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Mucosal Priming with a Replicating-Vaccinia Virus-Based Vaccine Elicits Protective Immunity to Simian Immunodeficiency Virus Challenge in Rhesus Monkeys

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Mucosal surfaces are not targeted by most human immunodeficiency virus type 1 (HIV-1) vaccines, despite being major routes for HIV-1 transmission. Here we report a novel vaccination regimen consisting of a mucosal prime with a modified replicating vaccinia virus Tiantan strain ($MVTT_{SIVgpe}$) and an intramuscular boost with a nonreplicating adenovirus strain ($Ad5_{SIVgpe}$). This regimen elicited robust cellular immune responses with enhanced magnitudes, sustainability, and polyfunctionality, as well as higher titers of neutralizing antibodies against the simian immunodeficiency virus $SIV_{mac1A11}$ in rhesus monkeys. The reductions in peak and set-point viral loads were significant in most animals, with one other animal being protected fully from high-dose intrarectal inoculation of SIV_{mac239}. Furthermore, the animals vaccinated with this regimen were healthy, while ~75% of control animals developed simian AIDS. The protective effects correlated with the vaccine-elicited SIV-specific $CD8^+$ T cell responses against Gag and Pol. Our study provides a novel strategy for developing an HIV-1 vaccine by using the combination of a replicating vector and mucosal priming.

Mucosal tissues are the major entry points and first line of host defense against human immunodeficiency virus/simian immunodeficiency virus (HIV/SIV) transmission (1–3). Entry can occur in only a few hours and leads to a productive infection in activated memory $CD4^+$ $CCR5^+$ T cells, which are abundant in the mucosal lymph tissues (1, 4, 5). Profound $CD4^+$ T cell lymphopenia soon develops, with viral persistence in the gut-associated lymphoid tissues (5–7). Therefore, a successful HIV/SIV vaccine must induce protective immunity to block initial acquisition and subsequent replication (8–10). The most important characteristics of sufficient protective immunity are the sustainable responses of cell-mediated and broadly neutralizing antibodies (bnAbs) (11–30). Several breakthroughs have been achieved based on nonhuman primate models in studies of various immunologic approaches against SIV challenge. For example, studies have shown passively infused bnAbs to be significantly more effective than nonneutralizing antibodies in reducing viral replication and protecting against viral transmission (31–36). However, candidate immunogens that could elicit such bnAbs are still being explored (37–39). Similar animal models have also been used to demonstrate that vector-based AIDS vaccines can induce robust, sustainable T cell and antibody responses, as well as effectively blocking viral acquisition, replication, and disease progression (12–15, 18, 22).

The strategies used in these studies, however, did not include replicating vectors with direct mucosal vaccination. Earlier work by the Robert-Guroff group showed that a mucosal prime with live recombinant adenovirus 5 (Ad5) plus protein or vector boosts can elicit potent T cell immunity, systemic and mucosal antibody-mediated neutralization, and antibody-dependent cellular cytotoxicity and transcytosis inhibition (30, 40–49). Unlike nonreplicating adenovirus vectors, which have a limited anatomic distribution, the replicating Ad5 vector can disseminate across multiple mucosal sites irrespective of delivery route (44). The major identified cell targets of the replicating Ad5 vaccine were tissue macrophages and myeloid dendritic cells (mDCs) (44). These and some of our earlier findings led us to postulate that initial vaccination with a replicating vector that engages the mucosal system in concert with a potent boosting agent may significantly bolster protective immunity against HIV/SIV mucosal transmission. To test this, we generated a modified replicating vaccinia virus Tiantan (MVTT) strain from its parental VTT strain through targeted gene knockout. The parental VTT strain was used from the 1950s to the 1980s to immunize approximately 300 million individuals during the smallpox eradication campaign in China. Compared with its parental VTT strain, MVTT has reduced neurotoxicity and pathogenicity (50–52). Furthermore, single-dose intranasal MVTT vaccination has clearly been shown to be safe and to effectively elicit protective immunity against pathogenic vaccinia virus Western Reverse (WR) challenge (52). Intranasal vaccination with MVTT expressing the severe acute respiratory syndrome corona-

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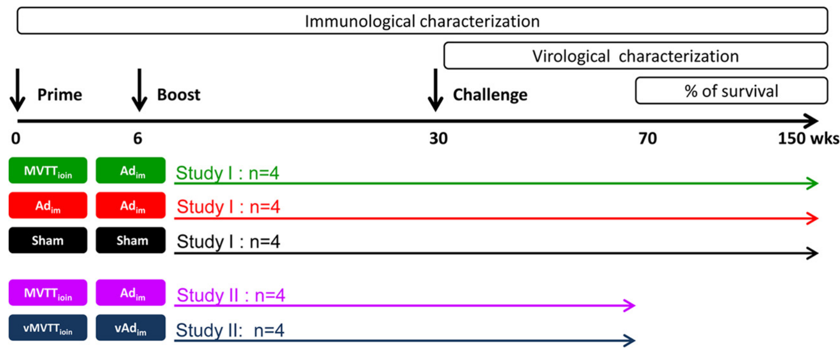

FIG 1 Immunization and challenge schedule for Chinese rhesus monkeys for study I and study II. Three groups ($n = 4$ per group) were used in study I, while two groups ($n = 4$ per group) were used in study II. Each group was color coded, and each animal is represented by a unique symbol in the other figures: emerald symbols represent the four animals in the $MVTT_{ioin}$+Ad_{im} group, red symbols represent the four animals in the Ad_{im}+Ad_{im} group, black symbols represent the four animals in the sham control group, purple symbols represent the four animals in the second $MVTT_{ioin}$+Ad_{im} group, and blue symbols represent the four animals in the vector control group.

virus (SARS-CoV) spike protein (MVTT-S) produced higher titers of neutralizing antibodies than vaccination with the nonreplicating vaccinia virus MVA-S (51, 53). Based on these findings, we developed a recombinant replicating vector, $MVTT_{SIVgpe}$, which expresses the three SIV_{mac239} structural proteins, Gag, Pol and Env, and investigated its protective immunogenicity *in vivo* in combination with an antigen-matched, nonreplicating vector, $Ad5_{SIVgpe}$.

MATERIALS AND METHODS

Vectors and virus. A recombinant modified replicating vaccinia virus Tiantan strain ($MVTT_{SIVgpe}$) and a recombinant, nonreplicating adenovirus type 5 strain ($Ad5_{SIVgpe}$) expressing the SIV_{mac239} Gag, Pol, and Env structural proteins were generated using the homologous recombination technology described previously (51, 54, 55). The original SIV_{mac239} stock was a generous gift of P. A. Marx and was further adapted in Chinese macaques by X. Wu (Chinese Academy of Medical Sciences, Beijing, China) prior to this study. The challenge stock was generated and titrated in Chinese rhesus monkey peripheral blood mononuclear cells (PBMCs).

Animals and vaccination. A total of 20 Chinese rhesus monkeys that weighed 3 to 5 kg and were 3 to 6 years of age were used in two separate studies: study I and study II (Fig. 1). In study I, 12 macaques were divided into three groups: (i) 4 monkeys received $MVTT_{SIVgpe}$ (10^9 PFU in 1 ml of phosphate-buffered saline [PBS]) through intraoral (i.o.) (0.5 ml) and intranasal (i.n.) (0.5 ml) routes and $Ad5_{SIVgpe}$ (10^{11} viral particles [vp] in 1 ml of PBS) through intramuscular (i.m.) injection ($MVTT_{ioin}$+Ad_{im} regimen); (ii) 4 monkeys received a homologous prime and boost with the $Ad5_{SIVgpe}$ vaccine (10^{11} vp in 1 ml of PBS) through intramuscular injection (Ad_{im}+Ad_{im} regimen); and (iii) 4 monkeys received PBS through intramuscular injection, as a sham control group. Study II was then conducted to support study I outcomes and to rule out vector-mediated, nonspecific protective effects. In the second study, eight monkeys were divided into two groups: (i) four monkeys received the $MVTT_{ioin}$+Ad_{im} testing regimen as in study I, and (ii) four monkeys received an empty MVTT control vector (10^9 PFU) through intraoral (0.5 ml) and intranasal (0.5 ml) routes and an empty Ad5 control vector (10^{11} vp in 1 ml of PBS) through intramuscular injection ($vMVTT_{ioin}$+vAd_{im} regimen) (Fig. 1). At either week 30 after the initial vaccination or week 24 after the final vaccination, each animal was challenged intrarectally with 5×10^5 50% tissue culture infective doses ($TCID_{50}$) of Chinese rhesus monkey-adapted and neutralization-resistant SIV_{mac239}. In all cases, the challenge virus stock was administered in 1 ml of PBS. Sequential peripheral blood samples were collected to evaluate the virologic and immunologic responses, following the experimental scheme highlighted in Fig. 1.

All animals were housed in the Animal Experimental Center of the Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, and were free of infection with simian immunodeficiency virus (SIV), simian T cell lymphotropic virus (STLV-1), and simian retrovirus (SRV) before the initiation of experiments. The experimental protocols were all approved by the Institutional Animal Care and Use Committee (IACUC). The monkeys with simian AIDS were sacrificed humanely by euthanasia in accordance with our IACUC protocols. The symptoms that defined simian AIDS were severe diarrhea, wasting, weight loss, muscular atrophy, severe ulceration, and inverted CD4/CD8 ratios. Additional indicators included huddled posture, immobility, ruffled fur, failure to eat, and hypothermia (colonic temperature of $<34°C$). The method of euthanasia was intravenous injection of pentobarbital sodium (80 mg/kg of body weight).

SIV peptide pools and cellular immune assays. Pools of overlapping SIV_{mac239}-specific peptides covering the entire sequences of Gag, Pol, Env, Nef, Vif, Vpx, Vpr, Rev, and Tat were obtained through the AIDS Research and Reference Reagent Program, National Institutes of Health (NIH). These peptide fragments are 15 amino acids in length, with 11 overlapping residues. Peptide pools containing Gag, Pol, and Env peptides were used to evaluate immune responses against vaccine antigens throughout the vaccination and after live SIV_{mac239} challenge. Peptide pools of Nef, Vif, Vpx, Vpr, Rev, and Tat fragments were used to assess the immune responses against nonvaccine antigens after challenge. Pools were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0.4 mg/peptide/ml before use. The cellular immune assays, conducted as previously described, included a gamma interferon enzyme-linked immunosorbent spot (IFN-γ ELISPOT) assay, multicolor intracellular cytokine staining (ICS) for T lymphocyte polyfunctionality, and carboxyfluorescein diacetate succinimidyl ester (CFSE) staining for *ex vivo* T cell proliferation (56). Multicolor ICS assays and detection of phenotypic markers of T central memory ($CD28^+$ $CD95^+$ cells) and T effector memory (T_{EM}; $CD28^-$ $CD95^+$ cells) were performed with the following monoclonal antibodies: anti-CD3–Pacific Blue, anti-CD4–AmCyan, anti-CD8–allophycocyanin (APC)–Cy7, anti-CD28–fluorescein isothiocyanate (FITC), anti-CD95–phycoerythrin (PE)–Cy5, anti-IFN-γ–PE, anti-tumor necrosis factor alpha (anti-TNF-α)–PE–Cy7, and anti-interleukin-2 (anti-IL-2)–APC (BD Pharmingen). Samples were analyzed with a FACSAria flow cytometer (BD Biosciences) and FlowJo software (version 7.6; Tree Star, Inc.). Numbers of circulating $CD4^+$ and $CD8^+$ T lymphocytes were determined using BD TruCount tubes according to the manufacturer's instructions (BD Biosciences).

Humoral immune assays. The serum-binding antibodies against SIV_{mac239} were determined by an enzyme-linked immunosorbent assay targeting lysed SIV_{mac239} particles as described previously (56). nAbs against SIV_{mac239} and $SIV_{mac1A11}$ were evaluated as a function of reduc-

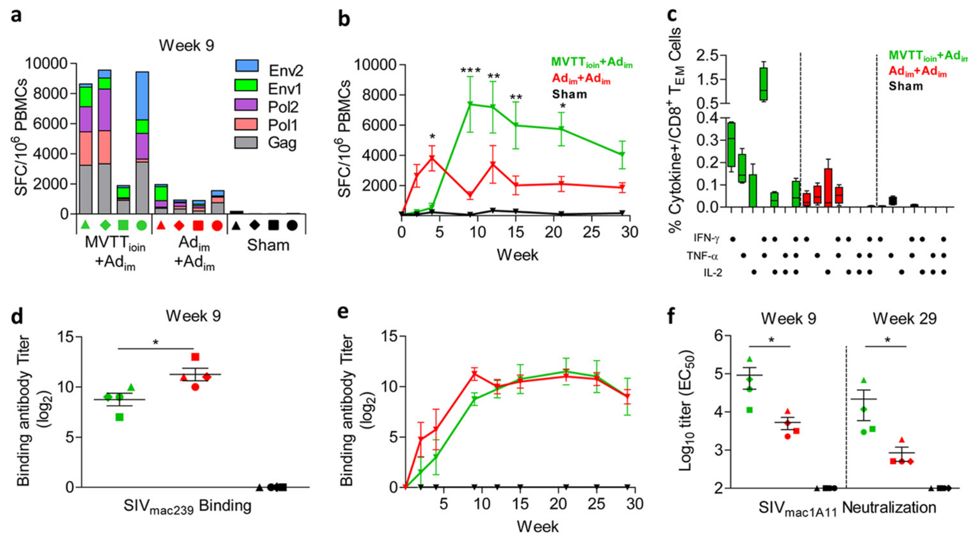

FIG 2 Immune characterization of the three groups in study I before challenge. (a and b) Temporal comparisons of SIV-specific spot-forming cells (SFC) per million PBMCs as measured by IFN-γ ELISPOT assay. (c) Comparison of percentages of $CD8^+$ T_{EM} cells in peripheral blood that secreted IFN-γ, TNF-α, and IL-2 after stimulation with the SIV Gag peptide pool. (d and e) Temporal comparisons of SIV-binding antibody responses (endpoint titers) against lysed SIV_{mac239} particles. (f) Comparison of nAb titers against $SIV_{mac1A11}$ at the peak and 1 week before challenge. EC_{50}, 50% effective concentration. Error bars in panels c to f represent standard errors of the means (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

tions in Tat-regulated luciferase reporter expression after a single round of infection of TZM-bl cells (AIDS Research and Reference Reagent Program, NIH), as described previously (54). Levels of nAbs against the Ad5 and MVTT vectors were measured as a 50% reduction in either reporter-secreted alkaline phosphatase (SEAP) or green fluorescent protein (GFP) activity, as described previously (52, 57).

Viral quantitation assay. Plasma and PBMCs were collected following standard protocols. Levels of SIV RNA in plasma were quantified by real-time PCR as described previously (56). The assay detection limit was 100 copies per 1 ml plasma.

Data analysis. Flow cytometry software analysis was performed using FlowJo 7.6 (Tree Star Inc.). Graphical representations were generated with GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA). The survival curves were analyzed by log rank (Mantel-Cox) tests, and immune correlates of protection were determined by Spearman rank correlation tests. Two-tailed *P* values were calculated for all analyses, and differences were considered statistically significant when *P* values were <0.05.

RESULTS

$MVTT_{ioin}+Ad_{im}$ regimen elicits potent immune responses in rhesus monkeys. The $MVTT_{ioin}+Ad_{im}$ regimen contained a combined intraoral (i.o) and intranasal (i.n.) mucosal prime with $MVTT_{SIVgpe}$ and an intramuscular (i.m.) $Ad5_{SIVgpe}$ boost, an approach designed to induce both cellular and humoral immune responses in Chinese rhesus monkeys. In study I, the $MVTT_{ioin}+Ad_{im}$ regimen was compared against either the homologous $Ad_{im}+Ad_{im}$ prime-boost with $Ad5_{SIVgpe}$ or the sham control regimen. All immunized macaques showed tolerance of the vaccination, with no signs of clinical illness. To evaluate cellular immune responses, ELISPOT assays for SIV-specific IFN-γ-secreting cells were performed on PBMCs obtained over the course of immunization (Fig. 2a and b). Four weeks after immunization, the initial priming with $MVTT_{ioin}$ induced 538 ± 557 IFN-γ-secreting cells per million PBMCs, whereas that with Ad_{im} elicited 3,805 ± 1,664 IFN-γ-secreting cells per million PBMCs (Fig. 2b) ($P < 0.05$). Following the boost with Ad_{im}, however, the frequency of positive responses increased and was maintained at significantly higher levels in $MVTT_{ioin}$-primed macaques than in the Ad_{im}-primed group over multiple time points (Fig. 2b) ($P < 0.05$). The frequency of positive responses in the $MVTT_{ioin}+Ad_{im}$ group peaked 9 weeks after initial immunization, at an average of 7,382 ± 3,685 IFN-γ-secreting cells per million PBMCs (Fig. 2b). Responses were predominately against Gag and Pol peptides, although an anti-Env response was also detected (Fig. 2a). In contrast, positive IFN-γ responses in the $Ad_{im}+Ad_{im}$ group declined slightly after boosting (Fig. 2b), likely due to the blocking effect by the high levels of vector-specific neutralizing antibodies induced during the priming step (see Fig. S1 in the supplemental material). Between this point and viral challenge, the frequency of positive responses in the $MVTT_{ioin}+Ad_{im}$ group remained significantly higher than that in the $Ad_{im}+Ad_{im}$ group, except for the time point of week 29 after initial priming (Fig. 2b) ($P < 0.05$). Furthermore, compared with the $Ad_{im}+Ad_{im}$ group, all animals but monkey 3 in the $MVTT_{ioin}+Ad_{im}$ group maintained significantly higher levels of Gag-specific $CD8^+$ T_{EM} cell polyfunctionality, particularly for IFN-γ and TNF-α, but not IL-2, release (Fig. 2c) ($P < 0.05$).

Additionally, both the $MVTT_{ioin}+Ad_{im}$ and $Ad_{im}+Ad_{im}$ regimens elicited equivalent levels of SIV_{mac239}-specific binding antibodies (Fig. 2d and e). Although higher levels of binding antibodies were found in the $Ad_{im}+Ad_{im}$ group than in the $MVTT_{ioin}+Ad_{im}$ group during the initial and boosting immunizations, such differences disappeared 12 weeks after the boost (Fig. 2d and e). However, 9 weeks after initial immunization and immediately preceding viral challenge (week 29), the $MVTT_{ioin}+Ad_{im}$ regimen generated significantly higher levels of nAbs against the tier 1 virus $SIV_{mac1A11}$ (Fig. 2f) ($P < 0.05$). No detectable nAbs against neutralization-resistant strain SIV_{mac239} were observed. Collectively, these results suggested that the $MVTT_{ioin}+Ad_{im}$ regimen elicited higher levels of nAbs and cellular immune responses with more enhanced magnitudes, sustain-

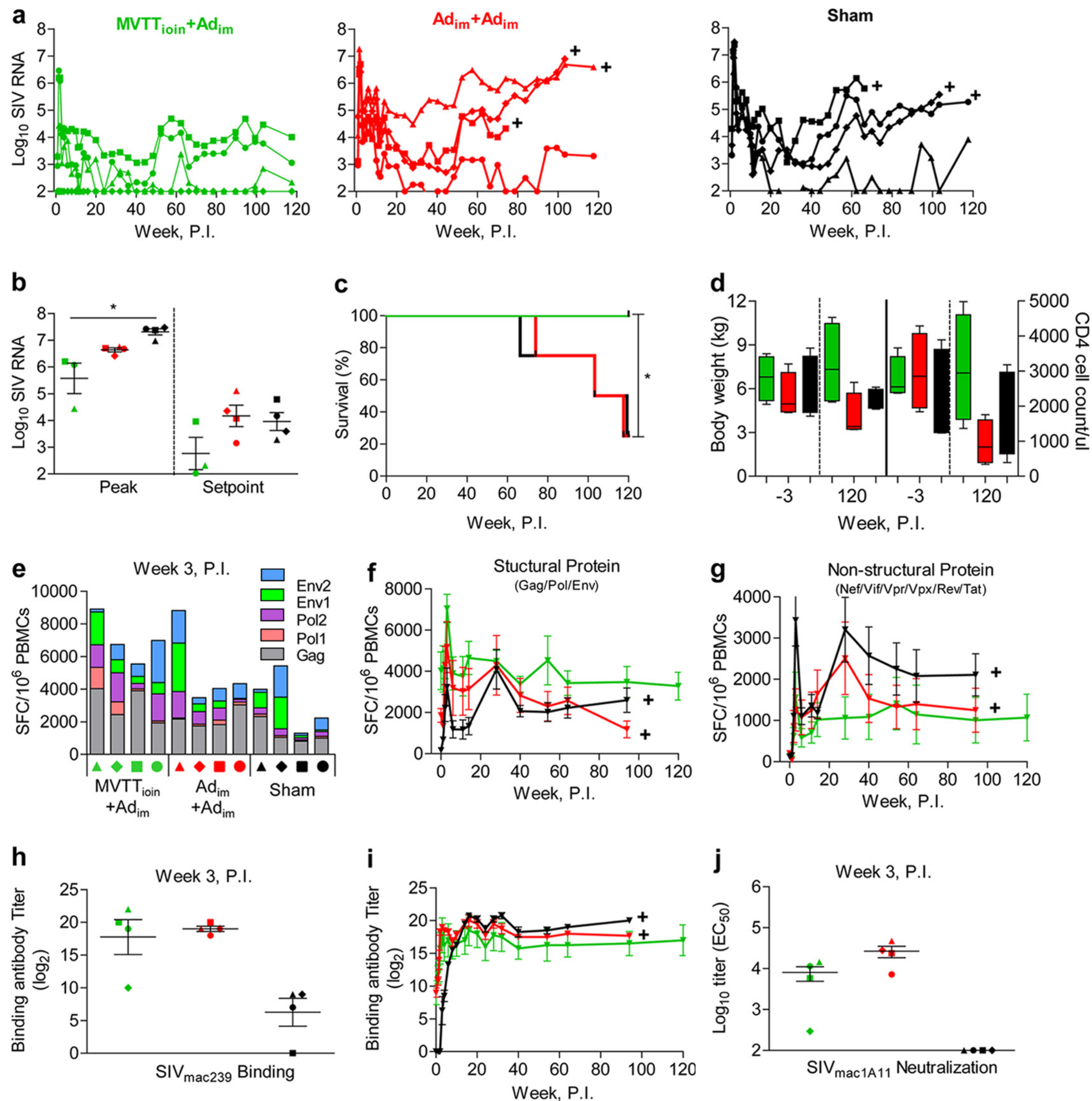

FIG 3 Comparison of protective effects among the three groups of animals in study I after challenge. (a and b) Temporal comparisons of plasma SIV RNA copies at peak and set point and over the course of infection, up to week 120 after challenge. (c) Comparison of disease progression and survival up to week 120 after challenge. (d) Changes in body weight and CD4 T cell count 3 weeks before and 120 weeks after challenge. Values for the last time point before sacrifice were used for animals that were euthanized before 120 weeks after challenge. Temporal changes in SIV-specific IFN-γ-secreting cells against the vaccine antigens Gag, Pol, and Env (e and f) or against the nonvaccine antigens Nef, Tat, Rev, Vif, Vpr, and Vpx (g), as measured by ELISPOT assay, are shown. Temporal analyses of SIV-specific binding (h and i) and neutralizing (j) antibody responses after challenge are also shown. Plus symbols represent time points when animals were humanely euthanized after the onset of simian AIDS. The error bars in panels f to j represent SEM. *, $P < 0.05$. P.I., postinfection.

ability, and polyfunctionality than those with the Ad$_{im}$+Ad$_{im}$ regimen in Chinese rhesus monkeys.

MVTT$_{ioin}$+Ad$_{im}$ regimen elicits protective immunity against SIV challenge in rhesus monkeys. We next sought to determine whether the superior cellular and humoral responses elicited by the MVTT$_{ioin}$+Ad$_{im}$ regimen would correlate with better control of pathogenic SIV$_{mac239}$ infection. Twenty-four weeks after the boost, each monkey was challenged intrarectally with a single high dose of Chinese monkey-adapted pathogenic SIV$_{mac239}$ (5×10^5 TCID$_{50}$ per animal) (Fig. 1). All animals became infected, except for monkey 2 in the MVTT$_{ioin}$+Ad$_{im}$ group. However, differences in peak and set-point viral loads were observed across all three groups (Fig. 3a). In the MVTT$_{ioin}$+Ad$_{im}$ group, the infected monkeys showed average reductions in the peak and set-point viral loads of 1.74 log (5.57 ± 0.98 versus 7.31 ± 0.23 log copies of SIV RNA) and 1.20 log (2.76 ± 1.04 3.96 ± 0.66 log copies of SIV RNA), respectively, compared with sham controls (Fig. 3b) ($P < 0.05$). No such differences were detected between the Ad$_{im}$+Ad$_{im}$ and sham control groups (Fig. 3b) ($P > 0.05$). This suggested a significant control of replication in the MVTT$_{ioin}$+Ad$_{im}$ group during both the acute and chronic phases of infection. More importantly, at 120 weeks postinfection, all four monkeys vaccinated with the MVTT$_{ioin}$+Ad$_{im}$ regimen remained clinically healthy, while three-fourths of the animals in the Ad$_{im}$+Ad$_{im}$ and sham groups developed simian AIDS (Fig. 3c) ($P < 0.05$), as defined by symptoms of diarrhea, wasting and body weight loss, and CD4 T cell decline (Fig. 3d).

To further assess immune protection, sequential PBMC and

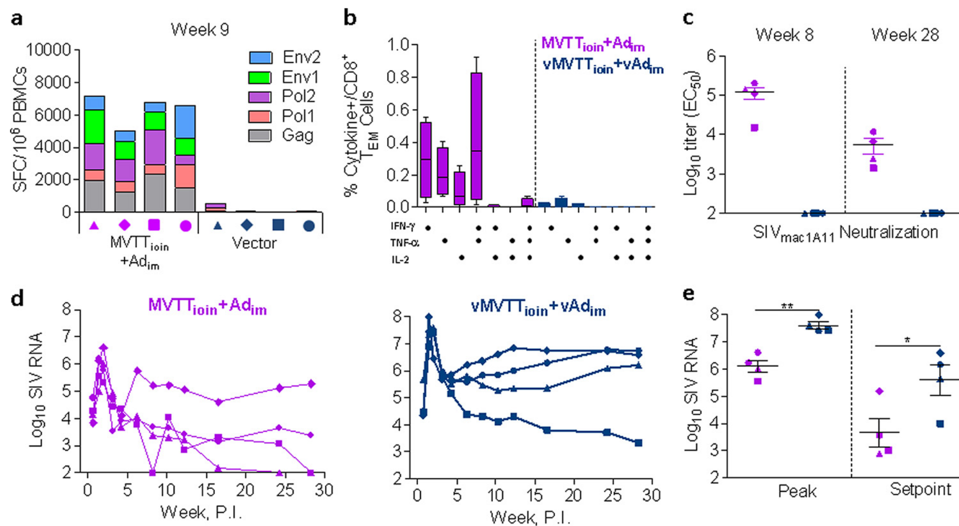

FIG 4 Immunologic and virologic characterization of two groups before and after challenge in study II. (a) Peak levels of SIV-specific IFN-γ ELISPOT responses to the $MVTT_{ioin}+Ad_{im}$ or control vector regimen. (b) Comparison of Gag-specific $CD8^+$ T_{EM} polyfunctional responses in the peripheral blood with secretion of IFN-γ, TNF-α, and IL-2 between the two groups before viral challenge. (c) Comparison of neutralizing antibody responses against $SIV_{mac1A11}$ between the two groups at peak and before viral challenge. (d and e) Temporal comparisons of plasma SIV RNA copies for up to 30 weeks after challenge or at the peak and set point. Error bars in panels b, c, and e represent SEM. *, $P < 0.05$; **, $P < 0.01$.

plasma samples were examined for SIV-specific cellular and humoral immune responses after infection. SIV_{mac239} infection boosted the vaccine-elicited antigen-specific $CD8^+$ T cell ELISPOT responses against the structural proteins Gag, Pol, and Env (Fig. 3e and f). Although the frequencies of positive responses for all three groups peaked at 3 weeks postinfection, response magnitudes were significantly higher in $MVTT_{ioin}+Ad_{im}$ group animals than in $Ad_{im}+Ad_{im}$ group animals (Fig. 3e and f). The frequency of positive responses at the peak was, on average, 7,045 ± 1,395 per million PBMCs for the $MVTT_{ioin}+Ad_{im}$ group, 5,171 ± 2,461 for the $Ad_{im}+Ad_{im}$ group, and 3,235 ± 1,839 for the sham control group (Fig. 3f) ($P < 0.05$). In the majority of animals, peak responses were primarily against Gag and Pol peptides, with Env responses detected in several animals (Fig. 3e). Furthermore, despite a decline in antigen-specific $CD8^+$ T cell ELISPOT responses after the peak responses, IFN-γ-secreting cell levels remained relatively stable in the $MVTT_{ioin}+Ad_{im}$ group, being, on average, above 3,801 ± 1,495 per million PBMCs throughout the subsequent phase of infection (Fig. 3f). It should be noted that there was a transient increase in IFN-γ-secreting cells in both the $Ad_{im}+Ad_{im}$ and sham groups at 28 weeks postinfection (Fig. 3f). This coincided with a rise in viral plasma loads in both groups of animals (Fig. 3a). Interestingly, such biphasic cellular immune responses were more apparent against the nonvaccine antigens Nef, Vif, Vpr, Vpx, Rev, and Tat (Fig. 3g), reflecting the elevated responses triggered by the rise of viral replication in the $Ad_{im}+Ad_{im}$ and sham groups. Collectively, these findings suggest that the $MVTT_{ioin}+Ad_{im}$ and $Ad_{im}+Ad_{im}$ vaccine regimens generated distinct immune response profiles, which may be a critical factor behind the observed differences in clinical outcomes. Finally, in terms of antibody responses after viral challenge, only minimal differences in anti-$SIV_{mac1A11}$ binding and neutralizing antibody responses were observed between the two vaccine regimens, except in monkey 2 (Fig. 3h, i, and j). In monkey 2, no viral load (Fig. 3a), anti-SIV ELISPOT responses against nonstructural antigens, or boosted binding and neutralizing antibodies against $SIV_{mac1A11}$ (Fig. 3h and j) were detectable, indicating a predisposed vaccine-boosted protection from infection.

To further assess the enhanced immunogenicity and protective capacity of the $MVTT_{ioin}+Ad_{im}$ regimen, study II was conducted, with the same vaccination and viral challenge schedule for an additional four macaques (Fig. 1). A second control group of four macaques was inoculated with empty MVTT and Ad5 viral vectors, following the previous routes and dosages (Fig. 1). As in study I, sequential PBMC and plasma samples were collected and used to monitor cellular and humoral immune responses during the trial period. Consistent with our initial results, the $MVTT_{ioin}+Ad_{im}$ regimen elicited high levels of $CD8^+$ T cell ELISPOT responses against Gag, Pol, and Env (Fig. 4a). The frequency of positive responses in the $MVTT_{ioin}+Ad_{im}$ group peaked 9 weeks after initial immunization, at an average of 5,336 ± 1,459 IFN-γ-secreting cells per million PBMCs (Fig. 4a). Responses were predominantly targeted to Gag and Pol peptides, with an appreciable portion found against Env (Fig. 4a). Equivalent to the results of study I, high levels of Gag-specific $CD8^+$ T_{EM} cells secreting both IFN-γ and TNF-α were identified (Fig. 4b). In terms of antibody responses, high levels of neutralizing antibody titers against the tier 1 virus $SIV_{mac1A11}$ were observed again at week 8 after initial immunization and preceding viral challenge (week 28) (Fig. 4c). More importantly, the regimen also reproduced the observed protection against the SIV_{mac239} intrarectal challenge, as demonstrated by 1.52-log (6.09 ± 0.44 versus 7.61 ± 0.27 log copies of SIV RNA) and 1.9-log (3.65 ± 1.06 versus 5.60 ± 1.14 log copies of SIV RNA) reductions in peak and set-point viral loads, respectively, compared with the vector control group (Fig. 4d and e) ($P < 0.05$). Together, studies I and II provide strong evidence that the $MVTT_{ioin}+Ad_{im}$ regimen induces a superior, more potent and antigen-specific immune response against pathogenic SIV_{mac239} infection in Chinese rhesus monkeys than that induced by the $Ad_{im}+Ad_{im}$ regimen.

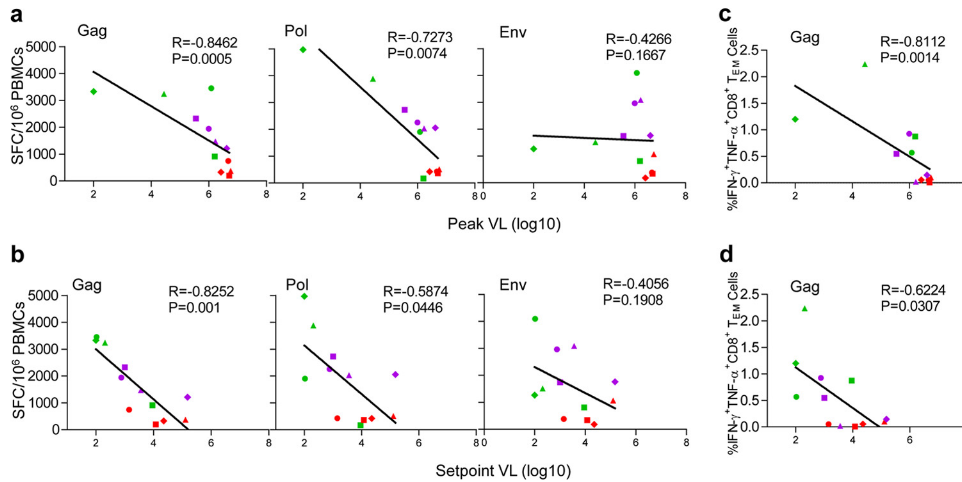

FIG 5 Analysis of immune correlates of protection in $MVTT_{ioin}$+Ad_{im}- and Ad_{im}+Ad_{im}-vaccinated animals. (a and b) Negative correlation between the peak and set-point viral loads and SIV-specific $CD8^+$ T cell ELISPOT responses against Gag and Pol but not Env. (c and d) Negative correlation between the percentages of Gag-specific IFN-γ- and TNF-α-positive $CD8^+$ T_{EM} cells and peak and set-point viral loads.

SIV-specific $CD8^+$ T cell-mediated immunity against Gag and Pol is associated with protection. Finally, we investigated the protective effects of the two regimens against SIV_{mac239} infection, with eight animals vaccinated with the $MVTT_{ioin}$+Ad_{im} regimen and four vaccinated with the Ad_{im}+Ad_{im} regimen. Regression analysis revealed that prior to viral challenge, the peak SIV-specific $CD8^+$ T cell ELISPOT responses against Gag and Pol, but not Env, were negatively correlated with both the peak viral load (Fig. 5a) ($P = 0.0005$, $P = 0.0074$, and $P = 0.1667$) and the set-point viral load (Fig. 5b) ($P = 0.0010$, $P = 0.0446$, and $P = 0.1908$). The percentage of Gag-specific IFN-γ^+ TNF-α^+ $CD8^+$ T_{EM} cells before challenge was also associated with virologic control (Fig. 5c and d) ($P = 0.0014$ and $P = 0.031$). These findings indicate that SIV-specific $CD8^+$ T cell-mediated immunity against Gag and Pol is associated with the control of SIV_{mac239} replication and attenuation of disease progression.

DISCUSSION

We report here a potent replicating vaccinia virus/adenovirus vector-based vaccine regimen that directly primes the mucosal system for robust and sustainable immunity against neutralization-resistant SIV_{mac239} challenge in monkeys. The novelty of this regimen lies in the replicating nature of MVTT, an attenuated version of the parental VTT strain that was used for the eradication of smallpox in China. With the proven safety and efficacy of VTT in humans, a replicating vector was hypothesized to improve both the quantity and the quality of vaccine antigens through *de novo* synthesis and multiple yet limited rounds of replication that highly mimic the early steps in a wild-type infection. This hypothesis is well supported by the results presented in this work and elsewhere for the use of a replicating recombinant Ad5 vector to prime the mucosal system (30, 40–49). Among the eight $MVTT_{ioin}$+Ad_{im}-vaccinated monkeys, one was completely protected from infection, while the others displayed significantly lower peak and set-point viral loads than those of the control groups. Although some other vector-based strategies have obtained partial protection against viral acquisition and/or replication (12–15, 25, 58, 59), no previous vaccine candidates have shown efficacy similar to that reported here against a single high-dose mucosal challenge (5×10^5 $TCID_{50}$ per animal). Indeed, prior studies applied multiple low-dose challenges that were about 1,000- to 10,000-fold lower than that in this report (12, 13, 27–30). The drastic dosage difference between our study and others highlights the exceptionally high levels of protective immunity induced by our vaccine regimen.

We found that SIV-specific $CD8^+$ T cell ELISPOT responses against Gag and Pol, but not Env, and Gag-specific IFN-γ^+ TNF-α^+ $CD8^+$ T_{EM} cell responses were associated with the virologic control after challenge. These findings are unique compared with other studies, for two reasons. First, the SIV-specific $CD8^+$ T cell ELISPOT responses induced by the $MVTT_{ioin}$+Ad_{im} regimen were about 2- to 3-fold higher than those in previous reports (12, 13, 15, 22, 58); second, the $MVTT_{ioin}$+Ad_{im} regimen combined key correlates induced by other promising vaccine approaches, such as persistently replicating cytomegalovirus-based vaccines, nonreplicating poxvirus and adenovirus vector-based vaccines (12, 13), and a recombinant yellow fever 17D (rYF17D) prime vaccine boosted with recombinant Ad5 (59). For instance, a SIV vaccine based on persistently replicating cytomegalovirus from rhesus monkeys (RhCMV) was able to induce persistent, high-frequency SIV-specific T_{EM} responses, which is one of the immunologic correlates identified for the robust control of SIV_{mac239} challenge (13, 14). In particular, the $CD8^+$ T_{EM} cells induced by RhCMV were associated with higher levels of IFN-γ and TNF-α but not IL-2 (13, 14), as in our study. This polarized T_{EM} phenotype may indicate cytotoxic potential upon antigen recognition and stimulation. Furthermore, SIV vaccine regimens based on nonreplicating poxvirus and adenovirus vectors have been used to induce protective immunity in monkeys, with protection against viral acquisition correlated with the Env-specific binding and neutralizing activity, as well as attenuated replication and disease progression with Gag ELISPOT responses (12). These changes are consistent with those induced by our vaccine strategy, although we identified $CD8^+$ T cell ELISPOT responses against Pol as an additional correlate of protection.

Several caveats of our study and related conclusions should be noted. First, the number of animals was relatively small, limiting

the statistical power of these findings. Future studies with a reasonably large number of animals are necessary to more concretely characterize the protective immunity associated with the $MVTT_{ioin}+Ad_{im}$ regimen. Furthermore, it would be ideal to examine the protective potential of the $MVTT_{ioin}+Ad_{im}$ regimen against virus swarms through repeated low-dose rather than single high-dose challenge, to better mimic viral inoculums during natural HIV/SIV transmission. Second, it is uncertain whether any of the observed SIV-specific immune protection against high-dose viral challenge was due to unique genotypic and/or phenotypic features of Chinese rhesus monkeys. In particular, the relatively low immunogenicity of the $Ad_{im}+Ad_{im}$ regimen in Chinese monkeys was somewhat surprising. High titers of neutralizing antibody against the Ad5 vector induced in the priming step may have attenuated the boosting effect, resulting in decreased rather than increased SIV-specific immune responses. Why this restriction was biased toward cellular but not humoral responses requires future investigation. Furthermore, clear differences in major histocompatibility complex (MHC) allele compositions (60), as well as rates of disease progression after SIV infection (61), have been reported between Indian and Chinese rhesus monkeys. In general, Indian monkeys have shown progression to AIDS in a relatively short period, whereas Chinese monkeys have demonstrated a more prolonged progression similar to that of HIV-1 infection in humans (61). We should note that none of our animals carried the protective allele, Mamu-A1*01, found in Indian monkeys. Third, it would be ideal to use a replicating recombinant Ad5 intraoral and intranasal prime plus a nonreplicating Ad5 intramuscular boost as an additional control for the $MVTT_{ioin}+Ad_{im}$ regimen, to more precisely evaluate the attributed immune responses induced by the replicating $MVTT_{SIVgpe}$ strain. Fourth, although intraoral and intranasal immunization was used in this study, we decided not to collect mucosal samples for virologic and immunologic characterization in order to maintain the integrity of mucosal surfaces for subsequent challenge. It is therefore uncertain whether immune responses specific to the mucosal surface correlated with protection and disease attenuation in infected animals. Fifth, although our results clearly show that the $MVTT_{ioin}+Ad_{im}$ regimen is superior to the $Ad_{im}+Ad_{im}$ regimen, it is uncertain to what extent this can be attributed to the use of heterologous virus vectors for the prime and boost versus mucosal priming with MVTT *per se*. Future studies comparing this approach with other routes of initial priming will provide a more definitive answer to this question. Nevertheless, the exceptional protective capability and unique immunologic correlates induced by the $MVTT_{ioin}+Ad_{im}$ regimen highlight the potential of a combination of mucosal priming with a replicating vaccinia virus and a nonreplicating adenovirus boost. It should serve as an important, novel strategy for the development of an effective vaccine against HIV-1 infection.

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