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.

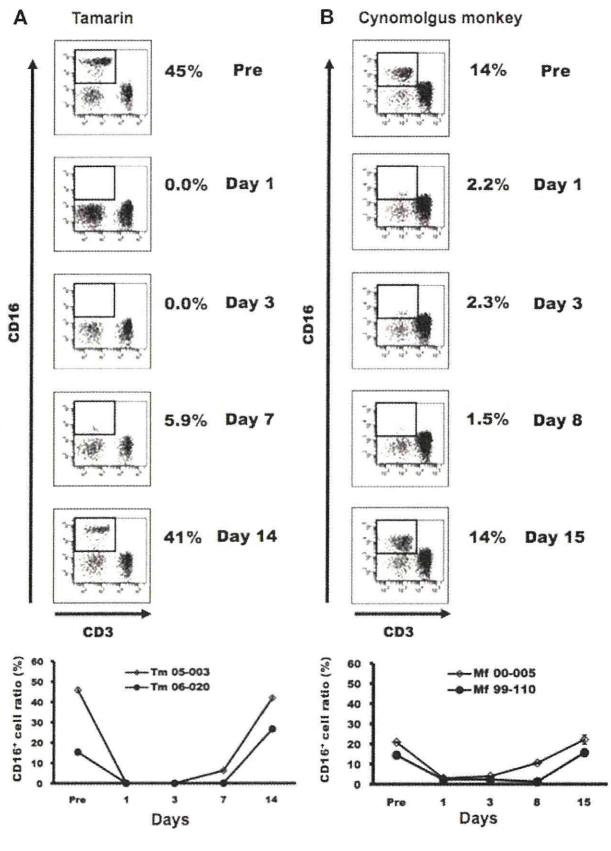

FIGURE 5 | *In vivo* depletion of CD16$^+$ NK cells using a murine anti-CD16 (3G8) mAb in tamarins. (A,B) Tamarins and cynomolgus monkeys were administered with 50 mg/kg of the 3G8 mAb. CD16$^+$ NK cell numbers were determined in whole blood specimens. Tamarins: Tm 05-003 and Tm 06-020. Cynomolgus monkeys: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

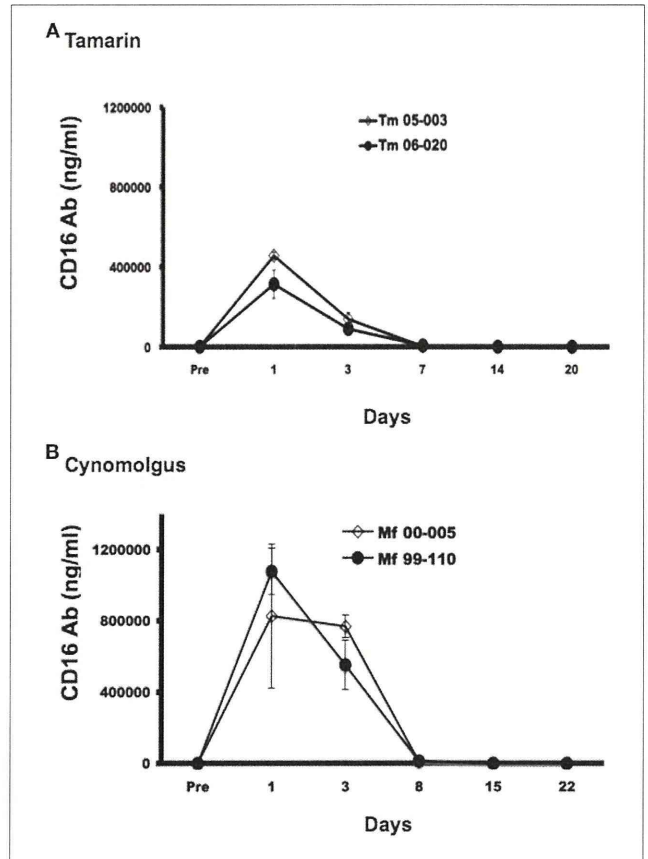

FIGURE 6 | Kinetics of concentration of CD16 (3G8) mAb *in vivo*. The concentration of CD16 (3G8) mAb in plasma was measured by ELISA as described in Section "Materials and Methods". Results shown are representative of three independent experiments. **(A)** Tamarins: Tm 05-003 and Tm 06-020. **(B)**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

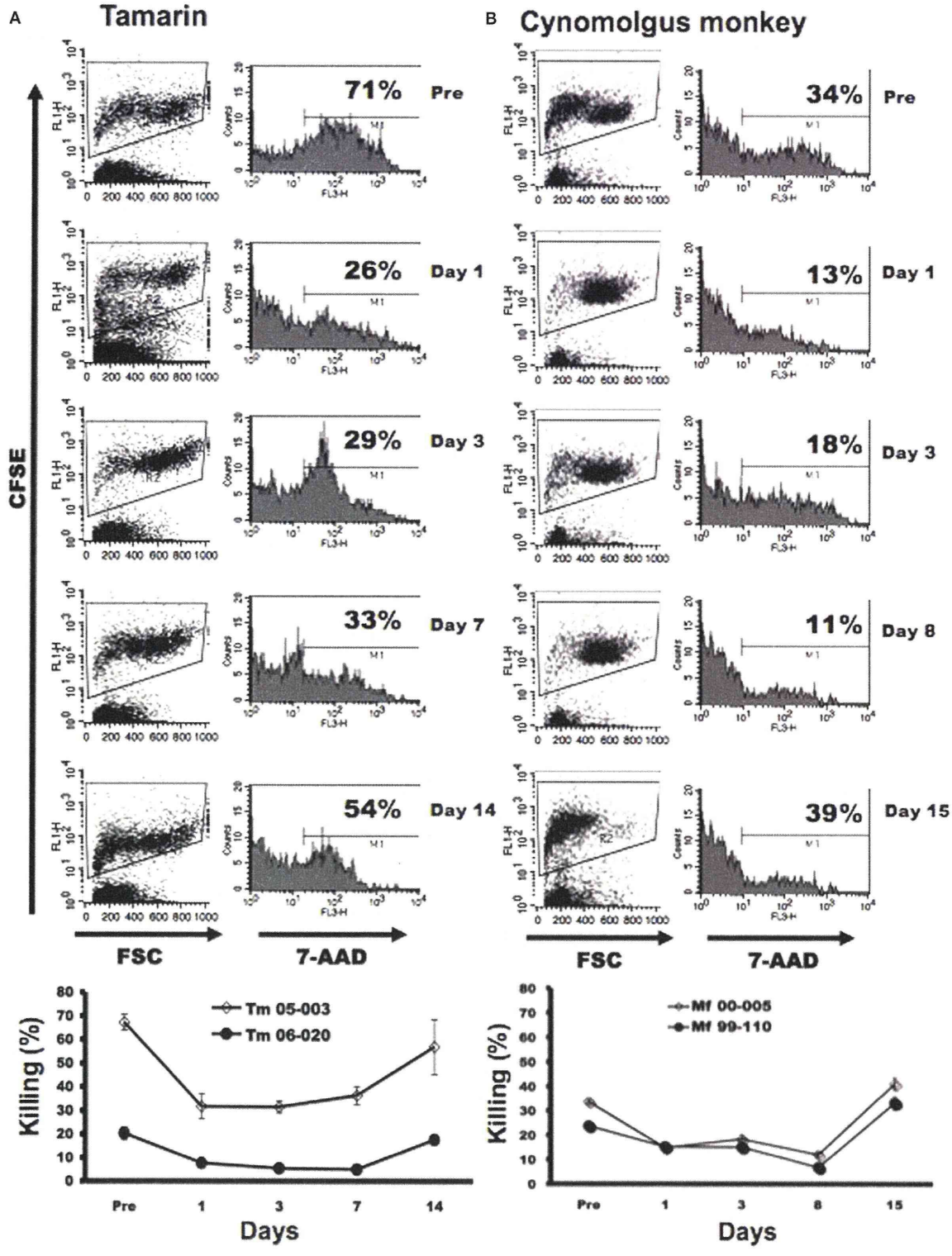

FIGURE 7 | Attenuation of natural killer activity by *in vivo* depletion of CD16$^+$ cells. (A,B) Tamarins and cynomolgus monkeys were administered 50 mg/kg of murine 3G8 mAb. The activity was determined in PBMCs of both monkeys. Tamarins: Tm 05-003 and Tm 06-020. **(B)** Cynomolgus monkeys: Mf 00-005 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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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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Acquisition of HIV-1 Resistance in T Lymphocytes Using an ACA-Specific *E. coli* mRNA Interferase

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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

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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.

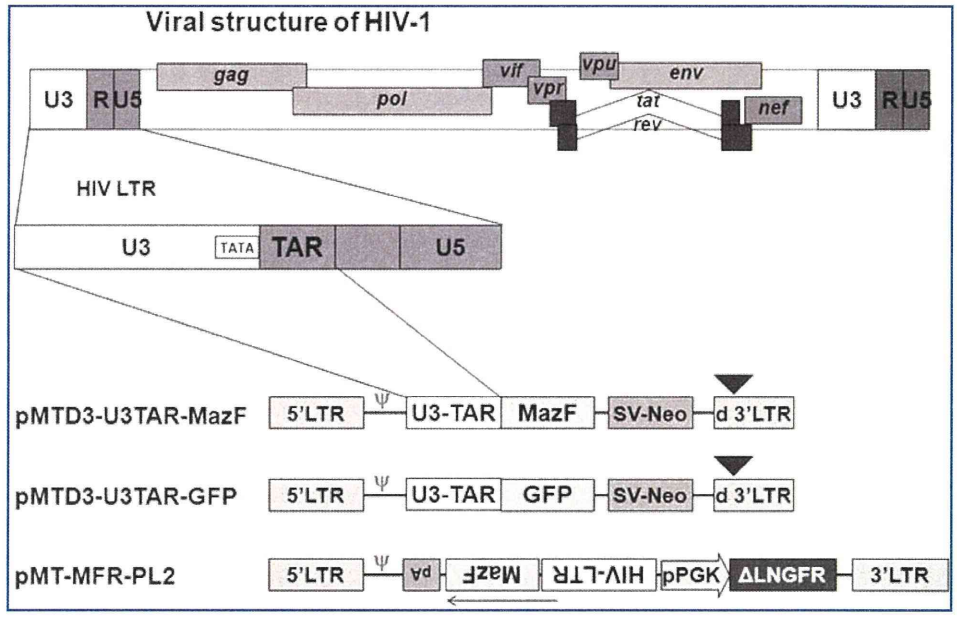

FIG. 1. Construction of retroviral vector under the control of HIV-LTR promoter. To remove promoter activity of the MoMLV LTR, the self-inactivating retroviral vector pMTD3 was constructed based on pMT (Lee *et al.*, 2004) by deleting a 276-bp fragment from its 3′LTR U3 region. A synthetic ACA-less *mazF* gene was then inserted downstream of HIV-1 U3-TAR resulting in the self-inactivating retroviral vector, pMTD3-U3TAR-MazF. As a control, the GFP gene was inserted in place of the *mazF* gene, which resulted in pMTD3-U3TAR-GFP. The self-inactivating retroviral vectors were generated using the transient transfection method with the packaging plasmids MoMLV-gag-pol, GALV-env, and the self-inactivating retroviral vector in 293T cells. The viral preparation was obtained 2 days after transfection by filtering the culture supernatant. To improve the viral titer for efficient gene transduction over an initial vector, HIV-LTR-MazF-polyA cassette was inserted in the opposite direction of the MoMLV-LTR at the multi-cloning site of pMT. A truncated form of the human low-affinity nerve growth factor gene (ΔLNGFR) (Verzeletti *et al.*, 1998) was used as a surface marker. The resultant vector plasmid was designated pMT-MFR-PL2. GALV-env retroviral vector was generated as described in Materials and Methods.

Retroviral transduction into primary rhesus macaque CD4+ T cells

Rhesus macaque CD4+ T cells were isolated from peripheral blood mononuclear cells (PBMC) using anti-CD4 monoclonal antibody-conjugated beads (Dynal CD4 Positive Isolation Kit; Invitrogen). Prior to gene transduction, the isolated CD4+ T cells were activated for 3 days with a combination of anti-monkey-CD3 clone FN-18 (BioSource, Camarillo, CA) and anti-human-CD28 monoclonal antibody clone L293 (BD Biosciences, Franklin Lakes, NJ)-conjugated beads at a cell-to-bead ratio of 1:1 in GT-T503 (Takara Bio) supplemented with 10% FBS and 200 IU of interleukin-2 (Chiron, Emeryville, CA). On day 3, activated CD4+ T cells were infected with the MazF retroviral vector (MT-MFR-PL2) in the presence of RetroNectin (Takara Bio) as per the manufacturer's instructions. The transduction was repeated again on day 4. The cells were further incubated for another 3 days. The genetically modified cells marked with the ΔLNGFR+ were concentrated with anti-CD271 monoclonal antibody-conjugated beads (CD271 MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany). Aliquots of the *mazF* gene-modified cells (designated as MazF-Tmac cells) were collected and cryopreserved until use. As a control, the nontransduced CD4+ T cells were also prepared using the same method as used above.

HIV infection

CEM-SS cells and CEM-SS cells transduced with MTD3-U3TAR-MazF or MTD3-U3TAR-GFP were infected with HIV-1 IIIB at the different multiplicities of infection (MOIs) of 0.07, 0.0007, and 0.00007. After infection, cells were washed with PBS and subsequently cultured in 10 ml of RPMI 1640 containing 10% FBS. HIV-1 p24 levels in the culture supernatant were calculated using the p24 ELISA kit (PerkinElmer, Waltham, MA). Viable cell numbers were measured using the trypan blue exclusion assay. The doubling time of cells was calculated by logistic regression analysis of each growth curve for the HIV-1 infection sets.

SHIV infection

The cryopreserved cells of the control CD4+ T and MazF-Tmac cells were recovered in GT-T503 medium supplemented with 10% FBS and 200 IU of interleukin-2 and reactivated with anti-monkey-CD3 and anti-human-CD28 monoclonal antibody-conjugated beads at a cell-to-bead ratio of 5:1. After a 6-day incubation, the cells were infected with simian/human immunodeficiency virus (SHIV) 89.6P (Reimann *et al.*, 1996) at the MOI of 0.01 and cultured for 6 more days. SHIV RNA levels in the culture supernatant and intracellular RNAs were determined by using quantitative real-time PCR (Thermal Cycler Dice Real Time System; Takara Bio Inc.) with a set of specific primers designed in the SHIV *gag* region (Miyake *et al.*, 2006).

Flow cytometry

Flow cytometry was used for the analysis of surface CD4 expression and transduction efficiency. Endogenous expression levels of CD4 in CEM-SS cells and CEM-SS cells transduced with MTD3-U3TAR-MazF were analyzed using phycoerythrin (PE)-labeled anti-human CD4 antibody (Beckman Coulter, Fullerton, CA). Intracellular p24 levels were analyzed using fluorescein isothiocyanate-labeled anti-p24 antibody (Beckman Coulter) after the cells were fixed and permeabilized for flow cytometric analysis.

Gene transfer efficiencies of the retroviral Tat expression vector into CEM-SS cells and CEM-SS cells transduced with MTD3-U3TAR-MazF were analyzed by detecting the ZsGreen marker fluorescence. Immediately before flow cytometry, propidium iodide (PI) was added at the concentration of 100 ng/ml to stain dead cells. Samples were run through a FACSCantoII flow cytometer (BD Biosciences), and data were analyzed using the FACSDiva software (BD Biosciences).

Genomic DNA analysis

Genomic DNA was extracted by phenol/chloroform extraction from CEM-SS cells and CEM-SS cells transduced with MTD3-U3TAR-MazF cells infected with HIV-1 IIIB at the MOI of 0.007. Two different regions of the HIV-1 *gag* gene (246–467 and 905–1046) were amplified by PCR at 14 days after HIV-1 IIIB infection. As a positive control, genomic DNA was amplified from H9 cells chronically infected with HIV-1 IIIB. Human mitochondrial DNA (mtDNA) was amplified as a control for the PCR.

Co-culture with chronically infected cells

The CEM-SS cell line chronically infected with HIV-1 IIIB (CH-1) was mixed with CEM-SS cells or CEM-SS cells transduced with MTD3-U3TAR-MazF. CH-1 cells were mixed at different ratios of 10, 1, or 0.1%. After 6 and 14 days of infection, intracellular p24 levels were analyzed by flow cytometric analyses.

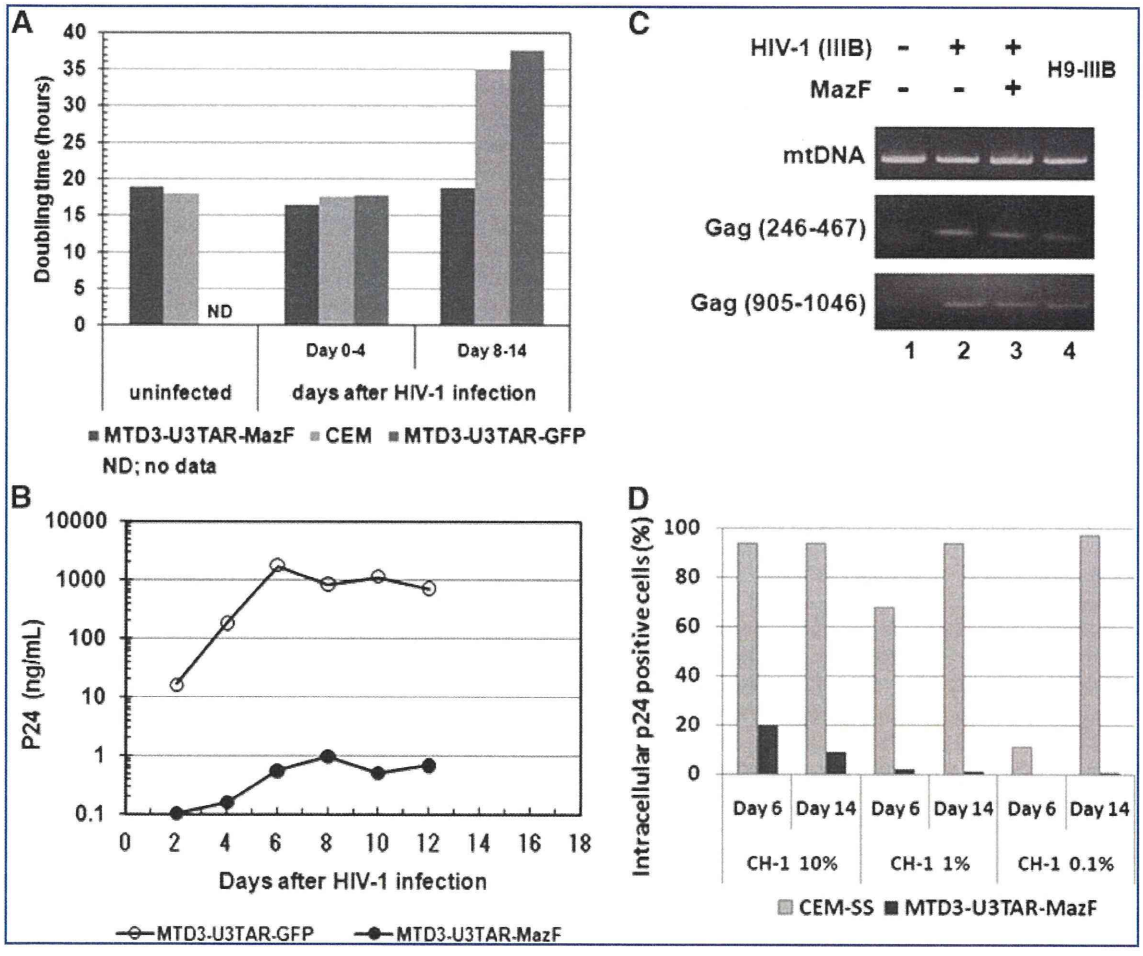

FIG. 2. Analysis of MazF-transduced CEM-SS cells after HIV-1 infection. (**A**) CEM-SS cells transduced with either the *mazF* gene or the GFP gene were infected with HIV-1 IIIB at an MOI of 0.07. After infection, the doubling time of the cells for each culture condition was calculated using linear regression analysis using Microsoft Excel software. The square of the correlation coefficient (R^2) between culture day and log (cell number) values was observed to be >0.97. (**B**) HIV-1 p24 levels in the culture supernatant were estimated using the p24 ELISA kit. Filled circles indicate p24 levels in the supernatant of CEM-SS cells transduced with MTD3-U3TAR-MazF. Open circles indicate p24 levels in the supernatant of CEM-SS cells transduced with MTD3-U3TAR-GFP. (**C**) Genomic DNA PCR analysis of CEM-SS cells and MazF-transduced CEM-SS cells infected with HIV-1 IIIB at an MOI of 0.007. Two different regions of the HIV-1 *gag* gene (246–467 and 905–1046) were amplified by PCR at 14 days after HIV-1 IIIB infection. As a positive control, the genomic DNA was amplified from H9 cells chronically infected with HIV-1 IIIB. Human mtDNA was amplified as a control for the PCR reaction. (**D**) Intracellular p24 levels were analyzed in the mixtures of CEM-SS cell lines chronically infected with HIV-1 IIIB (CH-1) using CEM-SS cells or MazF-transduced CEM-SS cells. CH-1 cells were mixed at different ratios of 10, 1, or 0.1%. After 6 and 14 days of infection, cells were stained with an anti-HIV-1 p24 antibody and subjected to flow cytometric analysis.

Results

We first constructed the retroviral vector system in which the gene for MazF was inserted downstream of the HIV-1 TAR sequence (Fig. 1). As the *E. coli mazF* gene contains nine ACA sequences in its open-reading frame, all of these ACA sequences were first engineered to other MazF-uncleavable sequences without altering the amino acid sequence of MazF to make the *mazF* mRNA resistant to MazF. The resulting self-inactivating retroviral vector (MTD3-U3TAR-MazF) was used to transduce CD4+ T lymphoid CEM-SS cells to create a system in which MazF induction in CEM-SS cells upon infection with HIV-1 effectively suppressed HIV-1 replication without causing apoptosis of infected T cells. The MTD3 retroviral vector contained an intact 5′ LTR and a mutated 3′ LTR that lacks most of the transcriptional elements present in U3. Cells transduced with the resulting retroviral vector contained the defective LTR at both ends (Yu *et al.*, 1986). The self-inactivating retroviral vector was transiently produced and subsequently transduced into the human T lymphoid line CEM-SS cells, which are highly susceptible to HIV infection. Transduced cells were subjected to G418 selection to obtain drug-resistant populations. A GFP-expressing retroviral vector under the control of HIV-LTR (MTD3-U3TAR-GFP) was also used as a control.

The growth rate of CEM-SS cells transduced with MTD3-U3TAR-MazF was comparable to that of the parental CEM-SS line (Fig. 2A), suggesting that MazF expression was tightly controlled and did not inhibit cell growth. Furthermore, the CD4 levels of MTD3-U3TAR-MazF-transduced CEM-SS cells were identical to those of the parental CEM-SS cells (Fig. 3A).

To investigate the effects of HIV-1 infection, MazF-transduced or GFP-transduced CEM-SS cells were infected with HIV-1 IIIB at different MOIs, specifically 0.07, 0.0007, and 0.00007 (Fig. 4). Levels of the HIV-1 p24 antigen in the culture media were examined 16 days post infection. As shown in Fig. 4, in MazF-transduced CEM-SS cells, HIV-1 replication was effectively suppressed. To more precisely investigate the antiviral effects of MazF, viral production and cell growth were measured every other day after HIV-1 IIIB infection at the MOI of 0.07. As shown in Fig. 2A, in the beginning of the culture from day 0 to day 4, cell growth was similar among CEM-SS cells, MazF-transduced CEM-SS cells, and GFP-transduced CEM-SS cells, as well as uninfected CEM-SS cells. CEM-SS cells harboring the *mazF* (ACA-less) gene grew at a normal rate throughout the time course of HIV-1 IIIB infection, whereas both GFP-transduced CEM-SS cells and the parental cell line showed aberrant growth rates due to HIV-1 infection in late cultures after day 8 (Fig. 2A). Indeed, a high level of p24 was detected in the GFP-transduced cell populations during the course of infection (Fig. 2B). In the case of MazF-transduced cells, however, levels of p24 were three orders of magnitude lower than those of GFP-transduced cells throughout the experiment (Fig. 2B). Notably, CD4 levels of MazF-transduced cells infected with HIV-1 IIIB were largely unaffected (Fig. 3B). Together with the fact that the HIV-1 IIIB infected cells harboring the *mazF* gene grew normally (Fig. 2A), these results suggest that HIV-1 IIIB gene expression in the HIV-1-LTR-regulated *mazF* (ACA-less)-transduced cells is effectively inhibited by blocking HIV-1 replication with little damage to cellular function.

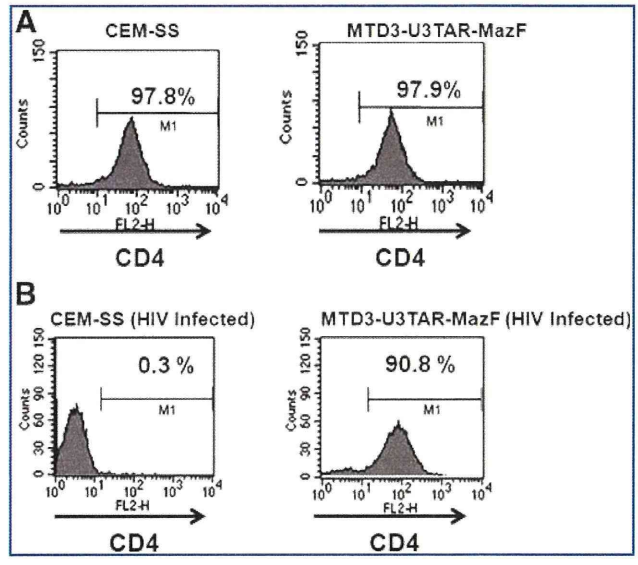

FIG. 3. CD4 levels in MazF-transduced cells. (**A**) Endogenous expression levels of CD4 were analyzed using PE-labeled anti-human CD4 antibody following flow cytometric analysis. (**B**) CEM-SS control cells and CEM-SS cells transduced with MTD3-U3TAR-MazF were infected with HIV-1 IIIB at an MOI of 0.007. After infection, the cells were maintained for 5 weeks and CD4 expression levels were analyzed using PE-labeled anti-human CD4 antibody following flow cytometric analysis.

Next, we examined if HIV-1 IIIB was integrated into the genome of MazF-transduced CEM-SS cells upon HIV-1 infection. Two different regions of the HIV-1 *gag* gene were amplified by PCR using genomic DNA 14 days after HIV-1 IIIB infection. As shown in Fig. 2C, both regions of the *gag* gene were detected in the genome of MazF-transduced CEM-SS cells, which were resistant to HIV-1 replication (lane 3). Similarly, HIV-1 DNA was detected in the genomes of CEM-SS cells (lane 2) and H9-IIIB cells (lane 4) (positive control H9 cells chronically infected with HIV-1 IIIB), whereas no bands were detected in noninfected cells (lane 1). We also established a CEM-SS cell line chronically infected with HIV-1 IIIB (CH-1). When this cell line was mixed with CEM-SS cells or MazF-transduced CEM-SS cells at a ratio of 10, 1, or 0.1%, CEM-SS cells were gradually infected with HIV-1 produced from CH-1 cells (Fig. 2D) and their cell growth was suppressed. Alternatively, MazF-transduced CEM-SS cells showed no growth inhibition (data not shown), indicating that HIV-1 replication was suppressed in MazF-transduced CEM-SS cells. As a result, the culture was eventually taken over by normally growing MazF-transduced CEM-SS cells over the slow-growing CH-1 cells. These data demonstrate that MazF-transduced cells are resistant to HIV-1 IIIB infection by blocking HIV-1 IIIB replication.

To investigate the *mazF* gene expression and subsequent effects more precisely, CEM-SS cells and CEM-SS cells transduced with MTD3-U3TAR-MazF were infected with the Tat-expressing retroviral vectors, M-LTR-Tat-ZG or H-LTR-Tat-ZG (Fig. 5A). Induction of the m*azF* gene in CEM-SS cells transduced with MTD3-U3TAR-MazF was monitored by real-time PCR, and the relative ratios were compared with mock infection (Fig. 5B). Infected cells were also subjected to

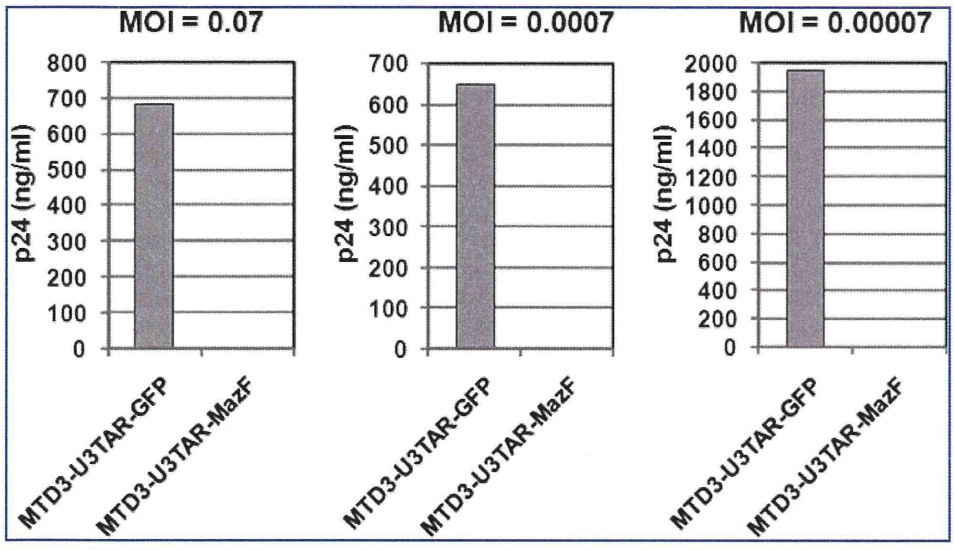

FIG. 4. HIV-1 IIIB infection using MazF-transduced CEM-SS cells at different MOIs. Polyclonal cell populations of CEM-SS resulting from gene transduction with retroviral vectors MTD3-U3TAR-MazF or MTD3-U3TAR-GFP were infected with HIV-1 IIIB at different MOIs (0.07, 0.0007, and 0.00007). Sixteen days after infection, HIV-1 p24 levels in the culture supernatant were estimated using the p24 ELISA kit (PerkinElmer). Given the cytopathic effect of HIV-1, the MTD3-U3TAR-GFP cell population showed delayed proliferation after HIV-1 infection in contrast to the MTD3-U3TAR-MazF population. The delay was more pronounced for the high-MOI group (0.07) than for the low-MOI group (0.00007) at later time points. On day 16 post infection, the accumulated cell number of the high-MOI group was threefold lower than that of the low-MOI group, so the difference in HIV-1 p24 levels between the two MOI groups (0.07 and 0.00007) reflects total cell numbers.

flow cytometry, and both Tat-positive (ZsGreen-positive) cells and dead cells (PI-positive) were monitored (Fig. 5B). As shown in Fig. 5B, strong induction of *mazF* expression was observed upon constitutive M-LTR-Tat-ZG vector transduction, and there was a significant decline in Tat-positive (ZsGreen-positive) cell population. On the other hand, *mazF* induction in HIV-LTR-driven Tat expression was lower, and the influence on cell death was also less than by MLV-LTR-driven Tat expression as observed in the PI-positive population. Although these experiments do not directly reflect HIV-1 replication, these data support the hypothesis that only low levels of MazF are expressed upon HIV-1 infection and MazF-positive cells can survive with HIV-1 provirus.

As the SIN-based retroviral vector contains the *mazF* gene in the normal orientation, the *mazF* gene is expressed from viral mRNA, resulting in the degradation of the viral RNA and thus significantly reducing the viral titer from this vector. On the other hand, when the MazF expression cassette is

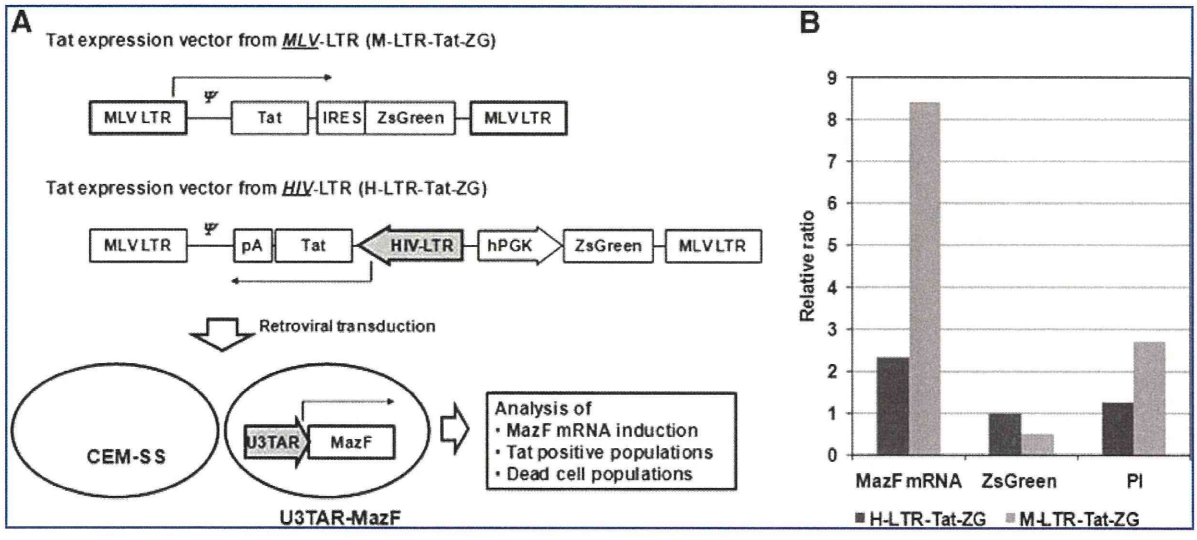

FIG. 5. Analysis of MazF induction upon Tat expression. (**A**) Outline of experimental procedure to analyze MazF induction upon Tat expression. (**B**) MazF mRNA levels were analyzed in MTD3-U3TAR-MazF transduced CEM-SS cells after Tat-expressing retroviral vector infection using real-time RT-PCR. The relative fold change is shown compared with that of mock infections. Tat-positive (ZsGreen-positive) cell populations and dead (PI-positive) cell populations in MTD3-U3TAR-MazF-transduced CEM-SS cells were analyzed by flow cytometry 2 days after different Tat retroviral vector transduction. The relative ratio is shown compared with that of CEM-SS cells.

inserted in the opposite direction from the retroviral genome, the viral titer increased and the gene transfer efficiency was improved more than 10 times (data not shown). To investigate the antiviral effect of the TAR-*mazF* system in the primary CD4+ T lymphocytes, the reversely orienting MT-MFR-PL2 vector was introduced into rhesus macaque primary CD4+ T cells from two individual monkeys (#14 and #15). The resulting *mazF*-containing cells were then infected with SIV/HIV-1 chimeric virus SHIV 89.6P. As the SHIV 89.6P harbors HIV-1-derived *env*, *rev*, *vpu*, and *tat* genes, the TAR-*mazF* system is expected to function when MazF-Tmac cells are infected with SHIV 89.6P. Indeed, efficient suppression of SHIV 89.6P replication was observed for both primary cell lines, #14 and #15 (Fig. 6A).

To evaluate further how well the retroviral *mazF* system is able to suppress viral RNA production, total cellular RNAs were extracted from MazF-Tmac cells to estimate quantitatively the amounts of SHIV RNA, as well as the mRNAs for ribosomal protein L13a (RPL13a, XM_001093017) and β-actin (NM_001033084), by real-time PCR. The relative ratios were normalized by using 18S rRNA (FJ436026), which is protected from MazF cleavage in ribosomes (Shimazu *et al.*, 2007). We obtained similar results in MazF-Tmac cells from both #14 and #15 primary cell lines. Representative results from MazF-Tmac cells from #14 are shown in Fig. 6B, where one can see that SHIV RNA was preferentially cleaved, whereas the cellular mRNAs were not affected. These results clearly demonstrate that MazF induction from the Tat system upon SHIV 89.6P infection leads to severe defect in maintaining SHIV 89.6P RNA but does not affect cellular mRNAs in SHIV-infected CD4+ T cells.

Discussion

This study demonstrates the distinct feasibility of RNase-based strategies for gene therapy. RNase-based strategies may be preferred over RNA-based strategies for HIV therapy, because RNases cleave HIV-RNA to cause permanent damage to HIV RNA function. Additionally, as RNases function as an enzymatic catalyst, they are required only at low concentrations in the cells to effectively block HIV proliferation. In the present study, the gene for MazF, an ACA-specific mRNA interferase, was engineered under the HIV-1 LTR promoter and inserted in the genome of the CD4+ T lymphoid cells so that MazF is expected to be produced only when the cells are infected with HIV-1 to produce the Tat protein. We demonstrated that *mazF*-Tmac cells indeed acquired resistance against SHIV replication, but cell growth was not inhibited after SHIV infection (data not shown), indicating that cellular mRNAs were not significantly affected. Notably, MazF was also able to function against the expression of SHIV proviral genome, because the production of SHIV in the culture supernatant was dramatically reduced.

Acquisition of HIV-1 resistance, and more remarkably the ability of MazF-transduced cells to suppress HIV-1 replication, may be explained as follows: Upon HIV-1 infection, Tat expression is first induced from the HIV-1 proviral genome. Tat then triggers the transcription of the *mazF* gene under the LTR promoter, as well as the full-length HIV proviral genome. The resulting induction of MazF expression leads to the cleavage of newly emerged HIV-1 mRNAs so that Tat protein synthesis is no longer sustainable. However, it is important to note that HIV-1 infection does not hamper cell growth and that the HIV-1 provirus genome is retained in the MazF-transduced cells. Therefore, the cellular level of Tat appears to be maintained at a very low level so that the level of MazF induction is also kept very low enough to cleave HIV-1 mRNAs, but not cellular mRNAs. Depending on the integration site and proviral copy number, there might be some MazF-transduced cells that were not resistant to HIV-1 replication. However, these cells could not survive due to HIV-1-induced cell death.

In mammals, virus infection is known to activate the interferon response to induce RNaseL, which mediates degradation of 28S and 18S ribosomal RNAs. This results in inhibition of protein synthesis as part of the host antiviral response (Silverman, 2003). An amphibian ribonuclease,

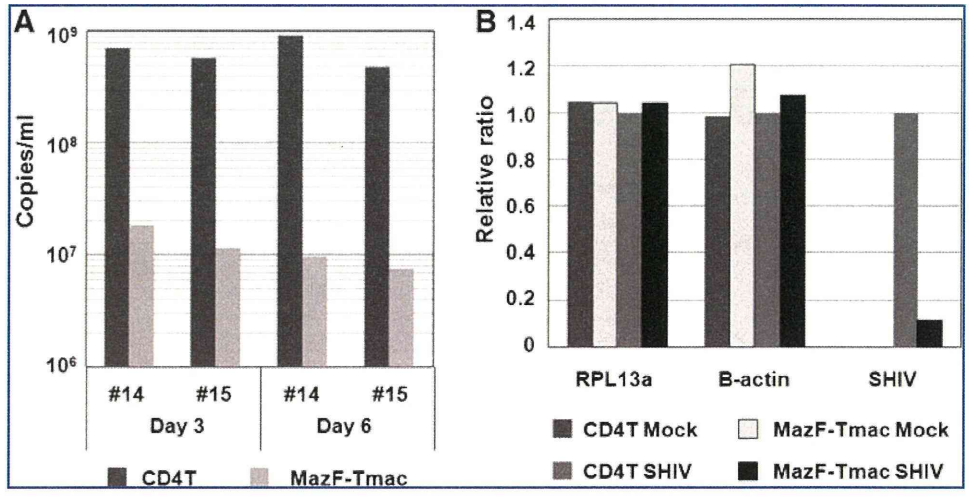

FIG. 6. Effect of MazF-induction into rhesus macaque primary CD4+ T cells on SHIV 89.6P replication. (**A**) Rhesus macaque primary CD4 T cells from two monkeys (#14 and #15) were activated and transduced with MT-MFR-PL2 vector. The MazF-transduced cells (MazF-Tmac cells) were reactivated with CD3/28 beads followed by infection with SHIV 89.6P. On days 3 and 6 post infection, culture supernatants were collected and evaluated for SHIV RNA copy by using the quantitative real-time PCR method. (**B**) Total cellular RNAs extracted from MazF-Tmac cells at 6 days post SHIV 89.6P infection were used to measure the amounts of SHIV RNA, as well as cellular housekeeping mRNAs, by using the quantitative real-time PCR method.

Onconase, is able to inhibit protein synthesis in mammalian cells and has been used as a protein drug. When it was added to the culture media of H9 cells persistently infected with HIV-1, HIV-1 replication was inhibited without blocking cell growth, as degradations of 18S and 28S rRNAs and cellular mRNAs were prevented (Saxena *et al.*, 1996). MazF induction in mammalian cells has shown to cause apoptotic cell death as a result of degradation of cellular mRNAs (Shimazu *et al.*, 2007). However, in the present study, MazF expression induced by HIV-1 Tat appears to be maintained at very low levels, just enough to cleave HIV-1 RNA but not cellular mRNAs, so that cells were able to grow normally. MazF expression may be autoregulated in the cell in such a way that when Tat-induced MazF eliminates invading HIV-1 RNA, Tat expression from the HIV-1 provirus is simultaneously stopped, resulting in simultaneous arrest of MazF production to recover normal cellular functions.

Targeting HIV RNA as a therapeutic strategy using antisense RNA (Levine *et al.*, 2006), ribonucleases (Agarwal *et al.*, 2006), and RNA interference (RNAi) technology (Morris and Rossi, 2004) has been attempted. However, the use of antisense RNA and RNAi technology has not been effective as an anti-HIV technology, as HIV can easily circumvent these RNA inhibitors by creating mutations at the target sequence regions (Lee and Rossi, 2004). On the other hand, the present strategy using MazF targets abundant ACA sequences in HIV-1 RNA (>240), so that it is not possible for HIV-1 to escape from MazF attack by mutations. Furthermore, because MazF has no homology to any mammalian ribonucleases, MazF mRNA interferase activity cannot be inhibited by ribonuclease inhibitors existing in mammalian cells.

In summary, the use of MazF appears to be a novel and highly effective tool for anti-HIV gene therapy. It is effectively able to suppress HIV-1 replication, preventing the emergence of mutated HIV-1. Importantly, MazF induction by invading HIV-1 shows little toxicity to host cells while it efficiently suppresses HIV-1 replication. Specific inhibition of HIV-1 replication by MazF without affecting cell growth is the key feature of MazF-based HIV-1 gene therapy. This may be the first step for RNase-based HIV-1 gene therapy with efficacy *in vitro*. The feasibility of the MazF-based *ex vivo* gene therapy may be verified using autologous CD4+ T lymphocytes from HIV-1 patients. To use our *mazF* vector system for gene therapy, its safety has to be critically evaluated and it should not have any negative impacts on T-cell function. For example, it needs to be shown that there is no alteration in the secretion of functionally important cytokines even though it was observed that MazF expression in HIV-infected CD4+ T cells does not inhibit cell growth. We are currently addressing this question.

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Author Disclosure Statement

No competing financial interests exist.

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Interleukin-1 Family Cytokines as Mucosal Vaccine Adjuvants for Induction of Protective Immunity against Influenza Virus▽

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A safe and potent adjuvant is needed for development of mucosal vaccines against etiological agents, such as influenza virus, that enter the host at mucosal surfaces. Cytokines are potential adjuvants for mucosal vaccines because they can enhance primary and memory immune responses enough to protect against some infectious agents. For this study, we tested 26 interleukin (IL) cytokines as mucosal vaccine adjuvants and compared their abilities to induce antigen (Ag)-specific immune responses against influenza virus. In mice intranasally immunized with recombinant influenza virus hemagglutinin (rHA) plus one of the IL cytokines, IL-1 family cytokines (i.e., IL-1α, IL-1β, IL-18, and IL-33) were found to increase Ag-specific immunoglobulin G (IgG) in plasma and IgA in mucosal secretions compared to those after immunization with rHA alone. In addition, high levels of both Th1- and Th2-type cytokines were observed in mice immunized with rHA plus an IL-1 family cytokine. Furthermore, mice intranasally immunized with rHA plus an IL-1 family cytokine had significant protection against a lethal influenza virus infection. Interestingly, the adjuvant effects of IL-18 and IL-33 were significantly decreased in mast cell-deficient *W/W*^{v} mice, indicating that mast cells have an important role in induction of Ag-specific mucosal immune responses induced by IL-1 family cytokines. In summary, our results demonstrate that IL-1 family cytokines are potential mucosal vaccine adjuvants and can induce Ag-specific immune responses for protection against pathogens like influenza virus.

Because most pathogenic viruses, including influenza virus, enter through a mucosal surface (18), preventing infection at the viral entry site by inducing mucosal immunity should be an effective strategy for combating such pathogens. A key aspect of mucosal immunity is production of secretory immunoglobulin A (sIgA), as well as induction of cytolytic T lymphocytes (CTLs) against epithelium-transmitted pathogens (5, 21). Therefore, it is important to develop mucosal vaccines that induce effective immune responses at mucosal surfaces (31).

However, protein subunit antigens (Ags) generally evoke only a weak or undetectable adaptive immune response when administered intramucosally (1). Therefore, to produce effective mucosal vaccines, it is necessary to develop an appropriate mucosal vaccine adjuvant (34). Cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin are known potent mucosal vaccine adjuvants and have been used in nonclinical experimental systems (9, 27). However, their clinical application as nasal adjuvants had to be discontinued because of side effects such as Bell's palsy (29). Therefore, mucosal vaccine adjuvants with high efficacy and safety for clinical application continue to be urgently required.

Cytokines are key molecules that trigger the innate and adaptive immune responses (including maturation of Ag-presenting cells, differentiation of Th1 and Th2 cells, and induction of cytotoxic natural killer [NK] cells and CTLs), resulting in protective layers against virus infection (11, 41, 43). Therefore, cytokines are promising vaccine adjuvants for enhancing the immune response against infectious pathogens. At present, more than 30 members of the interleukin (IL) cytokine/IL receptor family have been identified and found to be involved

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in regulating and maintaining homeostasis of the immune system (3, 14). Specific IL cytokines have been used as vaccine adjuvants to enhance primary and memory immune responses against some cancers and infectious diseases (2, 6). However, there has been no comparative study of IL cytokines as mucosal vaccine adjuvants.

Recently, it was pointed out that identification of the cellular targets of vaccine adjuvants is an important issue (12). Dendritic cells (DCs) are responsible for Ag uptake and presentation to naive T cells and represent a key target for adjuvant activity (22, 33). Recent reports have demonstrated that other accessory cells, such as mast cells (MCs) and NKT cells, act as immunosensors to initiate and modulate innate and adaptive immune responses (16, 40). It has been reported that MCs contribute to the induction of an adaptive immune response or accessory function and that the synthetic Toll-like receptor 7 ligand imiquimod acts as a mucosal vaccine adjuvant in an MC-dependent manner (19). However, it is still not clear whether MCs are promising cellular targets for cytokine adjuvants in mucosal vaccines.

In this study to develop effective and safe mucosal vaccine adjuvants, we identified promising cytokines with mucosal vaccine adjuvant activity by screening 26 different IL cytokines. We also investigated the mucosal and systemic immune responses induced by these cytokines in normal and MC-deficient mice. The IL-1 family cytokines (IL-1α, IL-1β, IL-18, and IL-33) were found to be effective mucosal vaccine adjuvants for induction of protective sIgA and CTL immunity against influenza virus. In addition, the adjuvant activities of IL-18 and IL-33 were MC dependent.

MATERIALS AND METHODS

Cytokines and Ags. CT was purchased from List Biological Laboratories (Campbell, CA). Twenty-six types of mouse recombinant IL cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-28B, IL-31, and IL-33) were purchased from R&D Systems (Minneapolis, MN). Baculovirus-expressed recombinant influenza virus hemagglutinin (rHA) derived from influenza virus A/New Caledonia/20/1999 (Protein Sciences, Meriden, CT) was used as the vaccine Ag.

Mice and immunization protocols. Female BALB/c mice and MC-deficient (WBB6F1 *W/W^v*) and congenic littermate control (WBB6F1 WT) mice were purchased from Japan SLC (Hamamatsu, Japan) and used at 6 weeks of age. All animal experimental procedures used in this study were performed in accordance with our institutional guidelines for animal experiments. Mice were immunized intranasally with rHA alone (1 μg/mouse), rHA (1 μg/mouse) plus CT (1 μg/mouse), or rHA (1 μg/mouse) plus one of the IL cytokines (0.1 μg, 0.3 μg, or 1.0 μg/mouse) on days 0 and 28.

Sample collection. Fourteen days after the final immunization, plasma and mucosal secretions (nasal washes, saliva, vaginal washes, and fecal extracts) were obtained as previously described (24).

Detection of Ab responses by ELISA. rHA-specific antibody (Ab) levels in plasma and mucosal secretions were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (24). Briefly, ELISA plates were coated with 2 μg rHA/ml of 0.1 M carbonate buffer and incubated overnight at 4°C. The plates were then incubated with blocking solution (Block Ace; DS Pharma Biomedical, Osaka, Japan) at 37°C for 2 h. Diluted plasma or mucosal secretions were added. After incubation at 37°C for 2 h, the coated plates were washed with phosphate-buffered saline (PBS)–polyoxyethylene sorbitan monolaurate (Tween 20; Wako Pure Chemical, Tokyo, Japan) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG solution to detect IgG in plasma or with a biotin-conjugated goat anti-mouse IgA detection Ab (Southern Biotechnology Associates, Birmingham, AL) solution to detect sIgA in mucosal secretions, at 37°C for 2 h. For detection of sIgA, the plates were incubated with HRP-coupled streptavidin (Zymed Laboratories, South San Francisco, CA) for 1 h at room temperature. After incubation, a color reaction was developed with tetramethylbenzidine (Moss, Inc., Pasadena, MD), stopped with 2 N H_2SO_4, and measured as the optical density at 450 to 655 nm ($OD_{450-655}$) in a microplate reader.

Multiplex cytokine assay. Splenocytes from immunized BALB/c, WBB6F1 *W/W^v*, or WBB6F1 WT mice were harvested 14 days after the final immunization and stimulated *in vitro* with 10 μg rHA/ml. After 72 h, culture supernatants from *in vitro* unstimulated and rHA-stimulated cells were analyzed by a Bio-Plex multiplex cytokine assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Samples were analyzed on a Luminex 100 analyzer (Luminex, Austin, TX). The mean concentrations of cytokines in supernatants from rHA-stimulated cells were calculated relative to those in unstimulated cells.

IFN-γ ELISPOT assay. Splenocytes from immunized mice were harvested 14 days after the final immunization and stimulated at a cell density of 1×10^7 cells/ml with a mixture of two H-2K^d-restricted class I HA peptides, $HA_{240-248}$ (IYSTVASSL) and $HA_{462-470}$ (LYEKVKSQL) (MBL, Nagoya, Japan), at a final concentration of 10 μg total peptide/ml complete RPMI (25). After 24 h of incubation at 37°C, plates were washed, and gamma interferon (IFN-γ)-producing cells were measured by use of an enzyme-linked immunospot (ELISPOT) assay kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Tetramer assay. Splenocytes from immunized mice were harvested 14 days after the final immunization and used as effector cells to determine $HA_{240-248}$-specific CTL responses. Splenocytes (7×10^6 cells) were added to wells in a 24-well plate, followed by addition of 1 ml of medium containing a CTL epitope peptide ($HA_{240-248}$; IYSTVASSL) at a final concentration of 1 μg/ml. After incubation at 37°C for 2 days, medium containing human recombinant IL-2 (rIL-2) (Shionogi Co., Osaka, Japan) was added to each well of CTL effector cells, to a final concentration of 10 U human rIL-2/ml. Effector cells were stained for tetramers after restimulation for 7 days. For analysis, 1×10^6 cells were treated with purified anti-mouse CD16/CD32 Ab (Fc-γ III/II receptor Ab; BD Biosciences Pharmingen, San Diego, CA) and then stained with phycoerythrin (PE)-conjugated H-2K^d-$HA_{240-248}$ peptide tetramer (MBL, Nagoya, Japan) for 20 min at room temperature. Fluorescein isothiocyanate (FITC)-conjugated CD8α (clone KT15; MBL, Nagoya, Japan) was added for an additional 20 min. Cells were analyzed with a FACS Canto flow cytometer (BD Biosciences Pharmingen). Data analysis was done with FlowJo (TreeStar, Eugene, OR) software.

Histopathological analysis. BALB/c mice were immunized intranasally with rHA (1 μg/mouse), with or without IL-1α, IL-1β, IL-18, or IL-33 (1 μg/mouse), on days 0 and 28. Fourteen days after the final immunization, the heads of the mice were severed from the bodies and placed in fixative solution (4% paraformaldehyde). The samples then were sectioned and stained with hematoxylin and eosin (H&E) or Luna stain and examined for pathological changes under a light microscope. Histopathological examination was performed by the Applied Medical Research Laboratory (Osaka, Japan).

Influenza virus infection *in vivo*. To examine the prophylactic effect of IL cytokine treatment against influenza virus, mice were immunized intranasally on days 0 and 28 with 1 μg PR8 HA vaccine (inactivated-product vaccine with influenza virus A/Puerto Rico/8/34) (Charles River, North Franklin, CT)/mouse plus 1 μg CT or IL-1 family cytokine/mouse. Fourteen days after the final immunization, mice were fully anesthetized by intraperitoneal injection of pentobarbital, and each was infected by intranasal application of 25 μl PBS containing 256 hemagglutinating units (HAU) of influenza virus A/PR/8/34 (H1N1) (kindly provided by the Research Institute for Microbial Diseases of Osaka University, Osaka, Japan) per mouse. This procedure produced upper and lower respiratory tract infections.

Statistical analysis. All results are expressed as means ± standard errors of the means (SEM). Differences were compared using Bonferroni analysis of variance (ANOVA).

RESULTS

Comparative analysis of rHA-specific Ab responses induced by 26 different IL cytokines. One potential advantage of successful mucosal immunization is the possibility of eliciting both systemic IgG and mucosal sIgA Ab responses against invading pathogens. Therefore, in this study, we tested 26 different IL cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-28B, IL-31, and IL-33)

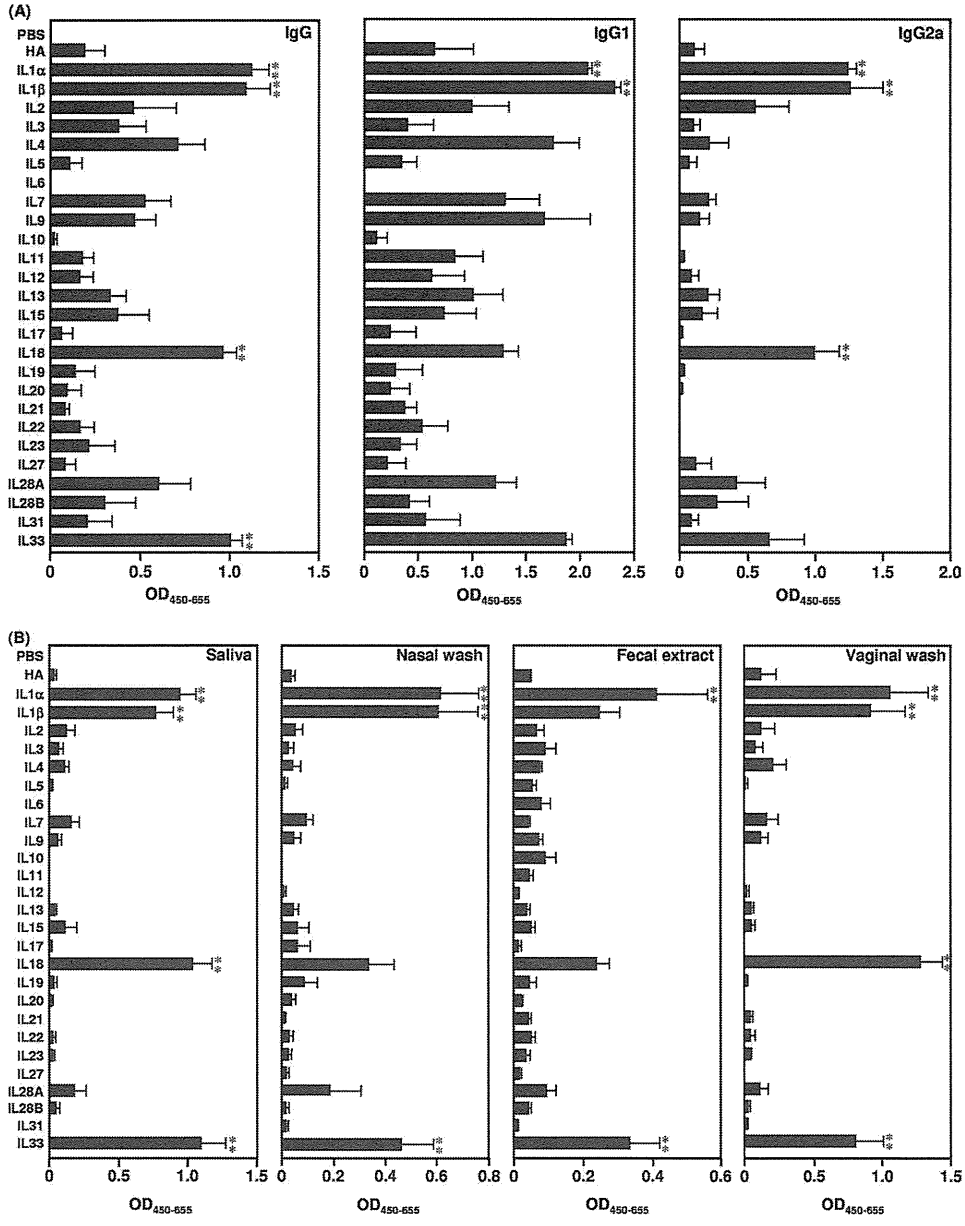

FIG. 1. Ab responses induced by IL-1 family cytokines. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone or rHA plus each interleukin. (A) Plasma was collected 14 days after the final immunization and analyzed by ELISA for rHA-specific IgG, IgG1, and IgG2a. (B) Saliva, nasal washes, fecal extracts, and vaginal washes were collected 14 days after the final immunization and analyzed by ELISA for rHA-specific sIgA. Data are presented as means ± SEM ($n = 5$). **, $P < 0.01$ compared to the value for the rHA-treated group.

as mucosal vaccine adjuvants. To examine the potential of these IL cytokines as mucosal vaccine adjuvants, BALB/c mice were immunized intranasally with 1 μg rHA plus 1 μg of an IL cytokine on days 0 and 28. Fourteen days after the final immunization, we examined the level of anti-rHA IgG in plasma by ELISA (Fig. 1A). Intranasal immunization with rHA plus 11 of the IL cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-7, IL-9, IL-13, IL-15, IL-18, IL-28A, and IL-33) induced higher rHA-specific IgG responses in plasma than those for mice immunized with rHA alone (Fig. 1A). In particular, immunization with rHA plus IL-1α, IL-1β, IL-18, or IL-33, referred to as IL-1 family cytokines, resulted in the highest rHA-specific IgG responses among the IL cytokines. The IgG subclass of the rHA-specific responses was then examined to assess the type of immune response induced by the 26 IL cytokines (Fig. 1A). Plasma Ag-specific IgG subclasses reflect the subset of $CD4^+$ T-helper cells induced by vaccination, with IgG1 and IgG2a corresponding to Th2 and Th1 responses, respectively. Consistent with the rHA-specific IgG responses, intranasal immunization with rHA plus IL-2, IL-3, IL-4, IL-7, IL-9, IL-13, IL-15, or IL-28A generally produced a greater rHA-specific IgG1 subclass response than immunization with rHA alone but a similar IgG2a response to that with rHA alone. In contrast, mice immunized with rHA plus IL-1 family cytokines showed