Multi-Low-Dose Mucosal Simian Immunodeficiency Virus SIVmac239 Challenge of Cynomolgus Macaques Immunized with “Hyperattenuated” SIV Constructs[▽]

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Hyperattenuated simian immunodeficiency virus SIVmac239-derived constructs Δ 5-CMV and Δ 6-CCI are an effort to render SIV incapable of, in practical terms, both reversion and recombination while maintaining the immune features of SIV as a retrovirus. Primary inoculation of cynomolgus macaques with 10^8 50% tissue culture infective doses (TCID₅₀) of Δ 5-CMV or Δ 6-CCI induced low-level humoral and cellular responses detectable in the absence of measureable *in vivo* replication. The first of three DNA boosts resulted in elevated gamma interferon (IFN- γ) enzyme-linked immunospot (ELISPOT) responses to Gag, Pol, and Env in the Δ 5-CMV vaccine group compared to the Δ 6-CCI vaccine group ($P = 0.001$). Weekly intrarectal challenge with a low dose of SIVmac239 followed by a dose escalation was conducted until all animals became infected. The mean peak viral load of the Δ 5-CMV-vaccinated animals (3.7×10^5 copies/ml) was ~ 1 log unit lower than that of the control animals. More dramatically, the viral load set point of these animals was decreased by 3 log units compared to that of the controls (<50 versus 1.64×10^4 copies/ml; $P < 0.0001$). Seventy-five percent (6/8) of vaccine recipients controlled virus below 1,000 copies/ml for at least 6 months, with a subset controlling virus and maintaining substantial CD4 T-cell counts for close to 2 years of follow-up. The correlates of protection from SIV disease progression may lie in the rapidity and protective value of immune responses that occur early in primary SIV infection. Prior immunization with hyperattenuated SIVmac239, even if sterilizing immunity is not achieved, may allow a more advantageous host response.

To date, the most promising approach to inducing sterilizing immunity in the macaque model has been through the use of live attenuated virus (LAV) vaccines based on simian immunodeficiency virus (SIV). A major advantage of an attenuated virus strategy for the development of a human immunodeficiency virus (HIV) vaccine is the ability of attenuated viruses to induce broad and persistent immunity (29, 51). In particular, SIV strains engineered with deletions of *nef* (SIV $\Delta*nef*) have afforded the most significant protection upon challenge with pathogenic SIV (13, 14, 29, 60, 65, 72). Numerous SIV-derived live attenuated vaccine models have been developed, many of which employ deletions in the viral accessory genes (3, 12, 14, 15, 25, 29, 30, 53, 64, 72). In many cases, vaccinations have$

been shown to substantially decrease viral burden during the acute phase of infection, maintain low to undetectable levels of virus during the chronic phase of infection, and limit the progression to AIDS. Although promising, a major caveat to the live attenuated virus vaccine approach is the potential for compensatory reversion and the observations that incompletely attenuated viruses may harbor residual pathogenicity (5, 10, 14). Even SIV constructs containing multiple deletions in *nef*, *vpr*, and the negative regulatory element (NRE) can cause AIDS-like disease in adult macaques and particularly in neonates (4, 5, 27, 53). This may be analogous to some human long-term nonprogressors infected by *nef*-deleted HIV variants in whom a slowly increasing viral burden has been accompanied by disease progression (22, 34, 37). Additional mutations can be engineered into vaccine vectors to generate highly attenuated viruses, but this often comes at the expense of their protective efficacy (8, 23, 30).

We previously made two series of novel live attenuated SIV vaccine models (25) in which the simplified SIV constructs retain all the structural viral proteins but have inactivating mutations for all viral accessory genes. These constructs retain significant antigenicity, without the pathogenic effects associated with accessory viral factors, thus limiting or eliminating the potential for reversion (25).

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TABLE 1. Prime-boost immunization and mucosal challenge schedule^a

Time	Group 1 (vaccinated with Δ5-CMV)	Group 2 (vaccinated with Δ6-CCI)	Group 3 (control)
Wk 0	Δ6-CCI virus i.v. (10 ⁸ TCID ₅₀)	Δ6-CCI virus i.v. (10 ⁸ TCID ₅₀)	Medium
Wk 9	Δ5-CMV virus i.v. (10 ⁸ TCID ₅₀)	Δ6-CCI virus i.v. (10 ⁸ TCID ₅₀)	Medium
Wk 79	Δ5-CMV plasmid DNA i.d. (2.5 mg via Bioject)	Δ6-CCI plasmid DNA i.d. (2.5 mg via Bioject)	Control plasmid DNA i.d. (2.5 mg via Bioject)
Wk 87	Δ5-CMV plasmid DNA i.d. (2.5 mg via Bioject)	Δ6-CCI plasmid DNA i.d. (2.5 mg via Bioject)	Control plasmid DNA i.d. (2.5 mg via Bioject)
Wk 114	Δ5-CMV plasmid DNA i.m. (2.5 mg via Bioject with CpG adjuvant) and Δ5-CMV virus i.m. (10 ⁸ TCID ₅₀ with CpG adjuvant)	Δ6-CCI plasmid DNA i.m. (2.5 mg via Bioject with CpG adjuvant) and Δ6-CCI virus i.m. (10 ⁸ TCID ₅₀ with CpG adjuvant)	Control plasmid DNA i.m. (2.5 mg via Bioject with CpG adjuvant) and medium i.m. with CpG adjuvant
Wk 118 (low-dose challenge)	SIVmac239 i.r. (250 TCID ₅₀)	SIVmac239 i.r. (250 TCID ₅₀)	SIVmac239 i.r. (250 TCID ₅₀)

^a The experimental design follows. Twelve cynomolgus macaques were randomly assigned into two experimental groups and one control group (4 monkeys in each group). Groups 1 and 2 received an initial priming immunization with infectious Δ6-CCI. Groups 1 and 2 received subsequent boosting immunizations with infectious virus (week 9) or plasmid DNA (weeks 79 and 87) derived from Δ5-CMV or Δ6-CCI construct. A final boost comprised of both virus and DNA, in association with CpG adjuvant, was administered at week 114. At week 118, the eight vaccinated animals and four control animals were challenged intrarectally with a low dose (250 TCID₅₀) of monkey-grown SIVmac239 (nef open). Inoculations were repeated on a biweekly basis until a detectable SIV infection was established as determined by nested RT-PCR. Abbreviations: i.v., intravenously; i.d., intradermally; i.r., intrarectally.

Whether administered parenterally or mucosally, conventional challenge trials in macaques have often utilized artificially high single-dose inocula in an effort to ensure that most, if not all, of the naive or placebo-immunized animal subjects become infected following a single exposure. The rationale for using a single massive challenge has been reconsidered in light of the possibility that vaccines with protective efficacy under physiologic challenge conditions may not be identified. This practice is now being replaced by an approach designed to better approximate the relatively low *in vivo* acquisition rates following a single sexual exposure to HIV (21, 45, 69) and should provide a more realistic assessment of vaccine efficacy in “real-world” situations. Importantly, recent studies using this approach have demonstrated viremia of magnitude and kinetics comparable to that seen following single high-dose mucosal inocula (47), and this approach has been used successfully in more recent challenge trials (31, 70). Here we are assessing the safety, immunogenicity, and protective efficacy of two hyperattenuated SIV vaccine candidates following a multi-low-dose intrarectal challenge with highly pathogenic SIVmac239 in the cynomolgus macaque model.

SIV-specific humoral immune responses were assessed at various time points postvaccination and postchallenge by Western blotting. Cellular immunogenicity was monitored by evaluation of peripheral T-cell responses (via gamma interferon [IFN-γ] enzyme-linked immunospot [ELISPOT] assay) following stimulation with peptide pools spanning the entire SIVmac239 proteome. The protective efficacy of the different vaccine candidates was assessed by classical endpoints, such as quantitative analysis of plasma viral load, quantitative immunophenotyping of lymphocytes, and clinical markers of disease progression. Even using extremely attenuated SIV constructs with only minimal evidence of replication, a modest immune response that can impact long-term disease progression is generated.

MATERIALS AND METHODS

Vaccine constructs. The construction of this panel of severely attenuated simplified SIV constructs has been described previously (23). This panel was originally generated to improve the safety characteristics of live attenuated

viruses (LAVs) by engineering novel “hyperattenuated” viruses in a manner that would eliminate the pathogenic accessory viral factors and preclude the possibility of reversion or recombination that may result in pathogenesis. Two constructs termed Δ5-CMV (Delta-5) and Δ6-CCI (Delta-6) demonstrating different degrees of attenuation were utilized in this study. Both variants were derived from full-length infectious clones of SIVmac239 (24, 32) and were engineered with gross and targeted inactivating deletions and mutations within the accessory genes. These genes are known to play important roles in immune modulation and HIV pathogenesis, and their removal was effected to allow for an increased margin of safety.

Animals. Twelve adult male cynomolgus macaques (*Macaca fascicularis*) from the Philippines, with a mean weight of 5.6 kg (range, 4.4 to 8.7 kg) were housed in biosafety level 2+ (BSL2+) containment facilities of the Scientific Services Division (Ottawa, Canada) in accordance with a protocol approved by the Institutional Animal Care Committee of Health Canada. Serological testing revealed that the animals were free from infection with SIV, simian retrovirus type D (SRV) (serotypes 1, 2, and 5), simian T-cell leukemia (STLV), and simian-type herpesvirus prior to initiation of the study. The food intake, stool consistency, and general well-being of the animals were monitored daily. On a monthly basis, the animals were weighed and examined for external signs of enlarged lymph nodes or rash. Animals were monitored closely for clinical signs of simian AIDS (SAIDS), including wasting (weight loss), diarrhea, generalized lymphadenopathy, pneumonia, encephalitis, and vascular thrombosis, concurrent to surveillance for secondary infections. Vaccine inoculations, sampling procedures, and mucosal challenge were conducted under anesthetic (10 mg/kg ketamine administered intramuscularly [i.m.]). The monkeys were randomly assigned into two vaccine groups, receiving either Δ5-CMV or Δ6-CCI vaccinations, and a control group as described below (Table 1) with all subsequent testing and analysis done in a blinded manner.

Prime-boost immunizations. DNA plasmid constructs were purified by standard protocols on CsCl gradients followed by passage through endotoxin-free columns (Qiagen). Infectious viral stocks were produced following Lipofectamine-mediated (Invitrogen) transfection of COS-7 cells. Viral supernatants were recovered by centrifugation, filtered through 2-μm filters, and stored at -80°C before the titers were determined. Two groups of four monkeys received a priming intravenous (i.v.) immunization with the most attenuated construct; the monkeys were given 10⁸ 50% tissue culture infective doses (TCID₅₀) of Δ6-CCI. A subsequent immunization with either Δ5-CMV or Δ6-CCI virus was delivered as indicated (Table 1). Following a 70-week period of observation for the safety of these viruses, animals were boosted twice with 2.5 mg of plasmid DNA administered using a Biojector 2000 device (Bioject, Portland, OR). A final boost of both plasmid DNA (via Biojector 2000) and virus in combination with CpG adjuvant (0.5 mg/animal) (Coley Pharmaceuticals, Kanata, Ontario, Canada) (via a needle) was administered intramuscularly into each thigh. Control animals received sham inoculations of medium, a plasmid control, or both as appropriate (Table 1).

TABLE 2. Multi-low-dose escalating mucosal challenge^a

Group or parameter	Animal identification no.	No. of challenges to infection	Cumulative dose (TCID ₅₀)	Escalating dose regimen (no. of doses × TCID ₅₀)
Group 1 (Δ5-CMV vaccinated)	C97061M	5	1,250	16 × 250; 1 × 500; 1 × 1,000
	C97062M	18	5,500	
	C98007M	9	2,250	16 × 250; 1 × 500; 1 × 1,000
	C98008M	18	5,500	
Avg		12.5	3,625	
Geometric mean		11	3,037	
Group 2 (Δ6-CCI vaccinated)	C97014M	3	750	
	C97060M	15	3,750	
	C98004M	3	750	
	C98006M	2	500	
Avg		5.8	1,438	
Geometric mean		4.1	1,013	
Group 3 (control)	C87017M	25	17,750	16 × 250; 1 × 500; 1 × 1,000; 7 × 1,750
	C94078M	20	9,000	16 × 250; 1 × 500; 1 × 1,000; 2 × 1,750
	C98009M	4	1,000	
	C98012M	8	2,000	
Avg		14.2	7,438	
Geometric mean		11.2	4,228	

^a A multiple low-dose mucosal challenge was performed. Animals were challenged every week with 250 TCID₅₀ of SIV intrarectally. SIV infection was monitored by PCR, and uninfected animals were rechallenged weekly until infected. A subset of animals followed an escalating dose regimen as indicated (C97062M, C98008M, C87017M, and C94078M). Multiple challenges resulted in SIV infection in all animals (mean, 10.8; range, 2 to 25).

Intrarectal challenge. Four weeks following the final boost, the animals were challenged intrarectally with a low dose (250 TCID₅₀) of pathogenic SIVmac239 (*nef* open) that had been propagated on phytohemagglutinin (PHA)-stimulated monkey peripheral blood mononuclear cells (PBMCs) using virus stock made by transfection of 293T cells with proviral DNA; the virus titers on primate cells (CEMx174) were determined. Challenge inocula were prepared in RPMI 1640 medium. A syringe silastic-catheter assembly housing the virus preparation was inserted nontraumatically into the rectum of the animal to a depth of approximately 5 cm. The virus solution was injected into the rectum, and the catheter was held in place for 30 to 60 s following expulsion of the liquid and then withdrawn with care. The animal's posterior remained elevated for 15 to 20 min to promote localized absorption of the inoculum. Animals were subsequently rechallenged intrarectally every week with 250 TCID₅₀ SIVmac239 (*nef* open) or an escalating dose regimen (Table 2) until the animals were infected.

Assessment of SIV infection and viral load determinations. A nested reverse transcription-PCR (RT-PCR) assay was used to determine the SIV infection status of the animals. Plasma samples were collected 5 days subsequent to each challenge, and 140 µl of freshly isolated plasma was used for recovery of viral RNA using a QIAamp viral RNA minikit (Qiagen). The first round of PCR amplification with SIV-specific primers (5'-GGAGATCTGCGACAGAGAC T-3' and 5'-CTCACTGATACCCCTACCAAGTC-3') was completed using standard PCR conditions. PCR product from the first PCR amplification was diluted 10-fold into a second PCR mixture containing a nested primer set (5'-GGAAGATGGATACTCGCAATCC-3' and 5'-CATCATCTTCTCAT CTATATCATCC-3') and subjected to a second round of amplification. PCR products were visualized by ethidium bromide staining following agarose gel electrophoresis.

Viral load determinations were determined by real-time PCR on an ABI 7000 using the QuantiTect Probe RT-PCR kit (Qiagen) and primers and probe sequences previously published by Horton et al. (28). All plasma viral loads were converted to log₁₀ values prior to further analysis. The plasma viral load quantitation assay was validated by direct comparisons with an established assay in the lab of Jonathan Heeney (Biomedical Primate Research Center, The Netherlands).

Immunophenotyping and fluorescence-activated cell sorting (FACS). Immunophenotypic analysis was performed using two four-color panels including the following antibodies (BD Biosciences): PerCP-conjugated CD45 (clone TU116), fluorescein isothiocyanate (FITC)-conjugated CD3 (SP-34), phycoerythrin (PE)-conjugated CD4 (L200), allophycocyanin (APC)-conjugated CD8 (CD8-APC) (RPA-T8), CD20-APC (2H7), R-phycoerythrin (PE)-conjugated CD16 (CD16-

RPE) (3G8), and CD56-RPE (MY31). Five thousand gated lymphocytes (CD45⁺; low side scatter [SSC]) were acquired per sample using a BD FACSCaliber and analyzed using CELLQuest ProSoftware (BD Biosciences). T-cell populations were identified and enumerated by staining with antibodies directed against CD3, CD4, and CD8. The pan-B-cell marker CD20 was used for identification of B lymphocytes. Natural killer (NK) cells (CD3⁻) were identified following costaining with antibodies directed against CD16 and CD56.

Immunoblotting. Humoral IgG immune responses directed against SIV proteins were monitored using an SIV Western blot assay kit (Zeptomatrix, Buffalo, NY). Briefly, SIV Western blot strips were exposed to a 1:100 dilution of plasma, and the reaction was developed according to the manufacturer's directions.

Cynomolgus PBMC isolation. Protocols routinely used for the isolation of PBMCs from humans and rhesus macaques are not amenable for use with cynomolgus macaque samples due to significant contamination of red blood cells and granulocytes. To obtain sufficiently pure populations of PBMCs, we have developed a novel methodology described briefly here. Plasma was isolated from blood samples via routine centrifugation (1,200 × g, 10 min). The buffy coat was collected and diluted to 8 ml in tissue culture medium or phosphate-buffered saline (PBS). The diluted buffy coat was then layered onto a 5-ml cushion of room temperature 60% Percoll in 1 × PBS. The stock of Percoll from which this dilution is made is a 9:1 mixture of Percoll and 10 × PBS which can be prepared in advance and stored at 4°C. The sample was centrifuged at 500 × g for 30 min with no brake. PBMCs were isolated at the interface, washed with PBS, and subjected to a final centrifugation step. Isolated PBMCs can be resuspended in medium and enumerated as desired.

Peptides. Peptides spanning the entire SIVmac239 proteome were obtained through the NIH AIDS Research and Reference Reagent Program (catalog numbers 6204, 6205, 6207, 6443, 6448, 6449, 6450, 6883, and 8762). Individual peptides were resuspended to 20 mg/ml in 80% dimethyl sulfoxide (DMSO) and subsequently grouped into 39 peptide pools comprised of 20 individual peptides at 1 mg/ml each. Peptide pools representing Gag were utilized at all time points assessed by ELISPOT assay, whereas peptides derived from Env, Pol, Tat, Nef, Vif, Rev, Vpr, and Vpx were incorporated into the study design for assessments of T-cell responses from week 77 of the vaccination schedule and throughout the follow-up to SIV infection.

ELISPOT assay. Peptide-specific gamma interferon (IFN-γ) secretion was measured by ELISPOT assay. Briefly, sterile 96-well flat-bottom plates (Multi-screen HTS and Immobilon-P membrane; both from Millipore) were used. Each well was coated with 0.5 µg of antibody (monoclonal antibody [MAb] GZ-4; Mabtech), and the plates were incubated overnight at 4°C. The plates were

FIG. 1. Detection of SIV-specific antibody responses by Western blotting. SIV-specific antibody responses against vaccine constructs and following SIVmac239 infection were elicited. Humoral immune responses to the vaccines and those that developed following SIV infection were monitored by Western blotting with a commercial kit. Plasma samples were obtained postvaccination (A) and at 5 weeks postinfection (B). The qualitative assay allowed for detection of antibodies directed against the structural genes (Gag, Pol, and Env) expressed by both Δ5-CMV and Δ6-CCI. The relative positions of SIV-derived antigens are indicated by the arrows to the left of the gels. Positive (+ve) and negative (-ve) reactivity control lanes are indicated in addition to a positive serum control band present within each lane.

washed six times with PBS and then blocked with 200 μ l of R10 buffer (RPMI 1640 medium plus 10% fetal calf serum [FCS]) at 37°C for 2 h. Freshly isolated PBMCs were routinely plated at 200,000 cells/well and stimulated in duplicate with peptide pools (2.5 μ g/ml/peptide). The total volume was 100 μ l. PBMCs stimulated with staphylococcal enterotoxin B (SEB) and DMSO were used as positive and negative controls, respectively. The plates were incubated at 37°C for 12 to 16 h in a 5% CO₂ environment and washed extensively with water and then with PBS containing 0.1% Tween 20 (PBS-T). The wells were incubated with biotinylated detection antibody (Mabtech) for 2 h at room temperature (RT). Streptavidin-AP was added to each well and incubated for 1 h at RT and washed with PBS-T. IFN- γ -secreting cells were identified by developing the plates with chromogenic AP substrate (Bio-Rad). The spots were enumerated using an automated reader system (CTL Analyzers, LLC) employing ImmunoSpot 3.1 software. Enumeration was done in a blinded manner such that the type of intervention or group number was unknown to the reader. The number of IFN- γ -producing cells was calculated following background subtraction and were expressed as spot-forming cells (SFC)/10⁶ PBMCs. Responses to individual peptide pools were considered positive if the number of SFC/10⁶ PBMCs of either one of the two replicate wells exceeded the highest value for the six DMSO control wells, if the average of the two replicates exceeded the cutoff value, and if the net SFC/ml exceeded 20 if the cutoff was less than 20. The cutoff was defined as the average of the DMSO controls plus two times the standard deviation.

Statistical analysis. Viral RNA levels (viral RNA equivalents/ml) were transformed to log₁₀ values for analysis. Peak viral loads were assigned as the highest measurement taken between weeks 0, 1, and 2 postinfection inclusive. The long-term viral load set point was determined as the mean of values taken for all animals within a group from weeks 15 to 55 postinfection. The baseline levels for lymphocyte populations for each group represent the mean levels as determined using at least two time points for each animal prior to the initiation of challenge. Basic statistical differences of the mean values between groups and subgroups with respect to peak viral load, set point, lymphocyte population, and IFN- γ ELISPOT results were assessed using the Student *t* test (SPSS software, version 11.0.2) following Levene's test for equality of variances. Due to the small sample size, more-complex statistical analyses were not pursued.

RESULTS

Immunogenicity of hyperattenuated vaccines. To address the immunogenicity and as an indirect measure of replication of the vaccine candidates, we employed Western blotting to

detect SIV-specific antibody responses longitudinally throughout the immunization schedule. Baseline levels were assessed prior to initiation of vaccinations and showed no detectable SIV-specific antibody responses (data not shown). SIV-specific antibodies were detectable at our initial assessment 4 weeks following the first priming immunization. Maturation of the response was seen between sampling at weeks 8 and 21 following the first boost and demonstrated reliable detection of SIV-specific antibodies in all of the vaccinated animals and none of the control animals (data not shown). The groups vaccinated with Δ5-CMV and with Δ6-CCI presented differences in their SIV-specific antibody repertoires. In general, antibodies directed against p17 and p27 were frequently detected in Δ5-CMV vaccinees but absent in Δ6-CCI-vaccinated animals. Antibodies directed against unprocessed p55 (Gag) were rarely seen in any animals prior to analysis at week 116, following the third boost with both virus and DNA (Table 1). This time point immediately prior to the initiation of challenge revealed the greatest breadth of responses directed against Gag (p17, p27, and p55) and Pol (p66) (Fig. 1A). At no time prechallenge were we able to detect specific antibody responses against Env antigens.

Further to our assessment of humoral immunity, we measured contributions from CD8 T-cell-specific responses via IFN- γ ELISPOT assay, using peptide pools spanning the entire SIVmac239 proteome. Of note, our analysis of time points surrounding the two initial viral primes were restricted to Gag-specific T-cell responses, whereas all other time points included the full panel of peptides. Gag-specific responses were assessed at 4 weeks following each of the two initial primes where both vaccinated groups initially received Δ6-CCI virus, and groups 1 and 2 received Δ5-CMV and Δ6-CCI, respectively, thereafter (Table 1). Δ6-CCI virus priming was capable of inducing modest Gag responses that were maintained for at

A. Group 1 ($\Delta 5$ -CMV)

B. Group 2 ($\Delta 6$ -CCI)

FIG. 2. Magnitude of SIV-specific IFN- γ ELISPOT responses following immunization. SIV-specific cytotoxic T-cell responses elicited during vaccination of the $\Delta 5$ -CMV vaccinated group (A) and the $\Delta 6$ -CCI vaccinated group (B). ELISPOT responses to SIV antigens were measured for 39 peptide pools (twenty 15-mers/pool) at the times indicated during the vaccination phase (Table 1). Positive responses to individual pools that met our criteria were used to determine the number of SIV-specific IFN- γ -secreting T cells responding to each of the nine SIV genes. Gag peptide pools were employed at all time points examined, whereas peptide pools representing the other SIV genes were introduced only following DNA vaccinations (i.e., 77, 83, and 93 weeks postvaccination). Responses generated against peptide pools for Tat, Nef, Ref, Vif, Vpr, and Vpx were pooled and tallied as “Other”.

least 4 weeks thereafter (Fig. 2). Following the second virus prime, Gag-specific responses were boosted in a subset of animals, but there were no significant differences between the two vaccine groups. When assessed immediately prior to DNA boosting, these responses had decreased substantially but remained detectable in a subset of animals (Fig. 2). The most profound difference between the two vaccinated groups came in the ability of responses to be significantly impacted following a single DNA boosting event. The $\Delta 5$ -CMV vaccine group had elevated Gag, Env, Pol, and total IFN- γ responses 4 weeks after the DNA boost (week 83) compared to the $\Delta 6$ -CCI recipients ($P = 0.03, 0.008, 0.04$, and 0.001 , respectively). This also manifested in greater breadth of response as measured by the number of pools recognized for each of Gag, Env, and total IFN- γ responses ($P = 0.07, 0.06$, and 0.05 , respectively). The second DNA boost at week 87 did not appear to result in further stimulation, with most $\Delta 5$ -CMV recipients demonstrating a reduction in SIV-specific responses within 4 weeks. This was in contrast to the $\Delta 6$ -CCI group, in which three of four animals (animals C97014M, C98004M, and C98006M) did demonstrate enhanced responses following the second DNA boost, where a substantial portion of the response was directed against Env. The fourth $\Delta 6$ -CCI animal (C97060M) demonstrated only minimal SIV-specific responses throughout the vaccination regimen and had no detectable responses following either of the two DNA boosts. As expected, the control group was devoid of any detectable cytotoxic T-lymphocyte (CTL)

responses when assessed at week 93 of the vaccination regimen and prior to the initiation of mucosal challenge. Although both $\Delta 5$ -CMV and $\Delta 6$ -CCI were essentially devoid of functional accessory proteins, the regions of these proteins that were retained in the constructs were sufficient to generate some detectable T-cell responses. This was especially true for Vif, in which case 6 of the 8 vaccinated animals responded to the Vif peptide pool at some point during the vaccination. Only the N-terminal 21 amino acids of Vif are retained in both vaccine constructs, suggesting there is at least a nominal epitope present in that region. It is likely that this response may be due to epitope Vif-RW9 (2) to which responses have been documented in rhesus macaques and where eight of the nine amino acids are present in our constructs. Two animals also presented with Nef-specific responses, and to date, only one Nef-specific CD8 T-cell epitope (RM9) has been mapped in cynomolgus macaques (9); however, this region was not present in our constructs, suggesting the presence of another, as yet unmapped epitope(s). **Impact of vaccination on intrarectal challenge.** Four weeks following the final immunization at week 114 (Table 1), we initiated a multi-low-dose mucosal SIV challenge trial. Vaccinated and control groups were challenged with 250 TCID₅₀ of SIVmac239 (nef open), administered intrarectally. Animals were assayed weekly for SIV infection by PCR, and uninfected animals were rechallenged on a weekly basis. There were four cases where animals remained uninfected following the initial

FIG. 3. Longitudinal plasma viral RNA levels in vaccinated and control groups following intrarectal SIVmac239 challenge. Plasma viral RNA levels were determined by quantitative RT-PCR and transformed to log₁₀ copies/ml for analysis. (A) Viral loads are shown for each animal after challenge. The animal numbers are shown in abbreviated form (for instance, 61 for C97061M) (see text or Tables 2, 3, and 4 for animal numbers). (B) Peak viral loads were assessed within the first 2 weeks postinfection. $\Delta 5$ -CMV-vaccinated animals demonstrated a reduction in peak viral loads of approximately 1 log unit compared to those of the $\Delta 6$ -CCI-vaccinated or control animals. Peak values are represented as means $\pm$ standard errors of the means (SEMs) (error bars). NS, not significant. (C) Set point viral loads were significantly decreased in the $\Delta 5$ -CMV-vaccinated group compared to the control group (~ 3 -log-unit decrease; $P < 0.001$). Data points represent the mean values for each group of animals postinfection $\pm$ SEMs. The smoothed curve is the estimated viral load following transformation with a smoothed local regression (LOESS) curve fit. Viral load set points as illustrated by the dotted lines were determined as the mean of data accrued between weeks 15 and 55 postinfection.

16 weekly low-dose challenges (total dose of 4,000 TCID₅₀). Subsequently, an escalating dose regimen was used for these animals (Table 2). All monkeys were infected following multiple low-dose intrarectal challenges as demonstrated by plasma viremia (Fig. 3A). The number of exposures and cumulative dose required to achieve infection varied considerably among the animals with no significant differences between the groups (Table 2). A subset of the animals (animal identification numbers C97062M, C98008M, C97060M, C87017M, and C94078M) required 15 to 25 inoculations before successful infection. In particular, animals C87017M and C94078M from

the control group appeared strongly resistant to challenge, requiring 25 and 20 inoculations of escalating dose, respectively, or the equivalent of a total dose of 17,750 and 9,000 TCID₅₀ (Table 2). Infection was achieved following a dose escalation to 1,750 TCID₅₀. For those animals requiring 15 or greater challenges, of which four required dose escalation regimens to effect SIV infection, there was predictive value in the infecting dose (ranging from 250 to 1,750 TCID₅₀) as to outcome as measured in viral load between weeks 15 and 25 where we had full viral load data on all of these animals ($P < 0.03$) (Table 2 and Fig. 3). An analysis of the two vaccine groups with

TABLE 3. Clinical outcome following SIVmac239 infection and progression toward simian AIDS^a

Group	Animal identification no.	Clinical SAIDS (wpi) ^b	Plasma RNA viral load (copies/ml) ^c	CD4 ⁺ T-cell count (wpi) ^d	% CD4 ⁺ T-cell depletion ^d
Group 1 (Δ 5-CMV vaccinated)	C97061M	None (97)	<50	754 (88)	16.7
	C97062M	None (83)	6,085	611 (74)	41.9
	C98007M	None (92)	55	623 (83)	25.4
	C98008M	None (78)	202,021	202 (69)	80.0
Group 2 (Δ 6-CCI vaccinated)	C97014M	Yes (89)	126,423	156 (59)	64.1
	C97060M	None (78)	2,993	160 (70)	80.2
	C98004M	None (98)	<50	775 (92)	25.0
	C98006M	Yes (25)	76,917	203 (25)	85.6
Group 3 (control)	C87017M	Yes (63)	847,332	148 (59)	77.4
	C94078M	None (80)	294,615	88 (70)	87.2
	C98009M	None (97)	11,965	80 (88)	92.5
	C98012M	Yes (80)	55,537	317 (52)	69.4

^a An examination of plasma RNA viral load and peripheral CD4 T-cell counts at euthanasia was performed. The time of necropsy or the latest time point sampled is indicated in parentheses. Percent of CD4 T-cell depletion reflects deviation from the mean baseline levels for each individual animal as determined prechallenge.

^b Onset of clinical simian AIDS (SAIDS) symptoms, requiring euthanasia. Animals showing no signs of SAIDS were sacrificed at the end of the predetermined study period. The week postinfection (wpi) of euthanasia is indicated in parentheses.

^c Plasma viral load as measured at necropsy.

^d CD4⁺ T-cell enumeration at the latest time point assessed. Sampling time point in weeks postinfection (wpi) is shown in parentheses. The percentage of peripheral CD4 T-cells lost was determined compared to the baseline level.

respect to the number of challenges to infection or the total dose to infection revealed no significant differences.

Viremia following SIV infection. Plasma viral loads were monitored longitudinally following SIV infection. The kinetics and magnitude of acute viremia were consistent with other studies following SIV mucosal challenge which have shown lower peak viral loads in cynomolgus monkeys compared to rhesus macaques of Indian origin (31, 63). Primary infection peaked within 1 to 2 weeks postinfection and ranged between 10^5 and 10^7 copies/ml in most animals (Fig. 3A). The mean peak viral load in the Δ 5-CMV-vaccinated animals (3.66×10^5 copies/ml; range, 7.4×10^2 to 1.15×10^6 copies/ml) was approximately 1 log unit lower than that in the control group (3.18×10^6 copies/ml; range, 4.76×10^5 to 4.94×10^6 copies/ml) ($P = 0.04$) (Fig. 3B). The Δ 6-CCI-immunized animals (4.08×10^6 copies/ml; range, 2.98×10^5 to 11.5×10^6 copies/ml) did not exhibit a similar decrease in peak viral load. One Δ 5-CMV-vaccinated animal (C97061M) controlled acute viral replication very well, as evidenced by a peak viral load 2 weeks postinfection of only 740 copies/ml (Fig. 3A). An examination of peak viral loads with respect to the number of challenges required to reach infection revealed no significant associations.

The transitional phase from acute to chronic infection demonstrated rapid control of viral loads 3 to 5 weeks postinfection with animals stabilizing viral loads and reaching individual set points by approximately weeks 10 to 15 (Fig. 3A) postinfection. Long-term set points were determined for the Δ 5-CMV and control groups based on the mean of viral load measurements taken during the chronic phase of infection (weeks 15 to 55 postinfection) prior to escape (Fig. 3C). Δ 5-CMV-immunized animals had a long-term set point approximately 3 log units lower than that established in the control group (28 versus 1.64×10^4 copies/ml, respectively; $P < 0.0001$) (Fig. 3C). Two very distinct courses of viremia were apparent within the Δ 6-CCI group, which either approximated that of the Δ 5-CMV-immunized animals (C97060M and C98004M) or that of the control group (C97014M and C98006M) which prevented any meaningful analysis of Δ 6-

CCI viral load set points (Fig. 3C). Of significance, the two Δ 6-CCI noncontrollers were infected more readily than the vaccinated controllers (2.5 versus 11.33 challenges; $P = 0.02$) (Table 2). Most significant was that 75% (6/8) of vaccinated animals maintained viral loads below 1,000 copies/ml for at least 6 months postinfection (Fig. 3A). Two Δ 5-CMV-vaccinated animals (C97062M and C98008M), and one Δ 6-CCI-vaccinated animal (C97060M) experienced a relapse in control of viremia at some point thereafter (Fig. 1A), exemplified by an increase in plasma viral loads. The remaining three vaccinated animals maintained very low or undetectable (<55 copies/ml) viral loads during approximately 2 years of follow-up (Fig. 3A and Table 3).

Peripheral lymphocyte dynamics following SIV infection. As T-cell populations are known to vary dramatically with age in cynomolgus monkeys as they do among other primate species (6, 50), we assessed baseline levels of CD4 and CD8 T cells in our animals. Absolute baseline CD4 counts were comparable between the groups and ranged from a mean of 859 cells/ μ l in the control animals to 950 cells/ μ l in the Δ 5-CMV-vaccinated group (Fig. 4A). This is in good agreement with recently published experiments in three macaque species following SIV mac251 infection (56) whose baseline level in cynomolgus monkeys was 920 cells/ μ l. Importantly, animal C98006M, whom we identified as a rapid progressor, had significantly elevated baseline CD4 T-cell levels compared with the mean values for the other animals (1,413 versus 865 cells/ μ l; $P = 0.03$). Furthermore, we observed a significant difference in baseline peripheral CD4 T-cell counts within the control group with respect to the number of challenges required to achieve SIV infection. Animals C87017M and C94078M who were "resistant" to infection, requiring 25 and 20 challenges, respectively (17,750 and 9,000 TCID₅₀), had lower baseline CD4 counts than the other two control animals (animal ID no. C98009M and C98012M) (670 versus 1,050 cells/ μ l; $P = 0.003$), who were more readily infected with only 4 and 8 doses, respectively (1,000 and 2,000 TCID₅₀, respectively) (Table 2). However, this phenomenon was not observed in the vaccinated

FIG. 4. Summary of lymphocyte populations following SIVmac239 infection. T cells were enumerated as either CD3⁺ CD4⁺ or CD3⁺ CD8⁺ T-cell populations at various times postinfection. (A) Data represent the absolute CD4⁺ T-cell counts/ μ l of PBMCs for each individual animal. $\Delta 5$ -CMV-vaccinated animals maintained normal CD4 counts throughout the study ($>560/\mu$ l), with the exception of animal C98008M, whose CD4 count had dropped below $250/\mu$ l by week 69 postinfection. (B) CD8⁺ T-cell enumeration demonstrates a brief expansion within this cell population followed by return to near-normal levels throughout the study. Baseline cell counts for each population are represented as dashed lines and were determined as the mean counts for each group taken prior to initiation of challenge.

animals. CD8 T-cell baseline values were consistent between the groups and ranged from a mean of 903 cells/ μ l in the $\Delta 5$ -CMV group to 1,010 cells/ μ l in the control group (Fig. 4B).

Initially following infection, a pronounced lymphopenia in the periphery was observed in a subset of animals, which included CD4 and CD8 T cells (Fig. 4), natural killer cells (CD16⁺ CD56⁺), and B cells (CD20⁺) (data not shown). This has been documented in other studies following SIV infection in rhesus macaques (46, 49). Recovery within the NK and B-cell populations to baseline levels occurred rapidly within approximately 1 week (data not shown). Concomitant and presumably in response to the peak of viral load was a variable increase in CD8 T-cell numbers, which resolved within a few weeks (Fig. 4B).

The extent and rate of CD4 loss at acute times postinfection were also examined (Fig. 5). Both the $\Delta 6$ -CCI-vaccinated and control groups experienced a significant decrease in CD4 T cells from the periphery by week 19 postinfection, amounting to a loss of approximately 50% from within this T-cell compartment. This was in contrast to the $\Delta 5$ -CMV-vaccinated animals who demonstrated only a modest loss in CD4 T cells ($\sim 22\%$ by week 19) compared to either the $\Delta 6$ -CCI-vaccinated animals ($P = 0.01$) or control animals ($P = 0.08$). By 40 weeks postinfection, peripheral CD4 counts in the control group had declined to below 500 cells/ μ l

(Fig. 4A). Half of all vaccinated animals, including three $\Delta 5$ -CMV recipients (C97061M, C97062M, and C98007M) and one $\Delta 6$ -CCI recipient (C98004M), were able to maintain significant T-cell levels (>550 cells/ μ l) for the duration of the study, and

FIG. 5. Acute peripheral CD4⁺ T-cell depletion following SIVmac239 infection. The loss of CD4 T lymphocytes was evaluated in PBMCs and expressed as the percentage remaining at each time point relative to baseline values. Data points represent the mean values for each group with error bars showing the SEMs. Linear regression analysis was employed to assess the rate of CD4 depletion within each group. At all time points assessed, the $\Delta 5$ -CMV-vaccinated group maintained a greater fraction of peripheral T cells than either the $\Delta 6$ -CCI-vaccinated or control group ($P < 0.05$).

TABLE 4. Postinfection SIV-specific IFN- γ -secreting T cells

Group	Animal identification no.	ELISPOT response to the following SIV antigen at the indicated time ^a :														
		Gag			Env			Pol			Other			Total		
		Wk 5	Wk 11	CPI	Wk 5	Wk 11	CPI	Wk 5	Wk 11	CPI	Wk 5	Wk 11	CPI	Wk 5	Wk 11	CPI
Group 1 (Δ 5-CMV vaccinated)	C97061M	38	591	435	40	986	756	0	103	1,596	64	1,541	704	142	3,221	3,491
	C97062M	3,355	4,142	2,277	729	410	1,923	550	225	887	184	148	948	4,818	4,925	6,035
	C98007M	82	299	0	22	433	553	22	190	1,963	158	963	304	284	1,885	2,820
	C98008M	625	574	444	350	378	1,610	0	302	2,996	171	304	220	1,146	1,558	5,270
Group 2 (Δ 6-CCI vaccinated)	C97014M	0	<u>438</u>	0	144	295	47	0	0	73	0	483	90	144	1,216	210
	C97060M	5,025	<u>698</u>	1,445	870	166	511	820	45	499	690	123	238	7,405	1,032	2,693
	C98004M	1,487	<u>891</u>	347	2,238	1,830	0	316	125	0	1,802	1,576	794	5,843	4,422	1,141
	C98006M	633	<u>1,297</u>	160	800	3,066	641	944	819	251	253	122	0	2,330	5,304	1,052
Group 3 (control)	C87017M	958	183	951	317	0	0	433	120	601	6,640	9,349	3,981	8,348	9,652	5,533
	C94078M	21	62	0	133	1,344	1,521	0	165	984	23	349	2,256	177	1,920	4,761
	C98009M	308	287	807	131	568	0	0	0	203	406	719	187	845	1,574	1,197
	C98012M	361	559	0	190	471	83	771	7,395	0	501	2,586	169	1,823	11,011	252

^a Magnitude of SIV-specific IFN- γ ELISPOT responses following SIVmac239 infection. ELISPOT responses to SIV antigens were measured for 39 peptide pools (twenty 15-mers/pool) spanning the entire SIVmac239 proteome at the times indicated. Measurements were taken during the acute (week 5) and transitional (week 11) phases. Measurements taken from the chronic phase of infection (CPI) represent data taken from different time points for each animal and ranged from 25 to 65 weeks postinfection. Responses to Tat, Nef, Rev, Vif, Vpr, and Vpx were pooled and tallied as "Other." Significant differences ($P < 0.05$) are shown. Values that are significantly different in the responses to Env, Pol, and Total responses between the two immunized groups are italicized, whereas values that are significantly different in the responses to Gag between Δ 6-CCI and control animals are underlined, and values that are significantly different from Pol between group 1 and control animals are shown in boldface font.

in some cases in excess of 750 cells/ μ l (Fig. 4A and Table 3). The mean CD4 levels in the Δ 5-CMV-vaccinated group during the chronic phase and prior to any late stage progression revealed a loss of only 27% of this cell population (Fig. 4A). The fourth Δ 5-CMV-vaccinated animal (C98008M) was able to maintain comparable CD4 counts until at least week 40, but by week 69 postinfection, CD4 counts had dropped to 202 cells/ μ l (Fig. 4A and Table 3). This animal demonstrated a loss of control of viremia after week 25 as judged by plasma viral load (Fig. 3A), potentiating the progressive CD4 loss (Fig. 4A and Table 3). Four animals, including two Δ 6-CCI recipients and two controls, developed signs of AIDS and were euthanized. The remaining animals remained clinically healthy over the approximately 2 years of follow-up and were sacrificed according to a predetermined study timeline (Table 3).

Postinfection humoral immune responses. Within 5 weeks postinfection with SIV, all of the vaccinated animals responded rapidly in the development of a full complement of antibodies against all SIV gene products (Fig. 1B). These strong recall responses indicate that significant memory B cells were generated by the vaccinations. This was in contrast to the control animals, whom within 5 weeks had developed only weak responses against a subset of gene products (Fig. 1B). However, by 10 weeks postinfection, the antibody responses exhibited by the control group were undistinguishable from those of either one of the vaccinated groups (data not shown).

Magnitude and breadth of SIV-specific T-cell responses postinfection. Following the initiation of our challenge protocol, we monitored CD8 T-cell-specific responses via IFN- γ ELISPOT assay, using peptide pools spanning the entire SIV mac239 proteome (see Materials and Methods). CTL responses were evaluated at intervals designed to represent acute (approximately week 5), transitional (approximately week 11) and chronic (more than 25 weeks) immune responses. Furthermore, we examined the breadth of responses based on both the number of SIV genes recognized and the number of peptide pools recognized for each gene.

Following infection, most animals exhibited an increase in IFN- γ response by week 5. Animal C87017M, who exhibited both the highest peak and set point viral loads within the control group, demonstrated dramatically elevated responses against the accessory proteins Vif, Nef, and Vpx, which remained elevated for the duration of the study. Although not reaching statistical significance, the Δ 6-CCI-vaccinated animals who were able to control their initial infection (animals C97060M and C98004M) exhibited increased total SIV-specific responses as measured at week 5 postinfection (6,624 versus 1,237 SFC/ 10^6 PBMCs; $P = 0.06$) compared to the remainder of their group. This was in marked contrast to the prechallenge immune responses present in these animals, which was undetectable at the end of the vaccination schedule.

It was anticipated that maturation in both the magnitude and breadth of SIV-specific T-cell responses throughout the transitional (week 11) and chronic phases of infection may influence disease progression. Gag-specific responses were elevated in the Δ 6-CCI-vaccinated group than in the control group at week 11 postinfection (831 versus 273 SFC/ 10^6 PBMCs; $P = 0.04$) (Table 4). Furthermore, the Δ 6-CCI controllers elicited greater IFN- γ responses directed against one particular Gag peptide pool (702 versus 79 SFC/ 10^6 PBMCs; $P = 0.04$) than the Δ 6-CCI progressors. Durability of responses (more than 25 weeks) was best maintained in the Δ 5-CMV-vaccinated group exhibiting elevated Pol-specific responses compared to the controls (1,860 versus 447 SFC/ 10^6 PBMCs; $P = 0.03$) (Table 4). The Δ 5-CMV-vaccinated group also had increased breadth against Pol at this time point compared to controls (9.75 versus 3.25 pools; $P = 0.007$). The Δ 5-CMV-vaccinated group also exhibited enhanced responses at times in the chronic phase of infection directed against Env (1,210 versus 300; $P = 0.05$) and against Pol (1,860 versus 206; $P = 0.01$) and total responses (4,404 versus 1,274; $P = 0.01$) (Table 4) compared to the other vaccine group.

Although postvaccination responses appearing early on following infection did not appear to influence disease progres-

sion, there were some apparent differences in the immune responses that matured following infection between “resistant” and “susceptible” animals which manifested at the transitional and chronic time points. “Susceptible” animals requiring 9 or less intrarectal challenges to become infected had elevated responses to Env peptide pool 8 (223 versus 62 SFC/10⁶ PBMCs; $P = 0.05$) at week 11. Of interest was the observation that “resistant” animals requiring 15 or more challenges to infection had marked differences in their chronic immune responses. These animals had elevated Gag peptide pool 3 responses (662 versus 90 SFC/10⁶; $P = 0.03$), Env-specific responses (1,113 versus 297 SFC/10⁶ PBMCs; $P = 0.04$), and total IFN- γ responses (4,858 versus 1,452 SFC/10⁶ PBMCs; $P = 0.002$). The breadth of Pol responses ($P = 0.06$) and the total number of genes recognized ($P = 0.02$) were also elevated in the “resistant” group (data not shown).

DISCUSSION

To date, the most successful protection from SIV challenge in the macaque model has been afforded by live attenuated vaccines (LAVs) (3, 14, 36). However, scientists have persistently been up against the assumed need to balance safety concerns associated with the potential for reversion and recombination versus assumptions that persistent infection is required and that abortive hyperattenuated SIV infection is synonymous with inadequate immunogenicity.

In an attempt to further increase the margin of safety for use of LAVs as viable vaccines against HIV, we evaluated the safety, immunogenicity, and protective efficacy of two “hyper-attenuated” SIV vaccine candidates and their ability to protect from pathogenic low-dose SIVmac239 mucosal challenge. Our SIV constructs display attenuation greater than that seen for SIV $\Delta 5$ (*vif*-deleted) (19). The $\Delta 5$ -CMV and $\Delta 6$ -CCI constructs have been shown to produce replication-competent virus-like particles containing all the viral structural proteins and possessing morphology typical of wild-type virus (23). $\Delta 6$ -CCI is highly attenuated yet is fully infectious in a variety of cell lines, including CEMx174, MT4, and monkey PBMCs, whereas $\Delta 5$ -CMV is less attenuated with increased capacity for growth in MT4 cells. It was hoped that deletions of the accessory genes, including *tat* and *rev*, which have been implicated in HIV pathogenesis (17, 41, 55, 66) might provide an improved level of safety. During extended passage of $\Delta 5$ -CMV and $\Delta 6$ -CCI in tissue culture, there was no evidence of reversion (23) as judged by no apparent increase in replicative capacity. In the current study, we assessed two vaccination regimens with both vaccine groups receiving an initial inoculation with the more attenuated construct ($\Delta 6$ -CCI) and subsequently boosted with the same construct or the less attenuated variant ($\Delta 5$ -CMV). The rationale for this approach was based on the premise that initial exposure to $\Delta 6$ -CCI may in effect lead to greater attenuation of $\Delta 5$ -CMV on subsequent boosts, while still maintaining a good safety profile. Our observations that both virus constructs are noninfectious (at most producing an abortive infection) *in vivo* (as no persistent infection was detected) suggest that they are safe *in vivo*.

This vaccination induced low but persistent humoral and T-cell immune responses. The observation that Gag-specific CTL responses were reproducibly detected following immuni-

zation suggests that even severely attenuated SIV constructs may be replicating at levels sufficient for the induction of immune responses. The induction of robust and rapid recall responses following infection indicated that memory responses were generated by the immunizations. However, vaccination failed to induce sterilizing immunity, as all animals ultimately became infected. The $\Delta 5$ -CMV vaccination schedule did have a statistically significant impact on disease and imparted an ~ 1 -log-unit decrease in peak viral load; all animals efficiently controlled viral loads to below 1,000 copies/ml, and in two cases below 100 copies/ml within a few weeks of infection. The viral load set point was decreased by approximately 3 log units in the $\Delta 5$ -CMV recipients. Two of the $\Delta 6$ -CCI vaccinees also controlled viral loads to below 100 copies/ml but without a reduction in peak viral load. A total of 3/8 vaccinated animals were able to control viral load to near or below the limit of detection and were able to maintain a significant fraction of peripheral CD4 T cells for close to 2 years of follow-up. None of the $\Delta 5$ -CMV vaccine recipients displayed symptoms of SAIDS, and they were all clinically healthy at the end of the study period.

The principal difference between the two vaccine constructs is the retention of *rev* within $\Delta 5$ -CMV, whereas it is inactivated within the $\Delta 6$ -CCI construct. Although the constitutive transport element (CTE) is thought to be a suitable replacement for the Rev/Rev response element (68, 76), it is possible that the lack of Rev may impact proteolytic processing and/or replicative capacity. The repertoire of antibody responses in the $\Delta 5$ -CMV-vaccinated group was skewed toward processed Gag antigens, with $\Delta 6$ -CCI antibodies directed more toward Pol antigens. With regard to cellular immunity, the $\Delta 5$ -CMV-vaccinated group also exhibited an improved ability to respond to a DNA prime as judged by the IFN- γ ELISPOT assay results. Compared to $\Delta 6$ -CCI vaccinees, the $\Delta 5$ -CMV vaccinees maintained an increase in total IFN- γ responses, including elevated Env- and Pol-specific responses at late times postinfection. Following SIV infection, the $\Delta 5$ -CMV-vaccinated group exhibited increased breadth and a more durable Pol-specific response during the chronic phase compared to the control group. An examination of overall virus control with respect to humoral or T-cell-mediated immunity revealed no significant correlations. The paucity of clear immunological parameters that would be predictive of protection is in accordance with recent efforts to elucidate immunological mechanisms differing between progressors and elite controller macaques (44) and in a recent report demonstrating a clear lack of definable characteristics of anti-SIV-specific CD8 T-cell responses in rhesus macaques following SIVmac239 infection (67).

One $\Delta 6$ -CCI recipient (C98006M), whom we classified as a “rapid progressor” required euthanasia at week 26 postinfection due to symptoms of SAIDS. Recent reports suggest that this “rapid progressor” phenotype can be associated with a lack of appropriate or sufficient anti-SIV humoral responses (7, 40, 75). Although the antibody neutralizing capacity was not measured directly here, we did not detect any gross differences in antibody profiles in this particular animal. Of note was the observation that this animal had a significantly elevated baseline CD4 T-cell level with a rapid and massive expansion occurring during the very earliest stages of infection. The expanded available pool of target cells may have a profound

impact upon disease progression, as has been demonstrated by Klatt et al. (35), who showed that the availability of activated CD4 T cells was the principal determinant of viral load set point in SIV-infected sooty mangabeys. Alternatively, major histocompatibility complex (MHC) haplotypes may also influence susceptibility and subsequent disease progression. Indeed, recent reports have demonstrated that both the H6 and H7 haplotypes in cynomolgus macaques from the Mauritius Island are associated with control and that the H5 haplotype is associated with poor outcome (16, 48). Recent advances in the understanding of MHC organization in cynomolgus macaques of Indonesian and Philippines origin (11, 54) have indicated much more genetically diverse MHC loci in these animals compared to animals of Mauritian origin. The potential impact of MHC haplotypes in cynomolgus macaques from the Philippines (used in this study), in regards to SIV resistance/susceptibility remains less well characterized (54).

Following the initiation of the challenge phase, a subset of animals, independent of vaccination status, appeared relatively resistant to challenge, and in some instances required an escalating dose regimen to produce infection. This group of five "resistant" animals represented a fraction of inherently resistant animals similar to those seen by Letvin et al. (38). Their study was designed to address the possibility that multiple subinfectious doses may generate measurable immune responses using standard methods among other goals. In our vaccine recipients, we were limited to examining this issue by looking at the development of CTL responses against the accessory proteins, and in accordance with Letvin et al. (38), we did not see any evidence of immune responses following multiple low-dose inoculations in the absence of detectable infection. Of note, and in contrast to their report (38), we did observe evidence of peripheral immune responses elicited following multiple low-dose challenges in uninfected control animals (C87017M and C94078M). Detectable IFN- γ responses were present in two animals following 23 (14,250 TCID₅₀) and 14 (3,500 TCID₅₀) low-dose challenges, respectively, in the absence of detectable SIV infection and were directed exclusively against Gag, Pol, and Env. These preinfection responses showed no correlation with improved pathogenesis and may have been detrimental, as both animals C87017M and C94078M had higher viral load set points and more pronounced peripheral CD4 depletion than the other control animals. It is currently unclear how the impact of multiple subinfectious doses might impact on disease progression following infection or why multiple doses appear to correlate with a more robust and durable CTL response during the chronic phase of infection. Our observations following multiple subinfectious challenges may be attributable to differences between macaque species, differences in challenge virus dose, and/or both. Indeed, compared to Indian rhesus macaques, infection of cynomolgus macaques requires approximately 10-fold-higher doses (52, 63). Furthermore, following infection, cynomolgus macaques exhibit lower peak and set point viral loads and follow a disease progression which approximates more closely that seen in humans (52, 56, 63).

The mechanism by which LAVs are able to offer control with respect to decreased peak viral loads and decreased viral load set points has yet to be elucidated and has remained an intense topic of research. Immune-mediated mechanisms ranging from humoral (72) to cell-mediated mechanisms (14, 59, 73) to in-

nate factors (18, 20) have been implicated in control. Viral interference or viral competition has also been implicated in potential control (61, 62). Recent mathematical modeling has provided additional credence to this idea (71) and provides an explanation for the often noted inverse correlation associated with the degree of attenuation and subsequent protection (30). Wodarz (71) suggests that the number of target cells available for the incoming challenge virus is the principal determinant in protection. Less attenuated or "hotter" strains are able to infect more target cells, in effect, limiting important reservoirs for the challenge virus. Thus, the role of immunity in protection afforded by LAVs may be of little consequence. Our study furthers the observations of Johnson et al. (30), as better protection was afforded by the less attenuated $\Delta 5$ -CMV construct compared to the $\Delta 6$ -CCI construct. Our observations are also reminiscent of studies done by one of our group and others with highly exposed persistently HIV-seronegative sex workers (HEPS) who had multiple positive ELISPOT assays and were suspected of having undergone prior abortive HIV infection as a means of protection from subsequent exposure (42, 43).

The most significant drawback to the use of live attenuated viruses as HIV vaccines in humans are inherent safety concerns with respect to reversion (1, 58) or recombination (33) and observations of residual pathogenicity in both adult and infant macaques (4, 5, 10, 14, 27, 53). Recently, Reynolds et al. (57) examined a group of rhesus macaques following vaccination with the prototypical live attenuated SIVmac239 Δ nef and challenged with a heterologous virus (SIVsmE660). Of concern was the observation that a significant subset of animals, including two animals harboring the protective *Mamu-B*17+* allele (74), displayed evidence of recombination between the vaccine and challenge strains. This demonstrates that in addition to the possibility of viral escape by changes at the amino acid level affecting CTL epitope recognition, gross changes on a larger genome-wide scale are also possible and may contribute to loss of control in LAV-vaccinated animals. In our study, we did not observe any sign of enhanced pathogenicity in the vaccinated groups. Of note, three vaccinated animals that originally controlled viral load of <1,000 copies/ml later experienced a steady increase in viral burden and concomitant loss of peripheral CD4 T cells until the end of the study. The administration of a homologous challenge virus made it unfeasible to address the issue of recombination between the vaccine and challenge strains. However, the inability of the vaccine virus to set up a persistent infection *in vivo* would suggest that it is unlikely to recombine with challenge virus. Inherent safety concerns and risk are a population-based concern, and the Western world's concerns about safety should not impede the development of vaccine approaches for settings where the benefits of such a vaccine can justify its use. Even if an attenuated viral vaccine approach will never be rendered completely safe, it is of paramount importance that the mechanism(s) of protection afforded by this approach be pursued with renewed vigor in light of recent clinical vaccine trial failures.

In conclusion, vaccination with nearly nonreplicative SIV that we have termed "hyperattenuated SIV" demonstrated good safety and induced weak antibody and cellular immune responses when measured by conventional methods after viral infection, but these responses were the key factors associated with protective immunogenicity in the form of reduced peak

viral load and decreased set point in some animals after DNA boosting with the same constructs. Recent observations on the role of peripheral effector memory T-cell responses (26, 39) highlight the complex interplay required and suggest that the characteristics of a priming dose are of absolute importance and relate to factors other than magnitude to generate subsequent protective immune responses. As might be predicted, many variables, such as antigenic targets, magnitude and breadth of response, and location and timing of the response, all play a part in determining outcome. As we demonstrated here, these changes may be subtle and not easily detected in the periphery by standard methodologies. In this study, very discrete priming with one hyperattenuated construct set the stage for a DNA boost that was ultimately very important in directing control of pathogenesis when analyzed both descriptively and from a statistical correlation. Further optimization of these constructs in conjunction with improved techniques to carefully carve out the specific type of priming event necessary to invoke an optimal, long-lasting, peripheral effector memory response, for instance with a thorough examination of vaccine-elicited responses in the mucosa, will be important in elucidating correlates of protection from SIV challenge.

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