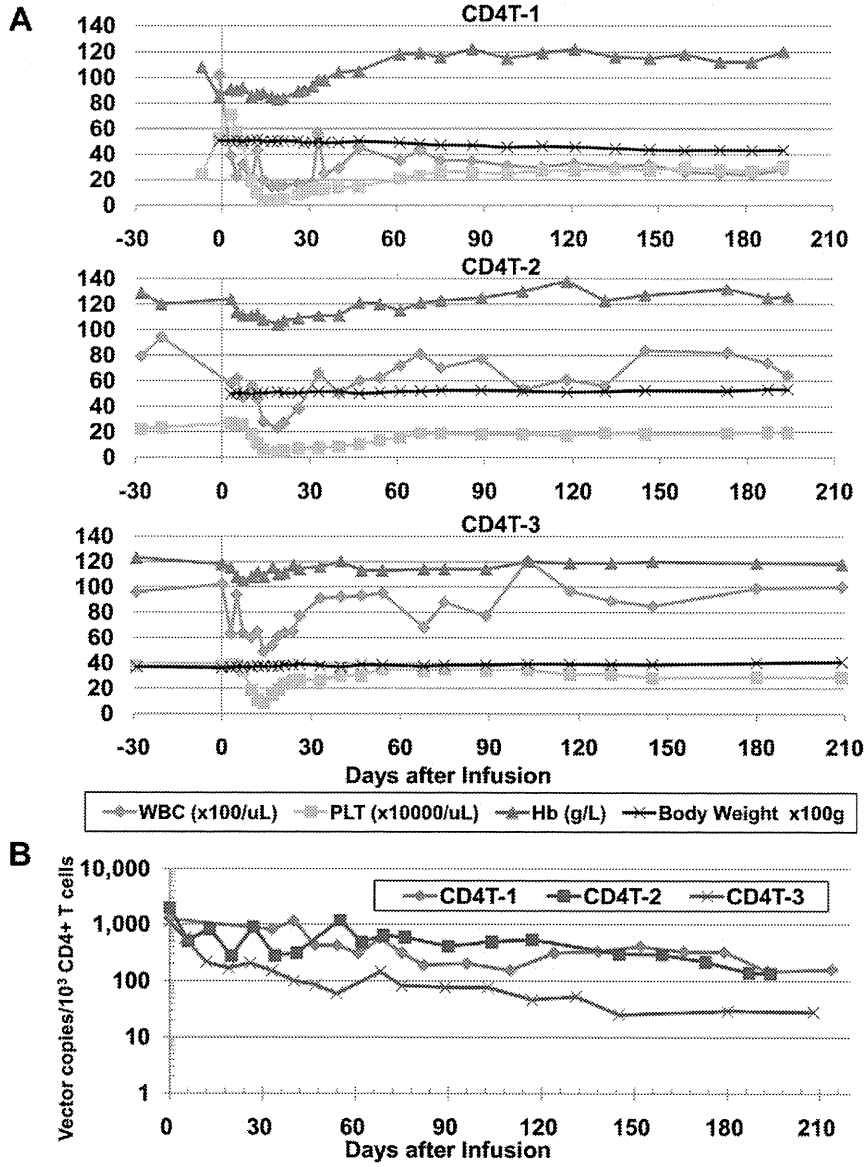

Figure 2. Hematological analysis and engraftment of the MazF-transduced CD4+ T cells. (A) The body weight and several hematological features were measured at the indicated time points, and the number of WBC, Hb, and PLT were represented. Each macaque was monitored throughout the study period. (B) The *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

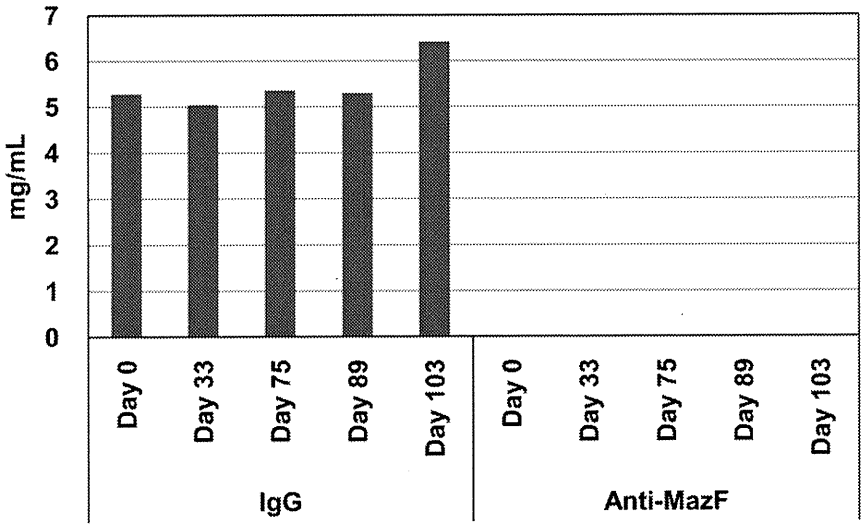

Figure 3. No detection of anti-MazF antibodies in monkey blood after transplantation of MazF-Tmac cells. Plasma samples were isolated from the monkey CD4T-2 at day 0, 33, 75, and 103 after transplantation and were used to detect anti-MazF antibodies on a MazF protein-immobilized microplate. The plasma 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

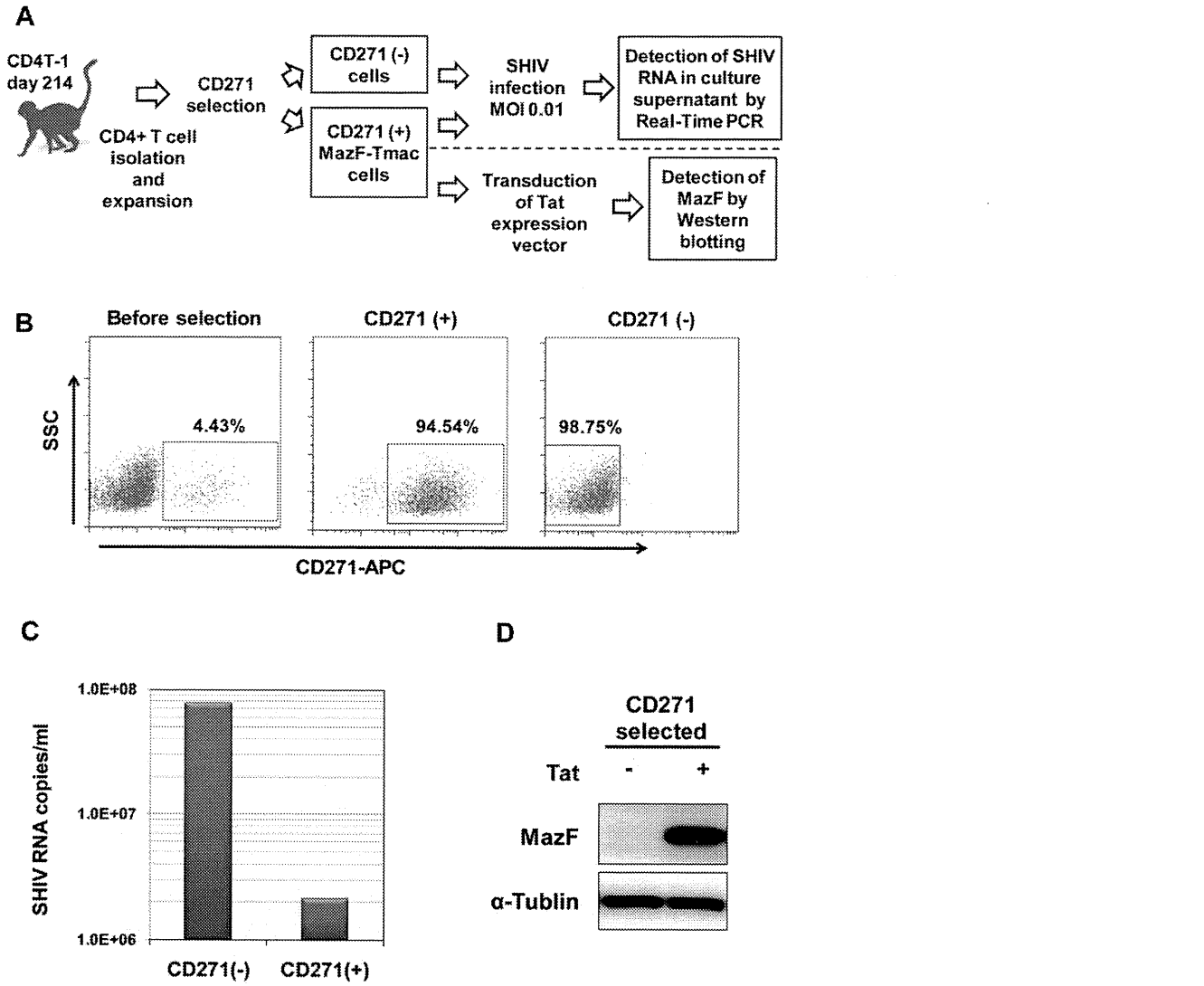

Figure 4. Examination of the anti-viral efficacy of MazF-Tmac cells harvested from the monkey. (A) Flow diagram of the experiment. CD4+ T lymphoid cells from CD4T-1 (214 days post-infusion of the MazF-Tmac cells) were stimulated and expanded *ex vivo*. The genetically modified cells expressing ΔLNGFR+ 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

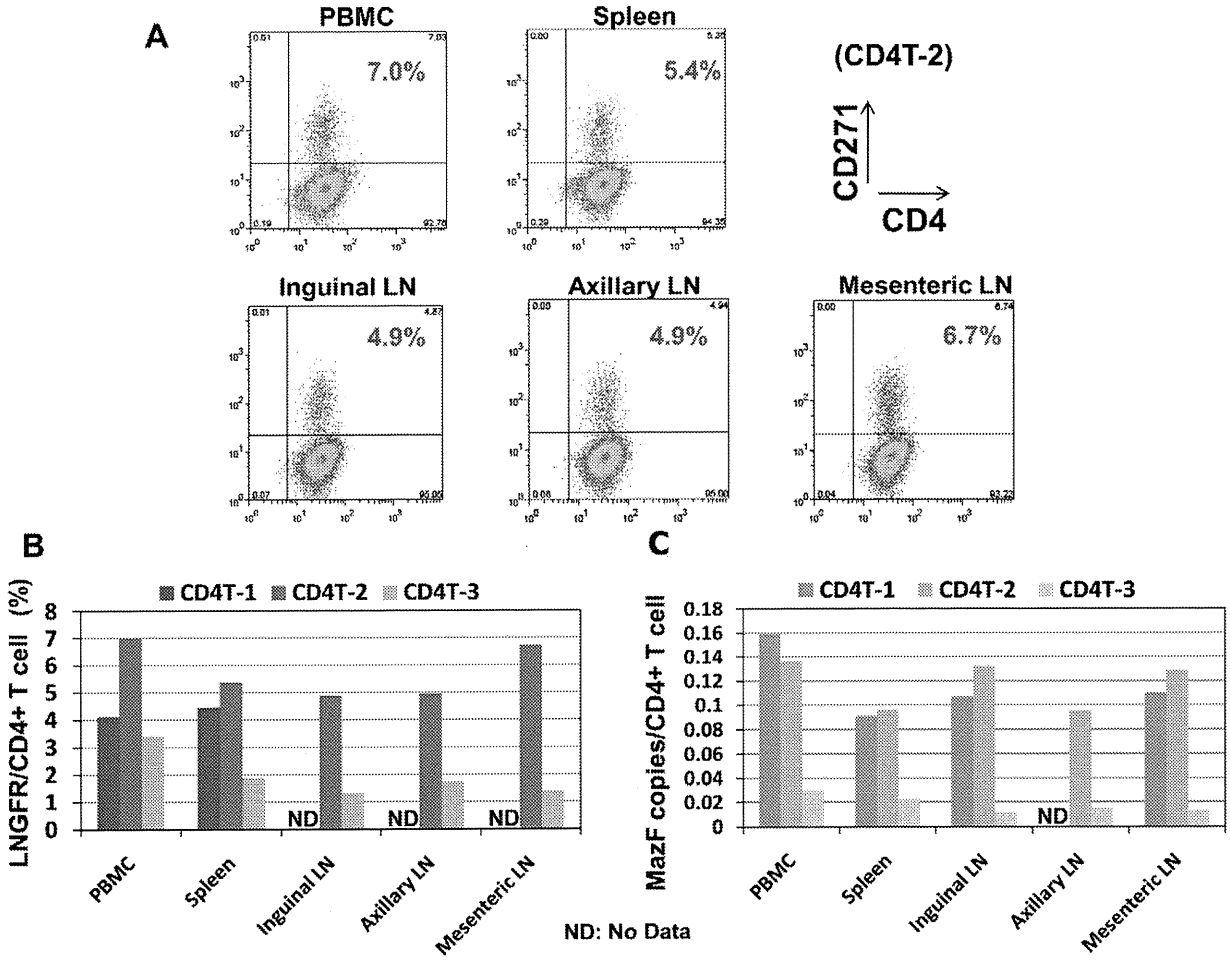

Figure 5. Analysis of the distribution of MazF-Tmac cells in several organs. (A) CD4+ T cells were isolated from lymphocytes separated from several organs, incubated 3–4 days, and stained with anti-CD4 and anti-CD271 antibodies. CD4T-2 is represented by a dot plot. (B) The percentage of CD271+ cells 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

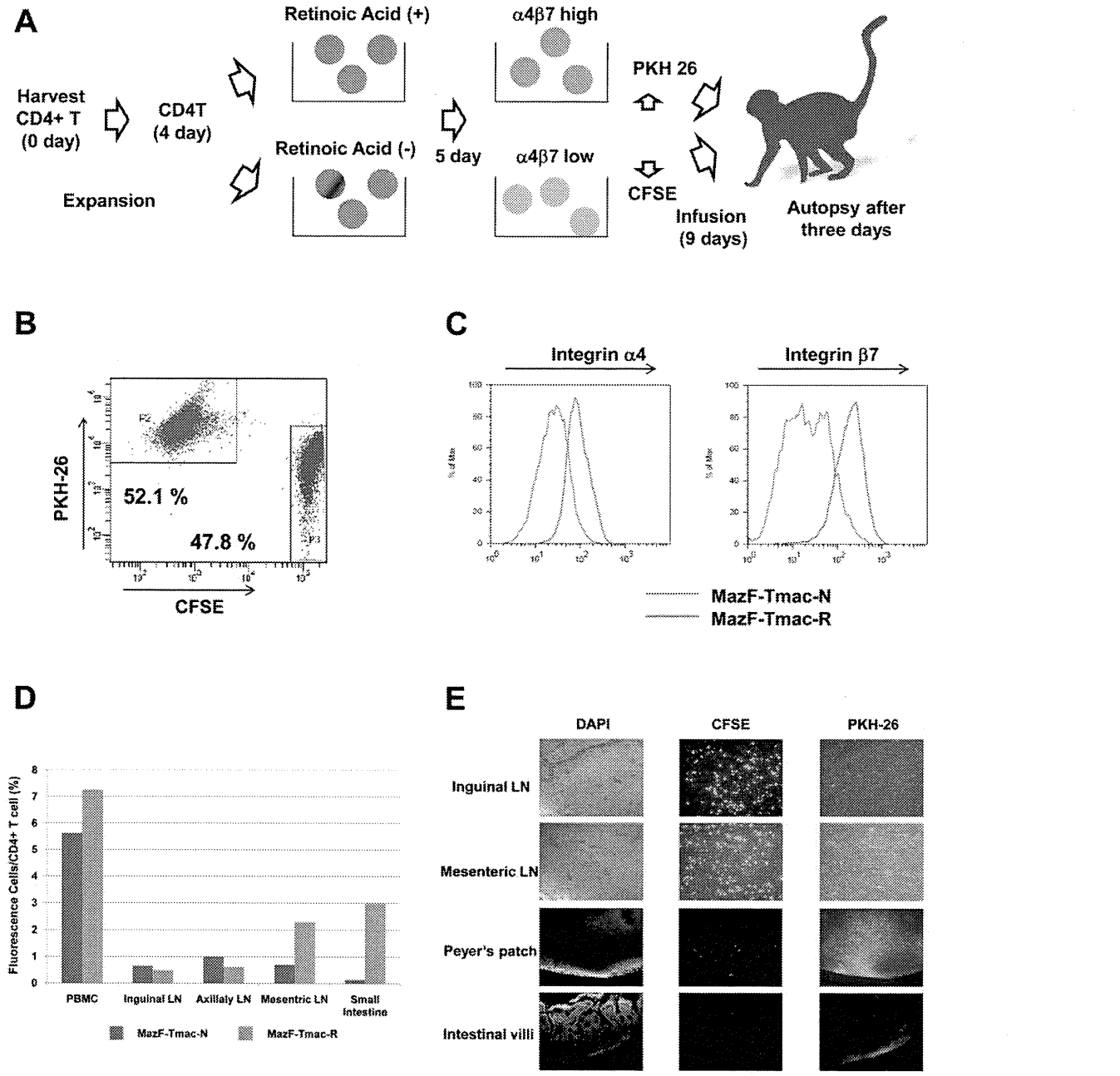

Figure 6. Comparison of the homing effect of MazF-Tmac cells treated with or without retinoic acid. (A) CD4+ T cells from the CD4T-4 monkey were stimulated with anti-CD3/CD28 beads, and MT-MFR-PL2 vector was transduced twice on days 3 and 4. After transduction, total lymphocytes were divided into two 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

cynomolgus macaques. Busulfan was orally administered to the macaques twice at 10 mg/kg each (CD4T-1 and CD4T-2) or 6 mg/kg each (CD4T-3) [38]. The expanded cells were harvested, washed three times with PBS, and re-suspended in PBS containing 10% autologous plasma. The collected cells were infused intravenously to monkeys at the speed of 1 ml per minute.

Flow cytometry analysis

The cell surface markers of the expanded cells and peripheral blood mononuclear cells (PBMC) were analyzed using FACSCalibur (BD Bioscience) and FACSCanto (BD Bioscience), and data analysis was performed using CellQuest software (BD Bioscience), FACSDiva software (BD Bioscience) or FlowJo software (Tree Star, Inc., Ashland, OR). The following antibodies were used for staining: anti-CD3 (SP34-2, PerCP), anti-CD4 (L200, FITC), anti-CD25 (2A3, FITC), anti-CD28 (CD28.2, PE), anti-CD95 (DX2, FITC), anti-CXCR4 (12G5, PE) and anti-integrin-β7 (FIB504, PE), which were obtained from BD Bioscience. The anti-CD49d (HP2/1, FITC) antibody was obtained from Beckman Coulter (Fullerton, CA), and the anti-CD271 (LNGFR, PE and APC) antibodies were obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Measurement of hematological data

Two ml of blood was prepared every week. Blood samples were used to measure the white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit values, mean corpuscular volume, mean cell hemoglobin concentration and platelet (PLT) count using a Sysmex K-4500 instrument (Toaiyouddenshi, Kobe, Japan). The concentrations of the biochemical markers in blood samples were also monitored including total proteins, albumin, blood urea nitrogen, glucose, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase, creatine phosphokinase, lactate dehydrogenase, creatine, sodium, potassium, chlorine and C-reactive protein using an AU400 instrument (Olympus Medical Systems, Tokyo, Japan).

Quantification of gene-modified CD4+ T cells

The existence and persistence of genetically modified CD4+ T cells were monitored by measuring the proviral genome of the transgene using quantitative real-time PCR. DNA samples were extracted from 2×10^6 PBMCs using a Gentra Puregene Blood Kit (QIAGEN, Hilden, Germany). The proviral copy number of the transgene was calculated from 400 ng of genomic DNA with quantitative PCR using a Cycleave RT-PCR Core Kit (Takara Bio) and Provirus Copy Number Detection Primer Set (Takara Bio) according to the manufacturer's instructions. The reaction was performed with the Thermal Cycler Dice Real Time System (Takara Bio), and the data was analyzed using Multiplate RQ software (Takara Bio). For each run, a standard curve was generated from the pMT-MFR-PL2 plasmid, whose copy numbers were already known. Based on the standard curve, the amount of infused cells was quantified.

Detection of anti-MazF antibodies in macaque blood after transplantation of MazF-Tmac cells

To examine whether anti-MazF antibodies can be generated after the transplantation of MazF-Tmac cells, the plasma isolated from the macaques was analyzed. In order to detect anti-MazF antibodies, purified MazF protein or anti-monkey IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) was precoated onto the wells of a 96-well microplate and subsequently blocked with PBS-1% BSA. The plasma samples were isolated from the CD4T-2 at day 0, 33, 75, and 103 after transplantation and were diluted to 500,000-fold, 50,000-fold, and 10,000-fold. Cynomolgus macaque IgG purified from normal macaque plasma with Melon Gel IgG purification Kit (Thermo Fisher Scientific, Rockford, IL, USA) was used as a control for this reaction. The two-fold serial dilutions of the IgG (1 ng/ml to 64 ng/ml) and the diluted plasma samples, as described above, were separately added to each well. After an overnight incubation at 4°C, the wells were washed with PBS-1% BSA. The POD-conjugated anti-monkey IgG (Nordic Immunological Laboratories) was then added to the wells. After 4 hours of incubation at room temperature, the wells were washed three times with PBS-1% BSA followed by the addition of the substrate solution (o-Phenylenediamine, Sigma). The optical density of each well was read at 490/650 nm using a 680XR microplate reader (Bio-Rad Laboratories, Hercules, CA) after stopping the reaction with H_2SO_4 stop solution (Figure S1).

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

To examine the function of the *mazF* gene in cells harvested from a MazF-Tmac-transplanted monkey, the frozen lymphoid cells from CD4T-1 at autopsy (214 days post-infusion of MazF-Tmac cells) were recovered, CD4+ T cells were selected using a CD4+ T Cell Isolation Kit (Miltenyi Biotec), stimulated with anti-CD3/CD28 beads at a cell-to-bead ration of 1:1, and expanded in GT-T503 medium supplemented with 10% FBS, 200 IU recombinant human interleukin-2, 2 mM L-glutamine, 2.5 μg/ml Fungizone, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 7 days of expansion, the genetically modified cells expressing ΔLNGFR+ were concentrated with an anti-CD271 monoclonal antibody (CD271 MicroBeads, Miltenyi Biotec) and expanded for 4 days. The cells from the CD271-negative fraction were also harvested and expanded as control non-gene modified CD4+ T cells. The expanded CD271-enriched cells and CD271-negative cells were infected with SHIV 89.6P at the MOI of 0.01 and cultured for 6 more days. Culture supernatants and cell pellets were harvested at 6 days post-infection. RNA in the culture supernatant was recovered with the QIAamp Viral RNA Mini Kit (QIAGEN) and SHIV RNA levels in the culture supernatant were determined by quantitative real-time PCR with a set of specific primers specific for the SHIV *gag* region [39]. In order to detect the Tat-dependent expression of MazF in the CD271-enriched MazF-Tmac cells harvested from the monkey, the cells were transduced with the Tat expression retroviral vector M-LTR-Tat-ZG [6] in the presence of RetroNectin® as per the manufacturer's instruction. Twenty hours after Tat transduction, the cells were harvested, counted by trypan blue exclusion assay, washed twice with PBS, and 5×10^5 cells were suspended in 50 μl of 1× SDS sample buffer. The cell samples were incubated at 95°C for 10 min, and 5 μl of each cell sample was used for western blot analysis. For gel electrophoresis of proteins, the sample solutions described above were loaded into the wells of a 4–20% Tris-Glycine gel (Atto, Tokyo, Japan). After completion of electrophoresis, the gel was transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) with papers containing transfer buffer using the semi-dry method at 60 mA (constant voltage) for 60 min. The membrane was cut in half horizontally around the 20 kDa protein band of the pre-stained protein marker (Bio-Rad Laboratories). The upper part of the membrane was used to detect the α-tubulin (50 kDa) as an internal standard, while the lower part of the membrane was used to detect MazF (12 kDa). After blocking, the membranes were then incubated overnight at 4°C in the blocking buffer (5% skim milk in PBS)

containing 1 μg/ml anti-α-tublin antibody (Cell Signaling Technology) and 1 μg/ml anti-MazF polyclonal antibody (rabbit, in-house preparation), respectively. Each membrane was washed three times and subsequently incubated at room temperature for 1 hour in 10 ml of the blocking buffer containing the 10,000-fold diluted goat anti-IgG rabbit antibody (peroxidase conjugated, Thermo Fisher Scientific). The membrane was washed five times by gentle shaking in the washing buffer at room temperature for 5 min. The membrane was soaked at room temperature for 5 min in substrate solution (SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate, Thermo Scientific). Protein signals were detected by a CCD camera (LuminoShot 400 Jr, Takara Bio), which captures a digital image of the western blot.

Collection of lymphocyte from several organs

Several organs were collected following euthanasia of the monkeys. After thoracotomy, the right atrium was incised, and 2 L of heparinized PBS was infused into the left ventricle using an 18-gauge needle. After perfusion, several organs were collected, and lymphocytes were separated using the following method: samples of spleen, thymus, liver, bone marrow, and axillary, inguinal and mesenteric LNs were minced and filtered through a 40 μm nylon filter (BD Bioscience); lymphocyte of the small intestine were collected by the Percoll (GE Healthcare, Castle Hill, Australia) density-gradient centrifugation method as described previously [39]; and lymphocytes obtained from each organ were used for the flow cytometric analysis, and extracted DNA was used for quantification PCR.

In vivo homing analysis

CD4T-4 was used for homing analysis. Isolated CD4+ T cells were stimulated with anti-CD3/CD28 beads and cultured in GT-T503 medium supplemented with 10% FBS, 200 IU IL-2, 2 mM L-glutamine, and 2.5 μg/ml Fungizone. After 4 days of expansion, activated CD4+ T cells were divided into two culture bags (ClutiLife Eva), and 10 nM retinoic acid (Sigma) was added to one of the bags. After an additional 5 days of incubation, expanded cells with or without retinoic acid were harvested and labeled with 2 mM PKH26 (Sigma) or 5 μM CFSE (Sigma), respectively, according to the manufacturer's instructions. Thereafter, the cells were washed three times with PBS, mixed in PBS containing 10% autologous plasma and infused into the macaque. Then, CD4T-4 was euthanized at 3 days after transplantation. Lymphocytes from several organs were collected as previously described, and the distributions of labeled lymphocytes were detected by flow cytometric analysis. The specimens from several organs were fixed in buffered formaldehyde and embedded in plastic. Serial sections were made using a diamond saw. The slides were then analyzed under a fluorescence microscope to detect the distribution of the expanded cells in the distal organ specimens.

Supporting Information

Figure S1 Raw data of 490/650 nm absorbance. The raw data of the optical density of each well at 490/650 nm was read using a microplate reader 680XR (Bio-Rad Laboratories, Hercules, CA) is represented.
(PDF)

Figure S2 Photographs of histopathological analysis. Individual photographic data of histopathological analysis of CD4T-1, -2, and -3 in Table 3 is represented.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HC NS YY KT JM IK. Performed the experiments: HC NS HT HS NA. Analyzed the data: HC NS HS NA. Contributed reagents/materials/analysis tools: NS HT HS NA. Wrote the paper: HC NS.

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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.

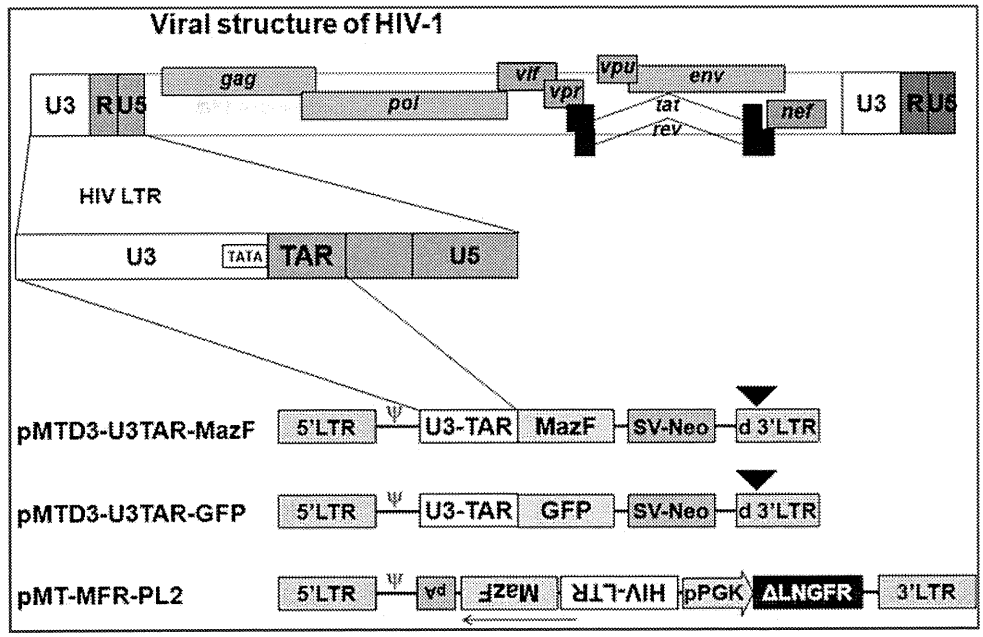

FIG. 1. Construction of retroviral vector under the control of HIV-LTR promoter. To remove promoter activity of the MoMLV LTR, the self-inactivating retroviral vector pMTD3 was constructed based on pMT (Lee *et al.*, 2004) by deleting a 276-bp fragment from its 3'LTR U3 region. A synthetic ACA-less *mazF* 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.

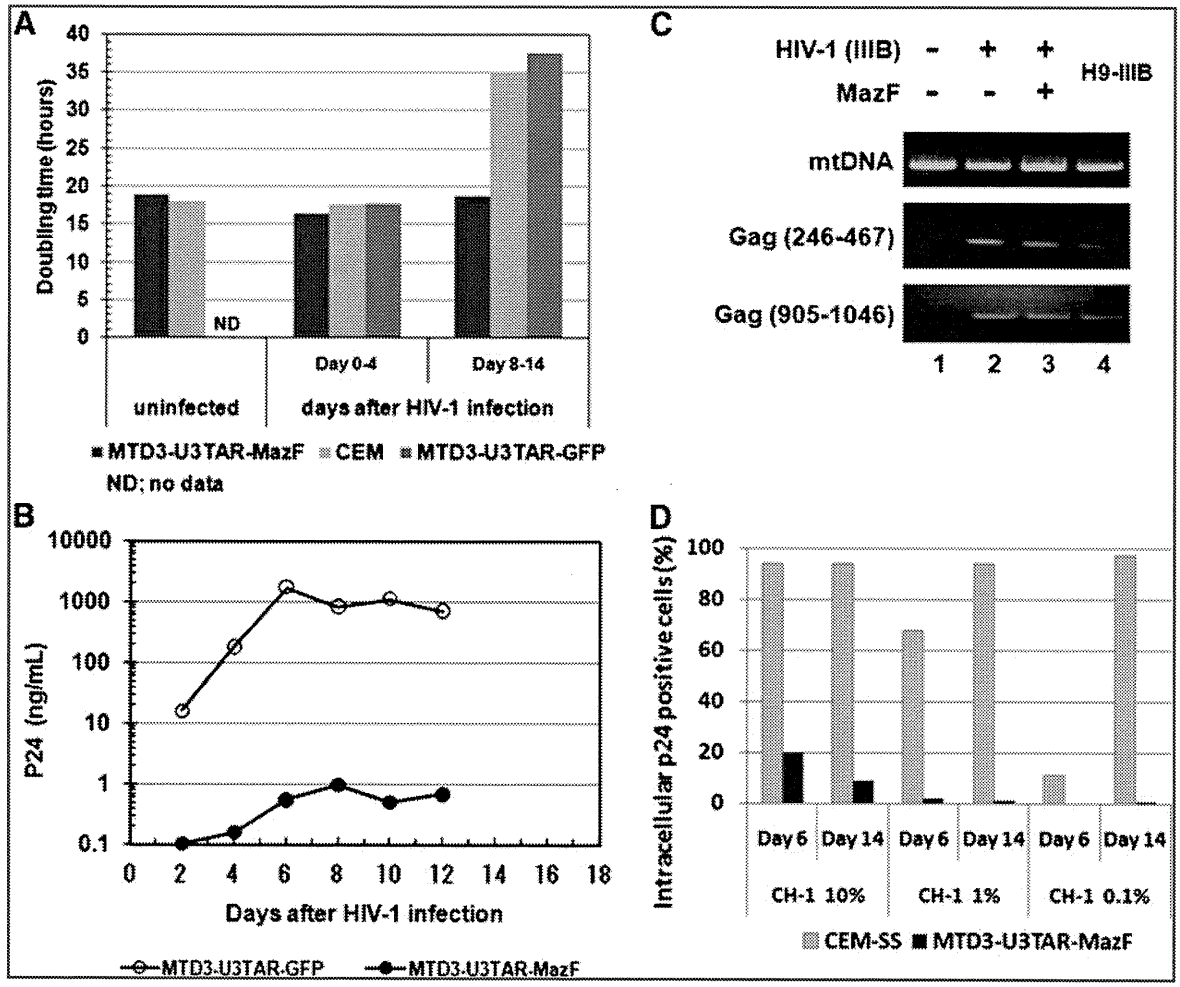

FIG. 2. Analysis of MazF-transduced CEM-SS cells after HIV-1 infection. (**A**) CEM-SS cells transduced with either the *mazF* gene or the GFP gene were infected with HIV-1 IIIB at an MOI of 0.07. After infection, the doubling time of the cells for each culture condition was calculated using linear regression 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.

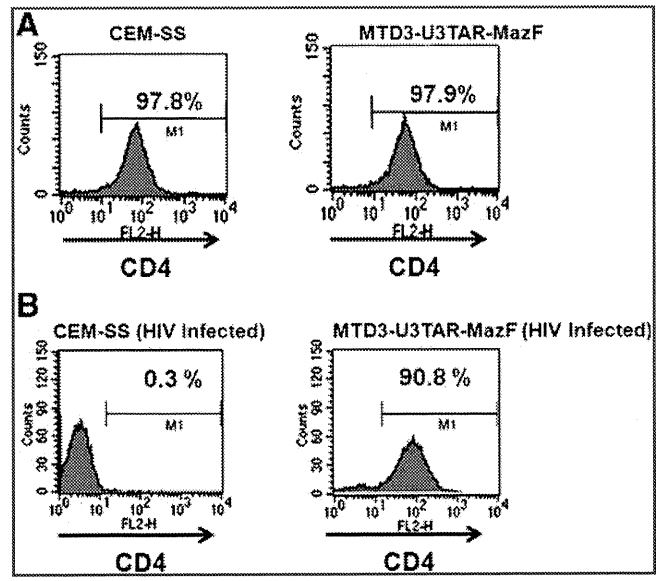

FIG. 3. CD4 levels in MazF-transduced cells. (**A**) Endogenous expression levels of CD4 were analyzed using PE-labeled anti-human CD4 antibody following flow cytometric analysis. (**B**) CEM-SS control cells and CEM-SS cells transduced with MTD3-U3TAR-MazF were infected with HIV-1 IIIB at an MOI of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

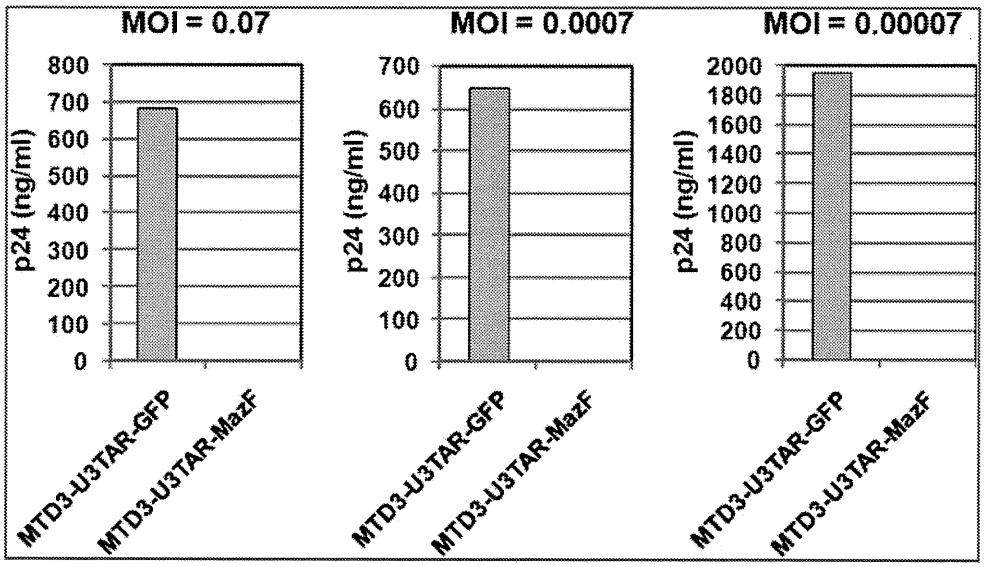

FIG. 4. HIV-1 IIIB infection using MazF-transduced CEM-SS cells at different MOIs. Polyclonal cell populations of CEM-SS resulting from gene transduction with retroviral vectors MTD3-U3TAR-MazF or MTD3-U3TAR-GFP were infected with HIV-1 IIIB at different MOIs (0.07, 0.0007, and 0.00007). Sixteen days 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

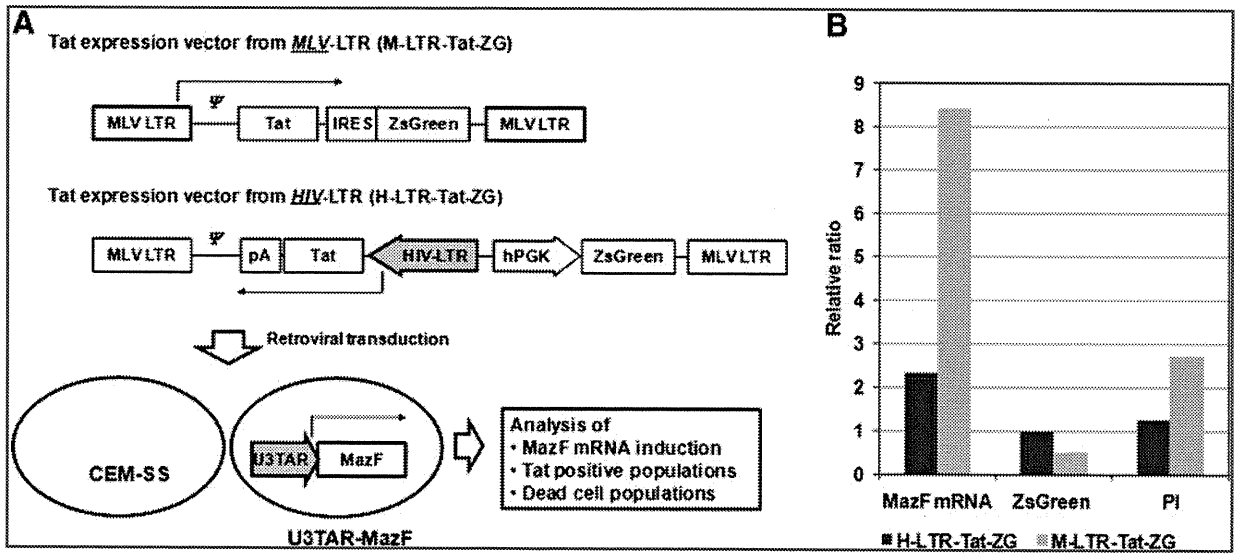

FIG. 5. Analysis of MazF induction upon Tat expression. (**A**) Outline of experimental procedure to analyze MazF induction upon Tat expression. (**B**) MazF mRNA levels were analyzed in MTD3-U3TAR-MazF transduced CEM-SS cells after Tat-expressing retroviral vector infection using real-time RT-PCR. The 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,

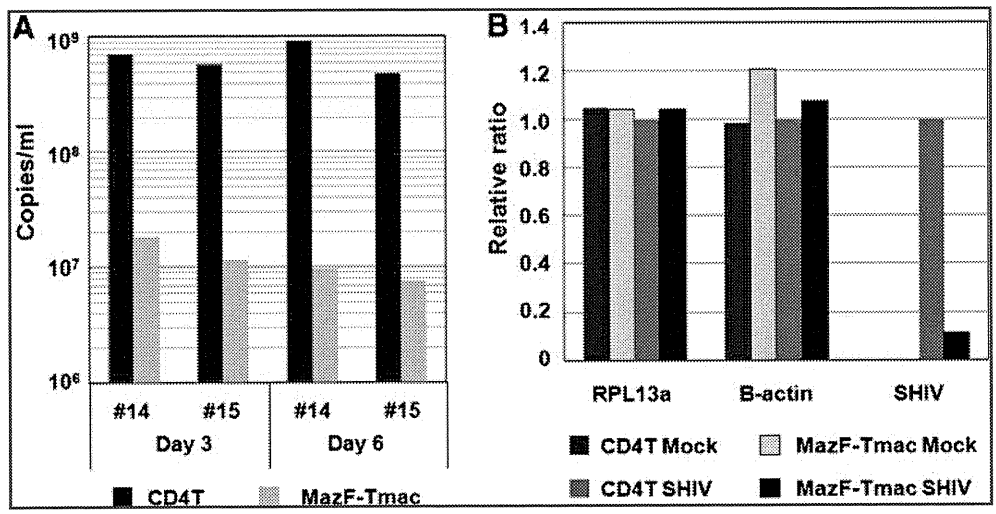

FIG. 6. Effect of MazF-induction into rhesus macaque primary CD4+ T cells on SHIV 89.6P replication. (**A**) Rhesus macaque primary CD4 T cells from two monkeys (#14 and #15) were activated and transduced with MT-MFR-PL2 vector. The MazF-transduced cells (MazF-Tmac cells) were reactivated with CD3/28 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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Review

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

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ABSTRACT

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

Contents

1. Introduction

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

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

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

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

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

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

1.1. First term (1978–1882)

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

1.2. Second and third terms (1983–1994)

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