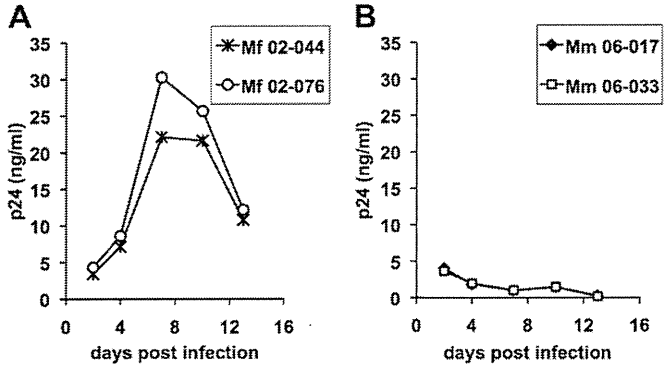

Fig. 2. Growth properties of the NL-DT5R in $CD8^+$ cell-depleted PBMCs from CM (A) and RM (B). The cells were infected with NL-DT5R and the viral replication was monitored by p24 antigen in the culture supernatants using a p24 quantitative ELISA kit. Animal identifications are indicated at the top of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

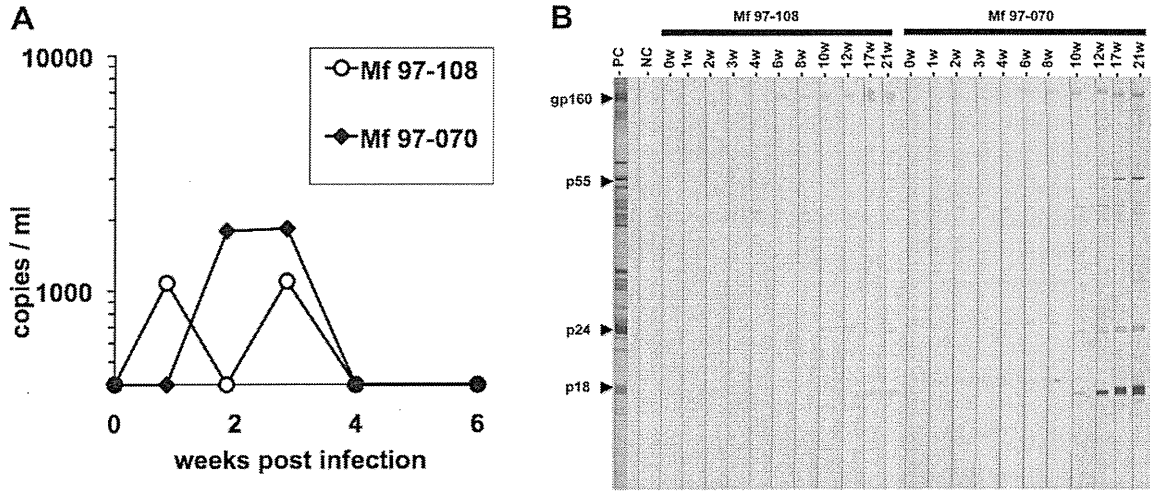

Fig. 3. Profiles of plasma viral RNA loads (A) and anti-HIV-1 antibody responses (B) in CM infected with NL-DT5R. Mf97-108 (open circles) and Mf97-070 (closed diamonds) were used in this study. Viral stocks for inoculation were prepared in $CD8^+$ cell-depleted PBMCs, and then 6.1 ng p24 of HIV-1 were 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.

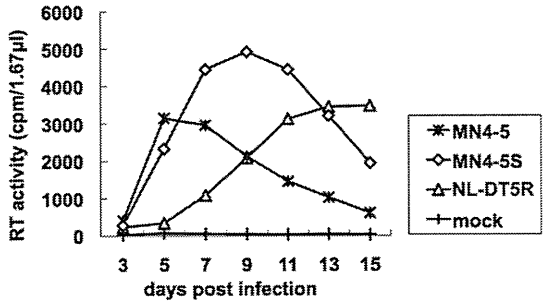

Fig. 4. Growth properties of HIV-1mt in HSC-F cells. The cells were infected with a series of HIV-1mt derivatives. The viral replication was monitored by RT activity in the culture supernatants.

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.

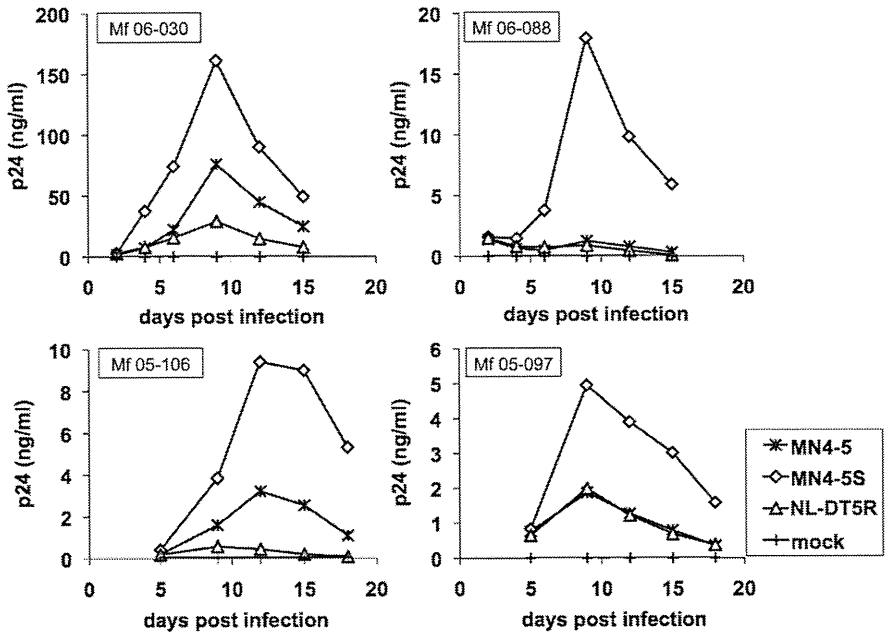

Fig. 5. Growth properties of HIV-1mt in $CD8^+$ cell-depleted PBMCs from four CM. The cells were infected with a series of HIV-1mt derivatives. The viral replication was monitored by p24 antigen in the culture supernatants using a p24 quantitative ELISA kit. Animal identifications are indicated at the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

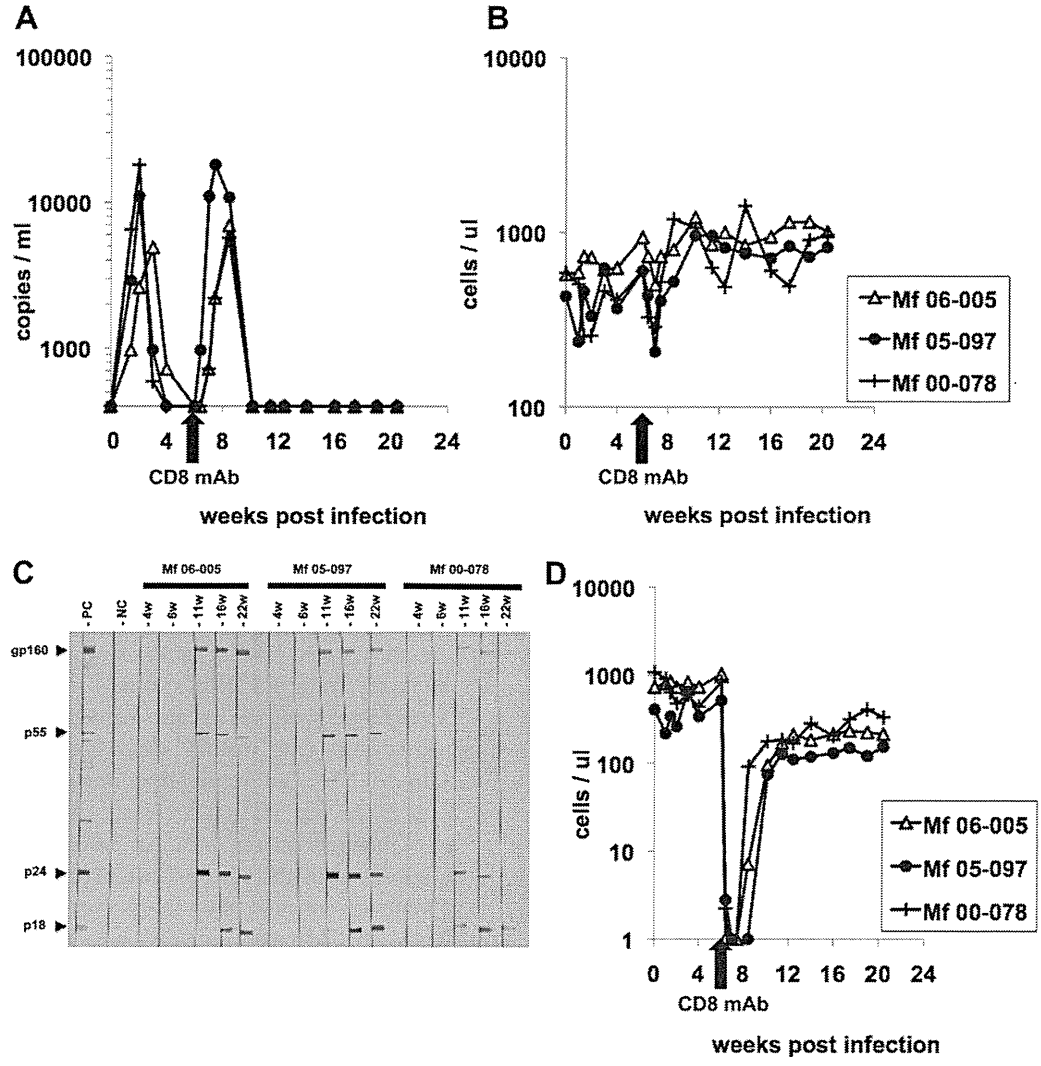

Fig. 6. Profiles of plasma viral RNA loads (A), circulating $CD4^+$ T lymphocytes (B), anti-HIV-1 antibody responses (C) and circulating $CD8^+$ T lymphocytes (D) in CM infected with HIV-1 derivatives. Viral stocks for inoculation were prepared in $CD8^+$ cell-depleted PBMCs and then 10 ng of p24 of 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.

Immunogenetics (2011) 63:417–428
DOI 10.1007/s00251-011-0519-7

ORIGINAL PAPER

Molecular evolution of immunoglobulin superfamily genes in primates

Hitoshi Ohtani · Toshiaki Nakajima · Hirofumi Akari · Takafumi Ishida · Akinori Kimura

Received: 1 September 2010 / Accepted: 17 February 2011 / Published online: 10 March 2011

Abstract Genes of the immunoglobulin superfamily (IgSF) have a wide variety of cellular activities. In this study, we investigated molecular evolution of IgSF genes in primates by comparing orthologous sequences of 249 IgSF genes among human, chimpanzee, orangutan, rhesus macaque, and common marmoset. To evaluate the non-synonymous/synonymous substitution ratio (ω), we applied Bn-Bs program and PAML program. IgSF genes were classified into 11 functional categories based on the Gene Ontology (GO) database. Among them, IgSF genes in three functional categories, immune system process (GO:0002376), defense response (GO:0006952), and multi-organism process (GO:0051704), which are tightly linked to the regulation of immune system had much higher values of ω than genes in the other GO categories. In addition, we estimated the average values of ω for each primate lineage. Although each primate lineage had comparable average values of ω, the human lineage showed the lowest ω value for the immune-related genes. Furthermore, 11 IgSF genes, *SIGLEC5*, *SLAMF6*, *CD33*, *CD3E*, *CEACAM8*, *CD3G*, *FCER1A*, *CD48*, *CD4*, *TIM4*, and *FCGR2A*, were implied to have been under positive selective pressure during the course of primate evolution. Further sequence analyses of *CD3E* and *CD3G* from 23 primate species suggested that the Ig domains of *CD3E* and *CD3G* underwent the positive Darwinian selection.

Keywords Natural selection · Immune system · Immunoglobulin domain · Comparative genomics · CD3 complex

Introduction

Comparative genomics is a promising approach for studying the biological development of the genome from the view point of evolution. Recently, large-scale genome sequences of human, chimpanzee, orangutan, rhesus macaque, and common marmoset have been made available (Consortium CSaA 2005; Gibbs et al. 2007), and the comparative genomic analyses among primates are crucial for addressing the issue of which genetic changes have made us uniquely human. In addition, such analyses are also useful for identifying the susceptibility genes for human diseases and for understanding the pathophysiological mechanisms of the diseases, because the biological differences among primates, such as differences in the disease susceptibility, have been reported (Lyashchenko et al. 2008; Song et al. 2005).

To identify the genes that have come under the pressure of natural selection in the course of primate evolution is of critical importance, because such genes would very likely be linked to biological function involved in the human

Electronic supplementary material The online version of this article (doi:10.1007/s00251-011-0519-7) contains supplementary material, which is available to authorized users.

H. Ohtani · T. Nakajima · A. Kimura (✉)
Department of Molecular Pathogenesis, Medical Research Institute, and Laboratory of Genome Diversity, Graduate School of Biomedical Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113–8510, Japan
e-mail: akitis@mri.tmd.ac.jp

H. Akari
Center for Human Evolution Modeling Research, Primate Research Institute, Kyoto University, Inuyama, Japan

T. Ishida
Unit of Human Biology and Genetics, Graduate School of Science, The University of Tokyo, Tokyo, Japan

diseases. In fact, comparisons of genomes between the human and chimpanzee and between the human and rhesus macaque have suggested that dozens of genes have emerged under the pressure of natural selection in the course of primate evolution, in particular, those which are involved in the host–pathogen interactions, reproduction, and sensory systems (Clark et al. 2003; Gibbs et al. 2007; Nielsen et al. 2005). These studies have also reported that the immunoglobulin superfamily (IgSF) genes are commonly observed among the genes in primates, which had come under the pressure of natural selection.

Members of the IgSF are defined by the presence of one or more regions homologous to the basic structural unit of immunoglobin (Ig) molecules. The Ig domain possesses a characteristic Ig fold, which is composed of two opposing anti-parallel beta-strands connected by disulfide bonds between cysteine residues (Halaby and Mornon 1998). The IgSF is a large group of cell surface, cytoplasmic, and serum proteins involved in the recognition, binding, and/or adhesion processes of cells (Lander et al. 2001). Members of the IgSF have a wide variety of cellular functions acting as cell surface antigen receptors, co-receptors and co-stimulatory molecules of the immune system, molecules involved in antigen presentation to lymphocytes, cell adhesion molecules, certain cytokine receptors, and intracellular muscle proteins. They are commonly ascribed to a role in molecular–molecular interactions (Barclay 2003; Lander et al. 2001; Otey et al. 2009).

Although the IgSF genes have been reported as showing evidence of positive selection, the phylogenetic analyses focused on the Ig domains of IgSF genes have not been conducted. The purpose of present study is to provide insights into the overview of the molecular evolution of the IgSF genes and to identify the IgSF genes under the positive selection in the course of five primate species.

Materials and methods

Sequence data collection

Selection of the IgSF genes was done by using the Conserved Domain Database v2.22 at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/sites/entrez). As in the previous studies (Gibbs et al. 2007; Kosiol et al. 2008), we identified orthologous genes for human IgSF genes from chimpanzee, orangutan, rhesus macaque, and common marmoset by using the UCSC/MULTIZ alignment program which is constructed by the synteny-based genome-wide multiple alignments (Blanchette et al. 2004; Kent et al. 2003). Sequence alignment was done by using the Clustal X program (Larkin et al. 2007). IgSF genes were classified based on the Gene Ontology (GO) database (http://www.geneontology.org/) (Ashburner et al. 2000).

Primate genomic DNA samples

DNA samples from 23 primate species including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), black gibbon (*Hylobates concolor*), white-handed gibbon (*Hylobates lar*), siamang (*Symphalangus syndactylus*), rhesus macaque (*Macaca mulatta*), crab-eating macaque (*Macaca fascicularis*), baboon (*Papio hamadryas*), black and white colobus (*Colobus guereza*), dusky lutong (*Trachypithecus obscurus*), silvered lutong (*Trachypithecus cristatus*), Central American spider monkey (*Ateles geoffroyi*), long-haired spider monkey (*Ateles belzebuth*), tufted capuchin (*Cebus apella*), common squirrel monkey (*Saimiri sciureus*), red-handed tamarin (*Saguinus midas*), cotton-top tamarin (*Saguinus oedipus*), golden lion tamarin (*Leonthopithecus rosalia*), common marmoset (*Callithrix jacchus*), and lesser galago (*Galago senegalensis*) were analyzed for *CD3E* and *CD3G* sequences.

PCR and sequencing analysis of *CD3E* and *CD3G*

Sequence information for coding regions of *CD3E* and *CD3G* were obtained by direct sequencing of gene segments amplified by polymerase chain reaction (PCR) from the genomic DNA samples. Primers for PCR were designed in the highly conserved non-coding regions among the genes from human, chimpanzee, orangutan, rhesus macaque, and common marmoset, referring the genomic sequences deposited in the UCSC Genome Browser (electronic supplementary material (ESM) Table 1). Primers for prosimian were designed by referencing the common marmoset sequences and whole-genome shotgun sequences from prosimians in NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers were used for both PCR and direct sequencing analyses of the genes. When sequence variations (heterozygous sequences) in specific species were detected, the sequences which were conserved among 23 primate species were considered as ancestral sequences and used in the statistical analyses.

PCR was performed in a reaction mixture of 15 μL containing 0.1 μL Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 1 μL of 50 ng/μL DNA template, 1.5 μL of 2.0 mM dNTPs, 0.5 μL of 10 μM each primer, 1.5 μL reaction buffer containing 20 mM $MgCl_2$, and sterile water. PCR condition was as follows: 94°C for 2 min, 35 cycles (94°C for 30 s, 55–60°C for 30 s, 72°C for 1 min), and 72°C for 5 min. PCR products were then purified and sequenced by the BigDye Terminator cycling system using an ABI3130× automated DNA sequencer (Applied Biosystems,

Foster City, CA, USA). Editing and assembly of sequences were performed using SEQUENCHER (Gene Codes, Ann Arbor, MI, USA). The *CD3E* and *CD3G* sequences determined in this study were deposited in DNA Data Bank of Japan under the following accession numbers: AB583139-AB583171 (ESM Table 2).

Statistical analyses

In this study, we used both Bn-Bs program and PAML program to reduce the chance of false-positive findings. A criterion for the gene under positive selection pressure was that the p values obtained by both the Bn-Bs and PAML programs were less than 0.05. The first screening of IgSF genes under the selection pressure was performed by using the Bn-Bs program. The genes showing p values less than 0.05 in the first screening were further analyzed by using the PAML program.

The Bn-Bs program estimates the values of non-synonymous substitution rate (dn) and synonymous substitution rate (ds) based on the modified Nei–Gojobori method (Nei and Gojobori 1986), where a phylogenetic tree is given (Zhang et al. 1998). The value of ω, an abbreviation for the value of dn/ds, is a criterion of natural selective pressure acting on the gene. Statistical significance of the difference between dn and ds were examined by Z test (Chatterjee et al. 2009). An ordinary least-squares method was used to estimate the branch lengths and variances for the evolutionary distances between two sequences (Rzhetsky and Nei 1993).

Investigation on the presence of branch-specific positive selection (the branch model) and site-specific positive selection (the site model) were performed by using CODEML, an application from PAML version 4.7 (Yang 2007). The branch model is used for evaluation of difference in the value of ω for each branch, and it is useful for detecting a positive selection acting on particular branch by using the likelihood ratio tests (Yang and Nielsen 2000). The site model treats ω allowing the variance among codons (Yang 2005; Yang and Nielsen 2000). Bayes empirical Bayes (BEB) method was used to detect the sites under the positive selection (Yang and Nielsen 2000; Wong et al. 2004; Yang et al. 2005; Yang 2005, 2007).

Results

Non-synonymous/synonymous substitution ratio of IgSF genes

Four hundred sixty-one IgSF genes were selected from the human genome, based on the Conserved Domain Database v2.22 at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Among them, 47 genes composed of MHC, KIR, and PSG genes (ESM Table 3) were excluded from the phylogenetic analysis, because there are many paralogous genes with high similarity in the sequences, which may lead to uncertainty to identify the reliable orthologous genes. Thus, a total of 414 IgSF genes were subjected to the following analysis. By using the UCSC Genome Browser (http://genome.ucsc.edu/), we attempted to identify orthologous genes for these 414 human IgSF genes in genomes from chimpanzee, orangutan, rhesus macaque, and common marmoset. We were unable to identify orthologs for 53, 55, 52, and 81 genes from the genome of chimpanzee, orangutan, rhesus macaque, and common marmoset, respectively, due to the alignment incompleteness (sequence identity of less than 80%), insertion/deletions accompanied by frameshift, or nucleotide substitutions resulting in a premature stop codon. After removing the IgSF genes of which the reliable orthologous genes were not identified in the non-human primates, remaining 249 IgSF genes were used in the study of positive selection.

The Bn-Bs program was applied to evaluate the non-synonymous/synonymous substitution ratio (Larkin et al. 2007), and the value of Σdn and Σds, which were the sum values of dn and ds, respectively, in seven primate lineages, human, chimpanzee, human-chimpanzee ancestor, orangutan, human–chimpanzee–orangutan ancestor, rhesus macaque, and common marmoset were calculated. The IgSF genes were classified into 11 functional categories based on the Gene Ontology database (http://www.geneontology.org/); GO:0002376: immune system process, GO:0006952: defense response, GO:0051704: multi-organism process, GO:0007166: cell surface receptor linked signaling pathway, GO:0007155: cell adhesion, GO:0007165: signal transduction, GO:0008219: cell death, GO:0030154 : cell differentiation, GO:0008283: cell proliferation, GO:0019222: regulation of metabolic process and GO:0050794: regulation of cellular process. When the Σdn/Σds ratios were calculated for the entire coding sequences, there was no evidence to support the presence of positive natural selection. The Σdn/Σds ratios from the analyzed genes, except for *LAIR1* (Σdn/Σds ratio= 1.00, statistically not significant), were lower than 1.0, implying that most of the IgSF genes had been under the pressure of negative selection in the course of primate evolution. Among the functional categories, GO:0002376: immune system process (median Σdn/Σds ratio=0.407; interquartile range (IQR), 0.285–0.632, $p=1.40\times10^{-6}$), GO:0006952: defense response (median Σdn/Σds ratio= 0.394; IQR, 0.287–0.506, $p=6.73\times10^{-3}$), and GO:0051704: multi-organism process (median Σdn/Σds ratio=0.400, IQR, 0.302–0.475, $p=2.46\times10^{-2}$) showed much higher values of Σdn/Σds ratio than the tested IgSF genes (median Σdn/Σds ratio=0.208, IQR, 0.107–0.440; Fig. 1a).

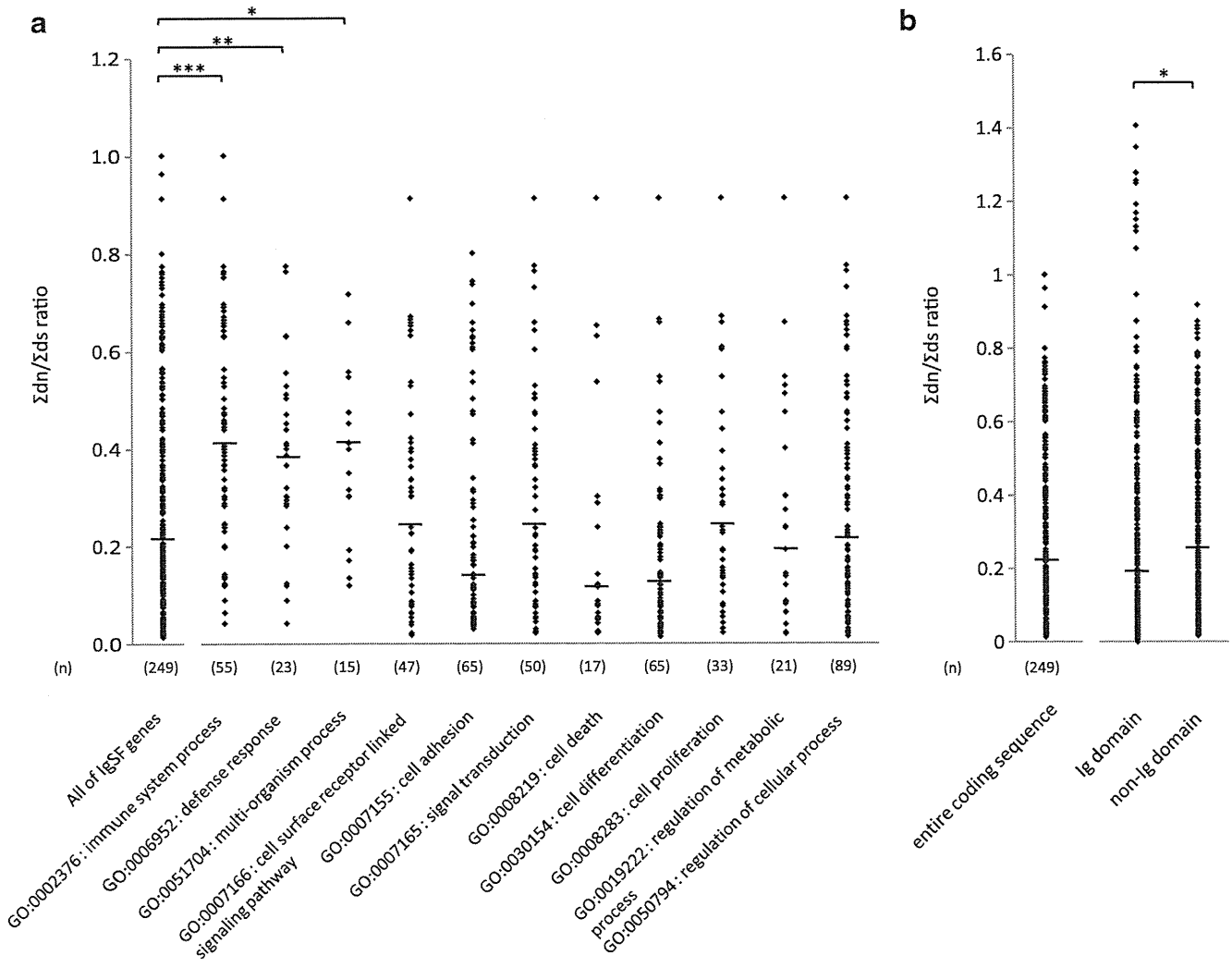

Fig. 1 Σdn/Σds ratio of IgSF genes. **a** The IgSF genes were categorized by gene ontology. **b** Σdn/Σds ratios for the entire coding region, Ig domain, and non-Ig domain. The Σdn/Σds ratios were calculated by Bn-Bs program. *Bars* indicate median values of Σdn/Σds ratio for each group. An *asterisk* indicates that there was significant difference between two groups (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

The coding segments of IgSF genes were divided into two segments in each gene; one was the segment encoding the Ig domain, whereas the other was the coding region other than the Ig domain (non-Ig domain). The Σdn/Σds ratios were also separately calculated for the Ig and non-Ig domains in the IgSF genes. As shown in Fig. 1b, the Σdn/Σds ratios for the Ig domains (median Σdn/Σds ratio=0.198; IQR, 0.070–0.420) were significantly lower than the Σdn/Σds ratios for the non-Ig domains (median Σdn/Σds ratio=0.242; IQR, 0.125–0.456, $p=2.10\times10^{-2}$). Interestingly, despite the lower levels of Σdn/Σds ratio for the Ig domains, the Σdn/Σds ratios of Ig domains from 11 genes, *LAIR1*, *CD3G*, *CD3E*, *CEACAM7*, *ICAM4*, *CD244*, *CD4*, *CD3D*, *CD7*, *SLAMF6*, and *BTLA*, were over 1.0 and higher than mean Σdn/Σds of the non-Ig domains, although none of them was statistically significant.

Non-synonymous/synonymous substitution ratios of IgSF genes in primate lineages

Average values of ω for different lineages, including human, chimpanzee, orangutan, rhesus macaque, and common marmoset lineages, were calculated. First, we made a long sequence by connecting the coding sequences from all IgSF genes to calculate the average ω value, because there were many IgSF genes in which the ds was 0, which made it impossible to determine the exact value of ω. Then, the values of ω at intervals of approximately 20,000 bases were calculated. The average values of ω for each branch of the five-specie phylogeny were also calculated by using the Bn-Bs program. It was found that the average values of ω for the entire coding region was 0.241 in the human lineage, 0.277 in the chimpanzee lineage, 0.225 in the orangutan lineage, 0.234 in the rhesus

lineage, and 0.281 in the marmoset lineage. We also estimated the average value for the immune-related IgSF genes, i.e. the IgSF genes categorized into GO:0002376, GO:0006952, and GO:0051704, and the average value for the other IgSF genes; 0.285 and 0.225 in the human lineage, 0.381 and 0.236 in the chimpanzee lineage, 0.307 and 0.193 in the orangutan lineage, 0.370 and 0.181 in the rhesus lineage, and 0.473 and 0.206 in the marmoset lineage, respectively. In addition, essentially identical results were obtained by using the PAML program (ESM Figs. 1 and 2).

IgSF genes under the pressure of positive natural selection

The dn and ds values for the entire coding regions of IgSF genes in each lineage were calculated by using the Bn-Bs program and plotted in Fig. 2, where the genes with dn values higher than ds values were distributed in the upper diagonal portion. We performed statistical tests by using both the Bn-Bs and PAML programs. When a statistically significant level (p value less than 0.05) was obtained by the Bn-Bs program, further analyses by using the PAML program were done. As the results, five IgSF genes were suggested to have been under the significant positive selection; *SIGLEC5* [Z score=2.70 (p=0.003), chi-square value=5.32 (p=0.021)], and *SLAMF6* [Z score=1.69 (p=0.046), chi-square value=3.93 (p=0.048)] in the human lineage, *CD33* [Z score=2.43 (p=0.008), chi-square value=4.90 (p=0.027)] in the chimpanzee lineage, and *CD3E* [Z score=2.67 (p=0.004), chi-square value=9.04 (p=0.003)] and *CEACAM8* [Z score=2.08 (p=0.019), chi-square value=6.52 (p=0.011)] in the human–chimpanzee–orangutan ancestor lineage (Table 1). No gene under the significant control of positive selection was identified in the human–chimpanzee ancestor lineage, the orangutan lineage, the rhesus lineage, or the marmoset lineage. We also calculated the dn and ds values in the seven lineages for the Ig and non-Ig domains in the IgSF genes. Five genes, *CD3E*, *CD3G*, *FCER1A*, *CD48*, and *CD4*, and four genes, *SIGLEC5*, *TIM4*, *FCGR2A*, and *CD3E*, were suggested to be under the positive natural selection in the Ig and non-Ig domains, respectively (Table 1, ESM Figs. 3 and 4).

It should be noted here that we did not perform a multiple-test adjustment, such as a strict Bonferroni correction, and thus the levels of statistical significance were marginal. Nevertheless, we obtained significant results in the analyses done by two different programs, the Bn-Bs and PAML programs, instead of performing the multiple-test adjustment. In the Bn-Bs program, statistical significance of the difference between the dn and ds values were examined by Z test. On the other hand, in the PAML program, detection of positive selection acting on particular branch was based on the likelihood ratio test.

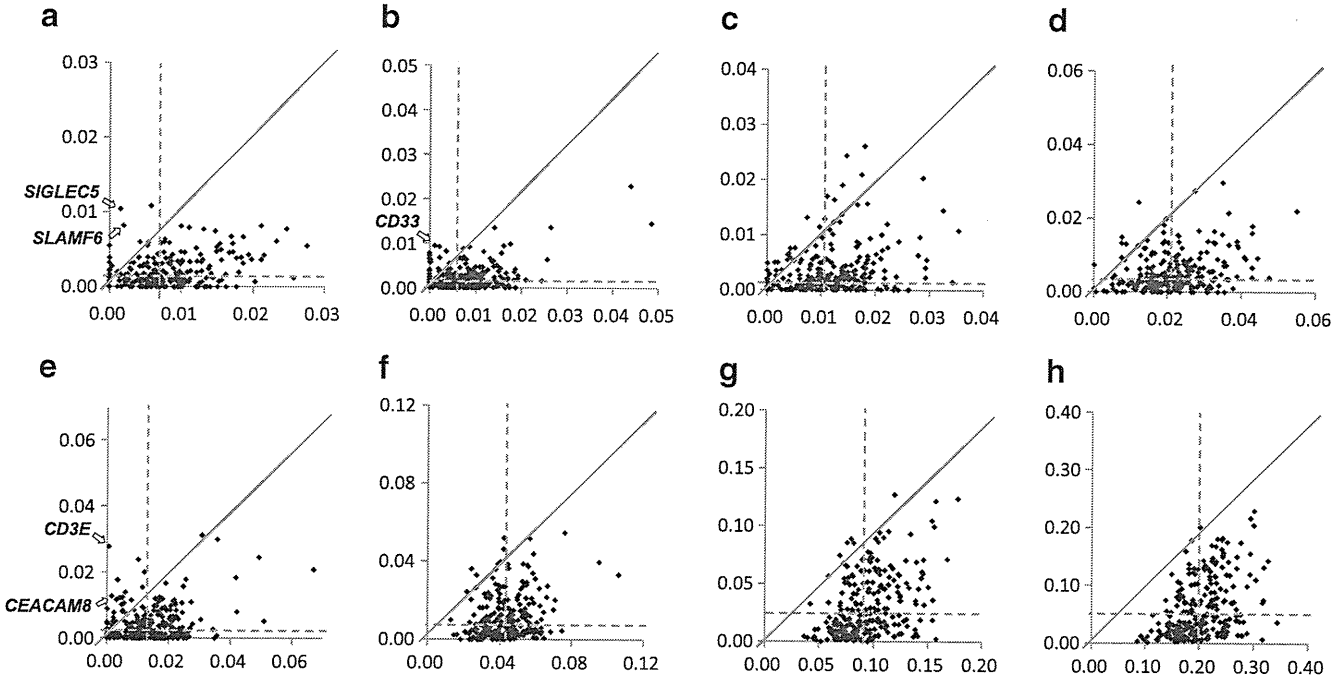

Fig. 2 Pairwise comparison plots of dn and ds values for the entire coding regions of IgSF genes in primate lineages. The values of dn (*vertical axis*) and ds (*horizontal axis*) for each primate lineage and their summation (Σ) were calculated by Bn-Bs program. *Dotted lines* indicate the average values of dn or ds. Arrows indicate the IgSF genes which were identified as to be under a positive selection by the analysis of both Bn-Bs and PAML program analyses. **a** Human lineage, **b** chimpanzee lineage, **c** human–chimpanzee ancestor lineage, **d** orangutan lineage, **e** human–chimpanzee–orangutan ancestor lineage, **f** rhesus lineage, **g** marmoset lineage, **h** Σ

Table 1 IgSF genes suggested to be under the positive selection in the course of primate evolution

Region	Gene name	Accession	BnBs			PAML			Lineage[c]
			ω (dn, ds)	*Z* score	*p* Value	ω	Chi-square	*p* Value	
Entire coding region	*SIGLEC5*	NM_003830	6.90 (0.010, 0.002)	2.70	0.003	nc	5.32	0.021	H
	SLAMF6	NM_052931	4.19 (0.008, 0.002)	1.68	0.046	nc	3.93	0.048	H
	FCGR3A	NM_000569	nc[a] (0.008, 0.000)	2.56	0.005	nc	2.80	ns[b]	C
	CD33	NM_001772	8.40 (0.009, 0.001)	2.43	0.008	nc	4.90	0.027	C
	TIM4	NM_138379	29.43 (0.006, 0.000)	2.12	0.017	nc	3.81	ns	C
	IL11RA	NM_001142784	nc (0.004, 0.000)	2.01	0.022	nc	2.24	ns	C
	FCGR2A	NM_021642	nc (0.004, 0.000)	1.99	0.023	nc	1.27	ns	C
	ICAM2	NM_000873	65.00 (0.006, 0.001>)	1.93	0.027	nc	2.16	ns	C
	AMICA1	NM_001098526	nc (0.004, 0.000)	1.77	0.039	nc	2.33	ns	C
	CD244	NM_001166663	4.34 (0.009, 0.002)	1.70	0.044	3.06	1.27	ns	C
	CD3E	NM_000733	48.67 (0.028, 0.001)	2.67	0.004	nc	9.03	0.003	HCO
	CEACAM8	NM_001816	8.73 (0.013, 0.001)	2.08	0.019	nc	6.52	0.011	HCO
	BTLA	NM_181780	5.30 (0.018, 0.003)	1.69	0.046	4.74	2.13	ns	HCO
Ig domain	*CD244*	NM_001166663	6.20 (0.022, 0.004)	1.96	0.025	nc	3.77	ns	H
	FCGR3A	NM_000569	19.98 (0.009, 0.001>)	1.89	0.029	nc	1.98	ns	H
	SLAMF6	NM_001184714	49.70 (0.011, 0.001>)	1.89	0.029	nc	1.95	ns	H
	CD244	NM_001166663	nc (0.017, 0.000)	2.51	0.006	nc	2.08	ns	C
	FCGR3A	NM_000569	nc (0.008, 0.000)	2.03	0.021	nc	1.69	ns	C
	BTN2A2	NM_006995	nc (0.007, 0.000)	1.77	0.038	nc	1.47	ns	C
	IGSF2	NM_004258	341.00 (0.007, 0.001>)	1.75	0.040	nc	2.35	ns	C
	VSIG10L	NM_001163922	nc (0.017, 0.000)	1.66	0.048	nc	0.63	ns	C
	LILRA4	NM_012276	nc (0.035, 0.000)	2.64	0.004	nc	2.50	ns	HC
	SLAMF6	NM_052931	nc (0.013, 0.000)	1.80	0.036	nc	2.58	ns	HC
	TIM4	NM_138379	206.00 (0.014, 0.000)	1.71	0.043	nc	1.11	ns	HC
	PECAM1	NM_000442	nc (0.009, 0.000)	2.31	0.010	nc	2.10	ns	O
	CEACAM7	NM_006890	292.40 (0.015, 0.000)	1.79	0.036	nc	1.75	ns	O
	BTLA	NM_181780	nc (0.025, 0.000)	2.43	0.008	nc	1.99	ns	HCO
	CD3E	NM_000733	16.07 (0.055, 0.003)	2.16	0.015	nc	4.43	0.035	HCO
	IGSF2	NM_004258	nc (0.009, 0.000)	1.76	0.039	nc	2.94	ns	HCO
	CD3G	NM_000073	nc (0.068, 0.000)	3.42	0.000	nc	4.74	0.029	R
	FCER1A	NM_002001	77.38 (0.029, 0.000)	2.86	0.002	nc	5.35	0.021	R
	ICAM4	NM_001544	nc (0.049, 0.000)	2.71	0.003	nc	2.89	ns	R
	CD3E	NM_000733	7.77 (0.085, 0.011)	2.62	0.004	nc	5.32	0.021	R
	SIGLEC11	NM_052884	2.08 (0.032, 0.015)	1.70	0.044	2.70	1.73	ns	R
	CD48	NM_001778	2.32 (0.197, 0.085)	2.31	0.010	2.65	4.23	0.040	M
	LRRC4	NM_022143	6.61 (0.051, 0.008)	1.79	0.037	3.59	1.69	ns	M
	CD4	NM_000616	1.92 (0.199, 0.104)	1.75	0.040	3.45	6.34	0.012	M
non-Ig domain	*SIGLEC5*	NM_003830	9.49 (0.012, 0.001)	2.66	0.004	nc	4.82	0.028	H
	TREML1	NM_178174	nc (0.005, 0.000)	1.75	0.040	nc	2.38	ns	H
	KAZALD1	NM_030929	nc (0.007, 0.000)	1.72	0.042	nc	1.72	ns	H
	CD33	NM_001772	24.03 (0.009, 0.001>)	2.17	0.015	nc	2.89	ns	C
	TIM4	NM_138379	27.66 (0.008, 0.001>)	2.09	0.018	nc	4.01	0.045	C
	IL11RA	NM_001142784	nc (0.005, 0.000)	1.79	0.037	nc	2.22	ns	C
	BTN1A1	NM_001732	52.71 (0.004, 0.001>)	1.72	0.043	nc	2.19	ns	C
	CD3E	NM_000733	154.60 (0.008, 0.001>)	1.67	0.047	nc	1.76	ns	HC
	SIRPB2	NM_001122962	10.29 (0.012, 0.001)	1.79	0.037	nc	2.89	ns	O
	FCGR2A	NM_021642	nc (0.017, 0.000)	2.64	0.004	nc	5.00	0.025	HCO
	CD3E	NM_000733	nc (0.017, 0.000)	1.66	0.048	nc	3.84	0.050	HCO

[a] *nc* Not calculated (Bs=0)

[b] *ns* Not significant ($p>0.05$)

[c] *H* human, *C* chimpanzee, *R* rhesus, *M* marmoset, *HC* human–chimpanzee ancestor, *HCO* human–chimpanzee–orangutan ancestor

Natural selection of *CD3E* and *CD3G* in primates

We further analyzed two genes, *CD3E* and *CD3G*, which were suggested to be under the positive natural selection. *CD3G* was the gene giving the lowest *p* value, and the Ig domain of CD3E underwent the positive selection. CD3E and CD3G tightly bound to each other (Xu et al. 2006). Because it is known that interacting protein pairs, such as receptor and its ligand, exhibit higher level of co-evolution than non-interacting protein pairs (Goh et al. 2000; Jothi et al. 2006; Li et al. 2005), we hypothesized that co-evolution might occur between *CD3E* and *CD3G*. To investigate the natural selection operated on these genes in the course of primate evolution, we determined protein coding sequences of *CD3E* and *CD3G* from 23 different primate species, including eight hominoids (human, chimpanzee, bonobo, gorilla, orangutan, black gibbon, white-handed gibbon, and siamang), six Old World monkeys (rhesus macaque, crab-eating macaque, hamadryas baboon, black and white colobus, silvered lutong, and dusky lutong), eight New World monkeys (common marmoset, cotton-top tamarin, red-handed tamarin, golden lion tamarin, common squirrel monkey, tufted capuchin, long-haired spider monkey, and Central American spider monkey), and one prosimian (lesser galago). After the alignment of nucleotide sequences and removal of alignment gaps, the values of dn and ds for the entire region, Ig domain, and non-Ig domain were calculated by using Bn-Bs program in each lineage of the phylogenic tree of primates.

The dn and ds values for *CD3E* in each primate lineage are indicated in Fig. 3. The dn values were larger than the ds values in several lineages which might be underwent positive selection pressure in the primate evolution. In particular, the dn values for the Ig domain were significantly

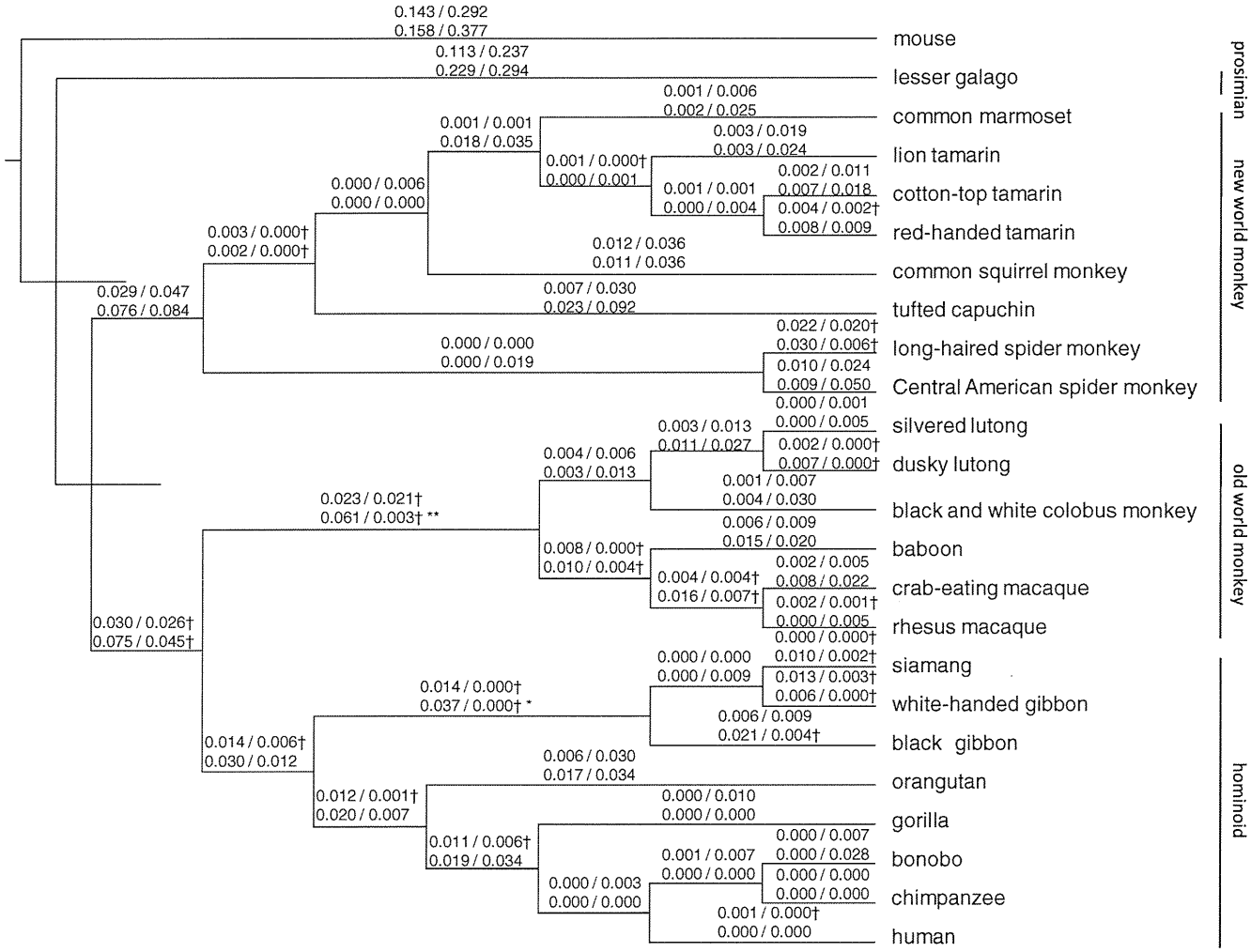

Fig. 3 Phylogenetic trees of *CD3E* in the primate evolution. *Values above branches* indicate estimated values of dn and ds per lineage by using Bn-Bs program. Values indicated in the upper parts are for the entire coding region, while values in the lower parts are for the Ig domain. *Daggers* indicate the value of dn higher than ds. An *asterisk* indicates that there was a significant difference between the dn and ds values (*$p<0.05$, **$p<0.01$, Z test)

larger than the ds values in two lineages: gibbon ancestor lineage [dn=0.061, ds=0.003, Z score=2.37 (p=0.009)] and Old World monkey ancestor lineage [dn=0.037, ds=0.000, Z score=1.94 (p=0.026)]. The significant positive selection on the Ig domains in the Old World monkey ancestor lineage [chi-square value=4.45 (p=0.035)] was confirmed by the PAML program, whereas it was not significant in the gibbon ancestor lineage [chi-square value=0.58 (p=0.446)]. Amino acid (AA) sequence alignment of CD3E in the primates is shown in Fig. 4. We identified three alignment gaps, all of which were in the Ig domain. Of 53 AAs of the Ig domain, approximately 30% (16/53) were evolutionary conserved among the primate species. On the other hand, approximately 70% (97/143) of AAs were conserved in the non-Ig domain, demonstrating that significantly more AA substitutions were distributed in the Ig domain ($p=2.16\times10^{-6}$). In addition, five AA sites in the Ig domain (positions at 51, 53, 72, 80, and 105 in the human sequence) were identified as possible target sites for the positive selection by the BEB method using the PAML program.

The dn and ds values for *CD3G* in each primate lineage were also measured by using the Bn-Bs program (ESM Fig. 5). The dn values for the Ig domain were significantly larger than the ds values in two lineages; Old World monkey ancestor lineage [dn=0.060, ds=0.000, Z score=3.30 (p=0.0005)] and hominoid and Old World monkey ancestor lineage [dn=0.042, ds=0.007, Z score=1.65 (p=0.049)]. The positive selection was confirmed by the PAML program in both lineages; Old World monkey ancestor lineage [chi-square value=5.32 (p=0.021)] and hominoid and Old World monkey ancestor lineage [chi-square value=4.17 (p=0.041)]. The AA alignment of CD3G from 23 primate species is shown in Fig. 4. Approximately 40% (25/56) of AAs in the Ig domain were conserved in the primate evolution, whereas

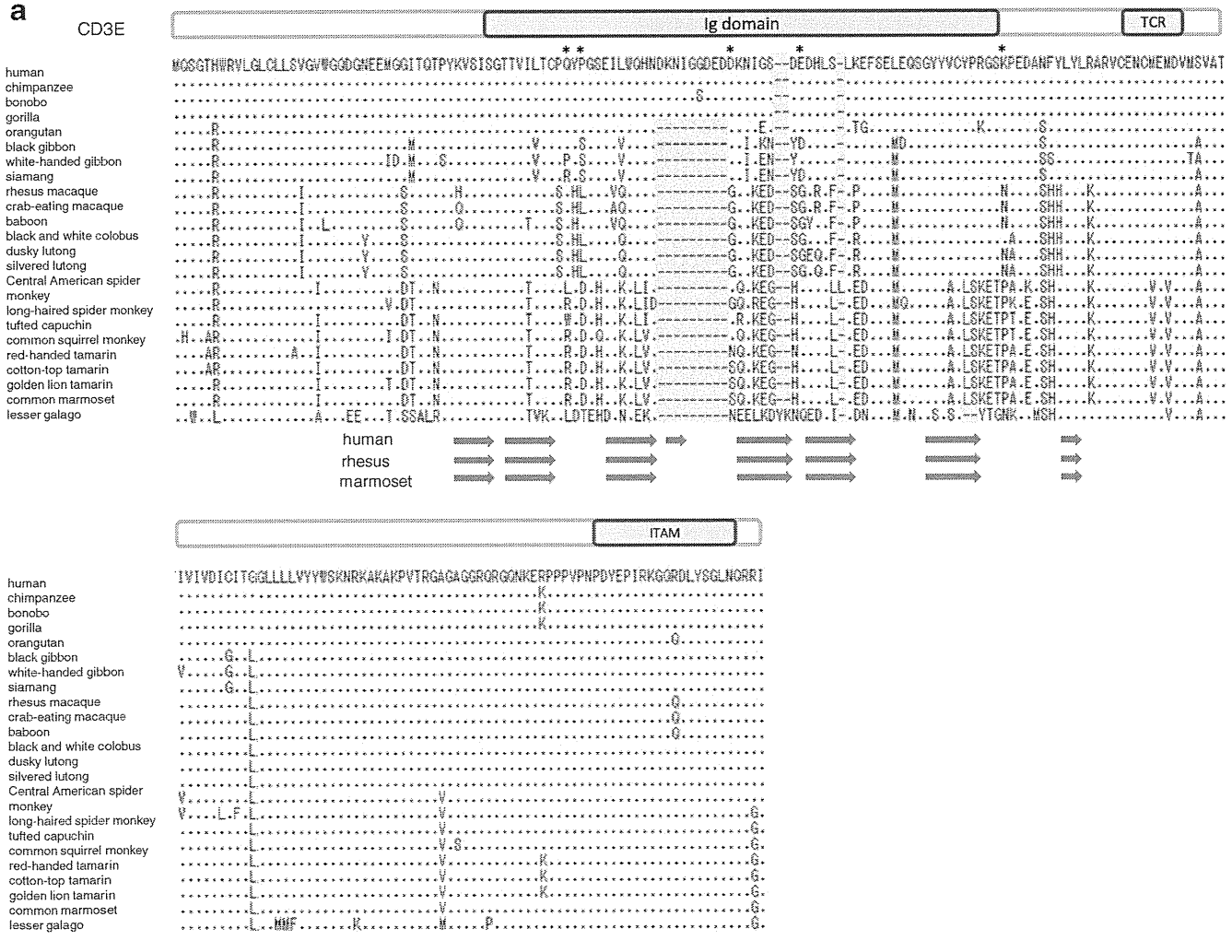

Fig. 4 Alignments of CD3E (**a**) and CD3G (**b**) amino acid sequences from 23 primate species. *Dots* indicate the identities to the human reference sequence, while hyphens indicate alignment gaps. TCR indicates amino acid sites at which the CD3 molecules interact with T cell receptor. ITAM represents the immune-tyrosine activation motif. *Arrows* indicated under the amino acid sequences are β-strand structures modeled by the SWISS-MODEL program. *Asterisks* indicate AA sites identified as being under the significant positive selection (p<0.05)

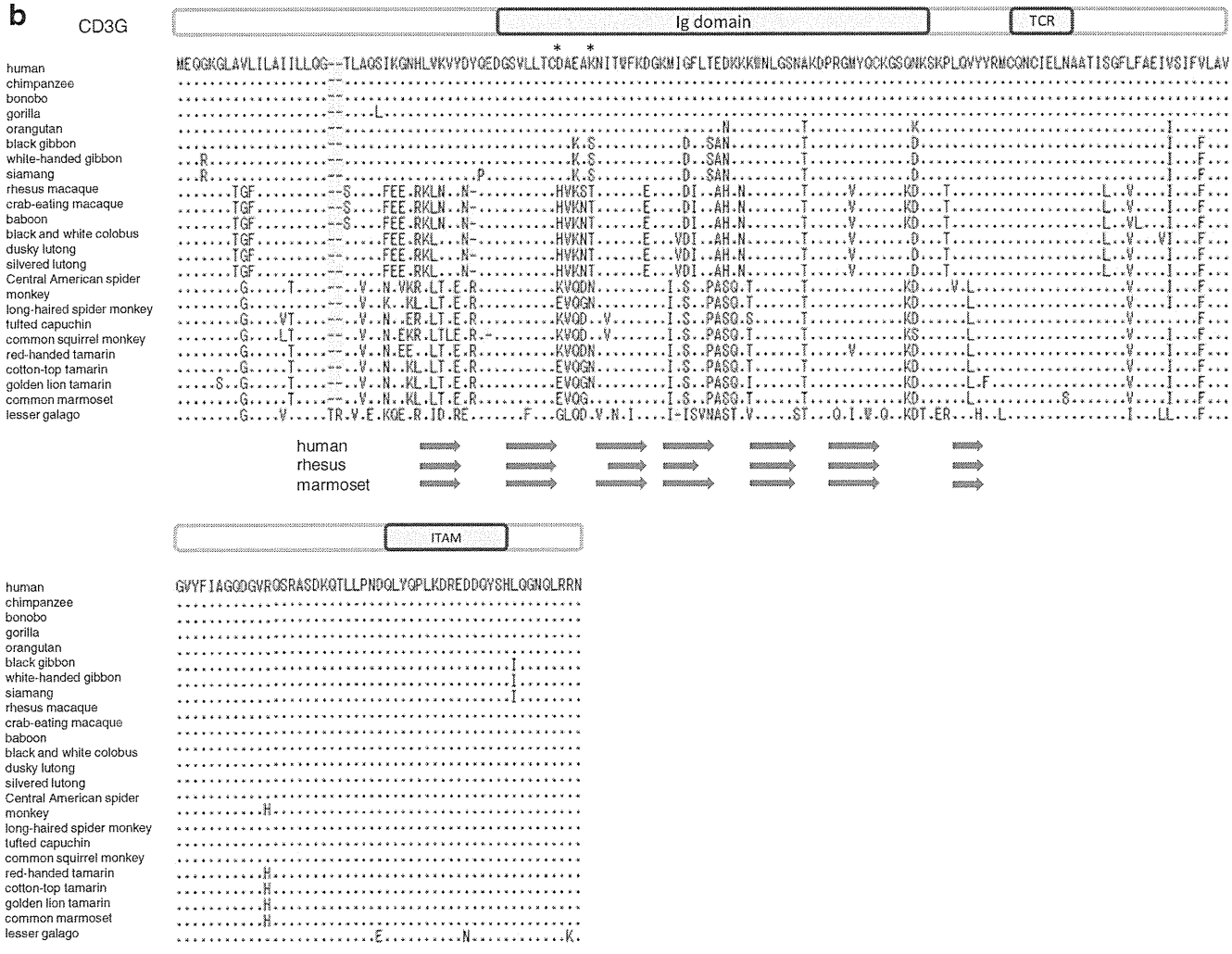

Fig. 4 (continued)

approximately 60% (81/128) of AAs in the non-Ig domain were conserved, demonstrating that the AA changes were significantly more frequent in the Ig domain (p=0.019). Two AA sites in the Ig domain (positions at 47 and 51 in the human sequence) were identified as significant target sites for the positive selection. These lines of evidence suggested that the pressure of positive Darwinian selection had shaped the structure of Ig domains in CD3E and CD3G during the course of primate evolution.

Discussion

Members of the IgSF have a wide variety of cellular activities and were classified into 11 functional categories based on the Gene Ontology database (http://www.geneontology.org/). When the association between the IgSF functional categories and the Σdn/Σds ratios were analyzed, three GO categories tightly linked to the immune system, i.e., GO:0002376: immune system process, GO:0006952: defense response, and GO:0051704: multi-organism process, showed much higher values for the Σdn/Σds ratio than the average value of the IgSF genes. It has been reported that the evolutionary rate of immune-related genes is higher than the other genes (Gibbs et al. 2007; Kosiol et al. 2008; Nielsen et al. 2005; Yu et al. 2006). The rapid evolution of immune-related genes might be a direct consequence of a complex selection pressure exerted by infectious diseases, autoimmunity, and tumors (Barreiro and Quintana-Murci 2010). On the other hand, as shown in Fig. 1a, the Σdn/Σds ratios of genes linked to three functional categories, GO:0007155: cell adhesion, GO:0007165: signal transduction, and GO:0008219: cell death, were comparable to those for the functional categories other than the immune-related genes. Genes in these three categories have also been reported to have a higher non-synonymous/synonymous substitution ratio than the genes in the other categories (Clark et al. 2003; Gibbs et al. 2007; Nielsen et al. 2005).

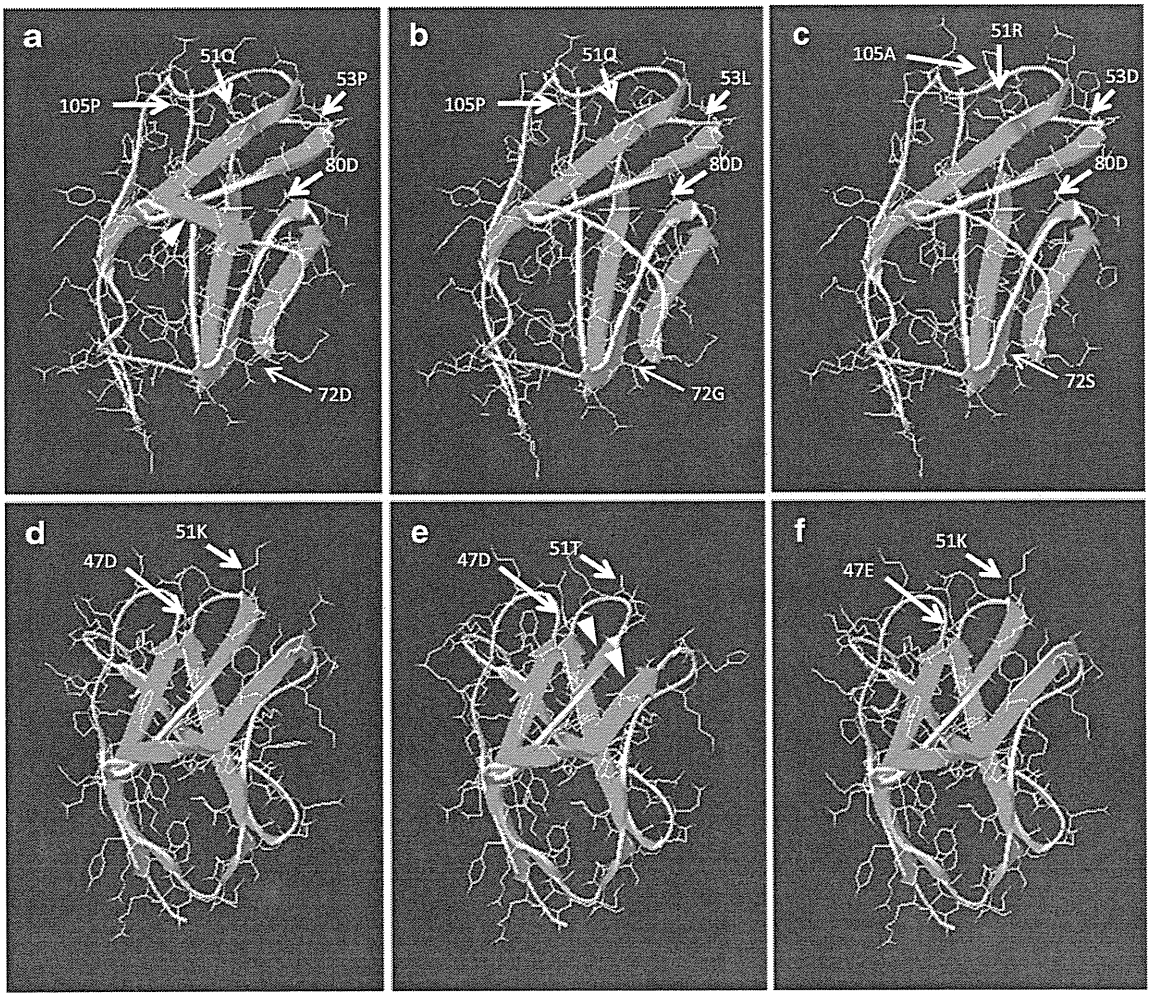

Fig. 5 Three-dimensional structures of CD3E and CD3G modeled by SWISS-MODEL. Arrows indicate amino acid sites identified as being under positive selection by using the BEB method in the PAML program. **a** Human CD3E. An *arrowhead* indicates a β-strand which is unique to human CD3E. **b** rhesus macaque CD3E, **c** marmoset CD3E, **d** human CD3G, **e** rhesus macaque CD3G. *Arrowheads* indicate short strands of β-strand which are unique to rhesus CD3G. **f** marmoset CD3G

It has been reported that the average value of ω in the human lineage is higher than that in the other primate lineages (Ellegren 2008; Gibbs et al. 2007; Kosiol et al. 2008). The differences in the ω among the primate lineages may be attributable to the differences in the effective population size during the course of evolution (Bakewell et al. 2007). Interestingly, in our study, the average value of ω for immune-related genes in the human lineage was the lowest among the primate lineages. Because previous studies suggest that the rapid evolution of the immune-related genes may be due to a direct consequence of complex selection pressure exerted by infectious reagents including microbes and viruses (Barreiro and Quintana-Murci 2010), the observation in our study led us to a hypothesis that in the course of human evolution there might be fewer challenges from pathogens than the other primates, in part due to a shorter course of human evolution. In support of this, it was reported that humans had faced relatively fewer challenges from retroviruses and that humans were consequently at present more susceptible to retrovirus infections than the other primates (Sawyer et al. 2006). However, such a slow evolution of human lineage might also be caused by other factors such as long generation time and small population size.

We identified 11 genes possibly having undergone positive selective pressure (Table 1). Among them, *SIGLEC5*, *CD33*, *CD4*, and *CD3E* have been reported to be genes under the pressure of positive selection in the primate evolution (Angata et al. 2004; Gibbs et al. 2007; Zhang et al. 2008). These genes play crucial roles in the innate and adaptive immune systems, and infectious pathogens might have exerted selective pressure on them (Angata 2006; Crocker et al. 2007), because SIGLEC5, CD33 (SIGLEC3), and CD4 are cell surface receptors for microorganisms.

CD3E and *CD3G* encode the components of T cell antigen receptor (TCR) complex, TCR–CD3 complex. The

TCR–CD3 complex plays a key role in the regulation of immune system through the recognition of antigenic peptides presented by MHC molecules, and mutations in either *CD3E* or *CD3G* are known to cause primary immunodeficiency in humans (Buckley 2004; de Saint et al. 2004; Recio et al. 2007; Sun et al. 2001). Furthermore, previous studies have revealed the role of Ig domains of CD3E and CD3G. For example, cell surface expression of the stable TCR on mature T cell, assembly of the components of T cell antigen receptor complex and T cell activity are regulated by the Ig domains of CD3E and CD3G (Dietrich et al. 1996; Guy and Vignali 2009; Sun et al. 2001).

Because we hypothesized that co-evolution might occur between *CD3E* and *CD3G*, protein coding sequences of these *CD3* genes from 23 primate species were determined, and it was suggested that the Ig domains of both CD3E and CD3G have undergone positive Darwinian selection pressure in the primate evolution, especially in the Old World monkey ancestor lineage. However, the role of Ig domains in the direct interaction of CD3E and CD3G has not been reported. Although the direct demonstration of functional interaction are needed to clarify the impacts of AA substitutions on the function of CD3E and CD3G, we modeled three-dimensional structures of the Ig domains of CD3E using SWISS-MODEL, an Automated Comparative Protein Modeling Server (http://swissmodel.expasy.org/SWISS-MODEL.html; Bordoli et al. 2009). As shown in Fig. 5, eight, seven, and seven β-strands were found in the Ig domains of CD3E from human, rhesus macaque, and common marmoset, respectively, and the three AA insertion/deletion observed in the Ig domain appeared to have a strong impact on the modeled structure. In addition, it was possible that five AA sites in the Ig domain, which were identified as target sites for the positive selection, would change the Ig domain structure. It has been reported that highly conserved CXXCXEXXX motifs in the CD3 family play an important role in the molecular interactions among components of the TCR–CD3 complex (Borroto et al. 1998; Xu et al. 2006). Because the Ig domains are localized just upstream of the N-terminal of CXXCXEXXX motifs, drastic structural changes in the Ig domains might affect the functional properties of CD3E and CD3G. It is likely that such structural changes would affect the stability of the TCR–CD3 complex and the expression level in mature T cells (Call and Wucherpfennig 2004; Guy and Vignali 2009; Wang et al. 1998).

What was the extent of selective pressure exerted on *CD3E* and *CD3G* in the course of primate evolution? Given that the TCR–CD3 complex plays a crucial role in the regulation of immune system, infectious diseases and autoimmunity have been postulated to be the strongest selective pressures (Robins et al. 2009; Sun et al. 2001). It is widely accepted that the susceptibility to infectious pathogens, such as *Mycobacterium tuberculosis bacilli* and HIV-1, are different among primate species (Lyashchenko et al. 2008; Song et al. 2005). Because the dn values for the Ig domains of *CD3E* and *CD3G* are significantly greater than the ds values in the Old World monkey ancestor lineage, their ancestors might have been exposed to powerful selective pressure. To clarify the selective pressure exerted on *CD3E* and *CD3G*, further study on phenotypic differences, such as the relative susceptibilities to infectious pathogens and/or autoimmune disease, among various primate species is needed.

In conclusion, we investigated the molecular evolution of IgSF genes in primates. The study has demonstrated that the immune-related IgSF genes have high non-synonymous/synonymous substitution rates, and those 11 IgSF genes, namely *SIGLEC5*, *SLAMF6*, *CD33*, *CD3E*, *CEACAM8*, *CD3G*, *FCER1A*, *CD48*, *CD4*, *TIM4*, and *FCGR2A*, may undergo the positive selective pressure in the primate evolution.

Acknowledgments This work was supported in part by research grants from 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, the program of Research on Publicly Essential Drugs and Medical Devices, by grant-in-aids for Scientific research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, by a grant from the Life Science Institute Foundation, Japan, and by grants for India–Japan Cooperative Science Program from Japan Society for the Promotion of Science (JSPS), Japan and Department of Science and Technology (DST), India.

References

Angata T, Margulies EH, Green ED, Varki A (2004) Large-scale sequencing of the CD33-related Siglec gene cluster in five mammalian species reveals rapid evolution by multiple mechanisms. Proc Natl Acad Sci USA 101:13251–6

Angata T (2006) Molecular diversity and evolution of the Siglec family of cell-surface lectins. Mol Divers 10:555–66

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25:25–9

Bakewell MA, Shi P, Zhang J (2007) More genes underwent positive selection in chimpanzee evolution than in human evolution. Proc Natl Acad Sci USA 104:7489–94

Barclay AN (2003) Membrane proteins with immunoglobulin-like domains—a master superfamily of interaction molecules. Semin Immunol 15:215–23

Barreiro LB, Quintana-Murci L (2010) From evolutionary genetics to human immunology: how selection shapes host defence genes. Nat Rev Genet 11:17–30

Blanchette M, Kent WJ, Riemer C, Elnitski L, Smit AF, Roskin KM, Baertsch R, Rosenbloom K, Clawson H, Green ED, Haussler D, Miller W (2004) Aligning multiple genomic sequences with the threaded blockset aligner. Genome Res 14:708–15

Bordoli L, Kiefer F, Arnold K, Benkert P, Battey J, Schwede T (2009) Protein structure homology modeling using SWISS-MODEL workspace. Nat Protoc 4:1–13

Borroto A, Mallabiabarrena A, Albar JP, Martinez AC, Alarcon B (1998) Characterization of the region involved in CD3 pairwise interactions within the T cell receptor complex. J Biol Chem 273:12807–16

Buckley RH (2004) The multiple causes of human SCID. J Clin Invest 114:1409–11

Call ME, Wucherpfennig KW (2004) Molecular mechanisms for the assembly of the T cell receptor-CD3 complex. Mol Immunol 40:1295–305

Chatterjee HJ, Ho SY, Barnes I, Groves C (2009) Estimating the phylogeny and divergence times of primates using a supermatrix approach. BMC Evol Biol 9:259

Clark AG, Glanowski S, Nielsen R, Thomas PD, Kejariwal A, Todd MA, Tanenbaum DM, Civello D, Lu F, Murphy B, Ferriera S, Wang G, Zheng X, White TJ, Sninsky JJ, Adams MD, Cargill M (2003) Inferring nonneutral evolution from human–chimp–mouse orthologous gene trios. Science 302:1960–3

Consortium CSaA (2005) Initial sequence of the chimpanzee genome and comparison with the human genome. Nature 437:69–87

Crocker PR, Paulson JC, Varki A (2007) Siglecs and their roles in the immune system. Nat Rev Immunol 7:255–66

de Saint BG, Geissmann F, Flori E, Uring-Lambert B, Soudais C, Cavazzana-Calvo M, Durandy A, Jabado N, Fischer A, Le Deist F (2004) Severe combined immunodeficiency caused by deficiency in either the delta or the epsilon subunit of CD3. J Clin Invest 114:1512–7

Dietrich J, Neisig A, Hou X, Wegener AM, Gajhede M, Geisler C (1996) Role of CD3 gamma in T cell receptor assembly. J Cell Biol 132:299–310

Ellegren H (2008) Comparative genomics and the study of evolution by natural selection. Mol Ecol 17:4586–96

Gibbs RA et al (2007) Evolutionary and biomedical insights from the rhesus macaque genome. Science 316:222–34

Goh CS, Bogan AA, Joachimiak M, Walther D, Cohen FE (2000) Co-evolution of proteins with their interaction partners. J Mol Biol 299:283–93

Guy CS, Vignali DA (2009) Organization of proximal signal initiation at the TCR:CD3 complex. Immunol Rev 232:7–21

Halaby DM, Mornon JP (1998) The immunoglobulin superfamily: an insight on its tissular, species, and functional diversity. J Mol Evol 46:389–400

Jothi R, Cherukuri PF, Tasneem A, Przytycka TM (2006) Co-evolutionary analysis of domains in interacting proteins reveals insights into domain–domain interactions mediating protein-protein interactions. J Mol Biol 362:861–75

Kent WJ, Baertsch R, Hinrichs A, Miller W, Haussler D (2003) Evolution's cauldron: duplication, deletion, and rearrangement in the mouse and human genomes. Proc Natl Acad Sci USA 100:11484–9

Kosiol C, Vinar T, da Fonseca RR, Hubisz MJ, Bustamante CD, Nielsen R, Siepel A (2008) Patterns of positive selection in six mammalian genomes. PLoS Genet 4:e1000144

Lander ES et al (2001) Initial sequencing and analysis of the human genome. Nature 409:860–921

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–8

Li Y, Wallis M, Zhang YP (2005) Episodic evolution of prolactin receptor gene in mammals: coevolution with its ligand. J Mol Endocrinol 35:411–9

Lyashchenko KP, Greenwald R, Esfandiari J, Chambers MA, Vicente J, Gortazar C, Santos N, Correia-Neves M, Buddle BM, Jackson R, O'Brien DJ, Schmitt S, Palmer MV, Delahay RJ, Waters WR (2008) Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife. Vet Microbiol 132:283–92

Nei M, Gojobori T (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol 3:418–26

Nielsen R, Bustamante C, Clark AG, Glanowski S, Sackton TB, Hubisz MJ, Fledel-Alon A, Tanenbaum DM, Civello D, White TJ, Sninsky JJ, Adams MD, Cargill M (2005) A scan for positively selected genes in the genomes of humans and chimpanzees. PLoS Biol 3:e170

Otey CA, Dixon R, Stack C, Goicoechea SM (2009) Cytoplasmic Ig-domain proteins: cytoskeletal regulators with a role in human disease. Cell Motil Cytoskeleton 66:618–34

Recio MJ, Moreno-Pelayo MA, Kilic SS, Guardo AC, Sanal O, Allende LM, Perez-Flores V, Mencia A, Modamio-Hoybjor S, Seoane E, Regueiro JR (2007) Differential biological role of CD3 chains revealed by human immunodeficiencies. J Immunol 178:2556–64

Robins HS, Campregher PV, Srivastava SK, Wacher A, Turtle CJ, Kahsai O, Riddell SR, Warren EH, Carlson CS (2009) Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. Blood 114:4099–107

Rzhetsky A, Nei M (1993) Theoretical foundation of the minimum-evolution method of phylogenetic inference. Mol Biol Evol 10:1073–95

Sawyer SL, Wu LI, Akey JM, Emerman M, Malik HS (2006) High-frequency persistence of an impaired allele of the retroviral defense gene TRIM5alpha in humans. Curr Biol 16:95–100

Song B, Javanbakht H, Perron M, Park DH, Stremlau M, Sodroski J (2005) Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. J Virol 79:3930–7

Sun ZJ, Kim KS, Wagner G, Reinherz EL (2001) Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. Cell 105:913–23

Wang B, Wang N, Salio M, Sharpe A, Allen D, She J, Terhorst C (1998) Essential and partially overlapping role of CD3gamma and CD3delta for development of alphabeta and gammadelta T lymphocytes. J Exp Med 188:1375–80

Wong WS, Yang Z, Goldman N, Nielsen R (2004) Accuracy and power of statistical methods for detecting adaptive evolution in protein coding sequences and for identifying positively selected sites. Genetics 168:1041–51

Xu C, Call ME, Wucherpfennig KW (2006) A membrane-proximal tetracysteine motif contributes to assembly of CD3deltaepsilon and CD3gammaepsilon dimers with the T cell receptor. J Biol Chem 281:36977–84

Yang Z (2005) The power of phylogenetic comparison in revealing protein function. Proc Natl Acad Sci USA 102:3179–80

Yang Z (2007) PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol 24:1586–91

Yang Z, Nielsen R (2000) Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. Mol Biol Evol 17:32–43

Yang Z, Wong WS, Nielsen R (2005) Bayes empirical bayes inference of amino acid sites under positive selection. Mol Biol Evol 22:1107–18

Yu XJ, Zheng HK, Wang J, Wang W, Su B (2006) Detecting lineage-specific adaptive evolution of brain-expressed genes in human using rhesus macaque as outgroup. Genomics 88:745–51

Zhang J, Rosenberg HF, Nei M (1998) Positive Darwinian selection after gene duplication in primate ribonuclease genes. Proc Natl Acad Sci USA 95:3708–13

Zhang ZD, Weinstock G, Gerstein M (2008) Rapid evolution by positive Darwinian selection in T-cell antigen CD4 in primates. J Mol Evol 66:446–56

Arch Virol (2011) 156:1073–1077
DOI 10.1007/s00705-011-0959-2

BRIEF REPORT

Serotype-specific and cross-reactive neutralizing antibody responses in cynomolgus monkeys after infection with multiple dengue virus serotypes

Mikako Ito · Yuko Katakai · Fumiko Ono · Hirofumi Akari · Ryo-zabro Mukai · Tomohiko Takasaki · Akira Kotaki · Ichiro Kurane

Received: 20 September 2010 / Accepted: 19 February 2011 / Published online: 16 March 2011

Abstract Neutralizing antibody responses were examined in monkeys after dengue virus infections. In monkeys that had been infected once or twice with DENV-2, neutralizing antibody was cross-reactive with all four serotypes after secondary or tertiary infection with DENV-3. In monkeys that had been inoculated with DENV-1 and DENV-2 in the primary and secondary infections, neutralizing antibody titers did not increase after tertiary infection with DENV-3. These results indicate that antibody responses after secondary and tertiary infections with different serotypes are cross-reactive with all four serotypes, consistent with what has been observed in humans, and suggest that monkeys are useful for determining neutralizing antibody responses.

Keywords Dengue virus · Monkey · Neutralyzing antibody · Serotype cross-reactivity

M. Ito · T. Takasaki · A. Kotaki · I. Kurane (✉)
Department of Virology 1, National Institute of Infectious Disease, 1-23-1 Toyama, Shinjyuku-ku, Tokyo 162-8640, Japan
e-mail: kurane@nih.go.jp

M. Ito
e-mail: mito@nih.go.jp

T. Takasaki
e-mail: takasaki@nih.go.jp

A. Kotaki
e-mail: ak@nih.go.jp

Y. Katakai · F. Ono · R. Mukai
The Corporation for Production and Research of Laboratory Primates, 1 Yahatadai, Tsukuba 305-0843, Japan
e-mail: katakai@primate.or.jp

F. Ono
e-mail: ninchan@primate.or.jp

R. Mukai
e-mail: mukai@primate.or.jp

H. Akari
Laboratory of Disease Control, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan
e-mail: akari@pri.kyoto-u.ac.jp

Present Address:
H. Akari
Section of Comparative Immunology and Microbiology, Center forHuman Evolution Modeling Research, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan

Dengue virus infections occur in most tropical and subtropical areas of the world. Dengue virus is transmitted by mosquitoes and causes dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1]. There are four serotypes of dengue virus: dengue virus serotypes 1, 2, 3 and 4 (DENV-1, DENV-2, DENV-3, and DENV-4) [2]. Four serotypes of dengue virus are circulating concurrently in most tropical and subtropical regions. This increases the possibility of sequential infections with two or more serotypes of dengue virus. Serotype specificity and cross-reactivity in neutralizing antibody responses after secondary and tertiary infections are complex.

It is important to define the serotype specificity and cross-reactivity in dengue virus infection, since neutralizing antibodies are considered to be the most important factor for protective immunity in humans. Studies using patients' sera are often obscure, because confirmation of time of infection or infection serotypes is usually difficult. In the present study, we analyzed serotype specificity and cross-reactivity of neutralizing antibodies induced after well-controlled primary, secondary and tertiary DENV infections in monkeys (*Macaca fascicularis*). This study

provides insight into the complexity of protection against the four serotypes of dengue virus.

DENV-1 strain 02-17, DENV-2 strain DHF0663, and DENV-3 strain DSS1403 were used. The 02-17 strain of DENV-1 was isolated from a traveler in Japan who came back from Indonesia. The DHF0663 strain of DENV-2 was isolated in Indonesia in 2001 from a case of DHF. The DSS1403 strain of DENV-3 was isolated in Indonesia during the 2001 DSS epidemic, propagated in C6/36, stored at −80°C, and used after the third passage in C6/36 cells. DENV-1, DENV-2 and DENV-3 were used after one, four and three passages in C6/36 cells, respectively, as described previously [3].

Eight female monkeys (*Macaca fascicularis*), aged 7 years and weighing 2,850-3,600 g, were used in the experiments. These monkeys were born and raised at the Tsukuba Primate Research Center, Tukuba, Japan. The monkeys were confirmed to be seronegative for antibodies to DENV. Monkeys were anaesthetized intramuscularly with ketamine HCl (5 mg/kg), and inoculated with dengue virus [3]. Monkeys were challenged intradermally with 0.5 ml of DENV-3 suspension containing 4.5×10^6 plaque-forming units (PFU)/ml.

Eight monkeys were separated into four groups. In group 1, KT1 and KT2 were inoculated with DENV-1 and DENV-2 in the primary and secondary infection, respectively, and then with DENV-3 in the tertiary infection. In group 2, KT4 and KT5 were inoculated two times with DENV-2 in the primary and secondary infections, and then with DENV-3 in the tertiary infection. In group 3, KT3 and KT6 were inoculated with DENV-2 and then with DENV-3 in the secondary infection. In group 4, KT7 and KT8 were infected with DENV-3 in the primary infection. Animal procedures were approved by the Committees on Biosafety and Animal Handling and Ethical Regulations of the National Institute of Infectious, Diseases, Japan.

Clinical manifestations such as volume of food and water consumed and appearance of feces were observed each day. Before and after infection, and at the time of each blood sampling, hematologic and serum chemistry, rectal temperatures and body weight were recorded. Blood samples were collected on days 0, 3, 5, 7, 10 and at weeks 2 and 4 after inoculation with DENV-3. Serum was separated from blood and stored at −70°C until use.

Serum samples were tested for dengue-virus-specific IgM antibody by IgM-capture enzyme-linked immunosorbent assay (ELISA) (FOCUS, Cypress, California, USA) [3]. The assays were performed in duplicate. Serotype-specific and cross-reactive neutralizing antibody titers to DENV-1 DENV-2, DENV-3 and DENV-4 were measured by a standard PRNT50 method using LLC-MK2 cells as described previously [4]. Briefly, 4- to 10-fold serial dilutions of sera were incubated for one hour at 37°C with a working dilution of virus to give 40-60 PFU/ml in the final volume of virus-serum mixture. After incubation, 100 μL of virus-serum mixture was added to a LLC-MK2 cell monolayer grown in 6-well polystyrene plates for seven days. After incubation for one hour at 37°C in an atmosphere of 5% CO_2, 3 ml of Eagle's MEM containing 1% methylcellulose with 2% FCS was overlaid onto the cells, and the plate was incubated at 35°C in 5% CO_2 for 7 days. The cells were fixed with 1 ml of 3.7% formaldehyde for one hour and stained with methylene blue tetrahydrate solution, and the visualized plaques were then counted. The serum dilution that resulted in a 50% reduction in plaque count was calculated by the Reed and Muench method [5].

There were no notable differences in behavior or consumption of food in any of the eight monkeys after inoculation with DENV-3. Similarly, specific changes in hematologic and serum chemistry, rectal temperatures or body weight were not seen in any of the animals.

Levels of neutralizing antibody were examined at weeks 2 and 4 after inoculation with DENV-3 in the primary (KT7 and KT8), secondary (KT3 and KT6) or tertiary (KT1, KT2, KT4 and KT5) infection (Table 1). Neutralizing antibody in KT7 (group 4) was specific for DENV-3. The neutralizing antibody titer in KT8 (group 4) was highest against DENV-3, but these antibodies were also cross-reactive with DENV-2 and DENV-4 at much lower levels. These results indicate that the neutralizing antibody response is generally specific for the inoculated serotype or cross-reactive, with the highest titer to the inoculated serotype after primary infection.

High levels of neutralizing antibody to DENV-2 were present in KT3 and KT6 (group 3) before the second inoculation with DENV-3 because of primary infection with DENV-2. Neutralizing antibody titers against all four serotypes increased after secondary infection with DENV-3. Neutralizing antibody titers were high to DENV-2 and DENV-3, and cross-reactive to DENV-1 and DENV-4 at lower levels. These results indicate that the neutralizing antibody response is serotype cross-reactive after infection with two serotypes.

High levels of neutralizing antibody to DENV-2 were present in KT4 and KT5 (group 2) because of two inoculations with DENV-2. After inoculation with DENV-3, neutralizing antibody titers to all four serotypes increased in KT4, and in KT5, neutralizing antibodies to DENV-1, DENV-3 and DENV-4 increased. The titers to DENV-2 increased only 2-3-fold in KT4. The results indicate that the neutralizing antibody response is serotype cross-reactive after two inoculations with DENV-2 and a third inoculation with DENV-3.

Cross-reactive neutralizing antibodies to all four serotypes were present in KT1 and KT2 (group 1) before

Table 1 Fifty-percent plaque reduction neutralizing antibody titer ($PRNT_{50}$) after the third dengue virus infection in monkeys

Serotype used for inoculation			Designation of monkey	Challenge virus for assessing $PRNT_{50}$	Titer before the third inoculation	Titer after the third inoculation	
First inoculation	Second inoculation	Third inoculation				2 weeks	4 weeks
DENV-1	DENV-2	DENV-3	KT1 (group 1)	DENV-1	6129	4938	6813
				DENV-2	3518	4001	2973
				DENV-3	367	745	638
				DENV-4	44	33	26
DENV-1	DENV-2	DENV-3	KT2 (group 1)	DENV-1	24	78	38
				DENV-2	284	346	460
				DENV-3	23	204	179
				DENV-4	18	51	ND
DENV-2	DENV-2	DENV-3	KT4 (group 2)	DENV-1	<10	33	45
				DENV-2	1402	2888	4704
				DENV-3	15	529	522
				DENV-4	24	240	215
DENV2	DENV-2	DENV-3	KT5 (group 2)	DENV-1	<10	24	26
				DENV-2	1836	3441	2558
				DENV-3	11	376	422
				DENV-4	20	207	236
None	DENV-2	DENV-3	KT3 (group 3)	DENV-1	<10	96	53
				DENV-2	271	5832	2128
				DENV-3	<10	1374	1093
				DENV-4	<10	258	255
None	DENV-2	DENV-3	KT6 (group 3)	DENV-1	<10	113	25
				DENV-2	283	2066	1454
				DENV-3	<10	3293	1142
				DENV-4	14	612	251
None	None	DENV-3	KT7 (group 4)	DENV-1	<10	<10	<10
				DENV-2	<10	<10	13
				DENV-3	<10	3804	3410
				DENV-4	<10	<10	<10
None	None	DENV-3	KT8 (group 4)	DENV-1	<10	<10	<10
				DENV-2	<10	43	90
				DENV-3	<10	675	917
				DENV-4	<10	25	15

inoculation with DENV-3 because of two inoculations with DENV-1 and DENV-3. Neutralizing antibody titers did not increase in KT1. In KT2, the titers to DENV-3 increased, but those to DENV-1, DENV-2, and DENV-4 did not.

These results, in general, indicate that antibody responses after primary infections are specific for the infecting serotype and that those occurring after secondary and tertiary infections with different serotypes are cross-reactive with all four serotypes. These results are consistent with those observed in humans [6]. However, it should be noted that there is variability in responses among monkeys, and this variability is consistent with what is seen in humans.

IgM responses were also examined in the eight monkeys for 6 weeks after inoculation with DENV-3 (Table 2). Specific IgM was detected in KT7 and KT8 (group 4), which were inoculated with DENV-3 in the primary DENV infection, in KT3 and KT6 (group 3), which had been infected with DENV-2 in the primary infection and DENV-3 in the secondary infection, and in KT4 (group 2), which had been infected two times with DENV-2 in the primary and secondary infections and with DENV-3 in the tertiary infection. IgM response was not detected in KT5 (group 2), which had been infected two times with DENV-2 in the primary and secondary infection and with DENV-3 in the tertiary infection, and in KT1 and KT2 (group 1), which had

Table 2 IgM responses after the third dengue virus infection in monkeys

Designation of monkey	5 days	7 days	14 days	21 days	28 days
Group 1					
KT1	1.04[a]	0.95	0.93	1.06	1.01
KT2	1.18	1.53	1.87	1.73	1.59
Group 2					
KT4	1.05	1.23	9.21[b]	6.43	4.71
KT5	0.99	1.06	1.90	1.76	1.37
Group 3					
KT3	1.37	1.85	6.06	3.27	2.29
KT6	1.29	2.03	14.36	8.75	5.04
Group 4					
KT7	1.20	1.23	6.84	5.39	3.39
KT8	1.28	3.49	26.94	20.92	13.46

[a] Data are presented as IgM index calculated by the formula: Optical density of samples collected before inoculation/Optical density of samples collected on designated days

[b] The IgM response was considered positive when the IgM index was equal to or higher than 2.00, as indicated by underlining

been infected with DENV-1 and DENV-2 in the primary and secondary infections and with DENV-3 in the tertiary infection. Thus, IgM was detected in two monkeys that were infected with DENV-3 in the primary DENV infection, in two monkeys that had been infected with DENV-2 in the primary infection and with DENV-3 in the secondary infection, and in one of the two monkeys that had been infected two times with DENV-2 in the primary and secondary infections and with DENV-3 in the tertiary infection. An IgM response was not detected in other monkeys. The results are consistent with previous reports of human IgM responses that an IgM response is apparent in primary infection but not in all of the cases with secondary infection [7, 8].

In the present study, serotype specificity and cross-reactivity in the neutralizing antibody response were analyzed after primary, secondary and tertiary infections with dengue virus in monkeys (*Macaca fascicularis*). Neutralizing antibody responses after primary infection are similar to those reported using *Aotus nancymae* monkeys [9]. The responses in monkeys after primary and secondary infections were also consistent with those reported in humans. After inoculation with DENV-3, neutralizing antibody titers to all four serotypes increased in KT4 (group 2), and in KT5 (group 2), neutralizing antibody titers to DENV-1, DENV-3 and DENV-4 increased. These results indicate that the neutralizing antibody response is serotype cross-reactive after two inoculations with one serotype and a third inoculation with the other serotype.

Cross-reactive neutralizing antibodies to all four serotypes were present in KT1 and KT2 before inoculation with DENV-3. Neutralizing antibody titers did not increase in KT1. In KT2, the titers to DENV-3 increased, but those to DENV-1, DENV-2, and DENV-4 did not. These results suggest that DENV-3 infection was not established in KT1, and only slightly in KT2. This result is consistent with the general opinion that tertiary infection does not usually occur in humans [10]. In humans, analysis of neutralizing antibody responses in tertiary infection is difficult. Symptomatic tertiary DENV infection is rare, and determination of the order of infecting serotypes in tertiary infections in humans is difficult. Thus, the results after tertiary infection in KT1 and KT2 (group 1) in the present study are unique, and this type of study can be only performed in animal models.

In dengue virus infection in humans, it is usually difficult to determine the infecting serotypes in secondary and tertiary infection. The advantage of using monkeys is that the timing of infections and infecting serotypes can be controlled. The results obtained in the present study suggests that monkeys (*Macaca fascicularis*) are suitable animals for analyzing antibody responses to dengue virus and that detailed serotype-specific and cross-reactive responses can be analyzed in detail after multiple dengue virus infections.

Acknowledgments Animal procedures were approved by the Committees on Biosafety and Animal Handling and Ethical Regulations of the National Institute of Infectious, Diseases, Japan. This work was supported by the Research on Emerging and Re-emerging Infectious Diseases from Ministry of Health, Labour and Welfare, Japan (H17-Shinkou-019 and H20-Shinkou-ippan-015), and the Research on Biological Resources and Animal Models for Drug Development from Ministry of Health, Labour and Welfare, Japan (H19-Seibutsushigen-ippan-003).

References

1. Halstead SB (1988) Pathogenesis of dengue: challenges to molecular biology. Science 239:476–481
2. Gubler DJ, Kuno G, Markoff L (2009) Flaviviruses. In: Knipe DM, Howley PM (eds) Fields Virology. Lippincott Williams & Wilkins, New York, pp 1155–1251
3. Ito M, Mukai RZ, Takasaki T, Kotaki A, Kurane I (2010) Antibody-dependent enhancement of dengue virus infection in vitro by undiluted sera from monkeys infected with heterotypic dengue virus. Arch Virol 155:1617–1624
4. Kelly EP, Greene JJ, King AD, Innis BL (2000) Purified dengue 2 virus envelope glycoprotein aggregates produced by baculovirus are immunogenic in mice. Vaccine 18:2549–2559
5. Reed LJ, Muench HA (1938) Simple method of estimating fifty percent endpoints. Am J Hyg 27:493–497
6. Innis BL (1997) Antibody responses to dengue virus infection. In: Gubler DJ, Kuno G (eds) Dengue and dengue hemorrhagic fever. Cab International, New York, pp 221–243
7. Chanama C, Anantapreecha S, A-nuegoonpipat A, Sa-gnasang A, Kurane I, Sawanpanyalert P (2004) Analysis of specific IgM