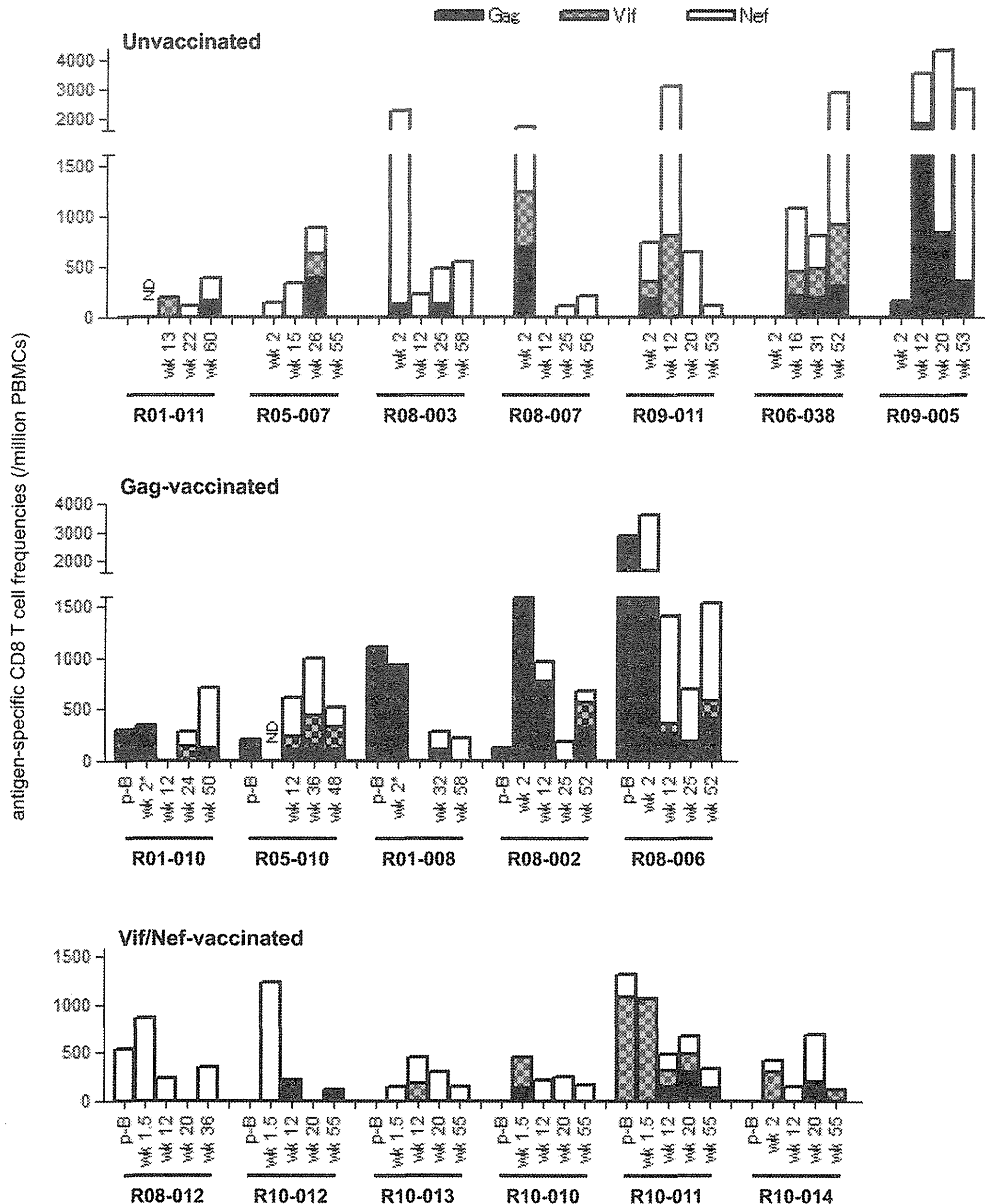

FIG 3 SIV Gag/Vif/Nef-specific CD8$^+$ T-cell responses in macaques. We examined CD8$^+$ T-cell responses specific for Gag, Vif, and Nef 1 week after SeV-Gag boost (p-B) and approximately 2 weeks, 3 months, 6 months, and 1 year after SIV challenge in unvaccinated (top), Gag-vaccinated (middle), and Vif/Nef-vaccinated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

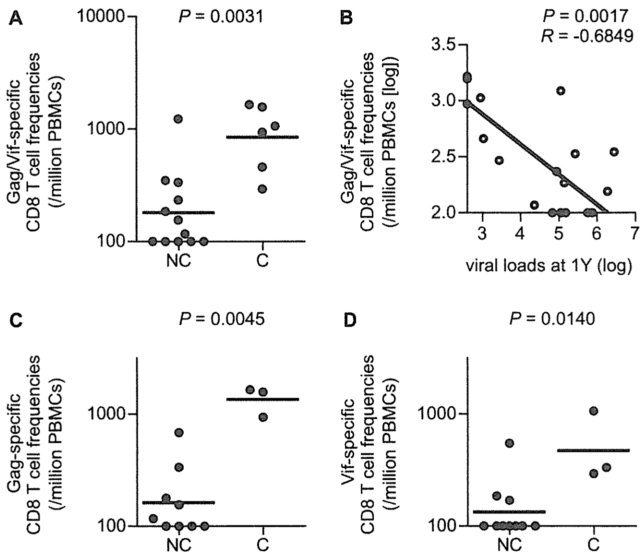

FIG 4 Comparison of Gag/Vif-specific $CD8^+$ T-cell frequencies in the acute phase between SIV controllers (C) and noncontrollers (NC). Data on Gag- and Vif-specific $CD8^+$ T-cell frequencies around week 2 postchallenge, which are shown in Fig. 3, were used. In macaques R01-011 and R05-010, samples 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

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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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A Novel Protective MHC-I Haplotype Not Associated with Dominant Gag-Specific CD8$^+$ T-Cell Responses in SIVmac239 Infection of Burmese Rhesus Macaques

Naofumi Takahashi[1,2], Takushi Nomura[1,2], Yusuke Takahara[1,2], Hiroyuki Yamamoto[1], Teiichiro Shiino[1], Akiko Takeda[1], Makoto Inoue[3], Akihiro Iida[3], Hiroto Hara[3], Tsugumine Shu[3], Mamoru Hasegawa[3], Hiromi Sakawaki[4], Tomoyuki Miura[4], Tatsuhiko Igarashi[4], Yoshio Koyanagi[4], Taeko K. Naruse[5], Akinori Kimura[5], Tetsuro Matano[1,2]*

1 AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan, **2** The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **3** DNAVEC Corporation, Tsukuba, Japan, **4** Institute for Virus Research, Kyoto University, Kyoto, Japan, **5** Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

Abstract

Several major histocompatibility complex class I (MHC-I) alleles are associated with lower viral loads and slower disease progression in human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections. Immune-correlates analyses in these MHC-I-related HIV/SIV controllers would lead to elucidation of the mechanism for viral control. Viral control associated with some protective MHC-I alleles is attributed to CD8$^+$ T-cell responses targeting Gag epitopes. We have been trying to know the mechanism of SIV control in multiple groups of Burmese rhesus macaques sharing MHC-I genotypes at the haplotype level. Here, we found a protective MHC-I haplotype, *90-010-Id* (D), which is not associated with dominant Gag-specific CD8$^+$ T-cell responses. Viral loads in five D$^+$ animals became significantly lower than those in our previous cohorts after 6 months. Most D$^+$ animals showed predominant Nef-specific but not Gag-specific CD8$^+$ T-cell responses after SIV challenge. Further analyses suggested two Nef-epitope-specific CD8$^+$ T-cell responses exerting strong suppressive pressure on SIV replication. Another set of five D$^+$ animals that received a prophylactic vaccine using a Gag-expressing Sendai virus vector showed significantly reduced viral loads compared to unvaccinated D$^+$ animals at 3 months, suggesting rapid SIV control by Gag-specific CD8$^+$ T-cell responses in addition to Nef-specific ones. These results present a pattern of SIV control with involvement of non-Gag antigen-specific CD8$^+$ T-cell responses.

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* E-mail: tmatano@nih.go.jp

Introduction

Virus-specific CD8$^+$ T-cell responses play a central role in the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication [1,2,3,4,5]. Genetic diversities of HLA or major histocompatibility complex class I (MHC-I) result in various patterns of CD8$^+$ T-cell responses in HIV-infected individuals. Cumulative studies on HIV infection have indicated the association of MHC-I genotypes with higher or lower viral loads [6,7,8,9,10]. In some MHC-1 alleles associating with lower viral loads and slower disease progression, certain CD8$^+$ T-cell responses restricted by these MHC-I molecules have been shown to be responsible for HIV control [11,12,13]. In rhesus macaque AIDS models, *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17* are known as protective alleles, and macaques possessing these alleles tend to show slower disease progression after SIVmac251/SIVmac239 challenge [14,15,16,17].

Recent studies have indicated great contribution of CD8$^+$ T-cell responses targeting Gag epitopes to reduction in viral loads in HIV/SIV infection [18,19,20,21]. Viral control associated with some protective MHC-I alleles is attributed to Gag epitope-specific CD8$^+$ T-cell responses [22,23,24]. 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 an escape mutation with viral fitness costs, leading to lower viral loads [22,24,25,26,27]. On the other hand, CD8$^+$ T-cell responses targeting SIV antigens other than Gag, such as Mamu-B*08- or Mamu-B*17-restricted Vif and Nef epitopes, have been indicated to exert strong suppressive pressure on SIV replication [28,29,30,31,32,33]. Accumulation of our knowledge on the potential of these non-Gag-specific as well as Gag-specific CD8$^+$ T-cell responses for HIV/SIV control should be encouraged for elucidation of viral control mechanisms.

We have been examining SIVmac239 infection in multiple groups of Burmese rhesus macaques sharing MHC-I genotypes at the haplotype level and indicated an association of MHC-I haplotypes with AIDS progression [21,34]. In our previous study, a group of macaques sharing MHC-I haplotype *90-120-Ia* (A) induced dominant Gag-specific $CD8^+$ T-cell responses and tended to show slower disease progression after SIVmac239 challenge [21]. Prophylactic immunization of these A^+ macaques with a DNA vaccine prime and a Gag-expressing Sendai virus (SeV-Gag) vector boost resulted in SIV control based on Gag-specific $CD8^+$

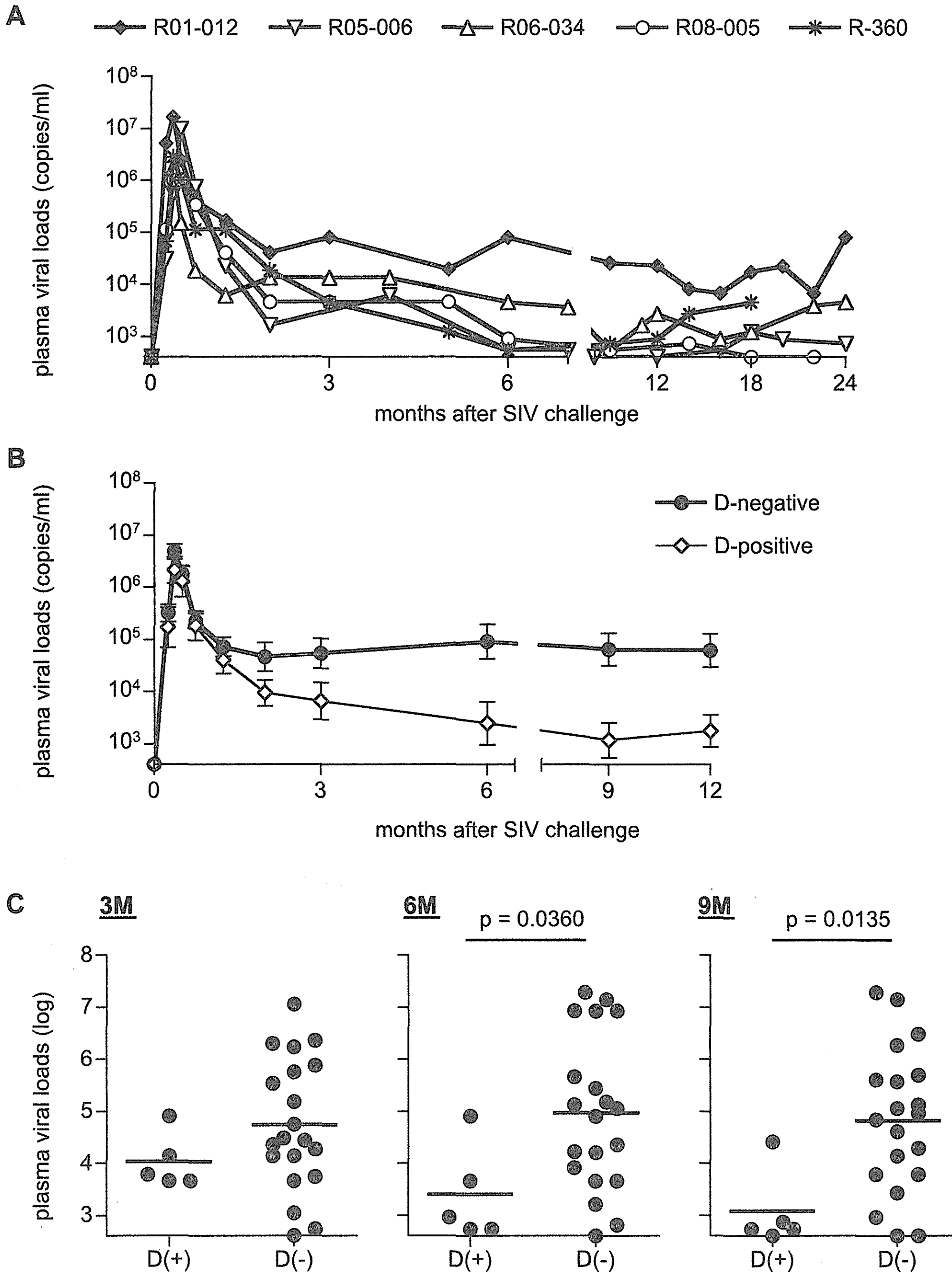

Figure 1. Plasma viral loads after SIVmac239 challenge in unvaccinated macaques. Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described previously [35]. The lower limit of detection is approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads after challenge in unvaccinated macaques possessing MHC-I haplotype D. (B) Changes in geometric means of plasma viral loads after challenge in five unvaccinated D^+ animals in the present study and twenty D^- animals in our previous cohorts [21]. Three of twenty D^- animals were euthanized because of AIDS before 12 months, and we compared viral loads between D^+ and D^- animals until 12 months. (C) Comparison of plasma viral loads at 3 months (left panel), 6 months (middle panel), and 9 months (right panel) between the unvaccinated D^+ and the D^- animals. Viral loads at 6 months and 9 months in D^+ animals were significantly lower than those in the latter D^- animals ($p = 0.0360$ at 6 months and $p = 0.0135$ at 9 months by t-test).
doi:10.1371/journal.pone.0054300.g001

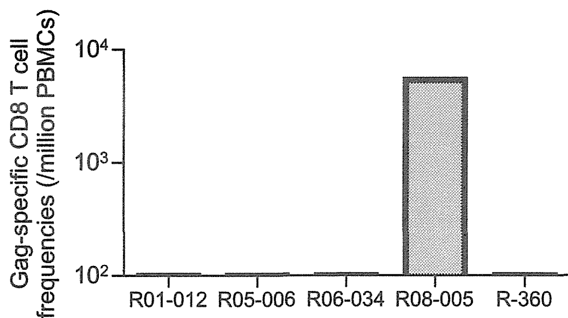

Figure 2. SIV Gag-specific CD8$^+$ T-cell responses in unvaccinated D$^+$ macaques at week 2 after SIVmac239 challenge.
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T-cell responses [35,36]. Accumulation of data on interaction between virus replication and T-cell responses in multiple groups of macaques sharing individual MHC-I haplotypes would provide great insights into our understanding of the mechanism for HIV/SIV control.

In the present study, we investigated SIVmac239 infection of a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-010-Id* (D), which was not associated with dominant Gag-specific CD8$^+$ T-cell responses. These animals had persistent viremia in the early phase but showed significant reduction of viral loads around 6 months after SIV challenge. Most D$^+$ animals showed predominant Nef-specific but not Gag-specific CD8$^+$ T-cell responses. This study presents a protective MHC-I haplotype, indicating the potential of non-Gag antigen-specific CD8$^+$ T-cell responses to contribute to SIV control.

Materials and Methods

Ethics Statement

Animal experiments were carried out in National Institute of Biomedical Innovation (NIBP) and Institute for Virus Research in Kyoto University (IVRKU) after approval by the Committee on the Ethics of Animal Experiments of NIBP and IVRKU in accordance with the guidelines for animal experiments at NIBP, IVRKU, 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 in 12 hours per day. Animals were fed with apples and commercial monkey diet (Type CMK-2, Clea Japan, Inc. Tokyo). Blood collection, vaccination, and SIV challenge were performed under ketamine anesthesia. The endpoint for euthanasia 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.

Animal Experiments

We examined SIV infections in a group of Burmese rhesus macaques (n = 10) sharing the MHC-I haplotype *90-010-Id* (D). The determination of 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 [21,34,37]. Macaques R01-012 and R01-009 used in our previous report [35] and macaques R03-021 and R03-016 used in an unpublished experiment were included in the present study. Five macaques R01-009, R06-020, R06-033, R03-021, and R03-016 received a prophylactic DNA prime/SeV-Gag boost vaccine (referred to as DNA/SeV-Gag vaccine) [35]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from an *env*-deleted and *nef*-deleted simian–human immunodeficiency virus SHIVMD14YE [38] 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 (CIUs) of F-deleted replication-defective SeV-Gag [39,40]. All animals were challenged intravenously with 1,000 $TCID_{50}$ (50 percent tissue culture infective doses) of SIVmac239 [41]. At week 1 after SIV challenge, macaque R03-021 was inoculated with nonspecific immunoglobulin G (IgG) and macaques R03-016 with IgG purified from neutralizing antibody-positive plasma of chronically SIV-infected macaques in our previous experiment [42].

Analysis of SIV Antigen-specific CD8$^+$ T-cell Responses

SIV antigen-specific CD8$^+$ T-cell responses were measured by flow-cytometric analysis of gamma interferon (IFN-γ) induction as described previously [43]. Autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) were established from peripheral blood mononuclear cells (PBMCs) which were obtained from individual macaques before SIV challenge [44]. PBMCs obtained from SIV-infected macaques were cocultured with autologous B-LCLs pulsed with peptides or peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Env, and Nef amino acid sequences. Alternatively, PBMCs were cocultured with B-LCLs infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (BD, Tokyo, Japan). Fluorescein isothiocianate-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, San Diego, CA) were used. Specific T-cell levels were calculated by subtracting non-specific IFN-γ$^+$ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell levels less than 100 cells per million PBMCs were considered negative.

Sequencing Analysis of Plasma Viral Genomes

Viral RNAs were extracted using High Pure Viral RNA kit (Roche Diagnostics, Tokyo, Japan) from macaque plasma samples. Fragments of cDNAs encoding SIVmac239 Gag and 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, Tokyo, Japan) as described before [45]. Predominant non-synonymous mutations were determined.

Statistical Analysis

Statistical analysis was performed using Prism software version 4.03 with significance levels set at a P value of <0.050 (GraphPad Software, Inc., San Diego, CA). Plasma viral loads were log transformed and compared by an unpaired two-tailed t test.

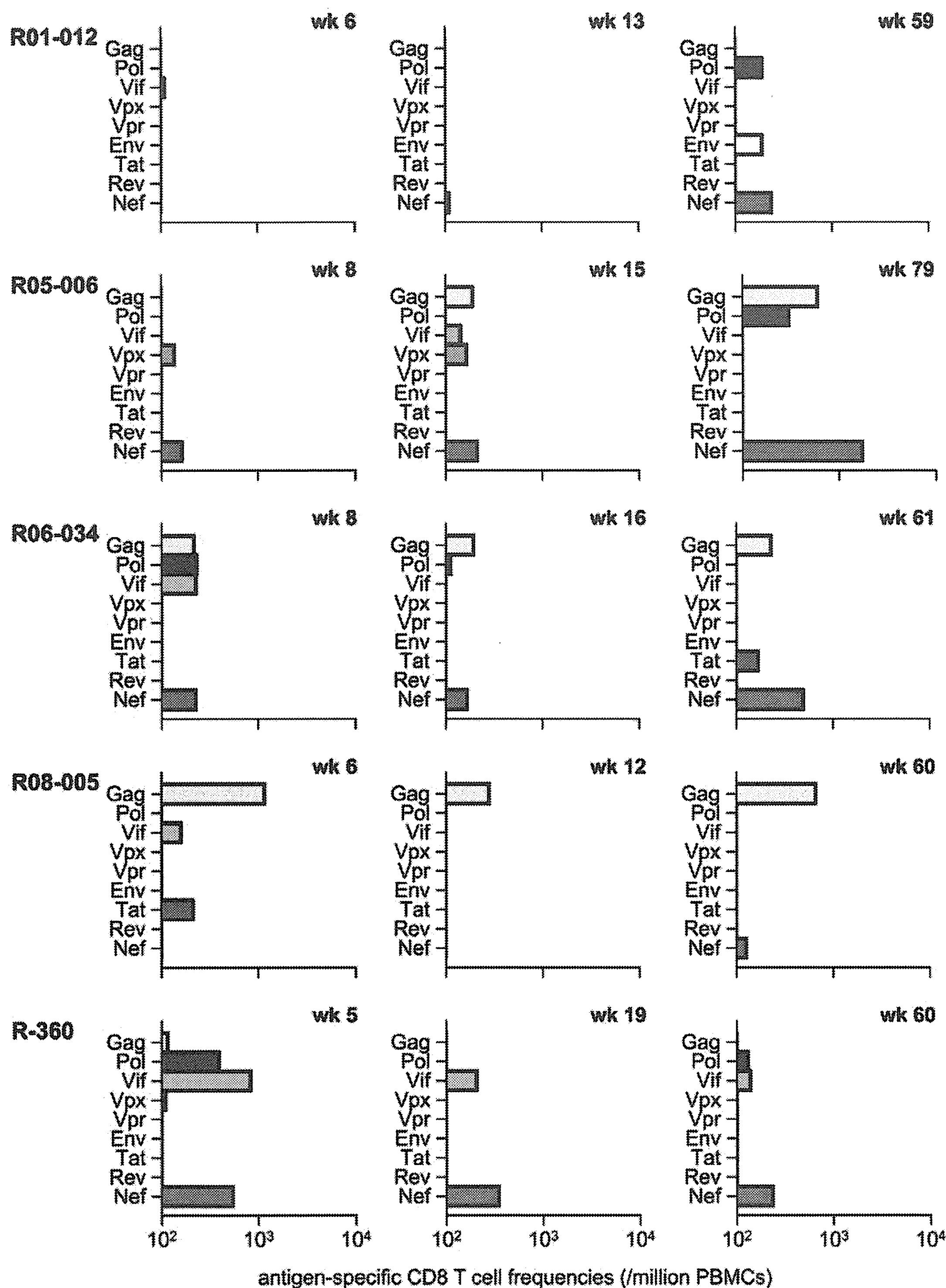

Figure 3. SIV antigen-specific CD8$^+$ T-cell responses in unvaccinated D$^+$ macaques. Responses were measured by the detection of antigen-specific IFN-γ induction in PBMCs obtained at indicated time points after SIVmac239 challenge.
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Results

Lower Viral Loads in D$^+$ Macaques in the Chronic Phase of SIV Infection

We first investigated SIVmac239 infection of five unvaccinated Burmese rhesus macaques sharing the MHC-I haplotype D (referred to as D$^+$ macaques). Confirmed MHC-I alleles consisting of this haplotype is *Mamu-A1*032:02*, *Mamu-B*004:01*, and *Mamu-B*102:01:01*. These animals showed lower set-point plasma viral loads (Fig. 1). Comparison of plasma viral loads between these five animals and our previous cohorts of SIVmac239-infected Burmese D-negative (D$^-$) rhesus macaques (n = 20) [21] revealed no

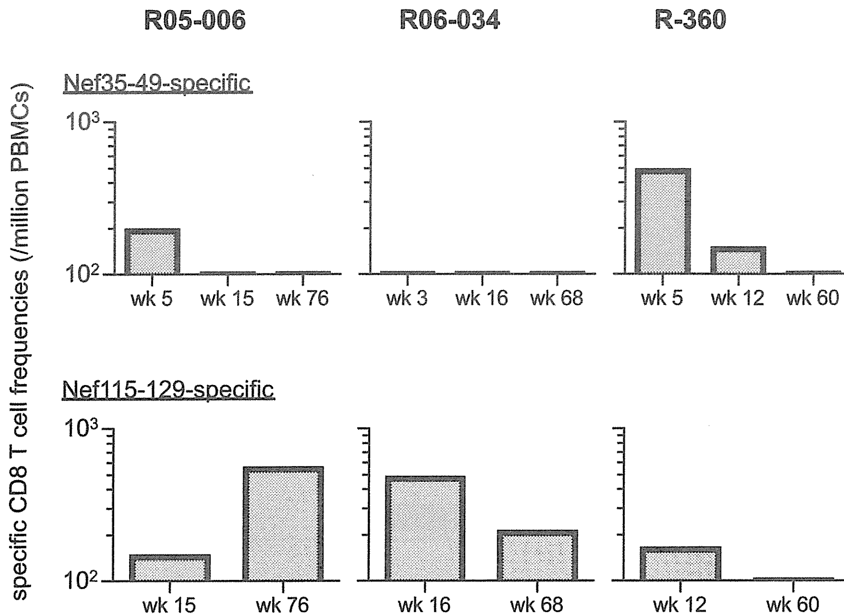

Figure 4. SIV Nef-specific CD8$^+$ T-cell responses in macaques R05-006, R06-034, and R-360. Nef_{35-49}-specific (upper panels) and $Nef_{115-129}$-specific (lower panels) CD8$^+$ T-cell responses were examined at indicated time points after SIVmac239 challenge.
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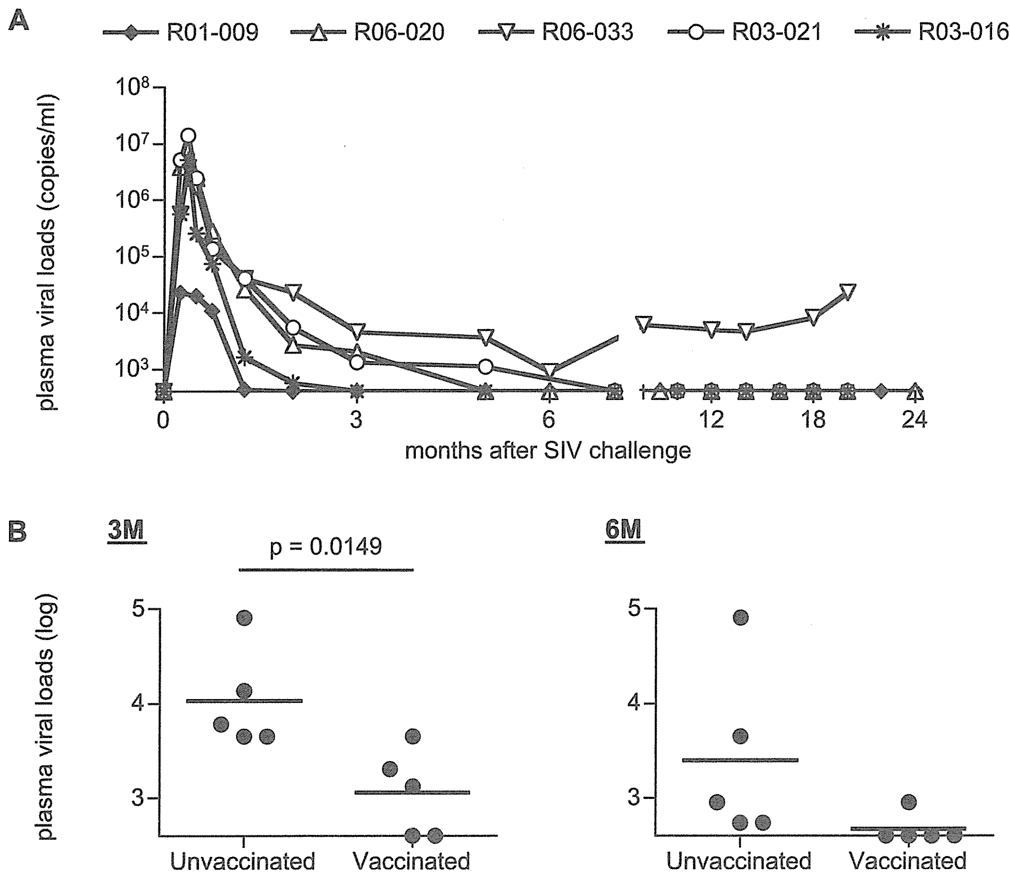

Figure 5. Plasma viral loads after SIVmac239 challenge in vaccinated D$^+$ macaques. (A) Changes in plasma viral loads after challenge vaccinated macaques possessing MHC-I haplotype D. (B) Comparison of plasma viral loads at 3 months (left panel) and 6 months (right panel) between five unvaccinated D$^+$ and five vaccinated D$^+$ animals. Viral loads at 3 months in vaccinated animals were significantly lower than those in the unvaccinated ($p = 0.0149$ by t-test).
doi:10.1371/journal.pone.0054300.g005

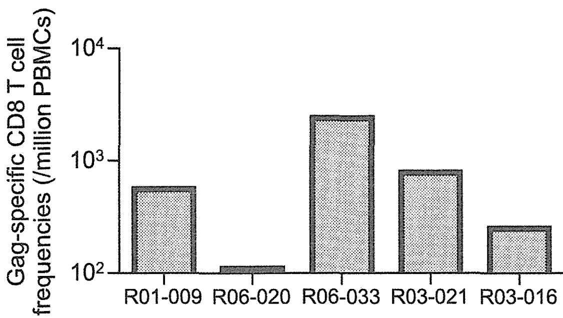

Figure 6. SIV Gag-specific CD8$^+$ T-cell responses in vaccinated D$^+$ macaques at week 2 after SIVmac239 challenge.
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significant difference at 3 months after SIV challenge ($p = 0.2436$ by t-test), but viral loads in the former D$^+$ animals became significantly lower than the latter after 6 months ($p = 0.0360$ at 6 months and $p = 0.0135$ at 9 months by t-test; Fig. 1). Four of these five macaques sharing MHC-I haplotype D showed low viral loads, less than 5×10^3 copies/ml, after 6 months, whereas macaque R01-012 maintained relatively higher viral loads.

Predominant Nef-specific CD8$^+$ T-cell Responses

We examined SIV antigen-specific CD8$^+$ T-cell responses by detection of antigen-specific IFN-γ induction. In the very acute phase, we did not have enough PBMC samples for measurement of individual SIV antigen-specific CD8$^+$ T-cell responses and focused on examining Gag-specific CD8$^+$ T-cell responses in most animals. At week 2 after challenge, Gag-specific CD8$^+$ T-cell responses were undetectable in four of five animals (Fig. 2).

Figure 7. SIV antigen-specific CD8$^+$ T-cell responses in vaccinated D$^+$ animals after SIVmac239 challenge. Samples for this analysis were unavailable in macaque R01-009.
doi:10.1371/journal.pone.0054300.g007

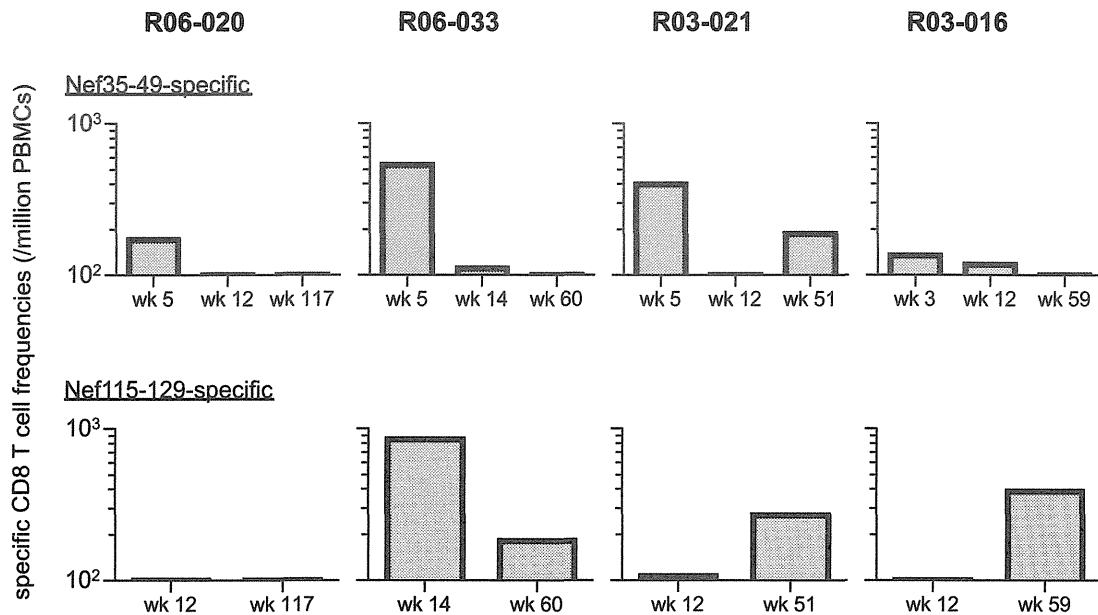

Figure 8. SIV Nef-specific CD8$^+$ T-cell responses in macaques R06-020, R06-033, R03-021, and R03-016. Nef_{35-49}-specific (upper panels) and $Nef_{115-129}$-specific (lower panels) CD8$^+$ T-cell responses were examined at indicated time points after SIVmac239 challenge.
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We then examined CD8$^+$ T-cell responses specific for individual SIV antigens in the early and the late phases (Fig. 3). Nef-specific but not Gag-specific CD8$^+$ T-cell responses were predominant in most D$^+$ animals. Gag-specific CD8$^+$ T-cell responses were dominantly induced in macaque R08-005 showing very low set-point viral loads. Macaque R01-012 having higher viral loads showed poor CD8$^+$ T-cell responses in the early phase.

Among four D$^+$ animals controlling SIV replication with less than 5×10^3 copies/ml of plasma viral loads after 6 months, Gag-specific CD8$^+$ T-cell responses were dominant only in macaque R08-005, while efficient Nef-specific CD8$^+$ T-cell responses were induced in the remaining three, suggesting possible contribution of Nef-specific CD8$^+$ T-cell responses to SIV control in these three controllers (R05-006, R06-034, and R-360). We then attempted to localize Nef CD8$^+$ T-cell epitopes shared in these animals and found Nef_{35-49}-specific and $Nef_{115-129}$-specific CD8$^+$ T-cell responses (Fig. 4), although we did not have enough samples for mapping the exact epitopes.

Reduction of Viral Loads in the Early Phase of SIV Infection by Prophylactic Vaccination

We also investigated SIVmac239 infection of additional five, vaccinated Burmese rhesus macaques sharing the MHC-I haplotype D. These animals received a prophylactic DNA/SeV-Gag vaccination. In four of these five vaccinated macaques, plasma viremia became undetectable after 6 months, while macaque R06-033 showed persistent viremia (Fig. 5A). Difference in viral loads between unvaccinated and vaccinated D$^+$ animals was unclear in the acute phase, but the latter vaccinees showed significant reduction in viral loads compared to those in the former unvaccinated at 3 months ($p = 0.0360$; Fig. 5B). After 6 months, unvaccinated animals also showed reduced viral loads, and the difference in viral loads between unvaccinated and vaccinated became unclear.

In contrast to unvaccinated D$^+$ animals, all five vaccinated animals elicited Gag-specific CD8$^+$ T-cell responses at week 2 after challenge (Fig. 6), reflecting the effect of prophylactic vaccination.

We then examined CD8$^+$ T-cell responses specific for individual SIV antigens in these vaccinated animals (Fig. 7). Samples for this analysis were unavailable in vaccinated macaque R01-009. Vaccinated animals except for macaque R06-020 showed dominant Gag-specific CD8$^+$ T-cell responses even at 1–2 months. However, Gag-specific CD8$^+$ T-cell responses became not dominant after 1 year, while Nef-specific or Vif-specific CD8$^+$ T-cell responses became predominant, instead, in most vaccinees except for macaque R03-016.

Like three unvaccinated macaques (R05-006, R06-034, and R-360), vaccinated D$^+$ animals induced Nef_{35-49}-specific and $Nef_{115-129}$-specific CD8$^+$ T-cell responses after SIV challenge (Fig. 8). In analyses of three unvaccinated (Fig. 4) and four vaccinated animals (Fig. 8), Nef_{35-49}-specific CD8$^+$ T-cell responses were induced in the early phase in six animals but mostly became undetectable in the chronic phase. $Nef_{115-129}$-specific CD8$^+$ T-cell responses were also induced in most animals except for macaque R06-020 which showed $Nef_{112-126}$-specific ones in the chronic phase (data not shown). Macaques R05-006, R03-021, and R03-016 showed efficient $Nef_{115-129}$-specific CD8$^+$ T-cell responses not in the early phase but in the chronic phase. In contrast, vaccinated animal R06-033 that failed to control viremia showed higher $Nef_{115-129}$-specific CD8$^+$ T-cell responses in the early phase than those in the chronic phase.

Selection of Mutations in Nef CD8$^+$ T-cell Epitope-coding Regions

To see the effect of selective pressure by Nef-specific CD8$^+$ T-cell responses on viral genome mutations, we next analyzed nucleotide sequences in viral *nef* cDNAs amplified from plasma RNAs obtained at several time points after SIV challenge. Nonsynonymous mutations detected predominantly in Nef_{35-49}-coding and $Nef_{115-129}$-coding regions were as shown in Fig. 9. Remarkably, all the unvaccinated and vaccinated D$^+$ animals showed rapid selection of mutations in the Nef_{35-49}-coding region in 3 months. On the other hand, mutations in the $Nef_{115-129}$-coding region were observed in the late phase in all the three

		Nef35-49					Nef115-129				
Nef		36	37	41	42	44	119	122	124	125	126
		E	D	Q	S	G	M	F	K	E	K
R01-012	1M		*G								
	3M		*G								
	14M	*G	*G								
	24M	*G	*G			*E					
R05-006	1M					*E					
	3M			R							
	16M			R							
	24M			R							R
R06-034	1M										
	3M	*G									
	10M	*G	*G			*E					*R
	18M	G				E					R
R08-005	1M										
	3M				*F						
	6M	G									
	14M				F						
	24M	*G			F						
R-360	1M										
	3M		*G								
	6M		G								
	12M		G				T	L			
	20M		G				T	L			
R06-020	1M										
	3M	*K									
	11M		G	R							
R06-033	1M										
	3M	*G									
	6M		*G								
	14M		G			*E				K	E
R03-021	1M										
	3M				*F						
	14M	G							R		
R03-016	1M	*K		*R							
	4M	K									
	12M	K									

Figure 9. Predominant non-synonymous mutations in Nef_{35-49}-coding and $Nef_{115-129}$-coding regions of viral cDNAs in D^+ animals after SIVmac239 challenge. Amino acid substitutions are shown. Detection of similar levels of wild-type and mutant sequences at the residue is indicated by asterisks. Samples for this analysis were unavailable in macaque R01-009.
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unvaccinated animals eliciting $Nef_{115-129}$-specific $CD8^+$ T-cell responses. These mutations were also detected in two of three vaccinated animals eliciting $Nef_{115-129}$-specific $CD8^+$ T-cell responses.

We also analyzed viral *gag* sequences to see the effect of Gag-specific $CD8^+$ T-cell pressure on viral genome mutations in vaccinated animals (data not shown). Our previous study [35] showed rapid selection of a mutation leading to a glutamine (Q)-to-lysine (K) change at the 58th residue in Gag (Q58K) at week 5 in vaccinated macaque R01-009, although no more samples were available for this sequencing analysis. This Q58K mutation results in escape from Gag_{50-65}-specific $CD8^+$ T-cell recognition. In the present study, macaque R03-016 showed rapid selection of a mutation leading to a K-to-asparagine (N) change at the 478th residue in Gag in 1 month. These results may reflect rapid disappearance of detectable plasma viremia in 1 or 2 months in these two vaccinees. Macaque R06-020 showed selection of a *gag* mutation in 3 months, while other two vaccinees (R06-033 and R03-021) selected no *gag* mutation in the early phase.

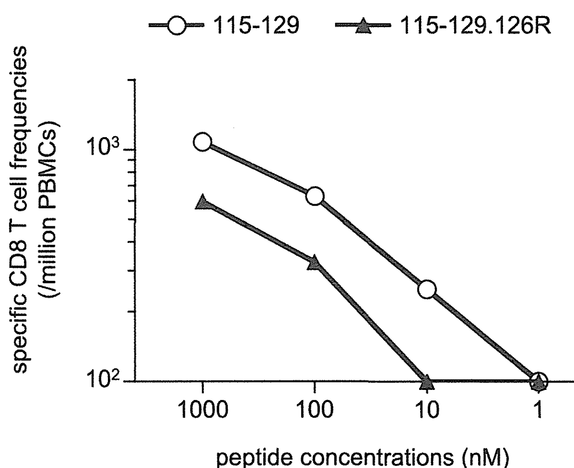

Figure 10. IFN-γ induction in $CD8^+$ T cells after stimulation with the wild-type or the mutant peptide. PBMCs obtained at week 31 from macaque R06-033 were stimulated by coculture with B-LCL pulsed with indicated concentrations of the wild-type $Nef_{115-129}$ peptide (open circles, 115–129, LAIDMSHFIKEKGGL) or the mutant $Nef_{115-129}$ peptide with a K126R alteration (closed triangles, 115–129.126R, LAIDMSHFIKERGGL).
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Discussion

HIV infection in humans with polymorphic MHC-I genotypes induces various patterns of viral antigen-specific $CD8^+$ T-cell responses. Previous studies have found several protective MHC-I alleles associated with lower viral loads and slower disease progression in HIV/SIV infection [7,13,14,16,17]. Elucidation of the mechanisms of viral control associated with individual protective MHC-I alleles would contribute to HIV cure and vaccine-based prevention. Because $CD8^+$ T-cell responses specific for some MHC-I-restricted epitopes can be affected by those specific for other MHC-I-restricted epitopes due to immunodominance [29,46,47], macaque groups sharing MHC-I genotypes at the haplotype level are useful for the analysis of cooperation of multiple epitope-specific $CD8^+$ T-cell responses. Previously, we reported a group of Burmese rhesus macaques sharing MHC-I haplotype *90-120-Ia* (A), which dominantly induce Gag-specific $CD8^+$ T-cell responses and tend to show slower disease progression after SIVmac239 challenge [21]. In the present study, we presented another type of protective MHC-I haplotype, which is not associated with dominant Gag-specific $CD8^+$ T-cell responses. Significant reduction of viral loads in unvaccinated macaques possessing this D haplotype compared to those in D^- macaques was observed after 6 months. Analysis of SIV infection in macaques sharing this protective MHC-I haplotype would lead to understanding of $CD8^+$ T-cell cooperation for viral control.

Analyses of antigen-specific $CD8^+$ T-cell responses after SIVmac239 challenge indicate that this MHC-I haplotype D is associated with predominant Nef-specific $CD8^+$ T-cell responses. Nef-specific $CD8^+$ T-cell responses were efficiently induced in all SIV controllers, whereas Gag-specific $CD8^+$ T-cell responses were dominant in only one of them. We found Nef_{35-49}-specific and $Nef_{115-129}$-specific $CD8^+$ T-cell responses shared in D^+ animals. We were unable to determine the MHC-I alleles restricting these epitopes, but these responses are not usually induced in our

previous D^- cohorts and considered to be associated with this MHC-I haplotype D.

Sequencing analysis of viral genomes showed rapid selection of mutations in the Nef_{36-44}-coding region within 3 months in all the D^+ animals. This is consistent with our results that Nef_{35-49}-specific $CD8^+$ T-cell responses were mostly induced in the early phase but undetectable in the chronic phase. These mutations were not consistently selected in our previous D^- cohorts and thus considered as MHC-I haplotype D-associated mutations. This suggests strong selective pressure by Nef_{35-49}-specific $CD8^+$ T-cell responses in the acute phase of SIVmac239 infection in D^+ macaques, although it remains undetermined whether these mutations result in viral escape from Nef_{35-49}-specific $CD8^+$ T-cell recognition.

$Nef_{115-129}$-specific $CD8^+$ T-cell responses were detected in six D^+ animals. In five of them, nonsynonymous mutations in the $Nef_{119-126}$-coding region were observed in the chronic phase. At least, we confirmed viral escape from $Nef_{115-129}$-specific $CD8^+$ T-cell recognition by a mutation leading to a K-to-arginine (R) (K126R) substitution at Nef residue 126 (Fig. 10). The number of nonsynonymous substitutions per the number of sites estimated to be nonsynonymous (dN) exceeded that estimated to be synonymous (dS) during the evolution process of $Nef_{115-129}$-coding region, but the value did not show statistically significant difference from that of neutral selection. Among three unvaccinated animals that controlled SIV replication without dominant Gag-specific $CD8^+$ T-cell responses, amino acid substitutions in the $Nef_{119-126}$-coding region were observed in a year in macaques R06-034 and R-360 but after 2 years in macaque R05-006. The former two animals tended to show earlier increases in plasma viral loads in the chronic phase, while the latter R05-006 maintained higher frequencies of $Nef_{115-129}$-specific $CD8^+$ T-cell responses. $Nef_{115-129}$-specific $CD8^+$ T-cell responses were efficient in the chronic phase in vaccinated controllers R03-021 and R03-016 but decreased in R06-033 that failed to contain SIV replication. Although a possible effect of this haplotype-associated factors other than $CD8^+$ T-cell responses such as NK activity on SIV infection [48,49,50] remains undetermined, these results imply involvement of Nef-specific $CD8^+$ T-cell responses in the SIV control associated with MHC-I haplotype D.

Unvaccinated macaque R08-005 dominantly elicited Gag antigen-specific $CD8^+$ T-cell responses and showed rapid selection of a mutation encoding Gag 257 residue, which was not observed in any other D^+ animals. Nef-specific $CD8^+$ T-cell responses were detectable only at week 2 in the acute phase (data not shown) and a mutation in the Nef_{42}-coding region was rapidly selected. It is speculated that those dominant Gag-specific $CD8^+$ T-cell responses associated with the second, non-D MHC-I haplotype were effective in this animal. Nef_{35-49}-specific $CD8^+$ T-cell responses may not be efficient due to immunodominance but exert some suppressive pressure on viral replication.

DNA/SeV-Gag vaccination resulted in earlier reduction of viral loads after SIV challenge. Vaccinees showed significantly lower viral loads at 3 months than those in unvaccinated animals. Gag-specific $CD8^+$ T-cell responses were elicited at week 2 in all the vaccinees but not in the unvaccinated except for one animal R08-005. No *gag* mutations were shared in the vaccinees in the acute phase, but three of them showed rapid selection of individual non-synonymous mutations in *gag*. Rapid selection of mutations in the Nef_{36-44}-coding region was consistently detected even in these vaccinees. These results suggest broader $CD8^+$ T-cell responses consisting of dominant vaccine antigen Gag-specific and inefficient naive-derived Nef-specific ones in the acute phase. In three vaccinated animals, Gag-specific $CD8^+$ T-cell responses became lower or undetectable, and instead, Nef-specific $CD8^+$ T-cell responses became predominant in the chronic phase.

In summary, we found a protective MHC-I haplotype not associated with dominant Gag-specific $CD8^+$ T-cell responses in SIVmac239 infection. Our results in D^+ macaques suggest suppressive pressure by Nef_{35-49}-specific and $Nef_{115-129}$-specific $CD8^+$ T-cell responses on SIV replication, contributing to reduction in set-point viral loads. DNA/SeV-Gag-vaccinated D^+ animals induced Gag-specific $CD8^+$ T-cell responses in addition to Nef-specific ones after SIV challenge, resulting in earlier containment of SIV replication. This study presents a pattern of SIV control with involvement of non-Gag antigen-specific $CD8^+$ T-cell responses, contributing to accumulation of our knowledge on HIV/SIV control mechanisms.

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Author Contributions

Performed animal experiments: HS TM TI YK. Performed MHC-I typing: TKN AK. Conceived and designed the experiments: NT TM. Performed the experiments: NT TN YT HY AT. Analyzed the data: NT HY T. Shiino TM. Contributed reagents/materials/analysis tools: MI AI HH T. Shu MH. Wrote the paper: NT TM.

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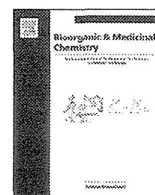

CXCR4-derived synthetic peptides inducing anti-HIV-1 antibodies

Chie Hashimoto[a], Wataru Nomura[a], Tetsuo Narumi[a], Masayuki Fujino[b], Toru Nakahara[a], Naoki Yamamoto[c], Tsutomu Murakami[b], Hirokazu Tamamura[a,*]

[a] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan
[b] AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan
[c] Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

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ABSTRACT

Despite almost 30 years since the identification of the human immunodeficiency virus type I (HIV-1), development of effective AIDS vaccines has been hindered by the high mutability of HIV-1. The HIV-1 co-receptors CCR5 and CXCR4 are genetically stable, but viral proteins may mutate rapidly during the course of infection. CXCR4 is a seven transmembrane G protein-coupled receptor, possessing an N-terminal region (NT) and three extracellular loops (ECL1-3). Previous studies have shown that the CXCR4-ED-derived peptides inhibit the entry of HIV-1 by interacting with gp120, an HIV-1 envelope glycoprotein. In the present study, antigenicity of CXCR4-derived peptides has been investigated and the anti-HIV-1 effects of induced antisera have been assessed. It was found that CXCR4-ED-derived antigen molecules immunize mice, showing that the linear peptides have higher antigenicity than the cyclic peptides. The L1- and L2-induced antisera inhibited the HIV-1 entry significantly, while anti-N1 antibodies have no inhibitory activity. This study produced promising examples for the design of AIDS vaccines which target the human protein and can overcome mutability of HIV-1.

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1. Introduction

The human immunodeficiency virus HIV-1 is the agent causing the acquired immunodeficiency syndrome (AIDS), discovered by Montagnier and colleagues in 1983.[1] HIV-1 infects human host $CD4^+$ cells and destroys immune systems, causing immunodeficiency. The detailed molecular mechanism of HIV-1 entry into its host cells is well understood.[2,3] First, an HIV-1 envelope (Env) glycoprotein gp120 interacts with the host cell receptor CD4.[4] This triggers conformational changes within viral Env proteins, and leads to the exposure of the co-receptor binding site of gp120, which is composed of the V3 loop and the bridging sheet of gp120.[5] The binding of gp120 to the extracellular domains of the co-receptors CCR5 and CXCR4 facilitates the insertion of another Env glycoprotein (gp41) into the cell membranes, causing the fusion of the viral and cell membranes and thereby enabling the entry of the viral core into the cell.[6] Since 1990, several anti-HIV-1 drugs including protease, reverse transcriptase and integrase inhibitors have been developed.[7] These drugs are ordinarily administered in a two- or three-drug cocktail therapy, which is designated as a highly active antiretroviral therapy (HAART). This therapy has enjoyed great success and engendered hope in the clinical treatment of HIV-1-infected patients. Anti-HIV-1 drugs, however, cannot eliminate HIV-1 completely from patients, and patients are required to take drugs indefinitely. During such long-term administration, drug-resistant variants emerge and consequently new drugs, which can inhibit viral entry by novel mechanisms, are required.

The HIV-1 co-receptors CCR5 and CXCR4 belong to a family of seven transmembrane G protein-coupled receptors (GPCR), which possess the extracellular N-terminal region (NT) and three extracellular loops (ECL1-3) as the extracellular domains (ED). The CCR5- and CXCR4-ED regions interact with their endogenous ligands together with the V3 loop and the bridging sheet of gp120, to comprise the co-receptor binding site of gp120. HIV-1 strains are classified as macrophage-tropic (R5), T-cell line-tropic (X4) and dual/mixed-tropic (R5X4).[8] R5-HIV-1 strains, which use CCR5 as a co-receptor, constitute a majority in the early stage of HIV-1 infection. X4-HIV-1 strains use CXCR4 as a co-receptor and are the major species in late stages of HIV-1 infection and AIDS, and R5X4-HIV-1 can use both CCR5 and CXCR4 as co-receptors.

Maraviroc (4,4-difluoro-*N*-[(1*S*)-3-[(3-exo)-3-[3-methyl-5-(1-methylethyl)-4*H*-1,2,4-triazol-4-yl]-8-azabicyclo[3.2.1]oct-8-yl]-1-phenylpropyl]-cyclohexanecarboxamide) is the first CCR5 antagonist to be approved by the FDA and has been used for the treatment of patients infected only with R5-HIV-1 strains.[9] Antibody-based therapy has been studied as an alternative route to small molecule anti-HIV-1 agents and antigen molecules based on the extracellular loops of CCR5/CXCR4 have been

* Corresponding author. Tel.: +81 3 5280 8036; fax: +81 3 5280 8039.
E-mail address: tamamura.mr@tmd.ac.jp (H. Tamamura).

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developed.[10–12] A humanized monoclonal antibody against CCR5, PRO-140 (CytoDyn/Progenics) has completed Phase II clinical trials,[13,14] and has no significant effect on the physiological function of CCR5,[15,16] suggesting that the HIV-1 co-receptor CCR5 is an effective preventative AIDS vaccine target. However, these therapies cannot be used for patients in the late stage of HIV-1 infection and AIDS, in which X4-HIV-1 strains are dominant. Accordingly, various CXCR4 antagonists including a 14-mer cyclic peptide T140,[17] a T140-derived 5-mer cyclic peptide FC131,[18] AMD3100,[19,20] a Dpa-zinc complex,[21] azamacrocyclic-zinc complexes,[22] KRH-1636[23] and other compounds have been developed.[24–29] Previously, we developed CXCR4-ED-derived peptidic inhibitors, NT-derived fragment peptides and cyclic peptides based on ECL1 and ECL2, which showed potent anti-HIV-1 activity[30] and suggested that CXCR4-ED peptides may interact with the V3 loop, thus blocking the gp120–CXCR4 interaction. Consequently, antibodies against these CXCR4-ED peptides might block the HIV-1 entry effectively. To date however, no AIDS vaccine candidate has been entered into Phase III efficacy trials despite enormous efforts. The main obstacle to this has been the genetic diversity of HIV-1 strains.[31–33] Antibody-based therapy and AIDS vaccines targeting the co-receptors are however valuable approaches because they are independent of viral mutation. In this study, the antigenicity of these CXCR4-ED peptides is investigated and the anti-HIV-1 activity of induced antibodies is evaluated.

2. Results and discussion

2.1. Design

CXCR4 possesses an N-terminal region (Met^1-Ile^{39}) and three extracellular loops (ECL1: Val^{99}-Ala^{111}, ECL2: Ala^{175}-His^{203} and ECL3: Asp^{262}-Lys^{282}) (Fig. 1A). It has been reported that an anti-ECL3 antibody, A80 induces lymphocyte agglutination and enhances HIV-1-mediated syncytium formation[34] and peptidic antigen molecules derived from the extracellular domains, excluding ECL3, have been synthesized.[30] The 39-mer Met^1-Ile^{39} NT was segmented into 3 NT-fragment peptides: **N1** (Met^1-Asp^{20}), **N2** (Asn^{11}-Arg^{30}) and **N3** (Tyr^{21}-Ile^{39}), with 10-mer overlapping sites. Two different types of cyclization strategies were used to synthesize cyclic peptides of ECL1 and ECL2 which mimic their loop architectures. A cyclic peptide based on ECL1 (**C1**) was designed to result from a cyclization reaction between the N-terminal chloroacetyl group and the C-terminal Cys thiol group in a dilute solution at pH 7.8 (Fig. 1B). A cyclic peptide based on ECL2 (**C2**) was designed to be produced in a head-to-tail cyclization using HOBt/HBTU/DIEA. As depicted in red in Figure 1B, the hydrophilic residues Arg and Glu were fused, Gly was introduced as a spacer and Cys was fused as a site for ligation with a carrier peptide; multi-antigen peptides (MAPs). The Cys residues involved in disulfide bonds in CXCR4-ED, between Cys^{28} on NT and Cys^{274} on ECL3, and between Cys^{109} on ECL1 and Cys^{186} on ECL2 were replaced by Ser, blue in Figure 1B, to avoid reaction with the chloroacetyl or bromoacetyl groups of the MAPs at this site.

In the design of antigen molecules, monomeric CXCR4-ED-derived peptides were conjugated to MAPs to increase inducibility.[35] In general, low molecular weight substances such as peptides are not immunogenic and require aid from carrier proteins or MAPs to stimulate a response from the immune system.[36] The MAPs were designed to introduce γ-aminobutyric acid (GABA) as spacers and monochloroacetic acid as the site of ligation with the thiol groups on the CXCR4-ED-derived peptides (Fig. 2A). However, the ligation reaction between 31-mer cyclic peptide based on ECL2 (C2) and chloroacetylated MAP experiences steric difficulties and is slow. Consequently, the MAP for the ligation of C2/L2 was designed to introduce longer GABA spacers than in the other CXCR4-ED-derived peptides and monobromoacetic acid was used to enhance the reactivity of the MAP.

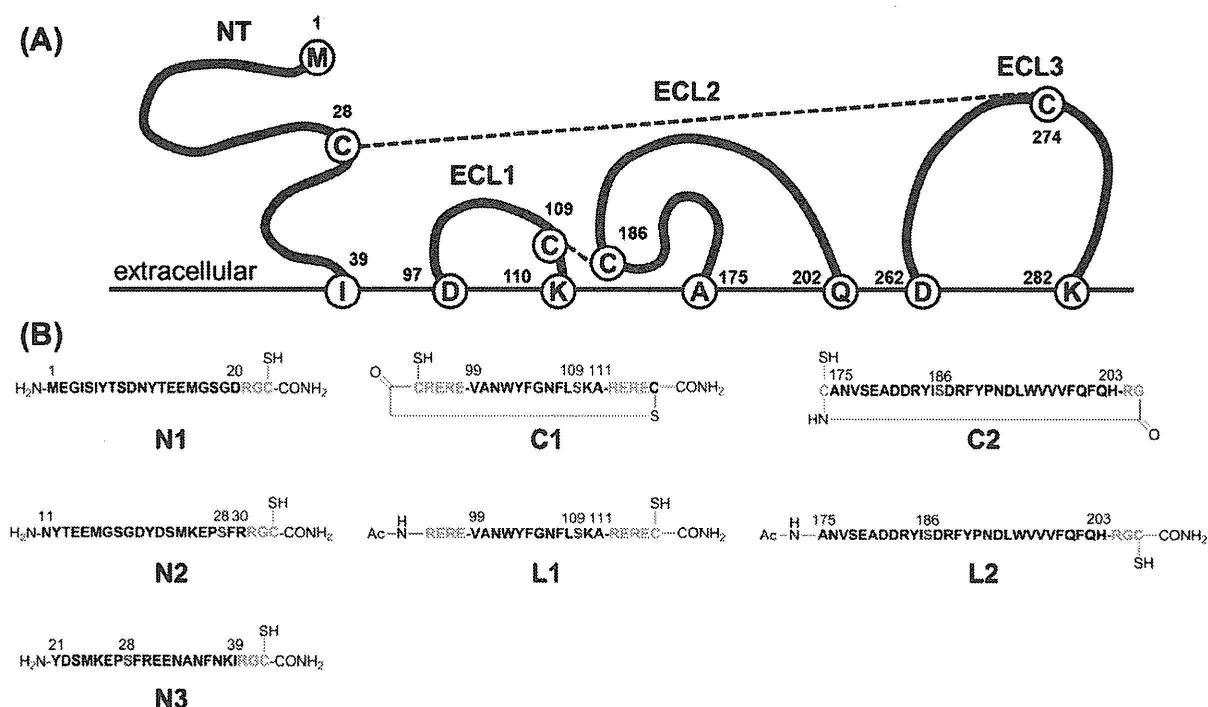
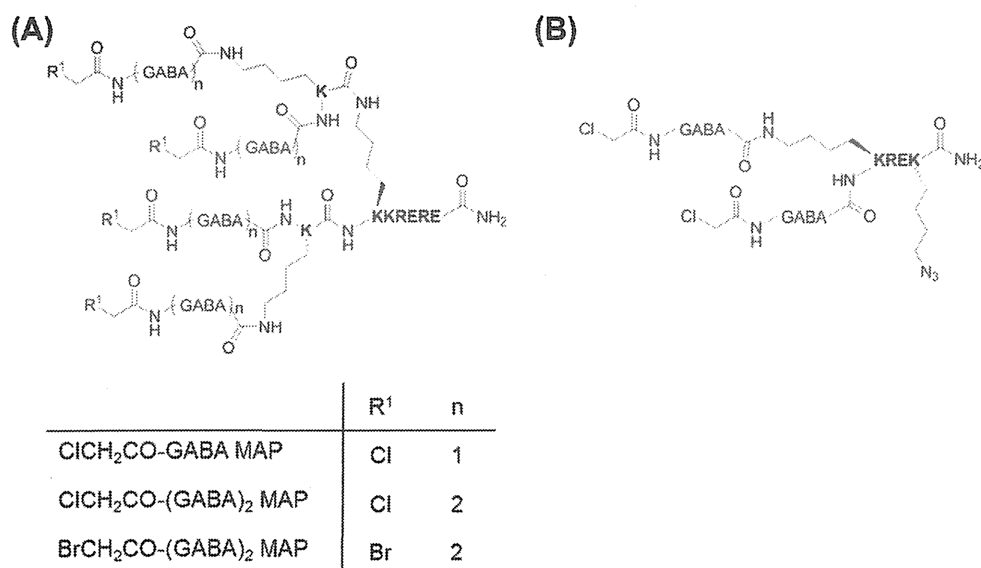


Figure 1. (A) Schematic structure of the extracellular domains of CXCR4 and (B) the structures of synthetic CXCR4-ED-derived peptides. Extracellular domains of CXCR4; the N-terminal region is NT, the extracellular loops are ECL1, ECL2 and ECL3. Two disulfide bonds formed between Cys^{28} on NT and Cys^{274} on ECL3, and between Cys^{109} on ECL1 and Cys^{186} on ECL2, are depicted as dashed lines (A). Cys$^{28,109,186\text{ and }274}$ are mutated to Ser (blue) to avoid reactions with MAPs at these sites. R and E (red), Arg and Glu, are added to increase solubility (B).



	R^1	n
$ClCH_2CO$-GABA MAP	Cl	1
$ClCH_2CO$-$(GABA)_2$ MAP	Cl	2
$BrCH_2CO$-$(GABA)_2$ MAP	Br	2

Figure 2. Structures of MAPs (A) and the 2-branched azide template (B). (A) The N-terminus is chloroacetylated or bromoacetylated (R^1 = Cl or Br) as a site for reaction with CXCR4-ED-derived peptides. GABA (n = 1 or 2) is fused as a spacer. R and E are added to increase solubility. (B) The N-terminus of the 2-branched template is chloroacetylated as a reaction site with the N1 peptide. The azide moiety is introduced by condensing Fmoc-Lys(N_3)-OH. GABA is fused as a spacer. R and E are added to increase solubility.

2.2. Chemistry

2.2.1. Synthesis of MAPs

MAPs were synthesized by standard Fmoc-solid phase peptide synthesis (Fmoc-SPPS) using a Rink amide resin or a NovaSyn® TGR resin. Fmoc-Lys(Fmoc)-OH was used in the branch position and the ligation sites with thiol groups on the synthetic CXCR4-ED-derived peptides were prepared by condensation with monochloroacetic acid or monobromoacetic acid. Coupling reactions were performed by treatment for 1–2 h with 5.0 equiv of Fmoc-protected amino acid, 5.0 equiv of diisopropylcarbodiimide (DIPCI) and 5.0 equiv of 1-hydroxybenzotriazole monohydrate ($HOBt{\cdot}H_2O$). Fmoc deprotection was achieved with 20% piperidine in *N,N*-dimethylformamide (DMF). The N-terminus was chloroacetylated or bromoacetylated by treatment for 1 h with 40 equiv of monochloroacetic acid or monobromoacetic acid, respectively, and with 40 equiv of DIPCI and 40 equiv of $HOBt{\cdot}H_2O$. Cleavage of peptides from resins and side chain deprotection was carried out by stirring for 1.5 h with a mixture of TFA, thioanisole, H_2O, *m*-cresol and triisopropylsilane (TIS) (10/0.75/0.5/0.25/0.1, v/v). After removal of the resin by filtration, the filtrate was concentrated under reduced pressure, and the crude peptides were precipitated in cooled Et_2O. Purification was performed by reverse phase HPLC to afford the expected products as colorless powders which were characterized by ESI-TOF-MS. HPLC profiles are provided in Supplementary data.

2.2.2. Conjugation of synthetic CXCR4-ED-derived peptides with MAPs

All CXCR4-derived peptides were previously synthesized by standard Fmoc-solid phase peptide synthesis.[30] The N-terminal chloroacetyl groups or bromoacetyl groups of the MAPs were conjugated to thiol groups of the Cys residues on the CXCR4-ED-derived peptides in 0.1 M sodium phosphate buffer at pH 7.8 or 7.2. Tetramers of the CXCR4-ED-derived peptides, with the exception of N1, were obtained successfully and characterized by ESI-TOF-MS. However, because of the aggregation tendency of the N1 monomeric peptide, the N1 tetramer was not obtained

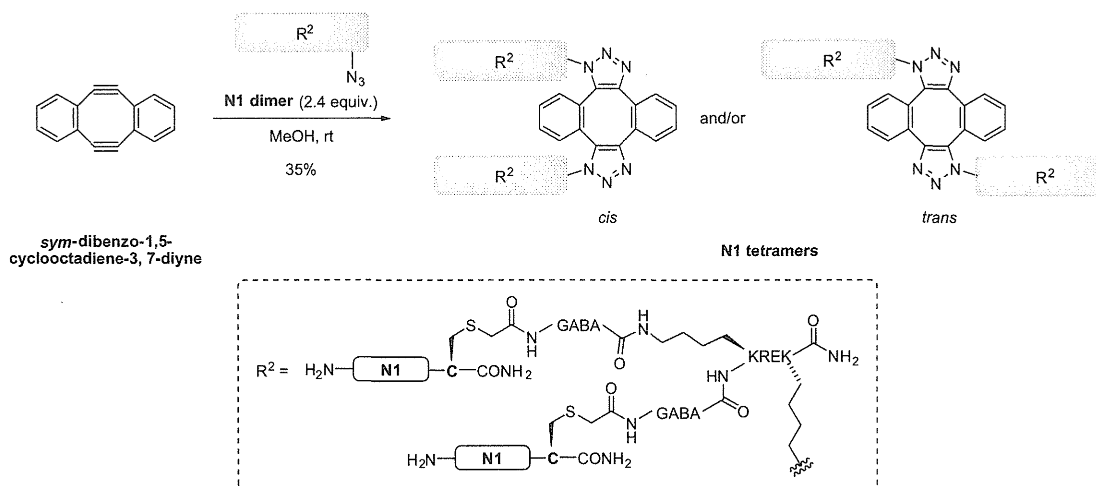
2.2.3. Synthesis of N1 antigen molecule (N1 tetramer)

In the ligation reaction of the N1 monomer and MAP, the N1 dimer, in which 2 of 4 linkers on MAP were reacted with N1 monomers, was detected by HPLC and ESI-TOF-MS. Hence, this N1 dimer, which was produced by the ligation of N1 monomeric peptides and 2-branched template with the azide moiety (Fig. 2B), was ligated to *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne which bears two alkyne moieties in a single molecule,[37] to produce the N1 tetramer by a strain-promoted azide-alkyne cycloaddition (SPAAC) (Scheme 1). A SPAAC between N1 dimers and diyne gave the N1 tetramer in *cis* and/or *trans* forms. In this study, CXCR4-ED-derived peptides were conjugated to MAPs in order to increase molecular weight and achieve recognition as antigens and isomerization is not a significant issue.

The azide template was synthesized by standard Fmoc-SPPS using a Rink amide resin. Fmoc-Lys(Fmoc)-OH was used in the branch position and Fmoc-Lys(N_3)-OH was used at the C-terminus for the reaction with the diyne. The N-terminus was chloroacetylated by treatment for 1 h with 40 equiv of monochloroacetic acid, 40 equiv of DIPCI and 40 equiv of $HOBt{\cdot}H_2O$. Cleavage of peptides from the resin and side chain deprotection was carried out by stirring for 1.5 h with a mixture of TFA, thioanisole, H_2O, *m*-cresol and triisopropylsilane (TIS) (10/0.75/0.5/0.25/0.1, v/v). Purification was performed by preparative HPLC affording the expected product which was characterized by ESI-TOF-MS. The N1 dimer was synthesized by the reaction of 1 equiv of the 2-branched azide template with 2 equiv of N1 in 0.1 M sodium phosphate buffer containing excess potassium iodide at pH 7.8. After stirring overnight at room temperature, the N1 dimer was purified by preparative HPLC and characterized by ESI-TOF-MS. The N1 tetramer was produced by the reaction of 1 equiv of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne with 2.5 equiv of the N1 dimer in dry MeOH stirred overnight at room temperature under N_2. The N1 tetramer was purified by semi-preparative HPLC and characterized by ESI-TOF-MS.

2.3. Immunization of mice

The CXCR4-ED-derived antigen molecules were used to investigate antibody induction in Balb/C mice. Six-week-old male Balb/C



Scheme 1. Conjugation reactions of N1 dimers and *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne. A strain-promoted azide-alkyne cycloaddition (SPAAC) between N1 dimer and *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne gives *cis* and/or *trans* isomers of N1 tetramers.

mice were maintained in an animal facility under specific pathogen-free conditions. All mice were bled one week before immunization. Each antigen molecule (100 μg) was dissolved in dimethyl sulfoxide (DMSO, 1 μL) and phosphate buffered saline (PBS, 49 μL). This solution was mixed with Freund's incomplete adjuvant (50 μL) and the emulsion was injected subcutaneously into anesthetized mice on days 0, 7, 14, 21 and 28. The mice were bled on days 5, 12, 19, 26 and 33. The serum was separated by centrifugation and inactivated by maintaining it at 56 °C for 30 min. ELISA was performed to determine whether antibodies were produced efficiently. The titer of sera collected from mice immunized with the N1 tetramer after the 5th immunization was increased, but there was no significant signal observed in the titers of sera collected from mice immunized with the N2 and N3 tetramers (Fig. 3). Each NT-fragment peptide has a 10-mer overlapping region. The overlapping region Tyr^{21}-Ile^{39} is therefore not responsible for antibody induction, but the Met^{1}-Asp^{20} region might be an epitope of the extracellular N-terminal region (NT).

When compared to the cyclic ECL1 (C1) and ECL2 (C2) tetramers, higher increases were observed in the titers of linear ECL1 (L1) and ECL2 (L2) tetramers (Fig. 4). The L1-induced antiserum showed higher antigenicity than the L2-induced antiserum and the C2-induced antiserum showed only slightly higher antigenicity than the C1-induced antiserum. Epitopes of linear peptides on MAPs are more efficiently presented on MAPs than the epitopes of cyclic peptides. Since cyclic peptides were located in close proximity to MAPs, it is possible that the region around the site of peptide conjugation and MAPs may be hidden. Hidden regions may not be exposed effectively as epitopes, and thus antibodies recognizing the hidden regions might not be produced. Other possible reasons for this result are (i) C1 and C2 might form structures similar to those of native murine ECL1 and ECL2, and consequently they might not be recognized reliably as foreign substances by macrophages, and (ii) peptide susceptibility may increase immunogenicity mediated by antigen-processing proteases.[38,39] Cyclic peptides are generally more stable and resistant to proteases[40] and the cyclic peptides C1 and C2 might be less susceptible to antigen processing proteases and their immunogenicities might be weaker than that of the linear peptides L1 and L2.

2.4. Anti-HIV-1 activity (p24 assay)

The HIV-1 inhibitory activity of antisera obtained by the immunization of CXCR4-ED-derived antigen molecules was assessed by p24 assays. The ECL-derived linear peptide-induced antisera showed significant HIV-1 inhibitory activity (Fig. 5) and anti-N1 antibodies were clearly produced but they had no significant HIV-1 inhibitory activity. Possible reasons for this are (i) N1-induced antibodies might bind to CXCR4 but have no significant inhibitory activity, and (ii) N1-induced antibodies bind to the N1-derived antigen region but, owing to structural flexibility of NT, they have no significant binding affinity for CXCR4. The HIV-1 inhibitory activity of antisera induced by immunization of the ECL-derived antigen molecules was quantified by further p24

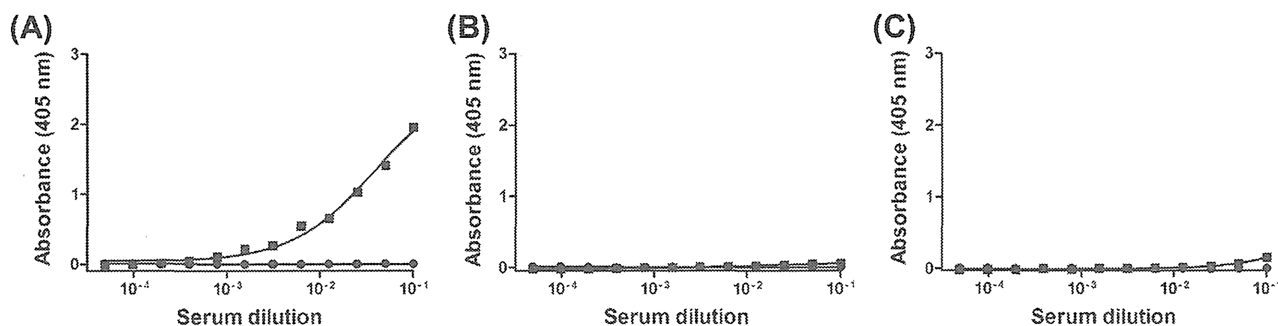


Figure 3. Results of serum titer ELISA of antisera collected from mice immunized with NT-derived antigens at one week before immunization (●) and one week after 5th immunization (■) to determine the immunogenicity of designed antigens. The titers were evaluated based on binding to each corresponding NT-derived monomer peptide; antiserum against (A) the N1 tetramer, (B) antiserum against the N2 tetramer, and (C) antiserum against the N3 tetramer.