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 endpoint. 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-γ), tumor necrosis factor alpha (TNF-α), interleukin-2 (IL-2), macrophage inflammatory protein 1β (MIP-1β), and CD107a in $CD4^+$ and $CD8^+$ T cells as described previously (1). Peripheral blood mononuclear cells (PBMCs) were cultured for 6 h in the absence or the presence of 10 μg/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 μg/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 CytofixCytoperm kit (BD) and the following monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-human IFN-γ (BD), phycoerythrin (PE)-conjugated anti-human MIP-1β (BD), peridinin chlorophyll protein-conjugated anti-human CD4 (BD), allophycocyanin (APC)-conjugated anti-human IL-2 (BD), PE-Cy7-conjugated anti-human TNF-α (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-γ, TNF-α, IL-2, MIP-1β, and CD107a, was performed using the FACSaria system (BD); 3×10^5 to 5×10^5 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 $CD4^+$ T-cell responses. Gag-specific $CD4^+$ 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 μg/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 μg/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 $CD8^+$ cells positively selected from PBMCs as described previously (46). In brief, PBMCs were separated into $CD8^+$ cells and $CD8^-$ cells by using Macs CD8 MicroBeads (Miltenyi Biotec, Tokyo, Japan). For preparing target cells, the $CD8^-$ cells negatively selected from PBMCs obtained before challenge were infected with SIVmac239 at a multiplicity of infection of $1{:}10^4$ $TCID_{50}$/cell and cultured in the presence of 2 μg/ml phytohemagglutinin L (Roche Diagnostics) and 20 IU/ml recombinant human IL-2 (Roche Diagnostics). Two days later, effector $CD8^+$ 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 $CD8^+$ 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 $CD8^+$ 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.

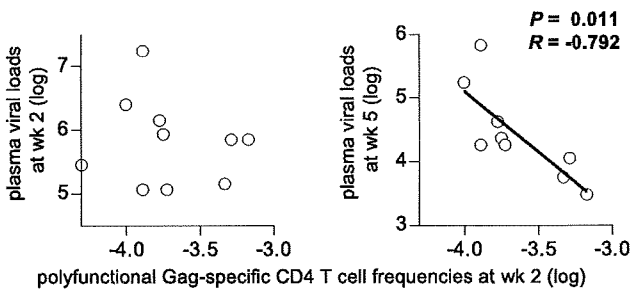

FIG. 3. Analysis of correlation between polyfunctional Gag-specific $CD4^+$ T-cell frequencies (log) at week (wk) 2 and plasma viral loads (log) at week 2 (left) or week 5 (right). Inverse correlation is shown on the right ($P = 0.011$; $R = -0.792$), but not on the left ($P = 0.694$; $R = -0.143$).

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 $CD8^+$ 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 1×10^4 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 $CD4^+$ 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-$TCID_{50}$ virus replication on MT-4 cells (data not shown). In NAb-immunized macaques, NAb responses were

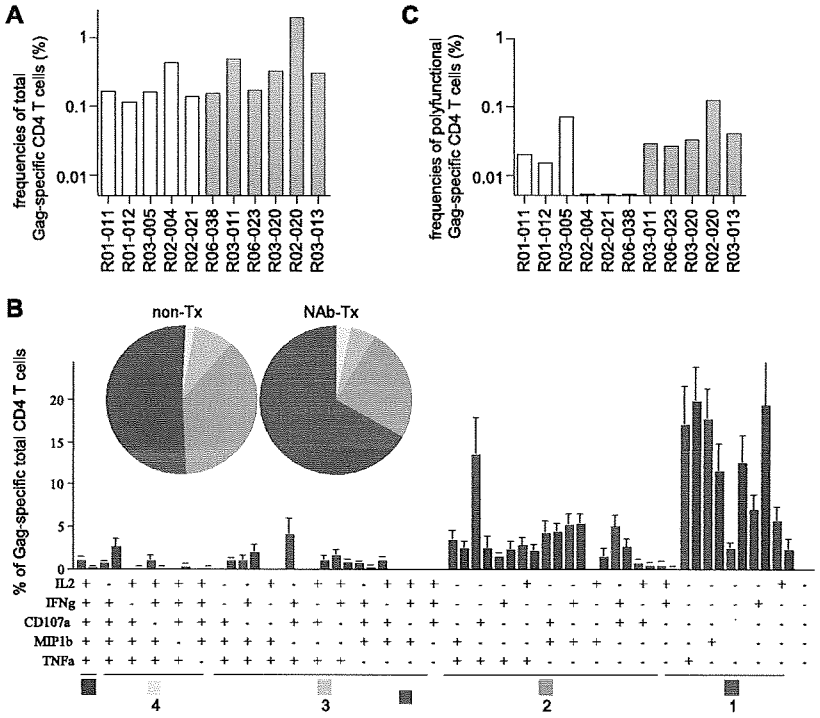

FIG. 4. Gag-specific CD4$^+$ T-cell responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific induction of IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a in CD4$^+$ T cells was examined. (A) Frequencies of total Gag-specific CD4$^+$ T cells. (B) Percentages of cells exhibiting Gag-specific induction of single or multiple marker cytokines in total Gag-specific CD4$^+$ T cells. See the legend to Fig. 2 for symbols. (C) Frequencies of polyfunctional Gag-specific CD4$^+$ T cells exhibiting Gag-specific induction of ≥3 marker cytokines in total CD4$^+$ 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$^+$ 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$^+$ 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-γ,

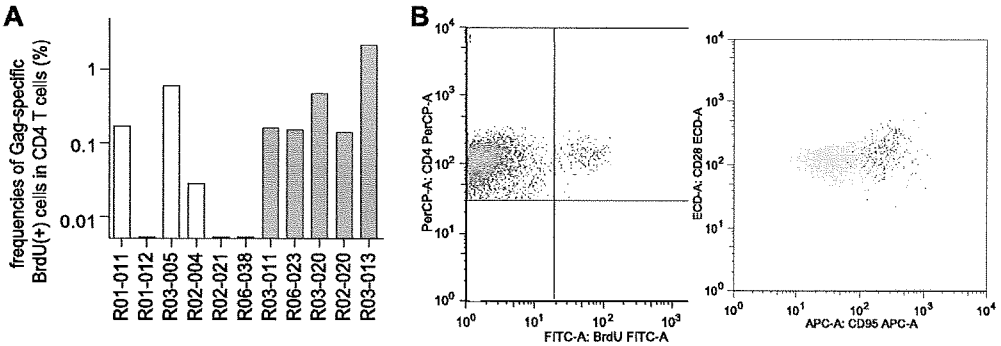

FIG. 5. Gag-specific CD4$^+$ T-cell proliferative responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific uptake of BrdU in CD4$^+$ T cells was examined. (A) Frequencies of Gag-specific BrdU$^+$ CD4$^+$ T cells in total CD4$^+$ T cells. The frequencies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

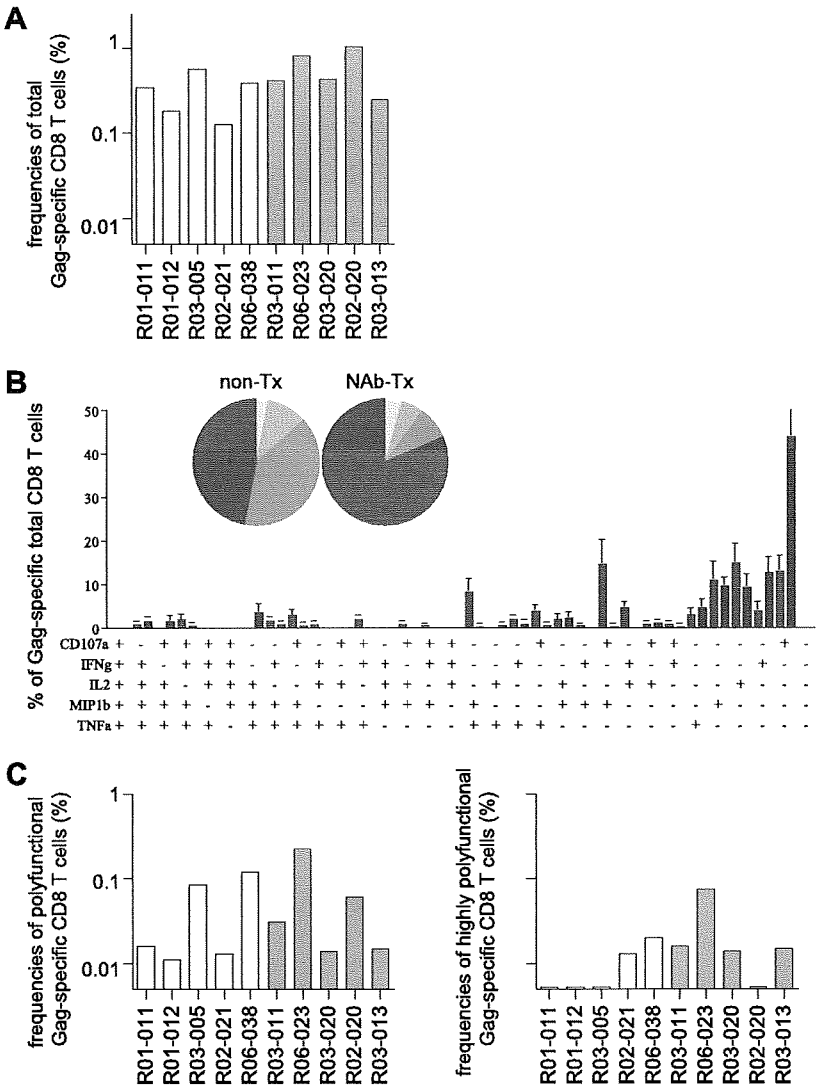

FIG. 6. Gag-specific $CD8^+$ T-cell responses in the acute phase. PBMCs at week 2 were stimulated with p55, and specific induction of IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a in $CD8^+$ T cells was examined. (A) Frequencies of total Gag-specific $CD8^+$ T cells. (B) Percentages of cells exhibiting Gag-specific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

TNF-α, IL-2, and MIP-1β and surface mobilization of CD107a (a degranulation marker) in $CD4^+$ T cells (Fig. 2A) (14, 25, 41). The Gag-specific responses of each factor, IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a, in $CD4^+$ 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 $CD4^+$ T cells (i.e., $CD4^+$ T cells exhibiting Gag-specific induction of one or more of the marker cytokines IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a) were observed between the two groups (Fig. 2B).

We examined the polyfunctionality of SIV Gag-specific $CD4^+$ T cells, as defined by their multiplicity of marker cytokines induced by Gag-specific stimulation (11, 41) (Fig. 2C). The mean percentage of cells producing ≥3 marker cytokines (Fig. 2C, sum of red, yellow, and green) in the Gag-specific $CD4^+$ 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 $CD4^+$ T cells within the $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ T cells with higher polyfunctionality at week 2.

The polyfunctional Gag-specific $CD4^+$ 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 $CD4^+$ T-cell responses in subsequent reduction of plasma viral loads in NAb-immunized macaques.

Polyfunctional Gag-specific $CD4^+$ T-cell responses in the chronic phase in NAb-immunized macaques. We then examined SIV Gag-specific $CD4^+$ T-cell responses in the chronic phase. Around week 30 after challenge, total Gag-specific $CD4^+$ 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 $CD4^+$ T cells showed no significant difference between the two groups (data not shown). The polyfunctionalities of these Gag-specific $CD4^+$ T cells (the percentage of cells producing ≥ 3 marker cytokines) within the total Gag-specific $CD4^+$ T-cell population in both groups were similar (Fig. 4B). However, the frequencies of these polyfunctional Gag-specific $CD4^+$ T cells as a fraction of total $CD4^+$ T cells in NAb-immunized macaques were higher than in unimmunized controls (Fig. 4C).

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

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

We then examined, by in vitro viral-suppression assays (13, 27, 46, 51), whether the $CD8^+$ 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, $CD8^-$ target cells prepared by CD8-negative selection from PBMCs were infected with SIVmac239 and cocultured with effector $CD8^+$ 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 $CD8^+$ cells from the majority of immunized animals, $CD8^+$ 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 $CD8^+$ 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 $CD8^+$ cells possessing higher anti-SIV efficacy.

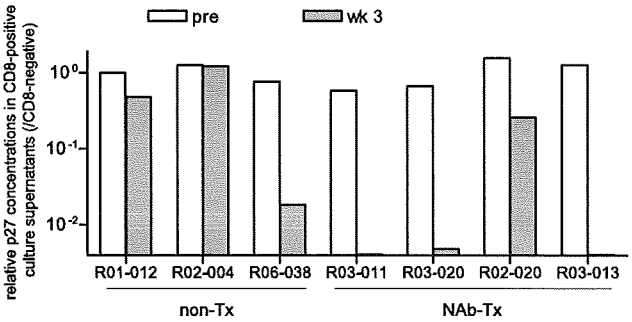

FIG. 7. Anti-SIV efficacy in vitro of $CD8^+$ cells. PBMC-derived $CD8^-$ (target) cells infected with SIVmac239 were cultured alone (no CD8) or cocultured with autologous PBMC-derived $CD8^+$ (effector) cells obtained prechallenge (pre) or at week 3 postchallenge (wk 3) at an E/T ratio of 1:4. The results 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-$CD8^+$ or week 3 $CD8^+$ cells to those without $CD8^+$ cells (CD8 negative) are shown. The coculture with either R03-011 week 3 $CD8^+$, R03-020 week 3 $CD8^+$, and R03-013 week 3 $CD8^+$ cells showed undetectable or marginal SIV p27 production after 8 days.

Polyfunctional Gag-specific $CD8^+$ T-cell responses in the chronic phase in NAb-immunized macaques. We next examined SIV Gag-specific $CD8^+$ T-cell responses in the chronic phase (Fig. 8). Around week 30 after challenge, the geometric means of total Gag-specific $CD8^+$ 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-γ responses in $CD8^+$ T cells (data not shown). There was no clear difference in polyfunctional Gag-specific $CD8^+$ T-cell responses between the two groups. However, highly polyfunctional Gag-specific $CD8^+$ T cells producing ≥ 4 marker cytokines were detectable in all NAb-immunized macaques, and the frequencies of these highly polyfunctional Gag-specific $CD8^+$ T cells in the total $CD8^+$ 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 $CD4^+$ T-cell responses with higher polyfunctionality were rapidly induced in NAb-immunized macaques. These results implicate more po-

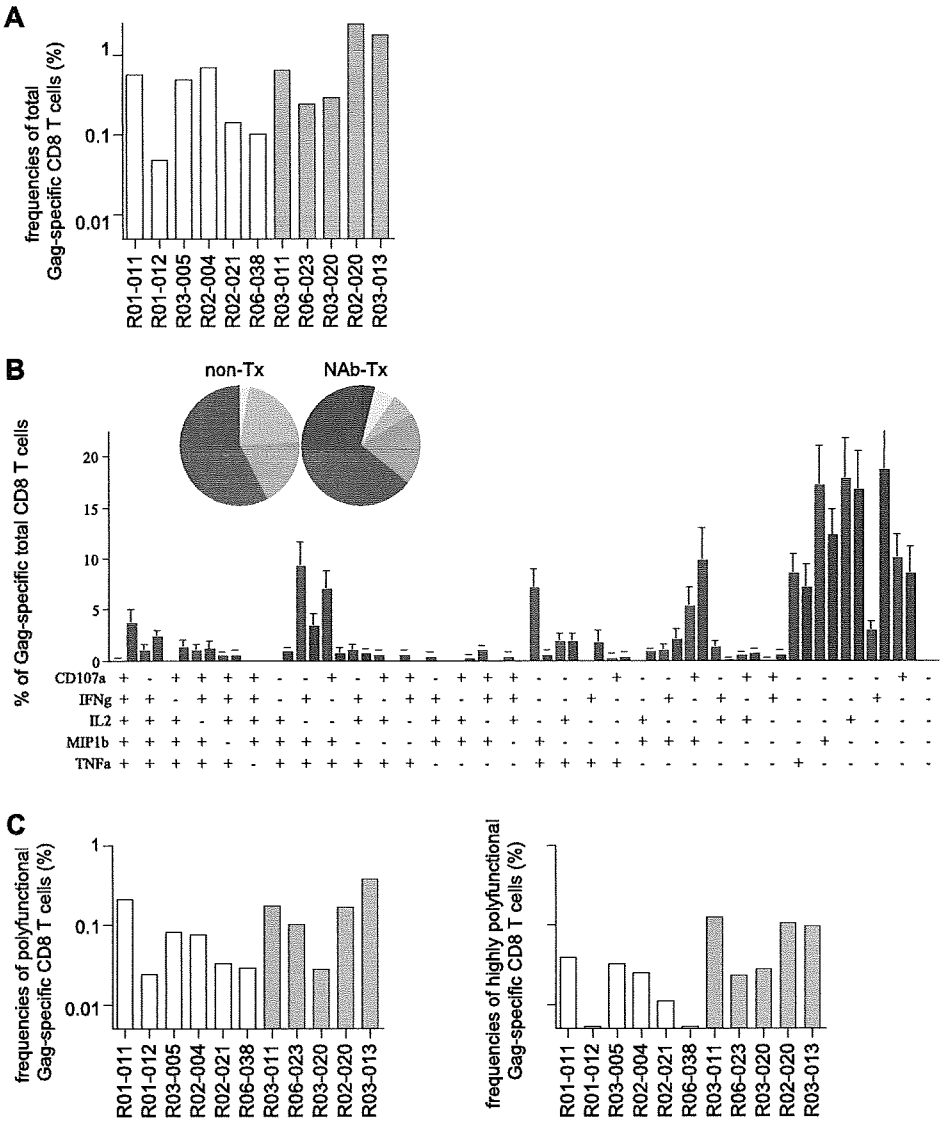

FIG. 8. Gag-specific $CD8^+$ T-cell responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific induction of IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a in $CD8^+$ T cells was examined. (A) Frequencies of total Gag-specific $CD8^+$ T cells. (B) Percentages of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

tent induction of functional virus-specific $CD4^+$ T-cell responses in this NAb-triggered SIV control.

All the NAb-immunized macaques had higher frequencies of polyfunctional Gag-specific $CD4^+$ T cells than any of the unimmunized controls at week 2, although the two groups possessed similar frequencies of total Gag-specific $CD4^+$ T cells. This implies higher polyfunctionality of Gag-specific $CD4^+$ T cells in the acute phase in NAb-immunized macaques than in unimmunized controls. HIV-1 is known to preferentially infect HIV-1-specific $CD4^+$ T cells (10); virus neutralization may therefore protect virus-specific $CD4^+$ T cells from SIV infection. However, it remains unclear whether NAbs preferentially protect polyfunctional virus-specific $CD4^+$ 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 $CD4^+$ T-cell responses in the acute phase. Thus, both NAb-mediated effects, i.e., enhancement of antigen presentation and protection of virus-specific $CD4^+$ T cells from viral infection, may contribute to the induction of polyfunctional virus-specific $CD4^+$ T cells in the acute phase.

It is thought that potent virus-specific $CD4^+$ 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 $CD4^+$

T-cell responses in the control of some viral infections (8, 11, 41). However, there has been no clear evidence indicating association of polyfunctional $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ T-cell responses facilitate induction of functional virus-specific $CD8^+$ T-cell responses (19, 42, 44). Stimulation with peptides would be optimal for analysis of $CD8^+$ T-cell responses, but in this study, our first priority was to analyze $CD4^+$ 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 $CD8^+$ T-cell responses after p55-specific stimulation but did not have enough cell samples for analyzing peptide-specific $CD8^+$ T-cell responses in the acute phase. In the acute phase, no significant enhancement of polyfunctional Gag-specific $CD8^+$ T-cell responses was detected after passive NAb immunization, but this does not exclude the possibility of functional $CD8^+$ T-cell induction in NAb-immunized animals, which may be detected by optimal analysis. Indeed, the viral suppression assay showed that $CD8^+$ 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 $CD8^+$ cell responses, which may be affected not only by $CD8^+$ 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-specific $CD8^+$ 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 $CD4^+$ 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.

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Impact of Cytotoxic-T-Lymphocyte Memory Induction without Virus-Specific CD4$^+$ T-Cell Help on Control of a Simian Immunodeficiency Virus Challenge in Rhesus Macaques$^\nabla$

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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$^+$ T cells may increase the target cell pool for HIV infection because the virus preferentially infects HIV-specific CD4$^+$ T cells. However, virus-specific CD4$^+$ helper T-cell responses are thought to be important for functional CD8$^+$ cytotoxic-T-lymphocyte (CTL) induction in HIV infection, and it has remained unknown whether HIV-specific memory CD8$^+$ T cells induced by vaccination without HIV-specific CD4$^+$ T-cell help can exert effective responses after virus exposure. Here we show the impact of CD8$^+$ T-cell memory induction without virus-specific CD4$^+$ 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 $Gag_{241\text{-}249}$ CTL epitope fused with enhanced green fluorescent protein (EGFP). Vaccination resulted in induction of SeV-EGFP-specific CD4$^+$ T-cell and $Gag_{241\text{-}249}$-specific CD8$^+$ T-cell responses. After a SIV challenge, the vaccinees showed dominant $Gag_{241\text{-}249}$-specific CD8$^+$ 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$^+$ T cells induced by vaccination without virus-specific CD4$^+$ 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$^+$ 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$^+$ T cells (5), induction of HIV-specific memory CD4$^+$ T cells by vaccination may increase the target cell pool for HIV infection and could enhance viral replication (42). However, CD4$^+$ helper T-cell responses are important for functional CD8$^+$ cytotoxic-T-lymphocyte (CTL) induction (11, 40, 43, 46), and it has remained unknown whether HIV-specific memory CD8$^+$ T cells induced by vaccination with non-virus-specific CD4$^+$ T-cell help (but without HIV-specific CD4$^+$ 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*. $Gag_{206\text{-}216}$ (IINEEAADWDL) and $Gag_{241\text{-}249}$ (SSVDEQIQW) epitope-specific CD8$^+$ T-cell responses were shown to be involved in SIV control in these vaccinated macaques (14, 16).

In the present study, focusing on CD8$^+$ T-cell responses directed against one of these epitopes, we have evaluated the efficacy of a vaccine expressing the $Gag_{241\text{-}249}$ 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$^+$ T-cell response and SeV-EGFP-specific CD4$^+$ T-cell responses after vaccination and showed rapid, dominant induction of potent secondary $Gag_{241\text{-}249}$-specific CD8$^+$ 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$^+$ T-cell memory without

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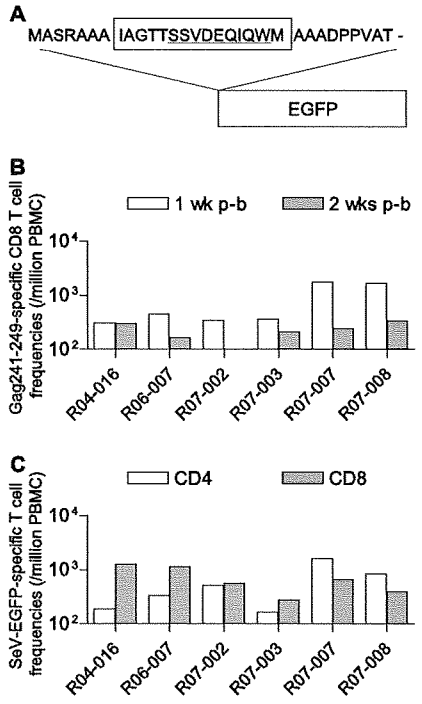

FIG. 1. $Gag_{241-249}$-specific $CD8^+$ T-cell induction by prophylactic vaccination. (A) Schema of the cDNA construct encoding the $Gag_{236-250}$-EGFP fusion protein. A DNA fragment that encodes a 31-mer peptide including the $Gag_{236-250}$ sequence was introduced into the 5′ end of the EGFP cDNA. (B) $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies 1 (open boxes) and 2 weeks (closed boxes) after F(−)SeV-$Gag_{236-250}$-EGFP boosting in group III macaques. (C) SeV-EGFP-specific $CD4^+$ (open boxes) or $CD8^+$ (closed boxes) T-cell frequencies 2 weeks after F(−)SeV-$Gag_{236-250}$-EGFP boosting in group III macaques. p-b, postboost.

virus-specific $CD4^+$ T-cell help by prophylactic vaccination can result in effective $CD8^+$ 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 $Gag_{236-250}$-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. $pGag_{236-250}$-EGFP-N1 DNA expressing a $Gag_{236-250}$-EGFP fusion protein was constructed from pEGFP-N1 DNA (BD, Tokyo, Japan). The fusion protein was designed to have 31 amino acids including SIVmac239 $Gag_{236-250}$-sequences (IAGTTSSVDEQIQWM) added to the amino-terminal portion of EGFP (Fig. 1A). The group III macaques received 5 mg of $pGag_{236-250}$-EGFP-N1 DNA intramuscularly and 6 weeks later received a single intranasal boost with 6×10^9 cell infectious units of F deletion-containing, replication-defective SeV (24) expressing the $Gag_{236-250}$-EGFP fusion protein (F[−]SeV-$Gag_{236-250}$-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 $CD8^+$ T-cell 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 (15, 27). In brief, peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines pulsed with 1 μM SIVmac239 $Gag_{241-249}$ or $Gag_{206-216}$ peptides for $Gag_{241-249}$-specific or $Gag_{206-216}$-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-γ staining was performed with a CytofixCytoperm 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-γ monoclonal antibodies (BD). Specific T-cell levels were calculated by subtracting nonspecific IFN-γ^+ 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 $Gag_{241-249}$-specific cytolytic $CD8^+$ T-cell responses. We analyzed $Gag_{241-249}$-specific induction of IFN-γ and CD107a in $CD8^+$ T cells. PBMCs were stained with custom-made, PE-conjugated $Gag_{241-249}$ epitope-Mamu-A*90120-5 tetrameric complexes, $Gag_{241-249}$-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 μM $Gag_{241-249}$ peptide for unstimulated controls or $Gag_{241-249}$-specific stimulation. In both cultures, anti-human CD28 and anti-human CD49d antibodies (5 μg/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-γ (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

$Gag_{241-249}$-specific $CD8^+$ 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 $Gag_{241-249}$-specific $CD8^+$ T-cell responses. We refer to groups I and II as naive controls in the present study. We constructed a plasmid DNA ($pGag_{236-250}$-EGFP-N1) and an F deletion-containing SeV (F[−]SeV-$Gag_{236-250}$-EGFP) vector both expressing an SIVmac239 $Gag_{236-250}$ (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 $pGag_{236-250}$-EGFP DNA injection, followed by a single intra-

TABLE 1. Macaques used in this study

Group	Animal identification codes	Vaccination[a]
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	$Gag_{241-249}$-specific vaccination [p$Gag_{236-250}$-EGFP-N1 DNA prime, F[(−)SeV-$Gag_{236-250}$-EGFP boost]

[a] All animals were challenged with SIVmac239.

nasal boost with the F(−)SeV-$Gag_{236-250}$-EGFP vector. Group II animals were administered pEGFP-N1 DNA and the F(−)SeV-EGFP vector, both expressing EGFP instead of $Gag_{236-250}$-EGFP, as a control vaccine.

We measured the antigen-specific $CD8^+$ T-cell responses in these macaques 1 or 2 weeks after the SeV boost by detection of specific IFN-γ induction. All group III macaques showed efficient $Gag_{241-249}$-specific $CD8^+$ T-cell induction after the F(−)SeV-$Gag_{236-250}$-EGFP boost (Fig. 1B). In these animals, we also confirmed SeV-EGFP-specific $CD8^+$ and $CD4^+$ T-cell responses (Fig. 1C) but did not detect $Gag_{206-216}$-specific $CD8^+$ T-cell responses, which are dominantly induced in *90-120-Ia*-positive macaques by Gag-expressing SeV vaccination (14). We have never found $Gag_{236-250}$-specific $CD4^+$ T-cell responses in any previously examined animals, and as expected, analyses with the $Gag_{236-250}$ peptide did not detect $Gag_{236-250}$-specific $CD4^+$ 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 $Gag_{236-250}$-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 ($Gag_{236-250}$-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 $Gag_{241-249}$ single-epitope-specific $CD8^+$ T-cell responses resulted in a significant reduction of peak and subsequent viral loads after the SIV challenge. No significant difference in peripheral $CD4^+$ T-cell counts was observed among these three groups (Fig. 2C).

Dominant $Gag_{241-249}$-specific $CD8^+$ T-cell responses in vaccinees after a SIV challenge. We assessed virus-specific $CD8^+$ T-cell responses at weeks 2 and 12 after a SIV challenge by measuring antigen-specific IFN-γ induction. $Gag_{241-249}$-specific $CD8^+$ 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, $Gag_{206-216}$-specific $CD8^+$ T-cell responses were induced equivalently or more efficiently than $Gag_{241-249}$-specific $CD8^+$ responses, whereas all of the group III animals showed dominant induction of $Gag_{241-249}$-specific $CD8^+$ T-cell responses. In these group III animals, $Gag_{206-216}$-specific $CD8^+$ T-cell responses were inefficient but frequencies of $CD8^+$ T cells exhibiting $Gag_{241-249}$-specific IFN-γ induction were significantly higher than in naive controls at week 2 (Fig. 3B) and week 12. $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies at week 2 inversely correlated with peak viral loads (Fig. 3C).

We also tested SIV-specific $CD8^+$ 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 $CD8^+$ T cells responding to SIVGP1-transduced cells (referred to as SIV-specific $CD8^+$ 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 $CD8^+$ 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 $Gag_{206-216}$-specific $CD8^+$ T-cell recognition at week 5, implicating $Gag_{206-216}$-specific $CD8^+$ T-cell responses (rather than $Gag_{241-249}$-specific $CD8^+$ T-cell responses) in viral control. In the present study, however, five of six $Gag_{236-250}$-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 $Gag_{241-249}$-specific $CD8^+$ T-cell responses in the acute phase play an important role in this vaccine-based SIV control.

Higher $Gag_{241-249}$-specific effector memory $CD8^+$ T-cell frequencies in vaccinees. We then examined $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies in these macaques by using PE-conjugated $Gag_{241-249}$-A*90120-5 tetramers. In group III animals, $Gag_{241-249}$-specific $tetramer^+$ $CD8^+$ 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 $tetramer^+$ $CD28^-$ $CD8^+$ T-cell frequencies after a challenge were especially marked (>30-fold) (Fig. 5B). Indeed, within the $tetramer^+$ cells, the ratio of $CD28^-$ cells increased after a challenge and these cells became predominant at week 2. Analysis of an effector memory subset delineated by the $CD95^+$ $CD28^-$ phenotype (29, 34) revealed significantly higher frequencies of $Gag_{241-249}$-specific $tetramer^+$ $CD95^+$ $CD28^-$ $CD8^+$ T cells in group III than in naive controls (Fig. 5C). These results suggest efficient responses of $Gag_{241-249}$-specific $CD8^+$ T cells with effector function in the acute phase in group III animals.

$Gag_{241-249}$-specific cytolytic $CD8^+$ T-cell responses in vaccinees. To further investigate the cytolytic quality of $Gag_{241-249}$-specific $CD8^+$ T-cell responses after a challenge, we examined

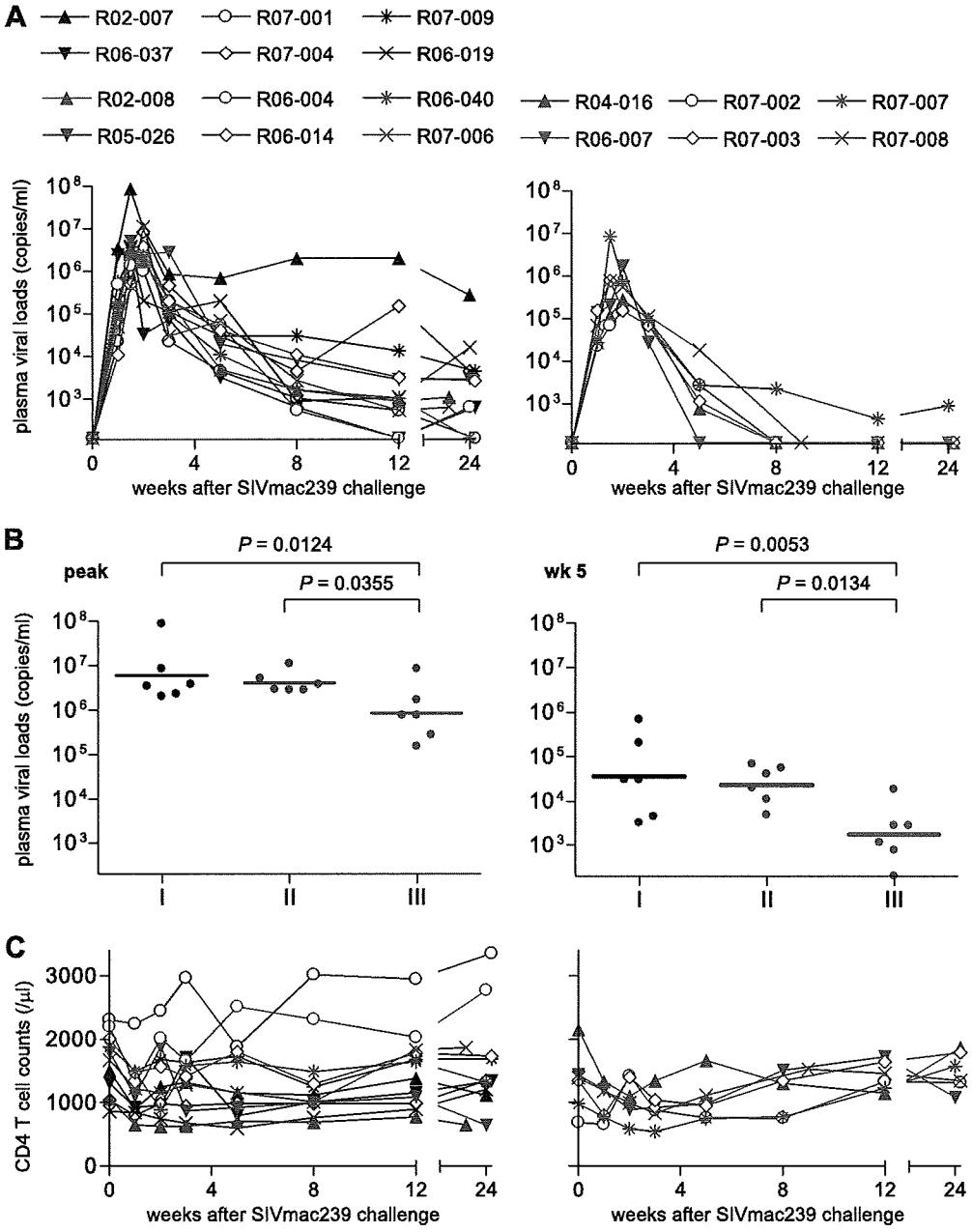

FIG. 2. Plasma viral loads and peripheral $CD4^+$ T-cell counts after a SIV challenge. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) in unvaccinated group I animals (black lines in the left panel), control-vaccinated group II animals (blue lines in the left panel), and $Gag_{236\text{-}250}$-vaccinated 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 4×10^2 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 $CD4^+$ T-cell counts (per μl) 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.

$Gag_{241\text{-}249}$-specific induction of CD107a (a degranulation marker), which is related to cytolytic activity (21, 38), in $CD8^+$ T cells at week 2. Frequencies of $CD8^+$ T cells exhibiting $Gag_{241\text{-}249}$-specific induction of CD107a, as well as IFN-γ, within the $CD8^+$ 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 $Gag_{241\text{-}249}$-specific $CD107a^+$ IFN-γ^+ $CD8^+$ T-cell responses, but further

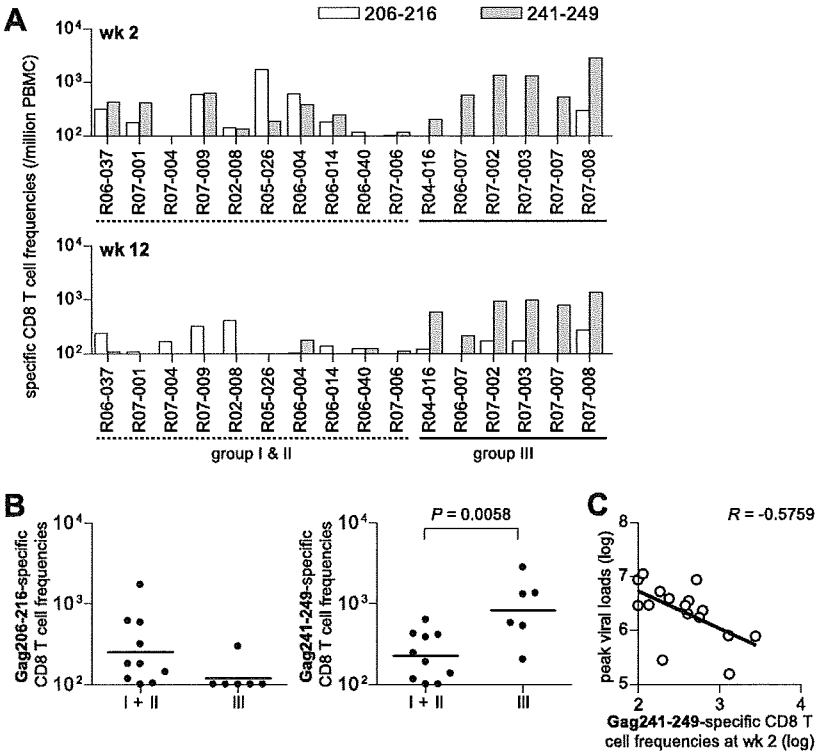

FIG. 3. Gag epitope-specific $CD8^+$ T-cell frequencies after a SIV challenge. (A) Frequencies of $CD8^+$ T cells (per million PBMCs) showing $Gag_{206\text{-}216}$-specific (open boxes) or $Gag_{241\text{-}249}$-specific (closed boxes) IFN-γ induction in naive controls and group III macaques at week 2 (upper panel) and week 12 (lower panel). (B) Comparison of the $Gag_{206\text{-}216}$-specific (left panel) or $Gag_{241\text{-}249}$-specific (right panel) $CD8^+$ 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 $Gag_{241\text{-}249}$-specific ($P = 0.0058$) but not $Gag_{206\text{-}216}$-specific ($P = 0.0922$) $CD8^+$ T cells in group III were significantly higher than in naive controls. The $Gag_{241\text{-}249}$-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 $Gag_{241\text{-}249}$-specific $CD8^+$ 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 $Gag_{241\text{-}249}$-specific granzyme B^+ IFN-γ^+ $CD8^+$ T cells. Indeed, group III animals had significantly higher frequencies of $Gag_{241\text{-}249}$-specific IFN-γ^+ $CD8^+$ T cells producing CD107a, granzyme B, or perforin ($P = 0.0076$; data not shown). These results indicate efficient induction of $Gag_{241\text{-}249}$-specific $CD8^+$ T cells with higher cytolytic activity in the acute phase in group III animals.

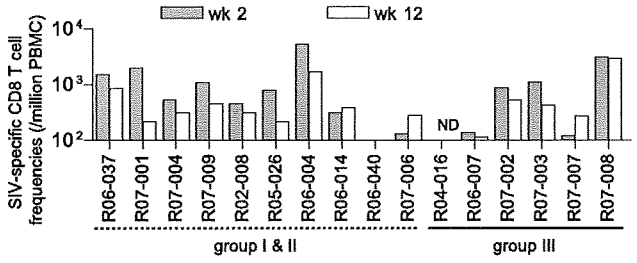

FIG. 4. SIV-specific $CD8^+$ T-cell frequencies after a SIV challenge. SIV-specific $CD8^+$ T-cell frequencies (per million PBMCs) in naive controls and group III macaques at week 2 (closed boxes) and week 12 (open boxes) are shown. ND, not determined.

DISCUSSION

In the present study, induction of $CD8^+$ T cells specific for a single $Gag_{241\text{-}249}$ 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 $CD8^+$ 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 $CD8^+$ T-cell memory response to a single epitope which can recognize the incoming HIV could facilitate HIV control.

Group III macaques showed more effective $CD8^+$ 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 $Gag_{206\text{-}216}$-specific $CD8^+$ T-cell recognition at week 5 (27). In contrast, the $Gag_{236\text{-}250}$ vaccination resulted in SIV control without *gag* mutation selection over 5 weeks in the present study, reflecting the fact that, rather than $Gag_{206\text{-}216}$-specific $CD8^+$ T-cell responses, dominantly induced $Gag_{241\text{-}249}$-specific $CD8^+$ T-cell responses played a central role in the reduc-

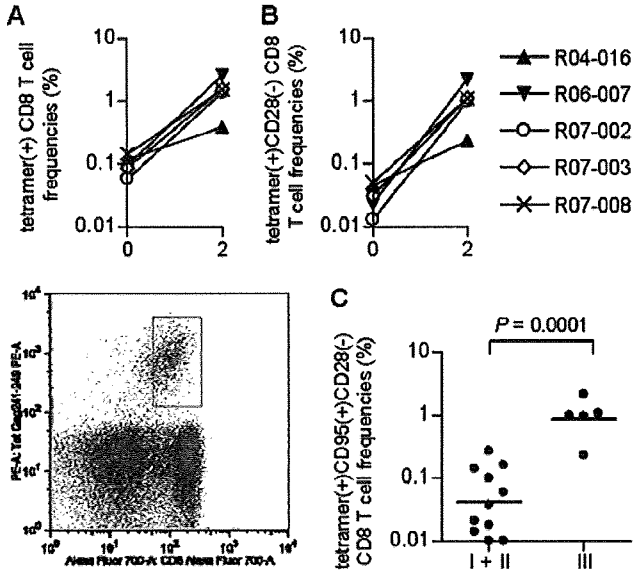

FIG. 5. Frequencies of $Gag_{241-249}$-specific $CD8^+$ T cells detected by $Gag_{241-249}$-Mamu-A*90120-5 tetramers after a SIV challenge. (A) Frequencies of $Gag_{241-249}$-Mamu-A*90120-5 $tetramer^+$ cells within $CD8^+$ T cells in group III animals before a challenge (week 0) or at week 2 after a challenge. A representative dot plot gated on $CD3^+$ lymphocytes for determining $tetramer^+$ $CD8^+$ T cells (*x* axis, CD8; *y* axis, tetramer) in macaque R07-008 is shown in the lower panel. (B) $Tetramer^+$ $CD28^-$ cell frequencies in $CD8^+$ T cells in group III animals at weeks 0 and 2. Data on $tetramer^+$ $CD95^+$ $CD28^-$ $CD8^+$ T-cell frequencies at week 0 are unavailable. (C) $Tetramer^+$ $CD95^+$ $CD28^-$ $CD8^+$ 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 $CD8^+$ T-cell responses and resulted in dominant induction of effective $Gag_{241-249}$-specific $CD8^+$ 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 $CD8^+$ T-cell responses might be important for viral control because the antiviral efficacy of $CD8^+$ T cells could be affected by MHC-I-restricted target epitopes (10, 19, 25, 35).

$Gag_{241-249}$-specific $CD8^+$ 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 $Gag_{241-249}$-specific effector memory $CD8^+$ T cells was especially marked. We did not examine polyfunctionality, but analyses of a cytolytic marker, CD107a, indicated higher frequencies of $Gag_{241-249}$-specific cytolytic $CD8^+$ 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 $Gag_{241-249}$-specific memory $CD8^+$ T cells showed induction of a high magnitude of $Gag_{241-249}$-specific $CD8^+$ 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

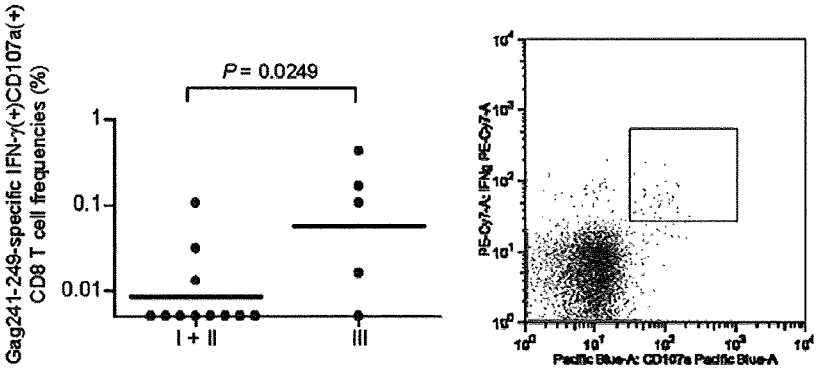

FIG. 6. $Gag_{241-249}$-specific cytolytic $CD8^+$ T-cell frequencies at week 2 after a challenge. PBMCs were cultured in the absence or the presence of the $Gag_{241-249}$ peptide for unstimulated controls or $Gag_{241-249}$-specific stimulation, and the frequencies of $CD8^+$ T cells exhibiting $Gag_{241-249}$-specific induction of both IFN-γ and CD107a in the total $CD8^+$ 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-γ (*y* axis) responses in $CD8^+$ T cells in macaque R07-008 after $Gag_{241-249}$-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 $Gag_{241-249}$-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 $CD8^+$ 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 $CD4^+$ T-cell memory induction on HIV/SIV replication.

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

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

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Contribution of RING domain to retrovirus restriction by TRIM5α depends on combination of host and virus

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ABSTRACT

The anti-retroviral restriction factor TRIM5α contains the RING domain, which is frequently observed in E3 ubiquitin ligases. It was previously proposed that TRIM5α restricts human immunodeficiency virus type 1 (HIV-1) via proteasome-dependent and -independent pathways. Here we examined the effects of RING domain mutations on retrovirus restriction by TRIM5α in various combinations of virus and host species. Simian immunodeficiency virus isolated from macaque (SIVmac) successfully avoided attacks by RING mutants of African green monkey (AGM)-TRIM5α that could still restrict HIV-1. Addition of proteasome inhibitor did not affect the anti-HIV-1 activity of AGM-TRIM5α, whereas it disrupted at least partly its anti-SIVmac activity. In the case of mutant human TRIM5α carrying proline at the position 332, however, both HIV-1 and SIVmac restrictions were eliminated as a result of RING domain mutations. These results suggested that the mechanisms of retrovirus restriction by TRIM5α vary depending on the combination of host and virus.

Introduction

Replication of retroviruses is influenced by several factors in host cells. Tripartite motif protein (TRIM) 5α has been identified as a restriction factor of human immunodeficiency virus type 1 (HIV-1) in rhesus monkey (Rh) cells (Stremlau et al., 2004). Rh TRIM5α potently restricts HIV-1 but only weakly does so simian immunodeficiency virus isolated from macaque (SIVmac) (Stremlau et al., 2004; Song et al., 2005), whereas African green monkey (AGM) TRIM5α can potently restrict both HIV-1 and SIVmac (Nakayama et al., 2005; Song et al., 2005). TRIM5α consists of the RING, B-box 2, coiled-coil, and SPRY (B30.2) domains (Reymond et al., 2001). Differences in the amino acid sequences in the SPRY domain of TRIM5α of different monkey species were shown to affect the species-specific restriction of retrovirus infection (Perez-Caballero et al., 2005a; Nakayama et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005). In addition, biochemical studies have shown that TRIM5α associates with retroviral capsid (CA) protein in detergent-stripped virions or with an artificially constituted core structure composed of capsid-nucleocapsid (CA-NC) fusion protein in a SPRY domain-dependent manner (Sebastian and Luban, 2005; Stremlau et al., 2006a). The SPRY domain is thus thought to recognize viral core. The coiled-coil domain of TRIM5α is important for the formation of homo-oligomers (Mische et al., 2005) and is essential for antiviral activity (Javanbakht et al., 2006). The intact B-box 2 domain is also required for TRIM5α mediated antiviral activity, since the restrictive activity of TRIM5α is diminished by amino acid substitutions in the B-box 2 domain (Javanbakht et al., 2005). RING containing proteins were frequently found to possess E3 ubiquitin ligase activity (Jackson et al., 2000). Indeed, Rh TRIM5α was poly-ubiquitinated and degraded rapidly via the ubiquitin-proteasome pathway, while disruption of the RING domain eliminated its auto-ubiquitination (Diaz-Griffero et al., 2006). Furthermore, it was demonstrated that TRIM5α is degraded via the ubiquitin-proteasome pathway during HIV-1 restriction (Rold and Aiken, 2008). However, deletion of the RING domain in TRIM5α only partially attenuates anti-HIV-1 activity (Javanbakht et al., 2005; Perez-Caballero et al., 2005b). Moreover, modulation of E1 ubiquitin-activating enzyme expression did not affect TRIM5α-mediated restriction activity in a temperature-dependent cell line (Perez-Caballero et al., 2005b) and finally, proteasome inhibitors did not affect TRIM5α mediated HIV-1 restriction (Anderson et al., 2006; Perez-Caballero et al., 2005b; Rold and Aiken, 2008; Stremlau et al., 2006a; Wu et al., 2006) even though they allowed HIV-1 to generate viral late reverse transcripts under TRIM5α mediated HIV-1 restriction (Anderson et al., 2006; Wu et al., 2006). The exact role of the TRIM5α RING domain in retrovirus restriction thus remains unclear.

In the study presented here, we investigated the effects of RING domain mutations on HIV-1 and SIVmac restrictions by TRIM5α and report that TRIM5α restricts HIV-1 and SIVmac differently.

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Results

Auto poly-ubiquitination of TRIM5α impaired by mutations in RING domain

The RING finger domain of TRIM5α comprises eight potential metal ligands and binds two atoms of zinc, with each zinc atom ligated tetrahedrally by either four cysteins or three cysteines and a single histidine. Based on the three-dimensional structure of the RING domains of TRIM5 (Abe et al., 2007) and the promyelocytic leukemia (PML) protein (Borden et al., 1995; Borden and Freemont, 1996), the first pair of metal ligands of the AGM TRIM5α RING domain (C15 and C18) would share a zinc atom with the third pair (C35 and C38), and the second (C30 and H32) and fourth pairs (C56 and C59) would share another zinc atom (Fig. 1A). To determine whether anti-HIV-1 and anti-SIVmac activities of AGM TRIM5α are similarly affected by RING domain mutations, several AGM TRIM5α constructs with mutations in the RING domain were generated (Fig. 1B). In the mutant TRIM5α constructs with C15AC18A, C30AH32A, or C15AC18AC30AH32A, two key amino acid residues in the first or second, or in both the first and

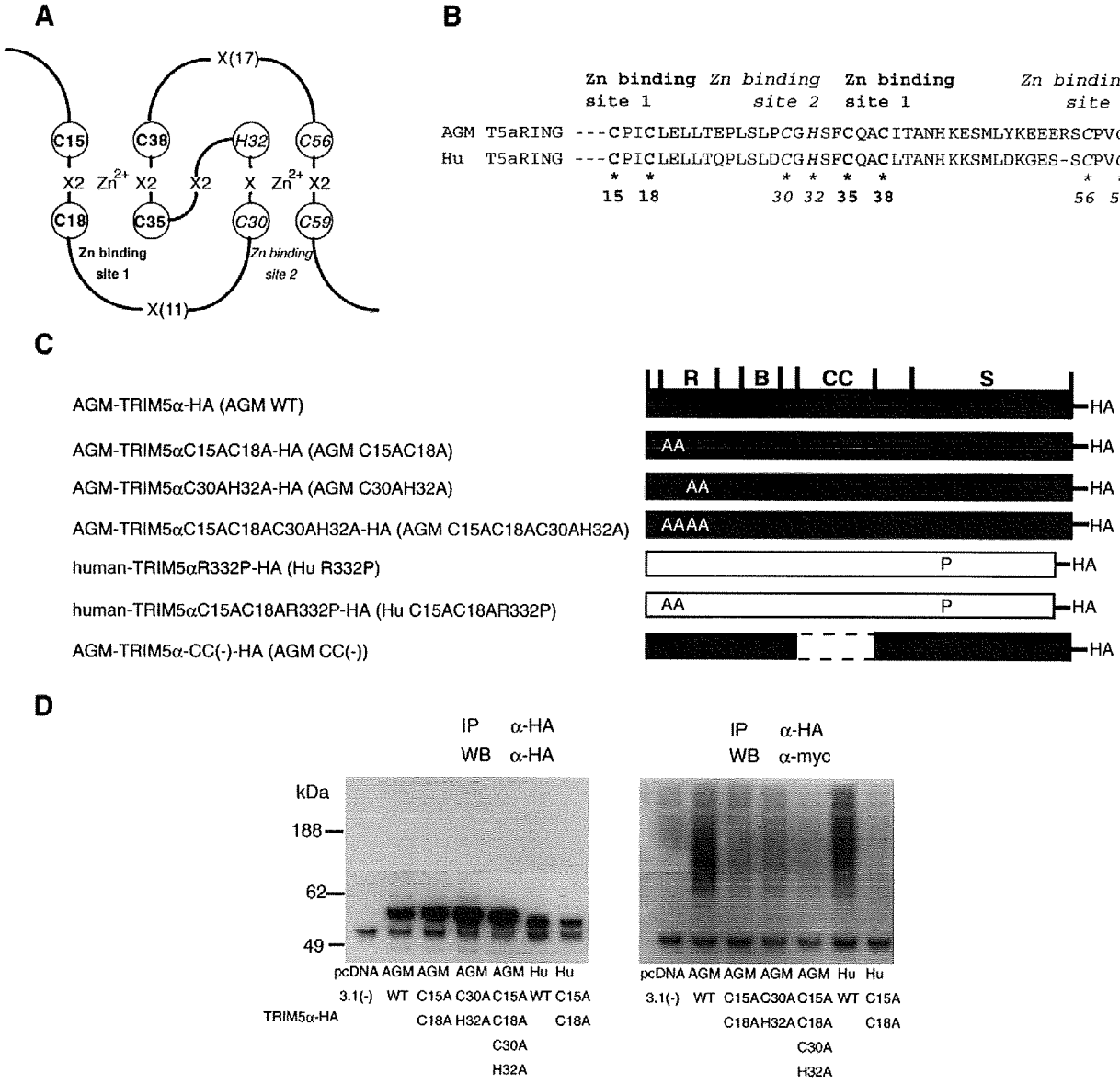

Fig. 1. Auto poly-ubiquitination of TRIM5α was impaired by RING domain mutations. (A) The RING finger zinc binding motif. The numbered AGM TRIM5α zinc-binding ligands are shown in circles. Each zinc atom is coordinated tetrahedrally by four ligands. Zinc-binding site 1 (**bold**) and site 2 (*italic*) are indicated. The numbers of amino acid residues between the zinc-binding cystein and histidine ligands in AGM TRIM5α are also indicated. (B) Primary amino acid sequences of the RING domains of AGM TRIM5α (AGM T5aRING) and human TRIM5α (Hu T5aRING) are aligned. Zinc-binding site 1 (**bold**), site 2 (*italic*), and cystein and histidine ligands (large numbers) are indicated. (C) Schematic representation of TRIM5α constructs. Black and white bars denote AGM and Hu sequences, respectively. Abbreviations for domains: R, RING; B, B-box 2; CC, Coiled-coil; S, SPRY. A dotted box denotes deletion of corresponding amino acid. The positions of individual amino acid changes are also indicated. (D) 293 T cells were transfected with plasmids encoding HA-tagged AGM TRIM5α (TRIM5α-HA) or its RING mutants together with a plasmid expressing myc-tagged ubiquitin (myc-Ub). Forty-eight hours after transfection, the cells were lysed and TRIM5α proteins in the lysates were precipitated with an anti-HA antibody. The immunoprecipitates were Western blotted and probed with anti-HA antibody for TRIM5α detection or with anti-myc antibody for ubiquitin detection. Abbreviations: WB, Western blot; IP, immunoprecipitation. The representative results of two independent experiments with similar results are shown.

second zinc-binding sites within the RING domain of AGM TRIM5α were replaced with alanine residues, respectively. All mutant TRIM5α constructs contained the HA-tag at their C-terminus (Fig. 1C).

To determine the effects of TRIM5α RING mutations on its ubiquitin ligase activity, 293T cells were transfected with plasmids encoding HA-tagged TRIM5αs together with plasmid expressing myc tagged ubiquitin. Forty-eight hours later, the cells were lysed and TRIM5α proteins were precipitated with the anti-HA antibody followed by Western blot analysis using anti-HA and anti-myc antibodies. Poly-ubiquitinated forms of the wild type AGM TRIM5α were observed (Fig. 1D). AGM TRIM5α with C15AC18A or C30AH32A was less poly-ubiquitinated than the wild type AGM TRIM5α, and AGM TRIM5α with C15AC18AC30AH32A was the least poly-ubiquitinated among the mutant constructs tested. These results confirmed the previously published report (Diaz-Griffero et al., 2006) that the TRIM5α RING zinc-binding site mutations impaired auto poly-ubiquitination of TRIM5α.

Contribution of RING domain to retrovirus restriction by AGM TRIM5α

We next examined anti-viral activities of zinc-binding site mutants of TRIM5α. The HA-tagged wild type and mutant AGM TRIM5α proteins were expressed by Sendai virus (SeV) in MT4 cells (Fig. 2A). CV1 cells were then used for a confocal microscopic examination of cytoplasmic bodies, since the cytoplasm of MT4 cells is not large enough for observation of cytoplasmic bodies. Each of the TRIM5αs with RING mutations formed uniformly larger cytoplasmic bodies than did the wild type (Fig. 2B), although the size of cytoplasmic

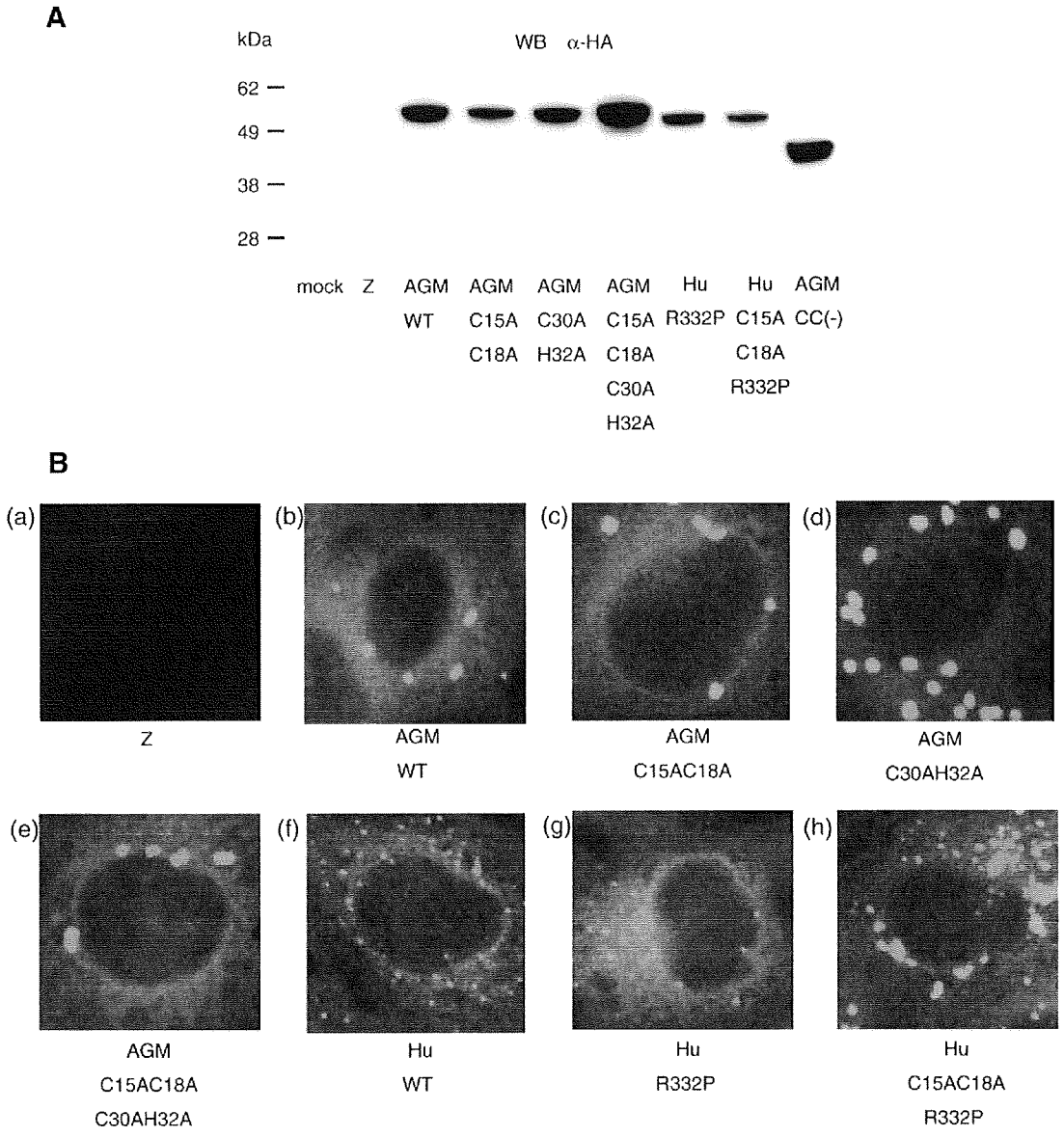

Fig. 2. Expression of RING mutant TRIM5α proteins. (A) Expression of various TRIM5αs. TRIM5α proteins in MT4 cells mock infected (mock) or infected with parental Z strain of SeV (Z), SeVs expressing AGM TRIM5α (AGM WT), AGM TRIM5α C15AC18A (AGM C15AC18A), AGM TRIM5α C30AH32A (AGM C30AH32A), AGM TRIM5α C15AC18AC30AH32A (AGM C15AC18AC30AH32A), human TRIM5α R332P (Hu R332P), human TRIM5α C15AC18AR332P (Hu C15AC18AR332P), or AGM-TRIM5α-Coiled-coil(−) (AGM CC(−)), were visualized by Western blotting with antibody to HA. (B) Subcellular localization of TRIM5αs. CV1 cells infected with SeV expressing HA-tagged TRIM5 proteins were analyzed as described in "Materials and methods". Representative confocal microscopic images are shown of parental Z strain of SeV (a), or with SeV expressing AGM WT (b), AGM C15AC18A (c), AGM C30AH32A (d), AGM C15AC18AC30AH32A (e), Hu WT (f), Hu R332P (g), or Hu C15AC18AR332P (h).

bodies slightly varied among different RING mutants of TRIM5α. These results confirmed the previous observations on Rh TRIM5α (Javanbakht et al., 2005). Specifically, AGM TRIM5α with C30AH32A showed the highest numbers of cytoplasmic bodies and the least levels of diffuse staining of cytoplasm among the three RING mutants (Fig. 2B).

For the viral replication assay, MT4 cells infected with SeVs expressing the wild type and mutant TRIM5αs were also superinfected with the NL43 strain of HIV-1, GH123 strain of HIV-2 or SIVmac239. Three days after infection, culture supernatants were collected and assayed for their levels of p24, p25 or p27 viral CA protein, respectively. AGM-TRIM5α-CC(−) was used as a negative control. AGM TRIM5α with C15AC18A, C30AH32A, or C15AC18A-C30AH32A moderately inhibited HIV-1 growth, while these variants completely lost their inhibitory effect on SIVmac growth (Fig. 3A). These results indicated that effects of cysteine substitutions in RING domain on anti-HIV-1 activity of AGM TRIM5α differ from those on anti-SIVmac activity, suggesting that SIVmac restriction by AGM TRIM5α was totally dependent on the intact RING domain of TRIM5α, while HIV-1 restriction was at least in part independent from this domain as reported previously (Javanbakht et al., 2005; Perez-Caballero et al., 2005b; Stremlau et al., 2004). It has been proposed that both proteasome-dependent and -independent pathways are involved in HIV-1 restriction by Rh TRIM5α, since disrupting the proteasome function by adding a proteasome inhibitor enabled the generation of normal levels of HIV-1 late reverse transcribed products, although HIV-1 infection and the generation of nuclear imports of 1-LTR and 2-LTR viral cDNA forms remained impaired by Rh TRIM5α (Anderson et al., 2006; Wu et al., 2006). We therefore concluded that AGM TRIM5α restricts SIVmac mainly via the RING-proteasome-dependent pathway.

We then tested the third virus, human immunodeficiency virus type 2 (HIV-2), which is more closely related to SIVmac than to HIV-1 (Gao et al., 1999). AGM TRIM5α clearly inhibited HIV-2 GH123 replication and all the RING domain mutants showed reduced anti-HIV-2 activity. AGM TRIM5α with C30AH32A completely lost its anti-HIV-2 activity (Fig. 3A). Unlike SIVmac, however, AGM TRIM5α with C15AC18A or C15AC18AC30AH32A still moderately inhibited HIV-2 GH123 growth (Fig. 3A). These results indicate that the RING domain contribution to HIV-2 restriction by TRIM5α was also distinct from its contributions to HIV-1 and SIVmac restrictions.

In a single round infection assay, MT4 cells infected with SeVs expressing the wild type or mutant TRIM5αs variants were superinfected with HIV-1-GFP or SIVmac-GFP. The wild type AGM TRIM5α potently restricted both HIV-1-GFP and SIVmac-GFP infection (Fig. 3B) as reported previously (Nakayama et al., 2005). On the other hand, AGM TRIM5α with C15AC18A, C30AH32A, or C15AC18A-C30AH32A only moderately inhibited HIV-1-GFP infection, while these variants completely lost their inhibitory effect on SIVmac-GFP infection (Fig. 3B). AGM C30AH32A exhibited the weakest anti-HIV-1 activity among the generated mutant constructs, probably due to its limited localization within the cytoplasm. However, the number of HIV-1-infected cells was still lower in AGM C30AH32A expressing cells than in those expressing negative control AGM-TRIM5α-CC(-) or cells infected with the parental SeV Z strain (Fig. 3B). The same results as above were obtained when we use canine Cf2Th cell line lacking endogenous TRIM5α expression (Sawyer et al., 2007) (Fig. 3C). These results confirmed our results in viral replication assay described in Fig. 3A.

Contribution of RING domain to retrovirus restriction by human TRIM5α with arginine-to-proline substitution at the 332nd position

An arginine-to-proline substitution at the 332nd position (R332P) in the SPRY domain reportedly conferred strong anti-HIV-1 and anti-SIVmac activities to human TRIM5α (Stremlau et al., 2005; Yap et al., 2005). To determine whether cystein residue substitutions in the RING domain of human TRIM5α with R332P (Hu-R332P) have similar effects on its anti-HIV-1 and anti-SIVmac activities to those of AGM TRIM5α described above, C15AC18A substitutions were introduced in Hu-R332P. The protein expression levels of Hu-R332P with C15AC18A were comparable to those of Hu-R332P without C15AC18A (Fig. 2B). In addition, Hu-R332P inhibited both HIV-1 and SIVmac infection (Fig. 3A, B and C), which confirmed previous findings (Stremlau et al., 2005; Yap et al., 2005). As expected, Hu-R332P with C15AC18A completely lost its auto poly-ubiquitination (Fig. 1D) and anti-SIVmac activity (Fig. 3A, B and C) indicating that SIVmac restriction by Hu-R332P also strongly depends on the intact RING domain of TRIM5α. In contrast to AGM TRIM5α, however, Hu-R332P with C15AC18A completely lost its anti-HIV-1 activity (Fig. 3A, B and C). These findings suggest that, unlike AGM TRIM5α, Hu-R332P TRIM5α restricted both HIV-1 and SIVmac mainly via a RING-proteasome-dependent pathway. Hu-R332P TRIM5α with C15AC18A also failed to restrict HIV-2 GH123 (Fig. 3A). Taken together with results on AGM TRIM5α described above, our results indicated that the extent of RING domain contribution to retrovirus restriction by TRIM5α could be determined by a combination of virus and host species. We speculate that the intact RING domain is required for the proteasome-dependent but not for the proteasome-independent pathway of TRIM5α restriction of retroviruses.

Effect of proteasome inhibition on antiviral activity of TRIM5α

For a direct investigation of whether AGM TRIM5α restricts SIVmac and Hu-R332P TRIM5α restricts both HIV-1 and SIVmac mainly via proteasome-dependent pathway, we used a proteasome inhibitor MG132. MT4 cells infected with SeVs expressing various TRIM5α were superinfected with HIV-1-GFP or SIVmac-GFP in the presence or absence of MG132. After infection, the cells were thoroughly washed and incubated in MG132-free medium. As shown in Fig. 4, MG132 had no effect at all on the anti-HIV-1 activity of AGM, Rh or cynomolgus monkey and of human/AGM chimeric TRIM5α carrying the SPRY domain of AGM TRIM5α and the RING, B-box 2, and coiled-coil domains of human TRIM5α. In contrast, and as expected, MG132 at least partially disrupted the anti-HIV-1 activity of Hu-R332P TRIM5α. Rh and cynomolgus monkey TRIM5α could not restrict SIVmac infection and that addition of MG132 did not affect the numbers of GFP-positive cells, indicating that the condition for MG132 treatment used in our study did not affect cell viability (Fig. 4). AGM, Hu-R332P and human/AGM chimeric TRIM5α restricted SIVmac infection while MG132 partially disrupted the anti-SIVmac activity of those TRIM5α. When we used Cf2Th cells, MG132 also disrupted the anti-HIV-1 activity of Hu-R332P TRIM5α at least partially (data not shown). These results support our conclusions that AGM TRIM5α restricted SIVmac mainly via the proteasome-dependent pathway, and that Hu-R332P TRIM5α restricted both HIV-1 and SIVmac mainly via the proteasome-dependent pathway (see Table 1 for summary of these results).

As described above, the previous studies have shown that disrupting the proteasome function by adding a proteasome inhibitor enabled the generation of HIV-1 late reverse transcribed products, even though HIV-1 infection and the generation of nuclear imports of 1-LTR and 2-LTR viral cDNA forms remained impaired by Rh TRIM5α (Anderson et al., 2006; Wu et al., 2006). We therefore examined levels of late reverse transcribed products and 2-LTR forms of HIV-1 cDNA in TRIM5α-expressing cells by real time PCR method. Mean C_T values (SD) of late reverse transcribed products were 29.80 (0.27), 29.30 (0.15), and 28.11 (0.10) in cells expressing Rh, AGM, and Hu-R332P TRIM5αs, respectively, while that in control cells was 24.73 (0.08). These results clearly indicated that synthesis of late reverse transcribed products were suppressed in cells expressing functional TRIM5α. When we added MG132, mean C_T values (SD) of late reverse