Citation: Molecular Therapy—Nucleic Acids (2014) 3, e168; doi:10.1038/mtna.2014.20

www.nature.com/mtna

CD4+ T Cells Modified by the Endoribonuclease MazF Are Safe and Can Persist in SHIV-infected Rhesus Macaques

Naoki Saito[1], Hideto Chono[1], Hiroaki Shibata[2], Naohide Ageyama[2], Yasuhiro Yasutomi[2] and Junichi Mineno[1]

MazF, an endoribonuclease encoded by *Escherichia coli*, specifically cleaves the ACA (adenine–cytosine–adenine) sequence of single-stranded RNAs. Conditional expression of MazF under the control of the HIV-1 LTR promoter rendered CD4+ T cells resistant to HIV-1 replication without affecting cell growth. To investigate the safety, persistence and efficacy of MazF-modified CD4+ T cells in a nonhuman primate model *in vivo*, rhesus macaques were infected with a pathogenic simian/human immunodeficiency virus (SHIV) and transplanted with autologous MazF-modified CD4+ T cells. MazF-modified CD4+ T cells were clearly detected throughout the experimental period of more than 6 months. The CD4+ T cell count values increased in all four rhesus macaques. Moreover, the transplantation of the MazF-modified CD4+ T cells was not immunogenic, and did not elicit cellular or humoral immune responses. These data suggest that the autologous transplantation of MazF-modified CD4+ T cells in the presence of SHIV is effective, safe and not immunogenic, indicating that this is an attractive strategy for HIV-1 gene therapy.
Molecular Therapy—Nucleic Acids (2014) **3**, e168; doi:10.1038/mtna.2014.20; published online 10 June 2014
Subject Category: Therapeutic proof-of-concept Gene insertion, deletion & modification

Introduction

Antiretroviral therapy (ART), which is based on a combination of different classes of inhibitors, is widely used for the treatment of human immunodeficiency virus type 1 (HIV-1) infection and effectively suppresses HIV-1 replication to low or undetectable levels, corresponding with a recovery of CD4+ T cell counts.[1–3] ART treatment dramatically improves the survival rate of HIV-1-infected individuals and has transformed HIV-1 infection into a controllable illness. However, the need for lifelong therapy and difficulties in adherence to medication regimes are likely to lead to the emergence of drug-resistant HIV-1 strains. Long-term side effects, such as cardiovascular disease, hepatotoxicity and dementia, have been reported in association with HIV-1 infection.[4–6] The most serious deficiency of ART is that it cannot eradicate latent virus.[7] Once the treatment is interrupted, a replicable HIV-1 reemerges. Thus, the benefits of current ARTs are limited. Therefore, it remains necessary to discover and develop novel approaches for the management of HIV-1 infection, including treatment options used in combination with the ART.[8]

The report of the "Berlin Patient," who appears to have been cured of HIV-1 infection by stem cell transplantation with HIV-1 resistant CCR5Δ32/Δ32 cells[9] has had a major impact. This patient developed acute myelogenous leukemia and received bone marrow transplantation with cells bearing a homozygous Δ32 mutation in the CCR5 gene. No HIV-1 was detectable in this patient, even in the absence of ART.[10] However, allogeneic bone marrow transplantation as a cure for HIV-1 infection is not a realistic strategy because there is a risk of death, and the long-term effects are unclear. In contrast, gene therapy for HIV-1 has steadily progressed as an alternative to antiretroviral drug regimens.[11,12] A number of strategies have been developed, including strategies involving dominant negative inhibitory proteins, fusion inhibitors, antisense RNA, aptamers, RNA decoys, ribozymes, RNA interference, and HIV-1 entry inhibition.[13–18] These protocols target autologous T cells or hematopoietic stem cells for gene modification using retrovirus, lentivirus, or adenovirus vectors to deliver anti-HIV-1 payloads. Some of these methods have progressed to clinical trials.[19,20]

Recently, we proposed a new approach for gene therapy for HIV-1 using the endoribonuclease MazF.[21] MazF is encoded by *Escherichia coli* and specifically cleaves the ACA sequence of single-stranded RNAs. MazF does not interfere with ribosomal RNAs. When overexpressed in mammalian cells, MazF preferentially cleaves mRNA but not rRNA.[22] Previous studies have demonstrated that the expression of MazF under the control of the HIV-1 LTR promoter was successfully induced upon HIV-1 replication and rendered CD4+ T cells resistant to HIV-1 and simian/human immunodeficiency virus (SHIV) without affecting cellular mRNAs.[21,23] Because HIV-1 RNA has more than 240 ACA sequences, viral RNA is assumed to be highly susceptible to MazF. A key regulator of MazF expression in this system is the HIV-1 Tat protein, which activates transcription from the HIV-1 LTR.[24] In this system, the Tat protein induces HIV-1 replication and MazF expression. Furthermore, the autologous transplantation of MazF-modified CD4+ T cells in cynomolgus macaques has been shown to be safe, and the modified cells showed little or no immunogenicity.[25] These results suggest that the conditional expression of MazF is an attractive strategy for anti-HIV-1 gene therapy.

To investigate the safety, persistence and efficacy of MazF-modified CD4+ T cells in a nonhuman primate model *in vivo* in the presence of viral infection, six rhesus macaques were

[1]Center for Cell and Gene Therapy, Takara Bio Inc, Seta, Otsu, Shiga, Japan; [2]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki, Japan. Correspondence: Junichi Mineno, Center for Cell and Gene Therapy, Takara Bio Inc., Seta 3-4-1, Otsu, Shiga, 520–2193, Japan. E-mail: minenoj@takara-bio.co.jp or Hideto Chono, Center for Cell and Gene Therapy, Takara Bio Inc., Seta 3-4-1, Otsu, Shiga, 520–2193, Japan. E-mail: chonoh@takara-bio.co.jp

Keywords: gene therapy; HIV-1; MazF; primate model; rhesus macaque; retroviral vector; SHIV
Received 25 February 2014; accepted 27 April 2014; published online 10 June 2014. doi:10.1038/mtna.2014.20

infected with a SHIV 89.6P.[26] Four rhesus macaques were transplanted with MazF-modified CD4+ T (MazF-Tmac) cells, and two were transplanted with control ZsGreen1-modified CD4+ T (ZsG-Tmac) cells. After transplantation of the gene-modified cells, changes in the CD4+ T cell count values, changes in plasma SHIV viral loads, and the persistence of gene-modified cells were monitored throughout the experimental period. The humoral and cellular immune responses elicited by MazF were assessed. At necropsy, distributions of the transplanted MazF-Tmac cells in the distal lymphoid tissues, including several lymph nodes and the spleen, were analyzed.

Results

Study protocol and SHIV challenge

Six rhesus macaques (#12, #13, #14, #15, #16, and #17) were used for this experiment. Each rhesus macaque was challenged with SHIV 89.6P, followed by transplantation with autologous CD4+ T cells transduced with the MazF retroviral vector MT-MFR-PL2 (#12, #13, #14, and #15) or the control vector MT-ZGR-PL2 (#16 and #17) (**Figure 1a**). The rhesus macaques were monitored over 6 months for changes in CD4+ T cell counts, changes in SHIV viral loads in the plasma, persistence of the gene-modified cells, and the immune responses elicited by the gene-modified cells. An outline of the experiment is shown in **Figure 1b**,**c**. The dose of SHIV, day of infusion and experimental period are summarized in **Supplementary Table S1**. Initially, rhesus macaque #15 was challenged with (5000) 50% tissue culture infective dose ($TCID_{50}$), which we speculated would be a proper dose based on our previous experience; however, the viral loads declined to the limit of detection. We, therefore, increased the dose of $TCID_{50}$ for the other experiments. However, #12, #16, and #17 showed high viral loads, whereas #13 and #14 showed low viral loads. Such differences might have been due to individual variation in the sensitivity of the rhesus macaques used in this experiment.

Gene-modified T cell manufacturing and transplantation

MazF- or ZsGreen1-modified cells were manufactured from previously collected CD4+ T cells and transplanted into each rhesus macaque 2 months after SHIV 89.6P infection. Repeated transplantations were performed at 2-month intervals. To transplant more than 10^9 MazF-Tmac or ZsG-Tmac cells, $1–2\times10^7$ primary CD4+ T cells were recovered, stimulated, and transduced either with the MT-MFR-PL2 vector or the MT-ZGR-PL2 vector, and expanded as described in **Supplementary Materials and Methods**. The numbers and characteristics of the gene-modified CD4+ T cells for each transplantation are summarized in **Table 1**. The transduction efficiencies of the MazF and ZsGreen1 vectors were 52.0–69.5% and 53.8–75.7%, respectively. The gene-modified cells marked with a truncated form of the human low-affinity nerve growth factor receptor (LNGFR/CD271) were concentrated with an anti-CD271 monoclonal antibody for the first transplantation of rhesus macaques #12 and #14, and the second transplantation of rhesus macaques #13 and #15. The gene-modified cells that were positively selected were over 97% pure. There was no selection of control ZsG-Tmac cells for transplantation. The majority of the expanded cells were CD3+ CD4+ T cells

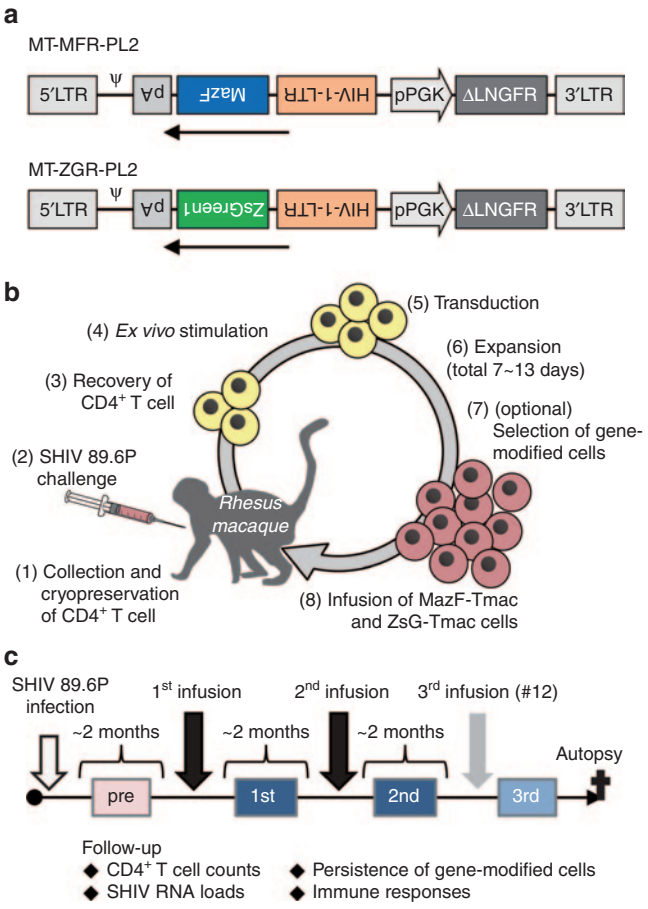

Figure 1 Diagram of autologous CD4+ T cell transplantation in a primate model. (a) Structures of the gamma-retroviral vectors MT-MFR-PL2 and MT-ZGR-PL2. LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; *LNGFR*, low-affinity nerve growth factor receptor gene; pPGK, phosphoglycerate kinase promoter. **(b)** Flow diagram of gene therapy in the rhesus macaques.[1] Peripheral blood was collected by apheresis; the CD4+ T cells were isolated and cryopreserved.[2] The rhesus macaques were challenged with SHIV 89.6P.[3,4] The CD4+ T cells were recovered and stimulated *ex vivo* with anti-CD3/CD28 beads.[5] The stimulated cells were transduced twice with retroviral vectors on days 3 and 4.[6] The transduced cells were expanded for an additional 3–9 days.[7] The ΔLNGFR-positive cells were selected at the second transplantation for rhesus macaques #13 and #15 and at the first transplantation for rhesus macaques #12 and #14.[8] On days 7–13, the expanded autologous cells were collected, washed and transplanted into the rhesus macaques intravenously. **(c)** Four rhesus macaques were transplanted with MazF-Tmac cells, and two were transplanted with control ZsG-Tmac cells. The rhesus macaques were followed over six months.

(>98%). More than 90% of these cells expressed CD95 and CD28, which are known markers of the central memory phenotype;[27] central memory cells generally have a longer lifespan than effector memory cells.[28] The expression levels of CXCR4, which is a known coreceptor for X4-tropic HIV-1 and SHIV entry, varied among animals and expansion periods.

Body weight and hematological data

There was no significant change in body weight throughout the experiment in the MazF-Tmac-transplanted rhesus macaques (see **Supplementary Figure S1a**). A gradual

Table 1 The summary of characteristics of gene-modified CD4+ T cells for each transplantation

	#12	#13	#14	#15	#16	#17
First transplantation						
Culture period (days)	13	9	13	9	9	9
Number of infused cells	2.1×10^9	3.0×10^9	2.3×10^9	1.6×10^9	1.6×10^9	1.1×10^9
CD271+ (gene-modified) (%)	97.2[a]	52.0	99.0[a]	54.1	53.8	75.0
CD3+ CD4+ (%)	98.3	99.9	99.0	99.8	99.2	99.8
CD28+ CD95+ CM (%)	93.4	97.5	95.4	98.7	N.D.	N.D.
CD28- CD95+ EM (%)	6.3	2.3	4.5	0.9	N.D.	N.D.
CXCR4+ (%)	36.3	63.2	8.9	65.5	67.9	52.6
Second transplantation						
Culture period (days)	9	13	9	13	10	10
Number of infused cells	2.7×10^9	3.3×10^9	1.8×10^9	2.7×10^9	3.8×10^9	2.8×10^9
Gene-modified (%)	69.5	96.3[a]	57.0	99.8[a]	55.2	75.7
CD3+ CD4+ (%)	99.0	99.9	98.7	99.8	99.5	99.6
CD28+ CD95+ CM (%)	96.9	90.8	97.7	97.6	N.D.	N.D.
CD28- CD95+ EM (%)	2.7	9.0	2.1	2.2	N.D.	N.D.
CXCR4+ (%)	55.4	47.8	35.7	84.0	63.5	63.4
Third transplantation						
Culture period (days)	7					
Number of infused cells	0.55×10^9					
Gene-modified (%)	65.4					
CD3+ CD4+ (%)	99.5					
CD28+ CD95+ CM (%)	96.9					
CD28- CD95+ EM (%)	3.1					
CXCR4+ (%)	77.0					

The autologous MazF-Tmac cells and the ZsG-Tmac cells were manufactured from the cryopreserved CD4+ T cells by stimulating the cells with anti-CD3/CD28 beads, and transducing them with the MT-MFR-PL2 and MT-ZGR-PL2 retroviral vectors. The CD3, CD4, CD28, CD95, CD271, and CXCR4 expression in the gene-modified cells at the time of transplantation were analyzed by flow cytometry.
[a]Gene-modified cells were concentrated based on their expression of the ΔLNGFR surface marker. CM, central memory; EM, effector memory; N.D., not determined.

decrease in body weight was observed in one ZsG-Tmac-transplanted rhesus macaque #16 by the end of the experiment (data not shown). Hematological data, including white blood cell count, hemoglobin concentration and platelets, were also analyzed, and significant changes were observed in rhesus macaque #16 at the end of the experiment (data not shown). Rhesus macaque #16 was sacrificed prior to the scheduled autopsy because of worsening symptoms. No significant changes were observed in any of the MazF-Tmac-transplanted rhesus macaques (see **Supplementary Figure S1b–d**). Thus, MazF-Tmac cells are considered safe based on the clinical observations.

CD4+ T cell counts in peripheral blood

To evaluate the impact of the autologous transplantation of gene-modified CD4+ T cells, the posttransplantation CD4+ T cell count values were compared with the values measured prior to transplantation. As shown in **Figure 2a**, the increases in the CD4+ T cell counts after transplantation of MazF-Tmac cells were significant in all four rhesus macaques, while no significant increases were observed in the two rhesus macaques transplanted with ZsG-Tmac cells. In the case of rhesus macaque #12, which was robustly infected with SHIV and had high viral loads (>10^5 copies/ml), the average CD4+ T cell count was 68 cells/μl before the gene therapy treatment, and increased to 107 cells/μl and 193 cells/μl after the first and second transplantation, respectively. Unfortunately, the mean CD4+ T cell count after the third transplantation decreased slightly to 139 cells/μl; this value was still higher than the baseline values.

The effectiveness was most clearly shown in the rhesus macaque #14, whose average CD4+ T cell count was 137 cells/μl before the gene therapy treatment, and subsequently increased to 329 cells/μl and 754 cells/μl after the first and second transplantations, respectively. To investigate the long-term safety of the treatment, this rhesus macaque was followed up for one and a half years. The other rhesus macaques (#13 and #15) had average CD4+ T cell counts of approximately 530 cells/μl before the gene therapy treatment that also increased to 1296 cells/μl and 856 cells/μl after the first transplantation, respectively, and retained these levels after the second transplantation. Thus, the transplantation of MazF-Tmac cells has the potential to increase CD4+ T cell counts.

Plasma SHIV viral loads

To investigate the influence of the transplantation of gene-modified cells, SHIV viral loads in the plasma were measured using quantitative PCR (qPCR). In rhesus macaque #15, the viral loads were below the detection limit at the time of gene therapy treatment. In rhesus macaques #13 and #14, the available data were limited to three time points due to a relatively short window in which they exhibited a stable set point before the infusion of MazF-Tmac cells. For this reason, we did not

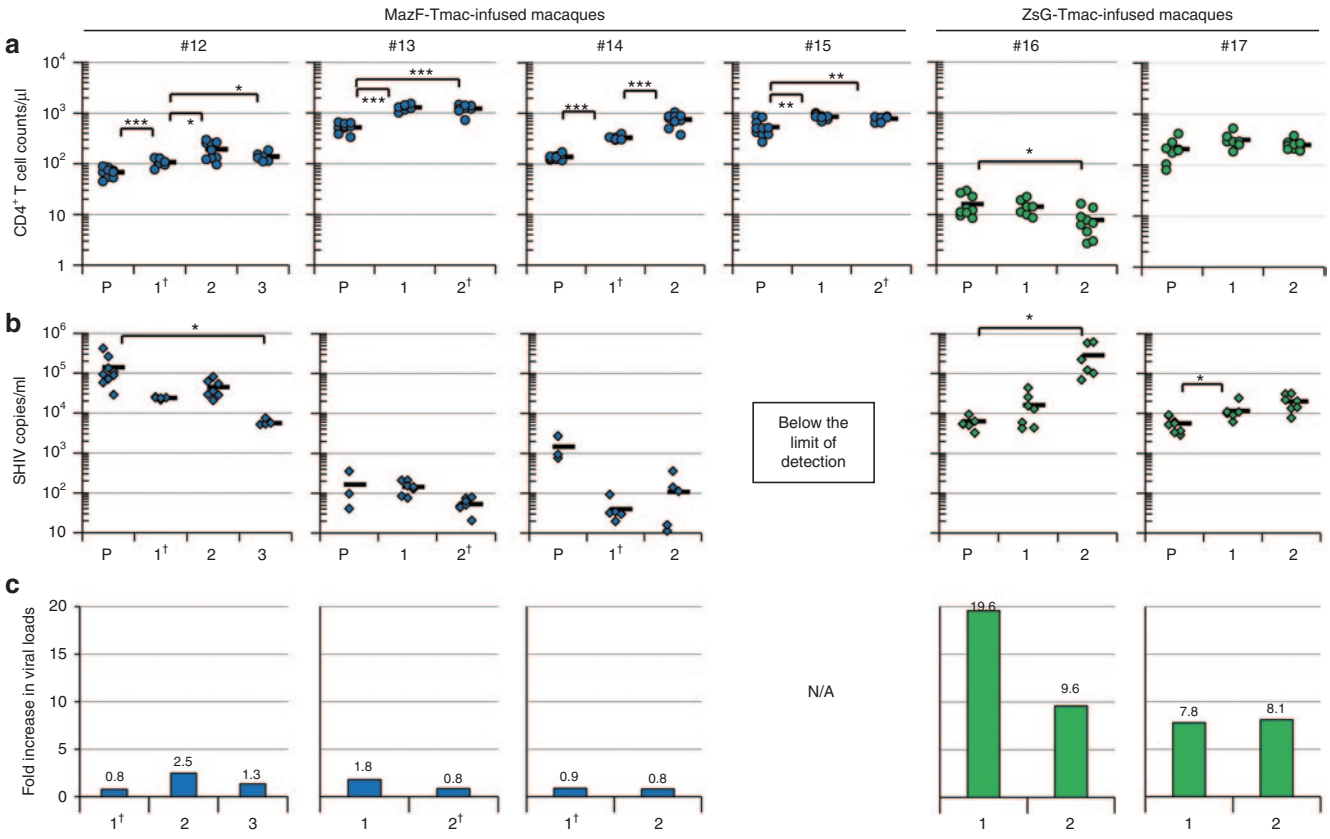

Figure 2 Changes in CD4⁺ T cell counts and viral loads. (a) The CD4⁺ T cell counts of MazF-Tmac- and ZsG-Tmac-transplanted rhesus macaques. The CD4⁺ T cell counts in the area of the viral load set point were averaged by multiple time point sampling 20 to 80 days after SHIV infection or transplantation. **(b)** Plasma SHIV viral RNA loads. The plasma SHIV viral RNA loads in the area of the set point were averaged by multiple time point sampling 20–80 days after SHIV infection or transplantation of gene-modified cells. **(c)** The fold increase of the plasma viral loads of MazF-Tmac- and ZsG-Tmac-transplanted rhesus macaques. The fold increase values were calculated by dividing [the average viral loads one week after transplantation of gene-modified cells] by [the average viral loads three weeks before transplantation of gene-modified cells]. P, pretransplantation of gene-modified cells; 1, after the first transplantation; 2, after the second transplantation; 3, after the third transplantation. Statistical significance indicated by Student's *t*-test (**P* < 0.05; ***P* < 0.01; and ****P* < 0.001). †Gene-modified cells were concentrated using the ΔLNGFR marker. N/A, not applicable due to undetectable viral loads.

employ statistical analyses between pre and postinfusion for these two macaques. However, there was no increase and tendency toward decrease in the viral loads after transplantation of MazF-Tmac cells (**Figure 2b**). For rhesus macaques #16 and #17, significant increases in the set point of SHIV viral loads were observed upon transplantation of ZsG-Tmac cells (**Figure 2b**). A dramatic rebound was detected immediately after transplantation (**Figure 2c**). These data indicate that the transplantation of ZsG-Tmac cells, which have no protective payload for SHIV, significantly impacted the viral loads. No remarkable rebound was observed after transplantation of MazF-Tmac cells (**Figure 2c**).

Longitudinal persistence of MazF-Tmac cells and ZsG-Tmac cells

To examine the *in vivo* persistence of transplanted MazF-Tmac cells and ZsG-Tmac cells, peripheral blood samples were collected to monitor the presence of gene-modified cells. The proviral copy number of the transduced retroviral vector was monitored by the qPCR method throughout the experiment. MazF-Tmac cells persisted for more than 6 months in the presence of SHIV. In particular, persistence for longer than one and a half years was observed in rhesus macaque #14. The ZsG-Tmac cells had nearly disappeared within two months after transplantation (**Figure 3a**). Similar results were obtained in flow cytometry analyses to detect the surface marker ΔLNGFR in the CD4⁺ T cells (**Figure 3b**). The calculated half-lives measured within 2 months of transplantation of the MazF-Tmac cells were much longer than that of the ZsG-Tmac cells (**Table 2**). The half-lives of the MazF-Tmac cells became even longer in the late phase of the experiment. Notably, in rhesus macaque #14, the half-life of the MazF-Tmac cells was 128.6 days when the period of analysis was extended to 9 months posttransplantation.

MazF antigen-specific interferon gamma (IFN-γ) enzyme-linked immunospot (ELISPOT) assay

To assess whether a cellular immune response was elicited by MazF-Tmac cells, an IFN-γ ELISPOT assay was performed. Peripheral blood mononuclear cells (PBMCs) from the MazF-Tmac-transplanted rhesus macaques were stimulated with a cocktail of MazF-overlapping peptides (see **Supplementary Figure S2**). As a negative control, PBMCs prepared from a normal (*i.e.*, untransplanted) rhesus

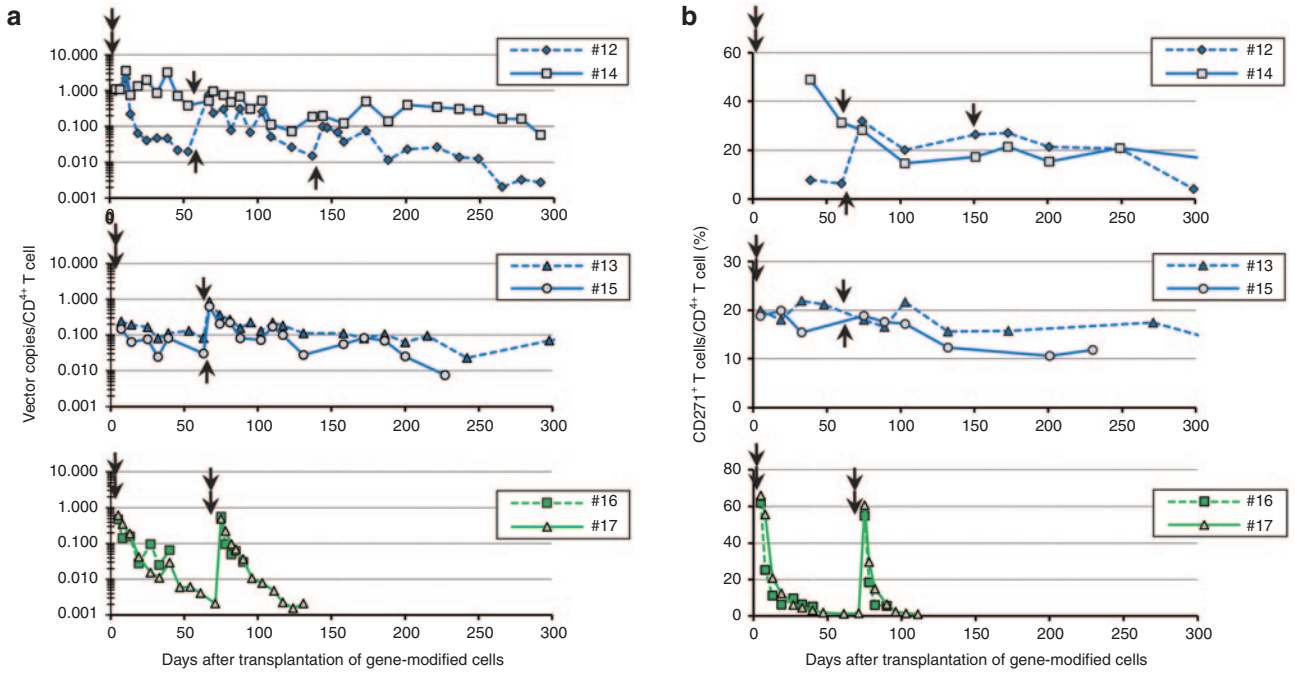

Figure 3 ***In vivo*** **persistence of MazF-Tmac and ZsG-Tmac cells. (a)** The persistence of MazF-Tmac cells and ZsG-Tmac cells was quantified using qPCR. The percentage of CD4+ T cells was analyzed using flow cytometry, and the proviral vector copy number was analyzed using qPCR. Using these data, the copy number of the transgene in CD4+ T cells was calculated. The arrows indicate the time point at which the gene-modified cells were transplanted. **(b)** The persistence of MazF-Tmac cells and ZsG-Tmac cells was quantified using flow cytometry. The percentage of gene-modified cells in the CD4+ T-cell population was determined after expanding the PBMCs with anti-CD3/CD28 beads stimulation for seven days, followed by antibody staining for CD4 and CD271 surface markers. The arrows indicate the time points at which the gene-modified cells were transplanted.

Table 2 The half-lives of gene-modified CD4+ T cells in each transplantation

	MazF-Tmac transplantation				ZsG-Tmac transplantation	
	#12	**#13**	**#14**	**#15**	**#16**	**#17**
First transplantation	7.7	42.8	35.6	32.4	3.9	4.6
Second transplantation	14.9	57.6	33.8	51.3	4.2	6.9
Third transplantation	29.0	N/A	N/A	N/A	N/A	N/A

The *in vivo* half-lives of the gene-modified cells in each rhesus macaque within a period of two months after transplantation was calculated by linear regression analyses using Microsoft Excel software.
N/A, not applicable.

macaque were stimulated. As shown in **Figure 4a**, no significant cellular immune responses related to the MazF-specific antigens were observed, indicating that the infused MazF-Tmac cells did not elicit a cellular immune response in the rhesus macaques in the presence of SHIV infection.

Detection of antibodies against MazF or ZsGreen1 in rhesus macaque blood

The evidence of longitudinal persistence of the MazF-Tmac cells supports the idea that these cells are not highly immunogenic; however, it is still important to assess the production of antibodies against MazF. As shown in **Figure 4b**, no significant production of antiMazF antibodies was detected in the blood samples from any of the rhesus macaques after transplantation with the MazF-Tmac cells. For the ZsG-Tmac cells, significant production of antibodies against ZsGreen1 was detected in rhesus macaque #17, while no antibody production was detected in rhesus macaque #16. The MazF-Tmac cells persisted for an extended period *in vivo*, and the MazF-Tmac-transplanted rhesus macaques produced no antibodies against MazF, indicating that the infused MazF-Tmac cells can be considered safe and not immunogenic in rhesus macaques in the presence of SHIV infection.

Distribution of gene-modified cells

At autopsy, lymphocytes isolated from several organs were analyzed for the distribution of the gene-modified cells using flow cytometry and qPCR. As shown in **Table 3**, ΔLNGFR-positive cells were detected by flow cytometry in the CD4+ T cells isolated from several lymph nodes, the spleen and the peripheral blood of the MazF-Tmac-transplanted rhesus macaques. A similar trend was observed in the qPCR analysis. The bone marrow, liver, and small intestine were also analyzed, but there were no detectable signs of gene-modified cells (data not shown). In the ZsG-Tmac-transplanted rhesus macaques, no gene-modified cells were detected in the organs or peripheral blood. These data strongly suggest that transplanted MazF-Tmac cells could circulate to the peripheral blood and the secondary lymphoid organs.

Histopathological analyses

It is advantageous to use primate models to investigate the safety of gene-modified cells because these animals can

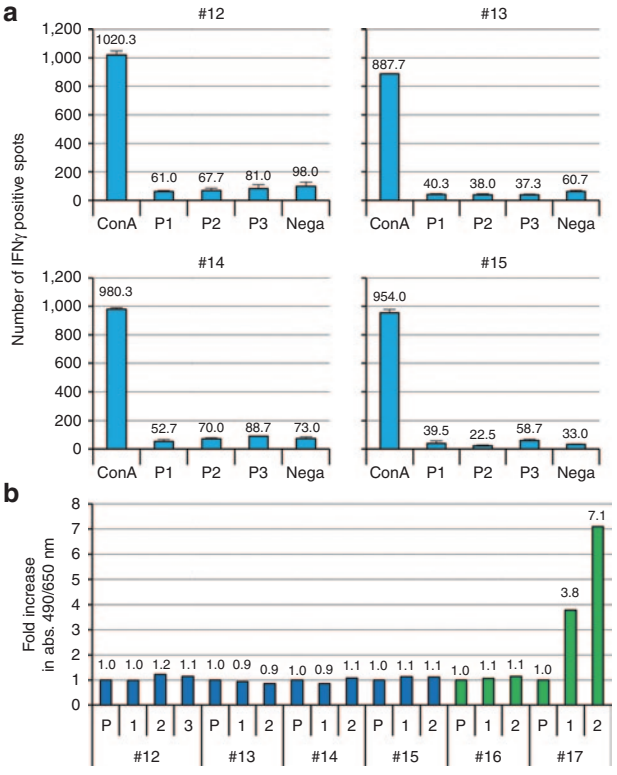

Figure 4 Detection of MazF-specific cellular and humoral immune responses. (a) IFN-γ enzyme-linked immunospot assay. The number of IFN-γ positive spots was measured under each stimulation condition. The PBMCs were prepared two months after the final MazF-Tmac cell transplantation. ConA: PBMCs from a MazF-Tmac-transplanted rhesus macaque were stimulated with concanavalin A. P1-3, PBMCs from a MazF-Tmac-transplanted rhesus macaque were stimulated with MazF peptide pools 1-3. Nega, nonstimulated PBMCs. Error bars represent the mean + SD. **(b)** Detection of MazF or ZsGreen1 specific humoral immune response using an enzyme-linked immunosorbent assay (ELISA). Plasma samples taken before transplantation (P), 4 weeks after the first transplantation,(1) 4 weeks after the second transplantation,(2) and 4 weeks after the third transplantation(3) of gene-modified cells were tested for the presence of antibodies against MazF in rhesus macaques #12, #13, #14, and #15 and for ZsGreen1 rhesus macaques #16 and #17. The relative fold increases in the absorbance values compared with the pretransplantation values are shown.

be used for surgical pathological analyses. We performed experimental autopsies at the end of the experiment. The histopathological findings of the specimens are summarized in **Supplementary Table S2**. Severe involution of the thymus was observed in rhesus macaque #12. This involution appeared to be the result of a physiological factor, such as aging, and was considered to be unrelated to the treatment of transplantation of MazF-Tmac cells. No serious adverse events related to MazF-Tmac cell transplantation were observed in any of the rhesus macaques in this treatment group.

Rhesus macaques #16 and #17 were affected by SHIV infection or transplantation of ZsG-Tmac cells, which have no SHIV resistance payload. As shown in **Supplementary Figure S3**, several disorders were observed in the axillary lymph nodes of the ZsG-Tmac-transplanted macaques,

Table 3 Distribution of gene-modified cells in lymphoid tissues

	MazF-Tmac transplantation							
	#12		#13		#14		#15	
	FCM (%)[a]	qPCR[b]	FCM (%)[a]	qPCR[b]	FCM (%)[a]	qPCR[b]	FCM (%)[a]	qPCR[b]
PBMC	4.1	2.9	15.4	18.0	4.8	11.1	11.8	26.4
Inguinal LN	N/A	3.0	13.2	16.8	4.2	8.1	7.3	17.1
Axillary LN	N/A	3.1	11.9	17.7	2.7	6.5	6.7	17.3
Mesenteric LN	N/A	0.54	13.0	18.2	4.0	10.1	5.6	14.3
Spleen	N/A	0.28	13.3	7.4	2.7	3.9	7.2	14.7

The lymphocytes isolated from several organs at autopsy were analyzed by flow cytometry and qPCR to determine the distribution of gene-modified cells.
FCM, flow cytometry; LN, lymph nodes; N/A, not applicable; PBMC, peripheral blood mononuclear cell.
[a]The percentage of ΔLNGFR-positive $CD4^+$ T cells was determined by flow cytometry. qPCR. [b]Proviral vector copy numbers per 10^2 $CD4^+$ T cells.

including a decrease in size, destruction of the structures, (see **Supplementary Figure S3a**) and a decrease in lymphoid cells (see **Supplementary Figure S3b**). These disorders were not observed in the MazF-Tmac-transplanted rhesus macaques. Rhesus macaque #16 was seriously affected by atrophy of the thymus, a decrease in lymphocytes in the inguinal and mesenteric lymph nodes, atrophy and lymphoid necrosis in the splenic marginal zone and a decrease in lymphoid cells in the periarterial lymphatic sheath and red pulp (see **Supplementary Table S2**). These observations were considered to be representative of changes relevant to the SHIV infection or transplantation of ZsG-Tmac cells. In rhesus macaque #17, atrophy of the thymus and a decrease in the lymphocytes in the inguinal lymph nodes were considered to be related to SHIV infection or the transplantation of ZsG-Tmac cells.

Function of persisting MazF-Tmac cells

To examine the Tat-dependent expression of MazF and its antiviral efficacy maintained in the rhesus macaques, $CD4^+$ T cells were isolated from the peripheral blood at 5 and 18 months after the first transplantation in rhesus macaques #13 and #15 and rhesus macaque #14, respectively. The expression of the MazF protein, the proviral copy numbers of SHIV and the production of SHIV in *ex vivo* culture were analyzed (**Figure 5a**).

A qPCR assay and Western blotting analysis were employed in PBMCs and isolated $CD4^+$ T cell samples from rhesus macaques #13 and #15 to detect the intact expression of MazF *in vivo*; however, there was no signal with either method. We speculate that the specific detection of MazF expression *in vivo* is challenging due to a limited number of MazF-Tmac cells in PBMCs and the low infectivity of SHIV *in vivo* compared with *in vitro* conditions. Next, the isolated $CD4^+$ T cells were expanded *ex vivo* for seven days, during which SHIV actively replicated, and Tat-dependent expression was expected to be induced in the MazF-Tmac cells in the $CD4^+$ T-cell population. The expression of MazF was observed in the expanded cells by Western blotting analysis (**Figure 5b**). Although the intact expression of MazF *in vivo* was below the detection limit by qPCR and Western blotting, the Tat-dependent conditional expression system was maintained long term after transplantation.

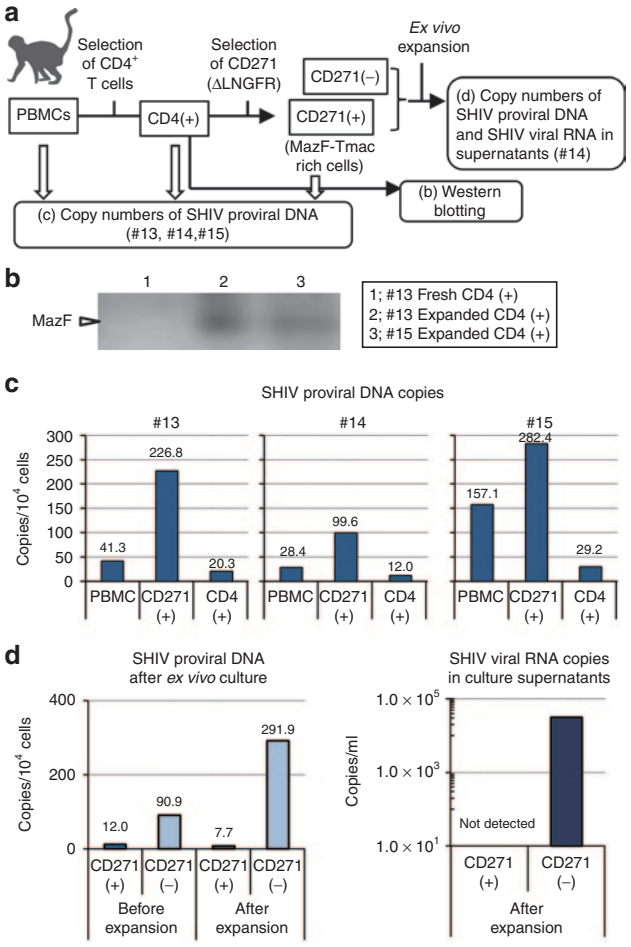

Figure 5 Function of long-term persisting MazF-Tmac cells. **(a)** Outline of the experiment. **(b)** CD4⁺ T cells were isolated from the peripheral blood of MazF-Tmac-transplanted rhesus macaques five months after the first transplantation. The isolated CD4⁺ T cells were expanded for seven days *ex vivo* and used for Western blotting analysis to detect the expression of MazF. **(c)** The CD271-positive and -negative cells were separated from the isolated CD4⁺ T cells of rhesus macaques #13, #14, and #15. DNA samples were collected from the PBMCs, the CD4⁺ T cells and the CD271⁺-MazF-Tmac-enriched population to analyze the proviral copy number of SHIV. **(d)** The CD271⁺-MazF-Tmac-enriched and CD271⁻-MazF-Tmac-negative populations isolated from rhesus macaque #14 were expanded *ex vivo* for 7 days. Changes in the SHIV proviral DNA levels in the cells and the SHIV RNA levels in the supernatants were analyzed in the CD271⁺-MazF-Tmac-enriched and CD271⁻MazF–Tmac-negative populations.

The persisted MazF-Tmac cells, which expressed the surface marker ΔLNGFR, were concentrated from a blood sample taken from rhesus macaques #13, #14, and #15 using an anti-CD271 monoclonal antibody. The number of proviral DNA copies of SHIV was measured in the PBMC population, CD4⁺ T-cell population and CD271⁺-MazF-Tmac-enriched population. As shown in **Figure 5c**, the number of proviral SHIV copies in the CD271⁺-MazF-Tmac-enriched population was one log lower than that in the CD4⁺ T-cell population in all three MazF-Tmac-transplanted rhesus macaques. To assess the function of the MazF-Tmac cells after transplantation, the CD271⁺-MazF-Tmac-enriched population and CD271⁻-MazF-Tmac-negative population isolated from rhesus macaque #14 at 18 months after transplantation were expanded *ex vivo* for 7 days in the absence of antiviral drugs. Changes in the SHIV proviral DNA level in the cells and the SHIV RNA level in the supernatants were analyzed. As shown in **Figure 5d**, the SHIV proviral DNA of the CD271⁻-MazF-Tmac-negative population increased by more than threefold after the expansion, indicating that SHIV was replicated in the CD271⁻-MazF-Tmac-negative population. The SHIV proviral DNA of the CD271⁺-MazF-Tmac-enriched population decreased slightly, indicating that replication of SHIV *ex vivo* was suppressed in the CD271⁺-MazF-Tmac-enriched population. The SHIV RNA copies accumulated in the supernatant of the CD271⁻-MazF-T-negative population in the absence of antiviral drugs, while there were no detectable SHIV RNA copies in the culture supernatants of the CD271⁺-MazF-Tmac-enriched population. These data indicate that the MazF-Tmac cells (CD271⁺ cells) are functional and possess the capability to suppress SHIV replication even one and a half years after transplantation.

Discussion

MazF is an endoribonuclease that specifically cleaves ACA sequences in RNA.[29] Because there are more than 240 ACA sequences in HIV-1 RNA, HIV-1 should have almost no chance to gain a MazF-related escape mutation. Therefore, anti-HIV-1 gene therapy using MazF is an attractive strategy to suppress a broad spectrum of HIV-1. HIV-1 Tat-dependent conditional expression of MazF in CD4⁺ T cells suppresses the replication of HIV-1 and SHIV 89.6P without affecting cellular mRNAs.[21,23] Because MazF is a bacterial protein and has never been tested in humans, it is important to assess the safety of the MazF-system *in vivo* using a nonhuman primate model. In a previous report, we showed the long-term persistence and safety of autologous transplantation of MazF-Tmac cells using cynomolgus macaques[25] that were not infected with a pathogenic virus. To obtain a better understanding of the MazF-modified CD4⁺ T cells in the presence of a viral infection *in vivo*, rhesus macaques were infected with pathogenic SHIV 89.6P and transplanted with autologous MazF-Tmac cells.

An engraftment of 1–2% of gene-modified cells in the peripheral circulation has been reported after the transplantation of approximately 10^{10} cells in adoptive T cell transfer gene therapy for humans,[13] and higher cell doses result in higher measurable engraftment levels.[30] We decided to transplant more than 10^9 cells in the primate model, reflecting one-tenth of the scale of the human gene therapy trials. To investigate the influence of repeated transplantations, transplantations were performed two or three times in this study.

The CD4⁺ T cell count values of the SHIV-infected rhesus macaques increased after the transplantation of the MazF-Tmac cells with statistical significance, while such increases were not observed in the ZsG-Tmac-transplanted rhesus macaques. The infused MazF-Tmac cells persisted for a long period *in vivo*, with half-lives ranging between 7.7 and 58 days. It is also possible that some of the MazF-Tmac cells were vigorously infected by SHIV and killed. To gain a longer

therapeutic benefit from the infusion of gene-modified T cells, gene-modified T cells are expected to expand *in vivo*; however, MazF-Tmac cells did not preferentially expand *in vivo* in this experiment. We are currently addressing this issue and attempting to confer self-expansion capability on MazF-Tmac cells in the presence of viral infections.

The viral load of MazF-Tmac-infused macaques did not decrease dramatically. In general, the major roles of CD4+ helper T cell are activation and regulation of the immune system. The helper T cell does not directly affect the viral load or infected cells as like cytotoxic T-lymphocytes or antibodies, so we speculate that the dramatic decrease of SHIV viral load was not observed though CD4+ T cell counts increased.

We used six rhesus macaques, which were divided in two arms—four rhesus macaques for MazF-modified T cell-treated arm and two macaques for ZsGreen1-modified T cell-treated arm. Only one rhesus macaque in the MazF-modified T cell-treated arm was highly infected with SHIV; the other three were weakly infected. However, SHIV proviral DNA was detected in the PBMCs of all of the macaques, and SHIV reproduced in culture medium when collected PBMCs were expanded *ex vivo* (**Figure 5**). The primary purpose of this experiment was to confirm the safety and persistence of MazF-Tmac cells in the presence of SHIV infection; thus, we used not only high viral load macaque but also weakly infected macaques for this study. No immune responses related to MazF were observed, and half-lives were extended after the repeated transplantation. The evidence of longitudinal persistence of MazF-Tmac cells suggests that MazF-modified T cells are not highly immunogenic. Because MazF remains at a constant low level upon viral infection,[23] MazF-Tmac cells are unlikely to activate an immune response. To obtain more safety information on MazF-modified CD4+ T cells in the presence of HIV infection, further investigations are needed, and a clinical trial entitled "A phase I, open label, dual cohort, single center study to evaluate the safety, tolerability and immunogenicity of autologous CD4 T cells modified with a retroviral vector expressing the *mazF* endoribonuclease gene in patients with HIV" is now ongoing in the United States (clinicaltrials.gov, identifier NCT01787994). Antiviral effect would also be assessed in the cohort 2 arm of this clinical trial.

In contrast, the infused ZsG-Tmac cells did not persist for an extended period *in vivo*, and the half-lives were not extended after repeated transplantation. The transient rebound of SHIV and marked decrease in the number of vector copies occurred simultaneously within 2 weeks after the transplantation of ZsG-Tmac cells; antiZsGreen1 antibodies developed gradually and reached their maximum level 40 days after the transplantation (see **Supplementary Figure S4**). We hypothesize that the ZsG-Tmac cells, which have no payload related to SHIV resistance, secreted a large amount of SHIV particles after infection and were destroyed by SHIV *in vivo*. The internalization of ZsGreen1 protein and antigen presentation by antigen-presenting cells induced the immune responses. In the case of rhesus macaque #17, ZsGreen1 protein might have been highly expressed by vigorous infection and triggered antibody production. In the case of rhesus macaque #16, ZsGreen1 protein was most likely expressed abundantly as in #17. Because this rhesus macaque exhibited severely reduced CD4+ T cell counts, we speculate that the B cells were not stimulated by Th2 effector cells due to a lack of CD4+ helper T cells; thus, no antibodies against ZsGreen1 were detected in rhesus macaque #16.

To address whether MazF-modified CD4+ T cells are associated with carcinogenicity *in vivo,* clonal expansion of the gene-modified cells was assessed using the linear amplification-mediated-PCR (LAM-PCR) method,[31] which traces the progeny of transduced cells by detecting the random insertion of the retrovirus or lentivirus vector. Although the preliminary data were collected from four macaques during a limited experimental period, there were no specific clonal expansions in any of the MazF-Tmac-transplanted rhesus macaques (see **Supplementary Figure S5**).

The histopathological analyses of the major organs, as well as the secondary lymphoid tissues, revealed that the transplantation of the MazF-Tmac cells was not associated with any carcinogenicity during the study period (see **Supplementary Figure S3** and **Supplementary Table S2**). A decrease in lymphocyte number was observed in the inguinal and axillary lymph nodes of the ZsG-Tmac-transplanted rhesus macaques (see **Supplementary Figure S3** and **Supplementary Table S2**). Although the transplanted ZsG-Tmac cells were not detected at the time of autopsy, the ZsG-Tmac cells might have migrated to the lymph nodes after transplantation, and the SHIV might have vigorously replicated in the migrated ZsG-Tmac cells, leading to cell death and, ultimately, bystander apoptosis of the neighboring uninfected cells and damage to the lymph nodes.[32] There was no such damage to the lymph nodes in rhesus macaque #12, which exhibited the highest SHIV viral loads and had undergone transplantation of MazF-Tmac cells three times.

Because gene therapy for HIV-1 should aim to reconstitute an HIV-1-resistant immune system, it is important for the gene-modified cells to not only inhibit viral replication but also maintain their distribution for long periods *in vivo*. Although the long-term persistence of gene-modified T cells or hematopoietic stem cells has been reported in the context of human gene therapy, it is difficult to obtain information about the distribution of these cells throughout the body. The use of primate models is advantageous for investigating the distribution pattern. At the time of the experimental autopsy, lymphocytes were isolated from the principal organs. MazF-Tmac cells were detected in the secondary lymphoid tissues, including several lymph nodes and the spleen, as well as the peripheral blood. A similar tendency was observed in previous data from cynomolgus macaques.[25] In contrast, ZsG-Tmac cells were not detected in any of the organs at the time of autopsy. The MazF-Tmac cells tend to persist primarily in the peripheral blood and secondary lymphoid tissues, regardless of the SHIV infection status.

The number of proviral SHIV DNA copies in the harvested CD271+-MazF-Tmac-enriched population was significantly lower than that in the CD4+ T-cell population (**Figure 5c**). The exact mechanism of low SHIV copies in MazF-Tmac cells *in vivo* remains unclear. We examined the expression of coreceptor CXCR4 in gene-modified cells at the time of infusion, and no difference in expression levels was observed between the gene-modified and unmodified cells. One possible hypothesis is that the majority of the SHIV-infected CD4+

T cells were infected at the acute infection stage but that the infused cells are less infected during chronic infection stage. Alternatively, leaky expression of MazF in the infused MazF-Tmac cells may inhibit the integration of SHIV. Another possibility is that vigorously infected MazF-Tmac cells died off after the over-induction of MazF expression. Further investigation is needed to reveal the mechanism.

We analyzed the function of the MazF-Tmac cells that persisted long after transplantation. Conditional MazF expression system was maintained and MazF protein expressed in T cells harvested from the rhesus macaques long after transplantation. In the freshly isolated samples, which are not expanded *ex vivo*, MazF signal was beyond detection (**Figure 5b**, lane 1). This phenomenon was considered due to low frequency of SHIV infection in MazF-Tmac cells (**Figure 5c**). However, we believe that low sensitivity to SHIV and low expression of MazF may contribute to the stable long-term persistence of MazF-Tmac cells, even in the presence of SHIV. Moreover, our qPCR analysis demonstrated that SHIV replication was blocked (**Figure 5d**). Although these data are from only one macaque, it appears that the MazF expression system was maintained, and the expressed MazF was functional long after transplantation.

Transplantation with MazF-Tmac cells contributed to an increase in the CD4$^+$ T cell counts, and the MazF-Tmac cells showed little or no immunogenicity in rhesus macaques in the presence of SHIV infection, suggesting that the autologous transplantation of MazF-modified CD4$^+$ T cells is an attractive strategy for HIV-1 gene therapy.

Materials and methods

General laboratory statement. Research sample processing and freezing were performed in a biosafety level (BSL) 3 laboratory at the Tsukuba Primate Research Center in the National Institute of Biomedical Innovation (NIBIO, Ibaraki, Japan). Laboratory analyses were performed in BSL2 laboratories at the Tsukuba Primate Research Center in NIBIO and at the Center for Cell and Gene Therapy of Takara Bio, which uses established standard operating procedures and protocols for sample processing, freezing, and analysis.

Study design. The animal study protocol was approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan (identifier 20–8156), and by the Animal Welfare and Animal Care Committee of the NIBIO (identifier DS20-98R3). 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,[33] and in accordance with the recommendations of the Weatherall report, "The use of nonhuman primates in research" and the "Rules for Animal Care and Management of the Tsukuba Primate Research Center."[34] The experimental design is diagrammed in **Figure 1**. Six rhesus macaques, #12, #13, #14, #15, #16, and #17, were used for this experiment. CD4$^+$ T cells were isolated from the blood samples taken from each rhesus macaque before the challenge with SHIV and cryopreserved as described below. After confirming the set point of the SHIV viral loads, the gene-modified CD4$^+$ T cells were manufactured and transplanted as described in **Supplementary Materials**. Autologous CD4$^+$ T cells were transduced with the MazF retroviral vector MT-MFR-PL2 (#12, #13, #14, and #15) or the control vector MT-ZGR-PL2 (#16 and #17).

Animals. The Burmese rhesus macaques were maintained at the Tsukuba Primate Research Center in NIBIO. All surgical and invasive clinical procedures were conducted 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, including blood sampling, gene-modified cell administration and clinical examinations or treatment.

SHIV 89.6P virus. A CXCR4-tropic SHIV 89.6P[26] was used for this experiment. SHIV 89.6P was propagated in rhesus macaque PBMCs. The culture supernatants were harvested, and the 50% tissue culture infective dose ($TCID_{50}$) was determined by infecting the CD4$^+$ human T-lymphoblastoid cell line M8166 with dilutions of the virus.[35] All the stock viruses were stored at −80°C until use. An intravenous challenge with SHIV 89.6P was performed at 5.0×10^3–1.8×10^5 $TCID_{50}$ (**Supplementary Table S1**).

Gibbon ape leukemia virus (GaLV)-enveloped gamma-retroviral vector MT-MFR-PL2 and MT-ZGR-PL2. The preparation of the retroviral vector used in this study has been previously described.[21] Briefly, an HIV-1-LTR-MazF-polyA cassette was introduced in the direction opposite of the MoMLV-LTR at the multiple cloning site of the retroviral vector plasmid pMT.[36] The Δ*LNGFR* gene[37] was introduced into the retrovirus vector as a surface marker. The Δ*LNGFR* gene is under the control of the human phosphoglycerate kinase (PGK) promoter. The resultant MT-MFR-PL2 was introduced into the packaging cell line PG13 (ATCC CRL-10686) and the GaLV-enveloped gamma-retroviral vector MT-MFR-PL2 was obtained by harvesting the culture fluid of the producer cells. For the control experiment, the *mazF* gene was replaced with the gene encoding the fluorescent ZsGreen1 protein (MT-ZGR-PL2). The GaLV-enveloped MT-ZGR-PL2 was obtained by harvesting the culture fluid of the PG13 derived producer cells.

CD4$^+$ T cells. Prior to the challenge with SHIV 89.6P, the peripheral blood of rhesus macaques was collected by apheresis as described previously.[38] The CD4$^+$ T cells were positively isolated using anti-CD4 antibody conjugated magnetic beads (Dynal CD4 Positive Isolation Kit, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The isolated CD4$^+$ T cells were cryopreserved and stored at −80°C until use.

Manufacturing autologous gene-modified CD4$^+$ T cells and transplantation into rhesus macaques. Refer to the **Supplementary Materials**.

Measurement of hematological data. Refer to the **Supplementary Materials.**

Flow cytometry analyses. Refer to the **Supplementary Materials.**

Quantification of gene-modified CD4+ T cells. Refer to the **Supplementary Materials.**

Analyses of the SHIV viral loads in plasma. Refer to the **Supplementary Materials.**

Detection of MazF antigen-specific IFN-γ secreting cells. Refer to the **Supplementary Materials.**

Detection of anti-MazF or anti-ZsGreen1 antibodies in rhesus macaque blood. Refer to the **Supplementary Materials.**

Collection of lymphocytes from several organs. Refer to the **Supplementary Materials.**

Examination of function and antiviral efficacy of persisting MazF-Tmac cells. Refer to the **Supplementary Materials.**

LAM-PCR. Refer to the **Supplementary Materials.**

Western blotting. Refer to the **Supplementary Materials.**

Supplementary material

Figure S1. Body weight and hematological data.
Figure S2. MazF peptides used for the IFN-γ enzyme-linked immunospot assay.
Figure S3. Histopathological analysis of axillary lymph nodes.
Figure S4. Changes in SHIV viral loads, ZsGreen1 proviral copy numbers and production of anti-ZsGreen1 antibodies of rhesus macaque #17.
Figure S5. Linear amplification-mediated (LAM)-PCR.
Table S1. Dose of SHIV 89.6P TCID50 used to infect each rhesus macaque and the time of transplantation of the gene-modified cells.
Table S2. Analysis of the in vivo safety (individual histopathological findings from the autopsy samples).
Materials and methods

Acknowledgments. The authors thank the staff of the Tsukuba Primate Research Center and Corporation for Production and Research of Laboratory Primates for their kind care and expert handling of the animals. The authors thank Keith A. Reimann of Harvard Medical School and Tomoyuki Miura of Kyoto University for providing the SHIV 89.6P. The authors thank the members of the Center for Cell and Gene Therapy of Takara Bio Inc., Koichi Inoue and Katsuyuki Dodo for their contributions and helpful advice, and Hiroshi Tsuda, Tomomi Sakuraba, and Mayumi Shimomura for technical support. The authors have no support or funding to report. The animal study was designed by Hideto Chono, Naoki Saito, Hiroaki Shibata, Naohide Ageyama, Yasuhiro Yasutomi and Junichi Mineno. Gene-modified T cells were manufactured by Naoki Saito and Hideto Chono. All surgical and invasive clinical procedures were conducted under the supervision of Naohide Ageyama. Blood samples were prepared by Hiroaki Shibata. The laboratory analyses were performed by Naoki Saito. The manuscript was written by Hideto Chono and Naoki Saito. All authors discussed and interpreted results. Naoki Saito, Hideto Chono and Junichi Mineno are employees of Takara Bio Inc. (http://www.takara-bio.com). European patent applications EP1921136B1 "Nucleic acid for treatment or prevention of immunodeficiency virus infection" and EP2138580B1 "Vector for gene therapy" were filed through Takara Bio Inc. These interests do not alter the authors' adherence to the Journal's policies regarding sharing data and materials. The other authors declared that they have no competing interests.

1. Kitahata, MM, Gange, SJ, Abraham, AG, Merriman, B, Saag, MS, Justice, AC *et al.* (2009). Effect of early versus deferred antiretroviral therapy for HIV on survival. *N Engl J Med* **360**: 1815–1826.
2. Maartens, G and Boulle, A (2007). CD4 T-cell responses to combination antiretroviral therapy. *Lancet* **370**: 366–368.
3. Geng, EH and Deeks, SG (2009). CD4+ T cell recovery with antiretroviral therapy: more than the sum of the parts. *Clin Infect Dis* **48**: 362–364.
4. Lekakis, J and Ikonomidis, I (2010). Cardiovascular complications of AIDS. *Curr Opin Crit Care* **16**: 408–412.
5. Núñez, M (2010). Clinical syndromes and consequences of antiretroviral-related hepatotoxicity. *Hepatology* **52**: 1143–1155.
6. Cheung, MC, Pantanowitz, L and Dezube, BJ (2005). AIDS-related malignancies: emerging challenges in the era of highly active antiretroviral therapy. *Oncologist* **10**: 412–426.
7. Siliciano, RF and Greene, WC (2011). HIV latency. *Cold Spring Harb Perspect Med* **1**: a007096.
8. Richman, DD, Margolis, DM, Delaney, M, Greene, WC, Hazuda, D and Pomerantz, RJ (2009). The challenge of finding a cure for HIV infection. *Science* **323**: 1304–1307.
9. Hütter, G, Nowak, D, Mossner, M, Ganepola, S, Müssig, A, Allers, K *et al.* (2009). Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med* **360**: 692–698.
10. Allers, K, Hutter, G, Hofmann, J, Loddenkemper, C, Rieger, K, Thiel, E *et al.* (2011). Evidence for the cure of HIV infection by CCR5Delta32/Delta32 stem cell transplantation. *Blood* **117**: 2791–2799.
11. Sarver, N and Rossi, J (1993). Gene therapy: a bold direction for HIV-1 treatment. *AIDS Res Hum Retroviruses* **9**: 483–487.
12. Dropulić, B and Jeang, KT (1994). Gene therapy for human immunodeficiency virus infection: genetic antiviral strategies and targets for intervention. *Hum Gene Ther* **5**: 927–939.
13. Levine, BL, Humeau, LM, Boyer, J, MacGregor, RR, Rebello, T, Lu, X *et al.* (2006). Gene transfer in humans using a conditionally replicating lentiviral vector. *Proc Natl Acad Sci USA* **103**: 17372–17377.
14. Morris, KV and Rossi, JJ (2006). Lentivirus-mediated RNA interference therapy for human immunodeficiency virus type 1 infection. *Hum Gene Ther* **17**: 479–486.
15. Rossi, JJ, June, CH and Kohn, DB (2007). Genetic therapies against HIV. *Nat Biotechnol* **25**: 1444–1454.
16. van Lunzen, J, Glaunsinger, T, Stahmer, I, von Baehr, V, Baum, C, Schilz, A *et al.* (2007). Transfer of autologous gene-modified T cells in HIV-infected patients with advanced immunodeficiency and drug-resistant virus. *Mol Ther* **15**: 1024–1033.
17. Li, MJ, Kim, J, Li, S, Zaia, J, Yee, JK, Anderson, J *et al.* (2005). Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol Ther* **12**: 900–909.
18. Hoxie, JA and June, CH (2012). Novel cell and gene therapies for HIV. *Cold Spring Harb Perspect Med* **2**: a007179.
19. Cannon, P and June, C (2011). Chemokine receptor 5 knockout strategies. *Curr Opin HIV AIDS* **6**: 74–79.
20. Tebas, P, Stein, D, Binder-Scholl, G, Mukherjee, R, Brady, T, Rebello, T *et al.* (2013). Antiviral effects of autologous CD4 T cells genetically modified with a conditionally replicating lentiviral vector expressing long antisense to HIV. *Blood* **121**: 1524–1533.
21. Chono, H, Matsumoto, K, Tsuda, H, Saito, N, Lee, K, Kim, S *et al.* (2011). Acquisition of HIV-1 resistance in T lymphocytes using an ACA-specific E. coli mRNA interferase. *Hum Gene Ther* **22**: 35–43.
22. Shimazu, T, Degenhardt, K, Nur-E-Kamal, A, Zhang, J, Yoshida, T, Zhang, Y *et al.* (2007). NBK/BIK antagonizes MCL-1 and BCL-XL and activates BAK-mediated apoptosis in response to protein synthesis inhibition. *Genes Dev* **21**: 929–941.
23. Okamoto, M, Chono, H, Kawano, Y, Saito, N, Tsuda, H, Inoue, K *et al.* (2013). Sustained inhibition of HIV-1 replication by conditional expression of the *E. coli*-derived endoribonuclease MazF in CD4+ T cells. *Hum Gene Ther Methods* **24**: 94–103.

24. Berkhout, B, Silverman, RH and Jeang, KT (1989). Tat trans-activates the human immunodeficiency virus through a nascent RNA target. *Cell* **59**: 273–282.
25. Chono, H, Saito, N, Tsuda, H, Shibata, H, Ageyama, N, Terao, K *et al.* (2011). *In vivo* safety and persistence of endoribonuclease gene-transduced CD4+ T cells in cynomolgus macaques for HIV-1 gene therapy model. *PLoS One* **6**: e23585.
26. Reimann, KA, Li, JT, Voss, G, Lekutis, C, Tenner-Racz, K, Racz, P *et al.* (1996). An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high *in vivo* replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. *J Virol* **70**: 3198–3206.
27. Pitcher, CJ, Hagen, SI, Walker, JM, Lum, R, Mitchell, BL, Maino, VC *et al.* (2002). Development and homeostasis of T cell memory in rhesus macaque. *J Immunol* **168**: 29–43.
28. Klebanoff, CA, Gattinoni, L, Torabi-Parizi, P, Kerstann, K, Cardones, AR, Finkelstein, SE *et al.* (2005). Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proc Natl Acad Sci USA* **102**: 9571–9576.
29. Zhang, Y, Zhang, J, Hoeflich, KP, Ikura, M, Qing, G and Inouye, M (2003). MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. *Mol Cell* **12**: 913–923.
30. Ranga, U, Woffendin, C, Verma, S, Xu, L, June, CH, Bishop, DK *et al.* (1998). Enhanced T cell engraftment after retroviral delivery of an antiviral gene in HIV-infected individuals. *Proc Natl Acad Sci USA* **95**: 1201–1206.
31. Schmidt, M, Zickler, P, Hoffmann, G, Haas, S, Wissler, M, Muessig, A *et al.* (2002). Polyclonal long-term repopulating stem cell clones in a primate model. *Blood* **100**: 2737–2743.
32. Ahr, B, Robert-Hebmann, V, Devaux, C and Biard-Piechaczyk, M (2004). Apoptosis of uninfected cells induced by HIV envelope glycoproteins. *Retrovirology* **1**: 12.
33. Primate Society of Japan (1986). Guiding principles for animal experiments using nonhuman primates. *Primate Res* **2**: 111–113.
34. Honjo, S (1985). The Japanese Tsukuba Primate Center for Medical Science (TPC): an outline. *J Med Primatol* **14**: 75–89.
35. Akiyama, H, Ido, E, Akahata, W, Kuwata, T, Miura, T and Hayami, M (2003). Construction and *in vivo* infection of a new simian/human immunodeficiency virus chimera containing the reverse transcriptase gene and the 3' half of the genomic region of human immunodeficiency virus type 1. *J Gen Virol* **84**(Pt 7): 1663–1669.
36. Lee, JT, Yu, SS, Han, E, Kim, S and Kim, S (2004). Engineering the splice acceptor for improved gene expression and viral titer in an MLV-based retroviral vector. *Gene Ther* **11**: 94–99.
37. Verzeletti, S, Bonini, C, Marktel, S, Nobili, N, Ciceri, F, Traversari, C *et al.* (1998). Herpes simplex virus thymidine kinase gene transfer for controlled graft-versus-host disease and graft-versus-leukemia: clinical follow-up and improved new vectors. *Hum Gene Ther* **9**: 2243–2251.
38. Ageyama, N, Kimikawa, M, Eguchi, K, Ono, F, Shibata, H, Yoshikawa, Y *et al.* (2003). Modification of the leukapheresis procedure for use in rhesus monkeys (Macaca mulata). *J Clin Apher* **18**: 26–31.

Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)