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# Immunogenicity and protection efficacy of subunit-based smallpox vaccines using variola major antigens

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**Abstract**

The viral strain responsible for smallpox infection is variola major (VARV). As a result of the successful eradication of smallpox with the vaccinia virus (VACV), the general population is no longer required to receive a smallpox vaccine, and will have no protection against smallpox. This lack of immunity is a concern due to the potential for use of smallpox as a biological weapon. Considerable progress has been made in the development of subunit-based smallpox vaccines resulting from the identification of VACV protective antigens. It also offers the possibility of using antigens from VARV to formulate the next generation subunit-based smallpox vaccines. Here, we show that codon-optimized DNA vaccines expressing three VARV antigens (A30, B7 and F8) and their recombinant protein counterparts elicited high-titer, cross-reactive, VACV neutralizing antibody responses in mice. Vaccinated mice were protected from intraperitoneal and intranasal challenges with VACV. These results suggest the feasibility of a subunit smallpox vaccine based on VARV antigen sequences to induce immunity against poxvirus infection.


*Keywords:* Variola major; Vaccinia virus; Smallpox; Subunit vaccine

## Introduction

The original smallpox vaccine, based on the live attenuated vaccinia virus (VACV), eradicated smallpox from the worldwide human population with the last case of natural smallpox infection occurring in Somalia in 1977 (WHO, 1980). However, due to safety concerns over the manufacture of the traditional VACV vaccine, the production of this type of vaccine, such as DryVax (Wyeth Laboratories, Inc.) in the United States and derived from the NYCBH strain of vaccinia virus, is no longer considered acceptable since termination of its production in 1982. Reports of adverse events including myocarditis that are associated with immunization with VACV (Casey et al., 2005) have prevented the completion of a 2002 re-vaccination campaign among non-military high-risk populations, such as healthcare professionals. Currently, VACV is still the main smallpox vaccine and a newer generation of live-attenuated VACV vaccines is being produced in tissue cultured cells to allow for stockpiling in hopes of being able to protect the general population in the event of a bioterrorist attack (Artenstein et al., 2005; Fang et al., 2006; Monath et al., 2004).

Recent studies have made great strides in demonstrating the use of subunit-based smallpox vaccines to induce protective immunity against smallpox. Poxviruses, which include variola major (VARV), vaccinia virus (VACV), monkeypox virus, ectromelia virus (ECTV) and others, belong to the *orthopoxvirus* genus and considerable cross-protection has been observed between these viruses. Poxviruses are large viruses with a genome that encodes about 200 proteins and it is this complexity which has partly delayed the identification of protective antigens against these viruses. Several potential targets of protective immunity have only recently been confirmed in well organized

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animal studies. There are two forms of infectious poxvirus: the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). Recent studies have shown that vaccinia virus IMV-specific antigens, A27, L1 and D8, and EEV-specific antigens, A33 and B5, are immunogenic and protective, albeit variably, against VACV infection in mice (Fogg et al., 2004; Galmiche et al., 1999; Hooper et al., 2000, 2004; Pulford et al., 2004; Sakhatskyy et al., 2006; Xiao et al., 2007), against ectromelia virus (ECTV) (Xiao et al., 2007) and against monkeypox in non-human primates (Heraud et al., 2006; Hooper et al., 2004). Furthermore, a four-gene combination DNA vaccine that encodes two IMV (A27 and L1) and two EEV (A33 and B5) antigens was protective against vaccinia virus challenge in mice and induced antibody responses against monkeypox virus in non-human primates (Hooper et al., 2003). However, these subunit vaccines were not as protective as the live-attenuated vaccinia virus vaccine unless polyvalent formulations and/or multiple immunizations are used to achieve comparable levels of protection inducible by one single vaccinia inoculation (Fogg et al., 2004; Hooper et al., 2000, 2003; Pulford et al., 2004; Sakhatskyy et al., 2006).

Why the live-vaccinia virus vaccine offers better protection against infection is unclear and studies have been conducted to examine the contributions of the two arms of the immune system in offering protective immunity against poxvirus infection by depletion of B- and T-cells prior to primary and/or secondary challenge with various poxviruses, offering conflicting results. Antibody-mediated depletion of B-cells, but not of CD4+ or CD8+ T-cells, prevented vaccine-induced protection from a lethal intravenous challenge with monkeypox virus (Edghill-Smith et al., 2005) and poxvirus in a mouse model (Belyakov et al., 2003) indicating that this protective response is primarily mediated by antibodies and that vaccinia-induced antibodies are necessary and sufficient for protection against a lethal poxvirus challenge. Additional results confirming the role of antibody responses show that passive administration of VACV antibodies confers protection from subsequent lethal monkeypox (Edghill-Smith et al., 2005; Hooper et al., 2004) and that type I/II IFN deficient, CD8+ depleted mice were able to survive a *secondary* infection with ECTV (Panchanathan et al., 2005). However, to determine immune responses in primary and secondary vaccination against smallpox in humans, a recent study has shown that positive CMI responses could be elicited 7 days after infection in secondary immunized volunteers (i.e., vaccinia non-naive) and that this response preceded increases in antibody titers (Kennedy et al., 2004). Furthermore, an important role for interferon (IFN) and less of a role for antibodies in conferring protection against poxvirus was observed following a *primary* infection (Panchanathan et al., 2005; Pulford et al., 2004). While the exact roles of each arm of the immune system in mediating poxvirus infection have not been completely elucidated, the majority of recent reports point to a greater role of antibodies against various poxvirus antigens in eliciting protection against poxvirus infection. Therefore, it appears as though subunit-based smallpox vaccines that induce poxvirus-specific antibodies would be effective in conferring protection from poxvirus infection.

Although poxviruses are highly conserved in the regions that encode protective antigens, it is not clear whether antibodies induced by variola antigens may confer a higher level of protection against homologous challenge when compared to those responses induced by vaccinia antigens. A recent study examining the differences between the major neutralizing B5 antigenic site on vaccinia virus and its variola virus ortholog B6 demonstrated that from a panel of 26 anti-B5 monoclonal antibodies only 16 cross-reacted with B6 protein and out of 10 that did not at least 3 were EEV neutralizing or blocked comet formation (Aldaz-Carroll et al., 2007). These results indicate that the production of a subunit-based vaccine using VARV antigens rather than those from VACV may confer greater protection against smallpox infection and there is no apparent reason not to use VARV antigens with a subunit-based vaccine. While safety issues over using the variola virus itself as a live attenuated vaccine lead to the use of antigens from the vaccinia virus, the production of a subunit-based vaccine does not incorporate live viruses into its design and therefore eliminates the safety concerns over using variola virus antigens. The goal of this current study was to demonstrate the feasibility of producing a subunit smallpox vaccine based on VARV antigen sequences. At least in theory, this approach should minimize the chance of reduced protection due to any sequence difference that may exist between VARV and VACV, particularly when antigen-specific immune responses, such as neutralizing antibody determinants and/or dominant T-cell epitopes, are involved. In the current study, three VARV antigens (A30, B7 and F8) were chosen based on the high immunogenicity and potential of protection from their VACV counterparts (A27, B5 and D8) as shown in the literature and from our recently published studies (Sakhatskyy et al., 2006).

## Results

### *Sequence homology between variola major and vaccinia protective antigens*

Amino acid sequences of three well-characterized protective antigens (A27, B5 and D8) from VACV were compared with orthologous proteins from the VARV virus (Fig. 1). For VACV sequences, two frequently used strains, Western Reserve (WR) and Copenhagen (COP), were included for the analysis. For VARV, the India 1967 (VARV-IND) and Bangladesh 1975 (VARV-BSH) strain sequences were used. The orthologous VARV protein for the VACV A27 antigen is A30 for VARV-IND and A31 for VARV-BSH; for the B5 antigen the orthologous protein is B7 for VARV-IND and B6 for VARV-BSH, and for the D8 antigen the orthologous protein for both VARV-IND and VARV-BSH is F8 (Shchelkunov, 1995; Shchelkunov et al., 1995). Sequences of these three proteins are highly homologous but not completely identical to the VACV antigen sequences: three amino acid differences exist between A27 and A30/A31, 23 amino acid differences between B5 and B6/B7 and 12 amino acid differences between D8 and F8 proteins (Fig. 1). Some of these differences occurred with only one of the two VARV strains, thereby further improving the overall level of homology.

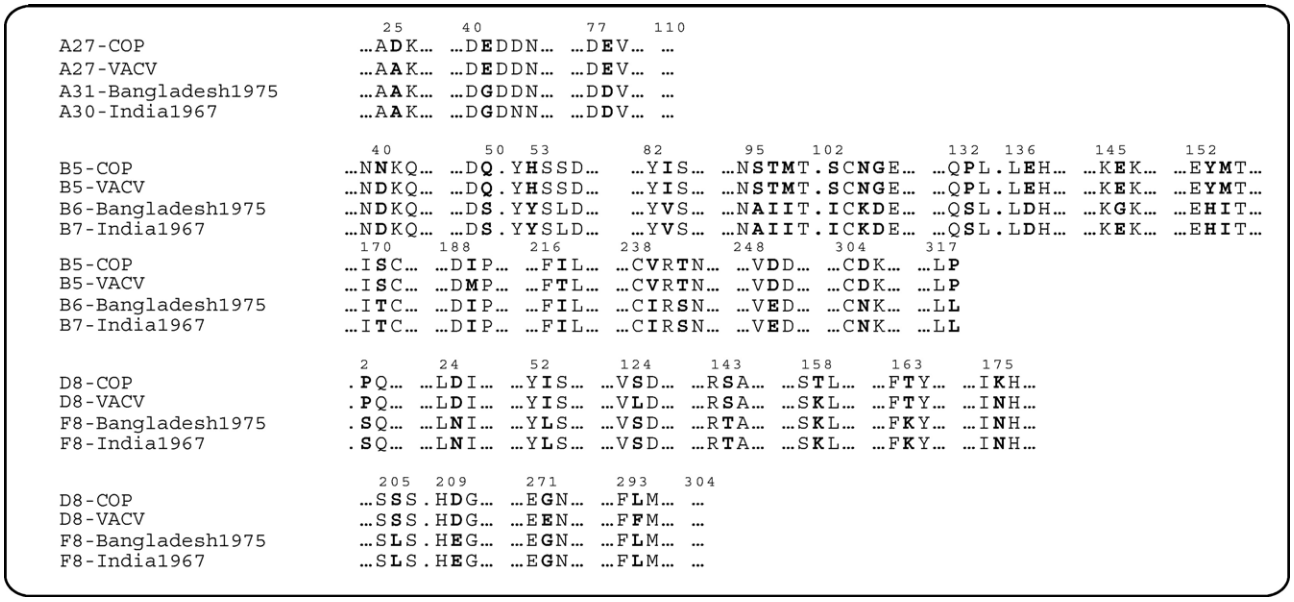


Fig. 1. Sequence analysis of the selected protective variola major and vaccinia antigens. Amino acid sequences of the proteins encoded by the WR and COP strains of the vaccinia virus and those encoded by variola India 1967 and Bangladesh 1975 were compared. The numbers at the top are amino acid positions. Amino acids that were different between these strains are shown in bold, and amino acids that are not shown but identical in all viruses are presented as dots.

*Construction of VARV DNA vaccines and production of recombinant A30, F8 and B7 by an E. coli expression system*

The codon modified A30L, B7R and F8L genes of VARV-IND were chemically synthesized without the involvement of actual variola virus. Although the final codons did not change what amino acids were coded, this procedure increases the frequency of codons that are used by mammalian cells and has been shown to potentially improve antigen expression and immunogenicity of DNA vaccines (Haas et al., 1996; Wang et al., 2006). Then the synthetic genes coding for the VARV A30, F8 or B7 proteins were individually cloned into the DNA vaccine vector pSW3891, which uses a CMV IE promoter to drive the expression of the coded antigen insert (Wang et al., 2005). In addition, they were all placed behind a human tissue plasminogen activator (tPA) leader sequence which was shown to be effective in improving the antigen expression and antigen immunogenicity for DNA vaccines (Wang et al., 2006). A30 and F8 DNA vaccines encode for the full-length proteins while B7 DNA vaccine expresses the ectodomain of B7 because the full-length B7 DNA vaccine does not achieve a good antigen expression (data not shown).

Expression of these codon-optimized VARV antigen DNA vaccines was verified in culture supernatants and cell lysates from 293T cells transiently transfected with each of the three VARV antigen DNA plasmids and examined by Western blot using a polyclonal anti-vaccinia virus serum (Fig. 2). All three antigens were present in both the cell lysate (L) and culture

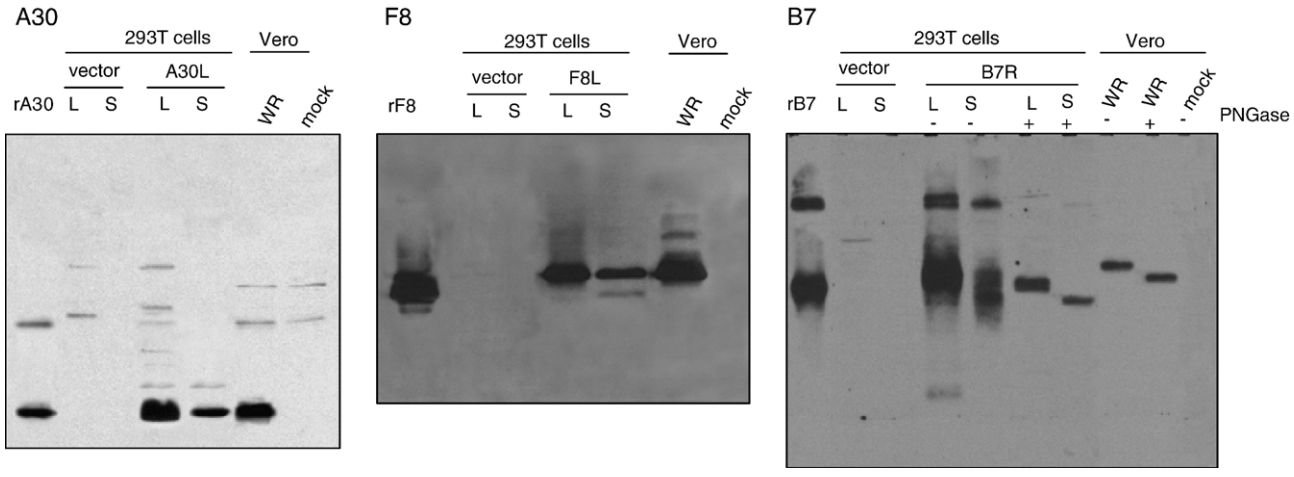


Fig. 2. Western blot analysis of transiently expressed A30, F8 and B7 proteins from the cell lysate (L) and culture supernatant (S) of 293T cells transfected with their respective variola major antigen-expressing DNA vaccine (A30L, F8L or B7R). Recombinant variola major proteins rA30, rB7 and rF8 produced in *E. coli* are also included in the analysis. Vaccinia WR strain, grown in Vero cells, is included as a positive control. The empty DNA vaccine vector is included as a negative control. The B7 proteins, which were glycosylated when expressed in 293T or Vero cells, were subjected to PNGase treatment.

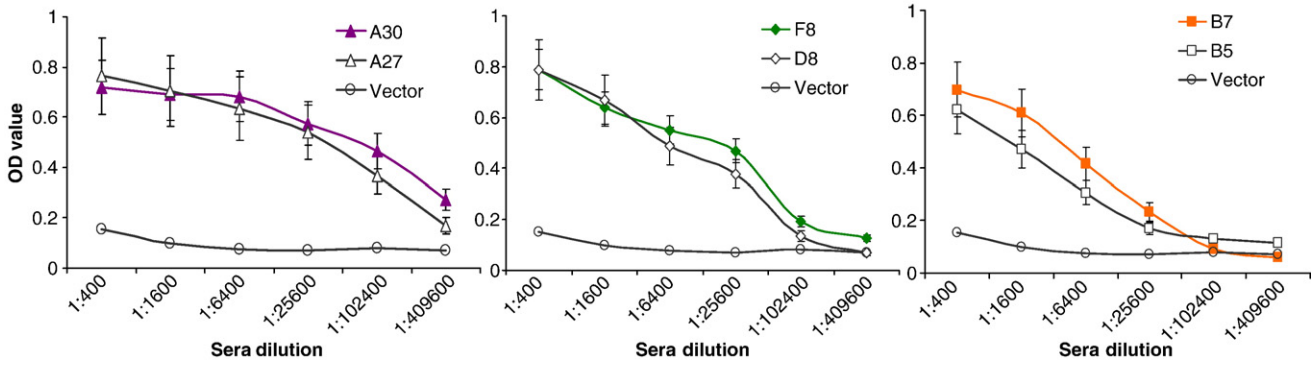


Fig. 3. Recognition of variola major and vaccinia antigens by ELISA using the same polyvalent mouse serum induced with the combination of three DNA vaccines expressing variola major antigens A30, B7 and F8. Each curve is the average of sera assayed from 10 mice that have received three monthly immunizations. Each panel shows one pair of ortholog antigens from both variola and vaccinia.

supernatant (S) and their molecular weights, in general, matched those from the VACV-WR strain grown in Vero cells, except for the B7 DNA vaccine which only expresses a smaller molecular weight ectodomain of B7 protein. The apparent molecular weight of B7 antigen in lysate was slightly larger than B7 in supernatant—indicating additional post-translational processing of B7 protein as an intermediate product prior to secretion. Non-transfected 293T cells and uninfected Vero cells did not show pox-specific antigens (Fig. 2). In addition, we show that recombinant A30, B7 and F8 proteins (rA30, rF8 and rB7) were successfully produced and purified from an *E. coli* expression system using the same synthetic A30, B7 and F8 genes (Fig. 2). These results also show that the B7 protein forms dimers in its non-denatured condition. According to sequence analysis, the B7 protein has potential sites for *N*-glycosylation which was confirmed by its sensitivity to PNGase treatment (Fig. 2).

### *Immunogenicity of the A30, F8 and B7 DNA vaccines*

Three monthly immunizations with VARV DNA vaccines elicited high levels of antigen-specific IgG antibody responses in mice (Fig. 3). The immunogenicity of each DNA vaccine is slightly different: A30 being the most immunogenic, followed by the F8 and B7 DNA vaccines. Antibodies induced by immunization with codon optimized variola DNA vaccines recognized both vaccinia and variola antigens expressed in the supernatant of transiently transfected 293T cells. Given the polyclonal nature of the immune sera, it was not surprising that we did not detect a significant difference in recognition of variola or vaccinia antigens by ELISA (Fig. 3).

Immunization with DNA vaccines, expressing the two individual VARV IMV antigens, F8 and A30, produced antibodies that were able to neutralize VACV in a plaque reduction assay that measures IMV antibodies (Fig. 4). The levels of neutralizing antibodies were similar to immune sera elicited by their individual VACV counterparts, D8 and A27. Similar to the previous report with VACV IMV antigens (Sakhatskyy et al., 2006), the subunit VARV IMV antigens were more effective than an intact vaccinia infection in eliciting IMV neutralizing antibodies in the current study.

### *Protection efficacy of VARV DNA vaccines expressing A30, F8 and B7 antigens against lethal VACV challenges in mice*

We tested the ability of mono- and polyvalent DNA vaccines that expressed VARV antigens to induce protection in two VACV challenge models. In the first challenge study, mice first received three monthly DNA immunizations, rested for 1 month and received another boost 2 weeks prior to challenge to ensure a complete protection in this pilot study. Mice were inoculated intraperitoneally (i.p.) with a lethal dose ($5 \times 10^7$ pfu) of VACV (WR). VARV antigen-specific antibodies after 1, 2 or 3 monovalent DNA vaccine inoculations were measured by ELISA (Fig. 5A). DNA vaccines expressing each of the three VARV antigens induced high titers of antibodies after the 2nd immunization and additional DNA vaccination was not needed

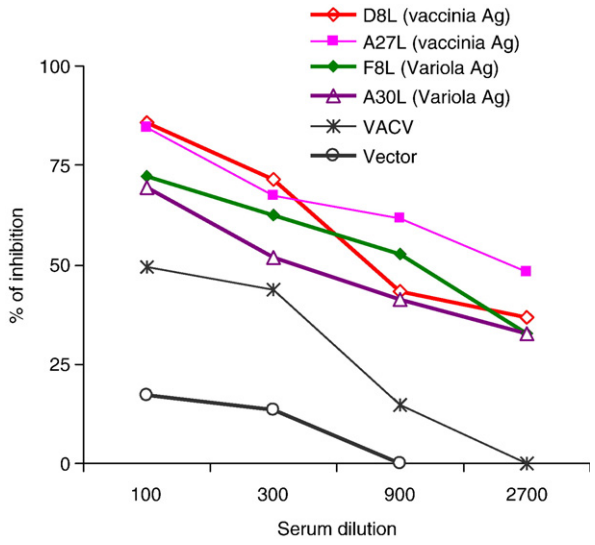


Fig. 4. Neutralizing antibody activities against the vaccinia intracellular mature virus (IMV) as measured by the plaque reduction test. Sera were collected from BALB/c mice after 3 monthly immunizations with DNA vaccines expressing either individual vaccinia antigens (A27L and D8L) or individual variola antigens (A30L and F8L). Sera from mice immunized with VACV served as a positive control. Sera from the group that received an empty DNA vaccine vector were used as the negative control. Data are shown as average titers from 10 mice per group.

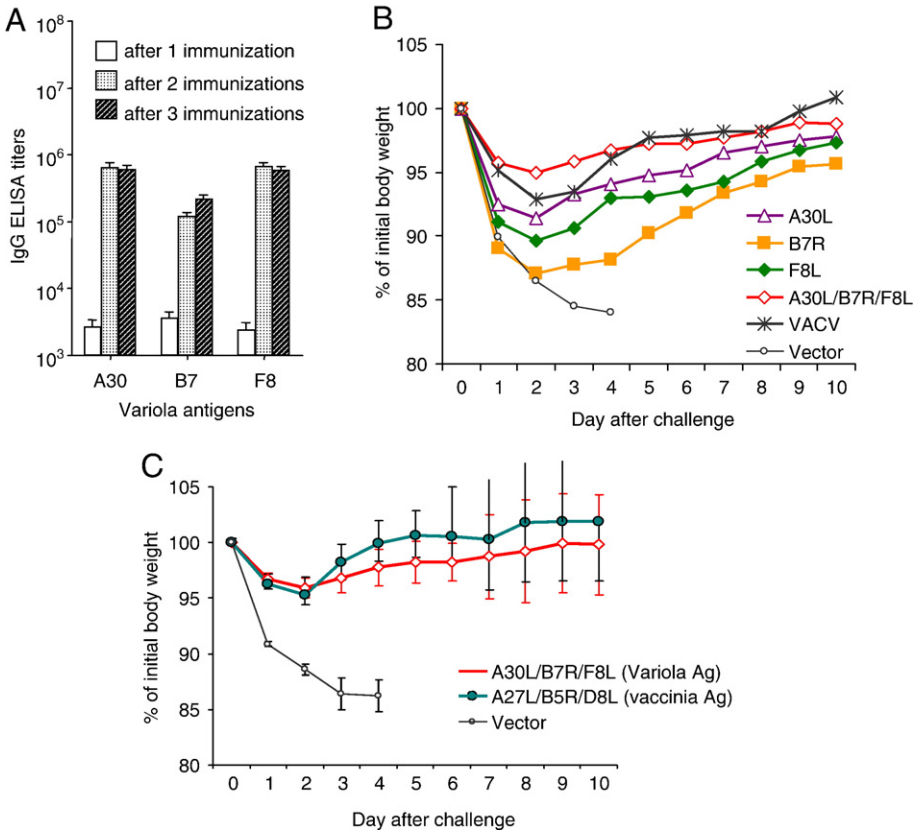


Fig. 5. Variola antigen-specific antibody responses and protection against lethal intraperitoneal (i.p.) VACV challenge in BALB/c mice immunized with DNA vaccines expressing variola antigens. (A) ELISA analysis of end titration IgG titers of mouse sera immunized with monovalent DNA vaccines expressing either A30, B7 or F8 against the autologous antigens expressed in 293T cells. Sera were collected after one, two or three monthly DNA immunizations. The data are shown as the geometric mean titers of 5 animals. (B) Protection against i.p. VACV challenge in mice immunized with either the monovalent or the combination of A30L, B7R and F8L DNA vaccines. Mice immunized with either the positive control vaccinia (WR) or the negative control empty DNA vaccine vector are also included. Body weight loss, shown as the percentage of pre-challenge weight, was measured daily. Each curve shows the group average weight loss (5 mice per group) after challenge. (C) Protection against i.p. VACV challenge in mice immunized with either combination of vaccinia DNA vaccines (A27L, B5R and D8L) or combination of variola DNA vaccines (A30L, B7R and F8L) or an empty DNA vaccine vector as the negative control. Body weight loss, shown as the percentage of pre-challenge weight, was measured daily. Each curve shows the group average weight loss ± standard deviation (5 mice per group) after challenge.

to further boost the levels of antibody responses (Fig. 5A). As an indicator of protection against lethal VACV in this i.p. challenge model, average weight loss of the surviving mice in each group was measured (Fig. 5B). All negative control mice inoculated with the DNA vector progressively lost weight and died by Day 5. Mice that received either the mono- or polyvalent VARV DNA vaccines survived the challenge and regained body weight. Mice that received the polyvalent VARV DNA vaccine recovered back to their initial body weight sooner than mice that received any of the monovalent DNA vaccines (Fig. 5B). The polyvalent VARV and polyvalent VACV formulations were able to achieve similar levels of protection (Fig. 5C).

Although intraperitoneal VACV challenge leads to lethal poxvirus infection, it does not represent the natural aerosol spread of the virus. The intranasal method of infection requires significantly less virus to produce a lethal infection and causes death at a later time point suggesting a different virus–host interaction. In addition, the intranasal mode of infection represents a more stringent challenge model (Sakhatskyy et al., 2006). Therefore, we investigated the efficacy of protection when subunit-based VARV antigens were delivered in the form of DNA or recombinant protein vaccines in an intranasal challenge model.

In this intranasal challenge study, groups of mice received 2 bi-weekly immunizations of either a polyvalent rA30, rB7 and rF8 recombinant protein vaccine or a polyvalent DNA vaccine expressing the A30, B7 and F8 antigens (Fig. 6). The number of immunizations was reduced to two based on the excellent immune responses after two immunizations as shown in the i.p. challenge studies (Fig. 5). Mice immunized with the vaccinia vaccine served as a positive control, and the negative control group received only empty DNA vector (Fig. 6). Antigen-specific antibodies induced by either the DNA or protein formulations and immunizations with vaccinia vaccine were analyzed by ELISA (Fig. 6A). Immunization with the polyvalent recombinant VARV protein vaccines was significantly more immunogenic than immunization with the polyvalent VARV DNA vaccines ($p<0.01$) (Fig. 6A) based on ELISA results. The protein vaccine was even more immunogenic than the live vaccinia vaccine in generating specific antibodies against three tested pox antigens. In contrast, immunization with the polyvalent DNA-based VARV vaccines induced only marginally higher antibody responses when compared to immunization

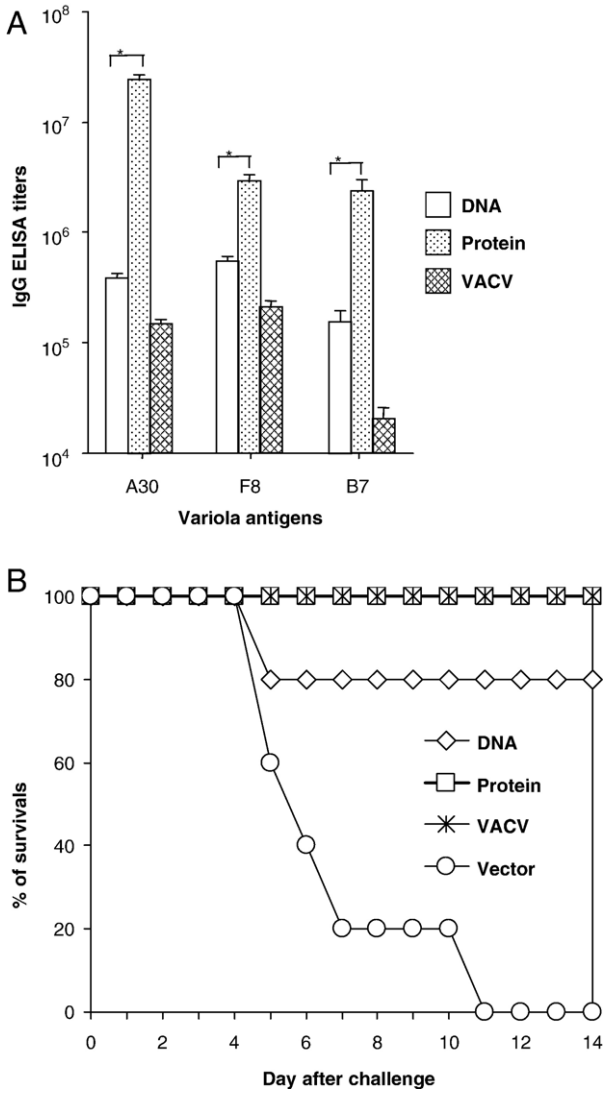


Fig. 6. Immunogenicity and protection efficacy of polyvalent recombinant variola protein vaccines and polyvalent DNA vaccines expressing variola antigens. (A) ELISA analysis of end titration IgG titers against A30, B7 or F8 antigens in immunized mice sera. Data are shown as the geometric mean titers of each group (5 per group) after two bi-weekly immunizations. Animals were immunized with either a combination of recombinant A30, B7 and F8 proteins (Protein), or a combination of three DNA vaccines expressing A30, B7 and F8 antigens (DNA). Control group animals received a one-time vaccinia (WR) immunization (VACV). The protein immunization elicited significantly higher antibody when compared to DNA immunization (* indicates $p<0.01$). (B) Protection against a lethal intranasal challenge of VACV (WR) in mice that received two bi-weekly immunizations of either the combination of three recombinant variola proteins (rA30, rB7 and rF8) or the combination of three DNA vaccines expressing A30, B7 and F8. Positive control animals received a one-time vaccinia (WR) immunization and negative control animals received three immunizations of an empty DNA vaccine vector. Each curve shows the daily percentage of survivals for each group (5 mice per group) after challenge.

with VACV, except for the B7-specific antibody, which probably occurred as a result of very low anti-B7 antibody responses in VACV immunized mice.

Mice were challenged with a lethal intranasal dose of VACV (WR) ($5\times10^6$ pfu) 2 weeks after the 2nd immunization. While all mice in the control group died by Day 11 (Fig. 6B), mice that received either the polyvalent recombinant VARV protein vaccination or the vaccinia vaccine immunization were fully protected, as indicated by a 100% survival rate following the intranasal challenge (Fig. 6B). Two immunizations with the polyvalent DNA vaccine induced partial protection with 4 out of 5 mice surviving by Day 14 (Fig. 6B). Both protein and DNA subunit vaccine formulations induced statistically significant greater levels of protection when compared to the vector control group ($p=0.0017$ and $p=0.0211$, respectively), as determined by a Kaplan–Meier survival test.

## Discussion

Over the past few years, progress has been made which establishes the use of subunit-based smallpox vaccines delivered in the form of DNA plasmids (Hooper et al., 2000, 2003, 2007, 2004; Pulford et al., 2004; Sakhatskyy et al., 2006) or recombinant proteins (Davies et al., 2005; Fang et al., 2006; Fogg et al., 2004; Galmiche et al., 1999; Heraud et al., 2006; Xiao et al., 2007) to induce antibodies and/or protection against poxvirus infection. This has been made possible through the identification of the immunogenic and protective vaccinia virus IMV-specific antigens (e.g., A27, L1 and D8) and EEV-specific antigens (e.g., A33 and B5). These antigens have shown to be immunogenic and protective against VACV infection in mice (Fogg et al., 2004; Galmiche et al., 1999; Hooper et al., 2000, 2004; Pulford et al., 2004; Sakhatskyy et al., 2006; Xiao et al., 2007), against ectromelia virus (ECTV) (Xiao et al., 2007) and against monkeypox virus in non-human primates (Heraud et al., 2006; Hooper et al., 2004). Despite this great progress, these previous studies used antigen genes cloned from VACV or monkeypox virus rather than those from VARV to induce broad and protective antibody responses mainly due to safety concerns over using VARV as a model and to the difficulty in producing VARV infection in species other than humans and some non-human primates. Although the sequences are very similar between these viruses (i.e., VACV or monkeypox virus) and VARV, they are not completely identical. In a recent study, the properties of the vaccinia virus EEV protein B5 and its Variola ortholog B6 were compared in order to determine whether B6 is a better choice as a subunit vaccine against smallpox. This study found that from a panel of 26 anti-B5 monoclonal antibodies only 16 cross-reacted with B6 protein and out of the 10 that did not, at least 3 were EEV neutralizing or blocked comet formation (Aldaz-Carroll et al., 2007). With current technology, it has become possible to produce synthetic gene sequences based on the known VARV protein sequences, which may be more immunologically desirable in producing immune responses against smallpox infection than the genes from another pox virus. It could be argued that VARV-based antigens should be more compatible with the invading smallpox infection than VACV antigens although the definitive proof can only be obtained in a VARV challenge study which is not readily available due to security control of VARV reagents.

In the current study, we produced subunit-based smallpox vaccines that are based on the three VARV protective antigens, A30, B7 and F8, which are orthologous of the IMV antigens A27 and D8 and the EEV antigen B5 from VACV. The sequences of

these three VARV proteins are homologous but not identical with the VACV antigen sequences, as only three amino acid differences exist between A27 and A30, 23 amino acid differences between B5 and B7 and 12 amino acid differences between D8 and F8. While these VACV antigens have been shown to elicit a good level of protection from intranasal VACV challenge (Pulford et al., 2004), there have been no reports showing whether DNA or recombinant protein vaccines based on the VARV protective antigens are able to induce neutralizing antibodies and protection against VACV.

Here, we show that immunization with codon optimized monovalent DNA vaccines that express the F8 and A30 VARV antigens elicited antibodies that were able to neutralize VACV, as measured in a plaque reduction assay. Furthermore, when these VARV antigens were given in combination, they induced neutralizing responses similar to those produced by their VACV counterparts, D8 and A27, when administered in combination (data not shown). This increase in immunogenicity after administration of a combination of antigens compared to when administered as a monovalent formulation is consistent with studies done with VACV antigens. Immunization with DNA vaccines encoding for a single VACV membrane protein induced only modest anti-VACV reactivity (Pulford et al., 2004) as compared to when these antigens were given in combination (Hooper et al., 2000, 2003). Mice that were vaccinated with polyvalent (i.e., A30, F8 and B7) VARV antigen DNA vaccines were protected from intraperitoneal challenge. When the VACV challenge was administered intranasally, mice that were immunized with either the polyvalent recombinant protein or DNA vaccines were protected. Furthermore, we observed that with only two immunizations, the protein-based subunit vaccine was more effective than DNA vaccines in eliciting higher antibody responses and better protection in the dose ranges tested in the current study with the adjuvant used for protein immunization. Since the current study was not designed to compare the difference between DNA and protein-based VARV vaccines, more dedicated studies, especially in non-human primates, are needed to further identify any qualitative and quantitative differences in immunogenicity and protective efficacy for these two types of VARV vaccines.

In summary, a vaccine based on antigens from the VARV virus can confer protective immunity against both intranasal and intraperitoneal challenge with the vaccinia virus and induce neutralizing antibodies against VACV. Given the decreased cross-reactivity of anti-B5 VACV antigens with B6 antigens of variola major (Aldaz-Carroll et al., 2007), our study demonstrated that it is feasible that a variola major antigen-based vaccine can be produced and may potentially confer more matched immune responses and/or greater protection following exposure to VARV. Results from this study show that there is no apparent advantage of substituting original VARV antigens for their orthologs from less virulent poxviruses, as indicated by the similar levels of neutralizing antibodies and levels of protection against VACV challenge between the VARV antigen-based vaccines, developed in this study, and the orthologous VACV antigens. Results from the current study suggest that future efforts in the development of subunit-based smallpox vaccines may be better suited to focus on optimizing the conditions necessary to elicit immunological responses from the variola major protective antigens themselves rather than those from the vaccinia virus.

## Materials and methods

### *Viruses and cells*

Western Reserve strain of vaccinia virus (VACV-WR) provided by Dr. Lisa Selin from 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).

### *Construction of DNA vaccines*

Individual variola (VARV) genes, with modified codon usage, were synthesized based on variola major India 1967 (VARV-IND) sequences. Sequences of orthopoxviruses were derived from NCBI genome database and aligned using MacVector 7.0. DNA inserts were then subcloned into pSW3891 immediately after the CMV immediate early (IE) promoter (Wang et al., 2005). For VARV DNA vaccines reported in this study, an additional human tissue plasminogen activator (tPA) sequence was used to enhance the antigen expression based on previous reports (Wang et al., 2006). The PCR amplified VARV genes were subcloned into the same vector downstream of the tPA leader sequence which was already included in the vector (Wang et al., 2004). 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, VARV antigen related vaccines are referred to as A30, B7 and F8, the recombinant VARV proteins are referred to as rA30, rB7 and rF8, and the VACV antigens are referred to as A27, B5 and D8.

### *Protein production*

Histidine-tagged antigen gene sequences of vaccinia (A27, D8, B5) or variola viruses (A30, F8, B7) were cloned into the pET-15b vector (Invitrogen). Constructs were than transformed into DE3 competent cells for expression. The transformed cells were grown overnight at 37 °C and inoculated at 5% into LB medium. The culture was grown until cell density measured at A600 reached O.D. range of 0.6 to 1.0. Then Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to reach a final concentration 0.4–0.8 mM for the induction of protein expression. Cells were harvested 3 h later and lysed by sonication.

Recombinant proteins were then loaded onto Ni column (Qiagen), according to the manufacturer's recommendations. After washing the unbound proteins with 20 mM imidazole, the target protein was eluted by 250 mM imidazole in 8 M urea lysis buffer. The purified proteins were refolded by dialysis against phosphate-buffered saline. Protein concentration and purity were determined by SDS gel.

*Immunization of BALB/c mice*

Six to eight week 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 and IBC approved protocols. The animals were immunized with a Helios gene gun (Bio-Rad) at the shaved abdominal skin as previously reported (Wang et al., 2004). Two DNA immunization schedules were used in this report (see figure legend for details). Each mouse received 4 monthly or 2 bi-weekly immunizations with six DNA shots of 2 μg each per immunization. Immunogenicity was studied in 10 mice per group and each challenge study group consisted of 5 mice. 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 of PBS by intradermal inoculation into the ear pinnae 1 month before challenge (Tscharke et al., 2002; Tscharke and Smith, 1999). Mice immunized with proteins received 2 bi-weekly immunizations with purified recombinant proteins (10 μg per injection) in PBS emulsified in Incomplete Freund's Adjuvant.

*Transient expression of antigens*

293T cells were transiently transfected with a calcium chloride co-precipitation method using 10 μg of plasmid DNA for $2\times10^6$ cells in a 60-mm dish. Cells were harvested 72 h later. Both supernatants and cell lysates were collected for ELISA or Western blot assays. Supernatants from multiple dishes were pooled and the amount of antigen was determined by Coomassie blue staining of SDS-polyacrylamide 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 (Wyatt et al., 2004). Serially diluted mouse sera (100 μl) were added to each well and assayed in duplicate after 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 2 M $H_2SO_4$, and the plates were read at OD450.

*Western blot analysis of in vitro expressed VACV antigens*

The same amount of transiently expressed antigens (10 ng of protein) was loaded for the SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto PVDF membranes (Bio-Rad, Hercules, CA) and incubated overnight at 4 °C in blocking buffer (0.2% I-block, 0.1% Tween-20 in 1× PBS). Membranes were incubated with a 1:200 dilution of rabbit sera immunized with the 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 per manufacturer's specifications. 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 (Sigma) for 5 min and plaques were counted. The neutralization was calculated as the percentage of the number of plaque counts reduced in the testing serum per assay compared to the mean number of the plaque counts for the three virus controls (without sera) in the same assay.

*Vaccinia virus challenge*

Age matched female BALB/c mice (5 per group) were used in all experiments. Challenges were conducted 2 weeks after the last immunization. For IP challenge study, mice were anesthetized intramuscularly with ketamine–xylazine (100/10 mg/kg) and then injected, intraperitoneally, with $5\times10^7$ pfu of VACV-WR in 100 μl of PBS. For intranasal challenge, mice were administered $5\times10^6$ pfu VACV-WR in 25 μl of PBS by intranasal inoculation. Mice were weighed and observed daily, as previously described (Selin et al., 1994). Death at the end of each following challenge was recorded. All experiments were done in compliance with protocols approved by the IACUC and IBC at UMMS.

*Statistical analysis*

Tests were performed using Epi Info™ software for Windows available from CDC web site. Survival curves were analyzed using the Kaplan–Meier test. Comparisons between the mean percentage body-weight changes for different groups at each day after challenge and between mean antibody titers 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 $p < 0.05$.

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## References

Aldaz-Carroll, L., Xiao, Y., Whitbeck, J.C., Ponce de Leon, M., Lou, H., Kim, M., Yu, J., Reinherz, E.L., Isaacs, S.N., Eisenberg, R.J., Cohen, G. H., 2007. Major neutralizing sites on vaccinia virus glycoprotein B5 are exposed differently on variola virus ortholog B6. J. Virol. 81, 8131–8139.

Artenstein, A.W., Johnson, C., Marbury, T.C., Morrison, D., Blum, P.S., Kemp, T., Nichols, R., Balser, J.P., Currie, M., Monath, T.P., 2005. A novel, cell culture-derived smallpox vaccine in vaccinia-naive adults. Vaccine 23 (25), 3301–3309.

Belyakov, I.M., Earl, P., Dzutsev, A., Kuznetsov, V.A., Lemon, M., Wyatt, L.S., Snyder, J.T., Ahlers, J.D., Franchini, G., Moss, B., Berzofsky, J.A., 2003. Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. Proc. Natl. Acad. Sci. U. S. A. 100 (16), 9458–9463.

Casey, C.G., Iskander, J.K., Roper, M.H., Mast, E.E., Wen, X.J., Torok, T.J., Chapman, L.E., Swerdlow, D.L., Morgan, J., Heffelfinger, J.D., Vitek, C., Reef, S.E., Hasbrouck, L.M., Damon, I., Neff, L., Vellozzi, C., McCauley, M., Strikas, R.A., Mootrey, G., 2005. Adverse events associated with smallpox vaccination in the United States, January–October 2003. JAMA 294 (21), 2734–2743.

Chen, H.D., Fraire, A.E., Joris, I., Brehm, M.A., Welsh, R.M., Selin, L.K., 2001. Memory CD8+ T cells in heterologous antiviral immunity and immunopathology in the lung. Nat. Immunol. 2 (11), 1067–1076.

Davies, D.H., McCausland, M.M., Valdez, C., Huynh, D., Hernandez, J.E., Mu, Y., Hirst, S., Villarreal, L., Felgner, P.L., Crotty, S., 2005. Vaccinia virus H3L envelope protein is a major target of neutralizing antibodies in humans and elicits protection against lethal challenge in mice. J. Virol. 79 (18), 11724–11733.

Edghill-Smith, Y., Golding, H., Manischewitz, J., King, L.R., Scott, D., Bray, M., Nalca, A., Hooper, J.W., Whitehouse, C.A., Schmitz, J.E., Reimann, K.A., Franchini, G., 2005. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. Nat. Med. 11 (7), 740–747.

Fang, M., Cheng, H., Dai, Z., Bu, Z., Sigal, L.J., 2006. Immunization with a single extracellular enveloped virus protein produced in bacteria provides partial protection from a lethal orthopoxvirus infection in a natural host. Virology 345 (1), 231–243.

Fogg, C., Lustig, S., Whitbeck, J.C., Eisenberg, R.J., Cohen, G.H., Moss, B., 2004. Protective immunity to vaccinia virus induced by vaccination with multiple recombinant outer membrane proteins of intracellular and extracellular virions. J. Virol. 78 (19), 10230–10237.

Frey, S.E., Newman, F.K., Cruz, J., Shelton, W.B., Tennant, J.M., Polach, T., Rothman, A.L., Kennedy, J.S., Wolff, M., Belshe, R.B., Ennis, F.A., 2002. Dose-related effects of smallpox vaccine. N. Engl. J. Med. 346 (17), 1275–1280.

Galmiche, M.C., Goenaga, J., Wittek, R., Rindisbacher, L., 1999. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. Virology 254 (1), 71–80.

Haas, J., Park, E.C., Seed, B., 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr. Biol. 6 (3), 315–324.

Heraud, J.M., Edghill-Smith, Y., Ayala, V., Kalisz, I., Parrino, J., Kalyanaraman, V.S., Manischewitz, J., King, L.R., Hryniewicz, A., Trindade, C.J., Hassett, M., Tsai, W.P., Venzon, D., Nalca, A., Vaccari, M., Silvera, P., Bray, M., Graham, B.S., Golding, H., Hooper, J.W., Franchini, G., 2006. Subunit recombinant vaccine protects against monkeypox. J. Immunol. 177 (4), 2552–2564.

Hooper, J.W., Custer, D.M., Schmaljohn, C.S., Schmaljohn, A.L., 2000. DNA vaccination with vaccinia virus L1R and A33R genes protects mice against a lethal poxvirus challenge. Virology 266 (2), 329–339.

Hooper, J.W., Custer, D.M., Thompson, E., 2003. Four-gene-combination DNA vaccine protects mice against a lethal vaccinia virus challenge and elicits appropriate antibody responses in nonhuman primates. Virology 306 (1), 181–195.

Hooper, J.W., Thompson, E., Wilhelmsen, C., Zimmerman, M., Ichou, M.A., Steffen, S.E., Schmaljohn, C.S., Schmaljohn, A.L., Jahrling, P.B., 2004. Smallpox DNA vaccine protects nonhuman primates against lethal monkeypox. J. Virol. 78 (9), 4433–4443.

Hooper, J.W., Golden, J.W., Ferro, A.M., King, A.D., 2007. Smallpox DNA vaccine delivered by novel skin electroporation device protects mice against intranasal poxvirus challenge. Vaccine 25 (10), 1814–1823.

Kennedy, J.S., Frey, S.E., Yan, L., Rothman, A.L., Cruz, J., Newman, F.K., Orphin, L., Belshe, R.B., Ennis, F.A., 2004. Induction of human T cell-mediated immune responses after primary and secondary smallpox vaccination. J. Infect. Dis. 190 (7), 1286–1294.

Monath, T.P., Caldwell, J.R., Mundt, W., Fusco, J., Johnson, C.S., Buller, M., Liu, J., Gardner, B., Downing, G., Blum, P.S., Kemp, T., Nichols, R., Weltzin, R., 2004. ACAM2000 clonal Vero cell culture vaccinia virus (New York City Board of Health strain)—a second-generation smallpox vaccine for biological defense. Int. J. Infect. Dis. 8 Suppl 2, S31–S44.

Panchanathan, V., Chaudhri, G., Karupiah, G., 2005. Interferon function is not required for recovery from a secondary poxvirus infection. Proc. Natl. Acad. Sci. U. S. A. 102 (36), 12921–12926.

Pulford, D.J., Gates, A., Bridge, S.H., Robinson, J.H., Ulaeto, D., 2004. Differential efficacy of vaccinia virus envelope proteins administered by DNA immunisation in protection of BALB/c mice from a lethal intranasal poxvirus challenge. Vaccine 22 (25–26), 3358–3366.

Sakhatskyy, P., Wang, S., Chou, T.H., Lu, S., 2006. Immunogenicity and protection efficacy of monovalent and polyvalent poxvirus vaccines that include the D8 antigen. Virology 355 (2), 164–174.

Selin, L.K., Nahill, S.R., Welsh, R.M., 1994. Cross-reactivities in memory cytotoxic T lymphocyte recognition of heterologous viruses. J. Exp. Med. 179 (6), 1933–1943.

Shchelkunov, S.N., 1995. Functional organization of variola major and vaccinia virus genomes. Virus Genes 10 (1), 53–71.

Shchelkunov, S.N., Massung, R.F., Esposito, J.J., 1995. Comparison of the genome DNA sequences of Bangladesh-1975 and India-1967 variola viruses. Virus Res. 36 (1), 107–118.

Tscharke, D.C., Smith, G.L., 1999. A model for vaccinia virus pathogenesis and immunity based on intradermal injection of mouse ear pinnae. J. Gen. Virol. 80 (Pt 10), 2751–2755.

Tscharke, D.C., Reading, P.C., Smith, G.L., 2002. Dermal infection with vaccinia virus reveals roles for virus proteins not seen using other inoculation routes. J. Gen. Virol. 83 (Pt 8), 1977–1986.

Wang, S., Heilman, D., Liu, F., Giehl, T., Joshi, S., Huang, X., Chou, T.H., Goguen, J., Lu, S., 2004. A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague. Vaccine 22 (25–26), 3348–3357.

Wang, S., Chou, T.H., Sakhatskyy, P.V., Huang, S., Lawrence, J.M., Cao, H., Huang, X., Lu, S., 2005. Identification of two neutralizing regions on the severe acute respiratory syndrome coronavirus spike glycoprotein produced from the mammalian expression system. J. Virol. 79 (3), 1906–1910.

Wang, S., Farfan-Arribas, D.J., Shen, S., Chou, T.H., Hirsch, A., He, F., Lu, S., 2006. Relative contributions of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine. Vaccine 24 (21), 4531–4540.

WHO, 1980. The global eradication of smallpox. Final report of the global commission for the certification of smallpox eradication. History of International Public Health, vol. 4. World Health Organization, Geneva.

Wyatt, L.S., Earl, P.L., Eller, L.A., Moss, B., 2004. Highly attenuated smallpox vaccine protects mice with and without immune deficiencies against pathogenic vaccinia virus challenge. Proc. Natl. Acad. Sci. U. S. A. 101 (13), 4590–4595.

Xiao, Y., Aldaz-Carroll, L., Ortiz, A.M., Whitbeck, J.C., Alexander, E., Lou, H., Davis, H.L., Braciale, T.J., Eisenberg, R.J., Cohen, G.H., Isaacs, S.N., 2007. A protein-based smallpox vaccine protects mice from vaccinia and ectromelia virus challenges when given as a prime and single boost. Vaccine 25 (7), 1214–1224.