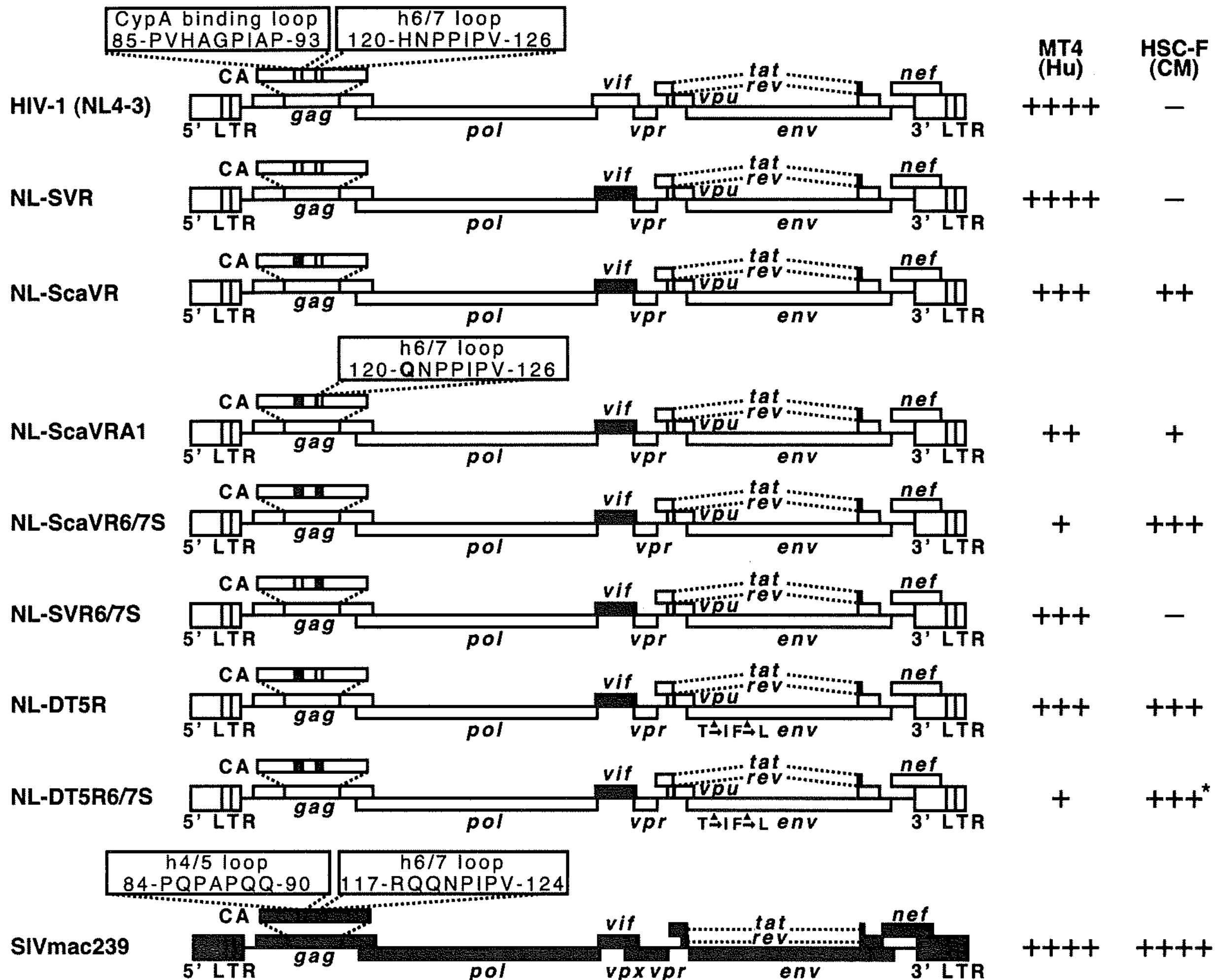

Figure 1

Structure of the chimeric HIV-1/SIVmac clones and a summary of their replication capabilities. White bars denote HIV-1 (NL4-3) and gray bars SIVmac239 sequences. ++++, +++, ++, +, and -denote the peak titer of virus growth in human (Hu) and cynomolgus monkey (CM) cells, respectively, to more than 1000 ng/ml, 100–1000 ng/ml, 10–100 ng/ml, 1–10 ng/ml, and less than 1 ng/ml concentration of capsid (CA) protein in the culture supernatants. * denotes that NL-DT5R6/7S replicated faster in HSC-F than did the parental NL-DT5R (see Fig. 2C).

ignated NL-ScaVR6/7S. The amount of RT per 1 ng of CA of NL-ScaVR (0.083 ng) was comparable to that of NL-ScaVR6/7S (0.081 ng), indicating that the replacement of L6/7 in HIV-1 with the corresponding loop of SIVmac did not affect the reactivity of CA antigen. Although NL-ScaVR6/7S grew slightly slower in MT4 cells, it could replicate more efficiently in HSC-F cells than the parental NL-ScaVR could (Fig. 2A). Similar results were obtained when we inoculated 20 ng of RT equivalent of NL-ScaVR or NL-ScaVR6/7S into HSC-F cells and measured the periodic RT production in culture supernatants (data not shown). These findings demonstrated that L6/7 CA of SIVmac improved the replication in CM cells of an HIV-1 derivative that already contained a SIVmac L4/5 and *vif*. We then generated NL-SVR6/7S, in which the L4/5 sequence was from HIV-1, but the L6/7 and *vif* came from SIVmac. NL-SVR6/7S showed better replication than NL-ScaVR6/7S in MT4 cells, but lost its replicative capability in HSC-F cells (Fig. 2B). NL-SVR, a virus with SIVmac *vif*, could replicate in MT4, but failed to do so in HSC-F (Fig. 2B). These results indicated that both L4/5 and L6/7 of SIVmac are required for efficient replication in HSC-F.

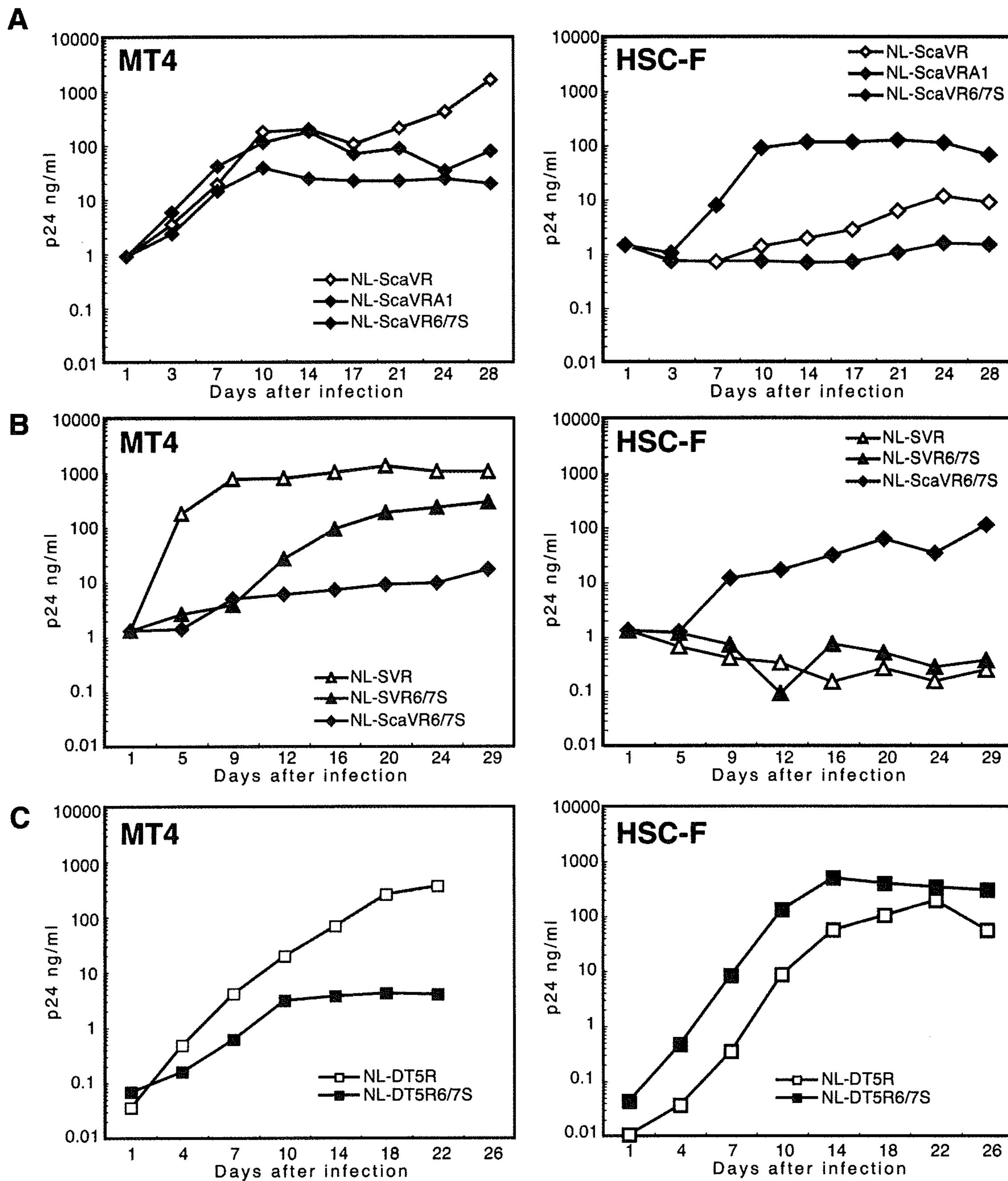

Figure 2
Replication properties of HIV-1 derivatives. Equal amounts of (A) NL-ScaVR (white diamonds: virus with SIVmac L4/5 and *vif*), and NL-ScaVRA1 (gray diamonds: virus with additional replacement of the 120th amino acid His with Gln in NL-ScaVR), and NL-ScaVR6/7S (black diamonds: virus with SIVmac L4/5, L6/7, and *vif*) (B) NL-SVR, NL-ScaVR6/7S, and NL-SVRS6/7S (gray diamonds: virus with SIVmac L6/7 and *vif*), and (C) NL-DT5R (white squares) and NL-DT5R6/7S (black squares), were inoculated into human MT4 or CM HSC-F cells, and culture supernatants were collected periodically. p24 antigen levels were measured by ELISA.

We then introduced SIVmac L6/7 into NL-DT5R, a molecularly cloned virus with two nonsynonymous changes in the *env* gene gained during long-term passages of NL-ScaVR in HSC-F cells [21]. The resultant virus was designated NL-DT5R6/7S. Although the peak titer of NL-DT5R6/7S was almost the same as that of NL-DT5R, NL-DT5R6/7S could replicate faster in HSC-F than the parental NL-DT5R (Fig. 2C). This finding confirmed that SIVmac L6/7 CA sequence improved the replication in CM cells of HIV-1 derivatives that contained SIVmac L4/5 and *vif*. The finding suggested that HIV-1 L6/7 and L4/5 CA sequences are important for intrinsic restriction in CM cells.

CypA incorporation into virus particles was not affected by replacement of HIV-1 L6/7 with that of SIVmac

Several studies have demonstrated that CypA augments HIV-1 infection in human cells [39], but inhibits its replication in OWM cells [18-20]. CypA was packaged in HIV-1 but not in SIVmac virus particles. To determine whether the replacement of HIV-1 L6/7 with that of SIVmac affects CypA binding of HIV-1 CA, we performed Western blot analysis of viral particles from HIV-1 derivatives. As shown in Fig. 3 (upper panel), CypA proteins were clearly detected in the NL-SVR particles (lane 1) but not in those of NL-ScaVR (lane 3), thus confirming that the L4/5 sequence of HIV-1 but not of SIVmac is required for CypA incorporation into viral particles. CypA proteins were detected in NL-SVR6/7S (lane 2) but not in NL-ScaVR6/7S (lane 4), indicating that the additional replacement of HIV-1 L6/7 with that of SIVmac had little effect on CypA incorporation. This finding suggests that the effect of L6/7 replacement on viral growth was independent from CypA binding of HIV-1 CA. When we used anti-p24 antibody (Fig. 3, lower panel), p55 Gag precursors and p24 proteins were clearly detected. There were no differences in the amount of p24 or the ratio of p24 to p55 among the four HIV-1 derivatives, indicating that the HIV-1 Gag precursor proteins with SIVmac L4/5 and L6/7 were processed normally by the viral protease.

Replacement of both L4/5 and L6/7 of HIV-1 CA with the corresponding loops from SIVmac impaired the CA binding activity of TRIM5α in Rh cells

It is known that the intrinsic restriction factors working against HIV-1 in CM and Rh cells can be saturated by inoculation of a high dose of HIV-1 particles [19,40-42]. To determine whether alteration in the CA of HIV-1 would affect its ability to saturate restriction factors, Rh LLC-MK2 cells were pre-treated with equal amounts of VSV-G pseudotyped HIV-1 particles that were with or without SIVmac L4/5 and/or L6/7 CA to saturate intrinsic restriction factor(s). The pre-treated cells were then infected with GFP-expressing HIV-1 carrying SIVmac L4/5 CA (HIV-1-L4/5S-GFP), since we wanted to exclude any effects of CypA on

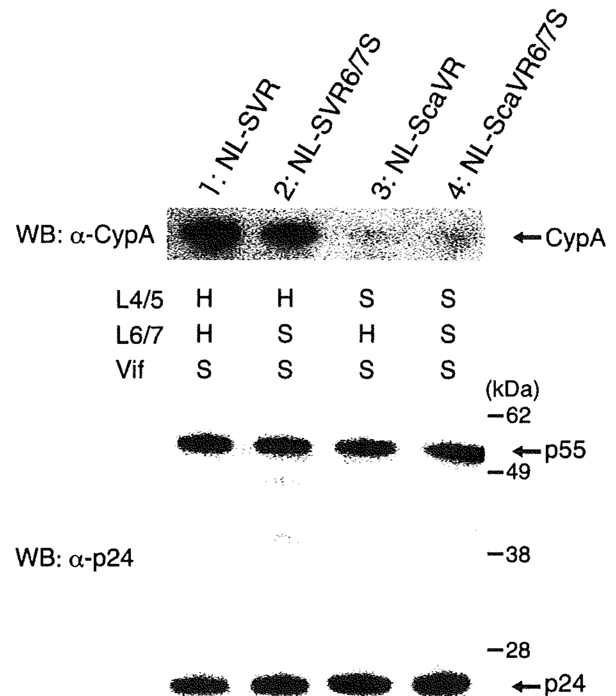

Figure 3
Western blot analysis of CA and CypA in particles of HIV-1 derivatives. The viral particles of NL-SVR (lane 1), NL-SVR6/7S (lane 2), NL-ScaVR (lane 3) and NL-ScaVR6/7S (lane 4) were purified by ultracentrifugation through a 20% sucrose cushion. CypA (upper panel) and p24 and p55 proteins (lower panel) were visualized by Western blotting (WB) using anti-CypA and anti-p24 antibody, respectively. "H" and "S" denote the amino acid sequences derived from HIV-1 and SIVmac, respectively.

the GFP expressing virus in LLC-MK2 cells. The susceptibility of particle-treated cells to virus infection was determined by the percentage of GFP-positive cells. The cells treated with the wild type (WT) particles showed greatly enhanced susceptibility to HIV-1 infection compared with non-treated cells (Fig. 4A, left), demonstrating that the intrinsic restriction factor(s) in LLC-MK2 cells were saturated by a high dose of particles. The cells treated with the particles carrying SIVmac L4/5 and those treated with particles carrying SIVmac L6/7 also showed enhanced susceptibility to HIV-1 infection (Fig. 4A, left). The cells treated with particles carrying both SIVmac L4/5 and L6/7 showed only slight enhancement of HIV-1 susceptibility (Fig. 4A, left; $p = 0.007$ compared by means of paired t test using all data points with the WT particle treated cells). Similarly, the cells treated with SIVmac particles showed only minor enhancement in HIV-1 susceptibility (Fig. 4A, left). Hamster TK-ts13 cells which lack TRIM5α expres-

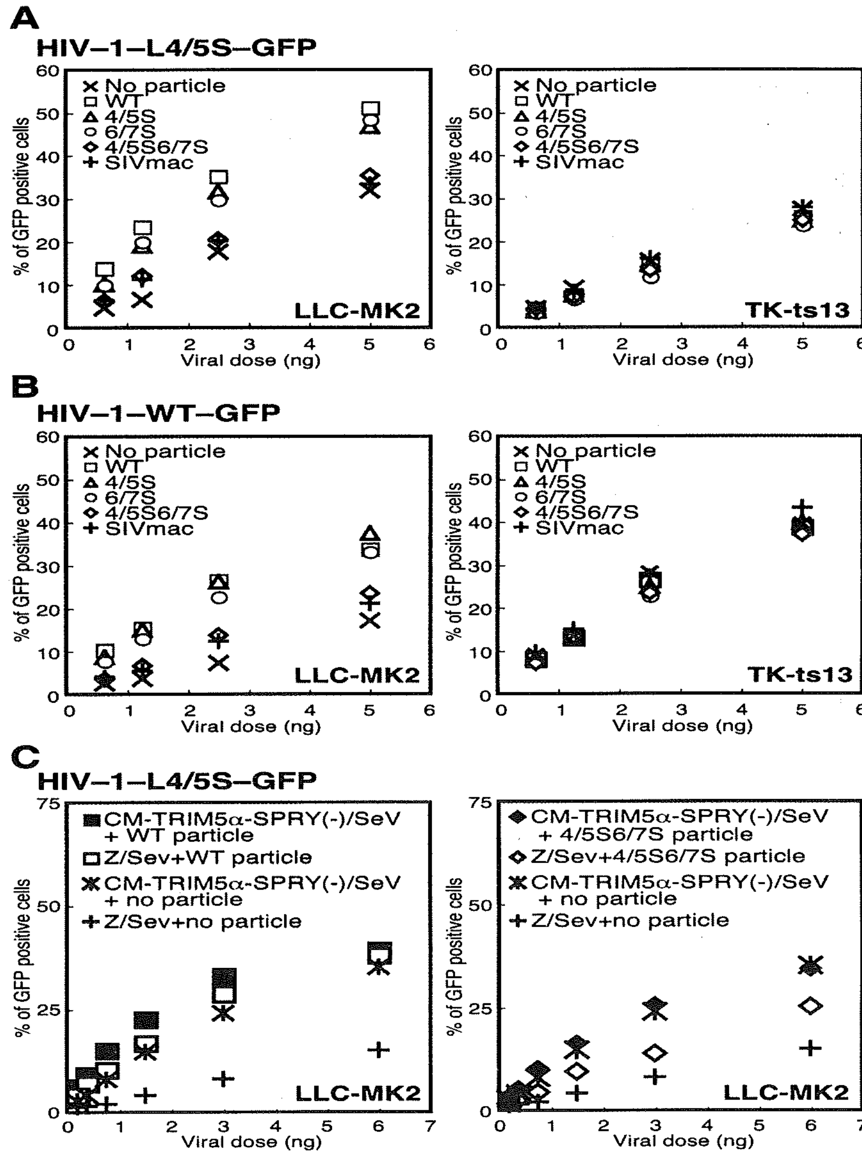

Figure 4
Saturation of intrinsic antiviral factors resulting from inoculation of high dose of virus particles. (A) Rhesus LLC-MK2 cells or hamster TK-ts13 cells were pre-treated with equal amounts of VSV-G pseudotyped particles with WT HIV-1 (white squares: Wt), with SIVmac L4/5 (white triangles: 4/5S), with SIVmac L6/7 (white circles: 6/7S), with SIVmac L4/5 and L6/7 (white diamonds: 4/5S6/7S), with SIVmac239 (pluses: SIVmac) or none (crosses) for 2 hours. The cells were then infected with the GFP expressing HIV-1 vector carrying SIVmac L4/5 (A: HIV-1-L4/5S-GFP) or GFP expressing HIV-1 vector with WT capsid (B: HIV-1-WT-GFP). Representative data of four independent experiments are shown. (C) Saturation activities were assessed in the presence or absence of functional TRIM5α. Before particle treatment, cells were infected with Sendai virus (SeV) expressing TRIM5 without the SPRY domain (black symbols), or an empty vector, parental Z strain of SeV (white symbols). Sixteen hours after SeV infection, cells were treated with particles for 2 hours and then infected with HIV-1-L4/5S-GFP. Representative data from six independent experiments are shown.

sion, on the other hand, showed no difference in HIV-1 susceptibility among cells treated with various HIV-1 derivatives or SIVmac particles (Fig. 4A, right). As shown in Fig. 4B, similar results were obtained when we used a GFP-expressing virus with WT HIV-1 capsid (HIV-1-WT-GFP). These results indicate that both HIV-1 L4/5 and L6/7 are important for CA binding to antiviral factor(s) in Rh cells. As described previously [20], HIV-1-WT-GFP could induce infection in only small numbers of LLC-MK2 cells. In contrast, more TK-ts13 cells were infected with HIV-1-WT-GFP than with HIV-1-L4/5-GFP. It is thus possible that CypA is a supporting factor for HIV-1 replication in hamster cells as well as in human cells.

Endogenous TRIM5α seems to be a likely candidate for the antiviral factor saturated by a high dose of HIV-1 particles (Fig. 4A and 4B). To confirm this, we assessed the ability of WT and mutant HIV-1 particles to saturate the intrinsic restriction factor in the presence or absence of functional TRIM5α. The dominant negative effect of an over-expressed TRIM5 mutant lacking SPRY domain [43] was used to suppress the function of cell endogenous TRIM5α. As shown in Fig. 4C, the infection of a recombinant SeV expressing TRIM5 without the SPRY domain caused marked enhancement of HIV-1-L4/5S-GFP virus infection without prior particle treatment (crosses vs. asterisks). This indicates that this dominant negative TRIM5 mutant successfully suppressed the restriction activity of endogenous TRIM5α. Treatment with the WT HIV-1 particles also saturated the restriction factors in the cells infected with the empty vector virus (parental Z strain of SeV), while the additional effect of the dominant negative mutant TRIM5α remained unclear (Fig. 4C left, white vs. black squares). These results suggest that the intrinsic factors saturated by the WT particles were mainly endogenous TRIM5α. In contrast to the effect of the WT particle treatment, the effect of the dominant negative TRIM5 mutant on HIV-1 infection was evident when we used particles with SIVmac L4/5 and L6/7 (Fig. 4C, right, white vs. black diamonds, p = 0.007, paired t test). These findings suggest that the diminished capability of particles with SIVmac L4/5 and L6/7 to saturate restriction factors was mainly due to their loss of interaction with TRIM5α. We, therefore, concluded that the ability of HIV-1 with SIVmac L4/5 and L6/7 to bind to TRIM5α is diminished in LLC-MK2 cells.

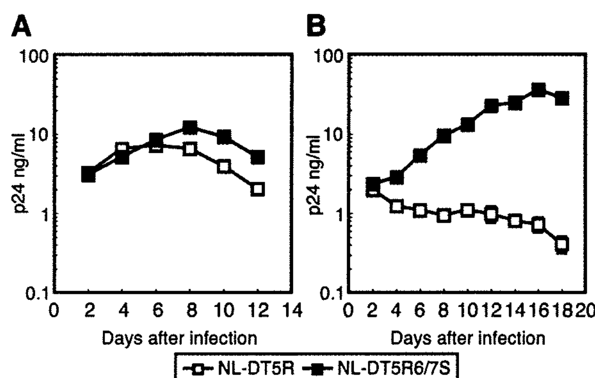

Figure 5
Replication capabilities of HIV-1 derivatives in peripheral blood mononuclear cells (PBMC) from CM. (A) PBMCs were obtained from CM, after which the CD8+ cells were removed, and the cells were stimulated with PHA-L for 1 day. (B) CD8-depleted CM PBMC were first stimulated with 1 μg/ml of PHA-L for 2 days and then with human IL2 100 U/ml for 2 more days. Equal amounts of p24 of NL-DT5R (white squares) or NL-DT5R6/7S (black squares) were inoculated, and the culture supernatants were collected periodically. p24 antigen levels were measured by ELISA. Values represent means with actual fluctuations of duplicate samples added. The values for mock infected cell culture supernatants were zero in the ELISA assay.

HIV-1 derivative with SIVmac L4/5, L6/7, and vif sequences can replicate efficiently in monkey primary cells

To verify the effect of additional replacement of HIV-1 L6/7 with that of SIVmac in primary CM cells, we prepared PBMCs from CM and removed CD8+ cells by means of magnetic beads. The cells were then stimulated for 1 day with 1 μg/ml of PHA-L. NL-DT5R6/7S showed more efficient replication than did the parental NL-DT5R in these cells and reached its peak titer 8 days after infection (Fig. 5A). For prolonged stimulation, CD8-depleted CM PBMCs were first stimulated with 1 μg/ml of PHA-L for 2 days and then with human IL2 100 U/ml for 2 more days. In these cells, NL-DT5R with HIV-1 L6/7 did not grow at all. On the other hand, NL-DT5R with SIVmac L6/7 (NL-DT5R6/7S) grew in CM primary cells in response to prolonged stimulation by PHA and IL-2 to reach titers, similar to those attained in cells with short stimulation, up to 8 days after infection (Fig. 5A and 5B). Furthermore, NL-DT5R6/7S continued to grow to much higher titers and reached its peak titer 16 days after infection; this higher peak may be due to better proliferation of these cells than those cells receiving short term stimulation (Fig. 5B). These results confirmed that the replicative capability of HIV-1 in CM cells was augmented by the additional replacement of L6/7 of CA with the corresponding sequence from SIVmac.

Discussion

We created simian-tropic HIV-1 with more efficient replication capability in CM cells using the knowledge obtained from our previous study of TRIM5α and HIV-2 capsid sequence variations [32] Introduction of the entire SIVmac L6/7 CA into the previously constructed version of HIV-1 derivatives containing SIVmac L4/5 CA and *vif* [21] caused only a four amino acid change in CA but

showed improved replication capability of HIV-1 in the CM cell line HSC-F. Introduction of the entire SIVmac L6/7 CA into NL-DT5R, which has two additional amino acid mutations in the *env* gene, enhanced replication in CD8+ cells-depleted CM PBMCs. After prolonged stimulation of CM PBMCs, replication of the original version of NL-DT5R was suppressed while that of NL-DT5R with SIVmac L6/7 was not. It would thus be of interest to test whether those HIV-1 derivatives with both L4/5 andL6/7 from SIVmac can induce infection of CM *in vivo*.

While the high-dose inoculation of WT HIV-1 particles into Rh cells saturated endogenous TRIM5α and enhanced subsequent infection with HIV-1, the introduction of HIV-1 particles that contained both L4/5 and L6/7 from SIVmac greatly impaired the ability of the particles to saturate TRIM5α. When we replaced either HIV-1 L4/5 or L6/7 with the corresponding sequence from SIVmac, these particles still saturated TRIM5α. These findings suggest that TRIM5α recognized the overall structure composed of both L4/5 and L6/7 of HIV-1 CA. Our previous results from computational 3D-structure modeling analysis of HIV-2 CA support this hypothesis [32]. The 120th amino acid of HIV-2 CA, which affects viral susceptibility to TRIM5α restriction, was located in L6/7. It is especially worth noting that the amino acid substitution at the 120th position was previously predicted to induce marked changes in the configuration of L6/7 and the L6/7 with the CM TRIM5α-sensitive Pro positioned most closely to L4/5 of HIV-2 [32]. It would, therefore, be interesting to investigate whether monkey TRIM5α proteins recognize CypA bound-L4/5 of HIV-1 CA.

During the preparation of our manuscript, Lin and Emerman reported that SIVagmTAN with both HIV-1 L4/5 and L6/7 was susceptible to Rh-TRIM5α restriction [44]. Our result is consistent with their finding, since the HIV-1 particles with both SIVmac L6/7 and SIVmac L4/5 showed reduced saturation activity for TRIM5α in Rh cells compared with HIV-1 particles with SIVmac L4/5 alone. Hatziioannou et al. very recently reported that stHIV-1 strains, which differ from HIV-1 only in the *vif* gene, could efficiently replicate in pig-tailed monkey and proposed a pig-tail monkey model of HIV-1 infection [45]. This is not surprising, since pig-tailed monkeys lack a TRIM5α protein, and the dominant form of TRIM5 expressed in this monkey species is a TRIMCyp fusion protein lacking anti-HIV-1 activity [46-48].

When we subjected CD8-depleted CM PBMC to prolonged stimulation, NL-DT5R6/7S grew efficiently but NL-DT5R did not. Since the expression levels of TRIM5α mRNA in human PBMC increased after stimulation with PHA and IL2 for 3 days (data not shown), we speculated that the higher expression levels of CM-TRIM5α in fully stimulated CM cells resulted in efficient restriction of NL-DT5R. However, no clear enhancement of CM TRIM5α mRNA expression could be detected in the CM cells subjected to prolonged stimulation (data not shown). The reason why NL-DT5R failed to grow in CM cells with prolonged stimulation is not yet clear, but it is possible that fully stimulated CM cells exerted stronger intrinsic inhibitory activity against HIV-1 infection than those with short-term stimulation.

NL-DT5R6/7S and NL-ScaVR6/7S replicated less efficiently in human MT4 cells than did the parental NL-DT5R and NL-ScaVR. One possible explanation is that the virus with SIVmac L6/7 became resistant to CM TRIM5α but became more sensitive to human TRIM5α, since the latter can restrict SIVmac more efficiently than HIV-1. Another possibility is that replacement of CA allowed the virus to evade the intrinsic inhibitory factors in CM cells but impaired viral replication *per se*.

We used the CM T cell line HSC-F and CD8+ cell-depleted PBMC from CM but not from Rh for our replication experiments. Although we observed an improvement of viral replication in CM cells, we cannot assume that the replacement of L4/5 and L6/7 is enough for HIV-1 to replicate to high titers in Rh cells since the CM TRIM5α resistant HIV-2 mutant virus GH123 (Q) was found to be restricted by Rh TRIM5α [34]. NL-DT5R6/7S and NL-ScaVR6/7S also showed less efficient replication capability than did SIVmac (Fig. 1). We are currently trying to adapt these viruses to CM and Rh cells by means of long-term passaging in the hope of introducing compensating mutations that can overcome these disadvantages and further augment their replicative capabilities in human and simian cells to reach a similar level as seen with SIVmac.

Conclusion

We have succeeded in improving simian-tropic HIV-1 for more efficient replication in CM cells by introduction of the SIVmac L6/7 CA sequence. It will be of interest to determine whether the HIV-1 derivatives with SIVmac L4/5 and L6/7 can induce infection in cynomolgus monkeys *in vivo*. Even if they fail to do so, further modification and/or adaptation of the current version of simian-tropic HIV-1 in monkey cells might be expected to lead to the development of an HIV-1 infection model in OWMs. This model has been long-awaited as a tool for vaccine development and as a model for better understanding of AIDS pathogenesis.

Abbreviations

OWM: old world monkey; CM: cynomolgus monkey; Rh: rhesus monkey; SHIV: HIV-1/SIV chimeric virus; CypA: cyclophilin A; TRIM: tripartite motif; CA: capsid; PBMC: peripheral blood mononuclear cell; GFP: green fluores-

cence protein; VSV-G: vesicular stomatitis virus glycoprotein; SeV: Sendai virus; L4/5: a loop between α-helices 4 and 5; L6/7: a loop between α-helices 6 and 7.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TS and EEN designed the research, AK, AS, YS, and EEN performed the research, TS, MN, AA, and EEN analyzed the data, and AA, HA, TS, and EEN wrote the paper.

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Transmission of Simian Immunodeficiency Virus Carrying Multiple Cytotoxic T-Lymphocyte Escape Mutations with Diminished Replicative Ability Can Result in AIDS Progression in Rhesus Macaques[▽]

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Cytotoxic T-lymphocyte (CTL) responses frequently select for immunodeficiency virus mutations that result in escape from CTL recognition with viral fitness costs. The replication in vivo of such viruses carrying not single but multiple escape mutations in the absence of the CTL pressure has remained undetermined. Here, we have examined the replication of simian immunodeficiency virus (SIV) with five *gag* mutations selected in a macaque possessing the major histocompatibility complex haplotype *90-120-Ia* after its transmission into *90-120-Ia*-negative macaques. Our results showed that even such a "crippled" SIV infection can result in persistent viral replication, multiple reversions, and AIDS progression.

Virus-specific CD8$^+$ cytotoxic T-lymphocyte (CTL) responses exert a suppressive effect on human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication (1, 10, 15, 21, 27). Under the CTL pressure, viral mutations resulting in viral escape from CTL recognition are frequently selected for, with viral fitness costs (2, 5, 8, 9, 12, 16, 19, 20, 24, 25, 26, 28). The transmission of the virus carrying a CTL escape mutation with lower viral fitness between major histocompatibility complex class I (MHC-I)-mismatched individuals can result in reversion of the mutation due to the absence of the CTL pressure (7, 14, 16, 17, 18). Such CTL escape mutations and their reversions have been suggested to be involved in viral evolution (3, 11, 13, 23).

We have developed a prophylactic vaccine using a Sendai virus vector expressing SIVmac239 Gag and shown its protective efficacy against SIVmac239 challenge in a group of Burmese rhesus macaques (*Macaca mulatta*) possessing MHC-I haplotype *90-120-Ia* (20). In these vaccinated macaques that are controlling SIVmac239 replication, $Gag_{206-216}$ epitope-specific CTL responses exerted strong selective pressure on the virus, and rapid selection of a mutant escaping from this CTL was observed at week 5 postchallenge. The virus, SIVmac239Gag216S, with this CTL escape mutation, GagL216S, leading to a substitution from leucine (L) to serine (S) at amino acid (aa) 216 in Gag showed lower replicative ability than the wild type (14, 20). Two of these vaccinees (macaques V3 and V5) showed an accumulation of additional viral CTL escape mutations in *gag* during the period of viral control and then the reappearance of plasma viremia around week 60 after SIVmac239 challenge (12). The SIV carrying these multiple CTL escape mutations showed lower replicative ability in vitro than the SIV carrying the single GagL216S mutation.

How such viruses with multiple CTL escape mutations replicate and evolve in the absence of the CTL pressure has not yet been well determined, while the reversion of CTL escape mutations has previously been shown by the transmission of viruses with single escape mutations (7, 14, 18). In the present study, we have examined the replication, in the absence of the CTL pressure in *90-120-Ia*-negative macaques, of the SIV with multiple *gag* CTL escape mutations that were accumulated in a *90-120-Ia*-positive macaque.

The induction of $Gag_{206-216}$-specific CTL, $Gag_{241-249}$-specific CTL, and $Gag_{373-380}$-specific CTL responses has previously been observed after SIVmac239 challenge in *90-120-Ia*-positive macaques (12). The *90-120-Ia*-positive vaccinees V5 and V3 showed rapid selection of the GagL216S mutation ($Gag_{206-216}$ CTL escape mutation) and then of an additional two mutations resulting in escape from $Gag_{241-249}$-specific CTL and $Gag_{373-380}$-specific CTL recognition, respectively, during the period of viral control. These were a $Gag_{241-249}$ CTL escape mutation leading to a GagD244E (aspartic acid [D] to glutamic acid [E] at aa 244 in Gag) substitution and a $Gag_{373-380}$ CTL escape mutation leading to GagA373T (alanine [A] to threonine [T] at aa 373) in vaccinee V5 or GagV375A (valine [V] to A at aa 375) or GagP376S (proline [P] to S at aa 376) in vaccinee V3. Viruses at the reappearance of viremia had one or two additional mutations in *gag*, GagI247L (isoleucine [I] to L at aa 247) and GagA312V (A to V at aa

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312) in vaccinee V5 or GagP172S (P to S at aa 172) or GagV145A (V to A at aa 145) in vaccinee V3. All of these mutations except for the $Gag_{373-380}$ CTL escape mutations resulted in amino acid changes in the Gag CA. We constructed molecular clones of SIVs with these *gag* mutations (12). The SIVs with three CTL escape mutations ($Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ CTL escape mutations) were referred to as group Q SIV mutants, and the SIVs with four or five *gag* mutations selected at the reappearance of viremia as group R SIV mutants. These group Q and R SIV mutants both showed lower replicative ability in vitro than SIVmac239Gag216S, while in the competition assay between groups Q and R, the viral replicative ability was not significantly affected by the GagP172S or GagV145A mutation but was reduced by the addition of the GagI247L and GagA312V mutations (12). These results do not support the possibility of compensation for loss of viral fitness from these mutations (4, 6). In the present study, we have examined the in vivo replication of the SIV carrying five *gag* mutations, GagL216S, GagD244E, GagI247L, GagA312V, and GagA373T, selected in macaque V5 at the reappearance of viremia, which was assumed to show the lowest replicative ability among group Q and R SIV mutants. The macaques were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (22).

We first compared the in vivo replication abilities of the SIV with a single GagL216S mutation and the SIVs with multiple CTL escape mutations in *90-120-Ia*-negative macaques (Fig. 1). In the competition between SIVmac239Gag216S and group Q SIV mutants, macaque R02-017 was coinoculated intramuscularly with molecular-clone DNAs of SIVmac239Gag216S and SIVmac239Gag216S244E373T and macaque R05-002 with molecular-clone DNAs of SIVmac239Gag216S and all three group Q SIV mutants. The results of the analysis of plasma viral *gag* genome sequences (Fig. 2) showed selection of SIV mac239Gag216S; i.e., all the mutations other than GagL216S became undetectable in 3 weeks postinoculation, indicating lower replicative abilities in vivo of group Q SIV mutants than of SIVmac239Gag216S, as indicated previously by in vitro competition (12). Further analysis revealed reversion of the selected GagL216S mutation to the wild-type sequence in a few months.

In the competition between SIVmac239Gag216S and group R SIV mutants, macaque R02-023, coinoculated with molecular clone DNAs of SIVmac239Gag216S and SIVmac239Gag 216S244E247L312V373T, showed selection of the former (Fig. 2). This macaque was euthanized at week 6 before exhibiting reversion of the GagL216S mutation. In macaque R02-022, coinoculated with molecular clone DNAs of SIVmac239Gag 216S and all three group R SIV mutants, almost all mutations other than GagL216S became undetectable rapidly but the GagV145A mutation was detected even at week 14. The GagL216S mutation was still dominant without reversion at week 14, and plasma viremia became undetectable after week 14 in this macaque. Both cases indicated a lower replicative ability in vivo of SIVmac239Gag216S244E247L312V373T than of SIVmac239Gag216S.

Additionally, macaque R03-022, coinoculated with the molecular-clone DNAs of SIVmac239Gag216S244E373T and SIV mac239Gag216S244E247L312V373T, showed selection of the

▲ R02-017 SIVmac239Gag216S
SIVmac239Gag216S244E373T (Q1)
▼ R05-002 SIVmac239Gag216S
SIVmac239Gag216S244E373T (Q1)
SIVmac239Gag216S244E375A (Q2)
SIVmac239Gag216S244E376S (Q3)
△ R02-023 SIVmac239Gag216S
SIVmac239Gag216S244E247L312V373T (R1)
▽ R02-022 SIVmac239Gag216S
SIVmac239Gag216S244E247L312V373T (R1)
SIVmac239Gag172S216S244E375A (R2)
SIVmac239Gag145A216S244E376S (R3)
○ R03-022 SIVmac239Gag216S244E373T (Q1)
SIVmac239Gag216S244E247L312V373T (R1)

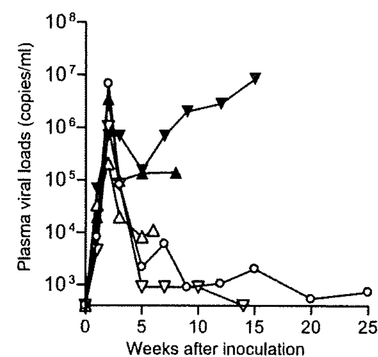

FIG. 1. Plasma viral loads of macaques used for in vivo competition assay (SIV *gag* RNA copies/ml plasma) after inoculation with SIV molecular-clone DNAs. Animals received 10 mg in total of DNAs consisting of an equal amount of each DNA; i.e., macaques R02-017, R02-023, and R03-022 were inoculated with 5 mg of each DNA, and macaques R05-002 and R02-022 with 2.5 mg of each DNA. Plasma viral loads were determined as described previously (20).

former (Fig. 2), indicating a lower replicative ability in vivo of SIVmac239Gag216S244E247L312V373T than of SIVmac239 Gag216S244E373T. In this macaque, reversion of the GagL216S mutation was observed in 6 months, while the GagD244E and GagA373T mutations were still dominant without reversion.

Next, we inoculated *90-120-Ia*-negative macaques with the SIV carrying multiple *gag* CTL escape mutations that was selected in *90-120-Ia*-positive macaque V5 (Fig. 3). The SIV carrying five gag mutations, GagL216S, GagD244E, GagI247L, GagA312V, and GagA373T, that was dominant at the reappearance of viremia in macaque V5, was propagated on rhesus macaque peripheral blood mononuclear cells to prepare the SIVmac239Gag216S244E247L312V373T challenge stock for macaques R05-001 and R06-016. Sequencing analysis confirmed no *gag* mutation except for the five mutations in the challenge virus. These two macaques were challenged intravenously with 1,000 50% tissue culture infective dose of SIVmac 239Gag216S244E247L312V373T. Both of them showed persistent viremia, although the levels of set-point plasma viral loads were low in macaque R06-016. Macaque R05-001, maintaining high viral loads, showed typical signs of AIDS, such as a reduction in peripheral $CD4^+$ T-cell counts, diarrhea, and general weakness, and was euthanized approximately 2 years postchallenge. Autopsy revealed postpersistent generalized

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Macaque R02-017 inoculated with molecular clones of
SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 373T

Wks p-c	aa sequences in Gag		
	216th	244th	373rd
1	Mt	**Wt**(mt)	**Wt**(mt)
3	Mt	**Wt**	**Wt**
8	**Wt**	**Wt**	**Wt**

Macaque R05-002 inoculated with molecular clones of
SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 373T
& SIVmac239 Gag 216S 244E 375A & SIVmac239 Gag 216S 244E 376S

Wks p-c	aa sequences in Gag				
	216th	244th	373rd	375th	376th
1	Mt	wt/mt	**Wt**(mt)	**Wt**(mt)	**Wt**
3	Mt	**Wt**	**Wt**	**Wt**	**Wt**
12	**Wt**	**Wt**	**Wt**	**Wt**	**Wt**

Macaque R02-023 inoculated with molecular clones of
SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 247L 312V 373T

Wks p-c	aa sequences in Gag				
	216th	244th	247th	312th	373rd
1	Mt	**Wt**	**Wt**	**Wt**	**Wt**(mt)
3	Mt	**Wt**	**Wt**	**Wt**	**Wt**(mt)
6	Mt	**Wt**	**Wt**	**Wt**	**Wt**

Macaque R02-022 inoculated with molecular clones of
SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 247L 312V 373T
& SIVmac239 Gag 172S 216S 244E 375A & SIVmac239 Gag 145A 216S 244E 376S

Wks p-c	aa sequences in Gag								
	145th	172nd	**216th**	244th	247th	312th	373rd	375th	376th
1	**Wt**(mt)	wt/mt	Mt	wt/mt	**Wt**(mt)	**Wt**(mt)	**Wt**	wt/mt	**Wt**(mt)
3	wt/mt	**Wt**	Mt	wt/mt	**Wt**	**Wt**	**Wt**	**Wt**(mt)	wt/mt
14	wt/mt	**Wt**	Mt	**Wt**	**Wt**	**Wt**	**Wt**	**Wt**	**Wt**

Macaque R03-022 inoculated with molecular clones of
SIVmac239 Gag 216S 244E 373T & SIVmac239 Gag 216S 244E 247L 312V 373T

Wks p-c	aa sequences in Gag				
	216th	**244th**	247th	312th	**373rd**
1	Mt	Mt	**Wt**	**Wt**	Mt
3	Mt	Mt	**Wt**	**Wt**	Mt
20	wt/mt	Mt	**Wt**	**Wt**	Mt
25	**Wt**	Mt	**Wt**	**Wt**	Mt

FIG. 2. Dominant viral genome sequences in competition assay. A *gag* DNA fragment was amplified from plasma RNA by reverse transcription and nested PCR and sequenced as described previously (20). The amino acid sequences at the positions where mutations were included in the inoculums are shown. Q and R groups of SIV mutants are described in the text. **Wt**, only the wild-type sequence was detected; **Wt**(mt), the wild-type sequence was dominant but the mutant was detectable (the mutant/wild-type ratio was less than 1/4); wt/mt, the wild type and the mutant were detected equally; Mt(wt), the mutant was dominant but the wild type was detectable (the wild-type/mutant ratio was less than 1/4); Mt, only the mutant was detected. Other than the residues indicated in this figure, no dominant mutation resulting in an amino acid change was detected in the *gag* region in macaque R02-017, R05-002, R02-023, or R02-022, but macaque R03-022 showed one amino acid change resulting in a GagV375M substitution at weeks 20 and 25. p-c, postchallenge.

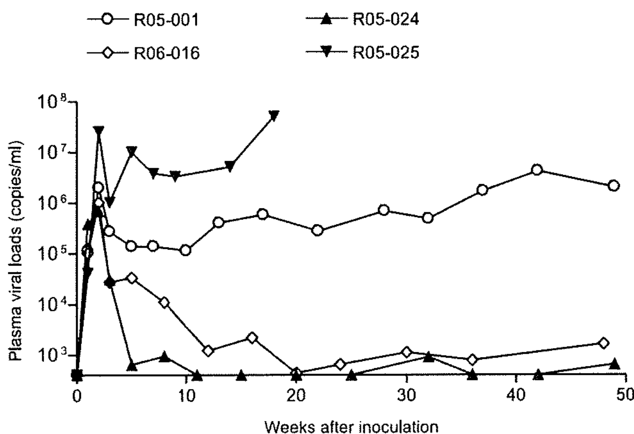

FIG. 3. Plasma viral loads (SIV *gag* RNA copies/ml plasma) in macaques after challenge with SIV carrying five *gag* mutations.

lymphadenopathy conditions and pneumocystis pneumonia. This macaque showed reversion of the GagD244E mutation in a few months, followed by reversion of the GagL216S, GagI247 L, and GagA312V mutations in a year postchallenge, while the GagA373T mutation remained dominant without reversion until euthanasia (Fig. 4). In contrast, macaque R06-016, with lower viral loads, showed no reversion of the five mutations. In the chronic phase, these two macaques showed additional Gag amino acid changes, including GagI140V (I to V at aa 140) and GagV375M (V to methionine [M] at aa 375) that were detected in both. Some of these mutations may contribute to the recovery of viral fitness.

To see the possibility of transmission of the viruses carrying the five gag mutations in the context of the polyclonal, V5-derived SIVs, macaques R05-024 and R05-025 were inoculated with plasma obtained from macaque V5 in the chronic phase of SIVmac239 infection (Fig. 3). For the challenge, plasma was obtained from macaque V5 at weeks 81, 87, 92, 100, and 113

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Macaque R05-001 infected with SIVmac239Gag216S244E247L312V373T

Wks	aa sequences in Gag					
p-c	**216th**	**244th**	**247th**	**312th**	**373rd**	other residues
1	Mt	Mt	Mt	Mt	Mt	
3	Mt	Mt	Mt	Mt	Mt	
10	Mt	**Wt**	Mt	Mt	Mt	I140V
22	wt/mt	**Wt**	Mt	Mt	Mt	I140V
37	wt/mt	**Wt**	Mt	Mt	Mt	V3A, I140V
42	**Wt**(mt)	**Wt**	Mt	Mt	Mt	V3A, (V68L/M), I140V
49	**Wt**	**Wt**	wt/mt	wt/mt	Mt	V3A, (V68L/M), I140V
55	**Wt**	**Wt**	**Wt**	**Wt**	Mt	V3A, (V68L/M), I140V, (V340M), D429N
86	**Wt**	**Wt**	**Wt**	**Wt**	Mt	V3A, (V68L/M), I140V, D429N
110	**Wt**	**Wt**	**Wt**	**Wt**	Mt	V3A, (V68L/M), (I140V), V375M, (D429E)

Macaque R06-016 infected with SIVmac239Gag216S244E247L312V373T

Wks	aa sequences in Gag					
p-c	**216th**	**244th**	**247th**	**312th**	**373rd**	other residues
5	Mt	Mt	Mt	Mt	Mt	
12	Mt	Mt	Mt	Mt	Mt	
30	Mt	Mt	Mt	Mt	Mt	S128P, I140V, V375M
54	Mt	Mt	Mt	Mt	Mt	I140V, V375M

Macaque R05-024 infected with V5-plasma

Wks	aa sequences in Gag										
p-c	3rd	68th	145th	**216th**	**244th**	**247th**	**312th**	**373rd**	390th	404th	other residues
1	Mt(wt)	wt/mt	Mt	Mt	Mt	Mt	Mt	Mt	wt/mt	wt/mt	
5	Mt(wt)	wt/mt	Mt	Mt	Mt	Mt	Mt	Mt	wt/mt	wt/mt	I257K
49	Mt	Mt	Mt	Mt	Mt	**Wt**	**Wt**	**Wt**	**Wt**	Mt	A222V, I257K, R485K

Macaque R05-025 infected with V5-plasma

Wks	aa sequences in Gag										
p-c	3rd	68th	145th	**216th**	**244th**	**247th**	**312th**	**373rd**	390th	404th	other residues
1	Mt	wt/mt	Mt	Mt	Mt	Mt	Mt	Mt	wt/mt	wt/mt	
5	Mt	wt/mt	Mt	Mt	Mt	**Wt**(mt)	**Wt**(mt)	Mt	wt/mt	wt/mt	
7	Mt	wt/mt	Mt	Mt	Mt	**Wt**	**Wt**	Mt	wt/mt	wt/mt	
14	Mt	wt/mt	Mt	Mt	Mt	**Wt**	**Wt**	Mt	wt/mt	wt/mt	
18	Mt	**Wt**(mt)	Mt	Mt	Mt	**Wt**	**Wt**	Mt	wt/mt	wt/mt	

FIG. 4. Dominant viral genome sequences after challenge with SIV carrying five *gag* mutations. The amino acid sequences at the residues where mutations were included in the inoculums and dominant amino acid changes at other residues in *gag* are shown. In the column of other residues, the predominant mutations with detectable wild-type sequence are shown in parentheses. **Wt**, **Wt**(mt), wt/mt, Mt(wt), Mt, and p-c are defined in the Fig. 2 legend.

post-SIVmac239 challenge and 0.2 ml of each was intravenously inoculated into these two macaques. In the challenge SIV plasma, the five *gag* mutations (GagL216S, GagD244E, GagI247L, GagA312V, and GagA373T) and GagV145A were dominant, and additional *gag* mutations were detected in the MA- and NC-coding regions. In macaque R05-024, exhibiting low viral loads, the SIV GagL216S and GagD244E mutations remained dominant, while reversion of the GagI247L, GagA312V, and GagA373T mutations was observed (Fig. 4). Macaque R05-025, exhibiting high viral loads, developed AIDS and was euthanized at week 18 postchallenge. Autopsy revealed lymphoatrophy and cytomegalovirus infection. This macaque showed rapid reversion of the SIV GagI247L and GagA312V mutations but maintained the GagL216S, GagD 244E, and GagA373T mutations until euthanasia.

In samples from these four macaques challenged with SIV mac239Gag216S244E247L312V373T or V5-derived plasma, we examined the virus-specific $CD8^+$ T-cell responses around 3 months postinfection by flow cytometric analysis of antigen-specific gamma interferon induction (data not shown) as described previously (14, 20). Analyses using vesicular stomatitis virus G-pseudotyped SIV-infected cells as a stimulator revealed SIV-specific $CD8^+$ T-cell responses in macaques R05-001, R06-016, and R05-024, but not in macaque R05-025, which may have contributed to the rapid AIDS progression in this animal. Macaque R05-024, exhibiting lower viral loads and rapid selection of a *gag* mutation resulting in an I257K (I to lysine [K] at aa 257) substitution, showed $CD8^+$ T-cell responses specific for the $Gag_{245-269}$ peptide mixture (a mixture of $Gag_{245-260}$, $Gag_{250-265}$, and $Gag_{255-269}$ peptides), suggesting a possibility of this mutation for viral escape from strong CTL pressure. None of these four macaques showed $CD8^+$ T-cell responses specific for the $Gag_{206-225}$ (a mixture of $Gag_{206-220}$ and $Gag_{210-225}$ peptides), $Gag_{206-225}$.216S ($Gag_{206-220}$.216S and $Gag_{210-225}$.216S), $Gag_{232-255}$ ($Gag_{232-246}$, $Gag_{236-250}$, and $Gag_{240-255}$), $Gag_{232-255}$.244E, $Gag_{236-255}$.244E247L, $Gag_{362-385}$ ($Gag_{362-377}$, $Gag_{367-381}$, and $Gag_{371-385}$), or $Gag_{362-385}$.373T peptide mixture, indicating that CTL responses were not involved in the reversion or nonreversion at residue 216, 244, 247, or 373 in these macaques.

The in vivo competition assay in the present study showed loss of viral fitness from the addition of the GagD244E and GagA373T mutations into SIVmac239Gag216S and further loss of viral fitness from additional GagI247L and GagA312V mutations. The reversion of GagD244E in macaque R05-001, GagA373T in macaque R05-024, and GagI247L and GagA312V in macaques R05-024 and R05-025 (Fig. 4) supports this notion. However, reversion was not observed in all the mutations after challenge with SIV carrying the five *gag* mutations. Challenge with SIVmac239Gag216S carrying the single GagL216S mutation has shown its reversion in 3 months (14), whereas the reversion of the GagL216S mutation was

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delayed or not observed after challenge with the SIV carrying five *gag* mutations. This may be due to the predominant selection of the reversion of other mutations or to lower viral replication efficiency in the latter case. Compensatory mutations can also be involved in this delay or nonreversion, but no additional *gag* mutation was observed in the early phase in macaque R06-016. The possibility of a contribution to this delay by GagI140V in macaque R05-001 and GagV145A in macaques R05-024 and R05-025 may be considered, while significant recovery of viral fitness by the latter mutation has not been observed (12).

It has been suggested that a reduction in viral fitness by CTL escape mutations may contribute to HIV/SIV control (19, 20, 28). Pressure by multiple epitope-specific CTLs may result in the selection of HIV/SIV with diminished replicative ability because of accumulating multiple escape mutations. The inefficient viral replication in macaques R02-022 and R03-022 (Fig. 1) and two of four macaques in the second experiment (Fig. 3) may reflect such a lower replicative ability of the mutant SIVs, but conversely, the results of the present study also showed efficient viral replication in macaques R05-001 and R05-025, indicating that the transmission of even such "crippled" HIV/SIV carrying multiple CTL escape mutations can result in persistent viral replication and AIDS progression. It remains unclear what host factors determined the viral replication efficiency in vivo in our study, while macaques with higher viral loads (R02-017, R05-002, R05-001, and R05-025) showed the first reversion earlier than those with lower viral loads (R02-022, R03-022, R06-016, and R05-024), suggesting an association of reversion with viral loads. Earlier reversion may result in the recovery of viral fitness, leading to higher viral loads, or conversely, higher viral loads may accelerate reversion.

Thus, our results suggest that in the transmission of HIV accumulating CTL escape mutations at the cost of viral fitness between MHC-mismatched individuals, even such crippled HIV infection can finally result in AIDS progression. Previous studies on SIVs with single CTL escape mutations showed their rapid reversion, but the present study on SIV with multiple CTL escape mutations indicates that the reversion of all the mutations was not required for the establishment of persistent viral replication or for the onset of disease. Furthermore, it suggests a possibility that CTL escape mutations resulting in viral fitness costs may not always revert rapidly even in the absence of CTL pressure after their transmission into MHC-mismatched hosts and can be transmitted further to other hosts. These results provide an important insight into HIV pathogenicity and evolution in human individuals with divergent MHC polymorphisms.

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Determination of a major histocompatibility complex class I restricting simian immunodeficiency virus $Gag_{241-249}$ epitope

Several major histocompatibility complex class I (MHC-I) alleles such as *HLA-B*57* have been shown to be associated with lower viral loads and better prognosis in HIV-1 infections, and MHC-I-restricted epitope-specific effective cytotoxic T lymphocyte (CTL) responses are found to play an important role in this reduction of viral loads [1–3]. Characterization of these effective CTLs could contribute to the development of an effective AIDS vaccine.

We have developed a prophylactic vaccine using a Sendai virus vector expressing simian immunodeficiency virus mac239 (SIVmac239) Gag (SeV-Gag) and have shown its protective efficacy against SIVmac239 challenge in a group of Burmese rhesus macaques (*Macaca mulatta*) sharing an MHC-I haplotype *90-120-Ia* [4]. Involvement of SIVmac239 $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses in this viral control have been indicated [5]. Interestingly, the SIVmac239 $Gag_{241-249}$ epitope is located in a region corresponding to the HLA-B*57-restricted HIV-1 $Gag_{240-249}$ epitope, TW10 (TSTLQEQIAW), and TW10-specific CTL responses have also been indicated to exert strong suppressive pressure on HIV-1 replication resulting in lower viral loads [6,7]. An SIVmac239 $Gag_{241-249}$-specific CTL escape mutation has been shown to result in a loss of viral fitness similarly with a TW10-specific CTL escape mutation [5]. In the present study, for further analysis of SIVmac239 $Gag_{241-249}$-specific CTL function, we have tried to determine the MHC-I that restricts this CTL epitope.

Among eight MHC-I alleles consisting of MHC-I haplotype *90-120-Ia* [4,8], expression of three alleles, *Mamu-A*90120-4*, *Mamu-A*90120-5*, and *Mamu-B*90120-6*, was predominant at RNA levels. We cloned cDNAs of these three alleles and established HLA-A/B/C-negative human 721.221 cell lines [9] expressing these cDNAs, respectively. These cells were pulsed with 10 nmol/l of $Gag_{241-249}$ peptide and used as target cells for the CTL assay using an SIVmac239 $Gag_{241-249}$-specific CTL clone as the effector. Measurement of cytotoxicity in standard ^{51}Cr release assay [5] revealed specific killing of $Gag_{241-249}$-pulsed cells expressing Mamu-A*90120-5, indicating restriction of this CTL epitope by the Mamu-A*90120-5 molecule (Fig. 1a).

Both of the Mamu-A*90120-5-restricted SIVmac239 $Gag_{241-249}$ epitope and the HLA-B*57-restricted HIV-1 TW10 epitope are considered to have the same anchor residues, serine (S) at position 2 and tryptophan (W) at the carboxyl terminus. Comparison of amino acid sequences of antigenic peptide-binding domains (α1 and α2 domains) in Mamu-A*90120-5 with those in HLA-B*5701 revealed limited similarities ($154/182 = 84.6\%$) between these two (Fig. 1b). This might be compatible with previous reports indicating that human and macaque MHC-I molecules with divergent peptide-binding grooves can bind similar or identical peptides [10,11]. MHC-I molecules form a peptide-binding groove including B-pocket and F-pocket that play a key role in determination of the binding peptide motif for its specific binding to the MHC-I. Mamu-A*90120-5 and HLA-B*5701 showed similarity in eight of 11 residues at 7, 9, 24, 25, 34, 45, 63, 66, 67, 70, and 99, which are considered to be anchor residues involved in B-pocket binding and in seven of eight residues at 77, 80, 81, 116, 123, 143, 146, and 147 involved in F-pocket binding [11–13].

In addition, TW10 epitope-specific CTLs, HLA-B*57-restricted HIV-1 $Gag_{147-155}$ [ISW9 (ISPRTLNAW)] epitope-specific CTLs have also been indicated to exert strong selective pressure on HIV-1 [14]. The SIVmac239 $Gag_{149-157}$ amino acid sequence corresponding to the HIV-1 $Gag_{147-155}$ epitope region is LSPRTLNAW, showing a difference at the amino terminus, and CTL responses specific for a peptide including the SIVmac239 $Gag_{149-157}$ amino acid sequence were not induced by SeV-Gag vaccination in Mamu-A*90120-5-positive macaques (data not shown). Interestingly, the SIVmac239 Gag 148th proline (P) and 149th leucine (L) correspond to the HIV-1 Gag 146th P and the 147th L, respectively that have been indicated to be selected in HIV-1-infected humans possessing HLA-B*57. Selection of the former 146th P has been shown to result in escape from ISW9-specific CTL recognition by disturbance in antigen processing [14]. Thus, it is speculated that the SIVmac239 $Gag_{149-157}$-derived peptide may not be presented by Mamu-A*90120-5 even if it has an ability to bind this peptide.

Both SIVmac239 $Gag_{241-249}$-specific CTLs and HIV-1 TW10-specific CTLs have been indicated to exert strong suppressive pressure on SIV/HIV-1 replication and select for a mutation resulting in escape from their recognition at the cost of viral fitness. Thus, this Gag region may be a promising CTL target for viral control, and SIVmac239 infection in Mamu-A*90120-5-positive macaques could be a unique model for examining viral replication in the

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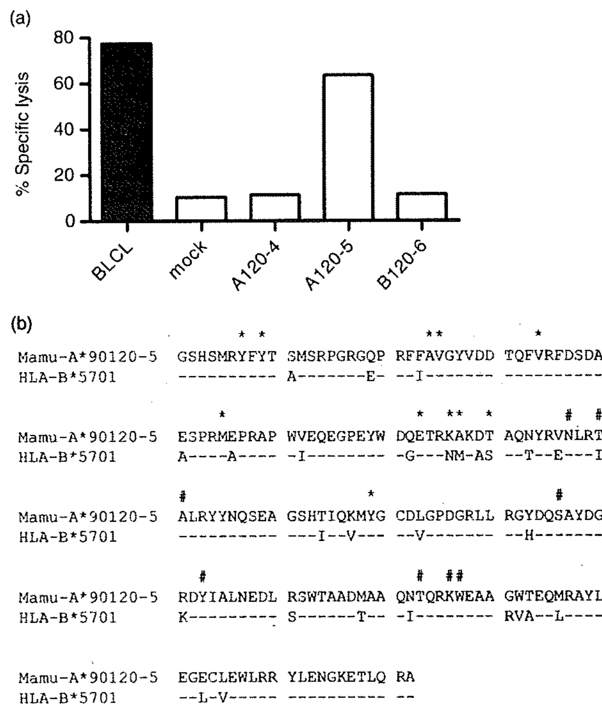

Fig. 1. Mamu-A*90120-5 that restricts the SIV $Gag_{241-249}$ epitope. (a) CTL assay using a $Gag_{241-249}$-specific CTL clone on a B-lymphoblastoid cell line derived from a macaque possessing *90-120-Ia* (BLCL), 721.221 cells (mock), and 721.221 cells expressing Mamu-A*90120-4 (A120-4), Mamu-A*90120-5 (A120-5), and Mamu-B*90120-6 (B120-6), respectively. (b) Amino acid sequences of the Mamu-A*90120-5 α1 and α2 domains in comparison with HLA-B*5701. The anchor residues involved in B and F-pocket binding are indicated by * and #, respectively.

presence of those CTLs targeting this region like TW10-specific CTLs. Finally, we obtained a phycoerythrin-conjugated $Gag_{241-249}$ epitope-Mamu-A*90120-5 tetramer for specific detection of $Gag_{241-249}$-specific CTLs. This could be useful for the analysis of $Gag_{241-249}$-specific CTL responses in Mamu-A*90120-5-positive macaques infected with SIVmac239.

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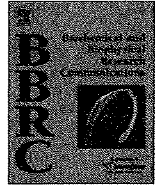

Antigen-specific T-cell induction by vaccination with a recombinant Sendai virus vector even in the presence of vector-specific neutralizing antibodies in rhesus macaques

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ABSTRACT

Recombinant viral vectors are promising vaccine tools for eliciting potent cellular immune responses against immunodeficiency virus infection, but pre-existing anti-vector antibodies can be an obstacle to their clinical use in humans. We have previously vaccinated rhesus macaques with a recombinant Sendai virus (SeV) vector twice at an interval of more than 1 year and have shown efficient antigen-specific T-cell induction by the second as well as the first vaccination. Here, we have established the method for measurement of SeV-specific neutralizing titers and have found efficient SeV-specific neutralizing antibody responses just before the second SeV vaccination in these macaques. This suggests the feasibility of inducing antigen-specific T-cell responses by SeV vaccination even in the host with pre-existing anti-SeV neutralizing antibodies.

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Virus-specific CD8$^+$ cytotoxic T lymphocyte (CTL) responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication [1–5]. Efficient CTL induction is a key strategy for AIDS vaccine development, and recombinant viral vectors are promising tools for its elicitation [6]. Most of the parental or related viruses of these vectors can induce natural infection in humans. Thus, pre-existing immunity against the vector virus itself could be an obstacle to viral vector-based efficient CTL induction in humans. Indeed, a clinical trial of a vaccine using adenovirus serotype 5 (AdV5) vectors has shown reduction in efficiency of vaccine-based CTL induction in people with pre-existing anti-AdV5 antibodies [7–9].

We have developed an AIDS vaccine using a recombinant Sendai virus (SeV) vector and have shown its potential for efficient CTL induction in macaques [10–12]. SeV, murine parainfluenza virus type 1 (PIV-1), is an enveloped virus with a negative-sense RNA genome. Its natural host is mice and its natural infection is not believed to occur efficiently in primates including humans [11,13]. However, antibodies against human PIV-1 (hPIV-1), whose natural infection frequently occurs in humans, are known to cross-react with SeV [14]. It can be expected that the presence of these cross-reactive antibodies at SeV vaccination may reduce its efficiency of CTL induction, but it remains unclear how much extent anti-hPIV-1 antibodies may have adverse effect on SeV-based CTL induction in humans.

Recently, we have vaccinated four rhesus macaques with SeV vectors twice at an interval of more than 1 year and examined antigen-specific CTL induction by the second SeV vaccination [15]. The second vaccination of macaques with an SIV Gag-expressing SeV (SeV-Gag) vector resulted in efficient induction of Gag-specific CTL responses. In the present study, we have established the method for measurement of SeV-specific neutralizing titers and examined SeV-specific neutralizing antibody responses at the second SeV-Gag vaccination in these four macaques. Our results revealed that Gag-specific CTL responses were induced by the second SeV-Gag vaccination in the presence of anti-SeV neutralizing antibodies.

Materials and methods

Samples. Plasma samples were obtained from four Burmese rhesus macaques (*Macaca mulatta*), R011, R012, R003, and R006, that received SeV vaccination twice as described previously [15]. In brief, macaques R011 and R012 received four times of vaccinations with an env- and nef-deleted simian-human immunodeficiency virus (SHIV) molecular clone DNA [16] and a single intranasal boost with a replication-competent SeV-Gag (F[+]SeV-

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Gag) [10,17], whereas macaques R003 and R006 were boosted intranasally with a recombinant SeV expressing HIV-1 Tat (F[+]SeV-Tat) after the DNA vaccinations [18]. Animals were challenged intravenously with SHIV89.6PD approximately 3 months after the SeV boost. Finally, these macaques received the second SeV vaccination; macaques R011 and R012 were vaccinated with replication-defective F-deleted SeV-Gag (F^{−}SeV-Gag) [19] at week 191 and euthanized at week 196 after the first F(+)SeV-Gag vaccination, whereas R003 and R006 were vaccinated with F(+)SeV-Gag at week 68 and euthanized at week 69 (R003) or 70 (R006) after the first F(+)SeV-Tat vaccination. These macaques were maintained in accordance with the Guideline for Laboratory animals of National Institute of Infectious Diseases and National Institute of Biomedical Innovation.

Measurement of plasma anti-SeV IgG levels. The plasma anti-SeV immunoglobulin G (IgG) levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Denka Seiken, Tokyo, Japan) using whole inactivated SeV (HVJ Z strain) particles and a peroxidase-conjugated anti-monkey IgG antibody [20].

SeV neutralization assay. A recombinant SeV expressing EGFP (SeV-EGFP) was obtained as described before [17]. Virus titer was determined by infecting LLCMK2 cells and counting the number

Table 1
SeV vaccination and Gag-specific CD8$^+$ T-cell responses

Macaques	SeV vaccination[a]		Gag-specific CD8 T-cell frequencies[b]	
	1st	2nd	Just before the 2nd SeV	1 week after the 2nd SeV
	At week 0	At week 191	At week 191	At week 192
R011	F(+)SeV-Gag	F(−)SeV-Gag	5.0×10^2	1.1×10^3
R012	F(+)SeV-Gag	F(−)SeV-Gag	1.6×10^2	1.3×10^3
	At week 0	At week 68	At week 68	At week 69
R003	F(+)SeV-Tat	F(+)SeV-Gag	5.0×10^2	2.2×10^3
R006	F(+)SeV-Tat	F(+)SeV-Gag	2.5×10^2	1.2×10^3

[a] Macaques R011 and R012 received the second SeV vaccination at week 191 after the first SeV vaccination, and R003 and R006 at week 68.
[b] Gag-specific CD8$^+$ T-cell frequencies per million peripheral blood mononuclear cells (PBMCs) reported previously [15] are shown.

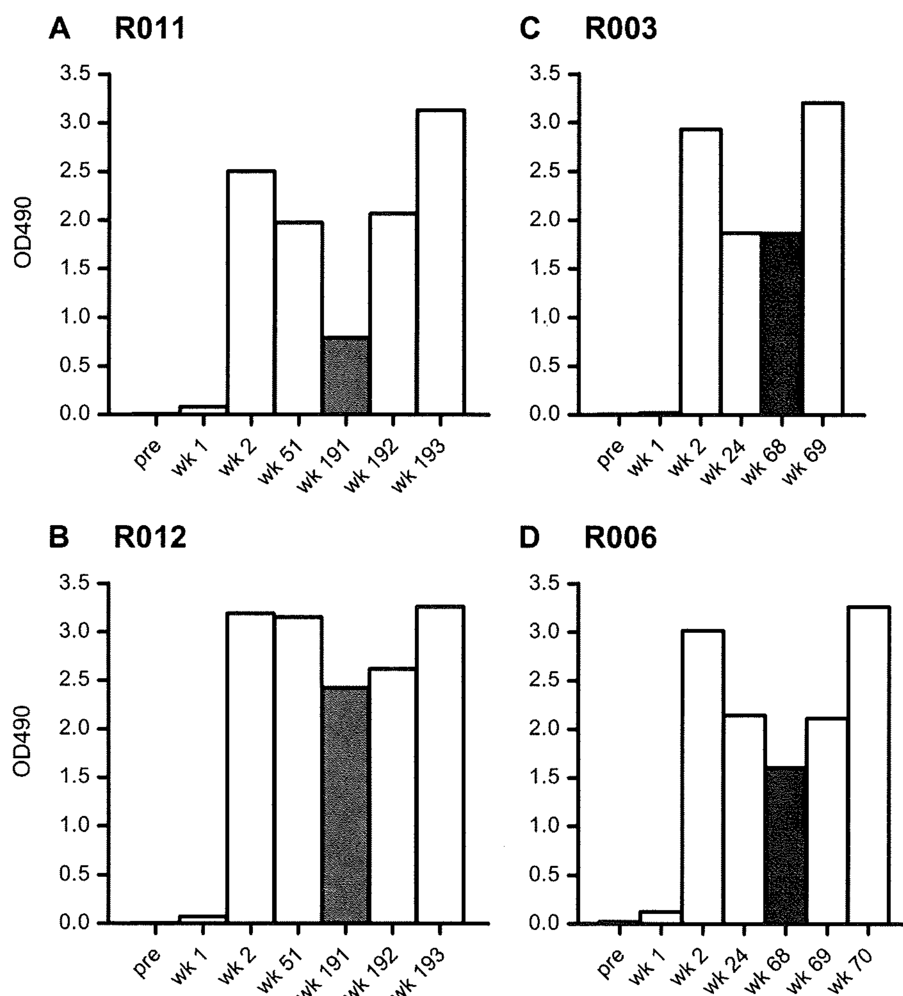

Fig. 1. SeV-specific IgG levels in plasma. Plasma samples obtained from macaques R011 (A), R012 (B), R003 (C), and R006 (D) before the initial DNA vaccination (pre) and at several time points after the first SeV vaccination were diluted by 1/5000 and subjected to ELISA assay. OD490, optical density at 490 nm.

of GFP-expressing cells. To assess the infectivity of SeV-EGFP on LLCMK2 cells, cells were plated at a density of 3.0×10^4 cells per well in 96-well plates, incubated overnight, and infected with serial twofold dilutions of SeV-EGFP. One day after the infection, cells were harvested by using 0.05% trypsin with 0.02% EDTA and subjected to flow-cytometric analysis for detection of EGFP-positive cells.

To measure SeV-specific neutralizing titers on LLCMK2 cells, cells were plated at a density of 3.0×10^4 cells per well in 96-well plates, incubated overnight, and infected with the mixture of SeV-EGFP and diluted plasma. For preparation of the mixture, 25 μl of virus solutions containing 8.3×10^4 cell infectious units (CIU) of SeV-EGFP were incubated with equal volume of serial twofold dilutions of heat-inactivated plasma samples at 37 °C for 1 h. One day after the infection, cells were harvested and subjected to flow-cytometric analysis for detection of EGFP-positive cells. Percent neutralizing activity was calculated by subtracting the percentage of the EGFP-positive cell number in the culture with plasma samples per that without plasma from 100%.

Results and discussion

In the present study, we examined SeV-specific antibody responses in plasma samples of four rhesus macaques that had received SeV vector vaccination twice as described previously [15]. At the second vaccination, macaques R011 and R012 received F(−)SeV-Gag at week 191 after the first vaccination, while macaques R003 and R006 received F(+)SeV-Gag at week 68. In all these macaques, Gag-specific CD8+ T-cell responses were augmented after the second vaccination (Table 1).

We first measured plasma anti-SeV IgG levels in these macaques (Fig. 1). All the macaques showed efficient induction of SeV-specific antibody responses 2 weeks after the first SeV vaccination. High levels of anti-SeV IgG were maintained until the second vaccination and enhancement of the SeV-specific antibody responses were observed after that. This enhancement appeared 1 week after the second SeV vaccination, indicating rapid secondary responses.

Next, we established a method for measurement of SeV-specific neutralizing titers on LLCMK2 cells by using SeV-EGFP. Titration of SeV-EGFP on LLCMK2 cells roughly exhibited a proportional relationship between viral titers and GFP-positive cell frequencies in the m.o.i. (multiplicity of infection) range from 0.2 (6.3×10^3 CIU/well) to 3.3 (1.0×10^5 CIU/well) (Fig. 1A). Then, in the neutralizing assay, 25 μl of virus solution containing 8.3×10^4 CIU of SeV-EGFP was mixed with 25 μl of diluted plasma and added into each well (m.o.i. = 2.8), and GFP-positive cell frequencies were measured.

Incubation of SeV-EGFP with serially diluted pre-immune plasma samples did not affect SeV infectivity and showed similar levels (approximately 75%) of GFP-positive cell frequencies (Fig. 2B). In contrast, neutralization of SeV infection was observed by incubation with plasma samples obtained at week 2 after the first SeV vaccination (Fig. 2B).

We then measured SeV-specific neutralizing titers just before the second SeV vaccination. We determined the end-point plasma titers required for 10-fold reduction of GFP-positive cell frequencies compared to the negative control without plasma (90% neutralization) (Fig. 3). Analyses revealed efficient SeV-specific neutralizing titers in plasma just before the second SeV vaccination in all four macaques. The 90% neutralization titers were 1:100 in macaques R003 and R006, 1:200 in macaque R011, and 1:800 in macaque R012. Higher neutralizing titer in macaque R012 compared to other three was compatible with their anti-SeV IgG levels. The second SeV vaccination resulted in increases in SeV-specific neutralizing titers by fourfold or more.

In macaques R011 and R012, Gag-specific CD8+ T-cell frequencies (cells/million PBMCs) after the first SeV-Gag vaccination were

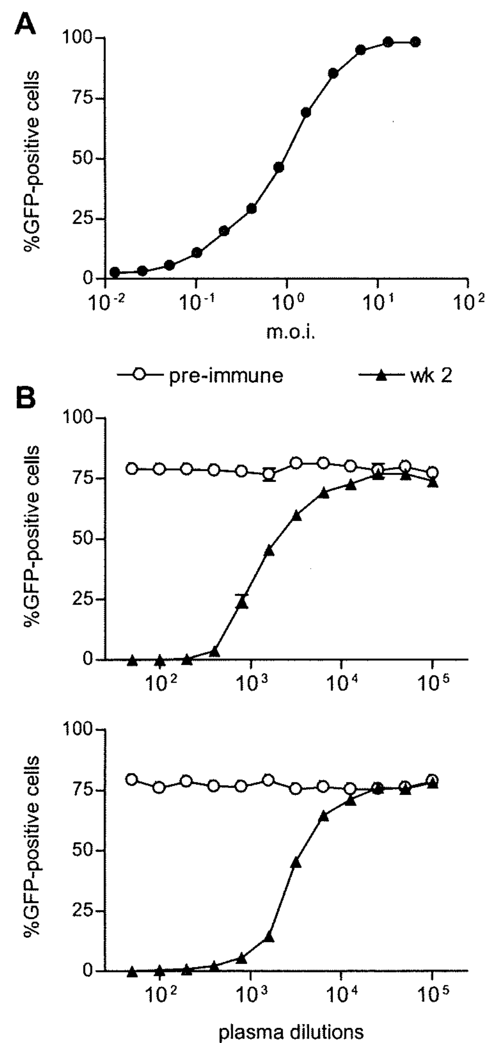

Fig. 2. GFP-positive cell frequencies after SeV-EGFP infection. (A) GFP-positive cell frequencies after infection with diluted SeV-EGFP. LLCMK2 cells were infected with serial twofold dilutions of SeV-EGFP. In case of m.o.i. of 3.3, 3.0×10^4 cells were plated per well in the 96-well plate, incubated overnight, and infected with 1.0×10^5 CIU of SeV-EGFP. The mean values obtained by duplicate experiments are shown. (B) GFP-positive cell frequencies after infection with the mixture of SeV-EGFP and diluted plasma samples. SeV-EGFP (8.3×10^4 CIU) was incubated with serially diluted plasma obtained pre-vaccination (pre-immune, open circles) or at week 2 after the first SeV immunization (closed triangles) from macaques R011 (upper panel) and R003 (lower panel) and added into LLCMK2 cells plated at 3.0×10^4 cells per well. The mean values obtained by duplicate experiments are shown.

approximately 1.0×10^3 [10] and those after the second SeV-Gag vaccination were just above 1.0×10^3 (Table 1) [15]. Granted that the priming conditions and the utilized vectors were different between the first and the second SeV vaccination, these results indicate efficient Gag-specific CD8+ T-cell induction even by the second SeV-Gag vaccination. Thus, our finding of SeV-specific neutralizing antibody responses just before the second SeV-Gag vaccination in the present study indicates the potential of recombinant SeV vectors to induce antigen-specific T-cell responses even in the presence of SeV-specific neutralizing antibodies, suggesting an important implication for development of an effective AIDS vaccine using viral vectors.

Because of the cross-reactivity of anti-SeV antibodies with hPIV-1, a clinical trial phase I of wild-type SeV vaccination against hPIV-1 infection has been performed [21]. However, the potential

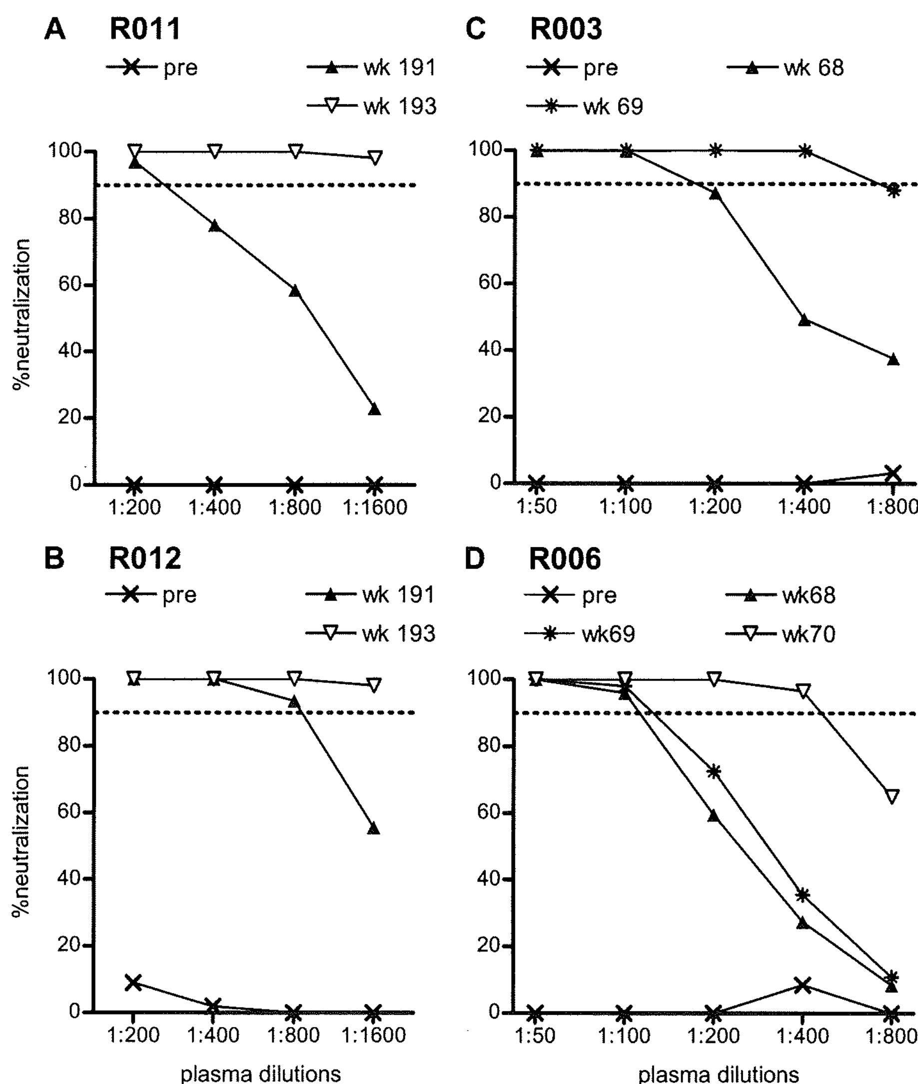

Fig. 3. SeV-specific neutralizing titers in plasma. We examined neutralizing titers in plasma samples obtained before the initial DNA vaccination (pre), just before the second SeV vaccination (at week 191 in R011 and R012 and at week 68 in R003 and R006), and after that in macaques R011 (A), R012 (B), R003 (C), and R006 (D). The mean values obtained by duplicate experiments are shown.

of SeV vaccination to induce hPIV-1-specific neutralizing antibody responses has not precisely evaluated. Conversely, anti-hPIV-1 antibodies may cross-react with SeVs, but how much extent these can neutralize SeVs remains unclear. While we do not know the frequency of anti-hPIV-1 antibody-positive individuals, the present study strongly suggests the feasibility of efficiently inducing antigen-specific T-cell responses by SeV vaccination even in the host with pre-existing anti-hPIV-1 antibodies. Precise evaluation of relationship between pre-existing SeV-specific neutralizing titers and efficiency of antigen-specific T-cell induction would contribute to estimation of SeV vector vaccine efficacy in anti-hPIV-1 antibody-positive individuals.

Acknowledgments

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