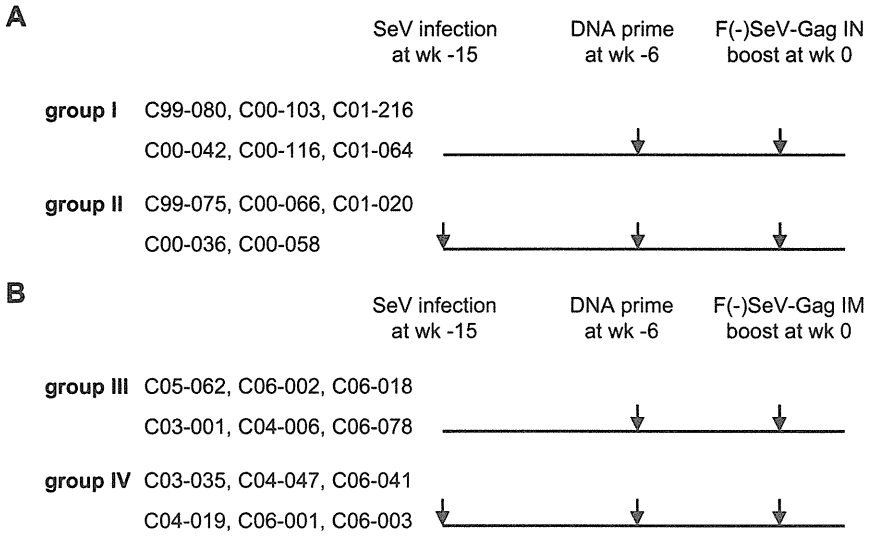

Fig. 2. Experimental protocols. (A) Groups I and II with intranasal F(−)SeV-Gag boost. Groups I ($n=6$) and II ($n=5$) received a DNA prime followed by an intranasal F(−)SeV-Gag boost. Group II animals were infected intranasally with SeV fifteen weeks before the boost. (B) Groups III and IV with intramuscular F(−)SeV-Gag boost. Groups III ($n=6$) and IV ($n=6$) received a DNA prime followed by an intramuscular F(−)SeV-Gag boost. Group IV animals were infected intranasally with SeV fifteen weeks before the boost.

fluorescent protein (SeV-EGFP) [30] as described before [26]. We determined the end-point plasma titers required for 10-fold reduction of SeV-EGFP infectivity compared to the negative control without plasma (90% neutralization titer; 90% effective concentration [EC_{90}]).

2.5. Statistical analysis

Statistical analysis was performed by Prism software version 4.03 with significance levels set at $p<0.05$ (GraphPad Software, Inc., San Diego, CA). $CD8^+$ T-cell and antibody levels were

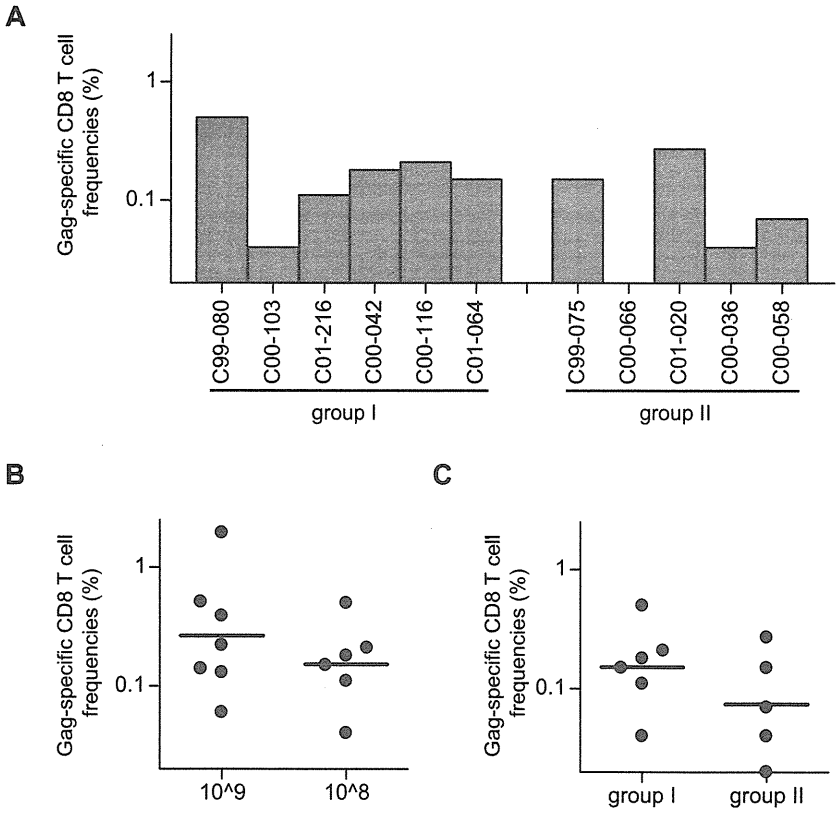

Fig. 3. Gag-specific $CD8^+$ T-cell frequencies after intranasal F(−)SeV-Gag boost in naive and pre-SeV-infected cynomolgus macaques. Gag-specific $CD8^+$ T-cell responses were examined by detection of IFN-γ induction after stimulation by B-LCLs infected with a vaccinia virus vector expressing SIVmac239 Gag. (A) Gag-specific $CD8^+$ T-cell frequencies (percent in $CD8^+$ T lymphocytes) two weeks after the boost in groups I and II. (B) Comparison of Gag-specific $CD8^+$ T-cell frequencies after boost in previously reported animals boosted with 6×10^9 of F(−)SeV-Gag ($10\hat{9}$) [31] and group II animals boosted with 6×10^8 of F(−)SeV-Gag ($10\hat{8}$) (geometric means: 0.266% in $10\hat{9}$ and 0.152% in $10\hat{8}$). (C) Comparison of Gag-specific $CD8^+$ T-cell frequencies after boost in naive (group I) and pre-SeV-infected (group II) animals (geometric means: 0.152% in group I and 0.074% in group II).

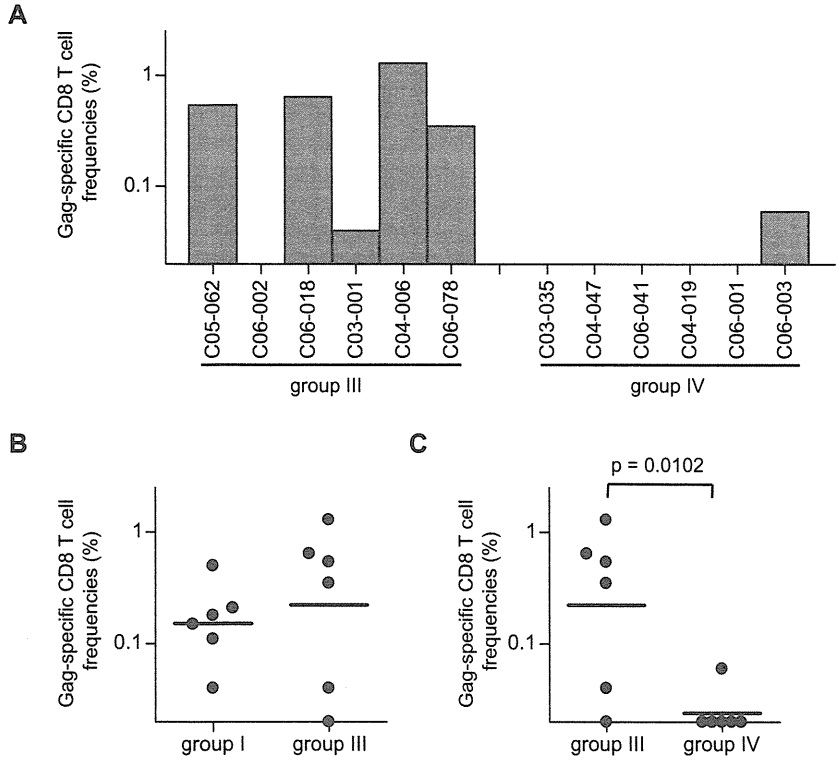

Fig. 4. Gag-specific CD8⁺ T-cell frequencies after intramuscular F(−)SeV-Gag boost in naive and pre-SeV-infected cynomolgus macaques. (A) Gag-specific CD8⁺ T-cell frequencies (percent in CD8⁺ T lymphocytes) two weeks after the boost in groups III and IV. (B) Comparison of Gag-specific CD8⁺ T-cell frequencies after boost in groups I and III. (C) Comparison of Gag-specific CD8⁺ T-cell frequencies after boost in groups III and IV (geometric means: 0.224% in group III and 0.024% in group IV; $p = 0.0102$ by unpaired t-test [$p = 0.0260$ by Mann–Whitney's test]).

log-transformed and compared by unpaired two-tailed t test and Mann–Whitney's test.

3. Results

3.1. Gag-specific CD8⁺ T-cell responses after intranasal F(−)SeV-Gag immunization

Our vaccine protocol consists of a single intramuscular DNA prime followed by a single boost with a replication-defective F-deleted SeV vector expressing SIVmac239 Gag, F(−)SeV-Gag, 6 weeks after the prime. In our previous studies, macaques were intranasally boosted with 6×10^9 CIU of F(−)SeV-Gag [28,31]. In the present study, we attempted vaccination with lower doses, 6×10^8 CIU (1/10 of usual dose), of F(−)SeV-Gag to sensitively examine the effect of anti-SeV antibodies on SeV-based CD8⁺ T-cell induction. In a preliminary experiment, we confirmed Gag-specific CD8⁺ T-cell induction by not only 6×10^8 CIU but also 6×10^7 CIU (1/100 of usual dose) of F(−)SeV-Gag boost in cynomolgus macaques (Fig. 1). Then, we examined the immunogenicity of 6×10^8 CIU of F(−)SeV-Gag in the present study.

Twenty-three cynomolgus macaques were divided into four groups. Groups I ($n = 6$) and II ($n = 5$) received a F(−)SeV-Gag boost intranasally whereas groups III ($n = 6$) and IV ($n = 6$) received it intramuscularly (Fig. 2). Groups II and IV were intranasally pre-infected with SeV fifteen weeks before the boost. No animals showed detectable Gag-specific CD8⁺ T-cell responses at week 0, just before the boost.

In group I, all six animals efficiently elicited Gag-specific CD8⁺ T-cell responses after the intranasal boost (Fig. 3A). There was no significant difference in Gag-specific CD8⁺ T-cell levels between the group I boosted with 6×10^8 CIU of F(−)SeV-Gag and the animals ($n = 7$) boosted with 6×10^9 CIU of F(−)SeV-Gag in our previous study [31] (Fig. 3B), confirming the immunogenicity of F(−)SeV-Gag boost at the dose of 6×10^8 CIU. In group II, efficient Gag-specific CD8⁺ T-cell responses were observed in four animals except for one (Fig. 3A). No significant difference in Gag-specific CD8⁺ T-cell levels was observed between groups I and II (Fig. 3C). These results indicate that the intranasal boost with the lower dose (6×10^8 CIU) of F(−)SeV-Gag can elicit Gag-specific CD8⁺ T-cell responses even in pre-SeV-infected macaques.

3.2. Gag-specific CD8⁺ T-cell responses after intramuscular F(−)SeV-Gag immunization

Five animals except for one in group III showed efficient Gag-specific CD8⁺ T-cell response after the intramuscular F(−)SeV-Gag boost (Fig. 4A). The Gag-specific CD8⁺ T-cell levels in group III were similar to those in group I (Fig. 4B), confirming the immunogenicity of intramuscular F(−)SeV-Gag boost. In contrast, group IV macaques failed to induce Gag-specific CD8⁺ T-cell responses efficiently; only one of six animals induced detectable responses (Fig. 4A). The Gag-specific CD8⁺ T-cell levels in group IV were significantly reduced compared to those in group III (Fig. 4C) and those in group II ($p = 0.0302$). These results indicate that the intramuscular F(−)SeV-Gag boost can elicit Gag-specific CD8⁺ T-cell responses efficiently in SeV-uninfected but not in pre-SeV-infected macaques.

3.3. SeV-specific antibody responses after F(−)SeV-Gag immunization

We then examined SeV-specific antibody responses. All pre-SeV-infected animals in groups II and IV had similar levels of SeV-binding antibodies in plasma at week 0, just before the F(−)SeV-Gag boost (Figs. 5 and 6). SeV-specific neutralization assay showed similar levels of SeV-specific NAb responses at week 0 in

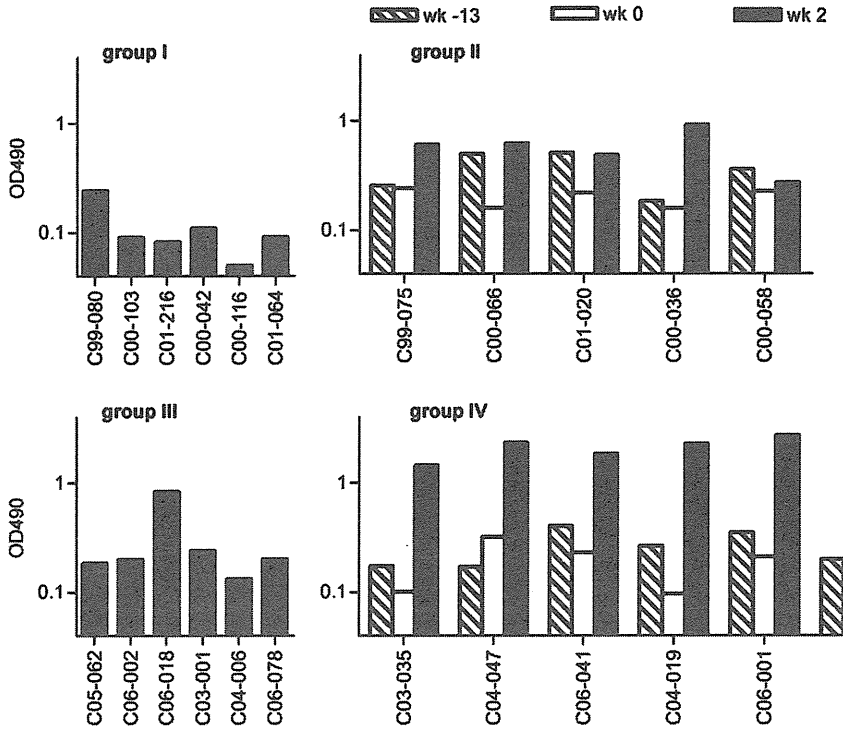

Fig. 5. SeV-specific IgG levels in plasma. Plasma samples obtained from group I and III animals at week 2 and those from group II and IV animals at weeks −13, 0 and 2 were diluted by 1/5000 and subjected to ELISA assay. OD490, optical density at 490 nm.

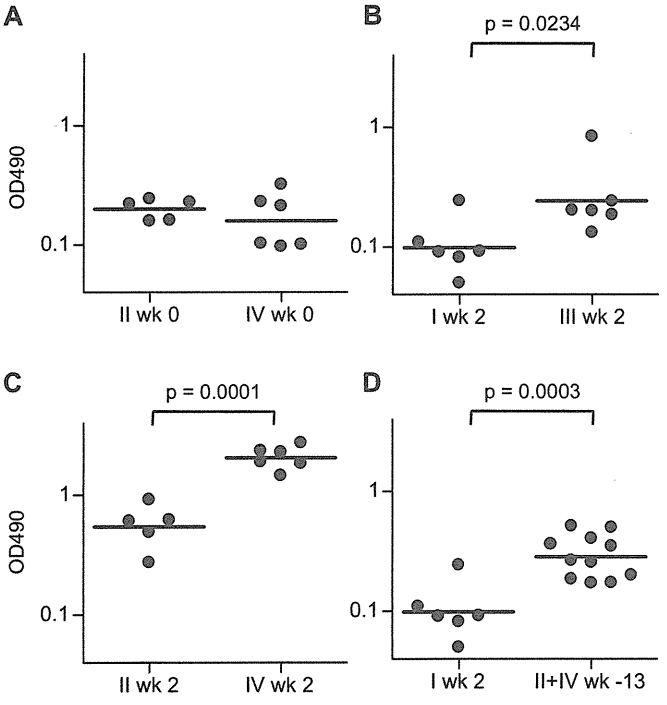

Fig. 6. Comparison of plasma SeV-specific IgG levels among groups. (A) Comparison of plasma SeV-specific IgG levels at week 0, just before F(−)SeV-Gag boost, in groups II and IV (geometric means: 0.199 in group II and 0.159 in group IV). (B) Comparison of plasma SeV-specific IgG levels at week 2, two weeks after the boost, in groups I and III (geometric means: 0.099 in group I and 0.242 in group III; $p = 0.0234$ by unpaired t-test [$p = 0.0411$ by Mann–Whitney's test]). (C) Comparison of plasma SeV-specific IgG levels at week 2 in groups II and IV (geometric means: 0.542 in group II and 2.051 in group IV; $p = 0.0001$ by unpaired t-test [$p = 0.0043$ by Mann–Whitney's test]). (D) Comparison of plasma SeV-specific IgG levels at week 2 in group I and at week −13, two weeks after SeV infection, in groups II and IV (geometric means: 0.285 in groups II and IV; $p = 0.0003$ by unpaired t-test [$p = 0.0042$ by Mann–Whitney's test]).

groups II and IV (Fig. 7); the 90% neutralizing titers were 25–100 and their geometric means were 57 and 56, respectively. Thus, even in the presence of these levels of anti-SeV NAbs, intranasal but not intramuscular administration with 6×10^8 CIU of F(−)SeV-Gag can efficiently elicit Gag-specific $CD8^+$ T-cell responses in macaques.

Plasma SeV-specific IgG levels at week 2, two weeks after F(−)SeV-Gag boost, in group I were significantly lower than those in group III (Fig. 6B). The F(−)SeV-Gag boost enhanced SeV-specific antibody responses in all the pre-SeV-infected animals. Plasma SeV-specific IgG levels two weeks after the boost in group II were significantly lower than in group IV (Fig. 6C). Neutralization assay confirmed these results; SeV-specific NAb titers two weeks after F(−)SeV-Gag boost in group I were significantly lower than in group III (Fig. 7B) and those in group II were significantly lower than in group IV (Fig. 7C). These results indicate that intranasal F(−)SeV-Gag vaccination induces plasma SeV-specific antibody responses less efficiently than intramuscular F(−)SeV-Gag vaccination. Finally, SeV-specific IgG levels and NAb titers at week −13, two weeks after SeV infection, in groups II and IV were higher than those at week 2, two weeks after intranasal F(−)SeV-Gag boost, in group I (Figs. 6D and 7D), suggesting less efficient induction of plasma SeV-specific antibody responses by intranasal replication-defective F(−)SeV-Gag immunization than replication-competent SeV.

4. Discussion

In the present study, we first confirmed that an intranasal boost even with a lower dose (6×10^8 CIU, one-tenth of that in our usual protocol) of F(−)SeV-Gag can induce Gag-specific $CD8^+$ T-cell responses efficiently in macaques. We then showed immunogenicity of the intranasal boost with this lower dose of F(−)SeV-Gag in the presence of SeV-specific NAbs in pre-SeV-infected macaques; Gag-specific $CD8^+$ T-cell responses were induced by the boost fifteen weeks after SeV infection.

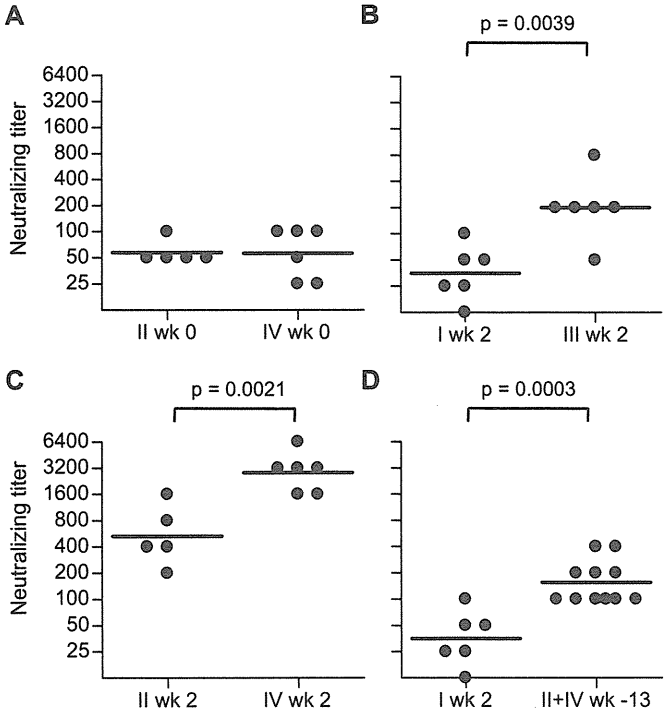

Fig. 7. Comparison of plasma SeV-specific NAb titers among groups. (A) Comparison of plasma SeV-specific NAb titers at week 0 in groups II and IV (geometric means: 5.7×10^1 in group II and 5.6×10^1 in group IV). (B) Comparison of plasma SeV-specific NAb titers at week 2 in groups I and III (geometric means: 3.5×10^1 in group I and 2.0×10^2 in group III; $p = 0.0039$ by unpaired *t*-test [$p = 0.0087$ by Mann–Whitney's test]). (C) Comparison of plasma SeV-specific NAb titers at week 2 in groups II and IV (geometric means: 5.3×10^2 in group II and 2.9×10^3 in group IV; $p = 0.0021$ by unpaired *t*-test [$p = 0.0087$ by Mann–Whitney's test]). (D) Comparison of plasma SeV-specific NAb titers at week 2 in group I and at week −13 in groups II and IV (geometric means: 1.6×10^2 in groups II and IV; $p = 0.0003$ by unpaired *t*-test [$p = 0.0029$ by Mann–Whitney's test]).

SeV has homology in viral genome sequences with hPIV-1, averaging 75% across the six viral genes [32]. Naturally acquired human antibody responses to hPIV-1 cross-react with SeV. A recent study investigating the prevalence of anti-SeV NAbs in humans in Africa, Europe, United States, and Japan [33] detected anti-SeV NAbs in 92.5% subjects with a median titer of 60.6; the 50% neutralization titers (EC_{50}) were measured on LLC-MK2 cells by determining the end-point plasma titers required for 2-fold reduction of SeV-GFP infection. The majority had titers less than 1000 with 71.7% less than 100. Therefore, it is inferred that, in more than 70% of people, anti-SeV NAb titers are no more than those observed just before the F(−)SeV-Gag boost in groups II in the present study. Although it remains unclear whether an intranasal immunization with the lower dose (6×10^8 CIU) or the usual dose (6×10^9 CIU) of SeV vector can work in those with 50% anti-SeV NAb titers of 100–1000, these results imply the potential of SeV vector to induce $CD8^+$ T-cell responses even in humans.

SeV vector has been used for gene transfer and efficient gene expression by its intramuscular inoculation has been shown in multiple studies [34–36]. While the immunogenicity of intramuscular SeV vector inoculation has not been determined, the present study, for the first time, has confirmed the potential of an intramuscular F(−)SeV-Gag boost to induce Gag-specific $CD8^+$ T-cell responses efficiently in SeV naive macaques. Interestingly, however, the intramuscular boost failed to elicit Gag-specific $CD8^+$ T-cell responses efficiently in pre-SeV-infected animals, indicating that both intranasal and intramuscular SeV administrations can induce antigen-specific $CD8^+$ T-cell responses equivalently in the absence of anti-SeV antibodies, whereas intranasal SeV vaccination is more immunogenic than intramuscular in the presence of plasma anti-SeV antibodies. These results possibly imply higher sensitivity of intramuscular SeV inoculation to plasma SeV-specific NAb responses, which may reflect the difference in the route and the mechanism for antigen presentation by intranasal and intramuscular SeV vector immunization in vivo. SeV-specific IgA was detectable in nasal swabs at week 0 in four of five group II macaques (except for macaque C00-058) (data not shown), although we were unable to quantify the IgA levels. Mucosal immune responses are considered important for protecting viral infection via the upper respiratory tract [37–39], but those mucosal responses at week 0 in group II did not significantly diminish $CD8^+$ T-cell induction by intranasal F(−)SeV-Gag boost in the present study.

This study showed less efficient induction of SeV-specific antibody responses by intranasal F(−)SeV-Gag immunization than intramuscular. Indeed, plasma SeV-specific IgG or NAb levels even after intranasal replication-competent SeV infection (at week −13 in groups II and IV) were not more than those after intramuscular replication-defective F(−)SeV-Gag boost (at week 2 in group III). Our results also indicated less efficient SeV-specific antibody induction by intranasal replication-defective F(−)SeV-Gag immunization than replication-competent SeV. Thus, intranasal SeV vector immunization may not induce plasma antibody responses efficiently. However, intranasal immunization with replication-defective F-deleted SeV vectors would be advantageous for repeated vaccination toward antigen-specific $CD8^+$ T-cell induction.

In summary, our results indicate that both intranasal and intramuscular SeV administrations are equivalently immunogenic in the absence of anti-SeV NAbs, whereas intranasal SeV vector vaccination is more immunogenic than intramuscular in the presence of anti-SeV NAbs. This study implies the potential of intranasal SeV vector vaccination to induce $CD8^+$ T-cell responses even in humans.

Acknowledgements

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Impact of Vaccination on Cytotoxic T Lymphocyte Immunodominance and Cooperation against Simian Immunodeficiency Virus Replication in Rhesus Macaques

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Cytotoxic T lymphocyte (CTL) responses play a central role in viral suppression in human immunodeficiency virus (HIV) infections. Prophylactic vaccination resulting in effective CTL responses after viral exposure would contribute to HIV control. It is important to know how CTL memory induction by vaccination affects postexposure CTL responses. We previously showed vaccine-based control of a simian immunodeficiency virus (SIV) challenge in a group of Burmese rhesus macaques sharing a major histocompatibility complex class I haplotype. $Gag_{206-216}$ and $Gag_{241-249}$ epitope-specific CTL responses were responsible for this control. In the present study, we show the impact of individual epitope-specific CTL induction by prophylactic vaccination on postexposure CTL responses. In the acute phase after SIV challenge, dominant $Gag_{206-216}$-specific CTL responses with delayed, naive-derived $Gag_{241-249}$-specific CTL induction were observed in $Gag_{206-216}$ epitope-vaccinated animals with prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory, and vice versa in $Gag_{241-249}$ epitope-vaccinated animals with single $Gag_{241-249}$ epitope-specific CTL induction. Animals with $Gag_{206-216}$-specific CTL induction by vaccination selected for a $Gag_{206-216}$-specific CTL escape mutation by week 5 and showed significantly less decline of plasma viral loads from week 3 to week 5 than in $Gag_{241-249}$ epitope-vaccinated animals without escape mutations. Our results present evidence indicating significant influence of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses, which affects virus control. These findings provide great insights into antigen design for CTL-inducing AIDS vaccines.

Human immunodeficiency virus (HIV) infection induces chronic, persistent viral replication leading to AIDS onset in humans. Virus-specific cytotoxic T lymphocyte (CTL) responses play a central role in the resolution of acute peak viremia (3, 4, 13, 22, 28) but mostly fail to contain viral replication in the natural course of HIV infection. Vaccination resulting in more effective CTL responses after viral exposure than in natural HIV infections would contribute to HIV control (30, 33). CTL memory induction by prophylactic vaccination may lead to efficient secondary CTL responses, but naive-derived primary CTL responses specific for viral nonvaccine antigens can also be induced after viral exposure. It is important to know how CTL memory induction by vaccination affects these postexposure CTL responses.

Cumulative studies on HIV-infected individuals have shown association of HLA genotypes with rapid or delayed AIDS progression (5, 14, 31, 34). For instance, most of the HIV-infected individuals possessing *HLA-B*57* have been indicated to show a better prognosis with lower viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in this viral control (1, 8, 23, 24). Indian rhesus macaques possessing certain major histocompatibility complex class I (MHC-I) alleles, such as *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17*, tend to show simian immunodeficiency virus (SIV) control (19, 25, 36). This implies possible HIV control by induction of particular effective CTL responses (2, 7, 12, 16, 27).

Recent trials of prophylactic T-cell-based vaccines in macaque AIDS models have indicated the possibility of reduction in postchallenge viral loads (6, 15, 17, 21, 35). We previously developed a prophylactic AIDS vaccine consisting of a DNA prime and a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (20). Our trial showed vaccine-based control of an SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90-120-Ia* (21). Animals possessing *90-120-Ia* dominantly elicited Mamu-A1*043:01 (GenBank accession number AB444869)-restricted $Gag_{206-216}$ (IINEEAADWDL) epitope-specific and Mamu-A1*065:01 (AB444921)-restricted $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses after SIV challenge and selected for viral *gag* mutations, GagL216S (leading to a leucine [L]-to-serine [S] substitution at amino acid [aa] 216 in Gag) and GagD244E (aspartic acid [D]-to-glutamic acid [E] at aa 244), resulting in escape from CTL recognition with viral fitness costs in the chronic phase (9, 26). Vaccinees possessing *90-120-Ia* failed to control a challenge with a mutant SIV carrying these two CTL escape mutations, indicating that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses play a crucial role in the vaccine-based control of wild-type SIVmac239 replication

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TABLE 1 Animals analyzed in this study

Group	No. of animals	Vaccination[a]	SIV-specific CTL response postboost
I	6	None	None
II	5	Gag (pCMV-SHIVdEN DNA prime, SeV-Gag boost)	Gag-specific CTL
III	6	$Gag_{241-249}$-specific (pGag$_{236-250}$-EGFP-N1 DNA prime, SeV-Gag$_{236-250}$-EGFP boost)	$Gag_{241-249}$-specific CTL
IV	5	$Gag_{206-216}$-specific (pGag$_{202-216}$-EGFP-N1 DNA prime, SeV-Gag$_{202-216}$-EGFP boost)	$Gag_{206-216}$-specific CTL

[a] All animals were challenged with SIVmac239.

(10). Furthermore, in an SIVmac239 challenge experiment with *90-120-Ia*-positive rhesus macaques that received a prophylactic vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), this single-epitope vaccination resulted in control of SIVmac239 replication with dominant induction of $Gag_{241-249}$-specific CTL responses in the acute phase postchallenge (32).

Thus, it is hypothesized that induction of single $Gag_{206-216}$ or $Gag_{241-249}$ epitope-specific CTL responses by vaccination may result in different patterns of CTL immunodominance and viral replication after SIV challenge. In the present study, we analyzed the impact of prophylactic vaccination inducing single $Gag_{206-216}$ epitope-specific CTL responses on SIV control in *90-120-Ia*-positive macaques and compared the results with those of vaccination inducing single $Gag_{241-249}$ epitope-specific CTL responses. This analysis revealed differences in CTL responses and patterns of viral control after SIV challenge between these vaccinated groups, indicating significant effects of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses.

MATERIALS AND METHODS

Animal experiments. Animal experiments were conducted through the Cooperative Research Program at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia. All animals were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases.

Five Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-120-Ia* (26) (group IV) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{206-216}$-specific CTL responses followed by an SIVmac239 challenge and were compared with three groups (I, II, and III) of *90-120-Ia*-positive animals reported previously (10, 32) (Table 1). Group I animals ($n = 6$) received no vaccination, while group II animals ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting Gag-specific CTL responses. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from a simian/human immunodeficiency virus ($SHIV_{MD14YE}$) molecular clone DNA with *env* and *nef* deleted (29) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx; SIVmac239–HIV-1 chimeric Vpr; and HIV-1 Tat and Rev (21). In group II animals, CTL responses were undetectable after DNA prime but Gag-specific CTL responses became detectable after SeV-Gag boost. Group III animals ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{241-249}$-specific CTL responses. A pGag$_{236-250}$-EGFP-N1 DNA and an SeV-Gag$_{236-250}$-EGFP vector, both expressing an SIVmac239 $Gag_{236-250}$ (IAGTTSSVDEQ IQWM)-EGFP fusion protein, were used for the group III vaccination. After the SeV-Gag$_{236-250}$-EGFP boost, group III animals induced $Gag_{241-249}$-specific CTL responses; the animals showed no $Gag_{236-250}$-specific $CD4^+$ T-cell responses but elicited SeV/EGFP-specific $CD4^+$ T-cell responses (32). For the group IV vaccination, A pGag$_{202-216}$-EGFP-N1 DNA and an SeV-Gag$_{202-216}$-EGFP vector, both expressing an SIVmac239 $Gag_{202-216}$ (IIRDIINEEAADWDL)-EGFP fusion protein, were used (Fig. 1). Approximately 3 months after the boost, all animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239 (11). In our previous study (32), the unvaccinated and the control-vaccinated

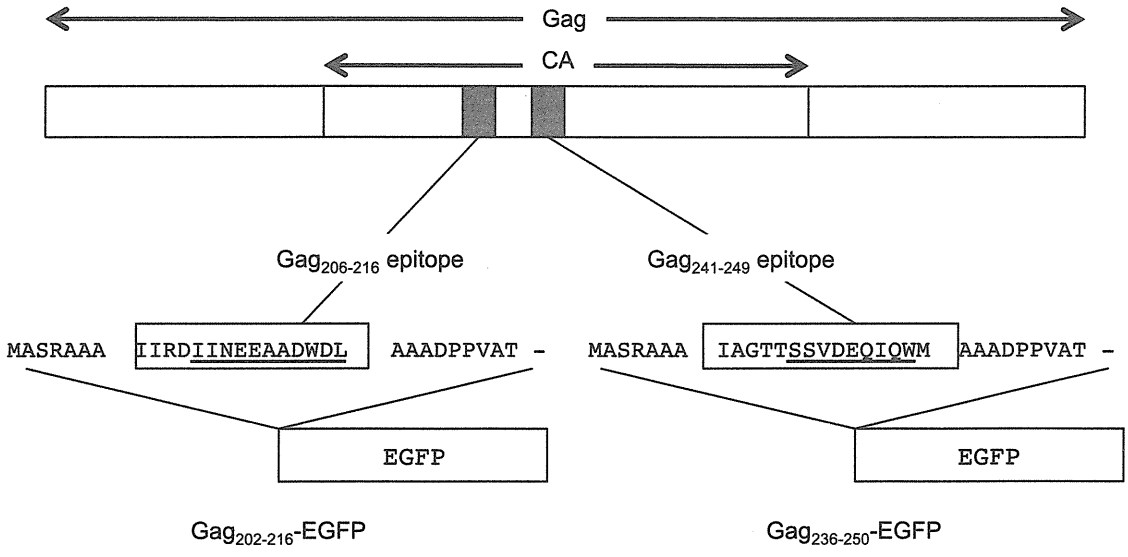

FIG 1 Schema of the cDNA constructs encoding $Gag_{202-216}$-EGFP and $Gag_{236-250}$-EGFP fusion proteins. A DNA fragment that encodes a 31-mer peptide (boxes) including the $Gag_{202-216}$ or $Gag_{236-250}$ sequence (underlining) was introduced into the 5′ end of the EGFP cDNA.

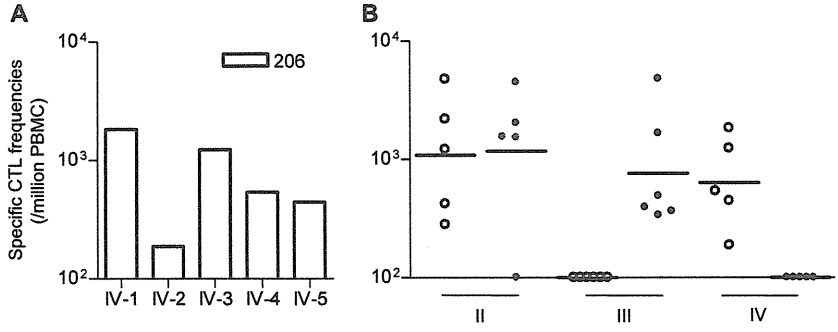

FIG 2 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after prophylactic vaccination. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies 1 week after SeV-$Gag_{202-216}$–EGFP boost in group IV macaques (open boxes). (B) $Gag_{206-216}$-specific (open circles) and $Gag_{241-249}$-specific (closed circles) $CD8^+$ T-cell frequencies 1 week after boost in group II (green), III (blue), and IV (red) macaques. The bars indicate the geometric mean of each group. No animal showed detectable Gag-specific CTL responses before the boost.

animals receiving a DNA and an SeV expressing EGFP showed no significant differences in viral loads after SIV challenge.

Analysis of antigen-specific CTL responses. We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (21). Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papioimmortalized B-lymphoblastoid cell lines pulsed with 1 μM SIVmac239 $Gag_{206-216}$ (IINEEAADWDL), $Gag_{241-249}$ (SSVDEQIQW), or $Gag_{367-381}$ (ALKEALAPVPIPFAA) peptide for $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, or $Gag_{367-381}$-specific stimulation. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (BD, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein-conjugated anti-human CD8 (BD), allophycocyanin (APC)-Cy7-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ (Biolegend, San Diego, CA) monoclonal antibodies. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell levels lower than 100 per million PBMCs were considered negative.

Sequencing of the viral genome. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). Fragments corresponding to nucleotides from 1231 to 2958 (containing the entire *gag* region) in the SIVmac239 genome (GenBank accession number M33262) were amplified by nested reverse transcription (RT)-PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using R software (R Development Core Team). Differences in geometric means of plasma viral loads were examined by one-way analysis of variance (ANOVA) and Tukey-Kramer's multiple-comparison test. Plasma viral loads at week 3 were examined for differences between group III and groups II and IV by analysis of covariance (ANCOVA) with week 5 viral loads as a covariate.

RESULTS

CTL responses after prophylactic vaccination. We previously reported the efficacy of vaccination eliciting whole Gag-specific or single $Gag_{241-249}$ epitope-specific CTL memory against SIVmac239 challenge (10, 32). In the present study, we examined the efficacy of prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory against SIVmac239 challenge and compared the results with those of the previous experiments.

Five Burmese rhesus macaques possessing MHC-I haplotype *90-120-Ia* received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{206-216}$ epitope-specific CTL responses. A plasmid DNA (p$Gag_{202-216}$-EGFP-N1) and an SeV (SeV-$Gag_{202-216}$-EGFP) vector, both expressing an SIVmac239 $Gag_{202-216}$-EGFP fusion pro-

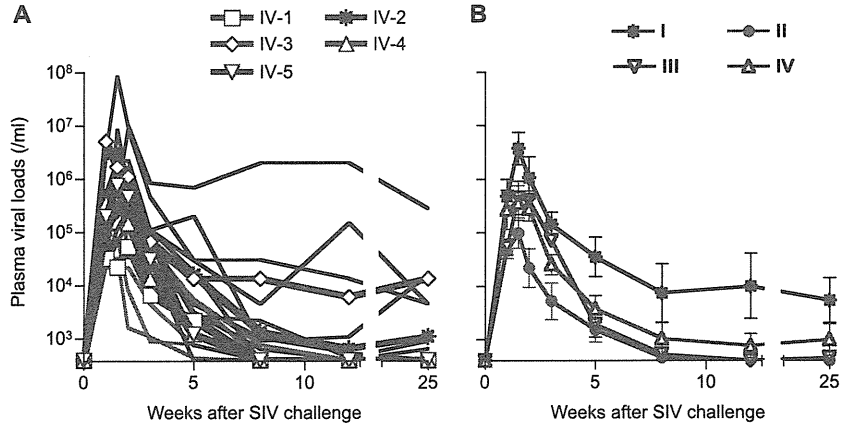

FIG 3 Plasma viral loads after SIVmac239 challenge. The plasma viral loads in group I, group II, group III, and group IV animals were determined as described previously (21). The lower limit of detection was approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) after challenge. (B) Changes in geometric means of plasma viral loads after challenge. Groups II and III (but not group IV) showed significantly lower set point viral loads than group I ($P = 0.0390$ between groups I and II, $P = 0.0404$ between groups I and III, and $P > 0.05$ between groups I and IV at week 25 by one-way ANOVA and Tukey-Kramer's multiple-comparison test).

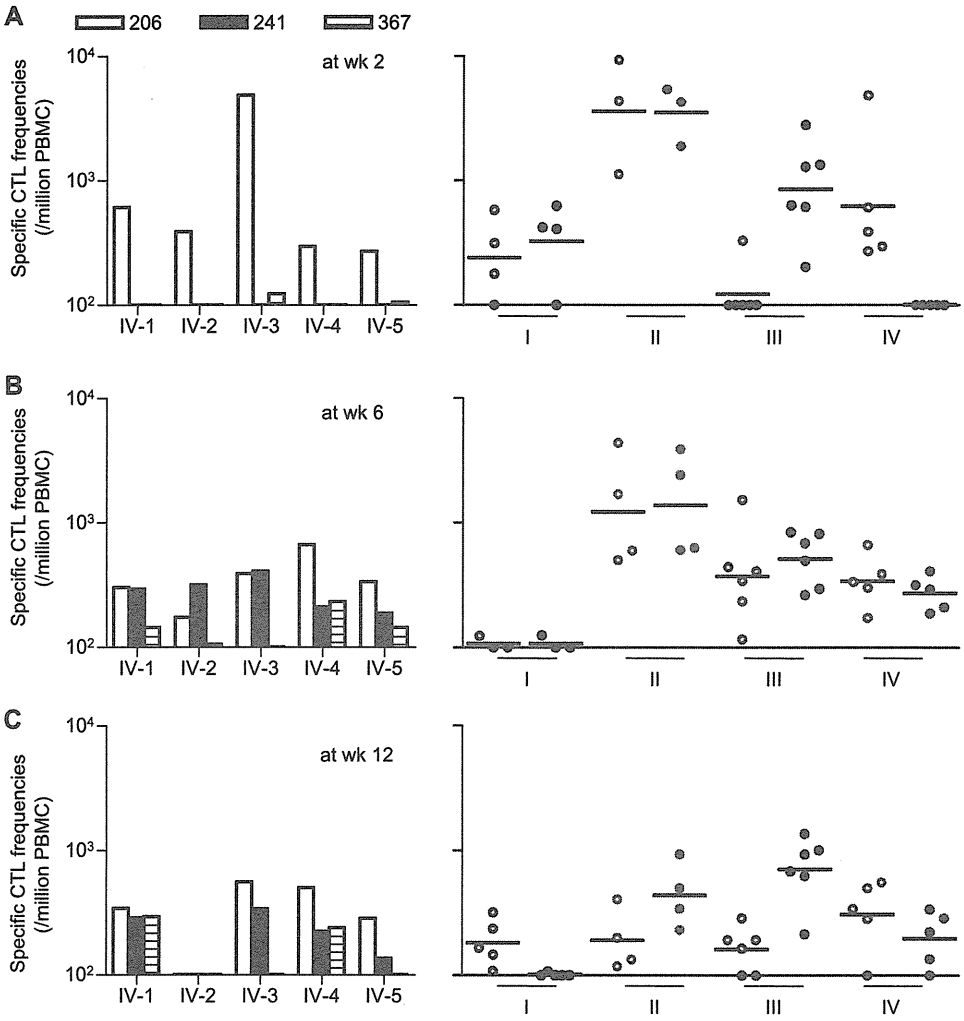

FIG 4 $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIVmac239 challenge. CTL responses at week 2 (A), week 6 (B), and week 12 (C) are shown. In the graphs on the left, $Gag_{206\text{-}216}$-specific (open boxes), $Gag_{241\text{-}249}$-specific (closed boxes), and $Gag_{367\text{-}381}$-specific (striped boxes) $CD8^+$ T-cell frequencies in group IV macaques are shown. On the right, $Gag_{206\text{-}216}$-specific (open circles) and $Gag_{241\text{-}249}$-specific (closed circles) $CD8^+$ T-cell frequencies in group I (black), II (green), III (blue), and IV (red) macaques are shown. The bars indicate the geometric mean of each group. Samples from macaques I-1, I-6, II-1, and II-3 at week 2; macaques I-1, I-2, I-6, and II-5 at week 6; and macaques I-1 and II-5 at week 12 were unavailable for this analysis. Statistical analyses among four groups at week 12 revealed significant differences in $Gag_{241\text{-}249}$-specific CTL levels (I and III, $P < 0.0001$; I and II, and III and IV, $P < 0.01$; I and IV, II and III, and II and IV, $P > 0.05$ by one-way ANOVA and Tukey-Kramer's multiple-comparison test) but not in $Gag_{206\text{-}216}$-specific CTL levels ($P > 0.05$ by one-way ANOVA).

tein, were used for the vaccination (Fig. 1). We confirmed $Gag_{206\text{-}216}$-specific CTL responses 1 week after SeV-$Gag_{202\text{-}216}$-EGFP boost in all five animals (Fig. 2A). As expected, no $Gag_{241\text{-}249}$-specific CTL responses were detected in these animals. No $Gag_{202\text{-}216}$-specific $CD4^+$ T-cell responses were detected in the animals except for one (IV-5) showing marginal levels of responses (data not shown).

Plasma viral loads after SIV challenge. We compared these five animals (referred to as group IV) with other groups (I, II, and III) of *90-120-Ia*-positive macaques reported previously (Table 1). Group I animals ($n = 6$) received no vaccination, group II ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting whole Gag-specific CTL responses, and group III ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{241\text{-}249}$ epitope-specific CTL responses. Both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses were detectable after SeV-Gag boost in four of five group II animals except for one animal (II-3), in which $Gag_{206\text{-}216}$-specific, but not $Gag_{241\text{-}249}$-specific, CTL responses were detected. In all group III animals, $Gag_{241\text{-}249}$-specific CTL responses were confirmed, while no $Gag_{206\text{-}216}$-specific CTL responses were detected after SeV-$Gag_{236\text{-}250}$-EGFP boost (Fig. 2B).

After SIVmac239 challenge, all animals were infected and showed plasma viremia during the acute phase. Plasma viremia was maintained in five of six unvaccinated animals in group I but became undetectable in one animal (I-2) at week 12. In contrast, all animals in groups II and III contained SIV replication with significantly reduced plasma viral loads compared to group I at the set point. In group IV, however, vaccine efficacy was not so clear; while three out of five animals contained SIV replication, the remaining two (IV-2 and IV-3) failed to control viral replication with persistent plasma viremia (Fig. 3).

Gag-specific CTL responses after SIV challenge. We then measured $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIVmac239 challenge by detection of peptide-

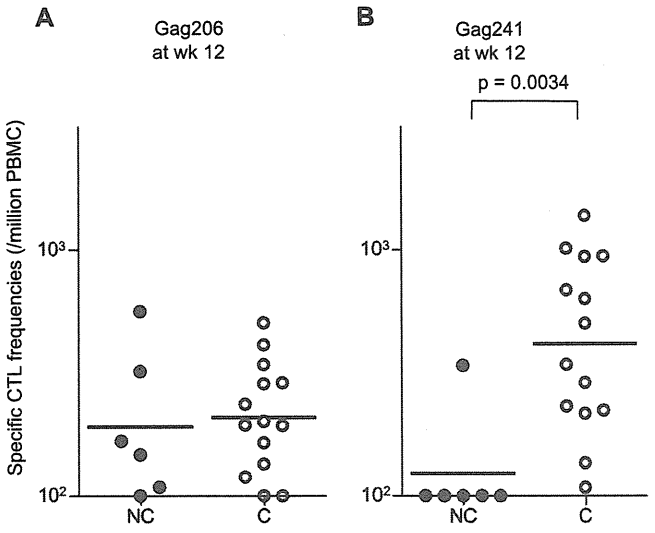

FIG 5 Comparison of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL responses in noncontrollers and controllers at week 12. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies in noncontrollers (NC; closed circles) and controllers (C; open circles). (B) $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies in noncontrollers and controllers. $Gag_{241-249}$-specific CTL levels in controllers were significantly higher than those in noncontrollers ($P = 0.0034$ by Mann-Whitney test). The bars indicate the geometric mean of each group. Data on a noncontroller (I-1) and a controller (II-5) were unavailable.

specific IFN-γ induction. At week 2 (Fig. 4A), most animals in groups I and II elicited both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses, whereas group III animals induced $Gag_{241-249}$-specific CTL responses dominantly. Remarkably, all animals in group IV showed efficient $Gag_{206-216}$-specific CTL responses without detectable $Gag_{241-249}$-specific CTL responses at week 2. These results indicate dominant $Gag_{206-216}$-specific CTL responses with delayed induction of $Gag_{241-249}$-specific CTL responses postchallenge in group IV animals with prophylactic $Gag_{206-216}$-specific CTL induction, and vice versa in group III animals.

At week 6 (Fig. 4B), efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were observed in all vaccinated animals in groups II, III, and IV, but not in group I. $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were induced equivalently even in groups III and IV. We also examined subdominant $Gag_{367-381}$ epitope-specific CTL responses, which were undetectable at week 2 but became detectable at week 6 in most group IV animals (Fig. 4, graphs on left). At week 12 (Fig. 4C), however, different CTL immunodominance patterns were observed among the groups. $Gag_{241-249}$-specific CTL levels were higher than $Gag_{206-216}$-specific levels in groups II and III but were reduced in groups I and IV. Interestingly, comparison between the animals with persistent viremia (referred to as noncontrollers) and those controlling SIV replication (referred to as controllers) revealed significant differences in $Gag_{241-249}$-specific CTL levels, but not in $Gag_{206-216}$-specific levels, at week 12 ($P = 0.0034$ by Mann-Whitney test) (Fig. 5).

Selection of a CTL escape mutation. Next, we examined viral genome *gag* sequences at weeks 5 and 12 after challenge to determine whether CTL escape mutations were selected in these animals (Table 2). At week 5, a mutation leading to an L-to-S substitution at the 216th residue in Gag (L216S) was selected in all the group II animals. This GagL216S change results in escape from $Gag_{206-216}$-specific CTL recognition, as described previously (21). All the group IV animals with $Gag_{206-216}$-specific CTL induction also showed rapid selection of this CTL escape mutation at week 5. Analysis at week 3 found the GagL216S mutation dominant in two (II-2 and II-5) group II and two (IV-1 and IV-3) group IV animals (data not shown). However, animals in group III showed no *gag* mutations at week 5, except for one animal (III-5) selecting a mutation leading to an L-to-F substitution at the 216th residue. Later, at week 12, the $Gag_{206-216}$-specific CTL escape mutation, GagL216S, was selected even in group III animals. No animals showed mutations around the $Gag_{241-249}$ epitope-coding region even at week 12. These results indicate that selection of this $Gag_{206-216}$-specific CTL escape mutation may be accelerated by prophylactic vaccination inducing $Gag_{206-216}$-specific CTL responses. On the other hand, in group III animals with single $Gag_{241-249}$ epitope-specific CTL induction, selection of a $Gag_{206-216}$-specific CTL escape mutation was delayed but was observed before selection of a $Gag_{241-249}$-specific CTL escape mutation, suggesting strong selective pressure by delayed $Gag_{206-216}$-specific CTL responses after SIV challenge.

In order to see the effect of rapid selection of the $Gag_{206-216}$-specific CTL escape mutation on SIV control, we compared plasma viral loads at weeks 3 and 5 between groups II and IV (referred to as group II+IV) with rapid selection of the GagL216S

TABLE 2 Selection of a CTL escape mutation

Group	Macaque ID	Amino acid change for Gag residues[b]: 206–216		241–249	
		Wk 5	Wk 12	Wk 5	Wk 12
I	I-1	None	ND	None	ND
	I-2[a]	None	L216S	None	None
	I-3	None	L216S	None	None
	I-4	None	None	None	None
	I-5	None	None	None	None
	I-6	None	None	None	None
II	II-1[a]	L216S	ND	None	ND
	II-2[a]	L216S	ND	None	ND
	II-3[a]	L216S	ND	None	ND
	II-4[a]	L216S	ND	None	ND
	II-5[a]	L216S	ND	None	ND
III	III-1[a]	None	L216S	None	None
	III-2[a]	None	L216S	None	None
	III-3[a]	None	NA	None	NA
	III-4[a]	None	NA	None	NA
	III-5[a]	L216F	L216S	None	None
	III-6[a]	None	L216S	None	None
IV	IV-1[a]	L216S	L216S	None	None
	IV-2	L216S	L216S	None	None
	IV-3	L216S	L216S	None	None
	IV-4[a]	L216S	L216S	None	None
	IV-5[a]	L216S	NA	None	NA

[a] Animals that controlled SIV replication at week 12 (controllers).
[b] Plasma viral gag genome mutations were examined at weeks 5 and 12. Amino acid substitutions in $Gag_{206-216}$ and $Gag_{241-249}$ epitope regions are shown. L216S results in viral escape from $Gag_{206-216}$-specific CTL recognition. It remains undetermined whether L216F results in CTL escape. ND, not determined; NA, not determined because Gag fragments were unable to be amplified from plasma RNA.

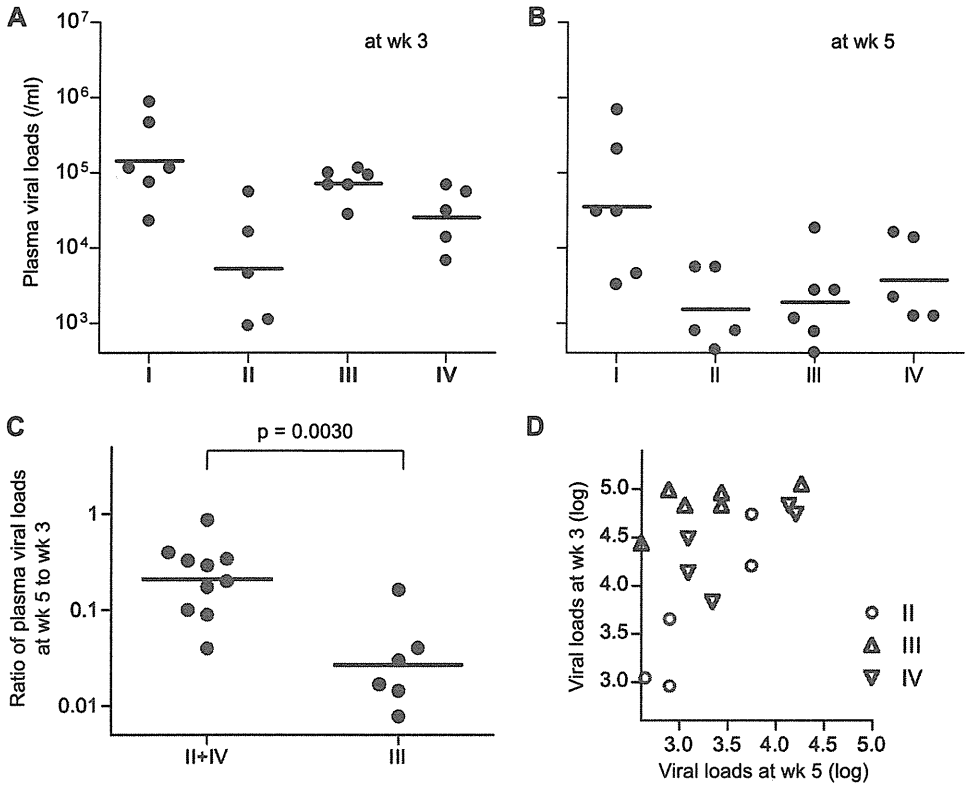

FIG 6 Comparison of plasma viral loads at weeks 3 and 5 among four groups. (A) Plasma viral loads at week 3 in group I, II, III, and IV animals. (B) Plasma viral loads at week 5 in group I, II, III, and IV animals. (C) Comparison of ratios of plasma viral loads at week 5 to week 3 in group II+IV animals and group III animals. The ratios in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test). The bars indicate the geometric mean of each group. (D) Scatter plots between plasma viral loads at weeks 3 and 5 in group II, III, and IV animals.

mutation and group III without the mutation at week 5 (Fig. 6). Ratios of plasma viral loads at week 5 to week 3 in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test) (Fig. 6C). To confirm this result, we examined the difference in week 3 viral loads between groups III and II+IV by ANCOVA, with week 5 viral loads as a covariate. This analysis revealed that week 3 viral loads controlled for by week 5 viral loads were significantly higher in group III than those in group II+IV (Fig. 6D and Table 3); i.e., the decline in viral loads from week 3 to week 5 was significantly sharper in group III than in group II+IV, possibly reflecting viral escape from suppressive pressure by $Gag_{206\text{-}216}$-specific CTL responses in the latter group during this period (from week 3 to week 5).

DISCUSSION

In the present study, we analyzed the impact of vaccination inducing single $Gag_{206\text{-}216}$ epitope-specific CTL memory on postchallenge CTL responses and SIV control in *90-120-Ia*-positive macaques and then compared the results with those of vaccination inducing single $Gag_{241\text{-}249}$ epitope-specific CTL responses. Our results indicate that these prophylactic vaccinations result in different patterns of $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL immunodominance and cooperation after SIVmac239 challenge.

Unvaccinated *90-120-Ia*-positive macaques (group I) showed both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIV challenge. In group IV animals with prophylactic induc-

TABLE 3 ANCOVA on week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV

ANOVA	Parameter	SS[a]	df[b]	MS[c]	*F*	*P* value
Homogeneity of slopes of regression	Group × slope	0.304	1	0.304	2.099	0.173
	Residual	1.735	12	0.145		
	Total	2.038	13	0.157		
Difference in week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV	Effect and group	1.106	1	1.106	7.052	0.020
	Residual	2.038	13	0.157		
	Total	3.144	14	0.225		

[a] SS, sum of squares.
[b] df, degrees of freedom.
[c] MS, mean squares.

tion of single $Gag_{206-216}$ epitope-specific CTL responses, $Gag_{206-216}$-specific CTL responses were induced dominantly but $Gag_{241-249}$-specific CTL responses were undetectable at week 2. In contrast, $Gag_{241-249}$-specific CTL responses were induced dominantly at week 2 in group III. Both groups showed $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 6. It may be difficult to compare these results with those in group II animals inducing whole Gag antigen-specific CTL and $CD4^+$ T-cell responses before challenge; the group II animals elicited $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 2. Our results indicate that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses and may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as nonvaccine antigens) after viral exposure.

A significant difference between groups III and IV is the pattern of selection of CTL escape mutation. All group IV animals showed rapid selection of a $Gag_{206-216}$-specific CTL escape mutation, while most group III animals showed no *gag* mutation at week 5 but selection of the $Gag_{206-216}$-specific CTL escape mutation later, at week 12. Thus, prophylactic vaccination may affect the patterns of viral genome diversification, possibly accelerating selection of CTL escape mutations. Interestingly, $Gag_{241-249}$-specific CTL mutations were not detected even at week 12 in group III animals, although a previous study observed not only the $Gag_{206-216}$-specific CTL escape mutation (GagL216S), but also a $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in the chronic phase of SIV infection in *90-120-Ia*-positive macaques (9). These results indicate that delayed, naive-derived $Gag_{206-216}$-specific CTL responses, as well as preceding $Gag_{241-249}$-specific CTL responses, exert strong suppressive pressure on SIV replication in group III animals, implying cooperation between vaccine antigen-specific and non-vaccine antigen-specific CTL responses for virus control.

Rapid selection of the $Gag_{206-216}$-specific CTL escape mutation (GagL216S) in group II and delayed selection of this mutation without a detectable $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in group III suggest that the virus with GagL216S (SIVmac239Gag216S) replicates more efficiently than the virus with GagD244E (SIVmac239Gag244E) under both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses. Our previous competition assay did not find a significant difference in viral fitness between these mutant viruses. Possibly, escape of SIVmac239Gag216S from $Gag_{206-216}$-specific CTL pressure may be more efficient than that of SIVmac239Gag244E from $Gag_{241-249}$-specific CTL pressure.

Our analysis revealed that the decline of plasma viral loads from week 3 to week 5 in group II+IV with rapid selection of the GagL216S mutation was significantly less than that in group III without the mutation at week 5, possibly reflecting viral escape from suppressive pressure by $Gag_{206-216}$-specific CTL responses in the former groups around weeks 3 to 5. Even the comparison between groups II and III, both showing dominant $Gag_{241-249}$-specific CTL responses at week 2, revealed a significantly sharper decline in the latter ($P = 0.0087$). Thus, our results suggest three patterns of $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL cooperation for virus control after SIVmac239 challenge. First, as observed in group II, dominantly induced $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses both work against wild-type SIV replication around week 2, but then a mutant virus escaping from the former CTL responses is selected, and the responses work against this mutant virus replication. Second, as observed in group III, dominantly induced $Gag_{241-249}$-specific CTL responses work against wild-type SIV replication around week 2 and then contribute to virus control, together with delayed, naive-derived $Gag_{206-216}$-specific CTL responses. Third, as observed in group IV, dominantly induced $Gag_{206-216}$-specific CTL responses work against wild-type SIV replication around week 2, but then a mutant virus escaping from $Gag_{206-216}$-specific CTL responses is selected, and delayed, naive-derived $Gag_{241-249}$-specific CTL responses instead work against this mutant virus replication. Viral loads at week 3 in group III looked higher than those in group IV, implying that $Gag_{206-216}$-specific CTL responses may exert a stronger suppressive effect on SIV replication in the acute phase than $Gag_{241-249}$-specific CTL responses. However, viral loads at week 5 in group III looked lower than those in group IV, and the comparison between the two groups showed significantly less decline in the latter ($P = 0.0303$). It is speculated that the third pattern observed in group IV is prone to failure in virus control. Indeed, two of five animals in group IV failed to control SIV replication. Even if vaccines are designed to express multiple antigens, of the vaccine-induced CTLs generated, only several epitope-specific cells may recognize the incoming HIV because of viral diversity and host MHC polymorphisms (18), and cooperation of these vaccine antigen-specific and non-vaccine antigen-specific CTL responses would be required for viral control. Thus, our results may imply a rationale of inducing escape-resistant, epitope-specific CTL memory by prophylactic AIDS vaccines.

In summary, this study showed dominant induction of vaccine antigen-specific CTL responses and delay in non-vaccine antigen-specific CTL responses in the acute phase of SIV infection, clearly describing the impact of prophylactic vaccination on CTL immunodominance and cooperation after virus exposure. Our results indicate that the patterns of cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses affect virus control and selection of CTL escape mutations. These findings provide great insights into antigen design in the development of a CTL-inducing AIDS vaccine.

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CTL escape and viral fitness in HIV/SIV infection

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Cytotoxic T lymphocyte (CTL) responses exert a suppressive effect on HIV and simian immunodeficiency virus (SIV) replication. Under the CTL pressure, viral CTL escape mutations are frequently selected with viral fitness costs. Viruses with such CTL escape mutations often need additional viral genome mutations for recovery of viral fitness. Persistent HIV/SIV infection sometimes shows replacement of a CTL escape mutation with an alternative escape mutation toward higher viral fitness. Thus, multiple viral genome changes under CTL pressure are observed in the chronic phase of HIV/SIV infection. HIV/SIV transmission to HLA/MHC-mismatched hosts drives further viral genome changes including additional CTL escape mutations and reversions under different CTL pressure. Understanding of viral structure/function and host CTL responses would contribute to prediction of HIV evolution and control of HIV prevalence.

Keywords: HIV, SIV, MHC, cytotoxic T lymphocyte, escape mutation, viral fitness, capsid

INTRODUCTION

Virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses play a central role in the control of HIV and simian immunodeficiency virus (SIV) replication (Borrow et al., 1994; Koup et al., 1994; Matano et al., 1998; Jin et al., 1999; Schmitz et al., 1999; Goulder and Watkins, 2008). CTLs recognize viral antigen-derived peptides (epitopes) presented by major histocompatibility class I (MHC-I) molecules on the surface of viral-infected cells. Under the CTL pressure, viral mutations in and around epitope-coding regions which result in viral escape from CTL recognition are frequently selected with the cost of viral fitness (Phillips et al., 1991; Borrow et al., 1997; Goulder et al., 1997; Price et al., 1997). Thus, analysis of structural and functional constraints in viral proteins could facilitate determination of effective CTLs that can limit viral escape options, contributing to immunogen design in development of CTL-inducing AIDS vaccines.

We previously developed an AIDS vaccine using a Sendai virus vector expressing Gag (SeV-Gag), which induces Gag-specific CTL responses efficiently. Our analysis showed vaccine-based control of a SIVmac239 challenge in a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-120-Ia* (Matano et al., 2004; Kawada et al., 2008). $Gag_{206–216}$ (IINEEAADWDL) epitope-specific CTL responses exert a suppressive effect on SIV replication and select for a CTL escape mutation, GagL216S, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) in Gag capsid (CA) with viral fitness costs (Kobayashi et al., 2005). Our studies starting with this finding revealed viral genome changes in persistent SIV infection, providing insights into HIV/SIV evolution.

LOSS OF VIRAL FITNESS BY ESCAPE MUTATIONS AND ITS RECOVERY BY COMPENSATORY MUTATIONS

In contrast to the SIVmac239 challenge experiment, *90-120-Ia*-positive vaccinees failed to control a challenge with another pathogenic SIV strain, SIVsmE543-3 (Hirsch et al., 1997), which has the same $Gag_{206–216}$ amino acid sequence with SIVmac239. SIVsmE543-3 has a different amino acid (glutamate [E]) from SIVmac239 (aspartate [D]) at Gag residue 205, and this GagD205E change resulted in escape from $Gag_{206–216}$-specific CTL recognition, leading to failure in control of SIVsmE543-3 replication in *90-120-Ia*-positive vaccinees (Moriya et al., 2008).

Theoretically, $Gag_{206–216}$-specific CTL responses can select for either GagD205E or GagL216S mutation. SIVmac239-infected *90-120-Ia*-positive macaques, however, select the latter GagL216S mutation but not GagD205E in a year postchallenge. This suggests a possibility that the GagD205E substitution in SIVmac239 results in larger reduction of viral fitness than GagL216S. Indeed, our analysis *in vitro* revealed much lower replicative ability of the virus with this GagD205E substitution, SIVmac239Gag205E, compared to the wild-type SIVmac239 (Inagaki et al., 2010). On LuSIV cells, which contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat, SIVmac239Gag205E infection showed significantly lower luciferase activity compared to wild-type SIVmac239, indicating suppression of the early phase of this mutant virus replication.

Further passage of SIVmac239Gag205E-infected culture supernatants *in vitro* found an additional mutation, GagV340M, resulting in a valine (V)-to-methionine (M) substitution at the 340th aa in Gag. Interestingly, SIVmac239 has V while SIVsmE543-3 has M at the Gag residue 340. SIVmac239Gag205E340M showed similar replication kinetics with wild-type SIVmac239, indicating compensation for loss of viral fitness in SIVmac239Gag205E by addition of the GagV340M substitution. Thus, CTL escape mutations resulting in loss of viral fitness could be selected with compensatory mutations. **Figure 1** is a schema indicating the interaction between escape and compensatory mutations.

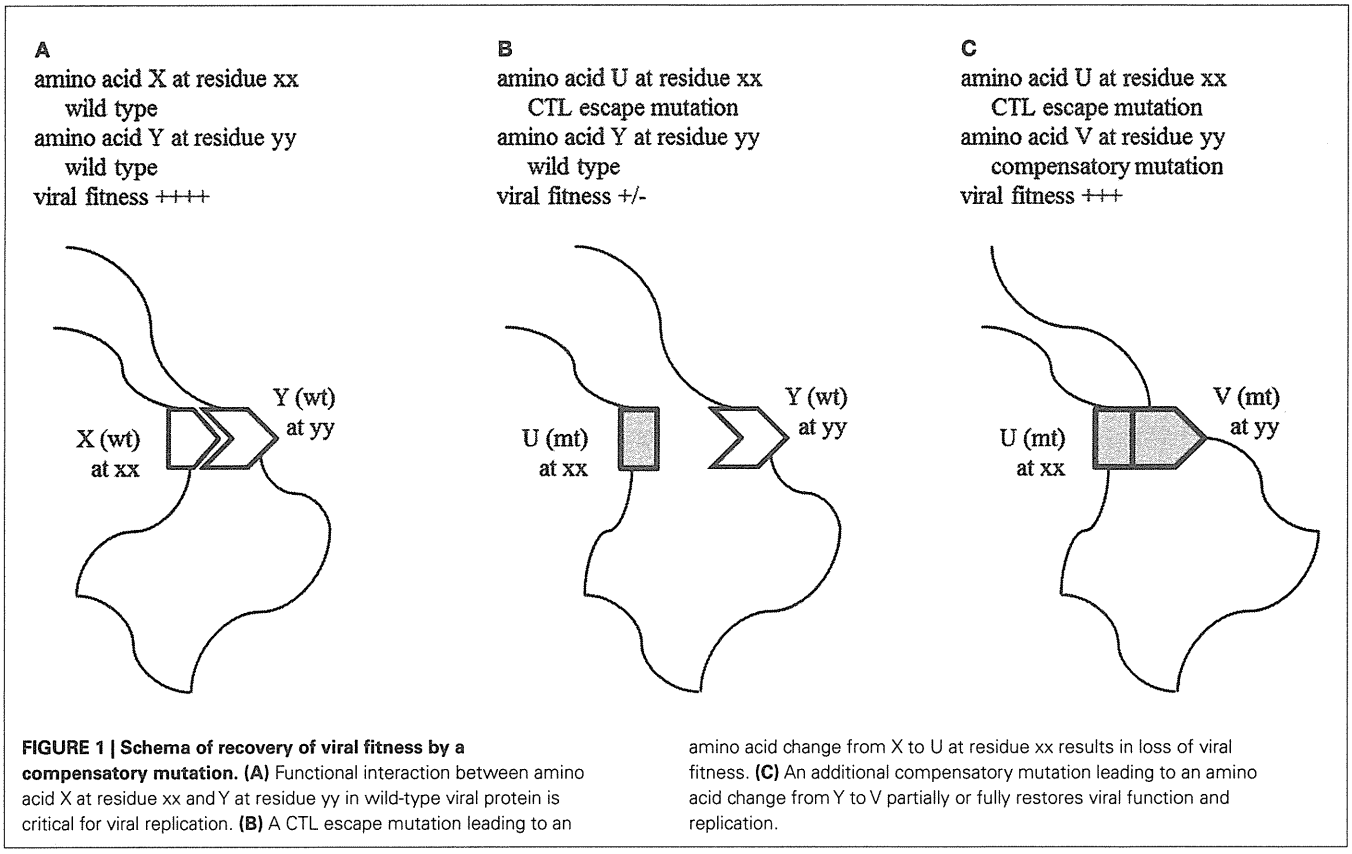

FIGURE 1 | Schema of recovery of viral fitness by a compensatory mutation. (A) Functional interaction between amino acid X at residue xx and Y at residue yy in wild-type viral protein is critical for viral replication. **(B)** A CTL escape mutation leading to an amino acid change from X to U at residue xx results in loss of viral fitness. **(C)** An additional compensatory mutation leading to an amino acid change from Y to V partially or fully restores viral function and replication.

GAG CA INTERMOLECULAR INTERACTION

The Gag CA is comprised of the N-terminal (NTD) and the C-terminal domains (CTD) (Momany et al., 1996; Gamble et al., 1997; Berthet-Colominas et al., 1999). Modeling of CA monomer structure showed that the Gag 205th residue is located in the helix 4 of CA NTD and the 340th is in the loop between helices 10 and 11 of CTD. A possibility of intramolecular contact between Gag residues 205 and 340 is not supported by this modeling. However, CA molecules are known to form hexamer lattice in mature virions (Ganser et al., 1999; Li et al., 2000; Ganser-Pornillos et al., 2007, 2008; Pornillos et al., 2009). Modeling of CA hexamer structure revealed that the Gag 205th residue is located in close proximity to the 340th of the adjacent CA molecule. The molecular model of CA hexamers incorporating the GagD205E substitution suggested shortening of the distance between Gag205 and Gag340 residues, which appeared compensated by GagV340M substitution. Thus, there may be intermolecular interaction between Gag residues 205 and 340 in CA hexamers. This is consistent with our results obtained by viral core stability assay. The core stability was reduced by the GagD205E substitution but recovered by the GagV340M substitution. Loss of viral fitness by GagD205E and its recovery by GagV340M implies a structural constraint for functional interaction between CA NTD and CTD involved in the formation of CA hexamers. In addition to previous reports on intramolecular compensation for loss of viral fitness by CTL escape mutations (Friedrich et al., 2004a; Crawford et al., 2007), our results present evidence indicating intermolecular compensation.

REPLACEMENT OF A CTL ESCAPE MUTATION WITH AN ALTERNATIVE ESCAPE MUTATION TOWARD HIGHER VIRAL FITNESS

As stated above, SIVmac239-infected *90-120-Ia*-positive macaques usually select the $Gag_{206–216}$-specific CTL escape mutation, GagL216S, but not GagD205E in a year postchallenge. After that, however, we found that the GagD205E mutation together with GagV340M became dominant instead of GagL216S in a *90-120-Ia*-positive macaque (Inagaki et al., 2010). In this macaque, neither GagD205E nor GagV340M was detected until week 123 after SIVmac239 challenge, but both became detectable at week 137 and were dominant at week 150. In contrast, the GagL216S mutation dominant until week 123 was undetectable at week 150. Thus, in this animal, SIVmac239Gag216S, whose replicative ability is lower than wild-type SIVmac239 but higher than SIVmac239Gag205E, became dominant under $Gag_{206–216}$-specific CTL pressure in the early phase, while in the later phase, this mutant virus was replaced with SIVmac239Gag205E340M, whose replicative ability is similar with the wild-type. This indicates replacement of a CTL escape mutation with an alternative escape mutation toward higher viral fitness in the chronic phase, implying persistent $Gag_{206–216}$-specific CTL pressure for more than 2 years after selection of the CTL escape mutation.

MULTIPLE VIRAL GENOME CHANGES UNDER CTL PRESSURE

In another study (Kawada et al., 2006), we observed accumulation of multiple CTL escape mutations in viral genomes in SIV-infected macaques. SeV-Gag-vaccinated animals possessing

MHC-I haplotype *90-120-Ia* elicited $Gag_{206-216}$-specific CTL responses and controlled viral replication with rapid selection of the GagL216S mutation after SIVmac239 challenge. Among these SIV controllers, two animals (V3 and V5) accumulated additional *gag* mutations and showed reappearance of plasma viremia around week 60 postchallenge. Both animals first selected a $Gag_{241-249}$ epitope-specific CTL escape mutation leading to a GagD244E (aspartic acid [D] to glutamic acid [E] at the 244th aa in Gag) substitution, and then, a $Gag_{373-380}$ epitope-specific CTL escape mutation leading to a GagA373T (alanine [A] to threonine [T] at the 373rd) or GagP376S (proline [P] to S at the 376th) substitution during the period of viral control. At the viremia reappearance, SIVmac239Gag216S244E247L312V373T with five *gag* mutations, L216S, D244E, I247L (isoleucine [I] to L at the 247th), A312V (A to V at the 312th), and A373T, became dominant in one of them (V5), and SIVmac239Gag145A216S244E376S with four *gag* mutations leading to V145A (V to A at the 145th), L216S, D244E, and P376S became dominant in the other (V3). These viruses with multiple *gag* mutations showed lower replicative ability *in vitro* than SIVmac239Gag216S carrying single GagL216S mutation. Indeed, SIVmac239Gag216S244E247L312V373T carrying five *gag* mutations had lower replicative ability *in vitro* compared to SIVmac239Gag216S244E373T carrying three *gag* mutations. These results suggest that selection of CTL escape mutations even with viral fitness costs could be advantageous for viral replication *in vivo* under CTL pressure.

SIV TRANSMISSION INTO MHC-MISMATCHED HOSTS DRIVES FURTHER VIRAL GENOME CHANGES

Previous studies (Friedrich et al., 2004b; Kobayashi et al., 2005; Loh et al., 2007) reported reversion of CTL escape mutations in the absence of CTL pressure by transmission of SIVs carrying single escape mutations between MHC-mismatched hosts. SIVs carrying CTL escape *gag* mutations selected in *90-120-Ia*-positive macaques showed lower replicative ability *in vitro*. We then examined *in vivo* replicative ability of those SIVs carrying CTL escape mutations in *90-120-Ia*-negative macaques (Seki et al., 2008). Coinoculation of macaques with SIVmac239GagL216S and SIVmac239Gag216S244E373T resulted in rapid selection of the former; i.e., D244E and A373T mutations were undetectable even in the acute phase, indicating lower replicative ability *in vivo* of the latter carrying three escape mutations than the former. Reversion of L216S was observed in a few months, confirming lower replicative ability *in vivo* of SIVmac239Gag216S than wild-type SIVmac239. Further competition indicated lower replicative ability *in vivo* of SIVmac239Gag216S244E247L312V373T carrying five *gag* mutations than SIVmac239Gag216S244E373T carrying three.

We next examined viral genome changes after challenge of *90-120-Ia*-negative macaques with SIVs carrying multiple CTL escape mutations selected in *90-120-Ia*-positive macaques. Challenge with SIVs carrying five *gag* mutations, L216S, D244E, I247L, A312V, and A373T, resulted in persistent viremia in all four *90-120-Ia*-negative macaques. Two animals exhibited higher viral

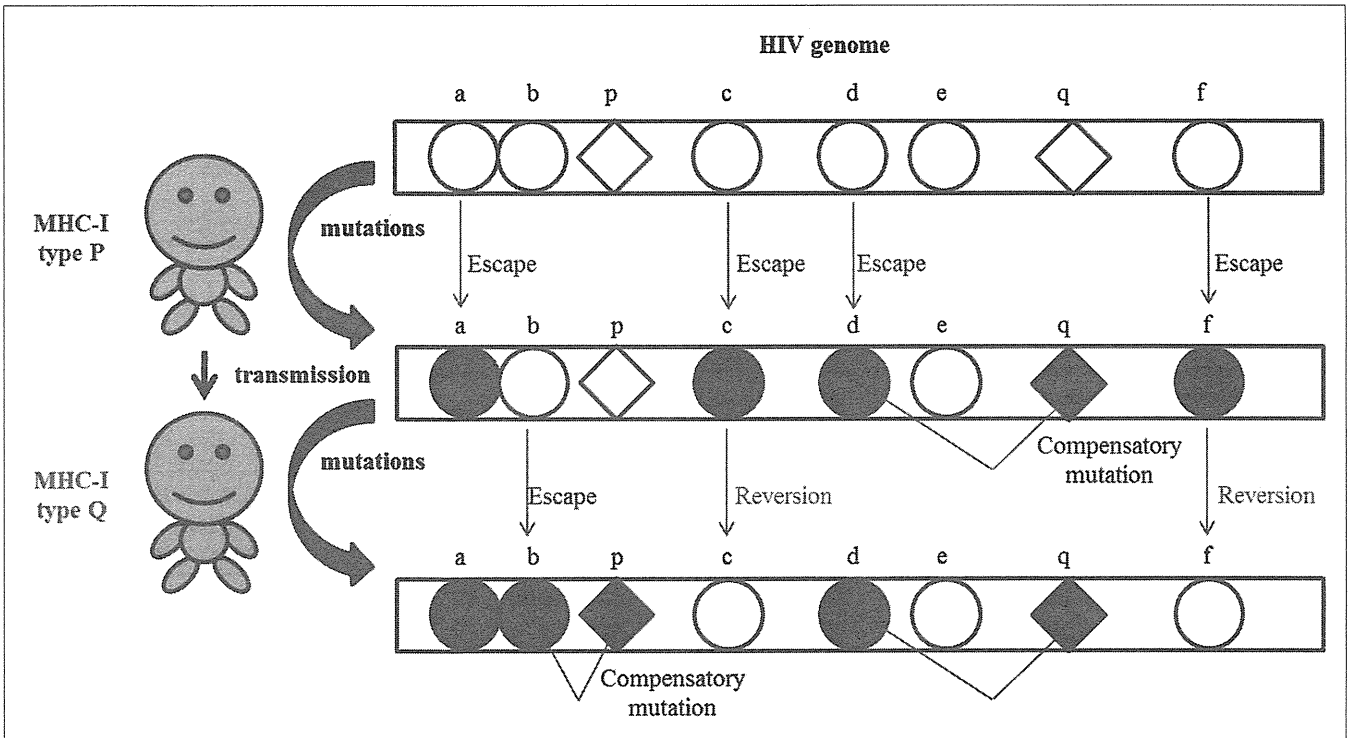

FIGURE 2 | Schema of HIV/SIV transmission resulting in accumulation of multiple viral mutations. Multiple CTL escape mutations resulting in viral fitness costs do not always revert rapidly even in the absence of CTL pressure after their transmission into HLA/MHC-mismatched hosts and such mutants can be transmitted further to other hosts. New escape mutations and compensatory mutations are also observed with transmissions. Thus, CTL affects HIV/SIV evolution in individuals with divergent HLA/MHC polymorphisms.

loads. One of them rapidly developed AIDS at week 18 while the other developed AIDS 2 years postchallenge. The former showed reversion of I247L and A312V but still had three CTL escape mutations, L216S, D244E, and A373T at AIDS onset. The latter showed reversion of four mutations in a year postchallenge, but the A373T mutation remained dominant without reversion until AIDS onset. In the remaining two animals that exhibited lower viral loads, multiple *gag* mutations including L216S and D244E were still dominant without reversion 1 year after challenge.

Thus, in the experiment of challenge with SIVs carrying multiple CTL escape mutations, the reversion of all the mutations was not required for AIDS onset, while transmission with SIVs carrying single CTL escape mutations showed their rapid reversion. This suggests that even HIVs accumulating multiple CTL escape mutations with viral fitness costs can induce persistent viral infection leading to AIDS progression after their transmission into HLA/MHC-mismatched individuals.

The reversion of the L216S mutation was delayed or not observed after challenge with SIVs carrying multiple *gag* mutations, whereas challenge with SIVmac239Gag216S resulted in its reversion in a few months. This may be due to the predominant selection of the reversion of other mutations, compensatory mutations, or to lower viral replication efficiency in the former case. Our results suggest that CTL escape mutations resulting in viral fitness costs may not always revert rapidly after their transmission into MHC-mismatched hosts and can be transmitted further to other hosts, driving further viral genome changes with accumulation of mutations (**Figure 2**). These results provide an important insight into HIV evolution in human individuals with divergent HLA/MHC polymorphisms.

CONCLUDING REMARKS

Cytotoxic T lymphocyte responses exert strong selective pressure on HIV and play a central role in viral evolution (Kaslow et al., 1996; Brander and Walker, 2003; Kiepiela et al., 2004; O'Connor et al., 2004). Correlation of frequencies of viral epitope variants with prevalence of restricting HLA alleles has been shown, indicating HIV adaptation to HLA polymorphisms at a population level (Kawashima et al., 2009). Loss of viral fitness by CTL escape mutations may contribute to HIV control (Martinez-Picado et al., 2006; Schneidewind et al., 2007), but our results indicate the potential of even such HIVs with lower viral fitness to induce AIDS progression. Elucidation of structural constraints of viral antigens for viral function would lead to determination of conserved, escape-resistant epitopes whose mutations largely diminish viral replicative ability (Dahirel et al., 2011), contributing to immunogen design in development of CTL-inducing AIDS vaccines.

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ORIGINAL PAPER

Molecular evolution of immunoglobulin superfamily genes in primates

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Abstract Genes of the immunoglobulin superfamily (IgSF) have a wide variety of cellular activities. In this study, we investigated molecular evolution of IgSF genes in primates by comparing orthologous sequences of 249 IgSF genes among human, chimpanzee, orangutan, rhesus macaque, and common marmoset. To evaluate the non-synonymous/synonymous substitution ratio (ω), we applied Bn-Bs program and PAML program. IgSF genes were classified into 11 functional categories based on the Gene Ontology (GO) database. Among them, IgSF genes in three functional categories, immune system process (GO:0002376), defense response (GO:0006952), and multi-organism process (GO:0051704), which are tightly linked to the regulation of immune system had much higher values of ω than genes in the other GO categories. In addition, we estimated the average values of ω for each primate lineage. Although each primate lineage had comparable average values of ω, the human lineage showed the lowest ω value for the immune-related genes. Furthermore, 11 IgSF genes, *SIGLEC5*, *SLAMF6*, *CD33*, *CD3E*, *CEACAM8*, *CD3G*, *FCER1A*, *CD48*, *CD4*, *TIM4*, and *FCGR2A*, were implied to have been under positive selective pressure during the course of primate evolution. Further sequence analyses of *CD3E* and *CD3G* from 23 primate species suggested that the Ig domains of *CD3E* and *CD3G* underwent the positive Darwinian selection.

Keywords Natural selection · Immune system · Immunoglobulin domain · Comparative genomics · CD3 complex

Introduction

Comparative genomics is a promising approach for studying the biological development of the genome from the view point of evolution. Recently, large-scale genome sequences of human, chimpanzee, orangutan, rhesus macaque, and common marmoset have been made available (Consortium CSaA 2005; Gibbs et al. 2007), and the comparative genomic analyses among primates are crucial for addressing the issue of which genetic changes have made us uniquely human. In addition, such analyses are also useful for identifying the susceptibility genes for human diseases and for understanding the pathophysiological mechanisms of the diseases, because the biological differences among primates, such as differences in the disease susceptibility, have been reported (Lyashchenko et al. 2008; Song et al. 2005).

To identify the genes that have come under the pressure of natural selection in the course of primate evolution is of critical importance, because such genes would very likely be linked to biological function involved in the human

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diseases. In fact, comparisons of genomes between the human and chimpanzee and between the human and rhesus macaque have suggested that dozens of genes have emerged under the pressure of natural selection in the course of primate evolution, in particular, those which are involved in the host–pathogen interactions, reproduction, and sensory systems (Clark et al. 2003; Gibbs et al. 2007; Nielsen et al. 2005). These studies have also reported that the immunoglobulin superfamily (IgSF) genes are commonly observed among the genes in primates, which had come under the pressure of natural selection.

Members of the IgSF are defined by the presence of one or more regions homologous to the basic structural unit of immunoglobin (Ig) molecules. The Ig domain possesses a characteristic Ig fold, which is composed of two opposing anti-parallel beta-strands connected by disulfide bonds between cysteine residues (Halaby and Mornon 1998). The IgSF is a large group of cell surface, cytoplasmic, and serum proteins involved in the recognition, binding, and/or adhesion processes of cells (Lander et al. 2001). Members of the IgSF have a wide variety of cellular functions acting as cell surface antigen receptors, co-receptors and co-stimulatory molecules of the immune system, molecules involved in antigen presentation to lymphocytes, cell adhesion molecules, certain cytokine receptors, and intracellular muscle proteins. They are commonly ascribed to a role in molecular–molecular interactions (Barclay 2003; Lander et al. 2001; Otey et al. 2009).

Although the IgSF genes have been reported as showing evidence of positive selection, the phylogenetic analyses focused on the Ig domains of IgSF genes have not been conducted. The purpose of present study is to provide insights into the overview of the molecular evolution of the IgSF genes and to identify the IgSF genes under the positive selection in the course of five primate species.

Materials and methods

Sequence data collection

Selection of the IgSF genes was done by using the Conserved Domain Database v2.22 at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez). As in the previous studies (Gibbs et al. 2007; Kosiol et al. 2008), we identified orthologous genes for human IgSF genes from chimpanzee, orangutan, rhesus macaque, and common marmoset by using the UCSC/MULTIZ alignment program which is constructed by the synteny-based genome-wide multiple alignments (Blanchette et al. 2004; Kent et al. 2003). Sequence alignment was done by using the Clustal X program (Larkin et al. 2007). IgSF genes were classified based on the Gene Ontology (GO) database (http://www.geneontology.org/) (Ashburner et al. 2000).

Primate genomic DNA samples

DNA samples from 23 primate species including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), black gibbon (*Hylobates concolor*), white-handed gibbon (*Hylobates lar*), siamang (*Symphalangus syndactylus*), rhesus macaque (*Macaca mulatta*), crab-eating macaque (*Macaca fascicularis*), baboon (*Papio hamadryas*), black and white colobus (*Colobus guereza*), dusky lutong (*Trachypithecus obscurus*), silvered lutong (*Trachypithecus cristatus*), Central American spider monkey (*Ateles geoffroyi*), long-haired spider monkey (*Ateles belzebuth*), tufted capuchin (*Cebus apella*), common squirrel monkey (*Saimiri sciureus*), red-handed tamarin (*Saguinus midas*), cotton-top tamarin (*Saguinus oedipus*), golden lion tamarin (*Leonthopithecus rosalia*), common marmoset (*Callithrix jacchus*), and lesser galago (*Galago senegalensis*) were analyzed for *CD3E* and *CD3G* sequences.

PCR and sequencing analysis of *CD3E* and *CD3G*

Sequence information for coding regions of *CD3E* and *CD3G* were obtained by direct sequencing of gene segments amplified by polymerase chain reaction (PCR) from the genomic DNA samples. Primers for PCR were designed in the highly conserved non-coding regions among the genes from human, chimpanzee, orangutan, rhesus macaque, and common marmoset, referring the genomic sequences deposited in the UCSC Genome Browser (electronic supplementary material (ESM) Table 1). Primers for prosimian were designed by referencing the common marmoset sequences and whole-genome shotgun sequences from prosimians in NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers were used for both PCR and direct sequencing analyses of the genes. When sequence variations (heterozygous sequences) in specific species were detected, the sequences which were conserved among 23 primate species were considered as ancestral sequences and used in the statistical analyses.

PCR was performed in a reaction mixture of 15 μL containing 0.1 μL Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 1 μL of 50 ng/μL DNA template, 1.5 μL of 2.0 mM dNTPs, 0.5 μL of 10 μM each primer, 1.5 μL reaction buffer containing 20 mM $MgCl_2$, and sterile water. PCR condition was as follows: 94°C for 2 min, 35 cycles (94°C for 30 s, 55–60°C for 30 s, 72°C for 1 min), and 72°C for 5 min. PCR products were then purified and sequenced by the BigDye Terminator cycling system using an ABI3130× automated DNA sequencer (Applied Biosystems,