MHC-I haplotype *90-120-Ia* elicited $Gag_{206-216}$-specific CTL responses and controlled viral replication with rapid selection of the GagL216S mutation after SIVmac239 challenge. Among these SIV controllers, two animals (V3 and V5) accumulated additional *gag* mutations and showed reappearance of plasma viremia around week 60 postchallenge. Both animals first selected a $Gag_{241-249}$ epitope-specific CTL escape mutation leading to a GagD244E (aspartic acid [D] to glutamic acid [E] at the 244th aa in Gag) substitution, and then, a $Gag_{373-380}$ epitope-specific CTL escape mutation leading to a GagA373T (alanine [A] to threonine [T] at the 373rd) or GagP376S (proline [P] to S at the 376th) substitution during the period of viral control. At the viremia reappearance, SIVmac239Gag216S244E247L312V373T with five *gag* mutations, L216S, D244E, I247L (isoleucine [I] to L at the 247th), A312V (A to V at the 312th), and A373T, became dominant in one of them (V5), and SIVmac239Gag145A216S244E376S with four *gag* mutations leading to V145A (V to A at the 145th), L216S, D244E, and P376S became dominant in the other (V3). These viruses with multiple *gag* mutations showed lower replicative ability *in vitro* than SIVmac239Gag216S carrying single GagL216S mutation. Indeed, SIVmac239Gag216S244E247L312V373T carrying five *gag* mutations had lower replicative ability *in vitro* compared to SIVmac239Gag216S244E373T carrying three *gag* mutations. These results suggest that selection of CTL escape mutations even with viral fitness costs could be advantageous for viral replication *in vivo* under CTL pressure.

## SIV TRANSMISSION INTO MHC-MISMATCHED HOSTS DRIVES FURTHER VIRAL GENOME CHANGES

Previous studies (Friedrich et al., 2004b; Kobayashi et al., 2005; Loh et al., 2007) reported reversion of CTL escape mutations in the absence of CTL pressure by transmission of SIVs carrying single escape mutations between MHC-mismatched hosts. SIVs carrying CTL escape *gag* mutations selected in *90-120-Ia*-positive macaques showed lower replicative ability *in vitro*. We then examined *in vivo* replicative ability of those SIVs carrying CTL escape mutations in *90-120-Ia*-negative macaques (Seki et al., 2008). Coinoculation of macaques with SIVmac239GagL216S and SIVmac239Gag216S244E373T resulted in rapid selection of the former; i.e., D244E and A373T mutations were undetectable even in the acute phase, indicating lower replicative ability *in vivo* of the latter carrying three escape mutations than the former. Reversion of L216S was observed in a few months, confirming lower replicative ability *in vivo* of SIVmac239Gag216S than wild-type SIVmac239. Further competition indicated lower replicative ability *in vivo* of SIVmac239Gag216S244E247L312V373T carrying five *gag* mutations than SIVmac239Gag216S244E373T carrying three.

We next examined viral genome changes after challenge of *90-120-Ia*-negative macaques with SIVs carrying multiple CTL escape mutations selected in *90-120-Ia*-positive macaques. Challenge with SIVs carrying five *gag* mutations, L216S, D244E, I247L, A312V, and A373T, resulted in persistent viremia in all four *90-120-Ia*-negative macaques. Two animals exhibited higher viral

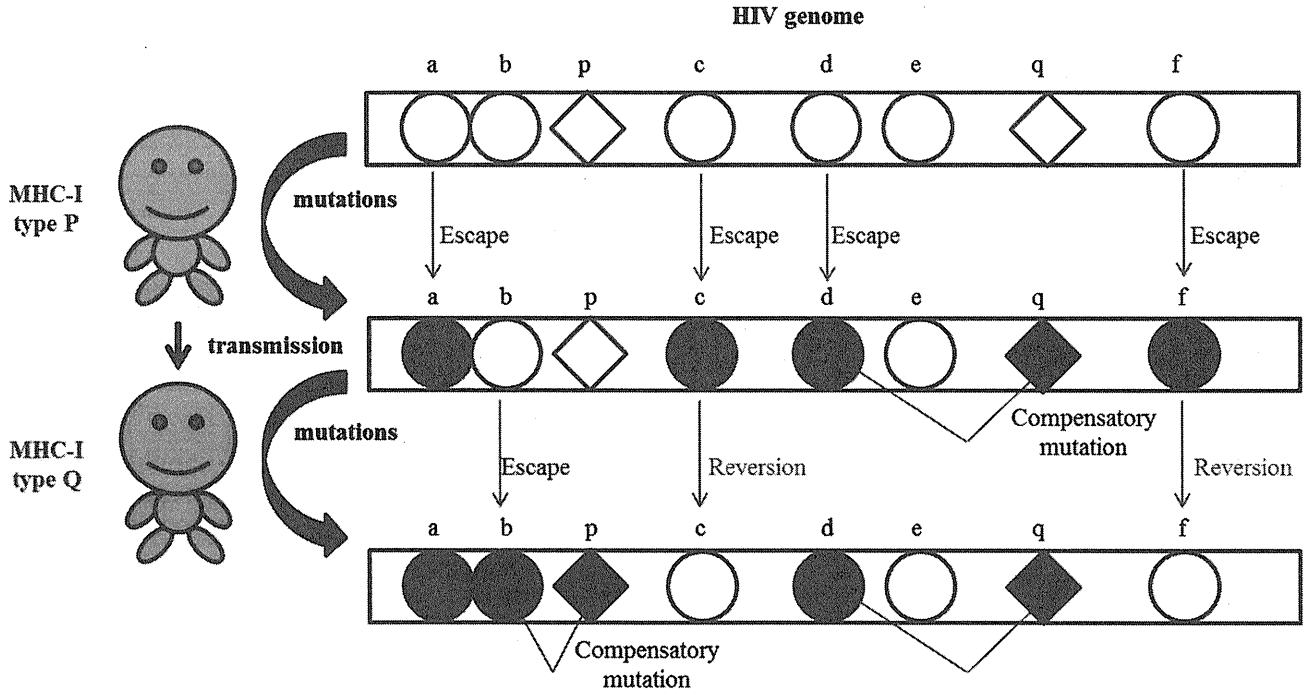


**FIGURE 2 | Schema of HIV/SIV transmission resulting in accumulation of multiple viral mutations.** Multiple CTL escape mutations resulting in viral fitness costs do not always revert rapidly even in the absence of CTL pressure after their transmission into HLA/MHC-mismatched hosts and such mutants can be transmitted further to other hosts. New escape mutations and compensatory mutations are also observed with transmissions. Thus, CTL affects HIV/SIV evolution in individuals with divergent HLA/MHC polymorphisms.

loads. One of them rapidly developed AIDS at week 18 while the other developed AIDS 2 years postchallenge. The former showed reversion of I247L and A312V but still had three CTL escape mutations, L216S, D244E, and A373T at AIDS onset. The latter showed reversion of four mutations in a year postchallenge, but the A373T mutation remained dominant without reversion until AIDS onset. In the remaining two animals that exhibited lower viral loads, multiple *gag* mutations including L216S and D244E were still dominant without reversion 1 year after challenge.

Thus, in the experiment of challenge with SIVs carrying multiple CTL escape mutations, the reversion of all the mutations was not required for AIDS onset, while transmission with SIVs carrying single CTL escape mutations showed their rapid reversion. This suggests that even HIVs accumulating multiple CTL escape mutations with viral fitness costs can induce persistent viral infection leading to AIDS progression after their transmission into HLA/MHC-mismatched individuals.

The reversion of the L216S mutation was delayed or not observed after challenge with SIVs carrying multiple *gag* mutations, whereas challenge with SIVmac239Gag216S resulted in its reversion in a few months. This may be due to the predominant selection of the reversion of other mutations, compensatory mutations, or to lower viral replication efficiency in the former case. Our results suggest that CTL escape mutations resulting in viral fitness costs may not always revert rapidly after their transmission into MHC-mismatched hosts and can be transmitted further to other hosts, driving further viral genome changes with accumulation of mutations (**Figure 2**). These results provide an important insight into HIV evolution in human individuals with divergent HLA/MHC polymorphisms.

## CONCLUDING REMARKS

Cytotoxic T lymphocyte responses exert strong selective pressure on HIV and play a central role in viral evolution (Kaslow et al., 1996; Brander and Walker, 2003; Kiepiela et al., 2004; O'Connor et al., 2004). Correlation of frequencies of viral epitope variants with prevalence of restricting HLA alleles has been shown, indicating HIV adaptation to HLA polymorphisms at a population level (Kawashima et al., 2009). Loss of viral fitness by CTL escape mutations may contribute to HIV control (Martinez-Picado et al., 2006; Schneidewind et al., 2007), but our results indicate the potential of even such HIVs with lower viral fitness to induce AIDS progression. Elucidation of structural constraints of viral antigens for viral function would lead to determination of conserved, escape-resistant epitopes whose mutations largely diminish viral replicative ability (Dahirel et al., 2011), contributing to immunogen design in development of CTL-inducing AIDS vaccines.

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*Review Article*

# *Mycobacterium bovis* Bacille Calmette-Guérin as a Vaccine Vector for Global Infectious Disease Control

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*Mycobacterium bovis* bacille Calmette-Guérin (BCG) is the only available vaccine for tuberculosis (TB). Although this vaccine is effective in controlling infantile TB, BCG-induced protective effects against pulmonary diseases in adults have not been clearly demonstrated. Recombinant BCG (rBCG) technology has been extensively applied to obtain more potent immunogenicity of this vaccine, and several candidate TB vaccines have currently reached human clinical trials. On the other hand, recent progress in the improvement of the BCG vector, such as the codon optimization strategy and combination with viral vector boost, allows us to utilize this bacterium in HIV vaccine development. In this paper, we review recent progress in rBCG-based vaccine studies that may have implications in the development of novel vaccines for controlling global infectious diseases in the near future.

## 1. Introduction

*Mycobacterium bovis* bacille Calmette-Guérin (BCG) is the only licensed vaccine that has substantially helped controlling tuberculosis (TB) for more than 80 years. This vaccine affords ~80% protection against TB meningitis and miliary TB in infants and young children [1]. However, the BCG-induced protective effects against pulmonary diseases over all ages are variable; the escalation of the worldwide TB epidemic is evidence that the vaccine does not work well to prevent pulmonary TB [2]. Recently, studies on the advanced molecular biology and genomics of mycobacteria have revealed that the BCG genome has various mutations and deletions compared with the original virulent strain of *Mycobacterium tuberculosis* and *M. bovis* [3]. Interestingly, there are substantial differences in the genomic DNA even among BCG substrains [4, 5] that can cause biological differences in the population of BCG vaccines.

Since a host-vector system in mycobacteria was developed in 1987 [6], recombinant BCG (rBCG) technology has been extensively applied in the development of vaccines against a variety of infectious diseases, including bacterial, viral, and parasitic infections in addition to TB [7, 8]. BCG is attractive as a vaccine vector because of its extensive safety record in humans, heat stability, low production cost, induction of long-lasting type 1 helper T cell (Th1) immunity, $CD8^+$ T-cell triggering, adjuvant activity, usability in newborns and its mucosal immune induction by oral administration. Taking the current situation of serious epidemics of emerging and reemerging diseases mainly in developing African and Asian countries into account, a new global vaccine should be affordable in such areas. Therefore, the low price and heat stability of BCG-based vaccines would be desirable. In this paper, we review various efforts to develop novel BCG vector-based vaccines mainly for controlling TB and HIV/AIDS.

## 2. Immunological Properties of BCG Vector

The immune responses induced by BCG are outlined in Figure 1. The most characteristic response to BCG is the induction of innate (nonspecific) immunity by cell wall components through toll-like receptors (TLRs) 2 and 4 on dendritic cells and macrophages [9]. After phagocytosis,

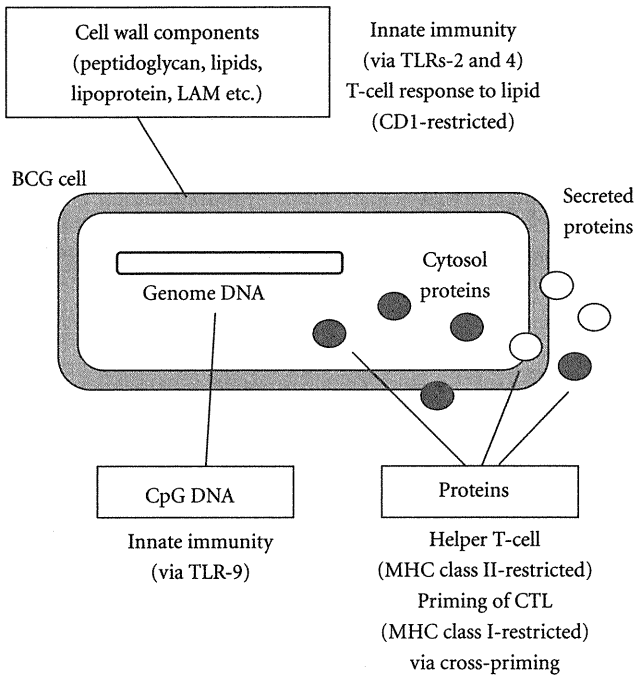


FIGURE 1: Outline of immune responses by BCG. Both innate immunity via TLRs and antigen-specific immunity via MHC- or CD1-restricted antigen presentation to T cells are induced by various BCG cell components.

BCG is degraded by lysosomal enzymes, and the processed antigen can be presented to the host immune system via various pathways. DNA fragments containing the CpG motif may activate innate immunity via the TLR9 route [10]. Lipids such as mycolic acid presented by CD1 stimulate CD1-restricted $CD8^+$ T cells [11]. Protein antigens, such as antigen 85 complex produced by BCG, induce Th1 response through presentation by major histocompatibility complex (MHC) class II. This pathway is the major route of BCG-induced responses and is indispensable for protective immunity against *M. tuberculosis* infection via protective cytokine interferon (IFN)-$\gamma$ production. On the other hand, the processing and presentation of protein antigens via the MHC class I pathway are also elicited in the BCG-infected antigen presenting cell (APC). As reported by Goonetilleke et al. [12], immunizing BCG-sensitized animals with recombinant vaccinia virus MVA expressing antigen 85A greatly enhances the MHC class I-restricted CTL response against antigen 85A, indicating that BCG priming could be a novel type of prime-boost vaccine. This immunological feature of BCG vector allows its application in vaccines against chronic viral infectious diseases such as HIV/AIDS. In addition, the strong Th1 induction by BCG would be favorable to aid the maturation and maintenance of CTL [13]. Thus, the BCG vector is expected to induce effective cell-mediated immunity against a targeted antigen.

## 3. TB Vaccine

*3.1. Background of the Global TB Epidemic.* TB kills 1.7 million people worldwide each year; someone dies from TB every 19 seconds [14]. Although the TB treatment protocol was established a long time ago, the recent increase of multidrug-resistant *M. tuberculosis* infection has generated a serious situation. New vaccines are urgently needed to eliminate TB as a public health threat and should be a major global public health priority. TB is a disease that is spread from person to person through the air. Furthermore, the terrible synergy between TB and HIV makes this disease even more dangerous, especially in sub-Saharan African countries. For instance, according to the World Health Organization's (WHO) Global TB report 2010 [14], South Africa had nearly 400,000 new TB cases in 2009 with an incidence rate of an estimated 806 cases per 100,000; TB is one of the leading causes of death in both adults and children of this country. The case fatality rate has increased from 3% in 1993 to 24.3% in 2007. A major reason for the increased fatality rate is South Africa's concurrent HIV epidemic. The prevalence of HIV infection in South Africa in 2009 was approximately 7%, which has been decreasing as a result of various efforts toward prevention. TB is a common opportunistic infection among people living with HIV, and 60% of new TB cases occurred in persons who were also infected with HIV in 2009 [14]. We can observe similar critical situations in the countries surrounding South Africa. Regarding the vaccination, such situation has raised concerns about the safety of using BCG vaccine in HIV-infected infants because between 10 and 30% of pregnant women are HIV infected in many sub-Saharan African countries.

*3.2. Current Efforts toward New TB Vaccine Development.* The global plan to stop TB 2011–2015 report [15] offers 7 objectives as follows: (i) to maintain a robust TB vaccine pipeline by supporting research and discovery, (ii) to conduct research to identify correlates of protection and preclinical studies to assess new TB vaccine candidates, (iii) to ensure the availability of vaccine production capacity by expanding manufacturing facilities for TB vaccines, (iv) to build capacity for large-scale clinical trials (phases II and III) of TB vaccine candidates at field sites in TB-endemic countries, (v) to conduct phase I, II, and III clinical trials of TB vaccine candidates, (vi) to develop delivery, regulatory, and access strategies for new TB vaccines, (vii) to build support for TB vaccine development and uptake through advocacy, communications, and resource mobilization. All these objectives are important to realize new TB vaccine development.

The main goal of vaccine development in the Global Plan to Stop TB 2006–2015 is for 2 vaccines to be in proof-of-concept trials by 2010 and that 1 new and safe vaccine is available by 2015. As of 2009, 12 TB vaccine candidates had entered clinical trials. Of these, 9 are still being tested (Table 1) : 5 are in phase I clinical trials, 2 are in phase II trials, and 2 are in phase IIb proof-of-concept trials [15]. One vaccine has produced estimates of safety and effectiveness in a targeted HIV-infected population. At least 6 TB vaccine candidates are in preclinical development, and at least 21 additional next-generation candidates are in the vaccine discovery phase [15]. As mentioned earlier, the current BCG vaccine has limited and variable effectiveness against TB.

Table 1: Summary of candidate TB vaccines in clinical trials 2009. Nine candidate preventive TB vaccines are currently in clinical phases.

| Status | Products | Product description | Sponsor |
|---|---|---|---|
| Phase IIb | MVA85A/AERAS-485 | Vaccinia virus MVA | OETC/AERAS |
| Phase IIb | AERAS-402/Crucell Ad35 | rBCG/adenovirus 35 | Crucell/AERAS |
| Phase II | Hybrid-I + IC31 | Ag85B/ESAT6 + adjuvant | SSI/TBVI |
| Phase II | M72 | Fusion protein + adjuvant | GSK/AERAS |
| Phase I | AdAg85A | adenovirus 5/Ag85A | McMaster Univ. |
| Phase I | VPM 1002 | rBCG/listeriolysin::ΔureC | Max Planck/TBVI |
| Phase I | Hyvac 4/AERAS-404 | Fusion protein + adjuvant | SSI/Sanofi/AERAS |
| Phase I | RUTI | Fragmented Mtb cell | Archivel Farma |
| Phase I | Hybrid-I + CAF01 | Ag85B/ESAT6 + adjuvant | SSI |

Abbreviations in the sponsors: AERAS, AERAS Global TB Vaccine Foundation; GSK, GlaxoSmithKline; OETC, The Oxford-Emergent Tuberculosis Consortium Ltd.; SSI, Staten Serum Institute; TBVI, Tuberculosis Vaccine Initiative.

Therefore, the first choice of strategy may be improving BCG by using recombinant DNA technology even though it may imply safety issue of vaccination in HIV-infected individuals. Overproduction against a protective antigen of TB in BCG (rBCG30) exhibited enhanced immunogenicity in humans [16]. Moreover, the expression of the listeriolysin gene in BCG (rBCG/*hly*$^+$::Δ*ureC*) is proven to be more potent in the induction of TB-specific cellular immune responses [17]. Another strategy for improving BCG vaccines is boosting BCG immunity with protein [18, 19] or viral vector vaccine such as modified vaccinia virus Ankara (MVA) strain [20] and adenovirus type 35 [21]. BCG-prime and recombinant MVA-antigen 85A boost regimen [22] exhibited efficient immune responses in humans and have entered the first phase IIb trial in newborns. Furthermore, a combination of such strategies in which 3 major antigens are overproduced and the perfringolysin gene is incorporated into BCG and boosted with a recombinant adenovirus vaccine has been developed [23]. However, it is unknown whether such strategies are relevant for developing vaccines that are effective against adult pulmonary TB. It is necessary to test whether these candidate vaccines effectively induce mucosal immunity and protect against lung disease.

## 4. HIV/AIDS Vaccine

*4.1. Background of the Global HIV Epidemic.* In 2009, there were an estimated 2.6 million people who became newly infected with HIV. This is more than 21% less than the estimated 3.2 million who became infected in 1997, the year in which annual new infections peaked. In 33 countries, the incidence of HIV has decreased by more than 25% between 2001 and 2009; 22 of these countries are in sub-Saharan Africa. This trend reflects a combination of factors including the impact of HIV prevention efforts and the natural course of HIV epidemics [24].

Although highly activated antiretroviral therapy apparently contributes to control HIV replication in infected individuals [25], several problems remain to be resolved. These problems include: (i) the following viral load recovers soon after the interruption of treatment; (ii) chronic toxicities cause abnormalities in lipid metabolism and mitochondria; (iii) drug-resistant viruses increase during long period of treatment; (iv) long-term treatment carries a risk of carcinogenesis [26]; (v) expensive drugs are still difficult to access in developing countries. Even in developed countries, the high cost of antiretroviral drugs produces a sense of impending crisis in public health policy [27]. In such circumstances, although the rate of new infections with HIV-1 is gradually decreasing, an effective preventive vaccine is still urgently needed to stem further spread of the virus [28]. Even though considerable recent progress has been made in the development of an HIV vaccine [29, 30], the immune correlate of viral protection is not fully elucidated due to the complicated interaction of viral, immunological, and genetic factors [31, 32]. Since it is known that some populations of HIV-1-infected people do not present disease progression when HIV-1 replication is regulated by host immunity [33, 34], targeted vaccine immunogens are designed to closely mimic the long-lasting protective immunity induced in the long-term human survivors of natural infection [35, 36]. Due to safety issues, a live-attenuated HIV vaccine is not practical. This inevitably led the trend of HIV vaccine development to component- and vector-based vaccines.

*4.2. Current Trends in HIV/AIDS Vaccine Research.* The first large-scale efficacy trial of an HIV/AIDS vaccine was conducted by a US company, Vaxgen Co., in which a genetically engineered surface envelope (Env) glycoprotein, gp120, vaccine was tested in humans. Although the vaccine was targeted toward inducing effective virus-neutralizing antibodies, the phase III efficacy trial revealed its ineffectiveness [37, 38]. The failure of the gp120 vaccine changed the trend of HIV/AIDS vaccine research from an antibody-targeted strategy to a cell-mediated immunity-targeted strategy. Because HIV-1 causes chronic infection due to its cell-associated features, cellular immunity especially virus-specific cytotoxic T lymphocyte (CTL) should be a more important arm of the host immune system. Indeed, immune deficiency virus-specific cell-mediated immunity has been suggested to effectively control viral replication during the natural course of viral infections [39–41]. Based on these findings, various vaccine modalities, including live viral vectors and DNA vaccines, have been used to elicit strong CTL and Th1 type

responses in nonhuman primate models. Although single-vaccine delivery systems sometimes exhibit insufficient immune responses, boosting with viral vector vaccines such as vaccinia virus [40, 41], adenovirus [42, 43], and Sendai virus [44] in DNA-primed individuals strongly amplified CTL responses and resulted in the effective control of simian immunodeficiency virus (SIV) replication. Among such viral vectors, adenovirus type 5 (Ad5) had the strongest CTL enhancement effect, and the DNA-prime and recombinant Ad5 boost vaccine strategy is recognized as the most promising. However, in 2007, Merck Co. reported that a recombinant Ad5 vaccine expressing HIV-1 Gag, Pol, and Nef antigens did not demonstrate any protective efficacy in a phase IIB clinical trial [45]. Surprisingly, the vaccinated group exhibited a significantly higher HIV-1 infection rate than the placebo group [45], suggesting that the recombinant Ad5 immunization may have some unknown effect in enhancing HIV-1 infection. Thus, we were aware that T-cell vaccine approaches may involve certain risks and limitations; this paradigm appears to have reached an impasse.

In September 2009, there was ground-breaking news that the RV144 large-scale efficacy trial in Thailand demonstrated a partial effect of reducing HIV-1 infection rate in the recipients of ALVAC (canarypox)/gp120 prime-boost vaccine [46]. Although the results demonstrated limited effects, they demonstrated the possibility of preventing HIV infection with the active immunization for the first time. Furthermore, although there was no apparent correlation between protection and virus-specific cellular immune response or neutralizing antibody levels in the vaccinees, more detailed analyses of the total host responses are expected in the future. Taking the vaccine formulation with the gp120 protein boost into account, some antibody-mediated reactions may be involved in this partial protection. On the other hand, a new T-cell-targeted vaccine also demonstrated protective efficacy in a macaque study in the same year. A rhesus cytomegalovirus-vectored vaccine expressing SIV Gag, Rev-Tat-Nef, and Env persistently infected rhesus macaques, primed, and maintained robust SIV-specific $CD4^+$ and $CD8^+$ effector memory T-cell responses in the absence of neutralizing antibodies [47]. The report suggests that T cell vaccines may have greater potential than previously estimated. Although the importance of broadly neutralizing antibody production would not change despite tremendous difficulties, cellular immunity-targeted candidate vaccines should be also clinically tested for proofs of concept.

*4.3. BCG-Vectored HIV Vaccine.* The most practical advantage of the BCG vector is its high safety. In addition to being effective at inducing protective immunity, an HIV-1 vaccine regimen must be shown to be safe, affordable, and compatible with other vaccines before it can be considered promising [39]. In this respect, vectors that have already been used in humans without serious complications and with low cost should be utilized for HIV vaccines. BCG is a unique live vaccine vector because of its easy antigen delivery to the professional APC to be presented to T cells. Therefore, this bacterium is expected to be an important vector for HIV vaccine development.

At the early stage of rBCG research in the 1990s, Aldovini and Young [48] demonstrated immunogenicity of rBCG against genetically engineered HIV-1 antigens in mice. We independently worked on an rBCG-vectored anti-HIV vaccine simultaneously. First, we demonstrated effective cellular immune induction against SIV Gag antigen by the rBCG vector in rhesus macaques [49, 50]. Furthermore, we cloned an extracellular $\alpha$ antigen (antigen 85B) gene from both BCG [51] and *Mycobacterium kansasii* [52], and established a foreign antigen secretion system in mycobacteria [53]. Based on this system, we extensively evaluated several rBCG constructs for candidate HIV vaccines and reported that an rBCG-HIV vaccine could induce protective humoral immune responses in guinea pigs [54]. These studies suggest that rBCG-based vaccines are feasible as AIDS vaccines. However, the CTL activity did not reach protective levels with a single injection of rBCG-HIV vaccine in the macaque model. To overcome the low immunogenicity of the rBCG vaccine in CTL induction, we utilized various strategies for enhancing the immune potential of the BCG vector.

*4.4. Prime-Boost Regimen for Enhancing Immune Responses.* The first strategy by which we tried to improve the potential of the rBCG-HIV vaccine was the use of a safe recombinant viral vector for a booster vaccine. With respect to safety, traditional live vaccines, which have been administered safely to both the healthy and the HIV-infected individuals, may be the vectors of choice for HIV-1 vaccines. To fully take advantage of the benefits of such traditional vaccines in the development of anti-HIV vaccines, we studied BCG Tokyo 172 strain and the replication-deficient vaccinia vaccine strain DIs [55, 56] both of which have been shown to be nonpathogenic when inoculated into immune-deficient animals as live recombinant vaccine vehicles [57]. The vaccinia virus DIs have been tested clinically as a smallpox vaccine in Japanese infants and proved to be quite safe. We chose this highly attenuated virus as a booster vaccine vector and constructed recombinant DIs (rDIs) expressing the HIV *gag* [58] or SIV *gag-pol* gene [59]. Both rDIs constructs were found to be effective in eliciting HIV- or SIV-Gag-specific immunity in mice. When they were administered as a booster antigen after priming with an SIV-DNA vaccine, the cellular immunity to SIV Gag was greatly enhanced [59]. In brief, we tested a new combination regimen: priming with rBCG-SIV Gag followed by boosting with rDIs-SIV Gag.

In the macaque study, we found that BCG/DIs vaccination induced a long-lasting and effective cellular immunity that was able to control a highly pathogenic virus SHIV C2/1 [60], after mucosal challenge [61]. A possible mechanism of effective Gag-specific cell-mediated immunity is shown in Figure 2. The strong Th1 response induced by the BCG vector may contribute to eliciting the Gag-specific CTL response. How these immune inductions are correlated with protective efficacy requires further investigation. In this study, the BCG/DIs vaccination developed high levels of cellular immunity in the macaques that were protected against the loss of $CD4^+$ T lymphocytes with reduced viral RNA levels after virus challenge. Furthermore, the BCG/DIs group showed no evidence of clinical diseases or mortality

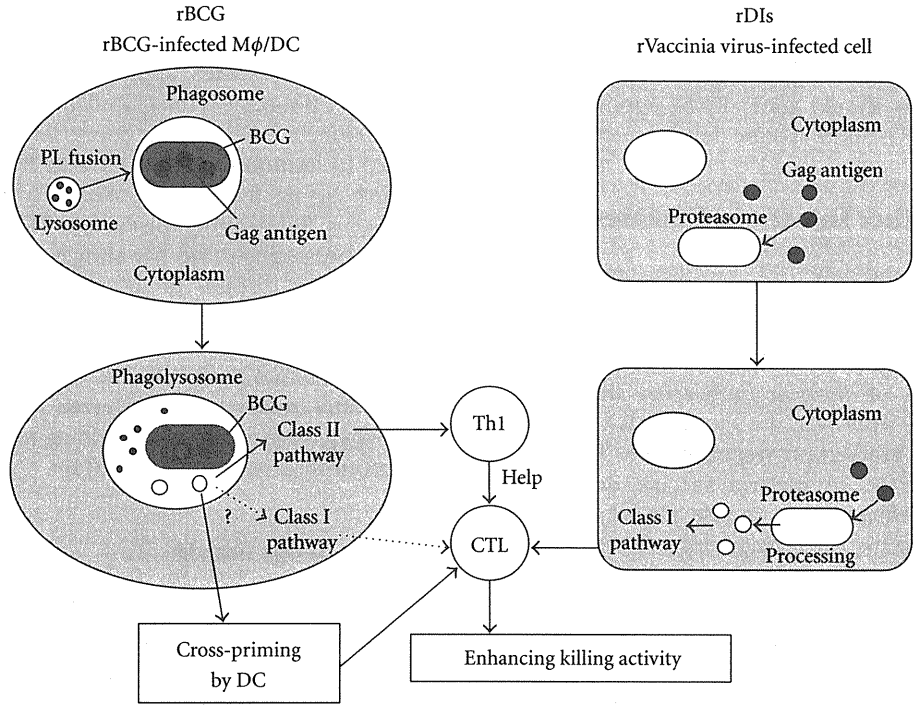


FIGURE 2: A possible mechanism of effective Gag-specific cell-mediated immunity induction with the rBCG/rDIs prime-boost vaccine. Abbreviations: DC, dendritic cell; Mϕ, macrophage; PL, phagosome-lysozome; Th1, type 1 helper T cell; CTL, cytotoxic T lymphocyte.

after viral challenge during the 1-year observation period [61]. These results suggest that the BCG/DIs prime-boost regimen might be a potential candidate for an effective and safe anti-HIV vaccine. Recent studies in macaques subjected to BCG/Ad5 [62] and BCG/MVA [63] regimens strongly support the effectiveness of the BCG vector. In the latter study, a hemolysin-expressing BCG strain, which was devised for more efficient antigen presentation to the CTL precursor, elicited a robust and broad range of HIV-1 specific T-cell responses along with recruitment of multiple T-cell clonotypes into the memory pool.

*4.5. Codon Optimization Strategy.* The major issue with BCG vehicle vaccines is the low expression level of the foreign antigen gene in BCG cells. In general, sufficient levels of foreign antigen-specific immune responses are obtained with high doses of rBCG between 10- and 100-fold greater than that needed for a practical dose against TB in humans [54]. This is considered the main limitation for the clinical use of rBCG-based vaccines. To address this substantial issue, we applied a codon optimization strategy for foreign genes in the rBCG system to increase its expression level. The aims of the study were to increase the immunogenicity of the foreign antigen, decrease inoculation dosages as small as the conventional BCG vaccine against TB, avoid adverse reactions, prevent possible association with Th2-type immune responses, and ward off the exacerbation of retroviral infections.

First, we determined the in vitro effects of codon optimization of the HIV gene in rBCG. Although the effect of codon optimization in mammalian cells is well documented [64–66], its effect in rBCG vehicle had never been fully elucidated. We targeted the HIV-1 *gag p24* gene as a model antigen to clarify the effect of codon optimization in the rBCG system. A specially designed synthetic p24 gene consisting of mycobacterial-preferred codons resulted in an increase in their GC content from 43.4% to 67.4%. Furthermore, codon-optimized rBCG was generated without any detectable changes in its characters including the growth rate. This rBCG exhibited a dramatic increase in Gag p24 antigen production approximately 40-fold greater than the non-optimized rBCG. Moreover, we successfully obtained data regarding the enhancement of immune responses in codon-optimized rBCG-immunized mice [67]. Inoculation of mice with a single low dose of the codon-optimized bacteria elicited effective cellular immunity. In the ELISPOT assay, the number of Gag-specific IFN-$\gamma$ spot-forming cells elicited by codon-optimized rBCG was significantly greater than that elicited by non-optimized recombinants [67]. These cellular immune responses would decrease if the $CD8^+$ T cells were depleted. The results also suggest that effective MHC-class I-restricted CTL responses are inducible by vaccination with codon-optimized rBCG. Furthermore, Gag-specific lymphocyte proliferative responses were also detected in the codon-optimized rBCG-immunized mice [67].

We also applied this strategy to an SIV Gag construct and successfully generated an rBCG harboring the codon-optimized SIV *gag* gene with an expression 10-fold greater than that of the native *gag* gene. In the macaque study, compared with a native *gag* gene construct, a low-dose ($10^6$ bacilli) injection of this construct induced optimal priming of Gag-specific $CD4^+$ and $CD8^+$ T cells and prolonged the maintenance of memory T-cell response after vaccinia DIs

boost [68]. These results imply that the quality of the priming vaccine is a critical factor for inducing a desirable immune response against immunodeficiency viruses. Thus, the codon optimization strategy should generally be applied to other foreign genes in rBCG-based vaccine development.

## 5. Vaccine for Other Infectious Diseases

There were various candidate rBCG vaccines targeting infectious diseases other than TB or HIV. Stover et al. [69] reported that the rBCG system would be useful in Lyme disease vaccine development; the vaccine incorporated with the surface protein of *Borrelia burgdorferi* first reached clinical phase I trials. However, the vaccine was rejected due to its low antibody production response [70]. Two groups [71, 72] applied rBCG in malaria vaccine development and demonstrated efficacy in a mouse model. Malaria is recognized as one of the three major infectious diseases as well as TB and AIDS. Although there is a long history of malaria vaccine development, we have not seen any licensed vaccine. The strategy to induce cellular immunity against conserved antigens using BCG vector could be effective to overcome substantial difficulties in producing vaccine due to antigenic diversity and unique life cycle of this parasite. In addition, BCG vector was tested for vaccine discovery against some viral diseases. A rBCG expressing the measles virus nucleoprotein demonstrated protection against measles virus pneumonia in macaques [73]. Furthermore, we demonstrated that a rBCG with a single hepatitis C virus (HCV) NS5 CTL epitope into antigen 85B induced HCV-specific CTL response in mice [74]. HCV is recognized as one of the major infectious pathogens of which the global infection rate is ~3%. Although the priority for preventive HCV vaccine development has become lower because of the remarkable progress in the treatment, BCG vector of targeting CTL induction may have implication for therapeutic vaccine against this disease. All these candidates at the early stage of rBCG study could not proceed to further development stages at those times. The rBCG-based vaccine development for these diseases should be reconsidered because the advanced technology that enhances the potential of BCG vectors has become currently available.

## 6. Conclusion and Future Perspective

As described in Section 3, several rBCG-based candidate vaccines are currently being evaluated for the development of TB vaccines. Such human trials would provide a greater insight into the paradigm of immune correlation in *M. tuberculosis* infection. In addition, the application of the codon optimization strategy enables us to utilize this bacterial vector as a primer of a heterologous prime-boost regimen for a preventive HIV vaccine. These results could suggest that the BCG vector is possible divalent vaccine controlling both TB and HIV/AIDS with a single construct; such study may help resolve the serious public health problem in the sub-Saharan African countries in which both diseases are highly prevalent [14].

Another potential outcome is the utility of the BCG vector for infant vaccines. One of the largest advantages of rBCG vaccines is their applicability to newborns. Because BCG as a TB vaccine is integrated into the expanded program on immunization in many countries, we have the earliest chance to immunize newborns with BCG within 3 months of birth before they are exposed to a variety of infectious pathogens. Substituting the current BCG with a novel rBCG vaccine possessing protective antigens against pathogens that cause serious diseases in infants, such as severe diarrhea and respiratory diseases, could be effective in developing countries. Such vaccine concepts should be also tested in appropriate animal models before they are tested in humans. Thus, after much trial and error in the last 2 decades, rBCG-based vaccines may contribute to the control of global infectious diseases in the near future.

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

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## Abstract

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

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

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

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## Introduction

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

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

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

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

## Results

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

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

### Transduction efficiency and cell surface markers of MazF-Tmac cells

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

### Longitudinal analysis of infused MazF-Tmac cells

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

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

### Detection of anti-MazF antibodies in monkey blood

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

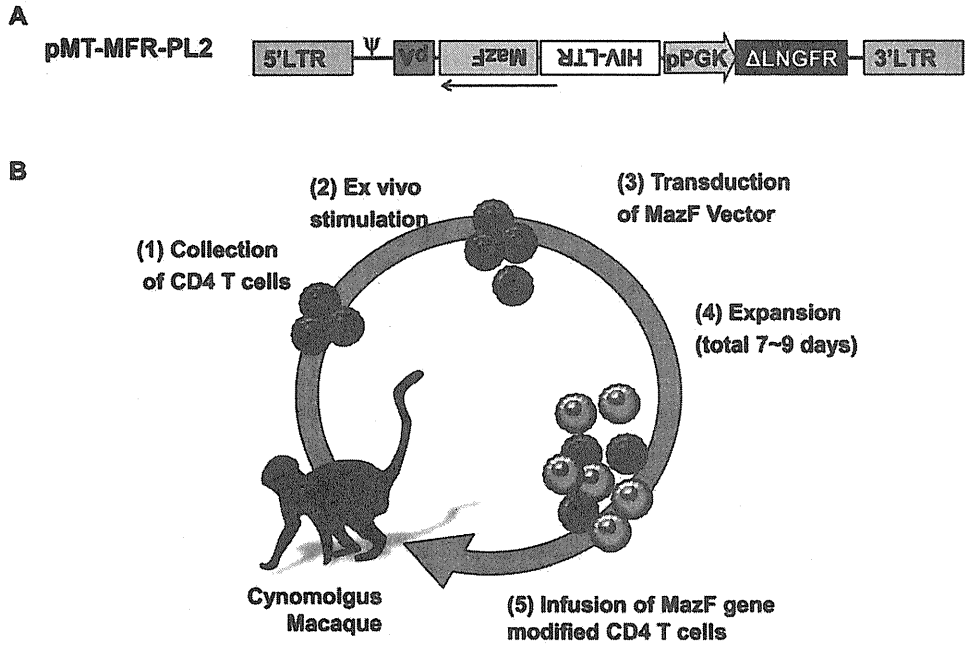


**Figure 1. Diagram of autologous CD4+ T cell transplantation using a non-human primate model.** (A) Design of gene transfer vector. The MazF gene derived from *E. coli* was inserted directly into the downstream of HIV-LTR sequence. The HIV-LTR-MazF-polyA cassette was introduced in the opposite direction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

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

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

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

doi:10.1371/journal.pone.0023585.t001

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

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

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

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

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

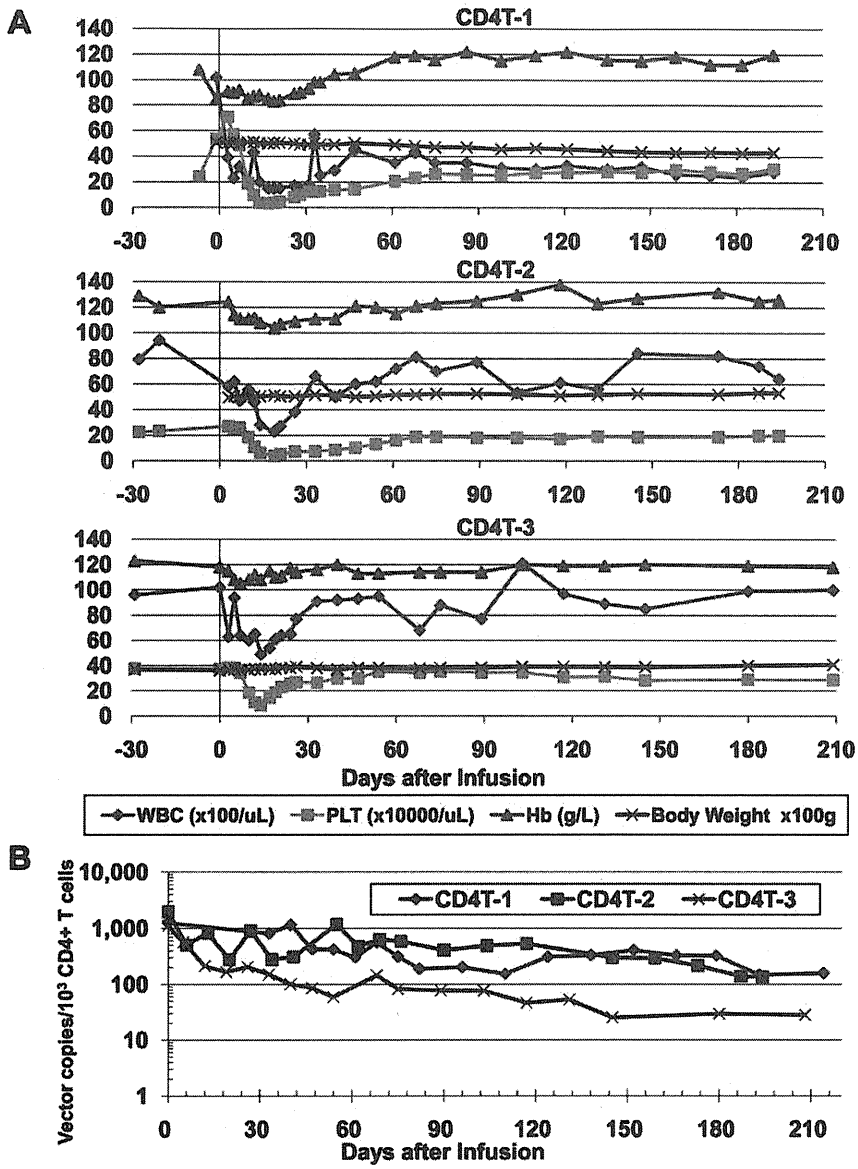


**Figure 2. Hematological analysis and engraftment of the MazF-transduced CD4+ T cells.** (A) The body weight and several hematological features were measured at the indicated time points, and the number of WBC, Hb, and PLT were represented. Each macaque was monitored throughout the study period. (B)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

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

## Distribution of MazF-Tmac cells

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

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

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

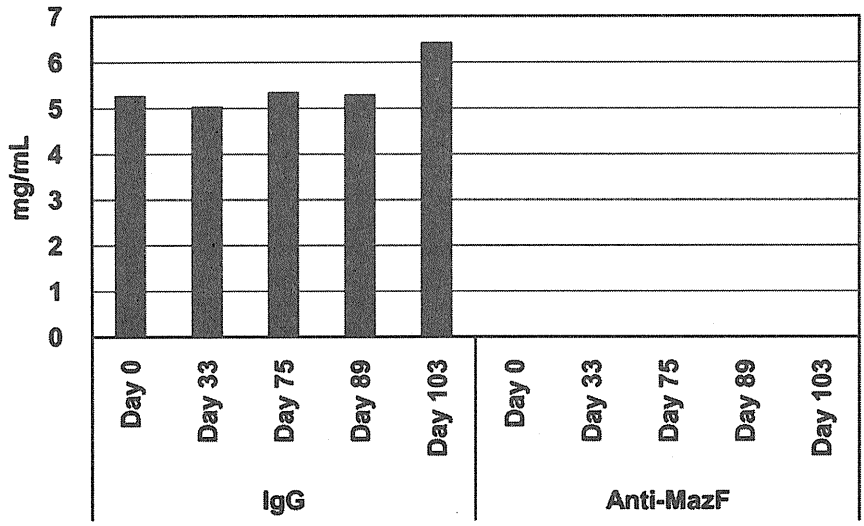


**Figure 3. No detection of anti-MazF antibodies in monkey blood after transplantation of MazF-Tmac cells.** Plasma samples were isolated from the monkey CD4T-2 at day 0, 33, 75, and 103 after transplantation and were used to detect anti-MazF antibodies on a MazF protein-immobilized microplate. The plasma samples were diluted to 500,000-fold, 50,000-fold, and 10,000-fold and added to each well. After the incubation, antibodies which reacted with immobilized MazF were tried to detect as described in Materials and Methods. No MazF-specific antibodies were detected.
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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 $6.8\times10^8$ 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

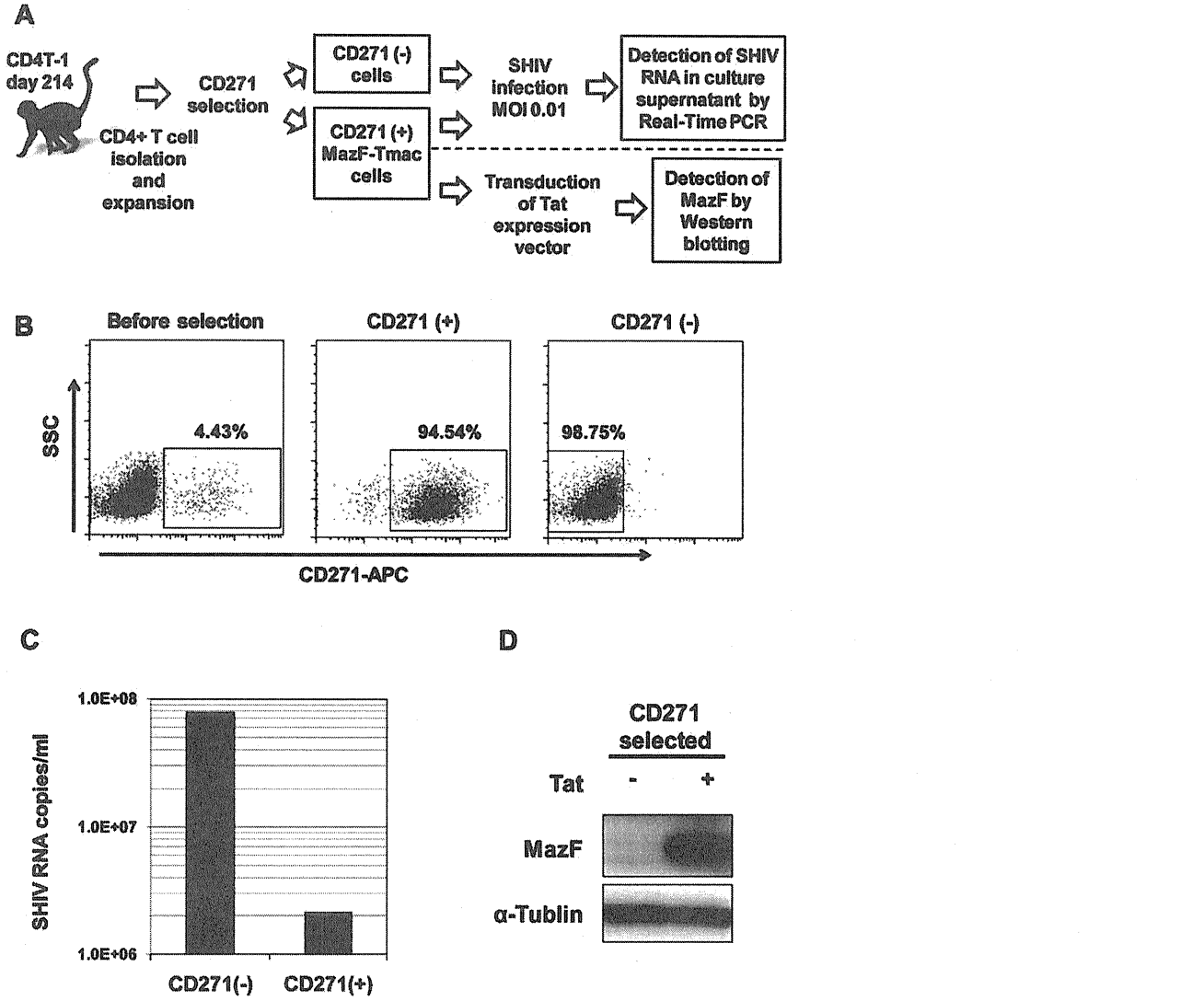


**Figure 4. Examination of the anti-viral efficacy of MazF-Tmac cells harvested from the monkey.** (A) Flow diagram of the experiment. CD4+ T lymphoid cells from CD4T-1 (214 days post-infusion of the MazF-Tmac cells) were stimulated and expanded *ex vivo*. The genetically modified cells expressing ΔLNGFR+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

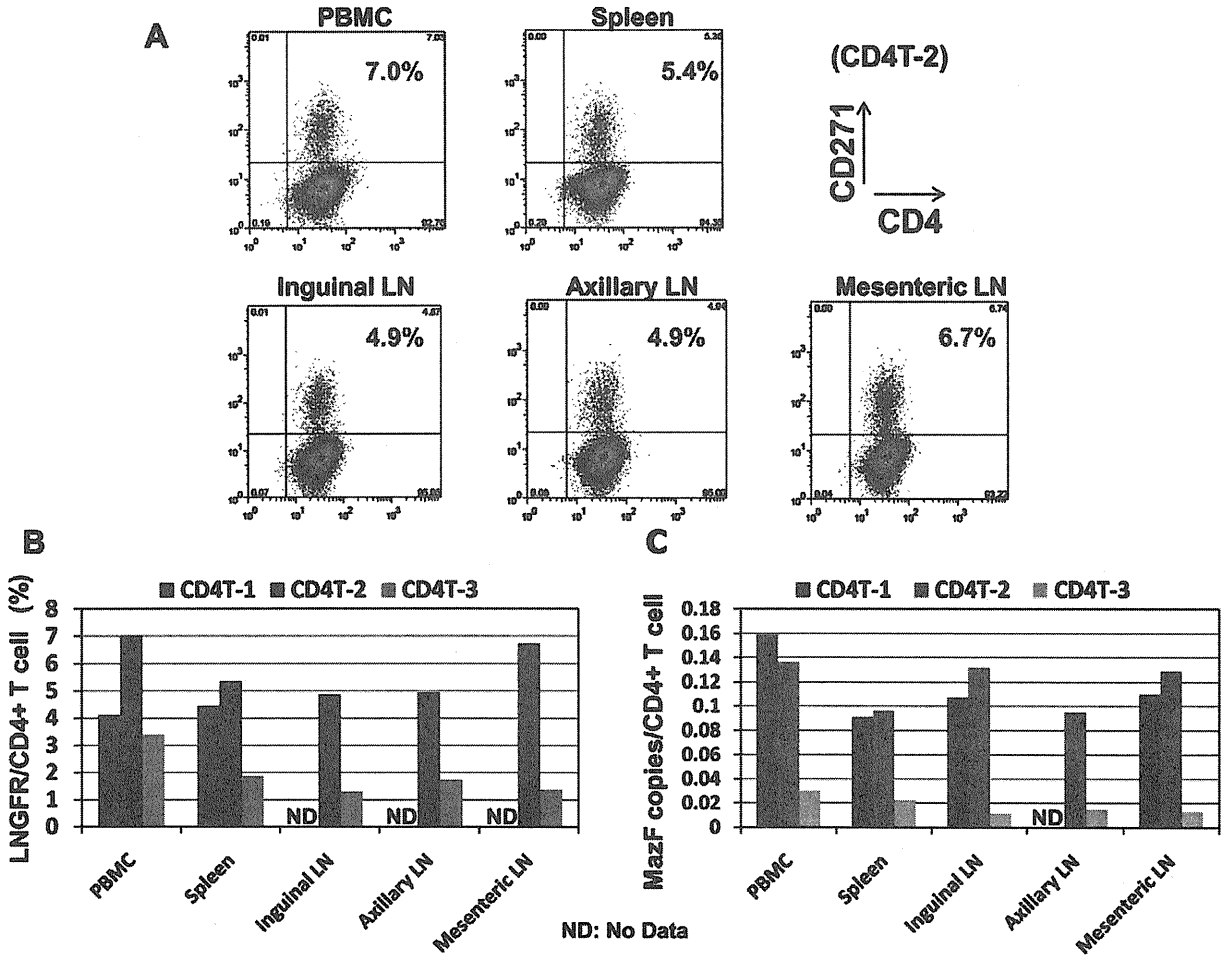


**Figure 5. Analysis of the distribution of MazF-Tmac cells in several organs.** (A) CD4+ T cells were isolated from lymphocytes separated from several organs, incubated 3–4 days, and stained with anti-CD4 and anti-CD271 antibodies. CD4T-2 is represented by a dot plot. (B) The percentage of CD271+ cells 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.
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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 $5\times10^9$ 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

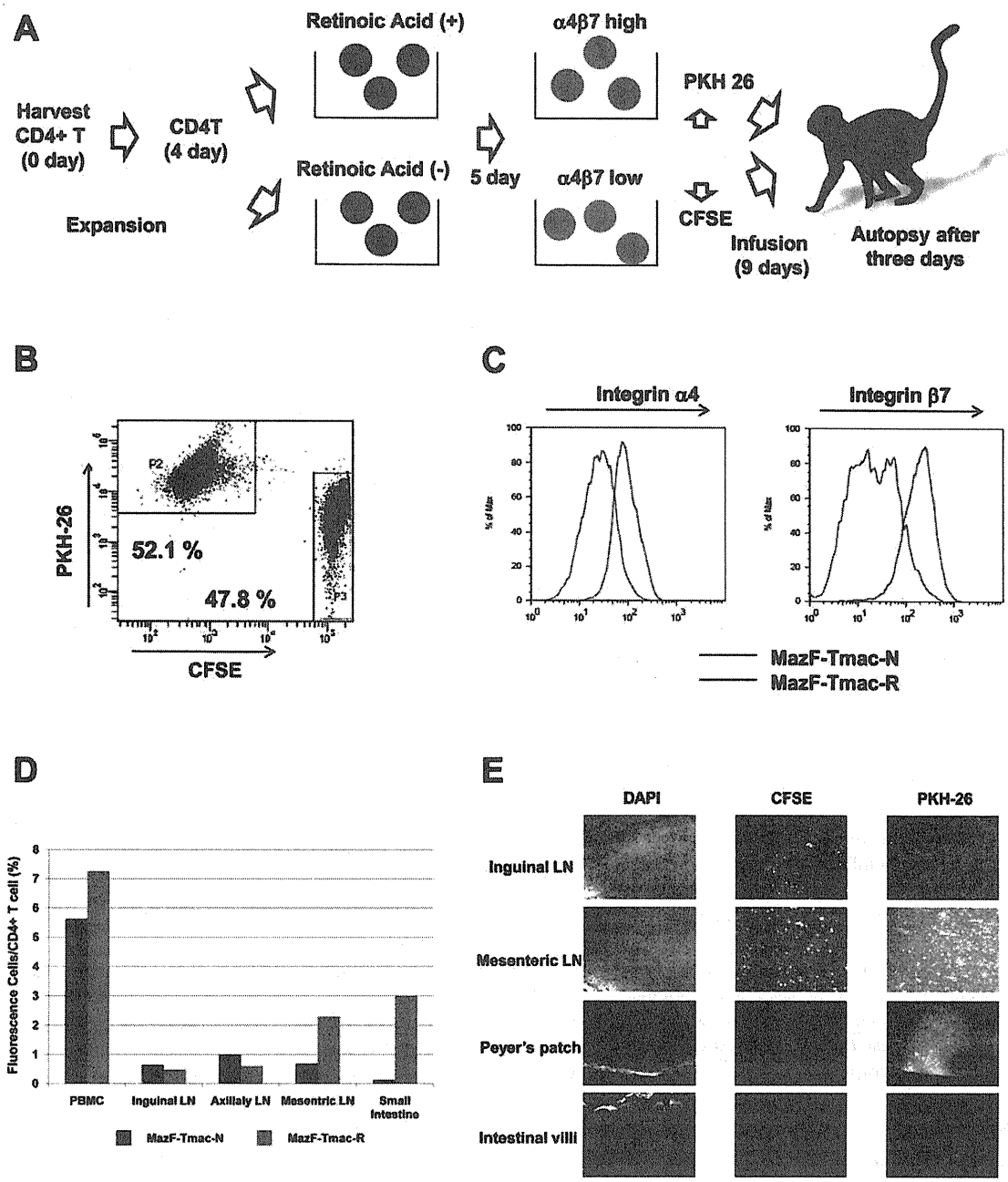


**Figure 6. Comparison of the homing effect of MazF-Tmac cells treated with or without retinoic acid.** (A) CD4+ T cells from the CD4T-4 monkey were stimulated with anti-CD3/CD28 beads, and MT-MFR-PL2 vector was transduced twice on days 3 and 4. After transduction, total lymphocytes were divided into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