Enhanced Control of Pathogenic Simian Immunodeficiency Virus SIVmac239 Replication in Macaques Immunized with an Interleukin-12 Plasmid and a DNA Prime-Viral Vector Boost Vaccine Regimen^{∇§}

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Received 10 May 2011/Accepted 28 June 2011

DNA priming has previously been shown to elicit augmented immune responses when administered by electroporation (EP) or codelivered with a plasmid encoding interleukin-12 (pIL-12). We hypothesized that the efficacy of a DNA prime and recombinant adenovirus 5 boost vaccination regimen (DNA/rAd5) would be improved when incorporating these vaccination strategies into the DNA priming phase, as determined by pathogenic simian immunodeficiency virus SIVmac239 challenge outcome. The whole SIVmac239 proteome was delivered in 5 separate DNA plasmids (pDNA-SIV) by EP with or without pIL-12, followed by boosting 4 months later with corresponding rAd5-SIV vaccine vectors. Remarkably, after repeated low-dose SIVmac239 mucosal challenge, we demonstrate 2.6 and 4.4 log reductions of the median SIV peak and set point viral loads in rhesus macaques (RMs) that received pDNA-SIV by EP with pIL-12 compared to the median peak and set point viral loads in mock-immunized controls ($P < 0.01$). In 5 out of 6 infected RMs, strong suppression of viremia was observed, with intermittent “blips” in virus replication. In 2 RMs, we could not detect the presence of SIV RNA in tissue and lymph nodes, even after 13 viral challenges. RMs immunized without pIL-12 demonstrated a typical maximum of 1.5 log reduction in virus load. There was no significant difference in the overall magnitude of SIV-specific antibodies or CD8 T-cell responses between groups; however, pDNA delivery by EP with pIL-12 induced a greater magnitude of SIV-specific CD4 T cells that produced multiple cytokines. This vaccine strategy is relevant for existing vaccine candidates entering clinical evaluation, and this model may provide insights into control of retrovirus replication.

The human immunodeficiency virus (HIV) epidemic is a major health problem, with more than 7,400 people infected every day, the majority of whom live in sub-Saharan Africa (56). Despite advances in HIV antiretroviral treatment and efforts to increase access, it remains the case that just 36% of HIV-infected people are receiving medication (58). Recent advances in the use of preexposure prophylaxis and microbicides, the implementation of policies for male circumcision, and education in the use of condoms in sub-Saharan Africa are

promising approaches to slow infection rates (18, 29); however, an HIV vaccine offers the most sustainable, affordable, and practical solution for controlling the HIV epidemic worldwide.

Only a few of the HIV vaccine candidates currently in clinical evaluation or under consideration for clinical development have demonstrated a significant and sustained impact on virus replication using analogous simian immunodeficiency virus (SIV) vaccines in a stringent nonhuman primate (NHP) challenge study (22, 34). Notably, a replicating rhesus cytomegalovirus (rhCMV) vector expressing the full SIV proteome recently demonstrated suppression of acute-phase virus replication and seemingly prevented the acquisition of infection in over half of the vaccinated rhesus macaques (RMs) following repeated low-dose challenge with the highly pathogenic simian immunodeficiency virus SIVmac239 (21, 22). Heterologous prime and boost vaccination of RMs with recombinant adenovirus (rAd) of serotype 26-Gag and rAd5-Gag decreased virus loads

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[∇] Published ahead of print on 6 July 2011.

[§] The authors have paid a fee to allow immediate free access to this article.

by 1.4 and 2.4 log at peak and set point, respectively, following a single intravenous challenge with SIVmac251 (34). Both the rhCMV and rAd26/rAd5 regimens were characterized by broad, robust, antigen-specific multifunctional CD4 and CD8 T-cell responses that effectively influenced early events in virus dissemination. These results indicate that vaccination can elicit sustained suppression of viral replication and reemphasize the potential importance of T-cell immunity, particularly in the wake of the unsuccessful Merck rAd5-HIV phase II trial (8, 13).

To date, vaccination of RMs with plasmid DNA (pDNA) followed by a boost with replication-defective rAd5 expressing SIV proteins has demonstrated limited efficacy following homologous SIV challenge, with a maximum reduction in viral set point of 1.5 log consistently observed (4, 7, 33, 62). DNA-based immunization strategies are attractive, particularly for HIV vaccines, because of ease of manufacture, quality control, safety, and stability for cold-chain delivery in developing countries. DNA-based vaccine approaches also avoid preexisting antivector immunity that is problematic for viral vector-based candidates that are in the HIV vaccine pipeline (13). The efficacy of pDNA/rAd5 vaccination has not been examined in an NHP SIV challenge protection study when using techniques that enhance the immunogenicity of pDNA priming. Immunogenicity has been improved by optimization of coding regions and the use of more efficient promoters to increase protein expression (43, 49), coexpression of cytokine or chemokine adjuvants, and the use of delivery methods that increase DNA uptake (5, 9, 16, 50). For example, electroporation (EP) combines an intramuscular (i.m.) DNA injection and simultaneous administration of short electrical pulses (37, 46, 47, 51, 66). Antigen presentation is improved by local inflammation and recruitment of antigen-presenting cells (APCs) (19, 20, 59), and protein production is increased, driven by more efficient uptake and expression of DNA (59). Additionally, plasmid-encoded interleukin-12 (pIL-12) has proven to be a promising candidate adjuvant that enhances cellular and humoral responses (53, 64). Combining DNA delivery by EP with pIL-12 further enhanced immune responses in RMs (24) and is currently undergoing phase I clinical assessment (<http://clinicaltrials.gov/ct2/show/NCT00991354?term=pennvax&rank=4>).

Here, we present data from an SIV challenge protection study, where we used a novel prime and boost combination to enhance the immunogenicity of vaccination. We proposed that codelivery of pDNA-SIV by EP with pIL-12 would augment the T-cell response and, therefore, increase the efficacy of DNA/rAd5-SIV vaccination. Strikingly, RMs that received a pDNA-SIV prime coadministered with pIL-12 by EP demonstrated greater than 2 and 4 log reductions in group median peak and set point virus loads compared to the median peak and set point loads in vaccine-naïve controls following repeated low-dose challenge with pathogenic SIVmac239. This level of viral control is unprecedented for the DNA/rAd5 prime-boost strategy and was not observed in RMs that received pDNA-SIV by EP without pIL-12.

MATERIALS AND METHODS

Vaccine construction. Plasmid DNA. The vaccine immunogens used in this study covered the full SIVmac239 (GenBank accession number M33262) genome. Open reading frame nucleotide sequences were optimized for high ex-

pression in mammalian cells and cloned into an expression vector (32). The SIV constructs were placed under the control of the human CMV promoter and the bovine growth hormone polyadenylation signal (51). Gag (12S), Pol (91S), Env (99S), and Nef (pCMV-Nef) were expressed as single proteins, while Vif, Vpr, Vpx, Tat, and Rev were expressed as a fusion protein (pCMV-VVVTR) (21). The Gag plasmid expressed a myristoylated Gag protein spanning amino acids 1 to 508, lacked 2 C-terminal amino acids, and was otherwise similar to a reported Gag plasmid (49). The Env plasmid expressed a native form of gp160, as previously reported (32, 49). A plasmid containing a gene encoding sequence-optimized Nef lacking a myristoylation signal was prepared with an insert amplified from the 179S plasmid (32). Deletions were introduced into Pol to inactivate protease ($\Delta_{25}\text{DTG}_{27}$), reverse transcriptase ($\Delta_{184}\text{YMDD}_{187}$), RNaseH (ΔE_{478}), and integrase (ΔD_{64} , ΔD_{116} , and ΔE_{152}). Large-scale plasmid production for DNA vaccine material was prepared by Aldevron LLC (Fargo, ND). The two subunits of rhesus IL-12 (plasmid AG157) were expressed from a dual promoter plasmid containing transcription units controlled by the human CMV immediate early promoter (hCMV) and bovine growth hormone polyadenylation signal and the simian CMV promoter (siCMV) and simian virus 40 (SV40) polyadenylation signal (R. Jalah, B. Felber, and G. Pavlakis, unpublished data).

Recombinant Ad5 vectors. The same optimized SIVmac239 sequences used for DNA-SIV plasmids were used to construct rAd5 vectors. The rAd5 vectors were constructed in a ΔE1A , partial ΔE1B , and ΔE3 genetic background using the AdEasy adenoviral vector system (Stratagene, La Jolla, CA). SIVmac239 genes were cloned into the E1 region of the rAd5 vector under the control of the hCMV immediate-early promoter-enhancer and the SV40 stop-polyadenylation signal. Ad5-Null was used as a negative-control vector and had an identical backbone but no transgene. All vectors were rescued in HEK 293 cells and purified by double cesium chloride centrifugation (52). Dosing was based on particle units (PU), determined by spectrophotometry (41). Large-scale rAd5 vector production was performed in HEK 293 cells by Puresyn, Inc. (Malvern, PA). Immunogen expression was verified by Western blotting from plasmids after transient transfection of HEK 293 cells or from rAd5 vectors after infection of A549 cells, using polyclonal antibodies against SIVmac239 Gag, Env, Nef, and Rev proteins prepared in rabbits and anti-SIVmac251 serum from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Study cohort, vaccination, and SIV challenges. SIV-negative Indian RMs (*Macaca mulatta*) were housed at the University of Pittsburgh in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and with the approval of the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC) standards/regulations. The protocols were approved and assigned the IACUC number 0803208. Animals were typed for major histocompatibility complex (MHC) type I alleles that have been associated with preferential control of SIV infection, namely, Mamu-A*01, -A*02, -A*11, -B*01, -B*03, -B*08, -B*17, and -B*29, for vaccine group allocation as previously described (28, 35, 65). No RMs positive for Mamu-B*17 were included in the study. Animals were also typed for TRIM5 α allele genotypes *TRIM5^Q*, *TRIM5^{CYP}*, and *TRIM5^{TFP}*, as combinations of these alleles have been associated with innate control of SIVsmE543 and E660 replication through binding of the capsid protein and inhibition of unfolding of the virus (31, 44, 48).

RMs were divided into 3 groups ($n = 8$ per group) equally distributed by age and MHC class I allele expression. At weeks 0, 8, and 16, pDNA encoding the whole SIVmac239 genome (*gag*, *pol*, *nef*, a *vif-vpx-vpr-rev-tat* fusion gene, and *env*) was administered by EP (Inovio, United States) or by EP plus 0.1 mg of rhesus pIL-12 at each vaccination site (0.4 mg pIL-12 in total). Constructs encoding *gag*, *pol*, and *env* were delivered into separate limbs, and the *nef* gene and VVVTR fusion gene were delivered into the same limb for a total of 4 vaccination sites per time point. The same receiving limb was used for each gene construct for pDNA and rAd5 vaccinations, with pIL-12 codelivered with each pDNA vaccination for the "with pIL-12" group. A negative-control group received saline by EP. At week 33, all animals were boosted with 2×10^{10} virus particles of rAd5 (rAd5-SIV) encoding all SIVmac239 genes. Negative controls received an Ad5-Null vector.

We used a repeated low-dose viral challenge regimen of SIVmac239 to assess vaccine efficacy (39). All animals were challenged intrarectally with a viral and saline inoculum of 800 50% tissue culture infective doses (TCID_{50}) of SIVmac239 (stock kindly supplied by D. Watkins, University of Wisconsin) every 2 weeks until infected. Blood was taken 1 week after each challenge, and Gag p27 in plasma measured by quantitative reverse transcription-PCR (qRT-PCR) to determine productive infection. The peak viral load was defined as the highest number of copies/ml detected within the first 3 weeks of infection. The set point viral load was defined as the mean viral load from 1 week after peak out to 40 weeks postinfection.

Sample collection. Peripheral blood was collected in sodium heparin tubes (BD Biosciences, United States), and peripheral blood mononuclear cells (PBMCs) isolated by density centrifugation on Ficoll (BioWhittaker, United States) using Accuspin tubes (Sigma, United States). Cells were frozen and stored in gaseous-phase liquid nitrogen. Blood was taken using SST tubes (BD Biosciences, United States) and stored overnight at 4°C for serum collection. Serum was heat inactivated for 35 min at 56°C and stored at -20°C. Inguinal or auxiliary lymph nodes were surgically removed, and cell suspensions were obtained by mechanical disruption of the tissue as previously described (25) and immediately used for detection of SIV. To obtain plasma, whole blood was collected in EDTA (BD, United Kingdom) tubes and centrifuged at 2,400 RPM for 10 min.

Peptides. Fifteen-mer peptides (Anaspec, United States) spanning the entire SIVmac239 proteome and overlapping by 11 amino acids were distributed into 10 open reading frame pools containing 50 to 125 peptides each. Peptides were used at 2 µg/ml in immunological assays. *Staphylococcus enterotoxin B* (SEB) was used as a positive control (1 µg/ml). The mock negative control consisted of 0.02% dimethyl sulfoxide to match peptide pools.

Intracellular cytokine staining. Between 600,000 and 1,000,000 PBMCs were incubated with SIVmac239 peptide pools or control peptides (see “Peptides”) for 9 h with brefeldin A (Sigma, United States), CD49d, and CD28 (BD Biosciences, United States) at 37°C with 5% CO₂. Cells were washed and stained with Live/Dead aqua viability fixable cell stain (Invitrogen, United States) and then washed and stained for extracellular markers with CD3-Pacific Blue, CD4-peridinin chlorophyll (PerCP) Cy5.5, and CD95-APC (BD Biosciences, United States). Cells were permeabilized with Fix Perm and stained for markers and intracellular cytokines with CD69-Texas Red, CD8-APC-H7, IL-2-phycoerythrin (PE), gamma interferon (IFN-γ)-Alexa Fluor 700, and tumor necrosis factor alpha (TNF-α)-PE-Cy7 (BD Biosciences, United States). Events were collected on an LSR-II (BD Biosciences, United States) and analyzed using FlowJo (Tree-star, United States), Pestre, and SPICE (provided by M. Roederer, Vaccine Research Center, NIAID, NIH). All data shown have had the mock-stimulated background responses subtracted.

Binding-antibody enzyme-linked immunosorbent assay (ELISA). Serial dilutions of heat-inactivated sera were incubated for 1 h on 96-well plates previously coated with SIV Gag p55 protein (Protein Sciences Corp, United States) or SIV Env gp130 (IAVI Neutralizing Antibody Consortium Repository, United States) and then blocked for 1 h with 3% bovine serum albumin (BSA)-phosphate-buffered saline (PBS). Plates were washed in 0.05% PBS-Tween and incubated for 1 h with alkaline phosphatase-conjugated secondary antibody. After washing, plates were incubated for 30 min with nitrophenylphosphate and optical density measured at 450 nm using SoftMAX Pro GxP version 5 (Molecular Devices, United States). All data shown have had the “no-serum” background subtracted.

qRT-PCR. One milliliter of plasma was spiked with 3.3×10^4 copies/µl of Armored RNA enterovirus (Asuragen, Austin, TX) and pelleted by centrifugation at $25,000 \times g$ for 1 h at 5°C. All liquid was removed, and total RNA extraction was performed as described in the RNeasy minikit instructions (Qiagen, Valencia, CA). RNA was eluted in 50 µl of RNase-free water, and real-time qRT-PCR was performed as previously described (25) with the following modifications. Duplicate reaction mixtures per sample were prepared, and synthesis of cDNA was performed using reagents from the Sensiscript reverse transcriptase kit (Qiagen, Valencia, CA). Briefly, to detect Gag p27 genomic RNA, a 20-µl reaction mixture was made containing 10 µl of total RNA, 400 nM Gag p27 reverse primer 5'-CACTAGKTGTCTCTGCACTATPTGTTT3', 2 µl of 10× reverse transcription buffer, 500 µM each deoxynucleoside triphosphate (dNTP), and 10 U of RNase inhibitor per µl (all reagents from Qiagen's Sensiscript RT kit). The reaction mixture was reversed transcribed at 50°C for 45 min, followed by heat inactivation of the reverse transcriptase at 95°C for 5 min. The real-time quantitative PCR was carried out using a QuantiTect multiplex PCR kit (Qiagen). A 30-µl reaction mixture was made with 25 µl of 2× QuantiTect multiplex PCR master mix, 400 nM (each) forward primer 5'-GTCTGCGTCATPTGGTG CAT3' and reverse primer plus 200 nM 6-carboxyfluorescein (FAM)-labeled minor groove binder (MGB) probe specific for Gag p27 (5'-6FAM-CITCPTC AGTKTGTTCAT-MGB-3'). This mixture was combined with the 20-µl cDNA mixture, and amplification and detection were performed with a Stratagene Mx3005P sequence detection system (La Jolla, CA) using the following conditions: 15 min at 95°C to activate the Hot Star Taq DNA polymerase, followed by 45 cycles of 15 s at 94°C and 60 s at 60°C. Copy numbers for test samples were determined by interpolation onto a standard curve of RNA standards.

Statistical analysis. Log rank tests were used to determine whether vaccination had an effect on infection rate. Nonparametric tests were performed to detect statistical differences between vaccine groups due to the small sample sizes. Kruskal-Wallis one-way analysis of variance (ANOVA) was used to com-

pare all 3 groups. Wilcoxon rank sum tests were performed to determine significant differences between the median values of group pairs, using an adjusted *P* value cutoff of <0.0167 for multiple comparisons. Peak viral load was defined as the highest viral load measured in the first 3 weeks of infection. Set point viral loads were defined using values from the first time point after peak viral load out to a maximum of 40 weeks postinfection. Uninfected animals were assigned viral load values of 0 for these analyses.

RESULTS

EP DNA codelivered with pIL-12 DNA and boosted with rAd5 elicits significant control of virus replication. To assess whether vaccination efficacy was improved if the immunogenicity of the DNA priming component was enhanced, we delivered the whole SIVmac239 genome in 5 separate pDNA-SIV plasmids (Gag, Pol, Env, Nef, and Vif/Vpr/Vpx/Tat/Rev fusion) in 3 successive primes delivered by EP with or without pIL-12 (at weeks 0, 8, and 16). The animals were boosted 4 months later with 5 corresponding separate rAd5-SIV constructs. Thus, the 24 RMs (*n* = 8 per group) were primed with pDNA-SIV, pDNA-SIV with pIL-12, or mock vaccination. We challenged the RMs repeatedly with the highly pathogenic virus clone SIVmac239 until they became infected or reached a maximum of 13 low-dose intrarectal challenges administered per animal (Fig. 1A). The RMs that received mock challenge or pDNA-SIV without pIL-12 immunization all became infected after 7 challenges (medians of 4 and 5, respectively) (Fig. 1B). In RMs that received pDNA-SIV codelivered with pIL-12, 6 became infected following a maximum of 9 challenges (median of 3), and the other 2 remained uninfected after all 13 challenges (Fig. 1B). We could not detect SIV RNA in the tissue or lymph nodes of the 2 uninfected RMs after all 13 challenges (data not shown). As expected due to the small sample sizes, we did not observe a difference between vaccine groups in the number of challenges required to acquire infection (Fig. 1B) (*P* > 0.05) (26, 27).

We did not detect any factor that could implicate an effect on infection or virus load between groups other than the vaccine administered. The number of challenges required to infect each of the RMs in the study did not affect the kinetics of infection or viral load (Fig. 1C). The presence of MHC class I allele Mamu-B*17 or -B*08 in RMs infected with SIV has been correlated with a reduction in plasma viremia (35, 36, 45, 65), as seen in humans infected with HIV type 1 (HIV-1) who express HLA-B*57 (17). In this study, we excluded animals that expressed Mamu-B*17, but each of the immunization and control groups included a single RM that expressed Mamu-B*08 (Fig. 1C). As expected, the Mamu-B*08-expressing RM in the mock-vaccinated group (RM-474) demonstrated a low virus load compared to the virus loads in the remainder of the unimmunized population (Fig. 1C). The effect of Mamu-B*08 was enhanced upon immunization with pDNA-SIV with or without pIL-12; RMs positive for this genotype (RM-614 and RM-622) controlled virus replication by >3 log following peak viremia (Fig. 1C). Furthermore, we did not observe an association between MHC class I allele Mamu-A*01 and lower virus loads as previously suggested (42); however, enhanced suppression of virus replication was observed in Mamu-A*01-positive RMs that coexpressed Mamu-B*08 or were immunized with pDNA-SIV with pIL-12 (Fig. 1C).

To determine the efficacy of each vaccination regimen, we

FIG. 1. (A) Study design. Numbers on time line indicate the vaccination time points and the first challenge, in weeks. See Materials and Methods for details. (B) Number of low-dose SIVmac239 intrarectal challenges required to infect rhesus macaques in each immunization group when the DNA prime components in the prime-boost regimen differ. (C) Kinetics of SIVmac239 infection from 0 to 40 weeks in each rhesus macaque. The animal identification number on each graph is followed by the MHC-I haplotype in parentheses. The number of challenges that occurred before virus was detectable in plasma is indicated above the graph for each immunization group. Dotted horizontal lines indicate group median set point viral loads. EP, electroporation delivery of pDNA-SIV; pIL-12, plasmid encoding IL-12; rAd5, recombinant Ad5-SIV boost.

FIG. 2. (A) Longitudinal virus load of each immunization group. (B and C) Peak (B) and set point (C) virus load of each immunization group. Gag p27 RNA in plasma was detected by RT-PCR. Group median viral loads are indicated by a solid horizontal black line in each box-and-whisker plot. Error bars show standard deviations. pIL-12, plasmid encoding IL-12; rAd5, recombinant adenovirus 5-SIV boost.

compared control of SIVmac239 replication at peak and set point viral loads. RMs that received pDNA-SIV alone (without pIL-12) and rAd5-SIV boost had modestly reduced group median plasma genome copy numbers (of approximately 1 log) at the peak and set point viral loads compared to the copy numbers in naïve controls (Fig. 2), which was a result similar to the results seen in previous work with conventional i.m. needle injection of pDNA/rAd5-SIV prime-boost followed by high-dose (7, 54) or low-dose (62) intrarectal pathogenic SIV challenge (Fig. 2). Coadministration of pDNA-SIV with pIL-12

significantly improved vaccination efficacy, with further reductions of median peak and set point viral loads by 1.5 log and 4 log, respectively, compared to the results for immunization in the absence of pIL-12. Strikingly, RMs vaccinated with pDNA-SIV with pIL-12 had median viral loads that were reduced by more than 2.6 log at peak and 4.4 log at set point compared with the results in mock-immunized controls ($P = 0.0013$ at peak, $P = 0.0027$ at set point) (Fig. 2B and C). This level of control of SIV replication is unprecedented compared to the results in other studies following either homologous or heterologous prime-boost vaccination with pDNA delivered intramuscularly and replication-defective rAd5 in a high-dose (4, 7, 54) or low-dose challenge (61, 62), and is comparable to the control of viremia recently observed following immunization with whole SIVmac239 genes vectored by replication-competent rhCMV (22). Given that all RMs received the same SIV genes delivered by EP and rAd5 boost, this result strongly implicates a critical role for the expression of IL-12 during the priming phase when generating an immune response capable of exerting sustained control of SIV replication.

We retrospectively examined the TRIM5 α genotype of all RMs, as polymorphisms at this locus can influence innate control of infection and replication as described for SIVsmE543 and E660 (44, 48). Briefly, there are 3 functional TRIM alleles that act by binding to capsid protein: *TRIM Q*, *TRIM TFP*, and cyclophilin A (*Cyp*). Combinations of these functional genotypes in RMs differentially influence uncoating of the virus during cell entry by binding to capsid protein, with *TRIM TFP/Cyp* genotypes inhibiting SIV infection to a greater extent than *TRIM Q/Q*, *Q/Cyp*, *Q/TFP*, or *TFP/TFP*. After evaluation of the genotypes, we confirmed that there was no association of TRIM5 α polymorphism with virus load or the number of exposures required to infect the individual RMs (Fig. 3) (31).

DNA priming by EP with pIL-12 preferentially affects T-cell responses. We examined both antibody and T-cell responses in RMs. Serum was analyzed by ELISA for SIV-specific binding IgG antibodies (Ab) directed against SIV Env gp130 and Gag p55 proteins. Gag- and Env-specific antibodies that were boosted in magnitude by rAd5 were detected in both immunization groups after pDNA-SIV priming (Fig. 4). Historically, coadministration of pIL-12 with pDNA has induced a higher

FIG. 3. Analysis of the acquisition and kinetics of infection in rhesus macaques, grouped by TRIM5 α polymorphisms. (A) Number of low-dose intrarectal challenges required for infection with SIVmac239. (B) Mean viral loads over time as measured by RT-PCR. Error bars show standard deviations.

FIG. 4. Mean binding antibody titers specific for SIV Env gp130 (A) and SIV Gag p55 (B) postvaccination and -infection in each immunization group. Dotted vertical lines indicate infectious challenge. Mean titers are shown for infected animals only postinfection. Error bars show standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; pIL-12, plasmid encoding IL-12; rAd5, recombinant adenovirus 5-SIV boost; ND, not done.

magnitude of IgG Ab responses than pDNA alone; however, we did not observe any difference here (Fig. 4). EP delivery of pDNA-SIV with or without pIL-12 elicited cytokine-producing CD4 and CD8 T-cell responses to each of the SIVmac239 vaccine immunogens (Fig. 5). This is consistent with data from other EP studies in NHPs and clinical assessment (30, 37, 50). Interestingly, rAd5-SIV preferentially boosted CD8 T-cell responses in both immunization groups with little or no boosting observed in the CD4 T-cell compartment, except for Gag responses (Fig. 5). pDNA-SIV priming with pIL-12 elicited a greater frequency of Gag-specific CD4 T cells and significantly more Env-, Nef-, and Pol-specific CD4 T cells than were seen in RMs that received pDNA-SIV alone (Fig. 5). EP of pDNA-SIV elicited overall a high frequency of multifunctional antigen-specific CD8 and CD4 T cells (Fig. 6). Notably, the frequency of multifunctional SIV-specific CD4 T cells was significantly higher in RMs that received pDNA-SIV and pIL-12, but these populations were not boosted in frequency following rAd5-SIV vaccination (Fig. 6).

DISCUSSION

The primary objective of this study was to determine whether codelivery of a plasmid expressing IL-12 with pDNA-SIV would increase DNA/rAd5 prime-boost vaccination efficacy, as assessed in a stringent NHP SIVmac239 challenge protection study. We demonstrate that 5 of 6 RMs that received pDNA-SIV priming vaccinations with pIL-12 controlled SIVmac239 replication to undetectable levels. Additionally, these RMs had a peak level of SIV replication that was reduced by almost 3 log and a set point virus load that was lower by approximately 4.5 log than the peak and set point virus loads in mock-immunized controls. Moreover, there was a 4 log difference in vaccine efficacy (as evidenced by median set point viral load) between this group and RMs primed with pDNA-SIV without pIL-12. To our knowledge, we are the first to prime conventional replication-deficient rAd5-SIV with pDNA-SIV codelivered by EP with pIL-12, and we show a significant improvement in vaccine efficacy. Although rAd5 is unlikely to be advanced into large-scale clinical trials due to preexisting immunity to the vector (40), this proof-of-concept study demonstrated that the efficacy of the pDNA prime vector boost vaccination platform was significantly increased when techniques previously shown to improve the immunogenicity of pDNA immunization were employed.

The kinetics and levels of virus control observed in this study, characterized by virus suppression immediately after infection and sustained undetectable virus loads with intermittent blips of replication, were similar to those detected in RMs immunized with live attenuated viruses, such as SIVmac239ΔNef or rhCMV (10, 22). Control of viral replication and potential protection from infection in the rhCMV vaccine model was associated with the induction of long-lived SIV-specific T cells with an effector memory phenotype (22) in the absence of neutralizing antibodies. Further analyses are planned to determine whether the presence of IL-12 modulated the phenotype of SIV-specific T cells induced in this study upon DNA priming, as well as functional antibody assays. Additionally, in both studies, RMs were immunized with each gene from the SIVmac239 genome, which may be advantageous in eliciting broad T-cell responses that exert immune pressure upon early virus replication, leading to sustained viral control. While other DNA/rAd vaccine regimens with various combinations of SIV genes have demonstrated limited efficacy following high-dose (4, 7, 38, 54) or low-dose (61, 62) challenge, this regimen has not previously shown control of early virus replication to the same extent as observed here, implicating an important role for IL-12.

The present study was not designed to determine whether vaccination reduced the frequency of infection, as many more animals are required to determine statistically significant protection (26, 27). Nevertheless, 2 RMs immunized with pDNA-SIV with pIL-12 at priming vaccination had no detectable virus loads following multiple exposures to pathogenic SIVmac239. Indeed, no SIV could be detected in lymph node tissues of these 2 RMs, indicating that they either cleared the virus or were refractory to SIV infection. We explored whether there could be genotypic differences between vaccinated and mock-vaccinated RMs to which viral control could be attributed. TRIM5α polymorphisms have been associated with failure of

FIG. 5. Frequency of SIV-specific CD8 (A) and CD4 (B) T cells producing any combination of IFN- γ , TNF- α , or IL-2 on stimulation with SIV peptide pools, as indicated on each graph, postvaccination and -challenge. Dotted vertical lines indicate infectious challenge. Mean percentages are shown for infected animals only postinfection. Error bars show standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; V/V/V, Vif/Vpr/Vpx; pIL-12, plasmid encoding IL-12; rAd5, recombinant adenovirus 5-SIV boost.

SIV to infect RMs expressing particular TRIM5 α variants that bind to virus capsid and prevent uncoating with certain virus isolates (48, 44). Here, we analyzed TRIM5 α alleles in our cohort and found that there was no association with TRIM5 α and resistance to SIVmac239 infection, specifically in the 2 RMs that remained uninfected. This confirms the *in vitro* finding that TRIM5 α variants do not bind efficiently to SIVmac239 capsid, due to amino acid substitutions in the capsid of the cloned virus (31).

We also examined the MHC genotypes of RMs to determine whether this influenced viral control in vaccinated animals. Control of SIV, like HIV, has been shown to be associated with the expression of particular MHC class I alleles, such as Mamu-B*17 and -B*08 and, to a lesser extent, Mamu-A*01 in Indian RMs (35, 36, 42, 65). While this effect on virus replication is not absolute (45, 63), we excluded RMs expressing the class I allele Mamu-B*17 from this study cohort. Regardless of TRIM5 α or MHC class I genotype, the entire mock-immunized study group became infected with typical peak virus loads for SIVmac239 following a median of 4 challenges. As expected, a single Mamu-B*08 RM had a 2 log reduction in virus load by 15 weeks postinfection. The remaining 7 RMs (including one expressing Mamu-A*01) failed to suppress virus replication at peak or set point. In RMs expressing the protective MHC class I molecule Mamu-B*08 that received pDNA-SIV with or without pIL-12, we observed early suppression of SIV compared to the results in the mock-immunized controls. Data from the Merck phase I STEP trial demonstrated that vaccinated individuals that expressed HLA-B*27, -B*57, or -B*58 had significantly lower virus loads following

infection than vaccinated individuals that expressed HLA alleles not associated with virus control (14). The results presented here also indicate that vaccination can enhance the capability of antigen-specific CD8 T-cell responses restricted by “protective” MHC class I alleles to recognize infected cells.

Additional studies using large cohorts of NHPs lacking any alleles associated with low virus loads are warranted to confirm early control of virus replication following immunization with pDNA-SIV with pIL-12 followed by rAd5. However, it is evident from the present study that the RMs lacking protective MHC class I alleles controlled SIVmac239 replication following EP SIV DNA plus pIL-12/rAd5 prime-boost, which suggests that the control observed was not the consequence of a fortuitous alignment of protective alleles in any particular vaccine group.

IL-12 is produced by activated dendritic cells (DCs) and macrophages following recognition of pathogens, and it enhances the activation of T cells, B cells, and natural killer (NK) cells (55, 57). It has been shown that the administration of recombinant rhesus IL-12 to RMs followed promptly by SIVmac251 challenge resulted in an enhanced immune response to challenge infection, characterized by increased serum IFN- γ and circulating SIV-specific CD8 and CD4 T cells and reduced virus loads (1). Immunization of RMs with pIL-12 and SIV DNA that encoded SIV Gag, Env, and Pol resulted in the reduction of SIVmac251 replication by 1.5 log (24). In a less rigorous simian-human immunodeficiency virus (SHIV)-89.6P challenge, there was a 2.4 log reduction in virus replication following the SIV DNA plus pIL-12 prime and vector boost vaccination with vesicular stomatitis virus expressing SIV

FIG. 6. Mean frequencies of SIV-specific CD8 (A) and CD4 (B) T cells producing 1, 2, or 3 cytokines (IFN- γ , TNF- α , or IL-2) on stimulation with SIV peptide pools spanning the whole proteome following SIV DNA priming and rAd5 boost. (C) Pie charts below the bar graphs indicate the relative proportions of SIV-specific CD4 and CD8 T cells at each time point that produced each combination of cytokines. Error bars show standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; pIL-12, plasmid encoding IL-12; rAd5, recombinant adenovirus 5-SIV boost.

Gag and HIV Env gp160 (12). In all these studies, viral control was associated with an increased antibody and IFN- γ T-cell response detected either by flow cytometry or enzyme-linked immunosorbent spot assay (ELISPOT). In agreement with and extending these earlier findings, we detected broad, multifunctional IFN- γ , TNF- α , or IL-2 CD4 and CD8 T-cell responses directed against each SIV protein. The antigen-specific CD8 T-cell responses were more sustained following vaccination codelivered with pIL-12. No difference in the magnitude of binding SIV-specific Ab was observed between vaccine groups; however, suppression of virus replication may have occurred in these animals through direct neutralization or antibody-dependent cell-mediated mechanisms, as has been described in HIV-1 infection (15, 23, 60). Work is ongoing to examine the neutralizing and nonneutralizing properties of SIV-specific Ab induced with and without pIL-12. Further studies that include a control group immunized with pIL-12 alone to examine non-specific effects of IL-12 on control of virus replication are needed to specify immune correlates of protection induced upon the incorporation of IL-12 into vaccination.

One clear feature of DNA priming with pIL-12 was the increased quantity and quality of SIV-specific CD4 T cells after DNA prime. There were 3-fold more SIV-specific CD4 T cells

detected in the group receiving pDNA-SIV with pIL-12 than in the group primed without pIL-12. Moreover, 50% of those cells produced 3 cytokines, compared to less than 20% of the SIV-specific CD4 cells induced in the other vaccine groups. CD4 T cells can mediate cytotoxicity (2, 6), as well as provide costimulatory signals to antigen-presenting cells for the activation and induction of effector and memory CD8 T-cell populations (3). It has been proposed that vaccines should avoid stimulating HIV-/SIV-specific CD4 T cells because HIV specifically targets activated HIV-specific CD4 T cells, and induction of these cells through vaccination could potentially lead to exacerbation of early infection (11). Our data demonstrate that vaccine-elicited antigen-specific CD4 T cells may be advantageous in maintaining immune control of virus replication.

This study adds to the developing evidence that control of SIV/HIV replication is achievable by vaccination. While the current study is underpowered to unequivocally demonstrate an effect on the susceptibility of infection, we believe there is a suggestion that vaccination can also reduce the rate of infection. Clearly, it would be highly desirable to achieve these effects in humans with a vaccine. The current study suggests several paths toward this goal. Further analysis of immune responses in controlling animals using protective vaccination

protocols such as the one demonstrated here or using rhCMV vectors should assist in the design of HIV vaccines that are capable of potent virus control. Second, it should be possible to adapt the vaccination approach shown here to human clinical testing. With this in mind, we plan to determine whether a vaccine encoding fewer proteins is equivalently effective and whether the rAd5 boost can be replaced with a more clinically relevant viral vector while maintaining efficacy.

ACKNOWLEDGMENTS

Statistical analyses were partially funded by NIH NIAID R01 grant no. AI054165-08. This research was supported in part by the Intramural Research Program of NIH, National Cancer Institute, Center for Cancer Research. Typing for major histocompatibility type I alleles was performed with support from NIH grant 5R24RR016038 awarded to David Watkins.

We thank Tom Friedrich for the kind donation of SIVmac239, Jeffrey Lifson and Mike Piatk for assistance in determining viral loads, Rosanne Boyle for administrative support, and Rebecca Powell, Ross Lindsay, Josephine Cox, Pat Fast, Richard Wyatt, and Michael Caulfield for reviews of the manuscript.

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