Published in final edited form as:

Vaccine. 2009 December 10; 28(1): 152–161. doi:10.1016/j.vaccine.2009.09.087.

Recombinant Proapoptotic *M. tuberculosis* Generates CD8⁺ T-cell Responses Against Human Immunodeficiency Virus Type 1 Env and *M. tuberculosis* in Neonatal Mice

Uma Devi K Ranganathan¹, Michelle H. Larsen^{2,3}, John Kim², Steven A. Porcelli^{2,3}, William R. Jacobs Jr.^{2,4}, and Glenn J. Fennelly^{1,5,*}

¹Department of Pediatrics, Albert Einstein College of Medicine, Bronx, New York 10461

²Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA, 10461

³Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA, 10461

⁴Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York 10461

⁵The Lewis M. Fraad Department of Pediatrics, Jacobi Medical Center, Bronx, New York 10461

Abstract

M. 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 *M. 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 lysA 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 IFN γ , and Gag-specific CD4⁺ T cells producing IFN γ within 3 weeks after immunization in adult mice; in addition, IFN γ 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

© 2009 Elsevier Ltd. All rights reserved.

*Corresponding author. Mailing Address: The Lewis M. Fraad Department of Pediatrics, Jacobi Medical Center, Bronx, NY 10461. Phone: (718) 918 4026, fennelly@aeom.yu.edu, Fax: (718) 918 4699.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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.

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 breastmilk 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 $\Delta lysA \Delta panCD$ Mtb and $\Delta RD1 \Delta panCD$ 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 $\Delta lysA \Delta secA2$ AMtb strain expressing HIV Env rapidly generated high-frequency Env-specific CD8⁺ T cells among

splenocytes, and significant Env-specific CD8⁺ T cell IFN γ and Mtb-specific CD4⁺ T cell IFN γ responses. In further studies using a $\Delta lysA \Delta secA2$ AMtb strain expressing an SIV Gag antigen, we demonstrated enhanced responses of Gag-specific CD8⁺ T cells producing perforin and IFN γ , as well as Gag-specific CD4⁺ T cells producing IFN γ and Mtb-specific CD8⁺ T cells producing perforin. These data suggest that $\Delta lysA \Delta secA2$ 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 *E. 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 (*oriM*) and the *E. coli* origin of replication (*oriE*), has been previously described [30] (Table 1). *E. coli* DH5 α 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 μ g/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 PstI and two ApaI sites, respectively in order to facilitate cloning), the RGPGRAFTI 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 19kDa 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 19kDa signal sequence upstream of *mgagp18* that was ligated in to pJH222 at the corresponding NheI sites to generate pJH222-*mgagp18* (designated pGAG).

Individual clones of mycobacteria were grown in Middlebrook 7H9 broth (Becton Dickinson, Franklin Lakes, NJ) at 37°C. All broth and agar media contained 10% oleic acid-albumin dextrose-catalase (OADC) (Becton Dickinson Microbiological Systems, Sparks MD), 0.05% Tween 80 (Fisher Scientific, Fair Lawn, NJ). The auxotrophic strain 5226 was supplemented with lysine (200 μ g/ml), 5222 with hygromycin (50 μ g/ml) and lysine, 6020 with both lysine and pantothenate (48 μ g/ml) (Sigma Chemical Co., St. Louis, MO), and 6030 with pantothenate. pENV or pGAG was electroporated into competent mycobacterial cells as previously described [32,33]. Strains 5222 and 5226 both contain an identical full-length *secA2* deletion plus a *lysA* deletion, that contains an inserted hygromycin resistance gene cassette, and is larger in the former strain (Table 1) [34]. Control recombinant mycobacterial strains were transformed with pJH222 without a gene insert. AMtb and BCG transformants were selected on Middlebrook 7H10 (Becton Dickinson, Sparks, MD) or Middlebrook 7H11 agar containing 20 μ g/ml of kanamycin (Sigma Chemical Co., St. Louis, MO). To prepare strains for mouse immunization, single colonies were grown in 7H9 broth containing kanamycin (20 μ g/ml) to mid log phase [OD₆₀₀ 0.6–0.8, that corresponds to an approximate concentration of 10⁸ CFU (colony forming units)/ml], washed twice with PBS containing

0.05% Tween 80, and then resuspended to the desired concentration in PBS. Cell counts were verified by plating serial dilutions of the inocula on Middlebrook 7H10 agar plates.

To confirm expression of recombinant HIV-1 Env or SIV Gag in mycobacteria, strains were grown in 7H9 media with shaking at 75 rpm to mid log phase. A 5 ml aliquot of each culture was harvested, washed twice with phosphate buffered saline (PBS) plus 0.05% Tween 80, resuspended in 200 µl of 2x protease inhibitor buffer (Roche Diagnostics, Germany) along with 200 µl of 0.1 mm silicon beads (BioSpec Products, Inc), subjected to FASTprep (Thermo Electron Corporation, MA) until grossly clear, and then incubated at 95° C for 45 min to kill viable mycobacteria. A 15 µl aliquot of each cell lysate was run on 12% (for BCG, 6020 and 6030 transformants) or 10–20% (for 5226 and 5222 transformants) gradient SDS-PAGE (Bio-Rad Laboratories, CA), electroblotted onto a nitrocellulose membrane, immunolabeled with an anti-HA monoclonal antibody (MAb) (clone 3F10) or V5 antibody-HRP, and then developed with a chemiluminescence detection kit according to the manufacturer's protocol (Perkin Elmer, MA). The CFU was estimated based on the optical density where an OD of 1 corresponds to 3×10^8 CFU. Relative levels of recombinant gp120 expression in mycobacterial lysates were estimated using ImageJ software, version 1.34n (Wayne Rasband; <http://rsb.info.nih.gov/ij/>).

2.2. Animals and Immunization

Four to six-week-old (adult) female BALB/c mice (National Cancer Institute) were housed and cared for according to the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine (AECOM). Four-to-five-day old (neonatal) male and female mice were bred at AECOM from BALB/c breeding pairs obtained from the National Cancer Institute and were housed under specific pathogen-free conditions.

Adult BALB/c mice were inoculated with 10^6 or 10^8 CFU of each strain (in 0.2ml PBS) via either the intraperitoneal or the subcutaneous route. Neonatal mice were inoculated with 10^4 CFU of each strain via the intraperitoneal route (10^2 in 50 µl PBS on either side at the peritoneum) using a 30 gauge needle. The various immunization schedules are represented in Fig 2.

2.3. Tetramer staining

To determine the frequency of gp120-specific tetrameric responses in peripheral blood mononuclear cells (PBMCs) or splenocytes, blood was obtained from the retro-orbital plexus and spleens were harvested at weekly intervals starting 1 week after inoculation for up to six weeks (Fig. 2, Groups A–C). H-2D^d tetrameric complexes folded with the P18 peptide (RGPGRAFVTI) [35], a sequence found in the V3 loop of HIV-1 HXBc2 envelope protein, and which was encoded in the native *env* sequence in plasmid pENV and synthetically fused downstream of the *gag* sequence in pGAG, were prepared as described previously [36]. Fresh blood samples (200 µl from each mouse) or splenocyte suspensions (recovered after passage through 70-µm nylon cell strainer) were recovered from individual mice, resuspended in RPMI-1640 (Gibco BRL; Invitrogen), treated with RBC lysis buffer, diluted in 3 ml RPMI medium with 40 U/ml heparin, and then layered over Ficoll-Hypaque (Lympholyte-M) before centrifugation at 800rpm for 20 min at 20°C. The mononuclear cell layer was carefully transferred to a fresh tube, diluted with 10 ml of PBS (GIBCO), pelleted, washed in 1 ml PBS with 2% fetal bovine serum (FBS), (Invitrogen, CA), and then resuspended in 200 µl PBS with 2% FBS. The cells were stained with P18-tetramer phycoerythrin, vortexed briefly, incubated at 20°C for 20 min, and then incubated with allophycocyanin conjugated with anti-CD8α MAb (Ly-2) (BD Pharmingen, CA) for 20 min at 20°C. To control for nonspecific fluorescence, an aliquot of each PBMC or splenocyte sample was incubated without monoclonal antibody, with APC anti-CD8 alone, and with phycoerythrin anti-CD4 alone. The cells were washed with 5

ml PBS plus 2% FBS, resuspended in 2% formaldehyde in PBS, incubated at room temperature for 1 hour, vortexed, sorted by BD with a FACSCalibur cytometer and then analyzed using cell quest software (Becton Dickinson, CA). A minimum of 10^6 cells were analyzed for each sample.

2.4. Intracellular cytokine staining

To determine the frequency of $CD3^+ CD4^+$ or $CD3^+ CD8^+$ T cells producing $IFN\gamma$, perforin and/or granzyme a/b against Gag, Env, BCG or Mtb antigens, splenocytes were harvested from individual mice 1 and 3 weeks after immunization (Fig. 2, Groups D and E) and were resuspended in complete RPMI 1640 media supplemented with 10% FBS, 25mM HEPES, penicillin and streptomycin (1%), essential (1%) and non-essential amino acids. Lymphocyte suspensions were stimulated with anti-CD28 mAb (2 μ g/ml) plus either a pool of 15-mer SIVmac239 Gag peptides (4 μ g/ml of each peptide; AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Germantown, MD; cat. #6204), P18 peptide (4 μ g/ml), TB10.3/4:20–28 peptide (an Mtb-derived H-2Kd restricted sequence, GYAGTLQSL, that is present in both Mtb and BCG), [37] (Sigma Aldrich) (4 μ g/ml), mc²5226 lysates (20 μ g/ml), BCG lysates (20 μ g/ml), granzyme a/b or media alone. Peptides were dissolved in dimethyl sulfoxide at 5mg/ml. $IFN\gamma$ production, perforin content and or granzyme a/b content in $CD4^+$ and $CD8^+$ T cells were detected by combined surface and intracellular staining. In brief, the cells were incubated at 37° C with 10% CO₂ for 2 hours and then treated with brefeldin-A (BFA) (Sigma Chemical Co., St. Louis, MO) at a final concentration of 10 μ g/ml and monensin A (2 μ M) (eBioscience). After another 4 hr incubation at 37° C, the cells were washed with PBS and treated with Live/Dead staining with blue fluorescent reactive dye (Invitrogen molecular probes, Oregon) incubated at 4° C, washed with PBS followed by cell surface staining with FITC anti-CD3, Pacific blue anti-CD8, APC-Cy7 anti-CD4 (eBioscience, San Diego), for 15 min on ice protected from light. The cells were then washed with PBS buffer, resuspended and treated with freshly prepared Fixation/Permeabilization solution (eBioscience) and incubated overnight at 4° C in dark followed by washing with permeabilization buffer (eBioscience). The cells were then blocked with anti-CD16/32 monoclonal antibody (eBioscience) in permeabilization buffer for 15min at 4° C and stained with PE-Cy 7 anti- $IFN\gamma$ (eBioscience) or APC anti-perforin (eBioscience) for 30 min at 4° C and washed with PBS followed by fixation with 2% paraformaldehyde for 1 hour. Multicolor flow analysis was performed using the BD LSR II and the Flowjo software (Tree Star).

2.5 Statistical analysis

Statistical tests were performed using Student's *t* test or Mann-Whitney analysis (Minitab, PA). *P* values of < 0.05 were considered significant.

3. Results

3.1. Expressing full-length HIV-1 Env or SIV Gag in recombinant AMtb

It has been shown previously that full-length heterologous viral proteins can be expressed in recombinant BCG and *M. smegmatis*, and that immunization with these constructs in animal models can elicit T-cell responses against a variety of viral pathogens including measles, [21,22] HIV-1 [24,30,38] and simian immunodeficiency virus (SIV) [39,40]. However expression of recombinant viral proteins has not to our knowledge been demonstrated previously in attenuated derivatives of Mtb. Following transformation with pENV, we detected expression of HIV gp120 protein in lysates of attenuated Mtb strains 6020, 6030 and 5226 and in BCG by Western immunoblotting (Fig. 1). Of note, the level of expression of HIV-1 Env in rBCG was estimated to be approximately eightfold higher than in the rAMtb strains by ImageJ version 1.40g analysis. Also, a higher proportion of break-down products was detected

in rAMtb compared to BCG. Gp120 or SIVmac239 Gag expression in strain 5222 were also confirmed by Western immunoblotting (data not shown).

3.2. Recombinant AMtb strain 6020(pEnv) engenders low-level Env-specific CD8⁺ T-cell responses in adult mice

To determine the feasibility of immunization with a live-attenuated Mtb, we compared the frequencies of gp120-specific tetrameric responses after immunization with either AMtb strains 6020(pENV) or 6030(pENV) or BCG(pENV) (Fig. 3). In a preliminary study, no gp120-specific responses above background levels were detected after immunization with 10⁶ CFU of these strains. In a separate experiment, after a higher dose (10⁸ CFU) inoculum, the frequency of P18 tetramer staining among CD8⁺ T cells in PBMCs recovered 1 week after immunization from groups of mice immunized with 6020(pENV) and BCG(pENV) (P=0.0003 and P=0.01 respectively), but not with 6030(pENV), (Fig. 3) was significantly higher than background frequencies detected among PBMCs from control-immunized groups.

3.3. Recombinant AMtb strain 5226(pENV) elicits high-frequency MHC class 1 restricted CD8⁺ T cell responses in adult and neonatal mice

The ability of BCG and other attenuated mycobacterial strains to persist within endosomes of infected macrophages and dendritic cells may restrict their ability to present antigens via the MHC class I pathway. Apoptotic macrophages infected with phagosome-enclosed pathogens appear to provide antigen to dendritic cells for cross-priming of CD8⁺ T cells via a detour pathway by which antigens derived from apoptotic vesicles act as cross-priming vehicles [41,42]. In order to engage this detour pathway, we used the AMtb strain 5226 in which the lysine auxotrophy was combined with a deletion of the *secA2* gene that is required for inhibition of host cell apoptosis by *M. tuberculosis* [43]. We compared the frequencies of gp120-specific tetrameric responses after immunization in adult and neonatal mice with rAMtb strain 5226 to the frequencies observed following immunization with either BCG or 6020 expressing HIV-1 Env in which the mechanisms for blocking apoptosis are intact. Remarkably, in adult mice inoculated with 5226(pEnv) the magnitude of the P18 tetrameric responses among splenocytes 1 week after immunization were sixfold higher than in mice inoculated with recombinant BCG (pEnv) (P<0.005) and twenty fourfold higher than in mice after 6020(pENV) inoculation (Fig. 4). In neonatal mice inoculated with 5226(pENV) at a 100-fold lower inoculum (10⁴), the magnitude of the P18 tetrameric responses among splenocytes 1, 2 (Fig. 5) and 3 (data not shown) weeks after immunization were significantly higher than in mice inoculated with BCG (pEnv) (P<0.05). No responses above background were observed in mice immunized with strain 3026 (Δ lysA H37Rv) expressing Env (data not shown).

3.4. Recombinant AMtb strain 5222(pENV) and 5222(pGAG) elicit CD4⁺ and CD8⁺ T-cell IFN γ and perforin responses against HIV, SIV and/or mycobacterial antigens in neonatal and adult mice

To determine whether live-attenuated Mtb generates mycobacteria-, HIV ENV- and SIV Gag-specific functional CD4⁺ and CD8⁺ T cell responses we evaluated antigen-specific CD4⁺ and CD8⁺ T cells for their functional maturation by assessing their expression of IFN γ , perforin and/or granzyme a/b using surface and/or intracellular staining with monoclonal antibodies and flow cytometric analysis. In adult mice, there were no antigen-specific responses detected 1 week after 5222(pGAG) immunization. However, 3 weeks after immunization, IFN γ and perforin producing CD8⁺ T cells specific for mycobacterial proteins (BCG lysate or TB10.3/4) were significantly elevated. In addition, Gag-specific CD8⁺ T cells producing perforin but not IFN γ were detected (Fig. 6A & 6B). Among CD4⁺ T cells significant levels of Gag-specific, but not mycobacteria-specific cells producing IFN γ were detected in splenocytes 3 weeks after immunization with 5222(pGAG) (Fig. 7A). In contrast, no mycobacterial-specific CD8⁺ (Fig.

6C & 6D) or CD4⁺ T cells producing IFN γ or perforin were detected after BCG immunization (Fig. 7B).

In neonatal mice immunized with 5222(pENV), significant levels of ENV P18 peptide-specific (but not mycobacterial specific) CD8⁺ T cells producing IFN γ were detected in the spleen one week after immunization (Fig. 8A). In contrast, no increase in P18-specific CD4⁺ T cells were detected, although IFN γ producing CD4⁺ T cells specific for mycobacterial antigens were augmented (Fig. 8B). No Mtb lysate-, TB 10.3/4- or P18-specific CD8⁺ T cells producing perforin were detected (Fig. 8C). For all experiments, P18 tetrameric responses among splenocyte CD8⁺ T cells 1 week after 5222(pGAG) or 5222(pENV) immunization were detected at frequencies of > 0.5% above levels of non-specific tetramer staining of splenocytes from groups of control immunized mice (data not shown).

4. Discussion

Safe and effective strategies to vaccinate against HIV and TB at the earliest possible time after birth to prevent breast milk HIV transmission and childhood tuberculosis are urgently needed. We studied the immunogenicity of attenuated rAMtb vectors for HIV, SIV and Mtb-specific T cell responses. We observed that with the highly attenuated 6020 (Δ lysA Δ panCD double auxotroph) or 6030 (Δ RD1 Δ panCD) strains expressing HIV-1 Env antigen, Env-specific responses were either undetectable or observed at low-frequencies and only after a high-dose inoculum. We postulated that the poor immunogenicity of these attenuated Mtb strains was due to the fact that Mtb complex bacteria including the natural mammalian pathogens Mtb and *M. bovis* (and its derivated, BCG) have evolved strategies to evade cell-mediated immune responses. These organisms persist in phagosomes of infected macrophages, prevent phagosomal maturation [44,45], and trigger apoptosis only weakly. Thus, they present antigen to the MHC class I pathway inefficiently. Taken together, these findings suggest that attenuated Mtb vectors could be made more effective by modifications that cause them to induce stronger apoptosis of infected cells.

In the present study, we observed that immunization with rAMtb strains 5226(pENV), 5222 (pENV) or 5222(pGAG) which contain a deletion in *secA2* along with the strongly attenuating Δ lysA mutation generate antigen-specific tetrameric responses by day seven after immunization in both adult and neonatal mice at frequencies that were dramatically higher than in mice vaccinated with either BCG or Δ lysA Δ panCD H37Rv expressing the same antigens, as well as functional CD4⁺ and CD8⁺ T cells producing IFN γ or perforin against both HIV or SIV and Mtb antigens. This is the first reported observation of heterologous vaccination with a rAMtb strain that is highly attenuated for virulence and persistence. The deletion of *lysA* markedly attenuates for virulence in the majority of SCID mice [34] and the deletion of *secA2* attenuates Mtb for virulence in immunocompetent and SCID mice only moderately relative to the wild type parental strain [46]. However, in order to minimize the potential that a Δ lysA Δ secA2 rAMtb strain would revert to virulence, a second attenuated deletion (for example Δ panCD [27]), would be required.

There are several advantages of a Δ lysA Δ secA2 H37Rv-based immunogen against HIV-1 and Mtb over candidate BCG-based immunogens including superior safety, ease of manipulation to obtain a pro-apoptotic phenotype, immunogenicity and antigenic relevance to Mtb. Safety in SCID mice is considered a reliable first-screen for safety in humans with immunosuppression. BCG has been observed by other workers and us to be consistently lethal for 100% of SCID mice. In contrast, a high proportion (85%) of SCID mice infected Δ lysA Δ secA2 Mtb survived for up to 642 days. (W.R. Jacobs, Jr. and M.H. Larsen, unpublished data). Although an auxotrophic Δ lysA BCG-based vaccine vector has been observed to be avirulent in SCID mice [34], single-dose recombinant Δ lysA BCG vaccination fails to elicit T-cell

responses against heterologous antigens in immunocompetent mice [24]. Of note, the deletion of *secA2* in BCG does not enhance its ability to induce apoptosis or elicit antigen-specific CD8⁺ T-cell responses (S.A. Porcelli and W.R. Jacobs, Jr., unpublished data). Also, 5222, 5226 and other *Mtb*-based strains contain the *RD1* region (deleted during attenuation of BCG) that encodes several antigens (including ESAT-6 and CFP-10) that appear to be targets for protective T cells.

Breast milk HIV exposure accounts for approximately 15–20% all cases of mother-to-child HIV transmission, resulting in an estimated 200,000 new infections in infants each year [3]. Neonatal immunization may be the best approach to prevent infection or reduce the severity of HIV-related disease in these infants. Moreover, infants with HIV infection are at high risk for exposure, infection and rapidly progressive disease from tuberculosis. Because of the high rate of disseminated BCG infection after neonatal BCG immunization in infants with HIV-1, a safer alternative is urgently needed. We demonstrate here that a recombinant live attenuated *Mtb* vaccine rapidly elicited high-frequency HIV-1 specific CD8⁺ T-cell responses after only a single dose in 4 or 5-day-old neonatal mice as well as HIV-specific functional CD4⁺ and *Mtb*-specific CD8⁺ T cells producing IFN γ . These responses may be relevant to protection against HIV and *Mtb* in humans. IFN γ plays a critical role in T-cell immunity against both HIV and *Mtb* via potent activation of macrophages [47,48] and CD8⁺ T cells producing perforin are important to control of both pathogens [49,50]. Others have observed that T cell responses against Gag alone may be sufficient to protect non-human primates against SIV infection [51] and to prevent progression to AIDS in humans after HIV-1 infection [52]. It is noteworthy that increases in the frequencies of CD4⁺ and CD8⁺ T cells producing IFN γ in response to *Mtb* antigens were observed three weeks after AMtb, but not after BCG immunization in adult mice. In BCG immunized animals higher frequencies of CD8⁺ and CD4⁺ T cells producing IFN γ spontaneously were detected among splenocytes, but these were not significantly enhanced by restimulation with mycobacterial antigens. This may reflect a non-specific immunostimulatory effect of BCG that, unlike 5222, continues to replicate after inoculation into mice, or may reflect a failure to detect specific responses with our assay. As suggested by data from human infants wherein BCG-specific T cells that produce IFN γ can be observed 10 weeks after neonatal BCG vaccination, [53] these responses may appear later.

The biological relevance of the rapidly induced HIV, and *Mtb*-specific CD4⁺ and CD8⁺ T cell responses that we observed following recombinant AMtb immunization in adult and neonatal mice will require further evaluation to determine the ability of these responses to protect against infection. Given the urgent need for safe and effective HIV and TB vaccines for infants in Africa, and the demonstrated ability to produce and administer live mycobacterial vaccines on a large scale, rAMtb-based HIV vaccines are an appealing platform for a human neonatal vaccine in lieu of BCG to prevent mother-to-child breast milk transmission of HIV. Vaccines designed on this platform could potentially establish immune memory for both HIV and TB that could be boosted with heterologous immunogens consisting of HIV or TB subunits [59, 60]. Ideally, such a rAMtb will need to be fully attenuated for virulence, while providing increased levels of immunogenicity compared to BCG.

Acknowledgments

We thank Ms. Karoline Biermann for providing the mc²5226 and mc²5222 knockout strains, Dr. Lydia Tesfa for assistance with FACS analysis, Dr. Jorge Sansarrry for guidance on statistical analysis and Dr Norman Letvin for providing the p18 tetramer. This work was supported by National Institutes of Health grants P01 AI052816 (P01 to GJF and WRJ), NDK 1R01DE019064-01 (R01 to GJF and MHL), AI 063537 (P01 to SAP and WRJ), the Center for AIDS Research at the Albert Einstein College of Medicine (AI51519) and the Einstein Cancer Center Flow Cytometry Core Facility (CA13330).

REFERENCES

1. UNAIDS, W.H.O. 2007 AIDS epidemic update. 2007.
2. De Cock KM, Fowler MG, Mercier E, de Vincenzi I, Saba J, Hoff E, et al. Prevention of mother-to-child HIV transmission in resource-poor countries: translating research into policy and practice. *JAMA* 2000 Mar 1;283(9):1175–1182. [PubMed: 10703780]
3. UNAIDS. AIDS epidemic update-special report on HIV/AIDS Geneva. Switzerland: 2006.
4. Adkins B, Leclerc C, Marshall-Clarke S. Neonatal adaptive immunity comes of age. *Nat Rev Immunol* 2004 Jul;4(7):553–564. [PubMed: 15229474]
5. Siegrist CA, Lambert PH. Maternal immunity and infant responses to immunization: factors influencing infant responses. *Dev Biol Stand* 1998;95:133–139. [PubMed: 9855423]
6. Siegrist CA. Neonatal and early life vaccinology. *Vaccine* 2001 May 14;19(25–26):3331–3346. [PubMed: 11348697]
7. Franchini M, Abril C, Schwerdel C, Ruedl C, Ackermann M, Suter M. Protective T-cell-based immunity induced in neonatal mice by a single replicative cycle of herpes simplex virus. *J Virol* 2001 Jan;75(1):83–89. [PubMed: 11119576]
8. Borkowsky W, Wara D, Fenton T, McNamara J, Kang M, Mofenson L, et al. Lymphoproliferative responses to recombinant HIV-1 envelope antigens in neonates and infants receiving gp120 vaccines. AIDS Clinical Trial Group 230 Collaborators. *J Infect Dis* 2000 Mar;181(3):890–896. [PubMed: 10720509]
9. Johnson DC, McFarland EJ, Muresan P, Fenton T, McNamara J, Read JS, et al. Safety and immunogenicity of an HIV-1 recombinant canarypox vaccine in newborns and infants of HIV-1-infected women. *J Infect Dis* 2005 Dec 15;192(12):2129–2133. [PubMed: 16288378]
10. McFarland EJ, Johnson DC, Muresan P, Fenton T, Tomaras GD, McNamara J, et al. HIV-1 vaccine induced immune responses in newborns of HIV-1 infected mothers. *AIDS* 2006 Jul 13;20(11):1481–1489. [PubMed: 16847402]
11. Cunningham CK, Wara DW, Kang M, Fenton T, Hawkins E, McNamara J, et al. Safety of 2 recombinant human immunodeficiency virus type 1 (HIV-1) envelope vaccines in neonates born to HIV-1-infected women. *Clin Infect Dis* 2001 Mar 1;32(5):801–807. [PubMed: 11229849]
12. McFarland EJ, Borkowsky W, Fenton T, Wara D, McNamara J, Samson P, et al. Human immunodeficiency virus type 1 (HIV-1) gp120-specific antibodies in neonates receiving an HIV-1 recombinant gp120 vaccine. *J Infect Dis* 2001 Nov 15;184(10):1331–1335. [PubMed: 11679925]
13. Rowland-Jones S, Sutton J, Ariyoshi K, Dong T, Gotch F, McAdam S, et al. HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. *Nat Med* 1995 Jan;1(1):59–64. [PubMed: 7584954]
14. Liu E, Law HK, Lau YL. BCG promotes cord blood monocyte-derived dendritic cell maturation with nuclear Rel-B up-regulation and cytosolic I kappa B alpha and beta degradation. *Pediatr Res* 2003 Jul;54(1):105–112. [PubMed: 12672905]
15. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 2006 Jul 10;203(7):1701–1711. [PubMed: 16818678]
16. Marchant A, Goetghebuer T, Ota MO, Wolfe I, Ceesay SJ, De Groote D, et al. Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination. *J Immunol* 1999 Aug 15;163(4):2249–2255. [PubMed: 10438968]
17. Murray RA, Mansoor N, Harbacheuski R, Soler J, Davids V, Soares A, et al. Bacillus Calmette Guerin vaccination of human newborns induces a specific, functional CD8+ T cell response. *J Immunol* 2006 Oct 15;177(8):5647–5651. [PubMed: 17015753]
18. Vekemans J, Lienhardt C, Sillah JS, Wheeler JG, Lahai GP, Doherty MT, et al. Tuberculosis contacts but not patients have higher gamma interferon responses to ESAT-6 than do community controls in The Gambia. *Infect Immun* 2001 Oct;69(10):6554–6557. [PubMed: 11553606]
19. Trunz BB, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* 2006 Apr 8;367(9517):1173–1180. [PubMed: 16616560]

20. Nascimento IP, Dias WO, Quintilio W, Christ AP, Moraes JF, Vancetto MD, et al. Neonatal immunization with a single dose of recombinant BCG expressing subunit S1 from pertussis toxin induces complete protection against *Bordetella pertussis* intracerebral challenge. *Microbes Infect* 2008 Feb;10(2):198–202. [PubMed: 18248757]
21. Zhu YD, Fennelly G, Miller C, Tarara R, Saxe I, Bloom B, et al. Recombinant bacille Calmette-Guerin expressing the measles virus nucleoprotein protects infant rhesus macaques from measles virus pneumonia. *J Infect Dis* 1997 Dec;176(6):1445–1453. [PubMed: 9395353]
22. Fennelly GJ, Flynn JL, ter Meulen V, Liebert UG, Bloom BR. Recombinant bacilli Calmette-Guerin priming against measles. *J Infect Dis* 1995 Sep;172(3):698–705. [PubMed: 7658061]
23. Bueno SM, Gonzalez PA, Cautivo KM, Mora JE, Leiva ED, Tobar HE, et al. Protective T cell immunity against respiratory syncytial virus is efficiently induced by recombinant BCG. *Proc Natl Acad Sci U S A* 2008 Dec 30;105(52):20822–20827. [PubMed: 19075247]
24. Im EJ, Saubi N, Virgili G, Sander C, Teoh D, Gatell JM, et al. Vaccine platform for prevention of tuberculosis and mother-to-child transmission of human immunodeficiency virus type 1 through breastfeeding. *J Virol* 2007 Sep;81(17):9408–9418. [PubMed: 17596303]
25. Ota MO, Vekemans J, Schlegel-Haueter SE, Fielding K, Sanneh M, Kidd M, et al. Influence of *Mycobacterium bovis* bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. *J Immunol* 2002 Jan 15;168(2):919–925. [PubMed: 11777990]
26. Hesselting AC, Marais BJ, Gie RP, Schaaf HS, Fine PE, Godfrey-Faussett P, et al. The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. *Vaccine* 2007 Jan 2;25(1):14–18. [PubMed: 16959383]
27. Sambandamurthy VK, Derrick SC, Jalapathy KV, Chen B, Russell RG, Morris SL, et al. Long-term protection against tuberculosis following vaccination with a severely attenuated double lysine and pantothenate auxotroph of *Mycobacterium tuberculosis*. *Infect Immun* 2005 Feb;73(2):1196–1203. [PubMed: 15664964]
28. Sambandamurthy VK, Jacobs WR Jr. Live attenuated mutants of *Mycobacterium tuberculosis* as candidate vaccines against tuberculosis. *Microbes Infect* 2005 May;7(5–6):955–961. [PubMed: 15914065]
29. Sampson SL, Dascher CC, Sambandamurthy VK, Russell RG, Jacobs WR Jr, Bloom BR, et al. Protection elicited by a double leucine and pantothenate auxotroph of *Mycobacterium tuberculosis* in guinea pigs. *Infect Immun* 2004 May;72(5):3031–3037. [PubMed: 15102816]
30. Cayabyab MJ, Hovav AH, Hsu T, Krivulka GR, Lifton MA, Gorgone DA, et al. Generation of CD8 + T-cell responses by a recombinant nonpathogenic *Mycobacterium smegmatis* vaccine vector expressing human immunodeficiency virus type 1 Env. *J Virol* 2006 Feb;80(4):1645–1652. [PubMed: 16439521]
31. Southern JA, Young DF, Heaney F, Baumgartner WK, Randall RE. Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics. *J Gen Virol* 1991 Jul;72(Pt 7):1551–1557. [PubMed: 1713260]
32. Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR Jr. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* 1990 Nov;4(11):1911–1919. [PubMed: 2082148]
33. Wards BJ, Collins DM. Electroporation at elevated temperatures substantially improves transformation efficiency of slow-growing mycobacteria. *FEMS Microbiol Lett* 1996 Nov 15;145(1):101–105. [PubMed: 8931333]
34. Pavelka MS Jr, Chen B, Kelley CL, Collins FM, Jacobs FM, Jacobs Jr WR Jr. Vaccine efficacy of a lysine auxotroph of *Mycobacterium tuberculosis*. *Infect Immun* 2003 Jul;71(7):4190–4192. [PubMed: 12819116]
35. Takahashi H, Nakagawa Y, Pendleton CD, Houghten RA, Yokomuro K, Germain RN, et al. Induction of broadly cross-reactive cytotoxic T cells recognizing an HIV-1 envelope determinant. *Science* 1992 Jan 17;255(5042):333–336. [PubMed: 1372448]
36. Staats HF, Bradney CP, Gwinn WM, Jackson SS, Sempowski GD, Liao HX, et al. Cytokine requirements for induction of systemic and mucosal CTL after nasal immunization. *J Immunol* 2001 Nov 1;167(9):5386–5394. [PubMed: 11673557]

37. Majlessi L, Rojas MJ, Brodin P, Leclerc C. CD8⁺-T-cell responses of Mycobacterium-infected mice to a newly identified major histocompatibility complex class I-restricted epitope shared by proteins of the ESAT-6 family. *Infect Immun* 2003 Dec;71(12):7173–7177. [PubMed: 14638811]
38. Honda M, Matsuo K, Nakasone T, Okamoto Y, Yoshizaki H, Kitamura K, 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. *Proc Natl Acad Sci U S A* 1995 Nov 7;92(23):10693–10697. [PubMed: 7479867]
39. Yasutomi Y, Reimann KA, Lord CI, Miller MD, Letvin NL. Simian immunodeficiency virus-specific CD8⁺ lymphocyte response in acutely infected rhesus monkeys. *J Virol* 1993 Mar;67(3):1707–1711. [PubMed: 8437240]
40. Kawahara M, Matsuo K, Honda M. Intradermal and oral immunization with recombinant Mycobacterium bovis BCG expressing the simian immunodeficiency virus Gag protein induces long-lasting, antigen-specific immune responses in guinea pigs. *Clin Immunol* 2006 Apr;119(1):67–78. [PubMed: 16386958]
41. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998 Mar 5;392(6671):86–89. [PubMed: 9510252]
42. Winau F, Kaufmann SH, Schaible UE. Apoptosis paves the detour path for CD8 T cell activation against intracellular bacteria. *Cell Microbiol* 2004 Jul;6(7):599–607. [PubMed: 15186397]
43. Hinchey J, Lee S, Jeon BY, Basaraba RJ, Venkataswamy MM, Chen B, et al. Enhanced priming of adaptive immunity by a proapoptotic mutant of Mycobacterium tuberculosis. *J Clin Invest* 2007 Aug;117(8):2279–2288. [PubMed: 17671656]
44. Via LE, Deretic D, Ulmer RJ, Hibler NS, Huber LA, Deretic V. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J Biol Chem* 1997 May 16;272(20):13326–13331. [PubMed: 9148954]
45. Via LE, Fratti RA, McFalone M, Pagan-Ramos E, Deretic D, Deretic V. Effects of cytokines on mycobacterial phagosome maturation. *J Cell Sci* 1998 Apr;111(Pt 7):897–905. [PubMed: 9490634]
46. Braunstein M, Espinosa BJ, Chan J, Belisle JT, Jacobs WR Jr. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of Mycobacterium tuberculosis. *Mol Microbiol* 2003 Apr;48(2):453–464. [PubMed: 12675804]
47. Sarol LC, Imai K, Asamitsu K, Tetsuka T, Barzaga NG, Okamoto T. Inhibitory effects of IFN-gamma on HIV-1 replication in latently infected cells. *Biochem Biophys Res Commun* 2002 Mar 8;291(4):890–896. [PubMed: 11866448]
48. Tascon RE, Stavropoulos E, Lukacs KV, Colston MJ. Protection against Mycobacterium tuberculosis infection by CD8⁺ T cells requires the production of gamma interferon. *Infect Immun* 1998 Feb;66(2):830–834. [PubMed: 9453650]
49. Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, et al. HIV-specific CD8⁺ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* 2002 Nov;3(11):1061–1068. [PubMed: 12368910]
50. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* 1998 Oct 2;282(5386):121–125. [PubMed: 9756476]
51. Liu J, O'Brien KL, Lynch DM, Simmons NL, La Porte A, Riggs AM, et al. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature* 2009 Jan 1;457(7225):87–91. [PubMed: 18997770]
52. Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, Moodley E, et al. CD8⁺ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med* 2007 Jan;13(1):46–53. [PubMed: 17173051]
53. Soares AP, Scriba TJ, Joseph S, Harbacheuski R, Murray RA, Gelderbloem SJ, et al. Bacillus Calmette-Guerin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. *J Immunol* 2008 Mar 1;180(5):3569–3577. [PubMed: 18292584]
54. Van Rompay KK, Abel K, Lawson JR, Singh RP, Schmidt KA, Evans T, et al. Attenuated poxvirus-based simian immunodeficiency virus (SIV) vaccines given in infancy partially protect infant and juvenile macaques against repeated oral challenge with virulent SIV. *J Acquir Immune Defic Syndr* 2005 Feb 1;38(2):124–134. [PubMed: 15671796]

55. Hawkrige T, Scriba TJ, Gelderbloem S, Smit E, Tameris M, Moyo S, et al. Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in healthy adults in South Africa. *J Infect Dis* 2008 Aug 15;198(4):544–552. [PubMed: 18582195]

FIG. 1. Expression of recombinant HIV-1 HXBc2 gp120 in rBCG and rAMtb strains

Each lane contains lysates of approximately 10^8 CFU of BCG, 6020, 6030 or 5226 harboring pEnv or pJH222, the corresponding control plasmid without the gp120 gene insert. Lysates were immunoblotted with HRP conjugated anti-HA antibodies and developed using chemiluminescence. The principal recombinant gp120 band migrates at approximately 55 kDa. Several lower molecular weight breakdown products, that are more prevalent in lysates of rAMtb than lysates of BCG, are also present.

FIG. 2. Figure summarizing the various vaccination schedules carried out in the study

FIG. 3. BCG-pEnv and rAMtb strain 6020-pEnv elicit Env-specific CD8⁺ T cells

Tetramer staining and flow cytometric analysis of splenocytes after intraperitoneal immunization of 4-to-6-week old BALB/C mice with approximately 10^8 of either rBCG, 6020 or 6030 harboring pEnv. As a negative control, groups of mice were inoculated with the same dose of rBCG or rAMtb harboring the control pJH222 plasmid. The mean ($\pm$ SEM) percent gp120 P18 tetramer-positive CD8⁺ T cells among splenocytes collected at the indicated time points is shown for each group of mice ($n = 6$ per group). The frequencies of CD8 and P18 tetramer staining responses were significantly higher after rBCG-pENV (at 1 and 2 weeks) and 6020-pEnv strain immunization (at 1 week) than after control rBCG and r6020 strain immunization ($P < 0.05$ [one asterisk]; $P < 0.005$ [two asterisks]).

FIG. 4. rAMtb 5226(pEnv) elicits Env-specific CD8⁺ T cells markedly more effectively than either rAMtb 6020-pEnv or rBCG-pEnv

Tetramer staining and flow cytometric analysis of splenocytes recovered 1 week after subcutaneous immunization of 4-to-6-week old BALB/C mice with approximately 10^6 of either 5226, 6020 or BCG harboring pEnv. As negative controls, groups of mice were inoculated with the same dose of rBCG or rAMtb harboring the control pJH222 plasmid. (A) Example of flow cytometric analysis from individual mice revealed that P18 tetramer-positive CD8⁺ T-cell responses were detected after immunization with 5226(pJH222), that were significantly higher than background frequencies detected from a corresponding control mouse, and that were dramatically higher than responses detected in a mouse after BCG(pENV)

immunization. Gating was exclusively on the total CD8⁺ T cell population. (B) The mean ($\pm$ SEM) percent gp120 P18 tetramer-positive CD8⁺ T cells among splenocytes collected 1 week after immunization is shown for each group of mice ($n = 5$ per group). The frequencies of CD8 and P18 tetramer staining responses were significantly higher after 5226-pENV than after either 6020-pEnv or BCG-pEnv immunization ($P < 0.05$ [one asterisk]; $P < 0.005$ [two asterisks]).

FIG. 5. rAMtb 5226(pEnv) elicits Env-specific CD8⁺ T cells markedly more effectively than rBCG-pEnv in neonatal mice

Tetramer staining and flow cytometric analysis of splenocytes after intraperitoneal immunization of 4-to 5 day old BALB/C mice with approximately 10^4 of either rAMtb strain 5226 or rBCG harboring pEnv. As negative controls, groups of mice were inoculated with the same dose of rBCG or rAMtb harboring the control plasmid, pJH222. The mean ($\pm$ SEM) percent gp120 P18 tetramer-positive CD8 T cells among splenocytes collected at the indicated time points is shown for each group of mice ($n = 5$ per group). The frequencies of P18 tetramer staining responses were significantly higher at 1 and 2 weeks after 5226(pENV) than after BCG(pENV) strain immunization. ($P < 0.05$ [one asterisks]; $P < 0.005$ [two asterisks])

FIG. 6. rAMtb 5222(pGAG) elicits functional mycobacteria-specific CD8⁺ T cells in adult mice
 Surface and intracellular staining of splenocytes after subcutaneous immunization of 4-to-6-week old BALB/C mice with approximately 10^6 of either rAMtb strain 5222 harboring pGAG (Fig. 6A & B) or BCG (Fig. 6C&D). The mean ($\pm$ SEM) percent of CD8 T+ cells among splenocytes collected at the indicated time points is shown for each group of mice ($n = 4-5$ per group). The frequencies of CD8+ T cells expressing IFN γ or perforin were significantly higher after stimulation with BCG lysates than after media only incubation ($P < 0.05$ [one asterisk]), as were the frequencies of CD8+ T cells expressing perforin after stimulation with BCG lysate, TB10.3/4- and Gag peptide 3 weeks after 5222(pGAG) immunization. ($P < 0.05$ [one asterisk]; $P < 0.005$ [two asterisks]). There was no significant differences in the frequency of CD8+ T cells producing IFN γ or perforin after BCG lysate or TB10.3/4 stimulation compared to incubation with media alone after either BCG immunization.

FIG. 7. rAMtb 5222(pGAG) elicits a Gag-specific CD4+ T cell IFN γ response in adult mice

Surface and intracellular staining of splenocytes was carried out after subcutaneous immunization of 4-to-6-week old BALB/C mice with approximately 10^6 of either rAMtb strain 5222 harboring pGAG (Fig. 7A) or BCG (Fig. 7B). The frequencies of CD4+ T cells expressing IFN γ were significantly higher after stimulation with a Gag peptide pool than after media only incubation ($P < 0.05$ [asterisk]). There was no significant difference in the frequency of CD4 + T cells secreting IFN γ after BCG stimulation compared to incubation with media alone after either 5222(pGAG) or BCG immunization.

FIG. 8. rAMtb 5222(pEnv) elicits Env- and Mtb-specific CD4+ and CD8+ T cell IFN γ responses in neonatal mice

Surface and intracellular staining of splenocytes for antigen-specific CD8+ T cells producing IFN γ (Fig 8A), granzyme a/b (Fig 8B) or CD4+ T cells producing IFN γ (Fig 8C) were carried out after intraperitoneal immunization of 4-to-5-day old BALB/C mice with approximately 10^4 of either rAMtb strain 5222 harboring pENV. The frequency of CD8+ and T cells producing IFN γ was significantly higher after stimulation with a Env P18 peptide than after media only incubation ($P < 0.05$ [one asterisk]) and the frequency of CD4+ T cells producing IFN γ was significantly higher after stimulation with Mtb lysates than after media only incubation ($P < 0.005$ [two asterisks]) There was no significant difference in the frequency of CD8+ T cells

producing granzyme a/b IFN γ after Mtb lysate or TB10.3/4 stimulation compared to incubation with media alone.

TABLE 1

Mycobacterial strains ^a

Strain	Abbreviation	Relevant Characteristics	Source or reference
<i>M. bovis</i> 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
<i>M. tuberculosis</i> H37Rv strains			
mc ² 6020	6020	$\Delta lys \Delta panCD$, 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	$\Delta RD1 \Delta panCD$, 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	$\Delta lys \Delta secA2$, 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	Δlys , 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	$\Delta lys \Delta secA2$, 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 **Abbreviations:** Hy^r, Hygromycin and superscript r Hygromycin resistance

A. Sambandamurthy et al., 2005

B. Sambandamurthy et al., 2006

C. Pavelka et al., 1999