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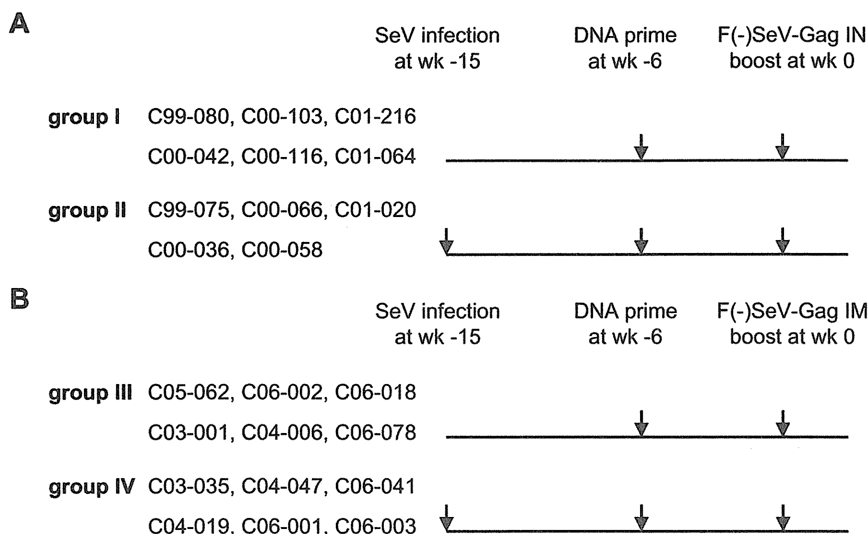

Fig. 2. Experimental protocols. (A) Groups I and II with intranasal F(−)SeV-Gag boost. Groups I ($n=6$) and II ($n=5$) received a DNA prime followed by an intranasal F(−)SeV-Gag boost. Group II animals were infected intranasally with SeV fifteen weeks before the boost. (B) Groups III and IV with intramuscular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

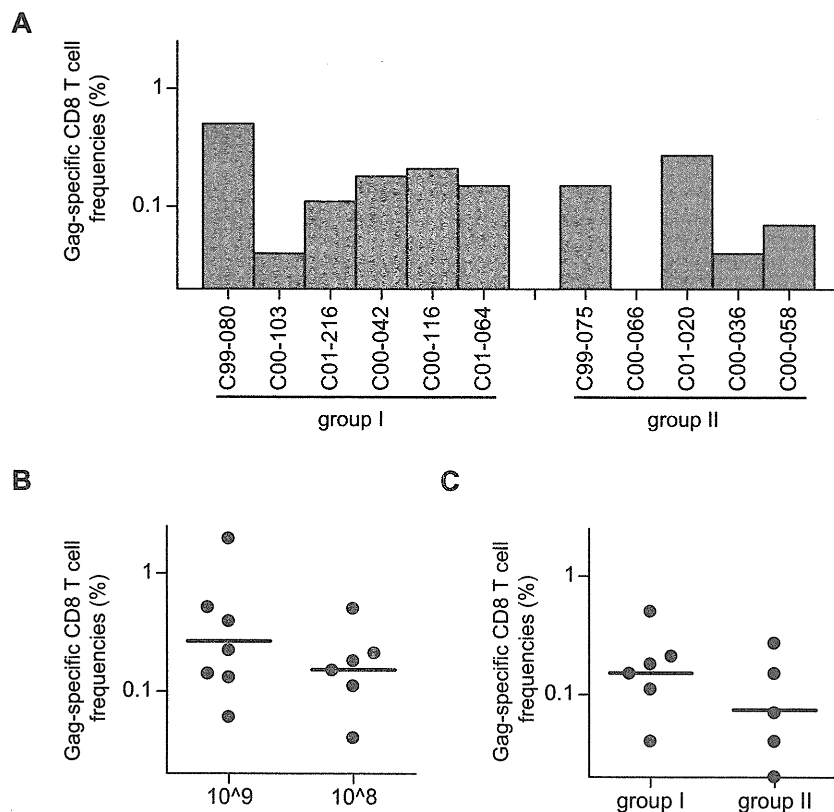

Fig. 3. Gag-specific $CD8^+$ T-cell frequencies after intranasal F(−)SeV-Gag boost in naive and pre-SeV-infected cynomolgus macaques. Gag-specific $CD8^+$ T-cell responses were examined by detection of IFN-γ induction after stimulation by B-LCLs infected with a vaccinia virus vector expressing SIVmac239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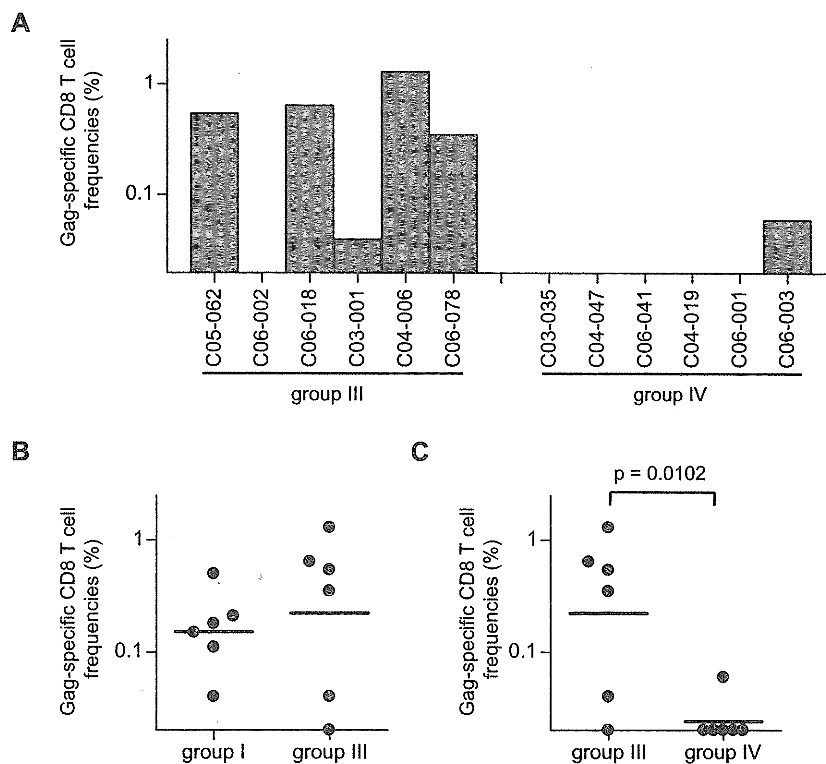

Fig. 4. Gag-specific CD8$^+$ T-cell frequencies after intramuscular F(−)SeV-Gag boost in naive and pre-SeV-infected cynomolgus macaques. (A) Gag-specific CD8$^+$ T-cell frequencies (percent in CD8$^+$ T lymphocytes) two weeks after the boost in groups III and IV. (B) Comparison of Gag-specific CD8$^+$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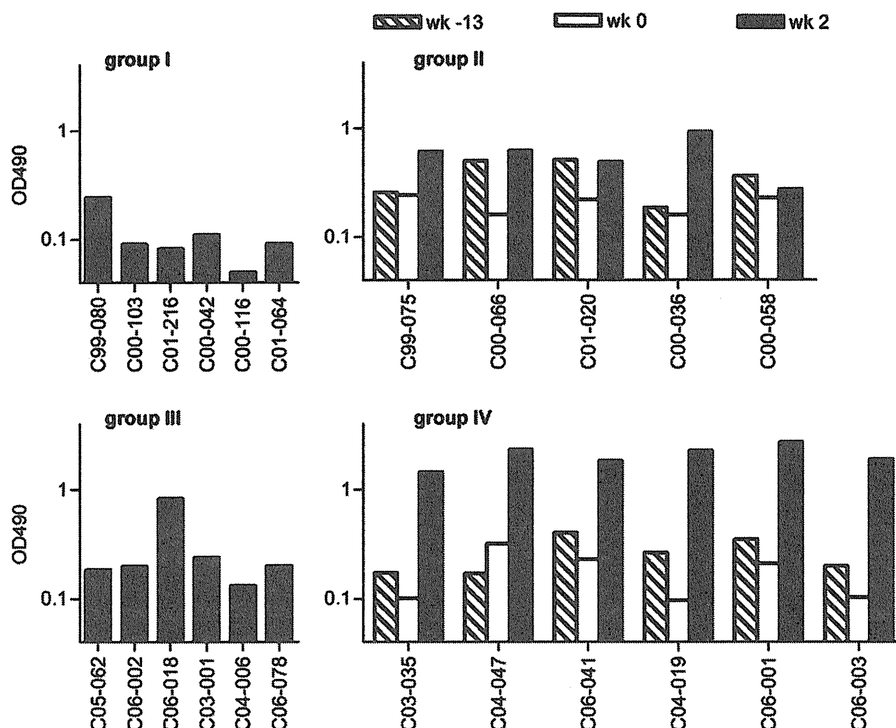

Fig. 5. SeV-specific IgG levels in plasma. Plasma samples obtained from group I and III animals at week 2 and those from group II and IV animals at weeks −13, 0 and 2 were diluted by 1/5000 and subjected to ELISA assay. OD490, optical density at 490 nm.

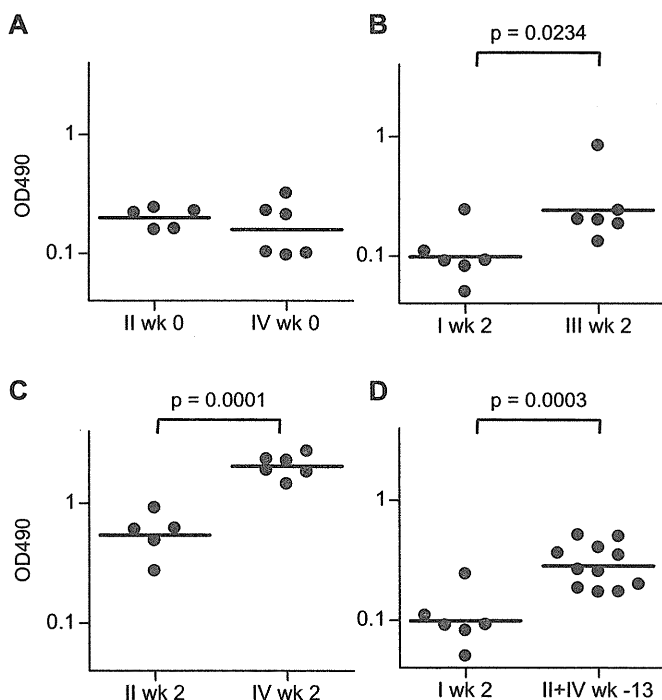

Fig. 6. Comparison of plasma SeV-specific IgG levels among groups. (A) Comparison of plasma SeV-specific IgG levels at week 0, just before F(−)SeV-Gag boost, in groups II and IV (geometric means: 0.199 in group II and 0.159 in group IV). (B) Comparison of plasma SeV-specific IgG levels at week 2, two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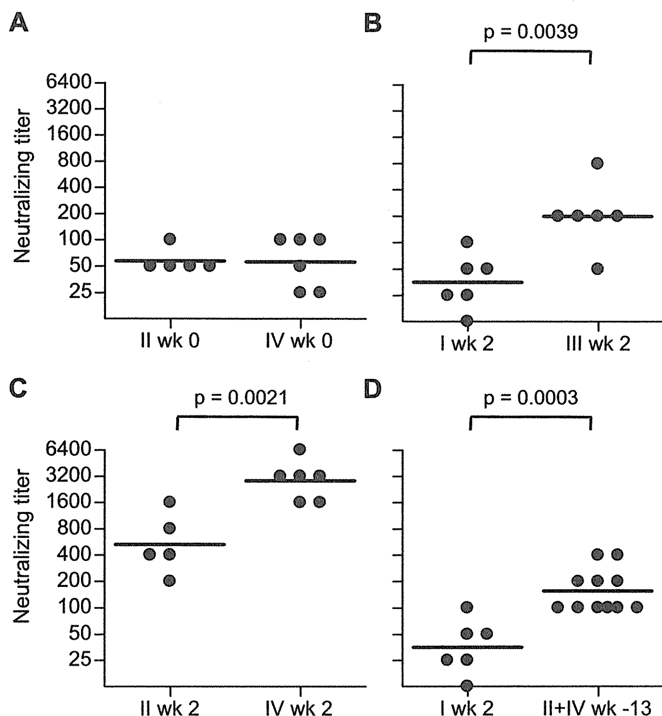

Fig. 7. Comparison of plasma SeV-specific NAb titers among groups. (A) Comparison of plasma SeV-specific NAb titers at week 0 in groups II and IV (geometric means: 5.7×10^1 in group II and 5.6×10^1 in group IV). (B) Comparison of plasma SeV-specific NAb titers at week 2 in groups I and III (geometric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

Diversity of MHC class I haplotypes in cynomolgus macaques

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Abstract Cynomolgus macaques are widely used as a primate model for human diseases associated with an immunological process. Because there are individual differences in immune responsiveness, which are controlled by the polymorphic nature of the major histocompatibility (MHC) locus, it is important to reveal the diversity of MHC in the model animal. In this study, we analyzed 26 cynomolgus macaques from five families for MHC class I genes. We identified 32 *Mafa-A*, 46 *Mafa-B*, 6 *Mafa-I*, and 3 *Mafa-AG* alleles in which 14, 20, 3, and 3 alleles were novel. There were 23 MHC class I haplotypes and each haplotype was composed of one to three *Mafa-A* alleles and one to five *Mafa-B* alleles. Family studies revealed that there were two haplotypes which contained two *Mafa-A1* alleles. These observations demonstrated further the complexity of MHC class I locus in the Old World monkey.

Keywords Cynomolgus macaque · MHC · *Mafa* class I gene · Haplotype · Polymorphism

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Introduction

Non-human primates are widely used for immunological research because their immune system is similar to that of humans. In particular, the Old World monkeys such as cynomolgus macaques (crab-eating macaques, *Macaca fascicularis*) became a useful model for human infectious diseases including acquired immunodeficiency syndrome (AIDS) (Wiseman et al. 2007), severe acute respiratory syndrome (Lawler et al. 2006), and influenza (Kobasa et al. 2007) as well as in the transplantation field (Wiseman and O'Connor 2007). In the AIDS research, cynomolgus and rhesus macaques are important animal models for the development of vaccines against human immunodeficiency virus (HIV) or studies for susceptibility to HIV infection and/or development of AIDS (Matano et al. 2004; Loffredo et al. 2008; Tsukamoto et al. 2008; Burwitz et al. 2009; Mee et al. 2009; Aarnink et al. 2011a). To fully evaluate the results of immunological experiments in the macaque models, it is essential to characterize the genetic diversity of immune-related molecules which may control the individual differences in the immune response against foreign antigens and/or pathogens.

The major histocompatibility complex (MHC) is well known to control the immune-responsiveness to foreign

antigens. There are two classes of MHC molecules: one is the MHC class I molecule presenting peptides of intracellular origin to CD8$^+$ T cell and the other is the MHC class II molecule binding extracellular-derived antigenic peptides for presenting to CD4$^+$ T cell. It has been reported that the complexity of MHC genes in the rhesus and cynomolgus macaques is higher than that in humans (Kulski et al. 2004; Watanabe et al. 2006; Gibbs et al. 2007; Otting et al. 2007, 2008; Doxiadis et al. 2011). For example, *MHC class I* configurations in macaques are usually composed of one copy of highly transcribed major *MHC-A1*gene (*Mamu-A1*or *Mafa-A1*) and several other minor *MHC-A* genes (*Mamu-A2~A7* or *Mafa-A2~A6*) in addition to several *MHC-B* genes (*Mamu-B* or *Mafa-B*) (Watanabe et al. 2006; Otting et al. 2007, 2008, 2009; Naruse et al. 2010; Doxiadis et al. 2011), whereas each one copy of *MHC-A* and *-B* genes (*HLA-A* and *-B*) can be found in human *MHC class I* locus. In addition, other *MHC* loci showing lower expression levels, i.e., *HLA-B*-like gene (*Mamu-I* or *Mafa-I*) and *HLA-G*-like non-classical gene (*Mamu-AG* or *Mafa-AG*) have been identified (Slukvin et al. 2000; Urvater et al. 2000). The extent of genetic diversity is different, in part, depending on the geographic areas, as we have previously reported for *MHC class I* genes in rhesus macaque (Naruse et al. 2010). As for the cynomolgus macaques, *MHC class I* allelic diversity was reported for Indonesian (Pendley et al. 2008; Wu et al. 2008; Kita et al. 2009; Otting et al. 2009), Malaysian (Otting et al. 2009; Aarnink et al. 2011b), Mauritian (Budde et al. 2010), Vietnamese (Wu et al. 2008; Kita et al. 2009), and Philippino (Campbell et al. 2009; Kita et al. 2009) macaques, but information about the *MHC class I* haplotype remains insufficient.

In the present study, we have analyzed *MHC class I* loci in cynomolgus macaques originated from Indonesia, Malaysia, and the Philippines to obtain information on haplotype configuration. We report here further the complex nature of *MHC class I* loci in the Old World monkey, i.e., the presence of unique haplotypes carrying two *Mafa-A1* genes.

Materials and methods

Animals

A total of 26 cynomolgus macaques from five families were the subjects. Each family was composed of one or two males with one or two females and their offspring. They were maintained in the breeding colonies in Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Japan. The founders of the colonies were captured in Indonesia, Malaysia, and the Philippines. All care including blood sampling of animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH Publication 85–23, revised 1985) and were subjected to prior approval by the local animal protection authority.

Sequencing analysis of cDNAs from *Mafa* class I genes

Total cellular RNA was extracted from whole blood by using RNAeasy (QIAGEN, Gmbh, Germany). Oligo(dT)-primed cDNA was synthesized using Transcriptor reverse transcriptase (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Full-length cDNAs

Table 1 Primers used in PCR or sequencing of *Mafa* class I genes

Primer ID	Application	Direction	Sequence (5′–3′)	Position	Reference
5′ MHC_UTR	PCR	Sense	GGACTCAGAATCTCCCCAGACGCCGAG	5′ UTR	Karl et al. 2008
3′ MHC_UTR_A	PCR	Antisense	CAGGAACAYAGACACATTCAGG	3′ UTR	Karl et al. 2008
3′ MHC_UTR_B	PCR	Antisense	GTCTCTCCACCTCCTCAC	3′ UTR	Karl et al. 2008
5A long	PCR	Sense	ATGGCGCCCCGAACCCTCCTCCTG	Exon 1	Tanaka-Takahashi et al. 2007
3A	PCR	Antisense	TCACACTTTCAAGCCGTGAGAGA	Exon 7	Tanaka-Takahashi et al. 2007
5ASSP	PCR	Sense	ATGGCGCCCCGAACCCTCCTCCTGG	Exon 1	Tanaka-Takahashi et al. 2007
4R	PCR	Antisense	CCAGGTCAGTGTGATCTCCG	Exon 4	Tanaka-Takahashi et al. 2007
P000044	PCR	Sense	GATTCTCCGCAGACGCCCA	5′ UTR	Wu et al. 2008
P000023	PCR	Antisense	GGAGAACCAGGCCAGCAAT	Exon 5	Wu et al. 2008
P000076	Sequencing	Sense	GAGCAGCGACGGGACCGCA	Intron 1	Wu et al. 2008
P000060	Sequencing	Antisense	CCTGGGGCTCTCCCGGGTCA	Intron 2	Wu et al. 2008
P000096	Sequencing	Sense	TGTACTGAGTCTCCCTGATGG	Intron 2	Wu et al. 2008
P000098	Sequencing	Antisense	TTCATTCCCTCAGAGATTTT	Intron 3	Wu et al. 2008
P000055	Sequencing	Sense	CCCAGGTRCCTSTGTCCAGGA	Intron 3	Wu et al. 2008
P000281	Sequencing	Antisense	AGAGGGGAAAGTGAGGGGT	Intron 4	Wu et al. 2008

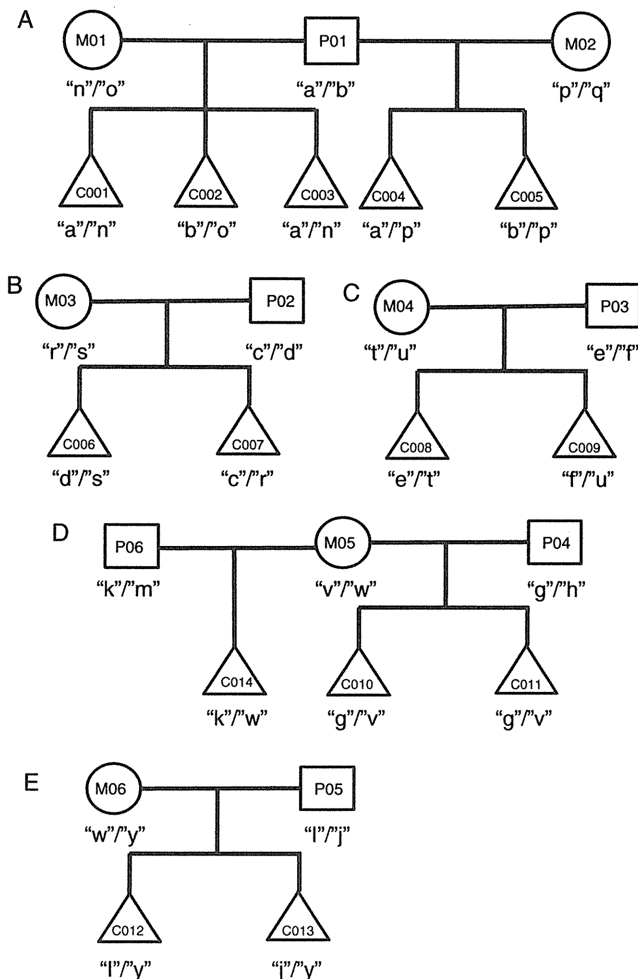

Fig. 1 Pedigree of cynomolgus macaques. The pedigrees of macaques analyzed in this study are shown. Founders were originated from Indonesia (**a**), Malaysia (**b**, **c**), and Philippines (**d**, **e**). *Open square*, *open circle*, and *open triangles* indicate father, mother, and offspring, respectively. The ID of each subject is noted in the symbol. *Mafa class I* haplotypes determined in this study are indicated under the subjects

for *Mafa* class I genes were amplified by polymerase chain reaction (PCR), as described previously (Tanaka-Takahashi et al. 2007; Naruse et al. 2010), by using locus-specific primer pairs as reported by Karl et al. (2008). Genomic gene and cDNA for *Mafa-A2* gene were analyzed according to the method described by Wu et al. (2008). The primers used in this study are listed in Table 1. To estimate the expression level of *Mafa-A* alleles, we also used an additional primer pair: MafaF (5′-TACGTGGACGA CACGCAGTT) and MafaR (5′-GGTGGGTCA CATGTGTCTTG). PCR was done under the condition of initial denaturation at 98°C for 10 s, 25 cycles of 98°C for 1 s, 64°C for 5 s, and 72°C for 20 s, followed by an additional extension at 72°C for 1 min, using Phusion Flash DNA polymerase (Finzymes, Espoo, Finland). The PCR products were cloned into pSTBlue-1 Perfectly Blunt vector (Novagen, WI, USA) according to the manufacturer's instructions and were transformed to NovaBlue Giga Singles™ competent cells (Merck Biosciences Japan, Tokyo, Japan). A total of 30 to 90 independent cDNA clones were obtained from each macaque for each locus and were sequenced on both strands by BigDye Terminator cycling system in an ABI 3730 automated sequence analyzer (Applied Biosystems, CA, USA).

Data analyses and nomenclature for *Mafa* class I allele

Nucleotide sequences of cDNA clones were aligned using the Genetyx software package (version 8.0, Genetyx Corp., Japan). When a cDNA sequence, which was represented by at least three clones, was independently obtained from at least two animals or repeatedly obtained from at least two independently prepared cDNAs from single animals, we considered it a real allele, not an artifact, and the sequences were submitted to the DNA Data Bank of Japan (DDBJ) database and to the Immuno Polymorphism Database for non-human primate MHC (http://www.ebi.ac.uk/ipd/mhc/sumit.html; Robinson et al. 2003) to obtain official nomenclature for the novel alleles of *Mafa-A* and *Mafa-B* genes. Neighbor-joining trees were constructed with Kimura's two-parameter method for a phylogenetic analysis of *Mafa-A* sequences spanning exons 2, 3, and a part of exon 4 obtained in this study by using the Genetyx software. Bootstrap values were based on 5,000 replications.

Table 2 *Mafa* class I alleles found in the cynomolgus macaques

Locus	Number of observed alleles	Number of novel alleles (%)	Number of observed alleles in macaques from different regions[a]		
			Indonesian	Malaysian	Philippino
Mafa-A	32	14 (43.7%)	9 (3), 33.3%	12 (8), 66.7%	11 (3), 27.3%
Mafa-B	46	20 (43.5%)	13 (5), 38.5%	20 (15), 75.0%	18 (1), 5.6%
Mafa-I	6	3 (50.0%)	2 (1), 50.0%	4 (3), 75.0%	2 (0), 0%
Mafa-AG	3	3 (100%)	0 (0), 0%	2 (2), 100%	1 (1), 100%
Total	87	40 (45.5%)	24 (9), 37.5%	38 (28), 73.7%	32 (5), 15.6%

[a] The number and frequency of novel alleles are indicated in parentheses

Results

Identification of *Mafa* class I alleles in cynomolgus macaques

We determined the nucleotide sequences of cDNA clones for *Mafa-A* and *-B* loci in 26 cynomolgus macaques from one family of Indonesian origin (six haplotypes), two families of Malaysian origin (eight haplotypes), and two families of Philippino origin (nine haplotypes) (Fig. 1). When the observed alleles were segregated in the family or when at least three clones with identical sequences were observed from two independent PCR for an individual, the nucleotide sequences were considered to be real and not artifacts. As shown in Table 2, 32 *Mafa-A*, 46 *Mafa-B*, 6 *Mafa-I*, and 3 *Mafa-AG* sequences were obtained in this study. Among them, 14 (43.7%), 20 (43.5%), 3 (50.0%), and 3 (100%) were novel alleles of *Mafa-A*, *Mafa-B*, *Mafa-I*, and *Mafa-AG* loci, respectively (Table 2).

Table 3 Alleles of *Mafa-A* locus identified in the cynomolgus macaques

Locus	Allele name	Novelty[a]	Accession number[b]	Origin[c]	Identical *Mamu* and/or *Mane* alleles[d]	Origin and reference of known alleles[e]
A1	*A1*001:01*		AM295828	Malaysian		Utrecht, Otting et al. 2007
A1	*A1*002:01:02*	Novel	AB569214	Indonesian		
A1	*A1*008:02*		EU392108	Philippino		Philippino, Campbell et al. 2009
A1	*A1*008:03-like*	Novel	AB647187	Philippino		
A1	*A1*018:06*		FM246489	Indonesian		Utrecht, Otting et al. 2007
A1	*A1*019:05*		AB447616	Indonesian		Indonesian, Kita et al. 2009
A1	*A1*023:01*	Novel	AB569216	Malaysian		
A1	*A1*032:05*	Novel	AB569215	Malaysian		
A1	*A1*052:02*		EU392105	Philippino	*Mamu-A1*052:01/03/06*	Philippino, Campbell et al. 2009
A1	*A1*054:01*		AB154771	Malaysian		Tsukuba, Uda et al. 2004
A1	*A1*056:02*	Novel	AB569218	Malaysian		
A1	*A1*062:05*	Novel	AB569219	Malaysian		
A1	*A1*068:02*	Novel	AB569217	Malaysian		
A1	*A1*074:02*		AB447606	Philippino		Philippino, Kita et al. 2009
A1	*A1*079:01*		AB154773	Malaysian		Tsukuba, Uda et al. 2004
A1	*A1*089:02*		EU392104	Philippino		Philippino, Campbell et al. 2009
A1	*A1*093:01*		EU392103	Philippino		Philippino, Campbell et al. 2009
A1	*A1*094:01*		EU392111	Philippino		Philippino, Campbell et al. 2009
A1	*A1*097:01*		AB447576	Indonesian	*Mamu-A1*109:01*	Indonesian, Kita et al. 2009
A1	*A1*103:01*	Novel	AB583236	Indonesian		
A1	*A1*124:01*	Novel	AB583237	Malaysian		
A2	*A2*05:13-like*	Novel	AB647189	Philippino		
A2	*A2*05:16*		AM295878	Indonesian		Utrecht, Otting et al. 2007
A2	*A2*05:34-like*	Novel	AB647190	Philippino		
A3	*A3*13:03*		EU392112	Philippino		Philippino, Campbell et al. 2009
A3	*A3*13:15*	Novel	AB583238	Malaysian		
A3	*A3*13:16*	Novel	AB583240	Indonesian		
A4	*A4*14:01*		AM295880	Indonesian		Utrecht, Otting et al. 2007
A4	*A4*14:02*		AM295881	Malaysian		Utrecht, Otting et al. 2007
A6	*A6*01:05*	Novel	AB583239	Malaysian		

[a] New alleles are indicated as novel

[b] Nucleotide sequences were submitted to a public database and given accession numbers

[c] Origin of cynomolgus macaques

[d] Identical sequences were found in *Mamu* or *Mane* alleles

[e] Origin and references in which each known allele was first reported. Utrecht and Tsukuba indicate that the alleles were found in colonies maintained in the University of Utrecht, The Netherlands, and Tsukuba primate center, Japan, respectively

Table 4 Alleles of *Mafa-B* locus identified in the cynomolgus macaques

Allele name[a]	Novelty[b]	Accession number	Origin[c]	Identical to *Mamu* and/or *Mane* alleles[d]	Origin and reference of known alleles[e]
*B*002:03*	Novel	AB569224	Indonesian, Malaysian		
*B*004:01*		EU203722	Indonesian		Indonesian, Pendley et al. 2008
*B*007:01:01*		AY958137	Philippino	*Mamu-B*007:02/03*	Mauritian, Krebs et al. 2005
*B*007:01:02*		EU392135	Philippino		Philippino, Campbell et al. 2009
*B*007:01:03*	Novel	AB569223	Indonesian		
*B*007:03*		FM212802	Philippino		Indonesian or Malaysian, Otting et al. 2009
*B*011:02*	Novel	AB569229	Malaysian		
*B*013:08*		EU392114	Indonesian, Philippino		Philippino, Campbell et al. 2009
*B*017:01*		EU392119	Philippino		Philippino, Campbell et al. 2009
*B*018:01*		AY958138	Indonesian	*Mamu-B*018:01*	Mauritian, Krebs et al. 2005
*B*030:02*		AY958134	Malaysian	*Mamu-B*030:03:01*	Mauritian, Krebs et al. 2005
*B*032:01*	Novel	AB569237	Malaysian		
*B*033:02*		EU392118	Philippino		Philippino, Campbell et al. 2009
*B*043:01*	Novel	AB569230	Malaysian	*Mamu-B*043:01*	
*B*056:01*		AY958131	Indonesian	*Mamu-B*056:01*	Mauritian, Krebs et al. 2005
*B*056:02*		EU392128	Philippino		Philippino, Campbell et al. 2009
*B*057:03*	Novel	AB569231	Malaysian	*Mamu-B*057:06*	
*B*060:04*	Novel	AB569226	Indonesian		
*B*061:01*		AB195445	Malaysian	*Mamu-B*061:04:01, Mane-B*061:01*	Tsukuba, Uda et al. 2005
*B*061:02*	Novel	AB569233	Malaysian		
*B*064:02*		FM212804	Philippino		Indonesian or Malaysian, Otting et al. 2009
*B*068:04*	Novel	AB569236	Malaysian	*Mamu-B*068:04 , Mane-B*nov078*	
*B*069:02*		FM212842	Malaysian		Indonesian or Malaysian, Otting et al. 2009
*B*074:01:02-like*	Novel	AB647188	Philippino		
*B*074:02*	Novel	AB569228	Malaysian	*Mamu-B*074:01 /02*	
*B*076:04*	Novel	AB569232	Malaysian		
*B*081:01*	Novel	AB569225	Indonesian		
*B*089:01:01*		EU392131/ FJ178820	Philippino		Philippino, Campbell et al. 2009
*B*089:01:02*		EU392125	Indonesian, Malaysian, Philippino	*Mamu-B*089:01, Mane-B*089:02*	Philippino, Campbell et al. 2009
*B*090:01*		AB195436	Malaysian		Tsukuba, Uda et al. 2005
*B*091:01*	Novel	AB569240	Malaysian	*Mamu-B*091:02*	
*B*092:01:01*	Novel	AB569227	Malaysian	*Mamu-B*092:02*	
*B*095:01*		EU392113/ AY958148	Philippino		Mauritian, Krebs et al. 2005 Philippino, Campbell et al. 2009
*B*104:03*		EU392126	Philippino		Indonesian, Pendley et al. 2008 Philippino, Campbell et al. 2009
*B*116:01*		EU392123	Philippino		Philippino, Campbell et al. 2009
*B*121:01*		AB195455	Indonesian		Tsukuba, Uda et al. 2005
*B*124:01:02*	Novel	AB569235	Malaysian		
*B*136:02*		EU203720	Indonesian,		Indonesian, Pendley et al. 2008
*B*137:03*		EU392117/ EU203723	Indonesian, Philippino		Indonesian, Pendley et al. 2008
*B*137:04*	Novel	AB569239	Malaysian		

Table 4 (continued)

Allele name[a]	Novelty[b]	Accession number	Origin[c]	Identical to *Mamu* and/or *Mane* alleles[d]	Origin and reference of known alleles[e]
*B*138:02*	Novel	AB569234	Malaysian		
*B*151:02:02*	Novel	AB569222	Indonesian		
*B*155:02*	Novel	AB569238	Malaysian		
*B*157:01*		EU392121	Philippino		Philippino, Campbell et al. 2009
*B*158:01*		EU392122	Philippino		Philippino, Campbell et al. 2009
*B*160:01*		EU606042	Philippino		-

[a] New alleles are indicated as novel

[b] Nucleotide sequences were submitted to a public database and given accession numbers

[c] Origin of cynomolgus macaques

[d] Identical sequences were found in *Mamu* or *Mane* alleles

[e] Origin and references in which each known allele was first reported. Utrecht and Tsukuba indicate that the alleles were found in colonies maintained in the University of Utrecht, The Netherlands, and Tsukuba primate center, Japan, respectively

The *Mafa-A* alleles found in this study are listed in Table 3, where 21 alleles were from the major *Mafa-A1* locus, while the remaining 11 alleles were from the minor *Mafa-A* loci, 3 from *Mafa-A2*, 3 from *Mafa-A3*, 2 from *Mafa-A4*, and 1 from *Mafa-A6* alleles (Table 3). The major *Mafa-A1*alleles were defined by the sequence similarity to the known *Mafa-A1* alleles to be given official nomenclatures by IPD, except for *Mafa-A1*008:03*-like allele, and we confirmed that the frequencies of cDNA clones for *Mafa-A1* alleles were over 10% in each macaque. Similarly, alleles of minor *Mafa-A* genes, *Mafa-A2*, *-A3*, *-A4*, and *-A6* were defined by sequence similarity to the known alleles. They, except for two novel *Mafa-A2* alleles, were also given official names by IPD. On the other hand, a total of 46 *Mafa-B* alleles (Table 4) as well as 6 *Mafa-I* and 3 *Mafa-AG* alleles (Table 5) were identified. It was found that 2 out of

Table 5 Alleles of *Mafa-AG* and *Mafa-I* locus identified in the cynomolgus macaques

Locus	Allele name[a]	Novelty[b]	Accession number[c]	Origin[d]	Identical to *Mamu* and/or *Mane* alleles[e]	Origin and reference of known alleles[e]
AG	*AG*04:03*	Novel	AB569221	Malaysian		
AG	*AG1 like-1*	Novel	AB569220	Malaysian		
AG	*AG1 like-3*	Novel	AB583241	Philippino		
I	*I*01:01:01*		EU392139	Philippino		Philippino, Campbell et al. 2009
I	*I*01:09/01:08*		AB195465/AB195464	Indonesian, Malaysian		Tsukuba, Uda et al. 2005
I	*I*01:15*		FM246493	Philippino	*Mamu-I*01:06* , *Mamu-I*01:08:01*	Indonesian or Malaysian, Otting et al. 2009
I	*I*01:15 like-1*	Novel	AB569241	Indonesian, Malaysian		
I	*I*01:15 like-2*	Novel	AB569242	Malaysian	*Mamu-I*03:01:01*, *Mamu-I*01:07:01* , *Mamu-I*01:06:05*	
I	*I*01:18 like*	Novel	AB569243	Malaysian		

[a] Official allele names were not obtained for *AG1 like-1*, *AG1 like-3*, *I*01:15 like-1*, *I*01:15 like-2*, and *I*01:18 like* due to the limited sequence information

[b] New alleles are indicated as novel

[c] Nucleotide sequences were submitted to a public database and given accession numbers

[d] Origin of cynomolgus macaques

[e] Identical sequences were found in *Mamu* or *Mane* alleles

[f] Origin and references in which each known allele was reported. Tsukuba indicates that the alleles were found in colonies maintained in the Tsukuba primate center, Japan

21 (9.5%) *Mafa-A1a* alleles and 12 out of 46 (26.1%) *Mafa-B* alleles had identical sequences to *Mamu-A1* and *Mamu-B* alleles, respectively, implying a genetic admixture of cynomolgus macaques with rhesus macaques during the evolution (Otting et al. 2007; Bonhomme et al. 2009; Otting et al. 2009). Because we determined the nucleotide sequences only for exons 2, 3, and 4, two novel *Mafa-AG* alleles and three novel *Mafa-I* alleles were not given official names. As for the geographic distribution of *Mafa* class I alleles, there was no overlapping of *Mafa-A* alleles originated from different regions (Table 3), while there were a few *Mafa-B* and *Mafa-I* alleles commonly observed in macaques from different regions (Tables 4 and 5, respectively). When we looked into the presence of novel alleles in the geographic distribution, most of the novel alleles were obtained from Malaysian macaques, while almost all of the alleles found in Philippino macaques were not novel (Table 2).

Mafa class I haplotypes identified in the family study

We could identify the *Mafa-A* and *Mafa-B* alleles composing 23 different haplotypes from the segregation studies (Table 6). It was found that one to three expressing *Mafa-A*

Table 6 *Mafa class I* haplotypes identified in the cynomolgus macaques

ID[a]	Origin[b]	Haplotype[c]	*Mafa-A1* (major)	*Mafa-A* (minor)	*Mafa-AG*	*Mafa-B* (major)	*Mafa-B* (minor)	*Mafa-I*
P01	Indonesian	"a"	A1*002:01:02	A3*13:16		B*136:02		I*01:09/01:08
		"b"	A1*103:01			B*007:01:03, B*121:01	B*151:02:02	
P02	Malaysian	"c"	A1*023:01			B*090:01	B*011:02, B*074:02	
		"d"	A1*068:02			B*043:01	B*030:02, B*057:03	I*01:15 like-2
P03	Malaysian	"e"	A1*001:01, A1*032:05		AG1 like-1	B*068:04, B*124:01:02	B*032:01, B*061:01, B*089:01:02	
		"f"	A1*079:01		AG*04:03	B*061:02, B*138:02	B*155:02	
P04	Philippino	"g"	A1*089:02	A2*05:13-like, A3*13:03		B*137:03		
		"h"	A1*008:02			B*104:03		
P05	Philippino	"i"	A1*094:01			B*007:01:02	B*160:01	
		"j"	A1*008:02		AG1 like-3	B*157:01	B*017:01, B*089:01:02, B*116:01	I*01:01:01, I*01:15
P06	Philippino	"k"	A1*08:03-like	A2*05:34-like		B*074:01:02-like		
		"m"	A1*089:02	A3*13:03		B*007:03, B*064:02	B*089:01:01	
M01	Indonesian	"n"	A1*018:06	A2*05:16, A4*14:01		B*002:03		I*01:15 like-1
		"o"	A1*097:01			B*056:01	B*089:01:02	
M02	Indonesian	"p"	A1*097:01			B*137:03	B*013:08	
		"q"	A1*019:05			B*018:01	B*004:01, B*060:04, B*081:01	
M03	Malaysian	"r"	A1*054:01			B*002:03		I*01:15 like-1
		"s"	A1*056:02	A4*14:02		B*076:04		I*01:18 like
M04	Malaysian	"t"	A1*062:05			B*069:02	B*137:04	
		"u"	A1*124:01	A3*13:15		B*091:01		
M05	Philippino	"v"	A1*074:02, A1*093:01			B*007:01:01, B*158:01		
		"w"	A1*093:01			B*007:01:02	B*160:01	
M06	Philippino	"w"	A1*093:01			B*007:01:02	B*160:01	
		"y"	A1*052:02			B*033:02, B*095:01		

[a] ID of founder animals as indicated in Fig. 1

[b] Origin of cynomolgus macaques

[c] Haplotypes were determined from studies of family as shown in Fig. 1

alleles and one to five expressing *Mafa-B* alleles consisted of *Mafa class I* haplotype, similar to the *Mamu class I* haplotypes in rhesus macaques (Naruse et al. 2010). Of particular interest was that there were two haplotypes, "e" (Malaysian founder P03) and "v" (Philippino founder M05), carrying two different *Mafa-A1* genes (Fig. 1; Table 6). Because previous studies have demonstrated that there is usually only one *Mafa-A1* allele on a chromosome (Otting et al. 2007), while the presence of two *Mamu-A1* alleles on the same haplotype was suggested in rhesus macaques (Naruse et al. 2010; Doxiadis et al. 2011), we performed further analyses.

The family studies showed that the *Mafa-A1*alleles consisting of haplotype "e", *Mafa-A1*001:01* and*Mafa-A1*032:05*, or haplotype "v", *Mafa-A1*074:02* and *Mafa-A1*093:01*, did not carry accompanying minor *Mafa-A* genes (Table 6). When we constructed a phylogenetic tree of *Mafa-A* alleles identified in this study (Fig. 2), it was found that *Mafa-A1*001:01* was mapped in the neighbor of *Mafa-A3*gene, raising a possibility that one of the two alleles on the same chromosome might be a minor *Mafa-A* allele and not the major *Mafa-A1* allele. To test the possibility, we investigate the expression level of *Mafa-A* alleles composing of haplotypes "e" and "v". For this purpose, other primer pairs were designed within the sequences completely shared by these alleles to amplify the *Mafa-A* cDNAs to avoid a possibility of affecting the efficacy of PCR by mismatches with the primer sequences. The cloning and sequencing analysis revealed that both *Mafa-A1*001:01* and *Mafa-A1*032:05* on the haplotype "e" were observed at similar frequencies among the cDNA clones of *Mafa-A* alleles in P03and C008 (Fig. 1): 29.7% and 33.3% in P03 and 22.5% and 17.5% in C008, respectively. Similarly, frequencies of haplotype "v" alleles, *Mafa-A1*074:02* and *Mafa-A1*093:01*, in cDNA clones were 59.5% and 40.5%, respectively, in M05, while those in C010 were 23.3% and 26.7% and 31.4% and 17.1% in C011, respectively. The frequencies of cDNA clones varied in different individuals presumably due to the allelic competition with the alleles of another haplotype in each individual (Fig. 1), but they were much higher than the frequencies of the minor *Mafa-A* allele (*Mafa-A3*13:03*) clones: 3.3% and 2.9% in C010 and C011, respectively. These observations indicated that two *Mafa-A* alleles were considered to be major *Mafa-A1* alleles in both haplotypes "e" and "v".

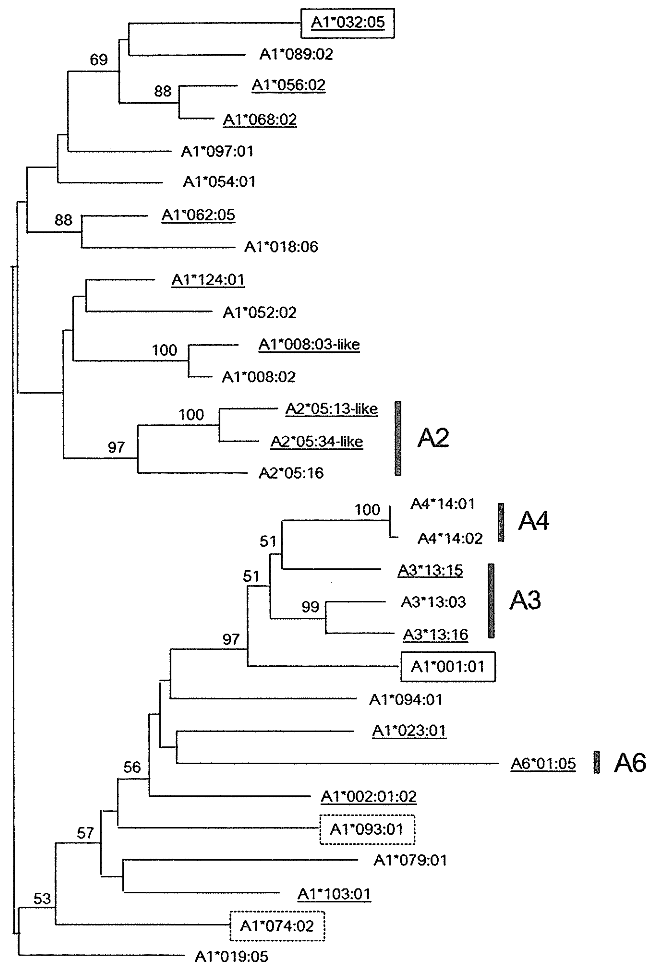

Fig. 2 Phylogenetic tree of *Mafa-A* alleles. A phylogenetic tree of the *Mafa-A* alleles detected in this study was constructed by using the neighbor-joining method with a bootstrap value of 5,000 replications. Values more than 50% are indicated as percentages. Novel alleles were *underlined*. *Mafa-A*1 alleles consisting of haplotype "e" are *boxed*, while the *stippled boxes* represent the alleles on haplotype "v". Alleles of minor *Mafa-A* genes, *Mafa-A2*, *A3*, *A4*, and *A6*, are also indicated

Discussion

Native cynomolgus macaques are widespread throughout the islands of Southeast Asia into mainland Asia. They are mainly found in Indonesia, Malaysia, and the Philippines, then Burma, India, Vietnam, Cambodia, Laos, and Thailand (Lang 2006). It was suggested that the founding population of Mauritian macaques was introduced from Indonesia (Pendley et al. 2008; Campbell et al. 2009). More than 40% of *Mafa* class I alleles observed in this study were novel, even though there have been many reports on the analysis of *Mafa class I* genes, demonstrating that the diversity of MHC in the cynomolgus macaques still needs to be investigated. When we considered the origin of founders, 73.7% (28/38) were novel in alleles found in Malaysian macaques, while only 15.6% (5/32) were novel alleles in Philippino macaques (Table 2). The geographic distribution of novel alleles may be due to the fact that the Malaysian macaques had not been extensively analyzed before (Otting et al. 2007;

Pendley et al. 2008; Kita et al. 2009). In the present study, *B*089:01:02* was found in individuals among Indonesian, Malaysian, and Philippino macaques in different *Mafa-B* haplotypes (Table 6). Likewise, *B*137:03* was found in Indonesian and Malaysian macaques (Table 4). In addition, shared alleles among the cynomolgus macaques, rhesus macaques, and pig-tailed macaques (*Macaca nemestrina*) were noted (Tables 3, 4, and 5). These observations indicated that the diversity of *MHC class I* genes is similar not only in the cynomolgus macaque population but also among the Old World monkeys, suggesting that the *MHC class I* polymorphisms might be generated before the divergence of Old World monkeys and/or there were admixtures of the Old World monkeys.

In this study, we determined the haplotype structure of *Mafa* class I locus by family studies and a total of 23 haplotypes were identified. Among them, haplotypes "i" and "w" carried identical *Mafa-B* alleles but different *Mafa-A* alleles (Table 6), suggesting that there were haplotypes originated by a recombination between the *Mafa-A* and *Mafa-B* loci. We showed that the *Mafa class I* haplotypes were usually composed of one to three *Mafa-A* alleles and one to five *Mafa-B* alleles, similar to the *Mamu class I* haplotypes, of which usually one *MHC-A1* gene and a few (one to three) *MHC-B* genes were highly transcribed (Otting et al. 2007, 2008; Naruse et al. 2010; Doxiadis et al. 2011). As for the *MHC-A* locus in the cynomolgus macaques, highly transcribed *Mafa-A1*gene and other minor *Mafa-A* genes, such as *Mafa-A2*, *-A3*, *-A4*, and *-A6* could be detected. It was reported that 87% of cynomolgus macaques had at least one *Mafa-A2* alleles (Wu et al. 2008). However, only 3 out of 23 (13.0%) haplotypes carried a *Mafa- A2* allele in this study (Table 6). We could not exclude a possibility that the strategy of our study might not be sufficient to detect the *Mafa-A* genes with low expression and/or the alleles with mismatches at the primer site, based on the number of clones within a PCR sample. Such a possibility is unlikely because we used the primer pairs which could cover the known *Mafa-A2* alleles, although there might be novel *Mafa-A2* alleles having different sequences at the primer binding sites. Therefore, we might underestimate the complexity of *Mafa* class I alleles in this study. High-throughput pyrosequencing methods may be a useful strategy to avoid the possibility of missing alleles, as described by several investigators (Wiseman et al. 2009; Budde et al. 2010; Aarnink et al. 2011b). In addition, because it was reported that the cell surface expression of Mamu class I molecule was varied depending on the locus and allelic structure (Rosner et al. 2010), locus- and allele-dependent expression of Mafa class I molecule at the cell surface will be required.

The most important finding in this study was that we demonstrated evidence for the presence of haplotypes carrying two major *MHC-A1* genes on the same chromosome from the family studies and additional cloning studies. Interestingly, we and others have reported similar phenomena in rhesus macaques (Naruse et al. 2010; Doxiadis et al. 2011). In addition, several haplotypes carried multiple major *Mafa-B1* alleles (Table 6), similar to the *Mamu-B1* locus (Otting et al. 2008; Doxiadis et al. 2011). The *raison d'etre* of multiple major *MHC class I* genes/alleles on the same chromosome may be that they play an immunological role as the "double lock strategy" (Doxiadis et al. 2011) in which the double *MHC-A1* alleles of high transcription level might be favorable to present peptide to CD8+ T cells. However, there is another unique haplotype which carries no *MHC-A1*allele in cynomolgus macaques (Otting et al. 2007) and maybe in rhesus macaques (Doxiadis et al. 2011). These observations suggested that the diversity of MHC in the Old World monkey is far more complicated than in humans.

In summary, we investigated 26 cynomolgus macaques from five families for the diversity of *MHC class I* alleles and haplotypes. A total of 87 alleles were identified, of which 40 were novel. There were 23 different haplotypes, and two of them carried two *MHC-A1* genes, demonstrating further the complexity of *MHC class I* locus in the Old World monkey.

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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 $Gag_{236-250}$ (IAGTTSSVDEQIQWM)-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

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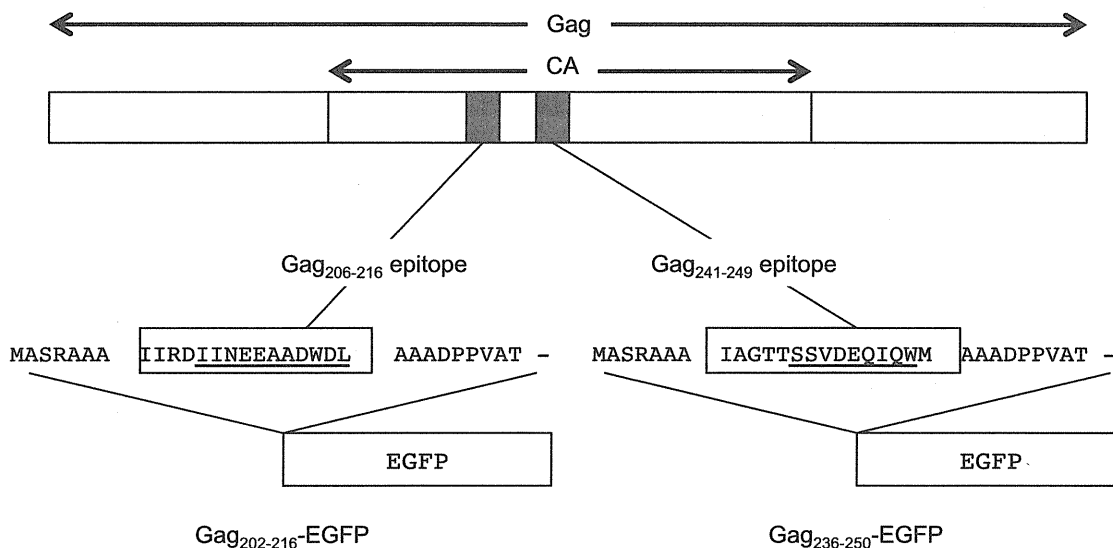

FIG 1 Schema of the cDNA constructs encoding $Gag_{202-216}$-EGFP and $Gag_{236-250}$-EGFP fusion proteins. A DNA fragment that encodes a 31-mer peptide (boxes) including the $Gag_{202-216}$ or $Gag_{236-250}$ sequence (underlining) was introduced into the 5′ end of the EGFP cDNA.

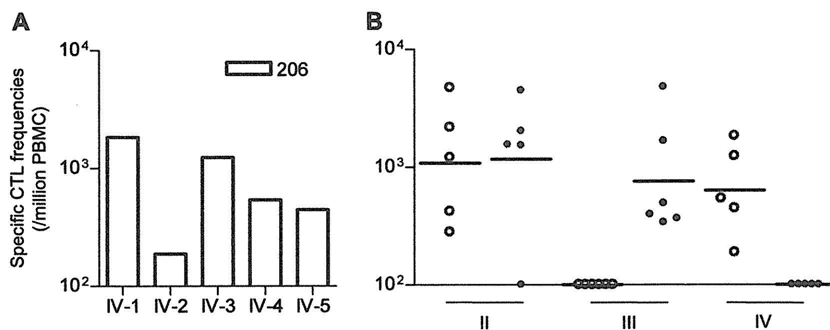

FIG 2 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after prophylactic vaccination. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies 1 week after SeV-$Gag_{202-216}$–EGFP boost in group IV macaques (open boxes). (B) $Gag_{206-216}$-specific (open circles) and $Gag_{241-249}$-specific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

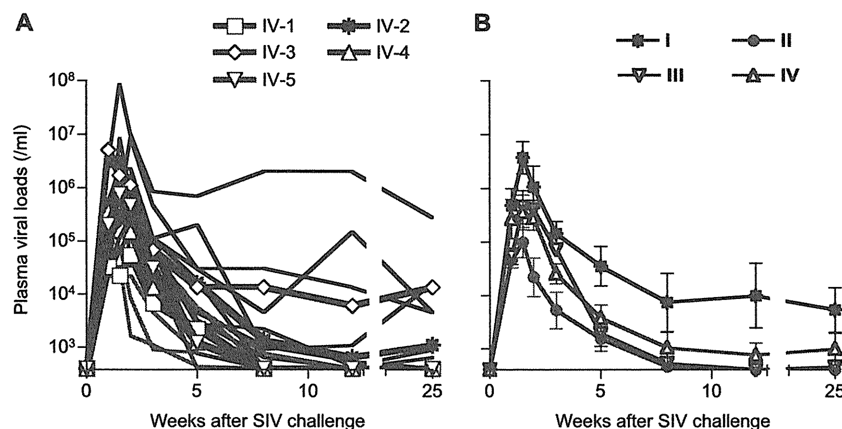

FIG 3 Plasma viral loads after SIVmac239 challenge. The plasma viral loads in group I, group II, group III, and group IV animals were determined as described previously (21). The lower limit of detection was approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma)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

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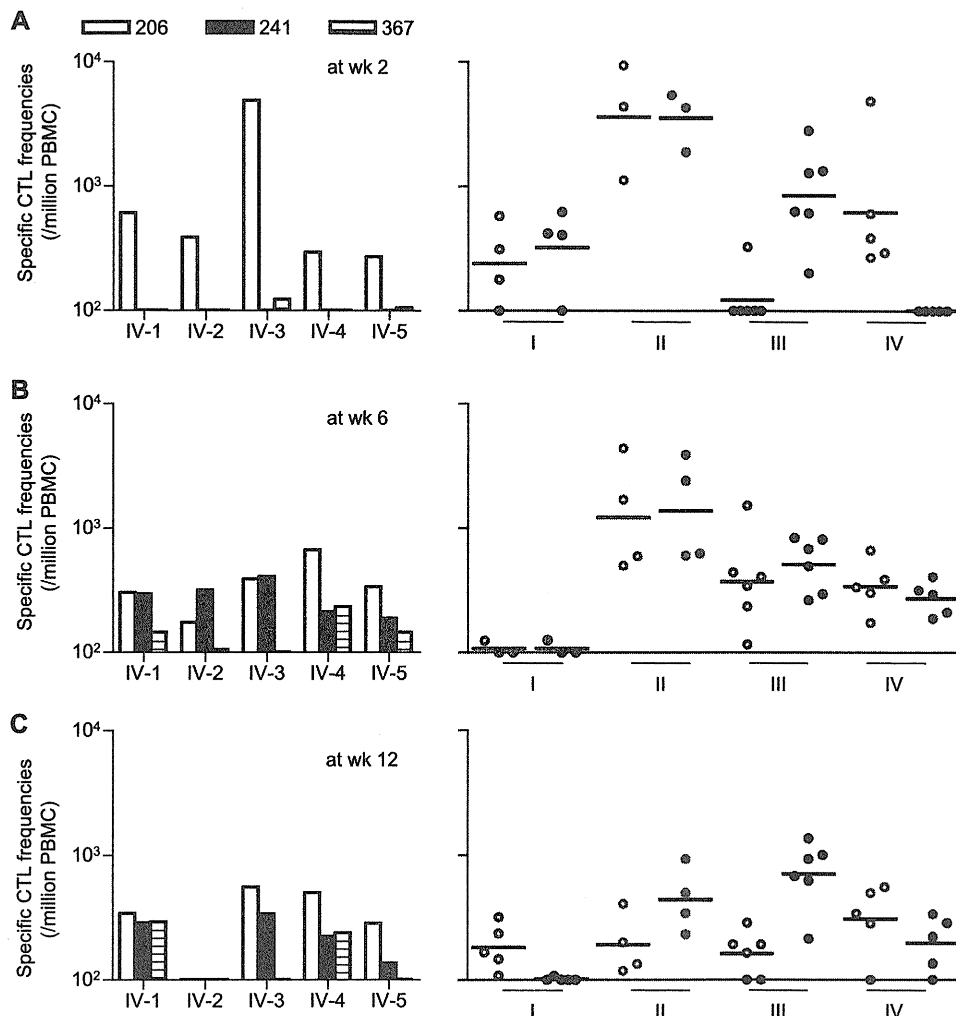

FIG 4 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after SIVmac239 challenge. CTL responses at week 2 (A), week 6 (B), and week 12 (C) are shown. In the graphs on the left, $Gag_{206-216}$-specific (open boxes), $Gag_{241-249}$-specific (closed boxes), and $Gag_{367-381}$-specific (striped boxes) $CD8^+$ T-cell frequencies in group IV macaques are shown. On the right, $Gag_{206-216}$-specific (open circles) and $Gag_{241-249}$-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 $Gag_{241-249}$-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 $Gag_{206-216}$-specific CTL levels ($P > 0.05$ by one-way ANOVA).

tein, were used for the vaccination (Fig. 1). We confirmed $Gag_{206-216}$-specific CTL responses 1 week after SeV-$Gag_{202-216}$-EGFP boost in all five animals (Fig. 2A). As expected, no $Gag_{241-249}$-specific CTL responses were detected in these animals. No $Gag_{202-216}$-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 $Gag_{241-249}$ epitope-specific CTL responses. Both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were detectable after SeV-Gag boost in four of five group II animals except for one animal (II-3), in which $Gag_{206-216}$-specific, but not $Gag_{241-249}$-specific, CTL responses were detected. In all group III animals, $Gag_{241-249}$-specific CTL responses were confirmed, while no $Gag_{206-216}$-specific CTL responses were detected after SeV-$Gag_{236-250}$-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 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after SIVmac239 challenge by detection of peptide-

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