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 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 Gal241-249 CTL epitope fused with enhanced green fluorescent protein (EGFP). Vaccination resulted in induction of SeV-EGFP-specific CD4+ T-cell and Gal241-249-specific CD8+ T-cell responses. After a SIV challenge, the vaccinees showed dominant Gal241-249-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.

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

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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 Gal241-249 CTL epitope fused with enhanced green fluorescent protein (EGFP). Vaccination resulted in induction of SeV-EGFP-specific CD4+ T-cell and Gal241-249-specific CD8+ T-cell responses. After a SIV challenge, the vaccinees showed dominant Gal241-249-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.

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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 Gag236-250-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, subsequently received with anti-human CD107a antibody (BD) for 6 h in the absence or presence of 1 μM Gag 236-249 peptide for stimulation. In both cultures, anti-human CD28 and anti-CD8 in CD8+ T-cells was performed with the FACSaria system (BD). Specific T-cell levels were calculated by subtracting nonspecific IFN-γ 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 virus-specific CD8+ T-cell responses.** We measured Gag 236-249-specific induction of IFN-γ and CD107a in CD8+ T-cells. PBMCs were stained with custom-made, PE-conjugated Gag 236-249, epitope-Mamu-A*0101-5 tetrameric complexes, Gag 241-249- and Gag 206-216 peptides for Gag 241-249-specific or Gag 206-216-specific stimulation, respectively. 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 envelopes from simian-human immunodeficiency virus molecular clone (SIVGP1) DNA (26, 41). Intracellular IFN-γ staining was performed with a CytoFluoropex kit and 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.

**RESULTS**

**Gag 236-249-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 elicits Gag 236-249-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-ΔSeV-Gag 236-250-EGFP) vector both expressing an SIVmac239 Gag236-250 (IAGTTSSVDEIQOWM)-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

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal identification codes</th>
<th>Vaccination</th>
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<tbody>
<tr>
<td>I</td>
<td>R02-007, R06-037, R07-001,</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>R07-004, R07-009, R06-019</td>
<td>Control vaccination</td>
</tr>
<tr>
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<td>R02-008, R05-026, R06-004,</td>
<td>[pEGFP-N1 DNA prime,</td>
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<tr>
<td></td>
<td>R06-014, R06-040, R07-006</td>
<td>F(−)SeV-EGFP boost]</td>
</tr>
<tr>
<td>III</td>
<td>R04-016, R06-007, R07-002,</td>
<td>Gag236-250-specific</td>
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<tr>
<td></td>
<td>R07-003, R07-007, R07-008</td>
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<td></td>
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<td>F(−)SeV-Gag236-250*</td>
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<td>EGFP boost]</td>
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* All animals were challenged with SIVmac239.

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 induction of Gag241-249-specific CD8+ T-cell responses after the F(−)SeV-Gag236-250-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 Gag206-216-specific CD8+ T-cell responses, which are dominantly induced in 90-120-La-positive macaques by Gag-expressing SeV vaccination (14). We have never found Gag236-250-specific CD4+ T-cell responses in any previously examined animals, and as expected, analyses with the Gag236-250 peptide did not detect Gag236-250-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 Gag236-250-specific CD8+ T-cell responses after the F(−)SeV-EGFP boost (data not shown).

Control of an SIV challenge in vaccinated animals. Group I (unvaccinated), II (control-vaccinated), and III (Gag236-250-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 Gag241-249 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 Gag241-249-specific CD8+ T-cell responses in vaccines 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. Gag241-249-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, Gag206-216-specific CD8+ T-cell responses were induced equivalently or more efficiently than Gag241-249-specific CD8+ T-cell responses, whereas all of the group III animals showed dominant induction of Gag241-249-specific CD8+ T-cell responses. In these group III animals, Gag206-216-specific CD8+ T-cell responses were inefficient but frequencies of CD8+ T cells exhibiting Gag241-249-specific IFN-γ induction were significantly higher than in naive controls at week 2 (Fig. 3B) and week 12. Gag241-249-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 vaccines (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-La-positive macaques vaccinated with Gag-expressing SeV contained SIV replication with rapid selection of a gag mutation (GagL216S), resulting in escape from Gag206-216-specific CD8+ T-cell recognition at week 5, implicating Gag206-216-specific CD8+ T-cell responses (rather than Gag241-249-specific CD8+ T-cell responses) in viral control. In the present study, however, five of six Gag236-250-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 Gag241-249-specific CD8+ T-cell responses in the acute phase play an important role in this vaccine-based SIV control.

Higher Gag241-249-specific effector memory CD8+ T-cell frequencies in vaccines. We then examined Gag241-249-specific CD8+ T-cell frequencies in these macaques by using PE-conjugated Gag241-249-A*90120-5 tetramers. In group III animals, Gag241-249-specific tetramer+ CD8+ T cells were still detectable just before the SIV challenge, and their frequencies increased greatly after the challenge; most of the vaccines 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 Gag241-249-specific tetramer+ CD95+ CD28+ CD8+ T cells in group III than in naive controls (Fig. 5C). These results suggest efficient responses of Gag241-249-specific CD8+ T cells with effector function in the acute phase in group III animals.

Gag241-249-specific cytolytic CD8+ T-cell responses in vaccines. To further investigate the cytolytic quality of Gag241-249-specific CD8+ T-cell responses after a challenge, we examined...
Gag \textsubscript{241-249}-specific induction of CD107a (a degranulation marker), which is related to cytolytic activity (21, 38), in CD8\textsuperscript{+} T cells at week 2. Frequencies of CD8\textsuperscript{+} T cells exhibiting Gag \textsubscript{241-249}-specific induction of CD107a, as well as IFN-\gamma within the CD8\textsuperscript{+} T-cell pool were significantly higher in group III than in naïve controls (\(P = 0.0249\) by unpaired \(t\)-test) (Fig. 6). One animal, R04-016, in group III did not show Gag \textsubscript{241-249}-specific CD107a\textsuperscript{+} IFN-\gamma\textsuperscript{+} CD8\textsuperscript{+} T-cell responses, but further
analysis revealed that this animal had Gag241-249-specific granzyme B^+/IFN-γ^+ CD8^+ T cells. Indeed, group III animals had significantly higher frequencies of Gag 241-249-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 241-249-specific CD8^+ T cells with higher cytolytic activity in the acute phase in group III animals.

DISCUSSION

In the present study, induction of CD8^+ T cells specific for a single Gag241-249 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, 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^+ macaques with rapid selection of the GagL216S mutation escaping from Gag206-216-specific CD8^+ T-cell recognition at week 5 (27). In contrast, the Gag236-250 vaccination resulted in SIV control without gag mutation selection over 5 weeks in the present study, reflecting the fact that, rather than Gag206-216-specific CD8^+ T-cell responses, dominantly induced Gag241-249-specific CD8^+ T-cell responses played a central role in the reduc-
induction of both IFN-γ and CD107a responses in CD8+ T cells 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.

FIG. 6. Gag241-249-specific cytolytic CD8+ T-cell frequencies at week 2 after a challenge. PBMCs were cultured in the absence or the presence of the Gag241-249 peptide for unstimulated controls or Gag241-249-specific stimulation, and the frequencies of CD8+ T cells exhibiting Gag241-249-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 Gag241-249-specific stimulation. Samples from macaques R06-019 in group I and R07-007 in group III were unavailable for this analysis.

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).

Gag241-249-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 Gag241-249-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 Gag241-249-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 Gag241-249-specific memory CD8+ T cells showed induction of a high magnitude of Gag241-249-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-Ia 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. 5. Frequencies of Gag241-249-specific CD8+ T cells detected by Gag241-249-Mamu-A*00120-5 tetramers after a SIV challenge. (A) Frequencies of Gag241-249-Mamu-A*00120-5 tetramer+ T 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.

Selection 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 Gag241-249-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).

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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 Gag241-249-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 pGag236-250-EGFP/F(−)SeV-Gag236-250-EGFP vaccination elicited Gag241-249-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 Gag241-249-specific CD8+ T-cell induction. The Gag241-249-specific memory CD8+ T cells induced by prophylactic vaccination without SIV-specific CD4+ T-cell help but without non-SIV-specific CD4+ T-cell responses responded efficiently to a SIV challenge, showing dominant Gag241-249-specific CD8+ T-cell responses resulting in SIV control; infection-induced SIV-specific CD4+ T-cell responses may be involved in Gag241-249-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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