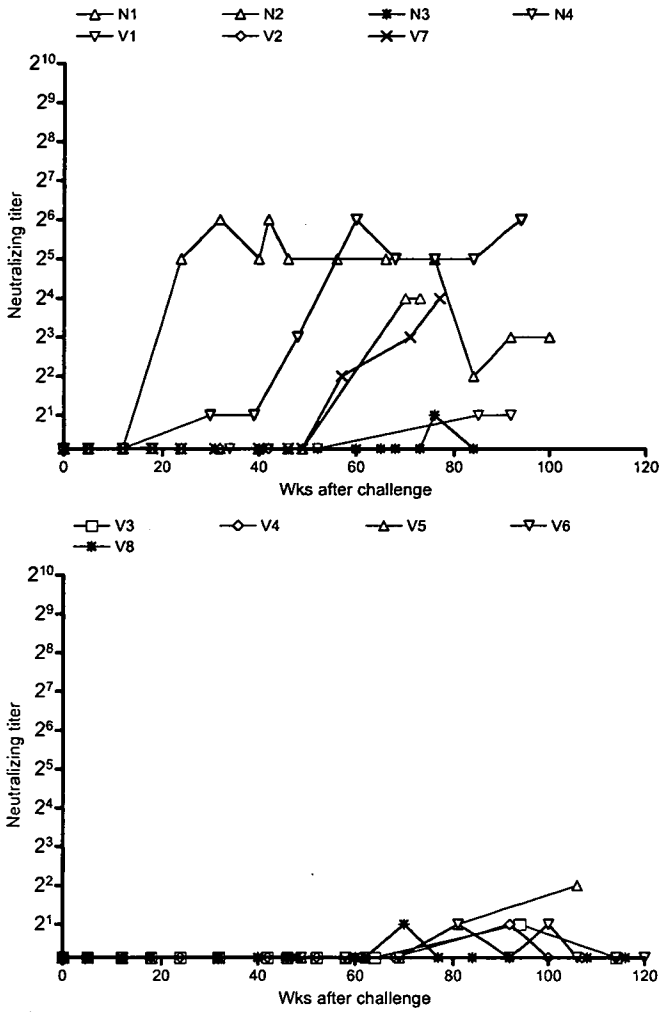

FIG. 2. SIVmac239-specific neutralizing antibody levels in plasma. Plasma titers for killing 10-$TCID_{50}$ SIVmac239 replication in the noncontrollers (top panel), including unvaccinated control animals, and in the controllers (bottom panel) are shown.

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

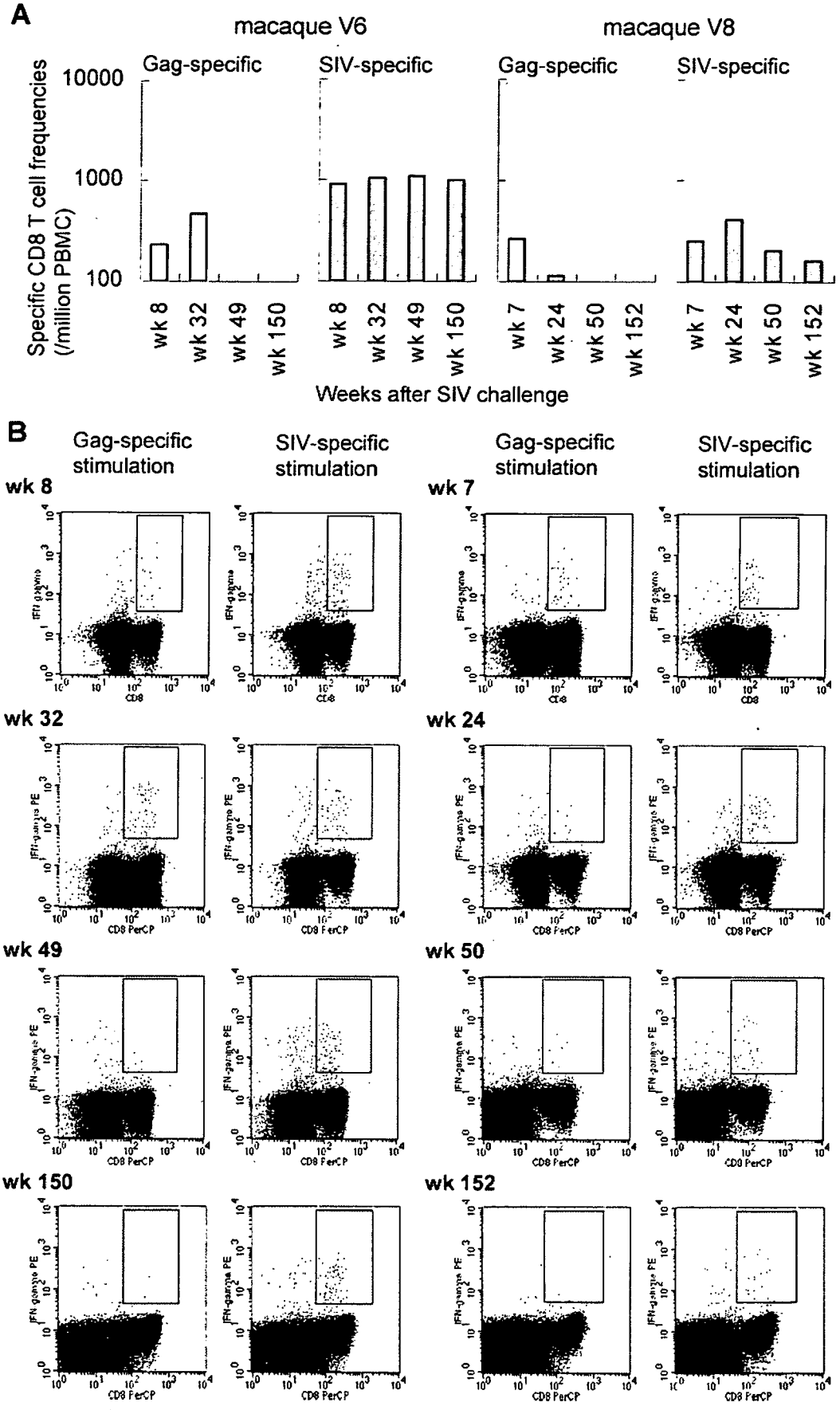

FIG. 3. Virus-specific $CD8^+$ T-cell responses in sustained controllers V6 (left panels) and V8 (right panels). (A) Gag-specific and SIV-specific $CD8^+$ T-cell frequencies in PBMCs. (B) Dot plots gated on $CD3^+$ lymphocytes after Gag-specific or SIV-specific stimulation.

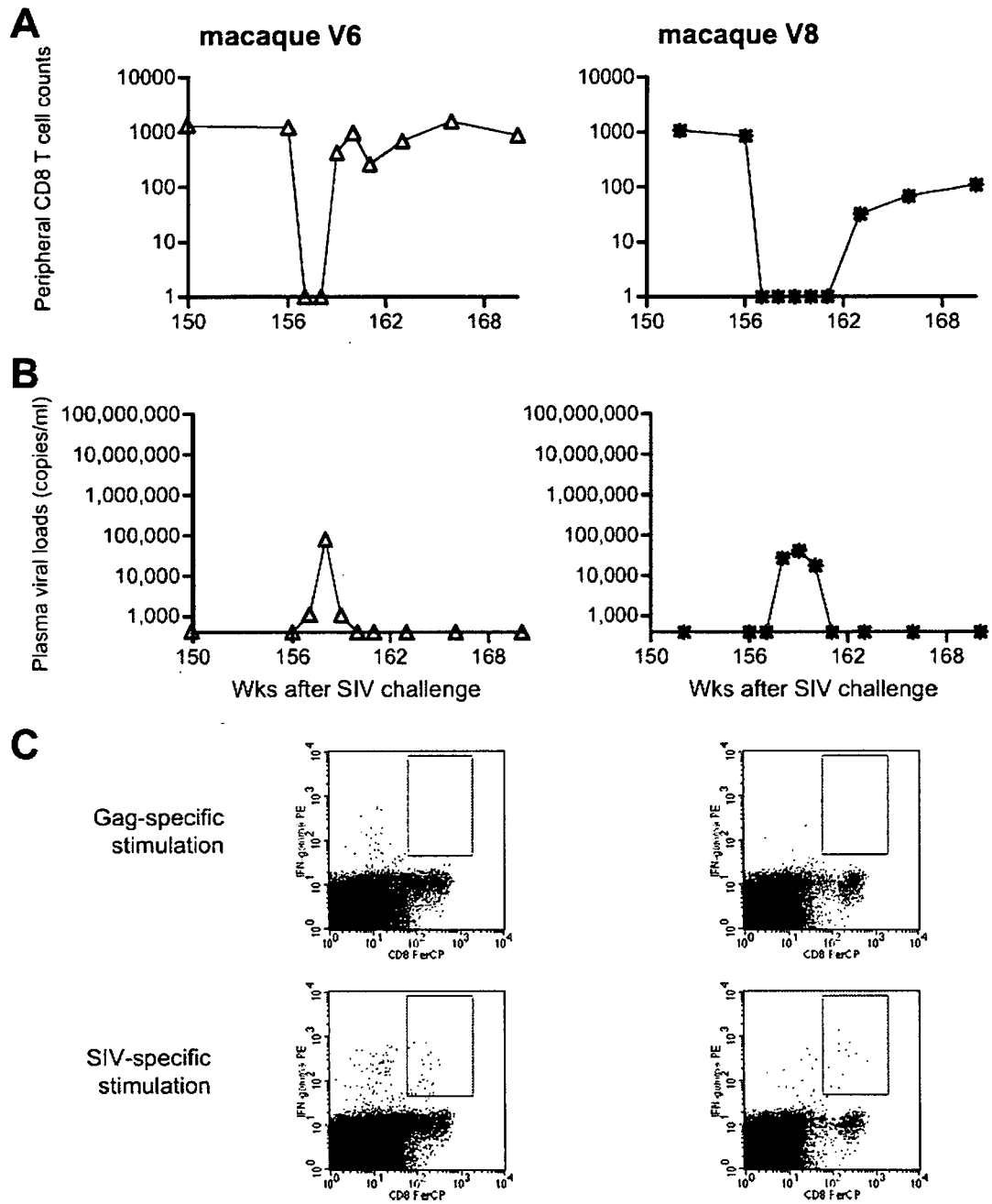

FIG. 4. $CD8^+$ cell depletion experiments starting at week 156 in sustained controllers V6 (left panels) and V8 (right panels). (A) Peripheral $CD8^+$ T-cell counts (per μl). (B) Plasma viral loads (viral RNA copies/ml plasma). (C) Virus-specific CTL responses at week 160 in V6 and at week 166 in V8.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

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

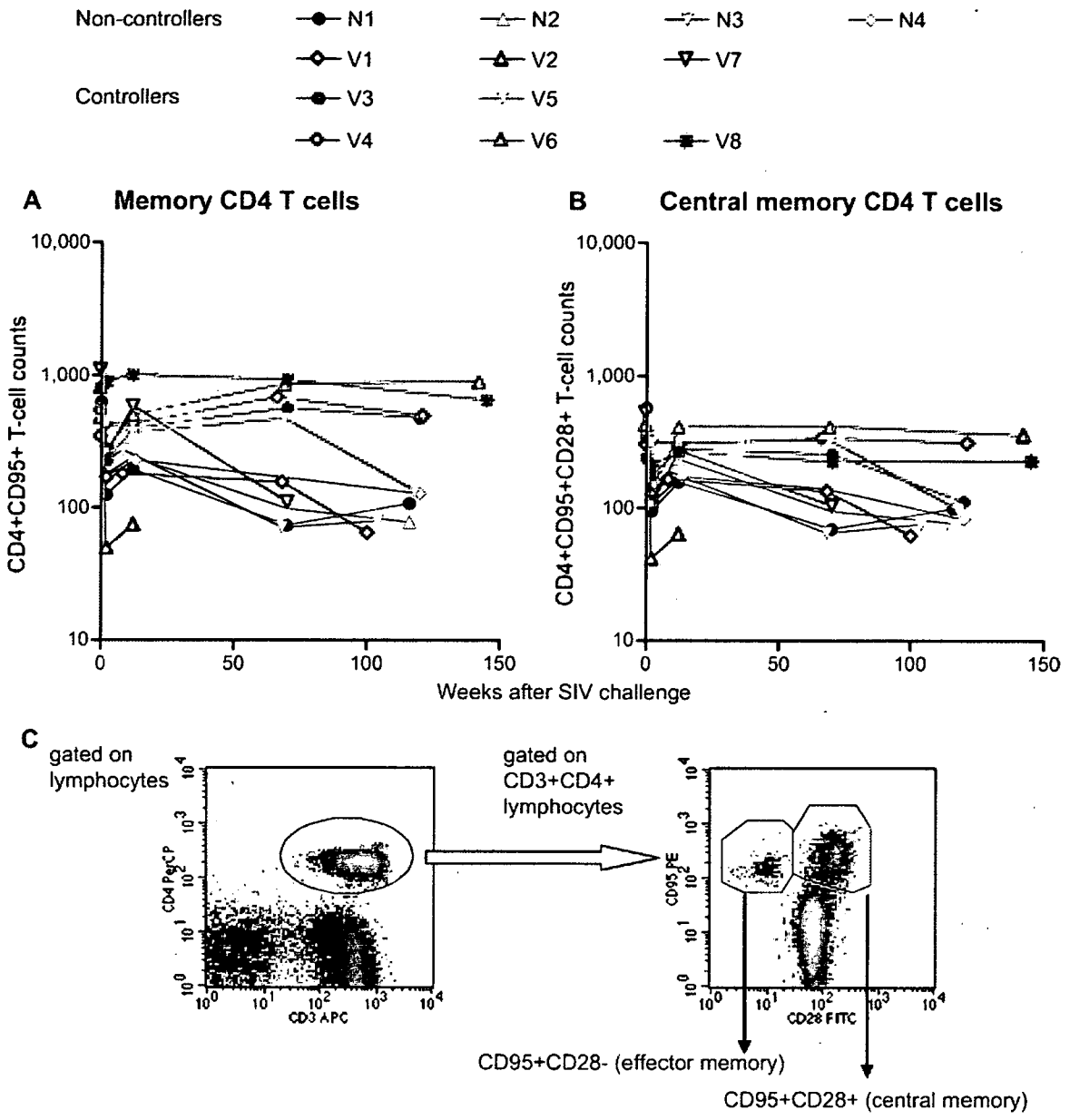

FIG. 5. Changes in peripheral memory CD4$^+$ T-cell counts. Noncontrollers are indicated in black or blue, and controllers are indicated in red. (A) Peripheral memory CD4$^+$ (CD4$^+$ CD95$^+$) T-cell counts (per μl). (B) Peripheral central memory CD4$^+$ (CD4$^+$ CD95$^+$ CD28$^+$) T-cell counts (per 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

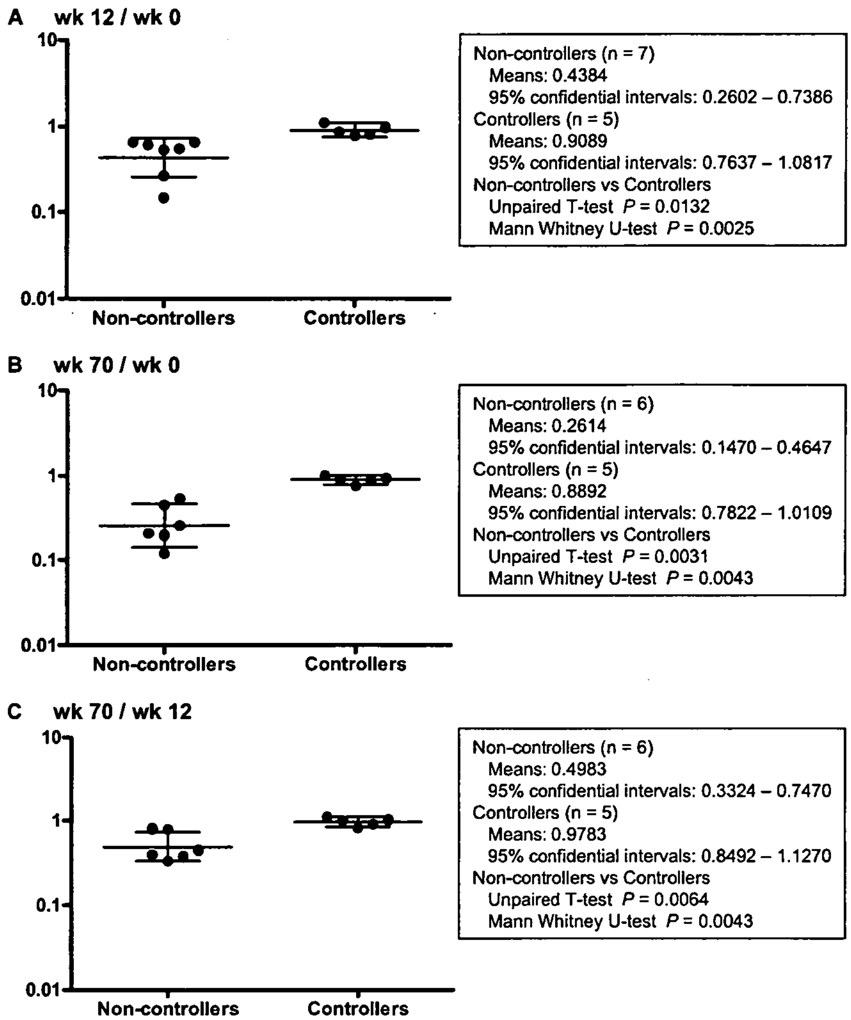

FIG. 6. Statistical analysis indicating preservation of central memory $CD4^+$ T-cell counts in the controllers. The ratios of central memory $CD4^+$ T-cell counts at week 12 to week 0 (A), week 70 to week 0 (B), and week 70 to week 12 (C) in the noncontrollers (except for rapid progressor V2 in panels 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-

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$^\nabla$

Tetsuo Tsukamoto,[1,2]† Mitsuhiro Yuasa,[1]† Hiroyuki Yamamoto,[1] Miki Kawada,[1,2] Akiko Takeda,[1] Hiroko Igarashi,[2] and Tetsuro Matano[1,2,3,4]*

International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-Ku, Tokyo 108-8639, Japan[1]; Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-Ku, Tokyo 113-0033, Japan[2]; AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan[3]; and Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan[4]

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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

* Corresponding author. Mailing address: International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-6409-2078. Fax: 81-3-6409-2076. E-mail: matano@m.u-tokyo.ac.jp.
† T.T. and M.Y. contributed equally to this work.
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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.

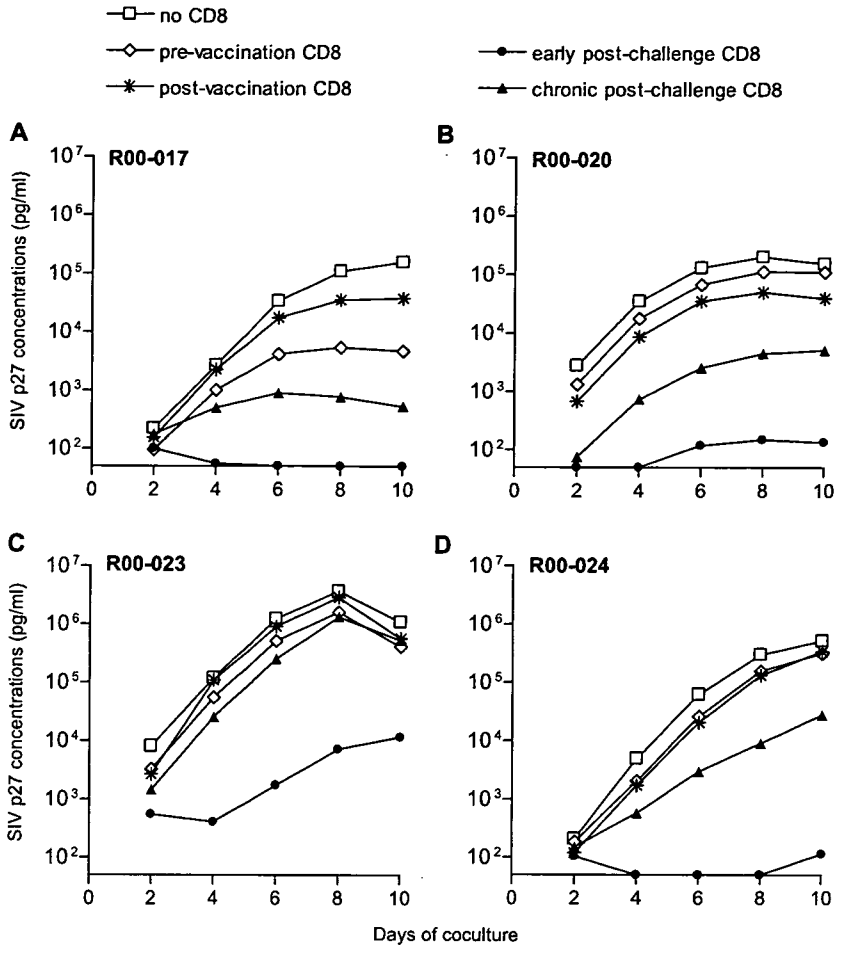
Macaques R00-023 and R00-024 were euthanized around 2 years after SHIV89.6PD challenge, while macaques R00-017 and R00-020 were followed up for more than 2 years. The latter two animals received monoclonal anti-CD20 antibody administration for $CD20^+$ cell depletion (starting at week 166 in R00-017 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



	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

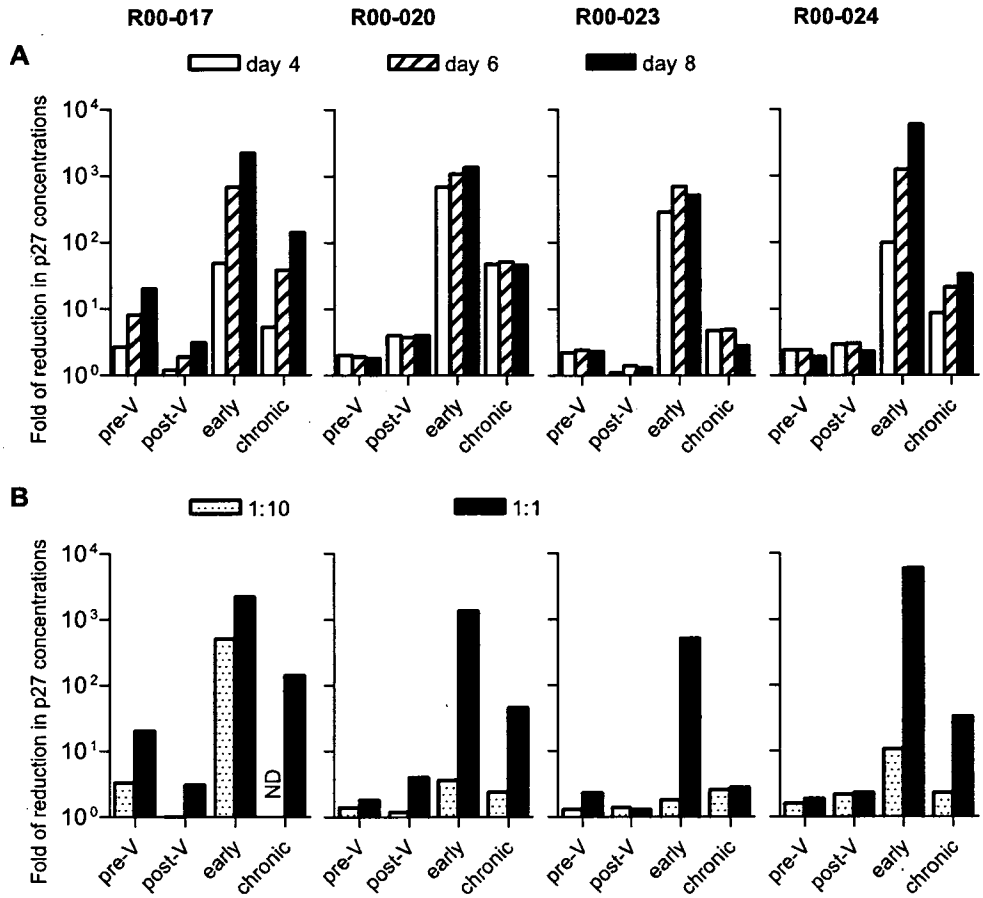


FIG. 2. Reduction in SIVmac239 production by addition of $CD8^+$ cells. The reduction (fold) in p27 concentration in the supernatant from coculture of SIV-infected $CD8^-$ cells with each group of $CD8^+$ cells compared to that from SIV-infected $CD8^-$ cell culture without $CD8^+$ cells is shown. (A) 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.

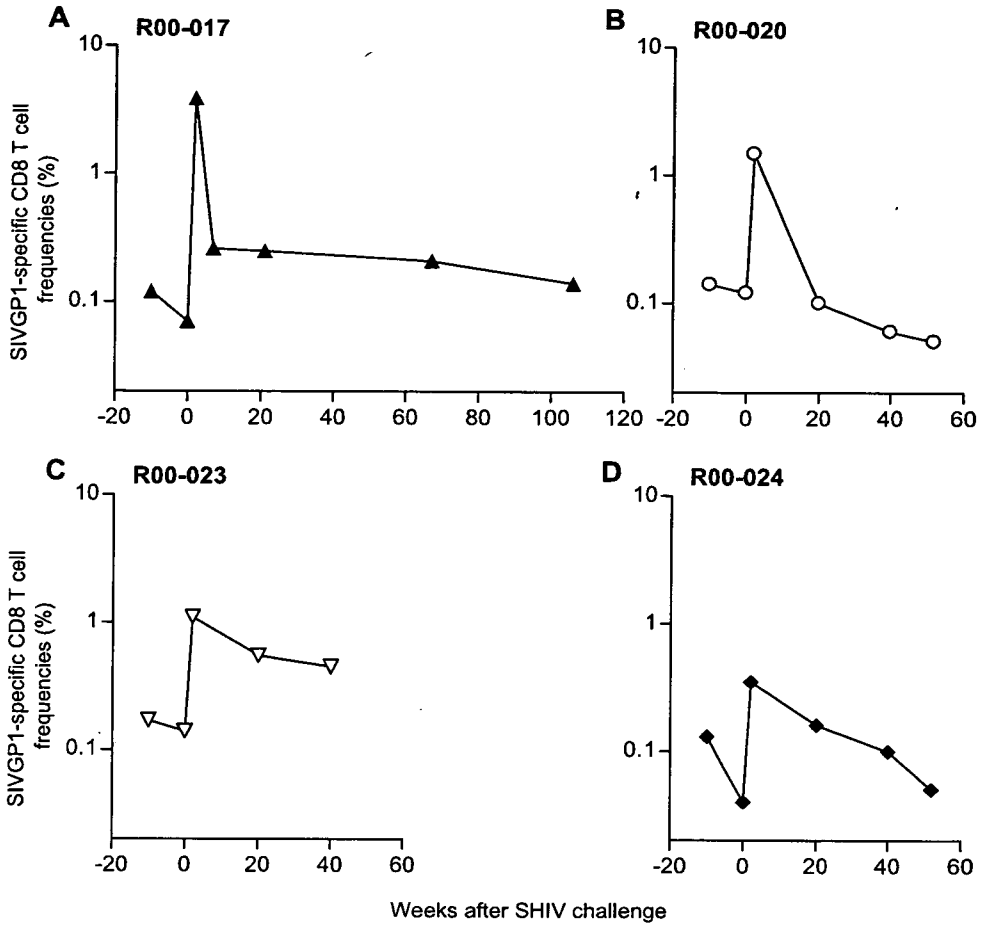


FIG. 3. SIVGP1-specific CD8$^+$ T-cell frequencies in macaques before and after SHIV89.6PD challenge. Frequencies of CD8$^+$ T cells showing SIVGP1-specific IFN-γ induction per total CD8$^+$ T cells in PBMCs are shown. The first time point prechallenge is 10 weeks before challenge.

CD20 depletion and SIVmac239 superchallenge in the SHIV controllers. Macaques R00-017 and R00-020 were further followed up and received monoclonal anti-CD20 antibody administration at week 166 (R00-017) or week 140 (R00-020) and SIVmac239 superchallenge at week 203 (R00-017) or week 151 (R00-020) (Table 1). Viral control was not abrogated, and plasma viremia remained undetectable after anti-CD20 antibody administration (Fig. 4). In both macaques, SHIV89.6PD-specific neutralizing antibodies (NAbs) were induced efficiently after SHIV89.6PD challenge and maintained at high levels in the chronic phase (54). The monoclonal anti-CD20 antibody administration resulted in rapid and prolonged depletion of peripheral CD20$^+$ lymphocytes, and more than a few months later, an approximately fourfold reduction in SHIV-specific NAb levels was observed (Fig. 5).

The following SIVmac239 superchallenge was contained in both macaques (Fig. 4). Macaque R00-017 did not show detectable plasma viremia even after SIVmac239 superchallenge, and macaque R00-020 showed only transient appearance of plasma viremia 1 week after SIVmac239 superchallenge. SIVmac239 *env* RNA but not SHIV89.6PD *env* RNA was detected in the transient plasma viremia (Fig. 6). SIVGP1-specific CD8$^+$ T-cell frequencies were at marginal levels just

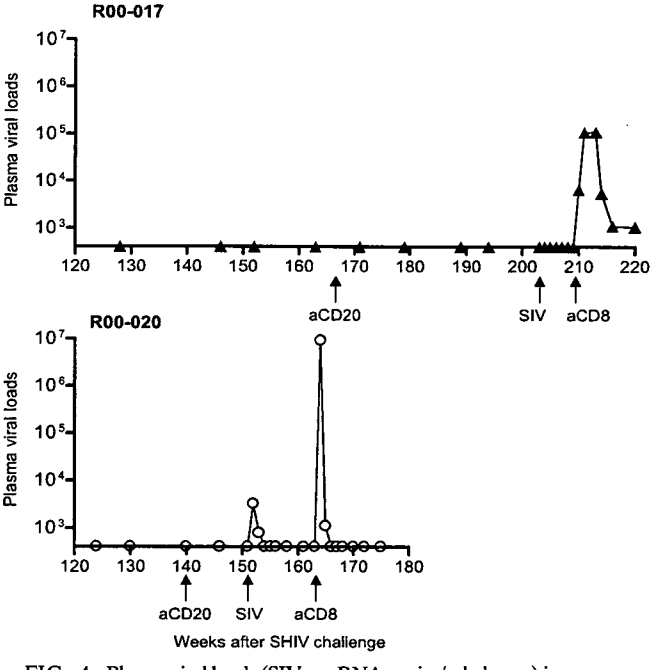


FIG. 4. Plasma viral loads (SIV *gag* RNA copies/ml plasma) in macaques R00-017 (upper panel) and R00-020 (lower panel) after week 120 post-SHIV challenge. aCD20 and aCD8, anti-CD20 and anti-CD8, respectively.

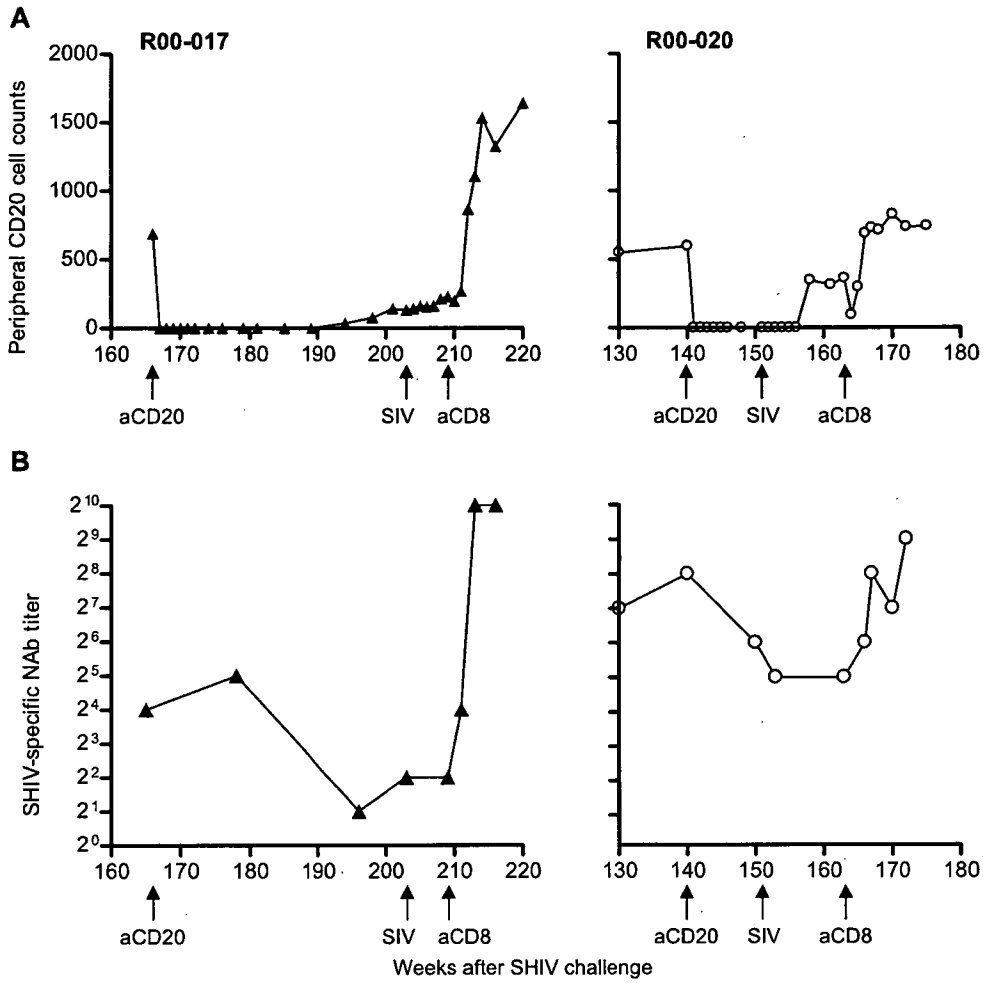


FIG. 5. Changes in SHIV89.6PD-specific NAb levels after monoclonal anti-CD20 antibody administration at week 166 in macaque R00-017 (left panels) and at week 140 in macaque R00-020 (right panels). (A) Peripheral $CD20^+$ cell counts (per μl). (B) SHIV89.6PD-specific neutralizing titers in plasma. aCD20 and aCD8, anti-CD20 and anti-CD8, respectively.

before SIVmac239 superchallenge but increased after the superchallenge (Fig. 7).

CD8 depletion after SIVmac239 superchallenge. Macaques R00-017 and R00-020 received monoclonal anti-CD8 antibody administration at week 209 (6 weeks after superchallenge) and week 163 (12 weeks after superchallenge), respectively (Table 1). Both macaques showed transient depletion of peripheral $CD8^+$ T lymphocytes and appearance of plasma viremia after the anti-CD8 antibody administration (Fig. 6).

In macaque R00-020, exhibiting a shorter period of $CD8^+$ T-lymphocyte depletion, plasma viremia was transient and detectable only at weeks 164 and 165, 1 and 2 weeks after the initial anti-CD8 antibody treatment. SIVmac239 *env* RNA but not SHIV89.6PD *env* RNA was detected in the transient plasma viremia. In macaque R00-017, exhibiting a longer period of $CD8^+$ T-lymphocyte depletion, plasma viremia appeared at week 210, 1 week after the initial anti-CD8 antibody treatment, and remained detectable during the observation period of 3 months. Interestingly, both SIVmac239 *env* RNA and SHIV89.6PD *env* RNA were detected; the former became detectable at week 210 and was detected during the observation period, whereas the latter was detected only at weeks 211 and 212. The former SIVmac239 *env* RNA levels peaked at week 213, and the latter SHIV89.6PD *env* RNA levels peaked at week 211.

SIVmac239-specific NAb responses were undetectable even after SIVmac239 superchallenge and CD8 depletion in both of the macaques (data not shown). SHIV89.6PD-specific NAb titers increased after the CD8 depletion not only in macaque R00-017 showing SHIV89.6PD viremia but also in macaque R00-020 without SHIV89.6PD viremia (Fig. 5). Both macaques showed increases in SIVGP1-specific $CD8^+$ T-cell frequencies after recovery from peripheral $CD8^+$ T-lymphocyte depletion (Fig. 7).

DISCUSSION

Previous $CD8^+$ cell depletion experiments in macaques using a monoclonal anti-CD8 antibody have indicated the importance of $CD8^+$ cell responses in SIV control in vivo (12, 29, 42). The present study evaluated the anti-SIV efficacy of these bulk $CD8^+$ cells in the vaccinated macaques that exhibited prophylactic SeV-Gag vaccine-based control of viral replication and showed induction of $CD8^+$ cells able to efficiently

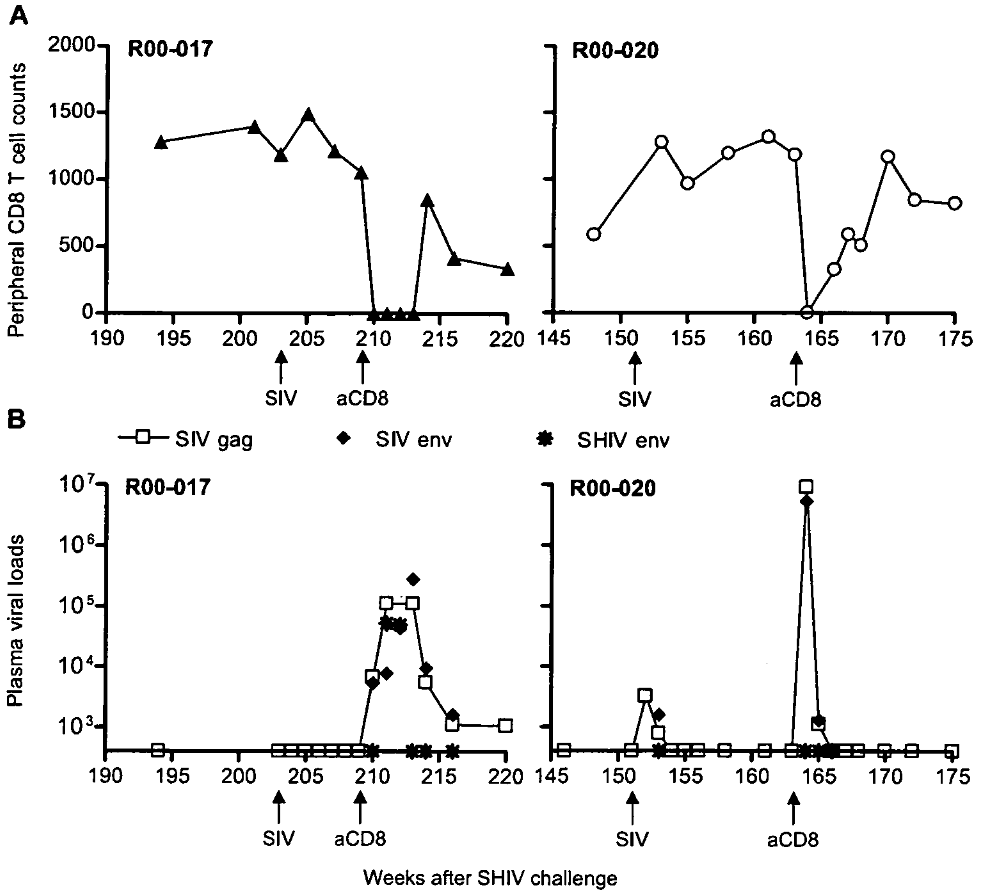


FIG. 6. SIVmac239 superchallenge and CD8$^+$ cell depletion in macaques R00-017 and R00-020. Macaque R00-017 received SIVmac239 superchallenge at week 203 and monoclonal anti-CD8 (aCD8) antibody administration starting at week 209, while macaque R00-020 received superchallenge at week 151 and anti-CD8 at week 163. (A) Peripheral CD8$^+$ T-cell counts (per μl) in macaques R00-017 (left panel) and R00-020 (right panel). (B) Plasma viral loads (copies/ml plasma) in macaques R00-017 (left panel) and R00-020 (right panel). In addition to SIV *gag* RNA levels, levels of SIV *env* RNA and SHIV *env* RNA at several time points are shown.

suppress SIV replication in vitro after SHIV challenge in these macaques. The difference in anti-SIV efficacies between postvaccination/prechallenge and postchallenge CD8$^+$ cells may explain why protective immune responses can be consistently induced not by current viral vector vaccination but by live virus infection.

These bulk CD8$^+$ cells are considered to include CD8$^+$ NK cells in addition to CD8$^+$ T lymphocytes. While previous studies using bulk CD8$^+$ cells or CTL clones (9, 24, 48, 55) have shown the importance of CTL activity on suppression of HIV/SIV replication, there may be a possibility that NK cells exert some suppressive effect on SIV replication, contributing to reductions in SIV production by prevaccination CD8$^+$ cells in the present study. The suppressive effect of postvaccination/prechallenge CD8$^+$ cells was not larger than that of prevaccination except for macaque R00-020. In contrast, postchallenge CD8$^+$ cells suppressed SIV replication more efficiently than those prevaccination and postvaccination. In the in vitro assay of SIV replication, individual macaques showed different sensitivities of target CD8$^-$ cells to SIV infection and different patterns of SIV replication kinetics in the absence of CD8$^+$ cells (Fig. 1). In macaque R00-023 showing higher levels of SIV production in the absence of CD8$^+$ cells, SIV infection at a lower MOI might exhibit a larger reduction in SIV production by addition of postchallenge CD8$^+$ cells.

Gag-specific CD8$^+$ T-cell levels peaked around 1 week after SeV-Gag vaccination and then decreased in the late phase after that (28). To prepare postvaccination/prechallenge CD8$^+$ cells, we used PBMCs in the late phase without those at week 1 post-SeV-Gag vaccination that include the peak levels of Gag-specific CD8$^+$ T cells. Thus, we compared anti-SIV efficacy of CD8$^+$ cells in the late phase postvaccination with that in the chronic phase post-SHIV challenge in this study. The postvaccination/prechallenge SIVGP1-specific CD8$^+$ T-cell frequencies roughly reflect Gag-specific CD8$^+$ T-cell ones because SIVGP1-specific CD8$^+$ T-cell responses were undetectable before SeV-Gag vaccination (data not shown). On the other hand, the postchallenge SIVGP1-specific CD8$^+$ T-cell responses are considered specific for SHIV antigens, including SIV-derived Gag, Pol, Vif, and partial Vpr. Therefore, our results shown in Fig. 3 suggest that SIV-specific CD8$^+$ T-cell frequencies in the chronic phase post-SHIV challenge were less than those post-SeV-Gag vaccination (prechallenge) in macaque R00-020. Interestingly, however, such postchallenge CD8$^+$ cells suppressed SIV replication more efficiently than postvaccination/prechallenge ones. Thus, SIV-specific CD8$^+$

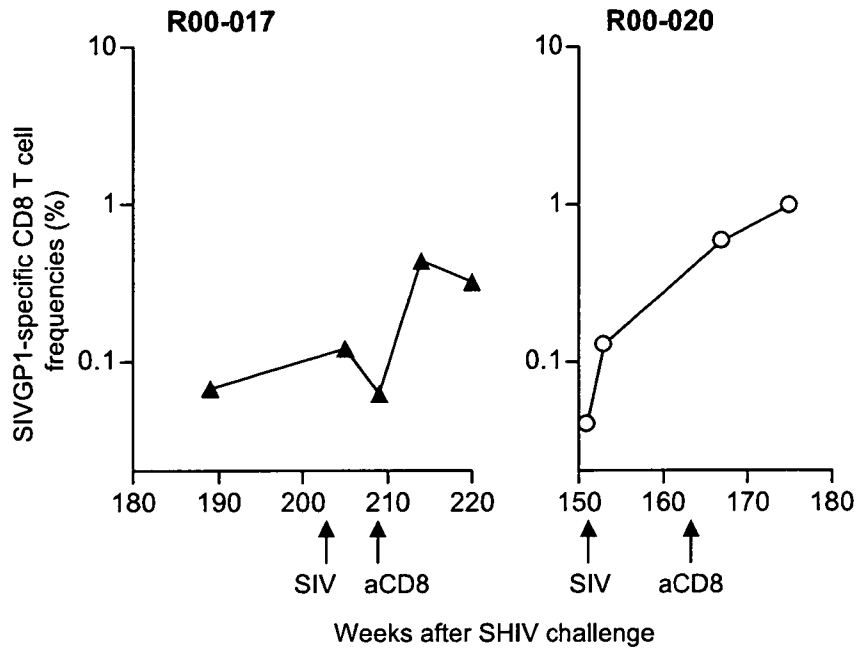


FIG. 7. SIVGP1-specific $CD8^+$ T-cell frequencies in macaques R00-017 (left panel) and R00-020 (right panel) before and after SIVmac239 superchallenge. Frequencies of $CD8^+$ T cells showing SIVGP1-specific IFN-γ induction per total $CD8^+$ T cells in PBMCs are shown. aCD8, anti-CD8.

T-cell frequencies may not always correlate with anti-SIV efficacy in vitro. It may be because postchallenge-induced, certain epitope-specific $CD8^+$ T cells may have higher anti-SIV efficacy in vitro than postvaccination/prechallenge $CD8^+$ T cells in this macaque. There may be a possibility of augmentation of anti-SIV efficacy by induction of broader $CD8^+$ T-cell responses after SHIV challenge.

A previous $CD8^+$ cell depletion study in macaques infected with live attenuated SIV has shown partial loss of superchallenged SIVmac251 control by monoclonal anti-CD8 antibody administration at the superchallenge and has suggested involvement of both cellular and humoral immune responses in this control (43). On the other hand, administration of monoclonal anti-CD8 antibody to macaques infected with live attenuated SIVmac239Δnef after SIVmac251 superchallenge resulted in the appearance of SIVmac239Δnef viremia without detectable SIVmac251 viremia (33). In contrast, the present study showed the appearance of superchallenged SIVmac239 viremia by monoclonal anti-CD8 antibody administration after superchallenge, suggesting that $CD8^+$ cells were crucial for the control of superchallenged SIVmac239 replication. It can be speculated that, in SIVmac239Δnef-infected animals, live virus replication levels before superchallenge were higher, resulting in more strict containment of superchallenge than that in our study. Additionally, neutralizing antibody responses may be involved in the containment of superchallenge in SIVmac239Δnef-infected animals but not in SHIV-infected ones. Thus, our results imply a more profound contribution of $CD8^+$ cells to control of SIV superchallenge in the absence of NAb help.

More than a few months after the anti-CD20 antibody administration, both macaques R00-017 and R00-020 showed fourfold reductions in SHIV-specific neutralizing titers, although it is unclear if these reductions were due to the $CD20^+$ cell depletion. Macaque R00-017 with a lower neutralizing titer showed transient appearance of SHIV viremia by $CD8^+$ cell depletion, but macaque R00-020 with a higher titer did not. These results were consistent with the previous study indicating involvement of humoral as well as cellular immune responses in the CXCR4-tropic SHIV control (26).

In summary, our results indicate that $CD8^+$ cells acquired the ability to efficiently suppress CCR5-tropic SIV replication in vitro by controlled CXCR4-tropic SHIV infection. While the levels of in vitro anti-SIV efficacy resulting in SIV control in vivo have not been determined, our results imply that such $CD8^+$ cell responses may be crucial for live attenuated vaccine-based containment of HIV/SIV superinfection.

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