Published in final edited form as:

Vaccine. 2011 July 26; 29(33): 5331–5339. doi:10.1016/j.vaccine.2011.05.088.

Enhancement of Immune Response to an Antigen Delivered by Vaccinia Virus by Displaying the Antigen on the Surface of Intracellular Mature Virion

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Abstract

Vaccinia virus (VACV) is the vaccine for smallpox and a widely-used vaccine vector for infectious diseases and cancers. The majority of the antibodies elicited by live VACV vaccination respond to virion structural proteins, including many integral membrane proteins on the intracellular mature virion (MV). Here, we showed that antibody response to an exogenous antigen delivered by VACV was greatly enhanced by incorporating the antigen as an integral membrane protein of MV. We constructed recombinant VACV expressing a *Y. pestis* protective antigen, LcrV, unmodified or fused with either a signal peptide or with the transmembrane domain of VACV D8 protein (LcrV-TM). Electron microscopy showed that LcrV-TM was displayed on the surface of MV. Importantly, VACV expressing LcrV-TM elicited a significantly higher titer of anti-LcrV antibody in mice than viruses expressing other forms of LcrV. Only mice immunized with LcrV-TM-expressing VACV were protected from lethal *Y. pestis* and VACV WR challenges. Antigen engineering through fusion with D8 transmembrane domain may be broadly applicable for enhancing the immune response to antigens delivered by a VACV vector. The recombinant virus described here could also serve as the basis for developing a vaccine against both smallpox and plague.

Introduction

Vaccinia virus (VACV), as the vaccine for smallpox, is one of the most successful vaccines in human history and remains the only vaccine that successfully eradicated a human disease. A single immunization with live VACV elicits robust antibody and cytotoxic T cell responses that last for decades in humans [1, 2]. The cytotoxic T cell responses predominantly recognize epitopes present in nonstructural, early VACV proteins [3, 4], while the antibody responses predominantly recognize structural, late VACV proteins [5, 6]. Major antibody targets include D8, H3 and L1 [6], all of which are anchored to the membrane of intracellular mature virions of VACV by a carboxy-terminal transmembrane domains [7]. The majority of the virions produced by VACV are intracellular mature virions (MV), while a small fraction of MV gain additional membranes through wrapping with

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Golgi cisternae and eventually exit the cells as the extracellular enveloped viruses (EV) [8, 9].

VACV has a wide host range and a very efficient gene expression system [9]. As such, VACV has also been used as a vaccine vector for infectious diseases such as AIDS and malaria, which have met with some success in animal trials and human clinical trials [10, 11]. Considering the success of VACV as the smallpox vaccine and as a useful vaccine vector, we recently initiated a study to use VACV as a vector to develop a vaccine that protects against not only smallpox but also plague, which is another grave concern for bioterrorism. Plague is caused by *Yersinia pestis*, a Gram-negative bacterium that is endemic in rodent reservoirs in many parts of the world [12]. Historically, *Yersinia pestis* has been a significant source of human morbidity and mortality, causing several global pandemics that killed 50–100 million people. Plague has several manifestations, but pneumonic and bubonic plague are most common. Pneumonic plague is the most feared form of the disease because it is an extremely aggressive and contagious pneumonia that must be treated within the first 24 hours of infection. Due to the contagious nature of pneumonic plague and the rapid disease course capable of causing death in several days, a prophylactic vaccine is highly desirable. Currently, there is no plague vaccine licensed for use in the U.S.A protein subunit vaccine based on the F1 capsular protein and the LcrV protein was shown to be protective in animal models [13–16]. Protection against plague generally correlates with serum titers of F1 and LcrV antibodies [13, 17]. In addition, passive immunization with anti-F1 or anti-LcrV antibodies protects against *Y. pestis* [18–22], suggesting that the mechanism of protection by active vaccination is largely provided by the antibody response. However, recent studies indicated that cell mediated responses also contribute to protection [23, 24] and that the subunit vaccine of F1 and LcrV failed to fully protect against *Y. pestis* in some species of nonhuman primates [25], suggesting that an alternative vaccination strategy may be necessary.

Initially, we attempted to generate a candidate plague vaccine by inserting an LcrV-expressing cassette into the ACAM2000 virus, the clonal smallpox vaccine that is currently licensed in the U.S. [26]. Although this recombinant virus elicited an antibody response to LcrV, the response was not able to protect mice from plague challenge. Since live VACV immunization elicits strong antibody responses to MV membrane proteins, we hypothesize that we may increase the antibody response to LcrV by targeting LcrV to the MV membrane. Our test of this hypothesis resulted in a recombinant virus that confers protective immunity in mice against lethal challenges with virulent *Y. pestis* and the WR strain of VACV. Our method of antigen engineering may also be beneficial for enhancing the immunogenicity of other antigens delivered by VACV.

Materials and Methods

Cell culture and Viruses

HeLa, BHK and CV-1 cells were cultured in Dulbecco's modified Eagle (DMEM) medium (Invitrogen) with 10% fetal bovine serum. All viruses were propagated on HeLa cells or CV-1 cells in DMEM medium with 1% FBS.

Recombinant virus construction—Recombinant viruses were generated through homologous recombination between the appropriate transfer plasmids and ACAM2000 strain of VACV [27] (obtained from Acambis Inc, Cambridge, Massachusetts). The transfer plasmid pSC11[28], which contains a LacZ cassette flanked by VACV thymidine kinase (TK) gene, was first modified by replacing LacZ with GFP, and the modified plasmid was used in all subsequent transfer vector construction. The full-length *Y. pestis* LcrV was amplified by PCR from pQE-Entero-LcrV [29] with primers LcrV-NheI (5'-

CTATCAGCTAGC ATGATTAGAGCCTACGAAC-3') and LcrV-BglII (5'-CGGAAGATCTTCATTTACCAGACGTGTCA-3'). The PCR product was then digested with NheI and BglII and cloned in the transfer vector immediately after VACV late promoter p11. The resulted plasmid (pYZ104) was further modified by inserting the tPA signal peptide sequence in-frame at the N-terminus of the LcrV sequence. The tPA signal peptide sequence was amplified by PCR with primers tPA-XbaI (5'-CCTCTAGAATGGATGCAATG-3') and tPA-NheI (5'-TGTTTCGTAGGCTCTAATCATGCTAGCCGAAACGAAGAC-3') from an existing vector [30]. The PCR product was digested with XbaI and NheI and cloned into the NheI site in pYZ104. The plasmid that contains LcrV fused with the D8 transmembrane domain (TM) was constructed as follows. LcrV and D8 TM sequences were amplified separately by PCR with primer pair LcrV-NheI (5'-CTATCAGCTAGCATGATTAGAGCCTACGAAC-3') and LcrV-end (5'-TTTACCAGACGTGTCATCTAGCAG-3'), and primer pair LcrV-D8L#1 (5'-GCTAGATGACACGTCTGGTAAAAAATATATCGA-3') and D8L-BglII (5'-CGGAAGATCTCTAGTTTTGTTTTCTCGC-3'), respectively. The two PCR products were then combined together by recombinant PCR with primers LcrV-NheI and D8L-BglII. The recombinant PCR product was digested and cloned into the NheI and BglII restriction sites of the transfer vector.

Recombinant virus construction was done according to a standard protocol [31]. Briefly, transfer vectors described above were transfected into CV-1 cells that had been infected with the ACAM2000 VACV at an MOI of 1. Recombinant viruses were isolated through at least three rounds of picking GFP-positive plaques and confirmed by PCR-amplifying TK flanking sequences.

Confirmation of LcrV expression by the recombinant virus

Monolayers of HeLa cells seeded in 12-well cluster plates were infected with the recombinant virus at an MOI of 5. Infected cells were harvested 24 hours post infection (hpi), suspended in 1x SDS-PAGE loading buffer and boiled. Samples were loaded on a SDS-PAGE gel, transferred to nitrocellulose, and the membrane was blotted with polyclonal serum against LcrV raised in rabbits (Dube, P et. al, unpublished) or monoclonal mouse antibody against VACV WR148 [32], followed by HRP-conjugated secondary antibodies (GE Healthcare). Chemiluminescence was visualized on a Biorad imager.

One-step growth curve

Monolayers of HeLa cells seeded in 12-well cluster plates were infected at an MOI of 10 for each of the recombinant viruses. At 12 or 24 hpi, infected cells were harvested, frozen three times and sonicated to lyse cells and release virions. Serial dilutions of cell lysates were titrated in triplicate on CV-1 cells.

VACV virion purification

Virion purification was performed as described [31]. All viruses used in the experiments were amplified in T-150 flasks of HeLa cells. After harvesting, cells were spun down and suspended in 10mM Tris-Cl (pH 9.0) and Dounce-homogenized. After centrifugation, cell lysates were collected, sonicated, and layered over 36% sucrose in 10mM Tris (pH 9.0). Sucrose cushioned lysates were centrifuged at 32,000g for 80 min, and the resulting pellet was suspended in 10mM Tris-Cl (pH 9.0). Sucrose-cushioned virions were sonicated and layered over a 24%–40% sucrose gradient and centrifuged at 26,000g for 50 min. The milky band of purified virions was collected, pelleted, and suspended in 1mM Tris-Cl (pH 9.0).

Immunogold labeling of virions and TEM

Sucrose-gradient purified virions were adsorbed to 200nm carbon-coated nickel grids and blocked with 5% BSA. Grids were then incubated with appropriate primary antibodies (polyclonal rabbit sera against LcrV or monoclonal mouse antibody against D8 [32]) overnight. After washing 3x with 0.1M Tris-Cl (pH 7.4), grids were incubated with secondary gold-conjugated antibodies (12nm anti-rabbit and 6nm anti-mouse, JacksonImmuno) overnight. Grids were washed 3x with 0.1M Tris-Cl and then fixed with 4% paraformaldehyde/2% glutaraldehyde. After washing 3X with 0.1M Tris-HCl, grids were rinsed with an ethanol gradient, dried on Whatman filter paper, and stained with uranyl-acetate. Grids were visualized at the UTHSCSA Electron Microscopy lab on a JEOL 1230 transmission electron microscope.

Detergent extraction of membranes from purified recombinant virus

Purified virions were incubated with 0.5% IGEPAL in 50 mM Tris-HCl (pH 8.0) with or without 50 mM dithiothreitol (DTT) for 30 min at 37°C. The soluble membrane-associated proteins and insoluble core protein fractions were separated by centrifugation at $20,000 \times g$ for 30 min. All virion fractions were analyzed by SDS-PAGE followed by Western blot with antibodies against LcrV and VACV H3L [32].

Immunofluorescence

BHK cells on glass coverslips were infected with either WT or recombinant ACAM2000 viruses at a MOI of 0.1. After 24 hours, infected cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-x-100, and blocked with 5% BSA in PBS (pH 7.4). Primary polyclonal serum against LcrV or monoclonal antibodies against VACV A14 and D8 [32] were added, followed by secondary anti-rabbit Alexafluor-568 and anti-mouse Alexafluor-488 antibodies (Invitrogen). The cells were stained with DAPI before they were visualized with an Olympus IX-81 fluorescent microscope using a 100x oil emersion objective.

Vaccination and serum collection

Groups of BALB/C mice (The Jackson Laboratory), 5 to 10 per group, were vaccinated with recombinant ACAM2000 viruses using the tail scratch method [33]. Mice were scratched with a 26ga. needle at the base of the tail 20 times, and 5 μ l purified virus was added to and worked into the wound. Control mice were vaccinated via the i.p. route with rLcrV incubated with an aluminum hydroxide adjuvant, or were unvaccinated. Four or five weeks post-vaccination, mice were bled from the tail to obtain serum.

Determination of α -LcrV and α -VACV serum antibody titers

Serum antibody titers from vaccinated mice were determined by ELISA with recombinant LcrV protein expressed in *E. coli* and purified virions of VACV WR. 96-well ELISA plates were coated with either recombinant LcrV protein or whole WR virions and blocked in 10% FBS in PBS (pH 7.0). The plates were incubated with serial dilution of the sera for 2 hrs, followed by incubation with HRP-conjugated anti-mouse IgG for 1 hr. For isotyping anti-LcrV antibody, isotype-specific goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 secondary antibodies (Sigma) were used, followed by a 1-hr incubation with HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnologies). After adding the substrate for HRP, the absorbance at 450nm was read on a microplate reader (Molecular Devices).

Challenge Experiments—Mice were challenged intranasally or intradermally with the wild-type *Y. pestis* strain CO92 as described [34–36]. *Y. pestis* challenge was performed in the UTHSCSA Biosafety Level-3 animal facility in accordance with established IBC and

IACUC protocols. Mice were challenged with 50 or 2000 $\times$ LD50 *Y. pestis* bacteria intradermally or 15 or 40 $\times$ LD50 intranasally and survival was monitored over the course of 28 days. Data is shown out to 8 days and no animals died or showed signs of disease after this point. VACV challenge was performed in the UTHSCSA Biosafety Level-2 animal facility. Mice were administered VACV strain WR at 1000 $\times$ LD50 (1×10^7 pfu/ml) intranasally as described [37]. Weight and survival were monitored over the course of 10 days. Survival curves significance was calculated using log-rank (Mantel-Cox) test of Kaplan-Meier curves (Graphpad Prism).

Results

Construction of recombinant Acam2000 vaccinia viruses expressing *Y. pestis* LcrV

Over the course of the studies, three recombinant ACAM2000 viruses expressing full-length *Y. pestis* LcrV protein were constructed (Fig. 1A). Initially, we constructed a recombinant ACAM2000 virus expressing unmodified LcrV, which was found to be inadequate at eliciting protection of mice from *Y. pestis* challenge (shown later). Therefore, in an effort to increase the immunogenicity of LcrV, we modified the LcrV expressed by ACAM2000. A previous report showed that anti-LcrV antibody response elicited by DNA vaccination was enhanced with the addition of a signal peptide to LcrV [30], so we constructed an ACAM2000 virus expressing LcrV with the signal peptide from plasminogen activating factor (referred to as s-LcrV). Finally, with the idea that incorporating LcrV as an integral membrane protein of MV may enhance anti-LcrV antibody response, we made another ACAM2000 virus expressing LcrV fused at its C-terminus with the transmembrane domain of VACV D8 protein (referred to as LcrV-TM). D8 is a major target of antibody responses elicited by the smallpox vaccine [5, 6, 38]. It has a C-terminal transmembrane domain but no signal peptide [39]. A previous study of VACV MV membrane protein A9 suggested that the transmembrane domain of MV membrane proteins was sufficient for targeting to MV envelope [40].

A late promoter of VACV was used to drive the expression of LcrV and the expression cassette was inserted into thymidine kinase gene of ACAM2000 through homologous recombination. The three recombinant viruses (referred to as LcrV, s-LcrV or LcrV-TM viruses) expressed LcrV at a similar level as demonstrated by Western blots of infected cell lysates (Fig. 1B). Multiple bands were detected by the polyclonal anti-LcrV antibody in s-LcrV- and LcrV-TM-infected cells, probably reflecting some degradation of the LcrV proteins. LcrV was also secreted to the media from cells infected with s-LcrV virus. The three viruses formed plaques of similar size (data not shown) and replicated with similar growth kinetics in cell culture as indicated by the one-step growth curves in HeLa cells (Fig. 1C).

LcrV-TM was displayed on the surface of VACV MV

To determine if LcrV-TM virus displays LcrV-TM protein on the surface of VACV MV, we performed immuno-electron microscopy studies of purified VACV virion particles. All three viruses were purified from infected cells through a sucrose cushion and a sucrose gradient. The purified virions were then stained with rabbit polyclonal antibodies against LcrV and mouse monoclonal antibodies against D8, followed by staining with secondary antibodies conjugated with gold-particles of different size. Electron microscopy showed that VACV D8 proteins (6 nM gold) were present on all three viruses. However, only virions from LcrV-TM virus were extensively stained with anti-LcrV antibody (12 nM gold), indicating that LcrV-TM is displayed on the surface of VACV (Fig. 2A and 2B). Quantitation of virions labeled with D8 antibody showed that approximately half of the D8-labeled virions were substantially labeled with LcrV antibodies, but there were also some D8-labeled virions that

were poorly labeled by LcrV antibodies (Table 1). LcrV-TM was also detected in purified virions by Western blot analysis (Fig. 2C). Similar to another VACV virion membrane protein, H3, LcrV-TM could be extracted from the virions by nonionic detergent NP-40, confirming that LcrV-TM is localized to virion membrane.

We also used immunofluorescence to examine the intracellular localization of the LcrV proteins in infected cells. BHK cells were infected with LcrV, s-LcrV or LcrV-TM viruses for 24 hrs. The cells were then stained with antibodies against LcrV as well as VACV MV membrane protein A14 (Fig. 3) to illustrate the localization of LcrV protein in relation to native VACV virion membrane proteins. As expected for structural proteins of VACV MV, A14 proteins predominantly localized to viral DNA factories [32]. LcrV-TM proteins were detected in viral DNA factories as well as in regions outside viral factories. In contrast, neither LcrV nor s-LcrV was found in viral DNA factories. LcrV exhibited a diffuse cytoplasmic staining, while s-LcrV demonstrated a vesicular staining pattern.

LcrV-TM virus elicited a significantly higher titer of anti-LcrV antibody in mice than other recombinant viruses—

LcrV and s-LcrV viruses were first tested for their abilities to induce antibody responses in mice. Groups of BABL/C mice (five per group) were immunized with the recombinant or WT ACAM2000 viruses. The immunization was performed with the tail scarification method in order to mimic the practice of smallpox vaccination in humans [33]. At 4 weeks post immunization, sera were collected from the mice and antibody titers against LcrV and VACV were assessed with ELISA. Similar levels of anti-VACV antibodies were detected in all mice (Fig. 4A). Anti-LcrV antibodies were also detected in mice immunized with the recombinant viruses, and mice immunized with the s-LcrV virus consistently had higher antibody titers than mice immunized with the LcrV virus. However, none of the mice survived subsequent intradermal challenge with virulent CO92 strain of *Y. Pestis* at a relatively low dose of $5 \times \text{LD}_{50}$ (data not shown).

Next, LcrV-TM virus was similarly tested for its ability to induce antibody responses against LcrV and VACV. As a positive control, a group of mice were immunized intraperitoneally with recombinant LcrV protein (rLcrV) coupled with an aluminum hydroxide (alum) adjuvant. At 5 weeks post immunization, the anti-VACV antibody titer was overall similar in mice immunized with different ACAM2000 viruses, although it was slightly higher in sLcrV-immunized mice than in LcrV-TM-immunized mice. This was probably due to a small variation in the amount of virus that was inoculated through the tail scarification method, as the difference was not consistently observed in subsequent experiments (data not shown). In contrast to the slightly lower anti-VACV antibody titer, a significantly higher titer of anti-LcrV antibody was detected in LcrV-TM-immunized mice than in sLcrV-immunized mice (Fig. 4B). The anti-LcrV titer was approximately the same as the titer detected in mice immunized with rLcrV with adjuvant (Fig. 4B). While the anti-LcrV antibodies elicited by rLcrV were predominantly of the IgG1 subtype (Fig. 4C) [41], LcrV-TM virus also elicited anti-LcrV of the IgG2a and IgG2b isotypes, suggesting a Th1 type of response.

Only LcrV-TM virus elicited protection against both *Y. pestis* and VACV WR challenge

Half of immunized mice shown in Fig. 4B (5 per group) were then challenged with $2000 \times \text{LD}_{50}$ dose of the highly virulent *Y. pestis* strain CO92 intradermally (Fig. 5A), while the other half (5 per group) were challenged with $1000 \times \text{LD}_{50}$ of virulent WR strain of VACV. Upon *Y. pestis* challenge, mice vaccinated with s-LcrV virus all died by day 4, while all mice immunized with LcrV-TM virus or rLcrV protein survived and showed minimal to no morbidity. In addition, mice immunized with LcrV-TM virus also survived the challenge

with VACV WR, whereas mice immunized with rLcrV all died by day 6 (Fig. 5B), as expected. Mice immunized with s-LcrV virus also survived the WR challenge.

The efficacy of the recombinant viruses in eliciting protection against *Y. pestis* was further tested by subjecting groups of similarly immunized mice to intranasal *Y. pestis* challenge. Upon challenge with $15 \times \text{LD}_{50}$ of *Y. pestis*, mice vaccinated with WT ACAM2000 virus all died by day 5, while mice immunized with LcrV-TM virus or rLcrV all survived and showed minimal to no morbidity (Fig. 5C). 20% of the mice immunized with LcrV or s-LcrV viruses also survived the challenge, which was statistically better than mice immunized with WT ACAM2000 (Log-rank test, $p=0.008$). However, upon challenge with a higher dose of *Y. pestis* ($40 \times \text{LD}_{50}$), a separate group of mice immunized with LcrV or s-LcrV viruses died by day 5, while mice immunized with LcrV-TM all survived (Fig. 5D).

Discussion

With the initial goal of developing a VACV-based candidate vaccine for plague, we constructed three recombinant VACV, which expressed *Y. pestis* LcrV at similar levels but differed in their ability to induce protective immune response, thus demonstrating that the immunogenicity of an antigen delivered by VACV is affected by the localization of the antigen in the virion and/or intracellular compartment. We first constructed a recombinant VACV that expressed unmodified *Y. pestis* LcrV, which was localized to cytoplasm of the infected cells. Immunization of mice with the recombinant virus induced an anti-LcrV antibody response, but the induced immune response was not sufficient to protect against a low dose challenge of virulent *Y. pestis*. Since adding a signal peptide to LcrV in a DNA vaccine against *Y. pestis* was previously reported to enhance the immune response to LcrV [30], we then constructed a second VACV that expressed LcrV with an N-terminal signal peptide. Indeed, the infected cells secreted LcrV, and the recombinant virus elicited a slightly better antibody response against LcrV in vaccinated mice. However, the immune response was still insufficient to protect against *Y. pestis* challenge. In recent years, the immunological responses to the smallpox vaccine have been studied extensively, and it has been found that the majority of the antibody responses are targeted against VACV virion structural proteins, especially a number of C-terminal-anchored virion membrane proteins [5, 6, 38]. With a hypothesis that dominant antibody responses to virion membrane proteins may partly be due to their localization on the virion membrane, we then aimed at targeting LcrV to the surface of the VACV virion. We fused the transmembrane domain of the VACV D8 protein to the C-terminus of LcrV. The fusion proteins were present in areas of virion assembly inside the infected cells and were exposed on the surface of purified virions. Immuno-electron microscopy also revealed the existence of virions that were not stained well with the anti-LcrV antibody, suggesting that the fusion proteins may not be targeted to virions as efficiently as the authentic virion membrane proteins. The plaque size and growth kinetics of the recombinant virus was similar to that of the WT ACAM2000, indicating that incorporating an exogenous antigen into VACV mature virions did not interfere with virion morphogenesis or the entry/fusion of the virions with the host cells. Most importantly, the virus elicited a significantly higher titer of anti-LcrV antibody in immunized mice and protected the mice in pneumonic and bubonic plague challenge models. The mechanism as to how the fusion protein induces a better antibody response remains to be determined. We speculate that clustering multiple copies of an antigen on a single virion results in a better antigen presentation to the immune cells than the monomeric form of the antigen. It has been demonstrated recently that the CD4^+ T cell responses to VACV mostly recognize epitopes on VACV virion structural proteins [42], so LcrV-TM as part of VACV virions may elicit a superior CD4^+ T cell help to B cells than other forms of LcrV proteins.

Several previous reports suggested that the immunogenicity of an antigen delivered by VACV could be enhanced by fusing the antigen with VACV proteins. VACV that expressed a fusion of the HIV envelope protein with VACV A27 or A4 protein resulted in a broader and more neutralizing anti-HIV antibody responses in mice [43]. In comparison to our current study, none of the A27 or A4 fusion proteins appeared to be displayed on the surface of the virion [43, 44]. VACV that expressed the fusion of HIV envelope or Gag protein with VACV B5 protein elicited better antibody or CD4⁺ T cell responses to the HIV antigens [45–47]. VACV B5 is a membrane protein on the VACV EV, and HIV proteins fused with B5 were preferentially targeted to the EV [46]. These reports also differ from our current study, which targeted the antigen to the surface of MV form of VACV. EV represents a small fraction of the virions produced by VACV during infection and tends to shed its fragile outer membrane [8], essentially becoming MV. Thus targeting an antigen to MV membrane may have the advantage of displaying the antigen on more virions than targeting the antigen to the EV membrane. Furthermore, because there was no direct challenge model for HIV in these previous studies, it was unclear whether the improvement in immune responses would be sufficient to protect against HIV infection. In our current study, since we used LcrV as the test antigen and there are unequivocal challenge models of both bubonic and pneumonic plague, we were able to demonstrate that fusing LcrV with D8 transmembrane domain clearly resulted in a superior immune response, which correlates with protection. The strategy of fusing the antigen with D8 transmembrane domain may be applicable for improving the immunogenicity of other antigens in VACV-based vaccines.

There have been extensive research efforts on the development of plague vaccine in recent years, as plague is considered as a potential bioterrorism threat and a licensed vaccine against plague does not exist. Current efforts have centered on the development of subunit vaccines using *Y. pestis* F1 and LcrV antigens. Plague vaccines based on DNA, bacterial or viral vectors have also been shown to be effective in animal models [14, 30, 48, 49]. Recently, a VACV that expressed F1-LcrV fusion protein was shown to elicit protection of mice against intranasal challenge of $10 \times \text{LD}_{50}$ of an attenuated *Y. pestis* strain [50]. Whether this virus could elicit protection against a fully virulent strain of *Y. pestis* remains to be determined. A MVA that expressed F1 or LcrV protein was also recently reported [51], but it did not always give 100% protection against *Y. pestis* even after prime and boost. The VACV we generated here elicits a protective immune response after only a single immunization. Compared to LcrV protein vaccine, LcrV-TM virus elicited a more Th1-like response, suggesting that it may have the advantage of eliciting cell-mediated response as well as the antibody response to LcrV. Additionally, the insertion of LcrV into ACAM2000 did not impair the ability of ACAM2000 to protect mice against virulent VACV WR challenge, suggesting that the recombinant virus is an effective vaccine against plague and smallpox. As VACV vaccine can be stockpiled in lyophilized form for years without losing efficacy and administering a single combinatorial vaccine is easier than administering multiple different vaccines sequentially, a single VACV-based vaccine against multiple bioterrorism agents may greatly simplify preparedness for bioterrorism agents.

Acknowledgments

This work was supported by NIH grants AI079217 (Y.X.), AI 067716 and AI 060789 (P.D.). A.E. was supported by the Translational Science Training (TST) Across Disciplines program at UTHSCSA, with funding provided by the University of Texas System's Graduate Programs Initiative.

References

1. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J Immunol.* 2003 Nov 15; 171(10):4969–73. [PubMed: 14607890]

2. Hammarlund E, Lewis MW, Hansen SG, Strelow LI, Nelson JA, Sexton GJ, et al. Duration of antiviral immunity after smallpox vaccination. *Nat Med*. 2003 Sep; 9(9):1131–7. [PubMed: 12925846]
3. Moutaftsi M, Peters B, Pasquetto V, Tschärke DC, Sidney J, Bui HH, et al. A consensus epitope prediction approach identifies the breadth of murine T(CD8+)-cell responses to vaccinia virus. *Nat Biotechnol*. 2006 Jul; 24(7):817–9. [PubMed: 16767078]
4. Oseroff C, Kos F, Bui HH, Peters B, Pasquetto V, Glenn J, et al. HLA class I-restricted responses to vaccinia recognize a broad array of proteins mainly involved in virulence and viral gene regulation. *Proc Natl Acad Sci U S A*. 2005 Sep 27; 102(39):13980–5. [PubMed: 16172378]
5. Davies DH, Liang X, Hernandez JE, Randall A, Hirst S, Mu Y, et al. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc Natl Acad Sci U S A*. 2005 Jan 18; 102(3):547–52. [PubMed: 15647345]
6. Davies DH, Molina DM, Wrammert J, Miller J, Hirst S, Mu Y, et al. Proteome-wide analysis of the serological response to vaccinia and smallpox. *Proteomics*. 2007 May; 7(10):1678–86. [PubMed: 17443847]
7. Condit RC, Moussatche N, Traktman P. In a nutshell: structure and assembly of the vaccinia virion. *Adv Virus Res*. 2006; 66:31–124. [PubMed: 16877059]
8. Smith GL, Vanderplasschen A, Law M. The formation and function of extracellular enveloped vaccinia virus. *J Gen Virol*. December 1; 2002 83(12):2915–31. [PubMed: 12466468]
9. Moss, B. Poxviridae: the viruses and their replication. In: Knipe, DM.; Howley, PM., editors. *Fields Virology*. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2905–46.
10. Bart PA, Goodall R, Barber T, Harari A, Guimaraes-Walker A, Khonkarly M, et al. EV01: a phase I trial in healthy HIV negative volunteers to evaluate a clade C HIV vaccine, NYVAC-C undertaken by the EuroVacc Consortium. *Vaccine*. 2008 Jun 13; 26(25):3153–61. [PubMed: 18502002]
11. Bejon P, Mwacharo J, Kai O, Mwangi T, Milligan P, Todryk S, et al. A phase 2b randomised trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. *PLoS Clin Trials*. 2006; 1(6):e29. [PubMed: 17053830]
12. Dennis, DT.; Meier, FT. Plague. In: Horsburgh, CR.; Nelson, AM., editors. *Pathology of Emerging Infections*. Washington DC: American Society for Microbiology; 1997. p. 21–47.
13. Heath DG, Anderson GW Jr, Mauro JM, Welkos SL, Andrews GP, Adamovicz J, et al. Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. *Vaccine*. 1998 Jul; 16(11–12):1131–7. [PubMed: 9682370]
14. Leary SE, Griffin KF, Garmory HS, Williamson ED, Titball RW. Expression of an F1/V fusion protein in attenuated *Salmonella typhimurium* and protection of mice against plague. *Microb Pathog*. 1997 Sep; 23(3):167–79. [PubMed: 9281474]
15. Leary SE, Williamson ED, Griffin KF, Russell P, Eley SM, Titball RW. Active immunization with recombinant V antigen from *Yersinia pestis* protects mice against plague. *Infect Immun*. 1995 Aug; 63(8):2854–8. [PubMed: 7622205]
16. Williamson ED, Flick-Smith HC, Lebutt C, Rowland CA, Jones SM, Waters EL, et al. Human immune response to a plague vaccine comprising recombinant F1 and V antigens. *Infect Immun*. 2005 Jun; 73(6):3598–608. [PubMed: 15908389]
17. Williamson ED, Vesey PM, Gillhespy KJ, Eley SM, Green M, Titball RW. An IgG1 titre to the F1 and V antigens correlates with protection against plague in the mouse model. *Clin Exp Immunol*. 1999 Apr; 116(1):107–14. [PubMed: 10209513]
18. Anderson GW Jr, Worsham PL, Bolt CR, Andrews GP, Welkos SL, Friedlander AM, et al. Protection of mice from fatal bubonic and pneumonic plague by passive immunization with monoclonal antibodies against the F1 protein of *Yersinia pestis*. *Am J Trop Med Hyg*. 1997 Apr; 56(4):471–3. [PubMed: 9158060]
19. Sato K, Nakajima R, Hara F, Une T, Osada Y. Preparation of monoclonal antibody to V antigen from *Yersinia pestis*. *Contrib Microbiol Immunol*. 1991; 12:225–9. [PubMed: 1935094]
20. Motin VL, Nakajima R, Smirnov GB, Brubaker RR. Passive immunity to yersiniae mediated by anti-recombinant V antigen and protein A-V antigen fusion peptide. *Infect Immun*. 1994 Oct; 62(10):4192–201. [PubMed: 7927675]

21. Hill J, Copse C, Leary S, Stagg AJ, Williamson ED, Titball RW. Synergistic protection of mice against plague with monoclonal antibodies specific for the F1 and V antigens of *Yersinia pestis*. *Infect Immun*. 2003 Apr; 71(4):2234–8. [PubMed: 12654847]
22. Hill J, Eyles JE, Elvin SJ, Healey GD, Lukaszewski RA, Titball RW. Administration of antibody to the lung protects mice against pneumonic plague. *Infect Immun*. 2006 May; 74(5):3068–70. [PubMed: 16622253]
23. Parent MA, Berggren KN, Kummer LW, Wilhelm LB, Szaba FM, Mullarky IK, et al. Cell-mediated protection against pulmonary *Yersinia pestis* infection. *Infect Immun*. 2005 Nov; 73(11):7304–10. [PubMed: 16239527]
24. Parent MA, Wilhelm LB, Kummer LW, Szaba FM, Mullarky IK, Smiley ST. Gamma interferon, tumor necrosis factor alpha, and nitric oxide synthase 2, key elements of cellular immunity, perform critical protective functions during humoral defense against lethal pulmonary *Yersinia pestis* infection. *Infect Immun*. 2006 Jun; 74(6):3381–6. [PubMed: 16714568]
25. Bashaw J, Norris S, Weeks S, Trevino S, Adamovicz JJ, Welkos S. Development of in vitro correlate assays of immunity to infection with *Yersinia pestis*. *Clin Vaccine Immunol*. 2007 May; 14(5):605–16. [PubMed: 17376861]
26. Nalca A, Zumbun EE. ACAM2000: the new smallpox vaccine for United States Strategic National Stockpile. *Drug Des Devel Ther*. 2010; 4:71–9.
27. Monath TP, Caldwell JR, Mundt W, Fusco J, Johnson CS, Buller M, et al. 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*. 2004 Oct; 8(Suppl 2):31–44.
28. Chakrabarti S, Brechling K, Moss B. Vaccinia virus expression vector: coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. *Mol Cell Biol*. 1985 Dec; 5(12):3403–9. [PubMed: 3939316]
29. Stacy S, Pasquali A, Sexton VL, Cantwell AM, Kraig E, Dube PH. An age-old paradigm challenged: old baboons generate vigorous humoral immune responses to LcrV, a plague antigen. *J Immunol*. 2008 Jul 1; 181(1):109–15. [PubMed: 18566375]
30. Wang S, Heilman D, Liu F, Giehl T, Joshi S, Huang X, et al. A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague. *Vaccine*. 2004 Sep 3; 22(25–26):3348–57. [PubMed: 15308359]
31. Earl, BMAP. *Current Protocols in Molecular Biology*. Vol. 1998. John Wiley and Sons, Inc; 1998. Overview of the vaccinia virus expression system; p. 16
32. Meng X, Zhong Y, Embry A, Yan B, Lu S, Zhong G, et al. Generation and characterization of a large panel of murine monoclonal antibodies against vaccinia virus. *Virology*. 2011 Jan 20; 409(2):271–9. [PubMed: 21056889]
33. Buller RM, Smith GL, Cremer K, Notkins AL, Moss B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature*. 1985 Oct 31; Nov 31; 317(6040):813–5. [PubMed: 4058585]
34. Bubeck SS, Cantwell AM, Dube PH. Delayed inflammatory response to primary pneumonic plague occurs in both outbred and inbred mice. *Infect Immun*. 2007 Feb; 75(2):697–705. [PubMed: 17101642]
35. Bubeck SS, Dube PH. *Yersinia pestis* CO92 delta yopH is a potent live, attenuated plague vaccine. *Clin Vaccine Immunol*. 2007 Sep; 14(9):1235–8. [PubMed: 17652523]
36. Cantwell AM, Bubeck SS, Dube PH. YopH inhibits early pro-inflammatory cytokine responses during plague pneumonia. *BMC Immunol*. 11:29. [PubMed: 20565713]
37. Meng X, Chao J, Xiang Y. Identification from diverse mammalian poxviruses of host-range regulatory genes functioning equivalently to vaccinia virus C7L. *Virology*. 2008 Mar 15; 372(2):372–83. [PubMed: 18054061]
38. Davies DH, Wyatt LS, Newman FK, Earl PL, Chun S, Hernandez JE, et al. Antibody profiling by proteome microarray reveals the immunogenicity of the attenuated smallpox vaccine modified vaccinia virus ankara is comparable to that of Dryvax. *J Virol*. 2008 Jan; 82(2):652–63. [PubMed: 17977963]
39. Niles EG, Seto J. Vaccinia virus gene D8 encodes a virion transmembrane protein. *J Virol*. 1988 Oct; 62(10):3772–8. [PubMed: 3418784]

40. Husain M, Weisberg AS, Moss B. Sequence-independent targeting of transmembrane proteins synthesized within vaccinia virus factories to nascent viral membranes. *J Virol*. 2007 Mar; 81(6):2646–55. [PubMed: 17192302]
41. Chichester JA, Musiychuk K, Farrance CE, Mett V, Lyons J, Yusibov V. A single component two-valent LcrV-F1 vaccine protects non-human primates against pneumonic plague. *Vaccine*. 2009 May 26; 27(25–26):3471–4. [PubMed: 19200825]
42. Sette A, Moutaftsi M, Moyron-Quiroz J, McCausland MM, Davies DH, Johnston RJ, et al. Selective CD4+ T cell help for antibody responses to a large viral pathogen: deterministic linkage of specificities. *Immunity*. 2008 Jun; 28(6):847–58. [PubMed: 18549802]
43. Collado M, Rodriguez D, Rodriguez JR, Vazquez I, Gonzalo RM, Esteban M. Chimeras between the human immunodeficiency virus (HIV-1) Env and vaccinia virus immunogenic proteins p14 and p39 generate in mice broadly reactive antibodies and specific activation of CD8+ T cell responses to Env. *Vaccine*. 2000 Jul 15; 18(27):3123–33. [PubMed: 10856792]
44. Rodriguez JR, Rodriguez D, Esteban M. Structural properties of HIV-1 Env fused with the 14-kDa vaccinia virus envelope protein. *Virology*. 1991 Apr; 181(2):742–8. [PubMed: 2014647]
45. Katz E, Moss B. Immunogenicity of recombinant vaccinia viruses that display the HIV type 1 envelope glycoprotein on the surface of infectious virions. *AIDS Res Hum Retroviruses*. 1997 Nov 20; 13(17):1497–500. [PubMed: 9390748]
46. Katz E, Wolffe EJ, Moss B. The cytoplasmic and transmembrane domains of the vaccinia virus B5R protein target a chimeric human immunodeficiency virus type 1 glycoprotein to the outer envelope of nascent vaccinia virions. *J Virol*. 1997 Apr; 71(4):3178–87. [PubMed: 9060681]
47. Kwak H, Mustafa W, Speirs K, Abdool AJ, Paterson Y, Isaacs SN. Improved protection conferred by vaccination with a recombinant vaccinia virus that incorporates a foreign antigen into the extracellular enveloped virion. *Virology*. 2004 May 1; 322(2):337–48. [PubMed: 15110531]
48. Palin A, Chattopadhyay A, Park S, Delmas G, Suresh R, Senina S, et al. An optimized vaccine vector based on recombinant vesicular stomatitis virus gives high-level, long-term protection against *Yersinia pestis* challenge. *Vaccine*. 2007 Jan 8; 25(4):741–50. [PubMed: 16959385]
49. Chiuchiolo MJ, Boyer JL, Krause A, Senina S, Hackett NR, Crystal RG. Protective immunity against respiratory tract challenge with *Yersinia pestis* in mice immunized with an adenovirus-based vaccine vector expressing V antigen. *J Infect Dis*. 2006 Nov 1; 194(9):1249–57. [PubMed: 17041851]
50. Bhattacharya D, Meccas J, Hu LT. Development of a vaccinia virus based reservoir-targeted vaccine against *Yersinia pestis*. *Vaccine*. 2010 Nov 10; 28(48):7683–9. [PubMed: 20875494]
51. Brewoo JN, Powell TD, Stinchcomb DT, Osorio JE. Efficacy and safety of a modified vaccinia Ankara (MVA) vectored plague vaccine in mice. *Vaccine*. 2010 Aug 16; 28(36):5891–9. [PubMed: 20638759]

Figure 1. Construction and characterization of recombinant ACAM2000 viruses expressing *Y. pestis* LcrV

A. Schematic diagram of the three different forms of LcrV antigen introduced into ACAM2000 virus through homologous recombination. B. Western blot analysis of cells infected by the recombinant viruses. Cells were infected by the indicated viruses for 24 h. Equivalent amount of either whole cell lysates or media were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with polyclonal rabbit sera against LcrV and mouse monoclonal antibody against WR148. C. One-step growth curve of the recombinant viruses in HeLa cells. HeLa cells were infected with each virus at a MOI of 5 and harvested at 0, 24, and 48 hours post infection. Harvested crude lysates were titrated in triplicate on CV-1 cells.

Figure 2. LcrV-TM is present on the surface of the MV envelope

A. Transmission electron microscopy analysis of purified virions. Sucrose gradient purified virions were adsorbed to 200nm carbon coated nickel grids. The grids were incubated with polyclonal rabbit sera against LcrV and monoclonal mouse antibody against VACV D8, followed by gold-conjugated 12nm anti-rabbit IgG and 6nm anti-mouse IgG to detect the presence of LcrV and D8 on the surface of virions. B. Gold particles per virion were quantified from at least 50 images of each recombinant virus. For LcrV-TM virus, only virions labeled substantially with anti-LcrV (~43% of the total virions, table 1) were included for gold particles quantification. C. Fractionation of purified virions. Sucrose gradient purified LcrV-TM virions were treated with IGEPAL extraction buffer in the presence or absence of DTT. Samples were centrifuged and supernatants and pellets containing membrane fractions and core fractions, respectively, were resolved by SDS-PAGE and transferred to a nitrocellulose membrane, and blotted with polyclonal rabbit sera against LcrV and monoclonal hybridoma supernatant (mouse) against VACV envelope protein H3L.

Figure 3. Intracellular localization of LcrV expressed by the recombinant viruses
 BHK cells seeded on glass coverslips were infected with the indicated viruses at MOI of 0.1. After 24 hours of infection, infected cells were fixed and permeabilized. Polyclonal rabbit sera against LcrV and monoclonal mouse hybridoma supernatant against VACV membrane protein A14 were added, followed by Alexafluor568-conjugated anti-rabbit and Alexafluor488-conjugated anti-mouse IgG, respectively. The cells were stained with DAPI before they were visualized with an Olympus IX-81 fluorescent microscope using a 100x oil emersion objective. The fluorescent signal from virus-expressed GFP was very weak compared to the signal from Alexafluor488 and thus did not appear in the image. Arrows indicate viral factories.

Figure 4. LcrV-TM virus elicited a high anti-LcrV antibody titer in vaccinated mice

A. Balb/c mice (5 per group) were immunized with WT (ACAM2000), LcrV or s-LcrV viruses. At 28 days post-vaccination, sera from blood were extracted from immunized mice and antibody titers against recombinant LcrV protein and purified VACV virions were determined by ELISA. B. Similar to A, Balb/c mice (10 per group) were immunized with s-LcrV or LcrV-TM viruses or recombinant LcrV protein (rLcrV) as control. At 35 days post immunization, serum antibody titers against LcrV and VACV were determined with ELISA. For (A) and (B), individual serum antibody titers are vertically plotted. Horizontal bars indicate average serum antibody titers. Statistics were determined using an unpaired student's t test with 95% confidence bounds, and the p values were shown. C. The isotypes of the anti-LcrV antibodies shown in B were determined with ELISA by using isotype-specific secondary antibodies. Average endpoint titers were plotted on a histogram.

Figure 5. LcrV-TM virus confers sterile protection against lethal *Y. pestis* and VACV WR challenges

A and B. Immunized mice shown in Fig. 4B were divided into two sets of 5 mice per group. One set of the mice were challenged intradermally with $2000 \times \text{LD}_{50}$ of *Y. pestis* strain CO92 (A) while the remaining 5 mice per group were challenged intranasally with a $1000 \times \text{LD}_{50}$ dose of wild-type VACV strain WR (B). Survival for each challenge set was followed over a period of 28 days (8 days shown). **C and D.** Groups of 10 (C) or 5 (D) mice were immunized with rLcrV protein or WT ACAM2000, LcrV, s-LcrV, or LcrV-TM viruses and challenged intranasally with $15 \times \text{LD}_{50}$ (C) or $40 \times \text{LD}_{50}$ (D) doses of *Y. pestis* strain CO92, and survival was monitored over the course of 28 days (8 days shown).

Table 1

Quantitation of LcrV positive virions.

Virus Name	total virions counted	LcrV positive virions [†]	% LcrV positive virions
LcrV-TM	104	45	43.3%
s-LcrV	85	0	0.00%
LcrV	83	0	0.00%

[†] cutoff for positive LcrV virions is >10 gold-particles against anti-LcrV antibody/virion