spanning the entire SIVmac239 Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Nef, and Env amino acid sequences. Intracellular IFN-γ staining was performed with a Cytofix-Cytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4 (Becton Dickinson), peridinin chlorophyll protein-conjugated anti-human CD8 (Becton Dickinson), allophycocyanin-conjugated anti-human CD3 (Becton Dickinson), and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (BioLegend, Tokyo, Japan). Specific CD8$^+$ T-cell frequencies were calculated by subtracting nonspecific IFN-γ^+ CD8$^+$ T-cell frequencies from those after peptide-specific stimulation. Specific CD8$^+$ T-cells counts of less than 100 per million PBMCs were considered negative.

RESULTS

In the present study, we used eight Burmese rhesus macaques consisting of four animals possessing MHC-I haplotype *90-010-Ie*, two possessing *89-075-Iw*, and two possessing *91-010-Is*. After a SIVmac239 challenge, all these animals failed to control viral replication and had high set-point plasma viral loads (geometric mean: 3×10^5 copies/mL) (Fig. 1).

We examined SIV-specific CD8$^+$ T cell responses at week 2 and week 6 or 12 after SIV challenge in these animals by detection of specific IFN-γ induction after stimulation using peptide mixtures (Figs. 2 and 3). At week 6 or 12, we examined CD8$^+$ T cell responses specific for the N-terminal half of Gag (Gag-N), the C-terminal half of Gag (Gag-C), Vif, Nef, the N-terminal half of Pol (Pol-N), the C-terminal half of Pol (Pol-C), Vpx, Vpr, the N-terminal half of Env (Env-N), the C-terminal half of Env (Env-C), Tat, and Rev. At week 2, however, we examined only Gag-N-, Gag-C-, Vif- and Nef-specific CD8$^+$ T cell responses because of limited availability of PBMCs.

In the first group of macaques, which possessed *90-010-Ie*, neither Gag- nor Vif-specific CD8$^+$ T cell responses were induced efficiently at week 2 (Fig. 2). Even at week 12, these responses were undetectable in most of the animals. In contrast, Nef-specific CD8$^+$ T cell responses were detected at week 2, 6, or 12 in all four animals. Env-specific CD8$^+$ T cell responses were detectable at week 12 in three of them. These results indicate that, during primary SIV infection in *90-010-Ie*-positive macaques, Gag- or Vif-specific CD8$^+$ T cell responses are not induced, however Nef-specific CD8$^+$ T cell responses are.

In the second group of macaques, which possessed *89-075-Iw*, Gag- and Vif-specific CD8$^+$ T cell responses were elicited efficiently (Fig. 3a). In the third group of macaques, which possessed *91-010-Is*, Gag-, Vif- and Nef-specific CD8$^+$ T cell responses were elicited efficiently (Fig. 3b). Other SIV antigen-specific CD8$^+$ T cell responses were not efficiently induced in these two groups except for Tat-specific CD8$^+$ T cell responses in macaque

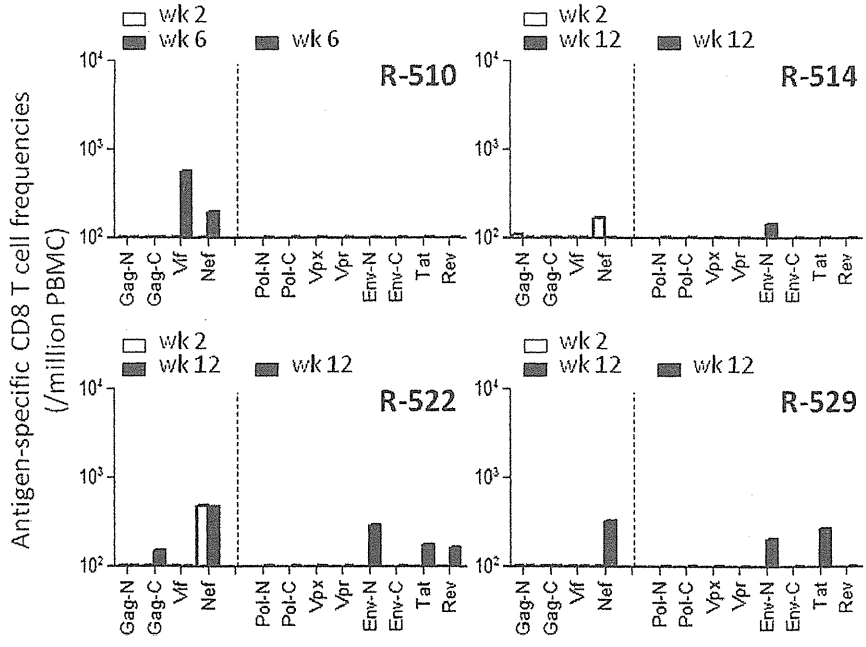

Fig. 2. SIV antigen-specific CD8$^+$ T cell frequencies in the first group of macaques, which possessed *90-010-Ie*. Gag-, Vif- and Nef-specific CD8$^+$ T cell frequencies at week 2 and Gag-, Vif-, Nef-, Pol-, Vpx-, Vpr-, Env-, Tat- and Rev-specific CD8$^+$ T cell frequencies at weeks 6 or 12 are shown.

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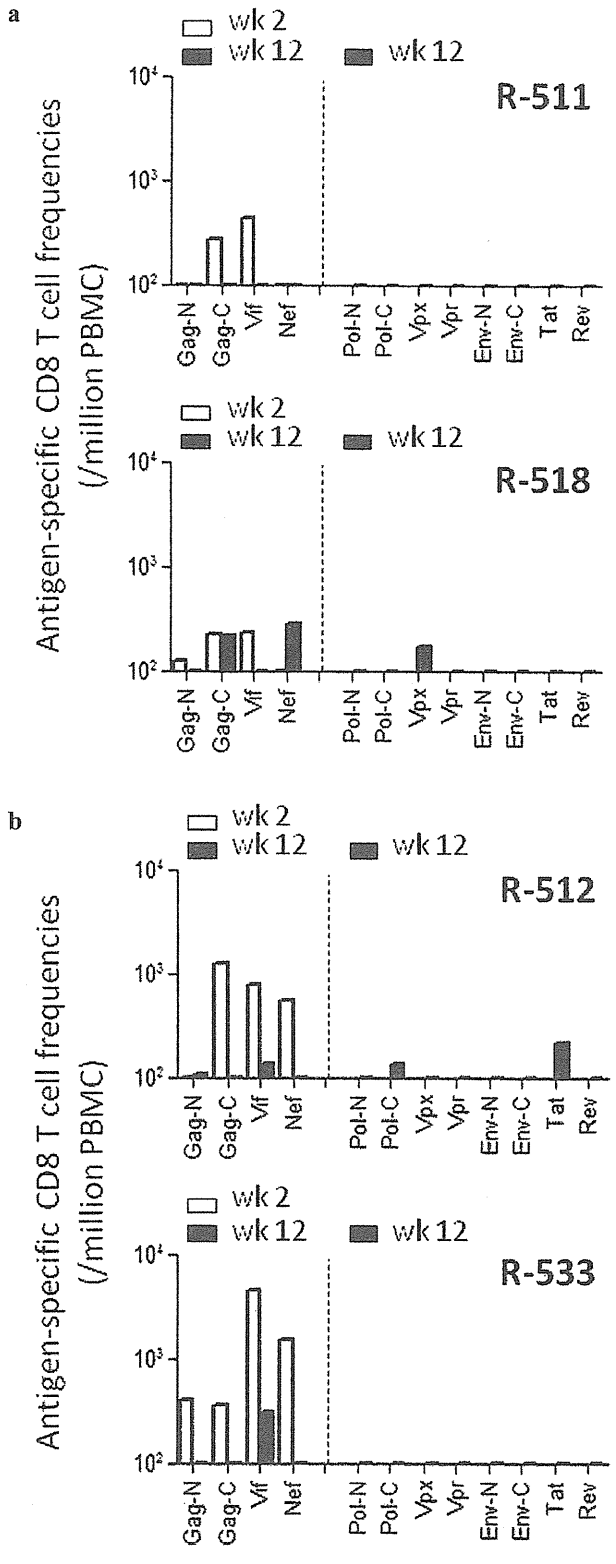

Fig. 3. SIV antigen-specific CD8$^+$ T cell frequencies in (a) the second group of macaques, which possessed *89-075-Iw* and (b) the third, which possessed *91-010-Is*.

R-512. Thus, in the four animals possessing *89-075-Iw* or *91-010-Is*, Gag- or Vif-specific CD8$^+$ T cell responses were induced more efficiently than Nef-specific ones at week 2. These responses in PBMCs were mostly diminished at week 12; possibly reflecting the considerable CTL consumption in the effector sites in animals with high viral loads.

DISCUSSION

Previous studies have indicated the potential of Gag-specific CTL responses to suppress HIV/SIV replication *in vivo* (12, 13, 16). Further, our recent study suggested the potency of Vif-specific CTL responses (17). Then, in the present study, we examined Gag- and Vif-specific CTL responses during primary SIV infection in three groups of animals, each group having a different MHC-I haplotype. Although the numbers of CTL frequencies differed between groups, the CTL responses tended have similar patterns.

Our previous study showed vaccine efficacy in a group of macaques with the MHC-I haplotype *90-120-Ia* (15, 16). Unvaccinated *90-120-Ia*-positive macaques predominantly induce Gag-specific CTL responses but fail to control viremia, while vaccinated ones show enhanced Gag-specific CTL responses and control SIV replication. $Gag_{206–216}$ epitope-specific and $Gag_{241–249}$ epitope-specific CTL responses were shown to be responsible for this vaccine-based viral control (16). However, some Gag-specific CTLs may be effective while others are not. Further analysis of this type of vaccine efficacy would contribute to understanding the requisites for vaccine-based viral control. Possibly, the *89-075-Iw*-positive or *91-010-Is*-positive animals presented in this study may be a candidate model for such analysis.

In primary SIVmac239 infection, it is speculated that some MHC-I haplotypes (referred to as type 1) are associated with Gag/Vif-specific CTL responses while others (referred to as type 2) are not. The MHC-I haplotype *90-120-Ia* described above belongs to type 1. In the present study, the second group, which possess MHC-I haplotype *89-075-Iw*, and the third, which possess *91-010-Is*, both showed efficient Gag- and Vif-specific CTL responses in primary SIV infection, although it remains undetermined whether these MHC-I haplotypes belong to type 1. In contrast, the first group of macaques, which possess MHC-I haplotype *90-010-Ie* did not show efficient Gag- or Vif-specific CTL responses in primary SIV infection. Instead, Nef-specific CTL responses were induced in all four animals. This suggests that the MHC-I haplotype *90-010-Ie* belongs to type 2; that is, primary SIV infection induces no predominant CTL responses specific for Gag/Vif epitopes

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restricted by *90-010-Ie*-derived MHC-I molecules. Our results imply that CTLs exerted selective pressure on SIV *gag* and *vif* in the second/third groups but not in the first group. Larger number of animals would enable us to compare those with type 1 and 2 MHC-I haplotypes, which would contribute to our understanding of the efficacy of Gag- and Vif-specific CTL responses against SIV infection.

In developing a prophylactic CTL-inducing AIDS vaccine, it would be important to induce CTL memory resulting in potent CTL responses post-HIV exposure, while prophylactic vaccination can affect the immunodominance patterns of CTL responses post-viral exposure (23, 24). Gag- and Vif-specific CTL memory induction may be a promising vaccine strategy, but the influence of prophylactic vaccination on the patterns of CTL responses post-viral exposure would be affected by MHC-I genotypes. In the hosts in which Gag- and Vif-specific CTL responses are induced during the natural course of SIV infection, Gag- and Vif-specific CTL memory induction by prophylactic vaccination would predominantly enhance these CTL responses. In contrast, in those in whom no Gag- or Vif-specific CTL responses occurred during the natural course of SIV infection, prophylactic vaccination inducing Gag- and Vif-specific CTL responses would result in broader CTL responses. Macaques in which both MHC-I haplotypes belong to type 2 may be ideal for evaluation of this type of vaccine efficacy, but it is very difficult to accumulate those animals. It would be reasonable to use groups of macaques possessing type 2 haplotypes such as the group 1 (*90-010-Ie*-positive macaques) presented in this study for such evaluation.

In summary, by focusing on Gag- and Vif-specific CTL responses, we found two types of rhesus macaques that showed different patterns of CTL responses during primary SIV infection; one elicited Gag- and Vif-specific CTL responses but the other did not. Accumulated analyses in both types of animals would contribute to understanding the impact of these potent CTL responses on primary SIV infection.

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Intranasal Sendai viral vector vaccination is more immunogenic than intramuscular under pre-existing anti-vector antibodies

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ABSTRACT

Viral vectors are promising vaccine tools for eliciting potent cellular immune responses. Pre-existing anti-vector antibodies, however, can be an obstacle to their clinical use in humans. We previously developed a Sendai virus (SeV) vector vaccine and showed the potential of this vector for efficient CD8+ T-cell induction in macaques. Here, we investigated the immunogenicity of SeV vector vaccination in the presence of anti-SeV antibodies. We compared antigen-specific CD8+ T-cell responses after intranasal or intramuscular immunization with a lower dose (one-tenth of that in our previous studies) of SeV vector expressing simian immunodeficiency virus Gag antigen (SeV-Gag) between naive and pre-SeV-infected cynomolgus macaques. Intranasal SeV-Gag immunization efficiently elicited Gag-specific CD8+ T-cell responses not only in naive but also in pre-SeV-infected animals. In contrast, intramuscular SeV-Gag immunization induced Gag-specific CD8+ T-cell responses efficiently in naive but not in pre-SeV-infected animals. These results indicate that both intranasal and intramuscular SeV administrations are equivalently immunogenic in the absence of anti-SeV antibodies, whereas intranasal SeV vaccination is more immunogenic than intramuscular in the presence of anti-SeV antibodies. It is inferred from a recent report investigating the prevalence of anti-SeV antibodies in humans that SeV-specific neutralizing titers in more than 70% of people are no more than those at the SeV-Gag vaccination in pre-SeV-infected macaques in the present study. Taken together, this study implies the potential of intranasal SeV vector vaccination to induce CD8+ T-cell responses even in humans, suggesting a rationale for proceeding to a vaccine clinical trial using this vector.

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1. Introduction

Virus-specific CD8+ T-cell responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication [1–6]. Efficient induction of virus-specific CD8+ T-cell responses is an important strategy for AIDS vaccine development, and recombinant viral vectors are promising vaccine tools for CD8+ T-cell induction [7,8]. Recent studies have indicated the potential of prophylactic viral vector immunization to induce virus-specific CD8+ T-cell responses and reduce postchallenge viral loads in macaque AIDS models [9–13]. Most of the parental or related viruses of these vectors can induce natural infection in humans. Thus, pre-existing antibodies against the vector virus itself could be an obstacle to viral vector-based CD8+ T-cell induction in humans. Indeed, a clinical trial of a vaccine using adenovirus serotype 5 (AdV5) vectors has shown reduction in efficiency of vaccine-based CD8+ T-cell induction in people with pre-existing anti-AdV5 antibodies [14–17].

We previously developed an AIDS vaccine using a recombinant Sendai virus (SeV) vector and showed that intranasal SeV vector immunization results in efficient induction of antigen-specific CD8+ T-cell responses in macaques [9,18,19]. SeV, murine parainfluenza virus type 1 (PIV-1), is an enveloped virus with a negative-sense RNA genome. SeV replication is localized in the airway because it requires a protease localized in the airway epithelium for envelope protein processing [20]. Thus, replication-competent SeV vectors [21] have been administered intranasally, while replication-defective SeV vectors [22] may be administered intramuscularly as well as intranasally. However, we have not

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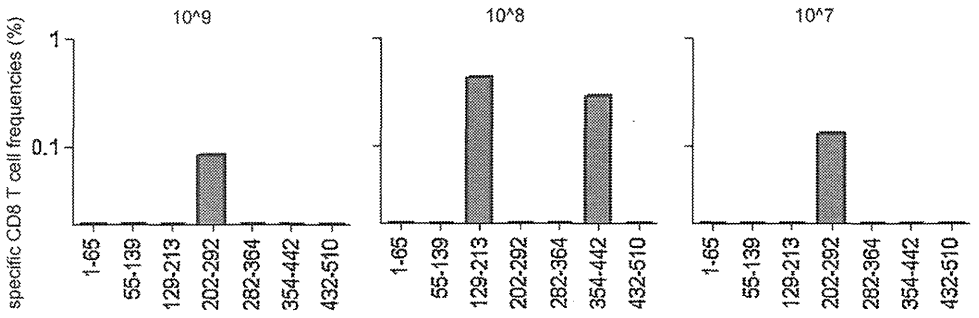

Fig. 1. Gag-specific CD8⁺ T-cell responses after intranasal boost with lower doses of F(−)SeV-Gag. Cynomolgus macaques received a DNA vaccination, and six weeks later, were intranasally boosted with 6×10^9 ($10\hat{9}$), 6×10^8 ($10\hat{8}$), or 6×10^7 ($10\hat{7}$) CIU of F(−)SeV-Gag, respectively. Gag peptide pool-specific CD8⁺ T-cell frequencies (percent in CD8⁺ T lymphocytes) two weeks after the boost are shown. A panel of overlapping peptides spanning the entire SIV Gag amino acid (aa) sequence was divided into 7 pools, 1–65 (corresponding to the 1st–65th aa in SIV Gag), 55–139 (55th–139th aa), 129–213 (129th–213th aa), 202–292 (202nd–292nd aa), 282–364 (282nd–364th aa), 354–442 (354th–442nd aa), and 432–510 (432nd–510th aa), and used for the stimulation to detect peptide pool-specific CD8⁺ T cells, respectively.

yet examined the immunogenicity of intramuscular SeV vector vaccination.

The natural host of SeV is mice and its natural infection has not been observed in primates including humans [20]. Antibodies against human PIV-1 (hPIV-1), whose natural infection frequently occurs in humans, are known to cross-react with SeV [23,24]. Our recent analyses in macaques showed efficient Gag-specific CD8⁺ T-cell induction by an intranasal immunization with 6×10^9 CIU of F(−)SeV-Gag more than one year after an initial SeV vector inoculation, suggesting a possibility of antigen-specific CD8⁺ T-cell induction by SeV vector administration in the presence of SeV-specific neutralizing antibody (NAb) responses [25,26]. However, it remains unclear to what extent SeV-specific NAbs could have adverse effect on CD8⁺ T-cell induction by SeV vector vaccination.

In the present study, we investigated antigen-specific CD8⁺ T-cell responses after intranasal or intramuscular immunization with a lower dose of SeV vector in macaques pre-infected with SeV to sensitively examine the effect of pre-SeV-infection on SeV-based CD8⁺ T-cell induction. Our results revealed that intranasal SeV administration is more immunogenic than intramuscular in the presence of anti-SeV NAbs and suggested the potential of this vector to induce antigen-specific CD8⁺ T-cell responses even in humans.

2. Materials and methods

2.1. Animal experiments

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation with the help of the Corporation for Production and Research of Laboratory Primates. All animals were maintained in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases. Blood collection and vaccination were performed under ketamine anesthesia. Cynomolgus macaques (*Macaca fascicularis*) of the TPRC breeding colonies derived from Indonesia, Malaysia, and the Philippines were used for this experiment. All animals received a DNA vaccine followed by a single boost with a replication-defective (non-transmissible) F-deleted SeV expressing SIVmac239 Gag, F(−)SeV-Gag, as described previously [9]. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA [27] and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV-1 chimeric Vpr, and HIV-1 Tat and Rev [19]. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals intranasally or intramuscularly received a single boost with 6×10^7, 6×10^8, or 6×10^9 cell infectious units (CIU) of F(−)SeV-Gag [22,28]. Group II and IV animals were intranasally infected with 1×10^8 CIU of replication-competent (transmissible) V-knocked-out SeV [18,21] nine weeks before the DNA prime.

2.2. Measurement of Gag-specific CD8⁺ T-cell responses

We measured Gag-specific CD8⁺ T-cell levels by flow-cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously [9]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) pulsed with peptide pools using panels of 117 overlapping peptides (mostly 15-mer) spanning the entire SIVmac239 Gag amino acid sequences [25] (Fig. 1) or a vaccinia virus vector expressing SIVmac239 Gag (Figs. 3 and 4) for Gag peptide pool-specific or Gag-specific stimulation. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (BD, Tokyo, Japan) and the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (BD, #556615, M-T477), peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD, #347314, SK1), allophycocyanin (APC)-conjugated anti-human CD3 (BD, #557597, SP34-2), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (BD, #557074, 4S.B3). Specific CD8⁺ T-cell levels were calculated by subtracting non-specific IFN-γ⁺ CD8⁺ T-cell frequencies from those after Gag peptide pool-specific or Gag-specific stimulation. Specific CD8⁺ T-cell levels less than 0.02% of CD8⁺ T lymphocytes were considered negative.

2.3. Measurement of anti-SeV IgG levels

The plasma anti-SeV immunoglobulin G (IgG) levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Denka Seiken, Tokyo, Japan) using whole inactivated SeV (HVJ Z strain) particles and a peroxidase-conjugated anti-monkey IgG antibody [29].

2.4. Measurement of anti-SeV neutralizing titers

We measured plasma SeV-specific neutralizing titers on LLC-MK2 cells using a recombinant SeV expressing enhanced green

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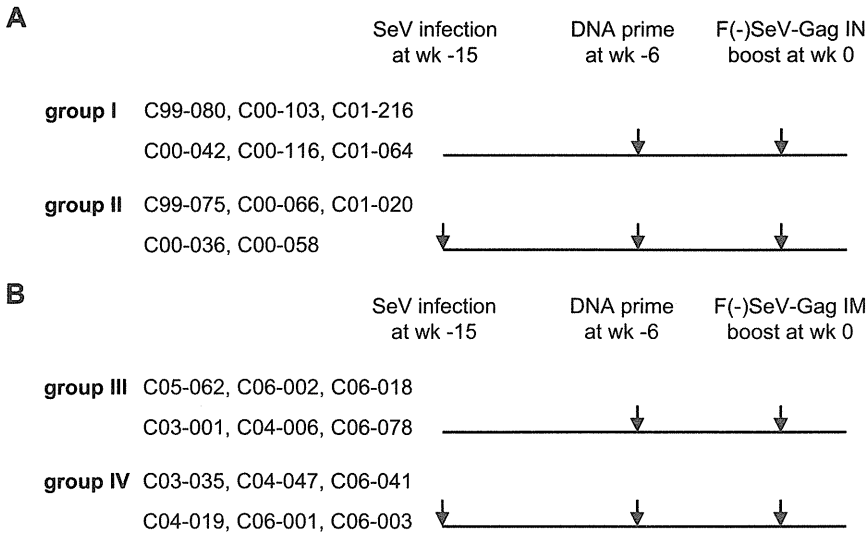

Fig. 2. Experimental protocols. (A) Groups I and II with intranasal F(−)SeV-Gag boost. Groups I ($n=6$) and II ($n=5$) received a DNA prime followed by an intranasal F(−)SeV-Gag boost. Group II animals were infected intranasally with SeV fifteen weeks before the boost. (B) Groups III and IV with intramuscular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

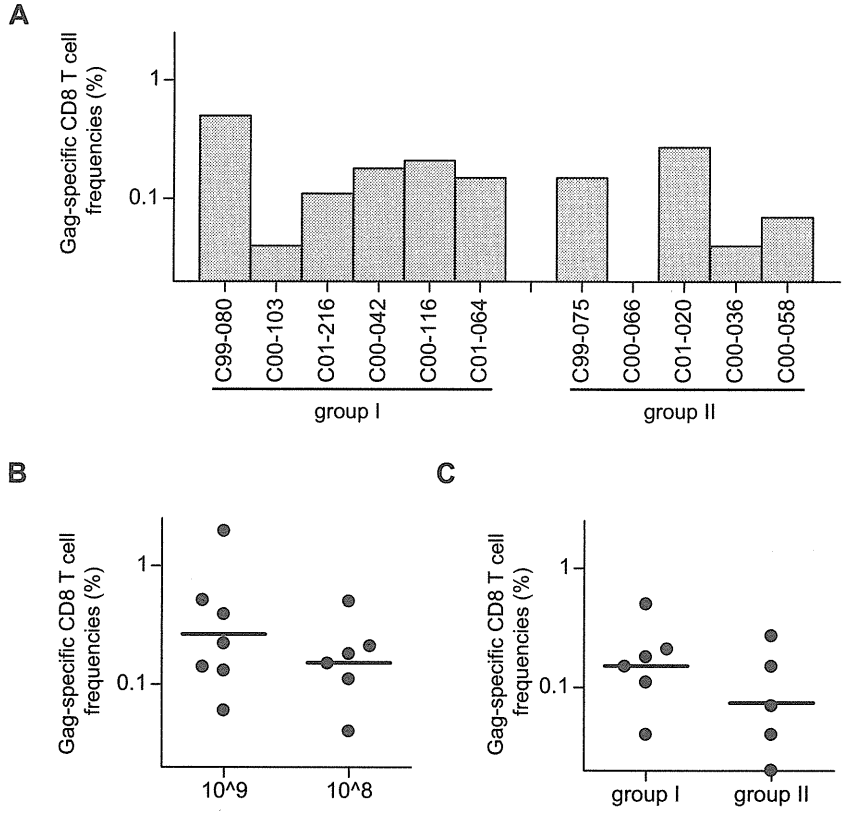

Fig. 3. Gag-specific $CD8^+$ T-cell frequencies after intranasal F(−)SeV-Gag boost in naive and pre-SeV-infected cynomolgus macaques. Gag-specific $CD8^+$ T-cell responses were examined by detection of IFN-γ induction after stimulation by B-LCLs infected with a vaccinia virus vector expressing SIVmac239 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).

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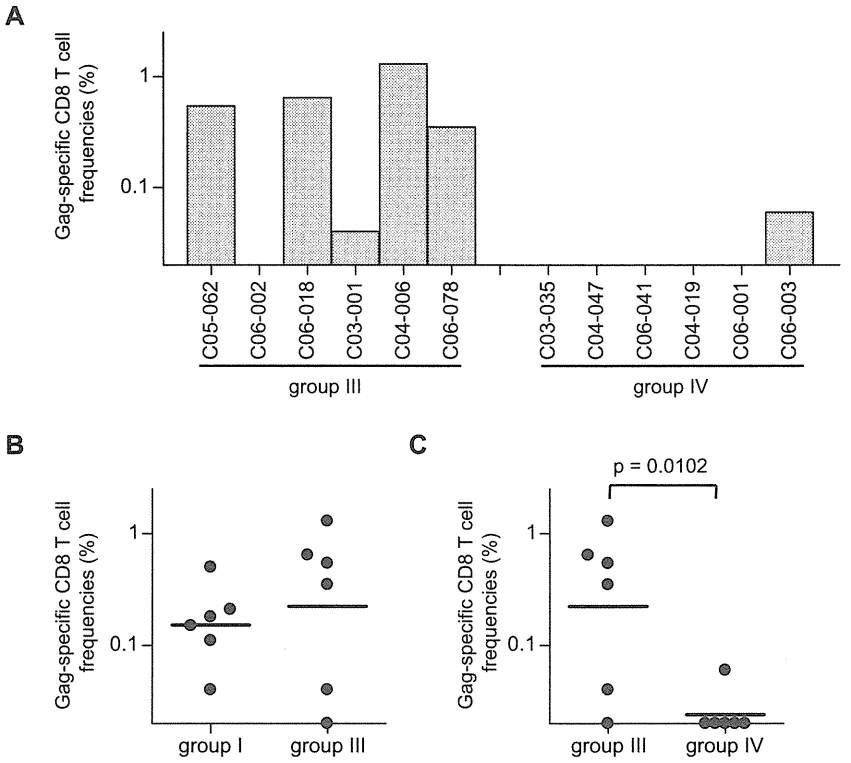

Fig. 4. Gag-specific CD8⁺ T-cell frequencies after intramuscular F(−)SeV-Gag boost in naive and pre-SeV-infected cynomolgus macaques. (A) Gag-specific CD8⁺ T-cell frequencies (percent in CD8⁺ T lymphocytes) two weeks after the boost in groups III and IV. (B) Comparison of Gag-specific CD8⁺ T-cell frequencies 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

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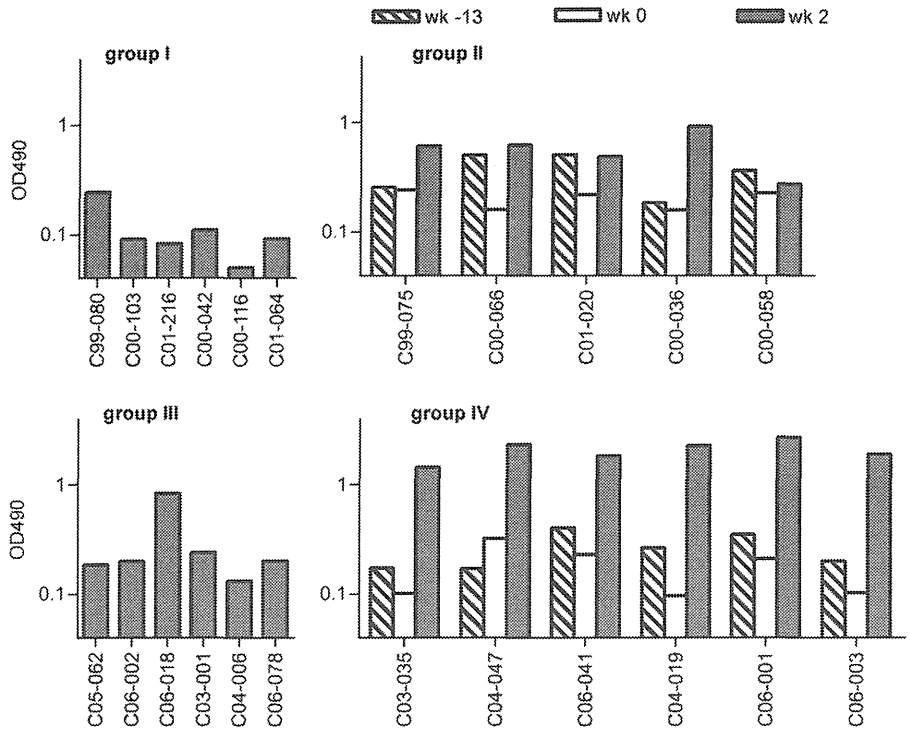

Fig. 5. SeV-specific IgG levels in plasma. Plasma samples obtained from group I and III animals at week 2 and those from group II and IV animals at weeks −13, 0 and 2 were diluted by 1/5000 and subjected to ELISA assay. OD490, optical density at 490 nm.

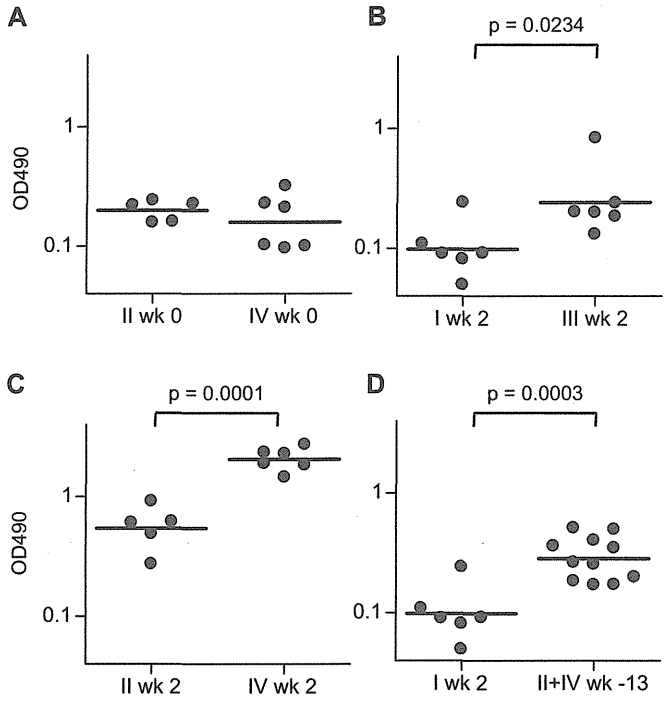

Fig. 6. Comparison of plasma SeV-specific IgG levels among groups. (A) Comparison of plasma SeV-specific IgG levels at week 0, just before F(−)SeV-Gag boost, in groups II and IV (geometric means: 0.199 in group II and 0.159 in group IV). (B) Comparison of plasma SeV-specific IgG levels at week 2, two 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.

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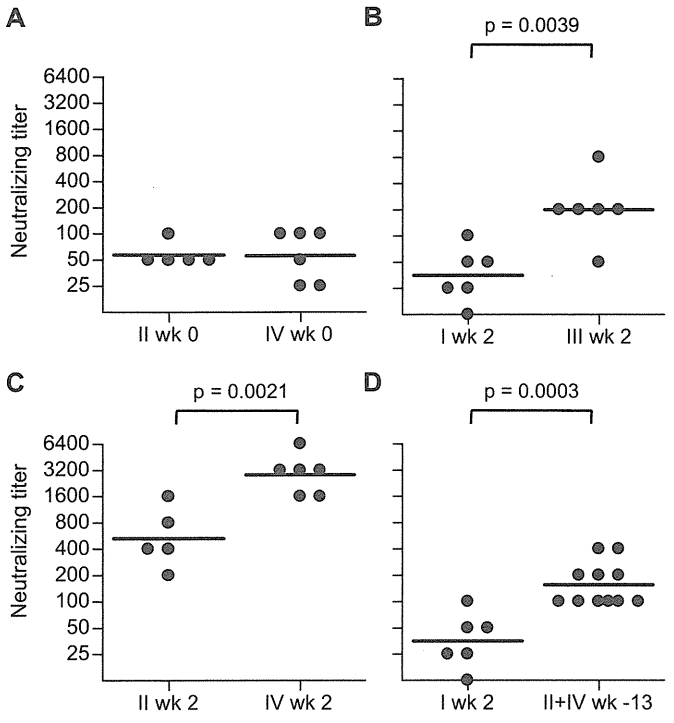

Fig. 7. Comparison of plasma SeV-specific NAb titers among groups. (A) Comparison of plasma SeV-specific NAb titers at week 0 in groups II and IV (geometric means: 5.7×10^1 in group II and 5.6×10^1 in group IV). (B) Comparison of plasma SeV-specific NAb titers at week 2 in groups I and III (geometric 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 post-challenge 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

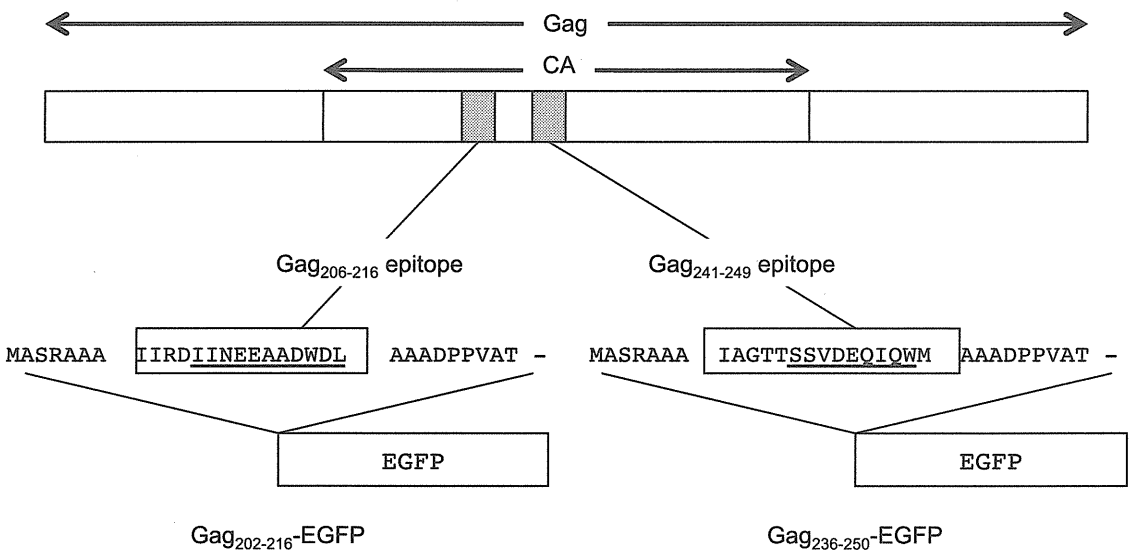

FIG 1 Schema of the cDNA constructs encoding $Gag_{202-216}$-EGFP and $Gag_{236-250}$-EGFP fusion proteins. A DNA fragment that encodes a 31-mer peptide (boxes) including the $Gag_{202-216}$ or $Gag_{236-250}$ sequence (underlining) was introduced into the 5′ end of the EGFP cDNA.

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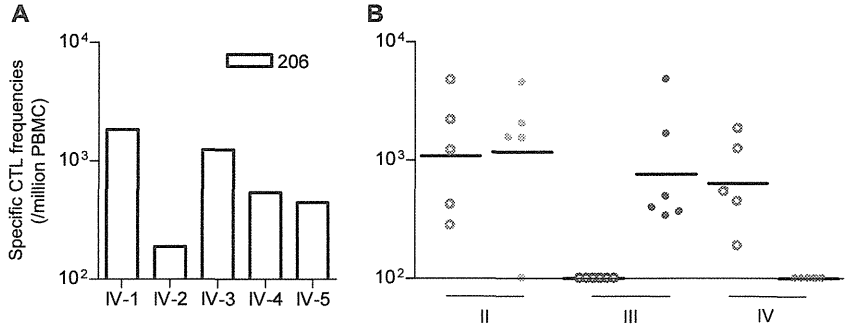

FIG 2 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after prophylactic vaccination. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies 1 week after SeV-$Gag_{202-216}$–EGFP boost in group IV macaques (open boxes). (B) $Gag_{206-216}$-specific (open circles) and $Gag_{241-249}$-specific (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-

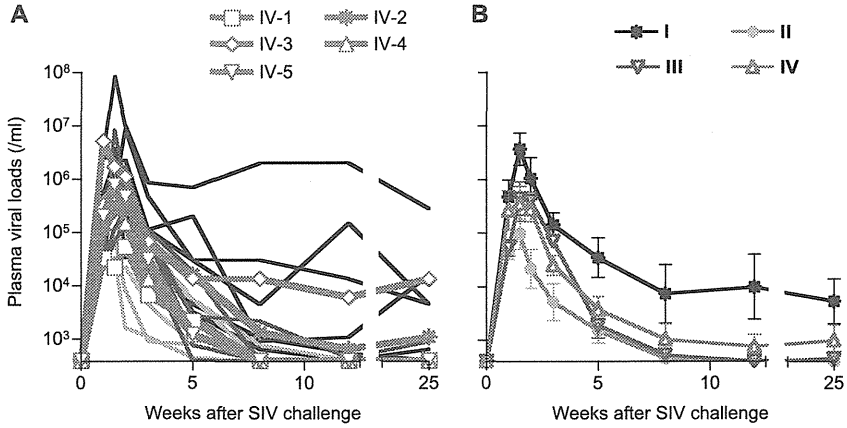

FIG 3 Plasma viral loads after SIVmac239 challenge. The plasma viral loads in group I, group II, group III, and group IV animals were determined as described previously (21). The lower limit of detection was approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) 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).

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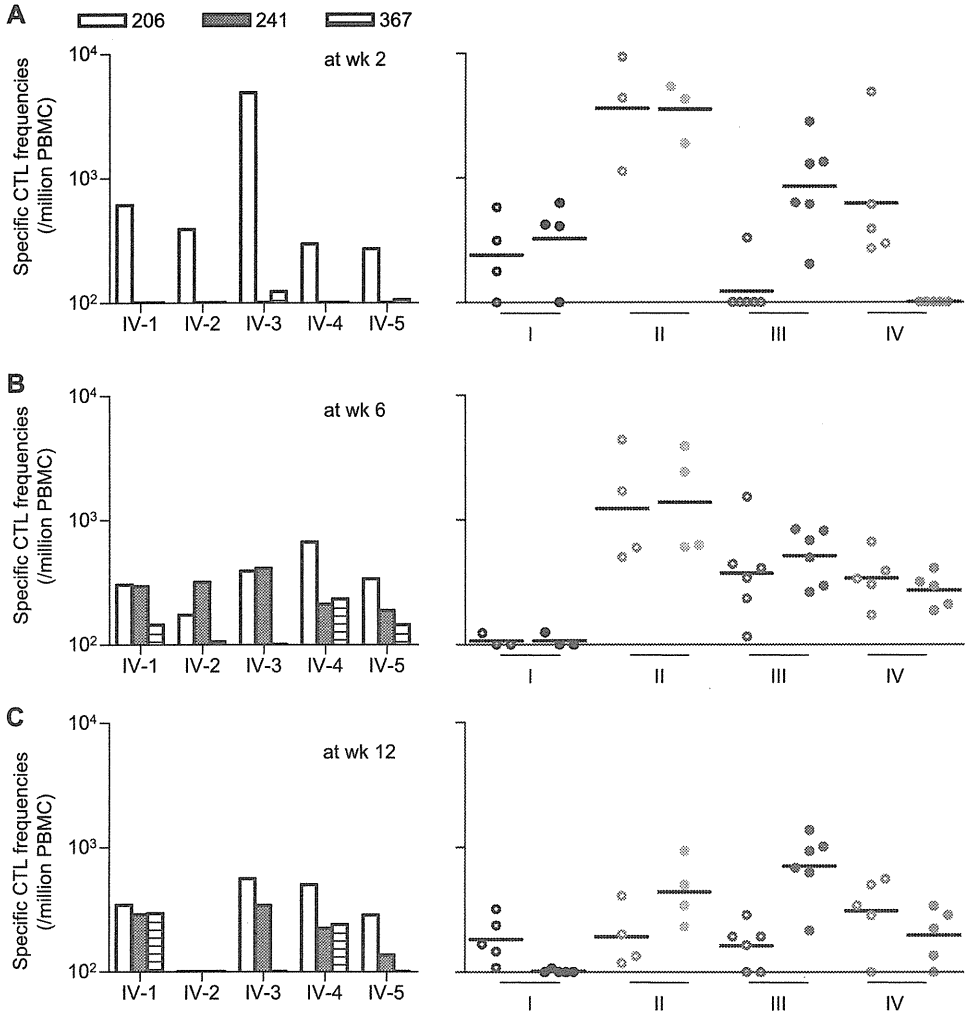

FIG 4 $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIVmac239 challenge. CTL responses at week 2 (A), week 6 (B), and week 12 (C) are shown. In the graphs on the left, $Gag_{206\text{-}216}$-specific (open boxes), $Gag_{241\text{-}249}$-specific (closed boxes), 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-

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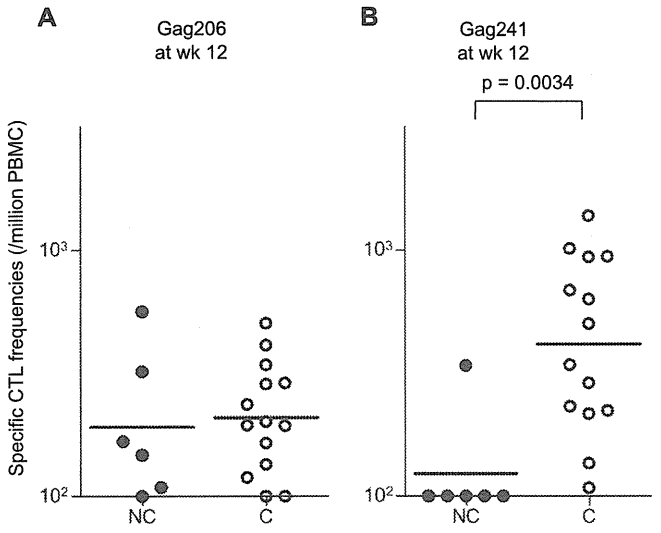

FIG 5 Comparison of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL responses in noncontrollers and controllers at week 12. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies in noncontrollers (NC; closed circles) and controllers (C; open circles). (B) $Gag_{241-249}$-specific $CD8^+$ T-cell 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 Wk 5	206–216 Wk 12	241–249 Wk 5	241–249 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.

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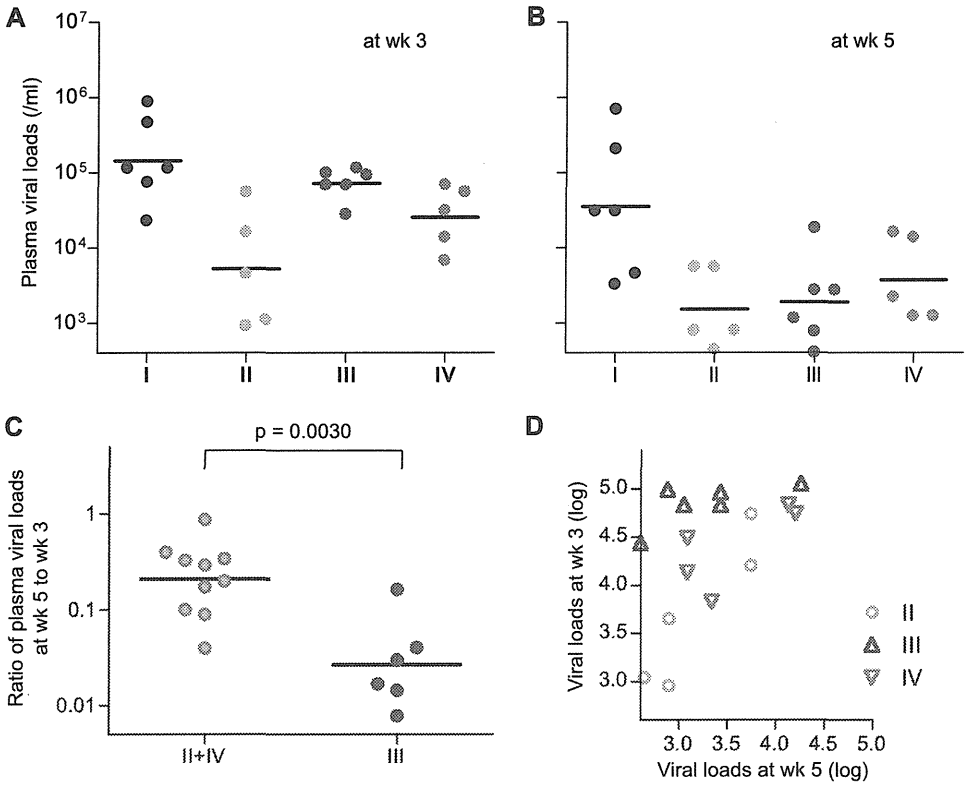

FIG 6 Comparison of plasma viral loads at weeks 3 and 5 among four groups. (A) Plasma viral loads at week 3 in group I, II, III, and IV animals. (B) Plasma viral loads at week 5 in group I, II, III, and IV animals. (C) Comparison of ratios of plasma viral loads at week 5 to week 3 in group II+IV animals 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.

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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.