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# **The potential of a novel Adenovirus vector for vaccination**

A thesis submitted for the degree of Doctor of Philosophy

Department of Biological Sciences

The University of Warwick

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October 2010

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## **Declaration**

I declare that all work presented in this thesis was conducted by me under the direct supervision of Dr. Hans-Gerhard Burgert, unless otherwise stated. None of the material reported here has at any time been submitted for examination for any other degree.



## **Acknowledgements**

I would like to thank my supervisor Dr. Hans-Gerhard Burgert for his help and support over the last 4 years.

Thank you to Dr. Zsolt Ruzsics for his expertise with the recombineering system

Thank you to Dr. Tomas Hanke and Dr. Anne Bridgeman for materials, their expertise and assistance with data gathered using the HIVA model system.

Thank you to Professor Andrew Easton, Helen Terry, Sam Dixon and Yashar Mohammed-Sadigh for materials and their assistance with the PVM mouse model system.

Thanks to Dr. Claire Harris and Dr. Andre Lieber for materials.

A special thank you to Dr. Susan Morris and Dr. Jen Cox for their help and support and to Jordan Wright and Helen Terry for keeping me sane.

Most of all I want to thank my mum, dad and sister. Without their love, patience and understanding I wouldn't have made it this far, in fact, I wouldn't be anything at all.

And finally, Lisette, who has lit up my life and made my future so much brighter. Thank you for sticking with me through this, I know it hasn't been easy! I love you with all my heart.

Dedicated to Gavin Speechley 1983-2002

‘An empty chair at all the tables’

## Abbreviations

A	Adenine
aa	Amino acid
A <sub>260</sub>	absorbance (at 260 nm)
Ab	Antibody
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
Ad	Adenovirus
AdN	Adenovirus serotype N (where N is a numerical integer)
ADP	Adenovirus death protein
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
Adpol	Adenovirus DNA polymerase
Amp	Ampicillin
APC	Antigen presenting cell
BAC	Bacterial artificial chromosome
BCG	Bacillus Calmette-Guérin
BD	Bekton Dickinson
bp	Base pair
bRSV	Bovine RSV
BSA	Bovine Serum Albumin
Cad	Chimpanzee Adenovirus
CAdV	Canine Adenovirus
CAR	Coxsackievirus Adenovirus Receptor
cDNA	Complementary Deoxyribonucleic acid
Cm	Chloramphenicol
CMC	Carboxymethyl Cellulose
CMI	Cell-mediated immunity
CMV	Cytomegalovirus
c.p.e.	Cytopathic effect
CTL	Cytotoxic T-lymphocyte (CD8+ T cell)
d	Distilled
DC	Dendritic Cell
DBP	DNA binding protein

DISC	Death Inducing Signalling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DOG	2-deoxy-galactose
DBP	DNA binding protein
DPPC	Dipalmitoylphosphatidylcholine
ds	double-stranded
DTT	Dithiothreitol
E	early
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetic acid
EGFR	Epidermal growth factor receptor
EKC	Epidemic keratoconjunctivitis
ELISA	Enzyme-linked Immunosorbant assay
ELISPOT	Enzyme-linked Immuno spot
ER	Endoplasmic Reticulum
EtBr	Ethidium Bromide
FACS	Fluorescence Associated Cell sorting
FADD	FAS associated death domain
FCS	Foetal Calf Serum
FIP	14.7K interacting protein
g	gram
<i>galK</i>	galactokinase
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GFP	Green Fluorescence Protein
GMEM	Glasgow Minimal Essential Medium
HBV	Hepatitis B virus
HCV	Hepatitis C virus
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

HPV	Human papillomavirus
HRP	Horseradish peroxidase
hRSV	Human RSV
HS	Heparan sulfate
HSV	Herpes simplex virus
HVR	hypervariable region
IAVI	International AIDS Vaccine Initiative
I/D	intradermal
iDC	immature DC
IFN	Interferon
IFN $\alpha$	Interferon alpha
IFN $\beta$	Interferon beta
IFN $\gamma$	Interferon gamma
Ig	Immunoglobulin
IL-6 (etc)	Interleukin 6 (etc)
I/M	intramuscular
I/N	intranasal
IP	Interferon gamma inducible proteins
I/P	intraperitoneal
ITR	inverted terminal repeat
Kan	Kanamycin
kb	kilobase
kDa	kilodaltons
L	late
LB	Luria-Bertani
LC	Langerhans cell
LPS	Lipopolysaccharide
LTR	long terminal repeat
M	Molar
m	milli
mCAR	mouse CAR
MCP	Membrane cofactor protein
MCPs	Monocytes chemoattractant proteins
mDC	mature DC

MHC	Major histocompatibility complex
MICA/B	Major histocompatibility complex class I chain-related proteins A and B (MICA and MICB)
MHC	MHC class II rich compartments
MIPs	Macrophage inflammatory proteins
mins	minutes
MLP	Major Late Promoter
MLTU	Major Late Transcription Unit
moDC	mouse DC
MOI	multiplicity of infection
MRC	Medical Research Council
mRNA	messenger Ribonucleic acid
MVA	Modified vaccinia Ankara
MyD88	Myeloid differentiation primary response gene 88
MyDC	Myeloid DC
μ	micro
NAbs	neutralising Antibodies
NEB	New England Biolabs
NK-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NHP	non-human primate
NK	Natural killer cell
nm	nanometer
OD <sub>(600)</sub>	optical density (at 600 nm)
ORF	open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDC	plasmacytoid DC
PEG	polyethylene glycol
pfu	plaque forming units
p.i.	post infection
PRR	Pathogen Recognition Receptors

pTP	precursor terminal protein
PVM	Pneumovirus of mice
rAd	Adenovirus vector
RANTES	regulated on activation, normal T cell expressed and secreted protein
RID	receptor for internalisation and degradation
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
SA	Sialic acid
S/C	subcutaneous
SDS	sodium dodecyl sulfate
SIV	Simian immunodeficiency virus
ss	single-stranded
SV40	Simian virus 40
T	Thymine
TBE	tris-borate EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TFB1 (etc.)	transformation buffer 1 (etc.)
TLR	toll-like receptor
Tn7	Transposon 7
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
V	volts
v	volume
VA	viral-associated
VCAM-1	Vascular cell adhesion molecule 1
<i>wt</i>	wild type
x	times
x g	times gravitational acceleration

## Abstract

Adenoviruses (Ads) have many attractive characteristics for use as agents of gene-based vaccines and therapies. The most frequently used Ad vectors in preclinical research are based on Ad5. However, in the clinical setting Ad5 vectors have severe limitations. About 90% of the population have neutralising antibodies against Ad5 and infection requires expression of the viral receptor CAR, which is not present on important cell types. Previous data from this laboratory suggested that the species D adenovirus, Ad19a, may overcome some of these limitations. Most relevant for vaccination is its high efficiency of infection of human dendritic cells (DCs), the most important antigen presenting cells. This highly effective DC targeting was retained in Ad19aGFP vectors. To investigate the potential of Ad19a vectors for vaccination further, two transgenes, the nucleocapsid gene from pneumovirus of mice (PVM-N), and a HIV polyprotein cassette (HIVA), were inserted into replication-deficient Ad5 and Ad19a vectors using recombineering. rAd19aPVM and rAd19aHIVA expressed a significantly higher amount of transgene compared with their Ad5 homologues. Encouraging results were obtained when the ability of rAd5PVM and rAd19aPVM to protect mice from lethal PVM challenge was examined using various prime/boost vaccinations. A dose of  $10^6$  pfu of rAd19aPVM, but not rAd5PVM, provided protection. rAd5PVM did, however, protect mice at the same dose when combined with rAd19aPVM in a heterologous prime boost schedule. Vaccination-induced IgG responses to PVM-N did not correlate with protection, implicating cell-mediated immune responses in protection. Utilising rAd19aGFP, evidence is also provided that Ad19a may use CD46 and to some extent CAR as a receptor on CHO cells, expanding our knowledge of the basic biology of this virus.

## Chapter 1: Introduction

### 1.1: Adenovirus Biology

#### 1.1.1: The Adenoviruses

The adenoviruses (Ads) were first discovered in 1953 when an agent isolated from adenoidal tissue was found to cause the degradation of cells in culture (Rowe *et al.*, 1953). Concurrently, the same viral agents were isolated from the respiratory secretions of military recruits suffering from a feverish respiratory illness (Hillman & Werner, 1954). These agents were subsequently shown to be related and induced cytopathic changes in human cells in culture (Huebner *et al.*, 1954). They were also identified as the agent responsible for other clinical diseases such as epidemic keratoconjunctivitis (EKC) and many other respiratory diseases which had no identified cause until the classification of the virus family which was named after the tissue it was initially isolated from, the adenoid (Enders *et al.*, 1956). They have therefore been classified as the *Adenoviridae* family, which has subsequently been further categorised into those viruses which only infect mammals, the *Mastadenoviridae*, those which infect only birds, the *Aviadenoviridae*, those which infect fish, the *Ichtadenoviridae*, and two groups with the ability to infect a wide range of hosts including mammals, birds, reptiles and marsupials, the first named for their high Adenine and Thymine (A & T) composition, the *Atadenoviridae*, and the remainder named the *Siadenoviridae* (Berk, 2007).

The *Mastadenoviridae* genus consists, among others, of fifty three described serotypes which infect humans, the fifty-second (Jones *et al.*, 2007) and fifty-third (Aoki *et al.*, 2008) being described only recently. The taxonomy of the human adenoviruses is based primarily on two criteria i) the differential ability of a virus to haemagglutinate red blood cells and ii) the differential ability to withstand neutralisation by antisera raised against known adenoviral serotypes (Rosen *et al.*, 1962), resulting in the placement of each serotype into one of six species labelled A-F (Table 1.1). Generally the lower the serotype number the better characterised the viruses have become. To date, the serotypes Ad2 and Ad5 of species C, which share high sequence identity, have been studied most extensively (Berk, 2007).

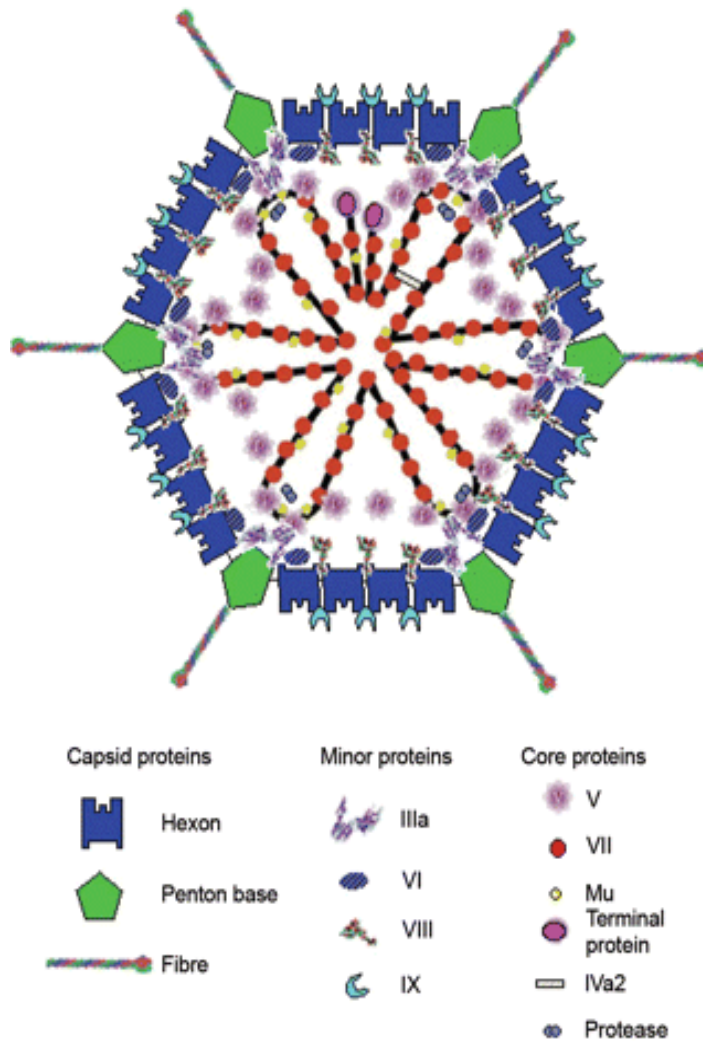


Species	Serotypes	Examples of diseases caused
A	12, 18, 31,	Gastroenteritis
B	3, 7, 11, 14, 16, 21, 34, 35, 50,	Acute respiratory and urethral diseases
C	1, 2, 5, 6,	Respiratory disease
D	8, 9, 10, 13, 15, 17, 19a, 20, 22-30, 32, 33, 36, 37-39, 42- 49, 51	Eye infections and Urogenital diseases
E	4	Acute respiratory disease
F	40, 41	Gastroenteritis

**Table 1.1: Classification of the human adenoviruses.** The classification of the 53 human Ad serotypes showing the allocation to species A-F and examples of the diseases caused by members of each species. Subtypes 52 and 53 have yet to be classified into a species although it has been suggested subtype 52 belongs to a new species, species G (Jones *et al.*, 2007).

### 1.1.2: Virion structure

Adenoviruses are non-enveloped DNA/protein particles of between 70-100nm in diameter with an icosahedral structure (Berk, 2007; Figure 1.1). The majority of the eleven structural polypeptides which constitute the Ad virions are encoded by the major late transcription unit (MLTU). The double stranded (ds) genome is packaged inside a capsid which is composed of seven of these polypeptides, the outer shell of which is arranged of 252 subunits (capsomeres) of which 240 trimers are of polypeptide II (hexon) and 12 are oligomers of polypeptide III (penton) which are surrounded by six and five identical neighbours respectively. The penton



**Figure 1.1: Schematic representation of an Ad virion** showing its structure and the location of the 11 virion associated polypeptides. Taken from Russell, 2009.

oligomer provides the base for the projecting trimer of polypeptide IV (fibre) whose length varies vastly between serotypes and terminates in a knob which plays a major role in cell receptor usage (Berk, 2007; Russell, 2009). The remaining capsid polypeptides consist of the ‘cement’ proteins: polypeptide IIIa which associates with those hexon proteins which surround the penton base and link adjacent facets of the capsid, polypeptide IX which associates with hexon and stabilises the hexon lattice polypeptide from the outside and polypeptides VI and VIII which bridge the capsid and core (Russell, 2009). The internal space created by the capsid is known as the core and the remaining four structural polypeptides reside here interacting with the genome: the terminal protein which covalently attaches to the ends of the viral DNA, mediates the attachment of the genome to the nuclear matrix and serves as a primer for DNA replication. Polypeptide X or  $\mu$ , the function of which is not fully understood, contacts viral DNA and is proposed to act as a DNA condensing agent (Anderson *et al.*, 1989). Polypeptide V binds to the penton base and bridges the core and capsid and polypeptide VII, the most abundant core protein, forms a histone like coat for the viral DNA to coil around. For the remainder of this thesis herein any mention of these proteins refers to their multimeric form and will be identified by their common names especially in the case of the hexon, penton and fibre proteins.

### **1.1.3: Genome organisation and composition**

Adenovirus genome lengths vary from 26,163 to 45,063 base pairs (bp) (Berk, 2007). All adenoviral genomes which have been described share an identical genome organisation with genes common between all genera located more centrally on the genome whilst genes specific to each particular genus often located closer to the DNA terminus (Davison *et al.*, 2003). The linear genome possesses two identical origins of DNA replication and a packaging sequence in the left end of the viral genome. Figure 1.2 displays the transcription map for Ad5. The genome encodes five early transcription units, (E1A, E1B, E2, E3 and E4), two delayed early transcription units (IX and IVa) and the MLTU whose primary transcript is alternatively processed to give five families of mRNA (L1-L5) which are all transcribed by RNA polymerase II. Finally, either one or two virus associated (VA) RNAs, dependent on serotype, are produced from the VA

Insert Figure 1.2 (2 pages)



transcription unit and are transcribed by RNA polymerase III. Generally, transcription units encode proteins with related functions in accordance with their temporal transcription (Chapter 1.1.4). The E1A/B and E3 proteins i.e. those which are deleted in Ad vectors (Chapter 1.1.7) are described in more detail below (Chapters 1.1.3.1 and 1.1.3.2). The E2 unit encodes the E2A-72kDa single stranded (ss) DNA-binding protein (DBP), the E2B-80kDa precursor terminal protein (pTP) and the E2B-140kDa DNA polymerase (Adpol) proteins which are essential for viral replication (Berk, 2007). The E4 unit encodes seven polypeptides termed Orf1, 2, 3, 4, 6, Orf3/4, and Orf6/7, all of which have been demonstrated to exist within infected cells except in the case of Orf3/4 which has only been predicted to exist from splicing analysis of the Ad mRNA (Virtanen *et al.*, 1984). The seven proteins have varied roles in lytic growth, host cell shutoff, mRNA stability and DNA replication (Leppard, 1997). The L1-L5 units encode the eleven structural polypeptides which make up the Ad virion and four non-structural regulatory proteins, L1-52/55K, L4-33K, L4-22K and L4-100K with various functions (Berk, 2007; Morris and Leppard, 2009).

### **1.1.3.1: The E1 region**

The E1 region is essential for virus replication and consists of two distinct genes, E1A and E1B. E1A produces three mRNAs through alternative splicing, 9S, 12S and 13S. The proteins produced by these mRNAs activate and repress transcription of both cellular and viral genes (Berk, 2007). They lack DNA binding properties and as such achieve this function by the modulation of cellular transcription factors. E1A proteins are principally responsible for the transcriptional activation of all subsequent early genes, activation of the expression of cellular genes which instigate progression of the cell cycle into S phase and inhibit the cellular response to interferon upon infection. Concurrently the E1B gene products, E1B/19K and E1B/55K are involved in the prevention of cell cycle arrest at the G<sub>1</sub>/S phase checkpoint caused by up-regulation of p53 produced by E1A modulation of transcription factors by the binding of E1B/55K to the activation domain of p53 and

the prevention of early apoptosis by E1B/19K acting as a homologue of the cellular anti-apoptotic protein Bcl-2.

### **1.1.3.2: The E3 region**

The E3 region is presumed to be required for Ad replication *in vivo* and can be deleted from the Ad genome without an effect on viral growth *in vitro*. The E3 gene products vary in number and function between species and are therefore assumed to be determinants of adenoviral pathogenicity and tropism. The composition of the E3 region in varying species and serotypes is reviewed in Burgert *et al.*, 2002. Most E3 proteins have been shown to have an immunomodulatory function and consist of transmembrane and intracellular proteins. With the exception of a single secreted protein, all E3 gene products act on the cell in which they were originally produced. The functions of the major E3 proteins described in species D Ads, including Ad19a (Chapter 1.3) are given below.

#### **1.1.3.2.1: E3/19K**

E3/19K is a type I transmembrane protein of between 25 and 35 kDa, represents the archetypical adenovirus immunomodulatory protein and has three known functions. Firstly, it binds to and retains newly synthesised MHC class I molecules in the endoplasmic reticulum (ER) preventing the transit of MHC class I molecules to the cell surface (Burgert & Kvist, 1985; Burgert *et al.*, 1987) where viral peptides would be presented to CD8<sup>+</sup> cytotoxic T-lymphocytes (CTLs), which would cause either perforin and granzyme release resulting in cell lysis (Trapani *et al.*, 2000) or induce Fas mediated apoptosis of the infected cell (Nagata & Golstein, 1995). Secondly, it has been proposed to bind to the antigen processing transporter TAP and prevents it from transferring peptides processed in the cytosol, such as viral peptides for MHC presentation, to the ER (Bennett *et al.*, 1999). Thirdly, it has more recently been shown that E3/19K also prevents major histocompatibility complex class I chain-related proteins A and B (MICA and MICB) from being expressed on the cell surface by intracellular sequestration (McSharry *et al.*, 2008; Sester *et al.*, 2010). MICA and MICB are ligands for the major Natural killer (NK) cell activating receptor NKG2D and their expression triggers activation of the NK cell (Gasser &

Raulet, 2006). The sequestration of MICA and MICB therefore prevents the killing of the infected cell by NK action which would otherwise occur on sensing down-regulation of MHC class I and up-regulation of MICA and MICB due to the expression of E1A (Routes *et al.*, 2005) and other Ad proteins (Tomasec *et al.*, 2005). Thus, E3/19K has a dual function: inhibition of T-cell recognition by the sequestration of MHC class I by ER retrieval and possibly the prevention of peptide transport by interaction with TAP and the prevention of NK cell action by the sequestration of MICA and MICB. E3/19K molecules are expressed by species B-E and may have differential ability to retain MHC I alleles (Deryckere & Burgert, 1996; Windheim *et al.*, 2004)

#### **1.1.3.2.2: E3/49K**

E3/49K was first discovered in Ad19a and was first thought to correlate with the ability to cause EKC but was later found to be expressed by all species D Ads regardless of EKC causing ability (Blusch *et al.*, 2002). E3/49K is an unusually large highly glycosylated 80-100 kDa type I transmembrane protein which is the only Ad protein to be secreted from the cell (Windheim & Burgert, 2002; Windheim *et al.*, unpublished data). The protein possesses unusual immunoglobulin-like folds and is thought to be secreted after proteolytic cleavage at the cell surface (Windheim, 2002). The secreted extracellular form of E3/49K has the ability to bind to NK cells and other lymphocytes (Windheim *et al.*, 2004; Windheim *et al.*, unpublished data). Inhibition of NK cell lysis suggests it has some immunomodulatory effect. It is also possible that it enhances transmission of species D Ads by other means.

#### **1.1.3.2.3: E3/10.4-14.5K**

The E3/10.4K and E3/14.5K proteins form a complex, named the receptor for internalisation for degradation (RID). E3/10.4K is produced as two isoforms, a transmembrane anchored protein of 91 aa (Burgert *et al.*, 2003) and an alternate form lacking an N-terminal signal sequence (Hoffman *et al.*, 1992). These two species of E3/10.4K form a disulphide-linked complex within the membrane and associate with E3/14.5K. E3/14.5K is another type I transmembrane protein which varies in length



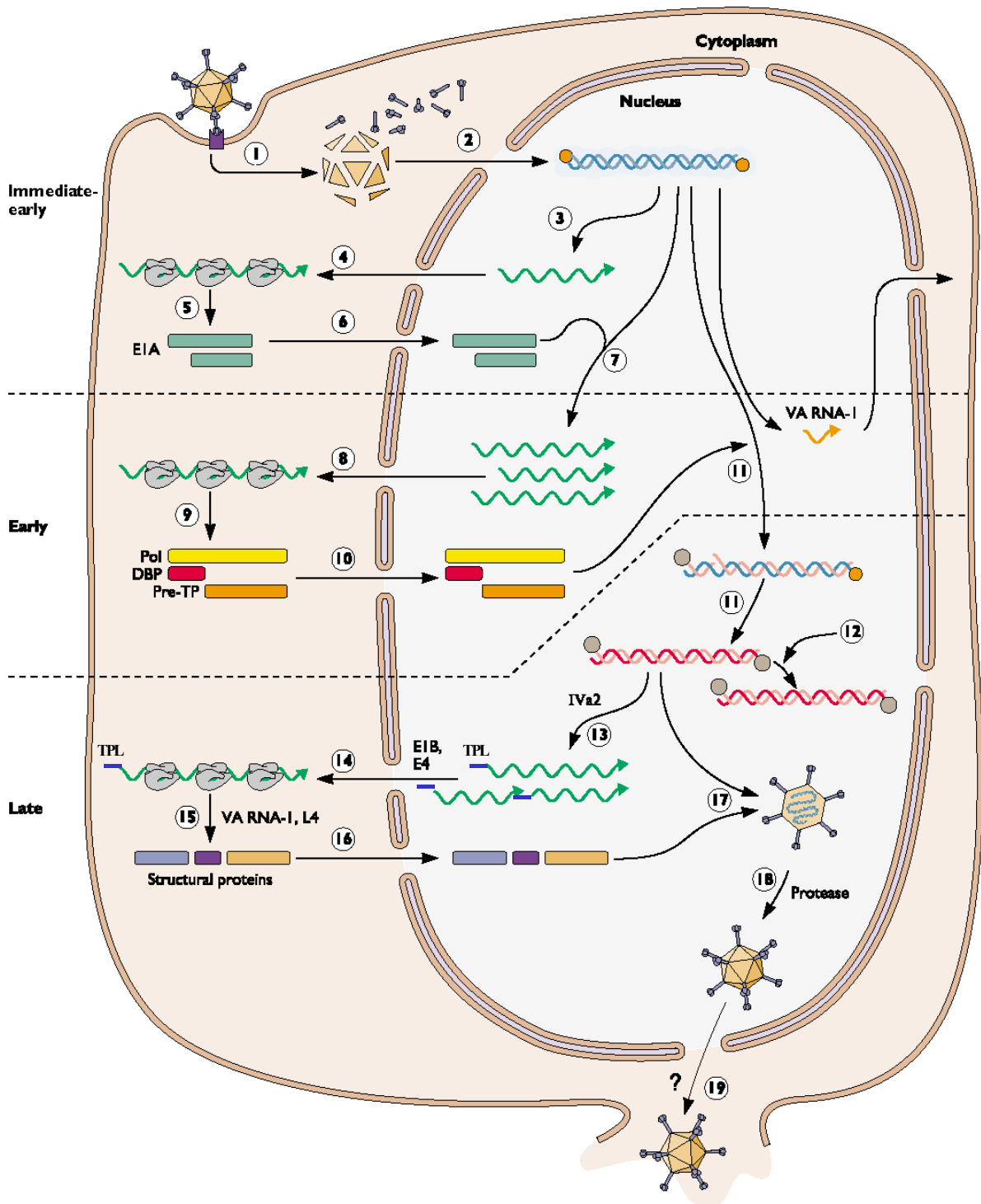
between 107 and 134 aa (Burgert and Blusch, 2000). A potential function of the complex was first suggested when the epidermal growth factor receptor (EGFR) was down-regulated and subsequently degraded during Ad infection (Carlin *et al.* 1989). Subsequent virus deletion mutant studies identified the viral proteins responsible as the RID complex (Tollefson *et al.* 1991; Elsing & Burgert 1998). The biological significance for the down-regulation and degradation of EGFR remains unknown. The RID complex was subsequently shown to also down-regulate the Fas receptor from the cell surface of Ad infected cells and target them for degradation in lysosomes, thus protecting the cells from Fas mediated apoptosis (Tollefson *et al.* 1998; Elsing and Burgert 1998). The RID complex also mediates down-regulation of other death receptors from the cell surface, such as the tumour necrosis factor (TNF) – related apoptosis inducing ligand (TRAIL) receptor 1 (Tollefson *et al.* 2001) and, in combination with the E3/6.7K protein, TRAIL-R2 (Benedict *et al.* 2001). These findings confirmed a possible role for the RID complex in evading apoptosis.

#### **1.1.3.2.4: E3/14.7K**

The E3/14.7K protein ranges in size between species and localises to the cytosol and nucleus (Gooding *et al.*, 1990). It is known to inhibit apoptosis induced by tumour necrosis factor (TNF). A yeast two hybrid screen identified a number of proteins that can bind 14.7K, which were termed 14.7K interacting proteins (FIPs) (Li *et al.*, 1997, 1998, 1999), prevented the internalisation of the tumour necrosis factor receptor (TNFR) from the cell surface (Schneider-Brachert *et al.*, 2006) and the formation of the death inducing signaling complex (DISC) (Schneider-Brachert *et al.*, 2006) but how it performs these functions remains to be confirmed.

#### **1.1.4: Replication and life cycle**

A diagram of the adenovirus replicative life cycle is shown in Figure 1.3. Briefly, the infecting virion binds first to its primary receptor (Chapter 1.1.6) and is endocytosed into the cell. The virion exits the endosome by pH-dependent lysis, the particle is uncoated and transported to the nucleus where the genome associates with the nuclear matrix (Berk, 2007). The Ad genes are transcribed in a temporal fashion and



**Figure 1.3: The adenovirus life cycle.** Adenovirus attachment to the host cell is mediated by the interaction between fibre and each serotype's specific receptor (see Chapter 1.1.6). The virus then enters the cell by endocytosis (1). Once the virion has transferred through the cell membrane it is uncoated and the viral genome, which remains associated with polyprotein VII, is imported into the nucleus (2). The early gene E1A is then transcribed by the host cell RNA polymerase II (RNA PolII) (3). The produced E1A mRNAs are alternatively spliced into their three constituents and exported to the cytoplasm (4), where the E1A proteins are then translated (5). Once translated, these proteins, which are first extensively phosphorylated are imported back into the nucleus (6), where upon they begin their function regulating the transcription of cellular and viral genes and progressing the cell cycle to S phase. The E1A proteins stimulate transcription of the other E1 genes by cellular RNA PolII (7). The remaining E1 mRNA species produced are processed as before and exported to the cytoplasm (8), translated into the remaining E1 proteins (9). These products include the proteins essential for viral replication which are imported into the nucleus (10) and induce viral DNA synthesis (11). The viral DNA molecules produced by replication events serve as further templates for replication (12) or for transcription of the MLTU (13). Efficient transcription of the MLTU requires the interaction of IVa2, which is produced upon viral replication. The late mRNAs are first processed and then selectively exported to the cytoplasm by a complex the E1B and E4 proteins (14). The translation of late mRNAs (15) requires the action of VA RNA I produced earlier to prevent breakdown of the late mRNAs by cellular attack. The structural polypeptides are translated from the late mRNAs and imported to the nucleus (16), where the capsid is assembled and progeny genomes packaged within to form immature virions (17). The structural proteins are matured by the action of the L3 protease to produce mature virions (18). Mature progeny virions are then released from the cell (19) either upon the lytic death of the cell or by some unknown process. Figure adapted and text taken from Flint *et al.*, 2000.

are divided into 'immediate early', 'early' and 'late' which refer to the time after cell infection when the genes involved in each of these phases are maximally expressed.

The 'immediate early' phase involves the transcription of the E1A region immediately after particle disassembly and entry into the nucleus due to its promoter being constitutively active (Berk, 2007). The E1A proteins, once translated, transactivate the 'early' genes. 'Early' phase gene expression begins on both strands of the genome with the E1, VA, major late transcription unit (MLTU) and E3 regions transcribed from the rightward strand and the E4 and E2 regions transcribed from the leftward strand. This organisation of gene clusters helps to control the timing of early gene expression with the E1 region being the first to be transcribed from rightward strand and the E4 region the first from the leftward strand, the proteins of both regions being required for DNA replication to begin (Berk, 2007). The 'late' phase begins upon viral DNA replication and continues with the transcription of the MLTU from either the original or progeny genomes. Replication begins from the origin of replication contained within one of the ITR and continues to the opposite end. Only one of the parental DNA strands serves as a template which results in the creation of a duplex containing one of the parental strands and a new daughter strand. The other parental strand is circularised by the binding of complementary 5' and 3' termini (to form a circular DNA molecule) with a short duplex which has become known as a 'panhandle'. This terminal duplex is identical to the ITR DNA duplex of the normal Ad genome and allows the remaining single strand to be replicated by the same process creating a second parent-daughter heteroduplex (Berk, 2007). The MLTU is under the control of the major late promoter (MLP), which is only weakly active during the 'early' phase of the Ad life cycle to prevent the structural proteins being produced too early. Once DNA replication has taken place, transcription of the MLTU from the MLP increases by an as yet unknown process (Berk, 2007). This allows the expression of the structural proteins and the four non-structural late proteins. The structural proteins are imported to the nucleus where they form the immature capsid which is packaged with a progeny genome, matured by proteolytic cleavage and the mature progeny virion exits the cell (Berk, 2007; Figure 1.3).

The time taken between virion entry and progeny exit from the cell varies dependent on the cell line, serotype and multiplicity of infection (MOI) used. When using an MOI of 10 in A549 cells the ‘immediate early’ and ‘early’ phases take between 5-6 hours after initial infection. The onset of late gene expression follows between 7-12 hours and the infectious cycle is complete in 20-24 hours.

### **1.1.5: Disease association**

Of the 53 Ad serotypes which infect humans only 22 (Ad1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 14, 18, 19a, 21, 31, 34, 35, 37, 40, 41, 52 & 53) have been associated with symptomatic disease (Wold & Horwitz, 2007; Jones *et al.*, 2007; Aoki *et al.*, 2008). In general Ad infections are acute and self-limiting with neutralising antibodies (Abs) quickly generated against the immunodominant hexon and penton proteins (Sumida *et al.*, 2005). Ad infection provides the host with life-long immunity to that serotype (Kojaoghlanian *et al.*, 2003). Disease and the site of infection vary greatly dependent on serotype (Table 1.1). Upper respiratory tract infections have been associated with species B, C and E Ads 1-6 whilst more severe lower respiratory tract infections have been associated specifically with the species B and E Ads 3, 7, 21 and 4 (Madisch *et al.*, 2006; Wold & Horwitz, 2007). Urinary tract infections and associated kidney infections of immunocompromised patients are associated with species B and D Ads 8, 11, 34, 35 and 37 (Swenson *et al.*, 1995; Wold & Horwitz, 2007). Gastrointestinal infections are associated with species A and F Ads 12, 18, 31, 40 and 41 along with the two more recently discovered Ad serotypes Ads 52 and 53. Ads 40 and 41 are considered to be the second most important viral cause of childhood gastrointestinal illness worldwide (Avery *et al.*, 1992; Madisch *et al.*, 2006). Finally eye infections are associated with species D Ads 8, 11, 19a and 37 with 8, 19a and 37 known to cause severe cases of epidemic keratoconjunctivitis (EKC) in densely populated areas and are frequently associated with nosocomial infections in Asia (Jernigan *et al.*, 1993; Aoki & Tagawa, 2002). If an Ad can be detected in the blood, replication was shown to take place from several serotypes in the liver, the urinary bladder, the pancreas, the myocardium, and the central nervous system (Collier *et al.*, 1966; Ginsberg *et al.*, 1991; Heemskerk *et al.*, 2005). Several studies have shown a link between the presence of anti-Ad36 antibodies and obesity

in humans although this remains unconfirmed (Pasarica *et al.*, 2008). Many of the human adenoviruses have been shown to transform rodent cells *in vitro* and some have been shown to be highly oncogenic, however, none have, to date, been associated with any malignancy in humans (Shenk, 1996; Endter & Dobner, 2005).

Ad infections can become persistent in immunocompromised patients, such as transplant recipients on immunosuppressive drugs or human immunodeficiency virus (HIV) infected hosts. Diseases associated with persistent infections of Ads include haemorrhagic cystitis by Ads 11, 34 and 35 (Wadell, 1999), hepatitis from Ad5 (Berthau *et al.*, 1996) and acute pneumonia (Marcos *et al.*, 2009). These infections are thought to be the result of transmission of an Ad from the donor to the recipient or re-activation of a previous infection due to immunosuppression. Re-activation of a previous infection confirms previous findings that Ads are capable of establishing persistent infections in immunocompetent hosts (Fox *et al.*, 1969, 1977) but whether or not they continue to replicate at an undetectable low level or they are truly latent and which cell type *in vivo* is supporting infection remains unknown. Persistence may be facilitated by the action of the immunodulatory E3 proteins (Chapter 1.1.3.2) and may, in fact, represent their primary function (Burgert *et al.*, 2002).

The reason behind the differential infection of human tissues by the Ad species and serotypes is thought to be dependent on various factors including the action of various Ad genes such as E3. One factor is the specificity of each serotype for a cell type which is thought to be dependent on the fibre knob protein and its specificity for a human cell receptor (Arnberg, 2009; Xia *et al.*, 1995).

### **1.1.6: Adenovirus receptors**

Many host molecules have been identified over the past 50 years which have been suggested to serve as primary receptors for the Ads. More recently, long-held theories as to the identities of the receptors for the most commonly used Ad serotypes (Ad2 and Ad5) have been challenged (Walters *et al.*, 1999, 2002). The current candidate human cellular proteins discussed as adenovirus receptors and the Ad serotypes which have been associated with them are described below. Major

difference between the fibre proteins of the different serotypes include their amino acid sequence and the number of fibre shaft repeats, and therefore the length of the fibre protein itself, both having significant effects on the ability of Ads to bind cellular receptors (Table 1.2; Arnberg, 2009).

A summary of the receptors proposed for each of the Ad species is given in Table 1.2.

Species	Serotypes	Proposed True receptors	Other attachment molecules	Fibre shaft repeats
A	12, 18, 31,	CAR, Integrins	Factor IX, Factor X	23
B1	3, 7, 16, 21, 50	CD46, CD80, CD86, Integrins, Receptor 'X'	Factor X	6
B2	11, 14, 34, 35	CD46, CD80, CD86, Integrins, Receptor 'X'	Factor X	6
C	1, 2, 5, 6,	CAR, VCAM-1, MHC, Integrins	Factor IX, Lactoferrin, DPPC, Heparan sulfate	22
D	8, 9, 10, 13, 15, 17, 19a, 20, 22-30, 32, 33, 36, 37-39, 42-49, 51	Sialic Acid, CD46, CAR, Integrins	Factor X	8
E	4	CAR, Integrins		12
F	40, 41	CAR, Integrins		12/22

Dipalmitoylphosphatidylcholine (DPPC), Vascular Cell adhesion molecule 1 (VCAM-1), Major Histocompatibility Complex (MHC). Receptor 'X' refers to an as-of-yet unidentified protein referred to in Tuve *et al.*, 2006

**Table 1.2: The proposed receptors used by human Ad species.** The table lists the species, the serotypes, the proposed receptors and the number of fibre shaft repeats in each fibre protein (Adapted from Arnberg, 2009).

## **CAR**

The coxsackie and adenovirus receptor (CAR) is a 40-46 kDa human protein with homologues in other mammalian species (Tomko *et al.*, 1997). It belongs to the immunoglobulin-like superfamily and can exist as extensively glycosylated forms (Tomko *et al.*, 1997). Its primary function in humans is thought to be as a component of tight junctions in epithelia (Cohen *et al.*, 2001). The protein was first named for the observed competition between the Coxsackie B virus and Ads 2 and 5 for its use *in vitro* (Lonberg-Holm, *et al.*, 1976; Bergelson *et al.*, 1997) and the murine homolog *in vivo* (Bergelson *et al.*, 1998). CAR has been subsequently shown, by sequence analysis and mutagenesis, to function as a receptor for Ads from species A, C, D, E and F *in vitro* (Roelvink *et al.*, 1998) but *in vivo* the use of CAR by several Ads has been contested. For example, there is an accumulating body of evidence that CAR is an efficient receptor for Ads *in vitro* on non-polarised epithelial cells such as A549s but the receptor's polarised expression *in vivo* (Walters *et al.*, 1999) and its lack of expression on some targeted tissue types, such as the liver and lymphocytes (Tomko *et al.*, 1997), suggest that it is not used *in vivo* although these findings can be explained by alternative mechanisms such as Factor X. Further evidence includes the use of a mutant Ad vector, unable to bind CAR, whose biodistribution *in vivo* was unaltered compared to a vector with the wild-type fibre knob (Alemany & Curiel, 2001). Other functions have now been suggested for the Ad/CAR interaction, such as viral escape (Walters *et al.*, 2002), and other human proteins are beginning to emerge as the functional receptors of Ads and these are discussed below.

## **Sialic Acid (SA)**

Sialic acid is a generic term for the N- or O-substituted derivatives of neuraminic acid (NANA), a nine-carbon monosaccharide. In mammalian cells, the most common sialic acids are NANA and *N*-glycolylneuraminic acid, although only the former is present in human cells (Ulloa & Real, 2001). The structural diversity of sialic acids arises not only from the nature of the monosaccharide but also from its



variable linkage to other sugars, which occurs in two main configurations:  $\alpha$ 2-3 and  $\alpha$ 2-6. SA has been shown to be a receptor for other viruses including, amongst others, influenza virus (Springer *et al.*, 1969), which utilises both the  $\alpha$ 2-3 and  $\alpha$ 2-6 form in humans (Palese & Shaw, 2007), and rotavirus, shown to utilise the  $\alpha$ 2-3 form (Delorme *et al.*, 2001). *In vivo*, SA has been shown to have two main functions. Firstly, SA acts as an anti-recognition agent by shielding sites such as other monosaccharides and macromolecules on cell membranes including receptor molecules. In this way SA contributes to cells being identified as 'self' and prevents attack from the immune system (Schauer, 2009). In fact, over expression of SA by tumour cells has been shown to prevent an immune response and increases malignancy (Varki, 2008). Secondly, SA can be a biological recognition site, functioning as ligands for a great variety of molecules such as hormones, lectins, antibodies, and inorganic cations (Varki, 2008).

The fibre-knob domains of the EKC-causing species D Ads 8, 19 and 37 have been shown to have an unusual positive surface charge which may facilitate binding to the negatively charged SA (Arnberg *et al.*, 1997) and the Ad37 fibre knob domain has been crystallised in complex with SA (Burmeister *et al.*, 2004). Subsequently, studies have shown that SA functions as the Ad37 receptor *in vitro* (Arnberg *et al.*, 1997, 2000a, 2000b, 2002; Johansson *et al.*, 2007). This was done either by utilising an enzyme which cleaves SA, neuraminidase, sialic-acid blocking lectins and/or CHO cells deficient in SA (Arnberg *et al.*, 2000). This data has been challenged, however, by other evidence which suggests the Ad37 receptor is CD46 (Wu *et al.*, 2001, 2003, 2004). It has even shown that the Ad37 fibre knob also binds to CAR with high affinity (Seiradake *et al.*, 2006). Neuraminidase treatment has subsequently been shown to affect both Ad8 (Lecollinet *et al.*, 2006) and Ad19a (Thirion *et al.*, 2006) infectivity *in vitro* providing more evidence for SA as a potential receptor for EKC-causing Ads. Ad37 and Ad19a possess identical fibres (Arnberg *et al.*, 1997) and as such it is thought that they may utilise the same receptor, however this has been disputed and will be examined within this thesis (Chapter 7). The receptor for the EKC-causing Ads therefore remains controversial and an investigation into the use of sialic acid *in vitro* by Ad19a will follow in this thesis.

## CD46

The membrane cofactor protein (MCP) or CD46 is a complement regulatory protein (Liszewski *et al.*, 1991) which, unlike CAR, is expressed apically on human epithelial cells *in vivo* and like SA has been shown to be used by other viruses such as the measles virus (Dhiman *et al.*, 2004; Cattaneo, 2004). Its primary function is to protect cells from complement attack by inactivation of complement components C3b and C4b (Arnberg, 2009) and CD46 down-regulation, by infection with an Ad35 vector, has been shown to trigger complement-mediated cell death (Sakurai *et al.*, 2007). There are four major splice variants of CD46 in humans, MCP1, MCP2, BC1 and BC2 (Liszewski *et al.*, 1991), with most investigations of viral receptor usage focusing on the MCP1 variant. CD46 is expressed on all nucleated cells in humans whereas in mice it is restricted to the testis (Sakurai *et al.*, 2008). The first Ads shown to utilise a receptor other than CAR *in vitro*, were from species B (Defer *et al.*, 1990; Roelvink *et al.*, 1998). It was first shown that Ads 11, 14, 16, 21, 35 and 50 (Gaggar *et al.*, 2003; Segerman *et al.*, 2003) but not Ad3 utilise CD46. Further work showed enhanced transduction of a CHO cell line expressing hCD46 and Ab blocking of hCD46 usage on A549 cells in Ads 11, 14, 16, 21, 34, 35 and 50 but not Ads 3 and 7 (Martilla *et al.*, 2005) and this was confirmed by blocking studies. This work has been countered by the finding that Ad3 infects cells expressing a different CD46 splice variant, BC1, when expressed on BHK cells and the Ad3 fibre can be isolated in a pull-down experiment bound to a soluble form of the same variant (Sirena *et al.*, 1994). Subsequently, the Ad7 fibre has also been shown by structural analysis to have affinity for CD46, however, it was shown to be significantly less than the affinity of the Ad11 fibre as a result of a differing orientation of a single arginine in the fibre head (Persson *et al.*, 2009). Further findings, however, suggest that Ad3 and Ad7 may utilise a receptor other than CD46. Ad3 fibres were shown to bind a protein of a significantly higher molecular weight than CD46 (130,000 compared to 50-70,000; Di Guilmi *et al.*, 1995) and the binding of <sup>3</sup>H labelled Ads 3, 7 and 14 was <48% blocked by anti-CD46 Abs in various highly CD46 expressing cell lines and their interaction with 293 cells was shown to be nearly independent of CD46 (Tuve *et al.*, 2006). It has been hypothesised that the high seroprevalance of

Ads 3 and 7 compared to the other species B Ads may also be linked to the utilisation of a receptor other than CD46 (Arnberg, 2009).

A great deal of structural work has now taken place on the species B Ad fibres and their interactions with CD46 and it is now generally accepted that CD46 functions as a primary receptor for the majority of the species (Persson *et al.*, 2008; Pache *et al.*, 2008; Cupelli *et al.*, 2010). It has also been noted that the binding of the Ad11 fibre to hCD46 is the first example of a viral receptor protein altering the conformation of its receptor (Persson *et al.*, 2007). As previously mentioned, CD46 has also become a candidate receptor for the EKC-causing species D Ads 19a and 37 (Wu *et al.*, 2001, 2003, 2004).

### **Heparan Sulfate proteoglycans (HSPG)**

Heparan sulphate (HSPG) is a glycosaminoglycan which is ubiquitously expressed on human cells, is known to have many functions including developmental processes, angiogenesis, blood coagulation and tumour metastasis (Dechecchi *et al.*, 2001) and serves as the cellular receptor for multiple viruses, including RSV (Hallak *et al.*, 2000). HSPG was first shown have a role in the infection of Ads 2 and 5 but not Ad3 when pre-incubation of the viruses with heparin prevented infection of A549 cells (Dechecchi *et al.*, 2001). The addition of Heparin *in vitro* also had an additive inhibitory effect on the transduction of human muscle cells by Ad5 vectors (Thirion *et al.*, 2006). A similar inhibitory effect on the transduction of Ad19a and Ad37 vectors has also been noted, however, the removal of SA was found to affect these vectors more profoundly (Thirion *et al.*, 2006). It was subsequently proposed that this mechanism, in Ad5, relied on a hypothetical motif in the Ad5 fibre shaft, the KKTK motif, when the mutation of the region containing this motif resulted in a significant decrease of Ad5 vector transduction of non-human primate (NHP) organs when administered systemically (Smith *et al.*, 2003). It is not known, however, if this finding was due to the inability of the Ad5 vector containing the KKTK mutation to interact with HSPG or whether it was due to reduced inflexibility of the Ad5 fibre as a result of the mutation (Nicklin *et al.*, 2005). The fibre shafts of Ads outside of species C do not possess the KKTK motif so this mechanism, if

confirmed, is most likely limited to species C Ads. The fibre knobs of Ads 3 and Ad35 have also been shown to have binding affinity for HSPG, however, the use of heparinase to remove HSPG from HeLa, CHO and Y79 cells had little effect on interaction with Ad3 or Ad35 (Tuve *et al.*, 2008). It is widely held that HSPG functions as an important co-receptor for certain Ad species C serotypes.

## **Integrins**

As can be seen in table 1.2 Integrins are listed as a proposed receptors for all Ad species, however, they do not function as a primary receptor, rather they are considered secondary receptors, required for Ad endocytosis to take place through an interaction between the Ad penton base proteins (Belin & Boulanger, 1993; Mathias *et al.*, 1994; reviewed in Nemerow *et al.*, 2009). Integrins refer to a group of 24 distinct proteins made up of  $\alpha$  and  $\beta$  subunits which function in cellular adhesion and cell signalling. A large number of viruses from diverse groups have now been shown to use integrins as secondary receptors and their effect on cell binding is not minor (Stewart & Nemerow, 2007). Ads from all major species except those of species E and F have been shown to have an exposed RGD (Arginine-Glycine-Aspartic acid) motif on their penton base protein and it is this motif which confers specificity for the use of integrins (Mathias *et al.*, 1994). Irrespective of their candidate receptors all Ads except Ads4, 40 and 41 retain the RGD motif (Arnberg, 2009) and this is thought to be one possible reason for their delayed uptake of Ad41 in A549 cells *in vitro* (Albinsson & Kidd, 1999). Upregulation of integrins on human monocytes and T lymphocytes and expression *in trans* on CHO cells has been shown to increase the transduction of an Ad5 vector (Huang *et al.*, 1995) and the use of anti-integrin antibodies prevented the transduction of a similar Ad5 vector in both normal CHO cells and CHO cells expressing hCAR (Salone *et al.*, 2003). Both these findings have led to the conclusion that integrins may be of importance *in vivo* (Arnberg, 2009). It is also thought that Ads may also utilise this interaction for initial attachment on certain cell types which lack their fibre knob primary receptor such as monocytic cells or dendritic cells (DCs) (Huang *et al.*, 1996).

## **Receptor X**

In 2006 a study revealed that the group B Ads, previously thought to use CD46 as their primary receptor could be separated into three groups: those that used CD46 solely (Ads 16, 21, 35 and 50) those that could use both CD46 and an unidentified alternate receptor termed 'X' (Ad 11) and those that solely use this new receptor (Ads 3, 7 and 14)(Tuve *et al.*, 2006, 2008; Wang *et al.*, 2009). Receptor X has been shown to be a glycoprotein more abundantly expressed on human cell types than CD46 but with less Ad binding affinity (Tuve *et al.*, 2006). Its identity, however, remains unknown.

## **Coagulation factors IX and X**

Coagulation factors IX and X are a serine protease and a serine peptidase, respectively, of the blood coagulation system in humans. Secreted by the liver, they act in the thrombin pathway with factor IX activating factor X which cleaves prothrombin to produce thrombin. Both have a half-life of ~ 40-45 hours in the human blood stream. In 2005 it was first shown that they may have a role in Ad tropism by co-precipitation and mass spectrometry which showed that the Ad5 fibre knob bound factor IX and formed a 'bridge' to either low-density lipoprotein receptor-related protein or heparan sulfate proteoglycans (HSPGs; Shayakhmetov *et al.*, 2005). In a subsequent study, factor X was also found to promote the transduction of hepatocytes of both Ad5 vectors which could utilise CAR and those in which the CAR binding site was ablated (Parker *et al.*, 2006). Factor X was also shown to be important for Ad5 liver transduction *in vivo* when, in MF1 mice, warfarin treatment, which leads to the production of an inactive factor X, was shown to prevent liver transduction (Waddington *et al.*, 2007). It has also shown that Ad 5 vectors pseudotyped with fibres from either species C and D showed tropism for Factor X regardless of the receptor specificity of the fibre (Parker *et al.*, 2007). The binding of these vectors to Factor X was subsequently found to be conferred by the binding of the Gla domain of FX to the central depression of the Ad5 hexon protein in a calcium dependent manner (Waddington *et al.*, 2008). Further experiments have shown that that other blood factors, including factor IX, bind to Ad5 hexon much

more weakly and do not enhance the transduction of hepatocytes as efficiently suggesting FX is the most important blood factor in Ad5 infection (Waddington *et al.*, 2008).

It has since been shown that the level of Factor X binding by the hexon protein of other Ad serotypes varies greatly. The hexon proteins of species B Ads 16 and 50 have been shown to have an even higher binding affinity for FX than Ad5 but others, such as the species D Ads 48 and 26 have no FX binding affinity (Waddington *et al.*, 2008). Surface Plasmon resonance (SPR) experiments have shown that an Ad35 vector or an Ad35 vector pseudotyped with the Ad5 fibre bound FX with tenfold lower affinity and in CD46 transgenic mice *in vivo* the addition of a FX binding protein upon systemic administration reduced liver accumulation for Ad5 based vectors (Grieg *et al.*, 2009).

These findings may help explain why a 18 year old male gene therapy patient died after treatment with an Ad5 vector due to liver complications (Raper *et al.*, 2003). Some studies have now recommended an investigation into the tropism of wt serotypes of Ads, i.e. not only fibre pseudotyped Ads, with various blood factors to help prevent problems *in vivo* downstream (Baker *et al.*, 2007; Arnberg, 2009).

### **Dipalmitoylphosphatidylcholine (DPPC)**

DPPC is a phospholipid which comprises 40-50% of lung surfactant and is essential for normal lung function (Arnberg, 2009). It is secreted by epithelial cells and its addition *in vitro* promotes gene delivery by an Ad5 vector in A549 cells (Balakireva *et al.*, 2003). DPPC binds the Ad5 hexon protein and may suggest a role in infection of the lungs by species C Ads (Balakireva *et al.*, 2003). It is now thought that DPPC may also play a role in respiratory infections caused by Ads outside of species C (Arnberg, 2009).

## **VCAM-1**

Vascular cell adhesion molecule 1 (VCAM-1) is an endothelial adhesion receptor which belongs to the immunoglobulin superfamily. Its main function is as an endothelial receptor for leukocytes (Elices *et al.*, 1990). VCAM-1 and CAR have significant homology and VCAM-1 expression on 3T3 cells increased Ad5 vector transduction (Chu *et al.*, 2001). VCAM-1, unlike CAR, is expressed apically on endothelial cells (Arnberg, 2009) and this may provide Ad5, and other CAR utilising Ads entry to these cells *in vivo*, however, this has yet to be investigated.

## **MHC-I**

The MHC-I complex consists of three domains  $\alpha 1$ , 2 and 3, which are exposed to the extracellular space, and are expressed on all nucleated cells. The most important function of MHC-I is the presentation of intracellular antigenic peptides to CTLs, the antigenic peptide being bound in a cleft between the  $\alpha 1$  and  $\alpha 2$  regions. When MHC-I expression was restored in Daudi cells, which otherwise lack cell surface MHC-I due to the loss of the  $\beta 2$  microglobulin gene, the cells became more permissive to Ad5 vector transduction and an MHC-I  $\alpha 2$  synthetic icosapeptide prevented Ad5 transduction of HeLa cells suggesting a role as a receptor target (Hong *et al.*, 1997). However, expression of MHC-I  $\alpha 2$  was subsequently shown not to enhance Ad5 vector transduction of CHO cells (Davison *et al.*, 1999; McDonald *et al.*, 1999). Research on the use of MHC as a receptor has not advanced since.

## **Lactoferrin (Lf)**

Lf is a globular glycoprotein of the transferrin family with a mass of ~80 kD which is found in various secretory fluids, such as milk, saliva, tears, and nasal secretions. It has multiple functions, acting simultaneously as an anti-bacterial, anti-fungal and anti-viral component by a number of different mechanisms including substrate sequestration to prevent bacterial growth, direct bacterial and fungal cell lysis by the generation of peroxides and the diversion of viral binding by competition for

lipoproteins. Lf has also been shown to bind some viruses, such as the hepatitis viruses (Nozaki *et al.*, 2003), directly to prevent virus-receptor interactions.

It was first found that tear fluid promoted wt Ad5 infection *in vitro* and subsequently shown that Lf was responsible for this activity (Johansson *et al.*, 2007). Lf was then shown to increase <sup>3</sup>H Ad5 particle binding to A549 cells alone in a dose-dependent manner and to increase transduction of an Ad5 vector (Johansson *et al.*, 2007). Similar to FX and DPPC, Lf is thought to act as a co-receptor, 'bridging' virus particles and host cells (Arnberg, 2009). All Ads of species C have shown Lf binding activity *in vitro* while representatives of other serotypes: species A (Ad31), B (Ad7 and Ad11), D (Ad37), E (Ad4), or F (Ad41) have not (Johansson *et al.*, 2007). Lf is also thought to promote Ad5 infection of T-lymphocytes in the tonsils and adenoids as evidenced by the high levels of Ad5 genomes in CAR-negative T cells (Garnett *et al.*, 2002). It has also been shown that DCs, which lack CAR, can be transduced by an Ad5 vector in the presence of lactoferrin presumably involving DC-SIGN (Adams *et al.*, 2009).

### **CD80/CD86**

CD80 and CD86, also known as B7.1 and B7.2, are proteins expressed on activated B cells, DCs and monocytes which bind CD28 and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) to provide the costimulatory signal necessary for T cell activation and survival. CD80 and CD86 were first proposed as receptors for the species B Ad3 due to the ability of the recombinant knob domain of the Ad3 fiber to specifically bind to CHO cells which heterologously express CD80 (CHO-CD80) or CD86 (CHO-CD86; Vasu *et al.*, 2003; Short *et al.*, 2004). Subsequently, Ad5 vectors pseudotyped with the Ad3 fibre transduced CHO-CD80 or CHO-CD86 whilst not transducing parental CHO cells (Short *et al.*, 2004). Ads 3, 7, 11, 14, 35, and 50 of both species B1 and B2 have since been shown to utilise CD80 and CD86 by enhanced infection and hexon accumulation in CHO-CD80 and CHO-CD86 cells (Short *et al.*, 2006).



The previous findings for Ad3, however, have been challenged recently in a separate study which showed that an rAd3GFP vector was unable to utilise CD80 or CD86 for HeLa cell entry (Hall *et al.*, 2009).

## **GD1a**

GD1a is a widely distributed ganglioside involved in the development, function and maintenance of the nervous system (Goodfellow *et al.*, 2005). GD1a's, however, are widely distributed on multiple human cell types. It has recently been suggested that GD1a might function as a receptor for the species D Ad37 (Arnberg, personal communication)

### **1.1.7: Adenovirus vectors**

Advances in molecular virology have allowed the manipulation of viruses to suit medical treatments such as gene therapy or vaccination (reviewed in Russell, 2009; Imperiale & Kochanek, 2004). Ads are considered good candidates for such uses due to their natural ability to infect human cells and the relatively simple manipulation of their large DNA genomes. To prevent them from causing their associated diseases during their use in humans (Chapter 1.1.5), their genomes were generally modified to prevent normal replication and pathogenesis from occurring.

The first generation of Ad vectors had their E1 region (Chapter 1.1.3.1; E1A and E1B inclusive) deleted by homologous recombination to create a vector that was theoretically replication incompetent in normal somatic cells (Haj-Ahmad & Graham, 1986). A beneficial by-product of this deletion was the increase of space for transgenic sequences which was later increased by the additional deletion of the E3 region whose products are non-essential for virus replication (Chapter 1.1.3.2; Jones & Shenk, 1978; Saito *et al.*, 1985; Xiang *et al.*, 1986). These vectors are grown in cell lines which express the E1 region in *trans* such as 293 cells or Per.C6 cells which were created by the transformation of human embryonic kidney cells with Ad5 (Graham *et al.*, 1977). With the deletion of the E1 and E3 region and dependent

on the serotype Ad vectors can accommodate an additional 6.5kb of foreign sequence.

To counteract some of the problems associated with E1 deleted vectors such as the increased possibility of an immune response after Ad protein expression and to further increase transgene capacity, research began to focus on a second generation of Ad vectors which possessed further deletions. Research first focussed on the E2 region. Deleting Adp01 and pTP resulted in reduced replication in non-complementing cell lines and a decrease in immune response in mice (Ding *et al.*, 2001). However, these deletions gave poor growth yields in complementing cell lines and it is possible that deletion of E2A in particular triggers enhanced expression of other viral genes, such as E4 (Rice and Klessig, 1985; Zhou *et al.*, 1996). Another candidate for deletion is the E4 region which would increase the packaging capacity to 10-11kb however new complementing cell lines would be required which are difficult to manufacture due to the cytotoxicity of E4 proteins when produced in *trans* and fail to completely complement Ad replication (Brough *et al.*, 1996). It would also appear that the deletion of E4 sequences can affect the level of transgene expression (Brough *et al.*, 1997; Lusky *et al.*, 1998). There is some evidence that E4 deletions may become necessary for *in vivo* human usage as E4-Orf1 has been shown to have transforming activity (Javier, 1994) and E4-Orf 6 blocks p53 activation (Dobner *et al.*, 1996). A further target for second generation vector deletion was polypeptide IX which allowed the growth of the vector in complementing cell lines and further increased transgene capacity (Caravokyri & Leppard, 1995).

A third generation of Ad vectors has been developed which are deleted of either multiple regions, such as E1, E2A, E3 and E4 (Gorzigilia *et al.*, 1999; Andrews *et al.*, 2001) or deleted entirely so that only the *cis*-elements required for DNA replication and the packaging signal are present, thus liberating the remainder of the genome to harbour transgenic sequences (Mitani *et al.*, 1995; Steinwaerder *et al.*, 1999). They have been developed to address the immunogenicity of first and second generation vectors by preventing expression of any Ad proteins thus making them less immunogenic and safer *in vivo*. Third generation vectors cannot be propagated in complementing cell lines, as currently no such cells exist to provide all the

necessary viral proteins *in trans* and their development is unlikely due to the level of complexity of Ad gene expression and regulation. They must therefore be grown in the presence of a packaging-incompetent, replication-defective helper virus, which expresses its viral genes, if grown in E1-complementing cells, in order to generate Ad virions. The helper virus is then removed from the vector stock by purification using the difference in virion density, although this is still relatively inefficient.

One danger in the growth of Ad vectors in 293 cells is homologous recombination allowing the vector to regain E1A functionality and become replication competent. A more recently developed cell line, PER.C6, attempts to prevent this by placing the Ad5 E1A and E1B sequences under the control of the human phosphoglycerate kinase promoter (Fallaux *et al.*, 1998). Lack of overlap with sequences outside the E1 transcription units in this cell line diminishes homologous recombination and thus the generation of a replication competent vector (Tatsis & Ertl, 2004). Other human adenoviral vectors, such as those based on Ad35, are not complemented by the E1 of Ad5 virus and thus necessitate modifications of available packaging cell lines, such as insertion of the Ad35 E1B gene into cell lines that carry the E1 of Ad5 virus (Vogels *et al.*, 2003). Furthermore, in the case of Ad35, the deletion of E1B is beneficial with only the E1B/55K protein being required for growth *in vitro* (Vogels *et al.*, 2003). The construction of other serotypes of adenoviral vectors, requires the endogenous E4 ORF6 (which binds to E1B) to be replaced by that of Ad5, for efficient replication in all cells (Tatsis & Ertl, 2004).

Regardless of which form of vector is used for vaccination or gene therapy, further research is required, especially in the use of Ads other than Ad5, to elucidate the growth characteristics of any created vector before it can safely advance to clinical trials.

## **1.2: Adenoviruses as vaccine vectors**

Adenovirus vectors were first developed for their use in gene therapy (for an example see Crystal *et al.*, 1994) but they are subject to immune responses directed against the capsid, genome, viral proteins and incorporated transgenes, and these can severely limit the efficacy of *in vivo* gene therapy (reviewed in Tatsis & Ertl, 2004;

Lasaro & Ertl, 2009; Nayak & Herzog, 2010). The same immune responses which were generated against gene therapy vectors maybe advantageous when used as vectors for vaccination where transgene expression may not need to be permanent and the vector can help to recruit immune cells to the target tissue and act as an adjuvant.

### **1.2.1: Ad5**

The first Ad vaccine vectors were generated based on Ad5; the most widely used and best understood Ad serotype. Very quickly, large numbers of transgenic antigens were inserted into Ad5 vectors and immune responses detected (Tatsis & Ertl, 2004; in Table 1.3 a non-comprehensive list is presented). Whilst highly promising results were gained demonstrating protection against various viral pathogens in pre-clinical non-human models (Shiver *et al.*, 2002; Sullivan *et al.*, 2000; Tang *et al.*, 2002; Schindler *et al.*, 1994; Casimiro *et al.*, 2003) it was soon discovered that Ad5 had severe limitations for use as a vaccine vector in humans.

#### **1.2.1.1: The problems with Ad5**

The major limitation of Ad5 vectors discovered was that the majority of the human population have pre-existing neutralising antibodies (NAbs) to Ad5 as a result of natural exposure over their life-time (Vogels *et al.*, 2007). This pre-existing immunity has been shown to substantially reduce the immunogenicity of rAd5 vaccines in several animal settings (Barouch *et al.*, 2003; Yang *et al.*, 2003; Casimiro *et al.*, 2003) and in humans (Barouch *et al.*, 2004). The immunogenicity could be recovered in part by increasing the dose given but toxic effects are induced. The full consequences of pre-existing immunity were not realised until the failure of a phase III clinical trial using an Ad5 vector in 2008. Three thousand uninfected volunteers were given a mixture of Ad5 based vaccines expressing either the gag, pol and nef genes of HIV1 and monitored for the vaccine's ability to reduce infection or viral load if HIV was acquired. Vaccination was successful in that HIV-specific CD8+ T cells were induced, however, it failed to protect Ad5 seronegative individuals from HIV infection (Buchbinder *et al.*, 2008) and more startlingly appeared to increase the rate

Pathogen	Transgene	Animal Model	Genes Deleted	Immune response
HIV/SIV	Gag, Pol, Nef Env	Rodents, NHP, human	E1, E3	CMI & Ab
Rabies	Glycoprotein	Rodents, dog	E1, E3	CMI & Ab
Dengue	Envelope	Rodents	E1, E3	CMI & Ab
Ebola	Glycoprotein, nucleoprotein	Rodents, NHP	E1, E3	CMI & Ab
SARS	Spike, nucleocapsid	Rodents, NHP	E1	CMI & Ab
HPV	Various	Rodents, NHP	E1	CMI & Ab
HCV	Various	Rodents	E1, E3	CMI & Ab
HBV	Surface antigen	Rodents, NHP, dog		Ab
Rotavirus	VP7	Rodents	E1, E3	Ab
Measles	Nucleocapsid, hemagglutinin, fusion protein	Rodents	E1, E3	CMI & Ab
RSV	Glycoprotein	Rodents, NHP, dog	E3	Ab
CMV	Glycoprotein B	Rodents	E1	Ab
HSV-2	Glycoprotein B	Rodents	E1, E3	CMI & Ab
EBV	Envelope Glycoprotein	Rodents	E1	CMI & Ab

Abbreviations: HIV, Human Immunodeficiency Virus; SIV, Simian Immunodeficiency Virus; HPV, Human papilloma virus; HCV, Hepatitis C Virus; HBV, Hepatitis B Virus; RSV, Respiratory syncytial virus; CMV, Cytomegalovirus; HSV-2, Herpes simplex 2 virus; EBV, Epstein-Barr Virus; NHP, Non-human primate; CMI, Cell mediated Immunity.

**Table 1.3: Vaccine vectors created using Ad5** showing the pathogen from which the transgene was isolated, the nature of the transgene takes, the animal models they

have been used in and the immune response generated. Adapted from Tatsis & Ertl, 2004.

of HIV infection in trial participants with high anti-Ad5 NAb titres (McElrath *et al.*, 2008). It was first thought that this response had been caused by the activation of a subset of Ad5 specific T cells which became susceptible to HIV infection, although this hypothesis has been challenged (O'Brien *et al.*, 2009).

Ad5 vectors have also been shown to have a strong tropism for the liver mediated by their interaction with FX (Chapter 1.1.6; Waddington *et al.*, 2008) which can have disastrous consequences as was seen in a phase 1 clinical trial when a patient with ornithine transcarbamylase deficiency was given  $3.8 \times 10^{13}$  particles of an Ad5 in the intra-hepatic artery. A large innate immune response was generated which, combined with the underlying liver problems associated with his condition, led to his death (Raper *et al.*, 2002).

Several strategies have been developed to allow Ad5 to circumvent pre-existing immunity. Initially it was thought that replacement of the Ad5 fibre with that of another serotype would suffice and there are multiple studies wherein the Ad5 vector has been pseudotyped with the fibre of another human Ad serotype (for examples see Cashman *et al.*, 2004; Denby *et al.*, 2004; Waddington *et al.*, 2007. Granio *et al.*, 2010) which have seen some success in altering the tropism of Ad5 vectors but liver toxicity and the vector targeting immune response was not significantly reduced.

As the majority of the anti-Ad5 immune response is directed against the hexon protein the goal is to eliminate the ability of the immune system to either recognise or access the capsid proteins. Genomic modifications have included the replacement of either the entire hexon gene or section of it with hexon proteins from other serotypes (Gall *et al.*, 1998; Youil *et al.*, 2002) but early results were not promising due to the use of other serotypes with high pre-existing immunity, such as Ad2. In contrast it was shown that by replacing the seven hypervariable regions (HVR) of the Ad5 hexon with those of a rare serotype, Ad48 allowed Ad5 to evade pre-existing immunity in the form of neutralising Abs in non-human primates and produce high levels of anti-transgene antibodies whilst a wt Ad5 vector could not (Roberts *et al.*, 2006).

Chemical methodologies have also seen early successes, such as; coating Ad5 particles with polyethylene glycol (PEG; reviewed in Kreppel & Kochanek, 2008) which reduced the level of anti-Ad5 immune response but did not affect liver transduction (Croyle *et al.*, 2005), encapsulating the particles in microspheres which also reduced the NAb response (Sailaja *et al.*, 2002) as did the formulation of Ad particles with anionic liposomes (Zhong *et al.*, 2010) but neither of the latter studies examined liver transduction.

A strategy is also under development for the prevention of Factor X mediated liver transduction which can be blocked by as little as a single mutation in HVR7 of the Ad5 hexon protein (Alba *et al.*, 2009).

Despite the partial success of these strategies, it has become generally accepted that research on other serotypes, particularly those with low seroprevalance in the population is vital to prevent problems from arising when Ad vectors are used in clinical settings. It is also important to develop vectors from other serotypes for use in heterologous prime-boost regimens as multiple infections of the same vector trigger strong anti-vector responses.

### **1.2.2: The use of other Ad serotypes**

The use of serotypes other than Ad5 was systematically approached by the examination of seroprevalance to each serotype in the adult human population. Examination of 100 serum samples from the Belgian population for Abs against 51 Ad serotypes showed that less than 20% of the population tested had pre-existing Abs against 27 of the human Ads (Vogels *et al.*, 2003). These findings suggested that using those Ads with significantly lower pre-existing immunity compared to Ad5, found to be as high as 80%, as the basis for vaccine vectors may prove useful. To date, there have been reports of vectors based on human Ad serotypes 2, 3, 4, 5, 6, 7, 11, 19, 24, 26, 34, 35, 36, 41, 48, 49, and 50 (reviewed in Stone & Lieber, 2006) and of those 9 have been examined and shown promising results for their ability to circumvent pre-existing Ad5 NAb in animal models, those being: Ad2 (Morrall *et al.*, 1999), Ad11 (Holterman *et al.*, 2004), Ad26 (Abbink *et al.*, 2007), Ad35 (Vogels

*et al.*, 2003), Ad41 (Lemiale *et al.*, 2007) Ad48 (Abbink *et al.*, 2007), Ad49 (Lemckert *et al.*, 2006) and Ad50 (Abbink *et al.*, 2007). However, those tested have so far proven to be less immunogenic than the homologous Ad5 vector in animals where no pre-existing immunity to Ad5 is present (Barouch *et al.*, 2004; Lemckert *et al.*, 2006; Lemckert *et al.*, 2005) and no studies have been carried out investigating the use of any of these vectors in animal models with pre-existing immunity to the tested serotype.

By far the largest body of work has been carried out on the species B Ad35 which has been shown to have very low seropositivity (Vogels *et al.*, 2003), evade pre-existing anti-Ad5 immunity in humans (Brouwer *et al.*, 2007) and pre-dosing of animals with Ad35 did not affect the subsequent use of an Ad5 vector (Shashkova *et al.*, 2009). Its usage of CD46 as a receptor has been shown to improve the transduction of dendritic cells (Chapter 1.5) and their subsequent activation compared to Ad5 vectors (Lore *et al.*, 2007) and produce almost no transduction of Non-Human Primate organs (Sakurai *et al.*, 2008; 2009) or the liver (Seshidhar Reddy *et al.*, 2003; Grieg *et al.*, 2009). Ad35 vectors have been used in protection studies using malaria (Ophorst *et al.*, 2006; Rodriguez *et al.*, 2009) and TB antigens (Radosević *et al.*, 2007) in all cases providing an equal or better level of protection as the homologous Ad5 vector and performing even better in heterologous prime/boost studies when the vectors were mixed.

Whilst there are promising features, some concerns in the use of Ads other than Ad5 including those which use CD46 as a receptor remain. In CD46 transgenic mice (a better model for humans) several serotypes induced high levels of toxicity at a  $10^{11}$  particles with death rates of 25% for Ad11, 75% for Ad3 and 100% for Ad4 (Stone *et al.*, 2007; Verhaagh *et al.*, 2006). Toxicity at a  $10^{11}$  dose and sequestration in the spleen or lungs has not been observed for an Ad5 vector and as such the application of Ad35 in humans in current clinical trials (Stone *et al.*, 2007), without further understanding of the nature of this toxicity, must be approached with caution.

CD4+ T cells against one human serotype have been shown to cross-react with other serotypes (Smith *et al.*, 1998; Heemskerk *et al.*, 2003) whilst CD4+ and CD8+ T cell responses were even shown to cross-react with Chimpanzee Ads 6 and 7 (Hutnick *et*



*al.*, 2010). However, one possible method for circumventing pre-existing immunity in humans completely is the creation of a vector from serotypes which infect different species.

### **1.2.3: Non-human Ad usage**

Ad vectors have, to date, been developed from bovine (Reddy *et al.*, 1999), ovine (Hoffmann *et al.*, 1999), porcine (Tuboly & Nagy, 2001), canine (Perreau & Kremer, 2006) and several simian serotypes (Farina *et al.*, 2001; Fitzgerald *et al.*, 2003; Reyes-Sandoval *et al.*, 2004; Roy *et al.*, 2006; Santra *et al.*, 2009; Reyes-Sandoval *et al.*, 2010). Research, for the majority of these serotypes, remains in the early stages or is aimed more at vectors for use in the species they were isolated from rather than humans but some have been investigated in more detail.

Canine Ad (CAAdV) serotype 2 was not neutralised by 98% of human sera, better than any human Ad and has potential as a gene therapy vector due to long term transgene expression in rats but has been shown to be incapable of transducing DCs and as such is unlikely to be applicable for vaccination in humans (Perreau & Kremer, 2006).

The largest body of research on a simian Ad has been performed with Ads isolated from Chimpanzees, CAd5, 6, 7 & 68. These Ads have been shown to be closely related to the species E Ads of humans; their level of seropositivity in the chimpanzee population is similar to Ad5 in humans (Cohen *et al.*, 2002) and CAd68 gains entry to cells by the use of CAR. They have been tested for transgene expression (Farina *et al.*, 2001), stimulation of anti-transgene antibody response (Xiang *et al.*, 2002) and stimulation of CD8<sup>+</sup> T cells (Fitzgerald *et al.*, 2003) and have been found to perform similarly to Ad5, however, they express 15 fold less transgene product in both human and mouse DCs and upon highly-efficient DC transduction induce the production of IFN- $\alpha$  and interleukin 6 (IL-6), something not observed with human Ads (Varnavski *et al.*, 2003). It is likely though that the use of Ads from other species may encounter regulatory problems when they enter clinical trials without a greater amount of research focussed on the effects of any simian-specific gene products they might produce on the human immune system.

## **1.3 Ad19a**

### **1.3.1: Isolation, identification and characterisation**

An Ad was first isolated in 1955 in a Saudi Arabian child with trachoma (Bell *et al.*, 1959, 1960), originally identified as the 19<sup>th</sup> human Ad discovered that did not cross-react with available antisera and thus was named Ad19. In subsequent years it became apparent that whilst the first isolation was made in a patient with an eye condition this original isolate did not cause eye disease (Robinson *et al.*, 2009). Prior to 1974, the major causative agent of EKC was thought to be Ad8 (Jawetz *et al.*, 1959; Dawson *et al.*, 1960) From 1974, Ad19-like Ads were isolated frequently in outbreaks of EKC (Desmyter *et al.*, 1974; Hierholzer *et al.*, 1974; Wadell & De Jong, 1980). These turned out to be a different genotype named Ad19a. The original isolate was subsequently termed the Ad19 proto-type virus (Ad19p). Ad19a was eventually placed in species D based on its haemagglutination profile.

In subsequent years it has been shown that a considerable number of cases of EKC, assumed to be caused by Ad19a based on serology, when analysed by haemagglutination inhibition, were actually caused by another species D Ad, Ad37 (Meng *et al.*, 1986). With Ad37 it was surprisingly found that the E3, E4 and fibre regions were 100% identical to Ad19a whereas the hexon and penton were very similar to Ad19p (Blusch *et al.*, 2002). Subsequent sequencing of the entire genome of Ad19a (Burgert & Ruzsics, patent; Robinson *et al.*, 2009) confirmed this view. These findings confirmed that Ad19a and Ad37 were closely related and it has been hypothesised that Ad19a may represent a virus which has undergone a prior recombination event, possibly between an Ad19p-like virus and Ad37 (Blusch *et al.*, 2002).

### **1.3.2: Disease Association**

As previously stated, Ad19a has been shown to be a major etiological agent responsible for EKC a highly-infectious condition which can cause severe visual sequelae (Butt & Chodosh, 2006) and the majority of the available literature on

Ad19a examines its isolation in clinical situations worldwide in patients suffering from EKC. The association with EKC prompted increasing interest to isolate the feature of Ad19a, Ad8 and Ad37 responsible. Principally it could be the result of a specific targeting of the EKC causing Ads to the eye, although other Ads cause eye infections, or a specific subset of immunological events that lead to this special disease state. A novel E3 protein, E3/49K (Burgert & Blusch 2002), which presents selectively in species D Ads might be a possible determinant. Interestingly, in 2003 an EKC isolate was discovered which possessed an unidentified restriction pattern. Further research revealed the majority of the hexon protein of this virus to be identical to Ad37 except for two loops which were identical to Ad22, which does not cause EKC, whilst the fibre knob was identical to Ad8 and the penton base identical to Ad37 (Aoki *et al.*, 2008). It has now been proposed that this isolate in particular, having been re-isolated in 16.1% of recent EKC cases in Japan, could represent a new Ad, Ad54 (Ishiko & Aoki, 2009). The characterisation of such intermediate Ad strains (Noda *et al.*, 1991; Engelmann *et al.*, 2006) may allow a better determination of the structural or genetic requirements for an EKC causing Ad and help to elucidate the reason for the disease association.

### **1.3.3: Vector development**

Ad19a became interesting for vector development when it was shown in a screen, along with other Ad serotypes, to have a high efficiency to infect DCs, an important target for vaccination. FACS staining for hexon revealed that >70% of DCs were infected by Ad19a whilst Ad2 only infected ~10% (Ruzsics *et al.*, 2006). This finding indicated Ad19a's ability to infect cells independently of CAR and, together with other data (T cells and B cells) suggested an interesting tropism for cells of the immune system and for these reasons was developed as a vector. Standard cloning methods (utilising either high or low copy plasmids) did not allow cloning of the genome. Only when the genome was inserted in a BAC vector could clones be isolated. Thus, an entirely new technology for genetic manipulation was required. The E3 region was deleted from the Ad19a genome and the E1 region replaced with an expression cassette consisting of green fluorescent protein (GFP) under the control of the CMV immediate-early promoter and the SV40 enhancer to create rAd19a $\Delta$ E1 $\Delta$ E3GFP (Ruzsics *et al.*, 2006). The vector was found to be stable in 293

cells and had a transduction pattern which contrasted with the homologous Ad5 vector used, efficiently transducing all the lymphoid cell lines tested (Thirion *et al.*, 2006).

Transgene expression and real-time PCR monitoring showed that rAd19aGFP transduced primary human myoblasts more efficiently than rAd5GFP but that this observation was not seen in the myoblasts of apes, pigs, mice and rats (Thirion *et al.*, 2006). Similar results were obtained in myotubes and further experiments suggested that Ad19a transduction of muscle cells was dependent on SA (Thirion *et al.*, 2006).

It was therefore concluded from the results of both studies that Ad19a represented a good candidate for use as both a vaccine vector, based on its infection of DCs, and a gene therapy vector for the treatment of human muscle disorders and that the vector warranted further investigation.

#### **1.4: Vaccination**

In 1972, Ads were found to be potent inducers of ‘interferon’ (Ustacelebi & Williams, 1972) and since then considerable research has been committed to identifying the makeup of the intrinsic responses to Ad infection. The field has grown substantially since genetic manipulation of Ads has allowed the creation of vectors for eventual use in humans with the intention of improving the safety and efficacy of these vectors in clinical applications.

As previously stated, Ad vectors are attractive candidates for vaccination due to their well characterised and easily manipulatable genome, broad tropism, their growth to high titres in tissue culture systems and their relative stability (Shiver & Emini, 2004; Tatsis & Ertl, 2004). Perhaps more importantly, they are also well suited for vaccination due to the immune responses they generate upon systemic inoculation *in vivo* (Tatsis & Ertl, 2004). Ad vectors are highly immunogenic, potently activating the innate immune response, initiating the production of cytokines and recruiting important antigen-presenting cells (APCs) to the site of infection (Yamaguchi *et al.*, 2007; Appledorn *et al.*, 2008) and subsequently activating the adaptive immune response, instigating the creation of CD8<sup>+</sup> T cells to both its own structural proteins

and heterologously expressed transgenes (Hensley *et al.*, 2005; Appledorn *et al.*, 2008).

#### **1.4.1: Immune responses to Ad vectors**

Due to their ability to stimulate strong innate and adaptive immune responses, Ads seem to act as adjuvants that facilitate immune responses against encoded transgenes. Table 1.3 shows the vaccine vectors generated based on Ad5 and as can be seen the majority have elicited antibody and cell mediated responses against their encoded insert. In pre-clinical studies, Ad vectors have been shown to generate transgene-specific T and B cell responses (Xiang *et al.*, 1996; He *et al.*, 2000). The generated T cells have been shown to consist mainly of CD8<sup>+</sup> T cells, although CD4<sup>+</sup> T cell responses have also been detected (Fitzgerald *et al.*, 2003). The full mechanism of how they generate these responses is still not fully understood (Tatsis & Ertl, 2004). It is likely that the responses to an expressed transgene are potentiated by the immune response to the vector itself and the transduction of subsequent APCs facilitates antigen presentation and the induction of an adaptive immune response.

A large majority of the studies on the immune response to Ad vectors have been performed *in vitro* (Hartman *et al.*, 2008) and the response *in vivo* is potentially much more complicated. Ad vectors elicit such strong innate immune responses that 90% of vector DNA is cleared from tissues within 24 hours of inoculation (Nayak & Herzog 2010), although this may also be linked to the transient nature of non-integrative vectors. Ads activate the innate immune response by the expression of pathogen-associated molecular patterns (PAMPs) which directly interact with pathogen recognition receptors (PRRs). A prominent group of PRRs is the toll-like receptor (TLR) family, however Ads activate immunity through both TLR-dependent and independent pathways (Yamaguchi *et al.*, 2007). TLR-independent pathways include the recognition of Ad DNA by the cytosolic cryopyrin NALP3, which triggers a pro-inflammatory cytokine response (Muruve *et al.*, 2008). There are two TLRs which have been directly linked to the innate response to Ad vectors, TLR-2 and TLR-9 (Iacobelli-Martinez & Nemerow, 2007; Appledorn *et al.*, 2008). TLR-9 is an endosomal receptor identified as a PRR for unmethylated DNA, like that of an Ad vector (Huang & Yang, 2009). TLR-2 is found on the cell surface and

is known to play a role in innate immunity (Appledorn *et al.*, 2008) but its Ad PAMP has yet to be identified (Nayak & Herzog, 2010). Signalling through these receptors is known to drive Th1 immunity by the induction of Interferon alpha (IFN- $\alpha$ ) and Interferon beta (IFN- $\beta$ ) which instigate the innate immune response to the vector and its transgene by first recruiting NK cells (Zhu *et al.*, 2008; Lasaro & Ertl, 2009). The activation of innate immune responses by Ad vectors relies on the activation of a universal adapter protein, Myeloid differentiation primary response gene 88 (MyD88), in both TLR-dependent and TLR-independent pathways. MyD88 is used by all the TLR family to activate the transcription factor NF- $\kappa$ B. NF- $\kappa$ B activation triggers the transcription and subsequent translation and secretion of monocyte chemoattractant proteins (MCPs), macrophage inflammatory proteins (MIPs), Interferons (IFN), Interleukins (IL), regulated on activation, normal T cell expressed and secreted protein (RANTES), interferon gamma inducible proteins, Granulocyte colony stimulating factor (G-CSF) and Granulocyte macrophage colony stimulating factor (GM-CSF) (Worgall *et al.*, 1997; Muruve *et al.*, 1999; Borgland *et al.*, 2000; Hartman *et al.*, 2007; Hartman *et al.*, 2008) which recruit macrophages and DCs to the site of transduction and subsequently activate them. Maturation of DCs upon Ad transduction or infection has been shown to be important for the initiation of an adaptive immune response and this will be covered in more detail in Chapter 1.5. Systemic inoculation of both mice and NHPs has been shown to trigger the rapid release of IL-6, IL-12 and TNF- $\alpha$  and lead to the accumulation of macrophages and DCs in the lymphatic tissues supporting the *in vitro* findings (Zhang *et al.*, 2001; Schnell *et al.*, 2001). The rapid increase of IFNs, ILs, RANTES, G-CSF, GM-CSF and the trafficking of APCs to the lymphatic tissue activates an adaptive immune response which generates CD4<sup>+</sup> and CD8<sup>+</sup> T cells and B cells (Barouch *et al.*, 2004; Nayak & Herzog, 2010). As previously mentioned, once a primary inoculation has been administered levels of neutralising antibodies to that serotype quickly rise and this has been shown to impair response to the transgene product (Fitzgerald *et al.*, 2003; Basis *et al.*, 2004; Tatsis & Ertl, 2004; Lasaro & Ertl, 2009).

Different pathogens are cleared by the human immune system by different pathways. For prophylactic vaccines to function effectively and for protection against the pathogen to be generated the encoded transgene must elucidate the correct immune response. Two types of CD4<sup>+</sup> T cell responses can be induced by the action of DCs

and other APCs, Th1 CD4+ T cells and Th2 CD4+ T cells and result in very different downstream pathways. Ad vectors are known to generate a Th1 response (Liu & Muruve, 2003) and as such may be more effective as a prophylactic vaccine for pathogens as a Th1 response generates CD8+ T cells which are essential for viral clearance and antibodies that can mediate antibody dependent cytotoxicity (Tatsis & Ertl, 2004; Lasaro & Ertl, 2009). Before experimentation in a clinical setting can take place in humans, experiments must be performed in animal model systems to evaluate the level of toxicity of the vector used, the amount of transgene expressed and whether immunogenicity has been triggered to protect against pathogen infection. Animal model systems can be used to monitor immune responses to a vector and its transgene and can be utilised to attempt prophylactic protection against a pathogen. In the latter examples it is possible that an animal species may not be found which can be successfully infected with the human pathogen to be examined. In these scenarios a related pathogen may be used, if one can be found, to monitor the efficacy of protection and the type of immune response generated. This thesis will use two examples of vaccine model systems and they are described herein.

#### **1.4.2: Vaccine model systems**

#### **1.4.3: HIV**

There are currently an estimated 40 million individuals in the world infected with HIV, and the World Health Organisation estimates AIDS has killed more than 25 million people since it was first recognised, making it one of the most destructive pandemics in recorded history (Derdeyn & Silvestri, 2005). Antiretroviral treatment reduces both the mortality and the morbidity of HIV infection, but routine access to antiretroviral medication is not available in all countries. Cheaper and easier to administer treatments are required to halt the growing pandemic (Letvin, 2006). A tremendous amount of effort and money has been spent on the search for an effective vaccine to control the growing AIDS pandemic long after the discovery and isolation of HIV 27 years ago (Barré-Sinoussi *et al.*, 1983).

HIV is transmitted both by sexual contact and haematogenously through contaminated needles and blood products. The virus can initiate infection by crossing a mucosal barrier or by direct entry into a T cell or monocyte/macrophage lineage cell in the peripheral blood (Valentin *et al.*, 1994; Letvin, 2006). HIV infection begins with primary infection representing a period of rapid viral replication that immediately follows the individual's exposure to HIV which leads to levels of HIV within peripheral blood commonly approaching several million viruses per ml (Barouch, 2008). During this period (usually 2–4 weeks post-exposure) most individuals (80 to 90%) develop an influenza-like illness called acute HIV infection, the most common symptoms of which may include fever, lymphadenopathy, pharyngitis, rashes, mouth and esophageal sores, and may also include, but less commonly, headaches, nausea, weight loss, and neurological symptoms (Kahn & Walker, 1998). Infected individuals may experience all, some, or none of these symptoms with symptoms lasting at least a week (Kahn & Walker, 1998). This phase is also characterised by a rapid and marked drop in the numbers of memory CD4<sup>+</sup> T cells that takes place mainly in mucosal tissues. This acute viremia is associated in virtually all patients with the activation of CD8<sup>+</sup> T cells which have the ability to kill HIV-infected cells, and a considerable time later with antibody production (seroconversion). Virus levels initially peak and then decline, as the CD4<sup>+</sup> T cell counts rebound to around 800 cells/ml (the normal value is 1,200 cells/ml). The early CD8<sup>+</sup> T cell response is thought to be important in controlling virus levels and has been linked to slower disease progression and a better prognosis (Pantaleo *et al.*, 1997). However, a clear immune correlate of protection remains elusive (Barouch, 2008).

Early attempts to develop a vaccine focused on the stimulation of Nabs using preparations of viral envelope proteins, such as the glycoprotein gp120. These were largely unsuccessful in eliciting antibodies that would bind to primary viruses, i.e., viruses that have not been passaged in cell lines (McMichael *et al.*, 2002). There are several reasons why the generated Abs are unable to bind primary virus isolates. Firstly, very few epitopes on the primary isolate envelope are accessible for antibody binding (McMichael *et al.*, 2002). Secondly, the existence of several conformational forms of the envelope proteins is a major complicating factor in vaccine design (Letvin, 2006). The NAb response to natural infection is directed to many different



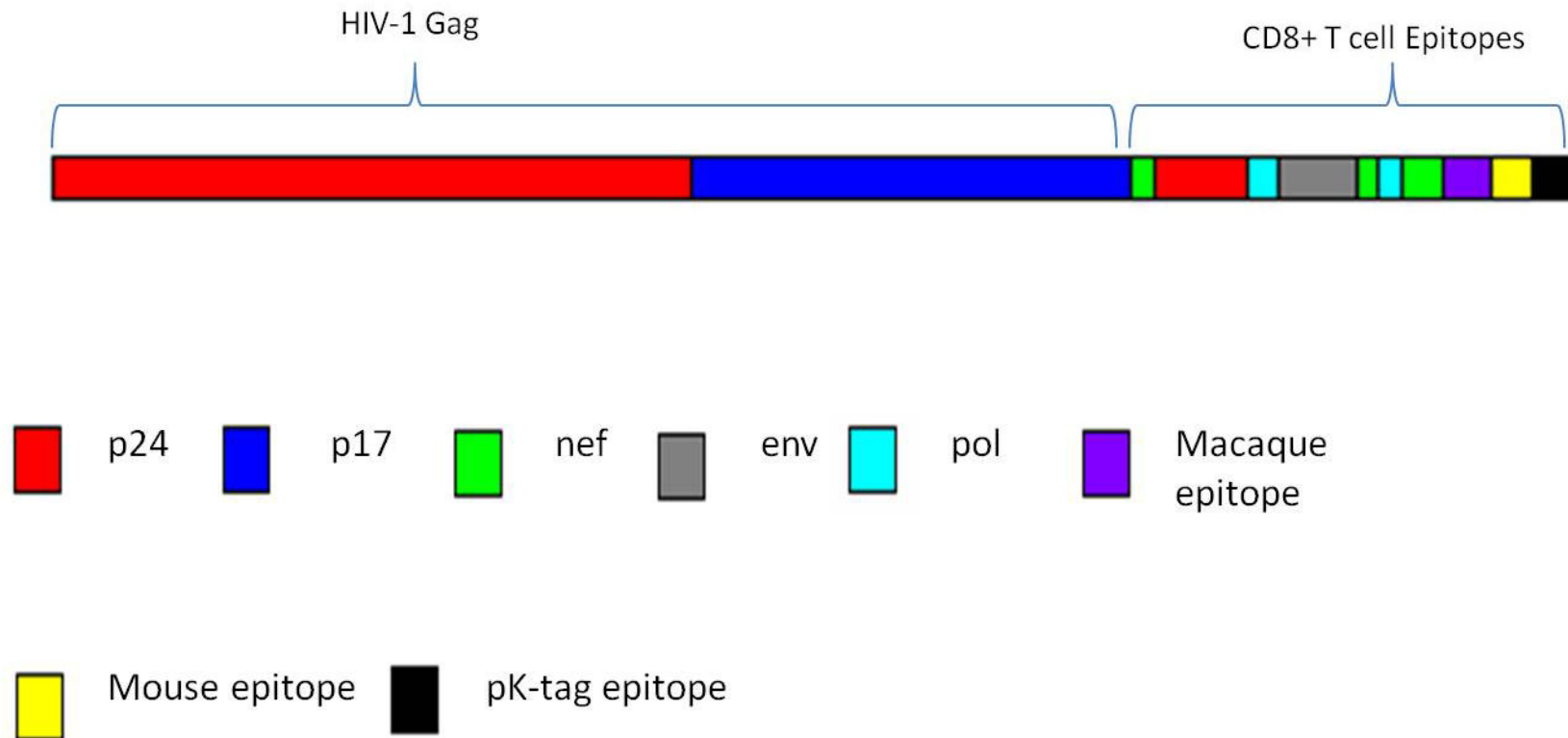
envelope epitopes, but only a very small fraction of this response is directed to epitopes of primary virus isolates. It is thought that the Ab response in a natural infection is directed not to the virus but to other conformations of the envelope proteins and in particular unprocessed gp160 (Haigwood *et al.*, 1996). Thirdly, HIV has a very high genetic variability as a result of its fast replication cycle, with the generation of about  $10^{10}$  virions every day, coupled with a high mutation rate of approximately  $3 \times 10^{-5}$  per nucleotide base per cycle of replication (Rambaut *et al.*, 2004). The key parts of the envelope, that bind to CD4 and CXCR4 and CCR5, the HIV receptors, are conserved however they are protected from Ab binding by hypervariable loops of polypeptide that easily mutate preventing Ab binding. Studies have shown that even minor variations in the hypervariable loops can dramatically alter antibody and CTL specificities in *in vitro* assays and immune control of persistent infections *in vivo* (Kusumi *et al.*, 1992; O'Connor *et al.*, 2001; Wei *et al.*, 2003; Lasaro & Ertl, 2009). Despite this evidence, Abs can protect animals from HIV infection *in vivo*. NAbs to the V3 loop of gp120 have been shown to completely protect chimpanzees from infection with T cell line adapted HIV (Emini *et al.*, 1992). NAbs isolated from long-term SIV infected macaques have also shown the ability to protect naïve macaques against HIV challenge (Haigwood *et al.*, 1996). In humans gp120 vaccine recipients could still be infected with HIV showing that a NAb response to envelope proteins is not sufficient to confer protection (McMichael *et al.*, 2002).

The CD8+ response against HIV has been very well characterised (reviewed in McMichael *et al.*, 2002). Although the variation of the virus is a huge challenge for developing an effective HIV vaccine based on Abs or CD8+ T cells, considerable advances have been made towards inducing stronger and broader responses in animal models. As Ad vectors have been shown to generate largely Th1 type immunity and a CD8+ T cell response some of these advances have come through implementing live adenoviral vectors, expressing HIV antigens such as gag, pol, nef and env, (Shiver *et al.*, 2002; Casimiro *et al.*, 2003; Pinto *et al.*, 2004). The majority of these vectors have been based on Ad5 and have encountered both predictable and unexpected problems in clinical trials (Barouch, 2008; Priddy *et al.*, 2008; McElrath *et al.*, 2008). In any case, these T cell vaccines are predicted to cause non-sterilising immunity, that does not protect from infection but may prevent disease progression.

There are several model systems for the investigation of anti-HIV1 immunity, including many which involve NHP models (reviewed in Shiver & Emini, 2004 and Barouch, 2008); however, for early investigations of vaccine research, particularly when using a new vector, it is essential to use a small animal model. Many different transgenes designed to generate anti-HIV immunity have been developed and expressed from a variety of vectors. One such transgene system is the HIVA transgene system, that has been extensively tested in immunogenicity and toxicity studies in mice, NHPs and humans (Hanke *et al.*, 2007).

#### **1.4.2.1.1 The HIVA transgene**

The HIVA immunogen was first developed in partnership between the UK Medical Research Council (MRC), the International AIDS Vaccine Initiative (IAVI) and the University of Nairobi. It consists of a consensus HIV-1 Gag p24/p17 sequence, multiple CD8<sup>+</sup> T cell epitopes and a monoclonal antibody tag (Figure 1.4; Hanke & McMichael, 2000). The immunogen was designed for use in a DNA and modified vaccine Ankara (MVA) heterologous prime/boost regimen which had been tested previously using a malaria antigen (Schneider *et al.*, 1998). Pre-clinical safety studies were carried out in BALB/c mice and the vaccine and transgene were found to be non-toxic (Hanke *et al.*, 2002). CD8<sup>+</sup> T cell generation was demonstrated by IFN- $\gamma$  ELISPOT in mice using both the DNA vaccine and MVA vaccine alone and in combination (Hanke & McMichael, 2000; Hanke *et al.*, 2002; Estcourt *et al.*, 2005). The HIVA immunogen became the first MVA based prophylactic HIV-1 vaccine candidate to enter clinical trials and the first with an HIV-1 derived string of CD8<sup>+</sup> T cell epitopes tested in humans (Hanke *et al.*, 2007). The immunogen has been used in both prophylactic (Mwau *et al.*, 2004; Cebere *et al.*, 2006; Goonetilleke *et al.*, 2006; Guimarães-Walker *et al.*, 2008; Jaoko *et al.*, 2008) and therapeutic trials (Dorrell *et al.*, 2005; 2006; 2007; Ondondo *et al.*, 2006; Yang *et al.*, 2009) and the knowledge gained has allowed the construction of improved immunogens such as RENTA (Nkolola *et al.*, 2004) and HIV<sub>consv</sub> (Rosario *et al.*, 2010). Both the DNA and MVA HIVA vaccines have been shown to induce HIV-1 specific T cell responses in the majority of vaccine recipients, when given therapeutically and prophylactically. The DNA vaccine has proved weak, whilst the MVA vaccine has consistently generated a strong CD4<sup>+</sup> and CD8<sup>+</sup> T cell response particularly when



**Figure 1.4: Composition of the HIV-1 polypeptide.** Sequences from Gag p24 (red), p17 (blue) and CD8+ T cell epitopes from nef (green), envelope (grey), polymerase (light blue), macaque (purple) and mouse (yellow) and a monoclonal antibody tag (black) are highlighted. Adapted from Hanke & McMichael, 2000

given therapeutically (Barouch, 2008). In prophylactic studies, however, a DNA/MVA heterologous prime/boost regime elicited a higher response than MVA alone (Barouch, 2008).

The HIVA immunogen has also been tested for the generation of CD8<sup>+</sup> T cell response in other vector systems such as Semliki Forest virus (alphavirus; Hanke *et al.*, 2003), bluetongue virus (Larke *et al.*, 2005), Bacillus Calmette-Guérin (BCG; Rosario *et al.*, 2010a), ovine atadenovirus (Bridgeman *et al.*, 2009; Rosario *et al.*, 2010b) and Ad5 (Bridgeman *et al.*, 2009). The immunogen has therefore proven extremely useful in comparative vector studies and represents an excellent model system for the testing of new vectors and the comparison of immune responses generated.

#### **1.4.2.2 RSV**

Respiratory syncytial virus (RSV) is the major cause of upper and lower moderate-to-severe respiratory tract infections worldwide in children (Ogra, 2004), the immunosuppressed and the elderly (reviewed in Falsey & Walsh, 2000). Almost all children have been infected by 3 years of age (Smith & Openshaw, 2006) and it is estimated, in the US, that there are up to 60,000 RSV-related hospitalisations and as many as 7,000 deaths each year due to RSV infection (Falsey & Walsh, 2000).

All efforts to formulate a safe and effective vaccine for use in humans have, so far, been unsuccessful (Olszewska & Openshaw, 2009). Formalin-inactivated vaccines were tested in the 1960s but failed to prevent the acquisition of the disease in children and in younger infants and actually enhanced symptoms upon subsequent natural infection (Kim *et al.*, 1969). Other vaccine strategies are being developed but have so far only been weakly immunogenic (Olszewska & Openshaw, 2009). The present form of prevention during RSV season is palivizumab, a monoclonal antibody directed against the RSV fusion protein, whilst ribavirin remains the only form of treatment at onset of infection (Olszewska & Openshaw, 2009). Early studies using ribavirin were promising although it is now well-recognised that it has little impact on the progression of disease (Rosenberg *et al.*, 2005) and does not halt

progression to severe bronchiolitis and RSV pneumonia which require hospitalisation for respiratory support (Broughton & Greenough, 2003).

One of the main reasons for the lack of an effective vaccine or viable therapeutics in humans is the lack of a good working animal model for RSV infection. Human RSV effectively replicates in African green monkey and chimpanzee but only naturally infects primates, however, it does not cause the same symptoms as in humans and suffers from the significant practical and ethical impracticalities of any NHP model (Maggon & Barrik, 2004).

The bovine form of RSV (bRSV) is closely related to hRSV but it too suffers from the impracticalities of such a large animal model and a reverse genetics system for the modification of bRSV remains elusive (Rosenberg *et al.*, 2005; Valarcher & Taylor, 2007). Small animal models such as mice and rats are generally not useful since hRSV replicates inefficiently within the murine host resulting in no symptoms homologous to human infection (Rosenberg *et al.*, 2005). There is a requirement, therefore, for a small animal model system which reproduces hRSV-like pathogenesis. It was proposed that Pneumovirus of mice (PVM) could be used as a surrogate virus (see below).

The correlates of protection against RSV infection are still not fully understood but it is known to involve both humoral and cellular effectors, including CD8<sup>+</sup> T cells (Maggon & Barrik, 2004). It is generally thought that an antibody response prevents infection of the upper or lower respiratory tract after the initial encounter but it is cell-mediated responses such as the CD8<sup>+</sup> T cell response to internal proteins, such as the hRSV nucleocapsid protein, which terminate infection and cause prophylactic protection from infection (reviewed in Maggon & Barrik, 2004). In contrast, a Th2 response does not result in effective viral clearance and it is now thought that hRSV evades a protective Th1 response by driving a Th2 response instead which actively inhibits the generation of a Th1 response (reviewed in Becker, 2006). For prophylactic protection to work, it is therefore believed to be vital for the vaccine to have triggered a Th1 response condition so that on subsequent infection a Th2 response is prevented.

In bRSV infection, CD8<sup>+</sup> T cells are directed against the surface glycoproteins (F & G), Polymerase (L), Matrix (M), ion channel (M2) and nucleocapsid (N) proteins (Gaddum *et al.*, 2003). The bRSV N protein has been shown to be the major target of CD8<sup>+</sup> T cells in cattle in a DNA prime/ protein boost strategy (Letellier *et al.*, 2008). An immune response against N has long been known to induce protection in other animal models also (King *et al.*, 1987). More recently the heterologous expression of the bRSV N protein has been shown to protect against subsequent challenge with the virus itself (Letellier *et al.*, 2008).

#### **1.4.2.2.1 The PVM challenge model**

Pneumovirus of mice (PVM) belongs to the same family, subfamily and genus as hRSV (Rosenberg *et al.*, 2005). It was originally isolated from healthy mice but passage of extracts of the lung tissue from these mice in further animals led to the isolation of a virus that caused lethal infection (Thorpe & Easton, 2005). A significant proportion of the human population have been shown to be seropositive for PVM or an antigenically closely related virus and it has been suggested it remains an undiagnosed cause of a small number of cases of respiratory disease in humans (Pringle & Eglin, 1986). Serial passage of the early PVM isolates has resulted in attenuation and the loss of disease in mice (Domachowske *et al.*, 2002), however a second strain, J3666, has been described which has been maintained by animal-to-animal passage and remains fully virulent in mice (Cook *et al.*, 1998).

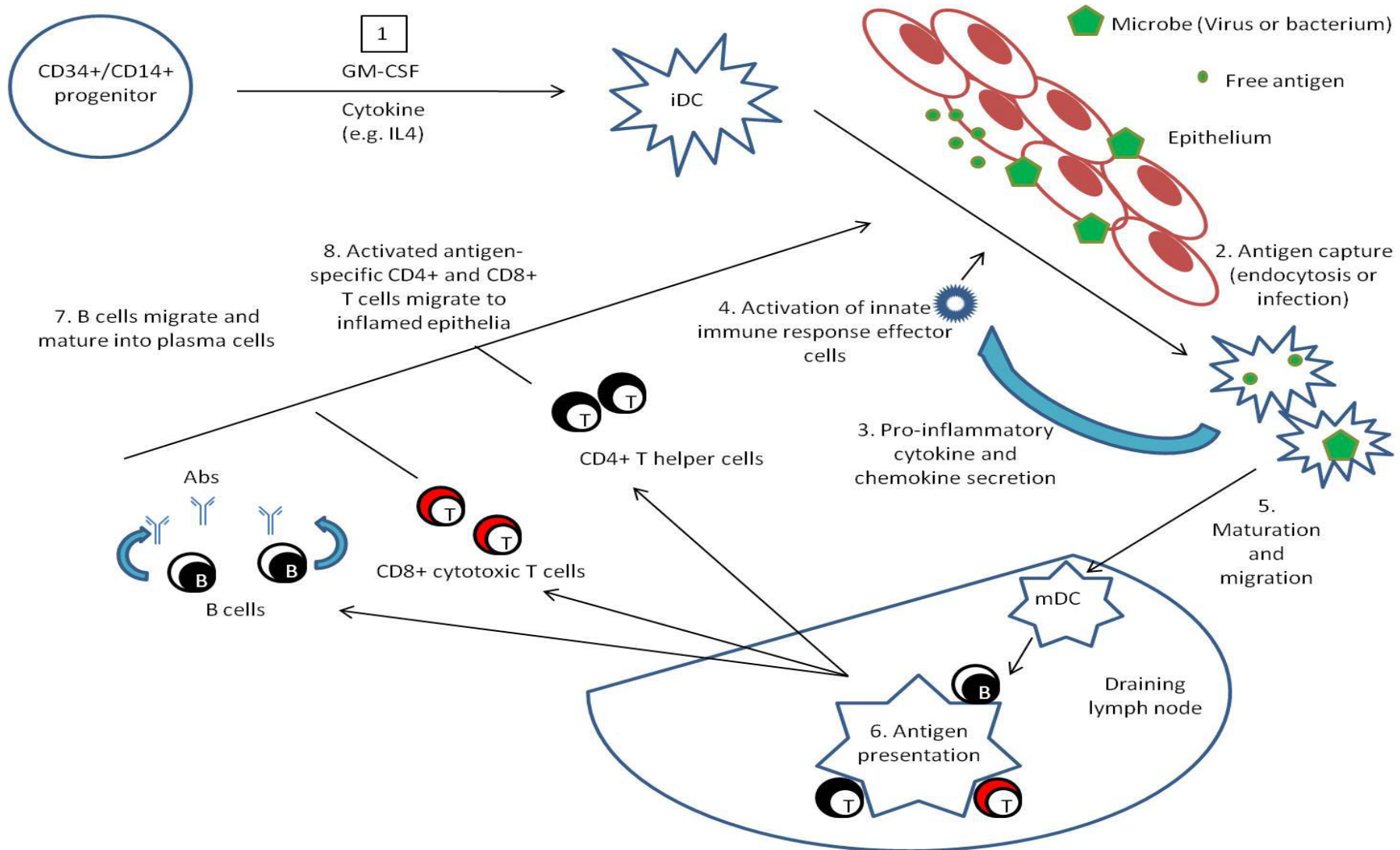
There is intensive research into using the pathogenesis of PVM J3666 in mice as a model of hRSV infection in humans due to the similar pathogenesis. The immediate response to both PVM infection in mice and RSV infection in humans is the recruitment of eosinophils to the lung tissue (Garofalo *et al.*, 1992 Garofalo *et al.*, 2001; Domachowske *et al.*, 2000) which results in the influx of granulocytes followed by a CD8<sup>+</sup> T cell response which results in viral clearance (Cook *et al.*, 1998). The production of MIP1 $\alpha$  has been shown to be responsible for severe hRSV infection (Garofalo *et al.*, 2001). The speed of progression of PVM infection, similar to hRSV, suggests that a NAb response is unlikely to be involved in resolving of disease (Thorpe & Easton, 2005).

An investigation was recently begun into the use of Ad vectors expressing PVM proteins to protect against lethal challenge with PVM. The F, M, N & L proteins of PVM have all been inserted into a transgene expression cassette within an Ad5 vector and all have shown the ability to protect mice against a lethal dose of PVM (Helen Terry, PhD 2010). So far the vaccine vector expressing the N protein has shown the greatest efficacy for protection which corroborates earlier evidence seen in bRSV and hRSV infections. Therefore, it was decided to also insert the N protein of PVM into an Ad19a based vector for comparisons of protection and immune responses generated between the Ad5 and Ad19a vectors.

## 1.5 Dendritic Cells

DCs are considered the most potent of all APCs to trigger adaptive immune responses (Steinmann and Hemmi, 2006). Lymphocytes such as T cells, B cells and NK cells and their products are controlled by their interactions with DCs (Banchereau & Steinmann, 1998). The term DC actually represents a number of distinct subsets with different localisations and differing functions. The localisation of each DC subset is usually linked to their function (Palucka *et al.*, 2008). DCs represent the major link between the innate and adaptive immune response due to their ability to sense foreign pathogens, to secrete chemokines and cytokines that activate the local innate immune response and act as APCs in the adaptive immune response. *In vitro* or *in vivo*, only a few DCs are necessary to provoke a strong T cell response (Banchereau & Steinman, 1998).

CD14<sup>+</sup> monocytes and/or pluripotential CD34<sup>+</sup> bone marrow cells can be stimulated to become immature DCs (iDCs; Figure 1.5), by the action of GM-CSF followed by encounter with a cytokine. The cytokine encountered specifies which form of iDC will form. The best studied iDC is stimulated by CD14<sup>+</sup> monocyte encounter with IL-4 to produce IL4-iDCs (Romani *et al.*, 1994; Palucka *et al.*, 2005), however, it is becoming clear that DCs derived from CD34<sup>+</sup> progenitors are better at eliciting CD8<sup>+</sup> T cell responses when exposed to IL4 (Ahlers & Belyakov, 2009). Other cytokines can produce whole spectra of iDCs whose functions are only beginning to be understood. For example, if a GM-CSF stimulated CD14<sup>+</sup> monocyte is incubated





**Figure 1.5: DC life cycle.** 1. Circulating progenitor cells are stimulated to become iDCs by the action of GM-CSF and cytokines. 2. iDCs encounter microbes or free antigen at peripheral tissue such as the epithelium and undergo antigen capture by phagocytosis or viral infection. 3. iDCs produce pro-inflammatory cytokines, such as IFN- $\alpha$ , and chemokines. 4. Produced chemokines and cytokines recruit and activate innate immune response effector cells, such as eosinophils, macrophages and NK cells at the site of iDC antigen encounter. 5. iDCs are activated, undergo maturation and migrate to the draining lymph node. 6. mDCs enter lymph organs and activate antigen-specific CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and B cells by the display of peptide-MHCI and/or peptide MHCII complexes. 7. B cells migrate and mature into plasma cells producing Abs to neutralise the pathogen. 8. Activated T lymphocytes migrate to the site of antigen encounter where they eliminate pathogens or pathogen infected cells.

with an immunosuppressive cytokine such as IL10 an IL10-iDC is formed which expands primarily regulatory T cells to suppress activation of the immune response (Steinbrink *et al.*, 2007).

iDCs are distributed in the blood, lymphoid tissues and all peripheral tissues that are in contact with the external environment, mainly the skin and the inner lining of the nose, lungs, stomach and intestines (Banchereau & Steinmann, 1998; Ueno *et al.*, 2010). In the blood, DCs exist as either myeloid DCs (myDCs) or plasmacytoid DCs (pDCs). pDCs release large amounts of type I IFN upon pathogen recognition and limit the spread of infection, they can also cross to the endothelial layer and activate the inflammatory response and the secretion of chemokines (Siegal *et al.*, 1999; Palucka *et al.*, 2008). pDCs have also been shown to have an important role in innate immunity as secretors of high levels of IFN- $\alpha$  upon contact with exogenous Ag (Cella *et al.*, 1999). The role of blood myDCs is not as well understood but it has been suggested that they represent the human equivalent of mouse ‘patrolling’ DCs which migrate from the blood to the dermis and into the lymph nodes (Ginhoux *et al.*, 2007). The peripheral tissues contain two further subsets of myDCs which represent more ‘classical’ DCs, epidermal Langerhans cells (LCs) and dermal (interstitial) DCs (Palucka *et al.*, 2008; Ueno *et al.*, 2010). It is these two subsets of DCs which, when immature, have high endocytic and phagocytic capacity.

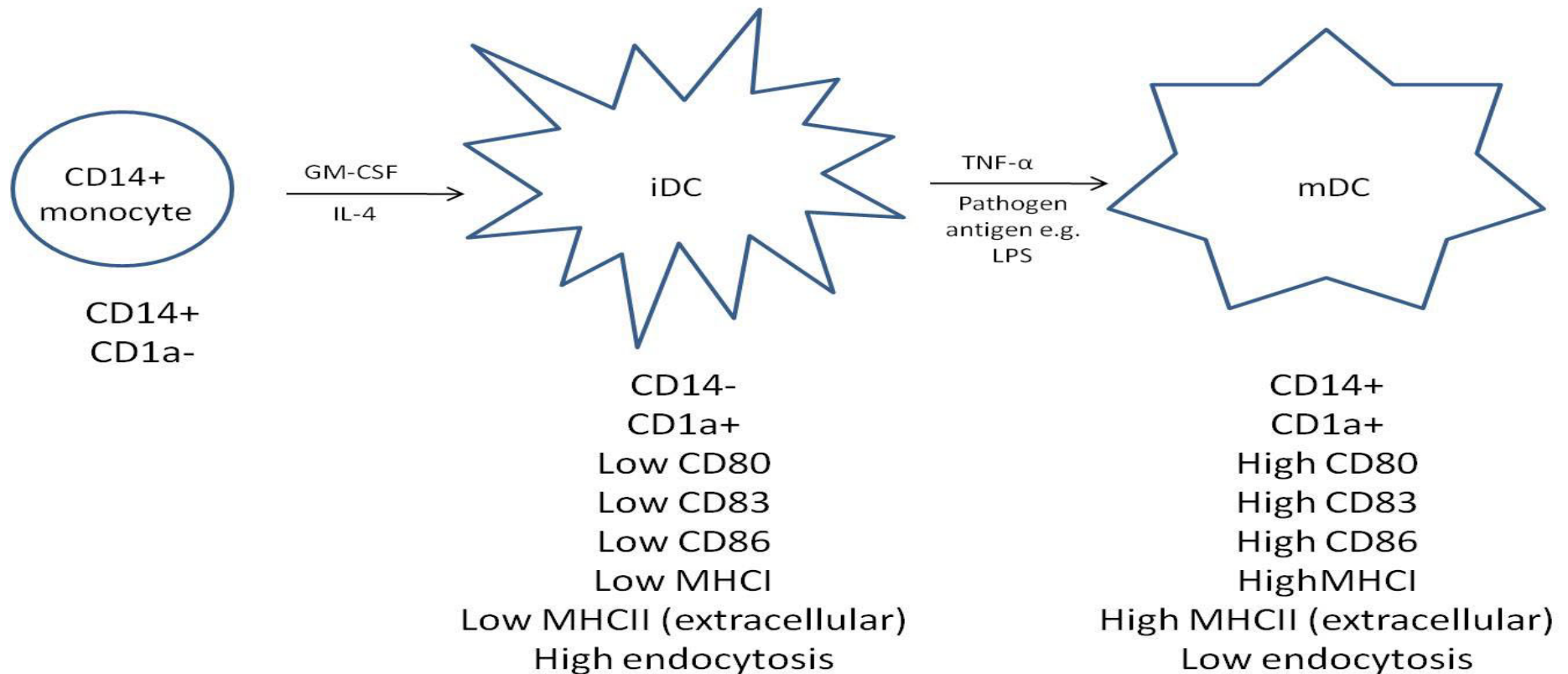
iDCs have several features that allow them to capture Ag; they can take up particles and pathogens by phagocytosis (Banchereau & Steinman, 1998), they can form large pinocytic vesicles to sample extracellular fluid for pathogens and particles in a process called macropinocytosis (Sallusto & Lanzavecchia, 1994) or they express PRRs, which can recognise PAMPs and subsequently endocytose microbial organisms via receptor mediated endocytosis (Jiang *et al.*, 1995). Macropinocytosis, the use of PRRs and the DC capacity for co-stimulatory molecule expression result in antigen presentation by DCs being so effective that even picomolar concentrations of Ags can suffice for stimulation, much lower than the micromolar amounts required by other APCs (Bhardwaj *et al.*, 1993).

The captured Ags enter the endocytic pathway of the cell. DCs are able to produce large amounts of MHC class II-peptide complexes. This is due to the specialised,

MHC class II rich compartments (MIICs) that are abundant in iDCs (Pierre *et al.*, 1997). During maturation of DCs, MIICs discharge their MHC II-peptide complexes on to the cell surface (Pierre *et al.*, 1997). This interaction generates a Th2 biased response leading to an inflammatory and Ab response.

A unique feature of DCs is their ability to deliver endocytosed Ags into the MHC class I presentation pathway in a process called cross-presentation (Brode & McCary, 2004; Heath *et al.*, 2004). This process is vital for the presentation of peptides to generate a CD8<sup>+</sup> T cell response against tumours or viruses which do not infect the DC itself which would otherwise only be presented to CD4 T cells on MHC class II resulting in an inflammatory and B cell response (Ueno *et al.*, 2010). As in all cell types, peptides can also enter the MHC class I presentation pathway if the iDC itself is infected with a virus or transduced with a vector. A dedicated peptide transporter, TAP, translocates these peptides from their site of generation in the cytosol to the endoplasmic reticulum, where they bind to MHC class I molecules. The peptide-bound MHC class I complexes are transported to the cell surface where they are displayed for CD8<sup>+</sup> T cell recognition. Thus, DCs have the capacity to activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Liu, 2003; Ueno *et al.*, 2010). DCs are therefore essential for an effective immune response against the majority of viruses due to their cross-presentation ability. Without the generation of a CD8<sup>+</sup> T cell response by MHC class I presentation most viral infections could not be cleared (Banchereau & Steinman, 1998).

After receiving maturation signals the DCs undergo a process of maturation which eventually allows them to traffic to the lymph nodes and become effective antigen presenters (Figure 1.5; 1.6). DCs can receive maturation signals through several pathways including, but not limited to, the binding of microbial organisms or products e.g. Lipopolysaccharide (LPS) to PRRs, cell products such as pro-inflammatory cytokines e.g. TNF- $\alpha$ , the products of dying cells or stimulation by NK cells (Palucka *et al.*, 2008). DC maturation is associated with several co-ordinated events beginning with the loss of endocytic/phagocytic capacity due to the downregulation of corresponding receptors, a change in cell morphology to allow for cell motility, the translocation of MHC molecules to the cell surface, in particular



**Figure 1.6: The growth and maturation of DCs.** Diagram highlights the stimulation of iDC formation from CD14+ monocytes by GM-CSF and IL-4 and the subsequent maturation of iDCs to mDCs in the presence of TNF- $\alpha$  or pathogen antigen such as Lipopolysaccharide *in vivo* or *ex vivo*. Given underneath each cell type is their receptor expression pattern at each stage of the DC life cycle which can subsequently be used to dissociate the cells from one another *in vivo*. MHCII (extracellular) denotes detectable cell surface MHCII rather than internal.

MHC II, the upregulation of B7-1 (CD80) and B7-2 (CD86) and the secretion of various cytokines for the generation of effector T cells (Palucka *et al.*, 2008; Ueno *et al.*, 2010). DCs migrate with their bound Ag from the peripheral tissue to the draining lymph nodes. Upon arrival the upregulation of CD80 and CD86 allows the effective activation of naïve CD8 and CD4 T lymphocytes via MHC class I or II (Banchereau & Steinman, 1998). If CD80 and CD86 are not present to bind to the CD28 molecules on T lymphocytes during MHC presentation and Ag recognition by the T cell receptor, the T lymphocyte will enter into a state of anergy (Groux *et al.*, 2004). Thus, it is in this way that primary immune responses can be initiated. Other APCs, such as macrophages, and to a lesser degree B cells also contribute to initiation of immune responses.

Following contact with mDCs via MHC class I or II, naive CD8<sup>+</sup> and CD4<sup>+</sup> T cells differentiate into CD8<sup>+</sup> cytotoxic T cells and helper CD4<sup>+</sup> T cells. Helper CD4<sup>+</sup> T cells support the differentiation and expansion of CD8<sup>+</sup> T cells and B cells.

When exposed to virally infected cells which are expressing their specific viral peptide on MHC class I, CD8<sup>+</sup> T cells release substances, primarily the cytotoxin perforin. Perforin creates a pore through which granzymes of the cytotoxic granules can enter the target cell cytosol where they cleave the central caspase 3 and other cellular targets resulting in the activation of apoptosis regulators (Bleackley, 2005). An alternative mechanism of CD8<sup>+</sup> killing involves the binding of activated CD8<sup>+</sup> T cells to the Fas receptor (CD95) expressed on target cells via apoptosis inducing ligands, primarily Fas ligand (FasL; CD95L). CD95 is expressed by all cells and is up-regulated when infected with viruses, damaged or dysfunctional. The interaction between CD95 and CD95L results in the recruitment of the death-inducing signalling complex (DISC) which interacts and translocates with the Fas-associated death domain (FADD) subsequently instigating a caspase cascade which results in the death of the cell (Berke, 1995).

As DCs can be generated *in vitro* by differentiation from monocytes their ability to stimulate naive T cells has been successfully exploited for various kinds of vaccination, against tumours (Banchereau *et al.*, 2001) and against infectious disease (Steinman & Banchereau, 2007). DCs can be loaded with Ag peptides or proteins, or

the genes for the corresponding Ag can be transferred by transfection or via transduction with viral vectors (Schuler *et al.*, 2003).

### 1.5.1 DCs as vaccine targets

Generating the correct kind of immune response can mean the difference between mortality or viral clearance and recovery (Banchereau *et al.*, 2009). There remain many pathogens for which no efficient vaccine is available, most causing chronic disease where a CD8<sup>+</sup> T cell response is critical for pathogen clearance. Due to their critical role in both the innate and the adaptive immune response there is now a large body of research into the targeting of vaccines to DCs to generate a protective or therapeutic response.

DCs can be targeted *in vivo* or *ex vivo*. *Ex vivo* peripheral blood leukocytes are obtained by leukaphoresis and monocytes isolated. CD14<sup>+</sup> monocytes are cultured for five days with GM-CSF and IL-4 to produce iDCs which are then targeted for antigen delivery by *ex vivo* specific methods, such as electroporation, or transduction with viral vectors before being infused back into the patient with activated T cells. Several studies have now investigated the targeting of an antigen itself to DCs by either coupling it to a DC specific ligand or a DC-receptor specific antibody. By targeting the antigen to a specific receptor it is possible to affect which MHC pathway the antigen is delivered to, however this does not induce the maturation of the DC and the induction of adaptive immune responses (Ahlers & Belyakov, 2009). Alternatively, the antigen itself, such as an envelope antigen incorporated into a surrogate vector envelope, or a DNA transgene encoding the antigen, can be incorporated into a vector system. Vector systems which have undergone DC trials include viral vectors (e.g. van de Ven *et al.*, 2009) and liposomes (e.g. Zheng *et al.*, 2010) and in several studies these have been targeted further by the integration of a receptor ligand or receptor-specific antibody (Ahlers & Belyakov, 2009). The search continues, however, for vectors which are naturally DC specific and therefore do not require further modification (Ahlers & Belyakov, 2009). *In vivo* targeting of DCs implies that the vector and antigen are administered and DC transduction takes place by encounters with iDCs at the site of application.

It is also apparent that different DCs may have different potential for vaccination. Therefore it is important to target the best DC subset most appropriate for triggering the immune response wished. *In vitro* generated DCs appear to differ in their capacity to activate lymphocytes (Palucka *et al.*, 2008). Interstitial DCs have been shown to effectively induce the differentiation of B cells (Caux *et al.*, 1997) whereas LCs are more effective activators of CD8<sup>+</sup> T cells (Ueno *et al.*, 2007). This has been proven in a murine model wherein a vector targeted specifically to LCs (CD8<sup>+</sup> DEC-205<sup>+</sup> DCs) produced a greater CD8<sup>+</sup> T cell response whilst the same vector targeted to interstitial DCs (CD8<sup>-</sup> DCs) produced an antibody response (Dudziak *et al.*, 2007). Of further interest, it has been shown that LCs and interstitial DCs display distinct cell receptor repertoires, suggesting different functions (Palucka *et al.*, 2008; Ueno *et al.*, 2010). For example, LCs express a wide range of TLRs but lack TLR4 and TLR5 which are important bacterial PRRs (Flacher *et al.*, 2006; van der Aar *et al.*, 2007) whereas interstitial DCs specifically express TLR 4 and 5 (van der Aar *et al.*, 2007). This may suggest that LCs represent a more virus/tumour specific DC whilst interstitial DCs are designed to handle bacterial infections.

#### **1.5.1.1 The maturation of DCs by vaccination**

Once effectively targeted there is a further requirement to mature DCs during vaccination as DC maturation has been shown to be a pre-requisite for induction of immunity (De Vries *et al.*, 2003; reviewed in Steinman & Banchereau, 2007). Many organisms including viruses such as HIV have the capacity to block DC maturation and prevent the formation of an adaptive immune response (Banchereau *et al.*, 2009) and research has shown that vaccines like the current yellow fever vaccine are only effective by transducing and subsequently maturing DCs (Querec *et al.*, 2006).

The timing of the maturation signal is very important, given too early and the maturation status of the DC will change before the vector reaches the cell and transduction may not occur, given too late, after the antigen has been processed, and tolerance towards that antigen may be induced (Wilson *et al.*, 2006). The best method for correctly maturing DCs would therefore rely on a vector which can not only transduce the DC effectively but also mature the DC without the requirement of another treatment. For this reason it has now become important for all candidate

vaccine vectors to be tested for their capacity to both transduce and mature DCs. Ad vectors, such as human Ads (Ad35; Rea *et al.*, 2001) and chimpanzee Ads (CAd68; Basner-Tschakarjan *et al.*, 2006), have shown increasing promise for use as DC targeting vectors for both their level of transduction of dendritic cells and resultant maturation (see Chapter 5). In contrast to Ad5, however, different serotypes have been shown to differ in their effective targeting of DCs.

## **1.6 Aims of the research**

Based on the interesting observation that Ad19a is particularly efficient at infecting DCs (Ruzsics *et al.*, 2006), the major aim of this work was to evaluate whether a vector derived from Ad19a has potential for vaccination. To this end, two antigens suitable for vaccination were inserted and basic features of this vector system (expression level of the transgene, transduction of DCs, potential receptors and the capacity to induce immune responses or protect against lethal challenge *in vivo*) were investigated.



## **Chapter 2: Materials and Methods**

### **2.1: Solutions, buffers and media**

All chemicals were of analytical grade and supplied by either Fisher Scientific or BDH unless otherwise stated. The solutions listed here are the stock solutions, the working concentrations are given in the methods section.

**2-deoxy-galactose (DOG):** 1g DOG in 10ml ddH<sub>2</sub>O.

**2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma):** 50mg/ml

**ABTS buffer (Roche):** 16.7g ABTS in 1L ddH<sub>2</sub>O

**Acrylamide solution (Roth GmbH, Germany):** rotiphorese®Gel30, 30% (w/v) acrylamide, 0.8% bis-acrylamide in distilled, de-ionised H<sub>2</sub>O.

**Blocking Buffer:** 5% skim milk powder (Merck), 0.02% NaN<sub>3</sub> in phosphate buffered saline (PBS) / 0.05% Tween 20.

**Blotting/Transfer Buffer:** 48mM Tris, 39mM Glycine, 0.037% sodium dodecyl sulphate (SDS), 20% Methanol in H<sub>2</sub>O.

**Caesium chloride 1.27g/ml (CsCl):** 18.47g CsCl dissolved in 50 ml PBS

**Caesium chloride 1.35g/ml (CsCl):** 22.71g CsCl dissolved in 50ml PBS

**Caesium chloride 1.42g/ml (CsCl):** 27.42g CsCl dissolved in 50ml PBS

**Carboxymethyl Cellulose (CMC) Agar:** 4g in 100ml ddH<sub>2</sub>O

**Chloroform/iso-amyl alcohol:** 96% (v/v) chloroform, 4% (v/v) iso-amyl alcohol

**Crystal Violet:** 1.5g in 100ml EtOH (dilute 1:40 for use)

**D-Biotin:** 0.2 mg/ml in H<sub>2</sub>O

**Dithiothreitol (DTT):** 0.5 M, 1.55 g in 20 ml H<sub>2</sub>O

**DNA gel loading buffer (6x):** 0.25% orange G, 40% (w/v) sucrose in distilled water.

**DNA marker ladders (New England Biolabs):** 0.25 µg/µl, dilute stock (0.5 µg/µl) 1:1 with 6x DNA gel loading buffer.

**dNTPs stock (Fermentas):** 100 mM solutions of dCTP, dTTP, dATP and dGTP

**Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL).**

**Dulbecco's modified Eagle's medium: F12 Nutrient mixture (F12) (Gibco BRL).**

**Ethidium bromide solution:** 10 mg/ml in H<sub>2</sub>O

**FACS buffer:** PBS (-Ca<sup>2+</sup>, -Mg<sup>2+</sup>), 3% FCS, 0.075% NaN<sub>3</sub>.

**FACS buffer + saponin:** PBS, 3% FCS, 0.075% NaN<sub>3</sub>, 0.1% saponin.

**Foetal Calf Serum (FCS) (Biosera, Sussex, UK) - heat inactivated.**

**Fixation buffer (BD Bioscience):** CellFIX™.

**Freezing buffer:** DMEM (supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamine), 25% FCS, 10% DMSO.

**G418-sulphate (Gibco):** 50 mg/ml in DMEM.

**Glasgow Minimal Essential Medium (GMEM) (Sigma Aldrich)**

**Gluteraldehyde:** 2g in 100ml PBS

**L-Glutamine (100x) (Gibco BRL):** 200 mM in H<sub>2</sub>O

**L-Leucine:** 10mg/ml, filter sterilised

**Luria-Bertani (LB) Medium (pH 7.0):** 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, made to a total volume of 1 litre with distilled H<sub>2</sub>O. Autoclaved at 15lb/sq.in. for 20 minutes on liquid cycle.

**LB-amp:** LB medium supplemented with 100 µg/ml ampicillin (amp).

**LB-kan:** LB medium supplemented with 25 µg/ml kanamycin (kan).

**LB-cm:** LB medium supplemented with 35 µg/ml chloramphenicol (cm).

**LB-cm/kan:** LB medium supplemented with 25 µg/ml kanamycin and 35 µg/ml chloramphenicol (cm/kan)

**LB agar-amp plates:** Prepare LB media as above and add 15 g/l bacto-agar prior to autoclaving. Allow to cool and supplement with 100 µg/ml amp before pouring into sterile Petri dishes.

**LB agar-kan plates:** Prepare LB media as above and add 15 g/l bacto-agar prior to autoclaving. Allow to cool and supplement with 25 µg/ml kan before pouring into sterile Petri dishes.

**LB agar-cm plates:** Prepare LB media as above and add 15 g/l bacto-agar prior to autoclaving. Allow to cool and supplement with 35 µg/ml cm before pouring into sterile Petri dishes.

**LB agar-kan plates:** Prepare LB media as above and add 15 g/l bacto-agar prior to autoclaving. Allow to cool and supplement with 25 µg/ml kan before pouring into sterile Petri dishes.

**LB agar-cm/kan plates:** Prepare LB media as above and add 15 g/l bacto-agar prior to autoclaving. Allow to cool and supplement with 25 µg/ml kan and 35 µg/ml cm before pouring into sterile Petri dishes.

**M63 Medium:** 2 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 13.6 g KH<sub>2</sub>PO<sub>4</sub>, 2.5 mg FeSO<sub>4</sub>.7H<sub>2</sub>O adjusted to pH 7.0 with 2M KOH and made to a total volume of 1 litre with distilled H<sub>2</sub>O. Autoclave at 15lb/sq.in. for 20 minutes on liquid cycle.

**M63 Agar plates:** 4 g bacto-agar in 200 ml ddH<sub>2</sub>O and autoclave at 15lb/sq.in. for 20 minutes on liquid cycle. Add 50 ml M63 medium (as prepared above), 0.5 ml 1M MgSO<sub>4</sub>, 1.25ml D-biotin, 1.1 ml L-Leucine, 5 ml 10% glycerol, 5 ml 10% 2-deoxy-galactose (DOG) and 12.5 µg/ml cm before pouring into sterile Petri dishes.

**M9 Medium:** 6 g Na<sub>2</sub>HPO<sub>4</sub>, 3 g KH<sub>2</sub>PO<sub>4</sub>, 1 g NH<sub>4</sub>Cl, 0.5g NaCl made to a total volume of 1 litre with distilled H<sub>2</sub>O. Autoclave at 15lb/sq.in. for 20 minutes on liquid cycle.

**MacConkey Agar-cm/kan plates:** 10g MacConkey agar in 225 ml ddH<sub>2</sub>O and autoclave at 15lb/sq.in. for 20 minutes on liquid cycle. Add 25 ml 10% Galactose, 12.5 µg/ml cm and 25 µg/ml kan before pouring into sterile plates.

**MACs buffer:** PBS, 0.5% BSA, 2mM EDTA

**Minimum essential medium alpha (Alpha MEM) (GibCO BRL)**

**PBS/0.05% Tween 20:** 0.5 ml Tween 20 in 1 litre PBS.

**10x PCR buffer + MgCl<sub>2</sub> (Roche) :** 100 mM Tris-HCl, 15 mM MgCl<sub>2</sub>, 500 mM KCl, pH8.3 at 20°C

**Penicillin/Streptomycin:**10,000 U/ml penicillin G sodium and 10,000 µg/ml Streptomycin sulphate in 0.85% saline.

**Phenol (Roth GmbH, Karlsruhe, Germany):** Phenol equilibrated in TE buffer pH 7.5-8.0

**Phenol/chloroform/iso-amyl alcohol:** 50% (v/v) phenol, 48% (v/v) chloroform, 2% (v/v) iso-amyl alcohol.

**Phosphate buffered saline (PBS) pH7.4:** 136 mM NaCl, 2.7 mM KCl, 1.3 mM  $\text{KH}_2\text{PO}_4$ , 1.4 mM  $\text{Na}_2\text{HPO}_4$ .

**Polyacrylamide gel markers (Fermentas):** High molecular weight range (10,000 – 250,000 kDa) PAGE ruler pre-stained protein ladder.

**FACS Quench solution:** 50 mM  $\text{NH}_4\text{Cl}$ , 20 mM glycine in PBS

**Polyacrylamide gel electrophoresis sample buffer (PAGE sample buffer):** 200 mM Tris pH 8.8, 1 M sucrose, 0.01% Bromophenol blue, 5 mM EDTA, and prior to use add 0.5M DTT to 37 mM, and 10% SDS to a final concentration of 3%.

**Restriction endonucleases:** Restriction digests were carried out according to manufacturer's instructions.

**RPMI Medium 1640 (RPMI)**

**Saponin (Calbiochem)** 5% w/v in PBS, 0.22 $\mu\text{m}$  filter sterilise.

**Sodium dodecyl sulphate (10%):** Dissolve 100 g in 900 ml  $\text{H}_2\text{O}$ , heat to 68°C and adjust to pH 7.2 with concentrated HCl (drop wise), adjust volume to 1 litre with  $\text{H}_2\text{O}$

**SDS electrophoresis buffer (10x):** 0.25 M Tris, 2.5 M glycine, pH8.3, dilute 1:10 and add SDS to 0.1%

**Transformation Buffer 1 (TFB1):** 30 mM KAc, 100 mM RbCl<sub>2</sub>, 10mM CaCl<sub>2</sub>, 50 mM MnCl<sub>2</sub>, 15% v/v glycerol, pH 5.8 using 0.2 M acetic acid.

**Transformation Buffer 2 (TFB2):** 10 mM MOPS, 75 mM CaCl<sub>2</sub>, 10 mM RbCl<sub>2</sub>, 15% v/v glycerol, pH 6.5 using 1 M KOH

**Tris-borate buffer (TBE) (10x):** 0.89 M Tris pH8, 0.89 M boric acid, 20 mM EDTA.

**Tris-HCl (1M) pH 8.0:** Dissolve 121.1g of Tris base in 800ml H<sub>2</sub>O, adjust pH to 8.0 by the addition of 42ml concentrated HCl and adjust the volume to 1 litre with H<sub>2</sub>O. Sterilise by autoclaving.

**Trypsin (Gibco) (1x):** 0.5% Trypsin in 0.02% EDTA in PBS

## 2.2: Materials

### 2.2.1: Cell lines

**Table 2.1: Mammalian cell lines and growth conditions**

Cell line	Source	ATCC No.	Growth conditions	Reference
HEK 293 (Passage 29)	Human Embryonic Kidney	CRL-1573	DMEM + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin	Harrison <i>et al.</i> , 1977
A549	Human Lung Carcinoma	CCL-185	DMEM + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin	Giard <i>et al.</i> , 1973
CHO	Chinese Hamster Ovary	CCL-61	DMEM + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin or F12 + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin (dependant on source)	Puck <i>et al.</i> , 1958
CHO-CD46	Chinese Hamster Ovary transfected with human CD46 and neomycin resistance gene	Not listed	DMEM + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin or F12 + 10% FCS, 2mM glutamine, 100 U/ml penicillin, 100µg/ml streptomycin and 225µg G418 (dependant on source)	Liszewski & Atkinson, 1991
CHO-CAR	Chinese Hamster Ovary transfected with human CAR	Not listed	DMEM + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin	Bergelson <i>et al.</i> , 1997
B cell	Human B lymphocyte	Not listed	RPMI + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin	Kindly donated by Tao Dong, University of Oxford
Jurkat	Human leukaemic T cell lymphoblast	TIB-152	RPMI + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin	Weiss <i>et al.</i> , 1984
Lec2	Chinese Hamster Ovary glycosylation mutant	CRL-1736	Alpha + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin	Deutscher <i>et al.</i> , 1984

Pro5	Chinese Hamster Ovary glycosylation mutant	CRL-1781	Alpha MEM + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin	Stanley <i>et al.</i> , 1975
BSC-1	African Green Monkey Kidney epithelial	Not listed	GMEM + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin	Hopps <i>et al.</i> , 1963
P2-2	African Green Monkey Kidney epithelial chronically infected with PVM	Not listed	GMEM + 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100µg/ml streptomycin	Personal communication

## 2.2.2: Viruses and vectors

**Table 2.2: Viruses and vectors used in this study**

<b>Virus</b>	<b>Family</b>	<b>Subgenus</b>	<b>Source</b>
Ad19awt	<i>Adenoviridae</i>	D	Obtained from Hans-Gerhard Burgert (University of Warwick)
Ad19aΔE1ΔE3GFP	<i>Adenoviridae</i>	D	Created in this study by reconstitution of linearised BAC
Ad19aΔE1ΔE3HIVA	<i>Adenoviridae</i>	D	Created in this study by reconstitution of linearised BAC
Ad19aΔE1ΔE3PVM-N	<i>Adenoviridae</i>	D	Created in this study by reconstitution of linearised BAC
Ad5ΔE1ΔE3GFP <sub>sv</sub>	<i>Adenoviridae</i>	C	Created in this study by reconstitution of linearised BAC
Ad5ΔE1ΔE3HIVA <sub>sv</sub>	<i>Adenoviridae</i>	C	Created in this study by reconstitution of linearised BAC
Ad5ΔE1ΔE3HIVA	<i>Adenoviridae</i>	C	Obtained from Tomas Hanke (University of Oxford), generated using the AdEasy Adenoviral vector system
Ad5ΔE1ΔE3PVM-N	<i>Adenoviridae</i>	C	Obtained from Helen Terry (University of Warwick), generated using the AdEasy Adenoviral vector system
Ad19aΔ19K	<i>Adenoviridae</i>	D	Created in this study by reconstitution of



			linearised BAC
Ad19aΔE3	<i>Adenoviridae</i>	D	Obtained from Hans-Gerhard Burgert (University of Warwick)
Ad5F35ΔE1ΔE3GFP	<i>Adenoviridae</i>	C (vector), B (fibre)	Obtained from André Lieber (University of Washington)
PVM	<i>Pneumoviridae</i>	Strain J3666	Obtained from Andrew Easton (University of Warwick)

### 2.2.3: Antibodies for FACS analysis

**Table 2.3: Antibodies used in flow cytometry analysis**

Antibody	Species	Target	Iso-type	Working dilution	Reference
W6/32	Mouse	HLA-A,-B,-C (class I)	IgG2a	100µl/sample (supernatant)	Barnstable <i>et al.</i> 1978
MALI*	<i>Lectin</i>	2→3 sialic acid	N/A	2µg/sample	Vector Labs
MALII*	<i>Lectin</i>	2→3 sialic acid	N/A	2µg/sample	Vector Labs
34-1-2	Mouse	mMHC K <sup>d</sup> D <sup>d</sup>	IgG2a	100µl/sample (supernatant)	Ozato <i>et al.</i> 1982
TW1.3	Mouse	Ad5/Ad2 E3/19K	IgG3	100µl/sample (supernatant)	Cox, J.H. <i>et al.</i> 1991
2Hx-2	Mouse	Ad2 Hexon	IgG2a	100µl/sample (supernatant)	Cepko <i>et al.</i> 1981
E1-1*	Mouse	Coxsackie and adenovirus receptor (CAR)	IgG1	40µl/sample (supernatant)	AbCam
B-G27	Mouse	Fas	IgG2a	1 µg/sample	Komada <i>et al.</i> 1999
J4.48*	Mouse	Human CD46	IgG1	2µg/sample	AbCam
4D1†	Rat	Ad19a E3/49K	IgG2a	100µl/sample (supernatant)	Windheim 2002
L243 (G46-6)*	Mouse	HLA-DR	IgG2a	2µg/sample	BD Pharmingen
M5E2*	Dog	Human CD14	IgG2a	2µg/sample	BD Pharmingen
HI149*	Mouse	Human CD1a	IgG1	2µg/ sample	BD Pharmingen
FM95*	Mouse	Human CD86	IgG1	2µg/sample	Miltenyi Biotech
HB15*	Mouse	Human CD83	IgG1	2µg/sample	Miltenyi Biotech
MOG35	Mouse	Human GD1a	IgG2a	1µg/sample	AnaSpec

Secondary antibody (when required) was FITC conjugated goat anti-mouse IgG (whole molecule) used at 20µg/ml except † when Phycoerythrin conjugated anti -rat IgG (whole molecule) was used at 25µg/ml.

\* Commercial reagents (some information not listed)

## 2.2.4: Primers

**Table 2.4: Primer List**

Primer Name	Sequence	Details
GalKFOR	5'-TAC TGG CTT ATC GAA ATT AAT ACG ACT CAC TAT AGG GAG ACC CAA GCT GGC CTG TTG ACA ATT AAT CAT CGG CA-3'	Forward primer for isolation of GalKKn <sup>R</sup> fragment with CMV promoter homologous ends for recombination from pGPSGalK/Kn <sup>R</sup>
GalKREV	5'-TAA GAT ACA TTG ATG AGT TTG GAC AAA CAA CTA GAA TGC AGT GAA AAG CCA GTG TTA CCA ATT AAC C-3'	Reverse primer for isolation of GalKKn <sup>R</sup> fragment with poly A tail homologous ends for recombination from pGPSGalK/Kn <sup>R</sup>
H1-19KO	5'-ATC TTT ATA TGC TGG GTA AGA CAT TGT GGG GAG GAA CTA TGA AGG GGC TCC CTG TTG ACA ATT AAT CAT CGG CA-3'	Forward primer for isolation of GalKKn <sup>R</sup> fragment with Ad19aE3/19K homologous ends for recombination from pGPSGalK/Kn <sup>R</sup>
H2-19KO	5'-AGC ACG ATA CGG ATC ACT GTA TTC ATG GTT CTG CGA AAA AGA AAA AGA ATG CCA GTG TTA CAA CCA ATT AAC C-3'	Reverse primer for isolation of GalKKn <sup>R</sup> fragment with Ad19aE3/19K homologous ends for recombination from pGPSGalK/Kn <sup>R</sup>
PVM-NIsolationFOR	5'-TGG CAG TAC ATC AAG TGT AT-3'	Forward primer for isolation of PVM-N transgene from pCMVPVM-N
PVM-NIsolationREV	5'-GCT GCA ATA AAC AAG TTA AC-3'	Reverse primer for isolation of PVM-N transgene from pCMVPVM-N
19Kfor	5'-ATC TTT ATA TGC TGG GTA AGA CAT TGT GGG GAG GAA CTA TGA AGG GGC TCT <b>AAG</b> CTT GCT GAT TAT CCT TTC CCT GGT G-3'	Forward primer for isolation of an inactivated form of the Ad19a E3/19K gene with an additional stop codon ( <b>bold</b> ) for recombination into Bad19aΔ19KGalK/Kn <sup>R</sup>
19Krev	5'-AGC ACG ATA CGG ATC ACT ACT GTA TTC- 3'	Reverse primer for isolation of the Ad19a E3/19K gene for recombination into BAD19aΔ19KGalK/Kn <sup>R</sup>

## 2.2.5: Bacterial Artificial Chromosomes (BACS)

**Table 2.5: BACs used in study**

Name	Use	Antibiotic resistance
Bad19awt	Ad19a wild type sequence	Cm
Bad19aΔE1ΔE3GFP	Ad19a E1 and E3 deleted vector sequence containing GFP expression cassette	Cm

Bad19aΔE1ΔE3GalK/Kn <sup>R</sup>	Ad19a E1 and E3 deleted vector sequence containing the GalK/Kn <sup>R</sup> cassette	Cm, Kn
Bad19aΔE1ΔE3HIVA	Ad19a E1 and E3 deleted vector sequence containing the HIVA polyprotein expression cassette	Cm
Bad19aΔE1ΔE3PVM-N	Ad19a E1 and E3 deleted vector sequence containing the PVM nucleocapsid expression cassette	Cm
Bad19aΔ19KGalK/Kn <sup>R</sup>	Ad19a wild type sequence containing the GalK/Kn <sup>R</sup> cassette replacing E319K	Cm, Kn
Bad19a19K*	Ad19a wild type sequence containing inserted stop codon to prevent E319K expression	Cm
Bad5ΔE1ΔE3GFP <sub>sv</sub>	Ad5 E1 and E3 deleted vector sequence containing the GFP expression cassette and additional SV40 enhancer element	Cm
Bad5ΔE1ΔE3GalK/Kn <sup>R</sup> <sub>sv</sub>	Ad5 E1 and E3 deleted vector sequence containing the GalK/Kn <sup>R</sup> cassette and additional SV40 enhancer element	Cm, Kn
Bad5ΔE1ΔE3HIVA <sub>sv</sub>	Ad5 E1 and E3 deleted vector sequence containing the HIVA polyprotein expression cassette and additional SV40 enhancer element	Cm

## 2.2.6: Plasmids

**Table 2.6: Plasmids used in study**

Name	Use	Antibiotic resistance
pEGFPN1	Backbone for generation of HIVA shuttle plasmid	Kn
pHIVAOx	Isolation of HIVA polyprotein for generation of HIVA shuttle plasmid	Amp
pGPSGalK/Kn <sup>R</sup>	PCR isolation of GalK/Kn <sup>R</sup> cassette with homologous ends for recombination	Kn
pCMVHIVAOx	Isolation of HIVA polyprotein expression cassette for recombination	Kn
pCMVPVM-N	Isolation of PVM nucleocapsid expression cassette for recombination	Kn

## 2.2.7: Bacterial Strains

**Table 2.7: Bacterial strains used in study**

Name	Use	Growth restrictions
SW102	Designed for BAC recombineering using <i>galK</i> positive/negative selection. Contains a fully functional <i>gal</i> operon, except the <i>galK</i> gene is deleted.	Growth at 32°C prevents recombination. Growth at 42°C activates temperature sensitive repressor and subsequently allows recombination
PIR1	Maintenance of pGPSGalK/Kn <sup>R</sup>	None
DH10B	Maintenance of BACs	None
DH5 $\alpha$	Maintenance of plasmids	None

## 2.2.8: Sequencing Primers

**Table 2.8: Primers used in sequencing studies**

Primer Name	Sequence	Details
19KSEQPRIME	5'-ACG AAG TGG TCG GGT ATT TG-3'	Primer for sequencing of 19K mutation
PVM-N/F/1	5'-GGT TTC GAT TGC GGT GTT TT-3'	Primer for sequencing of PVM nucleocapsid expression cassette
PVM-N/F/2	5'-CAG AGA TGA TAG CAT CAA GAT-3'	Primer for sequencing of PVM nucleocapsid expression cassette
PVM-N/F/3	5'-TCT CTT TGA CCA ATT GTC CT-3'	Primer for sequencing of PVM nucleocapsid expression cassette
HIVA/F/1	5'-TTC TGC AGT CAC CGT CCT TG-3'	Primer for sequencing of HIVA polyprotein expression cassette
HIVA/F/2	5'- ACC CTG GAG GAG ATG ATG AC-3'	Primer for sequencing of HIVA polyprotein expression cassette
HIVA/F/3	5'- TGA CCT ACA AGG CCG TGG AC-3'	Primer for sequencing of HIVA polyprotein expression cassette

## 2.2.9: Antibodies for Western Blot analysis

**Table 2.9: Antibodies used in western blot analysis**

Clone	Species	Target	Iso-type	Dilution	Reference
SV5-Pk1	Mouse	V5-TAG	IgG2a	1:3000	Southern <i>et al.</i> , 1991
R48	Rabbit	Ad19a E3/49K	Polyclonal	1:1000	Windheim 2002
R22612	Rabbit	A19a E3/19K	Polyclonal	1:500	Deryckere & Burgert 1996
R2052	Rabbit	PVM nucleocapsid	Polyclonal	1:1500	Barr, 1993
2-28-33	multiple	$\beta$ - tubulin	IgG1	1:200	Siddiqui <i>et al.</i> , 1989

## 2.2.10: Antibodies for Competition studies

**Table 2.10: Antibodies used in competition studies**

Clone	Species	Target	Iso-type	Source	Reference
$\alpha$ CD46FII	Mouse	CD46 (MCP1)	IgG1	Claire Harris (University of Cardiff)	Unpublished
$\alpha$ CD46POLY	Rabbit	CD46 MCP1-IgG4 Fusion	Polyclonal	Claire Harris (University of Cardiff)	Unpublished

## 2.2.11: Soluble proteins for Competition studies

**Table 2.11: Soluble proteins used in competition studies**

Protein	Description	Source	Reference
MCP-BC-IgG4	CD46 (MCP1) fused to human IgG4	Claire Harris (University of Cardiff)	N/A
rhCXADR/Fc	CAR (Leu20-Gly237) fused to human IgG1	R&D systems	N/A

## 2.3: Mammalian cell lines

### 2.3.1: Cell lines

See Table 2.1

### 2.3.2: Maintenance of mammalian cell culture

Mammalian cell lines were maintained at 37°C in a 5% CO<sub>2</sub> incubator in a humidified atmosphere using the appropriate media (chapter 2.2.1). All manipulations were performed under sterile conditions in a Class II Laminar Flow cabinet using standard aseptic techniques. All centrifugation steps were performed in an Eppendorf 5810R centrifuge unless otherwise stated.

#### 2.3.2.1: Adherent cell lines

When confluent, adherent cells were passaged as follows: cells were washed with PBS, incubated with Trypsin-EDTA for 5-10 mins and detached by mechanical motion and returned to original volume with appropriate medium. Typically the cell monolayer of a 25 cm<sup>2</sup> flask was washed with 5ml PBS, trypsinised with 0.7 ml Trypsin-EDTA and resuspended in 5.3ml of appropriate medium and then subsequently re-seeded at ratios between 1:4 and 1:10.

### **2.3.2.2: Non-adherent cell lines**

When confluent, non-adherent cells were passaged as follows: cells were centrifuged (350xg for 3 mins), resuspended in PBS, centrifuged (350xg for 3 mins) and resuspended in original volume of appropriate medium. Cells were re-seeded at ratios between 1:6 and 1:10.

### **2.3.2.3: Long term storage of Mammalian cell lines**

Sub-confluent cell monolayers were washed and detached as described previously (section 2.2.1/2.2.2). Resuspended cells were centrifuged (300xg for 5 min) and resuspended in freezing buffer and frozen in 1 ml aliquots by cooling slowly in a pre-chilled Styrofoam box placed in a  $-70^{\circ}\text{C}$  freezer, before being placed in liquid nitrogen for long-term storage.

### **2.2.3.4: Recovery of mammalian cell lines from long term storage**

Cells were taken out of liquid nitrogen and thawed rapidly in a  $37^{\circ}\text{C}$  water bath before being layered onto 3ml FCS and centrifuged (300xg for 5 min) to remove DMSO. The cells were re-suspended in pre-warmed 1ml appropriate media and split 0.7ml and 0.3ml into two flasks of 10ml complete medium.

## **2.4: Generation of virus stocks**

### **2.4.1: Generation of adenovirus stocks**

Infectious medium was generated by the addition of a calculated amount of virus to give a multiplicity of infection (MOI) of 10 pfu/cell to DMEM without FCS. Subconfluent A549 cells were then washed with PBS, and then infected (by the addition of the infectious medium in an appropriate volume for the vessel used) with Ad5, Ad19a, Ad19a $\Delta$ E319K or Ad19a $\Delta$ E3 and incubated at  $37^{\circ}\text{C}/5\% \text{CO}_2$  and monitored for cytopathic effect (c.p.e.). Once the c.p.e. was judged to have reached 100% i.e. 100% of the cells were rounded and no longer adherent, which generally took 48-72 hours, cells were harvested by centrifugation (350xg for 7 minutes at  $4^{\circ}\text{C}$ ) and the pellets resuspended in 2 ml of sterile 30mM Tris-HCL pH 8.0 per

175cm<sup>2</sup> flask. Virus was released by three to four freeze thaw cycles before centrifugation (1800xg for 7 min at 4°C) to remove cell debris. Virus aliquots were stored long term at -80°.

#### **2.4.2: Generation of adenovirus vector stocks**

Subconfluent 293 cells were infected at an MOI of 10 with rAd5HIVAsv, rAd5PVM-N, rAd5HIVAn, rAd5GFP, rAd19aHIVAsv, rAd19aPVM-N, rAd19aGFP or rAd5F35GFP and incubated at 37°C/5% CO<sub>2</sub> until c.p.e. reached 100%, which generally took 48-60 hours. Cells were harvested by centrifuging (350xg for 7 minutes) at 4°C and the pellets re-suspended in 2 ml of sterile 30mM Tris-HCL pH 8.0. Virus was released by three to four freeze/thaw cycles before centrifugation at 1800xg for 7 minutes at 4°C in an eppendorf 5810R centrifuge, to remove cell debris. Virus aliquots were stored long term at -80°.

#### **2.4.3: Generation of PVM virus stocks**

BSC-1 cells were infected at an MOI of 50. After the c.p.e. effect had reached 100%, here characterised by the formation of syncytia followed by the loss of adherence, the infected cells were removed from the flasks by mechanical motion, centrifuged (300xg, 7 min, 4°C) and re-suspended in GMEM + 0% FCS. Virus aliquots were stored long term at -80°.

#### **2.4.4: Adenovirus purification**

293 or A549 cells were grown to subconfluence in 15 large flasks and infected with an MOI of 1 with adenovirus vector or virus and grown until at least 75% c.p.e. was evident, generally 48-72 hours. Cells were re-suspended by mechanical motion, harvested by centrifugation (350xg for 5 min at 4°C), re-suspended in 2ml DMEM + 0% FCS per 175cm<sup>2</sup> flask, pooled, harvested again (as before) and re-suspended in 15 ml 30mM Tris pH8.0. The cell suspension was sonicated (using a 20 % duty cycle for 10 seconds at a micro tip limit of 2)(Jenson Scientific Ultrasonic Processor), followed by centrifugation (3200xg for 10 min). The supernatant was collected for further purification. Caesium chloride (CsCl) gradients were prepared in SW41 tubes (Beckmann Optima) by the addition of 3ml 1.42 g/ml CsCl to the base of an SW41

tube which already contained 5ml 1.27 g/ml CsCl using a Pasteur pipette and subsequently 5ml viral cell lysate was layered on top. The tubes were weighed and balanced to ensure equal weight and placed in an SW40 rotor (Beckman Optima). The gradients were centrifuged (154693 xg for 1 hour at 15°C; Beckman Optima L90K) with no brake, removed and clamped. The virus band was removed by an 18G needle and 5ml syringe by side puncture. Harvested virus was pooled in an SW50 tube (Beckmann Optima), and the tube filled with 1.35 g/ml CsCl, weighed and balanced and placed in an SW50 rotor (Beckman Optima). The gradients were centrifuged (151693 xg for 20 hours at 15°C; Beckman Optima L90K), removed, clamped, the virus band harvested by 18G needle and 5ml syringe by side puncture and made up to a total volume of 2.5 ml with PBS. PD-10 columns (GE Healthcare) were equilibrated according to manufacturer's instructions, the virus loaded onto the column, eluted with 3.5 ml PBS, disposing of the first 0.5 ml eluant, collecting the following 2 ml and disposing of any remaining eluant. 200 µl sterile 100% glycerol was added to the 2ml fraction, mixed, aliquoted and stored for later titration and use.

## **2.5: Adenovirus infection of mammalian cells**

### **2.5.1: Infection/transduction of adherent cell lines**

Cells were seeded into 60mm dishes and grown to subconfluence. Cells in three control dishes were detached and counted before each experiment. For infection varying pfu/cell of virus was added per dish and cells were incubated for 1.5h at 37°C/5% CO<sub>2</sub> in 1 ml DMEM without FCS. Thereafter, unless otherwise stated, the virus was removed and the cells incubated at 37°C/5% CO<sub>2</sub> in 5 ml DMEM containing 2% FCS for a further 44h.

### **2.5.2: Infection/transduction of non-adherent cell lines**

Cells were seeded into 5ml of their respective media in a 6cm dish and grown to subconfluence. 10 µl of cells from two control dishes were counted before each experiment. Cells were centrifuged (300xg, 5 min) and re-suspended in media containing 0% FCS and varying pfu/cell of virus then incubated for 1.5h at 37°C/5% CO<sub>2</sub> in a 15 ml Falcon tube. Thereafter the cells are returned to a 6cm dish with media containing 2% FCS and, unless otherwise stated, incubated at 37°C/5% CO<sub>2</sub> for a further 44h.



## **2.6: Flow cytometry**

### **2.6.1: Surface staining**

The cells were grown to ~90% confluence in 60mm cell culture plates and harvested by washing briefly with PBS, followed by treatment with 0.5% trypsin-EDTA to detach the monolayer. After re-suspension in 5 ml of media, the cells were centrifuged at 300xg for 7 minutes at 4°C. The cells were then counted and re-suspended in FACS-buffer (4°C) to give approximately 500,000 cells/sample in a volume of 30 µl. Each sample was incubated with the relevant antibody (see table 2.3) on ice for 50 minutes in a total volume of 130 µl, using a flat bottom 96 well tissue culture plate. Unbound antibody was then removed by washing three times with 200 µl FACS buffer (4°C), pelleting cells by centrifuging at 340xg for 3 minutes at 4°C between washes. The cells were incubated with secondary antibody in a total volume of 50 µl for 50 minutes on ice, followed by four washes as previously described. They were then either fixed in the plate by the addition of FACS-FIX buffer and placed at 4°C in the dark overnight, or re-suspended in 100 µl FACS buffer (4°C) and analysed immediately in a final volume of 400 µl using a Beckton Dickinson FACScan flow-cytometer and Cellquest™ analysis programme. Emission fluorescence was recorded at 470 nm unless otherwise stated.

### **2.6.2: Intracellular staining**

Following harvesting, cells were re-suspended in 0.5 ml medium, washed with ice-cold PBS and centrifuged as before. The supernatant was carefully removed and the cells re-suspended in 450 µl PBS at room temperature (RT) and transferred to eppendorf tubes. 50µl of BD cellFIX was added to each sample with gentle vortexing and the samples incubated at RT for 20 minutes with occasional vortexing. The samples were then washed with 600 µl PBS at room temperature, centrifuged for 3 minutes at 800xg in a micro-centrifuge, the supernatant was removed, and the cells were re-suspended in 500 µl FACS-quench buffer for a minimum of 30 minutes at RT. Next, the cells were washed with 600 µl of PBS at RT and centrifuged as before, the supernatant removed and the cells re-suspended in FACS buffer to give approximately 500,000 cells/sample, as described previously. The antibody incubation protocol was the same as that for the unfixed cells, except that cells were

incubated with antibodies in the presence of 0.1% saponin, and washed with FACS+Saponin buffer. The antibodies used for FACS analysis are listed in Table 2.3 below.

## **2.7: Preparation of virus infected cells for flow cytometry analysis**

The cells were grown to sub-confluence in 60 mm tissue culture plates, washed once with serum free DMEM and infected with either adenovirus or adenovirus vectors at varying MOIs. 48 hours post-infection (p.i.), cells were harvested by trypsinisation, any cells that were detached prior to trypsinisation were also harvested. Cells were pelleted by centrifugation at 300xg for 7 minutes at 4°C, the supernatant removed and cells either stained as in chapter 2.5.2. or examined for GFP fluorescence using a Beckton Dickinson FACScan flow-cytometer and Cellquest™ analysis programme. Emission fluorescence was recorded at 470 nm. Isotype controls were performed by using the W6/32 Antibody and negative controls for GFP staining by the analysis of uninfected cells which should be negative for GFP fluorescence.

## **2.8: Preparation of DNA constructs**

All DNA was quantified by Nanodrop ND-1000 spectrophotometer (Thermo Scientific) according to manufacturer's instructions.

### **2.8.1: Polymerase Chain reaction (PCR)**

A standard PCR was performed in a final volume of 100µl. This contained 150ng of template DNA, 0.5µM primer mixture, 1 X PCR mixture (2.5µl 10 X PCR buffer + MgCl<sub>2</sub>, 200µM dNTPs.), 2.5 units Taq polymerase (Invitrogen) and made up to a total volume with sterile water. The solution was subjected to a 'touchdown' programme consisting of 1 cycle of 95°C for 4 min and 55°C for 1 min 30 sec., 20 cycles of 72°C for 3 min., 95°C for 45 sec., and 45°C (increasing every 2 cycles by 1°C ending at 55°C) for 1 min 30 sec., 10 cycles of 72°C for 3 min, 95°C for 45 sec., 55°C for 1min 30 sec and 1 cycle 72°C for 10 min followed by a 4°C soak until the sample could be removed for processing. 5µl of resultant PCR mixture was then analysed by 0.8% agarose gel electrophoresis. In stated cases adjustments of the thermal cycling parameters were necessary to improve DNA yield.

### **2.8.2: Agarose Gel electrophoresis**

DNA was separated by agarose gel electrophoresis and subsequently visualised by staining with ethidium bromide (EtBr). Agarose powder (0.8-1.2% w/v) was dissolved by boiling in 1x TBE and EtBr was added to give a final concentration of 0.5 µg/ml. The gels were run using a buffer of 1xTBE containing 0.5 µg/ml EtBr, under a constant voltage of 100V. DNA samples were mixed with 1/6 of the sample volume of DNA gel loading buffer (6x) and loaded on to the gel. Gel Bands were visualised and images taken on an UVIDoc trans-illuminator. DNA bands were excised using a UVP trans-illuminator (UV products Incorporated, USA.) and DNA gel purification performed.

### **2.8.3: DNA gel purification**

Bands were excised from the agarose gel using a clean scalpel, and DNA purification was carried out using a GFX™ PCR DNA and Gel band purification kit (GE Healthcare), according to the manufacturer's instructions.

### **2.8.4: DNA restriction digest**

Restriction enzyme digests were performed under the manufacturers recommended conditions using the supplied 10X buffer solutions (section 2.1).

### **2.8.5: Ligation reactions**

Ligations were carried out with a 3:1 molar ratio of insert to vector using T4 DNA ligase (Invitrogen), following the manufacturer's guidelines for the ligation of cohesive ends

### **2.8.6: Bacterial Artificial Chromosomes (BACs)**

All BACs were either constructed in the study, as stated, or obtained from Z. Ruzsics, University of Munich. See Table 2.5

### **2.8.7: Plasmids**

Plasmids were either constructed in the study, as stated, or obtained from Z. Ruzsics, University of Munich. See Table 2.6.

## **2.9: DNA propagation in bacteria**

### **2.9.1: Bacterial strains**

All bacterial strains were obtained from Dr. Z. Ruzsics, University of Munich. See Table 2.7.

### **2.9.2: Preparation of competent cells**

A starter culture was prepared by inoculating 5 ml of LB medium with a single colony from a plate of bacteria, and incubating overnight at 37°C with shaking (Gallenkamp orbital shaker). This was used to inoculate 1 litre of LB medium. The bacteria were grown at 37°C (32°C SW102) as before until the OD<sub>600</sub> was between 0.42 and 0.45, mid-log phase. The culture of bacteria was then placed on ice for five minutes before harvesting at 6000xg, for 15 minutes, at 4°C in a Beckman JA10 rotor. The supernatant was removed and the pellet carefully resuspended in 100 ml of pre-chilled TFB1, before incubating on ice for 1 hour. The cells were harvested as before and resuspended in 20 ml of pre-chilled TFB2, 100 µl aliquots were then rapidly frozen in an isopropanol/dry ice bath and stored at -80°C. Cells were tested for competency prior to use.

### **2.9.3: Transformation of competent cells**

Competent bacteria were thawed on ice, 10 µl of DNA added to them, mixed gently and incubated on ice for 30 minutes. The bacteria were heat-shocked at 42°C for 1 minute 30 seconds, then 900 µl of pre-warmed LB medium was added and the cells incubated at 37°C (32°C SW102) for 1 hour with shaking. 100 µl of bacteria were spread onto an LB-Amp plate. The remaining 900 µl were centrifuged at 16,000xg for 1 minute in a micro-centrifuge, the supernatant removed, and the bacterial pellet resuspended in 100 µl of LB and spread on an LB plate supplemented with the

relevant antibiotic (section 2.7.6 or 2.7.7). Plates were then incubated at 37°C (32°C SW102) overnight.

#### **2.9.4: Small scale DNA preparation (mini-preps)**

Single transformed bacterial colonies were picked and grown overnight in 5 ml cultures of LB medium, supplemented with appropriate antibiotic (section 2.7.6 or 2.7.7), at 37°C (32°C SW102) with shaking (GallenKamp Orbital shaker). 1 ml of overnight culture was harvested by centrifuging at 16,000xg for 3 minutes in a microcentrifuge, before DNA was prepared using the Qiagen mini-prep kit according to the manufacturer's instruction.

#### **2.9.5: Large Scale DNA preparation (maxi-preps)**

A starter culture was prepared by inoculating 5 ml of LB medium supplemented with appropriate antibiotic (section 2.7.6 or 2.7.7) with a single colony from a freshly streaked plate and incubating at 37°C (32°C SW102) with shaking for approximately 8 hours. The starter culture was then diluted 1:500 into 200 ml of LB medium, supplemented with the relevant antibiotic (section 2.7.6 or 2.7.7) and incubated at 37°C overnight with shaking (GallenKamp orbital shaker). Bacteria were harvested by centrifugation at 6000xg, for 15 minutes, at 4°C in a Beckman JA10 rotor, before DNA was prepared using the Qiagen maxi-prep kit (plasmids) or Machery Nagel AX100 maxi-prep kit (BACs) according to manufacturer's instructions.

#### **2.10: Sequencing reactions**

250-500 ng of PCR amplified template or 1.5 µg BAC DNA was mixed with 5.5 pmol of appropriate primer in a total volume of 10 µl, made up with H<sub>2</sub>O. Sequencing reactions were carried out using the Applied Biosystems Big Dye Terminator Version 3.1 chemistry and analysed on an ABI PRISM 3130xl Genetic analyser by the in house molecular biology service. For sequencing primers used see Table 2.8.

## **2.11: Preparation of DNA for BAC transfection**

### **2.11.1: Linearisation of DNA**

DNA was linearised by cutting 1µg with 10U/µg PacI (NEB) in a total volume of 100µl at 37°C overnight.

### **2.11.2: Phenol/Chloroform extraction**

An equal volume of phenol/chloroform/iso-amyl alcohol solution was added to the DNA, and the sample vortexed for 1 minute, before being centrifuged for 2 minutes at 16,000xg in a micro-centrifuge. The upper layer was transferred to a clean tube and an equal volume of chloroform/iso-amyl alcohol solution added. The sample was vortexed for 1 minute and centrifuged as before. The upper layer was transferred to a clean tube and made up to 250 µl with distilled H<sub>2</sub>O, before adding 1/10 of the sample volume (25 µl) of Sodium Acetate pH5 and mixing. Finally 2.5x the sample volume (687.5 µl) of cold 100% ethanol was added and the tube inverted several times. The DNA was stored at -20°C until needed.

### **2.11.3: Re-suspension of DNA**

DNA was centrifuged at 16,000xg for 30 minutes at 4°C in a micro-centrifuge, the ethanol supernatant was removed by careful decanting, and the pellet washed with 600 µl of ice cold 70% ethanol, under sterile conditions. Samples were centrifuged at 16,000xg for 20 minutes at 4°C, and the ethanol was removed by careful decanting, and the pellet dried by aspirating. The pellet was re-suspended in sterile H<sub>2</sub>O (v/v) to give a concentration of approximately 100ng/µl.

## **2.12: Transfection of mammalian cells for vector reconstitution**

For transient transfections, 293 cells were grown to 80-85% confluence in 12 well plates and transfected with 1µg linear BAC DNA and 3µl Lipofectamine 2000 (Invitrogen) in 200µl OptiMEM. At 5 hours post-transfection, the DNA mix was removed and the cells overlaid with 2 ml DMEM 2% FCS and incubated for a further 96 hours before further passage until c.p.e. was seen and vector harvested.

### **2.13: SDS-Polyacrylamide Gel Electrophoresis (PAGE)**

Proteins were separated by electrophoresis on discontinuous SDS-polyacrylamide gels according to the method of Laemmli (1970). Cell samples were boiled in PAGE sample buffer for 5-10 mins. Samples and molecular weight PAGE ruler plus™ markers (Fermentas) were loaded onto an upper 5 % stacking gel (120 mM Tris-HCl pH 6.8, 5 % (w/v) acrylamide, 0.12 % (w/v) N.N'-methylene-bis-acrylamide, 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulphate and 0.6 µl/ml TEMED) and proteins were separated as they passed through a lower 10 % resolving gel (430 mM Tris-HCl pH 8.8, 10 % (w/v) acrylamide, 0.27 % (w/v) N.N'-methylene-bis-acrylamide, 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulphate and 0.3 µl/ml TEMED). Electrophoresis was performed for 90 min at 130 V in running buffer using the Mini PROTEAN® 3 cell system (Bio-Rad) according to the manufacturer's instructions.

### **2.14: Protein transfer and Western blotting**

Following separation by SDS-PAGE, proteins were transferred to a Hybond™-ECL nitrocellulose membrane (Amersham Life Science) using the Mini PROTEAN® 3 cell system (Bio-Rad) according to manufacturer's instructions at 300mA for 75 minutes. Identification of molecular weight markers on the membrane enabled transfer of protein to be assessed. Membranes were placed in blocking solution (PBS containing 2 % (w/v) milk powder and 0.05% (v/v) Tween-20) for 16 hours at 4 °C. The next day membranes were washed twice in PBS/0.05%Tween-20 at 4°C to remove excess blocking reagents, followed by incubation with primary antibody diluted in PBS/0.05%Tween-20 for 1 hour at RT on a roller. In order to remove excess primary antibody the membranes were washed several times in PBS/0.05%Tween-20, before incubation with the secondary antibody diluted in PBS/0.05%Tween-20 as before. Following extensive washes to remove excess antibody, the proteins were detected using the Pierce® ECL Western blotting detection system (Thermo Scientific) according to the manufacturer's instructions

## **2.15: Preparation of Dendritic cells**

### **2.15.1: Isolation of Peripheral Blood Mononuclear Cells (PBMCs)**

Buffy coats were obtained from the National Blood Service in Birmingham. Whole blood was transferred to Falcon tubes and diluted 1:2 with pre-warmed RPMI to a total volume of 30 ml. The resulting mixture was layered on to 15 ml of Lymphoprep (Axis-Shield) and centrifuged (800xg for 30min) with the brake de-activated. The defined white band of lymphocytes was removed using a Pasteur pipette, pooled, diluted to 40 ml with RPMI, centrifuged (500xg for 15 min), the supernatant removed, pellet re-suspended in 40 ml RPMI and the lymphocytes counted. CD14 positive monocytes were then isolated via one of two methodologies.

### **2.15.2: Isolation of CD14+ monocytes by MACS separation**

After counting, the cells were pelleted (350xg for 10 min) and re-suspended in 80µl MACS buffer and 20 µl CD14 microbeads for the isolation of human monocytes (Miltenyi Biotech) per  $1 \times 10^7$  cells, mixed and incubated for 15 min at 4°C. The cells were washed in 25 ml MACS buffer, pelleted (350xg for 3 min), supernatant removed and re-suspended in 3ml MACS buffer. An LS MACs column (Miltenyi Biotech) was placed in the magnetic field of a MACS separator (Miltenyi Biotech) and rinsed with 3 ml of MACS buffer before the cell suspension was applied to the column and allowed to pass through, the unlabelled cell fraction collected and the column washed three times with 3 ml MACS buffer with the eluted fractions added to the unlabelled cell fraction. The column was then removed from the separator, placed onto a 15 ml Falcon, 5 ml MACS buffer applied to the column and immediately flushed by applying the plunger supplied with the column. The cells were counted, adjusted to  $5 \times 10^5$  cells/ml to a 6 well dish in RPMI + 5% FCS, 800 U/ml Granulocyte-macrophage colony-stimulating factor (GM-CSF) and 1000 U/ml Interleukin 4 (IL-4) (Immunotools) added to each dish and cultured for 5 days before being assessed markers of immature Dendritic cells (iDCs). Subsequent treatment with 25 ng/ml tumor necrosis factor alpha (TNF $\alpha$ ) or 1 µg/ml Lipopolysaccharide (LPS) further differentiates the iDCs into mature dendritic cells (mDCs) within 48 hours.



### **2.15.3: Isolation of CD14+ monocytes by adherence**

After counting the number of cells (generally  $\sim 1 \times 10^8$ ) the cells were pelleted (350xg for 10 min) and resuspended in 10 ml RPMI + 5% FCS and placed in a 75cm<sup>2</sup> cell culture flask at 37°C + 5% CO<sub>2</sub>. After 30 minutes 4 ml of media was removed from the flask and replaced with an equal volume of fresh RPMI + 2% FCS, after a further 30 minutes 8 ml was removed and replaced as before. After a final 30 minutes the flask was agitated, the remaining media removed, replaced with PBS and the flask agitated again. This process was repeated twice before the remaining adherent cells were cultured as before in RPMI + 5% FCS, 800 U/ml GM-CSF and 1000 U/ml IL-4 (Immunotools) for 5 days before being assessed for markers of iDCs. Once again subsequent treatment with 25 ng/ml tumor necrosis factor alpha (TNF $\alpha$ ) or 1  $\mu$ g/ml Lipopolysaccharide (LPS) further differentiates the iDCs into mature dendritic cells (DCs).

### **2.16: Virus titration**

#### **2.16.1: Adenovirus plaque assays**

A limiting dilution plaque assay was used to determine the infective titre of Ad19a and Ad5 viruses in plaque forming units per millilitre (pfu/ml). A549 cells were utilised for Ad viruses and the E1A expressing 293 cell line used to complement E1A deleted Ad vectors. The cells were seeded into 60mm dishes and grown to subconfluence. Cells were washed once in PBS, infected with a ten-fold serial dilution of each virus preparation in DMEM without FCS and incubated for 1.5h at 37°C. The virus was removed and the cells overlaid with 4.5ml of overlay medium (2xDMEM, 20 mM HEPES, 4% FCS, 10% NaHCO<sub>3</sub>, 2% penicillin-streptomycin, 1% Glutamine) diluted 1x in 1.8% Agarose. The overlay was allowed to solidify and the cells were incubated at 37°C for five days when they were overlaid with another 4.5ml overlay medium. This was repeated twice at ten days and fifteen days followed by a 3ml overlay at twenty days. The plaques were counted at day twenty-one for Ad5 and day twenty-eight for Ad19a. Counts were performed by determining the numbers of plaques as a two plate mean at a chosen concentration (generally the dilution which gives between 10-100 plaques was chosen). The mean number of plaques is then multiplied by the dilution factor to give the resultant number of pfu/ml.

### **2.16.2: PVM plaque assays**

A limiting dilution plaque assay was also used to determine the infective titre of PVM virus. BSC-1 cells were seeded in 12 well plates and grown to subconfluence. Cells were washed once in PBS, infected with a ten-fold serial dilution of PVM in GMEM without FCS and placed at 31°C for one hour. The virus was removed and the cells overlaid with 2 ml of overlay medium (which was a 1:1 mixture of 4% CMC Agar and GMEM + 2% FCS) and incubated at 31°C for ten days. After 10 days the cells were fixed by addition of 1 ml 2% Gluteraldehyde to each well and left for 1 hour at room temperature. The overlay medium was then poured off, the plates dried and 1ml crystal violet added to each well for 15 min. The plates were then rinsed with water, dried at 37°C, the plaques counted and the viral titre determined.

### **2.16.3: Adenovirus particle number calculation**

Once purified the particle number of an Ad vector or virus was calculated based on a modified methodology of Mittereder *et al.*, 1996. Briefly, a sample of purified virus was taken, diluted 1:20 in TE + 0.1% SDS, incubated in a heat block at 56°C for 10 mins and a 260 nm optical density reading was taken using a nanodrop as previously described. This reading was multiplied by 20 (the original dilution factor) and compared by ratio with known particle number standards for Ad5 (where an OD<sub>260</sub> nm reading of 1 =  $1 \times 10^{12}$  particles/ml).

## **2.17: Recombineering**

Recombineering was performed using a modification of the methodology of Warming *et al.*, 2005.

### **2.17.1: GalK/Kn<sup>R</sup> recombination**

500 µl of an overnight culture of SW102 cells transformed with the BAC to be recombined was diluted in 25 ml LB medium with or without antibiotic selection (see chapter 2.7.6) in a 100 ml conical flask and grown at 30°C in a shaking incubator (Gallenkamp Orbital Shaker) to an OD<sub>600</sub> of 0.6. Then, 10 ml was transferred to another 100 ml conical flask and heat-shocked at 42°C for exactly 15 min in a

shaking waterbath (Techne SB-16). The 15 ml remaining culture was left at 32°C as the uninduced control. After 15 min the two samples were cooled in an ice bath and transferred to two 15 ml Falcon tubes and pelleted at 500xg (Eppendorf centrifuge 5810R) at 4°C for 5 min. The supernatant was removed, the pellet resuspended in 1 ml ice-cold ddH<sub>2</sub>O by gently swirling the tubes in a water bath, 9 ml ice-cold ddH<sub>2</sub>O was added and the samples pelleted again. The supernatant was removed, and the pellet resuspended in 100 µl ddH<sub>2</sub>O and electroporated in a 0.1 cm cuvette (GeneFlow Ltd.) at 25 µF, 2.5 kV and 200 Ω with 250 ng *galK/Kn<sup>R</sup>* PCR product. After electroporation the bacteria were recovered in 1 ml LB in a 1.5 ml eppendorf for 1 h in a 32°C shaking waterbath. Both the induced and uninduced cultures were plated on MacConkey agar-cm/kan plates and incubated overnight at 30°C. The following morning the plates were inspected and normally no colonies were present on uninduced control plates. Surviving red colonies on the induced plate were selected and checked by restriction digest for successful *GalK/Kn<sup>R</sup>* recombination.

### **2.17.2: Second stage recombination**

Second stage recombination was performed as in chapter 2.16.1 using SW102 cells transformed with the required *galK/Kn<sup>R</sup>* containing BAC until electroporation. The culture was electroporated with 1 µg PCR product or gel purified fragment. The bacteria were then recovered in 10 ml LB in a 100 ml conical flask and incubated for 4.5 h in a 30°C shaking incubator. After the recovery period, the bacteria were washed twice in M9 medium as follows: 1 ml culture was pelleted in an Eppendorf tube at 13 200 r.p.m. for 30 s, the supernatant was removed and resuspended in 1ml M9 medium. Serial dilutions were plated on M63 agar plates with required antibiotic selection (see chapter 2.7.6). Plates were sealed with Parafilm<sup>®</sup> and incubated at 30°C for 120 hours. The plates were inspected and surviving colonies on the induced M63 agar plates were selected and checked for successful second stage recombination.

### **2.18: Animal work**

Female BALB/c mice (*H-2<sup>d</sup>*), 5 to 8 weeks old, were purchased from B&K Universal and acclimated for 1 week prior to experimentation. Mice were kept in accordance

with UK Home Office welfare guidelines and project licence restrictions and weighed as a group every 48 hour prior to virus challenge and every 24 hours thereafter.

#### **2.18.1: Intra peritoneal anaesthetisation**

Mice were intraperitoneally anaesthetised with 100mg/kg Ketamine (Ketaset, Fort Dodge) and 0.1mg/kg Xylazine Hydrochloride (Sigma) and returned to a holding cage until under the anaesthetic's effect, normally 2-4 min.

#### **2.18.2: Intranasal inoculation**

Mice were intranasally inoculated with 50 µl of adenovirus vector or PBS by applying drops of the fluid to the left and right nostrils, allowing the fluid to be absorbed by normal respiration. Subsequently the mouse was laid on its side until recovery from the anaesthetic.

#### **2.18.3: Tail tip blood**

Whilst under the anaesthetic's effect, when appropriate, 1 mm of the tip of the tail was removed by scissors and approximately 20 µl of blood harvested by 'milking' the tail.

#### **2.18.4: Sacrifice**

Mice were sacrificed according to Home Office regulations by cervical dislocation. The mouse was held by its tail and placed on a surface that it could grip, a finger placed firmly across the back of the neck. A sharp pull on the base of the tail was then applied to dislocate the neck.

### **2.19: Indirect Enzyme-linked immunosorbent assay (Indirect ELISA)**

Indirect ELISAs were performed according to Sambrook *et al.*, 1989. Briefly, 96 well plates were coated with either 50 µl/well of 20 µg/ml BSC-1 or P2-2 lysate to assay for antibody response to PVM or 50 µl/well of 1 µg/ml purified Ad19a and Ad5 vectors to assay for antibody response to each vector and incubated O/N at 4°C.

The plates were washed five times with 200  $\mu$ l/well PBS/Tween using a Biotrak II plate washer (Amersham Biosciences). The plates were blocked with 100  $\mu$ l/well 5% Milk powder in PBS/Tween before washing again. Sera samples were diluted 1:70 and diluted 3 fold across a 96 well plate. The serum antibodies were incubated with Horse Radish Peroxidase (HRP) conjugated goat anti-mouse IgG (whole molecule) or HRP conjugated goat anti-rabbit IgG (whole molecule) where stated. The presence of antibodies was quantified by the measurement of flurogenic activity by the addition of ABTS substrate and detected at 605nm using a RC Mutliscan (Labsystems Inc.).

## **2.20: Enzyme-linked immuno spot (ELISPOT)**

ELISPOTs were performed at the John Radcliffe hospital (Oxford) by Dr. Anne Bridgemann according to Czerkinsky *et al.*, 1983.

## **2.21: Competition Assays**

### **2.21.1: Monoclonal or Polyclonal Antibody Competition Assay**

Mammalian cell lines were grown to 80% confluence in 96 well plates, the media removed, 50  $\mu$ l DMEM without FCS containing no antibody or varying concentrations of monoclonal or polyclonal antibodies (see table 2.8) added and the plates incubated at 4°C for 1 hour. After 1 hour 50  $\mu$ l of Ad5GFP, Ad5F35GFP or Ad19aGFP at an MOI of 3 or 10 was added, incubated at 4°C for 30 min, incubated at 37°C for 30 min, then the media removed, the cells washed with DMEM without FCS, 50  $\mu$ l DMEM + 2% FCS added and the plates incubated for 38-46 hours at 37°C/5% CO<sub>2</sub>. The cells were harvested and analysed for GFP fluorescence by flow cytometry (see chapter 2.6).

### **2.21.2: Soluble Protein Competition Assay**

Mammalian cell lines were grown to 80% confluence in 96 well plates. Ad5GFP, Ad5F35GFP or Ad19aGFP at an MOI of 3 and 10 was mixed and incubated with varying concentrations of soluble proteins (see table 2.11) and incubated at 4°C for 1 hour. The media was removed from the plates and replaced with 100  $\mu$ l of the virus/protein suspension, incubated at 4°C for 30 min and then raised to 37°C for 30

min. The media was subsequently removed; the cells washed with DMEM without FCS, 50  $\mu$ l DMEM + 2% FCS added to each well and the plates incubated for 38-46 hours at 37°C/5% CO<sub>2</sub>. The cells were harvested and analysed for GFP fluorescence by flow cytometry (see chapter 2.6).

## **2.22: Computer and Statistical Analysis**

### **2.22.1: Sequence Alignments**

DNA sequence alignments were performed in clone manager v7.04 (SciED central)

### **2.22.2: Plasmid Maps**

Graphical representation of plasmids, including genes and restriction enzyme sites (Appendix) were generated using clone manager v7.04 (SciED central)

### **2.22.3: Graphical Data**

All data, unless otherwise stated, was analysed and graphs produced using Microsoft Excel<sup>®</sup> (Microsoft, Redmond, United States).

### **2.22.4: Linear Regression analysis**

Linear regression analysis was performed using the Prism 4 software (GraphPad software).

## **Chapter 3: Generation of recombinant Ad19a and Ad5 viruses and expression vectors**

### **3.1: Introduction**

The use of adenoviruses other than those from subgroup C has been held back initially by the lack of fully sequenced viral genomes and subsequently by the requirement for a cloning and mutagenesis system which is simple, quick, inexpensive and can be used on all adenoviral serotypes. Commercial systems, such as the Stratagene AdEasy system<sup>®</sup>, currently only exist for Ad5 and as such cannot be used to create adenovirus vectors based on other serotypes. There have been several approaches used to generate recombinant Ads from other serotypes which all work on the basis of either modifying a subcloned fragment of the Adenovirus genome before cloning it back into the Adenovirus genome (Berkner, 1998) or homologous recombination between two or more co-transfected plasmids carrying complementary sequences of the Ad genome (Bett *et al.*, 1994; Chartier *et al.*, 1996). Both methods were, at first, inefficient due to either the lack of restriction enzyme sites around the area to be modified (Chartier *et al.*, 1996) or the requirement for the subcloning of the modified fragment into shuttle plasmids respectively (McVey *et al.*, 2002). The recombination methodology, however, has since been further developed to make it more efficient.

A novel recombination strategy was developed for the construction of Ad35, Ad11 and Ad49 vectors (Vogels *et al.*, 2003; Holterman *et al.*, 2004; Lemckert *et al.*, 2006). This system used an adapter plasmid containing the left end of the Ad genome and a multiple cloning site flanked by a Cytomegalovirus promoter (CMV) and the simian virus 40 poly(A) transcription termination signal (SV40 polyA) and a cosmid containing the remaining genome. The adapter plasmid contained a 2,547bp overlap with the cosmid allowing for homologous recombination between the two in Ad5 E1-complementing PER.C6 cells (Lemckert *et al.*, 2006). Any modification made to either the adapter plasmid or cosmid could then be reconstituted into the full-length Ad genome. This method, however, i) required the replacement of the E4-ORF 6 of each Ad vector being generated with that of Ad5 to allow the growth of the vector on

the PER.C6 cell line, ii) required the use of a large homologous sequence and iii) relies on the intrinsic efficiency of homologous recombination within PER.C6 cells, which is relatively low.

Concurrently, a novel recombination methodology had been developed, ET recombination (Zhang *et al.*, 1998), which used  $\lambda$  phage-derived proteins to recombine linear DNA fragments into homologous targets within the *E.coli* genome. This basic methodology was adapted for the modification of Ad genomes in *E.coli*, such as Ad19a, and had the advantage of requiring very short areas of homology between the DNA fragment and the target site (Ruzsics *et al.*, 2006) allowing the use of short PCR fragments or fully synthetic oligonucleotides. In this system, co-transfection of the pBAD $\alpha\beta\gamma$  plasmid, which encoded the  $\lambda$  phage protein pair Red $\alpha$ /Red $\beta$ , allows for recombination between complementary sequences with high efficiency (Zhang *et al.*, 1998; Zhang *et al.*, 2000). In a first step a kanamycin resistance cassette was inserted into the Ad genome using a transposon 7 (Tn7) derived in vitro transcription system (Biery *et al.*, 2000), so that in a second step the correct modification could be identified and then the resistance cassette removed by a transposase. This methodology was shown to be highly effective in introducing deletions, insertions, and point mutations in a recombinant Ad genome (Ruzsics *et al.*, 2006). Whilst highly efficient, this methodology required the use of transposase, which was expensive, a ligation step and the use of a helper plasmid which made it complex to perform.

A further recombination methodology, known as recombineering, was developed by Warming *et al.*, 2005. This methodology included the creation of an *E.coli* strain, SW102, which expressed the  $\lambda$  phage recombination proteins *exo*, *bet* and *gam* from a stably integrated  $\lambda$  prophage under the control of the temperature sensitive repressor *cl857* (Yu *et al.*, 2000) Using this repressor, *exo*, *bet* and *gam* are not expressed when the bacteria are kept at 32°C but by increasing the temperature to 42°C for 15 min, the genes are expressed to high levels and homologous recombination becomes very efficient. The SW102 strain also lacks a functional galactokinase gene (*galk*), which catalyses the first step in the galactose degradation pathway meaning the bacteria cannot be grown on galactose as a sole source unless the *galk* gene is provided in *trans*. The lack or gain of the *galk* gene in *trans* can be



used for both positive and negative selection of transformed bacteria. Positive selection by a colour change from white to red induced by galactose use on MacConkey agar when acid is produced which lowers the pH of the agar and negative selection based on the phosphorylation of the galactose analog 2-deoxy-galactose (DOG) to 2-deoxy-galactose-1-phosphate by *galK* which is toxic and cannot be further metabolised (Alper and Ames, 1975). Using this system the insertion and subsequent removal of a *galK* containing sequence by recombination into an existing BAC (Bacterial artificial chromosome) or plasmid can be easily perceived. This methodology was then further modified by Ruzsics for mutation of viruses (personal communication). This involved the use of a second selection marker in combination with the *galK* gene, a kanamycin resistance cassette, to further reduce incorrect selection. To prove the validity of this system for the modification of Ad genomes, this modified recombineering methodology was used here (summarised in Figure 3.1) to generate recombinant Ad19a and Ad5 expression vectors expressing different transgenes and to introduce point mutations.

### **3.2: Aims**

The specific aims of this area of research were three-fold: firstly, to construct *galK/Kn<sup>R</sup>* intermediate BACs for further modification; secondly, to construct shuttle plasmids for the recombination of vaccination relevant transgenes, such as the HIVA polyprotein expression cassette developed by Hanke and the nucleocapsid protein of PVM, into the *galK/Kn<sup>R</sup>* containing intermediates, and thirdly, to use both the created BACs, shuttle plasmids and PCR generated mutagenesis sequences for generation of recombinant Ad19a and Ad5 viruses and expression vectors.

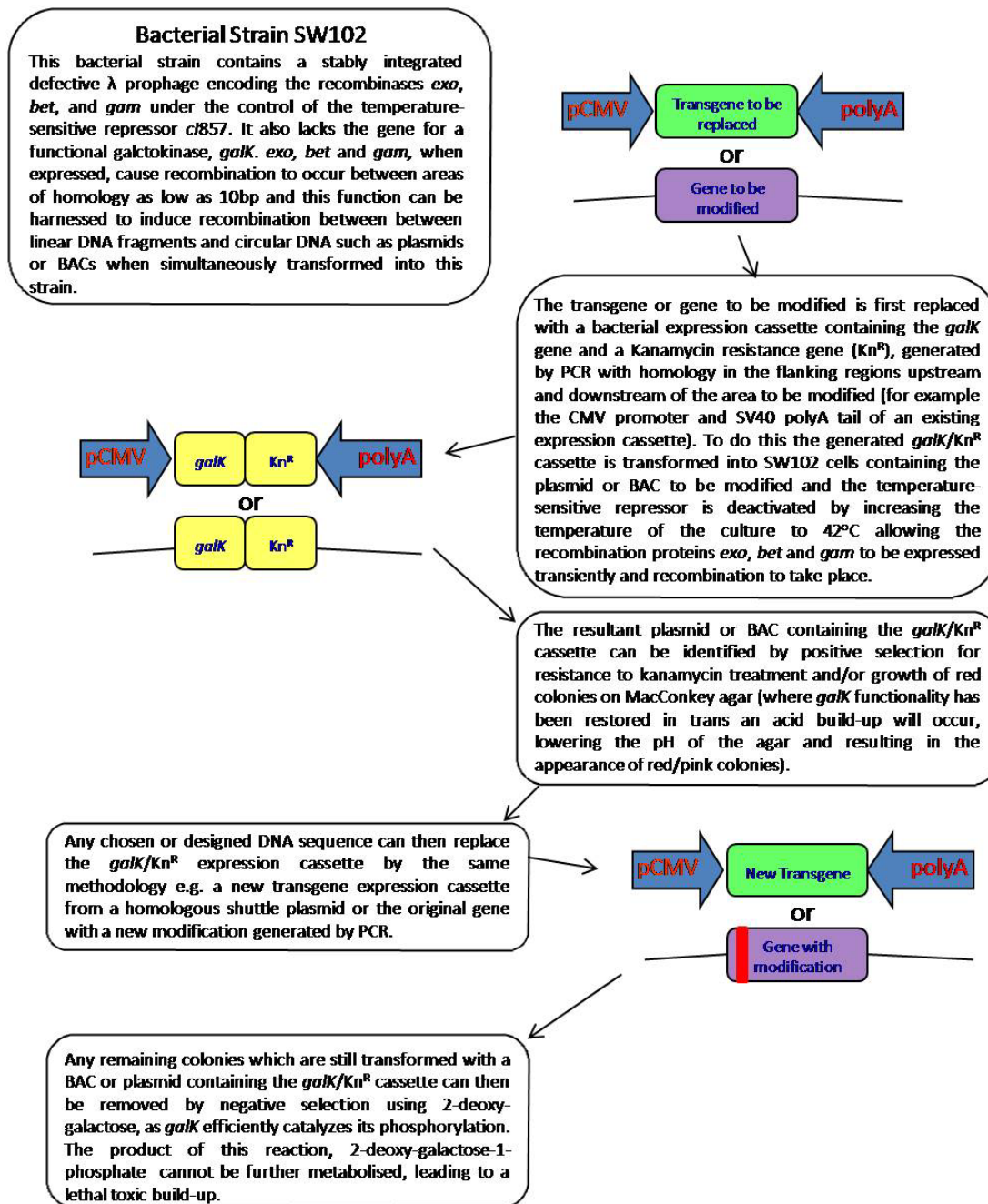


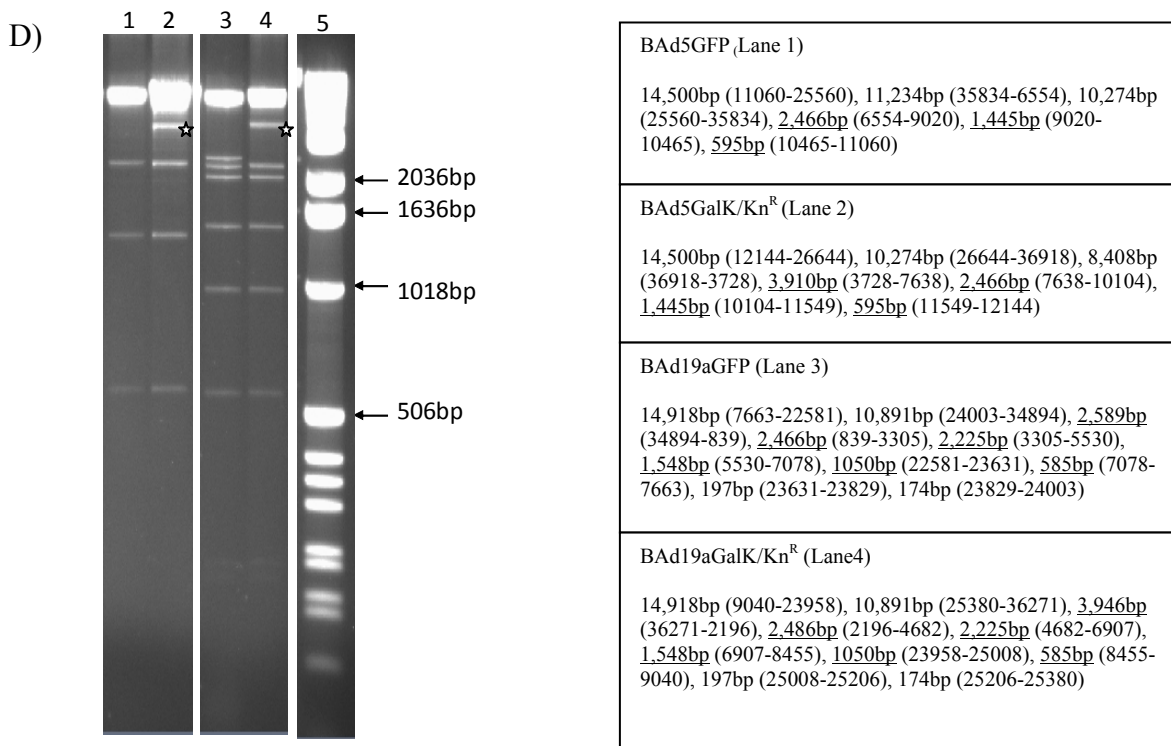
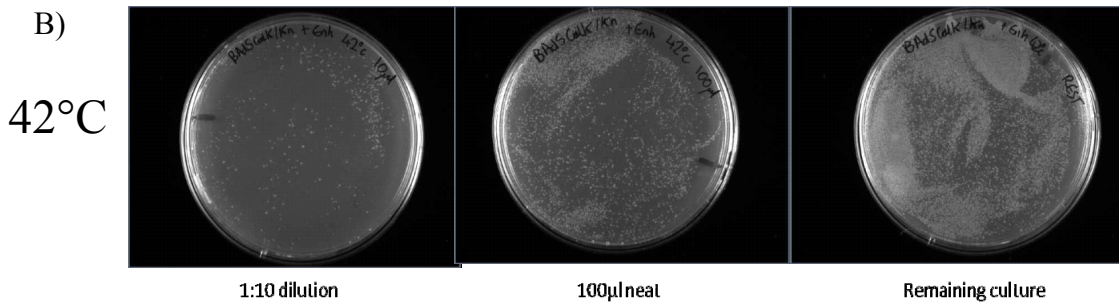
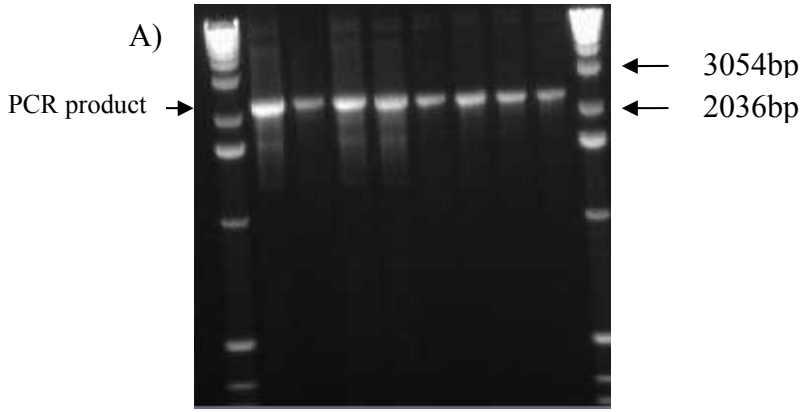
Figure 3.1.: The use of an adapted recombineering methodology to modify plasmids or BACs

### 3.3: Generation of recombinant *galK/Kn<sup>R</sup>* BACs

#### 3.3.1: Generation of BAd19aΔE1ΔE3*galK/Kn<sup>R</sup>* and BAd5ΔE1ΔE3*galK/Kn<sup>R</sup><sub>sv</sub>*

The *galK/Kn<sup>R</sup>* cassette with 50bp of homology to the CMV and SV40 polyA tail of BAd19aΔE1ΔE3GFP (BAd19aGFP; Appendix Figure 5 (Figure A5)) and BAd5ΔE1ΔE3GFP<sub>sv</sub> (BAd5GFP; Figure A3) was isolated by PCR from pGPS*galK/Kn<sup>R</sup>* (Figure A1) using the GalKFOR and GalKREV primers. The resultant PCR products were subjected to agarose gel electrophoresis, the expected 2,173bp band visualised (Figure 3.2 A) and subsequently purified by DNA gel purification. The purified DNA was recombined into the E1 region of BAd19aGFP or BAd5GFP, which had been previously transformed into SW102 cells, by Gal/*Kn<sup>R</sup>* recombination (Chapter 2.16.1). A map of the general genome organisation of the Ad recombinants generated is given in Figure 3.8. When plated on MacConkey agar-cm/kn plates colonies were only seen if the culture had been warmed to 42°C, at which the temperature-sensitive repressor is deactivated and the expression of *exo*, *bet* and *gam* allowed (Figure 3.2 B). No colonies were seen when the culture was left at 32°C (Figure 3.2C). Positive red clones were selected and checked by XhoI restriction digest for successful Gal/*Kn<sup>R</sup>* recombination. The resultant agarose gel of the restriction digests showed, by comparison to the expected banding patterns (although DNA fragments above 4kb are difficult to elucidate, bands below that level are sufficient to show a match in this case and all subsequent cases of BAC digestion) that both BAd19aΔE1ΔE3*galK/Kn<sup>R</sup>* (BAd19a*galK/Kn<sup>R</sup>* Figure A6) and BAd5ΔE1ΔE3*galK/Kn<sup>R</sup><sub>sv</sub>* (BAd5*galK/Kn<sup>R</sup>* Figure A4) were successfully generated (Figure 3.2 D).

Whilst the higher two bands (>4 kb) are not resolved, it is clear that all the separated Ad5 bands are identical except bands migrating at ~3910 and at ~8400 which are predicted after successful recombination (Figure 3.2 D, lanes 1 and 2). Similarly, the generated Ad19a recombinant shows the loss of the band migrating at ~2600 and the appearance of a band migrating at ~4000 which was also predicted (Figure 3.2 D, lanes 3 and 4).



**Figure 3.2: Generation of BAd19agalK/Kn<sup>R</sup> and BAd5galK/Kn<sup>R</sup>.** A) Agarose gel electrophoresis of PCR products using GalKFOR/GalKREV primers on pGPSgalK/Kn<sup>R</sup> alongside a 1kb DNA ladder showing the generated 2,173bp galK/Kn<sup>R</sup> fragment containing the galK and Kn<sup>R</sup> ORFs flanked by 50bp of homologous sequence to the CMV and SV40polyA sequences of BAd19aGFP or BAd5GFP. B) Colonies resulting from GalK/Kn<sup>R</sup> recombination after the culture was raised to 42°C deactivating the *cI857* temperature sensitive repressor and allowing recombination via the integrated  $\lambda$  prophage elements plated at either a 1:10 dilution, 100 $\mu$ l of undiluted culture or the remaining culture pelleted and re-suspended in 100 $\mu$ l. C) Colonies resulting from GalK/Kn<sup>R</sup> recombination when the culture was left at 32°C and plated as previously. D) Agarose gel electrophoresis of restriction digests with XhoI and expected restriction digest patterns of 1) BAd5GFP 2) BAd5galK/Kn<sup>R</sup> 3) BAd19aGFP 4) BAd19agalK/Kn<sup>R</sup> alongside 5) 1kb DNA ladder. Relevant visible bands are underlined in each case. For calculated banding patterns alongside relevant BAC maps and full DNA ladder map consult Figures A2, A3, A4, A5 and A6. Stars show the band denoting appearance of the GalK/Kn<sup>R</sup> cassette

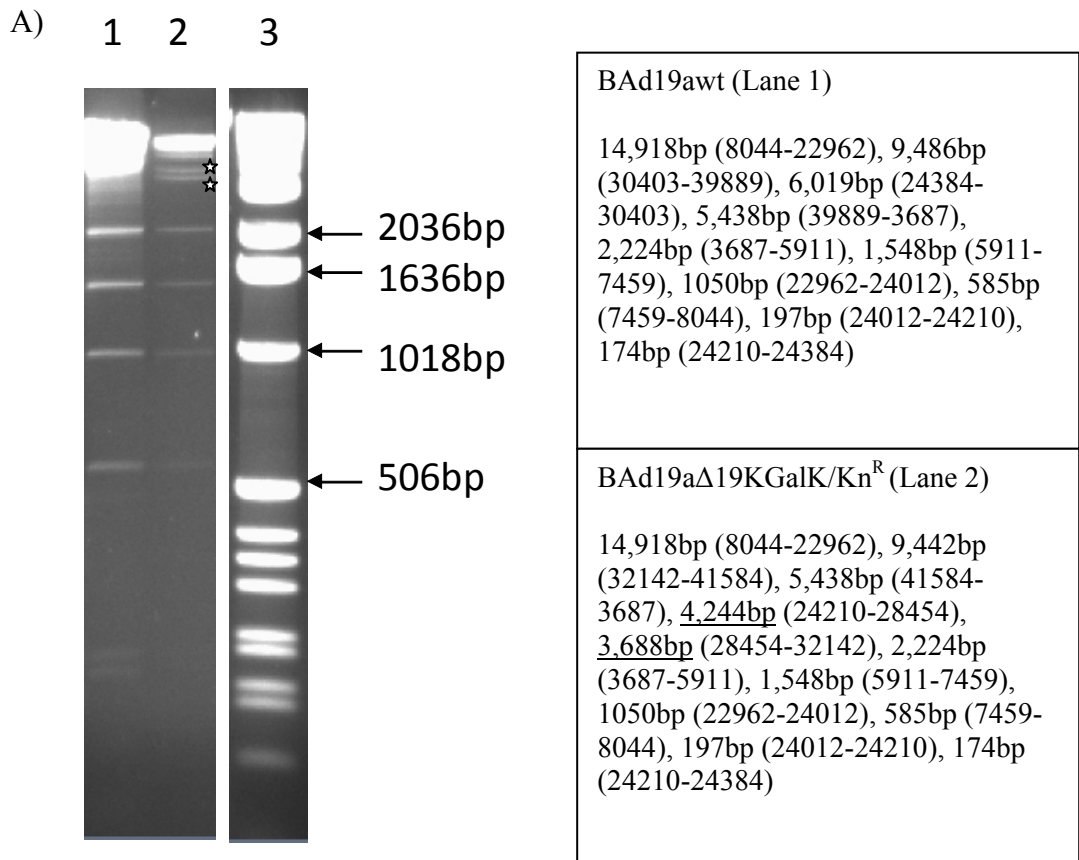
### 3.3.2: Generation of BAd19a $\Delta$ 19K*galK*/Kn<sup>R</sup>

The *galK*/Kn<sup>R</sup> cassette with 50bp of homology to the 5' and 3' flanking regions of the Ad19a E3-19K gene, was isolated by PCR from pGPS*galK*/Kn<sup>R</sup> (Figure A1) using the H1-19KO and H2-19KO primers. The resultant PCR products were subjected to agarose gel electrophoresis, purified and recombined into BAd19awt (Figure A7) using GalK/Kn<sup>R</sup> recombination, as previously. When plated on MacConkey agar-cm/kn plates colonies were only seen if the culture had been increased to 42°C indicating that the temperature sensitive recombination system has been induced. No colonies were seen when the culture was left at 32°C, as before. Positive red clones were selected and checked by XhoI restriction digest for successful recombination. The resultant agarose gel of the restriction digests matched the expected banding patterns (Figure 3.3) and showed that BAd19a $\Delta$ 19K*galK*/Kn<sup>R</sup> (Figure A8) was successfully generated.

### 3.4: Generation of pCMV shuttle plasmids

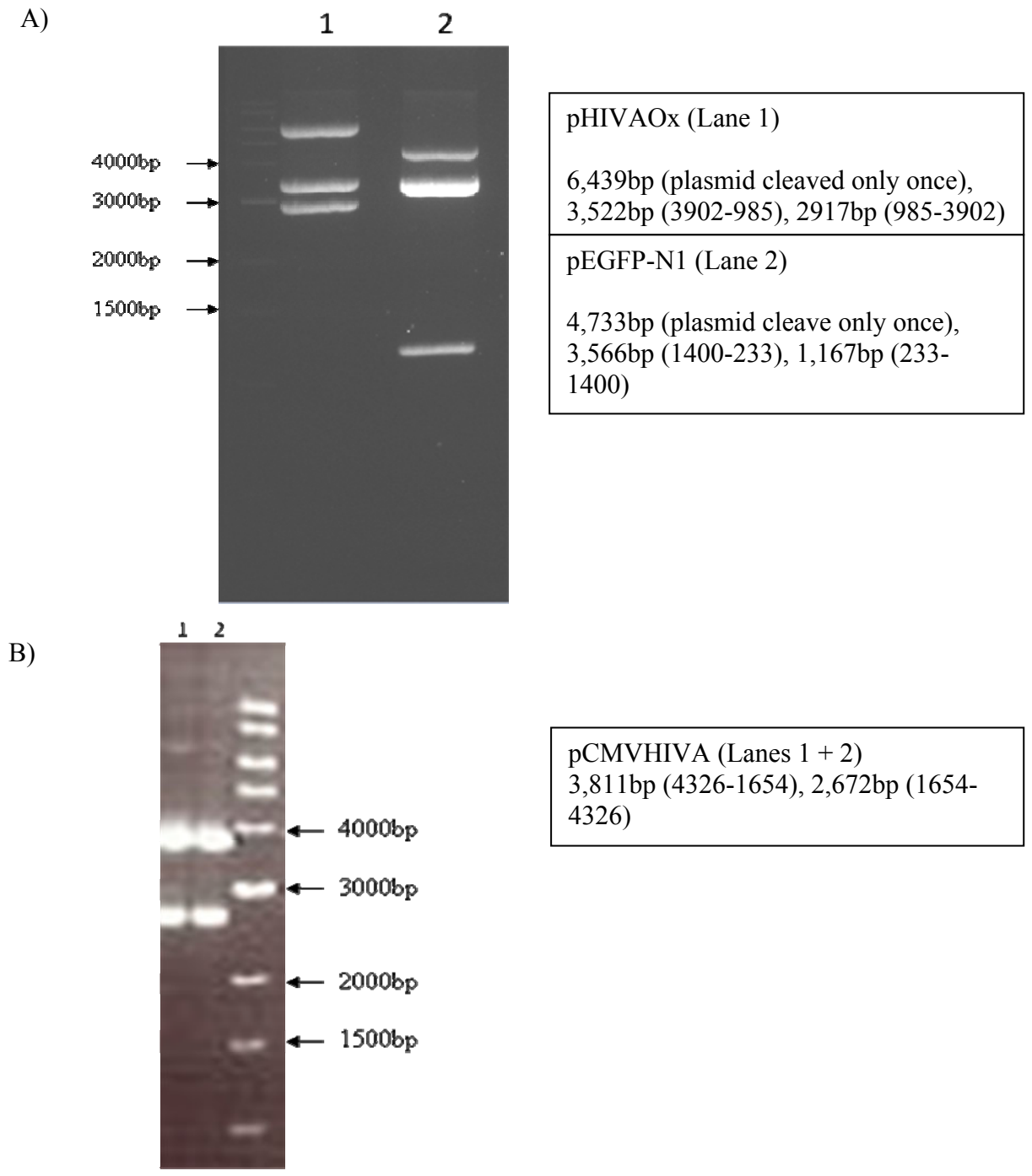
#### 3.4.1: Generation of pCMVHIVA

The pHIVAOx plasmid (obtained from T. Hanke; Figure A9) lacked the SV40 polyA signal required for recombination into the *galK*/Kn<sup>R</sup> intermediate BACs. A shuttle plasmid was therefore generated containing the HIVA polyprotein expression cassette with both the homologous CMV promoter and SV40 polyA tail as follows. pHIVAOx and pEGFP-N1 (Figure A10) were digested with NdeI and NotI (Figure 3.4A). The resultant 3,556bp band from pEGFP-N1 (shuttle plasmid backbone including SV40 polyA tail) and the 2,917bp band from pHIVAOx (CMV-HIVA fragment) were purified and then ligated to create pCMVHIVA. The generation of pCMVHIVA was confirmed by restriction digest with StuI generating the correct 3,811bp and 2,672bp bands (Figure 3.4B).



**Figure 3.3: Generation of BAd19aΔ19KgalK/Kn<sup>R</sup>**

A) Agarose gel electrophoresis of restriction digests with XhoI and expected restriction digest banding patterns of 1) BAd19awt 2) BAd19aΔ19KgalK/Kn<sup>R</sup> alongside 3) 1kb DNA ladder. Relevant bands are shown (stars) and highlighted by underlining in key. For calculated banding patterns alongside relevant BAC maps see Figures A7 and A8.



**Figure 3.4: Generation of pCMVHIVA**

A) Agarose gel electrophoresis and expected banding patterns of NdeI/NotI restriction digest of 1) pHIVAOx and 2) pEGFP-N1 alongside 1kb DNA ladder (NEB). For calculated fragments alongside relevant BAC maps see Figures A9 and A10. B) Agarose gel electrophoresis and expected banding patterns of StuI restriction digest of pCMVHIVA. For calculated banding patterns alongside relevant BAC maps consult Figure A11.



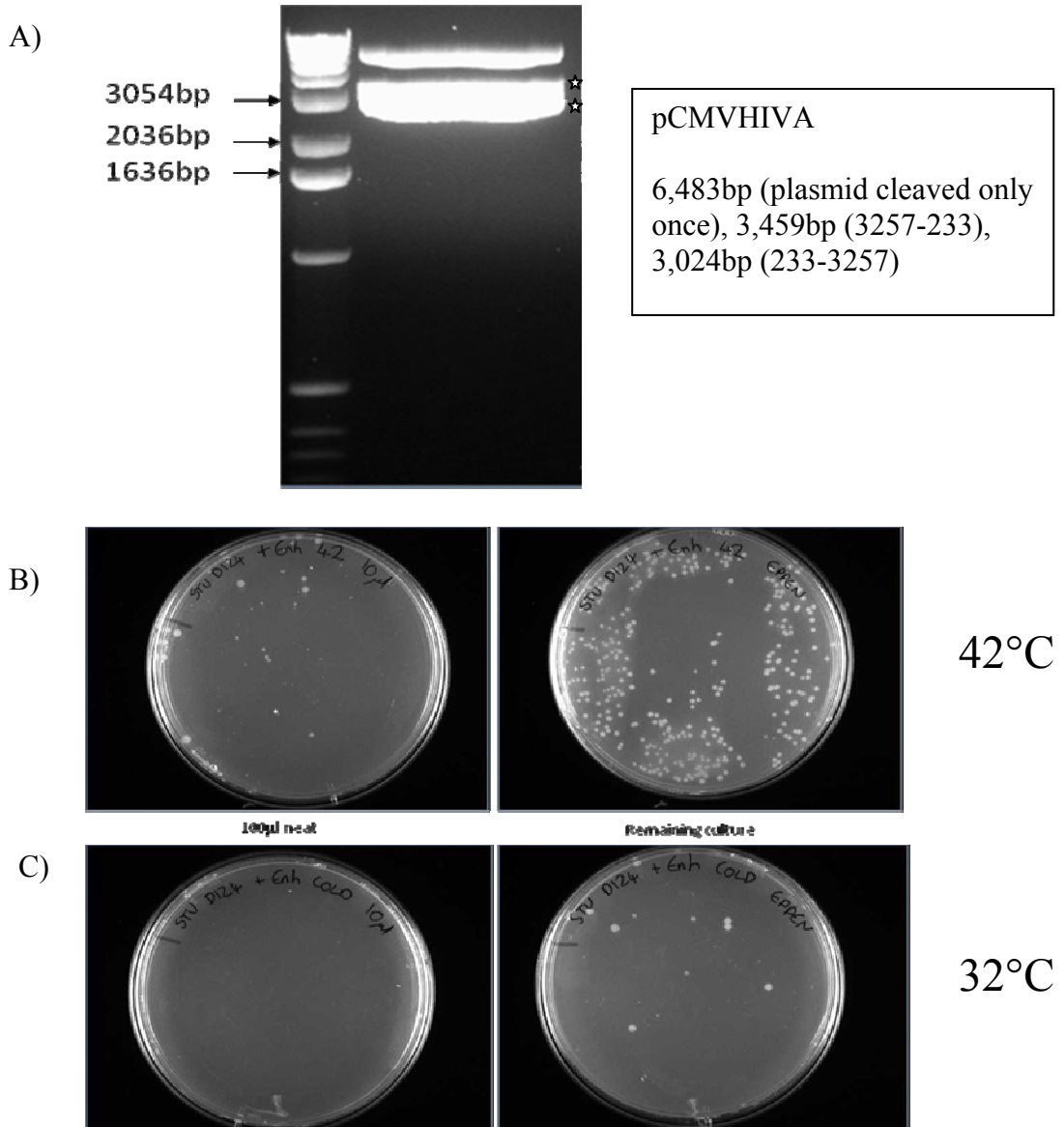
### 3.4.2: pCMVPVM-N

The pCMVPVM-N shuttle plasmid (Figure A12) containing the PVM-N transgene flanked by the CMV promoter and SV40 polyA tail was obtained from Helen Terry (University of Warwick)

### 3.5: Generation of recombinant Ad19a and Ad5 HIVA BACs

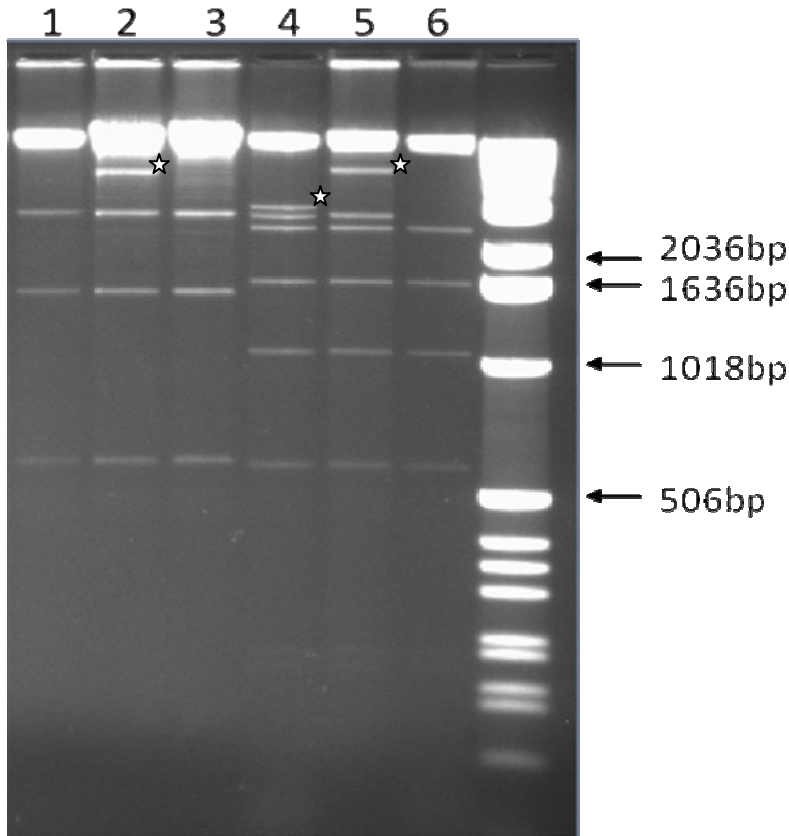
To generate BACs with Ad19a and Ad5 vector genomes containing the HIVA transgene within their respective expression cassettes requires the replacement of the existing *galK/Kn<sup>R</sup>* selection markers in BAd19*galK/Kn<sup>R</sup>* and BAd5*galK/Kn<sup>R</sup><sub>sv</sub>* with the CMV-HIVA expression cassette of pCMVHIVA (Chapter 3.4.1).

The pCMVHIVA plasmid was digested with NdeI and MfeI/MunI which generated the expected 3,024bp linear DNA fragment containing the HIVA transgene with 336bp of homology with the CMV promoter and 96bp of homology with the SV40 polyA tail within the corresponding regions in the *galK/Kn<sup>R</sup>* intermediate BACs (Figure 3.5A). A second stage recombination reaction (2.16.2), using the generated DNA, was then performed. To confirm generation of BAd19aΔE1ΔE3HIVA (BAd19aHIVA; Figure A13) and BAd5ΔE1ΔE3HIVA<sub>sv</sub> (BAd5HIVA; Figure A14) any surviving colonies on M63 plates, 120 hours after plating (Figure 3.5B), were analysed by XhoI restriction digest alongside their predecessors (Figure 3.6). The BACs were also sequenced (see chapter 2.9) across the HIVA transgene and SV40 polyA tail using sequencing primers HIVA/F/1, HIVA/F/2 and HIVA/F/3 and compared to the predicted BAC sequences (Figures A15 and A16). In both cases BAd19aHIVA and BAd5HIVA matched both the predicted restriction digest banding patterns and exhibited the correct sequence with no nucleotide mismatches confirming they were both successfully generated.



**Figure 3.5: Generation of recombinant Ad19a and Ad5 HIVA BACs**

A) Agarose gel electrophoresis of restriction digests with NdeI/MunI of pCMVHIVA 1kb DNA ladder with expected banding pattern. Two bands resulted from the digest, but due to the large amount of DNA used for later isolation and the proximity of the fragment sizes, both bands could not be resolved independently. For calculated banding patterns alongside relevant plasmid map see Figure A11. B) Colonies resulting from second stage recombination after 120 hours after the culture was raised to 42°C deactivating the *cI857* temperature sensitive repressor plated at either 100µl of undiluted culture or the remaining culture pelleted and re-suspended in 100µl. C) Colonies resulting from second stage recombination when the culture was left at 32°C plated as previously.



<p>BAd5GFP (Lane 1)</p> <p>14,500bp (11060-25560), 11,234bp (35834-6554), 10,274bp (25560-35834), 2,466bp (6554-9020), 1,445bp (9020-10465), 595bp (10465-11060)</p>
<p>BAd5GalK/Kn<sup>R</sup> (Lane 2)</p> <p>14,500bp (12144-26644), 10,274bp (26644-36918), 8,408bp (36918-3728), <u>3,910bp</u> (3728-7638), 2,466bp (7638-10104), 1,445bp (10104-11549), 595bp (11549-12144)</p>
<p>BAd5HIVA (Lane 3)</p> <p>14,500bp (12406-26906), 12,580bp (37180-7900), 10,274bp (26906-37180), 2,466bp (7900-10366), 1,445bp (10366-11811), 595bp (11811-12406)</p>
<p>BAd19aGFP (Lane 4)</p> <p>14,918bp (7663-22581), 10,891bp (24003-34894), <u>2,589bp</u> (34894-839), <u>2,466bp</u> (839-3305), 2,225bp (3305-5530), 1,548bp (5530-7078), 1050bp (22581-23631), 585bp (7078-7663), 197bp (23631-23829), 174bp (23829-24003)</p>
<p>BAd19aGalK/Kn<sup>R</sup> (Lane 5)</p> <p>14,918bp (9040-23958), 10,891bp (25380-36271), <u>3,946bp</u> (36271-2196), <u>2,486bp</u> (2196-4682), 2,225bp (4682-6907), 1,548bp (6907-8455), 1050bp (23958-25008), 585bp (8455-9040), 197bp (25008-25206), 174bp (25206-25380)</p>
<p>BAd19aHIVA (Lane 6)</p> <p>14,918bp (9603-24521), 10,891bp (25943-36834), 6,995bp (36834-5245), 2,225bp (5245-7470), 1,548bp (7470-9018), 1050bp (24521-25571), 585bp (9018-9603), 197bp (25571-25769), 174bp (25769-25943)</p>

**Figure 3.6: Generation of BAd19aΔE1ΔE3HIVA and BAd5ΔE1ΔE3HIVA<sub>sv</sub>**

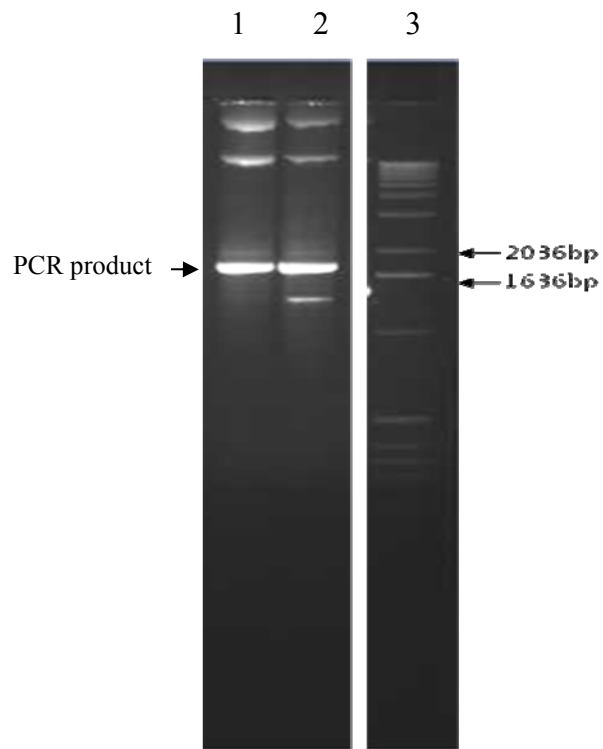
Agarose gel electrophoresis of restriction digests with XhoI and expected restriction digest patterns of 1) BAd5GFP, 2) BAd5*galK*/Kn<sup>R</sup>, 3) BAd5HIVA, 4) BAd19aGFP, 5) BAd19a*galK*/Kn<sup>R</sup> and 6) BAd19aHIVA alongside 1kb DNA ladder. Generation of BAd5HIVA has been highlighted by the loss of the 3,910 bp band from the corresponding *galK*/Kn<sup>R</sup> construct. Generation of BAd19aHIVA has been highlighted by the loss of the 3,946 bp and 2,486 bp band from the corresponding *galK*/Kn<sup>R</sup>. Important bands are highlighted (star) and underlined. For calculated banding patterns alongside relevant BAC maps and full DNA ladder map see Figures A2, A3, A4, A5, A6, A13 and A14.

### 3.6: Generation of a recombinant Ad19a PVM-N BAC

A recombinant BAC containing the PVM-N transgene was generated by the same method as BAd19aHIVA and BAd5HIVA except that the linear DNA fragment containing the PVM-N transgene expression cassette and homology with the CMV promoter and SV40 polyA tail of BAd19aGalK/Kn<sup>R</sup>, required for second stage recombination was generated by PCR rather than enzyme digest. The correct 1,686bp fragment was amplified using primers PVM-NIsolationFOR and PVM-NIsolationREV on pCMVPVM-N (Figure 3.7), isolated and used in the second stage recombination reaction. As before, after 120 hours BAC DNA of surviving colonies was extracted and sequenced across the insert (Figure A18) using the primers PVM-N/F/1, PVM-N/F/2 and PVM-N/F/3. No nucleotide mismatches were revealed in the sequenced fragment indicating correct generation of BAd19aΔE1ΔE3PVM-N (BAd19aPVM-N Figure A17) generation.

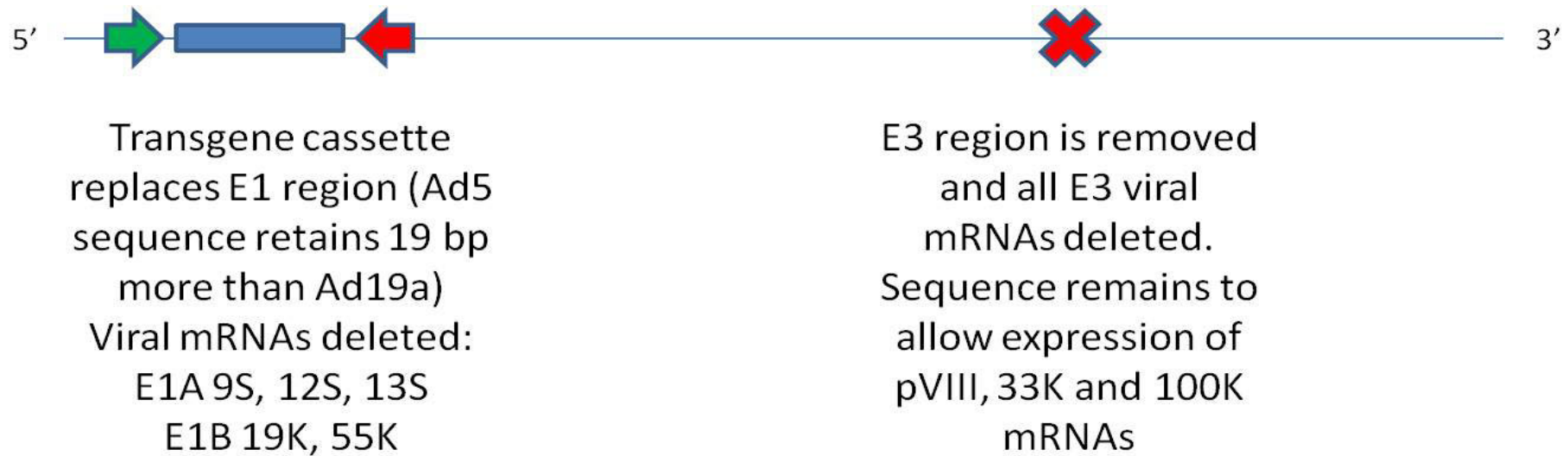
### 3.7: Generation of an E3/19K inactivated Ad19a BAC (BAd19a19K\*)

To show that the recombineering system is capable of creating seamless point mutations within BACs the *galK/Kn<sup>R</sup>* cassette within BAd19aΔ19K*galK/Kn<sup>R</sup>* was replaced with the Ad19a E3/19K gene containing a stop codon (taa) 4 codons after its start codon to create a nonsense mutation. The 564bp linear DNA fragment containing the E3/19K gene with the nonsense mutation and 50bp of homology at the 5' flanking end and 27bp of homology at the 3' flanking end of the wild-type E3/19K gene was generated using the 19Kfor and 19Krev primers on BAd19awt (Figure 3.9 A). The resultant PCR was subjected to agarose gel electrophoresis, isolated and used in a second stage recombination reaction. The surviving clones were analysed by XhoI restriction digest (Figure 3.9 B) and sequence alignment (Figure A20) using the primer 19KSEQPRIME. The restriction digest banding pattern was identical to that of BAd19awt indicating that the *galK/Kn<sup>R</sup>* cassette had apparently been replaced with the inactivated E3/19K. This was corroborated by sequence alignment which showed no nucleotide mismatches with the expected mutated E3/19K sequences (Figure A19).



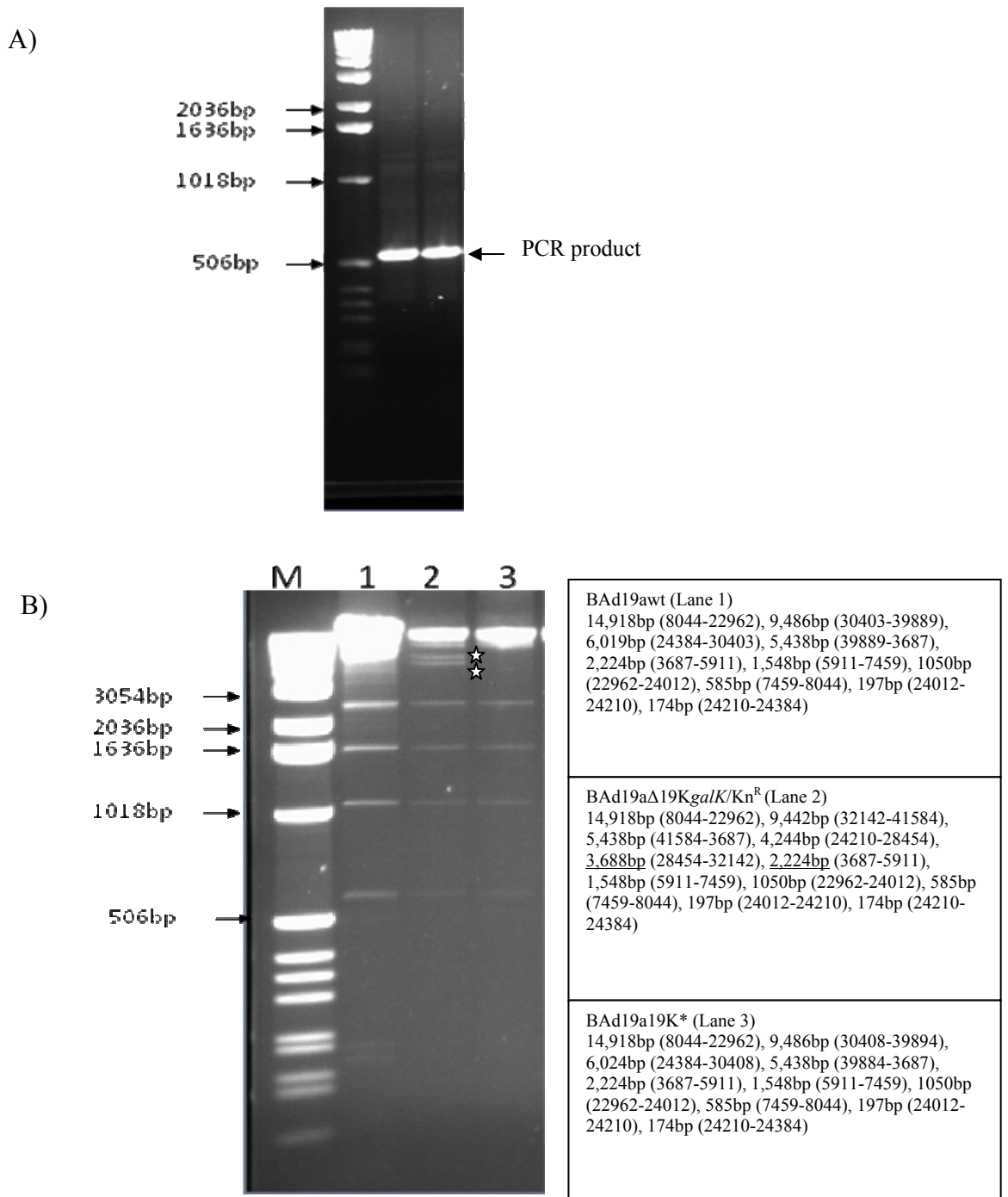
**Figure 3.7: Generation of BAd19aPVM-N**

Agarose gel electrophoresis of PCR performed on two samples (lanes 1 and 2) of pCMVPVM-N using primers PVM-NIsolationFOR and PVM-NIsolationREV generated the correct 1,686bp band for second stage recombination alongside 1kb DNA ladder (lane 3)



**Figure 3.8: Ad recombinant map**

Map to show genome organisation of all the Ad recombinants constructed in this chapter detailing the viral genes deleted. For functions of deleted genes see Chapter 1.1.3



### Figure 3.9: Generation of BAd19a19K\*

A) Agarose gel electrophoresis of PCR performed on BAd19awt using primers 19Kfor and 19Krev generated the correct 564bp band for second stage recombination. B) Agarose gel electrophoresis of restriction digests with XhoI and expected restriction digest patterns of 1) BAd19awt, 2) BAd19aΔ19KgalK/Kn<sup>R</sup> and 3) BAd19aΔ19K alongside 1kb DNA ladder. Correct generation of BAd19a19K\* is shown by the loss of the highlighted (stars) and underlined predicted bands at 3,688bp and 2,224 bp seen in BAd19aΔ19KgalK/Kn<sup>R</sup>. For correct banding patterns alongside relevant BAC map and full DNA ladder map see Figures A19 and A2.



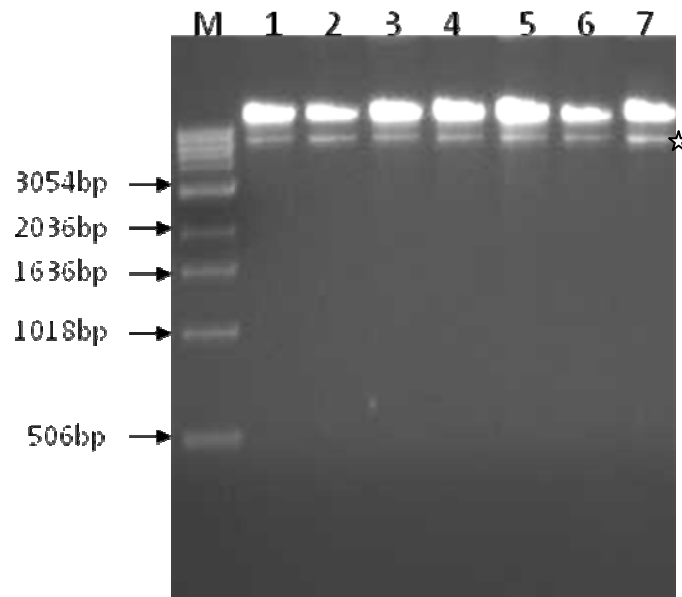
### **3.8: Reconstitution and purification of recombinant Ad19a and Ad5 viruses and expression vectors**

All BACs created or used in this study (except *galK/Kn<sup>R</sup>* intermediates) were reconstituted into their recombinant viruses or expression vectors as follows. BAd19awt, BAd19a19K\*, BAd19aGFP, BAd19aHIVA, BAd19aPVM-N, BAd5GFP and BAd5HIVA were linearised by restriction digest with PacI to remove the bacterial components (6415bp sequence) of the BAC backbone and generating a linear DNA fragment containing only the remaining Ad virus or vector genome. The digests were subjected to agarose gel electrophoresis (Figure 3.10), then the vector or virus band isolated and purified. 1µg of linear vector or virus DNA was then transfected into low passage HEK 293 cells (Chapter 2.11) and subsequent passage for between 1-3 weeks until a c.p.e was seen and then larger stocks of each virus or vector were generated (Chapter 2.3.1 (viruses); Chapter 2.3.2 (vectors) and titrated (Chapter 2.15.1). All viruses and vectors were then purified (Chapter 2.3.4) and titrated again.

### **3.9: Discussion**

Work presented in this chapter has shown the use of a modified recombineering strategy developed by Zsolt Ruzsics to alter or mutate BACs containing Ad vector and virus genomes.

The transgene expression cassettes in both Ad19a and Ad5 E1 and E3 deleted GFP vectors were successfully replaced with a PCR generated *galK/Kn<sup>R</sup>* selection cassette. The resultant BACs can now serve as intermediates for the insertion of any transgene into the expression cassette of Ad19a or Ad5 which has been cloned into a corresponding shuttle plasmid with homology to the CMV promoter and SV40 polyA tail of both BACs. Two vaccination-relevant transgenes, the HIVA polyprotein and the nucleocapsid protein of PVM, were first inserted into shuttle plasmids (if not already existent) and were then inserted into the expression cassette of the *galK/Kn<sup>R</sup>* intermediates in second stage recombination reactions. The inserts



**Figure 3.10: Reconstitution of recombinant Ad19a and Ad5 viruses and expression vectors**

Agarose gel electrophoresis of restriction digests with *PacI* 1) BAd19awt, 2) BAd19a $\Delta$ 19K, 3) BAd19aGFP, 4) BAd19aHIVA, 5) BAd19aPVM-N, 6) BAd5GFP and 7) BAd5HIVA alongside the 1kb DNA ladder (M). A 6415bp fragment (indicated by star) was generated containing the bacterial sequence from each BAC so that the remaining linearised virus or vector genomes could be isolated for reconstitution by gel isolation.

were generated by both restriction digest, providing > 90bp of homology, and PCR, providing < 50bp of homology, showing that both strategies were effective regardless of length of the homologous sequence. This strategy has created Ad19a and Ad5 E1 and E3 deleted vectors which express the HIVA and PVM-N transgenes when used to infect mammalian cells. The expression of these proteins, by the reconstituted viruses will be examined in Chapter 4.

To show that the recombineering system is capable of minimal modifications, a nonsense mutation, consisting of a 5bp sequence addition, was inserted into the E3-19K gene of Ad19awt. Both the required *galK/Kn<sup>R</sup>* selection cassette and the modified E3/19K gene were generated by PCR with the modified E3/19K gene only requiring 26bp of homology at its 3' end (in combination with 50bp of homology at its 5' end) for recombination to occur. This strategy has, upon reconstitution, created a virus that lost E3/19K production whilst having no effect on the expression of any surrounding E3 proteins tested (see Chapter 4). The effect of the nonsense mutation on E3/19K and surrounding E3 protein expression will also be examined in Chapter 4.

All BACs resulting from this study were checked by restriction digest and/or DNA sequencing and were found to be correct and were transformed into DH10B cells for long-term storage due to their lack of the SW102 recombination system.

This work has demonstrated that the modified recombineering strategy can be effectively used to make both large-scale and small-scale modifications to BACs containing Ad genomes. The use of the *galK/Kn<sup>R</sup>* selection cassette allowed for easy positive and negative selection of BACs during the process, however, it was found that some BACs underwent an unknown mutational event during second stage recombination which resulted in the loss of the *galK/Kn<sup>R</sup>* selection cassette without incorporating the transgene and therefore false-positive colony growth. However, the number of false-positive clones was consistently 30-100 fold lower than the number of correct clones. The false-positive clones could be easily identified by restriction digest and/or sequencing and discarded.

The second stage recombination process required for transgene insertion including downstream analysis for correct clones can be done in < 10days and therefore, once established, this technique provides a quick, simple and inexpensive methodology for vaccine or gene therapy vector creation.

More importantly, this technique can also be used to generate small mutations in an Ad genome or other large genomes allowing the targeted deletion or the expression of any gene or of any mutation. This allows the simple modification of existing viruses and vectors to examine the effect on virus phenotype, for example, the deletion of one or several E3 proteins in combination from an Ad genome for studies such as those published previously (Elsing and Burgert, 1998; Ruzsics *et al.*, 2006) or the modification of conserved amino acids within Ad proteins, as published previously, (Sester and Burgert; 1994; Hilgendorf *et al.*, 2003) which may lead to the design of better vectors for vaccination or gene therapy.

In conclusion, the work in this chapter has generated viruses and vectors which can be used to examine the potential of Ad19a as a vaccine vector in comparison to Ad5 in several settings and this work will be the subject of subsequent chapters.

## **Chapter 4: Examination of generated vector and virus phenotype including transgene expression in human cells**

### **4.1: Introduction**

This chapter will investigate the phenotypes of the viruses and vectors generated in Chapter 3.

#### **4.1.1: Particle/plaque forming unit ratios**

The adenoviruses, like most animal viruses, have ratios of particles to infectious units greater than one. Infectious units of Ads are recorded as plaque forming units (pfu) and are based on the number of plaques, marked areas of cell death, a known dilution of virus is able to generate on a monolayer culture of mammalian cells which support adenovirus infection and replication (Chapter 2.15.1). Ad particle number can be calculated by comparing the DNA concentration of a known dilution of disrupted Ad particles to known standards (Chapter 2.15.3). It has long been known that Ads differ dramatically in their particle/pfu ratios, ranging from 10:1 for the Ad5 and subgroup B Ads like Ad11 (Holterman *et al.*, 2004) to values several orders of magnitude higher for Ads from subgroup F such as Ad40 and Ad41 (Brown *et al.*, 1992). Published comparisons of Adenovirus particle/pfu ratios are rare. In 1967 it was shown that Ad19p had the second highest particle/pfu ratio of the entire catalogue of Ad serotypes discovered with a calculated ratio of 1600:1 compared to the Ad5 ratio of 20:1 (Green *et al.*, 1967). In 1978 two Ad19 isolates were calculated to have particle/pfu ratios of 9,600:1 and 25,000:1, respectively (Newland and Cooney, 1978). It is unclear what the reason is for this vast inefficiency of particle assembly. Comparison of different serotypes is therefore complicated, and dependent on the aspect of the Ad life cycle studied, pfu's or particles may be appropriate to quantitate Ads.

Currently, in a variety of vaccination and gene therapy settings both *in vitro* and *in vivo* Ad vector dose is given by particle number rather than by pfu and as such is not based on the number of virions capable of cell transduction but rather on the physical

number of virion particles. If particle numbers are used for comparative studies of different Ads to determine MOI or dose, transgene expression is adversely affected in an Ad with a high particle/pfu ratio, due to a lower number of pfu being used. Conversely, during *in vivo* studies, using identical pfu numbers rather than particle numbers may result in a larger immune response to the Ad with a higher particle/pfu ratio as a larger amount of immunogenic proteins is present. For a true comparison of different Ad serotypes the particle/pfu ratios must be taken into consideration. Therefore, we decided to base our assessment of the quality and quantity of the different Ads on their ability to infect/transduce cells, hence we measured their activity in a plaque forming assay.

#### **4.1.2: Adenovirus vector transgene expression**

Ad vectors based on differing serotypes, which contain expression cassettes with identical CMV promoters and SV40 polyA tails, should produce similar transgene expression. However, previous data suggested that sequences in *cis* and *trans* may influence transgene expression (Lusky *et al.*, 1999). In 2006, despite having identical CMV promoter driven expression cassettes, the Ad19a and Ad5 eGFP expressing vectors showed marked differences in eGFP expression at identical transduction efficiencies (Ruzsics *et al.*, 2006). The reasons remained unclear. One possibility was that an SV40 enhancer downstream of the expression cassette may have stimulated GFP expression. Therefore we have used an Ad5GFP vector with the same SV40 enhancer inserted downstream of GFP. As the expression level of the transgene is crucial for vector toxicity as well as for induction of immunity against the transgene, we tested transgenes other than eGFP to evaluate if we could observe the same enhanced expression profile. This examination of transgene expression from the created vectors is possible because expression of the inserted transgenes (HIVA and PVM-N) can be detected by western blot using antibodies SV5-Pk1 and R2052, respectively. In parallel, transduction efficiency can be monitored using FACS staining with the 2Hx-2 Ab, which recognises the hexon protein of any tested Ad (Cepko *et al.*, 1981).

## 4.2: Aims

The aims of this research were the examination of vector phenotype of the Ad vectors generated in Chapter 3 to include:

1. Calculation and consideration of particle/pfu ratios
2. Comparison of vector transgene expression in mammalian cells

## 4.3: Particle/plaque forming unit ratios

To assess the infectivity and transduction capacity of the virus vectors all virus and vector stocks, after reconstitution or passage, were examined in an Adenovirus plaque assay (Chapter 2.16.1). It was noted that Ad19a plaques were significantly larger than Ad5 plaques. Particle numbers were then calculated for one purified stock of each of the vectors used in this study and wild-type Ad controls by Adenovirus particle number calculation (Chapter 2.16.3). The resultant figures were then compared and a ratio of particles/pfu generated (Table 4.1).

**Table 4.1: Example of particle/pfu ratios of generated and existent viruses and vectors**

Virus or vector	Particle number (particles/ml)	Plaque forming units (pfu/ml)	Particle/pfu ratio	Number of preparations
Ad5wt	$2.9 \times 10^{12}$	$2 \times 10^{11}$	~14:1	1
Ad19awt	$1.1 \times 10^{13}$	$2.9 \times 10^9$	~3,793:1	2
rAd5GFP	$3.1 \times 10^{12}$	$6.15 \times 10^{10}$	~50:1	2
rAd19aGFP	$9.2 \times 10^{11}$	$5.9 \times 10^8$	~1,560:1	6
rAd5HIVA	$2.9 \times 10^{12}$	$7.95 \times 10^{10}$	~36:1	1
rAd19aHIVA	$8.5 \times 10^{12}$	$3.4 \times 10^8$	~25,117:1	4
rAd5PVM-N	$1.5 \times 10^{12}$	$2.9 \times 10^{10}$	~53:1	2
rAd19aPVM-N	$8.98 \times 10^{12}$	$1.378 \times 10^9$	~6,516:1	5

Particle/pfu ratios for Ad5 were similar and varied from 14:1 for the wild-type virus, 50:1 for rAd5GFP, 36:1 for rAd5HIVA, up to a maximum of 53:1 for rAd5PVM-N. These numbers were in the same range as previously published (Brown *et al.*, 1992; Ugai *et al.*, 2005) and matched expected rAd vector industrial manufacturing standards (<http://www.vectorbiolabs.com/vbs/faq-product.html>).

Ratios for Ad19a showed a greater variation and were significantly higher, at their lowest 1,560:1 for rAd19aGFP through 3,793:1 for Ad19awt, 6,516:1 for rAd19aPVM-N up to a maximum of 25,117:1 for rAd19aHIVA. The published figure for Ad19p wild-type, 1600:1, and other Ad19 isolates, 9600:1 and 25,000:1 (Newland and Coney, 1978) although calculated by different methodologies to the one used in this study, would suggest these findings are plausible.

It was decided that, due to the significant difference in particle/pfu ratio values, only pfu would be used to calculate the MOI for the remainder of the study as it measures the number of virions that are replication competent in 293 cells and as such is a model for the comparison between the two Ads.

#### **4.4: Examination of transgene expression of recombinant adenovirus vectors**

##### **4.4.1: Expression of GFP in fibroblasts**

rAd19aGFP and rAd5GFP were used to infect A549 cells, a human fibroblast cell line that does not complement Ad replication, using 1, 3 and 10 pfu/cell. The % of GFP positive cells and the mean fluorescence intensity of GFP expression were recorded for each infection and the results charted (Figure 4.1A & Figure 4.1B).

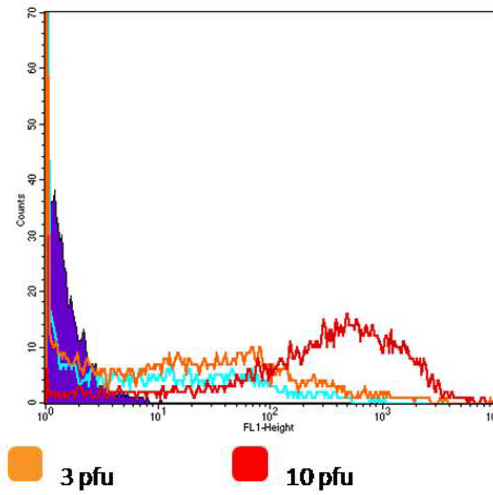
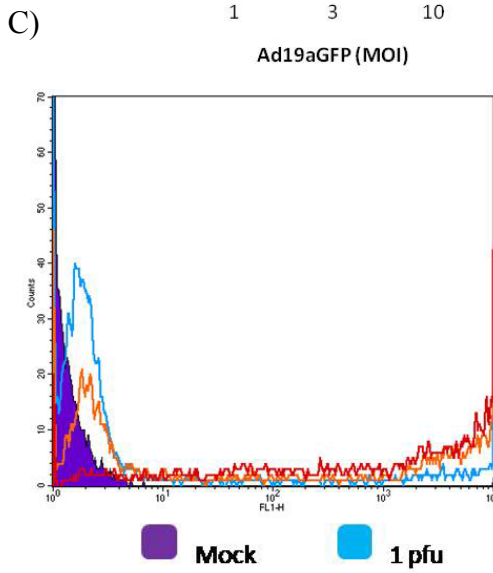
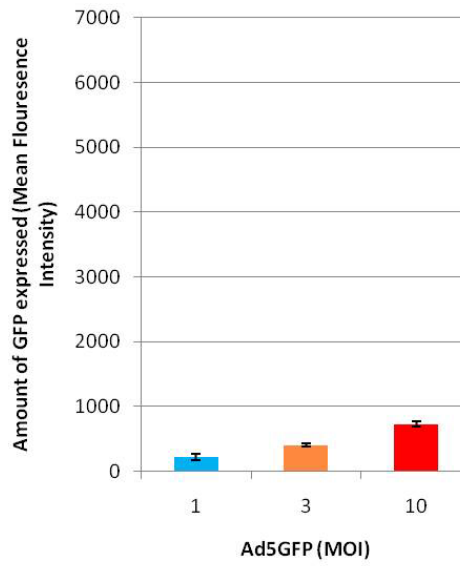
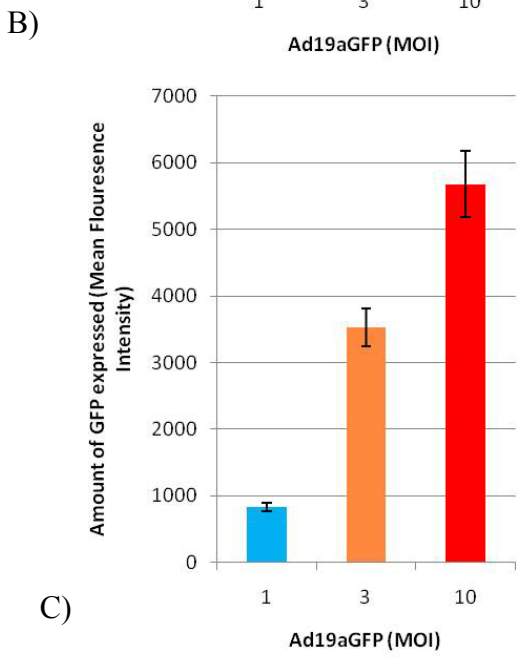
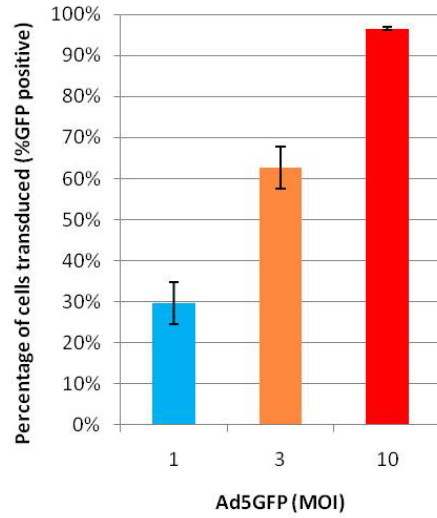
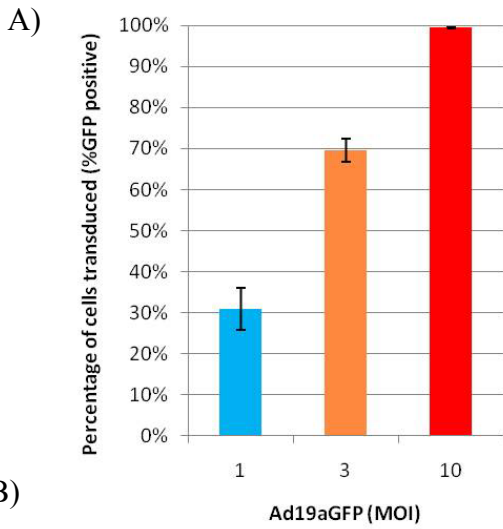
rAd19aGFP and rAd5GFP showed very similar transduction efficiencies across the range of MOI with means of 30.9%, 69.6% and 99.4% recorded for rAd19a and 29.5%, 62.6% and 96.5% recorded for rAd5. This finding confirmed that rAd19a and rAd5 vectors have similar transduction efficiencies in A549 cells using equal plaque forming units of each vector.

There was a marked difference, however, between the recorded GFP expression levels across the range of MOIs used. rAd19a produced considerably higher mean fluorescence intensities at each infection level when compared to rAd5 with means of 831, 3,529 and 5,680 recorded for rAd19a and 222, 408 and 729 recorded for rAd5. The rAd19a values were 3.7, 8.6 and 7.8x higher than the rAd5 values,



respectively. rAd19a transgene expression levels were, therefore, on average 6.7x higher than for rAd5.

This was taken as preliminary evidence that rAd19a expresses considerably more transgene than rAd5 at equal transduction efficiencies. This showed that several fold lower amounts of an rAd19a vector would be required to produce equal transgene expression levels as a comparable rAd5 vector.



**Figure 4.1: Examination of transduction efficiency and transgene expression of recombinant adenovirus GFP vectors by FACS analysis**

(A) Mean % transduction as measured by the percentage of GFP positive A549 cells upon infection with 1 MOI (blue), 3 MOI (orange) or 10 MOI (red) of rAd19aGFP (left) and rAd5GFP (right). Error bars show the standard error of the mean for 5 and 4 independent experiments, respectively. (B) Mean fluorescence intensity (FL1) of expressed GFP upon infection with rAd19aGFP (left) and rAd5GFP (right) as above. (C) Examples of FACS histograms showing rAd19aGFP transduction (left) and rAd5GFP transduction (right) either mock infected (purple fill) or infected with 1 MOI (blue line), 3 MOI (orange line) or 10 MOI (red line). % are calculated as the number of cells within a defined region over background controls (region not shown)

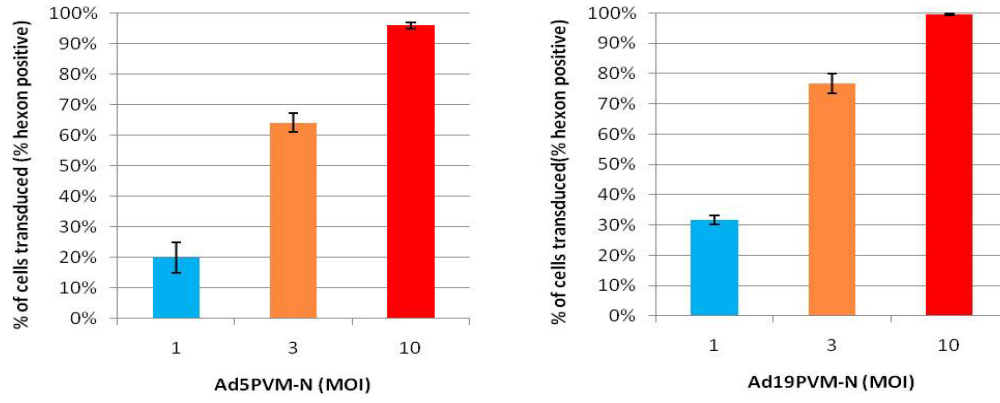
#### 4.4.2: Western blot analysis

To confirm previous findings of increased transgene expression by rAd19aGFP vectors and to extend those findings to other transgenes the generated PVM-N expressing rAd19a and rAd5 vectors were assessed for transgene expression by western blot alongside FACS using the 2Hx-2 Ab against hexon to confirm similar transduction efficiencies (Figure 4.2).

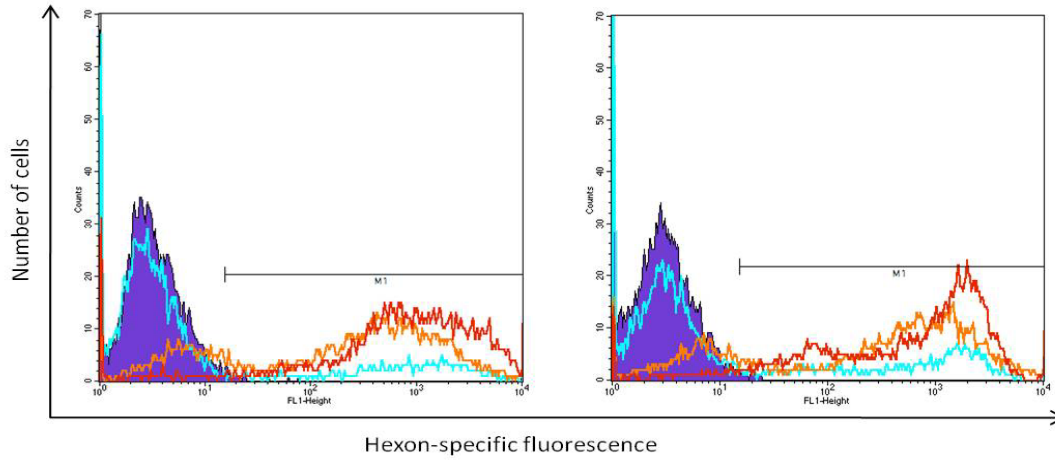
Extracts from equal numbers of A549 cells were loaded into each lane of the western blot as evidenced by the identical expression levels of the ~50kDa  $\beta$ -tubulin protein in all samples. Multiple protein species were detected in mock infected but particularly in infected cells (Figure 4.2 C). The transgene product was identified as PVM-N as it co-migrated with the protein expressed in persistently PVM infected P2-2 cells. As previously observed for GFP rAd19aPVM-N expressed considerably more of the 42 kDa PVM-N transgene product than the equivalent rAd5 vector at similar transduction efficiency, as illustrated by the accompanying FACS data (Figure 4.2 A & B). Simple densitometry analysis using the UVI DOC trans-illuminator software revealed that rAd19aPVM-N had expressed 3.6, 4.5 and 4.5x more transgene at each transduction level compared to rAd5 giving an average of 4.2x higher transgene expression by the rAd19a vector.

This data confirmed our previous conclusion that rAd19a expresses considerably more transgene than rAd5 at equal transduction efficiencies. Similar experiments were performed with the HIVA vectors using the SV5-Pk1 mAb and these results also showed a greater level of transgene expression from rAd19a vectors (data not shown).

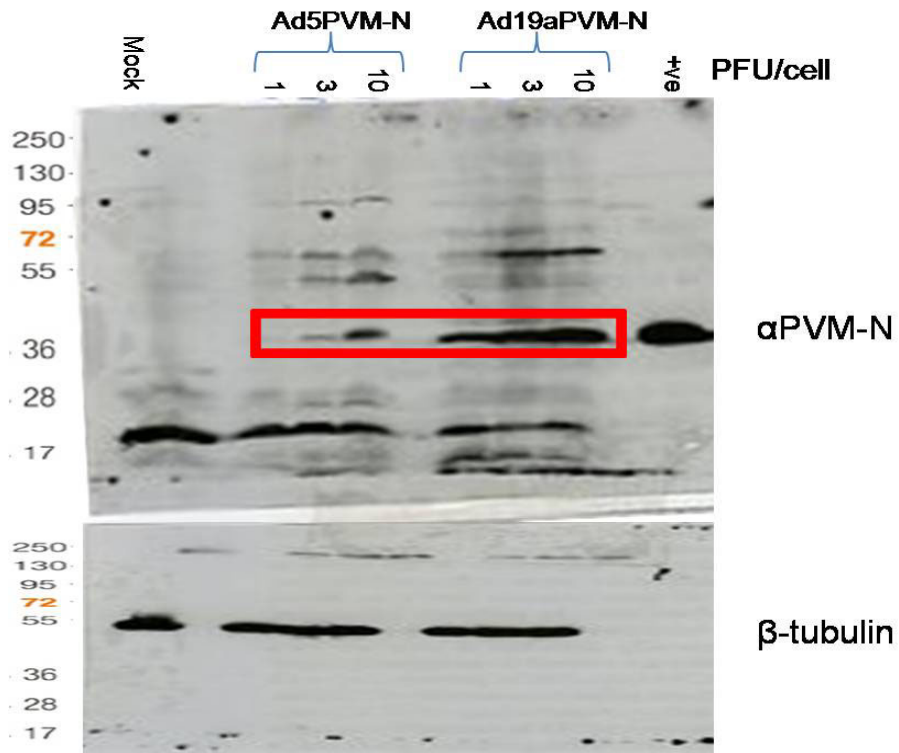
A)



B)



C)



**Figure 4.2: Examination of transduction efficiency and transgene expression of recombinant adenovirus PVM-N vectors by western blot and FACS analysis**

(A) Mean % transduction as measured by the percentage of A549 cells positive for 2Hx-2 staining when infected with rAd5PVM-N (left) and rAd19aPVM-N (right) at 1 MOI (blue bars), 3 MOI (orange) and 10 MOI (red). These are essentially all cells with fluorescence above the background staining produced in mock infected cells (see fig B). Error bars show the standard error of the mean for 3 independent experiments. (B) Examples of FACS histograms showing rAd5PVM-N transduction (left) and rAd19aPVM-N transduction (right) by 2Hx-2 staining as above (C) A549 cells were transduced with 1 MOI, 3 MOI or 10 MOI of rAd5PVM-N or rAd19aPVM-N, grown for 48 hours, lysed and subjected to western blot against PVM-N using R2052 antiserum (~42kDa highlighted in red box) or  $\beta$ -tubulin using mAb 2-28-33 (~50kDa) as a loading control. Samples were run alongside a lysed population of the P2-2 cell line as a positive control.

## **4.5: Evaluation of the phenotype of the putative E3/19K inactivated Ad19a virus**

### **4.5.1: Inactivation of E3/19K**

E3/19K protects Ad infected cells from cytotoxic T lymphocyte (CTL) mediated lysis by sequestering MHC class I in the endoplasmic reticulum (ER) (Chapter 1.1.3.2.1; Burgert & Kvist, 1985). Recently, we discovered a second function of E3/19K, protection from NK cell mediated lysis (McSharry *et al.*, 2008; Sester *et al.*, 2010). Both properties are obviously crucially important for its use as a vaccine vector where E3/19K should be eliminated to allow antigen presentation to T cells. To demonstrate the power of the recombineering protocol and to test that inactivation of E3/19K in Ad19a results in the up-regulation of MHC class I we introduced a reading frameshift by inserting 4 nucleotides with a stop codon at the beginning of the E3/19K ORF. We hoped that E3/19K expression is abrogated without affecting the expression of other E3 genes.

The expression of E3/19K can be monitored by FACS using several mAbs (Sester *et al.*, 1994). Moreover, on infection the E3/19K deleted Ad19a should also be unable to sequester MHC class I and hence this should be detectable on the cell surface by FACS with the W6/32 Ab. If the mutation has caused no further damage to the E3 region all other E3 genes should still produce functional proteins and their effects should still be observable. As examples, we tested for the expression of the E3/49K gene located directly downstream of E3/19K using a mAb and for the effect of the E3/10.4/14.5K complex by measuring the modulation of Fas (see Chapter 1).

#### **4.5.1.1: Aims**

To assess the success of the generation of an Ad19a virus with E3/19K inactivated. It will also be assessed if the inactivation has had an effect on the other E3 genes.

### **4.5.2: Phenotype evaluation**

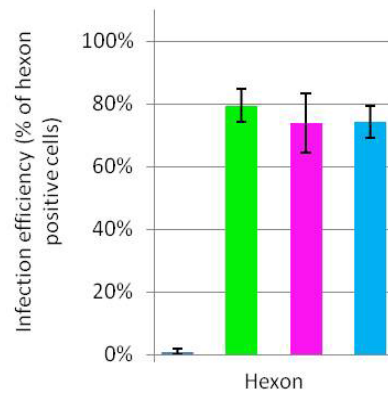
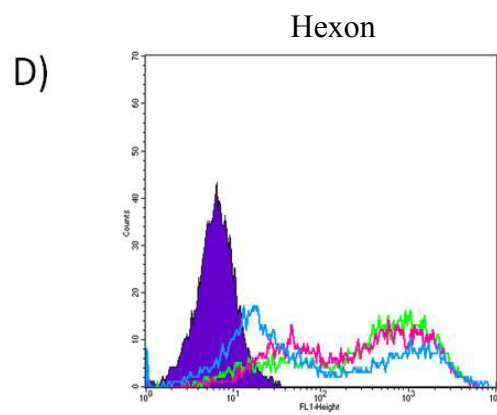
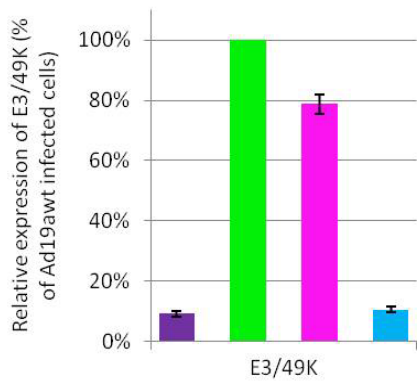
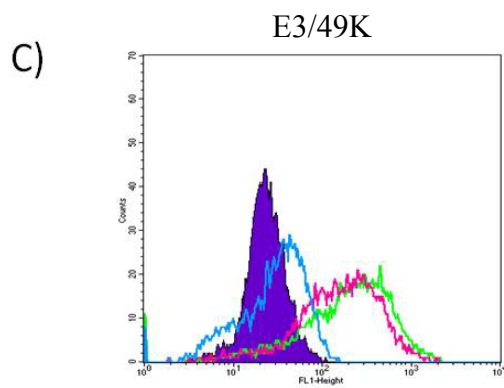
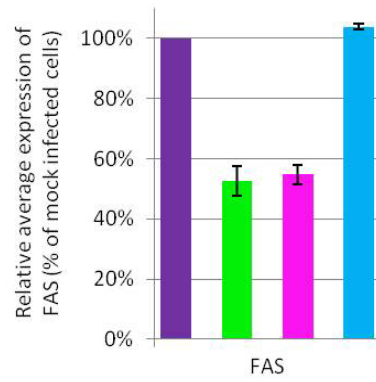
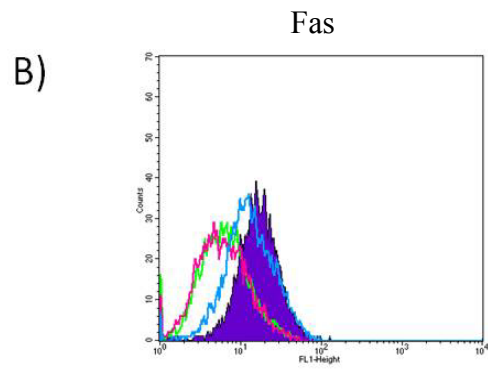
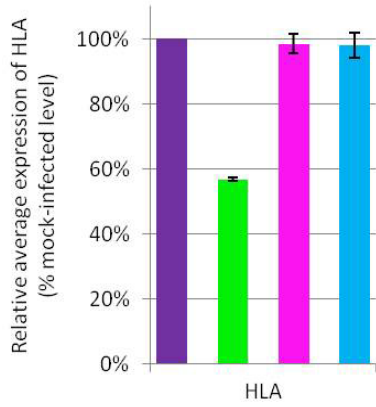
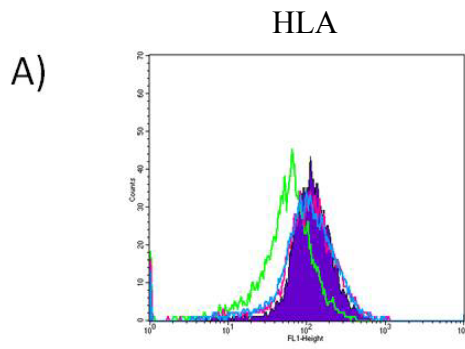
To assess whether the reconstituted Ad19a19K\* virus lost E3/19K expression, whilst retaining the functional expression of other E3 genes, infected 293 cells were examined by FACS analysis and western blot using various Abs against E3 products and their respective cellular targets. Each experiment was performed alongside 293 cells infected with 2 control viruses, Ad19awt which should have full expression of E3 genes and infected cells should show all E3 function remain and Ad19aΔE3 which has had the entire E3 region deleted preventing the expression of any of the E3 genes so infected cells should show none of their functional effects.

#### **4.5.3: FACS analysis**

An MOI of 10 of Ad19a19K\*, Ad19awt and Ad19aΔE3 was used to infect HEK 293 cells alongside a mock infection. After 24 hours the cells were examined by FACS for HLA (mAb W6/32), FAS (Ab BG-27), E3-49K (Ab 4D/1) and hexon (Ab 2Hx-2) expression (Figure 4.3). If E3/19K expression has been successfully eliminated from the Ad19a19K\* proteome down-regulation of HLA should be abrogated similar to cells infected with Ad19aΔE3. If the loss of E3/19K expression is confirmed it can then be examined if the insertion and frame-shift has caused a change to the expression of other E3 genes by monitoring the expression of E3/49K, which should still be detectable in cells infected with Ad19awt and Ad19a19K\* whilst not in cells infected with Ad19aΔE3. The function of another E3 gene E3/10.4/14.5K can be examined by monitoring the modulation of Fas which should be down-regulated in Ad19awt and Ad19a19K\* infected cells whilst not in cells infected with Ad19aΔE3.

Figure 4.3 shows that this is indeed the case. As expected, the inactivation of E3/19K prevented a detectable down-regulation of HLA matching previously published data (Deryckere & Burgert 1996; Figure 4.3 A) whilst not affecting the ability of E3/10.4/14.5K to modulate Fas (Figure 4.3 B). Also, the expression of the neighbouring E3/49K gene appears similar to Ad19awt (Figure 4.3 C). In all infections tested, all viruses showed similar mean infection efficiencies (Figure 4.3 D).





**Mock**
 **Ad19awt**
 **Ad19aΔ19K**
 **Ad19aΔE3**

**Figure 4.3: Mutation appears to selectively affect E3/19K functions and not those of other E3 genes**

(A) Example of a FACS histogram showing cell surface expression of HLA in cells infected with Ad19awt (green line), Ad19a19K\* (pink line) and Ad19a $\Delta$ E3 (blue line) or mock infected (purple fill). Below is a chart showing relative average expression of HLA on the cell surface of 293 cells compared to levels in mock infected cells, set to 100%, using the same colour scheme. (B) shows the cell surface expression of Fas upon infection with the various viruses, relative to mock infected cells (set to 100%), using the same colour code as above. (C) shows the average internal expression of E3/49K (as cell surface E3/49K is rapidly cleaved and secreted) upon infection with the various viruses, using the same colour code as above (D) shows the average internal expression of hexon, upon infection with the various viruses, using the same colour code as above. All error bars show the standard error of the mean for 3 independent experiments.

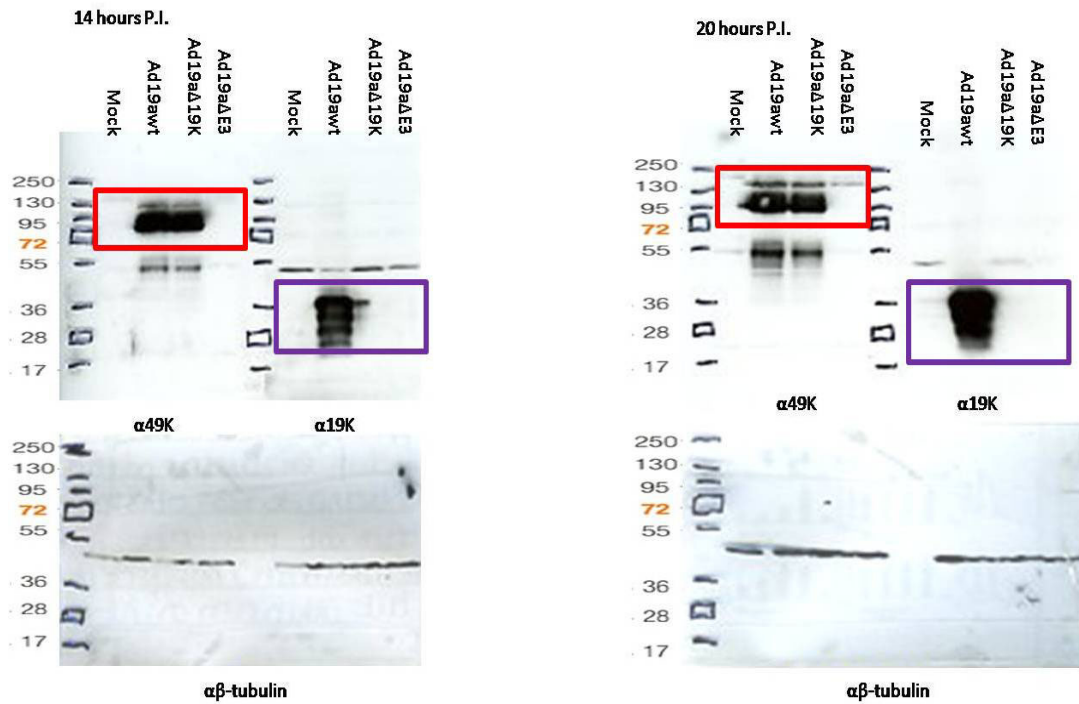
These findings, taken together, confirm that Ad19a19K\* has lost the ability to express a functional form of E3/19K and that this mutation has not impaired the function of either the immediately downstream E3/49K protein, but has had a minor effect on its expression level, or on the further downstream E3/10.4/14.5K genes which modulated Fas to similar levels as seen in Ad19awt infections. The reduction in E3/49K expression may have been caused by a reduction in translation of the E3/49K gene due to its immediate vicinity to the E3/49K start codon.

To investigate if Ad19a19K\* was still expressing a form of E3/19K which was non-functional and as such non-detectable in the FACS assay due to the lack of an Ad19aE3/19K mAb, a western blot was performed to detect for the presence of E3/19K and E3/49K using corresponding rabbit antisera.

#### **4.5.4: Western blot analysis**

293 cells were mock infected or infected with 10 pfu of Ad19awt, Ad19a19K\* or Ad19a $\Delta$ E3, left for 14 hours (left) or 20 hours (right) lysed and subjected to western blot for detection of E3/19K using antisera R22612, E3/49K using rabbit antisera R48 or  $\beta$ -tubulin using Ab 2-28-33 as a loading control (Figure 4.4). Equal amounts of 293 lysates were loaded into each lane of the western blot as evidenced by the identical expression levels of the ~50kDa  $\beta$ -tubulin protein in all samples. At both 14 hours and 20 hours only Ad19awt was shown to express a detectable form of E3/19K (purple frame) as evidenced by the 19-36kDa bands highlighted. Both Ad19a19K\* and Ad19awt expressed a detectable form of E3/49K as evidenced by the 88-95kDa bands highlighted. This finding shows that cells infected with Ad19a19K\* are not expressing a detectable form of E3-19K at either timepoint tested, as seen in cells infected with Ad19awt, but are expressing E3/49K.

In conclusion, the Ad19a19K\* virus is unable to express an E3/19K protein that is functionally active showing that the nonsense mutation inserted (see Chapter 3.7) has had the desired effect. Moreover, strong evidence is provided that the mutation selectively affects E3/19K but no other E3 products.



**Figure 4.4: Expression pattern of E3/19K and E3/49K in Ad19a19K\* as detected by western blotting**

293 cells were mock infected or infected with 10 pfu of Ad19awt, Ad19a19K\* or Ad19aΔE3. 14 h.p.i. (left) or 20 h.p.i. (right) cells were lysed and subjected to western blotting for E3/49K using antisera raised against its cytoplasmic tail (antisera R48, ~88-95kDa), E3/19K (antisera R22612, ~19-36kDa) or β-tubulin (mAb 2-28-33, ~50kDa) as a loading control. E3/19K and E3/49K specific protein species are highlighted by purple and red boxes respectively.

## 4.6: Discussion

The work presented in this chapter has detailed the phenotype of the viruses and vectors generated in Chapter 3 by recombineering, particle/pfu ratios, the expression of transgenes in mammalian cells by the vectors and the effect of the nonsense mutation inserted into Ad19a19K\*.

The results in section 4.3 showed that rAd19a, as had previously been seen, had a significantly higher particle/pfu ratio than Ad5. In the most extreme case, the Ad19aHIVA vector, this is almost 700x higher than the corresponding Ad5 vector. This should be taken into account when rAd19a vectors are used *in vivo* as the far larger number of immunogenic particles required to reach the same pfu may trigger some adverse inflammatory reactions, although we did not observe any negative side effects in our own *in vivo* experimentation (Chapter 7). The reason for the higher particle/pfu ratios could be either procedural or due to intrinsic differences in the biology of the serotypes.

Procedurally, a differential damage to the particles during purification, for example during sonication, could have occurred due to some more inherent instability of the Ad19a particles resulting in a large number of particles which are not capable of cell infection. The ratio could also be caused by a flaw in the methodology used to calculate the number of particles present because the figure for the conversion of OD 260nm DNA readings to particle number is based on Ad5 and other Ads may have a different DNA concentration per particle. This is unlikely, however, because use of Nanosight<sup>®</sup> technology has confirmed the particle number readings for Ad19a within less than 1 log (data not shown). Interestingly, the same methodology showed that, unlike rAd5 vectors, the rAd19a vector preparation had several particle size peaks suggesting distinct populations, perhaps caused by aggregation or the loss of fibre, which would both account for higher particle/pfu ratios. Also, 293 cells are known to produce Ad5 pIX protein (Ghosh-Choudhury, Haj-Ahmad and Graham, 1987) an important structural protein which may compete with the Ad19a equivalent for incorporation into Ad19a particles.

Biologically, the higher ratios could be natural due to some quirk of Ad evolution or caused by the growth conditions that Ad19a has been subjected to. If natural it may be due to Ad19a's tropism for the eye (Arnberg, Mei and Wadell, 1997). The surface of the eye, the known site of primary Ad19a infection, is cooler than the sites of infection used by other Ads. This could mean that the normal growth temperature of Ad19a is several degrees lower than 37°C meaning growth at this temperature may cause defects in particle formation. A simple experiment could be envisaged where Ad19a isolates were grown at temperatures lower than 37°C and their particle/pfu ratios then analysed to observe if a lower temperature prevents the high particle/pfu ratios from occurring. It may also have been caused by the growth of original ocular isolates of Ad19 clones over several passages in FT cells, KB cells and HeLa cells (Newland and Cooney, 1978) which are not a natural host for Ad19 infection which may have resulted in adaptive changes to the genome which generated a larger number of non-functional particles. Any adaptive biological changes in the Ad19 genome may have also been compounded by passage of Ad19a vectors in 293 cells which supply the E1A genes of Ad5 in *trans* which may cause some instability of the particles. This, however, would not explain the large particle/pfu ratio of Ad19awt which provides its own E1A genes.

In the meantime, electron microscopy (EM) has been performed on both Ad19a and Ad5 purified virus preps that had been frozen (data not shown) and many of the Ad19a particles were observed to have begun to disintegrate. Also higher aggregation was seen for Ad19a. Further experiments would need to be performed to make firm conclusions as to the likely cause of the high particle/pfu ratio. For example, the titres of Ad19a vectors purified using sonication versus freeze/thawing. If there was shown to be no difference resulting from the use of either procedure then further investigation into the biology of the virus would be required, perhaps by growth of the virus on different cell lines followed by particle calculation or the generation of a new cell line which provides the Ad19a E1A region in *trans* rather than the Ad5 one. It is certain that EM will be an excellent analytical tool to improve the procedure for production of stable Ad19a particles.

The lack of stable particle formation in Ad19a is in opposition to its known stability as an infectious agent. Several major Ad19a epidemics have been traced back to

surgical instruments used for eye examinations. This suggests that Ad19a is particularly stable in the environment compared to other serotypes.

Even though no negative effects have been observed to date, reactions to administration of a large number of particles have to be carefully monitored and may be a safety issue for regulatory boards.

It has been shown in this chapter that the generated PVM-N vector expresses its transgene and that, as previously observed for Ad19aGFP, rAd19a vectors express significantly more transgene than rAd5 vectors at equal transduction efficiencies. This is, of course, a very favourable feature of Ad19a vectors as less virus could be administered to obtain the expected therapeutic effect. As a result, toxicity issues would be expected to be reduced however the latter may be partially countered by the increased number of particles due to the high particle/pfu ratio.

The reason for the increased transgene expression remains unclear. Whilst the expression cassettes are identical, enhancer sequences or other *cis* elements in the vicinity of the inserted transgene which differ between Ad19a and Ad5 may have positively influenced the expression level of the transgene. Alternatively other parts of the Ad19a proteome i.e. the E4 proteins, may influence transgene expression which has been shown to occur in Ad5 (Lusky *et al.*, 1999). This could be examined by the deletion of one or all of the E4 ORFs using the same recombineering system and nonsense mutation insertion as described herein for Ad19a19K\*. If increased transgene expression persists after E4 modification then the enhanced expression is more likely to be related to aspects of Ad19a virus biology. For example, the presence of multiple copies of transgene has been shown to increase levels of transgene expression above the expected level considering the number of additional genes inserted (Takahashi *et al.*, 2010). In Ad19a aggregation of particles may result in a single receptor binding and cell entry event allowing multiple Ad19a particles and therefore multiple transgene copies entry which could account for the observed higher transgene expression. This conclusion is supported by the observed increased plaque size during Ad19a titration, compared to Ad5 plaques, which maybe the result of multiple copies of the genome gaining entry to a single cell after a single entry event.

Finally, the insertion of the nonsense mutation in Ad19a19K\* was shown to effectively prevent the expression of a functional E3/19K protein and was shown to have no effect on the function of E3/10.4/14.5K, two E3 ORFs which are further downstream. A slightly lower level of expression was noted for the E3/49K protein, which is encoded by the neighbouring ORF. However, it is not known if this change in expression is significant. If it is significant it is unlikely to be caused in transcription, as the preceding E3/19K ORF should be transcribed normally. It may instead be caused by affecting the splicing of the E3 transcription unit which has been shown to have an effect on the level of expressed E3 proteins (Scaria & Wold, 1994).

These findings have shown that the modification of Ad viruses or vectors on an individual gene basis is possible using the recombination system and allows for the selective mutation of individual genes which are detrimental to vector function whilst leaving intact other genes from the same region that may have more advantageous functions. This can eliminate the need to remove entire regions of the Ad genome. Further research on the functions of Ad genes may result in the discovery of additional gene products which would have a beneficial function in a vector designed for vaccination or gene therapy and should therefore be preserved whilst others are eliminated.

The next chapter will concentrate on the previously described enhanced transduction of Ad19a vectors in immune cell lineages including, and most importantly for vaccination, dendritic cells. Due to the results of this chapter particular interest will be placed on the ability of Ad19a to express high levels of transgene product in these important cell types.



## **Chapter 5: Adenovirus transduction of dendritic cells**

### **5.1: Introduction**

This chapter will investigate transduction of Ad vectors in immune cell lineages including, and most importantly for vaccination, dendritic cells.

#### **5.1.1: Dendritic cells**

For a full introduction to DCs see Chapter 1. As mentioned before, DCs are the most potent APCs known (Banchereau & Steinman, 1998). In the periphery, they are specialised to capture a range of antigens, soluble, particulate or even entire cells. If the material taken up is sensed foreign, they undergo phenotypic and functional changes allowing them to maximise their antigen presentation capacity. They migrate to T cell areas in the lymph node and activate both CD4<sup>+</sup> helpers and CD8<sup>+</sup> cytotoxic T lymphocytes, which are essential for an effective cell-mediated response to both viruses and tumours (Banchereau & Palucka, 2005).

Experimentally, DCs can be isolated from human blood by two distinct methodologies. Both initially require the separation of Peripheral Blood Mononuclear Cells (PBMCs) from human blood by the Ficoll-Hypaque gradient. To generate so-called monocyte derived DCs, CD14<sup>+</sup> monocytes may be isolated which can be differentiated into immature DCs (iDC). The CD14 antigen is strongly expressed on most monocytes and macrophages and weakly on neutrophils. One methodology separates CD14<sup>+</sup> monocytes from PBMCs using magnetic bead technology to isolate cells bound to a CD14 specific antibody (Chapter 2.14.2) whilst the other relies on the adherence of CD14<sup>+</sup> monocytes to a plastic surface (Chapter 2.14.3). In both methodologies the resulting cells are grown for 5 days in the presence of human Interleukin 4 (hIL-4) and human granulocyte macrophage colony stimulating factor (hGM-CSF). This produces non-adherent immature DCs which can be identified by the loss of monocyte differentiation markers and the upregulation of a range of molecules including CD1a (an MHC-1 like molecule), CD83 and CD86 (a co-stimulatory ligand necessary to initiate T cell activation).

Both methodologies were explored here and the results will be presented in this chapter.

To confirm the generation of iDCs the cells can then be differentiated to mDCs, over 2 days in the presence of either Lipopolysaccharide (LPS; Lutz *et al.*, 1999) or human TNF- $\alpha$  (hTNF- $\alpha$ ; Yamaguchi *et al.*, 1997). Maturation can then be confirmed by the further upregulation of CD86 and the concurrent upregulation of CD80 (a second co-stimulatory ligand necessary for T cell activation by APCs), HLAI, HLA II and adhesion molecules which can be detected by FACS. LPS, TNF- $\alpha$  and viral vector induced maturation of DCs was investigated.

### **5.1.2: Adenovirus vectors and dendritic cells**

Ad vectors can be used *ex vivo* to target DC populations for the stimulation of a strong antigen specific response which could be utilised to eliminate infectious agents or cancer cells.

*Ex vivo*, iDCs are generated from the patient's blood and then transduced with an Ad vector encoding an antigen of pathogens or tumour cells. The resulting matured dendritic cells are then re-injected back into the body where they traffic to the lymph nodes and activate a targeted immune response to the transduced gene (for an example see Mercier *et al.*, 2002).

Ad vectors have shown increasing promise for the transduction of dendritic cells for their use in cancer therapy (Sas *et al.*, 2008; Lundqvist & Pisa., 2002) or vaccination (Ranieiri *et al.*, 1999; Basak *et al.* 2004). Adenovirus transduction does not perturb normal DC function (Jenn, Schuler & Steinkasserer, 2001) and triggers maturation signals for the generation of highly immunostimulatory antigen presenting cells (Rea *et al.*, 1999; Morelli *et al.*, 2000). It has been noted, however that, when using Ads from subgroup C, very high MOIs are required to transduce 95% of immature DCs (Zhong *et al.*, 1999). Therefore, the ideal vector for *ex vivo* DC transduction would avoid vector-related immune responses, have relative specificity for transducing

DCs, induce high levels of transgene expression (Basak *et al.*, 2004) and act synergistically to induce DC maturation.

In 2001 a variety of Adenovirus fibres from subgroups A, B, D (not including the Ad19 fibre), and F, were pseudotyped onto Ad5 vectors expressing the Luciferase gene. These studies showed that the fibres of Ad16, Ad35 and Ad50 mediate increased tropism for immature DCs (Rea *et al.*, 2001). An Ad5F35 vector required a 10-100 fold lower MOI for transgene expression, produced enhanced antigen presentation in transduced DCs and increased expression of CD80, CD86 and HLAI.

Notably Ad16, 35 and 50 are all from subgroup B, the only subgroup known not to require CAR for transduction (Roelvink *et al.*, 1998) which suggests CAR usage may be the associated with the high MOI required for Ad5 transduction. Ad19a receptor usage will be investigated in Chapter 6.

Apart from receptor usage, the promoter driving transgene expression may be optimised for DC expression. It has more recently been shown that the CMV promoter is very effective at achieving high levels of transgene expression within a range of human DC populations (Papagatsias *et al.*, 2008). The use of the CMV promoter in both the Ad5 and Ad19a vectors created in this study should therefore provide an excellent choice for transgene expression in DCs.

### **5.1.2: Ad19a and dendritic cells**

We previously showed that Ad19awt is highly efficient in infecting DCs (Ruzsics *et al.*, 2006). However, it remained unclear whether this was related to a differential replication in DCs or different uptake mechanisms, therefore, it was crucial to investigate whether the replication deficient Ad19a vector retained the enhanced DC transduction. If the Ad19a vector is shown to efficiently transduce DCs then it would support the usage of Ad19a as an alternative vector for DC transduction.

## **5.2: Aims**

The aim of this section was to compare the efficiency of transduction and transgene expression in DCs with the Ad19a and Ad5 vector and to examine the maturation of iDCs upon Ad transduction. A secondary aim was to investigate which of the methods for iDC generation from CD14 + monocytes is more effective.

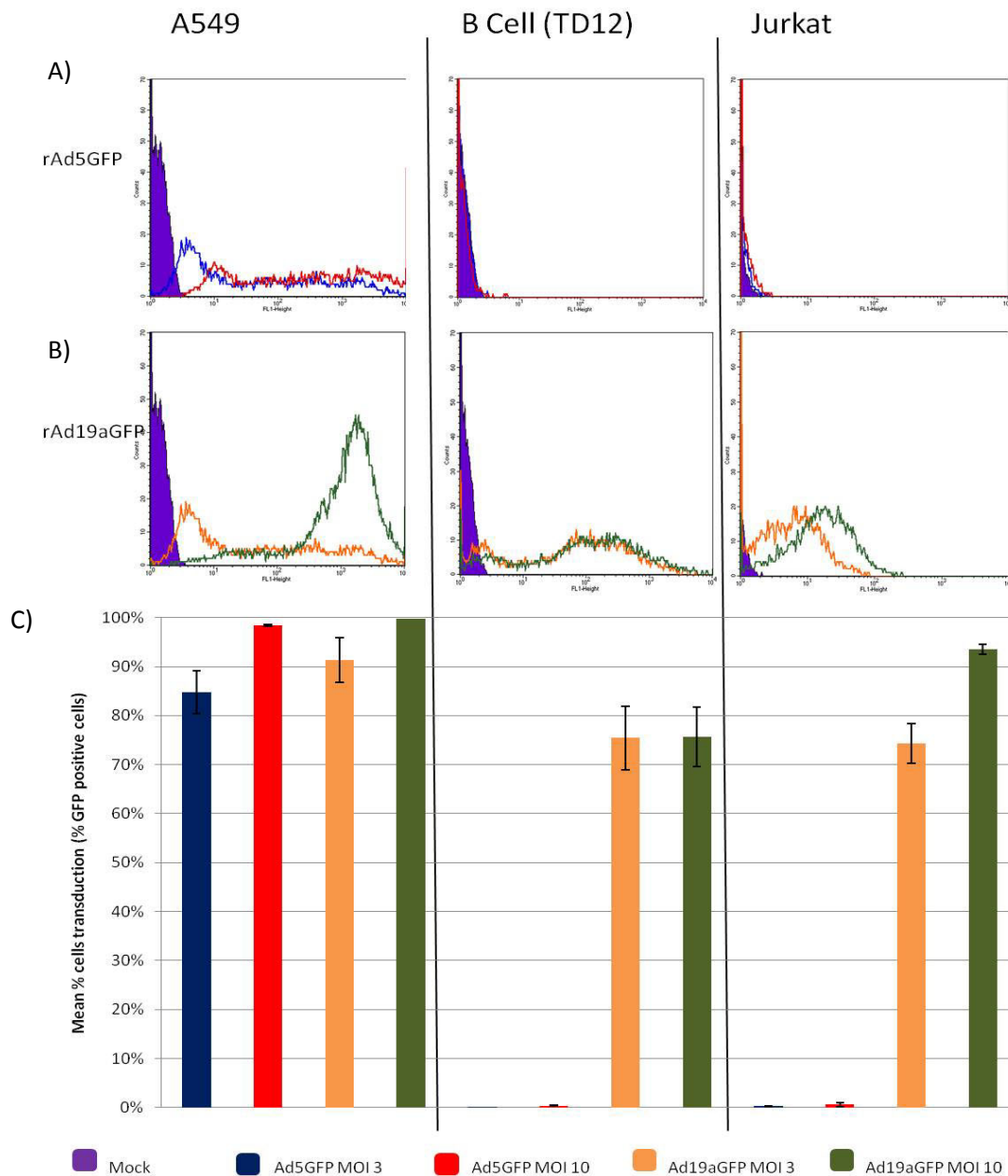
## **5.3: Adenovirus transduction of immune cell lineages**

It was first decided to confirm the previous finding (Ruzsics et al., 2006) that the Ad19a vector efficiently transduced immune lineage cells using FACS. As examples, Jurkat T cells and a human B cell line (TD12; Chapter 2) were used alongside control A549 cells to monitor Ad19a and Ad5 vector transduction.

### **5.3.1: FACS analysis**

rAd19aGFP and rAd5GFP were used to transduce A549 cells, TD12 cells and Jurkat T cells at an MOI of 3pfu/cell or 10pfu/cell then left for 48 hours. After 48 hours the transduced cells were analysed for expression of GFP by FACS analysis. Representative histograms upon vector transduction are shown for rAd5GFP (Figure 5.1 A) and rAd19aGFP (Figure 5.1 B). The results of three experiments are summarised in Figure 5.1 C.

rAd19aGFP and rAd5GFP showed similar levels of transduction in A549 cells at both MOIs tested, as seen previously, with mean % transduction levels of 91% (3pfu/cell) and 99% (10pfu/cell) and 85% (3pfu/cell) and 99% (10pfu/cell) respectively. In contrast, rAd5GFP failed to show efficient transduction of either TD12 cells or Jurkat cells at either MOI tested whilst rAd19aGFP transduced 75% of B cells at both 3pfu/cell and 10pfu/cell and 73% (3pfu/cell) and 94% (10pfu/cell) of Jurkat cells. It was noted, however, that in Jurkat cells in particular the mean fluorescence intensity was substantially decreased when compared to A549 cells suggesting that transgene expression from the CMV promoter was not as high as in A549 cells.



**Figure 5.1: Ad vector transduction of B and T cell lines**

(A) FACS histograms show transduction of A549 cells (column 1), B cells (column 2), or Jurkat cells (column 3) with rAd5GFP either mock treated (purple fill) or transduced with an MOI of 3 (blue line) or 10 (red line). (B) FACS histograms showing the same transduction as previously with rAd19aGFP, MOI of 3 (orange line) or 10 (green line). (C) Mean % transduction, measured by the percentage of GFP positive cells, of A549 cells (column 1), TD12 cells (column 2) and Jurkat cells (column 3) using the previous colour scheme showing poor transduction of B cells and Jurkat cells by Ad5GFP. Error bars indicate the standard error of the mean for three or four experiments.

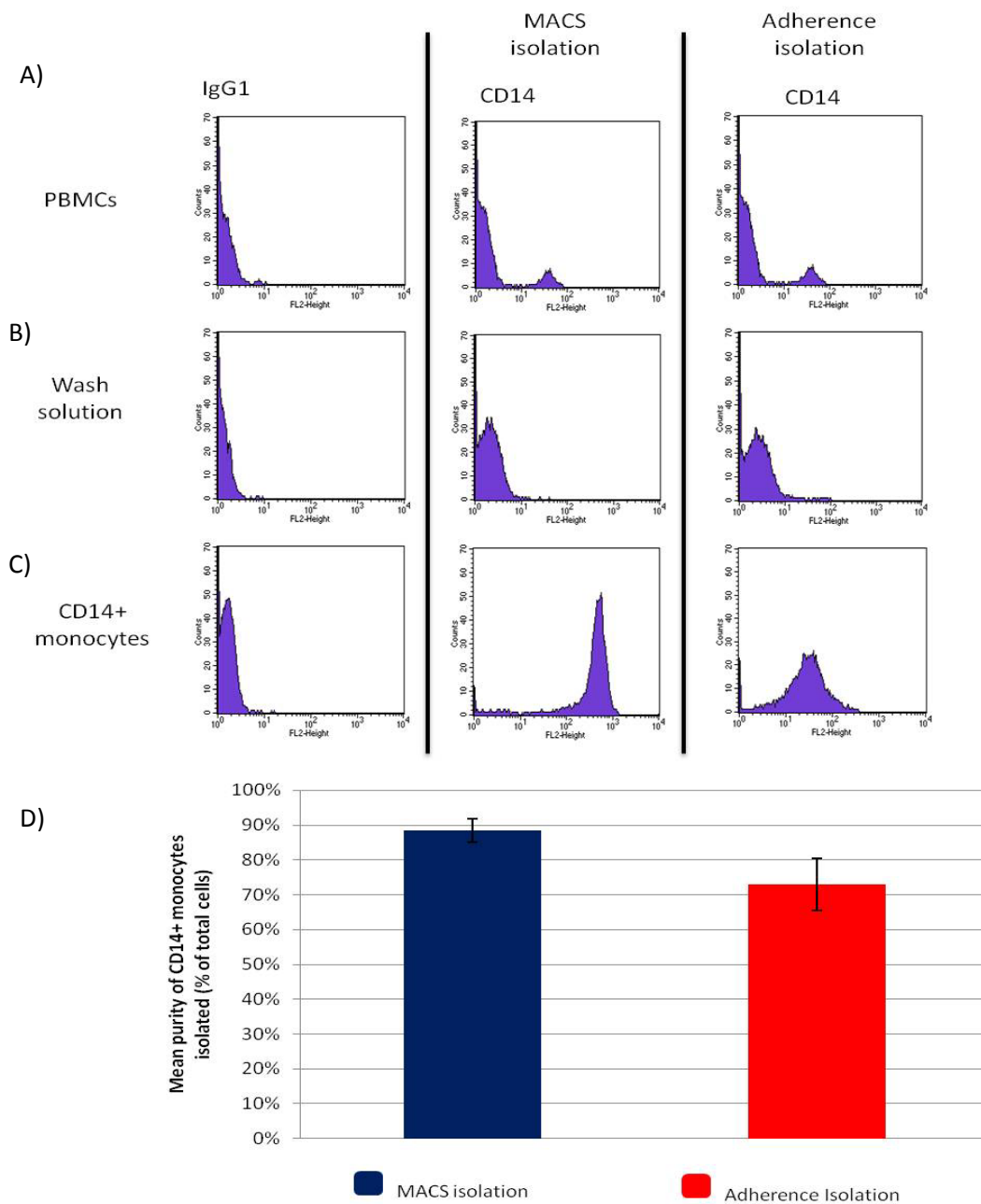
This data confirmed previous data that rAd19aGFP can efficiently transduce immune lineage cell types whilst rAd5GFP cannot (Ruzsics *et al.*, 2006). In the previous experiments the transduction of Jurkat cells by rAd5GFP was more efficient (15%). This is likely due to the higher MOI used (25 pfu/cell).

These results were taken as evidence that the rAd19aGFP and rAd5GFP vector preparations behaved as previously observed in B and T lymphoid cell lines and that Ad19a vectors may have distinct advantages in these cell types. We next wished to determine whether the increased efficiency of infection by Ad19a vs. Ad5 in DCs is preserved in the replication deficient vectors. A reliable system for the isolation of DCs from human PBMCs was therefore required.

## **5.4: Purification and maturation of dendritic cells**

### **5.4.1: Comparison of dendritic cell isolation methods**

The two methodologies outlined in Chapter 5.1.1 and explained in detail in chapters 2.14.2 and 2.14.3 were compared for the ability to isolate CD14<sup>+</sup> monocytes from human PBMCs. PBMCs were first isolated from human blood (Chapter 2.14.1) and then subjected to either MACS separation or adherence isolation. Cells were tested for the expression of the monocyte marker, CD14, by FACS with the M5E2 Ab prior to isolation (Figure 5.2 A), and following isolation (Figure 5.2 C). Cells in the wash solutions from both methodologies were also tested in order to evaluate if any CD14<sup>+</sup> cells were being lost during either process (Figure 5.2 B). The mean purity of CD14<sup>+</sup> monocytes purity achieved in all experiments using each method is given in Figure 5.2 D.



**Figure 5.2: Isolation of CD14+ monocytes from human blood by MACS or adherence separation.**

FACS histograms after staining of control cells or cells isolated by MACS isolation or adherence isolation showing human PBMCs (A; upper panel) Isolation Wash solutions (B; middle panel) or isolated CD14+ monocytes (C; lower panel) with an IgG1 control Ab (column 1) and a CD14 antibody (Columns 2 & 3). (D) Chart showing the mean purity of CD14+ monocytes isolated by MACS isolation (dark blue bar) or by adherence isolation (red bar) given as the % of CD14+. Error bars show the standard error of the mean for 7 and 4 experiments respectively.

When the same population of PBMCs, shown by almost equal CD14<sup>+</sup> staining prior to isolation (Figure 5.1 A), was subjected to the purification schemes some significant differences were observed. MACS separation resulted in a mean CD14<sup>+</sup> monocytes purity of 88%, whereas adherence isolation only resulted in 73% pure CD14<sup>+</sup> cells. In both protocols, loss of CD14<sup>+</sup> cells in the wash steps was minimal (Figure 5.2 B) but MACS separation resulted in a far purer final population of CD14<sup>+</sup> monocytes as evidenced by the narrow histogram of strongly positive CD14<sup>+</sup> cells following MACS separation when compared to the broader histogram and less bright CD14 staining following adherence isolation (Figure 5.2 C) which was observed consistently. These results suggest that although not many CD14<sup>+</sup> cells are being lost during adherence isolation there are more cells remaining in the flask which are not CD14 positive or express low levels of CD14 and do not therefore represent CD14<sup>+</sup> monocytes.

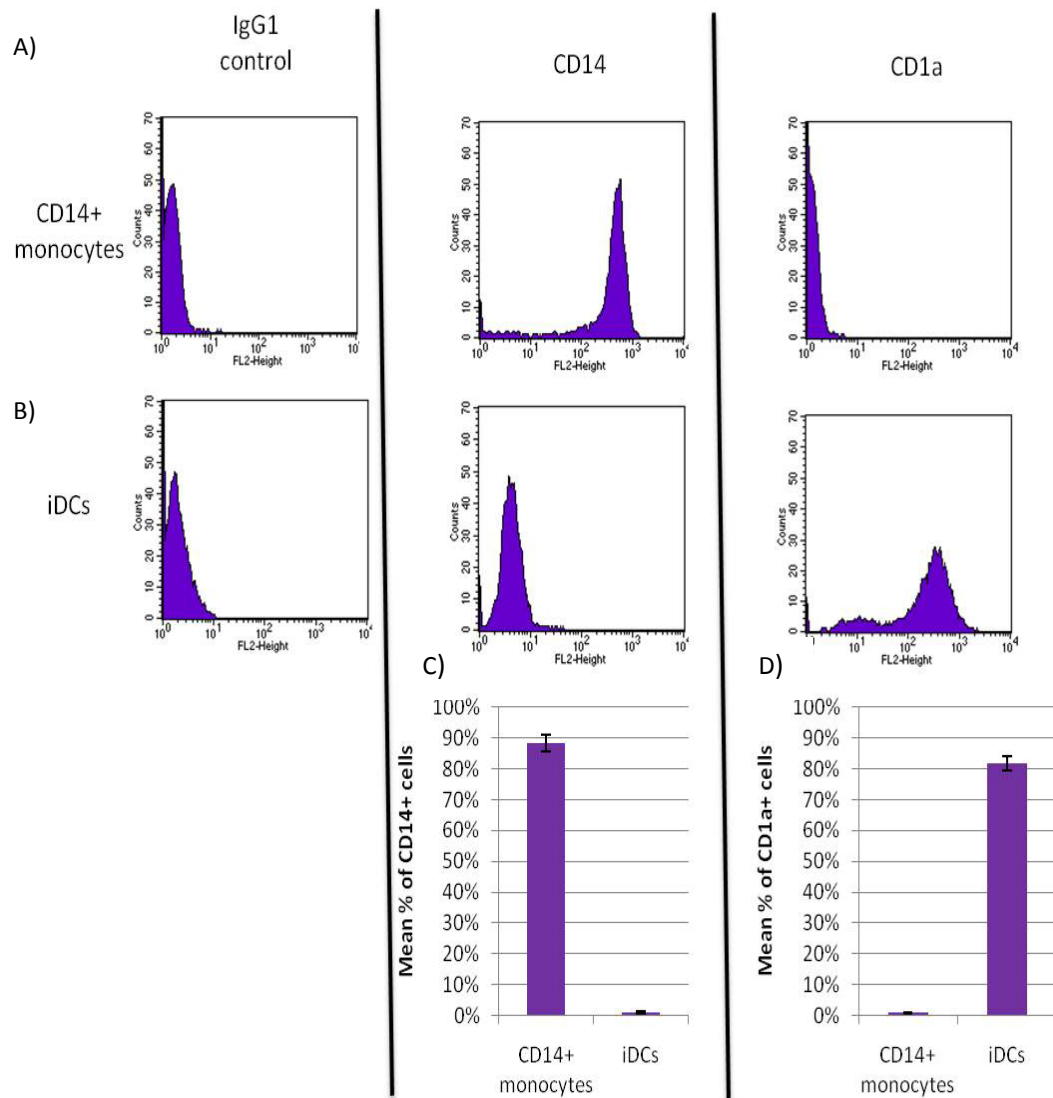
While more expensive, MACS separation was found to be quicker, simpler and resulted in purer populations of CD14<sup>+</sup> monocytes when compared to adherence isolation. It was decided that all subsequent experiments should be performed using MACS separation.

#### **5.4.2: Comparison of Dendritic cell maturation status**

Following CD14<sup>+</sup> monocyte isolation, the cells were grown in medium containing hIL-4 and hGM-CSF to stimulate their differentiation into iDCs. The resulting candidate iDCs can be tested for the loss of CD14 expression (monocyte marker) and the up-regulation of CD1a expression ((lymphocyte marker) see Chapter 1)) by FACS detection using the M5E2 (CD14) and HI149 (CD1a) Abs. (Figure 5.3).

To monitor successful DC differentiation a sample of cells was stained for CD14 and CD1a prior to application of hIL-4 and hGM-CSF and then 5 days after their application (Figure 5.3 A & B)





**Figure 5.3: Development of immature dendritic cells from CD14+ monocyte progenitors**

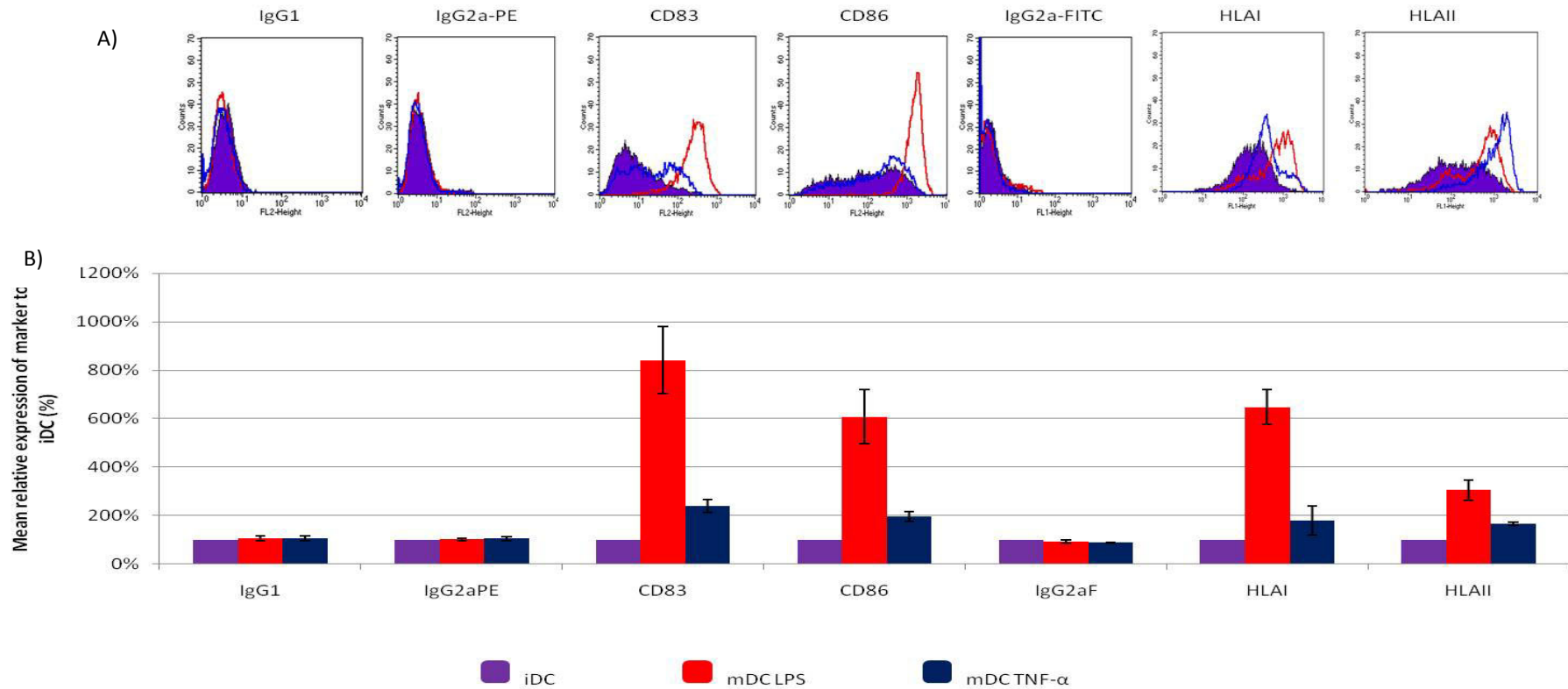
FACS histograms showing staining of CD14+ progenitor monocytes (A) or candidate iDCs (B) for IgG1 (Column 1; isotype control), CD14 (Column 2) and CD1a (Column 3) (B) Mean percentage of CD14+ cells detected in CD14+ progenitor monocytes or candidate iDCs upon staining for CD14 (C) or CD1a (D). Error bars show the standard error of the mean for 6 experiments.

The mean % of both CD14 and CD1a positive CD14<sup>+</sup> monocytes and iDCs were then recorded and summarised (Figure 5.3 C & D). Prior to every experiment, iDC formation was confirmed by the loss of CD14 expression from an average of 88% of cells to <1% and the up-regulation of CD1a expression from an average of <1% of cells to 82%. This data provided good evidence of differentiation into iDCs.

Further evidence that the cells grown in hIL-4/GM-CSF are indeed iDCs can be obtained by inducing their maturation by treatment with LPS or TNF $\alpha$ . iDC maturation was characterised by the up-regulation of CD83, CD86, HLAI and HLAII (see Figure 1.6). A control culture was left in the same cytokine cocktail as previously. Two methodologies for the maturation and subsequent characterisation of iDCs were trialled using either LPS or hTNF- $\alpha$  and the resulting mDCs compared by FACS analysis using the mAbs HB15 (CD83), FM95 (CD86), MCA81F (HLAI) and L243 (HLAII) (Figure 5.4). Confirmation of the previous data that iDCs had been successfully generated was obtained by their ability to mature. CD83, CD86, HLAI and HLAII were considerably up-regulated in mDCs vs. iDCs. The findings across all experiments also showed that LPS generated superior up-regulation of all four markers tested, demonstrating approximately 3.5x (CD83), 3.1x (CD86), 3.6x (HLAI) and 1.8x (HLAII) higher up-regulation when compared with DCs matured by hTNF- $\alpha$ . It should be noted, however, that hTNF- $\alpha$  resulted in an average of 1.5x higher expression of all markers tested when compared to iDCs and as such still generated mDCs. Both sets of data will be useful as controls for the investigation of the maturation of iDCs by Ad vectors.

## **5.5: Adenovirus vector transduction of immature dendritic cells**

Ad19awt has been previously shown to infect iDCs efficiently (Ruzsics *et al.*, 2006). However, it remained unclear whether this reflected better uptake or replication, therefore, it was important for further development of Ad19a vectors to examine whether the Ad19a vector retained this capacity.



**Figure 5.4: Maturation of immature dendritic cells by application of LPS or hTNF- $\alpha$**

(A) FACS histogram overlays showing staining of iDCs (purple fill), DCs matured with LPS (mDC LPS; red solid line) or DCs matured with hTNF- $\alpha$  (mDC TNF- $\alpha$ ; blue solid line) with mAbs against CD83, CD86, HLAI and HLAII or corresponding isotype controls. (B) Mean relative expression to iDC levels of each marker seen in 5 experiments. Error bars show the standard error of the mean for 5 experiments.

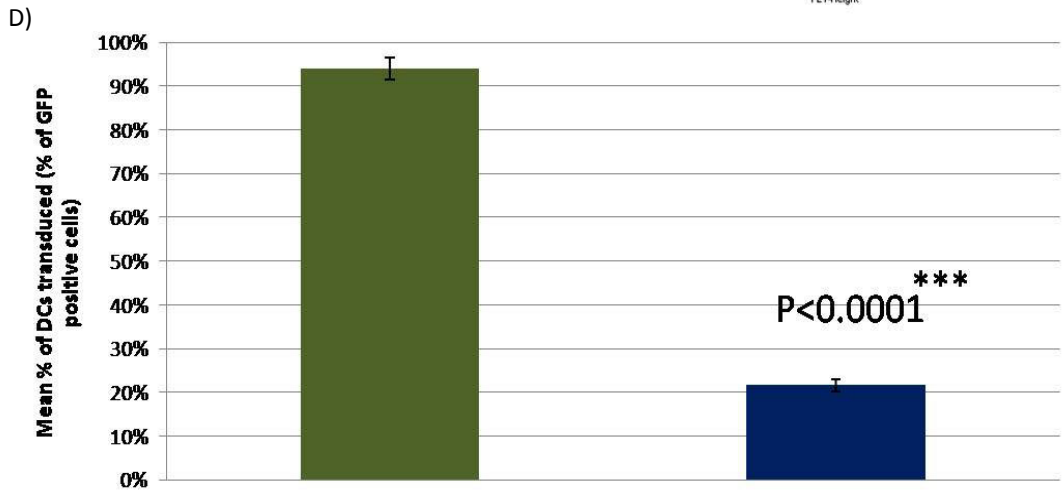
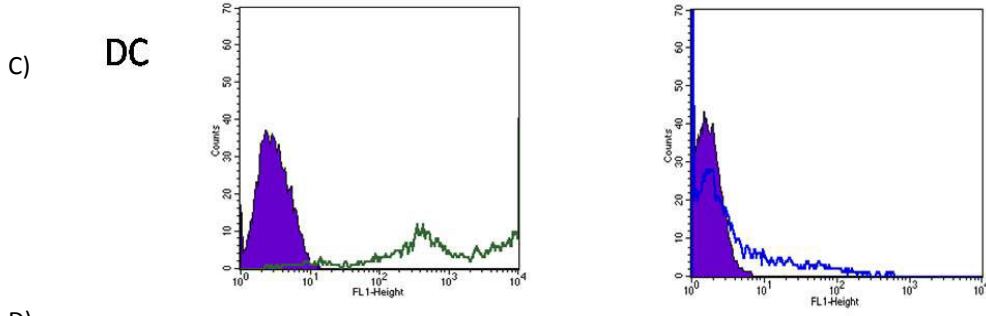
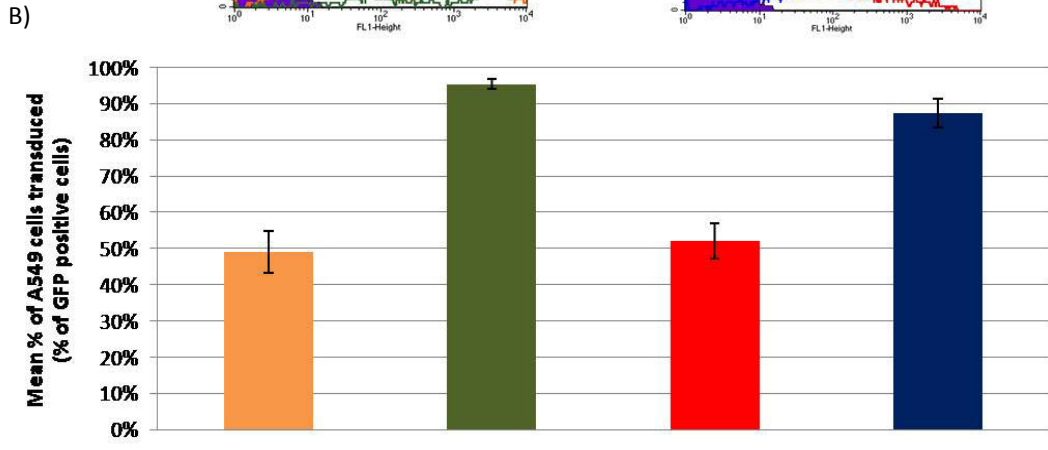
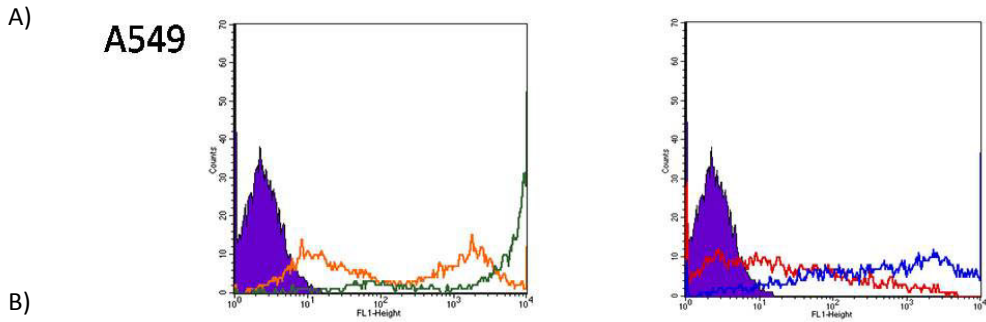
### **5.5.1: FACS analysis**

In each DC experiment control transductions of epithelial cells (A549 cells) were performed to confirm correct titration of vector stocks used. rAd19aGFP and rAd5GFP were used to transduce A549 cells at an MOI of 3pfu/cell or 10pfu/cell. The % of GFP positive cells after 48 hours was recorded (example shown in Figure 5.5 A) and the results of several experiments are summarised in Figure 5.5.

As expected, rAd19aGFP and rAd5GFP showed very similar transduction efficiencies in this cell type as seen previously (Chapter 4.4.1).

Concurrently to A549 controls, iDCs were transduced with rAd19aGFP or rAd5GFP at an MOI of 10pfu/cell (Figure 5.5 C & D) for 1.5 hours and returned to DC growth media for 48 hours before FACS analysis. As with the wild-type virus, rAd19aGFP was shown to efficiently transduce iDCs with a mean of 94% of iDCs staining positive for GFP after rAd19aGFP transduction. In comparison rAd5GFP was shown to be very inefficient at transducing iDCs with a mean of 22%. An unpaired t test on the two sets of data showed that rAd19aGFP transduces significantly more iDCs when compared to rAd5GFP.

This data was then confirmed in a western blot system using a different vector.



■ Mock    
 ■ Ad5GFP MOI 3    
 ■ Ad5GFP MOI 10    
 ■ Ad19aGFP MOI 3    
 ■ Ad19aGFP MOI 10

**Figure 5.5: Transduction of iDCs and control cells by GFP Ad vectors**

(A) FACS histogram overlaying the fluorescence patterns of A549 cells either mock transduced (purple fill) or transduced with an MOI of 3 (orange solid line rAd19aGFP, red solid line rAd5GFP) or 10 (green solid line rAd19aGFP, blue solid line rAd5GFP) (B) Chart showing the mean % of A549 cells transduced with an MOI of 3 or 10 of rAd19aGFP or Ad5GFP using the same colour scheme as before. Error bars show the standard error of the mean for 4 experiments. (C) FACS histograms overlaying the fluorescence patterns of iDCs mock transduced or transduced with an MOI of 10 of rAd19aGFP or rAd5GFP MOI using the same colour scheme as before. (D) Chart showing the mean % of iDCs transduced with an MOI of 10 of rAd19aGFP or rAd5GFP using the same colour scheme as before. Error bars show the standard error of the mean for 4 experiments. P value shown is from an unpaired t test performed on the iDC transduction data.

### 5.5.2: Western Blot analysis

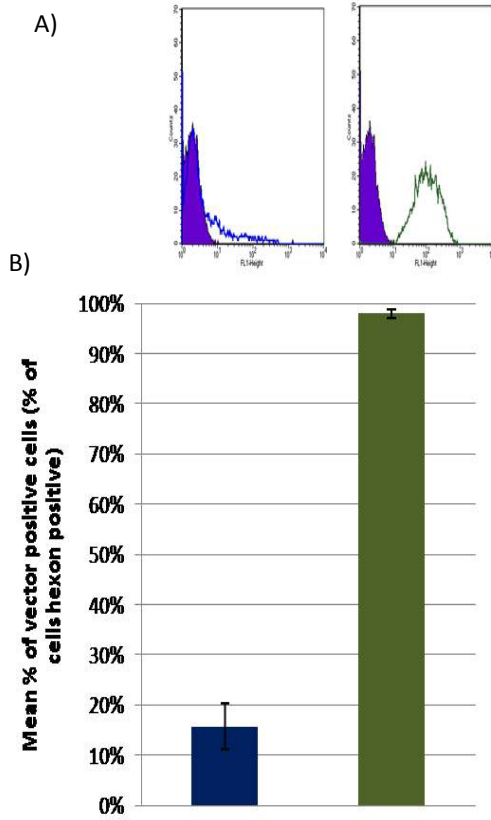
To investigate whether a different antigen could also be efficiently expressed, we transduced iDCs with the vaccine vector Ad19aHIVA and analysed expression semi-quantitatively by western blotting.

Control transductions of A549 cells were performed with rAd19aHIVA and rAd5HIVA to confirm the similar titre of rAd5 and rAd19aHIVA. iDCs were transduced with an MOI of 10 pfu/cell of both vectors. The % of hexon positive cells after 48 hours was recorded for each transduction by FACS and the results are summarised in Figure 5.6. Concurrently a sample of each transduction was lysed and subjected to western blot for the HIVA transgene using the SV5-Pk1 Ab to detect an epitope in the HIVA transgene. The same amount of lysate was analysed for the presence of  $\beta$ -tubulin as a loading control (Figure 5.6 E & F).

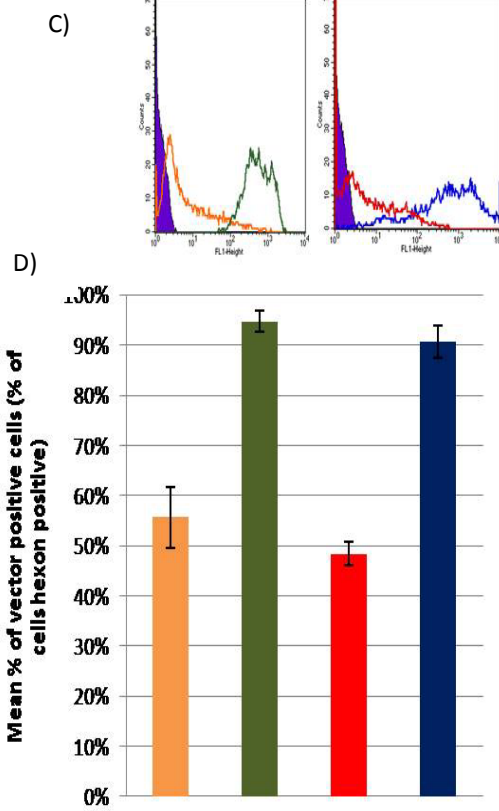
Hexon FACS analysis illustrated that rAd19aHIVA and rAd5HIVA performed similarly to the GFP vectors when they showing approximately equal transduction efficiencies in A549 cells but markedly different efficiencies in iDCs. This contrasting picture in iDC transduction was further evidenced by the observed detection of the HIVA transgene in cells transduced with rAd19aHIVA by western blot which was not observed in cells transduced with rAd5HIVA (Figure 5.6 E). By contrast the HIVA transgene was detected in all transductions of A549 cells and equal amounts of lysates were loaded into each lane as evidenced by the identical expression levels of the ~50kDa  $\beta$ -tubulin protein in all samples (Figure 5.6 F).

It was concluded that the Ad19a vector retained the ability of the Ad19a wild-type virus to efficiently infect/transduce DCs. Therefore, we propose the use of Ad19a vectors as an efficient transfer vector for DCs in both *in vivo* and *ex vivo* conditions. The data also showed that the Ad19a vector can efficiently transduce 95% of DCs at far lower doses than the 100 pfu/cell required for Ad5 vectors (Zhong *et al.*, 1999). This correlated with the expression of detectable amounts of transgene when transduced into iDCs at the doses tested which is expected to result in more efficient antigen presentation.

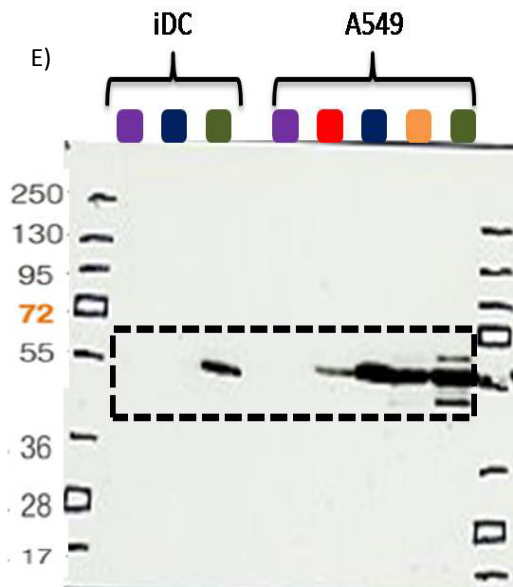
iDC



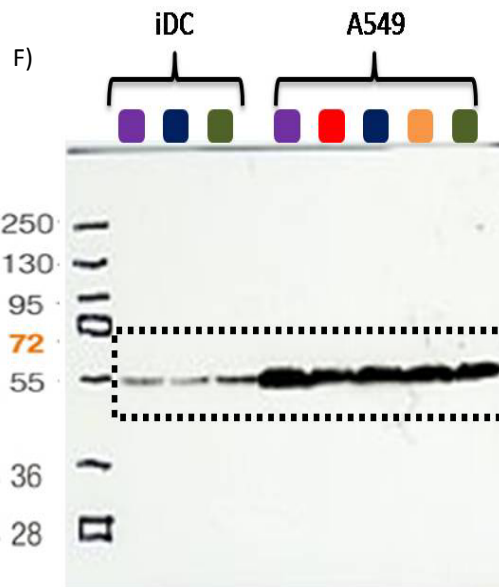
A549



■ Mock    
 ■ Ad5GFP MOI 3    
 ■ Ad5GFP MOI 10    
 ■ Ad19aGFP MOI 3    
 ■ Ad19aGFP MOI 10



$\alpha$ -HIVA



$\alpha$ - $\beta$ -tubulin



**Figure 5.6: Transduction of immature dendritic cells by HIVA Ad vectors**

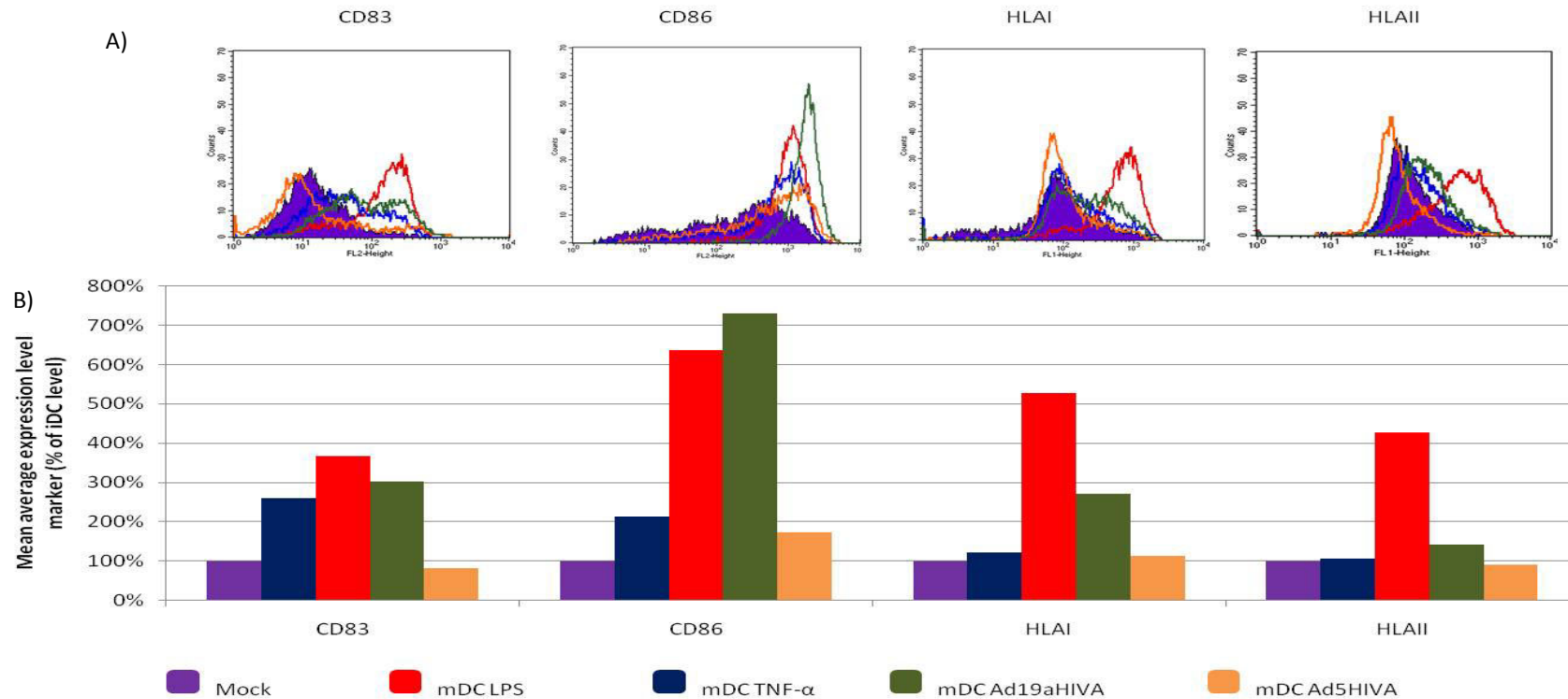
(A) FACS histograms overlaying HIVA vector hexon detection in iDCs either mock transduced (purple fill) or transduced with an MOI of 10 of rAd19aHIVA (green solid line) or rAd5HIVA (blue solid line). (B) Mean % of iDCs transduced with an MOI of 10 of rAd19aHIVA or rAd5HIVA using the same colour scheme as before. Error bars show the standard error of the mean for 3 experiments. (C) FACS histograms overlaying HIVA vector hexon detection as in (A) but for A549 cells showing mock transduction (purple fill) or transduction with an MOI of 3 (orange solid line Ad19a, red solid line Ad5) or 10 (green solid line Ad19a, blue solid line Ad5). (D) Mean % of A549 cells transduced with an MOI of 3 or 10 of rAd19aHIVA or rAd5HIVA using the same colour scheme as (C). Error bars show the standard error of the mean for 3 experiments. (E) iDCs and A549 cells were transduced with an MOI of 3 (A549 only) or 10 of rAd19aHIVA or rAd5HIVA, lysed and subjected to western blot for the HIVA transgene (SV5-Pk1, ~52kDa highlighted in dashed box) or  $\beta$ -tubulin (2-28-33, ~50kDa highlighted in spotted box) as a loading control.

## **5.6: The maturation of dendritic cells by Adenovirus vector transduction**

Ad vectors have been shown to mature dendritic cells upon transduction by the upregulation of expression of HLAI, HLAII, CD80 and CD86 (Morelli *et al.*, 2000), however, this data was generated with an Ad5 vector at an MOI of 100pfu/cell. Contemporaneously it was shown that an Ad vector from subgroup B, Ad35, also matured DCs upon transduction but did so at a 10-100 fold lower dose than Ad5 (Rea *et al.*, 2001). Here the ability of an Ad19a vector mature DCs upon transduction was investigated. In a single preliminary experiment, the maturation profile upon rAd19a or rAd5 transduction was compared to LPS and TNF- $\alpha$  treated DCs (Figure 5.7).

iDCs were transduced for 1.5 hours with rAd19aHIVA or rAd5HIVA at an MOI of 10pfu/cell and returned to normal DC growth medium or grown for 48 hours in the presence of LPS or hTNF- $\alpha$ . The latter deliberately matured DCs were then compared to iDCs and iDCs transduced by the viral vectors for the expression of CD83, CD86, HLAI and HLAII and the results summarised (Figure 5.7 B). IgG1 and IgG2a controls were performed as previously (data not shown).

LPS and TNF- $\alpha$  up-regulated the expression of all markers to approximately the same levels as observed previously. 10pfu/cell of rAd19a up-regulated the expression of all four markers tested to a greater level than hTNF- $\alpha$  (CD83 ~1.1x greater, CD86 ~3.4x greater, HLAI ~2.2x greater and HLAII ~1.3x greater), but less than LPS in the case of three markers (CD83 1.2x ~smaller, HLAI ~1.9x smaller and HLAII ~3.5x smaller). In contrast, 10pfu/cell of rAd5 increased the expression level of two of the four markers tested over iDC levels (CD86 ~1.7x greater and HLAI ~1.1x greater) only moderately and did not up-regulate either CD86 or HLAII. rAd5 failed to match or exceed the levels of up-regulation induced by hTNF- $\alpha$ . It was therefore concluded, albeit from a single experiment, that Ad19a vectors can efficiently cause maturation of iDCs at 10pfu/cell as identified by the few cell marker profiles available. Further investigation was not performed due to time constraints.



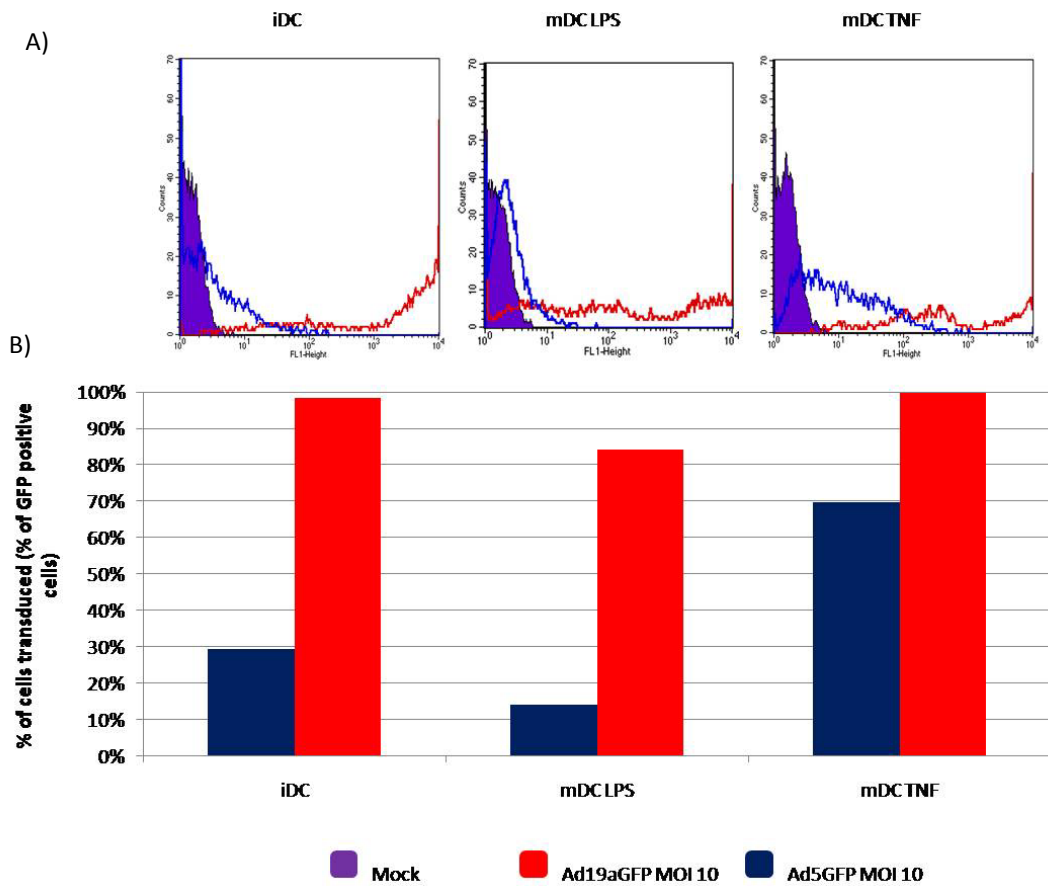
**Figure 5.7: Maturation of immature dendritic cells by Ad vectors**

(A) Overlays of FACS histograms showing the upregulation of various activation markers in mDCs. Purple fill represents the staining of iDCs, the red solid line DCs matured with LPS the blue solid line DCs matured with hTNF- $\alpha$ , the green solid line DCs matured with rAd19aHIVA and the orange solid line DCs matured with rAd5HIVA, with CD83, CD86, HLAI and HLAII antibodies. (B) Relative average expression to iDCs of each marker. Results are for 1 experiment only.

### **5.6.1: Transduction of mature dendritic cells**

It was then finally investigated if Ad vectors could transduce mDCs. In a single experiment, iDCs were matured with either LPS or hTNF- $\alpha$ , checked for successful maturation by marker staining as previously (data not shown) and when confirmed transduced with an MOI of 10 of either rAd19aGFP or rAd5GFP, alongside iDCs and returned to DC growth medium for 48 hours. Cells were then examined by FACS analysis for the level of GFP and the percentage of GFP positive cells and the results summarised in Figure 5.8.

Transduction efficiencies for both vectors in iDCs were as observed previously. Transduction efficiencies in LPS mDCs appear reduced when compared to iDC levels for both the Ad19a vector (~14% reduction in transduction efficiency) and the rAd5 vector (~50% reduction in transduction efficiency). In contrast, transduction efficiencies in hTNF- $\alpha$  mDCs were similar to iDCs for the Ad19a vector and greatly enhanced for the Ad5 vector (~40% increase in transduction efficiency). It was concluded that LPS maturation instigates a decrease in transduction efficiency in Ad vectors but that hTNF- $\alpha$  did not affect rAd19a transduction and actually enhanced rAd5 transduction over iDC levels. No further experiments could be performed due to time constraints.



**Figure 5.8: Infection of mature dendritic cells by Ad vectors**

(A) FACS histograms of iDCs (column 1), LPS matured DCs (column 2) or TNF- $\alpha$  matured DCs (column 3) either mock transduced (purple fill) or transduced with an MOI of 10 of rAd19aGFP (red solid line) or rAd5GFP (blue solid line). (B) Percentage of cells transduced in each transduction. Colour code as in (A). Results are for 1 experiment only.

## 5.7: Discussion

The work presented in this chapter has detailed the ability of the Ad19a vector to naturally target DCs, induce their maturation, express high levels of transgene and transduce mDCs. Taken together, these results may herald greater *in vitro* and *in vivo* immunogenicity induced by rAd19a than has been observed with rAd5.

The results in section 5.3 confirmed previous findings (Ruzsics et al., 2006) that the Ad19a vector efficiently transduced immune lineage cells. Jurkat T cells and TD12 B cells were shown to be efficiently transduced by the rAd19a vector but not by the corresponding rAd5 vector. These results were limited by the use of only two human immune lineage cell lines and as such cannot be conclusive of transduction of these cell types. However, previous data from this laboratory using other B and T cell lines support these claims (unpublished data). This suggests that rAd19a may utilise a cell receptor which is common on all immune lineage cell types. Further transduction experiments would be required, perhaps involving the isolation of primary immune lineage cells from human blood to confirm that rAd19a can infect all immune lineage cell types, but the results are promising for the *ex vivo* transduction of several immune cell types. If this holds true for the *in vivo* tropism this may allow therapeutic gene transfer in lymphocytes or possibly the development of oncolytic Ad19a viruses for targeting lymphomas or leukemic cells.

Most importantly, in section 5.5 it was shown that rAd19a possesses similar transduction efficiency of iDCs as has been reported previously for the wt virus (Ruzsics *et al.*, 2006) and that this transduction efficiency was significantly greater than the transduction efficiency of rAd5 at the same dose. This finding could suggest that Ad19a vectors would have a far greater ability to transduce DCs either *in vivo* or *ex vivo* allowing greater transgene or antigen expression at a lower dose and possibly, therefore, a greater immunostimulatory response. This data must be tempered by the findings in Chapter 4, specifically concerning particle/pfu ratios and this lower dose of rAd19a may contain a similar or greater number of particles as the rAd5 dose. It is important to remember, however, that rAd19a is known to use a cellular receptor other than CAR, much like rAd35 which shows similar transduction of DCs, when its fibre is pseudotyped onto Ad5 (Rea *et al.*, 2001). This differential

receptor usage may account for the increased transduction efficiency. The Ad19a receptor will be discussed in chapter 6 including its relationship to DC uptake.

The results in section 5.4 show the adoption of a simple and effective method for the isolation and growth of DCs from human blood. It also described two ways, using either LPS or hTNF- $\alpha$ , to induce maturation of DCs which have both been used previously (Lutz *et al.*, 1999; Yamaguchi *et al.*, 1997). In this study, LPS prompted greater up-regulation of CD83, the co-stimulatory molecule CD86 and the MHC components HLAI and HLAII. There are reasons that may account for this. It is important to note that hTNF- $\alpha$  production is itself induced *in vivo* by the presence of LPS (Dumitru *et al.*, 2000), therefore iDCs exposed to LPS may be responding to the presence of LPS itself and the hTNF- $\alpha$  and other cytokines produced by the other iDCs in the culture and this may account for the greater up-regulation of maturation markers. Additionally, the hTNF- $\alpha$  may have lost some activity during storage whereas LPS is much more stable. It was not investigated in this study if LPS and hTNF- $\alpha$  instigate differing changes in mDC function or phenotype but it was shown in section 5.6.1 that DCs matured by the two different immunostimulants have different susceptibilities to rAd transduction. Only one experiment was performed so the data is not conclusive, but LPS was shown to cause a decrease in both rAd19a and rAd5 transduction which may suggest that it caused the DCs to mature into a state which is less susceptible to virus infection, perhaps by down-regulation of virus receptor molecules. This may be accounted for by the creation of morphology or phenotype to manage a bacterial infection, induced by a bacterial cell wall component, LPS. In contrast, TNF- $\alpha$  may have a more generalised effect, as it is produced in reaction to bacterial antigen detection and viral infection, and therefore might produce a form of DC which is more susceptible to virus infection to potentially allow all circulating viruses *in vivo* to be taken up, even during DC transit to the lymph nodes, and their antigens presented to CD4<sup>+</sup> T cells. This function is unlikely to be due to the up-regulation of the Ad5 receptor, CAR, but may be due to the up-regulation of a non virus-specific viral uptake mechanism which has been hypothesised before for increased rAd5 transduction in mDCs (Lore *et al.*, 2007) and has been putatively identified as involving DC-SIGN in combination with lactoferrin (Adams *et al.*, 2009). Further work in this area would include repeating the rAd

transduction experiment from section 5.6.1 and attempting to block DC-SIGN to investigate if the increase in Ad5 transduction can be inhibited.

Interestingly, in section 5.6 in a single experiment, it was shown that, like LPS and TNF- $\alpha$ , rAd infection was capable of maturing DCs, as had been seen previously (Morelli *et al.*, 2000). It was also noted that rAd19a induced a significantly higher expression of DC maturation markers at the dose tested than Ad5, suggesting the delivery of stronger activation signals by Ad19a. This could be related to the larger number of particles introduced compared to Ad5 or an intrinsic property of Ad19a. This can only be resolved, therefore, by further titration studies (lowering the particle/pfu ratio of Ad19a preps by improving the quality of prep or by increasing the amount of Ad5 vector). The profound increase in MHC I expression is certainly a positive aspect for a vaccine vector as it supports efficient antigen presentation to CD8<sup>+</sup> T cells. In previous studies, rAd5 has been shown to induce the maturation of DCs. In these studies doses, >100 pfu were used (Rea *et al.*, 2001). This could explain the observed lack of maturation at the rAd5 dose tested here and add further weight to the hypothesis that rAd19a has a far higher immunostimulatory profile

A key question that's not addressed to date is whether the enhanced transduction and transgene expression by rAd19a correlates with enhanced antigen presentation by DCs. This could be done by co-culturing DCs transduced with the rAd19a and rAd5HIVA vectors with HLA-A2 restricted HIVA specific CTLs and analysing IFN- $\gamma$  secretion. This work is in progress in collaboration with Dr. T. Dong (University of Oxford). It is also unknown if rAd19a can target DCs *in vivo*, which is of course a major goal of any vaccine vector. Whilst this is beyond the scope of the current study, rAd19a's effectiveness in an *in vivo* setting in mice has been analysed (Chapter 7).

Efficient transduction of DCs has also been shown for rAd5 vector pseudotyped with the Ad35 fibre, which uses CD46 as its cellular receptor (Rea *et al.*, 2001) and that this successfully generates antitumour CTL responses *in vitro* (Slager *et al.*, 2004; Grujil *et al.*, 2006). As will be shown in Chapter 6, CD46 may also be one of the potential receptors for Ad19a. Thus, rAd19a may possess similar features and a



careful comparison between an Ad19a vector and an Ad35 vector for DC transduction may be useful in future studies.

rAd19a possesses relative specificity for DC transduction, induced high levels of transgene expression in iDCs and mDCs, up-regulated co-stimulatory and MHC molecules induced DC maturation. In conclusion, this chapter's findings have provided further progress towards the clinical use of rAd19a as a vaccine vector and would support the use of Ad19a vectors for *ex vivo* Ag delivery in DCs. Further work is required to examine if rAd19a transduces DCs *in vivo*.

## Chapter 6: The identity of the Ad19a receptor

### 6.1: Introduction

For a full introduction to Ad receptor usage and the natural function of each of the receptors mentioned herein please see Chapter 1.

Ad5 infection requires the presence of the Coxsackie and adenovirus receptor, CAR (Bergelson *et al.*, 1997), which is essential for transduction of cell lines *in vitro* (Hutchin *et al.*, 2000; Nalbantoglu *et al.*, 1999; Roelvink *et al.*, 1999), its presence can influence biodistribution *in vivo* (Seiradake *et al.*, 2009) and its engineered expression on cell lines can enhance transduction (Stockwin *et al.*, 2002). It is also known, however, that the use of other attachment molecules, such as Factor X, may have caused a fatal systemic inflammatory response in an 18 year old male gene therapy patient treated with an Ad5 vector (Raper *et al.*, 2003). It would therefore be desirable to characterise the identity of the Ad19a receptor to better understand the ranges of cell types which could be transduced by the vector *in vitro* and any complications the vector may encounter *in vivo*.

#### 6.1.1: The Ad19a receptor

Only subgroup B Ads and the subgroup D Ad, Ad37 have been definitively shown to use cell attachment receptors other than CAR (Roelvink *et al.*, 1998). Ad37 and Ad19a possess identical fibres (Arnberg *et al.*, 1997) and therefore the identity of the Ad37 receptor should shed some light on the identity of the Ad19a receptor. Perplexingly, the identity of the Ad37 receptor remains controversial.

The Ad37 fibre has been shown to bind CAR immobilized on a solid support with high affinity (Seiradake *et al.*, 2006), but has been shown to be too inflexible to allow CAR binding (Wu *et al.*, 2003; Nemerow *et al.*, 2009). Indeed, the fibre of Ad37 has been shown to be both short (~150 Å), when compared to the Ad5 fibre (~380 Å), and rigid by cryoelectron microscopy reconstruction (Chiu *et al.*, 2001) and in that regard is similar to the CD46-using Ad35 fibre which is also short (~85 Å) especially and only marginally flexible (Saban *et al.*, 2005). Previous data from

this group indicated that Ad19a can infect cells efficiently in the absence of CAR, and it has been concluded that CAR is unlikely to be the primary receptor for Ad37 (Arnberg, 2009) and Ad19a alike in the context of virus infection.

On Chinese hamster ovary (CHO) cells, Ad37 recognised  $\alpha$ 2-3 linked sialic acid (SA) displayed on surface glycoproteins (Arnberg *et al.*, 2000<sup>a</sup>; Arnberg *et al.*, 2002), treatment of cells with neuraminidase reduced Ad37 and Ad19a uptake (Arnberg *et al.*; 2000<sup>b</sup>; Thirion *et al.*, 2006) and multivalent molecules of sialic acid inhibited Ad37 from binding to and infecting human corneal cells (Johansson *et al.*, 2005; Johansson *et al.*, 2007). Ad5 pseudotyped with the Ad37 fibre has also been shown to require sialic acid for transduction (Cashman *et al.*, 2004). Sialic acid is now widely accepted to be a primary component of the Ad37 receptor but other receptors have been proposed as well.

CD46, a membrane cofactor protein, and the receptor for several human pathogens (Cattaneo, 2004) has been proposed as the Ad37 receptor, particularly on Chang C conjunctival cells (Wu *et al.*, 2001; Hsu *et al.*, 2005). This was mainly based on the fact that an antiserum against the N-terminal 19 amino acids of CD46 blocked Ad37 infection of human HeLa and conjunctival cells and expression of a 50kDa isoform of human CD46 in a CD46 null cell line increased cell binding by wild-type Ad37 and gene delivery by an Ad5 vector pseudotyped with the Ad37 fibre (Wu *et al.*, 2004). However, the study was not well controlled. If CD46 was the Ad19a receptor this would have dramatic implications for the choice of *in vivo* model as mice lack CD46 in the periphery (Cervoni *et al.*, 1992). The enhanced performance of an Ad vector requiring CD46 in a CD46 transgenic mouse line (Verhaagh *et al.*, 2006) underlines the restricted tissue distribution of CD46 in non-transgenic mice.

Recently, preliminary evidence was provided for a new candidate receptor linked to sialic acid, the GD1a sialic acid containing ganglioside GD1a (Arnberg, unpublished data).

The ongoing controversy in the field prompted further experiments to test which of the candidate receptors, if any, serve as the functional receptor for Ad19a. To this end, CHO cells expressing each of the candidate receptors, except GD1a, were

obtained. After verifying their expression alongside human control cell lines, the transduction efficiency of rAd19a alongside control viruses for each receptor was tested. The rAd5 vector served as a suitable control for CAR binding, and an Ad5 vector pseudotyped with the Ad35 fibre (generously provided by A. Lieber) and shown to bind CD46 (Gaggar *et al.*, 2003), was used as a positive control virus that binds CD46.

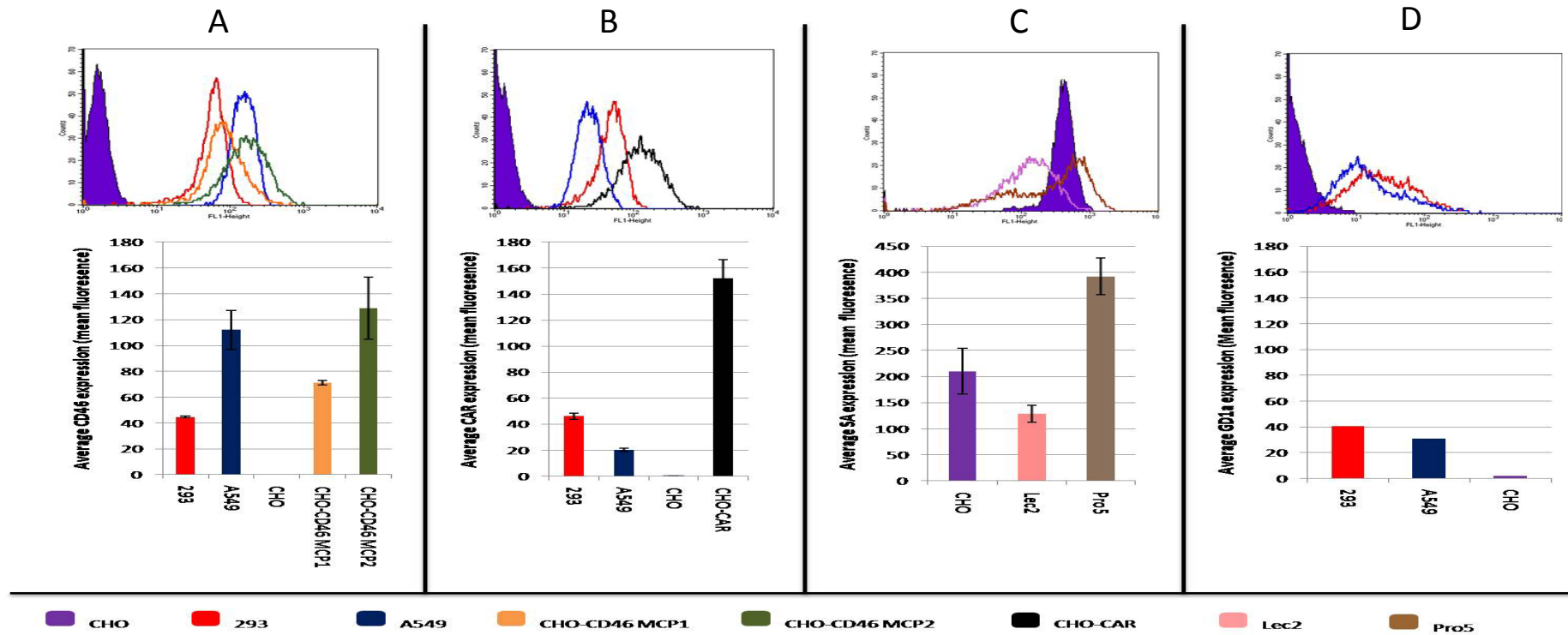
## **6.2: Aims**

The aims of this study were to investigate human receptor expression on modified CHO lines, transduce them with rAd19a, rAd5 and rAd5F35 and, if an enhanced transduction interaction was obtained, the attempted blocking of this transduction by Ab binding was attempted.

## **6.3: Cell Line Analysis**

Mock CHO cell lines, CHO cells expressing two isoforms of human CD46 (hCD46; CHO-CD46 MCP1 and CHO-CD46 MCP2), CHO cells expressing human CAR (hCAR; CHO-CAR) and CHO cells expressing more (Pro5) or less (Lec2) SA were obtained from various sources (Chapter 2) and were examined alongside 293 and A549 cells for the expression of each of the candidate receptors, CD46 (using mAb J4.48), CAR (mAb E1-1), SA (lectin MALI) and GD1a (mAb MOG35) (Figure 6.1).

hCD46 was found to be expressed only on 293, A549, CHO-CD46 MCP1 and CHO-CD46 MCP2 cell lines (Figure 6.1A). A549 cells expressed ~2.5x more hCD46 than 293 cells whilst CHO-CD46 MCP1 expressed ~1.6x more and CHO-CD46 MCP2 ~2.9x more than 293 cells. The highest level was expressed by CHO-CD46 MCP2, followed by A549 cells, CHO-CD46 MCP1 cells and finally 293 cells. All other cell lines in the study were negative (data not shown). The confirmed expression of hCD46 in the CHO-CD46 cell lines allowed their further use in this study.



**Figure 6.1: Cell Line analysis**

Top panel shows FACS histograms for the different Abs or lectins, CD46 (A), CAR (B), SA (C) or GD1a (D). The cell lines tested with each antibody or lectin are shown by the colour scheme: 293 (red), A549 (blue), CHO (purple), CHO-CD46 MCP1 (orange), CHO-CD46 MCP2 (green), CHO-CAR (black), Lec2 (pink) and Pro5 (brown). The lower panel shows bar charts summarising the experiments using the same colour code as above. The experiment in panel D was only performed once. Error bars represent the standard error of the mean for 3 experiments.

hCAR was found to be expressed on 293, A549 and CHO-CAR cells (Figure 6.1 B). A549 cells expressed  $\sim 2.3x$  less hCAR than 293 cells whilst CHO-CAR cells expressed  $\sim 3.3x$  more. All other cell lines tested negative and were not shown in the figure. The confirmed expression of hCAR on CHO-CAR cells, although substantially higher than the expression levels in human cell lines, allowed their further use in this study.

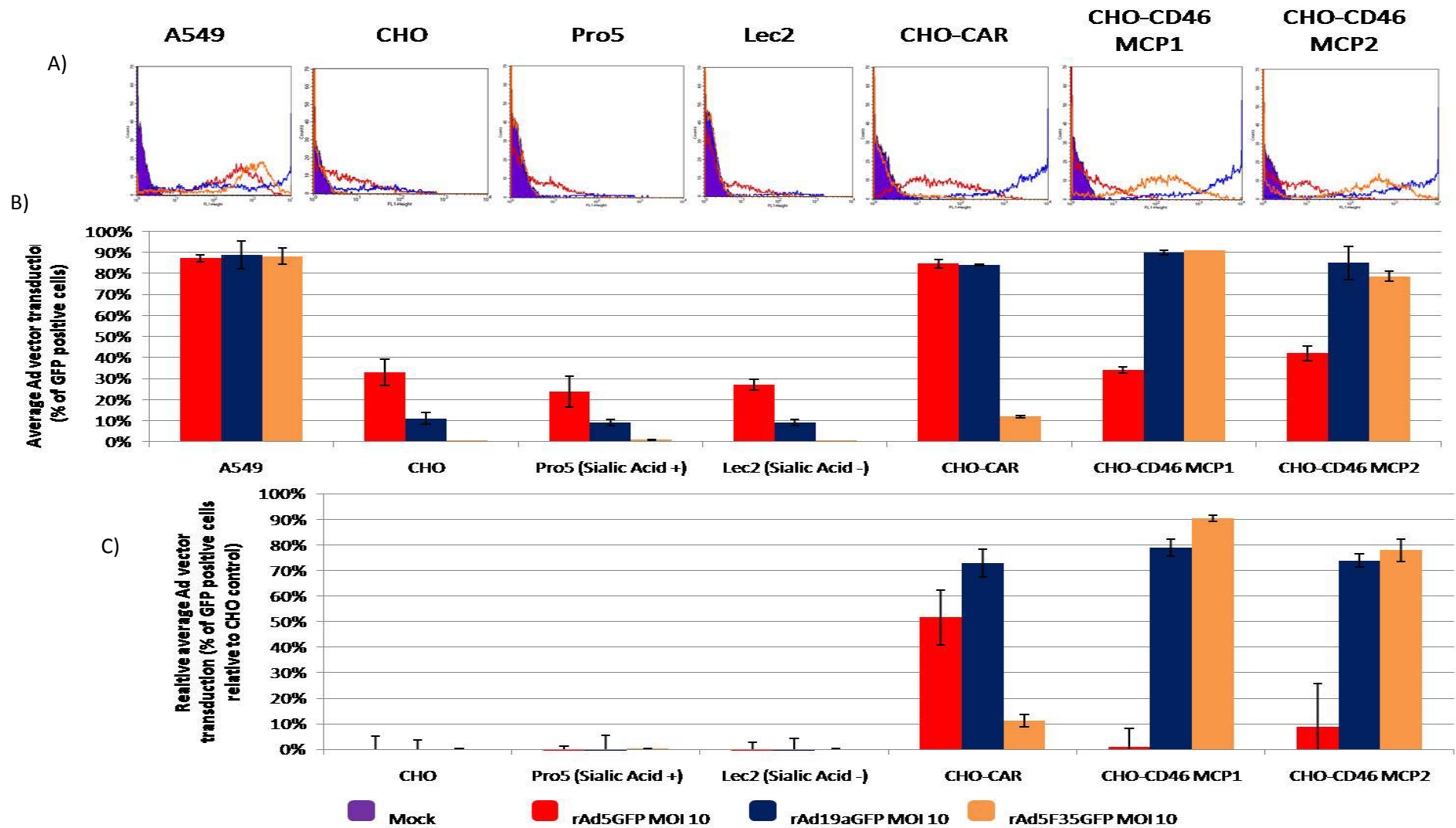
SA display was tested only on CHO, Lec2 and Pro5 cells as it is already well established that it is expressed on all mammalian cell types (Figure 6.1 C). Lec 2 expressed  $\sim 1.6x$  less SA than CHO cells whilst Pro5 expressed  $\sim 1.9x$  more confirming that they are good candidates to examine if the under-expression or over-expression of SA has any effect on rAd transduction.

In a single experiment, GD1a was found to be expressed on 293 and A549 cells only (Figure 6.1 D). There was little difference in expression between the two cell lines. All other cell lines tested negative.

With the confirmation of receptor expression on all of the acquired cell lines their transduction with Ad5, Ad19a and Ad5F35 vectors could be investigated.

#### **6.4: Adenovirus transduction of receptor modified CHO cell lines**

A549, CHO, Lec2, Pro5, CHO-CAR, CHO-CD46 MCP1 and CHO-CD46 MCP2 were grown in 12 well plates to 95% confluence and transduced with an MOI of 3pfu/cell (data not shown) or 10 pfu/cell of rAd5GFP, rAd19aGFP or rAd5F35GFP. 48 hours later they were examined for GFP fluorescence by FACS (Figure 6.2).



**Figure 6.2: Adenovirus transduction of receptor modified CHO lines**

A549, CHO, Pro5, Lec2, CHO-CAR, CHO-CD46 MCP1 and CHO-CD46 MCP2 cell lines were transduced with an MOI of 3pfu/ cell (data not shown) or 10pfu/cell rAd5GFP (red solid line), rAd19aGFP (blue solid line) and rAd5F35GFP (orange solid line). 48 hours later each cell line was examined for GFP fluorescence. A) Example FACS histogram overlays of each cell line and each transduction using the same colour scheme. B) Chart showing the mean transduction of each cell line with each virus using the same colour scheme as previously. Error bars show the standard error of the mean for 3 experiments. C) Chart showing the same data as (B) with the CHO background transduction deducted.



All 3 vectors exhibited approximately equal transduction efficiencies in the control cell line A549, confirming that the calculated titres for each vector were correct. In the control CHO cell line, i.e. in the absence of human receptor expression, both rAd5GFP and rAd19aGFP were capable of an average of 33% and 11% transduction respectively. Transduction of CHO cells with rAd5GFP has been noted before (Granio *et al.*, 2009), where a dose of 500 virus particles/cell resulted in 20-30% transduction. These are in line with the above results since 500 virus particles/cell is equivalent to an MOI of 10 due to rAd5GFP having a particle/pfu ratio of 50:1 (see Chapter 4.3). rAd5F35GFP was unable to transduce the control CHO cell line suggesting significant differences between the CD46 targeting Ad35 fibre and the targeting of the Ad19a fibre. Transduction of the CHO cell line was used as the background transduction level in CHO cell lines. In subsequent transduction experiments with CHO cell lines expressing various human receptors this background transduction level was deducted (Figure 6.2 C).

None of the three vectors used transduced either Lec2 or Pro5 cells above background levels (Figure 6.2 C) showing that, in this study, the reduced display of SA had no effect on rAd19aGFP transduction, arguing against it being a receptor component for Ad19a, despite a wealth of evidence for it being the Ad37 receptor.

Predictably, rAd5GFP transduction was recovered to nearly the same level as A549 transduction in CHO-CAR cells (Figure 6.1 B). Surprisingly though, rAd19aGFP transduction was also recovered to A549 levels. These findings suggested that rAd19aGFP was capable of using hCAR as a receptor during infection and the data showing the binding of the Ad37 fibre to hCAR (Seiradake *et al.*, 2006), that was discounted due to fibre inflexibility (Wu *et al.*, 2003; Nemerow *et al.*, 2009), may suggest that, in the case of Ad19a infection at least, that some other interaction is allowing the use of CAR. rAd5F35GFP also showed ~10% average transduction over background levels suggesting that hCD46 usage may also allow some level of hCAR usage.

As predicted, rAd5F35GFP transduced A549 cells to a similar level as CHO-CD46 MCP1 and CHO-CD46 MCP2 cells suggesting that CD46 is the primary receptor for Ad35. Unexpectedly, transduction of rAd19aGFP also returned to approximately

A549 transduction levels suggesting that hCD46 could also function as an Ad19a receptor in agreement with previous data for Ad37 (Wu *et al.*, 2004). rAd5GFP showed the same ~10% increase in average transduction over background levels as witnessed with rAd5F35GFP in CHO-CAR cells suggesting the prior finding that hCD46 usage allows some level of hCAR usage is also true in the reverse situation.

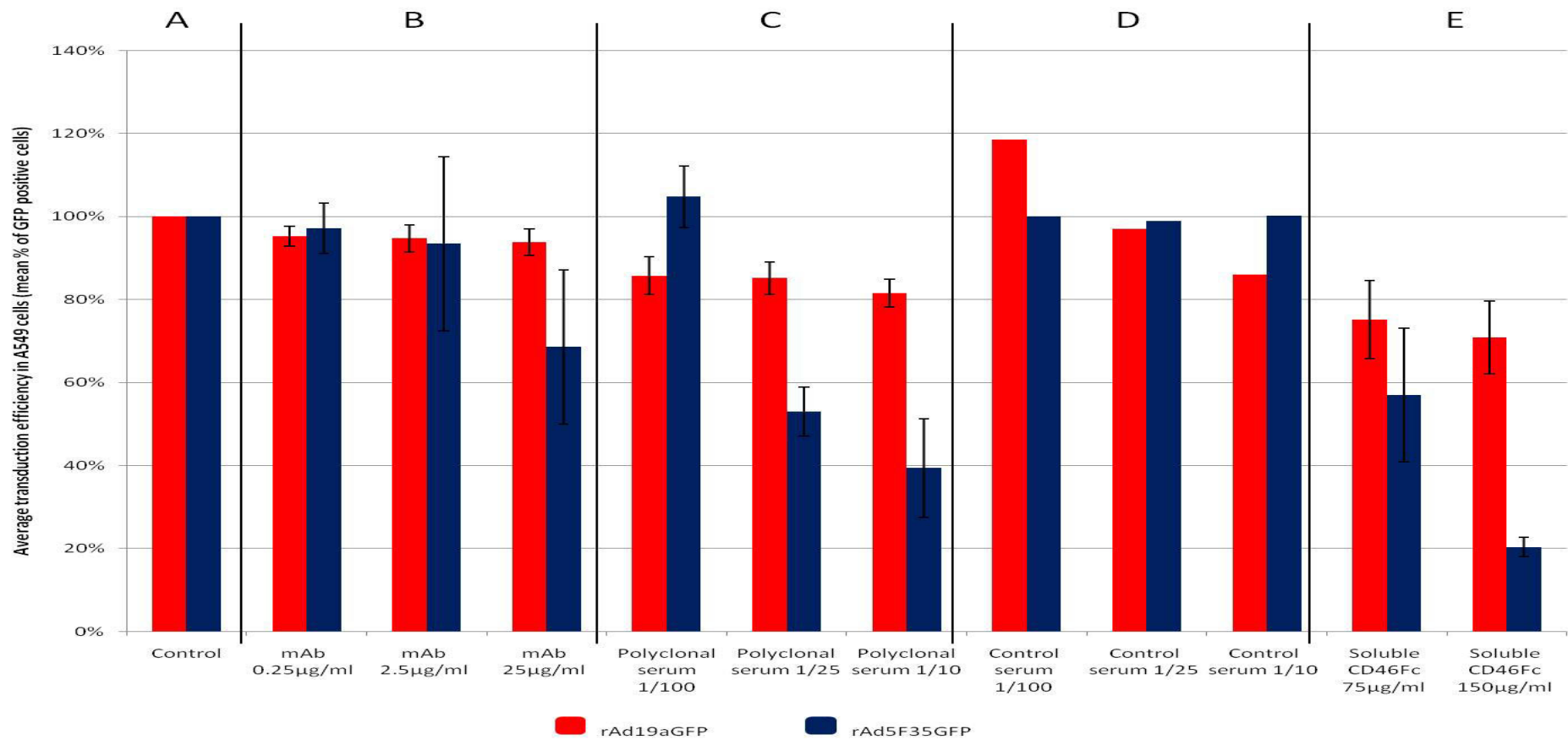
In conclusion, it was found that rAd19aGFP could utilise both hCAR and hCD46 for CHO cell transduction. Results using both an MOI of 10 and an MOI of 3 were identical. The control viruses rAd5GFP and rAd5F35GFP behaved predictably in the presence of hCAR and hCD46 and therefore the results with rAd19aGFP were deemed acceptable. For confirmation of those results, I attempted to block blocking of transduction of each cell line by the use of monoclonal or polyclonal Abs and soluble forms of the candidate receptor molecules was attempted.

## 6.5: Competition of transduction

Competition assays were performed as described in chapter 2.20. All stages were performed at 4°C to prevent receptor/antibody complex uptake and subsequent destruction by endosomal lysis.

### 6.5.1: Blocking of CD46 usage

Firstly, it was established that the system allows blocking of uptake of rAd5F35GFP, which is known to use CD46 (Figure 6.3). Representative FACS histograms for this work and all subsequent work in this chapter are shown in Figures A21-A24. A549 cell transductions were performed in parallel with both rAd19aGFP and rAd5F35GFP in each experiment and all subsequent competition represents the amount of transduction relative to these controls. Blocking of hCD46 in A549 cells by monoclonal antibody treatment ( $\alpha$ CD46FII) had little to no effect on rAd19aGFP and caused only a ~30% reduction in rAd5F35GFP transduction at the highest dose, although, as the error bars show, this reduction was not seen in all the experiments performed (Figure 6.3 B). Blocking by polyclonal antibody treatment ( $\alpha$ CD46POLY) caused a 47% reduction and 61% reduction in rAd5F35GFP average transduction at a 1/25 dilution and 1/10 dilution respectively. rAd19aGFP average transduction did not change significantly on increasing antibody dosage (Figure 6.3 C). Serum controls, i.e. rabbit sera from the same strain of rabbit used to generate the polyclonal antibody used in the previous treatment, had little or no effect on average rAd19aGFP or rAd5F35GFP transduction indicating that the inhibition was caused specifically by components of the serum raised against hCD46 in the polyclonal antibody and not by other contents of the serum. (Figure 6.3 D). Finally, blocking by pre-treatment of the vector particles with a soluble form of the CD46 receptor (MCP-BC-IgG4) resulted in a 43% and 80% reduction in average rAd5F35GFP transduction and a 25% and 29% reduction in average rAd19aGFP transduction at the two doses tested respectively (Figure 6.3 E). In comparison to the positive control vector, transduction by rAd19aGFP was only slightly, but consistently reduced by any of the CD46 blocking agents (highest for soluble hCD46 at 29%). Taken together these results show that rAd5F35GFP transduction of



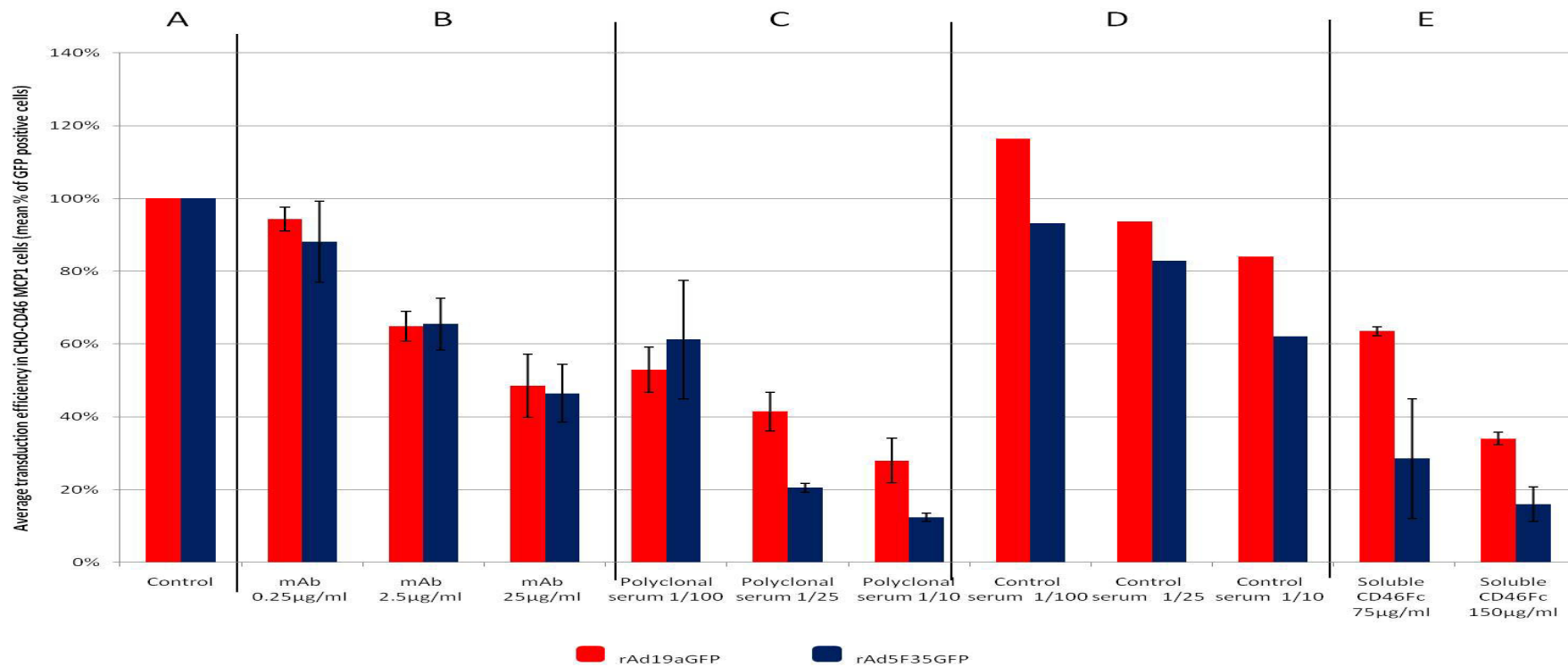
**Figure 6.3: Effect of CD46 blocking on transduction of A549 cells.** A chart showing A) the average mock transduction of A549 cells with either rAd19aGFP (red bars) or rAd5F35GFP (blue bars) and the effect of treatment with increasing concentrations of either B) mAb  $\alpha$ CD46FII, C) polyclonal antibody  $\alpha$ CD46POLY, D) Rabbit serum control or E) pre-treatment with of vector particles with soluble CD46 protein on that transduction. Error bars shown the standard error of the mean for three experiments.

A549 cells is dependent on the presence of hCD46 being present whereas rAd19aGFP transduction may be increased by the presence of hCD46 but it does not depend on it. These findings support the data from Chapter 6.3 that rAd19aGFP is capable of using both hCAR and hCD46, in the absence of any other human receptor entities, but this effect may be less relevant or irrelevant in a human cellular background.

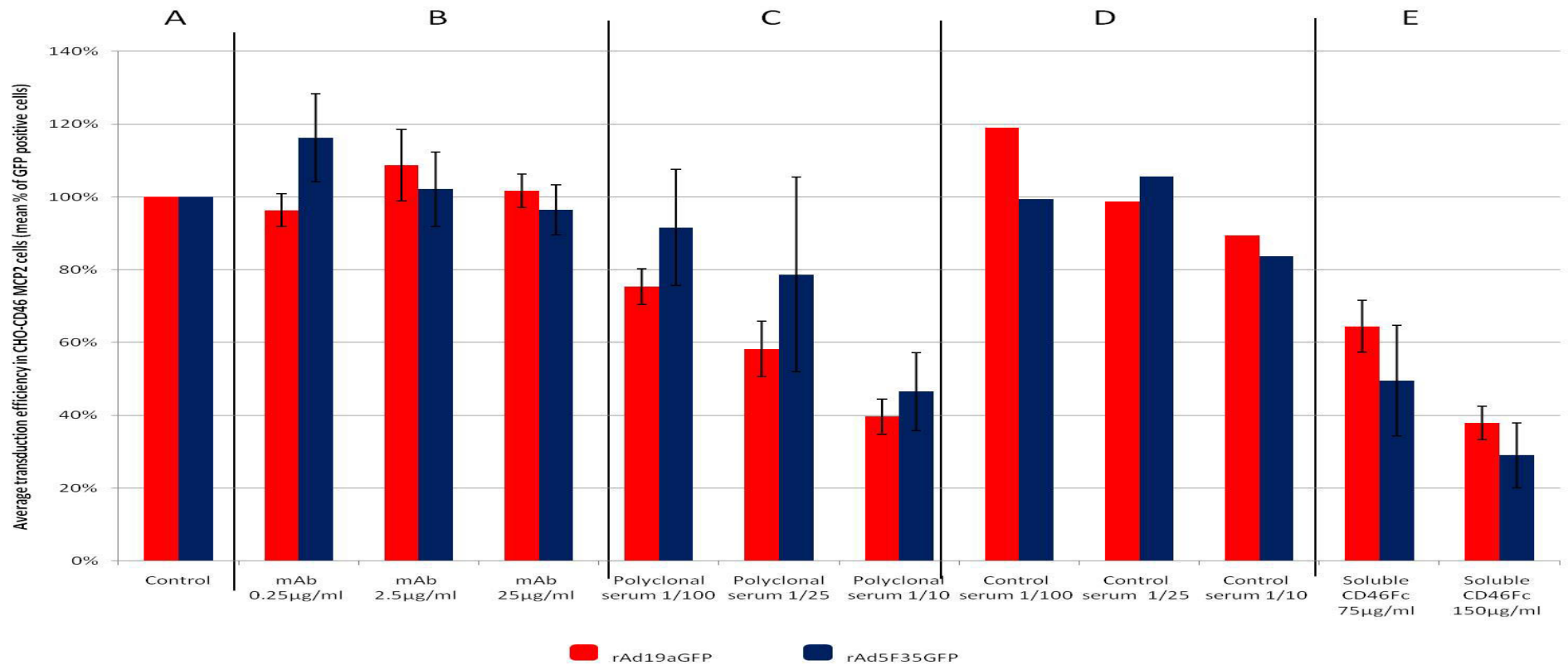
To investigate this hypothesis, the effect of blocking CD46 usage on CHO-CD46 MCP1 cells (Figure 6.4) and CHO-CD46 MCP2 cells (Figure 6.5) was investigated as previously. Blocking of hCD46 usage in CHO-CD46 MCP1 cells by monoclonal antibody treatment reduced rAd19aGFP transduction 6%, 35% and 51% on increasing dosage and had a similar 12%, 35% and 56% effect on rAd5F35GFP transduction (Figure 6.4 B). Polyclonal antibody treatment clearly reduced both rAd5F35GFP and rAd19aGFP transduction drastically whereas the control serum only caused <20% reduction in average transduction by either vector except in the case of rAd5F35GFP at the highest dosage where it resulted in a 38% reduction. However, this is still 50% less reduction than the same concentration of the polyclonal antibody and the results are only representative of a single experiment. Pre-treatment with soluble hCD46 resulted in a similar reduction in rAd19aGFP transduction and rAd5F35GFP transduction.

The blocking of hCD46 usage had a similar effect on both average rAd19aGFP and rAd5F35GFP transduction and it was therefore concluded that these results confirm that the transduction of CHO-CD46 MCP1 cells by rAd19aGFP is reliant on the availability of hCD46.

Finally, it was investigated whether the CD46 blocking agents would also affect the uptake of vectors in CHO-CD46 MCP2 (Figure 6.5). Blocking of hCD46 usage by the monoclonal antibody did not result in any significant reduction in either rAd19aGFP or rAd5F35GFP transduction whilst the CD46 antiserum and the CD46Fc fusion protein specifically inhibited their uptake. This had been expected to occur due to the targeting of the monoclonal antibody used against the MCP1 subtype of hCD46 and not the MCP2 isotype present on this particular cell line. This underscores the specificity of the blocking by the mAb.



**Figure 6.4: Effect of CD46 blocking on transduction of CHO-CD46 MCP1 cells.** A chart showing A) the average mock transduction of CHO-CD46 MCP1 cells with either rAd19aGFP (red bars) or rAd5F35GFP (blue bars) and the effect of treatment with increasing concentrations of either B) mAb  $\alpha$ CD46FII antibodies, C) polyclonal antibody  $\alpha$ CD46POLY, D) rabbit serum controls or E) pre-treatment of vector particles with soluble CD46 protein on that transduction. Error bars shown the standard error of the mean for three experiments.



**Figure 6.5: Effect of CD46 blocking on transduction of CHO-CD46 MCP2 cells.** A chart showing A) the average mock transduction of CHO-CD46 MCP2 cells with either rAd19aGFP (red bars) or rAd5F35GFP (blue bars) and the effect of treatment with increasing concentrations of either B) mAb  $\alpha$ CD46FII antibodies, C) polyclonal antibody  $\alpha$ CD46POLY, D) rabbit serum controls or E) pre-treatment of vector particles with soluble CD46 protein on that transduction. Error bars shown the standard error of the mean for three experiments.

It was concluded that as in the case of CHO-CD46 MCP1 cells, both rAd19aGFP and rAd5F35GFP transduction of CHO-CD46 MCP2 cells is reliant on the availability of hCD46.

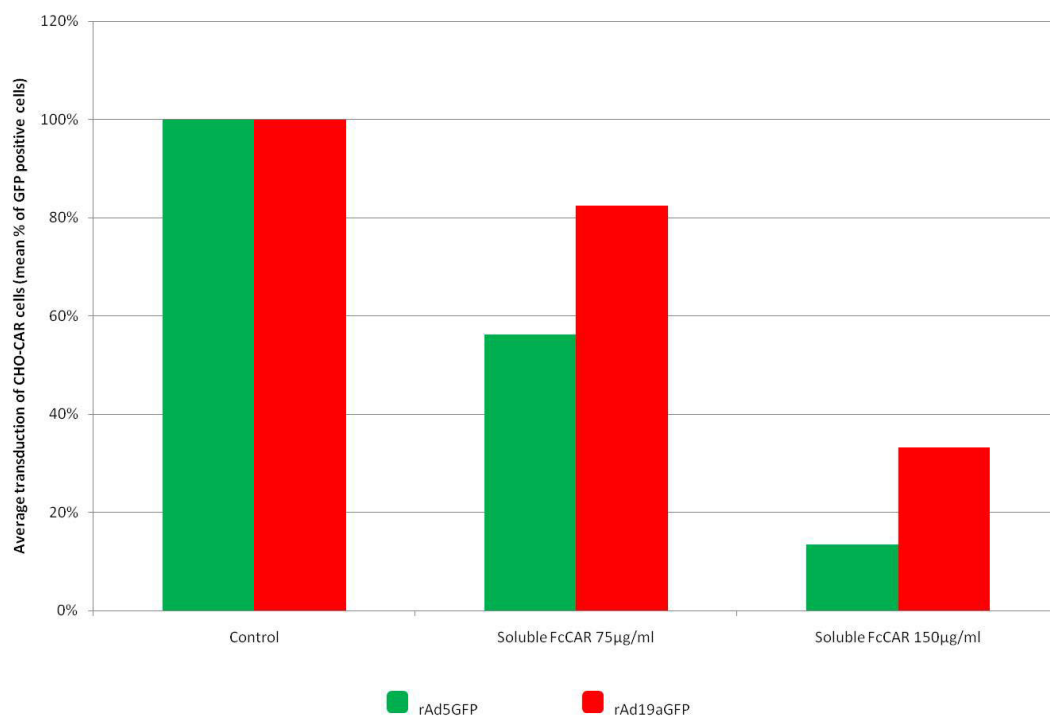
In summary, these results confirm the findings in Chapter 6.4 that rAd19aGFP can utilise hCD46 as a cellular receptor when expressed on the CHO cellular background. It remains open whether Ad19a may prefer the MCP2 isoform over MCP1 and this will require further investigation. Blocking of hCD46 usage in A549 cells had little to no effect on rAd19aGFP transduction whilst rAd5F35GFP transduction was significantly reduced. This is in line with the data for Ad37 (Wu *et al.*, 2003) and supports the hypothesis that, while under certain circumstances, rAd19aGFP can utilise hCD46 as a receptor it can also utilise at least one other receptor which may or may not be hCAR. These findings may or may not be relevant in the presence of a normal human receptor background.

### **6.5.2: Blocking of CAR usage**

Subsequent to hCD46 competition, attempts were made to block rAd19aGFP hCAR usage on CHO-CAR cells using rAd5GFP as a positive control. The first attempts at hCAR competition proved unsuccessful as several commercial antibodies against CAR proved incapable of blocking rAd5GFP transduction of CHO-CAR cells (data not shown) and as such could not be used to investigate the blocking of rAd19aGFP transduction. Pre-treatment of rAd5GFP with a soluble form of hCAR (rhCXADR/Fc) resulted in a 44% and 87% reduction in transduction on increasing dosage as hypothesised whilst the same treatment of rAd19aGFP also resulted in a reduction in transduction of 18% and 67% (Figure 6.6; Representative FACS histograms Figure A25 A)). This data, however, is only based on two experiments due to the low concentration and the highly expensive soluble hCAR.

These results represent preliminary findings that rAd19aGFP is reliant on hCAR in CHO-CAR cells but further experiments with more controls must be performed before a conclusion can be made.





**Figure 6.6: Effect of pre-treatment with soluble CAR on transduction of CHO-CAR cells.** Briefly, Ad vectors were incubated with the indicated concentration of fusion protein for 30 mins at 4°C. Subsequently they were used to transduce CHO-CAR cells at an MOI of 10. 24 hours later the cells were examined for GFP fluorescence by FACS analysis. The chart showing the average mock transduction of CHO-CAR cells with either rAd5GFP (green bars) or rAd19aGFP (red bars) and then the effect of pre-treatment of vector with soluble CAR protein on that transduction. Results are shown for two experiments

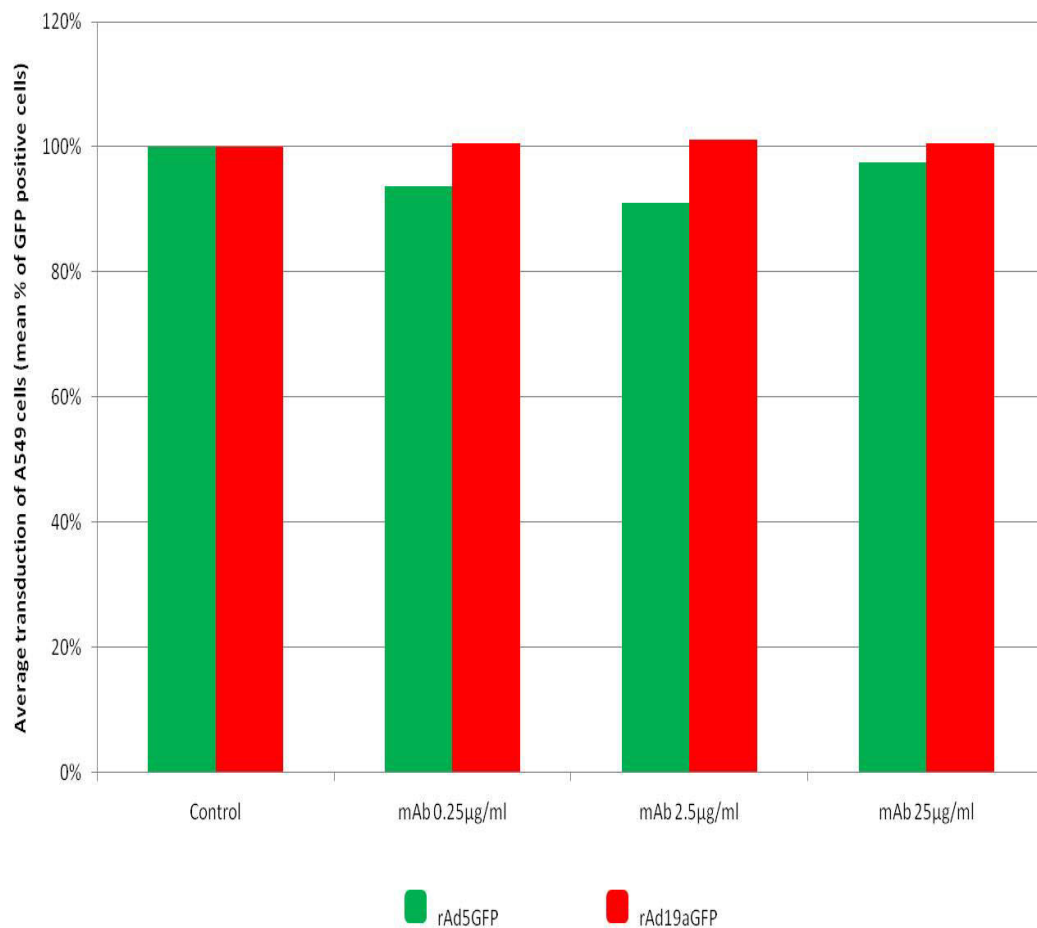
### 6.5.3: Effect of blocking GD1a usage

An attempt was made to investigate the impact of a  $\alpha$ GD1a monoclonal antibody (MOG35) together with relevant control antibodies on rAd19aGFP transduction of A549 cells. No useful vector control was available for this study as no virus or vector has been shown to use GD1a as a cell receptor apart from Sendai virus (Markwell *et al.*, 1981) or influenza (Bukrinskaya *et al.*, 1989). MOG35 or isotype controls had only a negligible effect on rAd5GFP transduction and no effect on rAd19aGFP transduction of A549 cells (Figure 6.7; Representative FACS histograms figure A25 B). It cannot, however, be concluded that rAd19aGFP does not utilise GD1a as a receptor as the antibodies used may not interfere with the region of the glycolipid which may or may not be responsible for any Ad19a binding and a larger panel of methods to compete GD1a usage would have to be used.

## 6.6: Discussion

This work presents the first attempt of a direct investigation into the tropism of Ad19a. It also represents the first attempt to investigate the tropism of the Ad19a or Ad37 fibre with the correct hexon and penton proteins being present rather than the use of an Ad5 vector pseudotyped with the Ad37 fibre (Cashman *et al.*, 2004) or non-human cell lines specifically transfected with receptor candidates rather than human cell lines (Johannson *et al.*, 2005; Johannson *et al.*, 2007). Moreover, many of the previous studies into the tropism of the Ad37 fibre have relied on fibre binding data rather than infection or transduction data.

After showing that the chosen non-human cell lines transfected with the candidate receptors indeed expressed their intended receptor (Chapter 6.3), it was then possible to utilise them for transduction experiments with the Ad19aGFP vector alongside controls for hCAR usage (rAd5GFP) and hCD46 usage (rAd5F35GFP)(Chapter 6.4). Somewhat surprisingly it was discovered that the rAd19aGFP vector was capable of utilising both hCAR and hCD46 and not SA. The binding of soluble Ad37/Ad19a fibre to hCAR had previously been established but it was thought that the fibre was too inflexible to allow hCAR binding. This finding would therefore suggest that



**Figure 6.7: Effect of GD1a blocking on transduction of A549 cells.** The chart shows the average mock transduction of A549 cells with either rAd5GFP (green bars) or rAd19aGFP (red bars) and the effect of treatment with 0.25µg/ml, 2.5µg/ml or 25µg/ml of the MOG35 monoclonal antibody. Results are shown for two experiments.

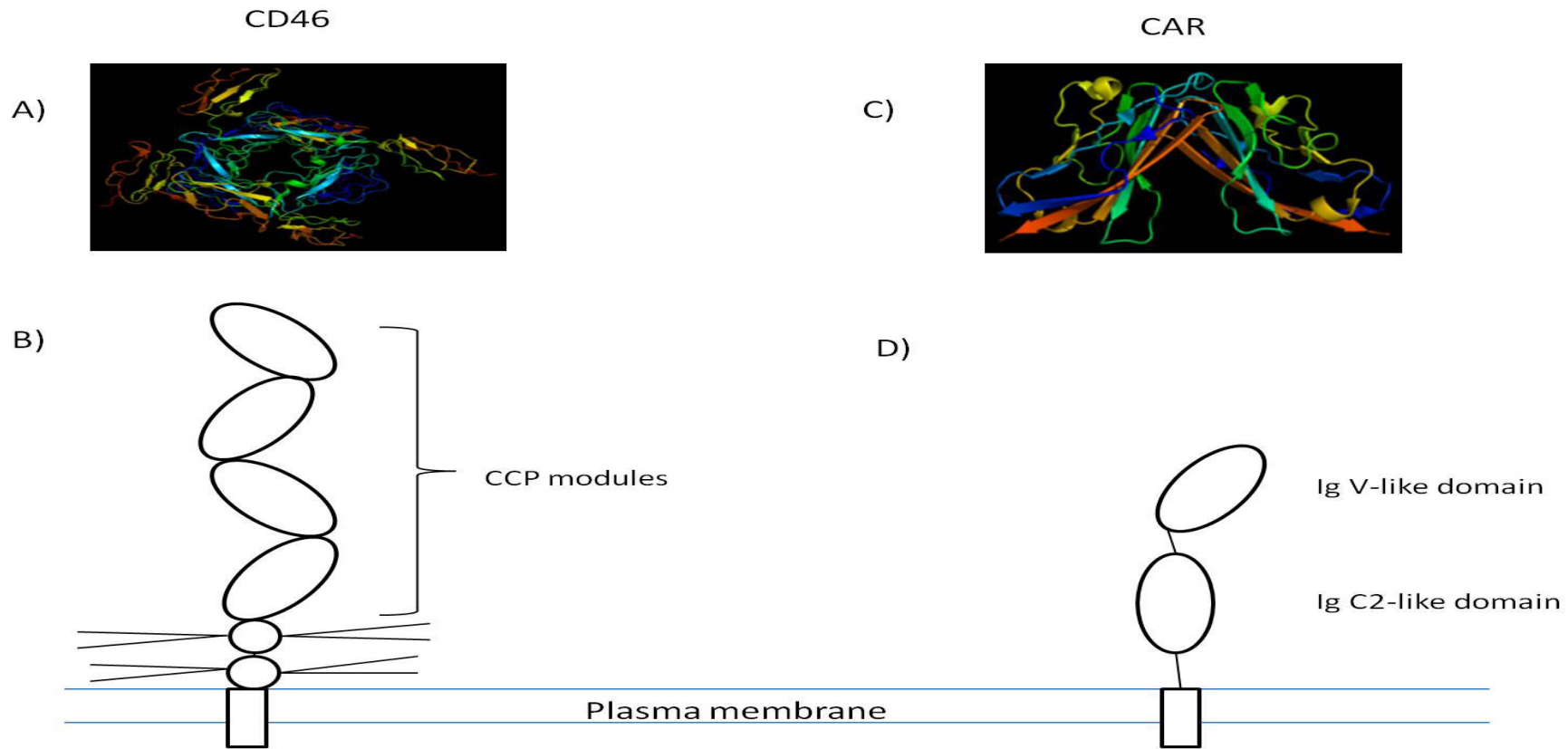
further work into the ability of an Ad37 vector to utilise hCAR in the same experimental set-up would be required.

The usage of more than one receptor by Ad species is not unusual with several subgroup B Ads known to use multiple receptors (Short *et al.*, 2004; Gaggar *et al.*, 2003; Marttila *et al.*, 2005) and the Ad37 fibre, which is identical to the Ad19a fibre, has already been shown to bind to both hCAR (Seiradake *et al.*, 2006) and hCD46 (Wu *et al.*, 2001). It is plausible that the differences between the hexon and penton proteins in Ad37 and Ad19a allow the viruses to have different tropisms but it is perhaps more likely that Ad37 is also capable of using CAR as a receptor and the inflexibility of the fibre is overcome by some as yet unknown mechanism. It is certainly possible that interactions between the penton base protein and integrins could play a role in CAR binding as penton base-integrin interactions have been shown to take place regardless of fibre tropism (Arnberg, 2009).

The finding that an increase or decrease in SA had no effect on the transduction efficiency of rAd19aGFP is unexpected considering the large amount of literature supporting this structure as the Ad37 receptor (Arnberg *et al.*, 2000<sup>a</sup>; Arnberg *et al.*, 2002; Arnberg *et al.*, 2000<sup>b</sup>; Thirion *et al.*, 2006; Johansson *et al.*, 2005; Johansson *et al.*, 2007; Cashman *et al.*, 2004). However, it has been noted that the Ad19p fibre only differs from the Ad19a/Ad37 fibre by two aa's (Arnberg *et al.*, 2002) and yet does not bind SA. Therefore it is plausible that the Ad37/Ad19a fibre is capable of binding SA when these two aa's are available when the fibre molecule alone is used, but some form of hindrance, perhaps from the other Ad structural components, prevents it from doing so in a whole virus system. It may also be conceivable that the binding of the Ad37/Ad19a fibre is only an early transient form of binding which only serves to allow the fibre to use another secondary receptor, such as hCD46 or hCAR, for actual cell entry. Evidence accumulated by the use of neuraminidase cleavage of SA or wheat germ agglutinin treatment preventing transgene expression (Thirion *et al.*, 2006) may be flawed as it could logically prevent the early interactions of Ads with the cells or cause hindrance to the actual receptor binding and subsequent entry event. Currently it is not possible to define whether Ad19a may use SA as a receptor, it can only be confirmed that, in the cell lines used, the reduction of SA in Lec2 cells did not have an impact on transduction. For this result

to be confirmed in further work, a positive control virus such as influenza, which does use SA as its primary receptor, would have to be utilised.

In Chapter 6.5 it was shown that the transduction of rAd19aGFP could be effectively competed in multiple cell lines, except human control cells, by increasing concentrations of either monoclonal or polyclonal antibodies against hCD46 or soluble forms of either hCD46 or hCAR. These results confirmed the finding from Chapter 6.3 that rAd19aGFP was capable of utilising hCD46 as a receptor due to the ability of all three methods tested to effectively reduce rAd19aGFP transduction of CHO-CD46 MCP1/2 cells. The results also suggest that the theory of dual receptor usage by Ad19a may be true due to only minor reduction in rAd19aGFP transduction in A549 cells when the same concentrations almost completely ablated rAd19aGFP transduction in CHO-CD46 MCP1/2 cells suggesting that rAd19aGFP was capable of using a second receptor on A549 cells when hCD46 was blocked. It remains unclear, therefore, if rAd19aGFP utilises hCD46 at all on normal human cells. In further support of the data, polyclonal antibodies prevented both rAd5F35GFP and rAd19aGFP transduction in both CHO-CD46 MCP1 and MCP2 cell lines but a monoclonal antibody directed against the MCP1 isotype could only prevent transduction of the CHO-CD46 MCP1 cell line but not the CHO-CD46 MCP2 cell line. It must be stated that the work on blocking the utilisation of hCAR is incomplete in comparison to the work on hCD46 due to the lack of antibodies which could reduce rAd5GFP control transductions and only two experiments were performed using the soluble form of CAR due to the expense and low concentration of the available supply. It should be noted that both CD46 and CAR have multiple domains (Figure 6.8) which both Ad19a and Ad5 could bind to. There is data, derived only from antibody competition experiments, that suggests different adenovirus strains may contact different CCP modules in CD46 (Figure 6.8; Cattaneo, 2004) and the same may be true of differing domains of CAR. The binding site of the antibody used to attempt blocking may, therefore, be a factor as an antibody able to block the binding of one adenoviral species may not block another. For example, the blocking of Ad19a and Ad35 usage of CD46 MCP-1 but not MCP-2 by the use of a monoclonal anti-CD46 MCP1 antibody may be due to a change in virus binding site and not antibody binding site, however, we lack the antibody binding domain information necessary to assess this. It is known that Ad5



**Figure 6.8. The domain structure of CD46 and CAR may affect antibody binding.** A diagram showing A) the protein database rendering of CD46, B) the structure of CD46 highlighting the four tandem complement control protein (CCP) modules, followed by one or two heavily O-glycosylated (thin lines) serine/threonine/proline-rich (STP) domains (circles) and the transmembrane region (rectangle) (adapted from Cattaneo, 2004), C) the protein database rendering of CAR and D) the structure of CAR highlighting the Ig V-like, Ig C2-like domain and the transmembrane region (adapted from Carson, 2001).

infectivity/transduction is difficult to block using antibodies and has been suggested that any attempt to do so would require integrin blocking alongside (Vivien Mautner, personal communication) to prevent unspecific transduction such as that observed in the parental CHO cells. To fully confirm if the blocking of hCAR usage can reduce both rAd5GFP and rAd19aGFP transduction a further supply of soluble protein would have to be acquired and used again on CHO-CAR cells and perhaps more importantly used on A549 cells to determine if a similar effect was obtained to that found when blocking hCD46 usage in the same cell line. Further controls would also be required, such as the use of another unrelated soluble Fc fusion protein to confirm that any effect detected with the soluble hCAR is caused by the CAR portion specifically and not a non-specific interaction of the Fc part.

Finally, a preliminary attempt was made to block rAd19aGFP usage of GD1a on A549 cells. In both experiments performed, a GD1a monoclonal antibody failed to reduce rAd19aGFP transduction when compared to isotype control antibodies. This finding suggests that rAd19aGFP does not utilise GD1a for infection/transduction of A549 cells. However, it is possible that Ad19a or Ad37 may use it for entry into other cell types. Further experiments in other human cell lines would be vital to form a conclusion and, as GD1a is expressed on many mammalian cell types, further investigation into the use of GD1a may be useful for further mouse studies.

If Ad19a can utilise both hCAR and hCD46 this may have important implications for the further use of the vector *in vitro* and *in vivo*. Staining of DCs has revealed high levels of CD46 expression (data not shown; see also Lore *et al.*, 2007) which may support Ad19a's enhanced transduction efficiency of DCs whilst CAR expression was negative which, similarly, would account for the low level of Ad5 transduction. However, in DCs many potential uptake mechanisms for Ads may exist with multiple receptors being involved. For example, Ad3 has been shown to use hCD80 and hCD86 as receptors (Short *et al.*, 2004) and as such it cannot be conclusively stated that hCD46 usage confers Ad19a's increased DC transduction efficiency. Further experiments with DCs could be performed to establish if Ad19a utilises one of its candidate receptors for transduction or whether it utilises a different mechanism, such as DC-SIGN. hCD46 usage would allow Ad19a to be used in cancer gene therapy applications as CD46 is expressed on many malignant human

tumour cells to help them escape complement attack (Mathis *et al.*, 2006) and has been proposed as a viable target for cancer gene therapy (Ulasov *et al.*, 2006). hCAR usage was originally implicated in the fatal outcome of an Ad5 vector clinical trial (Raper *et al.*, 2003) which may suggest that Ad19a's hCAR usage may confer the same negative effects, however, it has since been shown that liver tropism by Ad5 is primarily conferred by Ad5 hexon binding to human coagulation Factor Ten (FX) and is not caused directly by the tropism of the fibre protein (Waddington *et al.*, 2008). As such, it is unlikely that Ad19a could cause liver sequelae due to the considerable sequence differences between the Ad5 and Ad19a hexon proteins, although it cannot be discounted and further research on the 'full' serotypes of Ads from various serotypes rather than fibre-pseudotyped vectors, as reviewed in Baker *et al.*, 2007, would be required before entering human clinical trials. Current theories also suggest that, even in the case of Ad5, hCAR may not be a primary receptor *in vivo* for Ads because hCAR mRNA expression poorly matches the tropism of Ad5 vectors in an *in vivo* setting (Tomko *et al.*, 1997; Walters *et al.*, 1999) although a new CAR variant has been found on apical surfaces (Excoffon *et al.*, 2010). It has therefore been proposed that a putative function of the fibre-CAR interaction is to facilitate escape from the cell rather than entry. The fibre protein is produced in such large quantities that it cannot all be virus incorporated into virus particles; the excess fibre is shed and eventually reaches tight junctions where, as a consequence of CAR binding, it forms intercellular oligomers which facilitate the opening of tight junctions perhaps allowing virus escape (Walters *et al.*, 2002). Virus escape from epithelia could be a putative function for hCAR binding in Ad19a, although the employment of this function *in vivo* would be impossible using E1-deleted vectors as no replication takes place and no excess fibre is produced.

The work presented in this chapter has provided evidence for the enhanced ability of the Ad19a vector to transduce cells expressing hCD46 or hCAR, in the absence of other human molecules. This suggests that Ad19a has some intrinsic affinity for these molecules. However, in human cells, these interactions appear less relevant because other interactions may take place. As human cells are the relevant target, more experiments with such cell types and multiple blocking agents are required. Further experiments with human cell lines could include ocular cell lines, as these are the primary site of infection for Ad19a but not for Ad35 or Ad5 and this may



provide evidence as to which receptor Ad19a utilises for its primary infection at the ocular surface. An alternative approach would be to knock down proposed receptor molecules in human cells using the corresponding siRNA and analysing the effect. Unfortunately, the GD1a data is incomplete as information regarding its proposed usage by Ad37 became available relatively late in this study.

Further work must first involve gaining more *in vitro* data. Once candidate molecules have been isolated, *in vivo* experiments with KO or transgenic mice could be performed, as in Chapter 7, to assess if the presence or absence of these receptors has any effect on the effectiveness of a vaccine vector. The same mice could also be used with the GFP vectors to examine if changing the available receptor molecules has an effect on the *in vivo* distribution of Ad vectors after inoculation.

## **Chapter 7: T cell responses to rAd19aHIVA in a murine model and the effectiveness of rAd19aPVM-N to protect mice against lethal challenge with PVM**

### **7.1: Introduction**

Ad recombinants have been shown to induce potent transgene specific immune responses (Xiang *et al.*, 1995; Wang *et al.*, 1997) and have been used previously to protect animals against pathogenic challenge with various viral pathogens such as SHIV (Shiver *et al.*, 2002), Ebola virus (Sullivan *et al.*, 2000), swine influenza virus (Tang *et al.*, 2002), and measles virus (Schindler *et al.*, 1994). A full introduction to the use of recombinant Ads as vaccines is given in Chapter 1. This chapter will compare the use of Ad19a and Ad5 vectors in two animal models to assess *in vivo* any advantages an Ad19a vector may possess by analysing transgene specific immune response and protection against pathogenic challenge. One transgene, HIVA, was used to assess the CD8<sup>+</sup> T cell response while for the second, PVM-N, the antibody response was analysed.

#### **7.1.1: Use of the HIVA transgene**

The HIVA transgene, introduced in Chapter 1, was successfully inserted into identical expression cassettes of an Ad19a and an Ad5 vector (Chapter 3) and shown to express transgene (Chapter 4). The ability of each of these vectors to generate a CD8<sup>+</sup> T cell response in a mouse model was then examined by ELISpot analysis. To enable assessment of the immunogenicity of HIVA *in vivo* an H-2D<sup>d</sup>-restricted epitope P18-I10 (RGPGRAFVTI) (Takahashi *et al.*, 1988; Hanke *et al.*, 1998), herein designated epitope H, was intentionally inserted within the multi-epitope region of the HIVA transgene. A second epitope within the same region, the subdominant H-2K<sup>d</sup>-restricted epitope (IFQSSMTKI), herein designated epitope P, has also been shown to give a powerful immunological readout, however T cell responses to H have been shown to be generally > P (Im & Hanke, 2007). These two peptides can be utilised in the ELISpot assay to assess splenocytes harvested from inoculated BALB/c mice for the secretion of IFN- $\gamma$  on contact with their corresponding peptide alongside unspecific stimulation by PMA and Ionomycin.

### 7.1.2: The use of the PVM-N transgene

PVM, also introduced in Chapter 1, is a natural rodent Pneumovirus pathogen. Intranasal (I/N) inoculation of BALB/c mice with as few as 10pfu, leads to robust virus replication (Domachowske *et al.*, 2000) and doses as low as 60pfu can result in 20% mortality (Cook *et al.*, 1998). Depending on the quantity of the virus inoculum, mice have been shown to normally exhibit clinical signs of disease between 5-7 days post-challenge with the majority of mortality taking place before day 10. A system for monitoring the clinical signs of the pathogenic PVM J3666 (herein referred to as PVM; Cook *et al.*, 1998; Domachowske *et al.*, 2000; Bonville *et al.*, 2003) pathogenesis in mice has been previously established (Cook *et al.*, 1998; Figure 7.3) and the external clinical signs of disease in the mice can therefore be observed each day and a mean score calculated for each group. Advancing clinical signs have been shown to correlate with weight loss and this can also be monitored as initial signs, severity and subsequent recovery from disease.

Recently, it has been shown that an rAd5 vector expressing the nucleocapsid gene from PVM (PVM-N) is capable of protecting mice prophylactically, when given intranasally, against a lethal 250pfu PVM challenge in a prime/boost schedule at a  $10^7$  pfu dosage in a 50 $\mu$ l inoculum volume (Helen Terry, PhD thesis University of Warwick, 2010). It was decided to investigate if rAd19aPVM-N, generated in Chapter 3, can perform as well or better in the same model system. Protection has been shown not to correlate with an IgG response and this will also be investigated using a PVM-N specific ELISA.

## 7.2: Aims

The aims of this study were the investigation of the *in vivo* performance of Ad19a vectors, when compared to similar Ad5 vectors, in two vaccination model systems by either immunological readout or protection against lethal challenge.

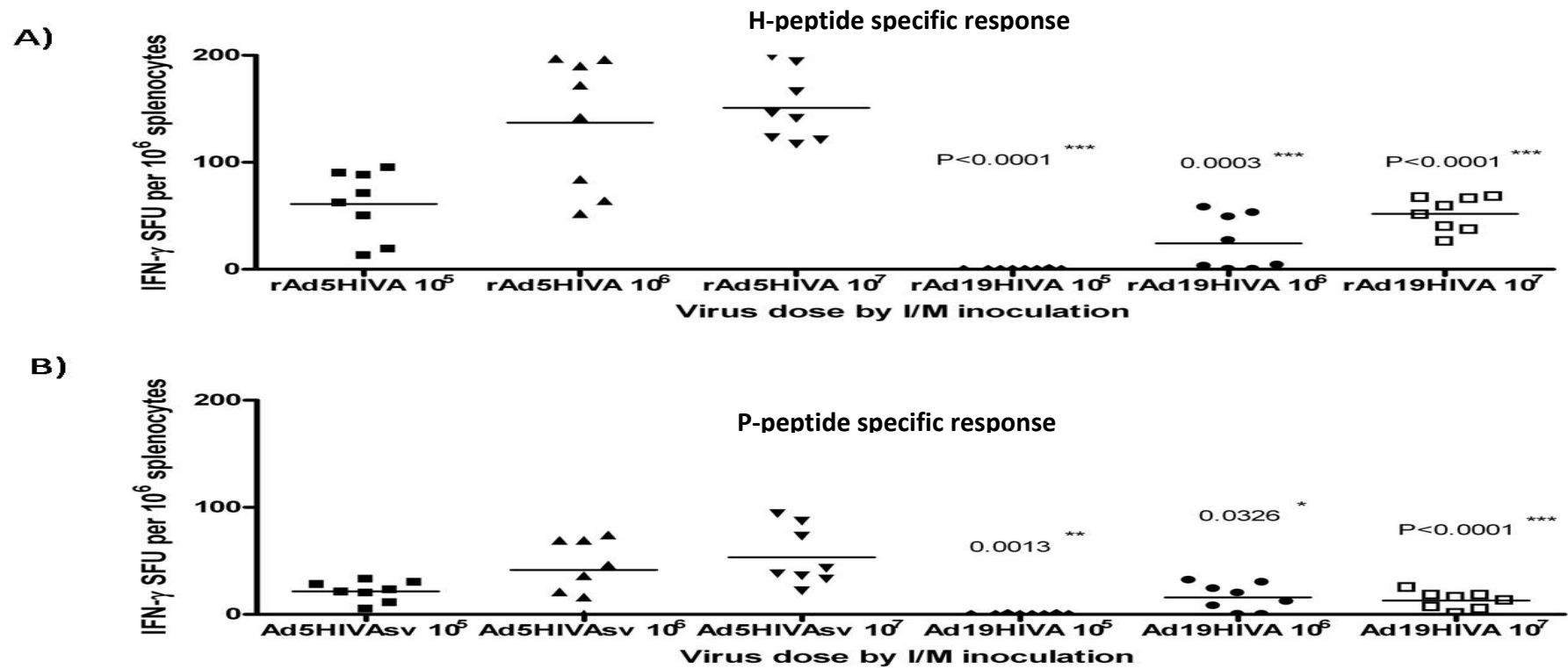
### **7.3: T cell responses to rAd19aHIVA in a murine model**

All work in Chapter 7.3 was carried out by Dr. Anne Bridgeman at the University of Oxford. Processing and presentation of results was done by the author. First, dose-escalation was used to investigate the toxicity of rAd19aHIVA and rAd5HIVA. Secondly, the potential of the two vectors to induce a T cell response was investigated. As a read-out, the ELISpot assay was used which analyses interferon gamma (IFN- $\gamma$ ) production at the single cell level. A spot forms at the site where secreted IFN- $\gamma$  is bound, is counted using an automated ELISPOT reader and then related to the number of cells added to the microtitre plate. This allows the frequency of cells secreting the cytokine to be calculated. One spot forming unit (sfu) is equal to one IFN- $\gamma$  secreting cell.

#### **7.3.1: rAd19aHIVA vs. rAd5HIVA *in vivo*: effect of dosage**

Groups of 4 mice were inoculated intramuscularly (I/M) with either  $10^5$ ,  $10^6$  or  $10^7$  pfu of either rAd5HIVA or rAd19aHIVA in a 10 $\mu$ l volume. The mice were left for 14 days before being sacrificed, their splenocytes harvested and subjected to an ELISpot assay (Chapter 2.19) in duplicate (Figure 7.1; Representative ELISpot assay Figure A 26). The number of cells secreting IFN- $\gamma$  on addition of 20ng of either the H peptide (Figure 7.1 A) or the P peptide (Figure 7.1 B) was calculated as sfu per 1 million splenocytes and summarised. The significance of the data was statistically analysed by unpaired t test.

Both vectors were found to be non-toxic at all doses tested as no external clinical signs of illness were observed in any of the mouse groups. rAd5HIVA produced a significantly higher IFN- $\gamma$  response than rAd19aHIVA to both the H peptide and P peptide at all three dosages tested. The H peptide was found to give a comparatively better immunological readout than the P peptide as has been seen previously (Im & Hanke, 2007).



**Figure 7.1: IFN- $\gamma$  response of splenocytes upon immunisation with  $10^5$ ,  $10^6$  and  $10^7$  pfu of rAd19aHIVA and rAd5HIVA.** Groups of 4 mice were given increasing dosages of rAd5HIVA or rAd19aHIVA intramuscularly. After 14 days the mice were sacrificed, splenocytes harvested and then assayed in duplicate for IFN- $\gamma$  responses to either (A) the H peptide (RGPGRAFVTI) or (B) the P peptide (IFQSSMTKI). Results are given as the number of IFN- $\gamma$  spot forming units (SFU) produced by 1 million splenocytes when stimulated. Numbers given are the comparative P values of an unpaired two-tailed t test between the same dosage of rAd5HIVA and rAd19aHIVA.

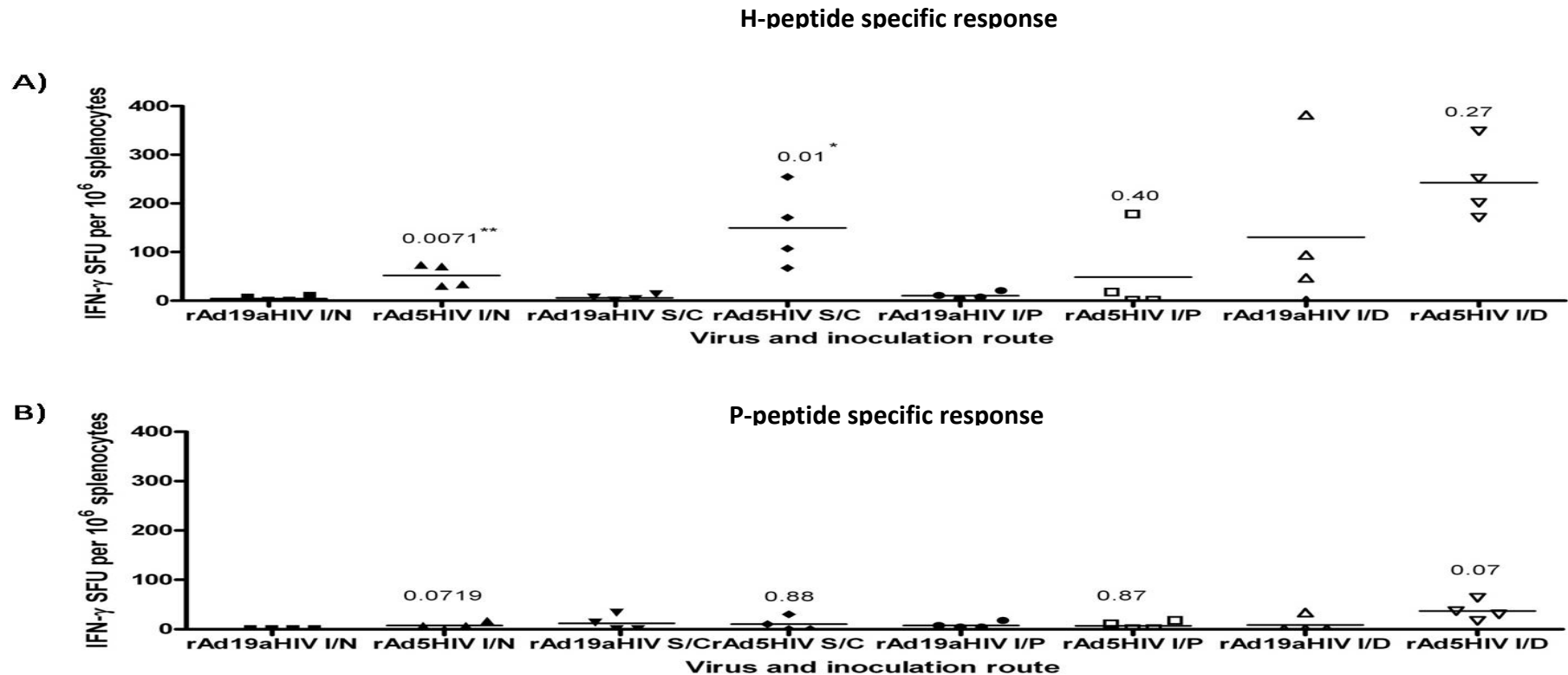
It was concluded that both vectors were safe and non-toxic in BALB/c mice up to  $10^7$  pfu and rAd5HIVA was capable of stimulating a greater IFN- $\gamma$  secreting T cell response than rAd19aHIVA when given I/M at the three doses tested. It was next decided to investigate if the greater immunological response stimulated by the Ad5 vector was reproducible when using other inoculation methodologies.

### **7.3.2: rAd19aHIVA vs. rAd5HIVA *in vivo*: influence of inoculation route**

Groups of 4 mice were given a single  $10^6$  pfu dose of rAd5HIVA or rAd19aHIVA by either intranasal (I/N), subcutaneous (S/C), Intraperitoneal (I/P) or Intradermal (I/D) inoculation. After 14 days the mice were sacrificed, splenocytes harvested and then assayed in duplicate as previously by ELISpot for response to either the H peptide (Figure 7.2 A) or the P peptide (Figure 7.2 B). Results are presented as previously.

Both vectors were once again found to be safe and non-toxic at the dosage tested in each inoculation methodology. All results were subjected to the same unpaired t test. A significant difference in the IFN- $\gamma$  response between the two vectors was only seen with the H peptide and only when administered I/N or S/C (Figure 7.2 A). No significant difference was observed when either vector was administered by I/P or I/D, or observed between any of the inoculation routes with the P peptide (Figure 7.2 B). In essentially all cases, the immune response against the P peptide was extremely low.

It was concluded that, at the  $10^6$  dosage tested, only I/M (Chapter 7.3.1), I/N or S/C administration produced a significantly higher T cell response in mice inoculated with the rAd5HIVA vector rather than the rAd19aHIVA vector. However, no immunisation route gave a robust T-cell response after the administration of the Ad19a vector. This data suggests that inoculation methodology clearly influences the extent of a generated T cell response and any advantage rAd5HIVA may have over the same Ad19a vector can be negated by using a different inoculation route.



**Figure 7.2: rAd19HIVA vs. rAd5HIVA *in vivo*: effect of inoculation route.** Groups of 4 mice were given a  $10^6$  dose of rAd5HIVA or rAd19aHIVA by either intranasal (I/N), subcutaneous (S/C), Intraperitoneal (I/P) or Intradermal (I/D) inoculation. After 14 days the mice were sacrificed, splenocytes harvested and then assayed in duplicate for IFN- $\gamma$  response to either (A) the H peptide (RGPGRAFVTI) or (B) the P peptide (IFQSSMTKI). Results are given as the number of IFN- $\gamma$  spot forming units (SFU) produced by 1 million splenocytes when stimulated. Numbers given are the comparative P values of an unpaired two-tailed t test between the same inoculation methodology for rAd5HIVA and rAd19aHIVA.






Secondly, rAd5HIVA can generally stimulate a greater transgene-specific T cell response in BALB/c mice; however, it is unknown whether this greater T cell response against a single epitope would correlate with better protection against the virus, in this case HIV. As protection against HIV cannot be tested in this mouse model we switched to the second vaccine antigen and investigated which vector was more effective at providing protection against lethal challenge with PVM.

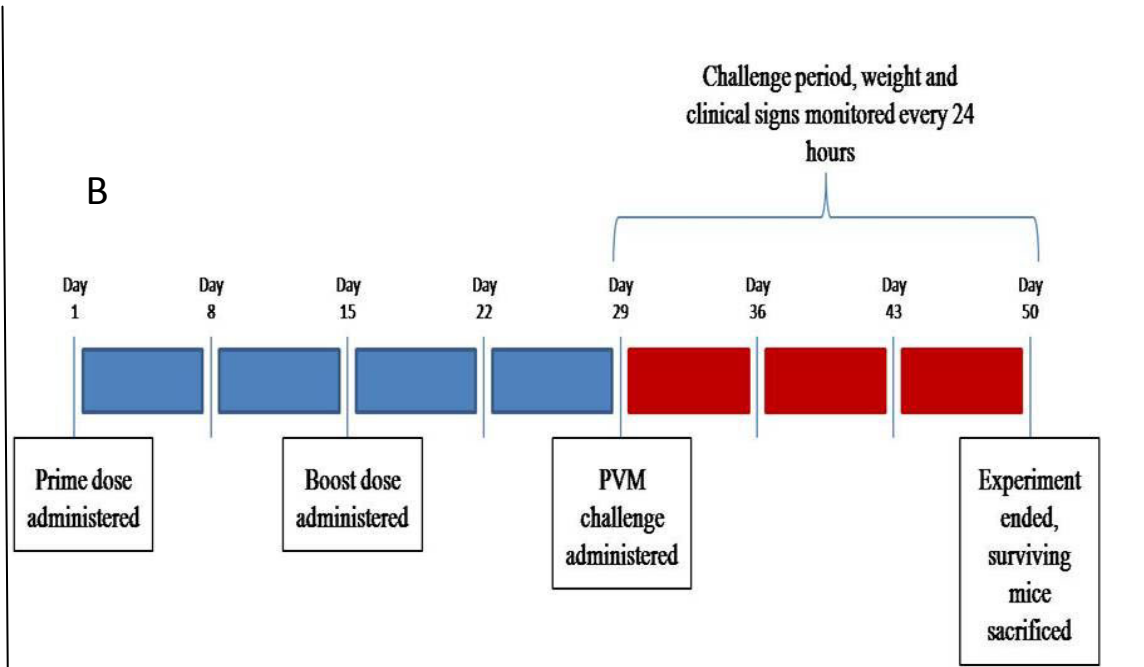
#### **7.4: Protective potential of rAd19aPVM-N and rAd5PVM-N against lethal challenge with PVM.**

A series of experiments were performed based on the Ad5 vector which had already been shown to provide protection (Helen Terry, PhD thesis, Warwick 2010). Therefore a newly generated corresponding Ad19a vector was tested, alongside GFP controls from both vectors, for protection against a lethal challenge by PVM. Unless otherwise stated, groups of five 5-8 week old female BALB/c mice were I/P anaesthetised (Chapter 2.17.1) and given an I/N 50 $\mu$ l (Chapter 2.17.2) prime dose of the vector, a boost dose 14 days later followed by a lethal 250 pfu dose of PVM 14 days thereafter (Figure 7.3 B). During the prime/boosting period, the weight of the mice was monitored up to day 29 when the PVM challenge was administered. Subsequently the weight and clinical score of the mice was monitored for the following 21 days when any surviving mice were culled and the experiment ended (Chapter 2.17.4). A diagram showing the clinical score scale used is shown in figure 7.3 A. Once a mouse had reached a clinical score of 5 it was sacrificed to prevent further suffering. Results are shown as the average clinical score for a group of 5 mice, including those which had been sacrificed which were given a score of 6. Mice that died for reasons unrelated to the PVM infection e.g. under anaesthetic were discounted. The average percentage bodyweight of the group of mice on each day was calculated based on the surviving mice in that group compared to the day of challenge.



**A**

Clinical Score	Clinical symptoms	
<b>1</b>	<b>Healthy, no clinical signs of illness</b>	
<b>2</b>	<b>Ruffled fur, especially on neck</b>	
<b>3</b>	<b>Ruffled fur extending beyond the neck, deeper breathing</b>	
<b>4</b>	<b>Ruffled fur, lethargy, hunched posture, laboured breathing</b>	
<b>5</b>	<b>As for 4, Inactive, emaciated, abnormal gait, huddle together, may show cyanosis of tail and ears</b>	
<b>6</b>	<b>Death</b>	



**Figure 7.3: Murine monitoring and vaccination schedule**

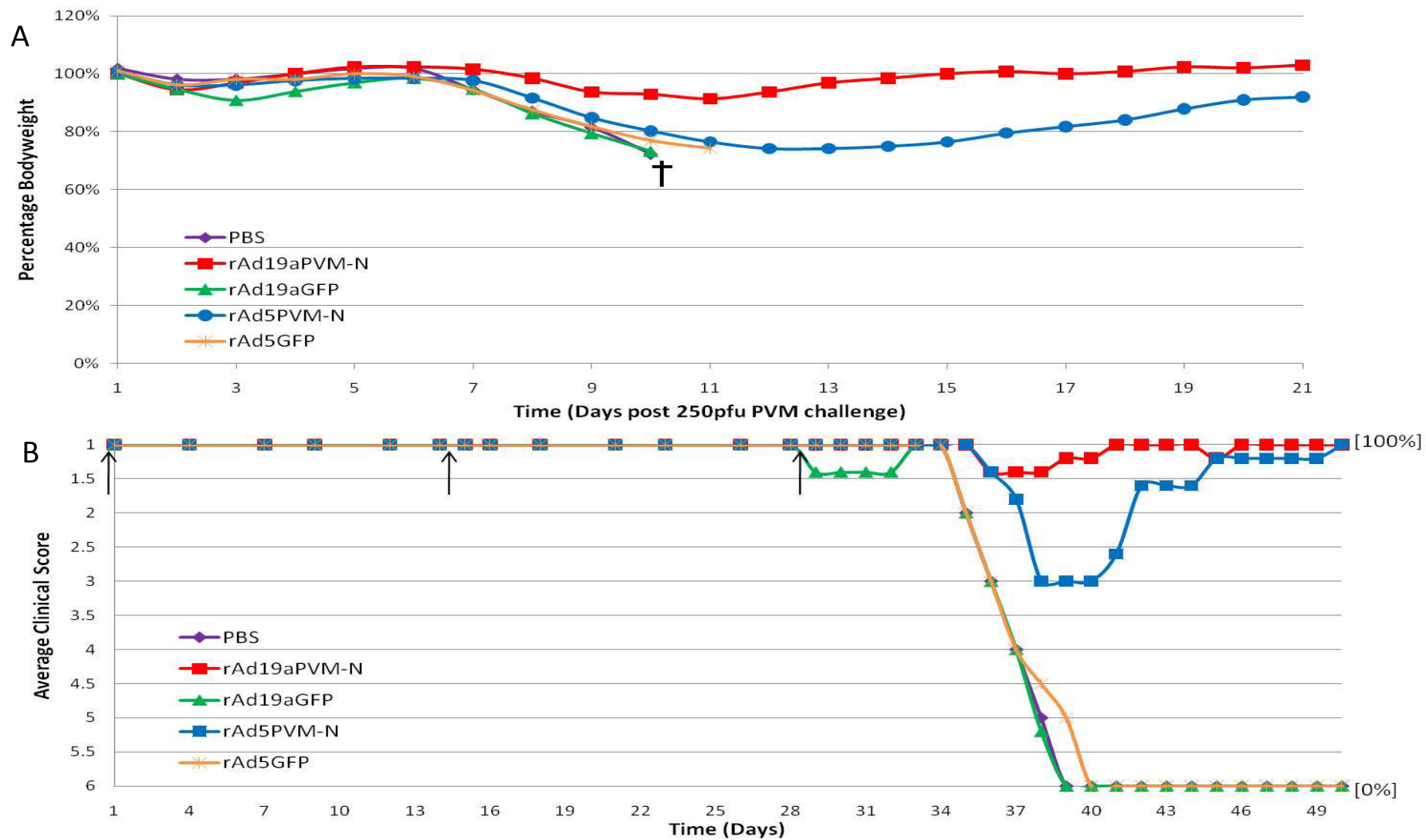
A) Clinical score alongside associated clinical symptoms and example photos of various clinical stages and B) Vaccination schedule showing the day of the prime dose, boost dose and PVM challenge administration and the experiment end. The portion of the schedule where weight and clinical score were monitored every 24 hours for sign of disease is highlighted in red.

#### **7.4.1: rAd19PVM-N and rAd5PVM-N protect mice against lethal challenge with 250pfu PVM at a $10^7$ intranasal dosage**

Previous experiments have shown that a  $10^7$  dose of pfu of rAd5PVM-N could protect mice against a lethal PVM challenge. To see if the same was true for rAd19aPVM-N groups of 5 female BALB/c mice were given a prime dose and a boost dose exactly 14 days later of PBS or  $10^7$  pfu of either rAd19aPVM-N, rAd19aGFP, rAd5PVM-N or rAd5GFP followed by a lethal dose of PVM 14 days after that. The percentage bodyweight of the mice after challenge and their average clinical score over the course of the whole experiment can be seen in Figure 7.4.

Both rAd5PVM-N and rAd19aPVM-N were shown to protect mice against a lethal PVM challenge at a  $10^7$  prime and boost dose, as shown by 100% survival in both groups by the end of the experiment. Mice inoculated with PBS or GFP control vectors lost ~25% of their original bodyweight in the ten days post challenge (Figure 7.4 A). This, combined with their rapid rise in clinical score resulted in all three groups being culled by day 10 (Figure 7.4 B). The rise in clinical score between days 29 and 32 in the rAd19aGFP group was attributed to fighting and signs of injury to one of the mice within that group. In comparison mice inoculated with rAd5PVM-N too lost ~26% of their original bodyweight by 13 days post challenge but their average clinical score did not exceed 3. The bodyweight of mice began to recover and their clinical score decrease from day 13 post challenge onwards and returned to a clinical score of 1 by day 21 post challenge. Surprisingly, mice inoculated with rAd19aPVM-N only lost 9% of their original bodyweight by day 11 post challenge and their clinical score reached a maximum of 1.4 on day 7 post challenge. The mice then quickly recovered to a clinical score of 1 by day 13 post challenge and their bodyweight was recovered to 100% of their original bodyweight by day 15.

It was concluded that both the Ad5 and Ad19a PVM-N vectors were capable of protecting mice against lethal challenge with PVM at a  $10^7$  I/N dose. Unexpectedly, mice inoculated with rAd19aPVM-N lost less bodyweight and reached a lower clinical score than those inoculated with rAd5PVM-N. This might suggest that rAd19aPVM-N is capable of stimulating an improved protective immune response



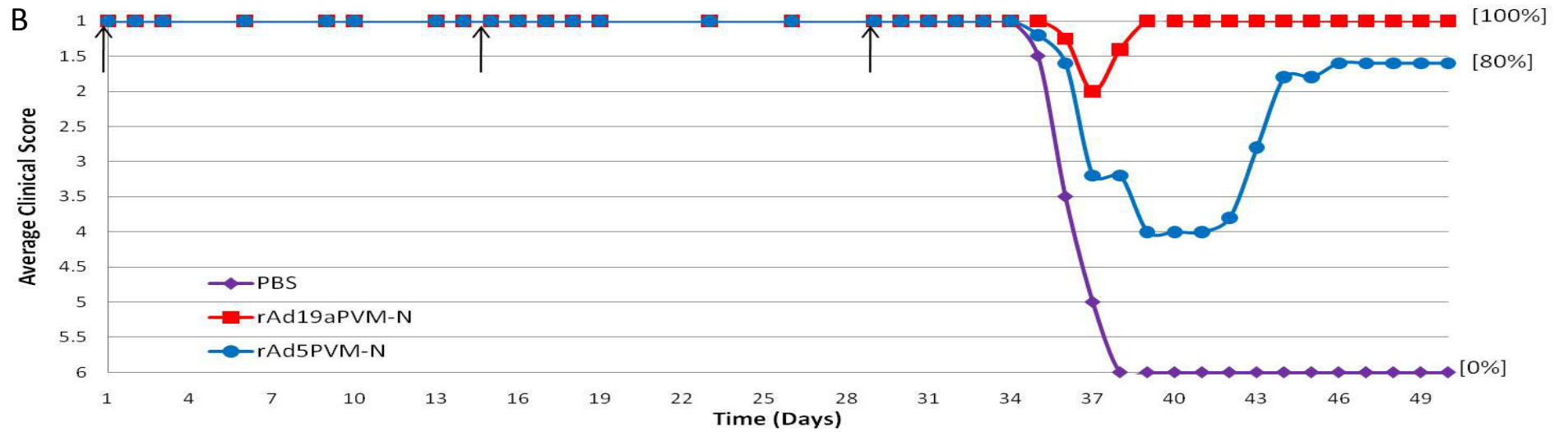
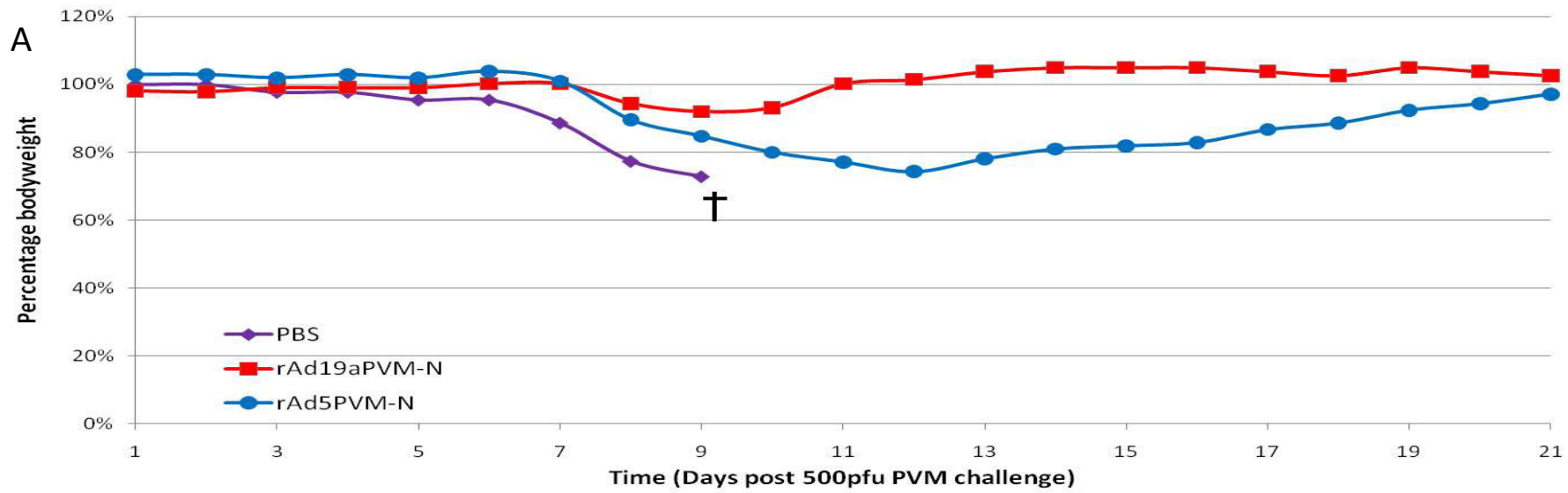
**Figure 7.4: rAd19PVM-N and rAd5PVM-N protect mice against lethal challenge with 250pfu PVM at a  $10^7$  intranasal dosage.** Groups of 5 female BALB/c mice were intranasally administered 50 $\mu$ l of a prime dose and a boost dose exactly 14 days later of PBS or  $10^7$  pfu of either rAd19aPVM-N, rAd19aGFP, rAd5PVM-N or rAd5GFP followed by a lethal dose of PVM 14 days after that. The mice were monitored for changes in bodyweight and clinical score and the figure shows (A) the percentage bodyweight of the surviving mice in relation to their bodyweight on day one post challenge (day of PVM challenge) for the subsequent 21 days and (B) the average clinical score of each mouse group over the 50 day course of the experiment. The numbers in square brackets show the number of surviving mice from each group at the end of the experiment. Arrows indicate timings of the prime dose, boost dose and PVM challenge in chronological order.

when compared to rAd5PVM-N. It was decided to investigate if this result could be substantiated by increasing the PVM challenge dose.

#### **7.4.2: rAd19PVM-N and rAd5PVM-N protect mice against super-lethal challenge with 500pfu PVM at a $10^7$ intranasal dosage**

In the above experiment the rAd5PVM-N vaccinated mice barely survive as they nearly reached the lower bodyweight limit for culling. In this experiment the lethal dose of 250 pfu of PVM was doubled to 500 pfu, a super-lethal dose, to examine if both the PVM-N vectors could still protect or a further differential regarding bodyweight or clinical score would be detected. As it has been shown that  $10^7$  pfu dose of either of the GFP vectors was unable to protect mice against a lethal 250pfu PVM challenge they were removed from the schedule for ethical reasons. Groups of 5 female BALB/c mice were intranasally administered with 50 $\mu$ l of a  $10^7$  pfu prime dose and boost dose exactly 14 days later of rAd19aPVM-N, rAd5PVM-N or PBS followed by a super-lethal 500pfu dose of PVM 14 days after that. The percentage bodyweight of the mice after challenge and their average clinical score over the course of the whole experiment can be seen in Figure 7.5.

Both rAd5PVM-N and rAd19aPVM-N vectors protected mice against a super-lethal PVM challenge at a  $10^7$  prime and boost dose as shown by 100% and 80% survival in both the Ad19a and Ad5 groups respectively by the end of the experiment. Mice inoculated with PBS lost ~27% of their original bodyweight by day 9 post challenge (Figure 7.5 A) and this combined with a rapid rise in average clinical score resulted in the whole group being sacrificed on day 9, one day earlier than with a 250 pfu challenge dose suggesting the higher dose did increase the rapidity of the onset of disease and rise in clinical score. Mice inoculated with rAd5PVM-N again lost ~26% of their original bodyweight by day 12 post challenge suggesting the increased dose had had little effect on bodyweight but the mice did have a markedly higher increase in clinical score over the previous dosage which had increased to 4 by day 11 post challenge and they did not fully recover, as indicated by a clinical score of 1.6 by the end of the experiment. This was attributed to one of the mice in the group not recovering from a clinical score of 3 which may have been suffering from a chronic



**Figure 7.5:  $10^7$  pfu of rAd19PVM-N and rAd5PVM-N intranasally administered protects mice against super-lethal challenge of 500pfu PVM** Groups of 5 female BALB/c mice were intranasally administered 50 $\mu$ l of a prime dose and a boost dose exactly 14 days later of PBS or  $10^7$  pfu of either rAd19aPVM-N or rAd5PVM-N followed by a 500pfu super-lethal dose of PVM 14 days after that. The mice were monitored for changes in bodyweight and clinical score and the figure shows (A) the percentage bodyweight of the surviving mice in relation to their bodyweight on day one post challenge (day of PVM challenge) for the subsequent 21 days and (B) the average clinical score of each mouse group over the 50 day course of the experiment. The numbers in square brackets show the number of surviving mice from each group at the end of the experiment. Arrows indicate timings of the prime dose, boost dose and PVM challenge in chronological order.

form of pneumovirus infection. Mice inoculated with rAd19aPVM-N only lost ~8% of their original bodyweight by day 9 post challenge confirming that the increased dose had had little effect on bodyweight as seen with the Ad5 vector. They also showed an increase in clinical score over the previous dosage to a maximum of 2 by day 8 post challenge but did recover by day 10 post challenge and they returned to 100% of their original bodyweight by day 11 post challenge.

It was concluded that increasing the PVM dosage to a super-lethal level had had no effect on the ability of either the rAd5 or rAd19a PVM-N vectors to protect. Whilst not causing any change to the amount of bodyweight lost over the course of the experiment it did, however, increase the average clinical score of both groups suggesting that the increased dosage had an effect on the symptoms of the disease.

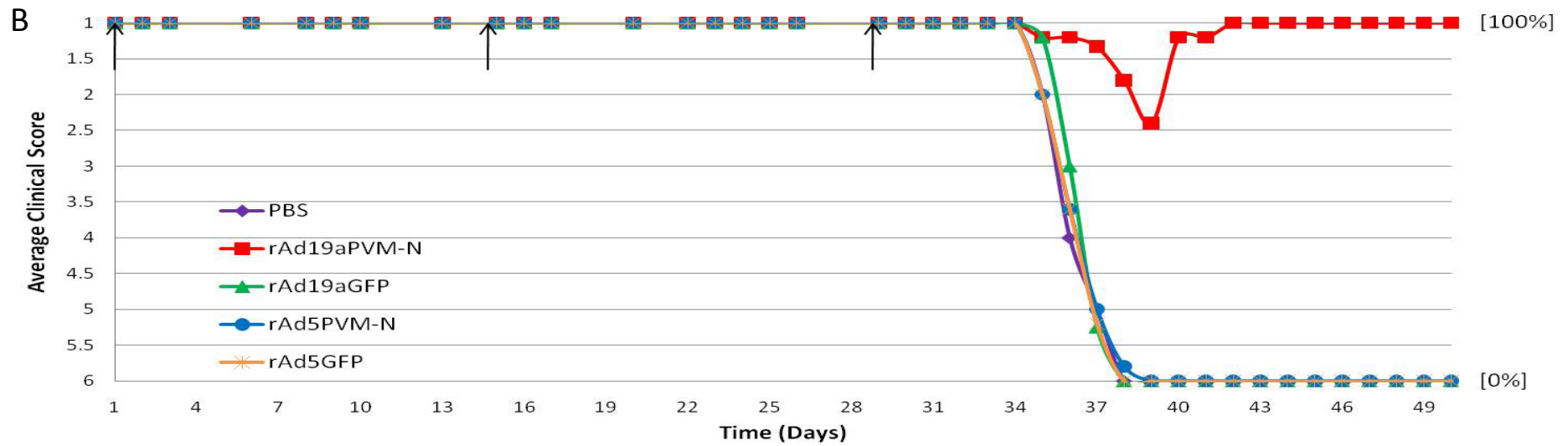
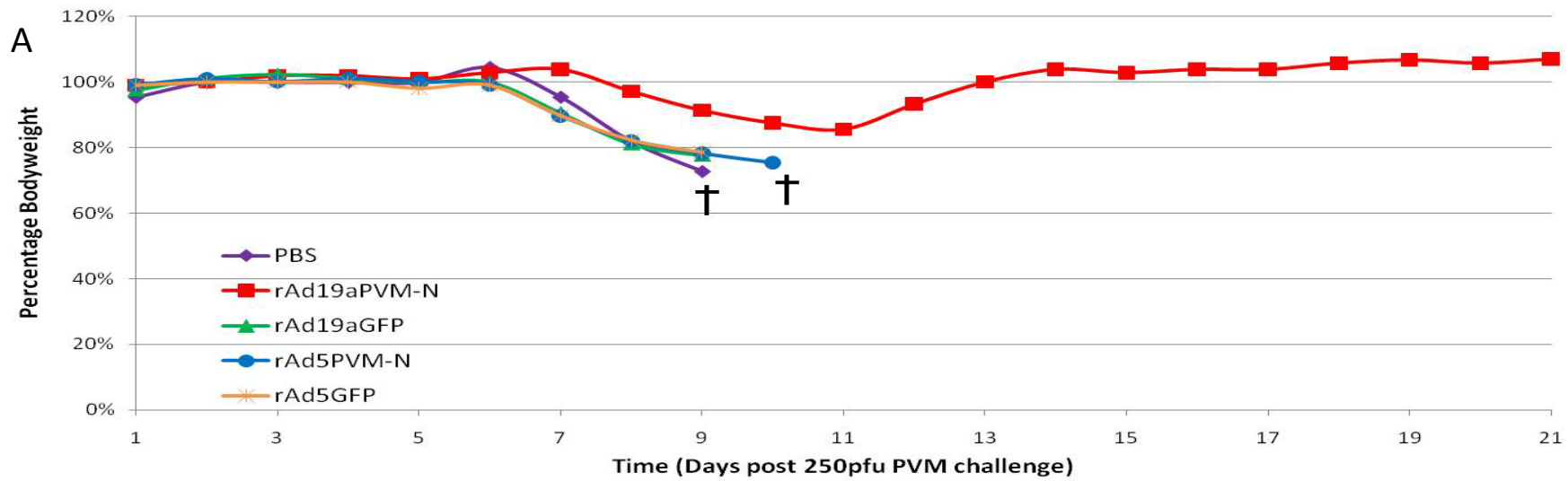
These results confirmed the earlier conclusion that rAd19aPVM-N induces an improved protective immune response when compared to rAd5PVM-N, however, increasing the challenge dose had failed to result in a difference in survival. It was therefore decided to lower the dose of the vectors ten-fold to observe if this had any effect on protection.

#### **7.4.3: rAd19PVM-N but not rAd5PVM-N protects mice against lethal challenge with 250pfu PVM at a $10^6$ intranasal dosage**

In the next experiment the dosage of each of the vectors was lowered to  $10^6$  pfu to examine if both the vectors were still capable of protection. The GFP vectors were included in the schedule for observation of any differences the drop in dosage may have on the controls. Results are shown in figure 7.6.

Only rAd19aPVM-N provided protection against lethal challenge with PVM at a  $10^6$  pfu dosage as shown by 100% survival at the end of the experiment. Mice inoculated with PBS or either of the GFP vectors lost ~21-27% of their bodyweight (Figure 7.6 A) by day 9 post challenge and this combined with their rapid rise in clinical score (Figure 7.6 B) resulted in their culling. In contrast to previous experiments, mice inoculated with rAd5PVM-N also lost ~25% of their bodyweight by day 10 post





**Figure 7.6: rAd19PVM-N but not rAd5PVM-N protects mice against lethal challenge with 250pfu PVM at a 10<sup>6</sup> intranasal dosage**  
Groups of 5 female BALB/c mice were intranasally administered 50µl of a prime dose and a boost dose exactly 14 days later of PBS or 10<sup>6</sup> pfu of either rAd19aPVM-N, rAd19aGFP, rAd5PVM-N or rAd5GFP followed by a 250pfu lethal dose of PVM 14 days after that. The mice were monitored for changes in bodyweight and clinical score and the figure shows (A) the percentage bodyweight of the surviving mice in relation to their bodyweight on day one post challenge (day of PVM challenge) for the subsequent 21 days and (B) the average clinical score of each mouse group over the 50 day course of the experiment. The numbers in square brackets show the number of surviving mice from each group at the end of the experiment. Arrows indicate timings of the prime dose, boost dose and PVM challenge in chronological order.

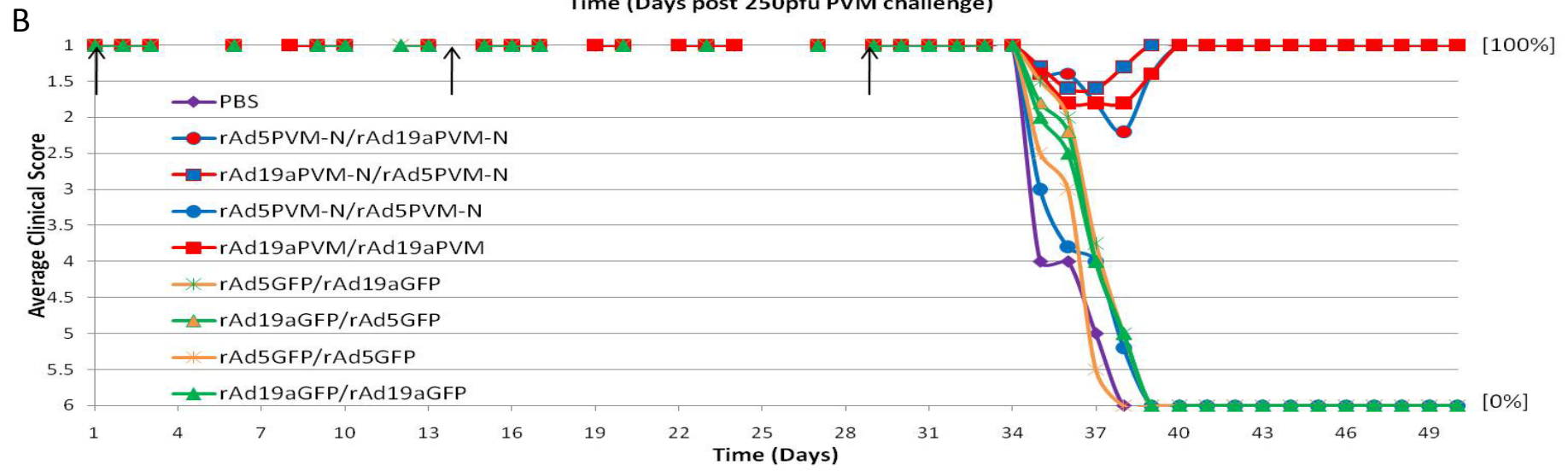
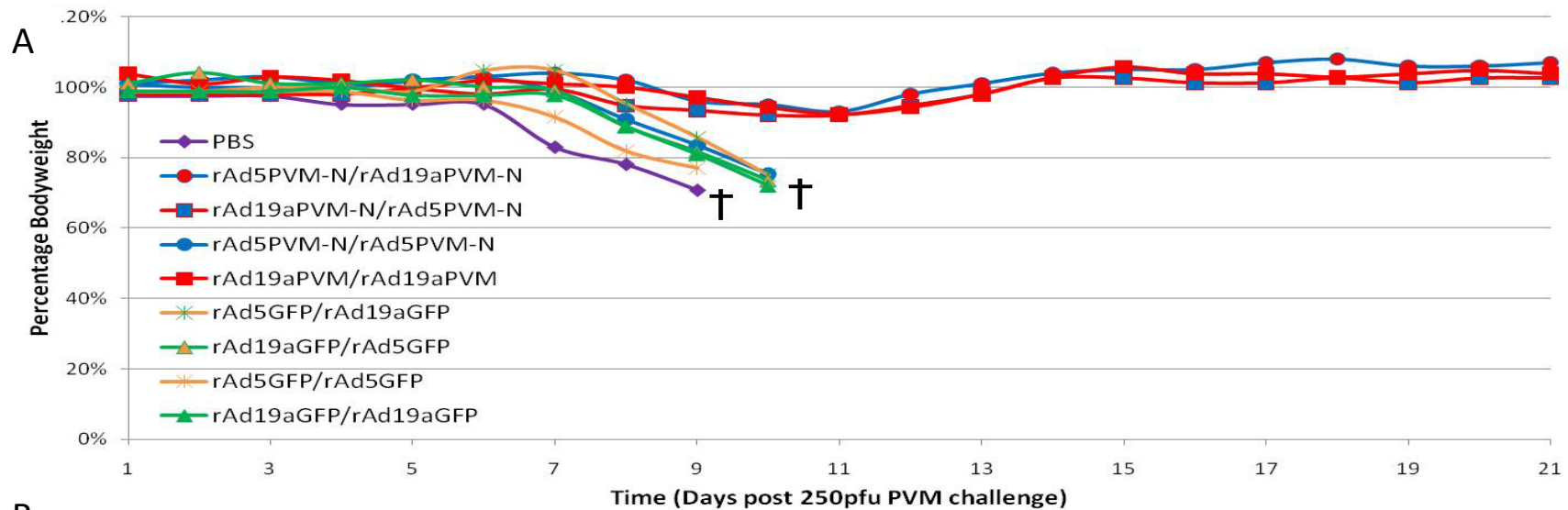
challenge and showed a similar swift rise in clinical score which resulted in their culling on the same day. Mice inoculated with rAd19aPVM-N lost 14% of their bodyweight by day 11 post challenge and reached a maximum average clinical score of 2.4 by day 10 post challenge. The mice quickly recovered, however, returning to an average clinical score of 1 by day 13 post challenge and recovering 100% of their original bodyweight on the same day.

It was concluded that decreasing the vector dosage had revealed a stark difference between the two vectors. Unexpectedly, as the ELISpot assays using the HIVA recombinants predicted Ad5 would be better than Ad19a at generating T cell responses and hence maybe protection,  $10^6$  pfu of rAd19aPVM-N was shown to protect mice against a lethal dose of PVM whilst a corresponding dose of Ad5 vector could not. It is not immediately obvious what the reason is for the different outcome, though there are several plausible scenarios. Firstly, the rAd19aPVM-N vector could be generating a larger T cell response than was witnessed for the rAd19aHIVA vector previously. Secondly, IFN- $\gamma$  may not be the best correlate of protection to observe for Ad19a vectors, particularly because the protection could be being mediated by another arm of the immune system, perhaps by an antibody response.

As rAd19aPVM-N had been shown to be superior in protection against PVM challenge to rAd5PVM-N at a  $10^6$  dose it was decided to examine if protection by rAd5PVM-N could be increased by mixing the two vectors in a heterologous prime/boost study.

#### **7.4.4: rAd19aPVM-N recovers rAd5PVM-N protection in a heterologous prime-boost study scheme**

In this experiment both the GFP and PVM-N versions of the rAd19a and rAd5 vectors were administered in different prime/boost combinations so that all possible configurations between the PVM vectors and the GFP vectors separately were administered. Results are shown in figure 7.7.



**Figure 7.7: rAd19aPVM-N recovers rAd5PVM-N protection in a heterologous prime-boost scheme against lethal challenge with 250pfu PVM at a 10<sup>6</sup> intranasal dosage** Groups of 5 female BALB/c mice were intranasally administered 50µl of a prime dose of PBS or 10<sup>6</sup> pfu of either rAd19aPVM-N, rAd5PVM-N, rAd5GFP or rAd19aGFP then the same groups given a boost dose of PBS, rAd5PVM-N, rAd19aPVM-N, rAd19aGFP and rAd5GFP exactly 14 days later followed by a 250pfu lethal dose of PVM 14 days after that. Control groups of mice were administered a prime and boost dose of the same vector, as previously. The mice were monitored for changes in bodyweight and clinical score and the figure shows (A) the percentage bodyweight of the surviving mice in relation to their bodyweight on day one post challenge (day of PVM challenge) for the subsequent 21 days and (B) the average clinical score of each mouse group over the 50 day course of the experiment. The numbers in square brackets show the number of surviving mice from each group at the end of the experiment. Arrows indicate timings of the prime dose, boost dose and PVM challenge in chronological order.

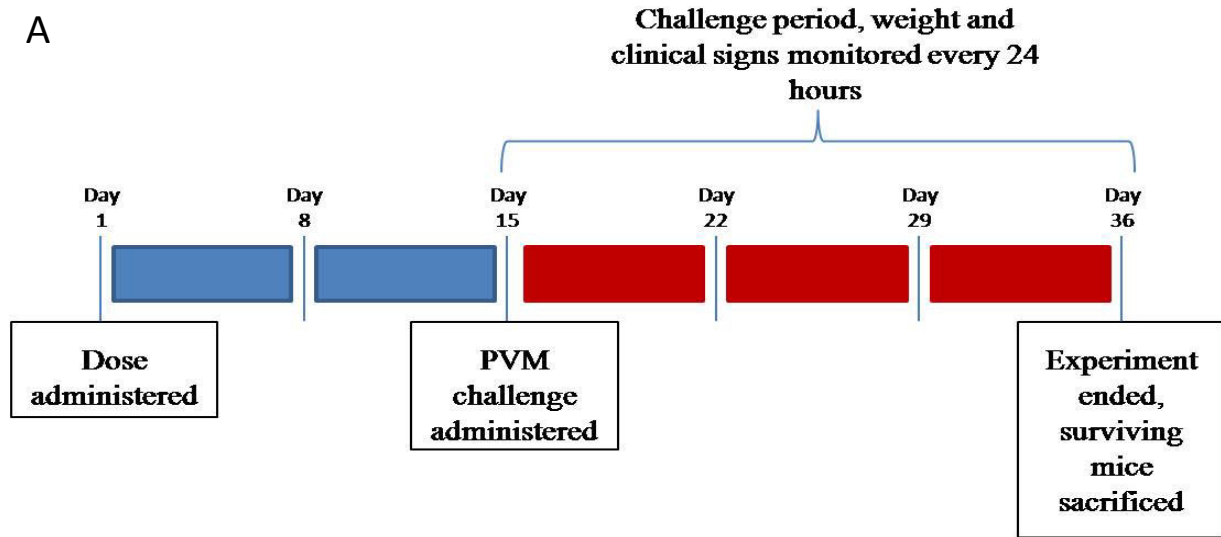
As before, an rAd19aPVM-N/rAd19aPVM-N prime/boost vaccination protected mice against lethal PVM challenge to a similar level as before whilst an rAd5PVM-N/rAd5PVM-N schedule did not. By day 9 or 10 post challenge, PBS controls and all GFP/GFP schedules lost ~25-29% of their original bodyweight (Figure 7.7 A) which, combined with the previously observed rapid rise in clinical score (Figure 7.7 B), resulted in all mice in these groups being culled by the day 10 post challenge. Interestingly, mice inoculated with rAd5PVM-N as a prime dose and rAd19aPVM-N as a boost dose or vice versa were protected against PVM challenge showing only a 7% and 8% reduction in bodyweight and rise in clinical score to 1.8 and 1.6 respectively. Even more interestingly mice inoculated with an rAd5PVM-N/rAd19aPVM-N schedule received a greater protective response (lower maximum clinical score) than the rAd19aPVM-N/rAd19aPVM-N control. Further experiments will need to be carried out to verify that this difference is significant.

It was concluded that mixing rAd19aPVM-N and rAd5PVM-N in heterologous prime/boost schedules allowed for protection against lethal PVM challenge when an rAd5PVM-N/rAd5PVM-N schedule did not. This was taken as early evidence that pre-existing immunity to the vector used to give the prime dose can have an effect on the efficacy of the boost dose and that Ad19a vectors may be used alongside Ad5 vectors in the future in prime/boost regimens to increase the generated immune response against a transgene. Alternatively, the successful protection in prime/boost studies could be explained by the fact that rAd19aPVM-N provides the same level of protection as the prime/boost schedules. This was investigated below.

#### **7.4.5: A single dose of rAd19aPVM-N but not rAd5PVM-N provides some protection for mice against lethal challenge with 250pfu PVM if challenged after 2 weeks but not after 4 weeks at a 10<sup>6</sup> intranasal dosage**

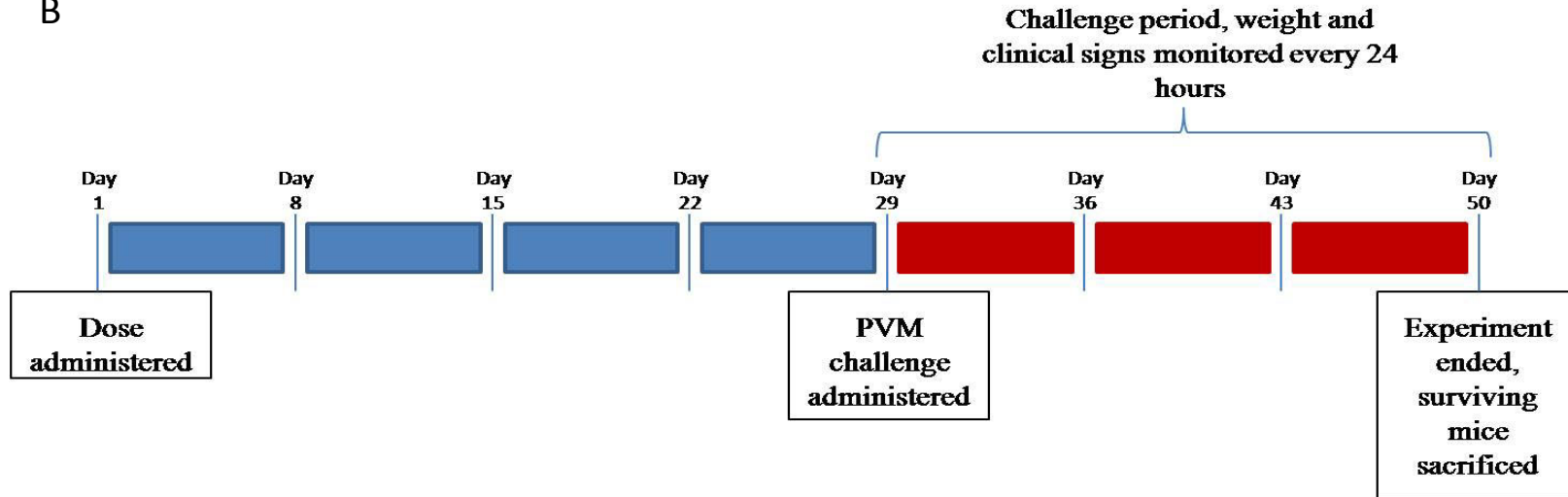
In the final *in vivo* murine experiment rAd19aPVM-N and rAd5PVM-N were administered as a single dose and then vaccinated mice were challenged either 2 or 4 weeks later (Figure 7.8) to establish if a single dose could have generated the protection seen in the previous experiments at a 10<sup>6</sup> vector dose.

A



**Figure 7.8: Single dose vaccination schedules** Vaccination schemes for A) Single dose 2 weeks and B) Single dose 4 weeks showing the day of vector dose followed by PVM challenge administration and the experiment end. The portion of the schedule where weight and clinical score were monitored every 24 hours for sign of disease is highlighted in red.

B

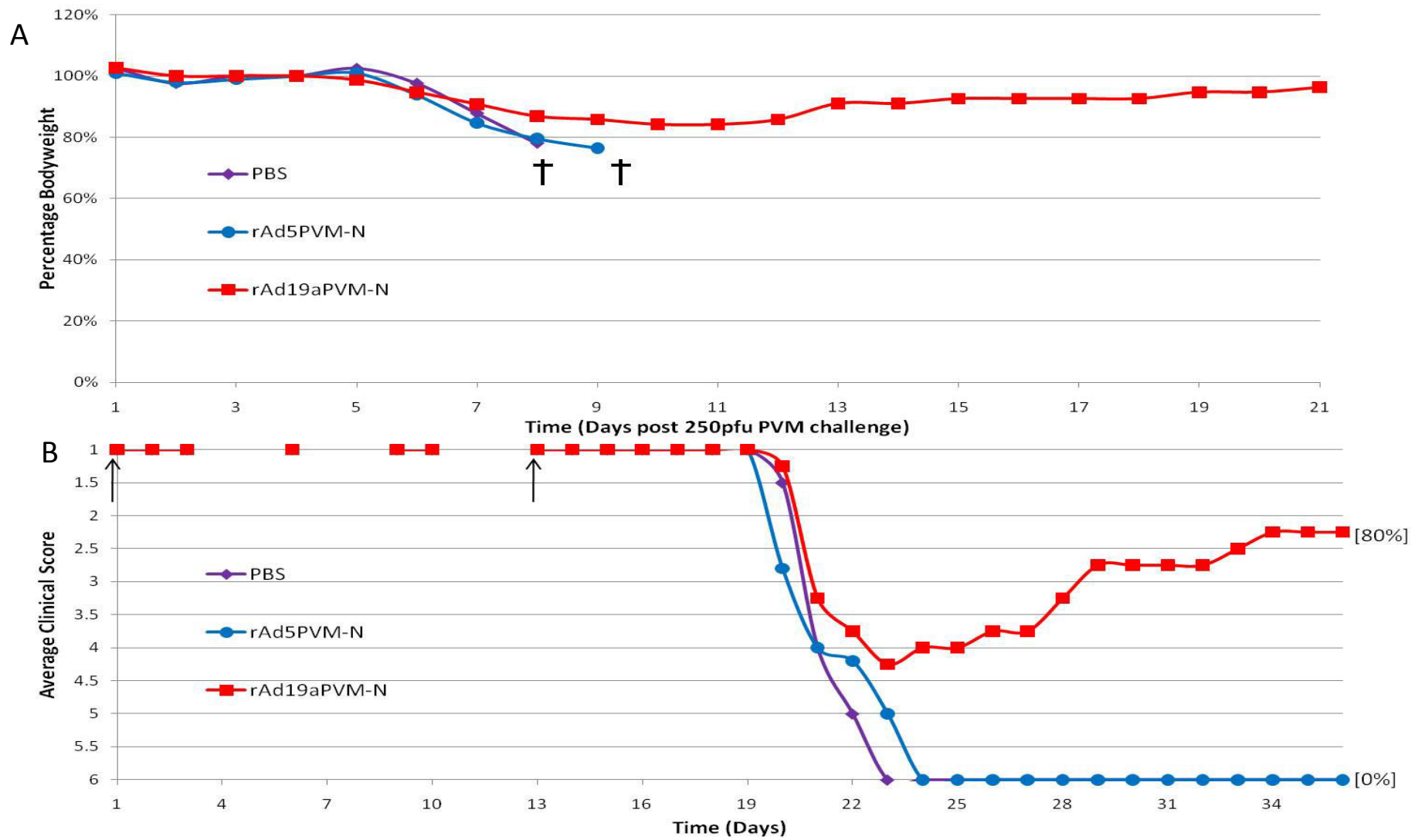


When mice were administered a single dose of either PBS or rAd5PVM-N and challenged with 250pfu PVM 14 days later they lost 22% and 23% of their original bodyweight (Figure 7.9 A) by days 9 and 10 post challenge, respectively, and once more their average clinical score rapidly rose (Figure 7.9 B) and both groups were culled on the same days. rAd19aPVM-N did display some protection, however only 80% of the mice survived with one mouse sacrificed on day 9 post challenge due to a clinical score of 5 being reached. The group lost 16% of their original bodyweight, higher than had been witnessed with an rAd19aPVM-N prime/boost schedule in previous experiments and their average clinical score rose as high as 4.25 on day 10 post challenge. It was concluded that whilst rAd19aPVM-N did protect most of the mice against PVM challenge after 14 days the clinical score rose significantly.

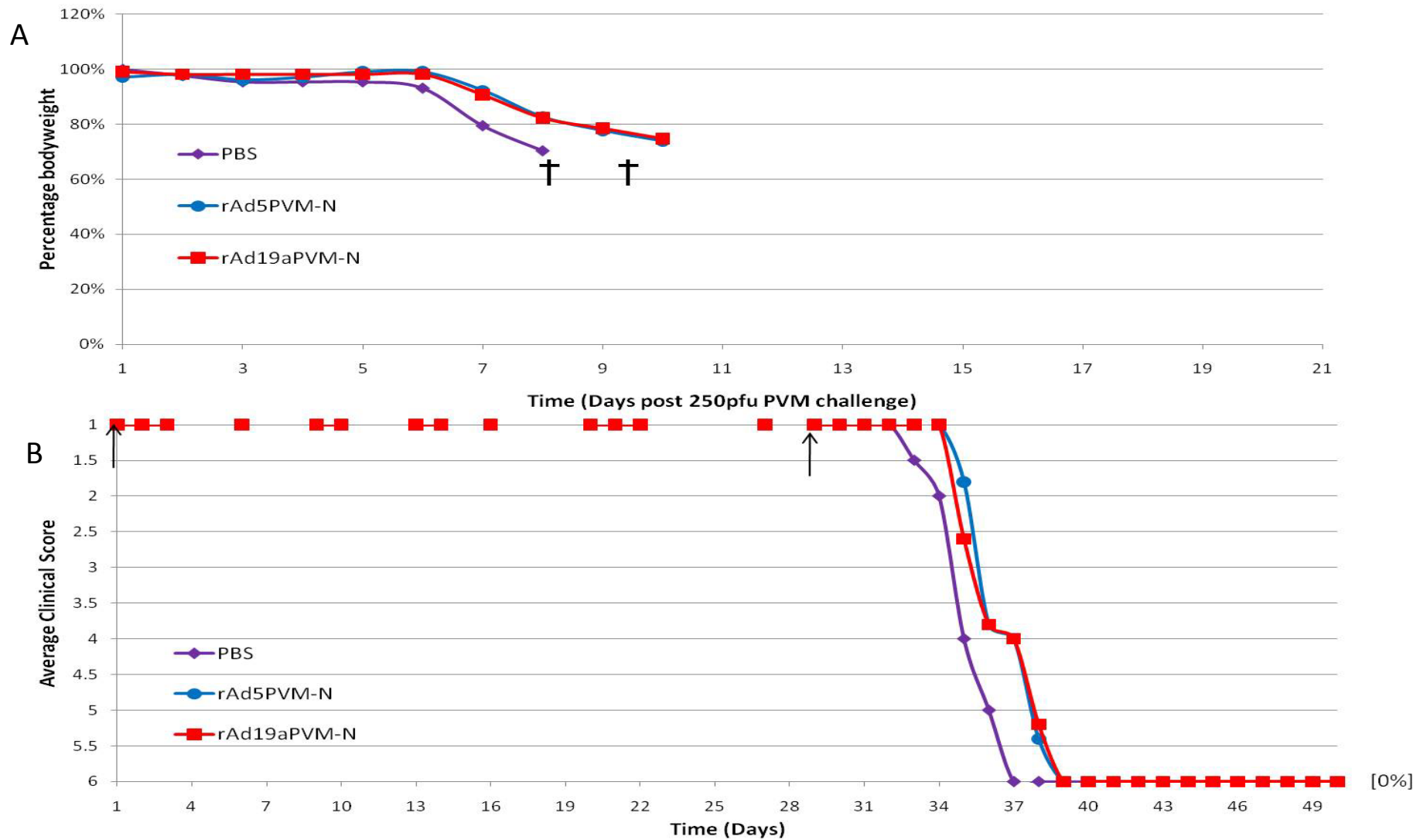
When mice were administered a single dose of either PBS, rAd5PVM-N or rAd19aPVM-N and challenged with 250pfu PVM 28 days later they lost 30%, 26% and 25% of their original bodyweight (Figure 7.10 A) by days 8 (PBS) or 10 (rAd5/19a) post challenge respectively and once more their average clinical score rapidly rose (Figure 7.9 B). The three groups were culled on day 8 (PBS only) or day 10. It was concluded that even rAd19aPVM-N could not protect mice against PVM challenge 28 days after a single  $10^6$  pfu dose.

It was further concluded that 100% rAd19aPVM-N protection at a  $10^6$  dose and prevention of a rise in average clinical score of more than 2 is dependent on both a prime and a boost dose being administered. The results suggest that protection after a single dose is short-lived as it has disappeared by 28 days after the dose, and a second dose is required to provide protection of all mice. What element of the immune system is primarily responsible for this protection is still unknown and it was decided to examine sera from the murine experiments for  $\alpha$ PVM-N IgG to establish if protection is IgG linked.





**Figure 7.9: rAd19PVM-N but not rAd5PVM-N provides some protection for mice against lethal challenge with 250pfu PVM at a 10<sup>6</sup> intranasal dosage if challenged 2 weeks after initial dose** Groups of 5 female BALB/c mice were intranasally administered a 50µl dose of PBS or 10<sup>6</sup> pfu of either rAd19aPVM-N or rAd5PVM-N followed by a 250pfu lethal dose of PVM 14 days after that. The mice were monitored for changes in bodyweight and clinical score and the figure shows (A) the percentage bodyweight of the surviving mice in relation to their bodyweight on day one post challenge (day of PVM challenge) for the subsequent 21 days and (B) the average clinical score of each mouse group over the 36 day course of the experiment. The numbers in square brackets show the number of surviving mice from each group at the end of the experiment. Arrows show the time of primary inoculation and PVM challenge chronologically

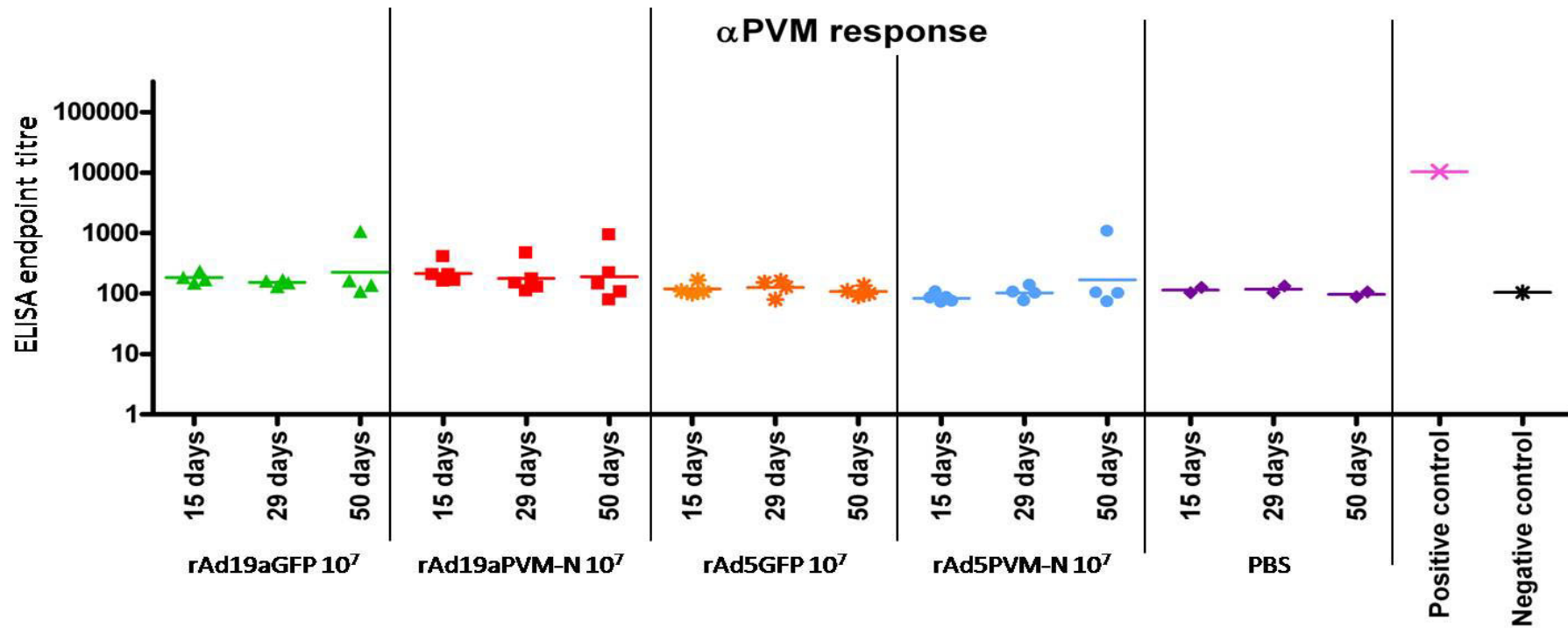


**Figure 7.10: Both rAd19PVM-N and rAd5PVM-N do not provide protection for mice against lethal challenge with 250pfu PVM at a 10<sup>6</sup> intranasal dosage if challenged 4 weeks after initial dose** Groups of 5 female BALB/c mice were intranasally administered a 50µl dose of PBS or 10<sup>6</sup> pfu of either rAd19aPVM-N or rAd5PVM-N followed by a 250pfu lethal dose of PVM 28 days after that. The mice were monitored for changes in bodyweight and clinical score and the figure shows (A) the percentage bodyweight of the surviving mice in relation to their bodyweight on day one post challenge (day of PVM challenge) for the subsequent 21 days and (B) the average clinical score of each mouse group over the 50 day course of the experiment. The numbers in square brackets show the number of surviving mice from each group at the end of the experiment. Arrows show the time of primary inoculation and PVM challenge chronologically

## 7.5: IgG response to rAdPVM-N vectors *in vivo*

In all experiments except those performed in chapter 7.4.5 and 7.4.6 tail sera (Figure 2.17.2) were taken from all surviving mice on days 15, 29 and the day of sacrifice, identified as day 50. Indirect ELISA analysis (Chapter 2.18) was then performed on the serum samples to test for an antibody response to the Ad vectors and the PVM-N transgene. Briefly, 96 well plates were coated with either 50  $\mu$ l/well of 20  $\mu$ g/ml BSC-1 or P2-2 lysate to assay for antibody response to PVM or 50  $\mu$ l/well of 1  $\mu$ g/ml purified Ad19a and Ad5 vectors to assay for antibody response to each vector and incubated O/N at 4°C. The plates were washed five times with 200  $\mu$ l/well PBS/Tween and blocked with 100  $\mu$ l/well 5% Milk powder in PBS/Tween before washing again. Sera samples were diluted 1:70 and diluted 3 fold across a 96 well plate. The serum antibodies were incubated with Horse Radish Peroxidase (HRP) conjugated goat anti-mouse IgG (whole molecule) or HRP conjugated goat anti-rabbit IgG (whole molecule). The presence of antibodies was quantified by the measurement of fluorogenic activity. Negative controls consisted of no primary antibody addition whilst  $\alpha$ PVM-N or  $\alpha$ Ad hexon antibodies were used as positive controls. Results were calculated as endpoint antibody dilution titres, i.e. the amount of dilution required of each sera sample for a negative ELISA response. This was performed by plotting the O.D.s of each serial dilution of sample on a scatter plot. An O.D. cut-off was then established (2 times the standard deviation of the mean of the negative control added to its' mean) and statistical software used to calculate at what  $\log_{10}$  dilution each sera sample and controls, both negative and positive, dropped below the cut-off giving a negative response. The resulting titres were then anti-logged and plotted on a vertical scatter plot. Endpoint titre means were subjected to an unpaired *t* test with Welch's correction (to correct for unequal variance) to compare vectors at each time point.

It was first investigated if the protection against 250pfu PVM challenge seen in the original protection study (Chapter 7.4.1) was linked to an  $\alpha$ PVM-N IgG response by monitoring the levels of such a response at each of the three time points. As can be seen in Figure 7.11 an  $\alpha$ PVM-N IgG response above the level of PBS immunised mice was rare and when it did occur it did not correlate with protection i.e. one mouse from the rAd19aGFP group had an IgG response above PBS background



**Figure 7.11: αPVM-N IgG responses induced by various vectors *in vivo***

Tail sera samples were removed from mice vaccinated with 10<sup>7</sup> pfu of either rAd19aGFP (Green triangles), rAd19aPVM-N (Red squares), rAd5GFP (Oranges asterisks), rAd5PVM-N (blue circles) and PBS (purple diamonds) at day 15, 29 or the day of sacrifice for each mouse (represented as day 50) in the standard vaccination schedule and examined for IgG response to PVM-N alongside a positive (αPVM-N mAb) and negative control.

levels similar to a single mouse in the rAd5PVM-N and rAd19aPVM-N groups however it was not protected. In addition, even after PVM challenge a general  $\alpha$ PVM-N IgG response was not seen, showing that such a response was not generated on wild-type virus challenge. To validate the observed results, the groups were statistically compared to each other at each time point and the results can be seen in Figure 7.12. As can be seen, a statistical difference between any of the PVM-N or GFP vectors and PBS IgG response was only seen on day 15 and only with the rAd19aPVM-N vector. At the successive two time points no statistical difference was observed between any of the vectors. There were statistical differences between some of the vector responses at both days 15 and 29, however, as these responses were largely negative they have no effect on conclusions.

Anti-Ad IgG responses which should be induced after vaccination were also measured. As expected, an Ad-specific IgG response was induced and this was non cross-reactive, as mice given an Ad19a vector only generated Abs that reacted against Ad19a but not Ad5 and vice versa (Figure 7.13). All mice vaccinated with PBS showed no anti-Ad19a or anti-Ad5 response at any time point. When compared statistically (Figure 7.14) a significant difference compared to PBS control mice was seen when mice were vaccinated with an Ad19a or Ad5 vector in anti-Ad19a or anti-Ad5 ELISAs respectively. Statistically significant differences between the response in rAd19aGFP and rAd19aPVM-N vaccinated mice were only seen on day 50 when the  $\alpha$ Ad19a response in the rAd19aPVM-N was significantly higher than the response in rAd19aGFP vaccinated mice perhaps due to the difference in particle/pfu ratio. Statistically significant differences were also seen, however, between the rAd5PVM-N and rAd5GFP responses at day 15 and 29, however, the rAd5PVM-N response was significantly higher than the rAd5GFP response on day 15 but on day 29 the reverse was true, suggesting that the response may be sensitive to minute differences in the dosage of the two vectors. The anti-Ad IgG response was monitored in all subsequent experiments, except those in Chapter 7.4.5 and 7.4.6, and very similar results were observed (data not shown) showing that in those experiments no errors were made with dosing, mice were only given either rAd19 or rAd5 vectors as intended in all cases.

15 days

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.0783	0.0320*	0.8411	0.1245
rAd19aGFP	0.0783		0.4211	0.0345*	0.0012**
rAd19aPVM-N	0.0320*	0.4211		0.0260*	0.0035**
rAd5GFP	0.8411	0.0345*	0.0260*		0.0503
rAd5PVM-N	0.1245	0.0012**	0.0035**	0.0503	

29 days

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.3055	0.2190	0.7366	0.5077
rAd19aGFP	0.3055		0.6023	0.3526	0.0399*
rAd19aPVM-N	0.2190	0.6023		0.3067	0.1074
rAd5GFP	0.7366	0.3526	0.3067		0.3377
rAd5PVM-N	0.5077	0.0399*	0.1074	0.3377	

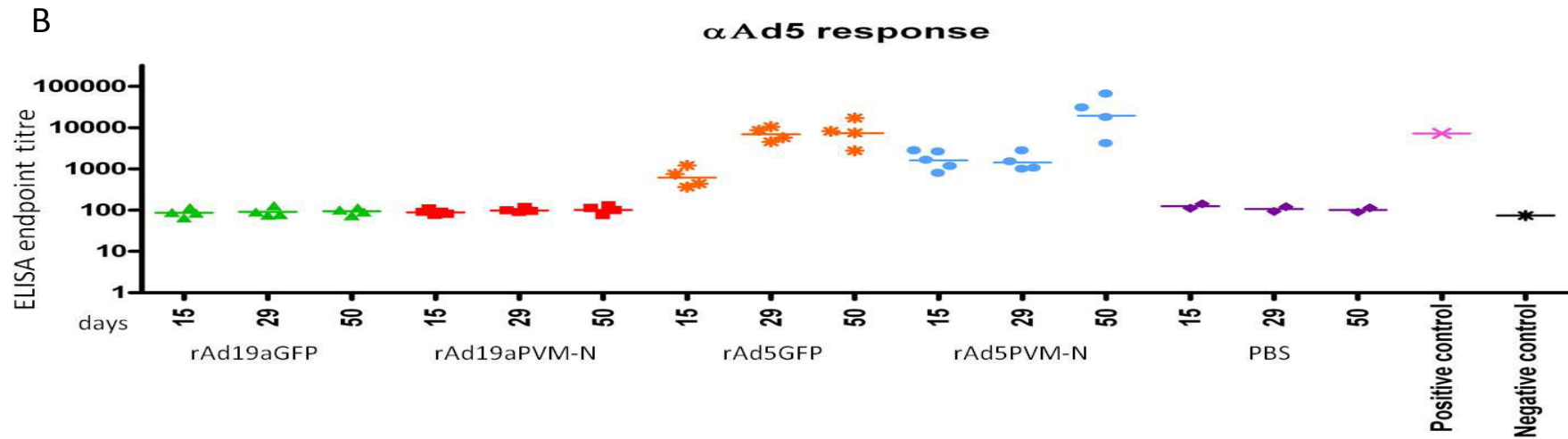
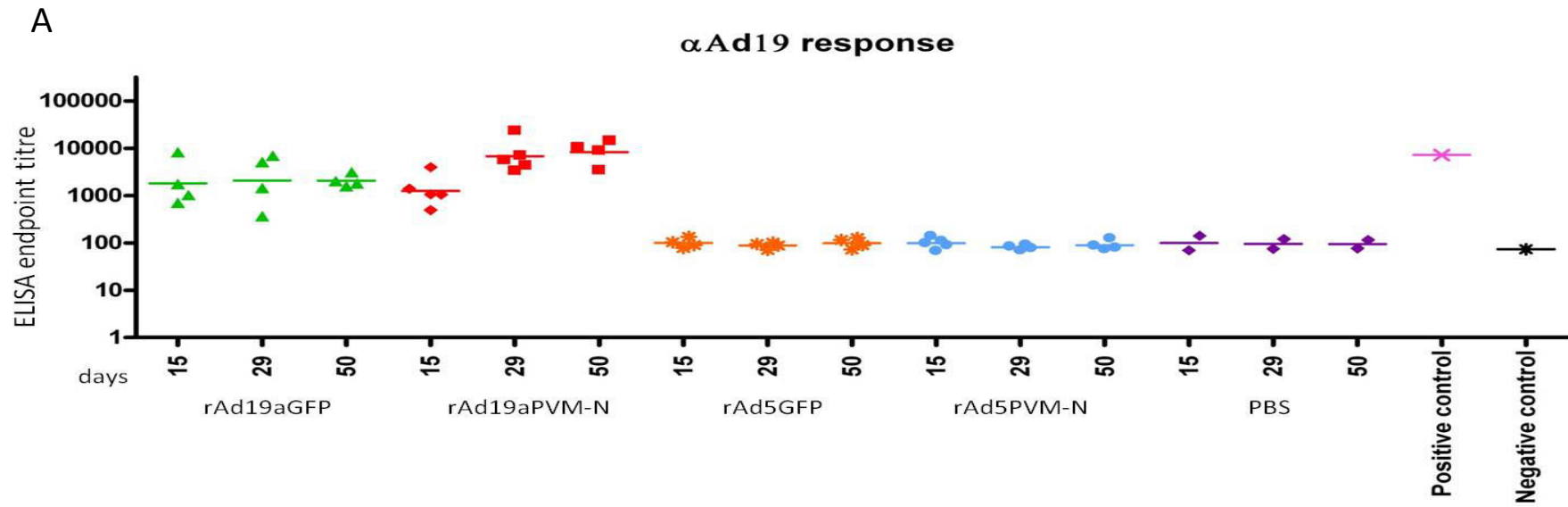
50 days

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.2107	0.2054	0.5073	0.4448
rAd19aGFP	0.2107		0.8048	0.2568	0.7308
rAd19aPVM-N	0.2054	0.8048		0.2689	0.8805
rAd5GFP	0.5073	0.2568	0.2689		0.5263
rAd5PVM-N	0.4448	0.7308	0.8805	0.5263	

**Figure 7.12: Statistical analysis of  $\alpha$ PVM-N IgG responses induced by various vectors *in vivo***

The means of  $\alpha$ PVM-N IgG responses to Ad vectors *in vivo* on day 15, 29 and 50 were compared by an unpaired t test with Welch's correction. Where statistical significance between two responses was shown, i.e. a P value of <0.05, it has been highlighted in green and the level of statistical significance given by either 1, 2 or 3 asterisks. If no statistical significance was detected it has been highlighted in red.





**Figure 7.13: Anti-Ad IgG responses to Ad vectors *in vivo***

Tail sera samples were collected from mice vaccinated with  $10^7$  pfu of either rAd19aGFP (green triangles), rAd19aPVM-N (red squares), rAd5GFP (orange asterisks), rAd5PVM-N (blue circles) and PBS (purple diamonds) at day 15 post vaccination, 29 or the day of sacrifice for each mouse (represented as day 50) in the standard vaccination schedule and examined for IgG response to (A) Ad19a or (B) Ad5 alongside a positive and negative control.

**A**

**Anti-Ad19a response**

15 days

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.0204*	0.0138*	0.9990	0.9857
rAd19aGFP	0.0204*		0.5981	0.0134*	0.0134*
rAd19aPVM-N	0.0138*	0.5981		0.0021**	0.0009***
rAd5GFP	0.9990	0.0134*	0.0021**		0.9645
rAd5PVM-N	0.9857	0.0134*	0.0009***	0.9645	

29 days

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.0228*	0.0005***	0.8063	0.6226
rAd19aGFP	0.0228*		0.1937	0.0185*	0.0170*
rAd19aPVM-N	0.0005***	0.1937		0.0002***	0.0002***
rAd5GFP	0.8063	0.0185*	0.0002***		0.4002
rAd5PVM-N	0.6226	0.0170*	0.0002***	0.4002	

50 days

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.0069**	0.0012**	0.8745	0.8856
rAd19aGFP	0.0069**		0.0151*	<0.0001***	<0.0001***
rAd19aPVM-N	0.0012**	0.0151*		0.0009***	0.0008***
rAd5GFP	0.8745	<0.0001***	0.0009***		0.6114
rAd5PVM-N	0.8856	<0.0001***	0.0008***	0.6114	

**B**

**Anti-Ad5 response**

15 days

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.1537	0.2305	0.0128*	0.0007***
rAd19aGFP	0.1537		0.8943	0.0027**	0.0001***
rAd19aPVM-N	0.2305	0.8943		0.0061**	0.0003***
rAd5GFP	0.0128*	0.0027**	0.0061**		0.0380*
rAd5PVM-N	0.0007***	0.0001***	0.0003***	0.0380*	

29 days

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.4696	0.6779	0.0004***	0.0024**
rAd19aGFP	0.4696		0.5889	<0.0001***	0.0005***
rAd19aPVM-N	0.6779	0.5889		0.0002***	0.0016**
rAd5GFP	0.0004***	<0.0001***	0.0002***		0.0036**
rAd5PVM-N	0.0024**	0.0005***	0.0016**	0.0036**	

50 days

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.6331	0.9849	0.0016**	0.0030**
rAd19aGFP	0.6331		0.6130	0.0015**	0.0029**
rAd19aPVM-N	0.9849	0.6130		0.0016**	0.0015**
rAd5GFP	0.0016**	0.0015**	0.0016**		0.6331
rAd5PVM-N	0.0030**	0.0029**	0.0015**	0.6331	

**Figure 7.14: Statistical analysis of  $\alpha$ Ad IgG responses to Ad vectors *in vivo***

The means of (A) anti-Ad19a and (B) anti-Ad5 IgG responses to Ad vectors *in vivo* on day 15, 29 and 50 were compared by an unpaired t test with Welch's correction. A difference between two responses was considered significant when the P value was <0.05. This is highlighted in green and the level of statistical significance given by either 1, 2 or 3 asterisks. If no statistical significance was detected it has been highlighted in red.

The  $\alpha$ PVM-N response as also monitored in the subsequent experiments and the results are shown in Figure A27 and A28. No significant difference in  $\alpha$ PVM-N IgG response over PBS controls was observed and so it was concluded that protection from lethal PVM challenge by either the rAd5PVM-N or rAd19aPVM-N vectors is not mediated by an IgG response which could be detected in this assay and as such it is likely mediated by another component of the immune response.

## 7.6: Discussion

In this chapter, we described for the first time the *in vivo* performance of two Ad19a based vectors in comparison to the well established Ad5 vector system. Whilst T cell responses against the incorporated HIVA antigen after a single I/M injection was less pronounced (as measured by IFN- $\gamma$  response to two peptides) than for the corresponding Ad5 vector, protection against a lethal dose of PVM, by an Ad19a vector incorporating a PVM antigen, was consistently more effective after I/N vaccination. Therefore, whether Ad19a vectors perform better than Ad5 vectors in an *in vivo* mouse model remains inconclusive and there are several possible interpretations of the data.

Firstly it is important to note that whilst transduction of human cells by Ad19a is superior over Ad5, in mice this is reversed in the case of mouse myoblasts (Thirion *et al.*, 2006). Thus, it is to be expected that I/M inoculation with Ad19a would not induce a more effective T cell response vs. Ad5. This was indeed the case (Figure 7.1). Varying the inoculation route did have an influence (Figure 7.2) and no difference in T cell response was seen at the same dose when the vectors were given I/P or I/D but in most cases there remained a small but significant improvement in T cell response with rAd5HIVA. It came as a surprise, therefore, that rAd19aPVM-N provided better protection against lethal PVM challenge than rAd5PVM-N when given I/N (Figure 7.5-7.7). The PVM challenge system provided an easy, clear readout (protection against PVM) to evaluate if the T cell response seen with the HIVA vectors correlated with protection in a surrogate system. In the experiments shown in Figures 7.3-7.10, when administered I/N, rAd19aPVM-N was shown to give increased protection against lethal PVM challenge over rAd5PVM-N at a  $10^7$  pfu dosage, continued to protect mice at a  $10^6$  pfu dosage whilst the Ad5 vector did

not and recovered rAd5PVM-N protection at a  $10^6$  pfu dosage when mixed in a prime/boost schedule. This data, combined with the data generated in Figure 7.2, that rAd5HIVA generated a significantly greater T cell response when given I/N would suggest, therefore, that PVM protection is mediated by another component of the immune response. It is certainly possible that IFN- $\gamma$  response is not the best correlate of protection for an Ad19a vector so it was consequently investigated if either of the vectors had generated an IgG antibody response to the PVM-N transgene *in vivo* and whether it was this antibody response which would correlate with protection. Neither of the vectors was shown, by an ELISA methodology, to generate an IgG response to the PVM-N transgene which was significantly greater than in mice inoculated with PBS. In those mice where a response was detected it did not necessarily correlate with protection. It is certainly plausible other immune components may be critical for the protection generated, perhaps IgA, but it is also possible that in the PVM setting the T cell response is different to the one generated by the HIVA vectors. Therefore an investigation by ELISpot into the T cell response to the PVM-N vectors is now vital for this work to be validated. It would also be useful to examine if an IgG response to the HIVA vectors is generated. There are several caveats to be taken into account when examining this data, however.

Firstly, there are differences between the two sets of vectors. In Chapter 4 it was shown that the rAd19aHIVA vector had a particularly poor particle/pfu ratio,  $\sim 25,000:1$ , when compared to the rAd19aPVM-N vector,  $\sim 6000:1$ . This difference may be as a result of the transgene and the further studies suggested in Chapter 4 may help to confirm this. In the meantime, a significantly higher particle/pfu ratio may suggest one reason why the rAd19aHIVA vector performed poorly against rAd5HIVA in the T cell experiments when rAd19aPVM-N generated a better protective response. Examining the IgG response to the Ad19a vector in the HIVA system may help to elucidate if the higher particle/pfu ratio generates a significantly higher immune response against the vector therefore preventing the generation of an anti-transgene response *in vivo*.

Secondly, the data suggests that inoculation route plays a role in the effectiveness of rAd vectors and that different transduction efficiency in different localised murine cell types could account for the observed differences in the resulting T cell response,

perhaps due to the lack of the Ad19a receptor, as discussed. Important information which could be gained by using the GFP vectors to examine the differences in cell transduction when administered by various inoculation routes in mice. The mice could then be sacrificed after 24-48 hours and their tissues examined for the presence of GFP fluorescence.

It must also be stated that there are differences between the methodologies used to generate the HIVA results and the PVM-N results. Firstly, they use different schedules with the mice immunised with the HIVA vectors only given a single dose and then examined 14 days later for their primary T cell response whilst in all cases except the final PVM-N experiment both a prime and boost dose were given. Also, in the experiment shown in Figures 7.9 and 7.10 it was shown that mice were only protected when given a single  $10^6$  dose of rAd19aPVM-N and that this protection was short-lived, as mice were only protected when challenged 2 weeks post inoculation and not after 4 weeks. Therefore the T cell response after a boost dose may be significantly different than those observed after a single dose in Figure 7.2. Secondly, both immunisation and the administration of the PVM lethal dose were given by the same I/N route and this may play an important role in the protection generated i.e. a theoretical localised innate immune response generated by the Ad19a vector due to its high particle/pfu ratio. It would be useful, therefore, to examine if protection can be generated by rAd19aPVM-N when administered by a different inoculation route.

Another caveat is that the Ad19a receptor may not be available in the mouse system such as CD46, shown in Chapter 6 to be a candidate receptor for Ad19a, which is only expressed in the testis in mice (Holers *et al.*, 1992). It would be important, therefore, to perform a thorough study on which of the other candidate Ad19a receptors, e.g. SA or GD1a, are expressed in mouse cells. To fully explore the effectiveness of any vector which may utilise hCD46, a non-human primate or a CD46 transgenic mouse model will be required (Sakurai *et al.*, 2008; Sakurai *et al.*, 2009). In contrast to hCD46, it has been shown that the expression of mouse CAR (mCAR) on CAR negative 3T3 cells confers susceptibility to Ad5 and Ad2 (Tomko *et al.*, 1997). Therefore, as a result of this, Ad5 and possibly Ad19a, also based on the results in Chapter 6, might be able to utilise mCAR if available *in vivo*, although

direct tests have not been carried out. It is also important to note that the transduction of mouse dendritic cells (moDC) has not been investigated and the effect of any similar enhanced transduction of an Ad19a vector seen in human DC's cannot therefore be reliably claimed until such an observation has been made. As has been previously discussed, DCs may use other uptake mechanisms for Ad vectors and this should also be taken into consideration. Overall, it can be assumed that the current system underestimates the potential of Ad19a vectors for vaccination in humans. However, the mouse system was valuable to evaluate toxicity and gave important early evidence into its potential in a mammalian system.

Continuing work in this area of experimentation should include the mixing of GFP vectors with the PVM-N vectors in heterologous prime/boost schedules like those in Chapter 7.4.4 to confirm that the GFP vectors do not also recover protection by rAd5PVM-N at a  $10^6$  dosage. It would also be beneficial to observe if the protection generated by a normal prime/boost rAd19aPVM-N scheme is as long-term as that generated by the rAd5 vectors perhaps by performing the prime/boost schedule as normal and then administering the lethal PVM dose after 2 weeks (as in the previous studies), 4 weeks, 8 weeks and 20 weeks and monitoring protection as previously described. Most importantly, it would be of great interest to observe the difference in protection when using hCD46 transgenic mice which have been used previously in studies with Ad35 vectors (Verhaagh *et al.*, 2006), when compared to the BALB/c mice used in this study. Similar to monkeys and humans, hCD46 transgenic mice express high-levels of hCD46 in their lungs and kidneys and might therefore represent a much better model for the use of Ad19a *in vivo* and would provide more significant data on the possible efficacy of Ad19a vectors in humans.

In conclusion, it has been shown that rAd19aPVM-N is capable of protecting mice against lethal challenge with PVM to a greater level than rAd5PVM-N despite mice not expressing one of the candidates for the Ad19a receptor (see Chapter 6), hCD46. Protection was shown not to correlate with an anti-PVM-N IgG response and might be assumed, therefore, to be generated by a T cell response but early data with the HIVA vectors in an ELISpot system cannot confirm this. In mice, Ad19a vectors provided a greater level of protection against a lethal challenge and were used in heterologous prime/boost studies to recover rAd5 protection which could be used in

humans to help circumvent pre-existing sero-positivity in prime/boost regimens  
Further work is now required in both animal model systems as well as cell culture  
with human cells to confirm that Ad19a vectors have real potential for vaccination.



## **Chapter 8: General Discussion**

### **8.1: The effectiveness of Ad19a as a vaccine vector**

Since their discovery in 1953 (Rowe *et al.*, 1953) Adenoviruses have played a crucial role in the characterisation of many important molecular processes, such as mRNA splicing (Berk, 2007). More recently they have emerged as extremely useful tools for the study of the interactions between viruses and the human immune system (Burgert *et al.*, 2002) as they produce several proteins with immuno-modulatory functions which may have a wide range of future applications (Burgert & Blusch, 2000). Moreover, Ads have been shown to possess many features that make them excellent candidates for their use as vectors for both gene therapy and vaccination, being non-integrative, transducing a wide range of cell types and establishing high levels of gene expression from expression cassettes.

The majority of Ad vectors to date are based on the Ad5 serotype which has been shown to have several drawbacks including high seropositivity in the human population and liver tropism. More recently, there has been an increased focus on the use of Ads other than Ad5 to evaluate if these can overcome such deficiencies.

This study has described the successful establishment of a methodology for the seamless modification of any Ad genome which was then validated by its utilisation to create several Ad vectors based on Ad5 and Ad19a and virus mutants (Chapter 3). Subsequent work concentrated on the assessment of some intrinsic biological features of the novel vector from subgroup D, Ad19a, and its potential as a vaccine vector.

The prototype Ad19 virus (Ad19p) was first identified in 1955 when isolated from a patient with trachoma which, subsequently, was shown to be caused by a bacterial infection. (Bell *et al.*, 1959; 1960). In 1978 an Ad was isolated from patients suffering from EKC that reacted with the Ad19 serum but had a different restriction pattern (Wadell & de Jong, 1980). This virus obviously was related to Ad19p but was distinct and was thus named Ad19a. Interestingly in all subsequent outbreaks of EKC linked to Ad19 it was this genotype which was isolated rather than Ad19p.

Ad19a has since become interesting for development as a vaccine vector. It has been shown to have a high efficiency to infect DCs, an important target for vaccination (Ruzsics *et al.*, 2006) and this finding, combined with data in T and B cells, indicated an interesting tropism for cells of the immune system. Standard cloning methods (utilising either high or low copy plasmids) did not allow cloning of the genome. Only when the genome was inserted into a BAC vector could stable clones be isolated and, as such a new technology for genetic manipulation was required. A basic non-replicating Ad19a vector was generated by deletion of the E1 region and replacing it with an expression cassette under the control of the CMV immediate-early promoter and the SV40 enhancer (Ruzsics *et al.*, 2006). In addition, 4.5kb of the E3 region was deleted.

In this study it was aimed to take these early findings further by establishing if Ad19a has potential for vaccination based on the infection efficiency of DCs. To this end a modified recombineering system based on a novel bacterial strain, SW102 (Warming *et al.*, 2005) and selection cassette (*galK/Kn<sup>R</sup>*; Chapter 2.17.1) was established in this laboratory for the insertion of vaccine model antigens into the Ad19a vector. These antigens, along with the existing GFP-expressing vector could then be compared to homologous Ad5 vectors for their ability to transduce various cell types, to induce an anti-transgene immune response and ultimately to protect mice against lethal challenge.

Remarkably, all Ad19a vectors tested showed the same high level of transduction of B cells, Jurkat T cells and dendritic cells (Chapter 5.3; 5.5) as was observed for the Ad19a virus (Ruzsics *et al.*, 2006; Burgert unpublished data). This transduction was significantly higher than the observed levels with the corresponding Ad5 vectors and similar to the levels reported for Ad35 (Rea *et al.*, 2001) and Ad11 (Holterman *et al.*, 2004). Ad19a vectors were shown to possess drastically higher relative specificity for DC transduction than Ad5 vectors, induced high levels of transgene expression in iDCs and mDCs and induced DC maturation as shown previously with vectors based on Ad35 (Rea *et al.*, 2001). Using 10 pfu/cell of Ad19aGFP (estimated ~15,000 virus particles/cell based on particle/pfu ratio) 90% of iDCs were transduced. This seems to be in a similar range or better than Ad35 (~40% iDC transduction at 5,000

virus particles/cell; Vogels *et al.*, 2003) and Ad11 (74.3% iDC transduction at 500 virus particles/cell; Holterman *et al.*, 2004). The transduction of other DC subsets such as CD11<sup>+</sup> myeloid dendritic cells (MyDCs) and CD123<sup>+</sup> plasmacytoid dendritic cells (Lore *et al.*, 2007) has not been eliminated and could be examined in further experiments. It is now crucial to gain quantitative data on the potential of Ad19a transduced DCs to activate specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells, in parallel with Ad19a, with other comparable vectors *in vitro*, such as Ad35 (Rea *et al.*, 2001). This work is being carried out in the Hanke lab (Oxford) using the HIVA Ad19a vector as in previous studies utilising CD8<sup>+</sup> and CD4<sup>+</sup> T cells specific for the epitopes of the HIVA transgene (Barouch, 2008). Another aspect that may be pursued in future is whether DC subtypes differ in their susceptibility to Ad19a. As previously mentioned, (Chapter 1.5.1) certain DC subsets such as LCs have been shown to express a cell receptor repertoire that makes them more susceptible for viral rather than bacterial uptake (Flacher *et al.*, 2006; van der Aar *et al.*, 2007) and are more effective activators of CD8<sup>+</sup> T cells (Ueno *et al.*, 2007). Whilst investigation of the effect of Ad19a mediated antigen expression in DCs is currently ongoing, the level of transgene expression was examined. It was found that Ad19a vectors expressed 4-7x higher amounts of transgene compared to Ad5 vectors in A549 cells (Chapter 4.4.1; 4.4.2). This finding gives further weight to the use of Ad19a as a vaccine vector as theoretically a lower dose would be required to generate the same level of transgene expression *in vivo* whilst simultaneously helping to prevent toxicity issues by the use of less vector.

The high level of transgene expression from Ad19a vectors remains unexplained and further studies will be required to elucidate whether it is linked to an intrinsic part of Ad19a biology. These results must be tempered by the finding that all generated Ad19a viruses and vectors had varying, but high particle/pfu ratios (Chapter 4.1). This was not unexpected; isolates of Ad19a have been shown to have large and varied particle/plaque forming unit ratios previously (Newland & Cooney, 1978). This finding may have significant implications for the use of an Ad19a vector *in vivo*. Whilst the Ad19a dosage could be lowered due to its high level of transgene expression, its particle/pfu ratio may actually result in the application of a greater number of virus particles than a significantly higher dose of Ad5. This may result in a strong innate immune response, which in some cases may be detrimental to the

health of the host or even fatal. In our *in vivo* experiments in mice (Chapter 7) we did not detect any toxicity caused by an Ad19a vector at doses of up to  $\sim 2.5 \times 10^{11}$  particles using the Ad19aHIVA vector at  $1 \times 10^7$  pfu. This does not preclude any toxicity, however, as the highest doses were given intranasally or intramuscular. It would now be important to perform dose escalating studies after intravenous infection because large doses of vector particles ( $1 \times 10^{11}$ ) from Ad3, 4 and 11 were shown to be fatally toxic when given intravenously in CD46-transgenic mice (Stone *et al.*, 2007). Therefore, before Ad19a can be used safely in humans further toxicity studies must be employed, especially in intravenous settings, and/or the reason for the high particle/pfu ratio must be discovered and/or rectified (discussed in Chapter 4.6). It has not been established, in this study, what causes the high particle/pfu ratio in Ad19a vectors. It is possible that the ratio is due to the intrinsic biology of Ad19a whilst also being possible that it is caused by the procedure used to purify the vector. It may be that this affects vectors more than the wild-type virus, perhaps because of the deletion of the E1 region. It could also be procedural as no-one has fully optimised the procedure for the purification of Ads. Downstream procedures could also be optimised by the use of electron microscopy to evaluate Ad19a preps (see Chapter 4).

It was hypothesised that the high particle/pfu ratio of Ad19a may have had an effect on the potential of Ad19a in a vaccination setting; however, it has been shown that this is not the case, although, some of the evidence gathered has been contradictory. For example, the HIVA transgene expressing Ad19a vector was shown, by ELISpot, to induce a significantly lower HIVA epitope specific CTL response when compared to a homologous Ad5 vector when administered at a range of dosages (Chapter 7.3.1). In contrast, the Ad19a vector expressing the nucleocapsid gene of PVM was shown to protect mice against lethal challenge with PVM at lower dosages than the corresponding Ad5 vector and was able to recover Ad5 protection at low dosages in heterologous prime/boost studies (Chapter 7.4). These findings could be linked to the very high particle number of the Ad19aHIVA vector which is  $\sim 4$ x higher than that of the Ad19aPVM-N vector. This could affect the effectiveness of the vector *in vivo* by focussing an immune response to the vector particles rather than the expressed antigen; however, the data suggests that inoculation route and vaccination schedule used may have a greater effect.

Firstly, it was shown that when administered through inoculation routes other than intramuscular or intranasal there was no significant difference in the CD8<sup>+</sup> T cell response generated by the Ad19a and Ad5HIVA vectors (Chapter 7.3.2) although no immunisation route gave a robust response after Ad19a administration. It should also be noted that both the inoculations and subsequent challenge in the PVM studies were given intranasally, therefore, the better protection seen with the Ad19a vector could, principally, be due to a localised immune response specific to Ad19a being detrimental to PVM replication in the lung tissue, although 14 days is a longer time period than is normally recorded for an innate immune response. A further experiment would be the evaluation of the ability of the Ad19a vector to induce protection against PVM compared to the Ad5 vector when administered by an alternative inoculation route.

Secondly, and perhaps more importantly, in the ELISpot studies utilising the HIVA transgene, the mice were only inoculated once before sacrifice 14 days later whereas in the PVM protection studies the mice were given both a prime and boost dose before sacrifice of the surviving mice 21 days later. These schedules are, therefore, vastly different both temporally, in terms of the time at which the response was evaluated after inoculation and, more importantly, procedurally as two separated doses were given. A prime/boost dose schedule is likely to induce a far stronger anti-transgene immune response due to the stimulation of immune memory established after the prime dose at the time of boost. However, in contradiction to this hypothesis, it was found that Ad19aPVM-N could protect mice against lethal challenge with PVM after a single prime dose if challenged 14 days later, in the same scenario as used in the HIVA ELISpot studies but it could not if challenged 28 days later (Chapter 7.4.5). These findings suggested that the immune response after a single dose vaccination is sufficiently high for protection to occur in the first 14 days; but is not effective after 28 days. As no reagents to examine CD8<sup>+</sup> T cell responses to PVM were available we concentrated on the IgG anti-transgene response using an indirect ELISA system. No significant difference in  $\alpha$ PVM-N IgG response over PBS controls was observed and so it was concluded that protection from lethal PVM challenge by either the rAd5PVM-N or rAd19aPVM-N vectors is not mediated by an IgG response which could be detected in this assay (Chapter 7.5).

There remain other arms of the immune system that could be investigated, but it should first be examined if the CD8<sup>+</sup> T cell response induced by the rAd19aPVM-N vector is higher than the response induced by the Ad19aHIVA vector. It is also important to note that the ELISpot assays in this study only measured a single parameter of effector T cells, IFN- $\gamma$ , and this may not be relevant. It is plausible that Ad19a induces T cells that produce more cytokines, such as IL-2 or hTNF- $\alpha$  and as such further ELISpot studies should evaluate other cytokines or the protocol could be changed to a killing assay to measure directly how effective the generated CD8<sup>+</sup> T cells are at killing Ad19a and Ad5 vector transduced cells.

Thirdly it must be noted that all of these studies have been performed in mice which may or may not express the equivalent of the human Ad19a receptor. For example, while mice express no homologue of hCD46 they do possess a homologue of hCAR (Tomko *et al.*, 2007). The Ad19a receptor remains controversial. The fibre of Ad19a, the protein responsible for cell targeting is identical to the fibre of another EKC causing Ad, Ad37 (Arnberg *et al.*, 2002). For this reason it has been assumed that Ad19a may use the same attachment receptor as Ad37. Ad37 has been shown to utilise hCAR (Seiradake *et al.*, 2006), sialic acid (Arnberg *et al.*, 2000<sup>a</sup>; 2000<sup>b</sup> 2002; Johansson *et al.*, 2005; 2007; Thirion *et al.*, 2006), hCD46 (Wu *et al.*, 2001, 2004; Hsu *et al.*, 2005) and other newly identified molecules such as GD1a (Arnberg, unpublished data). In this study, it was investigated which of these potential receptors is used by an Ad19aGFP vector *in vitro* by expressing each receptor on a non-human cell line to eliminate concurrent interactions with other human receptors. In our findings Ad19a was capable of utilising both hCAR and hCD46 for entry when expressed in non-human CHO cell lines, but not sialic acid or GD1a (Chapter 6.4), a finding which does not correlate with the majority of the findings for Ad37. Blocking experiments using monoclonal and polyclonal antibodies and soluble forms of the receptor pre-incubated with the vector, resulted in a reduction in transduction corresponding to control vectors that used hCAR and hCD46 (Chapter 6.5). They did not, however, have such a significant effect on the ability of Ad19a to transduce human A549 cells, suggesting that whilst Ad19a can utilise hCD46 or hCAR they alone are not responsible for Ad19a uptake in human cell lines. These experiments require further repetition for this conclusion to be fully validated but they represent a good working hypothesis.

It is not immediately obvious what differences in the experimental set-up could cause the discrepancy between the results for Ad19a and Ad37. It is especially strange as they possess identical fibre proteins. One plausible hypothesis would be that their divergent hexon and penton proteins affect tropism either in combination with fibre protein or by interacting directly with a cell receptor independent of the fibre protein. This hypothesis is in line with the finding that structural elements apart from the fibre protein itself can be responsible for changing tropism, as has been seen with the interaction of FX with the Ad5 hexon protein (Waddington *et al.*, 2008). Thus, future studies on Ad receptors should be performed using the native structure of the complete wt virion itself, as this represents the interactions *in vivo*, rather than binding assays with soluble or fibre knobs alone or Ad5 pseudotyped with the fibre proteins of different serotypes. It has also become apparent that Ads may be promiscuous in their use of receptors, with new receptor molecules being proposed all the time (reviewed in Arnberg, 2009) and as such it may be time to abandon the concept of a ‘primary’ receptor for each serotypes’ fibre protein and focus instead on Ad targeting and sequestration *in vivo* in humanised mice as a model for actual cell tropism. These findings may have important consequences for the evaluation of Ad19a’s effectiveness as a vaccine vector *in vivo*. For example, the largest body of research on the use of an Ad serotype outside of Ad5 has been on the subgroup B Ad, Ad35 which requires the use of hCD46 as a cell receptor (Gaggar *et al.*, 2003) and only with the use of CD46 transgenic mice (Verhaagh *et al.*, 2006) or NHPs (Sakurai *et al.*, 2008, 2009) has more relevant information for this serotype been gathered. A very recent study has shown that in hCD46 transgenic mice hCD46 is expressed on bone marrow derived mouse dendritic cells, as is the case in humans and this up-regulated rAd35 DC transduction in mice from ~25% to ~75% at 3,000 virus particles/cell and resulted in higher levels of serum interleukins than an Ad5 or Ad5F35 vector (Sakurai *et al.*, 2010). For this reason it is now important for Ad19a vectors to be evaluated in such models, as they might be clinically more relevant if hCD46 usage can be confirmed by other experimental approaches (siRNA).

## 8.2: Further work

This study has shown that Ad19a vectors may have advantages over Ad5 vectors for vaccination but it would be prudent to directly compare Ad19a with vectors based on Ad serotypes other than Ad5, such as Ad35, to address whether Ad19a vectors have any advantages over these. Studies such as those performed by Shott *et al.* in 2008 using Ad5 and Ad35 could be repeated to include an Ad19a vector expressing the same transgene to evaluate if Ad19a is capable of inducing a similar or stronger immune response than an Ad35 vector. Ad19a may have some advantages over Ad35, i.e. rAd19aGFP expresses more transgene (up to 8,043 MFI at 10 pfu/cell) than rAd35GFP (~3,500 MFI in iDCs after  $10^9$  virus particle injection into skin explants; de Gruijl *et al.*, 2006) in iDCs, although these experiments are not directly comparable due to the nature of the *in vitro* protocol and other literature does not evaluate transgene expression levels. Whilst it has been shown that Ad35 has a lower human seropositivity than Ad19a (~5% compared to ~20% for Ad19a/Ad19p; Vogels *et al.*, 2003), several other findings are very similar with Ad19a transducing iDCs, mDCs, T cells and B cells to similar or higher levels, although at incomparable infection efficiencies whilst being shown to have some properties which have not been evaluated in Ad35, such as the ability to protect non CD46-transgenic mice against lethal virus challenge.

If experiments confirm that Ad19a is a similarly effective or even better vector compared to other Ad vectors then its use could be established alongside these vectors in heterologous prime/boost systems such as those used previously (Rodriguez *et al.*, 2009) eliminating the requirement for an Ad5 vector and consequently the obstacles such as Ad5's high seropositivity and liver tropism.

Comparisons to other vector systems could also be investigated, especially using the HIVA transgene which has already been evaluated for CD8+ T cell generation by ELISpot in other vector systems such as MVA (Hanke *et al.*, 2002), semliki forest virus (alphavirus; Hanke *et al.*, 2003), bluetongue virus (Larke *et al.*, 2005), Bacillus Calmette-Guérin (BCG; <sup>a</sup>Rosario *et al.*, 2010), ovine atadenovirus (Bridgeman *et al.*, 2009; <sup>b</sup>Rosario *et al.*, 2010) and Ad5 (Bridgeman *et al.*, 2009). Ad19a may have



advantages over all of these vector systems due to its high transgene expression and efficiency of transduction of DCs. It is especially important that any comparisons are carried out in a human-like system, especially as MVA vectored HIVA vaccines have shown promising results *in vivo* in humans (see Chapter 1).

Before comparison studies can begin it is of vital importance that further immunogenicity studies are completed using the existing Ad19a HIVA and PVM-N vectors to establish the cause of the enhanced protection over Ad5 seen when using rAd19aPVM-N in the PVM challenge system and whether Ad19a is stimulating a different type of immune response by the evaluation of multiple cytokines in the HIVA system. It is especially important that further immunogenicity studies are carried out in more human-like *in vivo* systems such as CD46 transgenic mice and that the Ad19a receptor is further elucidated *in vitro* and *in vivo*. Parallel studies *in vivo* using KO mice that lack different gangliosides (GD1a etc.) would also be helpful to further promote the case of Ad19a as a vaccine vector.

### **8.3: Conclusions and Outlook**

This study has highlighted that whilst animal models are useful for the evaluation of the potential of Ad vectors for vaccination they have several flaws. Within this study alone, conflicting data has been generated based on two separate mouse models, although this could be explained by other causes as discussed. Any *in vivo* toxicity or efficacy studies performed in animal models which are not transgenic for the presence of the normal human receptor panels are not, therefore, good models for the effects of that vector in humans and any *in vitro* study using human cells is not informative of transduction profiles or sequestration in the *in vivo* host environment. The failure of the MERCK STEP trial (McElrath *et al.*, 2008) has called into question the validity of any efficacy data gathered in non-human models and further suggests that more early experimentation should take place in humans to truly evaluate the potential of any viral vector system.

In conclusion the Ad19a genome has been shown to be easily modified by a novel recombineering system allowing the seamless mutation of ORFs and the creation of

transgene expressing vectors. Ad19a vectors have been shown to transduce a greater range of human cells, including several immune lineage cell types, B cells, T cells and DCs, as has been seen previously for the wt virus (Ruzsics *et al.*, 2006; Burgert, unpublished data), and express greater amounts of transgene upon transduction when compared to Ad5 vectors. Finally, Ad19a vectors have been utilised in vaccine model systems and have been shown, in one model at least, to provide a greater level of protection against lethal challenge than a homologous Ad5 vector presumably by the stimulation of a greater cell mediated immune response. Some aspects of Ad19a biology require further evaluation before the vector could enter clinical trials but this study has highlighted Ad19a's potential and provided a solid basis for its continuing development as a vaccine vector.

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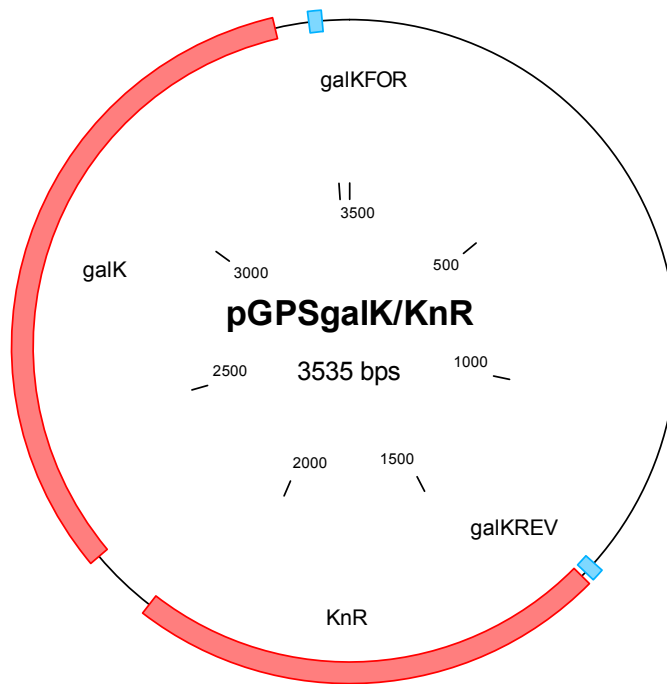
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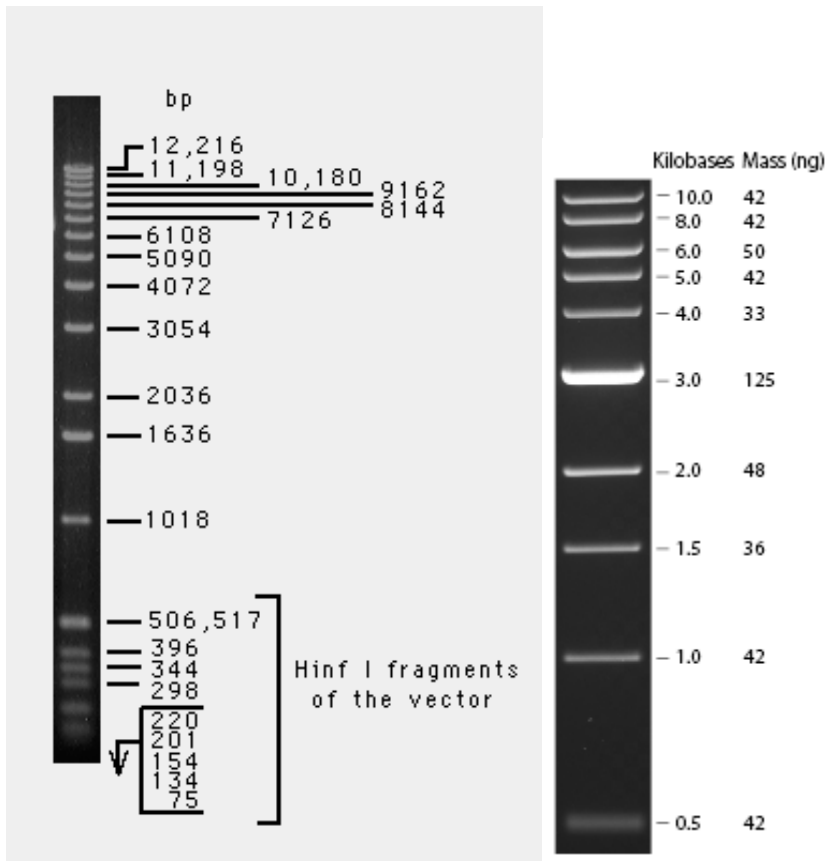
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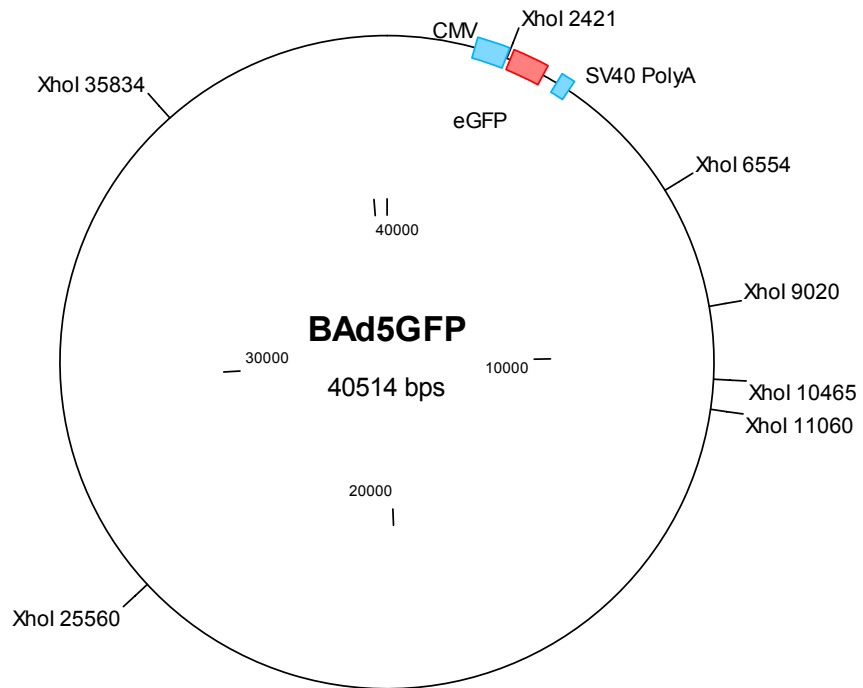
## Appendix



**Figure A1: Plasmid map of pGPSgalK/Kn<sup>R</sup>** with the *galK* and Kn<sup>R</sup> genes and primer binding regions for PCR primers GalKFOR/H1-19KO and GalKREV/H2-19KO indicated.

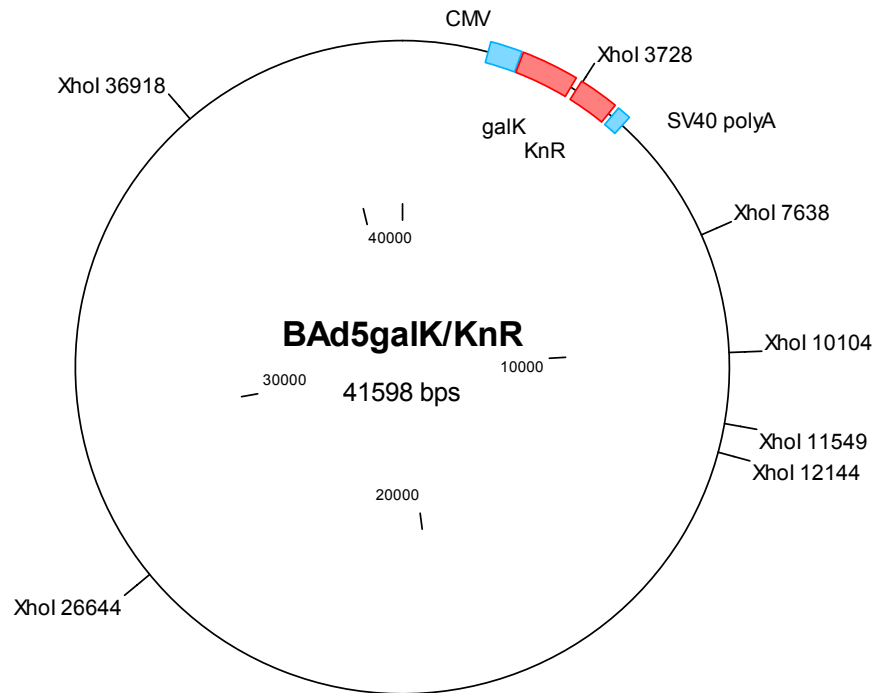


**Figure A2: Banding pattern of Invitrogen 1kb DNA ladder (left) and New England Biolabs 1kb DNA ladder (right)**



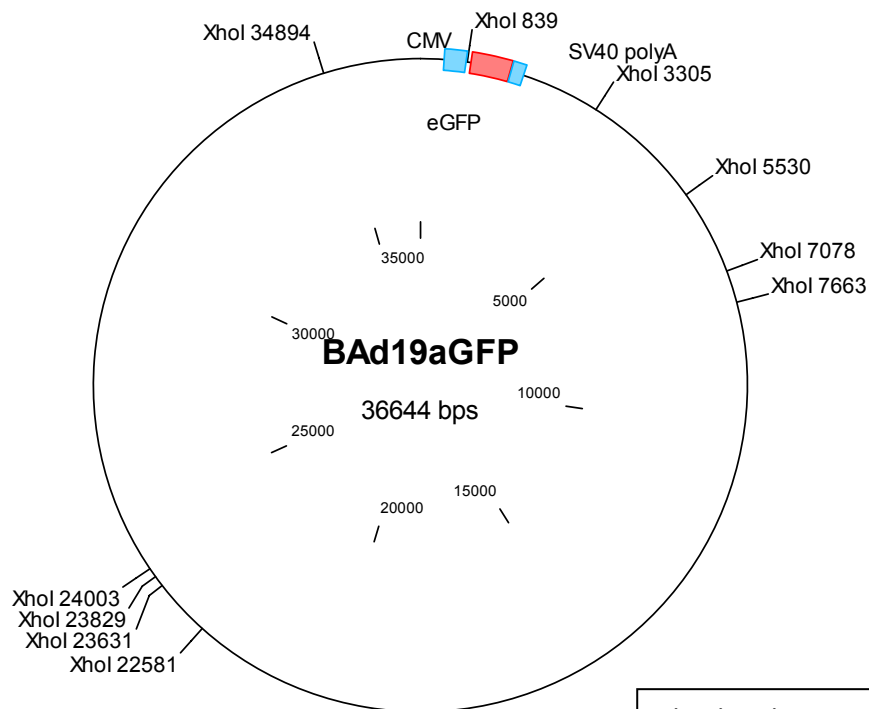
XhoI banding pattern	
14,500bp	(11060-25560)
11,234bp	(35834-6554)
10,274bp	(25560-35834)
2,466bp	(6554-9020)
1,445bp	(9020-10465)
595bp	(10465-11060)

**Figure A3: BAC map of BAd5GFP** with the CMV promoter, GFP transgene and SV40 polyA tail indicated. Also shown are XhoI restriction sites and the predicted XhoI digest banding pattern.



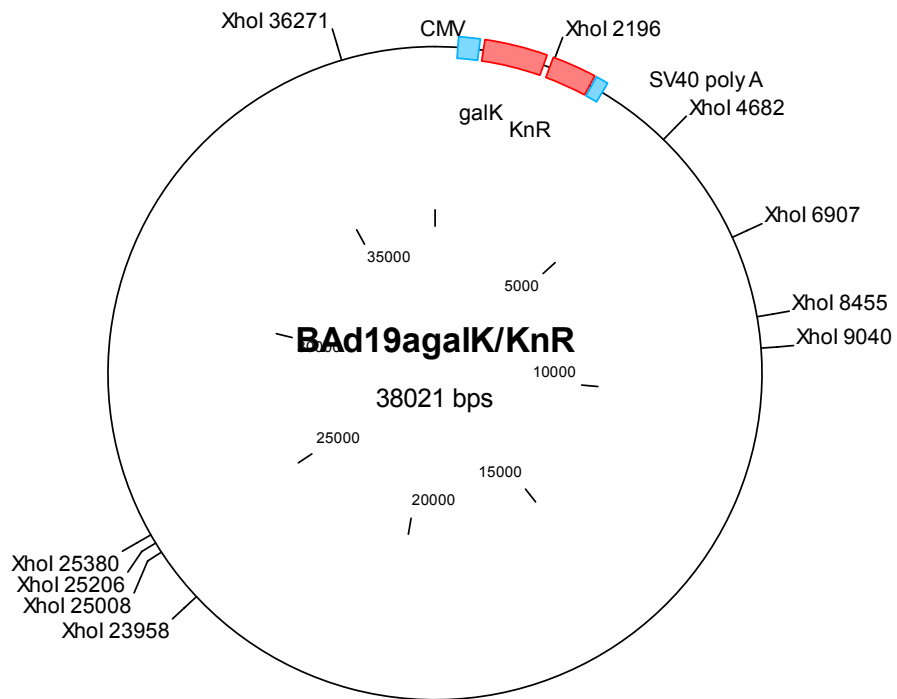
XhoI banding pattern
14,500bp (12144-26644)
10,274bp (26644-36918)
8,408bp (36918-3728)
3,910bp (3728-7638)
2,466bp (7638-10104)
1,445bp (10104-11549)
595bp (11549-12144)

**Figure A4: BAC map of BAd5galK/Kn<sup>R</sup>** with the CMV promoter, the *galK* and Kn<sup>R</sup> genes and the SV40 polyA tail indicated. Also shown are XhoI restriction sites and the predicted XhoI digest banding pattern.



XhoI banding pattern
14,918bp (7663-22581)
10,891bp (24003-34894)
2,589bp (34894-839)
2,466bp (839-3305)
2,225bp (3305-5530)
1,548bp (5530-7078)
1050bp (22581-23631)
585bp (7078-7663)
197bp (23631-23829)
174bp (23829-24003)

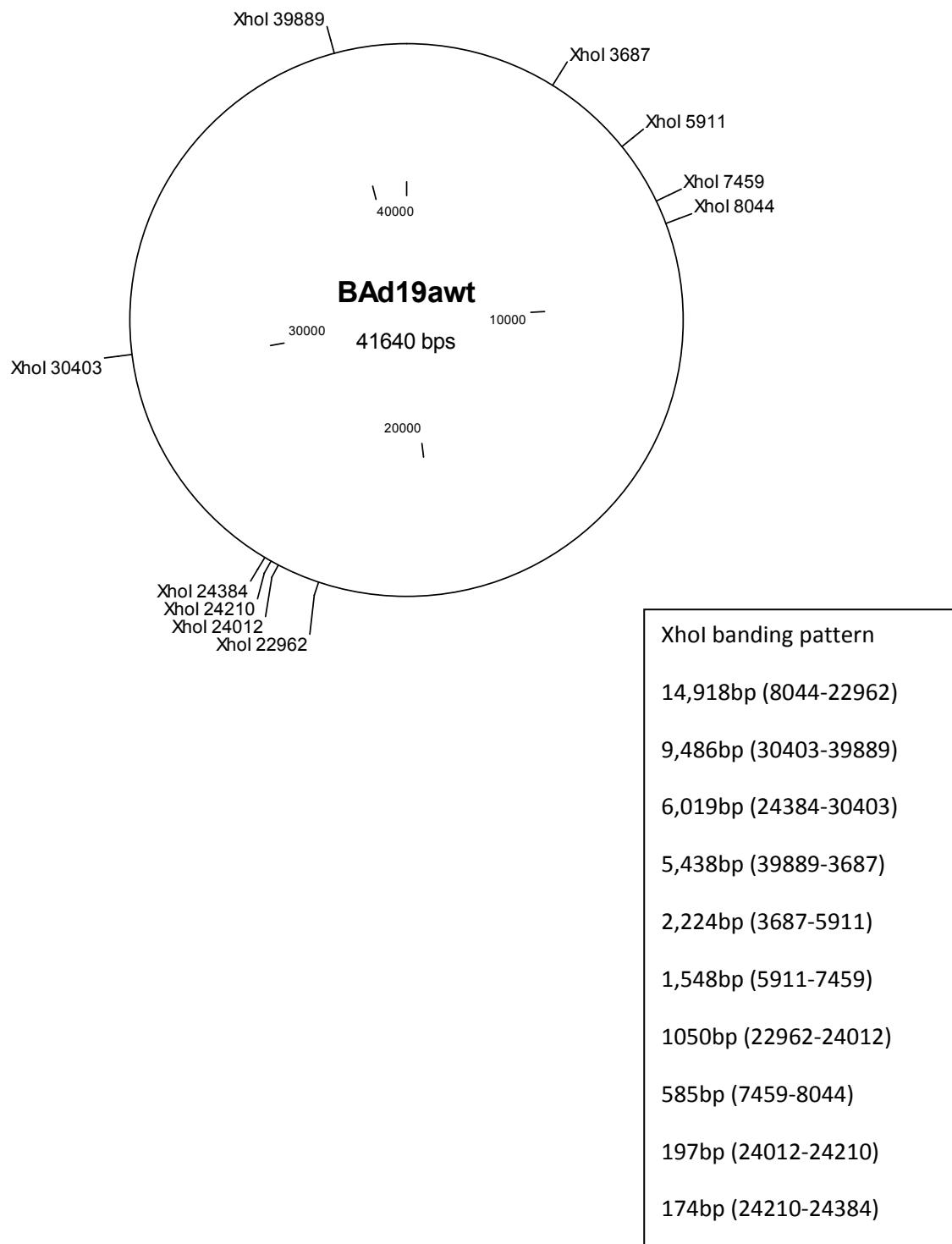
**Figure A5: BAC map of BAd19aGFP** with the CMV promoter, GFP transgene and SV40 polyA tail indicated. Also shown are XhoI restriction sites and the predicted XhoI digest banding pattern. BAd19aGFP lacks the E1 region from 141-424bp and all E3 genes.



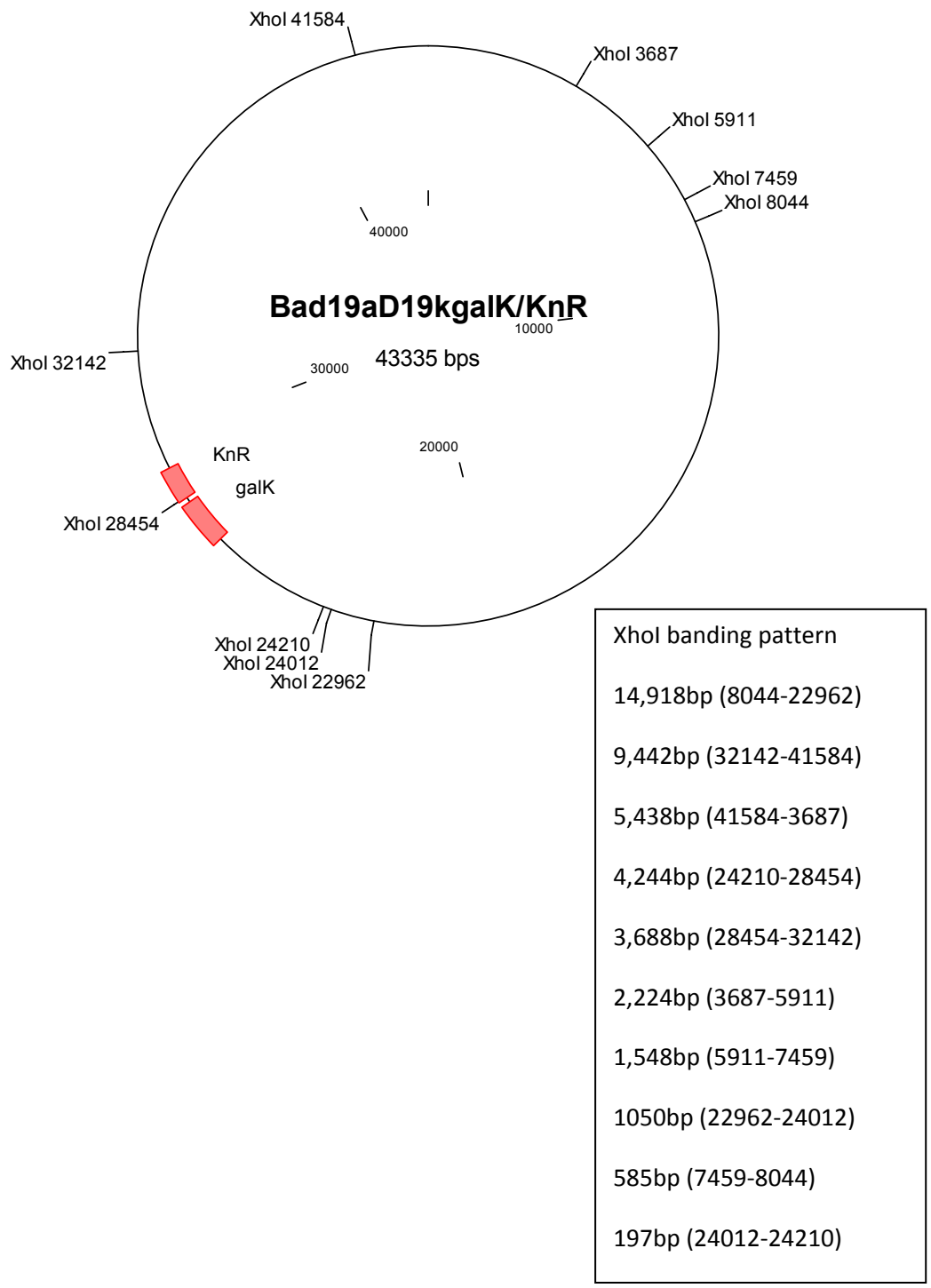
XhoI banding pattern
14,918bp (9040-23958)
10,891bp (25380-36271)
3,946bp (36271-2196)
2,486bp (2196-4682)
2,225bp (4682-6907)
1,548bp (6907-8455)
1050bp (23958-25008)
585bp (8455-9040)
197bp (25008-25206)
174bp (25206-25380)

**Figure A6: BAC map of BAd19agalK/Kn<sup>R</sup>** with the CMV promoter, galK and KnR genes and the SV40 polyA tail indicated. Also shown are XhoI restriction sites and the predicted XhoI digest banding pattern. BAd19agalK/Kn<sup>R</sup> lacks the E1 region from 141-424bp and all E3 genes.

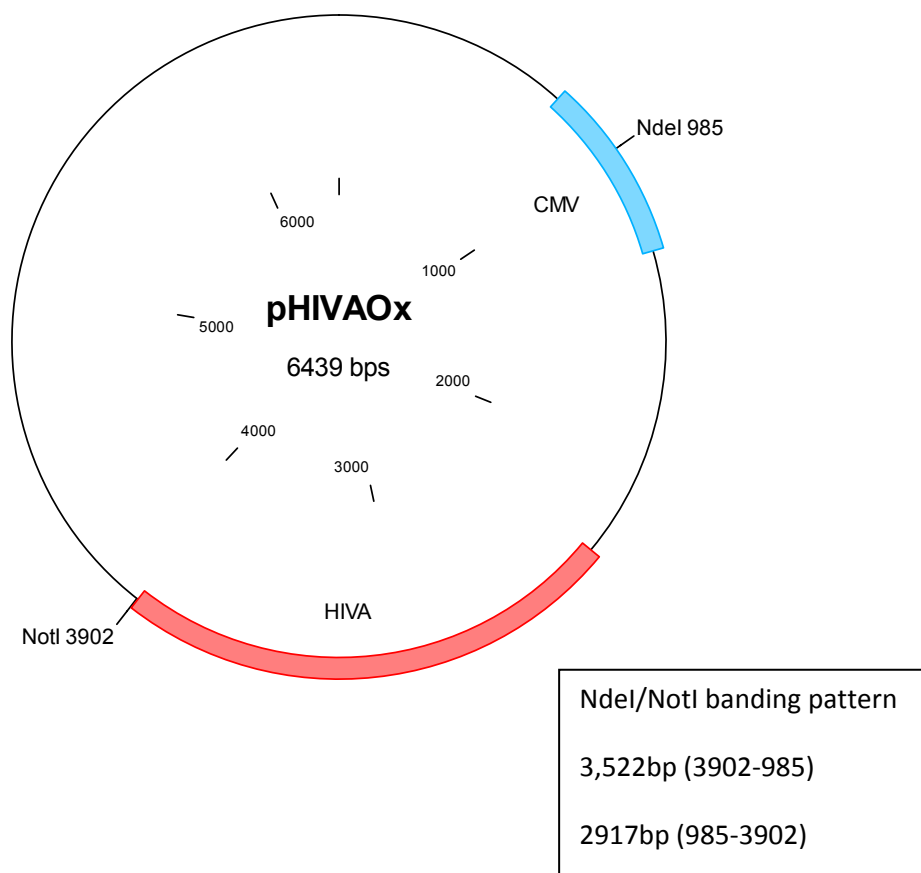




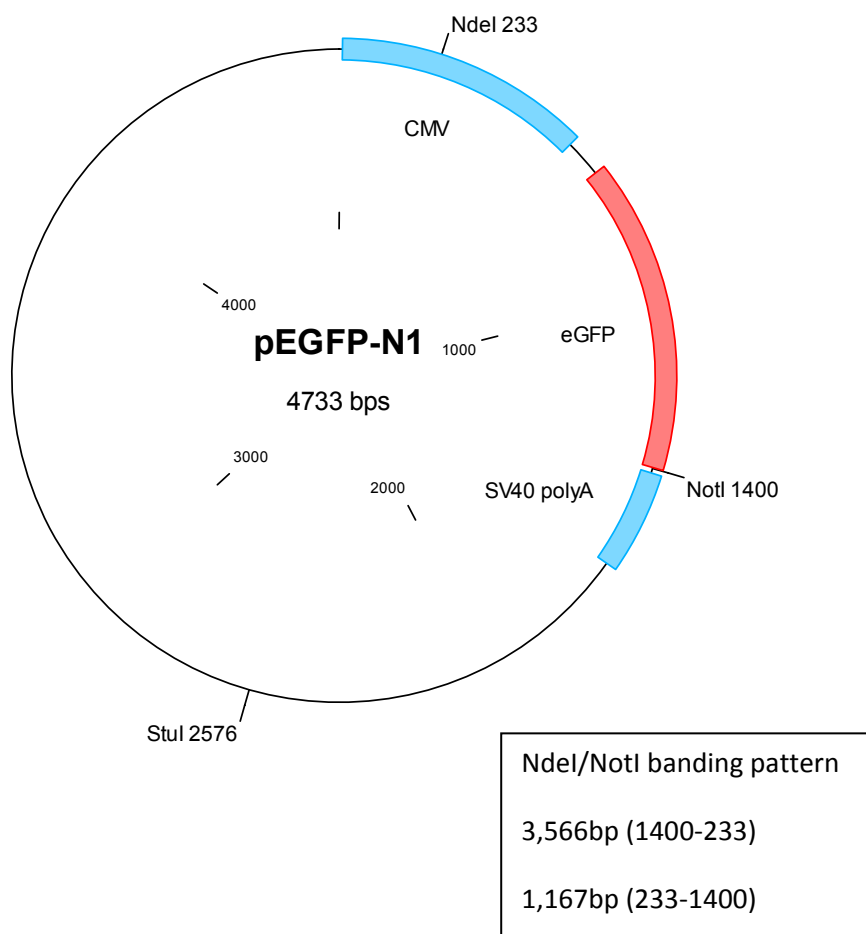
**Figure A7: BAC map of Bad19awt** showing XhoI restriction sites and the predicted XhoI digest banding pattern



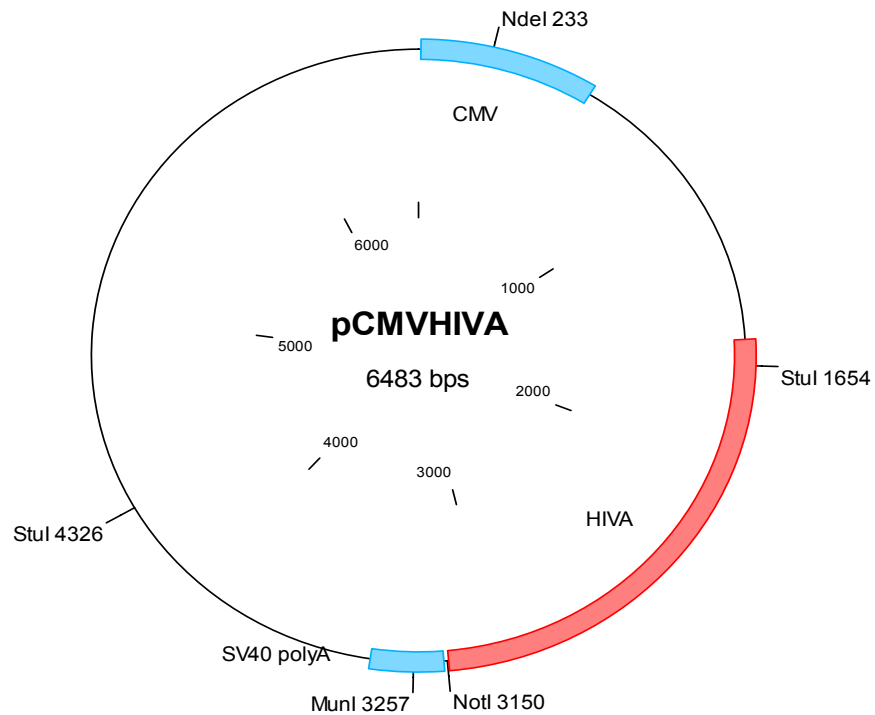
**Figure A8: BAC map of BAd19Δ19KgalK/Kn<sup>R</sup>** showing in red the replacement of Ad19a E3/19K with the *galK* and Kn<sup>R</sup> genes. Also shown are XhoI restriction sites and the predicted XhoI digest banding pattern.



**Figure A9: Plasmid map of pHIVAOx** with the CMV promoter and HIVA transgene indicated. Also shown are NdeI and NotI restriction sites and the predicted NdeI/NotI digest banding pattern

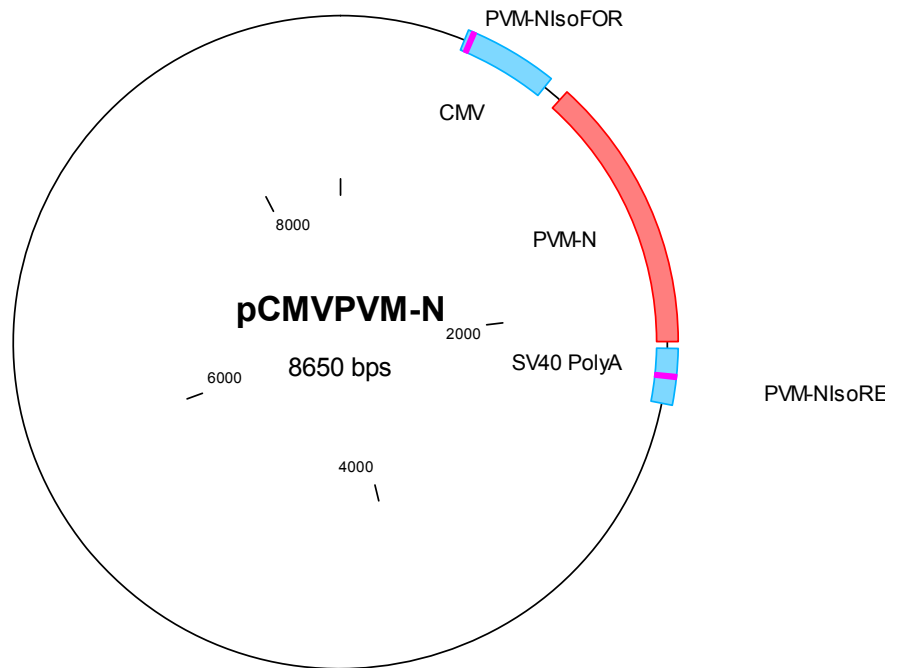


**Figure A10: Plasmid map of pEGFP-N1** with the CMV promoter, GFP transgene and the SV40 polyA tail indicated. Also shown are NdeI and NotI restriction sites and the predicted NdeI/NotI fragments.

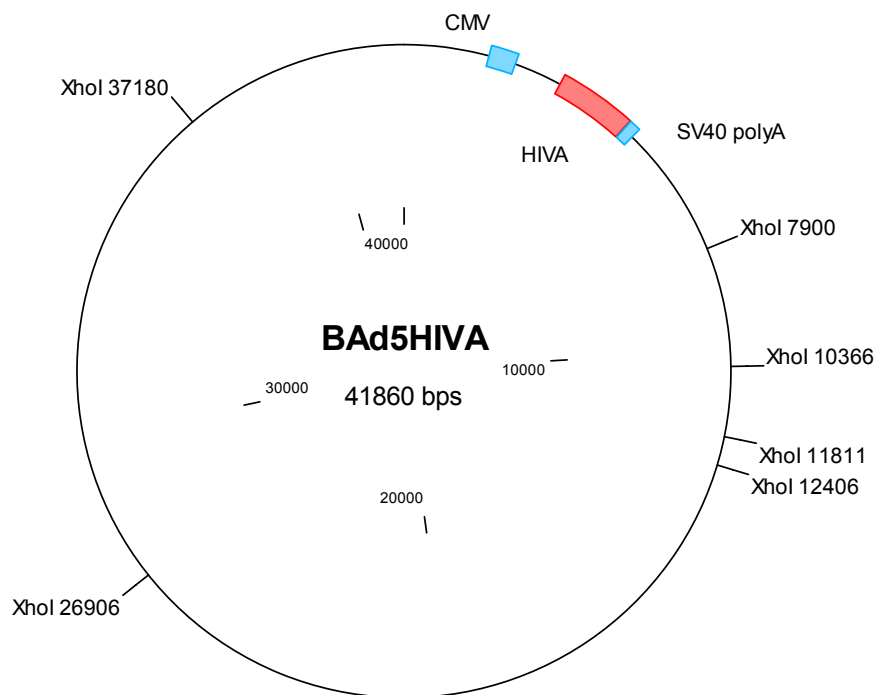


NdeI/MunI banding pattern
3,459bp (3257-233)
3,024bp (233-3257)
StuI banding pattern
3,811bp (4326-1654)
2672bp (1654-4326)

**Figure A11: Plasmid map of pCMVHIVA** with the CMV promoter, HIVA transgene, SV40 polyA tail and Kan<sup>R</sup> gene indicated. Also shown are NdeI, MunI, NotI and StuI restriction sites and the predicted NdeI/MunI and StuI digest banding patterns, respectively.

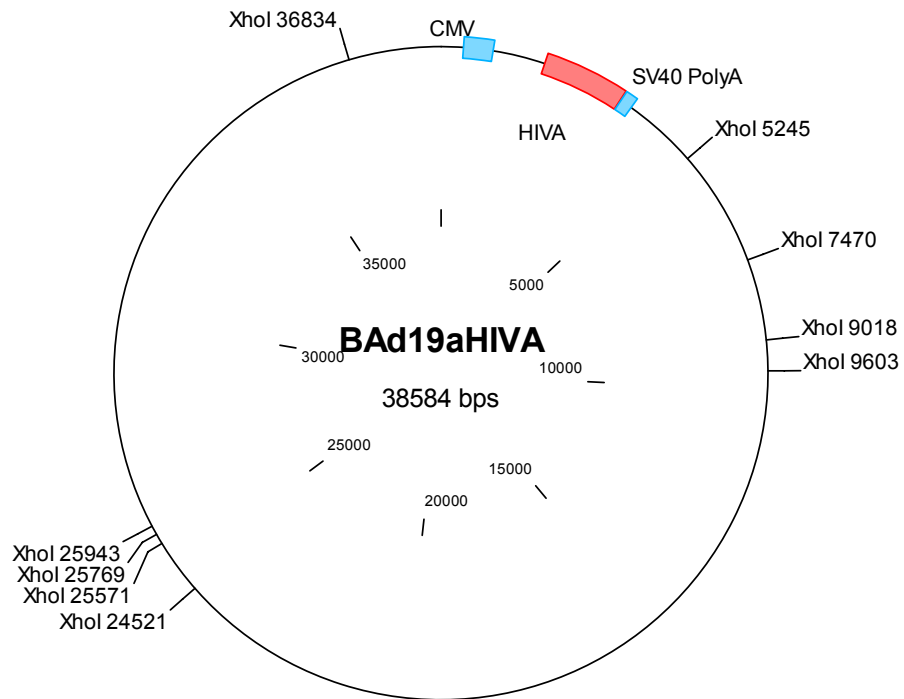


**Figure A12: Plasmid map of pCMVPVM-N** with the CMV promoter, PVM-N transgene and SV40 polyA tail indicated. Also shown are the primer binding sites (magenta) for the PVM-NisolationFOR and PVM-NisolationREV primers.



XhoI banding pattern
14,500bp (12406-26906)
12,580bp (37180-7900)
10,274bp (26906-37180)
2,466bp (7900-10366)
1,445bp (10366-11811)
595bp (11811-12406)

**Figure A13: BAC map of BAd5HIVA** with the CMV promoter, the HIVA transgene and the SV40 polyA tail indicated. Also shown are XhoI restriction sites and the predicted XhoI digest banding pattern.



XhoI banding pattern
14,918bp (9603-24521)
10,891bp (25943-36834)
6,995bp (36834-5245)
2,225bp (5245-7470)
1,548bp (7470-9018)
1050bp (24521-25571)
585bp (9018-9603)
197bp (25571-25769)
174bp (25769-25943)

**Figure A14: BAC map of BAd19aHIVA** with the CMV promoter, the HIVA transgene and the SV40 polyA tail indicated. Also shown are XhoI restriction sites and the predicted XhoI digest banding pattern.





**Figure A15: Sequencing alignment of BAd5HIVA** showing the predicted sequence of BAd5HIVA 200bp before the beginning of the HIVA transgene sequence to the end of the SV40 polyA tail (1795-2028) inclusive of the HIVA transgene (200-1795) (L1) aligned against the sequence generated by sequencing primers HIVA/F/1 (L2), HIVA/F/2 (L3) and HIVA/F/3 (L4). Matches between the predicted sequence and generated sequence are highlighted.

```

L1 BAd5HIVAHIVA      1 ctgagttgttgtgttctgataagagtcagaggtaactcccgttcggtgctgttaacggtggagggcagtgtagtctgagcagtactcgttgctgcccgg
L2 032009-014_H      -----
L3 032009-014_H      -----
L4 032009-014_H      -----

BAd5HIVAHIVA      101 cgcgccaccagacataatagctgacagactaacagactgttcctttccatgggtcttttctgcagtcaccgtccttgacacgaagcttcccgcgccacc
032009-014_H      -----
032009-014_H      -----
032009-014_H      -----

BAd5HIVAHIVA      201 atgcccatcgtgcagaacgccaggccagatgcaccaggccctgtccccccgcaccctgaaaggcctgggtgaaggtgatcgaggagaaggccttctccc
032009-014_H      1 -----nnnnnnnnnnnnnnnnctgggtgaaggtgatcgaggagaaggccttctccc
032009-014_H      -----
032009-014_H      -----

BAd5HIVAHIVA      301 ccgaggtgatccccatgttctcgcacctgtccgagggcgccacccccaggacctgaacatgatgctgaacatcgtgggcgccaccaggccgcatgca
032009-014_H      50 ccgaggtgatccccatgttctcgcacctgtccgagggcgccacccccaggacctgaacatgatgctgaacatcgtgggcgccaccaggccgcatgca
032009-014_H      -----
032009-014_H      -----

BAd5HIVAHIVA      401 gatgctgaaggacaccatcaacgaggaggccgcccagtgaggaccgctgcaccccgtgcacgcccggccccatccccccggccagatgcccagccccgc
032009-014_H      150 gatgctgaaggacaccatcaacgaggaggccgcccagtgaggaccgctgcaccccgtgcacgcccggccccatccccccggccagatgcccagccccgc
032009-014_H      -----
032009-014_H      -----

BAd5HIVAHIVA      501 ggatccgacatcgccggcaccacctccacctgcaggagcagatcggtggatgacctccaacccccccatccccgtgggcgacatctacaagcgctgga
032009-014_H      250 ggatccgacatcgccggcaccacctccacctgcaggagcagatcggtggatgacctccaacccccccatccccgtgggcgacatctacaagcgctgga
032009-014_H      -----
032009-014_H      -----

BAd5HIVAHIVA      601 tcatcctgggctgaacaagatcgtgcgcagtgtactcccccggtgctccatcctggacatccgccagggcccccaaggagcccttccgcgactacgtggaccg
032009-014_H      350 tcatcctgggctgaacaagatcgtgcgcagtgtactcccccggtgctccatcctggacatccgccagggcccccaaggagcccttccgcgactacgtggaccg
032009-014_H      -----
032009-014_H      -----

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032009-014_H 955 -----
032009-014_H 747 gaaggagcccgTgcacggcgTgtaccaccccgacatcgtgatctaccagTacatggacgacctgac-----ccccggcgTgcgctaccccctggcctgc
032009-014_H 29 gaaggagcccgTgcacggcgTgtaccaccccgacatcgtgatctaccagTacatggacgacctgacccccggccccggcgTgcgctaccccctggcctgc

BAAd5HIVAHIVA 1701 accccctacgacatcaaccagatgctgcgcgccccggcgcgcccttcgtgaccatccccaacccccctgctgggctggactgagcggcgcgactctag
032009-014_H -----
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032009-014_H 129 accccctacgacatcaaccagatgctgcgcgccccggcgcgcccttcgtgaccatccccaacccccctgctgggctggactgagcggcgcgactctag

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032009-014_H -----
032009-014_H -----
032009-014_H 229 atcataatcagccataccacatttTgtagaggTtttactTgctttaaaaaacctccacacctccccctgaaacctgaaacataaaatgaatgcaattgttg

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032009-014_H 1019 -----
032009-014_H 964 -----
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BAAd5HIVAHIVA 1999 ttgttccaaactcatcaatgtatcttaag-----
032009-014_H 1044 -----
032009-014_H 983 -----
032009-014_H 427 ttgttccaaactcatcaatgtatcttaagacgcgtaaatgtgaagcgTtaattattgaaaaaggaagagTcctgaggcggaagaaccagctg

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**Figure A16: Sequencing alignment of BAd19aHIVA** showing the predicted sequence of BAd19aHIVA 200bp before the beginning of the HIVA transgene sequence to the end of the SV40 polyA tail (1795-2028) inclusive of the HIVA transgene (200-1795) (L1) aligned against the sequence generated by sequencing primers HIVA/F/1 (L2), HIVA/F/2 (L3) and HIVA/F/3 (L4). Matches between the predicted sequence and generated sequence are highlighted.

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L1 BAd19aHIVAHI      1 ctgagttgtgtgttctgataagagtcagagtgtaactcccgttgcggtgctgttaacggtggagggcagtgtagtctgagcagtagctgctgctgcccgcg
L2 122007-378_H      -----
L3 122007-378_H      -----
L4 122007-378_H      -----

BAd19aHIVAHI      101 cgcgccaccagacataatagctgacagactaacagactggtcctttccatgggtcttttctgcagtcaccgtccttgacacgaagcttcccgcgccacc
122007-378_H      -----
122007-378_H      -----
122007-378_H      -----

BAd19aHIVAHI      201 atgcccatcgtgcagacgcgcagggccagatgcaccaggccctgtccccccgcaccctgaacgcctgggtgaaggtgatcgaggagaaggccttctccc
122007-378_H      1 -----nnnnnnnnngggccagatgcaccaggccctgtccccccgcaccctgaacgcctgggtgaaggtgatcgaggagaaggccttctccc
122007-378_H      -----
122007-378_H      -----

BAd19aHIVAHI      301 ccgaggtgatccccatggttctccgcctgtccgagggcgcccacccccaggacctgaacatgatgctgaacatcgtgggcggccaccaggccgcatgca
122007-378_H      87 ccgaggtgatccccatggttctccgcctgtccgagggcgcccacccccaggacctgaacatgatgctgaacatcgtgggcggccaccaggccgcatgca
122007-378_H      -----
122007-378_H      -----

BAd19aHIVAHI      401 gatgctgaaggacaccatcaacgaggaggccgcccagtgaggaccgctgcaccccgctgcacccggccccatccccccggccagatgcccagaccgcc
122007-378_H      187 gatgctgaaggacaccatcaacgaggaggccgcccagtgaggaccgctgcaccccgctgcacccggccccatccccccggccagatgcccagaccgcc
122007-378_H      -----
122007-378_H      -----

BAd19aHIVAHI      501 ggatccgacatcgccggcaccacctccacctgcaggagcagatcggtggatgacctccaacccccccatccccgtgggcgacatctacaagcgctgga
122007-378_H      287 ggatccgacatcgccggcaccacctccacctgcaggagcagatcggtggatgacctccaacccccccatccccgtgggcgacatctacaagcgctgga
122007-378_H      -----
122007-378_H      -----

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122007-378_H      387 tcatcctgggcctgaacaagatcgtgcgcatgtactccccctgtccatcctggacatccgccagggccccaaggagcccttccgcgactacgtggaccg
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122007-378_H      -----

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122007-378_H -----
122007-378_H 1 -----

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122007-378_H -----

BAD19aHIVAH1 1101 aagacctccgaggagctgaagtccctgttcaacacctggccaccctgtactgcgtgcaccagcgcacgcagtgaggacaccaaggaggccctggaca
122007-378_H -----
122007-378_H 237 aagacctccgaggagctgaagtccctgttcaacacctggccaccctgtactgcgtgcaccagcgcacgcagtgaggacaccaaggaggccctggaca
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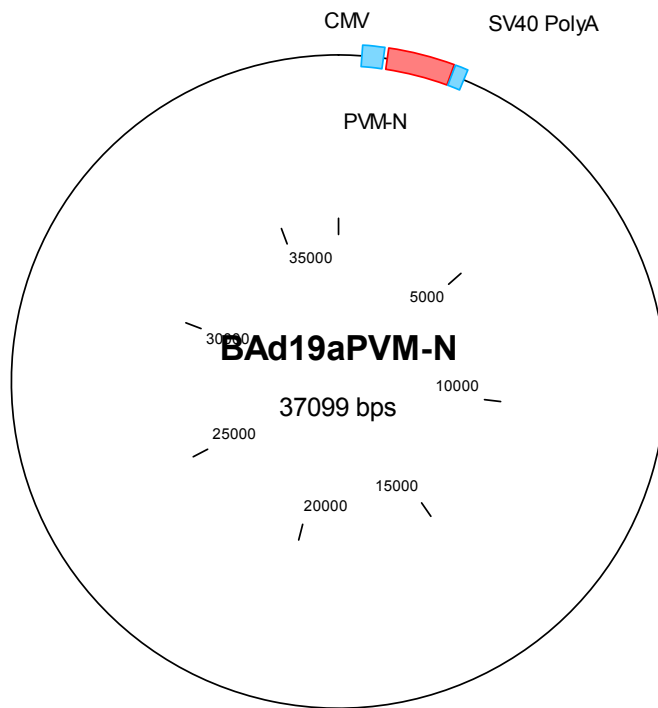
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**Figure A17: BAC map of BAd19aPVM-N** with the CMV promoter, the PVM-N transgene and the SV40 polyA tail highlighted.





**Figure A18: Sequencing alignment of BAd19aPVM-N** showing the predicted BAd19aPVM-N sequence from the beginning of the CMV promoter (1-392) to the end of the SV40 polyA tail (1665-1898) inclusive of the PVM-N transgene (468-1665) (Line 1(L1)) aligned against the sequence generated by sequencing primers PVM-N/F/1 (L2), PVM-N/F/2 (L3) and PVM-N/F/3 (L4). Matches between the predicted sequence and generated sequence are highlighted.

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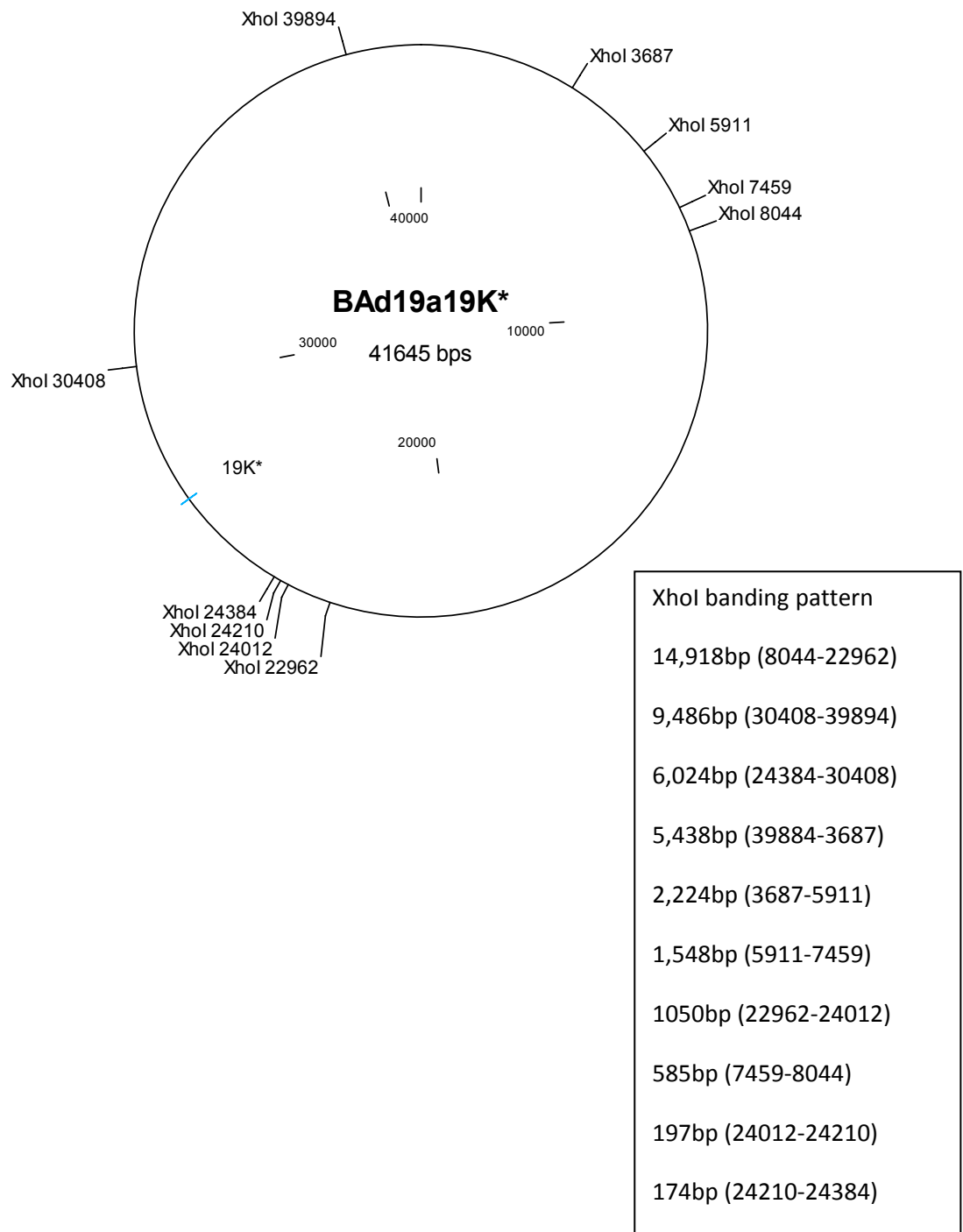
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```



**Figure A19: BAC map of Bad19a19K\*** with the site of stop codon insertion, XhoI restriction sites and the XhoI digest banding pattern indicated.



**Figure A20: Sequencing alignment of Bad19a19K\*** showing the Bad19awt sequence from 161bp prior to the E3-19K start codon (Line 1) aligned against the sequence generated by sequencing primer 19KSEQPRIME (Line 2). The successfully inserted nonsense mutation 9bp after the start codon is underlined.

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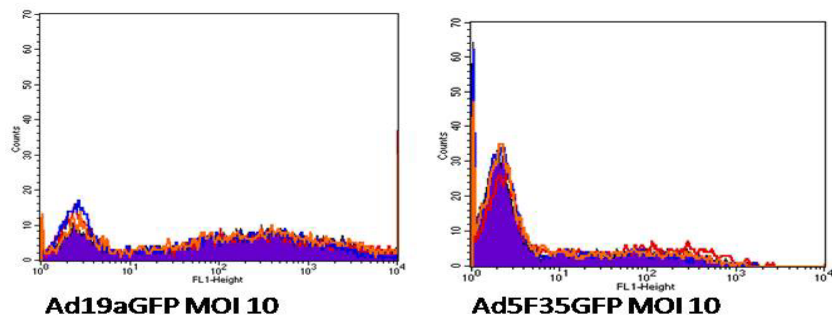
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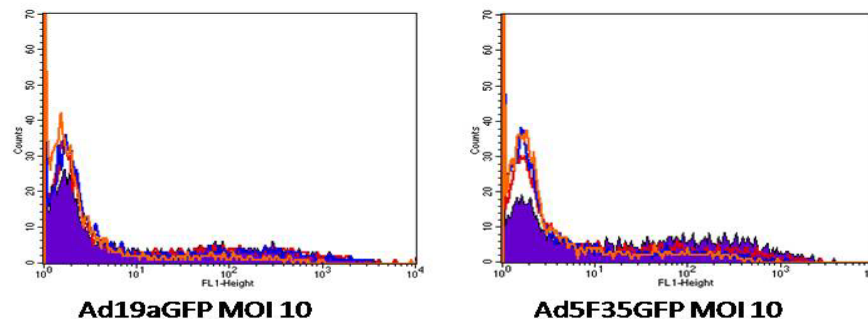




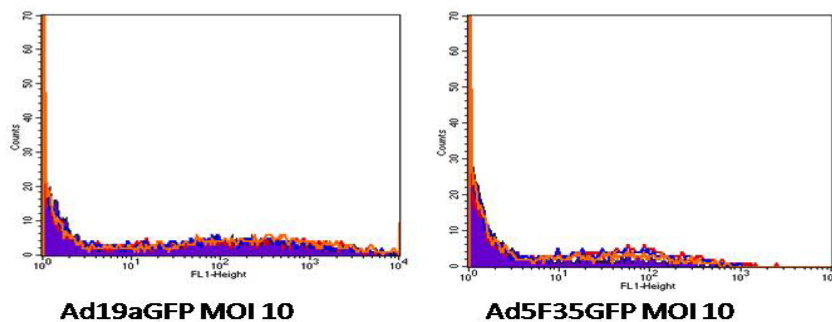
**A) A549**



**B) CHO-CD46 MCP1**



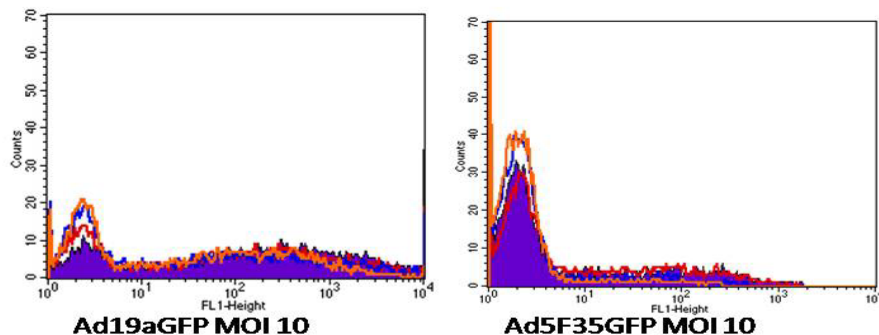
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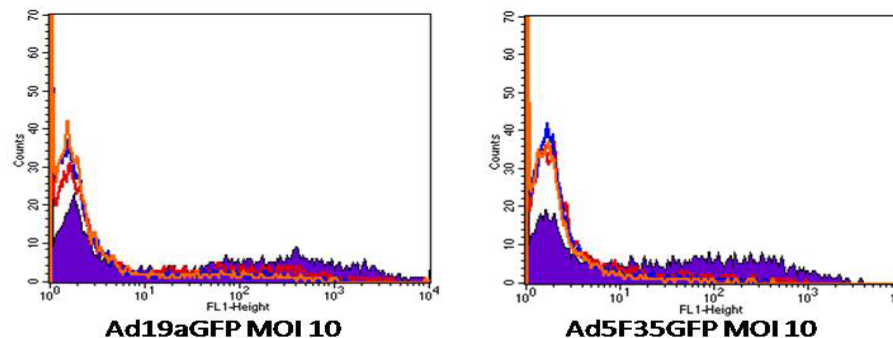
**Figure A21: Blocking of CD46 receptor usage by monoclonal antibody treatment** presenting example histograms showing the blocking of rAd19aGFP or rAd5F35GFP (as labelled) transduction by either 0.25µg/ml (red solid line), 2.5µg/ml (blue solid line) or 25µg/ml (orange solid line) αCD46FII monoclonal antibody in A) A549, B) CHO-CD46 MCP1 and C) CHO-CD46 MCP2 cells.

■ Mock infection   
 ■ αCD46FII 0.25µg/ml   
 ■ αCD46FII 2.5µg/ml   
 ■ αCD46FII 25µg/ml

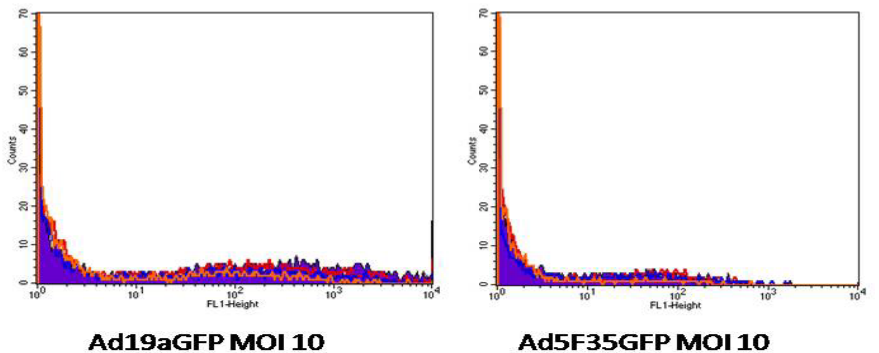
**A) A549**



**B) CHO-CD46 MCP1**



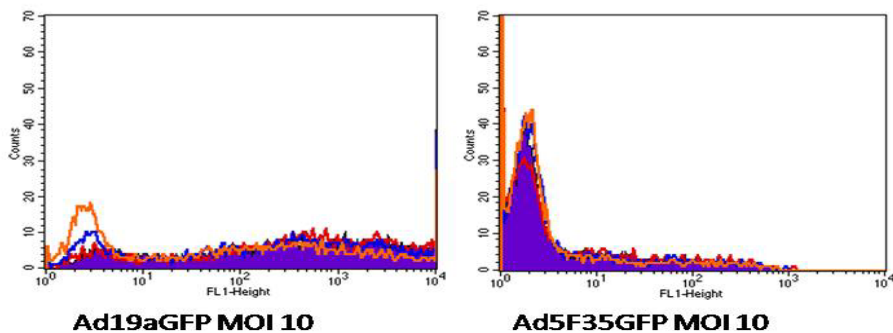
**C) CHO-CD46 MCP2**



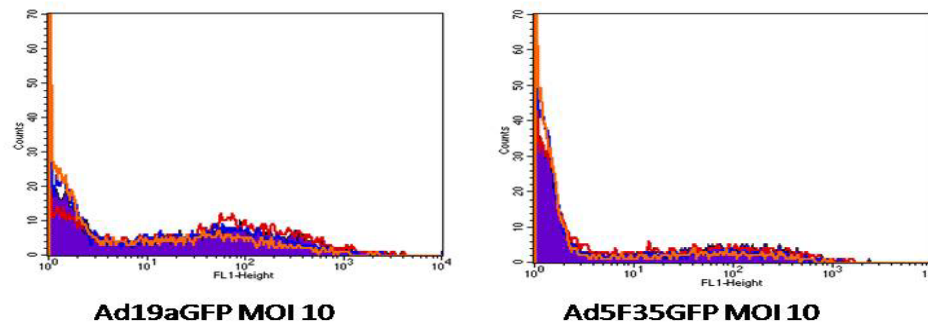
**Figure A22: Blocking of CD46 receptor usage by polyclonal antibody treatment** presenting example histograms showing the blocking of rAd19aGFP or rAd5F35GFP (as labelled) transduction by either a 1/100 (red solid line), 1/25 (blue solid line) or 1/10 (orange solid line) dilution of αCD46poly polyclonal antibody in A) A549, B) CHO-CD46 MCP1 and C) CHO-CD46 MCP2 cells.

■ Mock infection    
 ■ αCD46Poly 1/100 dilution    
 ■ αCD46Poly 1/25 dilution    
 ■ αCD46Poly 1/10 dilution

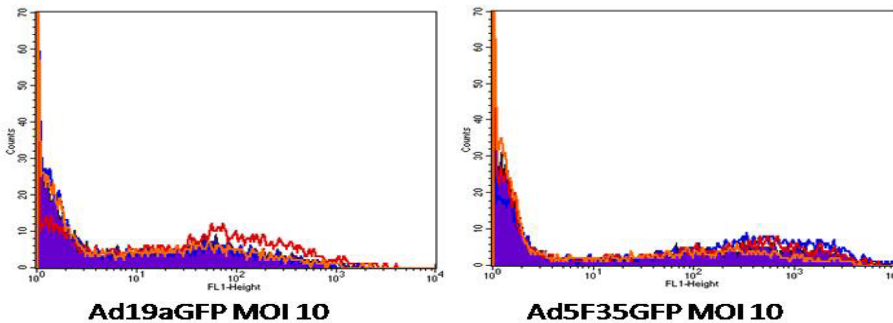
### A) A549



### B) CHO-CD46 MCP1



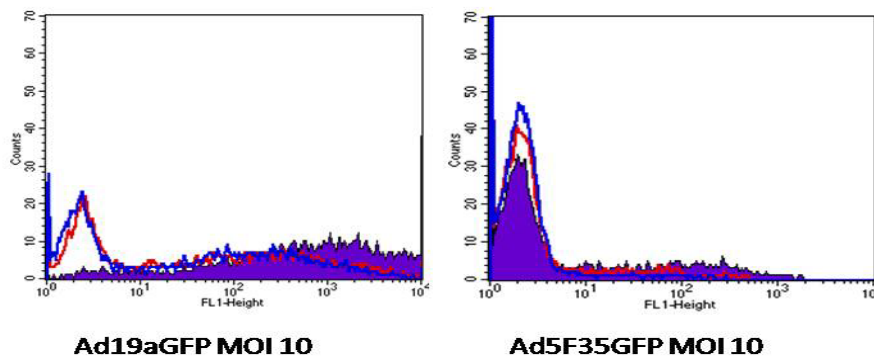
### C) CHO-CD46 MCP2



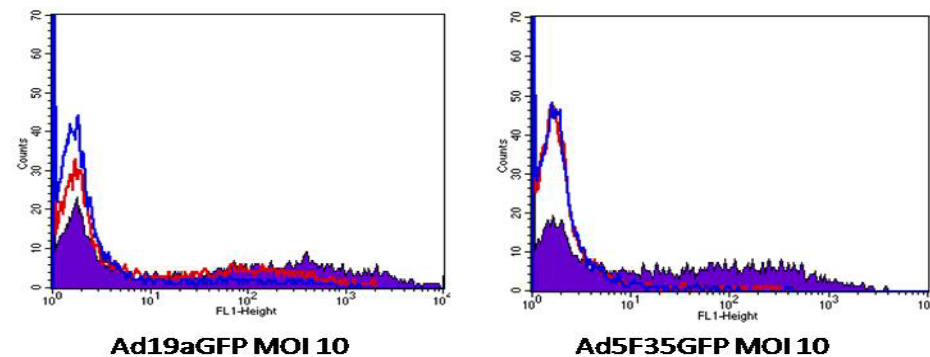
**Figure A23: Blocking of CD46 receptor usage by serum control antibody treatment** presenting example histograms showing the blocking of rAd19aGFP or rAd5F35GFP (as labelled) transduction by either a 1/100 (red solid line), 1/25 (blue solid line) or 1/10 (orange solid line) dilution of rabbit serum control in A) A549, B) CHO-CD46 MCP1 and C) CHO-CD46 MCP2 cells.

■ Mock infection    ■ Serum control 1/100 dilution    ■ Serum control 1/25 dilution    ■ Serum control 1/10 dilution

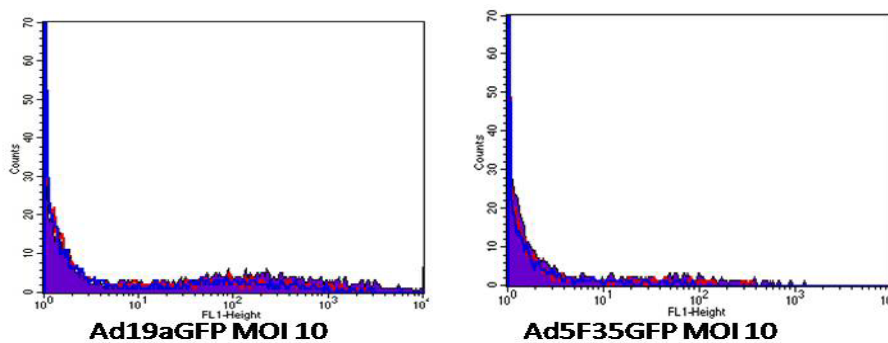
### A) A549



### B) CHO-CD46 MCP1

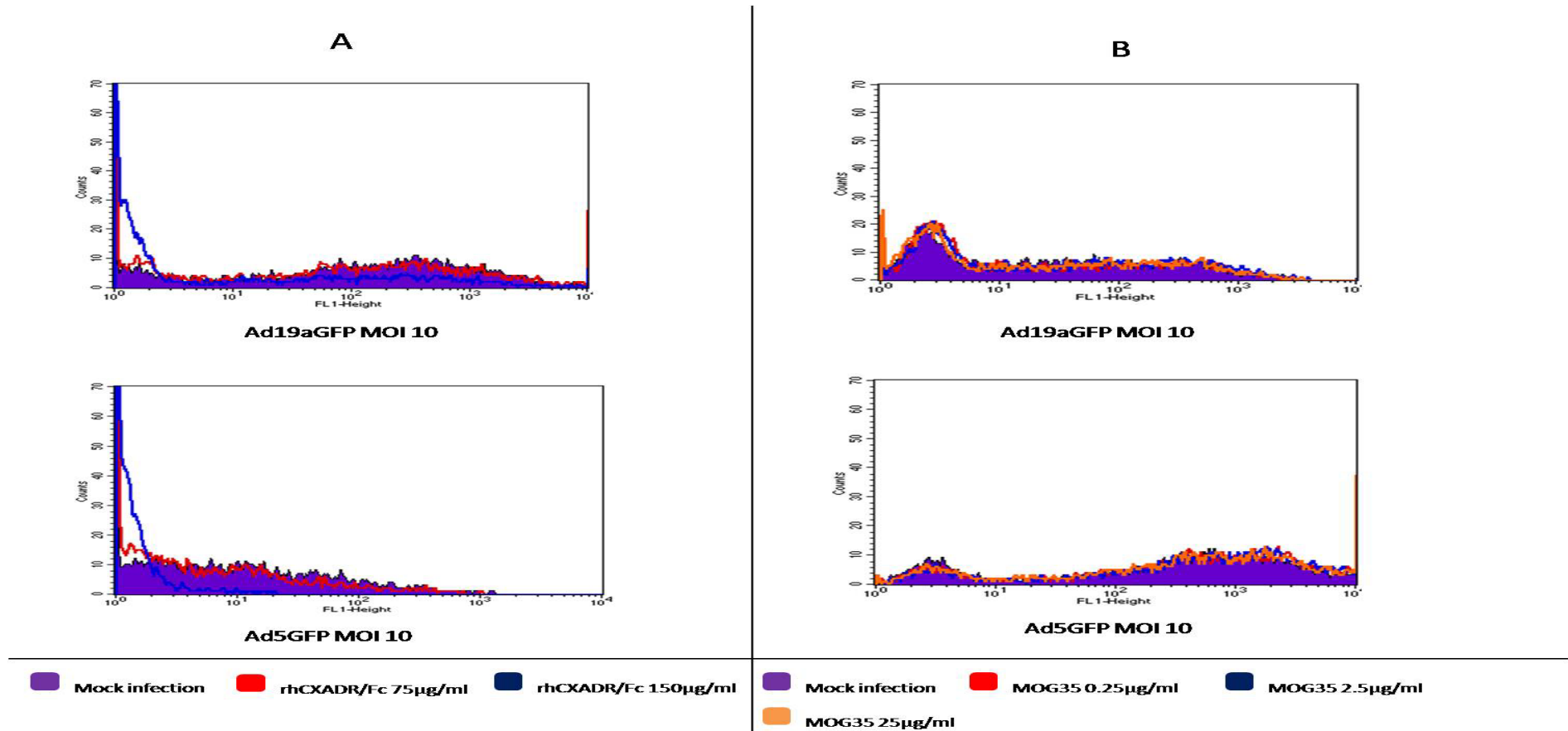


### C) CHO-CD46 MCP2

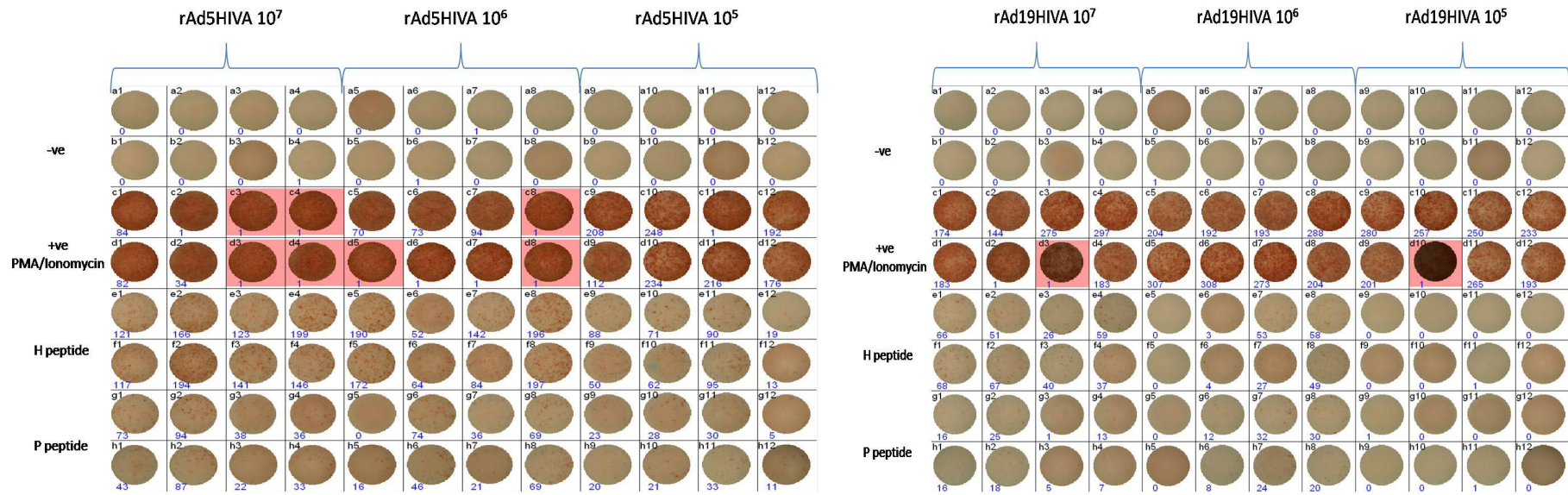


**Figure A24: Blocking of CD46 receptor usage by soluble CD46 treatment** presenting example histograms showing the blocking of rAd19aGFP or rAd5F35GFP (as labelled) transduction by pre-incubation of virus with either 75µg/ml (red solid line) or 150µg/ml (blue solid line) MCP-BC-IgG4 in A) A549, B) CHO-CD46 MCP1 and C) CHO-CD46 MCP2 cells.

■ Mock infection    ■ MCP-BC-IgG4 75µg/ml    ■ MCP-BC-IgG4 150µg/ml

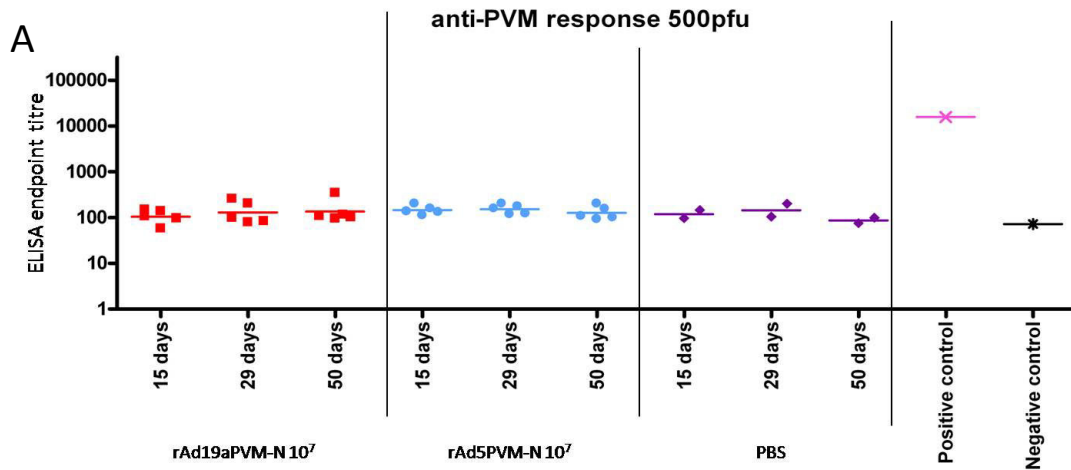


**Figure A25: Attempted blocking of CAR and GD1a usage** presenting example histograms showing A) the blocking of rAd19aGFP or rAd5GFP (as labelled) transduction by pre-incubation of virus with either 75µg/ml (red solid line) or 150µg/ml (blue solid line) rhCXADR in CHO-CAR cells and B) the attempted blocking of rAd19aGFP or rAd5GFP (as labelled) transduction with either 0.25µg/ml (red solid line), 2.5µg/ml (blue solid line) or 25µg/ml (orange solid line) MOG35 monoclonal antibody in A549 cells.



**Figure A26: Representative ELISpot assays** showing example ELISpot assays from the experiment performed in Figure 7.1. The assays show a splenocyte secreting IFN- $\gamma$  on stimulation with a peptide or the +ve PMA/Ionomycin control as a red spot which is then counted by an ELISpot reader and the number of spots identified in the lower left hand corner of each well. The splenocytes from 4 mice in each inoculation grouping were performed in duplicate (e.g. rAd5HIVA10<sup>7</sup> mouse 1 is represented by wells a1 and b1 on the first assay).





**B** 5 days

	PBS	rAd19aPVM-N	rAd5PVM-N
PBS		0.6719	0.5281
rAd19aPVM-N	0.6719		0.1327
rAd5PVM-N	0.5281	0.1327	

29 days

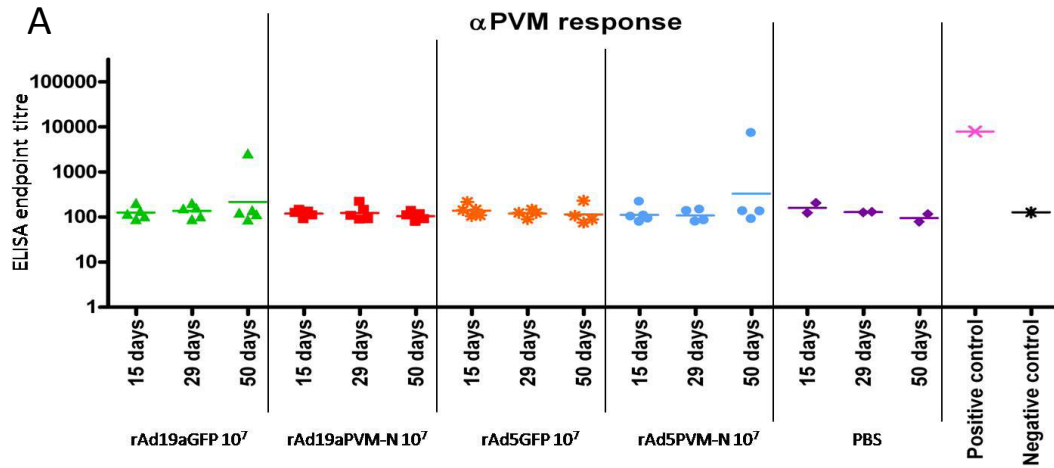
	PBS	rAd19aPVM-N	rAd5PVM-N
PBS		0.8121	0.8864
rAd19aPVM-N	0.8121		0.5411
rAd5PVM-N	0.8864	0.5411	

50 days

	PBS	rAd19aPVM-N	rAd5PVM-N
PBS		0.1853	0.1522
rAd19aPVM-N	0.1853		0.8385
rAd5PVM-N	0.1522	0.8385	

**Figure A27:  $\alpha$ PVM-N IgG responses to Ad vectors *in vivo* on super-lethal 500pfu PVM challenge and statistical analysis**

(A) Tail tip sera samples were removed from mice vaccinated with  $10^7$  pfu of either rAd19aPVM-N (red squares), rAd5PVM-N (blue circles) and PBS (purple diamonds) at day 15, 29 or the day of sacrifice for each mouse (represented as day 50) in the standard vaccination schedule and examined for IgG response to PVM-N alongside a positive and negative control. (B) The means of the above data were compared by an unpaired t test with Welch's correction. Where statistical significance between two responses was shown, i.e. a P value of  $<0.05$ , it has been highlighted in green and the level of statistical significance given by either 1, 2 or 3 asterisks. If no statistical significance was detected it has been highlighted in red.



**B**

**15 days**

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.5617	0.4670	0.7142	0.3717
rAd19aGFP	0.5617		0.7364	0.6148	0.6271
rAd19aPVM-N	0.4670	0.7364		0.3449	0.7807
rAd5GFP	0.7142	0.6148	0.3449		0.3596
rAd5PVM-N	0.3717	0.6271	0.7807	0.3596	

**29 days**

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.6824	0.7743	0.5212	0.3497
rAd19aGFP	0.6824		0.6166	0.4670	0.3097
rAd19aPVM-N	0.7743	0.6166		0.9153	0.6149
rAd5GFP	0.5212	0.4670	0.9153		0.6132
rAd5PVM-N	0.3497	0.3097	0.6149	0.6132	

**50 days**

	PBS	rAd19aGFP	rAd19aPVM-N	rAd5GFP	rAd5PVM-N
PBS		0.2795	0.7439	0.6182	0.3225
rAd19aGFP	0.2795		0.3150	0.3815	0.7385
rAd19aPVM-N	0.7439	0.3150		0.7779	0.3486
rAd5GFP	0.6182	0.3815	0.7779		0.3883
rAd5PVM-N	0.3225	0.7385	0.3486	0.3883	

**Figure A28: αPVM-N IgG responses to Ad vectors *in vivo* at 10<sup>6</sup> pfu dosage and statistical analysis**

(A) Tail tip sera samples were removed from mice vaccinated with 10<sup>7</sup> pfu of either rAd19aGFP (green triangles), rAd19aPVM-N (red squares), rAd5GFP (orange asterisks), rAd5PVM-N (blue circles) and PBS (purple diamonds) at day 15, 29 or the day of sacrifice for each mouse (represented as day 50) in the standard vaccination schedule and examined for IgG response to PVM-N alongside a positive and negative control. (B) The means of the above data were compared by an unpaired t test with Welch's correction. Where statistical significance between two responses was shown, i.e. a P value of <0.05, it has been highlighted in green and the level of statistical significance given by either 1, 2 or 3 asterisks. If no statistical significance was detected it has been highlighted in red.