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Enhanced T-Cell Immunogenicity and Protective Efficacy of a Human Immunodeficiency Virus Type 1 Vaccine Regimen Consisting of Consecutive Priming with DNA and Boosting with Recombinant Fowlpox Virus

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The induction of human immunodeficiency virus (HIV)-specific T-cell responses is widely seen as critical to the development of effective immunity to HIV type 1 (HIV-1). Plasmid DNA and recombinant fowlpox virus (rFPV) vaccines are among the most promising safe HIV-1 vaccine candidates. However, the immunity induced by either vaccine alone may be insufficient to provide durable protection against HIV-1 infection. We evaluated a consecutive immunization strategy involving priming with DNA and boosting with rFPV vaccines encoding common HIV-1 antigens. In mice, this approach induced greater HIV-1-specific immunity than either vector alone and protected mice from challenge with a recombinant vaccinia virus expressing HIV-1 antigens. In macaques, a dramatic boosting effect on DNA vaccine-primed HIV-1-specific helper and cytotoxic T-lymphocyte responses, but a decline in HIV-1 antibody titers, was observed following rFPV immunization. The vaccine regimen protected macaques from an intravenous HIV-1 challenge, with the resistance most likely mediated by T-cell responses. These studies suggest a safe strategy for the enhanced generation of T-cell-mediated protective immunity to HIV-1.

A safe and effective vaccine for human immunodeficiency virus type 1 (HIV-1) infection is urgently needed to curb the HIV-1 pandemic. The rational design of HIV-1 vaccines would be facilitated by a thorough knowledge of the immune correlates of protective immunity. Much circumstantial evidence suggests that HIV-1-specific T-cell responses may facilitate protective immunity. Individuals exposed to HIV-1 but who do not become persistently infected develop HIV-1-specific cytotoxic T lymphocytes (CTL) and T-helper (Th) lymphocytes without the generation of systemic HIV-1 antibodies, although mucosal HIV-1 antibodies have also been detected (34, 40). The generation of CTL and Th responses, but not antibodies, temporally correlates with the control of acute HIV-1 viremia in humans and macaques (26, 28, 39). The induction of HIV-1-specific CTL and Th responses is widely seen as critical to the success of an HIV-1 vaccine.

Early candidate HIV-1 vaccine regimens employed only nonreplicating compounds such as recombinant HIV-1 proteins. Vaccination of humans or nonhuman primates with recombinant proteins of HIV-1 or simian immunodeficiency virus (SIV) (a simian homologue of HIV-1) generated specific antibody responses but did not generally induce protective immunity in animal studies and resulted in significant numbers of breakthrough HIV-1 infections in small human trials (7, 45). Subsequent HIV-1 vaccine strategies attempting to induce both enhanced T-cell responses and antibody responses have focused primarily on recombinant vaccinia virus (rVV) and recombinant avian poxviruses (canarypox viruses and fowlpox viruses [FPVs]) genetically engineered to express HIV-1 proteins boosted by recombinant HIV-1 proteins (17). The use of recombinant poxvirus vectors has the theoretical advantage that expression of foreign genes from within the infected host cells allows the loading of major histocompatibility complex (MHC) class I molecules with immunogenic peptides and the stimulation of CTL responses. Unfortunately, vaccinations of humans and outbred nonhuman primates with poxvirus vectors expressing HIV-1 or SIV antigens and recombinant HIV-1 or SIV proteins, despite being theoretically attractive, have induced detectable HIV-1- or SIV-specific CTL responses in only a minority of recipients (9, 16, 18, 19, 25). Further, poxvirus-based regimens have demonstrated limited protective efficacy in SIV-macaque studies and have failed to prevent cases of HIV-1 infection in small human clinical trials (12, 19, 24). Considerable scope exists to improve the ability of poxvirus vectors to induce CTL responses and provide protective immunity.

Recombinant protein vaccinations, while facilitating a strong antibody response, stimulate primarily a particular subset of Th cells called Th2 cells, which are defined by their secretion of the cytokines interleukin-4 (IL-4), IL-5, and IL-10. Th2 cells and the cytokines they secrete may counteract any protective cell-mediated immunity (24, 43). In response to many pathogens and vaccines, humoral and cell-mediated immunities are mutually antagonistic; that is, the immune system supports either a strong Th1 response, (associated with IL-2 and gamma interferon [IFN-γ] production and enhanced CTL responses) or a strong Th2 response, each at least at the partial expense of the other. Although arguably desirable, it may not be feasible

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for an HIV-1 vaccine regimen to induce both strong, sustained antibody and CTL responses (41). A vaccine regimen that reproducibly induces predominantly Th1 and CTL responses to HIV-1 could potentially generate stronger T-cell responses than one that endeavors to induce both antibody and Th1-CTL responses.

Intramuscular (i.m.) or epidermal injection of purified plasmid DNA can induce immune responses to encoded antigens (46). Plasmid DNA vaccines, which are simple and inexpensive to produce, have the potential to revolutionize or reenergize many vaccine development fields, including that of HIV-1. i.m. injection of DNA encoding HIV-1 proteins into two chimpanzees generated HIV-1-specific CTL responses in one the animals and induced some protection from nonpathogenic HIV-1_{SF2} infection in both animals (3). When i.m. HIV-1 DNA vaccination of two macaques was boosted by recombinant protein vaccination, protection of the two macaques from nonpathogenic $SHIV_{HXB2}$ infection was observed (31). Although the antibody response was enhanced approximately 100-fold by recombinant protein boosting of macaques primed with i.m. DNA, the HIV-1-specific CTL precursor levels were augmented <2-fold by the recombinant protein boosting and remained at a low level (<15 CTL/10^6 peripheral blood mononuclear cells [PBMC]) (31). DNA vaccines alone have resulted in only very limited protection from pathogenic SIV or nonpathogenic $SHIV_{HXB2}$ infection of macaques (4, 32). Thus, although both DNA and avipoxvirus vectors show promise as HIV-1 vaccine candidates, considerable potential exists for novel strategies designed to enhance the T-cell immunogenicity and efficacy of both DNA and avipoxvirus vaccine vectors.

We have previously reported a consecutive immunization strategy involving priming by DNA vaccination and boosting with recombinant FPV (rFPV) vectors encoding common influenza virus antigens in attempts to generate improved specific immune responses (30). The rationale behind this vaccine strategy was that DNA immunization, which elicits low-level but persistent immunity, may prime for greatly enhanced T-cell responsiveness following boosting with another vaccine vector that expresses vaccine antigens from within host cells and therefore loads MHC class I molecules efficiently, such as rFPV. Recombinant avipoxvirus vectors may have advantages over rVV for use as a boosting vaccine vector. First, avipoxvirus vaccines, which cause an abortive infection in mammalian hosts, are safer, since wild-type VV can cause a lethal infection in immunodeficient humans. Second, antigenic competition from immune responses to vector antigens is likely to be lower for the weakly replicating avipoxviruses than for rVV, where the immune response to the vector is robust. Last, the lower level of antigen production from avipoxvirus vaccines compared to rVV may preferentially stimulate T-cell rather than antibody responses. For pathogens such as HIV-1, where T-cell-mediated responses may be required for protective efficacy, avoiding a marked enhancement of antibody-Th2 responses, as is typically observed following recombinant protein boosting, may facilitate enhanced Th1-CTL responsiveness and may therefore be desirable. We assessed an immunization strategy which employed priming the immune system with DNA encoding HIV-1 antigens and boosting with rFPV encoding shared HIV-1 antigens. We evaluated the immunogenicity and initial protective efficacy of this regimen in mice by using an rVV-based challenge model and in macaques by employing a nonpathogenic HIV-1 challenge system.

MATERIALS AND METHODS

Animals. Murine studies used groups of five or six specific-pathogen-free, 6- to 9-week-old CBA/H mice. Macaques (*Macaca nemestrina*, aged 8 to 16 months) were free from HIV-1/SIV/STLV/SRV infection and were anesthetized with Ketamine (10 mg/kg i.m.) prior to procedures. The studies were approved by the institutional Animal Experimentation and Ethics Committees. All macaques were vaccinated with three doses of tetanus toxoid (CSL, Parkville, Australia) i.m. prior to HIV-1 vaccinations. Macaques were evaluated once or twice weekly following HIV-1 challenge for the presence of a truncal rash or inguinal lymphadenopathy (>1.2 cm in diameter) associated with acute HIV-1 infection. Macaque B-lymphoblastoid cell lines (BLCL) were established from each macaque by infecting PBMC with supernatant from S394-1X1055 cells containing herpesvirus papio, a baboon herpesvirus, as previously described (26). BLCL could not be transformed from PBMC of one vaccinated animal (M4), and CTL data could not be generated from that animal.

Recombinant poxviruses. rVV expressing the *gag*, *env*, or *nef* gene of HIV-1_{LAI} or the *pol* gene of HIV-1_{HXB2} was used as previously described (23, 26). An rVV containing the HIV-1_{LAI} *env*, *gag*, and *pol* genes (denoted vac-env/gag/pol) and an rFPV containing HIV-1_{BH10} *env* (FP66) were kindly made available by D. Panicali, Therion Biologics, Cambridge, Mass. (35). For the murine challenge experiment, an rVV (WR strain) containing *gag* and *pol* was constructed by insertion of a chimeric promoter HIV-1_{SF2} *gag-pol* fragment (pGEM4z; Chiron) into the *Hin*dIIIJ (TK) region of VV (10). Expression of Gag and Pol by vac-gag/pol was confirmed by Western blotting. rVV titers in murine ovaries were assessed by a plaque assay on 143B human T-cell lines, with the limit of detection being 100 PFU (38).

An rFPV expressing *gag*, *pro*, and *pol* from HIV-1_{SF2} was constructed by using the insertion vector pAF09 and the parent FPV, FPV-M3, by techniques previously described (20). The *gag* and *pol* sequences were inserted under the control of the FPV P.E/L promoter such that the native *gag-pol* is expressed from the initiation of the P.E/L promoter. Western blotting and immunofluorescence analyses confirmed expression of the *gag* and *pol* genes. rFPV gag/pol was delivered intravenously to mice (10^7 PFU), and rFPV gag/pol and rFPVenv (10^8 PFU each) were delivered by skin scarification over left and right deltoid regions of macaques, respectively.

Immunizations. DNA immunizations employed the plasmid pNL4.3dpol, expressing *env* and *gag* genes of HIV-$1_{NL4.3}$ from a cytomegalovirus promoter, or control DNA containing *lacZ* (33). For gene gun immunizations, plasmid DNA (100 μg) was attached to gold particles by adding 100 μl of 0.1 M spermidine to a 1.5-ml centrifuge tube containing 50 mg of 0.95-μm-diameter gold beads (kindly provided by Powderject, Middleton, Wis.). The DNA and gold were coprecipitated with 200 μl of 2.5 M $CaCl_2$ during vortex mixing. The precipitate was washed and resuspended in ethanol (7.0 mg/ml). The DNA-gold suspension was sonicated and drawn up into Telzel-R tubing (McMaster-Carr, Los Angeles, Calif.), the ethanol was aspirated off with a peristaltic pump, and the gold particles were evenly smeared on the tubing by using a tube turner (Powderject) and then dried with nitrogen at 400 ml/min. The gold particle-lined tubing was cut into 1.25-cm pieces containing 0.5 mg of gold and 1 μg of DNA.

DNA-coated gold particles were delivered to shaved abdominal epidermis by using the hand-held, helium-driven Accell gene delivery system (Powderject). Animals were immunized at a helium pressure of 400 lb/in^2 (mice) or 350 lb/in^2 (macaques) with four nonoverlapping deliveries, each containing 1 μg of DNA. Control unimmunized mice received no DNA or rFPV vaccinations, and control macaques received pCMVlacZ DNA and FPV-M3 not expressing HIV-1 genes. Mice immunized i.m. with DNA received 50 μg of DNA in normal saline. Mice received two doses of DNA 4 weeks apart, followed after a further 8 weeks by one dose of rFPV, and were challenged 2 weeks later. Macaques received the DNA and rFPV vaccines at the times noted in Fig. 1.

Antibody and T-cell-proliferative responses. Macaque sera were assessed for antibodies to HIV-1 by three techniques: particle agglutination (Serodia-HIV, Fujirebio, Japan), competitive enzyme immunoassay (EIA) (Wellcozyme HIV Recombinant; Murex, Dartford, United Kingdom), and Western blotting with 200 μg of standard mixed HIV-1 protein stock (26). To detect immunoglobulin G2a (IgG2a) Gag antibody responses in mice, recombinant HIV-1_{SF2} p24 protein (a gift from Chiron, Emeryville, Calif.) was applied to plates (Dynatech, Chantilly, Va.) in bicarbonate buffer (pH 9.6) at 25 ng/well and left for 12 to 24 h at 40°C. After washing and blocking with 10% skim milk powder, serial serum dilutions starting at 1:10 were added at 20°C, left for 1 to 2 h, and subsequently washed. Biotinylated goat anti-mouse IgG2a antibody (Southern Biotechnology Associates, Inc.; 1:100 dilution) was added and washed off, and streptavidin-alkaline phosphatase conjugate (Amersham; 1:100 dilution) was added and left for a further 1 h. Color was developed by treatment with alkaline buffer solution (Sigma) for 30 min, and absorbance was determined at 405 nm.

Lymphoproliferative responses were assessed by a standard [^{3}H]thymidine incorporation assay as described previously (26). Briefly, macaque PBMC in triplicate wells at 10^5 cells/well were stimulated for 6 days with 10 μg of recombinant HIV-1_{MN} gp160 or HIV-1_{LAI} gp160 (MicroGeneSys Inc., Meriden, Conn.) or HIV-1_{SF2} gp120 or HIV-1_{SF2} p24 (Chiron) per ml in medium containing 10% autologous heat-inactivated serum and pulsed with [^{3}H]thymidine for 18 h before beta counting. PBMC were also incubated with medium alone or medium supplemented with 10 μg of baculovirus culture-derived control antigens (MicroGeneSys) per ml to assess unstimulated control responses and were stimulated with phytohemagglutinin (PHA) (10 μg/ml) or tetanus toxoid antigen (0.01 Lf/ml) as positive mitogenic and antigenic response controls. Proliferation is expressed as the stimulation index (SI) (mean [^{3}H]thymidine incorporation of

cells stimulated with antigen/mean incorporation in the absence of antigenic stimulation). Supernatants from selected lymphoproliferative cultures were assayed for the presence of IL-4 and IFN-γ by EIA (Genzyme, Cambridge, Mass.).

CTL responses. CTL activity in macaque PBMC was assessed in vitro by two weekly cycles of stimulation as previously described (26). Briefly, for the first cycle of stimulation, autologous PBMC stimulators were infected with vac-env/gag/pol and incubated with freshly isolated PBMC at a ratio of 10 responders to 1 stimulator cell for 7 days. Stimulation cycle 2 used autologous BLCL infected with vac-env/gag/pol as stimulators. Cytolytic activity was measured in a standard ^{51}Cr release assay. Autologous BLCL targets were infected with a panel of rVVs, labelled with ^{51}Cr, and added to the stimulated effector cells at various effector/target ratios to a total volume of 200 μl. After a 4-h incubation, 50 μl of each well was sampled and radioactivity was counted. Percent specific lysis of targets was calculated by the standard formula. The standard deviation for triplicate wells was less than 8%, and spontaneous release was less than 24% of the maximal release. Background lysis of control targets expressing VV antigens alone (<5% lysis in all cases) was subtracted to yield net percent specific lysis.

Quantification of CTL precursors. Analysis of CTL precursor responses to HIV-1 Env, Gag, and Pol antigens in PBMC of macaques was performed by a limiting-dilution analysis as described previously (26). Briefly, fresh PBMC were plated in 96-well round-bottomed plates in seven serial twofold dilutions of 1×10^5 to 1.5×10^3 cells/well in 24 replicates. Each well was stimulated with 10^4 autologous vac-env/gag/pol-infected PBMC and supplemented with 10 U of rIL-2 (Hoffman-La Roche, Nutley, N.J.) per ml every 3 to 4 days. After 10 to 14 days, cells in each well were divided and assayed for cytolytic activity against autologous BLCL targets expressing Env, Gag, and Pol or VV antigens alone. Wells were considered positive against a particular target if cytolysis exceeded the mean spontaneous release from that target by 3 standard deviations. CTL frequencies and 95% confidence intervals were determined by maximum-likelihood analysis with software provided by S. Kalams, Harvard Medical School (13).

HIV-1 challenge of macaques. HIV-1_{LAI} (provided by M. Agy, University of Washington, Seattle) was expanded separately in PHA-stimulated PBMC obtained prior to immunizations from the each of the eight macaques under study as described previously (26). HIV-1 titers in filtered supernatants were quantified on CEMx174 cells and ranged from $10^{5.1}$ to $10^{5.9}$ 50% tissue culture infective doses ($TCID_{50}$). The equivalent of 10^5 $TCID_{50}$ of cell-free HIV-1_{LAI} grown in autologous PBMC in 1 ml was administered to the femoral veins of all eight animals at 38 weeks following the initial DNA vaccination and 6 weeks after the last rFPV vaccination.

Plasma HIV-1 RNA was assessed by reverse transcription-PCR (Amplicor HIV Monitor with ultrasensitive specimen preparation; Roche Diagnostic Systems, Branchburg, N.J.) (limit of detection, 20 copies/ml) at 1 to 2 weeks following infection. Virus isolation was performed by cocultivating 10^6 macaque PBMC or lymph node mononuclear cells (LNMC) obtained between weeks 2 and 8 following infection with 10^6 PHA-stimulated pooled human PBMC in medium containing 50 U of rIL-2 per ml. Fresh medium and IL-2 were added to the cultures twice weekly, and PHA-stimulated human PBMC were added weekly, for 4 weeks. HIV-1 in culture supernatants was quantified by an HIV-1 p24 EIA (Abbott Laboratories, Abbott Park, Ill.).

HIV-1 *gag* and HLA-DQ DNAs were amplified from extracted DNA from PBMC and LNMC samples and quantified by using primer pairs SK38-39 and GH26-27 (Gibco-BRL), respectively, with PCR conditions as described previously (26). DNA from 10^6 PBMC or LNMC was standardized to the equivalent of 10^5 cells according to the DQ band density in comparison to 8E5 cell DNA (which contains one HIV-1 DNA copy per cell) and confirmed by measuring absorbance on a spectrophotometer (Ultrospec 3000; Pharmacia Biotech) at 260 nm. Positive PCR signals were confirmed to be HIV-1 specific by a nested PCR protocol whereby extracted DNA was first amplified with external HIV-1 primers A2 and B2 (0.5 μM; Gibco) prior to amplification with the internal primers SK38 and -39 (44). Negative PCR signals were confirmed to be negative by repeating the PCR with 5- to 10-fold more input DNA.

RESULTS

Immunogenicity and efficacy of an HIV-1 DNA-rFPV regimen in mice. The ability of a consecutive HIV-1 immunization strategy with DNA and rFPV to induce protective T-cell-mediated immunity was first evaluated in mice. The HIV-1 DNA priming component of the vaccine strategy was delivered either i.m. or epidermally to determine the relative immunogenicity of each delivery method. Groups of mice were given an intravenous booster inoculum of rFPV containing the *gag* and *pol* genes of HIV-1 4 weeks after DNA priming and were subsequently challenged (2 weeks later) with rVV expressing Gag and Pol antigens. This challenge system was chosen because the clearance of the rVV challenge is dependent on vaccine-induced T cells recognizing the genes expressed by the rVV. It was originally shown that priming of mice with influenza virus generates specific antiviral T cells that control subsequent infection with rVV expressing influenza virus proteins (14). Similarly, DNA and rFPV expressing influenza virus proteins induce T-cell-mediated protection against a challenge with rVV expressing influenza virus proteins (38). We found that a challenge with rVV encoding Gag antigens was efficiently controlled in mice which had been immunized consecutively with DNA and rFPV encoding Gag antigens (Table 1). Mice primed with DNA both i.m. and epidermally showed significant levels of protection against rVV challenge, indicating that T-cell responses were induced by both delivery methods, although the level of protection observed with the epidermal regimen was higher.

TABLE 1. Protection from challenge with rVV expressing HIV-1 Gag antigens following consecutive immunizations of mice with DNA and FPV expressing HIV-1 Gag

Immunization regimen	vac-gag/pol titer[d] following challenge
Control	6.8 ± 0.6
rFPV[a] alone	6.4 ± 0.3
DNA[b] i.m. alone	4.9 ± 0.7
DNA epidermally[c] alone	3.7 ± 0.6
DNA i.m. plus rFPV	3.9 ± 0.7
DNA epidermally plus rFPV	<2.0

[a] FPV expressing HIV-1_{SF2} *gag* and *pol*.
[b] pNL4.3dpol HIV-1 DNA vaccine (33).
[c] Delivered via gene gun to shaved epidermis.
[d] Titer of rVV expressing HIV-1_{SF2} *gag* and *pol* in ovaries. Results are $\log_{10}$ mean PFU ± standard error of the mean. The limit of detection is 2.0.

To address further the immunogenicity of the consecutive DNA and rFPV vaccine regimen in mice, HIV-specific IgG2a antibody levels, which reflect Th1 responses, were assessed (36). Groups of mice primed epidermally with DNA containing *env* and *gag* and boosted with rFPV containing *gag* and *pol* exhibited augmented IgG2a Gag antibody levels (mean titer, 1/36,400) compared to mice given *env-gag*-containing DNA epidermally alone (mean titer, 1/161) or rFPVgag/pol alone (mean titer, <1/10). DNA containing *env* and *gag* delivered by the i.m. route followed by rFPVgag/pol failed to induce detectable levels of anti-Gag IgG2a antibody (mean titer, <1/10).

DNA-rFPV HIV-1 vaccine regimen in macaques. (i) Antibody response. Murine experiments demonstrated enhanced immune responses to antigens following consecutive epidermal DNA and rFPV immunizations. We next assessed a DNA-rFPV immunization regimen in macaques, whose immune system more closely resembles that of humans. Juvenile macaques were injected with Env- and Gag-expressing DNA twice, 8 weeks apart, via the epidermal route by using a gene gun. We chose epidermal administration based on the enhanced immunogenicity of this approach in the murine studies. After a further 8 weeks, rFPV expressing Env and Gag-Pol was given three times, also 8 weeks apart. All vaccines were well tolerated. After the second DNA priming vaccination, HIV-1 antibodies were detected in all four actively vaccinated macaques, but in none of four control animals, by both whole-virus competitive EIA (optical density cutoff/sample ratio of >1) and particle agglutination assays (mean peak endpoint titer, 1:672) (Fig. 1). Immunoblotting demonstrated the presence of antibodies to both Env and Gag antigens in all animals (data not shown). Following each rFPV boost there was no increase in antibody titers. Indeed, a significant (>4-fold) gradual decline was observed in all HIV-1-vaccinated animals despite multiple rFPV vaccinations. Just prior to HIV-1 challenge, 6 weeks after the last rFPV vaccination, whole HIV-1 antibody titers

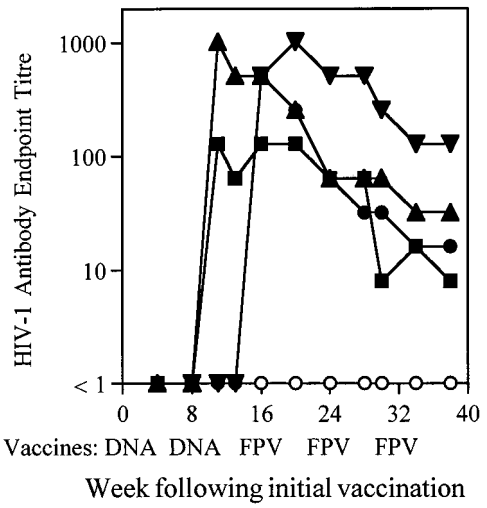

FIG. 1. HIV-1 antibodies in DNA-rFPV-vaccinated macaques. Four HIV-1-vaccinated macaques (M2, M3, M4, and M5) (closed symbols) seroconverted to HIV-1 after the second epidermal administration of plasmid DNA pNL4.3dpol as assessed by a particle agglutination assay. HIV-1 antibodies titers declined (>4-fold) thereafter despite three vaccinations with rFPV expressing HIV-1 Env and Gag-Pol antigens. Vaccination times are noted on the *x* axis. Four control macaques (M7, M8, M9, and M10) (open symbol) received DNA and FPV vaccines not expressing HIV-1 antigens and all had no detectable HIV-1-specific antibodies.

were 1:8 to 1:128 by the particle agglutination assay and were negative in three of four animals by competitive EIA. By immunoblotting both envelope and Gag antibody bands had also declined (≥4-fold decrease in density) in all animals.

(ii) T-cell immunogenicity. Th responses were assessed by antigen-specific proliferation of fresh PBMC obtained from DNA-rFPV-vaccinated macaques over time. Th proliferation in response to both Env and Gag antigens was detected after two initial HIV DNA priming vaccinations, although the response was modest (mean SI of 1.5 to 4) (Fig. 2A). Following rFPV boosting, an enhancement of the Th response to both Env and Gag antigens was observed, with a 6- to 17-fold increase in the mean SI for the HIV-1 antigens tested being detected. The Th response also recognized Env antigens from subtype B strains HIV-1_{MN} and HIV-1_{SF2}, which are heterologous to the immunizing Env subtype B strain HIV-1_{LAI}. The four control animals did not develop a Th response to HIV-1 Env or Gag antigens after either control DNA or FPV vaccinations (SI ≤ 1.6), although all HIV-1-vaccinated and control animals generated a tetanus-specific Th response following tetanus toxoid immunization (mean SI, 6.2; range, 3.5 to 10.1).

The finding that rFPV vaccination markedly enhanced DNA vaccine-primed Th responses, despite a fall in antibody titers, suggested that the rFPV immunogens not only were expressing Env and Gag antigens in vivo but also were preferentially promoting antigen-specific cell-mediated (Th1) rather than humoral (Th2) immunity. We had previously assessed cytokine secretion from HIV-1 and tetanus-specific Th cell cultures in HIV-1-inoculated *M. nemestrina*, demonstrating that IL-4 and IFN-γ could be readily detected in Th cell cultures and that IFN-γ secretion temporally correlated with the resolution of the acute infection process (26). We therefore assessed the cytokine secretion response of the HIV-specific Th cells following both DNA vaccine priming and rFPV boosting. High levels of IFN-γ, but minimal IL-4, were detected in supernatants of gp160-stimulated PBMC from all macaques receiving the consecutive DNA-rFPV HIV-1 vaccine regimen, indicative of a Th1 response (Fig. 2B). In contrast, the antigen-specific control Th response to tetanus toxoid vaccination was of a Th2 phenotype, indicating the capacity of the macaque PBMC under appropriate conditions to secrete IL-4. PBMC from control macaques receiving vaccines not expressing HIV-1 anti-

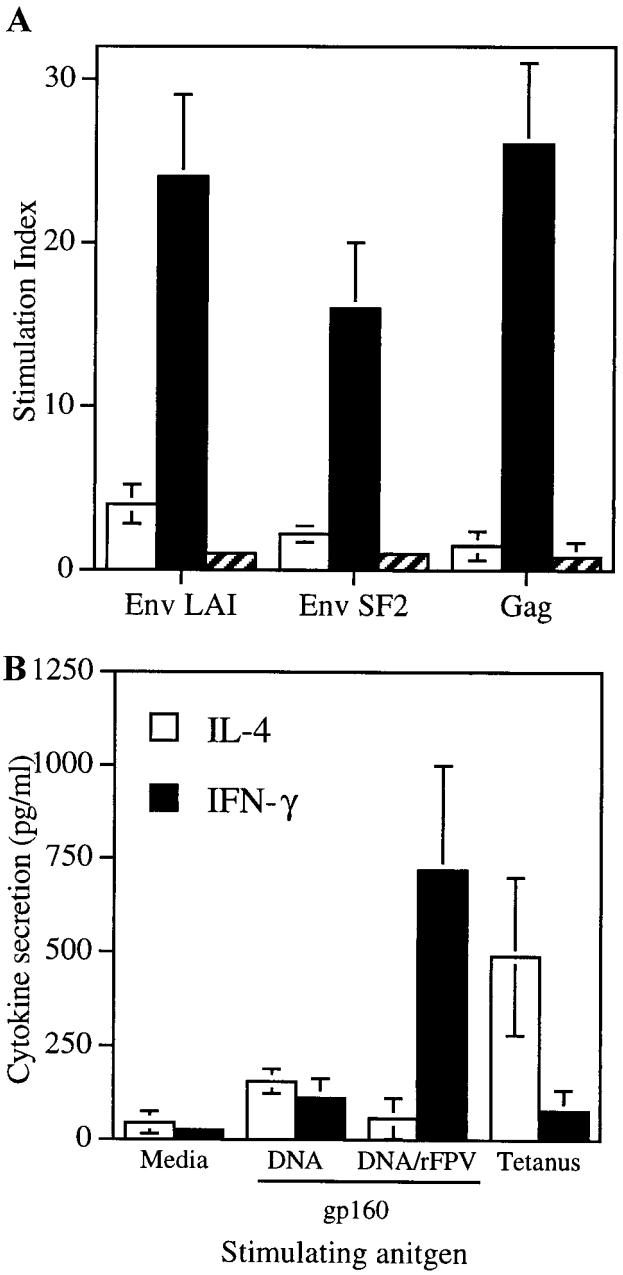

FIG. 2. HIV-1-specific Th responses following DNA-rFPV vaccination. (A) rFPV boosting of DNA-primed macaques resulted in a rise in lymphoproliferative responses to both Gag (p24) and homologous (LAI) and heterologous (SF2) subtype B HIV-1 Env antigens. The SIs (means ± standard errors of the means) of PBMC obtained from four macaques receiving HIV-1-expressing vaccines at week 10 following two DNA vaccinations (open bars) and at week 34 following three rFPV boosts (solid bars) are shown in comparison to those for four unvaccinated macaques (hatched bars). (B) PBMC from macaques vaccinated consecutively with HIV-1-expressing DNA and rFPV secrete significant IFN-γ but minimal IL-4 in response to recombinant HIV-1_{LAI} gp160 protein stimulation. The means ± standard errors of the means of triplicate measurements on the four vaccinated macaques (M2 to -5) after two DNA vaccinations alone (week 14) and after three rFPV boosts (week 38) are shown. PBMC from the same animals had an opposite pattern of cytokine secretion in response to stimulation with tetanus toxoid.

gens did not secrete either IFN-γ (<100 pg/ml) or IL-4 (<25 pg/ml) in response to HIV-1 protein stimulation.

We next evaluated the CTL response to HIV-1 antigens following DNA-rFPV immunization. CTL responses to either Env or Gag antigens (specific lysis of >5%) were detected after initial HIV-1 DNA priming in antigen-stimulated PBMC from two of three vaccinated animals studied (Fig. 3A). Following rFPV boosting, however, a CTL response to the vaccine antigens Env and Gag (common to both the DNA and rFPV vectors) was detectable in PBMC from all three evaluatable animals. No control animal developed an HIV-1-specific CTL response at any time point. Interestingly, only one of three animals developed a CTL response to Pol antigens, which were unique to the rFPV vaccinations, a proportion of CTL responders similar to that observed in human trials of avipoxvirus based vaccine regimens (16, 18). Thus, while neither vector alone was able to uniformly generate detectable CTL responses in this study of outbred nonhuman primates, the combination of both DNA and rFPV vaccines was successful in generating HIV-1-specific CTL responses.

We have previously shown that the generation of approximately one HIV-1 (Env, Gag, and Pol)-specific CTL precursor per 10^4 PBMC correlates temporally with the control of acute HIV-1 viremia in macaques (26). We therefore evaluated the frequency of HIV-specific precursor CTL in PBMC from macaques, after both DNA and rFPV vaccinations, to assess whether the immunogenicity of this vaccine regimen was likely to be sufficient to prevent or limit acute HIV-1 infection. Limiting-dilution assays demonstrated that the strength of the HIV-specific CTL response following rFPV boosting was 3.5- to 20-fold greater than that after DNA priming and approximated levels associated with control of acute HIV-1 infection of macaques (Fig. 3B).

(iii) HIV-1 challenge of macaques. A macaque model of acute HIV-1 infection, which provided clinical and laboratory evidence of acute HIV-1 infection, has been established (26). This challenge model results in low levels of HIV-1 RNA in plasma and is not pathogenic. Although this model does not provide an analysis of protective immunity against pathogenic lentivirus infections, it is suited to an initial evaluation of T-cell-mediated protection from acute HIV-1 infection, since T-cell responses appear to play a role in the control of acute infection in this model. Vaccinated and control macaques in this study were therefore challenged on the same day with cell-free HIV-1_{LAI} to assess the protective efficacy of the vaccine regimen. HIV-1_{LAI} was expanded in autologous PBMC of each challenged animal to limit recognition of foreign antigens coating the challenge inoculum. Previous dose titration studies of chimpanzee challenge stocks of the closely related HIV-1_{IIIB} strain in *M. nemestrina* suggest that the approximate challenge dose used in this study (10^5 $TCID_{50}$ of HIV-1_{LAI}) was ≥100 monkey infectious doses, where infection is defined by seroconversion to multiple HIV-1 antigens, HIV-1 isolation from PBMC, and repeated detection of HIV-1 DNA by PCR (2).

Following HIV-1 inoculation, all four control macaques not receiving the HIV-1 vaccines seroconverted to HIV-1 as determined by competitive EIA (optical density cutoff/sample ratio of >1), particle agglutination assay (endpoint titer, >1/128), and immunoblotting (bands to multiple HIV-1 proteins) by 2 to 6 weeks and demonstrated clinical signs of acute infection, including rash or lymphadenopathy (Table 2), similar to the case in earlier experiments (26). HIV-1 proviral DNA was detected in all serial PBMC samples (36 of 36 samples from the four control animals over 21 weeks) and in all four lymph node samples (at 4 weeks) taken following HIV-1 challenge. HIV-1

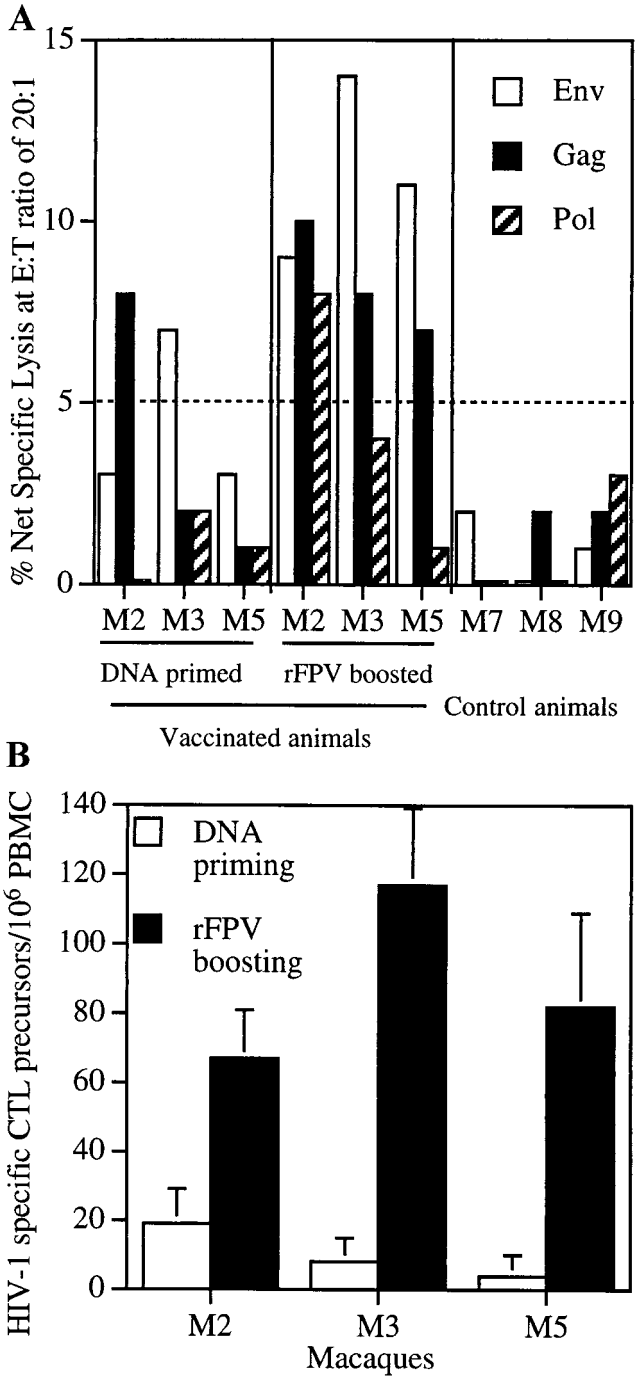

FIG. 3. HIV-1-specific CTL responses following DNA-rFPV vaccinations. (A) Net specific lysis of Env-, Gag-, or Pol-expressing autologous BLCL by antigen-stimulated bulk PBMC from three HIV-1-vaccinated macaques and three control macaques was assessed. CTL activities were determined after two DNA vaccinations alone (expressing Env and Gag antigens; week 10 following initial vaccination) or three subsequent rFPV boosting vaccinations (expressing Env, Gag, and Pol antigens; week 34 following initial vaccination). Greater than 5% net specific lysis (dotted line) was considered significant. E:T, effector/target. (B) Quantification of CTL precursors to Env, Gag, and Pol antigens was done by a limiting-dilution assay following DNA and rFPV vaccinations. CTL frequencies were assessed at week 14 (after DNA vaccinations alone) and week 34 (after rFPV boosting). Recognition of control targets expressing VV antigens alone was $<5/10^6$ PBMC and has been subtracted. CTL precursor frequencies and 95% confidence intervals were calculated by a maximum-likelihood analysis method.

TABLE 2. Protection of DNA-rFPV-vaccinated macaques from HIV-1 challenge

Animal	Acute-infection syndrome (rash/lymphadenopathy)	HIV-1 recovery by coculture[a] at wk:						Plasma HIV-1 RNA (copies/ml) at wk:		HIV-1 DNA detected in:	
		2	3	4	4 (LMNC)	6	8	1	2	PBMC[b]	LNMC[c]
HIV-1 vaccinated											
M2	−/−	−	−	−	−	−	−	<20	<20	0/9	No
M3	−/−	−	−	−	−	−	−	<20	<20	0/9	No
M4	−/−	−	−	−	−	−	−	<20	<20	0/9	No
M5	−/−	−	−	−	−	−	−	<20	<20	1/9	No
Unvaccinated controls											
M7	+/+	+ (14)	+ (7)	+ (7)	+ (7)	+ (14)	−	98	335	9/9	Yes
M8	+/+	−	+ (21)	−	+ (7)	+ (14)	−	258	77	9/9	Yes
M9	+/−	+ (14)	+ (7)	+ (7)	+ (14)	+ (7)	+ (14)	ND[d]	260	9/9	Yes
M10	+/+	−	+ (14)	+ (14)	+ (7)	+ (21)	+ (14)	125	31	9/9	Yes

[a] +, successful attempts (HIV-1 p24 level of >100 pg/ml in coculture supernatant) at recovery of HIV-1 in cocultures of macaque PBMC between weeks 2 and 8 following challenge. At week 4, LNMC were also analyzed. For positive cultures, the day of culture when p24 antigen was detected for the first time is noted in parentheses (culture supernatants were assessed at 7-day intervals to 28 days).
[b] No. of times detected (>1 copy/10^5 cells)/no. of attempts between weeks 1 and 21 following challenge.
[c] LNMC were analyzed at week 4 following challenge.
[d] ND, not done (the internal control RNA for the sample from M9 at week 1 did not amplify, and the result was therefore invalid).

could also be cultured from multiple (at least three of five) PBMC samples obtained between weeks 2 and 8 and from LNMC samples (at week 4) from all control animals following infection. HIV-1 RNA was also detected in the plasma of all control animals at a low level at 1 to 2 weeks following infection.

In contrast, the four animals receiving the HIV-1 antigen-expressing DNA and rFPV vaccines showed no clinical signs of acute HIV-1 infection, and neither HIV-1 RNA (<20 copies/ml) nor DNA (≤1 copy/10^5 PBMC) was detected in plasma or LNMC, respectively (Table 2). HIV-1 could not be cultured from either 10^6 LNMC (at week 4) or 10^6 PBMC sampled multiple times following infection. For the four HIV-1-vaccinated animals, only 1 of 36 serial PBMC samples had detectable HIV-1 DNA. Animal M5 had HIV-1 *gag* DNA (the equivalent of 1 to 3 copies/10^5 PBMC) detected at 2 weeks following challenge but did not have HIV-1 DNA in an LNMC sample taken 2 weeks later or in the seven subsequent PBMC samples taken.

(iv) HIV-specific T-cell responses postchallenge. Although a high degree of protection from HIV-1 infection in the DNA-rFPV-vaccinated animals was observed in this study, we have previously reported that protection from lentivirus challenge is likely to be nonsterilizing in nature and consistent with a role for T-cell responses in the clearance of the challenge virus (25). In the earlier study, however, CTL responses were not induced by the vaccine regimen (rVV priming and recombinant protein boosting). To determine whether the vaccine-induced immunity was sterilizing or nonsterilizing and to assess the potential roles of both vaccine-induced and virus-induced CTL responses following challenge, we assessed the quantitative HIV-1-specific CTL responses specific for the vaccine (and challenge virus) antigens (Env, Gag, and Pol) and, qualitatively, whether a CTL response to HIV-1 Nef antigens, expressed by the challenge virus but not by the vaccines, was generated early following HIV-1 challenge. A rise in HIV-1-specific CTL frequencies was observed in protected animals following challenge (Fig. 4A), suggesting that the CTL induced by the vaccine regimen recognized the challenge virus. Unvaccinated control animals also developed a CTL response to HIV-1 infection, as previously reported (26), although the CTL response was less vigorous than that of the animals with prechallenge anti-HIV-1 immune responses. Further, a CTL response to HIV-1 Nef antigens was detected early (2 weeks) following HIV-1 challenge in the two vaccinated animals studied, a time prior to the detection of Nef-specific responses in the two unvaccinated animals studied (Fig. 4B). Nef-specific CTL responses were not detected prior to challenge.

DISCUSSION

Vaccination with DNA and rFPV vectors offers great hope for improved immunoprophylaxis against infections caused by a wide range of pathogens, including HIV-1. Our studies have focused on the development of a vaccination strategy based on consecutive immunization with DNA plasmids and rFPV encoding a common vaccine antigen. Both vectors appear to be safe, but alone, they elicit relatively low levels of immunity and induce CTL responses in only a portion of outbred primate recipients (3, 16). In this study, the sequential use of DNA and rFPV vaccines elicited high levels of immunity to the encoded antigens and uniformly induced detectable HIV-1-specific CTL responses. One factor that may be important in the enhanced immunogenicity is the lack of host responses to vector-specific antigens that may minimize the prospects of antigenic competition, allowing the immune response to be directed almost entirely against the heterologous vaccine antigen. DNA vaccination may, perhaps because of continued low levels of antigens produced over time, provide a sustained priming effect for antiviral immune responses (27). A similar prime-boost strategy, using an attenuated rVV as the boosting vector, was recently reported to induce T-cell-mediated protection from malaria in a murine model (42). We found that epidermally delivered DNA vaccines were a particularly effective means of priming for T-cell responses, possibly due to the relatively small amounts of antigen expressed (which may preferentially stimulate T cells with higher-affinity receptors) and the richness of Langerhans cells, potent antigen-presenting cells, in the epidermis.

The strategy of priming with DNA and boosting with rFPV encoding HIV-1 antigens was found to generate primarily Th1 and CTL responses rather than antibodies, a potentially desirable property of an HIV-1 vaccine regimen given previous observations on the immune correlates of the control of HIV-1 in humans (40). When a DNA-rFPV HIV-1 vaccine approach was assessed in mice, an enhanced IgG2a response was de-

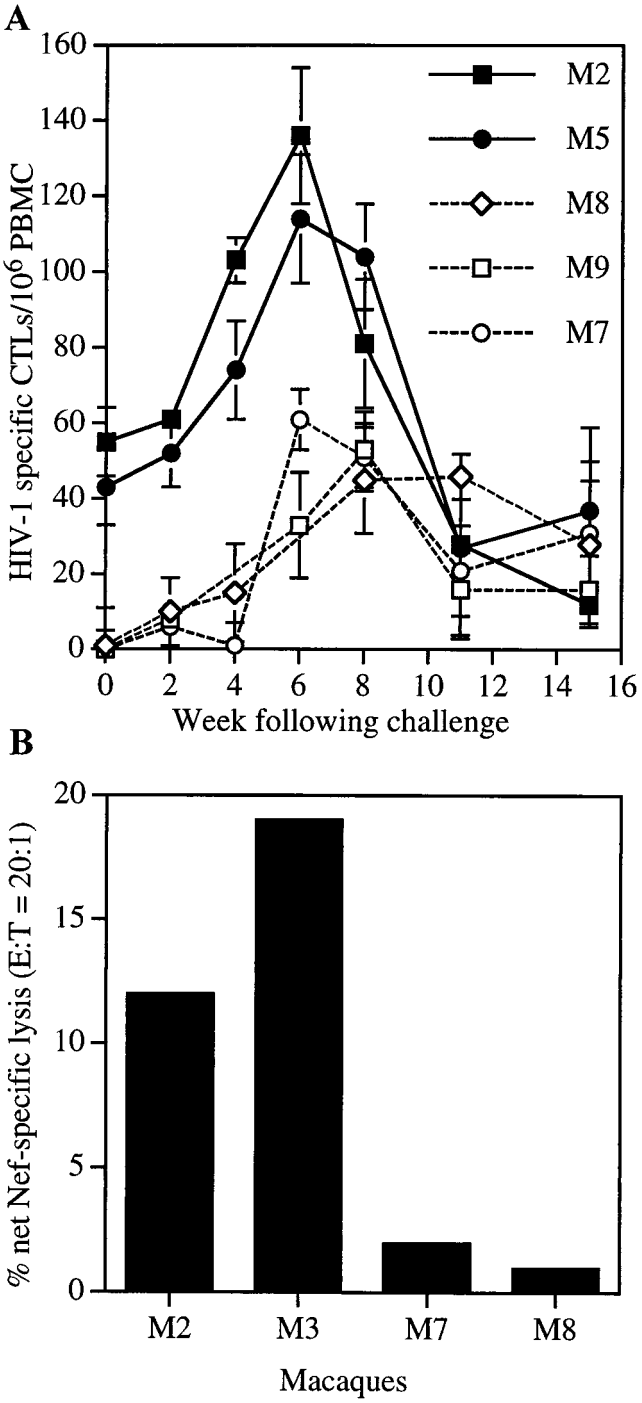

FIG. 4. HIV-1-specific CTL responses following HIV-1 challenge. (A) HIV-1 Env, Gag, and Pol CTL precursor frequencies in two HIV-1-vaccinated macaques (closed symbols) and three control macaques (open symbols) were assessed serially over time following HIV-1 challenge by limiting-dilution analysis. Error bars show 95% confidence intervals for CTL precursor estimation. (B) HIV-1 Nef-specific CTL were determined 2 weeks following HIV-1 challenge in bulk PBMC cultures from two vaccinated macaques (M2 and M3) and two control macaques (M8 and M9). E:T, effector/target.

tected, consistent with a Th1-based response, and this approach resulted in protection against a recombinant virus encoding HIV-1 antigens, which was previously shown to depend on T-cell responses (14, 38).

Our approach to HIV-1 vaccination of macaques had several features that facilitated an analysis of immunogenicity in primates. First, the macaques were prevaccinated with an irrelevant protein vaccine (tetanus toxoid) which generated a control antigen-specific Th2 response; this permitted the evaluation of vaccine-induced Th1 cytokine responses independent of concerns that Th2 cytokine responses could not be induced or detected in the model. Second, multiple HIV-1 genes were used in the vaccine, which we hypothesized would have a higher likelihood of generating a broadly reactive response than would the use of single proteins in outbred animals. Last, studying immune responses to HIV-1 proteins which were either shared by the DNA and rFPV vaccines (Env and Gag), unique to the rFPV vaccine (Pol), or unique to the challenge virus (Nef) permitted a dissection of the immunogenicity of each part of the vaccine regimen and the challenge virus with a limited number of macaque subjects.

The DNA component of the vaccine regimen primed both HIV-1-specific antibody and T-cell responses in macaques, although neither antibodies nor T-cell responses were of great magnitude, consistent with other reports on lentivirus DNA vaccines used alone (3, 32). Upon rFPV boosting, however, a decline in HIV-1 antibody titers and a coincident marked enhancement of HIV-specific CTL and Th responses occurred. This decline in antibodies in our study was somewhat surprising, given the previously reported potent (>100-fold) enhancement of antibody responses following recombinant protein boosting of rVV-primed humans and DNA-primed macaques (8, 31). The dichotomous nature of the humoral versus cell-mediated immune response observed in this study suggests that an HIV-1 vaccine strategy endeavoring to induce both strong antibodies and strong Th1-CTL responses, long believed to be desirable for candidate HIV-1 vaccines, may be very difficult to achieve. Indeed, recombinant protein vaccines load MHC class I molecules with peptides inefficiently, and therefore, significantly enhanced HIV-1-specific CTL levels are unlikely to be achieved by boosting with recombinant HIV-1 proteins. Continuously replicating attenuated lentiviruses may prove to be an exception to the inability of candidate HIV-1 vaccines to generate both strong, sustained antibody and T-cell responses. HIV-1 and SIV strains with Nef deleted produce both antibody and T-cell responses in humans and macaques, respectively, and protect against wild-type SIV challenge in macaques (6, 11, 15, 21). The ability of SIV Nef deletion strains to cause immunodeficiency in some neonatal and adult macaques is, however, not a concern with DNA or avipoxvirus vaccines (1, 5).

We chose a novel primate system to initially evaluate the efficacy of this vaccine strategy, employing autologous PBMC-grown HIV-1_{LAI} to challenge the pigtail macaques intravenously. We had previously demonstrated that acute HIV-1 infection could be detected by HIV-1 RNA, DNA, and coculture assays with this challenge model (26). Since the HIV-1_{LAI} challenge virus was grown separately in the autologous PBMC of each challenged animal, determining the precise monkey infectious dose of the challenge was not possible with this model. A previous titration of chimpanzee challenge stocks of the closely related HIV-1_{IIIB} in *M. nemestrina* (not grown in autologous cells) suggested that the 10^5 $TCID_{50}$ of the HIV-1_{LAI} challenge inocula used in this study represented approximately ≥100 monkey infectious doses (2). A macaque titration of HIV-1_{LAI} grown in nonautologous cells could also provide additional confidence in the challenge model employed in this study.

Protection from acute HIV-1 infection was assessed in multiple assays following HIV-1 challenge of the macaques, and all but a single early time point of HIV-1 *gag* DNA detection suggested that the vaccinated animals were protected from challenge, including the use of a sensitive plasma HIV-1 RNA

assay early following infection. It should be cautioned, however, that the HIV-1–macaque challenge system described is an acute-infection model, results in only low levels of plasma HIV-1 RNA, and is nonpathogenic. Whether the enhanced T-cell immunity induced by this vaccine strategy will be sufficient to protect against pathogenic lentivirus infections remains to be elucidated. DNA vaccines alone have provided minimal protection against virulent SIV challenge or nonpathogenic $SHIV_{HXB2}$ challenge (4, 32). The enhanced T-cell immunity generated by rFPV boosting of DNA vaccination observed in this study could facilitate more robust protection from pathogenic lentivirus models.

The nature of the protective immunity was of interest, since we had previously observed a pattern of nonsterilizing immunity in macaques protected from SIV (25). The generation of rapid and enhanced CTL responses to vaccine and nonvaccine antigens detected in the vaccinated animals following challenge indicates that the immunity was nonsterilizing and most likely dependent on T-cell responses for viral clearance. The presence of a single weak HIV-1 *gag* DNA signal in PBMC from one of four vaccinated animals early following challenge is, in this setting, perhaps not surprising, since viral clearance may be dependent on the rapid recognition of infected cells in the brief (perhaps 1- to 2-week) window period after infection but prior to widespread viral dissemination (22, 37).

In many models of viral immunity, $CD4^+$ Th responses facilitate the more rapid or sustained generation of $CD8^+$ CTL responses (47). The rapid generation of the de novo Nef-specific CTL response following HIV-1 challenge observed in this study may have been facilitated by the vaccine-induced Th response. The enhanced T-cell recognition of the incoming viral inoculum (as demonstrated by the Nef-specific CTL response in this study) should also facilitate the recognition of exposure to a divergent HIV-1 strain and its elimination, clearly a critical issue in HIV-1 vaccine development. Although both Th and CTL responses are likely to be important in the control or prevention of ongoing primate lentivirus infections, elucidating the precise roles of Th cells and CTL may ultimately require cell transfer studies with syngeneic macaques (28, 39).

This study suggests a primary role for T-cell responses in protection from HIV-1 challenge; however, we cannot exclude some role for the low levels of HIV-1 antibodies present prechallenge in this study. Further enhancement of the breadth and strength of the HIV-specific T-cell responses and abrogation of the antibody response by codelivery of a Th1 cytokine together with the DNA- and rFPV-encoded vaccine antigens could determine whether T-cell responses alone can control primate lentivirus infections in vivo (29). It is conceivable that even broader and greater HIV-specific T-cell responses will be required to protect against the diverse, virulent primary HIV-1 strains present throughout the world. Further exploration of this vaccine strategy in macaques with more-pathogenic lentiviruses is ongoing.

In summary, we have shown that consecutive immunization involving priming by DNA vaccination and boosting with an rFPV encoding HIV antigens elicits enhanced T-cell responses, which in macaques protects against a nonpathogenic HIV-1 challenge. This combination vaccine strategy could represent the basis of a safe and effective HIV-1 vaccine.

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