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A Recombinant Attenuated *Mycobacterium tuberculosis* Vaccine Strain Is Safe in Immunosuppressed Simian Immunodeficiency Virus-Infected Infant Macaques

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Many resource-poor countries are faced with concurrent epidemics of AIDS and tuberculosis (TB) caused by human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis*, respectively. Dual infections with HIV and *M. tuberculosis* are especially severe in infants. There is, however, no effective HIV vaccine, and the only licensed TB vaccine, the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine, can cause disseminated mycobacterial disease in HIV-infected children. Thus, a pediatric vaccine to prevent HIV and *M. tuberculosis* infections is urgently needed. We hypothesized that a highly attenuated *M. tuberculosis* strain containing HIV antigens could be safely administered at birth and induce mucosal and systemic immune responses to protect against HIV and TB infections, and we rationalized that vaccine safety could be most rigorously assessed in immunocompromised hosts. Of three vaccine candidates tested, the recombinant attenuated *M. tuberculosis* strain mc^{2}6435 carrying a simian immunodeficiency virus (SIV) Gag expression plasmid and harboring attenuations of genes critical for replication (*panCD* and *leuCD*) and immune evasion (*secA2*), was found to be safe for oral or intradermal administration to non-SIV-infected and SIV-infected infant macaques. Safety was defined as the absence of clinical symptoms, a lack of histopathological changes indicative of *M. tuberculosis* infection, and a lack of mycobacterial dissemination. These data represent an important step in the development of novel TB vaccines and suggest that a combination recombinant attenuated *M. tuberculosis*-HIV vaccine could be a safe alternative to BCG for the pediatric population as a whole and, more importantly, for the extreme at-risk group of HIV-infected infants.

About one-third of the world's population is infected with *Mycobacterium tuberculosis* (44). Every year, 8 to 10 million new individuals become infected with *M. tuberculosis* and almost 1.5 million people die of tuberculosis (TB) (44). The recent development of multidrug-resistant and extensively multidrug-resistant strains of circulating *M. tuberculosis* further underscores the need for novel approaches to combat TB. The only licensed TB vaccine, bacillus Calmette Guérin (BCG), is a live attenuated vaccine derived from *Mycobacterium bovis*. It is the oldest and most widely used vaccine worldwide. Although the BCG vaccine can induce potent cellular immune responses in infants and protect against disseminated TB in children (22, 25, 41), the duration of protection is questionable since immunity wanes with time in many vaccinated individuals and the vaccine shows only variable protection in adults (8, 29, 36). In addition, BCG vaccination offers little to no protection against pulmonary TB, cannot eliminate latent *M. tuberculosis*, and is ineffective at preventing subsequent TB.

TB is the leading cause of death in human immunodeficiency virus (HIV)-infected individuals (43, 44). Given the large geographical overlap between *M. tuberculosis* and HIV infection, BCG vaccination at birth was at one time recommended for all infants, because infants with HIV-induced immune suppression have a higher risk than adults of contracting TB (45). Recently, however, it became apparent that the annual risk for disseminated BCG disease in untreated HIV-infected infants (~0.42%), associated with a 75% mortality rate (12–14), clearly outweighs the potential benefits of BCG vaccination in children with HIV (13). Therefore, the WHO now advises against BCG vaccination of any infant infected with HIV or at risk for HIV infection (46). As a result, the number of infants coinfected with HIV and TB in resource-poor countries is expected to remain the same or even rise.

Alternative methods to control TB in infants infected with HIV are urgently needed. In response to this challenge, we aim to develop a novel infant combination HIV-TB vaccine based upon a safe, orally (p.o.) administrable attenuated *M. tuberculosis* strain expressing HIV antigens. Although the rate of *in utero* and perinatal mother-to-child-transmission of HIV has been significantly reduced with the introduction of antiretroviral therapy (ART) to mother and/or child (43), breast milk transmission of HIV remains a serious problem. Ideally, a vaccine to prevent p.o. HIV acquisition by breast-feeding should be administered p.o. BCG-based vaccines are advantageous because they can be administered at birth, are effective when administered p.o., and rapidly generate long-lived T cell responses against dually administered mycobacterial and coexpressed nonmycobacterial antigens when administered to human infants (27).

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TABLE 1 Recombinant attenuated *M. tuberculosis* vaccine strains used in this study

Vaccine strain	Groups	Deletion(s) attenuating: Replication	Immune evasion	Reference(s)
mc^{2}6020 (Δ*lysA* Δ*panCD*)[a]	A, B	Δ*lysA* Δ*panCD* (low)[d]	None	19, 30-33, 42, 49
mc^{2}5157 (Δ*nuoG* Δ*panCD* Δ*RD1* pSIV Gag)[b]	C–F	Δ*panCD* Δ*RD1* (intermediate)	Δ*nuoG*	28, 30
mc^{2}6435 (Δ*leuCD* Δ*panCD* Δ*secA2* pSIV Gag)[c]	G–L	Δ*leuCD* Δ*panCD* (low)	Δ*secA2*	28

[a] Δ*lysA* Δ*panCD*, deletions of the *lysA* and the *panCD* loci.
[b] Δ*nuoG* Δ*panCD* Δ*RD1*, deletions of the *nuoG*, *panCD*, and *RD1* loci; pSIV GAG, insertion of a full-length SIVmac239 Gag insert.
[c] Δ*leuCD* Δ*panCD* Δ*secA2*, deletions of the *leuCD*, *panCD*, and *secA2* loci.
[d] Replication levels are shown in parentheses.

To address the safety concern associated with the current BCG vaccine, we hypothesized that a rationally attenuated strain of human-adapted *M. tuberculosis* might be a better vaccine platform than bovine-adapted *M. bovis* BCG. We developed auxotroph mutants of human *M. tuberculosis* strain H37Rv in which mycobacterial genes important for replication and persistence were deleted or modified to attenuate replication. In addition, in an attempt to increase immunogenicity, several genes important for the evasion of host immune responses were deleted. The construction of these attenuated *M. tuberculosis* strains, their safety, and their immunogenicity profiles in comparison to those of the licensed BCG vaccine in SCID mice have been reported previously (16, 19, 28, 30–32). Some of these TB vaccine candidates were also characterized in nonhuman primates as an important step toward potential human clinical trials. Vaccine safety, immunogenicity, and efficacy data obtained with nonhuman primates would be expected to be highly relevant to humans (19). Attenuated *M. tuberculosis* vaccine strains mc^{2}6020 and mc^{2}6030 were safe and well tolerated in adult cynomolgus macaques and did not cause TB but provided only partial protection against an intrabronchial *M. tuberculosis* challenge (19). On the basis of these data, we developed novel, replication-attenuated *M. tuberculosis* vaccine strains with increased immunogenicity.

Because of obvious ethical concerns, pediatric HIV-TB vaccine safety assessments and challenge studies of efficacy cannot be performed with HIV-infected human infants. To account for the infant's relatively inexperienced and still developing immune system early after birth, we therefore chose to test vaccine safety in infant macaques that show immune system ontogeny after birth similar to that of human infants. In a first step toward the generation of a pediatric combination HIV-TB vaccine, we constructed attenuated *M. tuberculosis* strains that express the simian immunodeficiency virus (SIV) *gag* gene. The safety profiles of three distinct recombinant attenuated *M. tuberculosis*-SIV vaccine candidates with different degrees of attenuation of replication and/or immunogenicity (Table 1) were initially tested in healthy, non-SIV-infected infant rhesus macaques. None of these vaccine candidates induced clinical symptoms of TB. The vaccine strain (mc^{2}5157) that was attenuated predominantly for immune evasion and less for replication caused *M. tuberculosis* dissemination to multiple tissues and was therefore excluded as a potential pediatric vaccine. The safety of the two other vaccine candidates, recombinant attenuated *M. tuberculosis*-SIV mc^{2}6020 and mc^{2}6435, was then evaluated under even more stringent conditions with immunosuppressed SIV-infected infant macaques, analogous to HIV-infected human infants. The infant macaque model of SIV infection is a well-established animal model of pediatric HIV infection and is suitable for the testing of the safety and efficacy of intervention strategies for a wide range of infectious diseases (1, 3, 4, 24, 38). Vaccination of SIV-infected infant macaques with these two recombinant attenuated *M. tuberculosis*-SIV strains did not cause TB-like lung pathology. Importantly, in animals vaccinated with mc^{2}6435, local or systemic dissemination of mycobacteria did not occur, and live mycobacteria could not be recovered from any tissues under optimal culture conditions. The data represent an important step in the clinical testing of these novel live attenuated *M. tuberculosis* vaccine candidates and suggest that a combination recombinant attenuated *M. tuberculosis*-HIV vaccine could be a safe alternative to BCG for the pediatric population as a whole but more importantly for the extreme at-risk group of infants infected with HIV.

MATERIALS AND METHODS

Animals. Newborn rhesus macaques (*Macaca mulatta*) from the SIV-negative and type D retrovirus-free colony at the California National Primate Research Center (CNPRC; Davis, CA) were hand reared in a nursery. Animals were housed according to the *Guide for the Care and Use of Laboratory Animals* and the standards outlined by the American Association for Accreditation of Laboratory Animal Care; all animal protocols were reviewed and approved by the University of California at Davis Institutional Animal Care and Use Committee prior to study initiation. Animals were randomly assigned to the various study groups and were between 3 and 7 days of age at the first immunization (see Table 2). For vaccinations and blood collections, animals were immobilized by intramuscular injection of ketamine-HCl (Parke-Davis, Morris Plains, NJ) at 10 mg/kg of body weight. Trained veterinary staff monitored the animals daily for clinical symptoms associated with TB (e.g., breathing difficulty, coughing, increased mucus secretion, lethargic behavior, weight loss) and/or SIV infection.

Vaccine strains and immunization regimens. The three recombinant attenuated *M. tuberculosis* vaccine strains tested in the current study were rationally attenuated from wild-type *M. tuberculosis* strain H37Rv. *M. tuberculosis* H37Rv was modified through several deletions in genes supporting replication to increase vaccine safety and in genes important for mycobacterial immune evasion to enhance immunogenicity (5). As outlined in Table 1, strain mc^{2}6020 was predominantly attenuated for replication with deletions in the *lysA* and *panCD* loci (Δ*lysA* Δ*panCD*), no loci important for immune evasion were deleted. We demonstrated previously that deletion of *RD1* from *M. tuberculosis* H37Rv results in an attenuated strain with intermediate replication relative that of the parental strain (30). Strain mc^{2}5157 was designed primarily to test for enhanced immunogenicity (Δ*nuoG*) with attenuation for intermediate replication (Δ*panCD* Δ*RD1*) or no replication (Δ*leuCD* Δ*panCD* for mc^{2}6435). The construction of the recombinant attenuated *M. tuberculosis* vaccine strains has been previously described (19, 28, 31–33, 42, 49). In these prior studies, the deletions introduced were stable and reversions were not observed (19, 28, 31–33, 42, 49). Prior to their use in rhesus macaques, the safety and immunogenicity of all three recombinant attenuated *M. tuberculosis* strains were confirmed in mice (19, 28, 31–33, 42, 49). The recom-

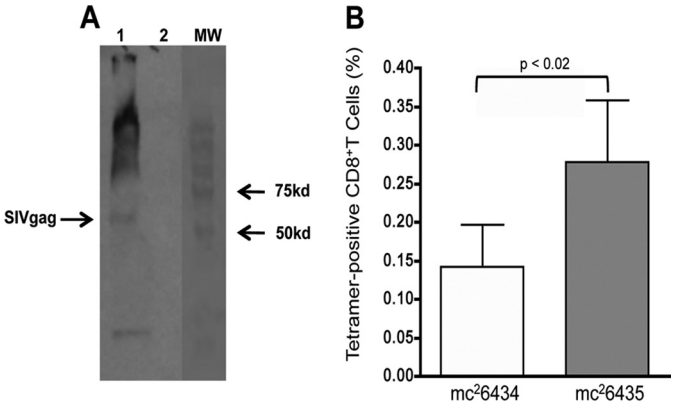

FIG 1 Vaccine-induced SIV-specific $CD8^+$ T cell responses in mice. (A) Western blotting result showing SIVgag protein expression in mc^26435 lysate (lane 1) and its absence in mc^26434 lysate expressing the control plasmid (lane 2). Lane 3 represents the molecular weight (MW) ladder. Recombinant attenuated *M. tuberculosis* whole-cell lysates were immunoblotted with HRP-conjugated anti-V5 antibodies and developed using chemiluminescence. The principal recombinant SIVgag band migrates at approximately 55 kDa. One lower-molecular-weight breakdown product is also observed in mc^26435 lysate. (B) C57BL/6 mice were immunized subcutaneously with 10^7 CFU of the attenuated *M. tuberculosis* vaccine strain mc^26434 ($n = 5$) or with mc^26434 that contained the SIV Gag expression cassette (named mc^26435, $n = 5$). One week after immunization, splenocytes were isolated and AL11-SIVgag tetramer-specific $CD8^+$ T cell responses were determined by flow cytometric analysis. Shown are the average percentages ± standard deviations of AL11-SIVgag tetramer-specific $CD8^+$ T cells. Note that mc^26435-immunized mice mounted AL11-SIVgag-specific $CD8^+$ T cell responses significantly higher than those of mc^26434-immunized mice ($P < 0.02$), albeit the absolute frequencies of tetramer-positive $CD8^+$ T cells were low.

binant attenuated *M. tuberculosis* mc^25157 and mc^26435 vaccine strains were further manipulated to incorporate a mycobacterial expression plasmid with a full-length SIVmac239 Gag insert (28). Expression of SIV Gag in vaccine preparations was confirmed by Western blotting immunolabeled with a horseradish peroxidase (HRP)-conjugated V5 antibody as previously described (Fig. 1A) (28). Immunogenicity was confirmed in C57BL/6 mice (Fig. 1B).

An overview of the vaccination schedule, including the route and dose, is provided in Table 2. Briefly, strain mc^26020 was administered both p.o. and intradermally (i.d.) at 1 week of age and animals were followed up for 6 months (group A). Animals vaccinated with mc^25157 were primed p.o. and received a homologous i.d. booster vaccination at either 2 (group C) or 3 (group E) weeks. Animals were euthanized at week 4 or 6, respectively, to test for vaccine-induced immune responses and *M. tuberculosis* dissemination in various tissues. The time intervals were selected on the basis of our previous pathogenesis and pediatric HIV/SIV vaccine studies in the infant macaque p.o. SIV infection model, in which we generally challenge at 4 weeks of age to mimic early breast milk transmission of HIV in humans (2, 23, 37, 38). Finally, we tested two heterologous prime-boost regimens using either recombinant adenovirus 5 expressing SIVmac239 Gag (rAd5-SIVgag; groups G and H) or recombinant modified vaccinia virus Ankara expressing SIVmac239 Gag, Pol, and Env (rMVA-SIVgpe; group J) that were kindly provided by the International AIDS Vaccine Initiative (Brooklyn, NY) and B. Moss (National Institute of Allergy and Infectious Diseases [NIAID], NIH, Bethesda, MD), respectively (23). The heterologous boosts were administered at 3 weeks (rAd5-SIVgag) or at 3 and 6 weeks (rMVA-SIV constructs) after the initial mc^26435 vaccination. In the studies described here, the vaccine boosts are reported solely for the purpose of revealing all of the study variables; vaccine immunogenicity will be reported separately (unpublished data). Note that all experiments were carried out using parallel mock-infected (saline), age-matched infant macaques as controls (Table 2, groups D, F, I, and K).

SIV infection. A subset of animals were infected with 10^3 50% tissue culture infective doses of SIVmac251 (stock 6/04) (23) by the intravenous (i.v.) route within 72 h of birth and then immunized 1 week later with mc^26020 (group B) or mc^26435 (group L) (Table 2). SIV-infected animals were euthanized when they met criteria established for retrovirus-infected animals (40).

Sample collection and preparation. EDTA blood samples were collected at week 0 (baseline) and then longitudinally as described in Table 2. Plasma was collected after centrifugation and stored in multiple small aliquots at −80°C for virological analysis and antibody testing. Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation as described previously (23). At the time of euthanasia, multiple tissues were collected, including tonsil, lymph node (LN; submandibular, retropharyngeal, bronchial, axillary, and mesenteric), lung, and intestinal tissues (ileum, colon). In addition, from animals that received an i.d. vaccination, we saved tissue from the dermal inoculation site (groups A, B, E, F, and I). Corresponding skin from p.o. vaccinated animals was collected as control tissue. Each tissue sample was divided and saved for multiple applications as follows: snap-frozen (*M. tuberculosis* culture), formalin fixed and paraffin embedded (pathology, *M. tuberculosis* stain-

TABLE 2 Study outline

Group	Size[a]	SIV (i.v.)	Vaccination								Sample collection points (wk)	Age at necropsy (wk)
			Prime strain	Dose(s) (CFU)	Route(s)	Age (wk)	Boost strain	Dose	Route(s)	Age (wk)		
A	3	No	mc^26020	10^9, 10^6	p.o., i.d.	1	None				0, 2, 4, 6, 8, 10, 12, 16, 20, 24	24
B	3	Yes	mc^26020	10^9, 10^6	p.o., i.d.	1	None				0, 2, 4, 6	6
C	5	No	mc^25157	10^9	p.o.	0	mc^25157	10^9 CFU	i.d.	2	0, 2, 4	4
D	2	No	Mock		p.o., i.d.	0	Mock		p.o., i.d.	2	0, 2, 4	4
E	5	No	mc^25157	10^9	p.o.	0	mc^25157	10^9 CFU	i.d.	3	0, 3, 6	6
F	2	No	Mock		p.o., i.d.	0	Mock		p.o., i.d.	3	0, 3, 6	6
G	6	No	mc^26435	10^9	p.o.	0	rAd5SIV	9×10^9 PFU	i.m.[b]	3	0, 3, 6, 9, 12, 16	16
H	6	No	mc^26435	10^6	i.d.	0	rAd5SIV	9×10^9 PFU	i.m.	3		16
I	4	No	Mock		p.o., i.d.	0	Mock		i.m.	3		16
J	8	No	mc^26435	10^9	p.o.	0	rMVASIV	10^8 PFU	i.m.	3, 6	0, 3, 6, 9, 12, 16	18
K	3	No	Mock		p.o.	0	Mock		i.m.	3, 6		16
L	3	Yes	mc^26435	10^9	p.o.	1	None				0, 1, 2, 4, 6, 8, 10	6–10

[a] Number of animals.
[b] i.m., intramuscular.

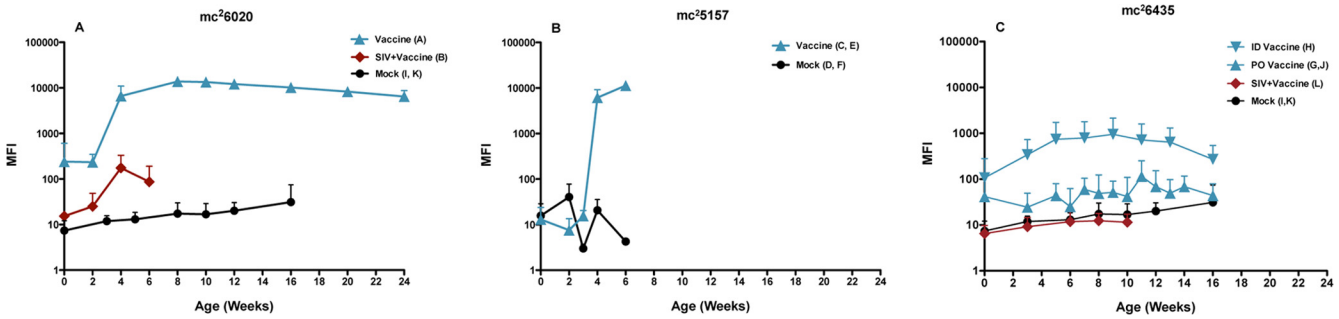

FIG 2 *M. tuberculosis*-specific plasma IgG antibody responses. Plasma antibody responses to PSTS1 antigen were measured by multiplex microbead array analysis. Average antibody levels plus standard deviations for mock-vaccinated animals (black lines, closed circles), SIV-infected and vaccinated animals (red lines, diamonds), and non-SIV-infected vaccinated animals (blue lines, triangles) are shown for each vaccine candidate. Data are reported as MFIs. The experimental groups are listed in parentheses in the top right corner of each panel. The mock-vaccinated animals used for groups A and B are the same animals used as controls for groups G, H, J, and L. Note that in panel A, one of three non-SIV-infected vaccinated animals had high PSTS1 antibody levels at day 0 (MFI of 666 compared to MFIs of 40 and 18 in the other remaining animals, and therefore, the average baseline antibody levels at day 0 appear higher than those of groups B, I, and K. Similarly, in panel C, 2 of 6 animals vaccinated i.d. and 2 of 14 animals vaccinated p.o. with mc²6435 had PSTS1 MFIs (antibody levels) of >100 at day 0, explaining the higher MFI values at day 0 than those of groups I, K, and L.

ing), and made into fresh tissue aliquots (immunogenicity). The isolation of cell populations from tonsil, LN, and intestinal tissues was performed as described previously (23).

CD4$^+$ T cell measurement. A complete blood count (CBC) was performed on an ABX Pentra 60+ electronic cell counter (ABX Diagnostics, Irvine, CA) with manual differential counts. Absolute counts and percentages of CD4$^+$ T cells in PBMC were determined using antibodies specific for rhesus macaque CD3 and CD4 by flow cytometric analysis and CBC values as described previously (2).

SIV replication. Plasma samples were analyzed for viral RNA by a quantitative reverse transcription-PCR assay as previously described (7).

***M. tuberculosis*-specific plasma antibodies.** The presence of IgG antibodies against the *M. tuberculosis* PSTS1 antigen in longitudinally collected plasma samples was determined by using a recently described multiplex microbead immunoassay based on the Luminex system (Luminex Corp., Austin, TX) (17). Each sample was tested in duplicate. Relative antibody levels are reported as mean fluorescence intensities (MFIs) (17). Sera from non-*M. tuberculosis*-infected and *M. tuberculosis*-infected rhesus macaques were used as negative and positive controls, respectively.

Pathology evaluation. Gross pathology evaluation was performed at necropsy. Formalin-fixed, paraffin-embedded tissues were cut into 5-μm sections and stained with hematoxylin and eosin (H&E) according to standard protocols. Lung sections from an adult macaque infected experimentally with virulent *M. tuberculosis* were kindly provided by P. Luciw for comparison (21). In addition, Ziehl-Neelsen-stained sections were examined for the presence of acid-fast bacilli (AFB). Tissue section slides were read in their entirety by a veterinary pathologist blinded with respect to treatment groups.

***M. tuberculosis* isolation.** Snap-frozen tissues, stored at −80°C, were shipped to the National Animal Disease Center (USDA-ARS, Ames, IA) to recover viable mycobacteria using three different culture methods, (i) the fast indicator tube test (MGIT), (ii) Middlebrook 7H12 medium (BacTec), and (iii) solid culture medium, to determine CFU counts. As recombinant attenuated *M. tuberculosis* auxotrophic mutants cannot grow in a standard mycobacterial growth medium culture, the medium was supplemented with pantothenate with or without lysine (mc²6020) or leucine (mc²6435). A tissue was considered positive if one of three culture methods yielded mycobacterial growth. *M. tuberculosis* positive-control samples were run in parallel for quality assurance.

Statistical analysis. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Antibody data at specific time points (see the text) were compared between two or more groups after $\log_{10}$ transformation using a nonparametric Mann-Whitney test or one-way analysis of variance (Kruskal-Wallis test with Dunn's comparisons), respectively. Area-under-the-curve analysis of antibody levels was also performed on $\log_{10}$-transformed data using GraphPad Prism Software. *P* values of ≤0.05 were considered significant.

RESULTS

Confirmation of vaccine exposure by the p.o. and i.d. routes. All three vaccine strains were attenuated for replication. We therefore wanted to confirm that they were able to induce seroconversion to validate the biological significance of our safety assessment that includes lack of mycobacterial dissemination in an immunocompromised host. Plasma antibodies to the *M. tuberculosis*-specific antigen PSTS1 were measured after p.o. and i.d. vaccination with the various recombinant attenuated *M. tuberculosis* vaccines. Independent of the vaccine strain, all non-SIV-infected vaccinated animals developed *M. tuberculosis*-specific plasma IgG antibodies to the PSTS1 antigen (Fig. 2), but the magnitude was dependent on the vaccine strain and the route of administration. Animals that received the mc²6020 vaccine via the p.o. and i.d. routes at 1 week of age (group A) developed relative antibody levels of up to 10^4 MFI by 4 weeks (Fig. 2A). Similar data were obtained with animals primed p.o. with mc²5157 and given a homologous i.d. boost (groups C and E; Fig. 2B). Consistent with more stringent attenuation of replication (deletions of the *leuCD* and *panCD* loci), in animals vaccinated p.o. with the mc²6435 vaccine strain (groups G and J), PSTS1 antibody levels were significantly lower ($P < 0.001$) during weeks 4 to 6 postimmunization than those in mc²6020- and mc²5157-vaccinated infant macaques. Furthermore, the route of vaccine administration influenced the magnitude of antibody induction in plasma, with i.d. mc²6435-vaccinated animals (group H) developing significantly higher ($P = 0.0119$) antibody levels than p.o. vaccinated animals (groups G and J). Although there was a trend toward higher PSTS1 antibody levels in p.o. mc²6435-vaccinated infants than in mock-immunized animals between weeks 5 and 8 postvaccination, this difference did not reach statistical significance. PSTS1 antibody levels, however, were significantly higher in mc²6435-vaccinated infants than in mock-immunized animals by area-under-the curve-analysis of antibody levels from week 0 to week 16 ($P = 0.0229$). The PSTS1 antibody levels in animals infected with SIV prior to vaccination with recombinant attenuated *M. tuberculosis* mc²6020

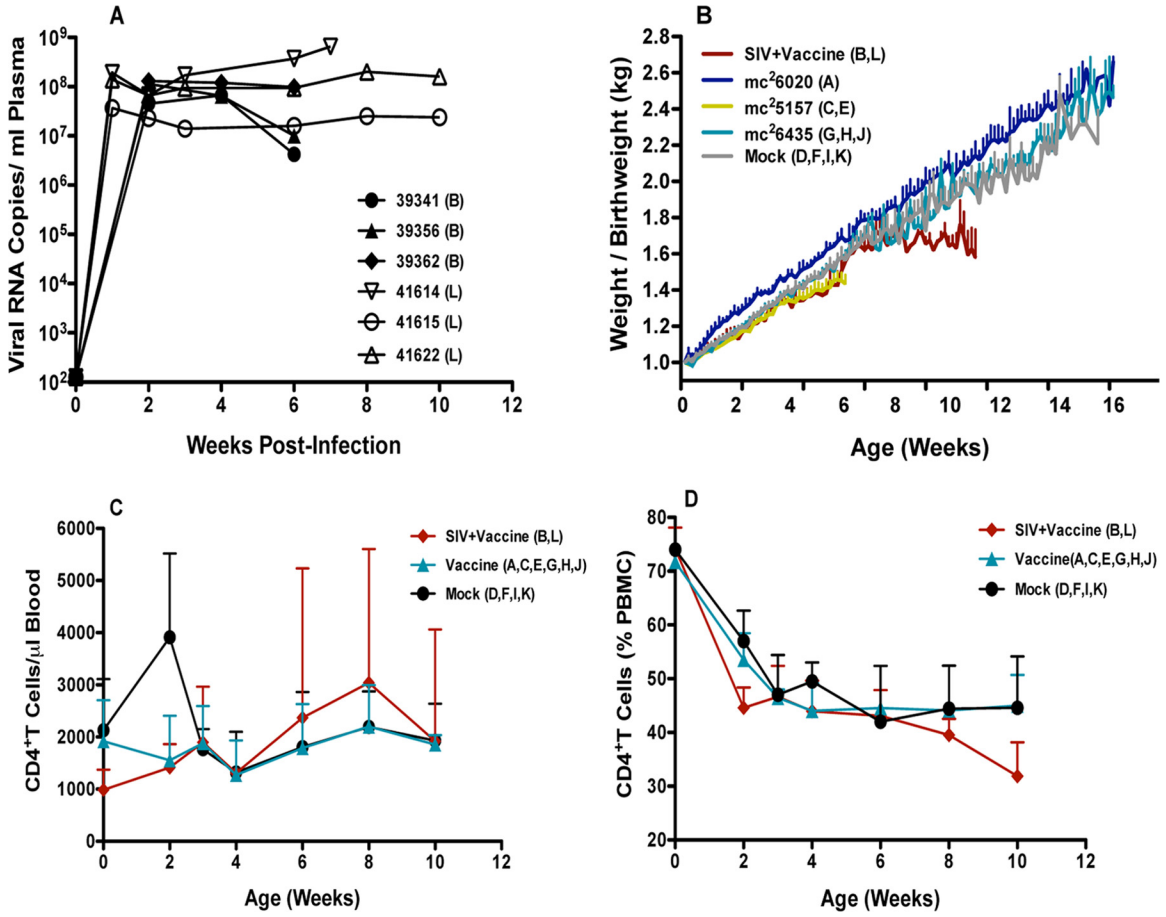

FIG 3 Plasma SIV viremia and clinical parameters. (A) Numbers of SIV RNA copies per milliliter of plasma in samples longitudinally collected from each animal are shown. Each symbol represents an individual animal. The experimental group of each animal is shown in parentheses. (B) The average increase in weight over the birth weight (kilograms) is shown for the animals in each of the three vaccine groups (dark blue, yellow, and light blue lines) and for SIV-infected and subsequently vaccinated animals (red line) in comparison to the weight gain of mock-vaccinated animals (gray line). The averages of absolute numbers ± standard deviations (C) and average percentages ± standard deviations of $CD4^+$ T cells (D) in peripheral blood are shown for animals vaccinated after prior SIV infection (red lines, diamonds) and for vaccinated non-SIV-infected (blue lines, triangles) animals in comparison to mock-vaccinated animals (black lines, circles). Note that panels C and D include the SIV-infected animals vaccinated with mc^26020 ($n = 3$) or mc^26435 ($n = 3$). For this analysis, vaccinated animals ($n = 33$) included animals from groups A, C, E, G, H, and J and mock-vaccinated animals ($n = 11$) included animals from groups D, F, I, and K.

(group B) or mc^26435 (group L) were lower than those of non-SIV-infected animals (Fig. 2A and C), likely because of SIV-induced immunosuppression.

Safety assessment of recombinant attenuated *M. tuberculosis*-SIV vaccine strains. The safety profile of the various recombinant attenuated *M. tuberculosis* vaccine strains was evaluated on the basis of a combination of (i) clinical observations (e.g., breathing difficulties), (ii) histopathological evaluation of multiple tissues, (iii) detection of AFB in tissues, and (iv) recovery of viable mycobacteria from tissues. A vaccine was considered to have no or only a minimal safety risk if AFB could not be detected, mycobacterial bacilli could not be recovered from any tissues, histopathology of lung and other tissues/organs appeared essentially normal, and no clinical symptoms were observed. In contrast, a vaccine strain was considered unsafe if one or more tissues were AFB positive, *M. tuberculosis* culture positive, or granuloma positive during histopathological examination.

We assessed the safety of the recombinant attenuated *M. tuberculosis* vaccine strains in a stringent two-step study. First, the recombinant attenuated *M. tuberculosis* vaccine strains were administered to immunologically immature infant macaques at 1 week of age. Next, if no clinical signs of *M. tuberculosis* infection were observed and no viable mycobacteria could be recovered from tissues, we tested the vaccine in infant macaques infected with highly pathogenic SIVmac251 at 1 week prior to recombinant attenuated *M. tuberculosis* vaccination as a model of HIV-induced immunosuppression in HIV-1-infected human infants.

(i) Clinical safety assessment. SIV infection (groups B and L) was confirmed by measuring plasma viral RNA levels. Consistent with our prior studies (2, 23, 38, 39), all six infant macaques developed high peak viremia ($>10^7$ copies/ml of plasma) (Fig. 3A) which persisted at high levels. The loss of $CD4^+$ T cells is a hallmark of HIV/SIV disease progression in adults but not a reliable clinical marker of virus-infected infants (2). Furthermore, as $CD4^+$ T cells represent the vast majority of T cells at birth, and $CD8^+$ T cell numbers continue to increase after birth, a decline in the percentage of $CD4^+$ T cells is characteristic of the normal developmental process after birth. In fact, a decline in $CD4^+$ T

cells was observed in all experimental groups and was not limited to only SIV-infected infants (2) (Fig. 3). Compared to age-matched control animals (mock-vaccinated animals), non-SIV-infected vaccinated animals showed similar peripheral blood $CD4^+$ T cell frequencies. In addition, the CBC values in non-SIV-infected, recombinant attenuated *M. tuberculosis*-vaccinated infants remained normal throughout the study period (data not shown).

A more critical factor in the evaluation of infant health is weight gain. All vaccinated non-SIV-infected infants showed a weight gain similar to that of age-matched control animals (Fig. 3). In contrast, consistent with previous studies (2, 24), SIV-infected animals showed poor weight gain (Fig. 3). Although wasting is also a common symptom of HIV/SIV-associated disease in adults (10), the lack of weight gain is much more detrimental in infants. Due to a failure to thrive and symptoms associated with rapid progression to simian AIDS (e.g., recurring episodes of diarrhea, poor appetite, and lethargic behavior), the SIV-infected animals were euthanized between 6 and 10 weeks after SIV infection (Fig. 3). Despite this apparent immunosuppression as a result of SIV infection, these animals did not show clinical symptoms typically associated with *M. tuberculosis* infection. Non-SIV-infected infants that received the mc^{2}6020 or mc^{2}6435 vaccine (group A or groups G, H, and J, respectively) showed normal weight gain (Fig. 3).

Clinically, no symptoms indicative of *M. tuberculosis* infection (e.g., breathing difficulties, coughing) were observed at any time during the study period. Similarly, no adverse signs of vaccination with recombinant attenuated *M. tuberculosis*-SIV vaccines were observed, with the exception of local reactivity in i.d. vaccinated animals. These animals showed a local inflammatory response following i.d. inoculations and developed indurations. In mc^{2}6020-vaccinated animals, the indurations resolved over time. Due to the short follow-up time in mc^{2}5157-vaccinated animals, it could not be determined whether the local inflammation would have resolved over time.

(ii) Histopathological evaluation. To thoroughly assess the safety of the recombinant attenuated *M. tuberculosis* vaccine strains in infants, we evaluated several tissues collected at necropsy for TB pathology, i.e., (i) the dermal inoculation site or corresponding skin samples from p.o. recombinant attenuated *M. tuberculosis*- or mock-vaccinated animals, (ii) axillary LNs that drained the dermal inoculation site, (iii) the lung as the primary site of TB-specific pathology, (iv) the lung-draining bronchial LNs, and (v) the spleen as a more distal lymphoid indicator of *M. tuberculosis* dissemination (Table 3).

Consistent with clinical signs of inflammation (see above), histopathological examination of mc^{2}5157-vaccinated animals at 4 or 6 weeks of age (groups C and E, respectively) revealed that moderate-to-severe pyogranulomatous dermatitis had persisted at the i.d. inoculation sites, whereas dermal tissues from mc^{2}6020-vaccinated animals appeared essentially normal (Table 3). In contrast to vaccination with mc^{2}6020 and mc^{2}5157, i.d. vaccination with mc^{2}6435 (group H) did not result in an inflammatory response at the site of inoculation (Table 3). The mild dermatitis that was observed in some of the vaccinees independent of the vaccine strain was occasionally noted in skin from p.o. or mock-vaccinated animals as well and thus was probably not due to vaccination (Table 3). Consistent with a potential spread of mycobacteria in mc^{2}5157-vaccinated animals (groups C and E), the majority of the axillary LNs of mc^{2}5157-vaccinated animals showed moderate lymphoid hyperplasia and pyogranulomatous inflammation, whereas non-SIV-infected animals vaccinated with mc^{2}6020 or mc^{2}6435 showed only mild histopathological changes (Table 3). Evidence of pulmonary TB lesions or granuloma formation in the lung indicative of *M. tuberculosis* infection was markedly absent from the vaccinated animals. In fact, the lungs of animals vaccinated with mc^{2}6020 (not shown) and mc^{2}6435 (Fig. 4E) were histologically indistinguishable from the lungs of mock-vaccinated infant macaques (Fig. 4C). Of the 10 animals vaccinated with mc^{2}5157, 6 developed small granulomas in their lungs (Fig. 4D). However, these lesions were smaller and less frequent than the granulomas induced by virulent *M. tuberculosis* infection (Fig. 4A and B).

In infants infected with SIV prior to recombinant attenuated *M. tuberculosis* vaccination, histopathological changes typical of SIV infection, such as mild lymphoid hyperplasia or subsequent lymphoid depletion, were commonly observed in various lymphoid tissues (Table 3). Although pneumonitis was detected in some SIV-infected, recombinant attenuated *M. tuberculosis*-vaccinated animals, the histopathology was not typical of *M. tuberculosis*-induced lung pathology. Importantly, SIV-infected animals did not develop granulomas in the lung or other tissues after vaccination with mc^{2}6020 or mc^{2}6435 (Fig. 4F). Thus, SIV-induced immunosuppression in infant macaques vaccinated with recombinant attenuated *M. tuberculosis* strains did not result in TB-induced disease or pathology.

(iii) Strain-dependent differences in mycobacterial dissemination. Miliary tuberculosis is one of the most severe complications of *M. tuberculosis* infection. Although the recombinant attenuated *M. tuberculosis* vaccine strains tested in the current study were replication attenuated, the degree of attenuation of replication and immune evasion differed between the strains (Table 1). Therefore, we tested the same tissues that were examined for TB-associated pathology for the presence of mycobacteria. First, tissue sections were stained with the Ziehl-Neelsen stain to detect AFB. However, even in pathogenic *M. tuberculosis* infection, detection of mycobacteria by AFB staining can be infrequent. Therefore, different culture methods using optimized growth medium specially supplemented for the auxotrophic strains were used to determine whether live mycobacteria could be recovered from any of the tissues. AFB were only rarely (1 or 2 bacilli per tissue), if at all, detected at dermal inoculation sites after mc^{2}6020 or mc^{2}6435 vaccination. Importantly, AFB were detected in tissue from only one of three SIV-infected infant macaques that received the mc^{2}6020 vaccine and only at one site, the axillary LN which drained the i.d. inoculation site. AFB were not detected by Ziehl-Neelsen staining in any other tissues of this animal. Live mycobacteria could be recovered from the same axillary LN of this animal but not from any other tissue samples. In contrast, all infant macaques vaccinated with mc^{2}5157, the recombinant attenuated *M. tuberculosis* vaccine strain that contained deletions that may increase its immunogenicity and yet result in replication higher than that of mc^{2}6020, tested positive for AFB in their dermal tissues, and in 6 of 10 animals, AFB were also detected in at least one other tissue type (Table 4). Furthermore, live mycobacteria could be recovered from several tissues of these animals (Table 4). Due to the widespread mycobacterial dissemination and more severe immunopathology in mc^{2}5157-vaccinated infant macaques, strain mc^{2}5157 was considered unsafe and not further pursued as a can-

TABLE 3 Histopathological evaluation

Group(s) (no. of animals)[a]	SIV infection	Vaccine strain (route[s])	Findings on tissue samples[b]					Pathology result[c]	
			Skin	Axillary LN	Lung	Bronchial LN	Spleen	TB	SIV
I, D, F, K (11)	No	Mock (p.o., i.d.)	7/11 normal; 3/11 very mild dermatitis; 1/11 no tissue	4/11 normal; 2/11 mild histiocytosis; 3/11 mild-to-moderate paracortical expansion; 2/11 no tissue	6/11 normal; 3/11 mild bronchiolitis or pneumonia; 2/11 no tissue	4/11 normal; 3/11 mild-to-moderate paracortical expansion; 2/11 mildly reactive; 1/11 mild eosinophilia; 1/11 no tissue	4/11 normal; 3/11 neutrophilia; 3/11 mild hyperplasia; 1/11 no tissue	No	No
A (3)	No	$mc^2$6020 (p.o., i.d.)	2/3 normal; 1/3 very mild dermatitis	1/3 mild hyperplasia; 1/3 mild lymphoid depletion; 1/3 no tissue	1/3 normal; 2/3 subacute mild pneumonitis	1/3 mild lymphoid hyperplasia; 2/3 no tissue	3/3 normal	No	No
B (3)	Yes	$mc^2$6020 (p.o., i.d.)	2/3 normal; 1/3 mild dermatitis	3/3 mild lymphoid depletion	2/3 normal; 1/3 mild pneumonitis	2/3 mild lymphoid depletion; 1/3 normal	3/3 mild-to-moderate lymphoid depletion	No	Yes
C, E (10)	No	$mc^2$5157 (p.o., i.d.)	10/10 moderate-to-severe pyogranulomatous dermatitis	2/10 normal; 3/10 mild-to-moderate hyperplasia; 5/10 pyogranulomatous lymphadenitis	4/10 normal; 6/10 moderate multifocal pneumonitis, some granulomatous inflammation	1/10 normal; 8/10 mild-to-moderate hyperplasia, rare granulomatous lymphadenitis; 1/10 no tissue	10/10 normal	Some	No
G, J (14)	No	$mc^2$6435 (p.o.)	14/14 normal	11/14 normal with mild paracortical expansion; 1/13 mild-to-moderate paracrotical expansion; 2/14 no tissue	8/14 normal; 5/14 mild interstitial pneumonitis, some congestion; 1/14 focal granuloma with foreign material	6/14 normal; 3/14 mildly reactive; 4/14 mild paracortical expansion; 1/14 mild extramedullary hematopoiesis	13/14 normal; 1/14 mild-to-moderate hyperplasia	No	No
H (6)	No	$mc^2$6435(i.d.)	6/6 normal	4/6 normal; 2/6 mild sinus histiocytosis	2/6 normal; 3/6 subacute pneumonitis; 1/6 mild arteriolitis	3/6 normal; 3/6 mildly reactive	4/6 normal; 1/6 increased neutrophils; 1/6 intraparenchymal hemorrhage	No	No
L (3)	Yes	$mc^2$6435(p.o.)	2/3 normal; 1/3 mild dermatitis	2/3 normal; 1/3 sinus histiocytosis	3/3 moderate pneumonia, hemorrhages, pneumocyte hyperplasia	3/3 normal	3/3 lymphoid hyperplasia, neutrophilia; 1/3 focal mineralization	No	Yes

[a] The experimental groups are described in the text and in Table 2.

[b] All of the observed histopathological findings are summarized for each tissue type. The value before the slash is the number of animals within a specific group that showed the pathology described, and the value after the slash is the total number of animals.

[c] The summary conclusion provided by the pathologist is shown as follows: no, negative; yes, positive; some, minor pathology due to SIV or *M. tuberculosis* infection.

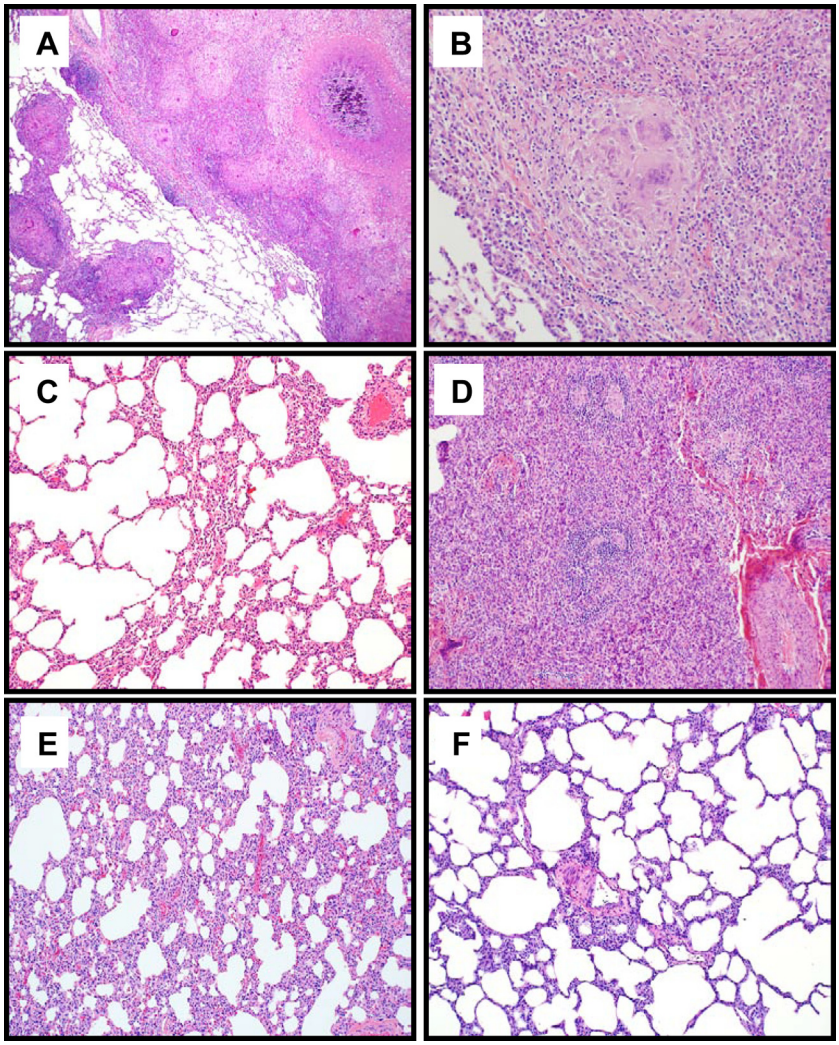

FIG 4 Pathology evaluation of lung tissue. Formalin-fixed, paraffin-embedded tissues were stained with H&E and blindly analyzed for histological changes due to vaccination and/or SIV infection. (A) Lung tissue (magnification, ×100) of an adult rhesus macaque infected with pathogenic *M. tuberculosis* (21). (B) The same tissue shown in panel A at ×200 magnification. Note the central necrosis, mineralization, and granuloma formation and the presence of multinucleate giant cells. (C) Lung section of a mock-immunized infant macaque. (D) Representative lung section of an mc^{2}5157-vaccinated infant macaque with granulomatous tissue and signs of extensive atelectasis. Panels E and F show essentially normal lung sections from non-SIV-infected (E) or SIV-infected (F) infant macaques after mc^{2}6435 vaccination. The sections in panels C to F are at ×100 magnification.

didate *M. tuberculosis*-SIV vaccine. Remarkably, all tissues from non-SIV-infected ($n = 20$) and also from SIV-infected ($n = 3$), mc^{2}6435-vaccinated animals were negative for AFB, and viable mycobacteria could not be recovered from any of these tissues by any of the culture methods used.

In summary, the data indicate that recombinant attenuated *M. tuberculosis*-SIV vaccine strains can be administered p.o. or i.d. to infant macaques during the first postnatal week. Although vaccine strain mc^{2}5157 caused dissemination of mycobacteria, the live attenuated vaccine strains mc^{2}6020 and mc^{2}6435 demonstrated a better safety profile in infant macaques. In particular, strain mc^{2}6435 was safe in non-SIV-infected (groups I and K) and in immunocompromised, SIV-infected infant macaques (groups G, H, and J) by all of the safety criteria used in this study.

DISCUSSION

An effective vaccine against HIV is not available, and the only approved TB vaccine, BCG, is not safe in immunosuppressed individuals. This safety concern is of particular importance in resource-poor countries affected by the dual epidemics of TB and AIDS. In particular, TB infection rates among children have risen in association with HIV prevalence, and about 1% of HIV-infected infants develop disseminated BCG disease after vaccination. Although ART coverage now extends to 42% of HIV-infected mothers, only about 23% of their newborn infants receive ART (43, 45) and the rest remain at risk for HIV acquisition by breast-feeding. These facts underscore the need for a novel safe vaccine to prevent pediatric HIV and TB infections. The current study represents an important first step toward the development of a p.o. administered highly attenuated *M. tuberculosis* vaccine expressing HIV antigens as a potential pediatric combination HIV-TB vaccine.

BCG was originally administered p.o., but this route was discontinued due to cervical lymphadenitis and parapharyngeal complications. The p.o. route may be more advantageous

TABLE 4 *M. tuberculosis* detection

Group(s) (strain), no. of animals, tissue type	AFB	MGIT[a]				BacTec[b]				Solid culture[c]				Result
		−	+	++	+++	−	+	++	+++	−	+	++	+++	
A ($mc^2$6020), 3														
Skin	0/3	NT[d]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Lung	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Axillary LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Spleen	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
B (SIV + $mc^2$6020), 3														
Skin	0/3	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Lung	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Axillary LN	**1/3**[e]	2/3	**1/3**	0/3	0/3	2/3	**1/3**	0/3	0/3	3/3	0/3	0/3	0/3	**1/3**
Spleen	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
C, E ($mc^2$5157), 10														
Skin	10/10	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	**2/10**	4/10	**1/10**	**3/10**	**2/10**	5/10	0/10	**3/10**	**2/10**	5/10	**1/10**	**3/10**	**1/10**	**5/10**
Lung	**1/10**	5/10	**1/10**	**1/10**	**3/10**	3/10	**5/10**	**1/10**	**1/10**	5/10	**1/10**	**1/10**	**3/10**	**7/10**
Axillary LN	**5/10**	2/10	**1/10**	**3/10**	**4/10**	2/10	**3/10**	**2/10**	**3/10**	3/10	**1/10**	**3/10**	**3/10**	**8/10**
Spleen	0/10	0/10	**5/10**	**5/10**	0/10	0/10	**10/10**	0/10	0/10	0/10	**4/10**	**4/10**	**2/10**	**10/10**
G, H, J ($mc^2$6435), 20														
Skin	0/20	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	0/20
Lung	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	0/20
Axillary LN	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	0/20
Spleen	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	0/20
L (SIV + $mc^2$6435), 3														
Skin	0/3	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Lung	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Axillary LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Spleen	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3

[a] MGIT results: −, no growth; +, ≥24 days; ++, 18 to 24 days; +++, <18 days.
[b] BacTec results: −, no growth; +, ≥24 days; ++, 18 to 24 days; +++, <18 days.
[c] Solid culture results: −, no growth; +, <5 CFU; ++, 5 to 50 CFU; +++, ≥50 CFU.
[d] NT, not tested.
[e] Samples positive for AFB or outgrowth of mycobacteria are in bold.

for a pediatric HIV-TB vaccine, because a mucosally administered vaccine could also induce local immune responses protective against HIV transmitted p.o. by breast-feeding. In fact, experiments with mice have shown that p.o. fed BCG can infect the submandibular lymph nodes and Peyer's patches (18) and that *M. tuberculosis* can bind to tonsillar M cells (6, 26). Thus, we assessed the safety of three distinct recombinant attenuated *M. tuberculosis* H37Rv strains, engineered with or without the SIV *gag* gene, in non-SIV-infected and in SIV-infected, and thus immunosuppressed, infant macaques by the p.o. and i.d. routes. BCG vaccination of SIV-infected macaques has previously been shown to exacerbate SIV disease progression and to cause dissemination of *M. bovis* bacilli (9, 35, 48). In accordance with a recent proposal to improve the classification of TB disease severity in human children by combining multiple factors ranging from disease pathogenesis criteria to bacteriological evaluation and clinical data (47), vaccine safety in infant macaques was rigorously assessed in the current study by comprehensive clinical examination, histopathological evaluation, and multiple *M. tuberculosis* culture methods.

For safety evaluation, animals were grouped by vaccine strain independent of the route of administration, immunization interval, or prime-boost regimen. The *panCD* deletion in strain $mc^2$5157 was not sufficient to prevent mycobacterial dissemination in infant macaques, and although granuloma size and frequency in the lung were limited in comparison to those of animals infected with pathogenic TB (21), the strain was deemed unsuitable for use in immunocompromised hosts. In contrast to the safety profile of $mc^2$5157, that of dual deletion strain $mc^2$6020 (Δ*panCD* Δ*lysA*) was improved. Only one of three SIV-infected infant rhesus macaques showed evidence of *M. tuberculosis* dissemination, and only in the axillary lymph node that drained the i.d. inoculation site. Whether or not this limited mycobacterial dissemination in immunosuppressed SIV-infected infant macaques correlates with a reduced risk of future dissemination or reactivation at other sites, however,

remains to be determined. Although we previously confirmed the safety of this strain, these studies were performed only with non-SIV-infected adult cynomolgus macaques (19). Thus, the current data support our hypothesis that auxotroph mutant *M. tuberculosis* strains can be developed as safe vaccine candidates for use in immunocompromised individuals. *M. tuberculosis* is a slowly replicating bacterium with genes that limit the host immune response, permitting bacilli to persist within a host, often in a latent state (11, 20). To be safe and effective, a live attenuated *M. tuberculosis* vaccine requires a balance between replication and immunogenicity. In infants with immature immunity, low-level replication may be required to induce *M. tuberculosis*-specific immune responses and promote persistent immune memory responses. However, if replication is too robust, attenuated *M. tuberculosis* strains may result in granuloma formation and bacillary dissemination, as was observed with mc^{2}5157. In addition to attenuating replication, deletions in mycobacterial genes that encode immune interference may be necessary to enhance attenuated *M. tuberculosis* immunogenicity.

On the basis of the results obtained with mc^{2}5157 and mc^{2}6020, strain mc^{2}6435 was designed with deletions of both the *panCD* and *leuCD* loci (Δ*panCD* Δ*leuCD*). An additional deletion was introduced into the *secA2* locus that interferes with apoptosis in *M. tuberculosis*-infected macrophages (5, 15). The deletion of this locus has been associated with both enhanced apoptosis of mycobacterium-infected macrophages *in vitro* and with increased antigen-specific CD8$^+$ T cell responses *in vivo*, the latter likely due to enhanced cross-presentation of mycobacterial antigens to dendritic cells (5, 15). A similar attenuated *M. tuberculosis* strain (Δ*lysA* Δ*secA2*) has previously been demonstrated to be safe and immunogenic in neonatal SCID mice (100% survival for up to 642 days), in which BCG is generally lethal (28). Our results here show that recombinant attenuated *M. tuberculosis* strain mc^{2}6435 was well tolerated by and did not cause any TB-like disease in 20 infant macaques vaccinated within the first week of life. Importantly, the safety of this strain was confirmed in infant macaques infected with highly pathogenic SIV prior to mc^{2}6435 vaccination. The tissue pathology of these SIV-infected infant macaques was consistent only with SIV infection with no signs of *M. tuberculosis* infection. Furthermore, there was no histopathological evidence of recombinant attenuated *M. tuberculosis* dissemination, and live mycobacteria could not be recovered from any of the tissues studied, even under optimal supplemented culture conditions for the attenuated *M. tuberculosis* strain.

No other TB vaccine candidate has been tested under such stringent conditions in infant macaques, a model of human infant TB vaccination. Analogous to our study, the safety of a Δ*leuCD* Δ*panCD* auxotroph *M. tuberculosis* vaccine strain was recently evaluated in non-SIV-infected and SIV-infected adult macaques (34). Vaccination of adult macaques with this attenuated *M. tuberculosis* strain did not cause adverse effects, AFB were not detectable, and viable mycobacterial bacilli could not be recovered from longitudinally collected blood samples or tissues collected at euthanasia (34). The adult SIV-infected animals were followed up for up to 1 year, further supporting the conclusion that auxotroph attenuated *M. tuberculosis* strains do not pose a safety risk to immunosuppressed individuals. The safety profile of recombinant attenuated *M. tuberculosis* strain mc^{2}6435 in SIV-infected infant macaques is consistent with this conclusion.

A potential caveat of our study is the relatively short follow-up time of SIV-infected infants. In HIV-infected children and SIV-infected neonatal macaques, disease is often more severe and progression is accelerated. In the current study, SIV-infected infants were euthanized between 6 and 10 weeks after SIV infection because of a failure to thrive. Therefore, vaccine-induced *M. tuberculosis*-associated pathology or dissemination had to manifest itself within a relatively short time period in SIV-infected animals to be detected and we cannot draw any conclusions about the long-term outcome. It should be emphasized, though, that *M. tuberculosis* dissemination was observed in multiple infant tissues collected at 4 or 6 weeks after vaccination with mc^{2}5157 by pathology, histology, and culture assays. The latter data imply that lung pathology and *M. tuberculosis* dissemination should be detectable within a 10-week time frame, especially in immunosuppressed SIV-infected animals.

To our knowledge, this is the first study demonstrating that an attenuated *M. tuberculosis* strain does not cause disease in an infant nonhuman primate model of neonatal TB vaccination and SIV infection that is highly relevant to human infants, including those at risk for perinatal HIV infection. Our data show that all three vaccines were able to induce persistent vaccine-specific antibody responses in infant macaques, albeit the magnitude was dependent on the level of replication attenuation (mc^{2}6020 = mc^{2}5157 > mc^{2}6435) and the route of vaccine administration (i.d. > p.o.). The persistence of these antibodies suggests that the recombinant attenuated *M. tuberculosis* vaccines primed the infant immune system. Despite the low plasma *M. tuberculosis*-specific PSTS1 antibody responses after p.o. and i.d. administration in infant rhesus macaques, recombinant attenuated *M. tuberculosis* vaccine strain mc^{2}6435 (which contains a mycobacterial expression plasmid encoding SIV Gag) was effective at inducing both SIV- and TB-specific CD4$^+$ and CD8$^+$ T cell immune responses in systemic and mucosal tissues (unpublished data). Therefore, recombinant attenuated *M. tuberculosis* strain mc^{2}6435 should be further explored and optimized as a valid TB vaccine candidate that could replace BCG, as well as function as a combination vaccine to protect infants against both HIV and TB infections. Considering that immune correlates of protection against HIV or TB acquisition are not well defined, these vaccines ultimately need to be tested for efficacy against an HIV/SIV and *M. tuberculosis* challenge.

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REFERENCES

1. **Abel K.** 2009. The rhesus macaque pediatric SIV infection model—a valuable tool in understanding infant HIV-1 pathogenesis and for designing pediatric HIV-1 prevention strategies. Curr. HIV Res. **7**:2–11.
2. **Abel K, et al.** 2006. Rapid virus dissemination in infant macaques after oral simian immunodeficiency virus exposure in the presence of local innate immune responses. J. Virol. **80**:6357–6367.
3. **Amedee AM, Lacour N, Ratterree M.** 2003. Mother-to-infant transmission of SIV via breast-feeding in rhesus macaques. J. Med. Primatol. **32**: 187–193.
4. **Amedee AM, Rychert J, Lacour N, Fresh L, Ratterree M.** 2004. Viral and immunological factors associated with breast milk transmission of SIV in rhesus macaques. Retrovirology **1**:17.
5. **Baena A, Porcelli SA.** 2009. Evasion and subversion of antigen presentation by Mycobacterium tuberculosis. Tissue Antigens **74**:189–204.
6. **Brandtzaeg P.** 1999. Regionalized immune function of tonsils and adenoids. Immunol. Today **20**:383–384.
7. **Cline AN, Bess JW, Piatak M, Jr, Lifson JD.** 2005. Highly sensitive SIV plasma viral load assay: practical considerations, realistic performance expectations, and application to reverse engineering of vaccines for AIDS. J. Med. Primatol. **34**:303–312.
8. **Colditz GA, et al.** 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA **271**:698–702.
9. **Croix DA, et al.** 2000. Effect of mycobacterial infection on virus loads and disease progression in simian immunodeficiency virus-infected rhesus monkeys. AIDS Res. Hum. Retroviruses **16**:1895–1908.
10. **Freeman LM, et al.** 2004. Body-composition changes in the simian immunodeficiency virus-infected juvenile rhesus macaque. J. Infect. Dis. **189**:2010–2015.
11. **Gill WP, et al.** 2009. A replication clock for Mycobacterium tuberculosis. Nat. Med. **15**:211–214.
12. **Hesseling AC, et al.** 2009. Disseminated bacille Calmette-Guérin disease in HIV-infected South African infants. Bull. World Health Organ. **87**:505–511.
13. **Hesseling AC, et al.** 2007. The risk of disseminated bacille Calmette-Guérin (BCG) disease in HIV-infected children. Vaccine **25**:14–18.
14. **Hesseling AC, et al.** 2006. Bacille Calmette-Guérin vaccine-induced disease in HIV-infected and HIV-uninfected children. Clin. Infect. Dis. **42**: 548–558.
15. **Hinchey J, et al.** 2007. Enhanced priming of adaptive immunity by a proapoptotic mutant of Mycobacterium tuberculosis. J. Clin. Invest. **117**: 2279–2288.
16. **Jacobs M, Fick L, Allie N, Brown N, Ryffel B.** 2002. Enhanced immune response in Mycobacterium bovis bacille Calmette Guérin (BCG)-infected IL-10-deficient mice. Clin. Chem. Lab. Med. **40**:893–902.
17. **Khan IH, et al.** 2008. Profiling antibodies to Mycobacterium tuberculosis by multiplex microbead suspension arrays for serodiagnosis of tuberculosis. Clin. Vaccine Immunol. **15**:433–438.
18. **Lagranderie M, Chavarot P, Balazuc AM, Marchal G.** 2000. Immunogenicity and protective capacity of Mycobacterium bovis BCG after oral or intragastric administration in mice. Vaccine **18**:1186–1195.
19. **Larsen MH, et al.** 2009. Efficacy and safety of live attenuated persistent and rapidly cleared Mycobacterium tuberculosis vaccine candidates in non-human primates. Vaccine **27**:4709–4717.
20. **Lin PL, Flynn JL.** 2010. Understanding latent tuberculosis: a moving target. J. Immunol. **185**:15–22.
21. **Luciw PA, et al.** 2011. Stereological analysis of bacterial load and lung lesions in nonhuman primates (rhesus macaques) experimentally infected with Mycobacterium tuberculosis. Am. J. Physiol. Lung Cell. Mol. Physiol. **301**:L731–L738.
22. **Marchant A, et al.** 1999. Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guérin vaccination. J. Immunol. **163**:2249–2255.
23. **Marthas ML, et al.** 2011. Partial efficacy of a VSV-SIV/MVA-SIV vaccine regimen against oral SIV challenge in infant macaques. Vaccine **29**:3124–3137.
24. **Marthas ML, et al.** 1995. Viral factors determine progression to AIDS in simian immunodeficiency virus-infected newborn rhesus macaques. J. Virol. **69**:4198–4205.
25. **Murray RA, et al.** 2006. Bacillus Calmette Guérin vaccination of human newborns induces a specific, functional CD^{8+} T cell response. J. Immunol. **177**:5647–5651.
26. **Neutra MR, Kozlowski PA.** 2006. Mucosal vaccines: the promise and the challenge. Nat. Rev. Immunol. **6**:148–158.
27. **Ota MO, et al.** 2002. Influence of Mycobacterium bovis bacillus Calmette-Guérin on antibody and cytokine responses to human neonatal vaccination. J. Immunol. **168**:919–925.
28. **Ranganathan UD, et al.** 2009. Recombinant pro-apoptotic Mycobacterium tuberculosis generates CD^{8+} T cell responses against human immunodeficiency virus type 1 Env and *M. tuberculosis* in neonatal mice. Vaccine **28**:152–161.
29. **Rodrigues LC, Diwan VK, Wheeler JG.** 1993. Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a meta-analysis. Int. J. Epidemiol. **22**:1154–1158.
30. **Sambandamurthy VK, et al.** 2006. Mycobacterium tuberculosis DeltaRD1 DeltapanCD: a safe and limited replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. Vaccine **24**:6309–6320.
31. **Sambandamurthy VK, et al.** 2005. Long-term protection against tuberculosis following vaccination with a severely attenuated double lysine and pantothenate auxotroph of *Mycobacterium tuberculosis*. Infect. Immun. **73**:1196–1203.
32. **Sambandamurthy VK, Jacobs WR, Jr.** 2005. Live attenuated mutants of Mycobacterium tuberculosis as candidate vaccines against tuberculosis. Microbes Infect. **7**:955–961.
33. **Sambandamurthy VK, et al.** 2002. A pantothenate auxotroph of Mycobacterium tuberculosis is highly attenuated and protects mice against tuberculosis. Nat. Med. **8**:1171–1174.
34. **Sampson SL, et al.** 2011. Extended safety and efficacy studies of a live attenuated double leucine and pantothenate auxotroph of Mycobacterium tuberculosis as a vaccine candidate. Vaccine **29**:4839–4847.
35. **Shen Y, et al.** 2001. Antiretroviral agents restore *Mycobacterium*-specific T-cell immune responses and facilitate controlling a fatal tuberculosis-like disease in macaques coinfected with simian immunodeficiency virus and *Mycobacterium bovis* BCG. J. Virol. **75**:8690–8696.
36. **Trunz BB, Fine P, Dye C.** 2006. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet **367**:1173–1180.
37. **Van Rompay KK, et al.** 2010. Immunogenicity of viral vector, prime-boost SIV vaccine regimens in infant rhesus macaques: attenuated vesicular stomatitis virus (VSV) and modified vaccinia Ankara (MVA) recombinant SIV vaccines compared to live-attenuated SIV. Vaccine **28**:1481–1492.
38. **Van Rompay KK, et al.** 2003. Immunization of newborn rhesus macaques with simian immunodeficiency virus (SIV) vaccines prolongs survival after oral challenge with virulent SIVmac251. J. Virol. **77**:179–190.
39. **Van Rompay KK, et al.** 1992. Simian immunodeficiency virus (SIV) infection of infant rhesus macaques as a model to test antiretroviral drug prophylaxis and therapy: oral 3′-azido-3′-deoxythymidine prevents SIV infection. Antimicrob. Agents Chemother. **36**:2381–2386.
40. **Van Rompay KK, et al.** 2004. The clinical benefits of tenofovir for simian immunodeficiency virus-infected macaques are larger than predicted by its effects on standard viral and immunologic parameters. J. Acquir. Immune Defic. Syndr. **36**:900–914.
41. **Vekemans J, et al.** 2001. Tuberculosis contacts but not patients have higher gamma interferon responses to ESAT-6 than do community controls in The Gambia. Infect. Immun. **69**:6554–6557.
42. **Waters WR, et al.** 2009. Efficacy and immunogenicity of Mycobacterium bovis DeltaRD1 against aerosol M. bovis infection in neonatal calves. Vaccine **27**:1201–1209.
43. **WHO.** 2011. Report on the global HIV/AIDS response. World Health Organization, Geneva, Switzerland. http://www.who.int/hiv/en/.
44. **WHO.** 2011. Global tuberculosis control 2011. World Health Organization, Geneva, Switzerland. http://www.who.int/tb/publications/global_report/en/.
45. **WHO.** 2011. World Health Organization HIV/TB facts 2011. World Health Organization, Geneva, Switzerland. http://www.who.int/hiv/topics/tb/hiv_tb_factsheet_june_2011.pdf.
46. **WHO.** 2007. Meeting of the immunization strategic advisory group of experts, April 2007—conclusions and recommendations. Revised BCG

vaccination guidelines for infants at risk for HIV infection. International Health Regulations. Wkly. Epidemiol. Rec. **82**:181–196.
47. **Wiseman CA, et al.** 2012. A proposed comprehensive classification of tuberculosis disease severity in children. Pediatr. Infect. Dis. J. **31**:347–352.
48. **Zhou D, et al.** 1999. Mycobacterium bovis bacille Calmette-Guérin enhances pathogenicity of simian immunodeficiency virus infection and accelerates progression to AIDS in macaques: a role of persistent T cell activation in AIDS pathogenesis. J. Immunol. **162**:2204–2216.
49. **Zimmerman DM, et al.** 2009. Safety and immunogenicity of the Mycobacterium tuberculosis DeltalysA DeltapanCD vaccine in domestic cats infected with feline immunodeficiency virus. Clin. Vaccine Immunol. **16**: 427–429.