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Control of Simian Immunodeficiency Virus Replication by Vaccine-Induced Gag- and Vif-Specific CD8$^+$ T Cells

Nami Iwamoto,[a,b] Naofumi Takahashi,[a,b] Sayuri Seki,[a,b] Takushi Nomura,[a,b] Hiroyuki Yamamoto,[a] Makoto Inoue,[c] Tsugumine Shu,[c] Taeko K. Naruse,[d] Akinori Kimura,[d] Tetsuro Matano[a,b]

AIDS Research Center, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo, Japan[a]; The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan[b]; DNAVEC Corporation, Ohkubo, Tsukuba, Ibaraki, Japan[c]; Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, Kandasurugadai, Chiyoda-ku, Tokyo, Japan[d]

For development of an effective T cell-based AIDS vaccine, it is critical to define the antigens that elicit the most potent responses. Recent studies have suggested that Gag-specific and possibly Vif/Nef-specific CD8$^+$ T cells can be important in control of the AIDS virus. Here, we tested whether induction of these CD8$^+$ T cells by prophylactic vaccination can result in control of simian immunodeficiency virus (SIV) replication in Burmese rhesus macaques sharing the major histocompatibility complex class I (MHC-I) haplotype *90-010-Ie* associated with dominant Nef-specific CD8$^+$ T-cell responses. In the first group vaccinated with Gag-expressing vectors ($n = 5$ animals), three animals that showed efficient Gag-specific CD8$^+$ T-cell responses in the acute phase postchallenge controlled SIV replication. In the second group vaccinated with Vif- and Nef-expressing vectors ($n = 6$ animals), three animals that elicited Vif-specific CD8$^+$ T-cell responses in the acute phase showed SIV control, whereas the remaining three with Nef-specific but not Vif-specific CD8$^+$ T-cell responses failed to control SIV replication. Analysis of 18 animals, consisting of seven unvaccinated noncontrollers and the 11 vaccinees described above, revealed that the sum of Gag- and Vif-specific CD8$^+$ T-cell frequencies in the acute phase was inversely correlated with plasma viral loads in the chronic phase. Our results suggest that replication of the AIDS virus can be controlled by vaccine-induced subdominant Gag/Vif epitope-specific CD8$^+$ T cells, providing a rationale for the induction of Gag- and/or Vif-specific CD8$^+$ T-cell responses by prophylactic AIDS vaccines.

Human immunodeficiency virus (HIV) infection induces persistent viral replication, leading to AIDS onset in humans. Virus-specific CD8$^+$ T-cell responses play a central role in the resolution of acute peak viremia (1–4) but mostly fail to contain viral replication in HIV infection. Prophylactic vaccination resulting in more effective CD8$^+$ T-cell responses postexposure than those in natural HIV infections might contribute to HIV control. Current trials in macaque AIDS models have shown that vaccine induction of T-cell responses can result in control of postchallenge viral replication (5–10). It is now critical to define the antigens that elicit the most potent responses for development of an effective T-cell-based AIDS vaccine.

Recent studies have implicated Gag-specific CD8$^+$ T cells in the control of HIV and simian immunodeficiency virus (SIV) replication (11–16). Several HLA or major histocompatibility complex class I (MHC-I) alleles have been shown to be associated with lower viral loads (17–25). Virus control associated with some of these protective MHC-I alleles is attributed to Gag epitope-specific CD8$^+$ T-cell responses (26–29). For instance, CD8$^+$ T-cell responses specific for the HLA-B*57-restricted $Gag_{240-249}$ TW10 and HLA-B*27-restricted $Gag_{263-272}$ KK10 epitopes exert strong suppressive pressure on HIV replication and frequently select for escape mutations with viral fitness costs, leading to lower viral loads (27, 30–33). Thus, certain individuals possessing MHC-I alleles associated with dominant Gag-specific CD8$^+$ T-cell responses could have a greater chance to control HIV replication than those without these alleles. For those individuals that do not express these MHC-I alleles, the question arises as to whether prophylactic vaccination inducing Gag epitope-specific CD8$^+$ T-cell responses might contribute to HIV control. Furthermore, recent studies have shown that CD8$^+$ T-cell responses targeting SIV antigens other than Gag, such as Mamu-B*08- or Mamu-B*17-restricted Vif and Nef epitopes, exert strong suppressive pressure on SIV replication (10, 34, 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) (36). 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* (5, 37). Unvaccinated animals possessing *90-120-Ia* dominantly elicited CD8$^+$ T-cell responses specific for the $Gag_{206-216}$ (IINEE AADWDL) and the $Gag_{241-249}$ (SSVDEQIQW) epitopes after SIV challenge (38, 39). DNA/SeV-Gag-vaccinated *90-120-Ia*-positive macaques showed enhanced $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell responses in the acute phase after SIV challenge, resulting in viremia control (37). This implies virus control by vaccine-based enhancement of Gag-specific CD8$^+$ T-cell responses in animals possessing MHC-I alleles associated with dominant Gag CD8$^+$ T-cell epitopes. However, we have not defined the efficacy of prophylactic vaccination inducing Gag-specific CD8$^+$ T-cell responses against HIV/SIV infection in the hosts pos-

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Address correspondence to Tetsuro Matano, tmatano@nih.go.jp.
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sessing MHC-I alleles not associated with dominant Gag $CD8^+$ T-cell epitopes.

In the present study, we first examined efficacy of prophylactic vaccination inducing Gag-specific $CD8^+$ T-cell responses against SIVmac239 challenge in a group of macaques that possess the *90-010-Ie* MHC-I haplotype (referred to as E) associated with dominant Nef-specific $CD8^+$ T-cell responses (39, 40). Furthermore, we examined the efficacy of prophylactic vaccination inducing Vif/Nef-specific $CD8^+$ T-cell responses in these E^+ macaques. Our results show SIV control in those vaccinees that mounted efficient Gag- or Vif-specific $CD8^+$ T-cell responses in the acute phase postchallenge.

MATERIALS AND METHODS

Animal experiments. Animal experiments were carried out in Tsukuba Primate Research Center, National Institute of Biomedical Innovation (NIBP), with the help of the Corporation for Production and Research of Laboratory Primates after approval by the Committee on the Ethics of Animal Experiments of NIBP (permission number DS21-28 and DS23-19) under the guideline for animal experiments at NIBP and National Institute of Infectious Diseases, which is in accordance with the Guidelines for Proper Conduct of Animal Experiments established by Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf). Blood collection, vaccination, and SIV challenge were performed under ketamine anesthesia.

We used Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-010-Ie* (E) (39, 40). The determination of MHC-I haplotypes was based on the family study in combination with the reference strand-mediated conformation analysis of *Mamu-A* and *Mamu-B* genes and detection of major *Mamu-A* and *Mamu-B* alleles by cloning the reverse transcription (RT)-PCR products as described previously (39–41). Confirmed MHC-I alleles consisting of the MHC-I haplotype E are *Mamu-A1*066:01*, *Mamu-B*005:02*, and *Mamu-B*015:04*. Unvaccinated R01-011, R05-007, R08-003, R08-007, R09-011, and R06-038 and Gag-vaccinated R01-010 and R01-008 used in our previous experiments (39, 42) are included in the present study. At week 1, unvaccinated macaque R06-038 was intravenously infused with 300 mg of nonspecific immunoglobulin G purified from uninfected rhesus macaques as described before (43). All animals were intravenously challenged with 1,000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (44).

Macaques R01-010, R05-010, R01-008, R08-002, and R08-006 received prophylactic DNA prime/SeV-Gag boost vaccination (referred to as Gag vaccination) (5). The DNA used for the vaccination, cytomegalovirus (CMV)-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian-human immunodeficiency virus SHIVMD14YE (45) molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIU) of F-deleted replication-defective Sendai virus (SeV) expressing SIVmac239 Gag (SeV-Gag) (46).

Macaques R08-012, R10-012, R10-013, R10-010, R10-011, and R10-014 received prophylactic DNA prime/SeV-VifNef boost vaccination (referred to as Vif/Nef vaccination). The Vif-expressing DNA used for the vaccination, pcDNA-SIVvif-opt, was constructed by introducing an optimized SIVmac239 Vif cDNA (GenScript) into pcDNA3.1. The Nef-expressing DNA used for the vaccination, pcDNA-SIVnef-G2A, has an SIVmac239 Nef cDNA with a mutation resulting in glycine (G) to alanine (A) at the 2nd amino acid (aa) in Nef. Animals intramuscularly received 3 mg of Vif-expressing DNA at the first DNA vaccination and 3 mg of Vif-expressing DNA and 3 mg of Nef-expressing DNA at the second DNA vaccination. Six weeks after the first DNA prime, animals received a single boost intranasally with 1×10^9 CIU of F-deleted SeV expressing Vif-opt (SeV-Vif) and 1×10^9 CIU of F-deleted SeV expressing Nef-G2A (SeV-Nef) (47).

Analysis of antigen-specific $CD8^+$ T-cell responses. We measured virus-specific $CD8^+$ T-cell frequencies by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (48, 49). Autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) were pulsed with each peptide (at a final concentration of 1 μM) or peptide pools (at a final concentration of 1 to 2 μM for each peptide) using panels of overlapping peptides spanning the entire SIVmac239 Gag, Vif, and Nef amino acid sequences (Sigma-Aldrich Japan) for 1 h. Peripheral blood mononuclear cells (PBMCs) were cocultured with these pulsed B-LCLs in the presence of GolgiStop (monensin; BD) for 6 h. Intracellular IFN-γ staining was performed with a Cytofix/Cytoperm kit (BD) 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 monoclonal antibodies (BioLegend). In the flow cytometric analysis, PBMCs were gated in forward scatter-side scatter dot plots, and B-LCLs were excluded in this step. Specific T-cell frequencies were calculated by subtracting nonspecific IFN-γ T-cell frequencies (less than 100 per million PBMCs) from those after peptide-specific stimulation. Specific T-cell frequencies lower than 100 per million PBMCs were considered negative.

Sequencing analysis of plasma viral genomes. Viral RNAs were extracted using the high pure viral RNA kit (Roche Diagnostics, Tokyo, Japan) from macaque plasma obtained around 1 year after challenge. Fragments of cDNAs encoding SIVmac239 Gag, Vif, and Nef were amplified by nested RT-PCR (25 cycles at the first RT-PCR using the PrimeScript one-step RT-PCR kit, version 2 [TaKaRa] and 30 cycles at the second PCR using KOD-Plus, version 2 [Toyobo]) from plasma RNAs and subjected to direct sequencing by using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan) as described before (39). Predominant nonsynonymous mutations were determined.

Statistical analysis. Statistical analysis was performed with Prism software version 4.03, with significance levels set at a P value of <0.050 (GraphPad Software, Inc.). Antigen-specific $CD8^+$ T-cell frequencies were compared by the nonparametric Mann-Whitney U test. Correlation was analyzed by the Pearson test.

RESULTS

Plasma viral loads after SIVmac239 challenge. We used a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-010-Ie* (E). In our previous study (39), unvaccinated E^+ macaques consistently showed persistent viremia after SIVmac239 challenge. $CD4^+$ T-cell percentage in PBMCs declined to less than 20% in a year. In the present study, we compared viral loads in vaccinated animals with those in these unvaccinated animals.

The first vaccine group of five E^+ macaques received a DNA prime and an SeV-Gag boost vaccination, followed by an SIVmac239 challenge. Two of these Gag-vaccinated animals failed to control viral replication, but the remaining three showed SIV control (Fig. 1). In the latter controllers, plasma viremia became undetectable in a few months. Macaques R01-008 and R08-006 rapidly controlled SIV replication and maintained high CD4 levels (Fig. 1).

The second group of six E^+ macaques received a DNA prime and an SeV-Vif/Nef boost vaccination, followed by an SIVmac239 challenge. The vaccine protocol first delivered Vif-expressing DNA, with the second vaccination consisting of Vif-expressing and Nef-expressing DNAs, and the third with Vif-expressing and Nef-expressing SeVs (SeV-Vif and SeV-Nef) with intervals of 3 weeks. After SIV challenge, three of these Vif/Nef-vaccinated an-

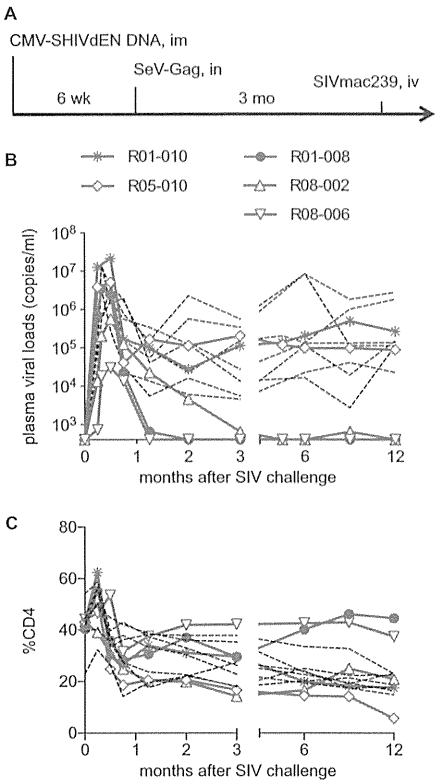

FIG 1 Viral loads and percentages of CD4 in Gag-vaccinated animals after SIVmac239 challenge. (A) Protocol of Gag vaccination and SIVmac239 challenge. (B) Plasma viral loads (SIV *gag* RNA copies/ml plasma) determined as described previously (5). The lower limit of detection is approximately 4×10^2 copies/ml. (C) Percentages of $CD4^+$ T cells in PBMCs. In panels B and C, data on unvaccinated animals ($n = 7$) are shown by dotted lines for comparison. Data on six unvaccinated (39) and two Gag-vaccinated (R01-010 and R01-008) (42) animals used in our previous studies are included.

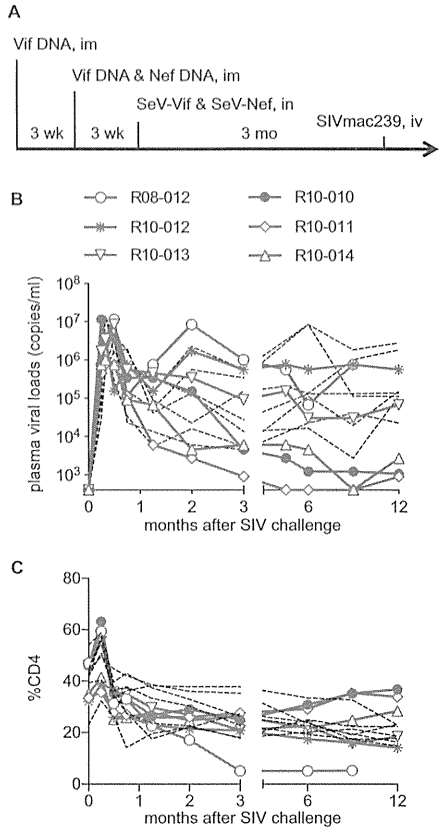

FIG 2 Viral loads and percentages of CD4 in Vif/Nef-vaccinated animals after SIVmac239 challenge. (A) Protocol of Vif/Nef vaccination and SIVmac239 challenge; (B) plasma viral loads; (C) percentages of $CD4^+$ T cells in PBMCs. In panels B and C, data on unvaccinated animals are shown by dotted lines for comparison.

imals failed to control viral replication and had high levels of set-point viral loads equivalent to those in unvaccinated macaques, but the remaining three showed SIV control with low levels of set-point viral loads (geometric mean of viral loads from 6 months to 1 year in each controller, $<2.0 \times 10^3$ copies/ml) and maintained higher CD4 levels (Fig. 2). Indeed, these six SIV controllers, consisting of three Gag-vaccinated and three Vif/Nef-vaccinated animals, showed significantly higher percentages of CD4 at 1 year than those in the remaining noncontrollers (see Fig. S1 in the supplemental material).

Gag-, Vif-, and Nef-specific $CD8^+$ T-cell responses in unvaccinated and vaccinated animals. We examined Gag-, Vif-, and Nef-specific $CD8^+$ T-cell responses in these animals. Unvaccinated macaques showed SIV-specific $CD8^+$ T-cell responses equivalent to those observed in Indian rhesus macaques (8) (Fig. 3). All of these E^+ unvaccinated macaques elicited immunodominant Nef-specific $CD8^+$ T-cell responses, consistent with our previous study analyzing other E^+ macaques (50). Gag-specific and Vif-specific $CD8^+$ T-cell responses were detected but were not immunodominant in these animals.

In contrast, all Gag-vaccinated E^+ macaques showed Gag-specific $CD8^+$ T-cell responses after the SeV-Gag boost and in the early phase after SIV challenge (Fig. 3). In these animals, Nef-specific $CD8^+$ T-cell responses mostly became immunodominant in the later phase. Importantly, all three animals that controlled SIV replication showed efficient Gag-specific $CD8^+$ T-cell responses in the acute phase postchallenge, suggesting a significant contribution of these Gag-specific $CD8^+$ T-cell responses to SIV control.

In the second group of Vif/Nef-vaccinated E^+ animals, analysis of Gag-specific, Vif-specific, and Nef-specific $CD8^+$ T-cell responses showed different patterns of responses between SIV controllers and noncontrollers (Fig. 3). In the acute phase after SIV challenge, the noncontrollers (R08-012, R10-012, and R10-013) elicited immunodominant Nef-specific $CD8^+$ T-cell responses, whereas the controllers (R10-010, R10-011, and R10-014) showed immunodominant Vif-specific $CD8^+$ T-cell responses. This suggests that the Vif-specific $CD8^+$ T-cell responses contributed to primary SIV control. In the chronic phase, Nef-specific $CD8^+$ T-cell responses were immunodominant except for one noncontroller, R10-012.

Thus, among 18 E^+ animals, consisting of seven unvaccinated, five Gag-vaccinated, and six Vif/Nef-vaccinated animals, three Gag-vaccinated and three Vif/Nef-vaccinated animals controlled SIV replication. Comparison between these six SIV controllers and the remaining 12 noncontrollers showed no significant difference in the sum of Gag-, Vif-, and Nef-specific $CD8^+$ T-cell fre-

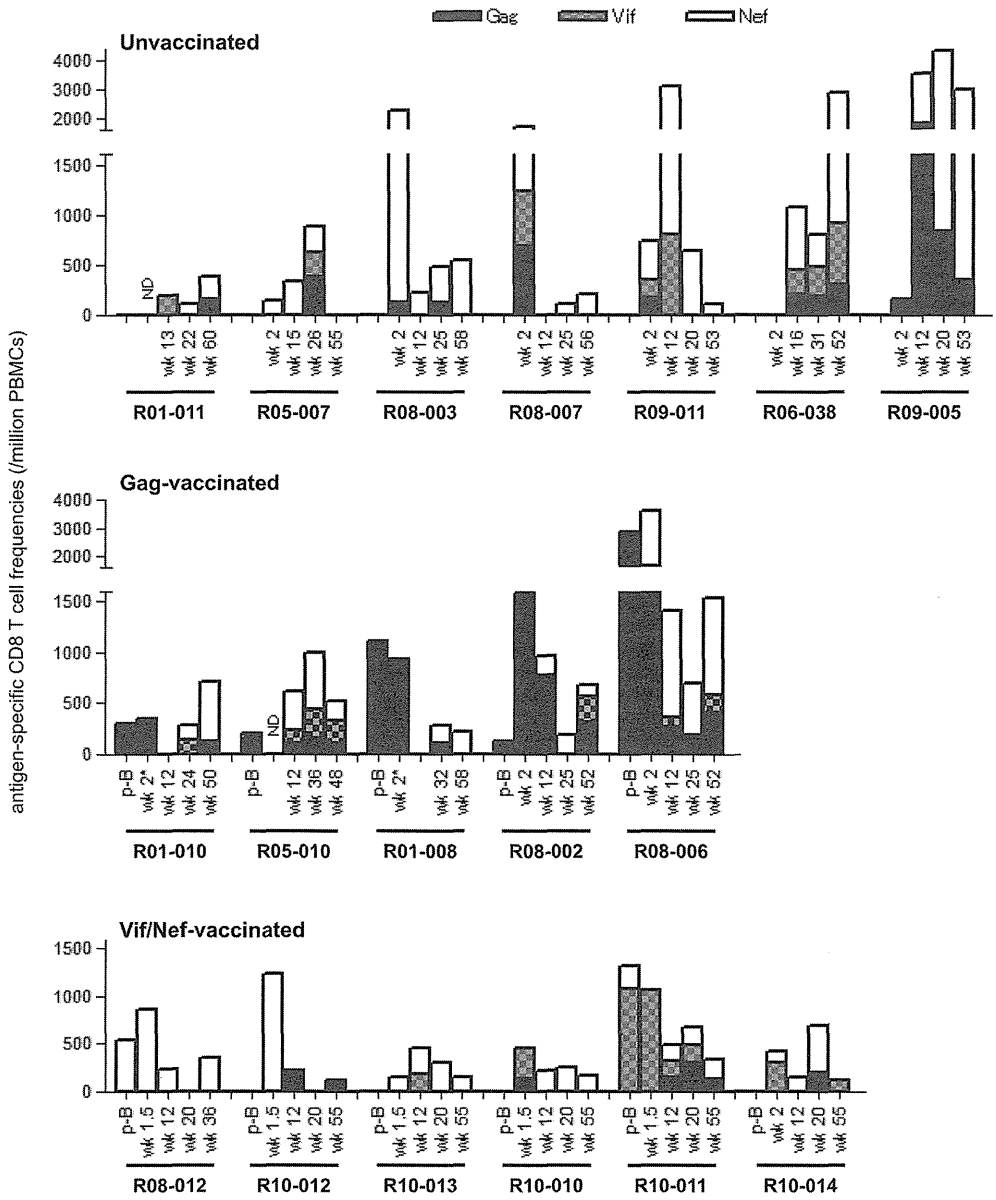

FIG 3 SIV Gag/Vif/Nef-specific CD8$^+$ T-cell responses in macaques. We examined CD8$^+$ T-cell responses specific for Gag, Vif, and Nef 1 week after SeV-Gag boost (p-B) and approximately 2 weeks, 3 months, 6 months, and 1 year after SIV challenge in unvaccinated (top), Gag-vaccinated (middle), and Vif/Nef-vaccinated (bottom) animals. We examined only Gag-specific CD8$^+$ T-cell responses but not Vif- or Nef-specific ones at week 2 in macaques R01-010 and R01-008 (indicated by asterisks). ND, not determined.

quencies in the acute phase (data not shown). The sum of Gag- and Vif-specific CD8$^+$ T-cell frequencies in the acute phase, however, was significantly higher in the controllers than in the noncontrollers ($P = 0.0031$ by Mann-Whitney U test) (Fig. 4A). Indeed, the sum of Gag- and Vif-specific CD8$^+$ T-cell frequencies in the acute phase was inversely correlated with postpeak plasma viral loads ($P = 0.0268$, $R = -0.5205$ with viral loads at 3 months [data not shown]; $P = 0.0017$, $R = -0.6849$ with viral loads at 1 year [Fig. 4B] by Pearson test). When we focused on seven unvaccinated and five Gag-vaccinated animals, three Gag-vaccinated controllers showed significantly higher Gag-specific CD8$^+$ T-cell frequencies in the acute phase than the remaining nine noncontrollers ($P = 0.0045$ by Mann-Whitney U test) (Fig. 4C). Also, in the analysis of seven unvaccinated and six Vif/Nef-vaccinated animals, Vif-specific CD8$^+$ T-cell frequencies in the acute phase were significantly higher in three Vif/Nef-vaccinated controllers than in the remaining 10 noncontrollers ($P = 0.0140$ by Mann-Whitney U test) (Fig. 4D). These results suggest that efficient Gag- or Vif-specific CD8$^+$ T-cell responses in the acute phase can result in SIV control.

Viral *gag*, *vif*, and *nef* mutations in vaccinated animals. We then tried to define the CD8$^+$ T-cell responses that might be contributing to the vaccine-based SIV control, although we were not able to map all of the CD8$^+$ T-cell epitopes because of sample

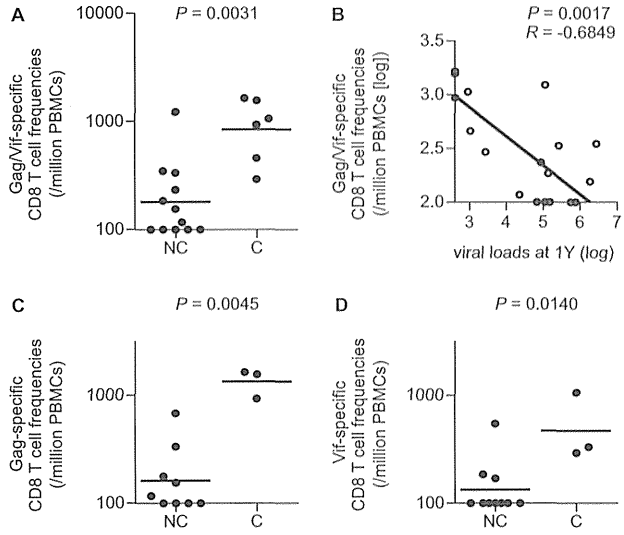

FIG 4 Comparison of Gag/Vif-specific CD8$^+$ T-cell frequencies in the acute phase between SIV controllers (C) and noncontrollers (NC). Data on Gag- and Vif-specific CD8$^+$ T-cell frequencies around week 2 postchallenge, which are shown in Fig. 3, were used. In macaques R01-011 and R05-010, samples at week 2 were unavailable, and data at week 12 were used. (A) Comparison of the sum of Gag- and Vif-specific CD8$^+$ T-cell frequencies (Gag/Vif-specific CD8$^+$ T-cell frequencies) between the controllers (three Gag-vaccinated and three Vif/Nef-vaccinated animals) and the noncontrollers in seven unvaccinated, five Gag-vaccinated, and six Vif/Nef-vaccinated animals ($n = 18$). The controllers showed significantly higher frequencies than the noncontrollers ($P = 0.0031$ by Mann-Whitney U test). (B) Correlation analysis of Gag/Vif-specific CD8$^+$ T-cell frequencies in the acute phase with plasma viral loads at 1 year. The frequencies were inversely correlated with the viral loads ($P = 0.0017$, $R = -0.6849$ by Pearson test). (C) Comparison of Gag-specific CD8$^+$ T-cell frequencies in seven unvaccinated and five Gag-vaccinated animals ($n = 12$). The three Gag-vaccinated controllers showed significantly higher frequencies than the noncontrollers ($P = 0.0045$ by Mann-Whitney U test). (D) Comparison of Vif-specific CD8$^+$ T-cell frequencies in seven unvaccinated and six Vif/Nef-vaccinated animals ($n = 13$). The three Vif/Nef-vaccinated controllers showed significantly higher frequencies than the noncontrollers ($P = 0.0140$ by Mann-Whitney U test).

limitation. Among three Gag-vaccinated controllers, R01-008, R08-002, and R08-006, our previous study found $Gag_{367-381}$-specific CD8$^+$ T-cell responses at week 5 in macaque R01-008 (5). This animal showed rapid selection of a mutation leading to an isoleucine (I)-to-threonine (T) change at the 377th aa (I377T) in SIV Gag, which results in escape from $Gag_{367-381}$-specific CD8$^+$ T-cell recognition. This suggests that these $Gag_{367-381}$-specific CD8$^+$ T-cell responses may have played an important role in SIV control. Analysis in the present study found $Gag_{385-400}$-specific CD8$^+$ T-cell responses in the acute phase with rapid selection of a mutation leading to an I-to-T change at the 391st aa (I391T) in Gag in macaque R08-006 (Fig. 5A). We confirmed that this I391T substitution results in escape from $Gag_{385-400}$-specific CD8$^+$ T-cell recognition (data not shown), suggesting a contribution of these $Gag_{385-400}$-specific CD8$^+$ T-cell responses to the control of SIV. Macaque R08-002 mounted $Gag_{273-292}$-specific CD8$^+$ T-cell responses but showed no *gag* mutation in the early phase. None of the noncontrollers selected *gag* mutations at week 5 or 6.

Among three Vif/Nef-vaccinated controllers, R10-010, R10-011, and R10-014 (Fig. 5B), macaque R10-010 mounted Vif_{65-76}-specific CD8$^+$ T-cell responses in the acute phase that resulted in the rapid selection of a mutation leading to a histidine (H)-to-tyrosine (Y) change at the 66th aa (H66Y) in Vif. Macaque R10-011 mounted $Vif_{113-132}$-specific and $Vif_{134-148}$-specific CD8$^+$ T-cell responses in the acute phase with rapid selection of a mutation leading to a Y-to-cysteine (C) change at the 143rd aa (Y143C) in Vif. We confirmed that this Y143C substitution results in escape from $Vif_{134-148}$-specific CD8$^+$ T-cell recognition (data not shown). None of the noncontrollers selected *vif* mutations at week 5 or 6. These suggest that Vif_{65-76}-specific and $Vif_{134-148}$-specific CD8$^+$ T-cell responses contributed to SIV control in macaques R10-010 and R10-011, respectively. Macaque R10-014 mounted $Vif_{113-132}$-specific CD8$^+$ T-cell responses but showed no *vif* mutation in the early phase.

In E$^+$ macaques, CD8$^+$ T-cell responses specific for Nef_{38-66} and $Nef_{101-138}$ regions were frequently observed (see Fig. S2 in the supplemental material). In all three Gag-vaccinated controllers, we confirmed both Nef_{38-66}-specific and $Nef_{101-138}$-specific CD8$^+$ T-cell responses in the chronic phase, although we did not have available samples for analysis of these responses in the acute phase. In five Vif/Nef-vaccinated animals, we confirmed Nef_{38-66}-specific CD8$^+$ T-cell responses in the acute phase, followed by $Nef_{101-138}$-specific CD8$^+$ T-cell induction. Nef_{38-66}-specific CD8$^+$ T-cell responses became undetectable at week 12 in all the three noncontrollers but were maintained at detectable levels in controllers R10-010 and R10-011.

Further mapping defined the Nef_{45-53} CD8$^+$ T-cell epitope. Mutations in the Nef_{45-53}-coding region were selected after 1 year in five of seven unvaccinated E$^+$ animals. Rapid selection of mutations at this Nef_{45-53}-coding region in a month after SIV challenge was observed in both Gag-vaccinated noncontrollers and all three Vif/Nef-vaccinated noncontrollers (Fig. 5C). In contrast, out of six Gag-vaccinated or Vif/Nef-vaccinated controllers, only one animal (R10-010) rapidly selected a mutation in this region. We confirmed that the leucine (L)-to-proline (P) substitution at the 53rd aa (L53P) in Nef results in escape from Nef_{45-53}-specific CD8$^+$ T-cell recognition (data not shown). Thus, Nef_{45-53}-specific CD8$^+$ T-cell responses may have exerted strong suppressive pressure on SIV replication in the acute phase in Gag-vaccinated or Vif/Nef-vaccinated noncontrollers.

DISCUSSION

In this study, we examined efficacy of prophylactic DNA-prime/SeV-boost vaccines against SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype E. Our previous study indicated that unvaccinated E$^+$ animals show typical courses of SIV infection and AIDS progression (39). However, three of five Gag-vaccinated and three of six Vif/Nef-vaccinated E$^+$ animals controlled SIV replication, indicating a possibility of virus control by prophylactic vaccination.

Unvaccinated E$^+$ animals showed high-frequency Nef-specific CD8$^+$ T-cell responses, particularly specific for the Nef_{38-66} and $Nef_{101-138}$ regions, after SIVmac239 challenge. The Nef_{45-53} region is a candidate for a CD8$^+$ T-cell target associated with MHC-I haplotype E, and the NefL53P mutation resulting in escape from Nef_{45-53}-specific CD8$^+$ T-cell recognition was often selected in E$^+$ animals. These results imply suppressive pressure on SIV replication by Nef-specific CD8$^+$ T-cell responses in macaques sharing this MHC-I haplotype.

Gag-vaccinated animals elicited detectable Gag-specific CD8$^+$ T-cell responses after SeV-Gag boost. All three Gag-vaccinated

A

Gag-vaccinated controllers	Gag CD8 T cell targets	gag mutations at wk 5
R08-002	Gag273-292	none
R08-006	Gag385-400	I391T

B

Vif/Nef-vaccinated controllers	Vif CD8 T cell targets	vif mutations at wk 6
R10-010	Vif65-76	H66Y
R10-011	Vif113-132 & 134-148	Y143C
R10-014	Vif113-132	none

C

		Nef45-53 GLDKGLSSL			Nef45-53 GLDKGLSSL
Unvaccinated					
R01-011	1 mo	--------F	R09-011	1 mo	---------
	6 mo	-S-------		6 mo	--------P
	1 yr	----C----		1 yr	--G-----P
R05-007	1 mo	---------	R06-038	1 mo	---------
	6 mo	---------		6 mo	---------
	1 yr	---------		1 yr	---------
R08-003	1 mo	---------	R09-005	1 mo	---------
	6 mo	--G------		6 mo	--G------
	1 yr	--------R		1 yr	--G-----H
R08-007	1 mo	---------			
	6 mo	-S-------			
	1 yr	-S-------			
Gag-vaccinated non-controllers			controllers		
R01-010	1 mo	--------P	R01-008	1 mo	---------
	6 mo	--G-----P	R08-002	1 mo	---------
	1 yr	--G------		6 mo	E--------
R05-010	1 mo	E--------		1 yr	E--------
	6 mo	E-G------	R08-006	1 mo	---------
	1 yr	--G------		6 mo	---------
				1 yr	---------
Vif/Nef-vaccinated non-controllers			controllers		
R08-012	1 mo	--------P	R10-010	1 mo	A--------
	6 mo	--------P		6 mo	E-------P
R10-012	1 mo	E----S---		1 yr	E--------
	6 mo	----D---P	R10-011	1 mo	---------
	1 yr	-----L--P		6 mo	---------
R10-013	1 mo	--------R		1 yr	---------
	6 mo	--------R	R10-014	1 mo	---------
	1 yr	--G------		6 mo	---------
				1 yr	---------

FIG 5 Predominant nonsynonymous mutations in CD8$^+$ T-cell target-coding regions. (A) Gag target regions for CD8$^+$ T-cell responses in the acute phase in Gag-vaccinated controllers. Macaque R01-008 induced $Gag_{367-381}$-specific CD8$^+$ T-cell responses and selected I377T mutation in 5 weeks as described before (5). (B) Vif target regions for CD8$^+$ T-cell responses in the acute phase in Vif/Nef-vaccinated controllers. (C) Nonsynonymous mutations in Nef_{45-53} CD8$^+$ T-cell epitope-coding regions of viral cDNAs at 1 month (1 mo), 6 months (6 mo), and 1 year (1 yr). Amino acid substitutions are shown.

controllers showed efficient Gag-specific CD8$^+$ T-cell responses in the acute phase after SIV challenge. In particular, macaques R01-008 and R08-006 showed rapid SIV control without detectable plasma viremia after week 5. $Gag_{367-381}$-specific CD8$^+$ T-cell responses with rapid selection of a $Gag_{367-381}$-specific CD8$^+$ T-cell escape mutation, I377T, were observed in R01-008, whereas $Gag_{385-400}$-specific responses were associated with an escape mutation, I391T, in R08-006. Our results suggest that the prophylactic Gag vaccination results in the efficient induction of these Gag-specific CD8$^+$ T-cell responses in the acute phase, which then played an important role in the control of primary SIV replication. The MHC-I haplotypes other than E (see Table S1 in the supplemental material) may be associated with these effective Gag epitope-specific CD8$^+$ T-cell responses. Nef-specific CD8$^+$ T-cell responses became predominant after 3 or 6 months.

Vif/Nef-vaccinated animals induced Vif- or Nef-specific CD8$^+$ T-cell responses in the acute phase after SIVmac239 challenge. Before challenge, detectable Vif-specific CD8$^+$ T-cell responses were elicited after SeV-Vif/Nef boost only in macaque R10-011. It should be noted, however, that all three Vif/Nef-vaccinated controllers showed high-frequency Vif-specific CD8$^+$ T-cell responses in the acute phase, while the three noncontrollers exhibited Nef-specific CD8$^+$ T-cell responses. In particular, our results implicate Vif_{65-76}-specific and $Vif_{134-148}$-specific CD8$^+$ T-cell responses in the control of primary viral replication in macaques R10-010 and R10-011, respectively. These CD8$^+$ T-cell responses may be associated with the second MHC-I haplotypes (see Table S1 in the supplemental material). Even Vif/Nef-vaccinated controllers inducing Vif-specific CD8$^+$ T-cell responses in the acute phase showed predominant Nef-specific CD8$^+$ T-cell responses in the chronic phase.

Vif/Nef-vaccinated noncontrollers showed no Vif-specific CD8$^+$ T-cell responses but mounted Nef-specific CD8$^+$ T-cell responses in the acute phase. All three noncontrollers rapidly se-

lected *nef* mutations in the Nef_{45-53}-coding regions, and Nef_{45-53}-specific $CD8^+$ T-cell responses were undetectable after 3 months postchallenge. Interestingly, both Gag-vaccinated noncontrollers also showed rapid selection of *nef* mutations in the Nef_{45-53}-coding regions. We speculate that, in these Gag-vaccinated or Vif/Nef-vaccinated noncontrollers, dominant Nef_{45-53}-specific $CD8^+$ T-cell responses may have exerted strong suppressive pressure on primary SIV replication without the help of other vaccine antigen-specific, effective $CD8^+$ T-cell responses, leading to failure in virus control with rapid selection of escape mutations. Unvaccinated macaque R08-007 elicited Gag- and Vif-specific as well as Nef-specific $CD8^+$ T-cell responses in the acute phase but failed to control SIV replication. The high magnitude of responses may reflect the highest peak viral loads (1.4×10^7 copies/ml) at day 10 in this animal among the unvaccinated. These naive-derived Gag- and Vif-specific $CD8^+$ T-cell responses may have been less functional and insufficient for SIV control. In contrast, in vaccinated controllers, prophylactic vaccination resulted in effective Gag- or Vif-specific $CD8^+$ T-cell responses postexposure, leading to primary SIV control, followed by Nef-specific $CD8^+$ T-cell responses possibly contributing to maintenance of virus control. Induction of $CD8^+$ T-cell responses specific for dominant Nef epitopes by prophylactic vaccination may not be good for SIV control in E^+ animals. Several studies have indicated contribution of subdominant $CD8^+$ T-cell responses to HIV or SIV suppression (51–53). Thus, induction of $CD8^+$ T-cell responses specific for subdominant but not dominant epitopes by prophylactic vaccination may be a promising AIDS vaccine strategy resulting in effective, broader $CD8^+$ T-cell responses postexposure.

In summary, this study demonstrates SIV control by prophylactic vaccination in hosts possessing MHC-I alleles associated with dominant non-Gag antigen-specific $CD8^+$ T-cell responses. Our results suggest that prophylactic vaccination resulting in effective subdominant Gag/Vif epitope-specific $CD8^+$ T-cell responses in the acute phase postexposure can lead to primary HIV control. This may imply a rationale of altering the hierarchy of postexposure $CD8^+$ T-cell immunodominance toward HIV control.

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Identification of SIV Nef CD8$^+$ T cell epitopes restricted by a MHC class I haplotype associated with lower viral loads in a macaque AIDS model

Takushi Nomura [a,*], Hiroyuki Yamamoto [a], Naofumi Takahashi [a], Taeko K. Naruse [b], Akinori Kimura [b], Tetsuro Matano [a,c,*]

[a] AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[b] Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan
[c] The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

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ABSTRACT

Virus-specific CD8$^+$ T-cell responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. Multiple studies on HIV-infected individuals and SIV-infected macaques have indicated association of several major histocompatibility complex class I (MHC-I) genotypes with lower viral loads and delayed AIDS progression. Understanding of the viral control mechanism associated with these MHC-I genotypes would contribute to the development of intervention strategy for HIV control. We have previously reported a rhesus MHC-I haplotype, *90-120-Ia*, associated with lower viral loads after SIVmac239 infection. $Gag_{206-216}$ and $Gag_{241-249}$ epitope-specific CD8$^+$ T-cell responses have been shown to play a central role in the reduction of viral loads, whereas the effect of Nef-specific CD8$^+$ T-cell responses induced in all the *90-120-Ia*$^+$ macaques on SIV replication remains unknown. Here, we identified three CD8$^+$ T-cell epitopes, Nef_{9-19}, Nef_{89-97}, and $Nef_{193-203}$, associated with *90-120-Ia*. Nef_{9-19} and $Nef_{193-203}$ epitope-specific CD8$^+$ T-cell responses frequently selected for mutations resulting in viral escape from recognition by these CD8$^+$ T cells, indicating that these CD8$^+$ T cells exert strong suppressive pressure on SIV replication. Results would be useful for elucidation of the viral control mechanism associated with *90-120-Ia*.

1. Introduction

In human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections, host immune responses fail to eradicate viruses and allow persistent infection, leading to AIDS progression. Unlike most acute virus infections, effective neutralizing antibody responses are not efficiently induced in early HIV/SIV infection [1]. Virus-specific CD8$^+$ T-cell responses play an important role in the control of HIV/SIV replication [2–6]. CD8$^+$ T cells recognize antigenic peptides bound to polymorphic major histocompatibility complex class I (MHC-I) molecules, whose genotypes affect CD8$^+$ T-cell responses [7,8]. Several MHC-I genotypes have been shown to be associated with lower viral loads and slower disease progression in HIV/SIV infections [9–14]. Understanding of the viral control mechanism associated with these protective MHC-I alleles would contribute to the development of intervention strategy for HIV control.

Recent vaccine trials in macaque AIDS models have shown a possibility of SIV control by effective CD8$^+$ T-cell responses [15–19]. It has been indicated that CD8$^+$ T cells targeting Gag are effective against HIV/SIV infection [20–23]. Furthermore, current studies have suggested that Nef- and Vif-specific CD8$^+$ T-cell responses can contribute to SIV control in macaque AIDS models [24,25].

We have previously reported a rhesus MHC-I haplotype, *90-120-Ia*, associated with lower viral loads after SIVmac239 challenge [14]. In that study, those Burmese rhesus macaques possessing *90-120-Ia* had lower set-point plasma viral loads (geometric mean at 1 year after SIV challenge: 1.5×10^4 copies/ml); two of them controlled viremia for more than 4 years while the remaining four developed AIDS in 4 years. Our vaccine trial has shown that all the *90-120-Ia*$^+$ macaques immunized with DNA-prime/Gag-expressing Sendai virus (SeV-Gag) vector-boost controlled a SIVmac239 challenge [26]. Mamu-A1*043:01-restricted $Gag_{206-216}$ (IINEEAADWDL) and Mamu-A1*065:01-restricted $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CD8$^+$ T-cell responses were responsible for this viral control

* Corresponding authors. Address: AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Fax: +81 3 5285 1165.
E-mail addresses: nomutaku@nih.go.jp (T. Nomura), tmatano@nih.go.jp (T. Matano).

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[26–28]. SIVmac239-infected *90-120-Ia*$^+$ macaques also elicited CD8$^+$ T-cell responses targeting Nef, which may be involved in viral control [14]. In the present study, we determined Nef CD8$^+$ T-cell epitopes associated with this MHC-I haplotype *90-120-Ia*.

2. Materials and methods

2.1. Samples

The present study used frozen plasma and peripheral mononuclear cell (PBMC) samples derived from ten Burmese rhesus macaques (*Macaca mulatta*) possessing MHC-I haplotype *90-120-Ia*. Our previous SIVmac239 challenge experiments using these animals [14,26–28] have been carried out in Tsukuba Primate Research Center in National Institute of Biomedical Innovation (NIBP) with the help of the Corporation for Production and Research of Laboratory Primates. These studies were approved by the Committee on the Ethics of Animal Experiments of NIBP under the guideline for animal experiments at NIBP and the National Institute of Infectious Diseases which is in accordance with the Guidelines for Proper Conduct of Animal Experiments established by the Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf).

Macaques R06-037, R07-004, and R07-009 were unvaccinated and intravenously challenged with SIVmac239 [14]. Macaques R03-018 and R07-007 received a DNA-prime/SeV-boost vaccine eliciting $Gag_{206-216}$- and $Gag_{241-249}$-specific CD8$^+$ T-cell responses, respectively, before SIVmac239 challenge as described before [27,28]. Macaques R06-035, R06-041, R05-004, R05-027, and R07-005 received a DNA-prime/SeV-Gag-boost as described before [26]. Macaques R06-035 and R06-041 were intravenously challenged with SIVmac239Gag216S244E, a SIVmac239 carrying two *gag* mutations, GagL216S and GagD244E, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) and an aspartic acid (D)-to-glutamic acid (E) substitution at the 244th aa in Gag, whereas macaques R05-004, R05-027, and R07-005 with SIVmac239Gag216S244E247L312V373T, a SIVmac239 carrying five *gag* mutations, GagL216S, GagD244E, GagI247L (isoleucine [I] to L at the 247th aa), GagA312V (alanine [A] to valine [V] at the 312th aa), and GagA373T (A to threonine [T] at the 373rd aa) [26].

The determination of macaque MHC-I haplotypes was based on the family study in combination with the reference strand-mediated conformation analysis of *Mamu-A* and *Mamu-B* genes and detection of major *Mamu-A* and *Mamu-B* alleles by cloning the reverse transcription (RT)-PCR products [14,29,30]. Confirmed MHC-I alleles consisting of the MHC-I haplotype *90-120-Ia* are *Mamu-A1*043:01* (GenBank accession number AB444869), *Mamu-A1*065:01* (AB444921), *Mamu-B*061:03* (AB430442), *Mamu-B*068:04* (AM902571), and *Mamu-B*089:01* (EF580172).

2.2. Sequencing analysis of plasma viral genomes

Viral RNAs were extracted using the High Pure Viral RNA kit (Roche Diagnostics) from plasma. Fragments of cDNAs encoding SIVmac239 Nef were amplified by nested RT-PCR from plasma RNAs and subjected to direct sequencing by using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems) [33].

2.3. Analysis of SIV peptide-specific CD8$^+$ T-cell responses

SIV peptide-specific CD8$^+$ T-cell responses were measured by flow-cytometric analysis of interferon-γ (IFN-γ) induction [25]. PBMCs (2.5×10^6 cells) were cocultured for 6 h with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs; 1.0×10^6 cells) pulsed with 1–5 μM or indicated concentrations of peptides designed for epitope mapping in 96-well V-bottom microwell plates. Intracellular IFN-γ staining was performed using a Cytofix Cytoperm kit (BD). Fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD), allophycocyanin Cy7 (APC-Cy7)-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (Biolegend) were used. Specific T-cell frequencies were calculated by subtracting nonspecific IFN-γ$^+$ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell frequencies less than 100 cells per million PBMCs were considered negative.

3. Results and discussion

3.1. Identification of three Nef CD8$^+$ T-cell epitopes associated with MHC-I haplotype 90–120-Ia

In our previous study [14], we examined viral genome sequences 1 year after SIVmac239 challenge in four groups of Burmese rhesus macaques possessing MHC-I haplotypes *90-120-Ia*, *90-120-Ib*, *90-010-Ie*, and *90-088-Ij*, respectively. Then, in the present study, we compared *nef* sequences in the four macaques possessing *90-120-Ia* with those in the remaining three groups ($n = 14$). Amino acid sequences revealed three regions in Nef, Nef12 (the 12th aa), Nef89/90 (the 89th or 90th aa), and Nef201/202 (the 201st or 202nd aa), which had substitutions in all *90-120-Ia*$^+$ animals but mostly not in others. Indeed, substitutions at Nef12 were observed only in two of the fourteen *90-120-Ia*-negative animals while substitutions at Nef89/90 or Nef201/202 were detected in none of them.

We tried to map CD8$^+$ T-cell epitopes around the regions described above to examine whether these *90-120-Ia*-associated *nef* mutations resulting in the Nef12, Nef89/90, and Nef201/202 amino acid substitutions were selected by CD8$^+$ T cells. Analysis using available samples derived from ten *90-120-Ia*$^+$ macaques identified three CD8$^+$ T-cell epitopes, Nef_{9-19} (RSRPSGDLRQR), Nef_{89-97} (DIDEEDDDL), and $Nef_{193-203}$ (YLMHPAQTSQW) (Fig. 1A). The endpoint peptide concentrations for CD8$^+$ T-cell responses were 10–100 nM against Nef_{9-19} epitope and 1–10 nM against $Nef_{193-203}$ (Fig. 1B). The endpoint was very low, less than 0.1 nM, for Nef_{89-97} epitope (Fig. 1B), indicating extremely high binding affinity of this epitope.

3.2. Determination of MHC-I alleles restricting CD8$^+$ T-cell epitopes

Nef_{9-19} epitope-specific CD8$^+$ T-cell responses in the early phase of SIV infection were examined in six *90-120-Ia*$^+$ animals, all of which showed positive responses (Fig. 2A), indicating that this epitope is associated with MHC-I haplotype *90-120-Ia*. On the other hand, Nef_{89-97} and $Nef_{193-203}$ epitope-specific CD8$^+$ T-cell responses in the early phase were detected not in all but in three of the seven and two of the four examined *90-120-Ia*$^+$ animals, respectively (Fig. 2A).

We then tried to determine *90-120-Ia*-derived MHC-I alleles restricting these CD8$^+$ T-cell epitopes. HLA-A/B/C-negative human 721.221 cell lines expressing *Mamu-A1*043:01*, *Mamu-A1*065:01*, and *Mamu-B*061:03* were available for the analysis. Nef_{89-97}-specific CD8$^+$ T-cell responses were detected on *Mamu-A1*043:01*-expressing 721.221 cells, whereas $Nef_{193-203}$-specific CD8$^+$ T-cell responses were detected on *Mamu-A1*065:01*-expressing 721.221 cells (Fig. 2B). These results indicate that the Nef_{89-97} and $Nef_{193-203}$ epitopes are restricted by Mamu-A1*043:01 and Mamu-A1*065:01, respectively. However, Nef_{9-19} epitope-specific CD8$^+$ T-cell responses were not detected on any of 721.221 cells expressing *Mamu-A1*043:01*, *Mamu-A1*065:01*, or *Mamu-B*061:03*,

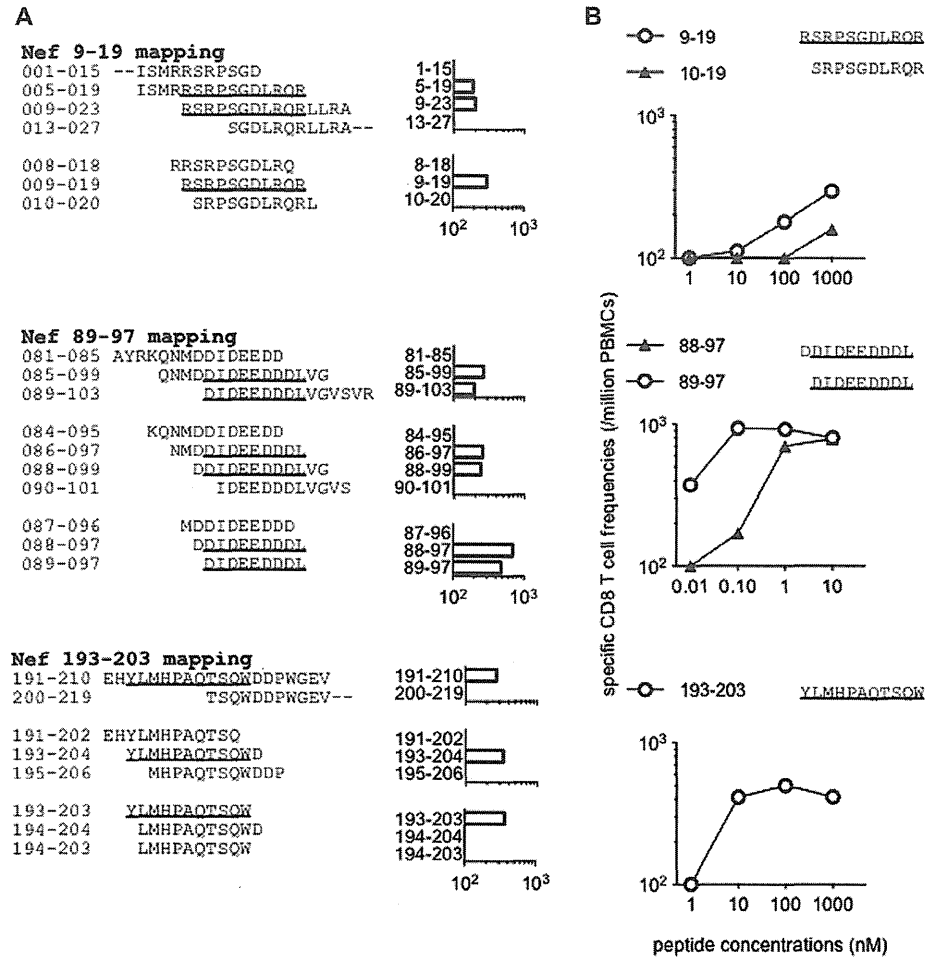

Fig. 1. Mapping of CD8$^+$ T-cell epitopes, Nef_{9-19}, Nef_{89-97}, and $Nef_{193-203}$. (A) Summarized data for mapping of Nef_{9-19} (top panels), Nef_{89-97} (middle), and $Nef_{193-203}$ (bottom) epitopes using PBMCs of SIV-infected *90-120-Ia*$^+$ animals. CD8$^+$ T-cell frequencies specific for the indicated peptides are shown (/million PBMCs). Representative results using PBMCs from R05-027 and R06-041 (top), R03-018 (middle), and R07-004 and R07-007 (bottom) are shown. (B) CD8$^+$ T-cell responses under the indicated concentrations of Nef_{9-19} and Nef_{10-19} (top panel), Nef_{88-97} and Nef_{89-97} (middle), and $Nef_{193-203}$ (bottom) peptides. Representative results using PBMCs from R05-004 (top), R03-018 (middle), and R07-009 (bottom) are shown.

implying that this epitope is restricted by a *90-120-Ia*-derived MHC-I molecule other than the above three (Fig. 2B).

3.3. Mutations resulting in viral escape from CD8$^+$ T-cell recognition

In our previous study [14], SIV-infected *90-120-Ia*$^+$ macaques had mutations resulting in Nef12, Nef89/90, and Nef201/202 amino acid substitutions as described above. The substituted amino acids were different at Nef89/90 in individual four animals, but three of the four had the same substitutions at Nef12 and Nef201, NefP12Q (proline [P]-to-glutamine [Q]) and NefS201Y (S-to-tyrosine [Y]), respectively. We examined whether these two *90-120-Ia*-associated *nef* mutations result in viral escape from CD8$^+$ T-cell responses specific for the epitopes we identified. Nef_{9-19} peptide-specific CD8$^+$ T-cell responses were reduced by the NefP12Q substitution (Fig. 3A). Also, the NefP12T substitution (a P-to-T substitution at the 12th aa in Nef) that was observed in the remaining one SIV-infected *90-120-Ia*$^+$ macaque resulted in viral escape from Nef_{9-19}-specific CD8$^+$ T-cell responses. $Nef_{193-203}$ peptide-specific CD8$^+$ T-cell responses were reduced by the NefS201Y (Fig. 3A). Selection of these escape mutations in SIV infection implies that these Nef_{9-19} and $Nef_{193-203}$ epitope-specific CD8$^+$ T cells exert suppressive pressure on SIV replication. The latter $Nef_{193-203}$ epitope overlaps with previously-reported MW9 ($Nef_{195-203}$) and HW8 ($Nef_{196-203}$) epitopes [32,33]. The MW9 is restricted by Mamu-B*17 [12], a protective MHC-I against SIVmac239 infection, while the HW8 is restricted by a MHC-I in a group of Mauritian cynomolgus macaques that frequently control SIVmac239 replication. Thus, this $Nef_{193-203}$ region may be a promising CD8$^+$ T-cell target for SIV control.

Further analysis of viral *nef* nucleotide sequences found relatively rapid selection of a mutation encoding Nef12, NefP12Q, NefP12S, or NefS13P, in two months after SIV infection in macaques R05-004, R05-027, R06-035, R06-041, and R07-005 (Fig. 3B). The NefP12S and NefS13P substitutions also resulted in viral escape from Nef_{9-19}-specific CD8$^+$ T-cell responses (Fig. 3A). However, no mutation was selected in the region encoding Nef_{89-97} or $Nef_{193-203}$ epitope in two months (Fig. 3B). In the early phase, Nef_{9-19}-specific CD8$^+$ T-cell responses were induced in all whereas Nef_{89-97}- and $Nef_{193-203}$-specific CD8$^+$ T-cell responses were detectable only in some of them, as described above. These results suggest that *90-120-Ia*$^+$ macaques predominantly elicit Nef_{9-19}-specific CD8$^+$ T-cell responses resulting in selection of Nef12/13 mutations in the early phase of SIV infection, followed by induction of Nef_{89-97}- and $Nef_{193-203}$-specific CD8$^+$ T-cell responses resulting in selection of Nef89/90 and Nef201/202 mutations in the chronic phase.

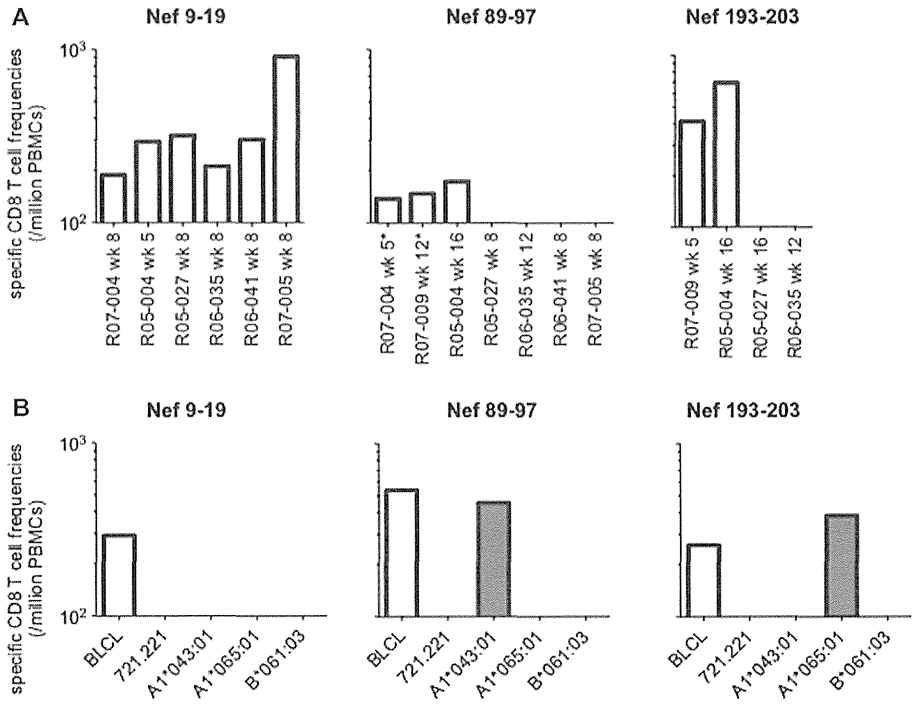

Fig. 2. Nef_{9-19}, Nef_{89-97}, and $Nef_{193-203}$ epitope-specific $CD8^+$ T-cell responses. (A) $CD8^+$ T-cell responses specific for Nef_{9-19} (left panel), Nef_{89-97} (middle), and $Nef_{193-203}$ (right) epitopes in the early phase of SIV infection in *90-120-Ia*$^+$ macaques. The asterisk indicates $CD8^+$ T-cell responses specific for Nef_{86-97} peptide. (B) Nef_{9-19}- (left panel), Nef_{89-97}- (middle), and $Nef_{193-203}$-specific (right) $CD8^+$ T-cell responses after coculture with peptide-pulsed B-LCLs, 721.221 cells, or 721.221 cells expressing *Mamu-A1**043:01*, *Mamu-A1**065:01*, or *Mamu-B**061:03*. These cells were pulsed with 1,000 nM Nef_{9-19} peptides (left), 1 nM Nef_{89-97} peptides (middle), and 100 nM $Nef_{193-203}$ peptides (right), respectively. Representative results using PBMCs from R06-035 for Nef_{9-19}, R03-018 for Nef_{89-97}, and R07-004 for $Nef_{193-203}$ are shown.

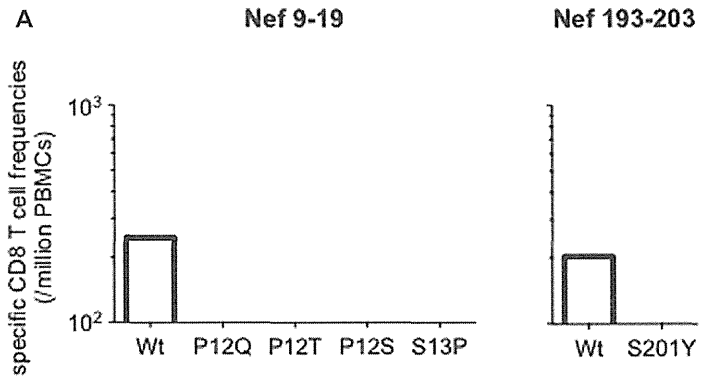

Fig. 3. $CD8^+$ T-cell escape mutations. (A) $CD8^+$ T-cell responses specific for the wild-type or mutant Nef_{9-19} peptides with the indicated substitutions (left panel) or the wild-type or a mutant $Nef_{193-203}$ peptide with S201Y substitution (right). Representative results using PBMCs from R07-005 for Nef_{9-19} and R07-004 for $Nef_{193-203}$ are shown. (B) Predominant nonsynonymous mutations in plasma viral *nef* regions encoding Nef_{9-19}, Nef_{89-97}, and $Nef_{193-203}$ epitopes in 3 months after SIV challenge in *90-120-Ia*$^+$ macaques. Amino acid substitutions are shown.

Our previous study [14] frequently found $CD8^+$ T-cell responses targeting Vif and a viral genome mutation resulting in VifP115S substitution (a P-to-S substitution at the 115th aa in Vif) in SIV-infected *90-120-Ia*$^+$ macaques. Then, in the present study, we identified a $CD8^+$ T-cell epitope, $Vif_{114-124}$ (FPCFTAGEVRR). The endpoint peptide concentration for $Vif_{114-124}$-specific $CD8^+$ T-cell

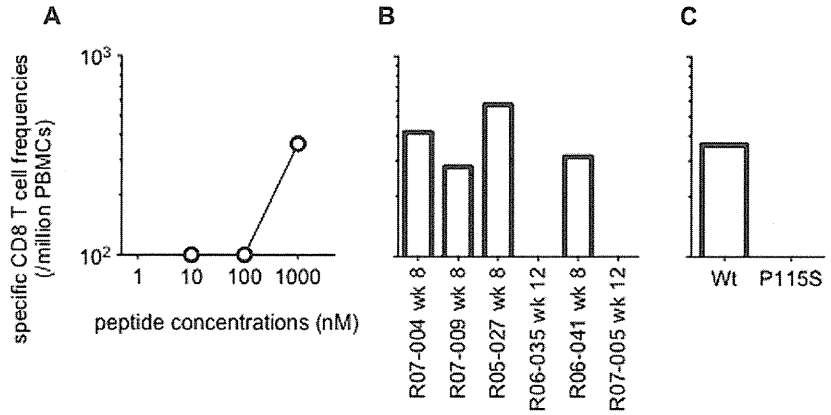

Fig. 4. Characterization of $Vif_{114-124}$ epitope-specific $CD8^+$ T-cell responses. (A) $CD8^+$ T-cell responses under the indicated concentrations of $Vif_{114-124}$ peptides. A representative result using PBMCs from R07-004 is shown. (B) $Vif_{114-124}$-specific $CD8^+$ T-cell responses in the early phase of SIV infection in *90-120-Ia*$^+$ macaques. (C) $CD8^+$ T-cell responses specific for the wild-type or a mutant $Vif_{114-124}$ peptide with P115S substitution. A representative result using PBMCs from R07-004 is shown.

responses was very high, more than 100 nM, indicating lower binding affinity of this epitope (Fig. 4A). $Vif_{114-124}$ epitope-specific $CD8^+$ T-cell responses were detected in the early phase in four of the six examined *90-120-Ia*$^+$ animals (Fig. 4B). The VifP115S substitution resulted in diminishment of $Vif_{114-124}$ peptide-specific $CD8^+$ T-cell responses, suggesting selective pressure by $CD8^+$ T cells targeting this epitope (Fig. 4C).

In summary, we identified three *90-120-Ia*-associated Nef $CD8^+$ T-cell epitopes, Nef_{9-19}, Nef_{89-97}, and $Nef_{193-203}$, in addition to the three previously-identified Gag epitopes, $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ [31]. Additionally, we identified a Vif $CD8^+$ T-cell epitope, $Vif_{114-124}$. In our previous study [26], all the *90-120-Ia*$^+$ macaques vaccinated with DNA-prime/SeV-Gag-boost controlled SIVmac239 replication without detectable viral loads after week 5 post-challenge, whereas those vaccinated animals (R05-004, R05-027, R06-035, R06-041, and R07-005) failed to show such rapid control of a challenge with SIVs carrying $Gag_{206-216}$-, $Gag_{241-249}$-, and $Gag_{373-380}$-specific $CD8^+$ T-cell escape mutations. This indicates that these $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitope-specific $CD8^+$ T-cell responses are responsible for the rapid SIVmac239 control. Macaques R05-004 and R05-027 showed persistent viremia and developed AIDS, whereas the remaining three (R06-035, R06-041, and R07-005) exhibited lower viral loads. The present study suggests involvement of Nef epitope-specific $CD8^+$ T-cell responses in this suppression of SIV replication in *90-120-Ia*$^+$ macaques.

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Generation of a monkey-tropic human immunodeficiency virus type 1 carrying *env* from a CCR5-tropic subtype C clinical isolate

Hiroyuki Otsuki, Mai Yoneda, Tatsuhiko Igarashi, Tomoyuki Miura *

Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, 53 Shogoin Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan

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ABSTRACT

Several derivatives of human immunodeficiency virus type 1 (HIV-1) that evade macaque restriction factors and establish infection in pig-tailed macaques (PtMs) have been described. These monkey-tropic HIV-1s utilize CXCR4 as a co-receptor that differs from CCR5 used by most currently circulating HIV-1 strains. We generated a new monkey-tropic HIV-1 carrying *env* from a CCR5-tropic subtype C HIV-1 clinical isolate. Using intracellular homologous recombination, we generated an uncloned chimeric virus consisting of at least seven types of recombination breakpoints in the region between *vpr* and *env*. The virus increased its replication capacity while maintaining CCR5 tropism after *in vitro* passage in PtM primary lymphocytes. PtM infection with the adapted virus exhibited high peak viremia levels in plasma while the virus was undetectable at 12–16 weeks. This virus serves as starting point for generating a pathogenic monkey-tropic HIV-1 with CCR5-tropic subtype C *env*, perhaps through serial passage in macaques.

Introduction

Nonhuman primate models with human-like immune systems are often employed to evaluate the efficacy of candidate vaccines against acquired immune deficiency syndrome (AIDS). However, human immunodeficiency virus type 1 (HIV-1) infects humans or chimpanzees (*Pan troglodytes*) but not rhesus macaques (*Macaca mulatta*), the most widely used primate species in biomedical research (Gibbs et al., 2007). Experimental infection of macaques with simian immunodeficiency virus (SIV) or simian-human immunodeficiency virus (SHIV) has been used extensively to investigate HIV-1 infection *in vivo*. Pathogenic infection with SIV allows insight into the mechanisms of pathogenesis and provides information for development of novel vaccination strategies. However, due to the marked antigenic difference in viral proteins between HIV-1 and SIV, macaque models with SIV are not suitable for evaluating the immune response directed against HIV-1 (Javaherian et al., 1992; Kanki et al., 1985; Murphey-Corb et al., 1986). SHIV, a chimeric virus carrying *tat, rev, vpu* and *env* from HIV-1 with an SIV genetic backbone, has been constructed and used widely to assess the immune response and pathogenicity directed against HIV-1 Env (Shibata and Adachi, 1992; Reimann et al., 1996; Harouse et al., 1999)

Highly pathogenic SHIV irreversibly depletes circulating $CD4^+$ T-lymphocytes, and cause rapidly AIDS-like symptoms in infected macaques. These properties are, however, different from the vast majority of circulating HIV-1 or SIV isolates, and the discrepancy would be attributed to the viral co-receptor preference (Nishimura et al., 2004). Entry of HIV-1 into cells is mediated through the interaction of viral envelope protein with cellular CD4 and subsequent binding to either the CCR5 or CXCR4 chemokine receptor or both receptors. The vast majority of HIV-1 clinical isolates preferentially utilize CCR5 as the co-receptor for entry (Choe et al., 1996). The CXCR4-tropic or dual-tropic viruses that utilize both CCR5 and CXCR4 emerge during late stages in the disease course (Doranz et al., 1996; Feng et al., 1996).

In addition to the co-receptor usage, it is necessary to consider the variation of *env* gene in SHIV construction. Most HIV-1 strains currently circulating belong to group M, consisting of subtypes A–D, F–H, J, K and their recombinants, and are largely responsible for the global AIDS pandemic (Hemelaar, 2012). Most of early SHIVs are generated by utilizing genes derived from subtype B

* Corresponding author. Tel.: +81 75 751 3984; fax: +81 75 761 9335.
E-mail address: tmiura@virus.kyoto-u.ac.jp (T. Miura).

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0042-6822/

viruses, which comprise an estimated 11% of the global prevalence of HIV-1. By contrast, subtype C is the dominant subtype, accounting for almost 50% of global infections. Subtype C viruses do not share the antigenicity of Env as the main target of neutralizing antibodies with subtype B viruses (Choisy et al., 2004; Gaschen et al., 2002). The V3 loop region of the subtype C envelope is less variable than that of other subtypes (Kuiken et al., 1999), and mutations appear to accumulate in the C3 and V4 regions, which are targets of autologous neutralizing antibody responses in individuals infected with subtype C viruses (Moore et al., 2008, 2009). The structure of these epitopes is dissimilar between subtypes B and C (Gnanakaran et al., 2007). There are pathogenic SHIVs that encode CCR5 tropic subtype C env gene (Ndung'u et al., 2001; Ren et al., 2013; Song et al., 2006).

Conventional SHIV that encodes SIV sequence in 5′ half of the genome has limited utility in the evaluation of cell-mediated immunity induced by a vaccine because it does not contain HIV-1 Gag in its genome; consequently, SHIV has different major epitopes for cytotoxic T lymphocytes (CTLs) known to be associated with lowering the plasma viral load in HIV-1 infection (Goulder and Watkins, 2004; Kiepiela et al., 2007). Recently, two major restriction factors were reported to block HIV-1 replication in monkey cells in a species-specific manner (Neil and Bieniasz, 2009). The restriction factor apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) protein is incorporated into viral particles and induces hypermutation in proviral DNA in target cells mediated by its cytidine deaminase activity (Sheehy et al., 2002). Macaque APOBEC3G proteins are counteracted by the SIV Vif protein but not by HIV-1 Vif (Mariani et al., 2003). The other major restriction factor that inhibits the viral replication cycle is tripartite motif 5α (TRIM5α) protein, which directly recognizes incoming viral capsid (CA) (Stremlau et al., 2004). HIV-1 CA can bind cyclophilin A (CypA), a ubiquitous cytosolic protein, to evade restriction by human TRIM5α, whereas the CypA-binding activity appears to enhance TRIM5α recognition in macaque cells (Berthoux et al., 2005; Keckesova et al., 2006; Stremlau et al., 2006). It is known that the host species barrier of Pig-tailed macaques (PtMs) (*Macaca nemestrina*) against HIV-1 is weaker than other macaques because they do not have the TRIM restriction (Brennan et al., 2008).

Based on these findings, derivatives of HIV-1 that has a remarkably different structure from the conventional SHIV were constructed by the introduction of minor genetic modifications into its genome to overcome the restriction factors in macaque cells. Hatziioannou et al. (2006) generated simian-tropic HIV-1 (stHIV-1) by replacing the entire *vif* gene of HIV-1 with that of SIVmac or HIV type 2. Kamada et al. (2006) reported the monkey-tropic HIV-1 (HIV-1mt) NL-DT5R, in which the CypA-binding motif of the CA protein is substituted by the corresponding sequence of SIVmac, and the entire *vif* gene is also substituted. Thippeshappa et al. (2011) generated HSIV-vif, a clone of HIV-1 by substituting the *vif* gene with that of a pathogenic SIVmne clone. These derivatives of HIV-1 established persistent infection in PtMs for months but were controlled thereafter (Hatziioannou et al., 2009; Igarashi et al., 2007; Thippeshappa et al., 2011). These monkey-tropic HIV-1 derivatives currently available are not CCR5-tropic; NL-DT5R and HSIV-Vif encode *env* from a CXCR4-tropic, and stHIV-1 encodes *env* from dual-tropic subtype B viruses.

In this study, we generated a new HIV-1mt strain carrying *env* from a CCR5-tropic subtype C HIV-1 clinical isolate. We employed intracellular homologous recombination (IHR) to produce the recombinant virus. Since the viral swarm generated by IHR did not show efficient replication in PtM primary cells, we conducted *in vitro* serial passages of the virus. Thus, we successfully generated a viral swarm that exhibited an enhanced replication capacity in PtM cells and established infection in PtMs with high peak viremia comparable to the currently available monkey-tropic HIV-1 derivatives.

Results

Generation of a new HIV-1mt carrying CCR5-tropic subtype C env through IHR

We employed IHR to generate recombinant viruses (Fujita et al., 2013). First, we prepared DNA fragments by polymerase chain reaction (PCR) amplification of a region spanning the 5′ long terminal repeat (LTR) to upstream of the V1/V2 region in *env* (nucleotide positions 1–6784 based on HXB2 numbering; accession number: K03455) using the plasmid DNA template encoding the full-length NL-DT5R proviral genome (fragment I in Fig. 1A). This fragment encodes a CypA-binding motif derived from the corresponding sequence of SIVmac239 to evade restriction from macaque TRIM5α, and the entire SIVmac239 *vif* gene to counteract the macaque APOBEC3G. Second, a region spanning the *vpr* gene to the R region of the 3′ LTR (nucleotide positions 5558–9625 based on HXB2 numbering) was amplified from the HIV-1 97ZA012 strain (fragment II in Fig. 1B). To increase the possibility to obtain a virus that can replicate in monkeys well, we thought that it was better to generate swarm viruses having variation without cloning. Resultant recombinant virus might fail to replicate normally if recombination occurred between fragments I and II that resulted in the 5′ LTR of subtype B and the 3′ LTR of subtype C. The discordance of the 3′ and 5′ LTR may disrupt successful translocation of the minus strand strong stop DNA to the plus strand genomic RNA during reverse transcription (Goff, 2007). To match the sequence of the 3′ LTR to that of the 5′ LTR, we prepared a third DNA fragment encoding a region spanning the 5′ LTR to the middle of *gag* (nucleotide positions 1–1433 based on HXB2 numbering) from the proviral DNA extracted from HIV-1 97ZA012-infected cells (fragment III in Fig. 1B). Fragments I and II

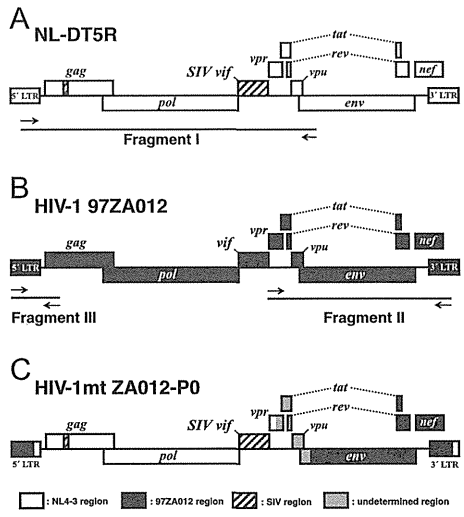

Fig. 1. Schematic representation of the genome organization of human immunodeficiency virus (HIV)-1 and monkey-tropic HIV-1 (HIV-1mt). Genome organizations of NL-DT5R (A), subtype C HIV-1 97Z012 (B) and HIV-1mt ZA012-P0 (C) are depicted. The horizontal line represents DNA fragments I, II and III, used for intracellular homologous recombination. Fragment I encodes a region from the 5′ LTR to *env* of NL-DT5R plasmid DNA. Fragment II encodes a region from the initiation of *vpr* to the R region of the 3′ LTR of the HIV-1 97ZA012 strain. Fragment III encodes a region from the 5′ LTR to upstream of the cyclophilin A-binding motif of the virus. Sequences from NL4-3 (open box), HIV-1 97ZA012 (filled box) and the SIVmac239 genome (diagonally striped box) are depicted. The gray box in HIV-1mt ZA012-P0 represents a gene that was not identified by direct sequence analysis.

had an overlapping region between the initiation of *vpr* to upstream of the *env* V1/V2 region, and fragments I and III had an overlapping region between the 5′ LTR to upstream of the CypA-binding site.

These amplified DNA fragments (fragments I, II and III) were co-transfected into C8166-CCR5 cells that are permissive to CCR5-tropic HIV-1. On day 8 post-transfection, we observed the formation of virus-induced cytopathic effects (CPEs), indicating the generation of replication-competent recombinant virus. The new recombinant virus was isolated and designated HIV-1mt ZA012-P0.

To determine the genomic organization of HIV-1mt ZA012-P0, we subjected the viral RNA isolated from the culture supernatant to direct sequencing. We found that the virus carried sequences of the U5 region of the 5′ LTR, *gag*, *pol* and *vif* derived from NL-DT5R and sequences of 3′ half of *env*, *nef*, and R and the U3 region of the 3′ LTR derived from 97ZA012 (Fig. 1C). First, the recombination breakpoint derived from fragments I and III was found to be located within the junction between the U5 and R region of the 5′ LTR (nucleotide positions 551–605 based on HXB2 numbering). However, additional recombination breakpoints between fragments I and II, encoding the *vpr–env* region, were not identified due to multiple peaks at the same locations in the analyzed sequence chromatograms. This result suggested that HIV-1mt ZA012-P0 represented a swarm that might contain several variants with various recombination breakpoints.

Increased replication competence of HIV-1mt ZA012 through long-term in vitro passage in CD8$^+$ cell-depleted pig-tailed macaque peripheral blood mononuclear cells (PBMCs)

We subsequently determined whether HIV-1mt ZA012-P0 replicates in CD8$^+$ cell-depleted pig-tailed macaque peripheral blood mononuclear cells (PtM PBMCs), in which the parental NL-DT5R replicated as described previously (Kamada et al., 2006). HIV-1mt ZA012-P0 from the culture supernatant of C8166-CCR5 was used to spinoculate CD8$^+$ cell-depleted PtM PBMCs, and the virion-associated reverse transcriptase (RT) activity was monitored in the culture supernatant (Fig. 2); however, no RT activity was detected in the culture supernatant after passage 1 (Fig. 2).

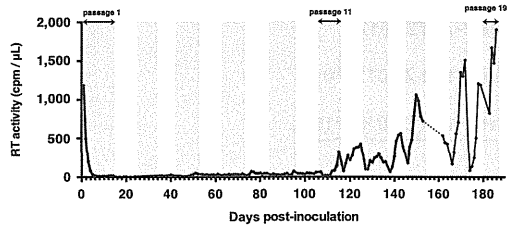

Fig. 2. Improved replication of HIV-1mt ZA012 throughout *in vitro* passages in CD8$^+$ cell-depleted PtM peripheral blood mononuclear cells (PBMCs). HIV-1mt ZA012-P0 was used to spinoculate CD8$^+$ cell-depleted PtM PBMCs, and virion-associated RT activity in the culture supernatant was monitored daily. Some of the infected cells were co-cultured with freshly prepared CD8$^+$ cell-depleted PtM PBMCs. One period of passage was indicated in the shaded grey or white zones. The dotted line indicates data not available.

Next we carried out *in vitro* serial passages to improve the replication competence of the virus as observed in the cases of HIV-1 (Freed and Martin, 1996; Willey et al., 1988). Infected cells were co-cultured with freshly prepared CD8$^+$ cell-depleted PtM PBMCs every 1 or 2 weeks. Although detectable RT activity was not observed during 10 successive passages (passage 1–10), a low level of viral replication was confirmed by the CPEs of C8166-CCR5 cells co-cultured with PBMCs taken from the passage (data not shown). A detectable peak of viral replication (319 cpm/μL) was observed at 115 days after the first inoculation (passage 11), and replication was maintained following passages, eventually resulting in enhanced replication in PtM PBMCs (1900 cpm/μL in passage 19). The resultant virus, isolated from the culture supernatant of passage 19, was designated HIV-1mt ZA012-P19.

To evaluate the replication capacity of the virus, the replication kinetics of HIV-1mt ZA012-P19 were compared to those of the parental NL-DT5R and HIV-1mt ZA012-P0. Each viral stock was normalized by the number of infectious units per cell (in this case, a multiplicity of infection (MOI) of 0.1) and used to inoculate CD8$^+$ cell-depleted PtM PBMCs isolated from two donor monkeys; virion-associated RT activity in the culture supernatant was monitored daily (Fig. 3). Although HIV-1mt ZA012-P19 exhibited a lower level of viral replication compared to that of SIVmac239, the virus showed more efficient replication than NL-DT5R and HIV-1mt ZA012-P0 in cells from both animals. Therefore, we successfully improved the replication capacity of the new HIV-1mt in PtM PBMCs by *in vitro* passaging.

Sequence analysis of HIV-1mt ZA012-P0 and ZA012-P19

It is likely that HIV-1mt ZA012-P0 acquired genetic changes and evolved to HIV-1mt ZA012-P19 through the serial passages in PtM PBMCs. To compare the genomic sequence of these viruses, we first performed single genome amplification (SGA) of viral RNA isolated from the culture supernatant to determine the nucleic acid sequences of the *vpr–env* region (nucleotide positions 5559–8795 based on HXB2) of HIV-1mt ZA012-P0. Subsequently, we identified the sequence of the region containing the expected recombination breakpoints generated by IHR between fragments I and II. Genetic analysis of 17 SGA clones revealed that these sequences had NL-DT5R sequences in the 5′ end and HIV-1 97ZA012 sequences in the 3′ end, with seven different recombination

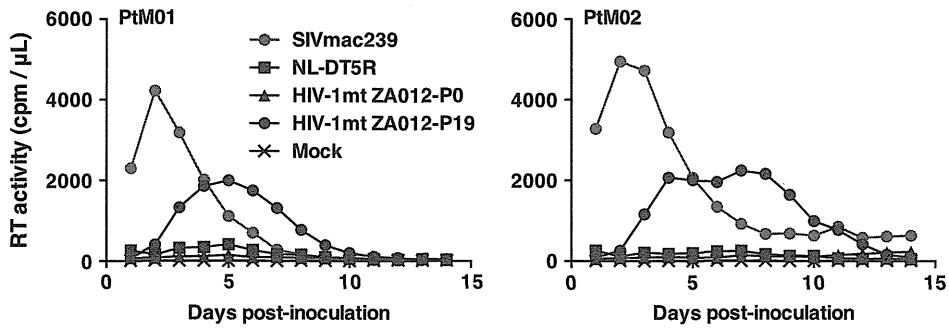

Fig. 3. Growth kinetics of HIV-1mt ZA012 in CD8$^+$ cell-depleted PtM PBMCs. Growth kinetics of HIV-1mt ZA012-P0, HIV-1mt ZA012-P19, SIVmac239 and NL-DT5R were compared in PBMCs from two PtMs. Each virus was used to spinoculate CD8$^+$ cell-depleted PtM PBMCs (MOI=0.1 $TCID_{50}$ per cell), and the virion-associated RT activity in the culture supernatant was monitored. The figure shown is representative of four independent experiments.