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Assessment of the Protective Effect of Imvamune and Acam2000 Vaccines against Aerosolized Monkeypox Virus in Cynomolgus Macaques

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To support the licensure of a new and safer vaccine to protect people against smallpox, a monkeypox model of infection in cynomolgus macaques, which simulates smallpox in humans, was used to evaluate two vaccines, Acam2000 and Imvamune, for protection against disease. Animals vaccinated with a single immunization of Imvamune were not protected completely from severe and/or lethal infection, whereas those receiving either a prime and boost of Imvamune or a single immunization with Acam2000 were protected completely. Additional parameters, including clinical observations, radiographs, viral load in blood, throat swabs, and selected tissues, vaccinia virus-specific antibody responses, immunophenotyping, extracellular cytokine levels, and histopathology were assessed. There was no significant difference ($P > 0.05$) between the levels of neutralizing antibody in animals vaccinated with a single immunization of Acam2000 (132 U/ml) and the prime-boost Imvamune regime (69 U/ml) prior to challenge with monkeypox virus. After challenge, there was evidence of viral excretion from the throats of 2 of 6 animals in the prime-boost Imvamune group, whereas there was no confirmation of excreted live virus in the Acam2000 group. This evaluation of different human smallpox vaccines in cynomolgus macaques helps to provide information about optimal vaccine strategies in the absence of human challenge studies.

Variola virus, the etiological agent of smallpox, is highly contagious and causes disease with a high mortality rate (1). Endemic smallpox was eradicated through a successful global immunization campaign by the World Health Organization more than 30 years ago (2), with the final natural case of smallpox recorded in Somalia in 1977 (3). Since the eradication, widespread vaccination against this pathogen has been discontinued, and so the majority of the world's population currently lacks protective immunity (4). As a consequence, the use of variola virus as a biological weapon poses a current major public health threat. Other orthopoxviruses, for example, human monkeypox, cowpox virus, and a variety of vaccinia virus-like viruses (5–8), also threaten public well-being. These orthopoxviruses are naturally occurring and usually spread to human beings by zoonotic infection. Since all of these orthopoxviruses pose a risk to public health, there is a renewed effort to develop and stockpile medical countermeasures such as safe, effective orthopoxvirus vaccines and therapeutic agents.

The traditional calf-lymph derived, smallpox vaccines (e.g., Dryvax) used in the eradication of smallpox are based on replicating vaccinia virus. They are highly efficacious; however, their use is associated with rare but severe side effects, particularly in immunocompromised individuals (9, 10). Adverse events include progressive vaccinia, eczema vaccinatum, myo/pericarditis, Stevens-Johnson syndrome, fetal vaccinia, encephalitis, and occasionally death (11). Second-generation smallpox vaccines, for example, Acam2000, have subsequently been developed and licensed. These vaccines are produced using the Lister-Elstree or New York City Board of Health vaccinia virus strains in qualified cell cultures according to Good Manufacturing Practice standards (12, 13). Although these qualified vaccine preparations are cleaner and appear to be as effective as earlier vaccines, there are still adverse events following vaccination (11). Thus, if these vaccines were used today, in a public health emergency, it is estimated that 25% of the general population would be at risk of developing complications (14).

Third-generation smallpox vaccines, such as Imvamune, manufactured by Bavarian Nordic (Martinsried, Germany), are currently being developed as safe and effective vaccines without the complications associated with traditional smallpox vaccines (15). Imvamune is based on a strain of the modified vaccinia Ankara (MVA) virus, which is a highly attenuated, replication-deficient strain of vaccinia virus. It was generated by more than 500 passages of vaccinia virus in chicken embryo fibroblasts, during which time it acquired multiple deletions and mutations and lost the capacity to replicate efficiently in people and most mammalian cells (16). In Germany, in the 1970s, MVA was tested in ~120,000 people. It was given as a preimmunization vaccine in combination with the Lister vaccine (a second-generation vaccine). Several high-risk groups were vaccinated, including young children with skin conditions (15–18), and there were no reports of serious adverse events using this two-step inoculation process (15).

It is not feasible to assess the protective efficacy of single or multiple doses of Imvamune vaccine in phase III human clinical trials because smallpox is no longer endemic in any part of the

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world. In order to progress licensing of effective medical countermeasures for biodefense, such as Imvamune, the U.S. Food and Drug Administration (FDA) has published the "Animal Rule" (19). This rule permits the approval or licensing of drugs and biological compounds based upon results obtained from an animal model that appropriately replicates the human condition. In the past, macaques have been used in studies employing both variola virus and monkeypox virus in order to model the ordinary disease presentation of smallpox infection in people (1). Since there are difficulties with working with variola virus, monkeypox virus infection in macaques has now become an acceptable surrogate model for human smallpox disease (20, 21), provided an appropriate dose and route of challenge such as aerosolization is used (1). Thus, this animal model is supported by the FDA and may provide valuable information on vaccine efficacy that could be used to aid licensing.

The purpose of the present study was to evaluate the protective effect of either a single dose of Imvamune, a prime and a boost of Imvamune, or a single dose of the licensed vaccine Acam2000 against disease following an aerosolized severe or lethal dose of the central African strain (Zaire 79) of monkeypox virus in cynomolgus macaques. Humoral and cell-mediated responses to vaccination were also examined.

MATERIALS AND METHODS

Experimental animals. Twenty-four captive bred, healthy, cynomolgus macaques (*Macaca fascicularis*) of Mauritian origin (12 male and 12 female) were obtained from a United Kingdom breeding colony for use in the present study. All of the animals weighed between 2.5 and 4.5 kg and were between 2 and 4 years of age at challenge. The monkeys were negative for neutralizing antibodies to orthopoxvirus prior to the start of the study. Animals were housed according to the United Kingdom Home Office *Code of Practice for the Housing and Care of Animals Used in Scientific Procedures* (1989) and the National Committee for Refinement, Reduction, and Replacement (NC3Rs) *Guidelines on Primate Accommodation, Care and Use, August 2006*. If a procedure required the removal of a primate from a cage it was sedated by intramuscular (i.m.) injection with ketamine hydrochloride (10 mg/kg; Ketaset, Fort Dodge Animal Health, Ltd., Southampton, United Kingdom). All procedures were conducted under a Project License approved by the Ethical Review Process of the Health Protection Agency, Salisbury, United Kingdom, and the United Kingdom Home Office. None of the animals had been used previously for experimental procedures.

Vaccines. Acam2000 Smallpox (vaccinia) vaccine was obtained from Acambis, Inc., Cambridge, MA. The freeze-dried vaccine was reconstituted in 0.3 ml of diluent, according to the manufacturer's instructions. Imvamune, modified vaccine virus Ankara-BN (MVA-BN), was manufactured by IDT Biologika GmbH (Germany) and was supplied by Bavarian Nordic A/S, Denmark, as a homogenous suspension. It was diluted in the diluent provided (Tris-buffered saline [TBS]) to give a final concentration of 2×10^8 50% tissue culture infective doses ($TCID_{50}$)/ml. The negative control for the experiment was TBS, the diluent used for the Imvamune vaccine.

Four treatment groups of six cynomolgus macaques were established. The first group of animals (TBS negative control) were inoculated with 0.5 ml of TBS 28 days prior to challenge. The second group of animals (Acam2000 ×1) were vaccinated with one dose of Acam2000 vaccine (2.5×10^5 to 12.5×10^5 PFU) at the same time. Both the TBS and the Acam2000 vaccines were delivered by scarification to the midscapular area with the use of a bifurcated-end needle. In the third group (Imvamune ×1), animals were vaccinated once with Imvamune (10^8 $TCID_{50}$ in a total volume of 0.5 ml) 28 days prior to challenge via the subcutaneous route. In the fourth group (Imvamune ×2), animals were vaccinated via the subcutaneous route with an Imvamune primer dose of 10^8 $TCID_{50}$ in a 0.5-ml total volume, 56 days prior to challenge, and an Imvamune booster dose (10^8 $TCID_{50}$ in a 0.5-ml total volume) 28 days prior to challenge. The distribution of male and female macaques in the study was as follows: the TBS negative control animals were male ($n = 6$), the Acam2000-vaccinated animals were female ($n = 6$), the Imvamune ×1-vaccinated animals were male ($n = 6$), and the Imvamune ×2-treated animals were female ($n = 6$). Each group of animals was kept separate to avoid cross contamination and/or spreading of the vaccine.

Monkeypox virus challenge strain. Monkeypox virus strain Zaire 79, NR-2324, was obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA). On the day of challenge, stocks of virus were thawed and diluted appropriately in minimum essential medium containing Earl's salts (Sigma, Poole, United Kingdom), 2 mM L-glutamine (Sigma), and 2% (vol/vol) fetal calf serum (Sigma).

Aerosol exposure and sampling. Monkeys were challenged with a target dose of 10^5 PFU of monkeypox virus using the AeroMP-Henderson apparatus; a flexible, highly configurable system in which the challenge aerosol was generated using a six-jet Collison nebulizer (BGI, Waltham, MA). The aerosol was mixed with conditioned air in the spray tube (22) and delivered to the nose of each animal via a modified veterinary anesthesia mask. Samples of the aerosol were taken using an SKC BioSampler (SKC, Ltd., Dorset, United Kingdom) and an aerodynamic particle sizer (TSI Instruments, Ltd., Bucks, United Kingdom); these processes were controlled and monitored using the AeroMP management platform (Biaera Technologies, LLC, Frederick, MD). To enable delivery of consistent doses to individuals each animal was sedated and placed within a "head-out" plethysmograph (Buxco, Wilmington, NC). The aerosol was delivered simultaneously with a measurement of the respiration rate. A back titration of the aerosol samples taken at the time of challenge was performed to calculate the presented/inhaled dose. The challenge was performed on 2 days and the mean presented dose on each day was 2.1×10^5 and 3.1×10^5 PFU/animal (the overall mean presented dose was 2.6×10^5 PFU).

Antibody concentrations, cellular-immune populations, and cytokines were monitored in the blood pre- and postchallenge. After challenge, the viral loads were monitored in the blood and throat. For the latter, a flocked swab (Copan Diagnostics, Murrieta, CA) was gently stroked six times across the back of the throat in the tonsillar area.

Lung imaging. Thoracic, dorsoventral, and ventrodorsal radiographs (SP VET 3.2; Xograph Imaging Systems, Ltd., Tetbury, United Kingdom) were acquired at day 9 postchallenge using Xograph mammography film. Lung pathology was evaluated by two consultant thoracic radiologists blinded to the animals' vaccination and clinical status, using a predetermined scoring system.

ELISA. Samples of blood were taken at various time points throughout the study. Serum was isolated and assayed for immunoglobulin G (IgG) serum antibodies to vaccinia virus using an enzyme-linked immunosorbent assay (ELISA). Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with a preparation of commercially prepared psoralen/UV-inactivated, sucrose density gradient-purified vaccinia virus (Lister strain; Autogen Bioclear UK, Ltd., Wiltshire, United Kingdom) in calcium carbonate buffer at 2.5 μg/ml. Unbound antigen was removed by washing the plates three times. The plates were blocked with blocking buffer (phosphate-buffered saline [PBS], 5% milk powder [Sigma], 0.1% Tween 20 [Sigma]) for 1 h at room temperature with shaking. Unbound blocking solution was removed by washing three times. Fourfold serially diluted serum samples (starting at 1:50) were added to the plate for 2 h at room temperature with shaking. Unbound antibodies were removed from the plate by three washes. The plates were then incubated for 2 h with shaking with horseradish peroxidase-labeled anti-monkey-IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Unbound detection antibody was removed by five washes and then developed using an ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)] peroxidase

substrate system (Kirkegaard & Perry). The development of the ELISA was stopped using the ABTS stop solution (Kirkegaard & Perry). ELISA titers were calculated and compared to a vaccinia virus immune globulin standard (BEI Research Repository Resource, Manassas, VA), which was used to convert the titer into arbitrary international units (AIU)/ml.

Flow cytometry. Whole blood was collected at time points throughout the study by using heparin as the anticoagulant. Antibodies to CD3e, CD4, CD20, and CD16 (BD Biosciences, Oxford, United Kingdom) and to CD8a (Invitrogen, United Kingdom) conjugated to R-phycoerythrin (PE)-cyanine dye (Cy7), allophycocyanin, PE, fluorescein isothiocyanate, and PE-Texas Red, respectively, were incubated with the blood for 30 min at room temperature. The red blood cells were removed from the whole blood by lysing them with Uti-Lyse reagent (Dako, Cambridgeshire, United Kingdom). Flow count beads (Beckman Coulter, High Wycombe, United Kingdom) were added to provide a standard to enable cell counts per μl of blood, before being acquired on the flow cytometer. The data were collected on an FC500 flow cytometer (Beckman Coulter) and analyzed with CXP analysis version 2.1 software (Applied Cytometry Systems).

Luminex analysis of cytokines. The concentrations of interleukin-6 (IL-6) and gamma interferon (IFN-γ) were determined in serum samples using a NHP 23 Plex kit (Merck Millipore, Billerica, MA) according to the manufacturer's instructions. Samples were acquired using a Luminex 200 system (Luminex, Austin, TX), and the data were analyzed using the Xponent software (version 3.0). The concentration of each cytokine in the serum was calculated based on a comparison with the corresponding standard curve generated using purified cytokines from the kit.

Monkeypox virus plaque assay. During the course of the study, EDTA-treated blood and throat swabs were collected and frozen at −80°C and, at necropsy, tissues were collected and snap-frozen in liquid nitrogen. Prior to testing, the tissue was thawed and homogenized in PBS by using a Precellys24 tissue homogenizer (Bertin Technologies, Villeurbanne, France). The titers of live infectious virus in the tissues, blood, and throat swabs were determined by plaque assay. Samples were incubated in 24-well plates (Nunc/Thermo Fisher Scientific, Loughborough, United Kingdom) with Vero E6 (ATCC CRL-1586; American Type Culture Collection, Manassas, VA) cell monolayers under MEM (Life Technologies, Foster City, CA) containing 1.5% carboxymethyl cellulose (Sigma), 5% (vol/vol) fetal calf serum (Life Technologies), and 25 mM HEPES buffer (Sigma). After incubation at 37°C for 72 h, the samples were fixed overnight with 20% (wt/vol) formalin-PBS, washed with tap water, and stained with methyl crystal violet solution (0.2% [vol/vol]; Sigma).

PRNT assay. Samples of blood were collected at designated time points prior to challenge and neutralizing, anti-vaccinia virus antibody titers were measured by plaque reduction neutralization (PRNT) assay. Heat-inactivated sera (56°C for 30 min) were serially diluted and incubated with ~50 PFU of wild-type Lister-Elstree vaccinia virus for 1 h at 37°C in 5% CO_2. The samples were then incubated with Vero E6 monolayers using the method described above. The neutralizing antibody titers were defined as the serum dilutions resulting in a 50% reduction relative to the total number of plaques counted without antibody, according to the Behrens-Karber formula (23). Titers were standardized to a standard preparation of human Vaccinia Immune Globulin CNJ-016 (BEI Research Repository Resource).

Virus detection by quantitative PCR. Tissue samples collected postchallenge and snap-frozen in liquid nitrogen were defrosted and homogenized in PBS using a Precellys24 tissue homogenizer. Viral DNA was isolated from homogenates by using a tissue kit (Qiagen, Crawley, West Sussex, United Kingdom) according to the manufacturer's instructions. Blood and throat swabs were processed using a Qiagen blood DNA minikit according to the manufacturer's instructions. Real-time PCR was performed using an Applied Biosystems 7500 Fast instrument (Life Technologies) with an in-house TaqMan assay targeted at the viral hemagglutinin (HA) gene and residues in the Z79 genome (GenBank accession no. HQ857562.I [V79-I-005]; monkeypox virus, residues 158734 to 158798, inclusive).

Clinical and euthanasia observations. The clinical observations were scored, and routine in-house welfare assessments were made at regular intervals pre- and postchallenge. These included measurements of the rectal temperature and body weight. These parameters also fed into a euthanasia scoring scheme. Both clinical and euthanasia scoring schemes used a scale to indicate severity (0 = none, 1 = mild, 2 = substantial, and 3 = intense). Clinical observation score sheets were used to record anorexia, behavioral changes (depression/unresponsiveness/repetitive activity), nasal discharge, cough, dyspnea, and rash/skin swelling, whereas euthanasia score sheets were used to record appearance and provoked and natural behavior. The criteria for immediate euthanasia included signs of severe systemic infection, >20% loss in body weight, convulsions, hemorrhagic rash, and persistent prostration. Postchallenge detailed clinical and euthanasia assessments were made on all animals every four to 6 h until recovery, at which time the frequency was reduced to twice daily.

Euthanasia and necropsy procedures. Animals were sedated with ketamine hydrochloride (10 mg/ml, i.m.; Fort Dodge Animal Health, Ltd.). Anesthesia was deepened using intravenous pentobarbitone sodium at 30 mg/kg (Sagatal; Rhone Merieux), and exsanguination was effected via the heart, before termination by injection of an anesthetic overdose (Dolelethal, 140 mg/kg; Vetquinol UK, Ltd.). A full necropsy was performed immediately to provide tissues.

Pathological studies. At necropsy, gross observations, including skin lesions, were recorded, and samples were collected of all lung lobes, trachea, heart, liver, kidneys, spleen, tongue, tonsil, esophagus, stomach, ileum, descending colon, lymph nodes (tracheobronchial, axillary, mesenteric, mandibular, and inguinal), adrenal gland, ovary or testis, skin (with or without lesion), and brain. The samples were placed in 10% neutral buffered formalin; fixed tissues were then processed routinely to paraffin wax, and sections cut at 5 μm and stained with hematoxylin and eosin (H&E).

Statistical analysis. Flow cytometry data and antibody titers as measured by ELISA and PRNT assays were compared across treatments using one-way Mann-Whitney tests. A Pearson product-moment correlation was performed on the transformed ($\log_{10}$) real-time PCR data set and the transformed ($\log_{10}$) PFU/ml data set for the blood and throat samples. All statistical analyses were performed using Minitab version 15.1. Differences were considered significant at P values of <0.05.

RESULTS

Local effects at site of vaccination. Red patches were observed on all animals at the vaccination site 4 days postvaccination by scarification with Acam2000. These developed into raised scabbed sites ~10 mm in diameter by day 6 postvaccination. Dry scabs persisted for ~3 weeks postscarification. No reactive signs were detected at the vaccination sites of any Imvamune-vaccinated animals. Similarly no vaccination-specific marks were seen on the TBS negative control animals.

Vaccine-induced humoral immune responses. Sera from MVA vaccinated (Imvamune), vaccinia-virus vaccinated (Acam2000), and TBS negative control animals were tested with a PRNT assay against one antigen, wild-type Lister-Elstree vaccinia virus to determine the levels of vaccinia virus-specific neutralizing antibodies (Fig. 1a) prior to challenge. Antibodies were induced and continued to rise after vaccination with Acam2000 (Fig. 1a), with a maximum median titer of 132 U/ml 6 days prior to challenge. Significantly lower levels ($P < 0.01$) of neutralizing antibodies were detected by PRNT assay in animals vaccinated with a single dose of Imvamune (13 U/ml, 6 days prior to challenge). Animals that received a second boost of Imvamune showed a rise in neutralizing antibodies after the booster vaccination (Imvamune

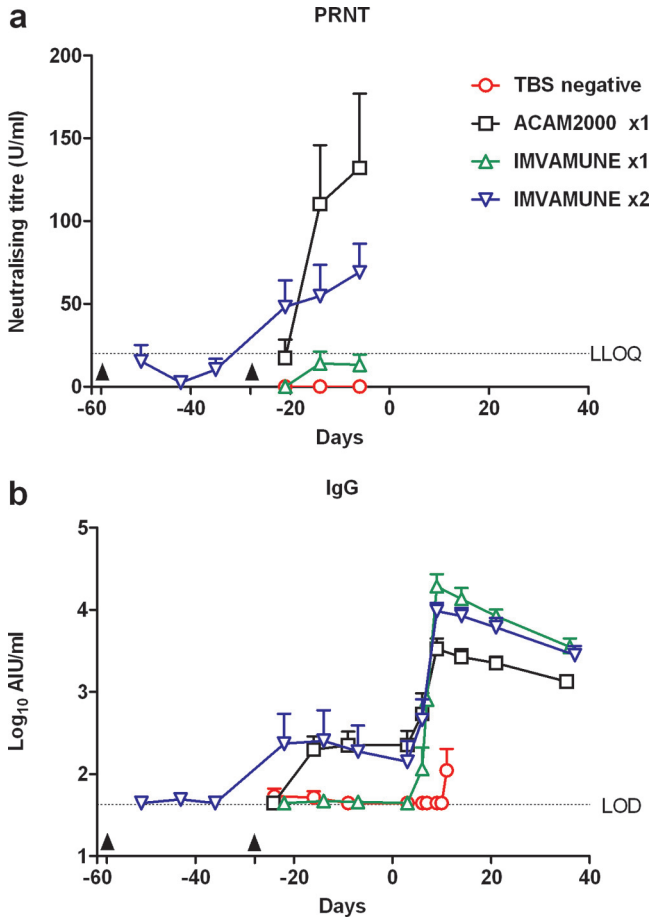

FIG 1 Vaccine-induced humoral immune responses. (a) Vaccinia virus-specific neutralizing serum antibody titres (median + 1 standard error [SE], $n = 6$) in samples collected on different days during the study, measured by PRNT assay. (b) Development of specific serum IgG responses in samples collected pre- and postchallenge, measured by ELISA (mean + 1 SE, $n = 6$), day 0, time of challenge with monkeypox. TBS negative, TBS negative control group; closed triangles, times of immunization; LOD, limit of detection; LLOQ, lower limit of quantification.

×2). This reached 69 U/ml 6 days prior to challenge and was significantly higher ($P < 0.05$) than the titer in single-dose Imvamune group. Although higher concentrations of neutralizing antibody were detected in the Acam2000 group, this was not significantly different from the amount of antibody detected in the two dose Imvamune group ($P > 0.05$) (Imvamune ×2) (Fig. 1a).

The levels of circulating IgG antibodies were detected by ELISA pre- and postchallenge using only one antigen, vaccinia virus. Prior to challenge, no rise in IgG antibody was seen in the TBS negative control or the single Imvamune dose group. In the Acam2000 group after vaccination, IgG rose to 2.4 $\log_{10}$ AIU/ml, 9 days prior to challenge (Fig. 1b). A similar rise in antibody was seen after the second dose of Imvamune in animals (Imvamune ×2) (2.3 $\log_{10}$ AIU/ml, 7 days prior to challenge) (Fig. 1b). After infection, the kinetics of the antibody responses for the Acam2000, Imvamune ×1, and Imvamune ×2 groups were similar; large increases in antibody titer from baseline levels were found which peaked at day 9 postchallenge and then decreased slowly to the end of the study. Vaccinia virus-specific IgG was not detected in the TBS negative control group until day 11 (2.0 $\log_{10}$ AIU/ml). Significantly ($P < 0.05$) higher levels of antibody were detected in the two-dose Imvamune group (4.0 $\log_{10}$ AIU/ml) on day 9 postchallenge than in the Acam2000 group, where a peak of 3.5 $\log_{10}$ AIU/ml was seen. Antibody concentrations in the single and two dose Imvamune groups remained significantly ($P < 0.05$) higher than the Acam2000 group on days 14 and 21 (Fig. 1b) postchallenge.

Vaccine-induced cell-mediated immune responses. The cell-mediated immune responses after vaccination and challenge were monitored by flow cytometry. Lymphocyte numbers rose in the TBS negative control, and Acam2000 groups after vaccination by scarification (Fig. 2a to c). These small peaks were probably caused by local irritation at the site of vaccination caused by scarification. Small rises in $CD3^+$, $CD4^+$, and $CD8^+$ cells were seen initially, following vaccination with one dose of Imvamune, but there was no marked increase following the second vaccination (Fig. 2). After challenge, however, there were noticeable rises in the different cell populations in animals that succumbed to disease in the TBS negative control group. For example, on day 9, there were significant differences ($P < 0.05$) between the TBS control and the Imvamune ×2 group. On closer inspection, these differences were caused by the significant rise in circulating NK cells in the TBS control ($P < 0.05$). By day 14, there were significantly higher ($P < 0.05$) numbers of B cells and $CD8^+$ T cells in the surviving animals (4/6) of the one dose Imvamune group compared to the Acam2000 group. Also, by day 14 significantly higher ($P < 0.05$) numbers of $CD4^+$ and $CD8^+$ cells were recorded in single Imvamune group than in the two-dose Imvamune group (Imvamune ×2).

Elevated concentrations of IFN-γ, as detected by Luminex assay, were seen in the TBS negative control group on day 6 after challenge (4630%) (Fig. 3a). In contrast, no rise in serum gamma-IFN was observed in animals in Acam2000 and two-dose Imvamune groups (Fig. 3a) as they were protected by vaccination. The single Imvamune dose group also had raised concentrations of serum gamma-IFN 6 days after challenge (Fig. 3a).

A significant rise in IL-6 cytokine was seen in the TBS negative control group following aerosol challenge, which continued to increase until the animals succumbed to infection on day 11 (4592% change from baseline) (Fig. 3b). Smaller increases in IL-6 were seen (day 6) in the single-dose Imvamune group (Fig. 3b).

Clinical signs of disease and mortality. Aerosol challenge with monkeypox virus at a mean presented dose of 2.6×10^5 PFU resulted in a severe or lethal infection in susceptible individuals. Most animals showed a decline in weight from their prechallenge weights (Fig. 4). This was most severe in the TBS negative control group, with a 10 to 18% loss in weight prior to euthanasia (Fig. 4a). All surviving animals in the vaccination groups—Acam2000, Imvamune ×1, and Imvamune ×2—had a consistent increase in body weight from day 14 postexposure, indicating recovery from the infection (Fig. 4b to d).

Signs of infection generally appeared from day 5 postchallenge. Animals in the TBS negative control displayed progressing depression, dyspnea, and nasal discharge. All six animals succumbed to infection between days 7 to 11 postchallenge. In the single-dose Imvamune group, two animals succumbed to infection on different days. Both animals displayed mild depression and dyspnea and were recumbent from day 6 postchallenge; animal M064F was found dead in cage on day 7 postchallenge, and animal I320I had

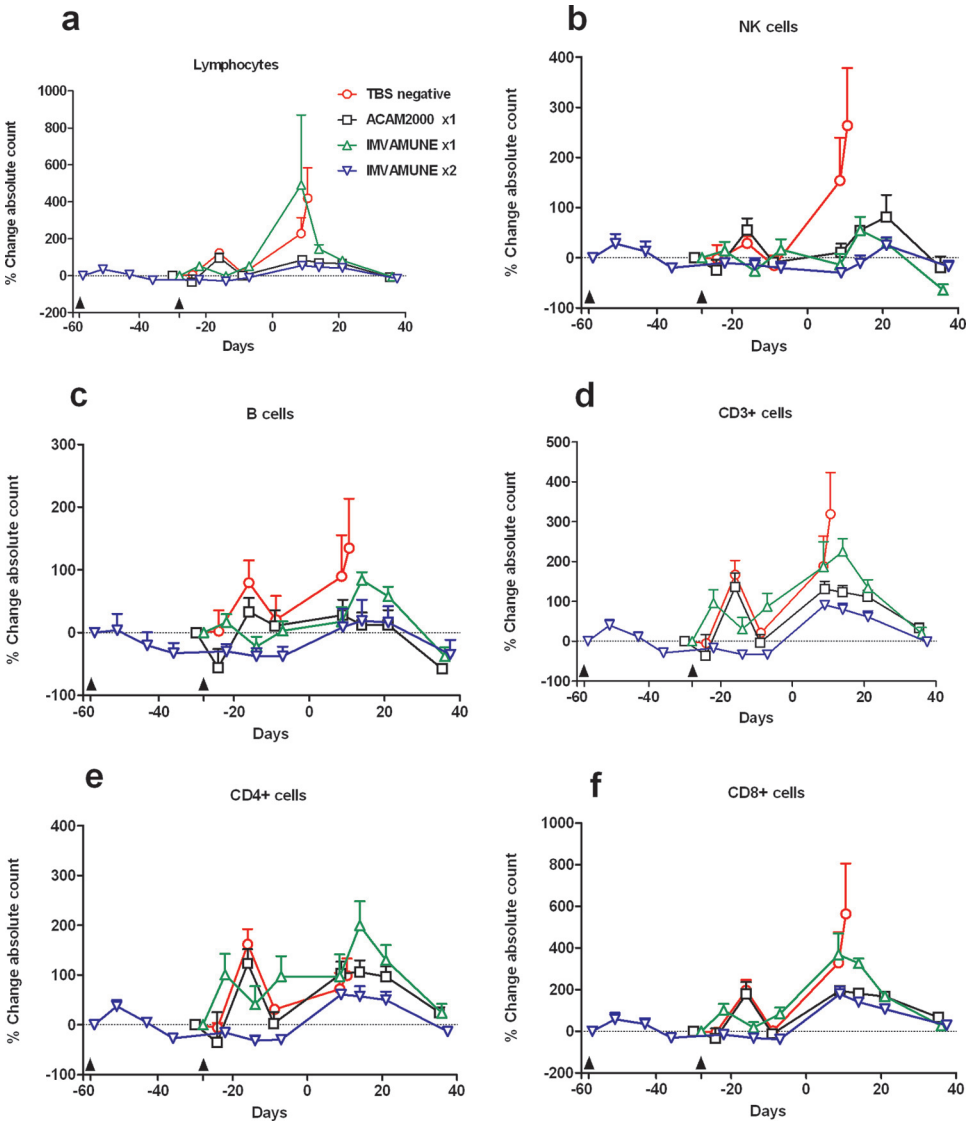

FIG 2 Cell-mediated immune responses, indicated as the mean percent (%) change compared to baseline levels (+ 1 SE, n = 6), in different cellular populations in whole blood on various days in the study. Different cellular populations were evaluated. (a) Lymphocytes; (b) natural killer (NK) cells; (c) B lymphocytes; (d) CD3+ T lymphocytes; (e) CD4+ T lymphocytes; (f) CD8+ T lymphocytes. Day 0, time of challenge with monkeypox; TBS negative, TBS negative control group; closed triangles, times of immunization.

clinical signs that progressed to severe and met the criteria for immediate euthanasia on day 9 postchallenge. The remaining four animals in the one-dose Imvamune group were generally free of clinical signs and survived to the end of the study (67% survival). All of the animals vaccinated with Acam2000 or two doses of Imvamune survived the monkeypox virus challenge. The animals appeared to be clinically normal, although there were differences in the level of protection afforded by these vaccines, as demonstrated in some of the test parameters, such as radiographs, lesion counts, and viral load (Table 1).

Skin lesions, as a result of monkeypox virus infection, first appeared at day 6 after challenge (Table 1). There was a peak in the mean number of lesions, across all vaccination and control groups at day 9. The greatest mean number of lesions was 51 per animal (range, 5 to 169) in the TBS negative control group (Table 1). Fewer lesions were detected in the vaccination groups. Vaccination with one or two doses of Imvamune led to fewer lesions on day 9, with means of 10 (range, 0 to 42) and 7 (range, 0 to 18) lesions per animal, respectively. The lowest mean number of lesions was 3 (range, 0 to 7) per animal in the Acam2000 treatment group.

Radiographs were taken postchallenge at the time when clinical signs were most severe (9 days). They were scored independently against a corresponding baseline image. Animals in the TBS negative control group generally displayed the most severe clinical signs (Table 1). Radiographs taken from animals in the Acam2000 vaccination group were normal. A wide spectrum of conditions was observed in the Imvamune single- and two-dose groups ranging from normal to severe edema (Table 1). One animal (Z385A) in the two-dose Imvamune group had moderate to severe pulmonary edema but recovered fully.

Whole blood, throat, and tissue viral loads of NHP exposed to

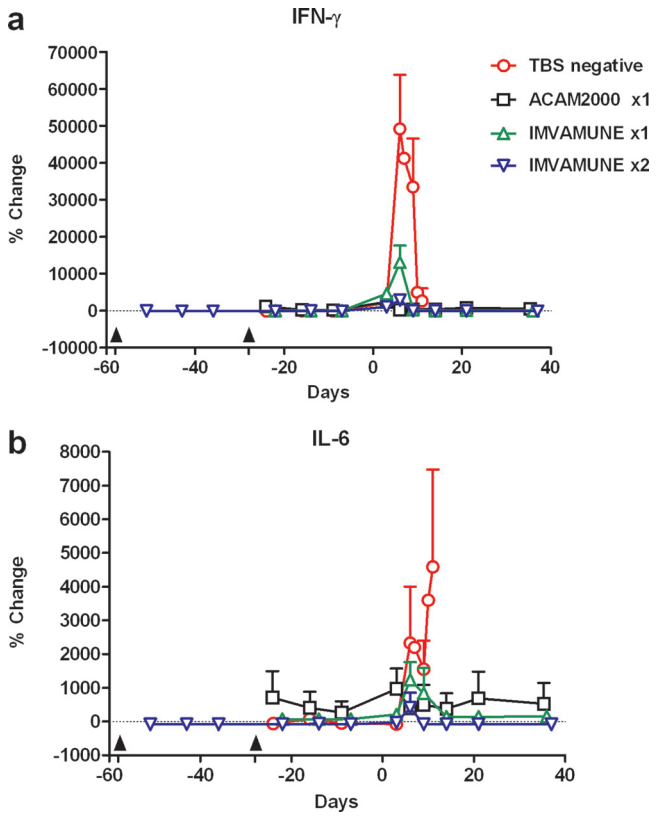

FIG 3 Cytokine profiles in the serum of cynomolgus macaques. The mean percent (%) change from baseline levels (+ 1 SE, $n = 6$) in cytokine levels, on various days during the study, was determined. The IFN-ã (a) and IL-6 (b) responses are shown. TBS negative, TBS negative control group; day 0, time of challenge with monkeypox; closed triangles, times of immunization.

aerosolized monkeypox virus: the monkeypox viral load of blood and throat swabs were assessed by plaque assay (PFU/ml) (Fig. 5a and b) and real-time quantitative PCR (genomes/ml) (Fig. 5c and d) (there was a strong correlation [$r = 0.746$; df = 26, $P < 0.001$, Pearson product-moment correlation] between plaque assay and real-time PCR data). The peak in the mean load of viral DNA (4×10^6 genomes/ml) in the blood of animals from the TBS negative control group (Fig. 5c) occurred on day 7 postchallenge. In contrast, no viral DNA was also detected in the group that received the Acam2000 vaccine on any day examined postchallenge. A peak in the mean level of viral DNA in the blood was detected in animals that had received one (6×10^7 genomes/ml; day 7 postchallenge) and two (1×10^4 genomes/ml; day 6 postchallenge) doses of Imvamune (Fig. 5c). Low levels of live virus were detected in the blood by day 3 postchallenge in all three vaccination groups and the TBS negative control group, ranging from 25 to 200 PFU/ml (Fig. 5a).

In the TBS negative control group, live virus (10^5 PFU/ml) and viral DNA (10^7 genome copies/ml) were detected in the throats of all animals challenged with monkeypox virus (Fig. 5b and d). Live virus (10^4 to 10^5 PFU/ml) was also detected in the throats of animals that had received a single dose of Imvamune (Fig. 5b and d). In the two-dose Imvamune group, four of six animals did not excrete virus in the throat (within the sensitivity of the plaque assay [<25 PFU/ml]). Live virus was detected at low levels (50 PFU/ml) in one of six animals, and one animal (Z385A) excreted high levels of virus that peaked (4.6×10^4 PFU/ml) on day 9 postchallenge. In contrast, live virus was not detected in the throats of animals (5/6 animals) vaccinated with Acam2000. Note that no plaque assay data were obtained for one remaining animal (M016D) in the Acam2000 group due to contamination of the cell monolayer.

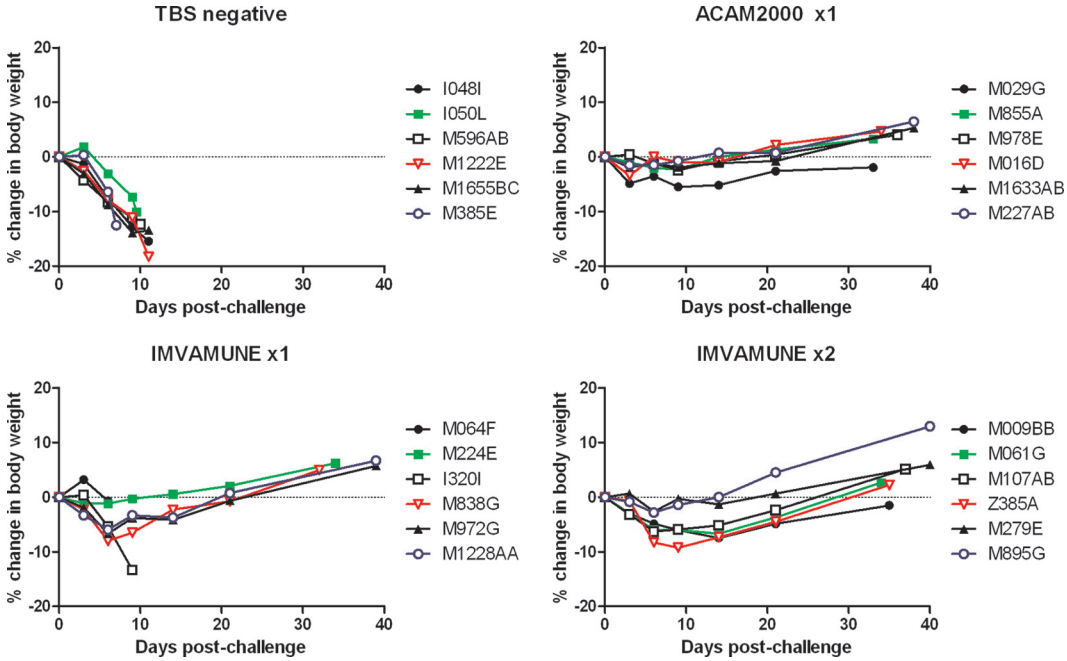

FIG 4 Change in the body weight of cynomolgus macaques. The percent (%) change in body weight compared to baseline levels in animals challenged with aerosolized monkeypox, over time, following vaccination, was determined. The results for individual animals are plotted. TBS negative, TBS negative control group.

TABLE 1 Clinical signs of monkeypox disease[a]

Measurement	Treatment group			
	TBS negative control	Acam2000 ×1	Imvamune ×1	Imvamune ×2
Temp	Normal	Normal	Normal	Normal
Clinical signs[b]	+++	+	++[c]	+
Mean no. of lesions (day 9)	51	3	10	7
Onset of lesions	Day 6	Day 9	Day 9	Day 6
Resolution of lesions	Not resolved	Within 5 days	Within 5 days	Within 5 days
Thoracic radiography[d]	+++	–	++	+
Survival (%)	0[e]	100	67[f]	100

[a] Clinical signs were monitored every 4 to 6 h until disease recovery and thereafter twice daily. Treatment groups are as described in Materials and Methods.
[b] Clinical signs: –, none; +, mild; ++, substantial; +++, intense signs.
[c] Two of the that animals succumbed to infection had intense clinical signs (+++).
[d] A thoracic radiograph was obtained 9 days postchallenge. Findings were scored as follows: –, normal; +, minor pulmonary edema; ++, mild pulmonary edema; +++, moderate pulmonary edema.
[e] All animals died between 7 and 11 days postchallenge and displayed high euthanasia scores.
[f] Two animals succumbed to infection on day 7 and day 9 postchallenge; the animal that died on day 7 had low euthanasia scores prior to death.

In addition to blood and throat swabs, tissues were collected postmortem and assayed by real-time quantitative PCR for viral load. The majority of tissues were positive for monkeypox virus in the TBS negative control group (Fig. 6a). The greatest viral loads were found in the tonsil and lung tissues, with between 10^6 and 10^7 genomes/mg. Two animals (M064F and I320I) vaccinated with a single dose of Imvamune also succumbed to monkeypox infection. These two animals also showed similar patterns of viral load, as seen in the TBS negative control group. Both tonsil and lung tissue reflected the greatest values of between 10^5 and 10^6 copies/mg (Fig. 6c). The remaining animals in the Imvamune ×1 group that survived to the end of the study (>30 days postchal-

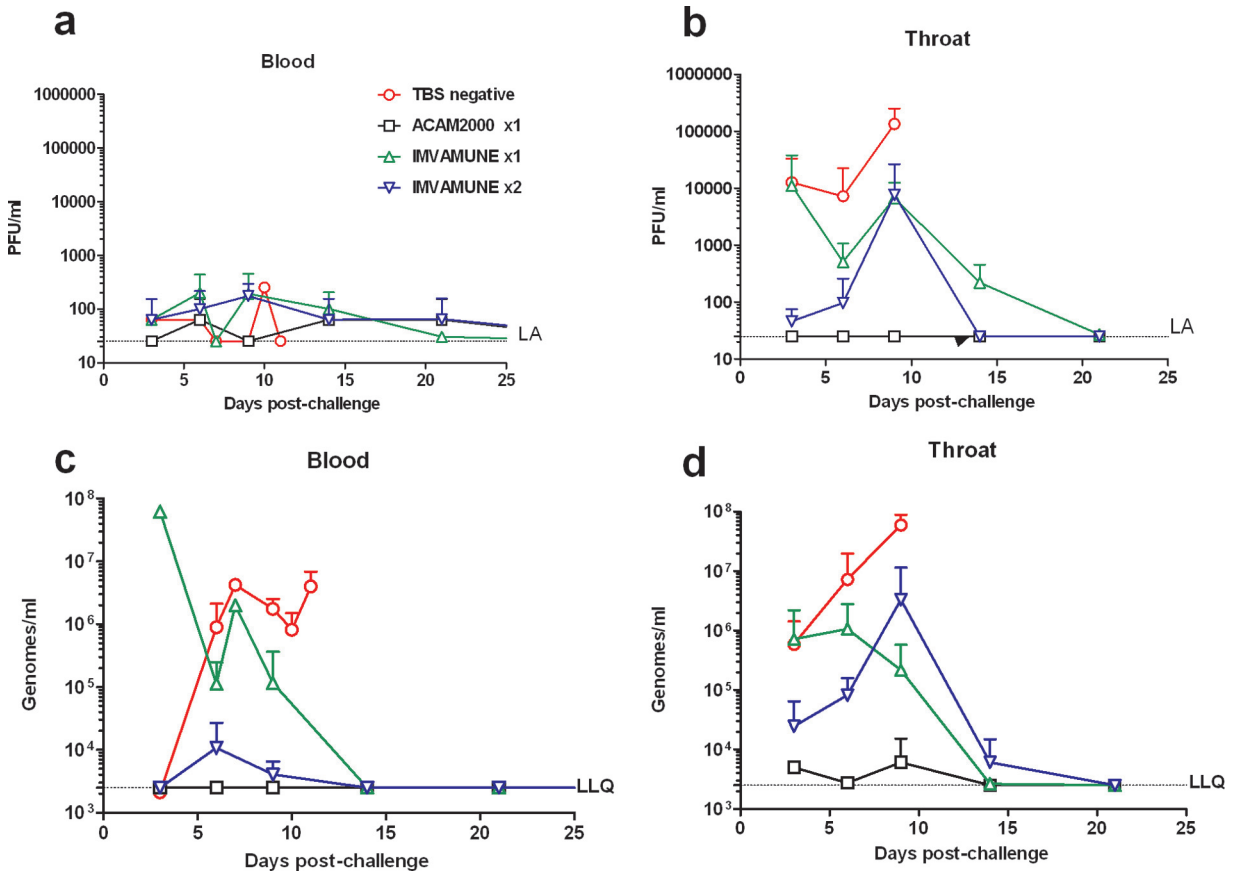

FIG 5 Mean detectable levels (+ 1 SD) of live monkeypox virus in the blood (a) and throats (b) of macaques after a challenge with aerosolized virus (the limit of plaque assay sensitivity was 25 PFU/ml). The numbers of viral genomes (HA gene), as determined from quantitative PCR analyses in the blood (c) and throats (d) in samples postchallenge, are shown. TBS negative, TBS negative control group; LLQ, the lower limit of quantification for quantitative PCR was 2,500 genomes/ml; LA, limit of plaque assay sensitivity. The plaque assay result for 1/4 animals in the Imvamune ×2 was lost due to monolayer contamination on day 14.

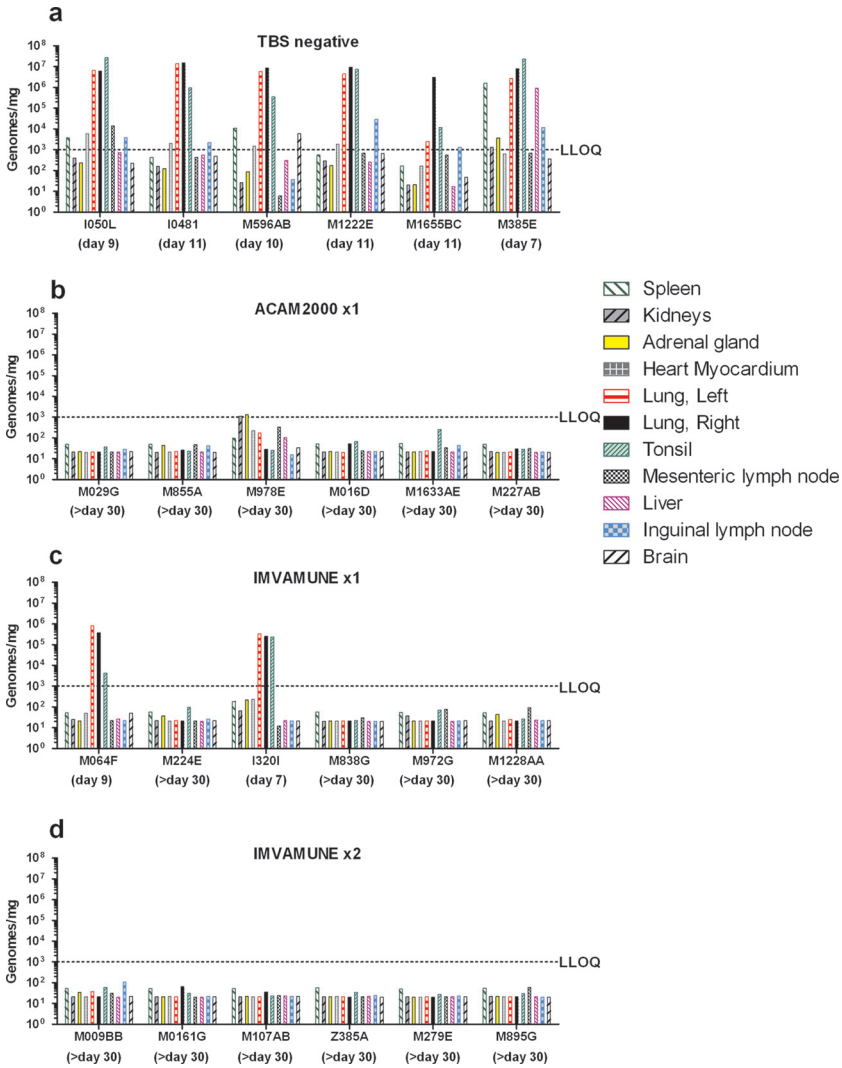

FIG 6 Viral load in tissues after challenge with monkeypox virus. The viral load, as determined by real-time PCR, in the tissues of different animals in the treatment groups. Individual animals in the TBS negative control (a), Acam2000 ×1 (b), Imvamune ×1 (c), and Imvamune ×2 (d) groups were evaluated. LLQ, lower limit of quantification was 1,000 genomes/mg (the time postmortem is given as the number of days postchallenge). TBS, TBS negative control group.

lenge) did not have any detectable viral loads by PCR in their tissue postmortem. No detectable viral loads were seen in the tissue of animals from the Acam2000 vaccination or the two doses of Imvamune (Fig. 6b and d). All of these animals also survived to the end of the study (between 30 and 40 days).

Pathological and histopathological findings. Gross findings on postmortem examination revealed that the main gross lesions associated with monkeypox infection consisted of lung consolidation in all animals in the TBS negative control and in two of the six animals in Imvamune ×1 group. An enlarged spleen was seen in five of six animals in the TBS negative control and in one of six animals in the Imvamune ×1 group.

On histological examination, changes consistent with acute monkeypox infection were observed in the TBS negative control group in the lungs, comprising (i) focal, acute necrotizing bronchitis and bronchopneumonia (Fig. 7a); (ii) focal, fibrinous, necrotizing alveolitis (Fig. 7b), often accompanied by edema; and (iii) focal acute vasculitis, sometimes together with thrombosis and perivascular edema. In addition, focal necrosis with or without neutrophil infiltration was observed in the trachea, larynx, and tracheobronchial lymph node. In the skin (with lesion), spleen, tonsils, the axillary, inguinal, and mandibular lymph nodes, and the descending colon, focal necrosis—with or without neutrophil infiltration—was observed.

In animals vaccinated with Acam2000, which were killed 33 to 38 days postchallenge, only mild, chronic lesions were observed. These comprised focal alveolar epithelialization and/or infiltration of alveolar walls by lymphocytes and macrophages, which were seen in two (M016D and M978E) of the six animals (Fig. 7c). Hyperplasia of bronchus-associated lymphoid tissue (BALT) was recorded in five of six animals.

Animals vaccinated with a single dose of Imvamune that died (M064F) or were killed for welfare reasons (I302I) (days 7 and 9 postchallenge, respectively) had lesions of acute disease in all lung lobes, similar to those described above in the TBS negative control group (Fig. 7d). In the trachea of one animal and in the larynx of another, focal necrosis, with or without neutrophil infiltration, was also observed. Hyperplasia of BALT was observed in one of

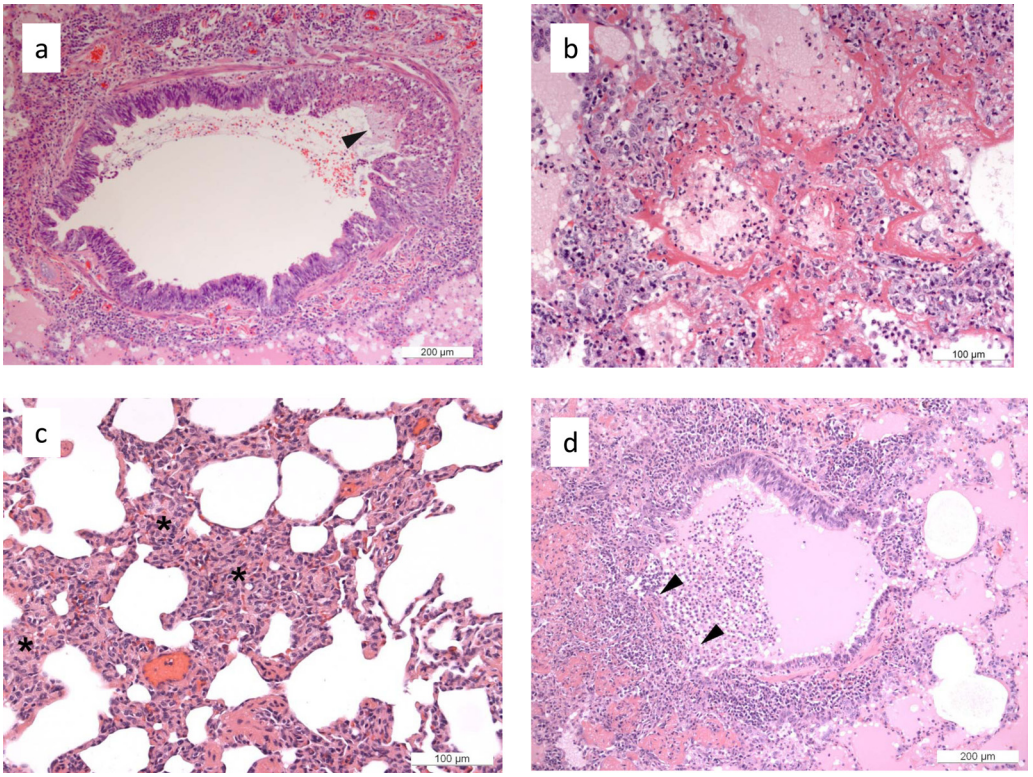

FIG 7 Histological lesions associated with monkeypox infection in the lung. (a) TBS negative control, animal M596AB (lung; focal, acute, and necrotising bronchiolitis [arrowhead]; H&E staining). (b) TBS negative control, animal M595AB (lung; focal, fibrinous, and necrotising alveolitis; H&E staining). (c) Acam2000 vaccination group, animal M016D (lung; patchy infiltration of alveolar walls by lymphocytes and macrophages [asterisks]; H&E staining). (d) Single Imvamune dose, animal I320I (lung; focal, acute, and necrotising bronchiolitis [arrowheads]; H&E staining).

four animals that were euthanized as scheduled 32 to 39 days after challenge. In all animals in the single-dose Imvamune group, mild changes of focal alveolar epithelialization and/or infiltration of alveolar walls by lymphocytes and macrophages, a finding consistent with chronic or resolving lesions, were recorded.

In animals that received two doses of Imvamune and were euthanized as scheduled 35 to 40 days after challenge, only mild lesions of chronic disease, comprising focal alveolar epithelialization and/or infiltration of alveolar walls by lymphocytes and macrophages were seen in two of the six animals. Hyperplasia of BALT was recorded in four of six animals.

Lesions attributable to monkeypox infection were not detected in the liver, kidney, heart, tongue, esophagus, stomach, ileum, mesenteric lymph node, adrenal gland, ovary, testis, or brain of any animal.

DISCUSSION

Since smallpox has been eradicated, the future licensing of a new generation of smallpox vaccines relies, in part, on the demonstration of efficacy in animal models of monkeypox (19). When monkeypox virus infects people as an epizootic pathogen, it presents a clinical disease similar to smallpox in that the time course and manifestation of disease is similar to that seen with human smallpox, particularly the rash which progresses through the macular, papular, vesicular, and pustular phases (24). Thus, a well-defined animal model using monkeypox virus should mimic the natural course of smallpox disease. Macaques have been used at various stages of smallpox vaccine and antiviral research (16, 25–36), and in each case the route of infection, dose, and choice of challenge strain have been key factors in determining whether the macaque model of monkeypox resembles human clinical variola virus infection.

Imvamune is a more recent smallpox vaccine and is being fast-tracked by the FDA for use in humans (37). This vaccine is currently stockpiled in the United States for use during an emergency, such as an imminent bioterrorist attack, to protect individuals who are at risk of developing side effects from older vaccines (37). Studies have been published demonstrating the safety, immunogenicity (4), and protective efficacy against vaccinia virus scarification in humans (38) of Imvamune delivered in a single- or two-dose regime. Several studies have also shown its protective potency in animals (33, 39, 40). In the present study, for the first time, the efficacy of both one and two doses of Imvamune vaccine was assessed in cynomolgus macaques following challenge with an aerosolized dose of 2.6×10^5 PFU of monkeypox Zaire Z9. Inhalation of aerosolized virus more closely resembles the natural route of infection of smallpox in humans (41) and therefore initiates the onset of clinical signs that are similar to human clinical disease (20, 42).

When aerosolized monkeypox virus was used at a dose (2.6×10^5 PFU) to produce severe or lethal disease in naive cynomolgus macaques, animals in the TBS negative control group succumbed to infection within 7 to 11 days. Pock lesions began to appear on day 6 postchallenge, and there was a peak in the number of lesions by day 9 (a mean of 51 lesions per animal). These data are in sharp

alignment with other natural history and pathology studies conducted at our laboratories, as well as with work performed by Nalca et al. (36). In contrast, in other vaccine trials where control animals have been challenged by a different route, such as the intravenous route with a dose 5×10^7 PFU (16) or 2×10^7 PFU (32), pock lesions ranging from 250 to >500 per animal appeared from days 3 to 6. These differences highlight the importance of the challenge route and the dose.

All of the animals that received the second-generation vaccine (Acam2000) survived the monkeypox virus challenge, although some signs of viral infection were observed, such as lesions (mean number of three per animal) on day 9 and low levels of viremia. The animals were generally well and lost very little weight. Both humoral and cell-mediated immune responses were primed, and high concentrations of neutralizing antibody and IgG antibody were detected after vaccination.

The optimal and intended vaccination regime for Imvamune is a prime-boost approach, and results from the present study highlight the importance of this vaccination strategy. The use of a prime-boost regime with Imvamune protected all of the animals challenged (100% survival). Both antibody and cell-mediated immune responses were stimulated, and high titers of neutralizing and IgG antibody were detected following the second dose of Imvamune. There was still some evidence of monkeypox virus infection in the group, as indicated by the presence of pock lesions on day 9 and minor pulmonary edema; nevertheless, this is comparable to the results from the Acam2000-vaccinated animals. In addition, however, there was evidence of virus excretion in the throats of two of six animals. Viral excretion in the throat after MVA vaccination has previously been shown when using the intratracheal (33) and intravenous (32) challenge routes (a different source of MVA and a different route of vaccination was used in the intravenous challenge study).

In the present study, a single dose of Imvamune did not protect all of the animals in the group, and two animals succumbed to infection. Postmortem, the virus was isolated from the lungs and tonsils of both animals. The titer of vaccinia virus-specific IgG antibody and neutralizing antibody prior to challenge was very low, and this may have contributed to the poorer outcome in this group. It should be noted that a single dose is not the optimal regime for Imvamune vaccination; however, one dose does give partial protection and thus may potentially be useful as a primer vaccine, in certain groups of people, which are then subsequently boosted during an emergency. Further work is clearly needed in this area.

Our data not only provide supportive information for the use of Imvamune as a vaccine against variola virus but also show that it could be useful as a vaccine to protect against human infections with monkeypox virus. Recent epidemiologic studies suggest that human monkeypox is currently exhibiting a robust emergence in the Democratic Republic of the Congo (7, 43, 44). Cessation of smallpox vaccination worldwide has resulted in diminished vaccine-induced orthopoxvirus immunity, creating a new "immunologic niche" for the emergence of human monkeypox (45). The use of next-generation smallpox vaccines for the prevention of human monkeypox is currently being discussed (45, 46).

Overall, we have demonstrated here that a prime-boost vaccination regime with Imvamune provides complete protection, as does the comparator vaccine Acam2000. Two doses of Imvamune should be used rather a single dose which only offers partial protection. This evaluation of different human smallpox vaccines in cynomolgus macaques helps to address questions about optimal vaccine strategies, in the absence of human challenge studies, during a time when the efficacy of Imvamune is being established under the "Animal Rule."

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