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Ⅲ. 研究成果の刊行物・別刷

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Human Immunodeficiency Virus Type 1 Derivative with 7% Simian Immunodeficiency Virus Genetic Content Is Able To Establish Infections in Pig-Tailed Macaques[▽]

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A human immunodeficiency virus type 1 (HIV-1) derivative ($HIV_{NL-DT5R}$) containing sequences encoding a 7-amino-acid segment of CA and the entire *vif* gene from simian immunodeficiency virus (SIV) was previously shown to establish spreading infections in cultured macaque peripheral blood mononuclear cells. To assess its replicative and disease-inducing properties in vivo, $HIV_{NL-DT5R}$ was inoculated into pig-tailed macaques. $HIV_{NL-DT5R}$ generated plasma viremia in all five of the monkeys and elicited humoral responses against all of the HIV-1 structural proteins but did not cause $CD4^+$ T-lymphocyte depletion or clinical disease. Additional adaptation will be required to optimize infectivity in vivo.

Because the host range of human immunodeficiency virus type 1 (HIV-1) is restricted to chimpanzees and humans, alternative systems such as the simian immunodeficiency virus (SIV) and simian/human immunodeficiency virus (SHIV)/macaque models have been developed and used extensively for vaccine and pathogenesis studies. However, both of these HIV-1 surrogates have shortcomings that diminish their usefulness as substitutes for HIV-1 in vivo. For example, although SIV has a genomic organization very similar to that of HIV-1, it elicits distinctive cellular and humoral immune responses that are SIV specific and exhibits sensitivities to antiretroviral drugs that are not observed for HIV-1 (26). SHIVs, which contain the HIV-1 *tat*, *rev*, *vpu*, and *env* genes inserted into the SIV genetic background, have been utilized in vaccine experiments to evaluate cellular immune responses directed against SIV Gag and humoral responses directed against the HIV-1 envelope glycoprotein (1, 2, 17, 18). The absence of the other HIV-1 genes in SHIV genomes precludes an evaluation of these virus-encoded proteins during progeny virus production or as antiviral targets in vivo.

We recently reported the construction and characterization of an HIV-1 derivative, designated $HIV\text{-}1_{NL-DT5R}$, which contains a 21-nucleotide SIV Gag CA element and the entire SIV *vif* gene inserted into the genetic background of $HIV\text{-}1_{NL4-3}$ (12). $HIV\text{-}1_{NL-DT5R}$ was able to establish spreading infections in a cynomolgus monkey T-cell line and CD8-depleted peripheral blood mononuclear cells (PBMC) from pig-tailed macaques and rhesus monkeys. Those experiments indicated that the presence of a total of 666 SIV nucleotides (6.7%) at these two specific locations within the full-length 9,894-nucleotide HIV-1 genome was sufficient to counteract innate restriction factors residing in simian cells, such as APOBEC3 and TRIM5α family members, which otherwise block HIV-1 replication (23, 24). Another recently described HIV-1 derivative (stHIV-1), which contains the entire SIV CA and Vif coding sequences, exhibited similar replication properties in macaque PBMC (6).

To ascertain whether the observed infectivity of $HIV\text{-}1_{NL-DT5R}$ for cultured macaque PBMC could be extended to virus-inoculated monkeys, an animal challenge stock was first prepared from $CD8^+$ T-cell-depleted pig-tailed macaque PBMC, infected with supernatant from 293T cells transfected with pNL-DT5R DNA (12). Virus released into the culture medium on days 8 and 9 postinfection (p.i.) was pooled, and the infectivity of the resulting $HIV\text{-}1_{NL-DT5R}$ stock was determined to be 1.9×10^5 50% tissue culture infective doses ($TCID_{50}$)/ml, as measured in human T-lymphoid MT4 cells (5). Four pig-tailed macaques were inoculated intravenously with 1.9×10^6 $TCID_{50}$ of virus. Animals were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (17a) and were housed in a biosafety level 2 facility; biosafety level 3 practices were followed. Two animals (A3P027 and A4P004) were treated with anti-human CD8 monoclonal antibody (MAb) cM-T807 on days 1 (10 mg/kg of body weight, subcutaneously), 4, and 7 (5 mg/kg, intravenously each day) p.i. to suppress the induction of early antiviral cellular immunity (21). Two monkeys (A3P017 and A3P023) were not treated with cM-T807. Virus replication was determined by measuring the levels of plasma $HIV\text{-}1_{NL-DT5R}$ RNA using real-time PCR with the following primers/probes specific for the $HIV\text{-}1_{NL4-3}$ *pol* gene: PNLPOL1 forward primer (GCAGTTCATGTAGCCAGTGGATAT at 4455 to 4478), PNLPOL1 reverse primer (TGGTGAAATTGCTGCCATTG at 4596 to 4577), and PNLPOL1 probe (CAGAGACAGGGCAA

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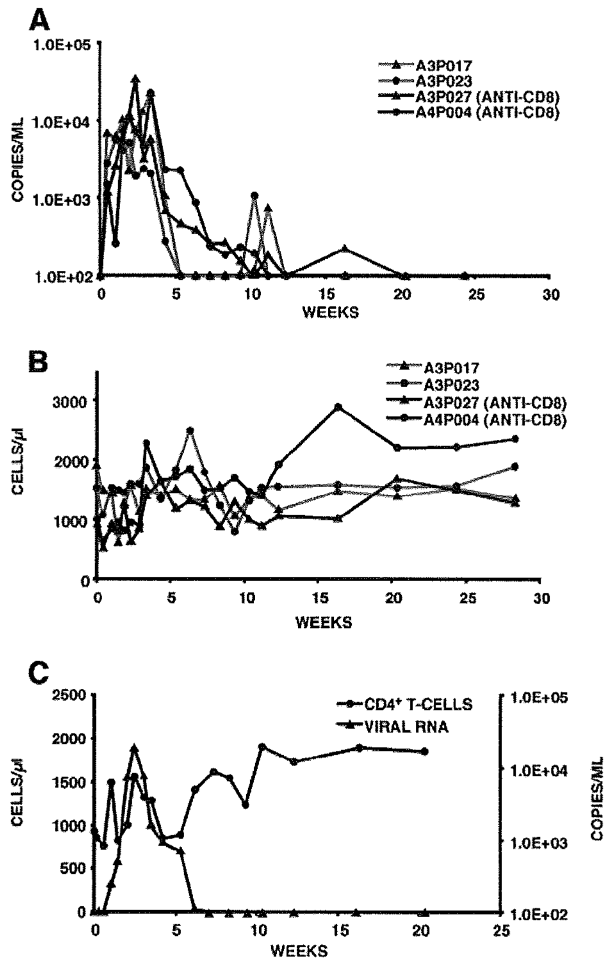

FIG. 1. Profiles of plasma viral RNA loads (A) and circulating $CD4^+$ T lymphocytes (B) in four pig-tailed macaques inoculated intravenously with 1.9×10^6 $TCID_{50}$ of HIV-$1_{NL-DT5R}$ and those of a single animal recipient of whole blood and lymph node cells prepared from the former four animals (C). The detection limit of our reverse transcription-PCR assay is 200 RNA copies/ml, and undetectable values are plotted as 100 RNA copies/ml of plasma.

GAAACAGCATACTTCC at 4501 to 4530) as previously described (3). The number of circulating $CD4^+$ T cells was monitored as a marker for virus-induced pathogenesis as described previously (3).

HIV-$1_{NL-DT5R}$ productive infections were established in all four animals with peak plasma viral loads ranging from 5.6×10^3 to 3.5×10^4 RNA copies/ml (Fig. 1A). No substantial difference was observed in the levels of peak viremia in the untreated and anti-CD8 MAb-treated monkeys. Plasma viral loads declined rapidly in the two untreated macaques and became undetectable by week 5 p.i. Viremia in the two animals treated with the cM-T807 MAb was maintained until weeks 10 to 11 at which point it fell below the limits of detection (200 viral RNA copies/ml). The prolonged viremia in the anti-CD8 MAb-treated macaques did not appear to reflect protracted suppression of $CD8^+$ T lymphocytes, since they returned to preinfection levels by week 2 postinoculation (data not shown). Although all four HIV-$1_{NL-DT5R}$-infected monkeys experienced modest declines in the numbers of circulating $CD4^+$ T lymphocytes during the initial weeks of the acute infection, presumably due to trafficking of T cells into lymphoid tissues, their levels rapidly returned to preinoculation values by week 5 p.i. (Fig. 1B). No evidence of clinical disease was observed in any of the virus-inoculated macaques during the first 6 months of their HIV-$1_{NL-DT5R}$ infections.

Extensive passaging of primate lentiviruses with impaired infectivities, both in vitro or in vivo, has resulted in the acquisition of genetic changes conferring augmented replicative properties (4, 8, 11, 13, 19, 22, 25). As an initial step in such a process, a starting virus inoculum was prepared by collecting lymph node and peripheral blood samples from each of the four HIV-$1_{NL-DT5R}$-infected monkeys at week 5 p.i. Lymph node cells (7.5×10^7 cells) were suspended in 20 ml of pooled whole blood, and the mixture was inoculated intravenously into another pig-tailed macaque (A3P024). This animal was treated with the anti-CD8 MAb on days 1, 4, and 7 at the same doses and routes as two of the monkeys in the initial infection. The plasma viral RNA levels in the recipient macaque peaked (1.9×10^4 RNA copies/ml) at week 2.4 p.i. and then rapidly declined, becoming undetectable at week 6 p.i. (Fig. 1C). The numbers of circulating $CD4^+$ T lymphocytes did not change appreciably during the initial 20 weeks of infection, and macaque A3P024 has thus far remained asymptomatic.

The infected monkeys responded to HIV-$1_{NL-DT5R}$ challenge by producing virus-specific antibodies as measured by immunoblotting of plasma samples collected during the initial 24 weeks of infection (Fig. 2). Commercially available diagnostic HIV-1 Western blotting strips (Cambridge Biotech HIV-1 Western blot kit; Maxim Biomedical Inc., Rockville, MD) were employed, and a plasma sample from an HIV-1-infected individual served as a positive control. The use of a different production lot of blotting strips resulted in the variability observed with samples from monkey A3P017 (Fig. 2). All the $HIV_{NL-DT5R}$-infected animals, regardless of anti-CD8 treatment, produced antibodies directed against HIV-1-encoded p17, p24, gp41, gp120, and gp160 (anti-gp160 as early as week 2 p.i. in macaque A4P004), and four of the five animals (A3P017, A3P027, A4P004, and A3P024) generated antibody against the HIV-1 p66 reverse transcriptase. In all five animals, the band intensities for each viral protein were maintained or increased over time, suggesting sustained virus replication, even after plasma viral RNA loads fell below the level of detection (Fig. 1). The weaker reactivity of plasma samples from monkey A3P023 was consistent with the lower values obtained with a commercially available enzyme-linked immunosorbent assay kit (Vironostika HIV-1 Microelisa system; bioMerieux Inc., Durham, NC) (data not shown).

To ascertain whether $HIV_{NL-DT5R}$ had established persistent infections in the animals, PBMC-associated viral DNA levels were measured at 45 weeks p.i. for pig-tailed macaques A3P017, A3P023, A3P027, and A4P004, and at 38 weeks p.i. for A3P024, since proviral DNA in PBMC can be detected even after plasma viral RNA loads fall below the limits of detection in animals effectively controlling virus replication (18). The same primer/probe pair and amplification conditions used to measure plasma viral RNA were employed for the detection of proviral DNA. Low levels of PBMC-associated viral DNA were detected in samples from all five animals (0.36

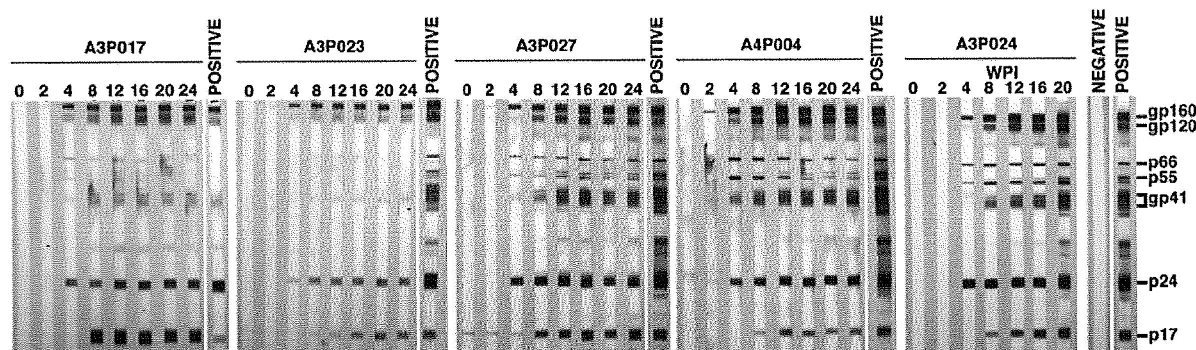

FIG. 2. Profiles of anti-HIV-1 antibody responses in pig-tailed macaques infected with HIV-$1_{NL-DT5R}$. Commercially available diagnostic HIV-1 Western blotting strips (Cambridge Biotech Western blot kit) were employed using a 1:68 dilution of pig-tailed macaque plasma samples. Normal human plasma was used as a negative control. Plasma from an HIV-1-infected individual was used as a positive control. The positions of HIV-1 proteins are indicated to the right of the blots. WPI, weeks postinfection.

copy/10^5 cells for A3P017, 0.14 copy for A3P023, 0.76 copy for A3P027, 0.70 copy for A4P004, and 0.21 copy for A3P024; the detection limit for this assay is 0.1 copy/10^5 cells). The proviral DNA loads measured in two of the anti-CD8-treated animals (A3P027 and A4P004) were somewhat higher than those in the untreated macaques (A3P017 and A3P023) and correlated with prolonged detection of plasma viremia in these monkeys. Taken together with the steady/increasing antibody responses and the presence of proviral DNA, these results indicate that $HIV_{NL-DT5R}$ is able to establish low-level persistent infections in pig-tailed macaques.

The results described in this report reinforce conclusions initially observed in transfection and single-cycle infectivity assays, which demonstrated that blocks to virus replication imposed by macaque TRIM5α and APOBEC3 cytidine deaminases markedly inhibited HIV-1 replication in simian cells (16, 24). We also reported that the restriction to the establishment of HIV-1 spreading infections in cultured monkey PBMC could be counteracted by HIV-$1_{NL-DT5R}$, which carries a short SIV Gag element and the entire SIV *vif* gene, but not by wild-type HIV-1 (12). The results from the present study now extend the previous observations to the organismal level and show that HIV-$1_{NL-DT5R}$ can replicate in vivo.

Although HIV-$1_{NL-DT5R}$ was able to establish infections in pig-tailed macaques, its replicative properties are reminiscent of first-generation SHIVs (10, 14, 15, 20). The latter generated modest levels of peak viremia during acute infections of macaque monkeys that were rapidly and durably suppressed and failed to induce any disease. Serial passaging of these SHIVs or the administration of $CD8^+$-T-cell-depleting MAbs during the primary virus infection resulted in the emergence of pathogenic virus, which replicated to high titers in infected animals and induced the systemic depletion of $CD4^+$ T lymphocytes and development of immunodeficiency (9, 11, 19). In this regard, it has also been reported that passaging of minimally cytopathic SIV_{MneCl8} (13) or even the pathogenic SIV_{B670} (8) gave rise to SIV variants exhibiting more robust replication phenotypes and augmented pathogenic properties. One could envisage using a similar approach for generating more fit HIV-$1_{NL-DT5R}$ variants for infections of macaque monkeys, including some carrying CCR5-utilizing *env* genes. These new derivatives could be used for analyses of cell-mediated immune responses directed against Gag and Pol proteins or to assess patterns of antiviral drug resistance against HIV-1-encoded proteins, not presently possible with SIV or currently available SHIVs.

Although it might be considered a step backward, one could argue that the direct substitution of additional SIV-specific sequences into the genetic backbone of HIV-$1_{NL-DT5R}$ might markedly improve its infectivity in monkeys. For example, the SIV long terminal repeat contains one (not two) NF-κB binding site, four (not three) SP1 binding sites, and unique PuB2, SF1-3, and peri-xB binding sites relative to the HIV-1 long terminal repeat (7). Similarly, the significantly larger SIV *nef* gene and the presence of both *vpr* and *vpx* genes in the SIV genome (rather than the single *vpr* gene in HIV-1) would suggest that the acquisition of nonhuman primate species-specific *cis*-acting elements and coding sequences may optimize virus infectivity in vivo. Both direct replacement and serial passaging strategies are being used to obtain HIV-$1_{NL-DT5R}$ variants with improved replicative potential in monkeys.

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Long-Term Control of Simian Immunodeficiency Virus Replication with Central Memory $CD4^+$ T-Cell Preservation after Nonsterile Protection by a Cytotoxic T-Lymphocyte-Based Vaccine[▽]

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Induction of virus-specific $CD8^+$ cytotoxic T-lymphocyte (CTL) responses is a promising strategy for AIDS vaccine development. However, it has remained unclear if or how long-term viral containment and disease control are attainable by CTL-based nonsterile protection. Here, we present three rhesus macaques that successfully maintained Env-independent vaccine-based control of simian immunodeficiency virus (SIV) mac239 replication without disease progression for more than 3 years. SIV-specific neutralizing antibody induction was inefficient in these controllers. Vaccine-induced Gag-specific CTLs were crucial for the chronic as well as the primary viral control in one of them, whereas those Gag-specific CTL responses became undetectable and CTLs specific for SIV antigens other than Gag, instead, became predominant in the chronic phase in the other two controllers. A transient $CD8^+$ cell depletion experiment 3 years postinfection resulted in transient reappearance of plasma viremia in these two animals, suggesting involvement of the SIV non-Gag-specific CTLs in the chronic SIV control. This sustained, neutralizing antibody-independent viral control was accompanied with preservation of central memory $CD4^+$ T cells in the chronic phase. Our results suggest that prophylactic CTL vaccine-based nonsterile protection can result in long-term viral containment by adapted CTL responses for AIDS prevention.

Human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections induce acute, massive depletion of $CCR5^+$ $CD4^+$ effector memory T cells from mucosal effector sites. This is followed by chronic immune activation with gradual immune disruption leading to AIDS (7, 15, 20, 25, 26, 33, 34). Acute depletion has an impact on disease course but does not dictate everything that happens in the chronic phase (7, 26). It has also been suggested that persistent viral replication-associated chronic immune activation may be critical for AIDS progression.

Virus-specific $CD8^+$ cytotoxic T-lymphocyte (CTL) responses are crucial for control of HIV and SIV replication (3, 8, 12, 18, 24, 29). Several vaccine regimens eliciting virus-specific CTL responses have been developed and evaluated in macaque AIDS models (6, 21). Some of them have shown protective efficacies leading to viremia control in a model of X4-tropic simian-human immunodeficiency virus (SHIV) infections (1, 16, 22, 23, 28, 31). However, assessment of the ability of vaccines to ameliorate disease progression requires analysis in macaque models of R5-tropic SIV infection (5). Although most CTL-based vaccine trials using rigorous SIV challenges in Indian rhesus macaques have failed, some of them have shown amelioration of acute memory $CD4^+$ T-cell depletion in the vaccinated animals with reduction in viral loads out to a year postinfection (4, 13, 19, 35). These findings have suggested that there may be a clinical benefit conferred by CTL-based AIDS vaccines. Unfortunately, it is still unclear as to how nonsterile protection conferred by prophylactic CTL-based vaccines can result in long-term viral containment and disease control.

We have previously developed a CTL-eliciting AIDS vaccine regimen using a DNA-prime/Gag-expressing Sendai virus (SeV-Gag) vector-boost (16, 32). Our regimen does not utilize Env immunogen that may induce neutralizing antibodies, although this antigen has been used in most of the vaccines except for a few cases (16, 31, 35). We have evaluated efficacy of this Env-independent vaccine against SIVmac239 challenge in Burmese rhesus macaques and found neutralizing antibody-independent, CTL-based control of primary SIV replication in five of eight vaccinees (17). In the present study, we have followed these macaques to examine if long-term viral containment without disease progression is possible by prophylactic CTL-based AIDS vaccines.

MATERIALS AND METHODS

Animal experiments. Twelve Burmese rhesus macaques (*Macaca mulatta*) used in our previous SIVmac239 challenge experiment (17) were followed in the

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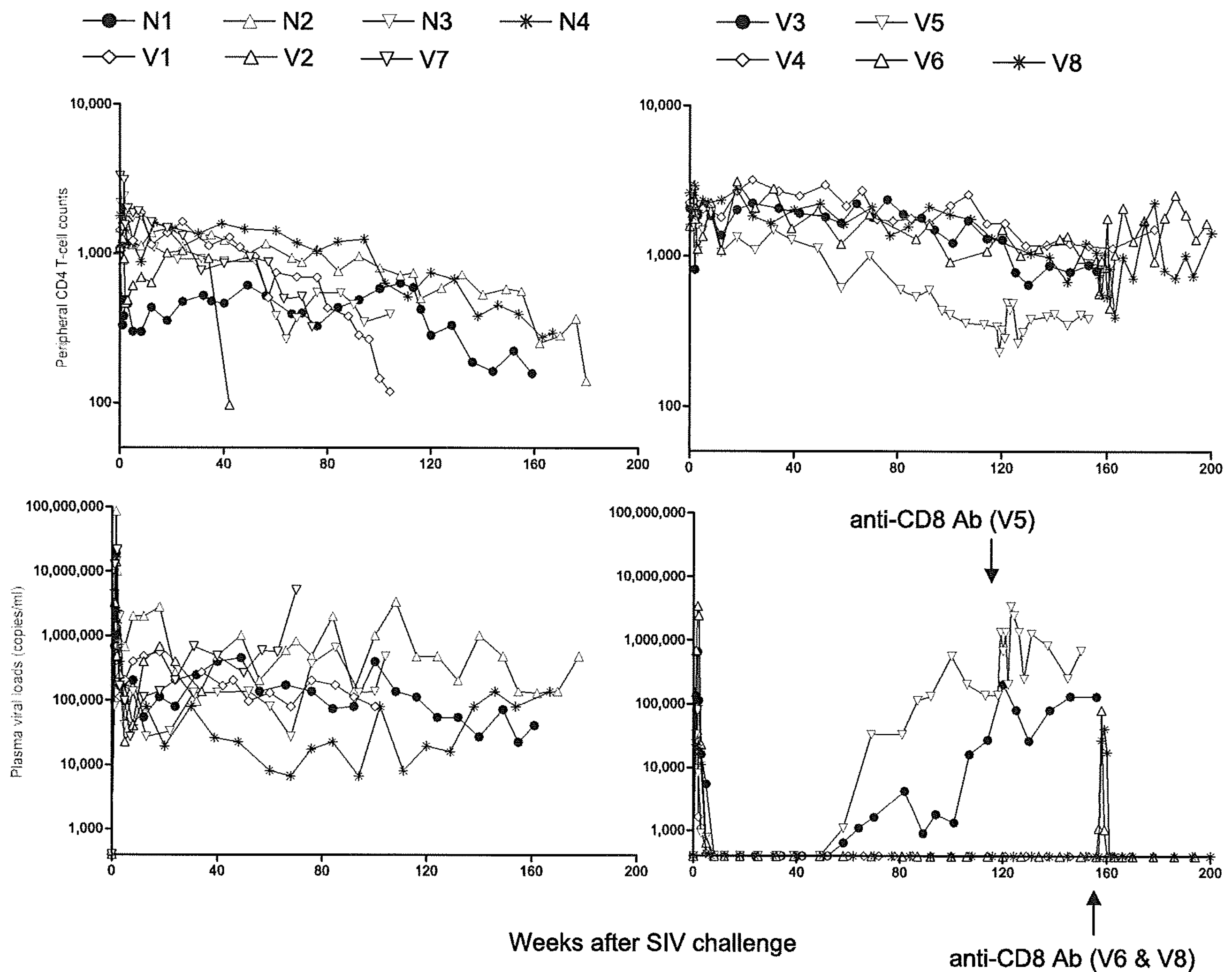

FIG. 1. Follow-up of the macaques after SIVmac239 challenge. Upper panels, peripheral $CD4^+$ T-cell counts (cells/μl); lower panels, plasma viral loads (viral RNA copies/ml plasma); left panels, the seven noncontrollers; right panels, the five controllers. All seven noncontrollers developed AIDS and were euthanized during the observation period (Table 1). Macaques V5, V6, and V8 received anti-CD8 antibody treatment starting from week 118, week 156, and week 156, respectively.

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present study. These macaques were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases and the National Institute of Biomedical Innovation. Four of them were naive, whereas the other eight macaques received a DNA vaccine followed by a single boost with SeV-Gag before an intravenous SIVmac239 challenge. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA (30) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV-1_{DH12} chimeric Vpr, and HIV-1_{DH12} Tat and Rev as described previously (17). At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals intranasally received a single boost with 1×10^8 cell infectious units of replication-competent SeV-Gag (V1, V2, V3, and V4) or 6×10^9 cell infectious units of F-deleted replication-defective F(-)SeV-Gag (9, 14, 32). Approximately 3 months after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (11).

For $CD8^+$ cell depletion, animals received a single intramuscular inoculation of 10 mg/kg of body weight of monoclonal anti-CD8 antibody (cM-T807) provided by Centocor (Malvern, PA) followed by three intravenous inoculations of 5 mg/kg cM-T807 on days 3, 7, and 10 after the first inoculation. The anti-CD8 antibody administration started at week 118 in macaque V5 and at week 156 in macaques V6 and V8. $CD8^+$ T-cell depletion in peripheral blood was confirmed by immunostaining using fluorescein isothiocyanate-conjugated anti-human CD8 antibody (DK25; Dako, Kyoto, Japan).

All the noncontrollers were euthanized when they showed typical signs of AIDS, such as reduction in peripheral $CD4^+$ T-cell counts, loss of body weight, diarrhea, and general weakness. Autopsy revealed lymphoatrophy or post-persistent generalized lymphadenopathy conditions consistent with AIDS.

Quantitation of plasma viral loads. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). Serial fivefold dilutions of RNA samples were amplified in quadruplicate by reverse transcription and nested PCR using SIV *gag*-specific primers to determine the endpoint. Plasma SIV RNA levels were calculated according to the Reed-Muench method as described previously (17). The lower limit of detection is approximately 4×10^2 copies/ml.

Measurement of virus-specific neutralizing titers. Serial twofold dilutions of heat-inactivated plasma were prepared in duplicate and mixed with 10 $TCID_{50}$ of SIVmac239. In each mixture, 5 μl of diluted plasma was incubated with 5 μl of virus. After a 45-min incubation at room temperature, each 10-μl mixture was added to 5×10^4 MT4 cells in a well of a 96-well plate. After 12 days of culture, supernatants were harvested. Progeny virus production in the supernatants was examined by enzyme-linked immunosorbent assay for detection of SIV p27 core antigen (Beckman-Coulter, Tokyo, Japan) to determine the 100% neutralizing endpoint. The lower limit of detection is a titer of 1:2.

TABLE 1. Summary of responses in macaques challenged with SIVmac239

Macaque group and no.	MHC-I haplotype[a]	VL		Status[c]	CD4 count[d] at euthanasia	Opportunistic infection at autopsy[e]
		Set point[b]	After wk 60			
Unvaccinated noncontrollers						
N1	*90-088-Ij*	$>10^4$	$>10^4$	Euthanized at wk 161	158	
N2	*90-120-Ia*	$>10^4$	$>10^4$	Euthanized at wk 180	141	PCP
N3	*90-122-Ie*	$>10^4$	$>10^4$	Euthanized at wk 104	393	
N4	*90-010-Id*	$>10^4$	$>10^4$	Euthanized at wk 167	296	CMV
Vaccinated noncontrollers						
V1	*90-088-Ij*	$>10^4$	$>10^4$	Euthanized at wk 105	119	
V2	*90-120-Ib*	$>10^4$		Euthanized at wk 42	97	PCP
V7	*90-122-Ie*	$>10^4$	$>10^4$	Euthanized at wk 77	323	
Vaccinated transient controllers						
V3	*90-120-Ia*	<400	$>10^3$	Alive >3 yr		
V5	*90-120-Ia*	<400	$>10^4$	Euthanized at wk 154*	384	
Vaccinated sustained controllers						
V4	*90-120-Ia*	<400	<400	Alive >3 yr		
V6	*90-122-Ie*	<400	<400	Alive >3 yr*		
V8	*90-010-Id*	<400	<400	Alive >3 yr*		

[a] MHC-I haplotype was determined by reference strand-mediated conformation analysis as described previously (2, 17). MHC class I haplotypes *90-120-Ia* and *90-120-Ib* are derived from breeder R-90-120, *90-122-Ie* is from R-90-122, *90-010-Id* is from R-90-010, and *90-088-Ij* is from R-90-088.

[b] Plasma viral load (VL, in RNA copies/ml plasma) around week 12.

[c] All seven noncontrollers exhibited reduction in peripheral CD4 T-cell count, loss of body weight, and general weakness and were euthanized and subjected to autopsy to be confirmed as AIDS. Macaques V5, V6, and V8 (indicated by asterisks) were administered an anti-CD8 antibody for CD8 cell depletion at weeks 118, 156, and 156, respectively.

[d] Peripheral CD4 T-cell counts.

[e] PCP, pneumocystis pneumonia; CMV, cytomegalovirus infection.

Measurement of virus-specific CTL responses. We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (17). In brief, peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIVGP1 for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a VSV-G expression plasmid and the SIVGP1 DNA, an *env*- and *nef*-deleted SHIV molecular clone DNA. Intracellular IFN-γ staining was performed using a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan). Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ^+ T-cell frequencies from those after Gag-specific or SIV-specific stimulation. Specific T-cell levels less than 100 cells per million PBMCs are considered negative.

Immunostaining of $CD4^+$ T-cell memory subsets. Frozen stocks of PBMCs were thawed and subjected to immunofluorescent staining by using fluorescein isothiocyanate-conjugated anti-human CD28, phycoerythrin-conjugated anti-human CD95, peridinin chlorophyll-conjugated anti-human CD4, and allophycocyanin-conjugated anti-human CD3 monoclonal antibodies (Becton Dickinson). Memory and central memory subsets of $CD4^+$ T cells were delineated by $CD95^+$ and $CD28^+$ $CD95^+$ phenotypes, respectively, as described previously (27).

Statistical analysis. Central memory $CD4^+$ T-cell counts just before SIV challenge (at week zero) were not significantly different between the noncontrollers ($n = 7$) and the controllers ($n = 5$) by unpaired t test. We calculated ratios of the counts at week 12 to week 0, week 70 to week 0, and week 70 to week 12 in each animal and performed an unpaired t test and nonparametric Mann-Whitney U-test between the noncontrollers and the controllers by using Prism software version 4.03 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Long-term viral containment without disease progression in the sustained controllers. We followed up on our vaccinated Burmese rhesus macaques used in the previous trial (17). These macaques were vaccinated using a DNA prime–SeV-Gag boost, and they were challenged with SIVmac239. Five of eight vaccinees controlled viral replication and had undetectable plasma viremia at week 8 postchallenge. The remaining three vaccinees (V1, V2, and V7) and all four unvaccinated macaques (N1, N2, N3, and N4) failed to control viral replication. Of the five controllers, two macaques V3 and V5 (referred to as transient controllers) exhibited viremia reappearance around week 60, but the other three, V4, V6, and V8 (referred to as sustained controllers), maintained viral control (10).

In the present follow-up study, all seven noncontrollers, including three vaccinees and four unvaccinated controls, exhibited persistent viremia and a gradual decline in peripheral $CD4^+$ T-cell counts (Fig. 1). All of them finally developed AIDS and were euthanized at week 42 to 180 postchallenge (Table 1), confirming that failure in control of SIVmac239 replication results in AIDS progression even in Burmese rhesus macaques. In contrast, all three sustained controllers maintained viral control and preserved peripheral $CD4^+$ T cells without disease progression for more than 3 years (Fig. 1).

We then examined SIVmac239-specific neutralizing antibody responses by determining the end point plasma titers for killing 10-$TCID_{50}$ virus replication on MT4 cells (Fig. 2). Our vaccine regimens did not utilize Env as an immunogen, and no neutralizing antibody responses were induced before challenge in any of the vaccinees. Even after challenge, none of the SIVmac239-challenged macaques showed detectable neutralizing antibody responses until 6 months. After that, neutralizing antibody responses became detectable in some of the noncontrollers. In contrast, no or little neutralizing antibody

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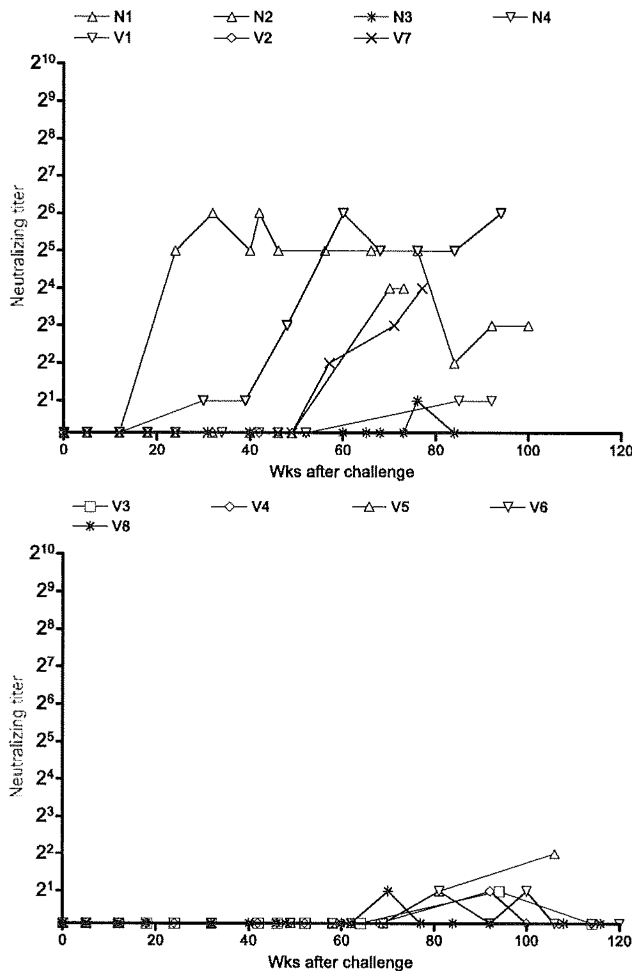

FIG. 2. SIVmac239-specific neutralizing antibody levels in plasma. Plasma titers for killing 10-$TCID_{50}$ SIVmac239 replication in the noncontrollers (top panel), including unvaccinated control animals, and in the controllers (bottom panel) are shown.

responses were induced in the controllers, even in the chronic phase.

Shift of antigens targeted by CTLs during the period of viral control. CTLs from all five controllers selected Gag CTL escape mutations soon after infection, indicating that vaccine-induced Gag-specific CTL responses were crucial for viral control in the early phase of SIV infection (17). In one sustained controller, macaque V4, possessing major histocompatibility complex class I haplotype *90-120-Ia*, $Gag_{206-216}$ (IINEEAAD WDL) epitope-specific CTLs and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses likely played a central role in control of viral replication in the chronic phase (10). We also analyzed virus-specific CTL responses in the remaining two sustained controllers, V6 and V8, to determine if vaccine-induced Gag-specific CTL responses played a role in control of viral replication in the chronic phase.

We measured Gag-specific and SIV-specific CTL frequencies in macaques V6 and V8 (Fig. 3). In both macaques, Gag-specific CTL frequencies were high around 2 months postchallenge but then decreased to below detection levels around 1 year postchallenge. In contrast, SIV-specific CTL responses against epitopes in other SIV proteins were still detectable 3 years postchallenge. These results suggest that the vaccine-induced Gag-specific CTL responses were diminished soon after challenge and that there was then a predominance of CTLs specific for SIV-derived antigens other than Gag in the chronic phase in both of the sustained controllers, V6 and V8.

Viremia reappearance by $CD8^+$ cell depletion in the sustained controllers. In the sustained controllers, V6 and V8, vaccine-induced Gag-specific CTLs involved in viremia control in the early phase became undetectable after approximately 6 months. CTLs specific for SIV-derived antigens other than Gag (referred to as SIV non-Gag-specific CTLs) were elicited or expanded after challenge, and these became predominant in the chronic phase. We then performed $CD8^+$ cell depletion experiments to examine if these SIV non-Gag-specific CTL responses played a role in the maintenance of viremia control in the chronic phase. Administration of the monoclonal anti-CD8 antibody, cM-T807, to macaques V6 and V8 at week 156 postchallenge resulted in transient depletion of peripheral $CD8^+$ T lymphocytes (Fig. 4A). In both macaques, plasma viremia reemerged in 1 or 2 weeks after the initial anti-CD8 antibody treatment and disappeared simultaneously with recovery of peripheral $CD8^+$ T lymphocytes in both of them (Fig. 4B). These results support the notion that, in the sustained controllers V6 and V8, these SIV non-Gag-specific CTL responses, rather than vaccine-induced Gag-specific CTL, played a crucial role in the control of SIV replication in the chronic phase. Analysis of the returning wave of virus-specific CTL responses revealed a predominance of SIV non-Gag-specific CTLs (Fig. 4C).

We also administered the anti-CD8 antibody to macaque V5, a transient controller, at week 118. In this macaque, accumulation of multiple Gag CTL escape mutations resulted in reappearance of plasma viremia around week 60. Transient $CD8^+$ cell depletion by the anti-CD8 antibody treatment resulted in a 1-log increase in plasma viral loads (Fig. 1), suggesting that CTLs still exerted pressure on the replication of the escaped viruses at week 118 in this animal.

Long-term central memory $CD4^+$ T-cell preservation in the sustained controllers. It has recently been suggested that vaccine-based transient control of viral replication can ameliorate central memory $CD4^+$ T-cell loss in the early phase of SIV infections. However, it is unclear if CTL-based sustained control of viral replication can contribute to memory $CD4^+$ T-cell preservation in the chronic phase. We, therefore, compared peripheral memory $CD4^+$ T-cell counts at several time points, prechallenge and around weeks 2, 12, 70, and 120 postchallenge, in the noncontrollers and the controllers (Fig. 5). All the noncontrollers showed significant but partial recovery of peripheral memory $CD4^+$ T-cell counts around week 12 after transient loss during the acute phase. However, memory $CD4^+$ T-cell counts, especially central memory $CD4^+$ T-cell counts at week 12, were lower than prechallenge levels in the noncontrollers. By contrast, such a reduction was not observed in the controllers, suggesting protection from acute memory $CD4^+$ T-cell depletion.

A continuous reduction in memory $CD4^+$ T-cell counts was observed in the noncontrollers. The controllers, however, showed no such reduction in memory $CD4^+$ T-cell counts out

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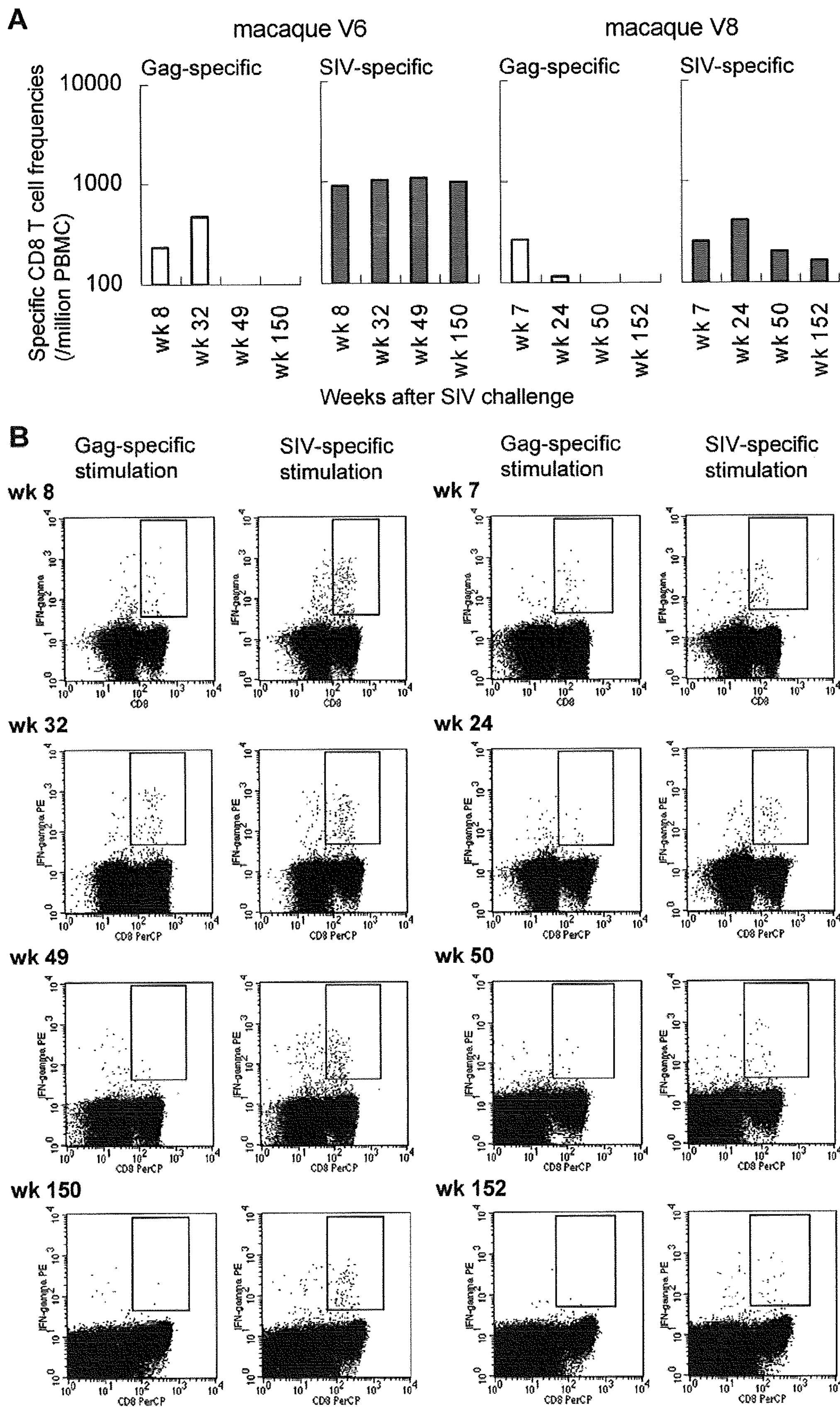

FIG. 3. Virus-specific $CD8^+$ T-cell responses in sustained controllers V6 (left panels) and V8 (right panels). (A) Gag-specific and SIV-specific $CD8^+$ T-cell frequencies in PBMCs. (B) Dot plots gated on $CD3^+$ lymphocytes after Gag-specific or SIV-specific stimulation.

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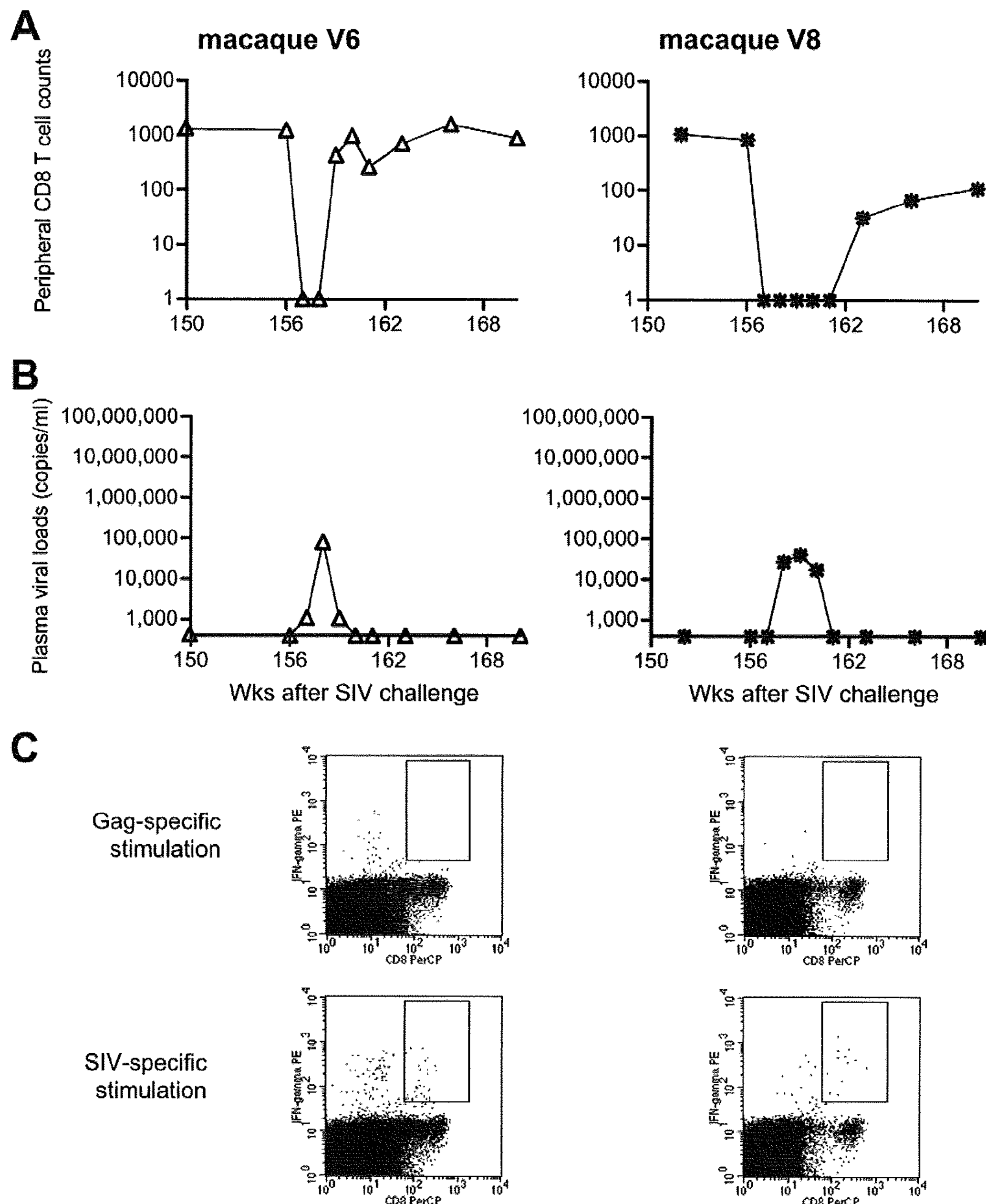

FIG. 4. CD8$^+$ cell depletion experiments starting at week 156 in sustained controllers V6 (left panels) and V8 (right panels). (A) Peripheral CD8$^+$ T-cell counts (per μl). (B) Plasma viral loads (viral RNA copies/ml plasma). (C) Virus-specific CTL responses at week 160 in V6 and at week 166 in V8. Dot plots gated on CD3$^+$ lymphocytes after Gag-specific or SIV-specific stimulation are shown.

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to week 70. At approximately week 120, all the sustained controllers still showed preservation of memory and central memory CD4$^+$ T cells. In contrast, both of the transient controllers, V3 and V5, experienced a reduction in central memory CD4$^+$ T-cell counts, although reduction in memory CD4$^+$ T-cell counts was observed in only one of them. These results suggest that CTL-based vaccines that control viral replication can also preserve central memory CD4$^+$ T cells even in the chronic phase. Finally, statistical analysis revealed that there was no significant reduction in central memory CD4$^+$ T cells during the period between weeks 12 and 70 in the controllers (Fig. 6). Thus, CTL vaccine-based, sustained viral control can result in preservation of central memory CD4$^+$ T cells in both the chronic phase as well as the acute phase.

DISCUSSION

Here we followed three Burmese rhesus macaques that maintained CTL vaccine-based control of SIVmac239 replication without disease progression for more than 3 years. The

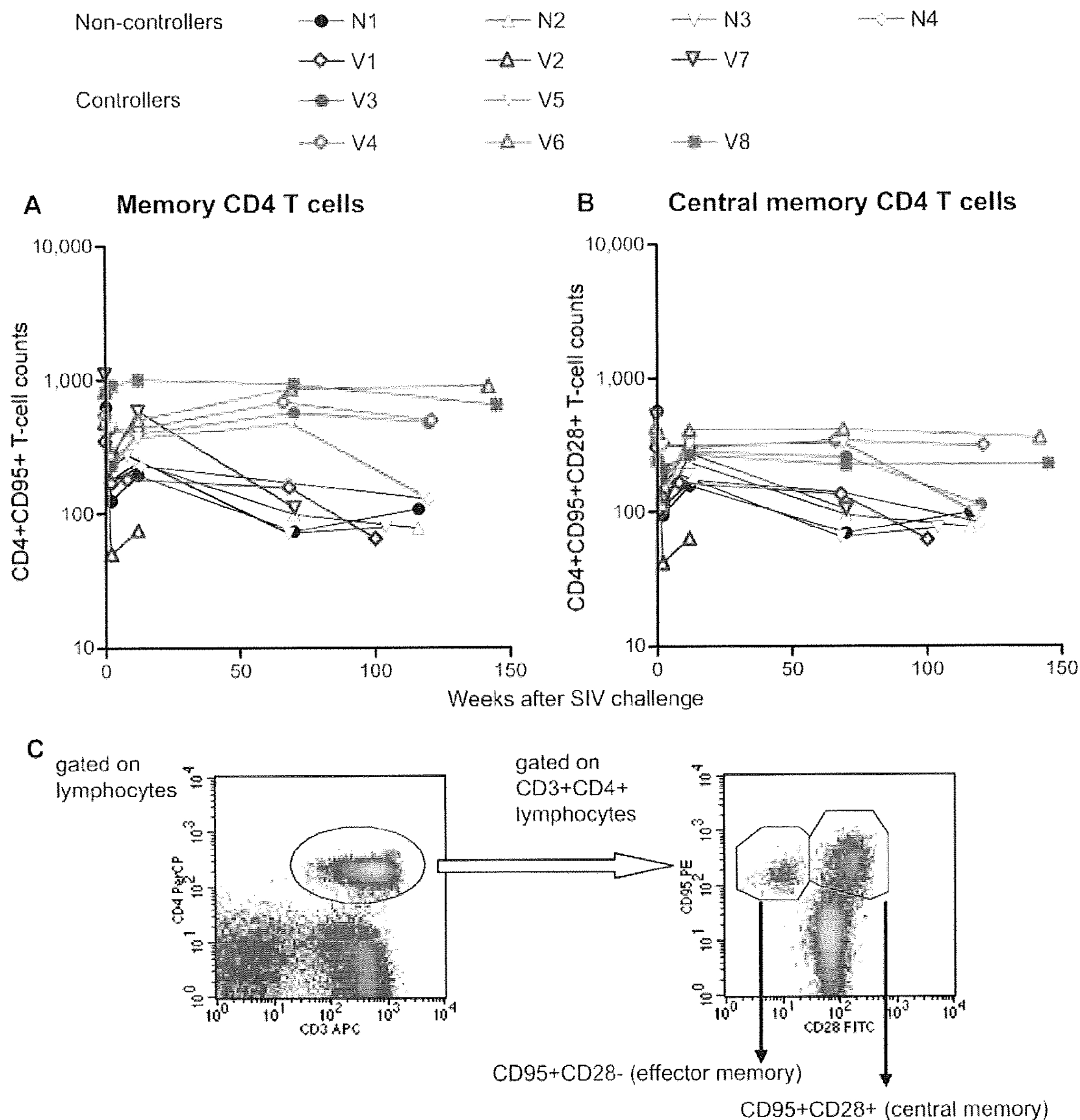

FIG. 5. Changes in peripheral memory $CD4^+$ T-cell counts. Noncontrollers are indicated in black or blue, and controllers are indicated in red. (A) Peripheral memory $CD4^+$ ($CD4^+$ $CD95^+$) T-cell counts (per μl). (B) Peripheral central memory $CD4^+$ ($CD4^+$ $CD95^+$ $CD28^+$) T-cell counts (per μl). (C) Representative density plots (macaque V4 prechallenge) for determining peripheral memory $CD4^+$ T-cell percentages. The left panel is a density plot gated on lymphocytes, and in this plot, $CD3^+$ $CD4^+$ lymphocytes are gated for the right panel of the density plot. In the right panel, we determined the percentages of central memory ($CD95^+$ $CD28^+$) $CD4^+$ T cells and memory ($CD95^+$ $CD28^+$ plus $CD95^+$ $CD28^-$) $CD4^+$ T cells.

set-point plasma viral loads in SIVmac239-infected Burmese rhesus macaques may be lower than those usually observed in SIVmac239-infected Indian rhesus but are higher than those typically observed in untreated humans infected with HIV-1. All four of the naive control animals along with three vaccinees failed to control viremia after SIVmac239 challenge. They also experienced peripheral $CD4^+$ T-cell loss and developed AIDS in 3 years, indicating that this model of SIVmac239 infection in Burmese rhesus macaques is adequate for evaluation of vaccine efficacies. Our finding of long-term control of viral replication and $CD4^+$ T-cell preservation in three vaccinees in this AIDS model underlines the potential of a prophylactic CTL-based vaccine for AIDS prevention.

Our previous study revealed rapid selection of Gag CTL escape mutations in all the controllers, indicating that vaccine-induced Gag-specific CTL responses played an important role in viral control in the early phase of SIV infection (17). In the chronic phase, neutralizing antibody induction was still inefficient, and our results suggest long-term CTL-based viral containment. Indeed, the vaccine-induced Gag-specific CTL responses have been shown to play a crucial role in viral control even in the chronic phase in one (V4) of three sustained

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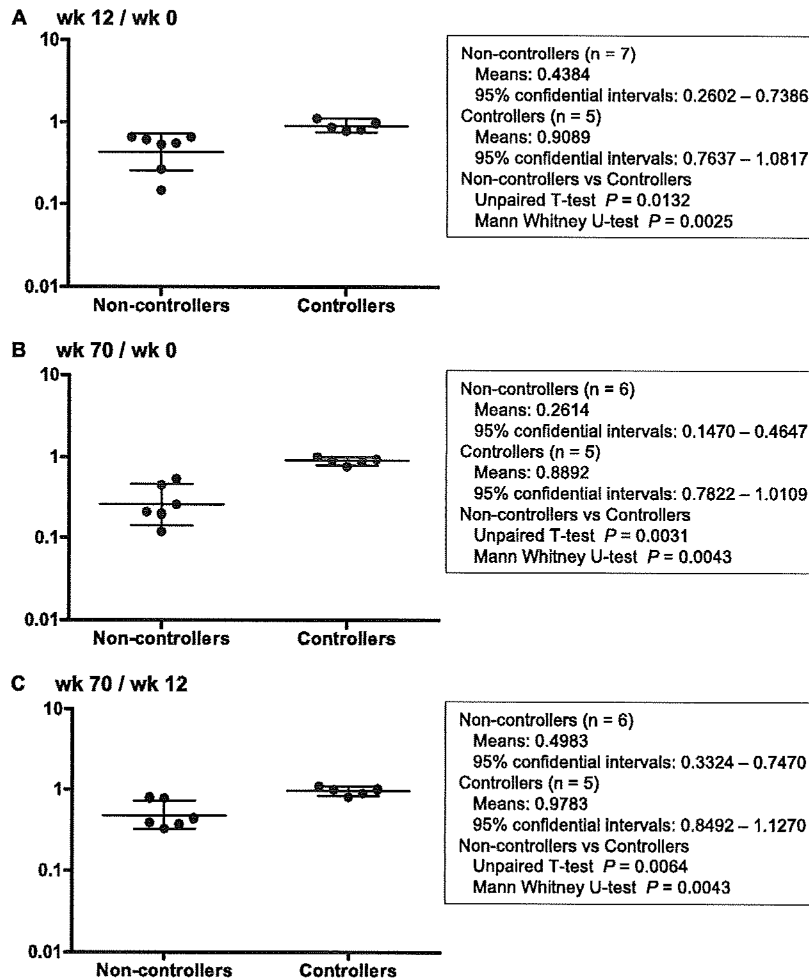

FIG. 6. Statistical analysis indicating preservation of central memory $CD4^+$ T-cell counts in the controllers. The ratios of central memory $CD4^+$ T-cell counts at week 12 to week 0 (A), week 70 to week 0 (B), and week 70 to week 12 (C) in the noncontrollers (except for rapid progressor V2 in panels B and C) and the controllers are plotted. The longer bars indicate geometric mean values, and the regions between the shorter bars indicate the 95% confidential intervals. Statistical analysis was performed with the *t* test and nonparametric Mann-Whitney U-test using the Prizm software.

controllers (10). In contrast, Gag-specific CTL responses became undetectable and SIV non-Gag-specific CTL responses, instead, became predominant in macaques V6 and V8. The results obtained from a $CD8^+$ cell depletion experiment are consistent with involvement of these SIV non-Gag-specific CTL responses in the long-term viral control in both sustained controllers, although there might be involvement of other components, such as NK and $CD4^+$ memory T cells. Thus, it can be speculated that vaccine-based control of primary SIV replication can preserve the ability of the immune system to elicit functional CTL responses, leading to reinforcement or adaptation of protective immunity by postchallenge induction or expansion of effective CTL responses. This may contribute to stable viral containment in the chronic phase.

In the natural courses of HIV and SIV infections, the infected hosts exhibit acute, massive depletion of $CCR5^+$ $CD4^+$ effector memory T cells from mucosal effector sites, and the chronic immune activation with gradual immune disruption that follows leads to AIDS (7, 15, 20, 25). The former acute memory loss may influence the latter chronic disease progression (25, 26). The acute depletion results in compromised immune responses at the effector sites and systemic proliferative responses that partially compensate for the loss of mucosal memory $CD4^+$ T-cell populations. Recent reports indicating amelioration of acute mucosal memory $CD4^+$ T-cell depletion and associated central memory $CD4^+$ T-cell loss in the early phase by CTL-based vaccines have suggested that vaccine-based amelioration of acute memory $CD4^+$ T-cell depletion in mucosal effector sites can delay AIDS progression (13, 19, 35). However, this acute memory $CD4^+$ T-cell depletion is not the only cause of chronic disease progression and persistent viral replication-associated immune activation may be responsible for chronic immune disruption leading to AIDS (7). Indeed, in both of the transient controllers, V3 and V5, central memory $CD4^+$ T cells were preserved during the initial, transient period of viremia control but decreased after the reappearance of plasma viremia. This suggests that there may be an association between persistent viral con-

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tainment and central memory CD4$^+$ T-cell preservation, even in the chronic phase.

Theoretically, protection by CTL-based AIDS vaccines is likely to be nonsterile, and it will be difficult to contain viral replication completely. Additionally, CTL-based viremia control would require CTL activation. Indeed, our CD8$^+$ cell depletion experiment indicated that persistent viral replication was inefficient but not completely contained in the absence of plasma viremia in sustained controllers V6 and V8. Transition of recognition of CTL epitopes from Gag to other non-Gag proteins in the chronic phase suggests that these "new" CTLs were either elicited or expanded by viral replication in the acute phase or by this inefficient persistent viral replication. Nevertheless, these macaques showed long-term viral control with central memory CD4$^+$ T-cell preservation, indicating that nonsterile protection by CTL-based vaccines can result in prevention of chronic central memory CD4$^+$ T-cell loss.

In summary, the present study shows that primary viral control by a CTL-based AIDS vaccine can result in long-term control of SIV replication by adapted CTL responses and preservation of central memory CD4$^+$ T cells without AIDS progression. Our results suggest that CTL-based vaccines can result in long-term viral containment and disease control.

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Induction of CD8$^+$ Cells Able To Suppress CCR5-Tropic Simian Immunodeficiency Virus SIVmac239 Replication by Controlled Infection of CXCR4-Tropic Simian-Human Immunodeficiency Virus in Vaccinated Rhesus Macaques[▽]

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Recent recombinant viral vector-based AIDS vaccine trials inducing cellular immune responses have shown control of CXCR4-tropic simian-human immunodeficiency virus (SHIV) replication but difficulty in containment of pathogenic CCR5-tropic simian immunodeficiency virus (SIV) in rhesus macaques. In contrast, controlled infection of live attenuated SIV/SHIV can confer the ability to contain SIV superchallenge in macaques. The specific immune responses responsible for this control may be induced by live virus infection but not consistently by viral vector vaccination, although those responses have not been determined. Here, we have examined in vitro anti-SIV efficacy of CD8$^+$ cells in rhesus macaques that showed prophylactic viral vector vaccine-based control of CXCR4-tropic SHIV89.6PD replication. Analysis of the effect of CD8$^+$ cells obtained at several time points from these macaques on CCR5-tropic SIVmac239 replication in vitro revealed that CD8$^+$ cells in the chronic phase after SHIV challenge suppressed SIV replication more efficiently than those before challenge. SIVmac239 superchallenge of two of these macaques at 3 or 4 years post-SHIV challenge was contained, and the following anti-CD8 antibody administration resulted in transient CD8$^+$ T-cell depletion and appearance of plasma SIVmac239 viremia in both of them. Our results indicate that CD8$^+$ cells acquired the ability to efficiently suppress SIV replication by controlled SHIV infection, suggesting the contribution of CD8$^+$ cell responses induced by controlled live virus infection to containment of HIV/SIV superinfection.

Live attenuated immunodeficiency virus infection can induce effective immune responses against pathogenic human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) replication, although concerns about conditions necessary for its safety as an AIDS vaccine have not been satisfied at present (3, 13, 19). In macaque AIDS models, infection of live attenuated viruses such as SIVmac239Δnef, SIVmac239Δ3, and simian-human immunodeficiency virus (SHIV) 89.6 have been shown to confer potent immune responses resulting in control of SIV superchallenge (7, 14, 35, 53). While involvement of virus-specific CD8$^+$ cytotoxic T-lymphocyte (CTL) responses has been indicated, it has remained unclear what immune responses play a key role in this control (19, 34).

Virus-specific cellular immune responses are crucial for control of HIV-1 and SIV infections (1, 4, 5, 10, 12, 20, 29, 38, 41, 42). Recombinant viral vector-based vaccines efficiently eliciting virus-specific cellular immune responses have been developed as promising AIDS vaccine candidates (32). These prophylactic vaccine trials in rhesus macaques have shown viral control and prevention of acute CD4$^+$ T-cell depletion after CXCR4-tropic SHIV challenge (2, 27, 36, 37, 40, 46). Unfortunately, however, trials of these vaccines have shown difficulty in containment of CCR5-tropic SIV infection that induces acute, massive depletion of CCR5$^+$ CD4$^+$ memory T cells and chronic disease progression like HIV-1 infection in humans (6, 8, 11, 21, 23, 28, 30, 31, 39, 49, 50, 52). Possibly, the specific immune responses responsible for SIV control might be induced by live SIV/SHIV infection but not consistently by recombinant viral vector vaccination. Previous CD8$^+$ cell-depletion experiments in macaques using a monoclonal anti-CD8 antibody have indicated the importance of CD8$^+$ cells in SIV control (12, 29, 42), but differences in antiviral efficacy between live SIV/SHIV infection-induced and recombinant viral vector vaccination-induced CD8$^+$ cells have not been determined.

Our previous trials of a prophylactic vaccine using a Gag-expressing Sendai virus (SeV-Gag) vector have shown control of CXCR4-tropic SHIV89.6PD replication in vaccinated rhesus macaques (27, 47). While this vaccination did not always result in CCR5-tropic SIVmac239 control (28), it was speculated that, after SHIV challenge, these vaccinees may possibly

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TABLE 1. Virus challenge and antibody administration schedule

Macaque	Prophylactic vaccination	Time (wk) of:			
		SHIV89.6PD challenge	Anti-CD20 monoclonal antibody administration	SIVmac239 superchallenge	Anti-CD8 monoclonal antibody administration
R00-017	SeV-Gag	0	166	203	209
R00-020	DNA prime with SeV-Gag boost	0	140	151	163
R00-023	DNA prime with SeV-Gag boost	0			
R00-024	DNA prime with SeV-Gag boost	0			

acquire the potential for controlling SIVmac239 superchallenge. In the present study, we have examined whether these SHIV controllers acquired $CD8^+$ cells effective against SIVmac239 replication. Our analyses have suggested that $CD8^+$ cell responses capable of suppressing SIVmac239 replication in vitro were induced by controlled SHIV infection and that these responses might be crucial for control of superchallenged SIVmac239 replication.

MATERIALS AND METHODS

Animal experiments. Four Burmese rhesus macaques (*Macaca mulatta*) used in this study (Table 1) were maintained in accordance with the *Guides for Animal Experiments Performed at National Institute of Infectious Diseases* (35a). Blood collection, vaccination, virus challenge, and antibody administration were performed under ketamine anesthesia. These macaques received prophylactic vaccination and SHIV89.6PD challenge as described in our previous studies (27, 47). Macaque R00-017 was vaccinated intranasally with 1×10^8 cell infectious units (CIU) of replication-competent SeV-Gag vector (15, 16), whereas macaques R00-020, R00-023, and R00-024 were primed intramuscularly with 5 mg of cytomegalovirus (CMV)-SHIVdEN DNA and then boosted intranasally with 6×10^9 CIU of replication-defective F-deleted SeV-Gag vector (22). The CMV-SHIVdEN DNA was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA (45) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx; SIVmac239-HIV-1_{DH12} chimeric Vpr; and HIV-1_{DH12} Tat and Rev as described previously (28, 47). These vaccinees were challenged intravenously with 10 50% tissue culture infective doses ($TCID_{50}$) of SHIV89.6PD (25) 13 weeks (in R00-020, R00-023, and R00-024) or 14 weeks (in R00-017) after SeV-Gag vaccination.

Macaques R00-023 and R00-024 were euthanized around 2 years after SHIV89.6PD challenge, while macaques R00-017 and R00-020 were followed up for more than 2 years. The latter two animals received monoclonal anti-CD20 antibody administration for $CD20^+$ cell depletion (starting at week 166 in R00-017 and week 140 in R00-020), intravenous superchallenge with 1,000 $TCID_{50}$ of SIVmac239 (18) (at week 203 in R00-017 and week 151 in R00-020), and monoclonal anti-CD8 antibody administration for $CD8^+$ cell depletion (starting at week 209 in R00-017 and week 163 in R00-020) (Table 1). For $CD20^+$ cell depletion, animals were inoculated intravenously with 10 mg/kg of monoclonal anti-CD20 antibody (Rituximab; Zenyaku Kogyo, Tokyo, Japan) four times every other week. Peripheral B-cell depletion was confirmed by immunostaining using anti-human CD19 antibody and anti-human CD20 antibody (Becton Dickinson, Tokyo, Japan). For $CD8^+$ cell depletion, animals received a single subcutaneous inoculation of 10 mg/kg of monoclonal anti-CD8 antibody (cM-T807) provided by Centocor (Malvern, PA) followed by three intravenous inoculations of 5 mg/kg cM-T807 on days 3, 7, and 10 after the first inoculation. Peripheral $CD8^+$ T-cell depletion was confirmed by immunostaining using anti-human CD8 antibody (DK25; Dako, Kyoto, Japan). Macaques R00-017 and R00-020 were euthanized 3 months after the anti-CD8 antibody administration.

Quantitation of plasma viral loads. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). For quantitation of plasma SIV/SHIV RNA levels, serial fivefold dilutions of RNA samples were amplified in quadruplicate by reverse transcription (RT) and nested PCR to determine the endpoint. SIV *gag*-specific primers (AGAAACTCCGTCTTGT CAGG and TGATAATCTGCATAGCCGC for the first RT-PCR and GATTA GCAGAAAGCCTGTTGG and TGCAACCTTCTGACAGTGC for the second DNA PCR) (Sigma-Aldrich, Tokyo, Japan) that recognize the *gag* region shared by SHIV89.6PD and SIVmac239 were used. Plasma SIV/SHIV RNA levels were calculated according to the Reed-Muench method as described previously (28). The lower limit of detection in this assay is approximately 4×10^2 copies/ml. After SIVmac239 superchallenge, plasma SIVmac239 RNA levels were measured by the LightCycler system (Roche Diagnostics) using SIVmac239 *env*-specific primers (AAGAATTGTTGCGACTGACC and CAGTAGTGTGGCA GACTTGTC) and probes (CATTCAGCTGCGCCTGGTCCTTTAAGTAC-Flu and LcRed-TCTTCGATGGCAGTGACCCTAGTCTGGAGG) (Nihon Gene Research Laboratories, Inc., Sendai, Japan) that recognize SIVmac239 *env* but not SHIV89.6PD *env*. SHIV89.6PD RNA levels were also measured using SHIV89.6PD *env*-specific primers (GGATGTTGATGATCTGTAGTGC and CCAATACTACTTCTTGTGGGTT) and probes (CAGTCTATTATGGGG TACCTGTGTGGAGAGAAGCA-Flu and LcRed-CCACCACTCTATTTT GTGCATCAGATGCTAAAGCC) that recognize SHIV89.6PD *env* but not SIVmac239 *env*. The lower limit of detection in this assay is approximately 1×10^3 copies/ml.

In vitro viral suppression assay. We examined SIVmac239 replication on CD8-depleted peripheral blood mononuclear cells (PBMCs) in the presence of $CD8^+$ cells positively selected from PBMCs. Macaque PBMCs prepared from blood at several time points were frozen and stored until use. Thawed PBMCs were separated into $CD8^+$ cells and $CD8^-$ cells by using MACS CD8 MicroBeads (Miltenyi Biotec, Tokyo, Japan). The purity of the former was more than 96%, while the latter included less than 3% of $CD8^+$ cells. To prepare target cells, one fifth of $CD8^-$ cells negatively selected from PBMCs obtained before SHIV89.6PD challenge were infected with SIVmac239 at a multiplicity of infection (MOI) of 1:10^4, and these infected cells and the remaining uninfected $CD8^-$ cells were cultured separately in the presence of 2 μg/ml phytohemagglutinin-L (Roche Diagnostics). After a 48-h culture, both infected and uninfected $CD8^-$ cells were collected, washed three times, and mixed to be used as target cells. Then, 4×10^5 target cells were cultured alone or cocultured with 4×10^5 (effector/target [E:T] ratio of 1:1) or 4×10^4 (E:T ratio of 1:10) $CD8^+$ effector cells positively selected from PBMCs in a well of 96-well flat-bottom plate and the culture supernatants were harvested every other day for measurement of SIV Gag CA p27 concentration by SIV core antigen enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter, Tokyo, Japan). RPMI 1640 medium (Invitrogen, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 20 IU/ml recombinant human interleukin-2 (Roche Diagnostics) were used for cell culture. All of the cocultures were in duplicate, and the mean value of p27 concentrations at each time point is shown.

Measurement of virus-specific $CD8^+$ T-cell responses. We measured virus-specific T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (27, 28). PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCL) (51) infected with vesicular stomatitis virus G (VSV-G)-pseudotyped SIVGP1 for SIVGP1-specific stimulation. The VSV-G-pseudotyped SIVGP1 was obtained by cotransfection of COS-1 cells with pVSV-G (Clontech, Otsu, Japan) and SIVGP1, an *env*- and *nef*-deleted $SHIV_{MD14}$ molecular clone DNA (28, 45). Intracellular IFN-γ staining was performed using a Cytofix-Cytoperm kit (Becton Dickinson). Peridinin chlorophyll-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting the IFN-γ T-cell frequencies after nonspecific stimulation from those after SIVGP1-specific stimulation.

Measurement of virus-specific neutralizing titers. We measured virus-specific neutralizing titers as described previously (17, 44). Serial twofold dilutions of heat-inactivated plasma were prepared in duplicate and mixed with 10 $TCID_{50}$ of SIVmac239 or SHIV89.6PD. In each mixture, 5 μl of diluted plasma was incubated with 5 μl of virus. After a 45-min incubation at room temperature, each 10-μl mixture was added to 5×10^4 MT-4 cells in a well of a 96-well flat-bottom

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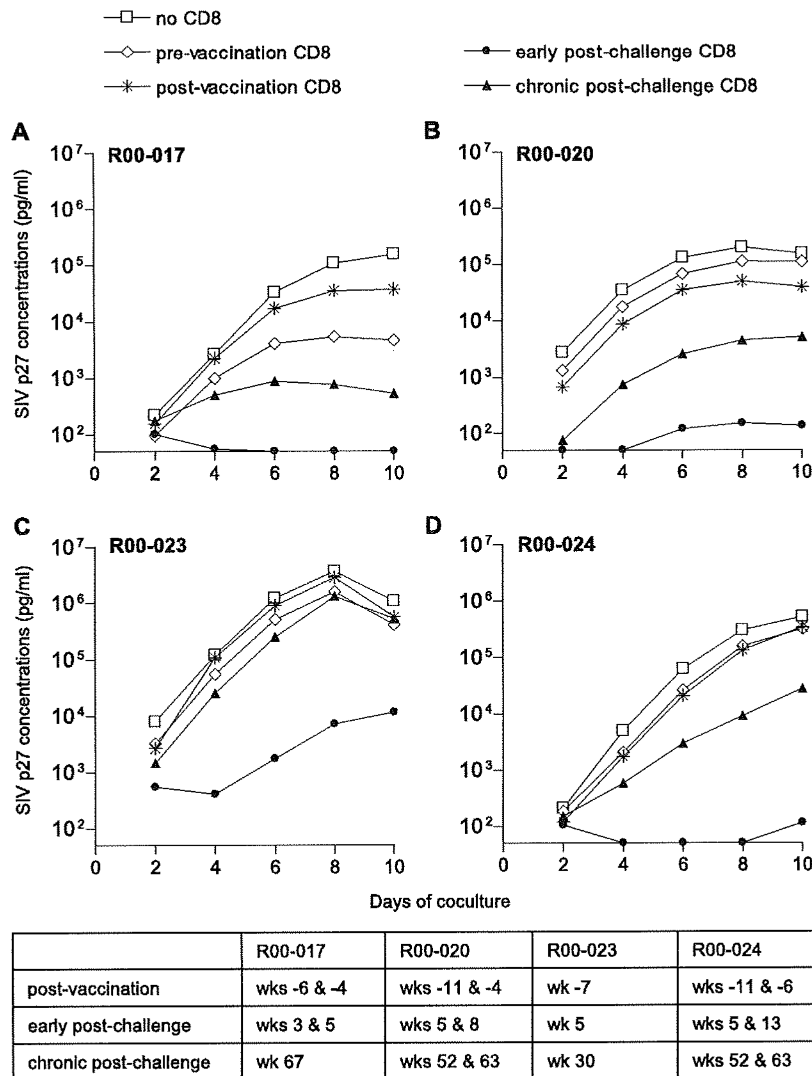

	R00-017	R00-020	R00-023	R00-024
post-vaccination	wks -6 & -4	wks -11 & -4	wk -7	wks -11 & -6
early post-challenge	wks 3 & 5	wks 5 & 8	wk 5	wks 5 & 13
chronic post-challenge	wk 67	wks 52 & 63	wk 30	wks 52 & 63

FIG. 1. SIVmac239 replication in vitro in the absence or the presence of $CD8^+$ cells in macaques R00-017 (A), R00-020 (B), R00-023 (C), and R00-024 (D). PBMC-derived $CD8^-$ (target) cells infected with SIVmac239 were cultured alone (no CD8) or cocultured with autologous PBMC-derived $CD8^+$ (effector) cells obtained prevaccination (pre-vaccination CD8), postvaccination and pre-SHIV challenge (post-vaccination CD8), in the early phase post-SHIV challenge (early post-challenge CD8), or in the chronic phase post-SHIV challenge (chronic post-challenge CD8) at an E:T ratio of 1:1. A representative result of two sets of experiments with similar patterns is shown in panels A and D, whereas the result of a single experiment is shown in panels B and C. Postvaccination and postchallenge $CD8^+$ cells were prepared from PBMCs obtained at different time points, as shown in the bottom table (weeks before [shown by minus] or after SHIV challenge), because of a limitation of available samples. SeV-Gag vaccination was performed 13 weeks (in R00-020, R00-023, and R00-024) or 14 weeks (in R00-017) before SHIV challenge. In some groups, $CD8^+$ cells at two time points were mixed to prepare enough cells. p27 concentrations in the culture supernatants were examined by ELISA.

plate. After 12 days of culture, supernatants were harvested. Progeny virus production in the supernatants was examined by SIV core antigen ELISA for detection of SIV p27 to determine the 100% neutralizing end point. The lower limit of detection is a titer of 1:2.

RESULTS

Potency of $CD8^+$ cells post-SHIV challenge for suppressing SIVmac239 replication in vitro. We established a method for examining SIVmac239 replication in vitro in the presence of $CD8^+$ cells and evaluated the effect of $CD8^+$ cells on SIVmac239 replication in vitro in four rhesus macaques that showed vaccine-based containment of SHIV89.6PD challenge (Table 1). One of them (R00-017) received a single intranasal SeV-Gag vaccination, while the other three (R00-020, R00-023, and R00-024) received a single intramuscular DNA priming followed by a single intranasal SeV-Gag booster before SHIV89.6PD challenge as described previously (27, 47). All four of these macaques controlled viral replication with undetectable plasma viremia after the acute phase for more than 2 years post-SHIV89.6PD challenge (54).

From each animal, we prepared four groups of bulk $CD8^+$ cells obtained prevaccination, post-SeV-Gag vaccination (pre-SHIV challenge), in the early phase post-SHIV challenge (weeks 3 to 8), and in the chronic phase post-SHIV challenge

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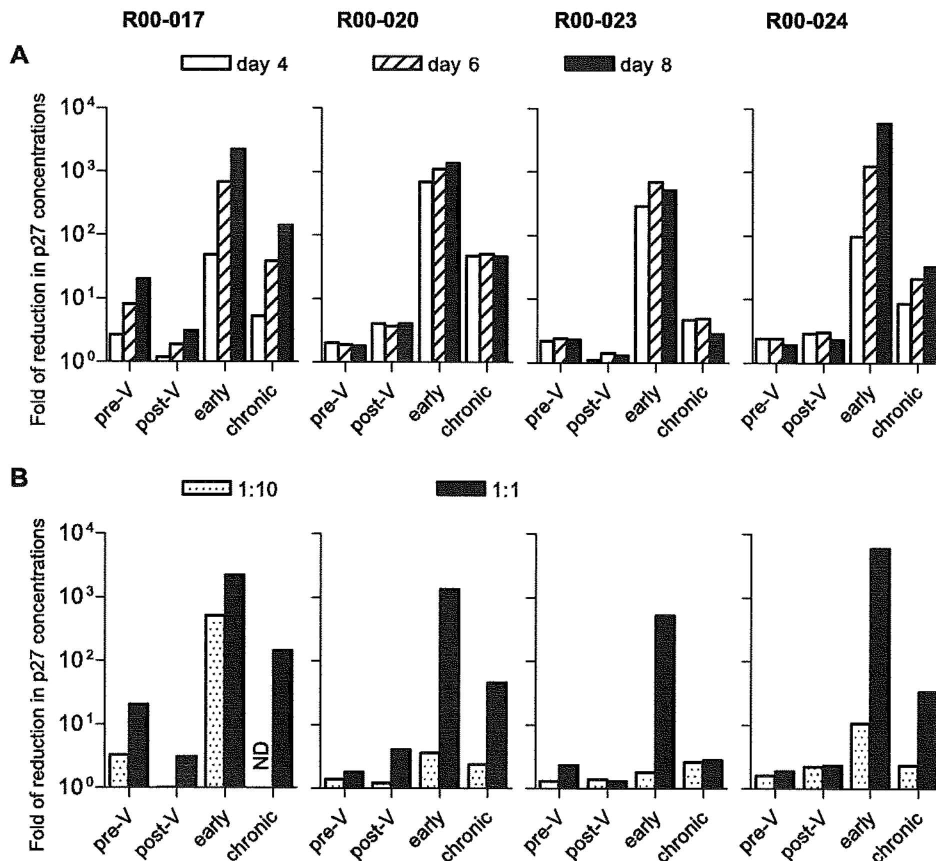

FIG. 2. Reduction in SIVmac239 production by addition of $CD8^+$ cells. The reduction (fold) in p27 concentration in the supernatant from coculture of SIV-infected $CD8^-$ cells with each group of $CD8^+$ cells compared to that from SIV-infected $CD8^-$ cell culture without $CD8^+$ cells is shown. (A) Reduction in p27 concentration on days 4, 6, and 8 of coculture at an E:T ratio of 1:1 (calculated from the data in Fig. 1). (B) Reduction in p27 concentration on day 8 of coculture at an E:T ratio of 1:1 (black bars) or 1:10 (dotted bars). pre-V, prevaccination CD8; post-V, postvaccination CD8; early, early postchallenge CD8; chronic, chronic postchallenge CD8 as described in the legend to Fig. 1. ND, not determined.

(weeks 30 to 67). These groups of effector $CD8^+$ cells were cocultured with SIVmac239-infected autologous target $CD8^-$ cells at the E:T ratio of 1:1, and p27 concentrations in the culture supernatants were measured for evaluation of SIVmac239 production (Fig. 1). Reduction in SIVmac239 production by addition of each group of $CD8^+$ cells was shown as reduction (fold) in p27 concentration compared to that in the supernatant from the SIVmac239-infected $CD8^-$ cell culture without $CD8^+$ cells (Fig. 2A).

Even addition of prevaccination $CD8^+$ cells resulted in reduction of SIV production. Especially, prevaccination $CD8^+$ cells derived from macaque R00-017 efficiently suppressed SIV replication, showing an approximately 20-fold reduction in viral production at day 8 of culture. In other three macaques, however, the reduction in SIV production by addition of prevaccination $CD8^+$ cells was less than threefold. In macaque R00-020, postvaccination/prechallenge $CD8^+$ cells suppressed SIV replication more efficiently than prevaccination ones, but in the other three macaques, the levels of suppression by postvaccination/prechallenge $CD8^+$ cells were not more than those by prevaccination cells.

In contrast, $CD8^+$ cells in the early phase postchallenge showed an efficient suppressive effect on SIV replication in all four macaques. Maximum reduction (fold) in SIV production by addition of these $CD8^+$ cells was more than 7×10^2. Addition of $CD8^+$ cells in the chronic phase postchallenge also resulted in efficient reduction of SIV production. The levels of reduction were lower than those by $CD8^+$ cells in the early phase postchallenge but higher than those by prechallenge $CD8^+$ cells. Thus, all four vaccinees, after SHIV challenge, acquired $CD8^+$ cells able to suppress SIVmac239 replication in vitro efficiently. Efficient reduction by early postchallenge $CD8^+$ cells was observed in some animals even at the E:T ratio of 1:10 (Fig. 2B).

We then measured SIVGP1-specific $CD8^+$ T-cell frequencies in PBMCs by detection of IFN-γ induction after stimulation with B-LCL expressing an *env*- and *nef*-deleted SHIV molecular clone (SIVGP1) DNA (27, 28) (Fig. 3). In all four macaques, SIVGP1-specific $CD8^+$ T-cell levels peaked during the acute phase post-SHIV challenge and gradually decreased after the set point. SIVGP1-specific $CD8^+$ T-cell frequencies after the acute phase were higher in macaques R00-017 and R00-023 compared to those post-SeV-Gag vaccination (prechallenge) but interestingly lower in macaque R00-020.

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