The development of recombinant Adenoviral vaccines to target pneumovirus infection

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Declaration

I hereby declare that the author, under the supervision of Prof. A.J. Easton and Dr K.N. Leppard, completed all the work presented in this thesis with the exception of two experiments. One performed by an undergraduate project student and the other by a PhD student colleague. Their contribution has been clearly acknowledged in the text where the experiments are discussed.

This thesis has not been submitted for a degree in any other institution.

List of Abbreviations

ABTS	2,2’-Azino-bis(3-ethylbeno-thiazoline-6-sulphonic acid)
Ad	Adenovirus
Ad5	Adenovirus serotype 5
Amp	Ampicillin
AP	alkaline phosphatase
APC	Allophycocyanin
APCs	Antigen presenting cells
APS	Ammonium persulfate
APV	Avian pneumovirus
ATP	adenosine 5’ -triphosphate
BAL	Bronchoalveolar lavage
BFA	Brefeldin A
BSA	Bovine serum albumin
CAR	Coxsackie adenovirus receptor
cDNA	Complementary deoxyribonucleic acid
CIAP	Calf Intestinal Alkaline Phosphatase
CMC	carboxymethyl cellulose agar
CMV	Cytomegalovirus
ConA	Concanavalin A
c.p.e.	Cytopathic effect
CTL	Cytotoxic T-lymphocyte
DAPI	4’-6-Diamidino-2-phenylinode
dATP	2’-deoxyadenosine 5’-triphosphate
dCTP	2’-deoxycytidine 5’-triphosphate
dGTP	2’-deoxyguanosine 5’-triphosphate
dTTP	2’-deoxythymidine 5’-triphosphate
DAIP	4’-6-diamidino-2-phenylindole
DBP	DNA binding protein
DC	Dendritic cell
DMDP	Dichloromethylenediphosphonic acid
DMEM	Dulbecco’s modified Eagle medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
DTT	Dithiothreitol
E1	E1 region of adenovirus genome
E3	E3 region of adenovirus genome
EDTA	diaminoethanetetra-acetic acid disodium salt
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunoabsorbent spot
ER	Endoplasmic reticulum
E.S	Encapsidation signal
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FI	Formalin inactivated
FITC	fluorescein isothiocyanate

GMEM	Glasgow minimal essential medium
GFP	Green fluorescent protein
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HL1	Hyperladder 1
hMPV	Human metapneumovirus
h.p.i	Hours post infection
HPV	Human papillomavirus
Hr	Hour
HRP	Horse-radish peroxidise
HSV	Herpes simplex virus
ICS	Intracellular staining assay
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMPDH	Inosine monophosphate dehydrogenase
Io	Ionomycin
I.N.	Intranasal
IFN	Interferon
IPS-1	IFN-β promoter simulator
ITR	Inverted terminal repeat
JEV	Japanese encephalitis virus
Kan	Kanamycin
kbp	Kilobase pair
kDa	KiloDalton
*Lac*Z	lactose operon gene Z
LB	Luria-Bertani medium
LITR	Left inverted terminal repeat
Lys	Lysine
MAVS	Mitochondrial antiviral signalling
MCP-1	Monocyte chemotactic protein 1
MCS	Multiple cloning site
Met	Methonine
MHC	Major histocompatability complex
Min	Minutes
MIP-1α	Macrophage inflammatory protein 1α
M.O.I	Multiplicity of Infection
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
NEAA	Non-essential amino acids
NK	Natural killer cells
NP40	nonidet p40
OD	Optical density
Ori	Origin of replication
pA	Poly A
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin

p.f.u.	plaque forming unit
PIV	Parainfluenza virus
PMA	Phorbol 12-myristate 13-acetate
PVM	Pneumonia virus of mice
rAd	Recombinant Adenovirus
RIG-1	Retinoic acid inducible gene-1
RITR	Right inverted terminal repeat
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complex
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institue (medium)
RSV	Respiratory syncytial virus
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
Secs	Seconds
SV40	Simian virus 40
Th	T-helper
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TCR	T-cell receptor
TEMED	N,N,N',N'-Tetramethylethylenediamine
T_h	T-helper cell
TLRs	Toll like receptors
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet light
Val	Valine
VLPs	Virus like particles
Vp	virus particles
VV	Vaccinia virus
VZV	Varicella zoster virus
WB	Western blot
WEEV	Western equine encephalitis virus
wt	Wild type

Abstract

Respiratory Syncytial Virus (RSV) is a member of the pneumovirus genus (family *Paramyxoviridae*, subfamily *Pneumovirinae*). RSV is an important respiratory virus of both infants and the elderly, representing an underappreciated burden on health care systems. In addition, re-infections can occur despite the presence of pre-existing immunity, suggesting that immunological memory to RSV is incomplete.

To date, treatment of RSV infection is limited to the provision of supportive care and no effective vaccine is available. Although several are currently under investigation, these candidates focus upon the delivery of the F and G antigens of RSV to stimulate the immune system, rather than the internal antigens, which may provide cross protection between different subtypes of RSV.

Vaccine development has been greatly hindered by the lack of an appropriate animal model in which to study vaccine efficacy and pneumovirus pathogenesis. Pneumonia virus of mice (PVM) is also a member of the Pneumovirus genus and, like RSV infection of humans, causes a bronchiolitis and fatal pneumonia in its natural host, the mouse. PVM has been proposed as an appropriate model system in which to both study pneumovirus pathogenesis and vaccine efficacy.

The PVM model system was adapted to investigate a potential vaccination strategy to address the lack of an available RSV vaccine. Replication deficient recombinant adenovirus serotype 5 (rAd5) vectors were constructed which expressed the F, M and N genes of PVM J3666, in addition to a control construct, which expressed the *Lac*Z gene of *E. coli.*

The constructs were administered via the intranasal route to BALB/c mice and were able to elicit complete protection against a lethal dose of pathogenic PVM J3666, in both short-term experiments and in a long-term experiment, up to 20 weeks post immunisation. The protection effect elicited by the constructs was observed when administered in a single dose, and in alternative mouse strains, C3H/He-mg and C57BL/6, which had differing immunity haplotypes.

The rAd5 vectors generated a PVM specific IgG humoral response to PVM and Ad5 antigen which did not correlate as the primary mediator of protection. The rAd5 candidate expressing the N gene of PVM was shown to induce IFNγ secreting T-cells. The use of a peptide library of PVM N protein determined that a specific response could be identified towards the amino acids $N_{41\text{-}90}$, $N_{81\text{-}130}$, $N_{161\text{-}210}$ and $N_{281\text{-}330}$. Thus, the PVM infection model of BALB/c mice provides an immunological platform to facilitate the study of RSV and PVM pathogenesis, immunology and vaccine development.

Chapter 1

Introduction

1.1 Introduction

Viruses are one of the most abundant life forms on our planet and have evolved to parasitize all known forms of cellular life. Since the 20th Century, humans have possessed the ability to tackle the threat of viral diseases, in the form of improved sanitary conditions, anti-viral drugs and vaccination of susceptible populations. In contrast to the wide range of antibiotics available to treat bacterial infections, the number of clinically available anti-viral drugs are fewer in comparison. As such, vaccination remains one of the most important tools for tackling viral infections.

Vaccines are biological tools that aim to prevent disease arising from an infectious agent. Vaccination is a way of stimulating the immune system, of a host in a controlled manner to clear the foreign organism. This process generates an immune memory so that when a natural infection does occur, the immune system is already primed to control and clear the infection, preventing disease. To date, smallpox remains the most successful example of this, and is the only human disease which has been eradicated from the population.

1.2 Approaches to vaccination

Vaccination has been used in human populations for over 200 years, since Edward Jenner discovered a method to protect against smallpox in 1796. Yet despite the advances made in science over this period, the majority of commercially available viral vaccines are still developed using traditional techniques. These vaccines are either live vaccines, where the virus has been attenuated to prevent disease, or inactivated vaccines, where the replication capacity of the virus has been destroyed by chemical or heat treatment. Several currently available vaccines are shown in Table 1.1. These vaccines retain the immunogenic properties of the virus and are able to directly stimulate an appropriate immune response.

Virus	Trade name	Type
Measles	M-M-R II	Live - attenuated
Mumps	Priorix	
Rubella	Tresivac	
	Trimovax	
Hepatitis A (HAV)	Havrix	Inactivated
	Avaxrim	Subunit
Hepatitis B (HBV)	Recombivax HB	Subunit
	Engerix-B	
Human papillomavirus (HPV)	Gardasil	Subunit
	Cervarix	
Influenza	LAIV	Live – attenuated
	TIV	Inactivated
	Pandemrix (H5N1)	
Japanese encephalitis (JEV)	JE-VAX	Inactivated
	IXIARO	
Polio	OPV (Sabin)	Live – attenuated
	IPV (Salk)	Inactivated
	Pediarix*	
Rabies	Rabipur	Live – attenuated
	Rabies Vaccine BP Pasteur Merieux	
Rotavirus	Rotarix	Live – attenuated
	RotaTeq pentavalent	Live - attenuated
Varicella zoster virus (VZV)	Priorix Tetra ($^{+}$MMR)	Live – attenuated
	ProQuad ($^{+}$MMR)	
	Zostervax	
Yellow fever	Arilvax	Live - attenuated

Table 1.1. A summary of approved human vaccines against viral pathogens in the UK. Although newer preparations have been developed, the majority of these vaccines rely on attenuation through repeat tissue culture passage or inactivation with heat or chemical treatments.

* Pentavalent preparation with Diptheria, Pertussis, tetanus and Haemopholis influenza B

Through increased knowledge of immunology and molecular virology, several advances in viral vaccine development have been made. These new strategies have encouraged the development of safer, more immunogenic vaccines and novel delivery systems. Such methods include inactivated subunit, peptide or DNA-based vaccines or live viral-vectored vaccines to express high levels of a viral antigen. Vaccines made by these new strategies have an advantage over traditional vaccines in that they can be tailored to stimulate a particular immune response; minimising adverse side effects whilst maximising immunogenicity.

The immunogenicity of a vaccine remains the most important aspect for its success. As described in more detail in Section 1.6.2, a strong, protective adaptive immune response should be stimulated following vaccination. This should include B-cells to secrete neutralising antibody and the stimulation of a mixed T-cell response (van Drunen Littel-van den Hurk, 2007). T-cells exist in two main populations: $CD8^+$ T-cells, which have a cytotoxic effector function; and $CD4^+$ T-cells, which drive and control the overall immune response. By stimulating these aspects of the immune system, immunity to a virus will develop, preventing disease in an individual who subsequently encounters the pathogenic virus/agent, and hence reducing disease incidence in a human population.

The ultimate aims for a successful vaccine are: to be highly efficacious, to ensure a robust immune response is generated in a mixed genetic population; generate long-lasting immunity; to be effective in the presence of maternal antibodies if it is to be given to infants; preferably be administered by non-invasive routes; to be heat- and light-resistant and easily manufactured to minimise development costs; to be safe and well tolerated to ensure maximum uptake of the vaccine within a population; and be sufficiently immunogenic to give protection following a small number, preferably one, or two doses.

1.2.1 Inactivated virus vaccines

Inactivated vaccines are formed from viruses which have been treated with heat or chemicals, to render them non-infectious. This allows specific proteins or whole-virus preparations to be used to directly immunise the host and generate an immune

response. The advantages of this method are that multiple vaccines can be delivered simultaneously and, due to the manufacturing methods, the risk of contaminating agents is small. However, multiple boosters are usually required to ensure adequate stimulation of the immune response. Also, such vaccines are often delivered via intramuscular routes and often do not stimulate a strong mucosal response. Such a response is important, as the majority of viruses enter the host via a mucosal surface, so stimulating this aspect of immunity is desirable (Yuki & Kiyono, 2009). An additional problem is that the methods of inactivating the virus may reduce the immunogenicity of the preparation, resulting in a need for larger immunisation doses, or it can stimulate an abnormal or inadequate immune response. This was most dramatically demonstrated during the formalin-inactivated (FI) respiratory syncytial virus (RSV) vaccine trial where the vaccine stimulated a strong inflammatory response, upon subsequent encounter with virus that resulted in tragedy (Kim et al., 1969). Furthermore, these vaccines are associated with a risk of inadequate inactivation and thus stringent procedures are required to ensure each batch is safe for administration. Unfortunately, the consequences of this were demonstrated during the Cutter incident in 1955, where a batch of polio vaccine was contaminated with live virus, which led to several fatalities (Offit, 2005).

The first polio vaccine (SALK) is an example of a vaccine made in this manner (Salk, 1954). The vaccine has since been updated to an inactivated polio vaccine (IPV) vaccine, which has enhanced potency over the original. Newer inactivated subunit vaccines have been developed, including the hepatitis A virus (HAV) vaccine, HAVRIX (Nothdurft, 2008) and the hepatitis B virus (HBV) vaccine Recombivax-HB (Venters et al., 2004) (Table 1.1). Virus-like particles (VLPs), represent another inactivated vaccine strategy. This utilises the ability of some viruses to spontaneously form viral capsids in certain conditions. These can be manipulated to incorporate peptides or genomic material of another virus and used to directly immunise an individual by the natural route of infection. The advantage of this method over subunit vaccines is that IgA antibodies and a balanced T-cell response are stimulated (van Drunen Littel-van den Hurk, 2007). One such preparation is currently undergoing clinical trials to tackle influenza virus (Pushko et al., 2010) and other VLPs include the human papillomavirus (HPV) vaccines, Cervarix and Gardasil (Szarewski, 2010) (Table 1.1). Other novel delivery strategies include using

transgenic plants expressing VLPs from potatoes to tackle norovirus, or rice expressing an immunogenic dose of enterotoxins (Yuki & Kiyono, 2009). VLPs can also be used to deliver genetic material, which has been shown to be successful in mice (Wolff, 1990), particularly when immunised in a prime-boost heterologous fashion (Plotkin, 2009).

1.2.2 Live attenuated virus vaccines

Live attenuated vaccines are essentially replication-competent pathogens, which can enter and thus infect a host cell but have lost the capacity to cause disease. This is achieved either through multiple passage in tissue culture or through genetic manipulation. Such vaccines are considered to be more favourable than inactivated candidates as they induce a more potent and robust immune response than killed or subunit vaccines (Collins, 1974). This is because the virus is able to mimic a natural infection, entering and replicating in the target cells. This stimulates both innate and adaptive immune responses, generating neutralising IgA and IgG antibodies and virus-specific $CD8^+$ T-cells in a way which more accurately reflects the normal response to the pathogen. The main disadvantages of these vaccines are that, depending on the attenuation method, they can demonstrate a reversion to virulence, especially if an individual is infected concurrently with the wild-type virus. Neither are they suitable for immunocompromised persons nor for those with underlying conditions as viral disease or other complications can arise. Pre-existing immunity, either from maternal antibodies in infants or from a previous natural infection, may affect the immunogenicity of the vaccine, in addition, the production of these vaccines is costly, as preparations must be stringently checked for contaminating agents or virulent virus. But these issues are overshadowed by the immunogenic potential of these vaccines, as they are able to stimulate strong, robust immune responses in fewer doses which minimises side effects, thus lowering the ‘per cost dose’. This enhances vaccine uptake within a population, resulting in more effective coverage.

1.2.2.1 Vectored vaccines

Due to the risks associated with the reversion to virulence of attenuated vaccines, viral-vectored vaccines are an attractive prospect. A genetically stable virus can be genetically manipulated to be replication-deficient. This generates a platform into which any genetic material can be incorporated. The viral vector will be able to infect its target cell efficiently, ensuring effective delivery of the antigen and stimulating innate and adaptive immune responses to the transgene. Incorporation of highly active mammalian promoters, such as the cytomegalovirus (CMV) immediate-early gene promoter, allows high levels of transgene expression, maximising immunogenicity. However, immune responses towards the viral vector are also induced, which reduces the durability of transgene expression and can prevent amplification of these responses with a boost dose. Several viruses are currently under investigation as vectors for this method, including members of the adenovirus, poxvirus and paramyxovirus families (Brun, 2008). DNA viruses in particular, such as members of the adenovirus and poxvirus families, are attractive candidates as these large viruses have stable genomes, capable of withstanding genetic manipulation and gene insertion.

1.2.3 Vaccine stimulation of mucosal immunity

The majority of licensed vaccines are used to immunise individuals systemically, and are usually delivered via the intramuscular route. They therefore do not stimulate a mucosal response. These vaccines rely instead on the ability of the immune system to mobilise appropriate immune effector cells to the site of infection, providing immunity to a virus wherever it is subsequently encountered. However, the principle entry route for viruses is via a mucosal membrane and therefore a vaccine that can stimulate a mucosal immune response, will be more efficient at preventing viral infection and disease, than a systemically targeted vaccine (Holmgren & Czerkinsky, 2005, Slatter, 2008, Vujanic et al., 2010, Yuki & Kiyono, 2009). In addition, stimulation of a mucosal response often results in the co-stimulation of a systemic response, allowing complete protection to be elicited towards a virus (Muller et al., 1995, Staats, 1994, Staats et al., 1996, Wu & Russell, 1998). Therefore, mucosal vaccination is a more appropriate strategy against viruses, particularly respiratory pathogens (Holmgren & Czerkinsky, 2005). This method of immunisation stimulates

a more ‘natural’ immune response towards the virus and can promote the retention of memory effector T-cells at mucosal sites, allowing more rapid stimulation of the immune response upon subsequent encounters (Yuki & Kiyono, 2009).

Potential mucosal immunisation includes nasal, oral, vaginal and anal delivery. However, the intranasal route of vaccine administration can successfully prime the immune response in both local and systemic compartments (Holmgren & Czerkinsky, 2005, Slatter, 2008, Yuki & Kiyono, 2009). One potential target for consideration for a mucosal delivered vaccine is RSV, an important pathogen of infants and elderly persons. The remainder of this review focuses on adenoviruses as potential viral vectors to tackle RSV disease, the immunopathology of RSV, and methods to develop a successful vaccine candidate.

1.3 Adenoviruses as vaccine vectors

1.3.1 Adenoviruses molecular biology

Adenoviruses are members of the *Mastadenovirus* genus of which there are more than 51 acknowledged serotypes, classified between six species, A-F. Species C viruses, which include human adenovirus 5 (Ad5), cause mild upper respiratory tract disease. They have been suggested as appropriate viral vector candidates for intranasal immunisation as they have a natural tropism to the mucosal surfaces of the lung, where there is extensive expression of their target receptor (Santosuosso et al., 2005). A comparison of intranasal immunisation with either an attenuated replication-competent vesicular stomatitis virus, Venezuelan encephalitis virus replicon particles, plasmid DNA, *Mycobacterium smegmatis,* or a replication-deficient Ad5 vector, determined that Ad5 was superior at eliciting an immune response towards an antigen (Barefoot et al., 2008). Ad5 vectors have been used in both oral (Sharpe et al., 2002) and intranasal immunisation routes (Van Kampen et al., 2005), of which the intranasal route was demonstrated to be more immunogenic and was able to overcome pre-existing immunity towards the vector (Barefoot et al., 2008, Schulte et al., 2009, Thacker et al., 2009). Studies suggest that Ad vectors induce a more robust mucosal immune response when used to immunise animals via

the intranasal route than other viral vectors and thus may be potential vectors for respiratory vaccines (Fu et al., 2009a).

Ads have non-enveloped, icosahedral shaped particles, which contain a linear double-stranded DNA genome which varies between 30-45kb in size depending on the serotype studied. Their large genome has a tightly regulated method of transcription (Fig. 1.1), and has a large coding capacity which can be genetically altered to allow foreign gene insertion (Russell, 2000, Tatsis & Ertl, 2004).

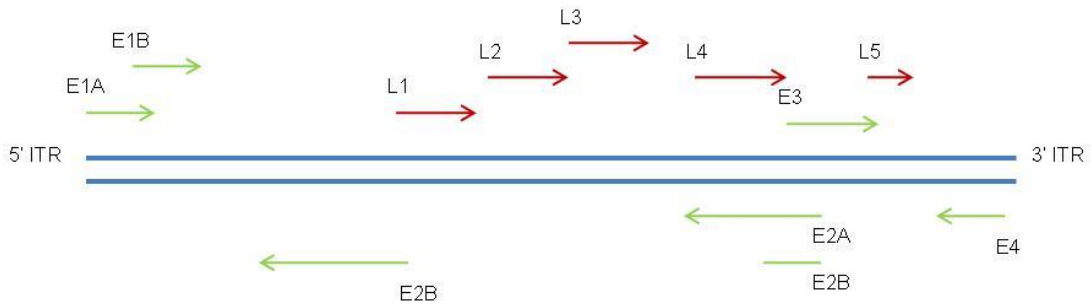

Figure 1.1 The main transcription products of the adenovirus genome (Ziff, 1980). The Ad5 genome encodes several proteins through a combination of alternative splicing and encoding proteins on both strands of the double stranded DNA genome. Upon entry to a host cell, replication is stimulated by early genes E- (green arrows), in a progressive manner. Early gene products encode transcriptional regulators and modulator proteins. These proteins stimulate late stage gene expression L- (red arrows). The late genes encode the structural proteins of the virus and are vital for capsid assembly.

Ad viruses have two stages to their replication, early and late. Early (E) gene expression is essential as it creates proteins and a cellular environment necessary for replication of the virus genome. The E1 and E4 genes encode regulators of gene expression which target various host proteins and further stimulate the expression of the other early gene regions, such as E2 (Fig. 1.1). The E2 region encodes proteins which are directly involved in Ad DNA replication, such as DNA binding protein (DBP) (Imler, 1995). The E3 region is non-essential for growth in tissue culture, however *in vivo,* E3 proteins have an important role in the Ad life cycle, as they are able to manipulate the hosts immune response, preventing recognition and clearance through adaptive immune responses. E3 expression can also be induced by NFκB, a

transcription factor involved in the induction of innate immune responses. This ensures that immune modulating genes are expressed in response to immune system stimulation. The E3 region encodes proteins such as E3-19K, which can bind to MHC class I molecules in the endoplasmic reticulum (ER) and prevent their transport to the cell surface (Burgert, 1985). This effectively down regulates MHC class I expression, hindering immune surveillance by $CD8^+$ T-cells (Sester et al., 2010). During late stage of replication, the late genes L1-5, are expressed (Fig. 1.1). These encode the 11 structural proteins which are required to construct the virus capsid and include the hexon, penton and fibre proteins.

1.3.2 The Ad5 vector system

Thorough understanding of Ad molecular biology has been essential for the successful generation of vaccine vectors. Ad5 is one of the most utilised vector systems due to its strong immunogenicity (Tatsis & Ertl, 2004).

Ads have a rigid protein capsid which dictates the genome size of the virus. This same principle limits the amount of additional genetic material which can be incorporated into an Ad vector. Ads can package up to 105% of their standard genome length, after which the constructs become highly unstable and are more likely to undergo rearrangement to lose the incorporated transgene (Bett et al., 1993, Ghosh-Choudhury, 1987). However, vector capacity can be increased through the deletion of certain regions of the Ad genome.

Standard Ad vectors for vaccination contain deletions in the E1 and/or E3 regions of the Ad5 genome, which increase the capacity for foreign DNA to 6.5kb. These were generated through molecular cloning and transfection of complementing cell lines, which ensures greater control of the Ad5 genome and eliminates the risk of potential contamination from helper viruses (Ghosh-Choudhury et al., 1986, Tatsis & Ertl, 2004). The removal of the E1 genes renders the virus replication-deficient, as it impairs the ability of the other early genes to be expressed halting the transcription cascade. This significantly impairs viral replication but prevents apoptosis of the host cell and thus premature vector removal, and improves the safety profile of the constructs (McCoy et al., 2007, Tatsis & Ertl, 2004). Deletions in the non-essential E3 region of the Ad5 genome further increase the vector's coding capacity for

foreign DNA and ensures that the E3 immune modulatory functions are not expressed and thus immunity towards the transgene can be induced. Complementing cell lines such as HEK293 cells (Table 2.1.5), have been developed which provide the missing E1 gene products *in trans*, allowing viruses to be grown to high titres (Graham et al., 1977).

Studies performed with first generation vectors ascertained that certain cellular factors, such as NF-IL6, were able to perform E1 like functions, thus enabling replication-deficient viruses to replicate in a host cell (Imperiale et al., 1984, Spergel et al., 1992). As such, second generation vectors were engineered primarily for gene therapy studies. These vectors have further deletions within the Ad genome to increase vector transgene capacity but also to decrease immunogenicity, and the risk of vector replication in complementing cell lines (Kindsmuller et al., 2009). In addition, third generation 'gutless' vectors have been developed to encode only minimal Ad genes within the genome.

1.3.3 Advantages and disadvantages of the Ad5 vector system

The Ad5 vaccine vector system has not, to date, yielded a successful vaccine platform for use in the clinic. However, their use as potential vaccine vectors has not subsided. A large variety of replication-deficient vaccine vectors exist with alternative gene deletions which reduces potential side effects associated with replication-competent vectors. This characteristic also ensures that they can be used to immunise immunocompromised individuals without the concern of overwhelming the immune system. The molecular cloning techniques have produced commercially available kits, such as the pAdEasy (Strategene) and pEntry (Invitrogen) systems. These kits allow the rapid generation and isolation of research grade Ad5 vectored vaccines to allow the rapid investigation of potential candidates.

Ads also have a broad cell tropism (Nanda et al., 2005). Ad5 in particular uses the Coxsackie adenovirus receptor (CAR) as its primary cell receptor which is expressed on many cell types, including myoblasts, hepatocytes and epithelial and endothelial cells (Tatsis & Ertl, 2004). In the airway, CAR is not expressed but Ad5 is able to utilise an alternative isoform to infect these cells (Excoffon et al., 2010). This characteristic ensures a large number of cells can be transduced, maximising

transgene antigen exposure to the immune system. Therefore, the route of immunisation can be chosen to best stimulate the type of immune response needed to provide protection against a pathogen. In addition, Ad5 can infect dividing and non-dividing cells (Tatsis & Ertl, 2004). This also ensures that transgene expression can be supported in a large number of cells.

Standard Ad5 vectors are amenable to large scale growth for commercial production as they can grow to high titres in a stable manner. The generation of stable replication-complementing mammalian cell lines, such as HEK293 cells, allows the replication-deficient viruses to be propagated in tissue culture. This reduces the potential for contamination with other viruses or helper viruses, which could lead to recombination and reversion to a replication-competent virus. Furthermore, Ad5 does not routinely integrate into the host cell chromosomes, improving the safety of the vector (Xu et al., 2009).

Furthermore, Ads naturally stimulate a strong immune response which is biased towards a T_h1 response both via systemic and mucosal immunisation routes as discussed below in Section 1.6.2 (Santra, 2005). Upon entry to a host cell, innate immune responses are triggered, which in turn stimulate the adaptive response. Ads stimulate high avidity and high titre neutralising antibodies against encoded transgenes, in addition to a strong $CD8^+$ T-cell response to both immunodominant and non-immunodominant epitopes (Barefoot et al., 2008, Santra, 2005, Tatsis & Ertl, 2004). This can greatly enhance the potency of the vaccine as, if an increased array of epitopes is recognised by the immune system, it decreases the chance of virus escape (Santra, 2005). Although this factor is not important for vaccines against more stable viruses, those with a high mutagenic rate, such as HIV and influenza, are therefore less likely to overcome the immune system and result in disease.

Pre-existing immunity towards Ad5 has been shown to have a negative impact on the immune response towards the transgene, reducing transgene expression from months to weeks (Zaiss, 2009). In addition, the immunogenicity of Ad5 can affect the efficacy of a vaccine if multiple doses are required, as these generate strong anti-Ad5 immune responses (Santra, 2005). Neutralising antibody typically targets the hexon, fibre and penton proteins and the cellular response is often directed to the E1a, E1b,

E2a and hexon proteins (Gahery-Segard et al., 1998, Thacker et al., 2009, Yang et al., 1995). Serological studies have indicated a high prevalence of anti-Ad5 immunity in the human population, which may hinder vaccine efficacy in these populations (Mast et al., 2009). However, some studies have indicated anti-Ad5 immunity may not impede an immune response towards a vaccine transgene (Casimiro et al., 2003). One particular study using a bovine Ad vector demonstrated that seropositive animals were able to mount an effective immune response against the vaccine transgene in spite of pre-existing immunity against the vector (Babiuk & Tikoo, 2000). Another group investigating this effect in humans also confirmed this finding (Gahery-Segard et al., 1998). Therefore, other groups suggested Ad5 could be used as part of a heterologous (rather than as a homologous) immunisation regime to minimise anti-Ad immunity issues (Natuk, 1994, Reyes-Sandoval, 2010, Santra, 2005, Shiver et al., 2002). Heterologous regimes involve immunising individuals with several different types of vaccine preparation such as live recombinant, DNA and subunit, whereas homologous regimes involve immunisation with the same vaccine preparation. In response to this issue, alternative Ad serotypes are currently being investigated as suitable vaccine vectors. Serological studies have suggested that Ad25, Ad11, Ad35 and Ad3 could be used as potential vectors, however, these have been shown to be less immunogenic than Ad5, in terms of the transgene response elicited (Abbink, 2007, Barouch et al., 2004, Lemckert et al., 2005, Li et al., 2009, Mast et al., 2009). Furthermore, these constructs are also less immunogenic than Ad5 when used in heterologous and homologous immunisation strategies (Santra et al., 2009). One solution is to replace the fibre knob of Ad5, the receptor attachment site, with that of another serotype, such as Ad35; this has been shown to retain immunogenicity but circumvent pre-existing immunity (Nanda et al., 2005). In addition, animal Ads particularly chimpanzee Ad serotypes such as, ChAd7, ChAd68 and ChAd1/5 are under consideration as potential vectors because humans do not have pre-existing immunity towards them (Dudareva et al., 2009, McCoy et al., 2007, Peruzzi et al., 2009).

1.3.4 Adenoviral vectored vaccines

Ad5 vectors have been investigated as a potential proof-of- principle vaccines for a number of viruses. A non-exhaustive list includes: HPV (Berg et al., 2007, Tobery et

al., 2003); rabies (Vos et al., 2001); human immunodeficiency virus (HIV) (Flanagan et al., 1997, Liu et al., 2008, Pinto et al., 2004); Japanese encephalitis virus (JEV) (Appaiahgari et al., 2006, Peng, 2008); dengue virus (Jaiswal et al., 2003, Khanam et al., 2006, Khanam et al., 2009, Raviprakash et al., 2008); severe acute respiratory syndrome (SARS) coronavirus (Kobinger et al., 2007, Ma, 2006, See et al., 2008); hepatitis C virus (HCV) (Martin et al., 2008); influenza A virus (Hoelscher et al., 2006, Naskalska et al., 2009); hantavirus (Safronetz et al., 2009); measles (Fooks et al., 1998, Fooks et al., 1995); western equine encephalitis virus (WEEV) (Wu et al., 2007); and rotavirus (Both et al., 1993, Gorziglia & Kapikian, 1992, Liu, 2005).

One of the most high profile Ad5 vaccine trials was the Merck Ad5-HIV vaccine STEP trial, which used an Ad5 E1-deleted vector expressing the *gag*, *pol* and *nef* genes of HIV-1 (Priddy, 2008). This vaccine underwent Phase III clinical trials, but was unsuccessful. Further investigation revealed that the vaccine did not prevent HIV infection or lower virus load during early infection. Of great concern was that the incidence of HIV infection was higher in the immunised group as opposed to the placebo recipients (Buchbinder et al., 2008), despite the stimulation of high levels of HIV specific $CD8^+$ T-cells (McElrath et al., 2008). Initially, it was assumed that the basis for this was that $CD8^+$ T-cell responses had also increased the $CD4^+$ T-cell population; as $CD4^+$ cells are the target for HIV, this would lead to enhanced infection rates. One group claimed this was unlikely as they could not demonstrate significant $CD4^+$ T-cell expansion (Koup et al., 2009), however, they used a different vaccine candidate which contained an additional E3/E4 deletion which may have affected the results. Another theory suggested that the degree of pre-existing antibody in the vaccine recipients was the cause, leading to premature removal of the vaccine, reducing transgene expression and thus the immunity induced to HIV. The vaccine stimulated high levels of HIV specific $CD8^+$ T-cells but this response did not correlate with protection (Buchbinder et al., 2008). A later study suggested that pre-existing immunity was unlikely to be the cause of this failed vaccine as neutralising epitopes towards Ad5 are different for 'natural' or 'vaccine' exposure (Cheng et al., 2010). This work did confirm that natural immunity was primarily directed to the fibre protein, so molecular manipulation of this protein may allow circumvention of the immune response. Regardless, the study suggested that a future vaccine must be capable of stimulating robust $CD8^+$ and $CD4^+$ responses to ensure a broad

stimulation of the immune response as $CD8^+$ T-cell responses alone may not be sufficient to protect against the establishment of an HIV infection (McElrath et al., 2008).

Despite this disappointing outcome, Ad5 still remains a vector of choice for development of potential vaccine candidates. One virus in particular, respiratory syncytial virus (RSV), has been of commercial interest for vaccine development. RSV is a pneumovirus and causes an acute respiratory infection. Producing a vaccine for this virus has a number of issues and difficulties surrounding its development, particularly the immunogenicity of RSV and of traditional vaccine candidates. Thus, recombinant delivery vectors represent an alternative option and Ad5 therefore represents a good system in which to generate a proof-of-principle vaccine for this virus.

1.4 Introduction to Pneumoviruses

1.4.1 Pneumovirus phylogeny

RSV is a member of the pneumovirus genus (family *Paramyxoviridae*, subfamily *Pneumovirinae* (Fig. 1.2)). Within this genus there are three members but only two are of importance for this study, RSV and pneumonia virus of mice (PVM). Recently, a pneumovirus like virus has been isolated from dogs (Renshaw, 2010).

The majority of information with regards to pneumoviruses has been derived from RSV but where comparisons have been made the features have proved to be applicable to other pneumoviruses. RSV and PVM share a high degree of similarity both at the molecular level in terms of gene order and function (Chambers et al., 1990, Thorpe & Easton, 2005), and at the cellular level in terms of the pathogenesis of RSV infection of humans and PVM infection of mice (reviewed by (Rosenberg & Domachowske, 2008). All pneumoviruses, indeed all paramyxoviruses have a negative sense, single-stranded RNA genome. They also have considerable commonality in gene content and gene order (Fig. 1.2). Members of the pneumovirus genus differ from other members of the subfamily *Pneumovirinae* through the presence of two extra genes, NS1 and NS2, situated at the 3' end of the RNA

genome. Also, the *Pneumovirinae* differ from the *Paramyxovirinae* through the presence of the M2 gene near the 5' end of the genome.

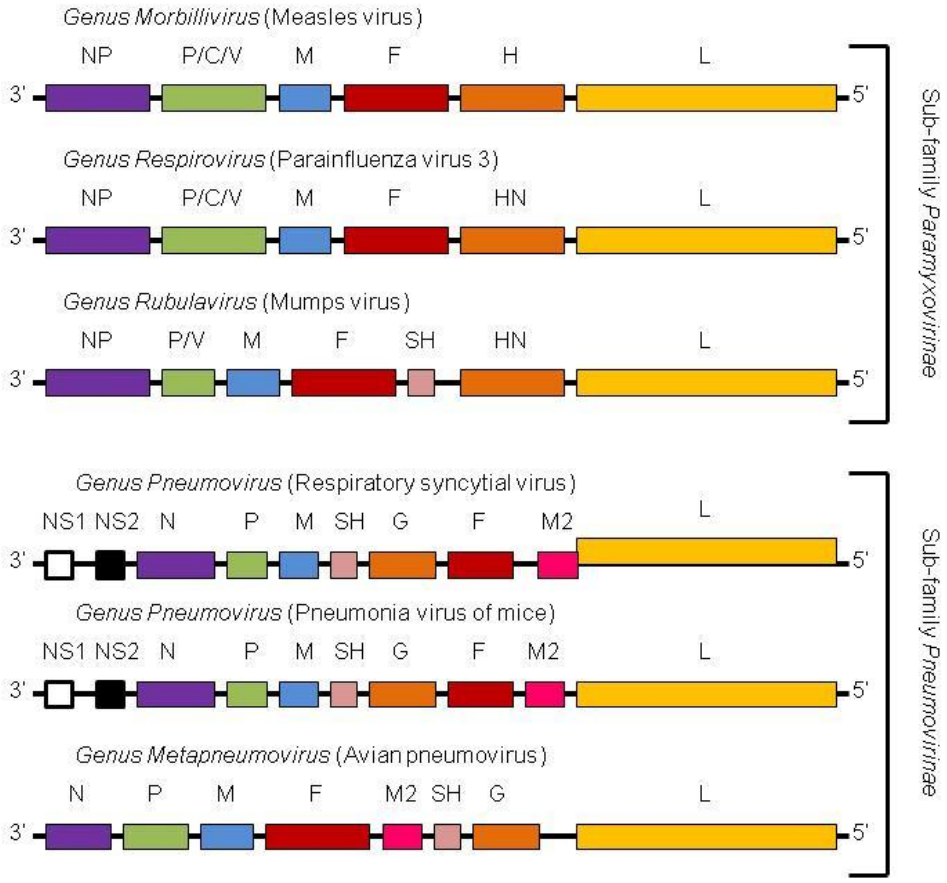

Figure. 1.2. The genome organisation of selected members of the virus family *Paramyxoviridae* ((Easton et al., 2004).
Viruses within the family *Paramyxoviridae* all have negative sense, single-stranded RNA genomes. The family is subdivided into the *Paramyxovirinae* and *Pneumovirinae* subfamilies, the members of which cause diseases in animals and humans. Members of the *Paramyxovirinae* include the Avulavirus, Henipavirus and TPMV-like virus genera but of importance to human health are the Morbillivirus, Respirovirus and Rubulavirus genera; of which measles virus, parainfluenza virus and mumps viruses are members respectively.
The second subfamily, *Pneumovirinae*, is further subdivided into two genera; Metapneumovirus and Pneumovirus. The Metapneumovirus genus contains two species, avian pneumovirus (APV) and human metapneumovirus (hMPV) while the pneumovirus genus contains RSV, bovine RSV and PVM. The genome of members of the *Paramyxoviridae* family have similar gene orders and functions. Members of the subfamily *Pneumovirinae* differ from the *Paramyxovirinae* in that they typically have 8-10 genes as opposed to 6-7. The additional genes include the M2 gene for all pneumoviruses, and the NS genes for the pneumovirus genus only. The genome organisation of representatives of each genus is shown and the genes are ordered in a 3'-5' manner and colour coded for similar functionality.

1.4.2 Pneumovirus gene function and lifecycle

Many reviews are available which deal with pneumovirus lifecycle and gene function in detail but for the purposes of this study, the lifecycle of the pneumoviruses will be summarised (Cowton et al., 2006, Easton et al., 2004, Easton et al., 2007).

The members of the pneumovirus genus have particles that are pleomorphic and can exist in filamentous and spherical enveloped forms at approximately 80-120μm in diameter (Compans, 1967), as illustrated diagrammatically in Fig. 1.3. The viruses have 8-10 genes, NS1, NS2, N, P, M, SH, G, F, M2 and L, which express their respective proteins of the same designation. For RSV, 11 proteins are translated from 10 genes.

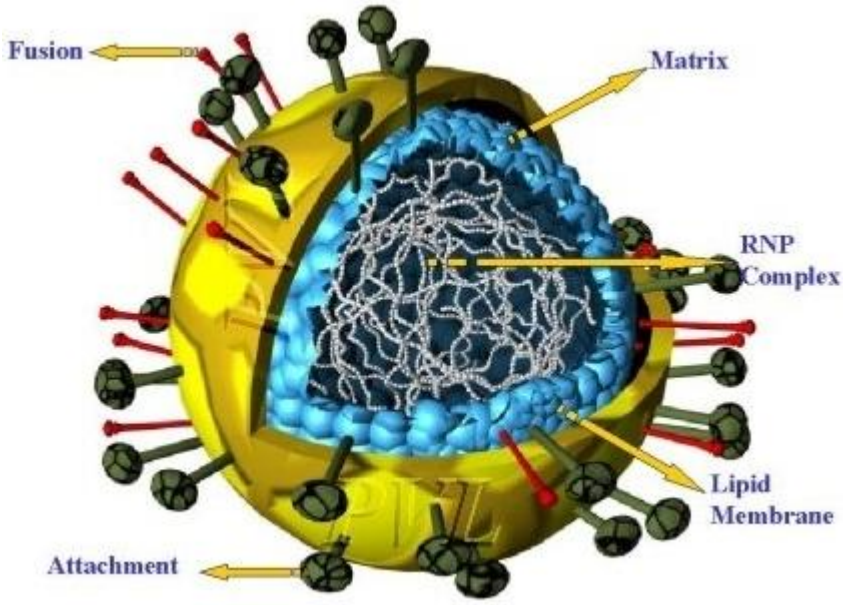

Figure 1.3. The typical structure of the pneumovirus virion (Kindly provided by A. Easton).

An illustration of the structure of the pneumovirus virion. The external fusion (F, red spikes), attachment (G, green protrusions) and small hydrophobic (SH) proteins are embedded in a lipid envelope derived from the host cell plasma membrane. The matrix (M, blue layer) protein interacts with the cytoplasmic tails of the F, G and SH proteins, and the ribonucleoprotein (RNP, white strands) complex to allow virus assembly. The RNP complex consists of the nucleoprotein (N), phosphoprotein (P), and viral polymerase (L) in complex with the RNA genome. The M2-1 and M2-2 proteins may also form part of the complex.

The lipid envelope of the virus is derived from the host cell membrane (Compans, 1967). Three external proteins are embedded in lipid rafts within this membrane the

fusion (F), attachment (G) and small hydrophobic (SH) proteins (Fleming et al., 2006, Low et al., 2008). The G protein (green spike, Fig. 1.3), is involved in the attachment of the virus to target cells and is highly glycosylated, (Easton et al., 2004). It is present in two forms in an infected cell, one being the full-length protein which is membrane-bound and the other a smaller molecule, generated from an internal initiation codon. This smaller form of the G protein can be secreted and is believed to have an immunomodulatory function (Easton et al., 2004). The PVM G protein has 11% amino acid sequence identity with that of RSV (Easton & Chambers, 1997) and is believed to be a main virulence factor, as attenuated PVM strain 15 has multiple differences in the G gene when compared to pathogenic strain J3666 (Krempl et al., 2007, Thorpe & Easton, 2005). The second external protein is the F protein (red spike, Fig. 1.3). This protein is involved in the fusion of the viral envelope and the host cell plasma membrane in a pH independent manner, and is believed to be sufficient to achieve viral entry and exit (Easton et al., 2004, Fleming et al., 2006, Kahn et al., 1999, Karron et al., 1997, Techaarpornkul et al., 2001). The F protein is synthesised as an F_0 70-kDa protein precursor which is processed by proteolytic cleavage in the *trans* Golgi network, generating two subunits F_1 and F_2, which are linked by a di-sulphide bridge (Chambers et al., 1992). The PVM F protein has 40% amino acid sequence identity with that of RSV and is believed to perform a similar function (Chambers et al., 1992, Easton & Chambers, 1997). The final external protein is the SH protein, which is also present on the external surface of the virus particle. The precise role of this protein is not known, however, it is thought that the SH protein of RSV is involved in the fusion process of the virus (Heminway et al., 1994), and it may have a role in modulating the T_h1 $CD4^+$ T-cell response through the inhibition of cytokine expression (Tripp et al., 2000). The SH protein of PVM has 22% amino acid sequence identity with that of RSV, and is significantly larger than its RSV counterpart (Easton & Chambers, 1997, Easton et al., 2007). It is unknown whether the difference in size corresponds to a difference in function between the two proteins.

Other viral genes encode the internal proteins of the particle or non-structural proteins. The matrix (M) protein (blue layer, Fig. 1.3), plays a role in viral assembly and viral genome transcription (Ghildyal, 2006). The protein forms a layer between the viral envelope and the ribonucleoprotein (RNP) complex (white strands, Fig. 1.3).

The M protein has an inhibitory role during viral transcription, and is imported to the nucleus during the early stages of infection (Ghildyal et al., 2003, Ghildyal, 2006). During the switch from transcription to replication of the viral genome, the M protein localises once again to the cytoplasm through interactions with the M2-1 protein (Ghildyal et al., 2009, Li et al., 2008). The M-M2-1 complex then associates with the RNP complex, allowing the M protein to interact with both the N protein and the cytoplasmic tails of the F and G proteins, hence allowing efficient assembly of the virus (Ghildyal et al., 2002, Teng & Collins, 1998). The M protein of PVM contains 42% amino acid sequence identity with that of RSV (Easton & Chambers, 1997).

The RNP complex is mainly comprised of the nucleocapsid (N), phosphoprotein (P) and the polymerase (L) proteins, complexed with a molecule of genomic RNA. The complex is involved in maintaining the genome structure to ensure efficient transcription can occur (Fig. 1.4). The PVM N protein has the greatest amino acid sequence similarity with its RSV counterpart at 60% (Barr et al., 1991). The N protein can aggregate and bind to viral RNA in a non-sequence specific manner, which is thought to induce a conformational change in the protein (Murphy et al., 2003). Amino acids $N_{352\text{-}369}$ at the C-terminus of the N protein have been identified as being essential for its function (Stokes et al., 2003), enabling the protein to interact with the P protein and preventing its self-aggregation. The P protein is an important co-factor for the polymerase and is one of the proteins which differs significantly between the PVM and RSV viruses; PVM P protein has 40.9% amino acid sequence identity with the RSV protein (Krempl et al., 2005). However, unlike RSV, the P gene of PVM contains an internal open reading frame (ORF), which encodes the P2-2 protein; synthesis of this protein is initiated through an internal start codon (Barr et al., 1994). The P2-2 protein is thought to inhibit viral transcription in a dose-dependent manner (Dibben et al., 2008). In addition, the RSV P protein also interacts with the M protein at a particular serine residue, S_{54}, the interaction of which is important for viral assembly upon exit from the cell and virus uncoating upon cell entry (Asenjo et al., 2008). The polymerase subunit is the final member of the RNP complex. This viral enzyme is required for genome replication and transcription upon host cell entry. The polymerase has a multifactorial role: the enzyme is able to transcribe mRNA from the genome, adding a 5’ cap and a 3’ poly(A) tail, synthesise an antigenome template and also, synthesises new viral genomes from this anti-

genome template (Edworthy & Easton, 2005, Poch et al., 1990). The L protein of PVM has 53% amino acid sequence identity with that of RSV (Easton & Chambers, 1997).

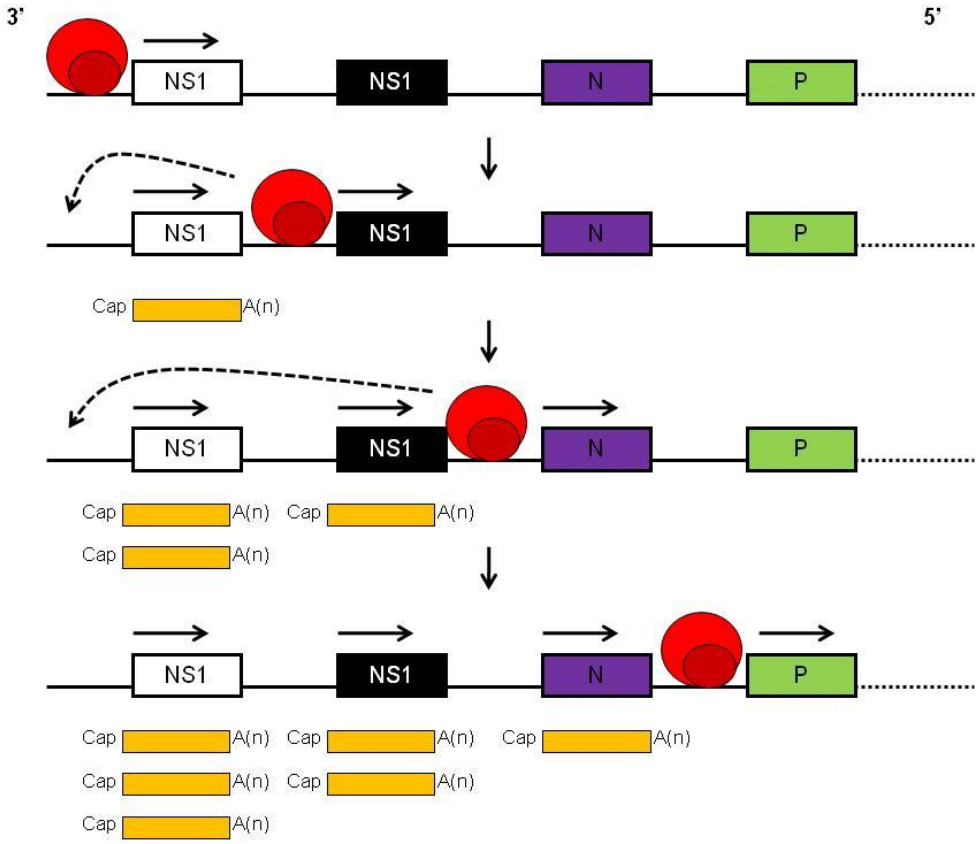

Figure 1.4. The method of transcription used by the pneumoviruses.
The transcription of the pneumovirus genome is similar to that described for other non-segmented, negative-sense RNA viruses. The polymerase complex (red) associates with the 3' leader sequence in the genome (Easton et al., 2004). The complex then initiates transcription of the first gene and synthesises a 5' cap on the mRNA (Liuzzi et al., 2005), stimulating mRNA transcription (top panel). Upon reaching a 'gene end' sequence the polymerase complex can dissociate from the genomic template, synthesising a poly(A) tail and return to the 3' leader sequence to re-initiate transcription (dotted line, panel two). Alternatively, the complex remains bound and scans through the intergenic region until the next 'gene-start' sequence is recognised (Kolakofsky, 2004); transcription of this gene then occurs. The second mechanism is not as efficient as the first, therefore generating a gradient of mRNA synthesis along the genome (bottom panel).

The M2 gene expresses two proteins, M2-1 and M2-2, through a novel, tightly regulated coupled translation mechanism (Ahmadian et al., 1999, Ahmadian et al.,

2000, Gould & Easton, 2005). Due to its position near to the 5' end of the viral genome, the M2 proteins are expressed at low levels (Ahmadian et al., 1999). The significant difference between the M2 gene of RSV and PVM is that the M2 gene in RSV overlaps with the L gene, which does not occur in the PVM genome (Thorpe & Easton, 2005). For RSV, the M2-1 protein is involved in viral RNA synthesis and acts as a transcriptional elongation factor (Teng & Collins, 1998, Tran et al., 2009) whereas the role of M2-2 is believed to be a negative regulator of RNA replication (Cheng et al., 2005, Collins, 1996). Thus, the RSV M2-2 protein may control the balance between genome transcription and replication (Bermingham & Collins, 1999). The PVM M2 proteins have 42% and 10% amino acid sequence identity respectively with their RSV counterparts (Easton & Chambers, 1997) and are associated, in part, with the RNP complex (García et al., 1993). Studies of the M2 protein of PVM have elucidated that the function of the M2-1 protein is similar to that of RSV, but at high levels decreases viral RNA replication (Dibben et al., 2008). The M2-2 protein of PVM has been demonstrated to inhibit RNA replication in a dose dependent fashion (Dibben et al., 2008). Therefore, in both viruses the control of these genes is critical for efficient viral replication.

Lastly, the virus encodes two non-structural proteins from its two 3' proximal genes, NS1 and NS2. These proteins are expressed first and most abundantly from the pneumovirus genome (Fig. 1.4). These proteins modulate the innate immune response and hence contribute to viral virulence (Bossert & Conzelmann, 2002, Buchholz et al., 2009, Wright, 2006); they also determine host range (Bossert & Conzelmann, 2002, Schlender et al., 2000). RSV mutants which lack these genes demonstrated an attenuated phenotype in chimpanzees (Jin et al., 2000, Whitehead et al., 1999). Likewise, PVM ΔNS1/NS2 have an attenuated phenotype in mice (Buchholz et al., 2009). NS1 and NS2 proteins suppress the innate type 1 interferon (IFN) response, through modulating the synthesis and function of these cytokines (Swedan et al., 2009), particularly NS2 which inhibits the IFN system through multiple pathways (Buchholz et al., 2009, Ling et al., 2009). This in turn can inhibit dendritic cell (DC) maturation and activation, which can further affect the immune response (Munir et al., 2008). In addition, the NS proteins of bRSV are sufficient to rescue an unrelated virus from an IFN response in bovine cells (Schlender et al., 2000). Furthermore, the proteins have been shown to have an anti-apoptotic function

through preventing TNF-α mediated apoptosis (Bitko et al., 2007). The NS proteins of PVM differ significantly in sequence from those of RSV with 15% and 20% amino acid sequence identity for NS1 and NS2 respectively (Krempl et al., 2005). The precise role of the NS1 protein of PVM has yet to be elucidated, while the NS2 protein has been shown to be an antagonist of the type I IFN response like its RSV counterpart (Buchholz et al., 2009).

1.5 Pathogenicity of pneumovirus

1.5.1 RSV disease

Respiratory disease is a significant health issue worldwide. RSV is a common causative agent of respiratory disease and globally, is estimated to cause 64 million infections and 160, 000 deaths annually (Cowton et al., 2006, van Drunen Littel-van den Hurk, 2007). RSV is a highly contagious winter pathogen in temperate climates, and is associated with seasonal epidemics during which two antigenic subtypes co-circulate (Falsey et al., 2005, Noyola et al., 2007).

RSV is more commonly associated as an infant respiratory pathogen, and is understood to be the single most important viral cause of respiratory tract infections (Bennett, 2007, Nicholson et al., 2006, Simoes, 1999). In infants, RSV causes bronchiolitis, a condition characterised by coughing, wheezing and breathing difficulties, which is associated with an acute infection in 50-90% of cases in infants (Bennett, 2007). The morbidity associated with RSV infection is high; however, the infection is resolved by innate and adaptive immune responses without further complications. In a few individuals, a severe RSV infection will develop. This is thought to occur due to the immaturity of the infant immune system, leading to an increased likelihood of an inflammatory response which is driven by the T_h2 $CD4^+$ T-cell response (Fig. 1.5) (Section 1.6.2). Severe infections are more common if infants are under two years of age, are born prematurely, or have an underlying medical condition. The immune response generated upon severe RSV infection is similar to that stimulated during episodes of asthma. This generates significant inflammation in the respiratory tract leading to breathing difficulties and a need for hospital care.

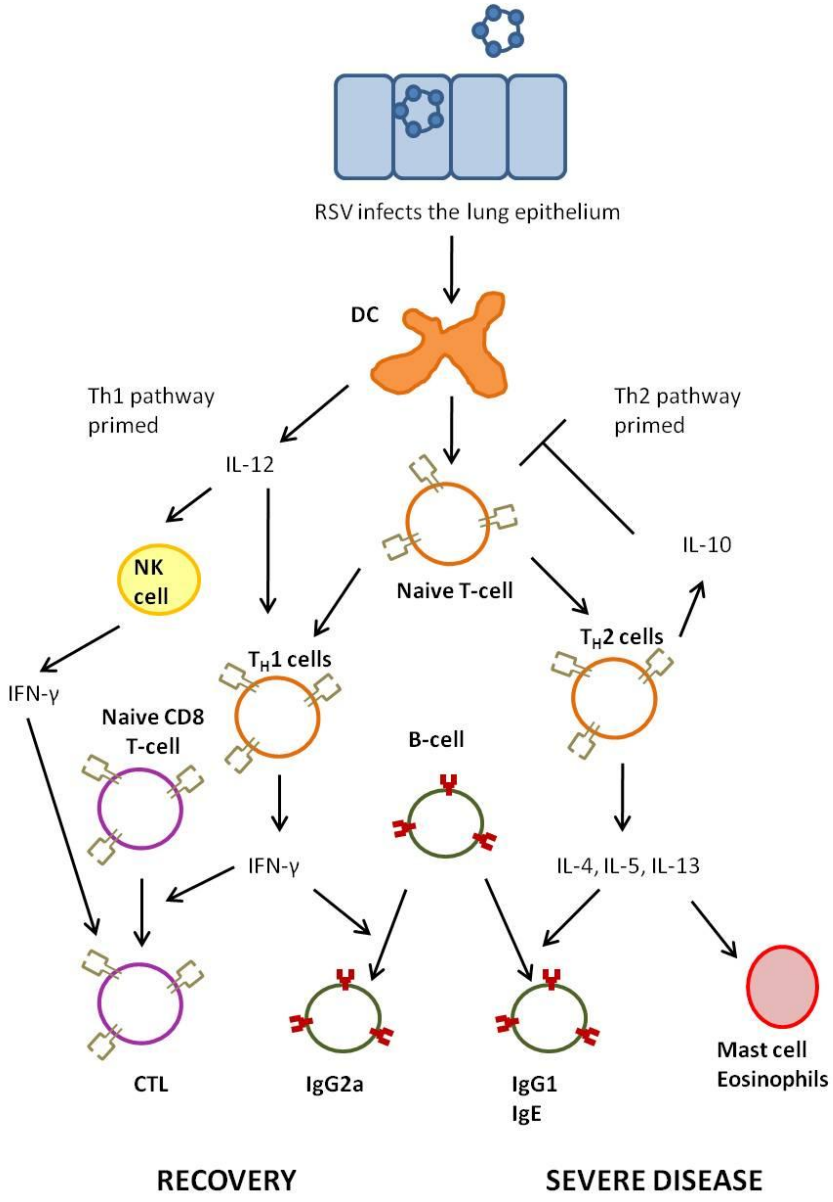

Figure 1.5. Immunopathogenesis of pneumovirus infection of infants (adapted from (van Drunen Littel-van den Hurk, 2007).

Pneumoviruses infect the epithelial cells of the respiratory tract. TLRs recognise virus specific patterns which triggers an innate immune response where IFNα/β is released to stimulate an anti-viral state (Faisca et al., 2006, Trinchieri & Sher, 2007). DCs, eosinophils, monocytes, lymphocytes and neutrophils are attracted by the cytokines released from the lung epithelium, such as TNF-α, IL-8, MCP-1, MIP-1α and RANTES. DCs can acquire RSV antigen and present it via MHC complexes to naive T-cells. Initially, a mixed T-cell response is stimulated. T_h1 $CD4^+$ T-cell are stimulated to release IFNγ, this in turn stimulates naive $CD8^+$ T-cells to mature into CTLs with cytotoxic capabilities and B-cells to secrete neutralising antibodies of the IgG2a isotype. T_h2 $CD4^+$ T-cells are also stimulated. These secrete IL-10 which inhibits DC activation of the T_h1 pathway. T_h2 T-cells release pro-inflammatory cytokines such as IL-4, IL-5 and IL-13. These attract mast cells and eosinophils and stimulate them to de-granulate and release histamine. This is an allergic immune response. In addition, B-cells are stimulated to secrete antibodies of the IgG1 and IgE isotypes. Both the T_h1 and T_h2 pathways release cytokines which inhibit the other pathway. It is the balance between these two pathways which determines the ultimate dominance of a particular pathway. If the T_h1 pathway dominates, the individual will recover and develop protective immunity. However, if the T_h2 pathway dominates, the individual will develop an enhanced disease pathology, which is allergic in nature.

In recent years, it has been elucidated that the cytokine and chemokine profile of the immune response determines the outcome of RSV disease, by affecting the outcome of $CD4^+$ T-cell differentiation. As described in Fig 1.5, a T_h1-biased response is associated with protection and clearance of a viral pathogen whereas a T_h2-biased response is associated with an immune response which is more allergic in nature. Both responses are characterised by a distinct cytokine profiles. The morbidity associated with severe RSV infections is great and it is estimated that 3% of infants admitted to hospital with severe RSV infections will succumb to the disease (Bennett et al., 2007).

A link has been established between viral respiratory infections, such as RSV, and the probability of the development and exacerbation of asthma in children (Hansbro et al., 2008). Asthma is a chronic inflammatory disease which is triggered upon exposure to particular allergens. This leads to severe airway hypersensitivity, airway narrowing and difficulties in breathing. Pro-inflammatory cytokines are released and the T_h2 $CD4^+$ T-cell population is activated, recruiting eosinophils to the airway. These release histamine, which further exacerbates the inflammatory response. Repeat episodes of asthma lead to damage to the mucosa of the lung epithelium which in turn triggers an excessive repair response. This results in airway remodelling, excessive mucus production and chronic disease (Hansbro et al., 2008), a response similar to that seen in a severe RSV infection (Kim et al., 2008, Taylor, 2007).

Evidence for a link between RSV infection and asthma includes: 80% of wheezing children have an associated viral infection (Johnston et al., 1995); RSV is frequently isolated from wheezing children (Johnston, 1999); a positive correlation has been demonstrated between persistent wheeze at 5 years of age and a history of an RSV infection with wheezing signs (Merci et al., 2007); RSV infection and allergic sensitisation to airborne allergens has been linked (Frick, 1979); and RSV bronchiolitis has been identified as an important risk factor for asthma development in children (Sigurs, 1995). In contrast, the link between an RSV and asthma is not always clear as it has been noted that the correlation between RSV infection and asthma development only occurs in a small proportion of infants (Merci et al., 2007, Murray, 1992, Pullan, 1982, Ramsey, 2007, Sims, 1978). This suggests that severe

infection or genetic factors may predispose individuals to asthma development (Mahalingam et al., 2006).

Furthermore, RSV has been identified as an important cause of respiratory disease in adults and in particular, the elderly (Falsey & Walsh, 2000, Hall, 2001b, Han, 1999). In the USA, it is estimated that RSV is thought to account for up to 10% of all winter respiratory hospital admissions (Falsey, 1995), a rate similar to that of influenza virus (Han, 1999). Adult infections present with a wide range of signs, from upper respiratory tract 'common cold'-like signs to respiratory failure, in contrast to the bronchiolitis presentation in young infants (Falsey et al., 2005, Falsey & Walsh, 2000, Han, 1999). As with infants, the severity of the illness is dependent upon the presence of underlying medical conditions and the general well-being of the individual (Walsh, 2004a, Walsh, 2004b). Healthy adults do not develop severe RSV disease, presumably because the immune system is mature and fully functional. However, re-infections can occur despite the presence of pre-existing immunity, suggesting that immunological memory to RSV is incomplete (Van der Poel et al., 1994).

1.5.2 RSV treatment

Currently, two antibody preparations are licensed for use in humans. The first is RSV-IGIV (RespiGam®), which is prepared from adult human sera, and to treat RSV infection the second is palivizumab (Synagis®), a monoclonal IgG antibody. Extensive use of RSV-IGIV has been discontinued as it is derived from blood and therefore poses a risk of transmitted blood borne pathogens. In addition, it required a large volume infusion which was not suitable for infants (Bennett et al., 2007). Palivizumab is a humanised monoclonal antibody which is directed against the F protein of RSV (Sidwell & Barnard, 2006). Motavizumab (Numax™) is a newer version of Palivizumab that is currently in Phase III clinical trials. It has been shown to reduce RSV disease with a lower dose than Palizumab (Bennett et al., 2007). Both antibodies aim to prevent the spread of RSV to the lower respiratory tract, preventing the more severe clinical manifestations of the disease.

Several anti-viral compounds intended to treat RSV are also being developed, these include: fusion/attachment inhibitors, siRNAs, N-protein inhibitors, viral polymerase inhibitors and inosine monophosphate dehydrogenase (IMPDH) inhibitors (Sidwell & Barnard, 2006). Ribavririn is currently the only anti-viral compound licensed which can be used to treat RSV infection. When phosphorylated, the drug inhibits IMPDH mRNA 5' cap formation and the viral RNA polymerase (Sidwell & Barnard, 2006). Ribavirin can inhibit RSV replication both *in vivo* and *in vitro*, however, it appears that the drug is not efficacious when used in infants suffering from severe RSV infection, as the clinical course of infection does not improve (Bennett et al., 2007, Rosenberg et al., 2005). Further studies suggested that the efficacy of ribavirin could be improved when used in combination with immunomodulator agents, such as anti-MIP-1α, Met-RANTES and montelukast (a cysteinyl leukotriene receptor antagonist), as these appear to limit leukocyte recruitment, reducing the inflammatory response and thus disease severity (Bonville et al., 2003, Bonville et al., 2004, Bonville et al., 2006b). Glucocorticoid (steroid) use has also been evaluated for the potential to reduce the inflammatory response towards pneumovirus infections. However, use of this treatment in the PVM infection model of pneumovirus disease has demonstrated that this leads to an enhanced pathology with accelerated mortality (Domachowske et al., 2001).

siRNA may provide a therapeutic treatment in the future. Effective siRNAs have been developed that inhibit the expression of the N and P proteins of RSV (Bitko et al., 2005, DeVincenzo et al., 2010), which have demonstrated the potential to generate an anti-viral effect by reducing RSV replication, allowing time for a strong $CD8^+$ T-cell response to be stimulated (Zhang & Tripp, 2008). Phase I clinical trials with ALN-RSV01, the siRNA directed against the RSV N gene, has shown promising results in that the siRNA was well tolerated in adults and demonstrated antiviral effects, irrespective of pre-existing neutralising antibody and proinflammatory cytokines levels (DeVincenzo et al., 2010).

Although these treatments are available, they are expensive and require administration in a clinical setting hence limiting their application. The anti-RSV antibody treatment is used prophylactically in high-risk infants to prevent RSV infection and ribavirin is reserved for severe infections only. Thus, supportive care, such as mechanical ventilation and supplemental oxygen therapy, remain the

standard treatment for RSV patients (Rosenberg et al., 2005). It has been observed that mechanical ventilation may exacerbate the inflammatory response towards the disease, as was demonstrated in the PVM infection model, and thus contributes to lung damage (Bem et al., 2009).

To date, no vaccine is clinically available either to treat RSV infection or promote the stimulation of a long-lived robust immune response. Several candidates have been investigated (Huang, 2009, Karron, 2005, Munoz, 2003, Plotnicky-Gilquin, 1999), but after the disaster of the FI-RSV vaccine in the 1960s (Kim et al., 1969), new candidates are pursued cautiously. A vaccine which could stimulate a long-term protective immune response against RSV would be advantageous as it could reduce the incidence of severe RSV disease and also may lead to a reduction in the development of childhood asthma, thus reducing the disease burden within healthcare settings. However, two main issues have prevented a successful candidate from reaching the clinic. The first is the failed FI-RSV inactivated vaccine, in which disease following infection was exacerbated with disastrous results (Kim et al., 1969), and the second is a lack of an amenable animal model within which to directly evaluate vaccine efficacy against RSV infection.

1.5.3 PVM infection in mice

PVM, a natural pathogen of rodents, was first isolated in 1939 during experiments to culture human respiratory viruses in mouse lung (Horsfall, 1940). The virus was isolated through the passage of lung samples from apparently healthy inbred mice into outbred healthy mice. These developed bronchiolitis signs, which progressed to a fatal pneumonia in 24% of the animals (Horsfall, 1940). Further study revealed that infection was only possible via the intranasal route (Horsfall, 1940). The virus is a common pathogen of laboratory animals if suitable precautions are not taken and once established, can cause an asymptomatic or mild infection in mice, making it difficult to eradicate from laboratory colonies (Horsfall, 1946). More recently, serological screening services and test kits have become available to test for the presence of PVM in laboratory colonies, resulting in a reduced prevalence of the virus. PVM is also a pathogen of wild rodents. One study estimated that several different species were seropositive for PVM, including voles and wood mice (Kaplan,

1980). Interestingly, analysis of human sera has identified that approximately 80% of the population is seropositive for PVM or an antigenically related virus (Horsfall, 1946, Pringle, 1986). This human seroconversion occurred by 2 years, a similar age as RSV, indicating that humans are readily exposed to the infection (Pringle, 1986). To date, the clinical relevance of these findings is unknown but the virus is not considered to be a burden to the health of the human population.

Currently, three strains of PVM have been identified and sequenced; PVM J3666 and PVM strain 15 (ATCC) are fully pathogenic whereas PVM strain 15 (Warwick) is an attenuated form, believed to have arisen through multiple passage in BS-C-1 cells (Krempl & Collins, 2004, Randhawa et al., 1995, Thorpe & Easton, 2005). As such, the infection of mice by PVM serves as an excellent model for RSV pathogenesis in humans.

1.5.4 Pneumovirus *in vivo* models

Cell culture systems have proved invaluable for virus characterisation and early evaluation of RSV treatments. However, these models lack an immune system and are thus inappropriate for vaccine efficacy studies. Potential vaccine candidates for RSV have been developed (Section 1.7), but these studies evaluated vaccine efficacy in an inappropriate *in vivo* model, the mouse. This is because mice are at best only semi-permissive for RSV infection. They can be infected but do not display outward signs of disease. Thus, efficacy must be measured through viral titre reduction, which does not correlate with clinical signs (Easton et al., 2004). Neither can RSV vaccine candidates be studied in children. Thus, an appropriate *in vivo* model for RSV needs to be developed.

1.5.4.1 RSV *in vivo* models.

The main advantage of *in vivo* models is that the disease process of a pathogen as a whole can be investigated, including toxicity and immune responses. In addition, the impact of genetic differences within a population on these processes can be investigated which would be problematic to study in cell culture.

Several species have been used to study RSV pathogenesis and also to evaluate vaccine candidates including chimpanzee and other primates, bovine, cotton rat,

guinea pig and mouse models (BALB/c, C3H and C57BL). Each model has its own advantages and disadvantages (Table 1.2). The main argument for the reduction in the use of animal models is that the findings within an animal do not necessarily reflect the situation in humans. Chimpanzees can be infected with RSV and exhibit upper respiratory tract illness but these are the most expensive and ethically problematic animal system; other primates also support RSV replication but do not demonstrate signs of disease (Belshe, 1977). The advantage of the chimpanzee model is that the immune responses of these animals is similar to humans, as opposed to rodents which do not always have the same immune genes (Moore & Peebles, 2006). Other primates can be used, but again, the immunological reagents available are extremely limited when compared to the rodent or chimpanzee model. Such animals also require a high inoculum of RSV in order to ensure that infection and viral replication occurs, which does not reflect the situation in humans (Simoes, 1999).

The cotton rat model for RSV infection at present remains the best rodent model for studying RSV. The main problem with this system is the lack of availability of reagents and genetic strains, for which the mouse model is superior. Cotton rats are susceptible to both upper and lower respiratory tract infection with RSV, similar to that in humans (Prince, 1978). In addition, the immune genes of the cotton rat are more similar to those of humans than those within the mouse model. The cotton rat genome encodes Mx genes which humans also have and in addition, they also show diminished immune responses with increased age (Boukhvalova et al., 2009).

The most commonly used animal model for RSV is the mouse, mainly due to its low cost, small size, availability of several genetic strains and wide range of available immunological reagents (Table 1.2). These animals are often used as the first stage of vaccine development. For some vaccines, successful evaluation in rodent models is sufficient to justify a move to human clinical trials. However, the knowledge of RSV immunosuppressive and sensitisation functions on the human immune system requires that potential candidates are thoroughly tested to ensure safety before use in humans. This often involves the use of rodents followed by primates before the studies are advanced to human Phase 1 clinical trials. However, despite the stringent evaluation of novel therapeutics before human trials, occasionally, the results from animals models do not reflect the effects within a human.

Type of model	Advantages	Disadvantages
Chimpanzee	Highly related to humans Immunological reagents available Similar RSV pathogenesis Vaccine enhanced disease observed Genetically varied	Expensive Limited availability of animals and facilities Genetically varied Require high inoculums, 10^4-10^6 p.f.u. Expensive, ethically problematic
Bovine (cow)	Use of natural pathogen (bRSV) Similar disease pathogenesis Bronchiolitis develops	Expensive, ethically problematic Limited immunological reagents available Bacterial superinfection common and can complicate findings No inbred strains available RSV requires 10^6 p.f.u. inoculum
Cotton rat	Inbred strains available Greater susceptibility to RSV than mice Vaccine enhanced disease observed Mx genes (these encode proteins which block the replication of some viruses)	Limited immunological reagents available Non-permissive for viral replication No knockout strains available Pneumonia disease rather than bronchiolitis RSV requires 10^5 p.f.u. inoculum
Guinea pigs	Juveniles develop bronchiolitis Inbred strains available Large size allows repeated measurements Suitable for asthma studies	Similar cost to mice Limited immunological reagents available No knockout strains available Pneumonia disease rather than bronchiolitis
Mouse	Multiple strains available: inbred, transgenic, knockout Large range of immunological reagents Low cost and easily bred Easy to manipulate large colonies Vaccine enhanced pathology Well defined immunology	Limited viral replication Large dose of RSV required No disease observed, only histopathology RSV requires 10^6 p.f.u. inoculums

PVM Mouse model	As above for mouse model Use of natural pathogen (PVM) Bronchiolitis disease develops Low dose of PVM is required to cause a fatal disease Extensive replication of PVM is observed Requires 10-60 p.f.u. inoculum	Regular screening of the colony required to ensure PVM-free Difficulties in using the virus in PVM-free environments (may require CAT3 conditions) Limited PVM-specific reagents available

Table 1.2. Comparison of *in vivo* models for evaluation of RSV treatments, including the PVM mouse model (adapted from (Moore & Peebles, 2006, Stark, 2006).

The immune response towards RSV has been extensively researched in mice, of which the BALB/c strain was determined to be the most susceptible (Jafri, 2004, Moore & Peebles, 2006, Prince, 1979). However, there are several disadvantages. A high dose of RSV is required to achieve infection, which results in only limited viral replication as the mouse is at best only semi-permissive to RSV (Bonville et al., 2006a, Jafri, 2004). In addition, the disease profile of RSV infection in mice is inappropriate for pathogenesis studies, as no overt signs of disease develop. More importantly for vaccine development, the enhanced disease observed in human infants is not replicated in the mouse model (Connors et al., 1992b).

1.5.5 PVM infection model

Despite the range of animal models available for investigating RSV and potential vaccine candidates (Table 1.2), no model is wholly appropriate to study RSV infection (Sidwell & Barnard, 2006). This is because these models rely on the infection of a host with a foreign, rather than a natural virus, and as such, the disease pathogenesis observed is different from RSV in humans. In addition, the adverse immune response seen with inactivated RSV means that any vaccine candidate must be extensively tested to ensure that no adverse priming of the immune response occurs. This would usually involve initial studies in a mouse model, followed by evaluation of candidates in chimpanzees prior to clinical trials in humans.

PVM infection of mice offers an attractive alternative model in which to study the pathogenesis of pneumoviruses. Importantly, both RSV and PVM cause a similar pathogenesis within their respective hosts and are highly similar at the genomic and molecular level (Section 1.4) (Cook et al., 1998, Cubie, 1997, Domachowske, 2000a, Rosenberg & Domachowske, 2008). Within their respective natural hosts, each virus stimulates bronchiolitis, which can also develop into a T_h2 driven enhanced disease (Barends et al., 2004). PVM has been shown to localise to the bronchiolar epithelium, similar to that of RSV in humans (Bonville et al., 2006a, Welliver, 2007). In both infections, granulocytes are recruited to the site of infection by MIP-1α and severe inflammation occurs (Domachowske, 2000a, Welliver, 2007). The PVM model recreates the severe natural RSV infection as observed in humans. High numbers of eosinophils are recruited to the lung, characteristic of the T_h2 response (Percopo et al.,

2009). PVM infection in mice therefore represents a good model in which to study respiratory virus in its relevant host (Rosenberg & Domachowske, 2008).

PVM infection of mice only requires small inoculums (10-200 p.f.u.) for disease signs to be observed and disease severity is dose-dependent (Bennett, 2007, Cook et al., 1998, Moore & Peebles, 2006). The virus takes 4-8 days to reach the maximum viral titre, which is again dose-dependent, reductions in titre can therefore be used to measure vaccine and anti-viral efficacy (Horsfall, 1951). Therefore, through identifying and developing effective vaccine candidates against PVM in the PVM infection model, it is highly likely that a similar candidate for RSV could be successful in humans. (Bonville et al., 2006a, Cook et al., 1998, Rosenberg & Domachowske, 2008).

1.6 Immune responses to pneumovirus infection

Immunity to viruses requires the immune system to recognise and destroy virus-infected cells, clearing the virus from the host. This involves the induction of both non-specific innate, and specific adaptive, immune responses. Studies of both RSV and PVM have concluded that they induce similar immune responses in their respective hosts (Collins & Graham, 2008, Faisca et al., 2006, Taylor, 2007). More information is available with regards to RSV due to its clinical significance. However, despite the wealth of information available, it is still disputed as to whether RSV pathogenesis is caused directly by the virus infection or is primarily mediated by the adaptive immune response towards it. Such information is useful for the development of a safe, effective vaccine to tackle RSV. For the purposes of this thesis, the immune responses to RSV and PVM will be briefly discussed but have been extensively reviewed elsewhere (Easton et al., 2004, Easton et al., 2007, Taylor, 2007).

1.6.1 Innate immune responses and pneumoviruses

Innate immunity is the first arm of the immune response that is stimulated to respond to RSV infection. This arm of the immune system does not recognise specific viruses,

but rather general viral ‘patterns’ to which it responds (Trinchieri & Sher, 2007). For PVM in particular, these responses are powerful enough to control the replication of ΔNS1/NS2 (attenuated) strains and render them apathogenic (Buchholz et al., 2009).

Upon infection, RSV enters the respiratory tract, where it infects lung epithelial cells. This stimulates Toll-like receptors (TLRs); TLR3/9 recognises double-stranded RNA and TLR7/8 recognise single-stranded RNA (Kawai, 2008). In particular, the F protein stimulates TLR4, triggering a signalling cascade (Faisca et al., 2006, Haeberle, 2002). Adapter molecules activate NF-κB and IRF-3/7 transcription factors, which travel to the nucleus and promote the transcription of type I IFNs and proinflammatory cytokines such as TNF-α (tumour necrosis factor), MCP-1 (monocyte chemotactic protein), MIP-1α/β (macrophage inflammatory protein), RANTES (regulated upon activation, normal T cell expressed and secreted) and interleukins e.g. IL-8, IL-12 and IL-6 (Domachowske, 2000c, van Drunen Littel-van den Hurk, 2007, Wang, 2007). The possibility of a protective function of TLR4 in response to RSV remains an unresolved issue. One group argued that TLR4-deficient mouse strains do not limit RSV replication (Ehl, 2004). Another argued that F protein stimulation of TLR4 and CD14 correlates with protective inflammatory responses (Kurt-Jones et al., 2000). TLR2/6 have also been linked with protective anti-RSV responses mediated through cytokine release (Groskreutz et al., 2006, Murawski et al., 2009). RSV is also believed to stimulate Retinoic acid inducible gene-1 (RIG-1) (Scagnolari et al., 2009). RIG-1 recognises viral RNA in the cytoplasm and undergoes a conformational change allowing it to interact with IFN-β promoter stimulator (IPS-1), also known as mitochondrial antiviral signalling (MAVS). This also leads to the recruitment of signalling molecules to promote type I IFN and proinflammatory cytokine transcription (Yoneyama, 2010).

One of the major cytokines released during the innate response is type I IFN (IFNα/β). IFNα/β signal the development of an ‘anti-viral’ state in a host cell. These soluble factors can promote this state in neighbouring uninfected cells or enhance the response in the same cell through a positive feedback mechanism (Fig. 1.6) (Haller et al., 2006). In brief, signalling pathways stimulate NF-κB and other transcription factors to upregulate particular genes, such as those encoding 2’, 5’ oligoadenylate synthetase, protein kinase R and RNase L, all of which interfere with viral replication

(Haller et al., 2006). Also upon RSV infection, cell surface molecules are upregulated to aid the recruitment of immune cells to the site of infection (Bueno et al., 2008). Major histocompatibility complex (MHC) class I molecules are also upregulated, increasing the antigen presentation for immune surveillance (Garofalo et al., 1996). IFNα/β promotes a T_h1-biased response (Durbin et al., 2002, Johnson et al., 2005, Schlender et al., 2000), but additional cytokine release from immune effector cells can weaken or alter this bias.

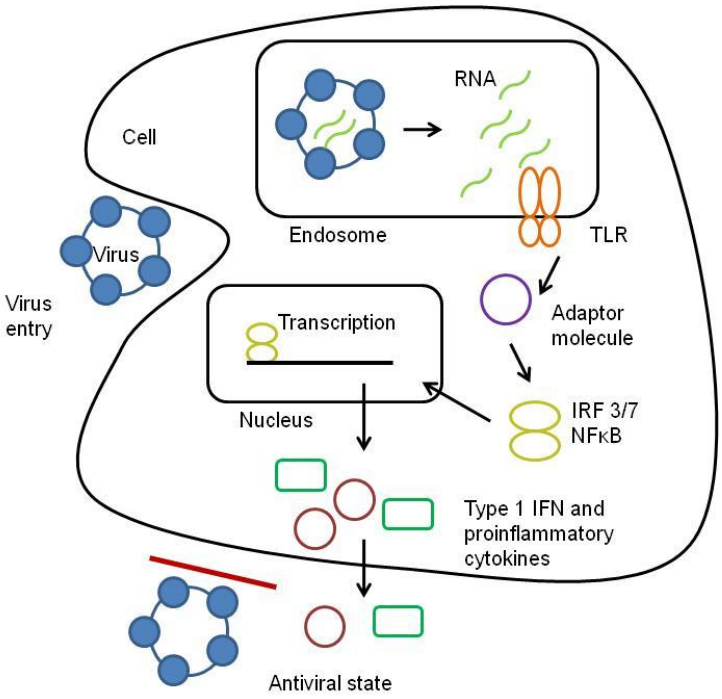

Figure 1.6. The generation of an antiviral state through induction of innate immune responses.
RSV enters a host cell through membrane fusion. Detection of the RNA genomic material stimulates immune responses. These trigger a signalling cascade through adaptor molecules which results in the stimulation of transcription factors such as IRF-3 and NF-κB. These transcription factors can enter the nucleus and up or down regulate the expression of specific mRNAs. mRNA for type 1 IFNα/β, along with pro-inflammatory cytokines such as IL-1, IL-12 and TNF-α which are also up-regulated. These cytokines are secreted by RSV-infected cells. Neighbouring cells can detect these cytokines and become stimulated to induce an 'anti-viral' state. However, infected cells are also capable of detecting these secreted cytokines, which therefore function as a positive feedback mechanism to further stimulate the induction of an antiviral state.

The cytokine and chemokine profile generated upon RSV infection is one of the most powerful responses of the immune system and links the innate and adaptive response together. Numerous studies have attempted to define the cytokine release profile following RSV infection in efforts to identify biomarkers to measure disease severity and anti-viral targets. The cytokine profile determines the character of the adaptive response generated towards RSV infection in mice and humans (Bennett, 2007). Evidence suggests that the cytokine response may mediate RSV immunopathogenesis (Culley et al., 2006, Hornsleth, 2001). This includes: infants with severe RSV disease had elevated MIP-1α and MCP-1 levels, stimulating a T_h2 $CD4^+$ biased response, whereas those with milder infections had reduced levels of these cytokines and a T_h1 $CD4^+$ biased response (Garofalo, 2001, McNamara, 2005). Additionally, during PVM infection, cytokine levels remained high after replicating virus was no longer detected, yet clinical signs persisted (Bonville et al., 2006a). Similar to RSV infection in humans, a more severe PVM infection of mice is associated with higher levels of MIP-1α (Domachowske, 2000a, Domachowske, 2000b). RSV-infected mice also produce MIP-1α but this is not associated with effector cell recruitment and protection (Domachowske, 2000c). However, blocking MIP-1α in PVM-infected mice was associated with increased mortality from greater viral replication (Domachowske, 2000a). Furthermore, in PVM-infected mice, MIP-1α in conjunction with IFNγ was required for efficient recruitment of adaptive effector cells such as neutrophils and eosinophils to the site of infection, which were associated with minimal disease signs (Bonville et al., 2009).

Cytokines released from RSV infected cells, activated professional antigen presenting cells (APCs) such as DCs or activated $CD4^+$ and $CD8^+$ cells and recruit eosinophils and neutrophils to the site of infection (Bonville et al., 2009, Domachowske, 2000b). The recruitment of eosinophils and the protective or non-protective role they may play is still unresolved. Eosinophils are typically associated with an allergic immune response, driven by T_h2 $CD4^+$ T-cells (Rosenberg et al., 2009a, Rosenberg et al., 2005, Rosenberg & Domachowske, 2001, Rosenberg et al., 2009b). They release enzymes that damage epithelial cells, whether RSV infected or not, which leads to airway damage and hypersensitivity to RSV (Rosenberg et al., 2009b). In addition, Garofalo *et al* demonstrated that eosinophils recruitment was greater in severe RSV infections than those with milder disease (Garofalo, 1992) and

Harrison *et al* also demonstrated eosinophils were actively recruited to the lung during severe RSV infection (Harrison et al., 1999).

Several studies suggest eosinophils play a protective role during pneumovirus infection. One study suggested that eosinophils reduced the infectivity of RSV for their target cells *in vitro* (Domachowske, 1998); another indicated that RSV and PVM can infect and replicate in eosinophils which was associated with reduced lung damage (Dyer et al., 2009, Kimpen, 1996). Furthermore, Castilow *et al* argued that although stimulated, eosinophils do not contribute to severe disease and that it is a T_h1- rather than a T_h2-driven response which correlated with disease signs (Castilow et al., 2008). Domachowske *et al* demonstrated that the use of glucocorticoids in PVM-infected mice reduced eosinophils but clinical signs remained (Domachowske et al., 2001, Domachowske, 2000a, Domachowske, 2000b). Neither were these cells able to clear RSV from infected mice, a result which may represent a fundamental difference between the infection of mice with RSV and PVM (Rosenberg et al., 2009b).

Other immune cells recruited to the sites of pneumovirus infection include natural killer (NK) cells, granulocytes and DCs (Domachowske, 2000b, Garofalo, 1992, Harrison et al., 1999). Arguably, the most important of these are the DC, which are professional APCs that are able to activate B-cells and $CD4^+$ and $CD8^+$ T-cells. They are recruited to the lung upon RSV infection where they acquire RSV antigen and migrate to lymphoid tissues to stimulate adaptive immune responses. At the site of infection, they themselves can release cytokines, such as IL-12, to stimulate NK cells. This cytokine can also modulate the differentiation of $CD4^+$ cells, discussed later in Section 1.6.2.2.2. IL-12 biases the differentiation of $CD4^+$ cells to the T_h1 pathway. RSV has been demonstrated to have anti-IL-12 effects, which therefore bias $CD4^+$ T-cell differentiation away from T_h1 and towards T_h2 pathway in mice (Bartz et al., 2003) and infants (Wang & Harrod, 2006). This cytokine is also associated with viral control as IL-12 deficient mice have a reduced ability to clear RSV due to impaired NK cell responses (Ehl, 2004).

1.6.2 Adaptive responses and pneumoviruses

The adaptive immune response provides an ability to control and overcome a wide variety of pathogens, concurrently stimulating memory towards those pathogens. There are two main effector cells types of the adaptive response, B-cells, which release antibodies, and T-cells, of which $CD8^+$ T-cells mediate cellular destruction functions and $CD4^+$ T-cells modulate the overall immune response to a pathogen through cytokine release. The adaptive response has been associated with immunopathogenesis of RSV (Openshaw & Tregoning, 2005), which is directly affected by age, genetics and overall wellbeing of an individual (Openshaw & Tregoning, 2005).

1.6.2.1 The humoral immune response

The humoral response is not essential for the resolution of primary infections, however it does protect against re-infections for most pathogens (Hacking & Hull, 2002). Usually, RSV infections can occur in the presence of pre-existing antibody suggesting that the induced immunity is transient (Sullender, 2000). B-cells are activated through recognition of viral peptide antigen presented by MHC class II molecules. Recognition is achieved either through DC or $CD4^+$ T-cells, by a process described in Fig. 1.7. Activated B-cells can then secrete antibodies against RSV antigen, the isotype of which is determined by the $CD4^+$ T-cell secreted cytokine profile.

IgG, IgM and IgA antibodies are produced in response to RSV infection and are found in both the lungs and blood (Brearey, 2007, Collins & Graham, 2008, Welliver, 1980). The antibody response is primarily directed against the F and G proteins, to which a neutralising antibody response is generated (Simoes, 1999). Non-neutralising antibodies are also raised against the N and P proteins (Connors et al., 1992a). Antibodies generated against the F protein are cross-reactive between different RSV strains, due to the highly conserved nature of the F1 protein subunit (Johnson et al., 1987, Muelenaer, 1991, Shao et al., 2009). In contrast, the G protein is antigenically variable as there is >20% amino acid variation within a subtype and 50% variation between the two RSV subtypes (Cane, 2001, Easton et al., 2004, Hacking & Hull, 2002). The C-terminus of the G protein is external on the virion and

is a target of the antibody response. Glycosylation of this region can mask the epitopes, preventing the antibody response from neutralising the virus (Cane, 1997). In addition, the G protein can exist in a truncated, soluble form. This is secreted from virus-infected cells and is believed to act as decoy to subvert the immune response in humans. In mice however, secreted G protein does not appear to act in this way, perhaps because RSV is not replication permissive in mice or because the protein is not produced in sufficient quantities (Bukreyev et al., 2008, Cane, 2001). Infants under 8 months of age do not develop anti-RSV neutralising antibodies to either the F or G protein, partly due to the presence of maternal antibody and partly due to the immaturity of the immune system (Crowe, 2003). However, the IgG response to these proteins increases with age upon each subsequent infection (Welliver, 1980).

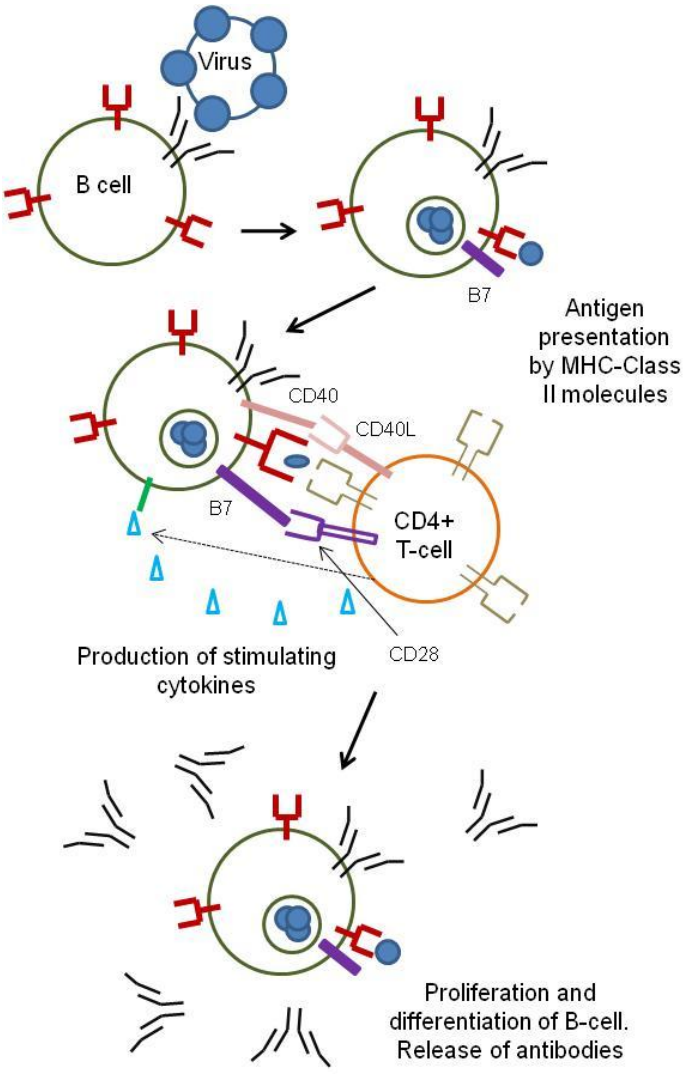

Figure 1.7. Stimulation of B-cells by T helper CD4$^+$ cells.

Viral antigen is processed by B-cells and displayed on the surface in complex with MHC Class II molecules (red). This also leads to up-regulation of co-stimulatory receptors. CD4$^+$ T-cells recognise the MHC-peptide complexes on the B-cell through the TCR (brown). The Th CD4$^+$ T-cell is further stimulated by interaction with co-stimulatory molecules such as CD40-CD40L, and B7-CD28 interactions. This in turn activates the CD4$^+$ T-cell, resulting in cytokine release, such as IL-2, IL-4 and IL-5. These cytokines promote B-cell differentiation and proliferation into antibody-secreting cells. The particular cytokine profile released can affect the antibody isotype produced by the B-cell. For example, IgA can be secreted by epithelial cells and is associated with mucosal immunity, whereas IgE is associated with allergic responses.

1.6.2.2 The cellular immune response

The cellular immune response is important for combating many viral infections, such as herpes simplex virus (HSV) (Khanna, 2003). This arm of the adaptive immune response has two main effector populations: $CD8^+$ T-cells which mature into cytotoxic T lymphocytes (CTLs), which mediate cell lysis effector functions; and $CD4^+$ T-cells which modulate the overall immune response. Both cell populations can only mediate their effector functions when stimulated by interaction with cell surface MHC molecules that are complexed with specific antigen peptide (Fig. 1.8).

Briefly, for the stimulation of T-cells, antigen is acquired by an APC either from external sources (through phagocytosis of apoptotic cells or antibody complexed with pathogens), or from internal sources, such as by direct infection of a cell or synthesis of viral proteins. Antigen is then processed by one of two pathways. Internally derived antigen enters protein degradation pathways (Fig. 1.8) and the resulting peptide fragments are transported to the ER, where they can bind to the peptide binding groove of MHC class I molecules. This triggers MHC transport to the cell surface where the stable antigen-MHC complexes are displayed for recognition by $CD8^+$ T-cells, APCs and NK cells. All cells express MHC class I molecules, including immune cells. Thus, MHC class I molecules are the primary immune surveillance complexes. Externally derived antigen is mainly complexed with MHC class II molecules, which are only found on immune cells, such as DCs and $CD4^+$ T-cells. Antigen is acquired by these cells and undergoes proteolytic cleavage in proteosomes. MHC class II molecules are assembled in the ER and are released into the cytoplasm within endosomes which eventually fuse with the vesicles containing the proteosome cleavage products, where peptide fragments can potentially bind (Fig. 1.8). Stable antigen-MHC class II complexes form and are transported to the cell surface for immune surveillance. APCs, such as DCs, are particularly important cells in the immune system as they can prime both $CD4^+$ and $CD8^+$ T-cells through the expression of both types of MHC molecule. Upon acquisition of antigen, DCs localise in lymphoid issues and activate T-cells using multiple receptor-ligand interactions (Fig. 1.9).

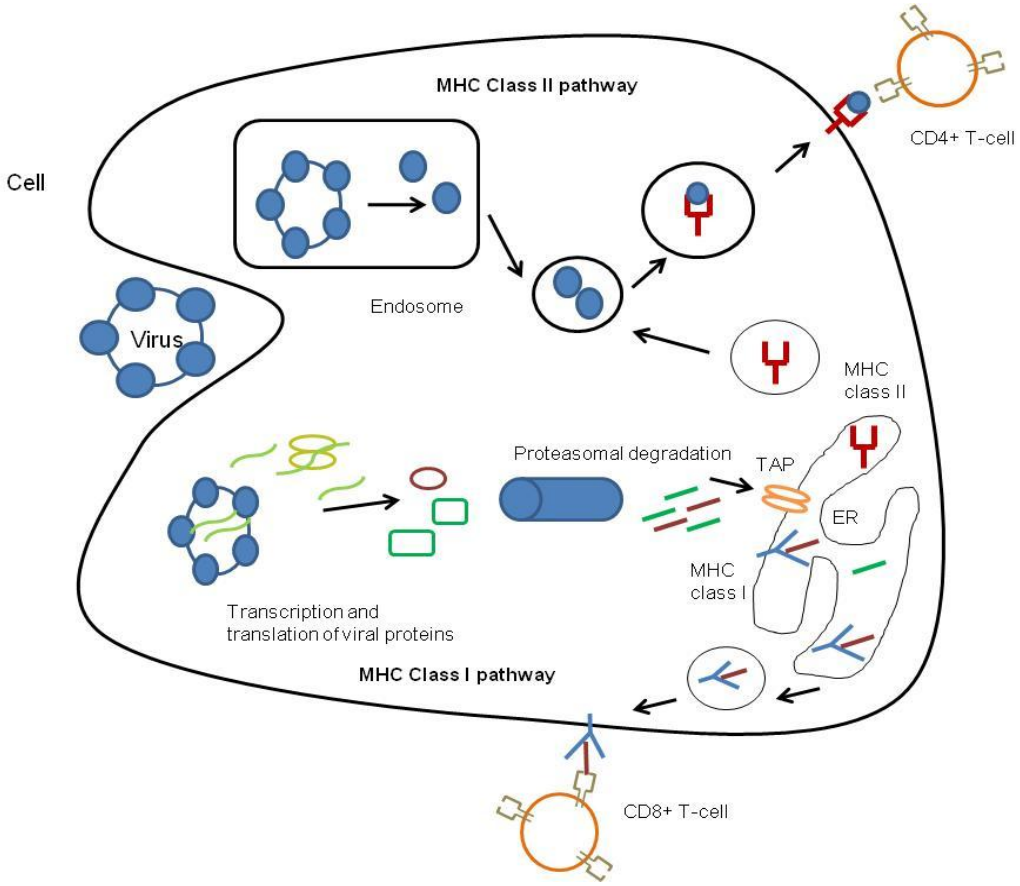

Figure 1.8. MHC Class I and II pathways for antigen presentation.
MHC Class II: Viruses in complex with neutralising antibody are recognised by APCs and enter the cells by endocytosis. The proteins are degraded into peptide fragments in endosomes, by enzyme such as cathepsins, and in lysosomes. The MHC class II molecules are synthesised, folded and assembled in the ER. They are then transported through and bud from the Golgi network in exocytic vesicles. The vesicles fuse with the endosome/lysosome vesicles containing the peptide fragments. To prevent the MHC class II molecules binding peptide in the ER, they associated with the invariant chain (Ii) protein which blocks the peptide binding groove. Once the exocytic vesicles fuse with the acidic endosomes, proteolytic enzymes digest the Ii leaving a protein called CLIP in the peptide binding groove. CLIP is removed through HLA-DM activity. This protein removes CLIP whilst simultaneously promoting peptide binding to the MHC class II molecule. The binding of peptide to the MHC molecule stabilises the complex, allowing it to be transported and displayed upon the cell surface for recognition by $CD4^+$ T-cells. **MHC Class I:** Some viruses can enter the cell via endocytosis or fusion with the cellular membrane. Once within the cell, they are able to utilise the host cellular machinery to replicate and translate their genomes. This leads to the synthesis of viral proteins which can become ubquitinated by host cellular processes. This signals their degradation by the proteosome. The peptides are then transported into the ER via a transporter molecule known as TAP. Newly synthesised MHC class I molecules are non-covalently bound to TAP allowing transported peptides to bind efficiently to the peptide binding groove. Once bound, the peptide stabilised the MHC class I molecule, it dissociates from TAP and exits the ER and Golgi via exocytic vesicles. The complex is transported to the cell surface where it can be recognised by $CD8^+$ T-cells.

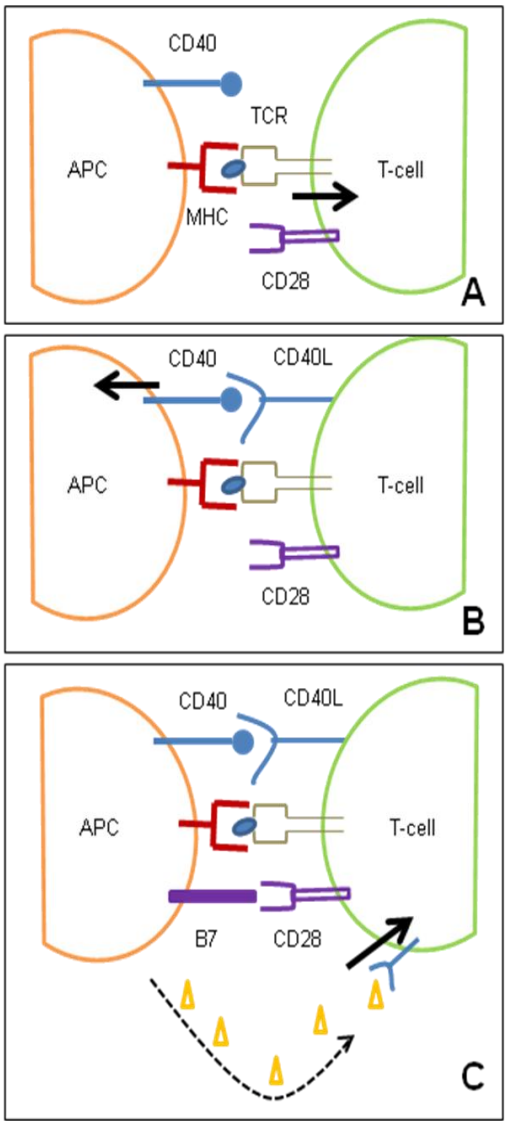

Figure 1.9. APC activation of T-cells. APCs process and thus present antigen on both MHC class I and II molecules for T-cell immunosurveillance. The T-cell receptor (TCR) on a T-cell binds in a weak and transient manner to a MHC molecule. If the TCR is specific for a peptide-MHC complex, the interaction becomes stronger and longer in duration (A). This triggers co-receptors such as CD40L and CD28 to be localised to the TCR-MHC complex (B). CD40L binding to CD40 on an APC stimulates that cell to increase the expression of B7 molecules and IL-12 secretion (C). B7 can bind to CD28 at the TCR complex which activates the T-cell to mediate its effector functions. It also stimulates proliferation, ensuring that sufficient numbers of that T-cell clone are available to mount a response. Other cytokines are released which can determine $CD4^+$ T-cell differentiation, specifically IFNγ for T_h1 and IL-4 for T_h2.

The cellular response mediates pneumovirus clearance and provides immunological memory against subsequent infection (Crowe, 2003). Therefore, stimulation of the cellular response is important to control pneumovirus infection, both in mice (Cannon, 1988, Graham, 1991) and humans (Fishaut, 1980, Hall, 1986). Evidence supporting this includes: individuals with T-cell deficiencies have severe and long-lasting RSV disease and adults with a strong T-cell response have milder RSV infections (Hall, 2001a, Simoes, 1999); vaccine candidate BBG2Na generated $CD4^+$ T-cell dependent immunity (Plotnicky-Gilquin et al., 2000); T-cell deficient mice fail to eliminate PVM and often become carriers in the absence of disease, and animals with limited T-cell numbers are associated with limited pathology (Frey et al., 2008); and RSV-specific memory T-cells can persist in the lung over the long-term and can

respond rapidly to subsequent re-infection (Ostler, 2001), indicating the role of these cells in the generation of pneumovirus immunopathology and immunity.

1.6.2.2.1 The role of CTLs in pneumovirus infection

CTLs are activated $CD8^+$ T-cells. They recognise specific antigen class I MHC complexes which triggers them to induce apoptosis in the cell displaying the complex through enzyme release. CTLs form part of the protective response that develops as part of a T_h1-biased $CD4^+$ response. They secrete IFNγ which promotes T_h1 and suppresses T_h2 responses. The CTL response is vital against RSV, as the virus can evade neutralising antibody responses by facilitating cell-cell fusion as a means of spread in the host (Ostler, 2002). For RSV, CTLs with specificity for the NS2, N, M, SH and F proteins have been identified, with the N protein being a dominant target, but none specific for G and P proteins were detected in humans (Alwan et al., 1993, Cherrie et al., 1992, Corvaisier, 1993, Srikiatkhachorn & Braciale, 1997). In BALB/c mice, the $M2_{82-90}$ epitope was dominant, and suppressed the establishment of other immunodominant clones (Kulkarni et al., 1995, Mok et al., 2008, Openshaw, 1990). F_{85-93} and N were other CTL epitopes recognised in both RSV-infected BALB/c mice (Chang et al., 2001, Pemberton et al., 1987) and bRSV-infected cows (Gaddum, 1996). For PVM, CTL epitopes $P_{261-269}$, M_{43-51} and $F_{302-312}$ have been identified experimentally in mice, in addition to a theoretical one in the N protein (Claassen et al., 2005).

Although the main role of CTLs is beneficial, CTLs are also thought to mediate immunopathologic effects, with indications that a strong CTL response led to fatal lung damage in RSV-infected mice (Cannon, 1988, Simmons et al., 2001). Activated CTLs also release IFNγ which is associated with both protection and airway obstruction in RSV infected mice (van Schaik, 2000). However, evidence also exists which suggests that CTLs are not primary mediators of pneumovirus pathogenesis. These include studies which demonstrated that CTLs were recruited to the lungs only after severe infection has passed (Chiba, 1989, Lukens et al., 2010). RSV-specific $CD8^+$ T-cells were shown to persist in local and systemic tissues long after disease signs had diminished (Ostler, 2002). Furthermore, a study in human infants suggested that severe RSV infection was characterised by an inadequate rather than a

robust, T-cell response (Welliver, 2007). Therefore, the balance between stimulation and suppression of the CTL response may be important for disease severity.

1.6.2.2.2 The role of $CD4^+$ cells in the response to pneumoviruses

$CD4^+$ T-cells modulate the overall immune response towards a pathogen. They achieve this through complex cytokine expression profiles and presentation of antigen to $CD8^+$ T-cells and B-cells. $CD4^+$ cells are initially activated by APCs that release cytokines which promote the establishment of either a T_h1 or T_h2-biased $CD4^+$ response (Fig. 1.10). IL-12 and IFNγ from DCs, infected cells and other APCs stimulate T_h1 activation and T_h2 suppression. T_h1 cells can then release similar cytokines, which activate CTLs, NK cells and macrophages. B-cells are stimulated to release IgA and IgG antibody isotypes. However, if eosinophils or mast cells are recruited to the site of infection, they release both T_h2 promoting and T_h1 suppressing cytokines such as IL-10, IL-4, IL-5 and IL-13. T_h2 $CD4^+$ T-cells can then release similar cytokines and activate eosinophils, mast cells and promote B-cell antibody isotype switching to IgE. This response is associated with inflammation and leads to sensitisation; such a response is stimulated in allergic responses, such as for asthma.

$CD4^+$ T-cells are thought to exert both protective and exacerbative roles in pneumovirus infected individuals; therefore a careful balance between the two must be established (Alwan, 1992). This balanced is determine by the cytokine secretion profile of the immune cells and locally infected cells (Becker, 2006, van Drunen Littel-van den Hurk, 2007). Pneumoviruses typically stimulate T_h1-biased responses which facilitate recovery and immunological memory towards the infection (van Drunen Littel-van den Hurk, 2007). It has been shown in the mouse model of RSV infection that initial infection stimulates a balanced T_h1/ T_h2 response, which usually later shifts to be T_h1 dominant (Matsuse et al., 2000b). However, in some individuals severe RSV disease can develop that is associated with a T_h2-biased response; sensitised animals initially develop T_h1 responses, but these become T_h2 dominant with subsequent infections (Dakhama, 2004, Matsuse et al., 2000a). This can occur through blocking of IFNγ secretion (Bartz et al., 2003).

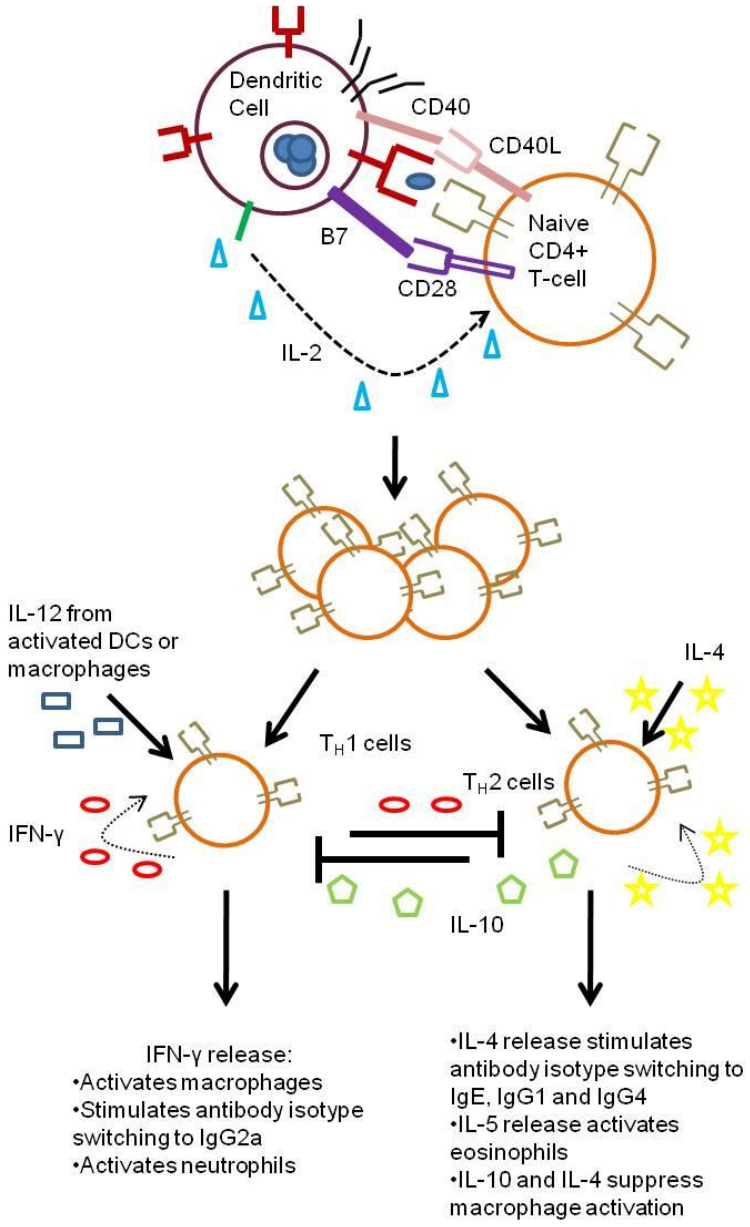

Figure. 1.10. Differentiation of Th $CD4^+$ T-cells. $CD4^+$ T-cells exist in separate subsets each associated with their own unique effector functions. Naive $CD4^+$ cells are stimulated by APCs displaying antigen. The release of IL-2 stimulates the naive cells to become activated and proliferate. The proliferating $CD4^+$ population relies on cytokine signals from the surrounding environment to determine the differentiation pathway stimulated. The cytokines can come from cellular sources or other activated APC cells. Cytokines such as IFNγ and IL-12 stimulate the T_h1 subset and inhibit the differentiation of the T_h2 subset. Conversely, the T_h2 subset is stimulated by IL-4 and inhibits T_h1 differentiation through IL-10 release. The differentiated T-cells use a positive feedback mechanism to further stimulate the differentiation pathway as they can respond to the cytokines they generate.

T_h2-biased responses are associated with high levels of eosinophils and proinflammatory cells in infected tissues in humans (Kim et al., 1969). This can lead to sensitisation to the virus, which leads in turn to stronger $CD4^+$ responses upon subsequent infections (Domachowske, 1999, Graham, 1993). Infants who suffer severe infections demonstrate decreased T_h1 and increased T_h2 responses towards RSV, so that with each subsequent infection, severe disease occurs (Castro, 2008).

T_h2-biased responses towards pneumoviruses have been associated with a higher risk of asthma development in children, which was linked to first age of infection rather than cytokine levels (Castro, 2008).

$CD4^+$ T-cell epitopes for RSV have been identified in the N, P, M and G proteins (Alwan et al., 1993, Liu et al., 2009). Experiments performed with the F, M and G proteins have identified the $M_{213-223}$ and $G_{381-385}$ epitopes which stimulated a T_h1-biased response (Claassen et al., 2007, Liu et al., 2009). Further studies demonstrated that T_h2-biased response was stimulated when G and SH proteins were investigated (Connors et al., 1992a, Elliott et al., 2004). Yet the G protein in particular appears to be able to stimulate a mixed T_h1 and T_h2 response (de Graaff, 2004, de Waal, 2004).

1.6.2.3 T-cell response impairment by pneumoviruses

Animal models of pneumovirus infection demonstrated that T-cells are able to differentiate into memory cells that remain in the local mucosa and can be reactivated in response to subsequent infection. In humans, as re-infections occur regularly in spite of neutralising antibody and T-cell driven responses, partly due to RSV subtype antigenic variation and partly suggesting memory T-cell reactivation was impaired. One study supported RSV-specific memory cell impairment. Although they can be reactivated in response to specific antigen, they do not produce IL-12 which is needed to promote the maintenance and differentiation of long-lived memory T-cells (Richter et al., 2005). T-cell impairment does occur naturally with age. This has been shown to correlate with decreased CD28 expression on memory T-cells (Effros, 2007). It has been suggested that these cells can have a suppressive effect on the general T-cell population, perhaps explaining why elderly persons are less able to respond to infection (Looney, 2002).

An interesting feature of pneumovirus pathogenesis is the ability of these viruses to impair pulmonary responses of both $CD4^+$ and $CD8^+$ T-cell populations (Effros, 2007, González, 2009). Other paramyxoviruses have been shown to demonstrate similar effects (Gray et al., 2005). Such an effect would be beneficial to the virus as it would delay viral clearance and ensure efficient viral replication. Studies have shown that RSV-infected mice generate large numbers of virus specific $CD8^+$ T-cells (Chang & Braciale, 2002, Chang et al., 2001), similar to other paramyxoviruses (Fulton et al., 2008). However, these cells are suppressed by replicating virus, either

through NS protein function or through an alternative mechanism such as T-cell exhaustion (Bucks, 2009, Chang & Braciale, 2002, Chang et al., 2001, Mueller, 2009). As a result, these cells do not persist in lymphoid tissues in the long term and only secrete low levels of IFNγ. In one study, RSV specific T-cells were shown to be functionally impaired in cytokine production (Chang & Braciale, 2002, Chang et al., 2001). Similar results were found using PVM (Claassen et al., 2005) and bRSV (Keles, 1999). Likewise, a study of M2 protein-specific T-cell responses to RSV, suggested that long-term T-cell mediated immunity was not stimulated and rapidly waned (Kulkarni, 1993). Also, the RSV F protein can directly inhibit T-cell proliferation by an unknown mechanism (Schlender et al., 2002). In the case of PVM, specific $CD8^+$ T-cell responses were found to be only partially activated, with only 10-20% of these cells producing IFNγ upon stimulation (Claassen et al., 2005). In addition, T_{reg} cells may play a role in T-cell suppression as one study showed that T_{reg} cells can lower the number of RSV-specific $CD8^+$ T-cells that survive into the memory phase and thus may decrease the immune memory of RSV (Ruckwardt et al., 2009). Also, cytokines such as IFNα and IFNλ have been linked to RSV $CD4^+$ T-cell suppression (Chi et al., 2006), as have healthy lung epithelial cells through their direct contact with RSV-specific T-cells preventing cytokine release (Wang et al., 2009). Clinically, these various mechanisms of T-cell suppression could explain the high rate of re-infection by RSV. Additionally, if RSV specific $CD8^+$ responses can be increased through immunisation for example, it could reduce T_h2 $CD4^+$ differentiation which improves the hosts immune response to RSV, allowing clearance from the virus, suggesting that the immunosuppressive effect of RSV could contribute to the severe disease observed (Olson, 2008, Srikiatkhachorn & Braciale, 1997). This suppressive effect has been overcome experimentally using IL-2, suggesting therapeutic potential for this cytokine (Chang & Braciale, 2002).

1.6.2.4 Summary

The immunopathogenesis of the pneumoviruses is due to several factors (Fig. 1.5). Cytokines play a role in determining the nature and intensity of the immune response particularly for $CD4^+$ T-cell mediated functions. Neutralising antibody does not exacerbate infections but neither does it appear to correlate with protection. In contrast, T-cells have been shown to be essential for the clearance of the virus, but

are also associated with the development of severe disease (Fig. 1.5). It is unknown whether the T-cells mediate the severe disease directly or whether the cytokines they release play a more dominant role. Interestingly, different viral proteins appear to prime different responses in infants and mice, even when delivered via the same route (Alwan et al., 1993). Therefore, the appropriate antigen must be chosen to ensure that the correct immune response is stimulated. It is likely that protective immunity to pneumoviruses requires $CD4^+$ and $CD8^+$ T-cell responses as $CD8^+$ responses can be improved with $CD4^+$ stimulation (Claassen et al., 2007). Therefore, controlled stimulation of $CD4^+$ T-cells by a vaccine could mediate a protective anti-pneumovirus response, through CTL and B-cell effector functions (Claassen et al., 2007). Thus, the immune response to pneumovirus relies on the complex interplay between multiple cell types and the cytokine profile they secrete (Fig. 1.5 and 1.10). This results in a delicate balance between protective $CD8^+$ and T_h1 $CD4^+$ T-cell responses and allergic eosinophils and T_h2 $CD4^+$ T-cell responses.

1.7 RSV vaccine development

The overall cellular response to pneumovirus infection is complex as illustrated in Fig. 1.5. The complexity of the immune response for RSV has direct implications for RSV vaccine development. This has been greatly hindered since the 1960s FI-RSV vaccine trial (Crowe, 2001). It is thought that the protective $CD8^+$ and T_h1 $CD4^+$ response was overwhelmed by a T_h2 $CD4^+$ response which led to severe disease (Tripp et al., 1999). An RSV vaccine must be able to stimulate a protective adaptive immune response which is T_h1-dominant or T_h1/T_h2 balanced. As mentioned previously, the particular pneumovirus protein used to induce a response and the delivery method can greatly influence the nature of the response. A vaccine must contain a strong $CD8^+$ T-cell epitope to ensure clearance and protection from the virus which would provide internal antigen processing (Fig. 1.8). However, appropriate $CD4^+$ T-cell stimulation is also required to maximise $CD8^+$ responses and prevent T_h2 allergic responses from being established. The stimulation of neutralising antibody does not appear to correlate with protection and therefore should not be the focus of an RSV vaccine.

From this understanding of the immune response, it is evident that an appropriate vaccine candidate would achieve expression of one or more full length RSV proteins directly in a cell, for example from an adenovirus vector. The T-cell response would naturally focus on the immunodominant epitope(s) and as the entire protein is expressed, antibodies may be induced as well. The use of a full length protein containing several epitopes would also circumvent the issue of variable MHC haplotype within the population.

A vaccine to be used in infants must be able to overcome any maternal immunity (Collins, 2007). This is likely to be achieved by a T-cell targeted vaccine. Coupled with this, infants have immature immune systems and cannot readily respond to pathogens as adults can. This was noted in a clinical trial of a vaccine candidate where infants under 6 months did not mount immune responses whereas older children did (Karron, 2005). Young children are also particularly vulnerable to developing a T_h2 biased immune response, which can be stimulated by certain vaccines as the FI-RSV trial demonstrated. Therefore, any vaccine candidate must be stringently tested to ensure that such a response is not mounted in this population. The vaccine itself must not interfere with other paediatric vaccines (van Drunen Littel-van den Hurk, 2007).

Live vaccines as opposed to inactivated vaccines have been demonstrated to be more appropriate for stimulating balanced T_h1 responses against RSV. The data from a study examining the difference in immune response stimulation using different prime-boost strategies with either killed or live RSV indicated that the resulting cytokine profile of the response was initiated through the prime vaccine dose, as killed vaccine generated a T_h2-biased response whereas the live virus stimulated a T_h1-dominant response (Tang, 2004). Vaccines containing a live viral element generated CTL responses and had reduced viral replication, with live vaccine prime and boost immunisation regimes stimulating the best immune response (Tang, 2004). The ideal RSV vaccine candidate would therefore consist of a live, attenuated virus which was able to replicate to a high titre, ensuring the high immunogenicity was retained. However, due to the difficulties with the vaccine-enhanced disease, more focus has been placed on subunit vaccines or live viral vectors expressing full length

proteins or peptides. The various previous attempts to develop an RSV vaccine are considered below.

1.7.1 Inactivated RSV vaccines

The first RSV vaccine candidate, a formalin-inactivated whole virus preparation (FI-RSV), was used in a clinical trial in the 1960s. The vaccine successfully immunised all individuals however the immune response was skewed towards a T_h2 $CD4^+$ T-cell response. Subsequent natural RSV infection was thus more likely to develop into severe RSV disease. During clinical trials, this led to the hospitalisation of 80% of the vaccines, compared to 5% of the control group (Girard et al., 2005, Kim et al., 1969, Kneyber, 2004). As a result of this, two infants died. Therefore, an understanding of the molecular mechanisms behind this tragedy has been greatly aided through the use of animal models. In summary, it has been demonstrated that the T_h2 $CD4^+$ response was greatly enhanced through the disruption of antigen by the inactivation process (Openshaw, 2002, Prince et al., 2001). A more recent study has suggested that the response was caused by the maturation of antibodies which had a low affinity for the RSV antigens, due to poor activation of TLRs. These antibodies were non-protective and promoted the development of a T_h2-biased response (Delgado et al., 2009).

1.7.2 RSV subunit vaccines

RSV subunit vaccines are attractive candidates as particular proteins can be used to immunise animals to ensure the stimulation of an appropriate immune response, without the need for the use of live virus. Subunit vaccines are often composed of the F and G proteins. These stimulate neutralising antibody, however they do not stimulate $CD8^+$ T-cells responses and thus are unlikely to be appropriate vaccines to stimulate lasting immunity to RSV (Kneyber, 2004). As a result, subunit vaccines are often used in conjugation with adjuvant agents such as alum and cholera toxin, which have had limited success.

RSV proteins can also be engineered to improve their immunogenicity. One such study fused a fragment of the G protein with Protollin adjuvant (Huang, 2009);

which promotes processing by the proteosome and hence antigen presentation. Intranasal delivery of the vaccine resulted in the stimulation of both IgG and IgA humoral responses towards the RSV G protein, but also reduced IL-13 expression which led to a decrease in eosinophil recruitment (Huang, 2009). Similarly, Protollin has been used as an adjuvant for an intranasal vaccine enriched with the F and G RSV proteins (eRSV). The eRSV vaccine with protollin adjuvant was successful in that intranasal immunisation in mice stimulated IgG and IgA antibody production and a T_h1 $CD4^+$-biased T-cell response, but the mice did have high IL-5 levels suggesting a T_h2 response was also stimulated (Cyr et al., 2007a, Cyr et al., 2007b). Liposome-encapsulated dichloromethylenediphosphonic acid (DMDP) vaccine, which are viral proteins contained within a liposome capsule, have also been investigated for these proteins (Benoit et al., 2006).

An alternative vaccine candidate used the RSV G and M2 proteins fused to a HSP70-like protein. Subcutaneous immunisation of mice revealed that this vaccine stimulated stronger $CD8^+$ T-cell and neutralising antibody responses than a non-conjugated version (Zeng et al., 2008). In addition, the $CD4^+$ T-cell response was T_h1/ T_h2 balanced, indicating that the vaccine did not prime for severe RSV disease in the animals (Zeng et al., 2006, Zeng et al., 2008). RSV $F_{412-524}$ protein has also been complexed with cholera toxin as an adjuvant. When used to immunise mice intranasally, IgA and IgG neutralising antibody responses were stimulated along with a T_h1-biased, but T_h1/T_h2 mixed $CD4^+$ T-cell response. These data correlated with protection and demonstrated that recombinant subunit vaccines could be used to stimulate adequate mucosal immunity (Singh et al., 2007). Furthermore, the $M2_{82-90}$ peptide of RSV has been fused with the *E.coli* heat-labile toxin and has been shown to stimulate $CD8^+$ T-cells responses to the epitope which correlated with protection from the virus, but also with enhanced weight loss during viral challenge (Simmons et al., 2001).

VLPs have been investigated as potential vaccines for RSV. One study used Newcastle disease virus, which is an avian paramyxovirus, to form VLPs which contained the G protein of RSV. Several intraperitoneal or intramuscular immunisations with this vaccine resulted in protection against RSV and the induction of moderate titres of neutralising antibody with no evidence of an enhanced

pathology (Murawski et al., 2010). Influenza A virus has also been used as the basis for an RSV subunit vaccine. Influenza virosomes were reconstituted with RSV F protein and used to immunise mice intramuscularly, resulting in the generation of neutralising antibody towards the F protein (Nallet et al., 2009). These candidates have yet to enter clinical trials. In addition, a lipopeptide-adjuvanted RSV virosome candidate has recently been demonstrated to induce high levels of neutralising antibody, coupled with a balanced T_h1/ T_h2 response in mice and cotton rats (Stegmann et al., 2010).

Two further strategies which have been trialled were the BBG2Na and PFP vaccines. BBG2Na is a G protein subunit vaccine which was able to induce neutralising antibodies but also biased the immune response to a T_h2 response through IL-4 release and stimulation of IgE. In addition, limited $CD8^+$ T-cell activation was observed and thus the candidate was discontinued after Phase III clinical trials (Collins, 2007, Kneyber, 2004, Plotnicky-Gilquin, 1999, Plotnicky-Gilquin et al., 2000). PFP-1, PFP-2 and PFP-3 are a range of peptide vaccines generated from purified F and G proteins that also gave poor results in trials. PFP-1 was first investigated in infants, where neutralising antibody was stimulated but immunisation was associated with upper respiratory tract signs which were unacceptable in such young infants (Kneyber, 2004). PFP-2 contains a greater amount of purified F protein than PFP-1. This was investigated for use in maternal immunisation programmes with the aim of stimulating the immunity of the foetus and infant through mother's milk. However, although the vaccine was well tolerated and safe, the vaccine was only slightly immunogenic (Munoz, 2003). PFP-3 underwent clinical trials in cystic fibrosis patients, where it was deemed to be well tolerated and safe. 67% of vaccinees demonstrated a rise in neutralising antibody titre, however, this was not statistically significant when compared to control groups (Polack, 2004).

1.7.3 Nucleic acid RSV vaccines

These vaccines comprise DNA copies of RSV sequences cloned within a plasmid vector, allowing the antigen to be expressed by cells of the vaccine in its natural state as opposed to a modified form due to inactivation methods. One study used RSV F DNA plasmids to immunise mice intramuscularly. This resulted in the generation of

neutralisation antibody responses including IgG and IgA, and a mixed T_h1/ T_h2 $CD4^+$ T-cell response which correlated with protection from RSV (Wu et al., 2009). Another study used chitosan-encapsulated DNA of the F, M2 and G genes of RSV within an expression vector. The DNA-chitosan complex formed nanoparticles which were confirmed to be more stable than naked DNA and which resulted in enhanced transgene expression compared to naked DNA, demonstrating that this technique could be valuable for immunisation purposes (Boyoglu, 2009). These methods of immunisation were also immunogenic in the presence of maternal antibody and studies comparing RSV F DNA delivery with delivery of F protein determined that the DNA was more immunogenic and stimulated strong $CD8^+$ T-cells without evidence of enhanced pathology (Martinez, 1999).

1.7.4 Attenuated RSV strains

Two attenuated RSV strains have been developed through passage in tissue culture at reduced temperatures. rA2cp248/404ΔSH has 5 missense mutations in the N and L genes due to cold-passage in tissue culture, a mutation at nucleotide position 248 within the L gene, a further mutation at nucleotide position 404 in the M2 gene and complete deletion of the SH gene. rA2cp248/404/1030ΔSH is similar but has an additional mutation at codon 1030 in the L gene (Karron, 2005). These vaccine candidates were attenuated in adults and well tolerated in children. rA2cp248/404/1030ΔSH did not replicate to high levels within children (Karron, 2005) suggesting it was over attenuated and only stimulated low levels of neutralising antibody (Bennett et al., 2007). Further study revealed that enhanced disease did not develop upon subsequent RSV infection (Wright et al., 2007). However, these vaccine candidates were found to be unstable after several rounds of tissue culture passage, suggesting that reversion to virulence *in vivo* may also occur (Lin et al., 2006). Vaccines developed using these methods are prone to failure from both under and over-attenuation as only *in vivo* studies allow the immunogenicity of the vaccine and pathogenesis from RSV challenge to be observed (Polack, 2004).

1.7.5 Recombinant viral vectors for RSV antigen delivery

Viral vectors are popular delivery vehicles for vaccination as they can infect the mucosa, stimulating a more natural immune response towards the transgene. Sendai virus is an example of such a vector. Its advantages are that it is a mouse pathogen and as such, pre-existing immunity within the human population is low. In addition, Sendai virus vectors have been shown to elicit strong immune responses towards the transgene product as well as being well tolerated in human clinical trials (Hurwitz, 1997, Slobod, 2004). As a direct result of these studies, an RSV Sendai virus vaccine candidate was developed which expressed the F protein (Zhan et al., 2007). This study demonstrated that neutralising antibody, IFNγ^+ T-cell responses towards the F protein and protection against challenge were induced in cotton rats following intranasal administration. Recombinant bovine parainfluenza virus 3 (bPIV3) has also been adapted for use in vaccination strategies. One candidate, MEDI-534, expressed the F and N proteins of human PIV3 and the RSV F protein. Experiments in the cotton rat model did not demonstrate the induction of enhanced RSV disease so this candidate has entered human clinical trials. MEDI-534 was shown to be replication restricted and did not generate adverse side effects. However, neutralising antibody titres were low in human adults (Tang et al., 2008).

Vaccinia virus (VV) is an alternative viral vector currently under consideration. VV is a large, stable DNA virus, amenable to genetic manipulation with a well understood safety profile. However, pre-existing immunity to VV is high in certain age groups due to its use as a smallpox vaccine in the past. In addition, life-long immunity is induced upon vaccination with a single dose, therefore the vector can only be used once in an immunisation regime. Due to the size of the virus, it is thought that it could be manipulated to express several antigens of different human pathogens, providing a 'one-shot' strategy to prevent a variety of diseases. For RSV, a VV construct expressing the F and G proteins was evaluated in chimpanzees (Collins et al., 1990). The results of this study were not encouraging. A similar construct expressing the G protein of RSV was also found to stimulate a T_h2 CD4$^+$-biased immune response in mice (Johnson et al., 2004a, Johnson et al., 2004b). Alternative candidates were generated; one encoded the entire M2 gene, and another the $M2_{82\text{-}90}$ CD8$^+$ T-cell epitope. It was determined that both candidates could

stimulate a strong $CD8^+$ T-cell response, which was discovered to be solely mediated by this epitope (Kulkarni et al., 1995, Kulkarni, 1993, Kulkarni et al., 1993).

1.7.6 Adenovirus vectored RSV vaccines

Adenovirus vectors have been proposed as useful vehicles to delivery immunogens. Several studies have explored its use in protection against RSV. However, all have focused on delivery of the F and G proteins as stimulators of antiviral antibody as opposed to other proteins which could stimulate cellular immune responses. In one study, different RSV vaccine candidates were compared in the cotton rat model including recombinant baculovirus expressing the F and G proteins of RSV (Bac-FG), vaccinia vectors expressing either the F or G proteins of RSV (Vac-F and Vac-G), adenoviral vector expressing the F protein of RSV (Ad-F) and FI-RSV (Connors et al., 1992a). It was shown that Vac-F and Ad-F stimulated neutralising antibody at levels higher than those stimulated by Bac-FG. In addition, Vac-F and Ad-F were associated with protection from the RSV challenge whereas Bac-FG and FI-RSV was less effective. It was concluded that recombinant vectors expressing RSV proteins could induce more robust protection than inactivated vaccines and Ad5 in particular is an appropriate candidate delivery vehicle (Connors et al., 1992a).

The group of Hsu *et al* investigated rAd4, rAd5 and rAd7 ΔE1/E3 viruses, which expressed the F or the G proteins or a recombinant FG fusion protein of RSV, in ferrets immunised intranasally. The constructs were tolerated at the highest dose, and did not cause lung histopathology. At 4 weeks, neutralising antibody responses were detected in the Ad4F- and Ad5F-immunised animals but not the Ad7F animals. The viruses were able to reduce RSV replication in the lung upon direct challenge, Ad5F being the most efficient. Animals were then immunised with Ad5F and Ad4F in combination, in a prime-boost regime. A low dose of AdF did not protect animals but was associated with neutralising antibody responses which increased over the 18 week experiment. In contrast, those animals immunised with the middle or high doses use in the study, saw a decrease in antibody titre over the experimental period but were protected from RSV challenge (Hsu, 1994).

The group of Yu *et al* investigated rAd5 ΔE1/E3 viruses generated using the pAdEasy system which expressed RSV G protein epitopes from a CMV promoter. The original RSV G protein sequence was codon-optimised for mammalian studies and multiple copies of the region, $G_{130\text{-}230}$, which includes multiple epitopes, cloned into the vector. This vaccine was used to immunise BALB/c mice via either the intranasal, intramuscular or oral routes with a single dose. It was found that, as expected, the codon-optimisation and incorporation of three copies of the protein fragment produced higher levels of G protein expression than a construct containing a single fragment. Intranasal and intramuscular immunisation induced strong IgG responses towards the G protein, with oral immunisation stimulating a much weaker response. Intranasal immunisation was also more efficient at stimulating a G protein-specific IgA response. Animals pre-exposed to the rAd vector did not have a reduced immune response towards the G protein, suggesting that pre-existing immunity does not affect the immunisation ability of this type of vaccine. Further analysis of the T-cell responses indicated that, in contrast to a similar construct expressed from VV, the rAd vaccine did not prime a specific $CD4^+$ T-cell response. The G protein fragment is known to contain an epitope which primes for enhanced disease after RSV challenge (Tebbey, 1998). Therefore, this result suggested that T_h2 $CD4^+$ responses were not primed by rAd5, presumably due to some feature of this vector system. Finally, intranasal immunised mice were shown to be protected from RSV challenge as the immunisation prevented detectable RSV replication within the lung of the animals; this protective effect remained for 10 weeks (Yu et al., 2008).

The same group investigated another vaccine candidate using the same recombinant adenoviral vector which expressed a codon-optimised F1 fragment of the RSV F protein, $F_{155\text{-}524}$. A single intranasal immunisation was sufficient to induce a strong mucosal IgA response and protection against subsequent RSV challenge in BALB/c mice. The group was unable to detect serum Ig or T-cell mediated responses towards the F1 fragment, concluding that the protective effect observed was primarily mediated by the IgA response. As before, immunisation via the intramuscular or oral routes was not associated with protection from RSV (Kim et al., 2010).

Fu *et al* also generated an rAd5 ΔE1/E3 virus with pAdEasy system, which expressed the full length F protein of RSV under control of a CMV promoter, FGAd-F. FGAd-F in a prime-boost regime in BALB/c mice, demonstrated enhanced CTL

activity against RSV when compared to an empty vector control construct. It also generated detectable IgG and IgA antibody titres which increased over the course of the immunisation period, suggesting that homologous prime-boost regimes are feasible when using adenovirus vectors. FGAd-F vaccinated-animals also showed diminished RSV replication in the lung when compared to the control group, suggesting that a protective effect was generated (Fu et al., 2009a).

Shao *et al* designed an rAd ΔE1/E3 vaccine expressing RSV strain B1 F protein in either a truncated form lacking the sequence encoding the transmembrane domain (rAd-F0ΔTM) or a full length copy (rAd-F0). When used to immunise BALB/c mice by the intranasal route, serum neutralising antibodies were detected at a similar level from both candidates. Following a booster dose via an alternative route, such as intranasal, subcutaneous or intraperitoneal, antibody titres increased for both vaccines; the immunisation route did not make a substantial difference to titres. Both rAd-F0 and rAd-F0ΔTM stimulated IFNγ secreting $CD4^+$ T-cells and some T_h2-associated cytokines, however the levels were lower than observed upon RSV challenge suggesting a balanced T_h1/ T_h2 response was induced. $CD8^+$ CTL were also induced to the F_{85-93} peptide epitope. Both rAd-F0 and rAd-F0ΔTM were able to reduce RSV titres upon subsequent challenge (Shao et al., 2009).

Similar results were obtained by Kohlmann *et al*, using rAd5 ΔE1/E3 constructs expressing either full length RSV F protein (rAdV-F) or a secreted form of the F protein (rAdV-Fsol). The vaccines elicited neutralising antibodies towards the F protein which were detectable by 35 weeks post-boost. Upon challenge, viral loads were reduced when compared to a control suggesting that these vaccines were protective. In addition, rAdV-Fsol was shown to generate IFNγ secreting $CD8^+$ T-cells (Kohlmann et al., 2009).

In summary, several studies have produced corroborating evidence to support the use of recombinant adenovirus as a delivery vehicle for vaccination against RSV. However, as discussed in Section 1.5, these studies have focused upon RSV pathogenesis in an inappropriate model, the mouse. Within this model RSV disease is absent or aberrant. Thus, RSV pathogenesis is monitored by viral lung titres. The absence of clinical signs in the RSV mouse model means that vaccine efficacy

cannot be completely determined, as it has been observed that clinical symptoms and cytokine release can persist during PVM infection, even after replicating virus is no longer detected (Bonville et al., 2006a). This issue can be addressed by using similar vaccine candidates expressing PVM proteins, for evaluation within the PVM infection model.

1.8 Aims

The first RSV vaccine to date entered ill-fated clinical trials in the 1960s. Now, in 2010, a suitable, effective vaccine has yet to be approved for clinical use. The molecular understanding of RSV and PVM has increased with the development of reverse genetics and the availability of complete sequences from several RSV and PVM strains. In addition, the understanding of both RSV and PVM pathogenesis in their respective hosts has enabled the development of anti-viral therapeutics, as well as dissecting the reasons behind the failed FI-RSV vaccine trial.

The overall aim of the project was therefore to advance the development of potential RSV vaccines by demonstrating that a live, replication-deficient, recombinant adenoviral vaccine expressing full-length specific proteins from a pneumovirus (PVM), can elicit protection against the same virus in its natural host, the mouse. In detail, the objectives were therefore to:

- Generate live, replication-deficient recombinant adenovirus vectors which express specific genes from PVM
- Evaluate the recombinant viruses for transgene expression
- Evaluate the constructs within the PVM infection model; optimising the immunisation schedule, dose and immunisation route
- Characterise the adaptive immune response towards the transgene

Chapter 2

Materials and methods

2.1 Materials, solutions, buffers and media

Tables 2.1.1-2.1.9 give details of the solutions, mammalian cell lines, virus and bacterial strains, animal strains and other biological reagents used in this thesis.

2.1.1 Solutions, buffers and media

Table 2.1.1. The composition of solutions used in this study.

Name of Solution	Composition
B-galactosidase substrate solution	5mM Potassium ferricyanide, 5mM Potassium ferrocyanide, 2mM Mg, 1mg/ml x-galactosidase in phosphate buffered saline (PBS)
Brefeldin A (BFA) stock	5mg was dissolved in dimethyl sulfoxide (DMSO) to give 1.25mg/ml. BFA solution was diluted in PBS to give a working stock of 200µg/ml
Blocking solution (Western blot)	ECL Advance™ Blocking Agent dissolved in PBS 0.005% (v/v) Tween®-20
CMC agar	4% (w/v) carboxymethyl cellulose (CMC) in sterile distilled water (SDW)
Coating solution (ELISA and ELISPOT)	20mM Na_2CO_3, 70mM $NaHCO_3$ in SDW to pH9.6
Dithiothreitol (DTT)	0.5 M, 1.55 g in 20 ml 0.01 M sodium acetate pH5.2
DOC lysis buffer	20% (v/v) ethanol, 100mM Tris pH9 and 0.4% (w/v) sodium deoxycholate in SDW
FACS buffer	1% FCS and 0.1% Sodium Azide in sterile PBS
Fixative solution	0.05% Glutaraldehyde, 2% formaldehyde in PBS
GTE solution	50mM glucose, 25mM Tris, 10mM diaminoethanetetra-acetic acid disodium salt (EDTA) pH8 in SDW, filter sterilised and stored at 4°C

GTY media	10% (v/v) glycerol, 0.125% (w/v) yeast extract and 0.25% (w/v) tryptone in SDW
Ionomycin stock (Io)	Ionomycin was made to 1mg/ml in DMSO. A working stock of 100µg/ml was made by dilution in PBS
Isotonic buffer (RT-PCR)	150mM NaCl, 10mM Tris-HCl pH7.6 and 1.5mM $MgCl_2$ in SDW and sterilised by autoclaving
Ketamine/Xylazine anaesthetic	Ketamine solution was diluted from a 100mg/ml stock in sterile PBS to give 75µg/g of mouse bodyweight. Likewise 180mg/ml of xylazine solution was diluted to give 15µg/g of mouse bodyweight in the same solution
Loading buffer (Agarose gel)	50% (v/v) glycerol, 50% (v/v) 2x Tris-borate-EDTA, 0.01% (w/v) bromophenol blue and 0.01% (w/v) xylene cyanol
Luria-Bertani medium (LB)	1% (w/v) bactotryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract in SDW and sterilised by autoclaving
Lysis Solution (Bacterial)	1% Sodium dodecyl sulphate (SDS), 0.2M NaOH in SDW
Neutralisation solution (Bacterial)	5M acetate and 3M potassium in SDW to pH4.8
Noble agar overlay	1x Dulbecco's modified Eagle medium (DMEM) with 1% (v/v) foetal calf serum (FCS), 0.375% (w/v) $NaHCO_3$ and 18% of 2.8% (w/v) noble agar
Noble agar feed	1x DMEM with 0.5% (v/v) FCS, 0.2% (w/v) $NaHCO_3$ and 19.5% of 2.8% (w/v) noble agar
Noble agar staining overlay	1x DMEM with 0.5% (v/v) FCS, 0.2% (w/v) $NaHCO_3$, 5% neutral red and 19.5% (w/v) noble agar
NZY+ medium	1% (w/v) NZ amine (casein hydrolysate), 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl in SDW to pH7.5 with 5M NaOH. The medium was sterilised by autoclaving. When cooled, 12.5mM $MgCl_2$, 12.5mM $MgSO_4$ and 0.4% (v/v) glucose (to a final concentration) were added
Penicillin/Streptomycin	10,000 U/ml penicillin G and 10,000 µg/ml Streptomycin sulphate in 0.85% PBS.
Phenol/Chloroform	Equal volumes of TE-saturated phenol and chloroform were mixed and stored at 4°C
Phorbol 12-myristate 13-acetate (PMA)	1mg PMA was dissolved in DMSO to 1mg/ml. A working stock of 10µg/ml was made by dilution in PBS
PMA/Io	10µg/ml PMA and 100µg/ml Io in sterile PBS
Resolving gel (10%)	9.9% (v/v) acrylamide, 425mM Tris pH8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS) and 0.08% (v/v) N,N,N',N'- tetramethylethylenediamine (TEMED) in SDW
RF1 buffer	100mM RbCl, 50mM $MnCl_2.4H_2O$, 30mM K acetate, 10mM $CaCl_2.2H_2O$, 15% (w/v) glycerol in SDW to pH5.8 with 0.2M acetic

	acid. The solution was filter sterilised and stored at 4°C
RF2 buffer	10mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH6.8, 10mM RbCl, 75mM $CaCl_2.2H_2O$, 15% (v/v) glycerol in SDW to pH6.8 with NaOH. The solution was filter sterilised and stored at 4°C
Running buffer (1x) (Protein gel)	25mM Tris, 190mM glycine and 0.1% (w/v) in SDS
Sample Buffer (2x)	0.2% (w/v) SDS, 1% (v/v) glycerol, 50mM Tris pH6.8 in SDW and a few crystals of bromophenol blue added
Sample Buffer (1x)	50% (v/v) 2x Sample Buffer and 100mM dithiothreitol (DTT) in SDW
Stacking gel	3.6% (v/v) acrylamide, 160mM Tris (pH6.8), 0.1% (w/v) SDS, 0.08% (w/v) APS, 0.12% (v/v) TEMED
TE buffer	10mM Tris-HCl (pH8) and 0.1mM EDTA in SDW
Transfer Buffer (1x)	190mM glycine, 25mM Tris and 20% (v/v) methanol in SDW
Trypsin solution	25% (v/v) trypsin in versene
Versene	0.002% (w/v) EDTA (disodium salt) and 0.002% (w/v) phenol red

2.1.2 Reagents and suppliers

All chemicals of analytical or molecular biology grade were supplied by Sigma-Aldrich (Poole, Dorset, UK) or BDH Laboratory supplies (Dorest, UK) unless otherwise stated in Table 2.1.2.

Table 2.1.2. Reagents used in this study

Supplier	Reagents
BD Biosciences (Oxford, UK)	BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit
BD Pharmingen	Allophycocyanin (APC) conjugated rat anti-mouse CD8a monoclonal (553085), Phycoerythrin (PE) conjugated rat anti-mouse CD4 monocloncal (553652), Fluorescein isothiocyanate (FITC) conjugated rat anti-mouse IFNγ monoclonal (554411)

BioLine (London, UK)	HyperLadder1
BioRad (Hemel Hempstead, UK)	APS, TEMED, Alkaline phosphatase (AP) conjugate substrate kit (170-6432)
BioSera (Sussex, UK)	FCS
Fermentas (Helena Biosciences, Sunderland, UK)	Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, GeneRuler™, dATP, dCTP, dGTP, dTTP, *Pfu* DNA polymerase, *Taq* DNA polymerase, 25mM $MgCl_2$, agarose (molecular grade), ribonuclease H, ribonuclease inhibitor, *Taq* DNA polymerase (recombinant), proteinase K, PageRuler™ Prestained Protein Ladder
Fort Dodge, Animal Healthcare Ltd. (Southampton, UK)	Ketamine solution 100mg/ml
GE Healthcare (Bucks., UK)	Hybond™ ECL™ nitrocellulose membrane, Hyperfilm™ ECL™, ECL™ Advance Western blotting reagents, Illustra GFX™ PCR DNA and Gel Band Purification Kit, DNase I
ICN Biomedicals, Inc (CA, USA)	Methionine (Met)-free DMEM
Invitrogen/Gibco BRL Life Technologies Ltd/Molecular Probes (Paisley, UK)	Restriction enzymes, DMEM, Opti-MEM®, trypsin, Lipofectamine™2000, AlexaFluor®-conjugated secondary antibodies, calf intestinal alkaline phosphatase (CIAP), SuperScript II RNase H-reverse transcriptase, proteinase K, 4'-6-diamidino-2-phenylindole (DAPI), Roswell Park Memorial Institute (RPMI) 1640+ GlutaMAX™-1 medium,
Mabtech (OH, USA)	Capture antibody – anti-mouse IFNγ monoclonal (AN18), Detection antibody – biotinylated anti-mouse IFNγ monoclonal (R46A2)
Merck (Nottingham, UK)	Ionomycin
Millipore (MA, USA)	MAIP plates (MAIPS4510) for ELISPOT
Mimotopes	PVM N peptide library
National Diagnostics (Hull, UK)	Protogel® (30% acrylamide/0.8% bis)
New England Biolabs (Herts., UK)	Restriction enzymes, bovine serum albumin (BSA)
Promega UK (Southampton, UK)	PureYield™ plasmid midiprep system reagents
QIAgen (Sussex, UK)	QIAprep® Spin Miniprep Kit, QIAprep® Maxiprep kit, QIAquick gel extraction kit,
Roche (Hamps, UK)	Buffer for 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid (ABTS), DNase I, Collagenase A

Sigma-Adlrich (Poole, Dorset, UK)	GenElute™ HP Plasmid Miniprep Kit, ATP, DTT, Tri-reagent®, spermine, ABTS, Xylazine, Nonidet p40 (NP40), Concanavalin A (ConA), histoplaque solution, β-mercaptoethanol, BFA, PMA
Stratagene (La Jolla, CA, USA)	AdEasy™ Adenoviral Vector System, BJ5183 electroporation competent cells, XL10-Gold® ultracompetent cells
Thermofisher (USA)	96 well flat bottom plates, 96 well U bottom plates
University of Warwick Biological Sciences media preparation service	Sterile PBS, 1x Tris-borate-EDTA (TBE) buffer, 5x Tris-acetate-EDTA (TAE) buffer, LB medium, LB agar plates, LB agar plates with 100µg/ml ampicillin (Amp), LB agar plates with 50µg/ml kanamycin (Kan), 100mg/ml Amp stock solution, 50mg/ml Kan stock solution, 10x running buffer (SDS-PAGE), 10x Tris-glycine transfer buffer (Western blotting), 1x DMEM, 2x DMEM, versene, 10% SDS, 0.5M EDTA pH8.0, 1x Glasgow minimal essential medium (GMEM) with non-essential amino acids (NEAA), 2x GMEM-NEAA, SDW, penicillin/streptomycin, neutral red, glutamine
Vector Laboratories Ltd	Mounting medium (Vectorshield®)

2.1.3 Virus strains

Table 2.1.3. Virus strains used in this study.

Virus strain	Phenotype	Reference
Ad5*dl*327 (Ad 327)	Wild type Ad5 containing the E3 deletion described for *dl*324	(Thimmappaya, 1982)
PVM strain J3666	The pathogenic strain of PVM. Restricted cell culture growth temperature at 32˚C	(Cook et al., 1998, Domachowske, 2002)
PVM strain 15 (Warwick)	Non-pathogenic strain of PVM, does not have a restricted growth temperature.	(Cook et al., 1998, Domachowske, 2002)
Influenza A/WSN/40	Wild-type influenza H1N1, the mouse neurotropic variant derived from A/WSN/31 (H1N1)	(Dimmock et al., 2008)

2.1.4 Bacterial strains and plasmids

Table 2.1.4. *E. coli* strains used in this study.

Strain	Description	Source/Reference
E. coli BJ5183	*end*A1 *sbc*BC *rec*BC *gal*K *met thi*-1 *bio*T *hsd*R (Str^r)	Stratagene (La Jolla, CA, USA) (Hanahan, 1983)
E. coli DH5α	F-*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 ($r_k^- m_k^+$) *sup*E44 *rel*A1 Δ(*lac*ZYA-*arg*F) U169 *deo*R *pho*A Λ^- Φ80d*lac*ZΔ*M*15	(Jessee, 1986)
E. coli XL-10 Gold	Tet^{r}Δ(*mcr*A) *183*Δ(*mcr*CB-*hsdSMR-mrr*) *173 end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac Hte* [F'*proAB lacI*^{q} *Z* Δ *M15 Tn*10 (Tet^r) AmyCam^r]	Stratagene (La Jolla, CA, USA) (Bullock, 1987)

2.1.5 Mammalian cells

Table 2.1.5. Mammalian cell lines used in this study.

Cell line	Description	Source/Reference
A549	Human lung carcinoma cells	(Girard et al., 2005)
BS-C-1	African green monkey kidney epithelial cells	(Hopps et al., 1963)
HEK293	Immortalised adenovirus transformed human embryonic kidney cells	(Graham et al., 1977)
HeLa	Human cervical epithelial carcinoma cells	(Scherer, 1953)
P2-2	BS-C-1 cell line persistently infected with PVM J3666	Kindly provided by A.Easton

2.1.6 Mouse strains

Table 2.1.6. Mouse strains used in this study.

Mouse strain	Description
BALB/c	The BALB/c mouse originated in 1923. This inbred strain is albino, with a MHC haplotype of $H2^d$.
C57BL/6	The mouse strain originated in 1921. This inbred strain is black and has a MHC haplotype of $H2^b$.
C3H/He-mg	This inbred mouse strain is agouti in colour and has an MHC haplotype of $H2^k$.

2.1.7 Antibodies

Table 2.1.7. Primary antibodies and secondary antibody conjugates used for Western blot, immunofluorescence and ELISA in this study.

Protein recognised	Description	Supplier/Reference	Dilution used		
			WB	IF	ELISA
PVM F	Rabbit anti-PVM F polyclonal clone 2018	Kindly provided by A Easton	1:10,000	1:1000	Neat
PVM N	Rabbit anti-PVM N polyclonal	(Barr et al., 1994)	1:1250	1:63	Neat
PVM P	Mouse monoclonal antibody, 26/11/B5	Kindly provided by A Easton (Barr et al., 1994)	1:400	1:40	Neat
PVM M	Mouse anti-PVM polyclonal	Kindly provided by R Ling (Ling, 1988)	N/A	1:100	Neat
PVM M	Mouse anti-PVM M (anti-M_RLing)	Kindly provided by R Ling (Ling, 1988)	N/A	1:100	N/A
Mouse IgG	Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG	Sigma Aldrich	1:5000	N/A	1:7500

Mouse IgG	Alexa488 goat anti-mouse IgG	Molecular Probes	N/A	1:500	N/A
Goat IgG	Alexa594 rabbit anti-goat IgG	Molecular Probes	N/A	1:500	N/A
Ad5 late proteins	AdJLB1 rabbit polyclonal serum	(Farley et al., 2004)	1:10,000	1:1000	1:850
DBP	Rabbit anti-DBP (DNA binding protein) polyclonal	(Harfst & Leppard, 1999)	N/A	1:100	N/A
DBP	Mouse anti-DBP monoclonal (mAb B6-8)	(Reich et al., 1983)	N/A	1:100	N/A
Rabbit IgG	HRP-conjugated goat anti-rabbit	Santa Cruz	1:200,000	N/A	1:10,000
Influenza A proteins	Mouse anti-influenza A polyclonal serum	Kindly provided by L Harvey-Smith	N/A	N/A	Neat
Mouse IgA	HRP-conjugated goat anti-mouse	Santa Cruz	N/A	N/A	1:10,000
Mouse CD8a	APC conjugated rat anti-mouse CD8a monoclonal (553085)	BD Pharmingen	N/A	N/A	N/A
Mouse CD4	PE conjugated rat anti-mouse CD4 monoclonal (553652)	BD Pharmingen	N/A	N/A	N/A
Mouse IFNγ	FITC conjugated rat anti-mouse IFNγ monoclonal (554411)	BD Pharmingen	N/A	N/A	N/A
Mouse IFNγ	Capture antibody – anti-mouse IFNγ monoclonal (AN18)	Mabtech	N/A	N/A	N/A
Mouse IFNγ	Detection antibody – biotinylated anti-mouse IFNγ monoclonal (R46A2)	Mabtech	N/A	N/A	N/A

2.1.8 Plasmids and primers

Table 2.1.8. Plasmids used in this study

Plasmid	Description	Gene of Interest	Source/Reference
pP2	This plasmid contains the T7 promoter, the Amp^R gene and the P gene of PVM strain J3666.	PVM P	Kindly provided by O. Dibben, University of Warwick
pN3	This plasmid contains the T7 promoter, the Amp^R gene and the N gene of PVM strain J3666.	PVM N	Kindly provided by O. Dibben.
P15-FL2G	This plasmid contains the T7 promoter, the Amp^R gene and the 10 genes of PVM strain 15	PVM F and M	Kindly provided by O. Dibben.
pBR322	General purpose cloning vector		Kindly provided by K. Leppard, University of Warwick (Bolivar et al., 1977)
pShuttle_CMV	The plasmid is provided by the AdEasy™ Adenoviral Vector System. It contains a multiple cloning site (MCS), located between the cytomegalovirus (CMV) promoter and the simian virus 40 (SV40), right-inverted terminal repeat (R-ITR), left-inverted terminal repeat (L-ITR), pBR322 origin, Kan^R gene and regions of sequence homology with pAdEasy-1 which encodes the adenoviral genome and allows recombination to take place.	None	Stratagene (La Jolla, CA, USA)
pShuttle_CMV_*Lac*Z (referred to throughout as pShuttle_CMV_Z)	This plasmid contains the features described for pShuttle_CMV. The *lacZ* gene was inserted into the MCS and serves as a control for the generation of recombinant adenoviral construct.	*Lac*Z	Stratagene (La Jolla, CA, USA)
pShuttle_CMV_N1 and _N4	This plasmid contains the features described for pShuttle_CMV. The PVM N gene cDNA was amplified by PCR from pN3 and inserted into the MCS.	PVM N	This study

pShuttle_CMV_P1	This plasmid contains the features described for pShuttle_CMV. The PVM P gene cDNA was amplified by PCR from pP2 and inserted into the MCS.	PVM P	This study
pShuttle_CMV_F1 and _F2	This plasmid contains the features described for pShuttle_CMV. The PVM F gene cDNA was amplified by PCR from p15-FL2G and inserted into the MCS.	PVM F	This study
pShuttle_CMV_M7	This plasmid contains the features described for pShuttle_CMV. The PVM M gene cDNA was amplified by PCR from p15-FL2G and inserted into the MCS.	PVM M	This study
pAdEasy-1	pAdEasy-1 contains the human Ad5 genome with the E1 and E3 genes deleted. The plasmid contains the pBR322 origin of replication and the Amp^R gene. A recombination event with pShuttle_CMV is required to generate a genome that is functional to create virus. The E1 gene deletion renders the rescued virus replication defective so missing E1 gene products are provided in *trans.* The E3 deletion prevents the virus from modulating the hosts immune response to allow evasion.	None	Stratagene (La Jolla, CA, USA)
pAdEasyP17	The plasmid contains the features described for pAdEasy-1. The pShuttle_CMV_P1 plasmid has recombined with the pAdEasy-1 plasmid at the right and left arms of homology, inserting the pShuttle_CMV_P1 pBR322 origin of replication, the Kan^R gene, R-ITR, L-ITR, CMV promoter and the PVM P gene. The pAdEasy-1 pBR322 origin of replication and Amp^R gene is displaced and lost during the recombination event.	PVM P	This study
pAdEasyP26	The plasmid contains the features described for pAdEasy_P17.	PVM P	This study
pAdEasyN3	The plasmid contains the features described for pAdEasy_P17 with the exception of the PVM N gene sequence downstream of the CMV promoter.	PVM N	This study
pAdEasyN20	The plasmid contains the features described for pAdEasy_N3.	PVM N	This study
pAdEasyF59	The plasmid contains the features described for pAdEasy_P17 with the exception of the PVM F gene sequence downstream of the CMV promoter.	PVM F	This study

pAdEasyF61	The plasmid contains the features described for pAdEasy_F59.	PVM F	This study
pAdEasyM2	The plasmid contains the features described for pAdEasy_P17 with the exception of the PVM M gene sequence downstream of the CMV promoter.	PVM M	This study
pAdEasyM5	The plasmid contains the features described for pAdEasy_M2.	PVM M	This study
pAdEasy*lacZ* (referred to throughout as pAdEasyZ)	The plasmid contains the features described for pAdEasy_P17 with the *lacZ* gene from pShuttle_CMV_*LacZ* downstream of the CMV promoter.	*LacZ*	This study
pUC18	The plasmid contains the Amp^R gene, the pMB1 replicon, MCS, a region of *E.coli* operon *lac* operon which contains the CAP protein binding site, *lac* promoter, *lac* repressor binding site and the 5'-terminal part of the *Lac*Z which encodes a β-galactosidase fragment.	None	Stratagene (La Jolla, CA, USA)

Table 2.1.9. Oligonucleotide primers used in this study.

Underlined regions indicate a restriction enzyme site and the sequence, direction and application of each primer is indicated.

Primer name	Sequence (5'-3')	Direction	Application
PVMFFor	GGGGTACCATGATTCCTGGCAG	Forward	Insertion of restriction enzyme site into the beginning of the gene sequence and PCR amplification.
PVMFRev	GGCTCGAGTCATGATAAAACTGTGAG	Reverse	Insertion of restriction enzyme site into the end of the gene sequence, RT-PCR and PCR amplification.
PVMMFor	GGGGTACCATGGAGGCCTACTTGGTA	Forward	As for PVMFFOR
PVMMRev	GGCTCGAGTCATCCACTTGTTGAGGA	Reverse	As for PVMFREV
PVMNFor	CCGCGGGTCGACATGTCTCTAGACAGATTGAAGC	Forward	As for PVMFFOR

PVMNRev	GGCTCGAGTTAAATATCATCATCAGGAGTGTC	Reverse	As for PVMFREV
PVMPFor	GGGGTACCATGGAGAAATTCGCCCCC	Forward	As for PVMFFOR
PVMPRev	GGCTCGAGTTAACTAAAAATTAATGCCCATGATG	Reverse	As for PVMFREV
N3	GGATTCTATCACATCAGAAAT	Forward	Sequencing the PVM N gene, kindly provided by O. Dibben.
J2	GTATCTCTAGATCAGCTAAAT	Reverse	Sequencing the PVM N gene, kindly provided by O. Dibben.
N5	TGAAAGCCGAGAAAGCCAGGT	Forward	Sequencing the PVM N gene, kindly provided by O. Dibben.
M1	CAGCCAACATATCACTAACTGTG	Forward	Sequencing the PVM M gene, kindly provided by O. Dibben.
M2	CACTGTACCCAGCATTACTCCCA	Forward	Sequencing the PVM M gene, kindly provided by O. Dibben.
P2-A	CTTTGTGGAACCCGAGGAG	Forward	Sequencing the PVM P gene, kindly provided by O. Dibben.
P2-B	CCCTGTACAAGCTAGCTCCCAATCCGACAA	Reverse	Sequencing the PVM P gene, kindly provided by O. Dibben.
J3	AGATGAAGAACCCGGCTCTTG	Reverse	Sequencing the PVM P gene, kindly provided by O. Dibben.
F1	AACCTCTTCTTCCTTTTGGACTTCAAGG	Reverse	Sequencing the PVM F gene.
F2	TGGCTGGTATTGTCACAATGCTGGCTCA	Forward	Sequencing the PVM F gene.
F7	TATTGAGTCATGCAAGAGCA	Forward	Sequencing the PVM F gene, kindly provided by O. Dibben.
F9	CATTGTAGGCGGCATGGCTG	Forward	Sequencing the PVM F gene, kindly provided by O. Dibben.
F11	ACTGTTTACTATCTTAGCAA	Forward	Sequencing the PVM F gene, kindly provided by O. Dibben.
F17	TCCACTGCACTACTATAGAT	Reverse	RT-PCR and PCR amplification, kindly provided by O. Dibben.
CMV1	TTTTGTGTTACTCATAGCGCGTAA	Forward	Sequencing the CMV promoter.
CMV2	TATTGACGTCAATGACGGTAAATGGCC	Forward	Sequencing the CMV promoter.
CMV3	TTTTGGCACCAAAATCAACGGG	Forward	Sequencing the CMV promoter.
F1	AGTCATGCAAGAGCAGCAACTCGTTA	Forward	Sequencing and amplification of F gene in RT-PCR.
F2	GCTGTTGTTAGCCTAACCAACGGCA	Forward	Sequencing and amplification of F gene in RT-PCR.

F3	TGTTAACGGACCGCGAACTCACCTC	Forward	Sequencing and amplification of F gene in RT-PCR.
F4	CGTCGCCAACGGATTGTGAGATC	Forward	Sequencing and amplification of F gene in RT-PCR.
F5	CAAAGGTATAATAAGGACTCTGCCAGAT	Forward	Sequencing and amplification of F gene in RT-PCR.
F6	TTCATTCTGTATAAGGTATTGAAAATGATCAGA	Forward	Sequencing and amplification of F gene in RT-PCR.
P2	CCAGAGCTGACACTGATGATGGATC	Forward	Sequencing the P gene.
M3	AGCAGGCTATAAGCTCCGATGTGG	Forward	Sequencing the M gene.
N1	AAGGAAAGGAATACAAAATACAAGTCCTAGATAT	Forward	Sequencing the N gene.
N2	GAAGGGCTTTAAATGTGCTGAAAGCC	Forward	Sequencing the N gene.
ActinF	TGGGTCAGAAGGATTCCTATGTGGGC	Forward	Amplification of the β-actin gene in RT-PCR.
ActinR	AGCACAGCTTCTCCTTAATGTCACGC	Reverse	Amplification of the β-actin gene in RT-PCR.

2.2 Techniques related to cloning DNA

2.2.1 DNA amplification by polymerase chain reaction

Primers (Table 2.1.9) were reconstituted to 100µM in SDW. Each reaction was preceded by a 2 min. denaturation step followed by cycle of 94°C for 30 sec., DNA annealing at 55°C for 1 min. and a DNA extension step at 72°C for 2 min. 35 cycles were carried out prior to a final extension step of 72°C for 7 min. Reactions were constituted as described in Table 2.2.1 and processed on the mastercycler gradients machine (Eppendorf). When colony screening was performed by PCR, reactions were constituted as described in Table 2.2.1 using a non-proof reading enzyme. *E. coli* colonies were picked from an LB agar plate and introduced into the reaction mixture to function as template DNA.

Proof reading reaction constituents	Final concentration	Non-proof reading reaction constituents	Final concentration
Primers	20 pmol each	Primers	20 pmol each
dNTP	250µM of each	dNTP	250µM of each
Pfu DNA polymerase	1µl (v/v) at 2.5U/µl	*Taq* DNA polymerase	0.2µl (v/v) at 5U/µl
Buffer*	10% (v/v)	Buffer**	10% (v/v)
SDW	Up to 50µl	$MgCl_2$	250µM
		SDW	Up to 20µl

Table 2.2.1: PCR reaction constituents.
* 20mM Tris-HCl pH8.8, 10mM $(NH_4)_2SO_4$, 10mM KCl, 0.1% (v/v) Triton ® X-100, 0.1mg/ml BSA, 2mM $MgSO_4$.
** 100mM Tris-HCl pH8.8, 500mM KCl, 0.8% (v/v) NP40

2.2.2 RNA amplification by reverse-transcriptase polymerase chain reaction

For each reaction to be established, a mastermix was prepared containing (at a final concentration): primer (20 pmol), 250µM of each dNTP, RNA 1µg, 10% (v/v) buffer (50mM Tris-HCl pH8.3, 75mM KCl, 3mM $MgCl_2$ and 10mM DTT), 1µl (v/v) SuperScript® II reverse transcriptase at 200U/µl and SDW up to 20µl. The reaction was incubated at 42°C for 1 hr. then heated to 72°C for 10 min. to inactivate the enzyme. The reaction was then incubated at 37°C for 30 min. with two units of ribonuclease H to remove remaining RNA before use as template for PCR.

2.2.3 DNA restriction digest

Digestion of DNA with individual enzymes was performed in the appropriate buffer according to manufacturer's instructions. Multiple enzyme digests were performed in the most optimal buffer or sequentially if the enzymes would not function acceptably under the same conditions. Restriction enzyme digestion reactions were performed in a total volume of 20-100µl and contained 1-10 units of enzyme per µg of DNA. Reactions were incubated at 37°C for 1-18 hr. and inactivated where appropriate at 65°C for 20 min.

2.2.4 DNA phosphorylation and dephosphorylation

1-20 pmol of DNA was phosphorylated prior to ligation using T4 DNA kinase according to manufacturer's instructions. The reaction was made up to 19µl in SDW and incubated at 37°C for 20 min. The reaction was inactivated through incubation at 75°C for 10 min. to denature the enzyme.

DNA was dephosphorylated prior to transformation using CIAP according to manufacturer's instructions. The reaction was made up to 50µl with SDW and incubated at 37°C for 30 min. EDTA, pH8.0 was added prior to incubation at 65°C for 3 min. to inactivate the CIAP.

2.2.5 Agarose gel electrophoresis

Agarose gels were prepared in 1x TBE at 0.5-1% agarose for small DNA fragments and 1x TAE at 1-1.5% agarose for larger DNA fragments. Ethidium bromide at 0.5µg/ml was added once the solution was lukewarm. The gel solution was poured into the gel apparatus and assembled according to manufacturer's instructions. Once set, the gel was placed into the tank and covered in 1% TAE or TBE. The samples were mixed with 2-5µl loading buffer and loaded onto the gel. The gel was run at 100-150V for 90-120 min. with either GeneRuler® or Hyperladder1 as a marker until the fragments were separated. DNA was visualised under ultraviolet (UV) illuminator.

2.2.6 DNA purification from agarose gel

DNA bands were visualised under UV light and cut from the gel using a sharp scalpel. The gel slice was extracted using either GFX™ PCR DNA and Gel Band Purification Kit or QIAprep® Spin Miniprep Kit according to manufacturer's instructions. DNA was eluted into 20-50µl SDW.

2.2.7 DNA purification from solutions

Proteins, buffers, and enzymes were removed by DNA extraction with phenol/chloroform. DNA samples were made up to 0.1ml with SDW and an equal volume of phenol: chloroform 1:1 solution was added. The sample was mixed by vortexing, prior to centrifugation at 16,100 x g for 3 min. The aqueous layer was removed into a new microfuge tube and the organic phase discarded.

2.2.8 DNA and RNA precipitation

DNA was precipitated by adding 0.1 volumes of 3M sodium acetate (pH5.2) and 2.5 volumes of ice-cold ethanol. The sample was mixed by vortexing and incubated at -20°C for 1 hr. The DNA solution was centrifuged at 16,100 x g for 15 min. and the supernatant was discarded. 1ml of 70% ethanol was added and the DNA was centrifuged at 16,100 x g for 5 min. This step was repeated prior to a 30 sec. centrifugation to remove any remaining ethanol. The tube was then incubated at 37°C for 15 min. to remove any traces of ethanol and resuspended in 10-50µl SDW.
RNA was precipitated using RNase-free isopropanol using the method described in above. The RNA was treated with DNase according to manufacturer's instructions to remove any remaining DNA and used directly for RT-PCR.

2.2.9 DNA extraction from adenovirus virus infected mammalian cell lysate

Adenovirus infected cell suspensions were centrifuged at 600 x g for 5 min. and the supernatant was discarded. The pellet was resuspended in 0.4ml TE pH9 with 10mM spermine and 0.4ml DOC lysis buffer and mixed. The solution was centrifuged at 12,000 x g for 15 min. The supernatant was transferred into fresh microfuge tubes and 60µl 10% (w/v) SDS, 20µl 0.5M EDTA and 20µl of 20mg/ml proteinase K was

added. The reaction was incubated at 37ºC for 1 hr. before the DNA was extracted from the solution as described in Section 2.2.8.

2.2.10 RNA extraction from transfected mammalian cell lysate

Transfected cells were harvested by scraping the cells from a tissue culture plate using small rubber pieces into 1ml PBS. Duplicates were pooled together and the cell suspension was centrifuged at 600 x g for 3 min. The pellet was resuspended in 0.5ml of isotonic buffer with 33µl of 10% (v/v) NP40. The suspension was incubated on ice for 10 min. prior to centrifugation at 600 x g for 3 min. The supernatant was transferred into RNase-free microfuge tubes to which 0.75ml Tri-Reagent® was added and the reaction was incubated at room temperature (RT) for 20 min. 0.1ml RNase-free chloroform was added to the reaction and a second incubation was performed at RT for 15 min. The reaction was then centrifuged at 16,000 x g for 15 min. and the upper aqueous phase was transferred to a fresh microfuge tube.

2.2.11 Plasmid extraction

For the purposes of routine analysis, plasmid DNA was extracted from 1-5ml of fresh overnight *E. coli* culture. The culture was centrifuged at 16,000 x g for 1 min. The supernatant was discarded and the pellet was resuspended in 0.1ml GTE. 0.2ml of lysis solution was added and the reaction was incubated on ice for 5 min. 0.15ml of neutralisation solution was then added prior to centrifugation at 16,000 x g for 10 min. The supernatant was transferred to a new microfuge tube and the DNA purified as described in Sections 2.2.7-8.

Any plasmid constructs which were required for sequencing or transformation were prepared using 1-5ml of fresh overnight culture using either GenElute™ HP Plasmid Miniprep kit or QIAgen® Spin Miniprep kit according to the manufacturer's instructions.

For the purposes of mammalian cell transfections, sequencing and transformation experiments, plasmid DNA was extracted from 50-250ml fresh overnight culture using Promega PureYield™ Midiprep System according to manufacturer's instructions. For cultures of 250-500ml in volume, a QIAgen® Maxiprep kit was used according to manufacturer's instructions.

2.2.12 DNA ligation

Ligation reactions were performed using T4 DNA ligase. For blunt end ligations, the reaction was set up with a molar ratio of 1:5 vector to insert, 10% (v/v) buffer (40mM Tris-HCl pH7.8, 10mM $MgCl_2$, 10mM DTT and 0.5mM ATP) and 10 units of T4 DNA ligase and made up to a final volume of 50µl with SDW. The reaction was incubated overnight at 16°C in a water bath and 10µl was used to transform *E. coli*.

2.2.13 DNA quantification

Approximate DNA concentrations were determined by agarose gel fluorescent band intensity, Sections 2.2.2 and 2.2.3, in comparison to Hyperladder1, or determined based on absorbance at 260nm ($1A_{260}$ = 50µg/ml) using the NanoDrop ND-1000 spectrophotometer.

2.2.14 DNA sequencing

DNA sequencing reactions contained 100ng of template plasmid, 5pmol of primer, and SDW to a final volume of 10µl. Sequencing reactions were carried out at the University of Warwick Molecular Biology services. Sequences were analysed using the Chromas software program and the SeqMan™II program.

2.3 Manipulation of *E. coli* strains

2.3.1 Growth and storage of *E. coli* strains

The *E. coli* strains outlined in Table 2.1.4 were cultured in LB medium at 37°C with vigorous shaking. Following plasmid transformation, bacteria were grown on LB agar plates or as liquid cultures and where appropriate, the cultures were supplemented with 100µg/ml Amp or 25µg/ml Kan. Bacterial stocks were maintained for short-term storage as streaked agar plates and stored at 4°C. For long-term storage, a 15% (v/v) mix of sterile glycerol and exponential phase liquid culture was prepared and stored at -70°C.

2.3.2 Production of chemically competent *E. coli* strains

The *E. coli* DH5α was cultured overnight from a colony in 5ml LB medium. 1ml was used to inoculate 100ml LB medium, which was grown to an OD_{600} 0.39 at 37°C with shaking. The culture was incubated on ice for 5 min. before centrifugation at 6000 x g for 10 min. at 4°C. The supernatant was discarded and the pelleted cells were resuspended in 40ml ice-cold RF1 buffer and incubated on ice for 1 hr. The cells were centrifuged at 6000 x g for 10 min. at 4°C and the supernatant was discarded. The pelleted cells were resuspended in 4ml ice-cold RF2 buffer and incubated on ice for 2-3 hr. Aliquots of cells were snap-frozen in a dry ice-ethanol bath before transfer to -70°C for long-term storage.

2.3.3 Production of electrocompetent *E. coli* strains

Electrocompetent *E. coli* BJ5183 cells were prepared by adding 25ml fresh overnight culture to 500ml LB medium. The cells were grown at 37°C to an OD_{600} 0.35-0.4. The cultures were incubated in an ice-cold water bath for 30 min. prior to centrifugation at 6000 x g for 15 min. at 4°C. The supernatant was discarded and the pelleted cells were resuspended in 500ml ice-cold pure SDW and centrifuged at 6000 x g for 20 min. at 4°C. The supernatant was discarded and the pelleted cells were resuspended in 250ml ice-cold 10% glycerol and centrifuged at 6000 x g for 20 min. at 4°C. The supernatant was discarded and the pelleted cells were resuspended in 1ml ice-cold GTY medium and diluted to OD_{600} 1.0 with GTY medium. The cells were aliquoted and snap-frozen in a dry ice-ethanol bath then transferred to -70°C for long-term storage.

2.3.4 Transformation of *E. coli* using purified DNA

Extracted and purified DNA was used to transform chemically competent *E. coli* strain DH5α. *E. coli* aliquots were thawed in a microfuge tube and mixed with a minimum of 50ng DNA prior to incubation on ice for 30 min. The reaction was incubated at 42°C for 45 sec. then incubated on ice for 2 min. 0.5ml of pre-warmed LB medium was added to the cells, which were incubated at 37°C for 1 hr. or 30°C for 1.5 hr. with shaking. The reaction was spread on selective agar plates and incubated at 37°C overnight or 30°C for 24 hr. 30°C growth temperature was used

for transformations of plasmids over 15kbp in size. For ligated DNA, 10µl of a ligation reaction was added to 0.1ml of competent cells and transformed as described above. XL10-Gold® cells were transformed and BJ5183 cells were electroporated according to manufacturer's instructions. Bacteria were spread onto LB Kan plates and incubated at 37°C overnight.

2.4 Tissue culture techniques

2.4.1 Maintenance of cell-lines

Mammalian cell lines were maintained as detailed in Table 2.4.1, at 37°C in 5% CO_2. All cells were cultured in 90mm tissue culture dishes or 175cm^2 tissue culture flasks and were passaged when the cellular monolayer reached 90-100% confluency. To remove the cells from the surface of the culture container, cells were washed with versene then incubated at 37°C with trypsin solution. Cells were regularly observed until all had become detached. The cells were then centrifuged at 340 x g for 3 min. with an equal volume of fresh growth medium. The supernatant was discarded and the pellet was resuspended in fresh growth medium and transferred into new tissue culture dishes, plates, flasks, or bottles at the appropriate density

Cell line	Medium	Split ratio
A549	DMEM with 10% (v/v) FCS	1:4
BS-C-1	GMEM with 5% (v/v) FCS	1:4
HEK293	DMEM with 10% (v/v) FCS	1:4
HeLa	DMEM with 10% (v/v) FCS	1:8
P2-2	GMEM with 15% (v/v) FCS, 2.5% (v/v) glutamine, 0.15% (v/v) Penicillin /Strepomycin	1:3

Table 2.4.1: Cell line maintenance and growth medium conditions

2.4.2 Storage and recovery of cell stocks

Mammalian cells were harvested from 175cm^2 tissue culture flasks using trypsin solution and centrifuged at 340 x g for 3 min. The pellet was resuspended in 90% (v/v) FCS and 10% (v/v) filter-sterilised DMSO. Approximately 1x10^7 cells/aliquot

were transferred to vials and frozen overnight at -70ºC prior to long term storage in liquid nitrogen.

To recover cell stocks, vials were thawed quickly from liquid nitrogen in a 37ºC water bath. The entire aliquot was introduced to a 175cm^2 tissue culture flask with full growth medium and maintained as described in Section 2.4.1. The media was changed 24 hr. later to remove any traces of DMSO.

2.4.3 Bulk culture of P2-2 cells

One 175cm^2 tissue culture flask of 80-90% confluent P2-2 cells was harvested as described in Section 2.4.1 and used to seed three large glass roller bottles each with 200ml of medium. CO_2 was introduced into the bottles for 30 sec., as the bottles were unvented, prior to incubation at 37ºC. Cells were harvested after four to seven days using mechanical agitation with glass beads. Lysates were pelleted by centrifugation at 340 x g for 3 min. before storage at -70ºC.

2.4.4 Transfection of cells in 12-well plates

HEK293 cells were transfected when cells were 80-90% confluent using Lipofectamine™ 2000 according to manufacturer's instructions. The mixture was added in a drop-wise manner to the cells in serum-free DMEM. After incubation at 37°C in 5% CO_2 for 5 hr., the serum-free media was replaced with DMEM with 2% (v/v) FCS and returned to the incubator. For larger or smaller densities of cells, all volumes were altered accordingly.

2.5 Virological Techniques

2.5.1 Growth of Adenovirus stocks

Mammalian cells were seeded into 90mm dishes to be 90% confluent the following day. Cells were infected with a multiplicity of infection (M.O.I) of 10 plaque forming units (p.f.u.)/ cell and incubated at 37°C in 5% CO_2 for 1 hr., rocking every 15 min. 10ml medium was then added to the cells. Full cytopathic effect (c.p.e.) was reached after approximately 2-3 days, the infected cells were harvested into a sterile

polypropylene tube and stored at -70ºC. Before use, virus preparations were freeze-thawed three times using a 37ºC water bath and dry ice to achieve rigid temperature cycles, so that viral particles were freed from cellular material.

2.5.2 Adenovirus plaque assays

The virus stock prepared as in 2.5.1 was centrifuged at 340 x g and the liquid aliquoted into a new tube to remove cell debris. The stock was serially diluted into 10-fold dilutions in serum free medium. Each virus dilution was used to infect monolayer 6-well cultures of HEK293 cells in triplicate, which were then incubated at 37°C for 1 hr. with rocking every 15 mins. to spread the inoculums. The virus solution was aspirated from the cells and 2ml of noble agar overlay was added to each well before incubation at 37°C in 5% CO_2. Every 3-4 days an additional 1ml of noble agar feed was added until plaques were visible, a larger or smaller volume was used for different tissue culture plate sizes. Plaques were stained with noble agar staining overlay allowing plaques to be counted and the virus titre determined.

2.5.3 β-galactosidase assays for constructs containing *Lac*Z

HEK293 cells were transfected or infected with either plasmid or virus containing the *Lac*Z gene as described in Sections 2.4.4 and 2.5.1. Cells were washed with PBS after 24-36 hr. and fixed using 1ml fixative solution for 5 min. at RT. Cells were washed twice in PBS prior to incubation at 37ºC overnight in 1ml β-galactosidase substrate solution. Cells were assessed for β-galactosidase activity under a light microscope by observing the cells changing to a blue colour.

2.5.4 Growth of PVM J3666 stocks

Confluent monolayers of BS-C-1 cells in GMEM NEAA with 2% (v/v) FCS at 37°C in 5% CO_2, were infected with stocks of PVM strain J3666 at an M.O.I of 10 p.f.u. Cells were incubated at 32°C and virus was harvested when c.p.e was observed. Virus was aliquoted and stored at -70°C.

2.5.5 PVM plaque assays

BS-C-1 cells were seeded at $5x10^5$ cells/ml into 12-well plates. The virus stock was thawed on ice and 10-fold serial dilutions were prepared in serum free media. 4% (w/v) CMC agar was diluted to 2% using 2x GMEM-NEAA with 2% (v/v) FCS, 0.15% (w/v) Penicillin/Streptomycin and 2% (w/v) glutamine. The dilutions were used to infect the cells in triplicate. After 1 hr. incubation at 32°C, 2ml of the CMC agar solution was added to each well of the plate. The assay was incubated at 32°C for 10-12 days until plaques were seen. To stain the assay, 2% (v/v) gluteraldehyde in PBS was added to each well and incubated at RT for 1 hr. The overlay was then removed and 1ml 1.5% (w/v) crystal violet in ethanol was added and the plate incubated at RT for 5 min. The stain was removed with SDW and dried at 37°C prior to observation of plaques under a light microscope.

2.6 Protein Detection

2.6.1 Immunofluorescence

12-well plates containing sterile cover slips were incubated with 0.1µg/ml polylysine for 1 hr. at 37°C before cells were seeded at $5x10^5$/well prior to the experiment. To fix the cells at the conclusion of the experiment, the cover slips were washed three times in 1ml PBS prior to incubation for 10 min. at RT with 10% (v/v) formalin in PBS. The cells were then washed three times with 1ml PBS and incubated for 10 min. at RT with 0.5% (v/v) NP40 in PBS to permeabalise the cells and permit antibody access to internal antigens. The cells were then washed three times in 1ml PBS and either stored at 4°C in 2ml PBS or used immediately in an immunofluorescence (IF) experiment.

The cells fixed onto cover slips were incubated for 1 hr. at RT with 0.5ml 1% (w/v) BSA in PBS prior to three 1ml washes in PBS. The cells were incubated for 1 hr. at RT with 0.25ml primary antibody (Table 2.1.7) diluted in 1% (w/v) BSA in PBS. The cells were washed three times with 1ml PBS prior to incubation for 1 hr. RT in the dark with 0.25ml secondary antibody (Table 2.1.7) diluted as before. The cells were then washed three times in PBS and the incubation steps were repeated with the

second primary antibody and the second secondary antibody. Once the final wash after staining was completed, the cells were incubated for 5 min. at RT in the dark with 1ml 0.001% (w/v) DAPI in PBS. The cover slips were removed from the wells and placed on tissue to remove excess DAPI stain. The cover slips were placed cell side down onto 4µl mounting solution and the edges sealed with clear nail varnish. Images were obtained using a Leica SP2 confocal system.

2.6.2 Radioactive labelling of mammalian cellular proteins using [^{35}S] methionine

Mammalian cells were infected with recombinant adenovirus (rAd) as described in 2.5.1. 20 hr. post-infection, the cells were pre-starved of methionine (Met) through addition of 0.5ml/well Met-free media for 20 min. Met-free media was then aspirated from the cells to which 0.1ml of [^{35}S]Met was added diluted to a final concentration of 50µCi/ml. Cells were incubated at 37°C for 30-60 min. prior to removal of the [^{35}S]Met. Cells were washed in 0.5ml PBS and lysed directly in 50µl 1x sample buffer.

2.6.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Cell extracts in 1x sample buffer were denatured in a boiling water bath for 10 min. and loaded onto a gel comprising of stacking gel plus 10% resolving gel. SDS-PAGE was performed using a Mini-PROTEAN 3 electrophoresis system (BioRad) with electrophoresis at 150V for 90 mins. The protein marker PageRuler™ Prestained Protein Ladder was used as a size marker for the proteins.

2.6.4 Western Blotting

Proteins were transferred from SDS-PAGE gels to nitrocellulose membranes at 4°C at either 80mA overnight or 350mA for 75 min. The membrane was blocked at RT for 1 hr. with blocking solution or at 4°C overnight. The membrane was then incubated for 1 hr. at RT with primary antibody (Table 2.1.7) diluted in blocking solution and shaken vigorously. The membrane was then washed in PBS-Tween for 1 hr. and with regular buffer changes. The membrane was then incubated for 1 hr. at

RT with secondary antibody appropriate to the primary antibody used (Table 2.1.7) diluted in blocking solution and shaken vigorously. The membrane was then washed in PBS-Tween for 1 hr. and as before changed regularly. Finally, the membrane was incubated for 5 min. at RT with 0.5ml ECL Advance™ Solution A and 0.5ml of ECL Advance™ Solution B (HRP chemiluminescent substrate). The membrane was placed on filter paper to remove excess substrate and exposed to X-ray film from 30 sec. to 5 min.

2.6.5 Exposure of radio labelled samples to X-ray film

Radio labelled samples were separated by SDS-PAGE as described in Section 2.6.4. The gel was fixed for 1 hr. in 250ml 25% (v/v) methanol and 7% (v/v) acetic acid in SDW prior to drying on a gel dryer (BioRad) for 2 hr. The gel was then exposed to X-ray film for 1-12 hr.

2.7 Immunogen preparation

2.7.1 Virus particle stock isolation

Crude adenovirus particle stocks were generated as described in Section 2.5.1. After full c.p.e. was reached, cells from five dishes were pelleted together at 340 x g for 3 min. The supernatant was discarded and the pellet stored at -70°C. The pellets were thawed on ice and resuspended in 5ml ice cold 0.1M Tris-HCl pH8. The cell suspension was sonicated in an ice bath with a microtip probe sonicator (Jencons Scientific Ltd) using two sets of 10x1 sec. separated by 30 sec. The suspension was centrifuged at 5000 x g for 10 min. and the resulting supernatant was layered over step CsCl gradients, prepared using 3ml 1.25g/ml (w/v) CsCl and 2ml 1.40g (w/v) CsCl in a Beckman SW41 ultra centrifuge tube. 0.1M Tris-HCl pH8 was added to each gradient to ensure they were of equal weight. Gradients were centrifuged at 35,000 rpm using a Beckman SW41 rotor for 1 hr. at 15ºC.

The band of virus particles was extracted from the gradient by bottom puncture and collected in a SW55 ultra centrifuge tube on ice. 1.35g/ml (w/v) CsCl was added to each tube to within 2-3mm of the rim and weighed to ensure each gradient was of

equal weight. Gradients were centrifuged at 40,000 rpm in a Beckman SW55 rotor for approximately 18 hr. at 15ºC. Virus particles were extracted from the gradient by bottom puncture and collected in a microfuge tube on ice.

Virus particles from CsCl gradient fractions were purified by dialysis in 400ml 20% (v/v) glycerol in PBS for 12-18 hr. at 4°C with one buffer change. The dialysis tubing was prepared by boiling for 20 min. in 2% (w/v) $NaHCO_3$, 10mM EDTA and rinsed in SDW. Tubing was boiled again for 5 min. in 1mM EDTA and autoclaved for 1 hr. Tubing was stored in 70% (v/v) ethanol and 1mM EDTA at RT.

2.7.2 Virus particle stock quantification

1µl of virus particle solution was diluted in 25µl 1xTE containing 0.1% (w/v) SDS and quantified by measuring absorbance at 260nm using the nanodrop spectrophotometer. The virion concentration is given by the relationship that 1 A_{260} = 10^{12} virions/ml (Tollefson, 2007). For each virus stock, a plaque assay was also performed as described in Section 2.5.1.2 to determine the infectious titre and DNA was isolated and amplified as described in Sections 2.2.5.2 and 2.2.1.1 respectively to confirm that the stocks retained the relevant PVM gene.

2.8 *In vivo* related techniques

2.8.1 Maintenance of mice

Pathogen free BALB/c mice were purchased from B and K Universal Ltd and were used to set up a breeding colony housed at the University of Warwick Biomedical Services Unit. Male and female mice were used in experiments at between five and seven weeks of age. Mice were maintained in filter-top cages and routinely monitored for specified pathogens, including PVM.

For all experiments, mice were housed within a category three isolated holding room once inoculated with PVM strain J3666. Room humidity and temperature, mouse weight and clinical signs were monitored daily for the duration of the experiment.

Mice were sacrificed if deemed appropriate due to health reasons, or at the end of the experiment, by cervical dislocation.

2.8.2 Administration of immunogens to mice

2.8.2.1 Administration of anaesthetic

Mice were anaesthetised using 0.15-0.2ml/mouse Ketamine/Xylazine solution by direct injection into the lower right abdomen. After procedures were completed, mice left to recover in low light conditions.

2.8.2.2 Inoculation with virus or vaccines

For intranasal immunisation, mice were anaesthetised and inoculated with 50µl of PVM strain J3666, Influenza A/WSN, or rAd constructs. The inoculum was evenly distributed between the nostrils and administered in small droplets.

For intraperitoneal immunisation, mice were inoculated with 200µl of rAd constructs directly into the abdomen as described for anaesthesia.

For subcutaneous immunisation, mice were anaesthetised and inoculated with 50µl of rAd constructs directly into the scruff of the neck. The inocula were evenly distributed by gently rubbing the area.

2.8.3 Sample collection

For serum sample collection, live mice were anaesthetised or restrained mechanically, to allow blood collection by removal and massaging of the tail tip. Samples were harvested from sacrificed mice by cardiac puncture and removal of serum using a syringe. The blood was incubated on ice for 2 hr. prior to centrifugation, then serum was transferred to a fresh sterile microfuge tube and stored at -20°C.

For lung and spleen collection, samples were harvested from sacrificed mice by removal of entire lung lobes or spleen. Samples were stored in 1ml PBS at -70°C for long-term storage. For use in ELISPOT assay, materials were stored until needed in 1ml PBS on ice.

For bronchoalveolar lavage (BAL) fluid collection, BAL samples were harvested from sacrificed mice. The entire chest cavity and trachea were exposed prior to

making a small incision at the top of the trachea. A blunt needle, previously loaded with 1ml PBS, was inserted into the trachea and secured using suture thread. The PBS was forced into, and withdrawn from the lungs and transferred to a sterile microfuge tube for storage at -70°C.

2.9 Immunological techniques

2.9.1 ELISA for detection of anti-PVM humoral immunity

Several lysates of the P2-2 cell line were grown as described in 2.4.3 and pooled together to form a stock lysate solution. The cell lysate was sonicated in an ice bath with a microtip probe sonicator (Jencons Scientific Ltd) using two sets of 10x1 sec. separated by 30 sec. This was diluted to 1mg/ml in coating solution and plated at 50µl/well into a flat-bottomed 96-well plate. The plate(s) were incubated at 4°C for 12 hr. The coating solution was removed from the plate, which was then incubated with 0.2ml/well 5% Marvel/PBS for 2 hr. at RT. Primary serum was typically diluted 1:85 for the initial dilution and then serially diluted 1:3 across the plate in 0.1ml/well. The plate was incubated at RT for 2 hr. prior to three 0.4ml/well PBS-Tween washes using a plate washer (Amersham Biosciences). Secondary HRP conjugated antibody was added at 0.1ml/well (Table 2.1.7) and the plate was incubated at RT for 2 hr. The three wash steps with 0.4ml/well PBS were repeated and 0.1ml/well of ABTS at 1.8mM, was added. The plate was incubated in the dark until a colour change was discernable prior to the absorbance being measured at 405-410nm on Labsystems multiskan RC plate reader.

2.9.2 ELISA for detection of anti-Ad humoral immunity

Ad327 particles were grown and purified as described in 2.5.1.1 and 2.7. Virions were exposed to UV light at 253.7nm for 5 min. using a transilluminator (Gelman Science Ltd UK) to inactivate them. These were then diluted to 1µg/ml in coating solution and plated at 50µl/well into a flat-bottomed 96-well plate. The protocol as described in Section 2.9.1.1 was then followed.

2.9.3 Murine lymphyocyte ELISPOT assay

ELISPOT plates (MAIPS4510) were coated aseptically with capture antibody in sterile coating solution to a final concentration of 15µg/ml at 50µl/well. The plates were incubated either overnight at 4°C or for at least 1 hr. at 37°C. Coating solution was then removed prior to addition of 100µl/well of RPMI with 10% FCS. The plates were then incubated at 37°C for at least 1 hr. to block the wells.

2.9.3.1 Preparation of murine splenocytes

Spleens were harvested as described in Section 2.8.3.2. Spleens from individual animals were pooled according to their group and mashed through a cell strainer. Cells were passed through the strainer several times to ensure all clumps were removed. The cell suspension volume was increased to 30ml with sterile PBS prior to the addition of 15ml Histoplaque solution underlay. The suspension was then centrifuged at 1200 x g for 30 min. at RT with no brake. Cells were removed from the interface between PBS and Histoplaque solution, and transferred to a fresh container. The cell suspension volume was increased to 40ml prior to centrifugation at 884 x g for 10 min. at RT. The supernatant was discarded and the cell pellet resuspended in 40ml PBS and centrifuged at 884 x g for 10 min. at RT; this step was repeated three times. The splenocytes were then resuspended in 1-5ml RPMI media with 10% FCS prior to counting using a haemocytometer.

2.9.3.2 Preparation of lung lymphocytes

Lungs were harvested as described in Section 2.8.3.2. and the lungs from individual animals were pooled according to their group. Tissue was chopped into very small pieces prior to addition of 15ml of DNase and Collagenase A at a final concentration of 1mg/ml and 2.4mg/ml respectively. This mixture was then incubated for a minimum of 30 min at 37°C with vigorous shaking, and then treated as previously described in Section 2.9.2.1.2.

2.9.3.3 Preparation of naive antigen presenting cells (APCs)

If required, an appropriate number of naive splenocytes were centrifuged at 884 x g for 10 min. prior to resuspension with PVM strain J3666 virus to give an M.O.I of 1 p.f.u./cell. APCs were incubated for 90 min. with rolling at 37°C prior to irradiation at 3000 rads for 15 min., then centrifuged at 884 x g for 10 min. APCs were then washed twice in sterile PBS before resuspending in an appropriate volume of RPMI with 10% FCS.

2.9.3.4 Preparation of target cell populations and antigen

Splenocytes or lung lymphocytes were diluted to an appropriate concentration in RPMI medium (RMPI containing 10% FCS, 0.1% β-mercaptoethanol, IL-2 at 5U/ml (final concentration), 10U/ml penicillin G and 10µg/ml streptomycin sulphate). Cells were added at $5x10^5$ cells/well in a 50µl volume to the ELISPOT plates.

Peptides were used at a final concentration of 5µg/ml per well whereas virus-infected or naive splenocytes were used neat at 50µl/well.

2.9.3.5 Development of ELISPOT plate

Cell and antigen suspension was removed and the plates washed six times with PBS-Tween. The secondary antibody, biotinylated rat anti-mouse IFNγ was diluted to 1µg/ml and added to the plate at 50µl/well. The plate was incubated in the dark for 2 hr. at RT. Plates were then washed six times with PBS-Tween prior to the addition of strepavidin AP conjugate at 1µg/ml at 50µl/well. The plates were incubated for 1 hr. at RT in the dark. Plates were then washed six times with PBS-Tween prior to the addition of AP conjugate substrate as per manufacturer's instructions at 50µl/well. Plates were incubated at RT for 5-20 min. in the dark to allow the colour to develop. The reaction was stopped by addition of water to the plates, which were then left to dry for at least 1 hr. prior to reading using an AID ELISPOT reader.

2.9.4 Intracellular staining assay (ICS) of murine lymphocytes

2.9.4.1 Spleen and lung lymphocyte preparation

Splenocytes and lung lymphocytes were prepared as described in Sections 2.9.3.1-2. The cell suspensions were either used directly in an ICS assay or diluted to $1.5x10^7$/ml and co-cultured in a $25cm^2$ tissue culture flask with $3x10^6$/ml of APCs, stimulated as described in Section 2.9.3.3. Cell suspensions were incubated at 37°C in 5% CO_2 for seven days. Cells were harvested and were pooled appropriately in a sterile polypropylene tube. The cells were centrifuged at 884 x g for 10 min. prior to resuspension in PBS. The cells were again centrifuged at 884 x g for 10 min. and resuspended in 1-5ml of RPMI medium with 10% FCS prior to counting with a haemocytometer.

2.9.4.2 Preparation of target cell populations and antigen

Splenocytes and lung lymphocytes were diluted to an appropriate concentration in RPMI medium (RMPI containing 10% FCS, 0.1% β-mercaptoethanol, IL-2 at 5U/ml (final concentration), 10U/ml penicillin G and 10µg/ml streptomycin sulphate). Cells were added at 50µl/well to a round bottom 96-well plate. Peptides were used at a final concentration of 5µg/ml per well whereas virus infected or virus naive APCs were added to the plate at 50µl/well.

The plate was then incubated for at least 3 hr. at 37°C in 5% CO_2, prior to the addition of PMA/Io solution and BFA (Table 2.1.1). BFA was added to inhibit protein transport to allow effective detection of IFNγ. The plate was then incubated for 3 hr. prior to immunostaining with appropriate antibodies (Table 2.1.7).

2.9.4.3 Intracellular fluorescent staining of lymphocytes

The cell suspension was pelleted by centrifugation at 250 x g for 2 min. then the cells were washed in 100µl/well of FACS buffer. The plate was centrifuged again at 250 x g for 2 min. prior to the addition of extracellular staining antibody, usually anti-mouse $CD4^+$ and anti-mouse $CD8^+$, both at a final concentration of 1µg/well in a 50µl volume (Table 2.1.7). The cell suspension was incubated for 20 min. at RT in the dark prior to centrifugation at 250 x g for 2 min. The cells were then washed

twice with 100µl/well of cold PBS, prior to addition of 100µl of BD Cytofix/Cytoperm™ Fixation/Permeabilization solution. The plate was incubated for 20 min. at 4°C, then washed twice with 1x BD Cytofix/Cytoperm™ Perm/Wash™ Buffer. Anti-mouse IFNγ (Table 2.1.7) was diluted to a final concentration of 1µg/well in 1x Perm/Wash™ buffer. The antibody was added to the cells in a 50µl volume and incubated for 30 min. at 4˚C. The cells were then washed twice with Perm/Wash™ buffer, before resuspension in 100µl/well cold FACS buffer. Cells were transferred to FACS tubes containing 300µl FACS buffer prior to analysis on FACSCalibur (BD Biosciences). Data was analysed using CellQuestPro software.

Chapter 3

Development of Adenovirus Serotype 5 Recombinant Viruses

3.1 Introduction

Adenovirus serotype 5 (Ad5) has been well characterised as a vector, both for gene therapy and vaccine development. Deletion of the E1 and E3 regions has increased the capacity of vectors to incorporate foreign DNA. These E1-E3 deleted vectors are replication-defective yet they are able to infect host cells, express genes, and thus stimulate the immune response to target cells in a natural manner. An aim of this study has been to clone the PVM F, M, N and P genes into an Ad5 vector using the AdEasy™ Adenoviral Vector System (Stratagene, USA). The system, developed by He and colleagues (He et al., 1998), utilises a double-recombination event in bacteria between the Ad5 genome backbone cloned in a plasmid, pAdEasy-1, and a shuttle plasmid, pShuttle_CMV, carrying the transgene. The resulting recombinant Ad genome can then be transfected into mammalian cells to generate infectious virus. This method eliminates the need for multiple plaque purification whilst generating a replication-defective virus. However, the E1-E3 deleted vector requires the E1 gene products for virus growth in tissue culture cells. The E1 gene products can be provided in *trans* by HEK293 cells, a transformed human embryonic kidney cell line that carries copies of the E1 genes in its genome.

In this chapter, the construction of the pShuttle_CMV vectors containing the PVM F, M, N and P genes and subsequent generation of Ad5 recombinant plasmids through homologous recombination with pAdEasy-1 is described. The expression levels and presence of the PVM proteins following recombinant virus infection were investigated using Western blot, immunofluorescence (IF) or RT-PCR. Finally, virus stocks were characterised by PCR to confirm retention of the relevant transgene and titred by plaque assay.

3.1.1 Overview of the AdEasy™ Adenoviral Vector System

The gene of interest can be cloned into the multiple cloning site (MCS) of pShuttle_CMV vector (Fig. 3.1A). The vector is then linearised by digestion with *Pme*I and co-transformed into BJ5183 cells with a plasmid containing the viral genome, pAdEasy-1. This promotes recombination between the two molecules of DNA (Fig. 3.1B). Transformed clones can then be screened for kanamycin resistance and through a restriction digest, using the *Pac*I enzyme. A diagnostic digest generates a DNA fragment which identifies recombinants. DNA from the recombinants is then purified prior to transformation into XL10-Gold® cells. XL10-Gold® cells are *rec*A$^-$, preventing further recombination events occurring and allowing the amplification of a stable plasmid. The *end*A mutation in the bacterial cell line improves the plasmid DNA quality as single stranded breaks in the DNA are less likely to be introduced. The DNA is then purified and re-screened using a diagnostic digest with the *Pac*I restriction enzyme. If the correct DNA fragment is identified, the DNA preparation is digested completely with the *Pac*I enzyme to liberate the rAd5 recombinant genome from the pAdEasy-1 plasmid. The liberated virus genome is then used to transfect HEK293 cells and with subsequent amplification steps within this cell line, the recombinant viruses can be isolated and grown to a high titre.

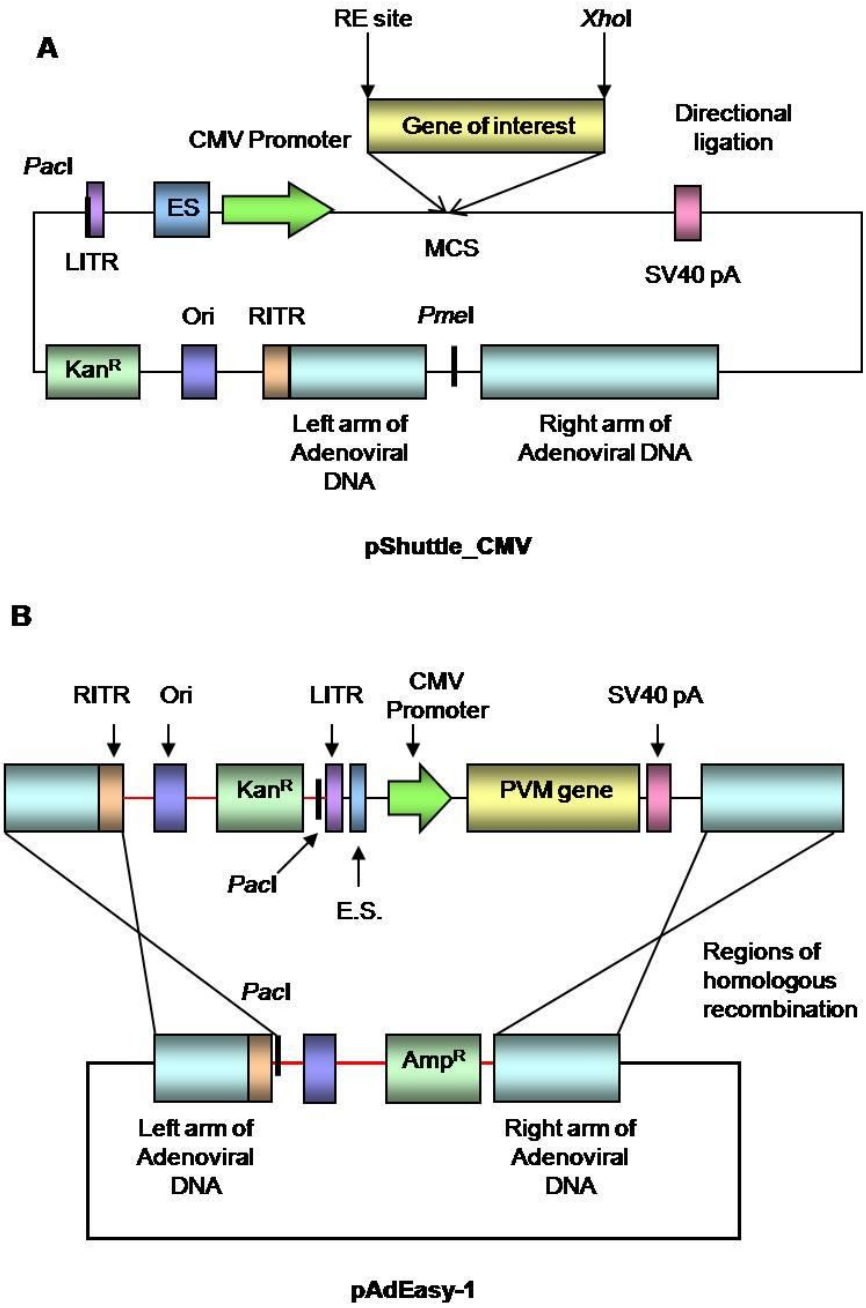

Figure 3.1. The overview of the AdEasy™ Adenoviral Vector System construction.
(A) The pShuttle_CMV plasmid is a basic vector which allows a recombination event to occur with another plasmid, pAdEasy-1 to generate an adenoviral genome within a bacterial plasmid backbone (App. B. Fig. B.1). The plasmid encodes left and right inverted terminal repeats (LITR and RITR respectively), an encapsidation signal (ES), pBR322 origin of replication (Ori), and kanamycin resistance (Kan^R) to allow for clone selection. A gene of interest, up to 6.6Kb in size can be inserted into the multiple cloning site (MCS) of the plasmid. The mammalian cytomegalovirus immediate-early promoter (CMV promoter) upstream of the MCS regulates the transgene expression. Downstream of the MCS is an SV40 polyadenylation signal (SV40 pA) which ensures polyadenylation of the gene of interest transcript.

Through restriction digest of pShuttle_CMV and the gene of interest, the gene of interest can be inserted into pShuttle_CMV through directional ligation. The vector is then linearised by restriction digest using the *Pme*I restriction enzyme.

(B) The pAdEasy-1 vector contains the human Ad5 genome, an ampicillin resistance gene (Amp^R), pBR322 origin of replication (Ori) and the left and right arms of adenoviral DNA. Linearised pShuttle_CMV containing the gene of interest can be co-transformed with pAdEasy-1 into *E. coli* strain BJ5183. This *rec*A^+ strain facilitates homologous recombination events between the plasmids in the left and right arms of Ad5 DNA. The recombination event is selected for by the loss of Amp^R from pAdEasy-1 and the acquisition of Kan^R and the gene of interest from pShuttle_CMV. The resultant recombinant Ad5 genome (black plasmid line) is released from the plasmid backbone through restriction digest with the *Pac*I enzyme.

3.2 Cloning the PVM genes

3.2.1 Construction of pShuttle_CMV plasmids containing the F, M and P genes of PVM

The initial cloning step amplified the F and M genes from plasmid p15FL-2G (Section 2, Table 2.1.8) by PCR. p15FL-2G contains cDNA of the genome of non-pathogenic PVM strain 15. The M proteins of PVM strain 15 and J3666 are identical with one non-coding nucleotide change, G700U. The F gene from PVM strain 15 has one non-coding change, G966U and two coding changes, A737G and C992U corresponding to amino acid changes Lys243Arg and Ala328Val respectively. The primers PVMFFOR and PVMFREV generated a cDNA fragment of the F gene, of 1616bp (Fig. 3.2A) whereas the primers PVMMFOR and PVMMREV generated a cDNA fragment of the M gene of 770bp (Fig. 3.2B).

The pP2 plasmid (Table 2.1.8) contains the P gene from PVM J3666. The P gene was amplified from the pP2 plasmid by PCR using the PVMPFOR and PVMPREV primers, generating a cDNA fragment of 894bp (Fig. 3.2C). All primers incorporated *Kpn*I and *Xho*I restriction sites at the 5' and 3' ends of the genes respectively. These restriction sites were present in the MCS of pShuttle_CMV with sufficient separation to allow their simultaneous digestion (Fig. 3.3).

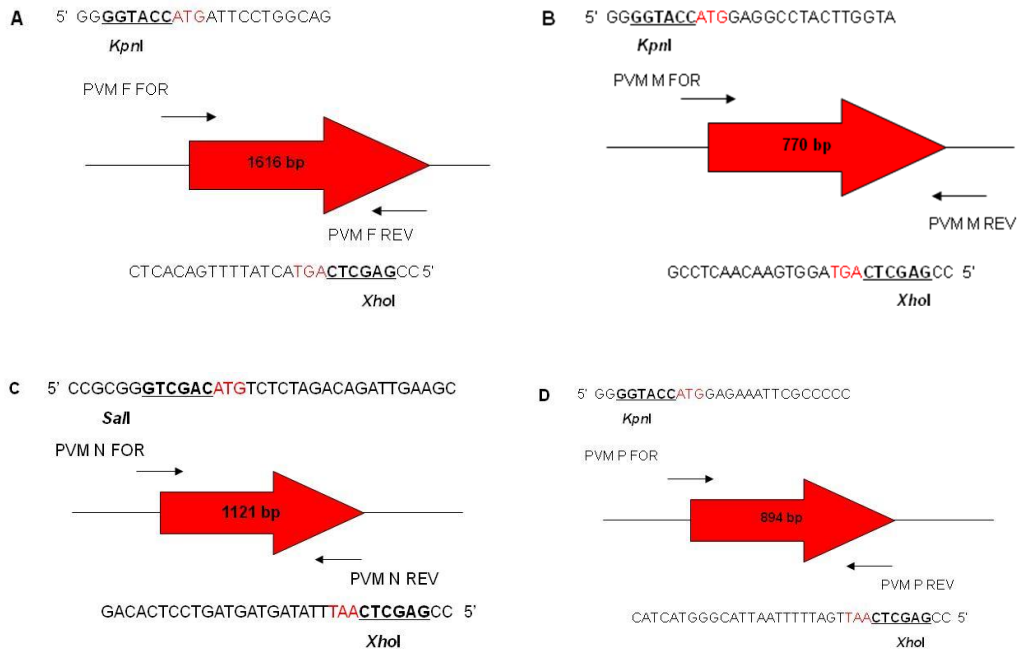

Figure 3.2. PCR amplification of the PVM genes.
The PVM F, M, N and P genes were amplified from their parental plasmids using the specific primer pairs: PVMFFOR and PVMFREV (**A**); PVMMFOR and PVMMREV (**B**); PVMNFOR and PVMNREV (**C**); and, PVMPFOR and PVMPREV (**D**). The restriction sites incorporated into the cDNA fragments are underlined whereas start and stop codons are identified in red.

The cDNA fragments and the pShuttle_CMV vector were purified prior to restriction digestion with *Kpn*I and *Xho*I enzymes. The DNA was then re-purified and the F, M and P gene cDNA's were separately ligated into the prepared pShuttle_CMV plasmid. The resultant reaction mixes were used to transform *E. coli* DH5α. The *E. coli* were screened for acquisition of kanamycin resistance using selective media. DNA from twenty randomly chosen colonies per construct was purified and confirmed to be correct by a diagnostic digest with *Kpn*I and *Xho*I restriction enzymes (Fig. 3.3).

DNA preparations from at least six positive clones for pShuttle_CMV_F and _M and one clone for pShuttle_CMV_P, were re-purified and sequenced using the original amplification primer pairs. Nucleotide sequence analysis confirmed no alterations were present within the pShuttle_CMV_M plasmid sequence as compared with the database sequence for this gene (AY729016.1, App. A. Fig. A.1). pShuttle_CMV_P was sequenced with two additional primers, P2-A and P2-B (Section 2, Table 2.1.9), which amplified regions within the PVM P gene. The pShuttle_CMV_P sequence

was compared with the database sequence for the P gene (AY573814, App. A. Fig. A.4) and no alterations were found to be present. Finally, pShuttle_CMV_F was sequenced with primers F1, F2, F7, F9 and F11, in addition to the original primers to ensure the entire gene was sequenced. The nucleotide sequence for the PVM F gene was compared with the database sequence for this gene (AY729016.1, App. A. Fig. A.2). Two non coding changes, T87C and T256C, and one coding change T849C corresponding to amino acid change P283L, were identified.

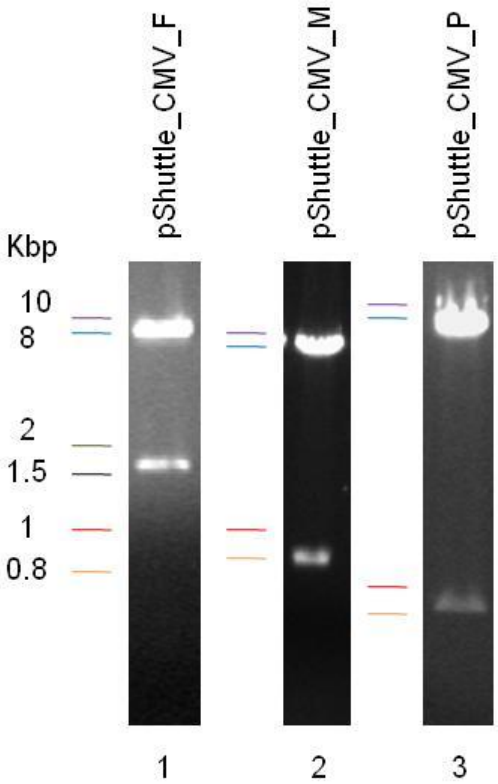

Figure 3.3. Screening the pShuttle_CMV clones for successful ligation of the PVM genes.
pShuttle_CMV plasmids containing the F, M and P genes of PVM were confirmed to be correct by a diagnostic digest with restriction enzymes *Kpn*I and *Xho*I. The digest liberated the PVM genes from the pShuttle_CMV vector, yielding a positive digestion pattern of bands approximately sized 1.6Kbp (lane 1), 0.77Kbp (lane 2), 0.89Kbp (lane 3) corresponding to the F, M and P genes respectively. Results are representative of several isolated clones.

3.2.2 Construction of pShuttle_CMV plasmids containing the N gene of PVM

The PVM N gene was amplified from the pN3 plasmid, which contains the N gene from PVM strain J3666 by PCR using the PVMNFOR and PVMNREV primers. A cDNA fragment of 1184bp was generated with *Sal*I and *Xho*I restriction sites incorporated into the 5' and 3' ends respectively (Fig. 3.2).

Several attempts were made to generate pShuttle_CMV_N encoding the N gene cDNA fragment in the correct orientation. All attempts were unsuccessful at cloning N cDNA in either orientation, therefore, it was concluded that the efficiency of the

*Sal*I digest was insufficient to generate a clonable product. To overcome this, the uncut N cDNA fragment was blunt ligated into the *Eco*RV site of pShuttle_CMV and the resultant reaction was used to transform *E. coli* DH5α, which were then selected for kanamycin resistance using selective media. Finally, DNA preparations from twenty randomly chosen colonies were purified and the correct orientation of the PVM N gene was confirmed through a diagnostic digest using the restriction enzyme *Bst*XI (Fig. 3.4). DNA from six of these correct colonies was re-purified and sequenced using the primers PVMNFOR, PVMNREV, N3, J2, and N5 (Table 2.1.9). Sequence analysis confirmed no alterations were present within the pShuttle_CMV_N plasmid sequence when compared with the database sequence for this gene (App. A. Fig. A.3 (AY573813)).

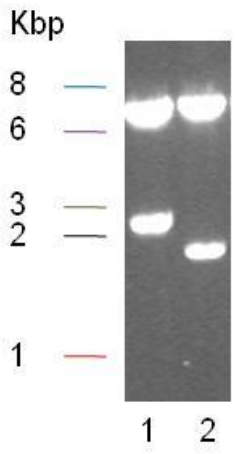

Figure 3.4. Screening pShuttle_CMV_N clones for the correct orientation of the PVM N gene.
A restriction digest with the *Bst*XI restriction enzyme identified the PVM N gene orientation. The enzyme cuts within the vector and within the PVM N gene which allowed the orientation to be determined. A positive digest pattern of bands of the sizes 6.4kbp and 2.1kbp visualised on an agarose gel, indicated that the N gene had been inserted into the MCS in the correct orientation 5'-3' (lane 1). A digest pattern of bands of the sizes 6.8kbp and 1.7kbp indicated N gene insertion into the MCS in the opposite orientation (lane 2).

3.2.3 Isolation of pAdEasy plasmids containing the F, M, N and P genes of PVM and the *Lac*Z gene of *E. coli*

The pShuttle_CMV plasmids containing PVM genes and pShuttle_CMV_Z which contains the *E. coli Lac*Z gene (Stratagene, USA) were digested with the restriction enzyme *Pme*I to linearise the plasmids. The purified plasmids were then co-transformed with pAdEasy-1 into *E. coli* BJ5183 by electroporation to generate pAdEasy_F, _M, _N, _P, and _Z by homologous recombination. The reactions were plated onto kanamycin selective agar, allowing positive clones to be identified by acquisition of kanamycin resistance.

DNA preparations from several clones for each construct were purified and screened for the recombination event using a *Pac*I restriction enzyme digest. DNA from clones F59, F61, M2, M5, N3, N20, P17, P26, Z2 and Z5 was then used to transform *E. coli* XL-10 GOLD®. DNA was purified from these clones and a diagnostic digest performed using the *Pac*I restriction enzyme. As illustrated in Fig. 3.1B, the *Pac*I restriction enzyme has two sites within the recombination product of pAdEasy with pShuttle_CMV. This is because the acquisition of pShuttle_CMV introduces an additional *Pac*I restriction site. The recombination event can occur between the right and left arms of homology (Fig. 3.1B), or between the pBR322 origins of replication and the left arms of homology. The former recombination event generates a DNA fragment of 4.5Kbp in size upon digestion with *Pac*I whereas if the origin of replication has been utilised as a recombination site, a DNA fragment of 3Kbp would be generated (Fig. 3.5). It was observed that during the screening process to identify recombinant plasmids, recombination between the origin of replication and the left arm of homology occurred less frequently, presumably because the origin of replication sequence is smaller in size than the right arm of homology and therefore is less likely to generate a recombination event.

DNA preparations were purified in bulk and the nucleotide sequences of the relevant transgenes reconfirmed using the same primers as described for pShuttle_CMV nucleotide sequencing. No additional alterations had occurred.

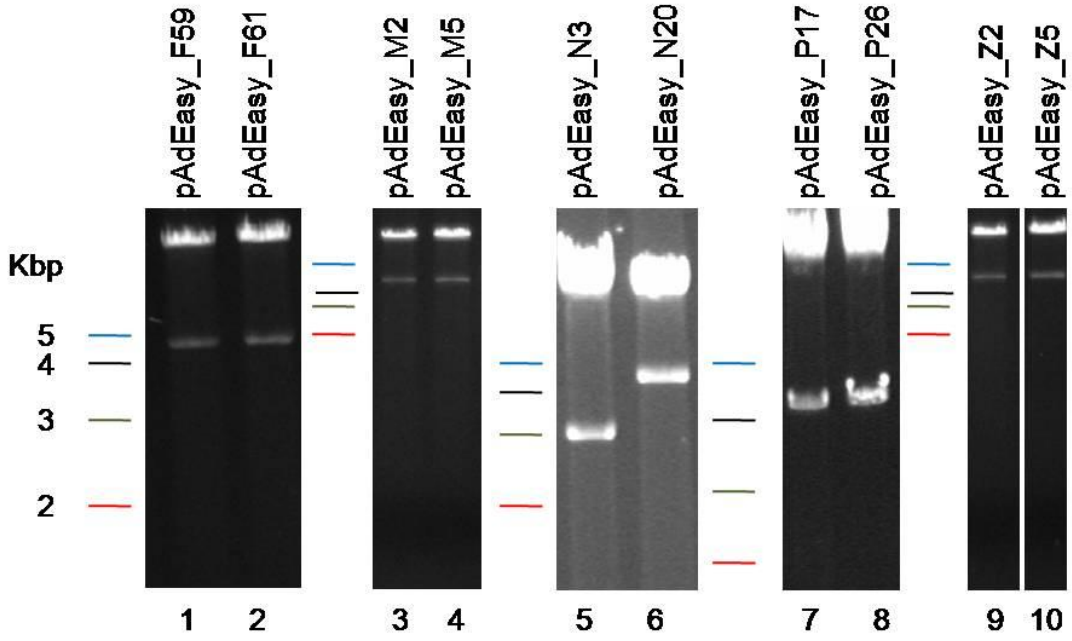

Figure 3.5. Confirmation of the recombination of pShuttle_CMV and pAdEasy. A *Pac*I diagnostic digest confirms the occurrence of the pShuttle_CMV and pAdEasy-1 recombination event through a positive DNA fragment pattern of a large fragment plus either a fragment of 4.5Kbp or 3Kbp in size. Positive results were obtained for pAdEasy_F59 and F61 (lanes 1 and 2), pAdEasy_M2 and M5 (lanes 3 and 4), pAdEasy_N3 and N20 (lanes 5 and 6), pAdEasy_P17 and P26 (lanes 7 and 8) and pAdEasy_Z2 and Z5 (lanes 9 and 10). The majority of the recombinants generated a 4.5Kbp fragment apart from pAdEasy_N3 (lane 5) (see text).

3.3 Growth and titration of recombinant Adenoviruses

To isolate viruses, recombinant Ad5 genomes released from the pAdEasy plasmid backbones by *Pac*I restriction digest were then used to transfect HEK293 cells. Cultures were then monitored for signs of cytopathic effect (c.p.e.). Cells and culture media were harvested after seven days and the cells lysed by freeze-thawing. 100µl of the lysate was used to infect a fresh 12-well culture of HEK293 cells. The cells and culture media were again harvested after seven days. The method was repeated until c.p.e was observed within a seven day period. The virus was then used to infect larger cultures with a high virus volume, which amplified the virus further. The recombinant viruses were then passaged in HEK293 cells until c.p.e. was observed with kinetics similar to a typical replication-competed Ad5 *dl*327 (Ad327, Section 2,

Table 2.1.3), usually within two to three days. At this stage, the recombinant viruses were characterised (Table 3.1) and viral titre was determined by plaque assay. Finally, large scale amplification was performed for selected viruses to generate purified stocks for subsequent experiments. rAdN20 and rAdP26 were not pursued further after passage four as it became evident that these recombinants grew much more slowly than rAdN, rAdP17 or wild-type Ad327. rAdF61, rAdM5 and rAdZ5 demonstrated comparable growth to Ad327 but were not characterised further.

Plasmid construct	Recombinant virus	Outcome
pAdEasy_F59	rAdF	Characterised
pAdEasy_F61	rAdF61	Not required
pAdEasy_M2	rAdM	Characterised
pAdEasy_M5	rAdM5	Not required
pAdEasy_N3	rAdN	Characterised
pAdEasy_N20	rAdN20	Not required
pAdEasy_P17	rAdP17	Characterised
pAdEasy_P26	rAdP26	Characterised*
pAdEasy_Z2	rAdZ	Characterised
pAdEasy_Z5	rAdZ5	Not required

Table 3.1: Designation of recombinant virus names used in this study.

* For transgene expression only

3.4 Characterisation of the Ad5 recombinants

3.4.1 Validation of recombinant virus replication

Late Ad5 protein expression was initially investigated to confirm that the presence of the PVM transgene in the recombinant viruses did not inhibit replication in the complementing cell line, HEK293. All characterised recombinant viruses demonstrated typical late Ad5 protein expression profiles by Western blot (Fig. 3.6). Late protein expression was detected with an antibody (AdJLB1, Section, Table 2.1.7), which detects several Ad5 late proteins, such as hexon, penton, protein V and protein VI. These are structural proteins required for construction of the viral capsid.

The late protein expression profile for the rAds (Fig. 3.6, lanes 1-4) has a high degree of similarity to that of Ad327 (Fig. 3.6, lane 6), confirming that the recombinants could express late proteins and thus enter late stage infection to generate infectious progeny when their E1 deficiency is complemented. Therefore, the presence of the relevant transgene did not inhibit rAd replication in HEK293 cells.

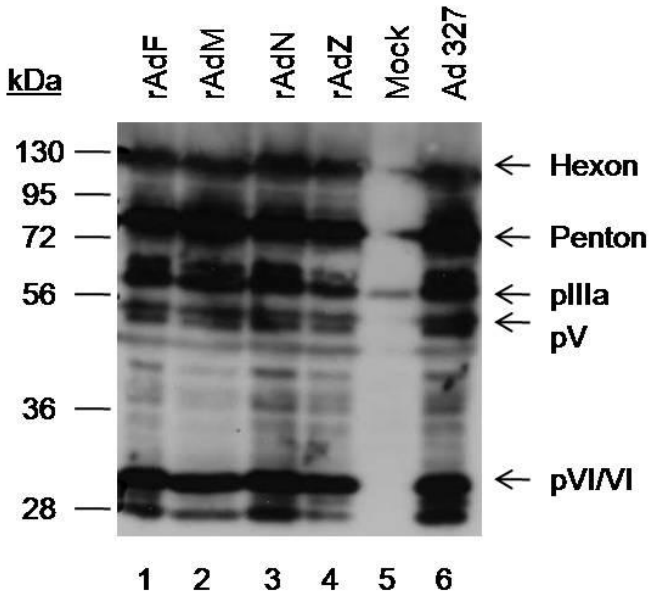

Figure 3.6. The presence of the transgene in the rAd viruses did not impede replication.

$1.5x10^6$ HEK293 cells in total, were mock-infected or infected with rAdF, -M, -N, -Z or Ad327 at a multiplicity of infection (M.O.I) of 10 p.f.u./cell. Cells were lysed 24 hours post infection (h.p.i) and lysates were separated by 10% SDS-PAGE prior to Western blotting. The membrane was probed with anti-AdJLB1 antibody which recognises late Ad5 proteins. The late protein expression profile of all rAds (lanes 1-4) was similar to wild-type control Ad327 (lane 6). The result is representative of two replicate experiments.

3.4.2 Validation of transgene expression from recombinant viruses

Transgene protein expression was investigated to ensure that the recombinant viruses expressed the relevant proteins under the control of the CMV promoter. Transgene expression was confirmed either by Western blot, IF, RT-PCR or by a β-galactosidase assay. As the final characterisation step, PCR was used to confirm the particle stocks generated for *in vivo* studies retained the gene of interest.

3.4.2.1 rAdF

Detection of PVM F mRNA expression was first attempted by RT-PCR using PVMFFOR and PVMFREV primers (Table 2.1.9 and Fig. 3.7). This combination of

primers should have enabled the detection of full length F cDNA from the mRNA extract. However, this reaction was unsuccessful, which may have been due to inefficient amplification of a long nucleotide fragment (data not shown). Therefore, the F17 and F2 primers were used in combination with PVMFFOR and PVMFREV respectively to detect PVMF mRNA via amplification of shorter segments.

Total mRNA was extracted from rAdF infected HeLa cells and F specific mRNA was converted to cDNA using the F17 or PVMFREV primers. For each primer, two reactions were performed, one with (RT^+) and one without (RT^-) the reverse transcriptase (RT) enzyme. The RT^- reaction acts as a control to identify contaminating rAd genomic DNA because no DNA fragments should be amplified from an RT^- reaction by the PCR step if mRNA was free from genomic DNA. The cDNA fragments generated were amplified by PCR using the same reverse primer and with either PVMFFOR (Fig. 3.7, lanes 1 and 2) or F2 primers (Fig. 3.7, lanes 3 and 4). β-actin cDNA was amplified from the total mRNA extract using actinF and actinR primers, to act as a mRNA loading control (Fig. 3.7, lanes 5 and 6). PVMFFOR and F17 amplified a 260bp fragment while F2 and PVMFREV amplified a 1190bp fragment (Fig 3.7B). This confirmed expression of mRNA from the F gene however the presence/absence of a small 164bp segment (Fig 3.7A) was not tested using these primer pairs. The β-actin gene fragment, of 500bp was amplified and confirmed that the RT-PCR and PCR reactions were successful. The RT^- reactions did not generate detectable DNA fragments, proving that the RT-PCR and PCR amplification occurred from mRNA rather than contaminating genomic DNA.

Attempts were made to detect PVM F protein expression using an anti-F polyclonal antibody (clone 2018, Table 2.1.7) in Western blots from rAdF-infected HEK293 cells (Fig. 3.8A) and pShuttle_CMV_F transfected HEK293 cells (Fig. 3.8B). The F protein is expressed as a large protein precursor known as F_0. Upon processing through the *trans*-Golgi network, F_0 is cleaved into F_1 and F_2. The anti-F polyclonal antibody detected F_1 expression in PVM infected positive control samples (Fig. 3.8, lanes 7 and 13), consistent with previously published data (Chambers et al., 1992). However, F protein expression was not detected from rAdF infected or pShuttle_CMV_F transfected cells (Fig. 3.8 lanes 1 and 8). The antibody did appear to react with a ~120kDa protein in cells infected with rAdZ and transfected with pShuttle_CMV_Z. As transfected cell samples could not have generated early or late

Ad5 proteins because they lack the Ad5 genome, the band is unlikely to be an Ad5 late protein. The antibody could have reacted with LacZ as the material used to generate the antibody was produced in bacteria, thus potentially, some LacZ protein may have contaminated the preparation and subsequently immunised the animals.

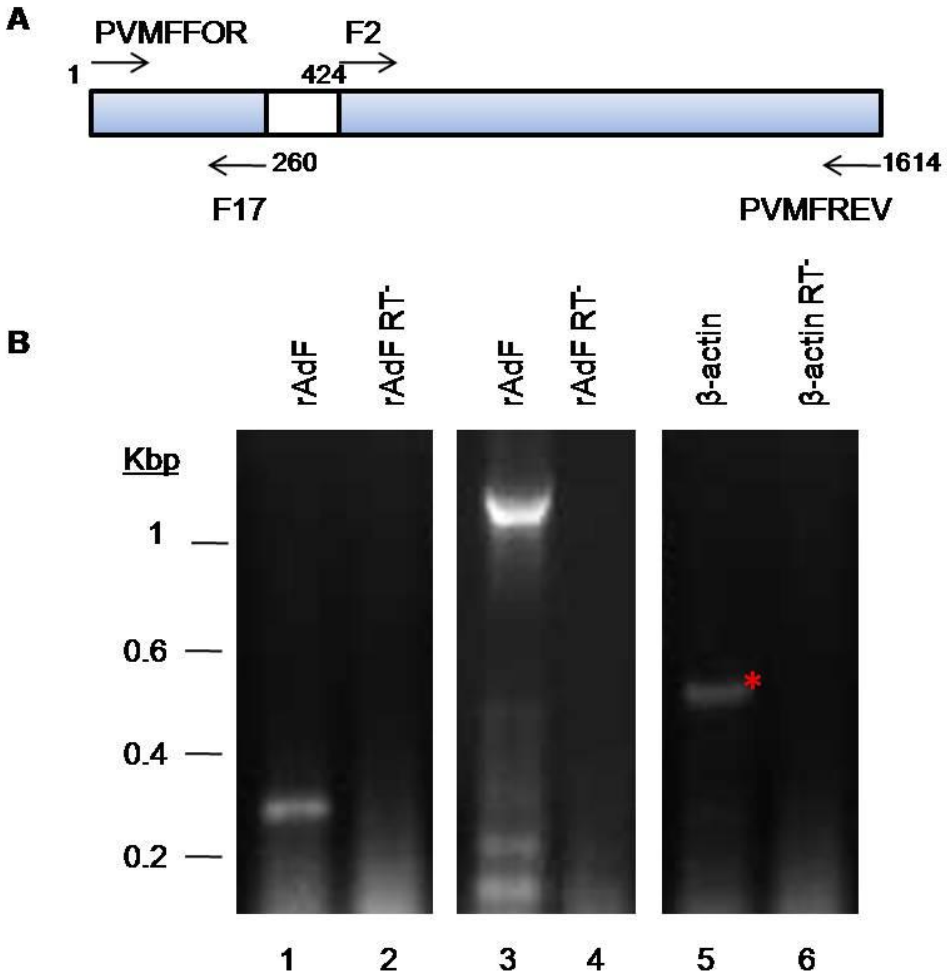

Figure 3.7. PVM F mRNA expression can be detected from rAdF infected cells.
(A) The nucleotide position of the primers used in the PCR reaction, PVMFFOR, F2, F17 and PVMFREV, in relation to the PVM F gene.
(B) $1.6x10^6$ HeLa cells in total were infected with rAdF at an M.O.I of 10 p.f.u./cell. Cells were lysed at 24h.p.i and total mRNA was extracted from the lysate. mRNA was then DNase treated to remove any contaminating rAd genomic DNA, repurified, and used directly in an RT reaction with the PVMFREV, F17 and actinR primers. RT negative reactions (lanes 2, 4 and 6), were included to confirm cDNA amplification from mRNA rather that rAdF genome. The resultant cDNA was treated with RNase H and was used directly in PCR reactions. Lane 1 and 2 indicate the fragments obtained from cDNA amplification using PVMFFOR and F17 and lanes 3 and 4 using F2 and PVMFREV. A fragment of β-actin (*) was amplified in lane 5 using actinF and actinR primers with the RT- control in lane 6 using the same primer pairs. The results illustrated in lanes 1, 2, 5 and 6 are representative of two repeat experiments.

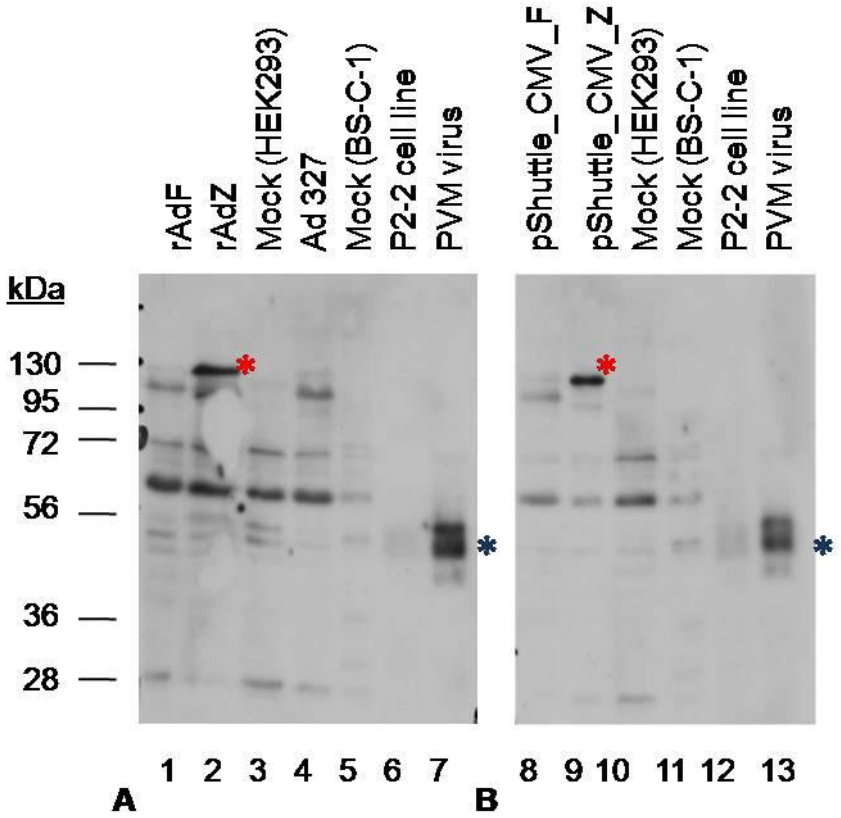

Figure 3.8. PVM F expression cannot be detected from rAdF infected or pShuttle_CMV_F transfected HEK293 cells.
(A) $1.5x10^6$ HEK293 cells in total were mock-infected or infected with rAdF, rAdZ or Ad327 at an M.O.I of 10 p.f.u./cell. Mock-infected BS-C-1 cells and P2-2 cells (a cell line persistently infected with PVM J3666) were seeded at $5x10^5$. Samples were lysed at 24h.p.i.
(B) $5x10^5$ HEK293 cells were mock-transfected or transfected with 1µg of pShuttle_CMV_F or –Z plasmid DNA. Samples were lysed at 48h.p.i. Controls as panel A.
Lysates and $1.3x10^5$ PVM J3666 virus (PVM positive control), were mixed with sample buffer and separated by 10% SDS-PAGE prior to Western blotting. The membrane was probed with anti-PVM F protein antibody (clone 2018, Table 2.1.7). Full length F_2 (the C-terminal product of the proteolytic cleavage of F protein) was detected at 39kDa in the positive controls P2-2 and PVM (lanes 6, 7, 12 and 13 (*)) and was absent from Ad327 and mock-infected samples (lanes 3-5, 10 and 11). F protein expression was not detected in rAd infected cells or pShuttle_CMV transfected cells (lanes 1,2,8 and 9), however the antibody appeared to detect a ~120kDa protein in samples containing LacZ (*).

Since it was not possible to prove expression of full length PVM F from rAdF by either RT-PCR or Western blot techniques, IF was performed to detect F protein expression in rAdF infected HeLa cells; cells were also stained for rAd5 DNA

binding protein (DBP), which is a marker for infection. F protein expression was detected in both PVM-infected BS-C-1 cells and rAdF-infected HeLa cells (Fig. 3.9) using the same anti-PVM F polyclonal antibody that was unable to detect F protein expression by Western blotting. This suggests that the Western blot technique is not sensitive enough to detect F protein expression, or that the antibody recognises a configuration of F protein that is removed by lysate processing.

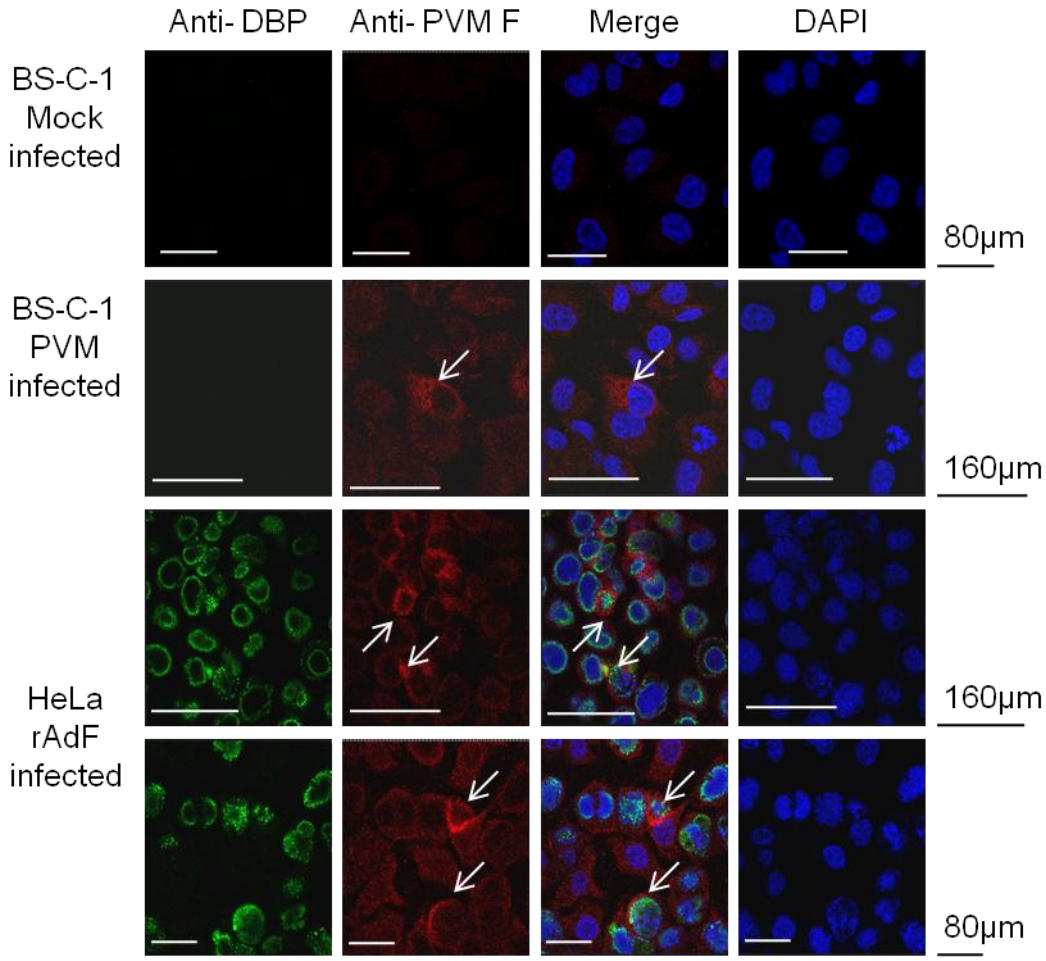

Figure 3.9. PVM F protein is expressed from rAdF.
$7.5x10^5$ HeLa cells were grown on coverslips and infected with rAdF at an M.O.I of 10 p.f.u./cell and $5x10^5$ BS-C-1 cells were mock infected or infected with PVM at an M.O.I of 10 p.f.u./cell. Virus-infected cell samples were fixed 24h.p.i and immunostained for Ad5 DNA binding protein (DBP, green) using mAb B6-8 (Table 2.1.7), and PVM F protein (red) using anti-PVM F antibody (clone 2018, Table 2.1.7), whereas DNA (blue) was visualised by DAPI staining. The merged panels show overlays of all three stains. Images were collected by confocal microscopy all at the same scale (scale bars 160µm (long) and 80µm (short). Arrows indicate cells that are positive for F protein staining.

PVM F protein expression was principally located in the cytoplasm of PVM or rAdF infected cells, with some rAdF-infected HeLa cells demonstrating strong F protein expression (Fig. 3.9 arrows). HeLa cells do not encode the missing Ad5 E1 gene products and thus should not complement rAdF growth and progression to late stage infection. However, several cells appeared to have progressed to late-stage Ad infection as judged by the DBP staining, which had altered from small nuclear bodies located within the nucleus to a ring-like structure at the boundaries of the nucleus. This suggested that the rAdF virus stocks might be contaminated with revertant wild-type rAd5 virus, a suggestion supported also by the observation that only a subset of the DBP-positive cells were positive for PVM F straining. The virus preparation might have become contaminated with a wild-type strain during stock passages, but this is thought unlikely. Alternatively, a subpopulation of rAdF might have re-acquired the E1A/B region present in the HEK293 cell line by recombination, allowing the virus to replicate in non-complementing cells. This problem has been documented previously in the use of HEK293 cells for recombinant virus growth (Lochmuller et al., 1994). F protein expression may be deleterious to Ad replication, which may have promoted loss of F gene expression and given a selective pressure for recombination with the E1A/B region of HEK293 cells.

To determine the relative amount of replication-competent Ad in the stock of rAdF, a radio-labelling experiment was performed to label all newly synthesised proteins with radioactive [^{35}S]Met (Fig. 3.10). The experiment was performed in two cell lines, A549 and HEK293 cells, respectively incapable and capable of complementing the E1-deficiences in rAd and thus allowing their full replication. It was hypothesised that comparing the extent of viral protein synthesis of each virus in the two cell types, the extend of any wild-type or replication-competent Ad5 would be discerned. In the complementing cell line, HEK293, the amount of key Ad protein expression from the rAds and Ad327 was indistinguishable (Fig, 3.10 lanes 7-12). This suggests that all the rAds are able to replicate equally within the complementing cells. In the non-complementing cell line, A549, Ad327 expressed as expected a large amount of presumably late-stage infection proteins (Fig. 3.10 lane 5). In contrast, rAdM, rAdN and rAdZ generated essentially no detectable viral late proteins (Fig. 3.10 lanes 2-4 and 6). Therefore, it was concluded that these virus stocks were unlikely to have a replication-competent subpopulation, however, the rAdF lysate did show small

amounts of the same proteins that were characteristic of Ad327 infection, confirming the presence of a replication-competent contaminant (Fig. 3.10 lanes 1 and 5). However, the quantity of their proteins was very low compared to that of wild-type virus and thus the revertant contamination is likely to be only a small proportion of the total virus present.

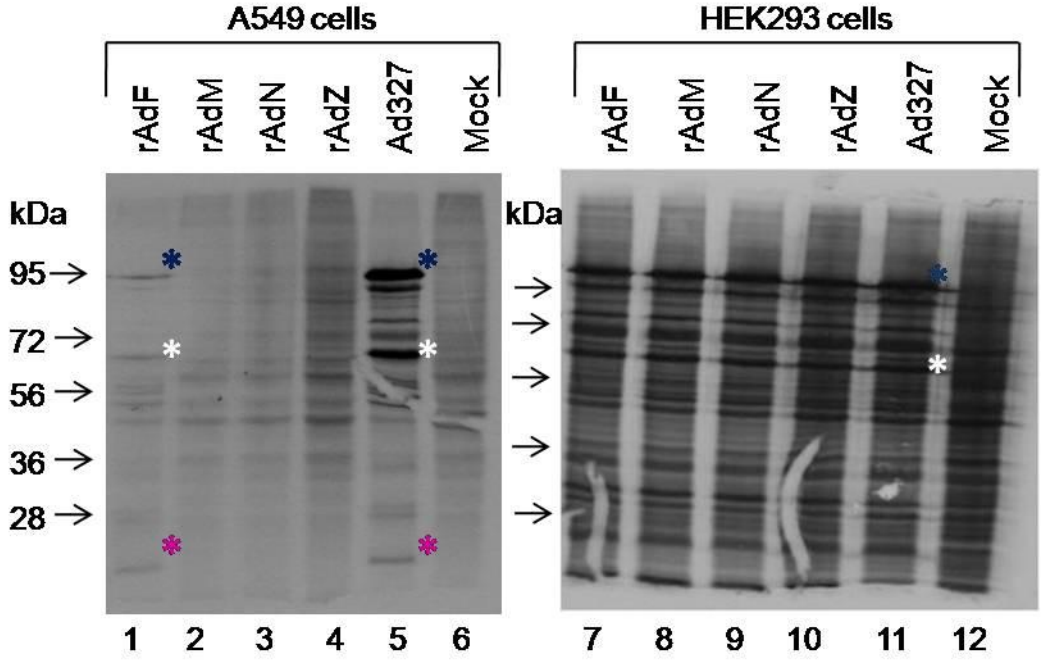

Figure 3.10. rAdF expresses late-stage infection proteins in a non-complementing cell line.
$5x10^5$ A549 or HEK293 cells were infected with rAdF, -M, -N, -Z and Ad327 at an M.O.I of 10 p.f.u./cell or mock-infected. 20h.p.i cells were pre-starved of methionine (Met) prior to incubation with [^{35}S]Met at 50µCi/ml. After one hour, samples were lysed and proteins were separated by 10% SDS-PAGE. Proteins were visualised by autoradiography. Key viral proteins include hexon (*), penton (* white) and pVI/VI (*). Both protein gels are representative of two repeat experiments.

3.4.2.2 rAdM

PVM M protein expression detection was attempted using an anti-PVM M protein mouse antibody expressed from a hybridoma (anti-PVM M polyclonal, Table 2.1.7). The antibody was used in a Western blot to try to detect M protein expression from rAdM infected and pShuttle_CMV_M transfected HEK293 cells. However, M protein expression was not detected in either the virus infected, plasmid transfected

or positive control samples with either this antibody or an alternative antibody (anti-PVM M_RLing, Table 2.17). This suggests that the epitope recognised by the antibodies is conformational, and hence undetectable by Western blotting, or that M protein was expressed at very low levels even by PVM infected cells (data not shown).

To test for transgene expression at the RNA level, full length PVM M mRNA was detected through RT-PCR using PVMMFOR and PVMMREV primers (Table 2.1.9) using total mRNA from rAdM-infected cells. The M specific primers successfully amplified full length M, generating a band of 770bp in size (Fig. 3.11 lane1). There appeared to be a similar band in the rAdM RT^- reaction (Fig. 3.11 lane 2) suggested slight contamination from genomic DNA. However, the amount of this product was very low in comparison to the result obtained following reverse transcription showing that the rAdM RT^+ product did come predominantly from mRNA.

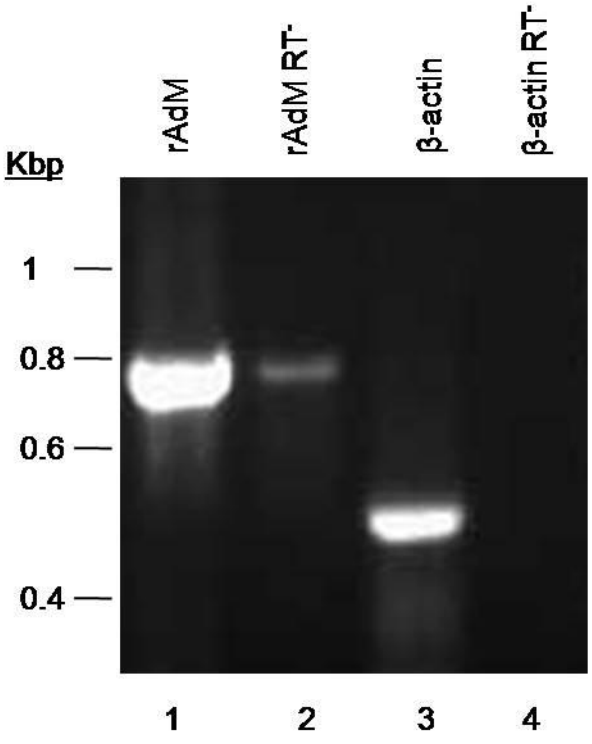

Figure 3.11. Full length M mRNA expression can be detected from rAdM infected cells.
$1.5x10^6$ HeLa cells were infected in total with rAdM at an M.O.I of 10 p.f.u./cell. Cells were lysed at 24h.p.i and total cytoplasmic mRNA was extracted, DNase treated and used directly in an RT reaction with the PVMMREV and actinR primers. RT negative reactions were included to confirm cDNA detection from mRNA rather than rAdM genome. The resultant cDNA was treated with RNase H and was used directly in a PCR reaction. Lanes 1 (RT^+) and 2 (RT^-) indicate the cDNA products from the M gene generated using PVMMFOR and PVMMREV primers. Lanes 3 (RT^+) and 4 (RT^-) indicate the cDNA products from the β-actin gene generated using the actinF and actinR primers. The results illustrated in this figure are representative of two repeat experiments.

Finally, PVM M protein expression was detected by IF on rAdM infected HeLa cells and PVM strain J3666 infected BS-C-1 cells (Fig. 3.12). M protein expression was principally located in the cytoplasm of PVM infected BS-C-1 cells (Fig. 3.12, arrows), an observation consistent with published data for RSV (Ghildyal et al., 2009), indicating progression to a late stage infection. This was the expected location for this protein. Immunostaining for the M protein in rAdM-infected HeLa cells did not produce a clear signal. M protein expression in these cells appeared to be predominantly in the nucleus, possibly because the lack of co-expression of other PVM proteins had disrupted its cytoplasmic localisation. Although the antibody did appear to stain weakly in a non-specific manner, more frequent and stronger immunostaining was present in the nuclei of rAdM-infected HeLa cells than in the control. RSV M protein localises in the nucleus during early stages of the infection cycle, supporting the evidence presented here (Ghildyal et al., 2003, Ghildyal et al., 2009). DBP immunostaining generated a large amount of cytoplasmic staining, together with the expected more intense staining present in the nuclei of most cells infected with rAdM. This diffuse nuclear staining is characteristic of cells in which Ad is not replicating. The presence of this additional cytoplasmic staining is characteristic of this polyclonal antibody (K.N Leppard, personal communication). These IF images, taken together with the absence of late protein expression from rAdM (Fig. 3.10), indicate that rAdM is unlikely to be contaminated with a replication competent revertant, while the weak positive signal for M protein, coupled with the evidence of M mRNA expression (Fig. 3.11), shows the transgene is expressed.

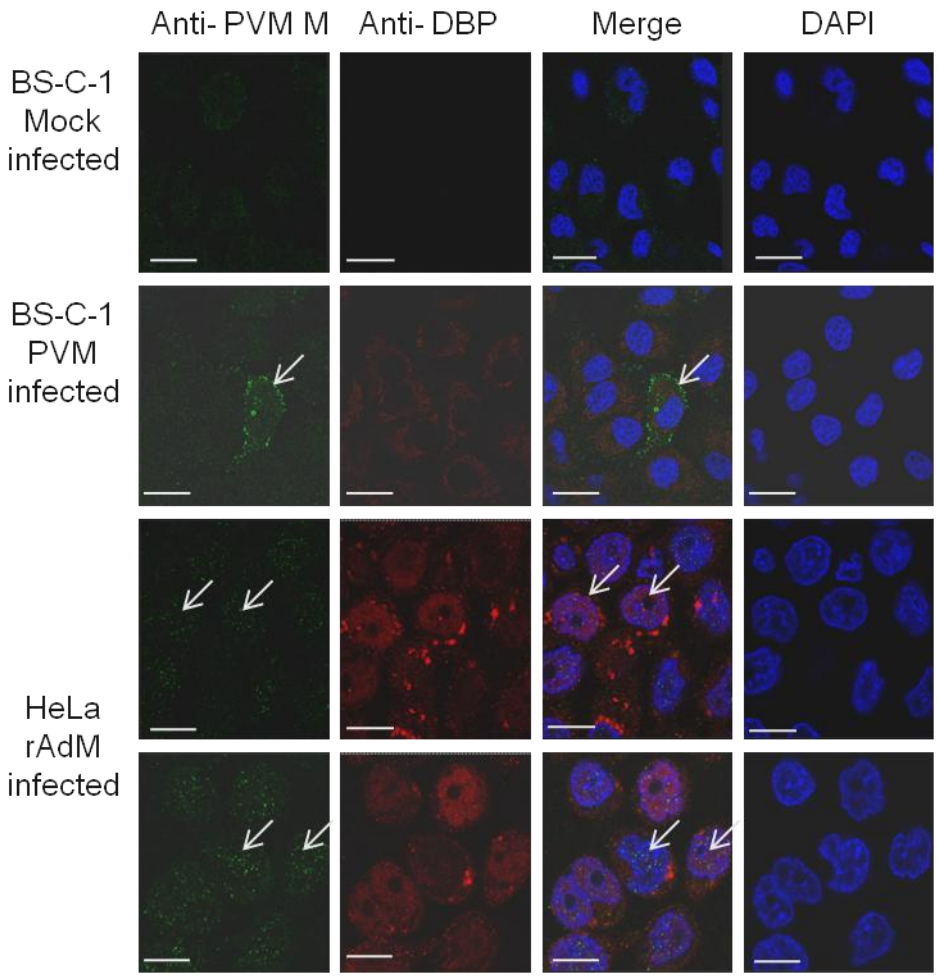

Figure 3.12. PVM M protein is expressed from rAdM.
$7.5x10^5$ HeLa cells were infected with rAdM at an M.O.I of 10 p.f.u./cell and $5x10^5$ BS-C-1 cells were mock-infected or infected with PVM J3666 at an M.O.I of 10 p.f.u./cell. Virus infected cells were fixed 24h.p.i and immunostained for PVM M protein (green) using mouse anti-PVM M_RLing, DBP (red) using rabbit polyclonal serum, whereas nuclei (blue) was visualised by DAPI staining. The merged panels show overlays of the images for each stain. Images were collected at the same scale by confocal microscopy. Scale bars: 80µm.

3.4.2.3 rAdN

PVM N mRNA expression from rAdN was detected by RT-PCR using PVMNFOR and PVMNREV primers (Table 2.1.9). These primers were able to detect full length N mRNA in rAdN infected cells (Fig. 3.13). RT negative reactions were included for both rAdN and the β-actin positive control reactions. No bands were amplified for the β-actin RT^- reaction which indicated that β-actin cDNA amplification was from mRNA rather than contaminating genomic DNA. The rAdN RT^- reaction generated

only a low intensity band, which suggested that although some contaminating genomic DNA was present, the product obtained from rAdN RT^+ reaction did come predominately from mRNA amplification (Fig. 3.13 lanes 1 and 2).

PVM N protein expression was also detected from rAdN-infected HEK293 cells by Western blot. N protein was expressed to a high level in the positive controls, P2-2 cells and PVM virus, and to a moderate level in the rAdN-infected samples (Fig. 3.14 *), at a size consistent with published literature (Barr et al., 1991). As expected no N protein expression was detected in rAdZ, mock-infected or Ad327-infected samples.

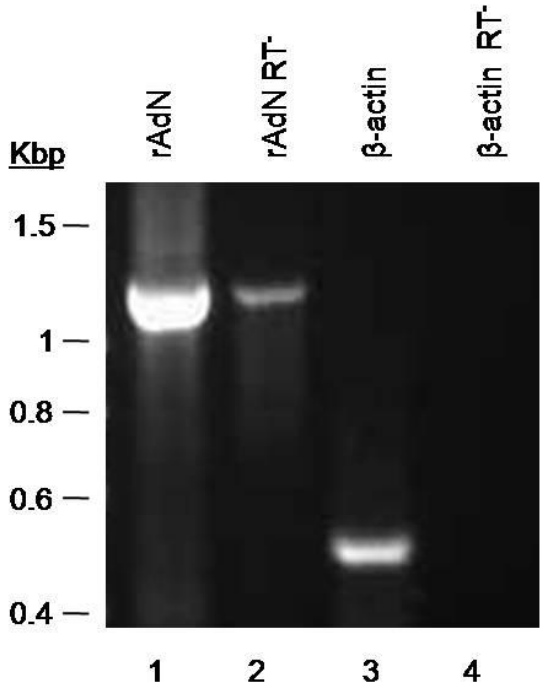

Figure 3.13. Full length PVM N mRNA expression can be detected from rAdN infected HeLa cells.
HeLa infection and RT reactions were performed as for Fig. 3.9, using rAdN and the N specific primer PVMNREV. Lanes 1 and 2 indicate the cDNA products from the N gene generated by PCR using PVMNFOR and PVMNREV primers. Lanes 3 and 4 indicate the cDNA products from the β-actin gene generated as previously described. The results are representative of two repeat experiments.

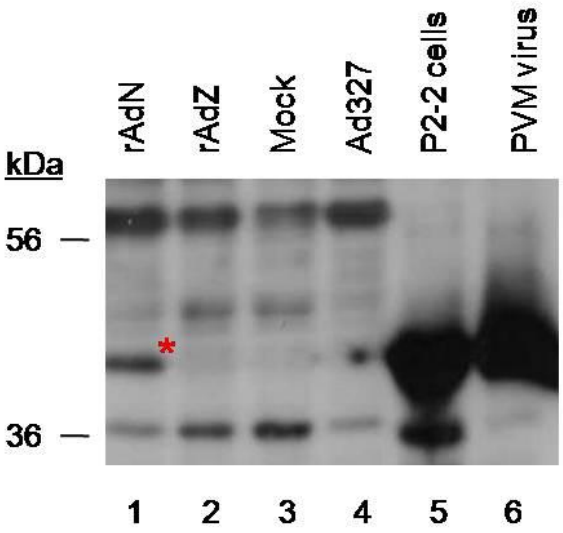

Figure 3.14. PVM N expression can be detected from rAdN infected HEK293 cells.
$1.5x10^6$ HEK293 cells were mock-infected or infected with rAdN, rAdZ or Ad327 at an M.O.I of 10 p.f.u./cell whereas P2-2 cells were seeded at $5x10^5$. Samples were lysed 24h.p.i and resuspended in sample buffer along with $1.3x10^5$ p.f.u./ml PVM J3666 virus as a PVM positive control. The lysates were separated by 10% SDS-PAGE prior to Western blotting. The membrane was probed with anti-N rabbit polyclonal (Table 2.1.7) which detected full length PVM N expression in rAdN-infected cells (*).

3.4.2.4 rAdP

During growth passages, rAdP demonstrated slower growth kinetics than the other rAd viruses. Consequently, the virus was examined at an early passage, passage four, to determine whether there was an apparent defect in the virus. Thus, a Western blot was performed to determine whether there was a defect in late Ad5 protein expression, which would result in inefficient replication and thus slower growth kinetics. The late protein expression profile of rAdP was similar to Ad327 (Fig. 3.15, lanes 1, 2 and 4). This indicated that the virus was able to progress to late stage replication in the complementing cell line, HEK293. The intensity of the penton band in particular was similar in both the rAdP and Ad327 samples, which suggested that the slower growth kinetics observed for the rAdP virus was not due to defective late gene expression and might therefore be due to an impediment in virion assembly and release.

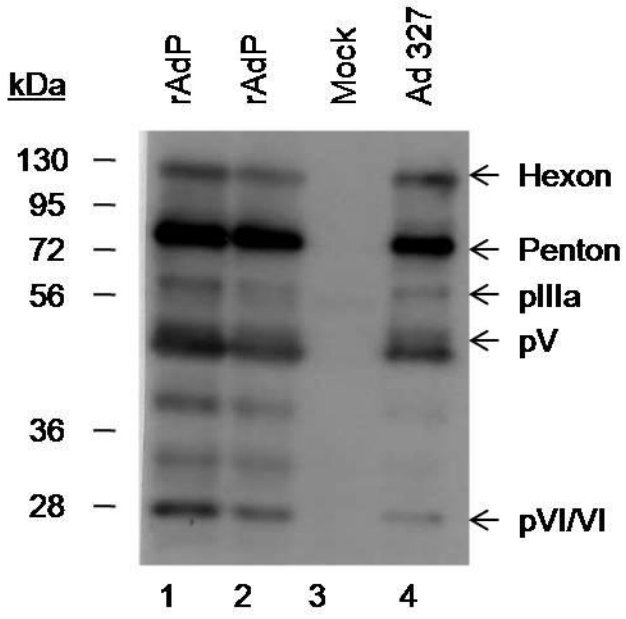

Figure 3.15. The PVM P transgene does not impede rAdP replication in a complementing cell line. $1.5x10^6$ HEK293 cells were mock-infected or infected with rAdP at an M.O.I of 10 and 1 p.f.u./cell (lanes 1 and 2 respectively) or Ad327 at an M.O.I of 10 p.f.u./cell (lane 4). Cells were lysed at 24h.p.i and lysates were separated by 10% SDS-PAGE prior to Western blotting. The membrane was probed with anti-adenoviral late protein AdJLB1 antibody (Table 2.1.7).

PVM P protein expression was investigated by Western blot analysis from rAdP infected HEK293 cells. A mouse anti-PVM P monoclonal antibody (Table 2.1.7) was used to detect P protein expression (Fig. 3.16A). Full length P protein expression was detected in the positive controls (Fig. 3.16A, lanes 1 and 2 (white *)) and was consistent with published data (Barr et al., 1994). However, no PVM P protein

expression was detected from rAdP-infected cells (Fig. 3.15 lanes 5 and 6). To investigate this further, a similar experiment was performed following transfection of pShuttle_CMV_P plasmid into HEK293 cells. Samples were harvested at 24 hour increments up to 72 hours post transfection (Fig. 3.16B, lanes 5-7). Again, P protein expression was detected in the positive control samples (Fig 3.16B lanes 1 and 2 (white *)), but no P protein expression was detected from Ad327, mock-transfected or pShuttle_CMV_P transfected cells (Fig. 3.16B, lanes 3-7). This is in contrast to equivalent experiments using rAdN (Fig. 3.14) and pShuttle_CMV_N (data not shown) in which N protein was readily detected.

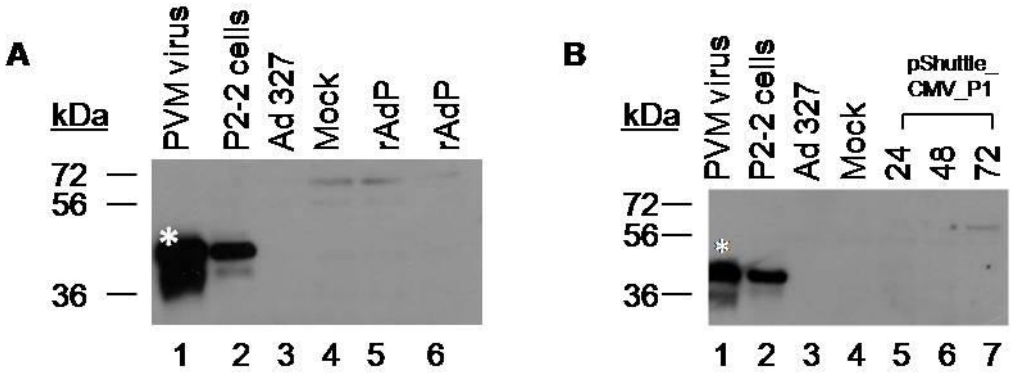

Figure 3.16. PVM P protein expression cannot be detected from rAdP-infected or pShuttle_CMV_P transfected, HEK293 cells.
(**A**) $1.5x10^6$ HEK293 cells in total were mock-infected or infected with rAdP or Ad327 at an M.O.I of 10 p.f.u./cell, lanes 3 and 6 or 1 p.f.u./cell, lane 5. P2-2 cells were seeded at $5x10^5$. Samples were lysed at 24h.p.i.
(**B**) $1.5x10^5$ HEK293 cells were mock-transfected or transfected with 1µg/well of pShuttle_CMV_P plasmid DNA whereas P2-2 cells were seeded at $5x10^5$. Samples were lysed at 24, 48 and 72 hours post transfection.
The lysates were separated by 10% SDS-PAGE along with $1.3x10^5$ p.f.u./100µl PVM J3666 virus stock which was resuspended in sample buffer. A Western blot was performed and the membrane was probed with a mouse anti-PVM P 26/11/B5 monoclonal antibody (Table 2.1.7).

To determine whether the inability to detect PVM P protein from rAdP infections using the Western blot technique was due to failure to express the protein or to produce mRNA, RT-PCR was performed to detect P mRNA expression. Thus, PVMPFOR and PVMPREV primers (Table 2.1.9) were unable to detect full length P mRNA expression from rAdP-infected HeLa cells (Fig. 3.17, lanes 1 and 2), while

the β-actin mRNA was successfully amplified as a control indicating that the RNA was of good quality. The experiment was repeated using both rAdP-infected HEK293 cells and pShuttle_CMV_P transfected cells with the PVMPFOR and PVMPREV primers and also an additional reverse internal primer, J3 (Table 2.1.9). Once again, PVM P mRNA expression was not detected with either primer pairs (data not shown). These results confirmed that the rAdP virus was non-functional and that this was likely due to an intrinsic defect in the P expression cassette since the pShuttle_CMV_P plasmid was also unable to express the PVM P mRNA or protein from the CMV promoter.

To test the possibility that some undetected error had occurred to prevent P protein expression in the construction of the shuttle plasmid used to isolate rAdP, a second construct containing the P gene was generated. pShuttle_CMV_P2 was made from an alternative parental plasmid using re-synthesised primer pairs PVMPFOR and PVMPREV, to account for possible errors within the original primer pairs. The plasmid was transfected into HEK293 cells and analysed for P protein and mRNA expression as described previously. However, PVM P expression was unable to be detected from the new construct by either assay (data not shown).

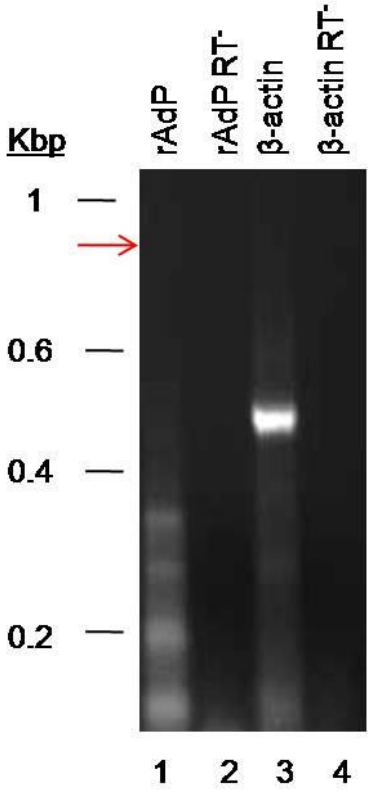

Figure 3.17. Full length P mRNA expression cannot be detected from rAdP-infected cells. $1.5x10^6$ HeLa cells were infected with rAdP at an M.O.I of 10 p.f.u./cell. Cells were lysed at 24h.p.i and total mRNA was extracted, DNase-treated, repurified and used directly in an RT reaction with the PVMPREV and actinR primers. RT-negative reactions were included to confirm cDNA detection from mRNA rather than rAdP DNA. The resultant cDNA was treated with RNase H and was used directly in PCR reactions. Lanes 1 (RT^+) and 2 (RT^-) indicate the cDNA products from the P gene generated using PVMPFOR and PVMPREV primers. Lanes 3 (RT^+) and 4 (RT^-) indicate the cDNA products from the β-actin gene generated using the actinF and actinR primers. The results illustrated in this figure are representative of two identical repeats. The red arrow indicates the expected size of the PVM P cDNA amplification product.

3.4.2.5 rAdZ

*Lac*Z transgene expression from the control virus rAdZ was confirmed by a β-galactosidase assay in HEK293 cells (Fig. 3.18). *Lac*Z specific enzymic activity increased in a dose-dependent manner in rAdZ infected samples, confirming that the virus was successfully expressing its transgene. The virus was used in *in vivo* experiments.

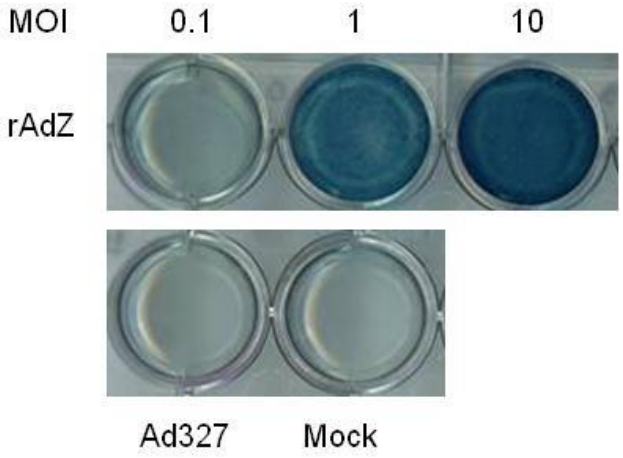

Figure 3.18. ***Lac*Z expression can be detected from rAdZ infected HEK293 cells**.
$5x10^5$ HEK293 cells were mock-infected or infected with rAdZ at an M.O.I of 0.1, 1 and 10 p.f.u./cell, and Ad327 at an M.O.I of 10 p.f.u./cell. Cells were fixed at 24 hours prior to staining for β-galactosidase activity.

3.4.3 Characterisation of recombinant virus stocks for *in vivo* studies

Each of the recombinant viruses was grown in bulk, isolated by caesium chloride gradient centrifugation and purified by dialysis (Section 2.7). Several stocks were generated for each rAd construct. Routinely, stocks were analysed by PCR to confirm that they retained the PVM gene of interest and titred by adenoviral plaque assay. All stocks were positive for their respective gene and after dialysis, were obtained at a concentration of at least $7x10^9$ p.f.u./ml (App. B. Table B.1).

As described in Section 2.7.2, the particle number for each virus preparation can be determined from the DNA content of the particle lysate. Some variation in the ratio of particle number (v.p) to p.f.u. was observed (App. B. Table B.1) due to variability in the preparation of the viral stocks. Wild-type Ad5 usually has a v.p:p.f.u. ratio of 20:1 (Schmick, 1998). All the rAd stocks had a greater v.p:p.f.u. ratio than typical wild-type Ad. This suggests that replication deficient rAds may not generate infectious particles as consistently as replication-competent Ads. In addition, there was some variability between the viral stocks for each construct, indicating

differences in their preparation. As such, only those stocks with similar v.p:p.f.u. infection ratios were used in *in vivo* studies. There are arguments in favour of standardising virus immunisation doses by either infectivity (p.f.u.) or particle (v.p). However since the PVM-specific immunogenicity of the rAd in this study depends on them infecting target cells to express their transgene, it was decided to standardise on infectious dose.

3.5 Discussion

The method used to generate recombinant Ad5 viruses has been demonstrated to be successful. rAdF, rAdM, rAdN and rAdZ viruses were grown to high titres and were stable. During the cloning procedure, no mutations were observed, presumably due to the use of the high fidelity enzyme *Pfu* turbo to generate the initial cDNA fragments. To further prevent mutations from arising in the pAdEasy genome clones, *E. coli* XL10-Gold™ was utilised to grow stocks of the plasmids which reduced the chance of unwanted recombination. Four recombinant viruses were generated using these methods, which were shown to have similar growth kinetics to wild-type Ad5 and no alterations were introduced into the transgene from the cloning procedure, preventing transgene expression from the CMV promoter. However, the same method generated a virus, rAdP, from which P mRNA and protein expression was undetectable. This same fault was also found in the pShuttle_CMV_P plasmid. To investigate the reasons for this, the CMV promoter was sequenced to determine whether a mutation had occurred that prevented P protein expression, but none were found. The constructs were re-isolated and the routine characterisation re-performed. Again, the second batch of isolates were negative for PVM P protein and mRNA expression. Thus, rAdP generation was discontinued and was not taken forward for *in vivo* studies.

Some additional insight into the failure of P protein expression from pShuttle_CMV_P has come from the work of C.Yu (personal communication), performed during her BSc Honours project in our laboratory. Two pShuttle_CMV_P constructs were generated, one containing Flag-tagged PVM P and one containing Flag-tagged PVM P with an intron sequence from SV40 inserted at the 3' end. No

Flag-tagged protein expression was detected from pShuttle_CMV_P (Flag), discounting the possibility that the P epitope/antibody combination was ineffective for detection of P protein expressed from rAdP. However, expression was detected from pShuttle_CMV_P (Flag/intron) (App. C. Fig. C.1). This result suggests that the P gene was not expressed from the CMV promoter (Section 3.6.2.4) because of aberrant RNA processing or export from the nucleus. Ad5 viruses are transcribed in the nucleus of cells which is not the natural expression environment for the PVM genes. Therefore, cryptic splice site may have prevented full length P mRNA or protein expression from the CMV promoter and therefore, P was not detected. The presence of an authentic pair of splice donor and acceptor sites outside the P open reading frame may have suppressed this cryptic splicing. Alternatively P mRNA expressed from the original, non-splicing, constructs may be very inefficiently polyadenylated and exported from the nucleus. Transient expression of some heterologous transgenes has been shown previously to be strongly stimulated by the inclusion of splicing cassettes (Huang & Gorman, 1990). In conclusion, the AdEasy™ Adenoviral Vector System has been used to generate rAd viruses containing and expressing specific genes from PVM. These viruses will be investigated *in vivo* as vaccine candidates for PVM in the following chapters.

Chapter 4

Development of an rAd PVM vaccination protocol and evaluation of rAd PVM construct efficacy in the PVM infection model

4.1 Introduction

PVM infection of mice has been proposed as an appropriate *in vivo* model in which to evaluate 'proof of principle' vaccine candidates for pneumoviruses. In this chapter, the efficacy of the rAd constructs over short and long term experiments is described.

4.1.1 The PVM infection model

As described in Section 1.4, PVM and human RSV are members of the virus family *Paramyxoviridae* and cause a similar pathogenesis within their natural hosts (Easton et al., 2004). PVM infection of mice is recognised as an appropriate model in which to study the pathogenesis of RSV and PVM (Cook et al., 1998, Domachowske et al., 2001), and therefore, the model can be employed to evaluate PVM vaccine efficacy. Horsfall and Hahn originally isolated PVM following the observation that serial passage of mouse lung material isolated from apparently healthy mice resulted in a fatal, transmissible pneumonia (Horsfall, 1940). This study documented the signs associated with a lethal PVM infection and determined that an infection was only possible through the intranasal route. For this reason, PVM inoculations in this study were delivered via this route.

The parameters of the clinical score for PVM infection were previously defined by A. J. Easton and colleagues (Easton, AJ personal communication, (Cook et al., 1998)). These parameters were redefined for the purposes of this study as illustrated in Fig. 4.1, because difficulties were encountered in discerning the difference between a clinical score of two or three, using the original scale. In addition, a direct relationship between increased clinical score and weight loss had previously been observed (Cook et al., 1998). Thus, bodyweight was monitored as well as clinical score throughout the PVM challenge period in all experiments. In addition to the characterisation of PVM infection, Cook and co-workers determined that the optimum volume for intranasal inoculation of PVM was 50µl (Cook et al., 1998), therefore, this inoculation volume was used throughout this study for both rAd and PVM viruses.

Clinical Score	Clinical symptoms
1	Healthy
2	Ruffled fur
3	Ruffled fur, lethargy, hunched posture, deeper breathing
4	As for 3, Inactive, emaciated, abnormal gait, huddle together, may show cyanosis of tail and ears
5	Death

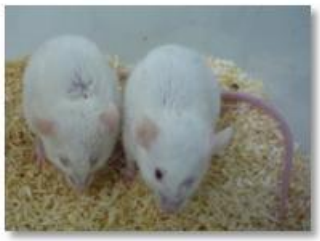

Figure 4.1. Clinical score parameters for mice infected with PVM strain J3666.
Animals inoculated with a lethal dose of PVM strain J3666 progress through five clinical stages of disease. Healthy animals score one on the scale, whereas a score of two denotes consistently ruffled fur, particularly in the neck region of the animal, as indicated in the area circled. A score of three indicates the animals have ruffled fur across their entire body, a characteristic hunched posture (as shown in the associated image), deeper breathing, and lethargy. Progression to a score of four denotes a severe infection. These animals retain the characteristics associated with a score of three, but appear emaciated and develop a 'pinched waist'. The animals are more likely to be inactive and 'huddle' together, with an abnormal 'waddling' gait observed when movement does occur. Animals that progress to a fatal infection may show cyanosis of the tail and ears and tremors before death occurs. If animals showed signs of cyanosis, and/or severe tremors in association with a loss of more than 25% of their initial bodyweight, they were culled for humane reasons, in accordance with the Home Office project licence under which the experiments were conducted. During some experiments, animals were culled prior to reaching a score of five, for humane reasons, particularly if there was no advantage for continuing the experiment.

4.1.2 Establishment of an immunisation strategy to evaluate rAd PVM vaccine efficacy

Several previous studies have utilised Ad5 as a vaccine vector for treatment to prevent RSV (Section 1.7.6) among other viruses. Several of these studies have employed a homologous prime-boost immunisation strategy. This indicates that animals have been immunised with the same (homologous) vaccine throughout the experiment rather than using the same immunogen but in a different format e.g. protein subunit or DNA, which is termed heterologous. The initial immunisation is referred to as the prime or priming dose whereas subsequent immunisations are known as a boost or booster dose. These studies demonstrated that animals immunised using this strategy generated a detectable immune response towards the constructs transgene product which, where tested, correlated with protection. The immunisation regime also allows the animals time to generate an immune response against the transgene product and, following a booster inoculation, to generate a greater magnitude of response towards it prior to challenge (Ellis et al., 2007). The rAd PVM constructs developed in this study were used in a similar way to immunise mice as detailed in Fig. 4.2, using the *in vivo* PVM infection model. This allowed the rAd constructs to be evaluated for efficacy directly against PVM in its natural host.

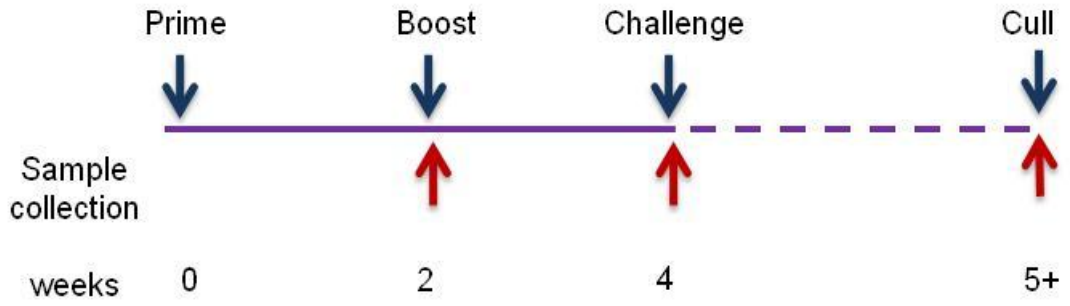

Figure 4.2. The standard immunisation regime for the rAd PVM constructs.
For the priming immunisation dose, mice were immunised with either a rAd PVM vaccine diluted to an appropriate concentration in a 50µl inoculum with PBS or 50µl of PBS as a control. After two weeks, the animals were boosted with an identical dose of the immunogen they had previously received. At four weeks, all mice were challenged with a lethal dose of PVM strain J3666, in a 50µl volume. Serum samples were collected from each animal at the two and four-week time points and upon termination of the experiment (red arrows), for further analysis.

Since PVM naturally infects the mucosal surfaces of the lung, it was likely that stimulation of an immune response via the mucosa would correlate with protection from the virus, as has been recorded for other respiratory viruses such as RSV, influenza and parainfluenza virus type 3 (Kohlmeier & Woodland, 2009, van Ginkel FW, 2000). Thus, the rAd PVM constructs were predominantly used to immunise animals via the intranasal route in order to stimulate this form of immunity.

4.2 Optimisation of PVM challenge model

To optimise the PVM challenge model the PVM J3666 virus stock (PVM), was titrated in BALB/c mice to determine its pathogenicity. Groups of mice were inoculated via the intranasal route with different quantities of PVM in a 50µl inoculum and the animals were observed for a period of three weeks, with the clinical score for each animal (Fig. 4.3A) and the total weight of each group (Fig. 4.3B) being monitored throughout.

For all inoculated groups, transient weight loss was observed on day one, which was attributed to the stress of the inoculation procedure affecting natural feeding patterns. Control animals were treated with PBS alone and were not challenged with PVM. These animals did not present with any clinical signs associated with PVM infection. For these animals, weight loss of up to 5% of initial bodyweight was observed on day's four to six; from day 7, onwards the animals maintained a stable bodyweight with no further weight loss.

Inoculation of 20 p.f.u. of PVM resulted in a mild illness. The mean clinical score increased from 1 to a peak of 1.8 on days 9 to 14 before returning to the baseline level. Weight loss coincided with observable clinical signs from days 9 to 16, reaching a maximum of 13% of bodyweight loss on day 12. Weight gain was observed from day 13 onwards, indicating recovery from PVM illness.

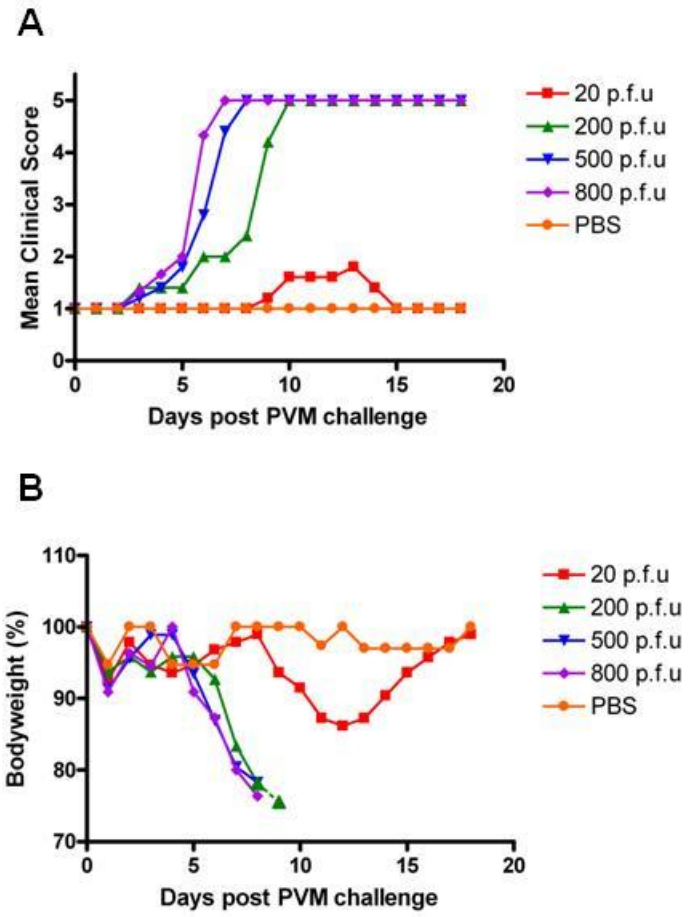

Figure 4.3. PVM strain J3666 titration in BALB/c mice.
Groups of five female BALB/c mice at five weeks of age were inoculated via the intranasal route with 20, 200, 500 or 800 p.f.u. of PVM strain J3666 in 50µl, as indicated. A further two mice were inoculated with 50µl of PBS via the same route. Two parameters were measured for the duration of the experiment, the clinical score (A) and the total bodyweight (B) of each inoculation group. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs.

Inoculation of 200, 500 or 800 p.f.u. of PVM resulted in a lethal infection. All groups presented with clinical signs from day 3 onwards, with the groups that received the 500 and 800 p.f.u. doses showing a more rapid disease progression than the 200 p.f.u. group. In the groups receiving 500 and 800 p.f.u., fatalities occurred on day 8, which coincided with the greatest weight loss of 21% for the 500 p.f.u. group, and 23% for the 800 p.f.u. group. In these groups, the onset of weight loss was observed one day

earlier, on day five than for the 200 p.f.u. group. One fatality occurred on day eight within the 200 p.f.u. group with the remaining 80% succumbing on day 10, which corresponded with the greatest weight loss of 24%. Overall, similar disease profiles were observed for the 200, 500 and 800 p.f.u. inoculum groups with more rapid progression of disease seen with increased doses. Based on this data, 250 p.f.u. of PVM strain J3666 in 50µl volume was chosen as the standard challenge dose to ensure full lethality in control groups without accelerated disease progression.

4.3 rAd PVM constructs can elicit protection against lethal PVM infection in the BALB/c mouse strain

BALB/c mice between 5 and 7 weeks of age were immunised via the intranasal route using a prime-boost regime (Fig. 4.2). For each rAd construct investigated, the animals were separated into three groups and immunised as detailed in Table 4.1. Animals treated with PBS served as a control group to monitor PVM pathogenesis.

Group	Prime dose (p.f.u./50µl)	Boost dose (p.f.u./50µl)	Animals/group
A	10^6	10^6	6
B	10^7	10^7	6
C	PBS	PBS	2

Table 4.1. The standard vaccine dosage for rAd vaccination.

Throughout the immunisation regime and prior to PVM challenge, no weight loss or clinical signs were observed for any animals (data not shown). Therefore, immunisation of BALB/c mice with the rAd based constructs by this route does not result in an observable pathogenesis.

4.3.1 rAdF-immunised mice are protected against lethal PVM infection

rAdF was investigated to determine whether this construct could protect immunised animals against lethal PVM infection. rAdF contains the F gene of PVM, which expresses the fusion (F) protein; an external membrane protein involved in viral entry.

Following standard challenge with PVM, all the animals immunised with either the 10^6 p.f.u. or 10^7 p.f.u. doses of rAdF (groups A and B respectively) survived the PVM challenge. When compared to the control group C, animals in group A were protected against the lethal effects of PVM infection, but did present with moderate clinical signs (Fig. 4.4A), and significant weight loss (Fig. 4.4B). The appearance of disease was delayed by two days when compared with control group C (on day 8), and reached a lower peak mean clinical score of 3.3 on day 9. Weight loss was observed in animals in group A from day 7 onwards and reached a maximum of 24% on day 11. From day 10 onwards, the clinical score decreased steadily until the baseline level was reached. However, unlike control group C, weight gain was observed in group A from day 13 onwards. The animals did not return to their pre-challenge weight, but this has been previously observed with animals recovering from PVM infection (A.J Easton personal communication).

Group B animals, which received the higher vaccine dose of 10^7 p.f.u., were fully protected against the PVM challenge, and presented with only mild transient clinical signs and minimal weight loss. The mean clinical score increased to a peak score of two on day 8 (Fig. 4.4A), at which time weight loss was initially observed (Fig. 4.4B). The clinical score returned to the baseline level by day 10, by which time the greatest weight loss, still only 3%, was observed. The animals began to gain weight thereafter and achieved 108% of their pre-challenge weight by the conclusion of the experiment. In contrast, the control group which had been treated with PBS (group C), presented with clinical signs and weight loss on day 6, with the former increasing in severity for three days (Fig. 4.4.A). All the animals died or were culled due to the PVM illness on day 9, which coincided with the greatest weight loss of 23% of initial average bodyweight (Fig. 4.4B). Thus, when compared to control group C, rAdF-immunised groups A and B were both protected against PVM infection.

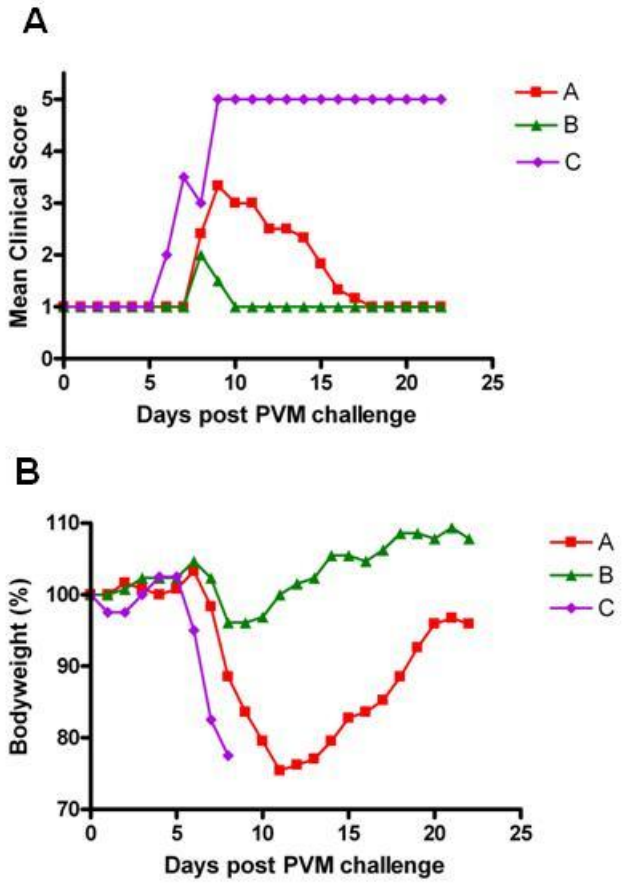

Figure 4.4. rAdF can elicit protection against lethal PVM challenge.
Female BALB/c mice were individually immunised with a prime dose of rAdF on day 1 and a boost dose on day 14 as detailed in Table 4.1. Mice in groups A and B received 10^6 p.f.u. and 10^7 p.f.u. of rAdF, respectively, whereas group C were immunised with PBS. Animals were challenged on day 31 with a lethal dose of PVM. Mean clinical score (A) and percentage bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). The data are representative of two repeat experiments.

4.3.2 rAdM-immunised animals are protected against lethal PVM infection

In light of the positive results achieved for the rAdF construct, the immunogenicity of rAdM was then investigated. The rAdM construct contains the M gene of PVM, which expresses the matrix (M) protein of PVM; an internal protein involved in maintaining the integrity of the virus and virus assembly.

This experiment, Fig. 4.5, was performed in parallel with that described in Section 4.3.1; using the same control PBS treated (group C) animals. Male mice were used in group A (immunised with 10^6 p.f.u. of rAdM) and had a minimum clinical score of two throughout the PVM challenge period. This was not observed for rAdF-immunised animals, which were all female. This difference has been attributed to the gender of the animals, as male mice are more aggressive than females and frequently fight with cage mates. In addition, male mice are frequently less meticulous in their grooming when compared to female mice. This affects the apparent clinical score, as fur ruffling is scored as a clinical sign of infection. Nevertheless, an effect of PVM infection was evident.

Group A presented with an increase in clinical score reaching a peak score of 4 by day 9 (Fig. 4.5A), which included three fatalities. Weight loss was observed from day 6 onwards and occurred at a rate similar to that of the control group C, reaching 28% weight loss on day 9 (Fig. 4.5B). Further fatalities were observed on day 10; one survivor maintained a high clinical score and did not regain weight. Therefore, this group was not significantly protected from PVM infection.

In contrast to group A animals, mice in group B (inoculated with 10^7 p.f.u. of rAdM) were protected against PVM infection though they did present with moderate clinical signs (Fig 4.5A) and substantial weight loss (Fig. 4.5B). This group demonstrated a delay in the onset of clinical signs when compared to the control group C. The mean clinical score reached a peak of 3 by day 9 (Fig. 4.5A), which coincided with a maximum weight loss of 19% (Fig. 4.5B) and one fatality. From day 10 onwards, the group recovered from the infection, appearing healthy from day 13 onwards. Thus, group B (immunised with 10^7 p.f.u. of rAdM) were protected from PVM infection when compared to test group A and control group C animals.

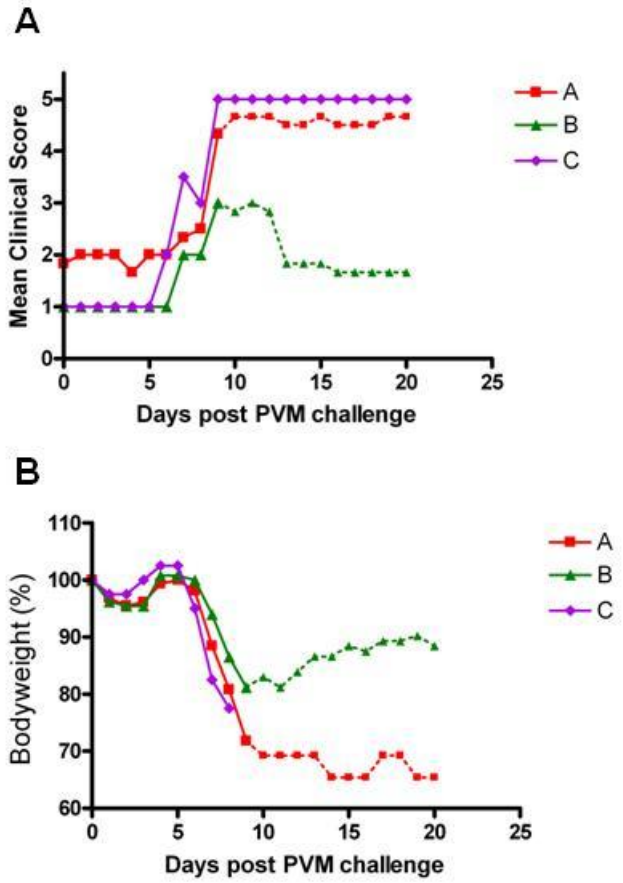

Figure 4.5. rAdM can elicit protection against lethal PVM challenge.
BALB/c mice were individually vaccinated and challenged as described in Fig. 4.4 and Table 4.1 with rAdM. Mice in groups A and B were immunised with 10^6 p.f.u. and 10^7 p.f.u. of rAdM, respectively, whereas group C received PBS. Mean clinical score (A) and percentage bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs. Group A and B consisted of male and female mice respectively.

4.3.3 rAdN-immunised mice are protected against lethal PVM infection

The third construct, rAdN, contains the N gene of PVM, which expresses the nucleotide (N) protein, which is involved in maintaining virus genome integrity and promotes replication. As before, this experiment was conducted concurrently with

those described in Sections 4.3.1 and 4.3.2 and the control animals were group C as previously described.

Mice inoculated with 10^6 p.f.u. of rAdN (group A) did not survive the PVM challenge dose. Clinical signs were observed from day 5 post PVM challenge and increased daily, reaching the maximum possible score on day 11 (Fig. 4.6A). Weight loss was observed from day 6 onwards (Fig. 4.6B), reaching a maximum of 29% by day 9. Therefore, this group was not protected against the PVM challenge dose. In contrast to group A, mice inoculated with the higher dose of rAdN (10^7 p.f.u. group B) were protected against PVM challenge, with all animals surviving. However, moderate clinical signs were observed; these commenced one day after groups A and C and reached a lower peak score of 3 on day 9 (Fig. 4.6A). This coincided with bodyweight loss, which began on day 7 and reached a maximum of 16% on day 10. From day 11 onwards, the group recovered from the PVM infection as their clinical score returned to baseline levels and weight gain was evident. As for mice immunised with the same dose of rAdM, group B rAdN-immunised animals did not regain bodyweight to pre-challenge levels.

4.3.4 Control rAdZ-immunised mice are not protected against lethal PVM infection

The final recombinant virus investigated was the control vaccine, rAdZ, which contains the *Lac*Z gene of *E. coli*, which expresses the β-galactosidase protein. As described above, mice that received 10^7 p.f.u. of rAdF, rAdM, or rAdN were protected against a lethal PVM infection with or without transient clinical signs of disease. To confirm that these positive effects were due to generation of immunity against the PVM proteins, rather than to some effect of the rAd vector, animals were immunised with 10^6 or 10^7 p.f.u. of rAdZ which did not contain any PVM gene sequences but instead expressed an irrelevant protein, β-galactosidase, from the *E. coli Lac*Z gene.

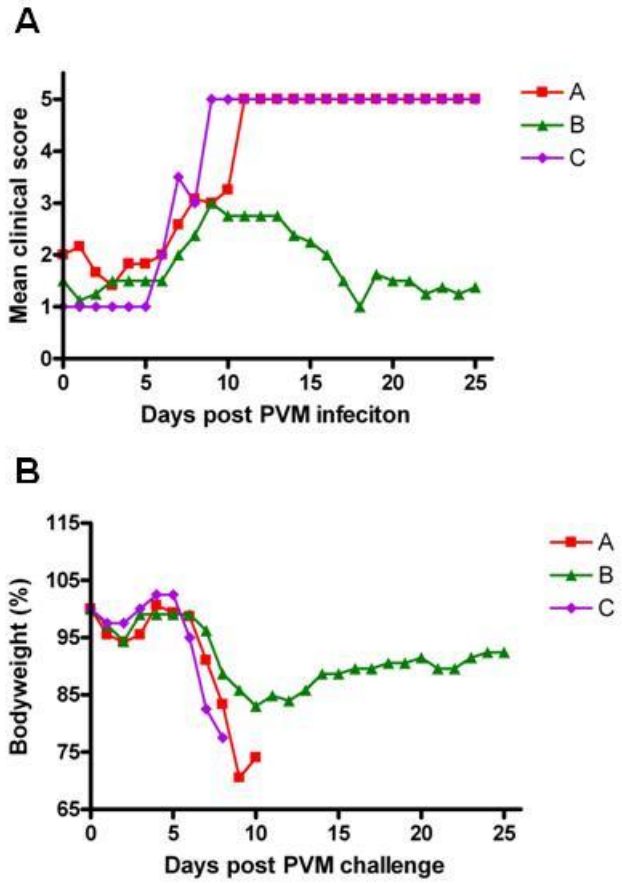

Figure 4.6. rAdN can elicit protection against lethal PVM challenge.
Male BALB/c mice were individually vaccinated and challenged as described in Fig. 4.4 and Table 4.1 with rAdN. Mice in groups A and B were immunised with 10^6 p.f.u. and 10^7 p.f.u. of rAdN, respectively, whereas group C received PBS. Mean clinical score (A) and percentage bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). The data are representative of two repeat experiments. In the experiment shown, group B comprised of only three animals due to anaesthesia complications.

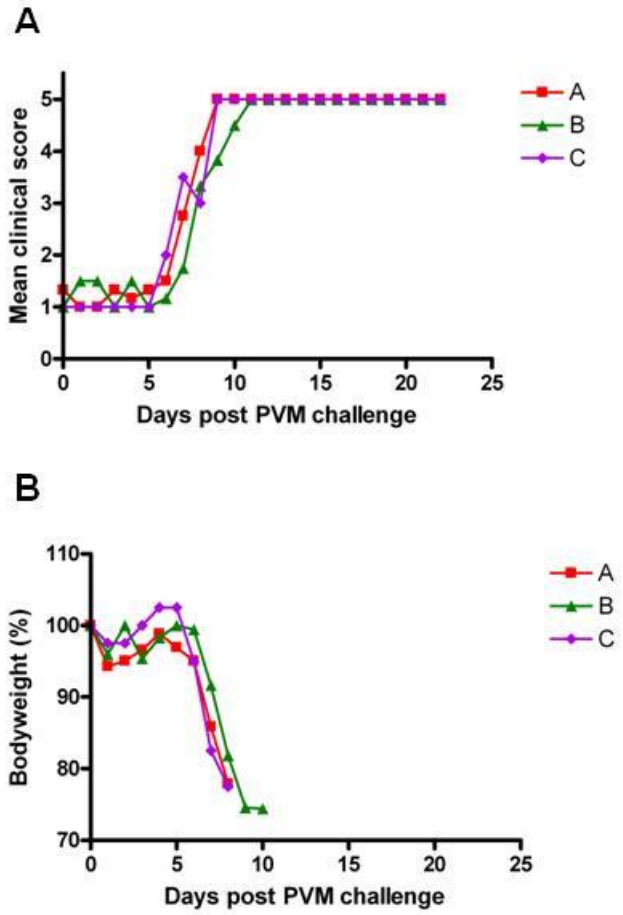

Figure 4.7. *Lac*Z does not protect mice from lethal PVM challenge.
BALB/c mice were individually vaccinated and challenged as described in Fig. 4.4 and Table 4.1 with rAdZ. Mice in groups A and B were immunised with 10^6 p.f.u. and 10^7 p.f.u. of rAdZ, respectively, whereas group C received PBS. Mean clinical score (A) and percentage bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Group A and B consisted of male and female mice respectively.

As seen in Sections 4.3.1-.3, the control animals in group C were as previously described. Mice inoculated with 10^6 p.f.u. or 10^7 p.f.u. of rAdZ (groups A and B respectively) were not protected against lethal PVM infection. Animals in group A developed observable clinical signs from day 6 onwards reaching the maximum possible score with the death of all mice on day 9 (Fig. 4.7A), similar to group C. Animals in group B presented with clinical signs one day later, on day 7, and reached the maximum score on day 11, two days after control group C. On day 9, 50%

mortality was observed for group B, with the remainder from that group dying on day 11. Animals in both groups A and B exhibited weight loss from day 5 post challenge and weight declined at the same rate witnessed for the control group C, reaching a maximum bodyweight loss of 25% for group A on day 9 and 27% for group B on day 11 (Fig. 4.7B).

4.3.5 Discussion

Each of the three PVM rAd constructs protected animals from subsequent PVM challenge when used at the 10^7 p.f.u. dose in the standard prime-boost immunisation regime. Only rAdF was also able to protect animals from lethal PVM challenge at the lower vaccine dose of 10^6 p.f.u. However, this vaccine stock was contaminated with a low level of a replication competent Ad5 virus, as described in Section 3.4.2.1. This may have affected the antigenicity of the vaccine preparation because the contaminant might support the replication of rAdF and if the recombinant virus were able to replicate then the animals would have received a greater dose of antigen. 16.6% of animals immunised with 10^6 p.f.u. of rAdM were protected in one experiment, whereas this dose failed to protect in a repeat experiment. Therefore, this was not judged to be significant protection against a lethal PVM infection. The protective effect of all three PVM rAd constructs at the 10^7 p.f.u. dose was specific to the rAd PVM constructs, as the rAdZ construct, expressing an irrelevant protein, did not protect mice from a lethal PVM challenge at this dose. Thus, the 10^7 p.f.u. dose was used as the standard immunisation dose in further experiments.

4.4 rAds can elicit protection against lethal PVM infection in different mouse strains

The BALB/c mouse strain is an inbred strain commonly used in *in vivo* experiments. The rAd PVM constructs were successfully able to elicit a protective response against lethal PVM infection in this mouse strain. This protection may be generated by either a humoral or a cellular immune response, as described in Section 1.6. The ability of the adaptive immune response to recognise and, thus respond, to antigen is

determined by the MHC genes. The gene region is highly polymorphic and has a large number of associated alleles. As mentioned in Section 1.6, the MHC complex directly influences the humoral and cellular arms of the immune response through the presentation of peptides and other antigens on the cell surface. The natural variation associated with this complex determines the ability of an MHC receptor to bind to antigen and thus to display the antigen for immune surveillance. As such, the expression of particular alleles may confer an advantage or disadvantage on an individual's ability to recognise specific antigens.

It is well known that the MHC haplotype of an individual affects their T-cell response towards an antigen. This occurs because genetic differences alter the range of antigens that can be recognised, and thus displayed, by the MHC complex. Therefore, this determines what antigens can be recognised and responded to by T-cells. Less well understood is the direct effect of MHC haplotype on the B-cell response towards an antigen. The MHC class II haplotype will determine whether an antigen is recognised by $CD4^+$ T-cells, which are involved in B-cell activation. Additionally, it is accepted that complement proteins (a group of proteins associated with innate immunity) are encoded within the MHC gene region. Thus, variation in this region may affect complement proteins, which can form cross-links with B-cell co-receptors and through this influence B-cell differentiation and antibody isotype switching (Carroll, 2008). Further evidence suggests that some innate immunity associated receptors such as TLRs, are linked to MHC haplotype (Rodo et al., 2006). Thus, co-receptor regulation has a direct effect on B-cell activation and proliferation towards a particular antigen.

In terms of vaccine development, it is the effect of MHC class I haplotype on T-cell activation which is the most important, particularly against viral pathogens. MHC class I complexes present antigen for recognition by CTLs, which recognise and destroy infected cells. Therefore, to be of value in an out-bred population, a vaccine such as the rAd PVM vaccines must generate a protective response against the transgene regardless of the MHC polymorphism of an individual. This is an important consideration during vaccine evaluation in mice as many laboratory mouse strains have been in-bred and therefore they will express an identical restricted set of MHC alleles. The BALB/c mouse strain, which has been primarily used in this study,

has an MHC haplotype of $H2^d$ (Section 2, Table 2.1.6). Therefore, it could be possible that the protection from rAd PVM constructs observed in this mouse strain could be linked to its MHC haplotype.

To investigate the ability of the rAd PVM recombinant viruses to elicit a protective response in mice with different MHC haplotypes, the C57BL/6 ($H2^b$) and C3H/He-mg ($H2^k$) (Section 2, Table 2.1.6) mouse strains were immunised with the rAd PVM vaccines to determine whether protection against PVM infection could be generated. The C57BL/6 and C3H/He-mg mouse strains are known to be susceptible to PVM infection and disease pathogenesis in these strains is similar to that observed in the BALB/c mouse strain (A. J. Easton, personal communication (Anh et al., 2006), though C57BL/6 mice have been reported to be slightly less susceptible to PVM infection than BALC/c mice (Anh et al., 2006).

Groups of animals between 5 and 7 weeks of age were immunised via the intranasal route as described in Table 4.2, using the standard prime-boost regime (Fig 4.2.). Animals treated with PBS served as a control group to monitor PVM pathogenesis during the challenge phase of the experiment. As previously observed in the BALB/c mouse strain, no weight loss or clinical signs were observed for any group during the immunisation period. This suggests that intranasal immunisation of mice with rAd based vaccines does not result in an observable pathogenesis, irrespective of strain background.

Group	Vaccine construct	Prime dose (p.f.u./50µl)	Boost dose (p.f.u./50µl)	Animals/group
A	rAdF	10^7	10^7	5
B	rAdM	10^7	10^7	5
C	rAdN	10^7	10^7	5
D	rAdZ	10^7	10^7	5
E	N/A	PBS	PBS	4

Table 4.2. The rAd vaccine dosage for protection studies against PVM in different mouse strains.

4.4.1 C3H/He-mg strain immunisation with rAd constructs

C3H/He-mg mice were immunised with rAd PVM recombinant viruses as described in Table 4.2. In contrast to the control group E, animals treated with rAdF or rAdN (groups A and C respectively), were both protected against lethal PVM infection. Animals in group A presented with a mild clinical score elevation, which was observable from day 7 onwards, reaching a peak score of 2 on day 9 (Fig. 4.8A), and declined to baseline levels from day 13 onwards. Weight loss was observed from day 3 onwards, reaching a maximum of 15% on day 9 (Fig. 4.8B). Mice in group A did not regain lost weight during the recovery period but maintained a bodyweight at 85% of their pre-challenge level. Animals in group C presented with slight clinical signs from day 7 onwards, which fluctuated slightly above the baseline level (Fig. 4.8A). Although the initial rate of weight loss, from day 6 was similar to control group E, mice in group C reached a maximum of only 9% bodyweight loss on day 7 and then weight gain resumed (Fig. 4.8B).

In contrast to animals treated with rAdF or rAdN (groups A and C), those treated with rAdM (group B) were only partially protected from PVM infection with some fatalities being observed. Clinical signs were delayed by two days when compared to the control group E, but were apparent from day 7 onwards reaching a peak score of 3 on day 9 (Fig. 4.8A), which coincided with the death of two animals. One animal did not present with clinical signs of PVM infection prior to death whereas the other presented with ruffled fur and wasting, characteristic of PVM infection. The remaining animals maintained a moderate clinical score of 3 for the remainder of the experimental period. Weight loss was observed for this group from day 6 onwards, reaching a maximum of 13% on day 9 (Fig. 4.8B). Unlike group A, surviving group B (rAdM-immunised) animals began to gain weight and therefore showed signs of recovery from day 10 onwards, returning to their pre-challenge bodyweight by day 13. Group D animals, which had received the control rAdZ construct, presented with signs from day 6 onwards, which then increased at a similar rate as for the PBS treated group E animals (Fig. 4.8A).

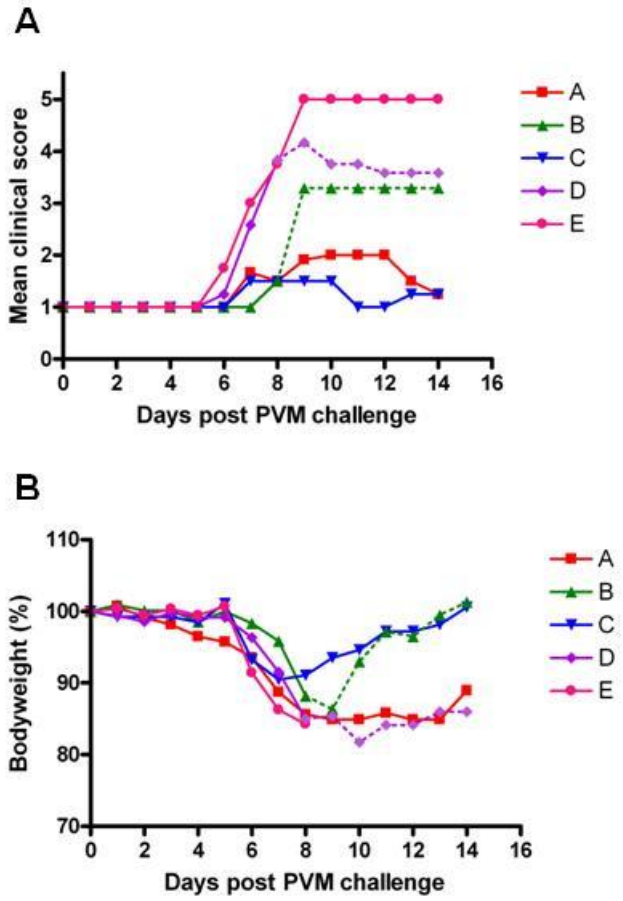

Figure 4.8. rAd constructs protect C3H/He-mg mice from lethal PVM challenge. C3H/He-mg mice from the one batch of animals were individually vaccinated as described in Table 4.2 using the standard immunisation protocol (Fig. 4.2), prior to challenge on day 28 with a lethal dose of PVM strain J3666. Animals were immunised with 10^7 p.f.u. of rAdF (group A), rAdM (group B), rAdN (group C), rAdZ (group D) or treated with PBS (group E). Mean clinical score (A) and percentage bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs. Group A-D consisted of three female and two male mice whereas group E consisted of two female and two male mice.

Similar to animals in control group E (treated with PBS), the peak clinical score for group D animals was reached on day 9; although it was lower than group E. This coincided with two deaths, with a further animal dying on day 10. The remaining mice maintained moderate clinical signs throughout the experimental period. Weight

loss was observed from day 6 onwards that was again equivalent to the control group E, reaching a maximum loss of 16% on day 10 (Fig. 4.8B). The surviving animals did not appear to regain weight and therefore did not fully recover from PVM infection. The pathogenicity of the challenge dose was confirmed by the weight-loss and clinical score of group E animals (Fig 4.8A, B).

4.4.2 C57BL/6 strain immunisation with rAd recombinant viruses

C57BL/6 mice were vaccinated with the rAd constructs as described in Table 4.2 and challenged with PVM as before. However, unlike the BALB/c and C3H/He-mg mouse strains, C57BL/6 animals in PBS-treated control group E, did not all succumb to PVM infection. Observable PVM-related signs were present in these animals from day 8 onwards and increased to a peak score of 4 by day 11 (Fig. 4.9A). This coincided with two fatalities while the remaining animals maintained a moderate clinical score of 3.5 for the remainder of the experiment. Weight loss in this group was evident from day 7 onwards, reaching a maximum of 25% on day 11 (Fig. 4.9B). This degree of weight loss was associated with high clinical scores and lethality or sacrifice for humane reasons in other mouse strains. The surviving animals did not fully recover from PVM infection as demonstrated by the absence of weight gain by the group for the remainder of the experiment. Thus, it appears that C57BL/6 mice are more tolerant of the pathogenic effects of PVM then are BALB/c or C3H/He-mg mice.

Animals in groups A, B, and C (inoculated with rAdF, rAdM, and rAdN, respectively) were fully protected against lethal PVM infection. No increase in clinical score was observed for these groups throughout the experiment (Fig. 4.9A) and all animals survived the PVM challenge. Only slight weight loss was observed in groups A and B, reaching a maximum loss of 7% on day 10 and 3% on day 9 respectively, while group C were completely unaffected (Fig. 4.9B). Mice in all three groups gained weight above their initial bodyweight and remained healthy for the duration of the experiment. This outcome is in clear contrast to the affect of the challenge dose on control group E animals. Surprisingly, group D animals (inoculated with rAdZ), were also protected against lethal PVM infection, however mild clinical signs and moderate weight loss was associated with PVM challenge for

this group. Clinical signs were observed from day 8, similar to that of control group E, but reached a lower average score of only 1.6 on day 9 (Fig. 4.9A) and quickly recovered to baseline levels. Weight loss was more severe, beginning on day 7 as for control group E, and reaching a maximum of 18.5% on day 9 (Fig. 4.9B). However, in contrast to group E, the group began to regain weight rapidly from day 12 onwards, but did not reach their pre-challenge bodyweight.

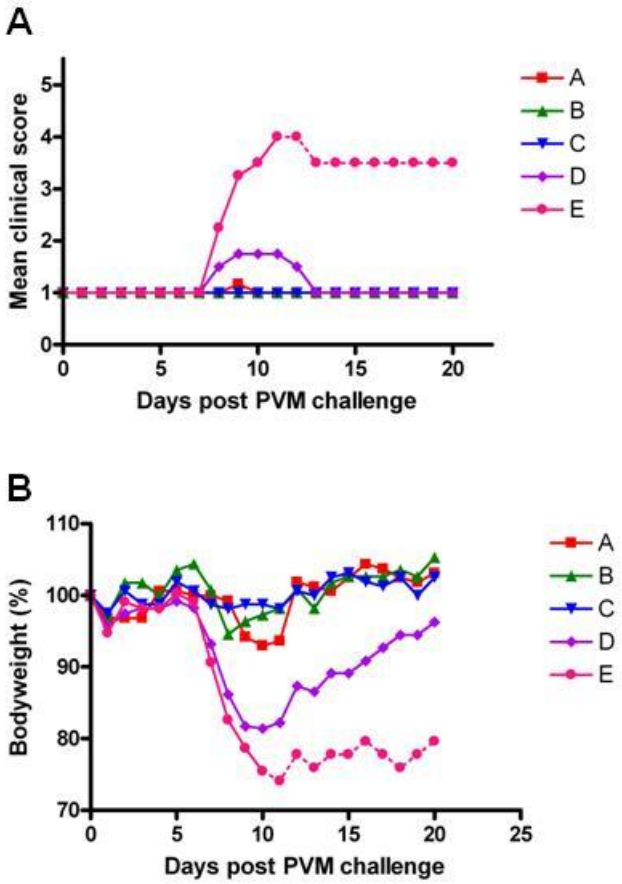

Figure 4.9. rAd constructs protect C57BL/6 mice from lethal PVM challenge. C57BL/6 mice were individually vaccinated and challenged as described in Fig. 4.8 and Table 4.2. Animals were immunised with 10^7 p.f.u. of rAdF (group A), rAdM (group B), rAdN (group C), rAdZ (group D) or treated with PBS (group E). Mean clinical score (A) and percentage bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs. Groups A and C consisted of three female and two male mice, groups B and D consisted of two female and three male mice, whereas group E consisted of two female and two male mice.

4.4.3 Discussion

The three PVM rAd vaccines were consistently able to protect different mouse strains from a lethal PVM infection. Thus, PVM transgene expression is able to stimulate a protective immune response regardless of MHC haplotype.

rAdF and rAdN constructs were able to protect BALB/c, C3H/He-mg and C57BL/6 mouse strains with the 10^7 p.f.u. dose, with no associated fatalities. BALB/c and C57BL/6 rAdF-immunised animals consistently presented with milder clinical signs and less weight loss following PVM infection, with the exception of the C3H/He-mg strain, where the animals did not regain bodyweight during the experimental period. rAdN-immunised animals varied in the degree of clinical signs and weight loss observed after PVM challenge dependent on the mouse strain. rAdM-immunised BALB/c and C3H/He-mg mice presented with moderate clinical signs and weight loss upon PVM challenge with at least one fatality in each group. C57BL/6 rAdM-immunised animals did not develop as severe PVM illness as the BALB/c and C3H/He-mg rAdM-immunised mice. PVM challenge in this group was associated with minimal weight loss and the absence of PVM related clinical signs.

The C57BL/6 mouse strain is reportedly more resistant to PVM infection than either the BALB/c or the C3H/He-mg mouse strains (Anh et al., 2006); the data presented here confirms this. The C57BL/6 mouse strain differed from the BALB/c and C3H/He-mg strains in that there was a discrepancy between the degree of weight loss observed upon PVM infection, and the clinical signs of the animals. For example, in the BALB/c and C3H/He-mg strains, PBS treated mice confirmed the pathogenicity of the PVM challenge dose, and all animals died as a result. PBS-treated C57BL/6 mice also served as a similar control; however, 50% of the animals survived lethal PVM infection (Fig. 4.9), although the survivors did not recover. In addition, BALB/c and C3H/He-mg mice immunised with rAdZ, were associated with 100% and 60% mortality, respectively, upon PVM challenge. In comparison, rAdZ-immunised C57BL/6 animals developed only mild clinical signs, but still showed moderated weight loss.

Unlike C57BL/6 mice, C3H/He-mg and BALB/c animals presented with a similar PVM pathogenesis progression. For the C3H/He-mg strain, clinical signs were more readily discerned and were observed for all immunised groups. Unlike the BALB/c

and C57BL/6 mouse strains, the C3H/He-mg strain did not tolerate substantial weight loss before death occurred. For example, PBS treated BALB/c mice tolerated approximately 23% bodyweight loss before deaths occurred (Fig. 4.4B). Equally, PBS treated C57BL/6 mice tolerated only approximately 25% bodyweight loss before fatalities occurred. Conversely, PBS treated C3H/He-mg mice tolerated only a 16% weight loss before deaths occurred, a result which was also observed for the rAd-immunised groups. This result indicates that the C3H/He-mg strain is less resistant to PVM challenge, succumbing to infection for reasons other than weight loss, or that the mouse strain cannot tolerate substantial bodyweight loss.

Unexpectedly, the control rAdZ-immunised C3H/H3-mg and C57BL/6 mice were partially protected against PVM infection. The rAdZ-immunised C57BL/6 mice presented with mild clinical signs and moderate weight loss whereas C3H/H3-mg mice developed severe clinical signs and moderate weight loss. This is in contrast to the result observed in BALB/c mice (Section 4.4, Fig. 4.7) where rAdZ-immunised animals were not protected at this vaccine dose and all succumbed to a PVM infection.

Therefore, these data confirm that the rAd vaccines can stimulate protective immunity regardless of the MHC haplotype of an animal. In addition, it was re-confirmed that the BALB/c mouse strain was more tractable than the other strains for further studies of these vaccines as no fatalities were observed in the rAd PVM vaccine immunised groups and no rAdZ-immunised animals were protected against a lethal PVM infection.

4.5 rAd PVM constructs can elicit long-term protection against lethal PVM challenge

Section 4.3 described how rAd PVM constructs were able to protect BALB/c mice against PVM infection when immunised with a 10^7 p.f.u. dose. The protective effect was specific to the PVM antigen expressed by the PVM rAd recombinant virus since no protection was conferred by an irrelevant protein expressed in the same way. This confirmed that the vaccines were able to stimulate a short-term immune response

specific for PVM. However, in a non-laboratory environment, an individual may encounter a pathogen weeks, months or years after the initial vaccination. Therefore, a good vaccine should stimulate an immunologic memory towards the specific pathogen to ensure long-term protection. To investigate whether the rAd PVM constructs could stimulate such a response, BALB/c mice were immunised with the rAdM, rAdN or control rAdZ vaccine constructs and challenged with a lethal PVM dose at specific time points over a 20-week period, as described in Fig. 4.10.

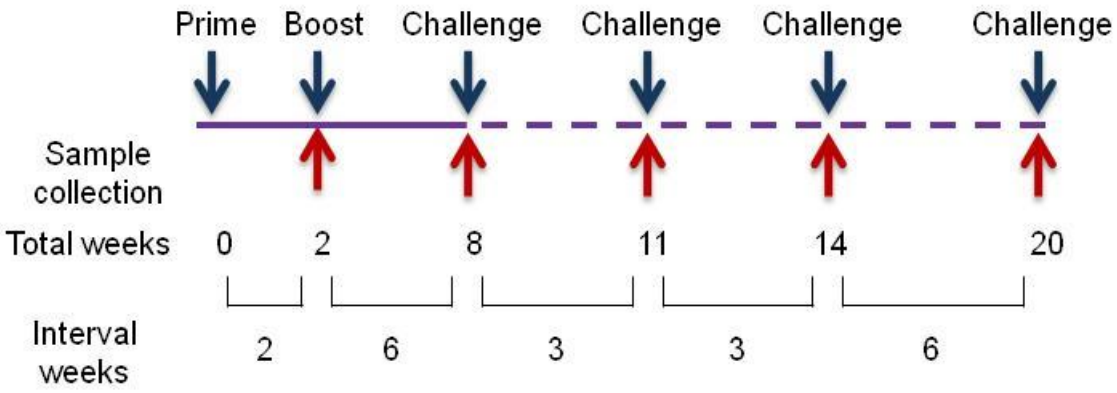

Figure 4.10. The rAd immunisation and PVM challenge regime for long-term protection studies.
BALB/c mice were immunised with 10^7 p.f.u. in 50µl of rAdM, rAdN or rAdZ, or treated with 50µl of PBS for the priming dose. After two weeks, the animals were given a booster dose of the same vaccine construct at 10^7 p.f.u. in 50µl and control animals were again treated with 50µl of PBS. Groups of animals were challenged with 250 p.f.u. of PVM strain J3666 in 50µl at 8, 11, 14 or 20 weeks after the priming immunisation. In addition to monitoring the clinical signs and weight, serum samples were collected from each individual animal (red arrows) for further analysis.

Animals between 5 and 7 weeks of age were immunised as described in Fig. 4.10 and Table 4.3. For each vaccine construct investigated, the animals were separated into four groups, split equally between males and females. Individual groups were challenged with a lethal dose of PVM and monitored for clinical score and weight loss. Each PVM group challenge contained six animals immunised with an rAd vaccine (groups A-D) and an additional two PBS treated animals (group E) which served as controls to monitor PVM pathogenesis.

Group	Prime dose (p.f.u./50µl)	Boost dose (p.f.u./50µl)	Challenge Time point (weeks)	Animals/group (♂:♀)
A	10^7	10^7	8	6 (3:3)
B	10^7	10^7	11	6 (3:3)
C	10^7	10^7	14	6 (3:3)
D	10^7	10^7	20	6 (3:3)
E	PBS	PBS	8, 11, 14, 20	8 (2/time point)

Table 4.3. The rAd vaccination regime for long-term protection studies.

4.5.1 rAdN-immunised BALB/c mice have long-lasting protection against PVM challenge.

Following PVM challenge, rAdN-vaccinated animals in groups A and C (challenged at 8 or 14 weeks after priming immunisation, respectively) did not present with any clinical signs associated with PVM infection (Fig. 4.11A), whereas animals in groups B and D (challenged at 11 and 20 weeks after inoculation respectively), presented with transient mild signs.

Mice in group D, with the longest delay between vaccination and challenge (20 weeks), developed the highest clinical score of all the rAdN-immunised groups, reaching an average peak score of 2.5 on day 12 (Fig. 4.11A). However, this was substantially below the clinical score of control animals (group E). Mild weight loss was associated with each group following PVM challenge, beginning on day 6 for group B, day 7 for groups C and D, and day 8 for group A (Fig. 4.11B).

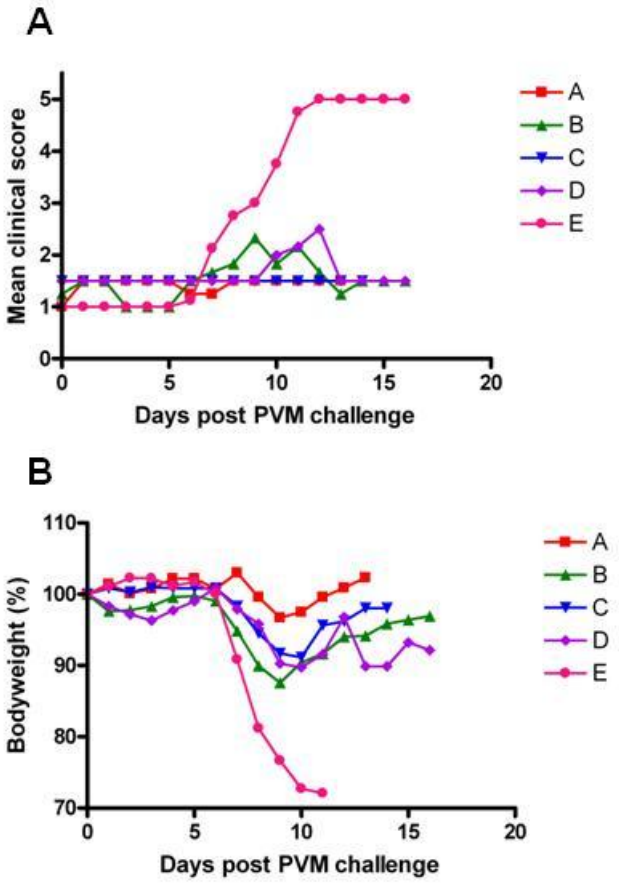

Figure 4.11. rAdN stimulates a long-term protective immune response against a lethal PVM challenge.
BALB/c animals were vaccinated as described in Fig. 4.10 and Table 4.3 with rAdN. Animals were challenged at 8, 11, 14 or 20 weeks post initial vaccine dose (groups A-D respectively) with 250 p.f.u. of PVM strain J3666 in 50µl. Mean clinical score (A) and percentage bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Data for animals treated with PBS for each time point were averaged to generate the data set shown for group E; there was no difference in the course of PVM pathogenesis for these animals challenged at different time points. All immunised male mice presented with ruffled fur before PVM challenge as described in Section 4.3.2, which resulted in a slight elevation in baseline clinical score for all groups.

No correlation was observed between length of time post-immunisation and the degree of weight loss associated with each group following PVM challenge, with group B animals losing the most weight, 12%, on day 9 (Fig. 4.11B). From day 9

onwards, the animals in groups A-C began to regain weight at a steady rate for the duration of the monitoring period. In contrast, animals in group D lost additional weight on day 13, prior to slight weight gain. However, in all these immunised groups, any observed weight loss was substantially less than was seen in control group E animals. These showed weight loss from day 6 onwards, reaching an averaged maximum of 28% by day 11 (Fig. 4.11B). There was no difference between as the course of PVM infection in control animals as the time course progressed. This confirmed the potency of the challenge dose used. Thus, the protection against lethal PVM infection induced by rAdN was long lasting.

4.5.2 rAdM-immunised BALB/c mice have long-lasting protection against PVM challenge

All rAdM-immunised groups of mice developed only mild clinical signs following lethal PVM challenge and mild or moderate weight loss (Fig. 4.12A). Unlike equivalent rAdN-immunised animals (Fig. 4.11), there appeared to be a possible correlation between the challenge time point post-immunisation and the severity of clinical signs observed. rAdM-immunised animals in group A (challenged 8 weeks after inoculation) did not develop clinical signs, other than a very small increase in clinical score on day 10 (Fig. 4.12A). In contrast, animals in group B, which were challenged 11 weeks after inoculation, presented with peak clinical scores of 2 on day 10, which declined thereafter, whereas animals challenged at 14 and 20 weeks after inoculation (groups C and D respectively) presented with peak clinical scores of 2.5 and 2.33, respectively, on day 9 (Fig. 4.12A). No fatalities were observed for any of the rAdM-vaccinated groups, in marked contrast to control group E onwards, which all succumbed to lethal PVM infection within the experimental period. The extended time course observed for the control group animals in this experiment was due to the increased age of the animals upon challenge. This would have provided them with a greater time to gain more bodyweight prior to PVM challenge than after a standard four-week challenge and in turn, this would provide the animals more reserves to combat the PVM infection for longer before death occurred.

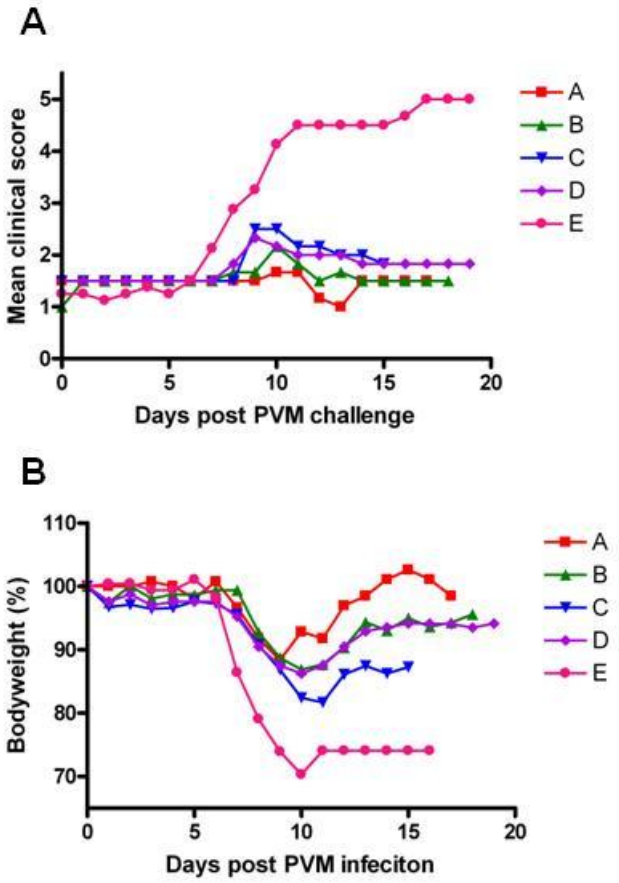

Figure 4.12. rAdM stimulates a long-term protective immune response against lethal PVM challenge.
BALB/c animals were vaccinated and challenged as previously described in Fig. 4.11 and Table 4.3. Animals were challenged at 8, 11, 14 and 20 weeks post priming vaccine dose (groups A-D respectively). Mean clinical score (A) and percentage bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Data for animals treated with PBS for each time point were averaged to generate the data set shown for group E; there was no difference in the course of PVM pathogenesis for these animals challenged at different time points. All immunised male mice presented with ruffled fur before PVM challenge as described in Section 4.3.2, which resulted in a slight elevation in baseline clinical score for all groups. In the experiment shown, group A comprised of only four animals due to death from anaesthesia complications and unknown causes.

As was observed for rAdN-immunised animals, mild to moderate weight loss was associated with PVM infection of all rAdM-immunised groups, but this was substantially less than for control group E animals and there appeared to be little

correlation between the degree of weight loss observed and the challenge time point post-immunisation. Weight loss began from day 6 for all groups, reaching a maximum of 11% on day 9 for group A, 13% for groups B and D on day 10, and 18% for group C on day 11 (Fig. 4.12B). After maximum weight loss was reached, all groups then began to regain weight, although only group A achieved their pre-challenge bodyweight during the experimental period.

4.5.3 rAdZ-immunised mice are not protected against immediate or delayed PVM challenge.

As a control for the long-term protection experiment using rAdN and rAdM recombinant viruses (Fig. 4.11, 4.12), an equivalent experiment was conducted with the rAdZ construct. As expected, in contrast to rAdN and rAdM-immunised animals, animals receiving rAdZ showed little protection against PVM infection, displaying a high fatality rate and severe clinical signs and weight loss at all challenge times (Fig. 4.13). PVM pathogenesis was similar for all rAdZ-immunised groups as control PBS-treated animals (group E). Clinical signs appeared from day 6 onwards, (Fig. 4.13A), peaking at a high score, of at least 4, during the monitoring period. Similarly, severe weight loss of between a maximum of 20-30% was observed for all rAdZ-immunised groups (Fig. 4.13B). The fatalities within all the rAdZ-immunised groups varied (Fig. 4.13C) and did not correlate with the length of time post immunisation and PVM challenge. As before, the pathogenicity of the PVM challenge dose was confirmed by the PBS-treated (group E) animals.

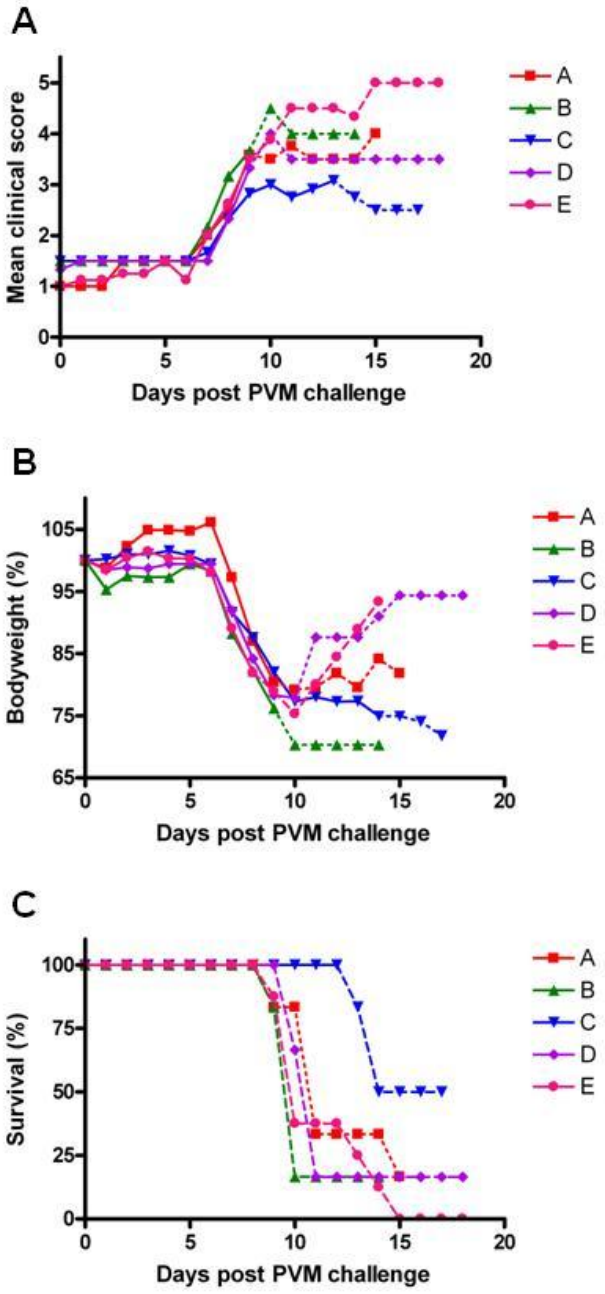

Figure 4.13. rAdZ did not stimulate a long-term protective immune response against lethal PVM challenge.
BALB/c animals were vaccinated and challenged as previously described in Fig. 4.11 and Table 4.3. Animals were challenged at 8, 11, 14 and 20 weeks post initial vaccine dose (groups A-D respectively). Mean clinical score (A), percentage bodyweight loss (B) and percentage survival (C) were monitored throughout the challenge period and calculated as described in Fig. 4.12. Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs. Data for animals treated with PBS for each time point were averaged to generate the data set shown for group E; there was no difference in the course of PVM pathogenesis for these animals challenged at different time points. All immunised male mice presented with ruffled fur before PVM challenge as described in Section 4.3.2, which resulted in a slight elevation in baseline clinical score for all groups.

4.5.4 Discussion

These data show that rAdM and rAdN vaccines were sufficiently immunogenic to elicit long-term protection against pathogenic PVM strain J3666. They were therefore able to induce immunologic memory to PVM. Animals, which received the rAd PVM constructs, had markedly reduced clinical signs and less weight loss compared with those which received the control vaccine, rAdZ. rAdM and rAdN immunised mice developed milder clinical signs when the time between the boost dose and PVM challenge was increased when compared to animals challenged only two weeks after boosting (group B mice; Fig. 4.5, 4.6). Maximum weight loss in these groups remained at a similar percentage of around 15% of initial bodyweight. This suggests that the animals were able to mount a more effective immune response when the time between the booster immunisation and challenge was increased. A small proportion of rAdZ-immunised animals did survive lethal PVM infection, however there appeared to be no correlation between time elapsed between vaccination and challenge, and the survival rate. Such sporadic survivals may have occurred because the animals challenged in this experiment were significantly older than those challenged in the experiment described in Fig. 4.7. The animals would have increased in weight prior to PVM challenge and this increased bodyweight may have provided some individuals with an advantage. Possibly, increased weight delays PVM lethality enabling a natural immune response to be mounted against PVM that ultimately facilitates recovery. Thus, the rAd PVM constructs are able to stimulate long-term protection against PVM in the BALB/c mouse strain.

4.6 A single immunisation with an rAd PVM construct protected BALB/c mice against lethal PVM challenge

The rAd PVM vaccines can provide long-lived protection against lethal PVM infection, as shown in Sections 4.4 and 4.5. To investigate the immunogenicity of these vaccines further, animals were immunised as described in Table 4.4, with a single dose of 10^7 p.f.u. of rAdM, rAdN or rAdZ, or treated with PBS as a control. Animals were challenged with a lethal dose of PVM strain J3666 six weeks after immunisation and monitored for clinical signs and weight loss.

Group	Prime dose (10^7 p.f.u./50µl)	Animals/group (♂:♀)
A	rAdM	6 (3:3)
B	rAdN	6 (3:3)
C	rAdZ	6 (3:3)
D	PBS	4 (2:2)

Table 4.4. The immunisation protocol for investigation of the efficacy of a single immunising dose of rAd PVM constructs

The data demonstrating the clinical signs and weight loss of mice are show in Fig. 4.14. In contrast to the control animals, rAdM-immunised animals (group A) were partially protected against the lethal effects of PVM challenge, but developed severe clinical signs and weight loss. One animal died two days after the PVM challenge, due to unknown causes. The remaining animals presented with PVM related signs from day 7 onwards, reaching a peak score of 3 on day 7 (Fig. 4.14A). Bodyweight declined from day 6 onwards at a similar rate to the animals in the control group D, reaching a maximum of 25% weight loss on day 11 (Fig. 4.14B). A further two animals died on days 10 and 11 (all male). The remaining animals (all female) recovered from PVM infection as evident through weight gain but still maintained a high clinical score.

rAdN-immunised animals (group B) were more robustly protected against PVM infection, with no associated fatalities. Mild clinical signs appeared from day 8 onwards, reaching a peak score of only 1.8 and decreasing thereafter (Fig. 4.14A). Three days post challenge, the animals appeared ruffled and this was attributed to the immunisation procedure. The group developed slight weight loss in response to PVM challenge, beginning on day 7 and then declining to a loss of 11% on day 9 (Fig. 4.14B), after which the group's weight increased indicating recovery from PVM infection.

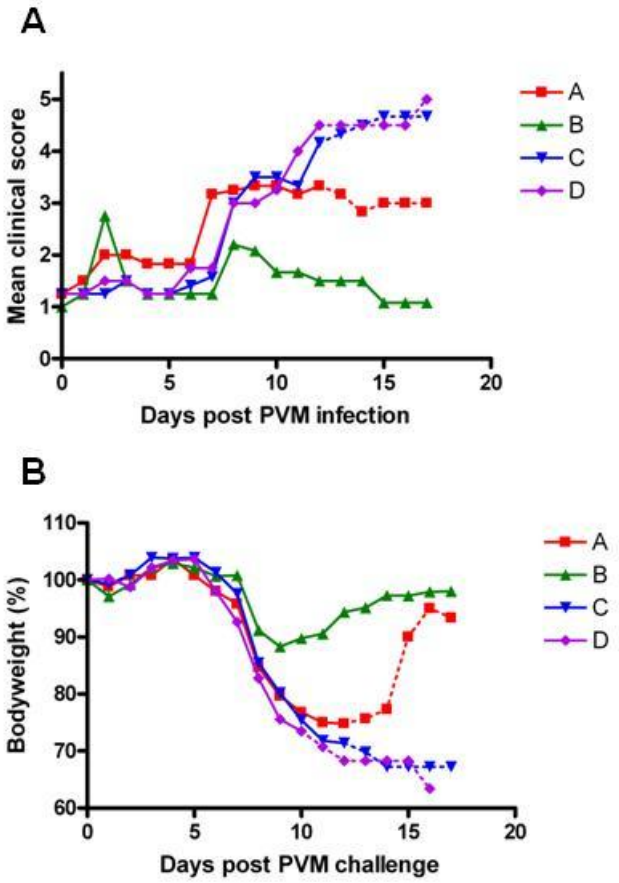

Figure 4.14. A single immunisation of rAdN can protect BALB/c mice against lethal PVM challenge.
BALB/c mice were vaccinated with a single dose of 10^7 p.f.u. of either rAdM (group A), rAdN (group B), rAdZ (group C) or PBS (group D), as described in Table 4.4. Six weeks after vaccination, the animals were challenged with a lethal dose of PVM strain J3666. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs.

In contrast to the animals that received rAdM or rAdN, rAdZ-immunised animals (group C) were indistinguishable from control group D in terms of clinical signs and weight loss and thus were not protected from PVM challenge. Both groups C and D showed signs of PVM-related illness from day 6 onwards, reaching a peak score of 4.33 and 5, respectively, on day 12 (Fig. 4.14A). Both groups presented with severe weight loss in response to lethal PVM challenge. Weight loss was evident from day 6

onwards (Fig. 4.14B), and reached a maximum loss from day 11 onwards. The mortality from both groups was 83%, with one animal in both groups surviving until termination of the experiment. These animals did not shown signs of recovery and maintained a high clinical score with no evidence of further weight gain. The mortality observed with this group of animals mirrored that of control group D, as two animals died unexpectedly on day 1, with a further two on day 12. An additional animal died on day 13, but the remaining mouse survived until the experiment was terminated. However, this animal did not recover from PVM infection, since it maintained a high clinical score and severe weight loss.

These data show that a single immunisation of 10^7 p.f.u. of rAdN was sufficient to confer protection and ensure the survival of all the animals in the group whereas only 50% of rAdM animals immunised at the same dose were protected. Interestingly, the animals which survived in this case were all female, suggesting a gender difference in the susceptibility of animals to PVM infection. This has been reported previously (Flandre et al., 2003, Krempl et al., 2007). This experiment indicated that the protective immune response stimulated by a single vaccine dose of the PVM rAd constructs can be sufficient to protect animals against a lethal PVM J3666 infection, although protection is likely to be less robust than is achieved by two-dose vaccination schedules.

4.7 Prime-boost immunisation with rAd vaccine mixtures is protective against lethal PVM infection

Immunisation of animals with the lower dose of 10^6 p.f.u. of rAdM, rAdN and rAdZ vaccines did not confer protection, whereas the 10^7 p.f.u. dose of the same recombinant virus achieved robust protection from lethal PVM infection. To investigate whether a particular vaccine construct was able to provide an immunogenic advantage, different combinations of vaccine constructs were used to immunise BALB/c mice.

BALB/c mice between 5 and 7 weeks of age were immunised via the intranasal route with the standard prime-boost regime (Fig. 4.2), with a mixture of two vaccine constructs at 10^6 p.f.u. each, or a $2x10^6$ p.f.u. dose of a single construct on day one. The animals were boosted on day 14 with the same immunisation dose and vaccine combination (Table 4.5). Animals treated with PBS served as a control group to monitor PVM strain J3666 (PVM) pathogenesis.

Throughout the immunisation regime and prior to PVM challenge, no weight loss or clinical signs were observed for any animals vaccinated with different recombinant virus combinations (data not shown). The animals were challenged with a lethal dose of PVM at day 28 and monitored for clinical signs and weight loss throughout the challenge period.

Group	Vaccine 1	Dose (p.f.u./50µl)	Vaccine 2	Dose (p.f.u./50µl)	Animals/group (♂:♀)
A	rAdN	$2x10^6$	N/A	N/A	(3:2)
B	rAdM	$2x10^6$	N/A	N/A	(3:2)
C	rAdZ	$2x10^6$	N/A	N/A	(3:2)
D	PBS	N/A	N/A	N/A	(0:2)
E	rAdN	$1x10^6$	rAdM	$1x10^6$	(3:2)
F	rAdN	$1x10^6$	rAdZ	$1x10^6$	(3:2)
G	rAdM	$1x10^6$	rAdZ	$1x10^6$	(3:2)

Table 4.5. The standard immunisation dose for vaccination with different recombinant virus combinations.

As described in Section 4.3, animals in a previous experiment that received a prime-boost immunisation regime of 10^6 p.f.u. of rAdM, rAdN or rAdZ were not protected against lethal PVM infection. Animals rapidly developed high clinical scores (Fig. 4.15A) and rapidly lost bodyweight in response to PVM infection (Fig. 4.15B).

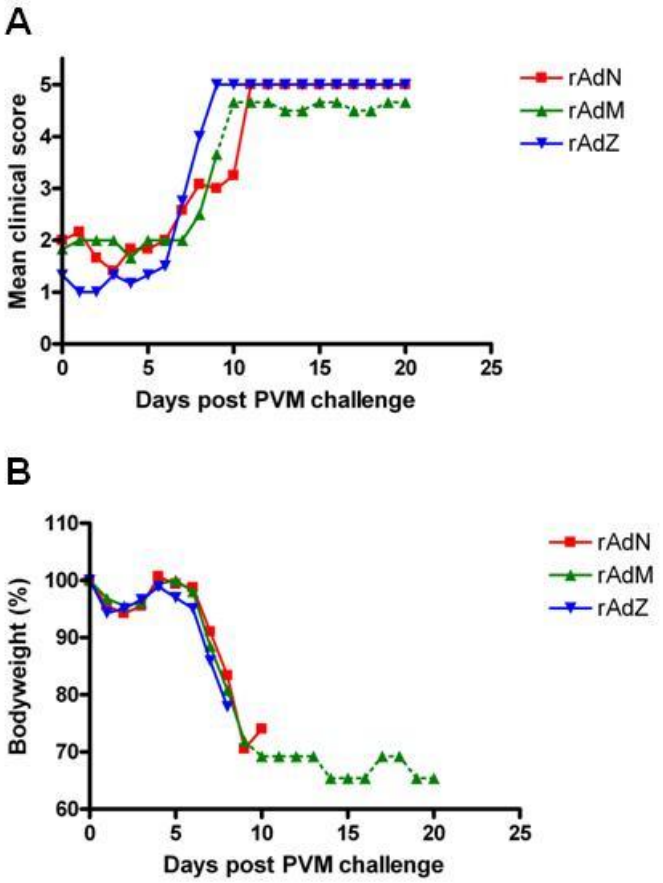

Figure. 4.15. Immunisation of BALB/c mice with a 10^6 p.f.u. dose of rAd PVM constructs does not confer protection against lethal PVM infection.
BALB/c mice were immunised with a prime-boost regime with 10^6 p.f.u. of a single rAd vaccine, rAdM, rAdN, or rAdZ, via the intranasal route. Animals were boosted on day 14 with the same dose and vaccine construct prior to challenge on day 31 with a lethal PVM infection. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs. This data was presented previously in Sections 4.3.2-4 and has been included here for ease of comparison.

Similar to animals receiving a single 10^6 p.f.u. dose of recombinant virus, animals that received a $2x10^6$ p.f.u. dose of rAdM (group B) or rAdZ (group C) were not protected against lethal PVM infection (Fig. 4.16). Group B animals had moderate clinical signs associated with PVM challenge. These appeared from day 7 onwards and reaching a peak score of 3 on day 10 (Fig. 4.16A). Significant weight loss was

observed for animals in this group, beginning on day 6 and decreasing steadily at a rate similar to control group D, reaching 20% on day 12, which coincided with the death of an animal (Fig. 4.16B). Further animals were lost on days 18 and 19. The remaining animals did not recover from PVM infection but had a greater average bodyweight than those, which had succumbed. rAdZ-immunised animals (Group C), developed severe signs, beginning on day 5 and reaching a peak score of 3.8 on day 12 (Fig. 4.16A). This group developed weight loss from day 5 onwards, similar to group B and control group D. Weight loss increased, reaching a maximum of 25% on day 12 which coincided with two fatalities. The remaining animals did not recover from PVM infection as evident from the lack of weight gain in the group and their persistently high clinical scores.

In contrast, animals receiving $2x10^6$ p.f.u. of rAdN (group A) were protected against PVM infection and developed only mild clinical signs These signs were observed from days 6 to 15 and reached a peak score of 2.33 on day 11 (Fig. 4.16A). As for groups B and D ($2x10^6$ p.f.u. rAdM and PBS treated, respectively), weight loss began on day 6 but in contrast to these non-protected groups, reached a peak of only 8% on day 12 (Fig. 4.16B). One fatality was observed on day 11, but the remaining animals recovered from PVM infection, as evident from their weight gain and a decrease in clinical score to baseline levels.

Animals were also immunised with different vaccine combinations in parallel with the single construct experiments described above, using the same PBS treated animals, group D (Fig. 4.17). Group G (immunised with both rAdM and rAdZ) was indistinguishable from the equivalent single vaccine groups (B and C) or the PBS control group D in terms of weight loss, and from group B in terms of clinical score.
Group G developed moderate clinical signs from day 7 onwards, reaching a peak mean score of 2.9 on day 11 (Fig. 4.17A), which coincided with one fatality. In contrast to this moderate clinical score, severe weight loss was observed for this group, beginning on day 5 and increasing to a maximum of 23% on day 12 (Fig. 4.17B). The remaining animals survived lethal PVM challenge but did not fully recover, maintaining a low bodyweight, with no indication of further weight gain and were therefore considered not to have been protected from PVM infection.

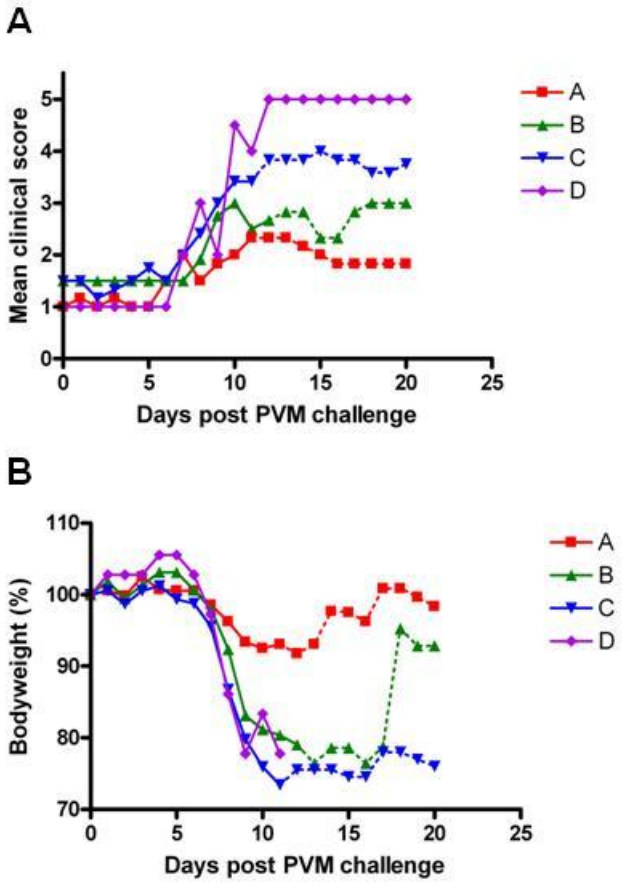

Figure 4.16. Immunisation of BALB/c mice with a $2x10^6$ p.f.u. dose of rAdN but not the rAdM or rAdZ constructs, is protective against lethal PVM infection.
BALB/c mice were immunised and challenged as described in Fig. 4.15 and Table 4.5 with $2x10^6$ p.f.u. of either rAdN (group A), rAdM (group B), rAdZ (group C) or PBS (group D). Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs.

In contrast, group E (immunised with rAdN and rAdM) and group F (immunised with rAdN and rAdZ) were protected against lethal PVM infection, both regimes being associated with only mild clinical signs and less bodyweight loss. Both groups developed mild clinical signs from day 8 onwards (Fig. 4.17A), with groups E and F reaching a peak mean score of 2.3 on day 15 and 2.6 on day 10 respectively. Weight loss was indistinguishable between these two groups, beginning on day 6 and initially decreasing at a similar rate to groups B, C, D and G. However, from day 12

their weight stabilised at 15% bodyweight loss, (Fig. 4.17B), a less severe loss than groups B, C, D or G. One animal in each of groups E and F died from PVM infection on day 15, with the remaining animals maintaining a moderate bodyweight loss with only a 3% weight gain over the remaining experimental period. Due to the mild signs, lower bodyweight loss and survival, groups E and F were considered to have been partially protected from lethal PVM infection.

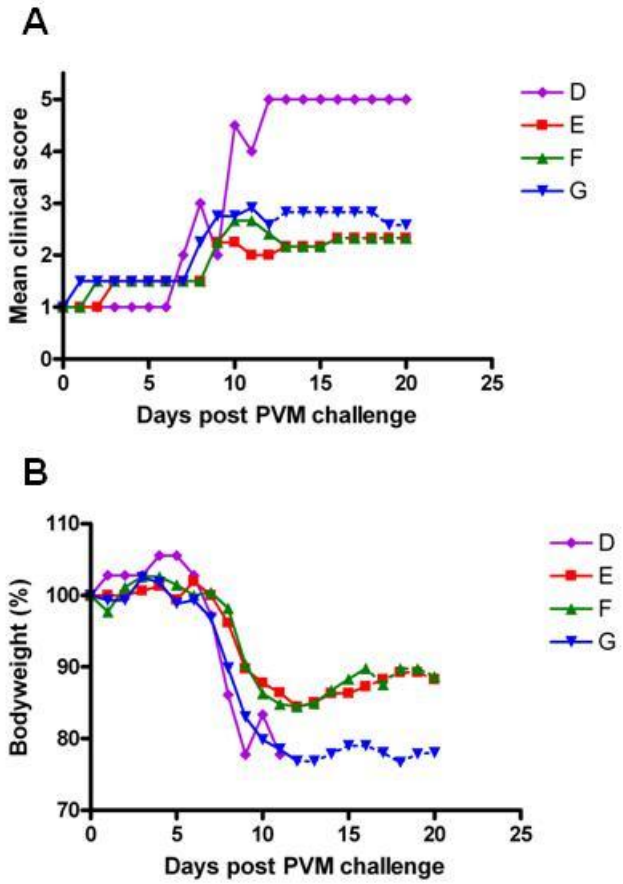

Figure. 4.17. Immunisation of BALB/c mice with some rAd PVM recombinant virus combinations can protect the animals against lethal PVM infection.
BALB/c mice were immunised and challenged as described in Fig. 4.16 and Table 4.5 with $2x10^6$ p.f.u. of rAd constructs in different combinations. Group D received PBS, group E received rAdN and rAdM, group F received rAdN and rAdZ and group G received rAdM and rAdZ. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs.

Although some animals survived in all rAd immunised groups in this experiment, the degree of PVM signs and weight loss associated with specific groups was different. Animals which were immunised with a $1x10^6$ p.f.u. dose of rAd constructs using a prime-boost immunisation regime (Section 4.3.1 and Fig. 4.15) were not protected against PVM infection and developed severe clinical signs and weight loss. In contrast, groups A, B and C which received a $2x10^6$ p.f.u. dose of a single vaccine construct were more likely to survive PVM infection. This could be attributed to an increased dose of immunogen, from $1x10^6$ to $2x10^6$, which may have sufficiently stimulated the immune response, even non-specifically (such as rAdZ), to reach the threshold of an innate immune response and promote an adaptive immune response to give protection against PVM. Regardless, groups B and C (immunised with $2x10^6$ p.f.u. of rAdM or rAdZ respectively) and group G (immunised with $1x10^6$ p.f.u. of both rAdM and rAdZ), developed a similar disease profile upon PVM infection as control group D. The animals maintained moderate to severe clinical signs and severe weight loss. No recovery was evident in these groups and groups B, C and G were not considered to have been protected against a lethal PVM infection. Group D received PBS and, as expected, was not protected against lethal PVM infection. These animals developed PVM related signs and weight loss at a similar rate and degree as other PBS treated animals in experiments, confirming the potency of the challenge dose used.

Group A animals were protected against a lethal PVM infection, developing only mild clinical signs and weight loss. In addition, groups E and F, which received a combination of either rAdM or rAdZ with rAdN, were also protected against PVM infection. This suggests that the rAdN recombinant virus is more effective at stimulating a protective immune response when present with or without the combination of the other rAd PVM constructs, than the other recombinant viruses.

4.8 Discussion

This chapter has described a number of experiments to examine the qualities of rAd PVM constructs. These data show that the rAd PVM recombinant viruses can

successfully elicit protection against lethal PVM challenge in both short and long-term experiments.

Animals immunised with the 10^7 p.f.u. dose of rAdF, rAdM or rAdN were protected against lethal PVM infection. This was true for the BALB/c, C57BL/6 and C3H/He-mg mouse strains, indicating that the vaccines conferred protection against PVM regardless of MHC haplotype.

The rAdF construct described in Section 4.3 was the only one to confer protection in BALB/c mice at a lower dose of 10^6 p.f.u. The virus was previously found to be contaminated with a replication competent virus (Section 3.6.2.1), therefore, because a small proportion of the virus stock was potentially able to replicate within the host, the rAdF vaccine was not evaluated in long-term experiments. Animals that received this virus developed milder clinical signs and suffered less weight loss than those which had received the alternative rAd PVM constructs. This may be due to the antigenicity of the PVM F protein or that the animals may have received a greater dose of PVM protein if replication occurred, supported by the contaminating replication-competent virus.

The rAdM construct was immunogenic and was able to elicit protection in animals when they were immunised with the 10^7 p.f.u. dose. However, protection was not always absolute, with a few fatalities occurring in BALB/c and C3H/He-mg mouse strains (Sections 4.3 and 4.4). When the time period between immunisation and PVM challenge was extended, as seen in the long-term experiments, no fatalities were observed for rAdM-immunised animals. This suggests that the standard immunisation-challenge schedule detailed in Fig. 4.2, did not allow sufficient time for the full protective effect of rAdM to be achieved. These data were similar to those for rAdN-immunised animals, with the exception that no fatalities were associated with rAdN construct at the 10^7 p.f.u. dose in any of the mouse strains used in this study. Both rAdM and rAdN-immunised animals were protected against PVM infection throughout the long-term experiments described in Section 4.5. The immunogenicity of those constructs also differed during the single immunisation experiment described in Section 4.6. BALB/c mice immunised with a single 10^7 p.f.u. dose of rAdN were protected from a lethal PVM infection six weeks after immunisation with only mild clinical signs and little weight loss. In contrast, rAdM-

immunised animals had moderate clinical signs and moderate weight loss with a 50% mortality rate (Fig. 4.14).

These data show that the rAd PVM constructs generated specific immunity against PVM infection, as the rAdZ control did not confer protection in BALB/c mice at either the 10^6 or 10^7 p.f.u. doses. rAdZ-immunised animals were not protected in the short, long-term, or single dose experiments in BALB/c or C3H/He-mg mouse strains and were associated with severe disease in the C57BL/6 mouse strain. Mouse strain differences in survival have been reported previously (Anh et al., 2006), but survivors in the long-term and single dose experiments were attributed to their greater bodyweight during the immunisation regime. These animals would therefore have greater energy reserves to allow them to survive longer without feeding during the later stages of PVM illness. This may have allowed time for a natural immune response to be developed towards PVM, allowing the recovery of some individuals as in the long-term experiments, or allowing virus replication to be kept in check, preventing death as in the single immunisation experiment.

The rAdM, rAdN and rAdZ constructs were also investigated for their immunogenicity when provided in a combination. The rAd PVM viruses were used to immunise animals in different combinations using the standard prime-boost regime. The recombinant viruses were used to immunise animals at the lower dose of 10^6 p.f.u. to ensure any additive protective effect could be clearly discerned, as previous experiments in Section 4.3 demonstrated this dose was not protective when single rAd PVM viruses were used. The experiment, detailed in Section 4.7, demonstrated that the rAdN virus was more immunogenic than rAdM. Immunisation with a double dose of rAdN was sufficient to protect animals from PVM infection. Interestingly, immunisation of a single dose of rAdN in combination with either rAdM or rAdZ vaccines also conferred protection against a lethal PVM infection, albeit with greater associated clinical signs and weight loss. In contrast, rAdM and rAdZ-immunised animals in combination or as a double dose, were indistinguishable from control animals. This data indicated that through increasing the dose of $1x10^6$ p.f.u. to $2x10^6$ p.f.u., the immune response is stimulated to protect against lethal PVM infection.

Throughout the *in vivo* studies, the dose of rAd PVM constructs used to immunise the animals was standardised using p.f.u. values considered in Section 3.6.3. The p.f.u. value was chosen to standardise the dose of rAd in the *in vivo* model because it directly relates to the amount of infectious virus in the preparation rather than the number of particles, which may or may not be infectious. As the immunisation of animals is likely to be directly affected by the number of infectious virus particles, the p.f.u. value was chosen to standardise the immunisation dose used. As described in Section 3.6.3, Ad5 had a particle: p.f.u. ratio of 20. The rAd viruses used in this study have higher ratios than this dependent upon the preparation used (App. B. Table B.1). For example, this can vary from 40 to 1132 for different rAdN virus preparations. For this reason, if a preparation with a high particle: p.f.u. ratio was used to immunise animals it might be able to stimulate a strong anti-vector response resulting in increased recombinant virus clearance. This in turn may affect the ability of an animal to mount a response towards the PVM transgene. For this reason, preparations with high particle: p.f.u. ratios were not used to immunise animals, such as rAdN stocks 5, 8 and 11 (App. B. Table B.1). This residual variation in the particle: p.f.u. ratio of the stocks used does not explain the differences observed in the immunogenicity of the viruses since rAdN-immunised animals were protected against PVM even when virus preparations with relatively high particle: p.f.u. ratios, such as stock 2, were used. In addition, rAdF had lower particle: p.f.u. ratios than rAdM and rAdN stocks, perhaps reflecting its contamination with a replication-competent revertant. rAdM and rAdN stocks used had a similar particle: p.f.u. ratio. As rAdN-immunised animals with these stocks were protected, it seems unlikely that rAdM-immunisation would result in virus clearance and therefore reduced immunogenicity. Therefore, differences in the particle: p.f.u. ratios of the various recombinant virus stocks do not explain the differences in the immunogenicity of the rAd vaccines in the *in vivo* model.

In conclusion, the rAd PVM constructs used in the experiments described here are capable of inducing protection from a challenge with a lethal PVM infection. The 10^7 p.f.u. dose was able to elicit this protective effect and was chosen as the standard immunisation dose for the majority of the PVM challenge experiments. The virus rAdN was proven to be particularly immunogenic and was able to protect animals against lethal PVM challenge with a single dose (Fig. 4.14) as well as with a prime-

boost regime with immunity being provided for both the short and long-term (Figs. 4.6 and 4.11). In addition, use of the rAdN construct consistently generated protection when used in a combination experiment, in contrast to the results observed with rAdM. This suggests that rAdN-immunisation of animals is more effective than rAdM or rAdF during single and short-term experiments. During long-term experiments, rAdN and rAdM-immunised animals were equally protected, suggesting that rAdM-immunisation may require a greater time period over which to generate sufficient immunological response and subsequent memory towards PVM. The evidence that the recombinant adenoviruses can establish long-term protection suggests that the protective mechanism is unlikely to be mediated by innate immunity. Thus, protection is most likely to be mediated by a long-lived humoral or cellular response. Overall, the rAd PVM constructs have been successfully used to demonstrate efficacy in the PVM *in vivo* modell.

Chapter 5

Further investigation of rAd recombinant virus immunisation in the PVM infection model

5.1 Introduction

The rAd PVM constructs have been shown to provide protection in mice against challenge with PVM when used to immunise mice with a 10^7 p.f.u. dose, as detailed in the previous chapter. The experiments focused upon an homologous prime-boost immunisation regime via the intranasal route. Immunisation via this route stimulated a strong immune response, most likely in the mucosa of the lung. However, although new vaccine delivery systems are in development, the majority of currently licensed vaccines are delivered intramuscularly, which predominantly stimulates a systemic rather than a mucosal immune response.

This chapter endeavours to further investigate rAd PVM vaccination in the PVM infection model. Mice were immunised via different routes to determine whether these can stimulate a protective immune response, similar to that observed via the intranasal route. In addition, a greater dose, of 10^8 p.f.u., was used to immunise animals in a variety of short and long-term experiments to determine whether PVM infection-associated signs would decrease in severity, whilst retaining the protective effect of the vaccine.

5.2 Immunisation via alternative routes is not protective against lethal PVM infection

As described in Sections 1.2 and 1.6, a vaccine must stimulate the correct immune response in order to be protective against a pathogen. Traditionally, most vaccines are used to immunise individuals intramuscularly with the view to stimulating a strong systemic IgG response towards the pathogen. However, the majority of viruses enter the host via a mucosal surface so the generation of mucosal immunity is often more important than a general systemic response (Crowe, 2003). Newer vaccine strategies now recognise that it is important to stimulate the mucosal immune response, to allow the secretion of specific IgA, which may prevent infection. As detailed in the previous chapter, using rAd via the intranasal

immunisation route generated a protective immune response against PVM. However, if a systemic immune response could be shown also to be protective, immunisation of individuals would be easier and allow immunity to be passed between mother and infant.

PVM rAd recombinant viruses were used to immunise BALB/c mice via intraperitoneal and subcutaneous routes to determine whether induction of systemic rather than local mucosal immunity could elicit protection against a lethal PVM infection. The subcutaneous route and intraperitoneal routes were investigated due to the relative ease of vaccine delivery by these routes on a small animal.

5.2.1 Subcutaneous immunisation route

BALB/c mice between 5 and 7 weeks of age were immunised via the subcutaneous route using the standard prime-boost regime (Section 4, Fig. 4.2). For each construct investigated, animals from the same breeding batch were separated into four groups and immunised as detailed in Table 5.1. Animals treated with PBS served as a control group to monitor PVM pathogenesis. Throughout the immunisation regime and prior to PVM challenge, no weight loss or clinical signs were observed for any of the animals. Therefore, immunisation of BALB/c mice with rAd viruses via the subcutaneous route does not result in an observable pathogenesis.

Group	Prime dose (p.f.u./50µl)	Boost dose (p.f.u./50µl)	Animals/group (♂:♀)
A	10^6	10^6	5 (2:3)
B	10^7	10^7	5 (2:3)
C	10^8	10^8	5 (2:3)
D	PBS	PBS	6 (3:3)

Table 5.1. The standard protocol for BALB/c immunisation via the subcutaneous route.

5.2.1.1 rAdM-immunisation of BALB/c mice

Animals in groups A, B and C (which received rAdM at 10^6 p.f.u., 10^7 p.f.u. or 10^8 p.f.u. respectively) were not protected against lethal PVM challenge. All animals developed clinical signs from day 6 onwards (Fig. 5.1A), with group A reaching the peak score on day 12, group B on day 10 and group C on day 13. The rate of development of clinical signs for rAdM-immunised groups was similar to that of control group D (PBS treated) animals. Substantial weight loss was also evident in each rAdM-immunised group and progressed at a rate similar to that of control group D. Group A (10^6 p.f.u. dose) reached a maximum of 30% weight loss on day 11, with one death on day 9, another on day 10 and the remaining three on day 11 (Fig. 5.1B). Group B (10^7 p.f.u. dose) had one fatality on day 8 and reached a maximum weight loss of 26% on day 9 which corresponded with the deaths of all the animals. Finally, group C (10^8 p.f.u. dose) had four fatalities on day 13, with the remaining animal dying on day 14. Weight loss was greatest on day 13, with 39% of initial bodyweight (Fig. 5.1B). Group D (PBS treated) presented with clinical signs from day 7, which increased in severity to the maximum score of 5 by day 12 (Fig. 5.1A). Weight loss was also observed from day 7 onwards, reaching a maximum of 29% on day 10 (Fig. 5.1B). Group D did not survive PVM challenge, with deaths occurring on day 9 and again on day 10; with the remaining three animals dying on day 12. This confirmed the pathogenicity of the virus stock. Thus, there was no evidence of any protective effect of delivering rAdM via the subcutaneous route.

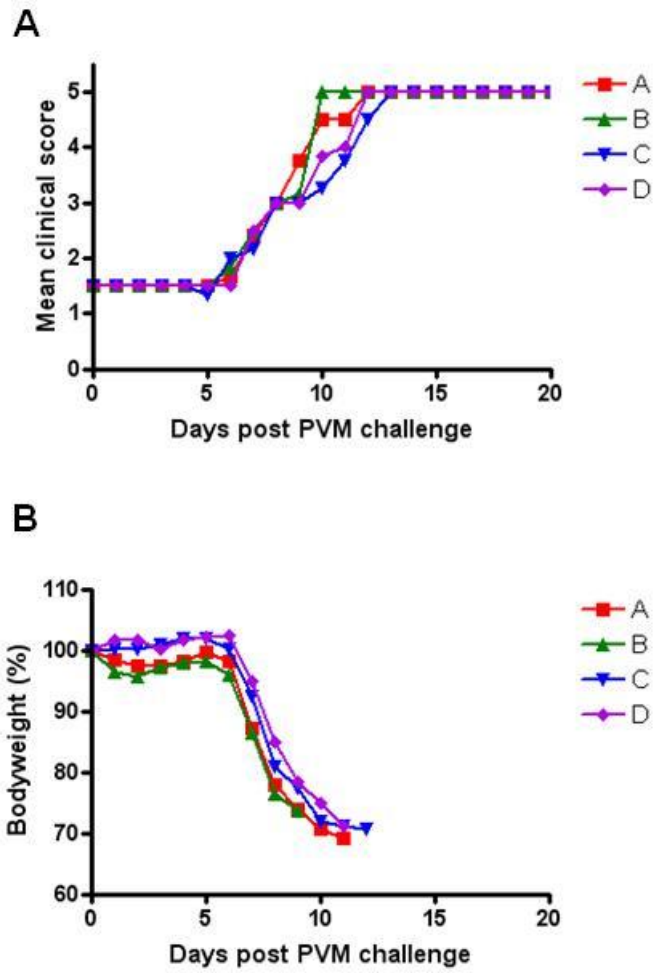

Figure 5.1 Subcutaneous immunisation with rAdM does not protect mice from a lethal PVM infection.

BALB/c mice were immunised using the standard prime-boost regime (Section 4, Fig. 4.2), via the subcutaneous route. Animals were primed on day 1 with the rAdM PVM vaccine at either a 10^6 p.f.u. (group A), 10^7 p.f.u. (group B) or 10^8 p.f.u. (group C) dose, or PBS (group D). Animals were boosted on day 14 with the same vaccine dose and construct as received for the priming dose. The animals were challenged with a lethal dose of PVM strain J3666 (250 p.f.u. in 50µl) on day 28 of the experiment. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%).

5.2.1.2 rAdN-immunised BALB/c mice

Animals were immunised concurrently with the experiment described in Section 5.2.1. The control animals were group D as previously described (Fig. 5.1), but have been included with these data for ease of comparison.

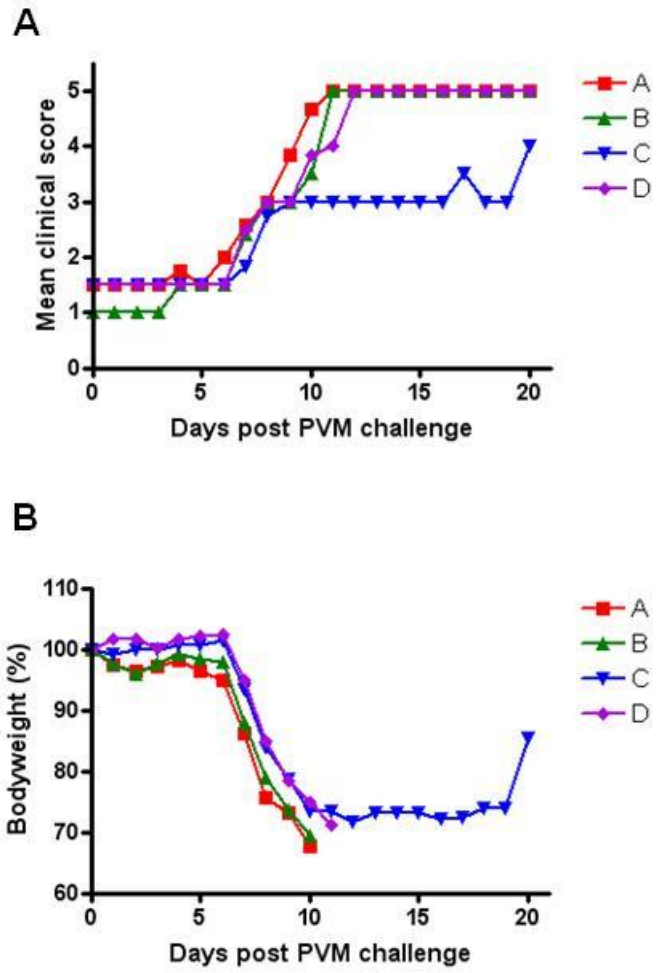

Figure 5.2 Subcutaneous immunisation with rAdN does not protect mice from lethal PVM challenge.
BALB/c mice were immunised and challenged as described in Fig. 5.1. Animals were immunised with rAdN PVM vaccine at either a 10^6 p.f.u. (group A), 10^7 p.f.u. (group B) or 10^8 p.f.u. (group C) dose, or PBS (group D). Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%).

Similarly to rAdM-immunised animals, groups A, B and C (immunised with 10^6, 10^7, or 10^8 p.f.u. rAdN respectively) were not protected against PVM challenge. Group A

presented with clinical signs from day 6 onwards, and reached the maximum score by day 11 (Fig. 5.2A). Weight loss was evident in this group from day 5 onwards, reaching a maximum of 32% on day 10 (Fig. 5.2B); with three animals dying on day 10 and the remaining two on day 11. Group B (immunised with 10^7 p.f.u.) developed clinical signs from day 4 onwards, reaching the peak score on day 11 (Fig. 5.2A). Similarly to group A, weight loss was observed from day 5 onwards and reached a maximum of 30% on day 10 (Fig. 5.2B). One fatality occurred on day 10 with the remaining animals succumbing to PVM infection on day 11. Clinical signs in group C (immunised with 10^8 p.f.u.) were observed from day 7 onwards and reached a peak score of 4 on day 20 (Fig. 5.2B). Weight loss declined as quickly as was observed for groups A, B and control group D, and reached a maximum of 28% on day 12 (Fig. 5.2B). The animals in group C survived until day 17 when two animals succumbed to PVM infection, with an additional animal dying on day 20. Two of the animals did survive PVM infection from this group but did not recover, as demonstrated by the lack of weight gain and consistently high clinical score. The slight increase in bodyweight at day 20 may reflect that these animals had lost less weight than those which had died. These animals are unlikely to be recovering from PVM infection due to their increase in mean clinical score. Therefore, the rAdN vaccine did not elicit protection against PVM when delivered by the subcutaneous route.

5.2.1.3 rAdZ-immunised BALB/c mice

As before, this experiment was conducted concurrently with those described above. The control animals were group D as previously described and have been included for ease of comparison. As expected, and similar to rAdM and rAdN-immunised animals, animals which were immunised with rAdZ via the subcutaneous route were not protected from PVM infection (Fig. 5.3).

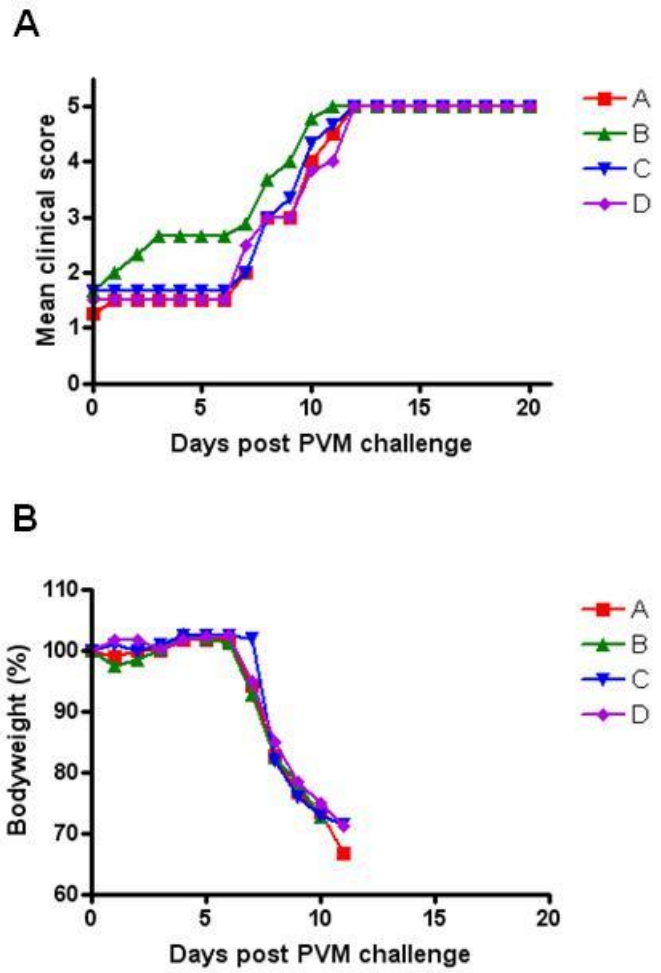

Figure 5.3. Subcutaneous rAdZ immunised mice are not protected from lethal PVM challenge.
BALB/c mice were immunised and challenged as described in Fig 5.1. Animals were immunised with rAdZ PVM vaccine at either a 10^6 p.f.u. (group A), 10^7 p.f.u. (group B) or 10^8 p.f.u. (group C) dose, or PBS (group D). Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%).

Groups A, B and C (immunised with 10^6, 10^7 and 10^8 p.f.u., respectively) were not protected against lethal PVM infection, as clinical score and weight loss was similar to that of control group D (PBS treated). Therefore, as for rAdM and rAdN-immunised animals, rAdZ-immunised animals were not protected from PVM challenge via this immunisation route.

5.2.2 Intraperitoneal immunisation route

BALB/c mice between 5 and 7 weeks of age were immunised with a high dose of 10^8 p.f.u. of rAd constructs via the intraperitoneal route using the standard prime-boost regime (Fig. 4.2), except that the periods between prime and boost, and between boost and challenge were increased to three weeks. The increased time-period between immunisations, along with the delivery of a high dose of recombinant virus, should have maximised the chance that a systemic immune response against the PVM antigen would be stimulated. The animals, drawn from the same batch, were separated into five groups and immunised as detailed in Table 5.2. Animals treated with PBS served as a control group to monitor PVM pathogenesis.

Group	Vaccine	Animals/group
A	rAdF	5 ♀
B	rAdM	5 ♂
C	rAdN	5 ♀
D	rAdZ	5 ♂
E	PBS	3 ♀

Table 5.2. The standard protocol for BALB/c immunisation via the intraperitoneal route.

Throughout the immunisation regime and prior to PVM challenge, no weight loss was observed for any animals in the experiment when vaccinated via the intraperitoneal route. The animals were challenged with a lethal dose of PVM at day 42 and monitored throughout the challenge period (Fig. 5.4). However, it was observed that, post-boost immunisation of all the constructs, a number of animals presented with non-PVM illness related abdominal signs. In particular, two animals which had received the rAdM construct were culled on the day of PVM challenge. These animals presented with very ruffled fur and had abdominal swelling. Post-mortem analysis of these mice showed that both animals had severe bloating of the stomach and upper intestine. The liver in these animals was shrunken, of a pale colour, and uneven in texture. It was concluded that these animals had suffered from

severe liver pathology, presumably due to the rAd vaccine vector. For the remainder of the experiment, the animals were monitored closely and culled immediately if these signs appeared.

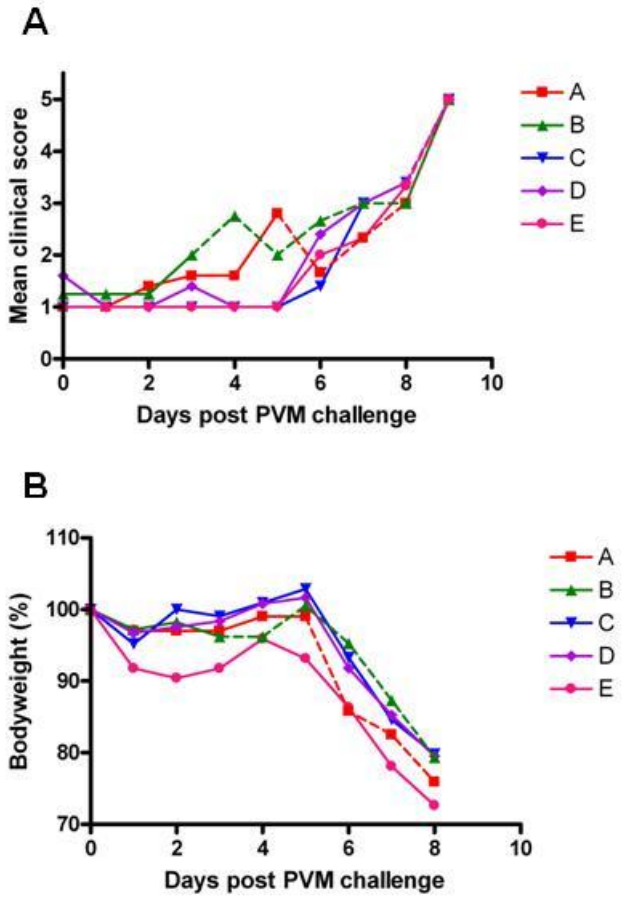

Figure. 5.4. Immunisation with rAd vaccines via the intraperitoneal route does not confer protection against a lethal PVM challenge.
BALB/c mice were immunised with 10^8 p.f.u. of each of the rAd vectors or treated with PBS. Animals were immunised with a prime dose of rAdF (A), rAdM (B), rAdN (C), rAdZ (D), or PBS (E), on day 1 and boosted on day 21 with the same dose of vaccine construct. Animals were challenged on day 42 with a lethal dose of PVM strain J3666. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs.

The remaining animals were challenged with a lethal dose of PVM at day 42 and monitored throughout the challenge period (Fig. 5.4). Similar to control group E, groups A-D (which received rAdF, rAdM, rAdN or rAdZ respectively) all developed weight loss associated with PVM illness from day 5 onwards, with groups B-D (rAdM, rAdN or rAdZ respectively) reaching a maximum of 20% and group A (rAdF-immunised) reaching a maximum of 25% on day 9. The rate and degree of weight loss was similar to that of the control group E (PBS treated). Clinical signs for groups C and D (rAdN and rAdZ-immunised animals respectively) were similar to that of group E, appearing from day 6 onwards and increasing rapidly until day 9. Signs were evident from as early as day 2 for groups A and B (rAdF and rAdM-immunised respectively), presumably due to the complications associated with the rAd vector. Control group E began to show clinical signs from day 6 onwards, which increased in severity through to day 9, when the maximum clinical score was reached (Fig. 5.4); which coincided with the death of all the animals. Weight loss was clearly evident from day 5 onwards although there was a slight decrease in bodyweight from day 1 to day 3 attributed to the immunisation procedure disrupting feeding. Weight loss reached a maximum of 27% on day 9. Thus, the rAd-immunised animals were not protected against lethal PVM infection when immunised via the intraperitoneal route.

5.2.3 Discussion

Immunisation of BALB/c mice via the subcutaneous or intraperitoneal route with any of the rAd PVM recombinants was not sufficient to elicit protection from lethal PVM challenge. Subcutaneous immunisation was not associated with adverse signs, nor was swelling visible at the site of the inoculation, indicating that the regime was well tolerated. However, rAdM and rAdN were not able to elicit protection, by this route, in contrast to the intranasal route (Section 4.3), even when a range of doses were delivered by this route suggesting the immune response was not adequately stimulated.

Intraperitoneal immunisation was subsequently performed with a higher dose of the rAd PVM constructs with increased time between immunisations to ensure the maximum chance of an immune response to be established. However, again

protection was not achieved against PVM for any of the vaccines. In addition, severe adverse reactions were observed in all rAd intraperitoneal immunised mice. These signs were not 'typical' of PVM infection and, for some animals, were present prior to PVM challenge. Moreover, PBS control mice did not present with the signs, which suggests that the severe liver pathology and gut related signs were a direct result of rAd vaccination. This result has been observed previously and is due to the natural liver tropism of Ad5 viruses (Flanagan et al., 1997, Yang et al., 1994). As neither of these alternative immunisation routes was associated with protection from PVM, stimulation of a local mucosal immune response may be important for protection from PVM infection. Thus, systemic immunisation is not sufficient to stimulate a protective immune response in the lung in time to prevent death from PVM infection, or that this regime may not have achieved systemic immunity and therefore, no protective effect would have been observed

5.3 Immunisation with a high dose of 10^8 p.f.u. of rAd constructs is protective against lethal PVM infection

In chapter 4, immunisation of animals using an intranasal prime-boost regime with vaccine doses of 10^6 and 10^7 p.f.u. respectively was investigated. As discussed in Section 4.3, animals which received the 10^6 p.f.u. dose of the constructs were not protected against lethal PVM infection. In contrast, those animals which received the immunisation dose of 10^7 p.f.u. were protected but still presented with some clinical signs and weight loss associated with PVM infection (Fig. 4.4-7). As such, it was hypothesised that if animals were immunised with a greater dose (10^8 p.f.u.), protection against lethal PVM infection would remain and PVM related signs might be reduced due to the increased dose of immunogen delivered. To investigate this hypothesis, animals were immunised with 10^8 p.f.u. of the rAd constructs via the intranasal route.

5.3.1 Immunisation of animals with a greater dose of rAd vectors is protective against lethal PVM infection

BALB/c mice between 5 and 7 weeks of age were immunised intranasally using the standard prime-boost regime (Fig. 4.2). For each recombinant Ad construct investigated, the animals were separated into groups and immunised as detailed in Table 5.3.

Group	Vaccine construct	Prime dose (p.f.u./50µl)	Boost dose (p.f.u./50µl)	Animals/group
A	rAdF	10^8	10^6	5 ♀
B	rAdM	10^8	10^8	5 ♀
C	rAdN	10^8	10^8	5 ♀
D	rAdZ	10^8	10^8	4 ♂
E	N/A	PBS	PBS	2 ♀

Table 5.3. The rAd immunisation protocol using the 10^8 p.f.u. dose.

Throughout the immunisation regime and prior to PVM challenge, no weight loss or clinical signs were observed for any animals vaccinated with rAdM, rAdN, rAdZ or treated with PBS. However, those immunised with the greater dose of the rAdF construct (group A) did present with ruffled fur and substantial weight loss post prime dose. These animals did not recover but due to these signs it was considered appropriate to reduce the boost dose for this group to 10^6 p.f.u. Upon boost with the lower dose, no vaccine related signs were observed. All animals were challenged with a lethal dose of PVM on day 31 and were monitored throughout the challenge period (Fig. 5.5). Animals treated with PBS served as a control group to monitor PVM pathogenesis.

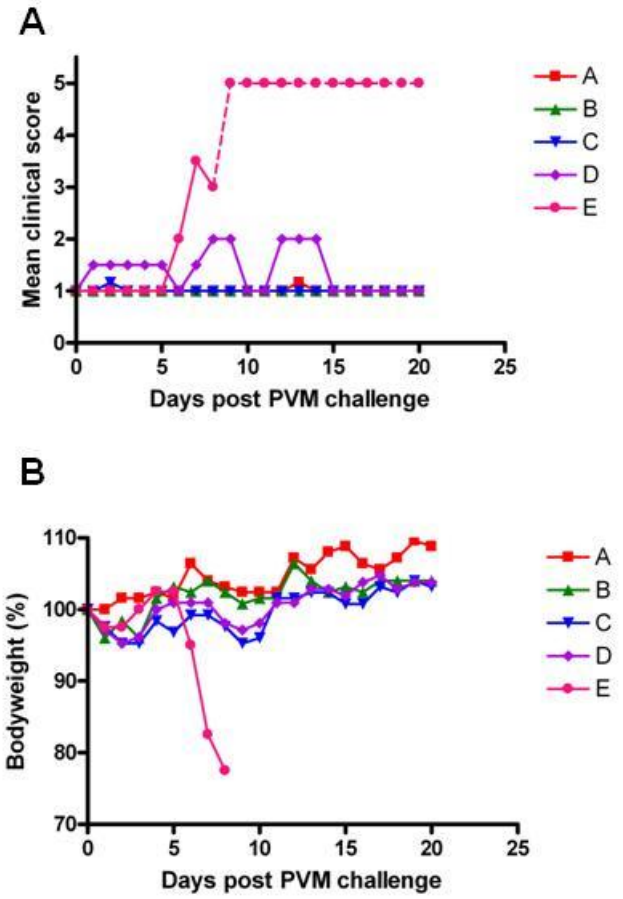

Figure. 5.5. Immunisation with rAd constructs confers protection against lethal PVM infection.
BALB/c animals were immunised as described in Table 5.3 using the standard prime-boost regime (Fig. 4.2). Animals were primed with a 10^8 p.f.u. dose of either rAdF (group A), rAdM (group B), rAdN (group C), rAdZ (group D) or PBS (group E). Animals were boosted on day 14 with the same vaccine dose and construct as received for the priming dose, apart from group A (rAdF) which received a lower dose of 10^6 p.f.u. The animals were challenged with a lethal dose of PVM strain J3666 (250 p.f.u. in 50µl) on day 31 of the experiment. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%).

Groups A, B and C (immunised with rAdF, rAdM, and rAdN respectively) did not present with weight loss or clinical signs following PVM challenge, all gaining weight to above their pre-challenge level. Group C presented with slight transient weight loss between days 7 and 10, which was not reflected in their clinical score.

These animals quickly recovered and gained weight above the pre-challenge level. Therefore, PVM rAd-immunised animals were protected from PVM challenge.

Unexpectedly, group D (rAdZ-immunised animals), were also protected against lethal PVM challenge. This group presented with mild clinical signs which increased to a peak score of 2 (Fig. 5.5A) and slight weight loss between days 7 and 10. All animals survived and recovered from PVM infection as demonstrated by a return to baseline levels of clinical score and weight gain (Fig. 5.5B). In contrast, control group E (treated with PBS) presented with clinical signs and weight loss from day 6, which increased in severity resulting in the deaths of all the animals on day 9. This result, for control group E, was similar to previous experiments with PBS treated animals. These data demonstrated that the apparent protection from PVM caused by rAdZ-immunisation was real, and not an artefact of lack of potency in the challenge dose used. Therefore, it appeared that animals immunised with rAd constructs at a high dose of 10^8 p.f.u., were protected against lethal PVM infection.

5.3.2 Immunisation of animals with a high prime dose and a low boost dose of rAd recombinant viruses is protective against lethal PVM infection

As hypothesised, animals immunised with the high, 10^8 p.f.u., dose of the PVM rAd constructs were protected from lethal PVM infection and presented with reduced signs and weight loss. Unexpectedly, protection towards a lethal PVM infection was also demonstrated in rAdZ-immunised animals, only when the high dose was used. This effect cannot be attributed to a change in PVM pathogenicity as previous experiments (Section 4.2), detailed that the 250 p.f.u. challenge dose of PVM is fully lethal in PBS control mice. In addition, all experiments described previously have included a PBS treated group to monitor PVM pathogenesis, and in all experiments, animals in this group have succumbed to PVM infection.

To investigate the unexpected protection observed with 10^8 p.f.u. rAdZ-immunised animals, the immunisation protocol was altered. BALB/c mice were immunised with a high dose of 10^8 p.f.u. of either rAdN (group A) or rAdZ (group B) or treated with PBS, as a control for PVM pathogenesis. Animals were boosted on day 14 with a low dose of 10^6 p.f.u. of the same construct and, prior to challenge on day 28 with a lethal dose of PVM and monitored for clinical signs and weight loss (Fig. 5.6).

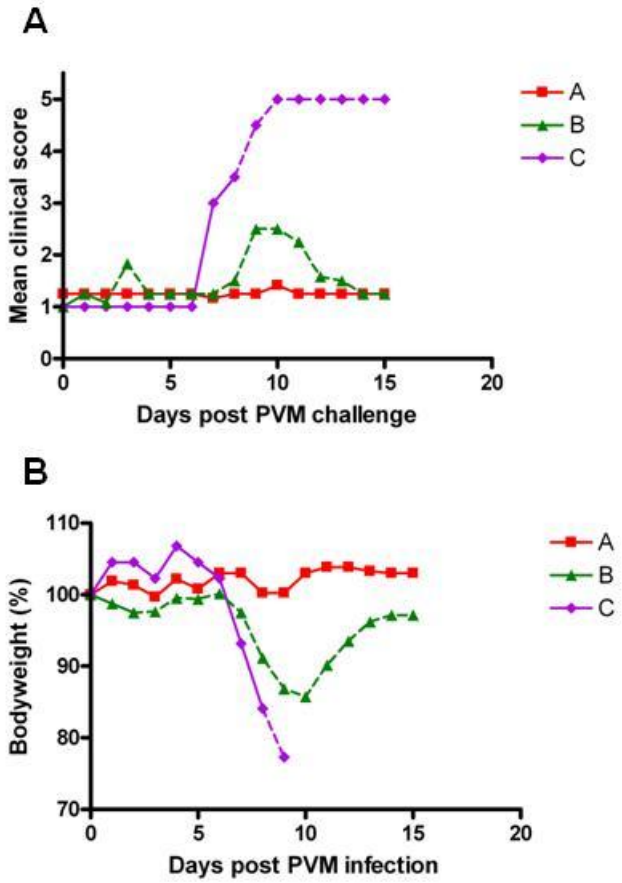

Figure. 5.6. A 10^8-10^6 p.f.u. prime-boost immunisation regime with an rAd construct confers protection against a lethal PVM infection.
BALB/c mice aged between 5 and 7 weeks of age were immunised with the standard prime-boost regime. However, a 10^8 p.f.u. prime dose and a 10^6 p.f.u. boost dose were used to immunise mice with either rAdN (group A) or rAdZ (group B) or treated with PBS (group C). Animals were challenged with a lethal dose of PVM strain J3666 on day 28. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs.

Group A (rAdN-immunised animals) did not present with clinical signs (Fig. 5.6A) or weight loss (Fig. 5.6B) following PVM challenge and thus were protected from lethal PVM infection. Group B (rAdZ-immunised animals) did present with mild clinical signs and moderate weight loss following PVM challenge. Clinical signs appeared from day 7 onwards (Fig. 5.6A), reaching a peak score of 2.5 on day 9 before decreasing to baseline levels by day 14. Weight loss was observed from day 7

onwards, reaching a maximum of 14% on day 10 (Fig. 5.6B), after which the animals gained weight and recovered from PVM infection. This was in contrast to the solid protection achieved with a 10^8 p.f.u. prime-boost regime with rAdZ (Fig. 5.5). One fatality was observed on day three and was attributed to anaesthesia complications. As expected, control group C (PBS treated) did not survive lethal PVM infection, presenting with severe clinical signs (Fig. 5.6A) and significant weight loss (Fig. 5.6B).

5.3.3 Discussion

As expected, rAd PVM constructs protected animals against lethal PVM infection with minimal signs. Unexpectedly, rAdZ-immunised animals were also protected, but only when immunised with the high dose, 10^8 p.f.u. To further investigate the threshold of protection elicited by the rAdZ construct, the prime-boost regime was altered so that animals were primed with a high, 10^8 p.f.u. dose and boosted with a low 10^6 p.f.u. dose of the construct. Protection was still achieved with the rAdZ construct using this protocol. However, a lower boost dose resulted in mild clinical signs and moderate weight loss (Fig. 5.6), in contrast to the absence of weight loss upon immunisation with a high boost dose (Fig. 5.5) in this rAdZ-immunised group. These data suggest that the protection against PVM achieved at the high dose is dose dependent and therefore is likely to be conferred by the rAd vector as opposed to the transgene.

5.4 Investigation into the immunogenicity of the 10^8 p.f.u. rAd construct dose

The protection conferred by the rAd constructs against PVM was observed in a short-term challenge experiment as previously described (Fig. 5.5). To determine the threshold of this protective effect further, animals were immunised using a prime-boost regime with a high vaccine dose and challenged with a lethal PVM dose after increasing time intervals in a long-term experiment. In addition, animals were immunised with a single high vaccine dose prior to PVM challenge.

5.4.1 rAdZ elicited protection against PVM is long-lasting

BALB/c mice between 5 and 7 weeks of age were immunised using a prime-boost regime (Section 4, Fig. 4.10). The animals were immunised with the high dose of 10^8 p.f.u. of either rAdN (group A) or rAdZ (group B) and were challenged at 8 and 11 weeks post prime immunisation with a lethal dose of PVM strain J3666. After 8 weeks, group A (rAdN-immunised animals) were fully protected against lethal PVM infection. No clinical signs or weight loss were observed in this group following PVM infection. Animals in group B (rAdZ-immunised) were also protected from lethal PVM challenge. The animals did present with mild clinical signs from days 9 to 13, but the peak clinical score did not increase above 2 (Fig. 5.7A). Slight weight loss was demonstrated from day 6 onwards, reaching a maximum of 8% on day 10 (Fig. 5.7B), after which the animals gained weight and recovered from PVM infection. In contrast PBS treated animals (group C) showed signs and weight loss characteristic of lethal PVM infection and were thus not protected against lethal PVM infection.

Even after 11 weeks from priming immunisation, both groups A and B (rAdN and rAdZ-immunised animals respectively) only developed mild clinical signs between days 7 and 12 following PVM challenge (Fig 5.8A). No weight loss was observed over the challenge period, and both groups maintained a constant bodyweight around the pre-challenge level (Fig. 5.8B). As before, group C (PBS treated) animals were not protected against PVM. Thus, when compared to control group C, both rAdN (group A) and rAdZ (group B) immunised animals were protected against lethal PVM infection for up to 11 weeks post initial immunisation.

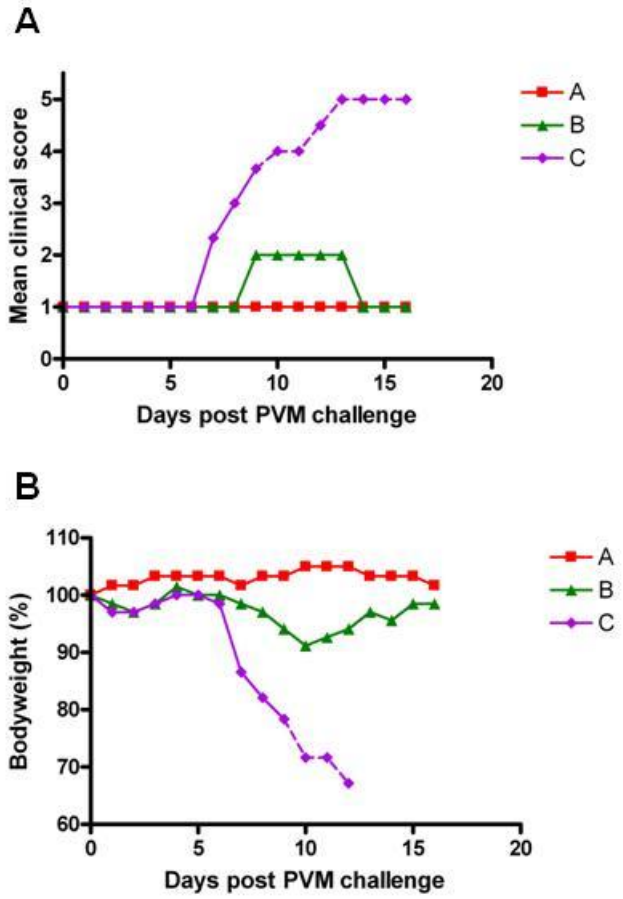

Figure. 5.7. rAd recombinant viruses can confer protection against lethal PVM infection 8 weeks after priming immunisation.
Female BALB/c mice between 5 and 7 weeks of age were vaccinated and challenged as described in Section 4, Fig. 4.10. Animals were immunised with a prime dose of 10^8 p.f.u. of either rAdN (group A) or rAdZ (group B) or treated with PBS on day 1. The animals were boosted with the same vaccine construct and dose on day 14. Animals were challenged at 8 weeks after initial immunisation with a lethal dose of PM strain J3666 (250 p.f.u. in 50µl). Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Upon a death of an animal in a group, the clinical score and bodyweight loss continued to be monitored, but are illustrated thereafter by dashed lines on the graphs.

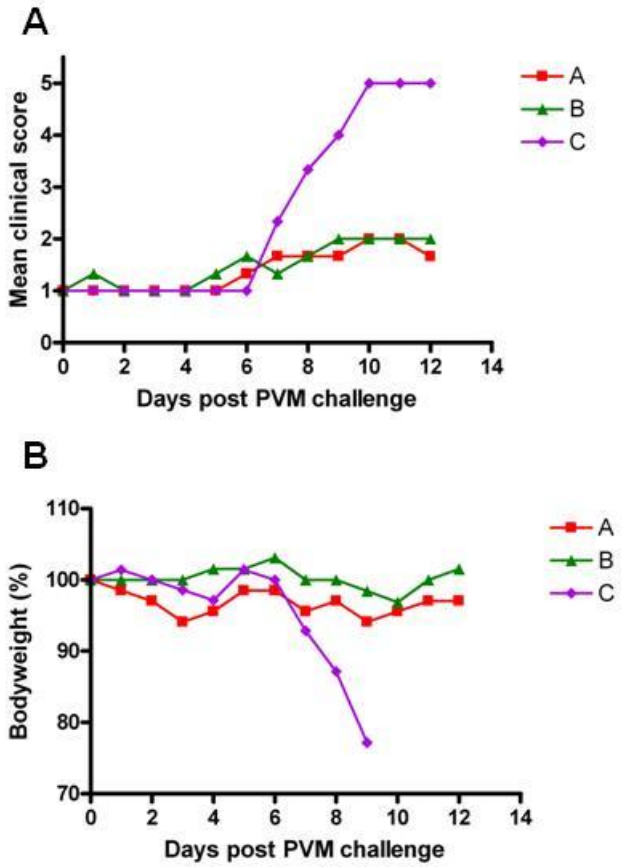

Figure. 5.8. rAd vaccines can confer protection against lethal PVM infection up to 11 weeks post prime dose.
Female BALB/c mice were vaccinated and challenged as described in Fig. 5.7. Animals were challenged at 11 weeks after initial immunisation with a lethal dose of PVM strain J3666. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%).

5.4.2 A single high dose of rAd vaccine confers protection against PVM

To determine the immunogenicity the 10^8 p.f.u. dose, groups of BALB/c mice between 5 and 7 weeks of age were immunised with a single, high dose of 10^8 p.f.u. of either rAdM (group A), rAdN (group B) or rAdZ (group C). PBS treated mice (group D) were included as a control for PVM pathogenesis. The animals were challenged with a lethal dose of PVM six weeks post immunisation and monitored for weight loss and signs as before (Fig. 5.9).

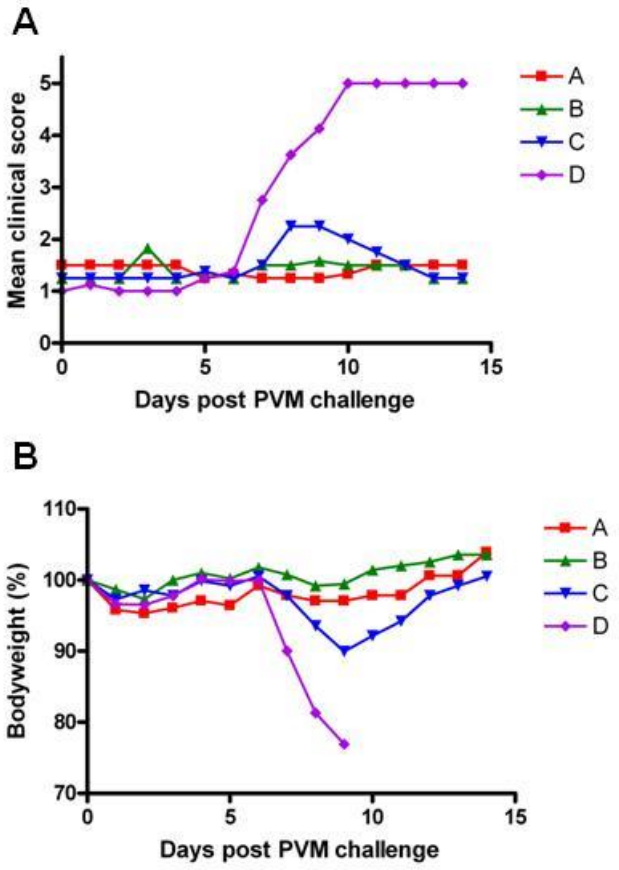

Figure. 5.9. Immunisation with a single dose of an rAd construct can confer protection against lethal PVM infection.
Groups of 6 (three male and three female) BALB/c mice aged between 5 and 7 weeks were immunised with a single dose of rAdM (group A), rAdN (group B), or rAdZ (group C) at 10^8 p.f.u. As a control, a group of two male and two female BALB/c mice was treated with PBS (group D). Six weeks post immunisation, the animals were challenged with a lethal dose of PVM strain J3666 (250 p.f.u. in 50µl). Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). One animal in group B was culled on day 4 due to immunisation complications and was not included in the final analysis.

Groups A and B (rAdM and rAdN-immunised, respectively) did not present with clinical signs or weight loss (Fig. 5.9), whereas group C presented with mild clinical signs (Fig. 5.9A), and slight weight loss (Fig. 5.9B) between days 7 and 12; however the animals recovered and gained weight. In contrast, group D (PBS treated) animals developed severe clinical signs and bodyweight loss (Fig. 5.9), coinciding with three

deaths on day 9 and the remainder on day 10. Therefore, animals were protected against lethal PVM infection after a single 10^8 p.f.u. immunisation dose of the rAd constructs.

5.4.2.1 Discussion

The protective effect of the rAd vectors was investigated in experiments where PVM challenge was delayed. First, rAd-immunised animals were challenged with PVM at 8 and 11 weeks post priming dose. The data in Section 4.5 demonstrated that the rAdM and rAdN constructs could elicit protection in animals immunised with 10^7 p.f.u. in a prime-boost regime for up to 20 weeks (Section 4, Figs. 4.11, 4.12). In contrast, rAdZ-immunised animals were not protected, even up to 8 weeks, using this regime (Section 4, Fig. 4.13). The data presented in this section demonstrates that rAd-immunised mice with a 10^8 p.f.u. prime-boost regime are protected against lethal PVM infection for up to 11 weeks. This result is not unexpected for rAdN-immunised animals, as protection was achieved in this manner with the 10^7 p.f.u. dose. However, rAdZ-immunised animals are protected for up to 11 weeks following immunisation with the high dose 10^8 p.f.u. only (Fig. 4.13, 5.7, 5.8). In addition, animals immunised with a single 10^8 p.f.u. dose of the rAd constructs were protected against lethal PVM infection. rAdZ-immunised animals did present with mild clinical signs and weight loss, which distinguished them from animals similarly immunised with rAdN, but they were ultimately protected against PVM infection.

These data confirm that the protective effect elicited by the rAd constructs is mediated by the adaptive immune response. The protective effect observed is not due to a stimulation of an innate, anti-viral immune response, as such a response would not be maintained for 11 weeks. Neither would the innate response be maintained for 6 weeks after a single immunisation dose. These data also indicate that the immunogenic effect of the high dose is strong, as a single dose can confer protection against a lethal PVM infection for 6 weeks and a double dose for up to 11 weeks.

5.5 Investigation into the immunogenicity of the rAdZ recombinant virus

Immunisation of mice with a 10^8 p.f.u. dose of rAdZ construct generated a protective response demonstrated up to six weeks post immunisation with a single dose and up to 11 weeks post immunisation with a prime-boost regime. The experiments described in Sections 5.3 and 5.4 do not elucidate whether the immunogen mediating the protective effect was the rAd vector or the *Lac*Z transgene. To investigate this further, BALB/c animals were immunised with additional rAd constructs. The rAd recombinant viruses were also investigated for their ability to confer protection against an unrelated respiratory virus, to determine whether the effect was virus-specific.

5.5.1 Immunisation with rAdEV and rAdGFP constructs confers protection against PVM with a high dose only

BALB/c mice between 5 and 7 weeks of age were immunised via the intranasal route with the high (10^8 p.f.u.) and middle (10^7 p.f.u.) doses of two additional rAd constructs. The first construct was generated using the AdEasy™ protocol (Section 3) and did not contain a transgene in the multiple cloning site of the vector and was known as rAdEV (Empty Vector). The other construct, rAdGFP, was kindly provided by Mr S. Martin. This construct was generated by an alternative method but the vaccine vector was of the Ad5 serotype and contained deletions within the E1 and E3 regions of the Ad genome broadly equivalent to those found in AdEasy-derived vectors. Thus, the construct was replication deficient as per the recombinant viruses generated in this study. rAdGFP contained green fluorescent protein gene (GFP), expressed under the control of the CMV immediate-early promoter. GFP expressed from rAdGFP therefore acts as an additional irrelevant protein to elucidate whether the LacZ protein or the rAd vector is the cause of the non-specific protection observed when using rAdZ as immunogen.

Animals were separated into five groups and were immunised as detailed in Table 5.4 using the standard prime-boost regime (Fig. 4.2). Throughout the immunisation regime and prior to PVM challenge, no weight loss or clinical signs were observed for any animals vaccinated with either the rAdEV or rAdGFP constructs.

Group	Vaccine construct	Prime dose (p.f.u./50µl)	Boost dose (p.f.u./50µl)	Animals/group (♂:♀)
A	rAdEV	10^7	10^7	5 (3:2)
B	rAdEV	10^8	10^8	5 (3:2)
C*	rAdGFP	10^7	10^7	5 (♂)
D*	rAdGFP	10^8	10^8	5 (♀)
E	N/A	PBS	PBS	2 (♀)

Table 5.4. The standard immunisation dose for vaccination with additional rAd constructs.
*Experiment performed by Mr S. Martin

Following PVM challenge, group A and B animals (immunised with rAdEV at the 10^7 and 10^8 p.f.u. doses, respectively) did not present with clinical signs or weight loss associated with PVM challenge (Fig. 5.10). Animals immunised with rAdGFP at the 10^8 p.f.u. dose (group D) presented with mild clinical signs, reaching a peak score of 2.5 on day 9 (Fig. 5.10A) and remaining constant at an average score of 2 for the duration of the experiment. Mild weight loss was observed from day 7 onwards, reaching a maximum of 5% on day 9 (Fig. 5.10B). Thereafter, the animals gained weight and recovered from PVM infection. In contrast, animals immunised with rAdGFP at 10^7 p.f.u. (group C) presented with severe clinical signs and weight loss that began on day 6 and reached the maximum of 27% on day 10 (Fig. 5.10B), which coincided with the deaths of all the animals. This pathogenesis was equivalent to that seen in the control group E animals (PBS treated). Thus, rAdGFP protected animals from lethal PVM challenge only at the high (10^8 p.f.u.) dose, similar to rAdZ.

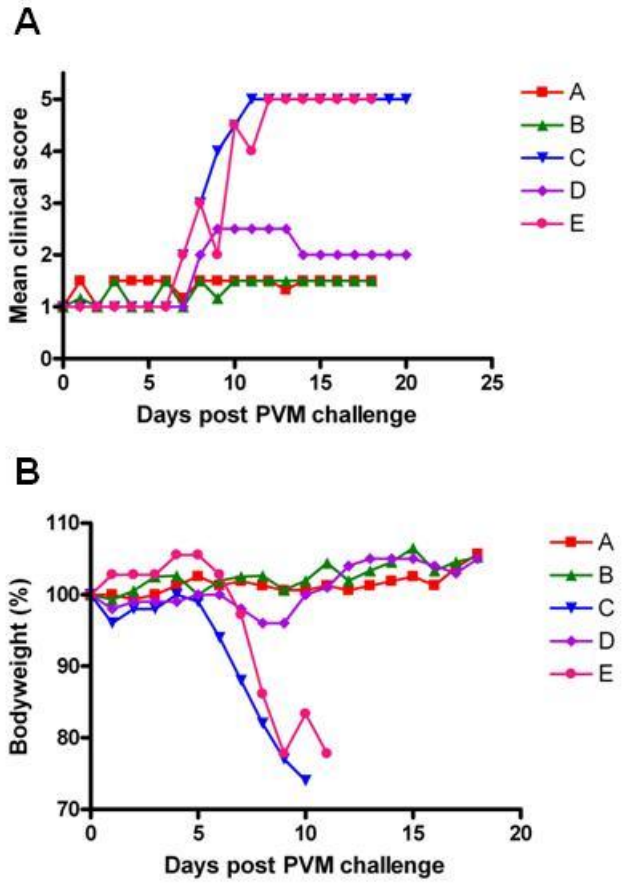

Figure. 5.10. rAdEV and rAdGFP can stimulate a protective immune response against lethal PVM challenge.
BALB/c mice were immunised on day 1 with rAdEV at 10^7 p.f.u. (group A) or 10^8 p.f.u. (group B) or PBS (group E). The animals were boosted on day 14 with the same vaccine construct and dose prior to challenge on day 28 with a lethal dose of PVM strain J3666. Animals from a different batch were vaccinated with rAdGFP at the 10^7 p.f.u. (group C) or 10^8 p.f.u. (group D) dose in a different experiment conducted by S. Martin; the animals were boosted and challenged as described for the rAdEV construct. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of PVM challenge (100%). Groups A and B immunised male animals showed signs of ruffled fur due to aggressive behaviour, therefore the average baseline clinical score for these groups was 1.5.

The equivalent protection from lethal PVM infection elicited by the high (10^8 p.f.u.) dose of rAdEV, rAdGFP and rAdZ, indicates that protection at this dose is attributed to the rAd vector, not a specific transgene. This is because it is highly improbable that two unrelated, irrelevant proteins, GFP and β-galactosidase, could confer a

protective response against PVM. As expected, animals immunised with the 10^7 p.f.u. dose of rAdGFP were not protected against lethal PVM infection; a result similar to that of rAdZ-immunised animals. Surprisingly, rAdEV immunised animals were also protected from PVM infection at this dose. This result may have occurred because the rAdEV preparation was later found to contain a low level of a replication-competent virus. This may have allowed the virus to replicate and thus, increase the dose of antigen received by the animals. Other rAds lacking a PVM transgene failed to protect immunised animals at this dose, which is in contrast to the rAd PVM constructs, which are protective at the 10^7 p.f.u. dose.

5.5.2 Immunisation of C3H/He-mg mice with rAd constructs does not elicit protection against influenza virus

The standard influenza A pathogenesis model in mice uses animals of strain C3H/He-mg (Morgan et al., 1993). Mice between 5 and 7 weeks of age were immunised with a 10^8 p.f.u. dose of either rAdEV or rAdZ vaccines via the intranasal route. The standard prime-boost regime was used (Fig. 4.2). As before, no weight loss or clinical signs were observed associated with the vaccination. On day 28, the animals were challenged with a previously determined lethal dose of influenza A/WSN virus (Section 2, Table 2.1.3). This lethal dose was equivalent to that used for PVM when analysed in a dose dependent manner (P.D. Scott, personal communication). Clinical signs of influenza illness are similar to that of PVM (Dimmock et al., 2008). Therefore, the same clinical score system was used to evaluate the animals, together with bodyweight loss, for the duration of the experimental period.

Groups A and B animals (rAdEV and rAdZ, respectively) developed a similar pathogenesis to control group C (PBS treated). They developed clinical signs from day 4 onwards reaching the peak score of 4.5 on day 7 (Fig 5.11A). Weight loss was evident from day 2/3 onwards, reaching a maximum of 28% for group A on day 7 and 27% for group B on day 6 (Fig. 5.11B). This coincided with the deaths of 83% of the animals in the groups. The pathogenesis seen in groups A and B was equivalent to that in PBS treated control mice (group C). As there was no protective difference between the rAd-immunised mice, groups A and B, and the control

animals (group C), the experiment was terminated on day 7 and it was concluded that the rAd vectors did not stimulate a protective response to an unrelated respiratory viruses. Thus, the protection against PVM elicited by recombinant Ad not expressing any PVM transgene is not a generalised protection, but is virus-specific to PVM.

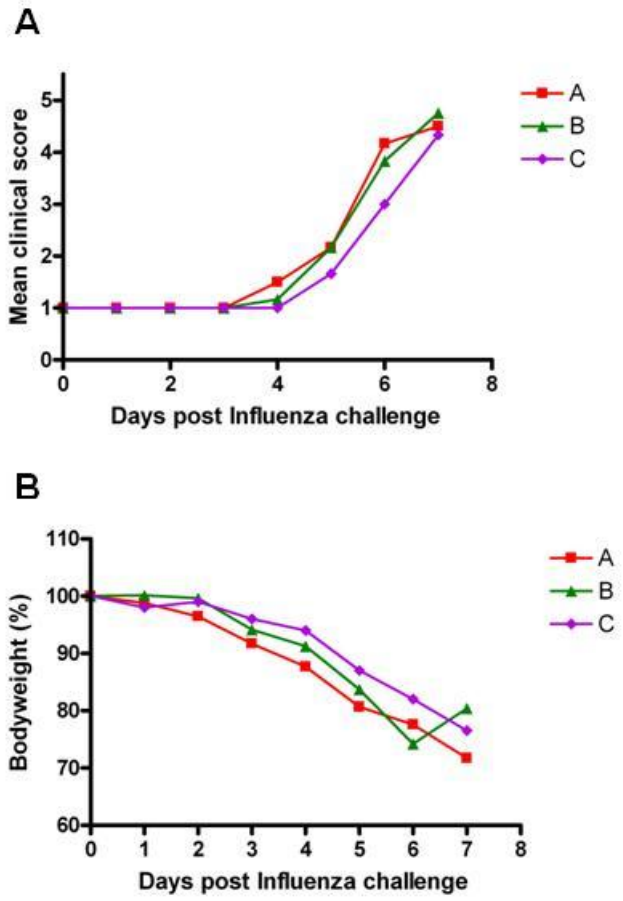

Figure. 5.11. rAd vaccine constructs do not protect C3H/He-mg mice from lethal influenza challenge.
C3H/He-mg mice from one batch of animals were vaccinated with a prime dose on day 1 of either 10^8 p.f.u. of rAdEV (group A) or rAdZ (group B) or PBS (group C). Groups A and B consisted of six animals, three males and three females whereas group C consisted of three males. The animals were boosted on day 14 with the same immunogen and dose prior to challenge with a lethal dose of $2.25x10^4$ p.f.u. in a 50µl volume of influenza A/WSN. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal and normalised to the weight on day 0 of influenza virus challenge (100%).

5.6 Discussion

In the previous chapter, rAd PVM vaccines were shown to elicit a protective immune response against a lethal PVM infection, that was long-lasting. In this chapter, the protective effect was investigated further through the evaluation of different immunisation routes and through an alternative dose.

While intranasal immunisation with rAd PVM constructs at the 10^7 p.f.u. dose was able to generate protection against a lethal PVM infection, immunisation via the subcutaneous route with either a 10^6, 10^7 or 10^8 p.f.u. dose was not associated with protection against PVM. Intraperitoneal immunisation also did not confer protection, and in addition, was associated with severe vaccine-related side effects. These side effects included severe liver pathology and gut related signs and have been observed before, even with replication-deficient Ad5 vectors (Vogels et al., 2003). Even if the vector is replication-deficient it will, when introduced into the peritoneal cavity, enter liver cells and stimulate a strong immune response which, results in clearance of the virus and consequent liver pathology. This might be circumvented by the use of alternative Ad serotypes as vaccine vectors. Interestingly, neither the subcutaneous nor the intraperitoneal immunisation route stimulated a protective immune response. This maybe because the immunisation dose was not sufficient to stimulate a response towards the PVM virus of sufficient magnitude to be protective. Alternatively, these results suggest that a systemic induced immune response may not be able to protect animals against a lethal PVM infection, perhaps indicating that a mucosal driven immune response is more effective at providing protection against this virus.

The focus of this chapter was an investigation of a high dose, 10^8 p.f.u. of the rAd vaccines and the effects such a dose elicited. As expected, the rAd PVM constructs were able to protect 10^8 p.f.u. immunised animals from a lethal PVM infection, given that they were able to achieve this with the lower 10^7 p.f.u. dose. As hypothesised, the increased dose did lead to a reduction in the clinical signs and weight loss observed upon PVM challenge, when compared to an equivalent experiment using a 10^7 p.f.u. dose. However, unexpectedly, the animals immunised with the 10^8 p.f.u. dose of rAdZ were also protected against PVM infection. This was not due to an altered pathogenicity of the PVM stock used for challenge as PBS control animals

progressed to a lethal infection as observed in previous experiments. Therefore, it was considered that the high dose may deliver a greater amount of antigen which was able to stimulate a transient short-term non-specific anti-viral state within the animal. To determine whether the protective effect was induced by the adenovirus vector or the *Lac*Z transgene, alternative vaccine constructs were used to immunise animals. One did not contain a transgene, rAdEV, and one contained an alternative irrelevant transgene, of GFP, rAdGFP. The data kindly provided by Mr S. Martin of rAdGFP-immunised animals, coupled with the data from rAdEV-immunised animals with the high vaccine dose (Fig. 5.10), demonstrated that both of the vaccines could elicit the protective effect against PVM challenge. Therefore, it was concluded that the rAd5 vector was the immunogen responsible for the specific protection against PVM virus. A similar finding has been reported previously by a different group (See et al., 2008). They discovered that an empty rAd5 replication-deficient construct, used to immunise ferrets by the intranasal route with a 10^9 p.f.u. dose, was able to confer a specific protective effect against SARS virus. They attributed this result to an altered lung mucosal morphology upon rAd vaccination. It was also suggested that the down-regulation of the CAR receptor, or PVM receptor, or an additional co-receptor, may confer an immunogenic advantage. However, such down-regulation would be unlikely to be maintained for a long period of time such as observed in the 11-week challenge experiment (Fig. 5.8). An alternative explanation is that a serum component common to the rAd and PVM virus stocks may stimulate a cross-protective immune response. However, this is deemed unlikely as both the rAd and PVM viruses were isolated by different methods and significantly diluted to obtain the immunisation and challenge doses, and in the case of the immunising rAd preparations, was highly purified prior to use.

The long-term experiments (Figs. 5.7, 5.8) where animals were immunised with a prime-boost regime of 10^8 p.f.u. of rAdN and rAdZ showed protection from PVM infection, persisting at least 11 weeks post-immunisation with rAdZ. In addition, a single 10^8 p.f.u. dose of the vaccines was able to confer protection for six weeks post-immunisation (Fig. 5.9). These data most likely suggest that an adaptive immune response may confer the protective effect elicited by the rAd vector. It is known that upon immunisation, cross reactive T-cells can be stimulated (Hutnick, 2010). It is possible that a cross-reactive T-cell population between rAd and PVM

was activated by rAd, and upon a booster immunisation dose, stimulated further. Thus, following challenge with PVM, this population would confer specific protection against the virus.

Additionally, evidence supporting an adaptive response for conferring the protective effect is illustrated in Appendix C. Fig. C.2. These data demonstrated that a 10^8 p.f.u. dose of rAdZ in a prime-boost regime was immunogenic enough to provide protection against a super lethal dose of PVM (500 p.f.u of PVM strain J3666). This was performed to ensure that any protective effect observed was robust as opposed to innate immunity simply attenuating the lethality of the PVM challenge dose, effectively allowing the animal an opportunity to generate a natural adaptive response towards PVM whilst viral replication was slowed. This further suggests that an innate immune response is unlikely to be mediating the protective effect.

However, the innate response may still be stimulated by the presence of the rAd vector. As vector persistence was not investigated in this study, it is unknown how long the rAd vector remains functional within the mouse lung. It is possible that the vector may persist within the mouse lung, providing recurrent stimulation of the innate immune system, perhaps through leaky gene expression (Imperiale et al., 1984, Spergel et al., 1992). This would lead to the possibility of an anti-viral state being induced, even up to 11 weeks post immunisation. Such a response may be demonstrated in Fig. 4.13C, where some rAdZ-immunised mice survived PVM challenge up to 20 weeks post immunisation. It is known with persistent human viruses, such as herpes simplex virus (HSV-1), that viral replication is inhibited upon infection by adaptive responses (Khanna et al., 2004). However, when the virus emerges from latency, it is the IFN response from both innate and adaptive sources which inhibit viral replication and prevent a lytic infection cycle. Therefore, leaky long-term expression of Ad proteins from the vector could stimulate innate responses in a cyclical manner, leading to the establishment of an anti-viral state which may have translated into apparent protection from PVM challenge by the rAd vectors as observed in Figs. 5.7-5.9. Furthermore, to investigate whether this protection was virus specific, animals were immunised with rAdZ and challenged with another respiratory virus, influenza. These animals were not protected against lethal influenza challenge (Fig. 5.11), thus, it can be concluded that the immune response

generated is specific against the PVM virus, or that adaptive responses are more important for control of influenza infection in mice.

In conclusion, although the results presented in this chapter suggest that an adaptive response is more likely to confer the protective effect observed upon high immunisation doses of the rAd vector, it does not discount the possibility of innate immune responses providing a protective role. As such, the cause of the specific protective effect towards PVM mediated by the rAd vector is as yet unidentified.

Chapter 6

Investigation into the immune responses generated by rAd construct immunisation

6.1 Introduction

The three rAd PVM constructs, rAdF, rAdM and rAdN described previously, were able to protect intranasally immunised mice from PVM infection. However, the experiments did not elucidate the mechanism behind the protective effect. As discussed in Section 1.6.2, the adaptive immune system involves the coordinated effects of both the humoral and cellular immune responses towards a pathogen. Anti-viral immunity requires a strong humoral or a strong cellular response to be effective at resolving the infection (Belyakov & Ahlers, 2009). The work described in this chapter attempts to identify whether the humoral and/or cellular response was involved in the protective effect observed upon PVM rAd-immunised BALB/c mice.

6.1.1 ELISA optimisation

One of the most effective methods to characterise the humoral response towards an antigen is through the use of an Enzyme-Linked Immunosorbent Assay (ELISA). An ELISA, appropriately configured, can determine the quantity of an unknown amount of either antigen or antibody in a sample. The assay used in this study was an indirect ELISA, which involved the addition of antigen into a microtitre plate to which the antibody in the serum samples can bind.

Two ELISA assays were developed, one to investigate the anti-PVM response and another to investigate the anti-Ad5 (vector) response. The anti-Ad5 ELISA had been previously optimised with UV inactivated Ad327 particles as antigen at a concentration of 1µg/ml (Flanagan et al., 1997). The anti-PVM ELISA utilised lysate from the persistently PVM infected P2-2 cell line as the PVM antigen. Because the P2-2 cell line is derived from BS-C-1 cells, this preparation is not of pure PVM antigen, but a mixture of PVM viral antigen and BS-C-1 cellular antigen. Therefore, BS-C-1 lysate, prepared in the same manner, was included in all ELISA experiments as a background antigen control. The assay values obtained for the BS-C-1 control lysate could then be used as the baseline level for the assay. Using serum from PVM immunised animals, the optimum concentration of P2-2 lysate was found to be 1mg/ml (App. C. Fig. C.3). To assess the sensitivity of the anti-PVM ELISA, serum

from animals that had recovered from a non-lethal dose of PVM was used to compare UV-inactivated PVM particles and P2-2 lysate as antigen in the assay (Fig. 6.1). The serum titre was found to be consistent between the P2-2 lysate and the UV inactivated particles. As PVM virus was difficult to bulk up in sufficient quantities for the number of ELISAs required, in this study lysate from the P2-2 cell line was deemed an appropriate substitute.

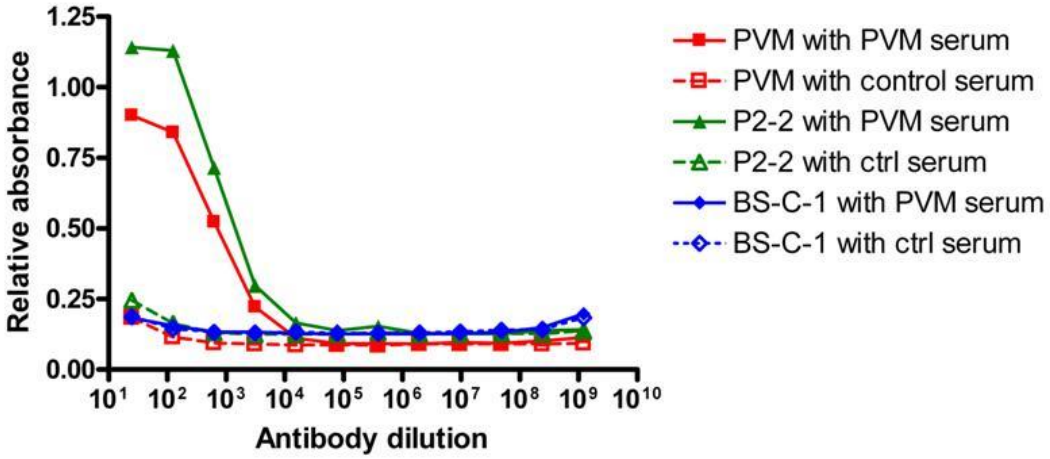

Figure 6.1. Anti-PVM ELISA to determine the sensitivity with different PVM antigens.
The PVM ELISA was performed as described in Section 2.9.11 using serum from animals immunised with a non lethal dose of 20 p.f.u. of PVM J3666. The serum was titrated against P2-2 lysate and UV inactivated PVM virus to compare the sensitivity of the two preparations.

Subsequently, to confirm that P2-2 lysate contained sufficient antigen to allow the detection of PVM-specific antibodies, polyclonal antibodies directed against the PVM F and N proteins (Section 2, Table 2.1.7) were evaluated using the anti-PVM ELISA (Fig. 6.2). These antibodies were readily detected in the assay, thus it was concluded that the P2-2 cell lysate was suitable for analysing serum samples from all rAd PVM immunised animals for the presence of such antibodies. In addition, the ELISA was shown to be specific for PVM antibodies only, as no signal was detected using control serum from influenza-immunised animals (App. C. Fig. C.4).

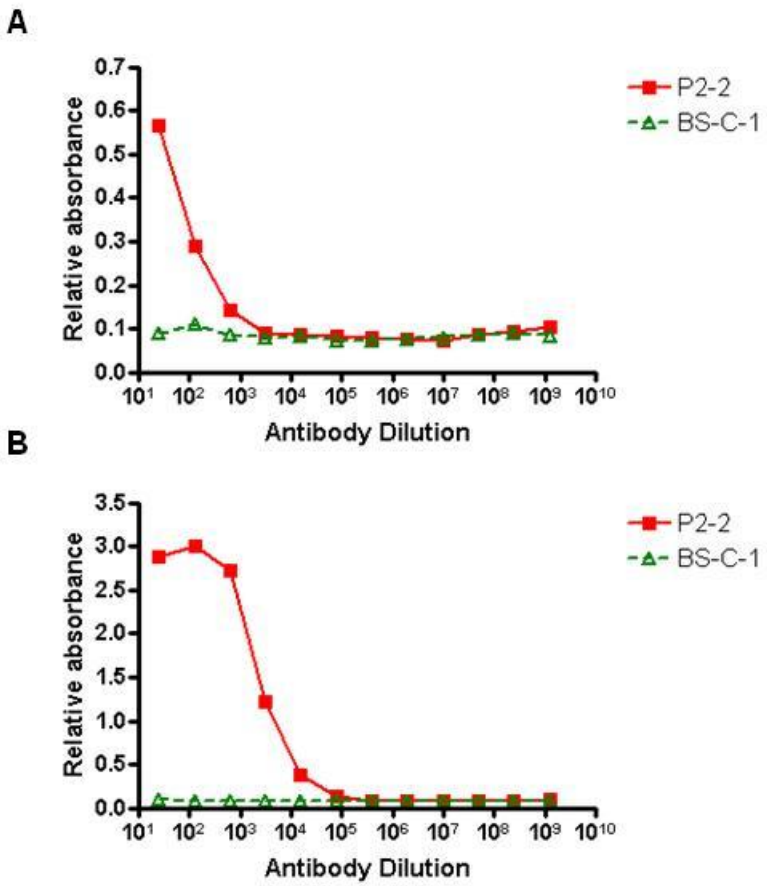

Figure 6.2. PVM protein monoclonal antibodies can be detected within the PVM ELISA.
The PVM ELISA was performed as described in Section 2.9.1.1, using the anti-PVM F polyclonal (clone 2018 Table 2.1.7) (A) and the anti-PVM N polyclonal (Table 2.1.7) (B) as the serum samples. The antibodies were used from a neat preparation and were successfully titrated within the assay, confirming specific responses to particular PVM proteins could be detected. The data are representative of two experimental repeats.

6.1.2 Detection of a PVM-specific response

To generate a bench mark for PVM antibody titres, four animals were inoculated with 20 p.f.u. of PVM strain J3666. Sera was collected at 14 days post infection and analysed using a PVM ELISA assay. The end point dilution was then calculated, where the response observed to P2-2 antigen declined to equal that observed in the BS-C-1 antigen control. The result from one such animal is detailed in Fig. 6.3, which describes how the end point dilution for a serum sample is calculated.

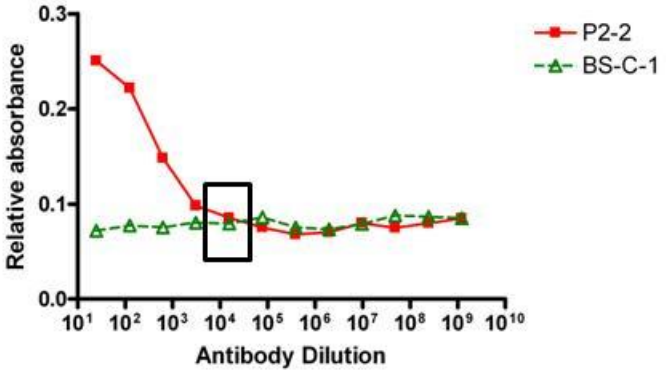

Figure 6.3. Determining the end point dilution for a serum sample.
A serum sample from an animal immunised with a non lethal dose of 20 p.f.u. of PVM strain J3666 was titrated by ELISA against P2-2 and BS-C-1 cell lysate antigen. The last data point against P2-2 antigen which remains above the BS-C-1 background control (black box), was taken as the end point dilution of the serum sample. The relative absorbance values for every dilution end point were averaged for PBS-treated animals. These values are experimental background in the ELISA assay, as they represent a non-specific response generated from serum components, towards the antigens used. Once calculated, the experimental background was removed from the values obtained from PVM infected or rAd-immunised animals for each dilution point. As such, they were normalised for experimental background.

The data for the animals was combined and demonstrated that all four animals produced the same anti-PVM antibody titre in response to mild PVM infection. This result represents an optimum response to PVM, producing a $\log_{10}$ end point titre of 3.5 (Fig. 6.4).

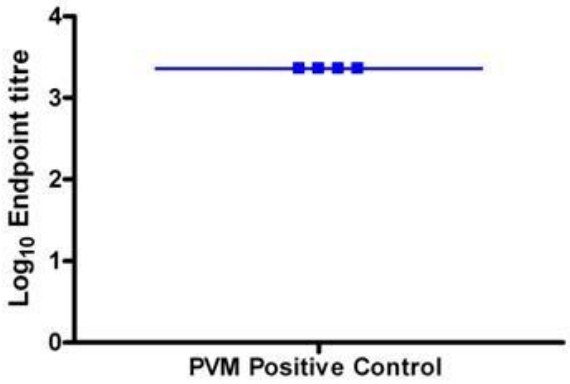

Figure. 6.4. The anti-PVM IgG titre from positive control animals.
Serum samples from four animals immunised with 20 p.f.u. of PVM strain J3666 were collected on day 14 post immunisation. All samples were analysed in the PVM ELISA and the antibody endpoint dilution values calculated as described in Fig. 6.3. The normalised values were then illustrated on a graph as $\log_{10}$ values, generating a positive value for PVM-recovered animals. The line represents the mean titre of the samples.

6.2 The humoral response of prime-boost rAd-immunised animals

Initially, the anti-PVM and anti-Ad5 IgG humoral responses were characterised for the rAd constructs. All animals immunised via the intranasal route with an rAd virus using the standard prime-boost strategy (Section 4, Fig. 4.2) were included in the analysis. Serum samples were obtained on day 14 (boost time point) and day 28 (PVM challenge time point), and analysed by ELISA for anti-PVM and anti-Ad IgG responses, using an anti-mouse IgG secondary antibody (Section 2, Table 2.1.7) to specifically detect IgG. Data for animals immunised with each of the rAd vaccine constructs were collated for the 10^6, 10^7 and 10^8 p.f.u. doses

6.2.1 Detection of PVM-specific antibody responses.

The anti-PVM ELISA did not detect high levels of PVM-specific IgG in the rAd PVM immunised animals (Fig. 6.5), when compared to the positive control PVM-immunised animals (Fig. 6.4).

One rAdF-immunised animal generated a detectable response towards PVM antigen when immunised with the first 10^6 p.f.u. dose (Fig. 6.5A, red symbols). Immunisation with the 10^7 p.f.u. dose did not increase the number of detectable responding animals (Fig. 6.5A, green symbols). A booster immunisation of animals at either of these doses did not increase either the number of detectable responders or the magnitude of the response. A greater number of animals immunised with the highest dose of rAdF, 10^8 p.f.u. (Fig. 6.5A, blue symbols), showed a rise in PVM-specific antibodies, particularly at the day 28 time point. However, the antibody titre was lower than that observed for the PVM-infected control animals.

rAdM-immunised mice also did not generate high levels of anti-PVM IgG antibodies at any of the time points or vaccine doses used (Fig. 6.5B). In contrast, rAdN-immunised animals demonstrated a positive relationship between the delivery of a boost dose and the magnitude of the anti-PVM response. Immunisation of animals with the highest dose of 10^8 p.f.u. (Fig. 6.6A, blue symbols), correlated with the greatest number of detectable responders, some of which generated a similar anti-PVM IgG titre to PVM infected animals (Fig. 6.4). Fewer animals showed a detectable positive response for either of the lower 10^6 or 10^7 p.f.u. doses (Fig. 6.6A,

red and green symbols), which was only observed at day 28. As expected, the rAdZ-immunised animals did not generate a detectable response towards PVM antigen at any immunisation dose or time point analysed (Fig. 6.6B). This confirmed that the irrelevant *Lac*Z transgene did not stimulate a detectable cross-reactive IgG immune response with PVM antigen in this assay.

These data suggest that the standard prime-boost immunisation strategy via the intranasal route is unable to stimulate robust, detectable anti-PVM serum IgG responses. It is known that mice require approximately 14 days in which to establish a primary antibody response (Black, 1974, Miller, 1973). Thus, the prime-boost strategy used in this study ensures sufficient time for a primary antibody response to develop and be expanded upon booster immunisation. The absence of a statistically significant relationship between a booster dose and an increase in the titre of detectable serum anti-PVM IgG antibodies suggests that serum IgG may not be the primary mediator of protection against PVM virus.

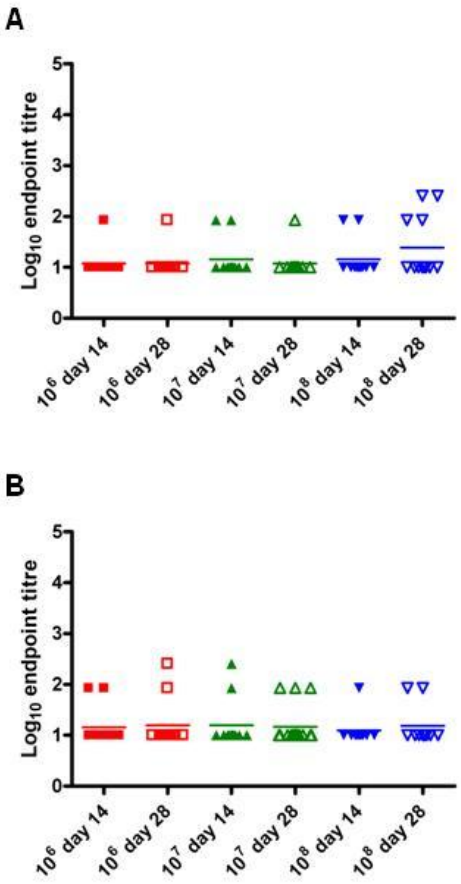

Figure 6.5. The anti-PVM IgG titre for rAdF and rAdM immunised animals.
BALB/c animals were immunised via the intranasal route using the standard prime-boost regime (Section 4, Fig. 4.2), with rAdF (A) or rAdM (B) vaccines at either the 10^6 p.f.u. (red symbols), 10^7 p.f.u. (green symbols) or 10^8 p.f.u. (blue symbols) dose. Serum samples were collected from individual animals at the day 14 (boost) and day 28 (PVM challenge) time points. All samples were analysed in a PVM ELISA and the antibody titres calculated as described in Fig. 6.3. The normalised values were then illustrated on the graph as $\log_{10}$ endpoint dilution values. The data are representative of all experiments performed using this immunisation regime. The lines represent the mean titre of the samples.

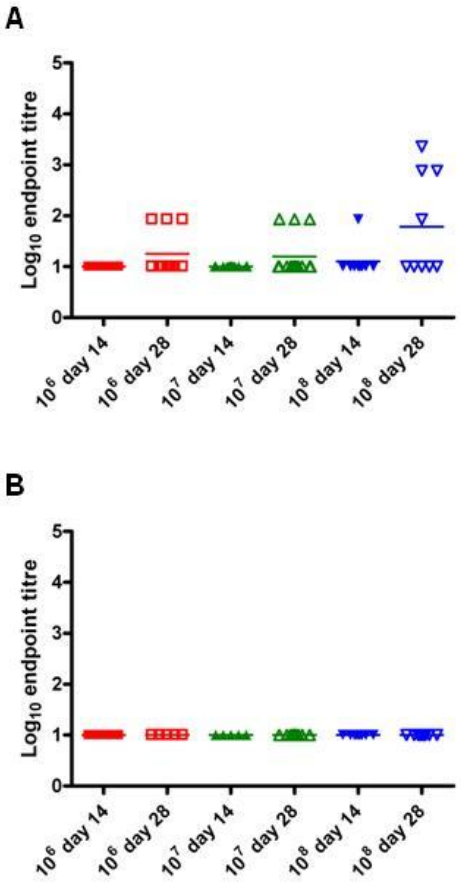

Figure 6.6. The anti-PVM IgG titre for rAdN and rAdZ immunised animals.
BALB/c animals were immunised as described in Fig 6.5, with rAdN (A) or rAdZ (B) vaccines at either the 10^6 p.f.u. (red symbols), 10^7 p.f.u. (green symbols) or 10^8 p.f.u. (blue symbols) dose. Serum samples were collected from individual animals at days 14 and 28 time points. All samples were analysed in a PVM ELISA and the antibody titres calculated as described in Fig. 6.3. The normalised values were then illustrated on the graph as $\log_{10}$ endpoint dilution values. The data are representative of all experiments performed with this immunisation regime. The lines represent the mean titre of the samples.

6.2.2 Detection of rAd-specific antibody responses

Since only a minority of animals receiving rAd PVM vaccine constructs mounted detectable anti-PVM antibody responses, it was unknown as to whether this reflected a general poor response to antigen delivery by rAd5 vectors via the intranasal route or a sensitivity issue with the anti-PVM ELISA assay. As such, it was of interest to determine whether the rAd-immunised animals mounted a response towards the Ad5 vector. To investigate this further, the same serum samples analysed in the PVM ELISA described above, were used in an Ad5 ELISA assay.

In contrast to the anti-PVM response, a greater number of animals generated a detectable response towards Ad5 antigen in the anti-Ad5 ELISA assay. There appeared to be a positive correlation between an increase in detectable antibody titre and a boost immunisation for all rAd constructs (Figs. 6.7 and 6.8). For rAdF-immunised animals, a boost dose for the 10^6 and 10^7 p.f.u. immunised groups correlated with a significant increase in anti-Ad5 titres at day 28 (p=0.001 and p=0.0001, App. D. Table D.1), whereas this was not apparent for the 10^8 p.f.u. immunised group. A boost dose also produced a significant increase in anti-Ad titres at all doses for rAdM-immunised animals (p=0.01, p=<0.0001 and p=<0.0001 App. D. Table D.2), the 10^7 and 10^8 p.f.u. doses for rAdN-immunised animals (p=<0.0001 and p=0.001, App. D. Table D.3) and the 10^8 p.f.u. rAdZ-immunised animals (p=<0.0001, App. D. Table D.4). These data indicated that the animals, including those that did not mount detectable anti-PVM IgG responses, had been successfully immunised with the viral constructs as they were able to produce detectable responses towards Ad5 antigen in the Ad5 ELISA assay.

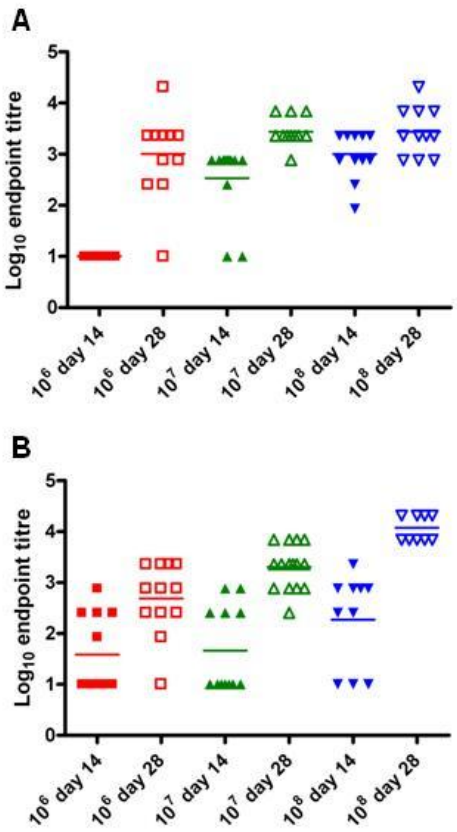

Figure 6.7. The anti-Ad5 IgG titre for rAdF and rAdM-immunised animals.
BALB/c animals were immunised and treated as described in Fig. 6.5 with rAdF (A) or rAdM (B) vaccines at either the 10^6 p.f.u. (red symbols), 10^7 p.f.u. (green symbols) or 10^8 p.f.u. (blue symbols) dose. All samples were analysed in an Ad5 ELISA and the antibody titres calculated as described in Fig. 6.3. The normalised values were then illustrated on the graph as $\log_{10}$ endpoint dilution values. The data are representative of all experiments performed with this immunisation regime. The lines represent the mean titre of the samples.

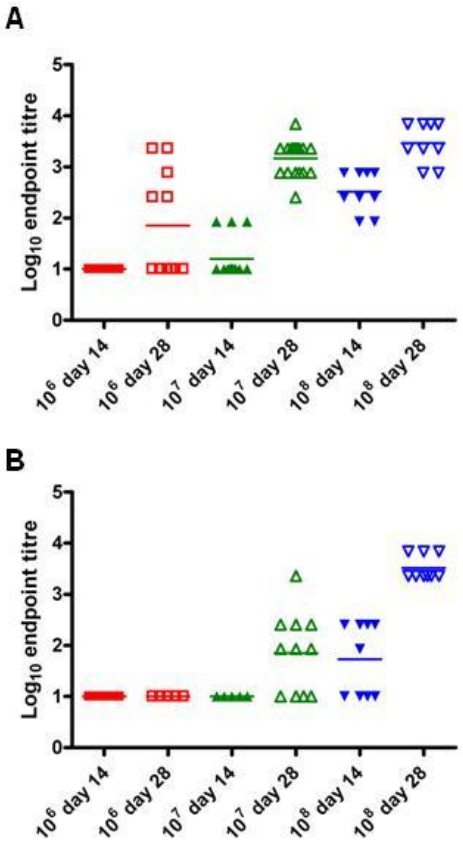

Figure 6.8. The anti-Ad5 IgG titre for rAdN and rAdZ-immunised animals.
BALB/c animals were immunised and treated as described in Fig. 6.5 with rAdN (A) or rAdZ (B) vaccines at either the 10^6 p.f.u. (red symbols), 10^7 p.f.u. (green symbols) or 10^8 p.f.u. (blue symbols) dose. All samples were analysed in an Ad5 ELISA and the antibody titres calculated as described in Fig. 6.3. The normalised values were then illustrated on the graph as $\log_{10}$ endpoint dilution values. The data are representative of all experiments performed with this immunisation. The lines represent the mean titre of the samples.

6.2.3 Summary

The anti-PVM IgG response generated by PVM rAd-immunised animals was lower than that observed in PVM infected mice. There appeared to be little correlation between the time course of the experiment and PVM-specific antibody titres. Additionally, an increase in vaccine dose did not appear to have an immunogenic effect on the rAdF and rAdM-immunised animals at the greatest dose (10^8 p.f.u., Fig. 6.5A and B, blue symbols), however, this effect was observed for rAdN-immunised animals (Fig. 6.6A). The antibody end point dilution values were compared with each other using the Mann-Whitney U test. This non-parametric test determined that all rAds produced levels of PVM-specific antibodies which were not significantly different from the control construct, rAdZ.

As such, the anti-Ad5 titre produced by the rAd constructs was investigated to determine whether these immunogens could elicit any response when delivered via the intranasal route. In contrast to the number of responders detected in the anti-PVM ELISA assay, the majority of animals tested generated a detectable response in the Ad5 ELISA assay. A boost dose significantly increased the anti-Ad5 titre for all of the rAd constructs at the majority of the immunisation doses. This indicated that the animals were able to be successfully immunised via the intranasal route with Ad5 vectors. Thus, the inability to detect strong anti-PVM responses in the PVM ELISA assay may be due to either a sensitivity issue or an inability of these particular PVM antigens to stimulate a strong humoral response by this route of delivery.

6.3 The longevity of the IgG immune response towards the Ad vaccines

6.3.1 The anti-PVM and anti-Ad response of long-term immunised animals

The short-term immunisation experiments described above were unable to generate robust, detectable anti-PVM titres. Although the mouse humoral response can be detected after 14 days post immunisation, the use of a replication-deficient vector which can infect cells and potentially express high levels of transgene could allow continuous immune stimulation for longer than 14 days. Thus, to determine how the antibody response profile altered over time, serum samples were obtained over a

long-term experiment as described in Section 4.5. Animals were immunised with a 10^7 p.f.u. dose of rAd constructs using the standard prime-boost regime (Fig. 4.2). Serum samples were obtained at weeks 4, 6, 8, 11, 14 and 20 post-primary immunisation for rAdM, rAdN and rAdZ-immunised animals and were analysed in both the PVM and Ad5 IgG ELISA systems. As detailed in Section 4.3, rAdF was not investigated due to construct purity issues.

rAdM and rAdN-immunised animals were fully protected from lethal PVM infection for up to 20 weeks post-immunisation, as described in Section 4.5. For rAdM-immunised animals, antibody titres were detectable from week 4 onwards, which corresponds to the day 28 time point in previous experiments. The response decreased to baseline levels by week 14, and remained undetectable up to week 20 (Fig. 6.9A). The response appeared to peak at week 8, where the greatest number of positive responding animals was observed.

The anti-PVM IgG response for rAdN-immunised animals peaked later, at 11 weeks (Fig. 6.9B), and was detectable in the ELISA from weeks 6 to 14. Similar to rAdM-immunised animals, the anti-PVM response was undetectable by week 20. As expected, serum from control rAdZ-immunised animals was uniformly negative in the assay (Fig. 6.9C). In contrast to the anti-PVM IgG titre, the antibody response towards the rAd5 vector was greater in magnitude and all rAd-immunised animals generated a detectable response over the 20 week period (Fig. 6.10). This indicated that the animals had been successfully immunised with the vectors via the intranasal route and that IgG responses to the vector were robust and prolonged.

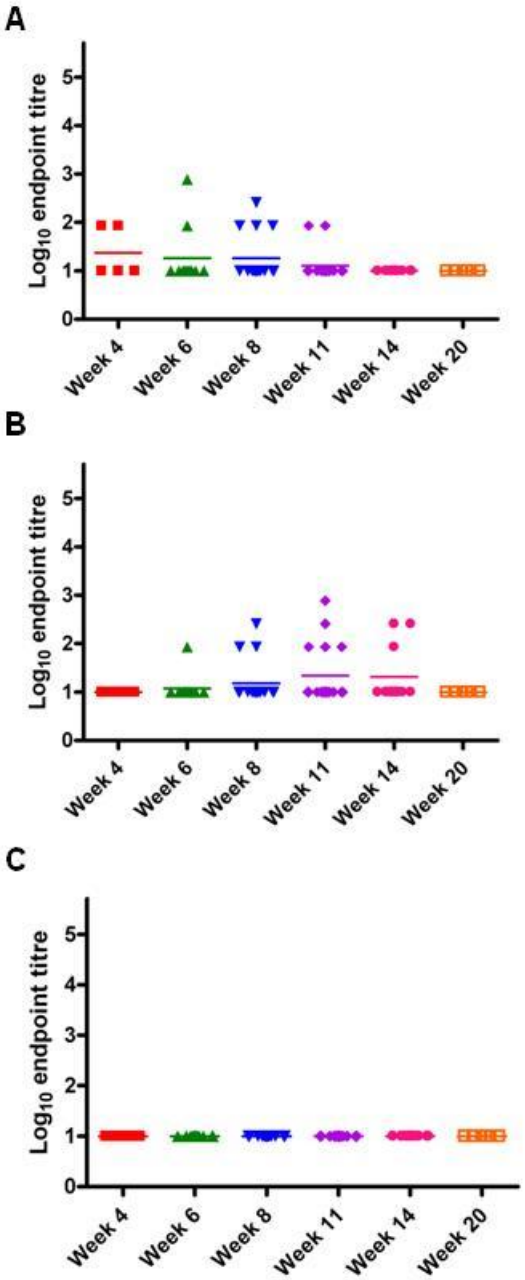

Figure 6.9. The anti-PVM IgG titre for long-term immunisation of rAd-immunised animals.
BALB/c animals were immunised as described in Section 4, Fig 4.10 with either rAdM (A), rAdN (B), or rAdZ (C) constructs at the 10^7 p.f.u. dose. Serum samples were collected at weeks 4, 6, 8, 11, 14 and 20 post primary immunisation. All samples were analysed in a PVM ELISA and the antibody titres were calculated as described in Fig. 6.3. The normalised values were then illustrated on the graph as $\log_{10}$ endpoint dilution values. The lines represent the mean titre of the samples.

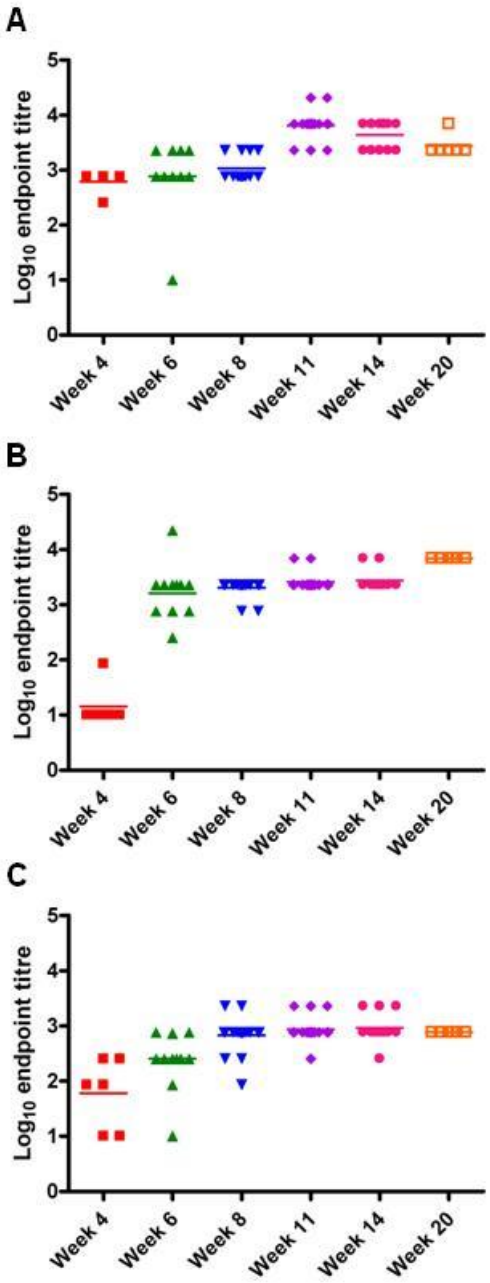

Figure 6.10. The anti-Ad5 IgG titre for long-term immunisation of rAd-immunised animals.
BALB/c animals were immunised and serum samples collected as described in Fig. 6.9 with either rAdM (A), rAdN (B) or rAdZ (C) vaccines. All samples were analysed in an Ad ELISA and the antibody titres were calculated as described in Fig. 6.3. The normalised values were then illustrated on the graph as $\log_{10}$ endpoint dilution values. The lines represent the mean titre of the samples.

6.3.2 Summary

The anti-PVM response observed during the long-term experiment was similar to that observed for the short-term experiment (Fig. 6.5 and 6.6). As before, the anti-PVM response detected by the ELISA assay was low compared to PVM positive controls (Fig. 6.4). Both rAdM and rAdN-immunised animals generated a different anti-PVM IgG response pattern. Detectable responders in the rAdM-immunised group were observed from 4 weeks post-primary immunisation, peaking at week 8.

The response then declined to baseline levels by week 14, indicating that rAdM-immunised animals continued to respond to PVM antigen for up to 11 weeks (Fig. 6.9). In contrast, rAdN-immunised animals were observed to respond to antigen from week 6 onwards, peaking at week 11 before returning to baseline levels by week 20. This indicated that the anti-PVM response towards N antigen took longer to become established but was detectable over a similar time period to that observed for the rAdM-immunised groups (Fig. 6.9). However, no statistical difference was evident within or between the rAd-immunised groups at each time point analysed. These data indicate that the anti-PVM IgG response continues to build for some weeks after the immunisation regime is completed. This is because the PVM antigen can be continuously expressed by the rAd vector and is therefore dependent on the level of vector persistence.

In contrast to the transient anti-PVM response, the anti-Ad IgG response was detectable in all rAd-immunised animals. The response was observed from week 4 onwards and was maintained over the time course of the experiment. Unlike the anti-PVM response, this response was long-lived and did not decline. This suggests that the rAd vector was able to persist within the mouse model for a substantial period before clearance. This is because if the antigen was cleared within the 20 week period, the antibody response would be expected to begin to show evidence of a decline, which it does not (Fig. 6.10). It is notable that the anti-PVM IgG response detected using the PVM specific ELISA did not correlate with protection against a lethal PVM infection as rAd PVM constructs were able to protect animals at 20 weeks (Section 4, Fig. 4.11 and 4.12), in the absence of a detectable anti-PVM IgG response (Fig. 6.9).

6.4 The IgG response of animals immunised with rAd constructs via alternative routes

The previous data suggested that a humoral response to PVM did not correlate with protection against a lethal dose of PVM J3666 in short or long-term experiments. Furthermore, the intranasal immunisation route did not achieve the generation of a

robust anti-PVM response similar to that observed in PVM infected animals (Fig. 6.4). In Section 5.2, animals were immunised with rAd constructs via alternative routes to determine whether the establishment of systemic immunity could protect animals against intranasal challenge. Concurrently, serum samples were obtained from immunised animals at the boost and pre-challenge time points and were analysed using an anti-PVM and anti-Ad ELISA to determine whether rAd immunisation via these routes could stimulate a detectable anti-PVM IgG response.

Animals were immunised with rAdM, rAdN or control rAdZ via the subcutaneous route using the standard prime-boost regime (Fig. 4.2). Serum samples were collected at 14 and 28 days. Subcutaneous immunisation with 10^6 p.f.u. or 10^7 p.f.u. of rAd PVM constructs did not generated a detectable anti-PVM IgG response (data not shown). Subcutaneous immunisation with the highest dose, 10^8 p.f.u., was associated with the generation of one detectable responder in both the rAdM and rAdN-immunised groups (Fig. 6.11A, red and green symbols). As expected, control rAdZ-immunised animals did not generate a detectable anti-PVM response (Fig. 6.11A, blue symbols). However, all animals generated a detectable anti-Ad response (Fig. 6.11B), indicating that they had been successfully immunised with the rAd vector.

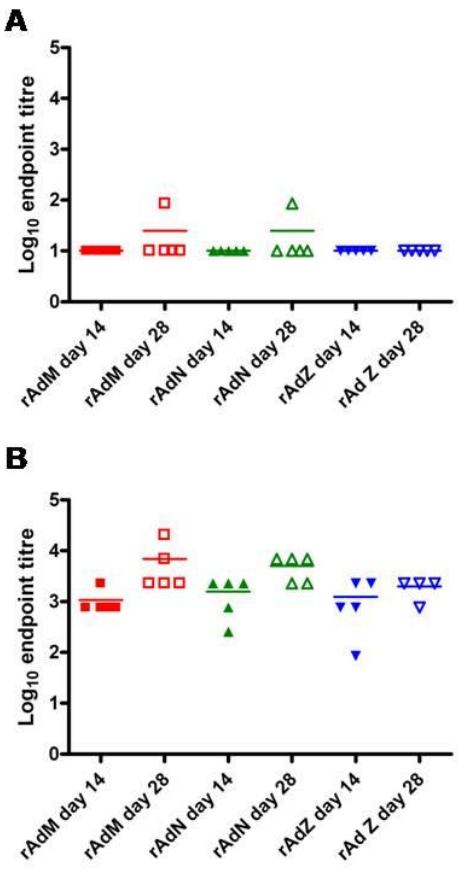

Figure 6.11. The anti-PVM and anti-Ad5 IgG titres for the subcutaneous immunisation route.
BALB/c animals were immunised with 10^8 p.f.u. of rAdM, rAdN or rAdZ using the standard prime-boost immunisation strategy (Fig. 4.2) via the subcutaneous route. Serum samples were collected at days 14 and 28 and were analysed in a PVM (A) and Ad5 (B) ELISA assay. The antibody titres were calculated as described in Fig. 6.3. The normalised values were then illustrated on the graph as $\log_{10}$ endpoint dilution values. The lines represent the mean titre of the samples.

Animals were also immunised with the highest dose, 10^8 p.f.u. via intraperitoneal route with either rAdF, rAdM, rAdN or control rAdZ. Only rAdM and rAdN-immunised animals generated a detectable response to PVM antigen (Fig. 6.12A), at a level similar to the subcutaneous experiment (Fig. 6.11). Similarly, all animals generated an anti-Ad response against the vector post boost immunisation indicating immunisation via this route was successful (Fig. 6.12B).

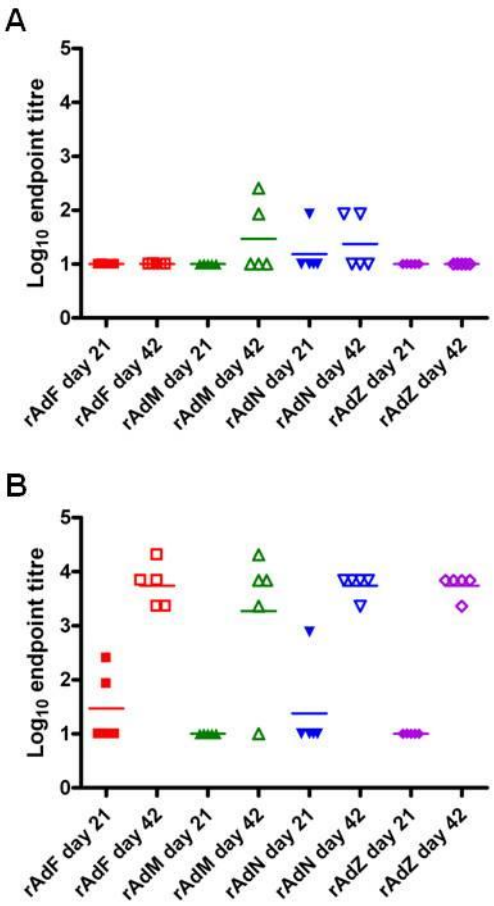

Figure 6.12. The anti-PVM and anti-Ad5 IgG titres for the intraperitoneal immunisation route.
BALB/c animals were immunised on days 0 and 21 with a 10^8 p.f.u. dose of rAdF, rAdM, rAdN or rAdZ via the intraperitoneal route. Serum samples were collected at days 21 and 42 and were analysed in a PVM (A) and Ad5 (B) ELISA assay. The antibody titres were calculated as described in Fig. 6.3. The normalised values were then illustrated on the graph as $\log_{10}$ endpoint dilution values. The lines represent the mean titre of the samples.

For the anti-PVM response, no statistical difference was evident within or between the rAd-immunised groups at each time point analysed. These data suggest that systemic immunisation routes for vaccination with rAd PVM constructs are no better at generating anti-PVM IgG antibody titres than the intranasal route (Fig. 6.5 and 6.6). The detection of anti-Ad5 responses similar to those observed upon intranasal

immunisation indicates that systemic immunisation routes can establish a response towards an antigen. However, as described in Section 5.2, immunisation of animals via these routes in contrast to delivery intranasally, did not result in protection against PVM challenge.

6.5 The anti-PVM IgA response of rAd-immunised animals

The data presented previously indicate that the anti-PVM serum IgG responses generated upon PVM rAd vaccination are relatively weak, transient and did not correlate with protection against lethal PVM challenge. Since protection following rAd construct immunisation correlated with delivery via a mucosal route, which did correlate with protection, it was possible that the main antibody response generated was of the IgA isotype. IgA is a class of antibody which can be secreted and is associated with mucosal immunity (Abbas, 2000). As such, the anti-PVM ELISA was adapted to investigate the IgA response generated upon rAd PVM vaccination.

Animals were immunised with a 10^7 p.f.u. dose of either rAdM, rAdN or control rAdZ via the intranasal route, using the standard prime-boost regime (Fig. 6.13). Groups of three animals were sacrificed on days 19, 22 or 25 post primary dose, to obtain bronchoalveolar lavage (BAL) samples (Fig. 6.13). Samples were analysed in anti-PVM and anti-Ad5 ELISA assays, modified to use an anti-mouse IgA secondary antibody to detect IgA specifically (Table 2.1.7).

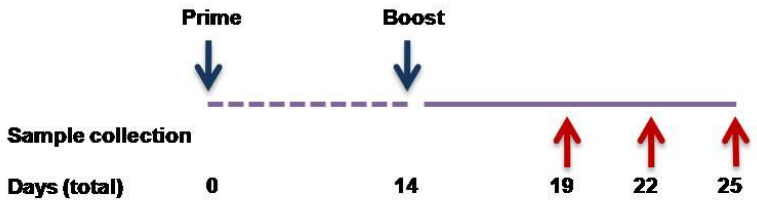

Figure 6.13. The immunisation regime for the investigation of the IgA response in rAd-immunised mice. For the priming immunisation dose, mice were immunised with either rAdM, rAdN or rAdZ constructs at a 10^7 p.f.u. dose in a 50µl inoculum with PBS. After two weeks, the animals were boosted with an identical dose of the immunogen they had previously received. At days 19, 22 and 25 post prime, groups of three mice for each vaccine construct were sacrificed and bronchoalveolar lavage fluid was sampled for each individual animal (red arrows), for further analysis.

Animals which received either the rAdM, rAdN or rAdZ constructs (Fig. 6.14, red, green and blue symbols respectively) did not generate a detectable anti-PVM IgA immune response in BAL samples at day 19. By days 22 and 25 a few animals generated a detectable anti-PVM response in the assay (Fig. 6.14), which was of a very low titre. This suggests that either the IgA response in rAd-immunised animals was not detectable in this assay or that little or no IgA response was generated at the time points analysed. As such, the sensitivity of either the anti-PVM IgA ELISA or the secondary anti-IgA antibody, may not be adequate to detect the IgA response in the samples obtained, particularly as obtaining BAL samples was technically challenging and the initial dilution from the animal is greater than that applied to serum.

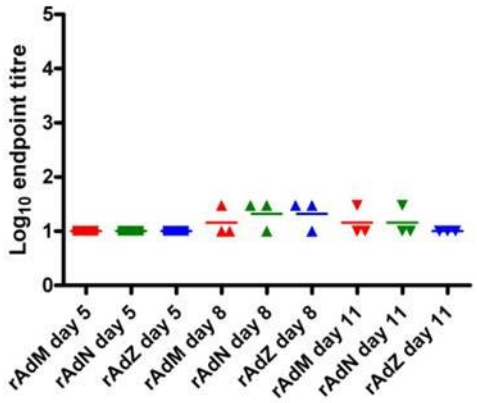

Figure 6.14. The anti-PVM IgA titre for rAd-immunised animals.
BALB/c animals between 5 and 7 weeks of age were immunised via the intranasal route using the standard prime-boost regime (Fig. 4.2). The mice were immunised with a 10^7 p.f.u. dose of either rAdM (red symbols), rAdN (green symbols) or rAdZ (blue symbols). Groups of three animals from each vaccine construct were sacrificed on days 19, 22 and 25 post boost. Bronchoalveolar lavage samples were obtained from each individual animal and analysed in the anti-PVM ELISA and the antibody titres calculated as described in Fig. 6.3. The normalised values were then illustrated on the graph as $\log_{10}$ endpoint dilution values. The lines represent the mean titre of the samples.

6.6 The cellular immune response towards the rAd vaccines

The data described previously demonstrates that the anti-PVM IgG response does not correlate with protection from lethal PVM infection. This is perhaps not surprising since as detailed in Section 1.6.2, the cellular immune response has been observed to be more important in combating RSV infection (Crowe, 2003). The internal antigens, such as the M and N proteins of PVM, are not the primary targets of the neutralising

antibody response. However, both the external and internal antigens are equally likely to elicit T-cell responses as they are all synthesised intracellularly and can all be presented by class I MHC molecules. Also, they can be equally presented by class II MHC molecules through virus particle or through infected cell internalisation by APCs such as DCs. Thus, the rAd PVM constructs described in this study are likely to contain immunogenic T-cell epitopes which may mediate the protective effect discussed in Sections 4 and 5. As the rAdN construct appeared to be more potent in the protection experiments, this vaccine construct was used to identify and characterise the cellular response elicited by the rAd vaccine constructs.

Two *ex vivo* assays were used to evaluate the cellular responses induced by the rAd5 PVM constructs, an IFNγ ELISPOT assay (Section 2.9.3), and an intracellular staining assay (ICS, Section 2.9.4). The ELISPOT assay analysed the number of antigen specific IFNγ secreting T-cells, whereas the ICS assay determined the frequencies of the $IFN\gamma^+$ T-cell subpopulation through the use of fluorescent-conjugated antibodies.

6.6.1 rAdN-immunised animals generate a PVM-specific T-cell response

To identify the cellular response towards the PVM rAdN construct, animals were immunised via the intranasal route using the standard prime-boost immunisation regime (Fig. 4.2) with a 10^7 p.f.u. dose of either the rAdN or rAdZ vaccine constructs. Control animals were either treated with PBS in accordance to the immunisation schedule, or infected on day 0 with 20 p.f.u. of PVM J3666. Animals were culled on day 20 and the spleens and lung lymphocytes were isolated and analysed for PVM-specific T-cell responses using the ICS assay. The lymphocytes were re-stimulated *in vitro* by infection with either PVM J3666 at an M.O.I of 1 p.f.u./cell, the same volume of BS-C-1 tissue culture medium as a negative control, or Phorbol 12-myristate 13-acetate (PMA)/ Ionomycin (Io), as a positive control.

All immunised animals provided splenocyte populations that had similar frequencies of $CD4^+$ T-cells (Fig. 6.15A), indicating that the T-cell subpopulations were comparable between the immunised groups. Both PVM J3666 infected and rAdN-immunised animals produced a specific $CD4^+$ IFNγ T-cell response against PVM

antigen, applying the criterion of a twofold greater frequency of IFNγ$^+$ cells than in the negative control stimulated population. This response was specific to these immunised groups as neither the control rAdZ-immunised or PBS treated animals produced such a response. Representaitive dot plots for this experiment are illustrated in Appendix C. Fig. C.6.

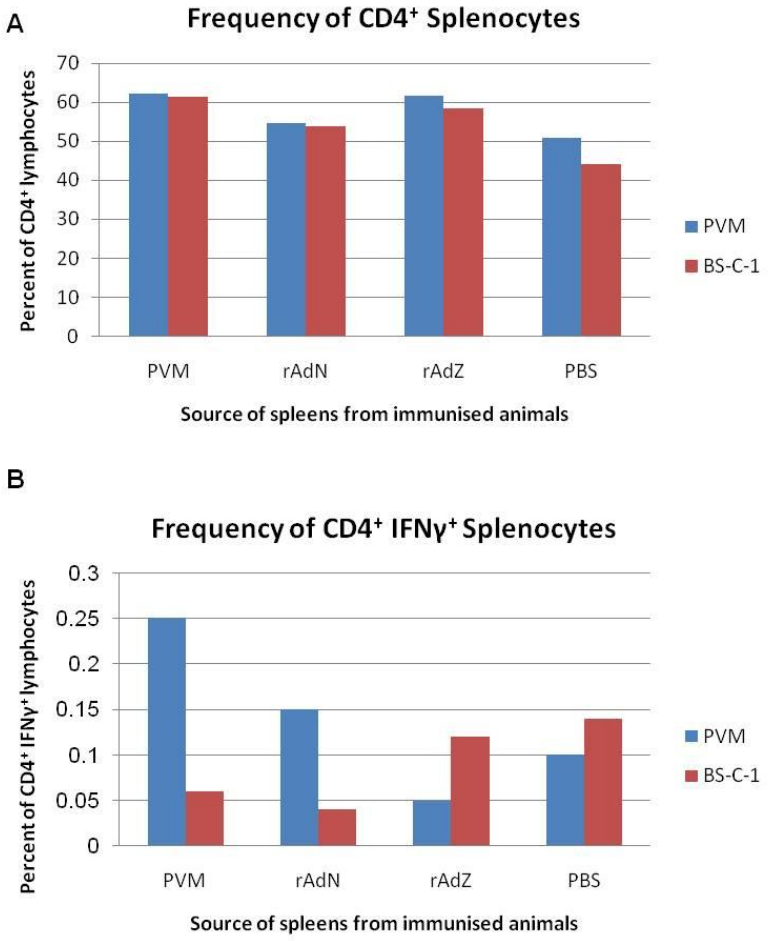

Figure 6.15. PVM-specific IFNγ$^+$-secreting CD4$^+$ splenocytes can be detected in rAdN-immunised animals.
BALB/c mice between 5 and 7 weeks of age were immunised with 10^7 p.f.u. of either rAdN or rAdZ, or were treated with PBS in accordance to the standard prime-boost regime (Fig. 4.2). In addition, four animals were infected with 20 p.f.u. of PVM J3666 via the intranasal route on day 0. Splenocytes were harvested on day 20, pooled for each immunisation group and re-stimulated *in vitro* with either PVM J3666 at an M.O.I of 1 p.f.u./cell (blue bars), the same volume of BS-C-1 control tissue culture medium (red bars), or PMA/Io as a positive control (data not shown). Splenocytes were stained for surface CD4 antigen with PE-conjugated anti-mouse CD4$^+$ (Table 2.1.7) and for intracellular IFNγ with FITC-conjugated mouse IFNγ$^+$ (Table 2.1.7) and analysed by flow cytometry using Cellquest software. The percentage of total CD4$^+$ lymphocytes (**A**) and total CD4$^+$ IFNγ$^+$ lymphocytes (**B**) and were expressed as a percentage of the total lymphocyte population.

The same cells were analysed for a PVM-specific $CD8^+$ $IFN\gamma^+$ response. All $CD8^+$ population frequencies were similar for each immunisation group indicating that the immunisation groups were comparable (data not shown). However, no specific $CD8^+$ $IFN\gamma^+$ response was detected for either the PVM J3666 infected or rAdN-immunised animals (Fig. 6.16).

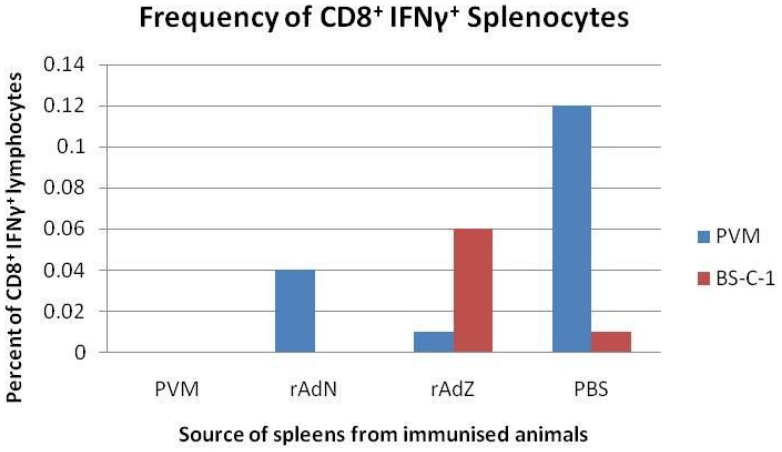

Figure 6.16. PVM-specific $IFN\gamma^+$-secreting $CD8^+$ splenocytes cannot be detected in rAdN-immunised animals.
Splenocytes from the same animals, as described in Fig. 6.15, were re-stimulated *in vitro* with either PVM J3666 at an M.O.I of 1 p.f.u/cell (blue bars) the same volume of BS-C-1 control tissue culture medium (red bars), or PMA/Io as a positive control (data not shown). Splenocytes were stained for surface CD8 antigen with APC-conjugated anti-mouse $CD8^+$ (Table 2.17) and for intracellular IFNγ with FITC-conjugated mouse $IFN\gamma^+$ and analysed as described in Fig. 6.15. The graph shows the $CD8^+$ $IFN\gamma^+$ T-cell population as a percentage of the total lymphocyte population.

The lung lymphocytes from the same animals were used in the ICS assay to detect the frequencies of $CD4^+$ $IFN\gamma^+$ (Fig. 6.17A) and $CD8^+$ $IFN\gamma^+$ (Fig. 6.17B) T-cells in the total lymphocyte populations. As before, the proportions of $CD4^+$ and $CD8^+$ cells in the lung lymphocyte populations were similar between samples and thus were comparable for each antigen stimulated group (data not shown). Cells from PVM J3666 infected animals generated the strongest $CD4^+$ $IFN\gamma^+$ response towards PVM J3666 re-stimulation (Fig. 6.17A), as observed previously (Fig. 6.15B). However, in contrast to splenocytes, lung lymphocytes from rAdN-immunised animals did not display a significant response towards PVM antigen (Fig. 6.17A). The frequency of $CD8^+$ $IFN\gamma^+$ lung lymphocytes for PVM re-stimulated PVM J3666 infected animals

was also significant (Fig. 6.17B). However, similar to the $CD4^+$ population, $CD8^+$ lung lymphocytes from rAdN-immunised animals did not display a specific response to PVM J3666 antigen (Fig. 6.17B).

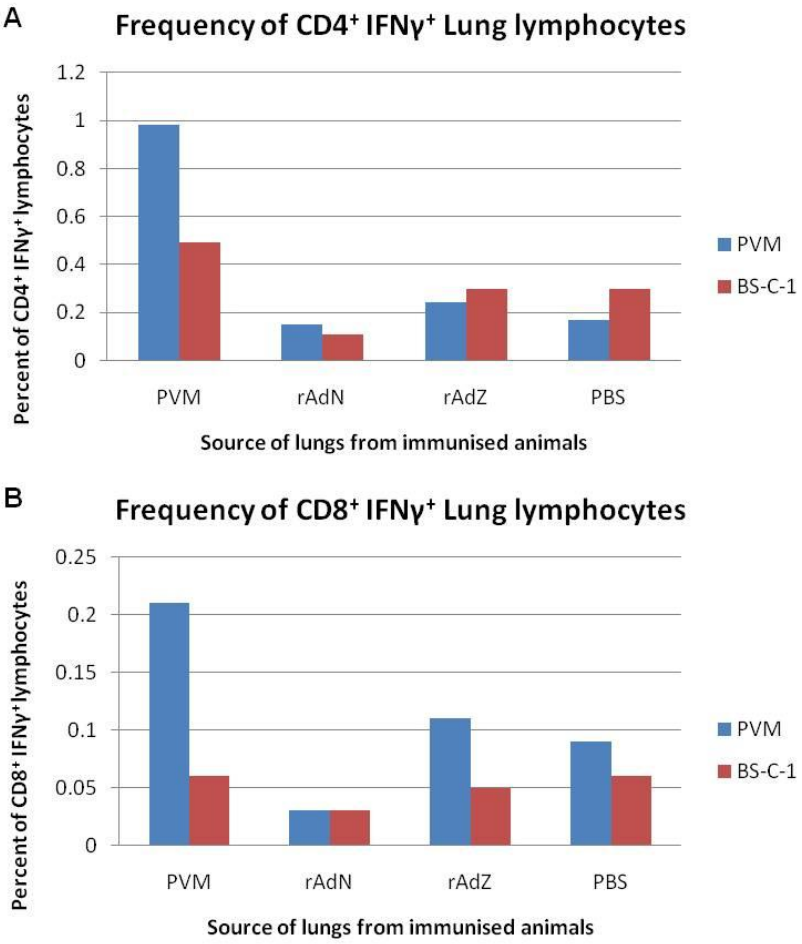

Figure 6.17. PVM-specific $IFN\gamma^+$-secreting $CD4^+$ and $CD8^+$ lung lymphocytes cannot be detected in rAdN-immunised animals.
Lung lymphocytes from the same animals as described in Fig. 6.15 were re-stimulated *in vitro* with either PVM J3666 at an M.O.I of 1 p.f.u/cell (blue bars) the same volume of BS-C-1 control tissue culture medium (red bars), or PMA/Io as a positive control (data not shown). Lung lymphocytes were stained and assayed for $CD4^+$ $IFN\gamma^+$ T-cells (**A**) as described in Fig. 6.15 and $CD8^+$ $IFN\gamma^+$ T-cells (**B**) as described in Fig. 6.16 and were expressed as a percentage of the total lymphocyte population.

These data collectively suggest that PVM J3666 infected animals respond to PVM antigen, producing both IFNγ-secreting $CD4^+$ and $CD8^+$ lung lymphocytes and IFNγ-secreting $CD4^+$ splenocytes. In contrast, no significant $IFN\gamma^+$-secreting $CD8^+$

population was detected in the rAdN-immunised animals in either the splenocytes or lung lymphocyte populations, although, a PVM antigen-specific $CD4^+$ $IFN\gamma^+$ response was observed in these animals. Thus, the ICS assay was able to detect PVM-specific T-cell responses in positive control (PVM infected) and rAdN-immunised animals. The frequencies of $IFN\gamma^+$ $CD4^+$ or $CD8^+$ T-cells were greater in the PVM J3666 infected group, presumably because the range of antigens to which a cellular response could be generated was greater. The response observed from rAdN-immunised animals was lower than that observed for the PVM-infected positive control presumably because only one PVM protein was present to stimulate the response.

In an attempt to increase the PVM-specific response observed in rAdN-immunised animals, an alternative immunisation regime was used (Fig. 6.18). The animals were immunised via the intranasal route with either rAdN or rAdZ at a 10^7 p.f.u. dose, or were PBS treated as a control, on days 0, 14 and 28. The additional boost dose was used to ensure that the T-cell populations were strongly stimulated with PVM antigen.

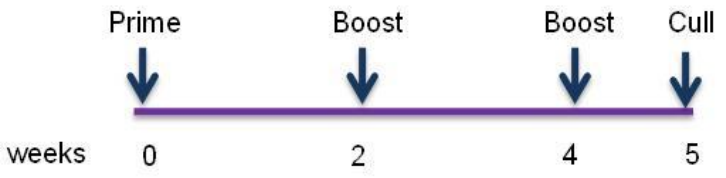

Figure. 6.18. The immunisation regime for the investigation of cellular response in rAd-immunised mice. For the priming immunisation dose, BALB/c mice between 5 and 7 weeks of age were immunised with either rAdN or rAdZ vaccines at a 10^7 p.f.u. dose in a 50µl inoculum or with PBS. After two and four weeks, the animals were boosted with an identical dose of the immunogen they had previously received. At five weeks post priming dose, the animals were sacrificed and the lung and spleens were removed. Splenocytes and lung lymphocytes were isolated (Section 2.8.3.2) for further analysis.

As before, the frequencies of $CD4^+$ and $CD8^+$ T-cells within the recovered lymphocyte populations were similar across all immunisation groups (data not shown). rAdN-immunised animals generated a PVM-specific $CD4^+$ $IFN\gamma^+$ response which was significant when compared to the BS-C-1 medium control and when compared to the response elicited by these antigens in the control immunised groups

(Fig. 6.19A). This result was expected as two-dose rAdN-immunised animals also generated such a response (Fig. 6.15B). However, unlike the two-dose rAdN-immunised animals, those receiving three doses produced a PVM-specific $CD8^+$ $IFN\gamma^+$ response which was significant when compared to the BS-C-1 media control antigen and the control immunised animals (Fig. 6.19B).

The extended immunisation regime did increase the frequency of $CD4^+/CD8^+$ $IFN\gamma^+$ T-cell populations for rAdN-immunised animals. The two-dose regime for rAdN gave 0.15% $CD4^+$ $IFN\gamma^+$ splenocytes and 0% $CD8^+$ $IFN\gamma^+$ splenocytes, whereas the three-dose regime for rAdN gave 1.1% $CD4^+$ $IFN\gamma^+$ splenocytes and 1.9% $CD8^+$ $IFN\gamma^+$ splenocytes. These data strongly suggest that rAdN-immunised animals can produce specific anti-PVM $CD4^+$ and $CD8^+$ T-cell responses that are detectable in the ICS assay. This supports the theory that the protective effect observed upon rAd PVM vaccination with the 10^7 or 10^8 p.f.u doses could be primarily mediated by a cellular response.

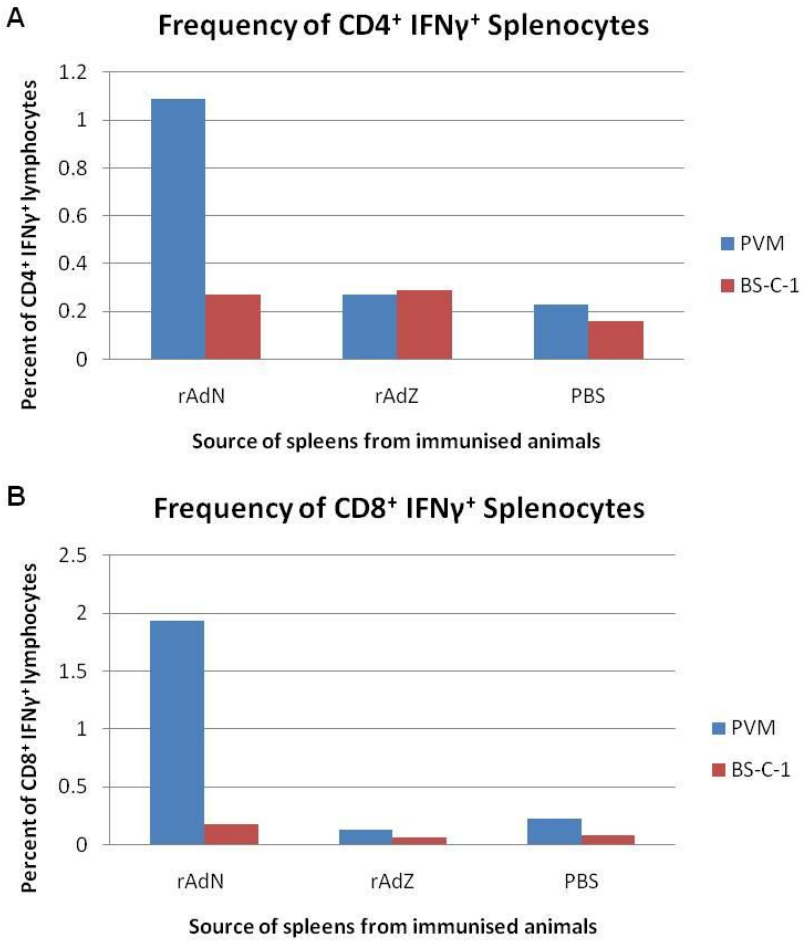

Figure 6.19. rAdN-immunised animals generate a PVM N-specific T-cell response.
BALB/c mice between 5 and 7 weeks of age were immunised as described in Fig. 6.18, with a 10^7 p.f.u. dose of either rAdN or rAdZ, or PBS, on days 0, 14 and 28. The splenocytes were harvested on day 35 and were pooled for each immunisation group. The cells were re-stimulated *in vitro* with either PVM J3666 at an M.O.I of 1 p.f.u./cell (blue bars), the same volume of BS-C-1 control tissue culture medium (red bars), or PMA/Io as a positive control (data not shown). Splenocytes were stained for $CD4^+$ and $CD8^+$ antigen and IFNγ as detailed in Figs. 6.15 and 6.16 respectively and then analysed by flow cytometry gated using Cellquest software for the lymphocyte population. Splenocytes were stained and assayed for $CD4^+$ $IFN\gamma^+$ T-cells (**A**) and $CD8^+$ $IFN\gamma^+$ T-cells (**B**) and were expressed as a percentage of the total lymphocyte population.

6.6.2 rAdN-immunised animals generate a PVM N-specific T-cell response

The precise nature of the antigen(s) stimulating the cellular response towards PVM during PVM infection is unknown, however, it has been shown that the RSV N protein contains T-cell epitopes (Bangham et al., 1986). The PVM and RSV N proteins share over 60% amino acid sequence identity (Barr et al., 1991), which

suggests that the PVM N protein may also be capable of inducing a T-cell response. In addition, a theoretic T-cell epitope has been identified in the PVM N gene (Claassen et al., 2005). In support of this, rAdN, which only expresses the N protein of PVM, was shown above to stimulate a PVM-specific T-cell response.

The cellular response towards PVM N protein was investigated with the aim of identifying immunodominant epitopes for the PVM N protein. Currently, no T-cell epitopes for either a $CD4^+$ or $CD8^+$ T-cell population have been characterised for the PVM N protein *in vivo*. Therefore, possible antigenic targets needed to be identified. To investigate this, a peptide library of the entire N protein was produced, comprising 77 15-mer peptides that overlapped by 10 amino acids (identified in App. E. Table E.1).

Animals were immunised via the intranasal route using the extended immunisation regime (Fig. 6.18), with 10^7 p.f.u. of either rAdN or rAdZ, or PBS (control). The splenocytes were harvested and pooled for each immunisation group. The peptides were combined into pools of eight and were used to re-stimulate the splenocytes *in vitro*, and the resulting responses were analysed by the ICS assay. As for previous experiments, the total frequency of the $CD4^+$ and $CD8^+$ T-cells in the lymphocyte population was consistent between immunisation groups.

When the peptide-specific $CD4^+$ response of rAdN-immunised animals was compared with control rAdZ-immunised and PBS treated animals, one peptide pool stimulated a specific $CD4^+$ $IFN\gamma^+$ T-cell response (Fig. 6.20A), applying the criterion of a twofold greater frequency of $IFN\gamma^+$ cells towards the PVM N peptides than in the negative control (rAdZ-immunised) populations. This included peptide pools 9-16 and 17-24 which corresponded to amino acids $N_{41\text{-}90}$ and $N_{81\text{-}130}$ respectively (Table 6.1 and App. E. Table E.1).

When the $CD8^+$ population was analysed, a larger range of peptide pools was shown to stimulate an $IFN\gamma^+$ response from rAdN splenocytes (Fig. 6.20B). rAdN-immunised animals produced $CD8^+$ T-cells that were activated specifically by peptide pools 9-16, 17-24, 25-32, 33-40, 49-56, and 57-64 (Fig. 6.20B and Table 6.1). As before, the result was judged positive if the T-cell population was twofold or

more greater for the rAdN-immunised animals then that observed for the control groups. These data indicated that the PVM N protein may contain several epitopes to which a T-cell response can be directed.

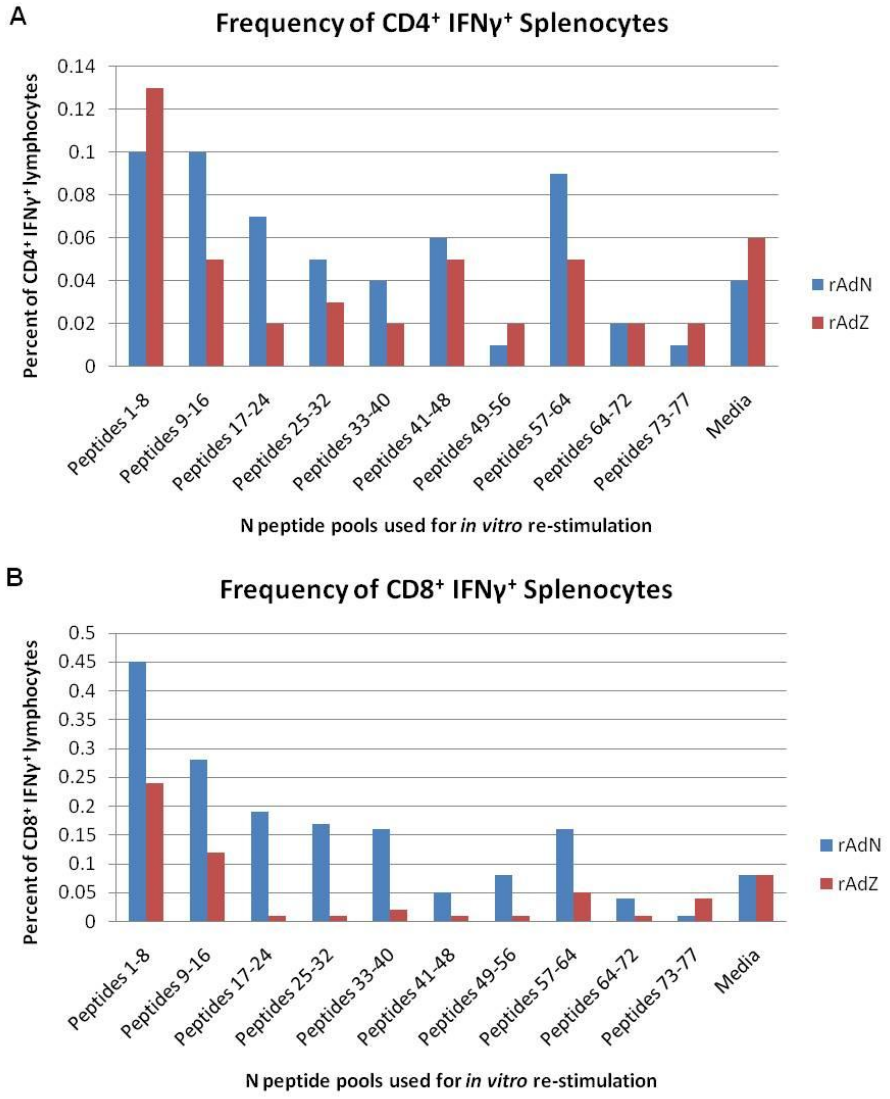

Figure 6.20. PVM N peptide-specific IFNγ$^+$-secreting CD4$^+$ and CD8$^+$ splenocytes can be detected in rAdN-immunised animals.
BALB/c mice were immunised with 10^7 p.f.u. of rAdN (blue bars) or rAdZ (red bars), or PBS treated (data not shown), as described in Fig. 6.18. Splenocytes were harvested and pooled for each immunised group. PVM N peptides were combined into pools of eight and were used to re-stimulate splenocytes *in vitro*, along with BS-C-1 tissue culture medium as a control. Splenocytes were stained for surface CD4 with PE-conjugated anti-mouse CD4$^+$, or APC-conjugated anti-mouse CD8$^+$, and with FITC-conjugated anti-mouse IFNγ$^+$ for intracellular IFNγ staining (Table 2.1.7) and analysed using Cellquest software. Total CD4$^+$ IFNγ$^+$ (**A**) and CD8$^+$ IFNγ$^+$ (**B**) cell frequencies were expressed as a percentage of the total lymphocyte population.

To corroborate the data observed in the ICS assay, the extended immunisation experiments were repeated but the re-stimulated T-cells were analysed via an alternative assay, an ELISPOT assay. The ELISPOT assay was optimised using animals immunised with the non-pathogenic PVM-strain 15 (Table 2.1.3), or PBS treated animals (App. C. Fig. C.5) and was shown to detect specific anti-PVM T-cell responses from PVM-infected animals. The ELISPOT assay can be more sensitive than ICS assays as translational regulation can occur, providing a more accurate insight into the directly secreted cytokine profile of a T-cell population. In addition, rare cell populations which produce high cytokine levels are captured by the membrane and thus allow rare populations to be visualised.

Animals were immunised with 10^7 p.f.u. of rAdN or rAdZ (control) via the intranasal route using the extended prime-boost immunisation regime (Fig. 6.18). Splenocytes and lung lymphocytes were harvested and pooled for each immunisation group. The lymphocytes were re-stimulated *in vitro* with the PVM N peptides, pooled into groups of eight as used previously.

As observed previously, a PVM-specific response was determined if T-cells from rAdN-immunised animals generated a response which was twofold or more greater towards the PVM N peptide pools than that observed for the rAdZ-immunised animals. On this basis, splenocytes from rAdN-immunised animals demonstrated a PVM-specific IFNγ^+ response to peptide pools 9-16, 17-24, 33-40, 41-48, 49-56, and 57-64 (Fig. 6.20A) although as discussed below, the media control value is high. Likewise, lung lymphocytes from the rAdN-immunised animals demonstrated a PVM specific IFNγ^+ response to peptide pools 9-16 and 33-40 (Fig. 6.20B).

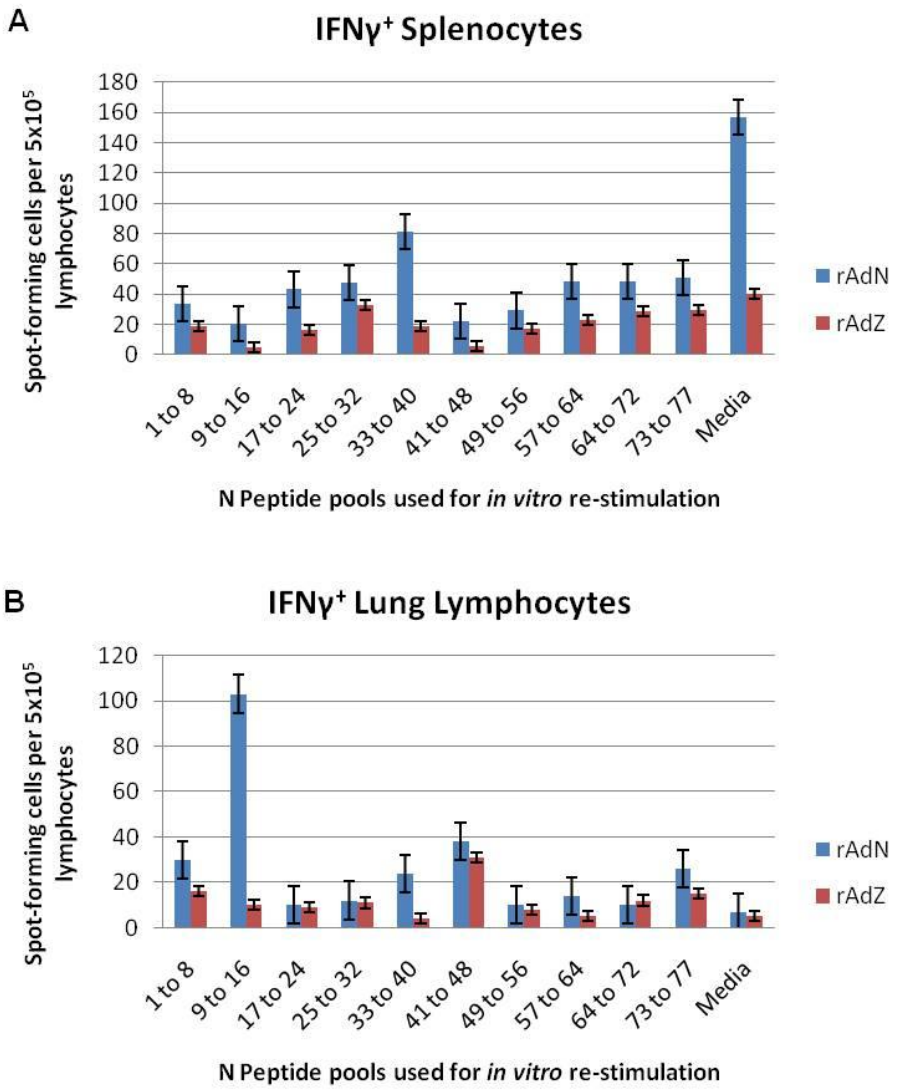

Figure 6.21. The PVM N peptide-specific T-cells can be detected in rAdN-immunised animals by IFNγ ELISPOT assay.
BALB/c mice between 5 and 7 weeks of age were immunised intranasally with 10^7 p.f.u. of rAdN (blue bars) or rAdZ (red bars) using the extended prime-boost immunisation regime (Fig. 6.18). The splenocytes and lung lymphocytes were harvested and pooled for the respective immunisation groups. Lymphocyte populations were re-stimulated *in vitro* with either the PVM N peptides, combined in pools of eight or BS-C-1 tissue culture medium control. The number of IFNγ$^+$ spot forming cells in $5x10^5$ splenocytes (**A**) or lung lymphocytes (**B**) was observed. Error bars represent standard error of the averaged value of two replicate wells.

These data confirmed that the ELISPOT assays for the lung lymphocytes and splenocytes were successful at identifying PVM-specific T-cell responses. However, the spots formed within the assay were small, indicating that IFNγ was not released in great quantities (data not shown). As pneumoviruses are known to functionally

impair T-cells (Section 1.6.2.3), IL-2 was included in the medium for all of these experiments. IL-2 is a cytokine which stimulates T-cell proliferation and increases cytokine synthesis for all T-cell populations (Abbas, 2000). IL-2 was also included to increase the spot size to ensure adequate detection in the assay.

For both assays, a medium control was incorporated in the assay (Fig. 6.21). For the lung lymphocyte assay, the background stimulation by medium was low for both rAdN and rAdZ-immunised animals (Fig. 6.21B). However, for splenocytes re-stimulation, rAdN-immunised animals generated a robust response towards the tissue culture medium control (Fig. 6.21A). Upon inspection of that particular well, the high background could either be attributed to contamination from the T-cell stimulator PMA/Io which was used elsewhere in the assay, or due to membrane damage from the pipette tip, resulting in darker areas to be generated on the ELISPOT membrane. As the result for rAdZ-immunised animals was low for medium stimulation, it confirms the medium used was not contaminated with PMA/Io or an equivalent compound. However, T-cells from rAdN-immunised animals still responded specifically to PVM antigen when compared to lymphocytes from rAdZ control immunised animals.

6.6.3 Summary

These data collectively demonstrate that similar peptide pools were identified as containing potential T-cell epitopes in both the ICS and ELISPOT assay. These peptide pools are summarised in Table 6.1. The peptide pools highlighted in bold are pools to which rAdN-immunised T-cells specifically responded in several experiments. These pools were from several regions of the N protein, amino acids N_{41-90}, N_{81-130}, $N_{161-210}$ and $N_{281-330}$. Two of these pools overlap and therefore they could detect the same T-cell epitope or distinct epitopes. Therefore, these data show that a specific T-cell response can be detected towards PVM N protein in rAdN-immunised animals and that the T-cell response is directed against multiple epitopes.

	IFNγ$^+$ CD4$^+$ Splenocytes (Fig. 6.20A)	IFNγ$^+$ CD8$^+$ Splenocytes (Fig. 6.20B)	IFNγ$^+$ Splenocytes (Fig. 6.21A)	IFNγ$^+$ Lung Lymphocytes (Fig. 6.21B)	N protein amino acid position
Peptide pool		1-8			1-50
	9-16	**9-16**	**9-16**	**9-16**	41-90
	17-24	**17-24**	**17-24**		81-130
		25-32			121-170
		33-40	**33-40**	**33-40**	161-210
			41-48		201-250
		49-56	49-56		241-290
	57-64	**57-64**	**57-64**		281-330

Table 6.1. N protein peptide pools to which cells recovered from rAdN-immunised animals responded specifically.
Pools highlighted in bold represent those for which a positive response was identified across several experiments. The amino acid positions represented by each pool are indicated in the right-hand column.

6.7 Discussion

Animals which received a low dose, 20 p.f.u., of PVM J3666 were observed to generate a robust serum IgG response towards PVM antigen in the anti-PVM ELISA (Fig. 6.4). In contrast short-term experiments with rAd PVM-immunised mice showed only weak responses in a minority of mice (Fig. 6.5 and 6.6). Furthermore, there was no statistically significant correlation between vaccine dosage and time, and an increase in the magnitude of the anti-PVM IgG response. This suggested that the standard immunisation regime was unable to stimulate detectable anti-PVM IgG responses similar to those of positive control PVM-infected animals. To determine whether the IgG response increased further during an extended time course, the anti-PVM IgG response was monitored over a 20 week period (Fig. 6.9). Again, these data indicated that the anti-PVM response did not reach the same magnitude as positive control PVM-infected animals (Fig 6.4). This suggested that the rAd PVM

constructs are less able to stimulate a detectable IgG response than a natural PVM infection.

It was unknown whether these observations were as a result of the immunisation route used (the intranasal route), or due to the sensitivity of the ELISA assay. As such, the immune responses for rAd immunised animals via alternative routes such as the subcutaneous and intraperitoneal routes were investigated. The results demonstrated that immunisation was successful via these routes (Fig. 6.11B and 12B), in that a response was detected against the rAd vector, but only a few animals made detectable anti-PVM responses (Fig. 6.11A and 6.12A). These data suggest that perhaps the systemic immunisation route was unable to stimulate a detectable anti-PVM response towards PVM antigen. Alternatively, the primary immune response directed towards PVM antigen expressed from rAd could have been an alternative isotype, IgA. However, no specific anti-PVM IgA responses were detected within BAL samples (Fig. 6.14) or serum (data not shown).

The anti-PVM ELISA assay was thoroughly optimised. It was demonstrated to be specific for PVM serum, (App. C. Fig. C.4), and to be capable of detecting antibodies to PVM F and N proteins through the use of monoclonal antibodies (Fig. 6.2). Thus, the PVM ELISA was demonstrated to be adequate for the studies described here and was deemed to be appropriately sensitive to detect PVM-specific IgG responses. A commercial anti-PVM ELISA is available from Charles River Laboratories (MA, USA). However, the PVM antigen used in this assay and the sensitivity of the assay when compared to the ELISA used in this study is unknown. If the anti-PVM ELISA used in this study does have a low threshold of sensitivity, it was not detected during optimisation, but may have decreased over time due to reagent degradation.

The results described here are interesting as rAd PVM-immunised animals are protected from lethal PVM infection at the 10^7 and 10^8 p.f.u. doses in short and long term experiments. However, the magnitude and duration of the anti-PVM IgG response did not correlate with the protection data. The majority of animals did generate anti-Ad5 responses, suggesting that animals could be successfully immunised by all the routes investigated. However, few of these animals also

generated detectable anti-PVM responses, regardless of the rAd construct immunisation dose and route. As such, the humoral response is not the primary mediator of the protective effect.

The lack of detectable anti-PVM IgG responses was unforeseen given that a response was detected for animals that had recovered from a mild PVM infection. However, this was achieved with a replication-competent virus. This increases the amount of antigen available for the immune response stimulation in contrast to the replication-deficient Ad vectors which rely on high transgene expression levels to generate sufficient antigen for an immune response. In addition, PVM infection presents a greater array of PVM antigens and epitopes for the immune system to respond to in contrast to the rAd constructs which present only a few epitopes within a particular protein.

The lack of correlation between antibody responses and protection suggested that the cellular response could be the primary mediator of this protective effect. To investigate this, ICS and ELISPOT assays were used to identify PVM-specific T-cell responses to both PVM protein (Figs. 6.15-19) and PVM N peptides (Figs. 6.20-21). The ICS assay successfully identified PVM-specific $CD4^+$ splenocytes in cells from rAdN-immunised animals (Figs. 6.15 and 6.19). This indicated that rAdN antigen may have been able to stimulate the cellular response in immunised animals which could correlate with the protective effect previously observed with immunisation of the 10^7 p.f.u. dose.

To identify specific epitopes within the N protein to which the T-cell populations were directed, PVM N protein peptides were pooled into groups of eight and used to re-stimulated rAdN-immunised splenocytes and lung lymphocytes. Several peptide pools were identified to which rAdN-immunised animals possessed an apparent specific response (Table 6.1). These pools were able to re-stimulate both $CD4^+$ $IFN\gamma^+$ and $CD8^+$ $IFN\gamma^+$ splenocyte populations from rAdN-immunised animals in an ICS assay and additionally identified $IFN\gamma^+$ secreting splenocytes and lung lymphocytes in an ELISPOT assay. Interestingly, four peptide pools were identified as able to re-stimulate $IFN\gamma^+$ secreting populations in both the ELISPOT and ICS assay which

were pools 9-16, 17-24, 33-40, and 57-64 (Table 6.1). Only pool 9-16 which corresponds to amino acids 41-90 was consistently identified across the different experimental assays for the two different lymphocyte populations. This suggests that this region of the N protein could contain an immunodominant epitope for the anti-PVM cellular response.

An unexpected finding with these data was that the CD4/CD8$^+$ IFNγ^+ populations were rare and much smaller in comparison to published data (Claassen et al., 2007, Claassen et al., 2005). A representative dot plot (Appendix C.6.), illustrated how rare this positive population was. A reason for the detection of such low CD4/CD8$^+$ IFNγ^+ T-cell populations may have been the impairment of T-cells by the PVM virus used to stimulate the T-cells *in vitro* (Section 1.6.2.3). Although the virus was irradiated, the degree of inactivation was not assessed; therefore there remains the possibility that the virus was not completely inactivated, which may have led to the impairment of the T-cell population. Neither was this impairment rescued by the inclusion of the IL-2 cytokine in the medium, which has been previously demonstrated to aid the rescue of impaired T-cells (Chang & Braciale, 2002). An additional issue was that intracellular staining with an anti-IFNγ antibody requires the cells to be fully permeabilised to allow the antibody to enter the cell and bind to the specific cytokine. Incomplete permeabilisation may have led to sub-optimal staining and thus the apparent detection of low CD4/CD8$^+$ IFNγ^+ populations in response to PVM virus or PVM N peptide *in vitro* stimulation. In this study, complete permeabilisation of the cells suspension was not demonstrated and thus suboptimal staining from the anti-IFNγ antibody may have occurred. Furthermore, the antibodies used within the ICS experiment may not have been effective. Although these antibodies were used in the study described by Claassen *et al,* (Claassen et al., 2005), no positive controls were used in this study to evaluate their effectiveness and optimise the concentrations used. This may also have resulted in the detection of fewer CD4/CD8$^+$ IFNγ^+ populations in the experiments described in this chapter.

Another issue with the results demonstrated here is that the positive and negative controls did not generate responses within expected ranges as previously published (Claassen et al., 2005, Kohlmann et al., 2009, Yu et al., 2008). For example,

CD4/CD8$^+$ IFNγ$^+$ T-cell populations were not strongly stimulated in response to PMA/Io treatment (data not shown), in addition, the responses generated from PBS treated animals restimulated with PVM *in vitro* were often greater than those observed from rAdN-immunised animals (Figs. 6.16-17). rAdN and rAdZ-immunised animals also demonstrated strong CD4/CD8$^+$ IFNγ$^+$ responses to medium only controls (Figs. 6.20-21). These data cannot be explained wholly by contamination of these controls with a positive stimulant, such as observed in Fig. 6.21A, and therefore, may represent a significant issue with the results from these assays. These results imply either that the responding CD4/CD8$^+$ IFNγ$^+$ population is so rare that any specific response could be masked by background or control responses in the assays illustrated in this chapter, or that due to the absence of assay repeats and replicates performed for the ICS assay, that the responses observed may not be a true representation of the CD4/CD8$^+$ IFNγ$^+$ T-cell populations stimulated upon rAd PVM construct immunisation of BALB/c mice.

In conclusion, the data presented in this chapter suggests that a cellular response as opposed to a humoral response is a primary mediator of protection against PVM in rAdN-immunised animals. Although IFNγ was the only cytokine investigated in this study, its detection could indicate that a balanced T-cell response is being elicited towards PVM since this cytokine is characteristic of a T_h1 CD4$^+$ T-cell response. Therefore, the rAd PVM constructs provide a successful immunisation strategy to protect animals against pneumoviruses.

Chapter 7

Final Discussion

7.1 General discussion

Since the 1960s, efforts to generate a successful vaccine against RSV have so far not yielded a clinically licensed candidate. In addition, vaccine efficacy has not been evaluated in an appropriate animal model. The studies presented in this thesis describe the development of 'proof of principle' vaccine candidates, to tackle pneumovirus infection. These have been generated using replication-deficient adenoviral vectors. The PVM infection model developed by Cook *et al*, was previously optimised to investigate the disease pathogenesis of PVM strain J3666 in the BALB/c mouse strain (Cook et al., 1998), and this model was used to allow the efficacy of recombinant adenoviral vaccines to be evaluated.

The adenoviral vaccine candidates were developed using the AdEasy™ Adenoviral Vector System (He et al., 1998). Plasmids were engineered to express full length F, M, N and P genes from PVM, and the *LacZ* gene from *E. coli.* Sequence analysis confirmed that the constructs were correct (App. Fig. A. 1-4). Expression of PVM F mRNA/protein or PVM N protein was detected for these constructs and corresponded to previously published data (Barr et al., 1991, Chambers et al., 1992). The PVM M protein was localised in both the nucleus and the cytoplasm (Fig. 3.17), consistent with published literature for RSV M protein (Ghildyal et al., 2009). The M protein is usually located in the nucleus during early stages of infection, but when the switch to genome replication occurs, the protein localises to the cytoplasm; a function believed to be controlled by the M2 protein (Ghildyal et al., 2009, Li et al., 2008). Expression of M protein in the absence of other PVM proteins, particularly M2, in rAdM-infected cells could explain why M localisation was detected in both the cytoplasm and nucleus of rAdM positive cells (Fig. 3.12).

Interestingly, PVM P protein expression from either pShuttle_CMV_P and pAdEasy_P plasmids, or rAdP virus was not detectable (Fig. 3.21, 3.22). The constructs retained the full-length P gene sequence and the virus was able to replicate in complementing cell lines (Fig 3.20). However, the growth kinetics were impaired when compared to the other rAd viruses or wild-type Ad5. To address this, new pShuttle_CMV_P plasmids were generated using new primer pairs to account for

possible sequence alterations in the primer sequence. Expression of the P protein from both of these plasmids was undetectable. PVM P mRNA expressed from rAdP will be produced in the nucleus. This may have a role in the subsequent failure to express P protein. This is possible as the PVM P gene is usually expressed in the cytoplasm.

rAd5 constructs have been generated using the AdEasy™ system for several RSV and non-RSV vaccine studies, and have been found to stimulate both humoral and cellular responses towards the transgene (Fu et al., 2009b, Guo et al., 2008, Jiang et al., 2008, Khanam et al., 2009, Kohlmann et al., 2009, Peng, 2008, Wu et al., 2007, Yu et al., 2008). In this study, rAdF, rAdM, rAdN constructs and a control construct rAdZ were used with the PVM infection model to determine vaccine efficacy *in vivo.* When delivered via the intranasal route, rAd PVM constructs were shown to protect several mouse strains with different MHC haplotypes against lethal PVM infection. This indicates that the immunity elicited by the vaccine constructs is likely to be protective in a mixed genetic population.

In BALB/c mice, intranasal immunisation with rAd PVM constructs gave protection up to 20 weeks post immunisation with a 10^7 p.f.u dose using the standard prime-boost regime, and up to 6 six weeks post immunisation with a single 10^7 p.f.u dose. Interestingly, while the rAd PVM constructs were able to elicit antigen-specific protection of mice at a 10^7 p.f.u dose, all of the constructs, including rAdZ, demonstrated protection in mice against lethal PVM challenge when immunised with a 10^8 p.f.u dose indicating an antigen non-specific effect. See *et al* have previously described such an effect, where a ΔE1/E3 replication-deficient empty Ad5 vector was used to immunise ferrets with a 10^9 p.f.u dose, via the intranasal route (See et al., 2008). The study indicated that ferrets immunised with the control construct demonstrated decreased SARS virus replication when compared to PBS-treated animals and they concluded that non-specific responses induced by the Ad5 vector interfered with SARS replication.

There are several differences between the present study and the one conducted by See *et al.* Firstly, they employed different host/challenge virus systems. Secondly, mice are fully permissive to PVM infection, whereas ferrets are only semi-

permissive to SARS. While ferrets exhibit signs similar to that observed in humans, (To et al., 2004), some studies have indicated that clinical signs are not always observed (Czub et al., 2005, Weingartl et al., 2004). This suggests that only a slight protective effect could be necessary in the ferret-SARS system to provide protection from virus replication.

In the light of the non-specific protection against PVM seen here with high doses of rAd, the rAdZ construct was evaluated within the influenza A virus mouse model (Dimmock et al., 2008), to determine how broadly acting this non-specific protective effect was. C3H/He-mg mice have been shown to be fully susceptible to influenza infection and were immunised with the rAdZ at the 10^8 p.f.u dose using the standard prime-boost regime, prior to challenge with a lethal dose of influenza A virus. The animals were not protected against the influenza challenge (Fig. 5.10), which suggested the protective effect may be linked to rAdZ stimulation of a particular immune response relevant to protection from PVM.

Animals immunised with the 10^8 p.f.u dose were still protected up to 11 weeks post prime dose after a prime-boost immunisation regime (Fig. 5.8), and up to 6 weeks with a single dose (Fig. 5.9). This could indicate that at the high dose the rAd vectors are able to persist for longer in the host. Previous studies investigating vector persistence indicated that Ad5 vectors do not persist longer than two weeks within the mouse model (Yang et al., 1995), and the length of time between immunisation and challenge in these experiments would suggest that innate immune responses are not mediating the anti-viral effects of rAdZ. One approach to investigate these theories would be to determine the longevity of the rAdZ and rAdGFP constructs in the mouse lung. Mice are usually non-permissive to Ad, but the virus can enter cells due to the expression of the mCAR receptor, the murine homologue of CAR, which allows transfection of a wide number of cell types including the brain, lungs, liver and kidneys (Bergelson et al., 1998, Kass-Eisler, 1994), however, the virus does not go through a full productive replication cycle due to problems with gene expression and regulation. As the brain is an immunoprivileged site, it implies that Ad5 may be capable of persistence in such sites, as has also been demonstrated for liver cells (Jager & Ehrhardt, 2009). This could allow continuous stimulation of the $CD8^+$ T-cell population by prolonged transgene expression (Finn et al., 2009), which could

elicit the protective effect observed at the high immunising dose. Although vector longevity and persistence were not measured in this study, this could be investigated using a β-galactosidase assay to measure *LacZ* expression in lung mucosa or the expression of Ad proteins through immunohistochemistry. Alternatively, transgene or Ad mRNA could be identified using *in situ* hybridisation.

To determine whether the *Lac*Z gene or the Ad5 vector was the source of the non-specific protection produced by high rAd doses, alternative transgenes were investigated using a PVM challenge. rAdGFP and rAdEV both generated a similar effect when used to immunise mice at the high dose only (Fig. 5.11). This suggested that the Ad5 vector was the source of this protection through either an antibody- or cellular-based response. The level of neutralising antibody generated towards the Ad5 vector was unlikely to be the source of the protective effect, because the IgG titres elicited by 10^7 or 10^8 p.f.u prime-boost doses were similar (Fig 6.6B). In addition, similar anti-Ad IgG titres for rAdZ and rAd PVM constructs were seen with the short and long-term experiments (Figs. 6.5, 6.6A, 6.8). Thus, the neutralising antibody response is unlikely to cross-react with PVM antigen and mediate the protective effect. This suggests that a T-cell response may be the cause of the non-specific protective effect observed.

It is known that PVM and influenza virus require $CD8^+$ and T_h1 $CD4^+$ T-cell responses to mediate viral clearance (Claassen et al., 2005, Doherty, 2009). This suggests that the Ad5 vector is not stimulating a cytokine profile where the T_h1 $CD4^+$ response or the $CD8^+$ response could be biased, providing protection against influenza. Therefore, the apparent PVM specific protection by Ad5 could be mediated by two mechanisms; the first being cross-reactive T-cells and the second a bystander effect occurring during PVM infection. Cross-reactive T-cells have been observed during RSV infection between two different strains (Kulkarni, 1993) and during adenovirus infection (Hutnick, 2010). Thus, a rare population of Ad-PVM cross-reactive T-cells may have been stimulated upon high levels of rAd5 immunisation. Alternatively, it has been shown that during viral infection large numbers of non-specific T-cells are actively recruited to sites of infection (Tough et al., 1996). This has been shown to be predominately the $CD4^+$ memory population and this response is mediated by the induced cytokine profile (Bangs et al., 2009).

Thus, Ad5 could stimulate the recruitment of T-cells which would contribute to a strong induction of inflammation through cytokine release, generating an anti-viral state at the site of infection. In addition, Ad has been shown to induce Ad-specific T-cells which are maintained in an effector state (Hutnick, 2010). If these cells were maintained in the lung mucosal tissue, they could enhance the development of a T_h1-biased $CD4^+$ response and promote the more rapid recruitment of immune effector cells to the site of infection, perhaps stimulating a protective environment against PVM. A third possibility is that the protective effect from a high dose of rAdZ was mediated through a threshold effect. The anti-Ad response could have reached a threshold, where it was able to reduce PVM replication at the high 10^8 p.f.u dose only. To address this, it would be of great interest to compare the titre of PVM in the mouse lung between the rAd PVM constructs and rAdZ construct immunised mice by plaque assay. If rAd PVM-immunised animals generated significantly lower PVM replication titres than rAdZ, it would suggest that the rAdZ construct generated a threshold of protective immunity, beyond which, innate responses were able to clear the infection.

The rAdF, rAdM and rAdN constructs did not stimulate a robust anti-PVM IgG or IgA response and the levels of IgG achieved did not appear to correlate with protection against PVM (Fig. 6.3, 6.4). The immune response was also monitored during the long-term experiments, where it was demonstrated that the IgG response peaked at week 11 for rAdN-immunised animals and week 8 for rAdM-immunised animals. The antibody response then declined to undetectable levels. Anti-Ad5 IgG responses were more robust, and detectable for all of the constructs. This was in contrast to the published literature where immunisation with an rAd5 vaccine expressing full length RSV F protein, was able to induce robust antibody responses towards the F or the G protein transgene (Fu et al., 2009b, Kohlmann et al., 2009, Shao et al., 2009, Yu et al., 2008); however, both of these studies differed from this investigation. Firstly, UV inactivated RSV particles were used as the antigen source in the ELISA assay, whereas this study used a persistently infected cell line, as PVM could not be grown in sufficient bulk. Secondly, the time difference in the immunisation protocol used in the study by Kohlmann *et al* was 3 weeks instead of the 2 weeks used in this assay. The three week period could have allowed increased

time over which a robust response could be generated. However, the second sample time point used in the study by Kohlmann *et al* would correspond to the 6 weeks immunisation sample point during the long-term experiment described here which did not generate a robust response towards the transgene. The rAdF construct used in this study was not investigated during the long-term experiment, so the anti-PVM IgG titre at this point is unknown. The study by Shao *et al* also used a 2-week prime-boost strategy, with sample collection on day-24 as opposed to day-28 used in this study. This suggests that the immunisation time course used in the experiments described in this thesis is unlikely to have a detrimental impact on the generation of antibody titres observed. The primary antibody response towards pneumoviruses is directed against the F and G proteins (Simoes, 1999). This is because as external proteins, they are more likely to be presented to the immune system in comparison to internal proteins, such as M and N. The absence of robust antibody titres directed against the M and N proteins is likely to reflect the level of the exposure of these proteins to the immune system rather than their immunogenicity. Interestingly, the longevity of the anti-RSV response was maintained at a higher level to the prime-boost experiment in the Kohlmann *et al* study. All animals generated a detectable response at all of the time points examined, and IgG was detectable up to 35 weeks after immunisation. This pattern was not replicated in this investigation, as firstly, not all animals generated a detectable anti-PVM IgG response at the time points examined, and secondly, the response had decreased to undetectable levels by 20 weeks. However, the titre of anti-PVM IgG response was, where detected, observed at a similar level to the prime-boost experiments.

The immunity elicited by the rAd PVM constructs using different immunisation routes was also compared in this study. It was observed that the rAd PVM constructs were most immunogenic when delivered via the intranasal route. Subcutaneous and intraperitoneal immunisation only generated antibody titres when the higher 10^8 p.f.u dose was used to immunise the animals. Neither did this response correlate with protection. This contrasts with other studies where immunisation of rAd constructs via routes other than intranasal were able to stimulate neutralising antibody responses (Kohlmann et al., 2009, Yu et al., 2008). However, it has been demonstrated that the intranasal immunisation route is superior at generating robust immune response towards the transgene in RSV virus systems (Kohlmann et al., 2009, Yu et al., 2008).

These findings confirm that the route of immunisation performed has a significant effect on the immune response induced and thus protection elicited against the pathogen.

In this study, immunisation with a prime-boost strategy failed to elicit a detectable mucosal anti-PVM IgA response. This is in contrast to studies performed by Shao *et al,* and Yu *et al*, where rAd vaccines expressing full-length F protein or codon-optimised G protein fragments were able to induce robust anti-RSV IgA titres against the transgene product. Again, a fundamental difference between these studies and the one described here is that the ELISA assay utilised purified RSV particles, as opposed to a persistently infected cell line, which may have increased the sensitivity of these assays. Thus, as described for the IgG response, the M and N proteins may not have been presented to the immune system in sufficient quantities in order to stimulate a robust, detectable response (Simoes, 1999). Alternatively, the lack of detectable PVM-specific IgA here may be due to problems with the BAL collection method. The BAL fluid was collected by one passage of PBS into the lung. Shao *et al* performed two washes and were able to detect PVM IgA in both BAL fluid and sera, whereas Yu *et al,* performed only one, similar to this study, and only immunised with a single $5x10^7$ p.f.u dose of the vaccine. For this study, the collection of BAL fluid was found to be difficult, thus one fluid passage was used which could have limited the recovery of IgA from the lung. In addition, due to time limitations, the sample size of the group was limited and neither was it possible to collect a positive control. Thus, the results from the IgA experiment may not represent the overall IgA response generated from rAd PVM construct immunisation.

One avenue that requires further exploration is the comparison of sensitivity of the ELISA assay described in this study, with commercially available screening tests. This study demonstrated that a functional anti-PVM ELISA assay was developed using a persistently PVM infected cell line P2-2. The individual proteins were shown to be detectable within the ELISA system (Fig. 6.2), however a quantitative measure of assay sensitivity was not investigated. Serum from animals which had received a low, non-lethal dose of PVM was used to evaluate the sensitivity of the ELISA. The samples generated positive titres within the assay (Fig. 6.4), however this response would have been generated across multiple epitopes and antigens, as opposed to a

single PVM protein. Animals immunised with the rAd PVM constructs are therefore likely to have a lower overall antibody titre. This is because the immune system of these animals was exposed to only one antigen and the epitopes within the protein, as opposed to the wide range of epitopes and antigens from the whole PVM virus. Furthermore, the rAd constructs were unable to replicate within the host, thus epitope exposure to the immune system is limited by the degree of transgene expression from the construct. Therefore, although the anti-PVM ELISA is able to detect antibody titres, it may not be sensitive enough to detect low titres of antibody, which may be induced upon vaccine administration or low levels of antibody maintained over the long-term experiments. It may therefore be useful to compare the samples with a serological testing kit such as the ELISA assay provided by Charles River Laboratories (MA, USA). However, an alternative explanation is that the immunisation regimes used in this study do not induce systemic neutralising antibody responses and perhaps the immune response is primarily localised to the lung mucosa. This would suggest that serological analysis of the lung tissue or BAL fluid may provide greater insight of the serological response towards PVM antigen generated by the rAd PVM constructs.

Immunisation with the rAd vector resulted in the generation of anti-Ad immunity which is consistent with several other studies using different virus systems (Shao et al., 2009, Yu et al., 2008). In these studies, anti-vector immunity did not appear to interfere with the establishment of transgene immunity, as 10^7 p.f.u doses were associated with either protection or the establishment of anti-RSV IgG and IgA responses. The findings in this study support this concept as is was observed that anti-vector immunity also did not appear to interfere with the development of transgene immunity. This was observed upon immunisation with the rAdM construct, as an example. A single 10^7 p.f.u dose was partially protective (Fig. 4.14) as a few fatalities were observed in this group. However, a prime-boost immunisation with the same dose was associated with full protection up to 18-weeks post boost, with no associated fatalities. Anti-Ad immunity was observed to increase over this time period (Fig. 6.8), as has been observed previously (Shao et al., 2009, Yu et al., 2008), but this did not interfere with protection from lethal PVM infection.

In the protection studies described in this study, the rAdN PVM construct appeared to be the most successful, in that protection against lethal PVM infection was always achieved using the 10^7 p.f.u immunising dose. The rAdN candidate was therefore further investigated with regards to the cellular response elicited by this vaccine construct.

BALB/c mice immunised with rAdN generated an IFNγ secreting $CD8^+$ and $CD4^+$ T-cell populations in response to lung lymphocyte and splenocytes re-stimulation with PVM virus and PVM N peptide pools. In this study, it was observed that immunisation with a prime-boost strategy with 10^7 p.f.u of the rAdN construct was able to induce large numbers of $CD4^+$ cells to the lung and spleen. Re-stimulation of rAdN-immunised splenocytes led to the recruitment of $CD4^+$ T-cells, which made up approximately 55% of the total number of cells analysed (Fig. 6.15). However, the total IFNγ-secreting T-cell population was only 0.15%. Similarly, 55% of lung lymphocytes analysed were $CD4^+$, and only 0.15% of the total lymphocyte population secreted IFNγ (Fig. 6.17). This suggested that either the population of $CD4^+$ T-cells stimulated were of the T_h2 type, which would not secrete IFNγ, or that the cells were active but not specific for PVM antigen, or lastly, that the PVM-specific T-cells were inactivated. All of these possibilities could have contributed to the results obtained in this study.

In the ICS experiments performed in this study using PVM virus to re-stimulate the T-cells, the frequency of IFNγ secreting T-cells was observed to be lower than previously published data (Kohlmann et al., 2009, Yu et al., 2008). Low frequencies of IFNγ secreting T-cells were also observed upon PVM peptide re-stimulation of T-cells. This is in contrast to the observations of Claassen *et al*, where 10-20% of the $CD8^+$ T-cells re-stimulated with PVM peptide from PVM-immunised mice were positive for IFNγ. This has also been observed with similar studies using RSV infected mice (Chang & Braciale, 2002, Gray et al., 2005). Furthermore, in humans a study suggested that 33% of $CD8^+$ T-cells secreted IFNγ in RSV infected infants (Lukens et al., 2010). Interestingly, all of these studies commented that the frequency of IFNγ secreting T-cell populations was low, though they are still greater than the numbers observed in this study using PVM antigens for both $CD8^+$ and $CD4^+$ T-cell populations. These findings suggest that there is a difference between the results

observed with RSV and PVM virus systems and other viruses. As such, it appears that pneumovirus infection could lead to significant T-cell suppression.

These data presented here indicate that the N protein of PVM is capable of inducing a cellular immune response in the BALB/c mouse model. In comparison to the cellular response observed for RSV, epitopes have been identified in the BALB/c mouse model include the dominant epitope $M2_{82\text{-}90}$ as well as epitopes within the N protein and the F, $F_{85\text{-}93}$ (Bangham et al., 1986, Chang et al., 2001, Kulkarni et al., 1995, Mok et al., 2008, Openshaw, 1990, Pemberton et al., 1987). Whereas for PVM, T-cell epitopes $P_{261\text{-}269}$, $M_{43\text{-}51}$ and $F_{302\text{-}312}$ have been identified experimentally in mice, in addition to a theoretic epitope in the N protein (Claassen et al., 2005). For bRSV, the F, G and N proteins have been shown to induce a protective cellular responses in calves (Gaddum, 1996, Taylor et al., 1997). However, none of these studies attempts to identify the epitope to which the cellular response is directed against in the N protein. Thus, the identification of a potential PVM, and therefore possibly an RSV, T-cell stimulating region has been performed in this study and is likely to be $N_{41\text{-}90}$.

To compensate for possible T-cell inactivation, the cytokine IL-2 was added to the external medium in both the ELISPOT and ICS assays. IL-2 has previously been demonstrated to rescue inactivated T-cells (Chang & Braciale, 2002). However, IL-2 addition did not significantly alter the total numbers of $CD4^+$ or $CD8^+$ T-cells, or the number of $IFN\gamma^+$ cells observed. Another possibility is that within the large population of $CD8^+/CD4^+$ T-cells detected within the ICS assay, a larger subpopulation could be Ad-antigen specific when compared to the PVM-specific population observed, suggesting that the immunogenic nature of Ad as a dominant effect over the immune response generated towards the N protein. One way to improve transgene immunogenicity would be to either use an Ad vector which expresses fewer Ad proteins as described in Section 1.3.4, or to codon-optimise the N gene, as previously described for the RSV G gene by Yu *et al* (Yu et al., 2008). As IFNγ is predominately a T_h1 response cytokine, it is possible that the cellular response to PVM is T_h2 biased. An avenue of interest not pursued in this study would be to investigate the cytokine profile induced upon rAdN-immunisation and whether this profile alters following PVM virus challenge. Cytokine release can be analysed using ELISA or ELISPOT assays. ELISPOT assays are inherently more sensitive as

they enable rare populations to be visualised by their ability to secrete IFNγ before the cytokine is diluted in the external medium. The rAdN construct used in this study was believed to stimulate a predominately T_h1 response as Ads naturally stimulate a T_h1 biased response which could also bias the response to the N protein to be of the T_h1 type (Santra, 2005). The expression profiles of the T_h2 response associated cytokines, such as IL-4, IL-5 and IL-13, could be measured by ELISPOT or ICS. If greater numbers of T_h2 cytokine secreting T-cells were detected for animals immunised with rAdN than those which were IFNγ positive, it would suggest the response is T_h2, as opposed to T_h1 biased.

Shao *et al* and Yu *et al* investigated the cytokine secretion levels following immunisation with rAd RSV vaccine candidates (Shao et al., 2009, Yu et al., 2008). The results suggested that the vaccine stimulated T-cell population secreted reduced levels of T_h2 associated cytokines leading to reduced levels of eosinophils recruitment when compared to RSV infected animals. They therefore suggested that the immune response was T_h1 biased. It is possible that a similar result would be obtained for the rAdN vaccine candidate used in this study.
As described in Section 1.6.2.3, pneumovirus infection can lead to T-cell suppression through inactivation of PVM-specific T-cells. This could be investigated by determining the level of extracellular CD28 expression. A low level of expression on the PVM-specific T-cell population would indicate an inactive population which would therefore not contribute to protective immunity towards PVM. However, this is unlikely to have occurred as only one protein was used to immunise animals as opposed to whole PVM virus,

A final possibility is that the method used to perform the IFNγ ELISPOT assay could result in variable numbers of $IFN\gamma^+$ T-cell populations observed, A study comparing *Mycobacterium tuberculosis* IFNγ secreting T-cell populations compared four different ELISPOT methods across several sites (Smith et al., 2009). Significant variation was observed between sites and between the protocols investigated. The ELISPOT method described in this study was adapted from that described by Claassen *et al* (Claassen et al., 2005), suggesting that the lower frequency of PVM-specific $IFN\gamma^+$ T-cells detected could either be due to reagent and user variability or

the quality of the lymphocyte populations obtained. Thus, the ELISPOT method used may have contributed to the results presented in this study.

Although no vaccine studies exist which utilise the N protein of a pneumovirus in an rAd vaccine construct, two studies have been described where a similar approach was used. One investigated bRSV N protein immunisation using plasmid DNA as the immunogen in a prime dose and purified N protein in a boost dose immunisation strategy (Carine et al., 2008). The results with this approach showed that IFNγ $CD8^+$ T-cells were stimulated and proliferated in response to vaccination which was enhanced upon boosting. Immunisation provided a protective effect against bRSV challenge with calves exhibiting reduced viral titres and reduced levels of pathological lung lesions. Although this study utilised N protein in a DNA and protein delivery system, rather than the recombinant viral vector system used here, it evaluated protection of a vaccine against a pneumovirus in its natural host. This protective effect was associated with the cellular response rather than neutralising antibody (Carine et al., 2008). The other study investigated measles virus, another member of the *paramyxoviridae* (Fig. 1.2). The N protein of measles virus has been expressed in a replication-deficient Ad5 vector as a potential immunogen. Immunisation of BALB/c mice with a 10^7 p.f.u dose with the rAd measles N construct via the intraperitoneal route resulted in the stimulation of N-specific antibody responses and a strong $CD8^+$ T-cell response (Fooks et al., 1995). The vaccine construct was shown to be protective against measles challenge, resulting in a reduction in virus titre. These studies confirm the findings presented in this investigation that an internal protein can stimulate protective immune responses in a host.

7.2 Future Experiments

To continue the investigation into the immunogenicity of the rAd PVM constructs it would be useful to generate and evaluate in the protection model an additional construct expressing the M2 gene of PVM, as the RSV M2 protein is known to contain epitopes such as $M2_{82\text{-}90}$ that is recognised in BALB/c mice, correlating with a protective $CD8^+$ T-cell response (Kulkarni et al., 1995, Mok et al., 2008, Openshaw, 1990).

The immune response towards the rAd PVM constructs requires further study. Several questions remain about the mechanism of protective immunity and the duration of the response. The longevity experiments only continued up to 20 weeks post-immunisation due to time constraints. The next step would be to extend this study for up to 52 weeks, to determine the extent of the long-term protection elicited by the 10^7 p.f.u. dose. It would also be prudent to incorporate a similar study using the 10^8 p.f.u dose to determine how long lasting is the antigen non-specific protective effect observed at this high dose. As was demonstrated for the 20-week experiment, PVM rAd constructs did not generate IgG antibody responses in an ELISA assay at 20 weeks, yet all animals were protected. Likewise, the anti-Ad IgG response was maintained at a high titre for the duration of the study. Thus, the immunological profile of protection could also be investigated further to determine if PVM-specific T-cells could be identified over a long-term experiment. If, for example, protection at the 10^7 p.f.u dose wanes over the 52 week period, it would suggest that either PVM specific memory T-cells are not maintained in the long-term, or are naturally impaired due to pneumovirus infection.

The mechanism of immunity could also be investigated by passive transfer of serum from rAd PVM and rAdZ-immunised mice, to non-immunised animals prior to PVM challenge. This would indicate whether a neutralising IgG or IgA response is required for the control of PVM infection. Such an experiment could also be performed using knockout mouse strains, such as the BALB/c nude mouse strain, which is T-cell deficient. Immunisation of this strain with the rAd PVM vaccines could determine whether protection is achieved through antibodies or innate immune responses. Innate responses could be investigated using SCID mice, as these animals are B and T-cell deficient, but have normal NK cell function. A SCID mouse strain is available which has defective NK cells, allowing the role of innate immunity in the development of protection against PVM to be investigated.

Another interesting line of work would be to compare rAd immunisation via the oral route and intranasal routes. Although the subcutaneous and intraperitoneal routes were investigated for potential routes of eliciting protection towards PVM, (Figs. 5.1-5.4); the subcutaneous route was not associated with protection at any dose investigated and the intraperitoneal route was associated with Ad vector-mediated

side effects. These routes primarily focus on systemic immunisation, which does not necessarily stimulate the mucosal response, reported to be important for protection against PVM (Kim et al., 2010). An investigation into the potential immunogenicity of the oral route for these vaccines would determine whether specific pulmonary localised mucosal responses are required for effective protection against PVM rather than a generalised mucosal response. Immunisation via this route was widely accepted for the oral polio vaccine (OPV) which was able to stimulate strong protective immunity towards poliovirus (Beale, 1991). However, oral immunisation has not yet been investigated for a respiratory pathogen.

A further interesting avenue would be to determine whether rAd PVM immunisation could overcome maternal immunity. This could be achieved by immunising female mice before and during pregnancy with a non-lethal dose of PVM, generating strong neutralising antibody responses towards PVM as described in Fig. 6.2. Groups of the pups born to mothers immunised with PBS or rAd PVM constructs could then be challenged with a lethal PVM infection. An indication that the rAd PVM immunised animals were statistically more likely to survive, would indicate that the vaccine could overcome maternal immunity. Similarly, the possibility of pre-sensitising animals to PVM could be explored. This has been achieved for both PVM- and RSV-infected mice using ovalbumin (Barends et al., 2004). This process stimulates a natural T_h2 $CD4^+$ T-cell repsonse towards PVM and RSV. It would be appealing to sensitise animals towards PVM, immunise with the rAd PVM constructs and then monitor the resultant disease and cytokine profile by ELISA or ELISPOT assay for cytokines Il-4, IL-5 and IL-13 levels. This would indicate whether the vaccine construct could skew the immune response away from a non-protective T_h2 response to a protective T_h1 response. If this were the case, then it would have great implications for infant vaccination, as such a vaccine could be given around six months of age to reduce the severity of subsequent RSV infection.

A final aspect worthy of further investigation is the effect of the pre-existing anti-Ad immunity on the establishment of transgene immune responses and thus protection. rAdEV or UV-inactivated wild-type virus could be used to pre-immunise animals before vaccination with the rAd PVM constructs to establish whether pre-existing immunity is detrimental to protection against PVM. Yu *et al* demonstrated that a 10^7

p.f.u dose does not have an effect on transgene immune responses, but higher doses may do (Yu et al., 2008). An alternative Ad serotype 19 ΔE1/E3 vaccine, rAd19-N, has been constructed which expresses the full-length N protein of PVM. This construct is also able to elicit protection in immunised mice in the PVM infection model (Mr S.N. Martin, personal communication). Constructs like rAd19-N could be used in conjunction with rAdN described in this study to determine the effect of a hetelogous prime-boost regime using alternative vaccine vectors. One study compared a prime-boost regime using different rAd vectors expressing the same genes of RSV (Hsu, 1994). This showed that different rAd immunisation serotype combinations exhibited different effects on the resulting immune responses generated. Therefore, the investigation of additional Ad serotype PVM constructs would be beneficial for further study.

This thesis has presented the first proof of principle rAd5 vaccine strategy against pneumovirus using a natural pathogen in its natural host. rAd PVM constructs generated protective immunity against lethal PVM infection which was long-lasting in duration. This work identifies a new method which may be extended for the evaluation of vaccine candidates for RSV. Through evaluation of PVM vaccines, successful candidates can be developed for RSV for clinical trials. Ultimately, the work described here provides a positive scenario suggesting not only that the development of vaccines against RSV is possible, but that the PVM infection model can be used as a platform to evaluate potential vaccine candidates for pneumovirus infections.

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Appendices

Appendix A: Nucleotide sequences

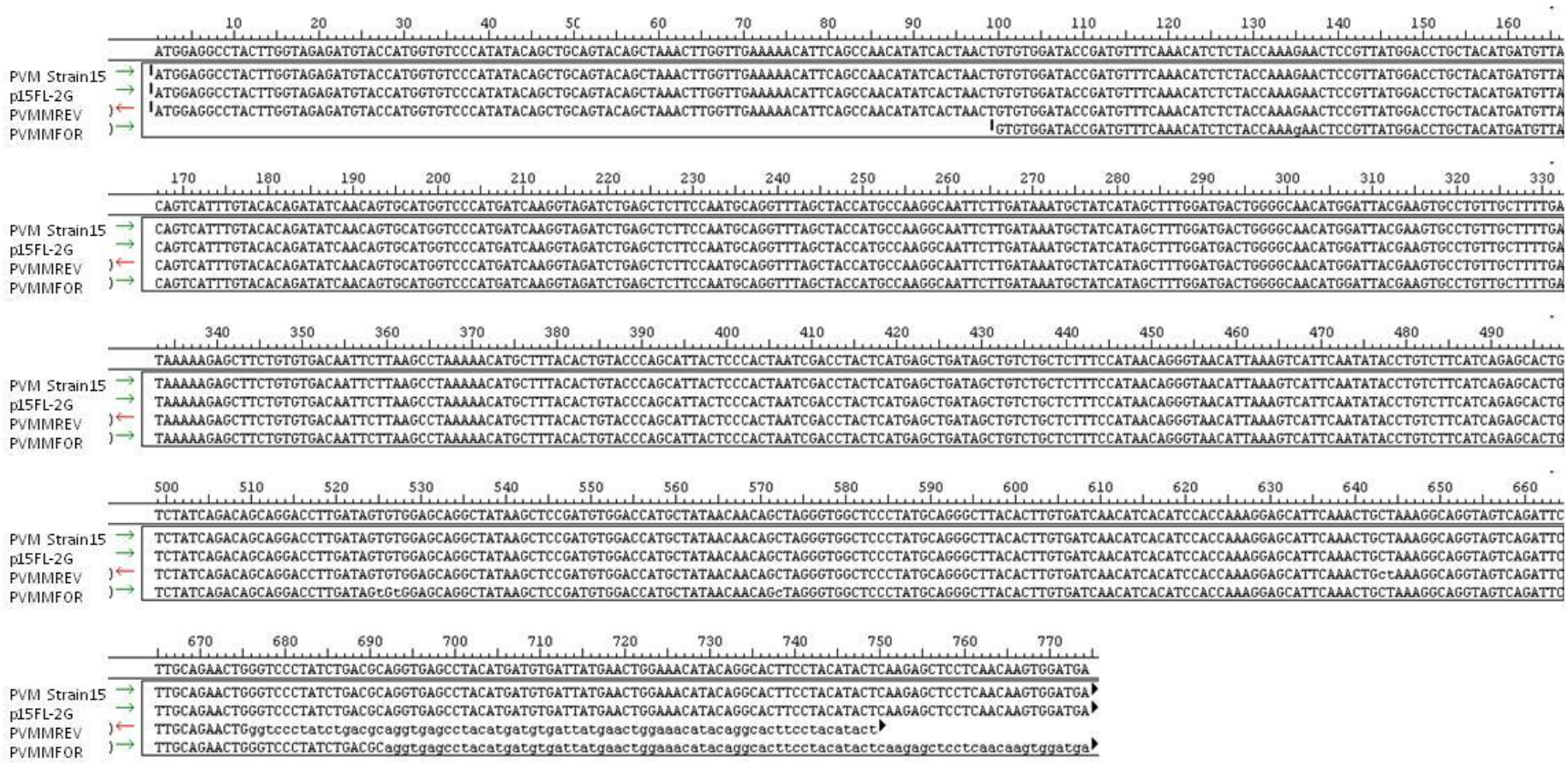
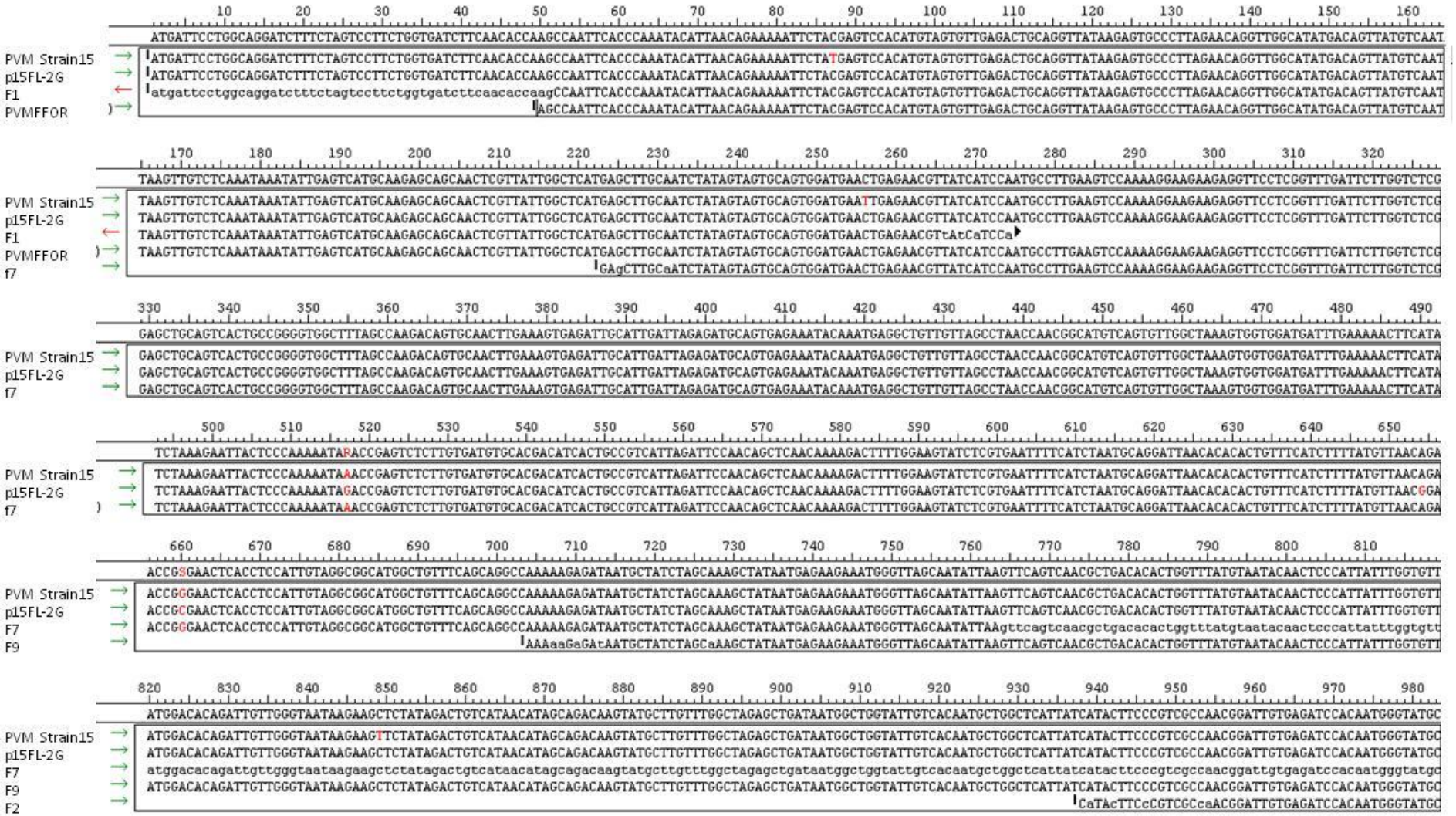


Figure A.1. Nucleotide sequence for analysis of pShuttle_CMV_M.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p15FL-2G
F1
PVMFFOR
f7
F7
F9
F2

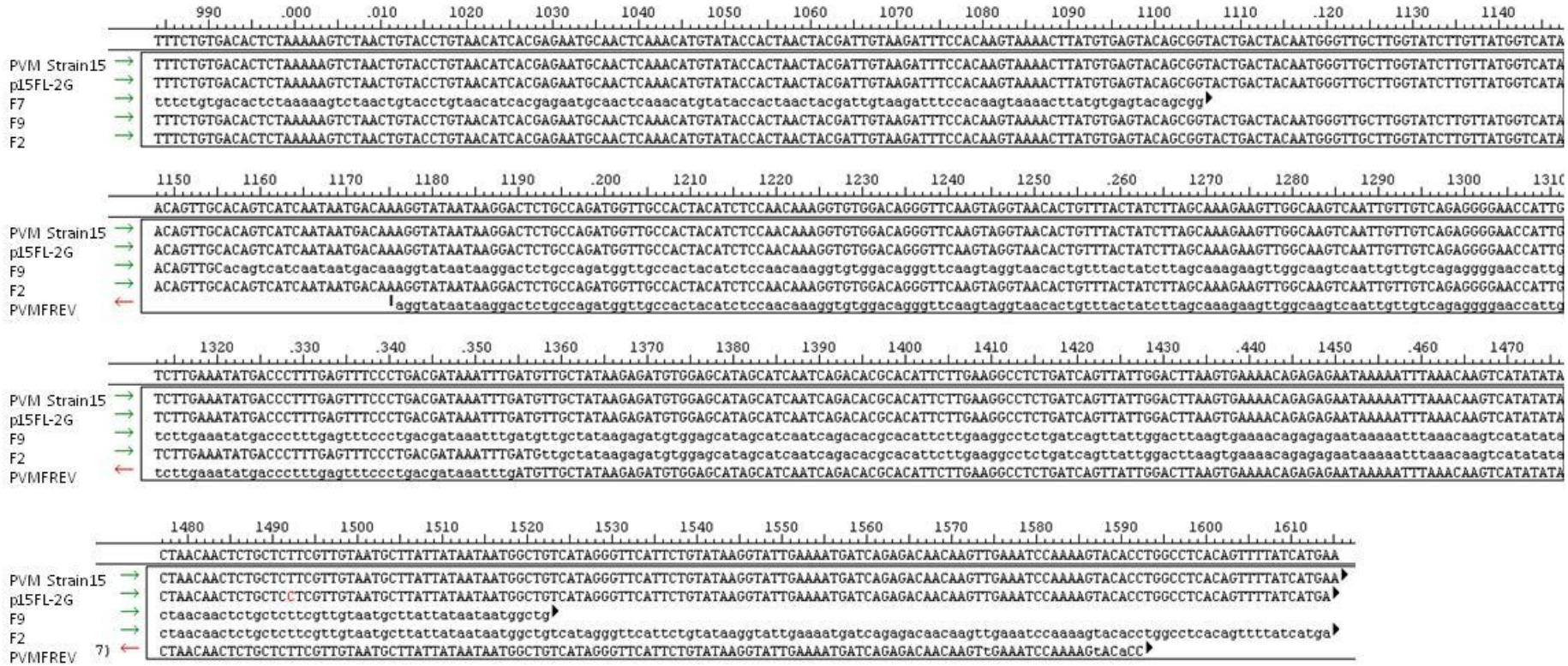
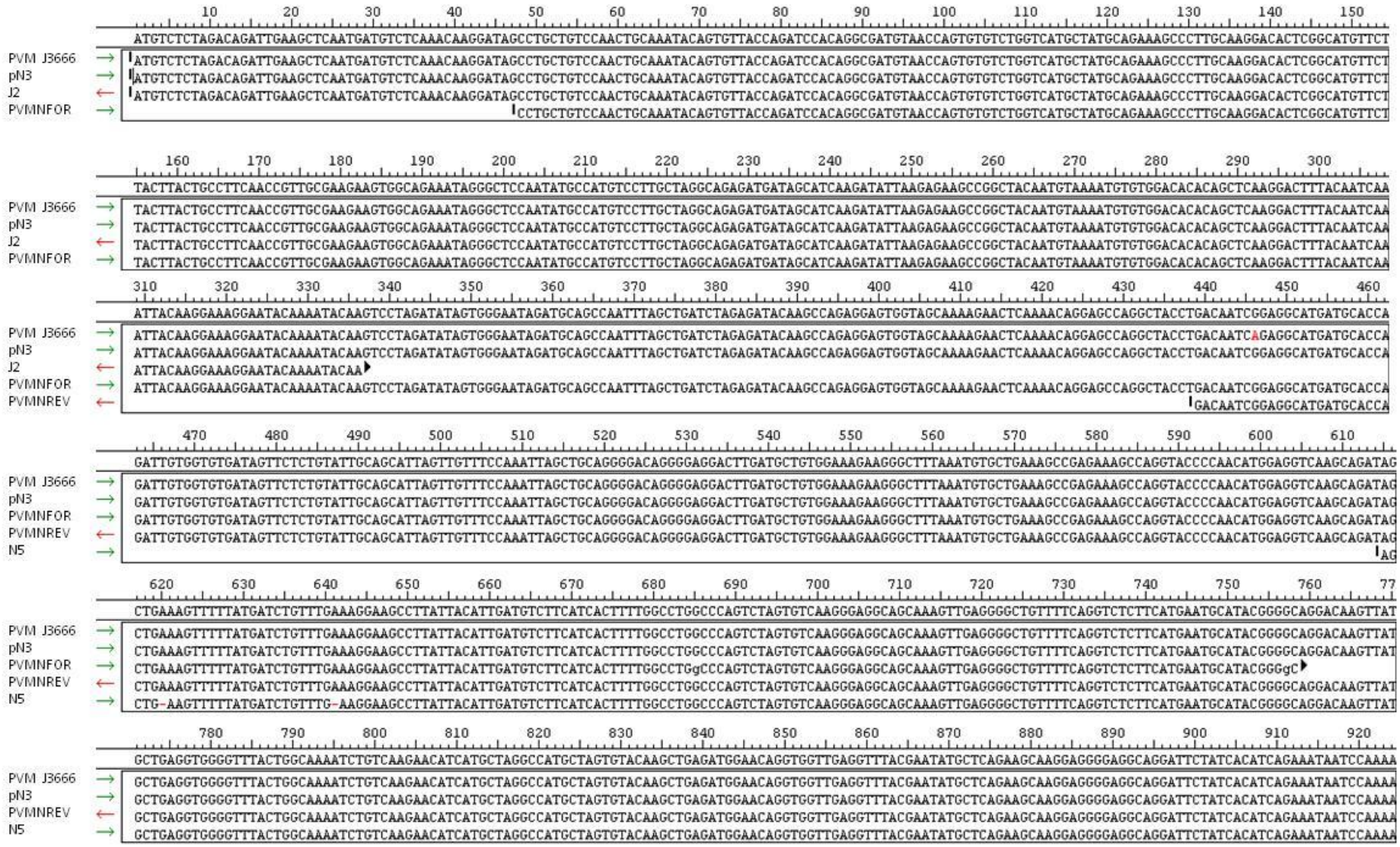


Figure A.2. Nucleotide sequence for analysis of pShuttle_CMV_F.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pN3
J2
PVMNFOR
PVMNREV
N5

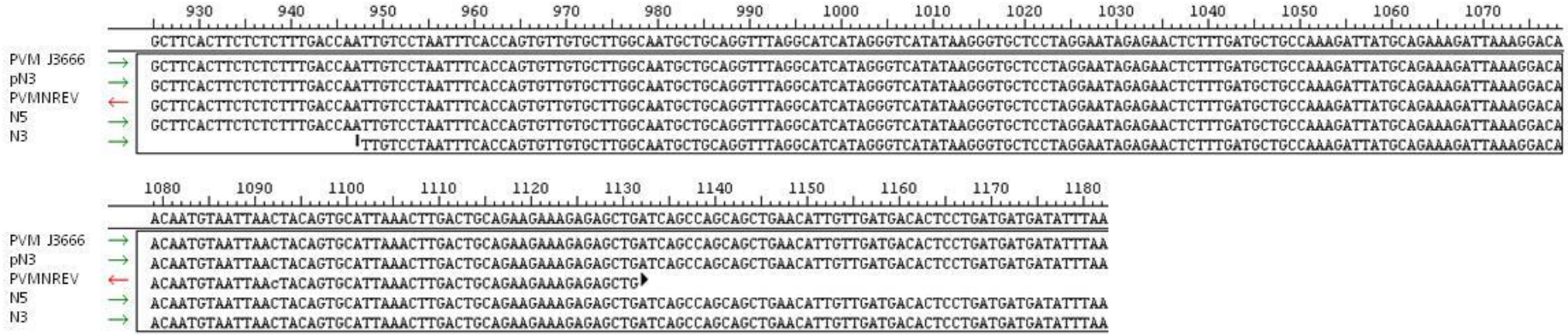

Figure A.3. Nucleotide sequence for analysis of pShuttle_CMV_N.

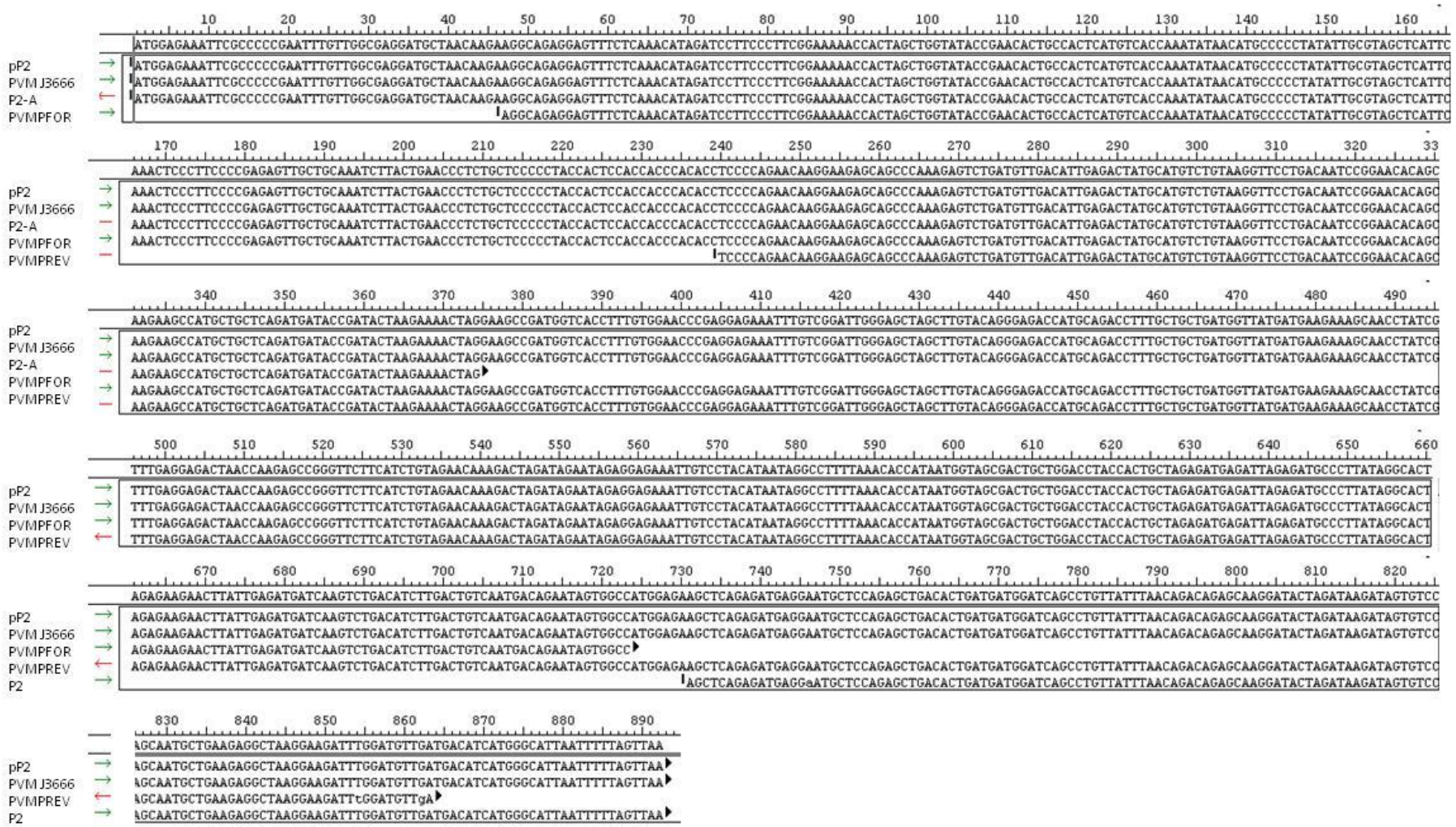

Figure A.4. Nucleotide sequence for analysis of pShuttle_CMV_P.

Appendix B: Plasmids and Virus stocks

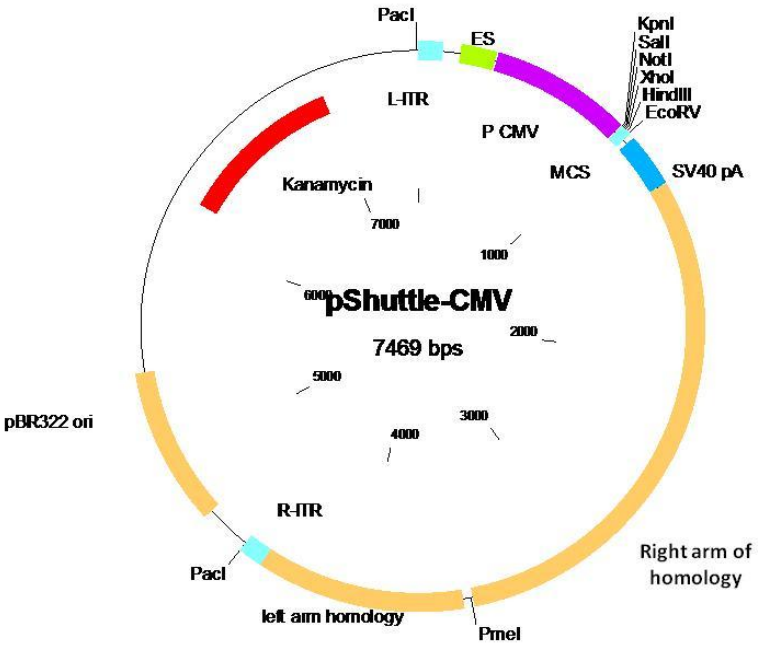

Figure B.1. pShuttle_CMV plasmid map.
The pShuttle_CMV plasmid encodes three regions of homology with pAdEasy, the left and right arms of homology which encode regions of the Ad5 genome, and the pBR322 origin of replication (orange). The plasmid also encodes Kan^R (red), inverted terminal repeats (ITR), encapsidation sequence (ES), CMV promoter (purple), SV40 polyA sequence (royal blue), and MCS (light blue).

Table B.1 Virus stock titres for *in vivo* studies.
The virus, stock number, adenoviral particle number, p.f.u./ml concentration, number of doses at 10^8 p.f.u./50µl and total number of doses per viruses is indicated.

Virus	Stock Number	Particle Number	P.f.u./ml	Particle/p.f.u. ratio	Number of doses at 10^8 p.f.u./50µl	Total number of doses
rAdF	1	$2.9x10^{12}$	$1x10^{11}$	29	430	
	2	$3.43x10^{12}$	$1x10^{11}$	34	500	
	3	$2.66x10^{12}$	$7x10^{10}$	38	252	1182
rAdM	1	$5.4x10^{11}$	$1.15x10^{10}$	47	82	
	2	$1.56x10^{12}$	$1.5x10^{10}$	104	75	
	3	$8.96x10^{11}$	$7.96x10^{9}$	113	28	
	4	$3.8x10^{12}$	$6.44x10^{10}$	59	289	
	5	$5.69x10^{12}$	$4.83x10^{10}$	118	246	
	6	$4.3x10^{12}$	$3.83x10^{10}$	112	145	868
rAdN	1	$8.18x10^{11}$	$1.9x10^{10}$	43	57	
	2	$1.24x10^{12}$	$8.3x10^{9}$	149	43	
	5	$4.299x10^{12}$	$2.25x10^{10}$	191	100	
	6	$3.278x10^{12}$	$4.2x10^{10}$	78	160	
	7	$3.175x10^{12}$	$8x10^{10}$	40	304	
	8	$6.0x10^{13}$	$5.3x10^{10}$	1132	270	
	9	$2.45x10^{12}$	$5.8x10^{10}$	42	240	
	10	$3.88x10^{12}$	$3x10^{10}$	129	153	
	11	$2.08x10^{12}$	$8.85x10^{9}$	235	41	
	12	$4.25x10^{12}$	$4.15x10^{10}$	102	155	1523
rAdZ	1	$5.75x10^{12}$	$6.33x10^{10}$	91	822	
	2	$1.25x10^{12}$	$1.17x10^{10}$	107	87	909

Appendix C: Additional *in vitro* and *in vivo* experimental results

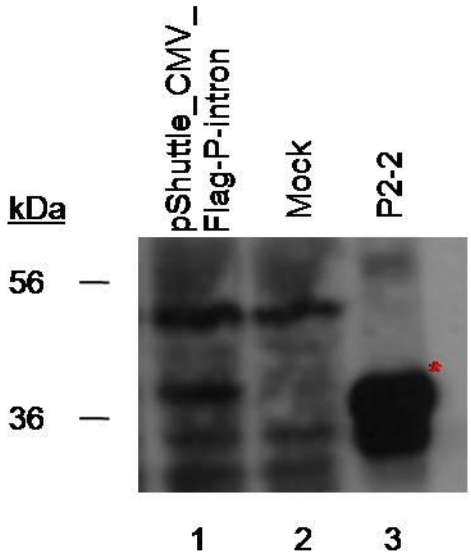

Figure C.1. PVM P protein expression can be detected from plasmid pShuttle_CMV_Flag-P-intron.
$1x10^6$ HEK293 cells were mock-transfected or transfected with 2µg of pShuttle_CMV_Flag-P-intron. P2-2 cells, a positive control, were seeded at $5x10^5$/well. Cells were lysed 72 hours later and lysates were separated by 10% SDS-PAGE prior to Western blotting. The membrane was probed with mouse anti-FLAG M2 monoclonal antibody (Sigma) used 1:10,000, which recognises the FLAG epitope. PVM P protein (*) was detected in both the pShuttle_CMV_Flag-P-intron and P2-2 cells (lanes 1 and 3), proving that PVM P protein is expressed from the construct.

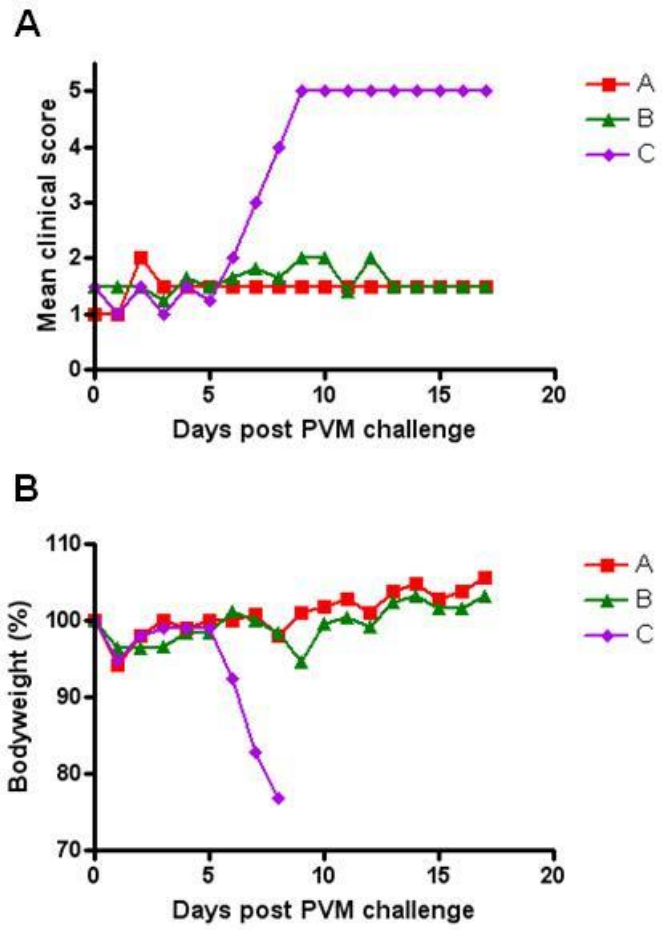

Figure. C.2. BALB/c mice immunised with a high dose of 10^8 p.f.u. of either rAdN or rAdZ are protected against a super lethal PVM infection.
BALB/c mice from one batch of animals were divided into groups of six, each with three male and three females per group. Animals were individually vaccinated with rAdN (group A) or rAdZ (group B) at10^8 p.f.u. dose or PBS (group C) via the intranasal route using a prime boost immunisation regime as previously described in Fig. 4.2. Animals were immunised with the same dose of immunogen on day 14, prior to inoculation with a super lethal dose of PVM strain J3666 of 500 p.f.u./50µl on day 28. Mean clinical score (A) and bodyweight loss (B) were monitored throughout the challenge period. Mean clinical score was calculated from the scores of each individual in an inoculation group. Bodyweight was calculated using the total weight per inoculated group, averaged per animal, with weight on day zero of PVM challenge, normalised to 100%. It should be noted that two animals were lost from group A prior to PVM challenge due to anaesthesia complications.

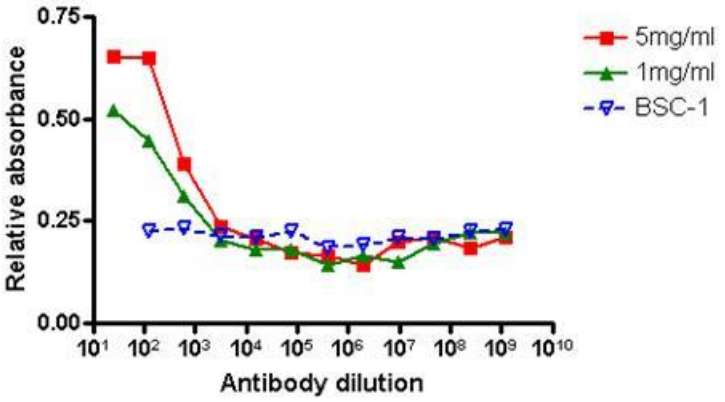

Figure. C.3. P2-2 cell lysate (PVM antigen) optimisation for PVM ELISA.
P2-2 cell lysate was cultured in large roller bottles as detailed in Section 2.4.3 and the ELISA was performed as detailed in Section 2.9.1. P2-2 cell lysate was used at a concentration of either 5mg/ml or 1mg/ml and control BS-C-1 lysate at 5mg/ml concentration. Serum was collected from previously PVM infected but recovered animals and was analysed to determine whether an IgG response towards PVM could be detected using an anti-mouse IgG HRP conjugated secondary antibody (Section 2, Table 2.1.7). A PVM specific IgG response was detected from the positive serum sample. The response detected at 1mg/ml was sufficient therefore, this concentration was used throughout the ELISA analysis. The data are representative of two experimental repeats.

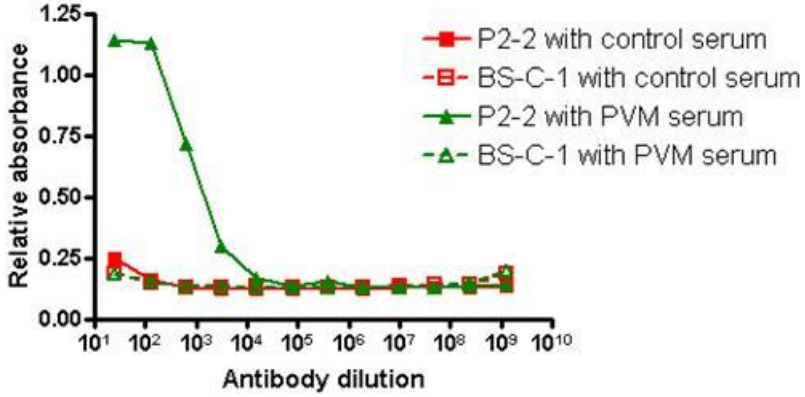

Figure. C.4. The PVM ELISA is specific for PVM antigen.
The PVM ELISA as performed as described in Section 2.9.1., using serum from either influenza infected but recovered animals (control serum, red) or PVM infected but recovered animals (PVM serum, green). Both serum samples were used from neat and only the PVM serum was able to generate a detectable anti-PVM IgG response within the ELISA, confirming that the ELISA was specific for PVM antigen.

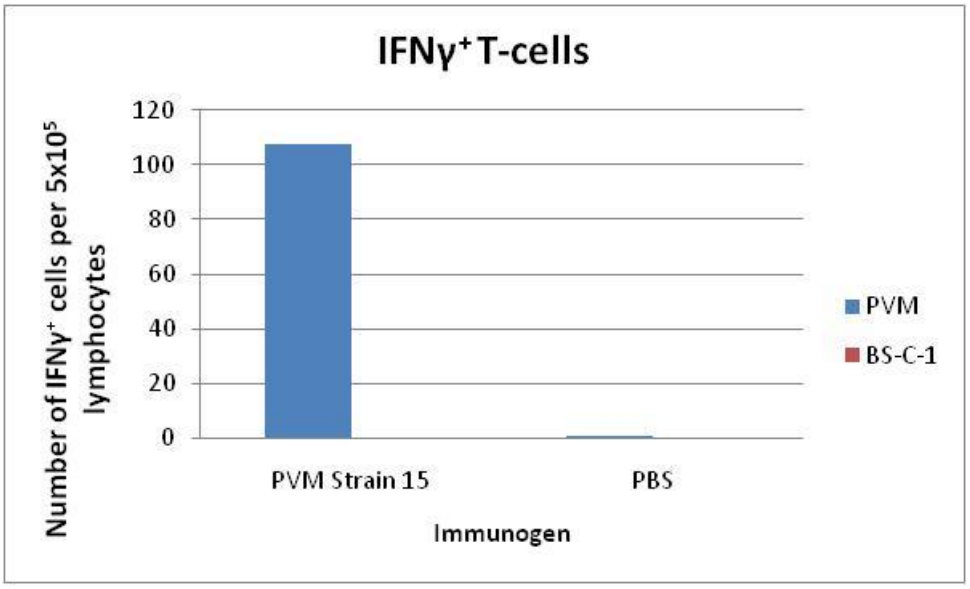

Figure. C. 5. PVM specific IFNγ$^+$ secreting splenocytes can be detected in the ELISPOT assay.
BALB/c mice between 5 and 7 weeks of age were immunised intranasally. with 500 p.f.u. non-pathogenic PVM strain 15 in a 50µl volume or with PBS (control). The animals were sacrificed at 21 days post immunisation and the spleens were collected from individual animals. Splenocytes were isolated for each individual animal and pooled for each immunisation group. The number of IFNγ$^+$ cells in 5x10^5 splenocytes is illustrated in response to re-stimulation with either PVM J3666 at an M.O.I of 1 or the same volume of BS-C-1 control tissue culture fluid.

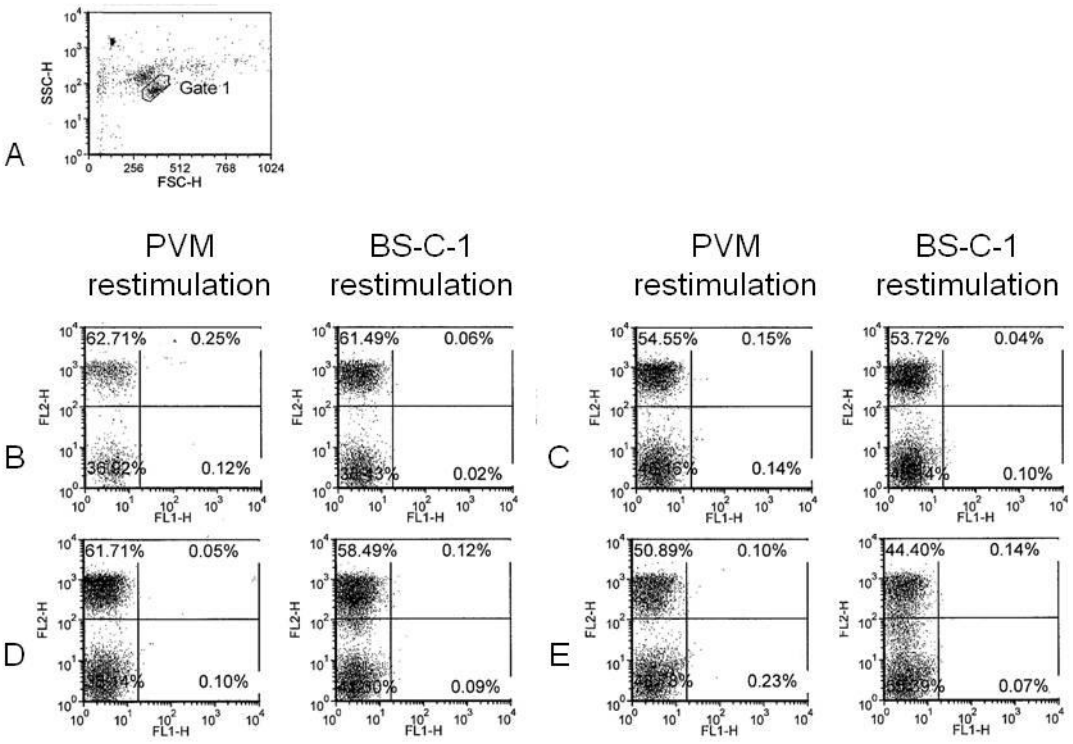

Figure. C. 6. Representative FACS fluorescent data profiles for CD4$^+$ IFNγ$^+$ and CD8$^+$ IFNγ$^+$ secreting splenocytes detected using the ICS assay.
Splenocytes were isolated from individual animals and pooled according to immunisation group. The cells were stained for CD4$^+$ and CD8$^+$ expression on the cell surface, prior to fixing and intracellular staining for IFNγ, using antibodies as detailed in Table 2.1.7. The samples were analysed using a flow cytometer and lymphocyte populations were gated using CellQuest Pro software. Gated lymphocyte populations were analysed for CD4/CD8$^+$ and IFNγ$^+$ expression (A). Representative dot plots for PVM-immunised animals (B), rAdN-immunised animals (C), rAdZ-immunised animals (D), and PBS treated animals (E) are shown.

Appendix D: Statistical analysis of antibody titres

Antibody titres from rAd vaccine immunised animals were compared by the mann-whitney U test, a non-parametric test using PRISM software. The resultant *p* values are represented in the tables below.

rAdF	10^6 day 14	10^6 day 28	10^7 day 14	10^7 day 28	10^8 day 14	10^8 day 28
10^6 day 14	xx	0.0002	0.0007	P<0.0001	P<0.0001	P<0.0001
10^6 day 28	0.0002	xx	0.0587	0.094	0.7925	0.1622
10^7 day 14	0.0007	0.0587	xx	0.0001	0.0479	0.0008
10^7 day 28	P<0.0001	0.094	0.0001	xx	0.0207	0.9301
10^8 day 14	P<0.0001	0.7925	0.0479	0.0207	xx	0.0581
10^8 day 28	P<0.0001	0.1622	0.0008	0.9301	0.0581	xx

Table D.1. Statistical comparison of rAdF-immunised animals for the anti-Ad IgG response at the $10^{6\text{-}8}$ p.f.u. dose at the 24 and 28-day time points.

rAdM	10^6 day 14	10^6 day 28	10^7 day 14	10^7 day 28	10^8 day 14	10^8 day 28
10^6 day 14	xx	0.0042	0.837	P<0.0001	0.073	P<0.0001
10^6 day 28	0.0042	xx	0.0099	0.0172	0.3199	P<0.0001
10^7 day 14	0.837	0.0099	xx	P<0.0001	0.1262	P<0.0001
10^7 day 28	P<0.0001	0.0172	P<0.0001	xx	0.0023	0.0003
10^8 day 14	0.073	0.3199	0.1262	0.0023	xx	P<0.0001
10^8 day 28	P<0.0001	P<0.0001	P<0.0001	0.0003	P<0.0001	xx

Table D.2. Statistical comparison of rAdM-immunised animals for the anti-Ad IgG response at the $10^{6\text{-}8}$ p.f.u. dose at the 24 and 28-day time points.

rAdN	10^6 day 14	10^6 day 28	10^7 day 14	10^7 day 28	10^8 day 14	10^8 day 28
10^6 day 14	xx	0.1068	0.5465	P<0.0001	0.0002	0.0002
10^6 day 28	0.1068	xx	0.1511	0.0014	0.1951	0.0018
10^7 day 14	0.5465	0.1511	xx	P<0.0001	0.0002	P<0.0001
10^7 day 28	P<0.0001	0.0014	P<0.0001	xx	0.0009	0.0797
10^8 day 14	0.0002	0.1951	0.0002	0.0009	xx	0.0005
10^8 day 28	0.0002	0.0018	P<0.0001	0.0797	0.0005	Xx

Table D.3. Statistical comparison of rAdN-immunised animals for the anti-Ad IgG response at the $10^{6\text{-}8}$ p.f.u. dose at the 24 and 28-day time points.

rAdZ	10^6 day 14	10^6 day 28	10^7 day 14	10^7 day 28	10^8 day 14	10^8 day 28
10^6 day 14	xx	1	1	0.0856	0.1898	0.001
10^6 day 28	1	xx	1	0.0856	0.1898	0.001
10^7 day 14	1	1	xx	0.0856	0.1898	0.001
10^7 day 28	0.0856	0.0856	0.0856	xx	0.9083	0.0005
10^8 day 14	0.1898	0.1898	0.1898	0.9083	xx	P<0.0001
10^8 day 28	0.001	0.001	0.001	0.0005	P<0.0001	xx

Table D.4. Statistical comparison of rAdZ-immunised animals for the anti-Ad IgG response at the $10^{6\text{-}8}$ p.f.u. dose at the 24 and 28-day time points.

Appendix E: PVM N peptide sequences

Table. E. PVM N peptide sequences

Peptide number	Peptide Sequence	Corresponding amino acids in PVM N protein
1	MSLDRLKLNDVSNKD	1-15
2	LKLNDVSNKDSLLSN	6-20
3	VSNKDSLLSNCKYSV	11-25
4	SLLSNCKYSVTRSTG	16-30
5	CKYSVTRSTGDVTSV	21-35
6	TRSTGDVTSVSGHAM	26-40
7	DVTSVSGHAMQKALA	31-45
8	SGHAMQKALARTLGM	36-50
9	QKALARTLGMFLLTA	41-55
10	RTLGMFLLTA FNRCE	46-60
11	FLLTA FNRCEEVAEI	51-65
12	FNRCEEVAEIGLQYA	56-70
13	EVAEIGLQYAMSLLG	61-75
14	GLQYAMSLLGRDDSI	66-80
15	MSLLGRDDSIKILRE	71-85
16	RDDSIKILREAGYNV	76-90
17	KILREAGYNVKCVDT	81-95
18	AGYNVKCVDTQLKDF	86-100
19	KCVDTQLKDFTIKLQ	91-105
20	QLKDFTIKLQGKEYK	96-110
21	TIKLQGKEYKIQVLD	101-115
22	GKEYKIQVLDIVGID	106-120
23	IQVLDIVGIDAANLA	111-125
24	IVGIDAANLADLEIQ	116-130
25	AANLADLEIQARGVV	121-135
26	DLEIQARGVVAKELK	126-140
27	ARGVVAKELKTGARL	131-145
28	AKELKTGARLPDNQR	136-150
29	TGARLPDNQRHDAPD	141-155
30	PDNQRHDAPDCGVIV	146-160
31	HDAPDCGVIVLCTAA	151-165
32	CGVIVLCTAALVVSK	156-170
33	LCTAALVVSKLAAGD	161-175
34	LVVSKLAAGDRGGLD	166-180
35	LAAGDRGGLDAVERR	171-185
36	RGGLDAVERRALNVL	176-190
37	AVERRALNVLKAEKA	181-195

38	ALNVLKAEKARYPNM	186-200
39	KAEKARYPNMEVKQI	191-205
40	RYPNMEVKQIAESFY	196-210
41	EVKQIAESFYDLFER	201-215
42	AESFYDLFERKPYYI	206-220
43	DLFERKPYYIDVFIT	211-225
44	KPYYIDVFITFGLAQ	216-230
45	DVFITFGLAQSSVKG	221-235
46	FGLAQSSVKGGSKVE	226-240
47	SSVKGGSKVEGLFSG	231-245
48	GSKVEGLFSGLFMNA	236-250
49	GLFSGLFMNAYGAGQ	241-255
50	LFMNAYGAGQVMLRW	246-260
51	YGAGQVMLRWGLLAK	251-265
52	VMLRWGLLAKSVKNI	256-270
53	GLLAKSVKNIMLGHA	261-275
54	VKNIMLGHASVQAE	266-280
55	LGHASVQAEMEQVV	271-285
56	VQAEMEQVVEVYEY	276-290
57	EQVVEVYEYAQKQG	281-295
58	VYEYAQKQGGEAGF	286-300
59	QKQGGEAGFYHIRN	291-305
60	EAGFYHIRNNPKAS	296-310
61	HIRNNPKASLLSLT	301-315
62	PKASLLSLTNCPNF	306-320
63	LSLTNCPNFTSVVL	311-325
64	CPNFTSVVLGNAAG	316-330
65	SVVLGNAAGLGIIG	321-335
66	NAAGLGIIGSYKGA	326-340
67	GIIGSYKGAPRNRE	331-345
68	YKGAPRNRELFDAA	336-350
69	RNRELFDAAKDYAE	341-355
70	FDAAKDYAERLKDN	346-360
71	DYAERLKDNNVINY	351-365
72	LKDNNVINYSALNL	356-370
73	VINYSALNLTAEER	361-375
74	ALNLTAEERELISQ	366-380
75	AEERELISQQLNIV	371-385
76	LISQQLNIVDDTPD	376-390
77	QQLNIVDDTPDDDI	379-393