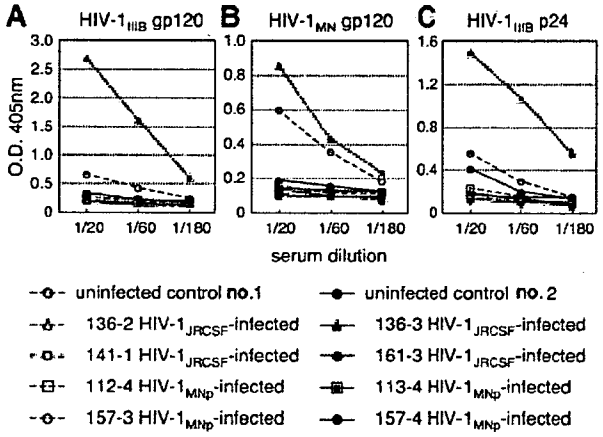

Figure 5. Detection of anti–HIV-1 antibodies from the plasma of HIV-1–infected mice. An ELISA assay was conducted by using plasma from 14 mice inoculated with either HIV-1_{JRCSF} or HIV-1_{MNp}, and from 2 uninfected control mice. Representatives (n = 8) of the 14 HIV-1–inoculated mice, and the 2 uninfected mice, are shown in the panels. Measurements of specific human antibodies for HIV-1_{IIIB} gp120 (A), HIV-1_{MN} gp120 (B), and HIV-1_{IIIB} p24 antigens (C) were shown. Results are expressed as the means from triplicate assays in 3 different experiments.

are initially activated in the xenogenic environment and then become anergic.[14] In contrast, SCID-hu (Thy/Liv) mice are more susceptible to X4 than to R5 strains[6] because HIV-1 infection is restricted mainly to the engrafted thymus that is primarily comprised of immature T cells, suggesting that this model may not be practical overt HIV infection. Our study represents the first attempt to infect NOG mice that received transplants of human hematopoietic stem cells with HIV-1. Very similar infection rates were seen for both R5 and X4 strains in the mouse model. Flow cytometry revealed both $CXCR4^+CD4^+$ and $CCR5^+CD4^+$ cells in PB, the spleen, and BM, but only CXCR4 on thymic $CD4^+$ T cells. It also showed the scattering of human macrophages, known to be susceptible to R5-tropic HIV-1 strains[28,29] and the source of HIV-1,[23,30-32] throughout various organs. $p24^+$ macrophage-like cells were detected in these organs after R5-tropic HIV-1_{JRCSF} infection. These data may help explain the susceptibility of hNOG mice to both R5- and X4-tropic HIV strains and also shed light on the active replenishment of these target cells in mice.

SCID mouse systems have been actively used in the evaluation of anti–HIV-1 drugs.[9,11,21] In most cases, HIV-1 detection levels reach a peak within a month after inoculation and level off, accompanied by $CD4^+$ T-cell depletion.[3,12,13] Although suitable for short-term experiments, it is also true that these models require large numbers of mice because of large variations in infection efficiency. In contrast, very stable infections were noted in our hNOG mice that were inoculated with a high dose of HIVs. They did not show rapid CD4/CD8 decrease in spite of high levels of viremia persisting for more than 40 days. Efficient hematopoiesis and thymopoiesis of human cells probably compensated for the loss of $CD4^+$ T cells, allowing for persistent infection. This capacity of the hNOG mouse system makes it attractive as a model for the long-term evaluation of anti–HIV-1 drugs. In addition to destroying mature blood cells, altered hematopoiesis in BM and the thymus has also been reported to be responsible for immunodeficiency in patients with AIDS.[33,34] To study hematopoietic abnormalities in HIV-1 infection, both SCID-hu (Thy/Liv) mice[8,35,36] and SIV- or SHIV-infected macaque models[20,37-39] have been used. The current hNOG mouse system, in which human cells are efficiently reproduced from stem cells and then settled into hematopoietic organs, offers a promising model for the study of events that occur after infection not only with R5-tropic HIV-1 but also with X4-tropic HIV-1. Indeed, the BM of hNOG mice infected with R5-tropic HIV-1 exhibited exceptionally elevated levels of HIV-DNA copies. On the other hand, the thymus of X4-tropic HIV-1_{MNp}–infected hNOG mice yielded large numbers of HIV-DNA copies, which seemed to correlate with the predominant expression of CXCR4 on the thymocytes. Thus, further observation is essential to address whether AIDS symptoms such as considerable $CD4^+$ T-cell depletion and hematopoietic abnormalities eventually occur in these mice.

It is noteworthy that human antibodies against both HIV-1 Env gp120 and Gag p24 antigens were detected in mice no. 136-3, no. 157-3, and no. 157-4 after exposure to high titers of HIV-1, suggesting that hNOG mice have the ability to respond to HIV-1 antigens. This encourages us to develop antibody-based HIV vaccine candidates, although additional modifications are required for the stable induction of immune responses. Importantly, since the seroconverted mice showed high viremia and high numbers of proviral DNA copies in the spleen, BM, and thymus, abundant viral production may stimulate human B-cell responses against HIV-1 and generate specific antibodies. These mice showed little or no detectable human IgG against HIV-1, as determined by Western blot analysis (data not shown), suggesting that very low levels of class-switching occurred in these mice, though further study is required.

In addition to the humoral immune responses, the induction of primary T-cell responses is critical for the study of HIV-specific immune responses and pathogenesis, as well as for vaccine development. Although we did not demonstrate the T-cell ability to respond to virus antigens, human T cells from the spleen proliferated when stimulated with anti–human CD3 antibodies (data not shown), indicating that the human T cells in the NOG mice that received transplants of hematopoietic stem cells are capable of responding to T-cell receptor–mediated signals and are expected to be able to elicit primary antigen-specific immune responses against foreign antigens. To address whether the specific T-cell responses may be induced will be one of the important studies.

In conclusion, the NOG mice that received transplants of human hematopoietic stem cells successfully achieved systemic and persistent infection with both R5-tropic and X4-tropic HIV-1, and generated humoral immune responses against HIV-1. These capacities of the hNOG mouse model may be very attractive for the study of HIV pathogenesis and humoral immune responses induced by HIV vaccine candidates.

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Authorship

Contributions: S.W., K.T., N.S., M.H., and N.Y. designed the study; S.W., K.T., S.O., S.H., M.Y., Y.S., M.Z.D., and Z.Y. carried out the research; M.I. contributed live mice; S.W., K.T., and T.M. analyzed the data; N.S., M.H., and N.Y. controlled the data; S.W. wrote the paper; and all authors checked the final version of the manuscript.

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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$^\nabla$

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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.

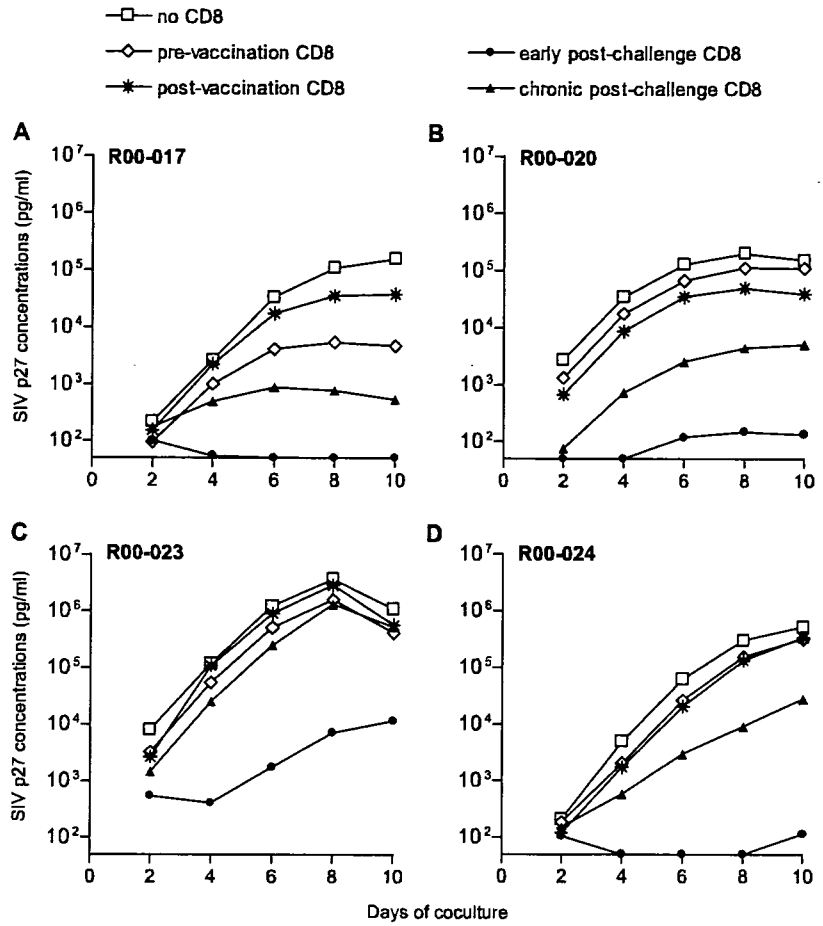
Macaques R00-023 and R00-024 were euthanized around 2 years after SHIV89.6PD challenge, while macaques R00-017 and R00-020 were followed up for more than 2 years. The latter two animals received monoclonal anti-CD20 antibody administration for $CD20^+$ cell depletion (starting at week 166 in R00-017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



	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

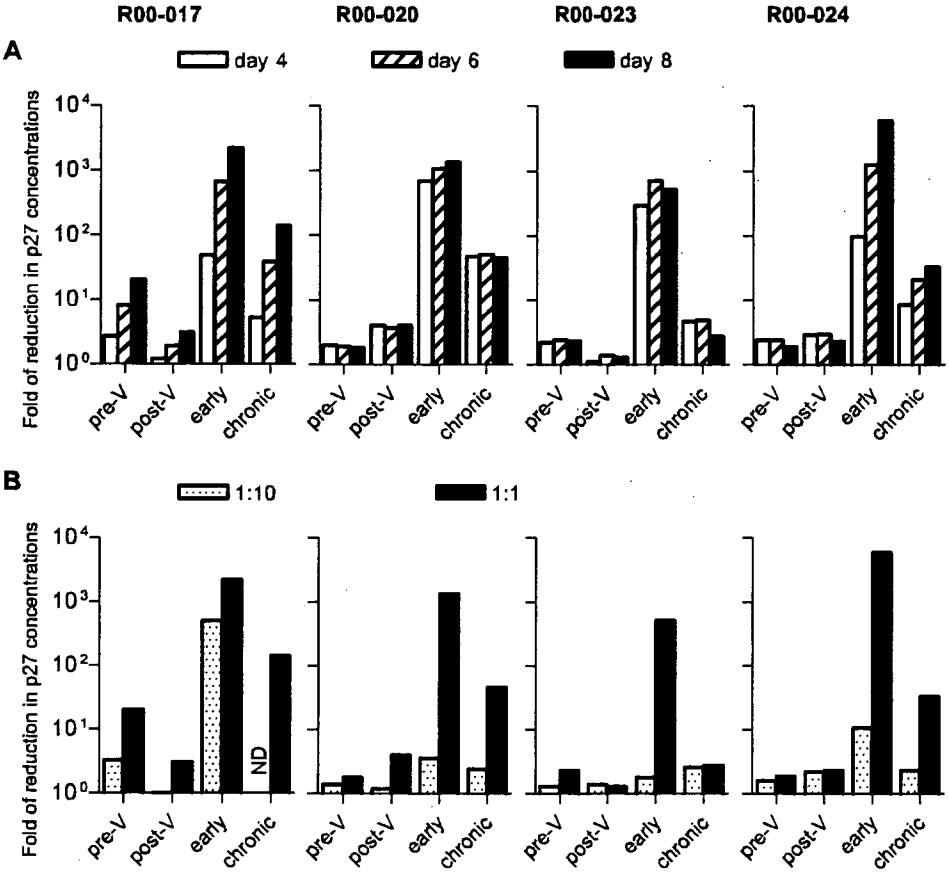


FIG. 2. Reduction in SIVmac239 production by addition of $CD8^+$ cells. The reduction (fold) in p27 concentration in the supernatant from coculture of SIV-infected $CD8^-$ cells with each group of $CD8^+$ cells compared to that from SIV-infected $CD8^-$ cell culture without $CD8^+$ cells is shown. (A)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

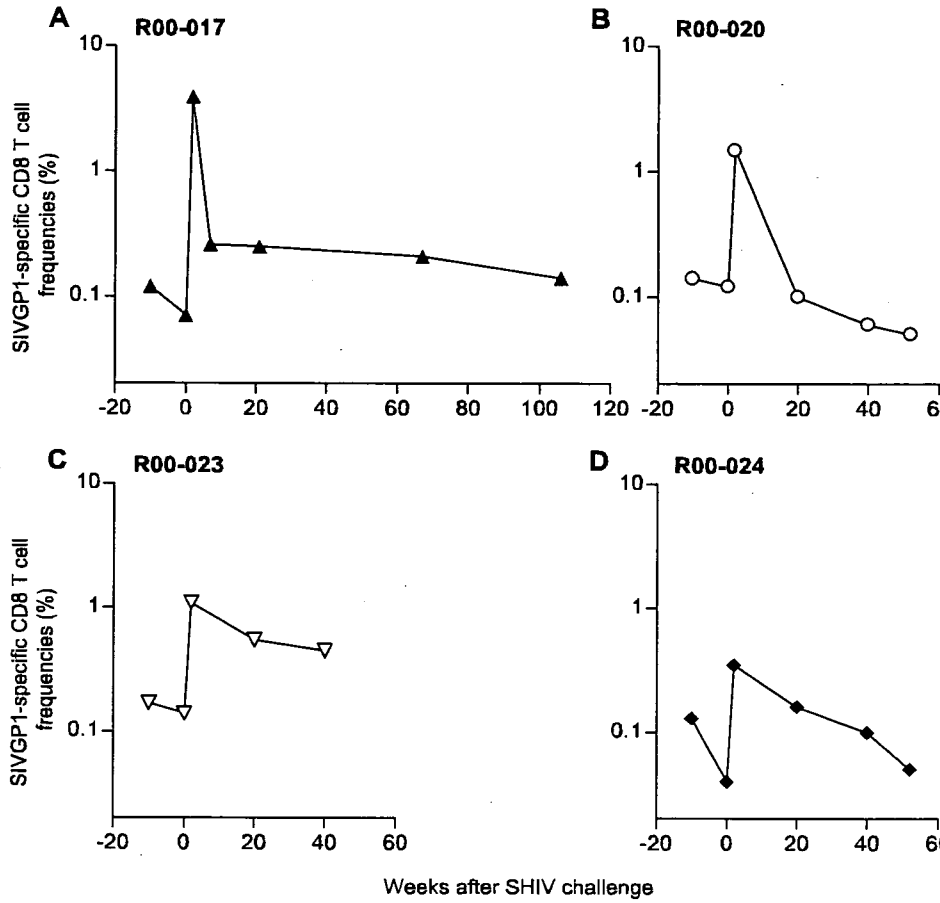


FIG. 3. SIVGP1-specific $CD8^+$ T-cell frequencies in macaques before and after SHIV89.6PD challenge. Frequencies of $CD8^+$ T cells showing SIVGP1-specific IFN-γ induction per total $CD8^+$ T cells in PBMCs are shown. The first time point prechallenge is 10 weeks before challenge.

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

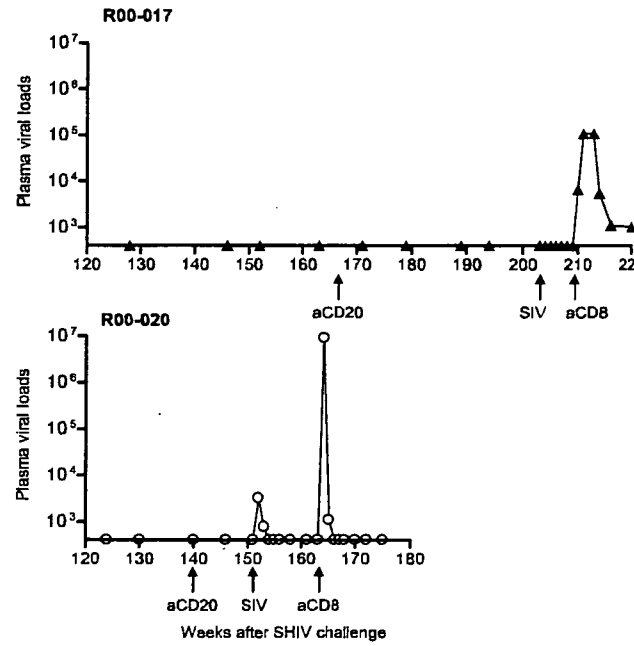


FIG. 4. Plasma viral loads (SIV *gag* RNA copies/ml plasma) in macaques R00-017 (upper panel) and R00-020 (lower panel) after week 120 post-SHIV challenge. aCD20 and aCD8, anti-CD20 and anti-CD8, respectively.

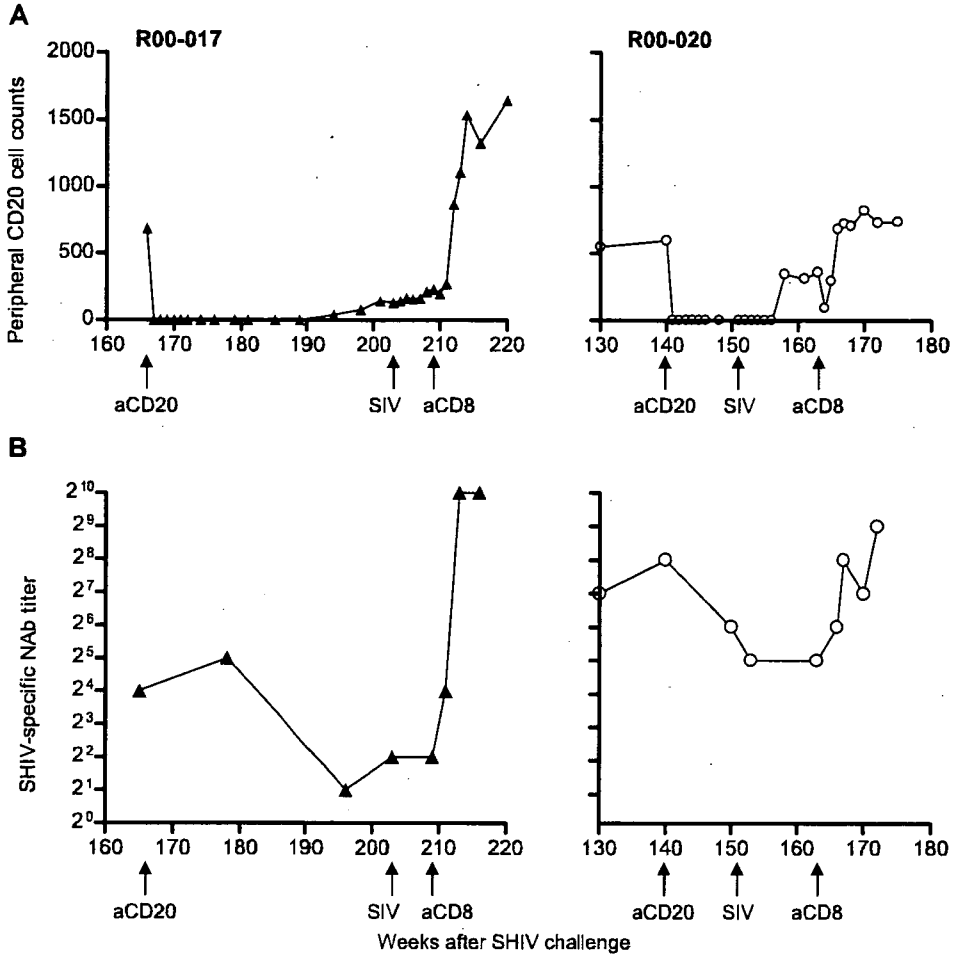


FIG. 5. Changes in SHIV89.6PD-specific NAb levels after monoclonal anti-CD20 antibody administration at week 166 in macaque R00-017 (left panels) and at week 140 in macaque R00-020 (right panels). (A) Peripheral $CD20^+$ cell counts (per μl). (B) SHIV89.6PD-specific neutralizing titers in plasma. aCD20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

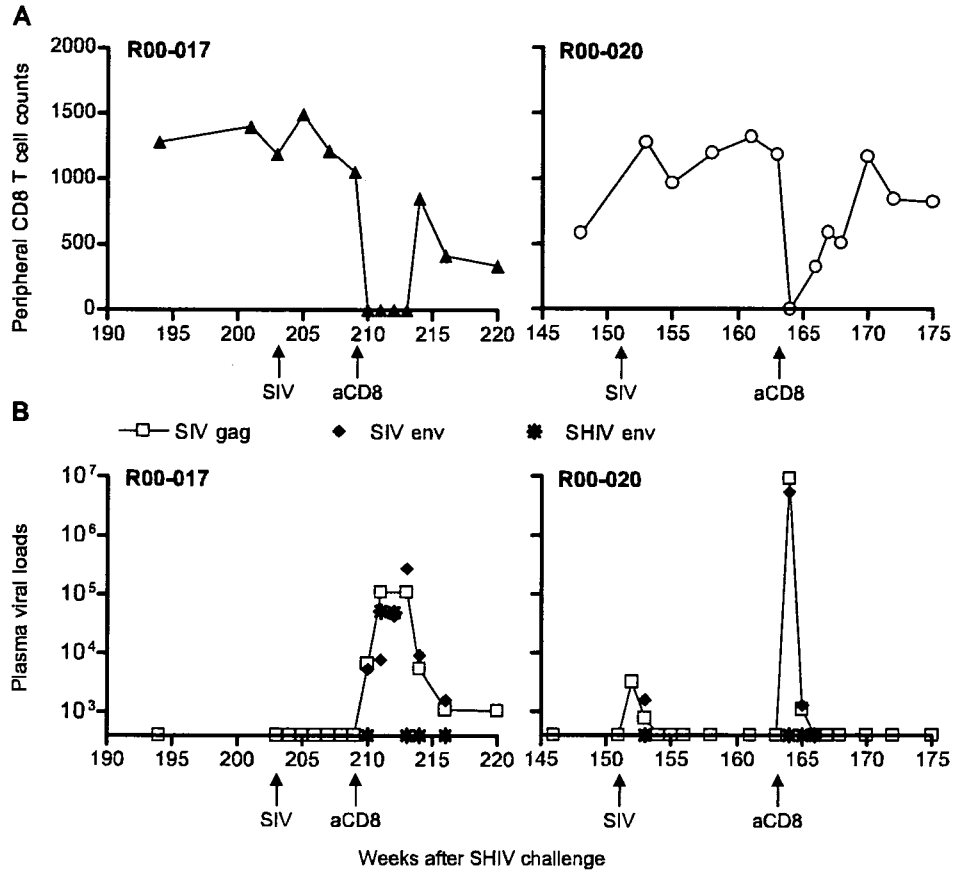


FIG. 6. SIVmac239 superchallenge and $CD8^+$ cell depletion in macaques R00-017 and R00-020. Macaque R00-017 received SIVmac239 superchallenge at week 203 and monoclonal anti-CD8 (aCD8) antibody administration starting at week 209, while macaque R00-020 received superchallenge at week 151 and anti-CD8 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 $CD8^+$

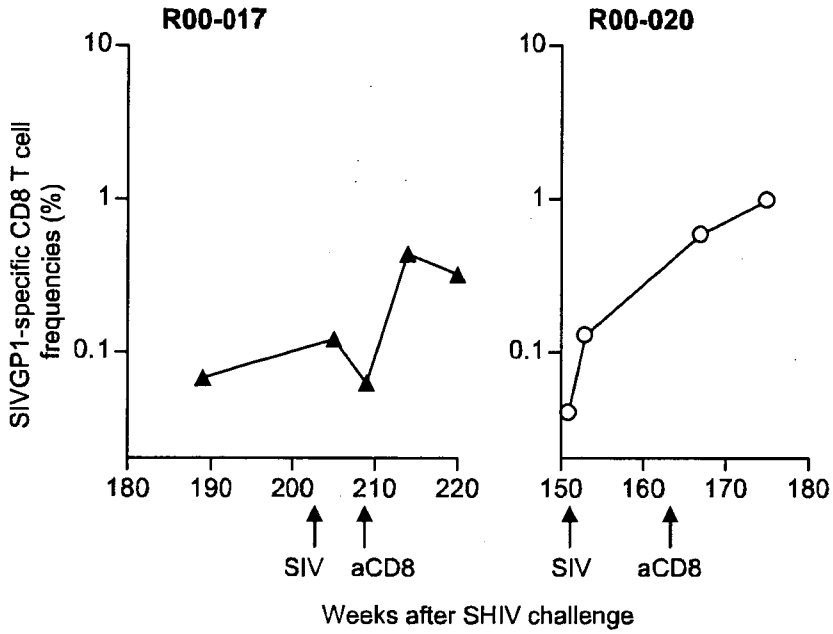


FIG. 7. SIVGP1-specific $CD8^+$ T-cell frequencies in macaques R00-017 (left panel) and R00-020 (right panel) before and after SIVmac239 superchallenge. Frequencies of $CD8^+$ T cells showing SIVGP1-specific IFN-γ induction per total $CD8^+$ T cells in PBMCs are shown. aCD8, anti-CD8.

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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Post-Infection Immunodeficiency Virus Control by Neutralizing Antibodies

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Background. Unlike most acute viral infections controlled with the appearance of virus-specific neutralizing antibodies (NAbs), primary HIV infections are not met with such potent and early antibody responses. This brings into question if or how the presence of potent antibodies can contribute to primary HIV control, but protective efficacies of antiviral antibodies in primary HIV infections have remained elusive; and, it has been speculated that even NAb induction could have only a limited suppressive effect on primary HIV replication once infection is established. Here, in an attempt to answer this question, we examined the effect of passive NAb immunization post-infection on primary viral replication in a macaque AIDS model. *Methods and Findings.* The inoculums for passive immunization with simian immunodeficiency virus mac239 (SIVmac239)-specific neutralizing activity were prepared by purifying polyclonal immunoglobulin G from pooled plasma of six SIVmac239-infected rhesus macaques with NAb induction in the chronic phase. Passive immunization of rhesus macaques with the NAbs at day 7 after SIVmac239 challenge resulted in significant reduction of set-point plasma viral loads and preservation of central memory CD4 T lymphocyte counts, despite the limited detection period of the administered NAb responses. Peripheral lymph node dendritic cell (DC)-associated viral RNA loads showed a remarkable peak with the NAb administration, and DCs stimulated in vitro with NAb-preincubated SIV activated virus-specific CD4 T lymphocytes in an Fc-dependent manner, implying antibody-mediated virion uptake by DCs and enhanced T cell priming. *Conclusions.* Our results present evidence indicating that potent antibody induction post-infection can result in primary immunodeficiency virus control and suggest direct and indirect contribution of its absence to initial control failure in HIV infections. Although difficulty in achieving requisite neutralizing titers for sterile HIV protection by prophylactic vaccination has been suggested, this study points out a possibility of non-sterile HIV control by prophylactic vaccine-induced, sub-sterile titers of NAbs post-infection, providing a rationale of vaccine-based NAb induction for primary HIV control.

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INTRODUCTION

In the natural courses of HIV infections, the host immune responses fail to contain the virus replication and allow persistent plasma viremia. While virus-specific cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on primary HIV replication [1–7], the contribution of virus-specific antibodies in clearance of primary HIV infection has remained unclear [8].

Neutralizing antibodies (NAbs) play a central role in control of most viral infections, but in HIV infections, NAb induction is not efficient in the early phase due to its unusual neutralization-resistant nature, such as the sophisticated masking of neutralizing epitopes in HIV envelope [8–11], and protective efficacies of post-infection NAbs in vivo have remained elusive. While evidence of virus escape implies NAb selective pressure to a certain extent [10,12–13], it has been speculated that post-infection NAbs could exert only a limited suppressive effect on primary HIV replication [14–16].

Post-infection passive NAb immunization studies in macaque AIDS models would contribute to elucidation of its protective role, in complementation with studies determining the requisites for sterile protection by pre-challenge administered NAb titers [14,16–21]. A model of CCR5-tropic simian immunodeficiency virus (SIV) infection that induces acute loss of memory $CD4^+$ T cells like HIV infections in humans [22–25] would be adequate for assessment of post-infection NAb efficacies in primary immunodeficiency virus infection.

In the present study, we examined the effect of passive NAb immunization at day 7 post-challenge on primary viral replication in a macaque AIDS model of CCR5-tropic SIVmac239 infection. Remarkably, our analysis revealed control of primary SIVmac239 replication by the passive NAb immunization post-infection.

METHODS

Animal experiments

Burmese rhesus macaques (*Macaca Mulatta*) were maintained in accordance with the Guideline for Laboratory Animals of National Institute of Infectious Diseases and National Institute of Biomedical Innovation. Major histocompatibility complex class I (MHC-I) haplotypes were determined by reference strand-mediated conformation analysis as described previously [6,26]. Blood collection, vaccination, virus challenge, passive immunization, and lymph node biopsy were performed under ketamine anesthesia. For vaccination, animals intramuscularly received a priming with 5 mg of CMV-SHIVdEN DNA encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV-1_{DH12}

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chimeric Vpr, and HIV-1_{DH12} Tat and Rev, followed by an intranasal booster six weeks later with 1×10^8 CIU (cell infectious units) of replication-competent Sendai virus expressing Gag (SeV-Gag) in macaque V5 or 6×10^9 CIU of F-deleted replication-defective SeV-Gag in other vaccinees as described previously [6]. Animals were challenged intravenously with 1,000 $TCID_{50}$ (50 percent tissue culture infective dose) of SIVmac239, three months after booster in case of vaccinees. For passive immunization, animals were intravenously administered with 300 mg of anti-SIV immunoglobulin G (IgG) or control IgG at day 7 post-challenge.

Antibody preparation

Pools of plasma showing SIVmac239-specific NAb titers of 1:4 to 1:64 were obtained from six SIVmac239-infected rhesus macaques with NAb induction in the chronic phase for preparing the IgG inoculums for passive NAb immunization. IgG was purified from the plasma after heat-inactivation and filtration by Protein G Sepharose 4 Fast Flow (Amersham) and concentrated by Amicon Ultra 4, MW50000 (Millipore) to 30 mg/ml. This IgG solution had SIVmac239-specific NAb titer of 1:16; i.e., 5 μl of 16-fold-diluted antibodies killed 5 μl of 10 $TCID_{50}$ SIVmac239 on MT-4 cells. Control IgG was prepared from non-infected rhesus macaques. Neutralizing F(ab')2 was obtained by pepsin digestion with Immunopure F(ab')2 purification kit (Pierce).

Quantitation of plasma viral loads

Plasma RNA was extracted using High Pure Viral RNA kit (Roche Diagnostics). Serial five-fold dilutions of RNA samples were amplified in quadruplicate by reverse transcription and nested PCR using SIVmac239 *gag*-specific primers to determine the end point. Plasma SIV RNA levels were calculated according to the Reed-Muench method as described previously [6]. The lower limit of detection is approximately 4×10^2 copies/ml.

Measurement of virus-specific neutralizing titers

Serial two-fold dilutions of heat-inactivated plasma or purified antibodies were prepared in duplicate and mixed with 10 $TCID_{50}$ of SIVmac239. In each mixture, 5 μl of diluted sample was incubated with 5 μl of virus. After 45-min incubation at room temperature, each 10-μl mixture was added into 5×10^4 MT-4 cells/well in 96-well plates. Day 12 culture supernatants were harvested and progeny virus production was examined by ELISA for detection of SIV p27 core antigen (Beckman-Coulter) to determine 100% neutralizing endpoint. The lower limit of titration is 1:2.

Measurement of virus-specific T-cell responses

Virus-specific T-cell levels were measured by flow-cytometric analysis of gamma interferon (IFN-γ) induction as described previously [6]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines infected with a vesicular stomatitis virus G (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 simian-human immunodeficiency virus (SHIV) molecular clone DNA. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (Becton Dickinson). Fluorescein isothiocianate-conjugated anti-human CD4, 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 non-specific IFN-γ^+ T-cell frequencies from those after SIV-specific stimulation. Specific T-cell levels less than 100 cells per million PBMC are considered negative.

Quantitation of cell-associated viral loads

Right and left inguinal lymph nodes and right and left axillary lymph nodes were obtained from macaques by biopsy at days 7, 8, 10, and 14 post-challenge, respectively. For measurement of dendritic cell (DC)-associated viral loads, $CD1c^+$ DCs were positively selected to over 99% purity using a macaque $CD1c^+$ DC magnetic sorting system (Miltenyi Biotech) from $CD20^-$ lymphocytes negatively-selected from lymph nodes. $CD1c^-CD20^-$ cells were used for measurement of non-DC-associated viral loads. Cell-associated viral RNA was extracted using RNeasy kit (Qiagen) and quantitated by LightCycler real-time PCR system (Roche Diagnostics) using SIV *gag*-specific primers and probes. The lower limit of detection is approximately 1,000 copies/10^6 cells.

Antigen presentation assay in vitro

PBMCs obtained in the chronic phase from SIVmac239-controllers were attached to culture plates for 4 h, and adhesive cells were cultured in the presence of 50 ng/ml GM-CSF (R&D Systems) and 5 ng/ml IL-4 (R&D Systems) for 5 days to obtain $CD1c^+CD83^+CD86^+HLA\text{-}ABC^+HLA\text{-}DR^+$ immature DCs [27]. Alternatively, $CD1c^+$ DCs were positively selected from CD20-depleted PBMCs as described above. For antigen presentation assay, 1×10^5 of the in vitro-generated DCs (Exp. 1, 2, and 3) or the positively-selected $CD1c^+$ DCs (Exp. 4) were pulsed for 17 h with 2,000 $TCID_{50}$ of SIVmac239 (corresponding to 2×10^6 SIV RNA copies and 3 ng of SIV p27) alone or preincubated for 45 min with 1.5 mg of either control IgG, neutralizing IgG, or neutralizing F(ab')2. Autologous PBMCs were cocultured with these pulsed DCs and then subjected to measurement of specific IFN-γ induction.

Statistical analysis

Statistical analysis was performed by Prism software version 4.03 (GraphPad Software, Inc.). Set point plasma viral loads and peripheral $CD95^+CD28^+$ central memory $CD4^+$ T-cell counts around 3 months after challenge of the naive controls ($n = 7$) and NAb-immunized macaques ($n = 4$) were log-transformed for improvement of normality and compared by two-tailed unpaired t test with significance levels set at $p<0.05$. Then their geometric means with 95% confidence interval were calculated. Due to the limited number of samples for each group providing difficulty for their normality testing, the two groups were additionally compared by nonparametric Mann-Whitney U test for confirmation of results. No significant difference in $CD95^+CD28^+$ central memory $CD4^+$ T-cell counts just before challenge was observed between the two groups ($p = 0.68$ by unpaired two-tailed t test with Welch's correction and $p = 0.31$ by Mann-Whitney U test) (data not shown).

RESULTS

SIV control by post-infection passive NAb immunization

While most SIVmac239-infected naive macaques usually fail to elicit NAb responses during the early phase of infection, some acquire detectable levels of NAbs against the challenge strain in the late phase. IgG purified from plasma pools of such SIVmac239-infected macaques with NAb induction, showing in

vitro SIVmac239-specific neutralizing activity of 1:16, was used for passive immunization as polyclonal anti-SIV NAbs. In the first part of this study, naive Burmese rhesus macaques were challenged intravenously with SIVmac239 followed by passive immunization with 10 ml of the polyclonal NAbs (300 mg IgG) at day 7 post-challenge (Figure 1A). Seven naive control macaques challenged with SIVmac239, including two infused with non-SIV-specific control antibodies, all failed to contain viral replication with persistent viremia (Figure 1B). These macaques showed peak plasma viral loads between days 7 and 14 post-challenge and most had set-point viral loads exceeding 1×10^4 SIV RNA copies/ml plasma. In contrast, four rhesus macaques passively immunized at day 7 with polyclonal NAbs showed significantly lower plasma viral RNA loads ($p=0.0033$ by unpaired t test and $p=0.0061$ by Mann-Whitney U test) compared with naive controls around 3 months post-challenge (Figures 1B&1C). Two of the NAb-immunized macaques, NA1 and NA4, controlled SIV replication with undetectable set-point plasma viremia. Thus, post-infection passive immunization of macaques with polyclonal NAbs had a significant suppressive effect on set-point viral replication.

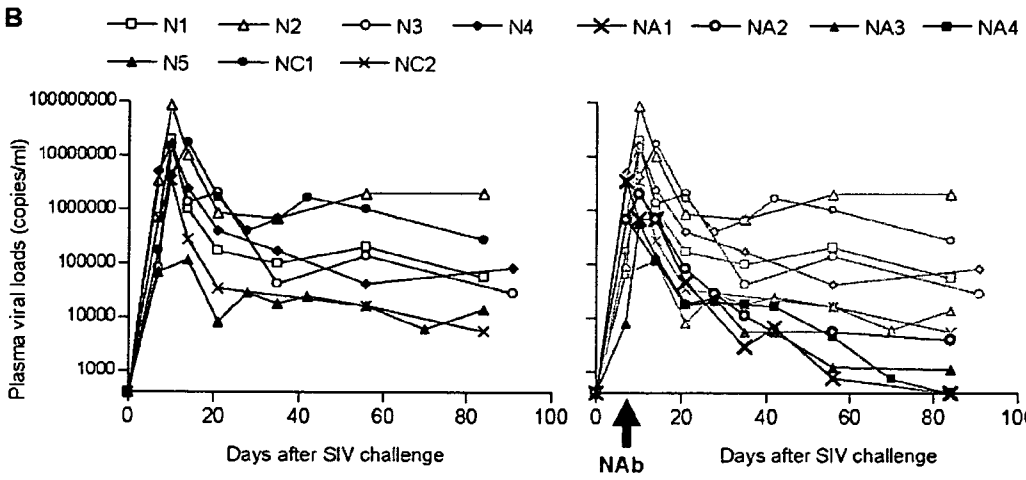
A

NAb Tx in unvaccinated macaques

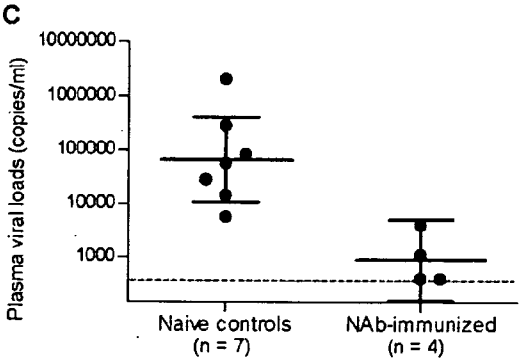
Group/macaque	Ab Tx at day 7
Naive controls	
N1*	-
N2*	-
N3*	-
N4*	-
N5	-
NC1	control Ab
NC2	control Ab

Group/macaque	Ab Tx at day 7
NAb-immunized	
NA1	anti-SIV NAb
NA2	anti-SIV NAb
NA3	anti-SIV NAb
NA4	anti-SIV NAb

B



C



D

NAb responses

Macaque	day 7	day 10	day 14	day 35	day 84
N1	-	-	-	-	-
N2	-	-	-	-	-
N3	-	-	-	-	-
N4	-	-	-	-	-
N5	-	-	-	-	-
NC1	-	-	-	-	-
NC2	-	-	-	-	-
NA1	-	+	+	-	-
NA2	-	+	-	-	-
NA3	-	+	-	-	-
NA4	-	+	-	-	-

Figure 1. Effect of post-challenge passive NAb immunization on primary SIV infection. (A) List of naive controls and NAb-immunized macaques. Experiments using macaques indicated by asterisk have previously been performed [6]. (B) Plasma viral loads after SIVmac239 challenge (SIV RNA copies/ml). Left panel, naive controls; right panel, NAb-immunized macaques shown by red lines and naive controls by gray lines for comparison. (C) Statistical analysis of plasma viral loads around 3 months post-challenge between naive controls (n = 7) and NAb-immunized macaques (n = 4). The geometric mean (indicated by the longer bar) of viral loads in naive controls is 6.5×10^4 copies/ml, and its 95% confidence interval (indicated by the shorter bars) is $1.1\times10^4-4.0\times10^5$ copies/ml. The geometric mean in NAb-immunized macaques is 9.1×10^2 copies/ml, and its 95% confidence interval is $1.6\times10^2-5.1\times10^3$ copies/ml. The difference between the two groups was statistically significant by unpaired two-tailed t test ($p=0.0033$) and by non-parametric Mann-Whitney U test ($p=0.0061$). Viral loads of macaques NA1 and NA4 were calculated as the lower limit of detection shown as the dashed line (400 copies/ml). (D) Plasma NAb responses after challenge. (+), positive; (−), negative. All detected titers were no more than 1:2.
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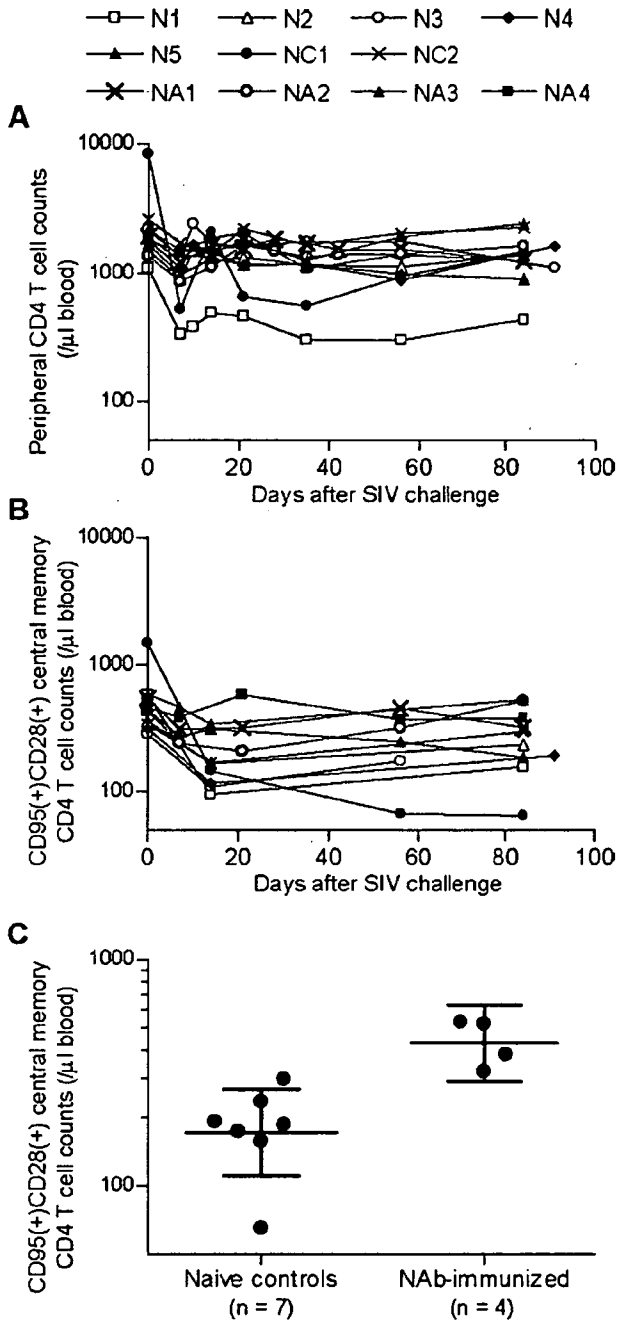

Figure 2. Central memory CD4$^+$ T-cell counts in naive controls and NAb-immunized macaques. (A) Peripheral CD4$^+$ T-cell counts (cells/μl). (B) Peripheral CD95$^+$CD28$^+$ central memory CD4$^+$ T-cell counts (cells/μl) [28]. (C) Statistical comparison of CD28$^+$CD95$^+$ central memory CD4$^+$ T-cell counts around 3 months post-challenge. The geometric mean (indicated by the longer bar) of central memory CD4$^+$ T-cell counts in naive controls is 1.7×10^2 counts/μl, and its 95% confidence interval (indicated by the shorter bars) is $1.1\times10^2-2.7\times10^2$ counts/μl. The geometric mean in NAb-immunized macaques is 4.3×10^2 counts/μl, and its 95% confidence interval is $2.9\times10^2-6.3\times10^2$ counts/μl. The difference between the two groups was statistically significant by unpaired two-tailed t test ($p=0.0066$) and by non-parametric Mann-Whitney U test ($p=0.0061$).
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Immune parameters in NAb-immunized macaques

Plasma NAb responses in the NAb-immunized macaques were detected marginally at day 10 post-infection but became undetectable within one week after the passive NAb immunization (Figure 1D), implying that the NAbs were rapidly exhausted for virus clearance. None elicited detectable *de novo* NAb responses past then. In the naive controls, no SIVmac239-specific NAbs were detected throughout the course. This discrepancy between the transient NAb detection and the persistent viremia control in the NAb-immunized macaques differed from previously-reported, dose-dependent establishment of sterile protection from CXCR4-tropic SHIV infection by pre-challenge passive NAb immunization [18–21].

Difference in total CD4$^+$ T-cell counts was not found throughout the course between the two groups (Figure 2A). Reductions in peripheral CD95$^+$ CD28$^+$ central memory CD4$^+$ T-cell counts [28–29] were observed in the naive controls after SIV challenge (Figure 2B). The NAb-immunized macaques, however, showed significantly higher central memory CD4$^+$ T-cell counts around 3 months post-challenge than those in the naive controls ($p=0.0066$ by unpaired t test and $p=0.0061$ by Mann-Whitney U test) (Figures 2B&2C), suggesting amelioration of central memory CD4$^+$ T-cell loss in the early phase of SIV infection by transient NAb responses around week 1 post-challenge. All of these NAb-immunized macaques showed efficient virus-specific CD8$^+$ T-cell induction at week 8 (Figure 3), although difference in the levels between the two groups was not significant, implying its possible enrollment in the observed viral control.

Post-infection passive NAb immunization in vaccinees

Our previous trial of a DNA-prime/SeV-Gag vector-boost vaccine in Burmese rhesus macaques has shown vaccine-based, NAb-independent control of SIVmac239 replication, suggesting association of MHC-I haplotype with this control [6,30]. We then examined possible synergy of post-challenge passive NAb immunization with the prophylactic CTL-based vaccination in suppression of SIV replication in two groups of macaques possessing MHC-I haplotype *90-088-Ij* and *90-120-Ia*, respectively (Figure 4A). In the former group of macaques possessing *90-088-Ij*, vaccinees failed to control SIV replication even after passive NAb immunization (Figure 4B). In the latter group of macaques possessing *90-120-Ia*, all 4 vaccinees without NAb immunization controlled SIVmac239 replication and had undetectable plasma viral loads after week 8 post-challenge (Figure 4B). All of them rapidly selected for a mutation escaping from $Gag_{206-216}$ epitope-specific CTL by week 5, suggesting a strong selective pressure on the virus by this CTL [6]. As for the

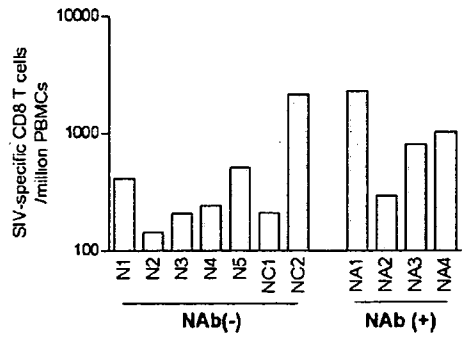

Figure 3. SIV-specific CD8$^+$ T-cell frequencies at week 8 post-challenge in naive controls and NAb-immunized macaques.
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two vaccinees VA2 and VA3 infused with NAbs, plasma viremia became undetectable by week 5 and rapid selection of CTL escape mutation was not observed (data not shown). SIV-specific $CD8^+$ T-cell frequencies at week 2 in the NAb-immunized vaccinees VA2 and VA3 were comparable with the vaccinees without NAb immunization, while SIV-specific $CD4^+$ T-cell induction at week 2 was observed in just one (V5) of the four vaccinees without NAb but in both of the NAb-immunized vaccinees (Figure 4C). These results suggest, even in the NAb-immunized vaccinees, a dominant effect of vaccine-induced cellular immune responses on control of SIV replication, although implying a possibility of NAb-mediated augmentation of CTL vaccine-based viral control.

Antibody-mediated virion uptake by DCs and T cell priming

In order to assess the possibility of altered virus distribution by NAbs, $CD1c^+$ DCs were isolated from peripheral lymph nodes of unvaccinated, SIVmac239-challenged macaques before and after passive NAb immunization, and DC-associated SIV RNA levels were quantified at the initial stage of infection. In three naive control macaques, accumulation of viral RNA to $CD1c^+$ DCs was undetectable at days 7, 8, and 10 post-challenge but became detectable at day 14 (Figure 5A). This elevation of DC-associated viral loads following peak viremia was consistent with previous immunohistochemistry reports on SIV and HIV-2 challenge experiments [31–32]. In marked contrast, both of macaques NA3 and NA4 immunized with NAbs at day 7 post-challenge showed immediate accumulation of viral RNA in $CD1c^+$ DCs at day 8 (one day after NAb immunization), suggesting antibody-mediated virion accumulation to DCs in vivo. Cell-associated viral loads in $CD1c^-CD20^-$ non-DCs were at comparable levels between the two groups, indicating that the rapid increase in DC-associated viral loads after NAb immunization was not due to changes in viral loads in lymph nodes.

Then an in vitro antigen presentation assay was performed to assume whether the early viral RNA accumulation in DCs could

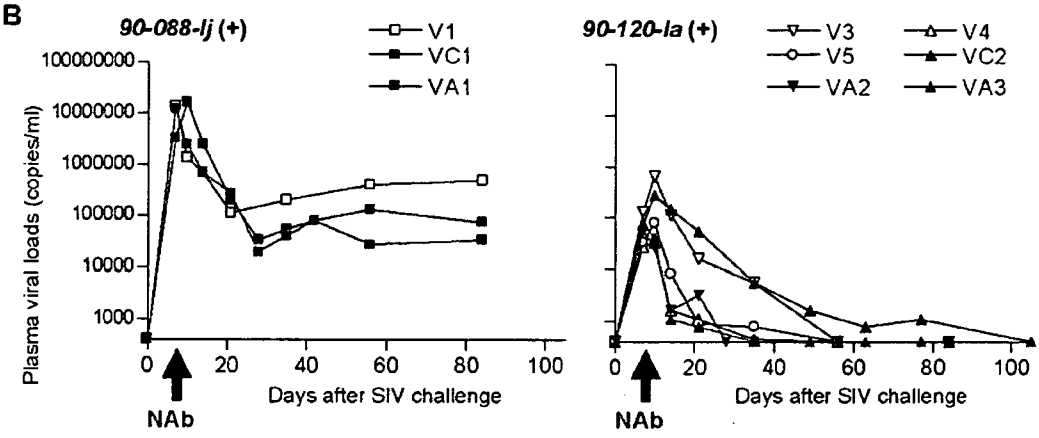
A

NAb Tx in vaccinees

MHC-I haplotype *90-088-Ij*-positive macaque	Ab Tx at day 7
V1*	-
VC1	control Ab
VA1	anti-SIV NAb

MHC-I haplotype *90-120-Ia*-positive macaque	Ab Tx at day 7
V3*	-
V4*	-
V5*	-
VC2	control Ab
VA2	anti-SIV NAb
VA3	anti-SIV NAb

B



C

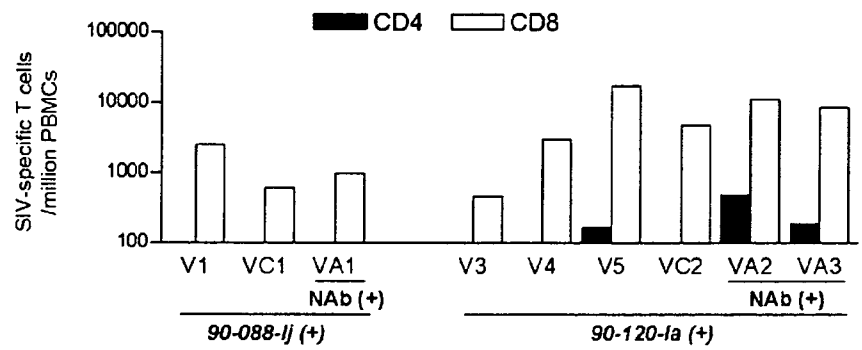


Figure 4. Effect of post-challenge passive NAb immunization in vaccinees. (A) List of vaccinees with or without passive immunization. (B) Plasma viral loads after challenge (SIV RNA copies/ml). Left panel, MHC-I haplotype *90-088-Ij*-positive macaques; right panel, *90-120-Ia*-positive macaques. Red lines represent NAb-immunized vaccinees. (C) SIV-specific $CD4^+$ T-cell and $CD8^+$ T-cell frequencies at week 2 post-challenge.
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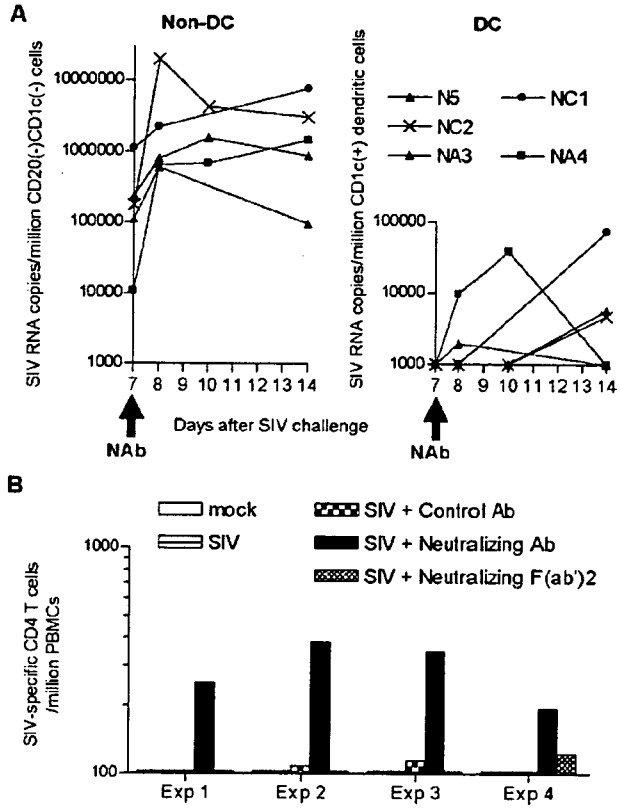

Figure 5. Antibody-mediated SIV uptake by DCs and T cell priming. (A) Peripheral lymph node-derived non-DC ($CD1c^-CD20^-$ lymphocytes)-associated (left panel) and $CD1c^+CD20^-$ DC-associated viral loads (right panel). (B) In vitro antigen presentation assay. Either in vitro-generated DCs (Exp. 1, Exp. 2, and Exp. 3) or positively-selected $CD1c^+$ DCs (Exp. 4) prepared from PBMCs were pulsed with SIV alone (SIV), SIV preincubated with control antibodies (SIV+Control Ab), SIV preincubated with NAbs (SIV+Neutralizing Ab), or SIV preincubated with Fc-depleted NAbs (SIV+Neutralizing F[ab']2). Autologous PBMCs were cocultured with these pulsed DCs and then subjected to measurement of specific IFN-γ induction.
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represent a correlation to T cell priming. DCs prepared from peripheral blood of macaques that controlled SIVmac239 replication were pulsed with antibody-neutralized SIV, and autologous PBMCs were cocultured with these pulsed DCs for measurement of specific IFN-γ induction. In all four sets of experiments, efficient IFN-γ induction in $CD4^+$ T cells was observed after stimulation by DCs pulsed with SIV preincubated with NAb but not by DCs pulsed with SIV alone, SIV preincubated with control antibodies, or SIV preincubated with Fc-depleted neutralizing F(ab')2 (Figure 5B). Efficient IFN-γ induction in $CD8^+$ T cells was not observed even after coculture with NAb-preincubated SIV-pulsed DCs except for one (Exp. 4). Overall, augmentation of virus-specific T-cell stimulation was observed by the coexistence of NAbs, suggesting their involvement in antigen presentation.

DISCUSSION

The present study showed suppression of primary SIV replication by passive NAb immunization post-infection, suggesting a possibility of HIV control by potent antibody induction during the acute phase of infection. It reversely follows that its absence may be involved in an increase in the burden of acute infectious viral loads and abrogation of virus-specific cellular immune responses, leading to initial control failure in HIV infections.

While this study does not exclude possibilities of additional antibody-mediated protective mechanisms such as antibody-dependent cell-mediated cytotoxicity or recently-reported complement virolysis [33], the non-sterile but consistent viral control at the set point by passive NAb immunization despite only transient detection of NAb responses during the acute phase coheres with involvement of cellular immune responses in this control [27,34–36]. Thus, results may provide additional interpretations to previous NAb passive immunization studies [14,16–21], which have mostly utilized CXCR4-tropic SHIV-challenged macaques and shown sterile protection by high titers of pre-challenge or very early post-challenge NAbs.

A technical confinement of this study is the use of polyclonal antibodies which may include not only NAbs but also non-neutralizing anti-SIV antibodies for passive immunization. However, our finding of primary SIV control by post-infection passive immunization with the anti-SIV inoculums with neutralizing activity presents significant evidence suggesting that potent antibodies post-infection can contribute to control of primary immunodeficiency virus infection. Whether neutralizing activity is required for the enhanced SIV control by passive immunization remains to be assessed in future studies. Our in vitro results suggest a possibility of virus-specific $CD4^+$ T-cell activation by NAbs, and neutralizing activity may contribute to protection of these virus-specific $CD4^+$ T cells from SIV *trans*-infection via DCs [37–38], possibly counteracting the abrogation of the optimal concert of adaptive immunity between $CD4^+$ T and $CD8^+$ T cells usually observed in the natural course of pathogenic immunodeficiency virus infection [23,25,39]. The possibility of failure in antibody-mediated priming of effective cellular immune responses by preexisting vaccine-induced dominant responses may account for lack of viral control in the NAb-immunized vaccinee possessing MHC-I haplotype *90-088-Ij*.

Despite suggested technical difficulties in achieving requisite neutralizing titers for sterile HIV protection by prophylactic vaccination, our results indicate a possibility of non-sterile HIV control by secondary expansion of prophylactic vaccine-induced, sub-sterile titers of NAbs post-infection, providing a rationale of vaccine-based NAb induction for primary HIV control. More understanding of the mechanism may lead to a more certain rationale for careful induction of NAbs and CTLs by vaccination, maybe potentially capable of synergistic HIV-1 control.

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Author Contributions

Conceived and designed the experiments: TM HY. Performed the experiments: TM HY MK AT HI. Analyzed the data: TM HY MK. Wrote the paper: TM HY MK. Other: Contributed to preparation of the passive immunization inoculums, experiments using DCs, and immunological analyses: HY. Contributed to immunological analyses: MK. Contributed to blood processing and immunological and virological analyses: AT. Contributed to blood processing and immunological and virological analyses: HI.

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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$^\nabla$

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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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