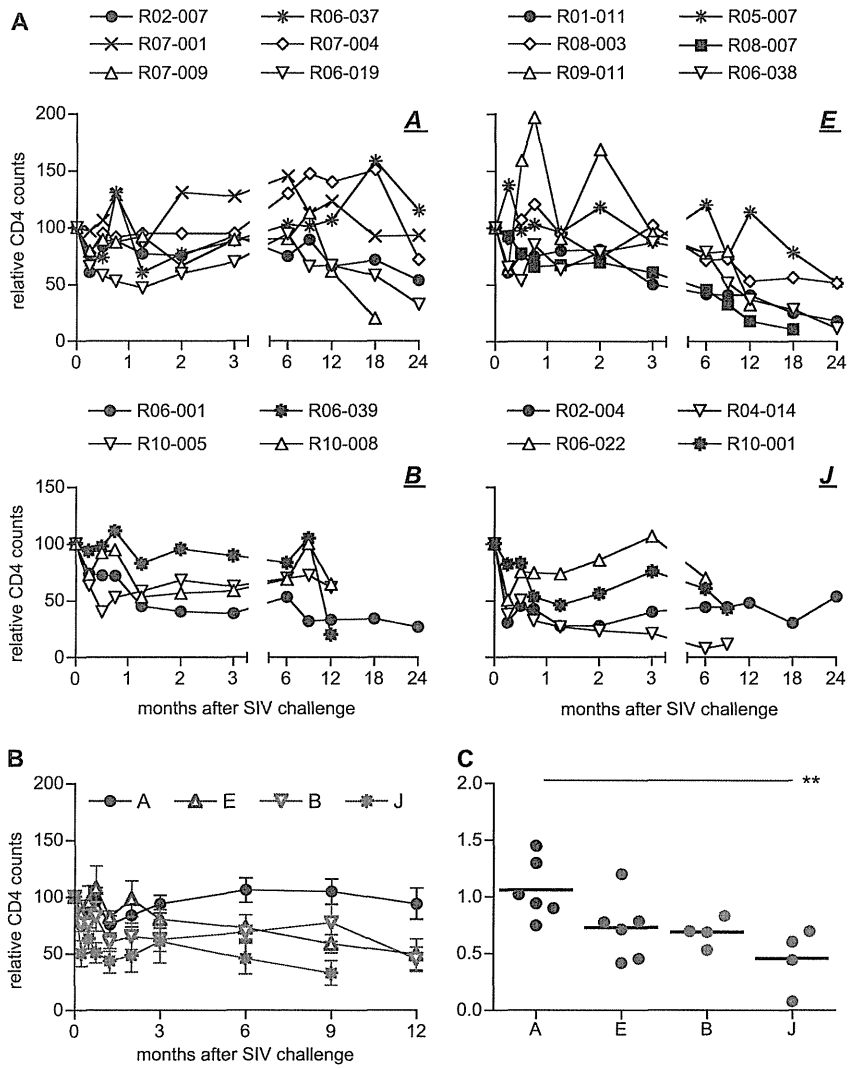

FIG 2 Relative $CD4^+$ T-cell counts after SIVmac239 challenge. (A) Relative $CD4^+$ T-cell counts after challenge in A^+ (upper left), E^+ (upper right), B^+ (lower left), and J^+ (lower right) macaques. For each animal, the peripheral CD4 counts relative to that at challenge (set at 100) are shown. (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^+

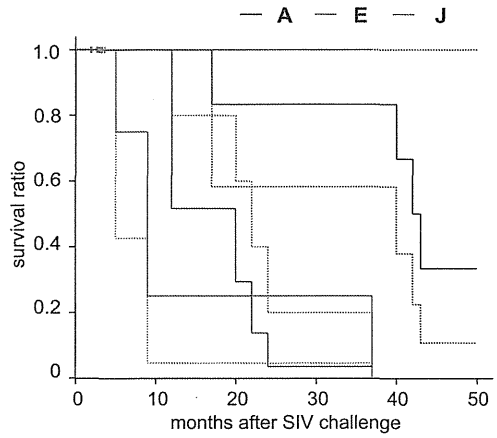

FIG 3 Kaplan-Meyer survival curves after SIVmac239 challenge in A^+, E^+, and J^+ macaques. Macaque R08-003, which is under observation, is not included. B^+ animals were excluded from this analysis because data on only two animals were available. We determined the Kaplan-Meyer estimate of the survival 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).

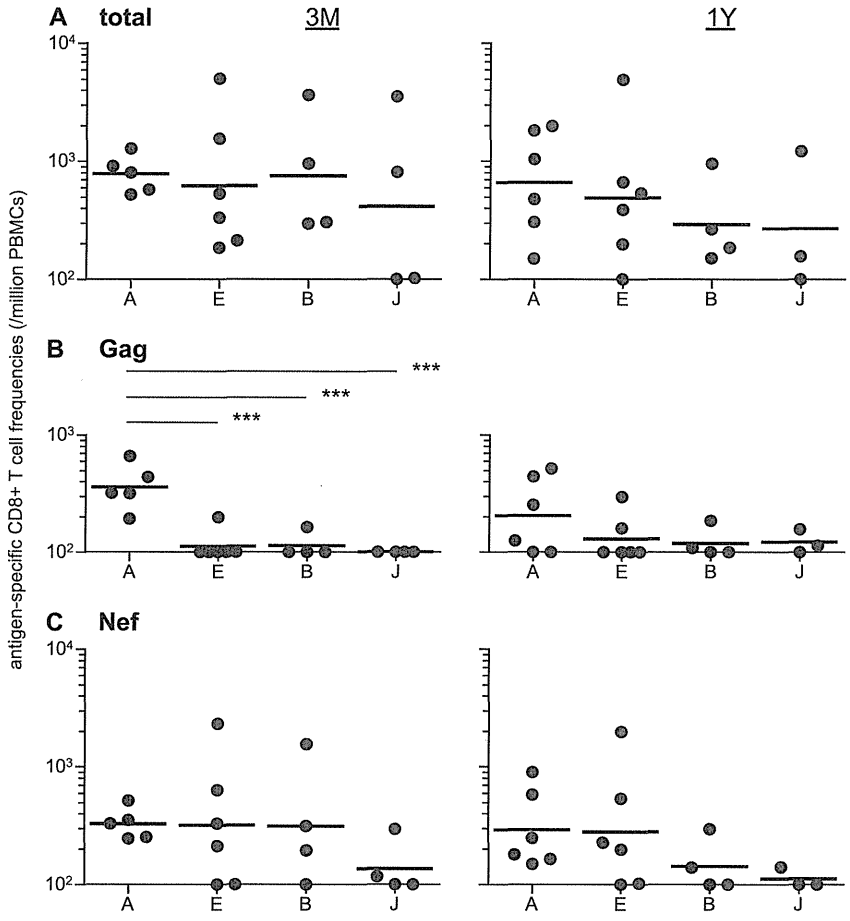

FIG 4 Comparison of SIV antigen-specific $CD8^+$ T-cell responses. Responses were measured by the detection of antigen-specific IFN-γ induction using PBMCs at 3 months (3 M; left) and at 1 year (1Y; right). (A) Whole SIV antigen-specific $CD8^+$ T-cell frequencies. The sum of Gag-, Pol-, Vif-, Vpx-, 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.

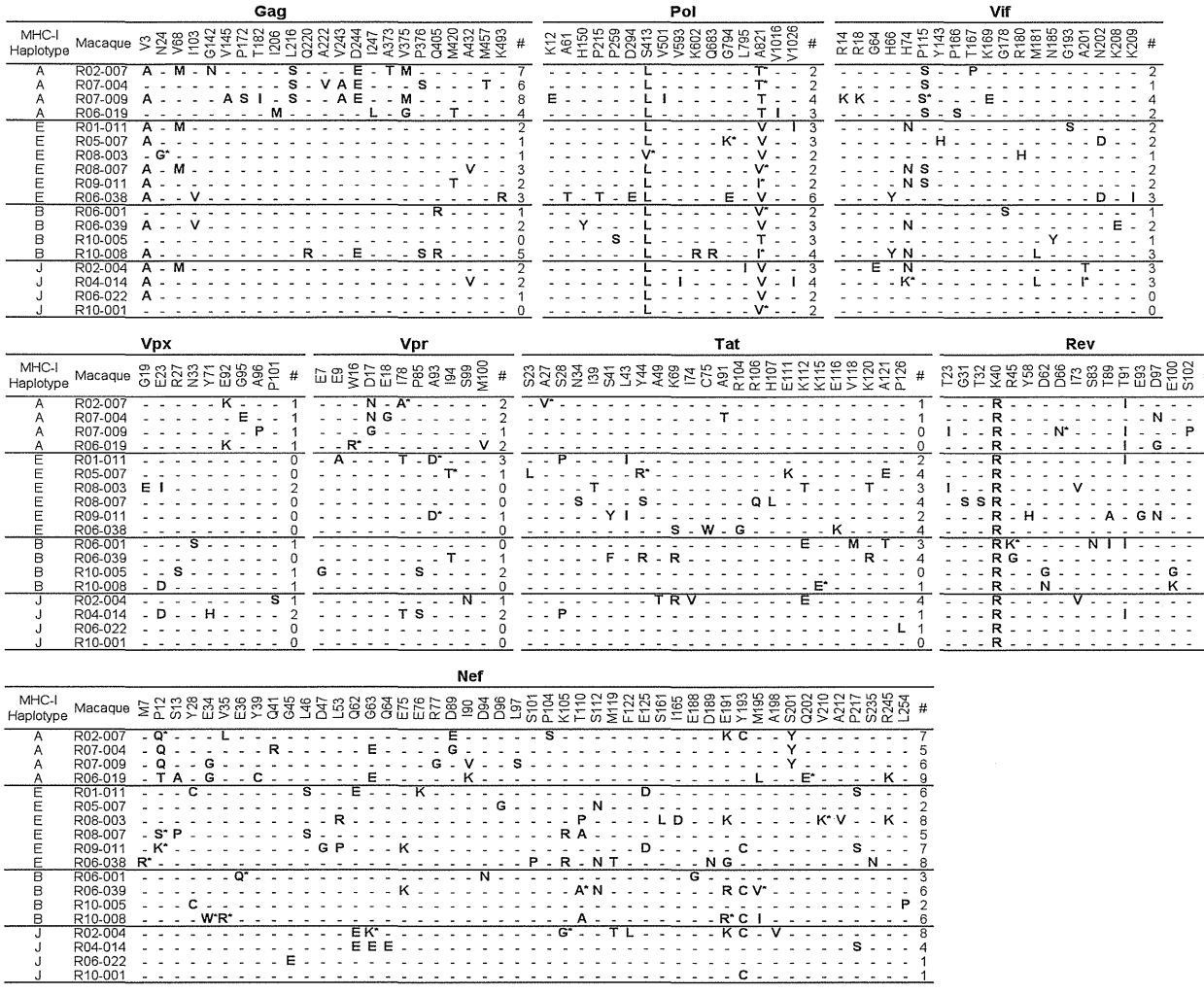

FIG 5 Predominant nonsynonymous mutations in viral cDNAs around 1 year after SIVmac239 challenge. Amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef are shown. In three animals, R04-014, R06-022, and R10-001 in group J^+, data on the samples obtained before their death at 5 or 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

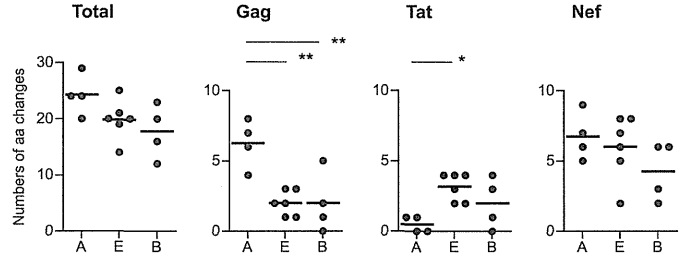

FIG 6 Comparison of the numbers of predominant nonsynonymous mutations in viral cDNAs around 1 year after SIVmac239 challenge among A^+, E^+, and B^+ macaques. J^+ animals were excluded from this analysis because three of them were euthanized by 1 year. The first graph (total) shows the sum of the numbers 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 ($P = 0.0140$).

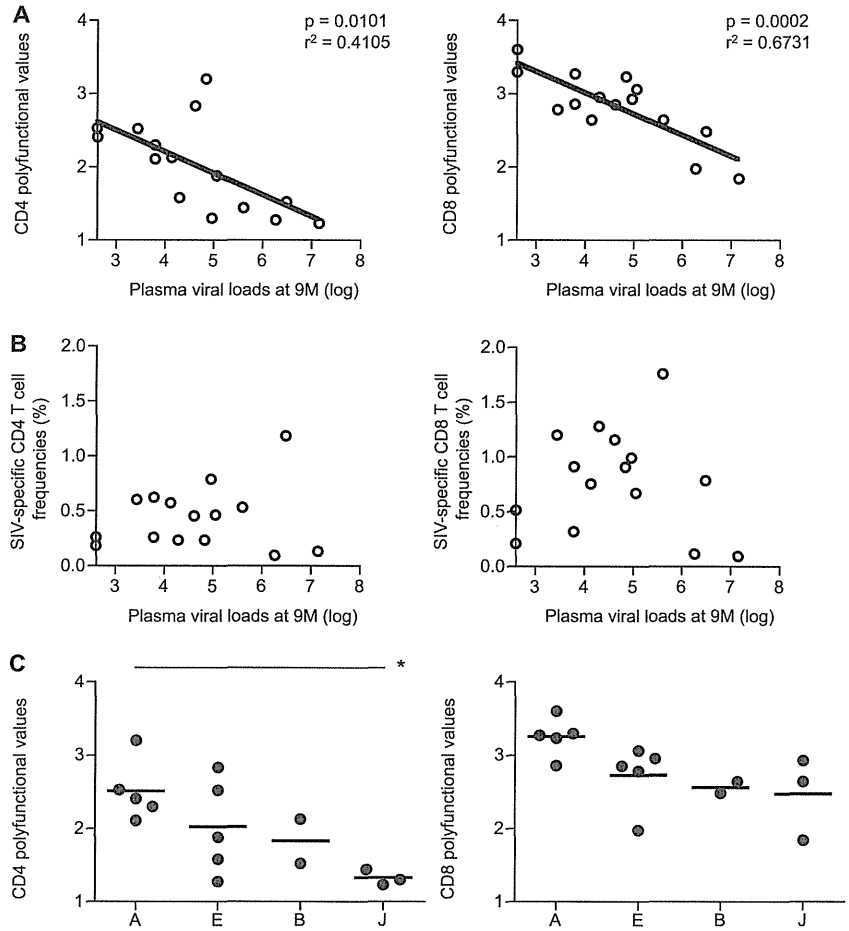

FIG 7 Polyfunctionality in SIV-specific $CD4^+$ and $CD8^+$ T cells around 8 months after SIVmac239 challenge. Samples of macaques R02-007 (A^+), R01-011 (E^+), R10-005 (B^+), R10-008 (B^+), and R10-001 (J^+) were unavailable. (A) Correlation analysis of plasma viral loads at 9 months with polyfunctionality (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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Impact of Vaccination on Cytotoxic T Lymphocyte Immunodominance and Cooperation against Simian Immunodeficiency Virus Replication in Rhesus Macaques

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Cytotoxic T lymphocyte (CTL) responses play a central role in viral suppression in human immunodeficiency virus (HIV) infections. Prophylactic vaccination resulting in effective CTL responses after viral exposure would contribute to HIV control. It is important to know how CTL memory induction by vaccination affects postexposure CTL responses. We previously showed vaccine-based control of a simian immunodeficiency virus (SIV) challenge in a group of Burmese rhesus macaques sharing a major histocompatibility complex class I haplotype. $Gag_{206-216}$ and $Gag_{241-249}$ epitope-specific CTL responses were responsible for this control. In the present study, we show the impact of individual epitope-specific CTL induction by prophylactic vaccination on postexposure CTL responses. In the acute phase after SIV challenge, dominant $Gag_{206-216}$-specific CTL responses with delayed, naive-derived $Gag_{241-249}$-specific CTL induction were observed in $Gag_{206-216}$ epitope-vaccinated animals with prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory, and vice versa in $Gag_{241-249}$ epitope-vaccinated animals with single $Gag_{241-249}$ epitope-specific CTL induction. Animals with $Gag_{206-216}$-specific CTL induction by vaccination selected for a $Gag_{206-216}$-specific CTL escape mutation by week 5 and showed significantly less decline of plasma viral loads from week 3 to week 5 than in $Gag_{241-249}$ epitope-vaccinated animals without escape mutations. Our results present evidence indicating significant influence of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses, which affects virus control. These findings provide great insights into antigen design for CTL-inducing AIDS vaccines.

Human immunodeficiency virus (HIV) infection induces chronic, persistent viral replication leading to AIDS onset in humans. Virus-specific cytotoxic T lymphocyte (CTL) responses play a central role in the resolution of acute peak viremia (3, 4, 13, 22, 28) but mostly fail to contain viral replication in the natural course of HIV infection. Vaccination resulting in more effective CTL responses after viral exposure than in natural HIV infections would contribute to HIV control (30, 33). CTL memory induction by prophylactic vaccination may lead to efficient secondary CTL responses, but naive-derived primary CTL responses specific for viral nonvaccine antigens can also be induced after viral exposure. It is important to know how CTL memory induction by vaccination affects these postexposure CTL responses.

Cumulative studies on HIV-infected individuals have shown association of HLA genotypes with rapid or delayed AIDS progression (5, 14, 31, 34). For instance, most of the HIV-infected individuals possessing *HLA-B*57* have been indicated to show a better prognosis with lower viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in this viral control (1, 8, 23, 24). Indian rhesus macaques possessing certain major histocompatibility complex class I (MHC-I) alleles, such as *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17*, tend to show simian immunodeficiency virus (SIV) control (19, 25, 36). This implies possible HIV control by induction of particular effective CTL responses (2, 7, 12, 16, 27).

Recent trials of prophylactic T-cell-based vaccines in macaque AIDS models have indicated the possibility of reduction in post-challenge viral loads (6, 15, 17, 21, 35). We previously developed a prophylactic AIDS vaccine consisting of a DNA prime and a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (20). Our trial showed vaccine-based control of an SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90-120-Ia* (21). Animals possessing *90-120-Ia* dominantly elicited Mamu-A1*043:01 (GenBank accession number AB444869)-restricted $Gag_{206-216}$ (IINEEAADWDL) epitope-specific and Mamu-A1*065:01 (AB444921)-restricted $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses after SIV challenge and selected for viral *gag* mutations, GagL216S (leading to a leucine [L]-to-serine [S] substitution at amino acid [aa] 216 in Gag) and GagD244E (aspartic acid [D]-to-glutamic acid [E] at aa 244), resulting in escape from CTL recognition with viral fitness costs in the chronic phase (9, 26). Vaccinees possessing *90-120-Ia* failed to control a challenge with a mutant SIV carrying these two CTL escape mutations, indicating that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses play a crucial role in the vaccine-based control of wild-type SIVmac239 replication

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TABLE 1 Animals analyzed in this study

Group	No. of animals	Vaccination[a]	SIV-specific CTL response postboost
I	6	None	None
II	5	Gag (pCMV-SHIVdEN DNA prime, SeV-Gag boost)	Gag-specific CTL
III	6	$Gag_{241-249}$-specific (pGag$_{236-250}$-EGFP-N1 DNA prime, SeV-Gag$_{236-250}$-EGFP boost)	$Gag_{241-249}$-specific CTL
IV	5	$Gag_{206-216}$-specific (pGag$_{202-216}$-EGFP-N1 DNA prime, SeV-Gag$_{202-216}$-EGFP boost)	$Gag_{206-216}$-specific CTL

[a] All animals were challenged with SIVmac239.

(10). Furthermore, in an SIVmac239 challenge experiment with *90-120-Ia*-positive rhesus macaques that received a prophylactic vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), this single-epitope vaccination resulted in control of SIVmac239 replication with dominant induction of $Gag_{241-249}$-specific CTL responses in the acute phase postchallenge (32).

Thus, it is hypothesized that induction of single $Gag_{206-216}$ or $Gag_{241-249}$ epitope-specific CTL responses by vaccination may result in different patterns of CTL immunodominance and viral replication after SIV challenge. In the present study, we analyzed the impact of prophylactic vaccination inducing single $Gag_{206-216}$ epitope-specific CTL responses on SIV control in *90-120-Ia*-positive macaques and compared the results with those of vaccination inducing single $Gag_{241-249}$ epitope-specific CTL responses. This analysis revealed differences in CTL responses and patterns of viral control after SIV challenge between these vaccinated groups, indicating significant effects of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses.

MATERIALS AND METHODS

Animal experiments. Animal experiments were conducted through the Cooperative Research Program at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia. All animals were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases.

Five Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-120-Ia* (26) (group IV) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{206-216}$-specific CTL responses followed by an SIVmac239 challenge and were compared with three groups (I, II, and III) of *90-120-Ia*-positive animals reported previously (10, 32) (Table 1). Group I animals ($n = 6$) received no vaccination, while group II animals ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting Gag-specific CTL responses. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from a simian/human immunodeficiency virus ($SHIV_{MD14YE}$) molecular clone DNA with *env* and *nef* deleted (29) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx; SIVmac239–HIV-1 chimeric Vpr; and HIV-1 Tat and Rev (21). In group II animals, CTL responses were undetectable after DNA prime but Gag-specific CTL responses became detectable after SeV-Gag boost. Group III animals ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{241-249}$-specific CTL responses. A pGag$_{236-250}$-EGFP-N1 DNA and an SeV-Gag$_{236-250}$-EGFP vector, both expressing an SIVmac239 $Gag_{236-250}$ (IAGTTSSVDEQ IQWM)-EGFP fusion protein, were used for the group III vaccination. After the SeV-Gag$_{236-250}$-EGFP boost, group III animals induced $Gag_{241-249}$-specific CTL responses; the animals showed no $Gag_{236-250}$-specific $CD4^+$ T-cell responses but elicited SeV/EGFP-specific $CD4^+$ T-cell responses (32). For the group IV vaccination, A pGag$_{202-216}$-EGFP-N1 DNA and an SeV-Gag$_{202-216}$-EGFP vector, both expressing an SIVmac239 $Gag_{202-216}$ (IIRDIINEEAADWDL)-EGFP fusion protein, were used (Fig. 1). Approximately 3 months after the boost, all animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239 (11). In our previous study (32), the unvaccinated and the control-vaccinated

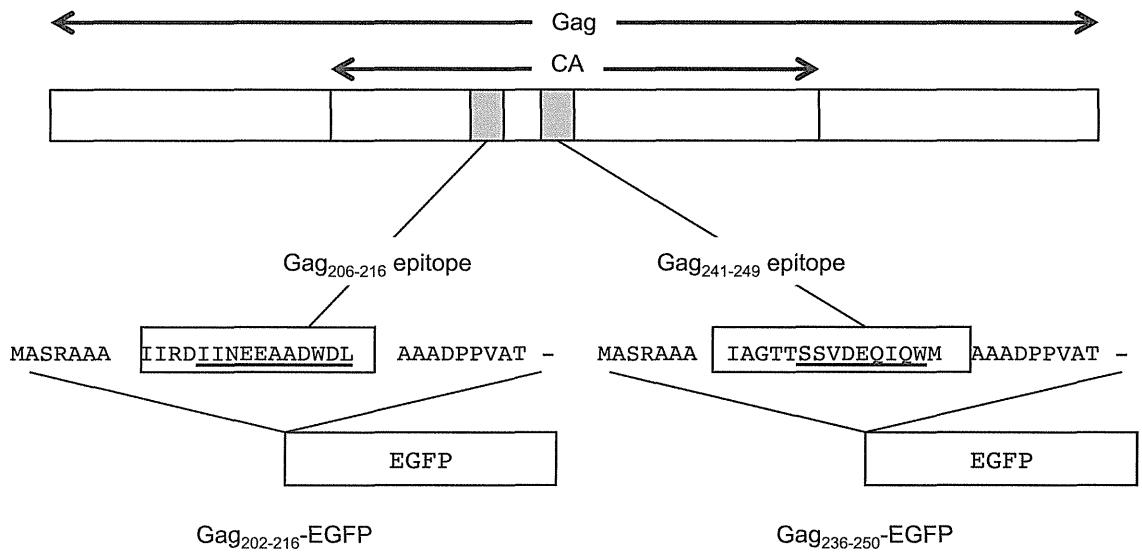

FIG 1 Schema of the cDNA constructs encoding $Gag_{202-216}$-EGFP and $Gag_{236-250}$-EGFP fusion proteins. A DNA fragment that encodes a 31-mer peptide (boxes) including the $Gag_{202-216}$ or $Gag_{236-250}$ sequence (underlining) was introduced into the 5′ end of the EGFP cDNA.

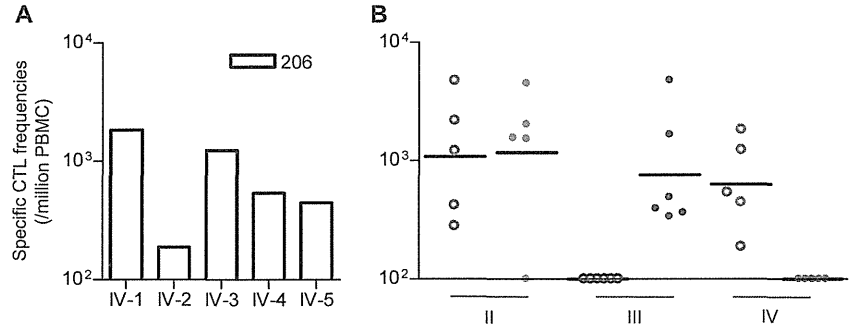

FIG 2 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after prophylactic vaccination. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies 1 week after SeV-$Gag_{202-216}$–EGFP boost in group IV macaques (open boxes). (B) $Gag_{206-216}$-specific (open circles) and $Gag_{241-249}$-specific (closed circles) $CD8^+$ T-cell frequencies 1 week after boost in group II (green), III (blue), and IV (red) macaques. The bars indicate the geometric mean of each group. No animal showed detectable Gag-specific CTL responses before the boost.

animals receiving a DNA and an SeV expressing EGFP showed no significant differences in viral loads after SIV challenge.

Analysis of antigen-specific CTL responses. We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (21). Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papioimmortalized B-lymphoblastoid cell lines pulsed with 1 μM SIVmac239 $Gag_{206-216}$ (IINEEAADWDL), $Gag_{241-249}$ (SSVDEQIQW), or $Gag_{367-381}$ (ALKEALAPVPIPFAA) peptide for $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, or $Gag_{367-381}$-specific stimulation. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (BD, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein-conjugated anti-human CD8 (BD), allophycocyanin (APC)-Cy7-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ (Biolegend, San Diego, CA) monoclonal antibodies. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell levels lower than 100 per million PBMCs were considered negative.

Sequencing of the viral genome. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). Fragments corresponding to nucleotides from 1231 to 2958 (containing the entire *gag* region) in the SIVmac239 genome (GenBank accession number M33262) were amplified by nested reverse transcription (RT)-PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using R software (R Development Core Team). Differences in geometric means of plasma viral loads were examined by one-way analysis of variance (ANOVA) and Tukey-Kramer's multiple-comparison test. Plasma viral loads at week 3 were examined for differences between group III and groups II and IV by analysis of covariance (ANCOVA) with week 5 viral loads as a covariate.

RESULTS

CTL responses after prophylactic vaccination. We previously reported the efficacy of vaccination eliciting whole Gag-specific or single $Gag_{241-249}$ epitope-specific CTL memory against SIVmac239 challenge (10, 32). In the present study, we examined the efficacy of prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory against SIVmac239 challenge and compared the results with those of the previous experiments.

Five Burmese rhesus macaques possessing MHC-I haplotype *90-120-Ia* received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{206-216}$ epitope-specific CTL responses. A plasmid DNA (p$Gag_{202-216}$-EGFP-N1) and an SeV (SeV-$Gag_{202-216}$-EGFP) vector, both expressing an SIVmac239 $Gag_{202-216}$-EGFP fusion pro-

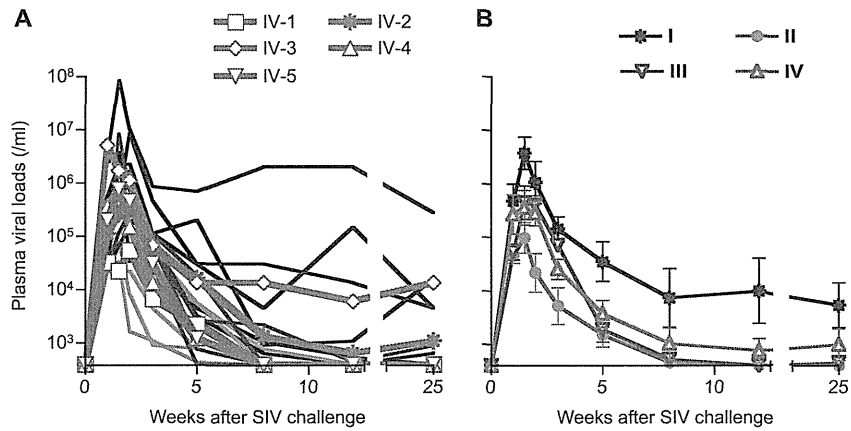

FIG 3 Plasma viral loads after SIVmac239 challenge. The plasma viral loads in group I, group II, group III, and group IV animals were determined as described previously (21). The lower limit of detection was approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) after challenge. (B) Changes in geometric means of plasma viral loads after challenge. Groups II and III (but not group IV) showed significantly lower set point viral loads than group I ($P = 0.0390$ between groups I and II, $P = 0.0404$ between groups I and III, and $P > 0.05$ between groups I and IV at week 25 by one-way ANOVA and Tukey-Kramer's multiple-comparison test).

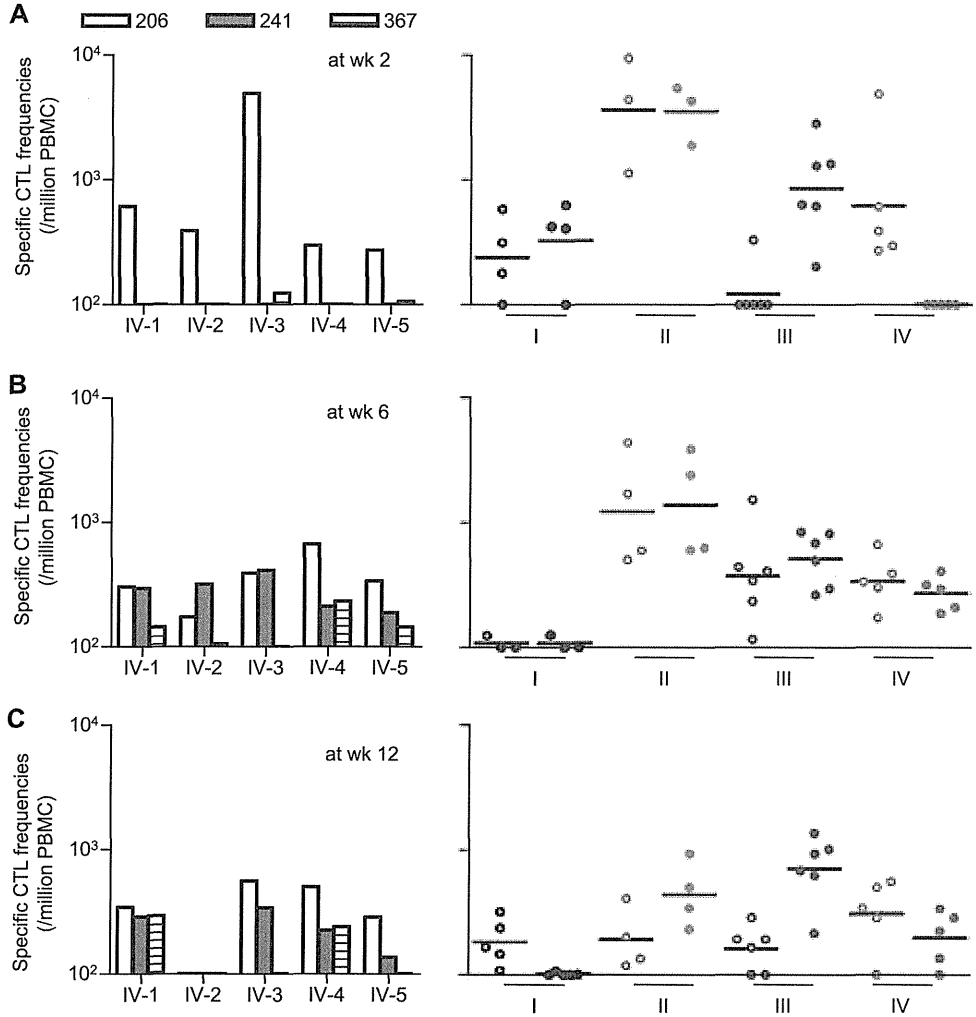

FIG 4 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after SIVmac239 challenge. CTL responses at week 2 (A), week 6 (B), and week 12 (C) are shown. In the graphs on the left, $Gag_{206-216}$-specific (open boxes), $Gag_{241-249}$-specific (closed boxes), and $Gag_{367-381}$-specific (striped boxes) $CD8^+$ T-cell frequencies in group IV macaques are shown. On the right, $Gag_{206-216}$-specific (open circles) and $Gag_{241-249}$-specific (closed circles) $CD8^+$ T-cell frequencies in group I (black), II (green), III (blue), and IV (red) macaques are shown. The bars indicate the geometric mean of each group. Samples from macaques I-1, I-6, II-1, and II-3 at week 2; macaques I-1, I-2, I-6, and II-5 at week 6; and macaques I-1 and II-5 at week 12 were unavailable for this analysis. Statistical analyses among four groups at week 12 revealed significant differences in $Gag_{241-249}$-specific CTL levels (I and III, $P < 0.0001$; I and II, and III and IV, $P < 0.01$; I and IV, II and III, and II and IV, $P > 0.05$ by one-way ANOVA and Tukey-Kramer's multiple-comparison test) but not in $Gag_{206-216}$-specific CTL levels ($P > 0.05$ by one-way ANOVA).

tein, were used for the vaccination (Fig. 1). We confirmed $Gag_{206-216}$-specific CTL responses 1 week after SeV-$Gag_{202-216}$-EGFP boost in all five animals (Fig. 2A). As expected, no $Gag_{241-249}$-specific CTL responses were detected in these animals. No $Gag_{202-216}$-specific $CD4^+$ T-cell responses were detected in the animals except for one (IV-5) showing marginal levels of responses (data not shown).

Plasma viral loads after SIV challenge. We compared these five animals (referred to as group IV) with other groups (I, II, and III) of *90-120-Ia*-positive macaques reported previously (Table 1). Group I animals ($n = 6$) received no vaccination, group II ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting whole Gag-specific CTL responses, and group III ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{241-249}$ epitope-specific CTL responses. Both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were detectable after SeV-Gag boost in four of five group II animals except for one animal (II-3), in which $Gag_{206-216}$-specific, but not $Gag_{241-249}$-specific, CTL responses were detected. In all group III animals, $Gag_{241-249}$-specific CTL responses were confirmed, while no $Gag_{206-216}$-specific CTL responses were detected after SeV-$Gag_{236-250}$-EGFP boost (Fig. 2B).

After SIVmac239 challenge, all animals were infected and showed plasma viremia during the acute phase. Plasma viremia was maintained in five of six unvaccinated animals in group I but became undetectable in one animal (I-2) at week 12. In contrast, all animals in groups II and III contained SIV replication with significantly reduced plasma viral loads compared to group I at the set point. In group IV, however, vaccine efficacy was not so clear; while three out of five animals contained SIV replication, the remaining two (IV-2 and IV-3) failed to control viral replication with persistent plasma viremia (Fig. 3).

Gag-specific CTL responses after SIV challenge. We then measured $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after SIVmac239 challenge by detection of peptide-

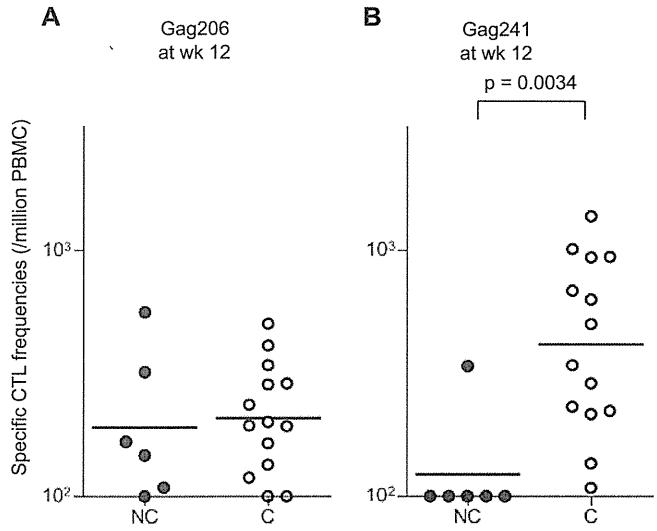

FIG 5 Comparison of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL responses in noncontrollers and controllers at week 12. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies in noncontrollers (NC; closed circles) and controllers (C; open circles). (B) $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies in noncontrollers and controllers. $Gag_{241-249}$-specific CTL levels in controllers were significantly higher than those in noncontrollers ($P = 0.0034$ by Mann-Whitney test). The bars indicate the geometric mean of each group. Data on a noncontroller (I-1) and a controller (II-5) were unavailable.

specific IFN-γ induction. At week 2 (Fig. 4A), most animals in groups I and II elicited both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses, whereas group III animals induced $Gag_{241-249}$-specific CTL responses dominantly. Remarkably, all animals in group IV showed efficient $Gag_{206-216}$-specific CTL responses without detectable $Gag_{241-249}$-specific CTL responses at week 2. These results indicate dominant $Gag_{206-216}$-specific CTL responses with delayed induction of $Gag_{241-249}$-specific CTL responses postchallenge in group IV animals with prophylactic $Gag_{206-216}$-specific CTL induction, and vice versa in group III animals.

At week 6 (Fig. 4B), efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were observed in all vaccinated animals in groups II, III, and IV, but not in group I. $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were induced equivalently even in groups III and IV. We also examined subdominant $Gag_{367-381}$ epitope-specific CTL responses, which were undetectable at week 2 but became detectable at week 6 in most group IV animals (Fig. 4, graphs on left). At week 12 (Fig. 4C), however, different CTL immunodominance patterns were observed among the groups. $Gag_{241-249}$-specific CTL levels were higher than $Gag_{206-216}$-specific levels in groups II and III but were reduced in groups I and IV. Interestingly, comparison between the animals with persistent viremia (referred to as noncontrollers) and those controlling SIV replication (referred to as controllers) revealed significant differences in $Gag_{241-249}$-specific CTL levels, but not in $Gag_{206-216}$-specific levels, at week 12 ($P = 0.0034$ by Mann-Whitney test) (Fig. 5).

Selection of a CTL escape mutation. Next, we examined viral genome *gag* sequences at weeks 5 and 12 after challenge to determine whether CTL escape mutations were selected in these animals (Table 2). At week 5, a mutation leading to an L-to-S substitution at the 216th residue in Gag (L216S) was selected in all the group II animals. This GagL216S change results in escape from $Gag_{206-216}$-specific CTL recognition, as described previously (21). All the group IV animals with $Gag_{206-216}$-specific CTL induction also showed rapid selection of this CTL escape mutation at week 5. Analysis at week 3 found the GagL216S mutation dominant in two (II-2 and II-5) group II and two (IV-1 and IV-3) group IV animals (data not shown). However, animals in group III showed no *gag* mutations at week 5, except for one animal (III-5) selecting a mutation leading to an L-to-F substitution at the 216th residue. Later, at week 12, the $Gag_{206-216}$-specific CTL escape mutation, GagL216S, was selected even in group III animals. No animals showed mutations around the $Gag_{241-249}$ epitope-coding region even at week 12. These results indicate that selection of this $Gag_{206-216}$-specific CTL escape mutation may be accelerated by prophylactic vaccination inducing $Gag_{206-216}$-specific CTL responses. On the other hand, in group III animals with single $Gag_{241-249}$ epitope-specific CTL induction, selection of a $Gag_{206-216}$-specific CTL escape mutation was delayed but was observed before selection of a $Gag_{241-249}$-specific CTL escape mutation, suggesting strong selective pressure by delayed $Gag_{206-216}$-specific CTL responses after SIV challenge.

In order to see the effect of rapid selection of the $Gag_{206-216}$-specific CTL escape mutation on SIV control, we compared plasma viral loads at weeks 3 and 5 between groups II and IV (referred to as group II+IV) with rapid selection of the GagL216S

TABLE 2 Selection of a CTL escape mutation

Group	Macaque ID	Amino acid change for Gag residues[b]: 206–216 Wk 5	206–216 Wk 12	241–249 Wk 5	241–249 Wk 12
I	I-1	None	ND	None	ND
	I-2[a]	None	L216S	None	None
	I-3	None	L216S	None	None
	I-4	None	None	None	None
	I-5	None	None	None	None
	I-6	None	None	None	None
II	II-1[a]	L216S	ND	None	ND
	II-2[a]	L216S	ND	None	ND
	II-3[a]	L216S	ND	None	ND
	II-4[a]	L216S	ND	None	ND
	II-5[a]	L216S	ND	None	ND
III	III-1[a]	None	L216S	None	None
	III-2[a]	None	L216S	None	None
	III-3[a]	None	NA	None	NA
	III-4[a]	None	NA	None	NA
	III-5[a]	L216F	L216S	None	None
	III-6[a]	None	L216S	None	None
IV	IV-1[a]	L216S	L216S	None	None
	IV-2	L216S	L216S	None	None
	IV-3	L216S	L216S	None	None
	IV-4[a]	L216S	L216S	None	None
	IV-5[a]	L216S	NA	None	NA

[a] Animals that controlled SIV replication at week 12 (controllers).
[b] Plasma viral gag genome mutations were examined at weeks 5 and 12. Amino acid substitutions in $Gag_{206-216}$ and $Gag_{241-249}$ epitope regions are shown. L216S results in viral escape from $Gag_{206-216}$-specific CTL recognition. It remains undetermined whether L216F results in CTL escape. ND, not determined; NA, not determined because Gag fragments were unable to be amplified from plasma RNA.

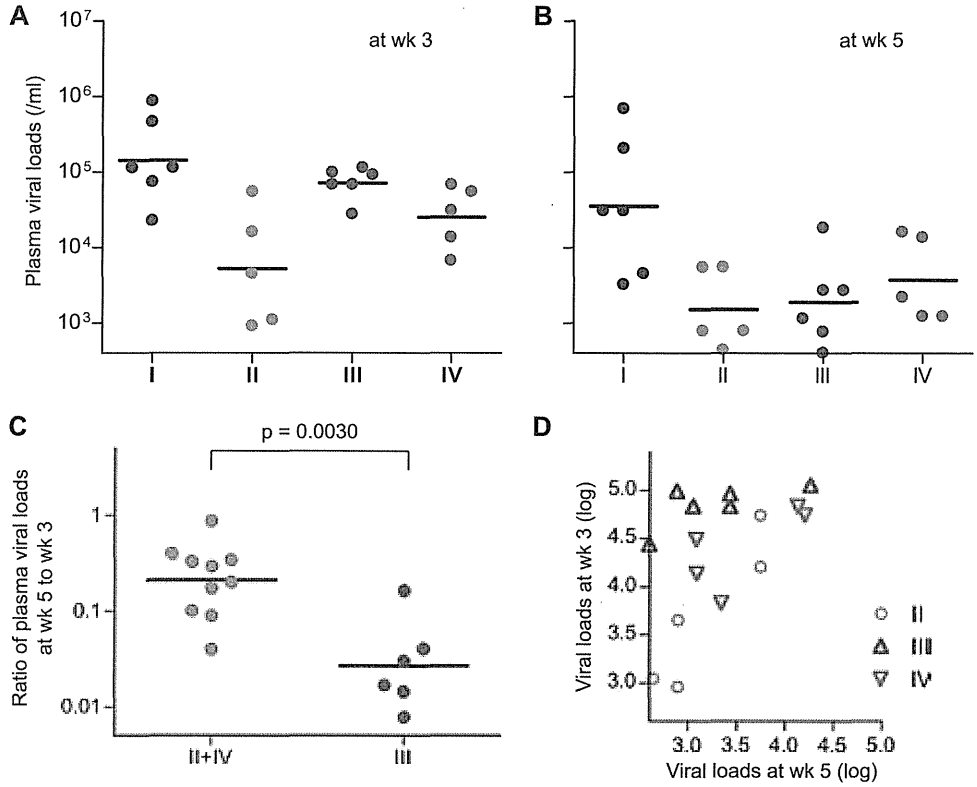

FIG 6 Comparison of plasma viral loads at weeks 3 and 5 among four groups. (A) Plasma viral loads at week 3 in group I, II, III, and IV animals. (B) Plasma viral loads at week 5 in group I, II, III, and IV animals. (C) Comparison of ratios of plasma viral loads at week 5 to week 3 in group II+IV animals and group III animals. The ratios in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test). The bars indicate the geometric mean of each group. (D) Scatter plots between plasma viral loads at weeks 3 and 5 in group II, III, and IV animals.

mutation and group III without the mutation at week 5 (Fig. 6). Ratios of plasma viral loads at week 5 to week 3 in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test) (Fig. 6C). To confirm this result, we examined the difference in week 3 viral loads between groups III and II+IV by ANCOVA, with week 5 viral loads as a covariate. This analysis revealed that week 3 viral loads controlled for by week 5 viral loads were significantly higher in group III than those in group II+IV (Fig. 6D and Table 3); i.e., the decline in viral loads from week 3 to week 5 was significantly sharper in group III than in group II+IV, possibly reflecting viral escape from suppressive pressure by $Gag_{206-216}$-specific CTL responses in the latter group during this period (from week 3 to week 5).

DISCUSSION

In the present study, we analyzed the impact of vaccination inducing single $Gag_{206-216}$ epitope-specific CTL memory on postchallenge CTL responses and SIV control in *90-120-Ia*-positive macaques and then compared the results with those of vaccination inducing single $Gag_{241-249}$ epitope-specific CTL responses. Our results indicate that these prophylactic vaccinations result in different patterns of $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL immunodominance and cooperation after SIVmac239 challenge.

Unvaccinated *90-120-Ia*-positive macaques (group I) showed both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after SIV challenge. In group IV animals with prophylactic induc-

TABLE 3 ANCOVA on week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV

ANOVA	Parameter	SS[a]	df[b]	MS[c]	*F*	*P* value
Homogeneity of slopes of regression	Group × slope	0.304	1	0.304	2.099	0.173
	Residual	1.735	12	0.145		
	Total	2.038	13	0.157		
Difference in week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV	Effect and group	1.106	1	1.106	7.052	0.020
	Residual	2.038	13	0.157		
	Total	3.144	14	0.225		

[a] SS, sum of squares.
[b] df, degrees of freedom.
[c] MS, mean squares.

tion of single $Gag_{206-216}$ epitope-specific CTL responses, $Gag_{206-216}$-specific CTL responses were induced dominantly but $Gag_{241-249}$-specific CTL responses were undetectable at week 2. In contrast, $Gag_{241-249}$-specific CTL responses were induced dominantly at week 2 in group III. Both groups showed $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 6. It may be difficult to compare these results with those in group II animals inducing whole Gag antigen-specific CTL and $CD4^+$ T-cell responses before challenge; the group II animals elicited $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 2. Our results indicate that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses and may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as nonvaccine antigens) after viral exposure.

A significant difference between groups III and IV is the pattern of selection of CTL escape mutation. All group IV animals showed rapid selection of a $Gag_{206-216}$-specific CTL escape mutation, while most group III animals showed no *gag* mutation at week 5 but selection of the $Gag_{206-216}$-specific CTL escape mutation later, at week 12. Thus, prophylactic vaccination may affect the patterns of viral genome diversification, possibly accelerating selection of CTL escape mutations. Interestingly, $Gag_{241-249}$-specific CTL mutations were not detected even at week 12 in group III animals, although a previous study observed not only the $Gag_{206-216}$-specific CTL escape mutation (GagL216S), but also a $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in the chronic phase of SIV infection in *90-120-Ia*-positive macaques (9). These results indicate that delayed, naive-derived $Gag_{206-216}$-specific CTL responses, as well as preceding $Gag_{241-249}$-specific CTL responses, exert strong suppressive pressure on SIV replication in group III animals, implying cooperation between vaccine antigen-specific and non-vaccine antigen-specific CTL responses for virus control.

Rapid selection of the $Gag_{206-216}$-specific CTL escape mutation (GagL216S) in group II and delayed selection of this mutation without a detectable $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in group III suggest that the virus with GagL216S (SIVmac239Gag216S) replicates more efficiently than the virus with GagD244E (SIVmac239Gag244E) under both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses. Our previous competition assay did not find a significant difference in viral fitness between these mutant viruses. Possibly, escape of SIVmac239Gag216S from $Gag_{206-216}$-specific CTL pressure may be more efficient than that of SIVmac239Gag244E from $Gag_{241-249}$-specific CTL pressure.

Our analysis revealed that the decline of plasma viral loads from week 3 to week 5 in group II+IV with rapid selection of the GagL216S mutation was significantly less than that in group III without the mutation at week 5, possibly reflecting viral escape from suppressive pressure by $Gag_{206-216}$-specific CTL responses in the former groups around weeks 3 to 5. Even the comparison between groups II and III, both showing dominant $Gag_{241-249}$-specific CTL responses at week 2, revealed a significantly sharper decline in the latter ($P = 0.0087$). Thus, our results suggest three patterns of $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL cooperation for virus control after SIVmac239 challenge. First, as observed in group II, dominantly induced $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses both work against wild-type SIV replication around week 2, but then a mutant virus escaping from the former CTL responses is selected, and the responses work against this mutant virus replication. Second, as observed in group III, dominantly induced $Gag_{241-249}$-specific CTL responses work against wild-type SIV replication around week 2 and then contribute to virus control, together with delayed, naive-derived $Gag_{206-216}$-specific CTL responses. Third, as observed in group IV, dominantly induced $Gag_{206-216}$-specific CTL responses work against wild-type SIV replication around week 2, but then a mutant virus escaping from $Gag_{206-216}$-specific CTL responses is selected, and delayed, naive-derived $Gag_{241-249}$-specific CTL responses instead work against this mutant virus replication. Viral loads at week 3 in group III looked higher than those in group IV, implying that $Gag_{206-216}$-specific CTL responses may exert a stronger suppressive effect on SIV replication in the acute phase than $Gag_{241-249}$-specific CTL responses. However, viral loads at week 5 in group III looked lower than those in group IV, and the comparison between the two groups showed significantly less decline in the latter ($P = 0.0303$). It is speculated that the third pattern observed in group IV is prone to failure in virus control. Indeed, two of five animals in group IV failed to control SIV replication. Even if vaccines are designed to express multiple antigens, of the vaccine-induced CTLs generated, only several epitope-specific cells may recognize the incoming HIV because of viral diversity and host MHC polymorphisms (18), and cooperation of these vaccine antigen-specific and non-vaccine antigen-specific CTL responses would be required for viral control. Thus, our results may imply a rationale of inducing escape-resistant, epitope-specific CTL memory by prophylactic AIDS vaccines.

In summary, this study showed dominant induction of vaccine antigen-specific CTL responses and delay in non-vaccine antigen-specific CTL responses in the acute phase of SIV infection, clearly describing the impact of prophylactic vaccination on CTL immunodominance and cooperation after virus exposure. Our results indicate that the patterns of cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses affect virus control and selection of CTL escape mutations. These findings provide great insights into antigen design in the development of a CTL-inducing AIDS vaccine.

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REFERENCES

1. **Altfeld M, et al.** 2003. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS 17:2581–2591.
2. **Berger CT, et al.** 2011. High functional avidity CTL responses to HLA-B-restricted Gag-derived epitopes associate with relative HIV control. J. Virol. 85:9334–9345.
3. **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.
4. **Goulder PJ, Watkins DI.** 2004. HIV and SIV CTL escape: implications for vaccine design. Nat. Rev. Immunol. 4:630–640.
5. **Goulder PJ, Watkins DI.** 2008. Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat. Rev. Immunol. 8:619–630.
6. **Hansen SG, et al.** 2009. Effector memory T cell responses are associated

with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nat. Med. 15:293–299.
7. **Julg B, et al.** 2010. Enhanced anti-HIV functional activity associated with Gag-specific CD8 T-cell responses. J. Virol. 84:5540–5549.
8. **Kaslow RA, et al.** 1996. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. Nat. Med. 2:405–411.
9. **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.
10. **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.
11. **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.
12. **Kiepiela P, et al.** 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat. Med. 13:46–53.
13. **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.
14. **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.
15. **Letvin NL, et al.** 2006. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. Science 312:1530–1533.
16. **Li F, et al.** 2011. Mapping HIV-1 vaccine induced T-cell responses: bias towards less-conserved regions and potential impact on vaccine efficacy in the Step study. PLoS One 6:e20479.
17. **Liu J, et al.** 2009. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. Nature 457:87–91.
18. **Liu Y, McNevin JP, Holte S, McElrath MJ, Mullins JI.** 2011. Dynamics of viral evolution and CTL responses in HIV-1 infection. PLoS One 6:e15639.
19. **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.
20. **Matano T, Kano M, Nakamura H, Takeda A, Nagai Y.** 2001. Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen. J. Virol. 75:11891–11896.
21. **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.
22. **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.
23. **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.
24. **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 [corrected] recognition. J. Virol. 83:2743–2755.
25. **Mothé 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.
26. **Naruse TK, et al.** 2010. Diversity of MHC class I genes in Burmese-origin rhesus macaques. Immunogenetics 62:601–611.
27. **Ndhlovu ZM, et al.** 2011. Mosaic HIV-1 Gag antigens can be processed and presented to human HIV-specific CD8+ T cells. J. Immunol. 186: 6914–6924.
28. **Schmitz JE, et al.** 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 283:857–860.
29. **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.
30. **Streeck H, et al.** 2009. Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells. J. Virol. 83:7641–7648.
31. **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.
32. **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.
33. **Turnbull EL, et al.** 2009. Kinetics of expansion of epitope-specific T cell responses during primary HIV-1 infection. J. Immunol. 182:7131–7145.
34. **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.
35. **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.
36. **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.