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Association of Major Histocompatibility Complex Class I Haplotypes with Disease Progression after Simian Immunodeficiency Virus Challenge in Burmese Rhesus Macaques

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Nonhuman primate AIDS models are essential for the analysis of AIDS pathogenesis and the evaluation of vaccine efficacy. Multiple studies on human immunodeficiency virus and simian immunodeficiency virus (SIV) infection have indicated the association of major histocompatibility complex class I (MHC-I) genotypes with rapid or slow AIDS progression. The accumulation of macaque groups that share not only a single MHC-I allele but also an MHC-I haplotype consisting of multiple polymorphic MHC-I loci would greatly contribute to the progress of AIDS research. Here, we investigated SIVmac239 infections in four groups of Burmese rhesus macaques sharing individual MHC-I haplotypes, referred to as A, E, B, and J. Out of 20 macaques belonging to A^+ ($n = 6$), E^+ ($n = 6$), B^+ ($n = 4$), and J^+ ($n = 4$) groups, 18 showed persistent viremia. Fifteen of them developed AIDS in 0.5 to 4 years, with the remaining three at 1 or 2 years under observation. A^+ animals, including two controllers, showed slower disease progression, whereas J^+ animals exhibited rapid progression. E^+ and B^+ animals showed intermediate plasma viral loads and survival periods. Gag-specific $CD8^+$ T-cell responses were efficiently induced in A^+ animals, while Nef-specific $CD8^+$ T-cell responses were in A^+, E^+, and B^+ animals. Multiple comparisons among these groups revealed significant differences in survival periods, peripheral $CD4^+$ T-cell decline, and SIV-specific $CD4^+$ T-cell polyfunctionality in the chronic phase. This study indicates the association of MHC-I haplotypes with AIDS progression and presents an AIDS model facilitating the analysis of virus-host immune interaction.

Virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) are major effectors against persistent virus infections (13, 44). In virus-infected cells, viral antigen-derived peptides (epitopes) are bound to major histocompatibility complex class I (MHC-I) molecules and presented on the cell surface. Viral peptide-specific CTLs recognize the peptide-MHC-I complexes by their T-cell receptors. CTL effectors deliver cell death via apoptosis as well as lysis (15, 48).

Human immunodeficiency virus type 1 (HIV-1) infection induces persistent viral replication leading to AIDS progression. CTL responses play a central role in the suppression of HIV-1 replication (6, 18, 25, 32, 43). Multiple studies on HIV-1-infected individuals have shown an association of HLA genotypes with rapid or delayed AIDS progression (14, 23, 27, 51, 54). For instance, HIV-1-infected individuals possessing *HLA-B*57* tend to show a better prognosis with lower viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in this viral control (3, 33, 34). In contrast, the association of *HLA-B*35* with rapid disease progression has been indicated (8).

Nonhuman primate AIDS models are important for the analysis of AIDS pathogenesis and the evaluation of vaccine efficacy (5, 35, 47). Models of simian immunodeficiency virus (SIV) infection in macaques are widely used currently (12, 22). Indian rhesus macaques possessing certain MHC-I alleles, such as *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17*, tend to show lower set point plasma viral loads in SIV infection (30, 36, 37, 59). Regarding MHC-I alleles, humans have a single polymorphic HLA-A, HLA-B, and HLA-C locus per chromosome, whereas MHC-I haplotypes in macaques have variable numbers of expressed polymorphic MHC-I loci (7, 9, 26, 41). Thus, the accumulation of multiple macaque groups, each sharing a different MHC-I haplotype, would contribute to the precise analysis of SIV infection.

We have been working on the establishment of an AIDS model using Burmese rhesus macaques sharing MHC-I haplotypes (38, 50). In the present study, we have focused on SIV infection in four groups of Burmese rhesus macaques, each consisting of four or more animals. These groups share MHC-I haplotypes *90-120-Ia* (referred to as A), *90-010-Ie* (E), *90-120-Ib* (B), and *90-088-Ij* (J), respectively. The analysis of SIVmac239 infection among these groups revealed differences in plasma viral loads, peripheral $CD4^+$ T cell counts, survival periods, virus-specific CTL responses, and T-cell polyfunctionality. Our results indicate the association of MHC-I haplotypes with disease progression in SIV infection and present a sophisticated model of SIV infection.

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TABLE 1 MHC-I haplotypes

MHC-I haplotype	Confirmed MHC-I allele(s)	
	Mamu-A	*Mamu-B*
A (*90-120-Ia*)	*A1*043:01, A1*065:01*	*B*061:03, B*068:04, B*089:01*
E (*90-010-Ie*)	*A1*066:01*	*B*005:02, B*015:04*
B (*90-120-Ib*)	*A1*018:08, A2*005:31*	*B*036:03, B*037:01, B*043:01, B*162:01*
J (*90-088-Ij*)	*A1*008:01*	*B*007:02, B*039:01*

MATERIALS AND METHODS

Animal experiments. We examined SIV infections in four groups of Burmese rhesus macaques having MHC-I haplotypes *90-120-Ia* (A) (n = 6), *90-010-Ie* (E) (n = 6), *90-120-Ib* (B) (n = 4), and *90-088-Ij* (J) (n = 4). Macaques R02-007, R06-037, R07-001, R07-004, R07-009, R01-011, R06-038, R06-001, R02-004, R04-014, and R06-022, which were used as controls in previous experiments (49, 53, 58), were included in the present study. 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 as described previously (31). Briefly, locus-specific reverse transcription-PCR (RT-PCR) products from total cellular RNAs were prepared and used to form heteroduplex DNAs with a 5′ Cy5-labeled reference strand (50). The heteroduplex DNAs were subjected to a 6% nondenaturing acrylamide gel electrophoresis to identify the patterns of MHC-I haplotypes. In addition, although recombination events could not be ruled out, major *Mamu-A* and *Mamu-B* alleles were determined by cloning the RT-PCR products and sequencing at least 48 clones for each locus from each subject as described previously (38). Because we used locus-specific primers in the RT-PCR, which were designed on the basis of known alleles (31, 38), MHC class I alleles harboring mismatches with the primer sequences or alleles of low expression would not be amplified well, hence there was a limitation that not all of the MHC class I alleles could be detected in our study. Confirmed *Mamu-A* and *Mamu-B* alleles in MHC-I haplotypes A, E, B, and

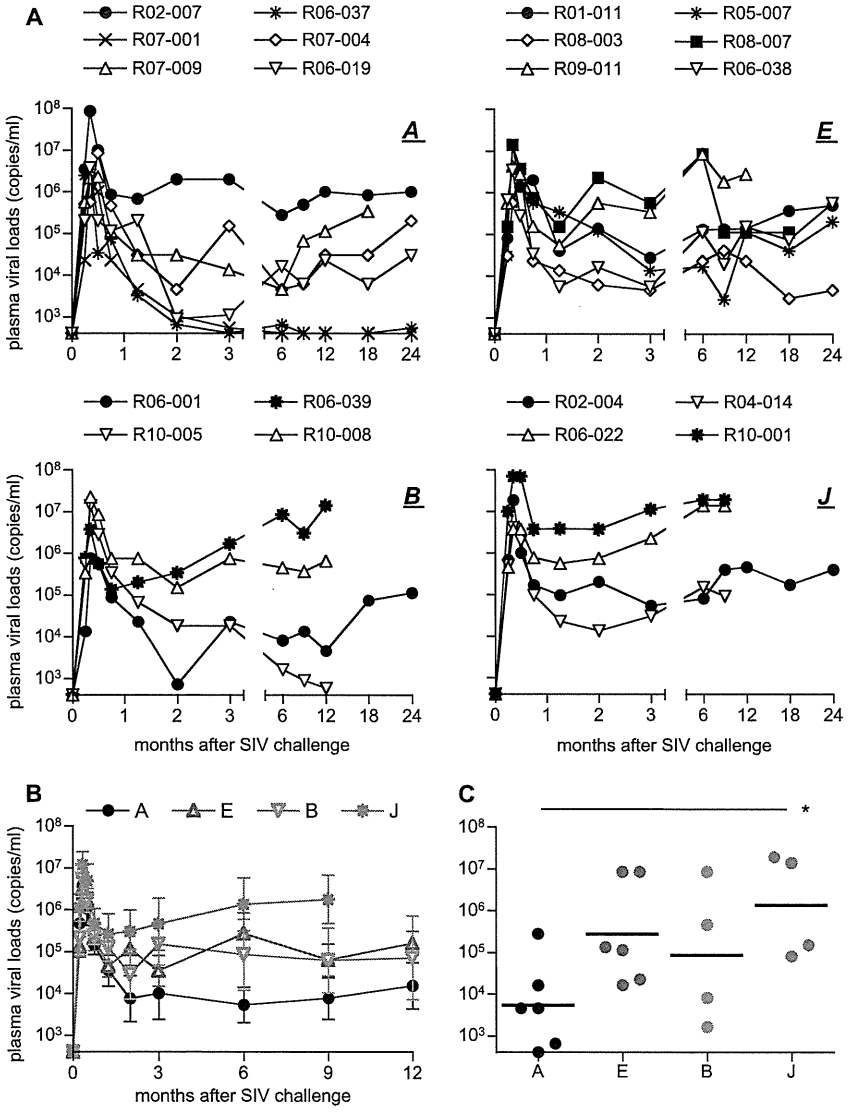

FIG 1 Plasma viral loads after SIVmac239 challenge. Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described previously (31). The lower limit of detection is approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads after challenge in A^+ (upper left), E^+ (upper right), B^+ (lower left), and J^+ (lower right) macaques. (B) Changes in geometric means of plasma viral loads after challenge in A^+ (black), E^+ (blue), B^+ (green), and J^+ (red) animals. (C) Comparison of plasma viral loads at 6 months among four groups. Those of A^+ animals were significantly lower than those of J^+ animals (P = 0.0444 by one-way ANOVA and Tukey-Kramer's multiple-comparison test).

J are shown in Table 1 (38). All animals were unvaccinated and challenged intravenously with 1,000 $TCID_{50}$ (50% tissue culture infective doses) of SIVmac239 (22). At 1 week after challenge, macaques R06-019, R06-038, and R10-008 were intravenously infused with 300 mg of nonspecific immunoglobulin G purified from uninfected rhesus macaques (57). Fifteen animals were euthanized when they showed typical signs of AIDS, such as reduction in peripheral $CD4^+$ T-cell counts, loss of body weight, diarrhea, and general weakness. Autopsy revealed lymphoatrophy or postpersistent generalized lymphadenopathy conditions consistent with AIDS (20). All animals were maintained in accordance with the guidelines for animal experiments at the National Institute of Biomedical Innovation and National Institute of Infectious Diseases.

Analysis of SIV antigen-specific $CD8^+$ T-cell responses. SIV antigen-specific $CD8^+$ T-cell responses were measured by the flow-cytometric analysis of gamma interferon (IFN-γ) induction as described previously (17). Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papioimmortalized B-lymphoblastoid cell lines (B-LCLs) pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Env, and Nef amino acid sequences. Intracellular IFN-γ staining was performed using a Cytofix Cytoperm kit (BD, Tokyo, Japan). Fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD), allophycocyanin Cy7 (APC-Cy7)-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (Biolegend, San Diego, CA) were used. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ^+ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell levels of less than 100 cells per million PBMCs were considered negative. Using PBMCs obtained from four SIV-infected macaques, we compared antigen-specific $CD8^+$ T-cell frequencies measured by this method (using peptide-pulsed B-LCLs) to those measured by the flow-cytometric analysis of IFN-γ induction after a pulse of PBMCs with peptides (without using B-LCLs). The levels of the former tended to be slightly higher than those of the latter. Specific $CD8^+$ T-cell responses, which were shown to be 100 to 200 cells per million PBMCs by the former method using B-LCLs, were undetectable by the latter method.

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, Pol, Vif, Vpx, Vpr, Tat, Rev, 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 (19). Predominant nonsynonymous mutations were determined. The Env-coding region, which is known to have multiple antibody-related mutations, was not included for the analysis.

Analysis of SIV-specific polyfunctional T-cell responses. To analyze polyfunctionality in SIV-specific T-cell responses, we examined the SIV-specific induction of IFN-γ, tumor necrosis factor alpha (TNF-α), interleukin-2 (IL-2), macrophage inflammatory protein 1β (MIP-1β), and CD107a in $CD4^+$ and $CD8^+$ T cells as described previously (58), with some modifications. Around 8 months after challenge, PBMCs were cocultured with B-LCLs infected with vesicular stomatitis virus G protein-pseudotyped SIVGP1 for the SIV-specific stimulation or mock-infected B-LCLs for nonspecific stimulation. The pseudotyped virus was obtained by the cotransfection of 293T cells with a vesicular stomatitis virus G protein expression plasmid and an *env* and *nef* deletion-containing simian-human immunodeficiency virus molecular clone (SIVGP1) DNA that has the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr (31, 46). Immunostaining was performed using a Fix & Perm fixation and permeabilization kit (Invitrogen, Tokyo, Japan) and the following monoclonal antibodies: APC-Cy7-conjugated anti-human CD3 (BD), PE-Texas red-conjugated anti-human CD4 (Invitrogen), Alexa Fluor 700-conjugated anti-human CD8 (BD), PE-Cy7-conjugated anti-human IFN-γ (eBioscience, San Diego, CA), Pacific blue-conjugated anti-human TNF-α (Biolegend), PerCP-Cy5.5-conjugated anti-human IL-2 (Biolegend), PE-conjugated anti-human MIP-1β (BD), and Alexa Fluor 647-conjugated anti-human CD107a (Biolegend). Dead cells were stained using Live/Dead Fixable Dead Cell Stain kit (Invitrogen). Analysis was carried out using PESTLE (version 1.6.1) and SPICE (version 5.2) programs as described previously (42). The polyfunctionality (polyfunctional value) was shown as mean numbers of induced factors among the five (IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a) per SIV-specific T cell.

Statistical analysis. Statistical analyses were performed using R software (R Development Core Team). Comparisons were performed by one-way analysis of variance (ANOVA) and Tukey-Kramer's multiple comparison test with significance levels set at $P < 0.05$. Correlation was analyzed by the Pearson test.

TABLE 2 List of macaques in this study

MHC-I haplotype	Macaque	Disease progression	Euthanasia time point (mo)
A	R02-007	AIDS	42
A	R06-037	No	49
A	R07-001	No	49
A	R07-004	AIDS	40
A	R07-009	AIDS	17
A	R06-019	AIDS	43
E	R01-011	AIDS	24
E	R05-007	AIDS	37
E	R08-003	Under observation (24 months)	
E	R08-007	AIDS	20
E	R09-011	AIDS	12
E	R06-038	AIDS	22
B	R06-001	AIDS	34
B	R06-039	AIDS	13
B	R10-005	Under observation (12 months)	
B	R10-008	Under observation (12 months)	
J	R02-004	AIDS	37
J	R04-014	AIDS	9
J	R06-022	AIDS	5
J	R10-001	AIDS	9

RESULTS

SIV infection in Burmese rhesus macaques. We accumulated four groups of unvaccinated, SIVmac239-infected Burmese rhesus macaques, groups A^+ ($n = 6$), E^+ ($n = 6$), B^+ ($n = 4$), and J^+ ($n = 4$), sharing MHC-I haplotypes A (*90-120-Ia*), E (*90-010-Ie*), B (*90-120-Ib*), and J (*90-088-Ij*), respectively, to compare SIV infections among these groups (Table 1). Out of these 20 animals, 18 showed persistent viremia (geometric mean plasma viral loads at 6 months of 1.6×10^5 copies/ml), while in the remaining two (A^+ macaques R06-037 and R07-001), plasma viral loads became less than 10^3 copies/ml or were undetectable at the set point (Fig. 1A). The former 18 animals are referred to as noncontrollers and the latter two as controllers in this study. Fifteen noncontrollers were euthanized with AIDS progression in 4 years (geometric mean survival period of 24 months), and the remaining three, after 1 or 2 years, are under observation (Table 2).

Group A^+ macaques, including two controllers, showed lower set point viral loads, whereas group J^+ macaques had higher viral loads (Fig. 1B). Viral loads in group E^+ and B^+ macaques were at intermediate levels. Multiple comparisons indicated significant

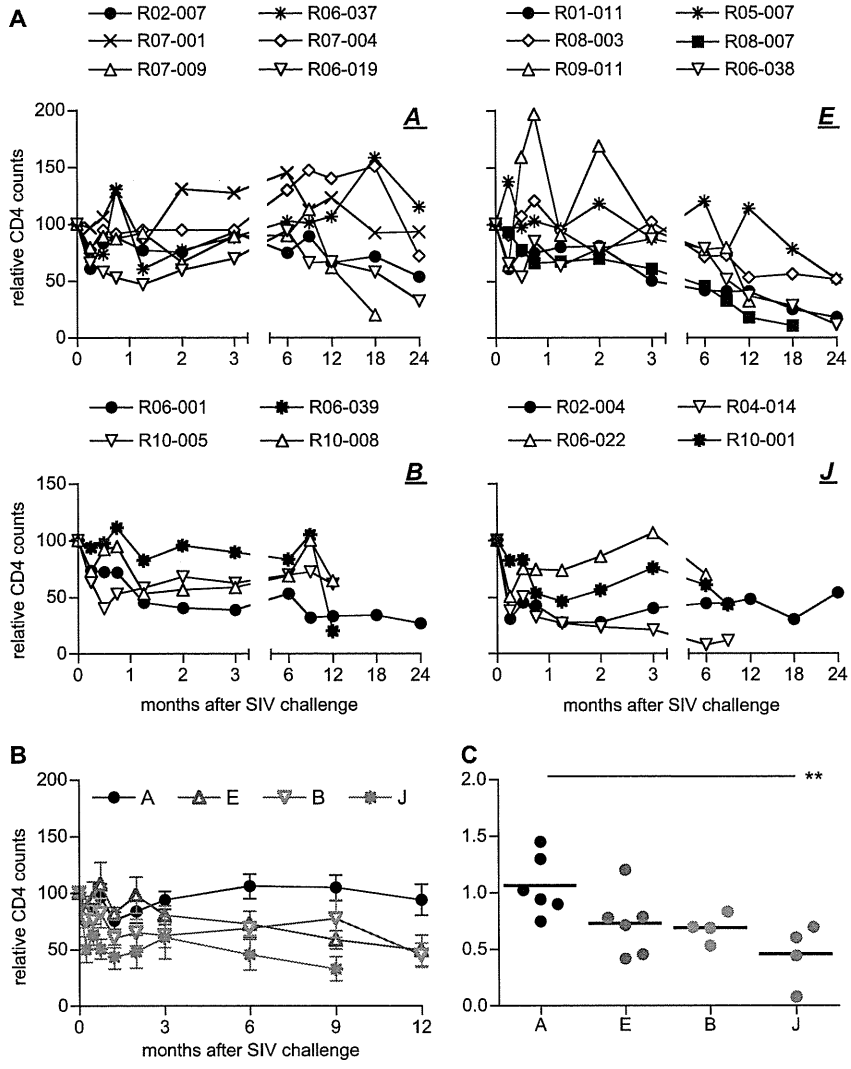

FIG 2 Relative $CD4^+$ T-cell counts after SIVmac239 challenge. (A) Relative $CD4^+$ T-cell counts after challenge in A^+ (upper left), E^+ (upper right), B^+ (lower left), and J^+ (lower right) macaques. For each animal, the peripheral CD4 counts relative to that at challenge (set at 100) are shown. (B) Changes in means of relative $CD4^+$ T-cell counts after challenge in A^+ (black), E^+ (blue), B^+ (green), and J^+ (red) animals. (C) Comparison of relative $CD4^+$ T-cell counts at 6 months among four groups. Those in J^+ animals were significantly lower than those in A^+ ($P = 0.0090$ by one-way ANOVA and Tukey-Kramer's multiple-comparison test).

differences in set point plasma viral loads between groups A^+ and J^+ (Fig. 1C).

Most noncontrollers showed a decline in peripheral $CD4^+$ T-cell counts (Fig. 2A). Relative $CD4^+$ T-cell counts in the chronic phase were the highest in group A^+ animals and the lowest in group J^+ animals. Multiple-comparison tests revealed significant differences in relative $CD4^+$ T-cell counts at 6 months between groups A^+ and J^+ (Fig. 2B and C). Furthermore, multiple comparisons among groups A^+, E^+, and J^+ found significant differences in survival periods, which were the longest in A^+ and the shortest in J^+ animals (Table 2 and Fig. 3). These results indicate an association of MHC-I haplotypes with AIDS progression after SIV challenge in Burmese rhesus macaques.

SIV antigen-specific $CD8^+$ T-cell responses. We analyzed SIV-specific $CD8^+$ T-cell responses at 3 months and 1 year after SIV challenge by the detection of antigen-specific IFN-γ induction to examine which antigen-specific $CD8^+$ T-cell responses were induced predominantly (Table 3). Analysis revealed the predominant induction of Gag-specific and Nef-specific $CD8^+$ T-cell responses in group A^+ animals and Nef-specific $CD8^+$ T-cell responses in groups E^+ and B^+. Vif-specific $CD8^+$ T-cell responses were detected in three J^+ animals but not macaque R06-022, which rapidly developed AIDS in 5 months without detectable SIV-specific $CD8^+$ T-cell responses.

There was no significant difference in whole SIV antigen-specific $CD8^+$ T-cell responses among these four groups, although those responses were marginal or undetectable in two of four J^+ animals (Fig. 4A). However, Gag-specific $CD8^+$ T-cell frequencies at 3 months were significantly higher in A^+ animals (Fig. 4B). The analysis of four groups revealed inverse correlations between Gag-specific $CD8^+$ T-cell frequencies and plasma viral loads at 3 months ($P = 0.0087$; $r^2 = 0.3407$; data not shown). Three groups of A^+, E^+, and B^+ animals tended to show higher Nef-specific $CD8^+$ T-cell responses than J^+ animals (Fig. 4C).

Viral genome mutations. We then analyzed mutations in viral cDNAs amplified from plasma RNAs of group A^+, E^+, and B^+

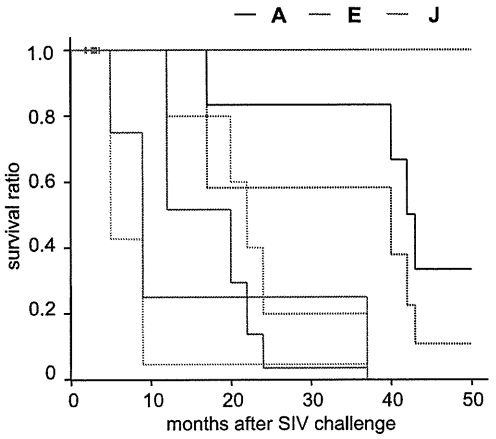

FIG 3 Kaplan-Meyer survival curves after SIVmac239 challenge in A^+, E^+, and J^+ macaques. Macaque R08-003, which is under observation, is not included. B^+ animals were excluded from this analysis because data on only two animals were available. We determined the Kaplan-Meyer estimate of the survival function of each group and then compared the three curves using the log-rank test (Mantel-Cox test). Analysis showed significant differences in survival curves (chi square, 9.9; $P = 0.007$ by log-rank test of Kaplan-Meyer estimates).

macaques around 1 year after SIV challenge. Nonsynonymous mutations detected predominantly were as shown in Fig. 5. Multiple comparisons among groups A^+, E^+, and B^+ (Fig. 6) showed no differences in total numbers of nonsynonymous mutations but revealed significantly higher numbers of *gag* mutations in A^+ animals. E^+ animals had higher numbers of *tat* mutations than A^+ animals. There was no significant difference in the numbers of mutations in other regions, including *nef*, among these groups. Group J^+ animals were not included in the multiple comparisons, because three of them were euthanized by 9 months. These three had lower numbers of nonsynonymous mutations before their death, possibly reflecting lower immune pressure.

Polyfunctionality in SIV-specific T-cell responses. Finally, we investigated T-cell polyfunctionality to compare T-cell functions (2, 4, 45) in these four groups having different viral loads. We analyzed the polyfunctionality of SIV-specific $CD4^+$ and $CD8^+$ T cells around 8 months after challenge by the detection of SIV-specific induction of IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a. SIV-specific $CD4^+$ T-cell polyfunctionality inversely correlated with plasma viral loads at around 9 months (Fig. 7A). We also found an inverse correlation between SIV-specific $CD8^+$ T-cell polyfunctionality and viral loads (Fig. 7A). However, there was no

TABLE 3 SIV antigen-specific $CD8^+$ T-cell responses[a]

MHC-I haplotype and time point after challenge	Macaque	$CD8^+$ T-cell response to:								
		Gag	Pol	Vif	Vpx	Vpr	Tat	Rev	Env	Nef
3 mo										
A	R02-007	ND	ND	ND	ND	ND	ND	ND	ND	ND
A	R06-037	657	—	104	—	—	—	—	—	520
A	R07-001	193	—	—	—	—	—	—	—	322
A	R07-004	316	—	137	—	—	—	—	—	353
A	R07-009	440	—	124	—	—	—	—	100	247
A	R06-019	322	—	—	—	—	—	—	—	253
E	R01-011	—	—	186	—	—	—	—	—	—
E	R05-007	—	—	—	—	—	203	—	—	330
E	R08-003	—	—	—	—	—	—	—	—	213
E	R08-007	—	—	—	—	—	—	—	335	—
E	R09-011	—	—	807	—	307	—	—	1,598	2,327
E	R06-038	199	—	248	—	—	249	—	234	634
B	R06-001	—	107	253	172	—	—	—	114	313
B	R06-039	—	—	—	—	—	—	—	110	195
B	R10-005	163	172	—	1,033	141	—	579	—	1,554
B	R10-008	—	—	—	133	—	—	165	—	—
J	R02-004	—	—	171	—	—	145	—	382	117
J	R04-014	—	534	625	280	440	290	1,060	—	296
J	R06-022	—	—	—	—	—	—	—	—	—
J	R10-001	—	—	102	—	—	—	—	—	—
1 yr										
A	R02-007	—	—	119	—	—	—	—	112	250
A	R06-037	515	—	124	272	178	—	—	—	906
A	R07-001	126	—	—	—	—	—	—	—	180
A	R07-004	—	—	—	—	—	—	—	—	150
A	R07-009	254	120	173	—	112	—	—	215	166
A	R06-019	444	155	284	—	188	—	—	174	583
E	R01-011	160	—	—	—	—	—	—	—	228
E	R05-007	—	—	—	—	—	—	—	—	—
E	R08-003	—	—	—	—	—	—	—	—	537
E	R08-007	—	—	—	—	—	—	—	—	199
E	R09-011	—	159	—	—	—	—	150	259	102
E	R06-038	298	174	611	—	—	406	387	1,052	1,982
B	R06-001	—	—	—	—	—	—	—	127	140
B	R06-039	—	—	—	—	—	151	—	—	—
B	R10-005	185	—	—	—	—	—	—	—	—
B	R10-008	109	232	—	—	—	—	325	—	296
J	R02-004	158	—	—	—	—	—	—	—	—
J	R04-014[b]	114	141	178	—	—	360	288	—	142
J	R10-001[b]	—	—	—	—	—	—	—	—	—

[a] Responses were measured by the detection of antigen-specific IFN-γ induction. Macaque R06-022, euthanized at 5 months, is not included in the lower portion. Antigen-specific $CD8^+$ T-cell frequencies (per 1 million PBMCs) are shown. ND, not determined; —, undetectable (<100).

[b] At 9 months (before euthanasia).

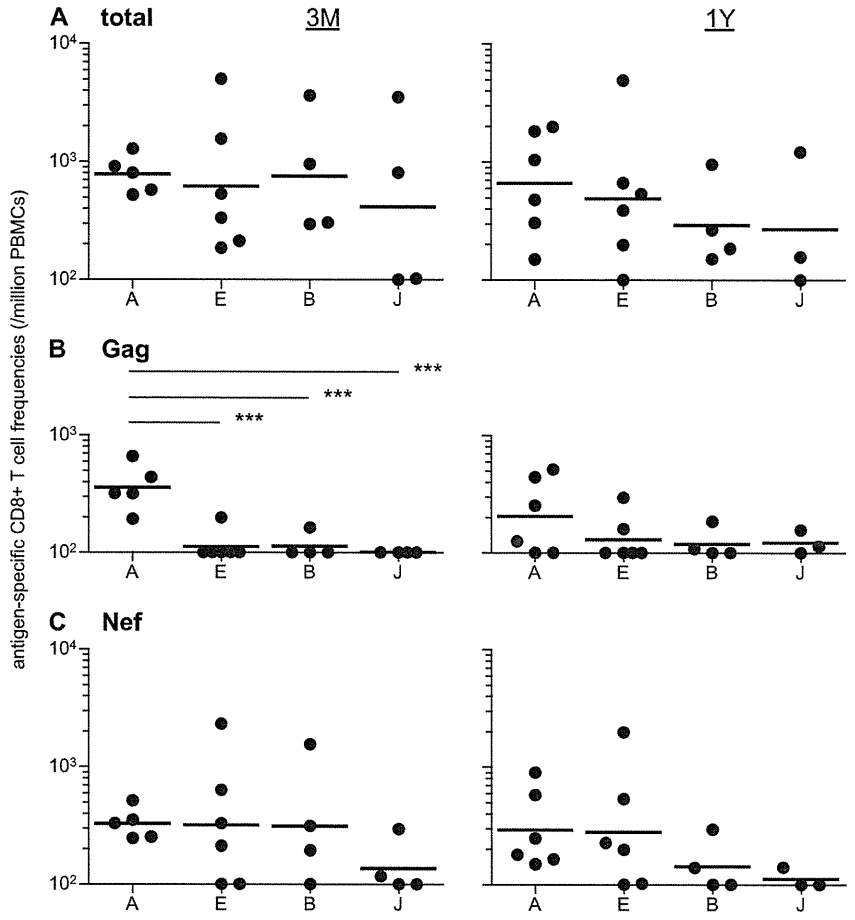

FIG 4 Comparison of SIV antigen-specific $CD8^+$ T-cell responses. Responses were measured by the detection of antigen-specific IFN-γ induction using PBMCs at 3 months (3 M; left) and at 1 year (1Y; right). (A) Whole SIV antigen-specific $CD8^+$ T-cell frequencies. The sum of Gag-, Pol-, Vif-, Vpx-, Vpr-, Tat-, Rev-, Env-, and Nef-specific $CD8^+$ T-cell frequencies in each animal is shown. (B) Gag-specific $CD8^+$ T-cell frequencies. The frequencies at 3 months in A^+ animals were significantly higher (A^+ and E^+, $P < 0.0001$; A^+ and B^+, $P = 0.0003$; A^+ and J^+, $P < 0.0001$ by one-way ANOVA and Tukey-Kramer's multiple-comparison test). (C) Nef-specific $CD8^+$ T-cell frequencies.

correlation between viral loads and total SIV-specific $CD4^+$ T-cell or $CD8^+$ T-cell frequencies (Fig. 7B). Polyfunctional T-cell responses tended to be higher in group A^+ and lower in group J^+. Multiple comparisons revealed significant differences in SIV-specific $CD4^+$ T-cell polyfunctionality with the highest in group A^+ and the lowest in group J^+ (Fig. 7C). These results may reflect difference in disease progression among these animals.

DISCUSSION

This study describes SIVmac239 infection in 20 Burmese rhesus macaques. Geometric means of set point plasma viral loads were approximately 10^5 copies/ml. The levels are considered lower than those usually observed in the widely used SIVmac239 infection model of Indian rhesus macaques (28, 55) but are higher than those typically observed in untreated humans infected with HIV-1. While two A^+ animals controlled SIV replication, the remaining 18 Burmese rhesus macaques failed to control viremia. Indeed, all of the animals in the three groups E^+, B^+, and J^+ showed persistent viremia. Those noncontrollers, including four A^+ animals, developed AIDS in 0.5 to 4 years. These results indicate that the SIVmac239 infection of Burmese rhesus macaques does serve as an AIDS model.

In the present study, we compared SIVmac239 infections among four groups sharing MHC-I haplotypes A, E, B, and J, respectively. These animals showed differences in plasma viral loads, peripheral $CD4^+$ T-cell counts, survival periods, patterns of viral antigen-specific $CD8^+$ T-cell responses, polyfunctionality of SIV-specific T-cell responses, and numbers of viral genome mutations. These results indicate the association of MHC-I haplotypes with AIDS progression. There has been a number of reports describing SIV infections in macaques sharing a single or a couple of MHC-I alleles, but few studies have examined SIV infection in macaques sharing an MHC-I haplotype (10, 11, 40). SIV infection induces multiple epitope-specific $CD8^+$ T-cell responses, and $CD8^+$ T-cell responses specific for some MHC-I-restricted epitopes can be affected by those specific for other MHC-I-restricted epitopes due to CTL immunodominance (16, 29, 52). Thus, the preparation of macaque groups sharing MHC-I genotypes at the haplotype level, as described in the present study, would contribute to the precise analysis of SIV infection. The establishment of groups sharing both MHC-I haplotypes (56) may be ideal, but the accumulation of macaque groups sharing even one MHC-I haplotype could lead to the constitution of a more sophisticated primate AIDS model.

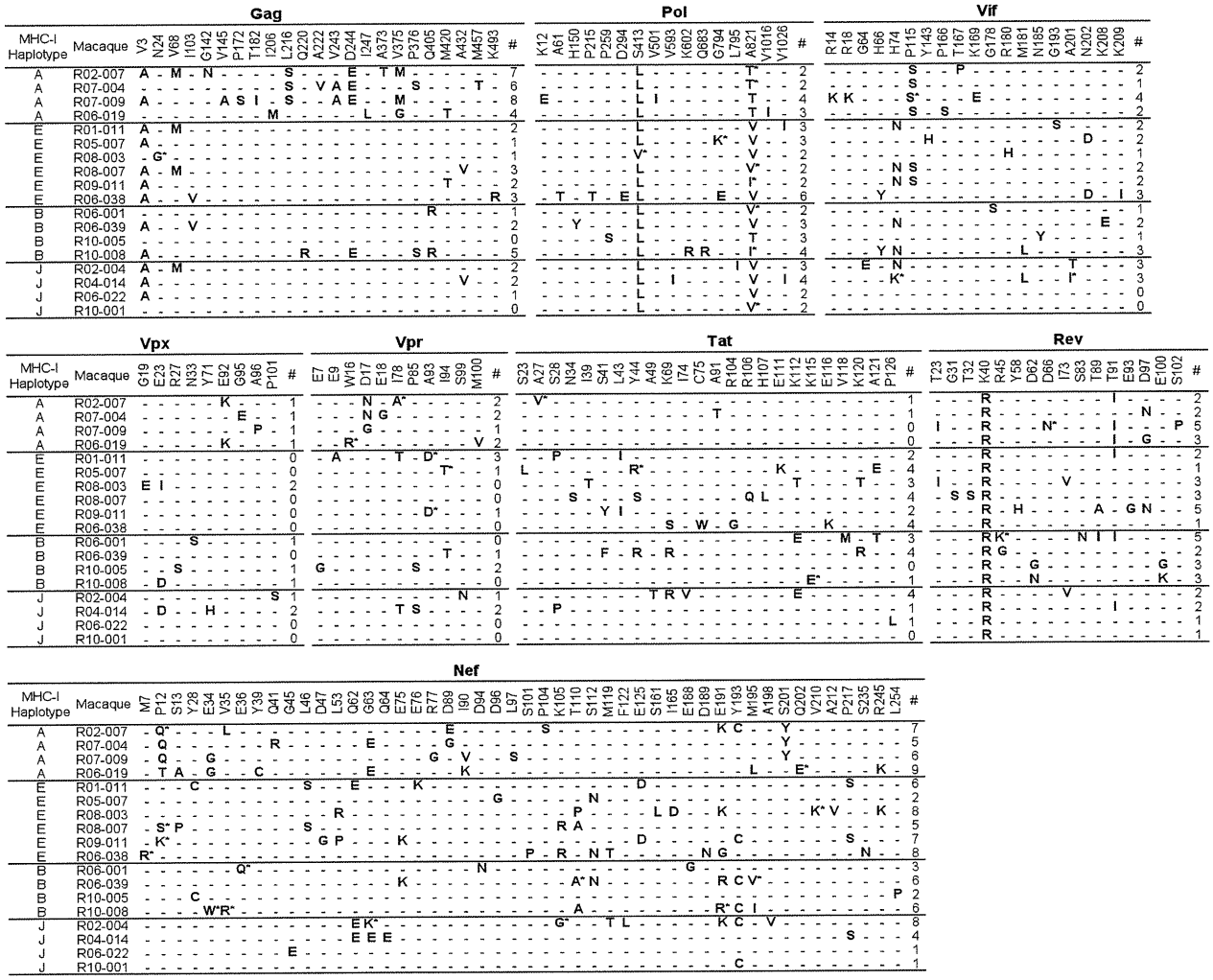

FIG 5 Predominant nonsynonymous mutations in viral cDNAs around 1 year after SIVmac239 challenge. Amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef are shown. In three animals, R04-014, R06-022, and R10-001 in group J^+, data on the samples obtained before their death at 5 or 9 months after challenge are shown. Asterisks indicate the residues where different mutations were detected. It is known that amino acid substitutions at the Pol residues 413 and 821 and the Rev residue 40 are frequently observed in SIVmac239 infection and contribute to higher viral fitness *in vivo* (1, 39).

Various MHC-I alleles that do not consist of the first MHC-I haplotypes (A in A^+, E in E^+, B in B^+, and J in J^+ animals) were detected as shown in Table 4. Two A^+ macaques, R02-007 and R07-004, had two major alleles, *Mamu-A1*008:01* and *Mamu-B*007:02*, of MHC-I haplotype J, although their second MHC-I haplotype was not determined as J on the basis of information on the family tree. Even when these two animals were excluded from the A^+ group, multiple-comparison analyses showed significant

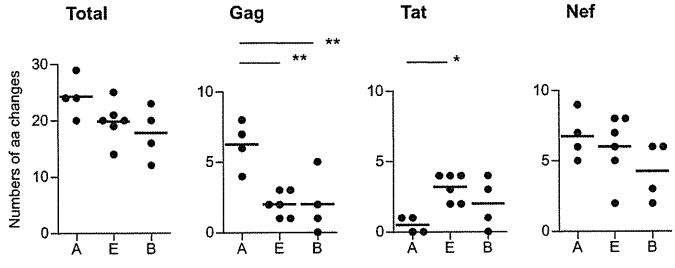

FIG 6 Comparison of the numbers of predominant nonsynonymous mutations in viral cDNAs around 1 year after SIVmac239 challenge among A^+, E^+, and B^+ macaques. J^+ animals were excluded from this analysis because three of them were euthanized by 1 year. The first graph (total) shows the sum of the numbers of amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef. Gag, Tat, and Nef graphs show the numbers of Gag, Tat, and Nef amino acid substitutions, respectively. The numbers of Gag amino acid substitutions in A^+ animals were significantly higher than those in E^+ and B^+ animals (A^+ and E^+, $P = 0.0037$; A^+ and B^+, $P = 0.0069$ by one-way ANOVA and Tukey-Kramer's multiple-comparison test). The numbers of Tat amino acid substitutions in E^+ animals were significantly higher than those in A^+ animals ($P = 0.0140$).

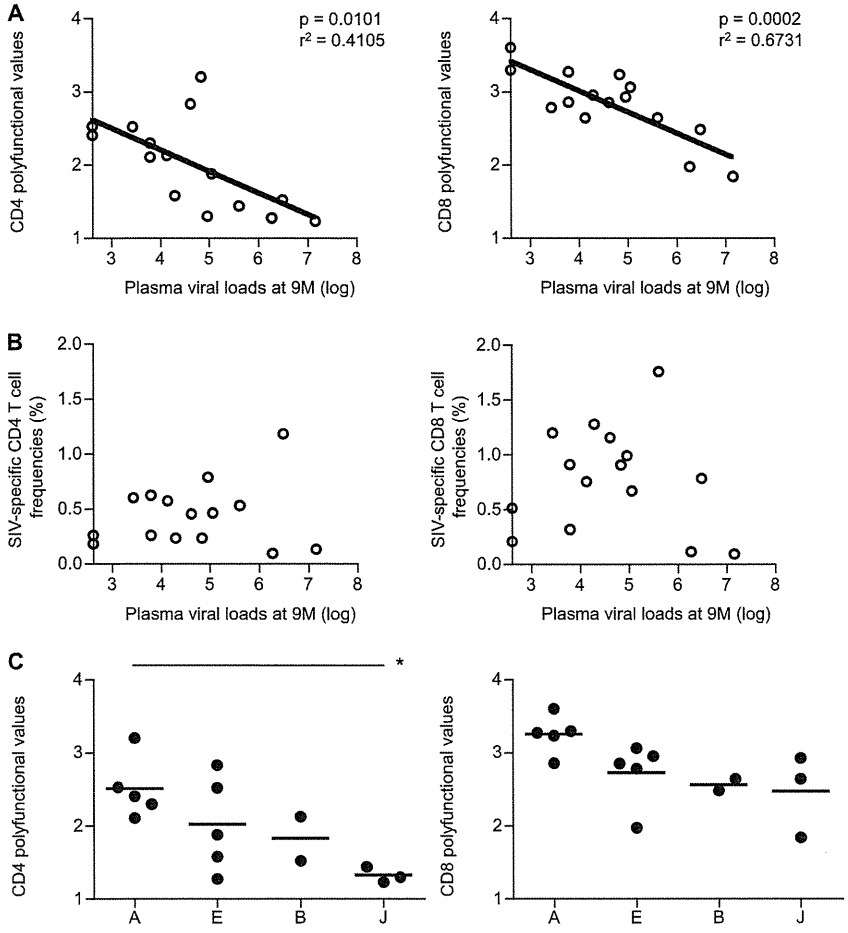

FIG 7 Polyfunctionality in SIV-specific $CD4^+$ and $CD8^+$ T cells around 8 months after SIVmac239 challenge. Samples of macaques R02-007 (A^+), R01-011 (E^+), R10-005 (B^+), R10-008 (B^+), and R10-001 (J^+) were unavailable. (A) Correlation analysis of plasma viral loads at 9 months with polyfunctionality (polyfunctional values) of SIV-specific $CD4^+$ (left) and $CD8^+$ (right) T cells. Viral loads inversely correlated with SIV-specific $CD4^+$ ($P = 0.0101$; $r^2 = 0.4105$) and $CD8^+$ ($P = 0.0002$; $r^2 = 0.6731$) T-cell polyfunctionality. (B) Correlation analysis of plasma viral loads at 9 months with SIV-specific $CD4^+$ (left) and $CD8^+$ (right) T-cell frequencies (frequencies of $CD4^+$ and $CD8^+$ T cells showing the SIV-specific induction of induction of IFN-γ, TNF-α, IL-2, MIP-1β, or CD107a). (C) SIV-specific $CD4^+$ (left) and $CD8^+$ (right) T-cell polyfunctionality in A^+ ($n = 5$), E^+ ($n = 5$), B^+ ($n = 2$), and J^+ ($n = 3$) macaques. Multiple comparisons among A^+, E^+, and J^+ animals (excluding the B^+ group with available data on only two animals) revealed significant difference in SIV-specific $CD4^+$ T-cell polyfunctionality (A^+ and J^+, $P = 0.0195$ by one-way ANOVA and Tukey-Kramer's multiple-comparison test).

differences in plasma viral loads, peripheral $CD4^+$ T-cell counts, survival periods, Gag-specific $CD8^+$ T-cell responses, and numbers of viral gag mutations. These two A^+ animals were noncontrollers, supporting the notion that CTL responses specific for Mamu-A1*008:01- or Mamu-B*007:02-restricted epitopes are not efficient or effective. In addition, several MHC-I alleles were shared in two or three animals, but the influence of these alleles on disease progression remains unclear.

In the group A^+ animals that showed lower viral loads and slower disease progression, Gag-specific $CD8^+$ T-cell responses were efficiently induced, and their frequencies were significantly higher than those in the other three groups. Furthermore, these A^+ animals had higher numbers of nonsynonymous *gag* mutations, possibly reflecting strong selective pressure by Gag-specific $CD8^+$ T-cell responses. Previously, $CD8^+$ T-cell responses specific for the $Gag_{206\text{-}216}$ (IINEEAADWDL) epitope restricted by MHC-I haplotype A-derived Mamu-A1*043:01 and the $Gag_{241\text{-}249}$ (SSVDEQIQW) epitope restricted by A-derived Mamu-A1*065:01 have been shown to exert strong suppressive pressure on SIV replication (19, 21). In the present study, most A^+ animals selected escape mutations from these $CD8^+$ T-cell responses, GagL216S (a mutation leading to a leucine [L]-to-serine [S] substitution at the 216th amino acid in Gag) and GagD244E (aspartic acid [D]-to-glutamic acid [E] substitution at the 244th amino acid) or I247L (isoleucine [I]-to-L substitution at the 247th amino acid). These results are consistent with recent findings suggesting the potential of Gag-specific $CD8^+$ T-cell responses to efficiently suppress HIV-1/SIV replication (24).

In SIV-infected A^+ animals, predominantly Nef-specific as well as Gag-specific $CD8^+$ T-cell responses were elicited. At 3 months postchallenge, all of the A^+ animals showed relatively similar levels of total antigen-specific, Gag-specific, and Nef-specific $CD8^+$ T-cell responses, and their deviations appeared to be less than those in the other three groups. This may reflect the diminished influence of the second MHC-I haplotypes in these A^+ animals in the early phase of SIV infection, i.e., $CD8^+$ T-cell responses specific for epitopes restricted by MHC-I molecules derived from the second haplotypes may be suppressed by dominant $CD8^+$ T-cell responses specific for A-derived MHC-I-restricted epitopes.

TABLE 4 Alleles in the second MHC-I haplotypes in macaques[a]

Group	Macaque	Allele(s)
A^+	R02-007	*A1*008:01, B*007:02*
A^+	R06-037	*A1*052:01, A2*005:13, B*089:02/03*[b]
A^+	R07-001	*A1*032:02, B*066:01*
A^+	R07-004	*A1*008:01, B*007:02, B*039:01*
A^+	R07-009	ND[c]
A^+	R06-019	*A1*032:02, A2*005:02, B*106:01, B*124:01*
E^+	R01-011	*A1*004:01, B*004:01, B*060:03, B*102:01*
E^+	R05-007	*A1*032:03, B*042:01, B*066:01, B*089:01*
E^+	R08-003	*B*074:02, B*101:01*
E^+	R08-007	*A2*005:10, B*054:02, B*061:04, B*063:02, B*124:01*
E^+	R09-011	*A1*041:02, B*061:02, B*068:04/05*[d]
E^+	R06-038	*A1*004:01, A-new,*[e] *B*001:01, B*007:02/03, B*017:03*
B^+	R06-001	*A1*008:01*
B^+	R06-039	*A1*032:02, B*004:01, B*033:01, B*066:01, B*102:01*
B^+	R10-005	*A1*003:01, B*019:01*
B^+	R10-008	*B*026:02, B*045:07, B*051:06*
J^+	R02-004	ND[f]
J^+	R04-014	*A4*014:03, B*071:01*
J^+	R06-022	*A5*030:06, B*102:01*
J^+	R10-001	*A1*004:01, B*026:02, B*043:01, B*073:01*

[a] Detected alleles not included in the first MHC-I haplotypes (A in A^+, E in E^+, B in B^+, or J in J^+ animals) are shown.
[b] The *Mamu-B* allele has sequences identical to *B*089:02* and *B*089:03* in exons 2 and 3.
[c] MHC-I alleles other than those consisting of the MHC-I haplotype A were not detected.
[d] The *Mamu-B* allele has sequences identical to *B*068:04* and *B*068:05* in exons 2 and 3.
[e] New *Mamu-A* allele 96% similar to *A1*018:03* by sequence homology in exons 2 and 3.
[f] MHC-I alleles other than those consisting of the MHC-I haplotype J were not detected.

Nef-specific $CD8^+$ T-cell responses were induced efficiently at 3 months or 1 year postchallenge in groups A^+, E^+, and B^+ but not in most J^+ animals, which showed higher viral loads and rapid disease progression. The former three groups had relatively higher numbers of nonsynonymous *nef* mutations, which correlated with Nef-specific $CD8^+$ T-cell responses at 1 year ($P = 0.0063$; $r^2 = 0.4765$; data not shown). Thus, these Nef-specific $CD8^+$ T-cell responses, whose suppressive pressure might be less than that of Gag-specific ones, may play roles in the suppression of SIV replication, while we have not determined Nef epitopes for those $CD8^+$ T-cell responses exerting strong suppressive pressure. No nef mutations common to each group were detected, which suggests multiple Nef epitope-specific $CD8^+$ T-cell responses. Regarding the Nef-specific $CD8^+$ T-cell responses in SIV-infected E^+ animals, some Nef epitopes are speculated to be restricted by E-derived MHC-I molecules. Our results, however, indicate that primary SIV infection induces no predominant $CD8^+$ T-cell responses specific for Gag epitopes restricted by E-derived MHC-I molecules in the early phase. In J^+ animals, we found no predominant $CD8^+$ T-cell responses specific for J-derived, MHC-I-restricted epitopes in the early phase of SIV infection.

This study indicates differences in the patterns of CTL immunodominance among these groups. Gag-specific $CD8^+$ T-cell responses were induced in group A^+, showing slower disease progression, and Nef-specific CTL responses were induced in those animals other than group J^+ animals, which showed rapid disease progression. These results can be reasonably explained by the differences in MHC-I haplotypes, although it is difficult to completely rule out the possibility of disease progression associating with other genes located around the MHC-I locus. In our previous study (21), the challenge of A^+ macaques with a mutant SIVmac239 carrying GagL216S and GagD244E mutations showed higher set point viral loads, indicating that these A-derived, MHC-I-restricted, $Gag_{206-216}$ and $Gag_{241-249}$ epitope-specific $CD8^+$ T-cell responses are responsible for lower viral loads in group A^+ animals.

Our analysis revealed differences in the target antigens for predominant $CD8^+$ T-cell responses but not in the magnitudes of SIV-specific $CD8^+$ T-cell responses among four groups. However, we found differences in polyfunctional SIV-specific $CD4^+$ T-cell responses in the chronic phase. Remarkably, plasma viral loads inversely correlated with the polyfunctionality of SIV-specific $CD8^+$ T cells as well as $CD4^+$ T cells. These results suggest stronger polyfunctional T cell responses in animals with lower viral loads, which, conversely, could contribute to the sustained suppression of viral replication in the chronic phase.

In summary, we examined SIVmac239 infection in four groups of Burmese rhesus macaques, with each group sharing different MHC-I haplotypes. Our results indicate the association of MHC-I haplotypes with disease progression. This study presents a robust AIDS model of SIV infection facilitating the analysis of virus-host immune interaction.

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REFERENCES

1. **Alexander L, Denekamp L, Czajak S, Desrosiers RC.** 2001. Suboptimal nucleotides in the infectious, pathogenic simian immunodeficiency virus clone SIVmac239. J. Virol. 75:4019–4022.
2. **Almeida JR, et al.** 2007. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. J. Exp. Med. **204**:2473–2485.
3. **Altfeld M, et al.** 2003. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS **17**:2581–2591.
4. **Betts MR, et al.** 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood **107**:4781–4789.
5. **Bontrop RE, Watkins DI.** 2005. MHC polymorphism: AIDS susceptibility in non-human primates. Trends Immunol. **26**:227–233.
6. **Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB.** 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. **68**:6103–6110.
7. **Boyson JE, et al.** 1996. The MHC class I genes of the rhesus monkey. Different evolutionary histories of MHC class I and II genes in primates. J. Immunol. **156**:4656–4665.
8. **Carrington M, et al.** 1999. HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. Science **283**:1748–1752.
9. **Daza-Vamenta R, Glusman G, Rowen L, Guthrie B, Geraghty DE.** 2004.

Genetic divergence of the rhesus macaque major histocompatibility complex. Genome Res. 14:1501–1515.
10. Evans DT, et al. 1999. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. Nat. Med. 5:1270–1276.
11. Evans DT, et al. 2000. Definition of five new simian immunodeficiency virus cytotoxic T-lymphocyte epitopes and their restricting major histocompatibility complex class I molecules: evidence for an influence on disease progression. J. Virol. 74:7400–7410.
12. Feinberg MB, Moore JP. 2002. AIDS vaccine models: challenging challenge viruses. Nat. Med. 8:207–210.
13. Goulder PJ, Watkins DI. 2004. HIV and SIV CTL escape: implications for vaccine design. Nat. Rev. Immunol. 4:630–640.
14. Goulder PJ, Watkins DI. 2008. Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat. Rev. Immunol. 8:619–630.
15. Guidotti LG, Chisari FV. 2000. Cytokine-mediated control of viral infections. Virology 273:221–227.
16. Ishii H, et al. 2012. Impact of vaccination on cytotoxic T lymphocyte immunodominance and cooperation against simian immunodeficiency virus replication in rhesus macaques. J. Virol. 86:738–745.
17. Iwamoto N, et al. 2010. Broadening of CD8+ cell responses in vaccine-based simian immunodeficiency virus controllers. AIDS 24:2777–2787.
18. Jin X, et al. 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J. Exp. Med. 189:991–998.
19. Kawada M, et al. 2006. Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. J. Virol. 80:1949–1958.
20. Kawada M, et al. 2007. Long-term control of simian immunodeficiency virus replication with central memory CD4+ T-cell preservation after nonsterile protection by a cytotoxic T-lymphocyte-based vaccine. J. Virol. 81:5202–5211.
21. Kawada M, et al. 2008. Gag-specific cytotoxic T-lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial. J. Virol. 82:10199–10206.
22. Kestler HW, III, et al. 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 65:651–662.
23. Kiepiela P, et al. 2004. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. Nature 432:769–775.
24. Kiepiela P, et al. 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat. Med. 13:46–53.
25. Koup RA, et al. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. 68:4650–4655.
26. Kulski JK, Anzai T, Shiina T, Inoko H. 2004. Rhesus macaque class I duplicon structures, organization and evolution within the alpha block of the major histocompatibility complex. Mol. Biol. Evol. 21:2079–2091.
27. Leslie A, et al. 2010. Additive contribution of HLA class I alleles in the immune control of HIV-1 infection. J. Virol. 84:9879–9888.
28. Letvin NL, et al. 2006. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. Science 312:1530–1533.
29. Loffredo JT, et al. 2008. Patterns of CD8+ immunodominance may influence the ability of Mamu-B*08-positive macaques to naturally control simian immunodeficiency virus SIVmac239 replication. J. Virol. 82: 1723–1738.
30. Loffredo JT, et al. 2007. Mamu-B*08-positive macaques control simian immunodeficiency virus replication. J. Virol. 81:8827–8832.
31. Matano T, et al. 2004. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J. Exp. Med. 199:1709–1718.
32. Matano T, et al. 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J. Virol. 72: 164–169.
33. Migueles SA, et al. 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc. Natl. Acad. Sci. U. S. A. 97:2709–2714.
34. Miura T, et al. 2009. HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte recognition. J. Virol. 83:2743–2755.
35. Morgan C, et al. 2008. The use of nonhuman primate models in HIV vaccine development. PLoS Med. 5:e173.
36. Mothe BR, et al. 2003. Expression of the major histocompatibility complex class I molecule Mamu-A*01 is associated with control of simian immunodeficiency virus SIVmac239 replication. J. Virol. 77:2736–2740.
37. Muhl T, Krawczak M, Ten Haaft P, Hunsmann G, Sauermann U. 2002. MHC class I alleles influence set-point viral load and survival time in simian immunodeficiency virus-infected rhesus monkeys. J. Immunol. 169:3438–3446.
38. Naruse TK, et al. 2010. Diversity of MHC class I genes in Burmese-origin rhesus macaques. Immunogenetics 62:601–611.
39. O'Connor DH, et al. 2002. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. Nat. Med. 8:493–499.
40. O'Connor SL, et al. 2010. MHC heterozygote advantage in simian immunodeficiency virus-infected Mauritian cynomolgus macaques. Sci. Transl. Med. 2:22ra18.
41. Otting N, et al. 2005. Unparalleled complexity of the MHC class I region in rhesus macaques. Proc. Natl. Acad. Sci. U. S. A. 102:1626–1631.
42. Roederer M, Nozzi JL, Nason MC. 2011. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A 79:167–174.
43. Schmitz JE, et al. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 283:857–860.
44. Seder RA, Hill AV. 2000. Vaccines against intracellular infections requiring cellular immunity. Nature 406:793–798.
45. Seder RA, Darrah PA, Roederer M. 2008. T-cell quality in memory and protection: implications for vaccine design. Nat. Rev. Immunol. 8:247–258.
46. Shibata R, et al. 1997. Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J. Infect. Dis. 176:362–373.
47. Sodora DL, et al. 2009. Toward an AIDS vaccine: lessons from natural simian immunodeficiency virus infections of African nonhuman primate hosts. Nat. Med. 15:861–865.
48. Stinchcombe JC, Bossi G, Booth S, Griffiths GM. 2001. The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity 15:751–761.
49. Takahara Y, et al. 2011. Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge. Biochem. Biophys. Res. Commun. 408:615–619.
50. Tanaka-Takahashi Y, et al. 2007. Reference strand-mediated conformation analysis-based typing of multiple alleles in the rhesus macaque MHC class I Mamu-A and Mamu-B loci. Electrophoresis 28:918–924.
51. Tang J, et al. 2002. Favorable and unfavorable HLA class I alleles and haplotypes in Zambians predominantly infected with clade C human immunodeficiency virus type 1. J. Virol. 76:8276–8284.
52. Tenzer S, et al. 2009. Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance. Nat. Immunol. 10:636–646.
53. Tsukamoto T, et al. 2009. Impact of cytotoxic-T-lymphocyte memory induction without virus-specific CD4+ T-cell help on control of a simian immunodeficiency virus challenge in rhesus macaques. J. Virol. 83:9339–9346.
54. Wang YE, et al. 2009. Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1. J. Virol. 83:1845–1855.
55. Wilson NA, et al. 2006. Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. J. Virol. 80:5875–5885.
56. Wiseman RW, et al. 2007. Simian immunodeficiency virus SIVmac239 infection of major histocompatibility complex-identical cynomolgus macaques from Mauritius. J. Virol. 81:349–361.
57. Yamamoto H, Kawada M, Takeda A, Igarashi H, Matano T. 2007. Post-infection immunodeficiency virus control by neutralizing antibodies. PLoS One 2:e540.
58. Yamamoto T, et al. 2009. Polyfunctional CD4+ T-cell induction in neutralizing antibody-triggered control of simian immunodeficiency virus infection. J. Virol. 83:5514–5524.
59. Yant LJ, et al. 2006. The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication. J. Virol. 80:5074–5077.

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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

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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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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 $CD8^+$

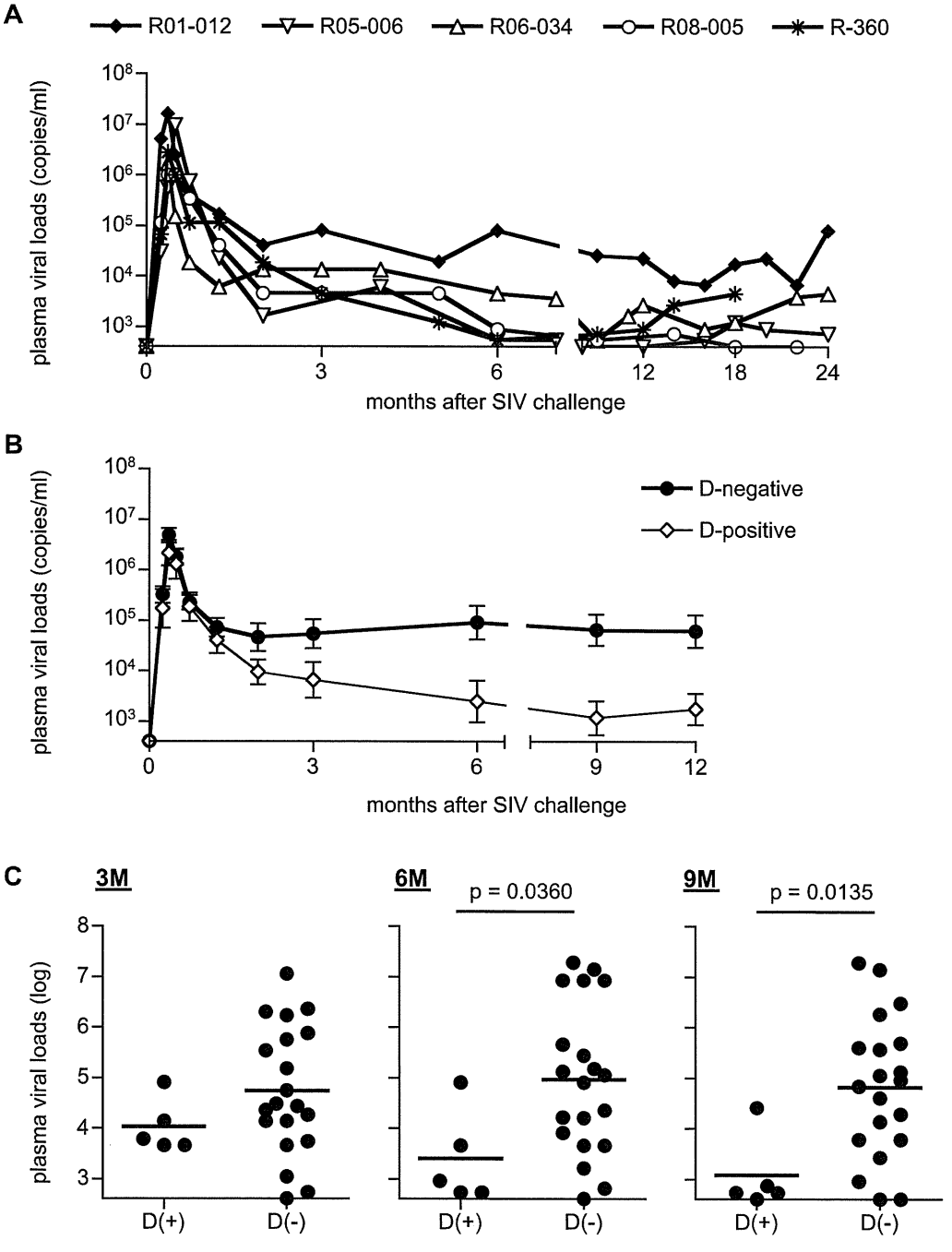

Figure 1. Plasma viral loads after SIVmac239 challenge in unvaccinated macaques. Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described previously [35]. The lower limit of detection is approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads after challenge in unvaccinated 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

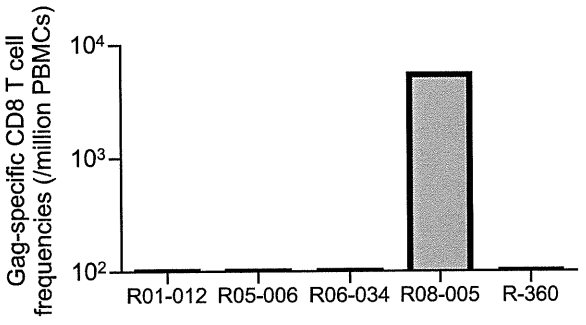

Figure 2. SIV Gag-specific CD8$^+$ T-cell responses in unvaccinated D$^+$ macaques at week 2 after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g002

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.

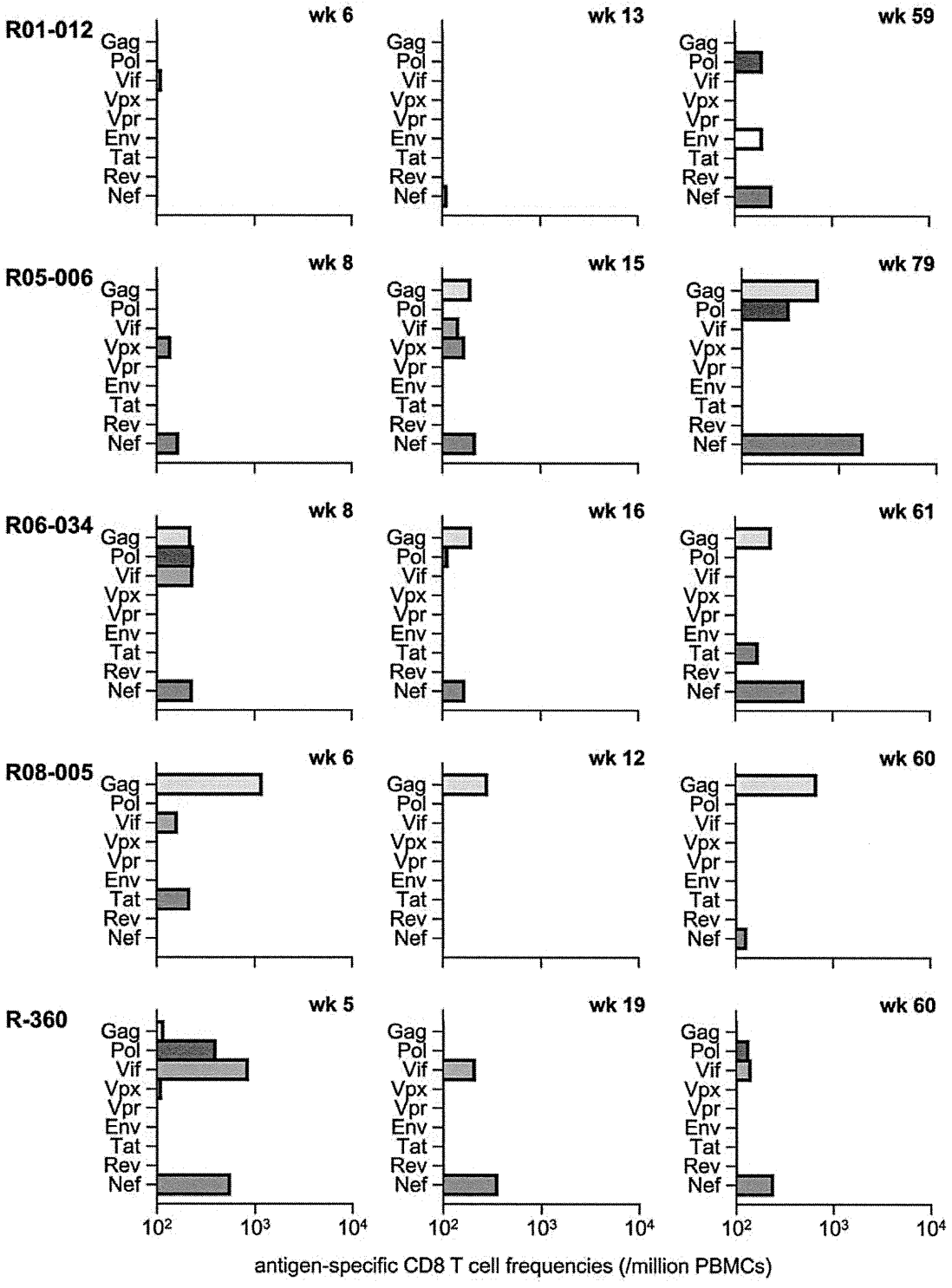

Figure 3. SIV antigen-specific CD8$^+$ T-cell responses in unvaccinated D$^+$ macaques. Responses were measured by the detection of antigen-specific IFN-γ induction in PBMCs obtained at indicated time points after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g003

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

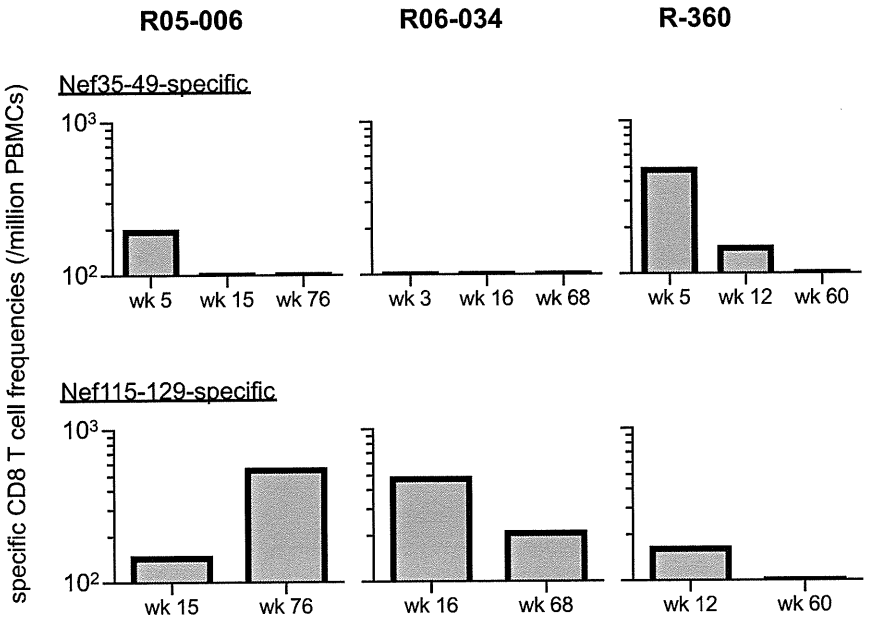

Figure 4. SIV Nef-specific CD8$^+$ T-cell responses in macaques R05-006, R06-034, and R-360. Nef_{35-49}-specific (upper panels) and $Nef_{115-129}$-specific (lower panels) CD8$^+$ T-cell responses were examined at indicated time points after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g004

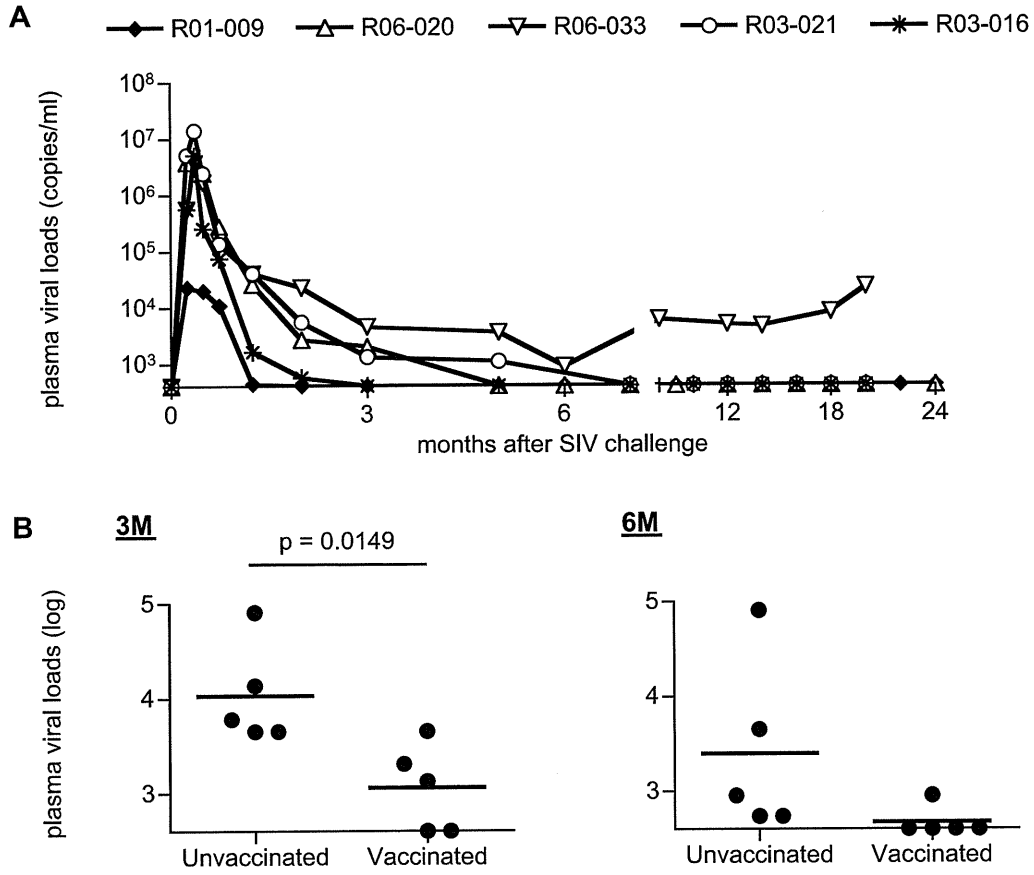

Figure 5. Plasma viral loads after SIVmac239 challenge in vaccinated D$^+$ macaques. (A) Changes in plasma viral loads after challenge vaccinated macaques possessing MHC-I haplotype D. (B) Comparison of plasma viral loads at 3 months (left panel) and 6 months (right panel) between five unvaccinated D$^+$ 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

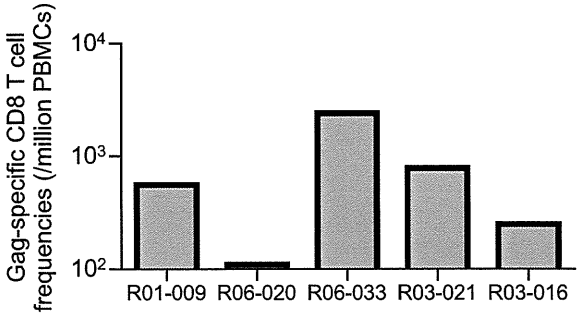

Figure 6. SIV Gag-specific CD8$^+$ T-cell responses in vaccinated D$^+$ macaques at week 2 after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g006

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).

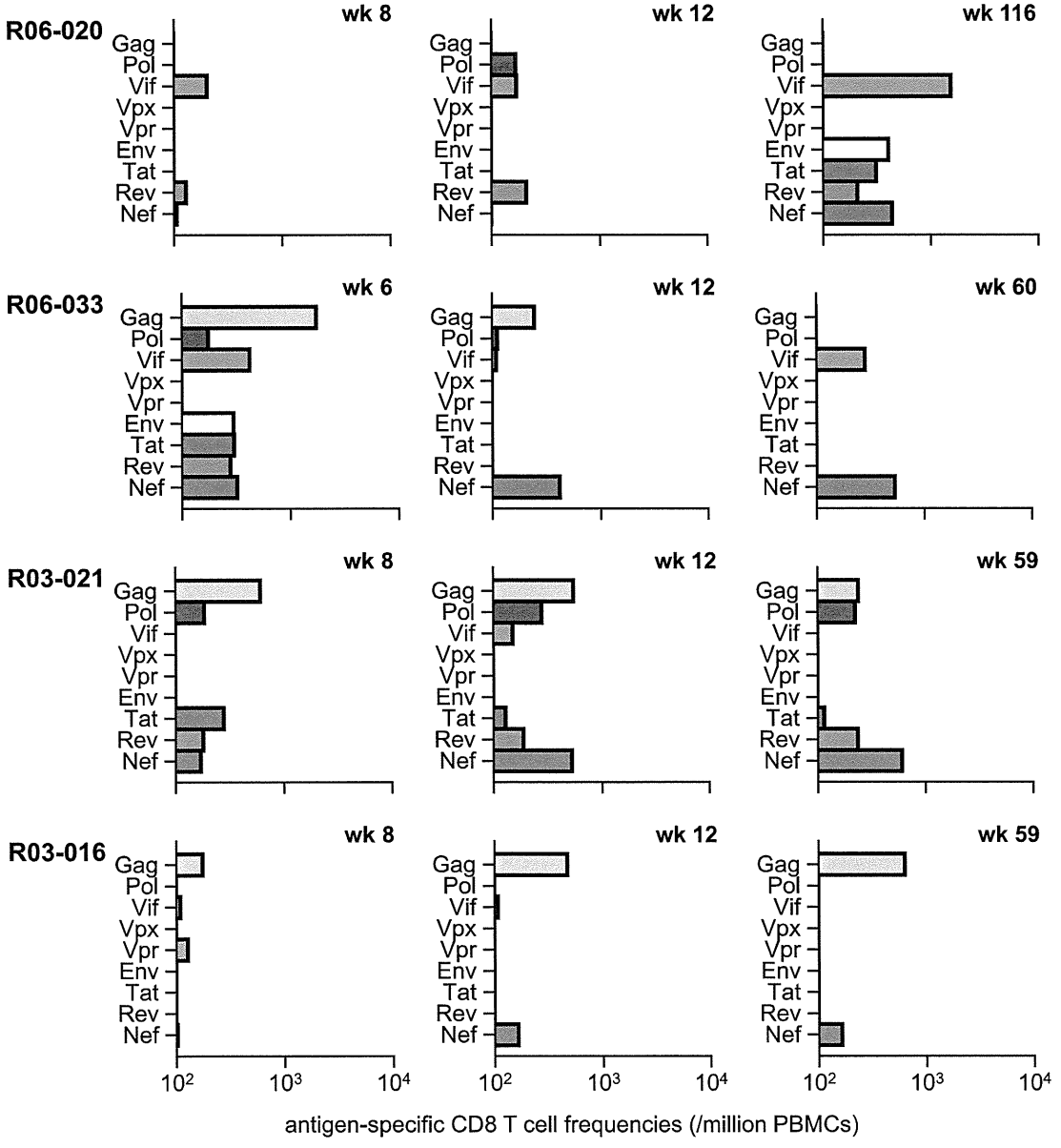

Figure 7. SIV antigen-specific CD8$^+$ T-cell responses in vaccinated D$^+$ animals after SIVmac239 challenge. Samples for this analysis were unavailable in macaque R01-009.
doi:10.1371/journal.pone.0054300.g007

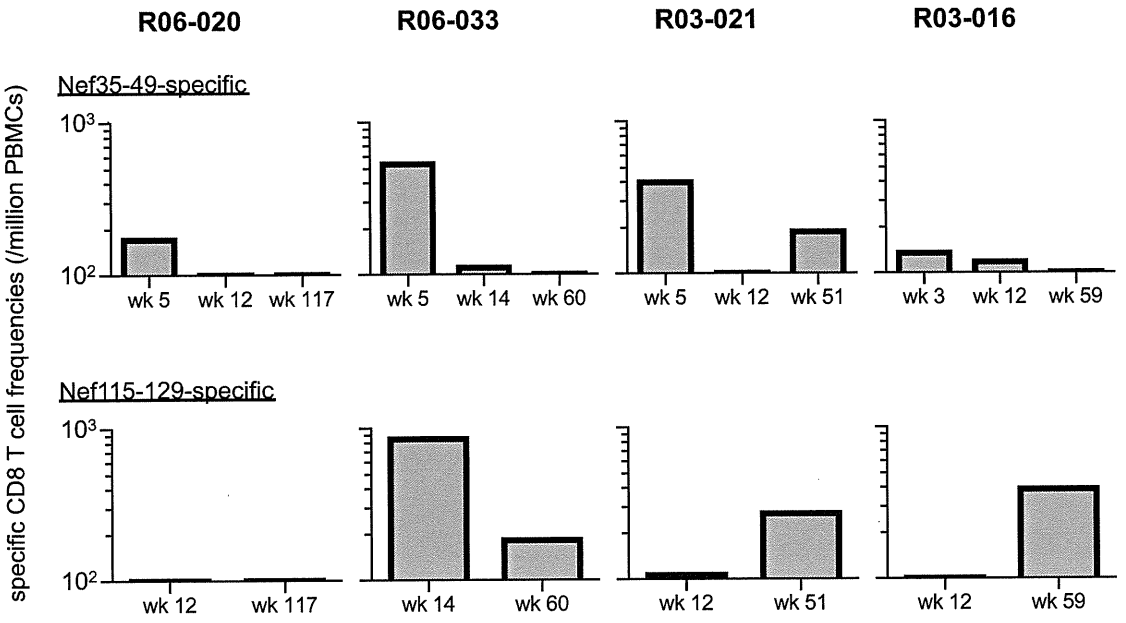

Figure 8. SIV Nef-specific CD8$^+$ T-cell responses in macaques R06-020, R06-033, R03-021, and R03-016. Nef_{35-49}-specific (upper panels) and $Nef_{115-129}$-specific (lower panels) CD8$^+$ T-cell responses were examined at indicated time points after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g008

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.
doi:10.1371/journal.pone.0054300.g009

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.

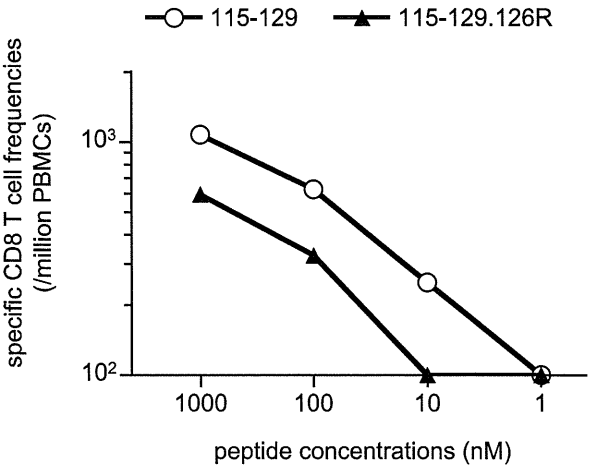

Figure 10. IFN-γ induction in $CD8^+$ T cells after stimulation with the wild-type or the mutant peptide. PBMCs obtained at week 31 from macaque R06-033 were stimulated by coculture with B-LCL pulsed with indicated concentrations of the wild-type $Nef_{115-129}$ peptide (open circles, 115–129, LAIDMSHFIKEKGGL) or the mutant $Nef_{115-129}$ peptide with a K126R alteration (closed triangles, 115–129.126R, LAIDMSHFIKERGGL).
doi:10.1371/journal.pone.0054300.g010

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.

References

1. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB (1994) Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 68: 6103–6110.
2. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, et al. (1994) Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 68: 4650–4655.
3. Matano T, Shibata R, Siemon C, Connors M, Lane HC, et al. (1998) Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J Virol 72: 164–169.
4. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, et al. (1999) Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med 189: 991–998.
5. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, et al. (1999) Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 283: 857–860.
6. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, et al. (1999) HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. Science 283: 1748–1752.
7. Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, et al. (2000) HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc Natl Acad Sci USA 97: 2709–2714.
8. Tang J, Tang S, Lobashevsky E, Myracle AD, Fideli U, et al. (2002) Favorable and unfavorable HLA class I alleles and haplotypes in Zambians predominantly infected with clade C human im- munodeficiency virus type 1. J Virol 76: 8276–8284.
9. Kiepiela P, Leslie AJ, Honeyborne I, Ramduth D, Thobakgale C, et al. (2004) Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. Nature 432: 769–775.
10. Leslie A, Matthews PC, Listgarten J, Carlson JM, Kadie C, et al. (2010) Additive contribution of HLA class I alleles in the immune control of HIV-1 infection. J Virol 84: 9879–9888.
11. Altfeld M, Addo MM, Rosenberg ES, Hecht FM, Lee PK, et al. (2003) Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS 17: 2581–2591.
12. Altfeld M, Kalife ET, Qi Y, Streeck H, Lichterfeld M, et al. (2006) HLA alleles associated with delayed progression to AIDS contribute strongly to the initial CD8+ T cell response against HIV-1. PLoS Med 3: e403.
13. Goulder PJ, Watkins DI (2008) Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat Rev Immunol 8: 619–630.

14. Muhl T, Krawczak M, Ten Haaft P, Hunsmann G, Sauermann U (2002) MHC class I alleles influence set-point viral load and survival time in simian immunodeficiency virus-infected rhesus monkeys. J Immunol 169: 3438–3446.
15. Mothe BR, Weinfurter J, Wang C, Rehrauer W, Wilson N, et al. (2003) Expression of the major histocompatibility complex class I molecule Mamu-A*01 is associated with control of simian immunodeficiency virus SIVmac239 replication. J Virol 77: 2736–2740.
16. Yant LJ, Friedrich TC, Johnson RC, May GE, Maness NJ, et al. (2006) The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication. J Virol 80: 5074–5077.
17. Loffredo JT, Maxwell J, Qi Y, Glidden CE, Borchardt GJ, et al. (2007) Mamu-B*08-positive macaques control simian immunodeficiency virus replication. J Virol 81: 8827–8832.
18. Edwards BH, Bansal A, Sabbaj S, Bakari J, Mulligan MJ, et al. (2002) Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. J Virol 76: 2298–2305.
19. Zuniga R, Lucchetti A, Galvan P, Sanchez S, Sanchez C, et al. (2006) Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. J Virol 80: 3122–3125.
20. Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, et al. (2007) CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 13: 46–53.
21. Nomura T, Yamamoto H, Shiino T, Takahashi N, Nakane T, et al. (2012) Association of major histocompatibility complex class I haplotypes with disease progression after simian immunodeficiency virus challenge in Burmese rhesus macaques. J Virol 86: 6481–6490.
22. Schneidewind A, Brockman MA, Yang R, Adam RI, Li B, et al. (2007) Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. J Virol 81: 12382–12393.
23. Emu B, Sinclair E, Hatano H, Ferre A, Shacklett B, et al. (2008) HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but such responses are not always necessary for long-term virus control. J Virol 82: 5398–5407.
24. Miura T, Brockman MA, Schneidewind A, Lobritz M, Pereyra F, et al. (2009) HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte recognition. J Virol 83: 2743–2755.
25. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, et al. (2004) HIV evolution: CTL escape mutation and reversion after transmission. Nat Med 10: 282–289.
26. Martinez-Picado J, Prado JG, Fry EE, Pfafferott K, Leslie A, et al. (2006) Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. J Virol 80: 3617–3623.
27. Crawford H, Prado JG, Leslie A, Hue S, Honeyborne I, et al. (2007) Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. J Virol 81: 8346–8351.
28. Friedrich TC, Valentine LE, Yant LJ, Rakasz EG, Piaskowski SM, et al. (2007) Subdominant CD8+ T-cell responses are involved in durable control of AIDS virus replication. J Virol 81: 3465–3476.
29. Loffredo JT, Bean AT, Beal DR, Leon EJ, May GE, et al. (2008) Patterns of CD8+ immunodominance may influence the ability of Mamu-B*08-positive macaques to naturally control simian immunodeficiency virus SIVmac239 replication. J Virol 82: 1723–1738.
30. Maness NJ, Yant LJ, Chung C, Loffredo JT, Friedrich TC, et al. (2008) Comprehensive immunological evaluation reveals surprisingly few differences between elite controller and progressor Mamu-B*17-positive simian immunodeficiency virus-infected rhesus macaques. J Virol 82: 5245–5254.
31. Valentine LE, Loffredo JT, Bean AT, Leon EJ, MacNair CE, et al. (2009) Infection with "escaped" virus variants impairs control of simian immunodeficiency virus SIVmac239 replication in Mamu-B*08-positive macaques. J Virol 83: 11514–11527.
32. Budde ML, Greene JM, Chin EN, Ericsen AJ, Scarlotta M, et al. (2012) Specific CD8+ T cell responses correlate with control of simian immunodeficiency virus replication in Mauritian cynomolgus macaques. J Virol 86: 7596–7604.
33. Mudd PA, Martins MA, Ericsen AJ, Tully DC, Power KA, et al. (2012) Vaccine-induced CD8+ T cells control AIDS virus replication. Nature 491: 129–133.
34. Naruse TK, Chen Z, Yanagida R, Yamashita T, Saito Y, et al. (2010) Diversity of MHC class I genes in Burmese-origin rhesus macaques. Immunogenetics 62: 601–611.
35. Matano T, Kobayashi M, Igarashi H, Takeda A, Nakamura H, et al. (2004) Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J Exp Med 199: 1709–1718.
36. Kawada M, Tsukamoto T, Yamamoto H, Iwamoto N, Kurihara K, et al. (2008) Gag-specific cytotoxic T-lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial. J Virol 82: 10199–10206.
37. Tanaka-Takahashi Y, Yasunami M, Naruse T, Hinohara K, Matano T, et al. (2007) Reference strand-mediated conformation analysis-based typing of multiple alleles in the rhesus macaque MHC class I Mamu-A and Mamu-B loci. Electrophoresis 28: 918–924.
38. Shibata R, Maldarelli F, Siemon C, Matano T, Parta M, et al. (1997) Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J Infect Dis 176: 362–373.
39. Li HO, Zhu YF, Asakawa M, Kuma H, Hirata T, et al. (2000) A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. J Virol 74: 6564–6569.
40. Takeda A, Igarashi H, Nakamura H, Kano M, Iida A, et al. (2003) Protective efficacy of an AIDS vaccine, a single DNA priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. J Virol 77: 9710–9715.
41. Kestler HW III, Ringler DJ, Mori K, Panicali DL, Sehgal PK, et al. (1991) Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 65: 651–662.
42. Yamamoto H, Kawada M, Takeda A, Igarashi H, Matano T (2007) Post-infection immunodeficiency virus control by neutralizing antibodies. PLoS One 2: e540.
43. Iwamoto N, Tsukamoto T, Kawada M, Takeda A, Yamamoto H, et al. (2010) Broadening of CD8+ cell responses in vaccine-based simian immunodeficiency virus controllers. AIDS 24: 2777–2787.
44. Voss G, Nick S, Stahl-Hennig C, Ritter K, Hunsmann G (1992) Generation of macaque B lymphoblastoid cell lines with simian Epstein-Barr-like viruses: transformation procedure, characterization of the cell lines and occurrence of simian foamy virus. J Virol Methods 39: 185–195.
45. Kawada M, Igarashi H, Takeda A, Tsukamoto T, Yamamoto H, et al. (2006) Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. J Virol 80: 1949–1958.
46. Tenzer S, Wee E, Burgevin A, Stewart-Jones G, Friis L, et al. (2009) Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance. Nat Immunol 10: 636–646.
47. Ishii H, Kawada M, Tsukamoto T, Yamamoto H, Matsuoka S, et al. (2012) Impact of vaccination on cytotoxic T lymphocyte immunodominance and cooperation against simian immunodeficiency virus replication in rhesus macaques. J Virol 86: 738–745.
48. Flores-Villanueva PO, Yunis EJ, Delgado JC, Vittinghoff E, Buchbinder S, et al. (2001) Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. Proc Natl Acad Sci USA 98: 5140–5145.
49. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, et al. (2002) Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet 31: 429–434.
50. Martin MP, Qi Y, Gao X, Yamada E, Martin JN, et al. (2007) Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. Nat Genet 39: 733–740.