# Immunogenicity and protection efficacy of monovalent and polyvalent poxvirus vaccines that include the D8 antigen

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## Abstract

Recent studies have established the feasibility of subunit-based experimental vaccines to protect animals from lethal poxvirus infection. Individual outer membrane proteins from intracellular and extracellular virions of vaccinia virus, when delivered in the form of either DNA vaccines or recombinant protein vaccines produced from baculovirus-infected insect cells, were able to protect mice from the vaccinia virus challenge and rhesus macaques from the monkeypox virus challenge. The polyvalent formulations with various combinations of the four poxvirus antigens (A27, L1, B5 and A33) achieved better protection than the monovalent formulation using only one of these antigens. However, it is not clear whether any of the remaining outer membrane poxvirus proteins can further improve the efficacy of the current polyvalent formulations. In this study, we conducted detailed analysis on the immunogenicity of D8, a previously reported protective antigen from intracellular mature virions. Our results indicated that D8 induced strong protective antibody responses and was effective in improving the efficacy of previously reported polyvalent poxvirus vaccine formulations. Therefore, D8 is an excellent candidate antigen to be included in the final polyvalent subunit-based poxvirus vaccines.
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*Keywords:* Smallpox; Vaccine; Membrane protein

## Introduction

Successful vaccination campaigns have led to the global eradication of smallpox in the 1970s. As a result, it is now estimated that more than half of the world's population is naive to any smallpox infections. This has raised public concerns of potential bioterrorism use of smallpox or other types of poxvirus as biological weapons (Henderson, 1999). At the same time, the original smallpox vaccines can no longer be produced due to the safety concerns over the traditional manufacturing process. Newer generations of vaccines made from further attenuated vaccinia viruses and grown in tissue cultured cells have shown promising immunogenicity in their initial studies (Artenstein et al., 2005; Earl et al., 2004; Weltzin et al., 2003; Wyatt et al., 2004). However, more studies are still needed to establish the scale-up production of these vaccines

and to confirm their safety profile in larger human populations. Although vaccinia virus is a potent vaccine and has the distinct ability to produce long lasting immunity in humans (Crotty et al., 2003; Demkowicz et al., 1996), the nature of its protective antigens has just begun to be revealed (Fogg et al., 2004; Hooper et al., 2004; Pulford et al., 2004). The corresponding epitopes involved in the activation of host immune responses at the B and T cell level that lead to protective immunity are much less understood.

Recent studies have demonstrated the exciting feasibility of developing subunit-based smallpox vaccines. Both DNA and recombinant protein vaccines using individual outer membrane proteins of vaccinia virus elicited protective antibody responses in both small animal and non-human primate models (Fogg et al., 2004; Hooper et al., 2004). A 4-antigen DNA vaccine formulation including two intracellular mature virion (IMV) antigens (encoded by *L1R* and *A27L* genes) and two extracellular enveloped virion (EEV) antigens (*A33R* and *B5R*) from vaccinia virus was able to protect mice against

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intraperitoneal vaccinia virus challenge (Hooper et al., 2003) and subsequently to protect rhesus macaques against monkey-pox virus challenge (Hooper et al., 2004). Similarly, the "ABL" formulation with 3 recombinant poxvirus proteins (A33, B5 and L1) produced from the baculovirus system was also able to protect mice against lethal intranasal vaccinia challenge (Fogg et al., 2004).

However, these studies also indicated that the current subunit-based formulations are still less effective than live attenuated smallpox vaccines (such as the DryVax). First, polyvalent formulations were needed for protection. None of these individual outer membrane proteins, when used as a monovalent vaccine, was able to elicit a protection in mouse model against poxvirus challenge as effectively as the vaccinia vaccine. Second, multiple immunizations were required to achieve protection. The efficacy of protection in mice receiving the 3 recombinant proteins ("ABL" formulation) became close to that of positive control using poxvirus vaccine (VV-Wyeth) only after 4 vaccinations (Fogg et al., 2004). Similarly, 4 to 5 immunizations were needed for the 4-valent DNA vaccine formulation in non-human primates to reduce the disease severity to mild or moderate levels while the one-time DryVax immunization could achieve more complete control of the disease progression (Hooper et al., 2004). Therefore, it is necessary to improve the current subunit-based poxvirus vaccines before committing them to a large scale human testing.

The exact mechanism by which a live attenuated vaccinia vaccine could generate better protection than the subunit-based vaccines is not clear. It is possible that a live attenuated vaccine can induce better T cell responses which may either provide T helper function to enhance the magnitude or quality of protective antibody responses or add cytotoxic T cell compo-nent to the mechanisms of protection. It is also highly likely that additional antigens which were not included in the recently reported subunit-based poxvirus vaccine studies may be able to further improve the protection against a complicated pathogen such as smallpox by expanding the breadth of protection when included in an improved polyvalent formulation.

Therefore, it is logical to identify additional poxvirus antigens which may provide better immune protection. Among nearly 200 proteins of vaccinia virus, at least six were reported to elicit neutralizing antibodies (Pulford et al., 2004). They are A27 (Rodriguez and Smith, 1990; Ramirez et al., 2002), L1 (Franke et al., 1990; Ichihashi and Oie, 1996; Wolffe et al., 1995), D8 (Hsiao et al., 1999), B5 (Law and Smith, 2001; Engelstad et al., 1992; Engelstad and Smith, 1993), H3 (Lin et al., 2000; Chertov et al., 1991; Davies et al., 2005) and A17 (Wallengren et al., 2001). Antibodies against A33 were able to inhibit comet formation (Galmiche et al., 1999). While most of these antigens (A27, L1, B5 and A33) have been studied in various subunit-based, polyvalent poxvirus vaccine formula-tions (Fogg et al., 2004; Hooper et al., 2003), D8 has not been included in such studies.

*D8L* gene in vaccinia virus encodes an IMV envelope protein that is involved in cell-surface binding and adsorption of IMV to the cells (Maa et al., 1990). Antibodies to D8 were detected in mice after immunization with vaccinia virus (Demkowicz et al.,

1992). Replication of vaccinia in brain tissues was significantly reduced when *D8L* gene was inactivated in recombinant viruses (Chernos et al., 1993). A recombinant D8 protein produced from *E. coli* expression system with modified gene sequence induced neutralizing antibodies that were able to block vaccinia infection to BSC40 cells (Hsiao et al., 1999). DNA immuniza-tion with *D8L* construct induced partial protection against vaccinia challenge in a mouse study although the overall immunogenicity of poxvirus DNA vaccines in that study appeared low with poxvirus-specific antibodies detected only in some but not all of *D8L* DNA-vaccinated mice (Pulford et al., 2004). No neutralizing antibody activities were reported with *D8L* DNA vaccine. More importantly, it is not clear whether D8 will be able to further improve the protection of other previously reported poxvirus antigens when included as part of a polyvalent formulation.

Here we report that immunization of mice with D8 in the form of an optimized DNA vaccine design was able to induce vaccinia neutralizing antibody responses, provided protection against lethal vaccinia challenge and was effective in further improving the protection efficacy of polyvalent poxvirus vaccine formulations.

## Results

### Construction and expression of DNA vaccines expressing D8 antigens

The vaccinia gene coding for D8 protein was cloned into a DNA vaccine vector pSW3891 which uses a CMV IE promoter to drive the expression of coded antigen insert (Wang et al., 2005). In order to produce a more immunogenic form of D8L DNA vaccines, two versions of *D8L* gene inserts were produced (Fig. 1A). The first one was the full length coding sequence of the wild type *D8L* gene (wtD8L). For the other version (tPA-D8L), the transmembrane (TM) region and its downstream cytoplasm tail were removed, and a human tissue plasminogen activator (tPA) leader sequence was added to the N terminus of *D8L* coding gene. The wild type *D8L* gene does not encode a natural leader sequence. In our previous DNA vaccine studies, tPA leader was able to increase the secretion of HIV-1 Env and SARS-CoV S proteins and their immunogenicity (Wang et al., 2005, 2006). A similar approach of adding a leader sequence and removing the TM region was reported effective in achieving a good expression of D8 as a recombinant protein (Hsiao et al., 1999).

Expression of D8 from the above DNA vaccines was examined by Western blot using culture supernatants and cell lysates from 293T cells transiently transfected with each of these two D8L DNA vaccine plasmids. It was clear that the tPA-D8L DNA vaccine had a higher level of expression than the wtD8L construct (Fig. 1B). The tPA-D8L was particularly effective in increasing the secretion of D8 antigen to super-natants. The apparent molecular weight of tPA-D8L protein was higher than wtD8L in supernatant, suggesting the involvement of more post-translational processing steps for this modified *D8L* gene design. One possibility is the glycosylation of D8
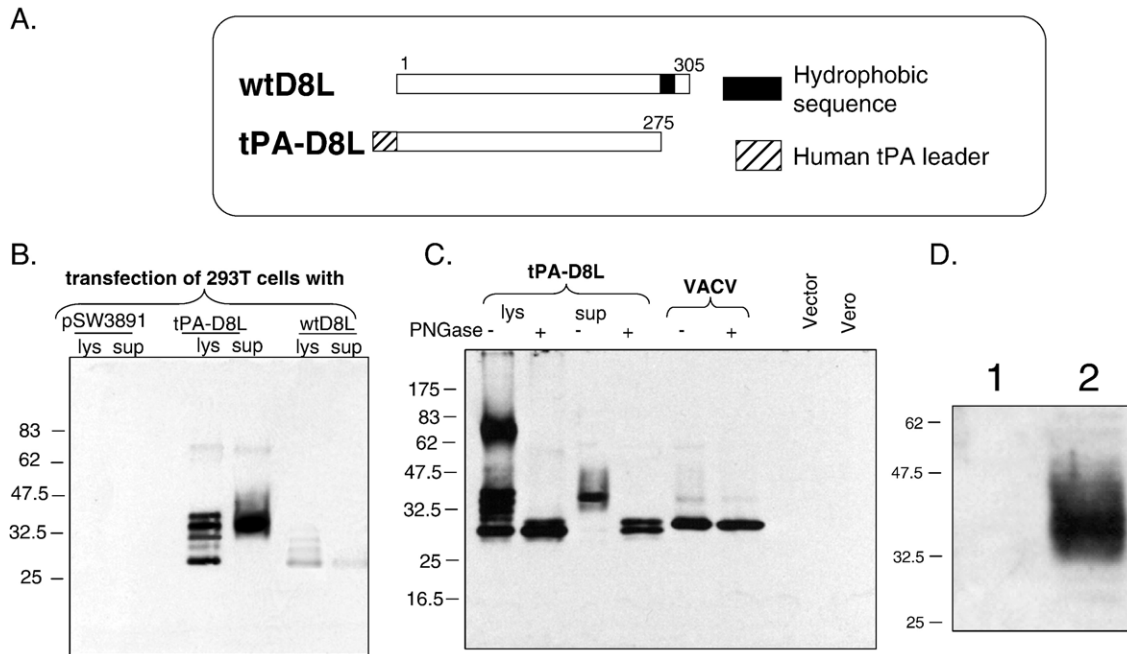
Fig. 1. (A) Schematic designs of D8L gene inserts. Transmembrane (TM) domain and the number of amino acid residues are marked. (B) Western blot analysis of transiently expressed D8 proteins from the supernatant (sup) and lysate (lys) of 293T cells transfected with two D8L DNA vaccine plasmids (tPA-D8L and wtD8L). The empty DNA vector (pSW3891) was included as the negative control. (C) Deglycosylation of D8 proteins with PNGase. D8 proteins, either produced in lysate (lys) or supernatant (sup) of 293T cells transfected with the tPA-D8L DNA plasmid, or from lysate of Vero cells infected with the vaccinia virus WR strain (VACV), were treated (+) or not treated (−) with PNGaseF before subjecting to SDS-PAGE and Western blot analysis. Vero cells without vaccinia infection (Vero) and 293T cells transfected with the empty DNA vector (vector) were also included as the negative controls. (D) Detection of D8 (lane 2) expressed in the supernatant of tPA-D8 DNA plasmid transfected 293T cells with mouse sera immunized with vaccinia virus WR strain. Supernatant 293T cells transfected with the empty DNA vector plasmid (lane 1) were included as the negative control. A D8-specific rabbit serum R274 (1:300 dilution) was used as the detecting antibody for Western blot analyses (B–C).

protein since tPA-D8L has three putative *N*-glycan sites (N29, N94 and N144). Further treatment of transiently expressed tPA-D8L proteins with PNGaseF indeed reduced the molecular weight of D8 both in lysate and supernatant to that of the precursor form of D8 (Fig. 1C), supporting that newly expressed tPA-D8L in 293T cells was glycosyalated. Interestingly, D8 expressed by vaccinia virus was mainly in its non-glycosylated form based on its apparent molecular weight and resistance to PNGaseF treatment (Fig. 1C). The identity of newly expressed D8 antigen was further confirmed by the strong reactivity between mouse sera immunized with vaccinia virus and the tPA-D8L protein expressed in 293T cells (Fig. 1D).

*Immunogenicity of the D8L DNA vaccines*

The immunogenicity of both forms of D8L DNA vaccines was tested in BALB/c mice. Animals received four bi-weekly gene gun-mediated DNA immunizations. The serum anti-D8 IgG responses went up with each immunization as measured by ELISA (data not shown) and they reached the peak level at 2 weeks after the fourth DNA immunization (Fig. 2). Both wtD8L and tPA-D8L DNA vaccines elicited positive anti-D8 IgG responses. The geometric mean titer of the tPA-D8L DNA vaccine group was higher than the wtD8L DNA vaccine group, against either the recombinant D8 antigen produced from 293T cells (Fig. 2A) or the lysates of Vero cells infected with the vaccinia virus (Fig. 2B).

As expected, the positive control mouse sera immunized with the vaccinia virus WR strain had higher titers against the cell lysates infected with the vaccinia virus than the recombinant D8 antigen due to the presence of multiple poxvirus antigens in the infected cell lysates. Likewise, the levels of D8-specific antibodies elicited by both D8L DNA vaccines were higher than that elicited by the vaccinia virus WR strain, presumably due to the fact that anti-D8 antibody is only part of the broad antibody responses against a wide range of antigens in sera immunized with vaccinia viruses.

The mouse sera elicited by D8L DNA vaccines were able to neutralize vaccinia virus as measured in a plaque reduction assay (Fig. 2C). The tPA-D8L DNA vaccine induced higher neutralizing antibodies than the wtD8L DNA vaccine. It is somewhat unexpected that the neutralizing titer of anti-tPA-D8L sera was almost the same as that of the mouse sera immunized with the vaccinia virus (Fig. 2C). It appears that either high titers of antibodies against one major poxvirus antigen (such as D8) or relatively lower titers of immune sera against multiple poxvirus antigens could achieve the same neutralizing effect as measured by this in vitro neutralization assay against the IMV form of poxvirus.

*Protection efficacy of D8L DNA vaccines in mice against lethal vaccinia challenges*

The protective potential of D8 antigen, either alone or in combination with other reported protective poxvirus antigens,
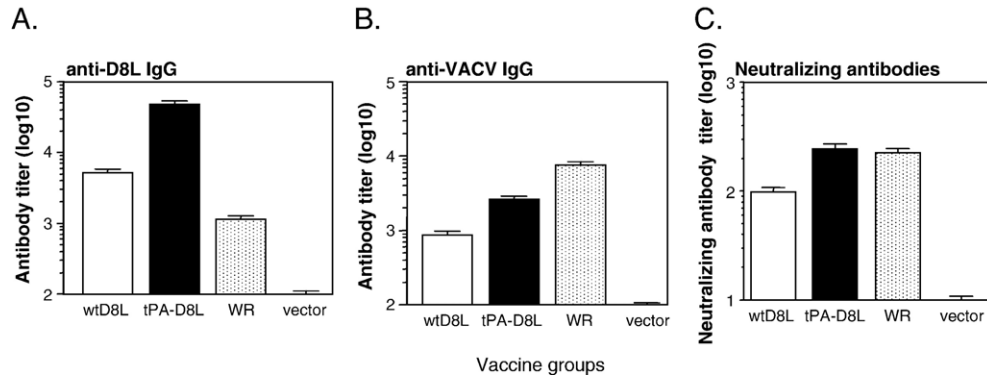
Fig. 2. Titers of antibody responses in immune sera of mice received either the tPA-D8L or wtD8L DNA vaccines. IgG antibody responses as measured by ELISA against either recombinant D8 antigen produced from 293T cells (A) or lysate of Vero cells infected with vaccinia virus WR strain (VACV) (B). Control groups of mice received either the empty DNA vector (Vector) or the vaccinia virus WR strain (WR). Each group included 5 mice and data shown are geometric means of the end titration titers for each group. (C) Titers of neutralizing antibodies for the same groups of mouse sera in an IMV-based, plaque reduction assay. Data shown are geometric means of neutralizing antibody titers which are the highest sera dilution that could inhibit 50% of virus infection in this assay.

was evaluated in BALB/c mice against lethal vaccinia challenge. In this series of challenge studies, each mouse received four bi-weekly gene gun-mediated DNA vaccinations with a fixed total dose of 12 μg DNA plasmids at each vaccination. Protection studies were first conducted against a lethal dose of vaccinia virus ($5 \times 10^7$ pfu per animal) delivered by intraperitoneal (i.p.) route. As a result of this high dose virus challenge, control mice inoculated with empty DNA vector were losing weight progressively until death as early as 3–4 days after challenge (Fig. 3A; Table 1).

Both forms of D8L DNA vaccines, when delivered alone, were able to protect mice from continuous weight loss and ultimate death as compared to the control group mice that received the empty DNA vector (Fig. 3A). None of the D8L DNA vaccinated mice died. The tPA-D8L DNA vaccine appears slightly more effective in controlling the weight loss than the wtD8L DNA vaccine, but neither of them were as effective as the positive control immunization with vaccinia virus WR strain (Fig. 3A). It is consistent with the previous reports that a single poxvirus antigen vaccine could not achieve the same level of
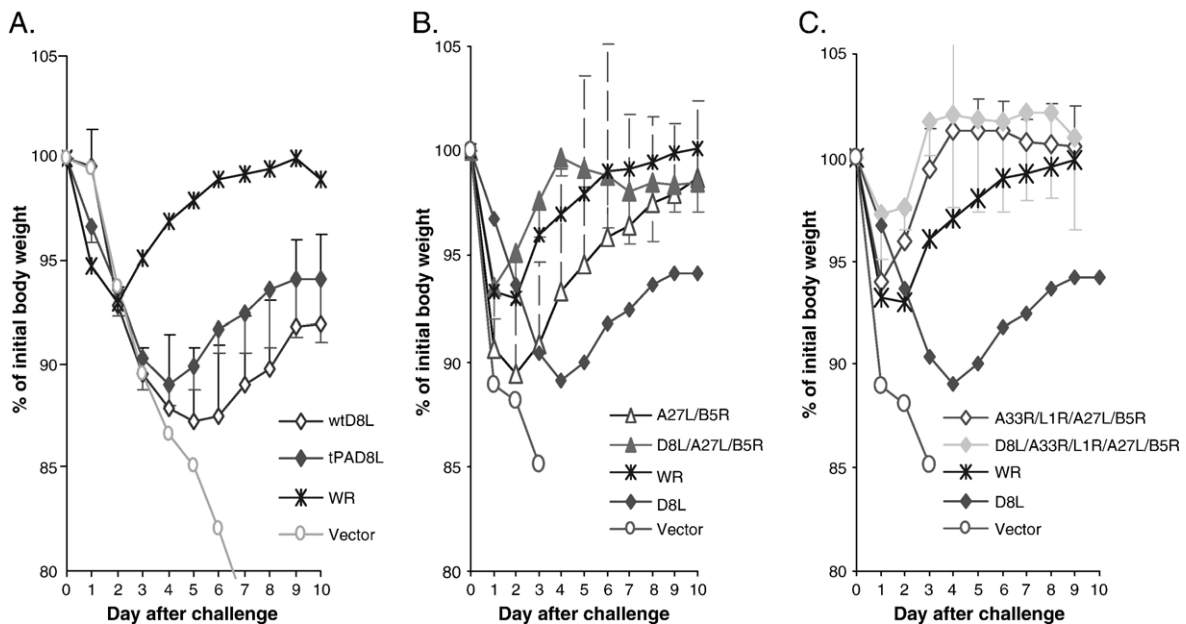


Fig. 3. Protection of BALB/c mice against challenge of vaccinia virus WR strain by the intraperitoneal route. Mice were challenged with $5 \times 10^7$ pfu of vaccinia virus WR strain 2 weeks after the final DNA immunization. The positive control group (WR) received immunization with $10^5$ pfu of vaccinia virus WR strain 2 weeks prior to challenge. The negative control group (vector) received the empty DNA vector. (A) Protection of mice immunized with individual D8L DNA vaccines. Body weight loss as the percentage of pre-challenge weight was measured. Each curve showed the group average weight loss (10 mice per group). (B) Protection of mice immunized with a bi-valent pox DNA vaccine formulation (A27L and B5R), with or without the third component (tPA-D8L DNA vaccine). Each curve showed the group average weight loss (10 mice per group). (C) Protection of mice immunized with a 4-valent formulation (A27L, B5R, L1R and A33R) with or without the addition of the fifth component (tPA-D8L DNA vaccine). Each curve showed the group average weight loss (10 mice per group).

Table 1
Average weight loss after VACV challenge

| Day | A27L/B5R | | D8L/A27L/B5R | | P value (paired Student's t test) |
|---|---|---|---|---|---|
| | % of initial weight | Standard deviation | % of initial weight | Standard deviation | |
| 0 | 100 | 0 | 100 | 0 | N/A |
| 1 | 90.6 | 2.5 | 93.6 | 1.5 | $p > 0.05$ |
| 2 | 89.5 | 3.5 | 95.2 | 1.8 | $p < 0.05$ |
| 3 | 90.8 | 3.8 | 97.7 | 1.7 | $p < 0.05$ |
| 4 | 93.4 | 5.8 | 99.7 | 0.8 | $p > 0.05$ |
| 5 | 94.6 | 8.9 | 99.2 | 0.9 | $p > 0.05$ |

protection provided by the live attenuated poxvirus vaccines (Fogg et al., 2004; Hooper et al., 2000; 2003; Pulford et al., 2004). Our data are the first report that D8 antigen alone was able to achieve a 100% protection against a lethal vaccinia challenge.

Next, we evaluated whether D8L DNA vaccine can be included in previously reported subunit-based polyvalent poxvirus vaccines (Fogg et al., 2004; Hooper et al., 2003) to further improve the efficacy of such formulations. DNA vaccine construct with the tPA-D8L insert was used in this study because measurements on both antibody response and weight loss indicated that the tPA-D8L DNA vaccine was more effective than the wtD8L DNA vaccine. DNA vaccines expressing individual poxvirus antigens (A27, B5, A33 and L1) were produced similar to previous reports (Hooper et al., 2000, 2003) except that the gene inserts were subcloned into the pSW3981 vector which is also used for the D8 DNA vaccines as described in this report. By using the same i.p. route challenge model as previously reported, the efficacy of bi-valent formulation with two well studied protective poxvirus antigens (A27 and B5) was significantly improved with the addition of

tPA-D8L DNA vaccine as the third component (Fig. 3B). Mice immunized with bi-valent formulation had lost 9.2% of weight on day 3 after challenge, whereas mice immunized with the 3-valent formulation including A27, B5 and D8 had lost only 2.3% ($p < 0.05$). Protection provided by this 3-valent formulation was similar to that provided with the vaccinia virus vaccination. Addition of D8 antigen also improved the protection efficacy of a previously reported 4-valent formulation (A27, B5, L1 and A33) by minimizing the weight loss during the first 1–2 days after challenge (Fig. 3C) when studied in the same i.p. challenge model. Mice immunized with the 5-valent formulation that included D8 antigen regained initial body weight at 2.5 days after challenge, as compared to 4.2 days in the 4-valent formulation group that did not include D8 ($p < 0.05$).

The above finding was further confirmed in an airway infection model where mice were challenged with $5 \times 10^6$ pfu of vaccinia virus delivered by the intranasal (i.n.) route as previously reported (Galmiche et al., 1999). In this study, mice that received the 5-valent formulation including D8 not only had stopped loosing weight earlier than those that received the 4-valent formulation (Fig. 4A), but also achieved 66% protection (10 out of 15 mice) as compared to the 26% protection (4 out of 15) by the 4-valent formulation against lethal challenge (Fig. 4). The difference of survival between two formulations was statistically significant ($p < 0.05$), but the difference in body weight loss was not.

*Antibody responses induced by polyvalent vaccine formulations in mice*

In order to further understand the specific antibody responses against individual pox antigens included in the polyvalent vaccine formulation and vaccinia vaccine, more detailed
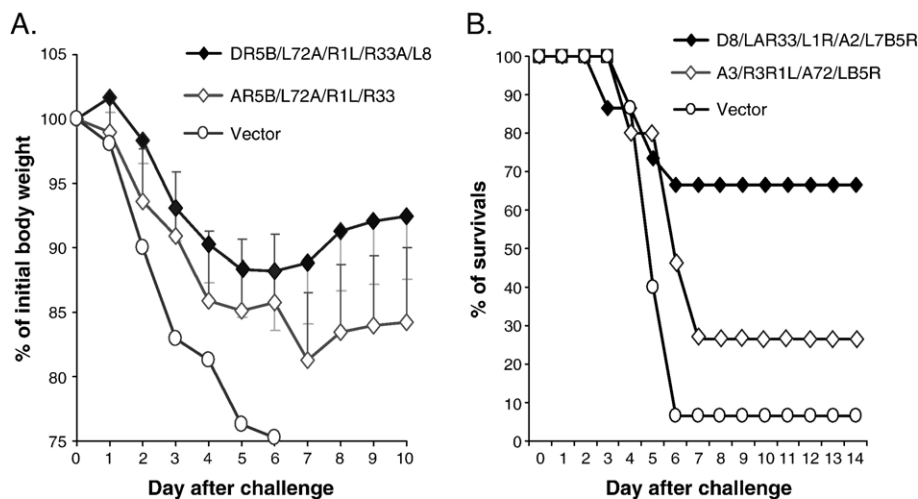


Fig. 4. Protection of BALB/c mice against lethal challenge of vaccinia virus WR strain ($5 \times 10^6$ pfu) by the intranasal route at 2-week after the last DNA immunization. Mice were immunized with a 4-valent pox DNA vaccine formulation (A27L, B5R, L1R and A33R) with or without the addition of the fifth component (tPA-D8L DNA vaccine). The negative control group (vector) received the empty DNA vector. (A) Body weight loss as the percentage of pre-challenge weight was measured. Each curve showed the group average weight loss of surviving mice (15 per group initially). (B) Survival curves of the same mice groups showing the percentage of mice alive at each day post-challenge.

analyses were conducted with mice sera immunized with different polyvalent vaccine formulations included in the above animal studies.

A27, D8 and L1 are highly immunogenic antigens. Anti-A27 antibody had the highest titers especially when mice received the bi-valent (A27 and B5) formulation (Fig. 5A). Anti-D8 antibody titers remained high in mice immunized with either 3-valent or 5-valent formulations (Fig. 5C). Similarly, anti-L1 antibody titers were high in mice immunized with either 4-valent or 5-valent formulations (Fig. 5D). B5 was less immunogenic with lower antibody titers against the autologous antigen (Fig. 5B). A33 was the least immunogenic among antigens included in the 4-valent and 5-valent formulations (Fig. 5E). It should be noted that since ELISA assays were carried out with crude lysates of cells transiently transfected with the DNA vaccines, low titers of anti-A33 antibodies may be attributed to either low level of specific IgG or to low level of A33 expression in 293T cells. However, induction of anti-vaccinia antibodies by A33R DNA immunization was indeed confirmed by Western blot against vaccinia virus (data not shown).

In our study, the total DNA vaccine dose was fixed no matter how many antigen components were included in various polyvalent formulations. There was less than 10-fold drop of anti-A27 titers when D8 was added to the bi-valent formulation containing A27 and B5. Otherwise, the levels of antigen-specific antibody responses only showed small drop when more poxvirus antigens were added, suggesting that the effect of antigen dilution due to the addition of new antigens was present but not significant. Mouse sera immunized with the vaccinia vaccine had positive antibodies against each of the 5 poxvirus antigens, confirming that these proteins were effective antigens during vaccinia immunization. However, the levels of such antibodies varied. Anti-D8 IgG titer was the highest and anti-A33 IgG titer was the lowest. Both vector control sera and sera lacking a particular antigen did not show specific reactivity against that antigen.

Sera from mice immunized with polyvalent DNA vaccines had high titers of IgG responses against vaccinia virus (VACV) antigens, and such titers increased when more antigens were added to the polyvalent DNA vaccine formulations (Fig. 5F). On the other hand, the anti-VACV titer for vaccinia virus
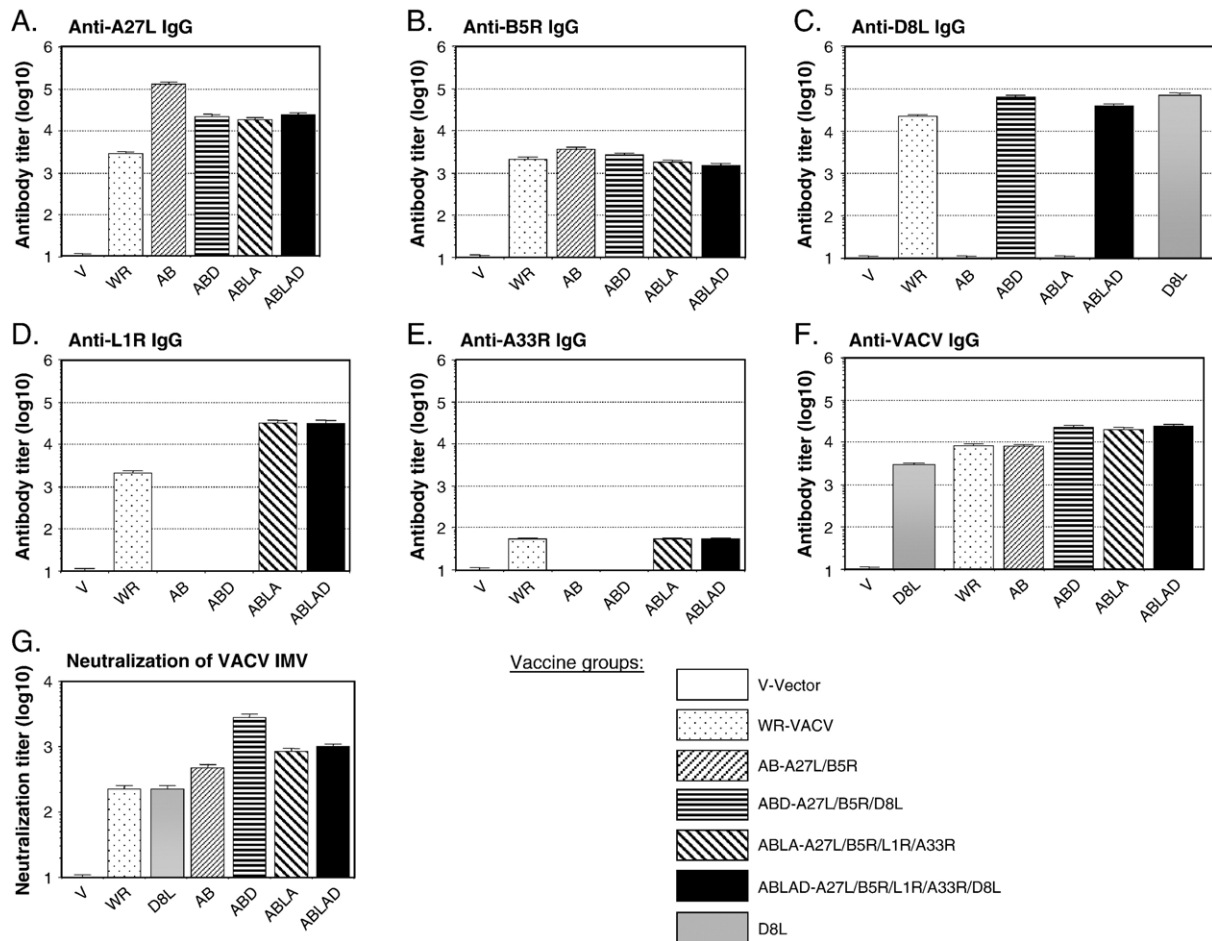


Fig. 5. Pre-challenge IgG antibody responses induced by individual D8L or polyvalent pox DNA vaccine formulations against either the individual pox antigens including A27 (A), B5 (B), D8 (C), L1 (D), A33 (E) or vero cell lysate infected with vaccinia virus (VACV) (F). Data are shown as the geometric means of end titration titers as determined by ELISA for each group (5 mice per group). Titers of neutralizing antibody responses against IMV were also shown (G) as the geometric means of highest serum dilutions that inhibit 50% of virus infection in the plaque reduction assay.

immunized mouse sera was lower than most of the polyvalent formulations. This finding suggested that while the vaccinia virus could induce antibody responses against very broad array of poxvirus antigens, subunit-based polyvalent formulations could develop higher total antibody responses even with less numbers of antigens. More importantly, the titer of anti-VACV antibody response did not correlate with protection (Figs. 3–5).

Levels of neutralizing antibody responses were also analyzed. In our assay, the neutralizing antibodies mainly targeted IMV form of poxvirus as previously described (Hooper et al., 2000). Each of the polyvalent formulations included in this report elicited positive neutralizing antibody responses at levels similar to or even better than that induced by the vaccinia vaccine (Fig. 5G). The 3-valent formulation including D8L elicited the strongest neutralizing antibody responses, presumably due to the combination of two strong IMV antigens, A27 and D8. The 5-valent formulation was less effective in generating neutralizing antibodies, most likely due to the diluting effect when the numbers of antigen components were increased.

## Discussion

Progress in the last several years has established the feasibility of subunit-based smallpox vaccines which can be delivered in the form of DNA plasmids (Hooper et al., 2003, 2004) or recombinant proteins (Fogg et al., 2004). While a single IMV or EEV antigen was able to provide various levels of protection, the combinations of these antigens in the form of polyvalent formulations afforded better protection in mice against vaccinia virus challenge and in non-human primates against monkeypox challenge (Fogg et al., 2004; Hooper et al., 2000, 2003, 2004). The polyvalent vaccine approach is attractive because broader immune responses can be generated by simultaneously targeting several antigens. The principle of polyvalent vaccines proved to be effective in licensed human vaccines such as those against polio, influenza virus and pneumococcus (Hurwitz et al., 2005).

Poxviruses are complex pathogens with potential protective antigen targets which have not been well studied. So far, even some antigens known to be able to induce neutralizing antibodies have not been included in the previously reported polyvalent formulations. It was also interesting to observe that reported polyvalent formulations were able to elicit better protection than the live attenuated poxvirus vaccines in mice but were less effective in protecting non-human primates (Hooper et al., 2003, 2004). Therefore, the effort of developing subunit-based poxvirus vaccines in the future needs to improve the polyvalent vaccines including the identification of additional protection antigens to enhance and broaden the protection efficacy.

In the current study, we produced a DNA vaccine expressing D8 protein, which is a known target for neutralizing antibody, but has not been included in the previously reported polyvalent poxvirus vaccines. D8 protein can bind to glycosaminoglycans on the cell surface and mediates the adsorption of intracellular mature virions to cells. Antibody raised against recombinant D8

protein neutralized IMV, soluble D8 protein inhibited viral absorption to the cells in a competitive manner and modified poxviruses with inactivated D8 demonstrated dramatically reduced infectivity (Hsiao et al., 1999). Immunization with recombinant D8 protein induced vaccinia-specific antibodies and proliferation responses (Demkowicz et al., 1992). Immunization with D8 DNA vaccine induced partial protection against intranasal challenge with IHD strain (Pulford et al., 2004). However, there were no reports of showing that D8 DNA vaccine was able to induce neutralizing antibodies and it is not clear whether D8 antigen can further improve the protection efficacy of reported polyvalent poxvirus formulations.

In this report, we demonstrated that D8L DNA vaccine-induced antigen-specific neutralizing antibodies. The levels of neutralizing antibody were similar to that induced by the vaccinia vaccine. Anti-D8 sera recognized vaccinia infected cell lysates, and mouse sera induced by vaccinia vaccine had significant levels of anti-D8 antibodies. Previous version of D8L DNA vaccine was not very immunogenic and not all immunized mice had positive anti-D8 antibody responses (Pulford et al., 2004). In the current study, we used an optimized DNA vaccine vector including an Intron A sequence downstream of the CMV IE promoter which was able to enhance the antigen expression and immunogenicity of DNA vaccines (Wang et al., 2006). Previous D8L DNA vaccine used the entire *D8L* gene insert but our data suggested that the modified D8L antigen is more immunogenic with the addition of tPA leader and the removal of C-terminal region including TM and cytoplasmic tail to facilitate its secretion. A similar *D8L* gene design for recombinant protein expression was reported by Hsiao et al. (1999) by removing the C-terminal region and adding a T7 tag to the N-terminus for bacterial expression of D8L antigen. There was no direct comparison between wild type D8 and modified D8 antigens in their study. Utilization of DNA vaccine as a tool made it easier for us to demonstrate that the modified D8L was more effective than the wild type D8L in antigen expression, antibody response and protection against lethal poxvirus challenge.

Our optimized D8L DNA vaccine construct alone was able to provide 100% survival to immunized mice against lethal poxvirus challenge by intraperitoneal route even though mice experienced more weight loss than those that received vaccinia vaccine. Addition of D8 antigen to previously reported 2-(A27+B5) or 4-valent (A27+B5+L1+A33) formulations further improved protective efficacy as measured by weight loss or survival. In the i.p. challenge model, animals experienced rapid weight loss as a result of receiving large amount of challenge virus. In contrast, the i.n. challenge model used by Fogg et al. for protein-based poxvirus vaccines, mimics the upper respiratory route of transmission of smallpox, requires lower amount of virus than i.p. challenge and takes more days to develop critical weight loss. Our study also showed that mice are more susceptible to i.n. vaccinia challenge than the i.p. challenge, and the lethal dose of virus is significantly reduced with the i.n. route. The i.n. challenged mice demonstrated high morbidity and mortality at a later time post challenge, suggesting a different dynamics of host and pathogen interaction from the

i.p. challenge. In our experience, the i.n. challenge represented a more stringent test since the same polyvalent formulations could not provide 100% survival as they did against the i.p. challenge. We used i.p. challenge model in the current study to be consistent with the results reported by Hooper et al. who also used DNA vaccines to deliver subunit poxvirus antigens. We then used the i.n. challenge model to further demonstrate the significant difference between the polyvalent formulations with or without D8 antigen. The polyvalent poxvirus vaccine formulation delivered by DNA immunization effectively induced protection regardless of the model used when compared to the negative controls. Given the many differences between mice and humans, the above results are highly suggestive of the protective potential of polyvalent poxvirus vaccines but their true efficacy will require protection study in non-human primates, and well organized human clinical trials with specified immunogenicity biomarkers which may correlate to protection.

In this study, we also compared the antibody responses against individual recombinant poxvirus antigens, antibody responses against entire vaccinia virus, and neutralizing antibody responses elicited by different DNA vaccine formulations and vaccinia vaccine. It was clear that the levels of antibody responses were partially related to the amount of DNA plasmids used for vaccination. By increasing the valency of the polyvalent formulation in a fixed total dosing regimen, the effective dose of individual components was reduced and the levels of antibody responses against individual antigens were lower. However, the levels of antibody responses against the vaccinia virus were increased with more antigens included in a polyvalent formulation, reflecting an additive effect of antibody responses against multiple antigens. This is similar to vaccinia vaccine which induces very broad antibody responses recognizing many poxvirus antigens but the titers of antibodies against individual poxvirus antigens may be lower than that induced by individual subunit-based vaccines.

In the case of neutralizing antibody responses, our results demonstrated a mixture of the above phenomena. When animals received vaccine formulations of 2–3 antigens, addition of another strong IMV antigen (such as D8) increased the levels of neutralizing antibody responses as measured by IMV mediated antibody responses. When more non-IMV antigens were added, the levels of neutralizing antibody responses actually were reduced. Interestingly, the protection was not correlated with the levels of IMV-mediated neutralizing antibody responses. Rather, better protection was achieved with more antigens included in the vaccine formulations, in a way mimicking vaccinia vaccine. At least two other protection mechanisms can be accounted for. First, the polyvalent poxvirus vaccine formulations and vaccinia vaccines also included EEV antigens and thus EEV-mediated neutralizing antibodies may play an important role for part of the protection as reported by previous studies (Appleyard et al., 1971; Boulter and Appleyard, 1973; Fogg et al., 2004; Galmiche et al., 1999; Hooper et al., 2003, 2004; Law and Smith, 2001). Second, cell-mediated immunity (CMI) may

also be critical for protection against smallpox infection (Demkowicz et al., 1996; Ennis et al., 2002; Frey et al., 2002; Kennedy et al., 2004). The finding that the increased IMV neutralizing antibody did not correlate with increased protection will have important impact to the design of subunit-based poxvirus vaccines.

In summary, we demonstrate in this report that D8 is an excellent candidate for subunit-based poxvirus vaccines. It was able to induce neutralizing antibodies and protection in both i.p. and i.n. challenge models in mice. Addition of D8 antigen did not interfere with the high immunogenicity of other antigen components in previously reported polyvalent poxvirus vaccine formulations. Our results proved that DNA immunization is a cost effective approach to screen and identify additional protective poxvirus antigens. The ultimate format of subunit-based poxvirus vaccines can be delivered in the form of DNA, protein or a combination of DNA prime plus protein boost which will be benefited from both delivery systems. Most importantly, results presented in this report support further search for optimal groups of protective poxvirus antigens so that polyvalent poxvirus vaccines can be finalized with the best protection efficacy. Since poxviruses are complex viruses, it may be reasonable to study the protection efficacy of additional antigens including those with less well studied protection mechanisms before finally selecting the candidate antigens to be included in subunit-based polyvalent poxvirus vaccine formulations.

## Material and methods

### Viruses and cells

WR strain of vaccinia virus (VACV, provided by Dr. Liisa Selin, University of Massachusetts Medical School) was propagated in Vero cells and clarified cell lysates were used for Western blot analysis and ELISA. VACV stock for challenge was prepared in L929 cells (Selin et al., 1994) and purified from serum contaminants by centrifugation on sucrose gradients (Chen et al., 2001). Viral titer assays were performed on Vero cells (Selin et al., 1994, 1998).

### Construction of DNA vaccines

Individual poxvirus genes (*D8L*, *A27L*, *B5R*, *L1R* and *A33R*) were PCR amplified from WR strain of VACV using pfu DNA polymerase (Stratagene, CA). DNA inserts were then subcloned into pSW3891 after the CMV immediately early (IE) promoter (Wang et al., 2005). For tPA-D8L construct, the PCR amplified genes were subcloned into the same vector downstream of the tPA leader sequence (Lu et al., 1998; Wang et al., 2004a, 2006). Each DNA vaccine plasmid transformed in *E. coli* (HB101 strain) was confirmed by restriction digestion and DNA sequencing before large amounts of DNA plasmids were prepared with a Mega purification kit (Qiagen, Valencia, CA). In this report, poxvirus proteins are referred as D8, A27, B5, L1 and A33 while their genes and the related DNA vaccines used *D8L*, *A27L*, *B5R*, *L1R* and *A33R*.

## Immunization of animals

Six to eight weeks old female BALB/c mice were purchased from Taconic Farms (Germantown, NY) and housed in the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS) in accordance with IACUC approved protocols. The animals were immunized with a Helios gene gun (Bio-Rad Laboratories, Hercules, CA) at the shaved abdominal skin as previously reported (Wang et al., 2004b). Each mouse received 4 bi-weekly immunizations with six DNA shots of 2 μg each per immunization. At least 5 mice were included in each group for immunogenicity study while 10 mice included in the protection study. The blood samples were collected peri-orbitally prior to the first immunization and 2 weeks after each immunization. Mice immunized with VACV received $10^5$ pfu of VACV in 10 μl PBS by intradermal inoculation into the ear pinnae 1 month before challenge (Tscharke et al., 2002; Tscharke and Smith, 1999).

A pilot rabbit immunization study was conducted to produce D8-specific rabbit sera which were used for the Western blot analysis of D8 antigen expression. New Zealand White (NZW) rabbits of 2–3 kg body weight were purchased from Millbrook Farm (Amherst, MA). Each rabbit received 3 bi-weekly immunizations of 36 μg D8L DNA vaccines at each immunization by a gene gun at the shaved abdominal skin. Sera were collected 2 weeks after the last immunization. D8-specific rabbit serum R274 was used for Western blot analysis in this report.

## Vaccinia virus-infected cell lysates

Vaccinia virus antigen for use in antibody binding assays was prepared as previously described (Fogg et al., 2004) with following modifications. Two T-150 flasks of confluent Vero cells were infected with WR strain of VACV at a multiplicity of infection (m.o.i.) of 1. After 3 days, infected cells were harvested and collected by centrifugation at 500 g for 5 min. The cell pellets were resuspended in 10 mM Tris–HCL (pH 8) and freeze-thawed 3 times, followed by Dounce homogenization. The homogenates were centrifuged at $500 \times g$ for 5 min and supernatants were aliquoted and stored at $-70$ °C.

## Transient expression of D8 antigen

The 293T cells were transiently transfected by a calcium phosphate co-precipitation method using 10 μg of plasmid DNA for $2 \times 10^6$ cells in a 60-mm dish. Cells were harvested 72 h later. Both supernatants and cell lysates were collected for ELISA or Western blot use. Supernatants from multiple dishes are pooled and the amount of D8 is determined by Coomassie blue staining of SDS-polyacryamide gel electrophoresis (SDS–PAGE) gels.

## Enzyme-linked immunosorbent assay (ELISA)

Vaccinia virus antigen-specific IgG responses in immunized mice were measured by ELISA using individual mouse sera from each animal group. ELISA plates were coated with 100 μl of the antigens at 1 μg/ml harvested from 293T cells transiently transfected with the DNA vaccine plasmids and incubated overnight at 4 °C. For anti-VACV assays, ELISA plates were coated with $10^5$ pfu of VACV per well and fixed with 2% paraformaldehyde (Wyatt et al., 2004). Serially diluted mouse (100 μl) were added to each well and assayed in duplicate after the blocking. The plates were incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) diluted at 1:1000 (100 μl per well), followed by horseradish peroxidase-conjugated streptavidin (Vector Laboratories) diluted at 1:2000 and finally developed with 3,3-,5,5-tetramethybenzidine (Sigma-Aldrich, St. Louis, MO) solution (100 μl per well). The reactions were stopped by adding 25 μl of 2M $H_2SO_4$, and the plates were read at OD450 nm. The endpoint titer was calculated as the serum dilution resulting in absorbance greater than 2 standard deviations above the absorbance in wells incubated with negative control mouse serum.

## Western blot analysis of in vitro expressed VACV antigens

The 293T cells were transiently transfected by a calcium phosphate co-precipitation method using 10 μg of plasmid DNA for $2 \times 10^6$ cells in a 60-mm dish, and were harvested 72 h later. Same amount of transiently expressed antigens (10 ng of protein) were loaded for the SDS–PAGE, then transferred onto PVDF membranes (Bio-Rad, Hercules, CA), and blocked overnight at 4 °C in blocking buffer (0.2% I-block, 0.1% Tween-20 in 1X PBS). Membranes were incubated with 1:200 dilution of rabbit sera immunized with corresponding DNA vaccines. After being washed, blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Tropix, Bedford, MA) at 1:5000 dilution for 1 h at room temperature, and signals were detected using a chemiluminescence Western-Light Kit (Tropix, Bedford, MA). For the glycosylation study, peptide N-Glycosidase F, PNGaseF (New England BioLab, Beverly, MA) was added to the denatured samples prepared from the supernatants of transiently transfected 293T cells according to manufacturer's instruction. After incubating overnight at 37 °C, samples were loaded onto SDS-PAGE, and analyzed by Western blot as described above.

## Plaque reduction neutralization assay

Fifty percent plaque reduction titer was determined by standard techniques (Frey et al., 2002). Briefly, sera from immunized animals were heat inactivated for 30 min at 56 °C and serial dilutions of antibodies were incubated with 50 pfu of VACV for 1 h at 37 °C. Confluent Vero cells monolayers were infected with antibody-virus mixtures for 1 h, washed with PBS and incubated under liquid overlay for 2 days. Monolayers were then stained with 0.5% of crystal violet for 5 min and plaques were counted. The neutralization was calculated as the percentage of the number of plaque counts reduced at the testing serum in an assay compared to the

mean of the plaque counts for the three virus controls (without sera) in the same assay.

## Vaccinia virus challenge

Age matched female BALB/c mice (10 per group) were used in all experiments. Two weeks after the last immunization, BALB/c mice were anesthetized intramuscularly with ketamine–xylasine and then injected with $5 \times 10^7$ pfu WR strain of VACV in 100 μl of PBS by intraperitoneal route. Alternatively, BABL/c mice at 2 weeks after the last immunization received $5 \times 10^6$ pfu WR strain of VACV in 25 μl of PBS by intranasal inoculation. Mice were weighed and observed daily as previously described (Selin et al., 1994). All experiments were done in compliance with protocols approved by the IACUC and IBC at the University of Massachusetts Medical School.

## Statistical analysis

Tests were performed using Epi Info™ software for windows available form CDC web site. Survival curves were analyzed using Kaplan–Meier test. Comparisons between the mean percentage body-weight changes for different groups at each day after challenge were performed using an unpaired, two-tailed Student's *t* test (Microsoft Excel software, version 2003) in consultation with a biostatistician. Significance levels were set at a *P* value less than 0.05.

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