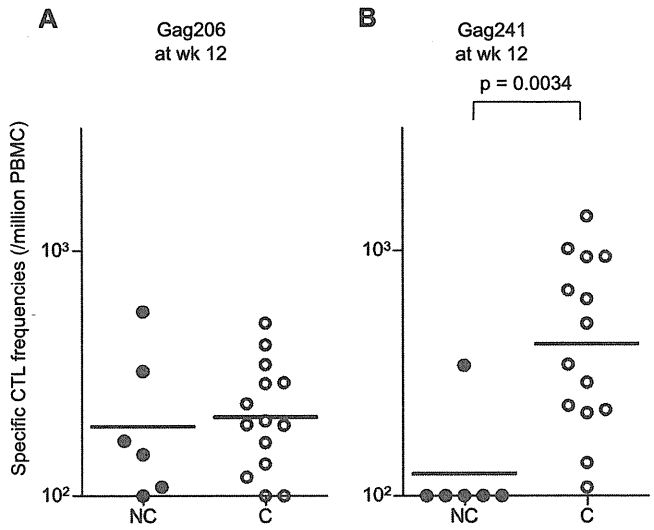

FIG 5 Comparison of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL responses in noncontrollers and controllers at week 12. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies in noncontrollers (NC; closed circles) and controllers (C; open circles). (B) $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies in noncontrollers and controllers. $Gag_{241-249}$-specific CTL levels in controllers were significantly higher than those in noncontrollers ($P = 0.0034$ by Mann-Whitney test). The bars indicate the geometric mean of each group. Data on a noncontroller (I-1) and a controller (II-5) were unavailable.

specific IFN-γ induction. At week 2 (Fig. 4A), most animals in groups I and II elicited both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses, whereas group III animals induced $Gag_{241-249}$-specific CTL responses dominantly. Remarkably, all animals in group IV showed efficient $Gag_{206-216}$-specific CTL responses without detectable $Gag_{241-249}$-specific CTL responses at week 2. These results indicate dominant $Gag_{206-216}$-specific CTL responses with delayed induction of $Gag_{241-249}$-specific CTL responses postchallenge in group IV animals with prophylactic $Gag_{206-216}$-specific CTL induction, and vice versa in group III animals.

At week 6 (Fig. 4B), efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were observed in all vaccinated animals in groups II, III, and IV, but not in group I. $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were induced equivalently even in groups III and IV. We also examined subdominant $Gag_{367-381}$ epitope-specific CTL responses, which were undetectable at week 2 but became detectable at week 6 in most group IV animals (Fig. 4, graphs on left). At week 12 (Fig. 4C), however, different CTL immunodominance patterns were observed among the groups. $Gag_{241-249}$-specific CTL levels were higher than $Gag_{206-216}$-specific levels in groups II and III but were reduced in groups I and IV. Interestingly, comparison between the animals with persistent viremia (referred to as noncontrollers) and those controlling SIV replication (referred to as controllers) revealed significant differences in $Gag_{241-249}$-specific CTL levels, but not in $Gag_{206-216}$-specific levels, at week 12 ($P = 0.0034$ by Mann-Whitney test) (Fig. 5).

Selection of a CTL escape mutation. Next, we examined viral genome *gag* sequences at weeks 5 and 12 after challenge to determine whether CTL escape mutations were selected in these animals (Table 2). At week 5, a mutation leading to an L-to-S substitution at the 216th residue in Gag (L216S) was selected in all the group II animals. This GagL216S change results in escape from $Gag_{206-216}$-specific CTL recognition, as described previously (21). All the group IV animals with $Gag_{206-216}$-specific CTL induction also showed rapid selection of this CTL escape mutation at week 5. Analysis at week 3 found the GagL216S mutation dominant in two (II-2 and II-5) group II and two (IV-1 and IV-3) group IV animals (data not shown). However, animals in group III showed no *gag* mutations at week 5, except for one animal (III-5) selecting a mutation leading to an L-to-F substitution at the 216th residue. Later, at week 12, the $Gag_{206-216}$-specific CTL escape mutation, GagL216S, was selected even in group III animals. No animals showed mutations around the $Gag_{241-249}$ epitope-coding region even at week 12. These results indicate that selection of this $Gag_{206-216}$-specific CTL escape mutation may be accelerated by prophylactic vaccination inducing $Gag_{206-216}$-specific CTL responses. On the other hand, in group III animals with single $Gag_{241-249}$ epitope-specific CTL induction, selection of a $Gag_{206-216}$-specific CTL escape mutation was delayed but was observed before selection of a $Gag_{241-249}$-specific CTL escape mutation, suggesting strong selective pressure by delayed $Gag_{206-216}$-specific CTL responses after SIV challenge.

In order to see the effect of rapid selection of the $Gag_{206-216}$-specific CTL escape mutation on SIV control, we compared plasma viral loads at weeks 3 and 5 between groups II and IV (referred to as group II+IV) with rapid selection of the GagL216S

TABLE 2 Selection of a CTL escape mutation

Group	Macaque ID	Amino acid change for Gag residues[b]: 206–216 Wk 5	206–216 Wk 12	241–249 Wk 5	241–249 Wk 12
I	I-1	None	ND	None	ND
	I-2[a]	None	L216S	None	None
	I-3	None	L216S	None	None
	I-4	None	None	None	None
	I-5	None	None	None	None
	I-6	None	None	None	None
II	II-1[a]	L216S	ND	None	ND
	II-2[a]	L216S	ND	None	ND
	II-3[a]	L216S	ND	None	ND
	II-4[a]	L216S	ND	None	ND
	II-5[a]	L216S	ND	None	ND
III	III-1[a]	None	L216S	None	None
	III-2[a]	None	L216S	None	None
	III-3[a]	None	NA	None	NA
	III-4[a]	None	NA	None	NA
	III-5[a]	L216F	L216S	None	None
	III-6[a]	None	L216S	None	None
IV	IV-1[a]	L216S	L216S	None	None
	IV-2	L216S	L216S	None	None
	IV-3	L216S	L216S	None	None
	IV-4[a]	L216S	L216S	None	None
	IV-5[a]	L216S	NA	None	NA

[a] Animals that controlled SIV replication at week 12 (controllers).
[b] Plasma viral gag genome mutations were examined at weeks 5 and 12. Amino acid substitutions in $Gag_{206-216}$ and $Gag_{241-249}$ epitope regions are shown. L216S results in viral escape from $Gag_{206-216}$-specific CTL recognition. It remains undetermined whether L216F results in CTL escape. ND, not determined; NA, not determined because Gag fragments were unable to be amplified from plasma RNA.

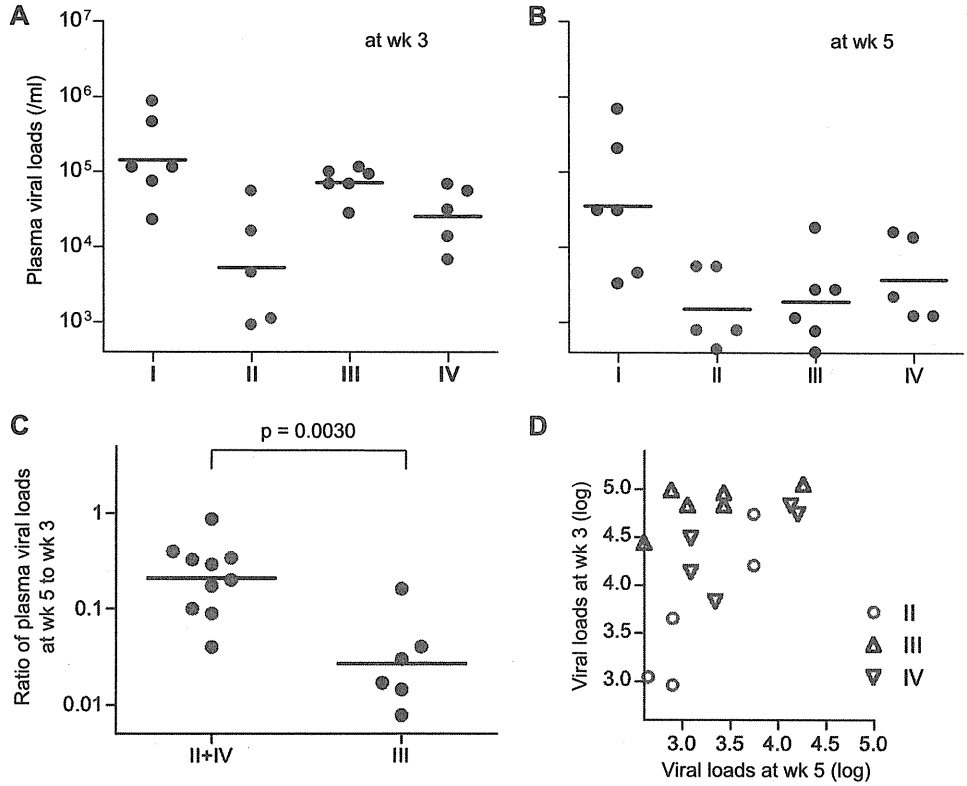

FIG 6 Comparison of plasma viral loads at weeks 3 and 5 among four groups. (A) Plasma viral loads at week 3 in group I, II, III, and IV animals. (B) Plasma viral loads at week 5 in group I, II, III, and IV animals. (C) Comparison of ratios of plasma viral loads at week 5 to week 3 in group II+IV animals and group III animals. The ratios in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test). The bars indicate the geometric mean of each group. (D) Scatter plots between plasma viral loads at weeks 3 and 5 in group II, III, and IV animals.

mutation and group III without the mutation at week 5 (Fig. 6). Ratios of plasma viral loads at week 5 to week 3 in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test) (Fig. 6C). To confirm this result, we examined the difference in week 3 viral loads between groups III and II+IV by ANCOVA, with week 5 viral loads as a covariate. This analysis revealed that week 3 viral loads controlled for by week 5 viral loads were significantly higher in group III than those in group II+IV (Fig. 6D and Table 3); i.e., the decline in viral loads from week 3 to week 5 was significantly sharper in group III than in group II+IV, possibly reflecting viral escape from suppressive pressure by $Gag_{206\text{-}216}$-specific CTL responses in the latter group during this period (from week 3 to week 5).

DISCUSSION

In the present study, we analyzed the impact of vaccination inducing single $Gag_{206\text{-}216}$ epitope-specific CTL memory on postchallenge CTL responses and SIV control in *90-120-Ia*-positive macaques and then compared the results with those of vaccination inducing single $Gag_{241\text{-}249}$ epitope-specific CTL responses. Our results indicate that these prophylactic vaccinations result in different patterns of $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL immunodominance and cooperation after SIVmac239 challenge.

Unvaccinated *90-120-Ia*-positive macaques (group I) showed both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIV challenge. In group IV animals with prophylactic induc-

TABLE 3 ANCOVA on week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV

ANOVA	Parameter	SS[a]	df[b]	MS[c]	*F*	*P* value
Homogeneity of slopes of regression	Group × slope	0.304	1	0.304	2.099	0.173
	Residual	1.735	12	0.145		
	Total	2.038	13	0.157		
Difference in week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV	Effect and group	1.106	1	1.106	7.052	0.020
	Residual	2.038	13	0.157		
	Total	3.144	14	0.225		

[a] SS, sum of squares.
[b] df, degrees of freedom.
[c] MS, mean squares.

tion of single $Gag_{206-216}$ epitope-specific CTL responses, $Gag_{206-216}$-specific CTL responses were induced dominantly but $Gag_{241-249}$-specific CTL responses were undetectable at week 2. In contrast, $Gag_{241-249}$-specific CTL responses were induced dominantly at week 2 in group III. Both groups showed $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 6. It may be difficult to compare these results with those in group II animals inducing whole Gag antigen-specific CTL and $CD4^+$ T-cell responses before challenge; the group II animals elicited $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses equivalently at week 2. Our results indicate that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses and may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as nonvaccine antigens) after viral exposure.

A significant difference between groups III and IV is the pattern of selection of CTL escape mutation. All group IV animals showed rapid selection of a $Gag_{206-216}$-specific CTL escape mutation, while most group III animals showed no *gag* mutation at week 5 but selection of the $Gag_{206-216}$-specific CTL escape mutation later, at week 12. Thus, prophylactic vaccination may affect the patterns of viral genome diversification, possibly accelerating selection of CTL escape mutations. Interestingly, $Gag_{241-249}$-specific CTL mutations were not detected even at week 12 in group III animals, although a previous study observed not only the $Gag_{206-216}$-specific CTL escape mutation (GagL216S), but also a $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in the chronic phase of SIV infection in *90-120-Ia*-positive macaques (9). These results indicate that delayed, naive-derived $Gag_{206-216}$-specific CTL responses, as well as preceding $Gag_{241-249}$-specific CTL responses, exert strong suppressive pressure on SIV replication in group III animals, implying cooperation between vaccine antigen-specific and non-vaccine antigen-specific CTL responses for virus control.

Rapid selection of the $Gag_{206-216}$-specific CTL escape mutation (GagL216S) in group II and delayed selection of this mutation without a detectable $Gag_{241-249}$-specific CTL escape mutation (GagD244E) in group III suggest that the virus with GagL216S (SIVmac239Gag216S) replicates more efficiently than the virus with GagD244E (SIVmac239Gag244E) under both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses. Our previous competition assay did not find a significant difference in viral fitness between these mutant viruses. Possibly, escape of SIVmac239Gag216S from $Gag_{206-216}$-specific CTL pressure may be more efficient than that of SIVmac239Gag244E from $Gag_{241-249}$-specific CTL pressure.

Our analysis revealed that the decline of plasma viral loads from week 3 to week 5 in group II+IV with rapid selection of the GagL216S mutation was significantly less than that in group III without the mutation at week 5, possibly reflecting viral escape from suppressive pressure by $Gag_{206-216}$-specific CTL responses in the former groups around weeks 3 to 5. Even the comparison between groups II and III, both showing dominant $Gag_{241-249}$-specific CTL responses at week 2, revealed a significantly sharper decline in the latter ($P = 0.0087$). Thus, our results suggest three patterns of $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL cooperation for virus control after SIVmac239 challenge. First, as observed in group II, dominantly induced $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses both work against wild-type SIV replication around week 2, but then a mutant virus escaping from the former CTL responses is selected, and the responses work against this mutant virus replication. Second, as observed in group III, dominantly induced $Gag_{241-249}$-specific CTL responses work against wild-type SIV replication around week 2 and then contribute to virus control, together with delayed, naive-derived $Gag_{206-216}$-specific CTL responses. Third, as observed in group IV, dominantly induced $Gag_{206-216}$-specific CTL responses work against wild-type SIV replication around week 2, but then a mutant virus escaping from $Gag_{206-216}$-specific CTL responses is selected, and delayed, naive-derived $Gag_{241-249}$-specific CTL responses instead work against this mutant virus replication. Viral loads at week 3 in group III looked higher than those in group IV, implying that $Gag_{206-216}$-specific CTL responses may exert a stronger suppressive effect on SIV replication in the acute phase than $Gag_{241-249}$-specific CTL responses. However, viral loads at week 5 in group III looked lower than those in group IV, and the comparison between the two groups showed significantly less decline in the latter ($P = 0.0303$). It is speculated that the third pattern observed in group IV is prone to failure in virus control. Indeed, two of five animals in group IV failed to control SIV replication. Even if vaccines are designed to express multiple antigens, of the vaccine-induced CTLs generated, only several epitope-specific cells may recognize the incoming HIV because of viral diversity and host MHC polymorphisms (18), and cooperation of these vaccine antigen-specific and non-vaccine antigen-specific CTL responses would be required for viral control. Thus, our results may imply a rationale of inducing escape-resistant, epitope-specific CTL memory by prophylactic AIDS vaccines.

In summary, this study showed dominant induction of vaccine antigen-specific CTL responses and delay in non-vaccine antigen-specific CTL responses in the acute phase of SIV infection, clearly describing the impact of prophylactic vaccination on CTL immunodominance and cooperation after virus exposure. Our results indicate that the patterns of cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses affect virus control and selection of CTL escape mutations. These findings provide great insights into antigen design in the development of a CTL-inducing AIDS vaccine.

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REFERENCES

1. **Altfeld M, et al.** 2003. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS 17:2581–2591.
2. **Berger CT, et al.** 2011. High functional avidity CTL responses to HLA-B-restricted Gag-derived epitopes associate with relative HIV control. J. Virol. 85:9334–9345.
3. **Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB.** 1994. Virus-specific CD8 cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. 68:6103–6110.
4. **Goulder PJ, Watkins DI.** 2004. HIV and SIV CTL escape: implications for vaccine design. Nat. Rev. Immunol. 4:630–640.
5. **Goulder PJ, Watkins DI.** 2008. Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat. Rev. Immunol. 8:619–630.
6. **Hansen SG, et al.** 2009. Effector memory T cell responses are associated

with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nat. Med. 15:293–299.
7. **Julg B, et al.** 2010. Enhanced anti-HIV functional activity associated with Gag-specific CD8 T-cell responses. J. Virol. 84:5540–5549.
8. **Kaslow RA, et al.** 1996. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. Nat. Med. 2:405–411.
9. **Kawada M, et al.** 2006. Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. J. Virol. 80:1949–1958.
10. **Kawada M, et al.** 2008. Gag-specific cytotoxic T-lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial. J. Virol. 82:10199–10206.
11. **Kestler HW III, et al.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 65:651–662.
12. **Kiepiela P, et al.** 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat. Med. 13:46–53.
13. **Koup RA, et al.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. 68:4650–4655.
14. **Leslie A, et al.** 2010. Additive contribution of HLA class I alleles in the immune control of HIV-1 infection. J. Virol. 84:9879–9888.
15. **Letvin NL, et al.** 2006. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. Science 312:1530–1533.
16. **Li F, et al.** 2011. Mapping HIV-1 vaccine induced T-cell responses: bias towards less-conserved regions and potential impact on vaccine efficacy in the Step study. PLoS One 6:e20479.
17. **Liu J, et al.** 2009. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. Nature 457:87–91.
18. **Liu Y, McNevin JP, Holte S, McElrath MJ, Mullins JI.** 2011. Dynamics of viral evolution and CTL responses in HIV-1 infection. PLoS One 6:e15639.
19. **Loffredo JT, et al.** 2008. Patterns of CD8 immunodominance may influence the ability of Mamu-B*08-positive macaques to naturally control simian immunodeficiency virus SIVmac239 replication. J. Virol. 82: 1723–1738.
20. **Matano T, Kano M, Nakamura H, Takeda A, Nagai Y.** 2001. Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen. J. Virol. 75:11891–11896.
21. **Matano T, et al.** 2004. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J. Exp. Med. 199:1709–1718.
22. **Matano T, et al.** 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J. Virol. 72: 164–169.
23. **Migueles SA, et al.** 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc. Natl. Acad. Sci. U. S. A. 97:2709–2714.
24. **Miura T, et al.** 2009. HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition. J. Virol. 83:2743–2755.
25. **Mothé BR, et al.** 2003. Expression of the major histocompatibility complex class I molecule Mamu-A*01 is associated with control of simian immunodeficiency virus SIVmac239 replication. J. Virol. 77:2736–2740.
26. **Naruse TK, et al.** 2010. Diversity of MHC class I genes in Burmese-origin rhesus macaques. Immunogenetics 62:601–611.
27. **Ndhlovu ZM, et al.** 2011. Mosaic HIV-1 Gag antigens can be processed and presented to human HIV-specific CD8+ T cells. J. Immunol. 186: 6914–6924.
28. **Schmitz JE, et al.** 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 283:857–860.
29. **Shibata R, et al.** 1997. Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J. Infect. Dis. 176:362–373.
30. **Streeck H, et al.** 2009. Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells. J. Virol. 83:7641–7648.
31. **Tang J, et al.** 2002. Favorable and unfavorable HLA class I alleles and haplotypes in Zambians predominantly infected with clade C human immunodeficiency virus type 1. J. Virol. 76:8276–8284.
32. **Tsukamoto T, et al.** 2009. Impact of cytotoxic-T-lymphocyte memory induction without virus-specific CD4+ T-cell help on control of a simian immunodeficiency virus challenge in rhesus macaques. J. Virol. 83: 9339–9346.
33. **Turnbull EL, et al.** 2009. Kinetics of expansion of epitope-specific T cell responses during primary HIV-1 infection. J. Immunol. 182:7131–7145.
34. **Wang YE, et al.** 2009. Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1. J. Virol. 83:1845–1855.
35. **Wilson NA, et al.** 2006. Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. J. Virol. 80: 5875–5885.
36. **Yant LJ, et al.** 2006. The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication. J. Virol. 80:5074–5077.

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CTL escape and viral fitness in HIV/SIV infection

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Cytotoxic T lymphocyte (CTL) responses exert a suppressive effect on HIV and simian immunodeficiency virus (SIV) replication. Under the CTL pressure, viral CTL escape mutations are frequently selected with viral fitness costs. Viruses with such CTL escape mutations often need additional viral genome mutations for recovery of viral fitness. Persistent HIV/SIV infection sometimes shows replacement of a CTL escape mutation with an alternative escape mutation toward higher viral fitness. Thus, multiple viral genome changes under CTL pressure are observed in the chronic phase of HIV/SIV infection. HIV/SIV transmission to HLA/MHC-mismatched hosts drives further viral genome changes including additional CTL escape mutations and reversions under different CTL pressure. Understanding of viral structure/function and host CTL responses would contribute to prediction of HIV evolution and control of HIV prevalence.

Keywords: HIV, SIV, MHC, cytotoxic T lymphocyte, escape mutation, viral fitness, capsid

INTRODUCTION

Virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses play a central role in the control of HIV and simian immunodeficiency virus (SIV) replication (Borrow et al., 1994; Koup et al., 1994; Matano et al., 1998; Jin et al., 1999; Schmitz et al., 1999; Goulder and Watkins, 2008). CTLs recognize viral antigen-derived peptides (epitopes) presented by major histocompatibility class I (MHC-I) molecules on the surface of viral-infected cells. Under the CTL pressure, viral mutations in and around epitope-coding regions which result in viral escape from CTL recognition are frequently selected with the cost of viral fitness (Phillips et al., 1991; Borrow et al., 1997; Goulder et al., 1997; Price et al., 1997). Thus, analysis of structural and functional constraints in viral proteins could facilitate determination of effective CTLs that can limit viral escape options, contributing to immunogen design in development of CTL-inducing AIDS vaccines.

We previously developed an AIDS vaccine using a Sendai virus vector expressing Gag (SeV-Gag), which induces Gag-specific CTL responses efficiently. Our analysis showed vaccine-based control of a SIVmac239 challenge in a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-120-Ia* (Matano et al., 2004; Kawada et al., 2008). $Gag_{206–216}$ (IINEEAADWDL) epitope-specific CTL responses exert a suppressive effect on SIV replication and select for a CTL escape mutation, GagL216S, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) in Gag capsid (CA) with viral fitness costs (Kobayashi et al., 2005). Our studies starting with this finding revealed viral genome changes in persistent SIV infection, providing insights into HIV/SIV evolution.

LOSS OF VIRAL FITNESS BY ESCAPE MUTATIONS AND ITS RECOVERY BY COMPENSATORY MUTATIONS

In contrast to the SIVmac239 challenge experiment, *90-120-Ia*-positive vaccinees failed to control a challenge with another pathogenic SIV strain, SIVsmE543-3 (Hirsch et al., 1997), which has the same $Gag_{206–216}$ amino acid sequence with SIVmac239. SIVsmE543-3 has a different amino acid (glutamate [E]) from SIVmac239 (aspartate [D]) at Gag residue 205, and this GagD205E change resulted in escape from $Gag_{206–216}$-specific CTL recognition, leading to failure in control of SIVsmE543-3 replication in *90-120-Ia*-positive vaccinees (Moriya et al., 2008).

Theoretically, $Gag_{206–216}$-specific CTL responses can select for either GagD205E or GagL216S mutation. SIVmac239-infected *90-120-Ia*-positive macaques, however, select the latter GagL216S mutation but not GagD205E in a year postchallenge. This suggests a possibility that the GagD205E substitution in SIVmac239 results in larger reduction of viral fitness than GagL216S. Indeed, our analysis *in vitro* revealed much lower replicative ability of the virus with this GagD205E substitution, SIVmac239Gag205E, compared to the wild-type SIVmac239 (Inagaki et al., 2010). On LuSIV cells, which contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat, SIVmac239Gag205E infection showed significantly lower luciferase activity compared to wild-type SIVmac239, indicating suppression of the early phase of this mutant virus replication.

Further passage of SIVmac239Gag205E-infected culture supernatants *in vitro* found an additional mutation, GagV340M, resulting in a valine (V)-to-methionine (M) substitution at the 340th aa in Gag. Interestingly, SIVmac239 has V while SIVsmE543-3 has M at the Gag residue 340. SIVmac239Gag205E340M showed similar replication kinetics with wild-type SIVmac239, indicating compensation for loss of viral fitness in SIVmac239Gag205E by addition of the GagV340M substitution. Thus, CTL escape mutations resulting in loss of viral fitness could be selected with compensatory mutations. **Figure 1** is a schema indicating the interaction between escape and compensatory mutations.

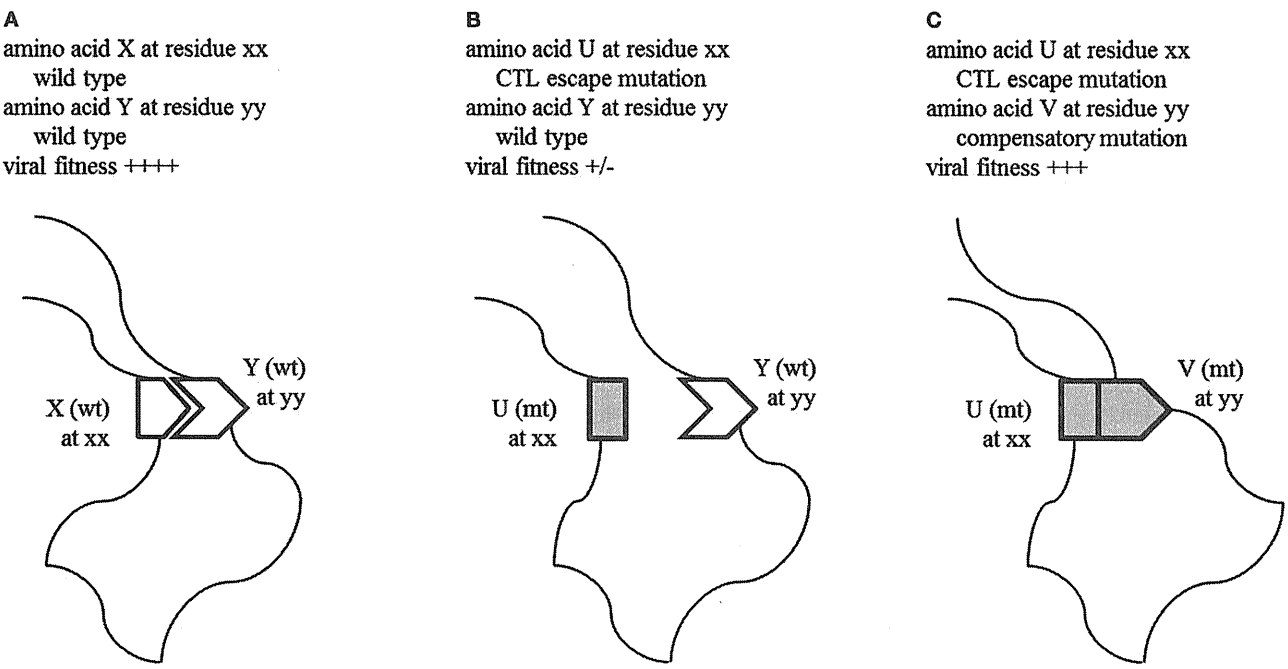

FIGURE 1 | Schema of recovery of viral fitness by a compensatory mutation. (A) Functional interaction between amino acid X at residue xx and Y at residue yy in wild-type viral protein is critical for viral replication. **(B)** A CTL escape mutation leading to an amino acid change from X to U at residue xx results in loss of viral fitness. **(C)** An additional compensatory mutation leading to an amino acid change from Y to V partially or fully restores viral function and replication.

GAG CA INTERMOLECULAR INTERACTION

The Gag CA is comprised of the N-terminal (NTD) and the C-terminal domains (CTD) (Momany et al., 1996; Gamble et al., 1997; Berthet-Colominas et al., 1999). Modeling of CA monomer structure showed that the Gag 205th residue is located in the helix 4 of CA NTD and the 340th is in the loop between helices 10 and 11 of CTD. A possibility of intramolecular contact between Gag residues 205 and 340 is not supported by this modeling. However, CA molecules are known to form hexamer lattice in mature virions (Ganser et al., 1999; Li et al., 2000; Ganser-Pornillos et al., 2007, 2008; Pornillos et al., 2009). Modeling of CA hexamer structure revealed that the Gag 205th residue is located in close proximity to the 340th of the adjacent CA molecule. The molecular model of CA hexamers incorporating the GagD205E substitution suggested shortening of the distance between Gag205 and Gag340 residues, which appeared compensated by GagV340M substitution. Thus, there may be intermolecular interaction between Gag residues 205 and 340 in CA hexamers. This is consistent with our results obtained by viral core stability assay. The core stability was reduced by the GagD205E substitution but recovered by the GagV340M substitution. Loss of viral fitness by GagD205E and its recovery by GagV340M implies a structural constraint for functional interaction between CA NTD and CTD involved in the formation of CA hexamers. In addition to previous reports on intramolecular compensation for loss of viral fitness by CTL escape mutations (Friedrich et al., 2004a; Crawford et al., 2007), our results present evidence indicating intermolecular compensation.

REPLACEMENT OF A CTL ESCAPE MUTATION WITH AN ALTERNATIVE ESCAPE MUTATION TOWARD HIGHER VIRAL FITNESS

As stated above, SIVmac239-infected *90-120-Ia*-positive macaques usually select the $Gag_{206–216}$-specific CTL escape mutation, GagL216S, but not GagD205E in a year postchallenge. After that, however, we found that the GagD205E mutation together with GagV340M became dominant instead of GagL216S in a *90-120-Ia*-positive macaque (Inagaki et al., 2010). In this macaque, neither GagD205E nor GagV340M was detected until week 123 after SIVmac239 challenge, but both became detectable at week 137 and were dominant at week 150. In contrast, the GagL216S mutation dominant until week 123 was undetectable at week 150. Thus, in this animal, SIVmac239Gag216S, whose replicative ability is lower than wild-type SIVmac239 but higher than SIVmac239Gag205E, became dominant under $Gag_{206–216}$-specific CTL pressure in the early phase, while in the later phase, this mutant virus was replaced with SIVmac239Gag205E340M, whose replicative ability is similar with the wild-type. This indicates replacement of a CTL escape mutation with an alternative escape mutation toward higher viral fitness in the chronic phase, implying persistent $Gag_{206–216}$-specific CTL pressure for more than 2 years after selection of the CTL escape mutation.

MULTIPLE VIRAL GENOME CHANGES UNDER CTL PRESSURE

In another study (Kawada et al., 2006), we observed accumulation of multiple CTL escape mutations in viral genomes in SIV-infected macaques. SeV-Gag-vaccinated animals possessing

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

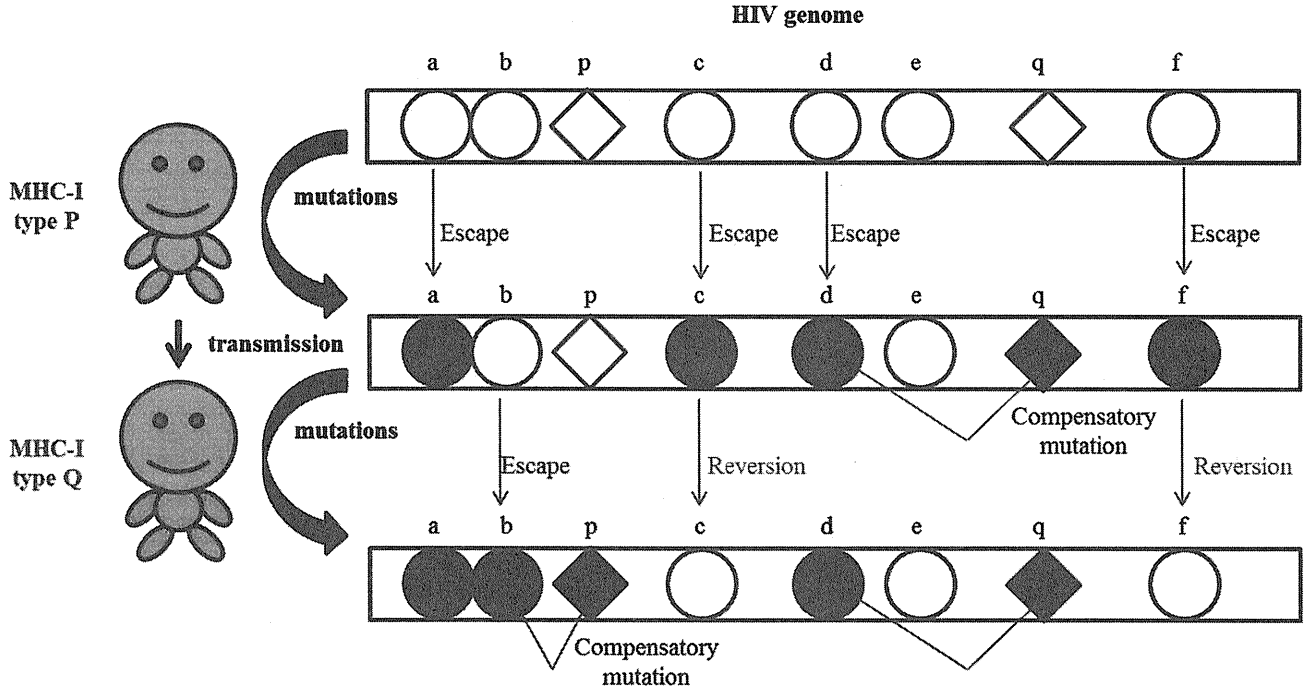

FIGURE 2 | Schema of HIV/SIV transmission resulting in accumulation of multiple viral mutations. Multiple CTL escape mutations resulting in viral fitness costs do not always revert rapidly even in the absence of CTL pressure after their transmission into HLA/MHC-mismatched hosts and such mutants can 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.

REFERENCES

Berthet-Colominas, C., Monaco, S., Novelli, A., Sibai, G., Mallet, F., and Cusack, S. (1999). Head-to-tail dimers and interdomain flexibility revealed by the crystal structure of HIV-1 capsid protein (p24) complexed with a monoclonal antibody Fab. *EMBO J.* 18, 1124–1136.

Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M., and Oldstone, M. B. (1994). Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* 68, 6103–6110.

Borrow, P., Lewicki, H., Wei, X., Horwitz, M. S., Peffer, N., Meyers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H., Oldstone, M. B., and Shaw, G. M. (1997). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTL) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat. Med.* 3, 205–211.

Brander, C., and Walker, B. D. (2003). Gradual adaptation of HIV to human host populations: good or bad news? *Nat. Med.* 9, 1359–1362.

Crawford, H., Prado, J. G., Leslie, A., Hué, S., Honeyborne, I., Reddy, S., van der Stok, M., Mncube, Z., Brander, C., Rousseau, C., Mullins, J. I., Kaslow, R., Goepfert, P., Allen, S., Hunter, E., Mulenga, J., Kiepiela, P., Walker, B. D., and Goulder, P. J. R. (2007). Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. *J. Virol.* 81, 8346–8351.

Dahirel, V., Shekhar, K., Pereyra, F., Miura, T., Artyomov, M., Talsania, S., Allen, T. M., Altfeld, M., Carrington, M., Irvine, D. J., Walker, B. D., and Chakraborty, A. K. (2011). Coordinate linkage of HIV evolution reveals regions of immunological vulnerability. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11530–11535.

Friedrich, T. C., Frye, C. A., Yant, L. J., O'Connor, D. H., Kriewaldt, N. A., Benson, M., Vojnov, L., Dodds, E. J., Cullen, C., Rudersdorf, R., Hughes, A. L., Wilson, N., and Watkins, D. I. (2004a). Extra-epitopic compensatory substitutions partially restore fitness to simian immunodeficiency virus variants that escape from an immunodominant cytotoxic T-lymphocyte response. *J. Virol.* 78, 2581–2585.

Friedrich, T. C., Dodds, E. J., Yant, L. J., Vojnov, L., Rudersdorf, R., Cullen, C., Evans, D. T., Desrosiers, R. C., Mothé, B. R., Sidney, J., Sette, A., Kunstman, K., Wolinsky, S., Piatak, M., Lifson, J., Hughes, A. L., Wilson, N., O'Connor, D. H., and Watkins, D. I. (2004b). Reversion of CTL escape-variant immunodeficiency viruses in vivo. *Nat. Med.* 10, 275–281.

Gamble, T. R., Yoo, S., Vajdos, F. F., von Schwedler, U. K., Worthylake, D. K., Wang, H., McCutcheon, J. P., Sundquist, W. I., and Hill, C. P. (1997). Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein. *Science* 278, 849–853.

Ganser, B. K., Li, S., Klishko, V. Y., Finch, J. T., and Sundquist, W. I. (1999). Assembly and analysis of conical models for the HIV-1 core. *Science* 283, 80–83.

Ganser-Pornillos, B. K., Cheng, A., and Yeager, M. (2007). Structure of full-length HIV-1 CA: a model for the mature capsid lattice. *Cell* 131, 70–79.

Ganser-Pornillos, B. K., Yeager, M., and Sundquist, W. I. (2008). The structural biology of HIV assembly. *Curr. Opin. Struct. Biol.* 18, 203–217.

Goulder, P. J., Phillips, R. E., Colbert, R. A., McAdam, S., Ogg, G., Nowak, M. A., Giangrande, P., Luzzi, G., Morgana, B., Edwards, A., McMichael, A. J., and Rowland-Jones, S. (1997). Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat. Med.* 3, 212–217.

Goulder, P. J. R., and Watkins, D. I. (2008). Impact of MHC class I diversity on immune control of immunodeficiency virus replication. *Nat. Rev. Immunol.* 8, 619–630.

Hirsch, V., Adger-Johnson, D., Campbell, B., Goldstein, S., Brown, C., Elkins, W., and Montefiori, D. (1997). A molecularly cloned, pathogenic, neutralization-resistant simian immunodeficiency virus, SIVsmE543-3. *J. Virol.* 71, 1608–1620.

Inagaki, N., Takeuchi, H., Yokoyama, M., Sato, H., Ryo, A., Yamamoto, H., Kawada, M., and Matano, T. (2010). A structural constraint for functional interaction between N-terminal and C-terminal domains in simian immunodeficiency virus capsid proteins. *Retrovirology* 7, 90.

Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C. E., Safrit, J. T., Mittler, J., Weinberger, L., Kostrikis, L. G., Zhang, L., Perelson, A. S., and Ho, D. D. (1999). Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiency virus-infected macaques. *J. Exp. Med.* 189, 991–998.

Kaslow, R. A., Carrington, M., Apple, R., Park, L., Muñoz, A., Saah, A. J., Goedert, J. J., Winkler, C., O'Brien, S. J., Rinaldo, C., Detels, R., Blattner, W., Phair, J., Erlich, H., and Mann, D. L. (1996). Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat. Med.* 2, 405–411.

Kawada, M., Igarashi, H., Takeda, A., Tsukamoto, T., Yamamoto, H., Dohki, S., Takiguchi, M., and Matano, T. (2006). Involvement of multiple epitope-specific cytotoxic T lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. *J. Virol.* 80, 1949–1958.

Kawada, M., Tsukamoto, T., Yamamoto, H., Iwamoto, N., Kurihara, K., Takeda, A., Moriya, C., Takeuchi, H., Akari, H., and Matano, T. (2008). Gag-specific cytotoxic T lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial. *J. Virol.* 82, 10199–10206.

Kawashima, Y., Pfafferott, K., Frater, J., Matthews, P., Payne, R., Addo, M., Gatanaga, H., Fujiwara, M., Hachiya, A., Koizumi, H., Kuse, N., Oka, S., Duda, A., Prendergast, A., Crawford, H., Leslie, A., Brumme, Z., Brumme, C., Allen, T., Brander, C., Kaslow, R., Tang, J., Hunter, E., Allen, S., Mulenga, J., Branch, S., Roach, T., John, M., Mallal, S., Ogwu, A., Shapiro, R., Prado, J. G., Fidler, S., Weber, J., Pybus, O. G., Klenerman, P., Ndung'u, T., Phillips, R., Heckerman, D., Harrigan, P. R., Walker, B. D., Takiguchi, M., and Goulder, P. (2009). Adaptation of HIV-1 to human leukocyte antigen class I. *Nature* 458, 641–645.

Kiepiela, P., Leslie, A. J., Honeyborne, I., Ramduth, D., Thobakgale, C., Chetty, S., Rathnavalu, P., Moore, C., Pfafferott, K. J., Hilton, L., Zimbwa, P., Moore, S., Allen, T., Brander, C., Addo, M. M., Altfeld, M., James, I., Mallal, S., Bunce, M., Barber, L. D., Szinger, J., Day, C., Klenerman, P., Mullins, J., Korber, B., Coovadia, H. M., Walker, B. D., and Goulder, P. J. (2004). Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* 432, 769–775.

Kobayashi, M., Igarashi, H., Takeda, A., Kato, M., and Matano, T. (2005). Reversion in vivo after inoculation of a molecular proviral DNA clone of simian immunodeficiency virus with a cytotoxic-T-lymphocyte escape mutation. *J. Virol.* 79, 11529–11532.

Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C., and Ho, D. D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* 68, 4650–4655.

Li, S., Hill, C. P., Sundquist, W. I., and Finch, J. T. (2000). Image reconstructions of helical assemblies of the HIV-1 CA protein. *Nature* 407, 409–413.

Loh, L., Batten, C. J., Petravic, J., Davenport, M. P., and Kent, S. J. (2007). In vivo fitness costs of different Gag CD8 T-cell escape mutant simian-human immunodeficiency viruses for macaques. *J. Virol.* 81, 5418–5422.

Martinez-Picado, J., Prado, J. G., Fry, E. E., Pfafferott, K., Leslie, A., Chetty, S., Thobakgale, C., Honeyborne, I., Crawford, H., Matthews, P., Pillay, T., Rousseau, C., Mullins, J. I., Brander, C., Walker, B. D., Stuart, D. I., Kiepiela, P., and Goulder, P. (2006). Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J. Virol.* 80, 3617–3623.

Matano, T., Kobayashi, M., Igarashi, H., Takeda, A., Nakamura, H., Kano, M., Sugimoto, C., Mori, K., Iida, A., Hirata, T., Hasegawa, M., Yuasa, T., Miyazawa, M., Takahashi, Y., Yasunami, M., Kimura, A., O'Connor, D. H., Watkins, D. I., and Nagai, Y. (2004). Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. *J. Exp. Med.* 199, 1709–1718.

Matano, T., Shibata, R., Siemon, C., Connors, M., Lane, H. C., and Martin, M. A. (1998). Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. *J. Virol.* 72, 164–169.

Momany, C., Kovari, L. C., Prongay, A. J., Keller, W., Gitti, R. K., Lee, B. M., Gorbalenya, A. E., Tong, L., McClure, J., Ehrlich, L. S., Summers, M. F., Carter, C., and Rossmann, M. G. (1996). Crystal structure of dimeric HIV-1 capsid protein. *Nat. Struct. Mol. Biol.* 3, 763–770.

Moriya, C., Igarashi, H., Takeda, A., Tsukamoto, T., Kawada, M., Yamamoto, H., Inoue, M., Iida, A., Shu, T., Hasegawa, M., Nagai, Y., and Matano, T. (2008). Abrogation of AIDS vaccine-induced cytotoxic T lymphocyte efficacy in vivo due to a change in viral epitope flanking sequences. *Microbes Infect.* 10, 285–292.

O'Connor, D. H., McDermott, A. B., Krebs, K. C., Dodds, E. J., Miller, J. E., Gonzalez, E. J., Jacoby, T. J., Yant, L., Piontkivska, H., Pantophlet, R., Burton, D. R., Rehrauer, W. M., Wilson, N., Hughes, A. L., and Watkins, D. I. (2004). A dominant role for CD8-T-lymphocyte selection in simian immunodeficiency virus sequence variation. *J. Virol.* 78, 14012–14022.

Phillips, R. E., Rowland-Jones, S., Nixon, D. F., Gotch, F. M., Edwards, J. P., Ogunlesi, A. O., Elvin, J. G., Rothbard, J. A., Bangham, C. R., Rizza, C. R., and McMichael, A. J. (1991). Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* 354, 453–459.

Pornillos, O., Ganser-Pornillos, B. K., Kelly, B. N., Hua, Y., Whitby, F. G., Stout, C. D., Sundquist, W. I., Hill, C. P., and Yeager, M. (2009). X-Ray Structures of the hexameric building block of the HIV capsid. *Cell* 137, 1282–1292.

Price, D. A., Goulder, P. J., Klenerman, P., Sewell, A. K., Easterbrook, P. J., Troop, M., Bangham, C. R., and Phillips, R. E. (1997). Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1890–1895.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L., and Reimann, K. A. (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283, 857–860.

Schneidewind, A., Brockman, M. A., Yang, R., Adam, R. I., Li, B., Le Gall, S., Rinaldo, C. R., Craggs, S. L., Allgaier, R. L., Power, K. A., Kuntzen, T., Tung, C. S., LaBute, M. X., Mueller, S. M., Harrer, T., McMichael, A. J., Goulder, P. J., Aiken, C., Brander, C., Kelleher, A. D., and Allen, T. M. (2007). Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. *J. Virol.* 81, 12382–12393.

Seki, S., Kawada, M., Takeda, A., Igarashi, H., Sata, T., and Matano, T. (2008). Transmission of simian immunodeficiency virus carrying multiple cytotoxic T-lymphocyte escape mutations with diminished replicative ability can result in AIDS progression in rhesus macaques. *J. Virol.* 82, 5093–5098.

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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,

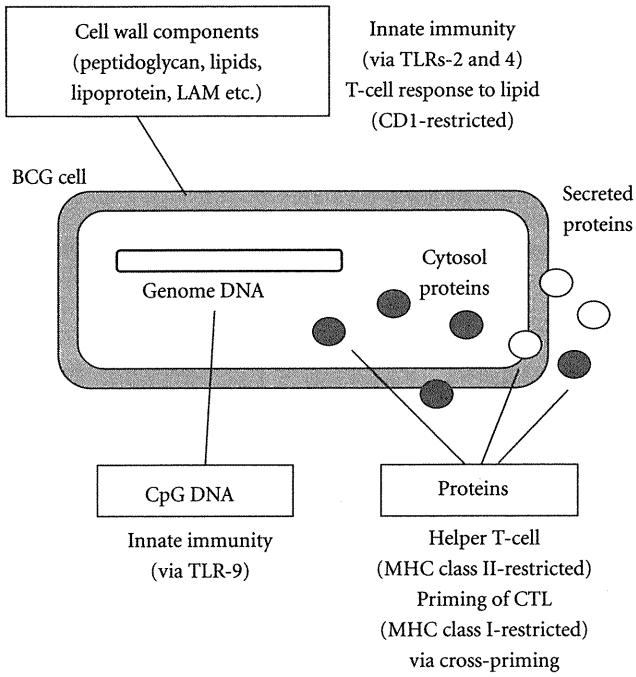

FIGURE 1: Outline of immune responses by BCG. Both innate immunity via TLRs and antigen-specific immunity via MHC- or CD1-restricted antigen presentation to T cells are induced by various BCG cell components.

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)-γ 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 α 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

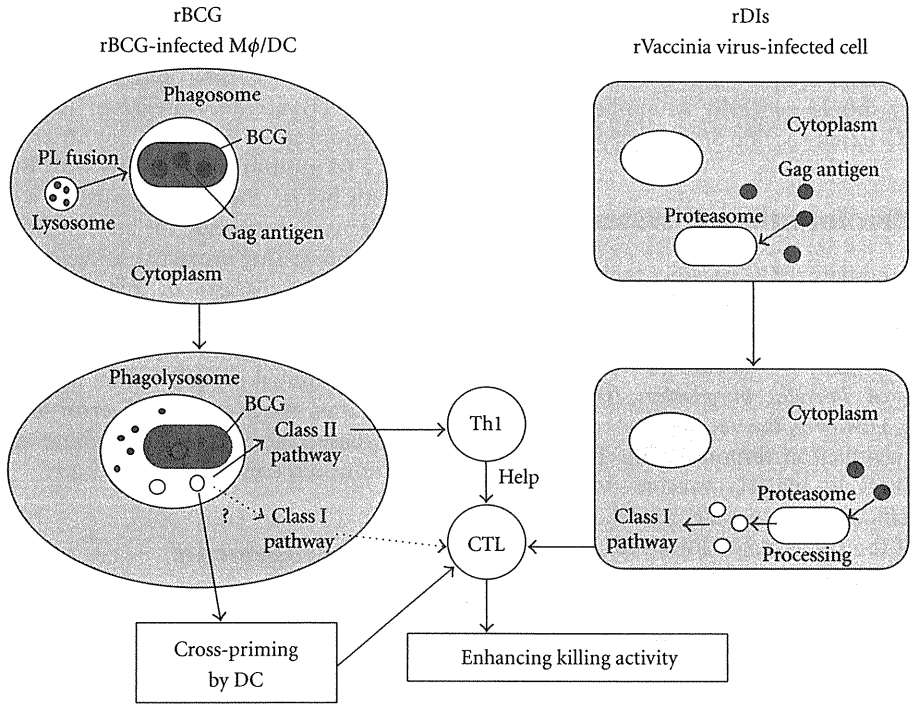

FIGURE 2: A possible mechanism of effective Gag-specific cell-mediated immunity induction with the rBCG/rDIs prime-boost vaccine. Abbreviations: DC, dendritic cell; Mϕ, macrophage; PL, phagosome-lysozome; Th1, type 1 helper T cell; CTL, cytotoxic T lymphocyte.

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-γ 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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References

[1] L. C. Rodrigues, V. K. Diwan, and J. G. Wheeler, "Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a metaanalysis," *International Journal of Epidemiology*, vol. 22, no. 6, pp. 1154–1158, 1993.

[2] G. A. Colditz, T. F. Brewer, C. S. Berkey et al., "Efficacy of BCG vaccine in the prevention of tuberculosis: meta-analysis of the published literature," *Journal of the American Medical Association*, vol. 271, no. 9, pp. 698–702, 1994.

[3] R. Brosch, S. V. Gordon, T. Garnier et al., "Genome plasticity of BCG and impact on vaccine efficacy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 13, pp. 5596–5601, 2007.

[4] S. M. Irwin, A. Goodyear, A. Keyser et al., "Immune response induced by three *Mycobacterium bovis* BCG substrains with diverse regions of deletion in a C57BL/6 mouse model," *Clinical and Vaccine Immunology*, vol. 15, no. 5, pp. 750–756, 2008.

[5] M. Seki, I. Honda, I. Fujita, I. Yano, S. Yamamoto, and A. Koyama, "Whole genome sequence analysis of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) Tokyo 172: a comparative study of BCG vaccine substrains," *Vaccine*, vol. 27, no. 11, pp. 1710–1716, 2009.

[6] W. R. Jacobs, M. Tuckman, and B. R. Bloom, "Introduction of foreign DNA into mycobacteria using a shuttle phasmid," *Nature*, vol. 327, no. 6122, pp. 532–535, 1987.

[7] R. Hernàndez-Pando, M. Castañòn, C. Espitia, and Y. Lopez-Vidal, "Recombinant BCG vaccine candidates," *Current Molecular Medicine*, vol. 7, no. 4, pp. 365–372, 2007.

[8] R. G. Bastos, S. Borsuk, F. K. Seixas, and O. A. Dellagostin, "Recombinant *Mycobacterium bovis* BCG," *Vaccine*, vol. 27, no. 47, pp. 6495–6503, 2009.

[9] J. Uehori, M. Matsumoto, S. Tsuji et al., "Simultaneous blocking of human toll-like receptors 2 and 4 suppresses myeloid dendritic cell activation induced by *Mycobacterium*

bovis bacillus Calmette-Guérin peptidoglycan," *Infection and Immunity*, vol. 71, no. 8, pp. 4238–4249, 2003.

[10] J. M. Roda, R. Parihar, and W. E. Carson III, "CpG-containing oligodeoxynucleotides act through TLR9 to enhance the NK cell cytokine response to antibody-coated tumor cells," *Journal of Immunology*, vol. 175, no. 3, pp. 1619–1627, 2005.

[11] T. Kawashima, Y. Norose, Y. Watanabe et al., "Cutting edge: major CD8 T cell response to live bacillus Calmette-Guérin is mediated by CD1 molecules," *Journal of Immunology*, vol. 170, no. 11, pp. 5345–5348, 2003.

[12] N. P. Goonetilleke, H. McShane, C. M. Hannan, R. J. Anderson, R. H. Brookes, and A. V. S. Hill, "Enhanced immunogenicity and protective efficacy against *Mycobacterium tuberculosis* of bacille Calmette-Guérin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara," *Journal of Immunology*, vol. 171, no. 3, pp. 1602–1609, 2003.

[13] E. A. Ramsburg, J. M. Publicover, D. Coppock, and J. K. Rose, "Requirement for CD4 T cell help in maintenance of memory CD8 T cell responses is epitope dependent," *Journal of Immunology*, vol. 178, no. 10, pp. 6350–6358, 2007.

[14] Global Tuberculosis Control Report, 2010, http://whqlibdoc.who.int/publications/2010/9789241564069_eng.pdf.

[15] The Global Plan to Stop TB 2011–2015, pp 81-88, 2010, http://www.stoptb.org/global/plan.

[16] D. F. Hoft, A. Blazevic, G. Abate et al., "A new recombinant bacille Calmette-Guérin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers," *Journal of Infectious Diseases*, vol. 198, no. 10, pp. 1491–1501, 2008.

[17] L. Grode, P. Seiler, S. Baumann et al., "Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guérin mutants that secrete listeriolysin," *Journal of Clinical Investigation*, vol. 115, no. 9, pp. 2472–2479, 2005.

[18] K. Von Eschen, R. Morrison, M. Braun et al., "The candidate tuberculosis vaccine Mtb72F/AS02A: tolerability and immunogenicity in humans," *Human Vaccines*, vol. 5, no. 7, pp. 475–482, 2009.

[19] J. T. van Dissel, S. M. Arend, C. Prins et al., "Ag85B-ESAT-6 adjuvanted with IC31 promotes strong and long-lived *Mycobacterium tuberculosis* specific T cell responses in naïve human volunteers," *Vaccine*, vol. 28, no. 20, pp. 3571–3581, 2010.

[20] H. McShane, A. A. Pathan, C. R. Sander et al., "Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans," *Nature Medicine*, vol. 10, no. 11, pp. 1240–1244, 2004.

[21] B. Abel, M. Tameris, N. Mansoor et al., "The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional $CD4^+$ and $CD8^+$ T cells in adults," *American Journal of Respiratory and Critical Care Medicine*, vol. 181, no. 12, pp. 1407–1417, 2009.

[22] T. J. Scriba, M. Tameris, N. Mansoor et al., "Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells," *European Journal of Immunology*, vol. 40, no. 1, pp. 279–290, 2010.

[23] R. Sun, Y. A. W. Skeiky, A. Izzo et al., "Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with *Mycobacterium tuberculosis*," *Vaccine*, vol. 27, no. 33, pp. 4412–4423, 2009.

[24] UNAIDS Report on the Global AIDS Epidemic, 2010, http://www.unaids.org/documents/20101123_GlobalReport_em.pdf.

[25] R. Granich, S. Crowley, M. Vitoria et al., "Highly active antiretroviral treatment as prevention of HIV transmission: review of scientific evidence and update," *Current Opinion in HIV and AIDS*, vol. 5, no. 4, pp. 298–304, 2010.

[26] A. E. Grulich, M. T. van Leeuwen, M. O. Falster, and C. M. Vajdic, "Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis," *Lancet*, vol. 370, no. 9581, pp. 59–67, 2007.

[27] K. A. Gebo, J. A. Fleishman, R. Conviser et al., "HIV Research Network. Contemporary costs of HIV healthcare in the HAART era," *AIDS*, vol. 24, no. 17, pp. 2705–2715, 2010.

[28] N. L. Letvin, D. H. Barouch, and D. C. Montefiori, "Prospects for vaccine protection against HIV-1 infection and AIDS," *Annual Review of Immunology*, vol. 20, pp. 73–99, 2002.

[29] S. H. E. Kaufmann and A. J. McMichael, "Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis," *Nature Medicine*, vol. 11, no. 4, pp. S33–S44, 2005.

[30] N. L. Letvin, "Progress toward an HIV vaccine," *Annual Review of Medicine*, vol. 56, pp. 213–223, 2005.

[31] G. Pantaleo and R. A. Koup, "Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know," *Nature Medicine*, vol. 10, no. 8, pp. 806–810, 2004.

[32] M. Z. Smith and S. J. Kent, "Genetic influences on HIV infection: implications for vaccine development," *Sexual Health*, vol. 2, no. 2, pp. 53–62, 2005.

[33] A. S. Fauci, S. M. Schnittman, G. Poli, S. Koenig, and G. Pantaleo, "Immunopathogenic mechanisms in human immunodeficiency virus (HIV) infection," *Annals of Internal Medicine*, vol. 114, no. 8, pp. 678–693, 1991.

[34] A. J. McMichael and T. Hanke, "HIV vaccines 1983–2003," *Nature Medicine*, vol. 9, no. 7, pp. 874–880, 2003.

[35] M. D. Daniel, F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers, "Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene," *Science*, vol. 258, no. 5090, pp. 1938–1941, 1992.

[36] J. D. Lifson, M. Piatak, J. L. Rossio et al., "Whole inactivated SIV virion vaccines with functional envelope glycoproteins: safety, immunogenicity, and activity against intrarectal challenge," *Journal of Medical Primatology*, vol. 31, no. 4-5, pp. 205–216, 2002.

[37] J. S. James, "First AIDS vaccine tested did not protect, but gives scientific leads," *AIDS Treatment News*, no. 389, p. 6, 2003.

[38] D. P. Francis, W. L. Heyward, V. Popovic et al., "Candidate HIV/AIDS vaccines: Lessons learned from the world's first phase III efficacy trials," *AIDS*, vol. 17, no. 2, pp. 147–156, 2003.

[39] R. R. Amara and H. L. Robinson, "A new generation of HIV vaccines," *Trends in Molecular Medicine*, vol. 8, no. 10, pp. 489–495, 2002.

[40] R. R. Amara, F. Villinger, J. D. Altman et al., "Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine," *Science*, vol. 292, no. 5514, pp. 69–74, 2001.

[41] I. Ourmanov, C. R. Brown, B. Moss et al., "Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV," *Journal of Virology*, vol. 74, no. 6, pp. 2740–2751, 2000.

[42] J. W. Shiver, T. M. Fu, L. Chen et al., "Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity," *Nature*, vol. 415, no. 6869, pp. 331–335, 2002.

[43] M. S. Seaman, L. Xu, K. Beaudry et al., "Multiclade human immunodeficiency virus type 1 envelope immunogens elicit broad cellular and humoral immunity in rhesus monkeys," *Journal of Virology*, vol. 79, no. 5, pp. 2956–2963, 2005.

[44] T. Matano, M. Kobayashi, H. Igarashi et al., "Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial," *Journal of Experimental Medicine*, vol. 199, no. 12, pp. 1709–1718, 2004.

[45] J. Cohen, "Did Merck's failed HIV vaccine cause harm?" *Science*, vol. 318, no. 5853, pp. 1048–1049, 2007.

[46] S. Rerks-Ngarm, P. Pitisuttithum, S. Nitayaphan et al., "Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand," *The New England Journal of Medicine*, vol. 361, no. 23, pp. 2209–2220, 2009.

[47] S. G. Hansen, C. Vieville, N. Whizin et al., "Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge," *Nature Medicine*, vol. 15, no. 3, pp. 293–299, 2009.

[48] A. Aldovini and R. A. Young, "Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines," *Nature*, vol. 351, no. 6326, pp. 479–482, 1991.

[49] Y. Yasutomi, S. Koenig, S. S. Haun et al., "Immunization with recombinant BCG-SIV elicits SIV-specific cytotoxic T lymphocytes in rhesus monkeys," *Journal of Immunology*, vol. 150, no. 7, pp. 3101–3107, 1993.

[50] Y. Yasutomi, S. Koenig, R. M. Woods et al., "A vaccine-elicited, single viral epitope-specific cytotoxic T lymphocyte response does not protect against intravenous, cell-free simian immunodeficiency virus challenge," *Journal of Virology*, vol. 69, no. 4, pp. 2279–2284, 1995.

[51] K. Matsuo, R. Yamaguchi, A. Yamazaki, H. Tasaka, and T. Yamada, "Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular α antigen," *Journal of Bacteriology*, vol. 170, no. 9, pp. 3847–3854, 1988.

[52] K. Matsuo, R. Yamaguchi, A. Yamazaki, H. Tasaka, K. Terasaka, and T. Yamada, "Cloning and expression of the gene for the cross-reactive α antigen of *Mycobacterium kansasii*," *Infection and Immunity*, vol. 58, no. 2, pp. 550–556, 1990.

[53] K. Matsuo, R. Yamaguchi, A. Yamazaki et al., "Establishment of a foreign antigen secretion system in mycobacteria," *Infection and Immunity*, vol. 58, no. 12, pp. 4049–4054, 1990.

[54] M. Honda, K. Matsuo, T. Nakasone et al., "Protective immune responses induced by secretion of a chimeric soluble protein from a recombinant *Mycobacterium bovis* bacillus Calmette-Guerin vector candidate vaccine for human immunodeficiency virus type 1 in small animals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 23, pp. 10693–10697, 1995.

[55] I. Tagaya, T. Kitamura, and Y. Sano, "A new mutant of dermovaccinia virus," *Nature*, vol. 192, no. 4800, pp. 381–382, 1961.

[56] T. Kitamura, Y. Kitamura, and I. Tagaya, "Immunogenicity of an attenuated strain of vaccinia virus on rabbits and monkeys," *Nature*, vol. 215, no. 5106, pp. 1187–1188, 1967.

[57] K. Takeya, K. Nomoto, S. Muraoka, S. Shimotori, T. Taniguchi, and T. Miyake, "Growth of two strains of *Mycobacterium bovis* (BCG) in a thymic mice," *Journal of General Microbiology*, vol. 100, no. 2, pp. 403–405, 1977.

[58] K. Ishii, Y. Ueda, K. Matsuo et al., "Structural analysis of vaccinia virus DIs strain: application as a new replication-deficient viral vector," *Virology*, vol. 302, no. 2, pp. 433–444, 2002.

[59] K. Someya, K-Q Xin, K. Matsuo, K. Okuda, N. Yamamoto, and M. Honda, "A consecutive priming-boosting vaccination of mice with simian immunodeficiency virus (SIV) gag/pol DNA and recombinant vaccinia virus strain DIs elicits effective anti-SIV immunity," *Journal of Virology*, vol. 78, no. 18, pp. 9842–9853, 2004.

[60] K. Shinohara, K. Sakai, S. Ando et al., "A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey," *Journal of General Virology*, vol. 80, no. 5, pp. 1231–1240, 1999.

[61] Y. Ami, Y. Izumi, K. Matsuo et al., "Priming-boosting vaccination with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin and a nonreplicating vaccinia virus recombinant leads to long-lasting and effective immunity," *Journal of Virology*, vol. 79, no. 20, pp. 12871–12879, 2005.

[62] M. J. Cayabyab, B. Korioth-Schmitz, Y. Sun et al., "Recombinant *Mycobacterium bovis* BCG prime-recombinant adenovirus boost vaccination in rhesus monkeys elicits robust polyfunctional simian immunodeficiency virus-specific T-cell responses," *Journal of Virology*, vol. 83, no. 11, pp. 5505–5513, 2009.

[63] M. Rosario, J. Fulkerson, S. Soneji et al., "Safety and immunogenicity of novel recombinant BCG and modified vaccinia virus Ankara vaccines in neonate rhesus macaques," *Journal of Virology*, vol. 84, no. 15, pp. 7815–7821, 2010.

[64] S. André, B. Seed, J. Eberle, W. Schraut, A. Bültmann, and J. Haas, "Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage," *Journal of Virology*, vol. 72, no. 2, pp. 1497–1503, 1998.

[65] M. Uchijima, A. Yoshida, T. Nagata, and Y. Koide, "Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium," *Journal of Immunology*, vol. 161, no. 10, pp. 5594–5599, 1998.

[66] D. L. Narum, S. Kumar, W. O. Rogers et al., "Codon optimization of gene fragments encoding Plasmodium falciparum merzoite proteins enhances DNA vaccine protein expression and immunogenicity in mice," *Infection and Immunity*, vol. 69, no. 12, pp. 7250–7253, 2001.

[67] M. Kanekiyo, K. Matsuo, M. Hamatake et al., "Mycobacterial codon optimization enhances antigen expression and virus-specific immune responses in recombinant *Mycobacterium bovis* bacille Calmette-Guérin expressing human immunodeficiency virus type 1 Gag," *Journal of Virology*, vol. 79, no. 14, pp. 8716–8723, 2005.

[68] M. Kanekiyo, Y. Ami, K. Matsuo et al., "A low-dose codon-optimized recombinant BCG-based HIV vaccine: prime-boost vaccination with recombinant BCG and replication-defective recombinant vaccinia virus DIs evokes SIV-specific immunity which overcomes the anamnestic BCG immunity in macaques," in *Proceedings of the 16th International AIDS Conference*, Toronto, Canada, August 2006.

[69] C. K. Stover, G. P. Bansal, M. S. Hanson et al., "Protective immunity elicited by recombinant bacille Calmette-Guerin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate Lyme disease vaccine," *Journal of Experimental Medicine*, vol. 178, no. 1, pp. 197–209, 1993.

[70] R. Edelman, K. Palmer, K. G. Russ et al., "Safety and immunogenicity of recombinant Bacille Calmette-Guerin (rBCG) expressing Borrelia burgdorferi outer surface protein A (OspA) lipoprotein in adult volunteers: a candidate Lyme disease vaccine," *Vaccine*, vol. 17, no. 7-8, pp. 904–914, 1999.

[71] S. Matsumoto, H. Yukitake, H. Kanbara, and T. Yamada, "Recombinant *Mycobacterium bovis* bacillus Calmette-Guerin secreting merozoite surface protein 1 (MSP1) induces protection against rodent malaria parasite infection depending on MSP1-stimulated interferon γ and parasite-specific antibodies," *Journal of Experimental Medicine*, vol. 188, no. 5, pp. 845–854, 1998.

[72] C. Zheng, P. Xie, and Y. Chen, "Recombinant *Mycobacterium bovis* BCG producing the circumsporozoite protein of Plasmodium falciparum FCC-1/HN strain induces strong immune responses in BALB/c mice," *Parasitology International*, vol. 51, no. 1, pp. 1–7, 2002.

[73] Y. D. Zhu, G. Fennelly, C. Miller et al., "Recombinant bacille Calmette-Guérin expressing the measles virus nucleoprotein protects infant rhesus macaques from measles virus pneumonia," *Journal of Infectious Diseases*, vol. 176, no. 6, pp. 1445–1453, 1997.

[74] S. Uno-Furuta, K. Matsuo, S. Tamaki et al., "Immunization with recombinant Calmette-Guerin bacillus (BCG)-hepatitis C virus (HCV) elicits HCV-specific cytotoxic T lymphocytes in mice," *Vaccine*, vol. 21, no. 23, pp. 3149–3156, 2003.

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

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Abstract

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

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

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

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Introduction

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

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

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

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

Results

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

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

Transduction efficiency and cell surface markers of MazF-Tmac cells

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

Longitudinal analysis of infused MazF-Tmac cells

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

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

Detection of anti-MazF antibodies in monkey blood

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