ACCEPTED VERSION, Oct 2015
10.1016/j.vaccine.2015.10.105

Intranasal immunisation with recombinant adenovirus vaccines protects against a lethal challenge with pneumonia virus of mice

Helen E. Maunder[a1], Geraldine Taylor[b], Keith N. Leppard[a†*], Andrew J. Easton[a†]

[a] School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK

[b] The Pirbright Institute, Pirbright, Woking GU24 0NF, UK

Email addresses:

a.j.easton@warwick.ac.uk (Andrew Easton); keith.leppard@warwick.ac.uk (Keith Leppard); H.Maunder@oxfordbiomedica.co.uk (Helen Maunder); geraldine.taylor@pirbright.ac.uk (Geraldine Taylor)

*Corresponding author Tel.: +44 2476 523579

[†] These authors contributed equally to the work.

Running title: Vaccine protection against PVM

[1] Present address: Oxford BioMedica (UK) Ltd, The Oxford Science Park, Oxford OX4 4GA

Abstract

Pneumonia virus of mice (PVM) infection of BALB/c mice induces bronchiolitis leading to a fatal pneumonia in a dose-dependent manner, closely paralleling the development of severe disease during human respiratory syncytial virus infection in man, and is thus a recognised model in which to study the pathogenesis of pneumoviruses. This model system was used to investigate delivery of the internal structural proteins of PVM as a potential vaccination strategy to protect against pneumovirus disease. Replication-deficient recombinant human adenovirus serotype 5 (rAd5) vectors were constructed that expressed the M or N gene of PVM pathogenic strain J3666. Intranasal delivery of these rAd5 vectors gave protection against a lethal challenge dose of PVM in three different mouse strains, and protection lasted for at least 20 weeks post-immunisation. Whilst the PVM-specific antibody response in such animals was weak and inconsistent, rAd5N primed a strong PVM-specific $CD8^+$ T cell response and, to a lesser extent, a $CD4^+$ T cell response. These findings suggest that T-cell responses may be more important than serum IgG in the observed protection induced by rAd5N.

Keywords: PVM; HRSV; intranasal immunisation; vaccine; adenovirus vector

1. Introduction

Viruses classified within the genus Pneumovirus include the human (HRSV) and bovine (BRSV) respiratory syncytial viruses and pneumonia virus of mice (PVM) [1]. HRSV is an important respiratory pathogen, causing approximately 30 million cases of acute lower respiratory tract disease in children under 5 annually, some 10% of which require hospitalization [2]. Most infants recover from a natural HRSV infection but some suffer a fatal outcome [3]. Since long-term immunity is not established, infections reoccur throughout life [4] and in elderly or immunocompromised individuals, these can lead to serious complications [5].

An early clinical trial of a formalin-inactivated HRSV vaccine led to exacerbation of disease on subsequent infection [6], associated with induction of low affinity, HRSV-specific antibodies [7], immune complex deposition and complement activation [8], and a Th2-biased immune response [9]. Subsequently, many HRSV vaccine strategies have been investigated [10-14], however no clinically successful candidate has emerged. HRSV vaccine candidates are often investigated in mice, although very high challenge doses are required to give disease and even then, pathogenesis does not match that seen in severely affected human infants [15]. In contrast, low doses of PVM in its natural rodent host give clinical signs ranging from upper respiratory tract infection to fatal pneumonia [16] with pathogenesis that closely resembles severe HRSV disease in humans, making it an appropriate system for investigating pneumovirus pathogenesis and immune responses [17, 18].

HRSV vaccine strategies have focused on stimulating a strong systemic humoral response against the F and G glycoproteins [11, 19]. However, the natural response that resolves

pneumovirus infection is primarily mediated through $CD8^+$ T-cells [20-24]. Recombinant adenoviruses (rAd) have been widely developed as vaccine candidates, including to deliver HRSV glycoproteins [13, 19, 25, 26], and typically elicit potent T_H1-biased responses [27]. We therefore evaluated intranasal (i.n.) delivery of human rAd type 5 (rAd5) PVM recombinants in the mouse as a model for protection against pneumoviruses. We selected this route since it was superior to intramuscular (i.m) delivery in providing protection against either lethal influenza A virus challenge or HRSV replication in the lung [13, 28]; it is also now an established route for human vaccination [29]. We focused on the internal proteins M and N since they contain potent cytotoxic T-cell (CTL) epitopes in HRSV [30, 31] and $H\text{-}2D^b$ and $H\text{-}2K^b$ restricted $CD8^+$ T cell epitopes in PVM [32, 33]. Furthermore, vaccinating calves with N protein vectors primed BRSV-specific T cells and conferred partial protection against BRSV challenge [34, 35] and mucosal immunisation of mice with HRSV N protein nanoparticles induced both specific antibody and T cell responses, and reduced HRSV pulmonary replication [36]. We show here that rAd5 containing the M or N gene of PVM is able to elicit long-term protection against lethal PVM infection in mice, correlating with the stimulation of PVM-specific CD4 and CD8 T-cell populations.

2. Materials and methods

2.1 Cell and virus culture

A line of persistently PVM-infected cells (designated P2-2) was established by infecting BSC-1 African green monkey kidney cells with PVM strain 15 at a multiplicity of infection of 0.01 pfu per cell [37]. Following the initial appearance of cytopathic effect and loss of cells, medium was replaced every 2-3 days. Within 4 weeks, growth of persistently infected cells was detected and these were subsequently passaged as normal. P2-2 cells show PVM gene

82 expression by immunofluorescence and western blotting, and continuously produce
83 infectious virus which is detectable in the growth medium.

84 BS-C-1 cells, P2-2 cells and HEK293 cells were cultured in Glasgow minimal essential media
85 (GMEM) plus 10% fetal bovine serum (FBS), GMEM plus 15% FBS or Dulbecco's modified
86 Eagle media (DMEM) plus 10% FBS respectively. PVM strain J3666 stocks were prepared and
87 titrated in BS-C-1 cells as described previously [17]. Ad5 *dl*327 [38] was propagated in
88 HEK293 cells cultured in DMEM plus 2% FBS. rAd5 stocks were amplified and titrated by
89 plaque assay on HEK293 cells.

90 *2.2 Construction and characterization of recombinant Ad5 vectors*
91 rAd5 with E3 deletions and E1 genes replaced by transgenes were generated using the
92 AdEasy™ Adenoviral Vector System (Stratagene) according to the manufacturer's protocol.
93 pShuttle-CMV plasmids, containing the M or N gene of PVM strain J3666 (Genbank
94 AY743909) amplified by PCR from cDNAs using primers that included appropriate restriction
95 enzyme sites, were used to generate rAd5M and rAd5N; the CMV promoters and transgene
96 regions were verified by sequencing [39, 40]. A plasmid containing the *Escherichia coli lac*Z
97 gene was used similarly to generate rAd5Z. Recombinant viruses were purified twice by
98 caesium chloride density gradient centrifugation and dialysed against 20% glycerol in
99 phosphate-buffered saline (PBS).

100 *2.3 Animal immunisation and processing*
101 Male and female BALB/c mice from the breeding colony at the University of Warwick were
102 confirmed PVM-free by serology and used at 5-7 weeks old. C3H/He-mg animals were
103 similarly sourced in-house [41]; 5-week old C57BL/6 mice were purchased from Charles
104 River. Animal care was carried out in accordance with the UK Animal Scientific Procedures

105 Act 1986 and was approved by the University of Warwick Ethical Review Board. Mice were
106 allocated to experimental groups with equal sex ratios, anaesthetised by intraperitoneal
107 injection with ketamine (75 µg/g bodyweight) and xylazine (15 µg/g bodyweight) and
108 inoculated i.n. with rAd doses in 50 µl PBS or PBS alone (control animals); repeat
109 immunisations were at two week intervals. Animals were challenged i.n. with a lethal dose
110 of 250 p.f.u. PVM strain J3666 in 50 µl PBS. Clinical signs were assessed as previously
111 described [17] and bodyweight measured daily. Animals were sacrificed for welfare reasons,
112 or at the end of the experiment, by cervical dislocation.

113 *2.4 ELISA*

114 Flat-bottom microtitre plates were coated overnight at 4 °C with 1 µg/ml of either purified
115 Ad5 *dl*327, or P2-2 or BS-C-1 cell lysate (cell material harvested and fragmented by agitation
116 with glass beads, then sonicated), then incubated for 2 h with blocking buffer (5% low fat
117 dried milk in PBS containing 0.02% (w/v) Tween 20 (PBS-T)). Serum, or broncho-alveolar
118 lavage (BAL) fluid obtained using 1.0 ml PBS per animal, was titrated in threefold dilutions
119 after an initial 1:85 dilution in blocking buffer. Antibody was bound for 2 h at room-
120 temperature (r.t.), wells washed 3x with PBS-T, then horseradish peroxidise conjugated goat
121 anti-mouse (Sigma) IgG –specific antibodies (Santa Cruz), diluted in blocking buffer, were
122 added for 2 h at r.t. After 3x washing, 100 µl/well of 0.01 mg/ml 2,2'-azino-bis(3-
123 ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS) solution (Sigma) was added
124 and absorbance measured at 405 nm using a Labsystems multiskan RC plate reader.
125 Antibody levels were determined by linear regression analysis and values expressed as Log_{10}
126 endpoint titres. Statistical analysis was performed using the Mann Whitney U test with
127 Prism software.

2.5 In vitro stimulation and analysis of PVM-specific lymphocytes

Spleens or lungs in 2 ml PBS were passed through cell strainers, generating single-cell suspensions that were centrifuged at 1200 x *g* over Histopaque 1086 (Sigma, Poole, UK). Mononuclear cells at the interface were harvested, washed 3x in PBS and resuspended in RPMI 1640 medium (Life Technologies) containing 10% FBS, 0.1% β-mercaptoethanol, 10 U/ml penicillin G and 10 µg/ml streptomycin sulfate (RPMI/10). Cells were re-stimulated *in vitro* with PVM-infected or mock-infected antigen-presenting cells (APCs) at a ratio of 10:1 in 96-well U-bottom plates containing 200 µl of RPMI/10 further supplemented with 5 U/ml recombinant human IL-2 (Roche) and 10 µg/ml Brefeldin A (Calbiochem®) for 16 h at 37 ^{o}C. APCs were naïve spleen cells, either PVM-infected (1 p.f.u. /cell) or mock-infected with BS-C-1 cell lysate for 90 min at 37 ^{o}C, irradiated (3000 rad) and washed. After re-stimulation, cells were surface stained with rat anti-mouse CD8α allophycocyanin or rat anti-mouse CD4 phycoerythrin mAbs (BD Pharmingen). Intracellular staining used CytoFix/CytoPerm solution and Perm/Wash buffer according to the manufacturer's instructions (BD Biosciences), and rat anti-mouse IFNγ-FITC (BD Biosciences). Cells were analysed on a FACSCalibur flow cytometer (BD Biosciences) and data analysed using CellQuest software.

3. Results

3.1 Immunisation with rAd5N or rAd5M protects mice against lethal PVM infection

Expression of PVM M and N by rAd5 in cell culture was confirmed by immunofluorescence or Western blotting, respectively (data not shown).To evaluate protection by rAd5M, rAd5N or rAd5Z, mice were immunised i.n. on day 0 and day 14, then challenged on day 28 with a lethal dose of PVM and monitored for 2-3 weeks for clinical signs and bodyweight loss as previously described [17]. Animals receiving 10^6 p.f.u. of rAd5M or rAd5N developed severe

clinical signs of disease and significant weight loss upon challenge, similar to PBS mock-immunised animals and were therefore not protected (data not shown). In contrast, all animals that received higher doses of rAd5M or rAd5N survived (Fig. 1). Animals receiving 10^7 p.f.u. of either recombinant showed transient signs of infection and weight loss but made a complete recovery (Fig. 1A, B) whereas animals immunised with rAd5Z developed severe disease after challenge, equivalent to mock-immunised animals (Fig. 1C). Thus, at the 10^7 p.f.u. dose, there was clear evidence of antigen-specific protection elicited by rAd5N and rAd5M. Survival data from these and subsequent experiments are summarised in Table 1.

Animals receiving 10^8 p.f.u. of rAd5M or rAd5N remained healthy after challenge (Fig. 1A, B). Surprisingly however, 10^8 p.f.u. of control rAd5Z was also protective (Fig. 1C) although, in contrast to those receiving specific immunogen, rAd5Z animals developed some signs of illness. Thus the 10^8 p.f.u. dose protected mice effectively from lethal challenge but a significant component of that protection was antigen-nonspecific. This non-specific protection is clearly distinct from that previously shown to be produced by defective-interfering (DI) influenza A virus against PVM. DI-based protection was undetectable 7 days post-administration of the protective virus and was interferon-dependent [41] whereas rAd5Z-based protection lasted for at least two weeks. Under our prime-boost protocol, and in contrast to its effect on PVM, 10^8 p.f.u. rAd5Z afforded no protection against influenza A virus (data not shown).

To investigate single dose efficacy, mice were immunised and challenged with PVM six weeks later. Mice given 10^7 p.f.u. rAd5M or rAd5N developed transient weight loss and elevated clinical scores but made a full recovery, stabilising with bodyweights only a few percent from the starting weight (Fig. 2A) whilst rAd5Z gave no protection. These data

174 indicate that even a single i.n. dose of 10^7 p.f.u. of rAd5M or rAd5N elicits PVM-specific
175 protection, although rAd5N was clearly more protective than rAd5M in this regimen. Similar
176 to the two-dose regimen, one 10^8 p.f.u. dose of rAd5M or rAd5N gave full protection against
177 disease whilst an equivalent single dose of rAd5Z also protected against lethal outcome,
178 albeit with some signs of disease (Fig. 2B).

179 Antigen-specific protection implied involvement of adaptive responses. Such responses vary
180 in outbred populations because epitopes must be presented by highly polymorphic major
181 histocompatibility complex (MHC) antigens. We therefore tested whether protection in
182 BALB/c mice (MHC haplotype $H2^d$) could be replicated in other mouse strains. C57BL/6 ($H2^b$)
183 and C3H/He-mg ($H2^k$) mice, immunised with 10^7 p.f.u. rAd in a two-dose regime, were fully
184 protected from PVM by rAd5N immunisation, as were C57BL/6 animals by rAd5M; in each
185 case animals remained healthy following challenge (data not shown). rAd5M only partially
186 protected C3H/He-mg animals with two of five succumbing during the challenge period,
187 although one of these occurred without prior elevation in clinical score and so may not have
188 been due to the challenge (data not shown). Thus, protection against lethal PVM infection
189 by rAd PVM recombinants extends to multiple MHC haplotypes.

190 Antigen-specific protection against PVM was observed at four and six weeks after the
191 primary immunisation. To determine the duration of protection, BALB/c mice were
192 challenged at 8, 11, 14 or 20 weeks after initial immunisation with 10^7 p.f.u. rAd in a two-
193 dose protocol (Fig. 3). Older animals are slightly less susceptible to PVM-induced disease
194 [42] and this was reflected in the survival of some PBS mock-immunised animals. However,
195 these control animals showed significantly greater weight loss and clinical scores than
196 immunised animals following PVM challenge at any time-point. rAd5N or rAd5M protected

against lethal PVM challenge up to 20 weeks post-immunisation (Fig. 3A, B). Whilst rAd5M-immunised animals showed some weight loss, particularly following prolonged delay between immunisation and challenge, this was transient and less severe than in rAd5Z-immunised mice, which showed severe disease similar to the mock-immunised animals (Fig. 3C). Thus, immunisation with 10^7 p.f.u. rAd5M or rAd5N can elicit long-lasting, antigen-specific protection in mice against severe or lethal PVM disease.

3.2 Immunisation with rAd5 PVM vectors induces a variable PVM-specific antibody response

Vaccination with 10^7 p.f.u. rAd5N elicited no detectable anti-PVM serum IgG by day 14 (Fig. 4A), and although a few mice showed some response at day 28, 2 weeks after the second immunisation, most still had no detectable PVM-specific antibody. Vaccination with 10^8 p.f.u. rAd5N generated a greater anti-PVM IgG response, with one animal responding at day 14 and an increased average titre among day 28 responding animals (Fig. 4B) although not all animals developed detectable antibody. This weak PVM-specific antibody response did not reflect an intrinsic problem in eliciting serum antibody responses by this vector and route of administration since robust anti-Ad5 serum antibody responses were induced by both 10^7 and 10^8 p.f.u. rAd (Fig. 4C, D). It was also not due to the assay failing to detect antibody directed against internal proteins of the virus, since the assay was highly effective in detecting binding of N-specific mAbs in comparison with either F-specific mAbs or polyclonal sera from PVM-infection mice (Fig. 4E).

To determine whether anti-PVM IgG might develop or increase over a longer-term experiment, animals were immunised with 10^7 p.f.u. rAd in a two-dose regime and serum analysed by ELISA at weeks 8, 11, 14 and 20. Although some rAd5M-immunised animals possessed anti-PVM IgG at weeks 6, 8 and 11 (Fig. 5A), at no time point did any more than a

minority show a response. For rAd5N, only one animal developed detectable anti-PVM IgG by week 6 but the numbers responding and magnitude of response increased in weeks 8 and 11 (Fig. 5B); again, only a minority showed a detectable response at any time point and anti-PVM IgG antibodies were no longer detectable by week 20. As expected, rAd5Z elicited no detectable anti-PVM IgG at any time (Fig. 5C). In contrast, all animals mounted a detectable anti-Ad5 response, confirming delivery of the immunogen (Fig. 5D-F). The absence of consistent anti-PVM antibody responses in the context of consistent protection from lethal PVM challenge suggested that an anti-PVM IgG response was not the primary mechanism of protection.

3.3 Immunisation with rAd5N vector elicits a PVM-specific T-cell response

rAd5N was selected to study T cell responses since it generated the more robust protection. Mice were immunised with 10^7 p.f.u in a two dose regimen and lymphocytes harvested for analysis on day 20. IFN-γ intracellular staining revealed that the majority of PVM-specific T-cells induced by rAd5N in the spleen and lungs were $CD4^+$ cells (0.2% $CD4^+$ $IFN\gamma^+$ in spleen and 0.08% $CD4^+IFN\gamma^+$ in lungs). In contrast, no PVM-specific $CD8^+$ T-cells were observed (data not shown). However, 7 days after immunisation with three doses of 10^7 p.f.u. of rAd5N, 2 weeks apart, the majority of PVM-specific T-cells induced by rAd5N in the spleen were $CD8^+$ T cells (9.2% $CD8^+IFN\gamma^+$ compared with 2.04% $CD4^+IFN\gamma^+$, Fig. 6). Notably, PVM-specific T-cells were not detected in lung lymphocytes from these mice (data not shown); this may have been related to the high background IFNγ response observed in lung lymphocytes from these animals. As PVM-specific T cells were not elicited by rAd5Z (Fig. 6), the PVM-specific $CD4^+$ and $CD8^+$ splenocytes in rAd5N-immunised animals demonstrate that rAd5N induces a significant PVM-specific T-cell response.

4. Discussion

Ad5 has a natural tropism for mucosal surfaces and is a potent stimulator of adaptive immunity [27, 43] making it an attractive candidate vector for recombinant pneumovirus vaccine development. We evaluated the properties of rAd5 vectors expressing PVM N or M proteins and found that i.n. immunisation with 10^7 p.f.u of either construct elicited protection against PVM lethal disease. This protection was maintained for 20 weeks in a two-dose regimen and for at least six weeks after a single immunisation. Protection also extended to mouse strains having three different MHC haplotypes.

Only weak and inconsistent anti-PVM serum IgG responses were detected in immunised animals that were reliably protected against lethal PVM challenge, indicating that there was no correlation between the humoral response and protection. Notably, anti-PVM IgG had declined to undetectable levels by 20 weeks post immunisation yet all animals were still protected. Specific protection was not explained by priming for a rapid PVM-specific antibody response as there was no difference in specific IgG titres between rAd5N-immunised and control vector or rAd5Z-immunised animals post-challenge (data not shown). PVM-specific antibody responses might be weak because PVM M and N proteins do not contain strong B-cell epitopes or because the route of administration could not elicit such responses. The latter is unlikely since rAd5 expressing HRSV antigens elicited good serum IgG responses when delivered by the i.n. route [13, 26] and our immunisations reliably elicited Ad5-specific IgG. We also considered whether our protocol might have elicited greater mucosal than serum antibody responses, however we did not detect any PVM-specific IgA in BAL fluid obtained from vaccinated mice. Therefore, whilst it remains

possible that rAd5N and rAd5M each elicited some form of antibody that our assays could not detect and which was the basis of protection, we consider this unlikely.

In contrast to the weak PVM-specific antibody responses, rAd5N immunisation produced strong PVM-specific $CD8^+$ and $CD4^+$ T cell responses in the spleen. PVM-specific T cells are known to protect against challenge [23] and we propose that this cellular response is a major factor mediating specific protection in our study. Since rAd5N elicited protection against PVM challenge in three different mouse strains, this suggests that PVM N contains T-cell epitopes that are recognised in multiple MHC backgrounds.

Previous studies with rAd HRSV vaccine candidates have included vectors expressing the F protein [25, 26, 44, 45], G protein elements [13] or F, N and M2-1 proteins [46] which elicited strong anti-HRSV serum antibody and potent T-cell responses in mice. In contrast to PVM rAd5 studied here, vaccination with a chimpanzee rAd (PanAd3) expressing F, N and M2-1 caused accelerated onset of weight loss in mice following HRSV challenge, a difference most likely due to the high titre HRSV challenge inoculum encountering a strong $CD8^+$ response induced by M2-1 [46]. However, whilst these studies demonstrate that various rAds and dosing regimens can inhibit HRSV replication in mouse lungs, the failure of even high-dose HRSV challenge of mice to fully replicate the pathogenesis of RSV infection in humans means the implications of these studies for protection of humans against HRSV disease are not clear.

Whilst 10^7 p.f.u. rAd5N and rAd5M clearly elicited antigen-specific protection against PVM, significant non-specific protection against PVM disease was seen with a 10-fold higher dose rAd5Z, albeit less effective than equivalent doses of rAd5N or rAd5M. A similar finding of non-specific protection was reported previously in ferrets immunised with high dose rAd:

288 though having no effect on SARS coronavirus replication in the nose, control rAd reduced
289 virus penetration into the lung and consequent pathology [47]. Many other studies of rAd
290 immunisation have not included both empty rAd and non-immunised controls so any non-
291 specific protection would not have been detected. In one further study that incorporated
292 both controls, non-specific protection in the airway was not seen after rAd delivery to mice
293 i.m. [48]; this difference from our findings likely reflects the distinct immunisation and
294 challenge target tissues preventing any non-specific local responses to the immunogen
295 being protective. Further investigation is needed to understand the nature and scope of
296 non-specific protection against respiratory infection following high-dose intranasal rAd
297 delivery.

298 One potential issue in extrapolating the present study to protection against HRSV in humans
299 is that widespread immunity to Ad5 might compromise immunisation of infants with rAd5
300 via maternal antibody [49]. However pre-existing circulating Ad5 antibody did not prevent a
301 response to mucosally delivered rAd5-Ebola antigen that was sufficient to protect mice
302 against lethal Ebolavirus challenge [50] so pre-existing antibody to rAd5 is not an absolute
303 barrier to effective use. Alternatively, vectors based on either human Ad types of lower
304 sero-prevalence, non-human Ads [45] or chimeric Ad5 vectors having substitutions in key
305 antigenic regions [51] may be used, although these alternative Ad types may not share the
306 strong immunogenicity of Ad5 [52].

307 In summary, we have shown that replication-deficient rAd5 vectors expressing internal
308 antigens of PVM elicit robust protection against PVM disease in multiple mouse strains
309 when delivered i.n. Protection can be generated with a single dose, and a two-dose regime
310 provides long-term protection. As PVM pathogenesis in rodents is similar to HRSV infection

311 in humans, our findings of successful protection against disease in this model suggest that a
312 similar strategy could be applied to protect against HRSV disease in humans.

313 **Acknowledgements**

314 The authors gratefully acknowledge the technical support provided by staff of the
315 Biomedical Support Unit, University of Warwick, Sara Wyld at The Pirbright Institute,
316 Compton, and Drs Paul Scott and Susan Morris for valuable discussions. HEM was supported
317 by a studentship from the Medical Research Council, U.K. The funder had no involvement in
318 the design of the study.

319 **Conflicts of Interest**

320 The authors declare no conflicts of interest.

321 **References**

322 [1] Easton AJ, Ling R. Mononegavirales. In: Mahy BWJ, Van Regenmortel MHV, editors.
323 Encyclopedia of Virology, 3rd edn. Oxford: Elsevier; 2008. p. 324-34.
324 [2] Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, et al. Global burden
325 of acute lower respiratory infections due to respiratory syncytial virus in young children: a
326 systematic review and meta-analysis. Lancet. 2010;375:1545-55.
327 [3] Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, et al. The
328 burden of respiratory syncytial virus infection in young children. New Engl J Med.
329 2009;360:588-98.
330 [4] Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection
331 with respiratory syncytial virus. J Infect Dis. 1991;163:693-8.
332 [5] Cowton VM, McGivern DR, Fearns R. Unravelling the complexities of respiratory
333 syncytial virus RNA synthesis. J Gen Virol. 2006;87:1805-21.

[6] Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol. 1969;89:422-34.

[7] Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med. 2009;15:34-41.

[8] Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H, et al. A role for immune complexes in enhanced respiratory syncytial virus disease. J Exp Med. 2002;196:859-65.

[9] Knudson CJ, Hartwig SM, Meyerholz DK, Varga SM. RSV Vaccine-Enhanced Disease Is Orchestrated by the Combined Actions of Distinct CD4 T Cell Subsets. PLoS Pathog. 2015;11:e1004757.

[10] Karron RA, Wright, P.F., Belshe, R. B., Thumar, B., Casey, R., Newman, F., Polack, F.P., Randolph, V.B., Deatly, A., Hackell, J., Gruber, W., Murphy, B.R., Collins, P.L. Identification of a recombinant live attenuated respiratory syncytial virus vaccine candidate that is highly attenuated in infants. J Infect Dis. 2005;191:1093-104.

[11] Murawski MR, McGinnes LW, Finberg RW, Kurt-Jones EA, Massare MJ, Smith G, et al. Newcastle disease virus-like particles containing respiratory syncytial virus G protein induced protection in BALB/c Mice, with no evidence of immunopathology. J Virol. 2010;84:1110-23.

[12] Wu H, Dennis VA, Pillai SR, Singh SR. RSV fusion (F) protein DNA vaccine provides partial protection against viral infection. Virus Res. 2009;145:39-47.

[13] Yu J-R, Kim S, Lee J-B, Chang J. Single intranasal immunization with recombinant adenovirus-based vaccine induces protective immunity against respiratory syncytial virus infection. J Virol. 2008;82:2350-7.

359 [14] Zeng R, Zhang Z, Mei X, Gong W, Wei L. Protective effect of a RSV subunit vaccine
360 candidate G1F/M2 was enhanced by a HSP70-like protein in mice. Biochem Biophys Res
361 Commun. 2008;377:495-9.
362 [15] Rosenberg HF, Bonville CA, Easton AJ, Domachowske JB. The pneumonia virus of
363 mice infection model for severe respiratory syncytial virus infection: identifying novel targets
364 for therapeutic intervention. Pharmacol Ther. 2005;105:1-6.
365 [16] Horsfall FLJ, Hahn RG. A latent virus in normal mice mapable of producing pneumonia
366 in its natural host. J Exp Med. 1940;71:391-408.
367 [17] Cook PM, Eglin RP, Easton AJ. Pathogenesis of pneumovirus infections in mice:
368 detection of pneumonia virus of mice and human respiratory syncytial virus mRNA in lungs
369 of infected mice by in situ hybridization. J Gen Virol. 1998;79:2411-7.
370 [18] Dyer K, Garcia-Crespo K, Glineur S, Domachowske J, Rosenberg H. The Pneumonia
371 Virus of Mice (PVM) Model of Acute Respiratory Infection. Viruses. 2012;4:3494-510.
372 [19] Shao H-Y, Yu S-L, Sia C, Chen Y, Chitra E, Chen IH, et al. Immunogenic properties of
373 RSV-B1 fusion (F) protein gene-encoding recombinant adenoviruses. Vaccine.
374 2009;27:5460-71.
375 [20] Claassen EA, van der Kant PA, Rychnavska ZS, van Bleek GM, Easton AJ, van der
376 Most RG. Activation and inactivation of antiviral CD8 T cell responses during murine
377 pneumovirus infection. J Immunol. 2005;175:6597-604.
378 [21] Ostler T, Ehl, S. Pulmonary T cells induced by respiratory syncytial virus are functional
379 and can make an important contribution to long-lived protective immunity. Eur J Immunol.
380 2002;32:2562-9.
381 [22] Simmons CP, Hussell T, Sparer T, Walzl G, Openshaw P, Dougan G. Mucosal delivery
382 of a respiratory syncytial virus CTL peptide with enterotoxin-based adjuvants elicits

383 protective, immunopathogenic, and immunoregulatory antiviral CD8+ T cell responses. J
384 Immunol. 2001;166:1106-13.
385 [23] van Helden MJG, van Kooten PJS, Bekker CPJ, Gröne A, Topham DJ, Easton AJ, et al.
386 Pre-existing virus-specific CD8+ T-cells provide protection against pneumovirus-induced
387 disease in mice. Vaccine. 2012;30:6382-8.
388 [24] Taylor G, Thomas LH, Wyld SG, Furze J, Sopp P, Howard CJ. Role of T-lymphocyte
389 subsets in recovery from respiratory syncytial virus infection in calves. J Virol.
390 1995;69:6658-64.
391 [25] Fu Y, He J, Zheng X, Wu Q, Zhang M, Wang X, et al. Intranasal immunization with a
392 replication-deficient adenoviral vector expressing the fusion glycoprotein of respiratory
393 syncytial virus elicits protective immunity in BALB/c mice. Biochem Biophys Res Commun.
394 2009;381:528-32.
395 [26] Kohlmann R, Schwannecke S, Tippler B, Ternette N, Temchura VV, Tenbusch M, et al.
396 Protective efficacy and immunogenicity of an adenoviral vector vaccine encoding the codon-
397 optimized F protein of respiratory syncytial virus. J Virol. 2009;83:12601-10.
398 [27] Santra S, Seaman, M.S., Xu, L., Barouch, D.H., Lord, C.I., Lifton, M.A., Gorgone, D.A.,
399 Beaudry, K.R., Svehla, K., Welcher, B., Chakrabarti, B.K., Huang, Y., Yang, Z., Mascola,
400 J.R., Nabel, G., Letvin, N.L. Replication-defective adenovirus serotype 5 vectors elicit
401 durable cellular and humoral immune responses in nonhuman primates. J Virol.
402 2005;79:6516-22.
403 [28] Park KS, Lee J, Ahn SS, Byun YH, Seong BL, Baek YH, et al. Mucosal immunity
404 induced by adenovirus-based H5N1 HPAI vaccine confers protection against a lethal H5N2
405 avian influenza virus challenge. Virology. 2009;395:182-9.

406 [29] Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, et al. Live
407 Attenuated versus Inactivated Influenza Vaccine in Infants and Young Children. New Engl J
408 Med. 2007;356:685-96.
409 [30] Alwan WH, Record FM, Openshaw PJ. Phenotypic and functional characterization of T
410 cell lines specific for individual respiratory syncytial virus proteins. J Immunol.
411 1993;150:5211-8.
412 [31] Cherrie AH, Anderson K, Wertz GW, Openshaw PJ. Human cytotoxic T cells stimulated
413 by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory
414 syncytial virus. J Virol. 1992;66:2102-10.
415 [32] Walsh KB, Sidney J, Welch M, Fremgen DM, Sette A, Oldstone MBA. CD8+ T-Cell
416 Epitope Mapping for Pneumonia Virus of Mice in H-2b Mice. J Virol. 2013;87:9949-52.
417 [33] Claassen EAW, van der Kant PAA, Rychnavska ZS, van Bleek GM, Easton AJ, van der
418 Most RG. Activation and inactivation of antiviral CD8 T cell responses during murine
419 pneumovirus infection. J Immunol. 2005;175:6597-604.
420 [34] Taylor G, Thomas LH, Furze JM, Cook RS, Wyld SG, Lerch R, et al. Recombinant
421 vaccinia viruses expressing the F, G or N, but not the M2, protein of bovine respiratory
422 syncytial virus (BRSV) induce resistance to BRSV challenge in the calf and protect against
423 the development of pneumonic lesions. J Gen Virol. 1997;78:3195-206.
424 [35] Carine L, Mathieu B, Laurent R, Jean-Francois T, Karl W, Stefan R, et al. Vaccination
425 of calves using the BRSV nucleocapsid protein in a DNA prime-protein boost strategy
426 stimulates cell-mediated immunity and protects the lungs against BRSV replication and
427 pathology. Vaccine. 2008;26:4840-8.
428 [36] Roux X, Dubuquoy C, Durand G, Tran-Tolla TL, Castagne N, Bernard J, et al. Sub-
429 nucleocapsid nanoparticles: a nasal vaccine against respiratory syncytial virus. PLoS ONE.
430 2008;3.

[37] Cook PM. Pathogenesis and persistence of pneumonia virus of mice. PhD thesis: University of Warwick; 1996.

[38] Thimmappaya B, Weinberger, C., Schneider, R.J., Shenk, T. Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. Cell. 1982;31:543-51.

[39] Barr J, Chambers P, Pringle CR, Easton AJ. Sequence of the major nucleocapsid protein gene of pneumonia virus of mice: sequence comparisons suggest structural homology between nucleocapsid proteins of pneumoviruses, paramyxoviruses, rhabdoviruses and filoviruses. J Gen Virol. 1991;72:677-85.

[40] Easton AJ, Chambers P. Nucleotide sequence of the genes encoding the matrix and small hydrophobic proteins of pneumonia virus of mice. Virus Res. 1997;48:27-33.

[41] Easton AJ, Scott PD, Edworthy NL, Meng B, Marriott AC, Dimmock NJ. A novel broad-spectrum treatment for respiratory virus infections: Influenza-based defective interfering virus provides protection against pneumovirus infection in vivo. Vaccine. 2011;29:2777-84.

[42] Bonville CA, Bennett NJ, Percopo CM, Branigan PJ, Del Vecchio AM, Rosenberg HF, et al. Diminished inflammatory responses to natural pneumovirus infection among older mice. Virology. 2007;368:182-90.

[43] Tatsis N, Ertl HCJ. Adenoviruses as vaccine vectors. Mol Ther. 2004;10:616-29.

[44] Fu Y-H, He J-S, Qiao W, Jiao Y-Y, Hua Y, Zhang Y, et al. Intranasal immunization with a helper-dependent adenovirus vector expressing the codon-optimized fusion glycoprotein of human respiratory syncytial virus elicits protective immunity in BALB/c mice. Virology Journal. 2013;10:183.

454 [45] Sharma A, Wendland R, Sung B, Wu W, Grunwald T, Worgall S. Maternal
455 immunization with chimpanzee adenovirus expressing RSV fusion protein protects against
456 neonatal RSV pulmonary infection. Vaccine. 2014;32:5761-8.
457 [46] Pierantoni A, Esposito ML, Ammendola V, Napolitano F, Grazioli F, Abbate A, et al.
458 Mucosal delivery of a vectored RSV vaccine is safe and elicits protective immunity in
459 rodents and nonhuman primates. Molecular Therapy — Methods & Clinical Development.
460 2015;2:15018.
461 [47] See RH, Petric M, Lawrence DJ, Mok CPY, Rowe T, Zitzow LA, et al. Severe acute
462 respiratory syndrome vaccine efficacy in ferrets: whole killed virus and adenovirus-vectored
463 vaccines. J Gen Virol. 2008;89:2136-46.
464 [48] Steitz J, Barlow PG, Hossain J, Kim E, Okada K, Kenniston T, et al. A Candidate H1N1
465 Pandemic Influenza Vaccine Elicits Protective Immunity in Mice. PLoS ONE.
466 2010;5:e10492.
467 [49] Mast TC, Kierstead L, Gupta SB, Nikas AA, Kallas EG, Novitsky V, et al. International
468 epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36
469 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV
470 vaccine trials. Vaccine. 2009;28:950-7.
471 [50] Croyle MA, Patel A, Tran KN, Gray M, Zhang Y, Strong JE, et al. Nasal delivery of an
472 adenovirus-based vaccine bypasses pre-existing immunity to the vaccine carrier and improves
473 the immune response in mice. PLoS ONE. 2008;3:e3548.
474 [51] Roberts DM, Nanda A, Havenga MJE, Abbink P, Lynch DM, Ewald BA, et al. Hexon-
475 chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity.
476 Nature. 2006;441:239-43.
477 [52] Abbink P, Lemckert, A.A.C., Ewald, B.A., Lynch, D.M., Denholtz, M., Smits, S.,
478 Holterman, L., Damen, I., Vogels, R., Thorner, A.R., O'Brien, K.L., Carville, A., Mansfield,

479 K.G., Goudsmit, J., Havenga, M.J.E., Barouch, D.H. Comparative seroprevalence and
480 immunogenicity of six rare serotype recombinant adenovirus vaccine vectors from subgroups
481 B and D. J Virol. 2007;81:4654-63.
482

Table 1 Survival data for animals challenged with PVM

Immunogen	BALB/c†				C3H-He-mg	C57BL/6
	10^7 p.f.u (x1)	10^8 p.f.u. (x1)	10^7 p.f.u. (x2)	10^8 p.f.u. (x2)	10^7 p.f.u. (x2)	10^7 p.f.u. (x2)
rAd5-N	6/6	5/5	3/3	6/6	5/5	5/5
rAd5-M	3/6	6/6	5/6	6/6	3*/5	5/5
rAd5-Z	1/6	6/6	0/6	4/4	2*/5	5*/5
PBS	0/2	0/2	0/4	2*/4	0/4	0/4

† Mice were inoculated i.n. with one (x1) or two (x2) doses of Ad5 immunogens, at day 0 and 14, and were challenged i.n. at day 42 (x1) or day 28 (x2) with a lethal dose of PVM

*Mice exhibited elevated clinical signs of disease

Figure Legends

Fig. 1. rAd5M and rAd5N protect BALB/c mice from lethal PVM challenge. Mice were immunised i.n. at 0 and 14 days with either 10^7 or 10^8 p.f.u. of rAd5N (A), rAd5M (B), or rAd5Z (C) or mock-immunised with PBS as a control. All animals were challenged at 28 days with a lethal dose of PVM, and then weighed (left panels) and assessed for clinical signs of PVM disease (right panels) daily for the duration of the experiment. Weights are expressed as a proportion of the initial weight of the relevant group of mice with the weight on day zero taken as 100%. Clinical signs are expressed on a scale of 1 (healthy) to 5 (moribund) [17]. Results are the means from five mice for each group and are representative of two separate experiments. A single PBS control group was studied within each experiment and data from this group are reproduced in panels A – C for ease of comparison.

Fig. 2. A single intranasal immunisation of rAd5M or rAd5N confers protection against PVM challenge. Mice were immunised with 10^7 p.f.u (A) or 10^8 p.f.u. (B) of either rAd5M, rAd5N or rAd5Z, or mock-immunised with PBS. Animals were challenged with PVM six weeks post immunisation and then weighed (left panels) and assessed for clinical signs of PVM disease (right panels) daily for the duration of the experiment. Dotted lines: mean weights or scores after the loss of one or more animals from the group. Other details as for Fig. 1.

Fig. 3. Protection from lethal PVM challenge by rAd5M and rAd5N is long lasting. Mice were immunised i.n. at 0 and 14 days with 10^7 p.f.u doses of rAd5N (A), rAd5M (B) or rAd5Z (C) or mock-immunised with PBS, and groups of six immunised animals plus two mock-immunised

511 animals were challenged with a lethal dose of PVM at each time point. Following challenge,
512 mice were weighed (left panels) and assessed for clinical signs of PVM disease (right panels)
513 daily. The graphs for PBS mock-immunised animals were generated by taking an average of
514 the data from the mice challenged at the various time points. Dotted lines: mean weights or
515 scores after the loss of one or more animals from the group. A single experiment was
516 conducted because of the numbers of animals involved. Other details as for Fig. 1.

517

518 **Fig. 4.** Antibody titres in sera from rAd5N-immunised mice. Mice were immunised i.n. at 0
519 and 14 days with either 10^7 p.f.u. (A, C) or 10^8 p.f.u (B, D) doses of rAd5N, rAd5Z or mock-
520 immunised with PBS as control. Sera from immunised animals were analysed for anti-PVM
521 IgG responses (A, B) and anti-Ad IgG responses (C, D) by ELISA. Results are expressed as the
522 geometric means of the Log_{10} endpoint titres, after correction of values for background,
523 with the background taken as the mean values in the assay from PBS control animal sera. (E)
524 Anti-N or anti-F monoclonal antibodies, or PVM-specific or control mouse sera, were
525 titrated in the ELISA using either PVM antigen-containing (P2-2) or control (BS-C-1) cell
526 lysate as antigens.

527

528 **Fig. 5.** Longevity of antibody titres in sera of rAd5M and rAd5N immunised mice. Mice were
529 immunised i.n. at 0 and 14 days with 10^7 p.f.u of rAd5M, rAd5N or rAd5Z or treated with PBS
530 alone. Sera from immunised animals were analysed for anti-PVM IgG responses (A-C) and
531 anti-Ad IgG responses (D-F) by ELISA. Antibody titres were calculated as for Fig. 4.

532

Fig. 6. Mucosal immunisation with rAd5N primes a PVM-specific T-cell response. Mice were immunised i.n. with 10^7 p.f.u of rAd5N or rAd5Z on days 0, 14 and 28 and spleens were harvested on day 35. Splenocytes from two mice from each group were pooled and stimulated *in vitro* with PVM or control cell lysate (BSC1) for 16 h, and responses were analysed by intracellular IFNγ staining of: (A) $CD4^+$ and (B) $CD8^+$ cells by flow cytometry. Frequencies of $IFN\gamma^+$ cells are shown as a percentage of total $CD4^+$ or CD8 T^+ cell numbers in the upper right quadrant. Representative data of groups of 4 mice are shown.

Figure 1

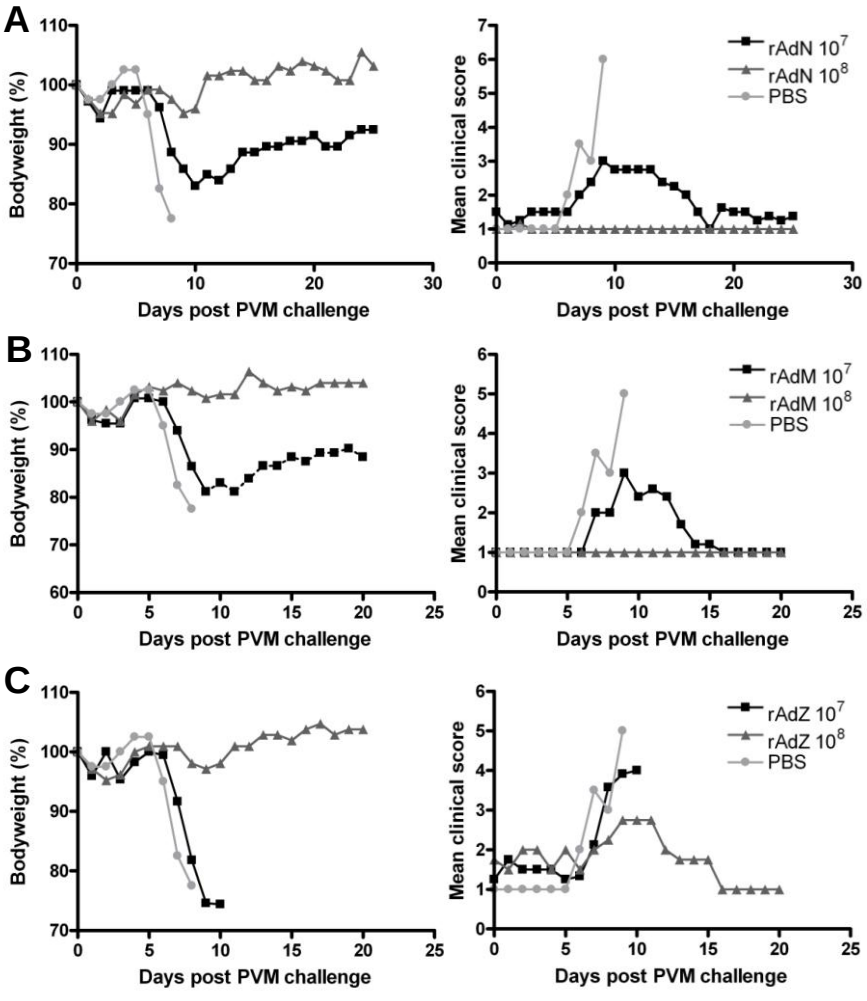

A
Bodyweight (%)
Days post PVM challenge
Mean clinical score
rAdN 10^7
rAdN 10^8
PBS
B
rAdM 10^7
rAdM 10^8
C
rAdZ 10^7
rAdZ 10^8

Figure 2

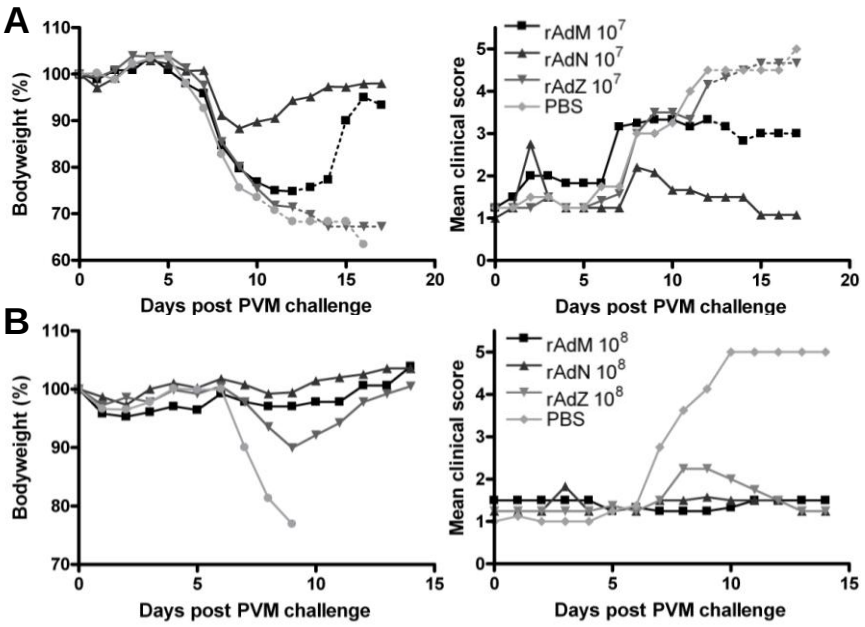

A
Bodyweight (%)
Days post PVM challenge
Mean clinical score
rAdM 10^7
rAdN 10^7
rAdZ 10^7
PBS
B
Bodyweight (%)
Days post PVM challenge
Mean clinical score
rAdM 10^8
rAdN 10^8
rAdZ 10^8
PBS

Figure 3

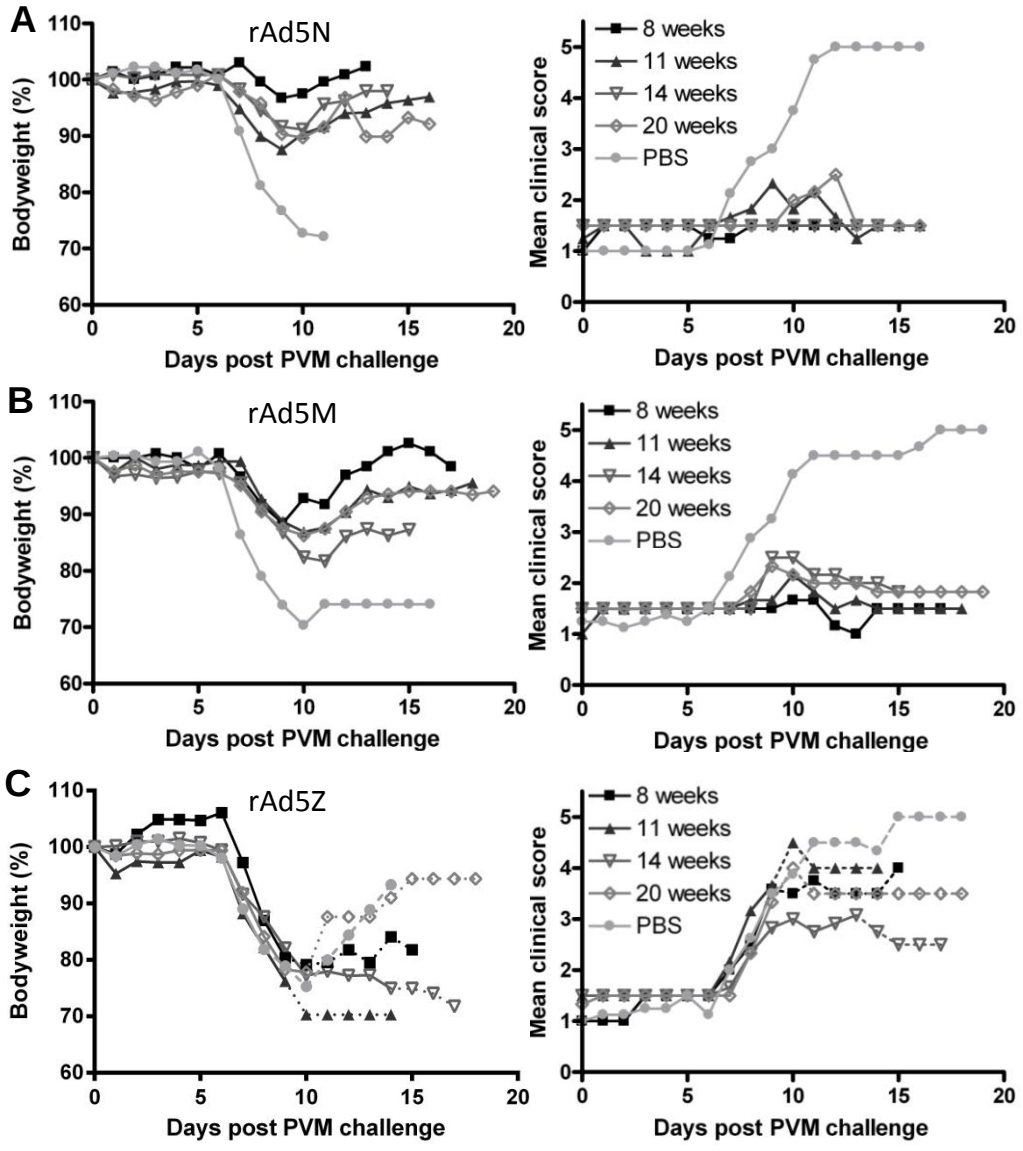

A
rAd5N
Bodyweight (%)
Days post PVM challenge
Mean clinical score
8 weeks
11 weeks
14 weeks
20 weeks
PBS
B
rAd5M
C
rAd5Z

Figure 4

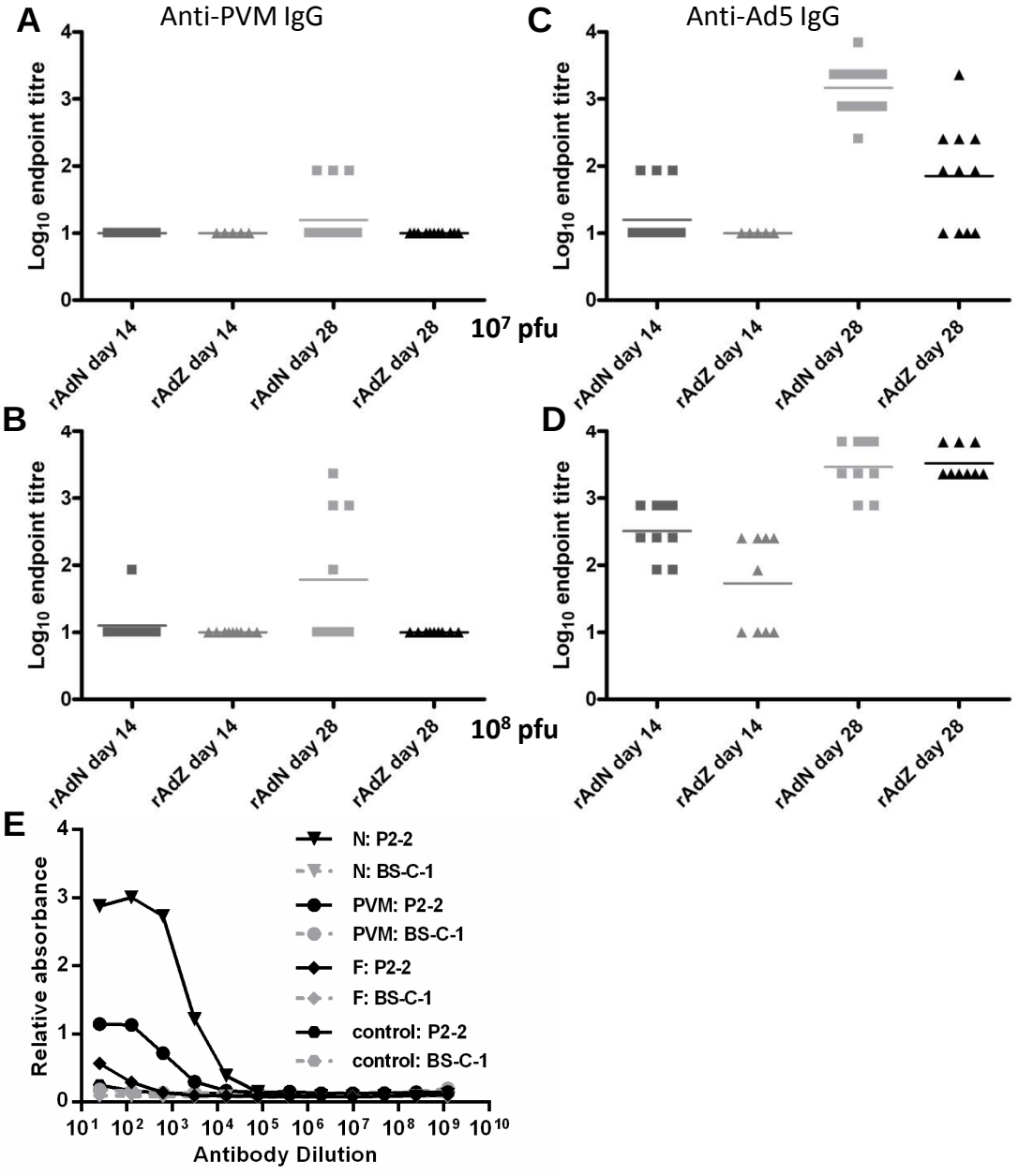

A
Anti-PVM IgG
C
Anti-Ad5 IgG
Log10 endpoint titre
10^7 pfu
rAdN day 14
rAdZ day 14
rAdN day 28
rAdZ day 28
B
D
10^8 pfu
E
Relative absorbance
Antibody Dilution
N: P2-2
N: BS-C-1
PVM: P2-2
PVM: BS-C-1
F: P2-2
F: BS-C-1
control: P2-2
control: BS-C-1

Figure 5

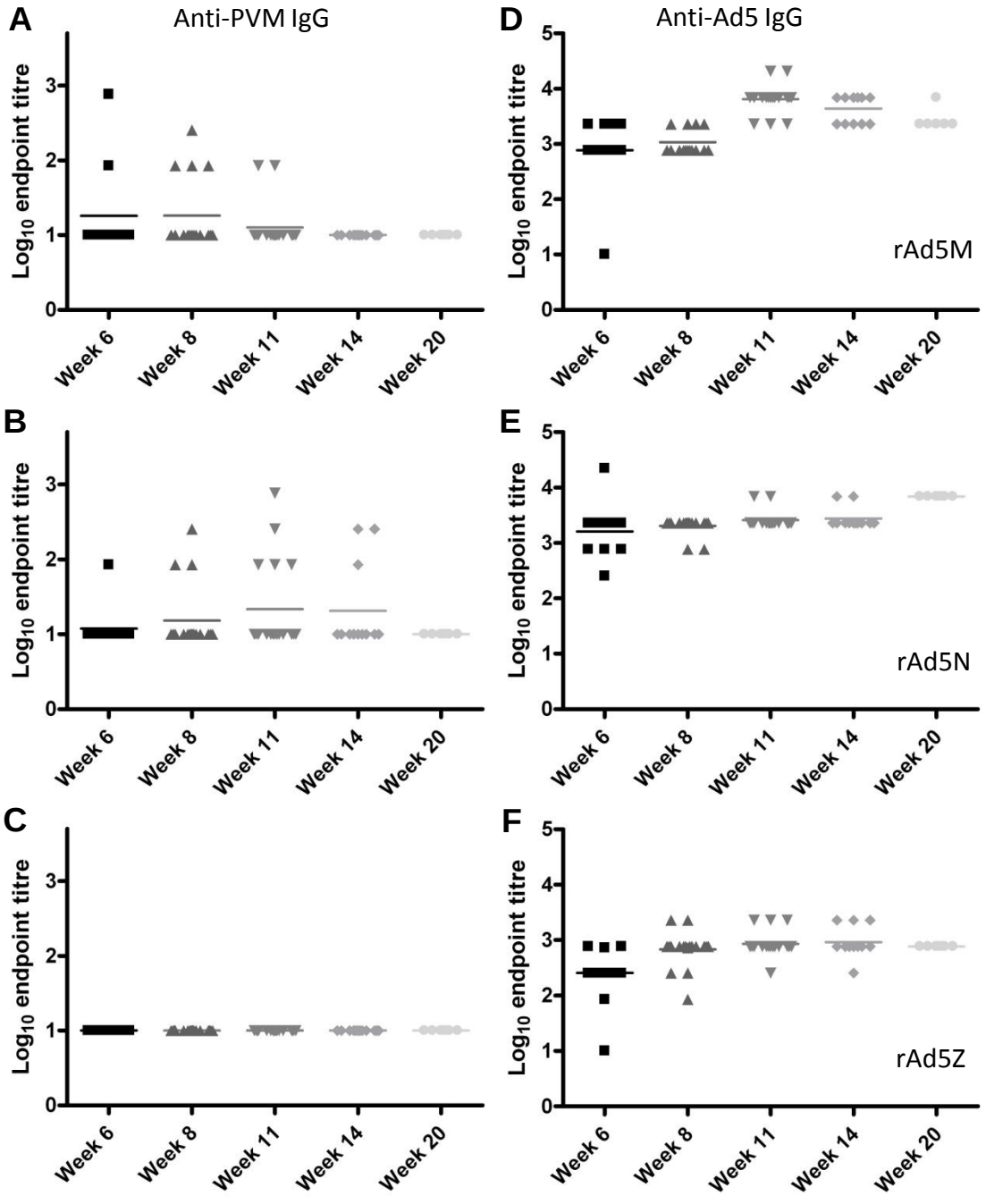
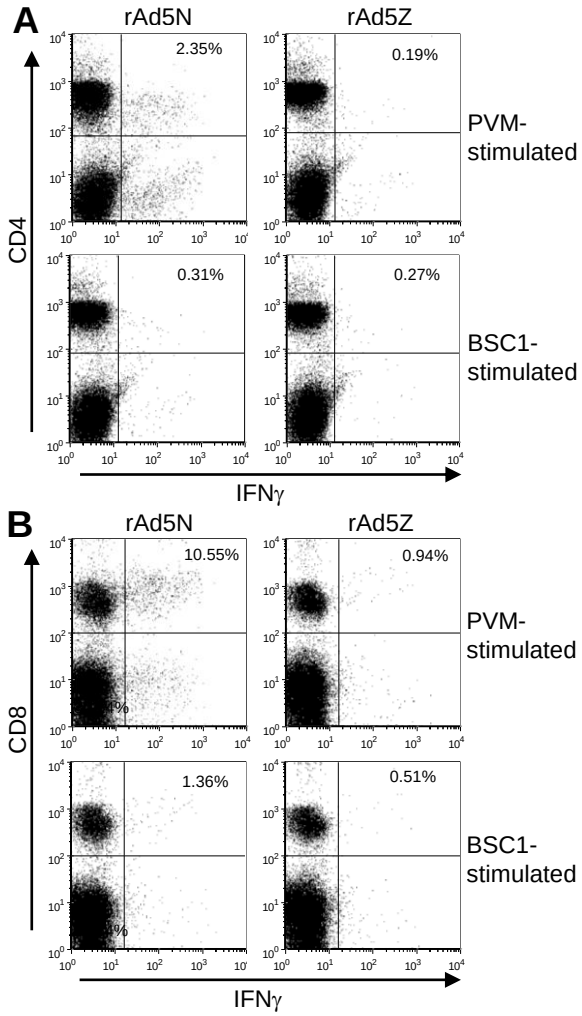

A
Anti-PVM IgG
D
Anti-Ad5 IgG
B
C
E
F
rAd5M
rAd5N
rAd5Z
Log10 endpoint titre
Week 6
Week 8
Week 11
Week 14
Week 20

Figure 6


A
rAd5N
rAd5Z
2.35%
0.19%
0.31%
0.27%
PVM-stimulated
BSC1-stimulated
CD4
IFNγ
B
rAd5N
rAd5Z
10.55%
0.94%
1.36%
0.51%
PVM-stimulated
BSC1-stimulated
CD8
IFNγ