed, in another mammalian species, cattle, HAdV-5-85A was shown to be less potent than ChAdOX1-85A at inducing 85A specific CD8+ T cell and antibody responses (Efrain Guzman, personal communication). It is therefore important that the mechanisms underpinning the difference in immunogenicity between vectors are fully understood to facilitate the rational design of vectors for use in humans. The subsequent chapters in this study investigate the mechanisms responsible for the superior immunogenicity of HAdV-5 based vectors in mice.

5 Investigating the mechanisms responsible for the difference in immunogenicity between vectors of different serotype in mice

5.1 Introduction

This chapter will focus on determining the mechanisms responsible for the differences in immunogenicity between HAdV-5 and chimpanzee adenovirus vectors from *Human adenovirus E*. Many potential factors could be involved, some of which are illustrated in Figure 5.1. Efficiency of *in vivo* infection could be superior with HAdV-5 vectors, leading to improved antigen delivery and a higher effective vaccine dose. Alternatively, or additionally, differences in tropism between the serotypes could mean that antigen delivery by HAdV-5 is targeted to cells and tissues that are more suited to antigen presentation to T cells and B cells. Data from the CD8+ T cell kinetic study (Figure 4.7) has already hinted that persistence of antigen could be greater after HAdV-5 administration. But is this persistence due to a higher initial antigen load immediately after infection, or more prolonged *de novo* antigen expression? And are there differences in the activity of the CMV promoter between vectors leading to differences in the level of antigen expression per infected cell? Adenoviruses are particularly suited to vaccine delivery, due to the strong innate stimulus provided by these vectors leading to maturation of antigen presenting cells [182,267]. However, an excessive innate response may lead to antiviral immunity and vector elimination by NK cells [268], or suppression of recombinant transgene expression [269].

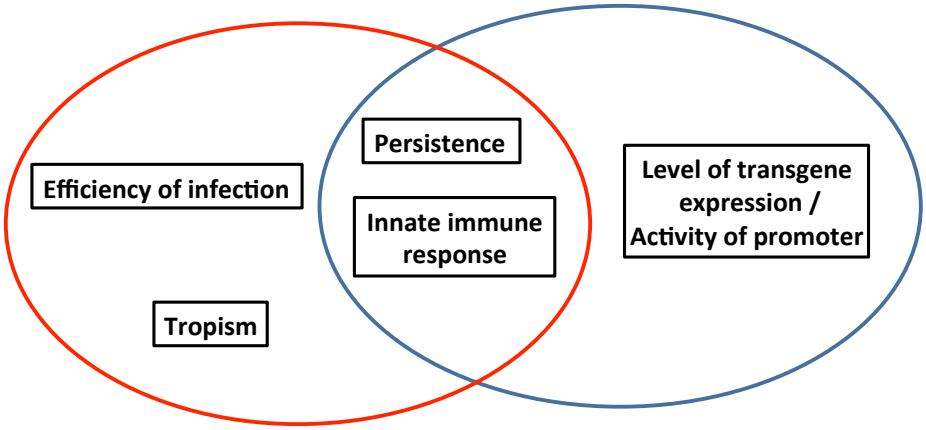
Differences in the innate immune response between vector serotypes may therefore have a significant impact on the magnitude of adaptive immune responses, and this is the specific focus for the next chapter (Chapter 6). It is also important to appreciate that these factors are not mutually exclusive; a combination of factors may be involved. Furthermore, some factors are likely to be interlinked. For instance the innate type I interferon response may down-regulate expression from the viral CMV promoter, as has been demonstrated in the context of intramuscular expression from adenovirus vectors in gene transfer studies [269,270].

The ultimate aim of this work is to develop more potent adenovirus vaccine vectors. An important focus will therefore be to determine the precise components of the vector responsible for the differences in immunogenicity between vectors. In particular, it will be important to determine whether components of the viral capsid, internal components of the virion, or factors expressed *de novo* post transduction, are primarily responsible (Figure 5.1). Capsid proteins could be particularly challenging to modify for vector improvement, since capsid assembly places structural constraints on these proteins. In addition, the major capsid proteins hexon and fiber are situated on the external face of the virion, and are primary targets for virus neutralizing antibodies [271]. Modification of these components may therefore effect *in vivo* neutralization of the virus, with implications for clinical application.

The BAC recombineering system employed in the construction of ChAdOX1 greatly facilitates the investigation of immune determinants, by enabling precise modification of the viral genome. It enables a reverse genetics approach to be taken to conclusively determine the specific effect of individual

viral components on vector immunogenicity. Using this approach, the effect of fiber-mediated infection on the immunogenicity and transduction efficiency of ChAdOX1 *in vivo* was investigated by replacing the native fiber knob and shaft domains with the corresponding regions from the HAdV-5 fiber protein. Construction of this chimeric vector supported an investigation into the link between vector cell entry, transgene expression, and immunogenicity, the prime focus for this forthcoming chapter.

Figure 5.1 – Potential factors involved in determining the immunogenicity of adenovirus vectors. Factors circled in red are likely to involve external viral capsid proteins, while those circled in blue could involve internal components of the virion.



5.2 Results

5.2.1 HAdV-5 vectors infect the CAR abundant A549 cell line more efficiently *in vitro* than ChAd vectors from *Human adenovirus E*.

Previous studies have confirmed that at least one member of *Human adenovirus E*, AdC68, can interact with both human and murine isoforms of the coxsackie and adenovirus receptor (CAR) to infect cells *in vitro* [272]. However, very few studies have addressed the molecular mechanisms by which members of *Human adenovirus E* infect cells either *in vitro* or *in vivo* [273] . In this study, transduction efficiency (the ability of vectors to enter cells and initiate transgene expression) was assessed in human lung carcinoma cell line A549, commonly used in the study of adenovirus biology due to the high surface expression of CAR [274]. Figure 5.2A indicates that HAdV-5 vectors transduce A549 cells more efficiently than ChAdOX1 vectors, across a range of MOIs. As a control, transduction efficiency was also tested in HEK293 cells, and was demonstrated to be equivalent between the two vectors (Figure 5.2B). The latter result is important, since it justifies the use of HEK293 cells to assess the infectious titer of both HAdV-5 and ChAdOX1 vector preparations. It is currently unclear why these vectors behave differently in the two cell lines. Though HEK293 cells also abundantly express human CAR, there may be elevated expression of alternative receptors to increase the efficiency of ChAdOX1 entry. Alternatively, the fact that the presence of E1 in HEK293 cells enables the vectors to replicate may reduce the effect of differences in the efficiency of cell entry on transduction (defined as the percentage of GFP positive cells in Figure 5.2 and 5.3), since transgene product will accumulate rapidly after replication of the viral genome.

As Figure 5.3 demonstrates, the efficiency of transduction of the A549 cell line by HAdV-5 is approximately a log greater than for AdC68 and ChAdOX1. This difference in transduction represents a clear biological difference in the efficiency of infection, cytoplasmic trafficking, or transgene expression between members of *Human adenovirus C* and *Human adenovirus E* in this cell line. Adenovirus attachment to the plasma membrane of cells is a co-operative process; initial receptor engagement promotes membrane clustering of receptors and facilitates entry of subsequent virions [275,276]. However, the co-operativity of infection was comparable between the two vectors, indicated by the slope of the transduction curve at intermediate MOIs in Figure 5.3A. Calculated Hill coefficients are similar between all three vectors (data not shown). Figure 5.3B indicates that fluorescence intensity, and therefore transgene product expression, is greater after HAdV-5 than AdC68 or ChAdOX1 at higher MOI. However, if fluorescence intensity is plotted against percentage transduction, the relationship between these parameters is similar between the three vectors (Figure 5.3C). It therefore seems that the higher fluorescence intensity after HAdV-5 incubation is likely due to infection of cells with multiple virions at high MOI, rather than greater protein expression within each singly infected cell. The data suggest instead that the difference in transduction may represent a difference prior to the onset of transcription, such as the affinity for a cell surface receptor (potentially the human isoform of CAR).

Figure 5.2: *In vitro* infectivity of lung carcinoma cell line A549 and HEK293 cell line with HAdV-5 and ChAdOX1 vectors. (A) A549 cells and **(B)** HEK293 cells were infected with HAdV-5 and ChAdOX1 vectors expressing TIPeGFP in 24 well plates at a range of MOI. After a 24 hour incubation, the percentage of cells expressing the GFP transgene product was assessed by flow cytometry. Bars show mean and SEM of two independent serial dilutions, and data presented is representative of at least three independent experiments.

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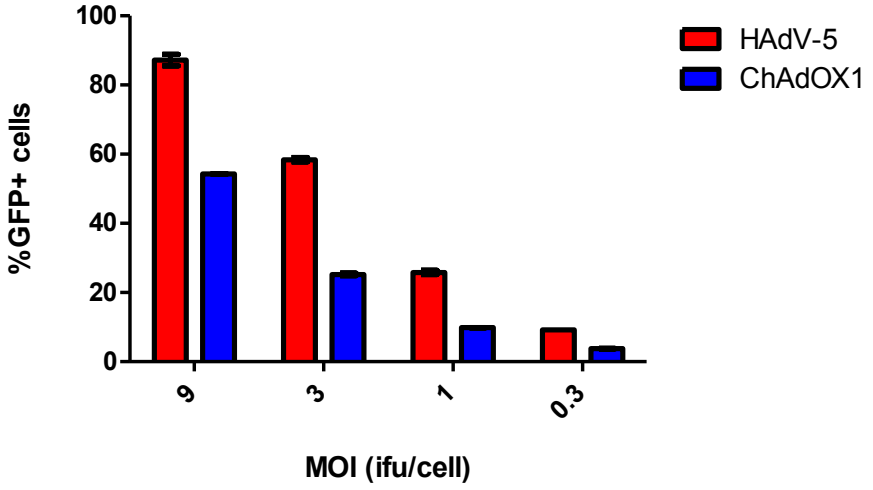

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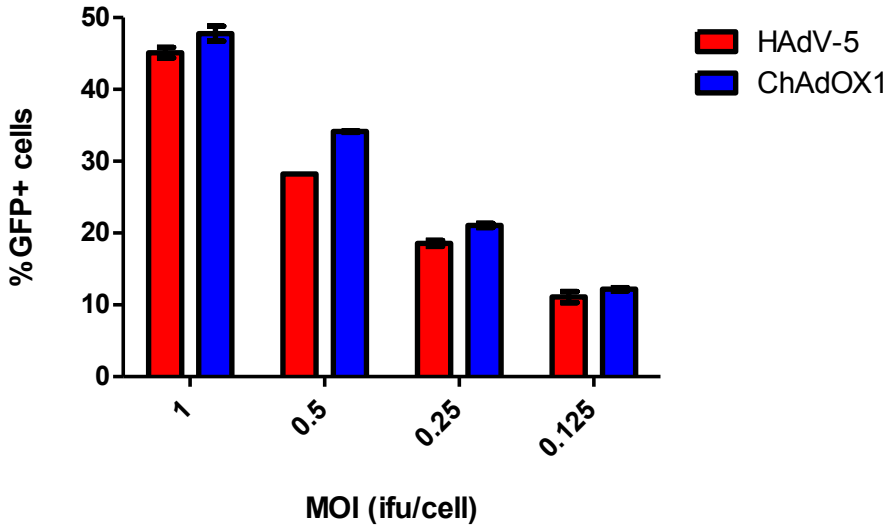
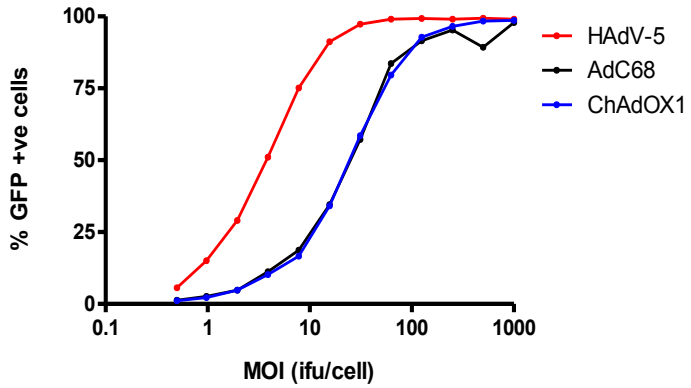


Figure 5.3: *In vitro* transduction efficiency of HAdV-5, AdC68 and ChAdOX1 vectors in A549 cells. Vectors expressing TIPeGFP were applied to a monolayer of A549 cells at MOI's ranging from 1000 ifu/cell to 0.5 ifu/cell. After 24 hours, cells were harvested. **(A)** The percentage of cells expressing GFP and **(B)** the mean fluorescence intensity of those GFP positive cells was estimated by flow cytometry. **(C)** The relationship between the percentage of transduced cells and the fluorescent intensity was also studied. A single dilution series is shown for each vector but data is representative of at least three independent experiments.

A



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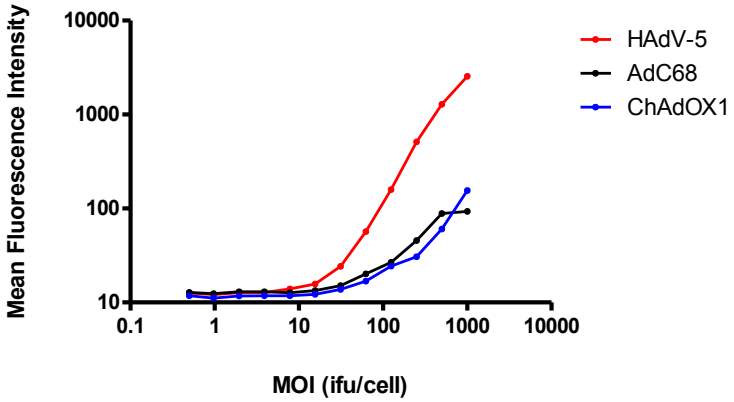
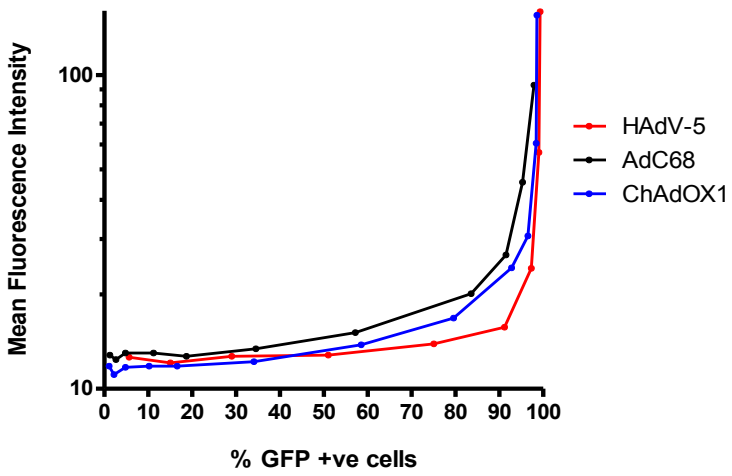


C



5.2.2 ChAdOX1 does not demonstrate Factor X mediated cell entry *in vitro*.

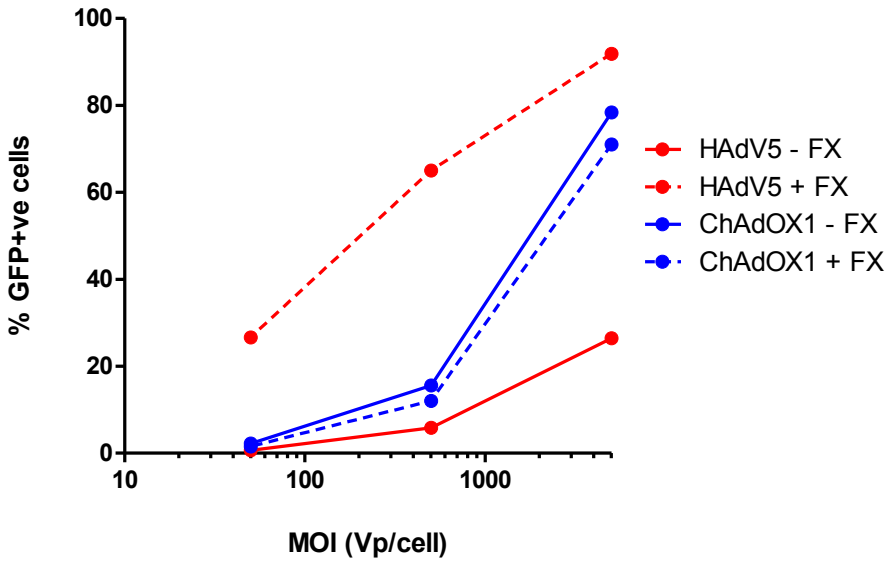
Recently, an alternative mechanism of hepatocyte cell entry for HAdV-5 has been described involving the blood coagulation factor, Factor X (FX). Factor X has been shown to interact with the adenoviral capsid of HAdV-5 through residues on the hyper-variable regions on the hexon, forming a molecular 'bridge' between the capsid and heparan sulphate glycosaminoglycans on the hepatocyte surface [120,277]. Though there is evidence that this interaction is not important in adenovirus infection of muscle tissue [120], it may still be relevant to vaccine hepatotoxicity since intramuscular injection invariably leads to a degree of systemic dissemination.

The efficiency of FX mediated infectivity was assessed *in vitro*, using the human ovarian carcinoma cell line, SKOV-3 and physiological plasma concentrations of recombinant FX [120]. Figure 5.4 demonstrates that the presence of FX has no effect on efficiency of ChAdOX1 transduction in SKOV-3 cells. It would therefore seem likely that the hexon protein of ChAdOX1 does not bind this coagulation factor. HAdV-5 transduction is significantly improved in the presence of FX in agreement with previous studies [120], though interestingly in the absence of FX, transduction of SKOV-3 cells is less efficient with HAdV-5 than ChAdOX1. SKOV-3 cell express moderate to low levels of CAR [278] suggesting that a receptor with greater affinity for the ChAdOX1 capsid than the HAdV-5 capsid may be present on these cells. Since transduction efficiency of both vectors in SKOV-3 cells is considerably lower than in A549 cells (Figure 5.3), this receptor could be an integrin; αvβ integrins have been shown to mediate the

lower affinity penton base interaction required for receptor-mediated endocytosis after initial virus attachment in CAR positive cells [105].

This data provides further evidence that despite the observation that both HAdV-5 and ChAd vectors from *Human adenovirus E* can utilize the CAR receptor for cell entry, the ChAd vectors display different behaviour to HAdV-5 *in vitro,* and may therefore behave differently *in vivo*. The fact that ChAdOX1 fails to bind to FX may reflect differences in the nature of hexon proteins between ChAdOX1 and HAdV-5. Indeed, the hydrophilic surface of the HAdV-5 hexon has a particularly strong negative charge [279], which may in part be responsible for differences in transduction efficiency both *in vitro* and *in vivo*.

Figure 5.4: Transduction efficiency of SKOV-3 cell line with HAdV-5 and ChAdOX1 vectors in the presence and absence of Factor X. SKOV-3 cells were infected at MOIs of 50, 500, or 5000 viral particles / cell with vectors HAdV-5 and ChAdOX1 expressing TIPeGFP in the presence or absence of 8μg/ml Factor X. After 90 min, the media was replaced and cells were incubated for a further 24 hours. The percentage of GFP positive cells was enumerated by flow cytometry. A single dilution series is shown for each vector but data is representative of at least two independent experiments.



5.2.3 *In vivo* transgene expression is superior after HAdV-5 administration in mice, and correlates with transgene product specific immune responses

Section 5.2.1 demonstrated that HAdV-5 vectors transduce A549 cells more efficiently than ChAdOX1, in a mechanism that may involve CAR receptor engagement. Since HAdV-5 is also more immunogenic than ChAdOX1 in mice, could the superior transduction efficiency of HAdV-5 in A549 cells be related to the superior magnitude of transgene product specific immune responses in mice? The CAR receptor was identified as a high affinity receptor for HAdV-2 and HAdV-5 through transfection of Chinese hamster ovary (CHO) cells with CAR cDNA [106]. However, the *in vivo* significance of this receptor interaction has been debated (Section 1.4.1) [107,108,109]. Furthermore, the murine CAR ortholog is slightly different from the human ortholog (83% identity), with a different tissue distribution to that of human CAR [280]. Expression of both murine and human isoforms of CAR in mature skeletal muscle tissue is low, which may be important in the context of intramuscular immunisation [280]. Expression has been reported to be down-regulated during development [281], confined within mature skeletal muscle to the neuromuscular junction [282].

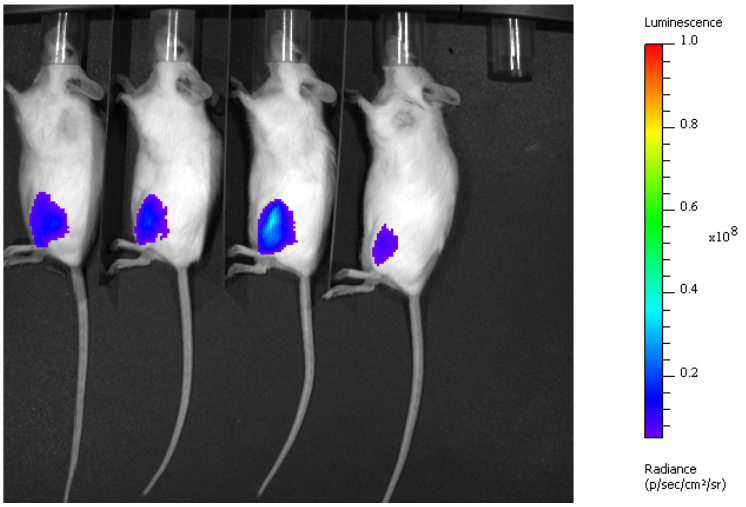
Therefore, to test the relevance of the *in vitro* work, the transduction efficiency of vectors HAdV-5, ChAdOX1, and ChAd63 expressing *Photinus pyralis* (firefly) luciferase were compared *in vivo* in real time using an IVIS 200 luminescence imaging system. Figure 5.5A-C indicates that luciferase expression was detected exclusively in the injected leg, and that, 24 hours post injection, the

bioluminescence signal after 10^8 ifu HAdV-5 administration was approximately a log higher than after 10^8 ifu ChAd63 or ChAdOX1 administration. Furthermore, Figure 5.5D illustrates that the log difference in expression between the 10^8 immunized groups is maintained throughout the two week period of study (though error bars overlap at the two week time point). Such is the difference in effective transduction efficiency between vectors, that a two log reduction in the dose of HAdV-5 to 10^6 ifu gives a comparable magnitude of transgene expression to ChAd63 and ChAdOX1 vectors at the 10^8 ifu dose. At the 10^8 dose, groups immunized with vectors ChAd63 and ChAdOX1 display comparable bioluminescence through the study, while at the 10^6 dose expression after ChAdOX1 injection appears to fall more rapidly than after ChAd63.

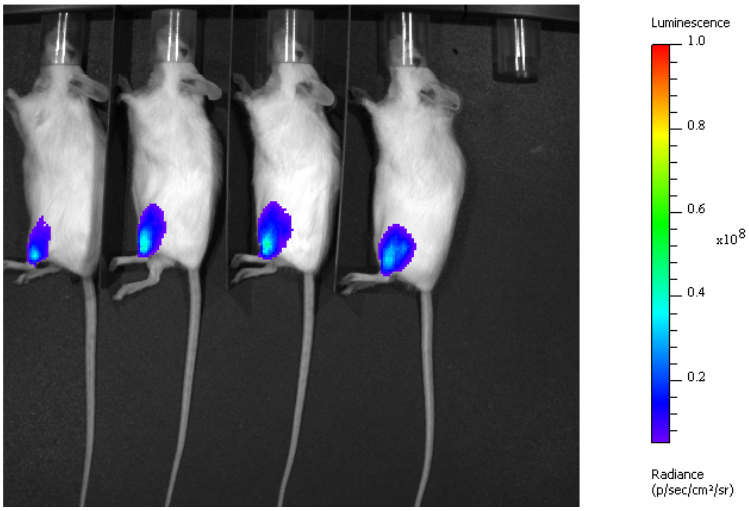
Importantly, these differences in transgene expression correlate with differences in transgene product specific immune responses (Figure 5.6). HAdV-5 vectors induced higher frequencies of anti-luciferase CD8+ T cells, and higher anti-luciferase IgG antibody titers than ChAd63 and ChAdOX1 vectors.

Figure 5.5: *In vivo* transgene expression after intramuscular injection of mice with ChAdOX1, ChAd63 and HAdV-5 vectors. Balb/c mice (4/group) were immunized intramuscularly with either 10^8 or 10^6 infectious units of **(A)** ChAdOX1, **(B)** ChAd63, or **(C)** HAdV-5 vectors expressing *Photinus* luciferase (images show 10^8 ifu dose). Bioluminescence (flux per second) was measured using an IVIS 200 live imaging system 24 hours post immunization after subcutaneous administration of luciferin substrate. **(D)** Graph displaying luciferase expression (measured on days 1, 3, 4, 7, 10 and 14 post immunization) with time after both 10^8 ifu (continuous line) and 10^6 ifu (dashed line) of each vector. Error bars display median and range.

A



B



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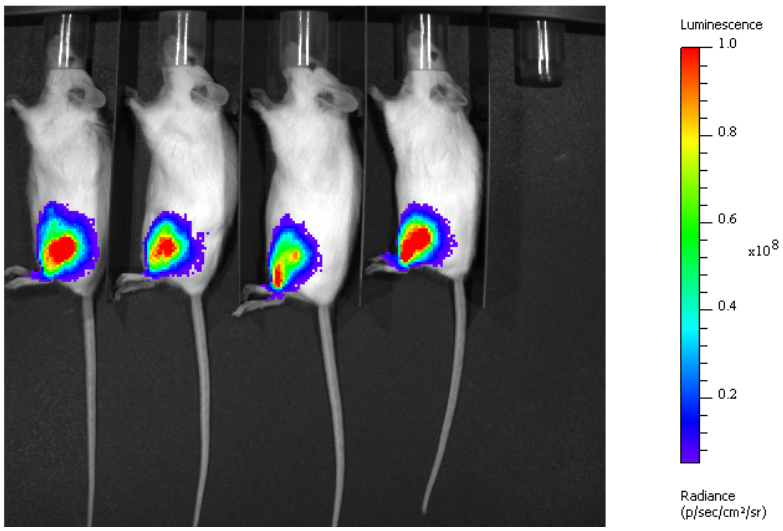

Luminescence
1.0
0.8
0.6
x10^8
0.4
0.2
Radiance
(p/sec/cm²/sr)

D

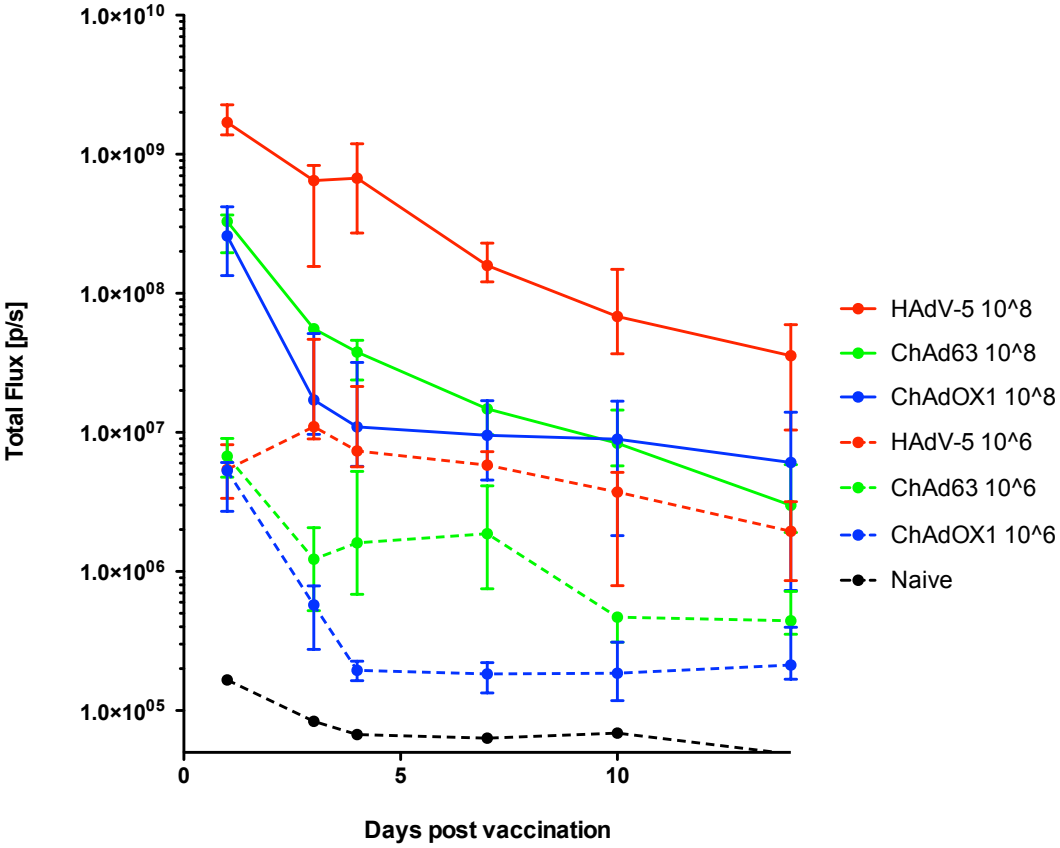
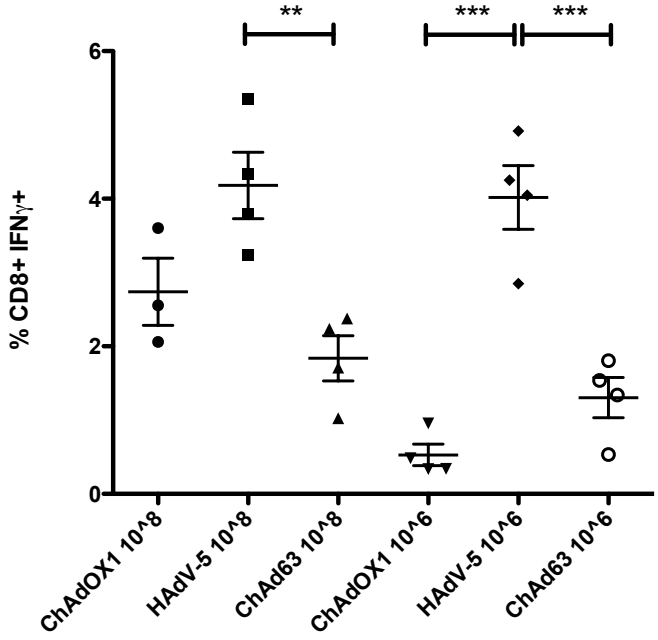
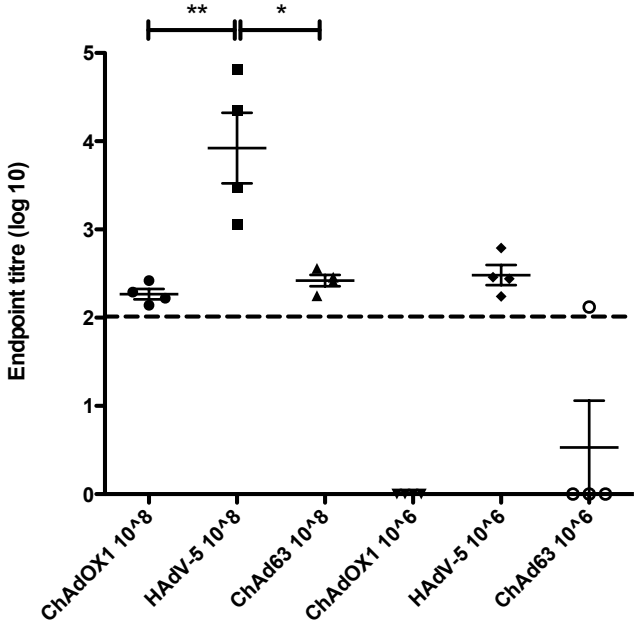

1.0×10^10
1.0×10^09
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
Total Flux [p/s]
0
5
10
Days post vaccination
HAdV-5 10^8
ChAd63 10^8
ChAdOX1 10^8
HAdV-5 10^6
ChAd63 10^6
ChAdOX1 10^6
Naive

Figure 5.6: Immunogenicity of HAdV-5, ChAd63, and ChAdOX1 correlates with *in vivo* transgene expression. Two weeks post immunization, the animals from Figure 5.5 were sacrificed. **(A)** CD8+IFNγ+ responses in the spleen to *Photinus* luciferase epitope GFQSMYTFV were measured by flow cytometry. **(B)** Anti luciferase IgG antibody titers were measured by endpoint ELISA. Dashed line indicates limit of detection. Error bars show mean and SEM. Tests for significance performed by one-way ANOVA with Bonferroni post test. *<0.05, **<0.01, ***<0.001

A



B



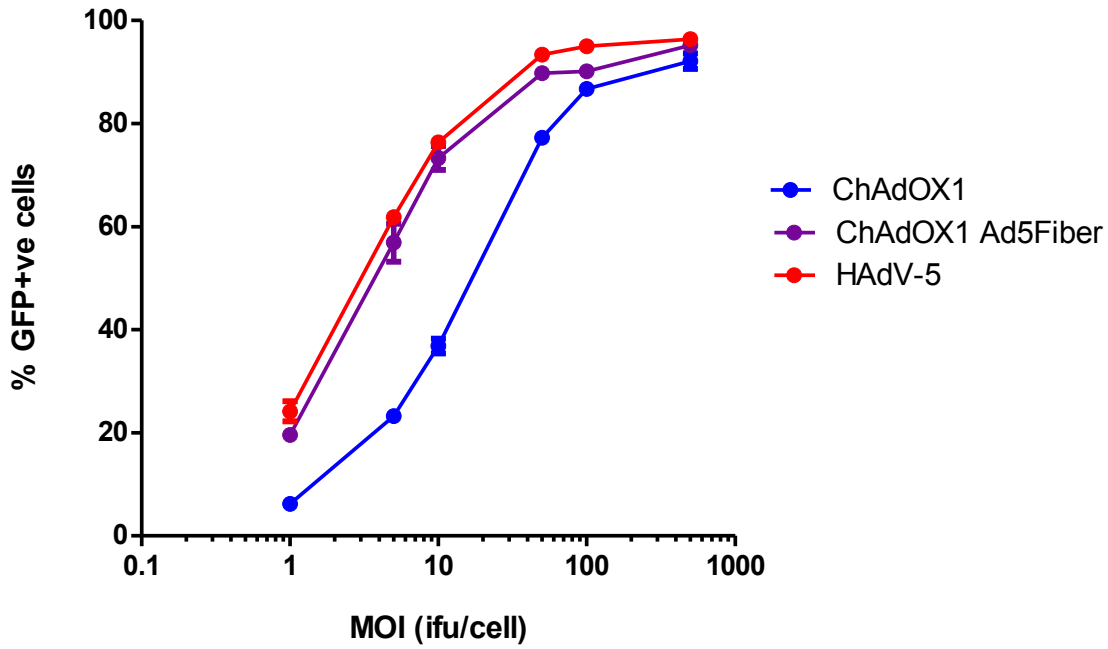
5.2.4 Exchange of the fiber protein between ChAdOX1 and HAdV-5 is sufficient to enable equivalent transduction efficiency in A549 cells.

In vitro studies presented above show that a significant difference in transduction efficiency exists between HAdV-5 and ChAd vectors from *Human adenovirus E* in A549 cells. (Section 5.2.1) The mechanism is hypothesized to be via a difference in affinity of the fiber protein for the CAR receptor, expressed abundantly on A549 cells. This has not been formally tested for ChAdOX1 through the use of CHO-CAR cells, but related vector ChAd63 did exhibit lower transduction efficiency compared to HAdV-5 in these cells (David Wyllie, unpublished data). *In vivo* studies presented above confirmed that transduction efficiency after intramuscular injection in mice is also different between these vectors, and the vector displaying the most efficient transduction *in vivo*, HAdV-5, elicits transgene product specific immune responses of superior magnitude than those obtained using ChAd63 or ChAdOX1 in agreement with the previous chapter. These results are consistent with an important role for fiber-mediated infectivity of vectors in the induction of potent vaccine responses *in vivo*.

Using BAC recombineering technology, the C-terminal shaft and knob domains of the ChAdOX1 fiber were replaced with the corresponding domains from HAdV-5, in order to determine whether the difference in structure of the fiber protein was primarily responsible for the difference in immunogenicity between these vectors (Section 2.3.4). Fiber proteins of all human adenovirus serotypes share a common architecture: an N-terminal tail domain facilitating

attachment to the capsid; a flexible shaft domain composed of repeating sequences, and a C-terminal globular knob domain for mediating receptor interactions [283]. During construction of the chimeric, the N-terminal tail region from ChAdOX1 was retained so as to minimize potential disruption of the capsid [284]. As a result, there was no difference in viral yield in HEK293 cells between the native ChAdOX1 vector and the ChAdOX1 Ad5Fiber chimeric during vector production (data not shown). Figure 5.7 shows that transduction efficiency in of the ChAdOX1 Ad5Fiber chimeric was equivalent to HAdV-5 in A549 cells. This result confirms that the difference between HAdV-5 and ChAdOX1 transduction in this cell line is solely fiber mediated. It is also significant since it demonstrates that the chimeric fiber protein is as functional as the native HAdV-5 structure in this assay.

Figure 5.7: Transduction efficiency of HAdV-5, ChAdOX1, and the chimeric ChAdOX1 Ad5Fiber vector in A549 cells. A549 cells were incubated with vectors expressing TIPeGFP at MOI's of between 1000 and 1 ifu/cell. After 24 hours, the percentage of GFP positive cells were enumerated by flow cytometry. Error bars display mean and SEM of two independent dilution series. Data representative of two independent experiments.



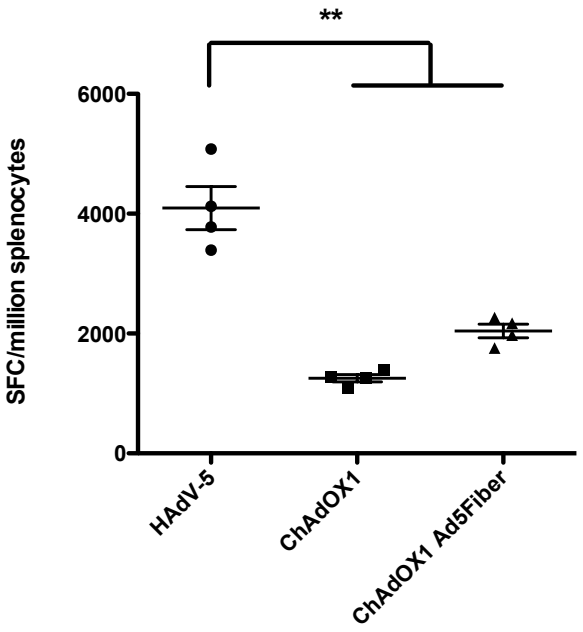
5.2.5 Replacing the fiber sequence from ChAdOX1 with the HAdV-5 fiber provides limited improvement in immunogenicity in mice.

The ChAdOX1 Ad5Fiber chimeric demonstrated fiber-mediated transduction efficiency equivalent to HAdV-5 in A549 cells. But is fiber-mediated transduction important after intramuscular administration *in vivo*? Is it responsible for the difference in potency between these vaccine vectors in mice? The observation of improved fiber mediated transduction *in vitro* demonstrates that the chimeric fiber protein is functional, and that this vector will enable the latter hypothesis to be reliably addressed.

Transgene product specific immune responses were evaluated in mice after intramuscular administration of HAdV-5, ChAdOX1, and ChAdOX1 Ad5Fiber vectors. Figure 5.8 shows that two weeks post immunisation there was no significant difference in the magnitude of CD8+ T cell responses between the ChAdOX1 and ChAdOX1Ad5Fiber immunized groups. There was a modest increase (approximately 5 fold) in anti-transgene product specific antibody titers which was significant by one-way ANOVA. However, antibody responses were still two logs higher after HAdV-5 administration than ChAdOX1Ad5Fiber administration. Therefore, if fiber mediated transduction does contribute to the difference in immunogenicity between theses vectors the contribution is a minor one.

Figure 5.8: Comparative immunogenicity of HAdV-5, ChAdOX1 and the ChAdOX1 Ad5Fiber chimeric. Balb/c mice (4/group) were immunized intramuscularly with 10^8 infectious units of vectors HAdV-5, ChAdOX1 or ChAdOX1 Ad5Fiber expressing TIPeGFP. Two weeks post immunisation, **(A)** CD8+ T cell responses against the immunodominant Pb9 epitope were measured in the spleen by IFNγ ELISpot. **(B)** Sera was harvested and anti-GFP antibody responses measured by endpoint ELISA. Error bars show mean and SEM. Tests for significance performed by one-way ANOVA with Bonferroni post test. *p<0.05, **p<0.01, ***p<0.001.

A



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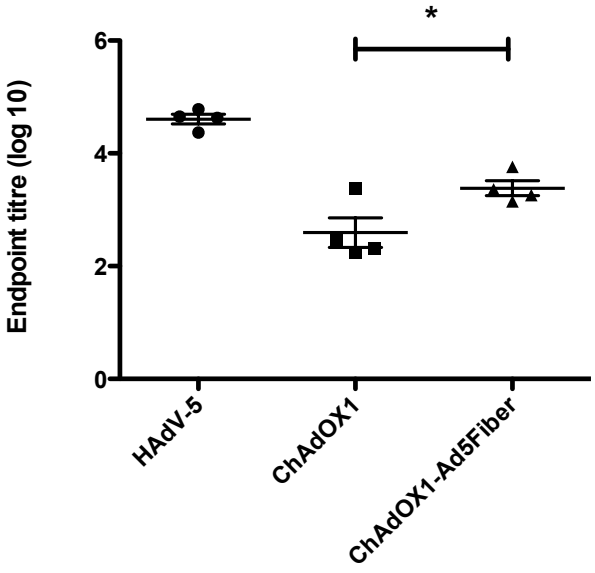


5.2.6 The chimeric ChAdOX1 Ad5Fiber vector offers only a modest improvement in *in vivo* transgene expression over ChAdOX1.

The chimeric ChAdOX1 Ad5Fiber vector provided only a modest increase in transgene product specific IgG antibody responses, and no significant improvement in CD8+ T cell responses (Section 5.2.5). A number of potential hypotheses may be drawn to explain these results. One might predict that, if fiber mediated transduction is important for intramuscular delivery *in vivo*, that ChAdOX1Ad5 Fiber would confer equivalent transgene expression *in vivo* to HAdV-5, as seen in A549 cells *in vitro*. One would therefore conclude from Section 5.2.5 that the difference in immunogenicity between these vectors is due primarily to factors other than transduction, such as the induction of innate immunity (Chapter 6). However, it is also possible that fiber mediated infectivity is not responsible for the difference in *in vivo* transduction efficiency observed in Section 5.2.3. Since CAR is expressed at low levels in mature skeletal muscle, and is not expressed on dendritic cells [191], fiber independent mechanisms of cellular entry may be more important in this context.

In order to address these hypotheses*, in vivo* imaging was employed to assess transgene delivery by the chimeric ChAdOX1 Ad5Fiber vector. A recombinant ChAdOX1 Ad5Fiber expressing *Photinus* luciferase was constructed, and comparable luciferase expression to a similar HAdV-5 based vector was demonstrated in A549 cells (data not shown). *In vivo* transgene expression after intramuscular administration was measured as described previously in Section 5.2.3. After 24 hours, transgene expression after injection of ChAdOX1 Ad5Fiber showed only a very modest improvement compared to the ChAdOX1 vector. The

bioluminescence signal was not significantly different between the two groups by one-way ANOVA, but was significant by student t-test ($p<0.0317$) and represented an approximate two-fold increase in mean flux per second. The trend for a higher luminescence signal was maintained throughout the period of study but the difference between groups was not statistically significant at any other timepoint. The log difference in bioluminescence between HAdV-5 and ChAdOX1 immunized groups was repeated in this study (Figure 5.9). It can therefore be confidently concluded that fiber-dependent interactions are not primarily responsible for the difference in transduction efficiency observed *in vivo* between HAdV-5 and ChAdOX1 immunized mice. Figure 5.10 shows that neither transgene product specific CD8+ T cell responses, nor anti-transgene specific antibody responses were significantly different between ChAdOX1 and ChAdOX1 Ad5Fiber vaccinated animals from the same experiment.

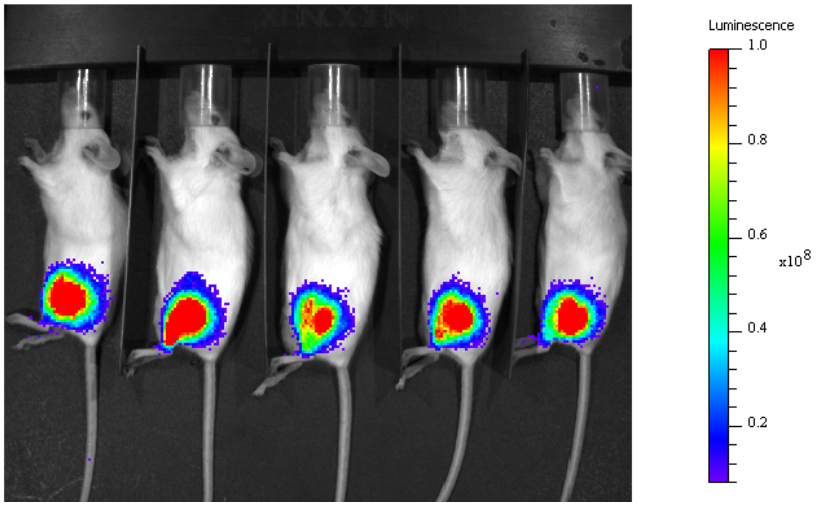
Both *in vivo* imaging studies suggest that luciferase expression after intramuscular administration is located primarily in the injected leg. Figure 5.11A displays a bioluminescence overlay of mice from Figure 5.9 imaged on their front, and confirms that detectable transgene expression was confined to the leg. This distribution of signal was the same in all groups studied (data not shown). However, the precise location of transgene expression within this region is unclear.

In a separate experiment, mice were injected intramuscularly with 10^8 ifu of either HAdV-5 or ChAdOX1 vectors expressing *Photinus* luciferase and 24 hours later muscle tissue and popliteal lymph nodes were removed for *ex vivo* detection of luciferase protein. Figure 5.11B-C indicate that luciferase signal is detectable in both muscle and lymph node tissue. However, the fold difference in

mean signal intensity in tissue infected by the two vectors is greater in the lymph node (33 fold) than in the muscle (2.8 fold). This perhaps implies that transduction of lymph node resident antigen presenting cells or lymph node epithelia may be particularly important for immunogenicity. The absolute magnitude of luminescence intensity is greater in the lymph node than the muscle tissue in Figure 5.11, but has been shown to vary between experiments (mainly due to difficulties in homogenization of muscle tissue) (data not shown). The fold difference in signal between vectors in lymph node and skeletal muscle tissue is, however, comparable between experiments.

Figure 5.9: *In vivo* transgene expression after intramuscular delivery of HAdV-5, ChAdOX1 and ChAdOX1 Ad5Fiber vectors. Balb/c mice (5/group) were immunized with 10^8 ifu of HAdV-5, ChAdOX1 or ChAdOX1 Ad5Fiber vectors expressing *Photinus* luciferase. **(A-C)** Bioluminscence was measured after 24 hours after administration of **(A)** HAdV-5, **(B)** ChAdOX1 or **(C)** ChAdOX1 Ad5Fiber as described in Figure 5.5. **(D)** Graph displaying luciferase expression (measured on days 1, 3, 7, and 14 post immunization) over time. Error bars indicate median and range.

A



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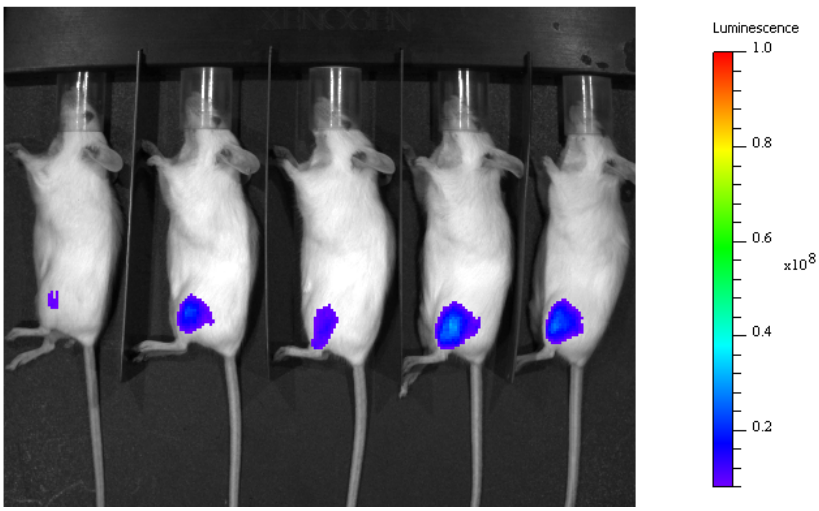

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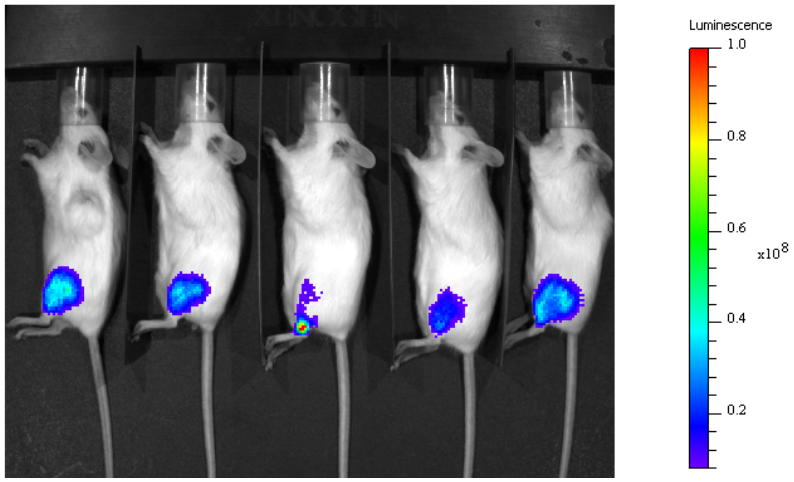

Luminescence
1.0
0.8
0.6
x10^8
0.4
0.2

D

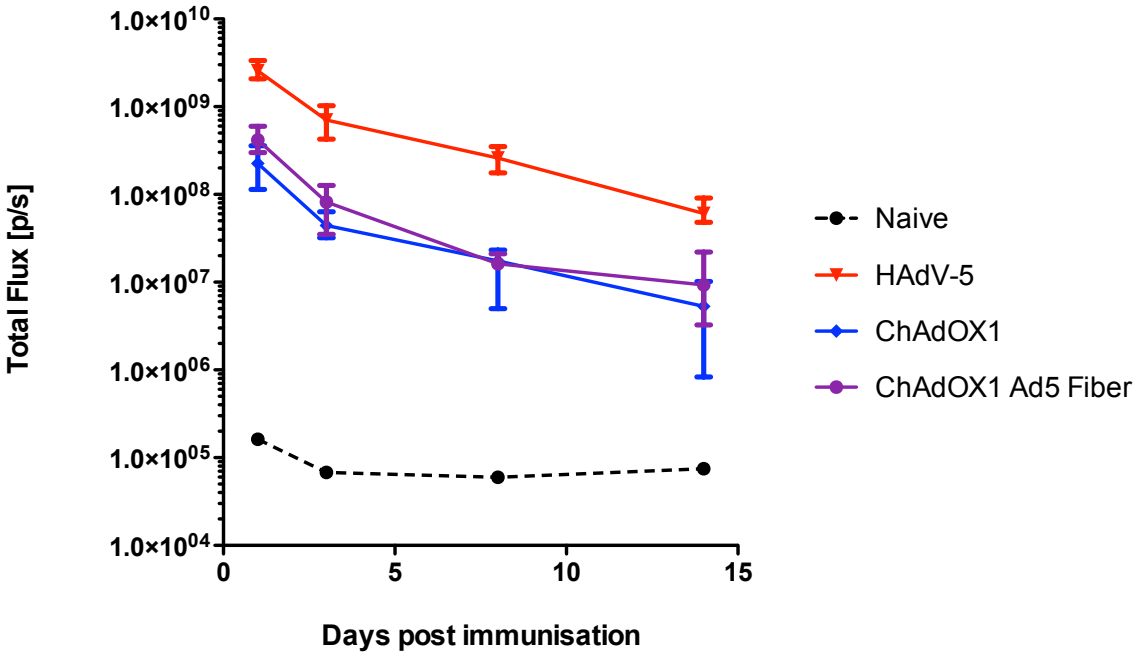
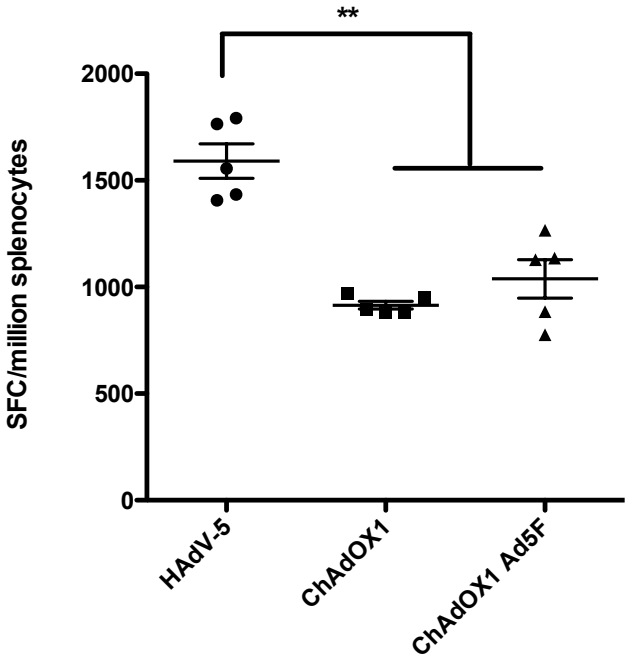

Total Flux [p/s]
1.0×10^10
1.0×10^09
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04
0
5
10
15
Days post immunisation
Naive
HAdV-5
ChAdOX1
ChAdOX1 Ad5 Fiber

Figure 5.10: Immunogenicity of HAdV-5, ChAdOX1 and ChAdOX1 Ad5Fiber vectors expressing *Photinus* luciferase. Two weeks post immunization, the animals from Figure 5.9 were sacrificed. **(A)** CD8+ T cell responses in the spleen to *Photinus* luciferase epitope GFQSMYTFV were measured by IFNγ ELISpot. **(B)** Anti luciferase IgG antibody titers in sera were measured by endpoint ELISA. Dashed line indicates limit of detection. Error bars show mean and SEM. Statistical analysis by one-way ANOVA with Bonferroni post test. **p<0.01, ***p<0.001

A



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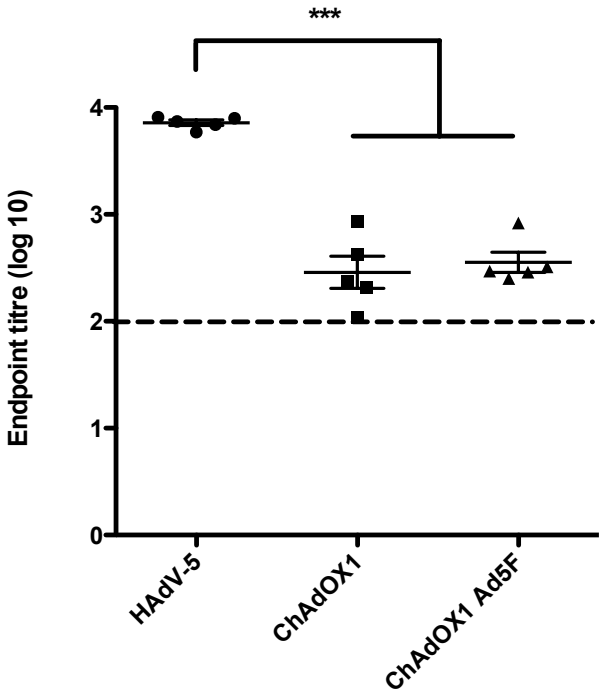
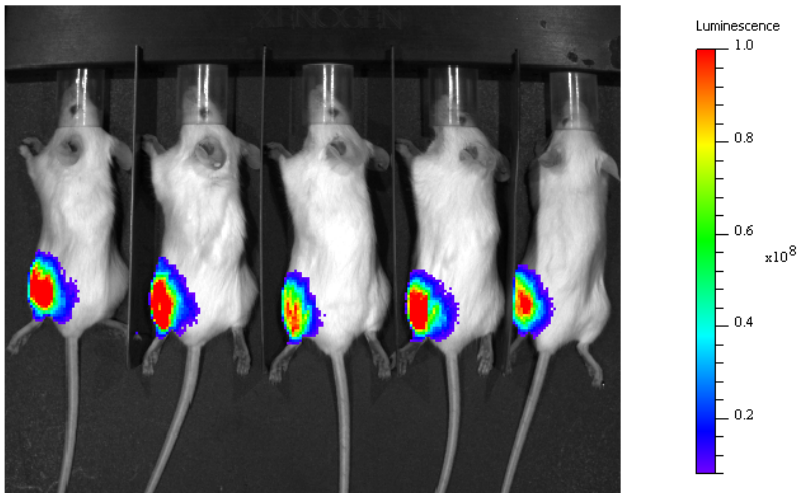
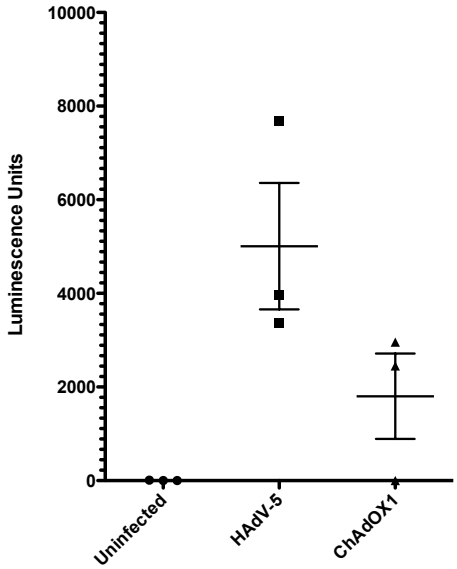


Figure 5.11: Location of transgene expression following intramuscular injection. **(A)** Animals from the HAdV-5 immunized group in Figure 5.9 were imaged for bioluminescence on their front as described previously. **(B-C)** Balb/c mice, (3/group) were immunized intramuscularly with 10^8 ifu HAdV-5 or ChAdOX1 vectors expressing *Photinus* luciferase. 24 hours later, mice were sacrificed, and popliteal lymph nodes and muscle tissue removed for extraction of luciferase protein (described in Section 2.7.4 and 2.7.5). Luciferase activity was assayed in vitro from **(B)** muscle and **(C)** lymph node extracts. Error bars show mean and SEM. Statistical analysis by student t-test. **p<0.01.

A



B



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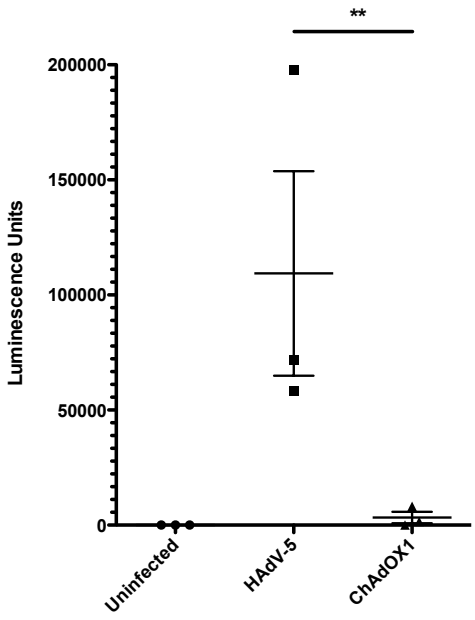


5.3 Discussion

The aim of this chapter was to provide an insight into the mechanisms responsible for the difference in immunogenicity in mice between replication defective adenovirus vectors of different serotype observed in the previous chapter. In particular, this chapter focused on the relationship between vector transduction (the ability of vectors to infect cells and express an encoded transgene), and the magnitude of the immune responses generated against the encoded transgene product.

Studies *in vitro* in the human lung carcinoma cell line A549 revealed a significant difference in transduction efficiency between HAdV-5 (*Human adenovirus C*) and members of *Human adenovirus E* (AdC68 and ChAdOX1). A ten-fold greater infectious titer of vectors AdC68 or ChAdOX1 was required to infect the same percentage of A549 cells compared to HAdV-5 at intermediate MOI (Figure 5.3). Transduction efficiency between the two ChAd vectors was however, virtually identical, suggesting that the superior transduction efficiency of HAdV-5 may be viral species related. No difference in the level of transgene expression within individual infected cells could be detected between HAdV-5 and the ChAd vectors (except at percentage transduction ≈100%, indicative of multiple infection by HAdV-5). This suggests that expression from the CMV promoter is equivalent in cells transduced by all three vectors, and indicates that the difference in transduction efficiency observed, at least in A549 cells, occurs prior to the onset of transcription.

The mechanism responsible for this marked difference in transduction efficiency *in vitro* is unclear, but later studies using a chimeric ChAdOX1 vector

harboring a HAdV-5 fiber protein indicated that the difference, at least between HAdV-5 and ChAdOX1 vectors in A549 cells, was entirely fiber mediated (Figure 5.7). These data would be consistent with the fiber protein from HAdV-5 displaying a higher affinity for a receptor on the surface of A549 cells; previous studies have shown that HAdV-5 infection in this cell line occurs primarily via the high affinity CAR receptor [274]. AdC68 has also been shown to utilize the CAR receptor for cell entry [272], as has ChAd63 (David Wyllie, unpublished data). However, since A549 cells express a variety of other potential adenovirus receptors including sialic acid [285], the affinity of CAR receptor binding to ChAdOX1 has not been formally tested. Indeed other studies have concluded that binding affinities to the CAR receptor were equivalent between *Human adenovirus C* member HAdV-2, and HAdV-4, the only member of species *Human adenovirus E* to be isolated in man. It is also possible that fiber interactions could influence the rate of cytoplasmic trafficking of the vectors; an *in vitro* study in which fiber proteins were exchanged between HAdV-5 and the Human adenovirus B member HAdV-7, revealed that differences in vector transduction in A549 cells were in part due to differences in the rate of trafficking post internalization [124].

Further *in vitro* studies in the SKOV-3 cell line demonstrated that, in a cell line expressing low levels of CAR, differences in transduction efficiency were still apparent between HAdV-5 and ChAdOX1 vectors (Figure 5.4). In this cell line transduction efficiency was superior after ChAdOX1 infection, although for both vectors transduction was considerably less efficient than in A549 cells. In agreement with previous studies, the addition of human coagulation factor X to media considerably improved the transduction efficiency of HAdV-5 [120].

However, the presence of Factor X had no effect on transduction with ChAdOX1, implying that the coagulation factor may have failed to bind to the hexon of ChAdOX1. Indeed, previous studies have indicated that the surface of the HAdV-5 hexon is different in nature and more negatively charged than the corresponding region from *Human adenovirus E* member HAdV-4 [279]. Though Factor X binding has been shown not to be important for transduction of tissues after intramuscular administration in mice [120], a difference in the nature of the hexon between the two vector capsids could conceivably affect transduction by this route of delivery *in vivo*.

Though these *in vitro* studies demonstrate a clear phenotypic difference between members of *Human adenovirus C* and *Human adenovirus E*, the direct relevance to *in vivo* transduction efficiency by intramuscular administration is less clear. Indeed, the mechanism of transduction of muscle tissue by adenovirus vectors is unknown [286], though pre-clinical gene therapy applications have successfully targeted skeletal muscle cells [287]. *In vitro* study of muscle tissue is particularly challenging; muscle is notoriously difficult to culture, and muscle cell lines such as murine C2C12 cells tend to be embryonic, forming cultures of immature myoblasts not mature myocytes. Furthermore, it is entirely possible that the difference in immunogenicity between vectors, particularly in relation to CD8+ T cell responses, is not primarily related to muscle transduction. Adenovirus vectors have, after all, been shown to infect dendritic cells *in vivo* [194] for direct priming of CD8+ T cell responses.

It was therefore important to study transduction of cells and tissues by adenovirus vectors after intramuscular delivery *in vivo*. At a dose of 10^8 infectious units, expression of a luciferase transgene *in vivo* was approximately a

log higher after HAdV-5 administration than after ChAd63 or ChAdOX1 (Figure 5.5). This result is in agreement with a previous study by Hensley *et al*, which demonstrated higher transgene expression *in vivo* after intramuscular administration of HAdV-5 than AdC68, though the data were not quantitative [187]. Furthermore, in the current study, transgene expression in the group immunized with HAdV-5 maintained elevated compared to the ChAd vector immunized groups throughout the two-week study period (Figure 5.5). Importantly, the elevated bioluminescence signal in the HAdV-5 immunized group was associated with higher frequencies of luciferase specific CD8+ T cell responses and IgG antibody responses, in agreement with previous observations (Figure 5.6). Of particular note, anti-luciferase IgG titers were comparable between the HAdV-5 10^6 ifu immunized group, and the 10^8 ifu immunized ChAd63 and ChAdOX1 groups. These three groups displayed comparable magnitudes of bioluminescence throughout the course of the experiment (Figure 5.5) perhaps indicating a stronger association between transgene expression and antibody titer than induction of CD8+ T cells.

Previous studies at the laboratory of Jonathan Bramson have demonstrated a direct causal link between the level of transgene expression and the magnitude of effector CD8+ T cell responses. The authors demonstrate, through doxycycline mediated regulation of transgene expression, that premature extinction of transgene, even after two weeks post vaccination, leads to a reduction in the magnitude of transgene product specific CD8+ T cells [198]. However, the mechanisms by which HAdV-5 vectors deliver superior transduction *in vivo* remain unclear. The current study sought to investigate this phenomenon, and to identify the region of the capsid or virion responsible for

the differences in transduction and immunogenicity between vectors. *In vitro* studies in A549 cells described above had identified the adenovirus fiber protein as an attractive candidate. Even if interaction with CAR is less important *in vivo*, adenovirus fiber proteins from various serotypes have been shown to mediate interactions with a wide variety of ligands including heparan sulphate proteoglycans [111], sialic acid [285], CD46 [113], and CD80/CD86 [117].

A chimeric ChAdOX1 vector expressing the fiber shaft and knob domains from HAdV-5 was constructed through BAC recombineering. However, despite possessing a functional HAdV-5 fiber (Figure 5.7), the chimeric vector, expressing the TIPeGFP antigen, failed to improve transgene product specific CD8+ T cell responses and gave only a modest increase in anti-GFP IgG antibody titers compared to the ChAdOX1 vector (Figure 5.8), which was not reproducible in subsequent studies (Figure 5.10). However, it was not clear from this experiment whether the exchange of fiber protein had failed to improve transduction, or whether transduction had indeed been improved but was not primarily responsible for the difference in immunogenicity between vectors. *In vivo* imaging confirmed that exchange of fiber proteins between ChAdOX1 and HAdV-5 failed to substantially improve transduction efficiency compared to the original ChAdOX1 vector *in vivo* (Figure 5.9). This result would be consistent with adenovirus vector transduction of tissue after intramuscular injection occurring via a fiber-independent mechanism.

Perhaps cell attachment is facilitated by integrins, in the absence of a higher affinity primary interaction such as the fiber-CAR interaction? Or perhaps an alternative entry mechanism exists that is yet to be discovered? However, it is important to clarify that the *in vivo* data presented in this study do not

conclusively demonstrate that cell entry after intramuscular injection is fiber-independent, nor do they confirm that cell entry by HAdV-5 vectors is more efficient than by ChAdOX1. Indeed, a recent study by Hensley *et al* suggested that the superior transgene expression conferred by HAdV-5 compared to AdC68 may be due to a difference interferon mediated regulation of the CMV promoter [187]. Contradictory to this hypothesis, a difference in transgene expression was observed in the same study between the two vectors in mice lacking the type I interferon receptor [187]. Nevertheless the hypothesis is plausible and is addressed in the following chapter (Chapter 6).

Ex vivo analysis of muscle tissue and draining lymph nodes after intramuscular injection of luciferase expressing vectors, revealed that a greater discrepancy between transduction expression after HAdV-5 and ChAdOX1 was observed in the draining popliteal lymph node (Figure 5.11). Though the identity of the infected cells within the lymph node is unclear, these data may indicate that differences in the direct infection of antigen presenting cells may contribute significantly to the difference in immunogenicity between these vectors. Transduction of dendritic cells is discussed in more detail in the following chapter. However, the work of Bramson and colleagues might predict that transgene expression within both muscle and lymph node tissues would contribute to the magnitude and maintenance of the induced CD8+ T cell response [199]. The authors found that although antigen presentation within draining lymph nodes was critical for priming an immune response, transgene expression within non-haematopoetic antigen presenting cells was sufficient to sustain the CD8+ T cell population following removal of the lymph nodes three weeks post immunization [197,199].

In conclusion, this study has highlighted the relationship between transgene expression *in vivo* and the induction of potent transgene specific CD8+ T cell and antibody responses. However, the mechanism responsible for the difference in transgene expression between HAdV-5 and members of *Human adenovirus E* is unclear. There may be a difference in fiber-independent binding to host cells, or intracellular trafficking to the nucleus post internalization. Equally, transgene expression induced after administration of the ChAd63 and ChAdOX1 could be down-regulated in response to an anti-viral mechanism such as the induction of type I interferon. This is the subject of the following chapter. However, what has been conclusively shown in this study is that neither the difference in transduction, nor the difference in immunogenicity between these vectors is due to differences in the structure of the fiber protein.

6 Assessing the effect of innate immunity on adenovirus vector immunogenicity

6.1 Introduction

The stimulation of innate immunity by adenovirus vaccine vectors is a critical factor in the ability of these vectors to induce potent transgene product specific immune responses. Blockade of pro-inflammatory cytokines TNFα and IL-6 reduces macrophage and NK cell recruitment and ultimately lowers anti-vector and anti-transgene humoral responses [288]. Furthermore, recent studies have shown that type I interferons can act directly on CD8+ T cells to promote cell survival during clonal expansion after an acute viral infection [289]. However, the relationship between induction of innate responses and vector immunogenicity is complex, and will be investigated further in this chapter.

A number of recent studies have shown that 'rare serotype' vectors, many of which have performed poorly in comparison to HAdV-5 in pre-clinical studies, often induce higher levels of type I IFN and pro-inflammatory cytokines than HAdV-5 based vectors [186,187,290]. Studies by Hensley *et al* found that chimpanzee adenovirus vector AdC68 induced higher levels of type I interferon, and more strongly promoted maturation of murine bone marrow derived DCs (BM-DCs) than HAdV-5 based vectors [193]. This was associated with a reduction in transgene expression, both *in vivo* after intramuscular immunization, and from BM-DCs after *in vitro* infection with the AdC68 vector [187]. Type I interferon induction by AdC68 was associated with lower transgene product specific antibody responses [187], but not CD8+ T cell

responses [193]. The authors demonstrated comparable viral DNA delivery to nuclei of infected BM-DCs, suggesting that the difference in transgene expression was independent of infection efficiency [187]. Interestingly, the mechanism of interferon stimulation was found to be different between the two vectors, with interferon induction by HAdV-5 but not AdC68 inhibited by wortmannin [193]. Wortmannin is an inhibitor of phosphoinositide-3-OH kinase, which is activated upon interaction of the HAdV-5 penton with cellular integrins, leading to pro-inflammatory cytokine secretion and DC maturation [267,291].

A recent study by Teigler *et al* has also implicated alternative receptor engagement with differential inflammatory profiles from adenovirus vectors [290]. The study showed that *Human adenovirus B* vector HAdV-35 and *Human adenovirus D* vectors HAdV-26 and HAdV-48 induce higher levels of pro-inflammatory cytokines than HAdV-5 vectors, both *in vivo* in rhesus macaques, and upon *in vitro* infection of human peripheral blood mononuclear cells (PBMCs). The authors demonstrated, through use of fiber chimeric viruses, that fiber-receptor interactions are at least partly responsible for differences in innate cytokine signaling between vectors [290]. The three lower seroprevalence vectors have all been shown to utilize CD46 rather than CAR as a primary receptor for cellular entry, though the use of CD46 by HAdV-26 is controversial [242,292]. Receptor usage for members of *Human adenovirus E* remains largely unknown, though one study has shown that the AdC68 vector can utilize both the human and murine isoforms of the CAR receptor for entry into Chinese hamster ovary (CHO) cells [272].

A recent study by Johnson *et al* also reported elevated induction of type I interferon with rare serotype vectors HAdV-35 and HAdV-28 (*Human adenovirus*

D) compared to HAdV-5 in both human and murine DCs [186]. Interestingly, in human PBMC the DC transduction efficiency of the vectors was the reverse of the trend seen in mice, with HAdV-5 being significantly less efficient than HAdV-35 and HAdV-28. Of note, the infection efficiency of human plasmacytoid DCs, a major source of type I IFN upon viral infection, by HAdV-5 vectors was particularly poor. The authors demonstrate through the use of mice defective in type I interferon signaling, that abrogation of the interferon response considerably improved the immunogenic potential of both HAdV-35 and HAdV-28 vectors at lower doses, elevating CD8+ T cell responses to magnitudes obtained after immunization with HAdV-5 [186]. The authors also demonstrate, as had been suggested previously [187], that type I interferon can modulate transgene expression in infected murine DCs directly. Application of exogenous IFNα achieved a two-fold decrease in transgene expression after HAdV-5 infection, while DCs from mice deficient in interferon signaling displayed a two fold increase in transgene expression after HAdV-35 and HAdV-28 infection [186]. It is also worth noting that type I interferon induction by adenovirus vectors may influence *in vivo* gene delivery indirectly, by stimulating NK cell mediated vector clearance [268]. Taken together, these data strongly implicate a role for innate immunity in determining the adaptive immunogenicity of vectors based on different viral serotypes.

The vector components responsible for induction of innate immunity are currently unclear, though many viral pathogen associated molecular patterns (PAMPs) have been described [180]. A number of studies have shown that UV/psoralen inactivated adenovirus vectors can induce secretion of pro-inflammatory cytokines, chemokines [293] and type I interferon [294], and

hence do not require viral transcription or translation to induce innate immunity. Other studies have also shown type I IFN induction to be independent of TLR signaling [193]. However, despite the lack of an absolute requirement for transcription from the viral genome, recent work has implicated the non-coding virus-associated RNA (VA-RNA) transcripts with induction of type I interferon (but not pro-inflammatory cytokines IL-12 or IL-6) after replication defective HAdV-5 vector infection of both murine fibroblasts and dendritic cells [141]. Induction of the interferon response by VA-RNA is believed to occur through cytosolic pattern recognition via RIG-I [185]. The sequences of both VA-RNA I, and VA-RNA II transcripts vary considerably between adenoviruses of different serotype [295,296], and it is possible that VA-RNA sequences from different serotypes may induce interferon with different sensitivity. It is therefore conceivable that differences in the sequences of the VA-RNAs between different virus serotypes may be responsible for differences in interferon induction and hence immunogenic potential of vectors.

The following chapter investigates the effect of innate immunity on vector performance, with a particular focus on type I interferon induction by vectors of different serotype. The interferon response after infection with HAdV-5 and the ChAd vectors is assessed, and in an extension of previous studies, the effect of VA-RNA on interferon induction and vector immunogenicity is addressed using a reverse genetics approach through BAC recombineering to exchange VA-RNA sequences between HAdV-5 and ChAdOX1. The effect of VA-RNA sequences and type I interferon induction on transgene expression from recombinant vectors is also investigated, both *in vitro* and *in vivo*.

6.2 Results

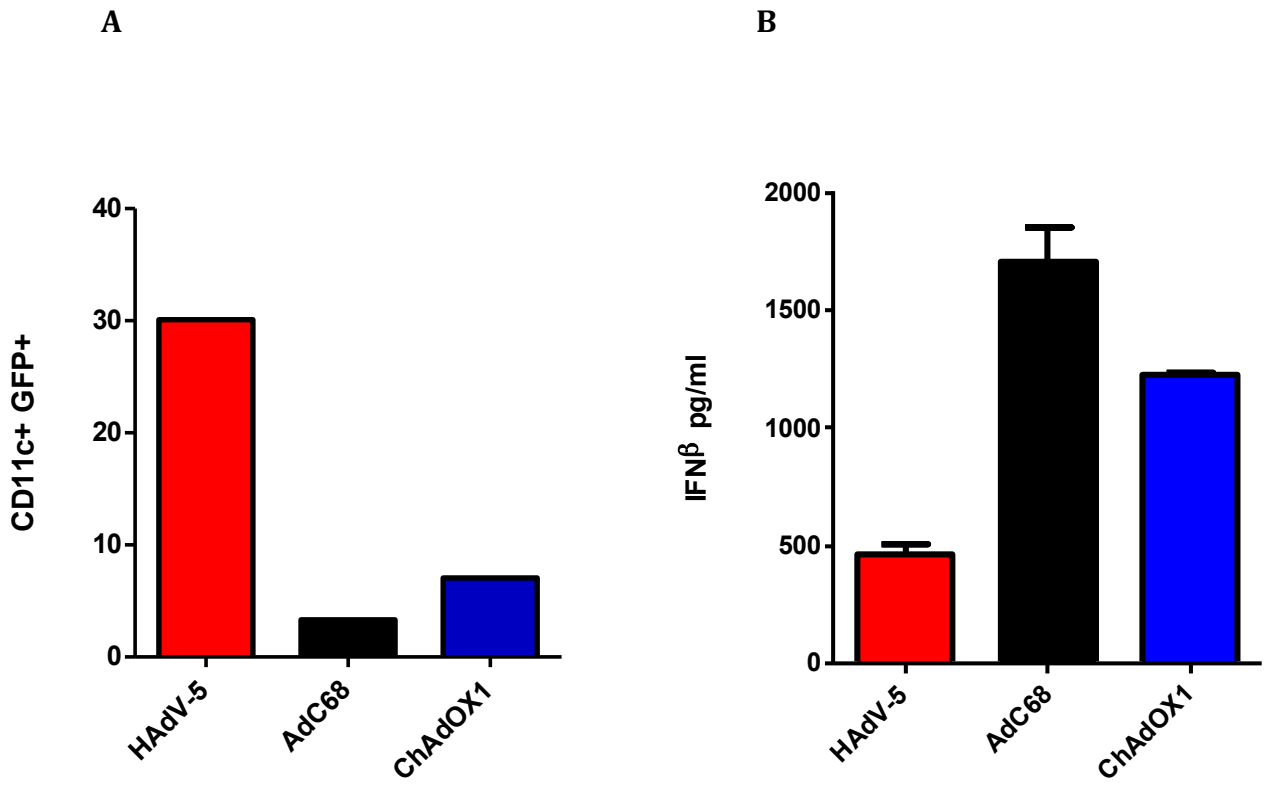
6.2.1 Chimpanzee adenoviruses from *Human adenovirus E* induce higher levels of type I interferon than HAdV-5 upon infection of murine dendritic cells

Previous studies have indicated that E1 deleted chimpanzee adenovirus vector AdC68 induces a stronger type I interferon response than HAdV-5 upon infection of murine DCs [187], and have correlated elevated interferon induction with lower transgene expression and transgene product specific immune responses [186,187]. This study sought to ascertain whether previous observations regarding type I interferon induction in mouse bone marrow derived DCs (BM-DCs) after infection with AdC68 and HAdV-5 vectors could be replicated. Given the high degree of sequence identity between AdC68 and ChAdOX1 (Section 3.1) one might also predict that the ChAdOX1 vector would induce a comparable interferon response to AdC68.

Cells extracted from mouse bone marrow were cultured in the presence of granulocyte macrophage colony stimulation factor (GM-CSF) and dendritic cells were identified as cells expressing elevated levels of surface marker CD11c [194,297]. Figure 6.1 shows that, in agreement with previous work [187,193], interferon-β induction upon infection of murine BM-DCs is stronger after AdC68 and ChAdOX1 infection than HAdV-5. Correspondingly, percentage transduction of DCs is lower after infection with AdC68 and ChAdOX1 vectors, than HAdV-5. Both transduction efficiency and interferon induction are comparable between the chimpanzee adenovirus vectors, and since the immunogenicity of both

vectors is comparable and lower than HAdV-5, these results would be consistent with a role for type I interferon in modulating vector immunogenicity.

Figure 6.1: Chimpanzee adenovirus vectors from *Human adenovirus E* induce higher levels of type I interferon and achieve a lower transduction efficiency upon infection of murine BM-DCs in comparison to HAdV-5. Cultured BM-DCs were infected with E1 E3 deleted recombinant adenovirus vectors expressing the model antigen TIPeGFP at an MOI of 10,000 infectious units per cell. **(A)** Transduction efficiency was assessed by quantifying the percentage of CD11c+ GFP+ cells by flow cytometry. **(B)** Mouse interferon-β expression was assessed by capture ELISA. Error bars display mean and SEM (n=2). Data is representative of at least three independent experiments.



6.2.2 Construction of a chimeric ChAdOX1 based vector expressing VA-RNA I and II from HAdV-5

Members of viral species *Human adenovirus C* and *Human adenovirus E* express two VA-RNAs, both approximately 160 nucleotides in length and transcribed by RNA Polymerase III. VA-RNA I is the major species, expressed rapidly upon infection and at levels approximately 20 fold higher than VA-RNA II [298]. Studies from the 1980's showed that VA-RNA I deleted viruses exhibited a log reduction in viral yield, while deletion of VA-RNA II had no effect on yield compared to wild type [299]. However, deletion of both VA-RNA I and II led to a 6-fold reduction in yield relative to the single VA-RNA I mutant, indicating that VA-RNA II can also facilitate viral replication, albeit in a minor capacity [300]. VA-RNA I transcripts, accumulating to 10^8 molecules per infected cell, bind to and inactivate the double stranded RNA-dependent protein kinase R (PKR), a key component of the antiviral blockade of protein synthesis [301]. VA-RNA II shows only limited ability to bind to and inhibit PKR [302]. However, both VA-RNA species are processed by Dicer into microRNAs (termed mivaRNAs) which saturate both Dicer and RNA induced silencing complex (RISC) complexes [303]. Therefore, VA-RNA I and II transcripts can also function as suppressors of cellular RNA interference, an important anti-viral defense system.

Adenovirus VA-RNA has been implicated in the induction of type I interferon by replication defective vectors [141] and it has been suggested that differences in the sequences of VA-RNA transcripts between vectors of different serotype may be responsible for differences in the magnitude of the interferon response [186]. Previous studies have shown that VA-RNA sequences can vary

considerably between members of different adenovirus species but often share a high degree of identity among members of the same species [295]. In this study, nucleotide sequences of VA-RNA I were compared between members of *Human adenovirus C* and *Human adenovirus E* (Figure 6.2). In agreement with previous studies, VA-RNA I sequences shared a high degree of identity between members of the same species; sequence identity was greater than 90% in all cases with many sequences differing by less than 2%. However, inter-species identity was much lower, typically around 60%. It is therefore entirely possible that VA-RNAs of different adenoviral species could induce the interferon response differently.

In order to test this hypothesis, both VA-RNA sequences from the ChAdOX1 vector were deleted and replaced by the corresponding sequences from HAdV-5, by BAC recombineering as outlined in Section 2.3.4. Transcription of both VA-RNA species by RNA polymerase III has been shown to occur at intragenic promoter regions [304]; hence the chimeric ChAdOX1 vector would contain the HAdV-5 VA regulatory elements in addition to the sequences of both VA-RNA species. In order to confirm that equivalent expression of the HAdV-5 VA-RNA species could be obtained from the chimeric vector, quantitative reverse transcriptase PCR (QRT-PCR) was performed on A549 cells infected with HAdV-5, ChAdOX1, and ChAdOX1-Ad5VA vectors *in vitro* (Figure 6.3).

Figure 6.2 Phylogenetic tree of VA-RNA I sequences from members of *Human adenovirus E* and *Human adenovirus C*.

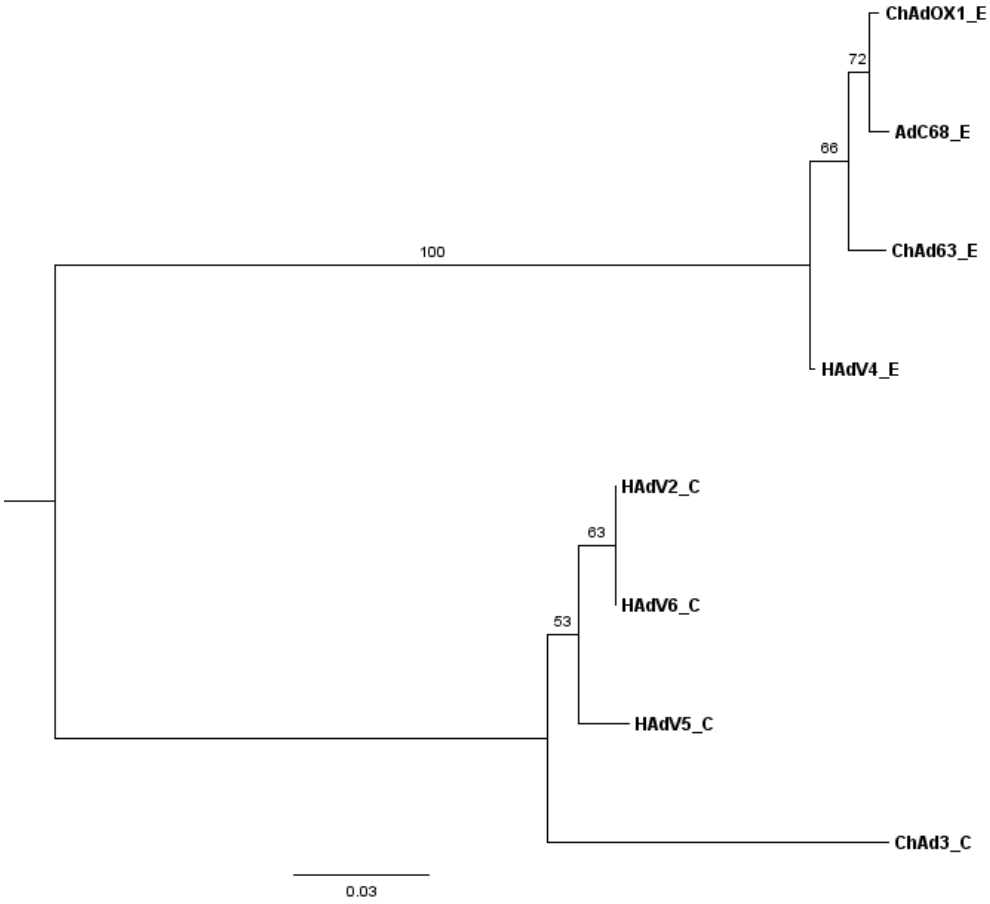
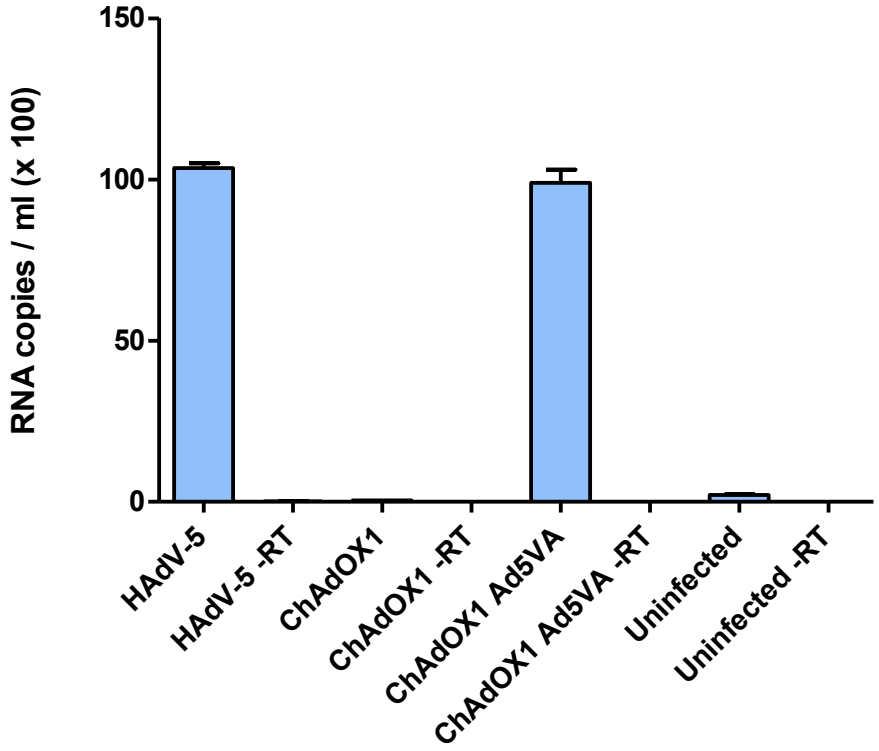


Figure 6.3 Expression of HAdV-5 VA-RNA I from HAdV-5 and chimeric ChAdOX1-Ad5VA vectors is equivalent. A549 cells were infected with HAdV-5, ChAdOX1, and ChAdOX1-Ad5VA vectors at an MOI of 500 ifu/cell. After 24 hours, RNA was extracted, cDNA synthesized, and RT-PCR performed to specifically amplify HAdV-5 VA-RNA I transcripts. PCR was also performed on samples without reverse transcriptase treatment (-RT) as a negative control. Bars show mean and SEM (n=2) and data is representative of two independent experiments.



6.2.3 ChAdOX1-Ad5VA does not confer superior immunogenicity over ChAdOX1 and has no effect on type I interferon production in BM-DCs.

It was hypothesised that replacing the VA-RNA sequences from ChAdOX1 with corresponding sequences from HAdV-5 may lead to a reduction in type I interferon induction, and hence an improvement in transgene product immune responses. However, Figure 6.4 shows clearly that, at least in mice, the chimeric ChAdOX1 Ad5-VA vector offers no improvement in either cellular or humoral immunogenicity compared to ChAdOX1, either at high dose (10^8 infectious units) or intermediate dose (10^6 infectious units).

Exchanging VA-RNA sequences also failed to reduce type I interferon induction upon infection of murine bone marrow derived DCs (BM-DCs) (Figure 6.5). The chimeric ChAdOX1 Ad5VA vector also failed to improve transgene expression within the infected CD11c+ cells. In addition, the chimeric ChAdOX1 Ad5Fiber vector (described in Chapter 5) induced comparable levels of type I interferon to the other ChAdOX1 vectors, despite the fiber protein being implicated in the initiation of innate responses by a recent study [290]. These results suggest that, at least in cultured murine BM-DCs, the difference in interferon induction between HAdV-5 and ChAdOX1 vectors is independent of both VA-RNA and fiber-receptor interactions.

Figure 6.4: Immunogenicity of chimeric vector ChAdOX1 Ad5-VA is equivalent to ChAdOX1. Balb/c mice were immunised intramuscularly with either 10^8 or 10^6 infectious units of vectors HAdV-5, ChAdOX1, or ChAdOX1 Ad5-VA expressing model antigen TIPeGFP. **(A)** CD8+ T cell responses to dominant epitope Pb9 were measured in the spleen two weeks post immunisation by IFNγ ELISpot. **(B)** Serum anti-GFP IgG antibody responses were measured two weeks post immunisation at the 10^8 dose, by endpoint ELISA. Dashed line indicates limit of detection. Error bars represent mean and SEM. Statistical analysis performed by one-way ANOVA with Bonferroni post test. **p<0.01, ***p<0.001

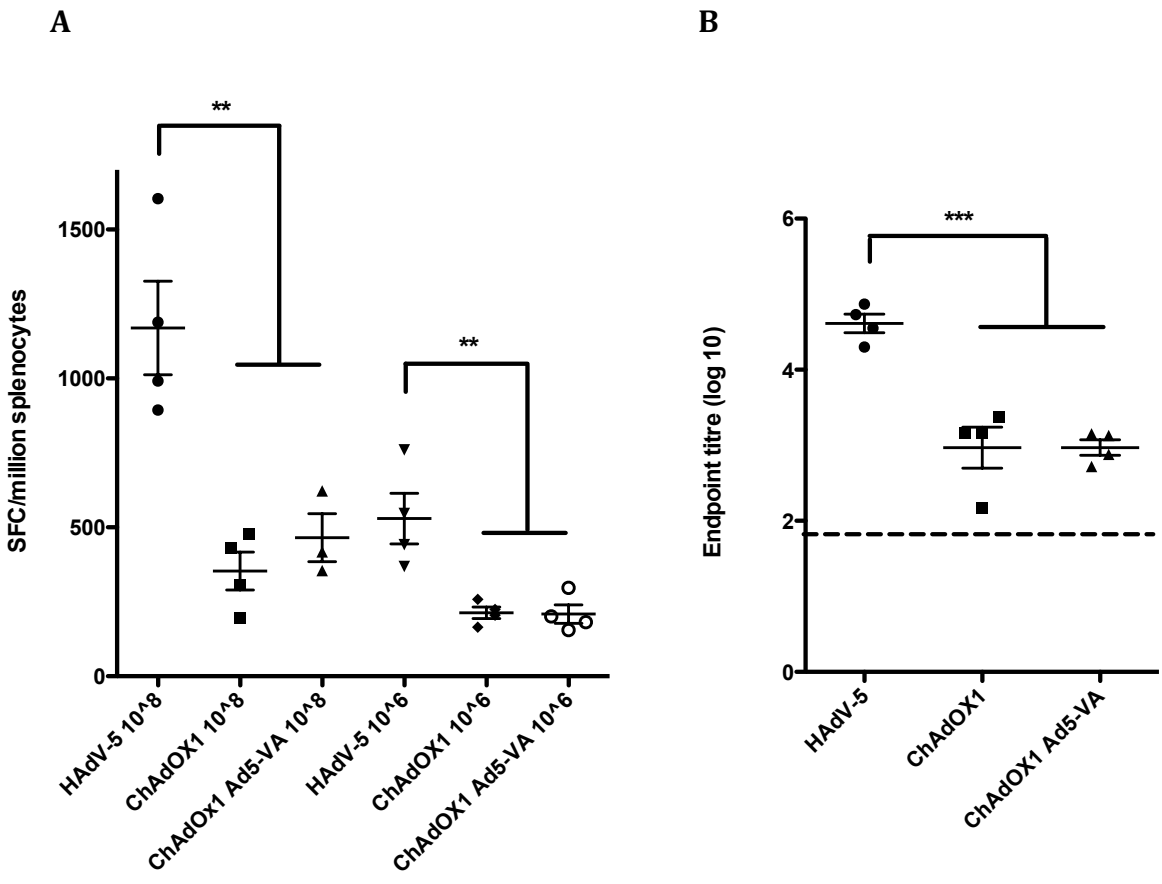
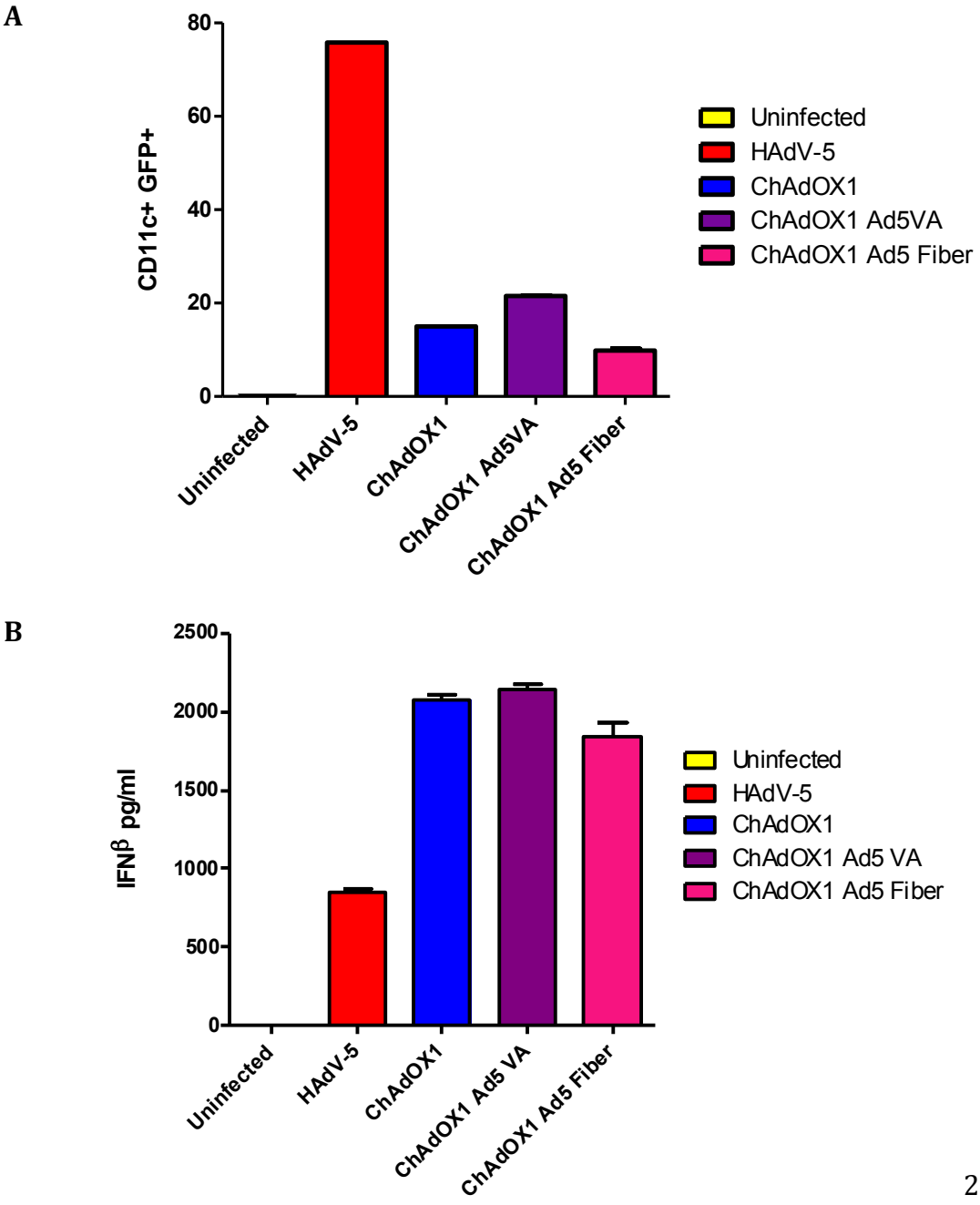


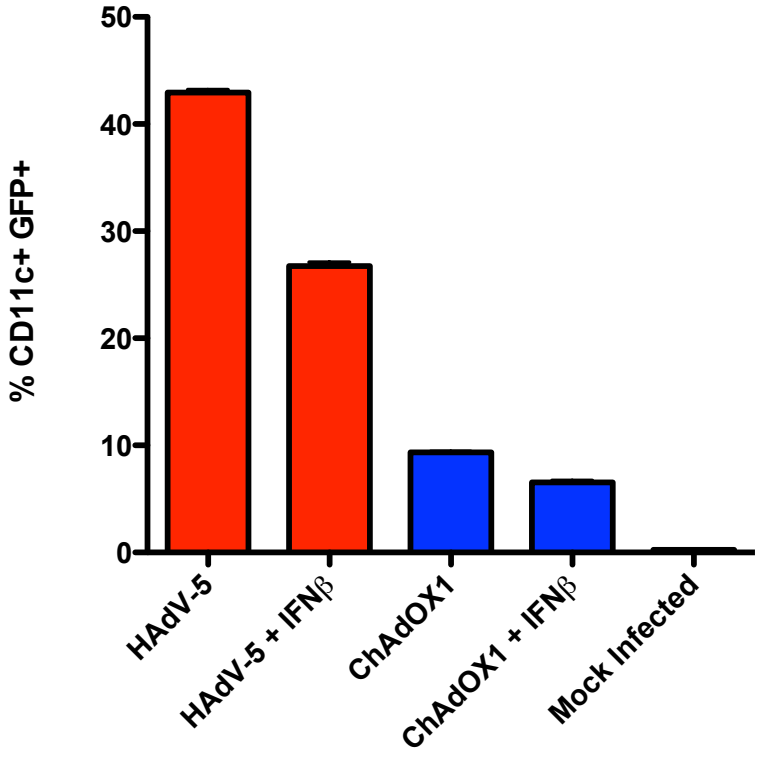
Figure 6.5: Exchanging VA-RNA and Fiber sequences to match the HAdV-5 vector fails to reduce the induction of type I interferon by ChAdOX1 in murine BM-DC. Murine bone marrow derived DCs were infected at an MOI of 5000 infectious units per cell with HAdV-5, ChAdOX1, and chimeric vectors ChAdOX1-Ad5VA and ChAdOX1-Ad5Fiber all expressing recombinant antigen TIPeGFP. After 24 hours, **(A)** the percentage of CD11c+ GFP+ cells 24 hours post infection were enumerated by flow cytometry, and **(B)** IFNβ was assayed from culture supernatants by capture ELISA. Bars represent mean and SEM (n=2), and data is representative of two independent experiments.



6.2.4 Treatment of murine BM-DC with recombinant interferon beta can reduce transgene expression from HAdV-5 vectors.

Since neither modification to the ChAdOX1 vector (VA-RNA or fiber) had an effect on the interferon response upon infection of murine BM-DC, the effect of type I interferon on transgene expression remained unclear. In an attempt to demonstrate a causal link between elevated IFNβ and reduced transgene expression, murine BM-DC were infected with HAdV-5 and ChAdOX1 vectors in the presence and absence of 2ng/ml IFNβ, corresponding to the level of IFNβ detected (Figure 6.5) after *in vitro* infection with ChAdOX1 at the same MOI. Figure 6.6 shows that addition of exogenous IFNβ upon infection with HAdV-5, reduces the number of transgene positive DCs by almost two fold. The effect of IFNβ on transgene expression from ChAdOX1 is much smaller, presumably because the ChAdOX1 vector naturally induces a robust interferon response. These results are in agreement with a recent study by Johnson *et al*, [186] which showed exogenous treatment of DCs with IFNα reduced transgene expression from HAdV-5 by approximately two fold, though the authors used a higher dose of 5ng/ml IFNα. These data indicate that type I interferon, at levels induced by ChAdOX1 *in vitro*, can modify transgene expression in DCs infected by HAdV-5 vectors. However, mechanisms underlying the reduction in transgene and the physiological relevance of this result remain unclear. Moreover, transgene expression is still higher in HAdV-5 infected cells even with interferon treatment, suggesting that interferon induction is not the sole contributor to the differences in transgene expression between these vectors.

Figure 6.6: Addition of exogenous IFNβ upon infection of murine BM-DC reduces transgene expression from HAdV-5 vectors. Murine BM-DC were infected with HAdV-5 and ChAdOX1 vectors expressing TIPeGFP at an MOI of 5000 infectious units per cell in the presence or absence of 2ng/ml recombinant mouse IFN-β. The percentage of CD11c+ GFP+ cells 24 hours post infection was measured by flow cytometry. Mock infection performed using a non-fluorescent HAdV-5 vector expressing Ag85A. Error bars display SEM (n=2).



6.2.5 Co administration of poly I:C can reduce cellular immunogenicity of adenovirus vectors but has no effect on *in vivo* transgene expression.

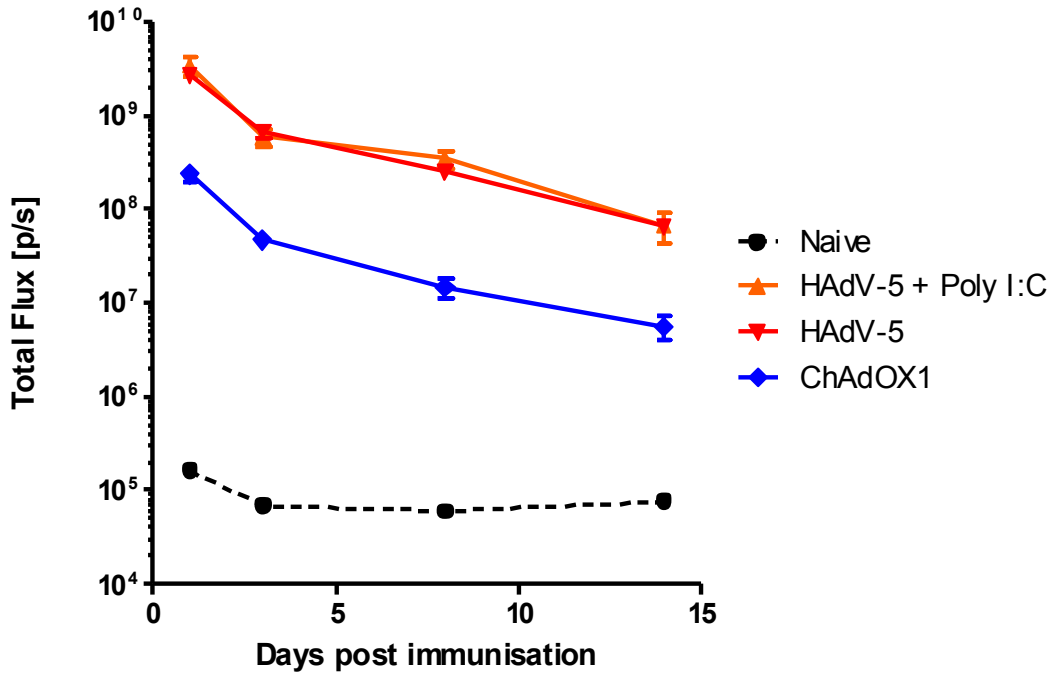
After the observation that addition of exogenous type I interferon could reduce transgene expression in murine BM-DC, it was important to determine whether a similar observation could be made *in vivo*. However, direct administration of recombinant IFNβ in animals could induce potentially lethal systemic inflammation. It was therefore decided to administer repeat subcutaneous (s.c.) injections of polyriboinosinic:polyribocytidylic acid (poly I:C), a synthetic TLR3 agonist known as a potent inducer of type I interferon [305,306] and whose administration in animals had been performed previously without side effects (Anna Goodman, DPhil thesis). Repeat administrations were given to mimic the continued induction of interferon from adenovirus vectors, which have been shown to be persistent *in vivo*[190]. Though an extensive time course is, to our knowledge, yet to be performed, there is certainly direct evidence to suggest that serum interferon levels remain elevated up to 18 hours post immunization with adenovirus vectors [307].

Vectors encoding *Photinus* (Firefly) luciferase were employed in order to measure transgene expression from immunized muscle in real time using the IVIS 200 imaging system. Figure 6.8A shows clearly however, that administration of poly I:C had no effect on transgene expression from the HAdV-5 vector. In agreement with previous observations (Section 5.2.3), transgene expression *in vivo* from HAdV-5 was approximately a log higher than from ChAdOX1 throughout the two-week period. However, a reduction in transgene

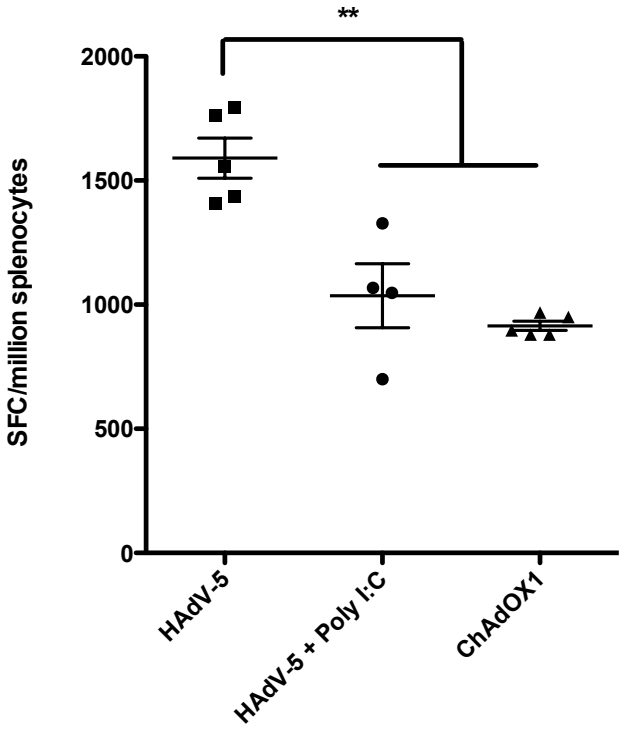
product specific T cell responses was seen in the HAdV-5 poly I:C group, such that the IFNγ ELISpot response was comparable to that obtained with ChAdOX1 (Figure 6.8B). No change was observed in the IgG antibody response after poly I:C co-administration (Figure 6.8C). In a separate experiment, serum IFNβ was measured by capture ELISA, 9 hours and 24 hours post intramuscular immunization with 10^8 infectious units of vectors HAdV-5, HAdV-5 + 30μg poly I:C (s.c.) and ChAdOX1 but IFNβ induction was unfortunately below the limit of detection in all individuals (data not shown).

Figure 6.7: Co-administration of poly I:C with adenovirus vectors reduces transgene product specific T cell responses but has no detectable effect on antibody responses or transgene expression *in vivo*. Balb/c mice (5/group) were immunized intramuscularly with 10^8 infectious units of HAdV-5 and ChAdOX1 vectors expressing *Photinus* luciferase. A third group of animals immunized with HAdV-5 (4/group) had additional subcutaneous injections of 30μg poly I:C at the time of vector administration and then 1 day, 3 days, and 7 days post immunization. **(A)** Luciferase expression was measured on an IVIS 200 imaging system at days 1, 3, 7, and 14 post immunization. **(B)** IFNγ ELISpot responses to luciferase CD8+ epitope GFQSMYTFV [308] and **(C)** anti-luciferase IgG antibody responses were measured two weeks post immunization. Dashed line indicates limit of detection. Error bars show mean and SEM. Statistical analysis by one-way ANOVA with Bonferroni post test. **p<0.01, ***p<0.001

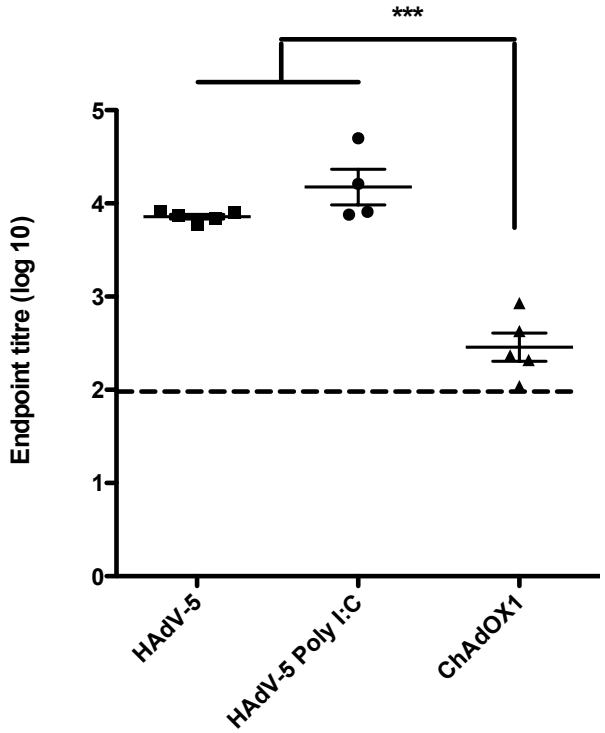
A



B


**
2000
1500
1000
500
0
SFC/million splenocytes
HAdV-5
HAdV-5 + Poly I:C
ChAdOX1

C


***
5
4
3
2
1
0
Endpoint titre (log 10)
HAdV-5
HAdV-5 Poly I:C
ChAdOX1

6.3 Discussion

The aim of this chapter was to explore the role of the type I interferon response in the induction of cellular and humoral transgene product specific responses by replication defective adenovirus vectors. Previous studies have observed that some vectors eliciting less potent vaccine responses induce higher levels of type I interferon [186,187] and these observations were supported in this study by the elevated secretion of IFNβ by murine BM-DC infected with AdC68 and ChAdOX1 vectors (Figure 6.1).

A reverse genetics approach was used in an attempt to identify the viral component responsible for the elevated induction of IFNβ in cells infected with AdC68 and ChAdOX1, but not HAdV-5. The non-coding VA-RNA transcripts had been previously implicated in the induction of type I interferon from E1 deleted adenovirus vectors [141] and were good candidates for eliciting a difference in interferon induction between vectors due to the low sequence identity between members of *Human adenovirus C* and *Human adenovirus E* (Figure 6.2), although sequence identity may not entirely reflect structural homology. Through BAC recombineering, both VA-RNA I and VA-RNA II transcripts from HAdV-5 were inserted into ChAdOX1, replacing the native ChAdOX1 VA-RNA sequences. The resulting ChAdOX1 chimeric expressed HAdV-5 VA-RNA I transcripts to levels comparable to that of the HAdV-5 vector, as assessed by quantitative reverse transcriptase PCR (Figure 6.3). However, replacing the ChAdOX1 VA-RNA sequences with those from HAdV-5 had no effect on either cellular or humoral transgene product specific responses, despite predictions otherwise [186] (Figure 6.4). Furthermore, the chimeric ChAdOX1 Ad5VA vector exhibited no

reduction in type I interferon induction or improvement in transgene expression upon *in vitro* infection of murine BM-DC (Figure 6.5).

Recent studies have also implicated fiber receptor interactions in the induction of innate responses. Indeed, replacement of the HAdV-5 fiber with the HAdV-35 fiber in a recent study by Teigler *et al* significantly increased induction of innate cytokines upon infection of human peripheral blood mononuclear cells (PBMC) including IFNα, IFNγ, IL-1β, IL-6 MIP-1α, MIP-1β and TNFα [290]. However, in the current study, replacement of the ChAdOX1 fiber with the HAdV-5 fiber had no effect on interferon induction or transgene expression in infected murine BM-DCs. The fact that HAdV-35 is a member of *Human adenovirus B* (and CD46 tropic), while ChAdOX1 belongs to *Human adenovirus E* could account for the apparent conflict with the Teigler study. It also entirely possible that steps in the viral entry pathway that are independent of the fiber protein may be responsible for the difference in interferon stimulation in this study. Certainly, the viral factors responsible for the induction of the type I interferon response remain unclear, though from this study it is clear that differences in the interferon response and immunogenicity between HAdV-5 and ChAdOX1 vectors are independent of VA-RNA sequences and the fiber protein.

The CpG content of adenoviral genomes has been shown to vary between members of different viral species, with HAdV-12 from *Human adenovirus A* particularly immunostimulatory, while HAdV-2 from Human adenovirus C far less immunostimulatory [309]. The capacity of the viral genome to activate the innate response may be determined in part by the ratio of immunostimulatory CpG-S motifs to neutralizing CpG-N motifs which block immune activation by CpG-S. However, upon analysis it appears that members of *Human adenovirus E*

contain a similar ratio of CpG-S and CpG-N motifs to members of *Human adenovirus C* (data not shown).

The relationship between the induction of type I interferon by recombinant adenovirus vectors and the efficiency of transgene delivery has been a subject of debate. Acsadi *et al* showed that both intraperitoneal and intramuscular pre-treatment of mice with recombinant IFNα reduced transgene product expression upon subsequent intramuscular administration of an E1 E3 deleted HAdV-5 vector [269]. A similar result was obtained in the same study through *in vitro* infection of IFNα pre-treated C2C12 myotubes, and in this experiment the reduction of transgene product was associated with lower transcriptional activity from the CMV promoter. In contrast, a recent study by Huarte *et al* found that while replication defective adenovirus vectors induce potent interferon responses, these innate responses failed to reduce expression of the encoded transgene product [188]. HeLa cells were infected with adenovirus vectors in the presence of human IFNα with no effect on transgene product delivery, while transgene expression was virtually abolished from a Semliki Forest Virus (SFV) vector in the same experiment. The same authors also demonstrated in mice that *in vivo* luciferase expression from a replication competent adenovirus vector was unaffected by prior immunization with a replication defective vector 24 hours earlier [188].

In the current study, and in agreement with previous observations [186], it was demonstrated that transgene delivery by HAdV-5 vectors in murine dendritic cells could be significantly lowered by exogenous administration of recombinant type I interferon *in vitro* (Figure 6.6). However, the *in vivo* relevance of type I interferon regarding activity of the CMV promoter remains

unclear. Repeated administration of poly I:C, a potent inducer of type I interferon, was sufficient to reduce CD8+ T cell responses in agreement with a previous study in our laboratory (Anna Goodman, DPhil thesis) but had no effect on *in vivo* transgene delivery (Figure 6.7). This result could reflect the fact that subcutaneous administration of poly I:C may have induced interferon systemically, but not specifically at the site of transgene expression. It is possible that the reduction in CD8+ T cell responses observed after poly I:C administration was due to a reduction in the capacity of DCs (activated though TLR3 mediated recognition of the dsRNA analog) to present vector delivered antigens to cognate CD8+ T cells.

The effect of type I interferon on activity of the CMV promoter is likely to be dose dependent; concentrations of recombinant interferon administered in the current and previous studies, both *in vitro* and *in vivo*, may be supra-physiological. Certainly the failure of prior vector administration to affect *in vivo* transgene expression in the study by Huarte *et al* might suggest this, even though the vector expressing luciferase was replication competent [188].

Unfortunately, since type I interferon was not detectable in the sera of animals immunized with HAdV-5 and ChAdOX1 it is not possible to draw firm conclusions regarding the role of type I interferon on adenoviral vector vaccine responses *in vivo*. However, the reduction of CD8+ T cell responses upon co-administration of poly I:C observed in this study, together with observations by Johnson *et al* [186], suggest that type I interferon induction has the potential to reduce CD8+ T cell responses, regardless of its effect on transgene expression *per se*. Identifying the factors responsible for the different induction of type I interferon by vectors of different serotype may therefore be important for the

development of future vaccine vectors. However, this study has established that differences in induction of type I interferon between HAdV-5 and ChAdOX1 seen *in vitro* are almost certainly unrelated to differences in the sequences of the viral VA-RNAs or the fiber protein. More importantly for vaccine design, this study has conclusively shown that exchanging the VA-RNA sequences between HAdV-5 and ChAdOX1 vectors has no impact on either cellular or humoral vaccine responses.

7 Discussion

Replication defective recombinant adenoviruses have emerged as promising vaccine delivery agents, particularly for induction of potent CD8+ T cell responses. The focus of vector development has recently shifted towards construction of vectors based on rare human or non-human serotypes [164,172], since pre-existing anti vector immunity may have reduced the efficacy of vaccines based on common human adenoviruses such as HAdV-5 [166]. In this study, the construction of a new replication defective vector based on a chimpanzee adenovirus, Y25, is described (Chapter 3). Chimpanzee adenovirus vectors have performed effectively in recent human vaccine trials, eliciting unprecedented magnitudes of transgene product specific CD8+ T cell responses [310]. However, to date only two chimpanzee adenovirus vectors, ChAd63 [256,310] and ChAd3 [204], have been tested in humans. An important aim of the current study was to generate a new vector with low human seroprevalence, which could be amplified to high titer and titered accurately to enable production of a clinical grade vaccine product for use in human trials. Through deletion of E1, E3, and extensive modification of the viral E4 region, a vector was generated to meet these requirements. The new vector, termed ChAdOX1, has recently entered clinical assessment in humans (Gilbert *et al*, manuscript in preparation).

The ChAdOX1 vector was constructed in a bacterial artificial chromosome (BAC). The single copy BAC vector, in addition to conferring genetic stability, enabled the use of BAC recombineering for precise and flexible modification of the viral genome. The BAC recombineering technique was employed during the

construction of ChAdOX1, but was also invaluable for generating chimeric vectors to investigate the viral determinants of vector immunogenicity.

Recent studies from our own laboratory have demonstrated that a careful consideration of the infectivity of vector preparations is required for a reliable assessment of comparative immunogenicity between vectors [216]. Therefore, basing vaccine doses on infectious titer, rather than viral particle count, a comparative assessment of immunogenicity after intramuscular immunisation was performed in mice between HAdV-5, ChAd63, AdC68 and ChAdOX1 vectors (Chapter 4). The current study has clearly demonstrated than HAdV-5 vectors (species *Human adenovirus C*) elicit higher transgene product specific T cell and IgG antibody responses than chimpanzee adenoviruses ChAd63, AdC68 and ChAdOX1 (all species *Human adenovirus E*) in mice. This difference in immunogenicity was independent of the nature of the encoded antigen, and mouse strain tested, and was primarily a quantitative difference in magnitude rather than a qualitative difference since the multi-functionality of CD8+ T cell responses and T_H1/T_H2 balance of IgG isotypes were comparable between vectors. That the immunogenicity of *Human adenovirus C* vectors is superior to *Human adenovirus E* vectors in mice is not in agreement with all previous studies, but does concur with the most comprehensive comparative study performed previously in mice to date [201]. However, not all previous studies administered vectors via the intramuscular route; different routes of administration would likely target different cell types and hence any conclusions drawn from the current study can only be applied to vaccination via the intramuscular route. Furthermore, despite the fact that mice are routinely used to screen new vectors for clinical application [201], the hierarchy of vector

immunogenicity in mice may not apply to other mammalian species. Indeed, comparative studies in cattle (by intramuscular immunisation) comparing HAdV-5 and ChAdOX1 vectors expressing antigen 85A have demonstrated superior 85A specific effector T cell and antibody responses in the ChAdOX1 vaccinated group (Efrain Guzman, personal communication). It is therefore important that the mechanisms responsible for the differences in immunogenicity between vectors are fully understood, to enable rational design of new vectors for use in humans.

In the current study, two potential hypotheses to explain the superior immunogenicity of HAdV-5 based vectors in mice were proposed and tested. In Chapter 5, the role of transduction efficiency *in vivo* was investigated. Modification of the ChAdOX1 capsid was performed to address the hypothesis that differences in the structure of the capsid, specifically the fiber protein, between HAdV-5 and ChAdOX1 vectors were responsible for a difference in transduction efficiency *in vivo* and hence immunogenicity. In Chapter 6, the role of innate immunity, specifically the induction of type I interferon, was investigated. Exchange of virus-associated RNA (VA-RNA) transcripts between vectors were performed in an attempt to lower the induction of type I interferon after ChAdOX1 administration, since high induction of type I interferon has previously been associated with reduced transgene product specific immune responses [186,187]. It is also important to consider that these factors are not mutually exclusive; indeed recent studies have implied that the level of transgene expression within vector transduced cells may be strongly associated with the induction of type I interferon upon vector administration [186,187,269].

In Chapter 5, a marked difference in transduction efficiency *in vivo* was demonstrated between HAdV-5 and the chimpanzee adenovirus vectors after intramuscular administration in mice. Importantly, the superior transduction efficiency of the HAdV-5 vector compared to ChAd63 and ChAdOX1, correlated with higher magnitudes of transgene product specific effector CD8+ T cells and IgG antibody titers. *In vitro* experiments had revealed that transduction of human lung carcinoma cell line A549 was more efficient by HAdV-5 than by AdC68 or ChAdOX1, and this difference was clearly shown to be fiber mediated. Transduction efficiency of a chimeric ChAdOX1 vector expressing the fiber shaft and knob domains from HAdV-5 was equivalent to that of the HAdV-5 vector, and approximately a log higher than the original ChAdOX1 vector. A549 cells express abundant levels of the coxsackie and adenovirus receptor (CAR)[274], identified as a high affinity receptor for HAdV-5 [106], and also demonstrated to enhance cell entry of *Human adenovirus E* vector AdC68 [272]. Interaction between the viral capsid and the CAR receptor is mediated via the fiber protein, and though the *in vivo* relevance of CAR has been questioned [107] and expression of the receptor within mature skeletal muscle is low [280], fiber proteins of different serotypes have been shown to interact with a variety of other receptors including heparan sulphate glycosaminoglycans, sialic acid, CD46 and CD80/CD86. A plausible hypothesis was therefore that differences in fiber-mediated infectivity of cells *in vivo* between vectors were responsible for the observed differences in both *in vivo* transgene expression and immunogenicity. However, the chimeric ChAdOX1 Ad5Fiber vector failed to offer a substantial improvement in either transgene expression or immunogenicity

over the ChAdOX1 vector *in vivo*. This study has therefore clearly established that HAdV-5 vectors offer superior transduction efficiency to *Human adenovirus E* ChAd vectors *in vivo*, and this difference in transduction efficiency correlates with immunogenicity. But neither differences in transduction efficiency, nor differences in immunogenicity relate to differences in the structure of the fiber protein between vectors.

However, the mechanism responsible for the superior transduction efficiency after HAdV-5 administration remains elusive. Indeed, it is unclear whether differences in observed transgene expression relate to differences in the efficiency of vector delivery of viral DNA to the nucleus, or the level of transcriptional and / or translational activity once viral DNA has reached the nucleus. To address this issue a PCR assay for detection of nuclear viral DNA in injected leg tissue was performed, but was confounded by the difficulty in isolating nuclear viral DNA away from extracellular viral DNA associated with non-infectious virions. Alternatively, a histological approach involving fluorescence *in situ* hybridization (FISH) could be employed to identify copies of viral DNA genomes within cell nuclei.

Another important question that this study has not addressed is the precise nature of the cell types infected by each of the vectors studied. The current study has hinted that the difference in transgene expression between vectors within the draining popliteal lymph nodes is significantly higher than within muscle tissue at the site of injection. However, it is unclear whether vectors display a difference in tropism within muscle and lymph node tissues. Again, one could address this question through histology, perhaps using vectors encoding a fluorescent marker gene such as GFP. Attempts were made to

phenotype infected dendritic cells isolated from draining lymph nodes after administration of vectors expressing enhanced GFP. However, populations of GFP expressing cells were too low to accurately quantify; pooling of lymph nodes from several individuals as described in Lindsay *et al* [194] may be required to perform this study. Are differences in direct presentation or cross presentation of antigen to dendritic cells primarily responsible for differences in CD8+ T cell induction between vectors? Both mechanisms of CD8+ T cell priming have been demonstrated in previous studies in mice [194,195]. This question could be addressed through the construction of vectors expressing transgenes under tissue specific promoters such as the skeletal muscle specific murine muscle creatine kinase (mMCK) promoter; although differences in promoter strength could affect conclusions drawn from such studies.

Chapter 6 investigated the relationship between the induction of innate immunity and immunogenicity of adenovirus vaccine vectors. This study confirmed previous reports that chimpanzee adenovirus vectors, including AdC68 [187,193], induce higher levels of type I interferon upon infection of murine bone marrow dendritic cells (BM-DC) than HAdV-5 vectors. Furthermore, in a link to the previous chapter and in agreement with previous studies, transgene expression within the infected DCs was higher after transduction by HAdV-5 [186,193]. And indeed, a direct causal relationship between type I interferon induction and transgene expression was demonstrated *in vitro*, after co-addition of recombinant murine IFNβ reduced the level transgene expression in BM-DCs transduced by a HAdV-5 vector. However, the *in vivo* relevance of these data remains unclear since type I interferon could not be detected in sera from vaccinated animals, contrary to previous studies [186,311].

Repeat subcutaneous co-administration of poly I:C in combination with an intramuscular HAdV-5 immunisation, lowered transgene product specific CD8+ T cell responses compared to HAdV-5 immunisation alone, but did not reduce the level of transgene expression *in vivo*. Though poly I:C, a synthetic dsRNA analog and TLR3 ligand, has previously been shown to induce type I interferon [193], the lack of serum cytokine data from this study precludes any firm conclusion from being drawn from this experiment. It is possible that additional pro-inflammatory cytokines induced by poly I:C may have been responsible for the observed reduction in immunogenicity, or that poly I:C induced supra-physiological levels of type I interferon that would not normally be associated with vector administration. Furthermore, the association between type I interferon and transgene expression *in vivo* is also unclear; subcutaneous administration of poly I:C may fail to induce interferon at the site of transgene expression (ie within infected muscle tissue or draining lymph nodes). It is worth noting that a previous study demonstrating type I interferon mediated reduction of intramuscular transgene expression performed injection of recombinant type I interferon directly into target muscles two days prior to vector administration [269]. Therefore, this study must conclude that it is still plausible that differences in transduction efficiency observed between vectors in Chapter 5, are not due to the efficiency of cell entry and trafficking, but are instead due to differences in the induction of innate immunity and consequent down-regulation of transgene expression. Certainly, the *in vitro* BM-DC would, at least in part, support this hypothesis, though the *in vivo* data is inconclusive. In order to extend the current study, a knockout mouse strain lacking the type I IFN receptor (IFNR-/-), featured in previous studies [186,187], could be utilized to

assess vector immunogenicity and transgene expression in the absence of interferon signaling. A thorough comparison of immunogenicity and quantitative *in vivo* transgene expression between multiple vectors in such a mouse strain could be highly informative.

Since the ultimate aim of this study is to improve adenovirus vaccine vectors for clinical use, it was important to attempt to identify the component of the virion or viral genome responsible for the potent induction of type I interferon by the *Human adenovirus E* ChAd vectors. Arguably the most obvious candidate viral components were the two virus associated RNA, (VA-RNA) transcripts. A recent study by Yamaguchi *et al* has identified adenovirus VA-RNA sequences as the components of E1 deleted HAdV-5 vectors responsible for inducing type I interferon upon *in vitro* infection of mouse embryonic fibroblasts and murine BM-DCs [141]. Although this study focused on interferon induction by HAdV-5, it was highly plausible that similar sequences present in *Human adenovirus E* vectors could also induce type I interferon, albeit at higher levels. Indeed, analysis of VA-RNA I sequences between members of *Human adenovirus C* and *Human adenovirus E* demonstrated a high degree (>90%) of identity between members of the same species, but only a modest (approximately 60%) degree of identity between members of different species. A chimeric ChAdOX1 vector was constructed by replacing both VA-RNA I and II sequences from ChAdOX1 with the corresponding sequences from HAdV-5. However, despite demonstration of equivalent expression of the HAdV-5 VA-RNA transcripts *in vitro*, the chimeric ChAdOX1 Ad5VA vector failed to improve transgene product specific responses compared to the original ChAdOX1 vector *in vivo*. Furthermore, the replacement of VA-RNA sequences had no effect on either type

I interferon induction or transgene expression after *in vitro* transduction of murine BM-DCs. So once again, this study has conclusively ruled out a highly plausible viral component from having a detectable effect on the magnitude of vector mediated transgene product specific immune responses. Fiber-receptor interactions have also been implicated in induction of innate cytokines upon infection of human peripheral blood mononuclear cells (PBMC) in a recent study comparing HAdV-5 and CD46 tropic vectors HAdV-35, HAdV-26 and HAdV-48 [290]. However, in the current study, the chimeric ChAdOX1 Ad5fiber vector also failed to reduce induction of type I interferon *in vitro*, implying that the difference in interferon induction between vectors is neither VA-RNA, nor fiber mediated.

In conclusion, the work presented in this thesis has led to the construction of ChAdOX1, a new chimpanzee adenovirus vaccine vector, with low human seroprevalence that has recently entered human clinical trials (Gilbert *et al*, manuscript in preparation). The study has revealed a clear difference in immunogenicity, both cellular and humoral, between HAdV-5 vectors and chimpanzee adenovirus vectors ChAd63, AdC68, and ChAdOX1 in mice. Potential factors responsible for the superior immunogenicity of HAdV-5 vectors include transduction efficiency (the ability of a viral vector to infect a cell and express an encoded transgene), and induction of innate immunity, which may lead to NK cell mediated vector clearance or down-regulation of transgene expression. These two factors are certainly not mutually exclusive, and indeed the latter point suggests that transduction efficiency could by definition be influenced by innate immunity. This study concludes that neither of these potential factors can be ruled out; indeed the mechanism behind the difference

in immunogenicity between vectors remains unclear. However, through reverse genetics, arguably the most plausible of candidate viral components, fiber and VA-RNA, have been conclusively proven not to be responsible for the difference in immunogenicity. The vector component(s) responsible remain elusive. Perhaps cell infectivity via non-fiber mediated interactions such as penton base-integrin attachment play an important role. Maybe the differences in capsid surface charge affect the tropism of these vectors. Or maybe the difference in type I interferon induction between vectors is important *in vivo*, and governed by factors other than VA-RNA expression and fiber mediated cell entry. Perhaps the difference in immunogenicity is due to a combination of all of these factors. Further studies will be required to answer to these important and intriguing questions.

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