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DNA and virus particle vaccination protects against acquisition and confers control of viremia upon heterologous simian immunodeficiency virus challenge

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We have previously shown that macaques vaccinated with DNA vectors expressing SIVmac239 antigens developed potent immune responses able to reduce viremia upon high-dose SIVmac251 challenge. To further improve vaccine-induced immunity and protection, we combined the SIVmac239 DNA vaccine with protein immunization using inactivated SIVmac239 viral particles as protein source. Twenty-six weeks after the last vaccination, the animals were challenged intrarectally at weekly intervals with a titrated dose of the heterologous SIVsmE660. Two of DNA-protein coimmunized macaques did not become infected after 14 challenges, but all controls were infected by 11 challenges. Vaccinated macaques showed modest protection from SIVsmE660 acquisition compared with naive controls (P = 0.050; stratified for TRIM5α genotype). Vaccinees had significantly lower peak (1.6 log, P = 0.0048) and chronic phase viremia (P = 0.044), with 73% of the vaccinees suppressing viral replication to levels below assay detection during the 40-wk follow-up. Vaccine-induced immune responses associated significantly with virus control: binding antibody titers and the presence of rectal IgG to SIVsmE660 Env correlated with delayed SIVsmE660 acquisition; SIV-specific cytotoxic T cells, prechallenge CD4+ effector memory, and postchallenge CD8+ transitional memory cells correlated with control of viremia. Thus, SIVmac239 DNA and protein-based vaccine protocols were able to achieve high, persistent, broad, and effective cellular and humoral immune responses able to delay heterologous SIVsmE660 infection and to provide long-term control of viremia. These studies support a role of DNA and protein-based vaccines for development of an efficacious HIV/AIDS vaccine.

virus acquisition | HIV-1 vaccine | viral vaccines/immunology | humoral immunity | HIV-1/immunology

The use of a combination vaccine consisting of the recombinant Canarypox ALVAC-HIV (vCP1521; containing Gag, PR, and Env) together with gp120 Env protein (AIDSVAX B/E) resulted in modest, but statistically significant protection from infection in the RV144 vaccine trial conducted in Thailand (1). The limited efficacy and the fact that the vaccine-induced responses waned over time suggest that improved vaccine designs are needed to achieve long-lasting cross-clade-specific immune responses able to prevent infection. Rhesus macaque simian immunodeficiency virus (SIV) challenge models provide an excellent system to test different vaccine modalities and to compare efficacy using different challenge viruses and infection routes.

DNA as priming immunization together with boosting by recombinant viral vectors is a vaccine platform widely used in the HIV/SIV field. DNA as the only vaccine component has been considered poorly immunogenic in humans, although recent results showed that in vivo DNA electroporation (EP) results in more efficient vaccine delivery, a higher frequency of responders, and higher, longer-lasting immunity than needle/syringe delivery (2). Similarly, the inclusion of DNA encoding the cytokine IL-12 as molecular adjuvant has been shown to be advantageous (3). These recent data suggest that DNA-only vaccination can be very immunogenic in humans. In an effort to further optimize DNA vaccines, we and others have previously focused on the use of DNA-only immunization and have reported successful induction of protective immune responses against SIV (4–8). DNA vaccination provides advantages, including simplicity, stability, versatility, and repeated administration without immunity against an exogenous vector, together with induction of high levels of antigen-specific immune responses upon improved delivery by in vivo EP (reviewed in refs. 9 and 10).

We hypothesized that the immunity induced by DNA vaccination could be improved by combining DNA immunization with simultaneous or sequential administration of inactivated viral particles as a source of viral proteins. The rationale for the addition of protein immunogens was to improve a key limitation of the SIV/HIV DNA vaccines, namely the relatively low levels of antibodies elicited against the virus. Here, we explored the protection afforded by this type of vaccination from virus acquisition upon repeated intrarectal challenge with a titrated dose of the heterologous SIVsmE660 and analyzed the immunological correlates of virus control.


Conflict of interest statement: KEB and NS are employed by Inovio Pharmaceuticals, Inc. and as such receive salary, bonuses and stock options as compensation. GNP and BFK are inventors on US government-owned patents and patent applications related to DNA vaccines and gene expression optimization that have been licensed to several companies. This article is a PNAS Direct Submission.

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Results

SIVmac239-Based Vaccine Protected Macaques from Heterologous SIVsmE660 Challenge. Rhesus macaques (n = 8 per group) (Table S1) were vaccinated four times (weeks 0, 8, 16, and 36) (Fig. 1A) with SIVmac239-derived vaccines using protocols that included DNA only (group 1), DNA+protein [aldrithiol-2 (AT-2) inactivated SIVmac239 viral particles] coimmunization (group 2), and sequential DNA prime-protein (viral particles) boost (group 3). All animals received the same optimized DNA vaccine, together with IL-12 DNA as adjuvant, via intramuscular injection followed by in vivo EP. The animals in all three groups developed strong and durable immune responses against SIV (see below). Inclusion of inactivated viral particles in the vaccine further increased the Ab levels, especially against Env (see below). After a rest period of 26 wk, the macaques were challenged intrarectally at weekly intervals with a titrated dose of a well-characterized heterologous SIVsmE660 (11). Within this swarm, the SIVsmE660 Env variants differ by ∼17% from the SIVmac239 Env sequence, mimicking the interclade Env diversity of HIV-1. The control group was expanded by 15 previously reported animals (11, 12), which were challenged with the same SIVsmE660 viral stock using an identical infection protocol (Table S2). After 11 challenges all control animals became infected, whereas after 14 challenges two of the eight animals from group 2 (DNA+protein coimmunization) and 14 of the 15 from group 3 remained uninfected (Fig. 1B). Because the TRIM5α genotype was reported to affect viremia after infection by SIVsmE660 (13), statistical analyses were performed after stratification to control for the restrictive TRIM5α genotype relative to the susceptible and moderate restrictive genotypes of the enrolled animals (Table S2). The analysis of virus acquisition was performed by combining all vaccinees (n = 24) (Fig. 1C) because they shared the SIVmac239 based DNA vaccine. We noted that the vaccinees and controls experienced similar infection frequencies over the first two challenges. After that point, the vaccinees showed slower acquisition than the controls, and the analysis of protection versus infection as a dichotomous outcome using the Cochran-Mantel-Haenszel test showed vaccine-induced protection against virus acquisition (P = 0.050). Additional evidence for a significant vaccine-induced delay of virus acquisition came from the analysis of vaccinees with mucosal SIV-specific IgG (see below). Thus, SIVmac239-derived vaccination-induced immunity able to protect from acquisition of the heterologous SIVsmE660.

Single genome amplification and direct sequencing of the SIV env gene from the plasma of the infected animals demonstrated that infections included variants from across the swarm of SIVsmE660-sequenced stock (Fig. S1). Transmitted/founder virus lineages were distributed widely throughout the phylogenetic tree of the challenge stock, suggesting that no selection or sieve effect could be identified at this level. Enumeration of the different Env sequences revealed that the infection of the controls and the vaccinees occurred with a median of one variant (Table S2), confirming that the titrated virus dose used for challenge in the present study and in Lai et al. (11) accurately modeled typical heterosexual HIV-1 transmissions (14, 15).

SIVmac239 Vaccinated Macaques Showed Long-Lasting Control of the Heterologous SIVsmE660 Challenge. To examine potential vaccine effects on control of viral replication, viral load (VL) was monitored over 40 wk of follow-up (Fig. 1D and Table S2), demonstrating lower viremia in the vaccinees. Peak VL was reached between weeks 1 and 3 for both the infected vaccinees and

Fig. 1. SIVmac239 vaccination schedule and challenge results after heterologous SIVsmE660 repeated low-dose challenge. (A) Macaques were immunized four times (V1 to V4) with DNA (group 1); DNA together with unadjuvanted AT–2–inactiveated SIVmac239 particles (group 2); DNA, followed by 2 AT–2 SIV particle vaccinations (group 3). After 26 wk, the animals were subjected to weekly intrarectal challenges using a titrated dose of the heterologous SIVsmE660 virus and were monitored for another 40 wk after infection. (B and C) Kaplan–Meier curves of the number of SIVsmE660 challenges to infection. (D) The three vaccine groups and (C) the combined group of vaccinated animals (n = 24) and controls (n = 23). For the combined group of vaccinees, analysis of protection versus infection as a dichotomous outcome using the Cochran-Mantel-Haenszel test showed vaccine-induced protection against virus acquisition (P = 0.050). (D) Median VL of the combined group of infected vaccinees (n = 22) and the controls (n = 8) monitored over the 40 wk of follow-up. (E) Comparison of peak VL of the combined group of vaccinees (n = 22) and the controls (n = 8). (F) Comparison of the chronic phase virus loads (AUC of weeks 7–40) of the combined group of infected vaccinees (n = 22) and the controls (n = 8).
controls. Vaccinees had significant lower peak VL (1.6 log; \(P = 0.0048\), stratified for TRIM5\(\alpha\)) with a median of log 5.99 compared with the controls with a median of log 7.58 (Fig. 1E). The majority of the vaccinees (73%) rapidly controlled viremia (Fig. S2). In the latter part of the chronic phase, the difference in VL between vaccinees and controls was >3 logs, with a median VL among the vaccinees at the detection limit (50 copies per milliliter of plasma). VL during the chronic phase of the infection (weeks 7–40) were analyzed and compared as censored area-under-the-curve (AUC) (Table S2), as a less variable statistical approach. The vaccinees had significantly lower chronic VL (\(P = 0.044\), stratified for TRIM5\(\alpha\)) (Fig. 1F) compared with the control animals. These data show potent long-lasting control of the heterologous SIVsmE660 by the vaccinees.

The peak VL also correlated directly with AUC (Fig. S3) (\(P = 0.0002\)), suggesting that peak VL was a good predictor of the virologic outcome. The AUC analysis suggested a grouping of the animals according to virological outcome. The groups were defined as: Elite Controllers, Viremic Controllers, and Noncontrollers, as denoted in Fig. S2 and Table S2. Elite Controllers (12 animals) were defined by peak VL of <6 log and chronic viremia below the threshold of detection for several weeks during the 40 wk of follow-up; Viremic Controllers (4 animals) were defined by peak VL of >6 log, detectable VL during the chronic phase and some occasional declines below the threshold of detection for several weeks during the 40-wk follow-up. Noncontrollers (6 animals) were defined by high peak and chronic VL and included one animal with the TRIM5\(\alpha\) restrictive genotype. The three-group trend analysis matched the significant relation between AUC and the peak VL (\(P = 0.0004\); Jonckheere-Terpstra), and was used for subsequent analyses. None of the 22 infected vaccinees showed any sign of disease progression (Fig. S2). From the control group (\(n = 8\)), four animals showed the typical course of high VL throughout the study, two animals showed spontaneous control of viremia (one had the TRIM5\(\alpha\) restrictive genotype), and the remaining two animals were rapid progressors and had to be killed before the end of the scheduled follow-up because of development of AIDS.

Two vaccinees (P082 and P090; group 2) had apparent sterilizing immunity with no evidence of infection even after 14 challenges, as judged by consistent negative plasma VL measurements and lack of anamnestic responses (Fig. S4C). In addition, no SIV DNA or RNA could be found in either peripheral blood mononuclear cells (PBMC) or lymph nodes, consistent with vaccine-induced sterilizing protection. To exclude the possibility that these animals were harboring a latent infection or had very low levels of compartmentalized viral replication (below the threshold of 50 copies per milliliter of plasma) as a result of immunological control, they were subjected to transient CD8+ cell depletion 17 mo after the last challenge. Treatment with anti-CD8 Ab rapidly depleted the CD8+ cells, but even under these conditions, no viral replication was detected during the 5 wk of follow-up using a more sensitive assay (detection threshold of seven copies per milliliter of plasma) (Fig. S4A). In contrast, rebounding viral replication was readily detected upon CD8+ cell depletion in macaque M911, an Elite Controller, which had suppressed viremia to undetectable levels and was included as positive control in the CD8 depletion experiment (Fig. S4B).

These data demonstrate that the SIVmac239 DNA-derived vaccination provided significant protection against the heterologous SIVsmE660 acquisition, with significant reduction in peak viremia during primary infection and potent, long-lasting (>40 wk) control of viral replication during the chronic phase without any signs of disease development.

**Humoral Immune Responses Correlate with Virus Acquisition.**

We examined the relationship between vaccine-induced immune responses and challenge outcome to investigate possible correlates of protection. Levels of anti-Env endpoint bAb titers to SIVmac239 and SIVsmE660 (Fig. 2A) were measured before the challenge (weeks 2 and 24 after the last vaccination). All vaccinees developed higher titers of anti-SIVmac239 Env bAbs (Fig. 2A, Left) (weeks 2 and 24, post-V4) that were cross-reactive with the heterologous SIVsmE660 Env (Fig. 2A, Right). Macaques coimmunized with DNA+protein (group 2) showed higher and more persistent humoral responses. A correlation was found between SIVsmE660 Env bAb titers (2 wk before the first challenge) and the number of virus challenges to infection (Fig. 2B) (\(P = 0.047\)), but not with bAb titers to SIVmac239 or SIVmac251. Avidity of the vaccine-induced Ab against SIVmac and SIVsmE660 Env proteins or their neutralizing capacity did not associate with virus acquisition. Upon infection, the vaccinees rapidly developed anamnestic responses with similar kinetics among the vaccine groups (Fig. S4). Peak bAb levels were reached by day 19 postinfection and were maintained over the 17-wk follow-up, independently of the virological outcome of the animal. The control animals displayed a slow increase in bAb levels, reaching peak responses at 17 wk postinfection. At day 12 postinfection anamnestic responses of the vaccinated animals were detected, whereas the control animals had not yet seroconverted. Both SIVsmE660 (Fig. 2C) (\(P = 0.0002\)) and SIVmac239 Env (\(P = 0.0045\)) bAb titers at day 12 postinfection significantly correlated with number of virus challenges to infection.

**Rectal IgG Ab Contributes to Protection.**

Humoral responses were also analyzed in rectal mucosa before challenge at vaccination.
4, week 2 (V4wk2). Antibodies against SIVsmE660 Env were detected in rectal wash samples from two of eight animals receiving the DNA-only vaccine, whereas four of the coimmunized and six of the animals that received particles as a boost scored positive for anti-SIV rectal IgG (Table S3). This finding is in overall agreement with the notion that the inclusion of protein in the DNA vaccine mixture resulted in higher humoral responses. Importantly, the presence of SIVsmE660 Env-specific IgG correlated with significant increase in the number of virus challenges to infection (Fig. 2D) ($P = 0.0241$), indicating that the vaccine-induced humoral responses at the portal of entry contributed significantly to the delay in SIVsmE660 virus acquisition.

Prechallenge and Anamnestic Cellular Immune Responses Correlate with Control of Viremia. Cellular immune responses were measured in cryopreserved PBMC stimulated with Gag and Env peptide pools followed by intracellular cytokine staining and polychromatic flow cytometry. Similar levels of cellular immune responses were found in the three vaccine groups after two DNA vaccinations (V2wk2) (Fig. S5A). We noted an animal-to-animal variation in the level of cellular immune responses, likely reflecting the different genetic make-up of outbred macaques. At V4wk2 and at V4wk24 (2 wk before the first virus challenge), the responses of the sequential DNA prime-protein boost group (group 3) were lower. This group received protein boosts after two DNA vaccinations, whereas the other two groups that received additional DNA boosts showed similar high responses. The DNA vaccinations induced SIV-specific CD4$^+$, CD8$^+$, and CD4$^+$CD8$^+$ double-positive (DP) memory T-cell responses with transitional (TM) and effector memory (EM) phenotype (Fig. S5B, Upper). Analysis of granzyme B-producing Gag- and Env-specific T cells (Fig. S5B, Lower) showed the presence of antigen-specific T cells with cytotoxic capability.

The contribution of cellular immunity to control of viremia was investigated by analyzing responses before challenge (V2wk2) and the anamnestic responses at day 12 postinfection. We found a significant inverse correlation between prechallenge SIV-specific T-cell responses and peak VL (Fig. 3A), but not with number of virus challenges to infection, indicating a critical contribution of these SIV-specific cellular immune responses in the containment of viral replication but not in the prevention of infection. To further identify the vaccine-induced cell subsets contributing to control of viremia, a detailed exploratory analysis of SIV-specific IFN-γ$^+$ T-cell types was performed to detect consistent patterns indicating cell types worthy of further investigation (Fig. 3A and Table S4). We found strong associations between control of viral replication (peak VL) and either the frequency of granzyme B$^+$ T lymphocytes or EM (CD28$^+$CD95$^+$CCR7$^-$) T cells, especially the CD4$^+$ subset (Fig. 3A, Lower). Significant association with virus control was also found with the CD4$^+$CD8$^+$ DP EM cell subset (Table S4). DP cells in HIV-1 infected persons have been associated with important immune function because of their high proliferative capability and multifunctionality (16, 17). The TM (CD28$^+$CD45RA$^+$CD95$^+$CCR7$^-$) T-cell subset also correlated, albeit less robustly with decreased peak VL (Table S4). No correlation was found with the CD8$^+$ T cells. The correlations between prechallenge cellular responses and control of acute viremia were attributed mainly to Gag-specific T cells.

Viral infection stimulated anamnestic responses, which were readily detectable at day 12 postinfection in vaccinated macaques. The anamnestic responses were characterized by the presence of polyfunctional effector T cells armed with granzyme and able to degranulate upon exposure to the antigen, and also by SIV-specific T cells producing TNF-α and IL-2. These anamnestic T-cell responses (measured as increase from prechallenge V4wk24 to day 12 postinfection) (Table S4) also showed strong inverse correlations with peak viremia (Fig. 3B), especially the granzyme B$^+$ T cells (Table S4). In contrast to the results described above (inverse correlation with CD4$^+$ effector T cells), the anamnestic response associated with control of viremia consisted largely of TM T cells, mainly the CD8$^+$ subset (Fig. 3B, Lower). Similarly, the absolute levels of these CD8$^+$

![Fig. 3.](image-url) Cellular immune responses correlate with control of viremia. The frequency of IFN-γ$^+$ producing SIV-specific T cells and T-cell subsets was determined. (A) Correlation of peak VL with prechallenge levels (V2wk2) of SIV-specific (Gag and Env) IFN-γ$^+$ total T cells and CD4$^+$EM T-cell subsets. The T-cell responses are presented as square-root transformation. (B) Correlation of postinfection responses (increase from V4wk24 to day 12 postinfection) of total T cells and CD8$^+$TM T-cell subsets. (C) Trend analysis of the postinfection responses (increase from day of first virus challenge to day 12 postinfection) of the total T cells and TM subset across the three groups defined according to virological outcome with $P$ values (Jonckheere-Terpstra).
Discussion

In this article, we show that SIVmac239-based vaccination in rhesus macaques confers significant protection against viral infection by a titrated dose of the heterologous SIVsmE660 virus, with repeated weekly challenges administered 26 wk after the last vaccination. Vaccine-induced protection was not restricted to protection from virus acquisition, with two animals having apparent sterile protection, but was also reflected by significant reduction of peak viral loads during primary infection and strong suppression of viral replication during the chronic phase of infection, with the majority of vaccinated animals having persistent control of viremia, at low or undetectable levels (<50 copies of viral RNA per milliliter of plasma) for the 40 wk of follow-up. These results extend our previous work, where we reported that SIVmac239-based DNA vaccine was effective in inducing strong and protective immune responses able to reduce viremia upon a single high-dose challenge using a broad (15) SIVmac251 stock administered 8 mo after the last vaccination (5). The results also are in agreement with our study of a DNA prime-recombinant Ad5 virus boost protocol in the presence of IL-12 DNA, where we reported potent control of peak and chronic viremia (18).

Antibody responses against Env have been implicated in protection from infection in some studies in the rhesus macaque/SIV model. Protection against viral acquisition has recently been demonstrated using a variety of vaccine regimens, including replicating vectors, and different challenge viruses. Lai et al. (11), using a combination of DNA prime and modified vaccinia Ankara boost, reported significant protection against infection with the heterologous SIVsmE660 in macaques receiving GM-CSF as vaccine adjuvant. Protection correlated with the avidity of Ab against Env of the challenge virus. Similarly, Barouch et al. (19), using different prime and boost heterologous regimens, demonstrated significant protection against viral acquisition with the heterologous and difficult to neutralize SIVmac251 challenge virus. This study demonstrated that the presence of Env in the vaccine was required to delay viral infection, and that this protection correlated with the anti-Env bAb titers and their neutralizing activity before the challenge. Similarly, Flatz et al. (20) recently reported that a SIVmac239-based vaccine expressing only Env could confer protection from virus acquisition upon repeated intrarectal challenge with a limiting dose of SIVsmE660, demonstrating that Env alone can be sufficient to protect against a heterologous SIV. As in previous studies, protection correlated with anti-Env Ab, especially neutralizing activity against the challenge virus measured in primary PBMC but not in reporter cell lines.

Ab levels elicited by DNA vaccination are generally lower than those obtained using vaccine regimens that include protein immunogens. Therefore, we reasoned that the DNA and protein coimmunization might lead to high levels of both humoral and cellular responses. To improve the level of protection achieved by our DNA-based vaccines (5), we tested two vaccination protocols combining DNA and AT-2–inactivated SIVmac239 particles as source of viral proteins. Previous studies showed that vaccination of pigtailed macaques with only AT-2–inactivated SIVmac239 particles was able to elicit both bAb and NAb responses and controlled a pathogenic homologous SIVmne challenge (21). In this article, SIVmac239 plasmid DNAs and AT-2–inactivated SIVmac239 particles were delivered simultaneously in the same muscle, or administered sequentially using a more conventional approach of two DNA prime vaccinations followed by two protein boosts. Both protocols induced higher Ab levels than DNA alone, although the coimmunization protocol showed more durable humoral responses and warrants further study. The plasma bAb to SIVsmE660 Env before the first virus challenge inversely correlated with the number of challenges required to acquire infection and, importantly, vaccinees with rectal anti-SIV IgG showed delayed virus acquisition, supporting the notion that humoral responses at the portal of entry potently prevent infection. It is interesting to note that the two animals that showed sterilizing immunity had among the highest bAb and NAb titers, which were remarkably maintained over time and, in addition, were also positive for rectal SIV-specific IgG. Thus, the inclusion of protein in the DNA vaccine greatly improved the quality of the humoral immune responses. Another problem associated with the development of an effective HIV vaccine is the transient nature of the Ab responses. Our results suggest that the described immunization protocols including DNA and protein are promising for further study because they can achieve durable (26 wk) high titers of Ab.

In addition to decreasing the number of HIV infections, an effective AIDS vaccine should also elicit immune responses that restrict or abolish systemic viral replication. The RV144 clinical trial showed modest protection against infection, and this protection apparently correlated with anti-Env bAb, especially IgG antibodies against the V1V2 region (22, 23), but no evidence of vaccine-induced virus control was found in the individuals who became infected. Clearly, a vaccine regimen able to prevent acquisition (11, 19, 20, 24) and reduce viral replication after infection has become possible (11, 19, 24) using the macaque model. In agreement with those findings, we report that our SIVmac239-based vaccine elicited systemic and mucosal anti-Env humoral immune responses able to significantly delay acquisition of the heterologous SIVsmE660, and SIV-specific cellular responses that efficiently contribute to postacquisition control of viral replication. Cellular immune responses are important for limiting the levels of systemic viral replication early after infection, and this control of primary viremia can be very efficient, even when Env is not part of the vaccine (19, 25, 26). In addition, using CMV-based viral vectors as vaccine, it was shown that strong cellular immunity distributed in mucosal effector sites could efficiently control viral replication locally, preventing pathogenic viral dissemination and establishment of systemic infection (27). This protection was found to be durable, mediated by EMT cells, and independent of antiviral humoral responses (28). In contrast to nonpersistent viral vectors, which induce cellular responses with central memory phenotype, the bias induced by CMV vectors toward cellular responses with EM phenotype was considered a substantial advantage, because EM cells react immediately upon encountering the antigen. Similarly, the cellular responses induced in the vaccinated macaques described in the present study were mainly EM T cells (both CD28+CCR7+ and CD28−CCR7−), and we have previously reported that these cellular responses efficiently migrate into mucosal sites, where they persist for long periods of time after the last vaccination (29).

In summary, our data demonstrate that DNA-based vaccines, in the absence of any replicating vectors, can protect against a heterologous mucosal challenge by inducing humoral responses able to delay viral acquisition, providing in some cases sterilizing immunity, and eliciting cellular responses with EM phenotype that contribute to control of viral replication and prevent the onset of AIDS in SIV-infected macaques. 

Methods

For a complete description of the source of materials and our methods, see SI Methods.
Vaccination. Macaques were vaccinated with a mixture of SIV DNA expressing Gag, Pol, Env, Nef, Tat, and Vif, together with rmlL-12 plasmid as DNA adjuvant (30). The DNA mixture was injected intramuscularly followed by in vivo EP (Inovio Pharmaceuticals). The animals received four vaccinations (V1–V4) using the following protocols: group 1, DNA only; group 2, DNA coadministered with adjuvant AT-2–inactivated SIVmac239 particles (21); group 3, two DNA vaccinations, followed by two AT-2 SIV particle vaccinations. The control group received empty vector or rmlL-12 DNA, respectively (n = 4 each). The animals were challenged intrarectally at weekly intervals using a well-characterized SIVsmE660 stock (11).

Humoral and Cellular Immune Responses. The Ab endpoint titers to SIVmac251, SIVmac239, and SIVsmE660 gp120 Env in plasma were measured. Mucosal SIVsmE660-specific IgG was measured as specific activity (specific binding antibody units/total IgG concentration) by multiplex binding assay. SIV-specific cellular immune responses were measured from PBMC by intracellular cytokine staining.

Viral Load. VLS over the course of the study were determined using the NASBA assay (31) with a threshold of detection of 50 copies per milliliter (Advanced Diagnostics). RNA was extracted from plasma or tissue samples using the Qiagen Viral RNA Mini kit (Qiagen). The viral load was determined using NASBA technology (32).

Statistical Analysis. AUcs were calculated using trapezoidal approximation of log viral loads between weeks 7 and 40 and are thus in units of log(VL)-weeks; calculations for animals killed before week 40 were completed using the Last Observation Carried Forward method. Correlation coefficients and significance levels were calculated using Spearman rank correlation. The stratified Wilcoxon rank sum test and the Jonckheere-Terpstra test were used for two-group comparisons and three-group tests of trend. For predefined null hypotheses, P ≤ 0.05 was considered significant and P ≤ 0.01 was considered highly significant. Correlation coefficients ≥0.50 in absolute value are indicative of strong trends.

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