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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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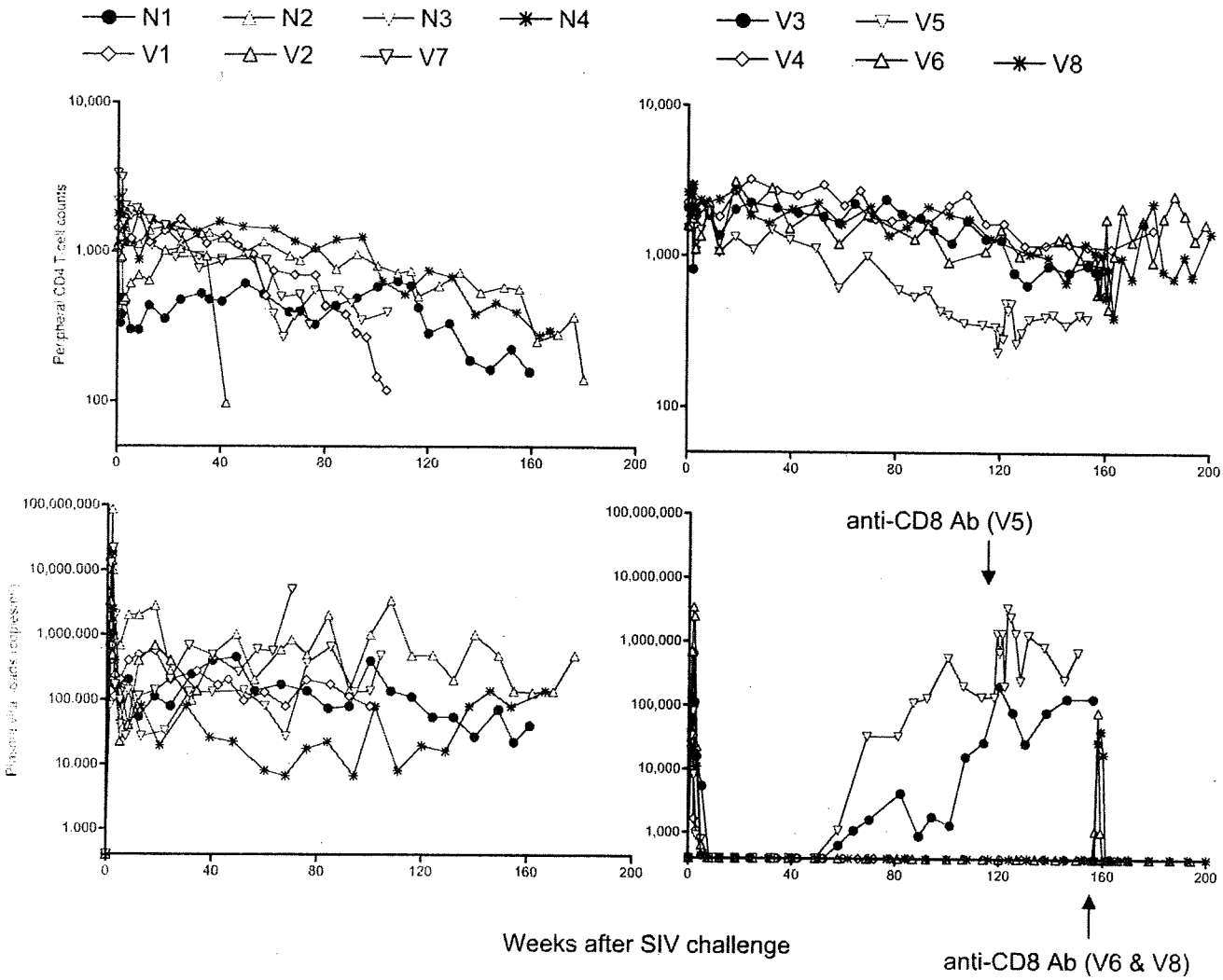


FIG. 1. Follow-up of the macaques after SIVmac239 challenge. Upper panels, peripheral $CD4^+$ T-cell counts (cells/μl); lower panels, plasma viral loads (viral RNA copies/ml plasma); left panels, the seven noncontrollers; right panels, the five controllers. All seven noncontrollers developed AIDS and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

present study. These macaques were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases and the National Institute of Biomedical Innovation. Four of them were naive, whereas the other eight macaques received a DNA vaccine followed by a single boost with SeV-Gag before an intravenous SIVmac239 challenge. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{SF162P3}$ molecular clone DNA (30) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV-$1_{DH12}$ chimeric Vpr, and HIV-$1_{DH12}$ Tat and Rev as described previously (17). At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals intranasally received a single boost with $1 \times 10^8$ cell infectious units of replication-competent SeV-Gag (V1, V2, V3, and V4) or $6 \times 10^9$ cell infectious units of F-deleted replication-defective F(-)SeV-Gag (9, 14, 32). Approximately 3 months after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (11).

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

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

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

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

TABLE 1. Summary of responses in macaques challenged with SIVmac239

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

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

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

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

[d] Peripheral CD4 T-cell counts.

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

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

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

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

## RESULTS

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

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

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

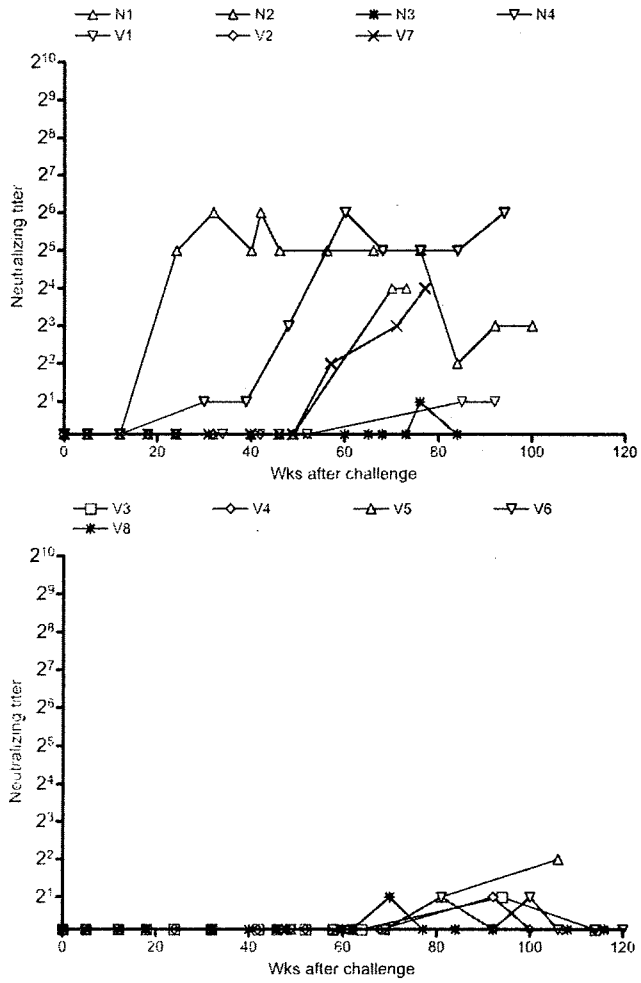


FIG. 2. SIVmac239-specific neutralizing antibody levels in plasma. Plasma titers for killing 10-$TCID_{50}$ SIVmac239 replication in the noncontrollers (top panel), including unvaccinated control animals, and in the controllers (bottom panel) are shown.

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

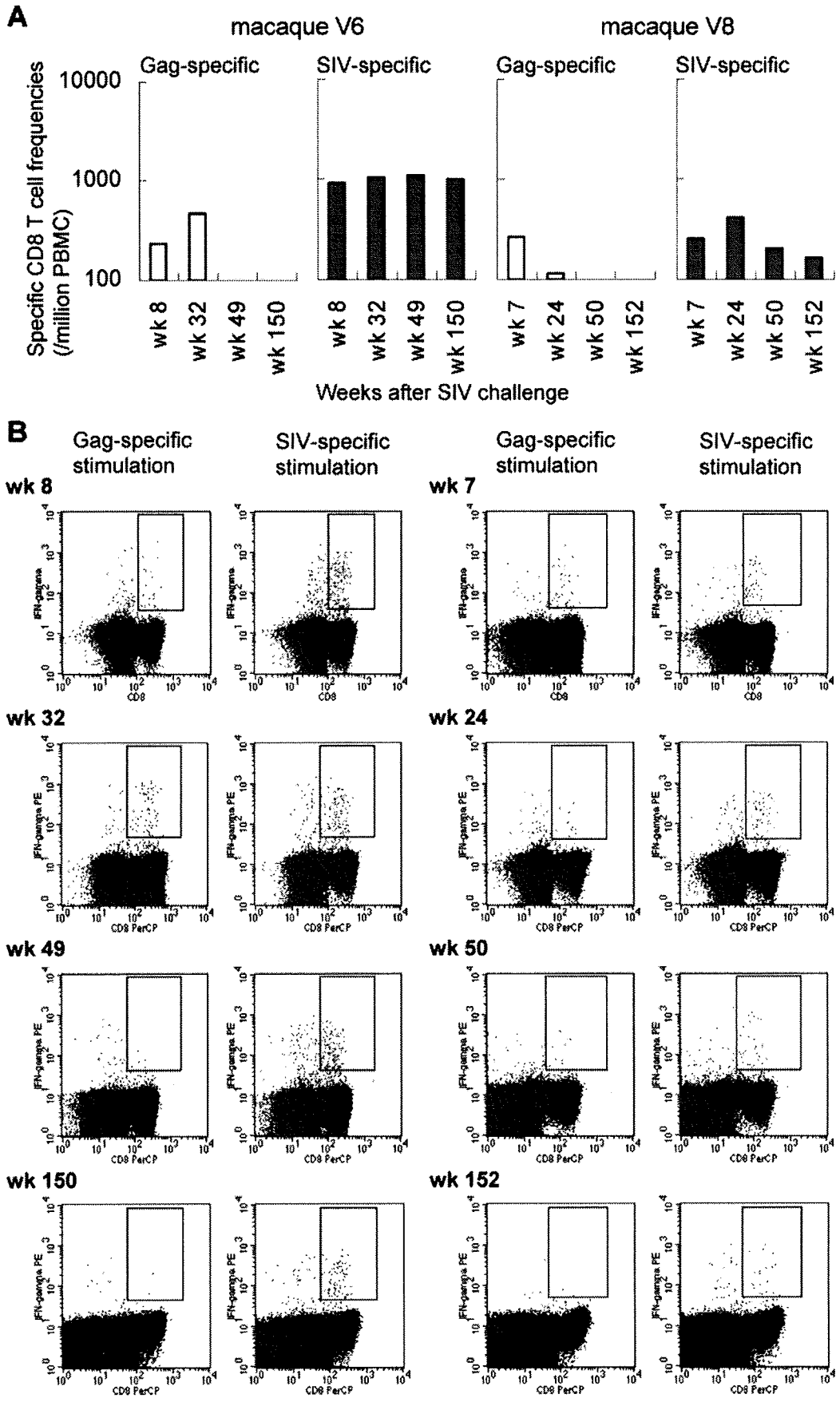


FIG. 3. Virus-specific $CD8^+$ T-cell responses in sustained controllers V6 (left panels) and V8 (right panels). (A) Gag-specific and SIV-specific $CD8^+$ T-cell frequencies in PBMCs. (B) Dot plots gated on $CD3^+$ lymphocytes after Gag-specific or SIV-specific stimulation.

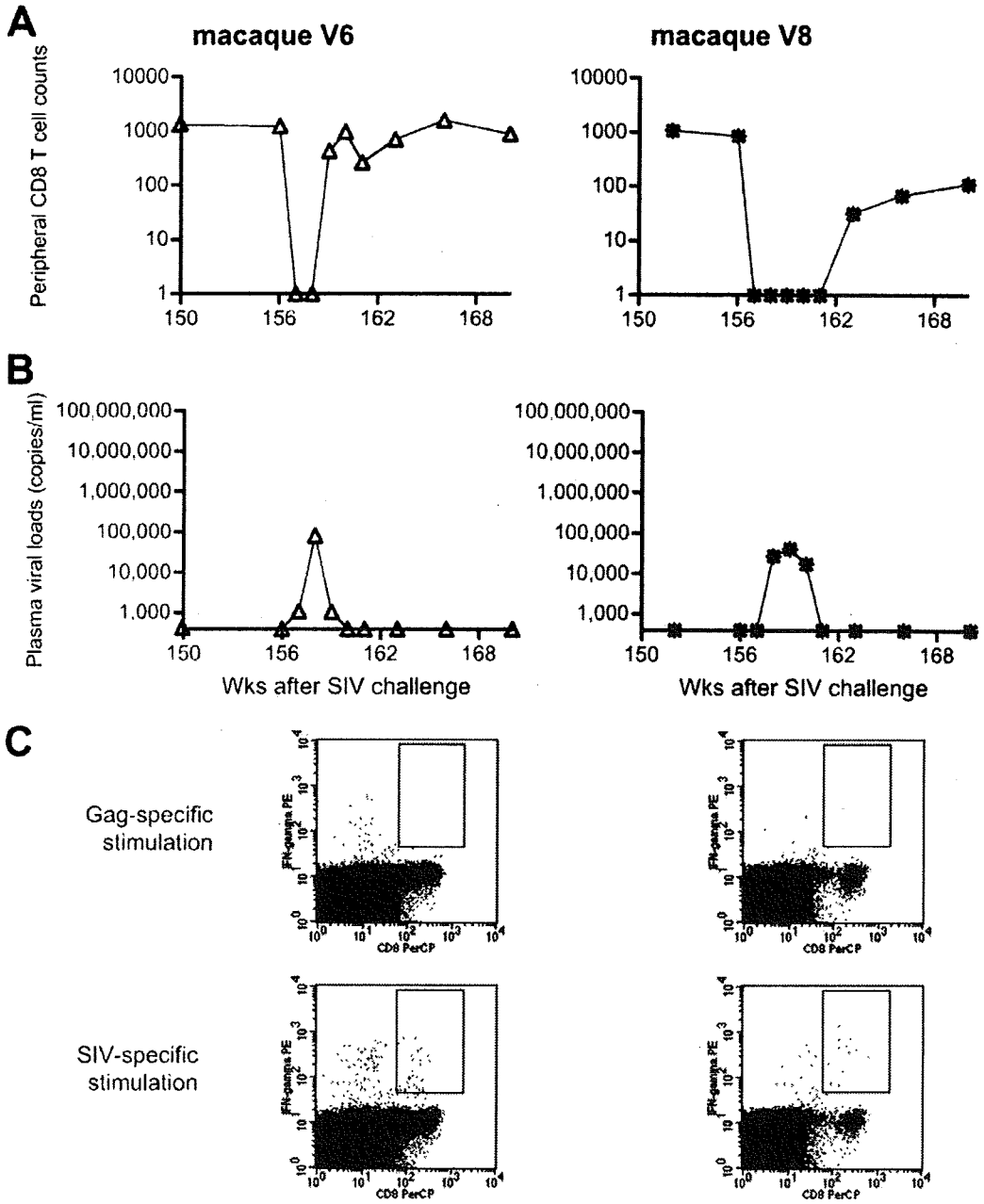


FIG. 4. $CD8^+$ cell depletion experiments starting at week 156 in sustained controllers V6 (left panels) and V8 (right panels). (A) Peripheral $CD8^+$ T-cell counts (per μl). (B) Plasma viral loads (viral RNA copies/ml plasma). (C) Virus-specific CTL responses at week 160 in V6 and at week 166 in V8. 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

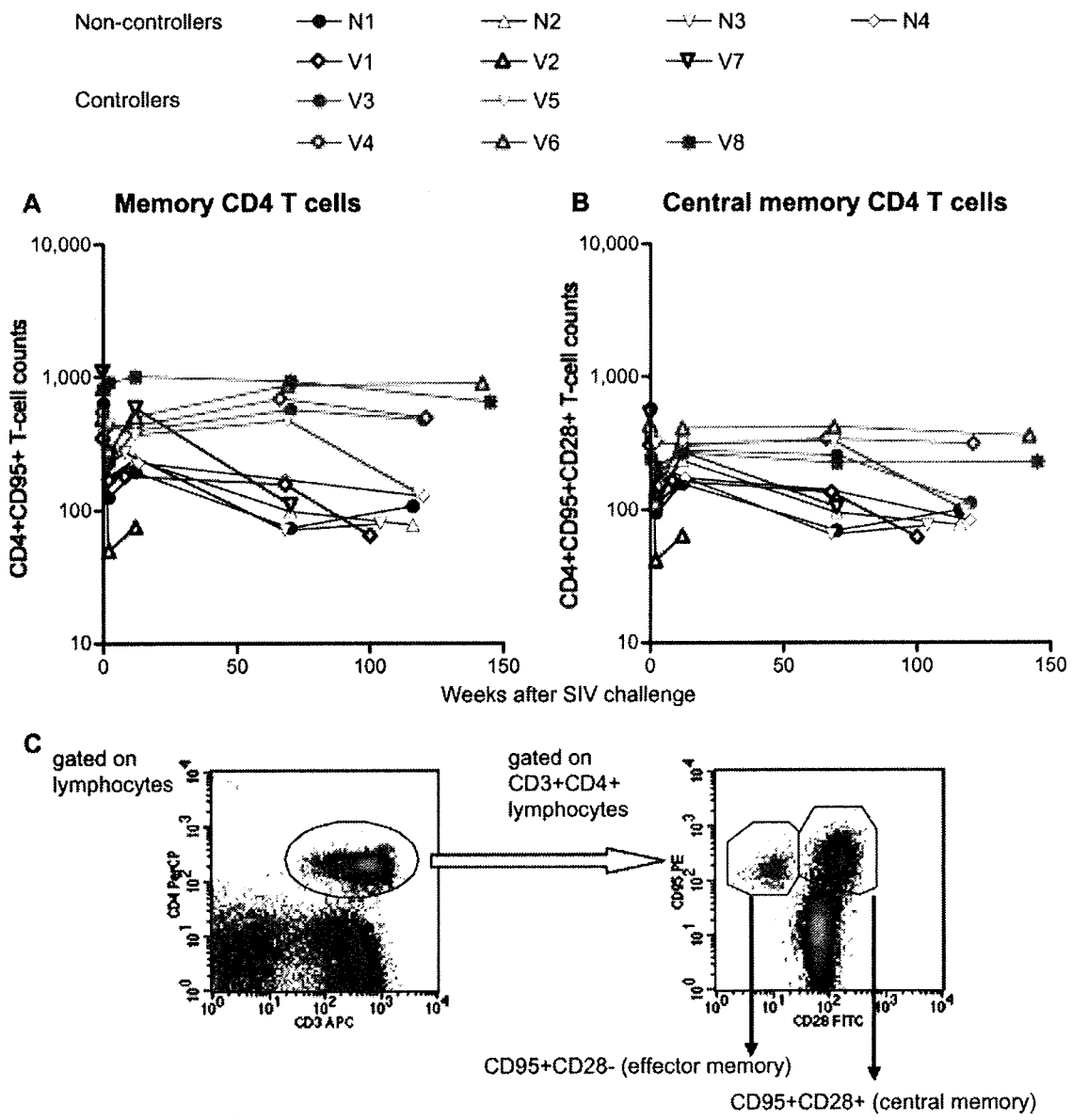


FIG. 5. Changes in peripheral memory $CD4^+$ T-cell counts. Noncontrollers are indicated in black or blue, and controllers are indicated in red. (A) Peripheral memory $CD4^+$ ($CD4^+$ $CD95^-$) T-cell counts (per μl). (B) Peripheral central memory $CD4^+$ ($CD4^-$ $CD95^+$ $CD28^+$) T-cell counts (per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

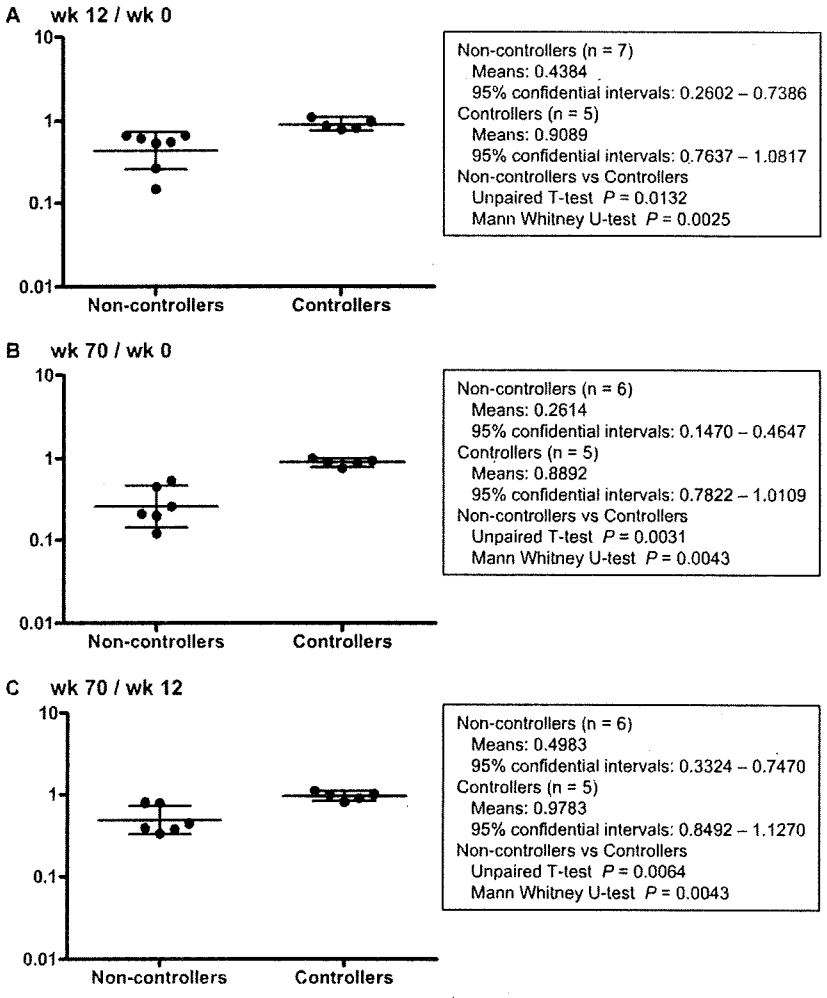


FIG. 6. Statistical analysis indicating preservation of central memory $CD4^+$ T-cell counts in the controllers. The ratios of central memory $CD4^+$ T-cell counts at week 12 to week 0 (A), week 70 to week 0 (B), and week 70 to week 12 (C) in the noncontrollers (except for rapid progressor V2 in panels 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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# 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 $1\times10^8$ CIU (cell infectious units) of replication-competent Sendai virus expressing Gag (SeV-Gag) in macaque V5 or $6\times10^9$ 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 $4\times10^2$ 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 $5\times10^4$ 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-$\gamma^+$ 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-$ABC^+$HLA-$DR^+$ immature DCs [27]. Alternatively, $CD1c^+$ DCs were positively selected from CD20-depleted PBMCs as described above. For antigen presentation assay, $1\times10^5$ 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 $2\times10^6$ 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 $1\times10^4$ 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.

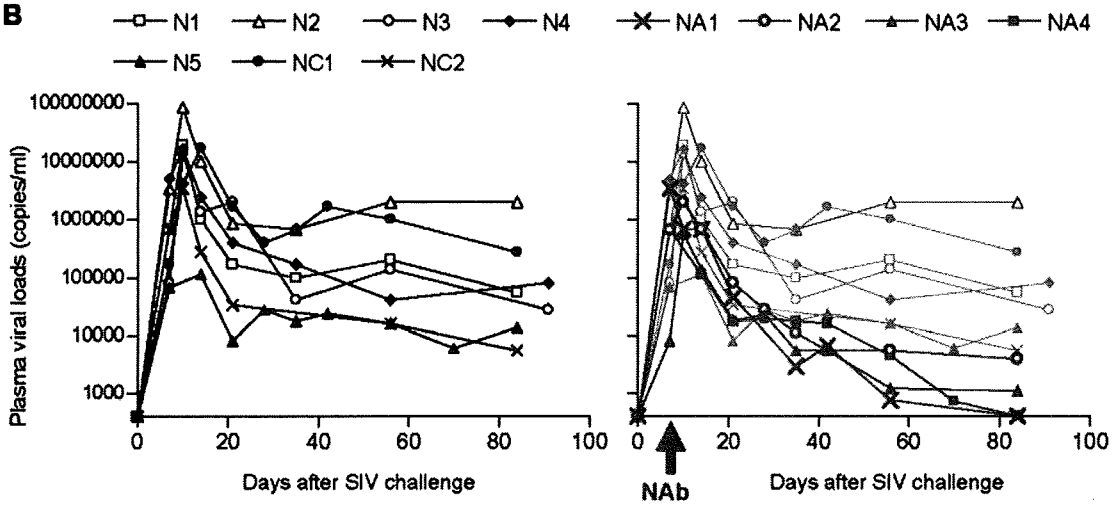
**A**

NAb Tx in unvaccinated macaques

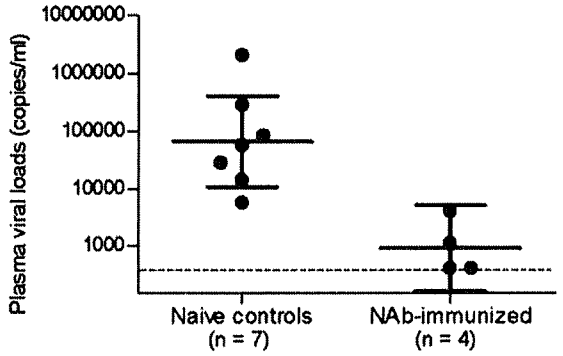
| Group/macaque | Ab Tx at day 7 |
|---|---|
| Naive controls | |
| N1* | - |
| N2* | - |
| N3* | - |
| N4* | - |
| N5 | - |
| NC1 | control Ab |
| NC2 | control Ab |

| 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 $6.5\times10^4$ 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 $9.1\times10^2$ 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.
doi:10.1371/journal.pone.0000540.g001

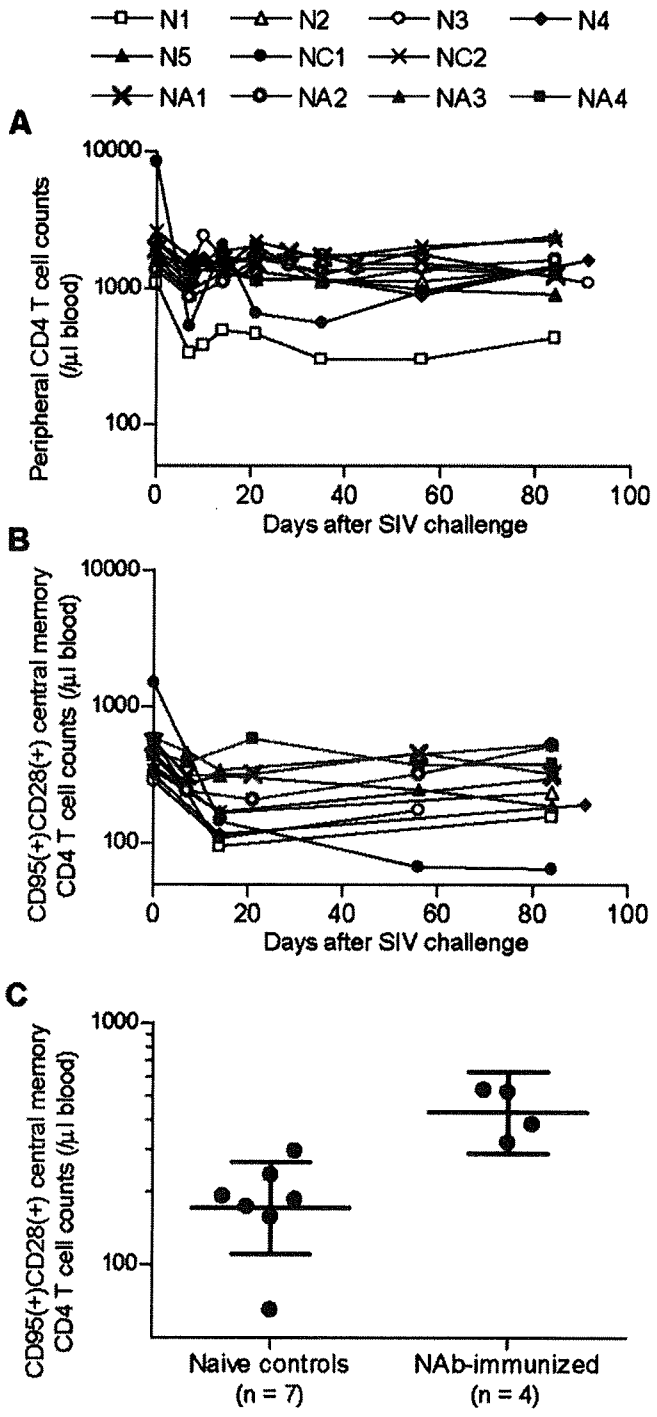



**Figure 2. Central memory CD4$^+$ T-cell counts in naive controls and NAb-immunized macaques.** (A) Peripheral CD4$^+$ T-cell counts (cells/μl). (B) Peripheral CD95$^+$CD28$^+$ central memory CD4$^+$ T-cell counts (cells/μl) [28]. (C) Statistical comparison of CD28$^+$CD95$^+$ central memory CD4$^+$ 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 $1.7\times10^2$ 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 $4.3\times10^2$ 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 ($p=0.0061$).
doi:10.1371/journal.pone.0000540.g002

## 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 $Gag_{206\text{-}216}$ epitope-specific CTL by week 5, suggesting a strong selective pressure on the virus by this CTL [6]. As for the

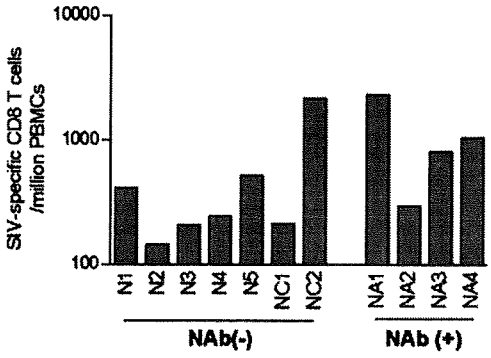



**Figure 3. SIV-specific CD8$^+$ T-cell frequencies at week 8 post-challenge in naive controls and NAb-immunized macaques.**
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two vaccinees VA2 and VA3 infused with NAbs, plasma viremia became undetectable by week 5 and rapid selection of CTL escape 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

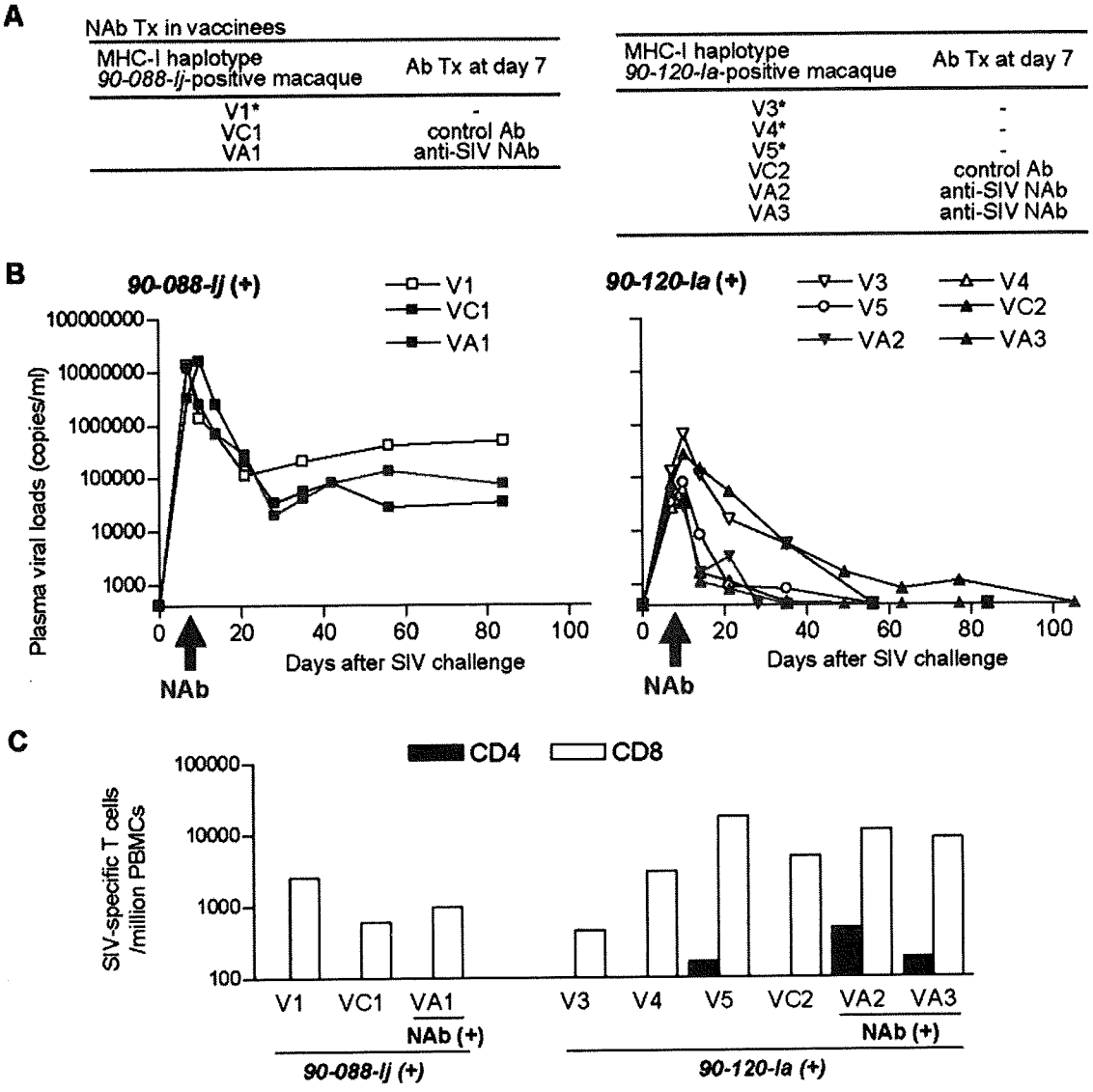


**Figure 4. Effect of post-challenge passive NAb immunization in vaccinees.** (A) List of vaccinees with or without passive immunization. (B) Plasma viral loads after challenge (SIV RNA copies/ml). Left panel, MHC-I haplotype *90-088-lj*-positive macaques; right panel, *90-120-la*-positive macaques. Red lines represent NAb-immunized vaccinees. (C) SIV-specific $CD4^+$ T-cell and $CD8^+$ T-cell frequencies at week 2 post-challenge.
doi:10.1371/journal.pone.0000540.g004

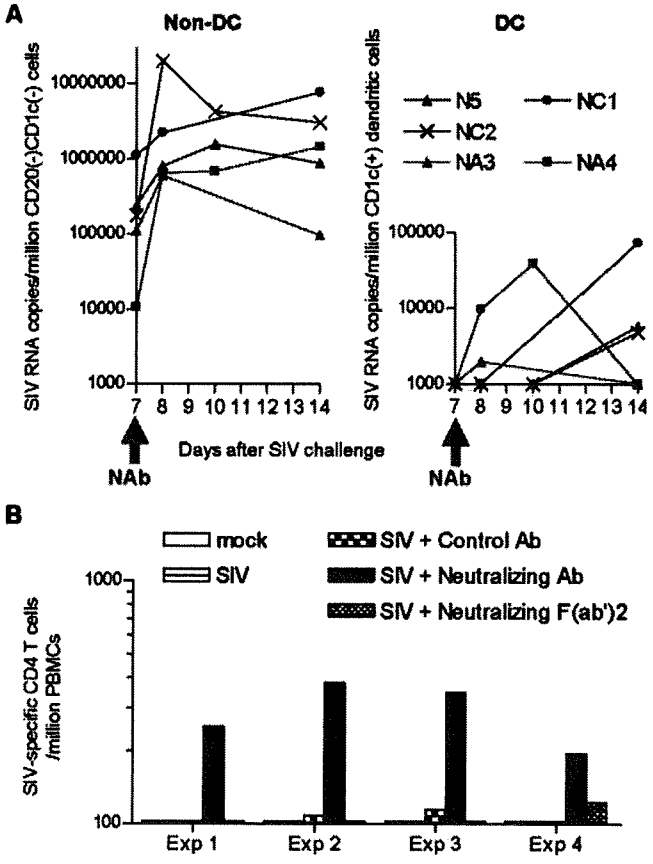


**Figure 5. Antibody-mediated SIV uptake by DCs and T cell priming.** (A) Peripheral lymph node-derived non-DC ($CD1c^-CD20^-$ lymphocytes)-associated (left panel) and $CD1c^+CD20^-$ DC-associated viral loads (right panel). (B) In vitro antigen presentation assay. Either in vitro-generated DCs (Exp. 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-$\gamma$ 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-$\gamma$ induction. In all four sets of experiments, efficient IFN-$\gamma$ 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-$\gamma$ 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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# Defect of Human Immunodeficiency Virus Type 2 Gag Assembly in *Saccharomyces cerevisiae*▽

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**We have previously shown that the expression of human immunodeficiency virus type 1 (HIV-1) Gag protein in *Saccharomyces cerevisiae* spheroplasts produces Gag virus-like particles (VLPs) at the plasma membrane, indicating that yeast has all the host factors necessary for HIV-1 Gag assembly. Here we expand the study by using diverse primate lentiviral Gags and show that yeast does not support the production of HIV-2 or simian immunodeficiency virus SIVmac Gag VLPs but allows the production of SIVagm and SIVmnd Gag VLPs. Particle budding was observed at the surfaces of cells expressing SIVagm and SIVmnd Gags, but cells expressing HIV-2 and SIVmac Gags showed only membrane-ruffling structures, although they were accompanied with electron-dense submembrane layers, suggesting arrest at an early stage of particle budding. Comparison of HIV-1 and HIV-2 Gag expression revealed broadly equivalent levels of intracellular Gag expression and Gag N-terminal myristoylation in yeast. Both Gags showed the same membrane-binding ability and were incorporated into lipid raft fractions at a physiological concentration of salt. HIV-2 Gag, however, failed to form a high-order multimer and easily dissociated from the membrane, phenomena which were not observed in higher eukaryotic cells. A series of chimeric Gags between HIV-1 and HIV-2 and Gag mutants with amino acid substitutions revealed that a defined region in helix 2 of HIV-2 MA (located on the membrane-binding surface of MA) affects higher-order Gag assembly and particle production in yeast. Together, these data suggest that yeast may lack a host factor(s) for HIV-2 and SIVmac Gag assembly.**

The major structural component of retroviruses is encoded by the *gag* gene, and Gag is the sole protein required for viral particle assembly. Three discrete Gag regions responsible for virus particle assembly have been identified and termed the membrane-binding (M), interacting (I), and late (L) domains. The M domain is located at the N-terminal matrix/membrane (MA) of Gag and contains a membrane-binding signal which directs the association of Gag with the membrane. The signal is largely composed of N-terminal myristoylation of MA in many mammalian retroviruses, including human immunodeficiency virus (HIV), and this modification is necessary for Gag targeting and subsequent binding to the plasma membrane (4, 14, 15). The I domain is essential for Gag-Gag interactions and spans from the central capsid (CA) to the nucleocapsid (NC) of Gag (7, 11, 24, 39). The L domain, responsible for pinching off viral particles from the membrane, is located at either the C-terminal domain of Gag or the MA-CA junction (16, 37).

Because Gag is sufficient for retroviral particle budding, many studies on particle assembly have used Gag expression and shown that expression of the Gag protein alone in higher eukaryotic cells produces a Gag virus-like particle (VLP) morphologically identical to the immature form of retroviral particles (14, 19, 44). The fact that Gag self-assembles into a viral particle suggests that Gag assembly is attributable to the intrinsic properties of Gag. This view is supported by in vitro studies in which purified Gag protein assembled into a spherical particle, analogous to a Gag VLP, in a test tube (5, 6, 22, 27). However, a number of recent studies clearly show that the Gag assembly process involves many host factors, some of which are indispensable for particle budding. These include endosomal sorting molecules, such as TSG101, Nedd4, AIP-1/ALIX, and AP-3 (9, 12, 46, 52, 53). Such host factors and protein sorting pathways appear to be commonly used machinery for intracellular trafficking of diverse retroviral Gags (21, 53). ABCE1/HP68 has also been identified as a host factor that supports multimerization of all primate lentiviral Gags (10, 56). In contrast, the host factors identified as host restriction factors, such as cyclophilin A and TRIM-5α, appear to be Gag type specific, although they are not involved in particle assembly but in uncoating and initiation of reverse transcription (2, 3, 20, 47, 50).

Recent studies on reverse genetics use small interfering RNAs, which specifically silence the expression of their corresponding genes. This new technology has made it possible to deplete a host factor of interest in mammalian cells. The study of genetics in eukaryotes has long been carried out with *Saccharomyces cerevisiae*, because yeast has the ability to replace the wild-type chromosomal copy of a gene with a mutant or deletion derivative, a property which is not available in other eukaryotic cells. Accordingly, many genetic mutants have been isolated in yeast and made available for the study of cellular factors and machinery. We previously developed a Gag VLP budding system with *Saccharomyces cerevisiae* in which the

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HIV type 1 (HIV-1) Gag protein simultaneously budded Gag VLPs from the plasma membrane, and we have suggested that a combination of this method and yeast genetics may be a powerful tool for the study of the host factors required for particle production (42). Here we expand this study by using diverse primate lentiviral Gags and show that yeast does not support the production of HIV-2 or simian immunodeficiency virus SIVmac Gag VLPs. Our data suggest that yeast may lack a host factor(s) required for tight membrane binding of HIV-2 Gag to facilitate higher-order assembly.

## MATERIALS AND METHODS

**Construction and expression of diverse primate lentivirus *gag* genes.** For expression in yeast, the full-length *gag* genes of HIV-1 (HXB2 strain), HIV-2 (ROD strain), SIVmac (mac239 strain), SIVagm (TY01 strain), and SIVmnd (GB1 strain) were amplified by PCRs using relevant forward and reverse primers. For the Gag-Flag fusion protein, the *gag* gene (truncated just before the termination codon) was amplified by PCR using a reverse primer containing a Flag epitope tag sequence. DNA construction of chimeric Gags between HIV-1 and HIV-2 and of Gag mutants containing amino acid substitutions was also carried out by PCRs using the relevant forward and reverse primers. The PCR fragments were cloned into the yeast expression vector pKT10 (48), which is a 2μm plasmid containing the *URA3* gene as a selective marker and the constitutive promoter for the yeast glyceraldehyde-3-phosphate dehydrogenase gene. The *S. cerevisiae* strain RAY3A-D (*MATa/α ura3/ura3 his3/his3 leu2/leu2 trp1/trp1*) (40) was transformed with the yeast expression plasmids.

For expression in higher eukaryotic cells, the *gag* genes of HIV-1 and HIV-2 were modified C-terminally with a Flag epitope tag and cloned into the higher eukaryotic expression vector pCAGGS (30), which contains the promoter for the actin gene. The codon usage of the HIV-1 *gag* gene was optimized. HeLa and 293T cells were transfected with the expression plasmids by using Lipofectamine 2000 (Invitrogen).

**Preparation of yeast spheroplasts and subcellular fractionation.** The procedure for yeast spheroplast formation was described previously (39). In brief, yeast transformants were grown at 30°C in synthetic defined medium without uracil (0.67% yeast nitrogen base, 2% glucose, and amino acid mixtures without uracil). Yeast cells were suspended in wash buffer (50 mM Tris [pH 7.5], 5 mM $MgCl_2$, and 1 M sorbitol) containing 30 mM dithiothreitol (DTT) and incubated at 30°C for 20 min with gentle shaking. The cells were resuspended in wash buffer containing 3 mM DTT and 0.4 mg/ml Zymolyase and incubated at 30°C for 20 min with gentle shaking for digestion of the cell wall. Following digestion, the cells were washed with 1 M sorbitol.

Subcellular fractionation of yeast cells was performed by a standard procedure (13). Yeast spheroplasts (10 optical density [OD] units) were resuspended in buffer (50 mM Tris [pH 8.0], 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A), with 150 mM NaCl or without salt, and homogenized with 15 strokes in a homogenizer. Following clarification at 500 × *g* for 5 min at 4°C, the cell lysates (whole-cell lysates) were subjected to centrifugation at 13,000 × *g* for 10 min at 4°C. The precipitates were stored as P13 fractions. The supernatants were centrifuged in a TLA100 rotor (Beckman Coulter) at 100,000 × *g* for 1 h at 4°C, and the precipitates (P100) and supernatants (S100) were separated.

**Sedimentation analysis.** Whole-cell lysates and subcellular fractions were applied to 20 to 70% (wt/vol) sucrose gradients in phosphate-buffered saline (PBS) and sedimented in an SW55 rotor at 120,000 × *g* for 2 h at 4°C, as described previously (28). Fractions of the gradients were collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting. The 80S ribosome and the immature form of HIV Gag VLPs purified from Gag-expressing HeLa cells were used as molecular weight markers for sedimentation analysis.

**Membrane and lipid raft flotation centrifugation.** Equilibrium flotation centrifugation with membranes was performed as described previously (32, 36), with minor modifications. The formation of yeast spheroplasts was carried out as described above. Yeast spheroplasts (10 OD units) were resuspended in buffer A (50 mM Tris [pH 8.0], 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A) containing 150 mM NaCl. Following a brief sonication, the cell lysates were clarified at 500 × *g* for 5 min at 4°C. The supernatants were adjusted to 70% (wt/vol) sucrose in PBS, layered at the bottom of 70%-65%-10% (wt/vol) sucrose step gradients in PBS, and subjected to equilibrium flotation centrifugation. Centrifugation was performed in an SW55 rotor (Beckman Coulter) at 4°C at 120,000 × *g* overnight. In some experiments, cells were resuspended in buffer A with 500 mM NaCl or without salt. For lipid raft flotation, the cell lysates, after sonication, were treated on ice with 0.5% Triton X-100 for 10 min. Following clarification, the supernatants were subjected to equilibrium flotation centrifugation. Fractions of the gradients were collected and subjected to SDS-PAGE followed by Western blotting. Membranes of higher eukaryotic cells were analyzed similarly by equilibrium flotation centrifugation.

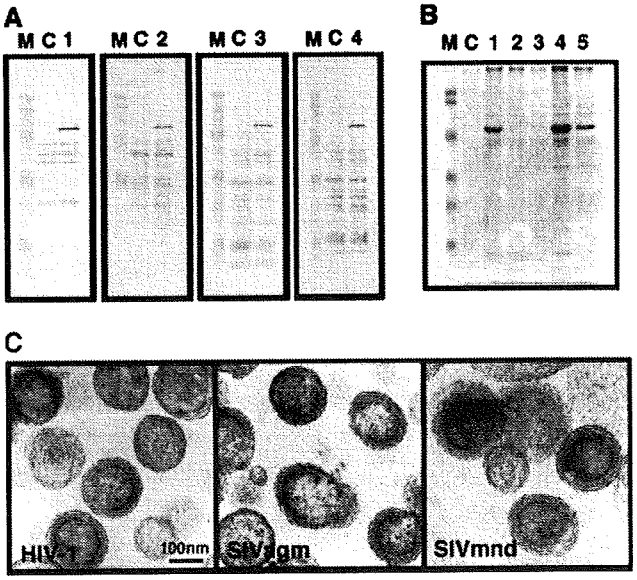


FIG. 1. Intracellular expression of diverse lentiviral Gags and production of Gag VLPs in yeast. Yeast cells were transformed with a pKT10 vector containing the full-length *gag* gene of HIV-1, HIV-2, SIVmac, SIVagm, or SIVmnd. (A) Intracellular Gag expression. Cells (0.5 OD unit) were subjected to SDS-PAGE followed by Western blotting using anti-HIV-1, anti-HIV-2, or anti-SIVmac CA antibody or anti-SIVagm monkey serum. Lanes: M, prestained molecular weight markers; C, cells transformed with the parental vector; 1 to 4, cells transformed with the vector containing the *gag* genes of HIV-1, HIV-2, SIVmac, and SIVagm, respectively. (B) Gag VLP production. Following removal of the cell wall, spheroplasts (200 OD units) were cultured in yeast extract-peptone-dextrose medium containing 1 M sorbitol overnight. Gag VLPs were purified from the culture medium by centrifugation on 20 to 70% sucrose gradients and analyzed by SDS-PAGE followed by CBB staining. Lanes: M, prestained molecular weight markers; C, mock fractions prepared from the culture medium of yeast spheroplasts transformed with the parental vector; 1 to 5, Gag VLP fractions purified from culture medium of yeast spheroplasts expressing HIV-1, HIV-2, SIVmac, SIVagm, and SIVmnd Gags, respectively. (C) Electron micrographs of Gag VLPs. Purified Gag VLP fractions were subjected to electron microscopic analysis. All micrographs are shown at the same magnification. Bar = 100 nm.

**Purification of Gag VLPs.** Purification of yeast-produced Gag VLPs was carried out as described previously (42). Briefly, the culture medium of yeast spheroplasts was clarified and then centrifuged through 30% (wt/vol) sucrose cushions in an SW28 rotor (Beckman Coulter) at 120,000 × *g* for 1.5 h at 4°C. The VLP pellets were resuspended and centrifuged in 20 to 70% (wt/vol) sucrose gradients in PBS in an SW55 rotor (Beckman Coulter) at 120,000 × *g* overnight at 4°C. Purification of Gag VLPs produced by higher eukaryotic cells was carried out by standard procedures.

**Protein detection.** Following SDS-PAGE, gels were subjected to either Coomassie brilliant blue (CBB) staining or Western blotting using an anti-HIV-1, anti-HIV-2, or anti-SIVmac CA mouse monoclonal antibody (Advanced Biotechnologies) or anti-SIVagm monkey serum. For the Gag-Flag fusion protein, Western blotting was carried out using an anti-Flag mouse monoclonal antibody (Sigma). In subcellular fractionation experiments, anti-Pep12 (for endosomes), anti-alkaline phosphatase (for vacuoles), and anti-phosphoglycerate kinase (for cytosol) mouse monoclonal antibodies (Molecular Probes) were used as or-

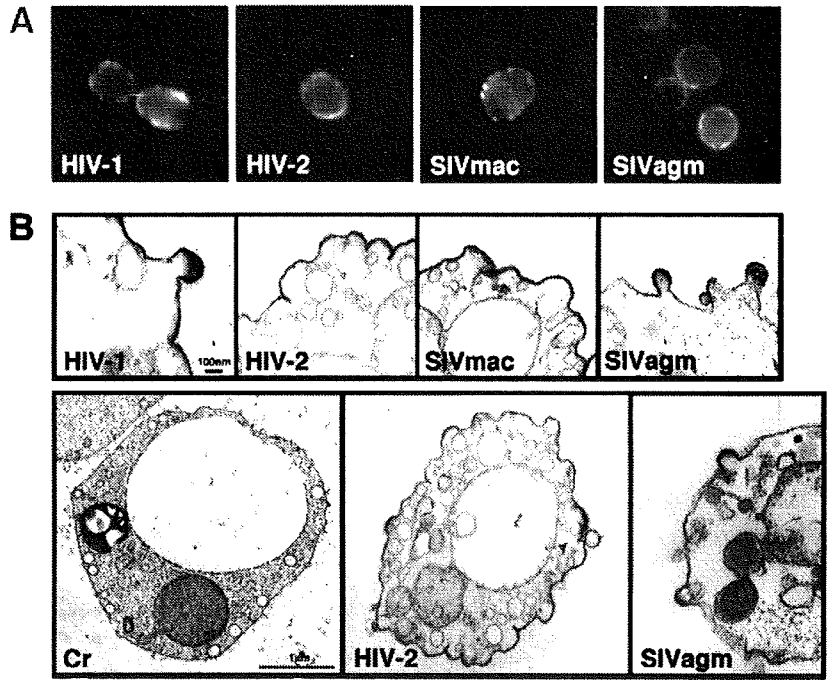


FIG. 2. Immunofluorescence staining and electron microscopy of yeast cells expressing diverse lentiviral Gags. (A) Immunofluorescence detection of Gag antigens. After fixation with 3.7% formalin, the cell wall was removed with Zymolyase and the membrane was permeabilized with 0.1% Triton X-100. Gag antigens were detected using anti-HIV-1, anti-HIV-2, or anti-SIVmac CA antibody or anti-SIVagm monkey serum. (B) Electron micrographs of yeast spheroplasts expressing each of the Gags. Micrographs in upper panels are shown at the same magnification (bar = 100 nm). Micrographs in lower panels show whole yeast cells taken at the same magnification (bar = 1 μm). Cr, yeast cell transformed with the parental vector.

ganelle markers. For the plasma membrane, yeast spheroplasts were incubated with cholera toxin subunit B (CTB), which binds to lipid rafts of the plasma membrane, at 4°C. Following a wash with 1 M sorbitol, subcellular fractionation was carried out as described above. The fractions were subjected to Western blotting using anti-CTB rabbit antibody (Molecular Probes).

For protein myristoylation, yeast cells were metabolically labeled with 500 μCi of [9,10(*n*)-$^3$H]myristic acid at 30°C for 30 min. Following SDS-PAGE, gels were subjected to fluorography.

**Immunofluorescence staining.** Yeast cells were fixed in 3.7% formalin in yeast extract-peptone-dextrose medium at 30°C for 30 min. Following removal of the cell wall, spheroplasts were treated with 0.1% Triton X-100 at room temperature for 5 min for membrane permeabilization. The cells were incubated first with an anti-HIV-1, anti-HIV-2, or anti-SIVmac CA mouse monoclonal antibody (Advanced Biotechnologies) or anti-SIVagm monkey serum and subsequently with anti-mouse immunoglobulin G–Alexa Fluor 488 (Molecular Probes) or anti-monkey immunoglobulin G–fluorescein isothiocyanate. For the Gag-Flag fusion protein, cells were incubated with an anti-Flag mouse monoclonal antibody (Sigma). For the plasma membrane, yeast spheroplasts were first incubated with CTB at 4°C (to label lipid rafts of the plasma membrane but not allow endocytosis) and subsequently with anti-CTB rabbit antibody (Vyrant lipid raft labeling kit; Molecular Probes). After fixation with 3.7% formalin, the spheroplasts were permeabilized with 0.1% Triton X-100 and costained with anti-Flag antibody for Gag-Flag.

**Electron microscopy.** Electron microscopy was carried out by standard procedures. Briefly, yeast spheroplasts were fixed in 2% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) for 2 h and postfixed with 1% osmium tetroxide for 1 h. Cell pellets were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope.

## RESULTS

**Yeast does not support HIV-2 or SIVmac Gag VLP production.** The initial goal of this study was to examine whether Gag proteins of diverse primate lentiviruses produce Gag VLPs from yeast spheroplasts, as does HIV-1 Gag (42). Primate lentiviruses are classified into the following five equidistant phylogenetic lineages: (i) HIV-1/SIVcpz, (ii) HIV-2/SIVmac/SIVsm, (iii) SIVagm, (iv) SIVmnd, and (v) SIVsyk (17). We used the *gag* genes from four different primate lentivirus lineages. Yeast cells were transformed with the yeast expression vector pKT10 containing the *gag* gene of HIV-1, HIV-2, SIVmac, SIVagm, or SIVmnd and were grown in synthetic defined medium without uracil. Western blotting using anti-HIV-1, anti-HIV-2, and anti-SIVmac CA antibodies and anti-SIVagm monkey serum revealed individual Gag proteins in the expressing cells but not in the cells transformed with a parental vector (Fig. 1A). We did not test the cells expressing SIVmnd Gag because an anti-SIVmnd antibody was not available.

Following removal of the cell wall, yeast spheroplasts were maintained under isotonic conditions overnight. For purification of Gag VLPs, the culture medium of the spheroplasts was subjected to centrifugation through a sucrose gradient and subsequent fractionation, as described previously (42). When equivalent volumes of the Gag VLP fractions were subjected to SDS-PAGE followed by CBB staining, Gag VLP production was observed for HIV-1, SIVagm, and SIVmnd Gags, although the yields of produced VLPs varied. In contrast, no Gag VLP production was observed for HIV-2 or SIVmac Gag (Fig. 1B). Western blotting using anti-HIV-2 and anti-SIVmac CA antibodies also failed to demonstrate Gag VLP production (data not shown). These findings were not specific to the yeast expression vectors or yeast backgrounds used (data not shown).