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# Sequential Priming with Simian Immunodeficiency Virus (SIV) DNA Vaccines, with or without Encoded Cytokines, and a Replicating Adenovirus-SIV Recombinant Followed by Protein Boosting Does Not Control a Pathogenic $SIV_{mac251}$ Mucosal Challenge▽

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**Previously, combination DNA/nonreplicating adenovirus (Ad)- or poxvirus-vectored vaccines have strongly protected against $SHIV_{89.6P}$, DNAs expressing cytokines have modulated immunity elicited by DNA vaccines, and replication-competent Ad-recombinant priming and protein boosting has strongly protected against simian immunodeficiency virus (SIV) challenge. Here we evaluated a vaccine strategy composed of these promising components. Seven rhesus macaques per group were primed twice with multigenic SIV plasmid DNA with or without interleukin-12 (IL-12) DNA or IL-15 DNA. After a multigenic replicating Ad-SIV immunization, all groups received two booster immunizations with SIV gp140 and SIV Nef protein. Four control macaques received control DNA plasmids, empty Ad vector, and adjuvant. All vaccine components were immunogenic, but the cytokine DNAs had little effect. Macaques that received IL-15-DNA exhibited higher peak anti-Nef titers, a more rapid anti-Nef anamnestic response postchallenge, and expanded $CD8_{CM}$ T cells 2 weeks postchallenge compared to the DNA-only group. Other immune responses were indistinguishable between groups. Overall, no protection against intrarectal challenge with $SIV_{mac251}$ was observed, although immunized non-Mamu-A*01 macaques as a group exhibited a statistically significant 1-log decline in acute viremia compared to non-Mamu-A*01 controls. Possible factors contributing to the poor outcome include administration of cytokine DNAs to sites different from the Ad recombinants (intramuscular and intratracheal, respectively), too few DNA priming immunizations, a suboptimal DNA delivery method, failure to ensure delivery of SIV and cytokine plasmids to the same cell, and instability and short half-life of the IL-15 component. Future experiments should address these issues to determine if this combination approach is able to control a virulent SIV challenge.**

AIDS is one of the greatest pandemics of our time, affecting the health and the social and economic foundations of countries worldwide. A potent human immunodeficiency virus (HIV) vaccine offers the best hope for controlling the spread of the virus. While a single immune correlate has not been identified, both antibodies and CD8 T-cell responses contribute to control of infection with HIV or the related simian immunodeficiency virus (SIV) and disease progression (5, 17, 23, 28, 35–39, 43, 46, 58). Appropriately designed envelope immunogens able to induce broad, potent neutralizing antibodies have not yet been achieved, but vaccine-elicited virus-specific cellular immune responses have been more readily elicited. Both DNA and recombinant viral vectors have emerged as prominent candidate vaccines for this purpose. Although DNA vaccines are not as immunogenic as other vectored vaccines, a variety of approaches can enhance their potency (20). Further, the striking observations that DNA priming followed by boosting with an adenovirus (Ad) or modified vaccinia virus Ankara (MVA)-vectored vaccine elicits enhanced immunity and protective efficacy (3, 59) suggest that among many available vectors (27) other DNA-vector combinations might be equally or more potent.

Increasing knowledge of cytokine networks and their influences on the immune system has provided new opportunities for vaccine design and propelled the field toward tailored immune responses. IL-12, first described as natural killer (NK) cell stimulatory factor (25), and IL-15 (18) are among promising candidate cytokine adjuvants for directing such tailored immune responses. Both interleukins have strong effects on NK cells and T cells, influencing the magnitude and quality of cellular responses (1, 4, 15, 30, 63). IL-12 acts as adjuvant for both CD4 and CD8 T-cell responses. When administered as a DNA expression vector in combination with other DNA vaccines, it increased cellular immunity in mice (24) and enhanced both humoral and cellular immune responses in rhesus macaques (11, 13, 57, 61). Recently, the addition of IL-12 DNA plasmids to a SIV*gag* DNA vaccine regimen and to a prime/boost DNA/vesicular stomatitis virus-SIV*gag* regimen increased protective efficacy against a $SHIV_{89.6P}$ challenge (11, 13). IL-15, in contrast, primarily increases cellular immunity and is important for development of memory T cells (44).

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TABLE 1. Immunization and challenge protocol

| Immunogen | Challenge, by group[a] | | | |
|---|---|---|---|---|
| | Group 1, DNA | Group 2, DNA/IL-12 | Group 3, DNA/IL-15 | Group 4, control |
| DNA,[b] wks 0 and 4, intramuscular | $SIV_{mac239}$*env*<br>$SIV_{mac239}$*gag*<br>$SIV_{mac239}$*rev/nef*<br>Control DNA | $SIV_{mac239}$*env*<br>$SIV_{mac239}$*gag*<br>$SIV_{mac239}$*rev/nef*<br>DNA/IL-12 | $SIV_{mac239}$*env*<br>$SIV_{mac239}$*gag*<br>$SIV_{mac239}$*rev/nef*<br>DNA/IL-15 | Control DNA |
| Ad5hr-SIV,[c] wk 12, intratracheal | $SIV_{smH4}$*env/rev*<br>$SIV_{mac239}$*gag*<br>$SIV_{mac239}$*nef*$_{\Delta 1\text{-}13}$ | $SIV_{smH4}$*env/rev*<br>$SIV_{mac239}$*gag*<br>$SIV_{mac239}$*nef*$_{\Delta 1\text{-}13}$ | $SIV_{smH4}$*env/rev*<br>$SIV_{mac239}$*gag*<br>$SIV_{mac239}$*nef*$_{\Delta 1\text{-}13}$ | Ad5hr-ΔE3 vector |
| Proteins,[d] wk 24 and 36, intramuscular | $SIV_{mac251}$ gp140<br>$SIV_{mac239}$ Nef | $SIV_{mac251}$ gp140<br>$SIV_{mac239}$ Nef | $SIV_{mac251}$ gp140<br>$SIV_{mac239}$ Nef | MPL-SE Adjuvant |
| Challenge,[e] wk 48, intrarectal | $SIV_{mac251}$ | $SIV_{mac251}$ | $SIV_{mac251}$ | $SIV_{mac251}$ |

[a] Groups 1 to 3 had seven macaques per group; group 4 had four macaques.
[b] At 2.5 mg DNA per dose, mixed together and administered at multiple sites in both thighs.
[c] At $5 \times 10^8$ PFU/recombinant; total Ad dose was $1.5 \times 10^9$ PFU.
[d] SIV gp140, 100 μg/dose; SIV Nef, 50 μg/dose.
[e] At 10 50% monkey infectious doses.

IL-15 may enhance central memory cells, while IL-12 may lead to greater terminal differentiation and development of effector memory cells (1). In nonhuman primates, IL-15 enhances both CD4 and CD8 effector memory T cells (41, 52) and, depending on the timing of administration, production of long-lived CD4 and CD8 memory T cells (62). However, potent, polyfunctional cytokines such as IL-15 must be administered cautiously. In macaques, for example, IL-15 prevented vaccine-induced control of viral replication (22), and in SIV-infected macaques it increased viral load and the rate of disease progression (40).

We are developing a replication-competent Ad recombinant vaccine approach (33), having demonstrated in chimpanzees that at the same or lower dose, priming with replicating Ad-HIV*env* followed by envelope protein boosting elicited better cellular and humoral immune responses than a similar regimen using a nonreplicating Ad-HIV*env* vaccine (50). A contribution of the HIV envelope immunogens to protection has been established using the $SHIV_{89.6P}$ model, in which prechallenge antibody titers elicited by a combination prime-boost regimen incorporating HIV Env and Tat in comparison to a multigenic regimen were associated with significantly stronger protection against the viral challenge (12). Furthermore, the value of an envelope protein component in the vaccine strategy was confirmed in a study showing better protection against $SHIV_{89.6P}$ elicited by an Ad-prime/protein boosting regimen in comparison to Ad priming alone (47). Importantly, in the rigorous $SIV_{mac251}$ challenge model, priming with multigenic Ad-SIV recombinants and boosting with envelope subunits potently protected 39% of rhesus macaques (49). The protection was durable, as shown in a subsequent rechallenge study 1 year later with no intervening booster immunization (32).

Priming by first-generation, unmodified DNA plasmids followed by boosting with a replication-competent Ad type 5 host range mutant (Ad5hr)-SIV recombinant did not enhance subsequent immunogenicity in a small pilot study (34). Here we revisited this question, using improved multigenic SIV DNA plasmid vaccines as priming vehicles, with and without additional plasmid DNAs encoding rhesus IL-12 and IL-15, to elicit stronger, long-lived immune responses. We structured the study around our standard immunization regimen (two mucosal administrations of replication-competent Ad recombinants followed by two boosts with envelope protein), which has elicited strong immunogenicity and long-lasting protection against $SIV_{mac251}$ (31, 32, 48, 49). We asked if two sequential DNA priming immunizations could substitute for the initial Ad recombinant priming. These were followed by a single boost with replicating multigenic Ad5hr-SIV recombinants and two boosts with SIV gp140 and SIV Nef proteins. The macaques were subsequently challenged intrarectally with pathogenic $SIV_{mac251}$.

## MATERIALS AND METHODS

**Immunization and challenge of macaques.** Twenty-five Indian rhesus macaques were housed at Advanced BioScience Laboratories, Inc. (ABL; Kensington, MD). The care and maintenance of the animals were in compliance with established guidelines, and the animal protocol received approval from the ABL Animal Care and Use Committee prior to study initiation. The macaques were immunized as outlined in Table 1. The three experimental immunization groups contained seven monkeys each, and the control group contained four. Eight Mamu-A*01-positive macaques were assigned to groups, two animals per group. Peripheral blood and tissue samples were obtained prior to immunization and periodically over the course of immunization and following challenge. At week 48 the macaques were challenged intrarectally with 10 50% monkey infectious doses of a rhesus peripheral blood mononuclear cell (PBMC)-grown $SIV_{mac251}$ challenge stock kindly provided by Ronald C. Desrosiers, New England National Primate Research Center, and made available by Nancy Miller, Division of AIDS, NIAID.

**Immunogens.** DNA plasmids used for vaccination were cytomegalovirus promoter-driven codon-optimized sequences of $SIV_{mac239}$*env*, C-terminally truncated $SIV_{mac239}$*gag* (p37), and $SIV_{mac239}$*rev/nef*. A dual promoter expression vector encoding the rhesus IL-12 p35 and p40 genes (13) and a rhesus IL-15 expression plasmid (11) contained codon-optimized genes for high expression in mammalian cells. The plasmids were manufactured and purified by Puresyn (Malvern, PA) and formulated in 0.15 M citrate buffer and 0.25% bupivicaine for intramuscular administration. The replication-competent Ad5hr recombinants encoding $SIV_{smH4}$*env/rev*, $SIV_{mac239}$*gag*, and $SIV_{mac239}$*nef*$_{\Delta 1\text{-}13}$ have been described previously (10, 48, 64) and were administered intratracheally in phosphate-buffered saline (PBS) at a dose of $5 \times 10^8$ PFU/recombinant. Empty Ad5hrΔE3 vector ($1.5 \times 10^9$ total dose) served as a control. Protein boosts consisted of $SIV_{mac251}$ gp140 and $SIV_{mac239}$ Nef (ABL) mixed with a 1/10

volume of monophosphoryl lipid A-stable emulsion (Corixa, Hamilton, MT) and administered intramuscularly, 100 μg and 50 μg per dose, respectively.

**Sample collection.** PBMCs were isolated using Ficoll-Paque Plus (GE Healthcare) and used fresh in all assays unless otherwise stated. Surplus cells were frozen in 90% fetal bovine serum (Invitrogen) and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and stored in liquid nitrogen until use. Lymph node biopsies and bronchoalveolar lavages (BAL) were collected periodically during the study. Lymph nodes were minced and passed through a 70-μm cell strainer (BD Pharmingen). The isolated cells were pelleted at 550 × *g* for 7 min and washed twice with PBS before use. BAL cells were obtained by flushing one bronchus with PBS and separating out the lymphocytes on a discontinuous Percoll (Sigma-Aldrich) gradient as described previously (47). The cells were maintained overnight at 37°C and 5% $CO_2$ in R10 (RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM L-glutamine) and used the next day.

**Cellular immune responses.** For the evaluation of virus-specific gamma interferon (IFN-γ)-secreting cells, the monkey IFN-γ enzyme-linked immunospot (ELISPOT) kit from U-CyTech Biosciences (Utrecht, The Netherlands) was used according to the manufacturer's protocol. PBMCs were distributed in triplicate wells of 96-well plates (100 μl at dilutions of $1 \times 10^6$ and $5 \times 10^5$ cells/ml) and stimulated by adding a single pool of Gag, Nef, or Env peptides at a final concentration of 1 μg/ml for each peptide. Concanavalin A (5 μg/ml) served as a positive control and R10 or R10 plus dimethyl sulfoxide (final concentration, 0.7%) as negative controls. Results are reported as spot-forming cells (SFC)/million PBMCs following subtraction of spots in negative control wells. $SIV_{smH4}$ Env and $SIV_{mac251}$ Nef peptides (ABL) and $SIV_{mac239}$ Gag peptides (AIDS Research and Reference Reagent Program, NIAID, NIH) were 15-mers overlapping by 11 amino acids.

A similar ELISPOT was conducted using the Mabtech anti-rhesus IFN-γ antibody (Mabtech, Sweden) at a concentration of 15 μg/ml in 0.1 M carbonate-bicarbonate solution (pH 9.6) in 96-well nitrocellulose membrane plates (Millipore, MA). Each sample was set up in triplicate at $2 \times 10^5$ cells per well. The samples were stimulated for 24 h with four separate pools of $SIV_{mac239}$ Env peptides (AIDS Research and Reference Reagent Program, NIAID, NIH) and three separate pools of $SIV_{mac239}$ Gag peptides. The responses were detected with a biotinylated anti-IFN-γ antibody followed by streptavidin-alkaline phosphatase. Spots were visualized with 5-bromo-4-chloro-3-indolylphospate–nitroblue tetrazolium substrate (Sigma-Aldrich). A positive response was defined as 2 SFC/$2 \times 10^5$ PBMCs above the control as well as background levels assessed at week zero. ELISPOT responses to $SIV_{mac239}$ Gag were also evaluated following CD8 depletion of PBMCs using anti-human CD8 antibody, cross-reactive with rhesus CD8, that was conjugated to magnetic Dynal beads according to the manufacturer's protocol (Dynal, Invitrogen, CA).

**Intracellular cytokine staining.** Intracellular cytokine staining for detection of SIV Gag-, Env (smH4)-, and Nef-specific IFN-γ-, IL-2-, and tumor necrosis factor alpha (TNF-α)-secreting $CD8^+$ and $CD4^+$ central and effector memory T cells was performed on freshly isolated PBMCs, BAL, and lymph node (LN) cells. Cells ($1 \times 10^6$) in 1 ml of R10 were either not stimulated or incubated with pools of Env, Nef, or Gag peptides (1 μg/ml each peptide) or concanavalin A (positive control) for 6 h at 37°C and 5% $CO_2$. One hour into the incubation, Golgi-Stop (BD Pharmingen) was added to all tubes. Poststimulation the cells were transferred into fluorescence-activated cell sorter tubes (BD) and washed twice with PBS (Invitrogen). A cocktail of the following surface antibodies was added: CD4-peridinin chlorphyll protein (clone L200; BD Pharmingen) or CD8β-R-phycoerythrin-Texas Red (clone 2ST8.5H7; Beckman-Coulter), CD95-phycoerythin (clone DX2; BD Pharmingen), and CD28-fluorescein isothiocyanate (clone CD28.2; BD Pharmingen). The cells were incubated in the dark for 25 min at room temperature, washed with PBS, and fixed in 125 μl of Fix and Perm solution A (Invitrogen) for 15 min. After further washing the cells were incubated in 125 μl Fix and Perm solution B containing a cocktail of anti-IFN-γ (clone B27), anti-IL-2 (clone MQ1-17H12), and anti-TNF-α (clone MAb11) antibodies (all from BD Pharmingen) coupled to allophycocyanin as described above. The cocktail approach was used since experiments were performed using a four-color flow cytometer. Cell numbers were also limited, precluding multiple staining reactions. The cells were washed in PBS and stored in PBS containing 3.7% formaldehyde solution at 4°C until analysis. Analysis was performed on a BD FACSCalibur using CellQuest software. A minimum of 50,000 events in the lymphocytic gate, based on forward and side scatter, were acquired. A positive response was defined as an increase in the percentage of IFN-γ-positive cells in stimulated PBMCs over unstimulated PBMCs that was significant at the two-tailed α level of 0.05 by the continuity-adjusted chi-squared test. The response comparison was excluded from the analysis if the harmonic mean of the gated central or effector memory event numbers in the comparison were less than 300, due to the substantial loss of power for detecting a response.

**Humoral immune responses.** Binding antibodies to SIV gp140 and SIV Nef were assessed by enzyme-linked immunosorbent assay as described previously (48). The antibody titer was defined as the reciprocal of the serum dilution at which the optical density of the test serum was two times greater than that of a naïve control macaque serum diluted 1:50.

**ADCC assay.** The antibody-dependent cellular cytotoxicity (ADCC) assay was performed as described in detail elsewhere (16) using heat-inactivated serum or plasma samples and human PBMCs as effectors. The target cells were CEM-NKr coated with 15 μg/ml of recombinant $SIV_{mac251}$ gp140. The ADCC assay results were acquired on a BD FACSCalibur machine and analyzed with WinMDI version 2.8.

**Virologic assays.** For the assessment of viral loads, the enhanced chemiluminescence-based nucleic acid sequence-based amplification assay with a sensitivity of 2,000 copies/input volume was used (55). To evaluate plasma samples consistently below this detection limit, a real-time nucleic acid sequence-based amplification assay with a sensitivity of <50 copies/input volume was used (32).

**Statistical analyses.** Analyses of ELISPOT responses, antibody titers, and viral loads used the exact Wilcoxon rank sum test for simple two-group comparisons, the exact Kruskal-Wallis test for comparisons across the three immunization groups or all four groups at once, and the exact Wei-Johnson test for two-group comparisons over multiple times. *P* values reported have been corrected for the multiple comparisons between groups, except as noted below for Fig. 6E.

## RESULTS

**Prechallenge ELISPOT responses.** Over the immunization course, IFN-γ ELISPOT responses to SIV Env, Gag, and Nef were elicited (Fig. 1). Responses to Rev were low throughout and are not reported here. Two sets of Env peptides were used for stimulating the PBMCs as shown (Fig. 1A and B): one matched the $SIV_{smH4}$*env* encoded in the Ad5hr recombinant and the other matched the $SIV_{mac239}$*env* DNA plasmid and was closely related to the $SIV_{mac251}$ Env protein boost. Only weak responses were observed following stimulation with either peptide pool following the two DNA immunizations, but a boosting effect was observed after administration of Ad5hr-$SIV_{smH4}$*env* (Fig. 1A and B). Despite the high background of the control animals at week 14 and the variability among animals as shown by the error bars (Fig. 1B), responses to both $SIV_{mac239}$ and $SIV_{smH4}$ Env peptides were observed, reflecting a priming effect by the $SIV_{mac239}$*env* plasmid inoculations. Following the first $SIV_{mac251}$ envelope immunization, $SIV_{mac239}$ Env responses were boosted, reflecting the initial $SIV_{mac239}$*env* DNA priming. PBMCs obtained after the second envelope boost were only available for assay with $SIV_{smH4}$ Env peptides. Overall, no significant differences in Env-specific IFN-γ secretion were seen between the immunization groups.

Responses to Nef and Gag exhibited similar patterns (Fig. 1C and D). High levels of ELISPOT responses were observed following the Ad5hr-SIV administrations at week 12, but administration of Nef protein at weeks 24 and 36 did not boost the number of Nef-specific IFN-γ-secreting cells. Both Nef- and Gag-specific responses declined prior to challenge. As with the Env responses, no significant differences were seen between the three immunization groups.

**CD8 depletion ELISPOT.** SIV Gag-specific IFN-γ-secreting PBMCs were further analyzed after depletion of CD8-positive cells. As shown in Table 2, DNA immunization elicited a predominantly CD4 response, which shifted to include more CD8 T-cell responses following the Ad5hr-SIV immunizations. By week 46, 2 weeks prior to challenge, the ELISPOT responses were again predominantly CD4. Overall, there were no significant differences in levels of CD4 or CD8 responses between immunization groups.

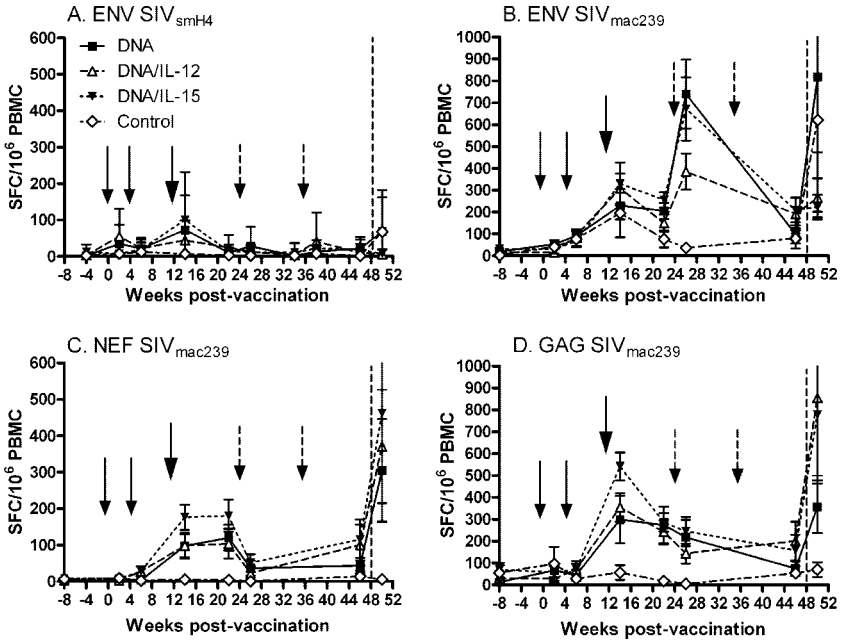


FIG. 1. IFN-γ ELISPOT responses to SIV Env, Nef, and Gag peptide pools. Mean SFC ± the standard errors of the means for each immunization group are shown for each immunogen. Env-specific responses were evaluated using both $SIV_{smH4}$ and $SIV_{mac239}$ Env peptide pools (A and B). Small arrows indicate times of DNA administration (weeks 0 and 4), the large arrow shows the time of Ad5hr-SIV recombinant immunization at week 12, and the broken arrows mark Env and Nef protein boosts at weeks 24 and 36. The vertical dashed lines indicate the time of challenge.

**Analysis of prechallenge memory T cells.** SIV Env-, Nef-, and Gag-specific effector and central memory CD4 and CD8 T cells in PBMCs and LN were examined by intracellular cytokine staining for production of IL-2, IFN-γ, and TNF-α in combination and in BAL cells for production of IFN-γ. Rectal pinch biopsies were obtained as well but yielded too few cells, precluding collection of meaningful data. While the ELISPOT results indicated a preponderance of vaccine-elicited IFN-γ-secreting SIV Gag-specific CD4 T cells in peripheral blood (Table 2), SIV Env-specific $CD8_{CM}$ T cells made up the greatest proportion of memory cells (Fig. 2C versus A, B, and D). This may have been due to the majority of effector memory cells homing to mucosal effector sites. Previously, we reported the presence of gut homing receptors on $CD8^+$ T cells induced by our vaccine regimen, as well as central and effector memory cells in BAL, an effector site (65). Here, the strongest responses prior to challenge (weeks 2 to 38) were elicited by $SIV_{smH4}$ Env peptides. In view of the weak Env-specific IFN-γ ELISPOT responses following the two DNA immunizations (Fig. 1A and B), one must assume these DNA vaccine-elicited $CD8_{CM}$ T cells were producing primarily IL-2 and/or TNF-α. Overall, none of the memory cell compartments displayed differences among immunization groups prior to challenge.

LN biopsies obtained at weeks 6 and 14 following the DNA and Ad5hr recombinant immunizations revealed low and sporadic SIV-specific CD4 and CD8 memory T-cell responses with

TABLE 2. Proportion of SIV Gag-specific $CD4^+$ IFN-γ-secreting T cells in peripheral blood over time as evaluated by ELISPOT, with and without $CD8^+$ T-cell depletion

| Wk | ELISPOT response to SIV Gag[a] | | | | | | | | | $CD3^+$ $CD4^+$ count[b] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Group 1, DNA | | | Group 2, DNA/IL-12 | | | Group 3, DNA/IL-15 | | | | | |
| | PBMC | $CD8^-$ PBMC | % $CD4^+$ PBMC | PBMC | $CD8^-$ PBMC | % $CD4^+$ PBMC | PBMC | $CD8^-$ PBMC | % $CD4^+$ PBMC | Group 1, DNA | Group 2, DNA/IL-12 | Group 3, DNA/IL-15 |
| Postvaccination | | | | | | | | | | | | |
| 2 | 66 ± 29 | 49 ± 25 | 74 | 28 ± 16 | 34 ± 10 | 122 | 59 ± 27 | 58 ± 28 | 98 | | | |
| 14 | 299 ± 107 | 223 ± 80 | 75 | 354 ± 65 | 205 ± 50 | 58 | 541 ± 64 | 417 ± 69 | 77 | | | |
| 22 | 274 ± 83 | 160 ± 53 | 59 | 242 ± 55 | 125 ± 41 | 52 | 285 ± 42 | 170 ± 28 | 60 | | | |
| 26 | 218 ± 79 | 126 ± 36 | 58 | 144 ± 45 | 103 ± 28 | 71 | 245 ± 66 | 133 ± 20 | 54 | | | |
| 46 | 74 ± 33 | 88 ± 39 | 118 | 158 ± 88 | 165 ± 89 | 104 | 158 ± 72 | 195 ± 70 | 124 | 863 ± 128 | 904 ± 119 | 1,069 ± 161 |
| Postchallenge | | | | | | | | | | | | |
| 2 | 357 ± 120 | 59 ± 33 | 16 | 854 ± 390 | 83 ± 27 | 10 | 778 ± 279 | 43 ± 15 | 6 | 1,132 ± 127 | 870 ± 171 | 1,006 ± 85 |

[a] The PBMC and $CD8^-$ PBMC data are reported as mean SFC ± the standard error of the mean; the percent $CD4^+$ PBMCs was calculated as follows: (mean $CD8^-$ PBMC SFC)/(mean PBMC SFC) × 100.

[b] Group mean CD4 cell counts ± standard errors of the means.

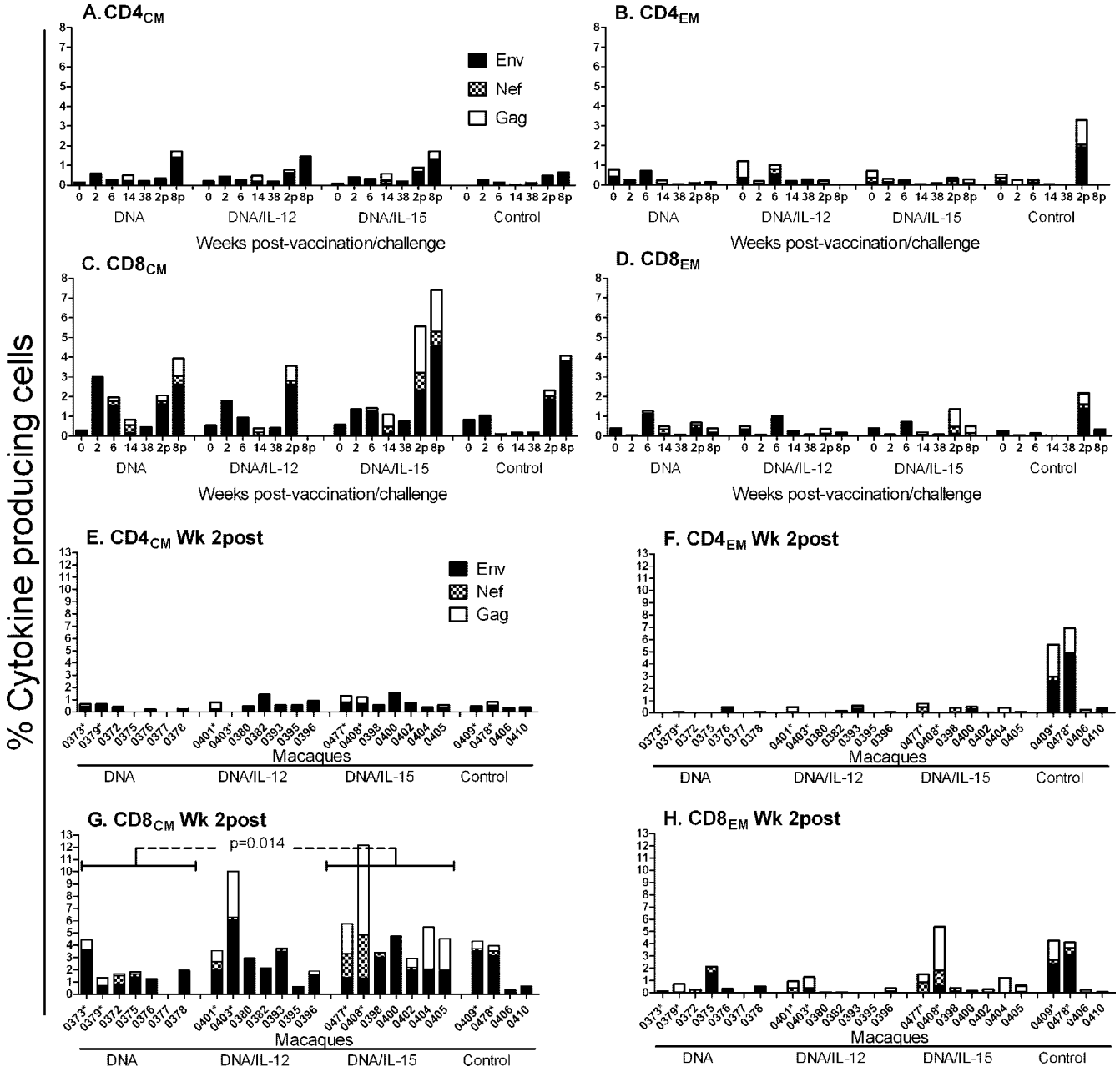


FIG. 2. Intracellular cytokine staining for SIV-specific CD4 and CD8 memory T cells secreting IFN-γ, IL-2, and TNF-α in PBMCs. (A to D) Stacked responses to SIV Env, Nef, and Gag peptide pools by CD4 and CD8 central and effector memory cells over time. Weeks 2 and 8 postchallenge are designated 2p and 8p, respectively. Mean responses for each group at each time point are plotted. (E to H) Stacked responses observed 2 weeks postchallenge for individual animals in each group. The asterisks denote Mamu-A*01-positive macaques. Macaque 0377 was not assayed at week 2 postchallenge.

no significant differences among groups (data not shown). In contrast, BAL cells, tested only for secretion of IFN-γ, exhibited strong $CD8_{CM}$ and $CD8_{EM}$ responses as expected at week 14 after the Ad5hr-SIV immunizations, which persisted through week 38 prior to challenge. The responses were mainly against Gag and Nef, reflecting the priming by the matched DNA immunizations (Fig. 3A and B). Once again, no differences were observed between immunization groups.

**Prechallenge humoral immune responses.** SIV gp140-specific binding antibodies were induced in all immunization groups following the Ad5hr-SIV immunizations at week 12 and were boosted to similar high titers following the envelope protein immunizations (Fig. 4A). Antibodies to Nef also appeared following the Ad5hr recombinant immunizations in all three vaccinated groups (Fig. 4B); however, in contrast to envelope-specific antibodies, macaques in group 3 primed in the pres-

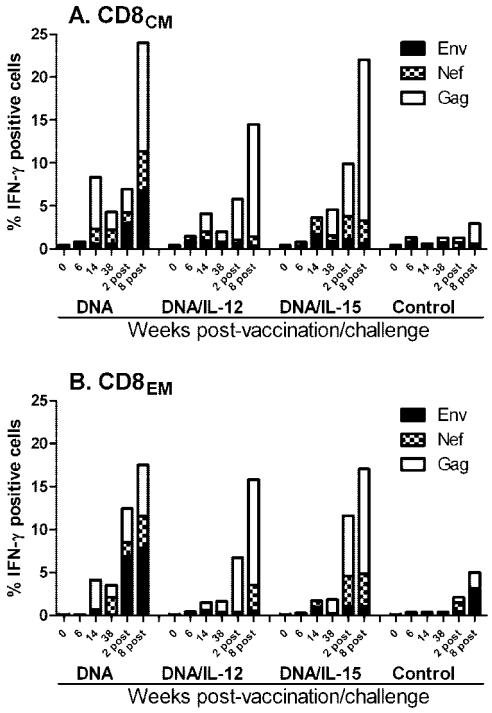


FIG. 3. Intracellular cytokine staining for SIV-specific $CD8_{CM}$ (A) and $CD8_{EM}$ (B) T cells secreting IFN-γ in bronchoalveolar lavage fluid. Stacked responses to SIV Env, Nef, and Gag peptide pools are shown over time. Mean responses for each group at each time point are plotted.

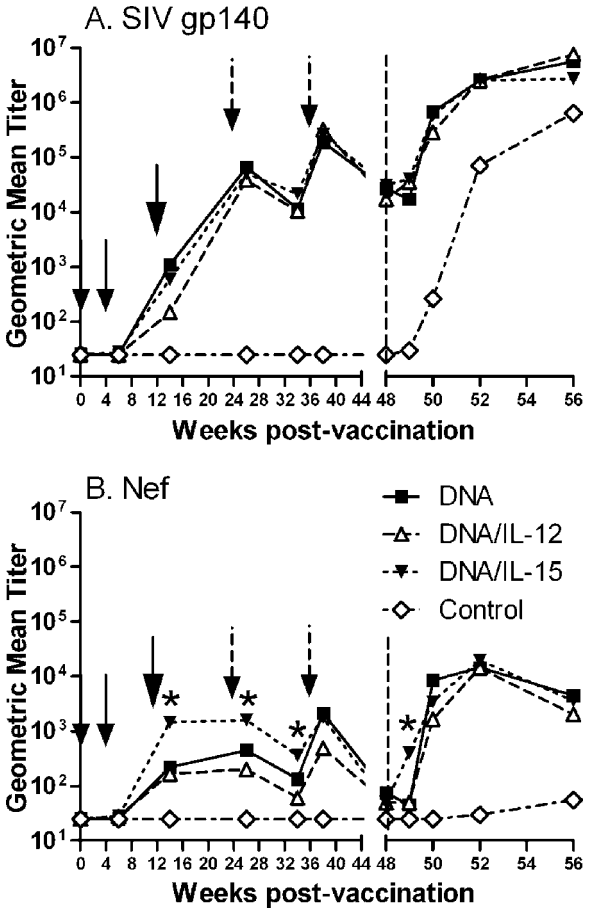


FIG. 4. Vaccine-induced antibody responses. Geometric mean antibody binding titers against SIV gp140 (A) and SIV Nef (B) are plotted over time. Small arrows indicate times of DNA administration (weeks 0 and 4), the large arrows show the time of Ad5hr-SIV recombinant immunization at week 12, and the broken arrows mark Env and Nef protein boosts at weeks 24 and 36. The vertical dashed lines indicate the time of challenge, and stars indicate significantly higher titers of the DNA/IL-15 group compared to groups 1 and 2 ($P$ = 0.04, 0.044, 0.029, and 0.014 at weeks 14, 26, 34, and 49, respectively).

ence of DNA encoding IL-15 exhibited anti-Nef titers significantly higher than for groups 1 and 2 at weeks 14, 26, and 34 ($P$ values of 0.04, 0.044, and 0.029, respectively). By the time of challenge, both envelope and Nef antibodies had declined to comparable values in all immunization groups.

**ADCC.** Previously, anti-envelope antibodies with binding titers of approximately $10^4$ at the time of challenge were not able to neutralize the primary $SIV_{mac251}$ challenge virus (49) but mediated ADCC activity, which is significantly correlated with reduced acute-phase viremia (17). Therefore, macaque sera were tested for the ability to mediate ADCC activity using $SIV_{mac251}$ gp140-coated target cells (Fig. 5). Sera from macaques in group 1 (DNA) and group 3 (DNA/IL-15) first showed ADCC activity after administration of the Ad5hr recombinants. While activity in all immunized macaque sera was strongly enhanced by the first protein boosts, ADCC titers remained low subsequently, with macaques in group 2 (DNA/IL-12) exhibiting the lowest titers. Overall, there were no significant differences between the three immunization groups.

**$SIV_{mac251}$ challenge outcome.** Following intrarectal challenge with $SIV_{mac251}$, all animals became infected. No protection was observed in the immunized macaques compared to controls (Fig. 6A). The majority of Mamu-A*01-positive animals controlled viremia better than their Mamu-A*01-negative counterparts, as expected (data not shown). However, when Mamu-A*01-negative and -positive animals were analyzed separately (Fig. 6B and C), the combined immunized Mamu-A*01-negative animals showed a clear protective effect, with a 1-log reduction in acute viremia (weeks 1 to 4) compared to the controls ($P$ = 0.037) (Fig. 6B). No difference was observed between immunization groups. Although the control non-Mamu-A*01 macaques continued to display viral loads approximately 1.5 logs higher than macaques in the DNA and DNA/

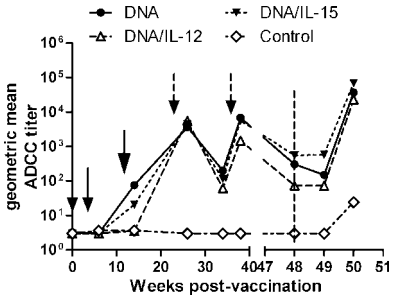


FIG. 5. Vaccine-induced ADCC. ADCC antibody titers were evaluated over the course of the study using target cells coated with SIV gp140. Small arrows indicate times of DNA administration (weeks 0 and 4), the large arrow shows the time of Ad5hr-SIV recombinant immunization at week 12, and the broken arrows mark Env and Nef protein boosts at weeks 24 and 36. The vertical dashed line indicates the time of challenge.

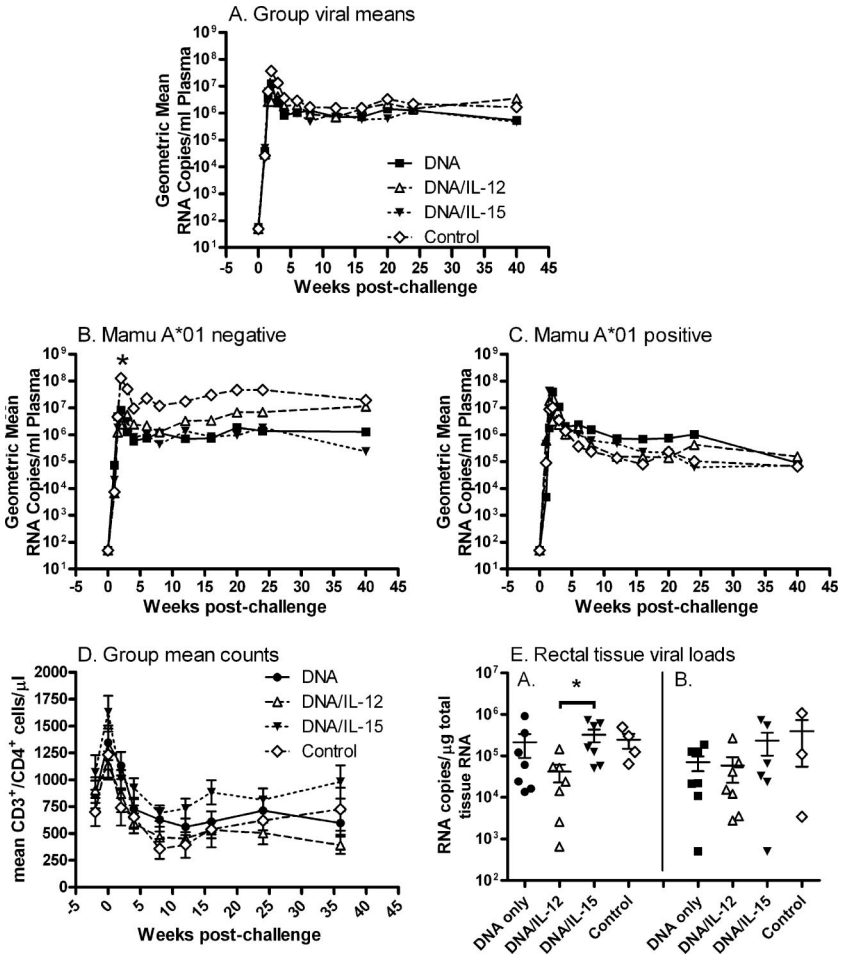


FIG. 6. Evaluation of protective efficacy following intrarectal challenge with $SIV_{mac251}$. (A) Viral loads by immunization group over time, including all macaques. (B and C) Viral loads by immunization group for Mamu-A*01-negative (B) and Mamu-A*01-positive (C) macaques only. The asterisk in panel B denotes a 1-log reduction in acute viremia of combined immunized macaques compared to controls ($P = 0.037$). (D) Mean CD4 counts by immunization group. Error bars indicate the standard errors of the means (SEM). (E) Viral loads in rectal biopsies 2 and 8 weeks postchallenge (graphs A and B, respectively). Viral loads are plotted for individual animals in each group. Group means (large bars) and SEM (small bars) are indicated. The DNA/IL-12 group showed a lower viral load compared to the DNA/IL-15 group at week 2 postchallenge (marked by the asterisk), which was marginally nonsignificant after correction for multiple comparisons ($P = 0.065$).

IL-15 groups during the chronic phase, these differences were not statistically significant. In contrast, the strong effect of Mamu-A*01 in the two control and six immunized macaques carrying this allele obscured any protective effect of the vaccine regimen, and the immunization groups were indistinguishable from the controls at all time points (Fig. 6C). Patterns of $CD3^+$ $CD4^+$ T-cell decline similarly revealed no differences in protective efficacy between the immunized and control macaques (Fig. 6D) regardless of whether the macaques were separated into Mamu-A*01-negative or -positive groups (data not shown).

Rectal pinch biopsies were obtained postchallenge for analysis of viral RNA (Fig. 6E). All samples tested were positive. At week 2 postchallenge the immunized groups taken together were not different from the controls. The IL-12-primed animals (group 2) exhibited a lower viral load than the IL-15-primed animals (group 3) ($P = 0.011$) and the controls ($P = 0.024$) (Fig. 6E), but when corrected for multiple comparisons, the differences became only borderline significant (group 2 versus group 3, $P = 0.065$; group 2 versus controls, $P = 0.073$). Group 2 macaques maintained the lowest rectal tissue viral load at week 8 postchallenge (Fig. 6E), but overall no significant differences among groups were observed at this later time point.

**Postchallenge cellular immune responses.** Env-specific recall responses in PBMCs were not observed at week 2 postchallenge by ELISPOT assay using either $SIV_{smH4}$ or $SIV_{mac239}$ Env peptides (Fig. 1A and B), perhaps reflective of the mixed immunizations, including $SIV_{mac239}$*env* encoded in DNA, $SIV_{smH4}$*env* encoded in Ad5hr-SIV, and $SIV_{mac251}$ gp140 envelope protein. However, strong recall responses were observed against both Nef and Gag peptides (Fig. 1C and D), but with no differences between the three immunization groups.

A strong expansion of SIV-specific $CD8_{CM}$ cells in PBMCs was also observed 2 and 8 weeks postchallenge (Fig. 2C and G), whereas little change was seen in the $CD8_{EM}$ or CD4 memory populations (Fig. 2A, B, D to F, and H). Further, 2

weeks postchallenge the macaques of group 3 (DNA/IL-15) exhibited significantly higher total (Env, Gag, Nef) SIV-specific $CD8_{CM}$ T-cell responses compared to macaques that received DNA without any cytokine (Fig. 2G) ($P = 0.014$). Across all the groups, the Mamu-A*01 macaques tended to develop the strongest recall $CD8_{CM}$ SIV-specific responses (Fig. 2G).

The expansion of memory CD8 T cells postchallenge was also implied by the sudden decrease in the percentage of SIV Gag-specific CD4 T cells detected by ELISPOT to only 6 to 16% of the overall ELISPOT response (Table 2). This decrease was not due to a loss of CD4 cells in blood, since the mean CD4 counts remained unchanged 2 weeks postchallenge compared to prechallenge values at week 46 (Table 2).

Recall responses were observed even more strongly in the BAL compartment (Fig. 3A and B). As BAL is an effector site, expansion of $CD8_{EM}$ as well as $CD8_{CM}$ cells was observed. In macaques that received only DNA priming, a homogeneous response to all three antigens, Env, Gag, and Nef, was observed. In contrast, the macaques that received DNA plus either IL-12 or IL-15 showed recall responses primarily to Gag and Nef. In BAL, as in peripheral blood, the strongest responses were among $CD8_{CM}$ rather than $CD8_{EM}$ cells. There were no significant differences between the immunized groups at weeks 2 and 8 postchallenge.

**Postchallenge humoral immune responses.** Postchallenge anamnestic antibody responses to Env and Nef rapidly developed in all three immunization groups (Fig. 4A and B). Elevated anti-Nef titers first appeared at week 49, 1 week postchallenge, in macaques of group 3 immunized with IL-15 DNA compared to all other macaques ($P = 0.014$) (Fig. 4B). By week 50, however, no differences among immunization groups were observed in either anti-Env or anti-Nef titers.

In parallel with the strong anamnestic response in anti-Env binding antibody, the ability of sera of the immunized macaques to mediate ADCC also increased sharply, compared to controls, by 2 weeks postchallenge (Fig. 5).

## DISCUSSION

Both IL-12 and IL-15 have been shown to modulate immune responses and augment the immunogenicity of vectored vaccines. Previously, for example, a 10-fold increase in Gag-specific antibodies and an approximate 5-fold increase in SIV Gag-specific IFN-γ-secreting cells followed administration of IL-12 DNA together with an SIV*gag* plasmid vaccine (57). IL-12 also has an activating function on murine and human B cells, resulting in their differentiation into immunoglobulin M-producing cells (2). Similarly, IL-15 may modulate antibody responses in addition to its well-known effects on T cells and NK cells, as it enhances germinal center B-cell proliferation (45). Therefore, we anticipated that the cytokine groups compared to the DNA-only group would exhibit both enhanced SIV-specific immunity as well as protective efficacy. Unexpectedly, with few exceptions, similar immune responses were seen in all immunization groups, and all three immunization regimens failed to achieve significant protection. Although regrouping the macaques according to their Mamu-A*01 status revealed a modest statistically significant 1-log reduction in acute viremia in immunized Mamu-A*01-negative macaques compared as a group with Mamu-A*01-negative control macaques, this effect was transient. The observation suggests the major histocompatibility complex class I haplotype effect was stronger than any vaccine-induced control.

The lack of protective efficacy in this study is puzzling, as all regimens were immunogenic, eliciting systemic and mucosal cellular responses and serum antibodies to Env and Nef. While low-level immune responses were observed following the DNA immunizations, as expected, the Ad5hr-SIV immunization boosted both cellular and humoral immunity. The humoral responses after the single Ad5hr-SIV immunization were higher than previously observed after one administration (12, 47), highlighting the good priming features of the two DNA immunizations. Of note, the mismatched Ad5hr-$SIV_{smH4}$*env*/*rev* recombinant boosted $SIV_{mac239}$ Env-specific IFN-γ SFC, primed by DNA encoding $SIV_{mac239}$ *env* and *rev* genes (Fig. 1B). This response was further boosted by the Env protein immunization. However, functional ADCC-mediating antibody titers remained lower than those elicited by two sequential Ad5hr-SIV recombinant immunizations and SIV gp120 boosting (49), which had been previously associated with control of acute-phase viremia (17). Overall, the cytokine DNA priming immunizations did not enhance immune responses compared to priming with DNA only, in contrast to previous reports (8, 11). We did observe higher anti-Nef titers in the DNA/IL-15 group prior to challenge (Fig. 4B) and a more rapid anti-Nef anamnestic response compared to the other immunization groups. However, these responses had no apparent effect on protective efficacy. Postchallenge $CD8_{CM}$ responses were also higher in the DNA/IL-15 group compared to DNA alone (Fig. 2G) but again did not improve the challenge outcome. Otherwise, immune responses were indistinguishable between groups. The lack of enhancement of immune responses in the DNA/IL-12 and DNA/IL-15 groups cannot be attributed to the quality or expression levels of the plasmids encoding the cytokines, since the same plasmids were used previously in other studies and were shown to increase IFN-γ-secreting cells and/or T-cell proliferative responses (7, 8, 11, 54). Rather, the number of immunizations was likely important, as effects in these earlier studies were seen after three or more immunizations.

The lack of protection was unexpected, in view of earlier results showing that priming rhesus macaques with DNA vaccines enhanced the protective efficacy of nonreplicating Ad- and MVA-vectored SIV vaccines (3, 59). As two sequential immunizations with replicating Ad5hr-SIV recombinants followed by envelope protein boosting elicits potent, durable protection against intrarectal $SIV_{mac251}$ challenge of rhesus macaques (32, 49), we anticipated even better protection when combining the two vaccine modalities, even without additional cytokines. In retrospect, however, the ability of DNA priming to strongly enhance protection was established using the $SHIV_{89.6P}$ challenge model. Few comparable studies have been performed in rhesus monkeys by using pathogenic SIV challenge models, and in these cases, results using DNA alone or in combination with other vectored vaccines have been less impressive. Nine sequential SIV DNA intramuscular immunizations resulted in reduced viral loads following intrarectal challenge with $SIV_{mac251}$ (42), while seven SIV DNA gene gun immunizations reduced initial viral loads in four of seven rhe-

sus macaques challenged with $SIV_{\Delta B670}$ (14). Using combination approaches, 10 DNA immunizations followed by 2 immunizations with MVA, both encoding multiple SIV genes, led to a transient reduction in acute viremia after intrarectal challenge with $SIV_{mac239}$ (21). Similarly, four SIV DNA intramuscular immunizations followed by three oral administrations of *Listeria monocytogenes* encoding SIV genes resulted in an initial reduction in viral burden after intrarectal $SIV_{mac239}$ challenge that was not sustained (9). Overall, the outcome for non-Mamu-A*01 macaques in this study, in which fewer immunizations were used, is in line with these results.

Evaluation of cytokine DNAs as vaccine adjuvants in rhesus macaques has also used primarily the $SHIV_{89.6P}$ challenge model. While a vaccine regimen comprised of six SIV DNA immunizations plus IL-15 DNA better controlled peak $SHIV_{89.6P}$ viremia than that in either control macaques or macaques that received the DNA vaccines without DNA/IL-15 (8), in general, comparative studies of IL-12 and IL-15 plasmids have shown vaccines incorporating IL-12 to be most effective. After four immunizations with a SIV*gag* plasmid, macaques that also received an IL-12 plasmid exhibited the greatest reductions in peak and set point viremia following $SHIV_{89.6P}$ challenge (11). Similarly, rhesus macaques that were primed with SIV plasmid DNA, including IL-12 DNA, prior to recombinant vesicular stomatitis virus-SIV administration exhibited the best protective outcome after $SHIV_{89.6P}$ challenge (13). With regard to a pathogenic SIV challenge model, priming three times with a multigenic SIV plasmid DNA plus IL-12 DNA followed by boosting with a multigenic nonreplicating Ad recombinant without IL-12 led to reduced viral burdens after intrarectal $SIV_{mac251}$ challenge (60). This study was promising although difficult to evaluate, as few animals were studied and rapid progressors, but not Mamu-A*01 macaques, were eliminated from statistical analysis. An experimental arm lacking IL-12 was not included. Administration of the DNA and Ad-SIV vaccines to the same intramuscular sites may have enhanced synergism between the different modalities. Here, following the intramuscular DNA immunizations, the Ad5hr-SIV vaccine was administered intratracheally, a more permissive site for replication of the host range mutant vector in macaques compared to the intramuscular or even the alternate mucosal intranasal route. This may have diminished a potential enhancing effect. It has been reported that IL-12 DNA needs to be administered at the same site as antigen for elicitation of an augmented cellular, but not humoral, response (57). Administration of Ad5hr-SIV vaccine intratracheally resulted in strong $CD8_{EM}$ and $CD8_{CM}$ responses in BAL fluid, an effector site that mirrors the small intestine (51), but this might have been at the expense of an IL-12 effect. It would be of interest to investigate administration of DNA with or without cytokine DNAs to the same site, followed by the replicating Ad5hr recombinants.

In addition to maintaining the same immunization route, other alterations in the vaccine strategy might lead to improved protection. Here, the DNA delivery method was not optimal. In comparison to intramuscular immunization, administration of DNA by electroporation greatly enhances cellular and humoral immune responses (19, 29). Further, since SIV *env*, *gag*, *rev*/*nef*, IL-12, and IL-15 were all encoded in different plasmids, there might have been poor cotransfection or coexpression of antigens and cytokines. Use of bicistronic vectors to provide simultaneous expression of cytokine and antigen in the same transfected cell might be optimal.

Further, although the goal of this study was to determine if two sequential DNA priming immunizations could substitute for an initial prime with replicating Ad-SIV recombinants, simply increasing the number of DNA immunizations, as mentioned above, might have improved the experimental outcome. Three immunizations with SIV*gag* DNA plus IL-12 DNA elicited a significant increase in IFN-γ-secreting cells compared to SIV*gag* DNA only; however, only two vaccinations were ineffective (7), in accord with our findings (Fig. 1).

The increasing understanding of the complex biology of IL-15 suggests that DNA expression plasmids should also express the IL-15 receptor, IL-15Rα, in order to achieve desired adjuvant effects. IL-15 is more stable and has a longer half-life when it is bound to IL-15Rα or is membrane associated and most effectively stimulates strong, durable CD8 memory responses if it is produced by cells that also present antigen and IL-15Rα (6, 56). Muscle cells express IL-15Rα (53) and could, following IL-15 DNA immunization, present de novo-synthesized IL-15 to lymphocytes according to the *trans*-presentation model of Ma et al. (30). Upon first priming, naïve T cells encounter expressed DNA; however, IL-15 has the most profound effect on differentiated effector and central memory T cells (52), which benefit most from IL-15 stimulation. Therefore, as memory cells home to lymph nodes, if IL-15 is to have an effect upon subsequent immunizations, it should be expressed with IL-15Rα for stability and trafficking to sites where memory cells reside. However, achieving the proper balance of IL-15 expression is also critical, since with constant dosing, the ability of T cells to respond to IL-15 wanes due to feedback inhibition (26) and overall is not beneficial (52, 62).

In summary, two DNA plasmid immunizations followed by a single administration of replication-competent Ad5hr-SIV recombinant vaccine did not substitute for two sequential Ad5hr-SIV recombinant immunizations with regard to protective efficacy against virulent $SIV_{mac251}$. Although the DNA/Ad regimen was immunogenic, the addition of DNAs encoding cytokine adjuvants did not augment immune responses to any appreciable extent. A number of factors might have contributed to the ineffectiveness of this vaccine regimen. In view of the advantages of focusing the immune response on gene inserts instead of vector components by the use of DNA priming, further exploration of vaccine strategies combining DNA and replicating Ad recombinant vaccines should proceed. The potential of cytokine adjuvants for tailored modulation of vaccine-elicited immune responses remains strong and should be exploited in future vaccine approaches.

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