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Recombinant pro-apoptotic *Mycobacterium tuberculosis* generates CD8$^+$ T cell responses against human immunodeficiency virus type 1 Env and *M. tuberculosis* in neonatal mice

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ABSTRACT

Mycobacterium bovis BCG is an attractive vaccine vector against breast milk HIV transmission because it elicits Th1-type responses in newborns. However, BCG causes disease in HIV-infected infants. Genetically attenuated *Mycobacterium tuberculosis* (Mtb) mutants represent a safer alternative for immunocompromised populations. In the current study, we compared the immunogenicity in mice of three different recombinant attenuated Mtb strains expressing an HIV envelope (Env) antigen construct. Two of these strains (Δ*lysA* Δ*panCD* Mtb and Δ*RD1* Δ*panCD* Mtb) failed to induce significant levels of HIV Env-specific CD8$^+$ T cell responses. In striking contrast, an HIV-1 Env-expressing attenuated Δ*lys*A Mtb containing a deletion in *secA2*, which encodes a virulence-related secretion system involved in evading adaptive immunity, generated consistently measurable Env-specific CD8$^+$ T cell responses that were significantly greater than those observed after immunization with BCG expressing HIV Env. Similarly, another strain of Δ*lysA* Δ*secA2* Mtb expressing SIV Gag induced Gag- and Mtb-specific CD8$^+$ T cells producing perforin or IFNγ, and Gag-specific CD4$^+$ T cells producing IFNγ within 3 weeks after immunization in adult mice; in addition, IFNγ-producing Gag-specific CD8$^+$ T cells and Mtb-specific CD4$^+$ T cells were observed in neonatal mice within 1 week of immunization. We conclude that Δ*lysA* Δ*secA2* Mtb is a promising vaccine platform to construct a safe combination HIV-TB vaccine for use in neonates.

1. Introduction

Tuberculosis is the major cause of morbidity and death among the estimated 33.2 million people with HIV-1 infection worldwide [1]. Infants in developing countries where HIV-1 and tuberculosis (TB) are highly endemic are at a high risk both for mother-to-infant HIV transmission (either through perinatal exposure to maternal secretions or subsequent exposure to breast milk) or *Mycobacterium tuberculosis* (Mtb) infection, as well as rapid progression to AIDS or disseminated tuberculosis after infection. Approximately 5–20% of babies born to HIV positive women in resource-limited countries are infected through breastfeeding [2] resulting in an estimated 200,000 cases of HIV-1 each year [3].

An effective pediatric vaccine against TB or HIV in Africa must be safe for infants at risk for HIV, will require a highly accelerated vaccine schedule and will likely need to elicit virus-specific neutralizing antibodies and cytotoxic T-lymphocyte (CTL) responses rapidly. CD8$^+$ T cell responses that may be critical to control intracellular pathogens including HIV and Mtb are inherently limited in human neonates. However, human and murine neonates generate functional CD8$^+$ T cells after infections with viruses or immunization with live-attenuated immunogens that deliver antigen into the cytoplasm of APCs [4–7]. Thus far, only two candidate vaccines to protect against breast milk HIV transmission (HIV-1 gp120 recombinant subunit and live-attenuated recombinant canarypox ALVAC vaccines) have been studied in human infants. These vaccines elicit HIV-1 specific T and B cell responses [8–10] that may contribute to control of HIV-1 infection [11–13]. However, antigen-specific immunity to these vaccines develops slowly, and only after repeated boosting. Therefore, there is an urgent need for a neonatal immunogen that generates HIV-specific immunity more rapidly.

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Intradermal *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccination in healthy newborns induces maturation of dendritic cells [14,15] and rapidly primes strong adult-level Th1-type $CD4^+$ and $CD8^+$ T cell responses against shared Mtb antigens [16–18] that may limit TB to pulmonary sites in infants [19]. Recombinant BCG (rBCG) has been developed as a candidate neonatal vaccine vector against pertussis [20], measles [21,22], RSV [23] and breast milk HIV transmission [24]. Vaccines based on BCG vectors have the advantages that they are administered at birth, can be given orally, and rapidly generate long-lived T cell responses against mycobacterial and co-expressed non-mycobacterial antigens when administered simultaneously to human infants [25]. Given the large geographical overlap between Mtb and HIV infection in Africa, BCG vaccination was until recently recommended at birth for all infants. However, it now appears that the annual risk for disseminated BCG disease in HIV-infected infants (~0.42%) clearly outweighs the potential benefits of BCG in children with HIV [26,19]. Therefore, the WHO now advises against BCG vaccination in any HIV-infected child [1]. Alternative methods to control TB in infants with HIV are urgently needed.

We and others have observed that candidate attenuated *M. tuberculosis* (AMtb) strains Δ*lys*A Δ*panCD* Mtb and Δ*RD1* Δ*panCD* Mtb that are avirulent in immunocompromised SCID and gamma-interferon knockout ($GKO^{-/-}$) mice confer protection against a lethal aerosol challenge with Mtb H37Rv in immunocompetent mice equal to that observed after BCG immunization [27–29]. To determine the effectiveness of these AMtb vectors as HIV-1 immunogens for $CD8^+$ T cell responses, in the present study we measured the frequency of $CD8^+$ T cell responses against HIV-1 Env or SIV Gag after immunization with recombinant mutant Mtb strains expressing these HIV-1 or SIV antigens. Of three attenuated Mtb strains used for vaccine construction, we found that one induced significantly augmented $CD8^+$ T cell responses to the expressed Env or Gag recombinant antigens. This strain combined a strongly attenuating auxotrophy mutation in the *lysA* gene with deletion of *secA2*, which encodes a virulence-related secretion system that has been shown to participate in evasion of innate and adaptive immunity by *M. tuberculosis*.

Our results showed that single-dose immunization in neonatal mice with a Δ*lysA* Δ*secA2* AMtb strain expressing HIV Env rapidly generated high-frequency Env-specific $CD8^+$ T cells among splenocytes, and significant Env-specific $CD8^+$ T cell IFNγ and Mtb-specific $CD4^+$ T cell IFNγ responses. In further studies using a Δ*lysA* Δ*secA2* AMtb strain expressing an SIV Gag antigen, we demonstrated enhanced responses of Gag-specific $CD8^+$ T cells producing perforin and IFNγ, as well as Gag-specific $CD4^+$ T cells producing IFNγ and Mtb-specific $CD8^+$ T cells producing perforin. These data suggest that Δ*lys*A Δ*secA2* Mtb could be developed further as a safe and effective neonatal combination HIV-TB vaccine platform.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Table 1 lists the mycobacterial strains used in this work. The multicopy *Escherichia coli*/mycobacterial shuttle episomal plasmid pJH222-*gp120* (designated pENV) that contains the Mtb-derived α antigen promoter that drives expression of a full-length human codon-optimized HIV-1 IIIB *gp120* envelope gene (HXBc2) fused to an N-terminus 19-kDa signal sequence of Mtb and a carboxy-terminus HA tag, an *aph* gene encoding kanamycin resistance, a mycobacterial origin of replication (*ori*M) and the *E. coli* origin of replication (*ori*E), has been previously described [30] (Table 1). *E. coli* DH5α was used for amplification of plasmid DNA, which was purified using QIAGEN midiprep columns (QIAGEN, Inc., Valencia, CA). *E. coli* transformants were grown at 37 °C in LB media supplemented with kanamycin (40 μg/ml). To generate pJH222 encoding SIV Gag, first, an insert that encodes the full-length SIVmac239 Gag sequence using the favored codon usage of *M. tuberculosis* (http://www.jcat.de/, with the exception of the following base pair modifications, 645—G to A, 805—C to T, 861—C to G and 1308—C to A that were introduced to ablate two Pst1 and two Apa1 sites, respectively in order to facilitate cloning), the RGPGRAFVTI sequence (an H2Dd-restricted epitope of the V3 loop of HXBc2 Env, designated P18), SIINFEKL and the V5 epitope of paramyxovirus, SV5 [31] the target of V5 antibody-HRP (to permit immunoblotting), and that contained a 5′ ApaI and a 3′ PstI restriction site, plus an additional TA base pairs (to permit in-frame expression of the peptides) fused at the 3′ end of the insert (designated *mgagp18*) was synthesized (Bio Basic Inc, Canada). Next, *mgagp18* was ligated into unique ApaI and PstI sites downstream of the alpha antigen promoter and the 19-kDa signal sequence in the integrative mycobacterial shuttle vector pBRL34. NheI enzyme digestion at two sites in this intermediate construct, released a fragment containing the alpha antigen promoter and 19-kDa signal sequence upstream of *mgagp18* that was

Table 1
Mycobacterial strains[a].

Strain	Abbreviation	Relevant Characteristics	Source or reference
M. bovis BCG strains			
Pasteur	BCG	Vaccine strain	
$mc^2$5151	BCG(pJH222)	BCG Pasteur with pJH222	This study
$mc^2$5152	BCG(pENV)	BCG Pasteur with pJH222::gp120	This study
M. tuberculosis H37Rv strains			
$mc^2$6020	6020	Δ*lys* Δ*pan*CD, lysine and pantothenate auxotroph, Hy^r; persists for <3 weeks in vivo	A
$mc^2$5153	6020(pJH222)	$mc^2$6020 with pJH222	This study
$mc^2$5154	6020(pENV)	$mc^2$6020 with pJH222::gp120	This study
$mc^2$6030	6030	Δ*RD1* Δ*pan*CD, RD1 knockout and pantothenate auxotroph, Hy^r; persists for 28 weeks in vivo	B
$mc^2$5155	6030(pJH222)	$mc^2$6030 with pJH222	This study
$mc^2$5156	6030(pENV)	$mc^2$6030 with pYUB1404	This study
$mc^2$5226	5226	Δ*lys* Δ*secA2*, lysine auxotroph (96 base pair deletion) and pro-apoptotic	This study
$mc^2$5127	5226(pJH222)	$mc^2$5226 with pJH222	This study
$mc^2$5128	5226(pENV)	$mc^2$5226 with pENV	This study
$mc^2$3026	3026	Δ*lys*, lysine auxotroph (331 base pair deletion)	C
$mc^2$5132	3026(pJH222)	$mc^2$3026 with pJH222	This study
$mc^2$5133	3026(pENV)	$mc^2$3026 with pENV	This study
$mc^2$5222	5222	Δ*lys* Δ*secA2*, lysine auxotroph (96 base pair deletion) and pro-apoptotic with hygromycin cassette	This study
$mc^2$5129	5222(pGAG)	$mc^2$5222 with pGAG	This study

A—Sambandamurthy et al. [27]; B—Sambandamurthy et al. (2006); C—Pavelka et al. (1999).

[a] *Abbreviations*: Hy^r, hygromycin; superscript r, hygromycin resistance.