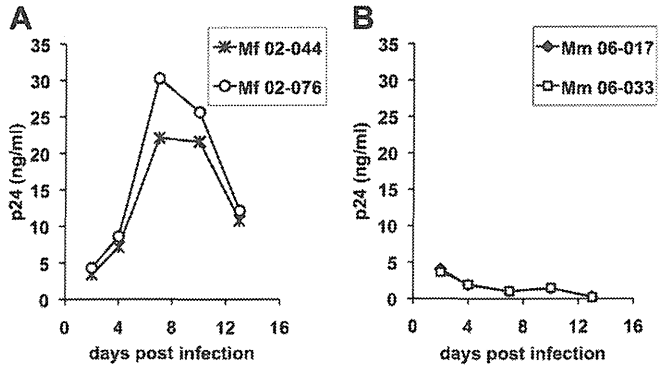

Fig. 2. Growth properties of the NL-DT5R in CD8$^+$ cell-depleted PBMCs from CM (A) and RM (B). The cells were infected with NL-DT5R and the viral replication was monitored by p24 antigen in the culture supernatants using a p24 quantitative ELISA kit. Animal identifications are indicated at the top of each panel.

These results indicated that although CM appeared permissive for NL-DT5R as compared with RM, the mutations introduced in NL-DT5R were not still sufficient to overcome the restriction by host factor(s) of these macaques.

3.2. MN4-5S showed improved replication capability in CM CD8$^+$ cell-depleted PBMCs

In order to improve the replication capability of HIV-1mt in CM, we conducted long-term passage of NL-DT5R in HSC-F cells. Additionally, NL-DT562, having an R5-tropic *env* gene on a background of NL-DT5R, was also passaged long-term in HSC-F cells. We found that the passaging improved the growth of the viruses (data not shown), and then viral clones were obtained after the long-term passaging and sequenced. Ten nucleotide substitutions were identified in the NL-DT5R-derived clone and 4 nucleotide substitutions (except for the *env* gene) in the NL-DT562-derived clone. These 14 nucleotide substitutions (7 of which were non-synonymous mutations) were assembled and introduced into NL-DT5R. The resultant clone was named MN4-5, and its structure is shown in Fig. 1. We previously found that insertion of an SIVmac loop between alpha helices 6 and 7 (L6/7) of CA into the corresponding region in HIV-1 significantly enhanced the viral replication in HSC-F cells and PBMCs of CM by relieving the inhibitory effect of TRIM5α [18]. We therefore inserted an SIVmac-derived L6/7 sequence into MN4-5. The resultant clone was named MN4-5S (Fig. 1). In order to examine the impact of these modifications on the viral replication, we analyzed the replication properties of this "adapted" virus in HSC-F cells and CD8$^+$ cell-depleted PBMCs of CM. MN4-5 showed higher replication as compared with NL-DT5R in both types of cells (Figs. 4 and 5). Moreover, MN4-5S showed enhanced growth capability in the cells as compared with the parental clones, NL-DT5R and MN4-5 (Figs. 4 and 5).

Notably, MN4-5S did not show any replication in RM cells (data not shown), indicating that the combination of the mutations introduced in NL-DT5R may be effective for escape from the restriction in CM cells but not in RM cells.

3.3. MN4-5S induced greater viremia in CM as compared with parental clone, NL-DT5R

Since MN4-5S showed enhanced ability to replicate in CM cells, we next examined the viral replication in vivo. The stock of MN4-5S virus was inoculated into 3 CM. MN4-5S induced 10-fold higher viremia in infected animals at 2–3 weeks after infection (Fig. 6A), as compared with that induced by NL-DT5R (see Fig. 3). This result was consistent with the in vitro result (Fig. 5) and demonstrated that the mutations inserted into NL-DT5R contributed to enhancement of viral replication in vivo. In addition, at the acute phase of infection a slight decrease of CD4$^+$ T cells was observed (Fig. 6B). The viremia became undetectable at 6 weeks after infection.

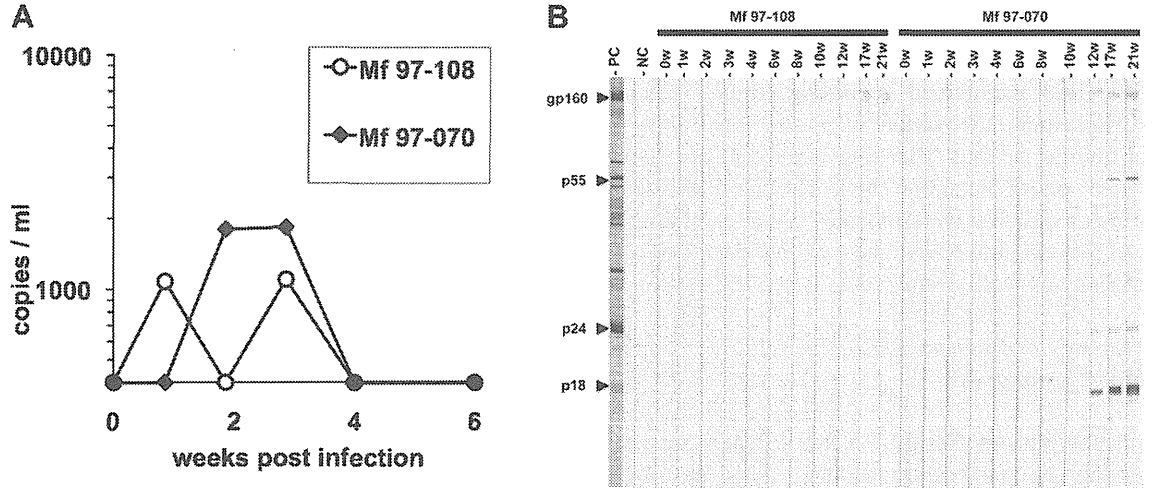

Fig. 3. Profiles of plasma viral RNA loads (A) and anti-HIV-1 antibody responses (B) in CM infected with NL-DT5R. Mf97-108 (open circles) and Mf97-070 (closed diamonds) were used in this study. Viral stocks for inoculation were prepared in CD8$^+$ cell-depleted PBMCs, and then 6.1 ng p24 of HIV-1 were inoculated into each animal. Commercially available diagnostic HIV-1 Western blotting strips were reacted with 100-fold diluted monkey plasma. Plasma from HIV-1-infected or uninfected individuals was used as a positive or a negative control, respectively.

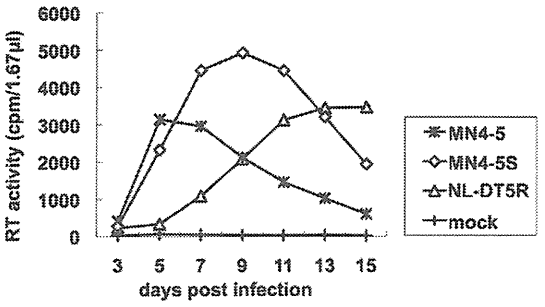

Fig. 4. Growth properties of HIV-1mt in HSC-F cells. The cells were infected with a series of HIV-1mt derivatives. The viral replication was monitored by RT activity in the culture supernatants.

Thereafter, antibody response against MN4-5S was observed in infected animals (Fig. 6C). As indicated by comparison with the lane of the positive control as a standard, the degree of antibody response seemed to be stronger than that against NL-DT5R (see Figs. 3B and 6C). Next we attempted to clarify the role of $CD8^+$ lymphocytes in the disappearance of viremia. We conducted in vivo depletion of $CD8^+$ cells by using a method reported previously [23]. We found that the reappearance of viremia was observed in all monkeys tested in parallel with the decline of $CD8^+$ T cells after the anti-CD8 mAb administration (Fig. 6A and D). This result indicated that $CD8^+$ T cells had a critical role in the control of HIV-1mt replication and suggested that the virus was able to infect latently in vivo.

4. Discussion

In the present study, we found that a modified HIV-1mt, MN4-5S, acquired greater ability to replicate in CM than NL-DT5R, and that both the SIVmac-derived L6/7 (HNP120-122 > RQQN120-123 of CA) and a series of substitutions identified by long-term passage of NL-DT5R in HSC-F cells contributed to this ability (Fig. 1). We recently showed that the substitution of L6/7 relieved the inhibitory effect of TRIM5α [18]. Additionally, our preliminary data suggest that non-synonymous mutations in the *integrase* and *env* genes are likely to be critical for the improved activity (Nomaguchi et al., manuscript in preparation). It is possible that these adaptive mutations may optimize the interaction between host and viral proteins.

It seemed that the growth kinetics of NL-DT5R in PM were comparable with those of MN4-5S in CM, which had peak levels in acute viremia of approximately 10^4 copies/ml [17]. It is therefore likely that PM may exhibit better susceptibility to HIV-1mt than CM. It is possible that the greater susceptibility of PM to HIV-1mt replication could be due to the genotype of TRIM5, because PM usually expresses a chimera between TRIM5α and CypA, so-called TRIM-Cyp, whose anti-HIV-1 activity is defective [24].

One unexpected finding in this study was that MN4-5S was unable to replicate in PBMCs of RM (data not shown), which was in contrast with the greater susceptibility of RM to SIV-mac infection. Our results suggested that RM was most resistant to HIV-1mt replication among the three macaque species. Since our HIV-1mt clones (NL-DT5R and MN4-5) were established on the basis of information obtained from serial passages of the viruses in CM-derived cells, it may be reasonable to consider that these viruses were consequently optimized to CM. Alternatively, it is also possible that anti-HIV-1 activities such as TRIM5α and APOBEC3 of RM could be greater than those of other macaques. Further studies are in progress to address these questions.

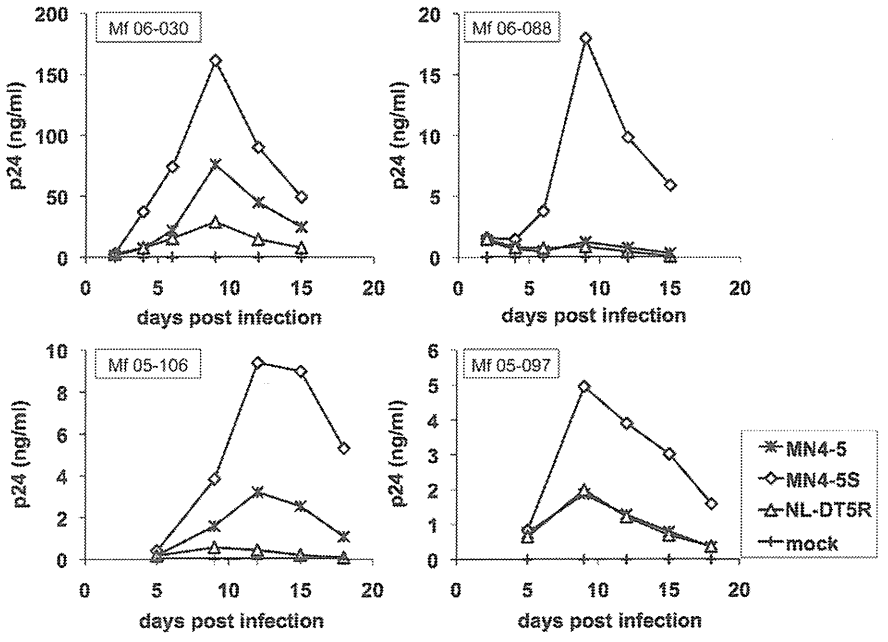

Fig. 5. Growth properties of HIV-1mt in $CD8^+$ cell-depleted PBMCs from four CM. The cells were infected with a series of HIV-1mt derivatives. The viral replication was monitored by p24 antigen in the culture supernatants using a p24 quantitative ELISA kit. Animal identifications are indicated at the top of each panel.

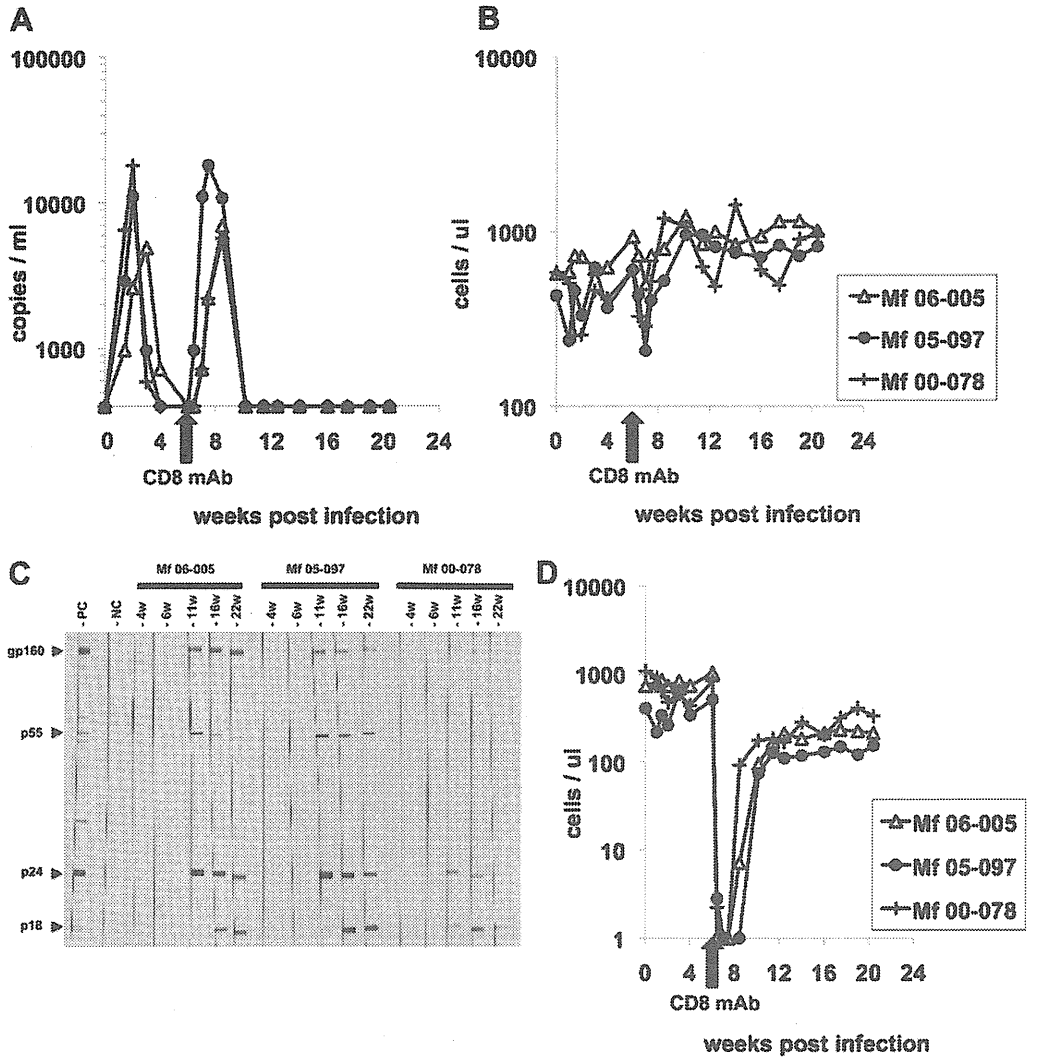

Fig. 6. Profiles of plasma viral RNA loads (A), circulating $CD4^+$ T lymphocytes (B), anti-HIV-1 antibody responses (C) and circulating $CD8^+$ T lymphocytes (D) in CM infected with HIV-1 derivatives. Viral stocks for inoculation were prepared in $CD8^+$ cell-depleted PBMCs and then 10 ng of p24 of HIV-1 were inoculated into each animal. Commercially available diagnostic HIV-1 Western blotting strips were reacted with 100-fold diluted plasma of each monkey. Plasma from HIV-1 infected or uninfected individuals was used as a positive or negative control, respectively. The black arrow indicates the day of anti-CD8 mAb (cM-T807) inoculation.

We demonstrated that the reappearance of viremia was observed in all monkeys tested in parallel with decline of $CD8^+$ T cells after anti-CD8 mAb administration (Fig. 6A and D). This result indicated that HIV-1-specific $CD8^+$ T cells had a critical role in the control of HIV-1mt replication and suggested that the virus may be able to infect latently in vivo. In order to establish a set point viremia and persistent infection, further modifications of HIV-1mt may be required to enable potent escape from the anti-viral immune response.

Further mechanistic characterization of anti-HIV-1 restriction factors will help in the construction of highly replicative and pathogenic HIV-1mt clones. As in the case of SHIV, in vivo passage of the virus could be a conventional and straightforward procedure for achieving such purposes [4]. However, the results of our study demonstrate that selective modification of HIV-1mt based on available knowledge regarding the molecular machineries is an alternative and powerful way. We are now in the process of developing the next generation of HIV-1mt that will acquire growth ability and pathogenicity in macaques as well as in humans.

Acknowledgements

The authors wish to thank T. Kurosawa, M. Fujita and M. Yasue for their helpful assistance. The authors also thank F. Ono, Y. Katakai, K. Komatsuzaki, A. Hiyaoka, K. Ohto, H. Ohto, and Y. Emoto for their support in animal experiments. We also thank M. Kaizu for his technical support. The anti-CD8 antibody used in the present study was provided by the NIH Nonhuman Primate Reagent Resource (R24 RR016001, N01 AI040101). This work was supported by grants from the Japan Health Sciences Foundation and the Ministry of Health, Labor, and Welfare in Japan and by Global COE Program A06 of Kyoto University.

References

[1] N.L. Letvin, M.D. Daniel, P.K. Sehgal, R.C. Desrosiers, R.D. Hunt, L.M. Waldron, J.J. MacKey, D.K. Schmidt, L.V. Chalifoux, N.W. King, Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III, Science 230 (1985) 71–73.

[2] H. Kestler, T. Kodama, D. Ringler, M. Marthas, N. Pedersen, A. Lackner, D. Regier, P. Sehgal, M. Daniel, N. King, et al., Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus, Science 248 (1990) 1109–1112.

[3] R. Shibata, M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, A. Adachi, Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells, J. Virol. 65 (1991) 3514–3520.

[4] K.A. Reimann, J.T. Li, R. Veazey, M. Halloran, I.W. Park, G.B. Karlsson, J. Sodroski, N.L. Letvin, A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys, J. Virol. 70 (1996) 6922–6928.

[5] M. Nomaguchi, N. Doi, K. Kamada, A. Adachi, Species barrier of HIV-1 and its jumping by virus engineering, Rev. Med. Virol. 18 (2008) 261–275.

[6] D. Sauter, A. Specht, F. Kirchhoff, Tetherin: holding on and letting go, Cell 141 (2010) 392–398.

[7] M. Stremlau, C.M. Owens, M.J. Perron, M. Kiessling, P. Autissier, J. Sodroski, The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys, Nature 427 (2004) 848–853.

[8] E.E. Nakayama, T. Shioda, Anti-retroviral activity of TRIM5 alpha, Rev. Med. Virol. 20 (2010) 77–92.

[9] A.M. Sheehy, N.C. Gaddis, J.D. Choi, M.H. Malim, Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein, Nature 418 (2002) 646–650.

[10] S. Henriet, G. Mercenne, S. Bernacchi, J.C. Paillart, R. Marquet, Tumultuous relationship between the human immunodeficiency virus type 1 viral infectivity factor (Vif) and the human APOBEC-3G and APOBEC-3F restriction factors, Microbiol. Mol. Biol. Rev. 73 (2009) 211–232.

[11] M.H. Malim, APOBEC proteins and intrinsic resistance to HIV-1 infection, Philos. Trans. R Soc. Lond B. Biol. Sci. 364 (2009) 675–687.

[12] S.J. Neil, T. Zang, P.D. Bieniasz, Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu, Nature 451 (2008) 425–430.

[13] J.L. Douglas, J.K. Gustin, K. Viswanathan, M. Mansouri, A.V. Moses, K. Fruh, The great escape: viral strategies to counter BST-2/tetherin, PLoS Pathog. 6 (2010) e1000913.

[14] R. Mariani, D. Chen, B. Schrofelbauer, F. Navarro, R. Konig, B. Bollman, C. Munk, H. Nymark-McMahon, N.R. Landau, Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif, Cell 114 (2003) 21–31.

[15] B. Jia, R. Serra-Moreno, W. Neidermyer, A. Rahmberg, J. Mackey, I.B. Fofana, W.E. Johnson, S. Westmoreland, D.T. Evans, Species-specific activity of SIV Nef and HIV-1 Vpu in overcoming restriction by tetherin/BST2, PLoS Pathog. 5 (2009) e1000429.

[16] K. Kamada, T. Igarashi, M.A. Martin, B. Khamsri, K. Hatcho, T. Yamashita, M. Fujita, T. Uchiyama, A. Adachi, Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells, Proc Natl Acad Sci U S A 103 (2006) 16959–16964.

[17] T. Igarashi, R. Iyengar, R.A. Byrum, A. Buckler-White, R.L. Dewar, C. E. Buckler, H.C. Lane, K. Kamada, A. Adachi, M.A. Martin, Human immunodeficiency virus type 1 derivative with 7% simian immunodeficiency virus genetic content is able to establish infections in pig-tailed macaques, J. Virol. 81 (2007) 11549–11552.

[18] A. Kuroishi, A. Saito, Y. Shingai, T. Shioda, M. Nomaguchi, A. Adachi, H. Akari, E.E. Nakayama, Modification of a loop sequence between alpha-helices 6 and 7 of virus capsid (CA) protein in a human immunodeficiency virus type 1 (HIV-1) derivative that has simian immunodeficiency virus (SIVmac239) vif and CA alpha-helices 4 and 5 loop improves replication in cynomolgus monkey cells, Retrovirology 6 (2009) 70.

[19] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J. Virol. 59 (1986) 284–291.

[20] T. Yamashita, N. Doi, A. Adachi, M. Nomaguchi, Growth ability in simian cells of monkey cell-tropic HIV-1 is greatly affected by downstream region of the vif gene, J. Med. Invest. 55 (2008) 236–240.

[21] H. Akari, K.H. Nam, K. Mori, I. Otani, H. Shibata, A. Adachi, K. Terao, Y. Yoshikawa, Effects of SIVmac infection on peripheral blood CD4+CD8+ T lymphocytes in cynomolgus macaques, Clin. Immunol. 91 (1999) 321–329.

[22] R.L. Willey, D.H. Smith, L.A. Lasky, T.S. Theodore, P.L. Earl, B. Moss, D.J. Capon, M.A. Martin, In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity, J. Virol. 62 (1988) 139–147.

[23] J.E. Schmitz, M.A. Simon, M.J. Kuroda, M.A. Lifton, M.W. Ollert, C.W. Vogel, P. Racz, K. Tenner-Racz, B.J. Scallon, M. Dalesandro, J. Ghrayeb, E.P. Rieber, V.G. Sasseville, K.A. Reimann, A nonhuman primate model for the selective elimination of CD8+ lymphocytes using a mouse-human chimeric monoclonal antibody, Am. J. Pathol. 154 (1999) 1923–1932.

[24] C.A. Virgen, Z. Kratovac, P.D. Bieniasz, T. Hatziioannou, Independent genesis of chimeric TRIM5-cyclophilin proteins in two primate species, Proc. Natl. Acad. Sci. U S A 105 (2008) 3563–3568.

TIM1 haplotype may control the disease progression to AIDS in a HIV-1-infected female cohort in Thailand

Nuanjun Wichukchinda[a,1], Toshiaki Nakajima[b,1], Nongluk Saipradit[a], Emi E. Nakayama[c], Hitoshi Ohtani[b], Archawin Rojanawiwat[a], Panita Pathipvanich[d], Koya Ariyoshi[e], Pathom Sawanpanyalert[a], Tatsuo Shioda[c,1] and Akinori Kimura[b,1]

Objective: To investigate association of TIM1 sequence variations with HIV/AIDS progression.

Introduction: HIV-1 infected individuals have wide variations in disease progression including AIDS. T cell immunoglobulin and mucin 1 (TIM1) is a cell surface protein involved in the regulation of Th1/Th2 immune response.

Materials and methods: We sequenced the highly polymorphic exon 4 of *TIM1* from 246 individuals of HIV-1 infected Thai female cohort to determine their *TIM1* haplotypes. Associations of *TIM1* haplotypes with baseline clinical data (sero-status, plasma viral load, CD4 cell count, and symptomatic AIDS) and survival status during 3 years of follow-up were evaluated.

Results: Seven *TIM1* haplotypes, D3-A, D4, D3-C, D1, W-A, W-C, and D3-C*, were found in the cohort. Patients possessing the D3-A haplotype showed trends towards higher $CD4^+$ cell count ($P = 0.06$) and lower proportion of AIDS-related symptoms ($P = 0.022$) than the other patients at the baseline. Kaplan–Meier analysis demonstrated that patients carrying the D3-A haplotype had a better survival rates ($P = 0.019$) than the others. D3-A haplotypes was tightly linked to the lower expression levels of *TIM1*.

Conclusion: *TIM1* D3-A haplotype is associated with the delay of disease progression to AIDS in the HIV-1 infected Thai females.

AIDS 2010, **24**:000–000

Keywords: disease progression, HIV-1, polymorphism, survival rate, TIM1

Introduction

T-cell immunoglobulin (Ig) and mucin domain (TIM) proteins, which expressed on T cells, play a central role in regulating Th1-cell and Th2-cell mediated immune response [1,2]. The genes for human TIM proteins are located on chromosome 5q31-33 and include three members; *TIM1*, *TIM3*, and *TIM4*. *TIM1* is called a hepatitis A cellular receptor 1 (*HAVCR1*) because it was originally identified as a receptor for hepatitis A virus [3]. *TIM1* is also known as kidney injury molecule 1 (*KIM1*) [4] and encodes for 359-amino acid membrane protein containing a putative signal sequence (residues 1–20), Ig domain (residues 21–121), mucin domain (residues 130–205), transmembrane (residues 291–311), and 50-amino acid long cytoplasmic tail [5].

[a]National institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand, [b]Department of Molecular Pathogenesis, Medical Research Institute, and Laboratory of Genome Diversity, Graduate School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, [c]Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, [d]Day Care Center, Lampang Hospital, Lampang, Thailand, and [e]Global COE Program and Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.

Correspondence to Akinori Kimura, Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan.

Tel: +81 3 5803 4905; fax: +81 3 5803 4907; e-mail: akitis@mri.tmd.ac.jp

[1] These authors equally contributed to this study.

Received: 15 November 2009; revised: 8 February 2010; accepted: 16 February 2010.

DOI:10.1097/QAD.0b013e32833a8e6d

ISSN 0269-9370 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins

TIM1 is associated with Th2 type immune responses and selectively expressed on Th2 cell [1,6]. Human *TIM1* exhibits a high degree of amino-acid variability in the extracellular mucin domain encoded by exon 4 [7]. Because mucin domain is involved in the recognition of molecules outside the cells, it was suggested that *TIM1* might have been under the selective pressure in the course of human evolution, and the variation in this gene might be involved in the difference in the susceptibility to immune-related diseases including infectious and autoimmune diseases. There are several reports on the association of polymorphisms in *TIM1* with allergic diseases [8–12]. In addition, Nuchnoi *et al.* [13] have recently reported that a *TIM1* promoter haplotype, which was linked to higher expression of *TIM1*, was associated with the resistance to cerebral malaria in Thais.

HIV-1 infection is characterized by its chronic disease course, in which $CD4^+$ T cells gradually decrease that is followed by the dysfunction of host immune system. During the course of HIV-infection, the balance between Th1 and Th2 immune responses played a crucial role in the control of viral replication [14,15]. Therefore, it is highly possible that polymorphisms of genes involved in the Th1 and Th2 immune responses potentially contribute to the disease progression to AIDS and/or the susceptibility to HIV-1 infection.

In this study, we investigated the association of *TIM1* polymorphisms with the disease progression of HIV/AIDS in 246 HIV-1-infected Thai females. It was found that the *TIM1* haplotype D3-A significantly delayed the progression to AIDS.

Materials and method

Individuals

We investigated a cohort composed of 246 HIV-1-infected women obtained from the HIV clinic in the Day Care Center of Lampang Hospital in north Thailand between July 6, 2000 and July 12, 2001 as described previously [16]. They were recruited when they visited the clinic. Although the dates of HIV infection and seroconversion were not assessed or estimated for the individuals, they were free from antiretroviral drug at the time of recruitment. We also investigated 74 exposed seronegative (ESN) Thai women [17]. For a control group, we collected blood samples from 119 female blood donors at the blood bank of the same hospital. EDTA-blood was taken from each individual at the time of recruitment, and separated for plasma and pack red cell. Plasma HIV-1 RNA copy number was measured by using a commercially available kit (Amplicor HIV-1 Monitor Test; Roche Molecular System, Inc. Branchburg, New Jersey, USA), which had a lower limit of detection at 400 copies/ml. $CD4^+$ cell count was measured by using FACScan (BD Biosciences, California, USA). The survival status of participants until 1 October 2003 was ascertained from the cohort database, mailing letters, and death certificates at the Lampang Provincial Health Office. Data were double entered and validated using the access program. This study was approved by the Ethical Review Committee for Research in Human individuals, Ministry of Public Health, Thailand in January 2000.

Genotyping

Genomic DNA was extracted and purified from the frozen buffy coat by using QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hiden, Germany). Each sample was analyzed for polymorphisms in the *TIM1*-exon 4 by direct sequencing. The primer pair, forward 5′-GGGCAATGACCAAGATTGAC-3′ and reverse 5′-ACCTTGATACAATGCCCTGG-3′, was used to amplify the 470-bp fragment of *TIM1*-exon 4 by polymerase chain reaction (PCR) that was performed in a total volume of 25 μl containing 20–50 ng of genomic DNA, 100 nmol/l of each dNTPs, 0.2 μmol/l of each primer, 2.5 mmol/l of $MgCl_2$, and 0.5 U of Taq DNA polymerase (Immolase DNA polymerase; Bioline USA, Inc. Massachusetts, USA). Amplification profile included initial incubation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 5 min. The PCR products were purified by sephadex gel centrifugation, and 1 μl of purified product was then used as a template for sequencing using BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA) in a total volume of 10 μl containing the PCR forward primer. Sequence analysis was done by ABI Prism 3100 Genetic Analyzer and SeqScape software version 2.5 (Applied Biosystems). Determination of *TIM1* haplotypes was base on the previous report [7]. Because there were four common insertion/deletion polymorphisms in *TIM1*-exon 4, each pattern of heterozygous samples was also analyzed by cloning the PCR product into a plasmid vector (TOPO™ TA Cloning Kit; Invitrogen, Carlsbad, California, USA) according to the manufacturer's instruction and six colonies of each sample were sequenced to further determine the *TIM1* haplotypes.

Quantification of *TIM1* transcript in human B lymphoblastoid cell lines

Human B lymphoblastoid cell lines were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum under 5% CO_2. Cells were collected at growing phase and approximately 10^7 cells were subjected to total cellular RNA preparation by RNeasy mini kit (Qiagen). One microgram of RNA was used to synthesize cDNA by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instruction. Quantitative real-time PCR used iCycler iQ Real-Time PCR Detection System (Bio-Rad) and iQ SYBR Green Supermix kit (Bio-Rad) to measure relative amount of

mRNA by cycle threshold. Expression level of *TIM1* was normalized by the expression level of *GAPDH* and the ratio of *TIM1/GAPDH* was arbitrarily expressed as arbitrary unit (AU) for comparison of the expression levels in different cell lines. Primers and probe for quantification of *TIM1* and *GAPDH* transcripts are as follows *TIM1* sense primer 5′-CCACCAGCTCACCA TTGTACT-3′; antisense primer 5′-TCTGCTTGGA CTTCCTTTTCA-3′. *GAPDH* primer sequences are as follows sense primer 5′-CTTCACCACCATGGAGA AGGC-3′; antisense 5′-GGCATGGACTGTGGTCAT GAG-3′. Relative expression level was expressed arbitrarily as the ratio of *TIM1/GAPDH*.

Statistical analysis

Association of *TIM1* haplotype with the disease progression was assessed with respect to baseline clinical data including plasma viral load, CD4 cell count, proportion of symptomatic AIDS, and survival status during the follow-up period for 3 years. Continuous variables of two groups with different genetic background were compared by a two-tailed nonparametric Kruskal–Wallis test. Qualitative variables of two groups were compared by a chi-squared test, and the statistical significance was not corrected for multiple testing. Significance in Kaplan–Meier analysis was assessed by a log-rank test. Statistical analyses were carried out using Epi Info version 3.01 (US-CDC). Crude and adjusted hazard (HR) and their 95% confidence intervals (CI) were calculated by Cox proportional hazard models using StatView (SAS Institute Inc., Cary, North Carolina, USA). For adjustment of CD4 cell count, patients were categorized into three groups, namely, those with CD4 cell count (cells/μl) below 50, those with 50–199, and those with 200 and over.

Result

TIM1-exon 4 haplotype distribution in Thais

We previously reported a total of eleven *TIM1* haplotypes in various ethnic groups [7]. In the present study, six out of the 11 *TIM1* haplotypes were found in the studied Thai population that was composed of 246 antiretroviral drug-free HIV-positives, 74 ESNs, and 119 controls, with the most prevalent haplotype D3-A (Table 1). In addition, we found a novel haplotype (D3-C*) in one individual carrying a frame-shift mutation, a single base (A) deletion at the 3rd codon of amino acid residue 207. The distribution of *TIM1* haplotypes did not differ among the HIV-positives, ESNs, and controls, suggesting that the *TIM1* haplotypes were not associated with the susceptibility or resistance to the HIV-1 infection (Table 1).

TIM1 haplotype and disease status of HIV-1 infection viral load, CD4 cell count, and clinical status

We analyzed viral load, CD4 cell count, and clinical status among the antiretroviral drug-free women at the recruitment by stratifying the individuals according to their *TIM1* genotypes. We found that patients homozygous or heterozygous for the D3-A haplotype showed a tendency towards higher $CD4^+$ cell count ($P = 0.06$) than patients with other genotypes. Further analyses revealed that the patients carrying the D3-A haplotype had lower proportion of AIDS-related symptoms ($P = 0.021$) than the other patients, although the difference was not statistically significant after the correction of P values for multiple testing (Table 2).

We also found that the D3-C haplotype behaved opposite way to the D3-A haplotype, although there were only two nucleotide differences between the D3-A and D3-C haplotypes (Table 1). CD4 cell count was lower in the patients homozygous or heterozygous for the D3-C haplotype than in those with the other genotypes, although the difference did not reach statistical significance ($P = 0.07$). Further analysis on the proportion of AIDS-related symptoms showed marginally significant difference ($P = 0.05$). The other *TIM1* haplotypes did not show any apparent association with the HIV-1 disease status.

TIM1 haplotype and survival status

Because the cross-sectional study demonstrated a possible association of *TIM1* D3-A haplotype with relatively benign disease status of HIV infection, we intended to

Table 1. TIM1 haplotypes found in the Thai population.

	Polymorphic site								Haplotype frequency		
TIM1 haplotype	T>C (Thr158Met)	3 bp deletion (Thr160del)	18 bp deletion (6AA 161-166 del)	C>T (Pro180Leu)	3 bp deletion (Thr201del)	1 bp deletion (frameshift fs207)	A>G (Thr208Ala)	G>T (Thr208Thr)	HIV-infected ($2n = 492$)	ESN[#1] ($2n = 148$)	Control[#2] ($2n = 238$)
W-A	T	w	w	C	w	w	G	G	0.018	0	0.008
W-C	T	w	w	C	w	w	A	G	0.002	0	0
D1	C	del	w	T	w	w	A	T	0.016	0.020	0.008
D3-A	C	w	del	C	w	w	A	G	0.652	0.676	0.697
D3-C	T	w	del	C	w	w	G	G	0.112	0.149	0.092
D3-C*	T	w	del	C	w	del	G	G	0.002	0	0
D4	T	w	w	C	del	w	A	G	0.205	0.155	0.193

#1; exposed seronegative individuals. #2; healthy blood donors.

Table 2. Clinical parameters of HIV-1 infected individuals stratified by the presence of each TIM1 haplotype.

	D1		D3-A		D3-C		D4		W-A	
	Presence $n=8$	Absence $n=238$	Presence $n=204$	Absence $n=42$	Presence $n=52$	Absence $n=194$	Presence $n=85$	Absence $n=161$	Presence $n=7$	Absence $n=239$
Median viral load (log_{10})	5.45	5.06	5.05	5.11	5.20	5.02	5.08	5.07	5.19	5.07
[IQR]	[4.95–5.90]	[4.28–5.55]	[4.33–5.57]	[4.28–5.51]	[4.54–5.55]	4.25–5.57]	[4.28–5.51]	[4.39–5.57]	[4.25–5.44]	[4.33–5.57]
P	0.0814		0.5975		0.2736		0.6323		0.9635	
Median CD4 cell count	169	266	278	169	174	275	270	262	258	262
cell/μl [IQR]	[48–356]	[71–422]	[90–437]	[50–371]	[52–374]	[94–422]	[76–416]	[66-421]	[25–354]	[71–422]
P	0.3909		0.0615		0.067		0.826		0.304	
HIV-1 related symptoms % (*n*)	62.5 (5)	38.2 (91)	35.8 (73)	54.8 (23)	50.9 (27)	35.8 (69)	40.0 (34)	38.5 (62)	57.1 (4)	38.5 (92)
P	0.155		0.021		0.05		0.819		0.2686	
Death (*n*)	3	62	49	16	18	47	24	41	1	64
PYO	12.58	511.93	443.47	81.03	105.60	418.90	179.94	344.56	17.33	507.17
Mortality rate (%)	23.85	12.11	11.05	19.75	17.05	11.22	13.34	11.90	5.77	12.62
(95%CI)	(−0.26,47.96)	(9.28,14.94)	(8.13,13.97)	(11.08,28.42)	(9.86,24.24)	(8.19,14.25)	(8.36,18.32)	(8.48,15.32)	(−5.31,16.85)	(9.73,15.51)

IQR; Interqualtile range, PYO; person years of observation. W-C and D3-C* were not shown because only one heterozygote was observed.

investigate the association of *TIM1* D3-A haplotype with the disease progression to AIDS. Among 246 HIV-positive individuals, we obtained follow-up information from 238 individuals (96.7%) with the median (interquaternary range) of follow-up day at 964 (495-1072) days. The details of follow-up information were reported in our previous study [16]. During the follow-up period, 65 cases died. When we used death as a marker for the disease progression to AIDS, we found that mortality rate of patients possessing the D3-A haplotype was slightly lower than the other patients (Table 2). Kaplan–Meier analysis showed significantly better survival for the D3-A haplotype-carriers than the others ($P=0.044$, log-rank test) (Fig. 1a).

In addition, during the follow-up period, 55 patients had started antiretroviral drug treatment. To adjust the possible effects of antiretroviral treatment on survival, we subtracted the number of days after the patients started the antiretroviral drug from the observation period. As shown in Fig. 1b, we obtained almost identical results to those shown in Fig. 1a. The statistical significance of better survival of patients carrying the D3-A haplotype was found ($P=0.019$), even after the adjustment for the effect of antiretroviral treatment. We also compared the survival prognosis between the homozygous and heterozygous D3-A haplotype carriers for the possible gene dosage effect, but no difference in the survival curves between them was observed (data not shown). These results suggested that the D3-A haplotype could exert a dominant effect on the HIV-1 diseases progression. On the contrary, Kaplan–Meier analysis showed that no other haplotypes significantly affected the survival prognosis in our population samples (data not shown).

The risk of death for patients carrying the D3-A haplotype during the untreated period was significantly lower than the other patients [hazard ratio (HR) = 0.51, Cox model; 95% confidential interval (CI) = 0.29–0.90]. HR conferred by the D3-A haplotype did not significantly change after the adjustment for age (HR = 0.52; 95% CI = 0.29–0.92), plasma viral load (HR = 0.51; 95% CI = 0.29–0.91), or the presence of other risk factors, *RANTES-28G* (HR = 0.56; 95% CI = 0.31–0.99) or *IL4–589T* (HR = 0.47; 95% CI = 0.27–0.84), in which we previously observed apparent protective effects against the disease progression in the same set of samples [16]. On the contrary, adjustment for the baseline CD4 cell count diminished the significant protective effect of D3-A haplotype (HR = 0.72; 95% CI = 0.40–1.28), suggesting that the protective effect conferred by the D3-A haplotype may be due to its effect on the CD4 cell count.

TIM1 D3-A haplotype and the expression of *TIM1*

We investigated the association between the *TIM1* haplotypes and the expression levels of *TIM1* in B

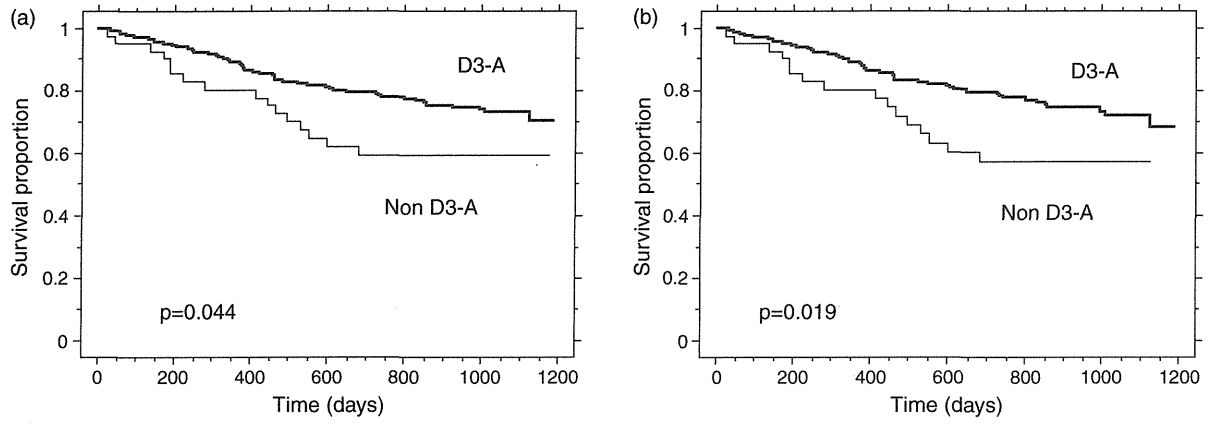

Fig. 1. Kaplan–Meier analysis of the effect of *TIM1* haplotype on the disease progression of HIV-1/AIDS. Kaplan–Meier analyses on the survival in the antiretroviral drug-free HIV-1 infected Thai females during the total follow-up period (a) or untreated period (b). In (a) and (b), patients were stratified by the presence and absence, respectively, of the D3-A haplotype.

lymphoblastoid cell lines. The amounts of *TIM1* mRNA were normalized by *GAPDH* mRNA among 22 cell lines. As shown in Fig. 2a, the cell lines homozygous or heterozygous for the D3-A haplotype expressed significantly lower levels of *TIM1* than those with other genotypes [D3-A carrier ($n=14$) vs. noncarrier ($n=8$); 5.49 ± 2.10 AU vs. 7.56 ± 1.85 AU, $P=0.042$, two-tailed Mann–Whitney test).

We also analyzed the associations of two tightly linked promoter sequence variations, rs7702919 and rs41297577, with the expression levels of *TIM1*. Although these two promoter SNPs were reported to be significantly associated with higher expression levels of *TIM1* [13], we could not replicate the associations; there was virtually no difference in the *TIM1* expression depending on the SNP genotypes or SNP haplotypes (Fig. 2b and c).

Discussion

We demonstrated that *TIM1* D3-A haplotype was associated with slower progression to HIV-related disease in a Thai cohort. Although the statistical significance for the association of D3-A haplotype with HIV-related symptoms in the cross sectional study was lost after the

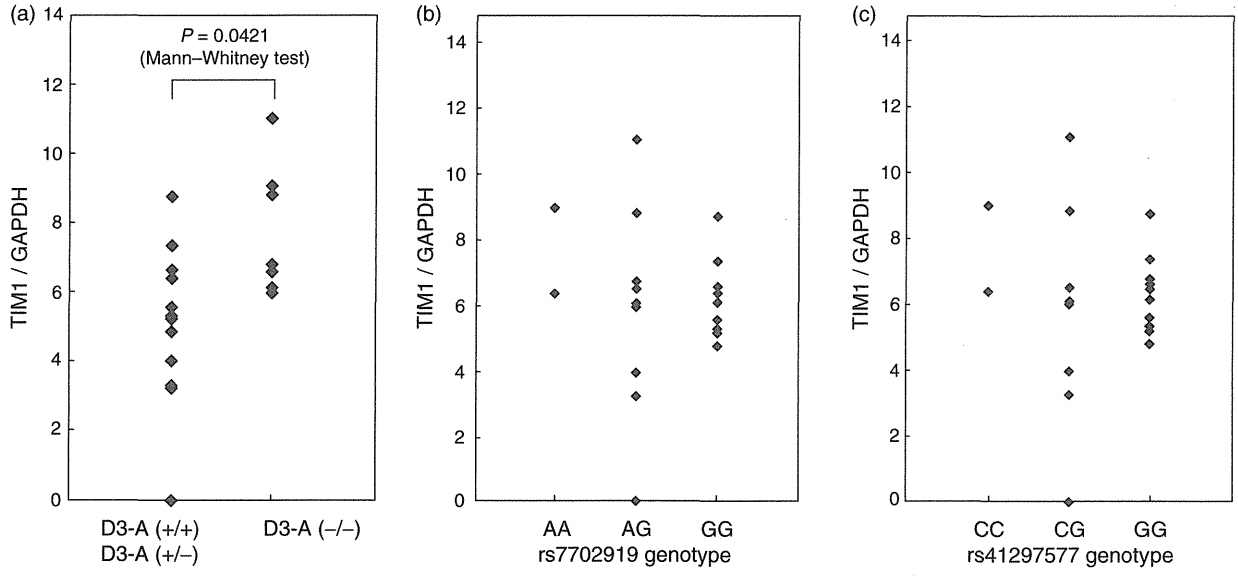

Fig. 2. Correlation between the *TIM1* polymorphisms and the expression levels of *TIM1* in B lymphoblastoid cell lines. Expression levels of *TIM1* were represented by cycle threshold values calculated from quantitative real-time PCR evaluation for *TIM1* transcripts normalized by the *GAPDH* transcripts. The ratio of *TIM1/GAPDH* was used to compare the expression levels in different B cell lines stratified by the presence or absence of D3-A haplotype (a), rs7702919 genotype (b), and rs41297577 genotype (c).

adjustment for multiple testing, Kaplan–Meier analysis showed that the patients carrying the D3-A haplotype followed a better survival prognosis during the untreated clinical course than the others. We also found that the *TIM1* expression level was lower in the cells with the D3-A haplotype than those without D3-A haplotype.

It has been reported that *TIM1* was expressed in the Th2 type $CD4^+$ cells and played crucial roles in the regulation of Th1/Th2 balance by promoting Th2 cells and augmenting Th2 cytokine production [1,2]. A six-amino-acid insertion/deletion polymorphism, which is tightly linked to the D3-A haplotype (Table 1), has been reported to be associated with the susceptibility to atopic disease in HAV-seropositive individuals [8–12]. It is suggested that the effect on Th1/Th2-subset differentiation appears to be a plausible mechanism how the *TIM1* polymorphism determines the susceptibility to atopic disease, because this polymorphism did not affect the infectious rates of HAV. It follows from these lines of evidence that the *TIM1* D3-A haplotype might be associated with low levels of Th2 promotion due to low expression of *TIM1*. Low levels of Th2 promotion would result in enhanced Th1 type responses. It is possible that the enhanced Th1 type immune responses in the HIV-1 infected individuals promote proliferation of CCR5-expressing $CD4^+$ cells, which can enhance the replication of HIV-1 in the infected cells and lead to the rapid progression to AIDS.

On the contrary, however, it is also possible that Th1 type immune responses may promote the proliferation of cytotoxic T cells that can suppress the HIV-1 replication in the infected individuals and lead to the slow progression to AIDS as observed in the study presented here. In the present study, we observed that individuals carrying the D3-A haplotype possessed relatively increased number of $CD4^+$ cells (Table 2) and $CD8^+$ cell (data not shown) at the baseline. It has been reported that in-vitro stimulation of $CD4^+$ T cells with a *TIM1*-specific monoclonal antibody enhanced the T-cell proliferation, suggesting that *TIM1* haplotypes and the expression levels of *TIM1* might be linked to $CD4^+$ and/or $CD8^+$ T-cell counts. It is thus necessary to analyze the number of $CD4^+$ T cells and the proportion of Th1/Th2 $CD4^+$ T cells in individuals with different *TIM1* haplotypes to clarify the effect of D3-A haplotype on the regulation of Th1/Th2 balance in the initial course of HIV-1 infection.

Nuchnoi *et al.* [13] reported an association of *TIM1* promoter haplotypes with the susceptibility to cerebral malaria. They also reported that the promoter haplotype associated with the resistance to cerebral malaria showed a higher expression of *TIM1*. We, however, could not replicate the associations of promoter SNPs with the *TIM1* expression in this study. We also studied the structure of linkage disequilibrium in *TIM1* by using three ethnic population samples from African–American, Caucasian, and Japanese. Sequence variations in exon 4 of *TIM1* were not in significant linkage disequilibrium with the promoter SNPs (data not shown). These observations suggested that the association between the D3-A haplotype and *TIM1* expression was not depending on the promoter SNPs.

Among 439 Thais analyzed in the present study, we identified a novel frame-shift mutation, a single base (A) deletion at the 3rd-codon of amino acid residue 207. This deletion causes a premature termination of TIM1 protein, resulting in a production of aberrantly truncated protein of 220 amino acids long. Therefore, individuals with this rare allele would produce nonfunctional TIM1, because the truncated protein should lack the transmembrane region and cytoplasmic tail. The effect of this frameshift mutation on the HIV-1 infection and/or disease progression to AIDS may be interesting, but we could not obtain definite findings, because there was only one case with this mutation. Further epidemiological studies using larger population samples are required to clarify the impact of the frameshift mutation on the susceptibility to HIV-1/AIDS.

There are several limitations in our study. First, accurate time of HIV infection and dates of seroconversion were not available in our HIV-infected individuals. To study the exact effects of *TIM1* haplotypes on the disease progression and survival time, information on the HIV infection and seroconversion will help further inspections. Second, this is the first report of the association. It is hence to investigate another cohort to replicate the finding. Third, our cohort was composed of HIV-infected women and there might be sex difference. Then, it is worth testing whether the effect of *TIM1* haplotypes could be found in male cohorts. Finally, this study was performed in a Thai population. Because the contribution of a genetic factor could be different depending on the races or ethnic groups, study in other ethnic groups is needed to further confirm the association of *TIM1* haplotypes with the disease status and/or disease progression of HIV/AIDS.

In conclusion, we revealed that a common *TIM1* haplotype D3-A was associated with the relatively slow disease progression in HIV-infected Thai women. We also demonstrated that this haplotype was linked to the decreased expression of *TIM1*. These observations suggested that the *TIM1* haplotype might have impacts on the susceptibility to HIV-1/AIDS via altered Th1/Th2-subset differentiation.

Acknowledgements

We are grateful to all the patients participated in the Lampang cohort and blood donors for their allowing us using their blood in this study and medical staff in Lampang Hospital for their cooperation. We thank Dr Taeko Naruse (Tokyo Medical and Dental University) for

kindly providing us with human B lymphoblastoid cell lines. This work was supported in part by research grants from the Department of Medical Sciences, Ministry of Public Health, Thailand, the Japanese Foundation for AIDS prevention, the Ministry of Health, Labor and Welfare, Japan, the Japan Health Science Foundation, the program of Founding Research Centers for Emerging and Reemerging Infection Disease, Grant-in-Aids for Scientific research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, and a grant from Heiwa Nakajima Foundation, Japan.

References

1. McIntire JJ, Umetsu SE, Akbari O, Potter M, Kuchroo VK, Barsh GS, *et al.* **Identification of *Tapr* (an airway hyperreactivity regulatory locus) and the linked *Tim* gene family.** *Nature Immunol* 2001; **2**:1109–1116.
2. Myers JH, Sabatos CA, Chakravarti S, Kuchroo VK. **The TIM gene family regulates autoimmune and allergic diseases.** *Trends Mol Med* 2005; **11**:362–369.
3. Feigelstock D, Thompson P, Mattoo P, Zhang Y, Kaplan GG. **The human homolog of HAVcr-1 codes for a hepatitis A virus cellular receptor.** *J Virol* 1998; **72**:6621–6628.
4. Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, Sanicola M. **Kidney injury molecule 1 (KIM1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain is up-regulated in renal cells after injury.** *J Biol Chem* 1998; **273**:4135–4142.
5. Curtiss M, Colgan J. **The role of the T-cell costimulatory molecule Tim-1 in the immune response.** *Immunol Res* 2007; **39**:52–61.
6. Khademi M, Illes Z, Gielen AW, Marta M, Takazawa N, Baecher-Allan C, *et al.* **T cell Ig- and mucin-domain-containing molecule-3 (TIM-3) and TIM-1 molecules are differentially expressed on human Th1 and Th2 cells and in cerebrospinal fluid-derived mononuclear cells in multiple sclerosis.** *J Immunol* 2004; **172**:7169–7176.
7. Nakajima T, Wooding S, Satta Y, Jinnai N, Goto S, Hayasaka I, *et al.* **Evidence for natural selection in the HAVCR1 gene: high degree of amino-acid variability in the mucin domain of human HAVCR1 protein.** *Genes Immun* 2005; **5**:398–406.
8. Gao PS, Mathias RA, Plunkett B, Togias A, Barnes KC, Beaty TH, Huang SK. **Genetic variants of the T-cell immunoglobulin mucin 1 but not the T-cell immunoglobulin mucin 3 gene are associated with asthma in an African American population.** *J Allergy Clin Immunol* 2005; **115**:982–988.
9. McIntire JJ, Umetsu SE, Macaubas C, Hoyte EG, Cinnioglu C, Cavalli-Sforza LL, *et al.* **Immunology: hepatitis A virus link to atopic disease.** *Nature* 2003; **425**:576–1576.
10. Graves PE, Siroux V, Guerra S, Klimecki WT, Martinez FD. **Association of atopy and eczema with polymorphisms in T-cell immunoglobulin domain and mucin domain-IL-2-inducible T-cell kinase gene cluster in chromosome 5q33.** *J Allergy Clin Immunol* 2005; **116**:650–656.
11. Chae SC, Song JH, Heo JC, Lee YC, Kim JW, Chung HT. **Molecular variations in the promoter and coding regions of human Tim-1 gene and their association in Koreans with asthma.** *Hum Immunol* 2003; **64**:1177–1182.
12. Noguchi E, Nakayama J, Kamioka M, Ichikawa K, Shibasaki M, Arinami T. **Insertion/deletion coding polymorphisms in hHAVcr-1 are not associated with atopic asthma in the Japanese population.** *Genes Immun* 2003; **4**:170–173.
13. Nuchnoi P, Ohashi J, Kimura R, Hananantachai H, Naka I, Krudsood S, *et al.* **Significant association between *TIM1* promoter polymorphisms and protection against cerebral malaria in Thailand.** *Ann Hum Genet* 2008; **72**:327–336.
14. Romagnani S, Del Prete G, Manetti R, Ravina A, Annunziato F, De Carli M, *et al.* **Role of TH1/TH2 cytokines in HIV infection.** *Immunol Rev* 1994; **140**:73–92.
15. Klein SA, Dobmeyer JM, Dobmeyer TS, Pape M, Ottmann OG, Helm EB, *et al.* **Demonstration of the Th1 to Th2 cytokine shift during the course of HIV-1 infection using cytoplasmic cytokine detection on single cell level by flow cytometry.** *AIDS* 1997; **11**:1111–1118.
16. Wichukchinda N, Nakayama EE, Rojanawiwat A, Pathipvanich P, Auwanit W, Vongsheree S, *et al.* **Protective effects of IL4-589T and RANTES-28G on HIV-1 disease progression in infected Thai females.** *AIDS* 2006; **20**:189–196.
17. Wichukchinda N, Nakayama EE, Rojanawiwat A, Pathipvanich P, Auwanit W, Vongsheree S, *et al.* **Effects of CCR2 and CCR5 polymorphisms on HIV-1 infection in Thai females.** *J Acquir Immune Defic Syndr* 2008; **47**:293–297.

Correspondence

Table 1. Associations between Apolipoprotein B Messenger RNA (mRNA) Editing Enzyme, Catalytic Polypeptide-Like 3B (*APOBEC3B*) Deletion and Susceptibility to Human Immunodeficiency Virus (HIV) Infection and AIDS

Population, genotype or allele	No. (%) of HIV-infected subjects or alleles	No. (%) of control subjects or alleles	I vs D		I/I + I/D vs D/D		I/I vs I/D + D/D	
			OR (95% CI)	P^a	OR (95% CI)	P^b	OR (95% CI)	P^a
Japanese			0.937 (0.653–1.343)	.791	0.650 (0.275–1.538)	.426	1.031 (0.645–1.648)	.995
I/I	45 (47.4)	128 (48.1)						
I/D	43 (45.3)	109 (41.0)						
D/D	7 (7.4)	29 (10.9)						
I	133 (70.0)	365 (68.6)						
D	57 (30.0)	167 (31.4)						
Indian			0.907 (0.637–1.291)	.651	1.215 (0.425–3.473)	.796	0.853 (0.569–1.281)	.508
I/I	181 (72.1)	139 (68.8)						
I/D	61 (24.3)	57 (28.2)						
D/D	9 (3.6)	6 (3.0)						
I	423 (84.3)	335 (82.9)						
D	79 (15.7)	69 (17.1)						

NOTE. CI, confidence interval; OR, odds ratio.

[a] χ^2 test.

[b] Fisher exact test.

No Evidence of an Association between the *APOBEC3B* Deletion Polymorphism and Susceptibility to HIV Infection and AIDS in Japanese and Indian Populations

To the Editor—The apolipoprotein B messenger RNA (mRNA) editing enzyme, catalytic polypeptide-like (APOBEC) family of genes for antiviral cytidine deaminases plays crucial roles in the intracellular defense mechanism against human immunodeficiency virus type 1 (HIV-1) [1–3]. *APOBEC3B*, a member of the APOBEC gene family and a 29.5-kb deletion that occurs between exon 5 of *APOBEC3A* and exon 8 of *APOBEC3B*, is commonly observed in the human population [4]. This deletion results in the complete loss of the *APOBEC3B* coding region in the genome [4].

It was reported by An et al [5] that individuals who are homozygous for the deletion allele of *APOBEC3B* had an increased the risk of HIV-1 infection and AIDS progression; this conclusion was based on studies of 2 ethnic populations, European Americans and African Americans. However, in their study the homozygotes were not observed in 724 European American control subjects and the distribution of genotypes among these control subjects significantly departed from the Hardy-Weinberg equilibrium. Thus, further replication studies are required to clarify the impact of *APOBEC3B* deletion on susceptibility to HIV infection and AIDS. Because the frequencies of the *APOBEC3B* deletion allele were found to be ~0.075 and ~0.039 in European Americans and African Americans, respectively, the expected numbers of individuals who are homozygous for the *APOBEC3B* deletion allele should be extremely low. On the other hand, the *APOBEC3B* deletion allele is more commonly observed in Asian populations [4], which are advantageous for studying the impact of the *APOBEC3B* deletion allele on susceptibility to HIV infection and AIDS.

We have investigated the association between the *APOBEC3B* deletion allele and susceptibility to HIV infection and AIDS in samples from 2 Asian populations, Japanese and Indian, which were previously analyzed for an association with polymorphisms in tripartite motif-containing 5α [6]. We obtained no evidence to support this association. The frequencies of the *APOBEC3B* deletion allele and of *APOBEC3B* deletion allele homozygotes did not differ between HIV-1–infected subjects and control subjects in either ethnic population (Table 1). In addition, a meta-analysis based on the results from the study of An et al [5] and from our study showed no statistical significance for the association between the *APOBEC3B* deletion and susceptibility to HIV infection and AIDS. The odds ratio for the *APOBEC3B* deletion allele was 1.046 (95% confidence interval, 0.896–1.221; $P = .572$), and that for *APOBEC3B* deletion allele homozygotes was 1.223 (95% confidence interval, 0.660–2.267; $P = .523$).

We also evaluated the effects of

APOBEC3B deletion on chronic HIV-1 infection. Ninety-five HIV-1–infected Japanese subjects with hemophilia were divided into 2 groups on the basis of their clinical courses during follow-up period of >15 years: one group of 48 subjects who maintained their $CD4^+$ T cell counts at ⩾200 cells/μL without antiretroviral treatment (nonprogressors), and another group of 47 subjects who required antiretroviral treatment because of depletion of their $CD4^+$ T cells below this level (slow progressors). When the frequencies of the *APOBEC3B* deletion allele and of homozygotes were compared between these 2 groups, there was no significant difference in the distribution of the allele (frequency, 0.323 in nonprogressors vs 0.277 in slow progressors) or homozygotes (frequency, 0.083 in nonprogressors vs 0.084 in slow progressors).

In addition, we found no effects of *APOBEC3B* deletion on HIV-1 load and $CD4^+$ T cell count. The data for HIV-1 load were available for 75 of 95 HIV-1–infected Japanese subjects and 76 of 251 HIV-1–infected Indian subjects. No significant correlations between the *APOBEC3B* genotypes and HIV-1 load were observed in either the Japanese population (mean ± standard error [SE] HIV RNA load for I/I genotype, 1.94 ± 0.30 $\log_{10}$ copies/mL; mean ± SE HIV RNA load for I/D genotype, 2.43 ± 0.30 $\log_{10}$ copies/mL; mean ± SE HIV RNA load for D/D genotype, 2.13 ± 0.77 $\log_{10}$ copies/mL) or the Indian population (mean ± SE HIV RNA load for I/I genotype, 4.33 ± 0.16 $\log_{10}$ copies/mL; mean ± SE HIV RNA load for I/D genotype, 4.14 ± 0.24 $\log_{10}$ copies/mL; mean HIV RNA load for D/D genotype, 5.02 $\log_{10}$ copies/mL). The data for $CD4^+$ T cell count were available for 203 of 251 HIV-1–infected Indian subjects, and there were no associations between the *APOBEC3B* insertion/deletion genotypes and the $CD4^+$ T cell count (mean ± SE cell count for I/I genotype, 332.3 ± 17.7 cells/μL; mean ± SE cell count for I/D genotype, 393.2 ± 35.5 cells/μL; mean ± SE cell count for D/D genotype, 344 ± 76.9 cells/μL).

It has been reported that APOBEC3G and APOBEC3F play major roles in the APOBEC3-mediated defense against HIV-1 infection [1–3]; however, HIV-1 has a defense against the anti-HIV effects of APOBEC3G and APOBEC3F through the Vif-induced degradation of APOBEC3 proteins. On the contrary, APOBEC3B has been reported to be resistant against the Vif-induced degradation of APOBEC3 [2, 7–9], which may be advantageous in the host defense against HIV-1 infection. However, the expression of *APOBEC3B* is usually absent in the lymphoid cells that serve as targets for HIV-1 infection [2, 9, 10], which may be the reason that the *APOBEC3B* deletion was not associated with the susceptibility to HIV infection and AIDS.

In conclusion, we found that the deletion polymorphism of *APOBEC3B* had no effect on susceptibility to HIV infection and AIDS in Japanese or Indian populations. Further studies will be required to clarify the impact of *APOBEC3B* deletion on the susceptibility to HIV infection and AIDS in still other populations.

Sakiko Itaya,[1] Toshiaki Nakajima,[1,2] Gurvinder Kaur,[4] Hiroshi Terunuma,[3] Hitoshi Ohtani,[2] Narinder Mehra,[4] and Akinori Kimura[1,2]

[1]Department of Molecular Pathogenesis, Medical Research Institute, and [2]Laboratory of Genome Diversity, School of Biomedical Science, Tokyo Medical and Dental University, and [3]Biotherapy Institute of Japan, Tokyo, Japan; [4]Department of Transplant Immunology and Immunogenetics, All India Institute of Medical Sciences, New Delhi, India.

References

1. Sheehy AM, Gaddis NC, Choi JD, Malim MH. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **2002**; 418:646–650.
2. Bishop KN, Holmes RK, Sheehy AM, Davidson NO, Cho SJ, Malim MH. Cytidine deamination of retroviral DNA by diverse APOBEC proteins. Curr Biol **2004**; 14:1392–1396.
3. Chiu YL, Greene WC. Multifaceted antiviral actions of APOBEC3 cytidine deaminases. Trends Immunol **2006**; 27:291–297.
4. Kidd JM, Newman TL, Tuzun E, Kaul R, Eichler EE. Population stratification of a common APOBEC gene deletion polymorphism. PLoS Genet **2007**; 3:e63.
5. An P, Johnson R, Phair J, et al. APOBEC3B deletion and risk of HIV-1 acquisition. J Infect Dis **2009**; 200:1054–1058.
6. Nakajima T, Nakayama EE, Kaur G, et al. Impact of novel TRIM5α variants, Gly110Arg and G176del, on the anti-HIV-1 activity and the susceptibility to HIV-1 infection. AIDS **2009**; 23:2091–2100.
7. Yu Q, Chen D, König R, Mariani R, Unutmaz D, Landau NR. APOBEC3B and APOBEC3C are potent inhibitors of simian immunodeficiency virus replication. J Biol Chem **2004**; 279:53379–53386.
8. Romani B, Engelbrecht S, Glashoff RH. Antiviral roles of APOBEC proteins against HIV-1 and suppression by Vif. Arch Virol **2009**; 154:1579–1588.
9. Doehle BP, Schäfer A, Cullen BR. Human APOBEC3B is a potent inhibitor of HIV-1 infectivity and is resistant to HIV-1 Vif. Virology **2005**; 339:281–288.
10. Jarmuz A, Chester A, Bayliss J, et al. An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. Genomics **2002**; 79:285–296.

Potential conflicts of interest: none reported.

Financial support: Ministry of Health, Labor, and Welfare of Japan; Japan Health Science Foundation; Program of Founding Research Centers for Emerging and Reemerging Infection Disease; Program of Research on Publicly Essential Drugs and Medical Devices; Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (Grants-in-Aid for Scientific Research); Heiwa Nakajima Foundation; Department of Biotechnology (grant to D.B.T.); Ministry of Science and Technology, Government of India; Indian Council of Medical Research (ICMR); India-Japan Cooperative Science Program, Japan Society for the Promotion of Science (JSPS) of Japan and Department of Science and Technology (DST) of India.

Reprints or correspondence: Dr Akinori Kimura, Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan (akitis@mri.tmd.ac.jp).

The Journal of Infectious Diseases 2010; 202(5):815–816

 0022-1899/2010/20205-0021$15.00
DOI: 10.1086/655227

Protection of Macaques with Diverse MHC Genotypes against a Heterologous SIV by Vaccination with a Deglycosylated Live-Attenuated SIV

Chie Sugimoto[1,2,3¤a], Satoru Watanabe[1,2¤b], Taeko Naruse[4], Eiji Kajiwara[5], Teiichiro Shiino[1], Natsuko Umano[5], Kayoko Ueda[1,2], Hirotaka Sato[1,2], Shinji Ohgimoto[6], Vanessa Hirsch[7], Francois Villinger[8,9], Aftab A. Ansari[8], Akinori Kimura[4], Masaaki Miyazawa[5], Yasuo Suzuki[3,10], Naoki Yamamoto[1¤b], Yoshiyuki Nagai[11], Kazuyasu Mori[1,2,3,8]*

1 AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan, **2** Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, Japan, **3** CREST, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan, **4** Division of Medical Science, Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo, Japan, **5** Department of Immunology, Kinki University School of Medicine, Osaka-Sayama, Osaka, Japan, **6** Department of Virology, Osaka City University Graduate School of Medicine, Abeno-ku, Osaka, Japan, **7** Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, United States of America, **8** Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia, United States of America, **9** Yerkes National Primate Research Center, Emory University, Atlanta, Georgia, United States of America, **10** Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai, Aichi, Japan, **11** Center of Research Network for Infectious Diseases, Riken, Chiyoda-ku, Tokyo Japan

Abstract

HIV vaccine development has been hampered by issues such as undefined correlates of protection and extensive diversity of HIV. We addressed these issues using a previously established SIV-macaque model in which SIV mutants with deletions of multiple gp120 *N*-glycans function as potent live attenuated vaccines to induce near-sterile immunity against the parental pathogenic SIVmac239. In this study, we investigated the protective efficacy of these mutants against a highly pathogenic heterologous SIVsmE543-3 delivered intravenously to rhesus macaques with diverse MHC genotypes. All 11 vaccinated macaques contained the acute-phase infection with blood viral loads below the level of detection between 4 and 10 weeks postchallenge (pc), following a transient but marginal peak of viral replication at 2 weeks in only half of the challenged animals. In the chronic phase, seven vaccinees contained viral replication for over 80 weeks pc, while four did not. Neutralizing antibodies against challenge virus were not detected. Although overall levels of SIV specific T cell responses did not correlate with containment of acute and chronic viral replication, a critical role of cellular responses in the containment of viral replication was suggested. Emergence of viruses with altered fitness due to recombination between the vaccine and challenge viruses and increased gp120 glycosylation was linked to the failure to control SIV. These results demonstrate the induction of effective protective immune responses in a significant number of animals against heterologous virus by infection with deglycosylated attenuated SIV mutants in macaques with highly diverse MHC background. These findings suggest that broad HIV cross clade protection is possible, even in hosts with diverse genetic backgrounds. In summary, results of this study indicate that deglycosylated live-attenuated vaccines may provide a platform for the elucidation of correlates of protection needed for a successful HIV vaccine against diverse isolates.

Citation: Sugimoto C, Watanabe S, Naruse T, Kajiwara E, Shiino T, et al. (2010) Protection of Macaques with Diverse MHC Genotypes against a Heterologous SIV by Vaccination with a Deglycosylated Live-Attenuated SIV. PLoS ONE 5(7): e11678. doi:10.1371/journal.pone.0011678

Editor: Douglas F. Nixon, University of California San Francisco, United States of America

Received March 22, 2010; **Accepted** June 28, 2010; **Published** July 20, 2010

Funding: This work was supported by AIDS research grants from the Health Sciences Research Grants, from the Ministry of Health, Labor, and Welfare in Japan, and from the Ministry of Education, Culture, Sports, Science and Technology in Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: mori@nibio.go.jp

¤a Current address: Division of Immunology, Tulane National Primate Research Center, Covington, Louisiana, United States of America
¤b Current address: Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Introduction

Molecular epidemiological studies have revealed the existence of an extensive degree of diversity of HIV-1 isolates [1]. HIV-1 is classified in three major groups (M, N, O) based on their geographical origin. Group M represents the predominant HIV-1 circulating through the world and has been divided into more than 10 subtypes (clades) as well as increasing number of circulating recombinant forms (CRF) primarily due to error-prone viral reverse transcriptase and the occurrence of super-infections. This diversity is continuously expanding worldwide and is a major obstacle for the successful development of an AIDS vaccine. While the generation of a vaccine capable to prevent transmission of HIV isolates endemic in a particular area remains an unfulfilled task, protection against phylogenetically distant viruses represents an even more formidable hurdle. The failure and dismal success of HIV-1 vaccine trials that have been conducted so far has prompted a re-emphasis for more basic studies concerning vaccine

design against heterologous challenge viruses, which can at present only be addressed in a macaque model. One of the pre-conditions for the objective assessment of the protective efficacy against a heterologous strain would be that the macaque model used should have the capacity to confer sterile or near-sterile immunity against the homologous virus challenge.

SIVmac239 infected rhesus macaques gradually develop AIDS after a variable period of chronic infection. In order to investigate the role and function of the glycan shield of the viral envelope, we previously developed a panel of deglycosylated mutants from this pathogenic SIVmac239 backbone [2]. Among these mutants, one mutant with five *N*-glycans deleted (Δ5G) was found to be profoundly attenuated in rhesus macaques. Thus, while the acute primary viremia showed viral peaks undistinguishable from those measured in animals infected with the wild-type SIVmac239 infection, viral load during the chronic phase was contained at or below the level of detection [3]. More importantly, these Δ5G "immunized" macaques during the chronic phase manifested near-sterile immunity when challenged with the homologous wild-type SIVmac239, and the animals showed neither evolution of pathogenic revertants nor clinical disease manifestation during a 10 year follow up period. While it is clear that similar live attenuated HIV-1 vaccines will not likely be utilized in humans, it is extremely important to have an animal model that shows protection against heterologous challenge virus so that minimally such a model can be exploited to identify reproducible immune correlates of protection. We therefore reasoned that our SIVmac239-deglycosylation platform may provide an unique opportunity to test and analyze protection against challenge with heterologous isolates.

The studies reported herein utilized a series of four deglycosylated SIVmac239 mutants as potential live attenuated vaccine viruses and the SIVsmE543-3 isolate [4] as the heterologous challenge virus. We submit that the diversities between the vaccine viruses and the challenge virus are equivalent to those found between major HIV-1 subtypes. Thus, this heterologous challenge model provides an ideal model to assess the potential of and define the conditions for cross-subtype (clade) protection against HIV.

The natural protective effects of select rhesus macaque (Mamu) MHC class I alleles such as Mamu B*08, Mamu B*17, Mamu A*01 and the MHC class I haplotype 90120-Ia have been shown to be associated with better control of SIV [5,6,7,8,9]. In sharp contrast, protection by the deglycosylated SIV mutants exhibited no such selectivity; protection was achieved in all 9 rhesus macaques tested so far, which were later found to be indeed genetically highly diverse. Previous human cohort studies revealed that individuals who demonstrated control of HIV infection without any treatment, called long-term non-progressors and elite controllers, have common genetic properties associated with susceptibility to HIV or anti-viral host responses [10,11,12]. However, candidate vaccines that are aimed at targeting outbred human population will have to show effectiveness in humans with diverse genetic backgrounds. In order to minimize the contribution of particular positive or negative genetic background, macaques possessing the above described elite genotypes were therefore eliminated from the studies reported herein. Furthermore, the macaques were grouped based on the genetic data so that each group comprised animals with an essentially similar genetically diverse background.

We herein report data from a series of studies that support the concept that cross-subtype control of HIV-1 is theoretically possible irrespective of genetic background. Data derived herein demonstrate a critical role that glycosylation plays in not only conferring attenuation of SIV/HIV but also the potential role glycosylation plays in conferring pathogenic properties to viruses that emerge following challenge with heterologous viruses.

Results

Genetic diversity of the challenge virus from the vaccine virus

SIVs are as diverse as the HIV-1 subtypes in group M, and at present a total of 9 different SIV lineages have been identified [13]. SIVmac239 belongs to lineage 8. We have generated a variety of modified candidate live vaccine strains by the introduction of deglycosylation mutations into multiple *N*-glycosylation sites of the gp120 of SIVmac239 (Fig. 1). The heterologous challenge virus used in this study is the molecularly cloned pathogenic strain SIVsmE543-3 that belongs to lineage 1. SIVmac239 and SIVsmE543-3 possess 23 and 22 *N*-glycosylation sites, respectively, and as seen their topologies in the gp120 protein backbones are almost the same (Fig. 1).

At first, we compared the amino acid sequence differences for the individual viral proteins between SIVmac239 and SIVsmE543-3 (Table 1). The genetic differences varied from 7.9% for Pol to 35.9% for Tat. We then compared the diversity between the 2 SIV strains utilized herein with the intra-subtype or inter-subtype diversities in the HIV-1 isolates and found that the differences between SIVsmE543-3 and SIVmac239 were significantly greater than any intra-subtype diversities of HIV-1 (Table 1). For the inter-subtype diversity analysis, we used subtypes B and C and a circulating recombinant CRF01_AE as reference strains that are predominantly circulating in Asian countries. The data indicated that the differences between the two SIV strains were as high or higher as those found among the three HIV-1 subtypes. These results validate the use of SIVmac239 as the parental virus for live attenuated vaccine virus and the SIVsmE543-3 as the heterologous challenge virus in the rhesus macaque model of human AIDS.

Properties of the 3 new deglycosylation mutants as live attenuated candidate vaccines

We previously reported that Δ5G, a SIVmac239 molecular clone with quintuple deglycosylation mutations behaved as a live-attenuated virus in vivo [2,3]. In addition to Δ5G, we tested three newly constructed deglycosylated mutants of SIVmac239 viruses, Δ5G-ver1, Δ5G-ver2 and Δ3G as potential candidate vaccines in this study (Fig. 1). They differ by the sites or numbers of N-glycosylation sites mutated in gp120 (Fig. 1). All four deglycosylated mutants replicated well in rhesus peripheral blood mononuclear cells (PBMC) in vitro, and the replication kinetics were similar to SIVmac239 ([2], and data not shown). However, differences were noted in the rate of replication in macrophage cultures and sensitivity to neutralizing antibodies (NAb) (data not shown). To investigate whether these differences translated into altered in vivo properties such as viral replication kinetics in rhesus macaques, reduced pathogenicity and potential vaccine properties, 12 animals were inoculated intravenously in groups of three with 100 $TCID_{50}$ of each of the four mutants (Fig. 2 A). Since the MHC types have been shown to significantly influence the outcome of HIV/SIV infection in their respective hosts, we chose macaques which did not inherit any of the known elite MHC alleles [5,6,7,8,9] (File S1). Furthermore, to minimize the possible influence of other MHC types, we distributed the animals evenly into vaccine and control groups such that each group comprised animals with randomized MHC alleles (File S1).

Consistent with our previous studies [3], the prototypic vaccine strain Δ5G, replicated as robustly as the SIVmac239 in macaques

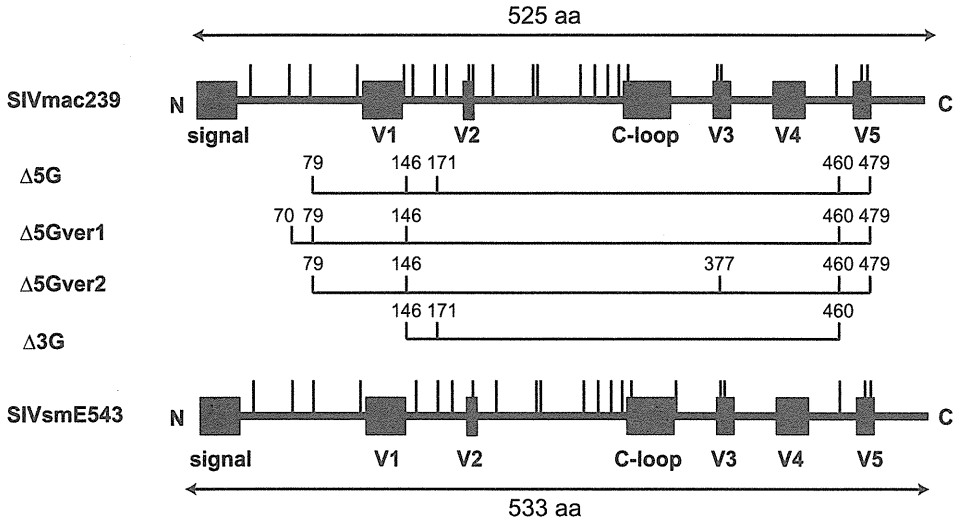

Figure 1. Live attenuated vaccines with deglycosylation mutations. N-glycosylation sites (vertical bars) localized within gp120 of SIVmac239 and SIVsmE543-3 are shown. SIVmac239 and SIVsmE543-3 have 23 and 22 *N*-glycosylation sites, respectively. The position of N-glycosylation sites mutated to remove the specific glycans for Δ5G, Δ5Gver-1, Δ5Gver-2, and Δ3G were indicated and constructed by site-directed mutagenesis based on SIVmac239. V1 to V5 indicate variable region 1 to 5 respectively. C-loop indicates the constant loop region within SIVmac239 [15].
doi:10.1371/journal.pone.0011678.g001

with peak plasma viral loads (VL) of $\sim 10^7$ copies/ml at 2 weeks post infection (pi) (Figs. 2A and 3). However, subsequently the VL of Δ5G rapidly declined to a level around or below the level of detection (100 copies/ml) whereas relatively high VL persisted in SIVmac239-infected macaques (Figs. 2A and 3). Essentially the kinetics of viremia observed with the three deglycosylation mutants, Δ5G-ver1, Δ5G-ver2 and Δ3G were similar to that seen with Δ5G (Fig. 2 A).

It has been well established that SIVmac239 elicits poor NAb in macaques [14]. In contrast, a deglycosylation mutant derived from SIVmac239 elicited higher NAb than SIVmac239, but levels of NAb responses varied among the animals [15]. Thus, we determined levels of potential NAb responses against each animal's respective infecting virus. Consistent with our previous results, most macaques infected with each of the deglycosylated SIVs induced NAb (Fig. 2 B). However, the levels of NAb responses differed among the four groups, with a decreasing order of magnitude for NAb responses from Δ5G-ver2>Δ5G-ver1>Δ3G>Δ5G. We detected no NAb response in two animals (Mm0301 in the Δ5G group and Mm0304 in the Δ3G group), and delayed and relatively weak responses in three animals (Mm0409 in the Δ5G group, Mm0511 in the Δ5G-ver1 group, and Mm0516 in the Δ3G group) (Fig. 2 B). Regardless of the levels of NAb, all 12 animals infected with the deglycosylation mutant viruses contained

Table 1. Differences between the vaccine and challenge SIV and inter-subtype differences of HIV-1.

Viral proteins	SIV[b]	HIV[a]						
	mac239 vs. smE543-3	Intra-subtype (A, B, C, D, F1, G, CRF01_AE, and CRF_02AG)	Inter-subtype					
			B vs. C		B vs. CRF01		C vs. CRF01	
			Mean	S.E.	Mean	S.E.	Mean	S.E.
Gag	11.1	4.3–7.1	10.2	1.2	11	1.4	12.8	1.5
Pol	7.9	2.8–6.5	7.8	0.7	8	0.8	7.5	0.7
Env	18.2	7.7–12.4	19.2	1.4	18.8	1.4	18.7	1.4
Nef	26.2	9.0–16.2	22.6	4.8	21.3	4.8	16.2	3.5
Tat	35.9	9.9–18.1	28.8	4.7	31.9	4.9	27.4	4.3
Rev	32.7	9.0–16.5	28.3	4.7	26.4	4.7	20.6	4.1
Vif	17.8	7.0–14.2	20.5	2.6	21.6	2.9	21.3	2.8
Vpr	14.9	5.4–10.6	13.1	3.2	13	3.4	5	3.5
Vpx	8.1	NA[c]	NA		NA		NA	
Vpu	NA	2.4–14.8	17.7	5.6	3.8	5.4	12.7	3

[a]Percentage amino acid sequence differences per site from averaging overall sequence pairs between the subtypes.
[b]Percent amino acid sequence differences per protein.
[c]Not applicable.
doi:10.1371/journal.pone.0011678.t001

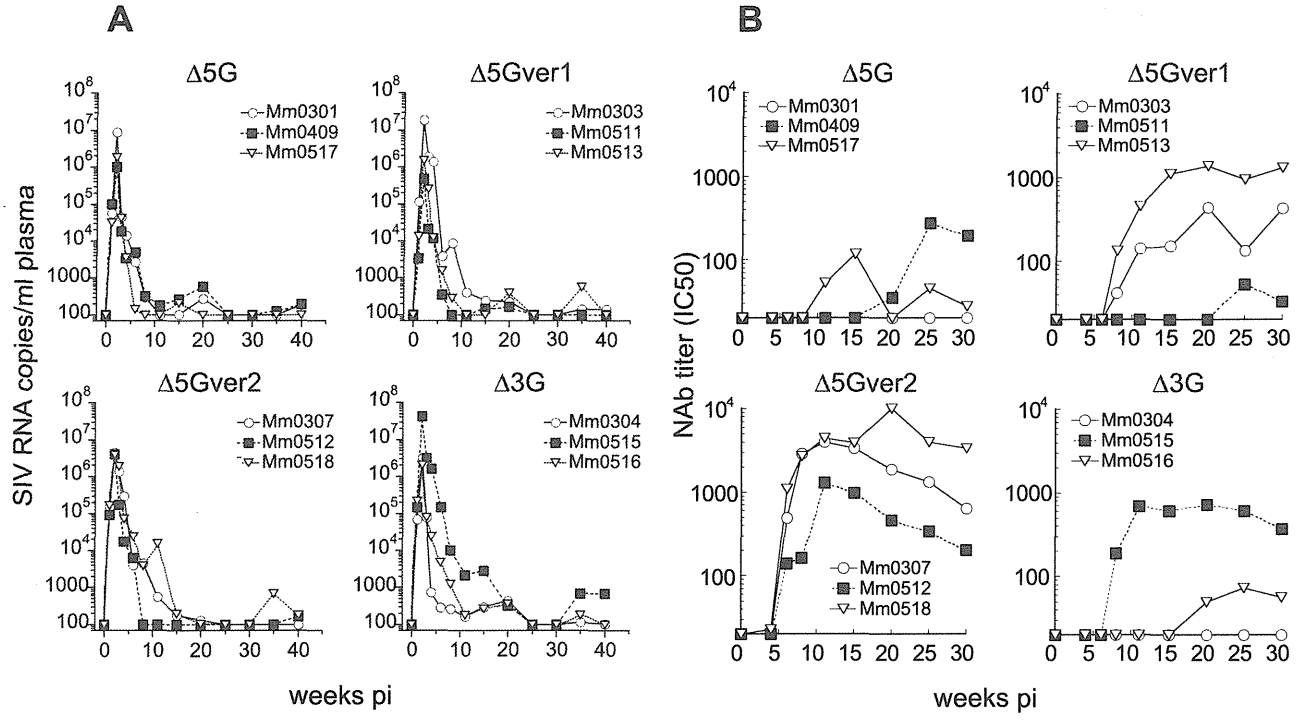

Figure 2. Viral loads and neutralizing antibodies in macaques infected with each of 4 deglycosylated SIV mutants. Twelve animals were divided into 4 groups consist of 3 animals each and infected with each of 4 deglycosylation mutants (Δ5G, Δ5Gver-1, Δ5Gver-2, and Δ3G). (A) Plasma viral loads were determined by real-time RT-PCR with SIVmac239 primers and probe set. (B) NAb responses against each respective infecting virus were measured in CEMx174/SIVLTR-SEAP system. NAb titers were indicated as the reciprocal of the dilutions of the plasma from the vaccinees yielding 50% inhibition (IC_{50}).
doi:10.1371/journal.pone.0011678.g002

primary infection with similar kinetics (Fig. 2 A) suggesting that NAb were most likely not a critical factor for containment of the acute infection in these animals.

We previously found that animals vaccinated with Δ5G completely resist infection when challenged with the parental pathogenic SIVmac239 [3], showing minimal if any replication of the challenge virus for more than 10 years. A similar homologous challenge was performed in a subset of animals that received the deglycosylation mutants in the present study. Thus, one of the three "immunized" animals from each group was challenged with a high dose (1000 $TCID_{50}$) SIVmac239 at 40 weeks following "vaccination" and plasma viral loads were determined (Fig. 3). As previously reported with Δ5G, a near-sterile immunity against challenge with SIVmac239 was not only noted with the Δ5G but also seen with our other three new deglycosylated SIV mutants, Δ5G-ver1, Δ5G-ver2 and Δ3G (Fig. 3). These results indicate that all 3 new vaccine versions possess similar equally high protective potential against the homologous, wild type SIVmac239 as the original Δ5G.

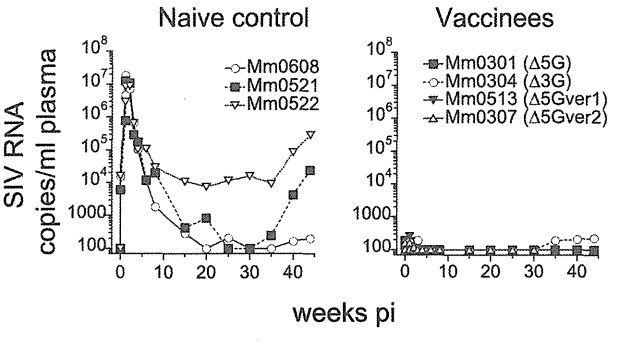

Figure 3. Plasma viral RNA loads in the homologous SIVmac239 challenge. Three naïve rhesus macaques (Mm0608, Mm0521, Mm0522) and 4 vaccinees (Mm0301, Mm0304, Mm0513, Mm0307), i.e. one animal from 4 deglycosylated SIV infection groups, were challenged intravenously with 1000 $TCID_{50}$ of SIVmac239. Plasma viral loads were determined by real-time RT-PCR with SIVmac239 primers and probe set.
doi:10.1371/journal.pone.0011678.g003

Protection of the vaccinated macaques against heterologous challenge infection

The remaining eight animals (2 per group) vaccinated with each of the 4 vaccine versions (Δ5G, Δ5G-ver1, Δ5G-ver2 or Δ3G) and 3 of the four animals that were vaccinated (described in the above paragraph) and challenged with SIVmac239 (Mm0307 died of SIV unrelated causes) were challenged with a high dose (1000 $TCID_{50}$) of SIVsmE543-3 delivered intravenously. Additional three naïve animals served as a control for this heterologous challenge experiment (Fig. 4 A). VL were monitored until 80 weeks post challenge (pc) using real time RT-PCR primer pairs and probes that distinguished the detection of SIVmac239 and SIVsmE543-3.

The 3 naïve control macaques infected with SIVsmE543-3 exhibited a peak VL of $\sim 10^7$ copies/ml at 2 weeks pi which is essentially similar to those we have routinely noted following infection with SIVmac239 with a few exceptions. Notably, the set point VL in SIVsmE543-3 was more than 10^5 copies/ml in 2 animals which is at least 1-log higher than that noted in animals infected with SIVmac239 (Figs. 3 and 4 A). We reason that SIVsmE543-3 is likely to be more pathogenic than SIVmac239 for

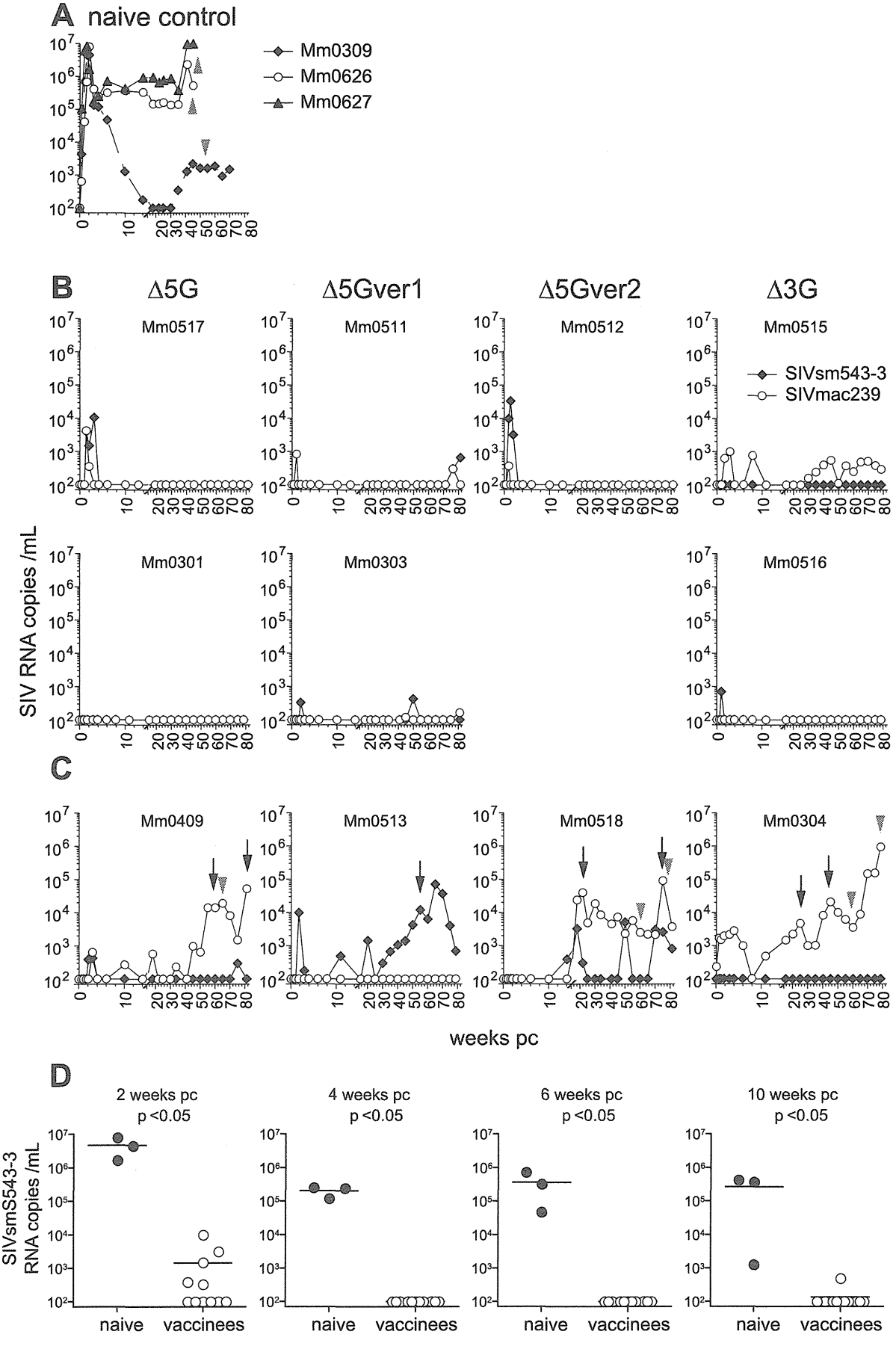
A naive control
Mm0309
Mm0626
Mm0627
B
Δ5G
Δ5Gver1
Δ5Gver2
Δ3G
Mm0517
Mm0511
Mm0512
Mm0515
SIVsm543-3
SIVmac239
Mm0301
Mm0303
Mm0516
SIV RNA copies /mL
C
Mm0409
Mm0513
Mm0518
Mm0304
weeks pc
D
2 weeks pc
p <0.05
4 weeks pc
p <0.05
6 weeks pc
p <0.05
10 weeks pc
p <0.05
SIVsmS543-3
RNA copies /mL
naive
vaccinees

Figure 4. Plasma viral RNA loads in the heterologous SIVsmE543-3 challenge. Three vaccine-naïve animals (Mm0309, Mm0626, Mm0627) (A) and 11 vaccinees (B, C) were challenged intravenously with 1000 $TCID_{50}$ of SIVsmE543-3. The vaccinees were divided into controllers (B) and non-controllers (C) based on control of vaccine and heterologous challenge infection. VL were determined by two sets of real-time RT-PCR for gag sequence of SIVsmE543-3 (closed diamonds) and SIVmac239 (open circles) respectively. For analysis of SIV sequence, PBMC and plasma were collected at the time-points indicated by arrows and arrowheads, respectively. (D) VL of the 11 vaccinees were statistically compared with those of 3 vaccine-naïve controls at 2, 4, 6 and 10 weeks pc. Significant difference between vaccinees and controls at each time points are shown (Mann-Whitney test).
doi:10.1371/journal.pone.0011678.g004

our Burmese rhesus macaques. Indeed, these 2 animals developed AIDS and were euthanized at 46 weeks pi, which is significantly faster than the time that we have noted for disease progression following SIVmac239-infection of these Burmese monkeys [3,8,15,16,17].

All 11 vaccinated animals contained the primary challenge with SIVsmE543-3 (Figs. 4A, B, and C). At 2 weeks pc, average VL for the vaccine groups were >3-log lower than those of naïve control animals (Fig. 4D). Thus, 5 of the 11 vaccinated animals (Mm0511, Mm0515, Mm0301, Mm0518, and Mm0304) contained the acute challenge below the level of detection (100 copies/ml) at all times, 3 animals (Mm0303, Mm0516, and Mm0409) showed marginal replication of SIVsm543-3 (329–700 copies/ml) during weeks 1–3 pc, while the other 3 animals (Mm0517, Mm0512, and Mm0513) showed viremia with $1–3\times10^4$ copies/ml during weeks 1–3 pc but these replication peaks were transient and by 4 weeks pc, all vaccinated animals controlled SIVsmE543-3 to undetectable levels (Figs. 4B, C and D). Interestingly, transient replication of the vaccine viruses was also detected in 6 animals after the SIVsmE543-3 challenge (Mm0517, Mm0511, Mm0512, Mm0515, Mm0409, and Mm0304) (Figs. 4 B and C), suggesting reactivation of the deglycoylated virus upon super-infection with SIVsmE543-3. Statistical analysis of these data led us to conclude that the vaccinated animals significantly controlled SIVsmE543-3 replication at least during the acute phase up to 10 weeks pc irrespective of their MHC genotypes (Fig. 4 D). These results, taken together indicate that each of the deglycosylated vaccines utilized has the potential of inducing protective immunity against a potentially highly pathogenic heterologous challenge virus.

We next evaluated the containment of the challenge virus infection during the chronic-phase compared with the one observed after homologous challenge with SIVmac239 (Fig. 3 and ref [3]). Based on longitudinal VL (of either vaccine or challenge virus), the vaccinated animals were divided into two groups. One group of 7 animals, which we termed as "controllers", comprised animals which controlled the challenge virus almost completely for the 80 weeks of follow-up pc (Fig. 4 B). Detailed analyses of VL showed complete control of the SIVsmE543-3 challenge in two of the animals (Mm0301 and Mm0515) over time. Similar potent antiviral control (except for small VL peaks during the acute-phase) were noted in three of the animals (Mm0512, Mm0516 and Mm0517). The last two "controllers" showed only occasional VL blips during the chronic-phase (Mm0303 and Mm0511). However, challenge with SIVsmE543-3 induced persistent low vaccine VL in Mm0515, while the challenge virus remained undetectable.

In what we termed as the non-controller group of 4 animals, VL gradually increased with time (Fig. 4 C). The evolving replicating viruses were found to consist of the challenge virus in Mm0513, whereas they were apparently vaccine viruses in the remaining three (Mm0409, Mm0518 and Mm0304). In the latter three, it appeared as if vaccine viruses were reactivated upon challenge with heterologous virus. These three eventually developed AIDS and were euthanized, whereas Mm0513 has not shown any disease manifestation >80 weeks pc. These four animals were regarded as poor or non-controllers.

These results indicate that the deglycosylated, live attenuated SIV viruses function as effective vaccines and possess potential to induce near-sterile, long-lasting immunity against the heterologous virus in a significant, albeit not all vaccinated animals. Also these results demonstrated that all 4 deglycosylation mutants exhibited similar vaccine efficacy based on the ratio of controllers and non-controllers (Figs. 4 B and C).

Adaptive immune responses in vaccinees

In efforts to investigate immune correlates of protection against the heterologous challenge in vaccinees during acute and chronic infection, we examined adaptive immune responses against vaccine and challenge viruses. As described, the levels of NAb responses against vaccine virus varied among the vaccinees, partly due to the differences in N-glycosylation in gp120 (Figs. 1 and 2B). The differences in the NAb responses in vaccinees were maintained even after challenge with the heterologous virus. Whereas the Δ5G-ver2-vaccinated animals elicited the highest level of NAb, the Δ3G-vaccinated animals elicited the lowest level of NAb. In addition, the Δ5G and Δ5Gver1-vaccinated animals elicited intermediate NAb responses (Fig. 5). Regardless of these differences, all of the vaccinees successfully contained acute-phase VL, before diverging into controllers and non-controllers during the chronic infection-phase (Figs. 4 B and C). Thus, vaccine induced NAb responses did not correlate with protection from challenge virus infection during either acute or chronic infection. In addition, we could not detect any appreciable NAb against SIVsmE543-3 in any of the vaccinees throughout the observation period (Fig. 5). Although NAb was reasoned to exert immune pressure driving the emergence of mutants with altered N-glycosylation in HIV/SIV infections [18,19,20], no significant association was observed between NAb responses and the emergence of the mutants in non-controllers (Figs. 4 and 5).

We next examined cellular responses specific to the viral proteins in the PBMC utilizing the IFN-γ ELISPOT assay against pools of peptides spanning the entire proteins of both SIVsmE543-3 and SIVmac239. Specific T cell responses to SIVmac239-peptides paralleled those to SIVsmE543-3-peptides in all of vaccinees, and therefore vaccine-elicited SIV specific T cells were assumed to cross-react with SIVsmE543-3 infected cells (Fig. 6). However, no obvious quantitative correlation was found between the overall specific T cell responses and either good, poor or the lack of control of viremia throughout the observation period (Fig. 4). It is of interest to note that more than half of the SIV specific T cell responses appeared directed against epitopes localized within the SIV-Gag protein in most of the vaccinated animals (Fig. 6) which suggests that a potential association exists between gag specific T cell response with control of viremia. These findings are consistent with previous reports that suggest that the magnitude of Gag-specific T cell response correlates with control of HIV/SIV viremia in not only HIV-1-infected cohorts [11,21] but also in macaques included in vaccine studies [8,16].

Taken together, whereas these results indicate no appreciable correlation between NAb response and control of heterologous challenge intravenous infection, there may exist a potential role of virus specific cellular responses in the control of viral replication.

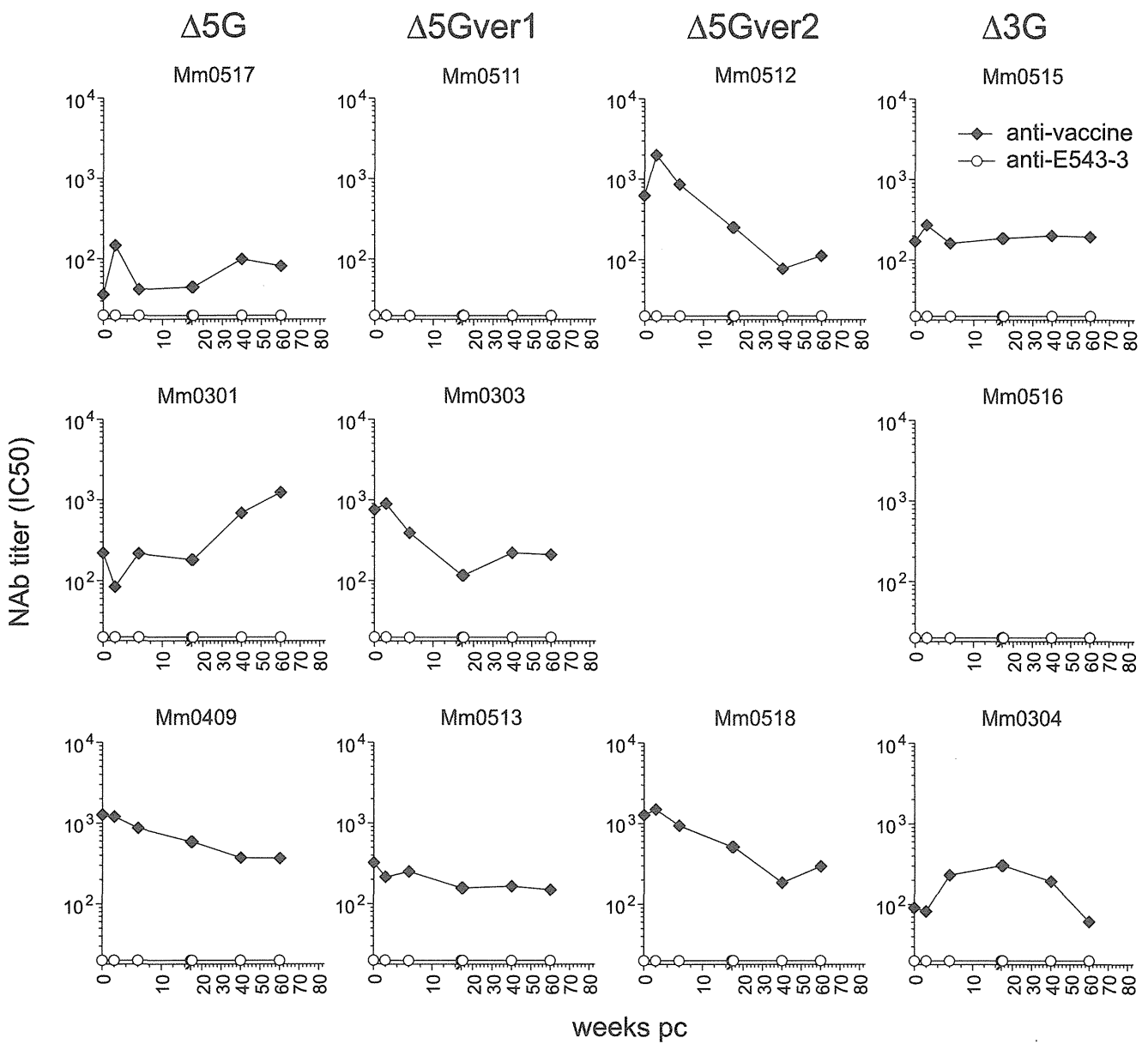

Figure 5. Neutralizing antibodies (NAb) response in the vaccine recipients. NAb responses against vaccine viruses (closed squares) and challenge virus (open circles) were measured in CEMx174/SIVLTR-SEAP system. NAb titers were indicated as the reciprocal of the dilutions of the plasma from the vaccinees yielding 50% inhibition (IC_{50}).
doi:10.1371/journal.pone.0011678.g005

Emergence of escape mutants with increased N-glycosylation sites in gp120 by recombination and single point mutations

In efforts to understand the mechanisms involved in the loss of control of viremia in the 4 non-controllers, we sequenced the emerging viruses using PBMC collected at time points indicated by arrows in Fig. 4 C. Sequence analysis of viruses isolated from Mm0513 confirmed that only the challenge virus with a 9 nucleotide deletion was replicating in this animal (Fig. 7). Whereas the vaccine virus was detected in the PBMC from Mm0304 collected at 25 weeks pc, a recombinant virus was predominantly present in the PBMC samples collected at 45 weeks pc from this animal (Fig. 7). Viruses with multiple recombinations were also found in Mm0409 and Mm0518 (Fig. 7). To examine if the recombination that we detected in the PBMC DNA was representative of the replicating viruses, we performed nested PCR utilizing primer pair sets aimed at the detection of putative recombination sites on RNA obtained from plasma from each animal (File S1). Consistent with the results obtained from PBMC DNA, the recombinants were also found in plasma RNA samples in all 3 animals, whereas only a few SIVsmE543-3 sequences were detected in Mm0518 (Table 2).

As noted above, while attenuated vaccine viruses have 18 or 20 N-glycosylation sites, the pathogenic strains, SIVmac239 and SIVsmE543-3 have 23 and 22 *N*-glycosylation sites, respectively (Fig. 1). We noticed that the gp120-encoding region of all replicating viruses in the chronic phase post challenge originated from the SIVsmE543-3 isolate regardless of recombination (Fig. 7). This resulted in restoration of N-glycosylation sites, the number of which was analyzed. We sequenced the PCR products amplified