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Parainfluenza virus 5-based vaccine vectors expressing vaccinia virus (VACV) antigens provide long-term protection in mice from lethal intranasal VACV challenge

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ABSTRACT

To test the potential for parainfluenza virus 5 (PIV5)-based vectors to provide protection from vaccinia virus (VACV) infection, PIV5 was engineered to express secreted VACV L1R and B5R proteins, two important antigens for neutralization of intracellular mature (IMV) and extracellular enveloped (EEV) virions, respectively. Protection of mice from lethal intranasal VACV challenge required intranasal immunization with PIV5-L1R/B5R in a prime-boost protocol, and correlated with low VACV-induced pathology in the respiratory tract and anti-VACV neutralizing antibody. Mice immunized with PIV5-L1R/B5R showed some disease symptoms following VACV challenge such as loss of weight and hunching, but these symptoms were delayed and less severe than with unimmunized control mice. While immunization with PIV5 expressing B5R alone conferred at least some protection, the most effective immunization included the PIV5 vector expressing L1R alone or in combination with PIV5-B5R. PIV5-L1R/B5R vectors elicited protection from VACV challenge even when CD8⁺ cells were depleted, but not in the case of mice that were defective in B cell production. Mice were protected from VACV challenge out to at least 1.5 years after immunization with PIV5-L1R/B5R vectors, and showed significant levels of anti-VACV neutralizing antibodies. These results demonstrate the potential for PIV5-based vectors to provide long lasting protection against complex human respiratory pathogens such as VACV, but also highlight the need to understand mechanisms for the generation of strong immune responses against poorly immunogenic viral proteins.

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Introduction

The respiratory tract can be a major entry site for many pathogenic viruses, including influenza virus, paramyxoviruses, coronaviruses, pox viruses and herpes viruses. The outcomes of these viral infections can be significantly influenced by immune responses at the mucosal surfaces of the respiratory tract, including the recruitment of innate immune cells, and the activation of T cells and antibody responses (Murphy, 1994; Virgin, 2007; Woodland and Randall, 2004). As such, there is intense interest in developing vaccination strategies and viral vectors that promote strong and long lasting protective immune responses against viral respiratory tract pathogens. This is particularly important for viral infections in different anatomical regions of the respiratory tract, since the mechanisms controlling immunity in these airway compartments can differ significantly (Woodland and Randall, 2004). The overall goal of the work described here was to determine the capacity of viral vectors

based on parainfluenza virus 5 (PIV5) to elicit protection against lethal respiratory tract infection by vaccinia virus (VACV).

Poxviruses such as variola virus, the causative agent of smallpox, highly fatal monkey poxvirus, and VACV can establish lethal infections through the respiratory tract (e.g., Buller and Palumbo, 1991; Kaufman et al., 2008). While a live attenuated form of VACV is currently used in the United States as a licensed smallpox vaccine, a number of concerns have been raised due to risk of adverse effects of this vaccine (e.g., Jacobs et al., 2009). VACV also presents major challenges to the development of alternative vaccination approaches that are based on purified VACV proteins and heterologous vectors expressing VACV antigens (Moss, 2006). First, VACV exists in two major infectious forms: the extracellular enveloped virion (EEV) and the intracellular mature virion (IMV). Importantly, the VACV antigens that are critical for neutralization of these two forms differ (Fogg et al., 2004; Hooper et al., 2000). For example, L1R is a myristoylated transmembrane protein in the IMV form and is an important target for IMV neutralization (Aldaz-Carroll et al., 2005b; Franke et al., 1990; Wolffe et al., 1995). B5R is a membrane-anchored VACV protein with an extracellular domain containing regions that are related to some complement regulatory proteins (Engelstad et al., 1992). Antibodies against B5R are important for neutralization of the EEV form (Aldaz-Carroll et al., 2005a; Bell et al., 2004; Galmiche et al., 1999). Because antibodies that neutralize the IMV do not neutralize the

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EEV, it is thought that immunization with antigens from both of these forms is necessary for maximum protection (Lustig et al., 2005).

A second challenge to development of vectors for immunization against poxviruses is that while VACV is itself highly immunogenic, the individual protein antigens themselves are poorly immunogenic outside of the context of VACV infections. Vaccination with purified VACV proteins or with DNA vaccines encoding VACV proteins requires multiple immunizations for protective responses (e.g., Berhanu et al., 2008; Fogg et al., 2004; Hooper et al., 2000, 2003). Finally, the VACV antigens that are important for control of infections initiated through the respiratory tract versus systemic routes (intravenous or intraperitoneal) can differ, and the immune mechanisms for protection from these different routes of infection are not completely understood (Belyakov et al., 2005; Kaufman et al., 2008).

Given the importance of developing safe and potent vaccination approaches against respiratory tract pathogens, we have developed the parainfluenza virus PIV5 as a vaccine vector (Arimilli et al., 2008; Capraro et al., 2008; Parks and Alexander-Miller, 2002). Our previous work in both mouse and ferret model systems has shown that intranasal (I.N.) delivery of PIV5 induces a potent antibody response, and that PIV5 is cleared from respiratory tissues by 7–9 days pi without evidence of systemic infection (Capraro et al., 2008). Likewise, PIV5 vectors expressing model antigens can elicit strong and long lasting T cell responses which can display a high avidity phenotype (Gray et al., 2003; Parks and Alexander-Miller, 2002). Importantly, vaccination of both ferrets and mice with recombinant PIV5 vectors did not result in overt pathology and there was no evidence of disease symptoms (Capraro et al., 2008). These desirable properties raise the potential use of PIV5 vectors as vaccines against complex human pathogens which infect the respiratory tract.

To test the hypothesis that PIV5-based vectors can elicit protective immunity in the respiratory tract against VACV infection, we engineered PIV5 vectors to express soluble forms of the VACV antigens L1R and B5R. These PIV5-L1R/B5R vectors elicited long term (>1.5 years) protection in mice from lethal I.N. VACV challenge, which was dependent on B cells but not on CD8+ cells. These results highlight the potential for PIV5-based vectors to provide protection against complex and highly lethal human pathogens such as variola virus, but also raise the critical issue of

how to generate strong immunity in airway compartments against poorly immunogenic proteins such as those found with VACV.

Results

Construction of PIV5 vectors expressing VACV proteins L1R and B5R

The open reading frames for VACV L1R and B5R proteins were modified by PCR to lack sequences coding for their transmembrane domains and to encode a C-terminal HA tag for detection. These modified L1R and B5R genes were individually inserted at the HN-L junction encoded in the PIV5 infectious cDNA clone such that they were flanked by PIV5 transcription signals (Fig. 1A). Viruses were recovered from cDNA clones and were designated PIV5-L1R and PIV5-B5R. Growth analyses of PIV5-L1R and PIV5-B5R did not show detectable differences in tissue culture cells compared to WT PIV5 and expression of L1R and B5R was stable during multiple passages (not shown).

As shown in Fig. 1B, western blot analysis of lysates from cells infected with the PIV5-L1R and PIV5-B5R viruses showed HA-tagged L1R and B5R expression consistent with predicted sizes of ~21 and ~35 kDa, respectively (Aldaz-Carroll et al., 2005a, 2005b). Similar to previous data with VSV-based vectors (Braxton et al., 2010), B5R was expressed at a somewhat higher level than L1R. Examination of extracellular media (Fig. 1B, right panel) showed very efficient time-dependent release of B5R. By contrast, most of L1R was retained within the cell similar to that seen in the case of the control cytoplasmic protein thymidine kinase (TK) expressed from a PIV5 vector (Parks et al., 2002). Longer exposures showed a low level of extracellular L1R protein, consistent with secretion of this protein when linked to a signal peptide (Shinoda et al., 2009).

Mice are protected from lethal VACV challenge after immunization with PIV5 vectors expressing L1R and B5R

To determine if the PIV5 vectors conferred protection from lethal VACV challenge, groups of 5 mice were immunized I.N. with PBS as a control or by the I.N. or I.M. route with 10^6 PFU of both PIV5-L1R and PIV5-B5R. On day 28, mice were given a booster immunization with PBS

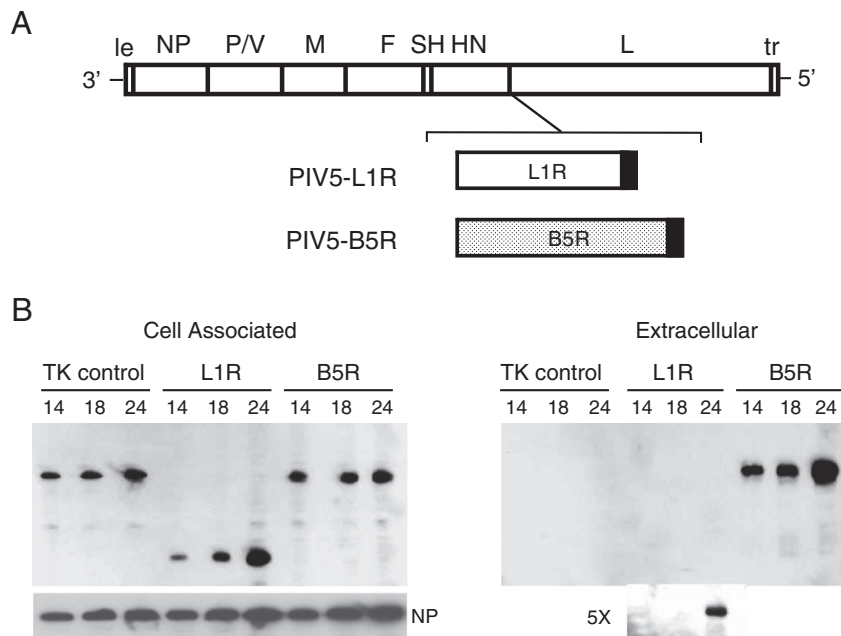


Fig. 1. Expression of L1R and B5R from recombinant PIV5 vectors. A) Schematic diagram of PIV5 viruses used in this study. The genome structure of PIV5 is shown schematically as negative sense RNA with the insertion site for L1R and B5R genes between HN and L. Black bar denotes HA tag added to the L1R and B5R open reading frames. le, leader; tr, trailer. B) Protein expression. A549 cells were infected at an MOI of 25 with PIV5 vectors expressing TK, L1R or B5R. At the indicated time pi, media and cell lysates were analyzed by western blotting for HA-tagged proteins or for NP. 5× denotes the L1R portion of the gel exposed 5 times longer.

I.N. or with the same dose of PIV5-L1R/B5R by either the I.N. or I.M. routes. As a positive control, mice received a single sublethal I.N. dose of VACV (10^2 PFU). Twenty one days after the boost, mice were challenged with VACV equivalent to 20 MTD₅₀ by I.N. administration, and lethality and weight were monitored daily.

As shown in Fig. 2A, positive control mice that received a sublethal dose of VACV were protected from death following VACV challenge (VACV control group). By contrast, mice that received PBS or only one priming dose of the PIV5-L1R and -B5R vectors (PBS and single I.N. and I.M. groups, Fig. 2A) succumbed to infection on day 7 or 8. Most importantly, mice that received an I.N. prime followed by I.N. boost with the PIV5 vectors (I.N.–I.N. group) all survived lethal VACV infection.

Although mice immunized with PIV5-L1R/B5R did not succumb to lethal VACV challenge, they did show signs of VACV-induced disease. This is evident in Fig. 2B which shows changes in body weight as a percentage of initial weight before challenge. Control mice immunized with a sublethal dose of VACV did not show changes in overall weight (VACV control). By contrast, animals in all other groups showed a substantial loss of weight that became evident at day 4 and maximal at day 7 or 8 post VACV challenge. Mice receiving PBS, the single dose of PIV5 vectors (I.N. and I.M. animals) or prime-boost by the I.M. route (I.M.–I.M. group) all lost substantial weight and either died or were removed from the study when loss of weight reached more than 30% of initial value. Importantly, mice given an I.N. prime and I.N. boost with the PIV5-B5R and PIV5-L1R vectors (Fig. 2B, I.N.–I.N., open

triangles) also lost weight, but weight loss was delayed compared to the PBS control and these I.N.–I.N. animals began to recover weight by about day 8 post challenge.

To determine the ability of the individual PIV5 vectors to confer protection, groups of mice were given prime-boost I.N. immunizations with 10^6 PFU of PIV5-L1R alone, PIV5-B5R alone or a combination of the two vectors. Mice were challenged with 20 MTD₅₀ of VACV by I.N. administration and lethality and weight were monitored daily. As shown in Fig. 2C, VACV-immunized control mice (closed triangles) survived VACV challenge, while all PBS-treated mice (open triangles) succumbed to challenge by day 7 or day 8. Approximately 70–80% of mice given a combination of L1R plus B5R vectors or given the L1R vector alone showed a high level of protection from death, while mice given the B5R vector alone showed a slightly lower level of protection (~40%) from lethal VACV challenge. These results on survival from challenge were supported by the results on weight loss shown in Fig. 2D, where mice given either PIV5-L1R only or a combination of PIV5-L1R plus PIV5-B5R lost the least amount of weight following lethal VACV challenge. These results indicate that while immunization with vectors expressing B5R alone conferred at least some protection, the most effective immunization included the PIV5 vector expressing L1R alone or in combination with B5R.

To determine the degree of pathology following VACV challenge, mice were immunized I.N. with PBS as a negative control, a sublethal VACV dose as a positive control, or with 10^6 PFU of both PIV5-L1R and

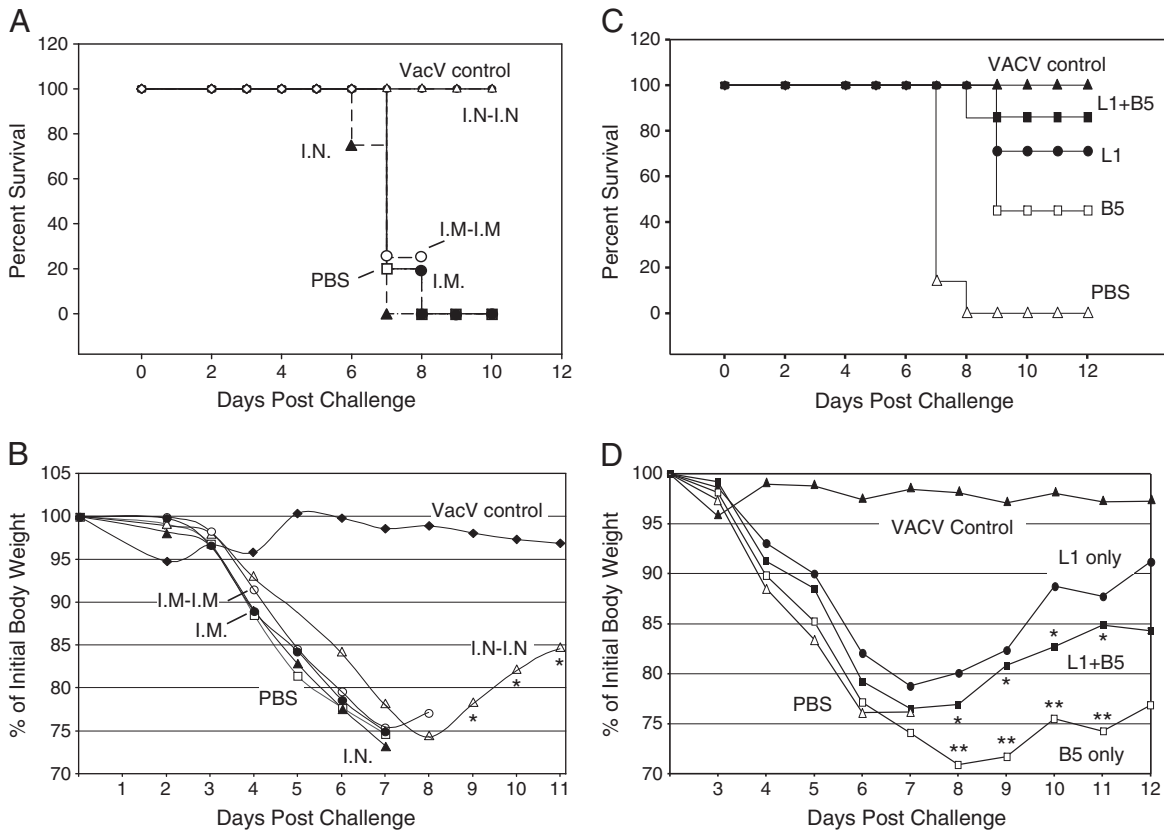


Fig. 2. Mice vaccinated with PIV5-L1R and PIV5-B5R are protected from lethal VACV challenge. Panels A and B) Groups of 5 mice each were immunized with PBS as a control or a combination of 10^6 PFU of PIV5-L1R plus PIV5-B5R through either the I.N. or I.M. route. On day 28, PIV5-vaccinated mice were boosted with either PBS as a control (I.N. or I.M. only) or with the same dose of PIV5 vectors by the I.N. or I.M. route (I.N.–I.N. or I.M.–I.M. groups). Control groups received either a sublethal level of VACV I.N. (closed diamond, VACV control) or prime-boost with PBS (open squares). Twenty one days after the boost, mice were challenged with VACV equivalent to 20 MTD₅₀ by I.N. administration. Mice were monitored daily to determine survival (panel A) and weight loss (panel B) as a percent of initial body weight. Mice were euthanized at an experimental end point when they had a loss of 30% of initial weight or development of severe disease characteristics that were indicative of terminal stages of disease. For panel A, $p < 0.003$ for comparison of I.N.–I.N. to I.N. only and also for I.N.–I.N. compared to I.M.–I.M. For panel B, * denotes $p < 0.001$ for comparison of I.N.–I.N. to VACV control. Panels C and D) Groups of 7 mice each were administered prime and boost I.N. doses of PBS (open triangle), a sublethal dose of VACV (VACV control, closed triangle), or 10^6 PFU of PIV5-L1R and PIV5-B5R alone (open square and closed circle) or in combination (closed square) on the same days described for panels A and B. Mice were then challenged with VACV and then monitored daily as in panel A. For panel C, $p < 0.02$ for comparison of VACV, L1 + B5, L1, and B5 groups compared to PBS control. For panel D, **; $p < 0.005$; *, $p < 0.05$ when comparing weight loss between animals immunized with PIV5-B5R alone to PIV5-L1R alone or PIV5-L1R plus PIV5-B5R, respectively.

PIV5-B5R in a prime-boost protocol as described for Fig. 2. On day 28 post boost, mice were challenged with VACV equivalent to 20 MTD₅₀ by I.N. administration. Nasal tissue and the olfactory lobes of the brain were analyzed on day 7 post challenge by staining with hematoxylin and eosin. Control mice immunized with a sublethal dose of VACV showed intact nasal epithelium and clear nasal cavity (Fig. 3, panels A and B). By contrast, PBS-treated mice showed inflammatory exudate (panel C) filling much of the nasal cavity with a bilateral extension through the cribriform plate into the adjacent olfactory lobes (white arrows, panel C). There was extensive destruction of the nasal epithelium (black arrows, panel D), with inflammatory cells being present in submucosa and in the nasal cavity. For mice immunized with the PIV5 vectors, VACV challenge resulted in small amounts of exudates seen in the nasal cavity but there was no extension into the brain region (Fig. 3, panel E). Higher magnification showed only very low levels of inflammatory cells in the exudates and only minimal disruption of the nasal epithelium (panel F). PIV5 immunized animals showed no significant lesions in lung tissue or other organs including heart, liver, kidney and spleen (not shown). Together, these data indicate that mice immunized with PIV5-L1R and -B5R vectors show relatively low levels of pathology in the nasal tissue at day 7 post VACV challenge compared to control animals that succumb to VACV infection.

Recovery of weight loss in PIV5-L1R and -B5R immunized mice correlates with clearance of challenge VACV from the lung tissue

To determine VACV load in lung tissue following challenge infection, groups of 5 mice were immunized as described in the legend to Fig. 2 and then challenged 20 MTD₅₀ of VACV I.N. at 21 day after the boost. On

days 4, 8 and 12 post challenge, the titer of VACV in lung tissues was determined by plaque assay. As shown in Fig. 4, no VACV was detected in tissues from control mice given the sublethal dose of VACV, and this was consistent with the lack of disease symptoms in these animals. PBS-treated mice had high VACV titers in the lung tissue until day 8 when they succumbed to infection (# in Fig. 4 denotes no animals survived). Importantly, mice immunized with the PIV5-L1R and -B5R vectors had VACV titers in lung tissues that were ~1 log lower than that seen in PBS treated control mice on days 4 and 8. By day 12 after challenge, there was no detectable VACV in lungs of mice immunized with PIV5 vectors (* samples at D12, Fig. 4). Given the kinetics of weight loss after VACV challenge of PIV5-immunized mice which begins by day 4, reaches a low point by day 8, and approaches normal levels by day 12, these results suggest that recovery from disease symptoms and protection from lethality correlate with clearance of VACV from lung tissue.

Protection from lethal VACV challenge elicited by the PIV5-L1R and -B5R vectors requires B cells but not CD8⁺ cells

To define the mechanism of protection mediated by the PIV5 vectors, groups of 7 mice were immunized I.N. as described above in a prime-boost protocol with PBS, sublethal VACV or a combination of both PIV5-L1R and PIV5-B5R vectors. Eighteen days after boosting with PIV5 vectors, CD8⁺ cells were depleted from mice for 4 consecutive days by daily administration of an anti-CD8 monoclonal antibody as detailed in Materials and methods. Flow cytometric analysis showed that anti-CD8 treatment resulted in a decrease of CD8⁺, CD3⁺ (CD8⁺ T cells) to less than 1% of total splenic cells (data not shown). After I.N. challenge with 20 MTD₅₀ of VACV, mice were monitored for lethality and disease

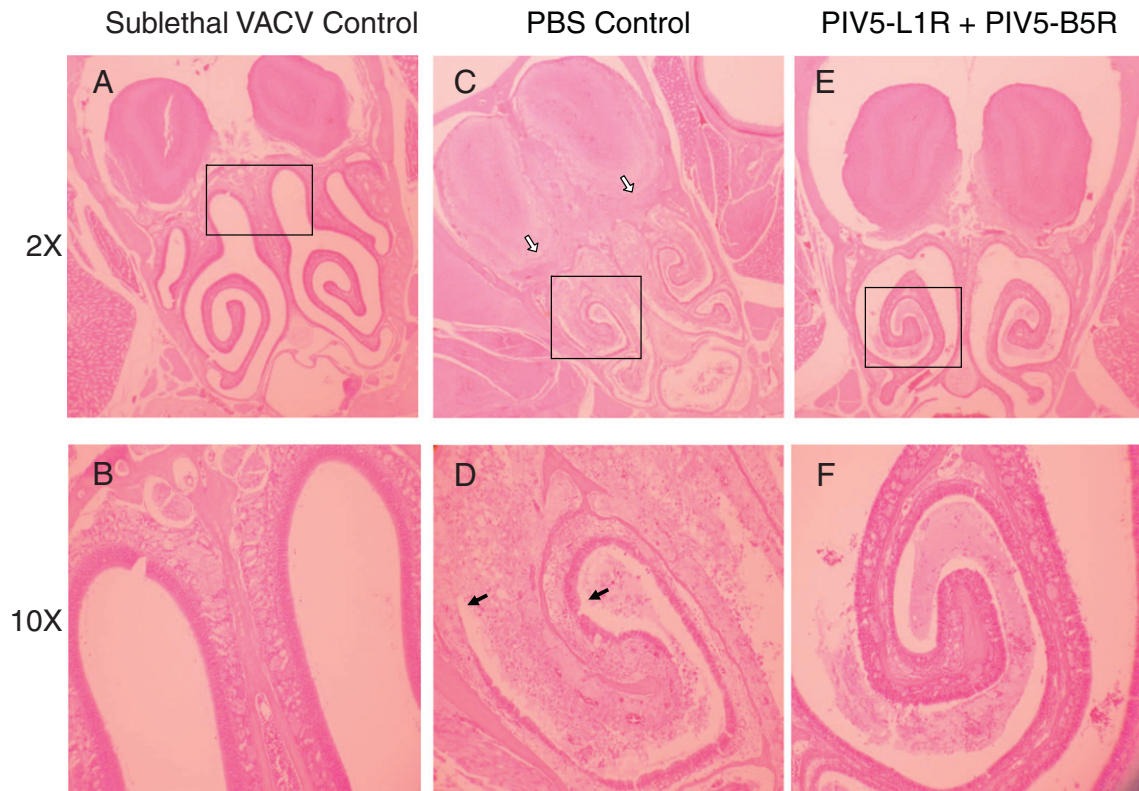


Fig. 3. Pathology in nasal tissue of vaccinated mice after lethal I.N. VACV challenge. Mice were vaccinated I.N. with PBS, a sublethal dose of VACV, or prime and boost with 10⁶ PFU of both PIV5-L1R and PIV5-B5R as described in the legend to Fig. 2. Twenty one days after the boost, mice were challenged I.N. with 20 MTD₅₀ of VACV. Seven d after challenge, tissues were evaluated histologically. Pictures represent H&E staining of a cross section of nasal cavities and olfactory lobes of the brain. Boxes in 2× pictures outline the area shown in higher magnification in the 10× panels. Panels C and D from the PBS-treated control mice show necrosis of the nasal epithelium with intense inflammation which extends into the brain through the cribriform plate. White arrows (2×) indicate the disruption of integrity of cribriform plate into the adjacent olfactory lobes and adjacent inflammation. The black arrows (10×) show areas of nasal epithelial ulceration. Panels E and F from mice immunized with the PIV5-L1R and PIV5-B5R show minimal fibrinous inflammatory exudate in the nasal cavity and minimal tissue disruption. Panels A and B from the sublethal VACV control animal are within normal limits.

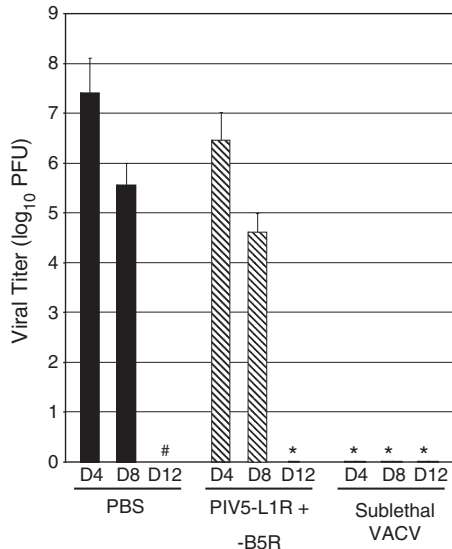


Fig. 4. VACV load in lungs of mice vaccinated with PIV5-L1R and PIV5-B5R vectors. Groups of 7 mice were vaccinated I.N. with PBS (black bars), a sublethal dose of VACV, or prime and boost with 10^6 PFU of both PIV5-L1R and PIV5-B5R (hatched bars) as described in the legend to Fig. 2. Twenty one days after the boost, mice were challenged I.N. with 20 MTD₅₀ of VACV and levels of VACV in lungs were determined by plaque assay. Standard deviations are indicated by the error bars. *, no detectable virus; #, mice succumbed to infection.

symptoms. As shown in Fig. 5A, PBS-treated control mice succumbed to lethal infection by days 8–10 (closed triangles), whereas all of the CD8-depleted mice that had been immunized with PIV5-L1R/B5R vectors were completely protected (open circles). Some CD8-depleted control mice that had received a single sublethal dose of VACV also succumbed to VACV challenge (closed squares), a finding that is consistent with previous reports for a role of cellular immunity in the case of some VACV infections (Belyakov et al., 2005; Wyatt et al., 2004). Although PIV5 immunized mice were protected from lethality they still showed disease symptoms such as hunching and loss of weight (data not shown). For PIV5-immunized mice, the magnitude of weight loss (~20–25%) and kinetics of weight loss (maximal at day 8 post challenge) were very similar to that seen without CD8 depletion (data not shown). Thus, protection from VACV lethality induced by PIV5-L1R/B5R does not depend on CD8+ cells.

To determine the role of B cells in protection elicited by the PIV5-based vectors, Jh mice which lack B cells were immunized I.N. in a prime-boost protocol as described above and then challenged I.N. 21 days after the boost with 20 MTD₅₀ of VACV. Lethality and disease symptoms were monitored daily. As shown in Fig. 5B, Jh mice immunized with the PIV5-L1R/B5R vectors succumbed to VACV challenge at a rate that was only slightly delayed compared to that seen with the PBS-treated control mice. By contrast, control mice that were immunized with a sublethal dose of VACV were fully protected. Jh mice that had been immunized with PBS or with the PIV5 vectors showed the appearance of disease symptoms such as loss of weight and hunching between day 4 and day 8 post VACV challenge (data not shown). Taken together, these data indicate that protection from VACV challenge that is elicited by the PIV5-L1R and PIV5-B5R vectors requires B cells but not CD8+ cells.

Antibody responses to infection with PIV5-L1R and PIV5-B5R vectors

To assay anti-L1R and anti-B5R antibody responses, mice were immunized I.N. with 10^6 PFU of either PIV5-L1R or PIV5-B5R and serum was collected at day 14 and day 21. Mice were then boosted with an equivalent amount of virus and serum was collected at day 4

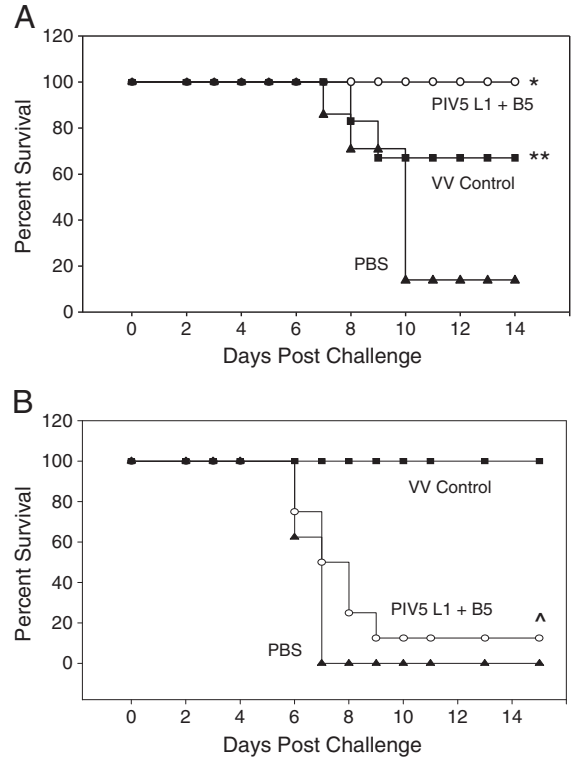


Fig. 5. Protection from lethal VACV challenge elicited by PIV5-L1R and PIV5-B5R vectors requires B cells but not CD8+ cells. A) Groups of 7 mice each were immunized by prime-boost I.N. with PBS (closed triangles), a sublethal dose of VACV (closed squares), or 10^6 PFU of PIV5-L1R and PIV5-B5R (open circles) as described in the legend to Fig. 2. CD8-positive cells were depleted by daily administration of anti-CD8 antibody for 4 days as described in Materials and methods. Mice were then challenged with 20 MTD₅₀ of VACV by I.N. administration, and then monitored daily to determine survival. * and ** denote $p < 0.001$ and $p < 0.04$ for comparison of PIV5 vaccinated and VV control vaccinated animals to PBS vaccinated animals, respectively. B) Jh B cell-deficient mice were vaccinated I.N. with as described in the legend to Fig. 2. Twenty one days after the boost, mice were challenged I.N. with 20 MTD₅₀ of VACV, and then monitored daily to determine survival. ^ denotes $p < 0.022$ for comparison of PIV5 vaccinated to VV control vaccinated animals.

post boost. On day 10 post boost, mice were sacrificed and bronchoalveolar lavage (BAL) fluid was collected. Levels of serum IgG specific for L1R, B5R and PIV5 were determined using previously described ELISAs based on reactivity against purified L1R, B5R or PIV5 particles (Braxton et al., 2010; Capraro et al., 2008; Delaney et al., 2010). Likewise, BAL fluid was tested in ELISA for antigen-specific IgA and IgG responses.

As shown in Fig. 6A, the PIV5-L1R and PIV5-B5R vectors elicited very low serum IgG levels on day 14 that were specific for L1R (open triangles) and B5R (closed squares). By 21 day post primary immunization, titers were slightly increased with a few animals failing to show sero-conversion (Fig. 6A). By day 4 post boost however, nearly all animals had seroconverted. This result contrasts with anti-L1R and anti-B5R responses elicited by sublethal VACV infection which has been shown previously in this same ELISA system to produce titers of 10^6 and 10^5 , respectively (Braxton et al., 2010). Similarly, the PIV5 vectors elicited anti-L1R and anti-B5R IgA and IgG titers in BAL that were at or below the limit of detection in this ELISA (Fig. 6B). This low BAL Ig titer against L1R and B5R contrasted with very strong anti-PIV5 IgA and IgG titers in BAL (Fig. 6C) which averaged 10^3 and 10^4 , respectively. These results are similar to those published previously showing that VSV vectors expressing L1R and B5R elicit low titers against these two VACV proteins (Braxton et al., 2010).

VACV can exist in two major infectious forms: the extracellular enveloped virion (EEV) and the intracellular mature virion (IMV) which

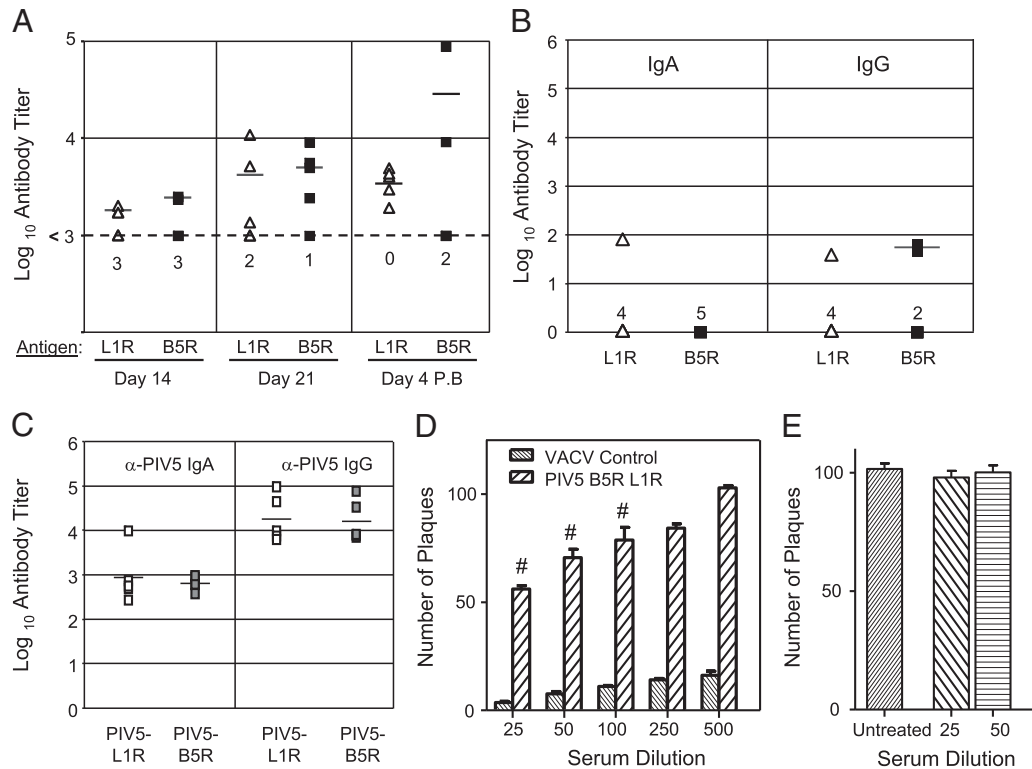


Fig. 6. PIV5-L1 and PIV5-B5 elicit neutralizing anti-L1 and anti-B5 antibodies. A–C) Groups of 5 mice were vaccinated I.N. with 10^6 PFU of either PIV5-L1 or PIV5-B5R and serum was collected at days 14 and 21. Mice were boosted with an equivalent dose of the same virus and serum and BAL was collected on days 4 and 10 post boost, respectively. Samples were analyzed by ELISA for anti-L1R (open triangles) or anti-B5R (closed boxes) IgG in serum (panel A). Alternatively, BAL was analyzed by ELISA for levels of IgA and IgG specific for L1R or B5R (panel B) or PIV5 (panel C). In panel A, the dotted line at 10^3 indicates background level for nonspecific reaction. Numbers below the symbols indicate the number of animals that did not sero-convert. Mean titers for a given set of animals are indicated by a horizontal bar. For panels A and B, titers were not statistically above that of negative controls. D and E) Sera from mice immunized with a combination of PIV5-L1R/B5R vectors (panel D) or from control mice immunized with PBS (panel E) were analyzed in an in vitro neutralization assay with 100 PFU of IMV VACV as described in [Materials and methods](#). Serum from mice given a sublethal dose of VACV was analyzed as a positive control. Results are the mean of sera from 5 mice with standard deviation shown by the bar. #, $p < 0.01$ when comparing between the 100 PFU of VACV used as starting infectivity versus the PFU remaining when treated with serum from immunized mice.

may differ in requirements for neutralization. (Fogg et al., 2004; Hooper et al., 2000). To determine if the PIV5 vectors elicited antibodies capable of neutralizing IMV form of VACV, sera from animals immunized with a combination of PIV5-L1R and -B5R were analyzed in an in vitro neutralization assay in which 100 PFU of the IMV VACV was incubated with dilutions of serum before determining remaining infectivity by plaque assay. As shown in Fig. 6D, sera from animals immunized with PIV5-L1R and -B5R vectors contained a statistically significant level of neutralizing activity (estimated at 1:20 dilution to reduce infectivity by 50%). It is noteworthy that assaying these dilutions of serum in vitro may give a biased view relative to the undiluted nature of blood. By contrast, sera from control mice immunized with PBS showed no significant reduction in plaques (Fig. 6E). These data indicate that while the ELISA shows low antibody titers against L1R and B5R in PIV5-immunized animals, these sera are capable of neutralizing VACV in a functional assay.

To determine if PIV5-based vectors elicited antibodies that were also capable of neutralizing EEV VACV, sera from animals immunized with a combination of PIV5-L1R and -B5R were analyzed in a plaque reduction assay with the EEV form of VACV. As shown in Fig. 7, sera from control mice immunized with PBS or with a control vector which lacked a foreign gene (PIV5) showed no neutralization of EEV VACV, whereas sera from control VACV immunized mice was effective in neutralization. Importantly, sera from mice immunized with PIV5-L1R and -B5R was capable of neutralizing the EEV form of VACV to a slightly lower extent compared to neutralization of the IMV form shown in Fig. 6. Thus, immunization with PIV5 vectors expressing B5R and L1R elicits neutralizing antibodies capable of inactivating both the IMV and EEV forms of VACV.

PIV5-based vectors provide long term protection from VACV challenge

The protection from VACV elicited in mice by PIV5-L1R and PIV5-B5R vectors was long lasting. This is demonstrated in Fig. 8 where mice were given a prime-boost immunization I.N. with PBS as a control, a

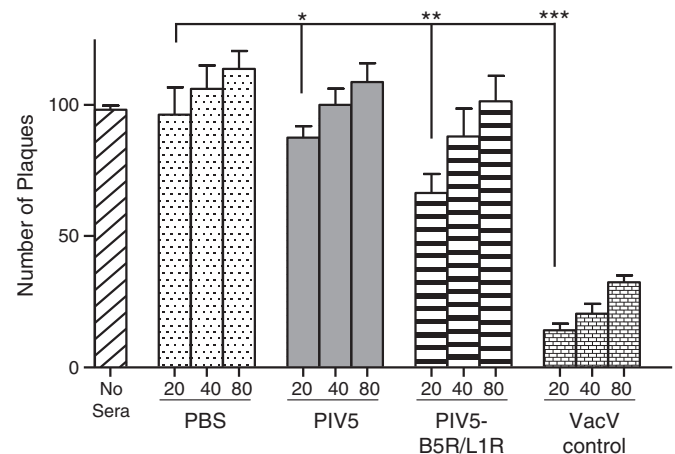


Fig. 7. Mice immunized with PIV5-L1R and PIV5-B5R vectors show low neutralizing antibody against the EEV form of VACV. Groups of 5 mice each were immunized by prime-boost I.N. with PBS, a sublethal dose of VACV, PIV5 lacking an inserted gene or a combination of 10^6 PFU of both PIV5-L1R plus PIV5-B5R as described in the legend to Fig. 2. Sera collected at 14 day post boost were diluted 1:20, 1:40 or 1:80 and then tested in a neutralization assay with 100 PFU of the EEV form of VACV. *, **, *** denote $p < 0.014$, $p < 0.0012$ and $p < 0.0001$ when compared to PBS control serum at 1:20 dilution.

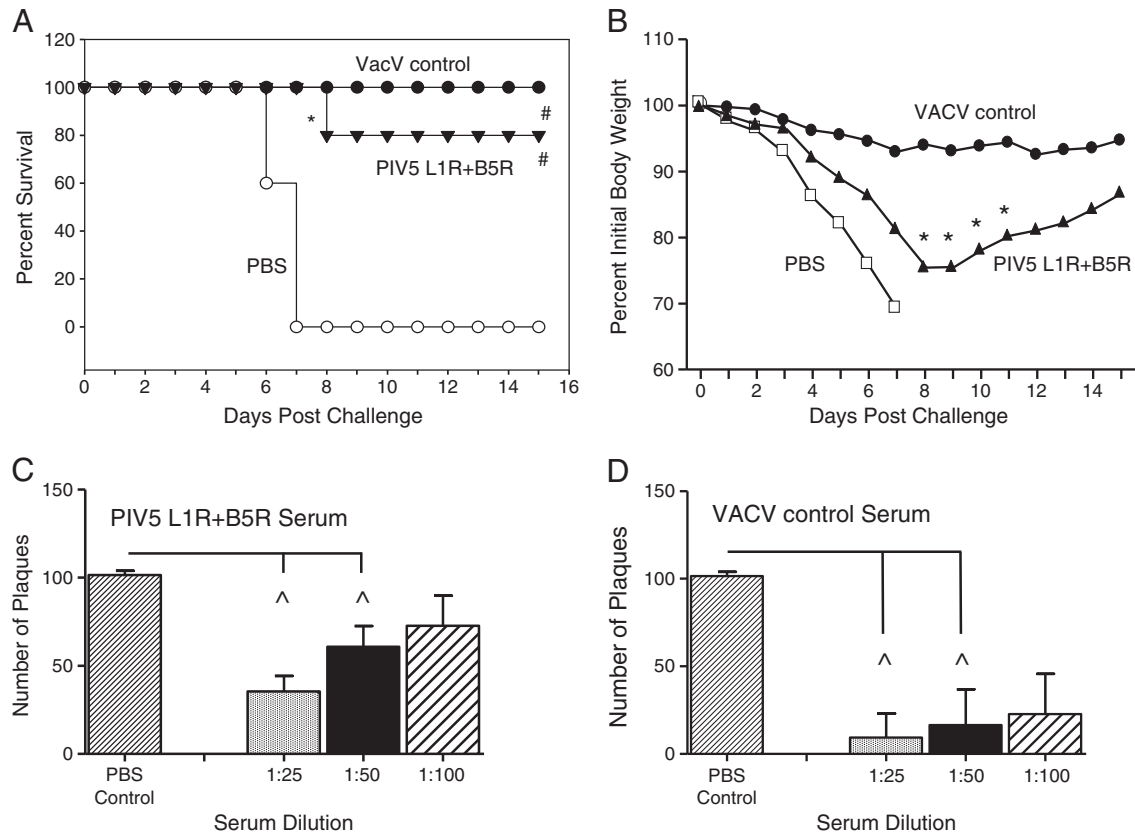


Fig. 8. Antibody responses and protection from lethal VACV challenge in mice 1.5 years after vaccination with PIV5-L1R and PIV5-B5R vectors. Groups of 5 mice each were immunized by prime-boost I.N. with PBS, a sublethal dose of VACV or a combination of 10^6 PFU of both PIV5-L1R plus PIV5-B5R as described in the legend to Fig. 2. After 1.5 years, mice were challenged with VACV equivalent to 20 MTD₅₀ by I.N. administration. Mice were monitored daily to determine survival (panel A) and weight loss (panel B) as a percent of initial body weight. In panel A, * denotes an animal that succumbed to an infection unrelated to VV challenge. # denotes $p < 0.003$ in comparison of immunized mice to PBS control. In panel B, * denotes $p < 0.01$, when comparing animals immunized with PIV5-L1R/B5R to VACV control. Panels C and D) Serum from mice vaccinated with PIV5-L1R and -B5R (panel C) or with sublethal VACV (panel D) were tested in an in vitro neutralization assay with 100 PFU of VACV as described in Materials and methods. Results are the mean values from 5 mice with standard deviations indicated by the error bars. ^, $p < 0.001$ when comparing immune serum to PBS control.

combination of 10^6 PFU of PIV5-L1R and PIV5-B5R or a single sublethal dose of VACV. Mice were then housed for 1.5 years before challenging I.N. with 20 MTD₅₀ of VACV. As shown in Fig. 8A, mice that had been immunized with sublethal doses of VACV or with the PIV5 vectors were protected from lethal challenge. One of these aged PIV5-immunized mice died during this experiment due to necrosis resulting from tail bleeding (star Fig. 8A), but this was not due to lack of protection from I.N. challenge. As with newly immunized and challenged animals, these mice that had been previously immunized with PIV5-L1R and PIV5-B5R and challenged at later points showed a loss of weight 8 day post challenge, but then rapidly regained weight to nearly 90% of initial values (Fig. 8B).

Serum from mice that had been immunized with the PIV5 vectors contained statistically significant levels of neutralizing antibodies out to 1.5 years after immunization. This is evident in Fig. 8C where a 1:25 dilution of serum from mice immunized with the PIV5-L1R and -B5R vectors reduced IMV VACV infectivity by ~70% compared to the ~90% seen with control sera from control mice that had received sublethal VACV infection (Fig. 8D). Taken together, the above data indicate the PIV5 vectors elicit protection from lethal I.N. VACV challenge that: 1) requires a prime-boost combination by the I.N. route, 2) is seen with either PIV5-L1R or PIV5-B5R, but is most effective when the L1R-expressing vector is included, 3) reduces VACV induced pathology in the nasal tissue, 4) requires B cells but is not affected by depletion of CD8⁺ cells and 5) is long lasting in mice out to at least 1.5 years after immunization.

Discussion

The goal of the work described here was to test the ability of PIV5-based vectors to elicit protective immunity against lethal I.N. VACV challenge. The I.N. infection route was chosen here based on the need for new vaccines to protect against aerosolized VACV which may have different requirements for protection from challenge by other routes (Kaufman et al., 2008) and the assumption that parainfluenza viruses would elicit the most robust responses by infection through the respiratory route. Our results indicate that PIV5 vectors expressing the VACV L1R and B5R proteins confer long lasting protective immunity against lethal I.N. VACV challenge. Protection from VACV-mediated lethality required a prime plus boost immunization protocol administered by the I.N. route, depended on B cells but not CD8⁺ cells and was capable of protecting mice for longer than 1.5 years after immunization. While immunization with vectors expressing B5R alone conferred at least some protection, the most effective immunization involved PIV5 expressing L1R alone or in combination with B5R. Taken together these results support the further development of PIV5-based vectors for immunization against highly pathogenic and complex human viruses such as poxviruses.

Mice responded to our PIV5 vectors by generating high ELISA titers of antibodies to the PIV5 vector and but relatively low ELISA titers of antibodies to VACV L1R and B5R proteins. We have previously shown that WT PIV5 elicits a very strong anti-PIV5 antibody response detected in serum (Capraro et al., 2008) and here we extend this analysis to

include very high anti-PIV5 IgG and IgA responses in BAL (Fig. 6). By contrast, results from ELISA data showed that the serum antibody responses to L1R and B5R were at low levels and were not detectable in BAL (Fig. 6). This is consistent with previous work showing that the L1R and B5R proteins are poorly immunogenic when expressed outside of the context of the native VACV. This is also reflected in the need for multiple vaccinations with pure L1R and B5R proteins to generate high antibody titers that provide protection (Berhanu et al., 2008; Braxton et al., 2010; Delaney et al., 2010; Fogg et al., 2004; Hooper et al., 2000; Xiao et al., 2007). It is known that there is an initial innate response in mouse lung to PIV5 infection. However, as we have shown that PIV5 is cleared from the respiratory tract by 9 days after infection (Capraro et al., 2008), it is unlikely that an innate response to the vector contributes substantially to protection from lethal VACV infection. This is further supported by the key findings that protection requires B cells and that mice are protected up to 1½ years after immunization with the PIV5 vectors. Importantly however, the PIV5 vectors elicited protection in mice that was dependent on B cells and generated functional anti-VACV antibody titers were easily detected during *in vitro* neutralization assays (Figs. 5 and 6). These data suggest that PIV5 vectors elicit memory B cells that respond to secondary exposure during VACV challenge by increasing levels of neutralizing anti-VACV antibodies. Alternatively, protection from VACV challenge could involve antibody-mediated cellular cytotoxicity (ADCC) or complement-mediated cell lysis, both of which are dependent on antibodies to VACV proteins. The role of complement in VACV protection has been shown previously (Benhnia et al., 2009), and is currently being tested in the context of PIV5-based vectors.

In contrast to recent work with VSV-based vectors expressing L1R and B5R where a single immunization provided protection (Braxton et al., 2010), our PIV5 vector-mediated protection from lethal I.N. VACV infection required a prime and a boost immunization administered by the I.N. route. This may reflect a higher level or prolonged expression of antigens in the airways of mice infected with VSV vectors compared to PIV5 vectors. In view of the finding that the anti-PIV5 antibody response to primary I.N. infection of mice is very strong (titer of $\sim 10^4$, Capraro et al., 2008), it is somewhat surprising that a boost with the same PIV5 vectors promoted protection from VACV challenge. This is particularly true of anti-PIV5 titers in the BAL, which were very high for IgA. Work is in progress to define the mechanism by which boosting with the PIV5 vectors influences protection from VACV challenge even in the face of a strong response due to prior exposure to PIV5.

While the PIV5 vectors clearly protected mice from succumbing to a lethal VACV infection, immunized animals displayed some signs of illness following VACV challenge. This was most notable in PIV5-immunized mice by the loss of weight starting at days 3–4, but this was also evident by signs of illness such as hunching, ruffled fur, conjunctivitis, labored breathing, lethargy and unresponsiveness to stimulus (not shown). Importantly, the kinetics of appearance of illness in PIV5-immunized mice was delayed and less severe compared to control PBS-treated animals, and the PIV5-immunized animals showed an improvement to near normal properties by day 12 post challenge. Similar results have been shown for animals vaccinated with purified VACV proteins (Delaney et al., 2010; Fogg et al., 2004) and with VSV-based vectors expressing L1R and B5R (Braxton et al., 2010), where immunized animals lost weight following VACV challenge even though they were protected from lethality. The disease symptoms in PIV5-immunized animals could reflect a delay in response of memory B cells in PIV5 immunized animals to VACV antigens during the challenge. Additional possibilities include a lower immune response to PIV5-based vaccination due to the fact that PIV5 immunized animals are exposed to only two of the multiple antigenic proteins that expressed during a VACV infection (Berhanu et al., 2008; Fogg et al., 2004; Hooper et al., 2000), or to alternative conformations of B5R and L1R when expressed from PIV5 versus bone fide VACV infection. In the latter case, we engineered expression of soluble versions of L1R and B5R to enhance

release from infected cells and possible uptake of secreted antigen. However, as the immunogenicity of L1R is particularly sensitive to conformational changes (Su et al., 2007), PIV5-expressed L1R may not elicit strong antibody responses due to its expression as an HA-tagged cytoplasmic protein.

There is intense interest in developing paramyxovirus-based vectors for vaccination and therapeutic applications (Bukreyev et al., 2006; Lamb and Parks, 2007; von Messling and Cattaneo, 2004), and PIV5-based vectors have attractive properties relative to other vectors based on viruses that are associated with human diseases. These desirable PIV5 properties including infections are largely noncytotoxic in most cell types, and that PIV5 is not associated with any disease in humans (Goswami et al., 1984). In ferrets and mice, PIV5 stimulates strong antibody and T cell responses, is cleared from respiratory tissues by 7–9 days pi without evidence of systemic infection (Capraro et al., 2008) and induces no overt pathology. As discussed previously (Capraro et al., 2008), the ferret model may be a more appropriate system for analysis of features of the PIV5 vectors, since the mouse is not a good predictor of viral permissiveness/tropism in humans. PIV5 can elicit strong systemic mucosal IgG and IgA responses, even when delivered by different routes. PIV5 is grown to very high titers ($> 10^{10}$ PFU/ml) in Vero cells, an approved cell line for vaccine work. Finally, anti-PIV5 sero-prevalence can be relatively low in many human populations (Hsiung, 1972; Johnson et al., 2008). A PIV5 vector was shown to elicit protective immunity in mice against influenza virus (Tompkins et al., 2007), although the mechanism of protection in that study was not addressed.

There have been previous reports of PIV5 association with human diseases, including Multiple Sclerosis (Goswami et al., 1984). At the time, these were attractive associations, largely due to PIV5's property of readily establishing persistent infections of cells with low levels of pathology (Choppin, 1964). Importantly, all of these claims have been disproven (McLean and Thompson, 1989) and it is currently thought that PIV5 is not associated with any human disease. PIV5 variants have been isolated from samples from human patients (Chatziandreou et al., 2004). The divergence of sequence between these PIV5 isolates indicates that these are not a common lab contaminant, but represent bone fide human infections. PIV5 does not establish persistent infections in mice or ferrets and viral load appears to be restricted to the respiratory tract (Capraro et al., 2008). Whether PIV5 establishes persistent infections in humans is not known.

Our work raises the important question of how virus-based vectors could be improved upon in order to generate a more complete protective immunity in different compartments of the respiratory tract and reduce disease symptoms. We have previously shown that engineered expression of the TLR5 agonist flagellin enhanced the ability of PIV5 to stimulate human dendritic cells and T cells (Arimilli et al., 2008). Likewise, we have shown that PIV5 vectors based on RNA synthesis mutants are potent activators of innate and adaptive immunity (Capraro et al., 2008; Manuse and Parks, 2009). These results with PIV5 variants support the general hypothesis that paramyxovirus vectors can be designed to express a target antigen along with immunomodulatory factors that elicit a broader response to protect against both lethality as well as reduce disease symptoms.

Materials and methods

Cells and viruses

The genes for VACV L1R and B5R have been described previously (Aldaz-Carroll et al., 2005a, 2005b). The L1R and B5R open reading frames were modified by PCR to encode a C-terminal HA tag, and then inserted into the PIV5 cDNA encoding the intergenic region at the HN–L junction. As described previously (He et al., 1997; Parks et al., 2002), the new genes were flanked by PIV5 transcription signals derived from the NP–P gene junction and were fully functional in directing transcription

of the foreign gene as an additional transcription unit. Further details of the cloning steps are available on request. Recombinant viruses individually expressing L1R and B5R were recovered as described previously (Parks et al., 2002) from cDNA plasmids kindly provided by Robert Lamb (Northwestern University) and Biao He (University of Georgia). The TK-expressing PIV5 has been described (Parks et al., 2002). For in vivo experiments, viruses were concentrated by centrifugation through a glycerol cushion (5 h; 25,000 RPM; SW28 rotor), and virus pellets were resuspended in a small volume of DMEM containing 0.75% BSA.

The IMV form of the Western Reserve (WR) strain of VACV was prepared and purified as described by Delaney et al. (2010). The EEV form of VACV was prepared exactly as described by Benhnia et al. (2009) and was stored at 4 °C prior to use in assays. Neutralization assays were carried out as described previously (Johnson et al., 2008). Briefly, 100 PFU of VACV were treated at 37 °C with varying concentrations of immune or control serum for 1 h. All sera were heat inactivated as described previously (Johnson et al., 2008). After incubation, viral titers were determined by plaque assays on CV-1 cells. Results were the average of six reactions, with the significance of data points calculated using the student's *t*-test.

Immunization of mice and VACV challenge

All research performed on mice in this study complied with federal and institutional guidelines set forth by Wake Forest University Animal Care and Use Committee. Female BALB/c mice (5–8 weeks of age) were purchased from Charles River. Jh B cell-deficient mice were of BALB/c origin. Mice were anesthetized with avertin and immunized intranasally (I.N.) as previously described (Capraro et al., 2008; Gray et al., 2003) with 10⁶ PFU of purified PIV5 vector in 20 µl of PBS. Control animals were given a sublethal dose of 0.1 Median Tolerated Dose (MTD₅₀) of VACV (100 PFU) by I.N. administration in 10 µl of PBS. As the Wake Forest University ACUC does not allow death as an endpoint, we used MTD₅₀ to denote a dose where 50% of the animals show signs of illness (e.g. loss of weight to 70% of initial) that are significant enough to warrant euthanasia. Thus, 50% of the animals tolerate this dose of virus. In prime-boost protocols, mice were given a second immunization with 10⁶ PFU of virus at the indicated days post primary immunization.

In challenge experiments, mice were anesthetized with avertin and infected I.N. with 1.7 × 10⁶ PFU of VACV in 10 µl of PBS, which was equivalent to 20 times the MTD₅₀. Mice were monitored and weighed daily to assess illness. Mice losing 30% or greater of their initial weight, or exhibiting severe disease signs indicative of impending death, were euthanized as required by IACUC guidelines of Wake Forest University Health Sciences. At the indicated days pi, mice were sacrificed, and the lungs were harvested by dissection, snap-frozen and stored at –80 °C. Tissues were homogenized using a PowerGen 700 tissue homogenizer (Fisher Scientific) followed by clarification of tissue debris at 450 g for 10 min as described previously (Capraro et al., 2008), and used in plaque assays as described above. Statistics for weight loss and survival were calculated as student's *t* test and Log-Rank, respectively.

Depletion of CD8+ cells from mice

Depletion of CD8+ cells was carried out as described previously (Braxton et al., 2010; Delaney et al., 2010). Briefly, to purify anti-CD8 antibodies clone 2.43 hybridoma cells were grown to confluency. Cells were pelleted by centrifugation, and the media was filtered and concentrated. The concentrated media was dialyzed into 20 mM sodium phosphate binding buffer and the antibody was purified using the Akta chromatography system, following elution with a 100 mM glycine elution buffer solution. Antibody was dialyzed in PBS and concentration was determined by BCA. To deplete CD8+ cells, mice were injected intraperitoneally for 4 consecutive days with 0.5 mg of anti-CD8 antibody in a total volume of 0.5 ml PBS. As a control, some

mice received an intraperitoneal injection of PBS. CD8 depletion was confirmed by flow cytometry, with depletion resulting in a loss of CD8⁺ and CD3⁺ cells (CD8⁺ T cells) to below 1% of total splenic cells.

ELISA and Western blotting

At the indicated days pi, mice were bled from the tail vein, and blood was allowed to clot overnight at 4 °C before clarification by centrifugation. ELISAs were carried out as described previously for PIV5-specific antibodies (Capraro et al., 2008) or for L1R and B5R (Braxton et al., 2010; Delaney et al., 2010). Proteins used in the ELISA were baculovirus-derived recombinant histidine-tagged B5R and L1R proteins generated in cultures of Sf9 cells and purified by metal affinity resin as described previously (Delaney et al., 2010).

For Western blotting, 6-well dishes of cells were infected as described in the figure legends. At each time point, media was collected, concentrated by TCA precipitation, and resuspended in 1% SDS. Cells were washed with PBS and lysed in 1% SDS. Protein concentration was determined by BCA assay (Pierce Chemicals). For each timepoint, the entire media sample and 2 µg of cell lysate was analyzed by gel electrophoresis under reducing conditions. Typically recovery of cell lysate was ~500–700 µg of protein. Thus, 2 µg of protein analyzed represented 0.3%–0.4% of the total intracellular sample. Western blotting was carried out with rabbit antiserum specific for the PIV5 NP protein or with a rat monoclonal antibody specific for the HA tag contained on the L1R and B5R proteins (Roche Inc). Blots were visualized by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Pierce Chemicals).

Pathology

The heads of the mice were removed and preserved whole in 10% neutral buffered formalin for at least 24 h, and then decalcified in Decalcifier-2 (Polysciences) for 6–8 h before cross sections were made through the noses at the level of the olfactory lobes of the brains. The tissues were returned to formalin, embedded in paraffin, processed routinely for histology, cut at 6 µm, and stained with hematoxylin and eosin (H and E). The sections were examined by a board certified veterinary pathologist and are representative of two experiments.

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