

FIG. 1. Follow up of NAb-immunized macaques. (A) Plasma viral loads (SIV *gag* RNA copies/ml plasma) in six unimmunized macaques (black lines) and five NAb-immunized animals (red lines) after SIVmac239 challenge. The plasma viral loads were measured as described previously (29). The lower limit of detection was approximately  $4 \times 10^2$  copies/ml. The MHC-I haplotypes are shown in parentheses following the macaque numbers as follows: E, haplotype *90-010-Ie*; D, *90-010-Id*; G, *90-030-Ig*; J, *90-088-Ij*; and H, *90-030-Ih*. Below are comparisons of plasma viral loads in unimmunized (non-Tx) and NAb-immunized (NAb-Tx) macaques at weeks (wk) 1, 2, 8, 30, and 60. The bars indicate the geometric mean of each group. The comparisons at weeks 8, 30, and 60 (indicated by asterisks) showed significant differences between two groups ( $P = 0.841$  at week 1,  $P = 0.238$  at week 2,  $P = 0.002$  at week 8,  $P = 0.005$  around week 30, and  $P < 0.001$  around week 60 by *t* test). (B) Peripheral CD4<sup>+</sup> T-cell counts (per  $\mu$ l) in unimmunized controls (black lines) and NAb-immunized macaques (red lines) after SIVmac239 challenge. The ratios of the counts around week 60 to those at week 0 in NAb-immunized macaques were significantly higher than in unimmunized controls ( $P = 0.028$  by *t* test).

for more than 1 year in the absence of detectable plasma NAbs, which was accompanied by potent Gag-specific T-cell responses. These results implicate virus-specific polyfunctional CD4<sup>+</sup> T-cell responses in this NAb-triggered primary and long-term SIV control.

#### MATERIALS AND METHODS

**Animal experiments.** We previously showed a reduction in set point viral loads by passive NAb immunization of rhesus macaques (*Macaca mulatta*) 1 week after SIVmac239 challenge (50). In the present study, we monitored these animals, including one additional NAb-immunized macaque (R06-023), for more than 1

year. Thus, five NAb-immunized rhesus macaques and six unimmunized controls were used in this study. Unimmunized macaque R02-021 was euthanized at week 32 because the animal showed loss of body weight, diarrhea, and general weakness. NAb-immunized macaque R02-020 was euthanized at week 42 because of a limitation on available cage numbers. Major histocompatibility complex class I (MHC-I) haplotypes were determined by reference strand-mediated conformation analysis as described previously (2, 29). A group of rhesus macaques possessing the MHC-I haplotype *90-120-Ia* with the potential to efficiently elicit potent Gag-specific CD8<sup>+</sup> T-cell responses (21, 29) were not included in this study. All animals were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (33).

For passive NAb immunization, immunoglobulin G (IgG) was purified from plasma samples from SIVmac239-infected macaques with SIV-specific NAb re-

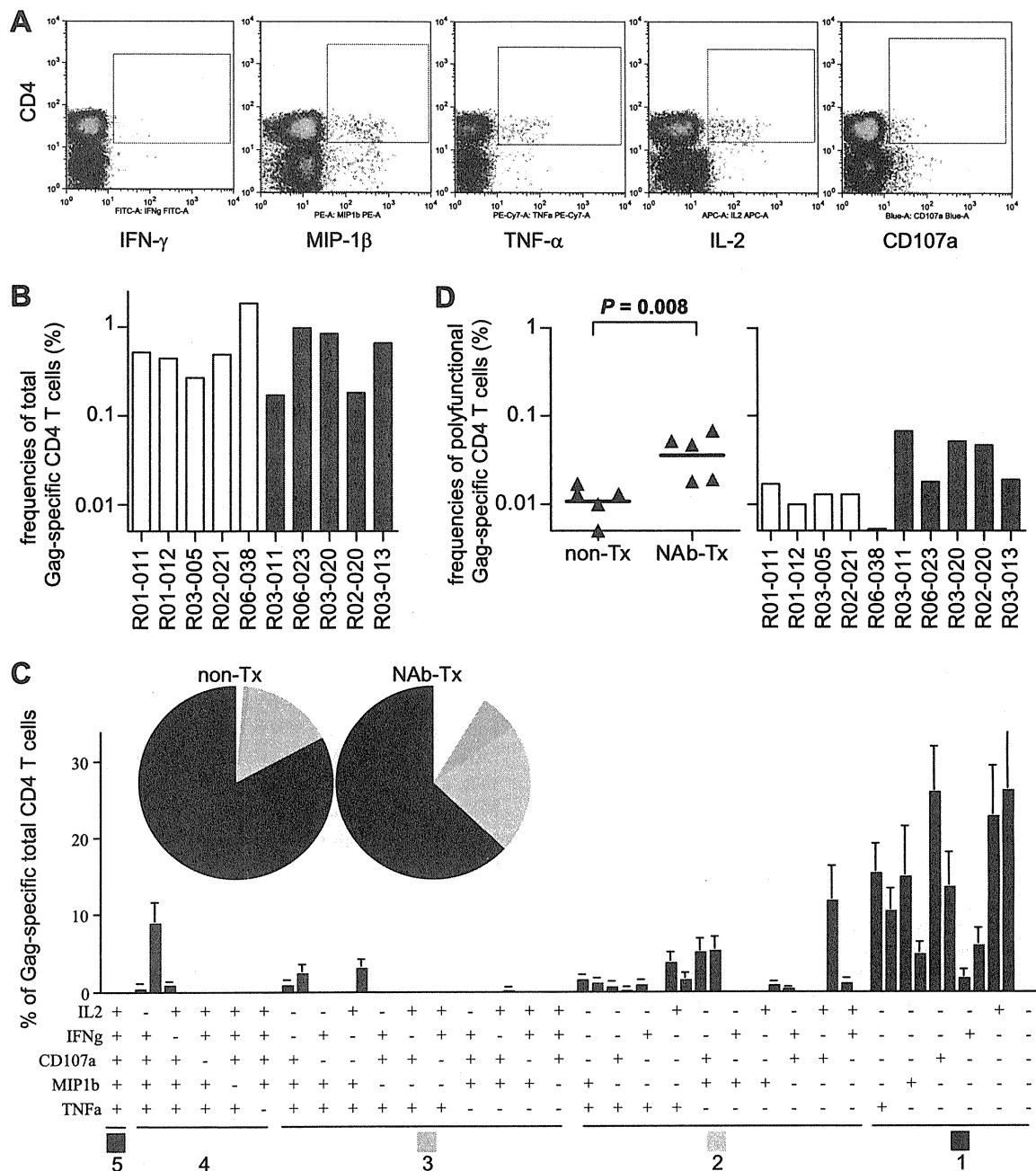


FIG. 2. Gag-specific CD4<sup>+</sup> T-cell responses in the acute phase. PBMCs at week 2 postchallenge were stimulated with a recombinant SIV Gag p55, and the specific induction of five marker cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , and CD107a) in CD4<sup>+</sup> T cells was examined. PBMCs from macaque R02-004 were unavailable and therefore could not be included in this analysis. (A) Representative dot plots showing responses of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , and CD107a in CD4<sup>+</sup> T cells in macaque R03-013 after Gag-specific stimulation. Gag-specific IFN- $\gamma$  responses were undetectable at week 2 in this animal. (B) Frequencies of total Gag-specific CD4<sup>+</sup> T cells that showed Gag-specific induction of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , or CD107a in total CD4<sup>+</sup> T cells. (C) Percentages of cells exhibiting Gag-specific induction of single or multiple marker cytokines in total Gag-specific CD4<sup>+</sup> T cells. (Top) Mean percentages of Gag-specific CD4<sup>+</sup> T cells producing one (dark blue), two (light blue), three (green), four (yellow), or five (red) marker cytokines in unimmunized (non-Tx) and NAb-immunized (NAb-Tx) macaques. (Bottom) Mean percentages of individual Gag-specific CD4<sup>+</sup> T-cell subsets divided by the patterns of marker cytokine induction in unimmunized (blue bars) and NAb-immunized (red bars) macaques. (D) Frequencies of polyfunctional Gag-specific CD4<sup>+</sup> T cells that showed Gag-specific induction of  $\geq 3$  marker cytokines in total CD4<sup>+</sup> T cells. On the left, the bars indicate the geometric mean of each group. The frequencies in NAb-immunized macaques ( $n = 5$ ) were significantly higher than in unimmunized controls ( $n = 5$ ) ( $P = 0.008$ ).

sponses in the chronic phase, as described previously (50). The SIVmac239-specific NAb titer of this IgG preparation (30 mg/ml) was 1:16 on MT-4 cells. Animals were intravenously infused with 300 mg of IgG 1 week after challenge with 1,000 50% tissue culture infective doses (TCID<sub>50</sub>) of SIVmac239 (22). Two unimmunized controls, R02-021 and R06-038, received 300 mg of control IgG

prepared from noninfected rhesus macaques at week 1. Neutralizing titers were measured as described previously (50). In brief, serial twofold dilutions of heat-inactivated plasma in duplicate were incubated with 10 TCID<sub>50</sub> of SIVmac239 for 45 min (5  $\mu$ l of diluted sample was incubated with 5  $\mu$ l of virus in each mixture) and added to  $5 \times 10^4$  MT-4 cells/well in 96-well plates. Progeny virus

production in day 12 culture supernatants was examined by enzyme-linked immunosorbent assays for detection of SIV p27 core antigen (Advanced BioScience Laboratories, Inc., Kensington, MD) to determine the 100% neutralizing end-point. The lower limit of titration was 1:2.

**Analysis of polyfunctional Gag-specific T-cell responses.** We analyzed Gag-specific induction of gamma interferon (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-2 (IL-2), macrophage inflammatory protein 1 $\beta$  (MIP-1 $\beta$ ), and CD107a in CD4<sup>+</sup> and CD8<sup>+</sup> T cells as described previously (1). Peripheral blood mononuclear cells (PBMCs) were cultured for 6 h in the absence or the presence of 10  $\mu$ g/ml of a recombinant SIV Gag p55 (Protein Sciences, Meriden, CT) for unstimulated controls or Gag-specific stimulation (12). They were incubated with anti-human CD28 and anti-human CD49d antibodies (5  $\mu$ g/ml) (BD, Tokyo, Japan) for costimulation and with anti-human CD107a antibody (BD) for immunostaining. Monensin (BD) and brefeldin A (Sigma-Aldrich, Tokyo, Japan) were added to the culture for the final 5 h of stimulation. Then, immunostaining was performed using a CytotfixCytoperm kit (BD) and the following monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-human IFN- $\gamma$  (BD), phycoerythrin (PE)-conjugated anti-human MIP-1 $\beta$  (BD), peridinin chlorophyll protein-conjugated anti-human CD4 (BD), allophycocyanin (APC)-conjugated anti-human IL-2 (BD), PE-Cy7-conjugated anti-human TNF- $\alpha$  (BD), APC-Cy7-conjugated anti-human CD3 (BD), energy-coupled dye-conjugated anti-human CD69 (Beckman Coulter, Tokyo, Japan), biotin-conjugated anti-human CD8 (BD), and anti-human CD107a (BD) conjugated with Pacific Blue using a Zeon mouse IgG1 labeling kit (Invitrogen, Tokyo, Japan). Flow-cytometric 10-color analysis of the induction of the five marker cytokines, IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , and CD107a, was performed using the FACSaria system (BD);  $3 \times 10^5$  to  $5 \times 10^5$  lymphocyte events were analyzed. The data were analyzed using FlowJo (version 8.2; TreeStar Inc., Ashland, OR) and FACSDiva (BD) software. Analysis of polyfunctional phenotypes of T cells was carried out using PESTLE (version 1.5.4) and SPICE (version 4.1.6) programs, which were generously provided by Mario Roederer (National Institutes of Health, Bethesda, MD). Specific T-cell levels were calculated by subtracting nonspecific T-cell frequencies from those after Gag-specific stimulation. Specific T-cell levels of less than 0.01% were considered negative.

**Analysis of proliferative Gag-specific CD4<sup>+</sup> T-cell responses.** Gag-specific CD4<sup>+</sup> T-cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation as described previously (9). In brief, PBMCs were cultured in the absence or the presence of 10  $\mu$ g/ml p55 for 6 days for unstimulated controls or Gag-specific stimulation. Then, the cells were incubated for 2 h with 100 ng/ml BrdU and immunostained using the following monoclonal antibodies: peridinin chlorophyll protein-conjugated anti-human CD4, APC-conjugated anti-human CD95 (BD), APC-Cy7-conjugated anti-human CD3, and energy-coupled dye-conjugated anti-human CD28 (Beckman Coulter, Tokyo, Japan) for surface staining and fluorescein isothiocyanate-labeled anti-human BrdU (BD) for intracellular staining. As a positive control, PBMCs were stimulated with 1  $\mu$ g/ml staphylococcal enterotoxin B for 3 days. Flow-cytometric analysis was performed using the FACSaria system, and the data were analyzed using FlowJo (version 8.2).

**In vitro viral suppression assay.** We examined SIVmac239 replication on CD8-depleted PBMCs in the presence of CD8<sup>+</sup> cells positively selected from PBMCs as described previously (46). In brief, PBMCs were separated into CD8<sup>+</sup> cells and CD8<sup>-</sup> cells by using Macs CD8 MicroBeads (Miltenyi Biotec, Tokyo, Japan). For preparing target cells, the CD8<sup>-</sup> cells negatively selected from PBMCs obtained before challenge were infected with SIVmac239 at a multiplicity of infection of  $1:10^4$  TCID<sub>50</sub>/cell and cultured in the presence of 2  $\mu$ g/ml phytohemagglutinin L (Roche Diagnostics) and 20 IU/ml recombinant human IL-2 (Roche Diagnostics). Two days later, effector CD8<sup>+</sup> cells positively selected from PBMCs obtained before challenge or at week 3 or 4 were added to the target cells at an effector/target (E/T) ratio of 1:4. The culture supernatants were harvested every other day. Reverse transcriptase activities in these supernatants were measured to confirm the peak of viral production in the control culture of target cells without CD8<sup>+</sup> cells around day 10 after SIV infection. SIV Gag capsid protein p27 concentrations in the supernatants after 8 days of coculture (i.e., at day 10 after SIV infection) were then measured by enzyme-linked immunosorbent assay. Results from macaques R01-011, R03-005, R02-021, and R06-023 were excluded because mean p27 concentrations in the control cultures without CD8<sup>+</sup> cells or in one of the duplicates were less than 50 ng/ml. The lower limit of p27 detection was approximately 0.2 ng/ml.

**Statistical analysis.** Statistical analysis was performed with Prism software version 4.03 with significance levels set at a  $P$  value of  $<0.050$  (GraphPad Software, Inc., San Diego, CA). Plasma viral loads and specific T-cell frequencies were log transformed and compared between unimmunized controls and NAb-immunized macaques by an unpaired two-tailed  $t$  test. Correlation was analyzed by the Pearson test.

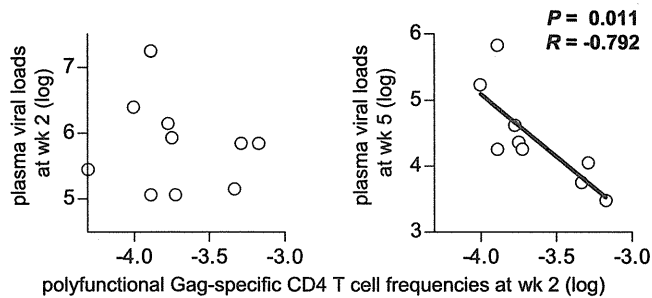


FIG. 3. Analysis of correlation between polyfunctional Gag-specific CD4<sup>+</sup> T-cell frequencies (log) at week (wk) 2 and plasma viral loads (log) at week 2 (left) or week 5 (right). Inverse correlation is shown on the right ( $P = 0.011$ ;  $R = -0.792$ ), but not on the left ( $P = 0.694$ ;  $R = -0.143$ ).

## RESULTS

### Long-term SIV control after passive NAb immunization

**postinfection.** In order to evaluate the long-term effect on SIV replication of a single passive NAb immunization in the acute phase, we monitored animals for more than 1 year after SIVmac239 challenge (Fig. 1). Five NAb-immunized rhesus macaques and six unimmunized controls, including two animals that received control antibodies at week 1, were followed up. Of these, NAb-immunized macaque R03-011 and two unimmunized controls, R01-011 and R06-038, shared the MHC-I haplotype *90-010-Ie*, and NAb-immunized R06-023 and unimmunized R01-012 shared *90-010-Id*. We previously reported that a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-120-Ia* mounted efficient Gag-specific CD8<sup>+</sup> T-cell responses and showed vaccine-based SIV control (21, 29), but those animals were not included in the present study.

The plasma viral loads of both NAb-immunized and unimmunized macaques were similar at week 1, just before NAb administration (Fig. 1A). At week 2 postchallenge, i.e., 1 week after NAb administration, the geometric mean of plasma viral loads in NAb-immunized macaques was slightly lower than in unimmunized controls, but this difference did not achieve statistical significance. At week 8, however, the difference became significant, with lower plasma viral loads in NAb-immunized animals (Fig. 1A). Thereafter, the NAb-immunized macaques maintained significantly reduced viral loads for more than 1 year. In the chronic phase, plasma viral loads were less than  $1 \times 10^4$  copies/ml in all five NAb-immunized macaques and were even undetectable in three of them. NAb-immunized macaque R03-011, possessing the MHC-I haplotype *90-010-Ie*, contained SIV replication with undetectable viremia, whereas unimmunized macaques R01-011 and R06-038, which shared this haplotype, had high viral loads. The NAb-immunized macaque R06-023, with MHC-I haplotype *90-010-Id*, contained SIV replication, whereas unimmunized macaque R01-012, which shared the same haplotype, failed to control viremia. Peripheral CD4<sup>+</sup> T-cell counts were maintained in the NAb-immunized macaques during the observation period (Fig. 1B).

We examined SIVmac239-specific neutralizing antibody responses by determining the end point plasma titers for inhibiting 10-TCID<sub>50</sub> virus replication on MT-4 cells (data not shown). In NAb-immunized macaques, NAb responses were

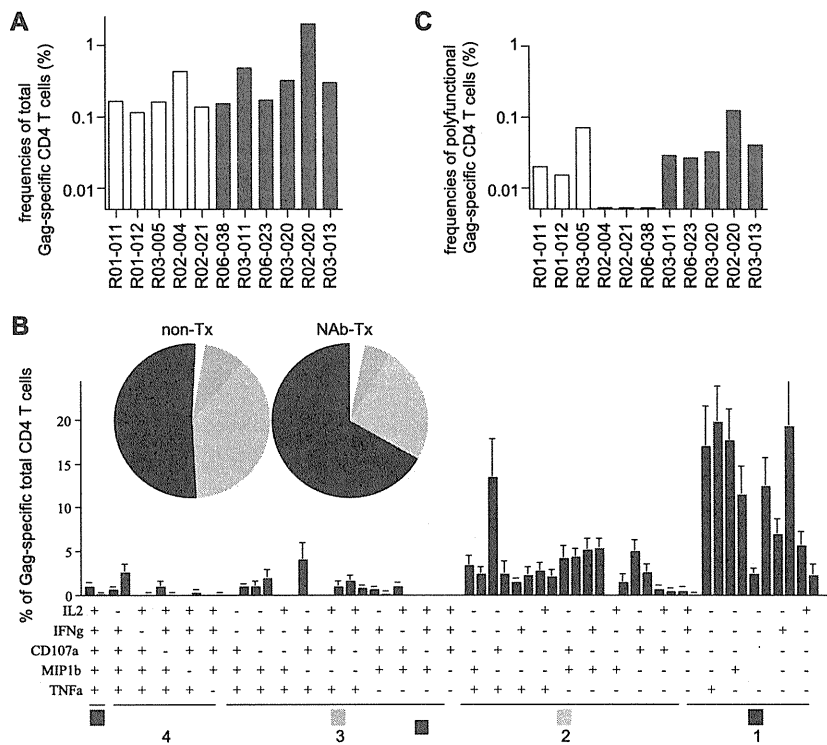


FIG. 4. Gag-specific CD4<sup>+</sup> T-cell responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific induction of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , and CD107a in CD4<sup>+</sup> T cells was examined. (A) Frequencies of total Gag-specific CD4<sup>+</sup> T cells. (B) Percentages of cells exhibiting Gag-specific induction of single or multiple marker cytokines in total Gag-specific CD4<sup>+</sup> T cells. See the legend to Fig. 2 for symbols. (C) Frequencies of polyfunctional Gag-specific CD4<sup>+</sup> T cells exhibiting Gag-specific induction of  $\geq 3$  marker cytokines in total CD4<sup>+</sup> T cells. The frequencies in NAb-immunized macaques ( $n = 5$ ) were significantly higher than in unimmunized controls ( $n = 6$ ) ( $P = 0.046$ ).

detected at day 10 postchallenge but became undetectable within 1 week of passive NAb immunization, as described previously (50), implying that the infused NAbs were rapidly exhausted for virus clearance. None of the animals had detectable de novo NAb responses even around week 40 after challenge. In unimmunized controls, SIVmac239-specific NAb responses were also undetectable, except in one animal, R01-012, after week 30. Thus, passive NAb immunization 1 week after SIV challenge resulted in a transient period of NAb

detection, followed by sustained virus control in the absence of detectable NAb responses.

**Polyfunctional Gag-specific CD4<sup>+</sup> T-cell responses in the acute phase in passively NAb-immunized macaques.** To investigate whether virus-specific T-cell responses were involved in this NAb-triggered SIV control, we first analyzed SIV Gag-specific CD4<sup>+</sup> T-cell responses in the acute phase. We stimulated PBMCs obtained at week 2 with a recombinant SIV Gag p55 protein and analyzed Gag-specific induction of IFN- $\gamma$ ,

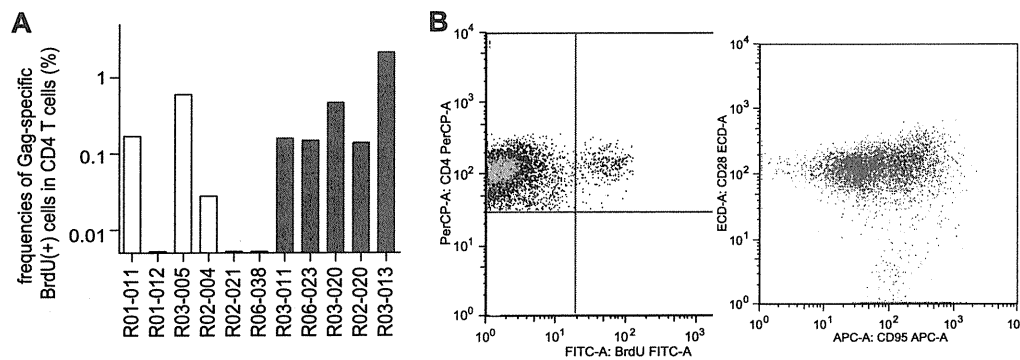


FIG. 5. Gag-specific CD4<sup>+</sup> T-cell proliferative responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific uptake of BrdU in CD4<sup>+</sup> T cells was examined. (A) Frequencies of Gag-specific BrdU<sup>+</sup> CD4<sup>+</sup> T cells in total CD4<sup>+</sup> T cells. The frequencies in NAb-immunized macaques were significantly higher than in unimmunized controls ( $P = 0.042$ ). (B) A representative density plot (gated on CD3<sup>+</sup> lymphocytes) showing BrdU<sup>+</sup> CD4<sup>+</sup> T-cell induction after Gag stimulation (macaque R03-013). Most Gag-specific BrdU<sup>+</sup> CD4<sup>+</sup> T cells gated in the left-hand plot were CD95<sup>+</sup> CD28<sup>+</sup> (indicated by red) in the right-hand plot gated on CD3<sup>+</sup> CD4<sup>+</sup> lymphocytes. FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll protein.

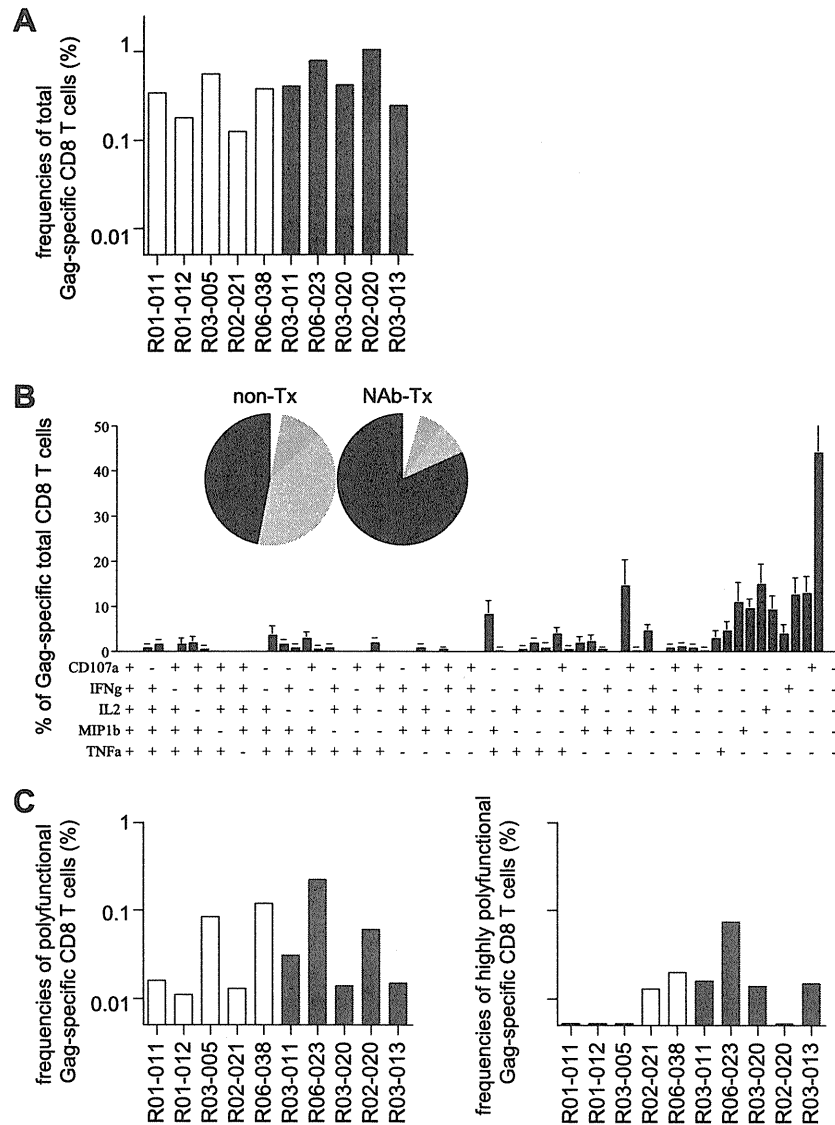


FIG. 6. Gag-specific CD8<sup>+</sup> T-cell responses in the acute phase. PBMCs at week 2 were stimulated with p55, and specific induction of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , and CD107a in CD8<sup>+</sup> T cells was examined. (A) Frequencies of total Gag-specific CD8<sup>+</sup> T cells. (B) Percentages of cells exhibiting Gag-specific induction of single or multiple marker cytokines in total Gag-specific CD8<sup>+</sup> T cells. See the legend to Fig. 2 for symbols. (C) Frequencies of Gag-specific CD8<sup>+</sup> T cells exhibiting Gag-specific induction of  $\geq 3$  marker cytokines (polyfunctional; left) or  $\geq 4$  marker cytokines (highly polyfunctional; right) in total CD8<sup>+</sup> T cells.

TNF- $\alpha$ , IL-2, and MIP-1 $\beta$  and surface mobilization of CD107a (a degranulation marker) in CD4<sup>+</sup> T cells (Fig. 2A) (14, 25, 41). The Gag-specific responses of each factor, IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , and CD107a, in CD4<sup>+</sup> T cells did not show significant differences between unimmunized and NAb-immunized animals (data not shown). We then analyzed these five factors to assess the polyfunctionality of virus-specific T cells and refer to them as marker cytokines in this study. No significant differences in the frequencies of total Gag-specific CD4<sup>+</sup> T cells (i.e., CD4<sup>+</sup> T cells exhibiting Gag-specific induction of one or more of the marker cytokines IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , and CD107a) were observed between the two groups (Fig. 2B).

We examined the polyfunctionality of SIV Gag-specific CD4<sup>+</sup> T cells, as defined by their multiplicity of marker cyto-

kines induced by Gag-specific stimulation (11, 41) (Fig. 2C). The mean percentage of cells producing  $\geq 3$  marker cytokines (Fig. 2C, sum of red, yellow, and green) in the Gag-specific CD4<sup>+</sup> T-cell pool was more than 15% in NAb-immunized macaques but less than 3% in unimmunized controls. The frequencies of these polyfunctional Gag-specific CD4<sup>+</sup> T cells within the CD4<sup>+</sup> T-cell pool were significantly higher in the immunized animals, with a solid difference ( $P = 0.008$  by  $t$  test) (Fig. 2D). Indeed, all the NAb-immunized macaques had higher frequencies of polyfunctional Gag-specific CD4<sup>+</sup> T cells than any of the unimmunized controls, indicating that passive NAb immunization 1 week after SIV challenge resulted in rapid induction of Gag-specific CD4<sup>+</sup> T cells with higher polyfunctionality at week 2.

The polyfunctional Gag-specific CD4<sup>+</sup> T-cell frequencies at

week 2 were inversely correlated with plasma viral loads at week 5 (Fig. 3). The inverse correlation, however, was not indicated with plasma viral loads at week 2. These results implicate rapidly induced polyfunctional Gag-specific CD4<sup>+</sup> T-cell responses in subsequent reduction of plasma viral loads in NAb-immunized macaques.

**Polyfunctional Gag-specific CD4<sup>+</sup> T-cell responses in the chronic phase in NAb-immunized macaques.** We then examined SIV Gag-specific CD4<sup>+</sup> T-cell responses in the chronic phase. Around week 30 after challenge, total Gag-specific CD4<sup>+</sup> T-cell frequencies in NAb-immunized animals were similar to or, if anything, higher than those in unimmunized controls (Fig. 4A). The Gag-specific responses of each marker cytokine in CD4<sup>+</sup> T cells showed no significant difference between the two groups (data not shown). The polyfunctionalities of these Gag-specific CD4<sup>+</sup> T cells (the percentage of cells producing  $\geq 3$  marker cytokines) within the total Gag-specific CD4<sup>+</sup> T-cell population in both groups were similar (Fig. 4B). However, the frequencies of these polyfunctional Gag-specific CD4<sup>+</sup> T cells as a fraction of total CD4<sup>+</sup> T cells in NAb-immunized macaques were higher than in unimmunized controls (Fig. 4C).

We also examined the SIV Gag-specific proliferative responses of CD4<sup>+</sup> T cells around week 30 by measurement of BrdU uptake after Gag-specific stimulation (Fig. 5A). This revealed higher proliferative responses of Gag-specific CD4<sup>+</sup> T cells in NAb-immunized macaques than in unimmunized controls. Gag-specific CD4<sup>+</sup> T-cell proliferative responses were detectable in all the NAb-immunized macaques but in only three of six unimmunized controls. Most of the BrdU<sup>+</sup> CD4<sup>+</sup> T cells after Gag-specific stimulation were of the central memory (CD95<sup>+</sup> CD28<sup>+</sup>) phenotype (36) (Fig. 5B). These results suggest that NAb-immunized macaques had potent Gag-specific CD4<sup>+</sup> T cells with efficient proliferative ability in the chronic phase.

**CD8<sup>+</sup> cells with high anti-SIV efficacy in NAb-immunized macaques.** The above-mentioned results revealed higher frequencies of polyfunctional Gag-specific CD4<sup>+</sup> T-cell responses in NAb-immunized macaques. We next analyzed Gag-specific CD8<sup>+</sup> T-cell responses in the acute phase (Fig. 6). At week 2, total Gag-specific CD8<sup>+</sup> T-cell frequencies were similar, and no clear difference in frequencies of Gag-specific CD8<sup>+</sup> T cells producing  $\geq 3$  or  $\geq 4$  marker cytokines was detected between the two groups.

We then examined, by *in vitro* viral-suppression assays (13, 27, 46, 51), whether the CD8<sup>+</sup> cells from these NAb-immunized macaques had the potential to control SIV replication more efficiently than those from the controls (Fig. 7). In this assay, CD8<sup>-</sup> target cells prepared by CD8-negative selection from PBMCs were infected with SIVmac239 and cocultured with effector CD8<sup>+</sup> cells prepared by CD8-positive selection from PBMCs at week 3. We obtained results from four NAb-immunized macaques and three unimmunized controls.

Three of four NAb-immunized macaques (R03-011, R03-020, and R03-013) showed more than 100-fold reduction in viral production at an E/T ratio of 1:4, although the remaining animal (R02-020) failed to show strong anti-SIV efficacy *in vitro*. Of the NAb-immunized animals, this individual R02-020 maintained the highest viral loads in the chronic phase. In contrast to CD8<sup>+</sup> cells from the majority of immunized ani-

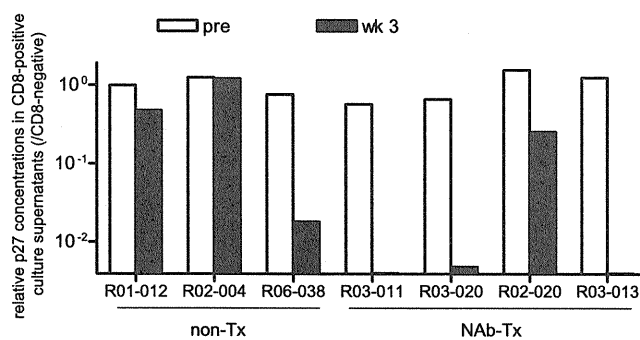


FIG. 7. Anti-SIV efficacy *in vitro* of CD8<sup>+</sup> cells. PBMC-derived CD8<sup>-</sup> (target) cells infected with SIVmac239 were cultured alone (no CD8) or cocultured with autologous PBMC-derived CD8<sup>+</sup> (effector) cells obtained prechallenge (pre) or at week 3 postchallenge (wk 3) at an E/T ratio of 1:4. The results were obtained from three unimmunized controls and four NAb-immunized macaques. The ratios of p27 concentrations in the culture supernatants after 8 days of coculture with pre-CD8<sup>+</sup> or week 3 CD8<sup>+</sup> cells to those without CD8<sup>+</sup> cells (CD8 negative) are shown. The coculture with either R03-011 week 3 CD8<sup>+</sup>, R03-020 week 3 CD8<sup>+</sup>, and R03-013 week 3 CD8<sup>+</sup> cells showed undetectable or marginal SIV p27 production after 8 days.

mals, CD8<sup>+</sup> cells from the unimmunized controls (R01-012, R02-004, and R06-038) showed weak anti-SIV efficacy. In fact, the reduction of virus production by CD8<sup>+</sup> cells from the unimmunized macaques R01-012 and R06-038 was less than 100-fold even in coculture at an E/T ratio of 1:1 (data not shown; not determined for R02-004). These results suggest that passive NAb immunization may facilitate the induction of potent CD8<sup>+</sup> cells possessing higher anti-SIV efficacy.

**Polyfunctional Gag-specific CD8<sup>+</sup> T-cell responses in the chronic phase in NAb-immunized macaques.** We next examined SIV Gag-specific CD8<sup>+</sup> T-cell responses in the chronic phase (Fig. 8). Around week 30 after challenge, the geometric means of total Gag-specific CD8<sup>+</sup> T-cell frequencies in NAb-immunized animals were higher than in unimmunized controls, but this difference did not achieve statistical significance. In particular, NAb-immunized macaques showed significantly higher levels of Gag-specific IFN- $\gamma$  responses in CD8<sup>+</sup> T cells (data not shown). There was no clear difference in polyfunctional Gag-specific CD8<sup>+</sup> T-cell responses between the two groups. However, highly polyfunctional Gag-specific CD8<sup>+</sup> T cells producing  $\geq 4$  marker cytokines were detectable in all NAb-immunized macaques, and the frequencies of these highly polyfunctional Gag-specific CD8<sup>+</sup> T cells in the total CD8<sup>+</sup> T-cell population were higher than in unimmunized controls.

## DISCUSSION

In our previous study (50), a single passive NAb immunization of rhesus macaques 1 week after SIVmac239 challenge resulted in significant reduction of set point viral loads. The present study has shown that this NAb-triggered virus control was maintained in the absence of detectable NABs in the chronic phase. Remarkably, virus-specific CD4<sup>+</sup> T-cell responses with higher polyfunctionality were rapidly induced in NAb-immunized macaques. These results implicate more po-

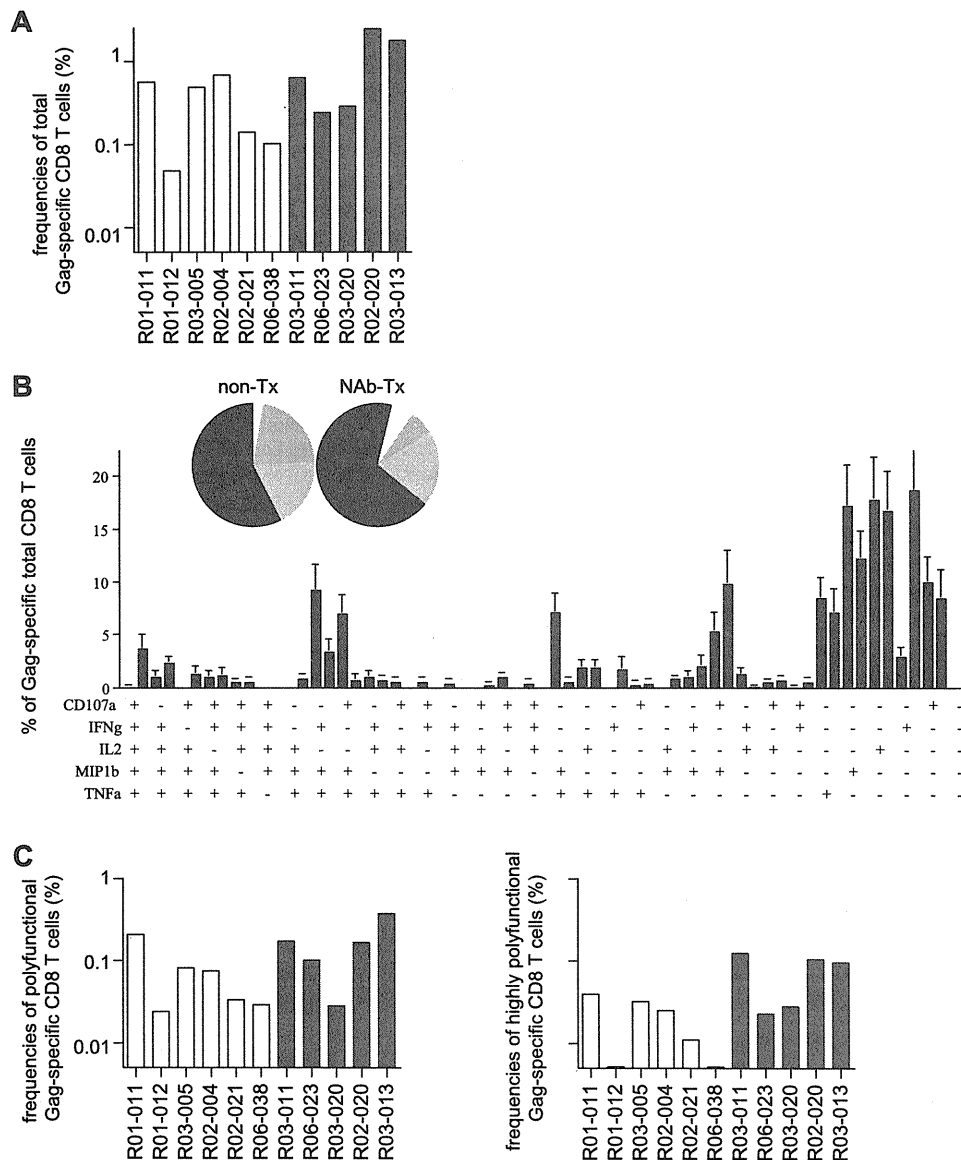


FIG. 8. Gag-specific CD8<sup>+</sup> T-cell responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific induction of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, MIP-1 $\beta$ , and CD107a in CD8<sup>+</sup> T cells was examined. (A) Frequencies of total Gag-specific CD8<sup>+</sup> T cells. (B) Percentages of cells exhibiting Gag-specific induction of single or multiple marker cytokines in total Gag-specific CD8<sup>+</sup> T cells. See the legend to Fig. 2 for symbols. (C) Frequencies of Gag-specific CD8<sup>+</sup> T cells exhibiting Gag-specific induction of  $\geq 3$  marker cytokines (polyfunctional; left) or  $\geq 4$  marker cytokines (highly polyfunctional; right) in total CD8<sup>+</sup> T cells. The highly polyfunctional Gag-specific CD8<sup>+</sup> T-cell frequencies in NAb-immunized macaques were significantly higher than in unimmunized controls ( $P = 0.023$ ).

tent induction of functional virus-specific CD4<sup>+</sup> T-cell responses in this NAb-triggered SIV control.

All the NAb-immunized macaques had higher frequencies of polyfunctional Gag-specific CD4<sup>+</sup> T cells than any of the unimmunized controls at week 2, although the two groups possessed similar frequencies of total Gag-specific CD4<sup>+</sup> T cells. This implies higher polyfunctionality of Gag-specific CD4<sup>+</sup> T cells in the acute phase in NAb-immunized macaques than in unimmunized controls. HIV-1 is known to preferentially infect HIV-1-specific CD4<sup>+</sup> T cells (10); virus neutralization may therefore protect virus-specific CD4<sup>+</sup> T cells from SIV infection. However, it remains unclear whether NABs preferentially protect polyfunctional virus-specific CD4<sup>+</sup> T

cells. Our previous study suggested augmentation of the Fc-mediated uptake of NAB-virion complexes into dendritic cells following passive NAB immunization (50). This may enhance antigen presentation and induction of polyfunctional virus-specific CD4<sup>+</sup> T-cell responses in the acute phase. Thus, both NAb-mediated effects, i.e., enhancement of antigen presentation and protection of virus-specific CD4<sup>+</sup> T cells from viral infection, may contribute to the induction of polyfunctional virus-specific CD4<sup>+</sup> T cells in the acute phase.

It is thought that potent virus-specific CD4<sup>+</sup> T-cell responses are important for the control of HIV-1/SIV replication (39). Recent studies analyzing the quality of T-cell responses suggested the possible involvement of polyfunctional CD4<sup>+</sup>



T-cell responses in the control of some viral infections (8, 11, 41). However, there has been no clear evidence indicating association of polyfunctional CD4<sup>+</sup> T-cell responses with HIV-1/SIV control. These cells are themselves targets for viral infection and killing (10), and most natural HIV-1/SIV infections fail to show efficient induction of potent virus-specific CD4<sup>+</sup> T-cell responses (52). In the present study, passive NAb immunization of rhesus macaques 1 week after SIV infection resulted in the induction of significantly higher levels of polyfunctional Gag-specific CD4<sup>+</sup> T-cell responses in the acute phase, followed by SIV control at the set point in the absence of NAb responses. The polyfunctional Gag-specific CD4<sup>+</sup> T-cell frequencies at week 2 were inversely correlated with plasma viral loads, not at week 2, but at week 5. These results indicate that NABs may facilitate the development and retention of polyfunctional virus-specific CD4<sup>+</sup> T-cell responses in the very early phase of HIV-1/SIV infection, contributing to subsequent virus control directly or indirectly. Thus, this is the first report documenting an association between polyfunctional CD4<sup>+</sup> T-cell responses in the acute phase and subsequent SIV control.

Previous studies of the chronic phase of HIV-1 infections have indicated an association between strong HIV-1-specific proliferative CD4<sup>+</sup> T-cell responses and HIV-1 control, as well as their impairment in HIV-1 infection with uncontrolled viremia (3, 17, 18, 32, 39). In the present study, compared to total Gag-specific CD4<sup>+</sup> T-cell frequencies in the acute phase, those in the chronic phase were reduced in unimmunized controls, but NAB-immunized macaques maintained similar frequencies in the chronic phase. This difference may reflect virus control in NAB-immunized macaques and high plasma viremia in unimmunized controls. Our analyses of polyfunctional and proliferative responses suggest that these animals maintained functional Gag-specific CD4<sup>+</sup> T-cell responses in the chronic phase. This may be due to virus control and, conversely, may contribute to sustained virus control.

It has been indicated that virus-specific CD4<sup>+</sup> T-cell responses facilitate induction of functional virus-specific CD8<sup>+</sup> T-cell responses (19, 42, 44). Stimulation with peptides would be optimal for analysis of CD8<sup>+</sup> T-cell responses, but in this study, our first priority was to analyze CD4<sup>+</sup> T-cell responses, and cell samples were used for the analysis of responses after stimulation with a recombinant Gag p55 protein. Therefore, we obtained results on polyfunctional CD8<sup>+</sup> T-cell responses after p55-specific stimulation but did not have enough cell samples for analyzing peptide-specific CD8<sup>+</sup> T-cell responses in the acute phase. In the acute phase, no significant enhancement of polyfunctional Gag-specific CD8<sup>+</sup> T-cell responses was detected after passive NAB immunization, but this does not exclude the possibility of functional CD8<sup>+</sup> T-cell induction in NAB-immunized animals, which may be detected by optimal analysis. Indeed, the viral suppression assay showed that CD8<sup>+</sup> cells able to efficiently suppress SIV replication *in vitro* were induced in the acute phase in those NAB-immunized macaques that contained SIV replication *in vivo*. These highly effective anti-SIV CD8<sup>+</sup> cell responses, which may be affected not only by CD8<sup>+</sup> T-cell polyfunctionality, but also by several other factors, are thus likely to be involved in NAB-triggered containment of SIV replication. Our analyses in the chronic phase indicated higher frequencies of highly polyfunctional Gag-spe-

cific CD8<sup>+</sup> T-cell responses in NAB-immunized macaques, consistent with the previously reported observation in HIV-1-infected nonprogressors (4).

Taken together, the present study indicates that passive NAB immunization of rhesus macaques in the acute phase may be able to trigger rapid induction of polyfunctional Gag-specific CD4<sup>+</sup> T-cell responses, followed by sustained SIV control in the absence of NAB responses in the chronic phase. These results highlight the importance of the synergy between NAB and T-cell responses in primary virus control, implying that the absence of potent NAB responses in the acute phase of HIV-1/SIV infection may be responsible for failure to control persistent viral replication.

Finally, induction of potent NAB responses is believed to be a promising strategy for AIDS vaccine development. While prechallenge passive NAB immunization studies have previously indicated the possibility of sterile protection against immunodeficiency virus infection in macaques, several studies have suggested difficulty in inducing high levels of NAB responses that are sufficient for sterile protection (16, 28, 35, 43, 48). Our results imply that prophylactic vaccination that elicits NAB responses, even if it does not achieve sterile protection, may contribute to HIV-1/SIV control by secondary NAB responses facilitating functional T-cell induction after viral exposure. Thus, this study indicates a potential for HIV-1/SIV control by synergy between NAB and T-cell responses, providing insights into the development of a prophylactic AIDS vaccine.

#### ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology; a grant from the Japan Health Sciences Foundation; and grants from the Ministry of Health, Labor, and Welfare of Japan.

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates. We thank H. Akari, F. Ono, K. Komatsuzaki, A. Hiyaoka, H. Ogawa, K. Oto, K. Ishikawa, T. Nakasone, and H. Igarashi for assistance with the animal experiments and M. Miyazawa, M. Yasunami, T. Naruse, and A. Kimura for determining MHC-I haplotypes. We also thank M. Roederer for providing the PESTLE and SPICE software and V. Appay for his technical support and helpful suggestions on analysis of Gag-specific T-cell responses.

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# Impact of Cytotoxic-T-Lymphocyte Memory Induction without Virus-Specific CD4<sup>+</sup> T-Cell Help on Control of a Simian Immunodeficiency Virus Challenge in Rhesus Macaques<sup>∇</sup>

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Received 2 June 2009/Accepted 29 June 2009

Despite many efforts to develop AIDS vaccines eliciting virus-specific T-cell responses, whether induction of these memory T cells by vaccination before human immunodeficiency virus (HIV) exposure can actually contribute to effective T-cell responses postinfection remains unclear. In particular, induction of HIV-specific memory CD4<sup>+</sup> T cells may increase the target cell pool for HIV infection because the virus preferentially infects HIV-specific CD4<sup>+</sup> T cells. However, virus-specific CD4<sup>+</sup> helper T-cell responses are thought to be important for functional CD8<sup>+</sup> cytotoxic-T-lymphocyte (CTL) induction in HIV infection, and it has remained unknown whether HIV-specific memory CD8<sup>+</sup> T cells induced by vaccination without HIV-specific CD4<sup>+</sup> T-cell help can exert effective responses after virus exposure. Here we show the impact of CD8<sup>+</sup> T-cell memory induction without virus-specific CD4<sup>+</sup> T-cell help on the control of a simian immunodeficiency virus (SIV) challenge in rhesus macaques. We developed a prophylactic vaccine by using a Sendai virus (SeV) vector expressing a single SIV Gag<sub>241-249</sub> CTL epitope fused with enhanced green fluorescent protein (EGFP). Vaccination resulted in induction of SeV-EGFP-specific CD4<sup>+</sup> T-cell and Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses. After a SIV challenge, the vaccinees showed dominant Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses with higher effector memory frequencies in the acute phase and exhibited significantly reduced viral loads. These results demonstrate that virus-specific memory CD8<sup>+</sup> T cells induced by vaccination without virus-specific CD4<sup>+</sup> T-cell help could indeed facilitate SIV control after virus exposure, indicating the benefit of prophylactic vaccination eliciting virus-specific CTL memory with non-virus-specific CD4<sup>+</sup> T-cell responses for HIV control.

Virus-specific T-cell responses are crucial for controlling human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication (3, 4, 12, 20, 28, 36, 37). Therefore, a great deal of effort has been exerted to develop AIDS vaccines eliciting virus-specific T-cell responses (23, 27, 30, 47), but whether this approach actually results in HIV control remains unclear (1, 6). It is important to determine which T-cell responses need to be induced by prophylactic vaccination for HIV control after virus exposure.

Because HIV preferentially infects HIV-specific CD4<sup>+</sup> T cells (5), induction of HIV-specific memory CD4<sup>+</sup> T cells by vaccination may increase the target cell pool for HIV infection and could enhance viral replication (42). However, CD4<sup>+</sup> helper T-cell responses are important for functional CD8<sup>+</sup> cytotoxic-T-lymphocyte (CTL) induction (11, 40, 43, 46), and it has remained unknown whether HIV-specific memory CD8<sup>+</sup> T cells induced by vaccination with non-virus-specific CD4<sup>+</sup> T-cell help (but without HIV-specific CD4<sup>+</sup> T-cell help) can exert effective responses after virus exposure. Indeed, the real

impact of prophylactic induction of CTL memory itself on HIV replication has not been well documented thus far.

We previously developed a prophylactic AIDS vaccine consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag (26). Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication whereas unvaccinated animals developed AIDS (15, 27). In particular, vaccination consistently resulted in control of SIV replication in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia*. Gag<sub>206-216</sub> (IINEEAADWDL) and Gag<sub>241-249</sub> (SSVDEQIQW) epitope-specific CD8<sup>+</sup> T-cell responses were shown to be involved in SIV control in these vaccinated macaques (14, 16).

In the present study, focusing on CD8<sup>+</sup> T-cell responses directed against one of these epitopes, we have evaluated the efficacy of a vaccine expressing the Gag<sub>241-249</sub> epitope fused with enhanced green fluorescent protein (EGFP) against a SIVmac239 challenge in *90-120-Ia*-positive rhesus macaques. The animals exhibited this single-epitope-specific CD8<sup>+</sup> T-cell response and SeV-EGFP-specific CD4<sup>+</sup> T-cell responses after vaccination and showed rapid, dominant induction of potent secondary Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses after a SIV challenge. Plasma viral loads in these vaccinees were significantly reduced compared to those of naive controls. These results indicate that induction of CD8<sup>+</sup> T-cell memory without

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<sup>∇</sup> Published ahead of print on 8 July 2009.

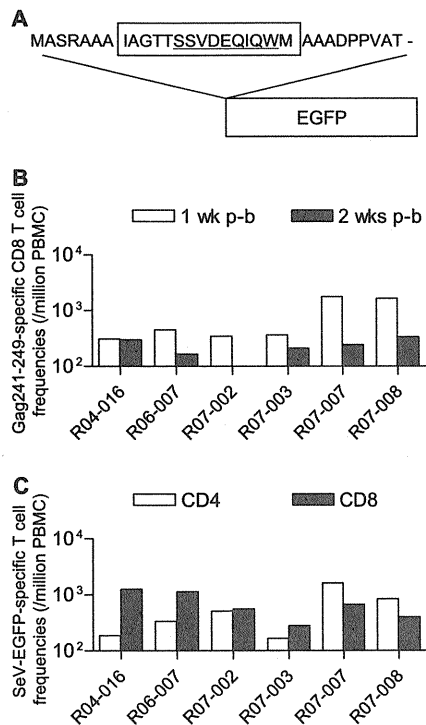


FIG. 1. Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell induction by prophylactic vaccination. (A) Schema of the cDNA construct encoding the Gag<sub>236-250</sub>-EGFP fusion protein. A DNA fragment that encodes a 31-mer peptide including the Gag<sub>236-250</sub> sequence was introduced into the 5' end of the EGFP cDNA. (B) Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell frequencies 1 (open boxes) and 2 weeks (closed boxes) after F(-)SeV-Gag<sub>236-250</sub>-EGFP boosting in group III macaques. (C) SeV-EGFP-specific CD4<sup>+</sup> (open boxes) or CD8<sup>+</sup> (closed boxes) T-cell frequencies 2 weeks after F(-)SeV-Gag<sub>236-250</sub>-EGFP boosting in group III macaques. p-b, postboost.

virus-specific CD4<sup>+</sup> T-cell help by prophylactic vaccination can result in effective CD8<sup>+</sup> T-cell responses after virus exposure.

#### MATERIALS AND METHODS

**Animal experiments.** Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-120-Ia* were divided into three groups: unvaccinated group I ( $n = 6$ ), control-vaccinated group II ( $n = 6$ ), and Gag<sub>236-250</sub>-vaccinated group III ( $n = 6$ ). The MHC-I haplotype was determined by reference strand-mediated conformation analysis as described previously (2, 27, 44). Macaque R06-019, administered nonspecific immunoglobulin G 1 week after a SIV challenge, and previously reported macaque R02-007 (15) were included in group I. pGag<sub>236-250</sub>-EGFP-N1 DNA expressing a Gag<sub>236-250</sub>-EGFP fusion protein was constructed from pEGFP-N1 DNA (BD, Tokyo, Japan). The fusion protein was designed to have 31 amino acids including SIVmac239 Gag<sub>236-250</sub>-sequences (IAGTTSSVDEQIQWM) added to the amino-terminal portion of EGFP (Fig. 1A). The group III macaques received 5 mg of pGag<sub>236-250</sub>-EGFP-N1 DNA intramuscularly and 6 weeks later received a single intranasal boost with  $6 \times 10^9$  cell infectious units of F deletion-containing, replication-defective SeV (24) expressing the Gag<sub>236-250</sub>-EGFP fusion protein (F[-]SeV-Gag<sub>236-250</sub>-EGFP). The group II macaques were primed with pEGFP-N1 DNA and boosted with F(-)SeV-EGFP instead. Approximately 3 months after the boost, these animals and the unvaccinated group I animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239 (17). All animals were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (32).

**Analysis of virus-specific CD8<sup>+</sup> T-cell responses.** We measured virus-specific CD8<sup>+</sup> T-cell levels by flow cytometric analysis of gamma interferon (IFN- $\gamma$ ) induction after specific stimulation as described previously (15, 27). In brief, peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines pulsed with

1  $\mu$ M SIVmac239 Gag<sub>241-249</sub> or Gag<sub>206-216</sub> peptides for Gag<sub>241-249</sub>-specific or Gag<sub>206-216</sub>-specific stimulation. Alternatively, PBMCs were cocultured with B-lymphoblastoid cell lines infected with vesicular stomatitis virus G protein-pseudotyped SIVGP1 for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a vesicular stomatitis virus G protein expression plasmid and *env* and *nef* deletion-containing simian-human immunodeficiency virus molecular clone (SIVGP1) DNA (26, 41). Intracellular IFN- $\gamma$  staining was performed with a Cytofix/Cytoperm kit (BD) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin (APC)-conjugated anti-human CD3, and phycoerythrin (PE)-conjugated anti-human IFN- $\gamma$  monoclonal antibodies (BD). Specific T-cell levels were calculated by subtracting nonspecific IFN- $\gamma$ <sup>+</sup> T-cell frequencies from those after peptide-specific or SIV-specific stimulation. Specific T-cell levels lower than 100 per million PBMCs were considered negative.

**Analysis of Gag<sub>241-249</sub>-specific cytolytic CD8<sup>+</sup> T-cell responses.** We analyzed Gag<sub>241-249</sub>-specific induction of IFN- $\gamma$  and CD107a in CD8<sup>+</sup> T cells. PBMCs were stained with custom-made, PE-conjugated Gag<sub>241-249</sub> epitope-Mamu-A\*90120-5 tetrameric complexes, Gag<sub>241-249</sub>-A\*90120-5 tetramers (Medical and Biological Laboratories Co. Ltd., Nagoya, Japan) (45), for 15 min at 37°C and subsequently incubated with anti-human CD107a antibody (BD) for 6 h in the absence or presence of 1  $\mu$ M Gag<sub>241-249</sub> peptide for unstimulated controls or Gag<sub>241-249</sub>-specific stimulation. In both cultures, anti-human CD28 and anti-human CD49d antibodies (5  $\mu$ g/ml) (BD) were added for costimulation and monensin (BD) and brefeldin A (Sigma-Aldrich, Tokyo, Japan) were used for inhibition of cytokine secretion. Immunostaining was performed with a Cytofix-Cytoperm kit and the following monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-human perforin (MABTECH), peridinin chlorophyll protein-conjugated anti-human CD4 (BD), APC-conjugated anti-human granzyme B (Invitrogen, Tokyo, Japan), PE-cyanine 7 (PE-Cy7)-conjugated anti-human IFN- $\gamma$  (BD), APC-Cy7-conjugated anti-human CD3 (BD), energy-coupled-dye-conjugated anti-human CD69 (Beckman Coulter, Tokyo, Japan), Alexa700-conjugated anti-human CD8 (BD), and anti-human CD107a conjugated with Pacific Blue with a Zeon mouse immunoglobulin G1 labeling kit (Invitrogen). Flow cytometric analysis was performed with the FACSAria system (BD). The data were analyzed with FlowJo (version 8.2; TreeStar Inc., Ashland, OR), FACSDiva (BD), PESTLE (version 1.5.4), and SPICE (version 4.1.6) software.

**Statistical analysis.** Statistical analysis of plasma viral loads in the acute phase (at the peak and week 5) was performed with R version 2.7.1 (R Development Core Team; <http://www.R-project.org/>). Data were log transformed, and a two-tailed one-way analysis of variance, followed by the Shaffer sequentially rejective method of multiple-comparison analysis (39), was performed to estimate differences among groups I, II, and III with overall significance levels set to  $\alpha = 0.05$  (two tailed). Statistical analysis of set point plasma viral loads was performed by the nonparametric Kruskal-Wallis test with the sequentially rejective pairwise Mann-Whitney exact test, because we did not assume residual normality and homoscedasticity in set point viral loads, which were mostly below the lower limit of detection in group III animals. Antigen-specific T-cell frequencies were log transformed and compared by unpaired two-tailed  $t$  test with significance levels set at  $P < 0.05$ , and correlation was analyzed by using Prism software version 4.03 (GraphPad Software, Inc., San Diego, CA).

#### RESULTS

**Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell induction following prophylactic vaccination.** Eighteen Burmese rhesus macaques possessing MHC-I haplotype *90-120-Ia* were divided into three groups of six animals each (Table 1). Group I received no vaccination, group II received a control vaccine, and group III received a vaccine eliciting Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses. We refer to groups I and II as naive controls in the present study. We constructed a plasmid DNA (pGag<sub>236-250</sub>-EGFP-N1) and an F deletion-containing SeV (F[-]SeV-Gag<sub>236-250</sub>-EGFP) vector both expressing an SIVmac239 Gag<sub>236-250</sub> (IAGTTSSVDEQIQWM)-EGFP fusion protein to be used for group III vaccination (Fig. 1A). SeV proteins and EGFP have no amino acid sequence identity with SIVmac239. These group III animals received a single intramuscular pGag<sub>236-250</sub>-EGFP DNA injection, followed by a single intra-

TABLE 1. Macaques used in this study

Group	Animal identification codes	Vaccination <sup>a</sup>
I	R02-007, R06-037, R07-001, R07-004, R07-009, R06-019	None
II	R02-008, R05-026, R06-004, R06-014, R06-040, R07-006	Control vaccination [pEGFP-N1 DNA prime, F(-)SeV-EGFP boost]
III	R04-016, R06-007, R07-002, R07-003, R07-007, R07-008	Gag <sub>241-249</sub> -specific vaccination [pGag <sub>236-250</sub> -EGFP-N1 DNA prime, F(-)SeV-Gag <sub>236-250</sub> -EGFP boost]

<sup>a</sup> All animals were challenged with SIVmac239.

nasal boost with the F(-)SeV-Gag<sub>236-250</sub>-EGFP vector. Group II animals were administered pEGFP-N1 DNA and the F(-)SeV-EGFP vector, both expressing EGFP instead of Gag<sub>236-250</sub>-EGFP, as a control vaccine.

We measured the antigen-specific CD8<sup>+</sup> T-cell responses in these macaques 1 or 2 weeks after the SeV boost by detection of specific IFN- $\gamma$  induction. All group III macaques showed efficient Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell induction after the F(-)SeV-Gag<sub>236-250</sub>-EGFP boost (Fig. 1B). In these animals, we also confirmed SeV-EGFP-specific CD8<sup>+</sup> and CD4<sup>+</sup> T-cell responses (Fig. 1C) but did not detect Gag<sub>206-216</sub>-specific CD8<sup>+</sup> T-cell responses, which are dominantly induced in 90-120-Ia-positive macaques by Gag-expressing SeV vaccination (14). We have never found Gag<sub>236-250</sub>-specific CD4<sup>+</sup> T-cell responses in any previously examined animals, and as expected, analyses with the Gag<sub>236-250</sub> peptide did not detect Gag<sub>236-250</sub>-specific CD4<sup>+</sup> T-cell responses in any of the group III animals in the present study. In group II animals, we detected SeV-EGFP-specific T-cell responses but not Gag<sub>236-250</sub>-specific T-cell responses after the F(-)SeV-EGFP boost (data not shown).

**Control of an SIV challenge in vaccinees.** Group I (unvaccinated), II (control-vaccinated), and III (Gag<sub>236-250</sub>-vaccinated) macaques were challenged intravenously with SIVmac239. Plasma viral loads in these animals were examined after the challenge (Fig. 2A). Most of the group I and II animals failed to contain SIV replication, although plasma viremia became undetectable at week 12 in one animal in group I (R06-037) and one in group II (R06-004). No significant differences were observed between groups I and II in plasma viral loads at the peak, at week 5, at week 12, or around week 24 after the challenge. In contrast, most group III animals contained SIV replication; plasma viral loads became undetectable after week 5 in five of the six animals (Fig. 2A). Plasma viral loads in these animals were significantly lower than those in unvaccinated group I and those in control-vaccinated group II at the peak, at week 5, and at the set point (Fig. 2B). Thus, the prophylactic vaccination inducing Gag<sub>241-249</sub> single-epitope-specific CD8<sup>+</sup> T-cell responses resulted in a significant reduction of peak and subsequent viral loads after the SIV challenge. No significant difference in peripheral CD4<sup>+</sup> T-cell counts was observed among these three groups (Fig. 2C).

**Dominant Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses in vaccinees after a SIV challenge.** We assessed virus-specific CD8<sup>+</sup> T-cell responses at weeks 2 and 12 after a SIV challenge by

measuring antigen-specific IFN- $\gamma$  induction. Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses were undetectable or marginal in some naive controls (group I and II) but were efficiently induced in all of the group III animals (Fig. 3A). In most of the naive controls, Gag<sub>206-216</sub>-specific CD8<sup>+</sup> T-cell responses were induced equivalently or more efficiently than Gag<sub>241-249</sub>-specific CD8<sup>+</sup> responses, whereas all of the group III animals showed dominant induction of Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses. In these group III animals, Gag<sub>206-216</sub>-specific CD8<sup>+</sup> T-cell responses were inefficient but frequencies of CD8<sup>+</sup> T cells exhibiting Gag<sub>241-249</sub>-specific IFN- $\gamma$  induction were significantly higher than in naive controls at week 2 (Fig. 3B) and week 12. Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell frequencies at week 2 inversely correlated with peak viral loads (Fig. 3C).

We also tested SIV-specific CD8<sup>+</sup> T-cell responses in these animals (Fig. 4). We used *env* and *nef* deletion-containing simian-human immunodeficiency virus molecular clone DNA SIVGP1 containing the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr and measured the frequencies of CD8<sup>+</sup> T cells responding to SIVGP1-transduced cells (referred to as SIV-specific CD8<sup>+</sup> T cells) as described previously (15). Naive controls (groups I and II) and vaccinees (group III) were found to possess similar levels of SIV-specific CD8<sup>+</sup> T cells at week 2 and week 12.

In our previous study (27), all of the 90-120-Ia-positive macaques vaccinated with Gag-expressing SeV contained SIV replication with rapid selection of a *gag* mutation (GagL216S), resulting in escape from Gag<sub>206-216</sub>-specific CD8<sup>+</sup> T-cell recognition at week 5, implicating Gag<sub>206-216</sub>-specific CD8<sup>+</sup> T-cell responses (rather than Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses) in viral control. In the present study, however, five of six Gag<sub>236-250</sub>-vaccinated animals controlled SIV replication and had undetectable set point viremia without selection of *gag* mutation over 5 weeks (data not shown). No *gag* mutation was selected at week 5 in naive controls, either. These results indicate that in the group III macaques, dominantly induced Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses in the acute phase play an important role in this vaccine-based SIV control.

**Higher Gag<sub>241-249</sub>-specific effector memory CD8<sup>+</sup> T-cell frequencies in vaccinees.** We then examined Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell frequencies in these macaques by using PE-conjugated Gag<sub>241-249</sub>-A\*90120-5 tetramers. In group III animals, Gag<sub>241-249</sub>-specific tetramer<sup>+</sup> CD8<sup>+</sup> T cells were still detectable just before the SIV challenge, and their frequencies increased greatly after the challenge; most of the vaccinees exhibited a >10-fold increase at week 2 compared to prechallenge levels (Fig. 5A). Increases in tetramer<sup>+</sup> CD28<sup>-</sup> CD8<sup>+</sup> T-cell frequencies after a challenge were especially marked (>30-fold) (Fig. 5B). Indeed, within the tetramer<sup>+</sup> cells, the ratio of CD28<sup>-</sup> cells increased after a challenge and these cells became predominant at week 2. Analysis of an effector memory subset delineated by the CD95<sup>+</sup> CD28<sup>-</sup> phenotype (29, 34) revealed significantly higher frequencies of Gag<sub>241-249</sub>-specific tetramer<sup>+</sup> CD95<sup>+</sup> CD28<sup>-</sup> CD8<sup>+</sup> T cells in group III than in naive controls (Fig. 5C). These results suggest efficient responses of Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T cells with effector function in the acute phase in group III animals.

**Gag<sub>241-249</sub>-specific cytolytic CD8<sup>+</sup> T-cell responses in vaccinees.** To further investigate the cytolytic quality of Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses after a challenge, we examined

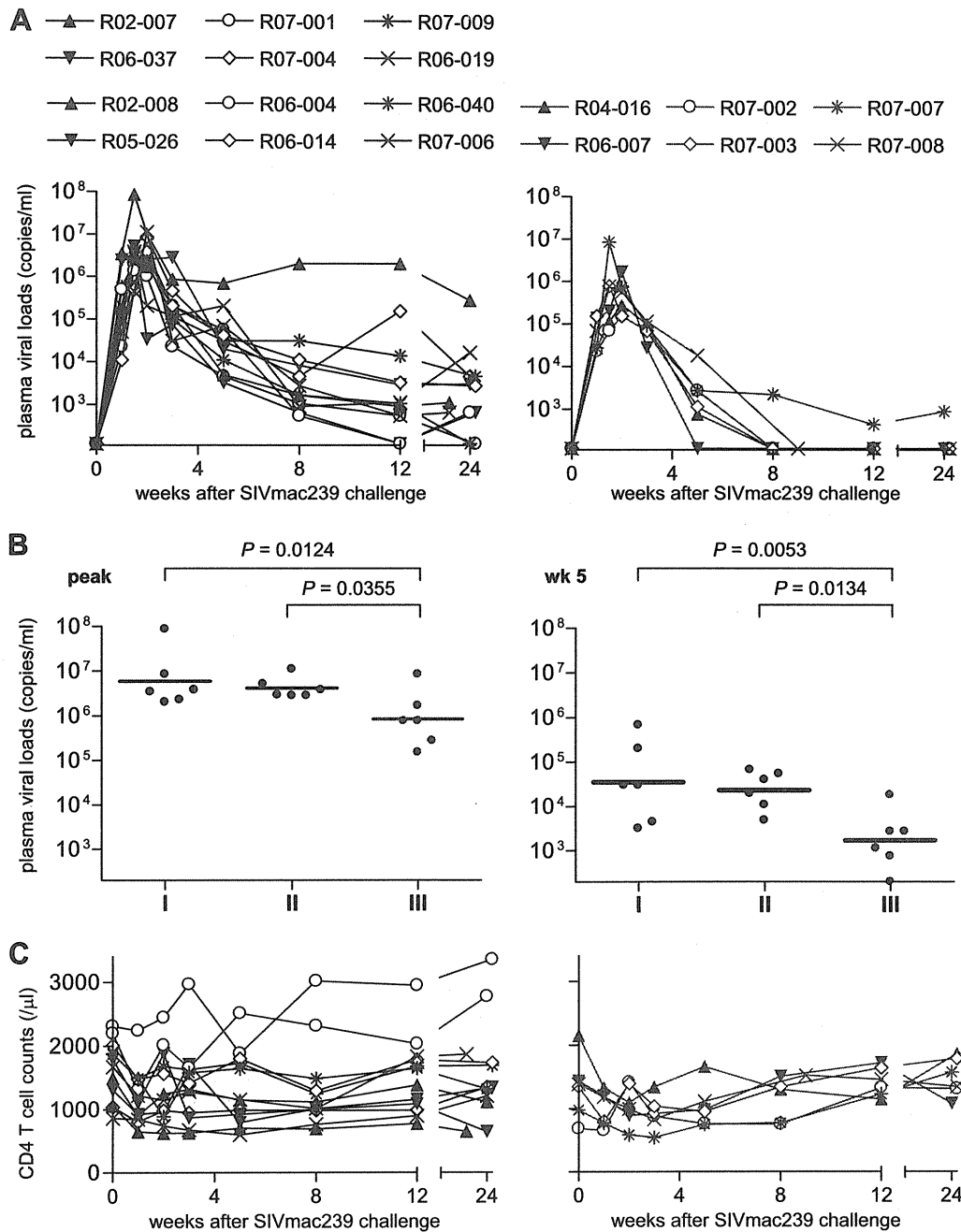


FIG. 2. Plasma viral loads and peripheral CD4<sup>+</sup> T-cell counts after a SIV challenge. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) in unvaccinated group I animals (black lines in the left panel), control-vaccinated group II animals (blue lines in the left panel), and Gag<sub>236-250</sub>-vaccinated group III animals (red lines in the right panel) after a SIVmac239 challenge. Plasma viral loads were determined as described previously (27). The lower limit of detection is approximately  $4 \times 10^2$  copies/ml. (B) Comparisons of plasma viral loads in groups I ( $n = 6$ ), II ( $n = 6$ ), and III ( $n = 6$ ) at the peak (left panel) and at week 5 (right panel). The bar indicates the geometric mean of each group. Viral loads at the peak and at week 5 in group III were significantly lower than in group I ( $P = 0.0124$  at the peak and  $P = 0.0053$  at week 5) and group II ( $P = 0.0355$  at the peak and  $P = 0.0134$  at week 5). There were no significant differences between groups I and II either at the peak or at week 5 ( $P = 0.6047$  at the peak and  $P = 0.6536$  at week 5). Set point viral loads in group III were significantly lower than those in group I and group II at week 12 by nonparametric analysis ( $P = 0.3939$  between I and II,  $P = 0.0152$  between I and III, and  $P = 0.0152$  between II and III;  $P = 0.1797$  between I and II,  $P = 0.0260$  between I and III, and  $P = 0.0411$  between II and III around week 24). (C) Changes in peripheral CD4<sup>+</sup> T-cell counts (per  $\mu$ l) in groups I (black lines) and II (blue lines) in the left panel and in group III (red lines) in the right panel after a SIVmac239 challenge.

Gag<sub>241-249</sub>-specific induction of CD107a (a degranulation marker), which is related to cytolytic activity (21, 38), in CD8<sup>+</sup> T cells at week 2. Frequencies of CD8<sup>+</sup> T cells exhibiting Gag<sub>241-249</sub>-specific induction of CD107a, as well as IFN- $\gamma$ ,

within the CD8<sup>+</sup> T-cell pool were significantly higher in group III than in naive controls ( $P = 0.0249$  by unpaired  $t$  test) (Fig. 6). One animal, R04-016, in group III did not show Gag<sub>241-249</sub>-specific CD107a<sup>+</sup> IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T-cell responses, but further

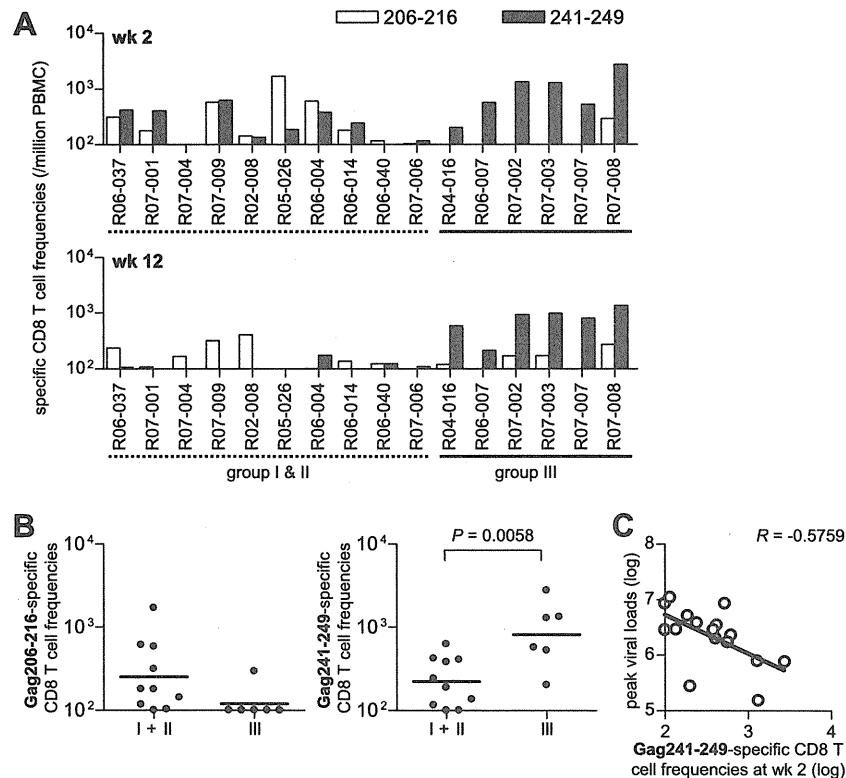


FIG. 3. Gag epitope-specific CD8<sup>+</sup> T-cell frequencies after a SIV challenge. (A) Frequencies of CD8<sup>+</sup> T cells (per million PBMCs) showing Gag<sub>206-216</sub>-specific (open boxes) or Gag<sub>241-249</sub>-specific (closed boxes) IFN- $\gamma$  induction in naive controls and group III macaques at week 2 (upper panel) and week 12 (lower panel). (B) Comparison of the Gag<sub>206-216</sub>-specific (left panel) or Gag<sub>241-249</sub>-specific (right panel) CD8<sup>+</sup> T-cell frequencies in naive controls ( $n = 10$ ) and group III animals ( $n = 6$ ) at week 2. The bar indicates the geometric mean of each group. Frequencies of Gag<sub>241-249</sub>-specific ( $P = 0.0058$ ) but not Gag<sub>206-216</sub>-specific ( $P = 0.0922$ ) CD8<sup>+</sup> T cells in group III were significantly higher than in naive controls. The Gag<sub>241-249</sub>-specific frequencies at week 12 in group III were significantly higher than those in naive controls ( $P < 0.0001$ ). (C) Analysis of the correlation between Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell frequencies (log) at week 2 and peak plasma viral loads (log). An inverse correlation is shown ( $P = 0.0196$ ,  $R = -0.5759$ ). Samples from macaques R02-007 and R06-019 in group I were unavailable for this analysis.

analysis revealed that this animal had Gag<sub>241-249</sub>-specific granzyme B<sup>+</sup> IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells. Indeed, group III animals had significantly higher frequencies of Gag<sub>241-249</sub>-specific IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells producing CD107a, granzyme B, or perforin ( $P = 0.0076$ ; data not shown). These results indicate efficient induction of Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T cells with higher cytolytic activity in the acute phase in group III animals.

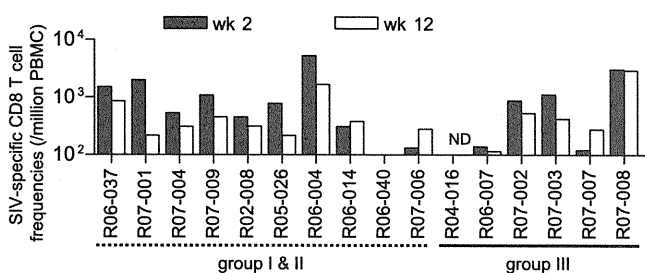


FIG. 4. SIV-specific CD8<sup>+</sup> T-cell frequencies after a SIV challenge. SIV-specific CD8<sup>+</sup> T-cell frequencies (per million PBMCs) in naive controls and group III macaques at week 2 (closed boxes) and week 12 (open boxes) are shown. ND, not determined.

DISCUSSION

In the present study, induction of CD8<sup>+</sup> T cells specific for a single Gag<sub>241-249</sub> epitope by prophylactic vaccination resulted in a significant reduction of plasma viral loads after a SIV challenge. Even if vaccines are designed to express multiple antigens, of the vaccine-induced CD8<sup>+</sup> T cells generated, at most one or only a few epitope-specific cells may recognize the incoming HIV because of viral diversity and host MHC polymorphisms (10). Our finding, however, implies that even a CD8<sup>+</sup> T-cell memory response to a single epitope which can recognize the incoming HIV could facilitate HIV control.

Group III macaques showed more effective CD8<sup>+</sup> T-cell responses than did naive controls after a SIV challenge. Our previous trial of a vaccine inducing Gag-specific T-cell responses resulted in SIV control in 90-120-*Ia*-positive macaques with rapid selection of the GagL216S mutation escaping from Gag<sub>206-216</sub>-specific CD8<sup>+</sup> T-cell recognition at week 5 (27). In contrast, the Gag<sub>236-250</sub> vaccination resulted in SIV control without *gag* mutation selection over 5 weeks in the present study, reflecting the fact that, rather than Gag<sub>206-216</sub>-specific CD8<sup>+</sup> T-cell responses, dominantly induced Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses played a central role in the reduc-



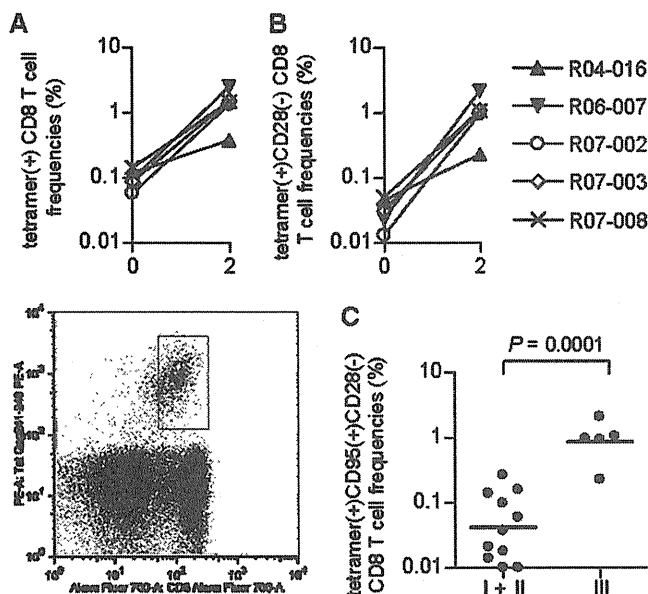


FIG. 5. Frequencies of Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T cells detected by Gag<sub>241-249</sub>-Mamu-A\*90120-5 tetramers after a SIV challenge. (A) Frequencies of Gag<sub>241-249</sub>-Mamu-A\*90120-5 tetramer<sup>+</sup> cells within CD8<sup>+</sup> T cells in group III animals before a challenge (week 0) or at week 2 after a challenge. A representative dot plot gated on CD3<sup>+</sup> lymphocytes for determining tetramer<sup>+</sup> CD8<sup>+</sup> T cells (x axis, CD8; y axis, tetramer) in macaque R07-008 is shown in the lower panel. (B) Tetramer<sup>+</sup> CD28<sup>-</sup> cell frequencies in CD8<sup>+</sup> T cells in group III animals at weeks 0 and 2. Data on tetramer<sup>+</sup> CD95<sup>+</sup> CD28<sup>-</sup> CD8<sup>+</sup> T-cell frequencies at week 0 are unavailable. (C) Tetramer<sup>+</sup> CD95<sup>+</sup> CD28<sup>-</sup> CD8<sup>+</sup> T-cell frequencies in naive controls (groups I and II) and group III animals at week 2. The bar indicates the geometric mean of each group. The frequencies in group III were significantly higher than those in naive controls ( $P = 0.0001$  by unpaired  $t$  test). Samples from macaques R06-019 in group I and R07-007 in group III were unavailable for this analysis.

tion of viral loads in the acute phase. These results suggest that this vaccination approach altered the dominance pattern of CD8<sup>+</sup> T-cell responses and resulted in dominant induction of effective Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses in the

acute phase after a SIV challenge, facilitating a reduction in peak viral loads. Selection of vaccine epitopes for induction of CD8<sup>+</sup> T-cell responses might be important for viral control because the antiviral efficacy of CD8<sup>+</sup> T cells could be affected by MHC-I-restricted target epitopes (10, 19, 25, 35).

Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell induction by prophylactic vaccination resulted in higher frequencies of these T-cell responses during the acute phase after the SIV challenge. The induction of Gag<sub>241-249</sub>-specific effector memory CD8<sup>+</sup> T cells was especially marked. We did not examine polyfunctionality, but analyses of a cytolytic marker, CD107a, indicated higher frequencies of Gag<sub>241-249</sub>-specific cytolytic CD8<sup>+</sup> T-cell responses, implying that these T cells originating from vaccine-induced memory may have higher cytolytic activity in the acute phase. These results suggest that group III animals with Gag<sub>241-249</sub>-specific memory CD8<sup>+</sup> T cells showed induction of a high magnitude of Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T cells with effector function after a SIV challenge, resulting in reduction of viral loads in the acute phase.

In this study, some 90-120-Ia-positive unvaccinated macaques showed lower viral loads. However, in our previous studies with Burmese rhesus macaques (reference 15 and unpublished data), all unvaccinated 90-120-Ia-negative animals failed to contain a SIVmac239 challenge and animals, including vaccinees, that failed to control SIVmac239 replication developed AIDS in 1 to 4 years; even R-90-120 descendants possessing the MHC-I haplotype 90-120-Ib but not 90-120-Ia (both 90-120-Ia and 90-120-Ib are derived from breeder R-90-120) showed high viral loads. Additionally, 90-120-Ia-positive animals failed to control the replication of SIVmac239 carrying CTL escape mutations (16). Thus, a SIVmac239 challenge of Burmese rhesus macaques mostly results in persistent viremia and progression to AIDS but some 90-120-Ia-positive animals may show lower viral loads due to 90-120-Ia-associated SIV-specific CTL responses. However, a previously reported 90-120-Ia-positive unvaccinated macaque, R02-007, developed AIDS around 3 years after a SIVmac239 challenge. Furthermore, two of the 90-120-Ia-positive vaccinees that controlled a SIVmac239 challenge but showed reappearance of viremia

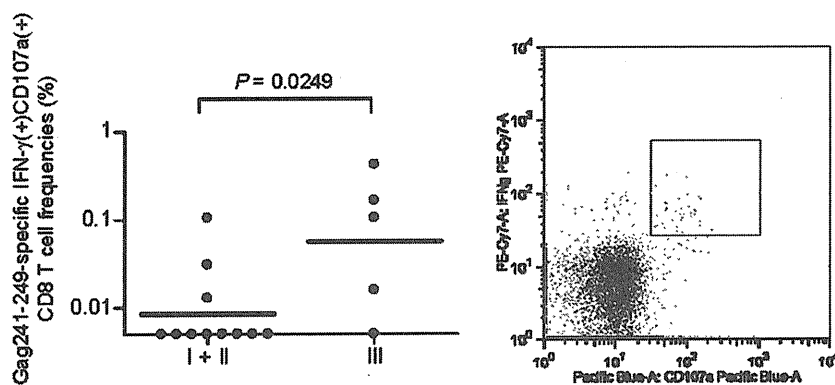


FIG. 6. Gag<sub>241-249</sub>-specific cytolytic CD8<sup>+</sup> T-cell frequencies at week 2 after a challenge. PBMCs were cultured in the absence or the presence of the Gag<sub>241-249</sub> peptide for unstimulated controls or Gag<sub>241-249</sub>-specific stimulation, and the frequencies of CD8<sup>+</sup> T cells exhibiting Gag<sub>241-249</sub>-specific induction of both IFN- $\gamma$  and CD107a in the total CD8<sup>+</sup> T cells were examined. The bar indicates the geometric mean of each group. The frequencies in group III were significantly higher than those in naive controls ( $P = 0.0249$  by unpaired  $t$  test). The right panel is a representative dot plot showing the CD107a (x axis) and IFN- $\gamma$  (y axis) responses in CD8<sup>+</sup> T cells in macaque R07-008 after Gag<sub>241-249</sub>-specific stimulation. Samples from macaques R06-019 in group I and R07-007 in group III were unavailable for this analysis.

around 1 year later developed AIDS (15). Thus, it is inferred that the majority of *90-120-Ia*-positive unvaccinated macaques develop AIDS after a SIVmac239 challenge. Several MHC-I alleles are known to be associated with lower viral loads in HIV and SIV infections, and potent CTLs directed against these MHC-I-restricted epitopes have been implicated in the suppression of viral replication (7, 8, 9, 10, 13, 18, 22, 31, 33, 48). The Gag<sub>241-249</sub>-specific CTL may also be naturally potent (10, 16), but the impact of memory induction of even these potent CTLs on viral control has not yet been determined. Thus, this is the first study documenting the benefit of single-epitope-specific memory CD8<sup>+</sup> T-cell induction by prophylactic vaccination for HIV/SIV control. Further analysis with a vaccine expressing a single helper epitope, as well as a CTL epitope, would contribute to evaluation of the impact of HIV/SIV-specific CD4<sup>+</sup> T-cell memory induction on HIV/SIV replication.

Because CCR5<sup>+</sup> memory CD4<sup>+</sup> T cells, especially HIV-specific CD4<sup>+</sup> T cells, are themselves targets of this virus, whether virus-specific CD4<sup>+</sup> T-cell induction by prophylactic vaccination can result in effective virus-specific CD4<sup>+</sup> T-cell responses postinfection and contribute to HIV control remains unclear. On the other hand, it has been unknown whether HIV-specific memory CD8<sup>+</sup> T cells induced by vaccination without HIV-specific CD4<sup>+</sup> T-cell help can elicit effective responses after virus exposure. In the present study, the pGag<sub>236-250</sub>-EGFP/F(-)SeV-Gag<sub>236-250</sub>-EGFP vaccination elicited Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses without SIV-specific CD4<sup>+</sup> T-cell help but possibly with EGFP-specific or SeV-specific CD4<sup>+</sup> T-cell help; i.e., SeV-EGFP-specific CD4<sup>+</sup> T cells would confer cognate help for Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell induction. The Gag<sub>241-249</sub>-specific memory CD8<sup>+</sup> T cells induced by prophylactic vaccination without SIV-specific CD4<sup>+</sup> T-cell help but with non-SIV-specific CD4<sup>+</sup> T-cell responses responded efficiently to a SIV challenge, showing dominant Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell responses resulting in SIV control; infection-induced SIV-specific CD4<sup>+</sup> T-cell responses may be involved in Gag<sub>241-249</sub>-specific CD8<sup>+</sup> T-cell induction postinfection. Therefore, this study documents that prophylactic vaccination eliciting virus-specific CD8<sup>+</sup> T-cell memory even without virus-specific CD4<sup>+</sup> T-cell responses (but with cognate non-virus-specific CD4<sup>+</sup> T-cell responses) can facilitate SIV control after a challenge.

Taken together, the present study demonstrates that induction of single-epitope-specific CD8<sup>+</sup> T-cell memory without virus-specific CD4<sup>+</sup> T-cell help by prophylactic vaccination can result in dominant potent CD8<sup>+</sup> T-cell responses and control of SIV replication after a challenge. These results imply possible HIV control by prophylactic vaccination eliciting virus-specific CD8<sup>+</sup> T-cell memory with non-virus-specific CD4<sup>+</sup> T-cell help and provide valuable insights into AIDS vaccine development.

#### ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology and grants from the Ministry of Health, Labor, and Welfare in Japan. The animal experiments were conducted through the Cooperative Research Program in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates.

We thank H. Akari, Y. Yasutomi, F. Ono, A. Hiyaoka, K. Komatsuzaki, K. Oto, K. Ishikawa, and T. Nakasone for their assistance in animal experiments; DNAVEC Corp. for providing SeV vectors; M. Roederer for providing the PESTLE and SPICE software; and Y. Tsunetsugu-Yokota, H. Igarashi, A. Kimura, T. Naruse, M. Miyazawa, K. Mori, N. Yamamoto, A. Nomoto, and Y. Nagai for their help.

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# Persistent Low-Level Viremia in HIV-1 Elite Controllers and Relationship to Immunologic Parameters

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**Background.** Human immunodeficiency virus type 1 (HIV-1) elite controllers are able to control virus replication to levels below the limits of detection by commercial assays, but the actual level of viremia in these individuals is not well defined. Here, we quantify plasma HIV-1 RNA in elite controllers and correlate this with specific immunologic parameters.

**Methods.** Plasma HIV-1 RNA levels were quantified in 90 elite controllers with use of a real time reverse-transcriptase polymerase chain reaction assay with a sensitivity of 0.2 copies/mL. HIV-1-specific immune responses and longitudinal CD4<sup>+</sup> T cell counts were examined.

**Results.** The median plasma HIV-1 RNA level was 2 copies/mL (interquartile range, 0.2–14 copies/mL). A longitudinal analysis of 31 elite controllers demonstrated 2–5-fold fluctuations in viremia in the majority of individuals; 6 had persistent levels below 1 copy/mL. Viremia correlated directly with HIV-1-specific neutralizing antibodies and Western blot reactivity but not with CD8<sup>+</sup> T cell responses. Absolute CD4<sup>+</sup> T cell decrease was more common among individuals with detectable viremia ( $P = .04$ ).

**Conclusions.** Low-level viremia is present in the majority of elite controllers and is associated with higher HIV-1-specific antibody responses. Absolute CD4<sup>+</sup> T cell loss is more common among viremic individuals, suggesting that even very low-level viremia has negative consequences over time.

In light of the recent failure of a prophylactic human immunodeficiency virus type 1 (HIV-1) vaccine trial [1], the rationale for current T cell–based HIV-1 vaccine approaches has been questioned. Understanding the relationships between low-level viremia, adaptive immune responses, and disease progression is of im-

portance, particularly in situations of natural HIV-1 control that are relevant to vaccine approaches based on protection from disease progression. Such situations of control of HIV-1 are seen in elite controllers, individuals who spontaneously control virus replication without antiretroviral drugs, to levels below the limits of detection by commercial assays [2]. Although some recent studies have indicated low-level viremia in these persons, the relationship to immunologic parameters, including CD4<sup>+</sup> T cell decrease remains to be determined [3–5].

We previously reported that low-level viremia can be detected by ultrasensitive quantitative polymerase chain reaction (PCR) in elite controllers and is associated with the expression of immunoregulatory receptors that affect CD4<sup>+</sup> T cell function [6]. In that study, the relationship between low-level viremia and other immunologic parameters was not examined. Low-level viremia and its relationship to HIV-1 antibody lev-

Received 29 January 2009; accepted 21 April 2009; electronically published 4 August 2009.

Potential conflicts of interest: none reported.

Presented in part: 15th Conference on Retroviruses and Opportunistic Infections, Boston, Massachusetts, February 2008 (abstract 349).

Financial support: National Institutes of Health (AI28568 and AI30914 to B.D.W.), the Bill and Melinda Gates Foundation, the International AIDS Vaccine Initiative, and the Mark and Lisa Schwartz Foundation.

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**The Journal of Infectious Diseases** 2009;200:984–90

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0022-1899/2009/20006-0019\$15.00

DOI: 10.1086/605446

els have recently been described in elite controllers, but semi-quantitative methods were used to measure both HIV-1 RNA and HIV-1 antibody levels, so the number of copies per mL of plasma was not defined [4]. Furthermore, the relationship between residual low-level viremia and Western blot reactivity, HIV-1-specific neutralizing antibodies, and CD8<sup>+</sup> T cell responses remains unknown.

Here, we used an assay described elsewhere [7] for the detection of HIV-1 RNA with single-copy sensitivity to quantitatively characterize the level of viral RNA in plasma of elite controllers. We examined the relationship between virus load, absolute CD4<sup>+</sup> T cell counts, and HIV-1-specific immune responses. We find that the majority of elite controllers maintain detectable levels of plasma viremia, with a median virus load of 2 copies/mL. Moreover, we show that this extremely low level of viremia correlates positively with HIV-1-specific neutralizing antibodies and full Western blot reactivity. We also show that CD4<sup>+</sup> T cell decrease over time is more common among individuals with low-level viremia.

## METHODS

**Study subjects.** HIV-1 elite controllers were randomly selected from a local cohort [8]. These subjects maintained plasma HIV-1 RNA levels below the limit of detection by commercial assays (<50 copies, by ultrasensitive PCR) without antiretroviral therapy. Definition of elite control was as described elsewhere [8]. The Massachusetts General Hospital Institutional Review Board approved all studies, and written informed consent was obtained from all participants prior to enrollment. Plasma and peripheral blood mononuclear cells (PBMC) were obtained as described elsewhere [8].

**HIV-1 RNA determination.** A single-copy assay for HIV-1 RNA detection was performed as described elsewhere [7], starting with 9 mL of plasma, which afforded a lower limit of detection of 0.2 RNA copies/mL. PCR amplification and sequencing of the HIV-1 *gag* region was performed for each subject from either plasma or PBMC samples, as described elsewhere [9], to rule out inefficient amplification by single-copy assay attributable to possible polymorphisms in the probe and/or primer sequences. Further details of optimum amplification conditions and performance characteristics, as well as quality control procedures to prevent artifactual amplification, are described elsewhere [7].

**Assessment of HIV-1-specific CD8<sup>+</sup> T cell responses.** In 53 individuals with available fresh PBMCs, interferon (IFN)- $\gamma$  enzyme-linked immunospot (ELISPOT) assays were performed as described elsewhere [10, 11]; the assays included 410 peptides 16–19 amino acids in length, overlapping by 10 amino acids, spanning the entire HIV-1 proteome. The same time point was used for ELISPOT and HIV-1 RNA detection. Positive responses were confirmed in a second assay after cell incubation

for 24 h. Responses were considered to be positive if experimental wells had at least 3 times the mean number of spot forming cells (SFCs) in the 6 negative control wells, there were at least 5 spots present in the experimental well, and the number of SFCs per  $1 \times 10^6$  PBMCs was >55. Responses to peptides were previously shown to be largely mediated by CD8<sup>+</sup> T cells [11, 12].

**Assessment of HIV-1-specific antibody responses.** Western blot was performed according to the manufacturer's instructions (GS HIV 1 Western Blot; Bio-Rad) for qualitative detection of HIV-1 antibodies to specific viral proteins (gp160, gp120, p65, p55, p51, gp41, p40, p31, p24, and p18). Neutralizing antibody responses against HIV-1 Env-pseudoviruses were measured as described elsewhere [4, 10]. Briefly, 3-fold serial dilutions of plasma samples were performed in a 96-well flat bottom plate, 200 median tissue culture infective doses (TCID<sub>50</sub>) of the virus was added to each well, and the plates were incubated for 1 h at 37°C. TZM.bl cells were then added. Following a 48-h incubation at 37°C, 100  $\mu$ L of Bright-Glo luciferase reagent (Promega) was added. The cells were allowed to lyse for 2 min, then the lysate was transferred to a 96-well black solid plate, and luminescence was measured using a Victor 3 luminometer (Perkin Elmer). The 80% inhibitory dose (ID<sub>80</sub>) titer was calculated as the serum dilution that caused an 80% reduction in relative luminescence units, compared with the virus control wells, after subtraction of cell control relative luminescence units.

**Statistical analysis.** Statistical analyses and graphical presentations were performed using GraphPad Prism, version 5.0 (GraphPad Software). A nonparametric Spearman test was used to calculate correlations. Significance when comparing 2 groups was determined with a 2-tailed nonparametric Mann Whitney *U* test. Changes in absolute CD4<sup>+</sup> T cell count over time were calculated using a linear regression model to calculate the value of the slope.

## RESULTS

**Low-level plasma viremia is detectable in the majority of HIV-1 elite controllers.** HIV-1 RNA levels were measured at a single time point in 90 HIV-1 elite controllers. Amplification of *gag* sequences was successfully obtained in 62 individuals. Polymorphisms in the primer and/or probe sequences that could affect amplification were present in 7 (11%) of 62 persons, which is consistent with our experience with this assay in large datasets [13]. All individuals with confirmed primer and/or probe mismatches were excluded, leaving 83 individuals in the analysis. The level of plasma viremia varied over a wide distribution (figure 1A), with a median of 2.3 copies/mL (interquartile range, 0.2–14 copies/mL). In the majority of individuals ( $n = 79$ ), the average HIV-1 RNA levels measured by single-copy assay were consistent with results of previous ul-