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Molecular Smallpox Vaccine Delivered by Alphavirus Replicons Elicits Protective Immunity in Mice and Non-human Primates

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Abstract

Naturally occurring smallpox was eradicated as a result of successful vaccination campaigns during the 1960s and 70s. Because of its highly contagious nature and high mortality rate, smallpox has significant potential as a biological weapon. Unfortunately, the current vaccine for orthopoxviruses is contraindicated for large portions of the population. Thus, there is a need for new, safe, and effective orthopoxvirus vaccines. Alphavirus replicon vectors, derived from strains of Venezuelan equine encephalitis virus, are being used to develop alternatives to the current smallpox vaccine. Here, we demonstrated that virus-like replicon particles (VRP) expressing the vaccinia virus A33R, B5R, A27L, and L1R genes elicited protective immunity in mice comparable to vaccination with live-vaccinia virus. Furthermore, cynomolgus macaques vaccinated with a combination of the four poxvirus VRPs (4pox-VRP) developed antibody responses to each antigen. These antibody responses were able to neutralize and inhibit the spread of both vaccinia virus and monkeypox virus. Macaques vaccinated with 4pox-VRP, flu HA VRP (negative control), or live-vaccinia virus (positive control) were challenged intravenously with 5×10^6 PFU of monkeypox virus 1 month after the second VRP vaccination. Four of the six negative control animals succumbed to monkeypox and the remaining two animals demonstrated either severe or grave disease. Importantly, all 10 macaques vaccinated with the 4pox-VRP vaccine survived without developing severe disease. These findings revealed that a single-boost VRP smallpox vaccine shows promise as a safe alternative to the currently licensed live-vaccinia virus smallpox vaccine.

INTRODUCTION

Eradication of smallpox as a naturally occurring disease is a monumental human accomplishment. This accomplishment, unfortunately, is tempered by the realization that the threat of smallpox as an unnaturally occurring disease remains. In fact, the cessation of

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smallpox vaccination programs has rendered much of the world population either unvaccinated, or vaccinated with waning immunity. Vaccination with a traditional smallpox vaccine (scarification with Dryvax), or cell-cultured derived version of that vaccine (ACAM2000), remains the most effective pretreatment strategy to prevent smallpox. However, the adverse events associated with the traditional smallpox vaccine make this vaccine contraindicated in persons with compromised immune systems, skin conditions, and evidence of heart disease [1,2]. Additionally, because of the potential for the vaccine virus to spread to non-vaccinated individuals, persons living with those who are contraindicated (e.g., due to skin conditions or pregnancy) are discouraged from being vaccinated. Furthermore, health professionals are advised to completely avoid contact with patients until a scab forms at the vaccination site. Many health professionals view vaccination with live-virus as an unacceptable risk, even in healthy individuals. Thus, much of the population, including many first responders and healthcare workers, remain susceptible to smallpox. An alternative vaccine that is safe, effective, and readily accepted by critical populations such as the military and first-line healthcare providers is needed to mitigate the potentially catastrophic threat posed by smallpox.

At the end of the eradication campaign, an effort to develop a safe alternative to the traditional smallpox vaccine was underway. Modified vaccinia Ankara (MVA) is a leading candidate as an alternative smallpox vaccine [3]. MVA was generated by extensive passage through a vian cells. The mutations selected for during the repeated passaging (many deletions) resulted in a highly attenuated virus that does not replicate efficiently in mammalian cells [4,5]. Recent studies indicate that two vaccinations with MVA can protect against vaccinia virus (VACV) and monkeypox virus (MPXV) in animal models, including the intravenous and intratracheal monkeypox nonhuman primate models [4,6]. Another highly attenuated vaccinia virus vaccine, called LC16m8, was developed in Japan at the end of the eradication campaign. LC16m8 is reported to be safer in humans and just as effective as the wild-type VACV vaccines in animal models [7,8]. Unlike MVA, Lc16m8 is able to replicate in mammalian cells. These vaccinia-virus vaccines show promise and are candidates for safe alternative for the licensed wild-type vaccinia virus vaccines. Nevertheless, these viruses have genomes encoding hundreds of proteins, including many immunoregulatory proteins and proteins of unknown function. They remain nebulous in terms of what viral components are necessary for protection and what components might elicit poorly understood adverse events, including myocarditis.

We and others have demonstrated that certain poxvirus open reading frames encode proteins that can contribute to protective immunity as measured by neutralizing activity *in vitro* and/or protection in animal models [9–23]. The identification of protective poxvirus immunogens has allowed development of safe and highly defined subunit vaccines. Thus far, subunit vaccine approaches have consisted of purified proteins, plasmid DNA vaccines, recombinant adenoviruses, and alphavirus replicons [12–16,18,22]. There are two infectious forms of orthopoxviruses; the intracellular mature virion (IMV) (also known as mature virion, MV) and the extracellular/cell-associated enveloped virions (EEV/CEV, also known as enveloped virion, EV) (reviewed in [24]). These two particle types are antigenically distinct and it has become clear that vaccines targeting combinations of both IMV and EEV immunogens are more protective than vaccines targeting individual immunogens on either particle [12,15,16, 20–22]. The likely explanation for this synergistic approach is that the immune responses to the IMV surface proteins neutralize input virus during the initial exposure, and released virus from disrupted EEV or lysed infected cells; whereas the immune responses to the EEV surface proteins inhibit the dissemination of EEV within the host. Currently, protective immunogens used in subunit vaccines have included the IMV surface proteins L1, A27, D8, H3, and the EEV surface proteins B5 and A33 [9,11–13,15–17,21,25,26]. There is some evidence that core proteins (i.e., A10L, A4L) and secreted proteins (i.e., type I interferon binding protein) may also play a role in protective immunity [10,27]. Additionally, several studies have demonstrated a critical role for antibody as the quintessential component of protection from secondary

poxviruses infections [34–37]. However, there is one report identifying a protective CD8+ T-cell epitope against VACV [28].

In our earlier work, we delivered the target immunogens as plasmid DNA using a gene gun or skin electroporation device [15–17]. We found that a combination of four vaccinia virus genes, L1R, A27L, A33R, B5R, produced functional antibodies against both the IMV and EEV. This four-immunogen combination was abbreviated using the term 4pox. The 4pox vaccine protected mice against both parenteral and intranasal lethal challenges with vaccinia virus, and can protect nonhuman primates against a lethal intravenous challenge with MPXV [16,17,26,29]. In mice, the 4pox vaccine can completely protect against a lethal intranasal challenge after two vaccinations [26]. Furthermore, a single vaccination significantly protected mice (>70% survival) (Golden J.W. and J.W. Hooper, unpublished findings). In the nonhuman primate studies, the macaques were vaccinated four times followed by a long-range boost [16].

We are interested in exploring strategies of improving the immunogenicity of gene-based molecular vaccines such that protective immunity can be produced in two or fewer vaccinations. Here, we describe an approach where alphavirus-based virus-like replicon particles (VRP) were used to deliver the 4pox vaccine antigens. VRP vaccines are single-cycle vectors capable of infecting cells and expressing heterologous genes, but are not capable of subsequent rounds of amplification because they lack the genes encoding viral structural proteins. We tested individual vaccinia virus genes and gene combinations for immunogenicity and protective efficacy first in mice, and then in nonhuman primates. We found that alphavirus replicons expressing a combination of four vaccinia virus genes protected cynomolgus macaques against a lethal MPXV challenge. Only two vaccinations were sufficient to confer protective immunity not only against lethality, but also against severe disease. Our findings advance the VRP vaccine platform as a viable orthopoxvirus vaccine candidate.

MATERIALS AND METHODS

Viruses and cells

VACV Connaught vaccine strain (derived from the New York City Board of Health strain) and VACV strain IHD-J (obtained from Dr. Alan Schmaljohn) were all maintained in VERO cell (ATCC CRL-1587) monolayers grown in Eagle minimal essential medium, containing 5% heat-inactivated fetal bovine serum (FBS), 1% antibiotics (100 U/ml penicillin, 100 µg/ml of streptomycin, and 50 µg/ml of gentamicin), 10 mM HEPES (cEMEM). BS-C-1 cells (ATCC CCL-26) were used for plaque reduction neutralization tests and comet inhibition tests.

Construction and selection of optimal 4pox replicons

Two replicon vector systems were used to express the VACV genes, pERK and pVEK. The pERK system is based on an attenuated strain of Venezuelan equine encephalitis (VEE) virus called V3014 [30] that is essentially the same as the system originally described by Pushko et al. [31] but it was modified for optimal gene-of-interest (GOI) expression by addition of a spacer-IRES sequence downstream of the 26S subgenomic promoter [32]. The pVEK vector is based on the current investigational new drug (IND) VEE virus vaccine (TC-83) [33] that was similarly modified for optimal GOI expression as described above. The VACV L1R, A33R, B5R, and A27L genes were PCR-amplified from existing DNA plasmids [16] using the primers and DNA templates indicated in Table 1. Each PCR product coded for *Xba*I restriction sites at the 5' and 3' end. The PCR products were then cloned into the *Xba*I site of the transfer vector pCDNA3.3 [32]. The orientation of the VACV gene in pCDNA3.3 was determined by restriction analysis and positive clones were sequenced to ensure no errors were introduced into the gene during PCR amplification. Each of the VACV genes were then subcloned as *Asc*I fragments into the *Asc*I site of the modified VEE replicon plasmids. The orientation of

the gene was determined by restriction analysis and clones in the sense orientation were selected. The newly constructed pERK and pVEK VACV-gene replicon vectors were then modified to contain random spacer fragments upstream of the encoded IRES element as described previously [32] and individual replicons were analyzed to determine the level of GOI expression by cell lysates ELISA analysis. Briefly, each replicon RNA (30 µg) was electroporated into 3×10^7 Vero cells, cells were diluted into OptiPro medium (GIBCO) and 3×10^6 cells were seeded into wells of 6-well plates. Electroporated cells were incubated overnight at 37°C in an atmosphere of 5% CO₂. Protein lysates were prepared from cells electroporated with each replicon ~18 hr post electroporation by exposing cells to 0.5 ml TX100 lysis buffer (10 mM Tris, 1 mM EDTA, 0.25 M NaCl, 1% Triton X-100) for 5 min at 4°C. Cell lysate supernatants were collected after microcentrifugation at 12,000 RPM for 10 min at 4°C. Total cellular protein concentration was determined for each sample using a BCA protein kit (Pierce, Rockford, IL). The relative VACV gene-specific protein expression level for each replicon was measured by cell lysate ELISA. Each 4pox, gene-specific, replicon cell lysate (40 µg cell lysate) was used to coat 96-well ELISA plates in triplicate (Nunc) overnight at 4°C using bicarbonate buffer (Sigma). VACV protein-specific primary antibodies were used to detect the expression level of each 4pox antigen in each replicon vector. Relative expression level was detected using an appropriate species-specific, alkaline phosphatase (AP)-conjugated antibody (Sigma-Aldrich, St. Louis, MO), and AP-specific color development solution (BIO-RAD) for visualization (data not shown). Two independent experiments were conducted for each complement of optimized 4pox-gene replicons. The 4pox, gene-specific replicon vector that expressed the highest average amount of each VACV protein, based on the optical density (O.D.) at 405 nm read on an ELISA plate reader, was selected for testing in animal studies.

RNA transcription, electroporation, and VRP production

The methods used to *in-vitro* transcribe replicon RNA, electroporate RNA into Vero cells, produce, and purify VRP vaccines were described previously [32]. The infectious unit (IU) titer of VRP was determined by immunofluorescence assay (IFA) using goat anti-VEE nsP2-specific polyclonal antiserum as the primary antibody and donkey anti-goat Alexa Fluor 488 (Invitrogen) as the secondary antibody on methanol-fixed cells using a Nikon Eclipse TE300 fluorescence microscope. The VRP were tested for the presence of contaminating replication-competent VEE (RCV) using two blind passages on VERO cells as described previously [32].

4pox antigen Western blot analysis

For Western blot analysis, cells were lysed in TX100 lysis buffer for 5 min at 4°C. After lysis, nuclei were removed by centrifugation and the total cell protein concentration for each sample was determined using a BCA protein assay kit (Pierce). Equivalent sample protein concentrations were heated with SDS sample buffer (with and without reducing reagent [nonreducing conditions are required to detect L1 by Western blot] and electrophoresed through a 4–12% Bis-Tris polyacrylamide gel (Invitrogen). Proteins were transferred to Immobilon-P PVDF membranes using the X-cell II blotting system and NuPage buffer (Invitrogen). Proteins were detected with monoclonal antibodies or antiserum specific for each VACV protein and (AP)-conjugated anti-mouse or rabbit antibody (Sigma) followed by development in AP-specific color development solution (Bio-Rad) for visualization.

Purification of VACV antigens for ELISA

Expression vectors were prepared. VACV, strain Connaught, A27L, L1R, A33R, and B5R open reading frames were cloned into the expression plasmids pET21d, pET16b, pET21a, and pET21a, respectively. The A27 construct (pET-A27L[VACV]) is described [29]. Three of the four genes, L1R, A33R, and B5R, were modified by removing predicted transmembrane

regions using a strategy essentially the same as that described for the MPXV orthologs [14]. The L1R construct (pET16b-L1R-181) contained nucleotides encoding L1 amino acids M (1)-G(181) fused to a 9xHis tag and 27 nucleotide spacer containing the factor Xa recognition site at the amino terminus. The A33R construct (pET21a-A33Recto) contained nucleotides encoding A33 amino acids N(60)-C(180) a 6xHis tag at the carboxy terminus. Likewise, the B5R construct (pET21a-B5Recto) contained nucleotides M(1) thru H(279) fused to a 6xHis tag at the carboxy terminus. In addition, the truncated B5R gene was subcloned into a VEE replicon vector for expression of the protein in mammalian cells (BHK cells).

A27, L1, and A33 were produced in *Escherichia coli* strain BL21(DE3)-RIL (Stratagene). The A27 was induced by diluting an overnight culture to 500 ml at an OD₆₀₀ of 0.05 with fresh Luria broth (LB) containing ampicillin, allowing the culture to replicate at 37°C to an OD₆₀₀ between 0.6 and 0.8, adding IPTG to a final concentration of 1mM, and continuing incubation for 3 hr at 37°C. The cells were harvested by centrifugation at 3,000 × g for 20 min after which they were lysed by the addition of 1ml of lysis buffer (0.1% NP-40, 50mM Tris-HCl, 300 mM sodium chloride, 1mg/ml Lysozyme (Sigma), 1X EDTA-free Protease Inhibitor tablet (Roche), pH 8.0) per 0.2 g wet weight. The solution was incubated at 4°C for 25 min then lysed by sonication using a Branson model 450 sonifier with the microtip attachment. The lysate was clarified by centrifugation at 30,000 × g for 15 min before being frozen at -20°C. To purify A27, the lysate was thawed, filtered through a 5-µm syringe filter, and applied to a 1.25-ml HisSelect cartridge (Sigma) pre-equilibrated with A Buffer (0.1% NP-40, 50 mM Tris-HCl, 300mM sodium chloride, pH 8.0). The column was washed with A Buffer containing 10 mM imidazole then A27 was eluted with a linear gradient of A Buffer containing imidazole from 10 mM to 250 mM over 16 column volumes. The eluted protein was buffer exchanged into B buffer (0.1% NP-40, 50mM Tris HCl, pH 8.0) using a HiPrep 10/26 desalting column (GE Healthcare) and polished by anion exchange on a 1-ml HiTrap Q HP column (GE Healthcare). For anion exchange, the protein was loaded onto the column, pre-equilibrated with B buffer, and eluted with a linear gradient of B buffer containing from 0 to 1M sodium chloride over five column volumes.

L1 protein was induced in BL21(DE3)-RIL *E. coli* cells by IPTG addition as described for A27 and was expressed as inclusion bodies. The cells were lysed and clarified as described for A27. The pellet obtained by clarification was resuspended three times with IB wash buffer (1% Triton X-100, 20mM Tris-HCl, 10mM EDTA, pH 7.5), sonicated, and clarified by centrifugation at 30,000 × g for 20 min. The washed inclusion bodies were solubilized using the Protein Solubilization kit (Novagen) to 20 mg/ml, clarified by centrifugation at 10,000 × g for 20 min, and dialyzed against 6 L of 20mM Tris-HCl, 0.1mM DTT, pH 8.5 using 3.5 MWCO Slide-A-Lyzer Big Buoy cassettes (Pierce). The protein solution was stored at 4°C after adding sodium azide to 0.05%.

The A33R gene product was produced by expression in BL21(DE3)-RIL *E. coli* cells by IPTG induction and purified utilizing immobilized metal affinity chromatography. Briefly, cells were induced with 1mM IPTG for 4 hr. The cells were pelleted by centrifugation and lysed on ice for 30 min in 20 ml of a buffer containing 20 mM NaPi, 500 mM NaCl, 10 mM imidazole, 0.1% NP40, 1.0% Triton X-100 and 1mg/mL lysozyme (pH 7.4). The cell/buffer solution was sonicated three times for 10 sec and then centrifuged for 15 min at 14,000 RPM at 4°C. The supernatant was collected and loaded onto a nickel chloride-charged, 5ml HiTrap chelating HP column (GE Healthcare) as described above. The histidine tagged A33 protein was eluted from the column with a linear gradient of imidazole (10 mM to 250 mM).

B5 was expressed in BHK-21 cells by transient transfection with *in vitro* transcribed replicon RNA. The transfected cell pellet was lysed by addition of 1 ml of 1% NP-40, 50mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, 1X EDTA-free Protease Inhibitor

tablet (Roche), pH 7.5) per 4×10^7 cells, incubated at 4°C for 30 min, and sonicated. The lysate was clarified by centrifugation at $20,000 \times g$ for 15 min and the supernatant was tandem filtered through a 47mm 5- μ m Durapore disc followed by a 25 mm 0.45- μ m depth filter. The filtrate was loaded onto a nickel chloride-charged 5 ml HiTrap chelating HP column (GE Healthcare) pre-equilibrated with C buffer (0.1% NP-40, 50 mM sodium phosphate, 300 mM sodium chloride, pH 7.5) containing 10 mM imidazole and eluted with a linear gradient of C buffer containing from 10 to 250 mM imidazole over 20 column volumes. The eluted protein was exchanged into D buffer (20 mM N-methylpiperazine HCl, 20 mM Bis-Tris HCl, 20mM Tris HCl, pH 6.0) using a 10DG Desalting Column (Bio-Rad) and applied to a Uno Q-1 column (Bio-Rad) pre-equilibrated with D buffer. The protein was eluted with a linear gradient of D buffer containing from 0 to 1M sodium chloride over 10 CV. and anion exchange chromatography using a Uno Q-1 column (Bio-Rad).

All proteins were assayed by BCA (Pierce) except B5, which required preparing the protein for assay using the Compat-Able Protein Assay Preparation Reagent Set (Pierce). Purified L1 protein was stored at 4°C. All other recombinant proteins were stored at -20°C.

Vaccination and challenge of mice

Female, 6–8 weekold, BALB/c, mice (Charles River Laboratory, Kingston, NY) were vaccinated with the appropriate vaccine by either the subcutaneous (s.c.) route (footpad inoculation) or by the intramuscular (i.m.) route (inner aspect of thigh) Blood was collected by retro-orbital bleeds for all groups at time points specified in each experiment. At the indicated number of weeks after the final vaccination, all animals were challenged with 2×10^6 PFU (high-dose) or 2×10^5 PFU (low dose) of VACV strain IHD-J via the intranasal route. Animals were anesthetized i.m. with anesthetic before viral challenge. Each mouse received 50 μ l of virus (diluted in sterile saline) by placing 25 μ l droplets onto each nares using a positive displacement pipettor. Individual mice were weighed daily after challenge and monitored for signs of morbidity (ruffled fur or hunched posture). Animals with a >30% weight loss were euthanized.

Immunogen-specific ELISA

ELISA was performed essentially as previously described [25,26]. Recombinant VACV antigens (see above) diluted in 0.1 M carbonate buffer, pH 9.6, were used to coat 96-well ELISA plates (100 μ l per well). A27 (50 ng/well), L1 (300 ng/well), A33 (50 ng/well), or B5 (50 ng/well) antigen was absorbed to ELISA plates overnight at 4 °C. An irrelevant HIS-tagged protein (c-terminal fragment of the heavy chain of botulinum neurotoxin A) at 50 ng/well was included as a negative control antigen. Plates were washed once with PBS + 0.05% tween-20 (wash buffer), blocked 1 hr at 37 °C with wash buffer containing 5% FBS + 3% goat serum (blocking buffer), washed once, and incubated 1 hr at 37°C with antibody diluted in blocking buffer containing 20 μ g/ml of *E. coli* lysate to reduce background. Plates were washed three times, incubated 1 hr at 37 °C with an appropriate species specific peroxidase-labeled antibody (anti-mouse or anti-monkey) (KPL, Inc, Gaithersburg, MD) diluted in blocking buffer, washed as before, and incubated in 100 μ l/well of 2, 2'-azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate. After 10–30 min at room temperature, the colorimetric reaction were stopped by adding 100 μ l/well of ABTS stop solution (KPL). The O.D. at 405 nm was determined by an ELISA plate reader. Non-specific binding was controlled for by subtracting O.D. values obtained on negative control antigen from O.D. values obtained on purified VACV antigens. End-point titers were determined as the highest dilution with a specific O.D. greater than the mean O.D. value from negative control serum samples wells (1:50 dilution) plus three standard deviations.

VACV-infected cell lysate ELISA

The preparation of VACV-infected cell lysate and its use as antigen in ELISA was performed as previously described [15,16].

VACV PRNT

VACV strain IHD-J infected-cell lysate was diluted in cEMEM to give approximately 1000 PFU/ml. Aliquots of viral suspension (100 μ l) were incubated with an equal volume of antibody diluted in cEMEM for 1 hr at 37°C (sera were heat inactivated, 56°C for 30 min, before dilution). The samples (180 μ l) were then adsorbed to BSC-1 monolayers in 6-well plates for 1 hr at 37°C in a 5% CO₂ incubator. Each well was then overlain with 2 ml of 1.5% methyl cellulose in Earl's basal minimal essential medium, 5% FBS, and antibiotics. After 4 days at 37°C in a 5% CO₂, monolayers were stained by adding Accustain Crystal Violet solution (Sigma). Plaques were counted and presented as the percent neutralization relative to plaque numbers in the absence of antibody.

Non-human primate vaccination

The 18 adult female cynomolgus macaques (*Macaca fascicularis*), used in this study were captive-bred, and were obtained from Three Springs Scientific, Inc., Perkasie, PA. On days 0 and 28, 1×10^8 IU of each VRP vaccine (i.e., 1×10^8 VRP for each of the four VACV genes in the 4pox vaccine for a total of 4×10^8 IU) diluted in 0.9 ml of 10 mM phosphate buffer pH 7.3 containing 1% human serum albumin and 4% sucrose was injected into the thigh muscle of each animal. Also on day 0, 2 animals were scarified with live vaccinia virus (DRYVAX) using a bifurcated needle dipped in the vaccine and used to administer the vaccine by 15 pricks. The experimental groups are outlined in Table 1.

Non-human primate MPXV challenge

On study day 56, all animals were anesthetized by i.m. injection with Ketamine HCL at approximately 10–30 mg/kg and subsequently inoculated with 1.0 ml medium containing 5×10^6 PFU/ml MPXV strain Zaire 79 by infusion into the saphenous vein using a 22 – 25 gauge needle. In order to confirm the delivered dose, the challenge inoculum was back-titrated on VERO cells using the standard plaque assay technique. Macaques were observed for clinical illness or changes in behavior twice daily. Expected signs of monkeypox viral infection included fever, poxvirus lesions, cough, lethargy, and appetite loss, beginning three or more days after infection with lesions progressing throughout the typical stages described for human monkeypox and smallpox. After challenge on study day 56, all study animals were evaluated specifically for temperature (°F), weight (kg), and lesion counts every 3 days. For all animals, blood was collected for a determination of complete blood counts (CBC), clinical chemistry values, viral load (real-time PCR), ELISPOT, and plaque-reduction neutralization (PRNT) assays. Hematological values of fresh, whole blood were determined using a Coulter AcT Series analyzer (Coulter Corp., Miami, FL).

Real-time PCR to measure genome viremia

At the time points indicated, blood samples and throat swabs were collected from all study animals for determination of viral load by real-Time PCR. Throat swabs were collected and processed in 0.3 ml of cEMEM as described in [29]. DNA was extracted from 0.2 ml of whole blood using Qiagen DNA mini kits as per the manufacturer's instructions. DNA was similarly extracted from throat swabs. MPXV genomes in extracted DNA was measured via the LightCycler Quantitative Pan-orthopox HA PCR assay. Primers and probes used for targeting the hemagglutination (HA) gene were: forward primers: OPHA F89 5'-ATGTACTATCTCAACGTAGTAG 3', reverse primer: OPHA R219 5'-CTGCAGAACATAAACTATTAATATG-3' and probe: OPHA-P143S-MGB 6FAM

AGTGCTTGGTATAAGGAG MGBNFQ. Viral load data are reported as the number of MPXV genome copies per ml of blood.

Statistical Analysis

For animal experiments involving the measurement of weight loss over time, Repeated Measures ANOVA of weight among groups with stepdown Bonferroni adjustment for pairwise comparisons was performed. Fisher's Exact tests were used to compare survival rates among groups. When evaluating differences in geometric mean titers (GMT) between groups, *t*-tests were performed with stepdown Bonferroni adjustment for multiple comparisons. Significance was set at a *p*-value < 0.05.

RESULTS

VRP containing VACV L1R, A27L, A33R, and B5R open reading frames express protein of appropriate size

We evaluated two alphavirus replicon vectors, pERK and pVEK, for a capacity to express target immunogens from VACV. The VACV L1R, A27L, A33R, and B5R open reading frames were cloned into both the pERK and pVEK replicon vectors as described in Methods. Expression of the protein-of-interest was evaluated by immunofluorescence (data not shown) and Western blot (Fig. 1). Proteins of the predicted molecular weight were expressed from both the pERK and pVEK replicon systems. There were no substantial differences between vector systems in the quantity or quality of the protein produced in cell culture.

Protective efficacy of VACV VRP in mice

pERK-based VRP expressing VACV immunogens were tested for protective efficacy in mice. The experiment consisted of single- and combination-immunogen VRP vaccines administered i.m. two or three times followed by a high-dose (2×10^6 pfu) or low-dose (2×10^5 pfu) intranasal challenge (Fig. 2A). The single-immunogen VRP vaccines were L1R-VRP, A27L-VRP, A33R-VRP, and B5R-VRP. The combination-immunogen VRP vaccine (4pox-VRP) consisted of all four aforementioned VRPs delivered simultaneously. Groups of mice vaccinated i.m. two or three times with irrelevant VRP or once by scarification with VACV were included in each experiment as negative and positive controls, respectively.

The outcome of the 2×10^6 pfu challenge is shown in Fig. 2B–C. All of the negative control mice became moribund and either succumbed to disease or were euthanized, whereas all of the scarified mice survived with minimal weight loss. All mice vaccinated two or three times with 4pox-VRP or A33R-VRP survived with negligible weight loss. The weight loss was not significantly (*p*-value > 0.2) different from the scarified group for any day after day 2. Although all of the mice vaccinated 2 or 3 times with B5R-VRP (Groups 2 and 9) survived challenge, weight loss was substantial and significantly different (*p*-value ≤ 0.029) from the scarified group on all days after day 2. Mice vaccinated two times with L1R-VRP or two or three times with A27L-VRP did not survive challenge. Due to a technical error, mice vaccinated three times with L1R-VRP were excluded from the study and protection was not assessed.

The outcome of the less rigorous 2×10^5 pfu challenge is shown in Fig. 2D–E. This challenge dose was used to detect subtle levels of protective immunity that were overwhelmed by the 2×10^6 pfu challenge. All of the mice survived challenge except three animals in the negative control group (survival data not shown). Four or more days after challenge, mice vaccinated three times with the 4pox-VRP, or A33R-, B5R-, L1R- or A27L-VRP, exhibited significantly (*p*-value ≤ 0.0141) less weight loss than the negative controls (Fig. 2D). Relative to the negative control VRP (Group 13), there was a significant (*p*-value ≤ 0.0172 on $\geq$ day 9) level of protection detected for all the VRP vaccines except A27L-VRP when administered only twice

(Fig. 2E). These weight loss data demonstrated that each component of the 4pox vaccine (L1, A33, B5 and A27) elicited some level of protection.

Immunogenicity of single-immunogen VRP in mice

Protection from secondary orthopoxvirus infection requires antibody [34–37]. Accordingly, we evaluated the humoral immune responses to the single-immunogen and combination VRP vaccines. Sera collected 1 week after the second vaccination, and 2 weeks after the third vaccination (time of challenge) were evaluated for binding antibody by VACV infected-cell-lysate antigen (Fig. 3A) and immunogen-specific ELISA (Fig. 3B). The mean infected-cell-lysate ELISA titer after two vaccinations with the B5R-VRP was greater than 2 (the lowest dilution of serum tested was 1:100 or log 2); however, the other three VRP elicited consistently measurable anti-VACV responses only after the third vaccination. The highest anti-VACV ELISA titers were elicited by B5R-VRP > L1R-VRP > A33-VRP > A27L-VRP. For the individual immunogen ELISA, all groups, except L1R-VRP, exhibited mean titers greater than log 2 after two vaccinations (Fig. 3B) indicating that the immunogen-specific ELISAs were more sensitive than the VACV infected-cell-lysate ELISA. Mice vaccinated with L1R-VRP developed detectable antibody responses after the third vaccination.

Serum collected after the second or third vaccinations were tested for IMV neutralizing antibodies by PRNT. Neutralizing antibody titers were low or undetected after the second vaccination, but after the third vaccination, there was neutralizing antibody detected in the mice vaccinated with either the A27L-VRP or L1R-VRP (Fig. 3C). Together these serological data demonstrated that detectable immune responses, including the production of neutralizing antibody for A27 and L1, were produced by vaccination with each of the four immunogens of interest.

Immunogenicity of 4pox-VRP

As reported above, all of the mice vaccinated with the 4pox-VRP vaccine survived challenge with minimal weight loss. To determine if antibody responses to all of the target immunogens (L1, A33, A27, and B5) were produced after vaccination with the combination vaccine, immunogen-specific ELISA and VACV-infected-cell lysate ELISA were performed on sera collected after two or three VRP vaccinations. The mean ELISA titers from a representative 4pox-VRP group and scarified group are shown (Fig. 4A–B). After two VRP vaccinations, the overall antibody titers, as measured by VACV infected-cell lysate antigen ELISA, were greater than three logs, which was similar to the mean titers produced after a single scarification with live VACV. The single-immunogen titers in the 2x 4pox-VRP vaccinated mice were greater than two logs for A33R and B5R, but < two logs for the IMV targets A27L and L1R. The responses against all four targets were greater than two after the third vaccination. With the exception of the anti-B5 titer, there were significant increases ($p < 0.05$) in ELISA titers after the third vaccination. Sera from mice scarified with VACV were collected at the same time the VRP mice sera after two or three vaccinations were collected. Titers against B5, L1, and A27 were not significantly different between 4pox-VRP vaccinated and scarified groups. A33 titers were significantly higher for 4pox-VRP vaccinated groups relative to the scarified group.

PRNT were performed on sera from mice vaccinated 2x or 3x with 4pox-VRP. After two vaccinations, only 25% sera tested had detectable levels of neutralizing antibodies (data not shown). After three vaccinations, the percentage of mice with detectable neutralizing antibody increased to 75%. The mean neutralizing antibody titer of mice vaccinated three times with the 4pox-VRP vaccine was greater than the titer in mice vaccinated once by scarification (Fig. 4C). These data demonstrated that the four VRPs (A33R-VRP, B5R-VRP, A27R-VRP, and L1R-VRP) could be effectively combined into a single vaccine without loss of immunogenicity toward each immunogen.

4pox VRP: comparison of pERK and pVEK replicon vector systems in mice

All previous experiments utilized the pERK VRP system. We next examined if a VRP vector based on the VEE virus vaccine strain (TC-83), pVEK, would similarly function as an efficacious 4pox vaccine vector. For this evaluation, groups of eight mice were vaccinated s.c. two or three times (rear footpad) with the 4pox gene combination delivered at doses of 10^6 or 10^7 using either the pERK or pVEK replicon system. Groups of mice vaccinated with negative control VRP were included. The experimental design is shown in Fig. 5A. Four weeks after the last vaccination, the mice were challenged intranasally with 2×10^6 pfu of VACV. Only one of the 16 mice vaccinated with the pERK or pVEK negative control VRP survived challenge (Fig. 5B). In contrast, all mice vaccinated with the pERK 4pox or pVEK 4pox survived regardless of 10^6 or 10^7 VRP dose. Thus the protection against lethal disease for all vaccine groups was highly significant (p -value < 0.0001). An ANOVA was used to detect differences between the average weight loss for the different groups on each day. Weight loss was similar in all experimental groups except group 5 (pERK 4pox) where weight loss was similar to the negative control groups (p -value was > 0.7). The weight loss in the pVEK 4pox and pERK 4pox groups vaccinated 2 or 3 times at high or low dose was not significantly different (p -values > 0.05) with one exception. Groups vaccinated only 2 times with the lower dose (Group 4 vs. 5) exhibited significantly different average weight loss on days 8 and 9 after challenge (p -value < 0.03), with the pERK 4pox exhibiting greater weight loss.

The prechallenge immune responses for the mice in groups 1–8 were evaluated by immunogen-specific ELISA (Fig. 5C). Mice vaccinated three times with a 10^6 pERK 4pox, or two times with a 10^7 pERK 4pox developed antibodies against all four immunogens. An anti-A27L response was not detected in sera from mice vaccinated with pVEK 4pox (groups 1, 3, 7). The pVEK 4pox did elicit antibody titers against the three other immunogens. Mice vaccinated only two times with pERK 4pox (group 5) had relatively low antibody titers. This is the same group that exhibited substantial weight loss after challenge (Fig. 5B).

Sera collected on weeks 3, 4, and 7 for each group were pooled and evaluated for neutralizing antibodies by PRNT. Neutralizing activity was not detected after one vaccination (week 3) (data not shown). However, neutralizing antibodies were detected 1 week after the second vaccination in sera from mice vaccinated with pVEK 4pox (groups 1, 4, and 7) or the high dose of pERK 4pox (group 8). When serum from week 7 was tested, all groups were positive for neutralizing antibodies except the group vaccinated twice with pERK 4pox (group 5), and the negative control groups. These data indicated that the pVEK system was as effective, or more effective, than the pERK system at inducing antibodies against the A33R, B5R, and L1R vaccine targets. The anti-A27 responses were low in general, the lowest being those generated by the pVEK VRP system.

Modification of L1R in VRP improves capacity of vaccine to elicit neutralizing antibodies

We recently reported that adding a tPA signal sequence to the L1R gene to target the L1 protein through the ER to the cell surface enhances neutralizing antibody responses of DNA vaccines containing L1R [26]. The likely reason for this improved immunogenicity is the correct formation of the disulfide bonds forming the critical neutralizing epitope [38]. We investigated the possibility that the tPA modification may enhance the L1 response generated by VRPs. To this end, an experiment was performed to test L1R-VRP and tPA-L1R alone (groups 1–4), or in the 4pox combination (groups 5–6) (Fig. 6A). In groups 1–4, eight mice were vaccinated s.c. twice at 3-week intervals with unmodified L1R-VRP or the tPA modified L1R-VRP (both pVEK and pERK versions). Serum collected 3 weeks after the first vaccination, and 1 week after the second vaccination, were evaluated for anti-L1 antibodies by ELISA and neutralizing antibodies by PRNT (Fig. 6 B–C). Adding the tPA significantly (p -value < 0.002) enhanced the anti-L1 antibody response after a single vaccination as measured by ELISA, and more

importantly, by PRNT. After the second vaccination, ELISA and neutralizing antibody GMT in the tPA-modified and unmodified L1-VRP groups were not significantly different. Because of the low A27 responses observed with both pERK and pVEK vectors (Fig. 5), a tPA-A27L pERK and pVEK VRP was also produced to attempt to enhance the immunogenicity of this target. The ELISA titers and neutralizing antibody titers were somewhat higher than the A27L VRP, but the differences were not statistically significant (data not shown). Nevertheless, the tPA-A27L VRP were used in the tPA-4pox experiments described below.

To test the protective efficacy of the modified VRP, groups of eight mice were vaccinated twice with pERK tPA-4pox or pVEK tPA-4pox, or negative control VRP (Fig. 6A). All of the mice vaccinated twice with the 4pox VRP (groups 1 and 2) were completely protected from intranasal challenge with VACV and weight loss was negligible (Fig. 6C). In contrast, control mice vaccinated with VRP containing flu HA (groups 2 and 4) all succumbed to infection after severe weight loss starting on day 3.

Serology was performed to evaluate the antibody responses elicited by the 4pox VRP containing the new tPA-modified genes (Fig. 6D–E). The immunogen-specific ELISA results indicated that both the anti-L1 and anti-A33 GMT were greater than 2 after the second vaccination. An anti-B5 response was not detected in any mice vaccinated with pERK tPA-4pox, and was detected in only three of eight mice vaccinated with pVEK tPA-4pox. An anti-A27L response was detected in six of eight mice vaccinated with tPA-4pox pERK and only two of eight mice vaccinated with tPA-4pox pVEK. Despite the low anti-A27 responses, the anti-L1 responses were sufficient to elicit neutralizing antibody. Neutralizing antibody responses were detected in all of the mice in groups 1 and 2 after a single vaccination (week 3) (Fig. 6E). PRNT titers increased several fold after the second vaccination (week 4). The PRNT₅₀ GMT for group 5 was 761 and for group 6 was 1226. A follow-up experiment was performed where mice were vaccinated only once with the tPA-4pox pVEK vaccine and then challenged intranasally with VACV. All eight mice lost weight but nevertheless survived challenge (data not shown). Together, these data demonstrated that the tPA modification of L1R delivered using the VRP platform significantly enhanced the level of neutralizing antibodies produced after vaccination with L1R, and this caused an overall improvement in the efficacy of the 4pox vaccine.

Immunogenicity of VACV VRP in nonhuman primates

To test the possibility that an alphavirus replicon smallpox vaccine could protect in a species that more closely models humans, we tested the immunogenicity of the candidate 4pox-VRP vaccines in cynomolgus macaques. Ten animals were vaccinated on week 0 and 28 with 4pox-VRP (five animals with pERK 4pox and five animals with pVEK 4pox). The tPA-L1R and tPA-A27L VRP were used in all work involving macaques although the tPA designation is not included in the 4pox-VRP nomenclature. As negative controls, six animals were vaccinated with VRP expressing influenza (A Wyoming) HA [39] (three animals with pERK HA and three animals with pVEK HA). As positive controls, two animals were vaccinated once with Dryvax by scarification. Sera were collected on weeks 0, 1, 4, 6, 8 (time of challenge), and the antibody responses were evaluated. The experimental design is shown in Fig. 7A and Table 2.

Individual sera were tested for binding antibodies by immunogen-specific ELISA (Fig. 7B). All 10 macaques vaccinated with the 4pox VRP developed antibodies against L1, A33, and B5 after the second vaccination, and many were positive after a single vaccination as measured by ELISA. The anti-A27 response was lower than the response to the other immunogens. This was especially true for the pERK 4pox group where only two of five macaques developed a detectable anti-A27 response. In contrast the macaques vaccinated with the flu Ha were generally negative with sporadic background titers of 2. The macaques vaccinated with Dryvax

had negligible anti-L1 responses but did have antibodies to A27, A33, and B5. The GMT for each group at the time of challenge (week 8) are shown (Fig. 7B).

PRNT were performed to measure anti-IMV neutralizing antibody responses. VACV-neutralizing antibodies were detected in all 10 of the macaques vaccinated with 4pox or Dryvax, but were not detected in any of the negative control animals (Fig. 7C). The geometric mean PRNT titer of the week 8 sera was greatest in the pERK 4pox group. To further evaluate the neutralizing antibody response, the time-of-challenge serum (week 8) was evaluated MPXV PRNT. All of the macaques vaccinated with 4pox or Dryvax had detectable levels of anti-MPXV neutralizing antibodies (Fig. 7D). As was the case for VACV PRNT, the MPXV PRNT titers were greatest in the pERK 4pox group.

In the earlier mouse studies, anti-EEV functional antibody was not measured. The larger volumes of sera collected in the nonhuman primate studies allowed us to perform assays to evaluate the capacity of sera to inhibit EEV spread. Comet inhibition assays were performed on week 6 and 8 sera. Macaques vaccinated with pERK 4pox vaccine clearly had a more robust functional anti-EEV response (four of five), than the animals vaccinated with pVEK 4pox (one of five⁵). Interestingly, only one of the macaques vaccinated with Dryvax exhibited detectable comet inhibition on week 6 (Fig. 7E).

ELISPOT data were generated from PBMC collected 6 days before challenge and on weeks 2, 4, and 6. VACV-specific IFN- γ -secreting T-cells were only detected in the Dryvax-vaccinated animals in group 5 (data not shown). These responses were first detected on week 2 and appeared to peak 4 weeks after vaccination by scarification.

Overall, the immunogenicity data in the nonhuman primates were similar to that predicted by the mouse studies. Both pVEK and pERK platforms produced antibodies against the targets. The anti-L1 and anti-A33 responses were more robust than the A27 and B5 responses. Functionally, the pERK platform resulted in greater levels of both IMV neutralizing antibodies and EEV spread-inhibiting antibodies.

4pox-VRP vaccines protect against monkeypox

To evaluate the protective efficacy of the vaccines, 4 weeks after the last vaccination, the nonhuman primates were challenged intravenously with 5×10^6 pfu of MPXV. All 10 macaques vaccinated with the 4pox VRP survived challenge (Table 3). Two animals vaccinated with the 4pox vaccine (one receiving pVEK 4pox and one receiving pERK 4pox) were completely protected from skin lesions. The other eight animals in groups 1 and 2 developed no more than 12 lesions after MPXV challenge. In contrast, four of the six animals vaccinated with the negative control VRP developed grave monkeypox and succumbed to disease, or were euthanized between days 9 and 12 postchallenge. The two surviving macaques in the negative control groups developed more than 100 lesions (severe disease). Both Dryvax-vaccinated animals did not develop skin lesions. The macaques vaccinated with the 4pox vaccine (groups 1 and 2) were compared to macaques vaccinated with the negative control vaccine (groups 3 and 4). Protection from lethal disease was significant (Fisher's Exact Test for survival rates between groups, p -value 0.0082).

The challenged animals were also monitored for other indicators of disease including changes in temperature, weight, hematology, and blood chemistry. The temperature data were unremarkable (data not shown). Only one animal (#4308) had a $>2^\circ\text{C}$ rise in base-line temperature (days 9, 12, 15). This animal survived with only 10 lesions. Of the 17 animals in the study, only four demonstrated a greater than 3% weight loss at two or more consecutive time points. All four of those animals (#s 4316 4317, 4318, 4319) were vaccinated with negative control VRP. However, weight loss was not always associated with disease because two of the

animals that succumbed to monkeypox did not exhibit significant weight loss (#4320 and #4321)(data not shown). There were above-normal levels of white blood cells in several animals during the course of the study, including both Dryvax-vaccinated animals and 3 of 10 4pox-vaccinated animals. Both macaques that survived severe monkeypox exhibited elevated levels of WBC on essentially every time point on or after day 9 (Table 3). The peak lesion number in macaques vaccinated with the 4pox vaccine (groups 1 and 2) were compared to macaques vaccinated with the negative control vaccine (groups 3 and 4) and the difference was significant (ANOVA p -value 0.0002).

Viral Load

MPXV genomes in the serum and in throat swabs were measured by quantitative PCR (Fig. 8A). For the protected animals in groups 1, 2 and 5, the level of virus genome in the blood was below the detection limit (<5000 genome copies/ml) of the assay. In contrast, the control animals in groups 3 and 4 demonstrated peak viral genome loads ranging between 5 to >6 logs between days 9 and 12. Four of these animals were euthanized. The remaining two animals controlled the infection as evidenced by clearance of the virus to undetectable levels, resolution of pock lesions, and survival to the end of the study. To obtain relative values for peak viremia, the two highest consecutive values were averaged for each macaque (Table 3). The maximum blood viremia in macaques vaccinated with the 4pox vaccine (groups 1 and 2) were compared to macaques vaccinated with the negative control vaccine (groups 3 and 4). Differences in the maximum blood viremia for the groups were significant (ANOVA p -value 0.0123). In addition, when comparisons between groups 1 and 3, and between 2 and 4, were made for each day after challenge, there were significant differences in the viremia on days 3, 6, 9, 12 (Fig. 8A).

MPXV genome loads were also measured in throat swabs (Fig. 8B). This assay was substantially more sensitive than the blood viremia assay, and MPXV genome was detected in all of animals, including the Dryvax-vaccinated macaques. Again, consistent with the clinical observations of groups 1, 2, and 5, viral genome loads in throat swabs were generally lower compared with those of groups 3 and 4. Three of the macaques vaccinated with the 4pox VRP had at least one time point with >6 logs of genome detected. All six of the negative control animals had high levels of viral genome detected in the throat swabs and all but one (#4321) had >six logs of virus detected in at least two consecutive time points. The mean for the two highest consecutive time points is shown in Table 3. The maximum throat swab viremia in macaques vaccinated with the 4pox vaccine (groups 1 and 2) were compared to macaques vaccinated with the negative control vaccine (groups 3 and 4). Differences in the maximum throat swab viremia for the groups were not significant (ANOVA p -value 0.1638). However, when comparisons between groups 1 and 3, and between 2 and 4, were made for each day after challenge, there were significant differences in the throat swab viremia on days 3, 9, and 12 (Fig. 8B). When the 4pox-vaccinated macaques (groups 1 and 2) were compared with the Dryvax-vaccinated macaques (group 5) for peak lesion number (ANOVA p -value 0.0876), maximum blood viremia (ANOVA p -value 0.6761), and maximum throat swab viremia (ANOVA p -value 0.7680) there were no significant differences.

DISCUSSION

This is the first time a virus-vectored molecular smallpox vaccine has been tested in nonhuman primates. Other virus-vector smallpox vaccine work including a VEE VRP cowpox study and a recently reported replication-incompetent recombinant adenovirus study were performed in mice [18,22]. In those studies, individual immunogens and combination immunogens were shown to confer protection against intranasal challenges with cowpox virus and vaccinia virus, respectively. Here we report that a four immunogen (4pox) alphavirus VRP vaccine was immunogenic and protected mice against an intranasal vaccinia virus challenge and, more

importantly, protected cynomolgus macaques against lethal intravenous MPXV challenge. Not only were the macaques protected against lethality, but also they were protected from severe disease. Impressively, two of the 10 4pox-vaccinated animals did not develop lesions after challenge, and the maximum number of lesions on any of the 10 animals was 12. In contrast, four of six macaques vaccinated with the flu HA VRP developed lethal monkeypox and the surviving two animals had too many lesions to count.

There have been three previously published studies investigating the efficacy of molecular smallpox vaccines in non-human primates. These include the evaluation of a plasmid DNA vaccine in rhesus macaques, a heterologous prime-boost (plasmid DNA followed by protein plus adjuvant) in rhesus macaques, and purified proteins plus adjuvant in cynomolgus macaques [14,16,18,22,40]. In all of the aforementioned nonhuman primate studies, at least three vaccinations were administered. In this current study, we generated protective immunity after only two vaccinations using VRP doses and injection methods directly applicable to human vaccination. This level of protection is similar to that provided by two vaccinations with MVA as measured by mortality, lesion count, and genome viremia [4,6]. Thus, the immune responses and short-term protection elicited by two vaccinations with VRP expressing four vaccinia virus proteins were as effective as two vaccinations with MVA expressing hundreds of virus structural and nonstructural proteins.

In all but one macaque vaccinated twice with the 4pox VRP or once with Dryvax, MPXV genomes were not detected in the sera. The one exception (#4315) had a minimally detectable level of genome on day15. This is in contrast to the negative control monkeys, all of which had five to six logs of genome copies in their sera within 1 week after challenge (Fig. 8). These results are similar to what was observed after two vaccinations with MVA; although genomes were detected in the blood of most of the animals in those studies [4,6]. We found that the throat swab samples were much more sensitive for detecting genome than the serum samples, as has been observed previously [16]. Genome was detected in the throat swabs of all challenged animals, including the Dryvax-vaccinated animals. Interestingly, some of these animals had no lesions but did have viral genome detected in their throat swabs. The level of genome detected in the negative control animals (that survived) remained high ($>10^6$) for several days, whereas the 4pox VRP and Dryvax-vaccinated animals only exhibited transient (single time-point) high levels of viremia, or levels remained below 10^6 (Fig. 8). Low levels of genome were detected in the throat swabs of several animals that survived challenge with MPXV even after lesions had resolved. Stittelaar et al. reported similar findings in an addendum to their 2005 paper where they detected genome in throat swabs but not in plasma in four of 12 macaques vaccinated twice with MVA 4 weeks after MPXV challenge [6].

Our VRP studies focused on four poxvirus immunogens: L1, A27, A33, and B5. In our initial mouse studies reported here, we confirmed that all four of these proteins were compatible with the VRP replicon system. All four elicited antibody responses as measured by ELISA, and the two IMV targets (L1 and A27) elicited neutralizing antibodies. As individual vaccines, all four contributed to protection in the mouse model as measured by lethality and weight loss. The most effective at protection was A33R-VRP followed by B5R-VRP, L1R-VRP, and finally A27L-VRP (see Fig. 2E).

A33R

A33 is a glycosylated 23–28 kDa type II integral membrane protein that forms dimers (55 kDa) via intermolecular disulfide bonds [41]. This EEV surface protein has been implicated in the formation of actin-containing microvilli and cell-to-cell spread [41,42]. In our mouse studies involving an intranasal challenge with VACV strain IHD-J, A33R-VRP was completely protective not only against lethality, but also against weight loss. The protection was complete even after only two vaccinations followed by a 2×10^6 pfu challenge (Fig. 2C). Others have

reported that vaccination with the A33R-based DNA vaccines or purified A33 protein plus adjuvant protected mice against VACV; however, there was always significant weight loss [12,13,16]. Similarly, vaccination with purified *E. coli*-expressed VACV A33, or the ectromelia virus ortholog EVM135, protected mice against lethal mousepox, but not against significant weight loss [11]. A33-specific monoclonal and polyclonal antibodies are protective in mouse models suggesting that the humoral response to A33 plays an important role in protection [12,13,15,43,44]. There is ambiguity in regards to the mechanism by which anti-A33 antibodies elicit protection. Cell culture experiments demonstrated that high concentrations of anti-A33 antibodies inhibit EEV spread as measured in comet inhibition assays [13]. Anti-A33 antibodies might also mediate destruction of EEVs [45] and infected cells (Schmaljohn A, personal communication) in a process involving complement. Future work in our laboratory is aimed at identifying the precise mechanism(s) of protection.

There is heterogeneity in A33 across orthopoxviruses and this can affect cross-protective immunity. We recently published a report indicating at least one important conformational A33 B-cell epitope is not conserved between MPXV and VACV and this difference can impact protective immunity [25]. The MPXV A33 ortholog differs from VACV and variola virus at positions 117, 118, and 120 and is not bound by a protective monoclonal antibody that bind both the VACV and variola virus A33. Thus, it is possible that the A33-VRP used in our study was suboptimal as a MPXV immunogen. As we did not vaccinate macaques with the A33R-VRP alone, we could not determine what role, if any, the VRP-A33R contributed to protective immunity. However, it was possible to assay the macaque sera for antibodies that bound the MPXV A33 by ELISA. We evaluated the macaque sera in a MPXV A33 ELISA and found that the animals vaccinated with the pERK or pVEK 4pox had antibodies that bound the MPXV A33 ortholog with titers essentially the same as the anti-VACV A33 titers (data not shown). Interestingly, we included control serum samples from macaques vaccinated with the MPXV A33 ortholog (from an unrelated study) and those sera had high anti-MPXV A33 titers but very low anti-VACV A33 titers. This suggests that there could be one-way cross-reactivity (i.e., antibody response against VACV A33 cross-reacts efficiently with MPXV A33 ortholog but antibody response against MPXV A33 ortholog did not react efficiently against VACV A33). For practical purposes these data indicated that the anti-A33 response elicited by vaccination with VACV A33 VRP did cross-react with the MPXV A33 ortholog and likely did contribute to the observed protective immunity.

B5R

The B5 protein is a 42 kDa glycoprotein found on the surface of the EEV [46,47]. It is involved in cell surface glycosaminoglycan-mediated disruption of the EEV outer membrane [48]. Molecular vaccines comprised of only a B5R DNA vaccine or purified B5 protein partially or completely protected certain animal models [13,16,20,22,49,50]. Monoclonal and polyclonal antibodies to B5 have been shown to neutralize EEV and to inhibit EEV spread [13,49–51]. In fact, a B5-specific chimpanzee/human monoclonal antibody was sufficient to protect mice from lethal disease after passive transfer up to 2 days after challenge [52]. Here, we found that vaccination with the B5R-VRP alone was not as protective in mice as the A33R-VRP; however, there was a significant level of protection observed in the low challenge dose experiment (Fig. 2D–E). Anti-B5 antibody was detected in most mice and macaques vaccinated with the B5R-VRP. However, in the experiment shown in Fig. 6, the anti-B5 GMT in mice vaccinated with the pERK or pVEK tPA-4pox were < 2. The B5R-VRP used in the pERK in the Fig. 6 experiment (and only in that experiment) included a GTX-IRES. The GTX was previously shown to have IRES activity in vitro [53,54], including in the context of pERK B5R (data not shown). However, our experimental data indicated that this sequence rendered the B5R-VRP non-immunogenic in mice. Specifically, eight of eight animals vaccinated with the pERK B5R-VRP did not develop anti-B5 antibody as measured by ELISA (data not shown). This explains

the undetectable anti-B5 response in mice vaccinated with pERK tPA-4pox but it does not explain the low anti-B5 response in the mice vaccinated with pVEK tPA-4pox (Fig. 6). It is possible that B5 expression levels were negatively impacted when delivered in combination with replicons expressing high levels of antigen (e.g., tPA-L1), or it is possible that the pVEK B5R-VRP used in that particular experiment were of lower potency than expected. Lower-than-expected anti-B5 responses were not an issue in the non-human primate experiment. All of the macaques vaccinated twice with the 4pox VRP had anti-B5 titers ≥ 2 . We found that one of five macaques vaccinated with pVEK 4pox and four of five macaques vaccinated with the pERK 4pox developed antibody responses that inhibited the spread of EEV as measured by comet inhibition assay (Fig. 7E). The increased anti-EEV activity in the pERK 4pox group corresponded with higher anti-A33 and anti-B5 ELISA titers. Because the inhibition of spread was not preformed on sera from mice vaccinated with individual A33- and B5-VRPs, we could not determine the relative contribution of the anti-A33 and anti-B5 responses to the EEV inhibitory activity. Based on our own experiences, it is likely that both the anti-A33 and anti-B5 responses contributed to the *in vitro* inhibition of comets, and *in vivo* protective immunity. Recently Benhnia et al. described the development of an EEV-spread inhibition assay that includes complement [55]. In those studies, anti-B5 monoclonal antibodies neutralized EEV in the presence of complement. Those same antibodies also directed complement-mediated killing of infected cells implicating anti-B5 responses in EEV neutralization and destruction of infected cells [55]. In future studies, we will investigate the capacity of sera produced after vaccination with A33 or B5 to direct complement-mediated neutralization of EEV and killing of poxvirus infected cells.

L1R

The orthopoxvirus L1 protein is a 23–29 kDa IMV myristoylated surface protein involved in a yet-to-be-identified post IMV attachment, pre-fusion event [56]. L1 is a target of exceedingly potent IMV neutralizing monoclonal antibodies [57]. When mice or monkeys are vaccinated with plasmids encoding L1 or purified L1 protein plus adjuvant, neutralizing antibodies are produced [12,15,16,40]. Mice vaccinated with cowpox VRP expressing L1 developed neutralizing antibodies as did adenovirus expression of vaccinia virus L1 ([18,22]. Recently, we discovered that DNA vaccination with L1R could be improved (i.e., higher levels of neutralizing antibodies) by the addition of a tPA sequence upstream of L1 [26]. The tPA directs the L1 protein into the ER where, presumably correct folding and disulfide bond formation occurs. These findings were recently supported by another study that targeted L1 to the ER [58]. The intramolecular disulfide bonds are involved in the structure of the conformational epitope bound by monoclonal antibody-7D11, a potent neutralizing antibody. A crystal structure of this epitope, bound by the 7D11FAB, has been solved [38]. Here we demonstrated that adding the tPA sequence to the L1R VRP dramatically enhanced the level of neutralizing antibodies elicited by vaccination in mice. Even after a single vaccination with tPA-L1R VRP, neutralizing antibodies could be detected. This confirms that adding the tPA sequence significantly enhanced the neutralizing antibody response elicited by vaccination with L1R.

A27L

A27 is a 14 kDa protein thought to be involved in an entry event [59]. The VRP that was the least impressive at contributing to protective immunity were those expressing the A27L protein. The relatively poor immunogenicity was especially apparent in the mice vaccinated with the pVEK A27L constructs. The reasons for the reduced immunogenicity of the A27L VRP are not known. The problem does not appear to be universal because some animals did mount a potent anti-A27 response. For example, two of the five macaques vaccinated with pERK 4pox vaccine produced high-titer, anti-A27 antibody, whereas the other three animals were negative. We attempted to improve the immunogenicity of the A27L VRP by including a tPA signal sequence, which resulted in increased secretion of A27 (data not shown). Adding

the tPA sequence to A27L in the pVEK system had negligible effect. However, adding the tPA sequence to A27L in the pERK system resulted in a modest, but significant, increase in the neutralizing antibody response (i.e., the GMT PRNT₅₀ titers for pERK A27L and pERK tPA-A27L were 27 and 190, respectively [data not shown]). For this reason the tPA-A27L VRP were used in the non-human primate study. Further improvement of the performance of the A27 immunogen is a focus of ongoing work.

Two replicon systems were used in this study: pERK and pVEK. The pERK system is based on the vector originally described by Pushko et al [31] that has been modified for optimal gene of interest expression [32]. The pERK replicon system is based on the attenuated V3014 strain of VEE virus [60] and has been used in preclinical studies for more than a decade in animals ranging from mice to nonhuman primates [39,61–68]. In addition, VRP generated with the pERK system have been evaluated in clinical trials and have been shown to be both safe and immunogenic in humans (Smith and Kamrud unpublished data). The pVEK replicon system is based on the vaccine strain of VEE virus (TC-83). The TC-83 virus vaccine is not FDA licensed but has been used in thousands of human volunteers over more than a 30-year time span [69,70]. Our non-human primate data indicate that VRP derived from both the pERK and pVEK systems induce immune responses capable of protecting animals against monkeypox challenge suggesting that either system would be effective for development of a smallpox vaccine.

In summary, the currently licensed smallpox vaccine is an infectious human pathogen. For healthy individuals, this pathogen causes no more than a skin lesion, generally a scar and possibly some minor discomfort including enlarged lymph nodes and fever. However, for individuals with compromised immune systems or skin disorders, this pathogen can cause serious disease and even death. In both cases, the live-virus can be transmitted to a person with close contact to the vaccinee and this can lead to serious, even life-threatening complications in the unintended recipient. While the need for an orthopoxvirus vaccine is generally perceived to be a requirement for protection against potential biological weapons, MPXV and other orthopoxviruses continue to naturally occur throughout the world. These factors combined with the biological weapons threat, implore the development of safe and effective orthopoxvirus vaccines. Alternative vaccines and vaccination strategies are needed if we are to effectively defend against intentional re-emergence of smallpox. Hyper-attenuated vaccinia virus vaccines such as MVA are one possible solution; however, an alternative solution is the use of highly defined gene- or protein-based molecular vaccines such as the 4pox VRP described here. A more defined vaccine has the benefit of permitting the future detection of non-vaccine target orthopoxvirus antibodies. In this regard, and similar to subunit vaccines against different viruses used on livestock, the capability of detecting exposure to orthopoxviruses would still remain. Such a capability may pose a particular necessity in the defense against biological weapons where it would be advantageous to know if populations, albeit vaccinated and protected, were being accidentally or purposely exposed to pathogenic orthopoxviruses. We have shown that only two vaccinations with a VRP-based 4pox vaccine platform protected 10 of 10 macaques against severe monkeypox after a lethal challenge. Thus, a VRP-based subunit vaccine has the ability to replace the current live-virus orthopoxvirus vaccine. Future studies investigating construct and dose optimizing strategies will be performed to further enhance the potency of this promising candidate future-generation smallpox vaccine.

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Figure 1. Expression of L1, A27, A33 and B5 in VRP infected cells

Equivalent sample protein concentrations from cell lysates were heated with SDS sample buffer with (A27, B5, and A33) and without (L1) reducing reagent, resolved by SDS-PAGE, and transferred to PVDF membranes using the X-cell II blotting system and NuPage buffer (Invitrogen). A27, A33, and L1 were detected with monoclonal antibodies specific for each VACV protein, MAb-4B4, MAb-1G10, MAb-10F5, respectively. B5 was detected with sera from rabbits vaccinated with B5R-VRP. The secondary antibody was anti-mouse or rabbit IgG conjugated to AP. Molecular mass markers are shown (kDa) on the left of each blot. tPA-A27L VRP and tPA-L1R VRP and the observed difference in the level of detected protein in the -tPA and +tPA for L1R is described later in the results section.

Figure 2. VACV challenge of VRP vaccinated mice

(A) Experimental design. Groups of eight mice were vaccinated two or three times with VRPs expressing L1, A27, A33, B5, or a combination of all four VRP. Negative control VRPs were used to vaccinate mice in groups 6 and 13. Scarified mice in group 7 were used as positive controls. Some mice did not survive the vaccination/bleed procedure. The final numbers of animals in each group that were challenged were: Group 1 $n=0$, Group 2 $n=5$, Group 3 $n=7$, Group 4, 5, and 7 $n=8$, Group 6 $n=7$. Survival and weight loss in mice vaccinated with VRP three times (B) or two times (C) and challenged intranasally with a high dose of VACV. (D) Weight loss in mice vaccinated with VRP three times and challenged with a low dose of VACV. (E) Weight loss in mice vaccinated two times and challenged with a low dose of VACV. The

percent weights of individual surviving mice were calculated relative to starting weights (day 0). Each symbol represents the mean $\pm$ standard deviations for each group on the indicated day. ANOVA with stepdown Bonferroni adjustments for multiple pairwise comparisons were performed to identify significant weight differences between groups on each day after challenge. Significant differences between groups are noted in the results section. The poison symbol indicates that all of the mice in the labeled group succumbed to infection.

Figure 3. Antibody responses against B5, A33, A27 and L1 in mice vaccinated with individual VRPs Sera from mice vaccinated two or three times as shown in Fig. 2A, groups 1–4, were evaluated by **A.** VACV infected-cell lysate ELISA and **B.** immunogen-specific ELISA using purified L1 (300 mg/well), B5 (50 ng/well), A27 (50 ng/well), and A33 (50 ng/well). Endpoint titers for individual mice were determined. Bars represent the geometric mean titer (GMT) for each group. **C.** Sera from individual mice in groups 1–4 (Fig. 2A) were evaluated for neutralizing antibodies by VACV PRNT. PRNT₅₀ GMT for groups vaccinated 2 or 3 times were plotted. Only the L1R-VRP group elicited neutralizing antibodies with a GMT greater than 40 (limit of detection for this assay). Samples with titers <40 were given a value of 20 for GMT calculation. VACV infected cell-lysate ELISA, immunogen specific ELISA, and VACV PRNT titers between groups of subjects receiving 2 or 3 vaccinations were compared (*t*-tests). An asterisk indicates difference in GMT between 2 and 3 vaccinations was significant (*p*-value was < 0.05).

Figure 4. Antibody responses against B5, A33, A27 and L1 in mice vaccinated with 4pox combination VRPs

Mice were vaccinated with the 4pox VRP as shown in Fig. 2A. Sera collected after two (A.) and three (B.) vaccinations was examined by immunogen-specific ELISA and VACV-infected cell lysate ELISA. Bars represent the GMT for each group. ELISA titers between groups vaccinated 2 times (A) or 3 times (B) were compared (*t*-tests). An asterisk indicates difference in GMT between 2 and 3 vaccinations was significant *p*-value was < 0.05). C. The presence of neutralizing antibodies in sera from mice vaccinated 3 times with 4pox VRPs or once by scarification was evaluated by VACV PRNT. Bars represent the GMT for each group. The lowest dilution tested was 1:40. Samples with titers <40 were given a value of 20 for GMT

calculation. T-tests of log-transformed PRNT₅₀ titers between groups of subjects receiving different vaccine types with stepdown Bonferroni adjustments for multiple comparisons were performed. An * indicates the difference in GMT was significant.

Figure 5. VACV challenge of mice vaccinated with 4pox immunogens expressed by different VRP vector systems

A. Groups of 8 mice were vaccinated once or twice with VRPs expressing the 4pox immunogens derived from the indicated vector (pERK or pVEK) at two different doses (low dose: 1×10^6 or high dose: 1×10^7 VRP). Vaccinated animals were subsequently challenged with 2×10^6 pfu of VACV strain IHD-J. **B.** Weights of individual VACV-challenged mice were monitored for 14 days after infection. The percent weights of surviving mice were calculated relative to starting weights (day 0) and plotted. Also shown is the percent survival for each group. **C.** Antibody titers for the 4pox immunogens were examined by immunogen-specific ELISA. Bars represent values for pooled sera for each group. **D.** Neutralizing antibody

titers on serum collected 1 week after two or three vaccinations were determined by VACV PRNT. Bars represent values for pooled sera for each group.

Figure 6. Characterization of the protective efficacy of a tPA-L1R VRP as compared to unmodified L1R VRP

A. Experimental design. Two experiments were performed. In the first experiment, groups of eight mice were vaccinated twice with VRP contain unmodified L1 or tPA-L1. In the second experiment, groups of eight mice were vaccinated with 4pox VRP or tPA-4pox VRP where both L1 and A27 were modified by addition of tPA and then challenged. **B.** L1 ELISA and **C.** VACV PRNT were performed on sera from mice in groups 1–4. GMT were determined and are represented by bars. Comparisons between the titers elicited by the different vaccines after 1 vaccination were made. An asterisk indicates that the difference in GMT was significant (*t*-test *p*-value < 0.05). Differences in ELISA and PRNT GMT after 2 vaccinations were not

significant (t -test p -value > 0.05). **D.** Vaccinated animals in groups 5–8 were challenged with 2×10^6 pfu of VACV strain IHD-J. Survival and weight loss data are shown. The weights of individual VACV-challenged mice were determined relative to their weight on day 0. Each symbol represents the mean $\pm$ standard deviation for each group. No statistical difference in weight loss was detected between pERK tPA-4pox and pVEK tPA-4pox groups (ANOVA p value > 0.1). All of the mice in the negative control groups lost weight and died or were euthanized (poison symbol). **E.** Immunogen-specific ELISA were performed on sera collected 1 week after the second vaccination (week 4) from mice in groups 5–8. Bars represent the GMT for each group. No statistical differences in ELISA titers were detected between pERK tPA-4pox and pVEK tPA-4pox groups after 2 vaccinations (t -test p -value > 0.05). **F.** VACV PRNT were performed on sera after 1 (week 3) or 2 (week 4) vaccinations. GMT were calculated using a value of 10 for samples with a titer less than 20. Comparisons between the GMT between groups of subjects receiving different vaccine types after 1 (week 3) or 2 (week 4) vaccinations were compared. An asterisk indicates that the difference in GMT was significant (t -test p -value was ≤ 0.002). For each vaccine there was a highly significant increase in the PRNT between weeks 3 and 4 (t -test p -value < 0.0001).

Figure 7. Immune responses in macaques vaccinated with VRPs expressing 4pox immunogens

A. Experimental design. Groups of five macaques were vaccinated twice with pERK or pVEK VRPs expressing the 4pox immunogens (tPA-L1R and tPA-A27L used). As negative control, groups of three macaques were vaccinated with VRPs expressing flu HA. As a positive control, two macaques were vaccinated once by Dryvax scarification. All animals were challenged 4 weeks after the last VRP vaccination. **B.** Antibody titers from individual vaccinated macaques against all four antigens were measured by immunogen-specific ELISA. Prebleed sera (day 0), and sera from weeks 2, 4, 6, and 8 were tested. GMT for week 8 for each group are shown. A value of 1 was given to samples with titers below detection. Repeated Measures ANOVA of log₁₀-transformed ELISA titers between groups was performed for each antigen over time,

and an ANOVA with stepdown Bonferroni adjustment for multiple pairwise comparison was used to detect group differences for each week. Significant differences (p -value < 0.05) between groups 1 and 3, between groups 2 and 4, and between group 5 both negative control groups 3 and 4, are shown with an asterisk. There was no significant difference between the 4pox-vaccinated (groups 1 and 2) and Dryvax-vaccinated animals (group 5). Individual animal identifiers are shown under the x-axis. **C.** Neutralizing antibody responses for each animal on weeks 0, 2, 4, 6, and 8 were evaluated by VACV PRNT. PRNT₅₀ titers are shown for individual macaques at each time point. The lowest dilution tested was 1:40. Samples with titers <40 were given a value of 20 for GMT calculation. The GMT for week 8 for each group are shown. Group comparisons and statistical analysis were performed as described in B above. **D.** MPXV PRNT was performed on the week 8 sera from each animal. GMT for each group are shown. An ANOVA with stepdown Bonferroni adjustments for multiple pairwise comparison was used to detect group differences. There was no significant difference between the 4pox-vaccinated (groups 1 and 2) and Dryvax-vaccinated animals (group 5). **E.** A comet assay, as described in Methods, was performed to evaluate the capacity of week 6 and 8 sera to inhibit the spread of VACV EEV in cell culture. All sera were tested in duplicate. Comet inhibition was scored blindly using a scale of 1–3 (where a score of 1 indicated no inhibition of spread, and a score of 3 indicated maximum inhibition). A representative plate with scores is shown. For each sera sample, duplicate scores were averaged and plotted.

Figure 8. Vaccination with VRP 4pox vaccine reduces circulating virus in macaques

A. Whole blood samples or **B.** throat swab samples collected at the time of MPXV challenge and at 3-day intervals thereafter were evaluated for the presence of MPXV genomes by quantitative Real-Time PCR. Viremia is plotted as genome copies per ml of blood, or throat swab suspension. An ANOVA was used to detect differences between groups on each day. Significant (p -value < 0.0182) differences between groups 1 and 3, and between groups 2 and 4 are indicated by an *.

Table 1

PCR primers used to amplify VACV to clone into VEE replicon vectors

Source of VACV gene	5' Forward primer 3'	5' Reverse primer 3'
pWRG/A33R	GCTCTAGAAATGATGACACCAGAAAACGACG	GCTCTAGATTAGTTCATTGTTTTAACAC
pWRG/A27L	GCTCTAGAAATGGACGGAACCTTTTCCCG	GCTCTAGATTACTCATATGGACGCCGTCCAG
pWRG/B5R	GCTCTAGAAATGAAAACGATTTCCGTTGTTAC	GCTCTAGATTACGGTAGCAATTTATGGAAC
pWRG/L1R	GCTCTAGAAATGGGTGCCGCAGCAAGCATAC	GCTCTAGAAATCAGTTTTGCATATCCGTGGTAG
pWRG/tPA-L1R	GTCTAGAATGGATGCAATGAAGAGAGGG	CTCTAGACTCAGTTTTGCATATCCGTGGTAGC

Table 2

Treatment groups and experimental design for nonhuman primate study

Group	VRP vaccine	Route	Dose	Vaccination schedule (Day)	Challenge (Day)*
1	pVEK 4pox 5	i.m.	1×10 ⁸ IU each VRP	0 and 28	56
2	pERK 4pox 5	i.m.	1×10 ⁸ IU each VRP	0 and 28	56
3	pVEK flu HA 3	i.m.	1×10 ⁸ IU	0 and 28	56
4	pERK flu HA 3	i.m.	1×10 ⁸ IU	0 and 28	56
5	Drvvax 2	Scarification	15 pricks	0	56

* postvaccination

Table 3

Results of MPXV iv challenge

Monkey ID	Group	Vaccine	Peak Lesion #	Blood viremia (genomes/ml)	Throat swab viremia (genomes/ml)	Disease	Day of Death
4306	1	pVEK 4pox	8	<	569,000	mild	Survived
4307	1	pVEK 4pox	12	<	36,180	mild	Survived
4308	1	pVEK 4pox	10	<	1,837,500	mild	Survived
4309	1	pVEK 4pox	8	<	247,200	mild	Survived
4310	1	pVEK 4pox	0	<	8,770	none	Survived
4311	2	pERK 4pox	2	<	840,500	mild	Survived
4312	2	pERK 4pox	8	<	390,000	mild	Survived
4313	2	pERK 4pox	4	<	25,370	mild	Survived
4314	2	pERK 4pox	0	<	1,123,000	none	Survived
4315	2	pERK 4pox	12	<6500	10,306,890	mild	Survived
4316	3	pVEK flu	TNTC [†]	338,900	13,770,000	grave	9
4317	3	pVEK flu	105	1,044,990	25,360,000	severe	Survived
4318	3	pVEK flu	TNTC	660,000	12,540,000	severe	Survived
4319	4	pVEK flu	TNTC [†]	981,000	83,100,000	grave	9
4320	4	pVEK flu	TNTC [†]	9,915,000	4,250,000	grave	12
4321	4	pVEK flu	TNTC	5,160,000	3,351,000	grave	12
4322	5	Drvxax	0	<	84,800	none	Survived
4323	5	Drvxax	0	<	136,500	none	Survived

[†] mean of two highest consecutive time points

^{††} moribund animal euthanized

na, not applicable

TNTC= Too numerous to count

< no genome detected (<5000 genomes/ml)