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Ⅳ.　研究成果の刊行物・別刷

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

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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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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-

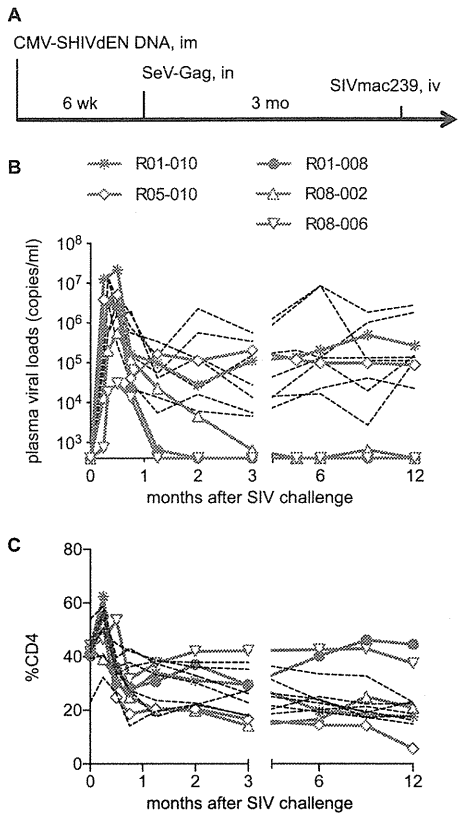

FIG 1 Viral loads and percentages of CD4 in Gag-vaccinated animals after SIVmac239 challenge. (A) Protocol of Gag vaccination and SIVmac239 challenge. (B) Plasma viral loads (SIV *gag* RNA copies/ml plasma) determined as described previously (5). The lower limit of detection is approximately 4×10^2 copies/ml. (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.

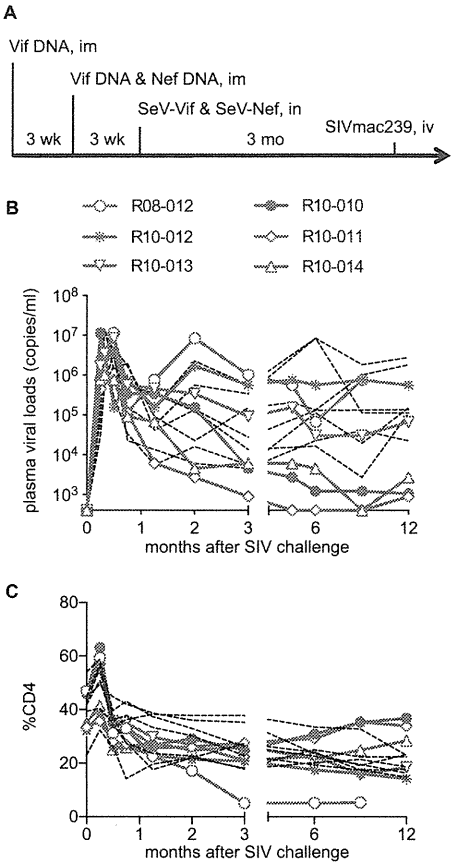

FIG 2 Viral loads and percentages of CD4 in Vif/Nef-vaccinated animals after SIVmac239 challenge. (A) Protocol of Vif/Nef vaccination and SIVmac239 challenge; (B) plasma viral loads; (C) percentages of $CD4^+$ T cells in PBMCs. In panels B and C, data on unvaccinated animals are shown by dotted lines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

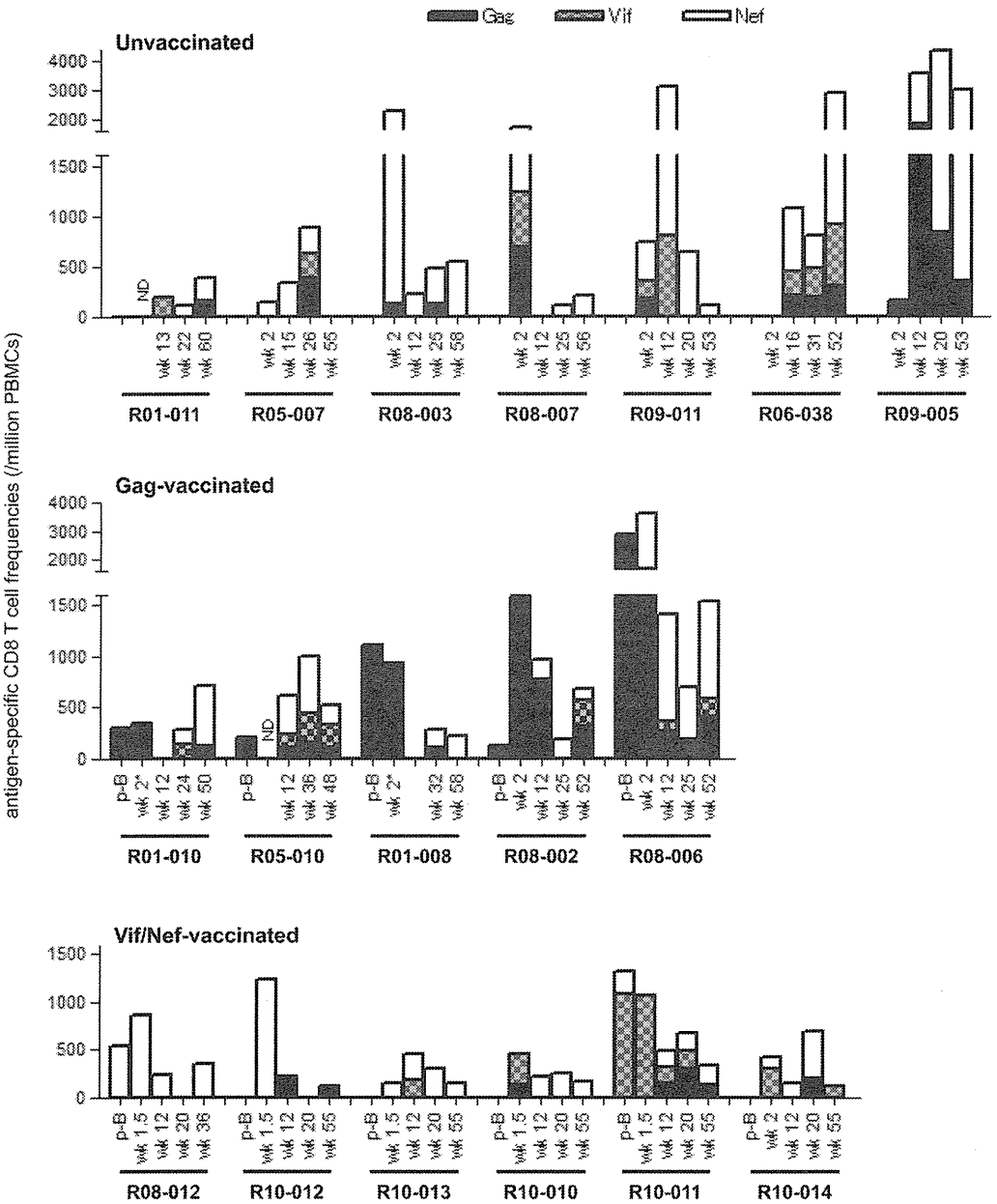

FIG 3 SIV Gag/Vif/Nef-specific $CD8^+$ T-cell responses in macaques. We examined $CD8^+$ T-cell responses specific for Gag, Vif, and Nef 1 week after SeV-Gag boost (p-B) and approximately 2 weeks, 3 months, 6 months, and 1 year after SIV challenge in unvaccinated (top), Gag-vaccinated (middle), and Vif/Nef-vaccinated (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

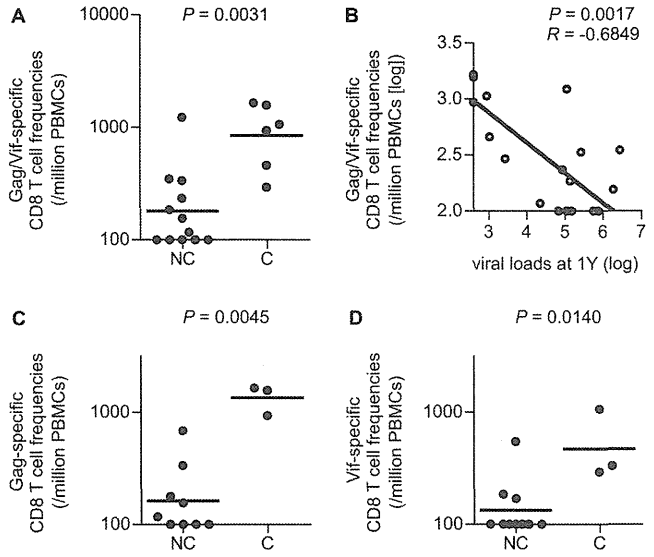

FIG 4 Comparison of Gag/Vif-specific $CD8^+$ T-cell frequencies in the acute phase between SIV controllers (C) and noncontrollers (NC). Data on Gag- and Vif-specific $CD8^+$ T-cell frequencies around week 2 postchallenge, which are shown in Fig. 3, were used. In macaques R01-011 and R05-010, samples 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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Limited Impact of Passive Non-Neutralizing Antibody Immunization in Acute SIV Infection on Viremia Control in Rhesus Macaques

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Abstract

Background: Antiviral antibodies, especially those with neutralizing activity against the incoming strain, are potentially important immunological effectors to control human immunodeficiency virus (HIV) infection. While neutralizing activity appears to be central in sterile protection against HIV infection, the entity of inhibitory mechanisms via HIV and simian immunodeficiency virus (SIV)-specific antibodies remains elusive. The recent HIV vaccine trial RV144 and studies in nonhuman primate models have indicated controversial protective efficacy of HIV/SIV-specific non-neutralizing binding antibodies (non-NAbs). While reports on HIV-specific non-NAbs have demonstrated virus inhibitory activity *in vitro*, whether non-NAbs could also alter the pathogenic course of established SIV replication *in vivo*, likewise via neutralizing antibody (NAb) administration, has been unclear. Here, we performed post-infection passive immunization of SIV-infected rhesus macaques with polyclonal SIV-specific, antibody-dependent cell-mediated viral inhibition (ADCVI)-competent non-NAbs.

Methods and Findings: Ten lots of polyclonal immunoglobulin G (IgG) were prepared from plasma of ten chronically SIV_{mac239}-infected, NAb-negative rhesus macaques, respectively. Their binding capacity to whole SIV_{mac239} virions showed a propensity similar to ADCVI activity. A cocktail of three non-NAb lots showing high virion-binding capacity and ADCVI activity was administered to rhesus macaques at day 7 post-SIV_{mac239} challenge. This resulted in an infection course comparable with control animals, with no significant difference in set point plasma viral loads or immune parameters.

Conclusions: Despite virus-specific suppressive activity of the non-NAbs having been observed *in vitro*, their passive immunization post-infection did not result in SIV control *in vivo*. Virion binding and ADCVI activity with lack of virus neutralizing activity were indicated to be insufficient for antibody-triggered non-sterile SIV control. More diverse effector functions or sophisticated localization may be required for non-NAbs to impact HIV/SIV replication *in vivo*.

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Introduction

Development of a successful vaccine is crucial for global human immunodeficiency virus (HIV) control. A recent clinical trial in Thailand has shown partial efficacy of an HIV vaccine regimen, RV144 [1]. Further analyses have suggested possible contribution of virus-binding antibodies to the protection from HIV infection [2,3]. Thus, understanding of the effect of virus-binding, non-neutralizing antibody (non-NAb) responses on the course of HIV/SIV infection may serve as one step for vaccine development.

In contrast to the constrained emergence of neutralizing antibodies (NAbs), non-NAbs are commonly induced in both the acute and chronic phase of HIV/SIV infection [4–6]. They are known to exhibit *in vitro* suppressive effects against virus replication, such as ADCC (antibody-dependent cellular cytotoxicity) and ADCVI (antibody-dependent cell-mediated virus inhibition) [7–10]. While several reports have suggested

inverse correlation between such effector functions and viral loads in HIV-infected individuals [5,6] and vaccinated SIV-infected macaques [11–14], the precise influence of non-NAb responses on viral replication control remains undetermined. Passive immunization studies in nonhuman primate AIDS models have shown partial protection from mucosal virus challenge by mucosal pre-challenge non-NAb infusion, suggesting limited protective efficacy of locally-distributed non-NAb responses [15,16]. In the present study, we focused on the effect of systemic distribution of non-NAbs on established primary viral infection, which is another practical vaccine correlate.

Passive immunization of polyclonal neutralizing antibodies (NAbs), which does not exclude coexistence of non-NAbs, has partially provided protective activity in nonhuman primate AIDS models [17–19]. Additionally, we have reported SIV control *in vivo* by post-infection administration of polyclonal NAbs, in which enhanced antigen presentation and subsequent augmented T-cell responses likely accounted for the control [20,21]. Since non-NAbs are potentially capable of supporting these suggested mechanisms, the protective activity of non-NAbs by themselves against established primary infection is important to be assessed. Here, we examined the effect of passive non-NAb immunization at day 7 post-challenge on primary SIV_{mac239} replication in rhesus macaques. Despite the virion-binding and ADCVI activity of non-NAbs having been confirmed *in vitro*, passive immunization of non-NAbs did not result in control of SIV replication *in vivo*.

Methods

Ethics Statement

Animal experiments were carried out in National Institute of Biomedical Innovation (NIBP) after approval by the Committee on the Ethics of Animal Experiments of NIBP in accordance with the guidelines for animal experiments at NIBP and National Institute of Infectious Diseases. To prevent viral transmission, animals were housed in individual cages allowing them to make sight and sound contact with one another, where the temperature was kept at 25 °C with light for 12 hours per day. Animals were fed with apples and commercial monkey diet (Type CMK-2, Clea Japan, Inc.). Blood collection, vaccination, and SIV challenge were performed under ketamine anesthesia. Three of eleven macaques, R10-001, R10-004, and R06-029, were euthanized during the observation period in the SIV challenge experiment of this study. Two of them (R10-004 and R06-029) were euthanized (at 7-10 months) after the minimum observation period required for this study (6 months) because of the limitation of available cage numbers. One macaque R10-001 was euthanized (at 9 months) at the endpoint for euthanasia, which was determined by typical signs of AIDS including reduction in peripheral $CD4^+$ T-cell counts (less than 200 cells/μl), 10% loss of body weight, diarrhea, and general weakness. At euthanasia, animals were deeply anesthetized with pentobarbital under ketamine anesthesia, and then, whole blood was collected from left ventricle.

Analysis of Virus-Specific Neutralizing Responses

Heat-inactivated plasma or purified antibodies were prepared in quadruplicate and mixed with 10 $TCID_{50}$ (50 percent tissue culture infective dose) of SIV_{mac239} [22]. In each mixture, 5 μl of diluted sample was incubated with 5 μl of virus. After 45 min incubation at room temperature, each 10 μl mixture was added into 5 x 10^4 HSC-F cells (macaque T cell line) [23] per well in 96-well plates. Day 10 culture supernatants were harvested and progeny virus production was examined by determining the supernatant reverse transcriptase activity to confirm the absence of neutralizing activity at 1:2.

Whole virus ELISA and immunoblotting. SIV virions used for the antigen were prepared by infecting HSC-F cells with SIV_{mac239} at MOI 0.01. Day 7 supernatant was collected and virus particles were purified by centrifugation at 35,000 rpm, 75 min on 20% sucrose in a SW41 rotor (Beckman Coulter), followed by 35,000 rpm, 75 min on 20%-60% sucrose in a SW55 rotor (Beckman Coulter) and 35,000 rpm, 75 min on 20% sucrose in a SW41 rotor. Precipitated SIV virions were diluted in phosphate buffered saline (PBS) and used to coat 96-Well Assay Plates (Becton Dickinson) at a concentration of 100 ng/ml p27 (0.1 ml per well) by overnight incubation at 4 °C. Wells were washed with PBS and blocked with 0.5% bovine serum albumin (BSA)/PBS. Purified anti-SIV immunoglobulin G (IgG) serially diluted in PBS (0.1 ml per well) were incubated for 2 hr at 37 °C. Plates were washed with PBS and virion-bound antibodies were detected with a horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG (H+L) (Bethyl Laboratory) and SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL). SIV-specific IgG activity in the purified IgG and plasma samples were detected using a Western blotting system for detection of SIV_{mac251} antigens (ZeptoMetrix) according to the manufacturer's instruction. Samples from animals (R02-006, R04-011, R04-014, and R06-005) with rapid AIDS progression showed lower antibody reactivity.

Antibody-dependent cell-mediated virus inhibition (ADCVI) assay. HSC-F cells (1 x 10^5) serving as MHC-mismatched targets were infected with SIV_{mac239} at MOI 0.001. After adsorption for 6 hr, cells were washed twice with medium and serially-diluted anti-SIV or control antibodies (1.0 or 0.1 mg/ml) were added to the target cells with 4 x 10^5 effector cells, rhesus peripheral blood mononuclear cells (PBMCs), at an E:T ratio of 4:1 in round-bottomed 96-well plates. Wells of target cells without antibodies or effector cells were set as negative controls. After 7 days of culture, supernatants were collected and measured for their Gag p27 concentrations by ELISA (ABL). The percentage of virus inhibition deriving from ADCVI was calculated as follows: % inhibition = 100x (1 - [p27p/p27c]); where p27p and p27c are the average p27 concentrations in wells with anti-SIV and control antibodies, respectively. Experiments were performed twice in duplicate.

Antibody preparation. Ten lots of IgG solutions were prepared from ten chronically SIV_{mac239}-infected rhesus macaques without detectable SIV_{mac239}-specific NAb responses, respectively. IgG was purified from the plasma after heat-inactivation and filtration by Protein G Sepharose 4 Fast Flow (Amersham) and concentrated by Amicon Ultra 4, MW50000

(Millipore) to 30 mg/ml. Purified IgG solutions were confirmed negative for SIV_{mac239}-specific neutralizing activity. Three lots prepared from three macaques (R06-007, R01-009, and R03-005) were mixed to obtain the IgG inoculums for passive non-NAb immunization. Five lots of IgG solutions were also prepared from five chronically SIV_{mac239}-infected rhesus macaques with detectable SIV_{mac239}-specific NAb responses, respectively. Control IgG (CAb) was prepared from pooled plasma of non-infected rhesus macaques.

Animal experiments. Burmese rhesus macaques (*Macaca mulatta*) were challenged intravenously with 1,000 $TCID_{50}$ of SIV_{mac239}. For passive immunization, animals were intravenously administered with 300 mg of anti-SIV non-NAb IgG or control IgG at day 7 post-challenge. The determination of major histocompatibility complex class I (MHC-I) haplotypes was based on the family study in combination with the reference strand-mediated conformation analysis (RSCA) 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 [24–27]. Data on control macaques R10-005, R10-008, and R10-001 have previously been reported [28].

Measurement of virus-specific T-cell responses. Virus-specific $CD8^+$ T-cell responses were measured by flow-cytometric analysis of gamma interferon (IFN-γ) induction as described previously [29]. PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) pulsed with overlapping peptide pools spanning the SIV_{mac239} Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Env, and Nef amino acid sequence. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (Becton Dickinson). Fluorescein isothiocianate-conjugated anti-human CD4, Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3 and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting non-specific $IFN\text{-}\gamma^+$ T-cell frequencies from those after SIV-specific stimulation. Specific T-cell levels less than 100 cells per million PBMCs are considered negative.

Sequencing. Viral RNAs were extracted using High Pure Viral RNA kit (Roche Diagnostics) from macaque plasma obtained at around 1 year after challenge. Fragments of cDNAs encoding SIV_{mac239} Env 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). Predominant non-synonymous mutations were determined.

Statistical analysis. Statistical analysis was performed by Prism software version 4.03 (GraphPad Software, Inc.). Comparison of viral loads, peripheral blood $CD4^+$ T-cell counts, peripheral blood central memory $CD4^+$ T-cell frequencies, and the number of non-synonymous mutations in Env-coding regions between non-NAb-infused and control animals was performed by nonparametric Mann–Whitney U test with significance levels set at $p < 0.05$.

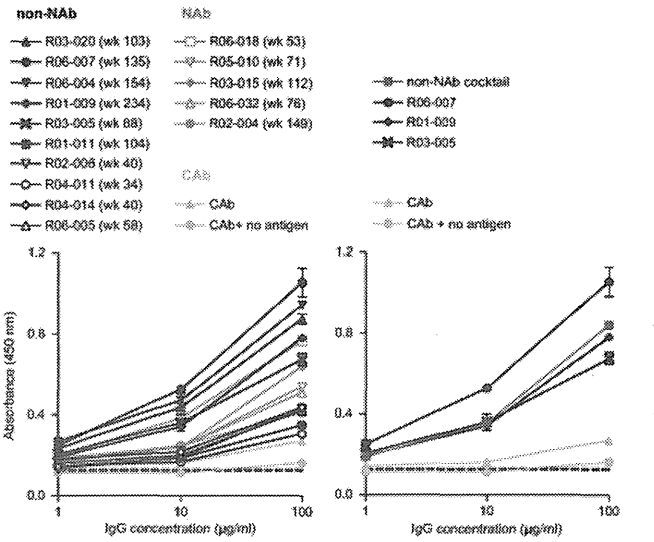

Figure 1. Binding properties of IgGs to SIV virions. Polyclonal IgGs purified from macaque plasma were subjected to whole virus ELISA using purified SIV_{mac239} virions as the antigen. Results on ten IgG lots derived from ten macaques without detectable neutralizing activity (non-NAbs; black lines), five with neutralizing activity (NAbs; red), and a control IgG (CAb; green) are shown in the left panel. Results on the non-NAb cocktail and three non-NAb lots composing the cocktail are in the right. The dotted line represents background absorbance. Time points of plasma sampling are shown in parentheses following the macaque IDs. A representative result, means and SDs of duplicate samples, from two experiments is shown.

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Results

In vitro virion binding and ADCVI activity of SIV-specific non-NAbs

Ten lots of polyclonal IgG were prepared from plasma of ten chronically SIV_{mac239}-infected, NAb-negative rhesus macaques, respectively. SIV_{mac239}-binding capacity was screened by whole virus ELISA using virions purified from culture supernatants of SIV_{mac239}-infected HSC-F cells (a macaque T-cell line) (Figure 1). The measured absorbance was proportionate with Env gp120 and Gag p27 reactivity examined by immunoblotting (Figure 2). Polyclonal IgG lots from three macaques (R06-007, R01-009, and R03-005) with intermediate to high virion-binding capacity, although what percentage of IgGs was SIV-specific are unknown, were pooled and further used as a non-NAb cocktail for passive immunization, whose virion-binding characteristics were also confirmed (Figure 1).

To examine the *in vitro* virus-suppressive activity of the non-NAb cocktail, ADCVI activity was evaluated using PBMCs as effectors and MHC-mismatched macaque HSC-F cells as infected targets (Figure 3). IgG lots with high virion-binding capacity showed high ADCVI activity, whereas those from macaques R04-011 and R06-005 with limited reactivity in

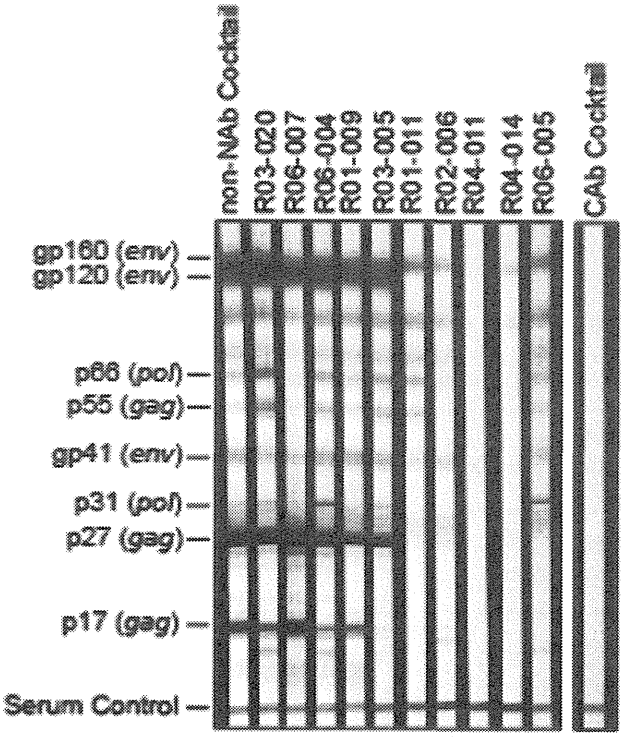

Figure 2. Binding properties of IgGs to SIV antigens. The non-NAb cocktail, ten non-NAb IgG lots derived from ten macaques, and CAb were subjected to immunoblotting (ZeptoMetrix). A representative result from two experiments is shown.

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ELISA and western blot exhibited low ADCVI activity. These results suggest that ADCVI activity is proportionate with overall virion binding. The non-NAb cocktail exerted more than 97% inhibitory activity even at 0.1 mg/ml IgG concentration. A 1.0 mg/ml IgG concentration approximates an estimated *in vivo* antibody concentration immediately after passive immunization (300 mg IgG in 300 ml body fluid), implying that the observed ADCVI activity is likely to occur *in vivo* after passive immunization.

In Vivo Effect of Non-NAb Passive Immunization in SIV Infection

Having confirmed the *in vitro* anti-viral property of the non-NAb cocktail, we performed the post-infection passive immunization. Five rhesus macaques were challenged intravenously with SIV_{mac239} followed by passive immunization with the non-NAb cocktail (300 mg IgG) at day 7 post-challenge. When we previously passively immunized rhesus macaques with polyclonal antibodies having anti-SIV neutralizing activity by this regimen (300 mg IgG i.v. at day 7), enhanced virus uptake by DCs, subsequent augmentation of SIV-specific $CD4^+$ T-cell responses, enhancement of *in vitro* virus-suppressive activity in $CD8^+$ cells, and set-point viremia control were observed [20,21]. The current passive immunization experiment contrasts this previous report by

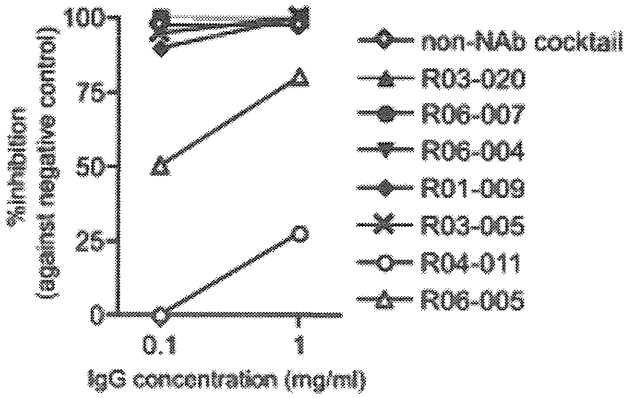

Figure 3. ADCVI activity of the non-NAb cocktail and non-NAb IgG lots. The reduction in SIV p27 concentration in the supernatant from SIV-infected cell culture with non-NAbs compared to that without antibodies is shown. A representative result, means of duplicate samples, from two experiments is shown.

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infusion of polyclonal antibodies with comparable SIV virion-binding capacity and ADCVI activity without anti-SIV neutralizing activity. The moment of passive immunization (day 7) also recapitulates the first time frame to detect anti-HIV/SIV antibodies after infection [30,31]. Six animals consisting of two without passive immunization and four with control IgG infusion at day 7 after SIV_{mac239} challenge were used as controls.

To examine the abundance of non-NAbs after infusion and *de novo* virus-specific antibody induction, plasma reactivity against SIV antigens was measured by immunoblotting (Table 1). SIV Env-specific antibodies were detected at week 1.5 post-infection exclusively in the non-NAb-infused animals. High reactivity in plasma in these animals resided up to week 3 post-infection. *De novo* induction of SIV-specific antibodies was comparably observed in both the non-NAb-infused and control groups from week 5 to week 12 post-infection. Collectively, the passive non-NAb immunization resulted in systemic distribution of SIV Env-specific antibodies around peak infection.

All five macaques infused with the non-NAbs failed to contain set-point viremia, similar to the six control animals (Figure 4). The non-NAb-infused and control groups exhibited comparable peak and set-point viral loads without significant difference. No significant difference in total $CD4^+$ T-cell counts was found throughout the course between these two groups (Figure 4). Peripheral $CD95^+CD28^+$ central memory $CD4^+$ T-cell counts at week 12 were also comparable between these two groups (data not shown).

Considering our previous study of NAb-triggered SIV control and facilitation of T-cell responses [20,21], we examined SIV antigen-specific $CD8^+$ T-cell responses in the chronic phase (Figure 5). Neither the responses to individual antigens nor the summation presented significant difference. Finally, to assess possible selective pressure on SIV by the passive non-NAb immunization, predominant nonsynonymous env mutations in the early phase (at week 12, data not shown) and in the

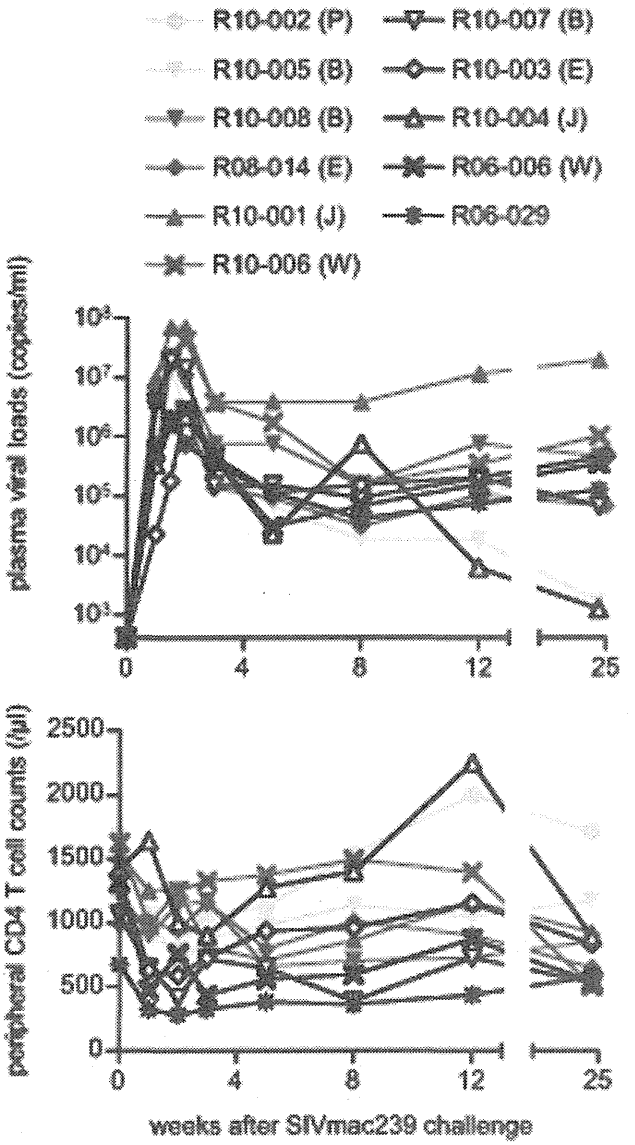

Figure 4. Passive non-NAb immunization in SIV infection. Upper panel: plasma viral loads after SIV_{mac239} challenge (SIV RNA copies/ml in plasma) in two unimmunized (green lines), four control IgG-immunized (blue), and five non-NAb-immunized macaques (black). Viral loads were determined as described previously [25]. The lower limit of detection is approximately 4 x 10^2 copies/ml. MHC-I haplotypes determined in individual animals are shown in parentheses as follows: B, haplotype *90-120-Ib*; E, *90-010-Ie*; J, *90-088-Ij*; P, *89-002-Ip*; W, *89-075-Iw*. Lower panel: peripheral $CD4^+$ T-cell counts after SIV_{mac239} challenge. No significant difference in viral loads or $CD4^+$ T-cell counts was observed between non-NAb-immunized and control animals.

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chronic phase were determined (Figure 6). Analysis at week 12 showed only one or two mutations, which were mostly observed also in the chronic phase. Mutations specific for the

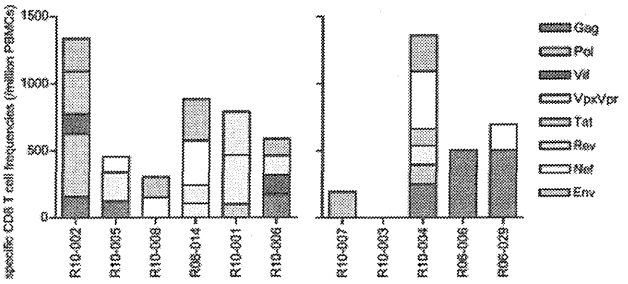

Figure 5. SIV antigen-specific $CD8^+$ T-cell responses. SIV Gag-, Pol-, Vif-, Vpx/Vpr-, Tat-, Rev-, Nef-, and Env-specific $CD8^+$ T-cell responses were measured by detection of antigen-specific IFN-γ induction using PBMCs at weeks 26-30 post-challenge.

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Table 1. SIV-specific antibody responses in plasma after SIV infection.

macaques	regimens[a]	plasma antibody responses[b]				
		wk 1	wk 1.5	wk 3	wk 5	wk 12
R10-002	-	-	-	-	+	++++
R10-005	-	-	-	-	+	++++
R10-008	CAb	-	-	-	++	++++
R08-014	CAb	-	-	+	++	++++
R10-001	CAb	-	-	-	+	+
R10-006	CAb	-	-	-	+	++++
R10-007	non-NAb	-	+++	++	++	++++
R10-003	non-NAb	-	++++	++	++	++++
R10-004	non-NAb	-	+++	++	+	++++
R06-006	non-NAb	-	++++	++	+	++++
R06-029	non-NAb	-	+++	++	++	++++

[a] Animals received no passive immunization (-), passive CAb immunization (CAb), or passive non-NAb immunization (non-NAb) at day 7 after SIV challenge. [b] Antibody responses were detected using a commercial Western blotting system (ZeptoMetrix). + Gag p27-positive; ++ Gag p27 and Env gp160-positive; +++ Gag p27, Env gp160, and one other Gag/Pol/Env-derived antigen-positive; ++++ Gag p27, Env gp160, and two or more other Gag/Pol/Env-derived antigen-positive.

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non-NAb-infused group, such as signs of ADCVI-induced escape [32], were not detected. A slight increase in predominant mutations in the Env V1-coding region was observed in the non-NAb-infused group, although the difference was not statistically significant (p = 0.08 by Mann–Whitney U test). Thus, the passive non-NAb immunization at day 7 post-challenge showed no significant impact on SIV replication *in vivo*.

Discussion

Whether augmentation of ADCVI without virus neutralizing activity may influence SIV replication control *in vivo* was a major interest in this study. Our results indicate that passive

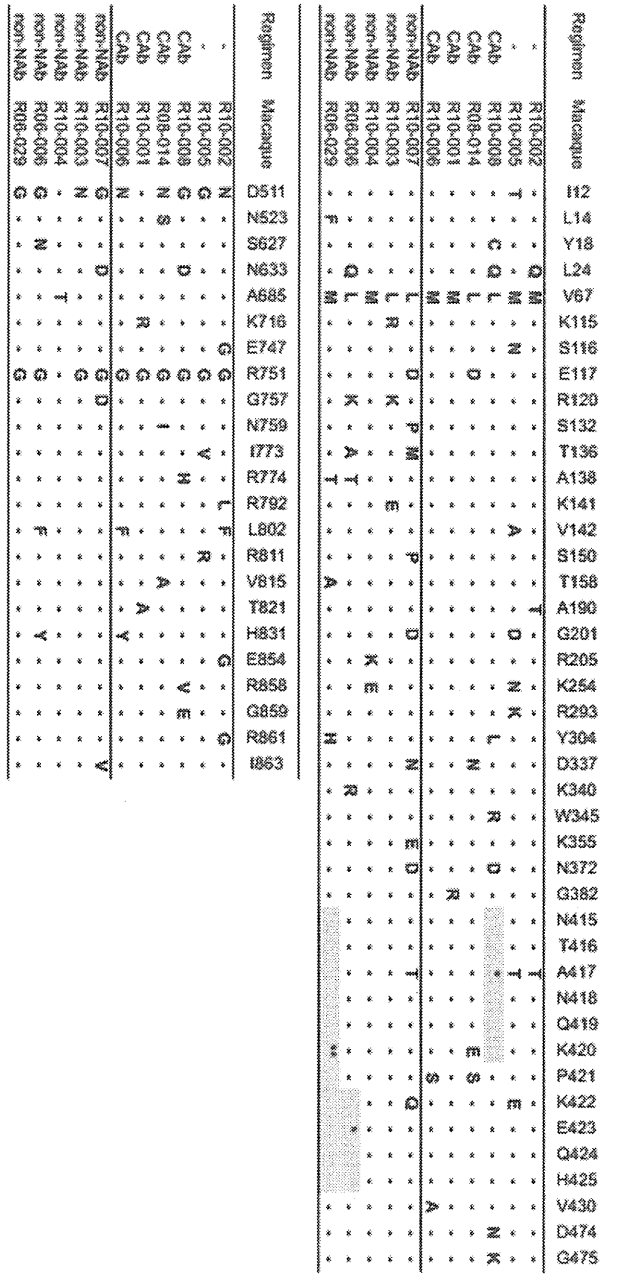

Figure 6. Predominant nonsynonymous env mutations. Viral cDNAs encoding Env were amplified from plasma RNAs obtained at 7-9 months (R10-001, R10-004, and R06-029) or 12 months (other animals) and subjected to sequencing analysis. Amino acid substitutions are shown. The asterisk (*) represents a deletion and the double asterisk (**) represents coexistence of multiple deletion patterns.

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non-NAb immunization does not influence primary SIV replication when administered at early post-infection. In agreement with the limited protective effect observed when non-NAbs were administered locally on mucosa before virus challenge [16], systemic distribution of non-NAbs post-infection did not correlate with suppression of SIV replication, despite antiviral activity of the non-NAbs observed *in vitro*.

For assessment of antibody binding affinity to antigens on virions, we utilized purified SIV virions instead of recombinant Env proteins [33] as the antigen for ELISA under detergent-free conditions. Virion-binding characteristics of antibodies showed a similar trend with ADCVI activity, as seen in other studies [13]. However, the non-NAb infusion did not result in SIV control *in vivo*, which consequently proposes the following notions.

First, this study indicates that augmentation of non-NAb-derived virus-suppressive activity does not alter SIV control course once infection is achieved. While previous studies on immunized macaques indicated inverse correlation between ADCC or ADCVI activity at virus challenge and acute plasma viremia [11–14], the degree of non-NAb contribution by itself was not clear since vaccination elicited multiple immune responses. In other reports, intracutaneously infused non-NAbs did not exert protection in neonatal macaques [15] and mucosal non-NAbs showed limited protective activity [16]. In coherence, even massive systemic distribution of non-NAbs at peak infection did not impact viral replication in the present study. Thus, antiviral non-NAb responses do not suffice for counteracting establishment of set-point viremia, although these responses may partially influence viral replication in the chronic phase, as indicated by a previous report showing that CD20 depletion in chronic SIV infection can result in accelerated viremia and disease course [34], similar to rapid progressors [35]. Taken together, our results suggest that non-NAbs may withhold a limited role in impeding virus spread *in vivo* in HIV/SIV infections, unlike in other chronic viral infection models [36].

Second, this study indicates the requirement of neutralizing activity of antibody for the suppression of primary SIV replication by passive NAb immunization post-infection, as observed in our previous study [20]. The NAb-triggered SIV control has been suggested to be attributed to antibody-mediated virion uptake by DCs and enhanced T-cell priming [21], which can also occur in the non-NAb-infused animals. Thus, the control failure in non-NAb-infused macaques implies that augmentation of antigen presentation alone may be insufficient for primary SIV control and that reduction of infectious virus burden and $CD4^+$ T-cell preservation is important for any immune augmentation [20,37,38].

In conclusion, the post-infection passive non-NAb immunization did not result in primary SIV control in a rhesus macaque AIDS model. Our results suggest that virion binding and ADCVI activity with lack of virus neutralizing activity in the acute phase are insufficient for giving an impact on primary HIV/SIV replication. Further sophistication of local and targeted induction of functional non-NAb responses may be required to impact HIV/SIV replication *in vivo*.

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Author Contributions

Conceived and designed the experiments: T. Nakane TM HY. Performed the experiments: T. Nakane T. Nomura SS MN. Analyzed the data: T. Nakane TM HY. Wrote the manuscript: T. Nakane TM HY. Performed MHC-I typing: TKN AK.

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A novel link of *HLA* locus to the regulation of immunity and infection: *NFKBIL1* regulates alternative splicing of human immune-related genes and influenza virus *M* gene

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ABSTRACT

HLA locus contains immune-related genes and genetically regulates immune responses against both foreign- and self-antigens in humans. Inhibitor of κB-like protein (IκBL), encoded by *HLA*-linked *NFKBIL1*, is a protein of unknown function, while genetic variations in *NFKBIL1* are known to associate with the susceptibility to inflammatory and/or autoimmune diseases. In this study, we found that IκBL suppressed exon exclusion in alternative splicing of human immune-related genes such as *CD45*. Yeast-two-hybrid screening and immunoprecipitation assay revealed molecular association of IκBL with CLK1, a serine/threonine and tyrosine kinase, which plays a role in the alternative splicing. Unexpectedly, we found that the regulation of alternative splicing in *CD45* by IκBL was independent from the kinase activity of CLK1. On the other hand, it was demonstrated that an SR protein, ASF/SF2, bound both IκBL and CLK1 at the RNA-recognition motifs of ASF/SF2, implying a competition of IκBL and CLK1 on SR protein. In addition, IκBL was found to regulate the CLK1-dependent synthesis of *M2* RNA, a splice variant of influenza A virus *M* gene. These observations suggest a functional involvement of IκBL in the regulation of alternative splicing in both human and viral genes, which is a novel link of *HLA* locus to the regulation of immunity and infection in humans.

1. Introduction

Human NF-κB inhibitor-like protein 1 gene (*NFKBIL1*) is located in the *HLA* class III region on the short arm of chromosome 6 and encodes a protein, inhibitor of κB-like protein (IκBL), which shows a limited homology to inhibitor of κB (IκB). A number of studies have demonstrated the association between genetic variations in *NFKBIL1* and susceptibility to inflammatory and/or autoimmune diseases, such as multiple sclerosis [1], rheumatoid arthritis (RA) [2], type I diabetes mellitus [3], Takayasu's arteritis [4], and chronic thromboembolic pulmonary hypertension [5]. It has been reported that the sequence variations in the promoter region of *NFKBIL1*, which showed the lowest and the highest promoter activity, would confer the susceptibility to RA and Takayasu's arteritis, respectively [4], implying that the altered expression of IκBL might contribute to the pathogenesis of immune-related diseases.

Alternative splicing is a crucial mechanism in the post-transcriptional control of gene expression in eukaryotes, in which target exons in pre-mRNA could be either excluded or included depending on the specific *cis*-regulatory elements and recognition by the splicing-related factors [6,7]. Pre-mRNAs of human immune-related genes are known to undergo extensive alternative splicing [8]. For example, resting T cells express larger mRNA isoforms of *CD45*, while target exons of *CD45* were selectively excluded to form shorter mRNA isoforms in activated T cells. Expression of each isoform generated by the alternative splicing is strictly regulated, whereas the abnormal alternative splicing events, which lead to altered expression of target mRNA isoforms, have been reported to associate with autoimmune diseases [9,10].

IκBL possesses nuclear localization sequences (NLS) at its N-terminus and localized in nuclear speckles [11,12], which are sub-nuclear structures enriched with pre-mRNA splicing factors [13]. In addition, IκBL was reported to associate with RNA [12]. These lines of evidence suggest that IκBL might play a role in the RNA splicing. In addition, it has been reported that CDC-like kinase 1 (CLK1),

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