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Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge

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ABSTRACT

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype *90-088-Ij* dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

1. Introduction

In human and simian immunodeficiency virus (HIV and SIV) infections, cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on viral replication but fail to control viremia leading to AIDS progression [1–5]. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. It is important to determine how prophylactic CTL memory induction affects CTL responses in the acute phase post-viral exposure.

We previously developed a prophylactic AIDS vaccine (referred to as DNA/SeV-Gag vaccine) consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag [6]. Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication [7]. In particular, vaccination consistently resulted in SIV control in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [8]; $Gag_{206-216}$ (IINEEAADWDL) and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses were shown to be responsible for this vaccine-based SIV control [9]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive macaques that received a prophylactic DNA/SeV vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), all the vaccinees controlled SIV replication [10]. This single epitope vaccination resulted in dominant $Gag_{241-249}$-specific CTL responses with delayed $Gag_{206-216}$-specific CTL induction after SIV challenge, whereas $Gag_{206-216}$-specific and

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$Gag_{241-249}$-specific CTL responses were detected equivalently in unvaccinated *90-120-Ia*-positive animals.

These previous results in vaccine-based SIV controllers indicate dominant induction of vaccine antigen-specific CTL responses post-challenge, implying that prophylactic vaccination inducing vaccine antigen-specific CTL memory may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as non-vaccine antigens) post-viral exposure. In these SIV controllers, the reduction of viral loads could be involved in delay of SIV non-vaccine antigen-specific CTL responses. Then, in the present study, we examined the influence of prophylactic vaccination on immunodominance post-challenge in those vaccinees that failed to control SIV replication. Our results showed dominant induction of vaccine antigen-specific CTL responses post-challenge even in these SIV non-controllers.

2. Materials and methods

2.1. Animal experiments

The first set of experiment used samples in our previous experiments of six Burmese rhesus macaques (Macaca mulatta) possessing the MHC-I haplotype *90-088-Ij* (macaques R02-004, R02-001, and R03-015, previously reported [7,11]; R04-014, R06-022, and R04-011, unpublished). Three of them, R02-001, R04-011, and R03-015, received a prophylactic DNA/SeV-Gag vaccine [7]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian–human immunodeficiency virus $SHIV_{MD14YE}$ [12] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [13,14]. All six *90-088-Ij*-positive animals including three unvaccinated and three vaccinated were challenged intravenously with 1000 50% tissue culture infective doses (TCID50) of SIVmac239 [15] approximately 3 months after the boost. At week 1 after SIV challenge, macaque R03-015 was inoculated with nonspecific immunoglobulin G as previously described [11].

In the second set of experiment, unvaccinated (R06-001) and vaccinated (R05-028) rhesus macaques possessing the MHC-I haplotype *90-120-Ib* were challenged intravenously with 1000 TCID50 of SIVmac239. The latter R05-028 were immunized intranasally with F-deleted SeV-Gag approximately 3 months before the challenge.

In the third, three rhesus macaques received FMSIV plus mCAT1-expressing DNA vaccination three times with intervals of 4 weeks. The FMSIV DNA was constructed by replacing *nef*-deleted $SHIV_{MD14YE}$ with Friend murine leukemia virus (FMLV) *env*, carrying the same SIVmac239-derived antigen-coding regions with SIVGP1, as described before [16]. Vaccination of macaques with FMSIV and a DNA expressing the FMLV receptor (mCAT1) [17] three times with intervals of a week was previously shown to induce mCAT1-dependent confined FMSIV replication resulting in efficient CTL induction while vaccination three times with intervals of 4 weeks in the present study resulted in marginal levels of responses (data not shown). These three DNA-vaccinated animals were challenged intravenously with 1000 TCID50 of SIVmac239 approximately 2 months after the last vaccination.

Some animal experiments were conducted in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates, in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases, and others were in Institute for Virus Research, Kyoto University in accordance with the institutional regulations.

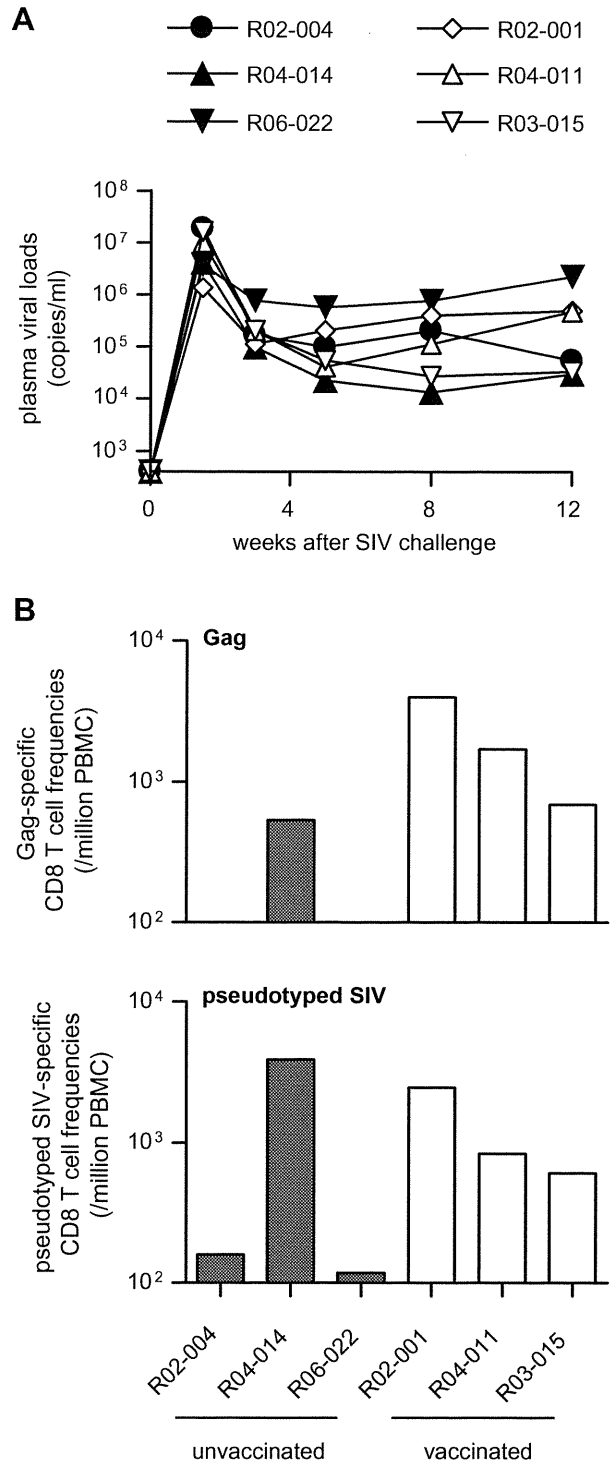

Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described previously [7]. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at week 2 after SIV challenge.

2.2. Analysis of virus-specific CTL responses

We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific

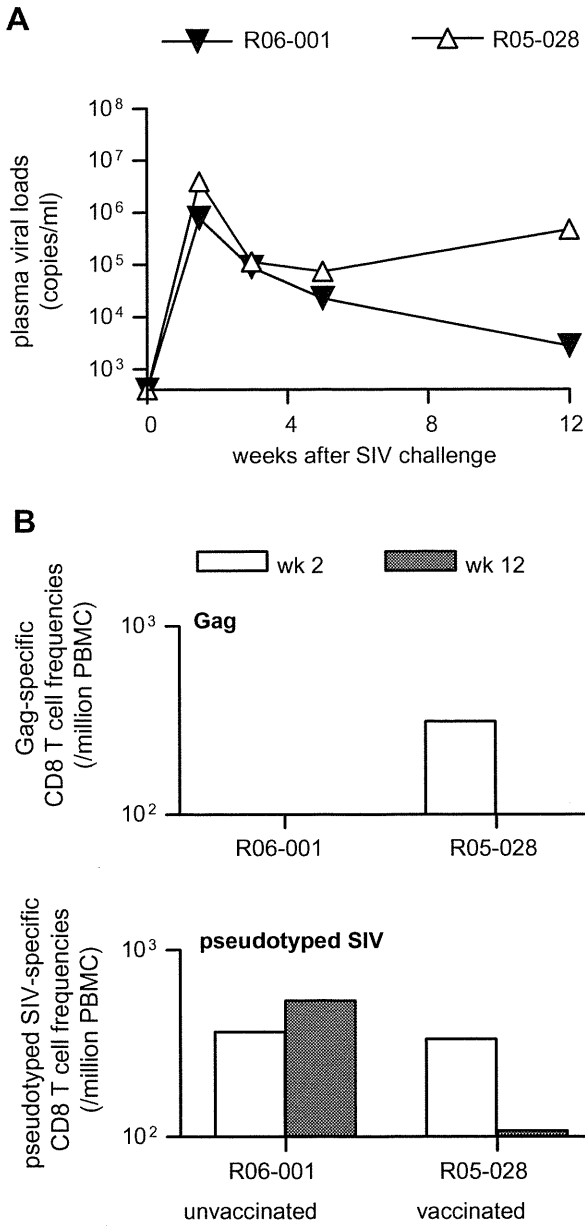

Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06-001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge.

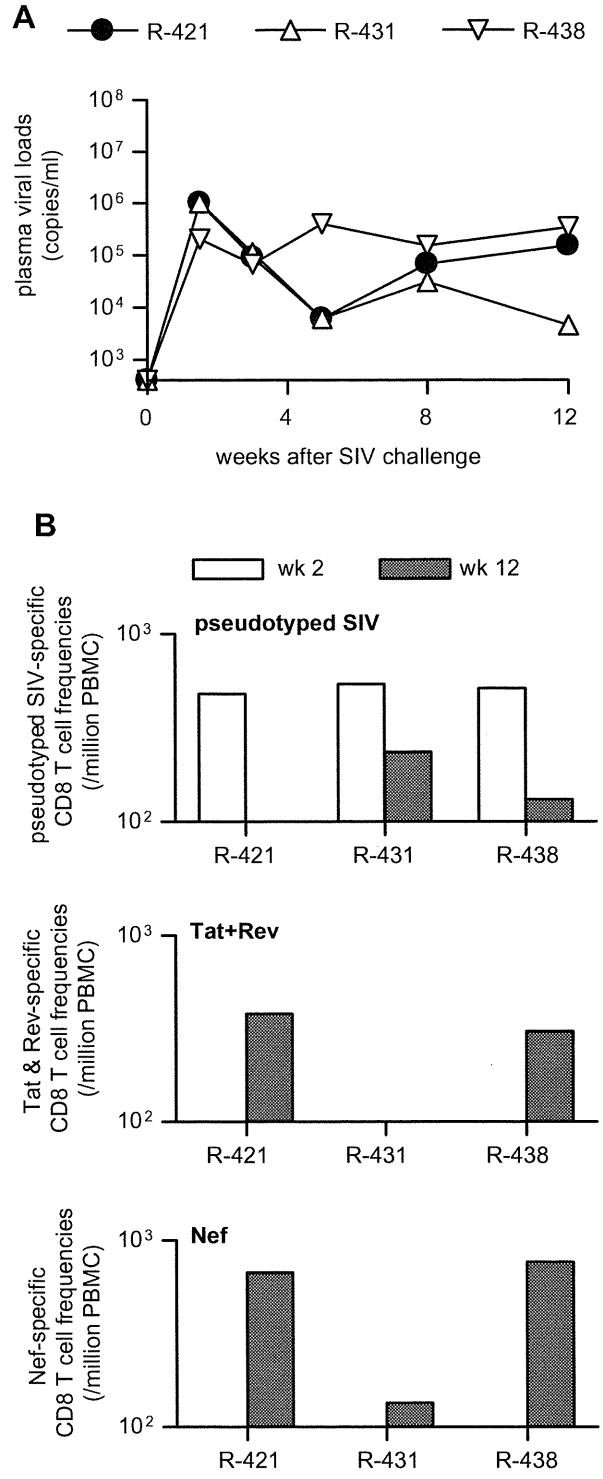

Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated macaques R-421, R-431, and R-438. (B) Vaccine antigen (pseudotyped SIV)-specific (top panel), Tat-plus-Rev-specific (middle panel), and Nef-specific $CD8^+$ T cell frequencies (bottom panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge. In macaque R-438, CTL responses at week 5 instead of week 12 are shown.

stimulation as described previously [18,19]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIV for pseudotyped SIV-specific stimulation. The pseudotyped SIV was obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA. Alternatively, PBMCs were cocultured with B-LCLs pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Tat, Rev, and Nef amino acid sequences. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (Becton Dickinson). Specific $CD8^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after Gag-specific, pseudotyped

	vaccine antigen					non-vaccine antigen										
	Gag				Vif	Vpr	Tat					Rev		Nef		
	165	333	375	376	143	73	23	115	120	122	125	45	50	63	100	124
wk 5																
R- 421					++											
R- 431					+											
R- 438	++		+							++						
wk 12																
R- 421		++			++				+		+	+	+			++
R- 431					+		+			++						
R- 438	++			++		+		++						++	++	

Fig. 4. Viral mutations in DNA-vaccinated macaques. Plasma viral genome sequencing was performed as described previously [18] to determine mutations resulting in amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef antigens (except for Env) at weeks 5 and 12 in DNA-vaccinated macaques. The amino acid positions showing mutant sequences dominantly (++) or equivalently with wild type (+) are shown. While we found a mutation leading to a lysine-to-arginine alteration at the 40th amino acid in Rev in all animals, this mutation is not shown because the wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo [18,23].

SIV-specific, or peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

3. Results and discussion

In our previous SIVmac239 challenge experiments, the prophylactic DNA/SeV-Gag vaccination did not result in viral control in rhesus macaques possessing the MHC-I haplotype *90-088-Ij*. These vaccinated animals showed similar levels of plasma viral loads as those in unvaccinated *90-088-Ij*-positive animals after SIV challenge (Fig. 1A). Analysis of virus-specific $CD8^+$ T-cell responses using PBMCs at week 2 after challenge showed equivalent Gag-specific and pseudotyped SIV-specific (Gag-, Pol-, Vif-, and Vpx-specific) CTL responses in all three vaccinees (Fig. 1B). Pseudotyped SIV-specific CTL responses were also detected in all three unvaccinated animals, but Gag-specific CTL responses were undetectable in two out of the three; even the Gag-specific CTL responses detected in macaque R04-014 were much lower than pseudotyped SIV-specific CTL responses, indicating dominant induction of CTL responses specific for SIV antigens other than Gag (Fig. 1B). Thus, in the acute phase of SIV infection, SIV non-Gag antigen-specific CTL responses were dominantly induced in unvaccinated *90-088-Ij*-positive macaques, whereas vaccine antigen (Gag)-specific CTL responses were dominant in *90-088-Ij*-positive vaccinees.

We then analyzed another vaccinees that failed to control a SIVmac239 challenge; these macaques were vaccinated with SeV-Gag alone or DNA alone. First, we compared post-challenge CTL responses in unvaccinated and SeV-Gag-vaccinated macaques possessing the MHC-I haplotype *90-120-Ib*. Both macaques failed to control SIV replication after challenge (Fig. 2A). In the unvaccinated animal R06-001, Gag-specific CTL responses were undetectable but pseudotyped SIV-specific CTL responses were induced efficiently at weeks 2 and 12 (Fig. 2B). In contrast, Gag-specific CTL responses were induced efficiently at week 2 in the SeV-Gag-vaccinated animal R05-028 (Fig. 2B). At week 12, Gag-specific CTL responses became undetectable while pseudotyped SIV-specific CTL responses were still detectable in this animal. These results indicate that, in the acute phase after SIVmac239 challenge, the unvaccinated *90-120-Ib*-positive macaque dominantly elicited SIV non-Gag antigen-specific CTL responses whereas the SeV-Gag-vaccinated *90-120-Ib*-positive macaque dominantly induced vaccine antigen (Gag)-specific CTL responses.

Next, we analyzed post-challenge CTL responses in three DNA-vaccinated macaques. These animals failed to control SIVmac239 replication after challenge (Fig. 3A). The DNA used for the vaccination and the pseudotyped SIV genome both have the same SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx, thus expected to induce pseudotyped SIV-specific CTL responses. Pseudotyped SIV-specific CTL responses, namely vaccine antigen-specific CTL responses, were induced efficiently at week 2 but diminished after that in all three animals (Fig. 3B). In contrast, Tat/Rev- and Nef-specific CTL responses were undetectable at week 2 but induced later (Fig. 3B). Again, vaccine antigen-specific CTL responses were dominantly induced in the acute phase after SIV challenge and non-vaccine antigen-specific CTL responses were elicited later.

All three animals showed viral genome mutations leading to amino acid substitutions in Gag or Vif at week 5 (Fig. 4). Further analysis indicated that viral mutations in vaccine antigen-coding regions appeared earlier than those in other regions. These results may reflect selective pressure on SIV by vaccine antigen-specific CTL responses dominantly induced in the acute phase, although it remains undetermined whether these mutations are CTL escape ones. Disappearance of vaccine antigen-specific CTL responses at week 12 may be explained by rapid selection of CTL escape mutations in vaccine antigen-coding regions. However, analysis using peptides found Gag-specific CTL responses in macaques R-421 and R-431 that had no gag mutations at week 5 (data not shown), suggesting involvement of immunodominance [20] in the disappearance of vaccine antigen-specific CTL responses at week 12.

In summary, the present study indicates that vaccine antigen-specific CTL responses are induced dominantly in the acute phase after viral exposure, with delayed induction of CTL responses specific for SIV non-vaccine antigens (SIV antigens other than vaccine antigens). While this delay previously-observed in vaccine-based SIV controllers [10] can be explained not only by immunodominance but also by reduction in viral loads, the delay in vaccinated non-controllers in the present study might reflect the immunodominance in CTL responses. Thus, in development of a prophylactic, CTL-inducing AIDS vaccine, it is important to select vaccine antigens leading to effective CTL responses post-viral

exposure [21,22]. These results imply a significant influence of prophylactic vaccination on the immunodominance pattern of CTL responses post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

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ORIGINAL ARTICLE

Major histocompatibility complex class I-restricted cytotoxic T lymphocyte responses during primary simian immunodeficiency virus infection in Burmese rhesus macaques

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ABSTRACT

Major histocompatibility complex class I (MHC-I)-restricted CD8$^+$ cytotoxic T lymphocyte (CTL) responses are crucial for the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. In particular, Gag-specific CTL responses have been shown to exert strong suppressive pressure on HIV/SIV replication. Additionally, association of Vif-specific CTL frequencies with *in vitro* anti-SIV efficacy has been suggested recently. Host MHC-I genotypes could affect the immunodominance patterns of these potent CTL responses. Here, Gag- and Vif-specific CTL responses during primary SIVmac239 infection were examined in three groups of Burmese rhesus macaques, each group having a different MHC-I haplotype. The first group of four macaques, which possessed the MHC-I haplotype *90-010-Ie*, did not show Gag- or Vif-specific CTL responses. However, Nef-specific CTL responses were elicited, suggesting that primary SIV infection does not induce predominant CTL responses specific for Gag/Vif epitopes restricted by *90-010-Ie*-derived MHC-I molecules. In contrast, Gag- and Vif-specific CTL responses were induced in the second group of two *89-075-Iw*-positive animals and the third group of two *91-010-Is*-positive animals. Considering the potential of prophylactic vaccination to affect CTL immunodominance post-viral exposure, these groups of macaques would be useful for evaluation of vaccine antigen-specific CTL efficacy against SIV infection.

Key words cytotoxic T lymphocyte, human immunodeficiency virus, major histocompatibility complex, simian immunodeficiency virus.

Virus-specific CD8$^+$ CTL responses are crucial for the control of HIV and SIV replication (1–5). CTLs recognize specific epitopes which are presented on the target cell surface by binding to the MHC-I molecule. There have been many reports indicating association of MHC-I (HLA class I) genotypes with rapid or delayed AIDS progression in HIV-infected people (6–8). For instance, most of the HIV-infected individuals possessing *HLA-B*57* have a better prognosis and smaller viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in control

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List of Abbreviations: CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; IFN-γ, gamma interferon; MHC-1, major histocompatibility complex class I; PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

of this virus (9, 10). Indian rhesus macaques possessing the MHC-I allele Mamu-B*17 tend to show smaller viral loads after SIVmac239 challenge (11). These findings imply possible HIV control by induction of particular effective CTL responses.

The potential of Gag-specific CTL responses to contribute to viral control was suggested by a cohort study indicating association of HIV control with the breadth of Gag-specific CTL responses (12). This was supported by an *in vitro* study indicating the ability of Gag-specific CTLs to respond rapidly to SIV infection (13). We previously developed a prophylactic AIDS vaccine using a Sendai virus vector expressing SIVmac239 Gag (14) and showed that Gag-specific CTL responses were responsible for vaccine-based SIV containment in a group of Burmese rhesus macaques possessing the MHC-I haplotype *90–120-Ia* (15, 16). Furthermore, our recent study analyzing the potential of $CD8^+$ cells to suppress SIV replication *in vitro* suggested association of *in vitro* anti-SIV efficacy with numbers of Vif-specific CTL frequencies (17). We also found weaker correlation between anti-SIV efficacy and numbers of Nef-specific CTL frequencies. These results imply the potency of Gag- and Vif-specific (and possibly Nef-specific) CTLs in suppressing HIV/SIV replication.

The immunodominance patterns of these potent CTL responses could be affected by host MHC-I genotypes (18, 19). Better understanding of these MHC-I-associated CTL immunodominance patterns during primary HIV/SIV infection would contribute to elucidation of the interaction between viral replication and host CTL responses. In the present study, we examined whether Gag- and Vif-specific CTL responses are efficiently induced during primary SIVmac239 infection in three groups of Burmese rhesus macaques possessing different MHC-I haplotypes. One group did not induce Gag- or Vif-specific CTL responses, whereas the other two groups elicited Gag- and Vif-specific CTL responses efficiently. These groups of macaques would be useful for analysis of the impact of Gag- and Vif-specific CTL responses on SIV replication *in vivo*.

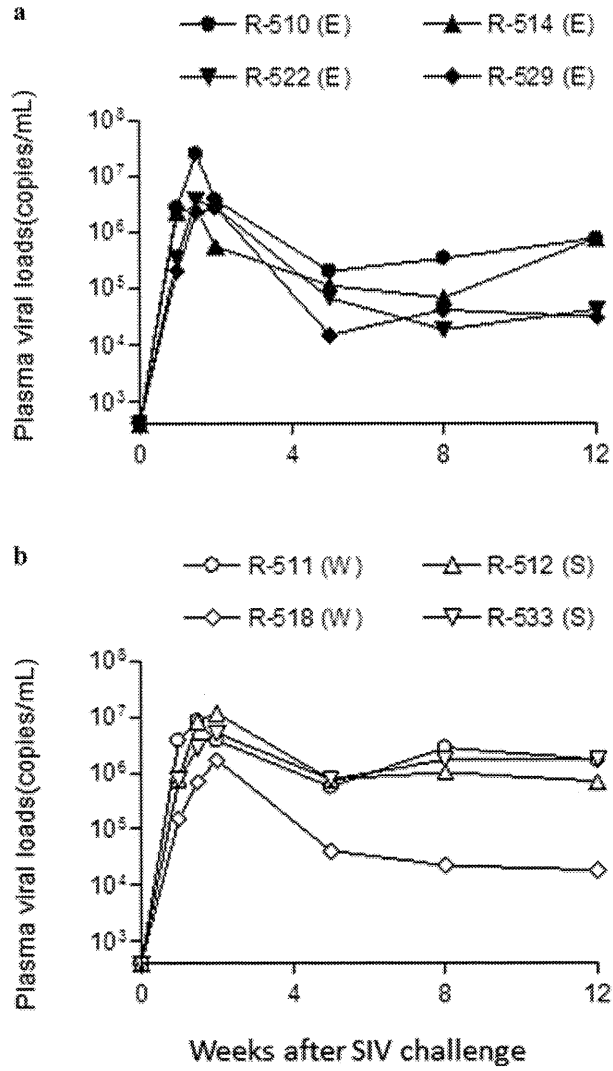

Fig. 1. Plasma viral loads after SIV challenge. (a) The first group of Burmese rhesus macaques, which possessed MHC-I haplotype *90-010-Ie* (R-510, R-514, R-522, and R-529) and (b) the second group, which possessed *89-075-Iw* (R-511 and R-518) and the third group, which possessed *91-010-Is* (R-512 and R-533) were challenged with SIVmac239. The viral loads (SIV gag RNA copies/mL) were determined as described previously (15).

MATERIALS AND METHODS

Animal experiments

Animal experiments using Burmese rhesus macaques (*Macaca mulatta*) possessing either the MHC-I haplotypes *90-010-Ie*, *89-075-Iw* or *91-010-Is* were performed in the Institute for Virus Research, Kyoto University, in accordance with the institutional regulations approved by the Committee for Experimental Use of Non-human Primates. The MHC-I haplotypes of macaques were determined as described previously (20, 21). These animals were challenged intravenously with 1000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (22).

Analysis of virus-specific cytotoxic T lymphocyte responses

Virus-specific $CD8^+$ T-cell frequencies were measured by flow cytometric analysis of IFN-γ induction after specific stimulation as described previously (17). PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines pulsed with peptide pools using panels of overlapping peptides

spanning the entire SIVmac239 Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Nef, and Env amino acid sequences. Intracellular IFN-γ staining was performed with a Cytofix-Cytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4 (Becton Dickinson), peridinin chlorophyll protein-conjugated anti-human CD8 (Becton Dickinson), allophycocyanin-conjugated anti-human CD3 (Becton Dickinson), and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (BioLegend, Tokyo, Japan). Specific $CD8^+$ T-cell frequencies were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after peptide-specific stimulation. Specific $CD8^+$ T-cells counts of less than 100 per million PBMCs were considered negative.

RESULTS

In the present study, we used eight Burmese rhesus macaques consisting of four animals possessing MHC-I haplotype *90-010-Ie*, two possessing *89-075-Iw*, and two possessing *91-010-Is*. After a SIVmac239 challenge, all these animals failed to control viral replication and had high set-point plasma viral loads (geometric mean: 3×10^5 copies/mL) (Fig. 1).

We examined SIV-specific $CD8^+$ T cell responses at week 2 and week 6 or 12 after SIV challenge in these animals by detection of specific IFN-γ induction after stimulation using peptide mixtures (Figs. 2 and 3). At week 6 or 12, we examined $CD8^+$ T cell responses specific for the N-terminal half of Gag (Gag-N), the C-terminal half of Gag (Gag-C), Vif, Nef, the N-terminal half of Pol (Pol-N), the C-terminal half of Pol (Pol-C), Vpx, Vpr, the N-terminal half of Env (Env-N), the C-terminal half of Env (Env-C), Tat, and Rev. At week 2, however, we examined only Gag-N-, Gag-C-, Vif- and Nef-specific $CD8^+$ T cell responses because of limited availability of PBMCs.

In the first group of macaques, which possessed *90-010-Ie*, neither Gag- nor Vif-specific $CD8^+$ T cell responses were induced efficiently at week 2 (Fig. 2). Even at week 12, these responses were undetectable in most of the animals. In contrast, Nef-specific $CD8^+$ T cell responses were detected at week 2, 6, or 12 in all four animals. Env-specific $CD8^+$ T cell responses were detectable at week 12 in three of them. These results indicate that, during primary SIV infection in *90-010-Ie*-positive macaques, Gag- or Vif-specific $CD8^+$ T cell responses are not induced, however Nef-specific $CD8^+$ T cell responses are.

In the second group of macaques, which possessed *89-075-Iw*, Gag- and Vif-specific $CD8^+$ T cell responses were elicited efficiently (Fig. 3a). In the third group of macaques, which possessed *91-010-Is*, Gag-, Vif- and Nef-specific $CD8^+$ T cell responses were elicited efficiently (Fig. 3b). Other SIV antigen-specific $CD8^+$ T cell responses were not efficiently induced in these two groups except for Tat-specific $CD8^+$ T cell responses in macaque

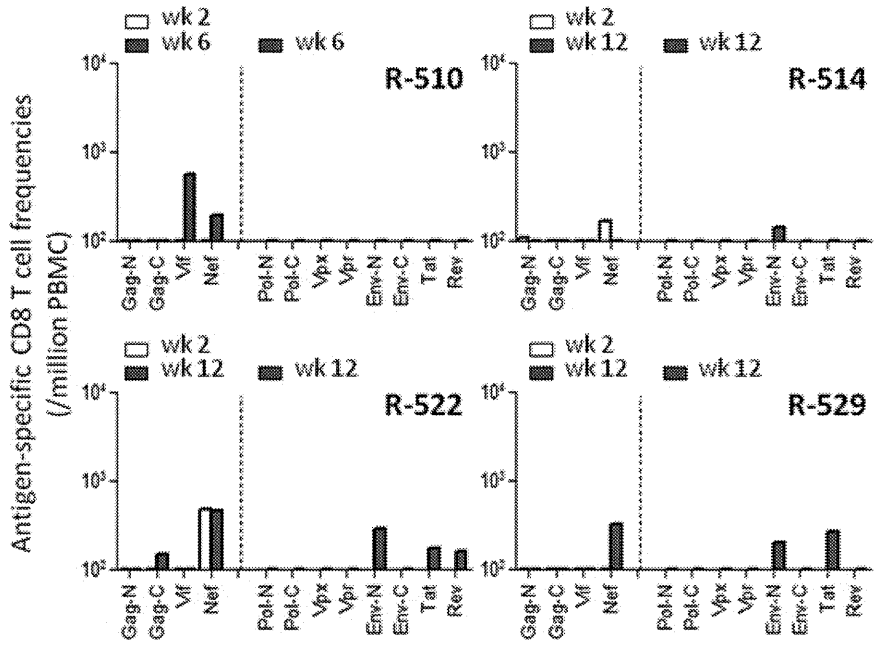

Fig. 2. SIV antigen-specific $CD8^+$ T cell frequencies in the first group of macaques, which possessed *90-010-Ie*. Gag-, Vif- and Nef-specific $CD8^+$ T cell frequencies at week 2 and Gag-, Vif-, Nef-, Pol-, Vpx-, Vpr-, Env-, Tat- and Rev-specific $CD8^+$ T cell frequencies at weeks 6 or 12 are shown.

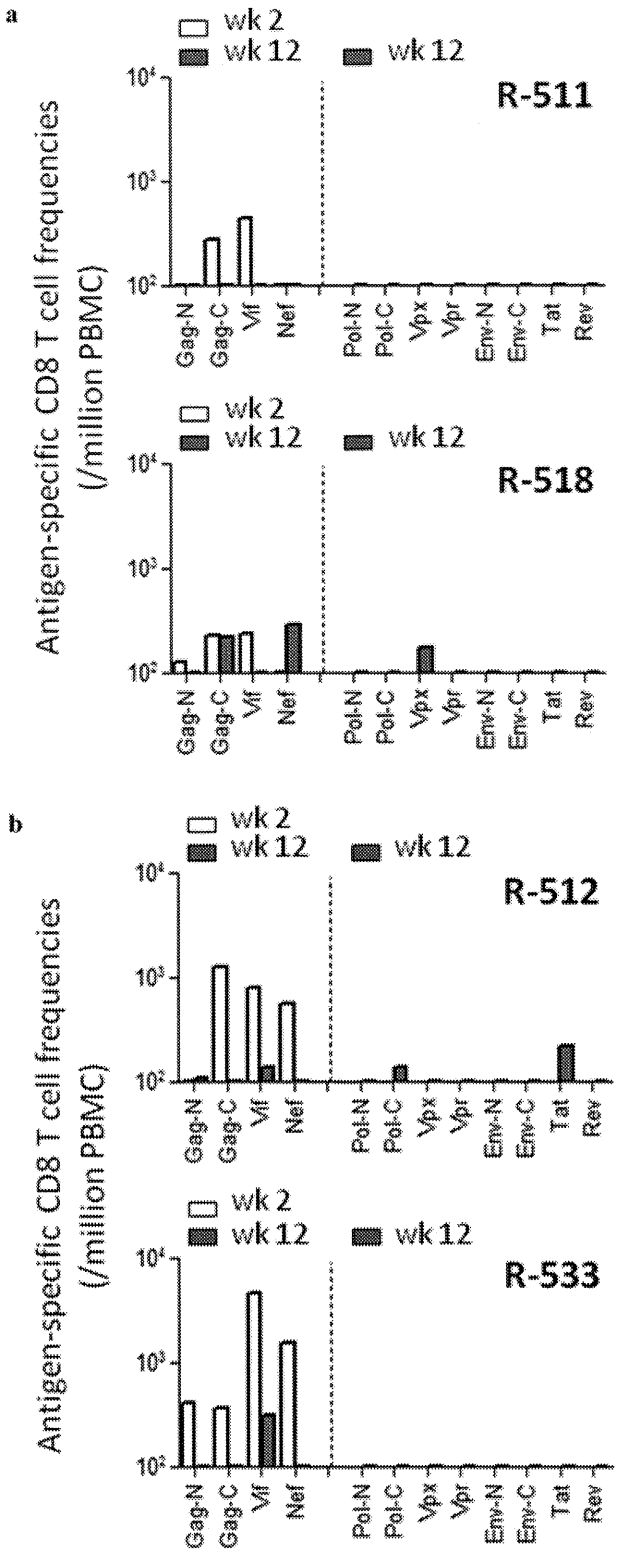

Fig. 3. SIV antigen-specific CD8$^+$ T cell frequencies in (a) the second group of macaques, which possessed *89-075-Iw* and (b) the third, which possessed *91-010-Is*.

R-512. Thus, in the four animals possessing *89-075-Iw* or *91-010-Is*, Gag- or Vif-specific CD8$^+$ T cell responses were induced more efficiently than Nef-specific ones at week 2. These responses in PBMCs were mostly diminished at week 12; possibly reflecting the considerable CTL consumption in the effector sites in animals with high viral loads.

DISCUSSION

Previous studies have indicated the potential of Gag-specific CTL responses to suppress HIV/SIV replication *in vivo* (12, 13, 16). Further, our recent study suggested the potency of Vif-specific CTL responses (17). Then, in the present study, we examined Gag- and Vif-specific CTL responses during primary SIV infection in three groups of animals, each group having a different MHC-I haplotype. Although the numbers of CTL frequencies differed between groups, the CTL responses tended have similar patterns.

Our previous study showed vaccine efficacy in a group of macaques with the MHC-I haplotype *90-120-Ia* (15, 16). Unvaccinated *90-120-Ia*-positive macaques predominantly induce Gag-specific CTL responses but fail to control viremia, while vaccinated ones show enhanced Gag-specific CTL responses and control SIV replication. $Gag_{206–216}$ epitope-specific and $Gag_{241–249}$ epitope-specific CTL responses were shown to be responsible for this vaccine-based viral control (16). However, some Gag-specific CTLs may be effective while others are not. Further analysis of this type of vaccine efficacy would contribute to understanding the requisites for vaccine-based viral control. Possibly, the *89-075-Iw*-positive or *91-010-Is*-positive animals presented in this study may be a candidate model for such analysis.

In primary SIVmac239 infection, it is speculated that some MHC-I haplotypes (referred to as type 1) are associated with Gag/Vif-specific CTL responses while others (referred to as type 2) are not. The MHC-I haplotype *90-120-Ia* described above belongs to type 1. In the present study, the second group, which possess MHC-I haplotype *89-075-Iw*, and the third, which possess *91-010-Is*, both showed efficient Gag- and Vif-specific CTL responses in primary SIV infection, although it remains undetermined whether these MHC-I haplotypes belong to type 1. In contrast, the first group of macaques, which possess MHC-I haplotype *90-010-Ie* did not show efficient Gag- or Vif-specific CTL responses in primary SIV infection. Instead, Nef-specific CTL responses were induced in all four animals. This suggests that the MHC-I haplotype *90-010-Ie* belongs to type 2; that is, primary SIV infection induces no predominant CTL responses specific for Gag/Vif epitopes

restricted by *90-010-Ie*-derived MHC-I molecules. Our results imply that CTLs exerted selective pressure on SIV *gag* and *vif* in the second/third groups but not in the first group. Larger number of animals would enable us to compare those with type 1 and 2 MHC-I haplotypes, which would contribute to our understanding of the efficacy of Gag- and Vif-specific CTL responses against SIV infection.

In developing a prophylactic CTL-inducing AIDS vaccine, it would be important to induce CTL memory resulting in potent CTL responses post-HIV exposure, while prophylactic vaccination can affect the immunodominance patterns of CTL responses post-viral exposure (23, 24). Gag- and Vif-specific CTL memory induction may be a promising vaccine strategy, but the influence of prophylactic vaccination on the patterns of CTL responses post-viral exposure would be affected by MHC-I genotypes. In the hosts in which Gag- and Vif-specific CTL responses are induced during the natural course of SIV infection, Gag- and Vif-specific CTL memory induction by prophylactic vaccination would predominantly enhance these CTL responses. In contrast, in those in whom no Gag- or Vif-specific CTL responses occurred during the natural course of SIV infection, prophylactic vaccination inducing Gag- and Vif-specific CTL responses would result in broader CTL responses. Macaques in which both MHC-I haplotypes belong to type 2 may be ideal for evaluation of this type of vaccine efficacy, but it is very difficult to accumulate those animals. It would be reasonable to use groups of macaques possessing type 2 haplotypes such as the group 1 (*90-010-Ie*-positive macaques) presented in this study for such evaluation.

In summary, by focusing on Gag- and Vif-specific CTL responses, we found two types of rhesus macaques that showed different patterns of CTL responses during primary SIV infection; one elicited Gag- and Vif-specific CTL responses but the other did not. Accumulated analyses in both types of animals would contribute to understanding the impact of these potent CTL responses on primary SIV infection.

ACKNOWLEDGMENTS

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In Vivo Safety and Persistence of Endoribonuclease Gene-Transduced CD4+ T Cells in Cynomolgus Macaques for HIV-1 Gene Therapy Model

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Abstract

Background: MazF is an endoribonuclease encoded by *Escherichia coli* that specifically cleaves the ACA sequence of mRNA. In our previous report, conditional expression of MazF in the HIV-1 LTR rendered CD4+ T lymphocytes resistant to HIV-1 replication. In this study, we examined the *in vivo* safety and persistence of MazF-transduced cynomolgus macaque CD4+ T cells infused into autologous monkeys.

Methodology/Principal Findings: The *in vivo* persistence of the gene-modified CD4+ T cells in the peripheral blood was monitored for more than half a year using quantitative real-time PCR and flow cytometry, followed by experimental autopsy in order to examine the safety and distribution pattern of the infused cells in several organs. Although the levels of the MazF-transduced CD4+ T cells gradually decreased in the peripheral blood, they were clearly detected throughout the experimental period. Moreover, the infused cells were detected in the distal lymphoid tissues, such as several lymph nodes and the spleen. Histopathological analyses of tissues revealed that there were no lesions related to the infused gene modified cells. Antibodies against MazF were not detected. These data suggest the safety and the low immunogenicity of MazF-transduced CD4+ T cells. Finally, gene modified cells harvested from the monkey more than half a year post-infusion suppressed the replication of SHIV 89.6P.

Conclusions/Significance: The long-term persistence, safety and continuous HIV replication resistance of the *mazF* gene-modified CD4+ T cells in the non-human primate model suggests that autologous transplantation of *mazF* gene-modified cells is an attractive strategy for HIV gene therapy.

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Introduction

Highly active anti-retroviral therapy (HAART) is widely used for human immunodeficiency virus (HIV) therapy and involves the combination of several drugs with different functions that are currently being evaluated in clinical trials; some of these drugs are currently available [1]. HAART treatment reduces plasma viral load to undetectable levels and recovers CD4+ T cells to clinically safe levels. Although HAART therapy has revolutionized the treatment of HIV-1 infection, the need for life-long therapy, difficulties with medication adherence and long-term medication toxicities have led to the search for new treatment strategies that will efficiently reduce the viral load and allow for stable immunological homeostasis. The number of patients who are HAART resistant has significantly decreased in the past 2 years due to newly available drugs, but based on previous experience, drug resistance is likely to increase again. Thus, additional approaches for the management of HIV infection, or approaches performed in combination with HAART therapy, are needed. Gene therapy for HIV-1 infection has been proposed as an alternative to antiretroviral drug regimens [2,3]. A number of different genetic vectors with antiviral payloads have been utilized to combat HIV-1, including antisense RNA against the HIV-1 envelope gene, transdominant protein RevM10, ribozymes, RNA decoys, single chain antibodies, and RNA-interference [4,5]. These protocols use T cells or hematopoietic stem cells as a target for gene modification. Autologous T cell transfer in HIV patients began in the mid 1990's, and since that time, no serious adverse events have been reported to be associated with infusions of autologous T cells, and infusions are well tolerated. The majority of these clinical trials used gene transfer by retrovirus or lentiviral vectors for the delivery of the anti-HIV payloads.

In order to develop a new approach for HIV therapy, we previously constructed an HIV-1 Tat-dependent expression retroviral vector in which the *Escherichia coli* (*E. coli*) endoribonuclease gene *mazF* was fused downstream of the trans-activation

response element (TAR) so that the gene expression of *mazF* is induced upon HIV-1 replication [6]. When MazF-transduced cells were infected with HIV-1 IIIB, the replication of HIV-1 was efficiently inhibited without affecting CD4+ T cell growth. MazF-transduced primary CD4+ T cells derived from monkeys also suppressed simian/human immunodeficiency virus (SHIV) replication [6]. Thus, autologous transfer of genetically modified CD4+ T cells conditionally expressing the MazF protein will be a promising strategy for HIV gene therapy. Generally, the shift from the chronic phase to the AIDS phase is due to the balance between viral growth and immune suppression, and the remarkable decrease in CD4+ T cells causes the subsequent deficiency of the immune system, the hallmarks of AIDS. The benefit of the MazF-based gene therapy strategy is that gene-modified CD4+ T cells may be protected from HIV-1-associated cell death and are therefore likely to help the immune system maintain a stable condition.

In this preclinical study, we examined the *in vivo* safety and persistence of MazF-transduced autologous CD4+ T cells (named MazF-Tmac cells) using a non-human primate model. Cynomolgus macaque primary CD4+ T cells were retrovirally transduced with the MazF vector, infused into the autologous monkeys, and the persistence and safety of the MazF-Tmac cells was monitored more than half a year. We found that infused MazF-Tmac cells were detected in the peripheral blood throughout the experimental period. Additionally, experimental autopsy revealed the distribution of the infused lymphocyte in total body.

Results

Manufacturing of MazF-transduced CD4+ T cells using *ex vivo*-expanded cynomolgus macaque CD4+ T cells

In order to infuse more than 1×10^9 MazF-transduced autologous cells, isolated primary CD4+ T lymphocytes were *ex vivo* stimulated, transduced with the MT-MFR-PL2 retroviral vector (Figure 1A), and expanded as described in the Materials and Methods. The resultant MazF-Tmac cells were transplanted into autologous monkeys via intravenous infusion (Figure 1B). We initially used concanavalin A (Con A) for the stimulation of CD4+ T cells (CD4T-1), but Con A only induced a 12-fold cell expansion after 7 days. In order to improve the *ex vivo* expansion, we used anti-CD3/anti-CD28 monoclonal antibody-conjugated beads (anti-CD3/CD28 beads), which are known to yield a more efficient cellular expansion [7,8]. As we expected, the fold expansion of CD4+ T cells (CD4T-2 and CD4T-3) stimulated with anti-CD3/CD28 beads was much higher than with Con A stimulation (Table 1). In order to improve the engraftment efficiency of CD4+ T cells, busulfan was orally administered to the macaques prior to the transplantation, and the gene-modified MazF-Tmac cells were infused into each monkey intravenously at $1.6–2.7\times10^9$ cells.

Transduction efficiency and cell surface markers of MazF-Tmac cells

The efficiency of MazF transduction and phenotype of cell surface markers of the MazF-Tmac cells were analyzed using flow cytometry. The MazF vector transduction efficiency of CD4T-2 and CD4T-3 cells was 61.8% and 60.0%, respectively, while only 34.5% for CD4T-1 (Table 1). As shown in Table 2, 99% of the expanded MazF-Tmac cells were CD3 and CD4 double-positive, and in these cells, more than 90% expressed CD95/CD28, which are known central memory phenotype markers [9]. Central memory cells generally have a longer life span compared to effecter memory cells [10]; thus, a higher percentage of central memory cells in MazF-Tmac cells is likely to result in longer persistence after transplantation. Furthermore, to assess the activation status of MazF-Tmac cells, we measured the expression of CD25, which is also known as IL-2 receptor alpha and is an activated T cell marker. CD25 expression of MazF-Tmac cells from CD4T-2 and CD4T-3 was low. In contrast, almost 100% of the CD4+ T cells were found to express CD25 with a higher expression level 2–4 days after stimulation (data not shown). Thus, these data indicate that a large number of MazF-Tmac cells entered into resting or non-activated states during the *ex vivo* culture. CXCR4, a co-receptor for X4 tropic HIV entry, was found to be expressed in expanded CD4T-2 and CD4T-3 MazF-Tmac cells. Furthermore, we observed that there was no significant difference in the measured cell surface markers between Con A- and anti-CD3/CD28 bead-stimulated MazF-Tmac cells (Table 2).

Longitudinal analysis of infused MazF-Tmac cells

To examine the *in vivo* safety and persistence of infused MazF-Tmac cells, peripheral blood from each monkey was collected to monitor the hematological effects and the proviral copy number of the transduced retroviral vector in the genome over six months. There was no significant change in the body weight of the monkeys throughout the experiment (Figure 2A). During the period of 2–4 weeks post-transplantation, severe reduction in the white blood cell (WBC) count, hemoglobin (Hb) concentration, and platelet (PLT) levels were observed in the monkeys CD4T-1 and CD4T-2, while only slight reduction was observed in CD4T-3. These negative effects are considered to be due to the effect of the busulfan treatment, which is known to cause partial bone marrow depletion and functional defects in blood-forming tissues. No other adverse events were observed throughout the experiments. The transient reduction of lymphocytes gradually recovered, and the cell number became stable two months after the transplantation (Figure 2A).

The percentage of persistent MazF-Tmac cells in CD4+ T cells was determined using real-time PCR and flow cytometric analyses. The percentage of MazF-Tmac cells gradually decreased in CD4T-1- and CD4T-2-transplanted monkeys, while in the CD4T-3-transplanted monkey, a drastic reduction of the infused MazF-Tmac cells was observed 3–4 weeks post-transplantation but was not observed at later time points (Figure 2B). Although the levels of MazF-Tmac cells gradually decreased over time, the infused MazF-Tmac cells were detected even after six months post-transplantation. It is reasonable to assume that a population of infused MazF-Tmac cells can persist for a long-term period, likely forming a resting condition.

Detection of anti-MazF antibodies in monkey blood

Although the levels of MazF-transduced CD4+ T cells gradually decreased in the peripheral blood, some were detected throughout the half-year experimental period, suggesting that MazF-Tmac cells showed little or no immunogenicity towards cynomolgus macaques. Because gene therapy for HIV is aimed at reconstituting an HIV-resistant immune system, genetically modified cells must not only inhibit virus replication, but also maintain their expected trafficking behavior and persist *in vivo*. Although the evidence of longitudinal persistence of MazF-Tmac cells supports the low immunogenicity of MazF-Tmac cells, it is important to assess the production of antibodies against MazF. As shown in Figure 3 and Figure S1, we detected no production of anti-MazF antibodies in the CD4T-2 monkey blood after transplantation of the MazF-Tmac cells.

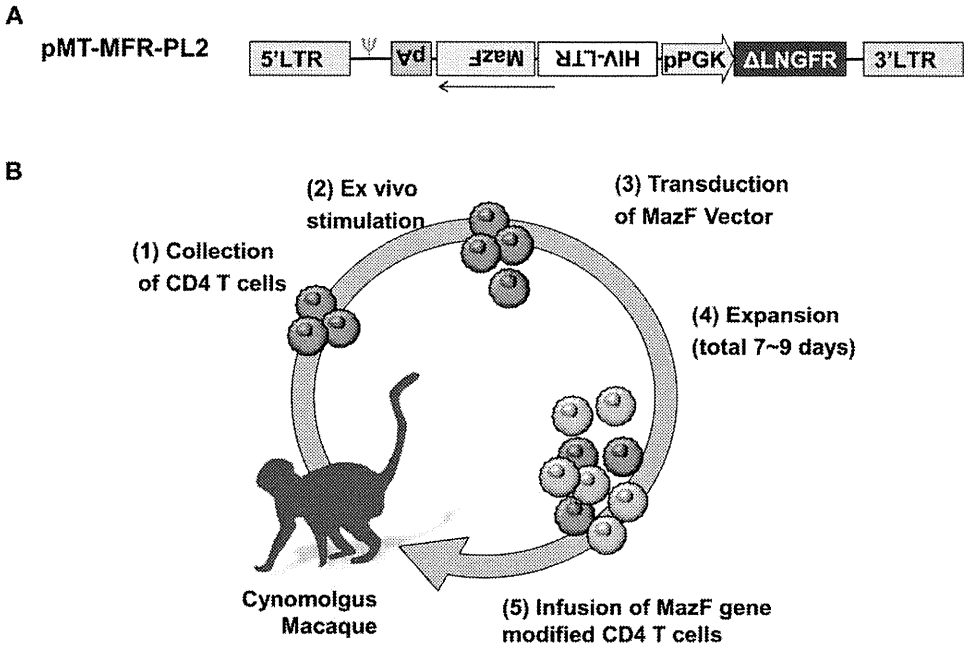

Figure 1. Diagram of autologous CD4+ T cell transplantation using a non-human primate model. (A) Design of gene transfer vector. The MazF gene derived from *E. coli* was inserted directly into the downstream of HIV-LTR sequence. The HIV-LTR-MazF-polyA cassette was introduced in the opposite direction of the MoMLV-LTR. A truncated form of the human ΔLNGFR was also introduced into the retrovirus vector as a surface marker. The ΔLNGFR gene is under the control of the human PGK promoter. (B) Flow diagram of gene-transduced CD4+ T cell manufacture. (1) Peripheral blood was collected by apheresis, (2) CD4+ T cells were selected by positive selection and stimulated *ex vivo* with Con A or anti-CD3/CD28 monoclonal antibody-conjugated beads. (3) The MT-MFR-PL2 vector was transduced twice on days 3 and 4. (4) The transduced cells were expanded for an additional 3–5 days until the total cell number reached more than 10^9. (5) On day 7–9, the expanded cells were collected, washed, and infused to the autologous macaques through venous blood.
doi:10.1371/journal.pone.0023585.g001

In vivo safety of MazF-Tmac cells

It is a great advantage to use primate models for investigating the safety of gene-modified cells, as they can be used for surgical pathological analysis. Therefore, we performed experimental autopsies six months after transplantation. To examine the safety of MazF-Tmac cells, specimens from several organs were fixed in buffered formaldehyde and embedded in plastic. Serial sections were made using a diamond saw. Slides were then stained with hematoxylin-eosin. Histopathological findings of the specimens were contracted with Bozo Research Center (Tokyo, Japan), and no severe adverse events relating to MazF-Tmac cell infusion was observed (Table 3 and Figure S2).

Table 1. Demographic data and summary of expansion fold and transduction efficiency.

	CD4T-1	CD4T-2	CD4T-3
Body Weight (kg)	5.25	5.18	3.7
Method for stimulation	Con A	Anti-CD3/CD28 Beads	Anti-CD3/CD28 Beads
Number of stimulated CD4+ T cells ($\times 10^7$ cells)	13.0	1.0	4.6
Days for expansion (days)	7	7	9
Number of infused MazF-Tmac cells ($\times 10^9$ cells)	1.6	1.7	2.7
Expansion Fold	12.3	170	58.7
Gene transfer efficiency (%)	34.5	61.8	60.0

doi:10.1371/journal.pone.0023585.t001

Examination of the anti-viral efficacy of MazF-Tmac cells harvested from monkey

In order to examine whether the Tat-dependent expression of MazF and anti-viral efficacy was maintained in the MazF-Tmac cells after infusion, CD4+ T lymphoid cells from a CD4T-1-transplanted monkey (214 days post-infusion of MazF-Tmac cells) were selected and expanded *ex vivo* (Figure 4A). After 7 days of expansion, the genetically modified cells expressing a truncated form of the human low affinity nerve growth factor (ΔLNGFR+) were concentrated with an anti-CD271 monoclonal antibody (Figure 4B). CD271-positive cells and CD271-negative cells were expanded for an additional 4 days. Both groups of expanded cells were infected with SHIV 89.6P [11] at the multiplicity of infection (MOI) of 0.01. Culture supernatants and cell pellets were analyzed at 6 days post-infection. As shown in Figure 4C, the replication of SHIV 89.6P was significantly suppressed in CD271-positive cells

Table 2. Cell surface markers of expanded MazF-Tmac cells.

	CD4T-1	CD4T-2	CD4T-3
CD3(+)/CD4(+) (%)	98.2	98.7	99.9
CD95(−)/CD28(+) (Naïve) (%)	0.7	1.2	0.4
CD95(+)/CD28(+) (CM) (%)	93.0	94.7	91.2
CD95(+)/CD28(−) (EM) (%)	6.2	3.9	8.3
CXCR4 (%)	N/A	92.0	79.4
CD25 (%)	N/A	30.4	24.5

CM: Central Memory, EM: Effector Memory.
doi:10.1371/journal.pone.0023585.t002

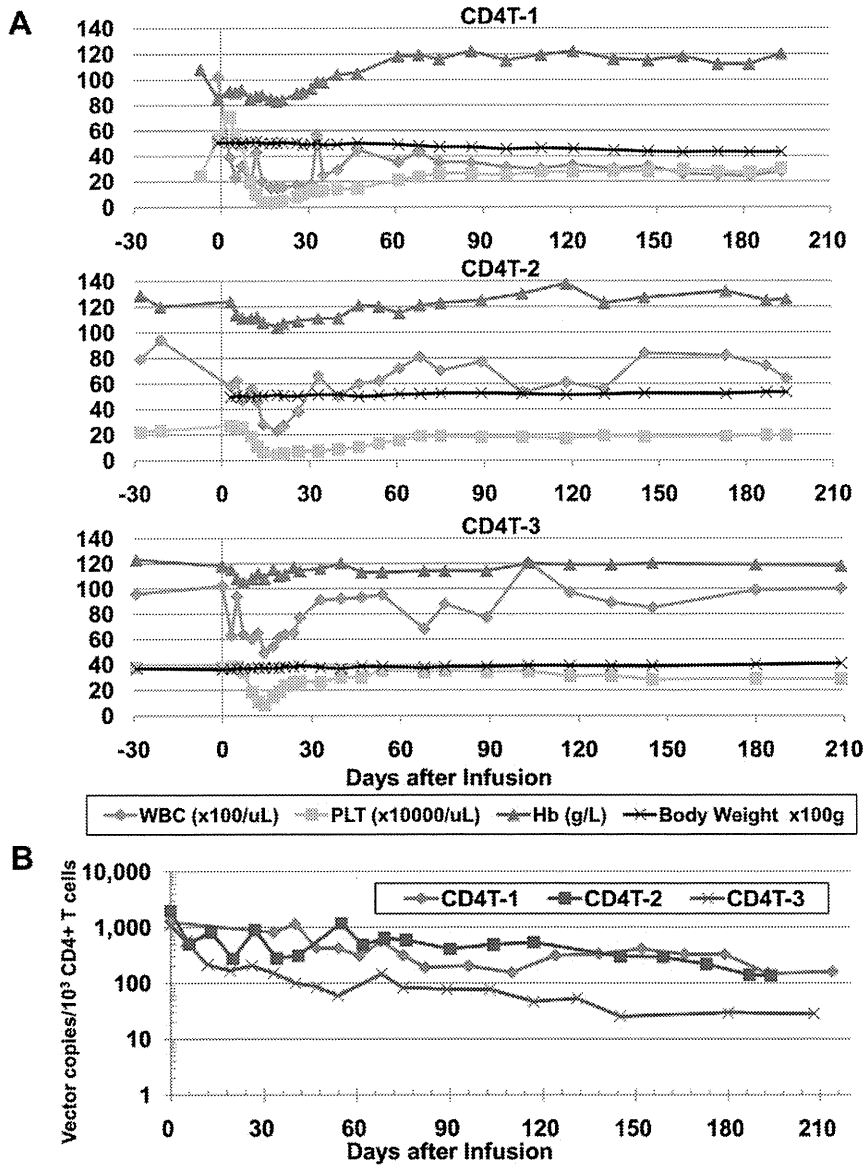

Figure 2. Hematological analysis and engraftment of the MazF-transduced CD4+ T cells. (A) The body weight and several hematological features were measured at the indicated time points, and the number of WBC, Hb, and PLT were represented. Each macaque was monitored throughout the study period. (B) The *in vivo* persistence of retroviral-transduced CD4+ T cells in the peripheral blood. PBMCs were collected at the indicated time points. The percentage of CD4+ T cells was analyzed using flow cytometry, and the proviral MazF vector copy was analyzed using real-time PCR. By compounding these two data, the copy number of the *mazF* gene in CD4+ T cells was calculated.
doi:10.1371/journal.pone.0023585.g002

in comparison with CD271-negative cells. Although western blot analysis managed to detect the expression of MazF, MazF was below the detection limit (data not shown). However, the expression of MazF was clearly induced when the same CD271-positive cells were transduced with the Tat expression retroviral vector M-LTR-Tat-ZG [6] (Figure 4D). These data suggest that the conditional expression system in MazF-Tmac cells is still active at 6 months post-transplantation.

Distribution of MazF-Tmac cells

To examine the distribution and persistence of the infused MazF-Tmac cells in a monkey, lymphocytes isolated from several organs were analyzed using flow cytometry and real-time PCR. As shown in Figure 5A and 5B, ΔLNGFR+ cells were detected in CD4+ T cells isolated from several lymph nodes (LNs), spleen, and peripheral blood. A similar tendency was obtained using real-time PCR (Figure 5C). In contrast, MazF-Tmac cells were not detected in the bone marrow, liver, thymus, and small intestine (data not shown). These data strongly suggest that infused MazF-Tmac cells mainly circulate in the secondary lymphoid organs.

In vivo distribution of MazF-Tmac cells treated with or without retinoic acid

Based on the findings that MazF-Tmac cells were well distributed among secondary lymphoid organs but not in small intestine, we performed additional experiment using one cynomolgus monkey (CD4T-4). In order to investigate the editing effect of the homing receptor to efficiently recruit the gene-modified cells to intestinal tissues in a non-human primate model, the distribution of retinoic acid-treated MazF-Tmac cells was

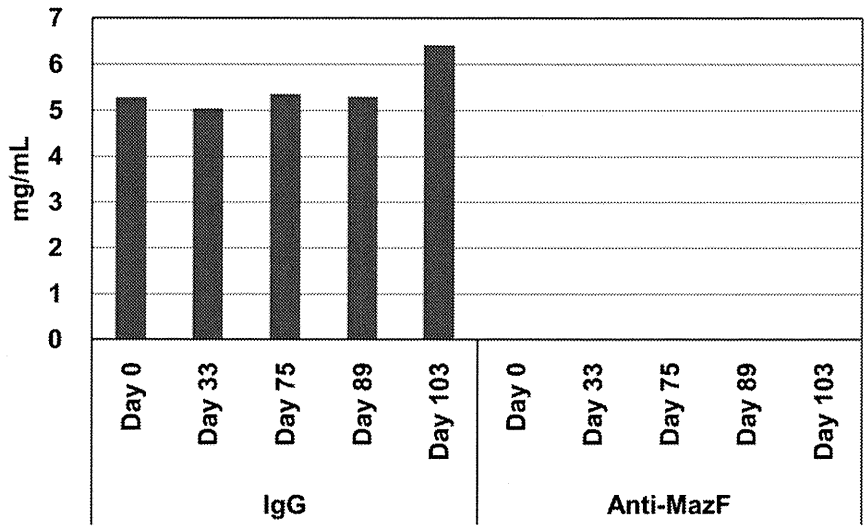

Figure 3. No detection of anti-MazF antibodies in monkey blood after transplantation of MazF-Tmac cells. Plasma samples were isolated from the monkey CD4T-2 at day 0, 33, 75, and 103 after transplantation and were used to detect anti-MazF antibodies on a MazF protein-immobilized microplate. The plasma samples were diluted to 500,000-fold, 50,000-fold, and 10,000-fold and added to each well. After the incubation, antibodies which reacted with immobilized MazF were tried to detect as described in Materials and Methods. No MazF-specific antibodies were detected.
doi:10.1371/journal.pone.0023585.g003

examined in a cynomolgus macaque. The experimental procedure is described in Figure 6A. Non-treated and retinoic acid-treated MazF-Tmac cells were designated as MazF-Tmac-N and MazF-Tmac-R, respectively. Expressions of integrin-α4 and integrin-β7 were remarkably increased in the presence of retinoic acid (Figure 6B). Thereafter, MazF-Tmac-N and MazF-Tmac-R were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and PKH26, respectively. The CFSE-labeled cells were mixed with an equal number of PKH26-labeled cells (Figure 6C), and 6.8×10^8 of the mixed cells were infused into a CD4T-4 monkey. Note that the transduction efficiency of the MazF vector was 65% (data not shown). Three days after the transplantation, experimental autopsy was performed to obtain samples of several organs as described in the Materials and Methods. Both the CFSE- and the PKH26-labeled CD4+ T cells were detected in the peripheral blood and several LNs by FACS analysis (Figure 6D). The percentage of the infused cells in the LNs was low compared to the peripheral blood, indicating that a large number of the infused cells did not migrate to the secondary lymphoid tissues and circulated in the peripheral blood at this time point. In the case of the inguinal and axillary LNs, the percentage of MazF-Tmac-R cells was low compared to MazF-Tmac-N cells. In contrast, a higher percentage of MazF-Tmac-R cells was observed in the mesenteric LN compared to MazF-Tmac-N cells. MazF-Tmac-N cells were evenly distributed in the three LNs analyzed, while the MazF-Tmac-R cells seemed to be preferentially distributed in the mesenteric LNs. Moreover, a large number of MazF-Tmac-R cells were distributed in the small intestine, while MazF-Tmac-N cells were not. To further evaluate the homing effect of the MazF-Tmac cells, the distribution of the labeled-MazF-Tmac cells in cryopreserved organs was analyzed using fluorescence microscopy (Figure 6E). A number of the PKH26-labeled MazF-Tmac-R cells were observed in the mesenteric LNs and in Peyer's patches. Taken together, retinoic acid-treated MazF-Tmac cells seem to be selectively recruited to mesenteric LNs and then transported to Peyer's patches. The distribution of MazF-Tmac-R cells in the intestinal villi remains to be determined.

Table 3. Analysis of *in vivo* safety (Histological finding about autopsy sample).

	CD4T-1	CD4T-2	CD4T-3
Lymph node	±	±	−
Spleen	−	−	±
Bone marrow	++**	−	−
Thymus	N/A	+*	−
Small intestine	−	−	−
Liver	−	−	−
Kidney	−	±	−
Pancreas	−	−	−
Stomach	−	−	±
Lung	−	±	−
Heart	−	−	±

−: No remarkable changes; ±: Minimal; +: Mild; ++: Moderate.
N/A: No equivalent sample available.
*Due to the Aging,
**Side effect due to the busulfan administration.
doi:10.1371/journal.pone.0023585.t003

Discussion

MazF is a toxin encoded by the *E. coli* genome and plays a role in growth regulation under stress conditions in *E. coli* [12]. MazF can act as an endoribonuclease (RNase) that specifically cleaves cellular mRNAs at ACA sequences [13]. Therefore, MazF induction in *E. coli* virtually eliminates almost all cellular mRNAs to completely inhibit protein synthesis. However, MazF-induced cells retain full capacity for protein synthesis, as MazF-induced cells are able to produce a protein at a high level if the prerequisite mRNA is engineered to be devoid of all ACA sequences without altering its amino acid sequence [14]. This indicates that RNA components involved in protein synthesis are protected from

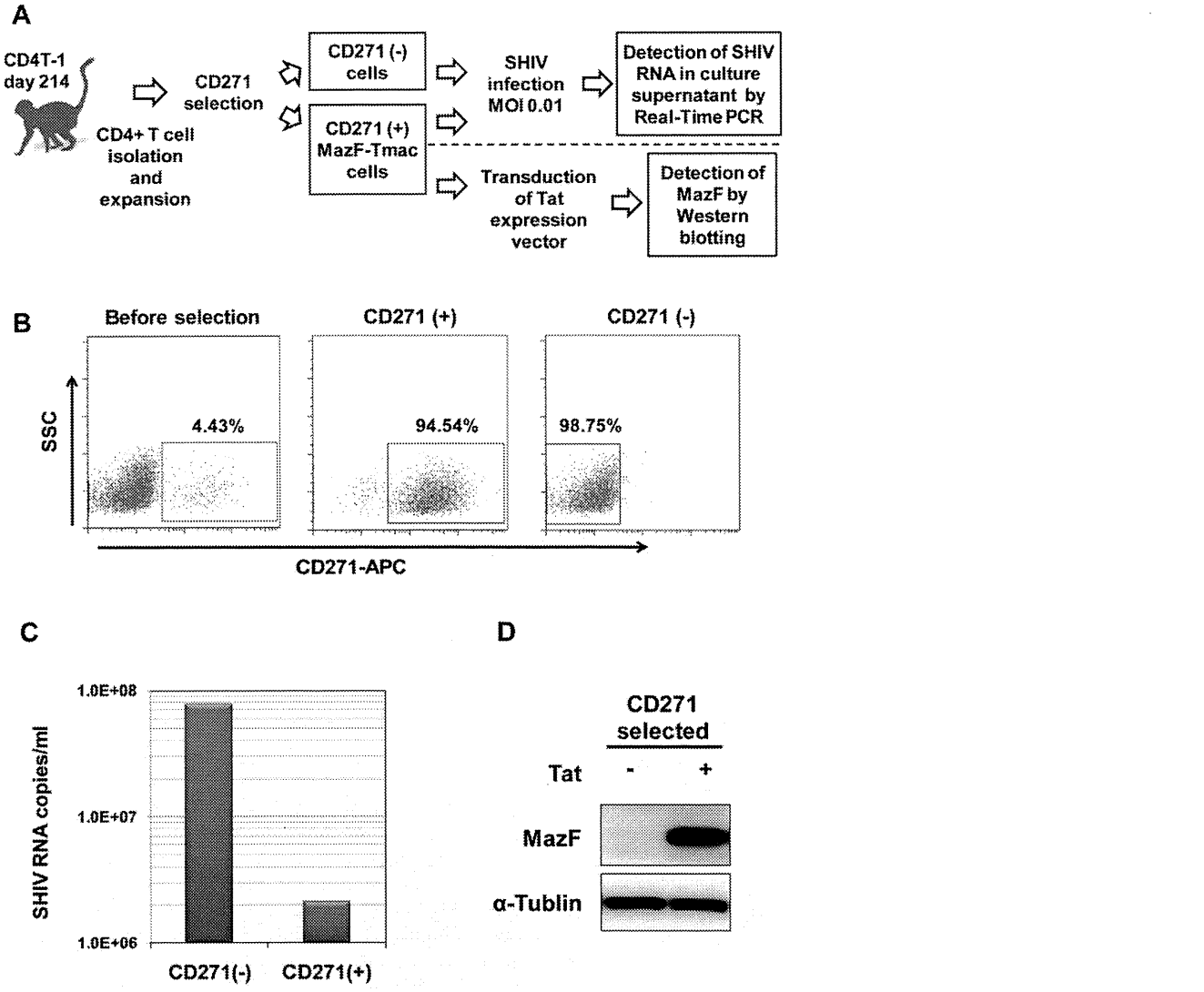

Figure 4. Examination of the anti-viral efficacy of MazF-Tmac cells harvested from the monkey. (A) Flow diagram of the experiment. CD4+ T lymphoid cells from CD4T-1 (214 days post-infusion of the MazF-Tmac cells) were stimulated and expanded *ex vivo*. The genetically modified cells expressing ΔLNGFR+ were concentrated with an anti-CD271 monoclonal antibody and expanded for 4 days. The expanded CD271-enriched cells and CD271-negative cells were infected with SHIV 89.6P. SHIV RNA levels in the culture supernatant were determined using quantitative real-time PCR. Expression of MazF was detected from the cell lysates by western blot analysis. Moreover, CD271-positive cells were transduced with the Tat expression vector. (B) CD271-positive and -negative cells were enriched using an anti-CD271 antibody, and dot plots of the flow cytometry analysis are presented. (C) The suppression of SHIV RNA in the culture supernatant at 6 days after infection was detected by real-time PCR analysis. (D) MazF-Tmac cells transduced with the Tat expression vector were harvested at 20 hours post-transduction and used for western blot analysis. Conditional expression of MazF in a Tat-dependent manner was observed.
doi:10.1371/journal.pone.0023585.g004

MazF cleavage. Indeed, ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are protected from MazF cleavage in *E. coli* [15].

RNase-based anti-HIV gene therapy is an attractive strategy to suppress HIV-1 RNA replication. In the case of MazF, there are more than 240 ACA sequences in HIV-1 RNA, suggesting that HIV has almost no chance to gain MazF-related escape mutations. This approach seems to have a substantial advantage over the other known antiviral strategies, including antiviral drug therapy, and RNA-based gene therapies, such as antisense RNA, ribozyme, and siRNA.

MazF overexpressed in mammalian cells preferentially cleaves messenger RNAs (mRNAs), but not ribosomal RNAs [16]. As HIV-1 RNA has more than 240 ACA sequences, we assumed that the viral RNA is highly susceptible to MazF, leading to inhibition of viral replication under a conditional expression system. Indeed, conditional expression of MazF with Tat suppresses replication of both HIV-1 IIIB and SHIV 89.6P without affecting cellular mRNAs, suggesting that this Tat-dependent expression system of MazF is an attractive payload for HIV gene therapy [6]. It is an intriguing phenomenon that viral RNAs are efficiently and preferentially cleaved without affecting cellular mRNAs, and we are now addressing this question. Meanwhile, MazF is a bacterial protein, and its expression is induced by Tat protein; thus, it is important to assess the safety and immunogenicity of *mazF* gene-modified cells *in vivo*. In order to determine the safety of our MazF-retrovirus system *in vivo*, we infused MazF-transduced CD4+ T cells into cynomolgus macaques. In human gene therapy trials, engraftment of 1–2% of genetically modified cells in the peripheral

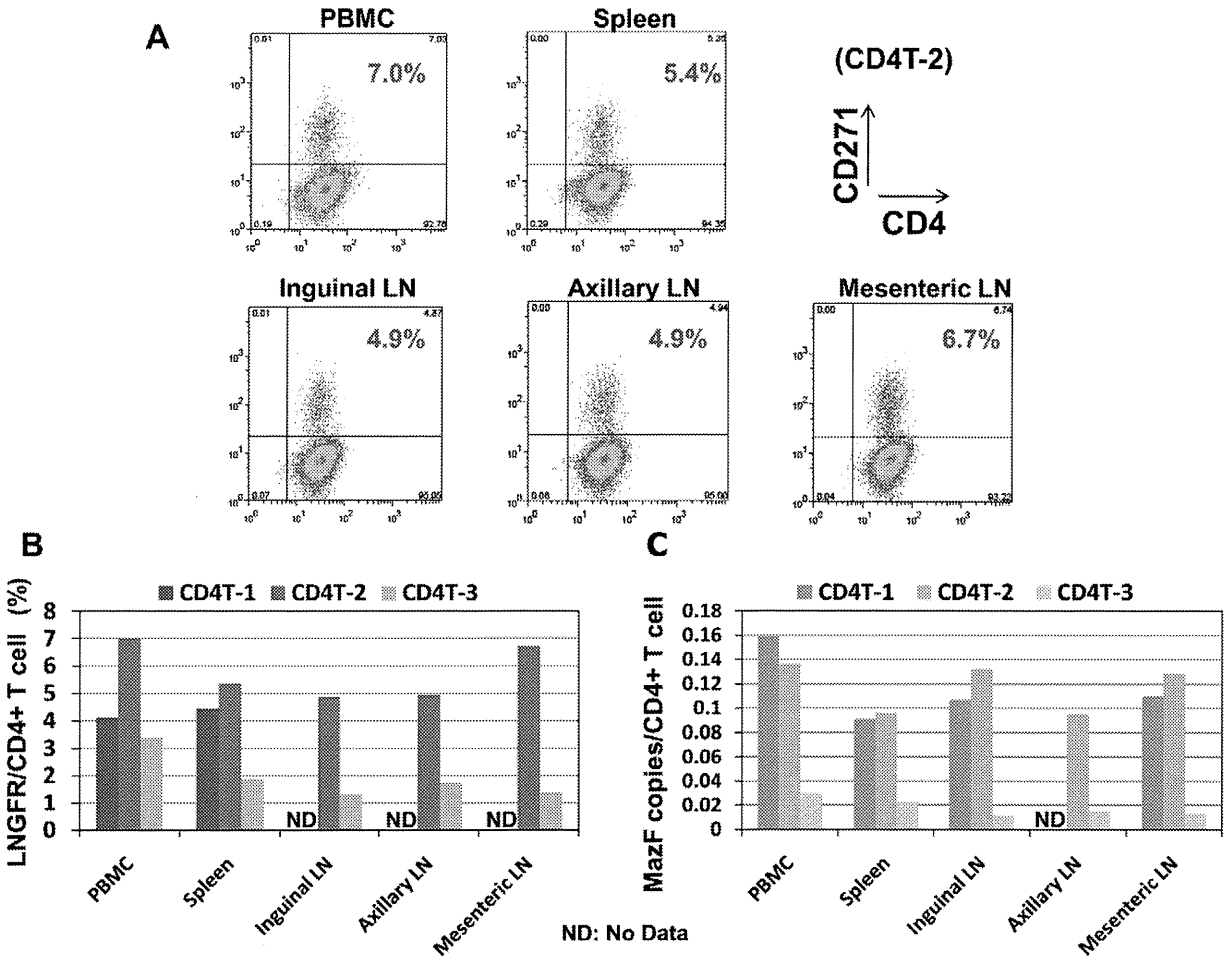

Figure 5. Analysis of the distribution of MazF-Tmac cells in several organs. (A) CD4+ T cells were isolated from lymphocytes separated from several organs, incubated 3–4 days, and stained with anti-CD4 and anti-CD271 antibodies. CD4T-2 is represented by a dot plot. (B) The percentage of CD271+ cells from three macaques is summarized. (C) The Copy number of the MazF gene in CD4+ T cells from each organ was calculated from real-time PCR and flow cytometric data.
doi:10.1371/journal.pone.0023585.g005

circulation has been observed following infusions of about 10 billion cells [17], and higher cell doses results in higher levels of engraftment [18,19]. Infusions of lower than 5×10^9 cells do not reliably result in measurable engraftment levels [19]. Therefore, we decided to infuse more than one billion cells into cynomolgus macaques, reflecting one-tenth of the scale of the human model. Indeed, the *mazF* gene-modified cells were detected over a six month period at a high level, and no histopathological disorders and no MazF-specific antibody production was observed during the experiment, demonstrating that MazF-Tmac cells showed little or no immunogenicity to monkeys. Moreover, MazF-Tmac cells harvested from the CD4T-1-transplanted monkey 6 months post-infusion showed resistance to the replication of SHIV 89.6P, indicating that the long-term persistent MazF-Tmac cells are functional. The expression of MazF in the SHIV-infected MazF-Tmac cells was below the limit of detection due to a low MOI such as 0.01, while in the MazF-Tmac cells transduced with the Tat expression retroviral vector M-LTR-Tat-ZG at 45% efficiency, expression of MazF was clearly induced, indicating that Tat dependent MazF expression system was maintained in the cells even 6 months after the autologous transplantation.

Because gene therapy for HIV is aimed at reconstituting an HIV-resistant immune system, genetically modified cells must inhibit virus replication and maintain persistence *in vivo*. Although *ex vivo* gene therapy targeting CD4+ T cells or CD34+ hematopoietic stem cells has been shown to promote long term persistence of infused cells in peripheral blood in human, it is difficult to obtain information about the distribution pattern of these cells in the whole human body. In order to obtain such information, the monkeys were sacrificed and lymphocytes were isolated from several organs after 6 months of monitoring. Importantly, the infused MazF-Tmac cells were detected in secondary lymphoid tissue, such as several LNs and spleen, and in peripheral blood, although individual differences between CD4T-1, -2, and -3-transplanted monkeys were observed. No histopathological disorders were observed in the organs containing MazF-Tmac cells, indicating that there were no lesions relating to MazF-Tmac cells. The distribution of MazF-Tmac cells in the lymphoid tissues of CD4T-3-transplanted monkey was lower compared to the CD4T-1 and -2-transplanted monkeys. One reason for this phenomenon is likely the lower dosage of busulfan used to treat the CD4T-3-transplated monkey. Busulfan is an alkylating agent with potent effects on hematopoietic stem cells that is commonly used for stem cell transplantation. In rhesus macaques, a low-dose of busulfan has an impact on bone marrow stem/progenitor cells with transient and mild suppression of peripheral blood counts

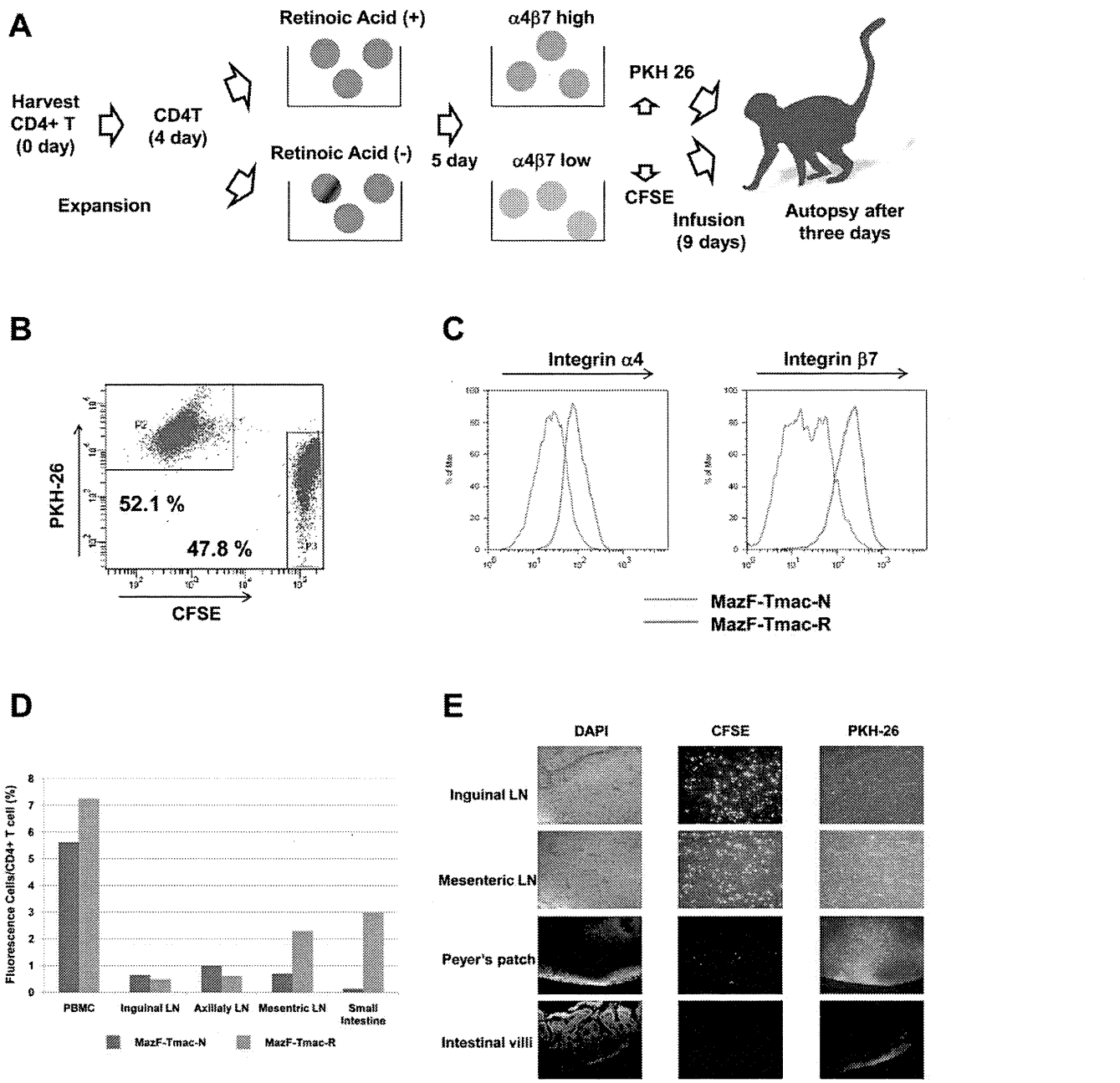

Figure 6. Comparison of the homing effect of MazF-Tmac cells treated with or without retinoic acid. (A) CD4+ T cells from the CD4T-4 monkey were stimulated with anti-CD3/CD28 beads, and MT-MFR-PL2 vector was transduced twice on days 3 and 4. After transduction, total lymphocytes were divided into two culture conditions in which retinoic acid was added to the one. After an additional 5 days of incubation, control and retinoic acid-treated cells were stained with CFSE and PKH26, respectively, mixed at nearly the same numbers, and infused into the autologous CD4T-4. Three days after the transplantation, experimental autopsy was performed. (B) A mixture of the two groups of MazF-Tmac cells stained with CFSE and PKH26 was analyzed using flow cytometry; the ratio of the two groups was almost same. (C) Up-regulation of the homing receptor was confirmed in the MazF-Tmac-R cells. The MazF-Tmac-N and MazF-Tmac-R cells are indicated by the blue line and red line, respectively. (D) Lymphocytes were collected from three lymph nodes (LNs) and small intestines, and a percentage of fluorescently-labeled cells were analyzed by flow cytometry. (E) Fluorescence microscope analysis of distal organ specimens.
doi:10.1371/journal.pone.0023585.g006

[20]. Thus, the lower engraftment efficiency of CD4T-3 (MazF-Tmac) cells might be due to the milder busulfan treatment.

In contrast to the LNs and spleen, a limited number of cells were detected in non-lymphoid tissues such as small intestine and liver. Considering HIV-1 infection, the gastrointestinal (GI) tract, which contains the vast majority of lymphoid tissues in the total body to protect mucosal membranes from foreign antigens, is the dominant site of HIV replication rather than LNs, which were originally thought to be the main infection sites [21]. In GI tract, CD4+ T cells are dramatically decreased during the acute phase of HIV infection [21,22,23]. In rhesus macaques, a similar depletion was also reported during the acute phase of simian immunodeficiency virus (SIV) infection, with CD4+ memory T cells specifically targeted [24,25]. Notably, the rate of mucosal CD4+

T cell depletion in pathogenic SIV-infected monkeys correlates with the disease progression in the rhesus macaque [26]. Indeed, recent studies provide evidence that the depletion of mucosal CD4+ T cells leads to damage of the gut mucosal layer resulting in translocation of microbial products, such as lipopolysaccharide (LPS), ultimately causing chronic and systemic immune activation, which is one of the hallmarks of HIV/SIV infection and one of the predictors of disease progression [27,28]. Although HAART therapy is effective in controlling viral replication and recovering CD4+ T cells in the peripheral blood, restoration of CD4+ T cells is delayed in the GI tract [21,29]. Thus, the repair of depleted CD4+ T cells using gene therapy might attenuate the breakdown of the mucosal layer and prevent mucosal immune system deficiency. To change the tissue distribution of infused CD4+ T cells, the enhancement of homing receptor expression in T lymphocyte is necessary. Integrin $\alpha4\beta7$ is known to facilitate the migration of lymphocytes from gut-inductive sites where immune responses are first induced (Peyer's patches and mesenteric LNs) to the lamina propria [30,31]. Expression of the homing receptor is induced by the addition of retinoic acid [32], which is produced mainly from retinol (vitamin A) by dendritic cells in the mesenteric LNs. As shown in Figure 6D and 6E, although these are preliminary data with only one monkey, editing of the homing receptors integrin-$\alpha4$ and integrin-$\beta7$ by retinoic acid enhanced the recruitment of MazF-Tmac cells to the mesenteric LNs, small intestine, and Peyer's patches. These results may indicate that MazF-Tmac cells treated with retinoic acid selectively accumulate in the mesenteric LNs and then migrate into Peyer's patches. It has been reported that the HIV-1 envelope protein gp120 binds to and signals through the activated form of integrin $\alpha4\beta7$ [33]; however, we expect that retinoic acid-treated MazF-T cells will persist in distal organs without the additional spread of HIV replication because of the HIV-1 resistance observed in the MazF-Tmac cells. Therefore, we speculate that the combination of several culture methods to edit the homing receptor will enhance the recruitment of MazF-Tmac cells to distal lymphoid organs, resulting in a more efficient therapeutic.

In summary, we showed long-term persistence, safety and continuous HIV replication resistance in the *mazF* gene-modified CD4+ T cells in a non-human primate model *in vivo*, suggesting that autologous transplantation of *mazF* gene-modified cells is an attractive strategy for HIV gene therapy.

Materials and Methods

Vector design and viral production

The GALV-enveloped gamma retroviral vector MT-MFR-LP2 was generated as previously described [6]. MT-MFR-PL2 expresses a truncated form of the human low affinity nerve growth factor gene (ΔLNGFR) [34] under the control of a functional PGK promoter and the MazF gene under control of the HIV-LTR promoter (Figure 1A). The ΔLNGFR is a surface marker that allows identification of transduced cells.

Animals

Four cynomolgus macaques (*Macaca fascicularis*, 6–7 years old), CD4T-1, CD4T-2, CD4T-3, and CD4T-4, were used in this experiment and were maintained at the Tsukuba Primate Research Center for Medical Science at the National Institute of Biomedical Innovation (NIBIO, Ibaraki, Japan). The study was conducted according to the Rules for Animal Care and the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [35] and in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research". The protocols for the experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (DS18-100). All surgical and invasive clinical procedures were conducted by trained personnel under the supervision of a veterinarian in a surgical facility using aseptic techniques and comprehensive physiologic monitoring. Ketamine hydrochloride (Ketalar, 10 mg/kg; Daiichi-Sankyo, Tokyo, Japan) was used to induce anesthesia for all clinical procedures associated with the study protocol such as blood sampling, gene-modified cell administration, clinical examinations and treatment.

Ex vivo expansion of CD4+ T cells, and transduction of the MazF vector

Peripheral blood from cynomolgus macaques was collected by apheresis as previously described [36]. For the dissolution of red blood lymphocytes, collected blood was treated with ACK lysing buffer (Lonza, Walkersville, MD) and was washed twice with phosphate buffered saline (PBS). Then, CD4+ T cells were isolated using anti-CD4 conjugated magnetic beads (Dynal CD4 Positive Isolation Kit, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Isolated CD4+ T cells were cultured at 5×10^5 cells/ml in GT-T503 (Takara Bio, Otsu, Japan) supplemented with 10% FBS (Invitrogen), 200 IU recombinant human interleukin-2 (IL-2; Chiron, Emeryville, CA), 2 mM L-glutamine (Lonza), 2.5 μg/ml Fungizone (Bristol Myers-Squibb, Woerden, The Netherlands) and activated for three days with either 5 μg/ml concanavalin A (Con A, Sigma Chemical, St. Louis, MO) for CD4T-1 or a combination of anti-CD3 clone FN-18 (Biosource, Camarillo, CA, USA) and anti-CD28 clone L293 (BD Biosciences, Franklin Lakes, NJ) monoclonal antibodies conjugated to M-450 epoxy magnetic beads (Invitrogen) at cell-to-bead ratio of 1:1 (CD4T-2 and CD4T-3). On day 3, the activated CD4+ T cells were transduced with the MazF retroviral vector MT-MFR-PL2 in the presence of RetroNectin® (Takara Bio) according to manufacturer's instructions. Transduction was repeated on day 4. CD4+ T cells were further expanded to day 7 to 9 until the total cell number reach more than 10^9. The closed system MazF-Tmac cell manufacturing was performed using gas permeable culture bags; Cultilife 215 (Takara Bio) and Cultilife Eva (Takara Bio) were used for CD4+ T cells expansion and Cultilife spin (Takara Bio) was used for transduction of the MazF retroviral vector.

Transplantation of expanded CD4+ T cells

Prior to the transplantation, each macaque was treated with busulfan (Ohara Pharmaceutical, Shiga, Japan). Busulfan has been used extensively as a preparatory regimen for allogenic hematopoietic stem cell transplantation based on its toxicity to hematopoietic stem cells. Furthermore, it has been reported that in non-human primates, hematopoiesis was significantly decreased after a single, clinically well-tolerated dose of busulfan, with slow, but almost complete, recovery over the next several months [20]. The effects of busulfan on lymphocyte engraftment, however, are not well documented. Although cyclophosphamide is widely used in immune gene therapy trials in humans for lymphocyte transplantation, there is no information available for cyclophosphamides effect on T-cell transplantation in the cynomolgus macaque. It should be noted that we have chosen busulfan for our CD4+ T cell transplantation because busulfan is shown to cause a reduction in the peripheral blood count in human trial [37], we have had success in using busulfan for cynomolgus macaque bone marrow transplantation and according to internal information, busulfan causes a reduction of the peripheral blood count in