A recombinant attenuated *Mycobacterium tuberculosis* vaccine strain is safe in immunosuppressed SIV-infected infant macaques

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ABSTRACT

Many resource-poor countries are faced with concurrent epidemics of AIDS and tuberculosis (TB), caused by 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 Bacille 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 (AMtb) containing HIV antigens could be safely administered at birth and induce mucosal and systemic immune responses to protect against HIV and TB infection, and we rationalized that vaccine safety could be most rigorously assessed in immunocompromised hosts.

Among three vaccine candidates tested, the recombinant AMtb strain mc26435 encoding an SIV Gag expression plasmid and harboring attenuations in genes critical for replication (panCD and leuCD) and immune evasion (secA2), was found to be safe after oral or intradermal administration in SIV-uninfected and SIV-infected infant macaques. Safety was defined as absence of clinical symptoms, lack of histopathological changes indicative of *M. tuberculosis* infection, and lack of mycobacterial dissemination. These data represent an important step in the development of novel TB vaccines and suggest that a combination rAMtb-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.
Introduction

About one third of the world’s population is infected with *Mycobacterium tuberculosis* (*Mtob*) (44). Every year, 8-10 million new individuals become infected with *M. tuberculosis*, and almost 1.5 million people die of tuberculosis (TB) (44). The recent development of multi-drug and extensively multi-drug resistant strains of circulating *M. tuberculosis* further underscores the need for novel approaches to combat TB. The only licensed TB vaccine, Bacille 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 infections.

TB is the leading cause of death in 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 in any infant infected with HIV or at risk for HIV infection (46). As a result, the number of infants co-infected 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 administrable attenuated *M. tuberculosis* strain expressing HIV antigens. Although the rate of in utero and perinatal mother-to-child-transmission (MTCT) 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 oral HIV acquisition by breast-feeding should be administered orally. BCG-based vaccines are advantageous because they can be administered at birth, are effective orally, and rapidly generate long-lived T cell responses against dually-administered mycobacterial and co-expressed non-mycobacterial antigens when administered to human infants (27).

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 the bovine-adapted *M. bovis* BCG. We developed auxotroph mutants of the 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* (*AMtb*) strains, their safety and their immunogenicity profile in comparison to the licensed BCG vaccine in SCID mice have been reportedly previously (16, 19, 28, 30-32). Some of these TB vaccine candidates were also characterized in nonhuman primates as an important step towards potential human clinical trials. Vaccine safety, immunogenicity and efficacy data obtained in nonhuman primates would be expected to be highly relevant to humans (19). The *AMtb* vaccine strains mc²6020 and mc²6030 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). Based on these data, we developed novel replication-attenuated *M. tuberculosis* vaccine strains with increased immunogenicity. Due to obvious ethical concerns, pediatric HIV-TB vaccine safety assessments and challenge studies for efficacy cannot be performed in HIV infected human infants. To account for the infant’s relatively inexperienced and still developing immune system early after birth, we therefore choose to test vaccine safety in infant macaques that show similar immune system ontogeny after birth compared to human infants. In a first step towards the generation of a pediatric combination HIV-TB vaccine, we constructed *AMtb* strains that express the SIV Gag gene. The safety profile of three distinct r*AMtb*-SIV vaccine candidates with different degrees of attenuation in replication and/or immunogenicity (Table 1) was initially tested in healthy, SIV-uninfected infant rhesus macaques. None of these vaccine
candidates induced clinical symptoms of TB. The vaccine strain (mc²5157) that was predominantly attenuated 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, rAMtb-SIV mc²6020 and mc²6435, was then evaluated under even more stringent conditions in 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 rAMtb-SIV strains did not cause TB-like lung pathology. Importantly, in animals vaccinated with mc²6435, 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 rAMtb-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 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 UC 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 10mg/kg body weight of ketamine-HCl (Parke-Davis, Morris Plains, NC). Trained veterinary staff monitored the animals daily for clinical symptoms associated with TB (e.g. difficulties in breathing, coughing, increased mucus secretions, lethargic behavior, weight loss) and/or SIV infection.

**Vaccine Strains and Immunization Regimens:** The three rAMtb vaccine strains tested in the current study were rationally attenuated from the 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 mc²6020 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 than the parental strain (30). The strain mc²5157 was designed primarily to test for enhanced immunogenicity (Δ*nuoG*) with attenuation...
for intermediate replication (Δ\text{panCD} Δ\text{RD1}), or no replication (Δ\text{leuCD} Δ\text{panCD}\text{176}) for mc\text{26435}). The construction of the r\text{A}\text{Mtb} vaccine strains has been previously described (19, 28, 31-33, 42, 49). In these prior studies, the introduced deletions were stable, and reversions were not observed (19, 28, 31-33, 42, 49). Prior to use in rhesus macaques, the safety and immunogenicity of all three r\text{A}\text{Mtb} strains was confirmed in mice (19, 28, 31-33, 42, 49). The r\text{A}\text{Mtb} mc\text{25157} and mc\text{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 blot immunolabeled with a V5 antibody-HRP, as previously described (Figure 1A) (28).

Immunogenicity was confirmed in C57BL/6 mice (Figure 1B).

An overview of the vaccination schedule including route and dose is provided in Table 2. Briefly, strain mc\text{26020} was administered both orally (PO) and intradermally (ID) at one week of age, and animals were followed for 6 months (Group A). Animals vaccinated with mc\text{25157} were primed orally and received a homologous ID booster vaccination at either 2 (Group C) or 3 weeks (Group E). Animals were euthanized at week 4 or 6, respectively, to test for vaccine-induced immune responses and \textit{M. tuberculosis} dissemination in various tissues. The time intervals were selected based on our previous pathogenesis and pediatric HIV/SIV vaccine studies in the infant macaque oral 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 (IAVI, Brooklyn, NY), and Dr. B. Moss (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 mc26435 vaccination. In the studies described here, the vaccine boosts are reported solely for the purpose of revealing all study variables; vaccine immunogenicity will be reported separately (manuscript in preparation). 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 TCID_{50} of SIVmac251 (stock 6/04; (23) by the intravenous (IV) route within 72 hours of birth, and then immunized one week later with mc^{2}6020 (Group B) or mc^{2}6435 (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. PBMC were isolated by gradient centrifugation as described (23). At the time of euthanasia, multiple tissues were collected, including tonsil, lymph nodes (LN: submandibular, retropharyngeal, bronchial, axillary, mesenteric), lung and intestinal tissues (ileum, colon). In
addition, from animals that received an ID vaccination, we saved tissue from the dermal inoculation sites (Groups A, B, E, F and I). Corresponding skin from orally vaccinated animals was collected as control tissue. Each tissue was divided and saved for multiple applications as follows: snap frozen (*M. tuberculosis* culture), formalin-fixed / paraffin-embedded (pathology, *M. tuberculosis* staining), and fresh tissue aliquots (immunogenicity). The isolation of cell populations from tonsil, LNs 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 (2). **SIV Replication:** Plasma samples were analyzed for viral RNA by a quantitative reverse transcription-PCR (qRT-PCR) assay as previously described (7). **M. tuberculosis -specific Plasma Antibodies:** The presence of IgG antibodies against the *M. tuberculosis* -PSTS1 antigen was determined in longitudinally collected plasma samples using a recently described multiplex microbead immunoassay based on the Luminex system (Austin, TX) (17). Each samples was tested in duplicate. Relative antibody levels are reported as mean fluorescence intensity (MFI) (17). Sera from *M. tuberculosis*-uninfected 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 micron 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 Dr. P. Luciw for comparison (21). In addition, Ziehl-Neelson 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 colony forming units (CFU). As rAMtb auxotrophic mutants cannot grow in standard mycobacterial growth media culture, the media 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 text) were compared between two or more groups after log10 transformation using a nonparametric Mann-Whitney test or One Way ANOVA analysis (Kruskal-Wallis test with Dunn’s comparisons), respectively. Area-under-the-
RESULTS

Confirmation of vaccine exposure by the oral and intradermal route

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 oral and intradermal vaccination with the various r*Amtb* vaccines. Independent of the vaccine strain, all SIV-uninfected vaccinated animals developed *M. tuberculosis*-specific plasma IgG antibodies to the PSTS1 antigen (Figure 2), but the magnitude was dependent on the vaccine strain and the route of administration. Animals that received the mc^2^6020 vaccine via the PO and ID route at 1 week of age (Group A) developed relative antibody levels reaching up to 10^4^ MFI by 4 weeks (Figure 2A). Similar data were obtained in animals orally primed with mc^2^5157, and receiving a homologous ID boost (Groups C and E; Figure 2B). Consistent with more stringent attenuation in replication (deletions of the leuCD and panCD loci), oral vaccination with the mc^2^6435 vaccine strain (Groups G and J) PSTS1 antibodies were significantly lower levels (P<0.001) during weeks 4-6 post immunization compared to levels in mc^2^6020 and mc^2^5157 vaccinated infant macaques. Furthermore, the route of vaccine administration influenced the
magnitude of antibody induction in plasma, with ID mc²6435 vaccinated animals (Group H) developing significantly higher (P=0.0119) antibody levels than PO vaccinated animals (Groups G and J). Although there was a trend towards higher PSTS1 antibody levels in PO mc²6435 vaccinated infants compared to mock-immunized animals between weeks 5-8 post vaccination, this difference did not reach statistical significance. PSTS1 antibody levels, however, were significantly higher in mc²6435 vaccinated infants compared to mock-immunized animals by area-under-the curve-analysis of antibody levels from week 0 to week 16 (P=0.0229). The magnitude of PSTS1 antibodies in animals infected with SIV prior to vaccination with rA\textit{Mtb} mc²6020 (Group B) or mc²6435 (Group L) was reduced compared to SIV-uninfected animals (Figures 2A and 2C), likely due to SIV-induced immunosuppression.

Safety assessment of rA\textit{Mtb}-SIV vaccine strains

The safety profile of the various rA\textit{Mtb} vaccine strains was evaluated based on the combined assessment 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 displayed any of the following: AFB positive, \textit{M. tuberculosis} culture positive, or granuloma detection during histopathological examination.
We assessed the safety of the rAMtb vaccine strains in a stringent two-step study. First, the rAMtb vaccine strains were administered to immunologically immature infant macaques at one 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 one week prior to rAMtb 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) (Figure 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) (Figure 3). Compared to age-matched control animals (mock-vaccinated animals), SIV-uninfected vaccinated animals showed similar peripheral blood CD4+T cell frequencies. In addition, the CBC values in SIV-uninfected rAMtb 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 SIV-uninfected infants showed weight gain similar to the age-matched control animals (Figure 3). In contrast, consistent with previous studies (2, 24), SIV-infected animals showed poor weight gain (Figure 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 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 (Figure 3). Despite this apparent immunosuppression as a result of SIV infection, these animals did not show clinical symptoms typically associated with *M. tuberculosis* infection. SIV-uninfected infants that received the mc²6020 or mc²6435 vaccines (Group A or Groups G, H, and J, respectively) showed normal weight gain (Figure 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 rAMtb-SIV vaccines were observed, with the exemption of local reactivity in ID vaccinated animals. These animals showed a local inflammatory response following ID inoculations and developed indurations. In mc²6020 vaccinated animals, indurations resolved over time. Due to the short follow-up time in mc²5157 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 rAMtb vaccine strains in infants, several tissues collected at necropsy were evaluated for TB pathology: (i) the dermal inoculation site or corresponding skin samples from orally rAMtb 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 mc²5157 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 ID inoculation sites, whereas dermal tissues from mc²6020 vaccinated animals appeared essentially normal (Table 3). In contrast to mc²6020 and mc²5157 vaccinated animals, ID vaccination with mc²6435 (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 orally or mock-vaccinated animals as well and thus was probably not due to vaccination (Table 3). Consistent with a potential spread of mycobacteria in mc²5157 vaccinated animals (Groups C and E), the majority of the axillary LNs of mc²5157 vaccinated animals showed moderate lymphoid hyperplasia and pyogranulomatous inflammation, whereas SIV-uninfected animals vaccinated with mc²6020 or mc²6435 showed only mild histopathological changes (Table 3). Evidence of pulmonary TB lesions or granuloma formation in the lung indicative
of \textit{M. tuberculosis} infection was markedly absent from the vaccinated animals. In fact, the lungs of animals vaccinated with mc\textsuperscript{2}6020 (not shown) and mc\textsuperscript{2}6435 (Figure 4E) were histologically indistinguishable from lungs of mock-vaccinated infant macaques (Figure 4C). Among the 10 animals vaccinated with mc\textsuperscript{2}5157, six developed small granulomas in their lungs (Figure 4D). However, these lesions were smaller and less frequent when compared to granulomas induced by virulent \textit{M. tuberculosis} infection (Figures 4A, 4B).

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

(iii) \textbf{Strain-dependent differences in mycobacterial dissemination}

\textbf{Miliary tuberculosis} is one of the most severe complications of \textit{M. tuberculosis} infection. Although the \textit{rMtb} vaccine strains tested in the current study were replication attenuated, the degree of attenuation in 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-Neelson 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 media 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 (1-2 bacilli per tissue), if at all, detected at dermal inoculation sites after mc26020 or mc26435 vaccination. Importantly, AFB were detected in tissue from only 1 of 3 SIV-infected infant macaques that received the mc26020 vaccine, and only at one site, the axillary LN which drained the ID inoculation site. AFB were not detected by Ziehl-Neelson staining in any other tissues in 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 mc25157, the rAMtb vaccine strain that contained deletions that may increase its immunogenicity and yet result in higher replication relative to mc26020, tested positive for AFB in their dermal tissues, and in 6 of 10 animals AFB were also detected in at least one other tissue (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 mc25157 vaccinated infant macaques, strain mc25157 was considered unsafe and not further pursued as a candidate *M. tuberculosis*-SIV vaccine. Remarkably, all tissues from SIV-uninfected (n=20) and also from SIV-infected (n=3) mc26435 vaccinated animals
were negative for AFB, and viable mycobacteria could not be recovered by any of the culture methods applied in any of these tissues.

In summary, the data indicate that rAMtb-SIV vaccine strains can be administered orally or intradermally to infant macaques during the first postnatal week. Although vaccine strain mc²5157 caused dissemination of mycobacteria, the live attenuated vaccine strains mc²6020 and mc²6435 demonstrated a better safety profile in infant macaques. In particular, strain mc²6435 was safe in SIV-uninfected (Groups I and K) and in immunocompromised SIV-infected infant macaques (Groups G, H, and J) by all safety criteria applied 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% 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 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 towards the development of an orally administered highly attenuated M. tuberculosis vaccine expressing HIV antigens as a potential pediatric combination HIV-TB vaccine.
BCG was originally administered orally, but this route was discontinued due to cervical lymphadenitis and parapharyngeal complications. The oral route may be more advantageous for a pediatric HIV-TB vaccine, because a mucosally administered vaccine could also induce local immune responses protective against HIV transmitted orally by breast-feeding. In fact, experiments in mice have shown that orally 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 SIV-uninfected and in SIV-infected, and thus immunosuppressed, infant macaques by the oral and intradermal 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 mc²5157 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 infected with pathogenic TB (21), the strain was deemed unsuitable for use in immunocompromised hosts. In contrast to mc²5157, the safety profile of the dual deletion strain mc²6020 (ΔpanCDΔlysA) 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 ID inoculation site. Whether or not this limited mycobacterial dissemination in immunosuppressed SIV-infected infant macaques correlates with reduced risk for 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 in SIV-uninfected 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 which encodes 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 *Mtb*-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 *mc²5157*. In addition to attenuating replication, deletions in mycobacterial genes
encoding immune interference may be necessary to enhance AMtb immunogenicity.

Based on the results with mc²5157 and mc²6020, the strain mc²6435 was designed with deletion in both the panCD and the leuCD loci (ΔpanCD ΔleuCD). An additional deletion was introduced in 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 AMtb strain (ΔlysA ΔsecA2) 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 the rAMtb strain mc²6435 was well tolerated and did not cause any TB-like disease in 20 of 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 mc²6435 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 rAMtb dissemination, and live mycobacteria could not be recovered from any 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 for human infant TB vaccination. Analogous to our study, the safety of a ΔleuCDΔpanCD auxotroph *M. tuberculosis* vaccine strain was recently evaluated in SIV-uninfected 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 for up to 1 year, further supporting the conclusion that auxotroph attenuated *M. tuberculosis* strains do not confer a safety risk in immunosuppressed individuals. The safety profile of the rAMtb strain mc²6435 in SIV-infected infant macaques is consistent with this conclusion.

A potential caveat of our study is the relatively short follow-up time in 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 due to failure to thrive. Therefore, vaccine-induced *M. tuberculosis*-associated pathology or dissemination had to manifest within a relatively short time period in SIV-infected animals to be detected, and we cannot draw any conclusions about 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 mc²5157 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 (mc²6020=mc²5157>mc²6435) and the route of vaccine administration (ID>PO). The persistence of these antibodies suggests that the rAMtb vaccines primed the infant immune system. Despite the low PSTS1 *M. tuberculosis*-specific plasma antibody responses after oral and intradermal administration in infant rhesus macaques, the rAMtb vaccine strain mc²6435 (that contains a mycobacterial expression plasmid encoding SIV Gag) was effective in inducing both SIV and TB-specific CD4⁺ and CD8⁺T cell immune responses in systemic and mucosal tissues (manuscript in preparation). Therefore, the rAMtb strain mc²6435 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 against both HIV and TB infection in infants. Considering that immune correlates of protection against HIV or TB acquisition are not well defined, these vaccines will ultimately need to be tested for efficacy against an HIV/SIV and *M. tuberculosis* challenge.
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The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.
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Figure Legends

Figure 1: Vaccine-induced SIV-specific CD8+T cell responses in mice. Panel A: Western blot result showing SIVgag protein expression in mc²6435 lysate (Lane 1) and its absence in mc²6434 lysate expressing the control plasmid (Lane 2). Lane 3 represents the molecular weight ladder. rAMtb 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 mc²6435 lysate. Panel B: C57BL/6 mice were immunized subcutaneously with 10⁷ CFU of the attenuated M. tuberculosis vaccine strain mc²6434 (n=5) or with mc²6434 that contained the SIV Gag expression cassette (named mc²6435, n=5). One week after immunization, splenocytes were isolated and AL11-SIVgag tetramer specific CD8+ T cell responses were determined by flow cytometric analysis. The figure shows the average percentages ± SD of AL11-SIVgag tetramer-specific CD8+T cells. Note that mc²6435 immunized mice mounted significantly higher AL11-SIVgag-specific CD8+T cell responses than mc²6434 mice (p<0.02), albeit absolute frequencies of tetramer positive CD8+T cells were low.

Figure 2: M. tuberculosis-specific plasma IgG antibody responses. Plasma antibody responses to PSTS1 antigen were measured by multiplex microbead array analysis. Average antibody levels ±SD for (i) mock-vaccinated (black lines, closed circles), (ii) SIV-infected and vaccinated animals (red lines, diamonds), and (iii) SIV-uninfected vaccinated animals (blue lines, triangles) are shown for
each vaccine candidate. Data are reported as mean fluorescence intensities (MFI). The experimental groups are listed in parentheses in the legends of each panel. Mock-vaccinated animals 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 3 SIV-uninfected vaccinated had high PSTS1 antibodies at day 0 (MFI of 666 compared to MFI of 40 and 18 in the other remaining animals, and therefore average baseline antibody levels at day 0 appear higher compared to Groups B, I, and K. Similarly, in Panel C, 2 of 6 ID and 2 of 14 PO animals vaccinated with mc^26435 had PSTS1 antibody levels of >100 MFI at day 0 explaining the higher MFI values at day 0 compared to Groups I, K, and L.

Figure 3: Plasma SIV viremia and clinical parameters. Panel A: SIV RNA copies per ml of plasma are shown for each animal in longitudinally collected plasma samples. Each symbol represents an individual animal. The experimental group of each animal is listed in parentheses. Panel B: The average increase in weight over birth weight (kg) 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 in mock-vaccinated (grey line) animals. The averages of absolute numbers ± SD (Panel C) and average percentages ± SD of CD4^+T cells (Panel D) in peripheral blood are shown for animals vaccinated after prior SIV infection (red lines, diamond) and for vaccinated SIV-uninfected (blue lines, triangle) animals in comparison to mock vaccinated animals (black lines, circle). Note that Panels C and D include the SIV-infected animals vaccinated with mc^26020 (n=3) and with
mc²6435 (n=3). For this analysis, vaccinated animals (n=33) included animals from Groups A, C, E, G, H, and J; and mock animals (n=11) included animals from Groups D, F, I and K.

**Figure 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. Panel A: Lung tissue (100x) of an adult rhesus macaque infected with pathogenic *M. tuberculosis* (21). Panel B: The same tissue shown in Panel B at 200x. Note the central necrosis, mineralization, granuloma formation, and the presence of multinucleate giant cells. Panel C: Lung section of a mock-immunized infant macaque. Panel D: Representative lung section of a mc²5157 vaccinated infant macaque with granulomatous tissue and signs of extensive atelectasis. Panels E and F show essentially normal lung sections after mc²6435 vaccination in SIV-uninfected (Panel E) or SIV-infected infant macaques (Panel F). Sections in Panels C-F are at 100x.
Table 1: rAMtb Vaccine Strains

<table>
<thead>
<tr>
<th>Vaccine Strain</th>
<th>Group</th>
<th>Mtb Attenuation for Replication</th>
<th>Immune Evasion</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>mc^6020 (ΔlysAΔpanCD)^1</td>
<td>A, B</td>
<td>ΔlysA, ΔpanCD (low replication)</td>
<td>none</td>
<td>19, 30-33, 42, 49</td>
</tr>
<tr>
<td>mc^5157 (ΔnuoGΔpanCDΔRD1:pSIV Gag)^2</td>
<td>C - F</td>
<td>ΔpanCD, ΔRD1 (intermediate replication)</td>
<td>ΔnuoG,</td>
<td>28, 30</td>
</tr>
<tr>
<td>mc^6435 (ΔleuCDΔpanCDΔSecA2:pSIV Gag)^3</td>
<td>G - L</td>
<td>ΔleuCD, ΔpanCD (low replication)</td>
<td>ΔsecA2</td>
<td>28</td>
</tr>
</tbody>
</table>

^1 ΔlysAΔpanCD = deletions of the lysA and the panCD loci

^2 ΔnuoGΔpanCDΔRD1 = deletions of the nuoG, panCD, and RD1 loci; pSIV GAG = insertion of a full length SIVmac239 Gag insert

^3 ΔleuCDΔpanCDΔSecA2 = deletions of the leuCD, panCD, and SecA2 loci
<table>
<thead>
<tr>
<th>Group</th>
<th>Size</th>
<th>SIV (IV)</th>
<th>Vaccination Prime Strain</th>
<th>Dose&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Route</th>
<th>Age (wks)</th>
<th>Boost Strain</th>
<th>Dose&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Route</th>
<th>Age (wks)</th>
<th>Sample Collection (weeks)</th>
<th>Necropsy Age (wks&lt;sup&gt;2&lt;/sup&gt;)</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>no mc'5020</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; and 10&lt;sup&gt;10&lt;/sup&gt; CFU</td>
<td>PO / ID</td>
<td>1</td>
<td>none</td>
<td>0</td>
<td>mc'5157</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>ID</td>
<td>2</td>
<td>0, 2, 4, 6, 8, 10, 12, 16, 20, 24</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>yes mc'5020</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; and 10&lt;sup&gt;10&lt;/sup&gt; CFU</td>
<td>PO / ID</td>
<td>1</td>
<td>none</td>
<td>0</td>
<td>PO / ID</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO / ID</td>
<td>2</td>
<td>0, 2, 4</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>no mc'5157</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO</td>
<td>0</td>
<td>mc'5157</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>ID</td>
<td>2</td>
<td>0, 2, 4</td>
<td>4</td>
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<tr>
<td>D</td>
<td>2</td>
<td>no mock</td>
<td>PO / ID</td>
<td>0</td>
<td>mock</td>
<td>PO / ID</td>
<td>2</td>
<td>PO / ID</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO / ID</td>
<td>2</td>
<td>0, 2, 4</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>no mc'5157</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO</td>
<td>0</td>
<td>mc'5157</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>ID</td>
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<td>0, 3, 6</td>
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<tr>
<td>F</td>
<td>2</td>
<td>no mock</td>
<td>PO / ID</td>
<td>0</td>
<td>mock</td>
<td>PO / ID</td>
<td>3</td>
<td>PO / ID</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO / ID</td>
<td>3</td>
<td>0, 3, 6</td>
</tr>
<tr>
<td>G</td>
<td>6</td>
<td>no mc'6435</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO</td>
<td>0</td>
<td>rAd5-SIV</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; PFU</td>
<td>IM</td>
<td>3</td>
<td>0, 3, 6, 9, 12, 16</td>
<td>16</td>
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</tr>
<tr>
<td>H</td>
<td>6</td>
<td>no mc'6435</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO</td>
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<td>rAd5-SIV</td>
<td>9x10&lt;sup&gt;6&lt;/sup&gt; PFU</td>
<td>IM</td>
<td>3</td>
<td>0, 3, 6, 9, 12, 16</td>
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<tr>
<td>I</td>
<td>4</td>
<td>no mock</td>
<td>PO / ID</td>
<td>0</td>
<td>mock</td>
<td>PO / ID</td>
<td>3</td>
<td>PO / ID</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO / ID</td>
<td>3</td>
<td>0, 3, 6, 9, 12, 16</td>
</tr>
<tr>
<td>J</td>
<td>8</td>
<td>no mc'6435</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO</td>
<td>0</td>
<td>rMVA-SIV</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; PFU</td>
<td>IM</td>
<td>3</td>
<td>0, 3, 6, 9, 12, 16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>3</td>
<td>no mock</td>
<td>PO</td>
<td>0</td>
<td>mock</td>
<td>IM</td>
<td>3</td>
<td>IM</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; PFU</td>
<td>IM</td>
<td>3</td>
<td>0, 3, 6, 9, 12, 16</td>
</tr>
<tr>
<td>L</td>
<td>3</td>
<td>yes mc'6435</td>
<td>10&lt;sup&gt;6&lt;/sup&gt; CFU</td>
<td>PO</td>
<td>1</td>
<td>none</td>
<td>0</td>
<td>mc'5157</td>
<td>9x10&lt;sup&gt;9&lt;/sup&gt; PFU</td>
<td>rAd5-SIV</td>
<td>3</td>
<td>0, 1, 2, 4, 6, 8, 10</td>
</tr>
</tbody>
</table>

<sup>1</sup> CFU = colony forming units; PFU = plaque forming units

<sup>2</sup> wks = weeks
Table 3: Histopathological Evaluation

<table>
<thead>
<tr>
<th>Group</th>
<th>SIV Infection</th>
<th>Vaccine Strain (Route)</th>
<th>Tissues</th>
<th>Pathology Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, F, K</td>
<td>No</td>
<td>MCAD (PO/ID)</td>
<td>1/11 normal; 3/11 very mild dermatitis; 3/11 no tissue</td>
<td>6/11 normal; 2/11 mild lymphoid depletion; 5/11 no tissue</td>
</tr>
<tr>
<td>T</td>
<td>(n=3)</td>
<td>No</td>
<td>M. tuberculosis (PO/ID)</td>
<td>1/3 normal; 1/3 mild hyperplasia; 1/3 very mild lymphoid depletion; 1/3 no tissue</td>
</tr>
<tr>
<td>E</td>
<td>(n=3)</td>
<td>Yes</td>
<td>M. tuberculosis (PO/ID)</td>
<td>2/3 normal; 1/3 mild dermatitis; 3/3 mild lymphoid depletion; 1/3 no tissue</td>
</tr>
<tr>
<td>C, E</td>
<td>(n=10)</td>
<td>No</td>
<td>M. tuberculosis (PO/ID)</td>
<td>1/10 normal; 3/10 moderate to severe pyogranulomatous dermatitis</td>
</tr>
<tr>
<td>H, J</td>
<td>(n=14)</td>
<td>No</td>
<td>M. tryptica (PO)</td>
<td>1/14 normal; 1/14 normal with mild paracortical expansion; 1/13 mild hyperplasia; 1/13 mild sinus histiocytosis</td>
</tr>
<tr>
<td>H</td>
<td>(n=6)</td>
<td>No</td>
<td>M. tryptica (ID)</td>
<td>1/6 normal; 5/6 mild sinus histiocytosis; 1/6 mild lymphoid depletion; 1/6 moderate paracortical expansion; 2/6 no tissues</td>
</tr>
<tr>
<td>L</td>
<td>(n=3)</td>
<td>Yes</td>
<td>M. tryptica (PO)</td>
<td>2/3 normal; 1/3 mild hyperplasia; 1/3 very mild sinus histiocytosis</td>
</tr>
</tbody>
</table>

The experimental groups as described in the text and in Table 2 are listed with the total number of animals provided parenthetically. All the observed histopathological findings are summarised for each tissue. The first number before the slash corresponds to the number of animals within a specific group that showed the described pathology out of the total number of animals (number behind the slash).

1 The summary conclusion provided by the pathologist is listed as: "-" negative, "+" positive, "-/+" some minor pathology for SIV or M. tuberculosis infection.

2 All the observed histopathological findings are summarised for each tissue.
Table 4: *M. tuberculosis* Detection

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal Tissue</th>
<th>M. tuberculosis Detection</th>
<th>AFB Culture</th>
<th>BacTec**</th>
<th>Solid Culture***</th>
<th>Result</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>MGIT*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Skin</td>
<td>0/3 NT</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bron. LN</td>
<td>0/3 NT</td>
<td>NT</td>
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</tr>
<tr>
<td></td>
<td>Lung</td>
<td>0/3 NT</td>
<td>NT</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Spleen</td>
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<td>NT</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>B</td>
<td>Skin</td>
<td>0/3 NT</td>
<td>NT</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Bron. LN</td>
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<td>NT</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Lung</td>
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<td>NT</td>
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<tr>
<td></td>
<td>Ax. LN</td>
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<tr>
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<td>NT</td>
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<tr>
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<td>Spleen</td>
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<td>NT</td>
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<tr>
<td>G, H, J</td>
<td>Skin</td>
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<td>0/3 NT</td>
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</tr>
</tbody>
</table>

MGIT: *no growth; **2 > 24 days; ***10 > 24 days*  
BacTec: *no growth; ++2 > 24 days; +++2 > 10 days*  
Solid Culture: *no growth; ++5 > 5 CFU; +++5 > 50 CFU; NT, not tested;  
2 samples positive for the detection of AFB or outgrowth of mycobacteria are bolded
Figure 2:

Abel et al.
Figure 3

Abel et al.
Figure 4; Abel et al.