Table 1 | Association of MHC-I haplotypes with disease progression in SIV infection (Nomura et al., 2012).

MHC-I haplotypes	Mean survival periods	Geometric means of setpoint plasma viral loads (copies/ml)	Peripheral $CD4^+$ T cell decline	Predominant CTL responses
90-120-Ia	>40 months	10^4	Slow	Gag/Nef
90-010-Ie	23 months	10^5	Intermediate	Nef
90-120-Ib	24 months	10^5	Intermediate	Nef
90-088-Ij	15 months	10^6	Rapid	-

In our study (Nomura et al., 2012), the group of Burmese rhesus macaques possessing MHC-I haplotype *90-010-Ie* (dominant MHC-I alleles: *A1*066:01* and *B*005:02*) exhibited a typical pattern of disease progression after SIVmac239 challenge (**Table 1**). These animals showed predominant Nef-specific CTL responses, approximately 10^5 copies/ml of setpoint plasma viral loads (geometric means), and 2 years of mean survival periods. Another group of macaques possessing *90-120-Ib* (dominant MHC-I alleles: *A1*018:08* and *B*036:03*) showed similar setpoint viral loads and survival periods. However, the group of Burmese rhesus macaques possessing MHC-I haplotype *90-088-Ij* (dominant MHC-I alleles: *A1*008:01* and *B*007:02*) showed higher setpoint plasma viral loads (geometric means: about 10^6 copies/ml) and shorter survival periods (means: about 15 months; **Table 1**). These animals mostly showed poor CTL responses.

In contrast, the group of Burmese rhesus macaques possessing MHC-I haplotype *90-120-Ia* (dominant MHC-I alleles: *A1*043:01* and *B*061:03*), referred to as A^+ animals, showed lower setpoint plasma viral loads (geometric means: about 10^4 copies/ml) and slower disease progression (means of survival periods: more than 40 months; **Table 1**). These animals predominantly elicited Gag-specific and Nef-specific CTL responses after SIVmac239 challenge. Mamu-A1*043:01-restricted $Gag_{206-216}$ (IINEEAADWDL) and Mamu-A1*065:01-restricted $Gag_{241-249}$ (SSVDEQIQW) were determined as dominant CTL epitopes. SIVmac239-infected A^+ animals selected viral escape mutations from these epitope-specific CTL responses with viral fitness costs in the chronic phase (Kobayashi et al., 2005; Kawada et al., 2006). These mutations are GagL216S, a mutation leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid in SIVmac239 Gag, and GagD244E, aspartic acid (D)-to-glutamic acid (E) at the 244th, or GagI247L, isoleucine [I]-to-L at the 247th. A^+ animals immunized with a prophylactic prime-boost vaccine consisting of a DNA prime followed by a boost with a recombinant Sendai virus vector expressing SIVmac239 Gag controlled an SIVmac239 challenge (Matano et al., 2004). However, vaccinated A^+ animals failed to control a challenge with a mutant SIVmac239 carrying GagL216S and GagD244E, indicating that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses are responsible for the control of the wild-type SIVmac239 replication (Kawada et al., 2006, 2008). Interestingly, the Mamu-A1*065:01-restricted SIVmac239 $Gag_{241-249}$ epitope is located in a region corresponding to the HLA-B*57-restricted HIV $Gag_{240-249}$ epitope TW10 and TW10-specific CTL responses have also been indicated to exert strong suppressive pressure on HIV replication. An SIVmac239 $Gag_{241-249}$-specific CTL escape mutation, GagD244E, results in loss of viral fitness similarly with an HIV TW10-specific CTL escape mutation. Both of the Mamu-A1*065:01-restricted SIVmac239 $Gag_{241-249}$ epitope and the HLA-B*57-restricted HIV TW10 epitope are considered to have the same anchor residues, S at position 2 and tryptophan (W) at the carboxyl terminus. Additionally, anchor residues of CTL epitopes presented by Mamu-B*17/Mamu-B*08 were indicated to be similar to those restricted by HLA-B*57/HLA-B*27 (Loffredo et al., 2009; Wu et al., 2011).

CONCLUDING REMARKS

Human HLA genotypes largely affect disease progression in HIV infection, reflecting that CTL responses play a central role in suppression of HIV replication. Animal AIDS models are required for understanding of the interaction between highly diversified viruses and the hosts with polymorphic MHC-I genotypes. SIV infection of Indian rhesus macaques are widely used as an AIDS model, and association of certain MHC-I alleles with slower disease progression has been indicated. We have recently reported SIV infection of Burmese rhesus macaques as a robust AIDS model and indicated association of MHC-I haplotypes with disease progression. Accumulation of those macaque groups sharing MHC-I haplotypes could lead to constitution of a more sophisticated AIDS model facilitating analysis of virus-host immune interaction.

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Original article

Immunogenicity of repeated Sendai viral vector vaccination in macaques

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Abstract

Induction of durable cellular immune responses by vaccination is an important strategy for the control of persistent pathogen infection. Viral vectors are promising vaccine tools for eliciting antigen-specific T-cell responses. Repeated vaccination may contribute to durable memory T-cell induction, but anti-vector antibodies could be an obstacle to its efficacy. We previously developed a Sendai virus (SeV) vector vaccine and showed the potential of this vector for efficient T-cell induction in macaques. Here, we examined whether repeated SeV vector vaccination with short intervals can enhance antigen-specific $CD8^+$ T-cell responses. Four rhesus macaques possessing the MHC-I haplotype 90-120-Ia were immunized three times with intervals of three weeks. For the vaccination, we used replication-defective F-deleted SeV vectors inducing $CD8^+$ T-cell responses specific for simian immunodeficiency virus $Gag_{206-216}$ and $Gag_{241-249}$, which are dominant epitopes restricted by 90-120-Ia-derived MHC-I molecules. All four animals showed higher $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses after the third vaccination than those after the first vaccination, indicating enhancement of antigen-specific $CD8^+$ T-cell responses by the second/third SeV vector vaccination even with short intervals. These results suggest that repeated SeV vector vaccination can contribute to induction of efficient and durable T-cell responses.

Keywords: AIDS vaccine; Viral vector; CTL; SIV

1. Introduction

Antigen-specific T-cell responses play a central role in the control of persistent infection with pathogens such as human immunodeficiency viruses (HIVs) [1–7]. Induction of efficient and durable T-cell responses is an important vaccine strategy, and recombinant viral vectors are promising vaccine tools for antigen-specific T-cell induction [8]. Many kinds of viral vectors including adenovirus (AdV) and poxvirus vectors have been shown to efficiently induce antigen-specific T-cell responses [9–14]. Repeated viral vector vaccination may induce enhanced and durable memory T-cell responses. Viral vector vaccination, however, elicits antibodies against the vector virus itself, which could be an obstacle to the potential of repeated viral vector vaccination [15].

We previously developed a vaccine system using Sendai virus (SeV) vectors to induce antigen-specific T-cell responses [16,17]. We have replication-defective (nontransmissible) F-deleted SeV, F(-)SeV, as well as replication-competent SeV vectors [18,19]. In our recent study [20], intranasal immunization even with a lower dose (6×10^8 CIU [cell infectious units]) of F(-)SeV vectors in SeV-infected macaques efficiently elicited antigen-specific $CD8^+$ T-cell responses in the presence of SeV-specific neutralizing titers of 1:50–1:100.

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Intramuscular F(-)SeV vector immunization can also induce antigen-specific $CD8^+$ T-cell responses efficiently in the absence of anti-SeV neutralizing antibodies, but intranasal F(-)SeV vector vaccination is more immunogenic than intramuscular in the presence of anti-SeV neutralizing antibodies.

Thus, repeated intranasal SeV vector immunization may have the potential to overcome anti-SeV antibody responses and induce more efficient and durable T-cell responses than those by single immunization. Our previous analyses in macaques [21] showed efficient antigen-specific $CD8^+$ T-cell induction by an intranasal immunization with 6×10^9 CIU of F(-)SeV vectors more than one year after an initial SeV vector inoculation, indicating the immunogenicity of repeated SeV vector vaccination with long intervals.

In the present study, we investigated whether repeated SeV vector vaccination with short intervals can enhance antigen-specific $CD8^+$ T-cell responses. Macaques received SeV vectors intranasally and intramuscularly at the second and the third vaccination at weeks 3 and 6 after the first intranasal SeV vector vaccination. While clear difference in immunogenicity was not shown between intranasal and intramuscular SeV vector administration, all the animals exhibited higher antigen-specific $CD8^+$ T-cell responses after the third vaccination than those after the first. Our results indicate that repeated SeV vector vaccination even with short intervals can contribute to induction of efficient, durable T-cell responses.

2. Materials and methods

2.1. Animals

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation with the help of the Corporation for Production and Research of Laboratory Primates. All animals were maintained in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases and TPRC. Blood collection and vaccination were performed under ketamine anesthesia.

In the present study, we used four Burmese rhesus macaques possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [22]; those *90-120-Ia*-positive macaques are known to dominantly elicit SIVmac239 $Gag_{206-216}$ (IINEEAADWDL) epitope-specific and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific $CD8^+$ T-cell responses after SIVmac239 challenge [23,24]. In our previous study [25–27], these four macaques were challenged with SIV after vaccination and controlled SIV replication in the chronic phase as follows. Macaque R04-016 received a prophylactic prime-boost vaccine eliciting single $Gag_{241-249}$ epitope-specific $CD8^+$ T-cell responses before SIVmac239 challenge [26]. Macaque R04-015 received a prophylactic prime-boost vaccine eliciting $Gag_{206-216}$ epitope-specific and $Gag_{241-249}$ epitope-specific $CD8^+$ T-cell responses [27,28]. Macaques R06-015 and R06-035 received a prophylactic DNA prime/F(-)SeV-Gag boost vaccine [25].

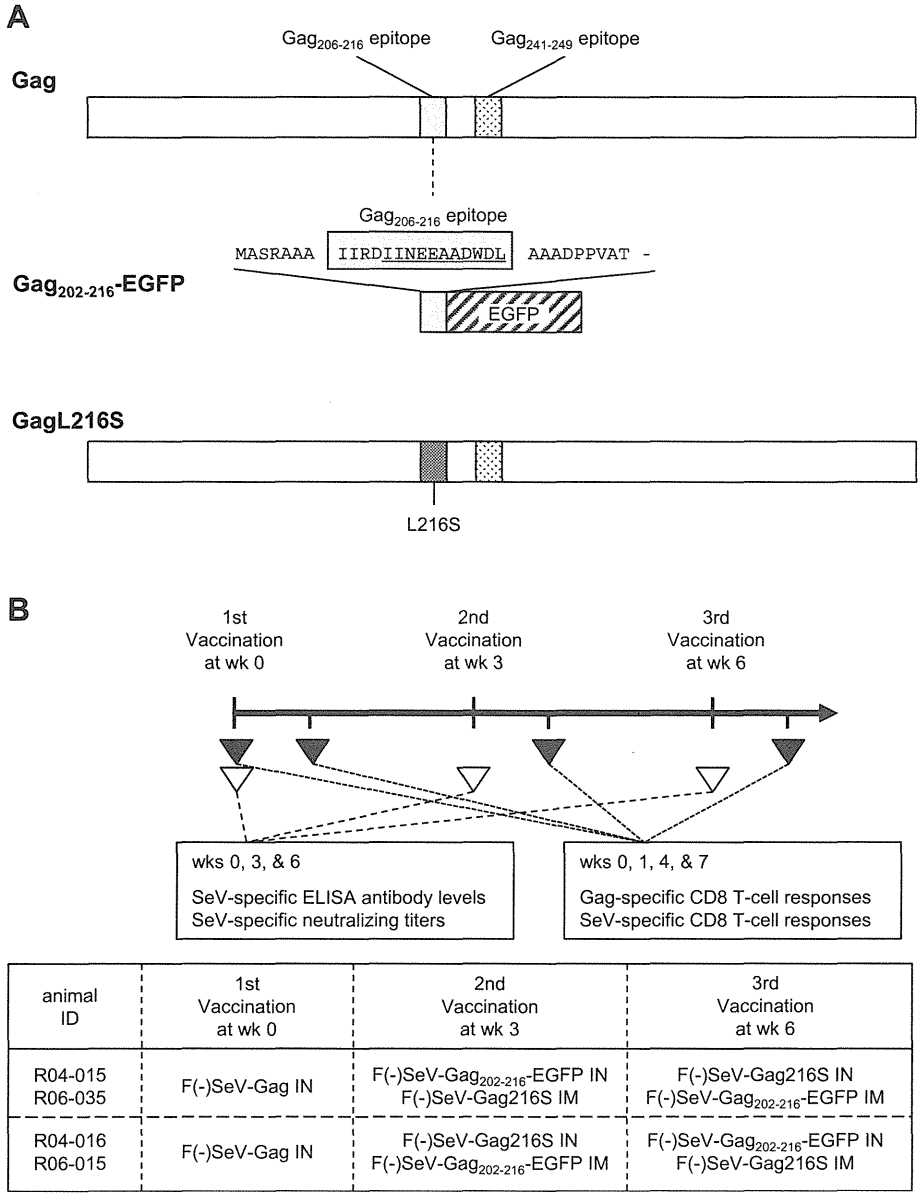
Macaques R04-015, R04-016, and R06-015 contained a challenge with SIVmac239 [29] approximately 3 months after the boost and a superchallenge with a mutant SIVmac239 carrying five gag mutations, SIVmac239Gag216S244E247L312V373T, in the chronic phase (at week 40 [R06-015] or 116 [R04-015 and R04-016] after SIVmac239 challenge) [27]. Macaque R06-035 was challenged with a mutant SIVmac239, SIVmac239-Gag216S244E, carrying two gag mutations, GagL216S and GagD244E leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid and an aspartic acid (D)-to-glutamic acid (E) substitution at the 244th in Gag [25]. These mutations result in escape from $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses. In this animal that failed to control the mutant SIV challenge, persistent viremia was observed for 8 months, but after that, plasma viremia became undetectable.

2.2. Vaccination

In the present study, macaques R04-015, R04-016, R06-015, and R06-035 received an intranasal F(-)SeV-Gag vector vaccination (referred to as the first vaccination) at weeks 128, 128, 52, and 67 after SIV challenge, respectively. At the second and the third vaccination, we used two kinds of F(-)SeV vectors, F(-)SeV-$Gag_{202-216}$-EGFP expressing $Gag_{202-216}$-EGFP fusion protein and F(-)SeV-Gag216S expressing SIVmac239 Gag with a mutation leading to escape from $Gag_{206-216}$-specific $CD8^+$ T-cell responses [23], for eliciting $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses, respectively (Fig. 1). The F(-)SeV-Gag216S is considered not to induce $Gag_{206-216}$-specific $CD8^+$ T-cell responses, because challenge of macaques with a SIV carrying this Gag216S mutation induced no $Gag_{206-216}$-specific $CD8^+$ T-cell responses in a previous study [25]. Macaques R04-015 and R06-035 received F(-)SeV-$Gag_{202-216}$-EGFP intranasally and F(-)SeV-Gag216S intramuscularly at the second vaccination three weeks after the first. At the third vaccination three weeks after the second, these animals received F(-)SeV-Gag216S intranasally and F(-)SeV-$Gag_{202-216}$-EGFP intramuscularly. On the contrary, macaques R04-016 and R06-015 received F(-)SeV-Gag216S intranasally and F(-)SeV-$Gag_{202-216}$-EGFP intramuscularly at the second vaccination and F(-)SeV-$Gag_{202-216}$-EGFP intranasally and F(-)SeV-Gag216S intramuscularly at the third. The dose of each vaccination was 6×10^9 cell infectious units (CIU). During the observation period in the present study, plasma viremia was undetectable in all four macaques.

2.3. Measurement of antigen-specific $CD8^+$ T-cell responses

We measured antigen-specific $CD8^+$ T-cell frequencies by flow-cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously [21,27]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) pulsed with 1 μM $Gag_{206-216}$ or $Gag_{241-249}$ peptide for $Gag_{206-216}$-specific or $Gag_{241-249}$-specific stimulation. For SeV-specific stimulation, PBMCs were cocultured with B-LCLs infected with SeV. Intracellular IFN-γ staining was performed using CytofixCytoperm kit



animal ID	1st Vaccination at wk 0	2nd Vaccination at wk 3	3rd Vaccination at wk 6
R04-015 R06-035	F(-)SeV-Gag IN	F(-)SeV-Gag$_{202-216}$-EGFP IN F(-)SeV-Gag216S IM	F(-)SeV-Gag216S IN F(-)SeV-Gag$_{202-216}$-EGFP IM
R04-016 R06-015	F(-)SeV-Gag IN	F(-)SeV-Gag216S IN F(-)SeV-Gag$_{202-216}$-EGFP IM	F(-)SeV-Gag$_{202-216}$-EGFP IN F(-)SeV-Gag216S IM

Fig. 1. Vaccination protocols. (A) Inserts of F(-)SeV vectors used for vaccination. F(-)SeV-Gag, F(-)SeV-Gag$_{202-216}$-EGFP, and F(-)SeV-Gag216S express the SIVmac239 Gag, a Gag$_{202-216}$-EGFP fusion protein, and a mutant Gag with a leucine-to-serine substitution at the 216th amino acid (L216S), respectively. The L216S substitution results in escape from Gag$_{206-216}$-specific CD8$^+$ T-cell recognition. (B) Protocols for macaque vaccination. Four *90-120-Ia*-positive macaques received F(-)SeV-Gag intranasally (IN) at the first vaccination. Macaques R04-015 and R06-035 received F(-)SeV-Gag$_{202-216}$-EGFP IN and F(-)SeV-Gag216S intramuscularly (IM) at the second vaccination and vice versa at the third. Macaques R04-016 and R06-015 received F(-)SeV-Gag216S IN and F(-)SeV-Gag$_{202-216}$-EGFP IM at the second vaccination and vice versa at the third.

(BD, Tokyo, Japan) and the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (BD, #556615, M-T477), peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD, #347314, SK1), allophycocyanin (APC)-conjugated anti-human CD3 (BD, #557597, SP34-2), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (BD, #557074, 4S.B3). Specific CD8$^+$ T-cell levels were calculated by subtracting non-specific IFN-γ$^+$ CD8$^+$ T-cell frequencies from those after Gag epitope peptide-specific or SeV-specific stimulation. Specific CD8$^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

2.4. Measurement of 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 [30].

2.5. Measurement of anti-SeV neutralizing titers

We measured plasma SeV-specific neutralizing titers on LLC-MK2 cells using a recombinant SeV expressing enhanced

green fluorescent protein (SeV-EGFP) as described before [31]. We determined the end-point plasma titers required for 10-fold reduction of SeV-EGFP infectivity compared to the negative control without plasma (90% neutralization titer; 90% effective concentration [EC_{90}]).

3. Results

3.1. Antigen-specific CD8$^+$ T-cell responses after repeated F(-)SeV vector vaccination

We used four SIV controllers possessing the MHC-I haplotype *90-120-Ia* [22,24]. In these rhesus macaques that were vaccinated and challenged with SIV in our previous studies [25,27], plasma viremia was undetectable in the chronic phase. In the present study, these four animals received SeV vector vaccination three times with intervals of three weeks in the chronic phase. Three kinds of SeV vectors, F(-)SeV-Gag inducing both $Gag_{206-216}$ epitope-specific and $Gag_{241-249}$ epitope-specific CD8$^+$ T-cell responses, F(-)SeV-$Gag_{202-216}$-EGFP inducing the former, and F(-)SeV-Gag216S inducing the latter, were used for the vaccination (Fig. 1). All four macaques received an intranasal F(-)SeV-Gag vector inoculation at the first vaccination. Macaques R04-015 and R06-035 received F(-)SeV-$Gag_{202-216}$-EGFP intranasally and F(-)SeV-Gag216S intramuscularly at the second vaccination and vice versa at the third. Macaques R04-016 and R06-015 received F(-)SeV-Gag216S intranasally and F(-)SeV-$Gag_{202-216}$-EGFP intramuscularly at the second vaccination and vice versa at the third.

Previously, macaques possessing the MHC-I haplotype *90-120-Ia* were shown to induce $Gag_{206-216}$ and $Gag_{241-249}$ epitope-specific CD8$^+$ T-cell responses dominantly in the early phase after SIVmac239 challenge [25,27]. In the present study, we examined these $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell responses one week after each vaccination (Fig. 2). The first F(-)SeV-Gag vaccination enhanced both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell responses in macaques R06-035 and R06-015 but not in R04-016. In macaque R04-015, efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell responses were detected at week 1 after the first vaccination, while PBMC samples were unavailable for analysis of responses just before the first vaccination.

At week 4, one week after the second vaccination, animals had similar or higher $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell frequencies compared to those at week 1. Also at week 7, one week after the third vaccination, animals had similar or higher $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell frequencies compared to those at week 4. Enhancement of these Gag-specific CD8$^+$ T-cell response was clear after the second vaccination (at week 4) in macaques R04-016 and R06-035 and after the third vaccination (at week 7) in macaques R04-015 and R06-015. Thus, all four animals showed higher Gag-specific CD8$^+$ T-cell responses at week 7 compared to those at week 1, indicating enhancement of Gag-specific CD8$^+$ T-cell responses by the second/third vaccination.

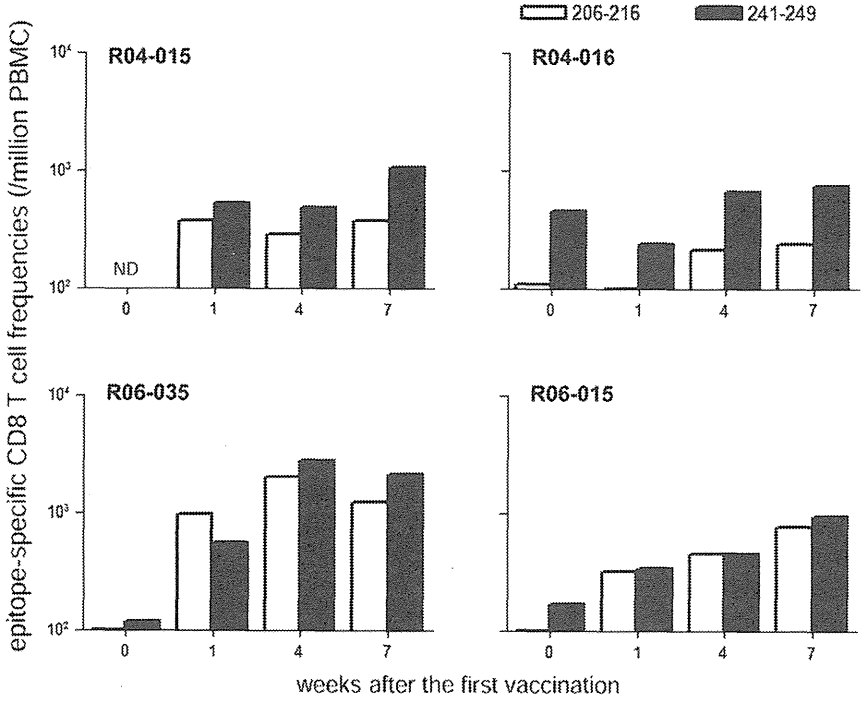


Fig. 2. Gag epitope-specific CD8$^+$ T-cell responses after repeated SeV vector vaccination. $Gag_{206-216}$-specific (open boxes) and $Gag_{241-249}$-specific (closed boxes) CD8$^+$ T-cell responses at week 0 (just before the first vaccination) and at weeks 1, 4, and 7 after the first vaccination (one week after each vaccination) were examined. ND, not determined.

3.2. SeV-specific CD8$^+$ T-cell responses after repeated F(-)SeV vector vaccination

We also examined SeV-specific CD8$^+$ T-cell responses one week after each vaccination (Fig. 3). SeV-specific CD8$^+$ T-cell responses were undetectable at week 0, just before the first vaccination. Vaccination mostly enhanced SeV-specific CD8$^+$ T-cell responses, and all animals showed efficient SeV-specific CD8$^+$ T-cell responses after three times of vaccination. Clear difference in the patterns of enhancement was not observed between $Gag_{206-216}$/$Gag_{241-249}$-specific and SeV-specific CD8$^+$ T-cell responses, suggesting little or no change in the immunodominance patterns between these CD8$^+$ T-cell responses by the second/third SeV vector vaccination.

3.3. SeV-specific antibody responses at repeated F(-)SeV vector vaccination

We then examined SeV-specific antibody responses just before each vaccination (Fig. 4 and Fig. 5). While these animals received a single SeV vector vaccination in previous studies more than a year before, ELIZA showed marginal levels of SeV-specific antibodies at week 0, just before the first vaccination. These animals exhibited undetectable or low levels of SeV-specific neutralizing antibody responses at week 0. At weeks 3 and 6, just before the second/third vaccination, all four animals had high levels of SeV-specific IgGs and neutralizing titers, indicating that the second/third SeV vector vaccination enhanced Gag-specific CD8$^+$ T-cell responses in the presence of high levels of SeV-specific neutralizing responses.

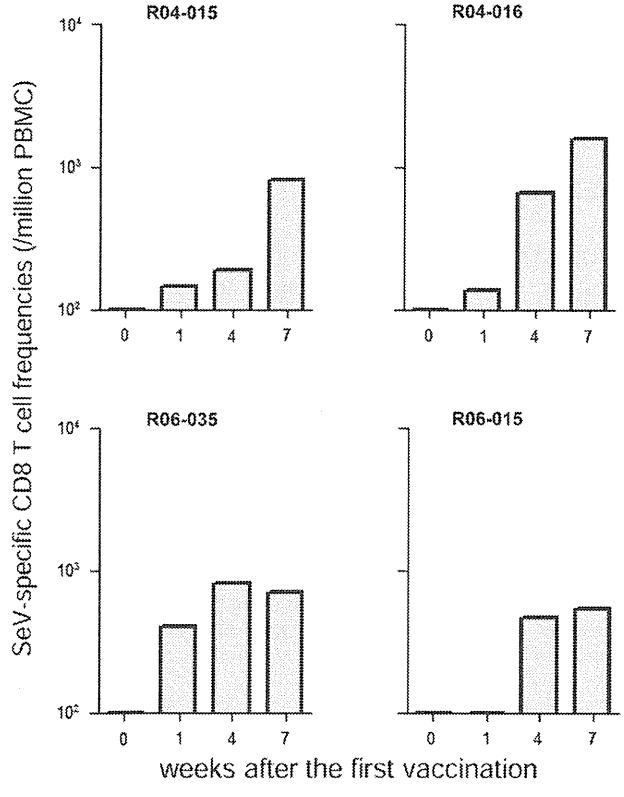


Fig. 3. SeV-specific CD8$^+$ T-cell responses after repeated SeV vector vaccination. SeV-specific CD8$^+$ T-cell responses at week 0 (just before the first vaccination) and at weeks 1, 4, and 7 after the first vaccination (one week after each vaccination) were examined.

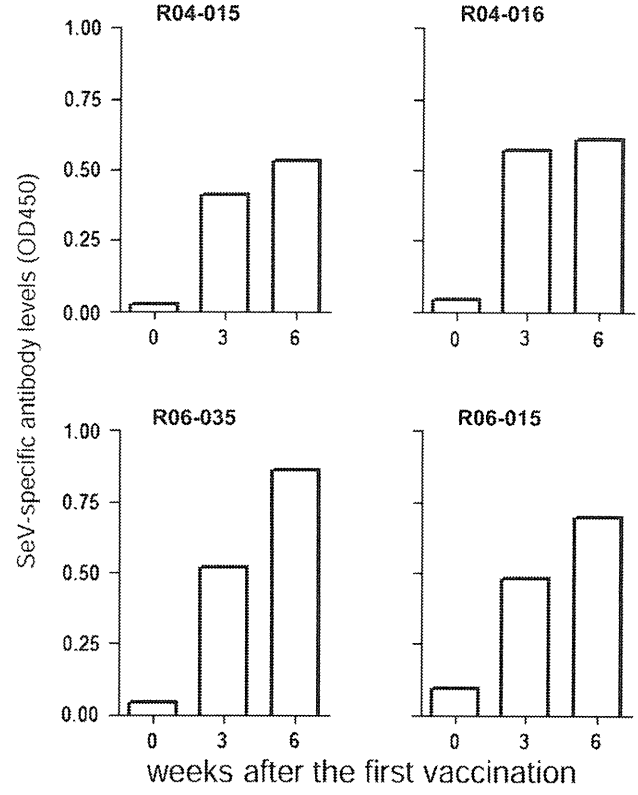


Fig. 4. SeV-specific IgG levels in plasma at repeated SeV vector vaccination. Plasma samples obtained at weeks 0, 3, and 6 after the first vaccination (just before each vaccination) were subjected to ELISA assay. OD450, optical density at 450 nm.

4. Discussion

Replication-defective viral vectors are promising safe tools for eliciting antigen-specific T-cell responses. A single inoculation of these vectors can induce efficient T-cell responses, which would peak within a couple of weeks and decline after that, although durable T-cell memory induction is important for vaccine efficacy. In the present study, we examined immunogenicity of three times of replication-defective SeV vector vaccination with intervals of three weeks in macaques and showed that antigen-specific CD8$^+$ T-cell responses after the third vaccination were higher than those after the first vaccination. Our results indicate the potential of repeated SeV vector vaccination to induce efficient and durable T-cell responses, providing a solution toward durable vaccine efficacy.

CD8$^+$ T-cells recognize MHC-I-restricted epitopes presented on target cells, and animals sharing MHC-I alleles would be useful for exact evaluation of vaccine immunogenicity. We confirmed $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell responses in macaques R04-015, R04-

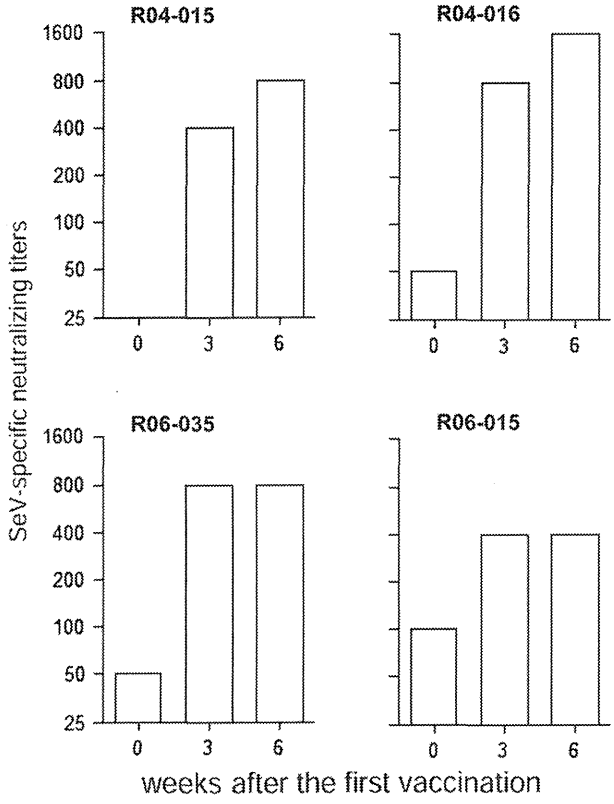


Fig. 5. SeV-specific neutralizing titers in plasma at repeated SeV vector vaccination. Plasma samples obtained at weeks 0, 3, and 6 after the first vaccination (just before each vaccination) were subjected to anti-SeV neutralizing assay. The end-point plasma titers required for 10-fold reduction of SeV-EGFP infectivity are shown.

016, R06-015, and R06-035 sharing MHC-I haplotype *90-120-Ia* after SIVmac239 challenge in our previous studies [25–27]. Then, we have focused on analyzing $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses for evaluation of vaccine immunogenicity in the present study.

Previous studies have shown that Gag-specific $CD8^+$ T-cell frequencies peaked around one week after a single F(-)SeV-Gag vaccination [19]. Indeed, Gag-specific $CD8^+$ T-cell responses became undetectable in a few months in five of six F(-)SeV-Gag-boosted macaques [32]. Then, it is inferred that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies would be much lower at weeks 4 and 7 than those at week 1 without repeated vaccination. In the present study, however, those frequencies at week 7 were higher than at week 1. $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies at weeks 4 and 7 were similar or rather higher than those at weeks 1 and 4, respectively. These results imply that each of the second and the third vaccination enhanced these $CD8^+$ T-cell responses, leading to augmented, durable $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses.

Pre-existing anti-vector antibodies can be an obstacle to the immunogenicity of viral vectors [15,33–35]. Viral vector vaccination induces immune responses against the vector virus itself, and so, anti-vector antibodies could inhibit induction of antigen-specific T-cell responses by repeated vaccination. Indeed, after the first vaccination, all four animals had high levels of SeV-specific neutralizing antibodies, which may have affected efficacy of T-cell induction by the second and the third SeV vector vaccination with short intervals. However, the second and the third SeV vector vaccination in the presence of high levels of SeV-specific neutralizing antibodies enhanced $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses.

Recently, we showed that intranasal SeV vector immunization is more immunogenic than intramuscular in the presence of SeV-specific neutralizing antibodies [20]. In that study, Gag-specific $CD8^+$ T-cell responses were induced not by intramuscular but by intranasal immunization with 6×10^8 CIU of F(-)SeV-Gag vectors in the presence of 1:100 of plasma SeV-specific neutralizing titers. We were unable to quantify SeV-specific IgA levels. In the present study, however, clear difference in immunogenicity was not shown between intranasal and intramuscular SeV vector vaccination. Enhancement of antigen-specific $CD8^+$ T-cell responses by the second, intramuscular vaccination was observed in macaques R04-016 and R06-035 (Fig. 2). Intramuscular immunization with higher doses (6×10^9 CIU) may overcome the inhibitory effect by SeV-specific neutralizing antibodies.

In summary, we examined antigen-specific $CD8^+$ T-cell responses in macaques after three times of SeV vector vaccination with intervals of three weeks. Antigen-specific $CD8^+$ T-cell responses did not decline but were enhanced by the second and the third vaccination even in the presence of high levels of SeV-specific neutralizing antibodies. These results indicate that repeated SeV vector vaccination even with short intervals can contribute to induction of efficient, durable T-cell responses.

Acknowledgments

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Original article

Gag-CA Q110D mutation elicits TRIM5-independent enhancement of HIV-1mt replication in macaque cells

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Abstract

HIV-1 is strictly adapted to humans, and cause disease-inducing persistent infection only in humans. We have generated a series of macaque-tropic HIV-1 (HIV-1mt) to establish non-human primate models for basic and clinical studies. HIV-1mt clones available to date grow poorly in macaque cells relative to SIVmac239. In this study, viral adaptive mutation in macaque cells, G114E in capsid (CA) helix 6 of HIV-1mt, that enhances viral replication was identified. Computer-assisted structural analysis predicted that another Q110D mutation in CA helix 6 would also increase viral growth potential. A new proviral construct MN4Rh-3 carrying CA-Q110D exhibited exquisitely enhanced growth property specifically in macaque cells. Susceptibility of MN4Rh-3 to macaque TRIM5α/TRIMCyp proteins was examined by their expression systems. HIV-1mt clones so far constructed already completely evaded TRIMCyp restriction, and further enhancement of TRIMCyp resistance by Q110D was not observed. In addition, Q110D did not contribute to evasion from TRIM5α restriction. However, the single-cycle infectivity of MN4Rh-3 in macaque cells was enhanced relative to the other HIV-1mt clones. Our results here indicate that CA-Q110D accelerates viral growth in macaque cells irrelevant to TRIM5 proteins restriction.

Keywords: HIV-1; HIV-1mt; Gag-CA; Macaque cells; Virus growth; Molecular modeling

1. Introduction

Mammalian cells express a variety of host restriction factors to defend themselves against pathogens. Viruses have evolved countermeasures to subvert their restriction and replicate efficiently in cells [1,2]. HIV-1, a causative agent of human AIDS, evades host restriction factors and replicates well in human cells. However, in macaques for experimental use, e.g. cynomolgus macaques (CyMs) and rhesus macaques (RhMs), HIV-1 replication is completely inhibited by host restriction factors present in their cells [3]. Construction of HIV-1 that overcomes species-barrier contributes much to understand the interaction of HIV-1 and its host as well as the establishment of HIV-1-infected macaque models [4,5].

Extensive molecular biological studies on the HIV-1/host interaction conducted to date have revealed main mechanical bases for the narrow host range exhibited by HIV-1. Macaque cells contain potent antiviral factors that effectively restrict or even abolish HIV-1 replication. These include APOBEC3 proteins (APOs), CyclophilinA (CypA), and TRIM5α/TRIMCyp

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(TRIM5 proteins). HIV-1 can indeed counteract human proteins corresponding to these restriction factors. APOs exhibit cytidine deaminase activity, and introduce lethal mutations into HIV-1 genome. HIV-1 Vif is able to neutralize the antiviral activity of human APOs, but not macaque APOs [6–8]. CypA acts on incoming HIV-1 core to regulate infection positively in human cells but negatively in macaque cells [9–11], though amino acid sequences of CypA from human and macaque are identical. Macaque TRIM5α recognizes and interacts with incoming HIV-1 core, and restricts virus infection in a less-defined mechanism [9–11]. Macaque TRIM5α is polymorphic, and has sequence variation in a C-terminal B30.2/SPRY domain important for capsid (CA) binding. Sequence variation in this domain causes modulation of host susceptibility to retrovirus infection [12,13]. Macaque TRIMCyp is a fusion protein resulted from replacement of a B30.2/SPRY domain with CypA. Both CyM and RhM cells express TRIMCyp, but affinity of these proteins to HIV-1 core is different due to amino acid substitutions in Cyp domains. Thus, CyM TRIMCyp restricts HIV-1 replication, but not RhM TRIMCyp [14,15].

Identification of host restriction factors in macaque cells and their target proteins in HIV-1 has prompted us to generate macaque-tropic HIV-1 (HIV-1mt) with a minimal modification of HIV-1 genome. We successfully constructed prototype HIV-1mt, NL-DT5R, by replacing CypA binding region on a loop between helices 4 and 5 (h4/5L) in *gag*-CA and entire *vif* genes with the corresponding regions of pathogenic SIVmac239 (Fig. 1) [16]. But growth potential of NL-DT5R was inferior to that of SIVmac239 both *in vitro* and *in vivo* [16,17]. These results indicated that genetic modifications in NL-DT5R were insufficient to confer the ability on the virus to grow efficiently in macaque cells [16–18]. In an attempt to improve growth potential of NL-DT5R, we adapted NL-DT5R and its R5-tropic version NL-DT562 to a CyM derived lymphocyte cell line HSC-F, and found a number of genetic substitutions in viral genomes of adapted viruses [19]. We introduced these mutations and CA h6/7L from SIVmac239 into NL-DT5R, and the resultant clone was designated MN4-5S (Fig. 1) [19]. MN4-5S exhibited enhanced growth potential in CyM both *in vitro* and *in vivo* compared to NL-DT5R [19]. But growth ability of MN4-5S was still lower than that of SIVmac239.

In this study, to further improve replication potential of HIV-1mt, we adapted MN4-5S in macaque cells and identified an adaptive mutation in CA that enhances growth ability in the cells. *In silico* structural modeling of the adaptive mutation predicted that Q110D mutation on helix 6 in CA (CA-Q110D) would promote viral replication in macaque cells. Indeed, a proviral clone carrying CA-Q110D, designated MN4Rh-3, exhibited marked enhancement of growth potential in macaque cells relative to all the other HIV-1mt clones we have constructed (Fig. 1). CyM TRIM5α/TRIMCyp susceptibility assays revealed that MN4Rh-3 completely evades from TRIMCyp restriction but not TRIM5α restriction as observed for the other HIV-1mt clones. While CA-Q110D contributed to neither endowment of further resistance to TRIMCyp nor evasion from TRIM5α restriction, CA-Q110D did lead to enhanced single-cycle infectivity to a macaque cell line compared with the other HIV-1mt clones. Our results here indicate that CA-Q110D accelerates viral growth in macaque cells independently of TRIM5 proteins restriction.

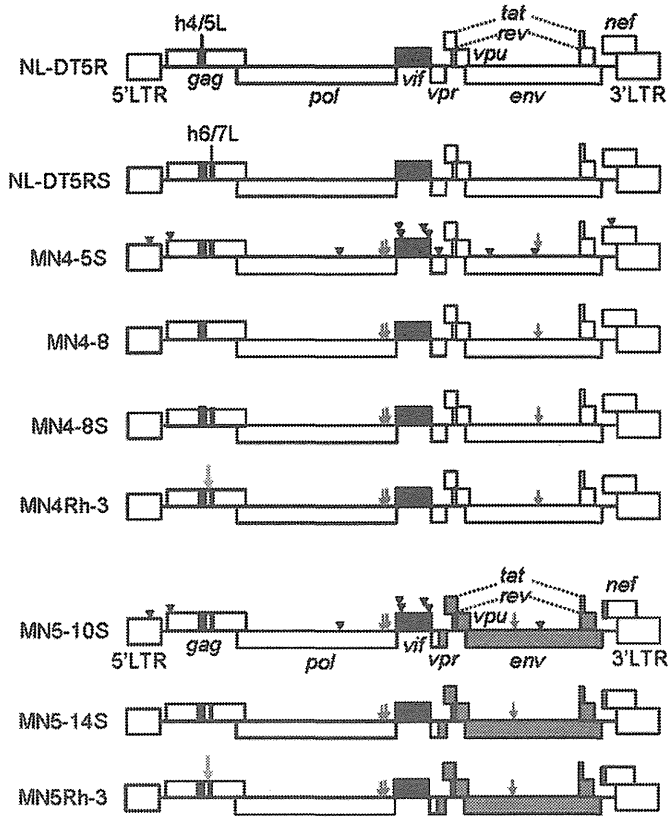


Fig. 1. Proviral genome structure of various HIV-1mt clones used in this study. HIV-1 NL4-3 [26] and SIVmac239 (GenBank: M33262) sequences are indicated by white and black areas, respectively. Gray areas in MN5-10S, MN5-14S and MN5Rh-3 show sequences from NF462 [21]. Blue arrows and black arrowheads show nucleotide substitutions that appeared in viral genomes of NL-DT5R and NL-DT562 during adaptation in HSC-F cells. Among nucleotide substitutions, adaptive mutations that enhance viral growth potential are indicated by blue arrows. Red arrows show the CA-Q110D mutation.

2. Materials and methods

2.1. Plasmid DNA

Construction of NL-DT5R, NL-DT562, NL-DT5RS, and MN4-5S were described previously [16,19–21]. MN4-5S carries all nucleotide substitutions that are present in adapted NL-DT5R and NL-DT562 clones except for mutations in the *env* gene of R5-tropic viruses (MN5-10S, MN5-14S, and MN5Rh-3 in Fig. 1) [19]. MN4-8S contains adaptive (growth-enhancing) mutations in MN4-5S but not the other mutations. MN4Rh-3 was constructed by introduction of the CA-Q110D mutation into MN4-8S. To construct R5-tropic viruses, 3′ halves of viral genomes (*Eco*RI in *vpr* to *Sph*I at the 3′ end of viral genome) of MN4-5S, MN4-8S, and MN4Rh-3 were replaced with the corresponding regions of adapted-NL-DT562,

and were designated MN5-10S, MN5-14S, and MN5Rh-3, respectively. For single-cycle infectivity assays to monitor viral susceptibility to TRIM5 proteins and to determine infectivity for CyM cells, *env*-deficient HIV-1mt variants encoding luciferase gene were constructed. NL-DT5R was cleaved with *Nde*I and *Nhe*I (both sites in *env* gene), blunt ended by T4 DNA polymerase, and resealed by T4 DNA ligase. The resultant clone was designated 5RΔEnv. Luciferase gene was then introduced into *nef* gene of 5RΔEnv as described previously [22], and the resultant clone was designated 5RΔEnv + Luc. A fragment containing the 3′ half genome was cut out from the 5RΔEnv + Luc, and introduced into the corresponding region in HIV-1mt variants (DT5R/4-3, NL-DT5RS, MN4-8, MN4-8S, and MN4Rh-3) to generate 5R/4-3ΔEnv + Luc, 5RSΔEnv + Luc, 4-8ΔEnv + Luc, 4-8SΔEnv + Luc, and 4Rh-3ΔEnv + Luc, respectively.

2.2. Cell culture

A human monolayer cell line 293T [23], a feline kidney cell line CRFK (ATCC CCL-94), and a CyM kidney cell line MK.P3(F) (JCRB 0607) were maintained in Eagles's minimal essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (hiFBS). CRFK cells expressing TRIM5α/TRIMCyp were maintained in MEM containing 10% hiFBS and 400 μg/mL G418 (SIGMA). Macaque lymphocyte cell lines, HSC-F [24] and HSR5.4 [25], were maintained in RPMI-1640 medium containing 10% hiFBS. Recombinant human IL-2 (AbD Serotec) was added to the medium (50 units/mL) for maintenance of HSR5.4 cells. A human lymphocyte cell line MT4/CCR5 (MT4 cells stably expressing CCR5) was maintained in RPMI-1640 medium containing 10% hiFBS and 200 μg/mL hyglomycine (SIGMA).

2.3. Virus replication assays

Virus stocks for infection were prepared from 293T cells transfected with proviral clones as described previously [16,19,26]. Virion-associated reverse transcriptase (RT) activity was measured as described previously [16]. HSC-F cells (10^6) were infected with equal RT units of viruses in the presence of IL-2. For infection of MT4/CCR5 cells (10^6), the spinoculation method [27] was used. Viral growth was monitored by RT activity released into the culture supernatants. We assessed the viral growth potential by the peak day of virus production, and if the viral growth kinetics are similar, by the production level on the peak day.

2.4. Generation and characterization of adapted viral clones

MN4-5S and MN5-10S viruses (Fig. 1) prepared from transfected 293T cells were inoculated into HSR5.4 cells (3×10^6) with an equal amount of viruses (5×10^7 RT units). The cultures were maintained in the presence of IL-2, and HSC-F cells were added on day 34 post-infection. The culture supernatants (collected on day 18 post-cocultivation, the peak day of virus production) were inoculated into fresh HSR5.4 cells, and total DNA was extracted from the cells on day 15 post-infection. Integrated proviruses were amplified from total DNA as two overlapping fragments by the polymerase chain reaction (PCR), and amplified products were cloned into MN5-10S as described previously [16]. Viruses were prepared from 293T cells transfected with the resultant clones, and inoculated into HSR5.4 cells. Only one clone exhibited a rapid growth kinetics compared to MN5-10S, and was designated Ad clone-25. To identify an adaptive mutation that enhances growth potential, each mutation found in the genome of Ad clone-25 was introduced into MN5-14S by site-directed mutagenesis (STRATAGENE). For screening, viruses prepared from transfected 293T cells were inoculated into HSC-F cells, and virus replication was monitored by RT activity released into the culture supernatants.

2.5. Molecular modeling of HIV CA N-terminal domain (NTD)

The crystal structure of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C [28]) was taken from the RCSB Protein Data Bank [29]. The three-dimensional (3-D) models of HIV-1 CA NTD were constructed by the homology modeling technique using 'MOE-Align' and 'MOE-Homology' in the Molecular Operating Environment (MOE) (Chemical Computing Group Inc., Quebec, Canada) as described [30–32]. 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 [33]. The final 3-D models were thermodynamically optimized by energy minimization using an AMBER99 force field [34] combined with the generalized Born model of aqueous solvation implemented in MOE [35]. Physically unacceptable local structures of the optimized 3-D models were further refined on the basis of evaluation by the Ramachandran plot using MOE.

2.6. Single-cycle infectivity assays

To generate CRFK cells expressing CyM TRIMCyp, the cDNA was isolated from HSC-F cells, and expression vector of FLAG-tagged CyM TRIMCyp was constructed as described previously [18]. The sequence of TRIMCyp from HSC-F cells was identical with Mafa TRIMCyp2 (GenBank: FJ609415). CRFK cell lines expressing CyM TRIMCyp were selected by G418 as described previously [18]. Expression and inhibitory effect of the selected cell clones were verified by Western blotting with anti-FLAG antibody (SIGMA) and by infection with vesicular stomatitis virus G protein (VSV-G) pseudotyped 5R/4-3ΔEnv + Luc, respectively. Assays using naïve CRFK, CRFK expressing CyM TRIM5α [18] or CyM TRIMCyp, and MK.P3(F) cells were similarly performed as described previously [36]. VSV-G pseudotyped virus stocks were prepared from 293T cells transfected with individual HIV-1mtΔEnv + Luc clones and pCMV-G (GenBank: AJ318514)

at a molar ratio of 1:1. Naïve CRFK, CRFK expressing TRIM5α/TRIMCyp and MK.P3(F) cells were infected with an equal titer of viruses (to generate approximately 10^7 relative luminescence (RLU) for naïve CRFK cells), and on day 2 post-infection, cells were analyzed for luciferase activity. Assays using recombinant Sendai virus (SeV)-CyM TRIM5α/TRIMCyp expression system were performed as described previously [31].

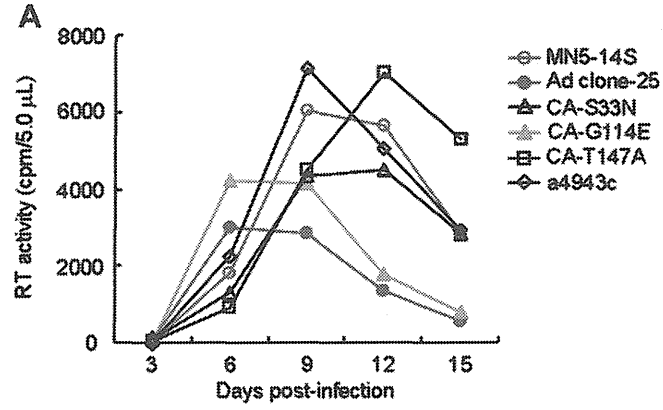
3. Results

3.1. An adaptive mutation G114E on helix 6 in CA (CA-G114E) enhances viral growth potential in macaque cells

An HIV-1mt variant MN4-5S replicated more slowly than SIVmac239 in macaque cells. In order to improve its growth potential, we carried out virus adaptation in a macaque lymphocyte cell line HSR5.4. Virus adaptation was performed by long-term culture of HSR5.4 cells infected with MN4-5S (X4-tropic) or its R5-tropic version MN5-10S (Fig. 1). Construction of proviral clones from adapted viruses was described in Materials and methods. We obtained only one clone (Ad clone-25) with enhanced growth potential from 100 proviral clones constructed and tested. We sequenced the entire genome of Ad clone-25, and found three non-synonymous mutations in CA (S33N, G114E, and T147A in Fig. 2A) and one synonymous mutation in integrase (IN)(a4943c in Fig. 2A). To identify an adaptive mutation that enhances growth potential, each mutation found in Ad clone-25 was introduced into a parental clone MN5-14S (Fig. 1). MN5-14S carries only growth-promoting mutations in MN5-10S, and the two clones exhibit similar growth potential in macaque cells. Viruses were prepared from 293T cells transfected with MN5-14S, Ad clone-25, or clones carrying individual mutations, and inoculated into HSC-F cells (Fig. 2A). Only one clone carrying CA-G114E exhibited similar growth kinetics to that of Ad clone-25 but not the others. This result indicates that CA-G114E is an adaptive mutation enhancing growth potential of HIV-1mt in macaque cells. This mutation is exactly the same as the previously found adaptive mutation, which enhanced growth of NL-4/5S6/7SvifS virus in human CEM-SS cells [37]. NL-4/5S6/7SvifS virus is a prototype HIV-1mt bearing the same CA with that of MN4-5S.

3.2. Molecular modeling of the CA NTD of HIV-1mt variants suggests that CA-G114E and CA-Q110D mutations have a similar positive effect on viral replication

The amino acid at position 114 is located in CA NTD. To obtain structural insights into impacts of the G114E substitution in order to improve growth capability of HIV-1mt variants in macaque cells, we conducted computer-assisted structural study: we constructed 3-D models of CA NTD of three HIV-1mt variants, CA-G114E, CA-G114Q, and MN4-5S, using homology-modeling technique (see Materials and methods). Main chain folds of the three models were indistinguishable, suggesting that 3-D position and type of side chain are critical



Nucleotide change	Region	Amino acid change in the region
g1283a	CA	S33N
g1526a	CA	G114E
a1624g	CA	T147A
a4943c	IN	None

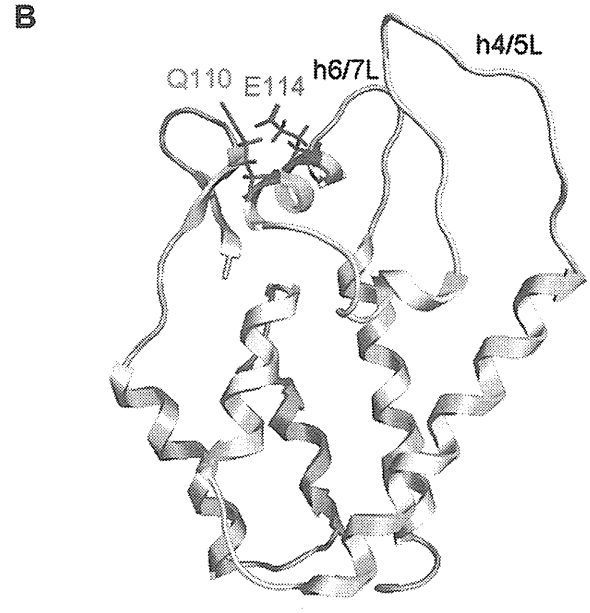


Fig. 2. Mutations in Gag-CA. (A) Identification of an adaptive mutation that enhances viral growth. Nucleotide substitutions found in the genome of Ad clone-25 are indicated at the bottom. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal RT units were inoculated into HSC-F cells. MN5-14S and Ad clone-25 served as controls. Virus replication was monitored by RT activity released into the culture supernatants. (B) 3-D structural models for CA NTD of HIV-1mt variants. Structural models of CA NTD of HIV-1mt variants were constructed by homology-modeling using "MOE-Align" and "MOE-Homology" in MOE as described previously [30–32]. Crystal structure of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C [28]) was used as template for homology modeling. Main chain folds were indistinguishable among the models, and only the model of G114E CA is shown as a representative. Magenta and red sticks: side chains of 110th and 114th amino acid residues, respectively, of the G114E CA NTD.

for the phenotypic change. The modeling study revealed that 114th residue of G114E CA NTD is located on helix 6 in CA NTD such that its side chain protrudes into the exposed surface of CA (Fig. 2B). A charged amino acid residue on a protein surface participates in determining physicochemical properties of interaction surface of the protein and thus influences its structural and functional properties. Therefore, we assumed that the protrusion of a negatively charged side chain from helix 6 into exposed surface could have somehow a positive effect on growth capability of the HIV-1mt variants in macaque cells. In this regard, especially worth noting is that 110th amino acid residue on helix 6 of the HIV-1mt variant CAs was positioned on the same helical face with 114th amino acid residue (Fig. 2B). Therefore, we predicted that substitution of glutamine (Q) at position 110 by acidic amino acid such as aspartic acid (D) and glutamic acid (E) may also have a positive effect on growth capability of the HIV-1mt variants in macaque cells as G114E does. SIVmac239 has aspartic acid and glutamine at the positions 110 and 114, respectively.

3.3. CA-Q110D promotes viral growth more efficiently in macaque cells than CA-G114E mutation but its enhancing effect is species-specific

To confirm our prediction described above, CA-Q110D mutation was introduced into MN5-14S (designated MN5Rh-3), and the growth property in HSC-F cells of MN5Rh-3 and a viral clone carrying G114E (CA-G114E in Fig. 2A) was compared. As shown in Fig. 3A, MN5Rh-3 grew better than CA-G114E, indicating that CA-Q110D further accelerates HIV-1mt replication in macaque cells compared with an adaptive CA-G114E mutation. We next constructed an X4-tropic proviral clone carrying the CA-Q110D (designated MN4Rh-3) (Fig. 1), and compared its growth property with MN5Rh-3 in HSC-F cells (Fig. 3B). MN4Rh-3 was found to exhibit higher growth ability than MN5Rh-3, and was therefore used for infection experiments hereafter.

While CypA and TRIM5α have inhibitory effect on HIV-1 replication in macaque cells, CypA promotes HIV-1 infection in human cells and human TRIM5α only weakly inhibits HIV-1 replication [38–40]. Since the CA-Q110D mutation (acquisition of negatively charged side chain), as predicted by structural modeling, could impact on the interaction of HIV-1 CA and its binding factor(s) by altering physicochemical properties of CA binding surface, it can be speculated that CA-Q110D may promote viral replication specifically in macaque cells. Thus, we analyzed the effect of CA-Q110D on viral growth in macaque and human cells. In this experiment, we used HIV-1mt variants (MN4-8, MN4-8S, and MN4Rh-3) that have distinct CA structures (Fig. 1). Viruses prepared from transfected 293T cells were inoculated into macaque HSC-F and human MT4/CCR5 cells, and examined for growth property (Fig. 3C). The introduction of SIVmac239 CA h6/7L (MN4-8S) resulted in enhanced and reduced viral growth in macaque and human cells, respectively, relative to MN4-8. MN4Rh-3 grew clearly better in macaque cells relative to MN4-8 and MN4-8S, but more poorly in human cells than the other twos. These results demonstrate that the CA-Q110D mutation enhances viral replication in a host cell species-specific manner.

3.4. CA-Q110D does not contribute to evasion from CyM TRIM5 proteins restriction

We predicted that the growth enhancement by CA-Q110D may come from the increased resistance to CyM TRIM5 proteins, and therefore examined the susceptibility of HIV-1mt variants to them by two independent assays.

First, assays were performed in feline kidney CRFK cells expressing TRIM5α or TRIMCyp by using VSV-G pseudotyped viruses encoding the luciferase gene (Fig. 4A–C). The sequence differences between HIV-1mt variants reside only in CA and IN (Figs. 1 and 4). Since adaptive mutations in IN contribute to enhancement of virion production but not early replication phase (manuscript in preparation), only the difference in CA affects the relative single cycle infectivity in this assay. A pseudotyped virus 5R/4-3 carries HIV-1 (NL4-3) CA without any modifications and served as negative control. While 5R and 4-8 have an identical CA structure carrying h4/5L from SIVmac239, 5RS and 4-8S have both h4/5L and h6/7L from SIVmac239 CA. 4Rh-3 carries CA-Q110D mutation in addition to h4/5L and h6/7L from SIVmac239 CA. Viral infectivity was measured by luciferase activity in infected cells and presented as RLU. Naïve CRFK and CRFK cells expressing TRIM5α were infected with an equal amount of viruses generating 10^7 RLU in naïve cells. As shown in Fig. 4B, the infectivity of 5R and 4-8 for cells expressing CyM TRIM5α was similar to that of a negative control 5R/4-3. However, higher infectivity was observed for 5RS and 4-8S relative to 5R and 4-8. These results were consistent with previous reports that h4/5L and h6/7L in HIV-1 CA are a part of determinant for TRIM5α restriction [20,36]. The sensitivity of 4Rh-3 to TRIM5α was similar to that of 5RS and 4-8S. This indicates that CA-Q110D did not contribute to increase the resistance to TRIM5α. It has been reported that CyM TRIMCyp has the ability to restrict HIV-1 replication [15]. To examine the susceptibility of HIV-1mt variants to TRIMCyp, we generated feline CRFK cells expressing TRIMCyp, and the cells were infected with pseudotyped viruses as described above. As shown in Fig. 4C, all the clones tested were more resistant to a similar extent to TRIMCyp than the control 5R/4-3. In agreement with a previous study showing that elimination of alanine at position 88 within h4/5L of HIV-1 CA confers the resistance on the virus to TRIMCyp [15], our results indicate that the replacement of HIV-1 CA h4/5L with that of SIVmac239 is sufficient for HIV-1mt to evade from the TRIMCyp restriction. Second, we performed another susceptibility assay using the recombinant SeV expression system. This system assures a very high expression level of target proteins in cells infected with the recombinant SeV. Therefore, the ability of viruses to completely counteract the restriction effect of TRIM5 proteins could be determined by MT4/SeV-TRIM5 expression system. Human MT4 cells were infected with recombinant SeV expressing CyM TRIM5α, TRIMCyp, or SPRY(−)TRIM5, and then super-infected with HIV-1

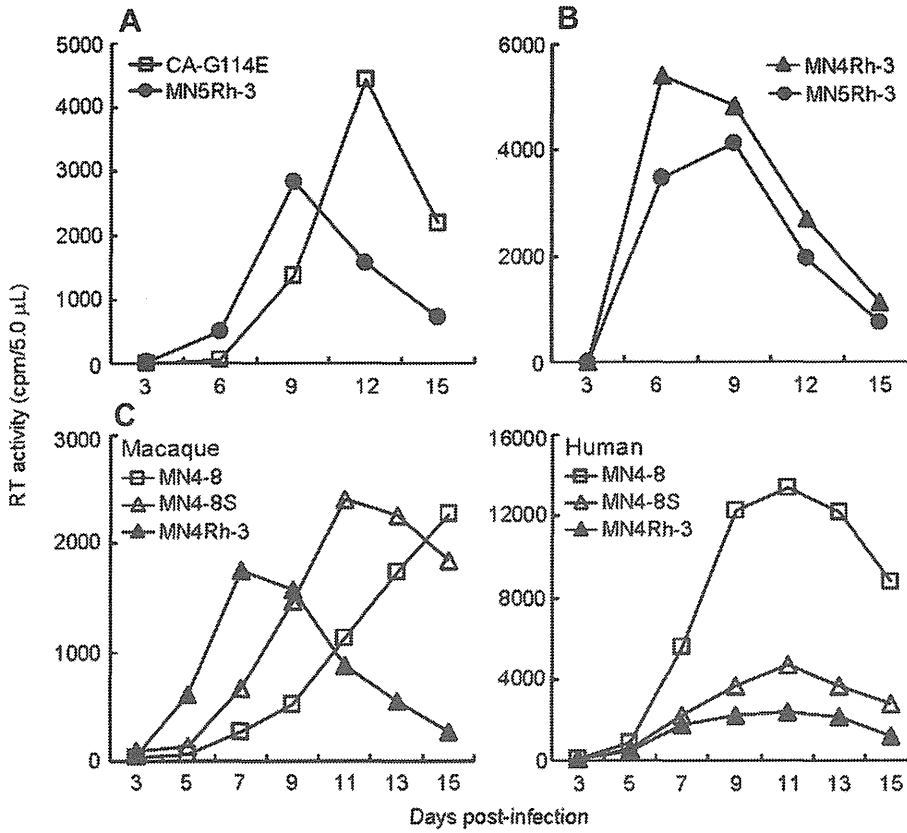


Fig. 3. Effect of CA modification on viral growth in macaque and human lymphocyte cell lines. (A and B) Growth kinetics of HIV-1mt clones carrying CA-G114E or CA-Q110D (MN5Rh-3 and MN4Rh-3) in CyM HSC-F cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (5×10^5 RT units) were inoculated into HSC-F cells (10^6). Virus replication was monitored by RT activity released into the culture supernatants. (C) Growth kinetics of MN4-8, MN4-8S, and MN4Rh-3 in HSC-F (Macaque) and MT4/CCR5 (Human) cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (10^6 RT units) were inoculated into HSC-F cells (10^6). For spinoculation of MT4/CCR5 cells (10^6), 6×10^5 RT units were used as inocula. Virus replication was monitored by RT activity released into the culture supernatants.

(NL4-3), SIVmac239, or HIV-1mt variants. SPRY(−)TRIM5 which can not bind to viral CA served as control. NL4-3 and SIVmac239 also served as controls for viral replication. As shown in Fig. 4D, NL4-3 replicated in cells expressing SPRY(−)TRIM5, but not in TRIM5α and TRIMCyp expressing cells. SIVmac239 exhibited similar growth kinetics in SPRY(−)TRIM5, TRIM5α and TRIMCyp expressing cells. All HIV-1mt variants replicated in TRIMCyp expressing cells similarly well in SPRY(−)TRIM5 cells. Together with assays in CRFK cells, these results showed that all HIV-1mt variants except for 5R/4-3 completely evade from TRIMCyp restriction. In contrast, the growth of all HIV-1mt variants was inhibited in CyM TRIM5α expressing MT4 cells. These results indicate that HIV-1mt variants do not evade from TRIM5α restriction as effectively as SIVmac239.

Results obtained by our two assay systems with respect to the susceptibility of HIV-1mt variants to CyM TRIM5α were apparently different (Fig. 4B and D), but this difference is most likely to be due to the TRIM5α expression level. In MT4 cells infected with recombinant SeV, TRIM5α is expressed at much higher level than that in transduced CRFK cells, masking the increase of resistance to TRIM5α detectable by the transduced CRFK system (Fig. 4B). Indeed, the growth enhancement of 5RS relative to 5R [20] can be explained by the results in Fig. 4B but not those in Fig. 4D. The apparent discrepancy of the sensitivity depending on TRIM5α expression level was also observed between B-LCL cells and transduced CRFK cells [41]. In sum, we can conclude here that MN4Rh-3 exhibits a partial resistance to TRIM5α insufficient for complete evasion as 5RS and 4-8S do, and that the CA-Q110D mutation is irrelevant to this property.

3.5. CA-Q110D enhances viral infectivity for macaque cells

Results so far showed that CA-Q110D does not contribute to evasion from TRIM5 proteins restriction in rather artificial systems using feline and human cells (Fig. 4). To investigate further how CA-Q110D enhances viral replication, we examined single-cycle viral infectivity in macaque cells. CyM kidney MK.P3(F) cells, which have heterozygote for TRIM5α and TRIMCyp, were infected with various VSV-G pseudoviruses and analyzed for their infectivity as described above. As shown in Fig. 5A, viral infectivity was increased by modification of h4/5L (compare 5R/4-3 and 5R&4-8). Modification of h6/7L in addition to h4/5L further augmented viral infectivity (compare 5R&4-8 and 5RS&4-8S). Introduction of the CA-Q110D mutation into 4-8S clone gave the highest infectivity among the viruses tested (see 4Rh-3). The results in

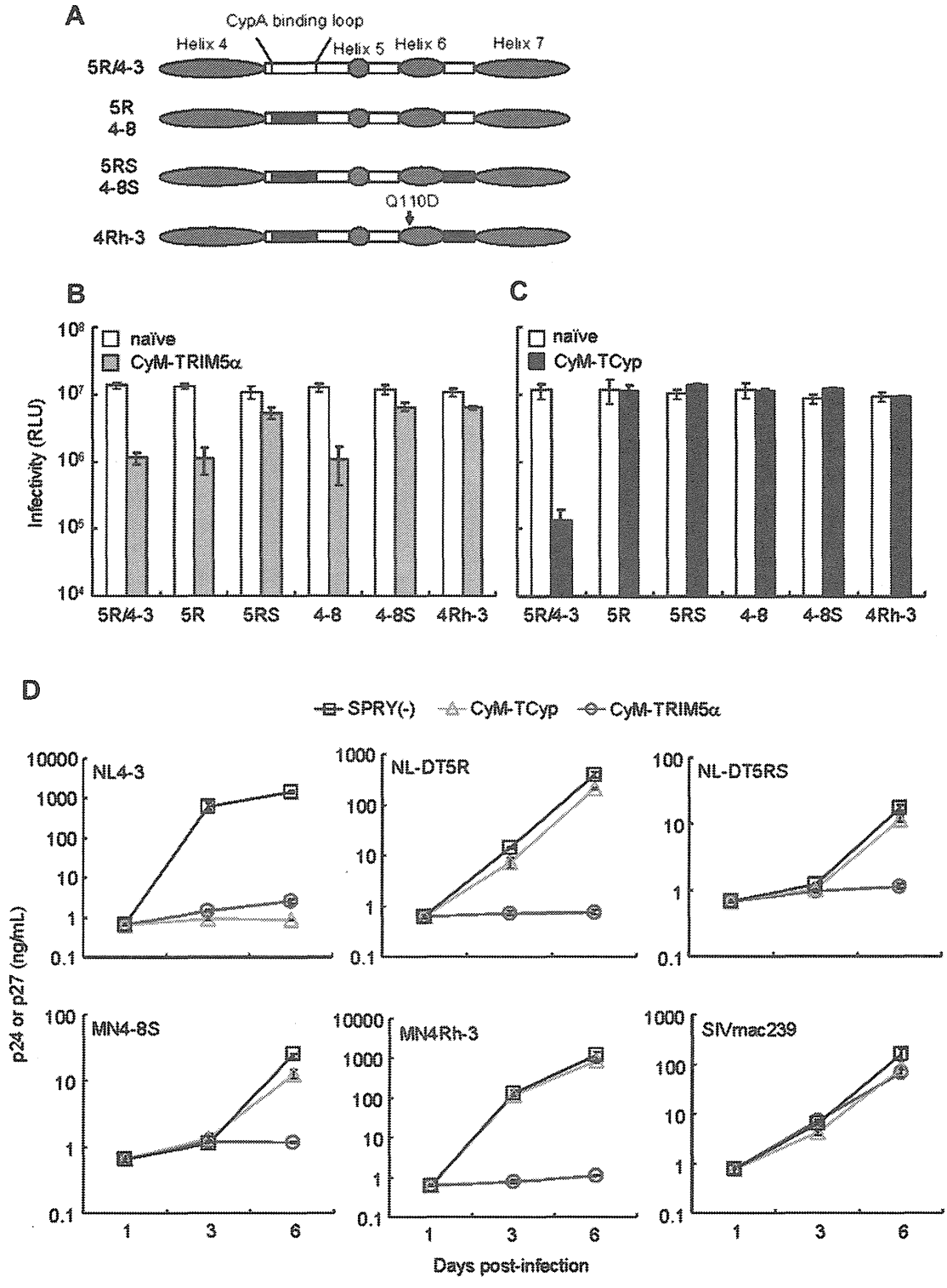


Fig. 4. Effect of CA modification in HIV-1mt variants on viral infectivity. (A) CA structure of viral clones used in TRIM5α/TRIMCyp susceptibility assays. Blue and white areas show helices and loops from HIV-1 NL4-3 CA, respectively. Sequences from SIVmac239 are indicated by black areas. (B and C) Susceptibility of HIV-1mt variants to CyM TRIM5 proteins as examined by CRFK system. Results for CyM TRIM5α (B) and for CyM TRIMCyp (TCyp) (C) are shown. VSV-G pseudotyped viruses were prepared from transfected 293T cells as input samples. Viruses generating 10^7 RLU in CRFK-naïve cells were inoculated into CRFK cells that express CyM TRIM5α or CyM TCyp. On day 2 post-infection, cells were analyzed for luciferase activity by a luminometer. (D) Susceptibility of HIV-1mt variants to CyM TRIM5 proteins as examined by SeV system. Human MT4 cells (10^5) were infected with recombinant SeV expressing CyM TRIM5α, TRIMCyp, or SPRY (−) TRIM5. Nine hours after infection, cells were super-infected with 20 ng (Gag-p24) of HIV-1 NL4-3, various HIV-1mt clones, or 20 ng (Gag-p27) of SIVmac239. Virus replication was monitored by the amount of Gag-p24 from NL4-3 and HIV-1mt clones or Gag-p27 from SIVmac239 in the culture supernatants. Error bars show actual fluctuations between duplicate samples. Data from one representative of three independent experiments are shown.

Fig. 5A show that CA-Q110D uniquely increases viral infectivity in macaque cells not observed in the other experimental systems (Fig. 4), and suggest that some factor(s) in CyM cells other than TRIM5α and TRIMCyp proteins is associated with this enhancement.

As shown in Fig. 5B, MN4Rh-3 displayed slower growth kinetics relative to those of SIVmac239 (note the peak day of virus production), although it grew better than the other HIV-1mt clones in CyM HSC-F cells. Approximately 100-fold more input virus (RT units) compared to SIVmac239 was required for MN4Rh-3 to exhibit similar growth kinetics with SIVmac239 (data not shown). These results have shown that even MN4Rh-3 grows more poorly in macaque cells than a standard SIVmac clone pathogenic for macaque monkeys.

4. Discussion

In this study, we have demonstrated that a single CA mutation (Q110D) greatly promotes HIV-1mt growth in macaque cells (Fig. 3). This enhancing effect was afforded independently of TRIM5 proteins restriction. The virus carrying the CA-Q110D mutation (MN4Rh-3) certainly overcame the anti-viral action of CyM TRIMCyp but not completely CyM TRIM5α. However, the mutation itself (Fig. 1) did not influence anti-TRIMCyp/TRIM5α activity of MN4Rh-3 reported here (Fig. 4). Notably, this mutation exquisitely enhanced viral growth in macaque cells (Fig. 3) by augmenting viral single-cycle infectivity (Fig. 5). The viral growth enhancement reported here is well reproduced in CyM peripheral blood mononuclear cells and in CyMs (manuscript in preparation).

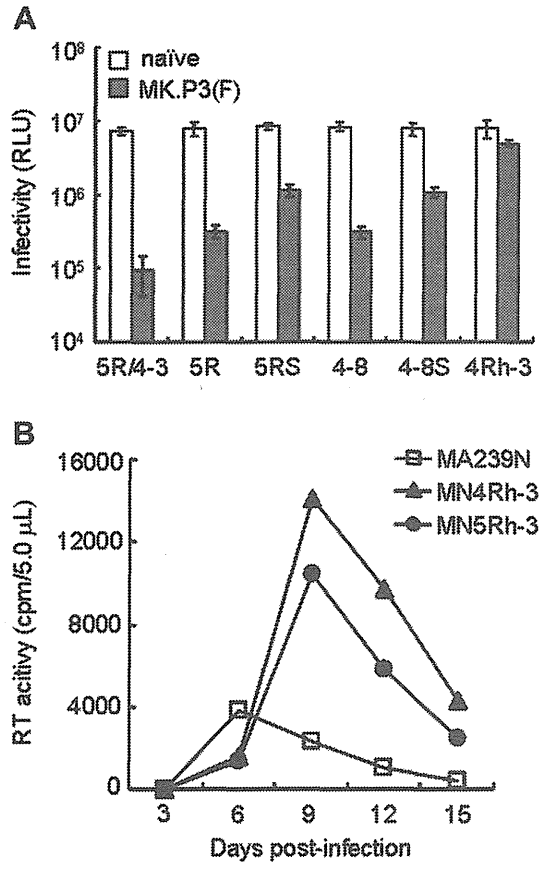


Fig. 5. Replication ability of various viruses in CyM cells. (A) Single-cycle infectivity of various HIV-1mt clones in CyM kidney MK.P3(F) cells. VSV-G pseudotyped viruses indicated were prepared from transfected 293T cells. MK.P3(F) cells were infected with an equal titer of viruses giving 10^7 RLU in CRFK-naïve cells. On day 2 post-infection, cells were analyzed for luciferase activity by a luminometer. (B) Multi-cycle growth kinetics of SIVmac and HIV-1mt viruses in CyM lymphocyte HSC-F cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (10^4 RT units) were inoculated into HSC-F cells (10^6). Virus replication was monitored by RT activity released into the culture supernatants. MA239N, an infectious clone of SIVmac239 with *nef*-open.

Regarding the mechanism for enhancement of viral growth by CA-Q110D, we initially thought a possibility that CA-Q110D compensates the disadvantage in HIV-1mt genome resulted from replacement of HIV-1 CA h4/5L and h6/7L with those of SIVmac239. However, this is highly unlikely because the enhancing effect is macaque cell-dependent (Fig. 3). Most feasible explanation is that CA-Q110D contributes to evade from a negative factor(s) in macaque cells such as CypA. Because HIV-1mt CA was designed not to bind to CypA, and the interaction between the two molecules was indeed undetectable by monitoring CypA virion-incorporation [18,20], we analyzed the binding by computer-assisted structural modeling. Homology modeling of the CA-CypA complexes was performed based on the crystal structure of HIV-1 CA NTD bound to CypA (PDB code: 1M9C [28]), and the binding energies, E_{bind}, were calculated using MOE as described previously [42,43]. As shown in Fig. 6, HIV-1 (NL4-3) CA was predicted to interact with CypA via its h4/5L (binding energy: −64.4 kcal/mol). The binding energy of CA and CypA was decreased by CA modifications, such as h4/5L replacement (NL-DT5R: −31.0 kcal/mol), h4/5L and h6/7L replacement (NL-DT5RS: −36.1 kcal/mol), and Q110D substitution in addition to h4/5L and h6/7L replacement (MN4Rh-3: −30.1 kcal/mol). Decrease in E_{bind} in NL-DT5R is consistent with the result that the h4/5L region directly interacts with CypA [28]. Notably, the E_{bind} for the NL-DT5RS CA was greater than that of the NL-DT5R and MN4Rh-3 CAs. These results suggest that not only h6/7L replacement but also Q110D substitution can influence structure of CypA binding surface of CA. The Q110D substitution is located on the exposed surface of helix 6 connecting to the h6/7L (Fig. 2B). CA helix 6 has been reported to interact with CypA binding region on h4/5L through hydrogen bonding [44,45]. Thereby it is reasonable that the local electrostatic change on the helix 6 by the Q110D substitution influenced structures of h4/5L via changes in fluctuation and conformation of h6/7L. This in turn could lead to reduction in stability of the MN4Rh-3 CA-CypA complex compared with NL-DT5RS CA-CypA complex, as predicted in Fig. 6. Our computer-assisted structural study suggests that the Q110D substitution can induce electrostatic modulation of the overall CA surface structure including h4/5L and h6/7L. Similar modulation mechanism of binding surface structures via charged amino acid substitution at distant site from the binding surface has been reported for Cyp domain of CyM TRIMCyp [15] and CD4 binding site of HIV-1 gp120 outer