(Tanaka Co., Ltd, Tokyo, Japan) and the tomato var. *Improved Pope* as previously published (Bhatia and Ashwath 2004; Sheeja et al. 2004). Briefly, the sterilized seeds were grown in MS medium and allowed to grow for 7–10 days. Callus induction of the explant material was performed in a MS medium containing 1.0 ppm zeatin for 7 days. Bombardment was carried out in tomato calli grown in MS medium containing 0.5 ppm zeatin, 1.0 ppm indole-butyric acid and 1.0 ppm giberillic acid. All reagents used for tomato transformation were purchased from Sigma.

Reverse transcription-PCR

Triplicates of bombarded tomato calli, regenerated leaf and shoot tissues were freshly obtained for mRNA extraction. The MicroFastTrack™ 2.0 mRNA Isolation Kit (Invitrogen) was used to isolate mRNA according to manufacturer's instructions. The Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) was used to synthesize cDNA according to manufacturer's instructions. The forward primer, TAT-F (5′-ATG GAA CCA GTT GAT CC-3′), used was based on the tomato codon-based HIV-1 *tat*, whereas, the reverse primer, GUS-R (5′-CGG TAT AAA GAC TTC GCG CTG-3′) was based on *gusA*. Both primers were synthesized by Invitrogen. The TOUCHDOWN PCR condition was performed using the TaKaRa *Taq*™ Hot Start Version (Takara Bio Inc., Japan) with an initial denaturation temperature of 95°C for 5 min proceeded by 5 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. This was followed by another 5 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 2 min. The last set of cycles consists of 25 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. A final 10 min extension at 72°C was also performed. Reverse transcription-PCR (RT-PCR) products were resolved on 1% agarose gel.

Protein extraction

Protein extracts were obtained from 2-weeks old transgenic tomato plants. Protein extraction was done on all transgenic samples using the P-PER Plant Protein Extraction Kit (Thermo Scientific) according to manufacturer's recommendation. Tomato protein extracts acquired (~100 µl) were divided for use in Western blot assay and Immunogenicity testing.

Western blot assay

Western blot using both antibodies against Tat and M2 (Sigma) was performed and amount of Tat expressed in bombarded tomato plants were estimated using the Bio-Dot Mircofiltration Apparatus (BIO-RAD) as previously published (Ota et al. 2005). The Tat protein standard used was a recombinant Tat (ImmunoDiagnostics, Inc.) with various dilutions.

Immunogenicity testing

Balb/c mice were intradermally immunized with the recombinant tomato protein extracts mixed in an incomplete Freund's adjuvant (IFA). The peptides used in this study were the Tat CTL epitope (Morris et al. 2001) and B cell epitope (Goldstein et al. 2001). Tat-specific antibody responses were measured by ELISA. Briefly, synthetic peptides for Tat and mutant Tat (mTat) diluted in PBS were coated in multiwell plates overnight at 4°C followed by 30 min of blocking with non-fat milk. Test samples were then added and incubated at room temperature for 1 h. After washing, the reacted antibodies were detected using the HRPO-labeled goat anti-mouse IgG (H + L) and ABTS substrate (Roche Diagnostics). The OD_{405} was recorded and used as a relative measure of antibody titer.

The number of Tat-specific IFN-γ secreting cells indicating specific CTL activity was determined by ELISPOT assay (Takamura et al. 2005). Briefly, a 96-well nitrocellulose plate (Millipore Corporation) was coated with anti-mouse IFN-γ mAb R4-6A2 (Phermingen) and incubated at 4°C overnight. After washing with PBS, complete medium with 10% fetal calf serum was added and incubated at 37°C for 1 h. Triplicate samples of $CD8^+$ T cells separated from the spleen of the immunized mice were plated in two-fold dilutions from 5×10^5 to 6.25×10^4 cells/well [29], added with Tat CTL peptide and incubated for 24 h at 37°C in 5% CO_2. After washing with PBS-T, biotinylated anti-mouse IFN-γ mAb XMG1.2 (Phermingen) was added and incubated overnight at 4°C. Plates washed with PBS-T were added with streptavidin-conjugated alkaline phosphatase (AP) (Mabtech AB) and visualized using AP color development buffer

(BIO-RAD) and counted by KS ELISPOT (Carl Zeiss, Inc.).

Results and discussion

Preferential expression of mTat/Tat-GUS fusion protein in tomato plant

Comparison between the control and transgenic tomato lines (Fig. 2a) showed that the transgenic lines were stunted in growth compared to the control tomato lines consistent with Tat expression (Ramirez et al. 2007; Karasev et al. 2005). Among 82 total calli bombarded (with either Tat or mTat), 55 (67.1%) survived with 14 (25.5%) confirmed plant regeneration. To confirm transformation, tomato extracts were used for Western blot analysis using anti-Tat and anti-M2 antibodies. A ~40 kDa protein, representing the mTat/Tat-GUS fusion protein (mTat/Tat is 14 kDa in size and GUS is 26 kDa in size), was detected instead of the expected 14 kDa size representing an mTat/Tat-only protein (Fig. 2b, c).

The indirect fusion between *mtat/tat* and *gusA* genes was designed to allow the tomato plant to selectively express either a fusion protein, a single protein or both. As seen in Fig. 2b, Tat and mTat were successfully expressed in all tomato extracts but only as a fusion protein and regardless of the stop codon found downstream of the *tat* gene which would have allowed a Tat-only protein to be expressed. Figure 2c illustrates the expected (Tat-only) and actual (Tat-fusion) proteins detected in the transgenic tomato lines. Expression of a fusion protein is suggestive of a codon read-though event (Tork et al. 2004) implying preferential expression of the fusion protein over the Tat-only protein in tomato plants. To resolve the transgenic nature of the Tat- and mTat-bombarded tomato calli, RT-PCR was performed. Figure 2d confirms the active transcription and presence of *mtat/tat* mRNA in all three sets of the transgenic tomato callus, shoot and leaf. In addition, since we were able to amplify a PCR product using a forward

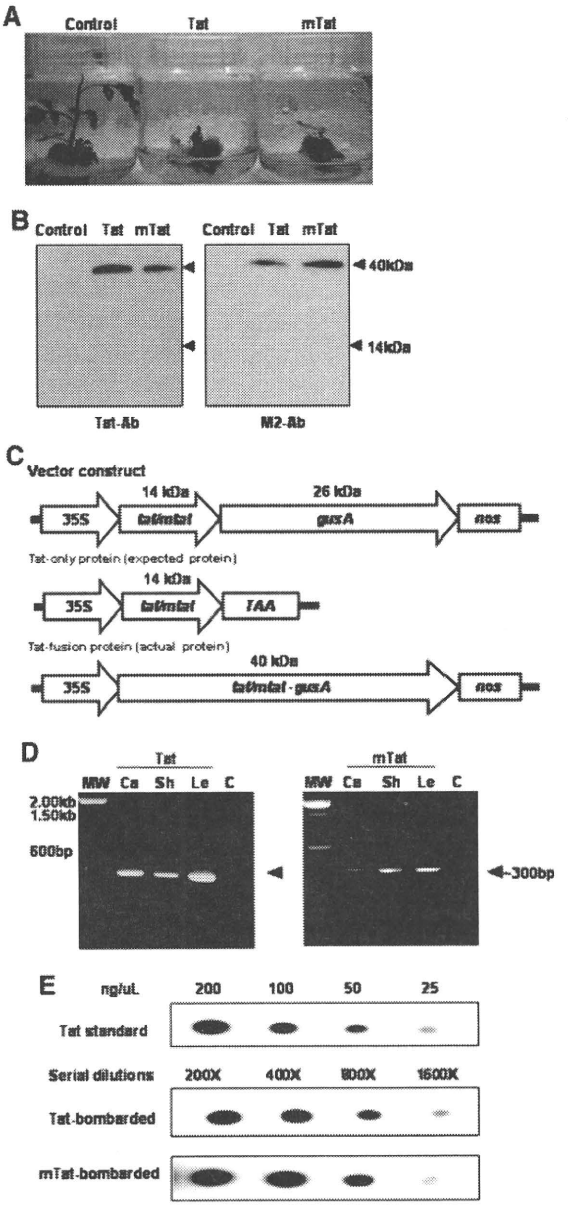

Fig. 2 Expression of both Tat and mTat proteins in tomato plant using particle-gun bombardment. **a** All tomato calli bombarded followed the same bombardment conditions and grown for 3 weeks. Control samples were bombarded with pBI121 vector only. Tat and mTat samples were bombarded with Tat- and mTat-pBI121 vectors, respectively. **b** Western blot assay using tomato extracts from the bombarded samples and detected with anti-Tat and anti-M2 antibodies as indicated in the *bottom*. Only a 40 kDa protein was clearly detected from either Tat- and mTat-pBI121 bombarded tomatoes representing a mTat/Tat-GUS fusion proteins. No 14 kDa protein was detected. **c** Schematic illustration of expected (Tat-only) and actual (Tat-fusion) protein transiently expressed. **d** Reverse transcription-PCR was performed using cDNA obtained from both Tat- and mTat-bombarded callus (*Ca*), shoot (*Sh*) and leaf (*Le*) tissues. Likewise, cDNA obtained from pBI121-bombarded tomato were used as controls. **e** Dot-blot assay providing estimated amounts of Tat expressed in both Tat- and mTat-bombarded tomato plants

primer based on the *tat* gene and a reverse primer based on the *gusA* gene, we show that a *tat-gusA* mRNA is transcribed further confirming production of Tat-GUS fusion protein. Furthermore, tomato extracts were found to contain ~2–4 μg mTat/Tat-GUS fusion protein per milligram plant protein (Fig. 2e) much higher than previous attempts (Ramirez et al. 2007; Karasev et al. 2005). This would imply that in tomato, Tat-GUS fusion protein is the protein form preferentially expressed allowing for a higher amount of protein production. Though the reason that drives the tomato plant to preferentially express the fusion protein is unclear, the significance of both the fusion protein and the amount produced in tomato plant was tested for its immunogenicity by injecting the tomato extracts into Balb/c mice.

Induction of antibody and CTL in Balb/c mice using recombinant tomato extracts

To test the immunogenicity of the fusion protein, Balb/c mice were intradermally injected with the recombinant tomato protein extracts and checked for immunogenic responses. Considering, previous attempts using a Tat-only protein have, thus far, successfully induced a humoral immune response (Ramirez et al. 2007; Karasev et al. 2005), we first established the consistency of humoral immune induction using Tat, in fusion form, in Balb/c mice. Humoral IgG immune responses were detected in the range of 1:10–1:160 titers before leveling-off at 1:320 for both mTat- and Tat-bombarded extracts (Fig. 3). The tomato extracts were found to induce a humoral immune response, regardless of the nominal amount used, and showed that Tat in fusion form could still induce an antibody response consistently with previous works using Tat-only protein (Ramirez et al. 2007; Karasev et al. 2005).

Interestingly, a cellular immune response, though minimal, was also detected. Cellular immune responses as detected by IFN-γ production were modestly induced at 22–24 cells/1 × 10^6 splenocytes using the recombinant tomato protein extracts (Fig. 4). It is noteworthy that Balb/c mice are normally used to test Th2 immune responses which are known to inhibit macrophage activation and instead stimulate antibody production (Mills et al. 2000), explaining the relatively low IFN-γ produced using our tomato extracts. Nevertheless, of greater

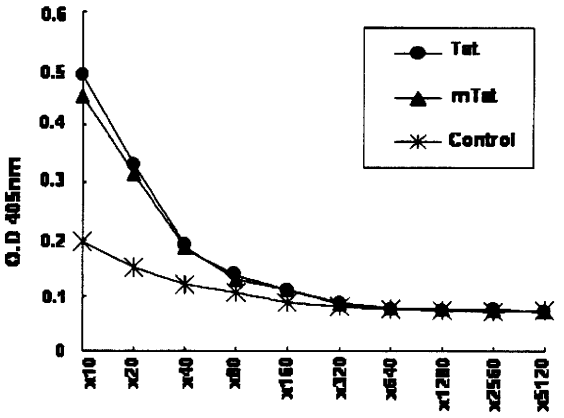

Fig. 3 Induction of IgG humoral immune response in Balb/c mice by recombinant tomato extracts. Plant extracts containing 5 μg Tat were inoculated in five mice intradermally. After 2 weeks, these mice were bled and the anti-Tat IgG antibody titer was measured by ELISA. The figures represent the mean and standard deviation of anti-Tat antibody response but the standard deviations could not be seen because of very small differences. Normal tomato extracts were used as control

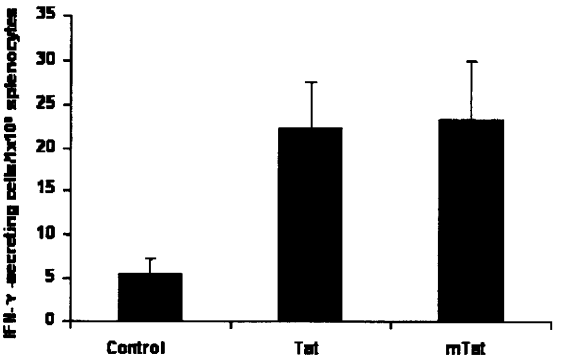

Fig. 4 Induction of cellular immune response in Balb/c mice by recombinant tomato extracts. IFN-γ secreting cells were determined by ELISPOT assay. $CD8^+$ cells were prepared from the spleen of each mouse and approximately 5–6.25 × 10^4 cells per well were incubated with the synthetic Tat peptide (Tat17-25) for 24 h, plates were washed by PBS-T, further incubated overnight at 4°C in the presence of 2 μg/ml of biotinylated anti-mouse IFN-γ monoclonal antibody, and the number of IFN-γ secreting $CD8^+$ T cells were visualized by adding streptavidin conjugated alkaline phosphatases. Data represent the mean and standard deviation of three independent experiments

importance is the ability of Tat protein, in fusion form, to induce a CTL response. The vaccine potential of utilizing HIV-1 Tat relies heavily in its ability to induce both humoral and cellular immunity in the host. Given that both IFN-γ and IgG were detected from the same induced Balb/c mice, it was

clearly demonstrated that a Tat-GUS fusion protein was preferentially expressed over the Tat-only protein in tomato plants. Furthermore, Tat in fusion form would seem to be ideal in inducing both humoral and cellular immune responses which coincidently are the requirements of a model HIV-1 vaccine (Walker and Burton 2008; Gaschen et al. 2002; Borrow et al. 1994). To our knowledge, this is the first report of induction of anti-Tat cellular immunity in Balb/c mice, using Tat protein in fusion form expressed in tomato plant.

No significant difference between wild-type and mutant Tat expressed in tomato plant

It is worth mentioning that tomato plants bombarded with either *mtat* or *tat* gene are both stunted in growth (Fig. 2a) and found to have no significant difference (Fig. 2b, c) implying that the mutations found in mTat are insufficient to distinguish it from Tat when expressed in tomato. With regards to immunogenicity, although previous findings (Kanazawa et al. 2000; Okamoto et al. 2000; Lilen et al. 2002) suggested that wild-type Tat might inhibit immune responses by downregulating the function of antigen presenting cells, the extent of immune responses elicited by either Tat or mTat did not apparently show any difference in mice. This is perhaps because murine Cyclin T1 does not bind to HIV-1 Tat (Bieniasz et al. 1998). Thus, a similar study using primates is warranted since, in human and primate cells, we expect a lower immunogenicity for Tat compared to mTat because of the recruitment of Cyclin T1 that is required for the action of class II transactivator expressing class II MHC molecules (Kanazawa et al. 2000; Okamoto et al. 2000; Lilen et al. 2002).

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Simian Betaretrovirus Infection in a Colony of Cynomolgus Monkeys (*Macaca fascicularis*)

Koji Fujiomto,[1,2,*] Jun-ichiro Takano,[1,2] Toyoko Narita,[1] Koji Hanari,[1] Nobuhiro Shimozawa,[2] Tadashi Sankai,[2] Takashi Yosida,[2] Keiji Terao,[2] Takeshi Kurata,[1] and Yasuhiro Yasutomi[2]

Of the 419 laboratory-bred cynomolgus macaques (*Macaca fascicularis*) in a breeding colony at our institution, 397 (95%) exhibited antibodies or viral RNA (or both) specific for simian betaretrovirus (SRV) in plasma. Pregnant monkeys (n = 95) and their offspring were tested to evaluate maternal–infant infection with SRV. At parturition, the first group of pregnant monkeys (n = 76) was antibody-positive but RNA-negative, the second group (n = 14 monkeys) was positive for both antibody and RNA, and the last group (n = 5) was antibody-negative but RNA-positive. None of the offspring delivered from the 76 antibody-positive/RNA-negative mothers exhibited viremia at birth. Eight of the offspring (including two newborns delivered by caesarian section) from the 14 dually positive mothers exhibited SRV viremia, whereas the remaining 6 newborns from this group were not viremic. All of the offspring (including 2 newborns delivered by caesarian section) of the 5 antibody-negative/RNA-positive mothers exhibited viremia at birth. One neonatal monkey delivered by CS and two naturally delivered monkeys that were viremic at birth remained viremic at 1 to 6 mo of age and lacked SRV antibodies at weaning. Family analysis of 2 viremic mothers revealed that all 7 of their offspring exhibited SRV viremia, 6 of which were also antibody-negative. The present study demonstrates the occurrence of transplacental infection of SRV in viremic dams and infection of SRV in utero to induce immune tolerance in infant monkeys.

Abbreviation: SRV, simian betaretrovirus.

Although simian betaretrovirus (SRV) causes symptoms of immunodeficiency, including anemia, tumors, and persistent refractory diarrhea, in some infected macaques,[1,7,10] most infected monkeys exhibit few or no clinical signs.[2] Macaques free of SRV are important in many types of experiments to avoid associated immunologic and virologic effects. Establishing an SRV-free breeding colony is paramount for a steady supply of appropriate monkeys for various experiments.[8]

We previously reported that SRV-T, a novel subtype of SRV, was found in the cynomolgus colony of our institution.[3] Approximately 20% of the colony monkeys tested in 2005 were viremic and shed SRV-T virus in saliva, urine, and feces.[4,5] The viruses shed by these monkeys are a potential source of horizontal SRV-T infection, as occurred in a rhesus monkey colony.[6,7] In the present study, we investigated the actual prevalence and transmission of SRV in the closed cynomolgus colony through several generations, to prevent the spread of the virus and to establish an SRV-free colony.

Materials and Methods

Animals. The Tsukuba Primate Research Center (Tsukuba, Japan) maintains approximately 1500 cynomolgus monkeys as a breeding and rearing colony and has been maintained as a closed colony for 30 y. All adult monkeys are kept in single cages. Pregnant monkeys are produced by timed mating system in which a female monkey is placed into a male monkey's cage for 3 d; pregnancy is confirmed by ultrasonography 5 wk after mating.

Dams nurse their offspring until weaning at approximately 6 mo. Weaned infants are paired with infants of similar size. Artificial nursing is performed when the dams do not exhibit appropriate nursing behavior.

The housing and care procedures of this study were approved by the Animal Welfare and Animal Care Committee of Tsukuba Primate Research Center of the National Institute of Biomedical Innovation.

Samples. Blood samples were collected from 419 breeders (female, 364; male, 55). All of these monkeys were born at Tsukuba Primate Research Center and are the second and third generations from the founder monkeys, which originated from the Philippines, Malaysia and Indonesia.

We selected 95 pregnant monkeys that exhibited SRV-specific antibodies by Western blotting or the virus as detected by RT-PCR (or both) as the subjects of the study. Blood samples from the mothers and the newborn infant monkeys were collected within 12 h after parturition.

Western blotting. SRV-specific Abs were assessed by Western blotting using SRV-T.[5] Purified virus for this analysis was obtained from the culture supernatant of cloned SRV-T–infected A549 cells by ultracentrifugation through a sucrose gradient; purified viruses were disrupted by 1% SDS for use as antigen in Western blotting. The criterion for a positive reaction was detection of 2 or more virion-specific bands (that is, Gag and Env proteins).

RT-PCR. RNA was extracted from serum of the monkeys (QIAamp Viral RNA Mini Kit, Qiagen, Tokyo, Japan, or MagNA Pure Compact Nucleic Acid Isolation Kit I, Roche, Mannheim,

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[1]The Corporation for Production and Research of Laboratory Primates and [2]Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation, Tsukuba, Japan.
[*]Corresponding author. Email: fujimoto@primate.or.jp

Germany). Reverse transcription was performed (ThermoScript RT-PCR System, Invitrogen, Tokyo, Japan) by using gene-specific reverse primers. PCR analysis was performed (Premix Ex*Taq* Hot-Start Version, Takara, Shiga, Japan) by using published sets of external primers (SRVenv1E and SRVenv2E) and nested primers (SRVenv3N and SRVenv4N).[9]

Results

SRV infection status of the 419 laboratory-bred breeders. Of the 419 (female, 364; male, 55) cynomolgus macaques evaluated, 22 were negative for both SRV-specific antibodies and RNA. Of the remaining 397 breeders, 340 were positive for SRV-specific antibodies but were not viremic, 29 were positive for both viral RNA and antibodies, and the remaining 28 monkeys had viremia without antibodies.

SRV infection status of 95 pairs of mothers and offspring at birth. RT-PCR and Western blotting of samples from 95 pairs of mothers and offspring at the time of birth revealed that the dams could be grouped into 1 of 3 categories based on the presence of SRV-specific antibodies and viremia.[2] Among the 95 dams, 76 developed SRV-specific Abs without viremia, 14 had both antibodies and viremia, and the remaining 5 were viremic without SRV-specific antibodies.

None of the offspring of the 76 dams that were antibody-positive but RNA-negative were viremic at birth. Eight infants (including 2 delivered by caesarian section) of the 14 dually positive dams were viremic at birth; the remaining 6 infants of dams in this group were viral RNA-negative. All 5 progeny (including 2 infants delivered by caesarian section) of viremic but antibody-negative dams were viremic at birth.

Plasma SRV-specific antibodies and RNA in viremic newborns during the first 6 mo. We then tested the SRV-specific antibody and RNA status of 3 representative viremic newborns at 1, 2, and 6 mo after birth (Table 1). All 3 of the dams exhibited SRV viremia at delivery, and 2 of them also were positive for SRV-specific antibodies. All 3 infants exhibited SRV-specific RNA at all time points, but none was antibody-positive at weaning.

Family analysis of two representative SRV-viremic dams. The SRV status of all 7 offspring born to 2 representative viremic mothers was verified in 2007. Dam 1319711082 and her 4 offspring (infant 1410311011, born 2003; infant 1420506016, born 2005; infant 1420608031, born 2006; and infant 1420709050, born 2007) all demonstrated SRV RNA in tests performed during 2007. In addition, this dam and her oldest infant (1410311011) were antibody-positive, unlike the 3 youngest siblings. Dam 1319710076 and her 3 offspring (infant 1410408017, born 2004; infant 1410508022, born 2005; and infant 1420701001, born 2007) were all RNA-positive but antibody-negative according to tests performed in 2007.

Discussion

In 2005, we reported that about 20% of the cynomolgus monkeys in the colony at our institution exhibited SRV-T viremia and that virus was present in saliva, urine, and feces from the viremic monkeys.[3-5] Because the virus secreted from these monkeys was a potential source of horizontal SRV-T infection, we performed the current large-scale survey of SRV infections in our laboratory-bred monkeys and assessed the transmission of SRV through the generations represented in the colony.

The present study validated our concerns about vertical and horizontal SRV infections in the colony, because more than 90% of the laboratory-born breeders were positive for SRV-specific antibodies or virus (or both). The rate of viremia in the present study (14%) was smaller than that (20%) in the earlier survey,[5] which involved 49 retired breeders. The rate of viremia in a colony may vary depending on the age distribution of animals and their countries of origin. In particular, we hypothesize that the 28 monkeys that exhibited SRV viremia without specific antibodies are immunotolerant to SRV because of being infected in utero, as is reported to occur in rhesus and pigtailed macaques.[7,12]

To evaluate transplacental maternal–infant transmission of SRV, we tested 95 pairs of mothers and newborns, including 4 infants delivered by caesarean section, by using SRV-specific RT-PCR. The results showed that all monkeys exhibiting SRV-specific antibodies without viremia produced newborns without viremia. However, the transplacental SRV infections observed in infants included 4 newborns delivered by cesarean section from viremic mothers. In pigtailed monkeys, SRV2 was detected in the tissues and amniotic fluid of fetuses and in the blood of newborns delivered from viremic mothers.[12] In other cynomolgus monkeys, SRV was transmitted through transfusion of blood from a viremic donor but not from a nonviremic donor.[13] These findings indicate that SRV viremia of the mother is essential to establishing transplacental infection of the fetus. However, the production of 6 SRV-negative newborns from 14 viremic dams with SRV-specific antibodies may indicate that these antibodies reduced the viral loads in the viremic mothers sufficiently to prevent transplacental infection with SRV. Further investigation to quantify SRV in blood and the occurrence of transplacental infections will resolve this question.

An important issue is whether SRV viremic newborns can convert to a nonviremic state after developing virus-specific antibodies. Three infants born from viremic mothers exhibited viremia, which was maintained at 1, 2, and 6 mo of age, with no antibodies at 6 mo of age. In addition, 7 offspring born from the representative 2 SRV-viremic mothers were all viremic, at ages of 6 mo to 4 y. Pigtailed monkey newborns infected transplacentally with SRV2 maintained a viremic state for 1 y without producing antibodies and harbored proviral DNA in many tissues.[11,12] A newborn rhesus monkey produced from a viremic mother was SRV1-positive within 24 h after birth and was antibody-negative for as long as 6 mo after birth.[7] These findings suggest that cynomolgus infants infected in utero with SRV and born from viremic mothers are immunologically tolerant to the virus and that they then become the source of SRV infection in the colony.

The cynomolgus monkey breeding colony at our institution has been maintained as SPF with regard to B virus, SVV, SIV, STLV1, and measles virus but not SRV. The cage system used during the first 25 y was a two-story type—monkeys were able to touch feces and urine of animals in adjacent cages. In addition, cages were washed with high-pressure water, perhaps helping to spread virus-contaminated waste and increasing the likelihood of horizontal infections. After redesigning the cage system to a single-story type that prevents monkeys from touching fecal and urine waste from another macaque, we anticipate that we will be able to establish an SRV-free colony by introducing SRV nonviremic monkeys into the breeding colony. Furthermore, elimination of viremic dams, which can become a source of transplacental infection, from the breeding colony is critical to establishing an

Table 1. SRV-specific antibodies and RNA in the plasma of viremic newborns during their first 6 mo

Infant ID	Method of delivery	Dam ID	Method of nursing	Status of dam at parturition		Status of infant at				
						0 d	1 mo	2 mo	Weaning (approximately 6 mo)	
				Antibodies	RNA	RNA	RNA	RNA	Antibodies	RNA
1310611144	Caesarean	1210003019	Artificial	+	+	+	+	+	−	+
1410508022	Natural	1319710076	Artificial	−	+	+	+	+	−	+
1420506016	Natural	1319711082	Maternal	+	+	+	+	+	−	+

Testing of infants for SRV-specific antibodies was delayed until weaning because transplacentally transferred maternal antibodies can persist at 2 mo of age.

SRV-free breeding colony. The establishment of an SRV-free cynomolgus breeding colony is paramount for supplying monkeys that are appropriate for many fields of investigation, including vaccine testing, gene therapeutics, organ transplantation, and infectious disease studies.

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Characterization of natural killer cells in tamarins: a technical basis for studies of innate immunity

Tomoyuki Yoshida[1,2†], Akatsuki Saito[2,3†], Yuki Iwasaki[1,4], Sayuki Iijima[1], Terue Kurosawa[1], Yuko Katakai[5], Yasuhiro Yasutomi[6], Keith A. Reimann[7], Toshiyuki Hayakawa[2] and Hirofumi Akari[1,2]*

[1] *Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, Japan*
[2] *Primate Research Institute, Kyoto University, Inuyama, Aichi, Japan*
[3] *International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan*
[4] *Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan*
[5] *Corporation for Production and Research of Laboratory Primates, Tsukuba, Ibaraki, Japan*
[6] *Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, Japan*
[7] *Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA*

Edited by:
Yasuko Yokota, National Institute of Infectious Diseases, Japan

Reviewed by:
Koji Ishii, National Institute of Infectious Diseases, Japan
Ikuo Shoji, Kobe University Graduate School of Medicine, Japan

***Correspondence:**
Hirofumi Akari, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan.
e-mail: akari@pri.kyoto-u.ac.jp

†*Tomoyuki Yoshida and Akatsuki Saito have contributed equally to this work.*

Natural killer (NK) cells are capable of regulating viral infection without major histocompatibility complex restriction. Hepatitis C is caused by chronic infection with hepatitis C virus (HCV), and impaired activity of NK cells may contribute to the control of the disease progression, although the involvement of NK cells *in vivo* remains to be proven. GB virus B (GBV-B), which is genetically most closely related to HCV, induces acute and chronic hepatitis upon experimental infection of tamarins. This non-human primate model seems likely to be useful for unveiling the roles of NK cells *in vivo*. Here we characterized the biological phenotypes of NK cells in tamarins and found that depletion of the CD16$^+$ subset *in vivo* by administration of a monoclonal antibody significantly reduced the number and activity of NK cells.

Keywords: CD16, cynomolgus monkey, tamarin, NK cell

INTRODUCTION

Natural killer (NK) cells are a component of the innate immune system that play a central role in host defense against viral infection and tumor cells. Much of the evidence for a role for NK cells in controlling viral infections has come from experiments with mice that were genetically modified (Lian and Kumar, 2002) or were treated with NK cell-depleting antibodies (Kasai et al., 1980) or from the study of humans with inherited NK cell deficiencies (Biron et al., 1989; Orange, 2002).

NK cells can be rapidly recruited into infected organs and tissue by chemoattractant factors produced by virus-infected cells and activated resident macrophages, which are also a major source of interferon (IFN), which induces NK cell proliferation, NK cell-mediated cytolysis of virus-infected cells, and the secretion of chemokines (Robertson, 2002). 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 antibody binding receptor CD16. For instance, human blood NK cells are cytotoxic against dengue virus-infected cells in target organs via direct cytolysis and antibody-dependent cell-mediated cytotoxicity (reviewed by Navarro-Sánches et al., 2005). Early activity of NK cells may be important for clearing acute infections such as that of dengue virus. However, the effect that NK cells may exert on chronic infections with viruses such as hepatitis C virus (HCV) is less clear.

HCV is the causative agent of chronic hepatitis C, cirrhosis, and finally liver cancer. In general, acquired and innate immunity induced by acute HCV infection is not sufficient for the viral clearance, and persistent HCV infection frequently leads to progression to chronic hepatitis (reviewed by Cheent and Khakoo, 2010). It was reported that dendritic cells (DCs) in HCV infection were not responsive to IFN-α, and thus failed to promote subsequent activation of NK cells as a primary innate immune response (reviewed by Kanto, 2008). This is in agreement with the finding that the killing activity of NK cells in patients with chronic hepatitis C is inactivated in *in vitro* studies (Deignan et al., 2002; Golden-Mason et al., 2008). These data suggest that the dysfunction of NK cells contributes to the persistent infection of HCV and chronic hepatitis. On the other hand, it was suggested that inappropriately activated NK cells caused liver injury after the viral infection (Liu et al., 2000). The population of NK cells is relatively minor in peripheral lymphoid organs but is abundant in liver, raising a question as to their function in the innate immune response to acute and chronic HCV infection in the liver. It is possible that NK cells partially regulate the replication of HCV in this organ during early infection whereas they promote the liver dysfunction in chronic HCV infection. To examine these possibilities, it is necessary to clarify the involvement of NK cells *in vivo* in HCV infection. However, it is questionable whether the results of *ex vivo* analyses of NK cells would reflect their actual roles *in vivo*. Therefore, it might be more informative to study the function of NK cells directly by means of *in vivo* depletion technique in animal models.

A chimpanzee model of HCV infection has frequently been employed to evaluate the role of acquired antiviral immune responses, although the involvement of NK cells has not been fully evaluated because of the limitations on the use of chimpanzees

due to ethical and financial restrictions (Cohen and Lester, 2007). Accordingly, New World monkeys infected with GB virus B (GBV-B) appear to be a promising model because (i) among viruses so far known, GBV-B is genetically the most closely related to HCV and can infect New World monkeys, including tamarins, marmosets and owl monkeys, but not Old World monkeys (reviewed by Akari et al., 2009), (ii) tamarins develop acute and chronic hepatitis after experimental GBV-B infection (Bukh et al., 1999; Sbardellati et al., 2001; Lanford et al., 2003; Martin et al., 2003; Ishii et al., 2007; Takikawa et al., 2010), (iii) the infection induces antiviral cellular immune responses (Woollard et al., 2008), and (iv) tamarins and marmosets are commercially available and easily handled, reared and bred. Moreover, tamarins, being primates, may have a similar immune system to humans, and therefore they may be useful for studying the function of NK cells against the hepatitis virus in this tamarin model.

Our final goal is to study the role of NK cells as a major player in innate immunity during the course of the progression of viral hepatitis. Since some basic information regarding the biological characteristics of NK cells still remains unclear, we initially sought to characterize NK cells in tamarins to provide a technical basis for further studies.

MATERIALS AND METHODS

ANIMALS

Five red-handed tamarins (*Saguinus midas*) and five cynomolgus monkeys (*Macaca fascicularis*) were used in this study. 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.

FLOW CYTOMETRY

Flow cytometry was performed as previously described (Akari et al., 1997) with a slight modification. Fifty microliters of whole blood from cynomolgus monkeys and tamarins was stained with combinations of fluorescence-conjugated monoclonal antibodies (mAb): anti-CD3 (SP34-2; Becton Dickinson), anti-CD4 (L200; BD Pharmingen), anti-CD8 (CLB-T8/4H8; Sanquin), anti-CD16 (3G8; BD Pharmingen), and anti-CD16 (DJ130c; Dako). Then, erythrocytes were lysed with FACS lysing solution (Becton Dickinson). After having been washed with sample buffer containing phosphate-buffered saline (PBS), 1% fetal calf serum (FCS), and 1% formaldehyde, the labeled cells were resuspended in the sample buffer. The expression of the immunolabeled molecules on the lymphocytes was analyzed with a FACSCanto II flow cytometer (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were separated from the blood of these monkeys by a Ficoll-Paque gradient method. The cells were resuspended in complete medium composed of RPMI-1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin, 2 mM HEPES and 55 μM 2-mercaptoethanol at 4°C until use. Fluorochrome-labeled mouse mAbs were reacted with 2×10^5 PBMCs at 4°C for 30 min. The labeled cells were washed with PBS containing 1% FCS, and resuspended in the sample buffer. The expression of the immunolabeled molecules on the lymphocytes was analyzed as mentioned above.

FLOW CYTOMETRIC 5-(AND 6)-CARBOXYFLUORESCEIN DIACETATE SUCCINIMIDYL ESTER (CFSE)/7-AMINO ACTINOMYCIN D (7-AAD) CYTOTOXIC ASSAY

Peripheral blood mononuclear cells were separated from the blood of these monkeys by a Ficoll-Paque gradient method. These PBMCs were then resuspended in complete medium at 37°C until use. The flow cytometric CFSE/7-AAD cytotoxicity assay was performed as previously described (Lecoeur et al., 2001) with slight modifications. K562 cells (3×10^6) were labeled with 500 nM CFSE (from a 1 mM stock solution in dimethyl sulfoxide [Sigma] stored at −20°C) in Hanks' Balanced Salt Solution for 8 min at 37°C in total of 2 ml. The cells were then washed twice in complete medium and used immediately for the cytotoxicity assay. The CFSE-labeled target cells (20,000 cells) were used at different E (effector):T (target) ratios (0:1, 3:1, and 9:1). After 24 h incubation, the cells were stained with 0.25 μg/ml of 7-AAD and incubated for 10 min at 37°C in a CO_2 incubator. The cells were washed twice with 1% FCS–PBS, resuspended in sample buffer and analyzed immediately by flow cytometry.

MAGNETIC CELL SEPARATION

Magnetic cell separation (MACS) was performed as previously described (Tenorio and Saavedra, 2005) with slight modifications. PBMCs (1×10^7) were washed with 3 ml of MACS buffer composed of PBS with 2 mM EDTA and 0.5% bovine serum albumin, and resuspended in 100 μl of the same buffer. Ten microliters of fluorescein isothiocyanate (FITC)-labeled anti-CD16 mAb (3G8) was added. The cells with or without the mAb were incubated for 10 min at 4°C, washed with 1 ml of MACS buffer, and resuspended in 80 μl of the same buffer. They were mixed with 20 μl of anti-FITC MicroBeads and incubated for 15 min at 4°C, washed with 1 ml of MACS buffer, and resuspended in 500 μl of the same buffer. The CD16-positive cells were separated by negative selection using LD columns and a MACS separation unit following the instructions provided by the manufacturer (Miltenyi Biotec). CD16-negative cells were resuspended in complete medium and co-cultured with K562 cells at 37°C for the NK cytotoxicity assay immediately.

DETECTION OF CIRCULATING ANTI-CD16 MAB (3G8)

Concentrations of an anti-CD16 antibody (3G8) in plasma samples were assessed using a mouse IgG_1 Quantitative ELISA Kit (Bethye Laboratory, Inc.). The assay was performed according to the manufacturer's instruction with a slight modification. To detect the mAb in monkey plasma, 96-well enzyme-linked immunosorbent assay (ELISA) plates were coated with a capture antibody and incubated for 1 h at 37°C and washed with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) three times. The plates were blocked with blocking solution (Postcoat) for 30 min at 37°C. Plasma samples from antibody-treated monkeys were diluted in dilution buffer (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin, 0.05% Tween 20, pH 8.0), applied to the wells in serial dilutions, incubated for 1 h at 37°C and washed with the wash solution five times. Goat anti-mouse IgG_1 conjugated with horseradish peroxidase and diluted 1:50000 in dilution buffer was added to each well and incubated for 1 h at 37°C. Each well was washed with the wash solution five times. Substrate solution was added to each well and incubated

for 10–15 min at room temperature, and then the reaction was stopped with H_2SO_4. Optical density was measured using an ELISA reader at 450 nm.

IN VIVO DEPLETION OF CD16 POSITIVE CELLS

Mouse anti-human CD16 (3G8) mAb (Fleit et al., 1982) was produced in serum-free medium and purified using protein A affinity chromatography. Endotoxin levels were lower than 1 EU/mg. The antibody was administered to tamarins (Tm 05-003, Tm 06-020) and cynomolgus monkeys (Mf 00-005, Mf 99-110) intravenously at 50 mg/kg at a rate of 18 ml/min using a syringe pump. Lymphocyte subsets were monitored for 3 weeks after the administration.

STATISTICAL ANALYSIS

Statistical analyses of lymphocyte ratios were performed using Student's *t*-test and single-factor ANOVA, followed by Fisher's protected least-significant difference *post hoc* test by using StatView software (SAS Institute, NC, USA). The results were confirmed in more than three independent experiments in tamarins and cynomolgus monkeys.

RESULTS

LYMPHOCYTE SUBSETS IN TAMARINS

First, we examined the lymphocyte subsets in tamarins as compared with cynomolgus monkeys (**Figure 1**). The percentages of T and B lymphocytes indicated as $CD20^-CD3^+$ and $CD20^+CD3^-$ subsets in the total lymphocytes were found to be 68.8% (range 41.9–68.8%) and 12.3% (range 11.8–12.6%) in tamarins and 68.4% (range 42.6–68.4%) and 10.2% (range 9.1–11.4%) in cynomolgus monkeys, respectively. The percentage of $CD4^+$ T cells in the $CD3^+$ subset was 45.5% (range 41.9–52.5%) and 55.3% (range 42.6–64.4%) while that of $CD8^+$ T cells was 41.0% (range 35.8–44.5%) and 31.2% (range 29.3–34.6%) in tamarins and cynomolgus monkeys, respectively. Next, the NK cell subset was determined as $CD3^-$ $CD16^+$ lymphocytes in this study. The percentage of NK cells was 30.5% (range 16.9–52.5%) and 18.9% (range 13.7–22.4%) in tamarins and cynomolgus monkeys, respectively. We analyzed statistically whether these lymphocyte ratios were different between tamarins and cynomolgus monkeys, and found that there were no significant differences of the lymphocyte ratios between them. We therefore concluded that the proportions of the major lymphocyte subsets in tamarins were relatively similar to those in cynomolgus monkeys.

FLUORESCENCE-BASED *IN VITRO* ASSAY FOR QUANTITATIVELY EVALUATING NATURAL KILLER ACTIVITY

Natural killer cell cytotoxic assays conventionally require considerable numbers of PBMCs, and this has been a major hurdle for analyzing the NK activity in small New World monkeys due to the limited availability of their blood. Therefore, we employed an alternative method using a fluorescence-based assay to assess the activity of NK cells in tamarins as previously described (Lecoeur et al., 2001) with slight modifications. When CFSE-stained K562 target cells were incubated with the effector PBMCs obtained from tamarins at an effector/target (E/T) ratio of 9:1, 42% of the K562 cells were positive for 7-AAD, which stains apoptotic cells (**Figure 2A**). We confirmed that the killing activity of NK cells was dose-dependent, and that the level in tamarins was higher than that in cynomolgus monkeys (**Figures 2B and 3**).

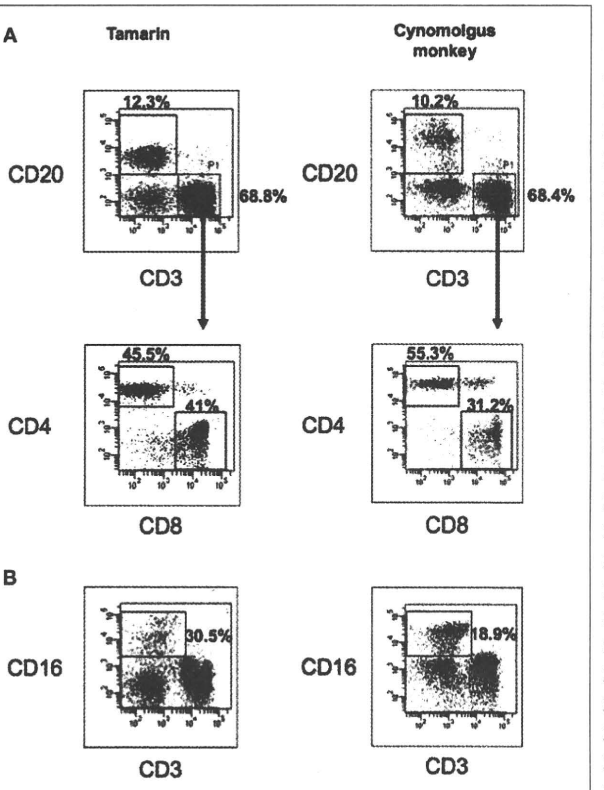

FIGURE 1 | Flow cytometric analysis of CD3, CD20, CD4, CD8, and CD16 expression on lymphocytes in cynomolgus monkeys and tamarins. Representative results in tamarins and cynomolgus monkeys are shown. **(A)** Flow cytometric analysis of CD3 and CD20 expression in lymphocytes (top panels) and CD4 and CD8 expression in $CD3^+$ population (bottom panels) of both monkeys. **(B)** Flow cytometric analysis of CD3 and CD16 expression in both monkey species. The numbers denote the percentages of lymphocytes. We analyzed statistically whether these lymphocyte ratios were different between tamarins and cynomolgus monkeys by using StatView software. There were no significant differences of the lymphocyte ratios between them.

Next, in order to examine if $CD16^+$ lymphocytes represent a major population with NK activity, $CD16^-$ PBMCs were obtained by negative selection using MACS (**Figure 4A**) in both tamarins and cynomolgus monkeys. We found that depletion of $CD16^+$ cells greatly attenuated the killing activity in both tamarins and cynomolgus monkeys (**Figure 4B**), indicating that $CD16^+$ lymphocytes are a major population with NK activity.

IN VIVO DEPLETION OF $CD16^+$ NK CELLS USING A MURINE ANTI-CD16 MAB

We next sought to establish a system to directly evaluate the role of NK cells in tamarins. We asked if the administration of an anti-CD16 (3G8) mAb could deplete $CD16^+$ lymphocytes *in vivo*. Tamarins were intravenously administered 3G8 or control mAb (MOPC-21) at a dose of 50 mg/kg. Using an anti-CD16 antibody that is not cross-blocked by 3G8 (clone DJ130c), it was found that at

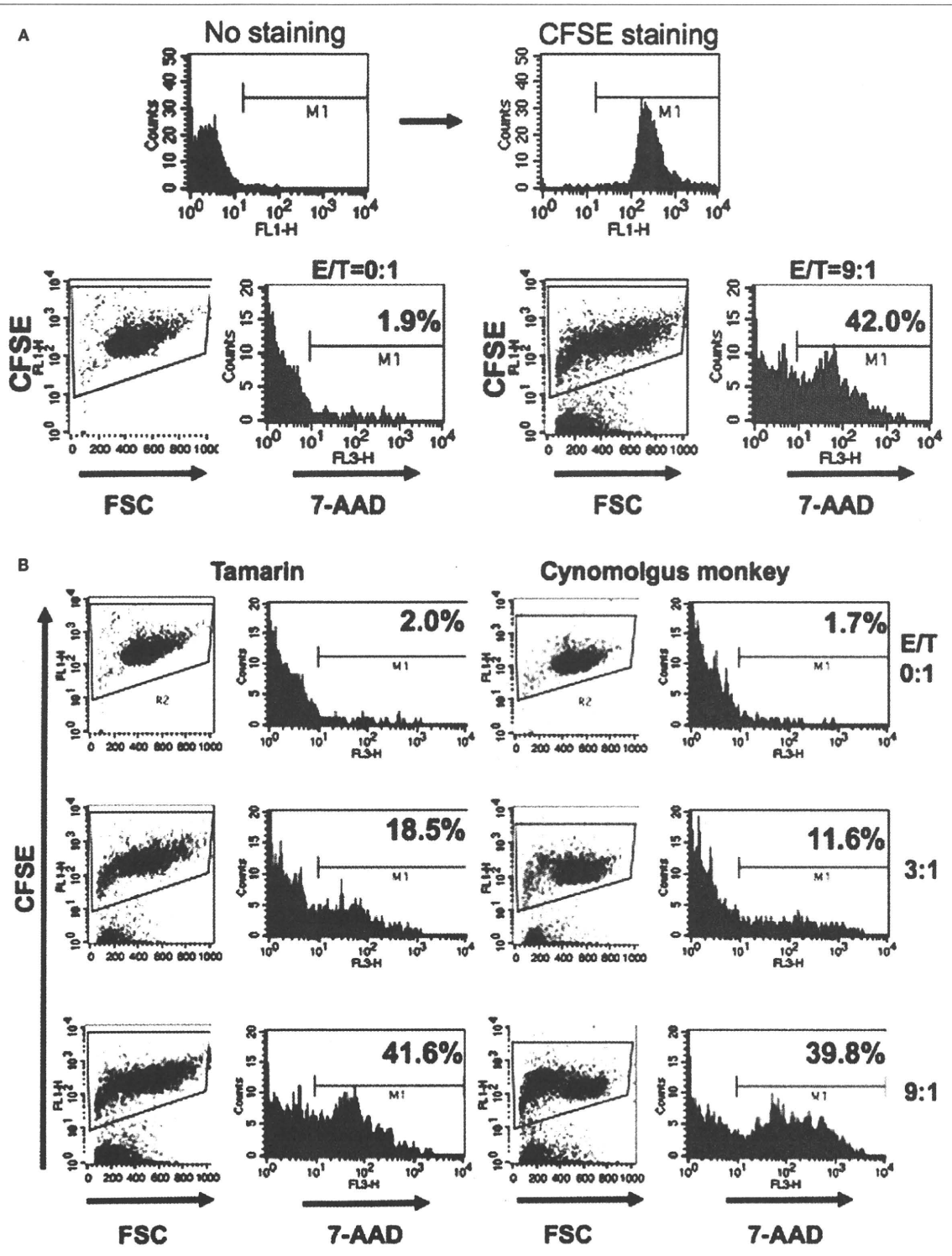

FIGURE 2 | Cytotoxicity assay by CFSE and 7-AAD staining. (A) CFSE-stained K562 cells were co-cultured with PBMCs at E/T ratios of 9:1 and 0:1. The CFSE-stained K562 cells were first gated by FSC and SSC characteristics, and 3000 events were captured. **(B)** CFSE-stained K562 target cells were killed by tamarin PBMCs using E/T ratios of 9:1, 3:1, and 0:1. The numbers in the panels denoted the percentages of 7-AAD positive cells. We analyzed statistically whether these killing activities were different between tamarins and cynomolgus monkeys by using StatView software.

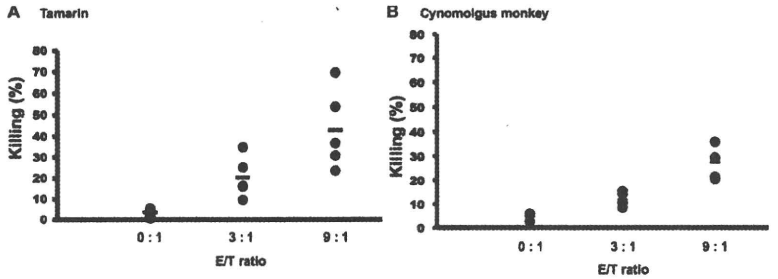

FIGURE 3 | Dose-dependency of killing activity of NK cells in tamarins. (A,B) K562 target cells were stained with CFSE and co-cultured with PBMCs as described in Section "Materials and Methods". CFSE-stained K562 target cells were killed by PBMCs of tamarins and cynomolgus monkeys in a dose-dependent manner. For all experiments, the number of observations used to calculate the mean were $n = 5$. We analyzed statistically whether these killing activities were different between tamarins and cynomolgus monkeys by using StatView software.

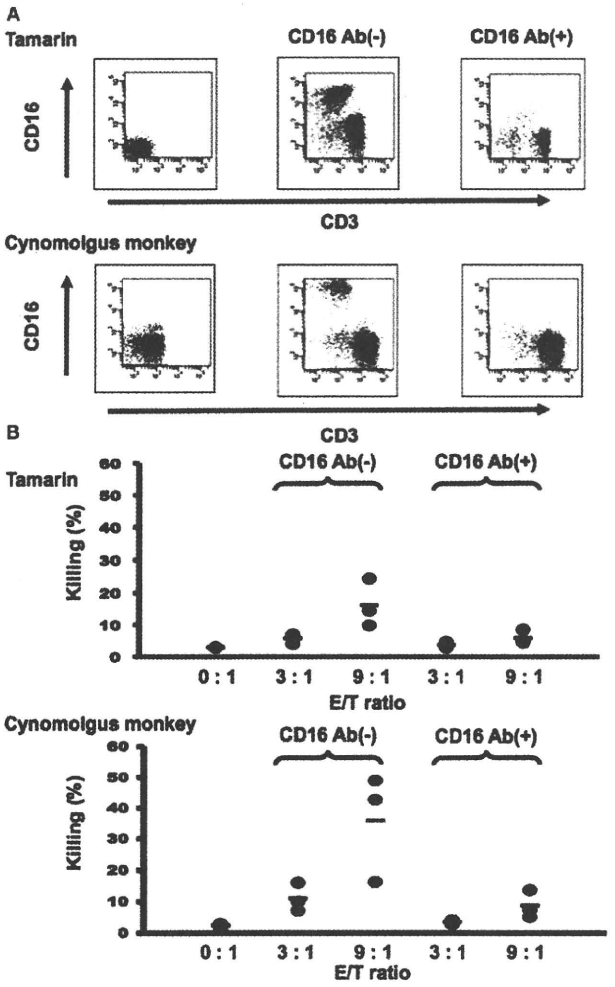

FIGURE 4 | CD16⁺ cells were a major population with natural killer activity in tamarins. (A) CD16⁺ cells were depleted from PBMCs by MACS as described in Section "Materials and Methods". CD16⁻ PBMCs were obtained by negative selection using MACS. **(B)** K562 cells were stained with CFSE and co-cultured with CD16-treated or untreated PBMCs as described in Section "Materials and Methods". Results shown are representative of three independent experiments.

1–3 days after the treatment CD16$^+$ cells were completely depleted, followed by recovery to the initial levels at around 2 weeks after the administration, which was consistent with the results in cynomolgus monkeys (**Figure 5B**). It is noteworthy that the numbers of CD4$^+$/CD8$^+$ T and B lymphocytes were not affected by the treatment and that administration of control antibody did not deplete CD16$^+$ cells during the period tested (data not shown), showing that the effect of 3G8 on CD16$^+$ cells was specific (data not shown). We also measured the concentration of the 3G8 mAb in the plasma of antibody-treated monkeys. As shown in **Figure 6**, the concentration of 3G8 reached a plateau at day 1, followed by a gradual decrease in both tamarins and cynomolgus monkeys, which was consistent with the kinetics of CD16$^+$ cells. In the case of MOPC-21 administration to tamarins, similar kinetics of its concentration with that of 3G8 were observed (data not shown).

ATTENUATION OF CD16$^+$ NK CELL FUNCTION BY *IN VIVO* DEPLETION OF CD16$^+$ CELLS

Finally, we tested whether depletion of the CD16$^+$ subset could attenuate the NK activity in PBMCs. The killing activity was reduced at day 1 and the reduction persisted for 1 week post-treatment in the 3G8-treated monkeys (**Figure 7**). These results showed that the administration of the 3G8 mAb significantly influenced the number and activity of CD16$^+$ lymphocytes in both tamarins and cynomolgus monkeys.

DISCUSSION

In this study, we attempted to establish a technical basis for the study of NK cells in tamarins. First, we characterized the NK cells in tamarins and showed that the anti-CD16 (3G8) mAb, an NK marker, cross-reacted with the PBMCs (**Figure 1**). Second, we assessed the killing activity of the CD16$^+$ NK cells in tamarins using our improved method (**Figures 2–4**) and demonstrated that CD16$^+$ NK cells were likely to be a major population with the killing activity in tamarins. Finally, to directly examine the role of CD16$^+$ NK cells *in vivo*, we assessed the effect of anti-CD16 (3G8) mAb *in vivo*. After administration of the mAb, CD16$^+$ NK cells were completely depleted and the killing activity was substantially attenuated in the treated monkeys (**Figures 5 and 7**). Our results suggest that our method for depletion of CD16$^+$ NK cells *in vivo* is useful for investigating the pivotal role of NK cells in the response against hepatitis viruses.

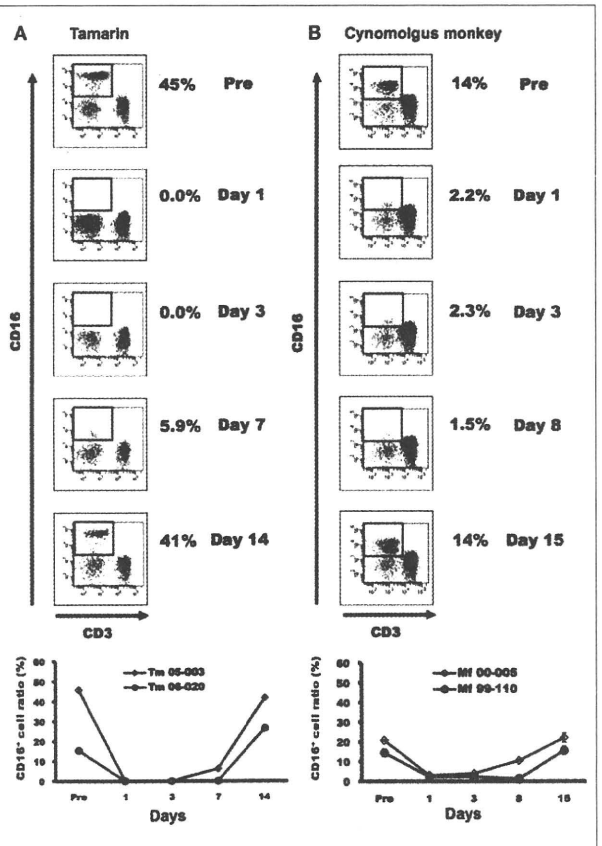

FIGURE 5 | *In vivo* depletion of CD16$^+$ NK cells using a murine anti-CD16 (3G8) mAb in tamarins. (A,B) Tamarins and cynomolgus monkeys were administered with 50 mg/kg of the 3G8 mAb. CD16$^+$ NK cell numbers were determined in whole blood specimens. Tamarins: Tm 05-003 and Tm 06-020. Cynomolgus monkeys: Mf 00-005 and Mf 99-110.

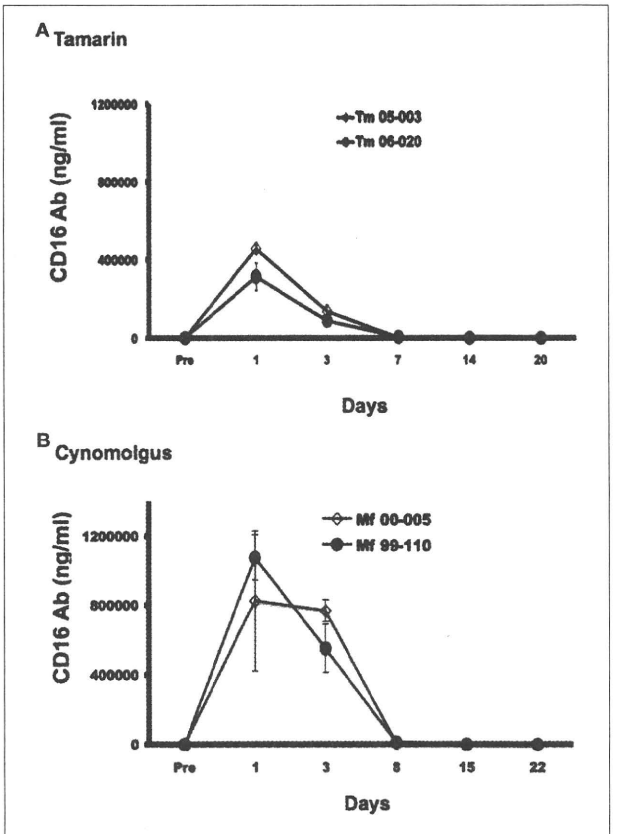

FIGURE 6 | Kinetics of concentration of CD16 (3G8) mAb *in vivo*. The concentration of CD16 (3G8) mAb in plasma was measured by ELISA as described in Section "Materials and Methods". Results shown are representative of three independent experiments. **(A)** Tamarins: Tm 05-003 and Tm 06-020. **(B)** Cynomolgus monkeys: Mf 00-005 and Mf 99-110.

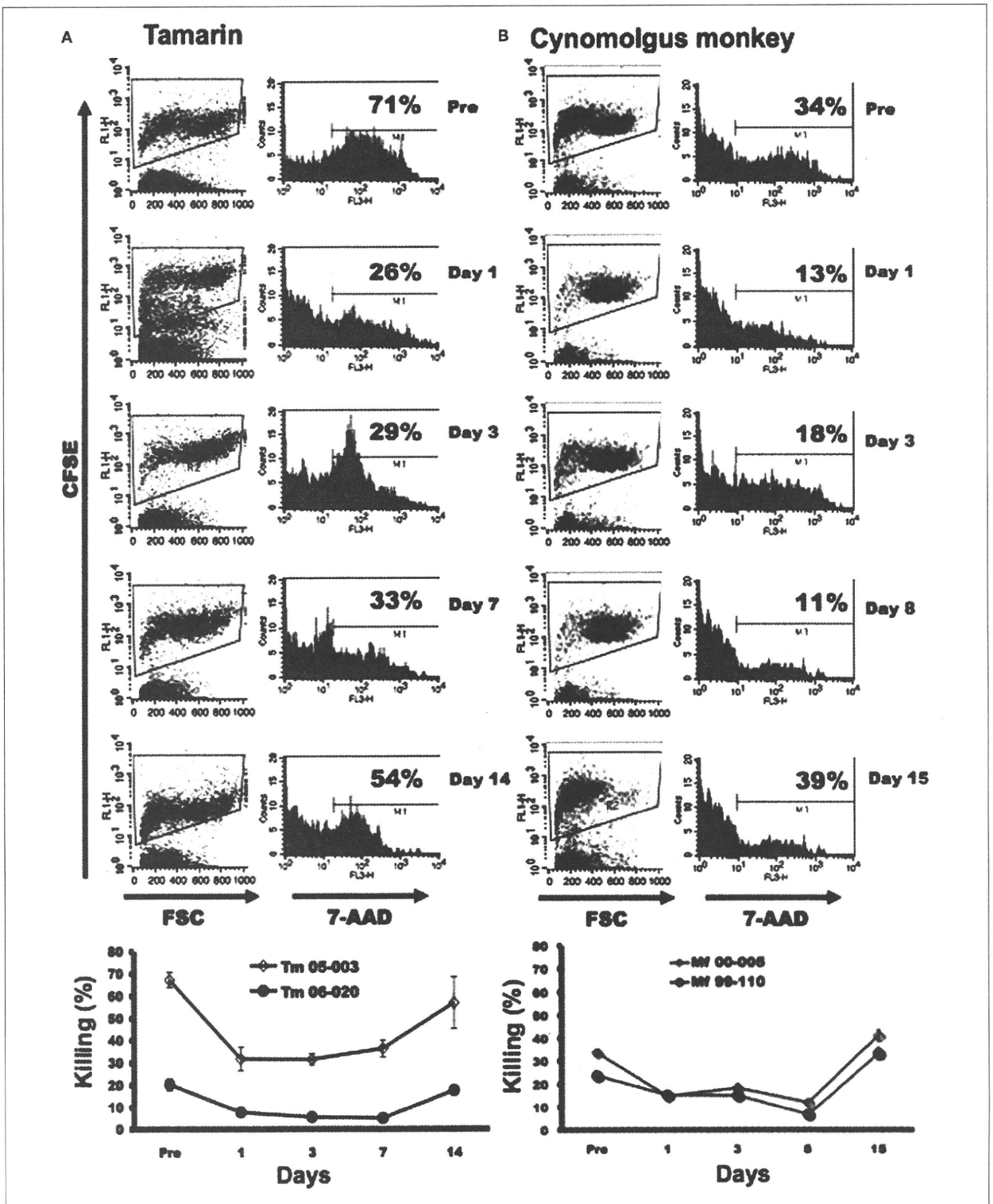

FIGURE 7 | Attenuation of natural killer activity by *in vivo* depletion of CD16⁺ cells. (A,B) Tamarins and cynomolgus monkeys were administered 50 mg/kg of murine 3G8 mAb. The activity was determined in PBMCs of both monkeys. Tamarins: Tm 05-003 and Tm 06-020. **(B)** Cynomolgus monkeys: Mf 00-005 and Mf 99-110.

Generally it is impossible to monitor the immunological status in humans pre- and post-infection with hepatitis viruses and to deplete specific subsets such as NK cells *in vivo*. Non-human primates have immune systems similar to that of humans and are suitable for the evaluation of innate and adaptive immune responses against hepatitis viruses (Woollard et al., 2008). GBV-B is most closely related to HCV. Since experimental infection with GBV-B induces acute and chronic hepatitis in tamarins, this model may be useful for the study of antiviral immunity. Moreover, we have also been developing a chimeric virus between HCV and GBV-B. Therefore, if the HCV/GBV-B chimeric virus is able to infect and replicate in tamarins, our method for *in vivo* depletion of $CD16^+$ NK cell in tamarins is very useful tool to understand the relationship between the chimeric virus and $CD16^+$ NK cells. Moreover, it is still unclear whether NK cells might play a pivotal role at the acute or chronic phase in hepatitis. Analyses to address this issue are in progress using our GBV-B model.

Unexpectedly, almost complete *in vivo* depletion of $CD16^+$ NK cells was not able to completely remove the NK activity in PBMCs, i.e., about one-third of the NK activity remained as compared with that before mAb treatment. This indicates that the $CD16^-$ subpopulation represents a substantial component of NK cells in monkeys. So far, mAbs recognizing tamarin's NK-specific CD markers other than CD16, such as CD56 and CD159A (Choi et al., 2008), are not available. Further characterization and phenotyping of NK cells in tamarins will be necessary to selectively and totally deplete NK cells *in vivo*.

Interestingly, tamarins have been used for the study of experimental infection with Rabies virus, Epstein–Barr virus, Hepatitis A virus and Herpesvirus as well as GBV-B (Mackett et al., 1996; Batista-Morais et al., 2000; Purcell et al., 2002; de Thoisy et al., 2003; Martin et al., 2003; Takikawa et al., 2010). Therefore we hope that our system may be useful for examining the role of NK cells in the control of viral infection as well as to develop novel antiviral strategies.

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Acquisition of HIV-1 Resistance in T Lymphocytes Using an ACA-Specific *E. coli* mRNA Interferase

Hideto Chono,[1,2] Kazuya Matsumoto,[1] Hiroshi Tsuda,[1,2] Naoki Saito,[1,2] Karim Lee,[3] Sujeong Kim,[4] Hiroaki Shibata,[5] Naohide Ageyama,[5] Keiji Terao,[5] Yasuhiro Yasutomi,[5] Junichi Mineno,[1] Sunyoung Kim,[3] Masayori Inouye,[6] and Ikunoshin Kato[1,2]

Abstract

Transcriptional activation of gene expression directed by the long terminal repeat (LTR) of HIV-1 requires both the transactivation response element (TAR) and Tat protein. HIV-1 mutants lacking a functional *tat* gene are not able to proliferate. Here we take a genetic approach to suppress HIV-1 replication based on Tat-dependent production of MazF, an ACA-specific endoribonuclease (mRNA interferase) from *Escherichia coli*. When induced, MazF is known to cause Bak- and NBK-dependent apoptotic cell death in mammalian cells. We first constructed a retroviral vector, in which the *mazF* (ACA-less) gene was inserted under the control of the HIV-1 LTR, which was then transduced into CD4+ T-lymphoid CEM-SS cells in such a way that, upon HIV-1 infection, the *mazF* gene is induced to destroy the infecting HIV-1 mRNA, preventing HIV-1 replication. Indeed, when the transduced cells were infected with HIV-1 IIIB, the viral replication was effectively inhibited, as HIV-1 IIIB p24 could not be detected in the culture medium. Consistently, not only cell growth but also the CD4 level was not affected by the infection. These results suggest that the HIV-1-LTR-regulated *mazF* gene was effectively induced upon HIV-1 IIIB infection, which is sufficient enough to destroy the viral mRNA from the infected HIV-1 IIIB to completely block viral proliferation in the cells, but not to affect normal cell growth. These results indicate that the T cells transduced with the HIV-1-LTR-regulated *mazF* gene acquire HIV-1 resistance, providing an intriguing potential for the use of the HIV-1-LTR-regulated *mazF* gene in anti-HIV gene therapy.

Introduction

RNASE-BASED STRATEGIES for anti-human immunodeficiency virus (HIV) gene therapy may be superior to RNA-based (antisense, ribozyme, or siRNAs) strategies, because the former strategies evade the effects of frequent resistant mutations in HIV-1. MazF is a unique sequence-specific endoribonuclease, or mRNA interferase, encoded by the *Escherichia coli* genome (Zhang *et al.*, 2003). It cleaves mRNA at ACA-specific sequences and effectively inhibits protein synthesis. To date, a number of MazF homologues have been found in various bacteria. These homologues have a wide range of sequence specificities and cleave three- to five-nucleotide RNA sequences in transcripts that play diverse roles in bacterial physiology (Zhu *et al.*, 2006, Yamaguchi and Inouye, 2009), including cell-growth regulation, specific gene regulation (Zhu *et al.*, 2009), and obligatory programmed cell death (Nariya and Inouye, 2008). Induction of *E. coli* MazF mRNA interferase in mammalian cells has been demonstrated to effectively induce Bak- and NBK-dependent apoptotic cell death (Shimazu *et al.*, 2007), indicating that MazF mRNA interferase may be a new and effective tool for gene therapy.

In the HIV-1 life cycle immediately after HIV-1 infection, Tat (transactivator of transcription), an early regulatory protein encoded by the HIV-1 genome, is produced, which subsequently binds to the TAR (transactivation response) sequence to induce the transcription of the HIV-1 genome leading to the expression of other HIV-1 proteins (Berkhout *et al.*, 1989). Therefore, for prevention of HIV-1 infection, it would be a best strategy to preferentially destroy the HIV-1 transcript upon HIV-1 infection. For this purpose, we constructed a Tat-dependent MazF expression system in a

[1]Center for Cell and Gene Therapy, Takara Bio Inc., Otsu, Shiga, 520-2193, Japan.
[2]Biotechnology Research Laboratories, Takara Bio Inc., Otsu, Shiga, 520-2193, Japan.
[3]Department of Biological Sciences, Seoul National University, Seoul 151-742, Korea.
[4]ViroMed Co. Ltd., Seoul 151-818, Korea.
[5]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, 305-0843, Japan.
[6]Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA.

retroviral vector, in which the *mazF* gene was fused downstream of the TAR sequence. As the *E. coli mazF* open-reading frame contains nine ACA sequences, all of them were engineered to MazF-uncleavable sequences without changing the amino acid sequence of MazF. This vector was then transduced into T cells so that MazF production is expected to be induced upon HIV-1 infection. Note that Tat protein produced upon HIV-1 infection induces not only the transcription of infected HIV-1, but also the transcription of the HIV-1 long terminal repeat (LTR)-regulated *mazF* (ACA-less) gene integrated into the genome of the T cells. In the present article, CD4+ T lymphoid line CEM-SS cells were used as T cells, which were transduced with the retroviral vector containing the Tat-inducible *mazF* (ACA-less) gene under the HIV-1-LTR promoter. When the transduced cells were infected with HIV-1 IIIB, the replication of the infected virus was effectively inhibited without affecting cell growth. Notably, the CD4 level after HIV-1 IIIB infection was not affected either. These results suggest that the HIV-1-LTR-regulated *mazF* (ACA-less) gene was effectively induced upon HIV-1 IIIB infection, which is sufficient enough to destroy the viral mRNA from the infected HIV-1 IIIB to completely block viral proliferation in the cells. However, the level of MazF induced is not enough to cause any serious cellular damage, thus maintaining normal cell growth and the CD4 level. These results suggest an intriguing potential for the use of the HIV-1-LTR-regulated *mazF* (ACA-less) gene in anti-HIV gene therapy.

Materials and Methods

Cell lines

293T (ATCC no. CRL-11268) cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, Steinheim, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). CEM-SS cells (Kim *et al.*, 1989) were cultured in RPMI-1640 (Sigma-Aldrich) containing 10% (v/v) FBS (Invitrogen). The doubling time of the cells for each culture condition was calculated by linear regression analysis using Microsoft Excel software (Microsoft, Seattle, WA).

Retroviral vectors

The self-inactivating retroviral vector pMTD3 was constructed by deleting a segment consisting of 267 nucleotides from the 3'LTR U3 region of pMT (Lee *et al.*, 2004). An ACA-less *mazF* gene was synthesized by engineering all nine ACA sequences in the original *E. coli mazF* gene to MazF-uncleavable sequences without changing the amino acid sequence of MazF. The HIV-LTR fragment was obtained from pQBI-LTRgagGFP (Quantum Biotechnologies Inc., Montreal, QC, Canada). To minimize the HIV-LTR sequence, U3-TAR fragments were obtained by PCR. The ACA-less *mazF* gene was inserted downstream of U3-TAR to obtain the final self-inactivating retroviral vector plasmid, pMTD3-U3TAR-MazF. As a control, the green fluorescent protein (GFP) gene was inserted into the vector to obtain pMTD3-U3TAR-GFP.

To mimic HIV replication, two kinds of retroviral vectors that express the HIV-1 Tat protein were constructed as follows: (1) Constitutitve Tat expression system from MLV-LTR. The HIV-1 *tat* gene was synthesized and inserted at the multiple-cloning site of pMT. To easily monitor the gene expression in transduced cells, an internal ribosome entry site (IRES) and a coding region for a fluorescent protein, ZsGreen, were fused downstream of the *tat* gene. Thus, the resulting plasmid, pM-LTR-Tat-ZG, expresses Tat as well as ZsGreen from MLV-LTR. (2) Tat expression system from the HIV-1 LTR. The HIV-LTR-*tat*-polyA cassette was inserted in the opposite direction of pMT, and the ZsGreen marker gene was expressed from a phosphoglycerate kinase (PGK) promoter in the normal orientation of pMT. The resulting vector plasmid was designated as pH-LTR-Tat-ZG.

To enhance the viral titer for efficient *mazF* gene transduction, the HIV-LTR-MazF-polyA cassette was introduced in the opposite direction of the MoMLV-LTR at the multicloning site of pMT plasmid (Lee *et al.*, 2004). A truncated form of the human low-affinity nerve growth factor gene (ΔLNGFR) (Verzeletti *et al.*, 1998) was also introduced into the retrovirus vector as a surface marker. The ΔLNGFR gene is under the control of human PGK promoter. The resultant vector plasmid was designated as pMT-MFR-PL2 (Fig. 1B).

Preparation of retroviral vectors

The self-inactivating retroviral vector was generated by the transient transfection method as follows: The GALV-*env* expression vector plasmid, pVM-GeR, was constructed by replacing the amphotropic-*env* gene of pVM-AE (Yu *et al.*, 2003) with the gibbon ape leukemia virus envelope gene. The GALV-env retroviral vector was produced by co-transfecting 293T cells with the retroviral *gag-pol* expression vector plasmid, pVM-GP (Yu *et al.*, 2003), pVM-GeR, and the self-inactivating retroviral vector plasmid. Two days after transfection, viral supernatant was harvested by filtration of the culture fluid from 293T cells with use of a 0.45-μm filter.

GALV-enveloped retroviral Tat expression vectors and MazF expression vector were also generated as follows: Ecotropic retroviral vectors were generated by the transient transfection method using the packaging plasmids pGP (MLV-*gag-pol*; Takara Bio, Otsu, Shiga, Japan) and pE-eco (ecotropic *env*; Takara Bio) with the retroviral vector plasmid pM-LTR-Tat-ZG, pH-LTR-Tat-ZG, or pMT-MFR-PL2. This was performed with use of human embryonic kidney 293T-derived G3T-hi cells (Takara Bio) by using the calcium phosphate co-transfection method. The GALV-*env* retroviral vector was obtained from PG13 packaging cells (ATCC no. CRL-10686) by infection with the ecotropic retrovirus vector as prepared above. After selection of the infected PG13 cells, the virus was collected from the growth medium by filtration of the supernatant with use of a low-protein binding filter (0.45 μm).

Retroviral transduction into CEM-SS cells

CEM-SS cells were infected with self-inactivating retroviral vectors in the presence of 8 μg/ml Polybrene (hexadimethrine bromide; Sigma-Aldrich). Polyclonal gene-transduced cell populations were obtained by selecting the cells with G418 (Invitrogen) at a concentration of 1 mg/ml.

CEM-SS cells or CEM-SS cells transduced with MTD3-U3TAR-MazF were infected with Tat expression retroviral vectors M-LTR-Tat-ZG or H-LTR-Tat-ZG in the presence of RetroNectin (Takara Bio) according to the manufacturer's protocol.