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

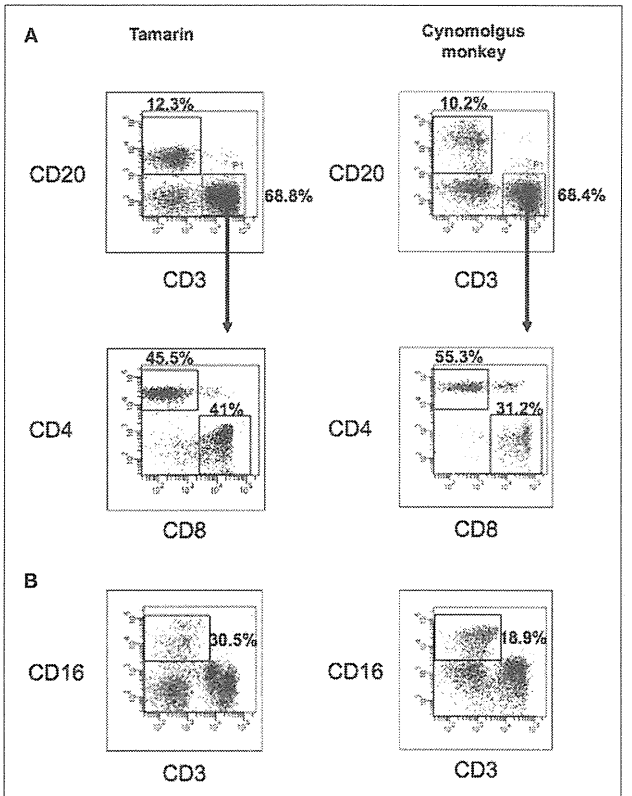

FIGURE 1 | Flow cytometric analysis of CD3, CD20, CD4, CD8, and CD16 expression on lymphocytes in cynomolgus monkeys and tamarins. Representative results in tamarins and cynomolgus monkeys are shown. **(A)** Flow cytometric analysis of CD3 and CD20 expression in lymphocytes (top panels) and CD4 and CD8 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.

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**).

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

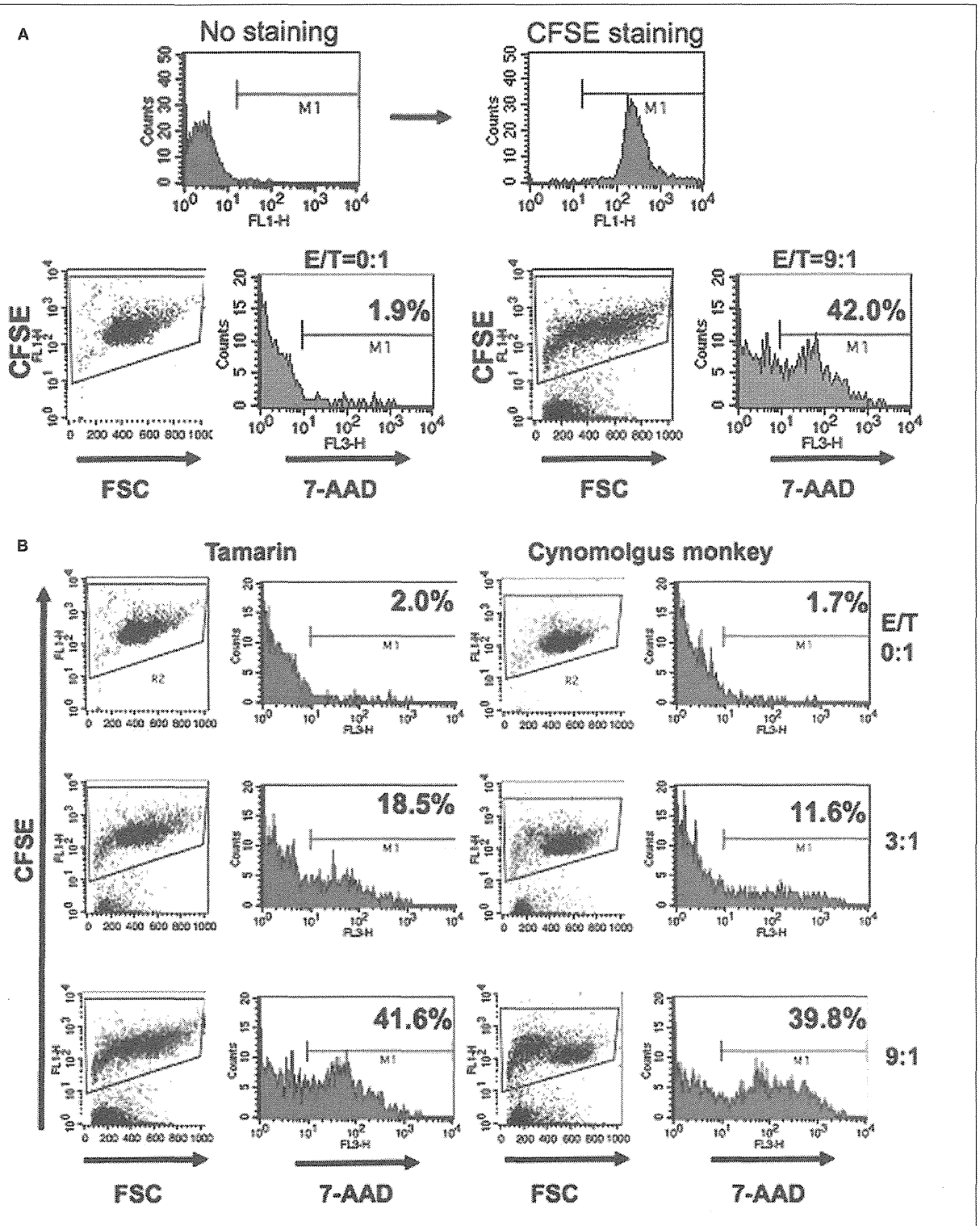

FIGURE 2 | Cytotoxicity assay by CFSE and 7-AAD staining. (A) CFSE-stained K562 cells were co-cultured with PBMCs at E/T ratios of 9:1 and 0:1. The CFSE-stained K562 cells were first gated by FSC and SSC characteristics, and 3000 events were captured. **(B)** CFSE-stained K562 target cells were killed 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.

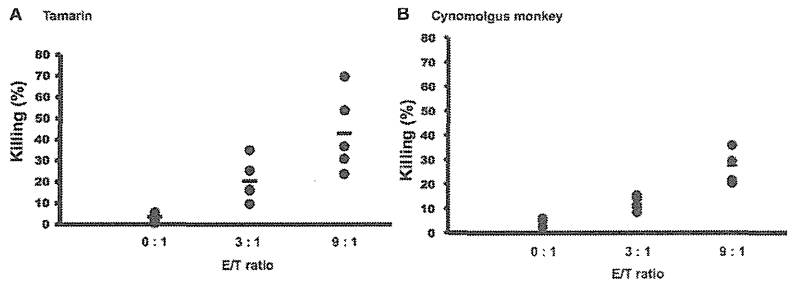

FIGURE 3 | Dose-dependency of killing activity of NK cells in tamarins. (A,B) K562 target cells were stained with CFSE and co-cultured with PBMCs as described in Section "Materials and Methods". CFSE-stained K562 target cells were killed by PBMCs of tamarins and cynomolgus monkeys in a dose-dependent 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.

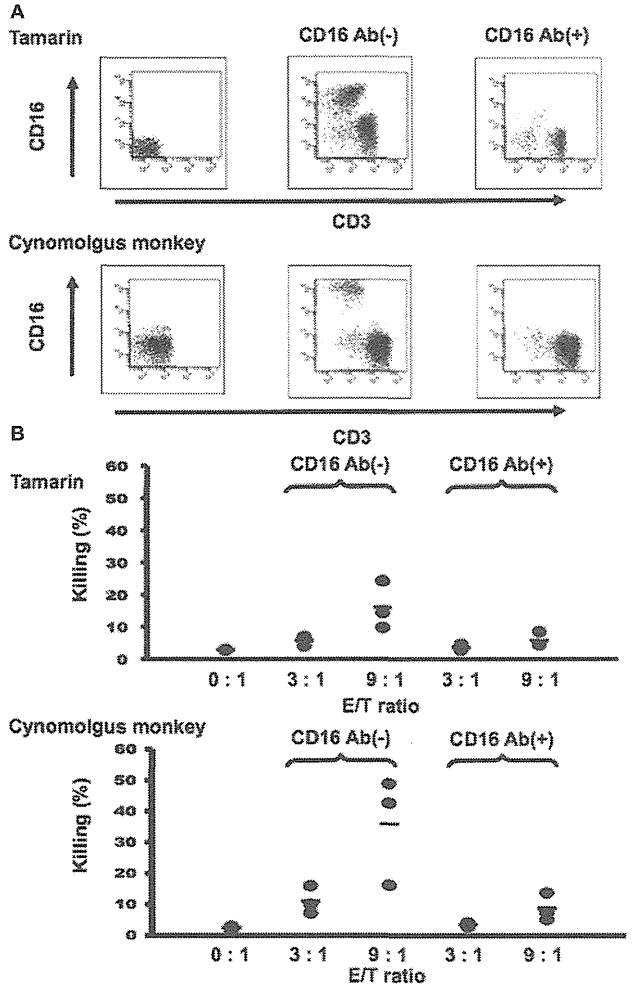

FIGURE 4 | CD16⁺ cells were a major population with natural killer activity in tamarins. (A) CD16⁻ cells were depleted from PBMCs by MACS as described in Section "Materials and Methods". CD16⁻ PBMCs were obtained by negative selection using MACS. **(B)** K562 cells were stained with CFSE and co-cultured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

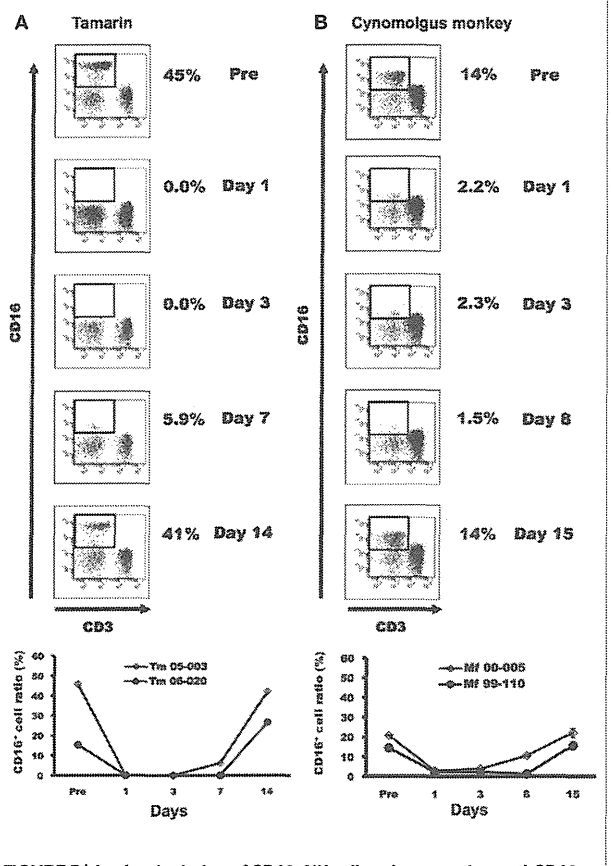

FIGURE 5 | *In vivo* depletion of CD16$^+$ NK cells using a murine anti-CD16 (3G8) mAb in tamarins. (A,B) Tamarins and cynomolgus monkeys were administered with 50 mg/kg of the 3G8 mAb. CD16$^+$ NK cell numbers were determined in whole blood specimens. Tamarins: Tm 05-003 and Tm 06-020. Cynomolgus monkeys: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

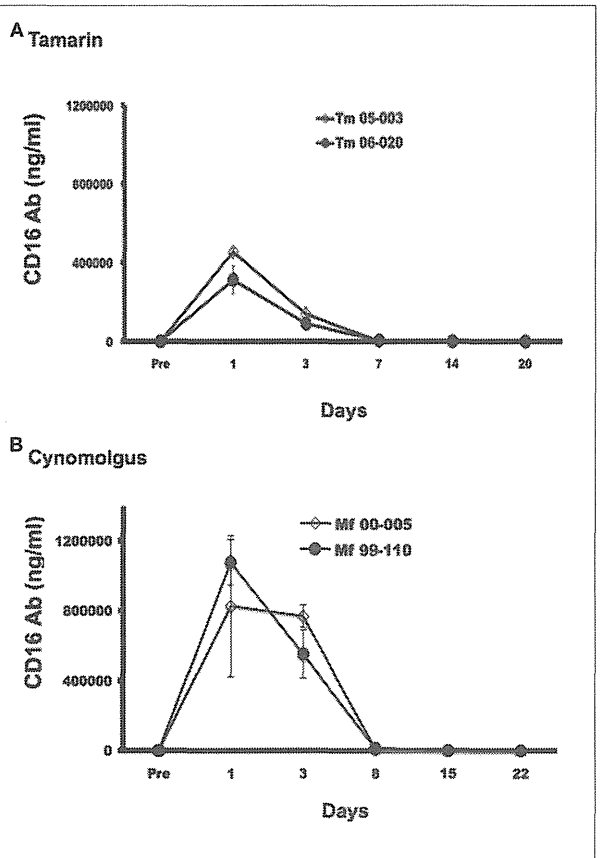

FIGURE 6 | Kinetics of concentration of CD16 (3G8) mAb *in vivo*. The concentration of CD16 (3G8) mAb in plasma was measured by ELISA as described in Section "Materials and Methods". Results shown are representative of three independent experiments. **(A)** Tamarins: Tm 05-003 and Tm 06-020. **(B)**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

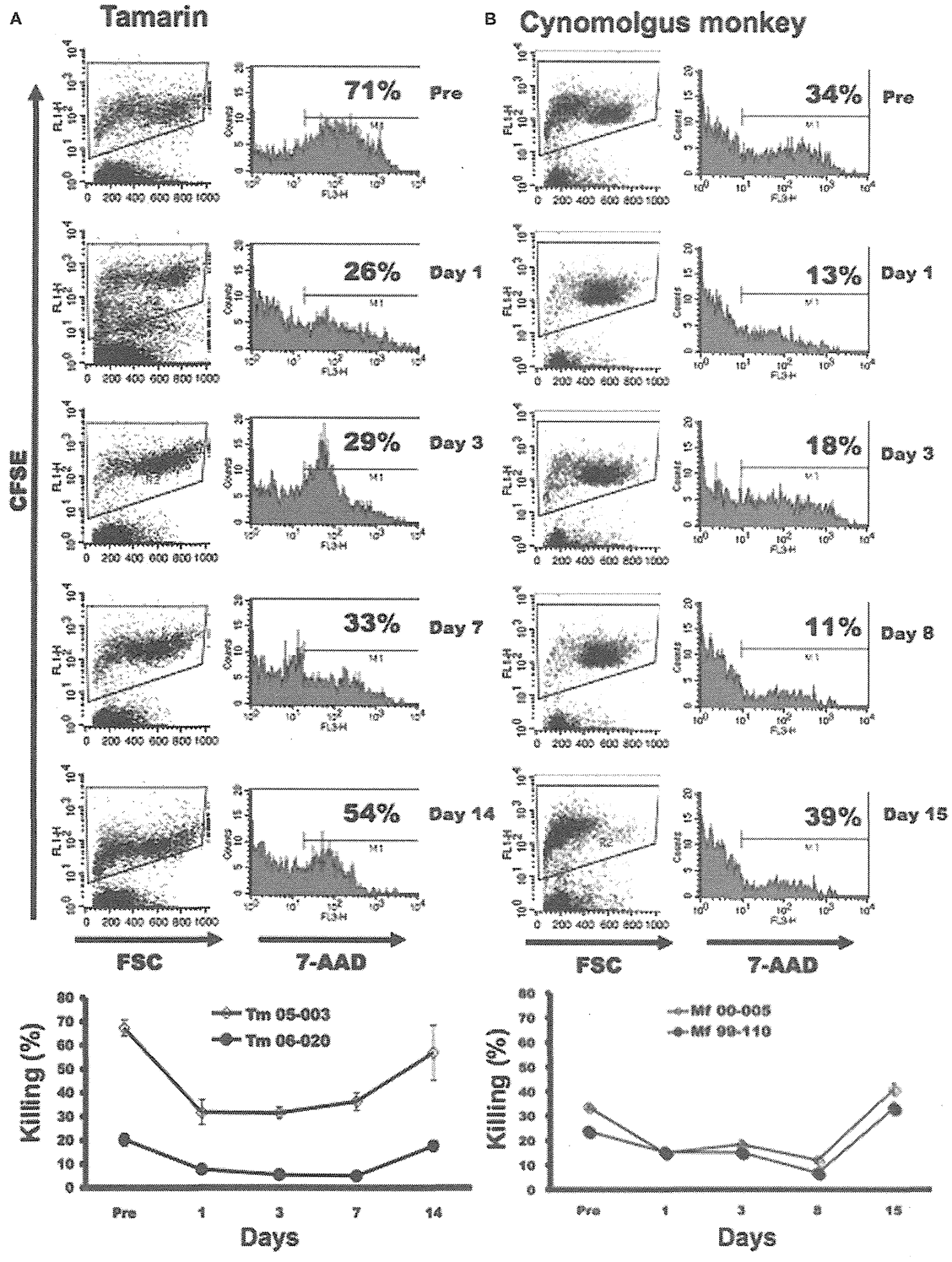

FIGURE 7 | Attenuation of natural killer activity by *in vivo* depletion of CD16$^+$ cells. (A,B) Tamarins and cynomolgus monkeys were administered 50 mg/kg of murine 3G8 mAb. The activity was determined in PBMCs of both monkeys. Tamarins: Tm 05-003 and Tm 06-020. **(B)** Cynomolgus monkeys: Mf 00-005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

Establishment of specific pathogen-free macaque colonies in Tsukuba Primate Research Center of Japan for AIDS research

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Virus

ABSTRACT

Cynomolgus monkeys have been maintained in indoor facilities as closed colony monkeys in Tsukuba Primate Research Center in Japan since 1978. Several microorganisms, including bacteria, parasites and viruses, were eliminated from the cynomolgus monkeys in this colony of TPRC. Various kinds of viruses (B virus, measles virus, simian varicella virus, simian immunodeficiency virus, simian T cell leukemia virus, simian D type retrovirus, simian cytomegalovirus, simian Epstein-Barr virus, and simian foamy virus), bacteria (*Shigella*, *Salmonella* and *Mycobacteria spp.*) and intestinal helminth were chosen as target microorganisms to establish a specific pathogen-free (SPF) colony. Except for a few pathogens (simian D type retrovirus, simian Epstein-Barr virus, and simian foamy virus), selected pathogens were completely eliminated from all monkeys in TPRC. In this review, the history of establishment of SPF cynomolgus monkey colonies in Japan is described.

Contents

1. Introduction

Nonhuman primates are critical resources for biomedical research. Macaque monkeys are one of the key nonhuman primate models that share nearly all characteristics with humans. Conditions of experimental animals are very important for biomedical experiments. The animals should not be infected with microorganisms because microorganism infection may affect results. Moreover, some pathogens are likely to harm not only monkeys but also humans in experiments involving macaques. For these reasons, there is a need for specific pathogen-free (SPF) macaque colonies for research purposes, biohazard avoidance and maintenance of health levels in established colonies (Table 1).

Tsukuba Primate Research Center (TPRC) in Japan has a large-scale breeding colony of experimental cynomolgus monkeys (approximately 1500 monkeys), which play a significant role in the development of pharmaceutical products and medical technologies. The center is the forefront facility in Japan that both supplies laboratory-bred monkeys, mainly cynomolgus monkeys, and performs medical research. Cynomolgus monkeys have been maintained in indoor facilities as closed colony monkeys in TPRC since 1978 [1]. In addition to quality control, supply, research resource development, and basic technology development involving the experimental monkeys, evaluation of state-of-the-art medical technology, evaluation of the efficacy of new drugs and safety assessments are also performed using the monkeys. The establishment of SPF macaques is therefore necessary in TPRC.

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Table 1
History of establishment of SPF cynomolgus monkeys in TPTC.

Year	Target microorganism	Complete elimination from TPRC
1978–1982	BV, MV, *Shigella*, *Salmonella*, *Mycobacteria*, helminth	MV, *Shigella*, *Salmonella*, *Mycobacteria*,
1983–1994	BV, SVV, SIV, STLV-1, SRV/D helminth	SIV, STLV-1, helminth
1995–2004	BV, SVV, SRV/D,	BV, SVV,
2004–Present	SRV/D (73%)[a], LCV (50%)[a], SFV (31%)[a]	CMV

[a] Infection rate of all cynomolgus monkeys in TPRC at present.

The cynomolgus monkeys in TPRC were obtained from Indonesia, Malaysia and Philippines [1]. The monkeys have been bred as pure blood of each origin without interbreed crossing. These pure blood monkeys should be important for comparison of various genetic effects in biological studies including vaccine development. The establishment of SPF colonies in TPRC is also important for this reason. These three pure blood colonies and one mixed blood colony each consist of approximately 100 SPF cynomolgus monkeys. In this review, attempts to establish SPF macaque colonies for advanced biomedical research are reported.

1.1. First term (1978–1882)

Several kinds of microorganisms were chosen for elimination from colony monkeys. Two viruses (B virus and measles virus), three species of bacteria (*Shigella*, *Salmonella* and *Mycobacteria spp.*) and intestinal helminths were selected as the first target pathogens for elimination in macaque colonies. B virus (BV, *Cercopithecine herpesvirus* 1) is an alphaherpesvirus that naturally infects macaque monkeys. In macaques, the virus typically causes a self-limiting disease similar to herepes simplex virus disease in humans [2]. In surprising contrast, BV infection in humans has resulted in the death of 80% of individuals [2]. Therefore, BV was firstly chosen as an SPF target pathogen for prevention of biohazard risks by this virus. The BV infections were detected by BV-specific antibody (Ab) response in sera using an ELISA system (BioReliance Co., USA). Prevention of the spread of BV in the macaque colony was carried out by early weaning of babies from mothers. Infection of the virus in plasma of the prematurely weaned monkeys was confirmed by a BV-specific Ab several times at intervals of 3–6 months. Measles, caused by measles virus (MV) infection, remains a major cause of infant mortality despite the availability of a safe and effective live attenuated virus vaccine. MV-free cynomolgus monkeys are required, since one of the purposes to supply cynomolgus monkeys in TPRC is certification tests for human measles vaccine. MV infection was examined in all monkeys by detection of specific Ab reaction in sera by ELISA and MV antigen (Ag) detected by RT-PCR. Although most of the cynomolgus monkeys from Asia were infected with MV, asymptomatic monkeys with MV excretion in plasma, urine and other biological fluid were not reproduced in TPRC. The MV-infected monkeys were eliminated by this breeding program. Two species of bacteria, *Salmonella* and *Shigella spp.*, were detected by cultivation of rectal or fecal swab samples. Monkeys having these bacteria received drug treatment (200 mg of sulfamethoxazole and 40 mg of trimethoprim once a day for 3 days by oral administration even to *Salmonella*, 200 mg of fosofomycin once a day for 3 days by oral administration even to *Shigella*) if they showed no clinical symptoms of infection with these bacteria. Infection with *Mycobacteria spp.* responsible for tuberculosis was examined by tuberculin (TB) skin tests, and monkeys with positive results of TB skin tests were eliminated. Infection with MV, *Salmonella*, *Shigella* or *Mycobacteria spp.* has not been detected in any monkeys in TPRC since 1982. Cynomolgus monkeys excreting helminth eggs in feces were given anthelimintics (ivermectin 200 μg/kg s.c twice for 2 weeks interval; metronidazol 40 mg/kg once a day for 5 days by oral administration; thiabendazole 50 mg/kg once a day for 3 days by oral administration and mebendazole 20 mg/kg once a day for 3 days by oral administration).

1.2. Second and third terms (1983–1994)

In addition to targeting BV and helminths for elimination from TPRC, simian immunodeficiency virus (SIV), simian T cell leukemia virus (STLV), simian D type retrovirus (SRV/D) and simian varicella virus (SVV) were newly targeted to establish SPF monkey colonies in 1983–1994. Although an AIDS model induced by SIV is very useful for AIDS studies, SIV is not present in macaques from Asia unless they have been experimentally exposed. In fact, natural infection with SIV was not seen in any of the monkeys in TPRC examined by ELISA for detection of SIV-specific Ab in sera. STLV is widely present in all New and Old World primate species. The incidence of STLV infection in most natural simian populations is 5–40%, but it can be much higher in wild monkeys [3,4]. STLV infection was detected in 11.7% of the monkeys in TPRC by IFA using MT-1 cells [5]. These monkeys were eliminated from TPRC over a period of several years. SVV is an alphaherpesvirus that causes varicella in Old World monkeys and establishes latent infection in ganglionic neurons [6]. Outbreaks in many animal facilities have been reported [7]. An outbreak of SVV infection occurred in TPRC during the period from November 1989 to April 1990. Varicella developed in almost 100 monkeys, and 67% of those monkeys died. The rate of infection with SVV in TPRC was 12.9% in 1990. SVV infection can usually be detected by SVV-specific Abs, even in asymptomatic monkeys, and SVV-infected monkeys were eliminated from TPRC in 2000. Attention must be paid to SRV/D both for its risk to macaque colony health and its negative effects on biomedical research. Monkeys infected with SRV/D eventually show symptoms that might be caused by SRV/D infection, such as diarrhea, weight loss and anemia, due to activation attributable to changing conditions of the individual [8–11]. This virus can be transmitted horizontally, vertically or sexually by symptomatic or asymptomatic animals. Moreover, some SRV/D-infected monkeys can become viremic yet remain Ab-negative, allowing infection to escape detection by routine Ab screening [12]. A new subtype of SRV/D, named SRV/D-T, was detected in the colony in TPRC in 2005 [13]. Certain monkeys were found to have plasma viremia of this subtype and did not develop any specific Abs to SRV/D-T. Cynomolgus monkeys in the colony showing SRV/D-T viremia secreted the virus in saliva, urine and feces, and the viruses secreted from these monkeys were thought to be a potential cause of horizontal infections of SRV/D-T. Moreover, there was a high rate of transmission of SRV/D-T infection between mothers and infants in TPRC. Screening for this virus infection was done by detection of both Ab (Western blot analysis) and virus (RT-PCR) in plasma [14]. STLV was completely eliminated from TPRC during the second and third terms.

1.3. Fourth and fifth terms to present (1995–2009)

Monkey infected with BV and SVV were completely eliminated from TPRC in the late 90s. Three viruses, simian cytomegalovirus (CMV), simian Epstein-Barr virus (EBV, simian lymphocryptoviruses (LCV)) and simian foamy virus (SFV), were added as target viruses in a new plan in 1995 to establish SPF monkey colonies. Simian CMV infections have been reported in various species of monkeys, including macaques [15]. This virus is readily transmitted in oral secretions, breast milk and urine [16], and 3% of adult monkeys in TPRC were infected with the virus. CMV infection was detected by IFA or an ELISA system using CMV Ag. Simian EBV has also been detected in several species of Old World and New World primates [17]. This virus is also readily transmitted, and serological surveys indicated that about 90% of adult cynomolgus monkeys in TPRC were infected. Detection of EBV infection was usually done by using commercial available human IFA kit. Infection with these two viruses, CMV and EBV, in macaques are opportunistic infections. Infection with the other virus, SFV, also does not seem to cause disease in nonhuman primates as natural hosts [18]. Humans can be infected with SFV, although the number of known SFV infection cases in humans is small [19]. SFV infection was detected by IFA using SFV Ag. Monkeys infected with SFV are fraught with hazards to workers in a primate center. The rate of infection with SFV in adult monkeys in TPRC was 80%. Detection of SFV was done by Ab response in sera using ELISA. Prevention of the spread of these three viruses, CMV, LCV and SFV, was performed by artificial nursing with feeding formula for baby monkeys that had been removed from their mothers immediately after birth. CMV infection in monkeys has not been detected in TPRC since 2005.

2. Conclusions

SPF nonhuman primate colonies are required for biomedical research with several beneficial effects such as animal health and occupational safety. High quality of laboratory animals is also required for advanced biomedical studies including vaccine research and development. Infectious agents frequently affect the results of animal experiments. The history of establishment of SPF cynomolgus monkeys in TPRC in Japan for evaluation of state-of-the-art medical technology, evaluation of the efficacy of new drugs and new vaccines, and safety assessments has been described in this review.

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Conflict of interest statement

The author states that they have no conflict of interest.

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

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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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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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BRIEF COMMUNICATION

Preferential expression and immunogenicity of HIV-1 Tat fusion protein expressed in tomato plant

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Abstract HIV-1 Tat plays a major role in viral replication and is essential for AIDS development making it an ideal vaccine target providing that both humoral and cellular immune responses are induced. Plant-based antigen production, due to its cheaper cost, appears ideal for vaccine production. In this study, we created a plant-optimized *tat* and mutant (Cys30Ala/Lys41Ala) *tat* (*mtat*) gene and ligated each into a pBI121 expression vector with a stop codon and a *gusA* gene positioned immediately downstream. The vector construct was bombarded into tomato leaf calli and allowed to develop. We thus generated recombinant tomato plants preferentially expressing a Tat-GUS fusion protein over a Tat-only protein. In addition, plants bombarded with either *tat* or *mtat* genes showed no phenotypic difference and produced 2–4 μg Tat-GUS fusion protein per milligram soluble plant protein. Furthermore, tomato extracts intradermally inoculated into mice were found to induce a humoral and, most importantly, cellular immunity.

Keywords AIDS · Antibody response · Cellular immune response · HIV-1 · Tat · Transgenic tomato

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Introduction

HIV-1 has already claimed millions of victims worldwide and despite billions of dollars spent on HIV-1/AIDS research annually (Walker and Burton 2008; Watkins et al. 2008), no promising candidate HIV-1 vaccine has been made to date due to: (a) specific viral characteristics including extreme genetic variability among various isolates collected worldwide and even within the infected individuals; (b) a high mutation rate allowing rapid escape of variants from immune responses; and (c) biological properties of HIV-1 regulatory proteins, such as Nef and Tat, which avoid immune responses (Walker and Burton 2008; Watkins et al. 2008; WHO 2008; Potts et al. 2008). As widely believed, these characteristics pose a major obstacle towards controlling AIDS (Gaschen et al. 2002; Moore et al. 2008).

An ideal strategy against HIV-1 is one that stimulates passive protection or neutralizing immunity by

producing both antibodies and cytotoxic T lymphocytes or CTLs (Walker and Burton 2008; Watkins et al. 2008; Addo et al. 2001). Earlier works have shown that CTLs can control HIV-1 replication in the absence of antibodies (Borrow et al. 1994) prompting several attempts to stimulate anti-viral CTL responses using a combination of varying HIV-1 proteins and their epitopes (Betts et al. 2005; Matano et al. 2004; Mwau et al. 2004). The Tat protein has been one of the well studied HIV-1 proteins (Barboric and Peterlin 2005; Emerman and Malim 1998; Goldstein et al. 2001; Okamoto and Wong-Staal 1986; Ramirez et al. 2007). It is a small regulatory protein composed of either 86 or 101 amino acid residues (14 or 18 kDa, respectively) encoded by two exons (Okamoto 1995). Among the HIV-1 proteins already studied, Tat shows great potential for CTL induction covering a wide variety of HIV-1 clones besides from little variability among distinct viral subtypes and is highly conserved in both inter- and intra-patient variants (Addo et al. 2001; Goldstein et al. 2001).

Over 4 million people become infected with HIV-1 each year (WHO 2008; Fox 2007) in third-world countries in particular (Flexner 2008). Cheap and affordable production of pharmaceutical products for third-world consumption has prompted the development of plant-made pharmaceuticals for often neglected diseases (Zahn et al. 2008), including HIV-1 (Ramirez et al. 2007; Flexner 2008; Shchelkunov et al. 2006; Webster et al. 2005). Previous attempts to utilize the tomato plant for HIV-1 Tat vaccine development in the form of an edible-vaccine was only successful in inducing antibodies or humoral immune response (Ramirez et al. 2007; Shchelkunov et al. 2006). At present, no report has been made with regards to induction of CTLs or cellular immune responses using Tat protein (Addo et al. 2001), more so, using a plant-expressed Tat protein.

In this study, we demonstrate the evidence of preferential expression of a Tat-GUS fusion protein over the Tat-only protein in tomato plant and is expressed much higher than previously reported (Ramirez et al. 2007). In addition, we were able to induce both humoral immune response and, surprisingly, cellular immune response using Balb/c mice when tomato extracts were intradermally introduced. To our knowledge, this is the first report of cellular immune induction using Tat expressed in a plant system.

Materials and methods

Vector construction and tomato transformation

The *tat* gene from the HXB2 strain of HIV-1 and *mtat* were synthesized following a specific codon-usage table based on tomato was used (www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4081). The plant-optimized M2 epitope directly fused to either *tat* or inactive *mtat* (Imai et al. 2005) genes with a stop codon, were individually ligated into a pBI121 expression vector (Clontech) upstream of a *gusA* gene (Fig. 1). Transformation was performed using a particle gun

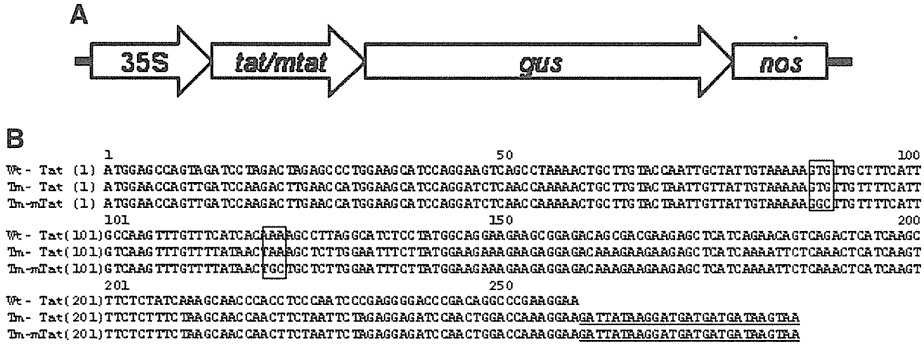

Fig. 1 Construction of the pBI121 plant expression vector containing either *tat* or *mtat* indirectly fused to *gusA* gene. **a** Expression was driven by 35S CaMV promoter and terminated with NOS termination signal located downstream of *gusA* gene. The inserted *tat/mtat* gene is located upstream of the *gusA* gene containing the termination codon (TAA) in between. **b** Codon-optimized *tat* and *mtat* were synthesized following the codon usage of tomato. The *boxed regions* represent point mutations at Cys30Ala and Lys41Ala found in *mtat* [23]. The *underlined segment* represents M2 epitope added to serve as an expression tag. Wt-Tat represents the Tat sequence from the HXB2 strain. Tm-Tat and Tm-mTat represents codon-optimized tomato Tat and mTat, respectively

(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 ($\sim$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

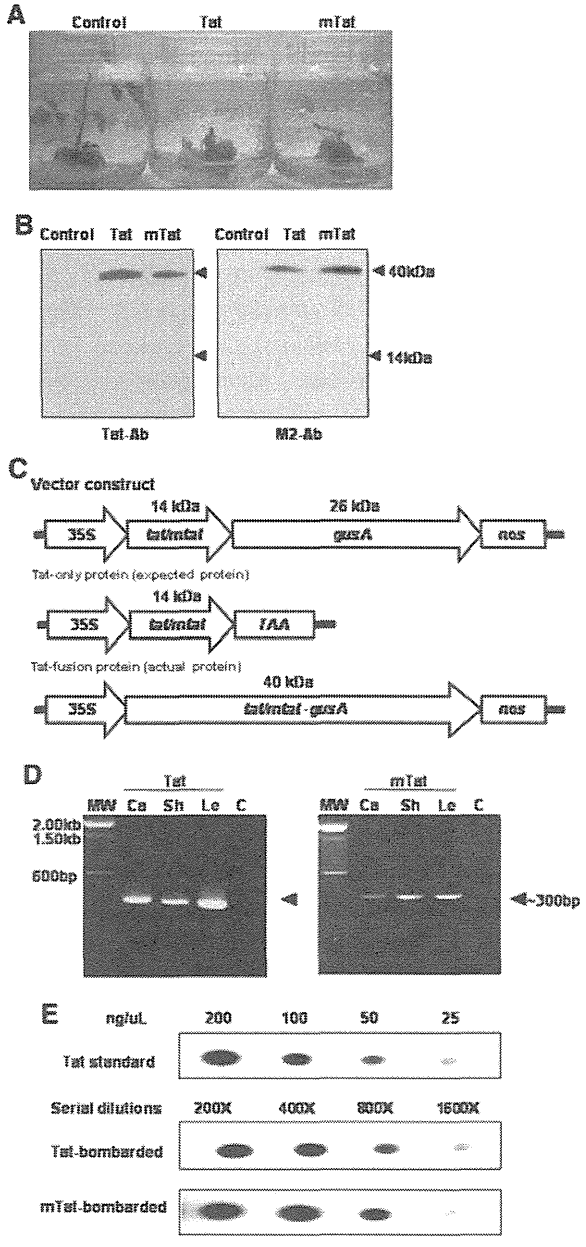

Fig. 2 Expression of both Tat and mTat proteins in tomato plant using particle-gun bombardment. **a** All tomato calli bombarded followed the same bombardment conditions and grown for 3 weeks. Control samples were bombarded with pBI121 vector only. Tat and mTat samples were bombarded with Tat- and mTat-pBI121 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

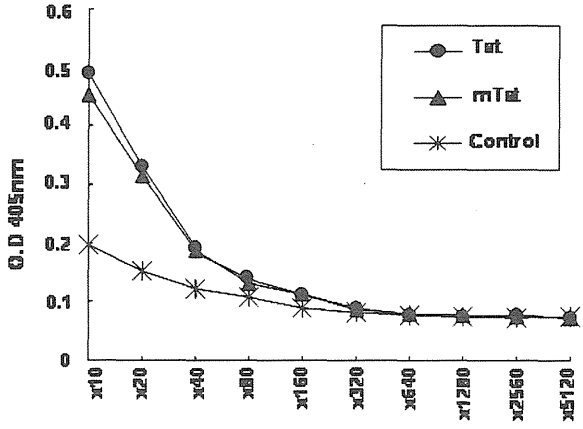

Fig. 3 Induction of IgG humoral immune response in Balb/c mice by recombinant tomato extracts. Plant extracts containing 5 μg Tat were inoculated in five mice intradermally. After 2 weeks, these mice were bled and the anti-Tat IgG antibody titer was measured by ELISA. The figures represent the mean and 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

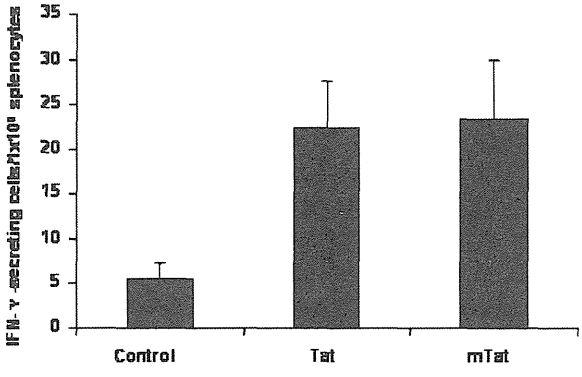

Fig. 4 Induction of cellular immune response in Balb/c mice by recombinant tomato extracts. IFN-γ secreting cells were determined by ELISPOT assay. $CD8^+$ cells were prepared from the spleen of each mouse and approximately 5–6.25 × 10^4 cells per well were incubated with the synthetic Tat peptide (Tat17-25) 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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