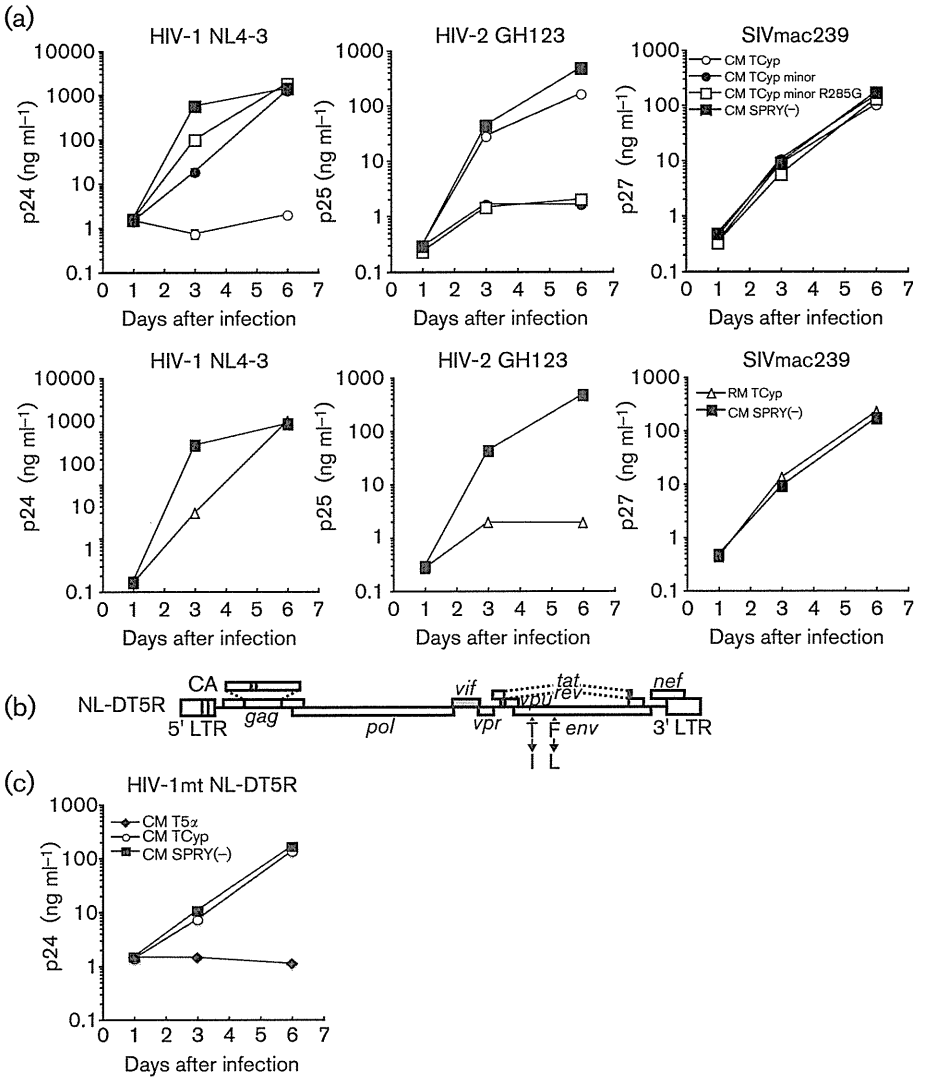

Fig. 4. Anti-lentiviral activity of various CM TRIMCyp. (a) MT4 cells were infected with recombinant SeV expressing CM TRIMCyp-major (DK) (CM TCyp; ○), CM TRIMCyp-minor (NE) (CM TCyp minor; ●), CM TRIMCyp-minor R285G (CM TCyp minor R285G; □), CM SPRY (−) (■) or RM TRIMCyp (RM TCyp; △). Data for CM SPRY (−) in the upper and lower panels were identical. Nine hours after infection, cells were superinfected with HIV-1 NL4-3, HIV-2 GH123 or SIVmac239. Culture supernatants were assayed separately for levels of p24, p25 or p27. (b) Structure of HIV-1mt NL-DT5R used in the experiment shown in Fig. 3(c). Open boxes denote HIV-1 (NL4-3) and shaded boxes denote SIVmac239 sequences. (c) MT4 cells were infected with recombinant SeV expressing CM TRIM5α (CM T5α; ◆), CM TRIMCyp-major (DK) (CM TCyp; ○) or CM SPRY (−) (■). Nine hours after infection, cells were superinfected with HIV-1mt NL-DT5R. Culture supernatants were assayed separately for levels of p24. Error bars show actual fluctuations between duplicate samples. Data from a representative of three (a) or two (c) independent experiments are shown.

SIV originated in African primates, it is unlikely that these viruses could contribute directly to this deviation, and some exogenous and endogenous retroviruses may thus play a critical role in this selection. Alternatively, it is possible that this deviation could come from bottleneck effects. It is estimated that the Philippine CMs were derived from Indonesian CM stocks via sea rafting or terrestrial access through Borneo during periods of low sea level in South-East Asia around 110 000 years ago (Abegg & Thierry, 2002; Blancher *et al.*, 2008; Kita *et al.*, 2009). Furthermore, phylogenetic analyses of mitochondrial DNA sequences of four CM populations distributed in South-East Asia suggested that Philippine CMs were derived from the small founding populations of Indonesian CMs, resulting in low genetic and nucleotide diversities (Blancher *et al.*, 2008). Importantly, however, as the Philippine CMs involved in this study at least originated from Luzon and Mindanao, the results in this study may reflect the frequency of TRIMCyp

in Philippine CMs as a whole, but do not represent local TRIMCyp distribution. In addition, hybridization with RMs may affect the prevalence of TRIMCyp. As Chinese RMs have been reported to have a low frequency of TRIMCyp (Newman *et al.*, 2008; Wilson *et al.*, 2008a), it is possible that interspecies mating with Chinese RMs might result in a lower prevalence of TRIMCyp in the Malaysian and Indonesian populations. In any case, it will be of great interest to determine the allele frequency of TRIMCyp in wild CMs to confirm whether our results reflect the observations in nature.

It is worth noting that the habitat of PMs is close to that of CMs, and in fact both species inhabit Indonesia; however, PMs reportedly express TRIMCyp but not TRIM5α (Brennan *et al.*, 2008; Liao *et al.*, 2007). In contrast, the allele frequency of TRIMCyp in Indonesian CMs was shown to be markedly lower (Table 1). This discrepancy in frequency of TRIMCyp between PMs and CMs suggests that the two species have independently evolved antiretroviral factors to counteract some pathogen(s) existing in their habitats. It is possible that unidentified co-factors that interact with TRIM5α/TRIMCyp may have a role in this discrepancy. Alternatively, the pathogen(s) could develop severe diseases in either monkey species. In the case of RMs, whilst the allele frequency of TRIMCyp was approximately 25 % in the Indian population, TRIMCyp was not detected in the Chinese population (Wilson *et al.*, 2008a). Although the precise reason(s) for these geographical deviations in CMs and RMs is still unknown, it is reasonable to speculate that the possible pathogens, including exogenous and endogenous retroviruses, are/were heterogeneously disseminated, depending on their habitats.

The amino acid sequence of the CypA domain of our CM TRIMCyp-major (DK) is identical to that of Mafa TRIMCyp2 cloned by Ylinen *et al.* (2010); thus, CM TRIMCyp-major (DK) showed almost identical antiviral properties to those of Mafa TRIMCyp2. However, CM TRIMCyp-major (DK) slightly restricted HIV-2 GH123, although Mafa TRIMCyp2 failed to restrict HIV-2 ROD. This discrepancy is possibly due to differences in assays; Ylinen and co-workers performed a single-round infection assay using replication-incompetent virus, whereas we performed a multiple-round replication assay using replication-competent virus and thus our assay could detect weak restriction activities. It is also possible that differences in HIV-2 strains or TRIMCyp amino acid differences outside the CypA domain could affect the result.

In the case of CM TRIMCyp-minor (NE), the amino acid sequence of the CypA domain was identical to that of RM TRIMCyp, and antiviral properties of CM TRIMCyp-minor (NE) were the same as those of RM TRIMCyp. In addition, exon 8 of both TRIMCyp genes showed a uniform sequence, identical to that of the Mamu 7 haplotype of RMs. Exon 8 of TRIMCyp would have been free from selection pressures, as it is absent from the mRNA due to splicing, and the ancestral sequences in exon 8 would have been preserved. Taken together, it is reasonable to speculate that this minor haplotype of CM TRIMCyp was the ancestor when CMs separated from RMs, and the major haplotype of CM TRIMCyp has arisen due to a specific evolutionary pressure on CMs. It should be noted that CM TRIM5α has Q at aa 339, where RM TRIM5α has a Q→TFP polymorphism. This Q→TFP polymorphism in the PRYSPRY domain also altered the spectrum of anti-lentiviral activity of TRIM5α (Kirmaier *et al.*, 2010; Kono *et al.*, 2008; Lim *et al.*, 2010; Wilson *et al.*, 2008b). Therefore, it is tempting to speculate that the selection pressure in CMs drove amplification and diversification in TRIMCyp, whilst that in RMs drove diversification of the PRYSPRY domain of TRIM5α.

In parallel with our study, Dietrich *et al.* (2011) recently reported the prevalence and functional diversity of TRIMCyp in CMs. They analysed populations from Indonesia, Indochina, Mauritius and the Philippines, and found that TRIMCyp was present in populations from Indonesia, Indochina and the Philippines, but not in populations from Mauritius. As they mentioned, the low genetic diversity, probably due to founder effects, may have led to the absence of TRIMCyp in the Mauritian population. In contrast, the small number of animals analysed may have resulted in the absence of TRIM5α in their Philippine population. They also analysed the effects of DK→NE substitution in CM TRIMCyp on antiretroviral activity by mutagenesis techniques. Furthermore, they found a unique individual with the DE haplotype in the CypA domain of TRIMCyp, whilst we did not identify such a haplotype in our study. Their results were essentially in accordance with ours, and we further demonstrated that Philippine CMs possessed TRIM5α as well as TRIMCyp, suggesting that maintenance of both TRIM5α and TRIMCyp in the CM population is beneficial to counteract challenges by retroviruses that are susceptible to TRIM5α and by those susceptible to TRIMCyp. Consistent with this, Reynolds *et al.* (2011) demonstrated that heterozygotes of RMs with TRIM5α and TRIMCyp showed higher resistance to repeated intrarectal challenge of SIVsmE660 compared with homozygotes for TRIM5α or TRIMCyp. Interestingly, this different outcome was not observed in the case of intrarectal challenge with SIVmac239. As RM TRIMCyp restricts SIVsm but not SIVmac (Kirmaier *et al.*, 2010), the combination of TRIM5α and TRIMCyp may function more efficiently as an antiviral factor against SIVsm.

We saw a small difference in anti-HIV-1 activity between CM TRIMCyp-minor (NE) and TRIMCyp-minor R285G. Dietrich *et al.* (2011) suggested that either of two polymorphic amino acid residues, K209E and R285G, might be responsible for attenuated anti-feline immunodeficiency virus activity of a certain haplotype of CM TRIMCyp. Our CM TRIMCyp-minor (NE) had K at aa 209, and an additional R285G mutation slightly attenuated the anti-HIV-1 activity of CM TRIMCyp-minor (NE). Residue 285 is in the linker region between the coiled-coil and CypA domains. The precise mechanism of how aa 285 affects anti-HIV-1 activity is unclear at present, but our result was consistent with those of Dietrich *et al.* (2011) and further revealed the importance of a single amino acid

substitution at aa 285 on the antiviral activity of CM TRIMCyp.

We showed that a prototypic HIV-1mt, named NL-DT5R, encoding L4/5 of SIVmac239 CA instead of that derived from HIV-1, evaded restriction by the major haplotype of CM TRIMCyp. As only HIV-1-derived L4/5 but not the SIVmac-derived L4/5 is expected to bind to CypA (Franke *et al.*, 1994), the substitution of L4/5 results in loss of binding of the capsid from CypA as well as TRIMCyp. Moreover, we recently demonstrated that HIV-1mt has the ability to grow in CMs (Saito *et al.*, 2011). Retrospective analysis of the *TRIM5* genotypes of the infected CMs revealed that they were homozygous for TRIMCyp (data not shown), suggesting that TRIMCyp homozygotes allow the replication of HIV-1mt *in vivo*. These findings will be helpful not only to understand the molecular mechanisms of the species barrier of primates to lentiviruses, but also to emphasize the importance of *TRIM5* genotypes for future studies regarding non-human primate models for HIV-1 infection.

METHODS

Sample collection. Blood samples were obtained from CMs kept in the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation, Tsukuba, Japan. CMs have been maintained in indoor facilities as closed colony monkeys in TPRC since 1978 (Honjo, 1985). CMs in TPRC were obtained from Indonesia, Malaysia and the Philippines. Although the detailed local information of their origin is unclear, more than 100 animals were introduced to each colony by dividing it several times. Basically, the monkeys have been bred as pure blood of each origin without interbreed crossing. The generation number of animals involved in this study ranged from two to four when we consider the wild-caught founders (introduced monkeys) as zero. These animals were maintained according to the rules of the National Institute of Biomedical Innovation and guidelines for experimental animal welfare. Bleeding was performed under ketamine hydrochloride anaesthesia.

PCR amplification and sequence analysis. Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) of 126 CMs using a QIAamp DNA Blood Mini kit (Qiagen). To test for the *CypA* insertion, the 3′ region of the *TRIM5* gene was amplified by PCR using LA *Taq* (TaKaRa) with primers TC forward (5′-TGACTCTGTGCTCACCAAGCTCTTG-3′) and TC reverse (5′-ACCCTACTATGCAATAAAACATTAG-3′), as described by Wilson *et al.* (2008a). The amplified products of *CypA* from 30 TRIMCyp homozygotes and 32 TRIMCyp/TRIM5α heterozygotes were gel-purified and subjected to direct sequencing using the forward and reverse primers.

To determine the sequences of the RING, B-box, coiled-coil and linker domains of TRIM5α and TRIMCyp, which span >15 kb of genomic DNA, we prepared phytohaemagglutinin (PHA)-stimulated PBMCs from six TRIMCyp homozygotes and three TRIM5α homozygotes. Total RNA was extracted from these cells using TRIzol (Invitrogen), and the RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) with TC reverse primer for TRIMCyp or TRIM5 reverse primer (5′-GAATT-CTCAAGAGCTTGGTGA-3′) for TRIM5α. The resultant cDNA was then PCR-amplified with LA *Taq* and forward primer TRIM5-235F (5′-GCAGGACCAGTGGAATAGC-3′). The amplified products were purified and subjected to direct sequencing using primers TRIM5-235F, TRIM-N (5′-AGGCAGAAGCAGCAGGAA-3′), TRIM-Nrev (5′-TTCCTGCTGCTTCTGCCT-3′) and TRIM-E (5′-ACCTCCCAG-TAATGTTTC-3′). As the direct sequencing results of exons 5 and 6 of TRIMCyp were ambiguous because of the existence of the other splicing variant containing exons 1–4 combined with *CypA* (Brennan *et al.*, 2008), amplified products were then cloned into the vector pCR-2.1TOPO (Invitrogen) and the nucleotide sequences of numerous independent clones (between three and nine) for each TRIMCyp were determined.

Exon 8 (PRYSPRY domain) was PCR-amplified from 12 TRIM5α homozygotes and seven TRIMCyp homozygotes by using TRIM-genotyping forward (5′-CTTCTGAACAAGTTTCCTCCCAG-3′) and reverse (5′-ATGAGATGCACATGGACAAGAGG-3′) primers. The amplified products were purified and subjected to direct sequencing using the TRIM genotyping forward and reverse primers.

Cloning and expression of TRIMCyp. cDNA of the major haplotype of CM TRIMCyp, CM TRIMCyp-major (DK), was amplified by RT-PCR of mRNA extracted from the TRIM5α/TRIMCyp-heterozygous CM T-cell line HSC-F using Not7TRIM5 (5′-GCGGCCGCAGCTACTATGGCTTCTG-3′) as the forward primer (*Not*I site underlined) and CypA Rev (5′-ACGGCGGTCT-TTTCATTCGAGTTGTCC-3′) as the reverse primer. RM TRIMCyp cDNA was amplified by RT-PCR of mRNA extracted from the TRIMCyp homozygous RM T-cell line HSR5.4 using Not7TRIM5 as the forward primer and CypA Rev as the reverse primer. The amplified products were then cloned into pCR-2.1TOPO and the authenticity of the nucleotide sequence was verified. To generate TRIMCyp cDNAs carrying a haemagglutinin (HA; YPYDVPDYAA) tag at the C terminus, the TRIMCyp cDNA clones were used as templates for PCR amplification with a primer including a *Not*I site and an HA tag.

To generate the minor haplotype, CM TRIMCyp-minor (NE), the C-terminal portion of RM TRIMCyp (*Sal*I–*Not*I) and the N-terminal portion of CM TRIMCyp-major (DK) (*Not*I–*Sal*I) were assembled in the pcDNA3.1 (−) vector (Invitrogen). CM TRIMCyp-minor R285G was generated by site-directed mutagenesis by a PCR-mediated overlap primer-extension method.

The entire coding sequences of these TRIMCyps were then transferred to the *Not*I site of the pSeV18+b (+) vector. Recombinant SeVs carrying various TRIMCyp were recovered according to a previously described method (Nakayama *et al.*, 2005). The viruses were passaged twice in embryonated chicken eggs and used as stocks for all experiments.

Virus propagation. Virus stocks were prepared by transfection of 293T cells with HIV-1 NL4-3, HIV-2 GH123, SIVmac239 and HIV-1mt NL-DT5R (Kamada *et al.*, 2006) using a calcium phosphate co-precipitation method. Virus titres were measured using p24 (for HIV-1 and HIV-1mt) or p27 (for HIV-2 and SIVmac239) RetroTek antigen ELISA kits (ZeptoMetrix).

Virus infection. Aliquots of 2×10^5 MT4 cells were infected with SeV expressing CM TRIM5α or each TRIMCyp at an m.o.i. of 10 and incubated at 37 °C for 9 h. Cells were then superinfected with 20 ng HIV-1 NL4-3 or HIV-1mt DT5R p24, 20 ng HIV-2 GH123 p25 or 20 ng SIVmac239 p27. The culture supernatants were collected periodically, and the levels of p24, p25 and p27 were measured with a RetroTek antigen ELISA kit.

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ORIGINAL PAPER

Diversity of MHC class I haplotypes in cynomolgus macaques

Yusuke Saito · Taeko K. Naruse · Hirofumi Akari · Tetsuro Matano · Akinori Kimura

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Abstract Cynomolgus macaques are widely used as a primate model for human diseases associated with an immunological process. Because there are individual differences in immune responsiveness, which are controlled by the polymorphic nature of the major histocompatibility (MHC) locus, it is important to reveal the diversity of MHC in the model animal. In this study, we analyzed 26 cynomolgus macaques from five families for MHC class I genes. We identified 32 *Mafa-A*, 46 *Mafa-B*, 6 *Mafa-I*, and 3 *Mafa-AG* alleles in which 14, 20, 3, and 3 alleles were novel. There were 23 MHC class I haplotypes and each haplotype was composed of one to three *Mafa-A* alleles and one to five *Mafa-B* alleles. Family studies revealed that there were two haplotypes which contained two *Mafa-A1* alleles. These observations demonstrated further the complexity of MHC class I locus in the Old World monkey.

Keywords Cynomolgus macaque · MHC · *Mafa* class I gene · Haplotype · Polymorphism

Yusuke Saito and Taeko K. Naruse contributed equally to this work.

Y. Saito · T. K. Naruse · A. Kimura (✉)
Department of Molecular Pathogenesis,
Medical Research Institute, Tokyo Medical and Dental University,
1-5-45 Yushima,
Bunkyo-ku, Tokyo 113-8510, Japan
e-mail: akitis@mri.tmd.ac.jp

Y. Saito · A. Kimura
Laboratory of Genome Diversity,
Graduate School of Biomedical Science,
Tokyo Medical and Dental University,
Tokyo, Japan

H. Akari
Primate Research Institute,
Kyoto University,
Inuyama, Japan

T. Matano
AIDS Research Center, National Institute of Infectious Diseases,
Tokyo, Japan

T. Matano
The Institute of Medical Science, The University of Tokyo,
Tokyo, Japan

Introduction

Non-human primates are widely used for immunological research because their immune system is similar to that of humans. In particular, the Old World monkeys such as cynomolgus macaques (crab-eating macaques, *Macaca fascicularis*) became a useful model for human infectious diseases including acquired immunodeficiency syndrome (AIDS) (Wiseman et al. 2007), severe acute respiratory syndrome (Lawler et al. 2006), and influenza (Kobasa et al. 2007) as well as in the transplantation field (Wiseman and O'Connor 2007). In the AIDS research, cynomolgus and rhesus macaques are important animal models for the development of vaccines against human immunodeficiency virus (HIV) or studies for susceptibility to HIV infection and/or development of AIDS (Matano et al. 2004; Loffredo et al. 2008; Tsukamoto et al. 2008; Burwitz et al. 2009; Mee et al. 2009; Aarnink et al. 2011a). To fully evaluate the results of immunological experiments in the macaque models, it is essential to characterize the genetic diversity of immune-related molecules which may control the individual differences in the immune response against foreign antigens and/or pathogens.

The major histocompatibility complex (MHC) is well known to control the immune-responsiveness to foreign

antigens. There are two classes of MHC molecules: one is the MHC class I molecule presenting peptides of intracellular origin to $CD8^+$ T cell and the other is the MHC class II molecule binding extracellular-derived antigenic peptides for presenting to $CD4^+$ T cell. It has been reported that the complexity of MHC genes in the rhesus and cynomolgus macaques is higher than that in humans (Kulski et al. 2004; Watanabe et al. 2006; Gibbs et al. 2007; Otting et al. 2007, 2008; Doxiadis et al. 2011). For example, *MHC class I* configurations in macaques are usually composed of one copy of highly transcribed major *MHC-A1*gene (*Mamu-A1*or *Mafa-A1*) and several other minor *MHC-A* genes (*Mamu-A2~A7* or *Mafa-A2~A6*) in addition to several *MHC-B* genes (*Mamu-B* or *Mafa-B*) (Watanabe et al. 2006; Otting et al. 2007, 2008, 2009; Naruse et al. 2010; Doxiadis et al. 2011), whereas each one copy of *MHC-A* and *-B* genes (*HLA-A* and *-B*) can be found in human *MHC class I* locus. In addition, other *MHC* loci showing lower expression levels, i.e., *HLA-B*-like gene (*Mamu-I* or *Mafa-I*) and *HLA-G*-like non-classical gene (*Mamu-AG* or *Mafa-AG*) have been identified (Slukvin et al. 2000; Urvater et al. 2000). The extent of genetic diversity is different, in part, depending on the geographic areas, as we have previously reported for *MHC class I* genes in rhesus macaque (Naruse et al. 2010). As for the cynomolgus macaques, *MHC class I* allelic diversity was reported for Indonesian (Pendley et al. 2008; Wu et al. 2008; Kita et al. 2009; Otting et al. 2009), Malaysian (Otting et al. 2009; Aarnink et al. 2011b), Mauritian (Budde et al. 2010), Vietnamese (Wu et al. 2008; Kita et al. 2009), and Philippino (Campbell et al. 2009; Kita et al. 2009) macaques, but information about the *MHC class I* haplotype remains insufficient.

In the present study, we have analyzed *MHC class I* loci in cynomolgus macaques originated from Indonesia, Malaysia, and the Philippines to obtain information on haplotype configuration. We report here further the complex nature of *MHC class I* loci in the Old World monkey, i.e., the presence of unique haplotypes carrying two *Mafa-A1* genes.

Materials and methods

Animals

A total of 26 cynomolgus macaques from five families were the subjects. Each family was composed of one or two males with one or two females and their offspring. They were maintained in the breeding colonies in Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Japan. The founders of the colonies were captured in Indonesia, Malaysia, and the Philippines. All care including blood sampling of animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH Publication 85–23, revised 1985) and were subjected to prior approval by the local animal protection authority.

Sequencing analysis of cDNAs from *Mafa* class I genes

Total cellular RNA was extracted from whole blood by using RNAeasy (QIAGEN, Gmbh, Germany). Oligo(dT)-primed cDNA was synthesized using Transcriptor reverse transcriptase (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Full-length cDNAs

Table 1 Primers used in PCR or sequencing of *Mafa* class I genes

Primer ID	Application	Direction	Sequence (5′–3′)	Position	Reference
5′ MHC_UTR	PCR	Sense	GGACTCAGAATCTCCCCAGACGCCGAG	5′ UTR	Karl et al. 2008
3′ MHC_UTR_A	PCR	Antisense	CAGGAACAYAGACACATTCAGG	3′ UTR	Karl et al. 2008
3′ MHC_UTR_B	PCR	Antisense	GTCTCTCCACCTCCTCAC	3′ UTR	Karl et al. 2008
5A long	PCR	Sense	ATGGCGCCCCGAACCCTCCTCCTG	Exon 1	Tanaka-Takahashi et al. 2007
3A	PCR	Antisense	TCACACTTTCAAGCCGTGAGAGA	Exon 7	Tanaka-Takahashi et al. 2007
5ASSP	PCR	Sense	ATGGCGCCCCGAACCCTCCTCCTGG	Exon 1	Tanaka-Takahashi et al. 2007
4R	PCR	Antisense	CCAGGTCAGTGTGATCTCCG	Exon 4	Tanaka-Takahashi et al. 2007
P000044	PCR	Sense	GATTCTCCGCAGACGCCCA	5′ UTR	Wu et al. 2008
P000023	PCR	Antisense	GGAGAACCAGGCCAGCAAT	Exon 5	Wu et al. 2008
P000076	Sequencing	Sense	GAGCAGCGACGGGACCGCA	Intron 1	Wu et al. 2008
P000060	Sequencing	Antisense	CCTGGGGCTCTCCCGGGTCA	Intron 2	Wu et al. 2008
P000096	Sequencing	Sense	TGTACTGAGTCTCCCTGATGG	Intron 2	Wu et al. 2008
P000098	Sequencing	Antisense	TTCATTCCCTCAGAGATTTT	Intron 3	Wu et al. 2008
P000055	Sequencing	Sense	CCCAGGTRCCTSTGTCCAGGA	Intron 3	Wu et al. 2008
P000281	Sequencing	Antisense	AGAGGGGAAAGTGAGGGGT	Intron 4	Wu et al. 2008

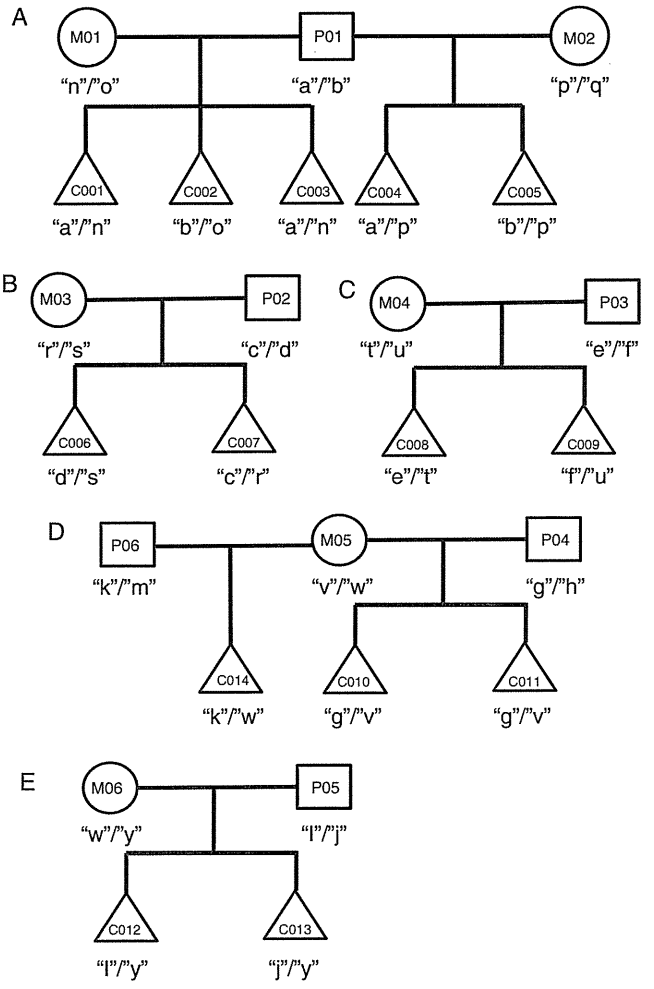

Fig. 1 Pedigree of cynomolgus macaques. The pedigrees of macaques analyzed in this study are shown. Founders were originated from Indonesia (**a**), Malaysia (**b**, **c**), and Philippines (**d**, **e**). *Open square*, *open circle*, and *open triangles* indicate father, mother, and offspring, respectively. The ID of each subject is noted in the symbol. *Mafa class I* haplotypes determined in this study are indicated under the subjects

for *Mafa* class I genes were amplified by polymerase chain reaction (PCR), as described previously (Tanaka-Takahashi et al. 2007; Naruse et al. 2010), by using locus-specific primer pairs as reported by Karl et al. (2008). Genomic gene and cDNA for *Mafa-A2* gene were analyzed according to the method described by Wu et al. (2008). The primers used in this study are listed in Table 1. To estimate the expression level of *Mafa-A* alleles, we also used an additional primer pair: MafaF (5′-TACGTGGACGA CACGCAGTT) and MafaR (5′-GGTGGGTCA CATGTGTCTTG). PCR was done under the condition of initial denaturation at 98°C for 10 s, 25 cycles of 98°C for 1 s, 64°C for 5 s, and 72°C for 20 s, followed by an additional extension at 72°C for 1 min, using Phusion Flash DNA polymerase (Finzymes, Espoo, Finland). The PCR products were cloned into pSTBlue-1 Perfectly Blunt vector (Novagen, WI, USA) according to the manufacturer's instructions and were transformed to NovaBlue Giga Singles™ competent cells (Merck Biosciences Japan, Tokyo, Japan). A total of 30 to 90 independent cDNA clones were obtained from each macaque for each locus and were sequenced on both strands by BigDye Terminator cycling system in an ABI 3730 automated sequence analyzer (Applied Biosystems, CA, USA).

Data analyses and nomenclature for *Mafa* class I allele

Nucleotide sequences of cDNA clones were aligned using the Genetyx software package (version 8.0, Genetyx Corp., Japan). When a cDNA sequence, which was represented by at least three clones, was independently obtained from at least two animals or repeatedly obtained from at least two independently prepared cDNAs from single animals, we considered it a real allele, not an artifact, and the sequences were submitted to the DNA Data Bank of Japan (DDBJ) database and to the Immuno Polymorphism Database for non-human primate MHC (http://www.ebi.ac.uk/ipd/mhc/sumit.html; Robinson et al. 2003) to obtain official nomenclature for the novel alleles of *Mafa-A* and *Mafa-B* genes. Neighbor-joining trees were constructed with Kimura's two-parameter method for a phylogenetic analysis of *Mafa-A* sequences spanning exons 2, 3, and a part of exon 4 obtained in this study by using the Genetyx software. Bootstrap values were based on 5,000 replications.

Table 2 *Mafa* class I alleles found in the cynomolgus macaques

Locus	Number of observed alleles	Number of novel alleles (%)	Number of observed alleles in macaques from different regions[a]		
			Indonesian	Malaysian	Philippino
Mafa-A	32	14 (43.7%)	9 (3), 33.3%	12 (8), 66.7%	11 (3), 27.3%
Mafa-B	46	20 (43.5%)	13 (5), 38.5%	20 (15), 75.0%	18 (1), 5.6%
Mafa-I	6	3 (50.0%)	2 (1), 50.0%	4 (3), 75.0%	2 (0), 0%
Mafa-AG	3	3 (100%)	0 (0), 0%	2 (2), 100%	1 (1), 100%
Total	87	40 (45.5%)	24 (9), 37.5%	38 (28), 73.7%	32 (5), 15.6%

[a] The number and frequency of novel alleles are indicated in parentheses

Results

Identification of *Mafa* class I alleles in cynomolgus macaques

We determined the nucleotide sequences of cDNA clones for *Mafa-A* and *-B* loci in 26 cynomolgus macaques from one family of Indonesian origin (six haplotypes), two families of Malaysian origin (eight haplotypes), and two families of Philippino origin (nine haplotypes) (Fig. 1). When the observed alleles were segregated in the family or when at least three clones with identical sequences were observed from two independent PCR for an individual, the nucleotide sequences were considered to be real and not artifacts. As shown in Table 2, 32 *Mafa-A*, 46 *Mafa-B*, 6 *Mafa-I*, and 3 *Mafa-AG* sequences were obtained in this study. Among them, 14 (43.7%), 20 (43.5%), 3 (50.0%), and 3 (100%) were novel alleles of *Mafa-A*, *Mafa-B*, *Mafa-I*, and *Mafa-AG* loci, respectively (Table 2).

Table 3 Alleles of *Mafa-A* locus identified in the cynomolgus macaques

Locus	Allele name	Novelty[a]	Accession number[b]	Origin[c]	Identical *Mamu* and/or *Mane* alleles[d]	Origin and reference of known alleles[e]
A1	*A1*001:01*		AM295828	Malaysian		Utrecht, Otting et al. 2007
A1	*A1*002:01:02*	Novel	AB569214	Indonesian		
A1	*A1*008:02*		EU392108	Philippino		Philippino, Campbell et al. 2009
A1	*A1*008:03-like*	Novel	AB647187	Philippino		
A1	*A1*018:06*		FM246489	Indonesian		Utrecht, Otting et al. 2007
A1	*A1*019:05*		AB447616	Indonesian		Indonesian, Kita et al. 2009
A1	*A1*023:01*	Novel	AB569216	Malaysian		
A1	*A1*032:05*	Novel	AB569215	Malaysian		
A1	*A1*052:02*		EU392105	Philippino	*Mamu-A1*052:01/03/06*	Philippino, Campbell et al. 2009
A1	*A1*054:01*		AB154771	Malaysian		Tsukuba, Uda et al. 2004
A1	*A1*056:02*	Novel	AB569218	Malaysian		
A1	*A1*062:05*	Novel	AB569219	Malaysian		
A1	*A1*068:02*	Novel	AB569217	Malaysian		
A1	*A1*074:02*		AB447606	Philippino		Philippino, Kita et al. 2009
A1	*A1*079:01*		AB154773	Malaysian		Tsukuba, Uda et al. 2004
A1	*A1*089:02*		EU392104	Philippino		Philippino, Campbell et al. 2009
A1	*A1*093:01*		EU392103	Philippino		Philippino, Campbell et al. 2009
A1	*A1*094:01*		EU392111	Philippino		Philippino, Campbell et al. 2009
A1	*A1*097:01*		AB447576	Indonesian	*Mamu-A1*109:01*	Indonesian, Kita et al. 2009
A1	*A1*103:01*	Novel	AB583236	Indonesian		
A1	*A1*124:01*	Novel	AB583237	Malaysian		
A2	*A2*05:13-like*	Novel	AB647189	Philippino		
A2	*A2*05:16*		AM295878	Indonesian		Utrecht, Otting et al. 2007
A2	*A2*05:34-like*	Novel	AB647190	Philippino		
A3	*A3*13:03*		EU392112	Philippino		Philippino, Campbell et al. 2009
A3	*A3*13:15*	Novel	AB583238	Malaysian		
A3	*A3*13:16*	Novel	AB583240	Indonesian		
A4	*A4*14:01*		AM295880	Indonesian		Utrecht, Otting et al. 2007
A4	*A4*14:02*		AM295881	Malaysian		Utrecht, Otting et al. 2007
A6	*A6*01:05*	Novel	AB583239	Malaysian		

[a] New alleles are indicated as novel

[b] Nucleotide sequences were submitted to a public database and given accession numbers

[c] Origin of cynomolgus macaques

[d] Identical sequences were found in *Mamu* or *Mane* alleles

[e] Origin and references in which each known allele was first reported. Utrecht and Tsukuba indicate that the alleles were found in colonies maintained in the University of Utrecht, The Netherlands, and Tsukuba primate center, Japan, respectively

Table 4 Alleles of *Mafa-B* locus identified in the cynomolgus macaques

Allele name[a]	Novelty[b]	Accession number	Origin[c]	Identical to *Mamu* and/or *Mane* alleles[d]	Origin and reference of known alleles[e]
*B*002:03*	Novel	AB569224	Indonesian, Malaysian		
*B*004:01*		EU203722	Indonesian		Indonesian, Pendley et al. 2008
*B*007:01:01*		AY958137	Philippino	*Mamu-B*007:02/03*	Mauritian, Krebs et al. 2005
*B*007:01:02*		EU392135	Philippino		Philippino, Campbell et al. 2009
*B*007:01:03*	Novel	AB569223	Indonesian		
*B*007:03*		FM212802	Philippino		Indonesian or Malaysian, Otting et al. 2009
*B*011:02*	Novel	AB569229	Malaysian		
*B*013:08*		EU392114	Indonesian, Philippino		Philippino, Campbell et al. 2009
*B*017:01*		EU392119	Philippino		Philippino, Campbell et al. 2009
*B*018:01*		AY958138	Indonesian	*Mamu-B*018:01*	Mauritian, Krebs et al. 2005
*B*030:02*		AY958134	Malaysian	*Mamu-B*030:03:01*	Mauritian, Krebs et al. 2005
*B*032:01*	Novel	AB569237	Malaysian		
*B*033:02*		EU392118	Philippino		Philippino, Campbell et al. 2009
*B*043:01*	Novel	AB569230	Malaysian	*Mamu-B*043:01*	
*B*056:01*		AY958131	Indonesian	*Mamu-B*056:01*	Mauritian, Krebs et al. 2005
*B*056:02*		EU392128	Philippino		Philippino, Campbell et al. 2009
*B*057:03*	Novel	AB569231	Malaysian	*Mamu-B*057:06*	
*B*060:04*	Novel	AB569226	Indonesian		
*B*061:01*		AB195445	Malaysian	*Mamu-B*061:04:01, Mane-B*061:01*	Tsukuba, Uda et al. 2005
*B*061:02*	Novel	AB569233	Malaysian		
*B*064:02*		FM212804	Philippino		Indonesian or Malaysian, Otting et al. 2009
*B*068:04*	Novel	AB569236	Malaysian	*Mamu-B*068:04 , Mane-B*nov078*	
*B*069:02*		FM212842	Malaysian		Indonesian or Malaysian, Otting et al. 2009
*B*074:01:02-like*	Novel	AB647188	Philippino		
*B*074:02*	Novel	AB569228	Malaysian	*Mamu-B*074:01 /02*	
*B*076:04*	Novel	AB569232	Malaysian		
*B*081:01*	Novel	AB569225	Indonesian		
*B*089:01:01*		EU392131/ FJ178820	Philippino		Philippino, Campbell et al. 2009
*B*089:01:02*		EU392125	Indonesian, Malaysian, Philippino	*Mamu-B*089:01, Mane-B*089:02*	Philippino, Campbell et al. 2009
*B*090:01*		AB195436	Malaysian		Tsukuba, Uda et al. 2005
*B*091:01*	Novel	AB569240	Malaysian	*Mamu-B*091:02*	
*B*092:01:01*	Novel	AB569227	Malaysian	*Mamu-B*092:02*	
*B*095:01*		EU392113/ AY958148	Philippino		Mauritian, Krebs et al. 2005 Philippino, Campbell et al. 2009
*B*104:03*		EU392126	Philippino		Indonesian, Pendley et al. 2008 Philippino, Campbell et al. 2009
*B*116:01*		EU392123	Philippino		Philippino, Campbell et al. 2009
*B*121:01*		AB195455	Indonesian		Tsukuba, Uda et al. 2005
*B*124:01:02*	Novel	AB569235	Malaysian		
*B*136:02*		EU203720	Indonesian,		Indonesian, Pendley et al. 2008
*B*137:03*		EU392117/ EU203723	Indonesian, Philippino		Indonesian, Pendley et al. 2008
*B*137:04*	Novel	AB569239	Malaysian		

Table 4 (continued)

Allele name[a]	Novelty[b]	Accession number	Origin[c]	Identical to *Mamu* and/or *Mane* alleles[d]	Origin and reference of known alleles[e]
*B*138:02*	Novel	AB569234	Malaysian		
*B*151:02:02*	Novel	AB569222	Indonesian		
*B*155:02*	Novel	AB569238	Malaysian		
*B*157:01*		EU392121	Philippino		Philippino, Campbell et al. 2009
*B*158:01*		EU392122	Philippino		Philippino, Campbell et al. 2009
*B*160:01*		EU606042	Philippino		-

[a] New alleles are indicated as novel

[b] Nucleotide sequences were submitted to a public database and given accession numbers

[c] Origin of cynomolgus macaques

[d] Identical sequences were found in *Mamu* or *Mane* alleles

[e] Origin and references in which each known allele was first reported. Utrecht and Tsukuba indicate that the alleles were found in colonies maintained in the University of Utrecht, The Netherlands, and Tsukuba primate center, Japan, respectively

The *Mafa-A* alleles found in this study are listed in Table 3, where 21 alleles were from the major *Mafa-A1* locus, while the remaining 11 alleles were from the minor *Mafa-A* loci, 3 from *Mafa-A2*, 3 from *Mafa-A3*, 2 from *Mafa-A4*, and 1 from *Mafa-A6* alleles (Table 3). The major *Mafa-A1*alleles were defined by the sequence similarity to the known *Mafa-A1* alleles to be given official nomenclatures by IPD, except for *Mafa-A1*008:03*-like allele, and we confirmed that the frequencies of cDNA clones for *Mafa-A1* alleles were over 10% in each macaque. Similarly, alleles of minor *Mafa-A* genes, *Mafa-A2*, *-A3*, *-A4*, and *-A6* were defined by sequence similarity to the known alleles. They, except for two novel *Mafa-A2* alleles, were also given official names by IPD. On the other hand, a total of 46 *Mafa-B* alleles (Table 4) as well as 6 *Mafa-I* and 3 *Mafa-AG* alleles (Table 5) were identified. It was found that 2 out of

Table 5 Alleles of *Mafa-AG* and *Mafa-I* locus identified in the cynomolgus macaques

Locus	Allele name[a]	Novelty[b]	Accession number[c]	Origin[d]	Identical to *Mamu* and/or *Mane* alleles[e]	Origin and reference of known alleles[e]
AG	*AG*04:03*	Novel	AB569221	Malaysian		
AG	*AG1 like-1*	Novel	AB569220	Malaysian		
AG	*AG1 like-3*	Novel	AB583241	Philippino		
I	*I*01:01:01*		EU392139	Philippino		Philippino, Campbell et al. 2009
I	*I*01:09/01:08*		AB195465/AB195464	Indonesian, Malaysian		Tsukuba, Uda et al. 2005
I	*I*01:15*		FM246493	Philippino	*Mamu-I*01:06* , *Mamu-I*01:08:01*	Indonesian or Malaysian, Otting et al. 2009
I	*I*01:15 like-1*	Novel	AB569241	Indonesian, Malaysian		
I	*I*01:15 like-2*	Novel	AB569242	Malaysian	*Mamu-I*03:01:01*, *Mamu-I*01:07:01* , *Mamu-I*01:06:05*	
I	*I*01:18 like*	Novel	AB569243	Malaysian		

[a] Official allele names were not obtained for *AG1 like-1*, *AG1 like-3*, *I*01:15 like-1*, *I*01:15 like-2*, and *I*01:18 like* due to the limited sequence information

[b] New alleles are indicated as novel

[c] Nucleotide sequences were submitted to a public database and given accession numbers

[d] Origin of cynomolgus macaques

[e] Identical sequences were found in *Mamu* or *Mane* alleles

[f] Origin and references in which each known allele was reported. Tsukuba indicates that the alleles were found in colonies maintained in the Tsukuba primate center, Japan

21 (9.5%) *Mafa-A1a* alleles and 12 out of 46 (26.1%) *Mafa-B* alleles had identical sequences to *Mamu-A1* and *Mamu-B* alleles, respectively, implying a genetic admixture of cynomolgus macaques with rhesus macaques during the evolution (Otting et al. 2007; Bonhomme et al. 2009; Otting et al. 2009). Because we determined the nucleotide sequences only for exons 2, 3, and 4, two novel *Mafa-AG* alleles and three novel *Mafa-I* alleles were not given official names. As for the geographic distribution of *Mafa* class I alleles, there was no overlapping of *Mafa-A* alleles originated from different regions (Table 3), while there were a few *Mafa-B* and *Mafa-I* alleles commonly observed in macaques from different regions (Tables 4 and 5, respectively). When we looked into the presence of novel alleles in the geographic distribution, most of the novel alleles were obtained from Malaysian macaques, while almost all of the alleles found in Philippino macaques were not novel (Table 2).

Mafa class I haplotypes identified in the family study

We could identify the *Mafa-A* and *Mafa-B* alleles composing 23 different haplotypes from the segregation studies (Table 6). It was found that one to three expressing *Mafa-A*

Table 6 *Mafa class I* haplotypes identified in the cynomolgus macaques

ID[a]	Origin[b]	Haplotype[c]	*Mafa-A1* (major)	*Mafa-A* (minor)	*Mafa-AG*	*Mafa-B* (major)	*Mafa-B* (minor)	*Mafa-I*
P01	Indonesian	"a"	A1*002:01:02	A3*13:16		B*136:02		I*01:09/01:08
		"b"	A1*103:01			B*007:01:03, B*121:01	B*151:02:02	
P02	Malaysian	"c"	A1*023:01			B*090:01	B*011:02, B*074:02	
		"d"	A1*068:02			B*043:01	B*030:02, B*057:03	I*01:15 like-2
P03	Malaysian	"e"	A1*001:01, A1*032:05		AG1 like-1	B*068:04, B*124:01:02	B*032:01, B*061:01, B*089:01:02	
		"f"	A1*079:01		AG*04:03	B*061:02, B*138:02	B*155:02	
P04	Philippino	"g"	A1*089:02	A2*05:13-like, A3*13:03		B*137:03		
		"h"	A1*008:02			B*104:03		
P05	Philippino	"i"	A1*094:01			B*007:01:02	B*160:01	
		"j"	A1*008:02		AG1 like-3	B*157:01	B*017:01, B*089:01:02, B*116:01	I*01:01:01, I*01:15
P06	Philippino	"k"	A1*08:03-like	A2*05:34-like		B*074:01:02-like		
		"m"	A1*089:02	A3*13:03		B*007:03, B*064:02	B*089:01:01	
M01	Indonesian	"n"	A1*018:06	A2*05:16, A4*14:01		B*002:03		I*01:15 like-1
		"o"	A1*097:01			B*056:01	B*089:01:02	
M02	Indonesian	"p"	A1*097:01			B*137:03	B*013:08	
		"q"	A1*019:05			B*018:01	B*004:01, B*060:04, B*081:01	
M03	Malaysian	"r"	A1*054:01			B*002:03		I*01:15 like-1
		"s"	A1*056:02	A4*14:02		B*076:04		I*01:18 like
M04	Malaysian	"t"	A1*062:05			B*069:02	B*137:04	
		"u"	A1*124:01	A3*13:15		B*091:01		
M05	Philippino	"v"	A1*074:02, A1*093:01			B*007:01:01, B*158:01		
		"w"	A1*093:01			B*007:01:02	B*160:01	
M06	Philippino	"w"	A1*093:01			B*007:01:02	B*160:01	
		"y"	A1*052:02			B*033:02, B*095:01		

[a] ID of founder animals as indicated in Fig. 1

[b] Origin of cynomolgus macaques

[c] Haplotypes were determined from studies of family as shown in Fig. 1

alleles and one to five expressing *Mafa-B* alleles consisted of *Mafa class I* haplotype, similar to the *Mamu class I* haplotypes in rhesus macaques (Naruse et al. 2010). Of particular interest was that there were two haplotypes, "e" (Malaysian founder P03) and "v" (Philippino founder M05), carrying two different *Mafa-A1* genes (Fig. 1; Table 6). Because previous studies have demonstrated that there is usually only one *Mafa-A1* allele on a chromosome (Otting et al. 2007), while the presence of two *Mamu-A1* alleles on the same haplotype was suggested in rhesus macaques (Naruse et al. 2010; Doxiadis et al. 2011), we performed further analyses.

The family studies showed that the *Mafa-A1*alleles consisting of haplotype "e", *Mafa-A1*001:01* and*Mafa-A1*032:05*, or haplotype "v", *Mafa-A1*074:02* and *Mafa-A1*093:01*, did not carry accompanying minor *Mafa-A* genes (Table 6). When we constructed a phylogenetic tree of *Mafa-A* alleles identified in this study (Fig. 2), it was found that *Mafa-A1*001:01* was mapped in the neighbor of *Mafa-A3*gene, raising a possibility that one of the two alleles on the same chromosome might be a minor *Mafa-A* allele and not the major *Mafa-A1* allele. To test the possibility, we investigate the expression level of *Mafa-A* alleles composing of haplotypes "e" and "v". For this purpose, other primer pairs were designed within the sequences completely shared by these alleles to amplify the *Mafa-A* cDNAs to avoid a possibility of affecting the efficacy of PCR by mismatches with the primer sequences. The cloning and sequencing analysis revealed that both *Mafa-A1*001:01* and *Mafa-A1*032:05* on the haplotype "e" were observed at similar frequencies among the cDNA clones of *Mafa-A* alleles in P03and C008 (Fig. 1): 29.7% and 33.3% in P03 and 22.5% and 17.5% in C008, respectively. Similarly, frequencies of haplotype "v" alleles, *Mafa-A1*074:02* and *Mafa-A1*093:01*, in cDNA clones were 59.5% and 40.5%, respectively, in M05, while those in C010 were 23.3% and 26.7% and 31.4% and 17.1% in C011, respectively. The frequencies of cDNA clones varied in different individuals presumably due to the allelic competition with the alleles of another haplotype in each individual (Fig. 1), but they were much higher than the frequencies of the minor *Mafa-A* allele (*Mafa-A3*13:03*) clones: 3.3% and 2.9% in C010 and C011, respectively. These observations indicated that two *Mafa-A* alleles were considered to be major *Mafa-A1* alleles in both haplotypes "e" and "v".

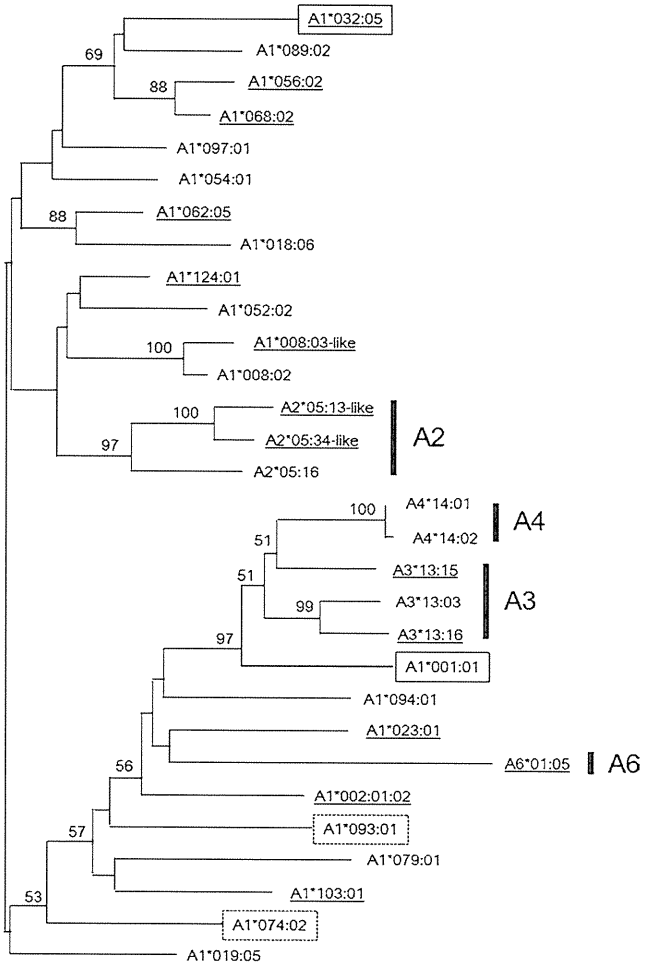

Fig. 2 Phylogenetic tree of *Mafa-A* alleles. A phylogenetic tree of the *Mafa-A* alleles detected in this study was constructed by using the neighbor-joining method with a bootstrap value of 5,000 replications. Values more than 50% are indicated as percentages. Novel alleles were *underlined*. *Mafa-A*1 alleles consisting of haplotype "e" are *boxed*, while the *stippled boxes* represent the alleles on haplotype "v". Alleles of minor *Mafa-A* genes, *Mafa-A2*, *A3*, *A4*, and *A6*, are also indicated

Discussion

Native cynomolgus macaques are widespread throughout the islands of Southeast Asia into mainland Asia. They are mainly found in Indonesia, Malaysia, and the Philippines, then Burma, India, Vietnam, Cambodia, Laos, and Thailand (Lang 2006). It was suggested that the founding population of Mauritian macaques was introduced from Indonesia (Pendley et al. 2008; Campbell et al. 2009). More than 40% of *Mafa* class I alleles observed in this study were novel, even though there have been many reports on the analysis of *Mafa class I* genes, demonstrating that the diversity of MHC in the cynomolgus macaques still needs to be investigated. When we considered the origin of founders, 73.7% (28/38) were novel in alleles found in Malaysian macaques, while only 15.6% (5/32) were novel alleles in Philippino macaques (Table 2). The geographic distribution of novel alleles may be due to the fact that the Malaysian macaques had not been extensively analyzed before (Otting et al. 2007;

Pendley et al. 2008; Kita et al. 2009). In the present study, *B*089:01:02* was found in individuals among Indonesian, Malaysian, and Philippino macaques in different *Mafa-B* haplotypes (Table 6). Likewise, *B*137:03* was found in Indonesian and Malaysian macaques (Table 4). In addition, shared alleles among the cynomolgus macaques, rhesus macaques, and pig-tailed macaques (*Macaca nemestrina*) were noted (Tables 3, 4, and 5). These observations indicated that the diversity of *MHC class I* genes is similar not only in the cynomolgus macaque population but also among the Old World monkeys, suggesting that the *MHC class I* polymorphisms might be generated before the divergence of Old World monkeys and/or there were admixtures of the Old World monkeys.

In this study, we determined the haplotype structure of *Mafa* class I locus by family studies and a total of 23 haplotypes were identified. Among them, haplotypes "i" and "w" carried identical *Mafa-B* alleles but different *Mafa-A* alleles (Table 6), suggesting that there were haplotypes originated by a recombination between the *Mafa-A* and *Mafa-B* loci. We showed that the *Mafa class I* haplotypes were usually composed of one to three *Mafa-A* alleles and one to five *Mafa-B* alleles, similar to the *Mamu class I* haplotypes, of which usually one *MHC-A1* gene and a few (one to three) *MHC-B* genes were highly transcribed (Otting et al. 2007, 2008; Naruse et al. 2010; Doxiadis et al. 2011). As for the *MHC-A* locus in the cynomolgus macaques, highly transcribed *Mafa-A1*gene and other minor *Mafa-A* genes, such as *Mafa-A2*, *-A3*, *-A4*, and *-A6* could be detected. It was reported that 87% of cynomolgus macaques had at least one *Mafa-A2* alleles (Wu et al. 2008). However, only 3 out of 23 (13.0%) haplotypes carried a *Mafa- A2* allele in this study (Table 6). We could not exclude a possibility that the strategy of our study might not be sufficient to detect the *Mafa-A* genes with low expression and/or the alleles with mismatches at the primer site, based on the number of clones within a PCR sample. Such a possibility is unlikely because we used the primer pairs which could cover the known *Mafa-A2* alleles, although there might be novel *Mafa-A2* alleles having different sequences at the primer binding sites. Therefore, we might underestimate the complexity of *Mafa* class I alleles in this study. High-throughput pyrosequencing methods may be a useful strategy to avoid the possibility of missing alleles, as described by several investigators (Wiseman et al. 2009; Budde et al. 2010; Aarnink et al. 2011b). In addition, because it was reported that the cell surface expression of Mamu class I molecule was varied depending on the locus and allelic structure (Rosner et al. 2010), locus- and allele-dependent expression of Mafa class I molecule at the cell surface will be required.

The most important finding in this study was that we demonstrated evidence for the presence of haplotypes carrying two major *MHC-A1* genes on the same chromosome from the family studies and additional cloning studies. Interestingly, we and others have reported similar phenomena in rhesus macaques (Naruse et al. 2010; Doxiadis et al. 2011). In addition, several haplotypes carried multiple major *Mafa-B1* alleles (Table 6), similar to the *Mamu-B1* locus (Otting et al. 2008; Doxiadis et al. 2011). The *raison d'etre* of multiple major *MHC class I* genes/alleles on the same chromosome may be that they play an immunological role as the "double lock strategy" (Doxiadis et al. 2011) in which the double *MHC-A1* alleles of high transcription level might be favorable to present peptide to CD8+ T cells. However, there is another unique haplotype which carries no *MHC-A1*allele in cynomolgus macaques (Otting et al. 2007) and maybe in rhesus macaques (Doxiadis et al. 2011). These observations suggested that the diversity of MHC in the Old World monkey is far more complicated than in humans.

In summary, we investigated 26 cynomolgus macaques from five families for the diversity of *MHC class I* alleles and haplotypes. A total of 87 alleles were identified, of which 40 were novel. There were 23 different haplotypes, and two of them carried two *MHC-A1* genes, demonstrating further the complexity of *MHC class I* locus in the Old World monkey.

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BRIEF REPORT

CD16$^+$ natural killer cells play a limited role against primary dengue virus infection in tamarins

Tomoyuki Yoshida · Tsutomu Omatsu · Akatsuki Saito · Yuko Katakai · Yuki Iwasaki · Sayuki Iijima · Terue Kurosawa · Masataka Hamano · Shinichiro Nakamura · Tomohiko Takasaki · Yasuhiro Yasutomi · Ichiro Kurane · Hirofumi Akari

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Abstract CD16 is a major molecule expressed on NK cells. To directly assess the role of natural killer (NK) cells in dengue virus (DENV) infection *in vivo*, CD16 antibody-treated tamarins were inoculated with a DENV-2 strain. This resulted in the transient depletion of CD16$^+$ NK cells, whereas no significant effects on the overall levels or kinetics of plasma viral loads and antiviral antibodies were observed in the treated monkeys when compared to control monkeys. It remains elusive whether the CD16$^-$ NK subpopulation could play an important role in the control of primary DENV infection.

Keywords Dengue virus · Tamarin · NK cells · CD16

T. Yoshida and T. Omatsu contributed equally to this study.

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T. Yoshida · Y. Iwasaki · S. Iijima · T. Kurosawa · M. Hamano · Y. Yasutomi · H. Akari
Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan

T. Yoshida (✉) · A. Saito · H. Akari (✉)
Center for Human Evolution Modeling Research, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan
e-mail: ytomoyuki@pri.kyoto-u.ac.jp

H. Akari
e-mail: akari@pri.kyoto-u.ac.jp

T. Omatsu · T. Takasaki · I. Kurane
Department of Virology I, National Institute of Infectious diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

DENV is one of the most serious mosquito-borne virus affecting humans, with 2.5 billion people at risk in tropical and subtropical regions around the world each year [12]. A wide variety of clinical manifestations have been noted, which range from asymptomatic, mild febrile illness (dengue fever [DF]) to dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), a life-threatening illness. It has been shown that humans with a secondary heterologous DENV infection are at a higher risk of contracting severe dengue disease [10, 26]. DHF/DSS occurs in infants during primary DENV infection, predominantly in the second half of the first year of life, when maternal antibodies have low residual neutralizing activity [11, 17].

NK cells are a component of the innate immune system that plays a central role in host defense against viral infection and tumor cells. It has been shown that infection by some viruses, such as herpes simplex virus-1, influenza virus or ectromelia poxvirus, can be controlled by NK cells in mice [15]. Yet the most compelling evidence for a role

A. Saito
International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

Y. Katakai
Corporation for Production and Research of Laboratory Primates, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan

S. Nakamura
Research Center for Animal Life Science, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga 520-2192, Japan

of NK cells in early defense against viruses was obtained in a study showing increased susceptibility to murine cytomegalovirus (MCMV) after NK cell depletion and increased resistance after adoptive transfer of NK cells [23]. Defects in NK cell activity, such as decreased production of interferon (IFN)-γ or cytotoxicity, render mice more susceptible to MCMV infection [23]. NK cells can kill virus-infected cells by using cytotoxic granules or by recognizing and inducing lysis of antibody-coated target cells (antibody-dependent cell cytotoxicity) via an Fc-binding receptor such as CD16 [21].

Early activity of NK cells may be important for clearing primary DENV infection [24]. In a DENV mouse model, mice experimentally infected with DENV showed increased NK cell levels [24]. A significant increase in the frequency of NK cell circulation was also shown in patients who developed an acute dengue disease [2]. In addition, patients with a mild dengue disease have elevated NK cell rates when compared to those with severe dengue diseases [9, 27]. Moreover, Kurane *et al.* [14] reported that human blood NK cells are cytotoxic against DENV-infected cells in target organs via direct cytolysis and antibody-dependent cell-mediated cytotoxicity. It was also shown that the intracellular cytotoxic granule, TIA-1, was up-regulated early in NK cells in the acute phase of DENV infection and that NK-activating receptor NKp44 was involved in virus-mediated NK activation through direct interaction with DENV envelope protein [2, 13]. These results suggest that the early activation of NK cells contributes to the prevention of the severe dengue disease. However, based on quantitative and functional analyses in animal models *in vivo*, defining the contribution of NK cells to suppression of DENV replication *in vivo* has been necessary.

We have recently reported that common marmosets (*Callithrix jacchus*) are highly permissive to DENV infection [22]. These New World monkeys, being nonhuman primates, are considered to have an immune system similar to that of humans [28, 29]. The present study was initiated to investigate the role of NK cells in controlling DENV during primary infection in our nonhuman primate model.

The animals were cared for in accordance with National Institute of Biomedical Innovation rules and guidelines for experimental animal welfare, and all protocols were approved by our Institutional Animal Study Committee. Eight tamarins (*Saguinus midas* and *Saguinus labiatus*) were used in this study. As marmosets and tamarins are closely related monkey species and are classified as members of the Callitrichinae, we expected that tamarins would also be permissive to DENV infection, like marmosets. To check the permissiveness of tamarins to DENV, 2 tamarins were infected with DENV-2 (DHF0663 strain: $6.7x10^7$ PFU/ml) subcutaneously or intravenously (Fig. 1).

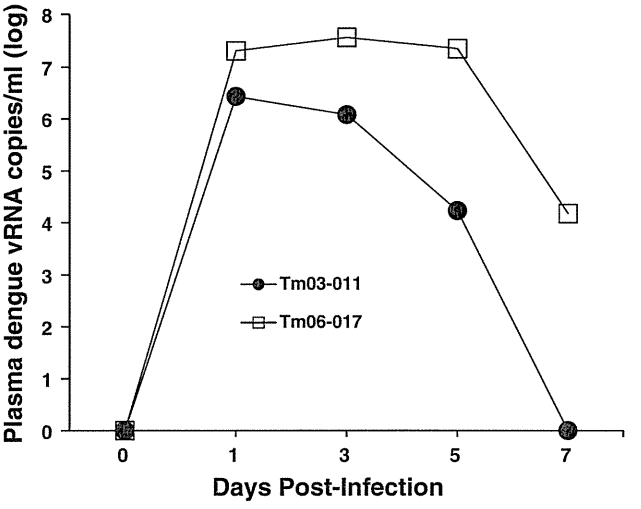

Fig. 1 Levels of vRNA in DENV-infected tamarins. Tamarins were infected subcutaneously or intravenously with DENV at a dose of $6.7x10^7$ PFU/ml. The vRNAs were detected in plasma by real-time PCR. Tm03-011, subcutaneous infection; Tm06-017, intravenous infection

Dengue viral RNA (vRNA), which was quantified using real-time PCR as previously described [22], was detected in plasma samples from the tamarins on day 1 post-infection. For each of the two tamarins (Tm03-011, Tm06-017), the plasma vRNA levels reached $2.7x10^6$ copies/ml and $2.0x10^7$ copies/ml on day 1 post-infection, respectively, and were detectable on days 3 and 5. These results indicate that tamarins are also permissive to DENV infection, which is consistent with the results obtained by using marmosets [22].

Next, we sought to assess the role of NK cells in DENV infection *in vivo*. In this regard, in vivo depletion of NK cells by the administration of NK-specific monoclonal antibody (mAb) was considered to be straightforward to directly address the question. We employed a new method by which an anti-CD16 mAb 3G8 [7] but not a control mAb MOPC-21 efficiently depleted a major NK population expressing CD16 in tamarins, as we recently reported [29]. The mouse anti-human CD16 mAb 3G8 was produced in serum-free medium and purified using protein A affinity chromatography. Endotoxin levels were confirmed to be lower than 1 EU/mg. Four red-handed tamarins and two white-lipped tamarins (*Saguinus labiatus*) were used in this experiment. Three tamarins were intravenously administered 3G8 at a dose of 50 mg/kg, while others were given a control mAb MOPC-21. One day later, both mAb-treated tamarins were subcutaneously inoculated with $3x10^5$ PFU/ml of DENV-2 DHF0663 strain on the basis of a previous report that a single mosquito might inject between 10^4 and 10^5 PFU of DENV into a human [20]. It was confirmed that at 1-3 days after the 3G8 mAb treatment, $CD16^+$ cells were almost completely depleted in the

tamarins followed by recovery to the initial levels at around 2 weeks after administration, while the cells were maintained at the initial levels in the monkeys with MOPC-21 (Fig. 2a). In addition, it is noteworthy that the ratios of CD4$^+$ and CD8$^+$ T cells and CD20$^+$ B cells were not affected by the administration of the 3G8 mAb (Supplementary Figure 1). In the case of administration of mAb MOPC-21, we confirmed no significant effect on CD16$^+$ cells (Supplementary Figure 2). The killing activities of the peripheral blood mononuclear cells (PBMCs) taken from the 3G8-treated monkeys were reduced at day 1 post-antibody-treatment, followed by an increase irrespective of depletion of CD16$^+$ NK cells at day 2 post-antibody-treatment (1 day after DENV inoculation), suggesting that the CD16$^-$ NK population may be activated by DENV infection (Fig. 2b). Plasma viral loads in both mAb-treated monkeys rose to 10^5 copies/ml by day 1 after infection and then reached a peak at 10^6 copies/ml on day 3 or day 7, followed by a rapid decline, with values dipping below the detectable level by day 14 after infection (Fig. 2c). These results suggested that CD16$^+$ NK cells apparently did not contribute to DENV replication in the acute phase in our tamarin model.

It was reported previously that non-structural glycoprotein NS1 is essential for flavivirus viability and that the NS1 protein circulates during the acute phase of disease in the plasma of patients infected with DENV [1]. Epidemiological studies have demonstrated that secreted NS1 levels are correlated with viremia levels and are higher in cases of DHF than in dengue fever (DF) early in illness [16]. Thus, it has been suggested that NS1 might be a useful marker as an indicator of the severity of dengue disease. We have used the level of the NS1 antigen as an alternative diagnostic marker to examine the effects of CD16 antibody treatment on DENV replication. The NS1 was measured by Platelia Dengue NS1 Ag assay (BioRad). Antigenemia was observed in these infected monkeys between 3-14 days post-infection. Serum IgM and IgG specific for DENV antigens were measured by ELISA. DENV-specific IgM or IgG antibody was equally detected in both mAb-treated monkeys (Fig. 3).

We recently demonstrated that marmosets are permissive to DENV infection [22]. In this study, we found that tamarins are also permissive to DENV infection (Fig. 1). Moreover, we also investigated the role of NK cells against early DENV infection using *in vivo* depletion of CD16$^+$

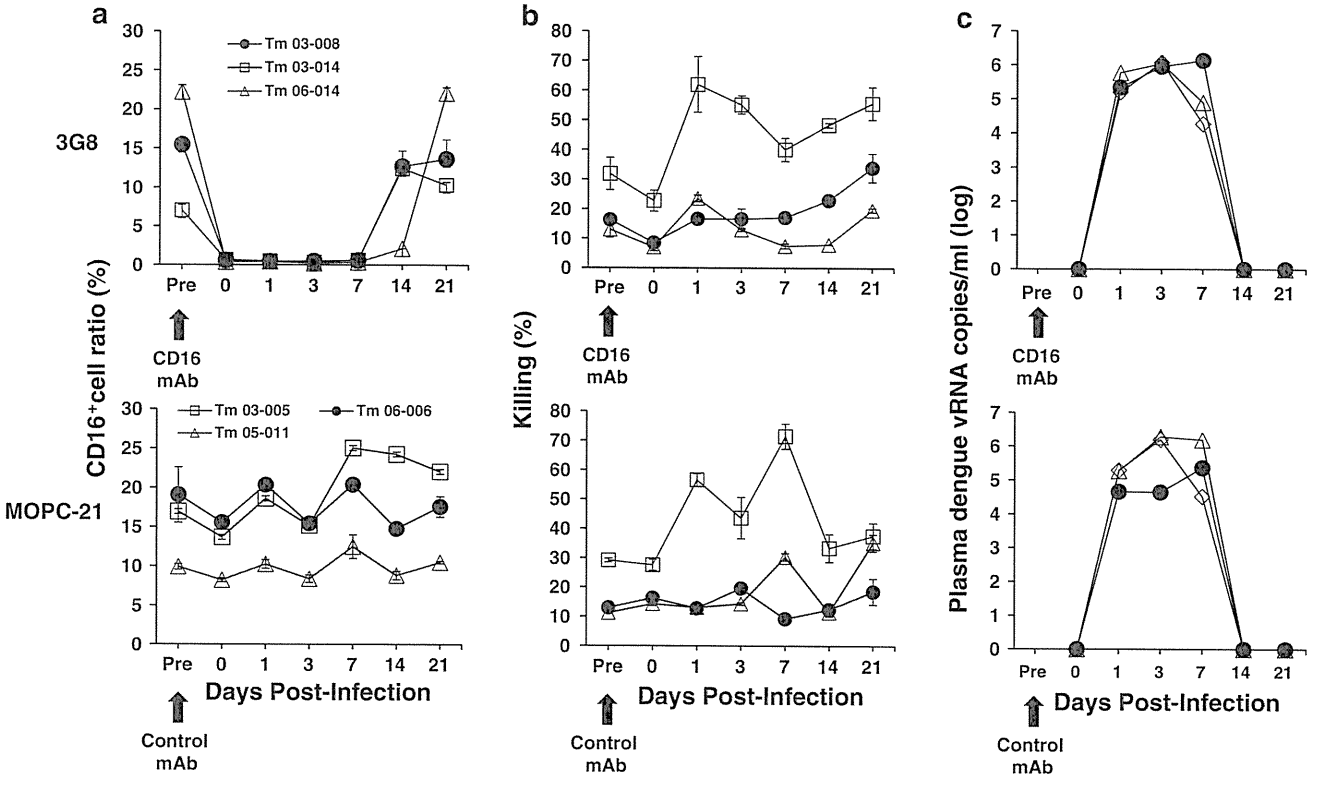

Fig. 2 Ratios of CD16$^+$ NK cells, killing activity of PBMCs, and vRNA in DENV-infected tamarins after treatment with 3G8 or MOPC-21 mAb. Tamarins were infected subcutaneously with DENV at a dose 3x10^5 PFU/ml after treatment with 50 mg/kg of 3G8 or MOPC-21 mAb. **a** Ratios of CD16$^+$ NK cells were determined in whole-blood specimens. **b** The activities of NK cells were determined in PBMCs of tamarins by NK cytotoxic assay. **c** The vRNAs were detected in plasma by real-time PCR

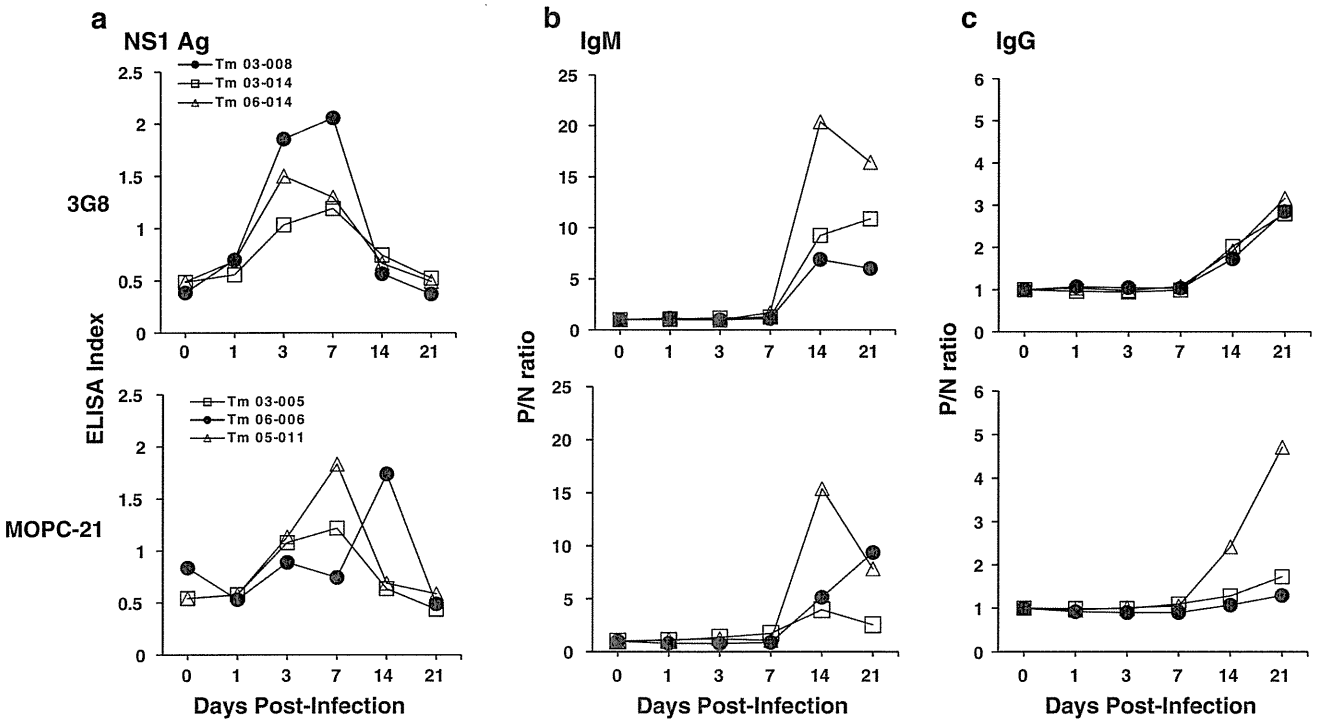

Fig. 3 Levels of NS1 antigen and DENV-specific IgM and IgG in plasma samples from DENV-infected tamarins after treatment with 3G8 or MOPC-21 mAb. The levels of NS1 antigen and DENV-specific IgM and IgG in plasma were measured by ELISA. **a** ELISA index of NS1 antigen, **b** positive/negative (P/N) ratio of DENV-specific IgM, **c** P/N ratio of DENV-specific IgG in plasma samples from DENV-infected tamarins after administration of the 3G8 or MOPC-21 mAb. The P/N ratio was calculated as the optical density of the test sample divided by that of a negative sample. P/N ratios <2 and ≥ 2 were considered to be negative and positive, respectively. Top, 3G8; bottom, MOPC-21 mAb

NK cells in tamarins and found that the depletion of $CD16^+$ NK cells had almost no effect on DENV replication (Fig. 2), indicating that this NK subpopulation is unlikely to contribute to controlling DENV replication. Interestingly, these results imply that the $CD16^-$ NK subpopulation may have a critical role of controlling DENV infection *in vivo*.

Using our model, we investigated the role of NK cells *in vivo* against DENV infection, which remains to be elucidated in several aspects. We previously reported that almost complete *in vivo* depletion of the $CD16^+$ NK subpopulation was not able to completely remove the NK-mediated cytotoxic activity in tamarins [29]. In this study, despite a transient but substantial reduction in the $CD16^+$ NK cell number following 3G8 treatment in tamarins, DENV replication was comparable to that in monkeys that received the control mAb. The NK-mediated cytotoxic activity was augmented in both study groups, indicating that $CD16^-$ NK cells were responsible for the cytotoxic activity and suggesting that they might play a role in controlling DENV replication.

The next question is how $CD16^-$ NK cells may regulate DENV infection. One possibility regarding $CD16^-$ NK cells is that $CD56^+$ or $CD57^+$ NK cells are involved in controlling DENV infection. Human NK cells are classically divided into two functional subsets based on their cell-surface density of CD56 and CD16, i.e., $CD56^{bright}CD16^-$ immunoregulatory cells and $CD56^{dim}CD16^+$ cytotoxic cells. Both subsets have been characterized extensively regarding their different functions, phenotypes, and tissue localization [8]. The NK cell number is maintained by a continuous differentiation process associated with the expression of CD57 that results in NK cells with poor responsiveness to cytokine stimulation but high cytolytic capacity [3, 18]. The second possibility is that $CD16^-$ NK cells have a non-cytolytic helper function. Generally, it is well known that NK cells possess both a cytolytic and a non-cytolytic helper function. It has been suggested that cytokine production is carried out by $CD56^{bright}CD16^-$ NK cells [4–6]. Interferon (IFN)-γ secreted by NK cells has shown potent antiviral effects against DENV infection in early phases [25]. One aspect of the NK helper function arises from recent evidence indicating that NK cells can be induced to function as non-cytotoxic helper cells following stimulation with interleukin-18 [19]. This cytokine induces IFN-γ secretion from NK cells and thus enables dendritic cells (DCs) to secrete IL-12, leading to Th1 polarization [19]. It is possible that $CD16^-$ NK cells, which have poor

cytotoxic activity but an enhanced ability to secrete cytokines and then lead to a Th1 response, are preserved during 3G8 administration. The persistence of this minor CD16$^-$ NK cell subpopulation could exert an antiviral effect through INF-γ-mediated pathways despite the depletion of CD16$^+$ NK cells. The third possibility is that CD16$^+$ NK cells of tamarins play pivotal roles against bacterial infections and cancer progression but not DENV-infected cells. We will address these possibilities for the roles of the NK subpopulation in the future studies.

In conclusion, this study provides a DENV *in vivo* replication model in tamarins and new information on the possible role of CD16$^+$ NK cells in DENV replication *in vivo*. It remains elusive whether the CD16$^+$ and CD16$^-$ NK subpopulations could play an important role in the control of primary DENV infection.

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Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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