Control of a Mucosal Challenge and Prevention of AIDS by a Multiprotein DNA/MVA Vaccine

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Heterologous prime/boost regimens have the potential for raising high levels of immune responses. Here we report that DNA priming followed by a recombinant modified vaccinia Ankara (rMVA) booster controlled a highly pathogenic immunodeficiency virus challenge in a rhesus macaque model. Both the DNA and rMVA components of the vaccine expressed multiple immunodeficiency virus proteins. Two DNA inoculations at 0 and 8 weeks and a single rMVA booster at 24 weeks effectively controlled an intrarectal challenge administered 7 months after the booster. These findings provide hope that a relatively simple multiprotein DNA/MVA vaccine can help to control the acquired immune deficiency syndrome epidemic.

Cellular immunity plays an important role in the control of immunodeficiency virus infections (*1*). Recently, a DNA vaccine designed to enhance cellular immunity by cytokine augmentation successfully contained a highly virulent immunodeficiency virus challenge (*2*). Another promising approach to raising cellular immunity is DNA priming followed by recombinant poxvirus boosters (*3*). This heterologous prime/boost regimen induces 10- to 100-fold higher frequencies of T cells than priming and boosting with DNA or recombinant poxvirus vaccines alone. Previously, we showed that boosting a DNA-primed response with a poxvirus was superior to boosting with DNA or protein for the control of a nonpathogenic immunodeficiency virus (*4*). Here we test DNA priming and poxvirus boosting for the ability to protect against a highly pathogenic mucosal challenge. The 89.6 chimera of simian and human immunodeficiency viruses (SHIV-89.6) was used for the construction of immunogens and its highly pathogenic derivative, SHIV-89.6P, for challenge (*5*). SHIV-89.6 and SHIV-89.6P do not generate cross-neutralizing antibody (*6*) and allowed us to address the ability of vaccine-raised T cells and nonneutralizing antibodies to control an immunodeficiency virus challenge. Modified vaccinia Ankara (MVA) was used for the construction of the recombinant poxvirus. MVA has been highly effective at boosting DNA-primed CD8 T cells and enjoys the safety feature of not replicating in human or monkey cells (*3*).

To ensure a broad immune response, both the DNA and recombinant MVA (rMVA) components of the vaccine expressed multiple immunodeficiency virus proteins. The DNA prime (DNA/89.6) expressed simian immunodeficiency virus (SIV) Gag, Pol, Vif, Vpx, and Vpr and human immunodeficiency virus–1 (HIV-1) Env, Tat, and Rev from a single transcript (*7–9*). The rMVA booster (MVA/89.6) expressed SIV Gag, Pol, and HIV-1 Env under the control of vaccinia virus early/late promoters (*10*). Vaccination was accomplished by priming with DNA at 0 and 8 weeks and boosting with rMVA at 24 weeks (Fig. 1A) (*11*). Four groups of six rhesus macaques each were primed with either 2.5 mg (high-dose) or 250 μg (low-dose) of DNA by intradermal (i.d.) or intramuscular (i.m.) routes with a needleless jet injection device (Bioject, Portland, Oregon) (*12*, *13*). Gene gun deliveries of DNA were not used because these had primed nonprotective immune responses in our 1996–98 trial (*4*). The MVA/89.6 booster immunization [2×10^8 plaque-forming units (pfu)] was injected with a needle both i.d. and i.m. A control group included two mock immunized animals and two naïve animals. The challenge was given at 7 months after the rMVA booster to test for the generation of long-term immunity. Because most HIV-1 infections are transmitted across mucosal surfaces, an intrarectal challenge was administered.

DNA priming followed by rMVA boosting generated high frequencies of virus-specific T cells that peaked at 1 week after the rMVA booster (Fig. 1). The frequencies of T cells recognizing the Gag-CM9 epitope were assessed by means of Mamu-A*01 tetramers (*14*), and the frequencies of T cells recognizing epitopes throughout Gag were assessed with pools of overlapping peptides and an enzyme-linked immunospot (ELISPOT) assay (*15*, *16*). Gag-CM9 tetramer analyses were restricted to macaques that expressed the *Mamu-A*01* histocompatibility type, whereas ELISPOT responses did not depend on a specific histocompatibility type. As expected, the DNA immunizations raised low levels of memory cells that expanded to high frequencies within 1 week of the rMVA booster (Fig. 1) (*17*). In *Mamu-A*01* macaques, CD8 cells specific to the Gag-CM9 epitope expanded to frequencies as high as 19% of total CD8 T cells (*17*). This peak of specific cells underwent a 10- to 100-fold contraction into the DNA/MVA memory pool (Fig. 1A) (*17*). ELISPOTs for three pools of Gag peptides also underwent a major expansion [frequencies up to 4000 spots for 1×10^6 peripheral blood mononuclear cells (PBMC)] before contracting from 5- to 20-fold into the DNA/MVA memory response (Fig. 1B). The frequencies of ELISPOTs were the same in macaques with and without the *A*01* histocompatibility type ($P > 0.2$) (*18*). At both peak and memory phases of the vaccine response, the rank order for the height of the ELISPOTs in the vaccine groups was 2.5 mg i.d. > 2.5 mg i.m. > 250 μg i.d. > 250 μg i.m. (Fig. 1B). The interferon-γ (IFN-γ) ELISPOTs included both CD4 and CD8 cells (*19*). Gag-CM9–specific CD8 cells had good lytic activity after restimulation with peptide (*19*).

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The highly pathogenic SHIV-89.6P challenge was administered intrarectally at 7 months after the rMVA booster (*20*), when vaccine-raised T cells were in memory (Fig. 1). The challenge infected all of the vaccinated and control animals (Fig. 2). However, by 2 weeks after challenge, titers of plasma viral RNA were at least 10-fold lower in the vaccine groups (geometric means of 1×10^7 to 5×10^7) than in the control animals (geometric mean of 4×10^8) (Fig. 2A) (*21–23*). By 8 weeks after challenge, both high-dose DNA-primed groups and the low-dose i.d. DNA-primed group had reduced their geometric mean loads to about 1000 copies of viral RNA per milliliter. At this time, the low-dose i.m. DNA-primed group had a geometric mean of 6×10^3 copies of viral RNA and the nonvaccinated controls had a geometric mean of 2×10^6. By 20 weeks after challenge, even the low-dose i.m. group had reduced its geometric mean copies of viral RNA to 1000. Among the 24 vaccinated animals, only one animal, animal number 22 in the low-dose i.m. group, had intermittent viral loads above 1×10^4 copies per milliliter (Fig. 2D).

By 5 weeks after challenge, all of the nonvaccinated controls had undergone a profound depletion of CD4 cells (Fig. 2B). All of the vaccinated animals maintained their CD4 cells, with the exception of animal 22 in the low-dose i.m. group (see above), which underwent a slow CD4 decline (Fig. 2E). By 23 weeks after challenge, three of the four control animals had succumbed to AIDS (Fig. 2C). These animals had variable degrees of enterocolitis with diarrhea, cryptosporidiosis, colicystitis, enteric campylobacter infection, splenomegaly, lymphadenopathy, and SIV-associated giant cell pneumonia. In contrast, all 24 vaccinated animals maintained their health.

Containment of the viral challenge was associated with a burst of antiviral T cells (Figs. 1 and 3A). At 1 week after challenge, the frequency of tetramer$^+$ cells in the peripheral blood had decreased, potentially reflecting the recruitment of specific T cells to the site of infection (Fig. 3A). However, by 2 weeks after challenge, tetramer$^+$ cells in the peripheral blood had expanded to frequencies as high as, or higher than, after the rMVA booster (Figs. 1 and 3A). The majority of the tetramer$^+$ cells produced IFN-γ in response to a 6-hour peptide stimulation (Fig. 3B) (*24*, *25*) and did not have the "stunned" IFN-γ negative phenotype sometimes observed in viral infections (*26*). The postchallenge burst of T cells contracted concomitant with the decline of the viral load. By 12 weeks after challenge, virus-specific T cells were present at about one-tenth of their peak height (Figs. 1A and 3A) (*19*). In contrast to the vigorous secondary response in the vaccinated animals, the naïve animals mounted a modest primary response (Figs. 1B and 3A). Tetramer$^+$ cells peaked at less than 1% of total CD8 cells (Fig. 3A), and IFN-γ–producing ELISPOTs were present at a mean frequency of about 300 as opposed to the much higher frequencies of 1000 to 6000 in the vaccine groups (Fig. 1B) ($P < 0.05$) (*18*). The tetramer$^+$ cells in the control group, like those in the vaccine group, produced IFN-γ after peptide stimulation (Fig. 3B). By 12 weeks after challenge, three of the four controls had undetectable levels of IFN-γ–producing ELISPOTs (*19*). This rapid loss of antiviral T cells in the presence of high viral loads may reflect the lack of CD4 help.

T cell proliferative responses demonstrated that virus-specific CD4 cells had survived the challenge and were available to support the antiviral immune response (Fig. 3C) (*27*). At 12 weeks after challenge, mean stimulation indices for Gag-Pol-Env or Gag-Pol proteins ranged from 35 to 14 in the vaccine groups but were undetectable in the control group. Consistent with the proliferation assays, intracellular cytokine assays demonstrated the presence of virus-specific CD4 cells in vaccinated but not control animals (*19*). The overall rank order of the vaccine groups for the magnitude of the proliferative response was 2.5 mg i.d. > 2.5 mg i.m. > 250 μg i.d. > 250 μg i.m.

At 12 weeks after challenge, lymph nodes from the vaccinated animals were morphologically intact and responding to the infection, whereas those from the infected controls had been functionally destroyed (Fig. 4). Nodes from vaccinated

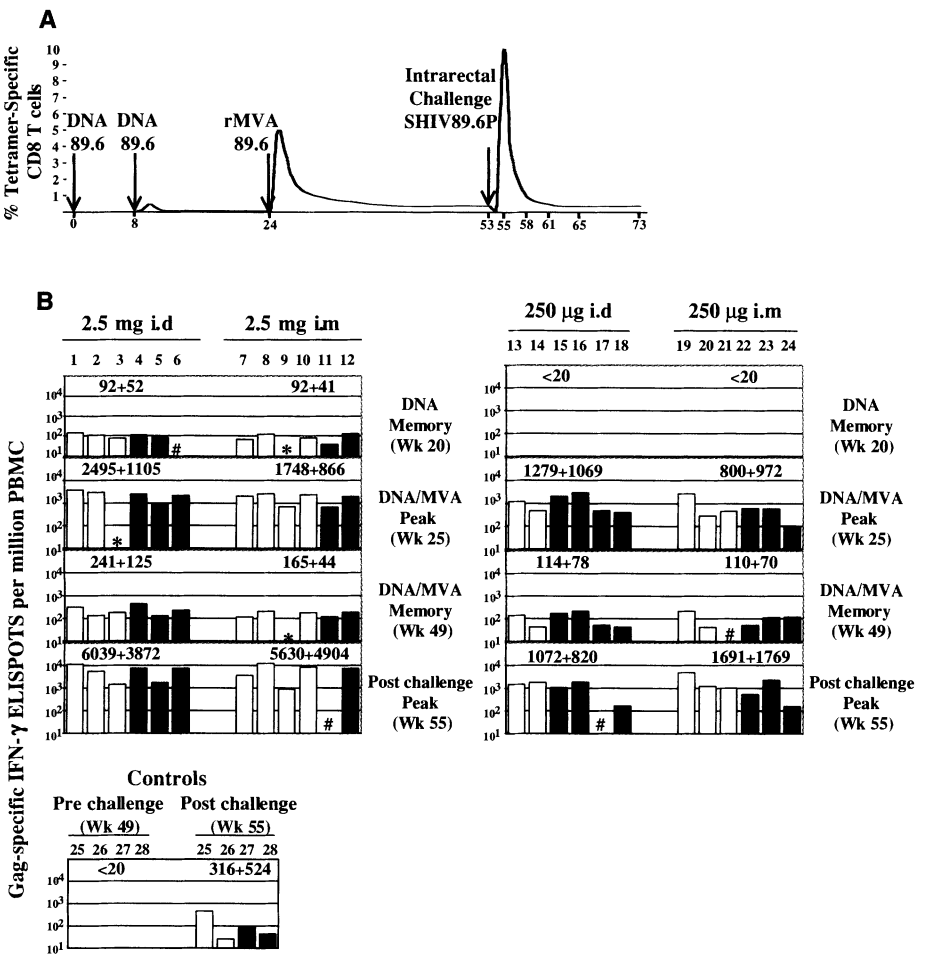

Fig. 1. Temporal frequencies of Gag-specific T cells. (**A**) Gag-specific CD8 T cell responses raised by DNA priming and rMVA booster immunizations. The schematic presents mean Gag-CM9–tetramer data generated in the high-dose i.d. DNA-immunized animals. (**B**) Gag-specific IFN-γ ELISPOTs in *A*01* (open bars) and non-*A*01* (filled bars) macaques at various times before challenge and at 2 weeks after challenge. Three pools of 10 to 13 Gag peptides (22-mers overlapping by 12) were used for the analyses. The numbers above data bars represent the arithmetic mean ± the SD for the ELISPOTs within each group. The numbers at the top of the graphs designate individual animals. *, data not available; #, <20 ELISPOTs per 1×10^6 PBMC. Temporal data for Gag-CM9–Mamu-A*01 tetramer-specific T cells can be found in the supplementary data (*17*).

animals contained large numbers of reactive secondary follicles with expanded germinal centers and discrete dark and light zones (Fig. 4A). By contrast, lymph nodes from the nonvaccinated control animals showed follicular and paracortical depletion (Fig. 4B), whereas those from unvaccinated and unchallenged animals displayed normal numbers of minimally reactive germinal centers (Fig. 4C). Germinal centers occupied <0.05% of total lymph node area in the infected controls, 2% of the lymph node area in the uninfected controls, and up to 18% of the lymph node area in the vaccinated groups (Fig. 4D). More vigorous immune reactivity in the low-dose than the high-dose DNA-primed animals was suggested by more extensive germinal centers in the low-dose group (Fig. 4D). At 12 weeks after challenge, in situ hybridization for viral RNA revealed rare virus-expressing cells in lymph nodes from 3 of the 24 vaccinated macaques, whereas virus-expressing cells were readily detected in lymph nodes from each of the infected control animals (*19*). In the controls, which had undergone a profound depletion in CD4 T cells, the cytomorphology of infected lymph node cells was consistent with a macrophage phenotype (*19*).

The prime/boost strategy raised low levels of antibody to Gag and undetectable levels of antibody to Env (Fig. 5). Postchallenge, antibodies to both Env and Gag underwent anamnestic responses with total Gag antibody reaching heights approaching 1 mg/ml and total Env antibody reaching heights of up to 100 μg/ml (*28*). By 2 weeks after challenge, neutralizing antibodies for the 89.6 immunogen, but not the SHIV-89.6P challenge, were present in the high-dose DNA-primed groups (geometric mean titers of 352 in the i.d. and 303 in the i.m. groups) (Fig. 5C) (*29*). By 5 weeks after challenge, neutralizing antibody to 89.6P had been generated (geometric mean titers of 200 in the high-dose i.d. and 126 in the high-dose i.m. group) (Fig. 5D) and neutralizing antibody to 89.6 had started to decline. By 16 to 20 weeks after challenge, antibodies to Gag and Env had fallen in most animals.

Discussion. Our results demonstrate that a multiprotein DNA/MVA vaccine can raise a memory immune response capable of controlling a highly virulent mucosal immunodeficiency virus challenge. Our levels of viral control were more favorable than have been achieved with only DNA (*30*) or rMVA vaccines (*31*) and were comparable to those obtained for DNA immunizations adjuvanted with interleukin-2 (*2*). All of these previous studies have used more than three vaccine inoculations, none have used mucosal challenges, and most have challenged at peak effector responses and not allowed a prolonged postvaccination period to test for "long-term" efficacy.

The dose of DNA had statistically significant effects on both cellular and humoral responses ($P < 0.05$), whereas the route of DNA administration affected only humoral responses (*18*). Intradermal DNA delivery was about 10 times more effective than i.m. inoculations for generating antibody to Gag ($P = 0.02$) (*18*). Neither route nor dose of DNA appeared to have a significant effect on protection. At 20 weeks after challenge, the high-dose DNA-primed animals had slightly lower geometric mean levels of viral RNA (7×10^2 and 5×10^2) than the low-dose DNA-primed animals (9×10^2 and 1×10^3).

The DNA/MVA vaccine controlled the infection, rapidly reducing viral loads to near or below 1000 copies of viral RNA per milliliter of blood. Containment, rather than prevention of infection, affords the opportunity to establish a chronic infection (*4*). By rapidly reducing viral loads, a multiprotein DNA/MVA vaccine will extend the prospect for long-term nonprogression and limit HIV transmission (*32*, *33*).

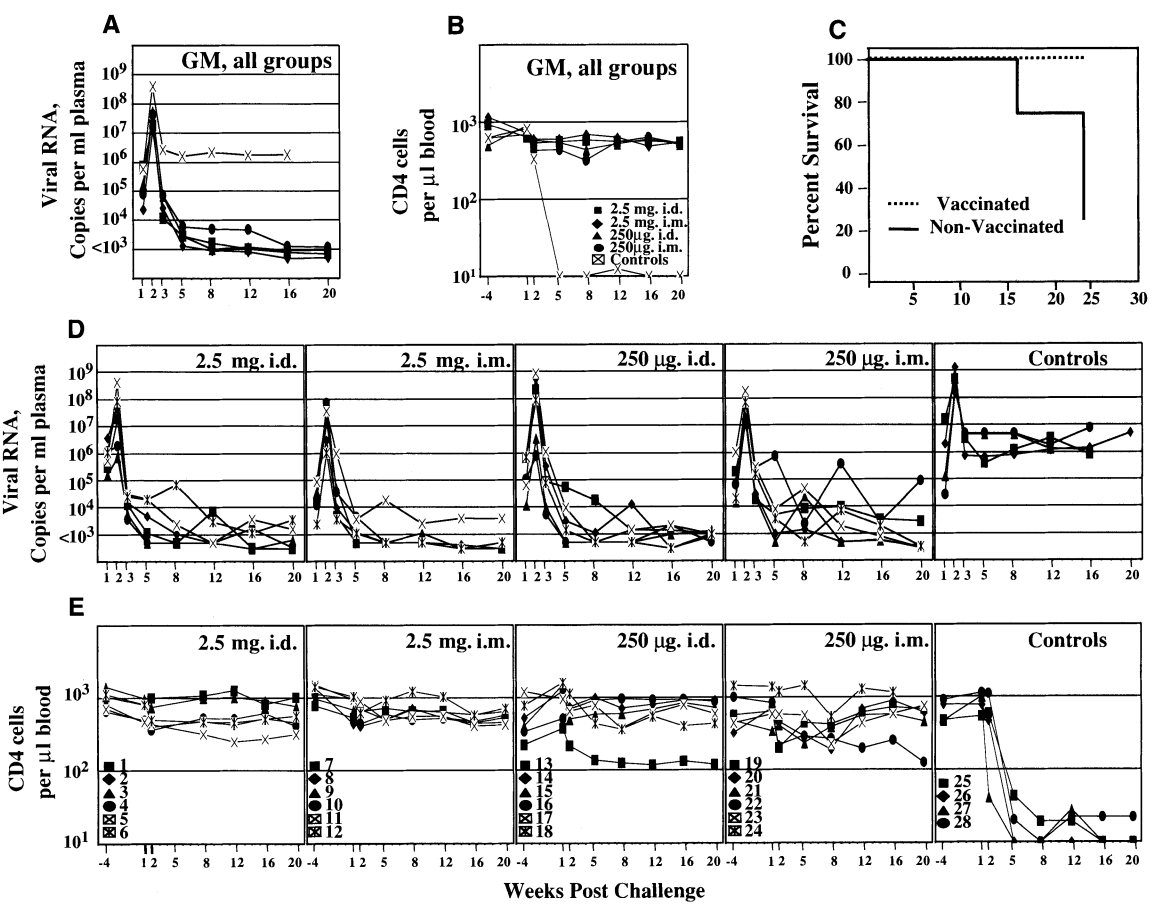

Fig. 2. Temporal viral loads, CD4 counts, and survival after challenge of vaccinated and control animals. (**A**) Geometric mean viral loads and (**B**) geometric mean CD4 counts. (**C**) Survival curve for vaccinated and control animals. The dotted line represents all 24 vaccinated animals. (**D**) Viral loads and (**E**) CD4 counts for individual animals in the vaccine and control groups. The key to animal numbers is presented in (E). Assays for the first 12 weeks after challenge had a detection level of 1000 copies of RNA per milliliter of plasma. Animals with loads below 1000 were scored with a load of 500. For weeks 16 and 20, the detection level was 300 copies of RNA per milliliter. Animals with levels of virus below 300 were scored at 300.

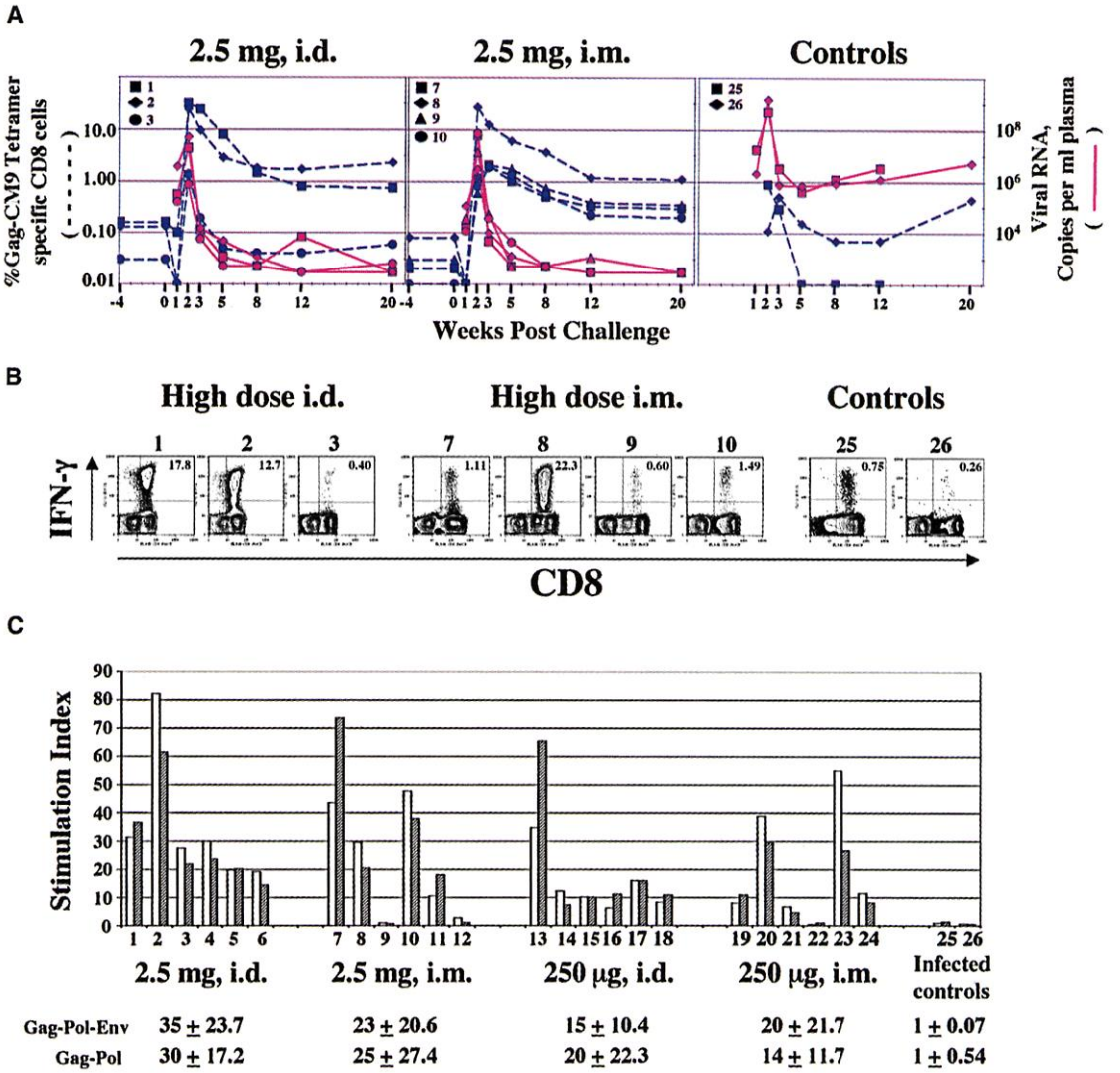

Fig. 3. Postchallenge T cell responses in vaccine and control groups. (**A**) Temporal tetramer$^+$ cells (dashed blue line) and viral loads (solid pink line). (**B**) Intracellular cytokine assays for IFN-γ production in response to stimulation with the Gag-CM9 peptide at 2 weeks after challenge. This ex vivo assay allows evaluation of the functional status of the peak postchallenge tetramer$^+$ cells displayed in Fig. 1A. (**C**) Proliferation assay at 12 weeks after challenge. Gag-Pol-Env (open bars) and Gag-Pol (hatched bars) produced by transient transfections were used for stimulation. Supernatants from mock-transfected cultures served as control antigen. Stimulation indices are the growth of cultures in the presence of viral antigens divided by the growth of cultures in the presence of mock antigen.

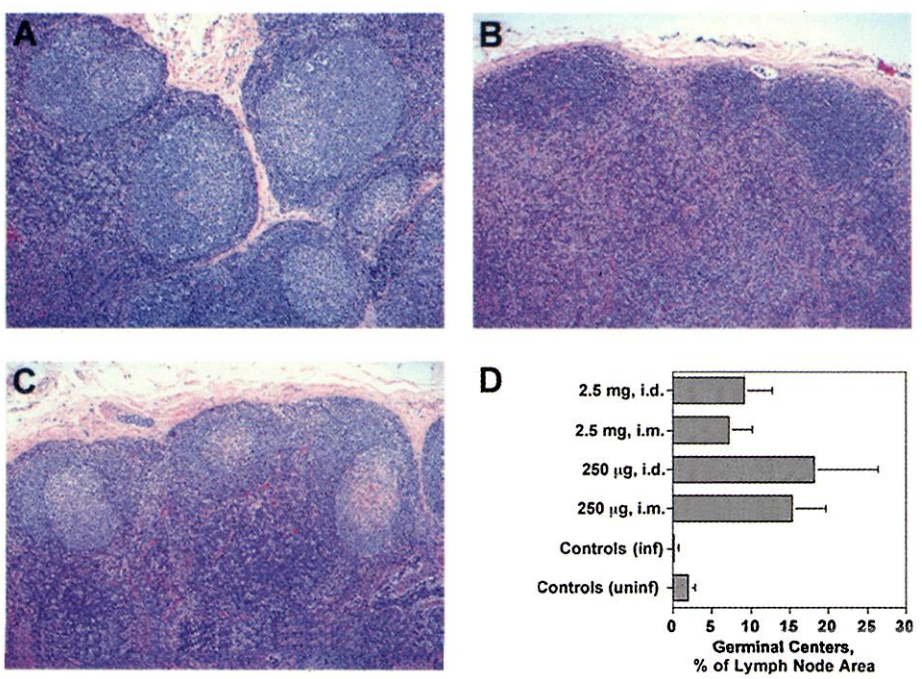

Fig. 4. Lymph node histomorphology at 12 weeks after challenge. (**A**) Typical lymph node from a vaccinated macaque showing evidence of follicular hyperplasia characterized by the presence of numerous secondary follicles with expanded germinal centers and discrete dark and light zones. (**B**) Typical lymph node from an infected control animal showing follicular depletion and paracortical lymphocellular atrophy. (**C**) A representative lymph node from an age-matched, uninfected macaque displaying nonreactive germinal centers. (**D**) The percentage of the total lymph node area occupied by germinal centers was measured to give a nonspecific indicator of follicular hyperplasia. Data for uninfected controls are for four age-matched rhesus macaques.

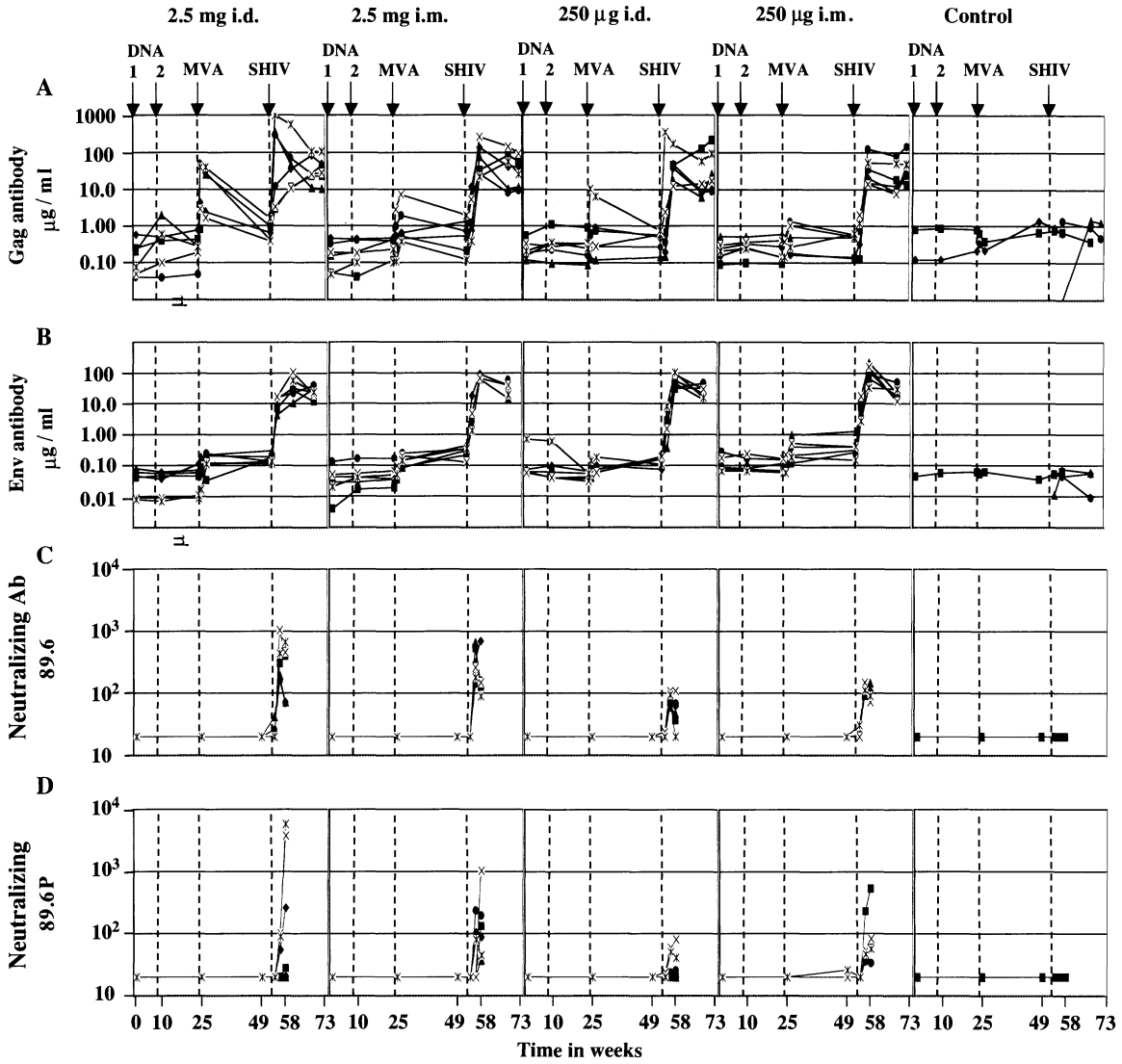

Fig. 5. Temporal antibody responses. Micrograms of total Gag (**A**) or Env (**B**) antibody were determined with ELISAs. The titers of neutralizing antibody for SHIV-89.6 (**C**) and SHIV-89.6P (**D**) were determined with MT-2 cell killing and neutral red staining (*29*). Titers are the reciprocal of the serum dilution giving 50% neutralization of the indicated viruses grown in human PBMC. Symbols for animals are the same as in Fig. 2.

References and Notes

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9. Molecularly cloned SHIV-89.6 sequences (kindly provided by J. Sodroski) were cloned into the vector pGA2 with Cla I and Rsr II sites. This cloning deleted both long terminal repeats (LTRs) and *nef*. The SHIV-89.6 sequences also were internally mutated for a 12–base pair region encoding the first four amino acids of the second zinc finger in nucleocapsid. This mutation renders SHIV viruses noninfectious (*7*). A mutation in gp41 converted the tyrosine at position 710 to cysteine to achieve better expression of Env on the plasma membrane of DNA-expressing cells (*8*). pGA2 uses the CMV immediate early promoter without intron A and the bovine growth hormone polyadenylation sequence to express vaccine inserts. Vaccine DNA was produced by Althea (San Diego, CA). In transient transfections of 293T cells, DNA/89.6 produced about 300 ng of Gag and 85 ng of Env per 1×10^6 cells.
10. The MVA double recombinant virus expressed both the HIV 89.6 Env and the SIV 239 Gag-Pol, which were inserted into deletion II and deletion III of MVA, respectively (L. S. Wyatt and B. Moss, unpublished results). The 89.6 Env protein was truncated for the COOH-terminal 115 amino acids of gp41. The modified H5 promoter controlled the expression of both foreign genes.
11. I.d. and i.m. DNA immunizations were delivered in phosphate-buffered saline (PBS) with a needleless jet injector (Bioject, Portland, OR) to deliver five i.d. 100-μl injections to each outer thigh for the 2.5-mg dose of DNA or one i.d. 100-μl injection to the right outer thigh for the 250-μg dose of plasmid. I.m. deliveries of DNA were done with one 0.5-ml injection of DNA in PBS to each outer thigh for the 2.5-mg dose and one 100-μl injection to the right outer thigh for the 250-μg dose. 1×10^8 pfu of MVA/89.6 was administered both i.d. and i.m. with a needle. One 100-μl dose was delivered to each outer thigh for the i.d. dose and one 500-μl dose to each outer thigh for the i.m dose. Control animals received 2.5 mg of the pGA2 vector without vaccine insert with the Bioject device to deliver five 100-μl doses i.d. to each outer thigh. The control MVA booster immunization consisted of 2×10^8 pfu of MVA without an insert delivered i.d. and i.m. as described for MVA/89.6.
12. T. M. Allen *et al.*, *J. Immunol.* **164**, 4968 (2000).
13. Young adult rhesus macaques from the Yerkes breeding colony were cared for under guidelines established by the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals" with protocols approved by the Emory University Institutional Animal Care and Use Committee. Macaques were typed for the *Mamu-A*01* allele with polymerase chain reaction (PCR) analyses (*30*, *31*). Two or more animals containing at least one *Mamu-A*01* allele were assigned to each group. Animal numbers are as follows: 1, RBr-5*; 2, RIm-5*; 3, RQf-5*; 4, RZe-5; 5, ROm-5; 6, RDm-5; 7, RAj-5*; 8, RJi-5*; 9, RAl-5*; 10, RDe-5*; 11, RAi-5; 12, RPr-5; 13, RKw-4*; 14, RWz-5*; 15, RGo-5; 16, RLp-4; 17, RWd-6; 18, RAt-5; 19, RPb-5*; 20, RIi-5*; 21, RIq-5; 22, RSp-4; 23, RSn-5; 24, RGd-6; 25, RMb-5*; 26, RGy-5*; 27, RUs-4; and 28, RPm-5. Animals with the *A*01* allele are indicated with asterisks.
14. For tetramer analyses, about 1×10^6 PBMC were surface-stained with antibodies to CD3 conjugated to fluorescein isothiocyanate (FITC) (FN-18; Biosource International, Camarillo, CA), CD8 conjugated to peridinin chlorophyl protein (PerCP) (SK1; Becton Dickinson, San Jose, CA), and Gag-CM9 (CTPYDINQM)–Mamu-A*01 tetramer conjugated to allophycocyanin (APC), in a volume of 100 μl at 8° to 10°C for 30 min. Cells were washed twice with cold PBS containing 2% fetal bovine serum (FBS), fixed with 1% paraformaldehyde in PBS, and analyzed within 24 hours on a FACScaliber (Becton Dickinson, San Jose, CA). Cells were initially gated on lymphocyte populations with forward scatter and side scatter and then on CD3 cells. The CD3 cells were then analyzed for CD8 and tetramer-binding cells. About 150,000 lymphocytes were

acquired for each sample. Data were analyzed with FloJo software (Tree Star, San Carlos, CA).
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16. For IFN-γ ELISPOTs, MULTISCREEN 96-well filtration plates (Millipore, Bedford, MA) were coated overnight with antibody to human IFN-γ (Clone B27, Pharmingen, San Diego, CA) at a concentration of 2 μg/ml in sodium bicarbonate buffer (pH 9.6) at 8° to 10°C. Plates were washed two times with RPMI medium and then blocked for 1 hour with complete medium (RPMI containing 10% FBS) at 37°C. Plates were washed five more times with plain RPMI medium, and cells were seeded in duplicate in 100 μl of complete medium at numbers ranging from 2×10^4 to 5×10^5 cells per well. Peptide pools were added to each well to a final concentration of 2 μg/ml of each peptide in a volume of 100 μl in complete medium. Cells were cultured at 37°C for about 36 hours under 5% CO_2. Plates were washed six times with wash buffer (PBS with 0.05% Tween-20) and then incubated with 1 μg of biotinylated antibody to human IFN-γ per milliliter (clone 7-86-1; Diapharma Group, West Chester, OH) diluted in wash buffer containing 2% FBS. Plates were incubated for 2 hours at 37°C and washed six times with wash buffer. Avidin–horseradish peroxidase (Vector Laboratories, Burlingame, CA) was added to each well and incubated for 30 to 60 min at 37°C. Plates were washed six times with wash buffer and spots were developed with stable DAB as substrate (Research Genetics, Huntsville, AL). Spots were counted with a stereo dissecting microscope. An ovalbumin peptide (SIINFEKL) was included as a control in each analysis. Background spots for the ovalbumin peptide were generally <5 for 5×10^5 PBMCs. This background when normalized for 1×10^6 PBMC was <10. Only ELISPOT counts of twice the background (≥20) were considered significant. The frequencies of ELISPOTs are approximate because different dilutions of cells have different efficiencies of spot formation in the absence of feeder cells (*15*). The same dilution of cells was used for all animals at a given time point, but different dilutions were used to detect memory and acute responses.
17. See supplementary data, available on *Science* Online at www.sciencemag.org/cgi/content/full/1058915/DC1.
18. Simple linear regression was used to estimate correlations between postbooster and postchallenge ELISPOT responses, between memory and postchallenge ELISPOT responses, and between logarithmically transformed viral loads and ELISPOT frequencies. Comparisons between vaccine and control groups and *A*01* and non-*A*01* macaques were performed by means of two-sample *t* tests with logarithmically transformed viral load and ELISPOT responses. Two-way analyses of variance were used to examine the effects of dose and route of administration on peak DNA/MVA ELISPOTs, on memory DNA/MVA ELISPOTs, and on logarithmically transformed Gag antibody data.
19. R. R. Amara *et al.*, data not shown.
20. The challenge stock (5.7×10^9 copies of viral RNA per milliliter) was produced by one intravenous followed by one intrarectal passage in rhesus macaques of the original SHIV-89.6P stock (*5*). Lymphoid cells were harvested from the intrarectally infected animal at peak viremia, CD8-depleted, and mitogen-stimulated for stock production (F. Villinger *et al.*, in preparation). Before intrarectal challenge, fasted animals were anesthetized (ketamine, 10 mg/kg) and placed on their stomach with the pelvic region slightly elevated. A feeding tube [8Fr (2.7 mm) by 16 inches (41 cm); Sherwood Medical, St. Louis, MO] was inserted into the rectum for a distance of 15 to 20 cm. After insertion of the feeding tube, a syringe containing 20 intrarectal infectious doses in 2 ml of RPMI-1640 plus 10% FBS was attached to the tube and the inoculum was slowly injected into the rectum. After delivery of the inoculum, the feeding tube was flushed with 3.0 ml of RPMI without FBS and then slowly withdrawn. Animals were left in place, with pelvic regions slightly elevated, for a period of 10 min after the challenge.
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23. For the determination of SHIV copy number, viral RNA from 150 μl of ACD anticoagulated plasma was directly extracted with the QIAamp Viral RNA kit (Qiagen), eluted in 60 μl of AVE buffer, and frozen at −80°C until SHIV RNA quantitation was performed. Five microliters of purified plasma RNA was reverse-transcribed in a final 20-μl volume containing 50 mM KCl, 10 mM tris-HCl (pH 8.3), 4 mM $MgCl_2$, 1 mM each deoxynucleotide triphosphate (dNTP), 2.5 μM random hexamers, 20 units of MultiScribe RT, and 8 units of ribonuclease inhibitor. Reactions were incubated at 25°C for 10 min, followed by incubation at 42°C for 20 min, and inactivation of reverse transcriptase at 99°C for 5 min. The reaction mix was adjusted to a final volume of 50 μl containing 50 mM KCl, 10 mM tris-HCl (pH 8.3), 4 mM $MgCl_2$, 0.4 mM each dNTP, 0.2 μM forward primer, 0.2 μM reverse primer, 0.1 μM probe, and 5 units of AmpliTaq Gold DNA polymerase (all reagents from PerkinElmer Applied Biosystems, Foster City, CA). The primer sequences within a conserved portion of the SIV *gag* gene are the same as those described previously (*21*). A PerkinElmer Applied Biosystems 7700 Sequence Detection System was used with the PCR profile: 95°C for 10 min, followed by 40 cycles at 93°C for 30 s, and 59.5°C for 1 min. PCR product accumulation was monitored with the 7700 sequence detector and a probe to an internal conserved *gag* gene sequence: 6FAM-CTGTCTGCGTCATTTG-GTGC-Tamra, where FAM and Tamra denote the reporter and quencher dyes. SHIV RNA copy number was determined by comparison with an external standard curve consisting of virion-derived SIVmac239 RNA quantified by the SIV bDNA method (Bayer Diagnostics, Emeryville, CA). All specimens were extracted and amplified in duplicate, with the mean result reported. With a 0.15-ml plasma input, the assay has a sensitivity of 10^3 RNA copies per milliliter of plasma and a linear dynamic range of 10^3 to 10^8 RNA copies ($R^2 = 0.995$). The intraassay coefficient of variation was <20% for samples containing $>10^4$ SHIV RNA copies per milliliter and <25% for samples containing 10^3 to 10^4 SHIV RNA copies per milliliter. To more accurately quantitate low SHIV RNA copy number in vaccinated animals at weeks 16 and 20, we made the following modifications to increase the sensitivity of the SHIV RNA assay: (i) Virions from ≤1 ml of plasma were concentrated by centrifugation at 23,000*g* at 10°C for 150 min before viral RNA extraction, and (ii) a one-step reverse transcriptase PCR method was used (*22*). These changes provided a reliable quantification limit of 300 SHIV RNA copies per milliliter and gave SHIV RNA values that were highly correlated to those obtained by the first method used ($r = 0.91$, $P < 0.0001$).
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25. For intracellular cytokine assays, about 1×10^6 PBMC were stimulated for 1 hour at 37°C in 5-ml polypropylene tubes with 100 μg of Gag-CM9 peptide (CTPYDINQM) per milliliter in a volume of 100 μl RPMI containing 0.1% bovine serum albumin (BSA) and 1 μg of antibody to human CD28 and 1 μg of antibody to human CD49d (Pharmingen, San Diego, CA) per milliliter. Then, 900 μl of RPMI containing 10% FBS and monensin (10 μg/ml) was added, and the cells were cultured for an additional 5 hours at 37°C at an angle of 5° under 5% CO_2. Cells were surface stained with antibodies to CD8 conjugated to PerCP (clone SK1, Becton Dickinson) at 8° to 10°C for 30 min, washed twice with cold PBS containing 2% FBS, and fixed and permeabilized with Cytofix/Cytoperm solution (Pharmingen). Cells were then incubated with antibodies to human CD3 (clone FN-18; Biosource International, Camarillo, CA) and IFN-γ (Clone B27; Pharmingen) conjugated to FITC and phycoerythrin, respectively, in Perm wash solution (Pharmingen) for 30 min at 4°C. Cells were washed twice with Perm wash, once with plain PBS, and resuspended in 1% paraformaldehyde in PBS. About 150,000 lymphocytes were acquired on the FACScaliber and analyzed with FloJo software,
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27. About 0.2 million PBMC were stimulated in triplicate for 5 days with the indicated antigen in 200 μl of RPMI at 37°C under 5% CO_2. Supernatants from 293T cells transfected with DNA expressing either SHIV-89.6 Gag and Pol or SHIV-89.6 Gag, Pol and Env were used directly as antigens (final concentration of ~0.5 μg of p27 Gag per milliliter). Supernatants from mock DNA (vector alone)–transfected cells served as negative controls. On day six, cells were pulsed with 1 μCi of tritiated thymidine per well for 16 to 20 hours. Cells were harvested with an automated cell harvester (TOMTEC, Harvester 96, Model 1010, Hamden, CT) and counted with a Wallac 1450 MICROBETA Scintillation counter (Gaithersburg, MD). Stimulation indices are the counts of tritiated thymidine incorporated in PBMC stimulated with 89.6 antigens divided by the counts of tritiated thymidine incorporated by the same PBMC stimulated with mock antigen.
28. Enzyme-linked immunosorbent assays (ELISAs) for total antibody to Gag antibody used bacterially produced SIV gag p27 to coat wells (2 μg per milliliter in bicarbonate buffer). ELISAs for antibody to Env antibody used 89.6 Env produced in transiently transfected 293T cells and captured with sheep antibody against Env (catalog number 6205; International Enzymes, Fairbrook CA). Standard curves for Gag and Env ELISAs were produced with serum from a SHIV-89.6–infected macaque with known amounts of immunoglobulin G (IgG) specific for Gag or Env. Bound antibody was detected with peroxidase-conjugated goat antibody to macaque IgG (catalog # YN-GMOIGGFCP, Accurate Chemical, Westbury, NY) and TMB substrate (Catalog # T3405, Sigma, St. Louis, MO). Sera were assayed at threefold dilutions in duplicate wells. Dilutions of test sera were performed in whey buffer (4% whey and 0.1% tween 20 in 1× PBS). Blocking buffer consisted of whey buffer plus 0.5% nonfat dry milk. Reactions were stopped with 2 M H_2SO_4 and the optical density read at 450 nm. Standard curves were fitted and sample concentrations were interpolated as μg of antibody per ml of serum with SOFTmax 2.3 software (Molecular Devices, Sunnyvale, CA)
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