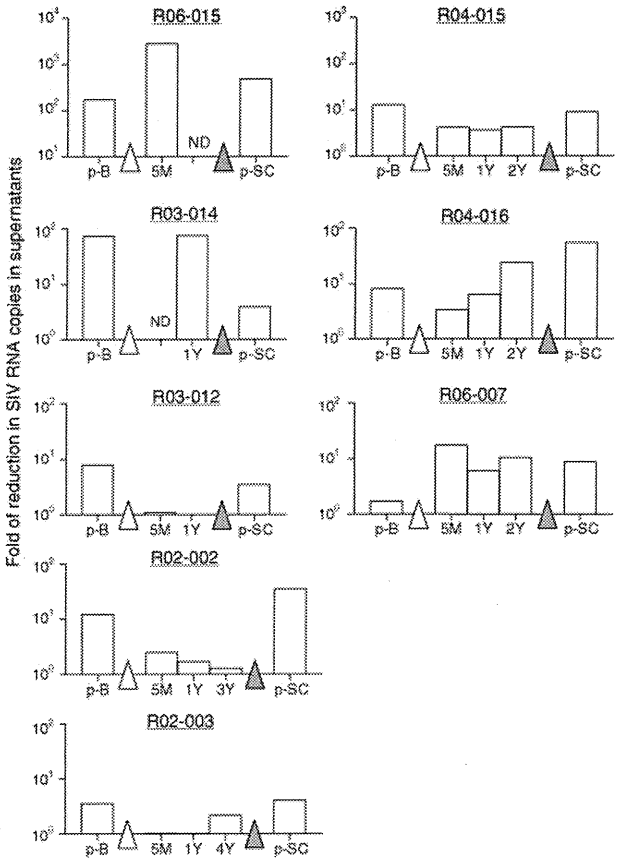

Fig. 2. Anti-SIVmac239 efficacy *in vitro* of CD8$^+$ cells in simian immunodeficiency virus controllers. PBMC-derived CD8$^-$ (target) cells infected with SIVmac239 were cultured alone or cocultured with autologous PBMC-derived CD8$^+$ (effector) cells at several time points at an $E:T$ ratio of 1 : 4. The ratios of viral RNA levels in the supernatants from the coculture to those without CD8$^+$ cells are shown. ND: not determined. p-B: 1 week after boost; 5M, 1Y, 2Y, 3Y, and 4Y: 5 months, 1, 2, 3, and 4 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

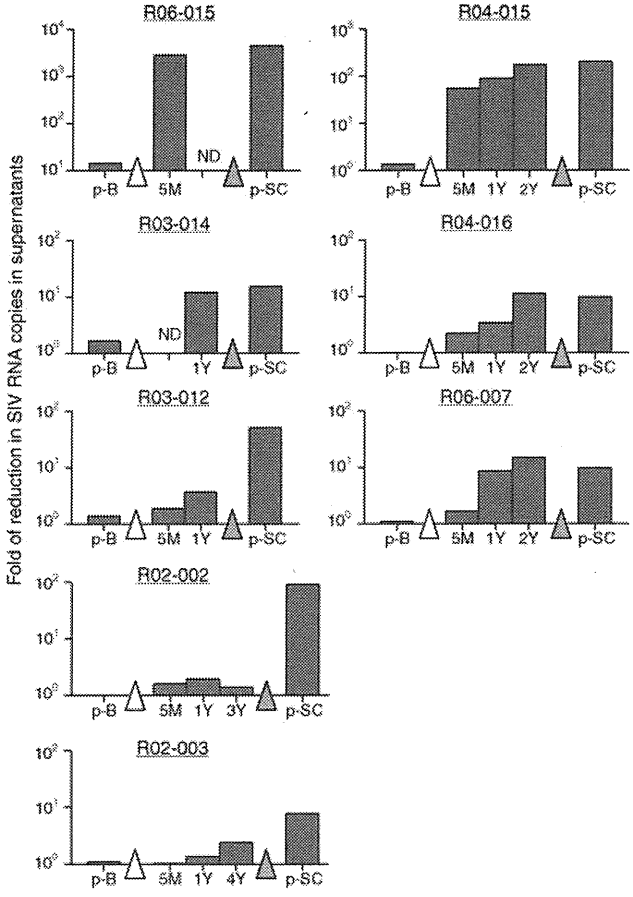

Fig. 3. Anti-SIV-G64723mt efficacy *in vitro* of CD8$^+$ cells in simian immunodeficiency virus controllers. PBMC-derived CD8$^-$ cells infected with SIV-G64723mt were cultured alone or cocultured with autologous PBMC-derived CD8$^+$ cells at several time points at an $E:T$ ratio of 1 : 4. The ratios of viral RNA levels in the supernatants from the coculture to those without CD8$^+$ cells are shown. ND: not determined. p-B: 1 week after boost; 5M, 1Y, 2Y, 3Y, and 4Y: 5 months, 1, 2, 3, and 4 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

ratio of 1 : 4, and viral production in culture supernatants was examined to assess suppressive effect of CD8$^+$ cells on viral replication in vitro.

CD8$^+$ cells 1 week after boost mostly suppressed wild-type SIVmac239 replication efficiently. In contrast, these postboost CD8$^+$ cells failed to show efficient suppressive effect on SIV-G64723mt replication. These results suggest that $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTL responses play a central role in the suppression of SIVmac239 replication by postboost CD8$^+$ cells.

After SIVmac239 challenge, all these animals showed efficient in-vitro anti-SIV-G64723mt efficacy (more than two-fold reduction in viral production) of CD8$^+$ cells, sooner or later, in the chronic phase. The levels of in-vitro anti-SIV-G64723mt efficacy of CD8$^+$ cells tended to become higher in the chronic phase. Anti-SIVmac239 efficacy of CD8$^+$ cells was not associated with anti-SIV-G64723mt efficacy. For instance, some CD8$^+$ cells efficiently suppressed SIV-G64723mt but not SIVmac239 replication. After all, all SIV controllers acquired CD8$^+$ cells able to suppress the mutant SIV-G64723mt replication *in vitro* in the chronic phase.

Control of a mutant simian immunodeficiency virus superchallenge

These animals were superchallenged with a mutant SIV, SIV-G64723mt, that has five *gag* mutations resulting in

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escape from recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTLs around 1 year (R06-015, R03-014, and R03-012), 2 years (R04-015, R04-016, and R06-007), 3 years (R02-002), or 4 years (R02-003) after SIVmac239 challenge. The replicative ability of SIV-G64723mt is significantly lower than that of wild-type SIVmac239, but SIV-G64723mt challenge of naive *90-120-Ia*-negative rhesus macaques can result in persistent viral replication and AIDS progression [23,28]. It has previously been shown that *90-120-Ia*-positive macaques vaccinated with DNA-prime/SeV-Gag-boost are unable to contain a SIV-G64723mt challenge, whereas they can control replication of wild-type SIVmac239 [24]. Indeed, we confirmed that $CD8^+$ cells obtained from these *90-120-Ia*-positive vaccinees before challenge efficiently suppressed wild-type SIVmac239 but not SIV-G64723mt replication *in vitro*. In the present study, however, all eight wild-type SIV controllers contained the SIV-G64723mt superchallenge without detectable viremia (Fig. 1b). SIVmac239-specific neutralizing antibody responses were undetectable around the superchallenge in any of these controllers (Fig. 1a). These results indicate that, after SIVmac239 challenge, the SIV controllers acquired the potential to control SIV-G64723mt replication in the absence of neutralizing antibody responses, although to what extent $CD8^+$ cell responses may contribute to this containment of SIV-G64723mt superchallenge remains unclear. Postsuperchallenge $CD8^+$ cells suppressed both SIVmac239 and SIV-G64723mt replication *in vitro* efficiently (Figs. 2 and 3).

Simian immunodeficiency virus Gag-specific cytotoxic T lymphocyte responses in simian immunodeficiency virus controllers

Then, in these SIV controllers, we examined $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTL responses, which have previously been indicated responsible for control of SIVmac239 replication in *90-120-Ia*-positive vaccinees [24] (Fig. 4a). In DNA/SeV-Gag vaccinated animals (R06-015, R03-014, R03-012, and R02-002), SIV-specific CTL responses were undetectable before SeV-Gag boost (data not shown), but $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific responses were efficiently induced 1 week after the boost. After SIVmac239 challenge, these animals showed efficient responses of these CTLs in the acute phase. These CTL levels were reduced in the chronic phase, but $Gag_{241-249}$-specific CTL responses were detectable even 1 year after challenge. In macaque R04-015 vaccinated with DNA/SeV-$Gag_{202-216}$-EGFP and DNA/SeV-$Gag_{236-250}$-EGFP, $Gag_{206-216}$-specific CTL responses were induced dominantly 1 week after boost and 2 weeks after SIVmac239 challenge, whereas $Gag_{241-249}$-specific CTL responses were detected predominantly in the chronic phase. In macaques R04-016 and R06-007 vaccinated with DNA/SeV-$Gag_{236-250}$-EGFP, $Gag_{241-249}$-specific CTL responses were induced dominantly 1 week after boost and 2 weeks after SIVmac239 challenge and were maintained in the chronic phase. No significant enhancement of these CTL responses was observed after SIV-G64723mt superchallenge.

We also examined Gag-specific CTL responses in SIV controllers at several time points by using a panel of overlapping peptides (Gag peptide pools 1–10) spanning the entire SIVmac239 Gag (Fig. 4b). Group I macaques vaccinated with DNA/SeV-Gag elicited CTL responses directed against not only Gag peptide pool 5 (including $Gag_{206-216}$ and $Gag_{241-249}$) and 7 (including $Gag_{367-381}$) but also other Gag peptide pools after boost and after challenge; some peptide pool-specific CTLs were diminished, whereas others appeared in the chronic phase. In contrast, group II macaques eliciting CTL responses directed against single $Gag_{206-216}$ (R04-015) or $Gag_{241-249}$ (R04-016 and R06-007) epitope after boost showed predominant Gag peptide pool 5-specific CTL responses after challenge and accumulated multiple Gag epitope-specific CTL responses in the chronic phase. These results indicate dynamics of postchallenge Gag-specific CTL responses in vaccine-based SIV controllers. After SIV-G64723mt superchallenge, changes in the pattern of Gag-specific CTL responses were observed in some animals.

Simian immunodeficiency virus non-Gag antigen-specific cytotoxic T lymphocyte responses in simian immunodeficiency virus controllers

Next, in SIV controllers, we examined CTL responses directed against SIV non-Gag antigens by using panels of overlapping peptides spanning the entire SIVmac239 antigens other than Gag (Fig. 5a). These SIV controllers showed SIV non-Gag-specific CTL responses from the early phase after challenge. After SIV-G64723mt superchallenge, broadening or changes in the pattern of these CTL responses were observed in some animals; Vif-specific or Nef-specific CTL responses were detected predominantly, although we did not find common CTL epitopes in Vif or Nef.

Correlation of antigen-specific cytotoxic T lymphocyte levels with in-vitro antivirus efficacy levels

Finally, we analyzed correlation of antigen-specific CTL levels with in-vitro anti-SIVmac239 or anti-SIV-G64723mt efficacy levels of $CD8^+$ cells (Fig. 5b). We found a correlation of anti-SIVmac239 efficacy levels with $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL levels but not with total Gag-specific CTL levels. The anti-SIVmac239 efficacy levels did not correlate with either $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL levels alone (data not shown), although our previous study [25] indicated inverse correlation between peak plasma viral loads and the levels of $Gag_{241-249}$-specific CTLs dominantly induced in DNA/SeV-$Gag_{236-250}$-EGFP-vaccinated animals in the acute phase after

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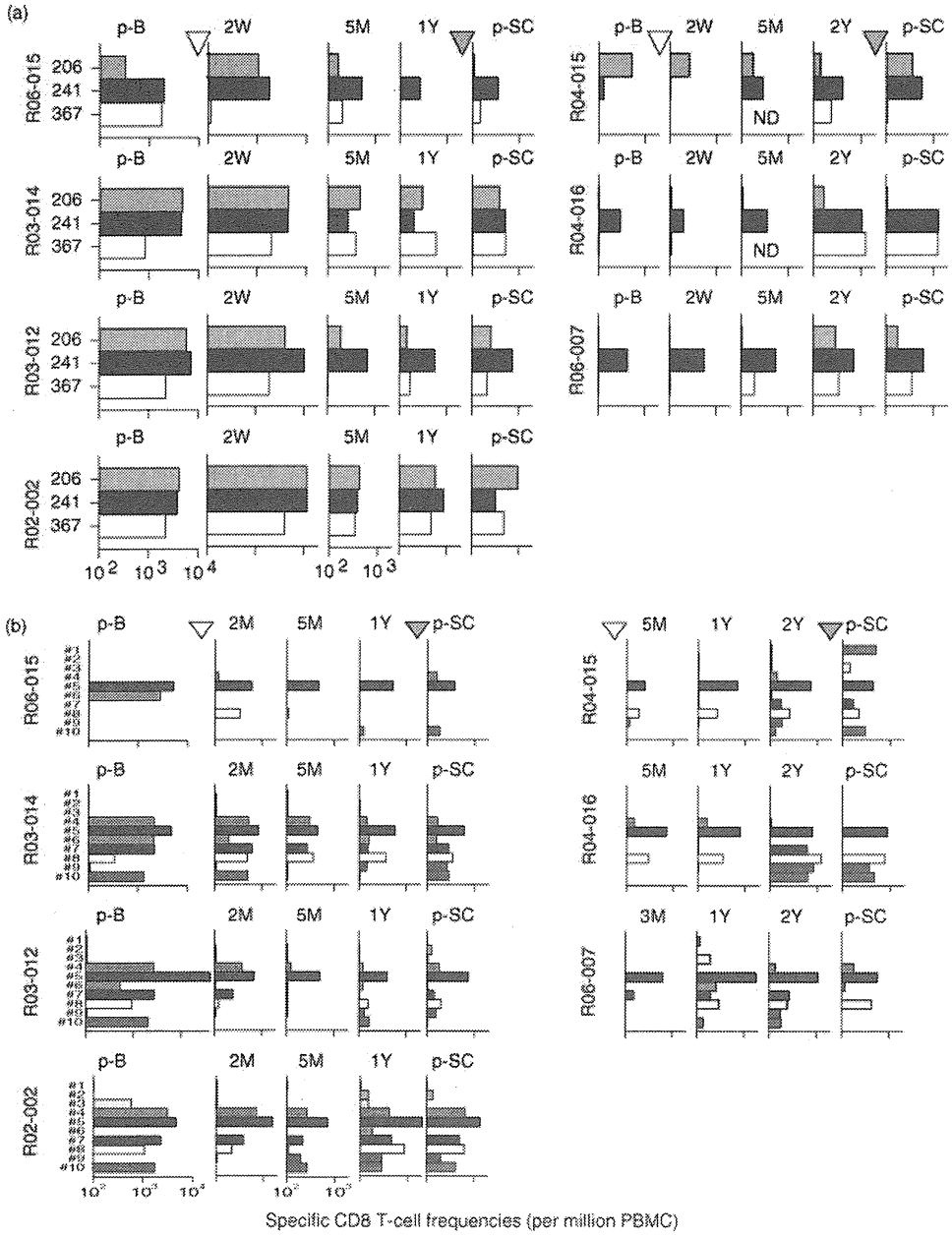

Fig. 4. Gag-specific CD8$^+$ T-cell responses in simian immunodeficiency virus controllers. (a) $Gag_{206-216}$-specific (206), $Gag_{241-249}$-specific (241), and $Gag_{367-381}$-specific (367) CD8$^+$ T-cell frequencies at several time points are shown. Regarding macaque R02-003, we confirmed efficient responses of these CTLs after boost and in the acute phase as reported previously [24] but did not have enough PBMC samples for the analyses in the chronic phase. (b) A panel of 117 overlapping peptides (15–17 amino acid in length and overlapping by 10–12 amino acid) spanning the entire SIV Gag amino acid sequence was divided into the following 10 pools (each consisting of 11 or 12 peptides): pool 1, first to 65th amino acid in SIV Gag; pool 2, 55th to 114th amino acid; pool 3, 104th to 165th amino acid; pool 4, 155th to 213th amino acid; pool 5, 202nd to 265th amino acid; pool 6, 255th to 316th amino acid; pool 7, 306th to 364th amino acid; pool 8, 354th to 416th amino acid; pool 9, 406th to 464th amino acid; and pool 10, 453rd to 510th amino acid. These Gag peptide pool-specific CD8$^+$ T-cell frequencies at several time points are shown. ND: not determined. p-B: 1 week after boost; 2W, 5M, 1Y, and 2Y: 2 weeks, 5 months, 1, and 2 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. CTL, cytotoxic T lymphocyte; PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

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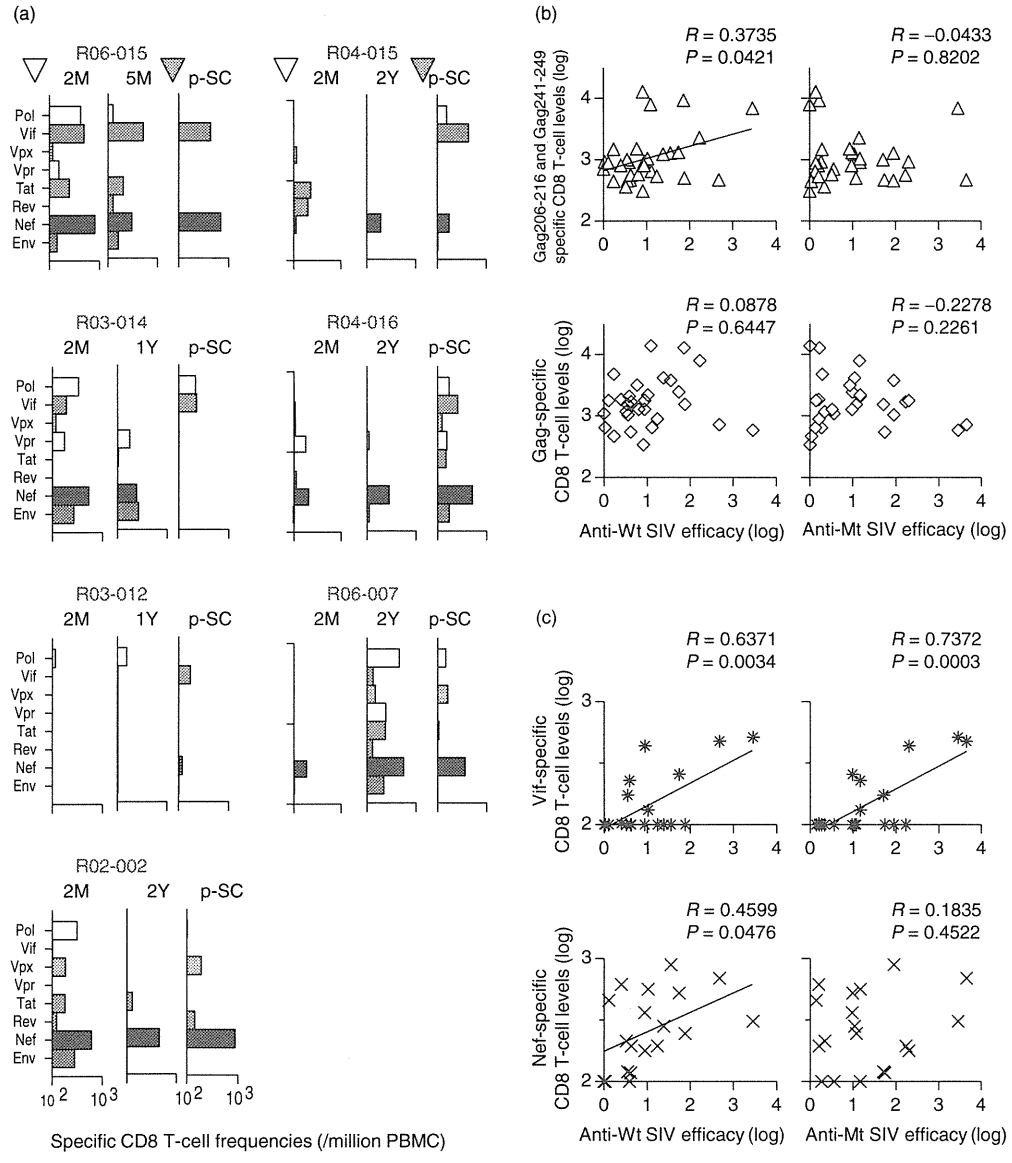

Fig. 5. Analysis of correlation between anti-SIVmac239 or anti-SIV-G64723mt efficacy *in vitro* and simian immunodeficiency virus antigen-specific CD8$^+$ T-cell levels in simian immunodeficiency virus controllers. (a) SIV non-Gag antigen-specific CD8$^+$ T-cell responses. Pol-specific, Vif-specific, Vpx-specific, Vpr-specific, Tat-specific, Rev-specific, Nef-specific, and Env-specific CD8$^+$ T-cell frequencies at several time points were measured by using panels of overlapping peptides spanning the entire SIVmac239 Pol, Vif, Vpx, Vpr, Tat, Rev, Nef, and Env amino acid sequences, respectively. R02-003 PBMC samples were unavailable. 2M, 5M, 1Y, and 2Y: 2, 5 months, 1, and 2 years after challenge, respectively; p-SC: 1 or 2 months after superchallenge. Open triangles indicate the time points of SIVmac239 challenge and closed triangles SIV-G64723mt superchallenge. (b) Analysis of correlation between anti-SIVmac239 (Wt SIV) efficacy (left panels) or anti-SIV-G64723mt (Mt SIV) efficacy (right panels) levels and $Gag_{206\text{-}216}$-specific plus $Gag_{241\text{-}249}$-specific CTL (upper panels) or Gag-specific CTL (lower panels) levels ($n = 30$ in each panel). A correlation between anti-SIVmac239 efficacy levels and $Gag_{206\text{-}216}$-specific plus $Gag_{241\text{-}249}$-specific CTL levels is indicated ($P = 0.0421$, $R = 0.3735$). (c) Analysis of correlation between after challenge anti-SIVmac239 efficacy (left panels) or anti-SIV-G64723mt efficacy (right panels) levels and Vif-specific CTL (upper panels) or Nef-specific CTL (lower panels) levels ($n = 19$ in each panel). Correlations of anti-SIVmac239 efficacy levels with Vif-specific CTL ($P = 0.0034$, $R = 0.6731$) and with Nef-specific CTL levels ($P = 0.0476$, $R = 0.4599$) and a strong correlation between anti-SIV-G64723mt efficacy levels and Vif-specific CTL levels ($P = 0.0003$, $R = 0.7372$) are indicated. CTL, cytotoxic T lymphocyte; SIV, simian immunodeficiency virus.

challenge. Correlations of anti-SIVmac239 efficacy levels after challenge with Vif-specific CTL levels and with Nef-specific CTL levels were indicated. On the contrary, anti-SIV-G64723mt efficacy levels after challenge strongly correlated with Vif-specific CTL levels, although any correlation of these levels with other SIV antigen-

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specific CTL levels was not indicated. These results suggest that Vif-specific CTL induction may contribute in part to acquisition of the potential to suppress SIV-G64723mt replication efficiently.

Discussion

We have previously shown that *90-120-Ia*-positive macaques eliciting Gag-specific CTL responses by vaccination can control SIVmac239 replication but are unable to contain a challenge with a mutant SIV, SIV-G64723mt, carrying multiple *gag* mutations that result in escape from recognition by $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTLs [24]. The present study revealed, by in-vitro viral suppression assay, that those *90-120-Ia*-positive vaccinees can acquire, after wild-type SIVmac239 challenge, $CD8^+$ cells able to suppress the mutant SIV replication. Induction of these $CD8^+$ cell responses may have some supportive effect on the maintenance of viral control after the initial viral containment [4,26,27]. Such dynamics of anti-SIV responses have not been shown clearly even in live attenuated SIV infection [41–44]. Recently, HIVs have been suggested to accumulate mutations escaping from dominant CTL responses [45–51], but our results imply a possibility of induction of cellular immune responses effective against even those HIV variants escaping from dominant CTL responses.

The group I animals induced multiple Gag epitope-specific CTL responses after boost (before challenge) and after challenge, whereas the group II animals elicited only $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL responses before challenge and showed induction of additional CTL responses directed against Gag epitopes other than $Gag_{206-216}$ and $Gag_{241-249}$ after challenge. Furthermore, both groups elicited SIV non-Gag-specific CTL responses after challenge. These results indicate post-challenge accumulation of broader CTL responses. The in-vitro anti-SIVmac239 efficacy levels correlated with Vif-specific and Nef-specific CTL as well as $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL levels but not with total Gag-specific or total SIV-specific CTL levels, suggesting that not all but some particular epitope-specific CTL responses were involved in suppression of SIVmac239 replication. Nef-specific CTL responses were detected more frequently than Vif-specific ones, whereas the latter showed stronger correlation with antiviral efficacy levels (Fig. 5). We did not find common CTL epitopes in Vif or Nef. These may imply higher frequencies of effective CTLs in Vif-specific ones; conversely, Nef-specific CTLs may include effective ones but with higher frequencies of ineffective ones.

Postboost $CD8^+$ cells able to suppress SIVmac239 replication failed to show suppressive effect on SIV-G64723mt replication. We confirmed it also in two *90-120-Ia*-positive vaccinated animals that had failed to control the mutant SIV challenge in our previous studies [24] (data not shown). However, $CD8^+$ cells in the chronic phase suppressed SIV-G64723mt replication efficiently. This indicates postchallenge induction of $CD8^+$ cells with the potential to suppress SIV-G64723mt replication in vaccine-based SIVmac239 controllers, although it remains unclear whether these $CD8^+$ cells with antimutant SIV efficacy are responsible for the control of mutant SIV superchallenge *in vivo*. The in-vitro anti-SIV-G64723mt efficacy levels correlated with Vif-specific CTL levels and $CD8^+$ cells with detectable Vif-specific CTL responses showed suppressive effect on SIV-G64723mt replication. These results implicate Vif-specific CTL responses in the suppression of SIV-G64723mt replication *in vitro* by $CD8^+$ cells in the chronic phase, although other factors may also be involved in this suppression. Preservation of memory $CD4^+$ T cells by vaccine-based SIV control [26] may contribute to induction of these effective CTL responses.

We found dynamics of cellular immune responses during viral control in vaccine-based SIV controllers, but the exact mechanism for broadening or changes in dominance patterns of CTL responses remains unclear. All the group I animals and macaque R04-015 showed rapid selection of a CTL escape *gag* mutation, L216S, at week 5 after challenge, whereas no *gag* mutations were selected at week 5 in macaques R04-016 or R06-007 (data not shown). We failed to recover viral genome cDNAs for sequencing from plasma after week 5 due to undetectable viral loads, but selection of viral CTL escape mutations and reversions [23,28,52–57] under undetectable levels of viral replication may contribute to induction of broader CTL responses in SIV controllers.

It is difficult to directly compare anti-SIVmac239 and anti-SIV-G64723mt efficacy of $CD8^+$ cells because of difference in their replicative ability, but the ratios of the latter level to the former 1 year after challenge were higher than those after boost in all animals. Indeed, $CD8^+$ cells 1 year after challenge in macaques R03-012 and R02-003 showed suppressive effect on SIV-G64723mt but not on wild-type SIVmac239 replication, although R03-012 $CD8^+$ cells at 5 months and 1 year after challenge efficiently suppressed SIVmac239 replication at higher E/T ratio of 1 : 1 (R02-003 $CD8^+$ cells in the chronic phase for this analysis were unavailable). Because no SIV controllers elicited CTL responses specific for peptides with mutated amino acid sequences (data not shown), all CTLs specific for SIV-G64723mt antigens in SIV controllers are expected to recognize SIVmac239 antigens also. Thus, our observation that some post-challenge $CD8^+$ cells showed efficient suppressive effect on SIV-G64723mt but not on SIVmac239 replication *in vitro* may be explained by higher replicative ability of SIVmac239 compared with SIV-G64723mt; it could

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be more difficult for $CD8^+$ cells to suppress replication of the wild-type SIVmac239 than the mutant SIV-G64723mt, implying a possible requirement of more potent CTL responses for SIVmac239 control than for SIV-G64723mt control.

In summary, this study showed dynamics of postchallenge cellular immune responses in vaccine-based SIV controllers. Our results suggest that, during persistent viral control, vaccine-based SIV controllers can acquire $CD8^+$ cells with the potential to suppress replication of SIV variants carrying CTL escape mutations. Elucidation of the mechanism for induction of broader responses in these controllers may contribute to development of a vaccine effective against highly diversified HIV infection.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, a grant-in-aid from the Japan Society for the Promotion of Science, grants-in-aid from the Ministry of Health, Labor, and Welfare, and a grant from Takeda Science Foundation in Japan. N.I. is a Research Fellow of the Japan Society for the Promotion of Science. The animal experiments were conducted through the Cooperative Research Program in the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates (CPRLP). We thank Dr H. Akari, A. Saito, Y. Yasutomi, A. Hiyaoka, K. Komatsuzaki, K. Oto, and F. Ono for their assistance in animal experiments, T. Naruse and A. Kimura for MHC-I haplotyping, and DNAVEC Corporation for providing SeV vectors.

Author contributions: N.I., T.T., M.K., and T.M. designed the study. T.M. ordered animal maintenance and experimental support to TPRC and CPRLP. N.I., T.T., and M.K. contributed to vaccination and challenge experiments. A.T. contributed to blood processing and measurement of plasma viral loads. N.I., T.T., and H.T. contributed to analyses of anti-SIV efficacy of $CD8^+$ cells. N.I., T.T., M.K., and H.Y. analyzed SIV-specific immune responses. N.I. and T.M. analyzed the data and wrote the article.

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A structural constraint for functional interaction between N-terminal and C-terminal domains in simian immunodeficiency virus capsid proteins

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Abstract

Background: The Gag capsid (CA) is one of the most conserved proteins in highly-diversified human and simian immunodeficiency viruses (HIV and SIV). Understanding the limitations imposed on amino acid sequences in CA could provide valuable information for vaccine immunogen design or anti-HIV drug development. Here, by comparing two pathogenic SIV strains, SIVmac239 and SIVsmE543-3, we found critical amino acid residues for functional interaction between the N-terminal and the C-terminal domains in CA.

Results: We first examined the impact of Gag residue 205, aspartate (Gag205D) in SIVmac239 and glutamate (Gag205E) in SIVsmE543-3, on viral replication; due to this difference, $Gag_{206-216}$ (IINEEAADWDL) epitope-specific cytotoxic T lymphocytes (CTLs) were previously shown to respond to SIVmac239 but not SIVsmE543-3 infection. A mutant SIVmac239, SIVmac239Gag205E, whose Gag205D is replaced with Gag205E showed lower replicative ability. Interestingly, however, SIVmac239Gag205E passaged in macaque T cell culture often resulted in selection of an additional mutation at Gag residue 340, a change from SIVmac239 valine (Gag340V) to SIVsmE543-3 methionine (Gag340M), with recovery of viral fitness. Structural modeling analysis suggested possible intermolecular interaction between the Gag205 residue in the N-terminal domain and Gag340 in the C-terminal in CA hexamers. The Gag205D-to-Gag205E substitution in SIVmac239 resulted in loss of in vitro core stability, which was recovered by additional Gag340V-to-Gag340M substitution. Finally, selection of Gag205E plus Gag340M mutations, but not Gag205E alone was observed in a chronically SIVmac239-infected rhesus macaque eliciting $Gag_{206-216}$-specific CTL responses.

Conclusions: These results present in vitro and in vivo evidence implicating the interaction between Gag residues 205 in CA NTD and 340 in CA CTD in SIV replication. Thus, this study indicates a structural constraint for functional interaction between SIV CA NTD and CTD, providing insight into immunogen design to limit viral escape options.

Background

One of the characteristics of human immunodeficiency virus (HIV) is to induce persistent viral replication resulting in AIDS progression. HIV has enormous capacity to mutate and escape from host immune recognition, driving genetic diversification of the circulating viruses [1-3]. The Gag capsid (CA), comprising the N-terminal (NTD) and the C-terminal domains (CTD) [4-6], is one of the most conserved proteins in highly-diversified HIVs [7]. Understanding structural constraints in such viral proteins could provide valuable information for immunogen design in AIDS vaccine development.

Virus-specific cytotoxic T-lymphocyte (CTL) responses play a central role in the control of immunodeficiency virus infection [7-12]. CTLs exerting strong suppressive pressure on HIV replication select for viral mutations resulting in escape from CTL recognition [13-16]. Escape mutations in viral proteins with structural constraints are often selected with viral fitness costs, possibly facilitating subsequent immune control

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[3,17-23]. Thus, conserved viral proteins such as CA can be a promising antigen for vaccine-based CTL induction toward HIV control.

We previously showed vaccine-based control of a simian immunodeficiency virus mac239 (SIVmac239 [24]) challenge in a group of Burmese rhesus macaques possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [19,25]. $Gag_{206-216}$ (IINEEAADWDL) epitope-specific CTL responses play an important role in this control and select for a CTL escape mutation, GagL216S, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) in Gag (CA) with the cost of viral fitness [26]. However, *90-120-Ia*-positive vaccinees failed to control a challenge with another pathogenic SIV strain, SIVsmE543-3 [27], that has the same $Gag_{206-216}$ epitope sequence with SIVmac239; $Gag_{206-216}$-specific CTLs did not show responses against SIVsmE543-3 infection due to an aspartate (D)-to-glutamate (E) change, GagD205E, at Gag residue 205 [28].

Thus, the GagD205E substitution in SIVmac239 could result in viral escape from $Gag_{206-216}$-specific CTL recognition. However, in our previous analyses of *90-120-Ia*-positive animals eliciting $Gag_{206-216}$-specific CTL responses for one or two years postchallenge, we observed selection of GagL216S, but not GagD205E mutation in SIVmac239 infection, suggesting a possibility that the GagD205E substitution results in larger reduction of viral replicative ability than GagL216S. In the present study, we first constructed a mutant SIVmac239, SIVmac239Gag205E, with the GagD205E substitution and examined its replication ability in vitro. We found that this amino acid change in the CA NTD results in loss of viral fitness, which can be recovered by an additional amino acid change in the CA CTD. Further analyses presented in vitro and in vivo evidence for a structural constraint in the functional interaction between SIV CA NTD and CTD.

Results

Compensation for loss of viral fitness in SIVmac239Gag205E by additional GagV340M substitution

We first constructed a mutant SIVmac239 molecular clone DNA with a mutation of a D-to-E substitution at the 205th aa in Gag (CA NTD) to obtain the mutant virus, SIVmac239Gag205E (Figure 1). Analysis of viral replication kinetics on HSC-F, a macaque T cell line, revealed delayed peak of the mutant SIVmac239Gag205E replication, indicating its lower replicative ability compared to the wild-type SIVmac239 (Figure 2).

We further followed up SIVmac239Gag205E replication on HSC-F cells and explored a possibility of viral reversion or additional mutations (Figure 3). No additional gag mutation became dominant on day 10 after SIVmac239Gag205E infection. Interestingly, however, in the second culture after passage of the first culture supernatants on day 10 into uninfected HSC-F cells, an additional mutation, GagV340M, resulting in a valine (V)-to-methionine (M) substitution at the 340th aa in Gag (CA CTD), became dominant in two of four sets of experiments; SIVmac239 has V while SIVsmE543-3 has M at the Gag residue 340. The GagD205E mutation remained dominant, and no other mutations were detected in the CA-coding region even in the second culture.

We then constructed a mutant SIVmac239 molecular clone DNA by introducing the GagV340M mutation into the SIVmac239Gag205E CA-coding region to obtain SIVmac239Gag205E340M (Figure 1). This mutant SIV showed similar replication kinetics on HSC-F cells with the wild-type SIVmac239, indicating compensation for loss of viral fitness in SIVmac239Gag205E by addition of the GagV340M substitution (Figure 2). These results imply that SIV CA with Gag205D-340V or Gag205E-340M combination is functional whereas the CA with Gag205E-340V is less functional.

Possible interaction between Gag residues 205 and 340 in SIV CA hexamers

Recovery of viral fitness of SIVmac239Gag205E by the GagV340M substitution suggests a possibility of interaction between Gag residues 205 in the NTD and 340 in the CTD. Modeling of CA monomer structure, however, showed that the Gag 205th residue is located in the helix 4 of CA NTD, while the 340th is in the loop between helices 10 and 11 of CTD, which does not support a possibility of intramolecular contact between Gag residues 205 and 340 (data not shown).

CA molecules are known to form hexamer lattice in mature virions [29-33]. Modeling of CA hexamer structure revealed that the Gag 205th residue in the NTD is located in close proximity to the 340th in the CTD of the adjacent CA molecule (Figure 4). These observations support a possibility of intermolecular interaction between Gag residues 205 and 340 in CA hexamers.

In addition, the 312th residue in the loop between helices 8 and 9 of CTD is located in close proximity to the 205th in the NTD of the adjacent CA molecule. Because SIVmac239 and SIVsmE543-3 have different amino acids at this residue 312, alanine (A) in the former and proline (P) in the latter, we also constructed a mutant SIVmac239 molecular clone DNA by introducing the GagA312P mutation resulting in A-to-P substitution at the 312th aa in Gag into the SIVmac239Gag205E CA-coding region to obtain SIVmac239Gag205E312P (Figure 1). Analysis of replication kinetics on HSC-F cells indicated recovery of viral fitness by the additional GagA312P substitution in SIVmac239Gag205E (Figure 2).

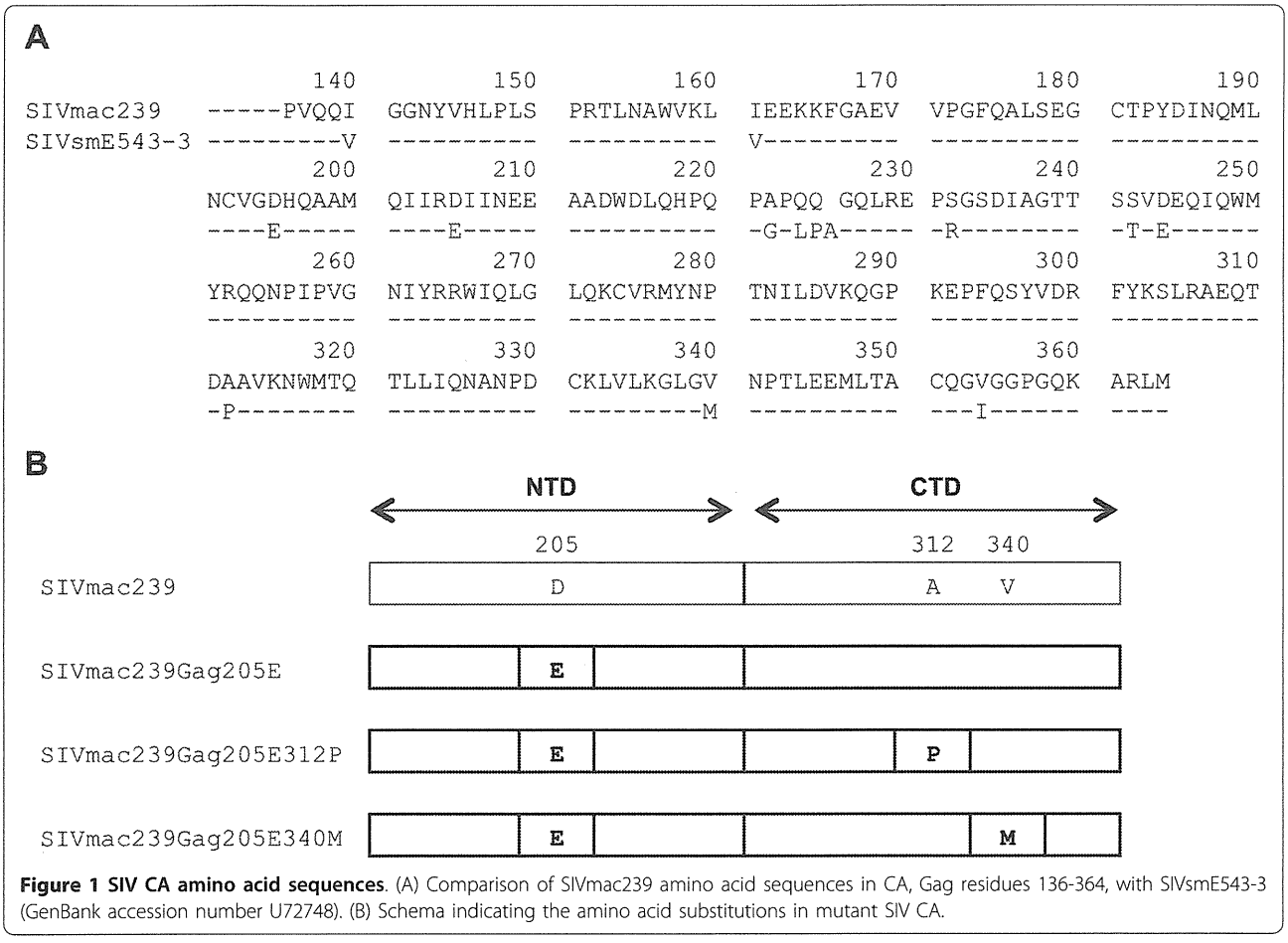

Figure 1 SIV CA amino acid sequences. (A) Comparison of SIVmac239 amino acid sequences in CA, Gag residues 136-364, with SIVsmE543-3 (GenBank accession number U72748). (B) Schema indicating the amino acid substitutions in mutant SIV CA.

Full recovery of viral fitness in SIVmac239Gag205E340M

We then focused on analyzing the possibility of functional interaction between Gag residues 205 in CA NTD and 312/340 in CA CTD. To confirm differences in viral fitness among SIVmac239, SIVmac239Gag205E, SIVmac239Gag205E312P, and SIVmac239Gag205E340M, we compared their replicative ability by viral competition assay (Table 1). The competitions confirmed lower viral fitness of SIVmac239Gag205E compared to wild-type SIVmac239, SIVmac239Gag205E312P, and SIVmac239Gag340M. SIVmac239Gag205E312P showed lower viral fitness than SIVmac239, whereas replication ability of SIVmac239Gag205E340M was no less than the wild-type. These results indicate that the GagD205E substitution in SIVmac239 reduced viral fitness, which was recovered partially by an additional GagA312P and fully by an additional GagV340M substitution. The competition between SIVmac239 and SIVmac239Gag205E340M at the ratio of 1:1 resulted in selection of the latter, suggesting that SIV CA with Gag205E-340M combination observed in SIVsmE543-3 may be slightly more functional than that with Gag205D-340V in SIVmac239.

Inhibition of the early phase of SIVmac239Gag205E replication

We examined whether the GagD205E substitution affects the early or late phase of SIVmac239 replication. On LuSIV cells, SIVmac239Gag205E infection showed significantly lower luciferase activity compared to wild-type SIVmac239, SIVmac239Gag205E312P, or SIVmac239Gag205E340M, indicating suppression of the early phase of SIVmac239GagD205E replication (Figure 5). In contrast, we did not find a significant difference in viral production among SIVmac239, SIVmac239Gag205E, SIVmac239Gag205E312P, and SIVmac239Gag205E340M (Figure 6). These results indicate that the loss of viral fitness by the GagD205E substitution is mainly due to inhibition of the early phase of viral replication.

Loss of in vitro core stability in SIVmac239Gag205E

If the GagD205E substitution disturbs intermolecular CA interaction for hexamer formation, it may affect SIV core stability. To assess the core stability in vitro [34], concentrated viruses were separated into three fractions by ultracentrifugation under gradient sucrose

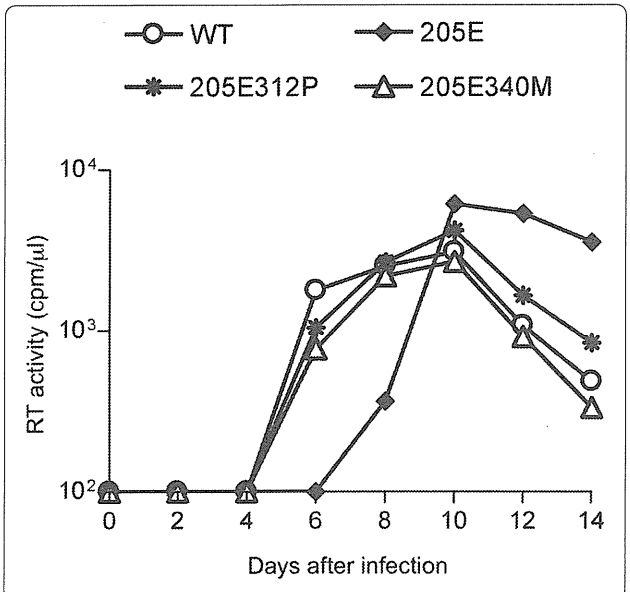

Figure 2 Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (WT, open circles), SIVmac239Gag205E (205E, closed diamonds), SIVmac239Gag205E312P (205E312P, asterisk), or SIVmac239Gag205E340M (205E340M, open triangles). Virus production was monitored by measuring RT activity in the culture supernatants. A representative result from five sets of experiments is shown.

concentrations in the presence of Triton X-100 and each fraction was subjected to Western blot analysis to detect CA p27 proteins (Figure 7). In the absence of Triton X-100, CA proteins were detected in the bottom fraction, whereas those in the presence of 1% Triton X-100 were sensitive to the detergent and detected not in the bottom but only in the top fraction (data not shown). We compared the in vitro viral core stability between SIVmac239 and SIVmac239Gag205E in the presence of 0.6%, 0.9%, and 1.35% Triton X-100, respectively, and found a difference in the presence of 0.6% Triton X-100. Additional experiments revealed that SIVmac239Gag205E core was more sensitive to 0.6% Triton X-100 treatment than SIVmac239, SIVmac239Gag205E312P, and SIVmac239Gag205E340M (Figure 7). These results suggest that viral core stability may be reduced by GagD205E substitution but can be recovered by additional GagA312P or GagV340M substitution.

Selection of GagD205E plus GagV340M mutations in a SIVmac239-infected macaque

The GagD205E substitution results in viral escape from $Gag_{206\text{-}216}$-specific CTL recognition. Finally, we examined whether this substitution can be selected in the chronic phase of SIVmac239 infection in *90-120-Ia*-positive macaques eliciting $Gag_{206\text{-}216}$-specific CTL responses using plasma samples obtained in our previous experiments [35,36]. SIVmac239-infected *90-120-Ia*-positive macaques select the GagL216S mutation resulting in viral escape from $Gag_{206\text{-}216}$-specific CTL recognition, but we found selection of both GagD205E and GagV340M mutations in viral genomes in one animal, R01-007 (Table 2). In this animal, GagD205E and GagV340M mutations were undetectable at week 123 after SIVmac239 challenge, but both became detectable at week 137 and were dominant at week 150. In contrast, the GagL216S mutation dominant at week 123 was not detected at week 150. These results present in vivo evidence indicating functional interaction between the Gag 205th residue in NTD and the 340th in CTD of SIV CA.

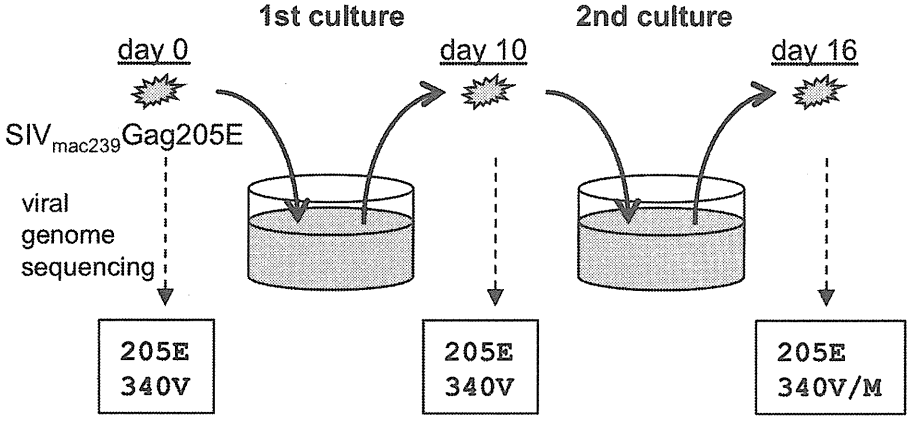

Figure 3 Passage of SIVmac239Gag205E culture supernatants. HSC-F cells were infected with SIVmac239Gag205E. The culture supernatant on day 10 was added to fresh HSC-F cells to start the second culture. Viral RNAs were extracted from the first culture supernatant on day 10 and the second culture supernatant on day 16 after the initial infection and subjected to sequence analyses. Dominant amino acid at the 340th residue remained V on day 10 in all cases but was M on day 16 in two of four sets of experiments (Gag340M was detectable on day 10 in these two sets of experiments). No other amino acid change was observed in the CA-coding region.

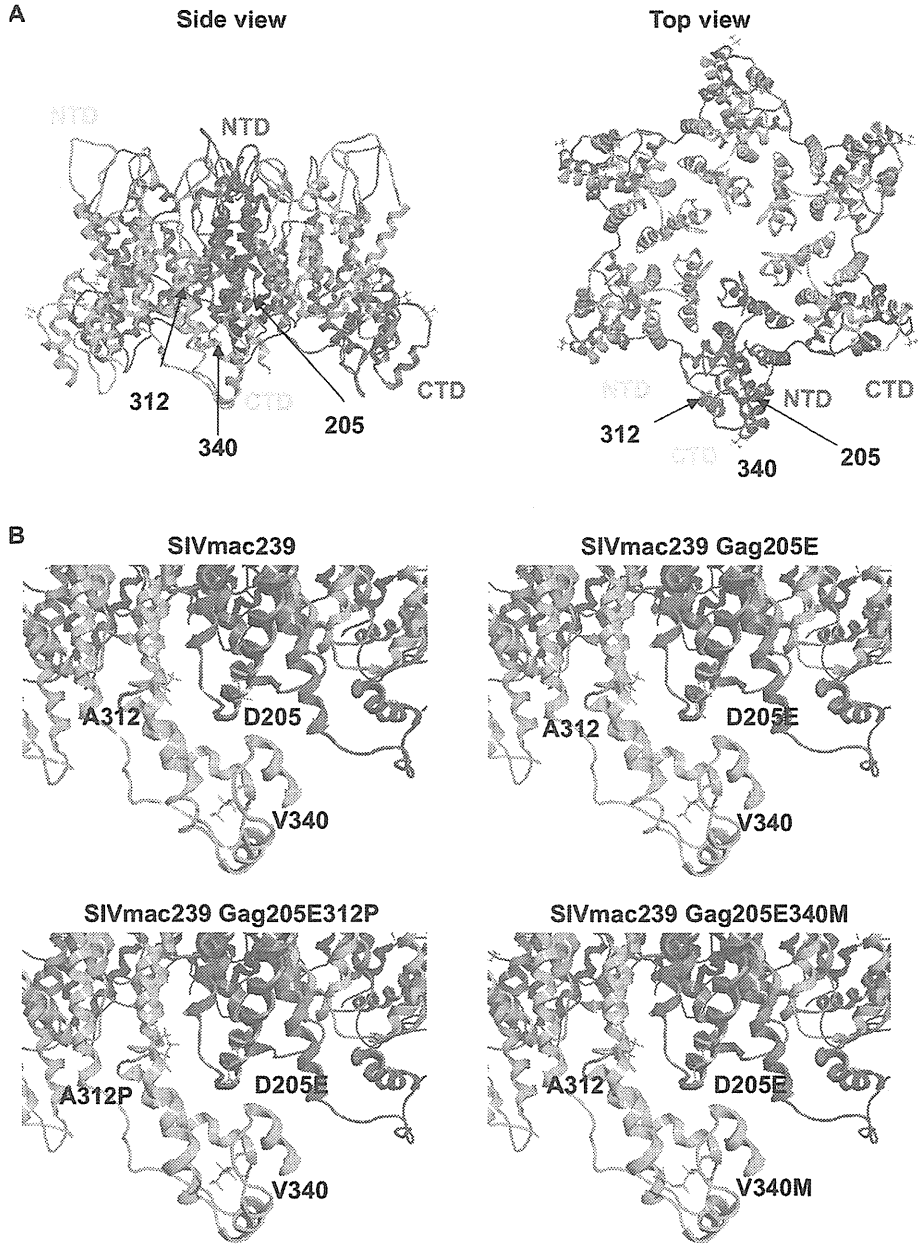

Figure 4 Structural models of SIVmac239 CA hexamer. The hexameric SIVmac239 CA models were constructed by homology-modeling using a crystal structure of the hexameric HIV-1 CA at a resolution of 1.90 Å (PDB code: 3H47[33]) as a modeling template. "MOE-Align" and "MOE-Homology" in MOE version 2008.1002 were used for the modeling. The side chains of the 205th, 312th, and 340th aa in Gag are shown as orange sticks. (A) Overall structure of SIVmac239 CA hexamer. (B) The hexameric structures near positions 205, 312, and 340 of wild-type and mutant SIVmac239 CAs.

Discussion

The Gag CA which is one of the most conserved proteins in HIV and SIV may be a promising immunogen for CTL-based AIDS vaccines. However, the limitations imposed on amino acid sequences in CA are not fully understood. In the present study, we found that the GagD205E substitution in SIVmac239 CA NTD reduces viral fitness, which is recovered by additional GagA312P or GagV340M substitution in the CTD. SIVmac239-Gag205E passaged in cell culture often resulted in selection of an additional GagV340M mutation. Furthermore, selection of Gag205E plus Gag340M mutations, but not Gag205E alone, was observed in a chronically SIVmac239-infected rhesus macaques. These results provide evidence indicating a functional interaction between Gag residues 205 in CA NTD and 340 in CA CTD,

Table 1 Competition between SIV mutants[a]

SIVs in competition	Ratio of inoc. titers[b]	Exp. no.	Dominant aa sequences[c]			
			day 6		day 18	
SIVmac239 & SIVmac239Gag205E	4:1	#1	205D		205D	
		#2	205D		205D	
	1:1	#1	205D		205D	
		#2	205D		205D	
	1:4	#1	205D		205D	
		#2	205D		205D	
SIVmac239 & SIVmac239Gag205E312P	4:1	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
	1:1	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
	1:4	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
SIVmac239 & SIVmac239Gag205E340M	4:1	#1	205D	340V	205D	340V
		#2	205D	340V	205D	340V
	1:1	#1	205D/E	340V/M	205E	340M
		#2	205D/E	340V/M	205E	340M
	1:4	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
SIVmac239Gag205E & SIVmac239Gag205E312P	4:1	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
	1:1	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
	1:4	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
SIVmac239Gag205E & SIVmac239Gag205E340M	4:1	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
	1:1	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
	1:4	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M

[a]HSC-F cells were coinfected with two kinds of SIVs indicated. Viral *gag* fragments were amplified by RT-PCR from viral RNAs from the culture supernatants on days 6 and 18 postinfection and then sequenced. Results from two sets of experiments (Exp. #1 and #2) are shown.

[b]The ratio of the dose (RT activity) of the virus indicated at the top to that at the bottom at coinfection.

[c]Dominant amino acid sequences at the positions where mutations were included in the inoculums are shown. 205D/E, D and E were detected equally at the 205th aa in Gag; 340 V/M, V and M were detected equally at the 340th aa in Gag.

presenting a structural constraint for functional interaction between SIV CA NTD and CTD.

HIV and SIV Gag proteins are expressed as unprocessed polyproteins, which are assembled and incorporated into the virions. Concomitant with viral budding, incorporated Gag polyproteins are proteolytically cleaved by viral protease into processed proteins including MA (matrix), CA, and NC (nucleocapsid), participating in mature infectious virion formation [37,38]. Recent structural analyses [31-33,39-41] indicated that CA proteins form hexamer lattice in matured virions; in the mature CA core, the intermolecular NTD-NTD and NTD-CTD interfaces are involved in the formation of CA hexamers, while the intermolecular CTD-CTD interface connects neighboring hexamers. Our modeling analyses did not support a possibility of intramolecular interaction but indicated possible intermolecular interaction between Gag205 in CA NTD and Gag312/340 in CA CTD, which may affect CA hexamer formation during viral maturation. This is consistent with our results in Figure 5 indicating that the GagD205E substitution results in inhibition of the early phase of SIVmac239 replication, which can be overcome by additional GagA312P or GagV340M substitution. This possibility is supported also by our results on viral core stability in vitro, although it remains unclear how much extent the

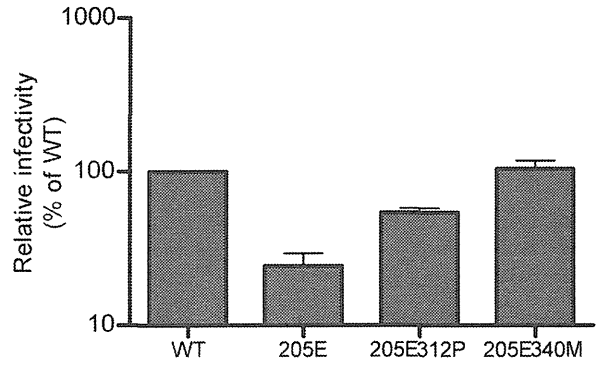

Figure 5 Mutant SIV infection. LuSIV cells were infected with SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340M (205E340M). Luciferase activity was measured 24 hr after infection. Relative infectivity is shown as the ratio (%) of the luciferase activity to that of SIVmac239 (WT). Mean values in three sets of experiments are shown.

core stability in vitro can reflect the one in vivo [42]. There has been no report suggesting the influence of the Gag 205 residue on SIV sensitivity to tripartite interaction motif 5α (TRIM5α). A previous report on HIV CA lattice [31,43] indicated a potential interaction between the helix 4 of NTD and the loop connecting helices 10 and 11 of CTD in the adjacent molecule. Our results suggest the possible involvement of Gag205 and Gag340 residues in this intermolecular NTD-CTD interaction in CA hexamers.

The molecular model of CA hexamers incorporating the GagD205E substitution suggested shortening of the distance between Gag205 and Gag340 residues, which looked to be compensated by GagV340M substitution (Figure 4). The modeling can draw a hydrophobic pocket between Gag205 and Gag340 residues in

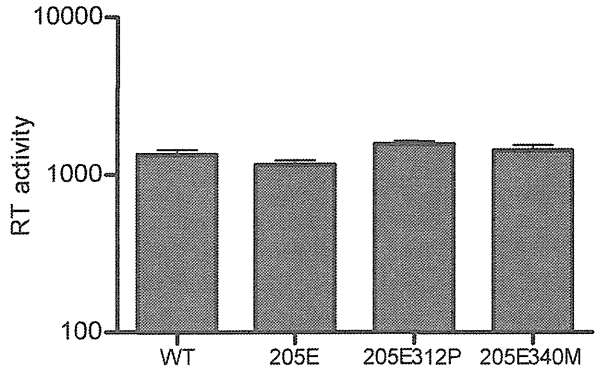

Figure 6 Mutant SIV production. COS-1 cells were transfected with molecular clone DNAs of SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340 M (205E340 M). RT activity of the culture supernatants two days after transfection was measured. Mean values in five sets of experiments are shown.

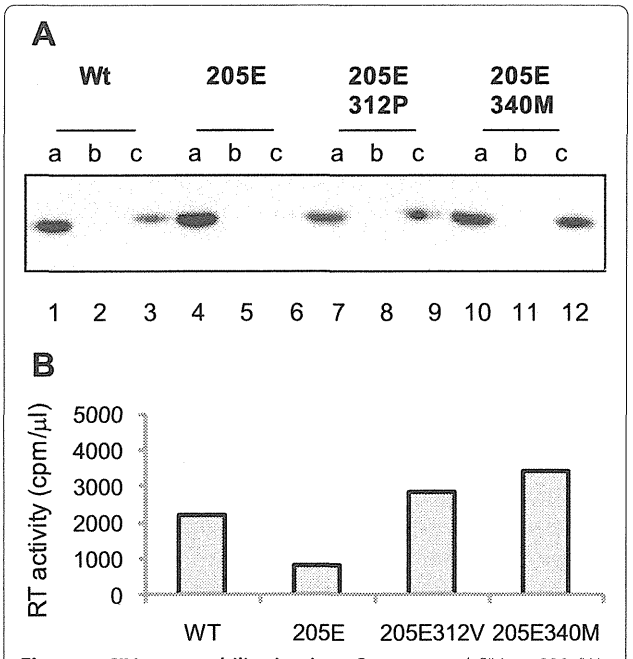

Figure 7 SIV core stability in vitro. Concentrated SIVmac239 (Wt; lanes 1-3), SIVmac239Gag205E (205E; lanes 4-6), SIVmac239Gag205E312P (205E312P; lanes 7-9), or SIVmac239Gag205E340M (205E340 M; lanes 10-12) was separated into three fractions (top [a], middle [b], and bottom [c]) by ultracentrifugation under gradient sucrose concentrations in the presence of 0.6% Triton X-100. Each fraction was subjected to Western blot analysis to detect SIV CA p27 proteins (A). A representative result from three sets of experiments is shown. The bottom (c) fractions were also subjected to RT assay (B).

SIVmac239Gag205E340M as well as SIVmac239, but not in SIVmac239Gag205E CA hexamers. Thus, this pocket may be a target candidate for anti-viral drugs.

Both GagL216S and GagD205E mutations can result in escape from $Gag_{206\text{-}216}$-specific CTL recognition [19,28], but the former is usually selected in SIVmac239-infected *90-120-Ia*-positive macaques probably

Table 2 Viral gag sequences in macaque R01-007 infected with SIVmac239[a]

Wks after challenge	Amino acid sequences[b]		
	at 205th	at 216th	at 340th
123	D	S	V
137	D (E)	S (L)	V (M)
150	E	L	M

[a]Viral RNAs were extracted from plasma obtained from a 90-120-Ia-positive macaque R01-007 at weeks 123, 137, and 150 after SIVmac239 challenge. Viral *gag* fragments were amplified by RT-PCR from viral RNAs and then sequenced. This animal showed efficient $Gag_{206\text{-}216}$-specific CTL responses and vaccine-based control of a SIVmac239 challenge with rapid selection of the GagL216S escape mutation (at week 5), but accumulated viral mutations in the chronic phase, leading to reappearance of plasma viremia around week 60 after challenge as described previously [19,35].

[b]Dominant amino acid sequences at the 205th, 216th, and 340th aa in Gag are shown. Parentheses indicate the sequences that are not dominant but detectable.

because the latter reduces viral fitness more severely than the former. In this study, we found selection of GagD205E plus GagV340M mutations in the chronic phase of SIVmac239 infection in a *90-120-Ia*-positive macaque. In this animal, the CTL escape GagL216S mutation first selected after SIVmac239 challenge became undetectable and was replaced with the CTL escape GagD205E mutation in combination with GagV340M in the chronic phase. This may imply that the GagD205E plus GagV340M mutations might be more advantageous than the GagL216S mutation for SIVmac239 replication in the presence of $Gag_{206-216}$-specific CTL pressure.

We observed the addition of GagV340M mutation but not a Gag205E-to-Gag205D reversion in SIVmac239-Gag205E passage. This may be due to difference in frequencies between purine-to-purine (guanine-to-adenine) change in the former and purine-to-pyrimidine (adenine-to-thymine) change in the latter. The appearance of additional GagV340M mutation in SIVmac239-Gag205E passaged in cell culture as well as the selection of GagD205E plus GagV340M mutations in an animal provides key evidence indicating functional interaction between Gag residues 205 in CA NTD and 340 in CA CTD. The Gag is a promising candidate as a vaccine immunogen for CTL induction, because cumulative studies have indicated the efficacy of Gag-specific CTL responses against HIV and SIV infection [7,25,44,45]. However, viral mutational escape from CTL recognition is a major challenge for AIDS vaccine design. Thus, the information on the structural constraint presented in this study might be helpful for immunogen design in AIDS vaccine development.

Conclusions

Our results present in vitro and in vivo evidence implicating the interaction between Gag residues 205 in CA NTD and 340 in CA CTD in SIV replication. SIV CA with Gag205D-340V (observed in SIVmac239) or Gag205E-340M combination (observed in SIVsmE543-3) is functional whereas the CA with Gag205E-340V is less functional. Thus, the present study indicates a structural constraint for functional interaction between SIV CA NTD and CTD, providing valuable information for immunogen design to limit viral escape options.

Methods

Analysis of mutant SIV replication

SIV molecular clone DNAs with gag mutations were constructed by site-directed mutagenesis from the wild-type SIVmac239 molecular clone DNA [24]. Virus stocks were obtained by transfection of COS-1 cells with wild-type or mutant SIV molecular clone DNAs using Lipofectamine LTX PLUS (Invitrogen, Tokyo, Japan). Viral titers were measured by reverse transcription (RT) assay as described previously [46]. For analysis of viral replication kinetics, HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) [47] were infected with wild-type or mutant SIVs (normalized by RT activity), and virus production was monitored by measuring RT activity in the culture supernatants. To examine viral infectivity, LuSIV cells, which are derived from CEMx174 cells and contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat, were cultured for 24 hr after viral infection and then lysed in a reporter lysis buffer (Promega Corp., Tokyo, Japan) for measurement of the luciferase activity in a luminometer (GloMax™ 96 Microplate Luminometer, Promega Corp.).

Viral competition assay

HSC-F cells were coinfected with two SIVs at a ratio of 1:1 or 1:4, and the culture supernatants harvested every other day were used for RT assays. On day 6, the supernatant was added to fresh HSC-F cells to start the second culture. Similarly, on day 12 after the initial coinfection, the second culture supernatant was added to fresh HSC-F cells to start the third culture. RNAs were extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan) from the initial culture supernatant on day 6 and from the third culture supernatant on day 18 post-coinfection. The fragment (nucleotides 1231 to 2958 in SIVmac239 [GenBank accession number M33262]) containing the entire gag region was amplified from the RNA by RT-PCR and sequenced to determine dominant sequences as described previously [19].

Molecular modeling of hexameric SIVmac239 CA

The crystal structures of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C[48]), HIV-1 CA CTD at a resolution of 1.70 Å (PDB code: 1A8O[5]), and hexameric HIV-1 CA at a resolution of 1.90 Å (PDB code: 3H47 [33]) were taken from the RCSB Protein Data Bank [49]. Three-dimensional (3-D) models of monomeric SIVmac239 CA were constructed by the homology modeling technique using 'MOE-Align' and 'MOE-Homology' in the Molecular Operating Environment (MOE) version 2008.1002 (Chemical Computing Group Inc., Quebec, Canada) as described [50,51]. We obtained 25 intermediate models per one homology modeling in MOE, and selected the 3-D models which were the intermediate models with best scores according to the generalized Born/volume integral methodology [52]. The final 3-D models were thermodynamically optimized by energy minimization using an AMBER99 force field [53] combined with the generalized Born model of aqueous solvation implemented in MOE [54]. Physically unacceptable

local structures of the optimized 3-D models were further refined on the basis of evaluation by the Ramachandran plot using MOE. The structures of hexameric SIVmac239 CA were generated from the monomeric structures by MOE on the basis of the assembly information of hexameric HIV-1 CA crystal structure [33].

Analysis of viral CA core stability in vitro

Detergent treatment of wild-type and mutant SIV particles was performed essentially as described previously [34]. Briefly, viruses from COS-1 cells transfected with viral molecular clone DNAs (normalized by RT activity) were concentrated by ultracentrifugation at 35,000 × rpm for 75 min at 4°C in a SW41 rotor (Beckman Instruments, Tokyo, Japan) through a cushion of 20% sucrose in phosphate buffered saline (PBS). The concentrated viral pellets were suspended in PBS. Sucrose step gradients were prepared in SW55 centrifuge tubes with the 2.0 ml layer of 60% sucrose on the bottom and 2.1 ml layer of 20% sucrose overlaid. Then, 0.1 ml of Triton X-100 in PBS and 0.5 ml of concentrated viruses were overlaid and ultracentrifuged at 35,000 × rpm for 60 min at 4°C in a SW55Ti rotor (Beckman Instruments). Three fractions (top [a], middle [b], and bottom [c]) of 1.1 ml each were collected from the top and subjected to Western blot analysis using plasma from a simian-human immunodeficiency virus 89.6PD-infected rhesus macaque [55] and RT assay.

Acknowledgements

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, a grant-in-aid from the Japan Society for the Promotion of Science, grants-in-aid from the Ministry of Health, Labor, and Welfare, and a grant from Takeda Science Foundation in Japan. NI is a Research Fellow of the Japan Society for the Promotion of Science.

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Authors' contributions

NI and TM designed the study. NI, HT, and AR performed virological analyses in vitro. MY and HS performed structure modeling analyses. HY and MK examined viral genome sequences. NI and TM analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 23 August 2010 Accepted: 18 October 2010
Published: 18 October 2010

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doi:10.1186/1742-4690-7-90
Cite this article as: Inagaki *et al.*: A structural constraint for functional interaction between N-terminal and C-terminal domains in simian immunodeficiency virus capsid proteins. *Retrovirology* 2010 7:90.

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Microbes and Infection 13 (2011) 58–64

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Original article

Improved capacity of a monkey-tropic HIV-1 derivative to replicate in cynomolgus monkeys with minimal modifications

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Received 31 July 2010; accepted 1 October 2010
Available online 16 October 2010

Abstract

Human immunodeficiency virus type 1 (HIV-1) hardly replicates in Old World monkeys. Recently, a mutant HIV-1 clone, NL-DT5R, in which a small part of *gag* and the entire *vif* gene are replaced with SIVmac239-derived ones, was shown to be able to replicate in pigtail monkeys but not in rhesus monkeys (RM). In the present study, we found that a modified monkey-tropic HIV-1 (HIV-1mt), MN4-5S, acquired the ability to replicate efficiently in cynomolgus monkeys as compared with the NL-DT5R, while neither NL-DT5R nor MN4-5S replicated in RM cells. These results suggest that multiple determinants may be involved in the restriction of HIV-1 replication in macaques, depending on the species of macaques. The new HIV-1mt clone will be useful for studying molecular mechanisms by which anti-viral host factors regulate HIV-1 replication in macaques.
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Keywords: HIV-1; Old World monkey; TRIM5α

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) productively infects only humans but not Old World monkeys (OWM) such as cynomolgus monkeys (CM) or rhesus monkeys (RM), whereas RM-derived simian immunodeficiency virus (SIVmac) can efficiently replicate in OWM. Because of this species barrier, alternative monkey models using SIVmac or simian/human immunodeficiency viruses (SHIV) have been used for AIDS research [1–4]. However, detailed analyses of molecular mechanisms of the pathogenesis of HIV-1 have been hampered by the lack of appropriate non-human primate models for HIV-1 infection.

The mechanistic basis for the inability of HIV-1 to replicate in OWM cells has remained unclear. Recently, a number of intrinsic anti-HIV-1 cellular factors, including tripartite motif protein 5α (TRIM5α), Cyclophilin A (CypA), apolipoprotein B mRNA-editing catalytic polypeptide (APOBEC3) family and Tetherin were discovered in OWM cells [5,6]. TRIM5α strongly suppresses HIV-1 replication, mainly by affecting the viral disassembly step, resulting in a decrease of reverse transcription products [7,8]. CypA acts as a regulator promoting TRIM5α-mediated restriction of HIV-1 [8]. APOBEC3 is also a major regulator of HIV-1 replication [9,10]. APOBEC3 exerts its inhibitory effect mainly by inducing G to A hypermutation

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doi:10.1016/j.micinf.2010.10.001

into the viral genome due to its cytidine deaminase activity, while hypermutation-independent inhibitory activity at the stage of reverse transcription is also evident [11]. Tetherin, also referred to as a BST-2, was identified as an intrinsic anti-viral factor that restricts the egress of HIV-1 by tethering virions to the host cell surface [12,13]. Importantly, HIV-1 can counteract human APOBEC3 activity by utilizing the viral accessory protein Vif, whereas it cannot counteract OWM APOBEC3 [14]. Similarly, HIV-1 counteracts human Tetherin activity by utilizing another viral accessory protein Vpu, whereas HIV-1 does not counteract OWM Tetherin activity [15].

In an attempt to generate a monkey-tropic HIV-1 (HIV-1mt), Kamada et al. constructed an HIV-1 variant carrying minimal SIVmac-derived sequences to overcome the restriction factors [16]. The prototype HIV-1 clone NL-DT5R had a sequence encoding an SIVmac loop between alpha helices 4 and 5 (L4/5) of *capsid* gene (CA) and the entire *vif* gene, which relieved the inhibitory effects on viral replication by restriction factors CypA, TRIM5α and APOBEC3. NL-DT5R was able to replicate in pigtail monkeys (PM) in vivo as well as in vitro, as reported by Igarashi et al. [17]. Although NL-DT5R induced immune responses in infected animals, the virus did not establish persistent infection.

In the present study, we sought to adapt NL-DT5R to CM by performing long-term passage in CM-derived HSC-F cells. We successfully obtained a modified HIV-1mt clone having several mutations. Additionally, we inserted an SIVmac loop between alpha helices 6 and 7 (L6/7) of CA [18]. The resultant clone named MN4-5S was found to replicate efficiently and to induce strong immune responses in infected CM, suggesting the impact of viral adaptation.

2. Materials and methods

2.1. Plasmid construction

The HIV-1 derivatives were constructed on a background of an infectious molecular clone, NL4-3 [19]. NL-DT5R, a cloned virus containing SIVmac239 L4/5 and the entire *vif* gene, was reported previously by Kamada et al. [16]. In addition, NL-DT562, a virus having an R5-tropic SF162-derived *env* gene on a background of NL-DT5R, was used in this study [20]. After long passage of NL-DT5R and NL-DT562 in cynomolgus T cell line HSC-F [21], several mutations were appeared in both viral genomes, and then all of them were inserted into NL-DT5R by gene-engineering techniques. Consequently, a clone having 14 nucleotide substitutions in its genome was constructed and named MN4-5. Among these substitutions, 7 were non-synonymous mutations. The structure of the clone is shown in Fig. 1. A part of L6/7 of CA (aa residues 120–122; HNP) of MN4-5 was also replaced with the corresponding segment of SIVmac239 CA (aa residues 120–123; RQQN) by means of site-directed mutagenesis as described previously in Ref. [18]. The resultant construct was designated MN4-5S.

2.2. Cells and viruses

Human embryonic kidney cell line HEK293T was maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin (Sigma). Monkey peripheral blood mononuclear cells (PBMCs) were separated with a standard Ficoll density gradient separation method and cultured in R-10 composed of

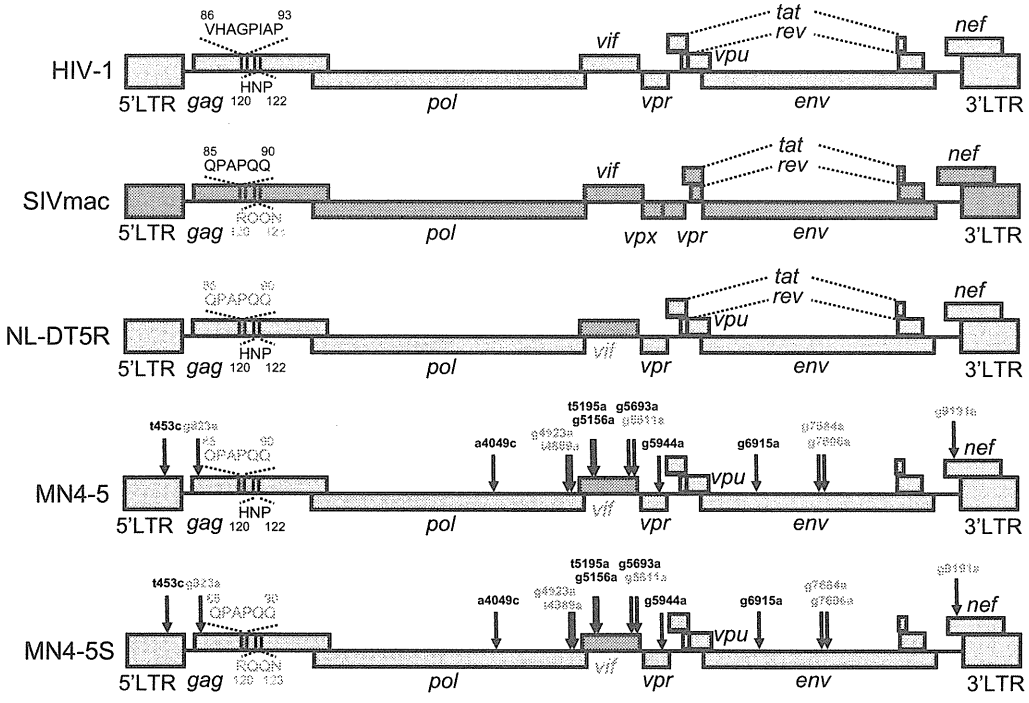

Fig. 1. Structure of HIV-1mt clones used in this study. The positions of nucleotide mutations are indicated by arrows in this figure. Among nucleotide substitutions, the positions of non-synonymous mutations are indicated in red.

RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin (Sigma). The growth kinetics of each HIV-1 clone were examined in activated $CD8^+$ cell-depleted PBMCs. Briefly, separated PBMCs were reacted with a PE-labeled anti-CD8 antibody and then treated with anti-PE magnetic beads. After washing, $CD8^+$ cell-depleted PBMCs were negatively separated by using MACS columns (Miltenyi Biotec). For stimulation, $CD8^+$ cell-depleted PBMCs were first cultured in R-10 containing 1 μg/ml of concanavalin A (Sigma) for 2 days followed by culture in R-10 supplemented with 100 U/ml IL-2 (Shionogi) for more 2 days. The cells were then infected with 100 ng of p24 of HIV-1 and the culture supernatant was collected periodically. HSC-F, a cynomolgus monkey-derived $CD4^+$ T cell line [21], was cultured in R-10.

Virus stocks were prepared as follows: sub-confluent HEK293T cells were transfected with proviral DNA using Lipofectamine2000 reagent according to the manufacturer's instructions. At 42 h after transfection, culture supernatants were centrifuged, filtrated with a 0.45 μm filter, and aliquoted as virus stocks for in vitro experiments. For preparation of viral stocks for in vivo experiments, $CD8^+$ cell-depleted PBMCs were infected with the HEK293T-derived stocks as described above. After washing, the cells were maintained for several days and the culture supernatants were collected and stored as described above.

2.3. Reverse transcription (RT) assay

Virion-associated RT activity was measured as described previously in Ref. [22]. HSC-F cells (1×10^6) were infected with equal amounts of viruses (1×10^7 RT units). Viral growth kinetics was determined by RT production in the culture supernatants.

2.4. Animal experiments

Healthy adult cynomolgus monkeys were used in this study. All animals were confirmed to be negative for simian retrovirus and were housed in individual isolators in a biosafety level 3 facility and maintained according to the National Institute of Biomedical Innovation rules and guidelines for experimental animal welfare. Bleeding and viral inoculation were performed under ketamine hydrochloride anesthesia. Viral stocks for inoculation were inoculated into each animal. The profiles of plasma viral RNA loads, circulating $CD4^+$ and $CD8^+$ T lymphocytes were evaluated as described below.

2.5. Flow cytometry and immunophenotyping of peripheral blood lymphocytes

Immunophenotyping of freshly isolated PBMCs was performed according to standard procedures using multicolor flow cytometry performed with a FACSCantoII (Becton Dickinson). $CD4^+$ and $CD8^+$ T cells were identified using monoclonal antibodies (mAbs) to CD3 (clone SP34-2, BD Pharmingen), CD4 (clone L200, BD Pharmingen) and CD8 (clone DK25, DAKO). Flow cytometric acquisition and analysis of samples was performed on at least 10,000 events collected by a flow cytometer driven by FACSDiVa software.

2.6. Analysis of anti-viral antibody response

Plasma samples from infected animals were first heat-inactivated at 56 °C for 30 min. Then, 100-fold diluted samples were reacted with commercially available anti-HIV-1 antibody detection strips (New LAV Blot I, Bio-Rad) according to the manufacture's instructions.

2.7. In vivo depletion of $CD8^+$ lymphocytes

Infected animals received an anti-CD8 mAb (cM-T807) as follows: 10 mg/kg (body weight) inoculation subcutaneously at 42 days post infection (DPI), followed by 5 mg/kg inoculation intravenously at 45, 49, and 52 DPI. The cM-T807 mAb was provided by the NIH Nonhuman Primate Reagent Resource. To repeatedly confirm the depletion of $CD8^+$ cells in the presence of cM-T807, an anti-CD8 mAb (clone DK25, DAKO) was used as reported previously in Ref. [23].

2.8. Quantification of viral RNA

Total RNA was collected from monkey plasma using a High Pure Viral RNA Kit (Roche Diagnostics) according to the manufacturer's instructions. Viral RNA was quantified with a quantitative real-time PCR system using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems). The primers and probe used in this study were as follows: Forward primer: HIVgag683 (+) (5′-CTCTCGACGCAGGACTCGGCTTGCT-3′); Reverse primer: HIVgag803 (−) (5′-GCTCTCGCACCCATCTCTCTCCTTCTAGCC-3′); Probe: HIVgag TaqMan 720R748 (FAM-GCAAGAGGCGAGRGGCGGCGACTGGTGAG-TAMRA). The quantification and data analysis were performed using the iQ5 Real-Time PCR Detection System (Bio-Rad). The detection limit of this assay was 400-copies/ml plasma.

3. Results

3.1. Growth properties of prototype HIV-1mt clone, NL-DT5R in macaques in vitro and in vivo

We first examined the replication properties of prototype HIV-1mt NL-DT5R in $CD8^+$ cell-depleted PBMCs of CM and RM. NL-DT5R replicated in the cells of CM but not in those of RM (Fig. 2). We next examined the in vivo replication properties of NL-DT5R in CM. Viral stocks for inoculation were prepared with $CD8^+$ cell-depleted CM PBMCs as described above. Then, two monkeys were infected with NL-DT5R intravenously and bled periodically. As shown in Fig. 3A, NL-DT5R established infection as indicated by detectable levels of plasma viremia and an anti-HIV-1 antibody response, although the viral level was marginal (about 1×10^3 copies/ml) and disappeared at 4 weeks post infection.